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Use of Products Containing a Phage in Food Industry as a New Method for *Listeria monocytogenes* Elimination from Food (*Listeria monocytogenes* Phages in Food Industry) – a Review

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Abstract

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Increased detection records of *Listeria monocytogenes* in foods have been recently observed. Standard methods of *L. monocytogenes* elimination from food products including pasteurisation or acidification may, however, be unreliable. The elimination of *L. monocytogenes* using specific bacteriophages which are natural antagonists of these pathogenic bacteria has become an innovative method that does not affect the taste, smell or appearance of the product. The use of phage preparations in food production is becoming an increasingly popular method for the effective and safe elimination of *L. monocytogenes* from food products.

Keywords: *Listeria monocytogenes* bacteriophages; food safety

Issues concerning the occurrence of *Listeria monocytogenes* in food

According to data of 2007–2009 concerning the percentage rates of pathogenic microorganism occurrence in food, *L. monocytogenes* is the second microbe after *Salmonella* spp., reported in the Rapid Alert System for Food and Feed. The proportion of *L. monocytogenes* cases in a group of food-borne pathogens increased from 10% in 2007 to 20% in 2009 (Ścieżyńska *et al.* 2014). In 2012, 1601 cases of listeriosis were reported in humans, which was less by 3.2% compared to 2009, when the number of listeriosis cases reached 1654. In Poland, the incidence was observed to increase from 32 cases in 2009 to 59 cases in 2010 (Zadernowska *et al.* 2012).

These data indicate the significance of the problem of food contamination with *L. monocytogenes*. Infections with these pathogens cause a high mortality rate that varies between 20 and 30% of deaths among patients with listeriosis (Farber & Losos 1988). The approximate infective dose of *L. monocytogenes* is estimated at 10 to 100 million colony forming units (CFU) in healthy hosts, and only 0.1 to 10 million CFU in individuals at high risk of infection (Farber *et al.* 1996). The acceptable limits for the colony forming units per gram of a food product according to EU regulations shall not exceed 100 in products unfavourable for *Listeria* development in the entire shelf life. In the case of products facilitating *Listeria* development and in infant formulas, they may be released onto the market only if *L. monocytogenes* is undetectable in 25 g of the sample (Norrung 2000; Sip & Gardo 2011a; Zadernowska *et al.* 2012). In turn, the Food and Drug Administration (FDA) in the USA applies a "zero tolerance" policy regarding *L. monocytogenes*, as it does not allow any level of *L. monocytogenes* cells in food products to be released onto the market.

L. monocytogenes is the common saprophytic microflora of foodstuffs (Freitag *et al.* 2009). In some cases, *L. monocytogenes* can be part of the so-called

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"environmental microflora" of a particular raw material, hence the control of its presence and elimination from food are even more important (Sip & Gardo 2011a). This is particularly true of "ready-to-eat" products, e.g. sausages, salads, juices, and raw products, including fish, raw beef, and vegetables (mainly roots) (Sokolowska & Przestrzelska 2003).

Selected methods for *L. monocytogenes* elimination from food products

Currently, certain physical, chemical, biological or combined methods are applied to eliminate or reduce the counts of *L. monocytogenes* in food (Sip & Gardo 2011b). Describing the effective elimination of *L. monocytogenes* from food, one should consider problematic issues related to thermal processing, surface treatment of food products with chemicals and/or ultraviolet radiation, environment acidification, treatments with chlorine compounds and bacteriocins (Sip *et al.* 2009).

In the case of *L. monocytogenes,* thermal processing may turn out ineffective. In theory, heating milk that contains this bacterium at the temperature of 69°C for 16 s should be sufficient (Pearson & Marth 1990). However, some *L. monocytogenes* strains have been reported resistant to even higher temperatures. Moreover, heating milk in a higher temperature range does not ensure the complete eradication of *L. monocytogenes*, due to a possibility of secondary contamination (Łobacz *et al.* 2008). In addition, noteworthy is a growing interest in dairy products made of unpasteurised milk (like cheeses) owing to their higher organoleptic qualities.

L. monocytogenes is a halophilic bacterium (it can survive in a sodium chloride concentration of up to 30%), therefore the addition of sodium chloride as a preservative does not ensure the product protection against bacteria of the genus *Listeria* (Hudson 1992; McLauchlin *et al.* 2004). Other preservatives, which in theory could be successfully applied due to their high efficiency, such as chlorine dioxide or oxidative water (reduction of the *L. monocytogenes* count by 3 and 5 orders of magnitude), are not recommended due to the risk of carcinogenic chlorine formation (Dzwolak 2008). An alternative solution is to use preparations containing lysozyme, but in this case there is no such specificity of action against selected groups of bacteria like in preparations containing the bacteriophages (which will be described later in this article).

L. monocytogenes cells are highly resistant to ultraviolet radiation (Rowan *et al.* 1999). Cells that form biofilms on the production surfaces exhibit even greater resistance. The number of cells not forming a biofilm can be reduced by 4 orders of magnitude as a result of UV-C 275 nm radiation of 750 μW/min/cm^2, whereas a reduction by 5 orders of magnitude has been reported as a result of the radiation beam of UV-C 254 nm at 100 μW/min/cm^2. However, these results cannot be applied to biofilm-forming bacterial cells (Yousef & Marth 1988).

Reducing the counts of *L. monocytogenes* by acidification of the environment may cause a decrease in the number of colony forming units up to three logarithmic cycles (Greer & Dilts 1995). It is worth mentioning that the use of organic acids (more potent bacteriostatics than inorganic acids) may promote the occurrence of strains exhibiting greater resistance to acidification (Lou & Yousef 1997). The acidification of food products does not protect them against further development of *L. monocytogenes* in refrigerated conditions. The use of lactic acid bacteria (LAB) seems a better solution (Sip *et al.* 2009) as they are antagonists of *L. monocytogenes* in competition for nutrients and modification of their environment. The strongest antagonists of *L. monocytogenes* include bacteria that produce Class IIa bacteriocins of the genera *Lactobacillus*, *Pedicoccus*, *Enterococcus*, and *Carnobacterium* (Ennahar *et al.* 2000; Drider *et al.* 2006; Sip *et al.* 2009). The use of the antagonistic strains can inhibit the growth of *L. monocytogenes*. The efficiency of bacteriocin production by antagonistic strains depends on various factors. Although formulations containing bacteriocin are often used in the industry, they involve a risk of bacteriocin reaction with food components or inactivation due to the action of proteolytic enzymes present in food (Sip & Gardo 2011a, b).

Bacteriophages and their application in the agriculture and food industry

The above-described problems related to the use of physical, physico-chemical, and microbiological methods have generated a great interest in the use of alternative techniques. The use of bacteriophages – viruses capable of infecting bacterial cells, to eliminate *L. monocytogenes* from food is becoming increasingly common, which is observed in the activity of companies that produce preparations containing *L. monocytogenes* phages such as Intralytix and Micreos.

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Characteristics of bacteriophages

Bacteriophages belong among the most common viruses in the natural environment. Bacteriophages that are used to eliminate certain bacterial strains need to be virulent bacteriophages. A virulent type of phage means that phages proliferate after entering the bacterial cells, which leads to the death of the host and release of the progeny phages. This development cycle is called the lytic cycle. In contrast, temperate bacteriophages, which are only a small fraction of phages, go through the lysogenic cycle in the bacterial cell and remain there as prophages. A prophage is an inactive form of the bacteriophage integrated into the genetic material of bacteria or remaining as a plasmid in the cell. Thus, the lysogenic cycle does not lead to host's death. Temperate bacteriophages may participate in the horizontal transfer of genetic material between bacterial cells by transduction, which may result in the acquisition of new genes by infected cells (Alisky *et al.* 1998; Kutter & Sulakvelidze 2004).

Phages can infect only a limited number of strains, but *L. monocytogenes* phages in this manuscript are exceptions from this rule. This ensures specificity, meaning that only certain bacteria are eliminated from a particular raw material/product and desirable strains remain unaffected. Phage proteins involved in the adhesion to host cells are responsible for this specificity. These proteins form long structures called tail fibres. Adhesion of bacteriophages to the cell walls of specific bacterial cells is related to the structure of the cell wall. Therefore, the acquisition of resistance to phages by bacteria would involve changes in the cell wall structure, which could be lethal for the bacterial cell (Kutter & Sulakvelidze 2004). One of the reported cases of acquisition of resistance to bacteriophages was associated with a simultaneous decrease in the virulence of the strain which has developed a defence mechanism. The specificity of phage action involves no risk to the natural microflora of the gastrointestinal tract and cultures of bacterial strains naturally inhabiting the area of bacteriophage activity (Carlton 1999; Hunter 2012).

Historical and current significance of bacteriophages in the control of bacterial infections

Bacteriophages were discovered and described already in 1915 by Frederick Twort as "microorganisms" having the ability to kill bacterial cells. The first successes associated with the use of bacteriophages to treat bacterial infections date back to 1919. Isolated bacteriophages were applied by Félix d'Hérelle to treat a patient with dysentery. In the 1930s, phage preparations were produced on a massive scale, but the discovery of antibiotics led to abandonment of further research on bacteriophages. Antibiotics were cheaper, easier to produce, and initially very effective against bacterial infections (Adams 1959; Brock 1961).

The interest in bacteriophages has begun to grow along with an increasing number of bacterial strains exhibiting resistance to a broad spectrum of antibiotics, including methicillin-resistant strains of *Staphylococcus aureus*. Mechanisms of resistance to antibiotics generally rely on enzymatic inactivation processes, mechanisms that limit the access of antibiotics to intracellular structures and the formation of "alternative" substrates for antibiotics. There is no data available showing that the mechanisms of bacterial resistance to bacteriophages such as RM, superinfection exclusion, CRISP/Cas and abortive infection are correlated with mechanisms of resistance to antibiotics. Acquisition of antibiotic resistance by the strain does not preclude the lack of sensitivity to bacteriophages (Carlton 1999). Currently, investigations on the use of bacteriophages in the treatment of antibiotic-resistant bacterial infections are conducted, *inter alia*, by the Eliava Institute in Tbilisi at the Institute of Microbiology and Virology in Georgia, and the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of Polish Academy of Sciences in Wroclaw (Houbsy & Mann 2009; Sulakvelidze 2011; Brussow 2012).

In recent years, bacteriophages have also found applications in the food industry as a new "means/method" to ensure food safety. Phage preparations are already used for the preservation of foods with a short shelf life, decontamination of raw fish, poultry, fruits and vegetables. Listex P100 marketed by EIB Food Safety and LMP102 ListShield by Intralytix are widely used in dairy industry, fish industry and in the production of ready-to-eat meals. Listex P100 and ListShield are relatively easily available commercial formulations. The use of phage formulations is not limited only to *L. monocytogenes*. There are also formulations like EcoShield, containing phages specific to *Escherichia coli*, and SalmoShield, with phages specific to *Salmonella* spp. (Carlton *et al.* 2005; Bren 2007).

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ListShield (Intralytix, Inc., Baltimore, USA) is a mixture (cocktail) of six phages that provide protection against *L. monocytogenes*. This product could be highly efficient. The efficacy is highly dependent on the multiplicity of infection. The manufacturer claims that the level of contamination with *L. monocytogenes* may be reduced by 99% to as much as 100% compared to the initial number of colony forming units. ListShield was approved by the U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA) for use directly on food. It was also approved by the EPA for direct use on processing surfaces in food factories and other establishments (Intralytix ListShield).

Listex™ P100 is a preparation containing bacteriophage P100, which is a virulent bacteriophage against *L. monocytogenes* bacteria. This preparation has the Generally Recognized as Safe (GRAS) status. Its use is recommended primarily for soft cheeses, fish and meat products. So far no cases have been reported of *Listeria* strain bacteria resistance to phage P100 (Micreos Listex™ P100).

Characteristics of *Listeria monocytogenes* bacteriophages

Among the available phage genomes of *L. monocytogenes* described in the databases of the National Centre for Biotechnology Information (NCBI), USA, bacteriophages A511, P100, and A118 are well characterised (Dorscht *et al.* 2009). Bacteriophage A511 has been isolated from a sewage sample, it is a temperate bacteriophage capable of infecting up to 95% of *L. monocytogenes* strains. A511 genome consists of 137 619 bp, and this bacteriophage has a retractile inelastic tail. Bioinformatic analyses showed that it was related to the phage P100 (Loessner & Scherer 1995).

The bacteriophage P100 genome consists of 131 384 bp and contains 174 open reading frames (ORFs). No similarities have been found in the genome to sequences potentially encoding toxins, virulence factors, and allergens.

Another known bacteriophage of *L. monocytogenes* A118, contains the 40 834 bp genome that consists of 72 ORFs. Twenty-six of them have known functions that include encoding proteins associated in the packaging of genetic material. They also participate in the lysis of bacterial cells (Bren 2007).

The use of bacteriophages for the elimination of *Listeria monocytogenes* from food products

Effectiveness of phage preparations is correlated with the type of food in which we want to reduce or completely eradicate *L. monocytogenes*. It has been proved that the count of *L. monocytogenes* cells in liquid foods (milk, mozzarella brine), treated with phage preparations, can be reduced below the detection threshold. In solid foods (i.e. seafood, poultry, sausages, cabbage), the count of *L. monocytogenes* cells can be reduced by 5 orders of magnitude (Carlton *et al.* 2005).

The titre of the phage in a solution (preparation) applied and the count of *L. monocytogenes* at the initial contamination are important for the efficacy of the preparation. The time at which bacteriophages are applied or introduced into the raw material or food is also taken into consideration. The phage should be applied in the early stages of the raw food processing.

Other important factors determining the efficacy of phage action are the storage time and temperature of the products treated with phage preparations. The majority of studies examine the lytic ability of the phage at the optimum growth temperature for the pathogen. However, in order to obtain information for the potential application of a phage, efficacy studies should be performed at the temperature at which the foodstuff is prepared, processed or stored. Considering the importance of time, it needs to be emphasised that the phage application during food processing must be precisely timed to correlate with the possible time points of *L. monocytogenes* entry into the product. If the phage were applied too late, after the bacterial growth has been initiated, the bacterial population might reach higher cell densities which might reduce the efficacy of phage treatment (Coffey *et al.* 2010; Guenther & Loessner 2011; Mahony *et al.* 2011).

Effective elimination of *L. monocytogenes* from food products by means of selected bacteriophages

The use of bacteriophage P100 in the production of soft cheeses results in a reduced count of *L. monocytogenes* cells on the surface of the cheese below the detection threshold (one-time use of the preparation with a phage titre of 3×10^9) (Guenther & Loessner 2011). Reduction in the phage titre down to 2×10^6 plaque forming units (PFU) in the

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formulation reduces the number of *L. monocytogenes* CFU by 3 log units (Guenther *et al.* 2009). In both cases, the initial level of colony forming units of *L. monocytogenes* was 10^3 per gram.

In the case of solid matrices such as fresh fish fillets, and application of a solution with the P100 phage of 2×10^7 titre, the elimination of *L. monocytogenes* colony forming units was at the level of 2 log units (Soni & Nannapanesi 2010). In the case of raw salmon fillets, 3.5 log reduction of colony forming units was obtained, but only when a phage preparation with a titre of 10^8 PFU/g had been applied. The same preparation used at the same level of *L. monocytogenes* contamination but in a liquid matrix (broth model system) caused the complete inhibition of *L. monocytogenes* growth (Soni *et al.* 2010).

The bacteriophage FWLLm1 (morphologically similar to the phage A511) in a preparation of 7 log PFU/g reduced the concentration of *L. monocytogenes* by 2.5 log on the surface of poultry meat, while a decrease in the titre of the formulation to 6 log PFU caused a reduction in the number of colony forming units only by approximately 1 log (Bigot *et al.* 2011).

Solid matrices, such as low-processed vegetable and fruit products, an example of which can be cut pieces of fresh apples and melons, were tested for the efficiency of LMP-102. The LMP-102 preparation is a commercially available cocktail of phages specific for *L. monocytogenes*. In the initial phase of the study, artificially contaminated pieces of apple and melon (at 10^5 and 10^6 CFU) were treated with a phage preparation of a titre higher by 2 log units than the level of contamination of the treated sample (Leverentz *et al.* 2003). A reduction in *L. monocytogenes* colony forming units was observed in samples treated with the LMP-102 formulation in the range from 2.0 to 4.6 log units. A similar study published in 2004 showed that the bacterial colony forming units in artificially contaminated fruits can be reduced by even 6.8 log units (Leverentz *et al.* 2004). The study focused on optimising the process of eliminating *L. monocytogenes* from the surface of melon tissue. Key factors in reducing the level of matrix contamination in this case were: time, titre of phage preparation, and pH of the matrix tested. It turned out that the best results were obtained in the treatment of contaminated matrix with a preparation of 10^8 PFU titre, added maximally within one hour after melon cutting and contamination.

Listex™ P100 was highly effective in eliminating *L. monocytogenes* in melon juice, as the observed reduction (10^{11} PFU) amounted to even 8 log units in an 8-day incubation cycle at 10°C. A reduction in pear juice was smaller, but still satisfactory, at the level between 2.10 and 2.80 log units (conditions the same as above). No reduction of *L. monocytogenes* CFU was observed in apple juice. The differences in the effectiveness of Listex™ P100 between apple, pear, and melon juices were most likely due to variations in pH values of the analysed food matrices (melon pH 5.77–5.92; pear pH 4.61–4.91; apple pH 3.70–3.76) (Oliveira *et al.* 2014).

Mixed methods of *L. monocytogenes* elimination with bacteriophage preparations

Bacteriophage preparations can be used in combination with selected methods of *L. monocytogenes* elimination from food.

Phage preparations with phage P100 (5×10^7 PFU/cm^2) in combination with protective bacterial cultures of *Lactobacillus sakei* (10^3 CFU/cm^2) were applied to inhibit the growth of *L. monocytogenes* in an artificially contaminated (10^3 CFU/cm^2) cooked ham. The use of specific phages alone led to a rapid decline in the number of colony forming units of *L. monocytogenes* by 1 log unit, while not ensuring any visible inhibition of growth after 14-day incubation. In contrast, the coupled use of bacteriophages and *Lactobacillus sakei* ensured *L. monocytogenes* growth inhibition at the level of 2 log units after 14-day incubation (Holck & Berg 2009).

In those studies, the elimination of *L. monocytogenes* from the surface of the fruit tissue was enhanced in a low pH environment by the addition of $MnCl_2$ solution. This compound showed no adjuvant activity only in the case of apple tissues (Leverentz *et al.* 2004).

The legal status of preparations containing bacteriophages

When describing the legislative framework of preparations containing bacteriophages one should focus on two previously mentioned products, i.e. Listex P100 and ListShield™, because these preparations are relatively easily available and have the appropriate certificates that will be described below.

The country, in which Listex P100 and ListShield™ are already widely applied as processing aids in the

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food production cycle, is the USA. These preparations have the GRAS (generally recognised as safe) status issued by the FDA and USDA [FSIS Form 260-9 (6/86)]. This means that they are considered safe preparations that do not change in any way the characteristics (taste, smell, texture) of the product to which they are added. Listex P100 and ListShield™ are also permitted by the Organic Materials Review Institute for use in the organic food production (OMRI Listed Product Number ebi-1855). ListShield was also approved for use in the production of kosher (K-certified) and "halal" food (Islamic Food and Nutrition Council of America).

In Canada, Listex P100 is permitted for use in the production of foods susceptible to the increased occurrence of *L. monocytogenes*.

In Europe, so far only the Ministry of Health in Denmark and Switzerland (Swiss Bundesamt für Gesundheit) have issued an official statement allowing the use of the Listex P100 preparation as an additive in food production.

Other European countries have not yet delivered an official position on the use of Listex P100. However, the legal record one may refer to when attempting to implement this preparation is the framework Directive 89/107/EEC on additives. According to Art. 1 Subsection 3, Directive 89/107/EEC defines food additives as follows: "Substances that are not consumed as food components, intentionally used in the processing of raw materials, foodstuffs or their components, to achieve a certain technological purpose during treatment or processing, which may result in the unintentional but technically unavoidable presence of residues of the substance or its derivatives in the final product, provided that these residues do not present any health risk and do not have any technological effect on the final product" (Council Directive 89/107/EEC).

Classification of a product as a food additive means that the manufacturer is not obliged to include information on the product label about the use of the above-discussed preparation. However, the food additive may include only agents that have no technological effects upon the final product (Teufer *et al.* 2007).

CONCLUSION

Phage preparations, in comparison with the majority of the methods used to eliminate *L. monocytogenes*, are relatively new and little known on a worldwide scale. This is largely due to the absence of certain legal regulations in some countries, particularly in Europe. Bacteriophages that are components of the preparations discussed are derived from environmental isolates, and from the biological point of view they are not genetically modified material, however, they are subject to patent protection. This gives rise to some kind of conflict due to the fact that the countries which have no legal regulation regarding the use of these preparations may treat bacteriophages as genetically modified material.

The results of the studies discussed in this publication show that bacteriophages can be one of the most effective, yet non-intrusive, safe methods to ensure the elimination of *L. monocytogenes* from different food matrices. The listed effects of bacteriophage applications show that the best matrices for the use of phages are those with pH in the range of 5.77–5.92 (melon). With decreasing pH, a decline is observed in the effectiveness of phage preparations. Products in which the *L. monocytogenes* elimination can be very effective due to their similar pH range are fresh cucumbers, leeks, horseradish, onions, parsnips, green peppers, sweet potatoes, zucchini, and mangoes.

The contamination with *L. monocytogenes* very often occurs during food cutting with strains present in the form of biofilms on cutting surfaces (Sip 2010). The increasing demand for products which include raw milk, fish meat, vegetables, and fruits have an impact on the increasing demand for rapid and safe methods of pathogen elimination from food. Bacteriophage preparations may belong among the few methods for bacteria elimination from food products that do not affect the taste, smell, texture, and appearance of the "preserved product".

The use of bacteriophage preparations should be perceived as an integral part of the hygiene-ensuring program, and not as its substitute. An effective solution would be a preventive treatment with phage preparations of raw materials with an increased risk of contamination with *L. monocytogenes* strains.

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