BIOPRESERVATION OF FOOD USING BACTERIOCINS, BACTERIOPHAGES AND ENDOLYSINS

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Abstract: The loss of faith of consumers in the chemical preservatives due to safety concerns associated with them and considering toxic effects in the human body in some cases, has led to their gradual withdrawal and replacement by biopreservatives. Also, food-borne outbreaks and increasing prevalence of antibiotic-resistant micro-organisms has led to a search for novel preservation techniques. This has prompted the quest for new natural antimicrobial compounds from different origins. Bacteriocins have been widely recognized as natural food biopreservatives but latest advances on bacteriocin biology have opened new fields to explore. On the contrary, the use of bacteriophages and endolysins in the food industry has only been considered in the last ten years and recent developments have produced promising perspectives. This report provides an overview of the current as well as foreseen applications of bacteriocins, bacteriophages and phage-encoded endolysins along the entire food chain and distinctly highlights their mode of action.

Keywords: Bacteriocins, bacteriophages, endolysins, mode of action, current and foreseen applications, mode of action.

Introduction

Today, people are extremely cautious and more aware of what they eat and drink. The concept of wellness and good health is spreading like a wildfire. There is a growing demand for minimally processed foods which can increase the risk of microbial contamination. Among the search for alternative technologies of preservation the preservation devoid of chemicals and their replacement by molecules and or microbes which are friendly and not toxic to our body's biosynthetic pathways, is being specially focussed upon.

Since 6000 BC a variety of foods have been produced as a result of fermentation like cheeses, breads, wines, beers and yoghurts.

Besides the synthesis of new products, fermentation also plays a very important role in the preservation through the generation of an acidic environment and a wide variety of antagonistic primary and secondary metabolites including organic acids, diacetyl, CO2 and even antibiotics.

Biopreservation of perishable vegetables is a native skill of Northeast Indian women. Lactic acid fermentation is the actual mechanism involve in the biopreservation process of perishable vegetable and bamboo shoots. Some ethnic fermented vegetables of Northeast India are gundruk, sinki, goyang, inziangsang, khalpi, anishi, etc. and ethnic fermented bamboo shoot products are mesu,

soidon, soibum, soijim, ekung, eup, hiring, and lungsie (Buddhiman & Jyoti Prakash Tamang, 2009)

However, in 1928 AD a new peptide which was shown to have antimicrobial activity against food pathogens like *Listeria monocytogenes* and *Clostridium botulinum* was discovered. Since then it has found its way in many dairy products as an effective antimicrobial agent.

The successful incorporation of nisin as a preservative has given rise to increase in research for newer bacteriocins which could possibly have a broader antimicrobial spectrum, better potency and stability in food systems.

Over the last ten years, the bacteriophages which were previously always considered to be a threat to the food industry notoriously known for their ability to inhibit the lactic acid starter cultures, hence not allowing the final product to have the desired quality; are now being relooked upon as a boon due to their host specific potential to destroy bacteria and hence serve as an preservative in food systems. However, in Europe it remains uncertain whether phages can be considered as processing aids or as decontaminants/ additives (Teufer & Von Jagow, 2007). In addition, the need to select a virulent phage to avoid transduction, the threshold requirements of the host and the potential development of bacterial strains resistant to phages and the main obstacles when considering phage as an anti-microbial for the food industry.

The endolysins produced by these phages however lack these disadvantages and therefore represent a promising alternative for controlling food borne pathogens.

1. Bacteriocins

The bacteriocins were first characterized in gram-negative bacteria. The colicins of *E.coli* are the most studied (Lazdunski, 1988). The colicins constitute a diverse group of antibacterial proteins, which kill closely related bacteria by various mechanisms such as inhibiting cell wall synthesis, permeabilizing the target cell membrane, or by inhibiting RNase or DNase activity. Among the gram-positive bacteria, the lactic acid bacteria have been comprehensively exploited as a reservoir for antimicrobial peptides with food applications.

They are ribosomally synthesized and inhibit closely related bacteria (Klaenhammer, 1993). Since bacteriocins are isolated from foods such as meat and dairy products, which normally contain lactic acid bacteria, they have unknowingly been consumed for centuries. Nisin is approved for use in over 40 countries and has been in use as a food preservative for over 50 years. Though nisin is currently the only bacteriocin approved for use in the United States, many bacteriocins produced by members of the LAB have potential application in food products.

1.1. Alternatives to Nisin

1.1.1. Lacticin 3147

Producing strain isolated from a Kefir grain (employed to produce a fermented product). Like nisin, the producer is a *Lactococcus lactis* (DPC3147 strain) Lacticin 3147 is a two peptide lantibiotic. This is particularly of interest due

to its broad spectrum of inhibition and better potency compare to Nisin (Table 1.2). The genes are encoded on a large plasmid which can be mobilised.

Strain	Nisin (U/ml (nM))	Lacticin 3147 (U/ml (nM))	Increased Activity (U/ml)
Listeria monocytogenes			
F 5817	12.57 (3750)	1.9 (312.5)	13
33413	6.28 (1875)	1.9 (312.5)	3
Bacillus cereus			
B.Cereus APC58	16.7 (5000)	3.8 (625)	4
Enterococci			
E. Facecium 5119	16.7 (5000)	1.9 (312.5)	8
Staphylococcus aureus			
S. Aureus 5971	4.1 (1250)	30.9 (5000)	0.13
S. Aureus Farm 1	4.1 (1250)	1.9 (312.5)	2

Table 1.1 Comparison of potency of Lacticin 3147 with Nisin
Source: Clare Piper et al. 2011, *Microbial Biotechnology*, 4 (3), 375–382

The Food Grade introduction of the bacteriocin genes into cheese starters was carried out. Lacticin 3147 producing starters have been used to control the pathogen *Listeria monocytogenes* on the surface of mould ripened cheese. Lacticin 3147 producing starters have been used to control the non-starter lactic acid bacteria complement in Cheddar cheese during the ripening process. A novel starter system using a bacteriocin (lactococcin)- producing adjunct has been designed which gives increased cell lysis during Cheddar cheese manufacture while ensuring that efficient acid production is not compromised.

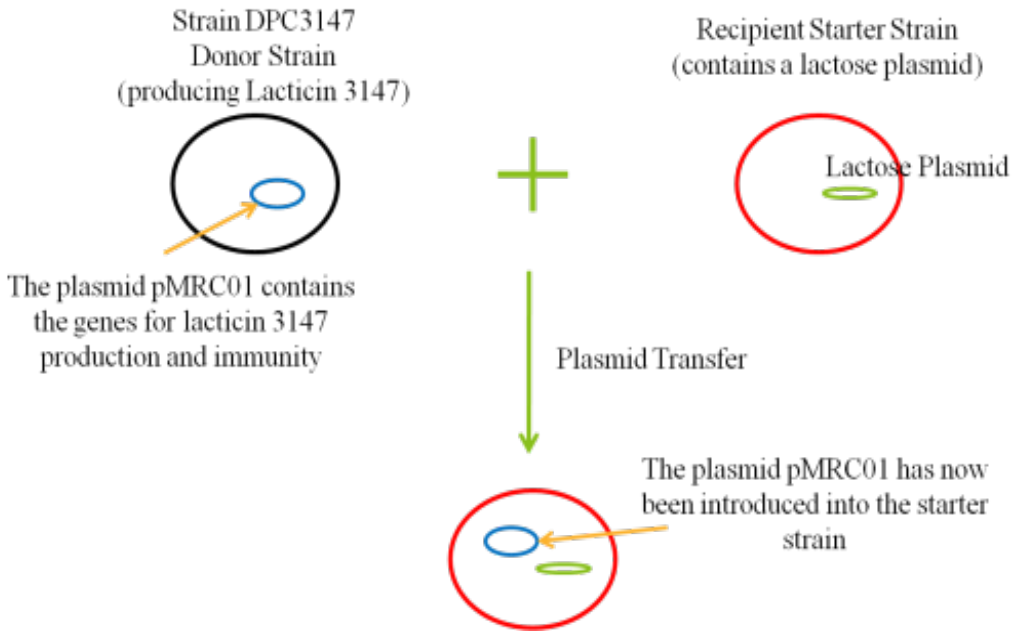
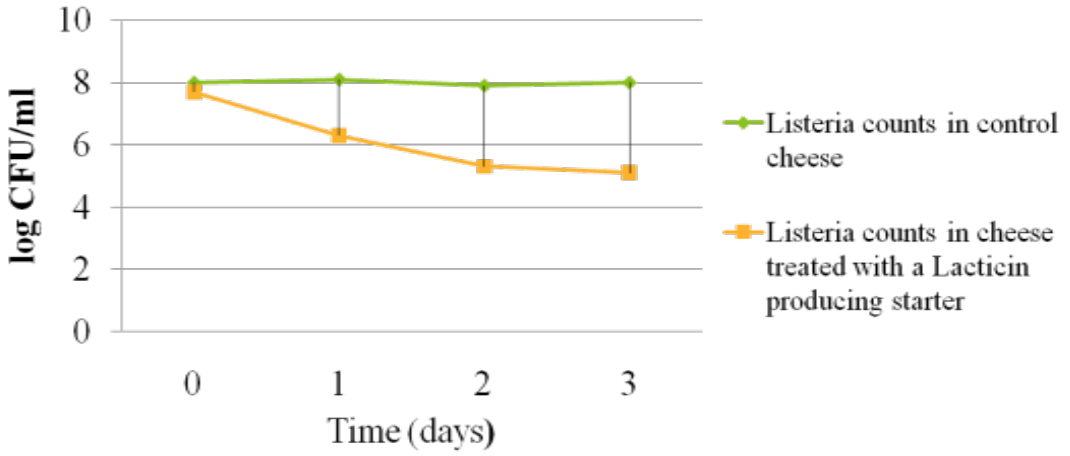


Figure 1.1 Development of a starter culture incorporating Lacticin 3147

Source: M.P. Ryan et al, *1996*, Proceedings of the fifth symposium of lactic acid bacteria: genetics, metabolism and applications, FEMS Publication

In Cheddar cheese it is desirable to have control over flavour- development. Since non-starter lactic acid bacteria contribute to off flavour defects, control of their growth is desirable. Gives the manufacturer control of flavour development in cheese, and prevents economic losses which may occur if off flavours develop. The presence of the bacteriocin is correlated with a reduction in non-starter lactic acid bacteria numbers. Besides, certain mould ripened cheeses may be at risk of contamination with pathogenic bacteria such as Listeria. During ripening the pH at the surface of these cheeses can exceed pH 7, which provides a suitable environment for the proliferation of many undesirable bacteria such as *Listeria*.



Graph 1.1 Potential of Lacticin 3147 for cheese safety

Source: M.P. Ryan et al, *1996*, Proceedings of the fifth symposium of lactic acid bacteria: genetics, metabolism and applications, FEMS Publication

1.1.2. Food grade Nisin derivatives:

An alternative route to address the deficiencies of Nisin is the application of bioengineered derivatives of the peptide which, despite differing only subtly, possess enhanced capabilities of commercial value.

1.2. Applications in Food Industry

Table 1.2 Examples of patented food applications of bacteriocins

Author	US Patent	Patent Title	Use
Vandenbergh et al.	5,817,362 (10.06.98)	Method for inhibiting bacteria using a novel lactococcal bacteriocin	A method for inhibiting Gram-positive bacteria in foods by using a novel bacteriocin produced by Lac. lactis NRRL-B-18535
Vedamuthu	5,445,835 (08.29.95)	Method of producing a yoghurt product containing bacteriocin PA-1	A yogurt product with increased shelf life containing a bacteriocin derived from a *P. acidilactici*
Boudreaux et al.	5,219,603 (06.15.93)	Composition for extending the shelf life of processed meats	Use of a bacteriocin from P. acidilactici and a propionate salt to inhibit bacterial growth and to extend shelf life of raw and processed meat
Collison et al.	5,015,487 (05.14.91)	Use of lanthionines for control of post-processing contamination in processed meat	Inhibiting the contamination of processed meat products by pathogenic or spoilage microorganisms by treating the surface of the meat product with a lantibiotic

1.3. Commercial Status

Subtilin, Cerein, Thuricin, Plantaricin have been isolated and characterized from different bacteriocin producing strains. But they are yet to acquire a commercial status. Nisin (*Lactobacillus lactis*) and Pediocin (*Pediococcus acidilactici*) are the only commercially produced bacteriocins. Lenticin is a novel strain from *Bacillus lentus.*

2. Bacteriophages

Bacteriophages or phages are the most abundant microorganisms on Earth (10^{31} particles) and widely spread including foods of various origins (Brüssow and Kutter, 2005). They are obligate

intracellular parasites that infect bacteria and reproduce by hijacking their host's biosynthetic pathways. They are viruses that specifically infect and multiply in bacteria. Thus, they are harmless to humans, animals, and plants.

2.1. Classification

Phages are classified as either lytic or lysogenic based upon their replication strategy. A virulent phage infects its bacterial host, replicates its DNA, and produces progeny that are immediately released for further infection, destroying its host in the process.

A temperate phage, on the other hand, follows the lysogenic cycle wherein it embeds itself into the genome of its bacterial host, establishing a stable relationship with the bacteria that it has infected. This stable relationship is maintained until some stressor, such as DNA damage, disrupts it. After this, the phage moves into the lytic cycle. These phages are capable of transferring genes for toxin production or pathogenicity factors between bacterial populations.

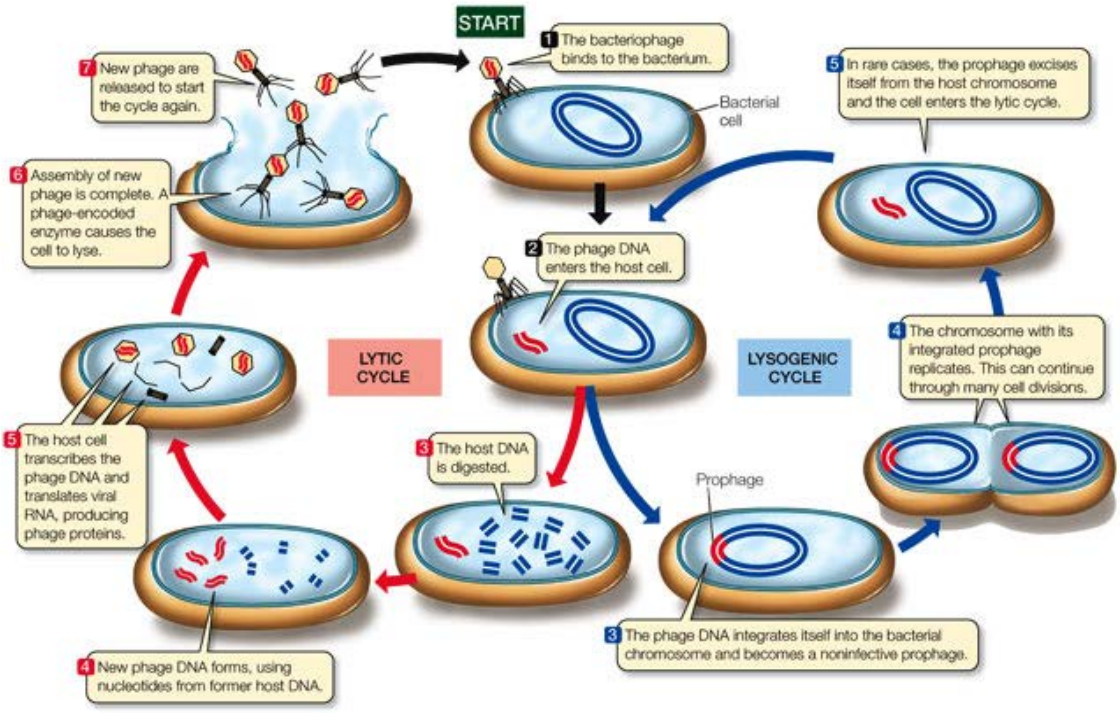


Figure 1.2 Phage life-cycle leading to cell lysis serves as the mechanism for bio-preservation

2.2. Applications in Food Industry

2.2.1. *Escherichia Coli* Bacteriophages

By their nature, ready-to-eat (RTE) foods pose additional safety risks as they receive no further processing prior to consumption (e.g., washing, cooking, etc.). Any bacteria

present in the RTE material will remain viable into the gastrointestinal tract. This added risk was demonstrated recently by the severe illness and death resulting from the consumption of packaged spinach contaminated by *E. coli* O157:H7.15 *E. coli* O157:H7 is a highly virulent food borne pathogen naturally found in the gastrointestinal tract of ruminants and other mammals (e.g., cattle, sheep, pigs, etc.). (FDA Statement, on Foodborne E.Coli O157:H7 Outbreak in Spinach, 2006) As a result, these bacteria are difficult to eradicate and frequently enter the human food chain via contact with contaminated faecal material of animals. Bacteriophage therapy is a likely strategy for reducing the presence of these dangerous microbes. EcoShield^{TM} manufactured by Intralytix is a unique and proprietary blend of three individual phages that provides broad protection specifically against pathogenic strains of *Escherichia coli* O157:H7.

2.2.2 *Listeria monocytogenes* Bacteriophages

The ubiquitous presence of this bacterium in food materials and factory environments, coupled with its ability to grow at low temperatures, render it uniquely capable of infecting refrigerated RTE foods.

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. (Kathy Walker, 2006, Food Regulation in the United States). The preparation as described consists of a combination of six individual lytic phages, selected for activity against different *L. monocytogenes* strains. It is an aqueous, phage preparation wit 0.1 ppm concentration. This cocktail is to be sprayed directly on the surface of the RTE food prior to packaging at a level of approximately 1 milliliter (mL) per 500 square centimetres of surface area. The bacteriophages will remain dormant unless their specific target, *L. Monocytogenes*, is encountered, triggering a full infection and destruction cycle. Because they are lytic phages, no viable *Listeria* will remain, and there will be no transfer of problematic genes associated with lysogenic phages. Clearly with this amendment, the FDA has demonstrated their belief that bacteriophages are safe for use in the human food chain as antimicrobial food additives.

2.3. Precautionary Steps

Although the FDA has now approved the use of a bacteriophage preparation as a food additive, several factors related to the general use of phage deserve

consideration. Great care should be taken in determining which bacteriophages are selected for use, how they are to be manufactured, and the manner in which they are used.

2.3.1. Industrial Preparation

The specific nature of bacteriophage infection requires the presence of the pathogenic host bacteria if industrial quantities of the phage are to be produced. The initial presence of such a pathogen necessitates the development of adequate separation and/or sterilization technologies to ensure the complete absence of the pathogen in the final bacteriophage preparation.

2.4.2. Selection of Bacteriophage

Only lytic phages should be used as food additives. Lysogenic bacteriophages have the potential to carry genes to their host bacteria that are associated with toxin production and pathogenicity. One example of this would be the transfer of shiga toxin from enterohemorrhagic E. coli to non-pathogenic E. coli by bacteriophages.

2.4.3. Immunity

Bacteria are known to develop resistance to bacteriophage over time, reducing the effectiveness of their antimicrobial properties. This requires constant vigilance and substitution of new phages for "old" phages to which the bacteria have become immune. This cycle is well known in the dairy foods industry.

3. Endolysins

3.1. Structure and mode of action

Bacteriophages have developed two basic ways to release the new virions from the infected bacterial cells. In filamentous bacteriophages the progeny is continuously extruded from bacteria cells without killing, whereas non-filamentous bacteriophages destroy the cell wall of the host bacterium by phage-encoded lytic enzymes. Small RNA and DNA phages encode specific proteins that interfere with host enzymes responsible for peptidoglycan biosynthesis. In large DNA phages, endolysins (also termed lysins) are produced during the late phase of gene expression in the lytic cycle and are responsible of the enzymatic cleavage of peptidoglycan (Young, Wang & Roof, 2000; Loessner, 2005). Endolysins are also capable of degrading the peptidoglycan of Gram positive bacteria when applied externally to the bacterial cell, thereby acting as antibacterial agents.

3.2. Challenges faced by the industry

3.2.1. Costs

The production costs of endolysins are expected to be high with the current technology, which may constitute the most significant barrier to their application as an alternative to phages or antibiotics. However, in the food industry, a cost-efficient production of enzymes should not

be considered an insurmountable obstacle, since a wide variety of enzymes are commercially available and produced in kg to ton quantities for application as enzyme supplements (baking, brewing, cheese flavoring, etc.). Technological developments for more efficient expression systems would make the option to use endolysins as food control agents a financially appealing one.

3.2.2. Expanding host range

Endolysins only cleave PGN linkages that are exclusively present in bacteria; however by displaying dissimilar lytic spectra they can be exploited differently. When a wide range of bacteria has to be controlled, endolysins with a broad host range will be required. For instance, in agriculture, endolysins targeting different bacterial taxa could play an important role as biopesticides, preventing tomato scabs, wilts and spots caused by *Streptomyces scabies,* *Clavibacter michiganensis* and *Xanthomonas campestris*, respectively. In milk processing plants, endolysins could act as food sanitizers targeting thermoduric bacteria (e.g. Bacillus spp. and Paenibacillus spp.) and psychrotolerant Pseudomonas spp., preventing spoilage of pasteurized milk and extending the shelf-life of food products.

3.2.3. Enhance activity in food systems

Potential problems in food processing units associated with bacterial biofilms e the formation of an aggregate of microorganisms as a result of bacterial surface adherence can be overcome by the use of endolysins.

3.2.4. Avoidance and resistance

Repetitive exposure of bacteria grown on agar plates to low concentrations of lysin did not lead to the recovery of resistant strains; neither did bacterial resistance occur after several cycles of exposure to low concentrations of enzyme in liquid conditions (Loeffler et al., 2001). It has been postulated that the lack of bacterial resistance toward endolysins is due to their unique mode of action. To avoid being trapped inside the host, phages have evolved to produce enzymes such as endolysins, targeting essential molecules that cannot be altered by bacteria. As a result, bacterial resistance is rare (Fischetti, 2004, Loeffler et al., 2001).

Table 1.3 Challenges faced for incorporating endolysins in food

Challenge	Action
Lack of undesirable traits	▪ Better knowledge of gene flow phage-host ▪ Blocking gene dissemination systems
Large-scale safer production systems	▪ Use of non-virulent hosts
Enhance activity in food systems	▪ Modelling phage behaviour ▪ Case-by-case study ▪ Same environnent as phage source
Expanding host range	▪ Use of phage mixtures
New phage-derived Antimicrobials	▪ Endolysins ▪ Peptidoglycan hydrolases - Inhibitors of host metabolism

Conclusion

Bacteriocins, bacteriophages and endolysins are all specific to bacteria only; however studies from some strains of Lactococcus species have shown to inhibit spoilage fungi as well. Using phages to target pathogenic "bad" bacteria in our foods without affecting the beneficial bacteria is a breakthrough in the food safety area. By conducting genomic studies on host-phage interactions and by genetic engineering the challenge of incorporating virulent phages in food systems needs to be done. Potential of endolysins needs to be exploited as they are unlikely to develop resistance and hence unlike bacteriophages will require no replacement.

Table 1.4 A Consolidation of the different bio-preservatives

	BACTERIOCIN	**BACTERIOPHAGE**	**ENDOLYSIN**
Definition	Ribosomally synthesized peptides	Virus specific to bacteria	Cleavage enzymes isolated from phages
Killing Spectrum	**Traditionally Narrow** Closely related to producing organisms	**Very Specific** Every Bacteriophage has a specific bacteria	Generally **Broad** Cleave PGN linkages in bacteria only

Antimicrobial Efficiency	1-2 hours	Virulent multiplication	Nanogram quantities eliminate bacteria in a few seconds
Production Cost	**Moderate**	**Cheap**	**Very high** With current technology
Resistance	**No development**	**Development** New Phages to replace old phages	**No development**
Consumer's Acceptance	Already commercialized and widely accepted	Increasing acceptance but GMO phages are not welcome	Use of GMOs as cell factories for endolysin production

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References:

1. Buddhiman & Jyoti Prakash Tamang, January *2009* "Traditional knowledge of biopreservation of perishable vegetable and bamboo shoots in Northeast India as food resources", Indian Journal of Traditional Knowledge, Volume 8, 89 - 95
2. Clare Piper, Colin Hill, Paul D. Cotter, R. Paul Ross' *2011* "Bioengineering of a Nisin A-producing *Lactococcus lactis* to create isogenic strains producing the natural variants Nisin F, Q and Z", *Microbial Biotechnology*, Volume 4, Issue 3, 375–382
3. Eduardo Marcos Balcuiunas, Fabio Andres, Castillo Martinez, *2013*, "Novel Biotechnological Applications of bacteriocins", *Food Control ,* 32, 134-142
4. Hui Zhang, Hongduo Bao, Craig Billington, J. Andrew Hudson, Ran Wang, 2012, Isolation and lytic activity of the *Listeria* bacteriophage endolysin LysZ5 against *Listeria monocytogenes* in soya milk, *Food Microbiology*, 31(1) ,133–136
5. Hugo Oliveira, Joana Azeredo, Rob Lavigne and Leon. D. Kluskens, *2012* "Bacteriophage endolysins as a response to emerging foodborne pathogens",

Trends in Food Science and Technology, (28), 103-115

6. JP Higgins, SE Higgins, KL Guenther, W Huff, AM Donoghue, DJ Donoghue and BM Hargis, 2005, Use of a specific bacteriophage treatment to reduce Salmonella in poultry products, *Poultry Science*, 84 (7), 1141-1145
7. Kathy Walker, "Use of Bacteriophages as Novel Food Additives" , October 29, 2006 Food Regulation in the United States, Michigan State University
8. M.A. Franchi, G.E. Serra, M.Christianini, 2003 "Use of Biopreservation in the control of bacterial contaminants o sugarcane alcohol fermentation", *Journal of Food Science*, 68 (7), 2310-2315
9. Nivedita Sharma, Ambika Attri, Neha Gautam and R.K. Gupta, "Role of Lenticin – A Bacteriocin as Biopreservative in different Food Products to enhance their safety", *Beverage and Food World, October 2012*
10. O'Flynn, R. P. Ross, G. F. Fitzgerald and A. Coffey, 2004, Evaluation of a Cocktail of Three Bacteriophages for Biocontrol of *Escherichia coli* O157:H7, *Applied and Environmental Microbiology*, 70 (6), 3417-3424
11. Patcharin Siringan, Phillippa L. Connerton, Robert J. H. Payne, and Ian F. Connerton, 2011, Bacteriophage-Mediated Dispersal of *Campylobacter jejuni* Biofilms, *Applied and Environmental Microbiology*, 77(10), 3320–3326
12. Pilar Garcia, Lorena Rodriguez, Ana Rodriguez and Beatriz Martinez, 2010, "Food Biopreservation: Promising strategies using bacteriocins, bacteriophages and endolysins", *Trends in food Science and technology,* 21, 373 – 382
13. P. Garcia, B. Martinez, J.M. Obeso and A. Rodriguez, "Bacteriophages and their application in food safety", *Letters in Applied Microbiology*
14. Piper C, Draper LA, Cotter PD, Ross RP, Hill.C, 2009, "A comparison of the activities of lacticin 3147 and nisin against drug-resistant Staphylococcus aureus and Enterococcus species", *Journal of Antimicrobial Chemotherapy*, 64, 546–551.
15. Raul R. Raya, Peter Varey, Rebecca A. Oot, Michael R. Dyen, Todd R. Callaway, Tom S. Edrington, Elizabeth M. Kutter and Andrew D. Brabban, 2006, "Isolation and characterization of a new T-even bacteriophage, CEV1, and determination of its potential to reduce Escherichia coli O157: H7 levels in sheep", *Applied and Environmental Microbiology*, Vol. 72 (9), 6405 – 6410
16. R.Modi, Y. Hirvi, A.Hill, M.Griffiths, 2001, Effect of Phage on Survival of *Salmonella* Enteritidis during Manufacture and Storage of Cheddar Cheese Made from Raw and Pasteurized Milk, *Journal of Food Protection*, Volume 64(7), 927-933
17. Ryan, M.P., Rea, M.C., Hill, C. and Ross, R.P. *1996*, "An application in Cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, Lacticin 3147", Applied Environmental Microbiology, 62, 612.
18. S. Ananou, M. Maqueda, M. Martínez-Bueno1 and E. Valdivia, "Biopreservation,

an ecological approach to improve the safetyand shelf-life of foods", *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*

19. T.Jacobsen, B.B.Budde, A.G.Koch, 2003, "Application of Leuconostoc carnosum for biopreservation of cooked meat products", *Journal of Applied Microbiology*, 95, 242 – 249
20. U.Schillinger, R.Geisen and W.H.Holzapfel, May 1996, "Potential of Antagonistic microorganisms and bacteriocins for the biological preservation of foods", *Trends in Food Science and Technology,* Vol.7
21. Vandana Bali, Parmjit S. Panesar, Manab B.Bera "Biopreservation: An Emerging Technigue in Food Processing Industry", *Beverage and Food World*, October 2012
22. Sofia Fernandes, Daniela Proenc¸, Catia Cantante, Filipa Antunes Silva, Clara Leandro, Sara Lourenc¸, Catarina Milheiric, Herminia de Lencastre, Patricia Cavaco-Silva, Madalena Pimentel, 2012, Novel Chimerical Endolysins with Broad Antimicrobial Activity Against Methicillin-Resistant Staphylococcus aureus, *Microbial Drug Resistance,*18(3)