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Bacteriophages and competitive cultures: A new vista for food preservation

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Abstract
In the recent years there has been a gradual shift in consumer preferences from use of chemical preservatives to utilization of natural antimicrobial compounds for food preservation. Bacteriophages and competitive cultures are the natural antimicrobial agents that are very efficient in reducing bacteria in food items. The phage products accepted by USFDA and USDA are being utilised in agricultural/ horticultural products, farm animals or animal products such as carcasses, meats. In the food industry utilisation of specific phages and competitive cultures avert putrefaction of products and spread of bacterial diseases. Therefore bacteriophages and competitive cultures endorse secure environments in animal food production, processing and handling.

Keywords: Bacteriophages, bacteriocin, competitive cultures, antimicrobial packaging

Introduction
A great threat is being posed to humans from microbes that are causing spoilage of food items. Spoilage render the foods unsuitable for human consumption due to undesirable changes that affect sensory attributes of food [1]. The most frequent form of food spoilage is microbial type. Microbial spoilage leads to loss of approximately 25% of total food produced each year. This food loss occurs even after following existing preservation techniques, good manufacturing practices, quality control and hygienic measures [2]. Food companies have to constantly meet up the consumer expectations for preparation of pathogen free and desired quality products with no artificial preservatives. Thus an effort is being made to exploit natural antimicrobials to reduce microbial contamination.

Bacteriophages are one such natural antimicrobial agent that have well recognized biotherapeutic potential throughout the world. Approval to the first phage product i.e ListShield TM to control *Listeria monocytogenes* in meat and poultry products was the main landmark in phage history of western world that was accomplished in 2006 [3]. Thereafter, various phage products have also been commended for use as biotherapeutics in foods [4].

Various new and innovative technologies are being developed throughout the world that include biological antimicrobial systems referred to as biopreservation. Biopreservation is a method that involves utilization of natural or controlled microflora, antimicrobials for food preservation and extension of its storage life.

Biopreservation of food involves utilization of lactic acid bacteria that cause inhibition of food spoilage microbes. The fermentation products produced by beneficial bacteria or bacteria itself are utilised in biopreservation to inactivate pathogens in food and control spoilage. Different ways by which beneficial microbes interfere with the growth of spoilage microbes are by causing decline in pH due to organic acid production and by various undissociated acid molecules that exhibit antimicrobial activity. The other biopreservative agents being used are bacteriophages and yeast.

Food Preservation by Bacteriophages
Viruses that infect bacteria with high specificity and efficacy are bacteriophages. These phages have double stranded DNA that is enclosed inside polyhedral head and major proportions of them are included under order Caudovirales [5]. Bacteriophages are present ever where in the environment. These can be isolated from human or animal related micro-organisms. Bacteriophages are the most numerous biological creatures in environment as there are approximately 10^{31} phage particles which is about ten times more than bacterial estimates on earth [6]. These viruses are not detrimental to animals, humans and plants [7].

Bacteriophages are the obligatory parasites which upon multiplication take over host protein machinery. Bacteriophages infect host cells in two ways viz. lysogenic and lytic pathway. In lysogenic pathway, genetic information is integrated into bacterial chromosome with no cell death and in lytic pathway there is discharge of newly formed virus from host cells by cell lysis [7]. Sternly lytic phage from food safety perspective is one of safest antibacterial approaches available. Lytic bacteriophages produce hydrolytic enzymes called lysins/endolysins in the final stage of lytic cycle that cause degradation of peptidoglycan layer of Gram positive bacteria [8].

Bacteriophages present advantages as biocontrol agents [7].

a. Phages are favored over other antimicrobial agents as they donot cause collateral damage to commensal microflora. This is due to their high specificity to target host cells which is attributed to bacterial cell wall receptors.
b. Phages exhibit self-duplication and self-limiting property which means that low or single amount of phage multiplies till a host threshold is attained.
c. Phages have a tendency to get accustomed to the altered host systems even if bacterial host cells develop phage defense mechanisms for their continued existence.
d. Phages have low innate toxicity as they are made up of nucleic acids and proteins.
e. Phages are simple to isolate, propagate and have prolonged shelf life.
f. Phages have the ability to survive food processing environmental stresses including various physiochemical conditions.
g. Phages are available in abundance in various foods and have been isolated from various raw products, processed food, fermented products and seafood. The various methods of application of phages along the food chain are presented in Fig: 1 [9].

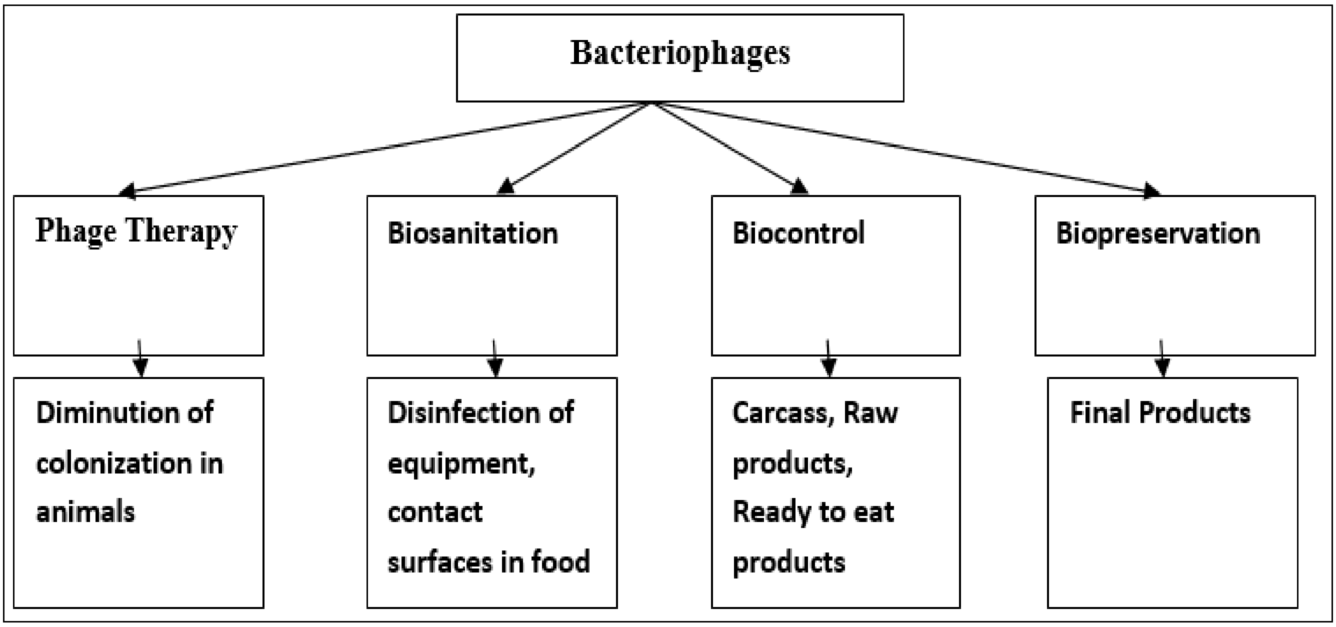

Fig 1: Various methods of application of phages along the food chain [9].

- Phage therapy involves reduction of pathogen colonization and is a approach followed prior to slaughter or during animal growth with an aim to decrease the probability of cross-contamination during food processing. Phages are included in drinking water or food to control *Campylobacter* and *Salmonella* in poultry. Phages are also sprayed on poultry to target *E. coli* and given orally/rectally for control of *E. coli* in ruminants.
- Biocontrol includes application of phages directly on the food surfaces like meat surface, processed foods, fresh produce or mixed into the raw milk.
- Biosanitation involves use of phages in destruction of bio-films formed on the surface of equipment by pathogenic bacteria.
- Biopreservation: Phages play a vital role as biopreservative agents as they function at a temperature ranging from very low i.e 1 °C to normal room temperature. Bacteriophages lyse host cells at temperature as low as 1 °C, thereby restricting the pathogenic and spoilage bacteria growth on refrigerated foods [7].

Guidelines related to use of Phages in food industry

i. **Proper Selection of Bacteriophage:** Lytic phages are commonly used for preservation of food. Lysogenic phages are not used as they contain toxic genes that can affect their host.
ii. **Immunity:** Bacteria tends to develop resistance to bacteriophage with time. Therefore, there should be replenishment of new phages with old one.
iii. **Industrial Preparation:** In case of production of industrial quantities of the phages, bacteriophage infection requires presence of pathogenic host bacteria.

Applications in Food Industry: The application of bacteriophages in various food products is highlighted in Table 1.

Table 1: The application of bacteriophages in various food products is highlighted

S. No.	Food type	Phage type	Outcome	Ref.
1.	Processed food (milk curd)	Cocktail (Φ88 and Φ35)	Addition of bacteriophages in pasteurized whole milk led to disappearance of *S. aureus* from acid curd after 4 hours of incubation at 25 °C. A total abolition of *S. aureus* occurred at 30 °C within 1 hour of incubation in renneted curd. Cocktail of phages added to milk before acid and enzymatic curd manufacture led to reduction of *S. aureus* by up to 6 log units.	[10]
2.	Ready to eat foods (pasteurized milk)	Cocktail (Φ35, Φ88) with nisin	When nisin along with cocktail of phages was mixed in milk, it was observed that phage-nisin combination reduced *S. aureus* by 1 log unit more than each antimicrobial substance alone at 37 °C for 24 hours.	[11]
3.	Chicken skin (Surface)	-	Phages application on chicken skin surface led to 1–1.3 log reduction in *Camplylobacter jejuni* within 24 hours.	[12]
4.	Beef	Phage mixture	Three-phage mixture was efficient in decreasing and ultimately eliminating *E. coli O157:H7*from the beef.	[13]
5.	Cheddar cheese	SJ2	Efficiency of bacteriophage SJ2 against *Salmonella enterica* in cheddar cheese prepared from raw and pasteurized milk during various stages of manufacture, ripening and storage was evaluated. It was observed that no pathogen was seen after 89 days of storage at 8 ˚C.	[14]
6.	Meat (raw and cooked beef)	Cj6	*Campylobacter* was reduced to great extent on both raw and cooked beef at 51 °C over a duration of 8 days by *campylobacter* bacteriophages.	[15]
7.	Meat (catfish fillets)	P100	*Listeria* phages have been efficient in diminution of colony forming unit (CFU) by 1.4–2.0 log units at 4 °C, 1.7–2.1 log at 10 °C and 1.6–2.3 log at 22 °C.	[16]

***E. coli* Bacteriophages**: *E. coli* O157:H7 is a highly pathogenic food borne bacteria that is naturally present in the gastrointestinal tract of ruminants. These bacteria commonly enter the human food chain via contact with contaminated faecal material of animals and are difficult to eradicate. Bacteriophage therapy can be used for reducing these dangerous microbes. The commercial preparation Eco Shield TM manufactured by Intralytix is a blendof three individual phages that provides broad protection specifically against pathogenic strains of *E. coli O157:H7*.

***L. monocytogenes* Bacteriophages**: Listeria is capable of infecting refrigerated ready to eat foods. Food additive regulations to allow the safe utilisation of a phage preparation as an anti-listerial agent in poultry products and ready to eat meat has been altered by FDA [17]. The preparation (0.1ppm concentration) including combination of six lytic phages having activity against different *L. monocytogenes* strain is directly atomized on surface of ready to eat meat. Bacteriophages on encountering their specific target, *L. monocytogenes* trigger a full infection and destruction cycle [8].

Commercial Phage Preparations

- "ListShield" Intralyx- It is efficacious against various strains of *L. monocytogenes.*
- SalmoFresh and SalmoLyse is used for controlling *S. enterica* *(*http://www.intalytics.com)
- BacWash product line is effective against *Salmonella omnilytics* (http://www.omnilytics.com/news/news019.html).
- Listex_ P100 by Micreos in Netherlands has been given Generally Regarded as Safe (GRAS) status by the FDA.

Food Preservation by Competitive Cultures

Competitive/antagonistic/protective cultures are food grade bacteria's and one of the major groups of the antagonistic cultures include lactic acid bacteria [18]. Their antagonistic activity involves primary or secondary metabolites production and competition for nutrients for inhibition of other microorganisms [19]. Some of compounds produced by the bacteria are lactic acid, hydrogen peroxide, fatty acid, bacteriocin and carbon dioxide.

Factors Affecting Protective Culture Performance

Temperature Effect: Growth rate and bacteriocin production by microorganisms is affected by temperature. The viability of protective cultures is to be maintained in products under refrigerated temperatures. The growth rate of protective cultures ought to be faster than the food poisoning and spoilage organisms.

Inoculum effect: Inoculum size affects the antimicrobial substance production rate, sensory attribute of product and cost-effectiveness of method. The initial load of other microorganisms and the uniqueness of the protective cultures utilized affect the inoculation level of protective cultures [20].

Food Effect: Protective cultures growth is supported or inhibited by composition and structure of food. Example includes salts, spices and added preservatives that have synergetic effect with protective cultures. Food contains certain ingredients such as glucose, yeast extract, biotin that promote the growth of protective cultures. Production of bacteriocin and increase in its activity is favored in product of low pH [18].

Food Preservation by Lactic acid bacteria

LAB group consisting of various bacteria such as *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus and Streptococcus* are being used as protective cultures in fermented foods. LAB are described as aerotolerant Gram-positive cocci or rods that ferment carbohydrates to produce energy and lactic acid [21]. LAB have been given Generally Recognized As Safe (GRAS) status and they play an essential role in food fermentation. These bacteria inhibit spoilage and pathogenic organisms growth, maintain the nutritive quality and improve the storage life of foods due to the production of inhibitory agents [22].

Food Preservation by Bacterial Metabolites

Organic Acid: Lactic acid is synthesized from hexose by homofermentative lactic acid bacteria and acetic acid, lactic acid by heterofermentative lactic acid bacteria. Lactic acid bacteria ensure food safety and quality by decreasing pH with these acids. In meat and dairy products, lactic acid bacteria have antimicrobial effect on spoilage and pathogenic bacteria such as *E. coli, S. aureus.*

Acetic acid is synthesized commercially by *Acetobacter aceti.* The bacteriostatic activity is evident at acetic acid level of 0.2% and bacteriocidal activity above 0.3%. The acid is more effectual against Gram-negative bacteria and bactericidal effect is more distinct at low pH (below pH 4.5).

Propionic acid produced by *Propionibacterium spp.* is capable of hindering the Gram-positive and Gram-negative bacterial growth. It is used for inhibiting the growth of molds in cheeses, butter, bakery products and also hampering the growth of bacteria, yeasts in syrup and fruits [23].

Hydrogen peroxide (H_2O_2) is an oxidizing component that is synthesized during aerobic growth by lactic acid bacteria. It has antimicrobial effect against vegetative cells and spores of majority of microorganisms [23]. It depicts its antimicrobial effects by altering the chemical structure and biochemical characteristics of enzymes of target organisms.

Fatty Acids that are produced by lipolytic LAB affects sensory attributes of fermented foods. Its antimicrobial activity varies according to chain length. Researches have depicted that with 10 mM of the fatty acids and monoglycerides, the yeast cell growth was repressed due to production of capric and lauric acid [24].

Diacetyl (2,3- butanedione) is synthesised during citrate fermentation by some lactic acid bacteria [24]. The antimicrobial effects of diacetyl is evident at low pH. Gram negative bacteria is susceptible at pH of 5.0 or less. Diacetyl antagonises utilization of arginine by reacting with arginine binding sites on the proteins of gram negative bacteria.

Antimicrobial Compounds including reuterin, reutericiklin and 2-pyrolidone-5-carboxylic acid are produced by certain species of lactic acid bacteria. These compounds are active in low pH and are thermostable. Reutericiklin that is produced by *L. reuteri* behaves as a proton ionophore, separate the cytoplasmic membrane due to its hydrophobicity and disrupts the transmembrane pH. Reuterin is a potent antimicrobial compound produced by *L. reuteri*, during the metabolism of glycerol to 1,3-propanediol and its inhibitory action is due to inactivation of ribonucleotide reductase.2-pyrrolidone-5-carboxylic acid produced by *Lactobacillus casei ssp. casei.* has inhibitory activity to *B. subtilis, E. cloacae.*

Bacteriocin: Bacteriocins are biologically active peptides synthesized by ribosomes with antimicrobial actions against closely related species of bacteria [25]. Bacteriocins were first discovered by A. Gratia in 1925.The bacteriocins from *E. coli* are called colicins and one of the oldest recognized colicin was called colicin V, now called as microcin V. The difference between bacteriocin and antibiotics is depicted in Table 2 [26].

Table 2: Difference between Bacteriocin and Antibiotics

Factors	Bacteriocin	Antibiotics
Application	It is used clinically and in foods.	It is used clinically.
Synthesis	It is synthesised in ribosome	It is produced as secondary Metabolite
Spectra of Bioactivity	It has mostly narrow spectrum activity.	It has mostly broad spectrum activity
Intensity of Bioactivity	It is active at nano to micromolar range.	It is active at micro to millimolar range.
Proteolytic Enzyme Degradability	It has high proteolytic enzyme degradability.	It has moderate to high proteolytic enzyme degradability.
Thermal Stability	Highly thermostable	Less thermostable
Active pH range	Active at wide pH range	Active at narrow pH range
Taste/Color	It has no taste/color	It has taste/color
Mode of Action	It leads to pore formation on cell membrane and also inhibits cell wall synthesis.	It has cell membrane or intercellular targets.
Toxicity towards Eukaryotic Cell	It has relatively no toxicity towards eukaryotic cells	It has relatively no toxicity towards eukaryotic cells.
Residual effect in consumers	It has comparatively less residual effect in consumers	It has comparatively high residual effect in consumers

Some bacteriocin-producing strains are applied as protective cultures in a various food products

LAB bacteriocins has certain characteristics due to which it can be used as food preservative. These include proteinaceous and non-toxic nature, non-immunogenicity to laboratory animals, inactivity against eukaryotic cells, thermo-resistance, bactericidal activity against majority of Gram-positive and Gram-negative bacteria [21].

LAB bacteriocins classification is as follows

Class 1 Lantibiotics: These bacteriocins include certain unusual amino acid such as lanthione or beta- methyl lanthionine. Nisin is one of the essential member of Class 1 Lantibiotics which is produced by several strains of *Lactobacillus lactis.* It inhibits the growth of various Gram-positive bacteria. These lantibiotics depending on the structure and charge of compound are classified into two subgroups

1) Group I a comprise of amphipathic, small cationic peptides that by non-specific interaction with the target cell produce voltage-dependent pores in the membrane.

2) Group I b consists of anionic or neutral peptides. The various antibiotics included under this group are mersacidin, actagardin, cinnamycin and mutacin A [27].

- Class 2 Small heat stable peptides includes small, thermo-stable, non lanthionine containing membrane active peptides. Pediocin, an important bacteriocin of this group is produced by *Pediococcus acidilactici* and is widely used in the fermentation of meat. Pediocin creates hydrophilic pores in the cytoplasmic membrane, thereby dissipating ions and hindering transport of amino acids in sensitive cells [28].
- Class 3 Large heat labile proteins are higher molecular weight proteins. Its two subclasses viz. subclass IIIa or

bacteriolysins cause cell wall degradation and IIIb alter membrane potential.

- Class 4 Complex proteins: This group comprise of glycoproteins (lactocin 27) or lipoproteins (lacstrepcins) that exhibit their activity in presence of non-protein moieties [27].
- Application of bacteriocin in food industry: Bacteriocins are effective against various Gram-positive bacteria like *Staphylococcus aureus, Listeria monocytogenes.* Bacteriocins also inhibit several Gram-negative bacteria either alone or in combination with heat, cold and chemicals [28]. The combination of hydrostatic pressure and bacteriocins is used for killing pathogenic *Staphylococcus* and *Listeria*. Nisin has been given GRAS (Generally Recognized As Safe) status and accepted for use in the United States. Thus, organisms producing nisin like *Lactococcus lactis* subspecies *lactis* are being used for manufacturing ripened cheese, gouda cheese and camembert cheese [28].

Application in Meat: The bacteriocins most common in meat and meat products include nisin, enterocin AS-48, enterocins A and B that are used alone or together with various physicochemical treatments, modified atmosphere packaging, chemical preservatives to control the proliferation of various pathogens [29]. Various bacteriocinogenic LAB have been used in food manufacturing processes as bioprotective cultures to control various pathogens [30]. Pediocin PA-l is more appropriate than nisin for utilisation in meat and meat products.

Application in Fish: Fresh fish spoilage is usually caused by Gram-negative microorganisms. In vacuum packed seafood and fresh fish, spoilage is caused by pathogenic microbes such as *Clostridium botulinum* and *L. monocytogenes*. Live bacteriocin producing cultures or concentrated bacteriocin preparations have been incorporated into these products [31]. The nisin and Microgard combination is effective in decreasing the total aerobic bacteria populations of fresh chilled Salmon and increasing its shelf-life [32].

Yeast: Biopreservative effect of yeast due to their antagonistic activity relies on nutrient competition and pH alterations in the medium due to organic acid production. Commercially yeast is used as bio-control agent to avert spoilage of fruits after harvest. The product “Aspire”containing *C.oleophila* has been used in the U.S. Aspire is used to prevent fruit deterioration during storage by either spraying or immersing in solution having a certain concentration of yeast [23].

Antimicrobial Packaging: Surface contamination of foods by microbes occurs during post processing handling. Various efforts have been made to delay spoilage and enhance safety by use of antibacterial dips or sprays. Antibacterial substances directly applied onto the food surface have less benefits because active substances quickly diffuse from surface into the food mass or are neutralized on contact. Antimicrobial food packaging materials decrease the microorganisms growth rate by extending the lag phase. Therefore, this antimicrobial packaging helps to maintain safety, quality of the product and enhance its shelf life.

Antimicrobially active food packages are made by addition and immobilization of antimicrobial agents or by surface modification and coating. Antimicrobial agents are added into the packaging materials that migrate through diffusion in food. Antimicrobial agents contained in packaging films are made more competent by slow migration of these agents into product surface from the packaging material.

Antimicrobial films are of 2 types

(1) Films that contain antimicrobial agents with large molecular structure to maintain its activity on the microbial cell wall despite being bound to the plastic. These antimicrobial agents including enzymes, other antimicrobial proteins migrate from films to the surface of food and exhibit their activity.

(2) Films that are effective against surface microbes without undergoing migration [33].

Bacteriocins incorporation in packaging films to control pathogenic organisms

Bacteriocins are being widely utilized for control of food spoilage and pathogenic organisms by adding them into packaging films. Antimicrobial packaging film contact with food surface ensures bacteriocins diffusion to the surface [33]. In comparison to dipping and spraying foods with bacteriocins, the steady release of bacteriocins from a packaging film to the surface of food is more efficacious. In the dipping and spraying methods, antimicrobial efficacy is decreased or completely abolished due to inactivation of the bacteriocins by food components or dilution below active concentration.

Two commonly used methods to formulate packaging films with bacteriocins are

- Bacteriocins are directly incorporated into polymers such as nisin incorporated into biodegradable protein films. Successful addition of nisin into soy protein and corn zein films have been performed by two packaging film forming methods such as heat press and casting. The films produced via both methods were of excellent that inhibited the growth of *L. plantarum* [33].
- Bacteriocin are coated or adsorbed to polymer surfaces like nisin adsorption on polyethylene, polypropylene films exhibited their effect by inhibiting the growth of *L. monocytogenes* [33].

Conclusion

Biological agents are found to be suitable alternative to physical and chemical preservative techniques. Bacteriophages are being used to improve food safety by serving as bio control agents as they have high specificity to target their host, low inherent toxicity and withstand various food physiochemical conditions. Competitive cultures being used exhibit antagonistic activity by producing primary or secondary metabolites and competing for nutrients with other harmful microorganisms. Among the various metabolites, bacteriocin has major application in food industry. A new approach antimicrobial packaging is becoming vital as it represents a lower risk to the consumer. With these preservative methods, products in natural state and in minimally processed form can reach consumers.

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