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BENEFICIAL FACE OF BACTERIOPHAGES: APPLICATIONS IN FOOD PROCESSING

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Abstract: *Foods are processed to make them available at all places; consequently, our awareness regarding hygiene measures in food production has also increased dramatically over the last decades. In many countries cases associated with foodborne infectious are increased. However, available techniques are unable to effectively control the problem. Further, exploring novel methods and technologies for ensuring the safety of food with effective quality control approaches are under research. Phages are the natural enemies of bacteria, and are more specific to host renders them ideal candidates for applications designed to increase food safety during the production process. Scientific findings are available showing the possibility to use as biocontrol agents against various pathogens without interfering with the natural microflora or the cultures in fermented products. Furthermore, phages or phage derived proteins can also be used to detect the presence of unwanted pathogens in food or the production environments, which allows quick and specific identification of viable cells. Bacteriophages are natural, found in various environments including water; foods etc. and are not found significantly influence the human cells.*

Keywords: *Bacteriophage, biocontrol, food-borne pathogens, quality control, biosensor*

1. INTRODUCTION

The word "Bacteriophage" describe a "Microbe" that attacked bacteria and was capable of killing them. Moreover, presence of spoilage or pathogenic microorganisms raises question about safety and acceptability of food and providing a safe quality food product to the consumers is a prime responsibility of food processors. Many process methodologies are available to destroy and monitor such microbes that enter food during various processing steps to make food safe for consumption. However, all the processes are suffering with one or more disadvantages. The detection and identification of pathogens in food products and drinking water supplies continue to mostly rely on conventional microbiological culture techniques. These tests are based on assessing a bacterium's ability to grow in plates or tubes containing a variety of media (solid or Liquid) under various conditions. While detection of a small number of bacteria possible by incubation, growth of bacteria to number sufficient for identification can take several days. In addition, further biochemical and serological tests are required to confirm the identity of the agent **[1]**. Molecular techniques such as Polymerase Chain reaction (PCR) may also be used to amplify a small amount of genetic material from bacteria. Alternatively, bacterial identification using Enzyme Linked Immunosorbent Assay (ELISA) is conducted by testing antibody-antigen interaction with the targeted bacterium and can be performed within a working day. However, these techniques still require an enrichment step during which bacteria are grown to the levels required for detection. In addition, problems associated with enzyme inhibition and DNA extractions have made direct detection of low numbers of bacteria in foods by PCR difficult to achieve. In addition, to meet the food safety management systems requirements, dairy and food processing industries need more rapid, accurate tools to control the quality of processed foods. Recently, there is an increased interest in the use of bacteriophages, viruses that infect and usually kill bacteria, as a means of inactivating food borne pathogens and spoilage organisms in food products **[2]**. Bacteriophages are obligate intracellular parasites that infect bacteria, reproduce by hijacking their host biosynthetic pathway and are host specific. Phages are classified as either lytic or lysogenic based upon their replication strategy.

2. LIFE CYCLES OF BACTERIOPHAGES

Bacteriophages infection in bacteria may cause two different consequences, are lytic and lysogenic cycles. The lytic phage pathway starts when the virion interacts with the hosts' cell surface receptor molecules. After phage adsorption to these molecules, the cell wall is made penetrable and the nucleic acid is transported into the cell, whereas the capsid remains outside the cell.

Inside the host occur several steps which include gene expression, genome replication and morphogenesis i.e., the formation of the capsids (and tails) and the packaging of the genomes into the capsids. Phages are reproduced very quickly, forming new virion particles and this reproduction phase ends with the lysis of bacteria. With the host lysis, hundreds of new phages are released from each infected bacteria **[3]**. The number of new phages produced, or progeny depends on the species and conditions, nevertheless each "parent" phage is able to produce in average 50-200 "daughter" phages per lytic cycle **[4]**. Lytic phage infection results in clear plaques on the respective host bacterial lawns.

Lysogeny, or the lysogenic cycle, is one of two phases of viral reproduction (the lytic cycle is the other). Lysogeny is characterized by integration of the bacteriophages nucleic acid into the host bacterium's genome. The newly integrated genetic material, called a prophage can be transmitted to daughter cells at each subsequent cell division, and a later event (such as UV radiation) can release it, causing proliferation of new phages via the lytic cycle. Certain types of viruses replicate by the lysogenic cycle, but also partly by the lytic cycle (mixed cycles). Some DNA phages, called temperate phages, only lyse a small fraction of bacterial cells; in the remaining majority of the bacteria, the phage DNA becomes integrated into the bacterial chromosome and replicates along with it. In this lysogenic state, the information contained in the viral nucleic acid is not expressed. The model organism for studying lysogeny is the lambda phage. Lytic phages bring about rapid lysis and death of the host bacterial cell, whereas temperate phages spend part of their life cycle in a quiescent state called prophage **[5]**.

3. BACTERIOPHAGES AND DAIRY FOODS

To summarize the extensive literature, different approaches in various environments yielded a remarkably constant rate of virus-mediated bacterioplankton mortality of about 15% per day. Phages are also present in the food we eat. Many food products from our daily life are the result of fermentation processes by lactic acid bacteria. Cheese factories using ***Lactococcus lactis*** can be contaminated with high levels of phages; one study reported up to 10^9 phage $ml^{-1}$of whey and 10^5 phage m^{-3} was found in the air. Furthermore, bacteriophages are ubiquitous in different environments, in the human gastro-intestinal tract, in water and in food products, unable to infect human cells and, consequently, consequently, they have great potential for use as biocontrol agents in foods **[2, 6, 7]**. Lactic acid bacteria (LAB) have been the focus of substantial research because of their economic importance in food fermentation. LAB comprises lactococci, lactic streptococci, leuconostoc, pediococci and lactobacilli. *Lactococcus lactis* is the major starter bacterium in the cheese industry, and a co-culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii,* ssp. *bulgaricus* for example, is used in the production of yogurt. An important problem in industrial milk fermentation is bacteriophage attack. Milk is the natural habitat of *S. thermophilus* **[8], i**n fact, this environment is, however, not small. About one third of the annual world output of around 500 million tons of milk is transformed into fermented products **[7]**. Two thirds of all processed milk is fermented by mesophilic starter bacteria (*L. lactis*), and thermophilic cultures (*S. thermophilus* and lactobacilli) account for a major part of the rest. Bacteriophages infection leads to the lysis of the starter cells and thereby interrupts the fermentation of milk sugar (lactose) into lactic acid by the starter bacteria. Lactic acid production plays an important role in the creation of the food matrix and its conservation against food spoilage organisms. The consequences of a phage infection are therefore a fermentation delay, an alteration of the product quality, and in severe cases the loss of the product leading to economic losses.

The first priority of dairy phage research has therefore been the development of phage-resistant starter strains. For *L. lactis* ssp. *lactis* many phage-resistance functions were derived from plasmids possessing natural antiphage functions. Comparative genomics identified related *cos*-site and *pac*-site phages, respectively, in lactococci, lactic streptococci and lactobacilli. Each group was represented with closely related temperate and virulent phages **[6, 7, 8]**. Lytic phages are the most significant cause of fermentation failures in the dairy industry worldwide. This review aims to provide an impact of Bacteriophages in terms of usefulness and harmful effects in the area of dairy and food sector.

4. BACTERIOPHAGES APPLICATION TO CONTROL PATHOGENS IN FOODS

For both pre and postharvest application of phages to control unwanted bacteria in food the term "biocontrol" is used to that of "phage therapy" **[9]**. The potential of phages for controlling food borne pathogens is reflected in several recent studies.

Table 1 presents various studies exploiting bacteriophages to control undesirable pathogenic bacteria in various foods. Various factors determine the efficacy of the phage application are the stability of the phage(s) under the physicochemical conditions of the food (pH, aW), under its storage conditions (temperature) and the ratio of phages to host cells (Multiplicity of infection-MOI) **[2]**. Furthermore, emergence of phage resistance and phage host range are

other two important issues to consider in the design of phage applications **[10]**.

The application of the host-specific bacteriophage φ2 at approximately $5x10^6/cm^2$ to the surface of chicken skin resulted in a significant 1 $\log_{10}$ reduction in the number of inoculated *Campylobacter jejuni* cells ($5x10^5/cm^2$), when stored at 4°C **[7].** O'Flynn *et al.* **[11]** demonstrated the efficacy of a three-phage cocktail (multiplicity of infection (MOI) - the ratio of phages to host cells - was 10^6 pfu/cfu) in reducing *E. coli* O157:H7 on inoculated steak meat at 37°C but simultaneously reported that no lysis occurred in the absence of growth of the host at 12°C. Two phages, phage Felix O1 and a variant, were applied (MOI of $1.9x10^4$ pfu/cfu) to frankfurters inoculated with *Salmonella typhimurium* and could reduce growth of the pathogen with ± 2 $\log_{10}$ after 24h at 22°C **[10]**. Goode *et al.* **[12]** was able to inactivate partly or completely, depending on the MOI, *S. enterica* and *C. jejuni* on chicken skin stored at 4°C for 48h.

Application of phage LH7 to two *L. monocytogenes* isolates inoculated onto vacuum packaged beef, which was stored at 4°C, had no effect compared to a control because of the MOI that had not been optimized **[13]**. When phage P100 used, an antilisterial effect (3.5 $\log_{10}$ reduction) was obtained in surface-ripened red-smear soft cheese **[14].** Garcia *et al.* **[15]** applied a cocktail of lytic phages ϕ88 and ϕ35 and milk-isolated temperate phages, ϕH5 and ϕA72 at multiplicity of infection (MOI) of 100, found a complete elimination of $3X10^6$ cfu mL^{-1} of the pathogen in ultra-high-temperature (UHT) whole milk at 37^0C. Furthermore, the lytic phage derivatives, added to milk, were able to decrease rapidly the viable counts of *S. aureus* during curd manufacture and the frequency of emergence of bacteriophage-insensitive mutants was reduced up to 200-fold in the presence of the two lytic phages compared with that detected with the combination of the temperate counterparts. In another study Kim *et al* **[16]** described the successful control of *Cronobacter sakazakii* in reconstituted infant formula by two newly isolated phages.

Combinatorial approach biocontrol studies employed of bacteriophages and their lysins, together with natural antimicrobial substances has been recently published. Recently, Garcia *et al.* **[17]** demonstrated synergistic effects between the endolysin LysH5, encoded by the *S. aureus* phage phi-SauS-IPLA88, with the bacteriocin nisin to effectively inhibit *S. aureus* in pasteurized milk. Mahony *et al*. **[18]** have recently reviewed the recent advances in the research of phages that target food pathogens and that promote their use in biosanitation, while also discussed its limitations.

Table 1: Some studies related to biocontrol by bacteriophage application

Pathogen	**Food matrix**	**Reference**
C. jejuni	Fresh cut fruit	**[44]**
	Chicken skin	**[6, 12]**
Salmonella spp	Cheese	**[45]**
	Chicken Frankfurters	**[10]**
E. coli O157:H7	Steak meat	**[11]**
L. monocytogenes	Beef	**[13]**
	Fruit	**[46, 47]**
	Cheese	**[43]**
	Ready-to-eat foods	**[48]**
S. aureus	Ultra-high-temperature (UHT) whole milk	**[15]**
	Pasteurized milk	**[17]**
E. sakazakii	Infant formula	**[16]**

5. BACTERIOPHAGES AS BIORECOGNITION MOLECULES IN BIOSENSORS DEVELOPMENT

Increasing public health concerns related to bacterial diseases, as well as the need to monitor food and water supplies have prompted interest in the development of low cost and low footprint pathogen detection system **[19]**. Therefore, there has been sustained interest towards the development of biosensing systems that would circumvent the limitation of conventional techniques. A typical biosensor platform couples physical transducers (electrochemical, mechanical, thermal, or optical) with a specific recognition probe. Bacteriophage offers such potential probe for specific biosensing. They are viruses that recognize specific receptors on the bacterial surface to which they bind and inject their genetic material. Such injection allows replication of the phage and release a new generation while killing the bacteria. These viruses recognize target bacteria through functional receptors located on their tail extremity **[20]**. This recognition is routinely employed in phage typing where a group of phages are used to differentiate between different bacteria. This unique level of specificity also presents remarkable possibilities for biosensors development. For instance, the use of a lytic phage for the SPR

detection of *Staphylococcus aureus* was recently reported **[21]**. However, the attachment of phages was accomplished by simple non-oriented physisorption of the viruses onto the sensor surfaces. Recently, Tolba *et al.* **[22]** the use of immobilized biotin carboxyl carrier protein gene (BCCP)-T4 bacteriophage (Recombinant bacteriophage) for an Escherichia coli B assay using a phage multiplication approach and real-time PCR allowed detection of as few as 800 cells within 2 h. However, phage head modification resulted in a decreased burst size and an increased latent period. It was shown that recombinant bacteriophages form specific and strong bonds with their respective solid support and are able to specifically capture and infect the host bacterium. Ag-p8MMM phage-modified sensor was recently reported for detection of glucose content **[23]**.

6. APPLICATION OF PHAGES TO NANOTECHNOLOGY

Phage display was first described many decades ago and has been defined as 'a simple functional genomic methodology for screening and identifying protein - ligand interactions'. Application of phage display is found in antibody engineering, screening for receptor antagonist and the atlas of protein expression project. The phage display process has been revised extensively. The selection of application of phage display in nanotechnology is described below.

Nanowires are important in nanotechnology, with potential applications in Microcircuit and optoelectronic **[24].** However, it can be difficult to fabricate these regular structures, and the processes required are often expensive or produce toxic byproducts. Although, use of phages in the synthesis of nanostructures is still in development, their use in bacterial detection is more advanced. Many methods of detecting specific bacteria are available, but the low cost and ready production of large number of phage, added to their specificity for a target bacterial species, makes them ideal for bacterial detection.

Applications of these bacterial detection systems are found in the detection of food and water-borne pathogens, in bioterrorism and disinfection in hospital and agriculture. Classically, bacterial pathogens were identified by phage typing and by enrichment on selective growth media, in addition to a suite of biochemical tests. These can be lengthy procedure, and can be delays can be problematic where perishable foods are under scrutiny. Recombinant phages are often used in phage based detection systems. Upon infection of their hosts, these engineered phage deliver reporter genes, such as luxAB genes from *Vibrio harveyi* **[25]**. Replication of the Viral genome results in many copies of the reporter genes being produced, and subsequent expression of these genes ensures the amplification of the initial phage-bacterium interaction into a signal that can be readily detected bioluminescence in the case of luxAB. Recently, Lee *et al.* **[26]** reviewed the principles of *in vitro* design of the bacterial virus phi29 DNA packaging motor and its potential nanotechnological and medical applications.

7. USE OF BACTERIOPHAGES FOR BIOFILM CONTROL

Pathogenic and spoilage bacteria are consistently found living in sessile communities attached to a wide range of biotic and abiotic surfaces, these communities better known as biofilms. In most food and dairy industries, pathogenic and spoilage bacteria tend to attach to equipment surfaces, and form Biofilm. These are important reservoirs of microbial contamination that may lead to equipment damage, energy losses, spoilage of finished products and transmission of food pathogen that may cause diseases. Bacterial biofilm formation in food industries has been the focus of some reviews. Biofilms tend to form on the surfaces of equipment used for example in food handling, storage, or processing, especially in sites that are not easy to clean or sanitize (ex. Dead ends, joints, corners, valves, and gaskets in tubing systems). Corroded areas of equipment surfaces are also ideal places for the development of biofilms. Besides stainless steel and Teflon, which are common equipment materials in industrial environments, biofilms are also found on a diversity of packaging and other equipment surfaces such as plastic, rubber, glass, wood etc. and they can exist also in food products **[27].**

It is a known fact that biofilms are the predominant bacterial lifestyle in surfaces. However, most research with phage is being performed with their planktonic counterparts and not with biofilm communities. Different phages have been used to infect a variety of bacterial biofilms and in general, all these phage-biofilm interaction studies reveal that phages are capable of decreasing the bacterial populations. The treatment of biofilms using phages is a complex process and only strictly lytic phages should be used. Like in phage infection of planktonic cells, there are several essential steps that need to occur. The first and crucial step in phage infection is the adsorption of phages to the receptors of the target bacteria. The Exopolysaccharide (EPS) matrix, in which bacteria are embedded in, can constitute a problem for phages, as it needs to be penetrated so that phages can reach and adsorb to the specific receptors located on the target hosts' surface. However, it has been reported that phages are well capable of penetrating through the EPS matrix by diffusion or due to the presence of phage associated enzymes. These enzymes have the role of destroying the matrix so that the phages can get in contact with

lipopolysaccharides, outer membrane proteins or other receptors necessary for the start of the host infection. The activity of polysaccharide depolymerase enzymes has been reported in biofilms of *E. agglomerans* infected with phage SF153b and also hypothesis, based on the visible degradation observed, in *P. fluorescens* biofilms infected with phage φS1. Although the presence of polysaccharide depolymerase enzymes in phages has been reported, this characteristic is not commonly observed in most naturally isolated phages. The difficulty in isolating phages possessing EPS degrading enzymes has lead to the reconstruction of phages, such as the T7. The gene-engineered T7 phage was built specifically to express a biofilm-degrading enzyme once the phage starts to infect and reproduce daughter particles inside a host. This genetic manipulation of the phage resulted in a decrease, about two orders of magnitude superior, of the bacterial biofilms when compared to the non-engineered phage. Once the adsorption step has occurred, the phages start using the hosts' machinery to produce hundreds of new phage particles that will be released through burst of the host cell. These progeny phages can start a new cycle of host infection. Phages are capable to kill early stage biofilms (or adhered cells). Sillankorva *et al.* **[28]** reported that single cells adhered to glass surfaces of a parallel plate flow chamber 22 during 60 minutes and under laminar flow regime, were efficiently killed with phage φS1. Cell removal was fast (20 minutes) and efficient leading to a biomass reduction of approximately 90%. Furthermore they reported that surfaces exposed to phages where impossible to be re-colonized by the bacteria. Another strategy studied and proven to reduce biofilm formation by *Staphylococcus epidermidis* is the pre-treatment of catheter surfaces with phages. The proximity of host cells in biofilm communities can be an advantage in biofilm treatment using phages, as the released phages stay concentrated in close proximity and therefore can start infecting a neighboring cell much faster than in planktonic cultures where cells are not as accessible. Despite the ability phages have in reducing the host cells present in biofilms, there are several factors which can influence the lytic performance of phages (ex. a change in temperature, growth media, flow, the EPS matrix, among other parameters and lead to a decreased phage killing of their target hosts in biofilms. Also, the metabolic state of the hosts in biofilms poses a problem for phage treatment as exponentially growing cells are faster attacked than cells at the later growth phases. Failure of phage infection of biofilms can also be caused by other factors. Doolittle *et al.* **[29]** have reported that a *P. aeruginosa* phage was unable to reach the host in the deeper layers of a biofilm, suggesting that the phage could not penetrate through the biofilm matrix. Unsuccessful phage infection can furthermore be due to an inactivation of the phages caused by the presence of proteolytic enzymes in the biofilm matrix; however this is clearly a host dependent parameter which will reflect in different matrix components excreted. Overall, the biofilm-phage interaction studies have demonstrated that single species biofilms can be controlled using lytic phages. Although total eradication was not observed in most of the studies reported, in general all these experiments describe a significant biomass decrease and cell number reduction. Prolonged phage experiments can lead to the appearance of bacterial resistance. Tait *et al.* **[30]** reported that after an extended exposure of cells to phages, the bacteria and phage started co-existing in the biofilm communities. The effect of pretreating hydrogel-coated catheters with *Pseudomonas aeruginosa* bacteriophages (phage M4) on biofilm formation by *P. aeruginosa* in an *in vitro* model was studied **[31]**. They suggested the potential of applying phages, especially phage cocktails to the surfaces of indwelling medical devices for mitigating biofilm formation by clinically relevant bacteria.

8. BACTERIOPHAGES APPLICATION IN WATER TREATMENT

Bacteriophages are viruses that infect bacteria, and those that infect *E. coli* are called Coliphages. Somatic Coliphages are viruses that infect host cells through the outer cell membrane. The host bacterium and its density, temperature, pH, and other variables affect the incidence, survival, and behavior of phage in different water environments **[32].** The impacts of these variables affect the consistency of data and comparisons of bacteriophages in water environments. Coliphages have been proposed as virus surrogates for water disinfection and treatment studies. The theory behind the use of Coliphages as an indicator of water quality is based on the premise that these viruses will behave more like human enteric viruses than do bacterial indicators. In addition, they have also been proposed as sewage indicators because of their constant presence in feces, sewage, and polluted waters. Leclerc *et al.* **[33]** indicates that somatic coliphage may be found in conditions unrelated to presence of a health risk. Enteric viruses have been detected in treated drinking water that was negative for bacteriophages **[34].** In one recent study, 41.2% of pathogen positive samples occurred with no detectable levels of somatic coliphage, while 47.1% of pathogen positive samples contained more than 25 PFU/100ml, thus indicating no significant correlation between pathogens and somatic Coliphages **[35].**

Bacteriophages of *Bacteroides fragilis* are viruses that infect *B. fragilis* and two in particular may be useful as drinking water quality indicators. The phage to *B. fragilis* HSP40 are found only in human feces and have not been isolated from feces of animals **[36].** However, *B. fragilis* RY2056 phage are more numerous and are not human-specific.

Since bacteriophages are natural infectious for some of the functionally important bacteria in wastewater they enhanced bacteria removal from wastewater **[37].** The occurrence of phages according to host type is most natural treatment and keeps the environment clean by their influences over to kill the bacteria. In the present study, an attempt has been made in soba stabilization station to analyze the degree of degradation of bacteria by their phages **[38].** The incidence of phages in water samples generally indicates pollution by human or animal feces **[33].** A relationship with bacterial numbers and activity implies that the majority of aquatic viruses may be phages. It remains to be seen that phages also have the potential to optimize wastewater treatment processes. With a greater understanding of the microbial ecology of wastewater treatment systems, phage treatments may become effective solutions to wastewater treatment problems and optimisation **[39].** Thomas *et al*. **[40]** have already investigated that biocontrol in wastewater treatment by using phages is possible through phage induced bacterial lysis.

9. BACTERIOPHAGES AS FOOD ADDITIVES

The Federal Food, Drug, and Cosmetic Act defines a food additive as "any substance the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food". Class I Preservatives comprise a major class of food additives. Unlike the previous generation of antimicrobial preservatives, bacteriophages are viruses - living entities that are the natural predators of bacteria. However, because they are viruses, potential issues exist with their usage that requires careful evaluation. In addition, if history is any indication, consumer acceptance of bacteriophage usage may present something of a challenge to the food industry **[41]**. The FDA recently amended the food additive regulations to permit the safe use of a bacteriophage preparation as an anti-listerial agent in RTE meat and poultry products. The preparation as described consists of a combination of six individual lytic phages, selected for activity against different *L. monocytogenes* strains. Bacteriophages are bacterial viruses that are ubiquitous in the environment. For almost every bacterial species, there exists at least one bacteriophage that can specifically infect and ultimately destroy that particular bacterial group. They do not harm human or animal cells. Given these characteristics, bacteriophages have proven to be valuable allies in mankind's fight against disease and show great promise as alternatives to traditional antimicrobials in the control of foodborne pathogens.

10. CONCLUSIONS

Phages are ubiquitously available from different environments, are specific in action and unable to influence human cells. Bacteriophages target only the pathogens of interest and the normal gut microflora are not affected. Increasingly available scientific studies involving phage-derived technologies can play guaranteed role in bacterial detection, industrial processes, therapeutics, nanotechnology, and, undoubtedly, in other fields still to be imagined. Their application has already become as an interesting tool to fight antibiotic resistant bacteria **[42]**. Moreover, spontaneous occurrence of phage resistant mutants is not likely to significantly influence the phage treatment efficacy **[43]**.

Complete phage genome analysis is required to ensure safety and effectiveness of use of phages. Finally, food industry acceptance and consumer preference are critical hurdles to be overcome for their commercial application. As such bacteriophages are considered ideal antibacterial agents for use in foods to enhance the safety.

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