Comprehensive REVIEWS in Food Science and Food Safety

Listeria monocytogenes: A Target for Bacteriophage Biocontrol

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Abstract: *Listeria monocytogenes* is a growing concern in the food industry as it is the causative agent of human listeriosis. There are many research articles concerning the growth, survival, and diversity of *L. monocytogenes* strains isolated from food-related sources, elucidating the difficulty in controlling these bacteria in a food-processing facility. Bacteriophage biocontrol of *L. monocytogenes* strains was introduced in 2006, through the first commercial bacteriophage product targeting *L. monocytogenes* ListShield^{TM}. This review focuses on the use of bacteriophage biocontrol to target *L. monocytogenes* in the food industry, specifically direct application of the bacteriophages to food products. In addition, we discuss characteristics of these bacteria that will have a significant influence on the effective treatment of bacteriophages such as genetic diversity between strains prevalent in one facility. There are many positive results of phage treatments targeting *L. monocytogenes* in food; however, success of *in vitro* studies might not be reproducible in practice. Future studies should focus on creating experimental design that will imitate the conditions found in the food industry, such as a stressed state of the targeted bacteria. *In situ* evaluation of bacteriophage treatment of *L. monocytogenes* will also be necessary because the presence of these bacteria in a processing facility can vary greatly regarding genetic diversity. The potential use of phages in the food-processing facility as a biosanitizer for *L. monocytogenes*, as well as the use of lysins to target these bacteria should also be explored. Despite the exciting research avenues that have to be explored, current research shows that biocontrol of *L. monocytogenes* is feasible and has potential to positively impact the food industry.

Keywords: bacteriophages, biocontrol, *Listeria monocytogenes*

Introduction

Listeria monocytogenes can cause listeriosis when transmitted to humans, usually via contaminated food products (Farber and Peterkin 1991). These pathogens are ubiquitous in nature and can contaminate the food processing line at any point. Food products that are traditionally prone to *L. monocytogenes* contamination include raw or processed dairy products, fish, meat, and vegetables (Farber and Peterkin 1991). Recently, ready-to-eat (RTE) food products, as well as fresh fruit have been implicated in *L. monocytogenes* contamination because the biggest outbreak of listeriosis in the United States was due to contaminated cantaloupes (FDA 2011). Food products that cause the biggest concern are those products that do not undergo a heat treatment or rely on refrigeration for control of *L. monocytogenes* because these bacteria can grow at refrigeration temperatures and even survive at −0.4 °C (Farber and Peterkin 1991).

In most countries, strict regulations for microbial standards are established to prevent retail of contaminated food products. However, control of unwanted bacteria in the food industry is extremely difficult because the food products provide a nutrient-rich niche for growth (Hagens and Loessner 2010). In addition, consumers pressure the industry for "natural" food products, free of chemicals and preservatives (Xi and others 2011). Undoubtedly, the pressure for natural products and the high microbiological standards for food products have increased difficulty in controlling contamination by *L. monocytogenes* (Ivanek and others 2005).

Bacteriophages, the viruses of bacteria, can act as natural antimicrobials against food pathogens in the food industry (Goodridge and Abedon 2003). These phages infect specific bacteria and use the genomic material of the bacteria to produce new phages, ultimately destroying the bacterial cell. The first 2 commercial phage products approved by the U.S. Food and Drug Administration (FDA) target *L. monocytogenes* in food products (Sulakvelidze 2013). These bacteria are one of the most studied foodborne pathogens, and therefore useful to evaluate the antimicrobial potential of bacteriophages (Cossart 2007). This review aims to evaluate the presence of *L. monocytogenes* in the food industry and the feasibility of using bacteriophages for biocontrol.

L. monocytogenes regulation

The rate of *L. monocytogenes* infection is not as high as that by other foodborne pathogens, but the mortality rate of listeriosis has been reported to be the third highest in the United States, making it a very serious public health threat (Scallan and others 2011). In 1991, it was suggested that listeriosis will be the leading fatal foodborne infection in the United States (Gellin and others 1991).

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Governmental bodies responded to this threat by implementing strict regulation for food products concerning *L. monocytogenes* contamination. The U.S. Dept. of Agriculture (USDA) has a strict zero tolerance policy (no viable cells detected in 25 g) for *L. monocytogenes* in RTE food products. The current regulation states that contamination with *L. monocytogenes* is adulteration of food products, thus giving the USDA grounds for legal action against food manufacturers (Kraiss 2008) According to the commission regulation of the European Union (EU; European Commission 2005), the concentration of *L. monocytogenes* should be kept below 100 cfu/g in food products and be absent in 25-g samples of RTE food products that can support the growth of the bacteria or is intended for infants or special medical purposes. Regulations in Canada, New Zealand, and Australia also differentiate between RTE food products that can support the growth of *L. monocytogenes* (absent in 25-g sample) and other food products (<100 cfu/g; Jami and others 2014).

Regardless of these strict regulations, many countries have reported increases in cases and outbreaks of listeriosis. The Advisory Committee of Microbiological Safety of Food (ACMSF) reported an increase of listeriosis in individuals older than 60 y in the U.K. since 2000 (ACMSF 2009). Surveillance of listeriosis in France showed the highest incidence in 2007 since reporting listeriosis became mandatory in 1998 (Goulet and others 2008). Allerberger and Wagner (2009) reported an increase of invasive listeriosis in Austria, as well as other European countries such as the Republic of Ireland and Germany. In an attempt to assess the global burden of listeriosis, de Noordhout and others (2014) used a meta-analysis to assess epidemiological data of listeriosis incidences from 1990 to 2012. They estimated that, in 2010, listeriosis resulted in 23150 illnesses and 5463 deaths worldwide. In addition, *L. monocytogenes* is responsible for an estimated US$2.6 billion cost of illness in the U.S., as well as a loss of 9400 quality-adjusted life-years per annum (Hoffmann and others 2012).

The regulations regarding *L. monocytogenes* also have a major impact on the food industry and in 2005 it was estimated that the cost of product recalls due to *L. monocytogenes* contamination in the United States was between US$ 1.2 and 2.4 billion (Ivanek and others 2005). In addition, many food manufacturers treat a positive result for *Listeria* spp. as a positive result for *L. monocytogenes*, which greatly increases the loss of food products and the economic burden. Clearly, additional control measures are needed to lessen the economic and health burdens of this pathogen.

L. monocytogenes in the food industry

The genus *Listeria* contains 10 species, namely *L. monocytogenes*, *Listeria marthii, Listeria innocua, Listeria seeligeri, Listeria welshimeri, Listeria ivanovii, Listeria grayi, Listeria rocourtiae* (Graves and others 2010; Leclercq and others 2010), *Listeria fleischmannii* (Bertsch and others 2013), and *Listeria weihenstephanensis* (Halter and others 2013). Three *Listeria* strains isolated from cheese in Switzerland were designated as a novel species, *L. fleischmanni*. Interestingly, cell wall binding domains from *Listeria* phage endolysins could bind to these strains, indicating the relatedness to the *Listeria* genus (Bertsch and others 2013). At the same time, 2 strains isolated from a fresh-water plant were designated as novel species, *L. weihenstephanensis* (Halter and others 2013). Strains from these novel species did not display hemolysis and can be classified as avirulent (Leclercq and others 2010; Bertsch and others 2013; Halter and others 2013). Thus far, virulence factors have only been identified in *L. monocytogenes*, *L. ivanovii*, and *L.* seeligeri (Gouin and others 1994). However, only *L. monocytogenes*, which is a very diverse species with 4 lineages and 13 serotypes, is associated with human illness (Orsi and others 2011). The differences between strains of *L. monocytogenes* is of importance because not all are virulent, and they have different abilities to adapt to processing environments. Isolation and characterization of *L. monocytogenes* is mainly performed to determine the prevalence of this pathogen in the food industry, as well as to identify different serotypes, genetic diversity, presence of virulence genes, and the antimicrobial resistance of these strains. This information is very useful in risk assessment studies, especially because it sheds some light on the differences between *L. monocytogenes* strains. We will now discuss studies that characterized *L. monocytogenes* strains isolated from food-related environments since 2010, referring to prevalence, genetic variability, and influence of bacteriophages (Table 1).

Prevalence of *L. monocytogenes* in the food industry

Prevalence of *L. monocytogenes* in food products is mostly low as 14 studies (Table 1: 2, 6, 8, 10, 11, 12, 13, 15, 18, 21, 22, 23, 25, and 27) indicated positive samples below 10.0% with the lowest incidence at 1.42% ($n = 6270$; Table 1: 15) positive samples. Only 5 studies (Table 1: 16, 17, 19, 21, and 26) indicated *L. monocytogenes* contamination of samples between 10% and 20% and 1 study reported a 55% ($n = 100$; Table 1: 24) incidence. Interestingly, the highest incidence of *L. monocytogenes* was found in blue-veined cheese rind ($n = 100$) (Table 1: 24), whereas the lowest incidence was reported from 6270 samples taken from various sources in cheese-producing facilities over at least 4 y (Table 1: 15). The size of prevalence studies and the variation in sources such as food product, swabs from personnel and food processing facility (food contact areas and nonfood contact areas), as well as the nearby environment have an influence on the results and make it difficult to compare independent studies. However, the highest level of contamination for specifically cheese product samples in study 15 was 13.6%, which is still pointedly lower than the 55% reported in study 24 (Table 1). This may indicate that the cheese rind tested in study 24 might be very susceptible to *L. monocytogenes* contamination because the cheese pulp from the same samples was not contaminated.

The samples tested in these studies included various food products, including raw meat, seafood, dairy products, fresh produce, and RTE products. Recently, a review regarding *L. monocytogenes* contamination of seafood products was published, describing a diverse prevalence of these bacteria (Jami and others 2014). In the group of studies discussed in this paper, the frequency of *L. monocytogenes* also varied significantly in the same type of food product. In some cases (Table 1: 7 and 25), seafood products had the highest contamination levels when compared to other products tested, and then in other cases (Table 1: 2 and 18), the contamination levels of seafood was lower. Similar inconsistencies were reported for meat and poultry products (Table 1: 1, 13, 14, 20, 23, 25, 27, and 29). Fresh produce is very difficult to compare because there are so many variations in these food products, but overall there seems to be a high prevalence of *L. monocytogenes* among vegetables. Leafy greens (2), salads (12 and 17), and vegetable dishes (6) had some of the highest incidences of *L. monocytogenes* contamination when compared to other food products tested (Table 1). Interestingly, 1 study (Table 1: 11) reported that salad mixes and lettuce had a lower percentage of *L. monocytogenes* than mixed vegetables. It is likely that increased manipulation during preparation of the food

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Table 1–Studies describing the prevalence of *L. monocytogenes.*

Number	Isolation source	Positive *L. monocytogenes* samples (%)	Reference
1	Raw meat	97	Shen and others (2013)
2	Various food products	20 (3.9)	Cetinkaya and others (2014)
3	Avocado processing facility and guacamole	140	Strydom and others (2013)
4	Various environmental and food sources	103	Lomonaco and others (2011)
5	Various food products	46	Chen and others (2011)
6	RTE food samples	10 (6.3)	Chen and others (2013)
7	RTE food samples, processing plants, human listeriosis cases	166 (4.6)	Lambertz and others (2013)
8	Dairy processing facilities	25 (4.6)	Parisi and others (2013)
9	Cheese	47	Acciari and others (2011)
10	Raw milk	18 (4.1)	Jamali and others (2013b)
11	RTE vegetable products	16 (3.1)	Sant'Ana and others (2012)
12	Various food products	554 (2.6)	Kramarenko and others (2013)
13	Duck meat and environment	15 (2.8)	Adzitey and others (2013)
14	Turkey meat	37 (20.5)	Erol and Ayaz (2011)
15	Cheese processing facility and environment	34 (1.4)	Almeida and others (2013)
16	Various food products	65 (12.4)	Wang and others (2013)
17	Raw and RTE food products	45 (11.4)	Jamali and others (2013a)
18	Various food products	59 (6.2)	Yu and Jiang (2014)
19	Raw and RTE food products	23 (16.4)	Marian and others (2012)
20	Chicken	202	Alonso-Hernando and others (2012)
21	Raw and RTE food products, environment, personnel swabs	4.83% (raw), 14.5% (RTE)	Fallah and others (2013)
22	RTE seafood	12 (4.8)	González and others (2013)
23	Raw and RTE meat products	66 (5.3)	Modzelewska-kapitu and Maj-sobotka (2014)
24	Blue-veined cheese rinds	100 (55)	Bernini and others (2013)
25	RTE meat and fish products	2 (5)	Kovačević and others (2012)
26	Raw and RTE chicken	51 (18.2)	Osaili and others (2011)
27	RTE poultry products	9 (3)	Meyer and others (2012)
28	Various samples	222	Fox and others (2012)
29	Beef samples	191 (17)	Khen and others (2014)
30	Water, clinical and milk	20 (2.8)	Soni and others (2013)

products can lead to a higher probability of contamination and, therefore, higher levels of the bacteria. Raw and processed dairy products mostly have a lower *L. monocytogenes* incidence when compared to other products (Table 1: 2, 6, and 7), although the highest prevalence reported in this paper was from cheese rind (Table 1: 24). The variation in prevalence in similar food products indicates that the food matrix is not the only factor in the persistence of these pathogens. The implementation and management of a quality control system and integrity of the cold chain also plays important roles in controlling *L. monocytogenes* contamination of processing facilities and food products.

Food processing plants and environmental areas were also included in some of the studies (Table 1: 3, 4, 6, 7, 8, and 21). It is suggested that contamination of food products occurs mainly in the processing plant (Miettinen and Wirtanen 2006; Lomonaco and others 2009). In a few cases, cross-contamination from resident strains, which survive in harborage sites, was suggested to be the source of contamination, rather than raw ingredients (Table 1: 3, 7, and 21). In a recent review, harborage sites was found to be the main reason why *L. monocytogenes* strains persist in food processing facilities (Carpentier and Cerf 2011). The processing facility is usually more contaminated than the final products (Pak and others 2002). For instance, a higher level of *L. monocytogenes* contamination was found on nonfood contact areas (18.75%; $n = 32$) when compared to food products (2.4%; $n = 249$) and food contact areas (4.88%; $n = 266$; Table 1: 8). Floor drains, equipment, and personnel have all been implicated in cross-contamination of *L. monocytogenes* (Carpentier and Cerf 2011; Jami and others 2014; Table 1: 3, 4, 7, 8, and 21). This emphasizes the necessity to monitor food-processing facilities very closely for the presence of *L. monocytogenes*. However, other sources of contamination or at least initial contamination of a facility should not be ignored. Ruminants shed *L. monocytogenes* in their feces and can spread this pathogen further along the food chain (Hutchison and others 2004; Lyautey and others 2007). Filiousis and others (2009) found identical clones of *L. monocytogenes* in unrelated food products that were isolated from open-air markets in Greece. This may be due to contamination at the markets or the prevalence of dominant clones in the region. Either way, it remains important to avoid circumstances where cross-contamination can take place, whether it is during processing or retail.

Genetic variability in *L. monocytogenes* strains isolated from the food industry

General opinion is that serotyping of *L. monocytogenes* strains is not of particular use in subtyping studies because strains associated with human infection or isolated from food sources are mostly in lineage I and II, which harbors serotypes 1/2a, 1/2b, and 4b (McLauchlin and others 2004; Parisi and others 2013). However, all the major outbreaks of listeriosis involve strains from serotype 4b, which is not as frequently isolated compared to serotype 1/2a (Doumith and others 2004). It might, therefore, be of use to screen strains implicated in a listeriosis outbreak with serotyping before using pulsed-field gel electrophoresis (PFGE) in epidemiological investigations (Swaminathan and others 2001). In the studies described here (Table 1), the preferred method for serotyping is by multiplex PCR that separates only the major serotypes, namely: 1/2a (3a), 1/2b (3b, 7), 1/2c (3c), and 4b (4d, 4e; Doumith and others 2004). As only serotypes 1/2a, 1/2b, 1/2c, and 4b are commonly found in food-related or clinical sources many researchers

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assume that these are the identities of the specific strains that were classified in the serotype groups described by Doumith and others (2004). Another method used is the conventional serotyping based on the commercially prepared antisera against somatic (O) and flagellar (H) antigens (Farber and Peterkin 1991).

Results form 12 independent studies (Table 1: 1, 2, 7, 8, 9, 10, 12, 16, 18, 21, 27, and 29) indicated that serotype 1/2a was most frequently isolated, regardless of isolation source or typing method. In all cases, serotype 4b was also isolated except from studies 9 and 27 (Table 1). Three studies indicated that the majority of the strains belonged to serotype 4b (Table 1: 6, 11, and 14). These strains were isolated from RTE foods in China (6), RTE vegetable products in Brazil (11), and fresh turkey meat in Turkey (14). This is of serious concern, especially because a ribotype was also identified in the Brazilian *L. monocytogenes* strains that have been implicated in listeriosis cases all over the world.

Although prevalence studies do not focus on serotyping of *L. monocytogenes* strains, some studies determine the serotypes of the bacterial strains when describing sensitivity or resistance to bacteriophages. Host ranges of 114 phages against strains, representing all the serotypes of *L. monocytogenes* ($n = 13$), indicated that strains belonging to serotype 4 and 1/2 were more susceptible to phages compared to strains in other serotypes (Vongkamjan and others 2012). These strains were all isolated from dairy silage and it is reasonable to assume that bacterial strains that are prevalent in food-related sources such as 1/2 and 4 will correlate with the bacteriophages in these environments. Another study also reported serotype 4 strains particularly sensitive to phages (Kim and others 2008). Interestingly, 9 of 12 phages were able to infect multiple *L. monocytogenes* serotypes, as well as other *Listeria* spp., including *L. invanovii, L. wellshimeri, L. seeligeri,* and *L. innocua*. However, strains belonging to *L. monocytogenes* serotype 1/2 often displayed resistance to these broad host range phages. At this point, there are not enough data to perceive a relationship between susceptibility of *L. monocytogenes* strains to bacteriophages and specific serotypes, but susceptibility of serotype 4 strains is, however, encouraging as these strains are most associated with listeriosis.

A more specific differentiation between *L. monocytogenes* strains than serotyping is needed to understand the ecology of these bacteria in the food industry. The ability to understand the contamination sources and routes inside a processing facility is necessary to implement control strategies, not to mention the identification of strains (and the sources of those strains) implicated in listeriosis outbreaks. Molecular subtyping methods such as PFGE have been critical in identifying the sources of listeriosis outbreaks (Graves and others 2005) and have also been used to evaluate the genetic diversity of the strains isolated from the food industry. In fact, 10 of 13 studies (Table 1: 1, 3, 4, 5, 7, 9, 13, 16, 18, and 27) described in this paper used PFGE to differentiate between strains. In addition, almost all of the studies used the Pulsenet-prescribed method, which includes both *ApaI* and *AscI* digestion (Swaminathan and others 2001). This is, however, time-consuming and expensive and other methods have been compared to PFGE results. Amplified fragment length polymorphism (AFLP; Table 1: 4, 5, 8, and 24), ribotyping (Table 1: 11) and restriction fragment length polymorphism (Table 1: 3) have all been reported to deliver good results compared to PFGE-typing of *L. monocytogenes* strain.

The genetic diversity of the *L. monocytogenes* strains reported by these studies seems contradicting, as some have reported very high diversities (Table 1: 1, 4, 5, 16, and 24) and others reported very low diversities (Table 1: 7, 9, 13, 18, and 27). The strains in these studies were isolated from different product types (Table 1: 1, 5, 7, 16, 18, and 27) and locations (Table 1: 1, 4, 5, 7, 9, 13, 16, 18, and 27) and included environmental (Table 1: 4, 7, and 13) and clinical (Table 1: 5) isolates. Furthermore, most of the strains were isolated over a period of at least 12 mo. Interestingly, the only study (Table 1: 24) that described the genetic variation in isolates from similar product types (cheese rinds produced from different cheese types produced in one facility) had a very high diversity according to AFLP results. This is also the study that presented the highest prevalence of *L. monocytogenes* and, therefore, it is probable that this specific facility had a high level of contamination with these bacteria. Regardless, it appears that the genetic diversity of *L. monocytogenes* also varies, like the dispersion of the pathogen in the food industry. This may present a problem for bacteriophage biocontrol because bacteriophages are host specific and it is advisable that the genetic diversity of the *L. monocytogenes* strains in a specific location be determined as a preliminary step to biocontrol.

Apart from prevalence and genetic variability within the *L. monocytogenes* species, there also exist differences between the pathogenicity of these strains. *L. monocytogenes* lineage I (particularly serotype 4b) is overrepresented in clinical strains, even when compared to lineage II, whereas lineages III and IV are rarely isolated and mostly from animal origin (Orsi and others 2011). It has been reported that lineage I strains have a 100-fold increased risk of causing listeriosis over lineage II and that strains from lineage II carry stop codons in the gene, *inlA*, which lowers the risk of infection (Chen and others 2006). The variability in pathogenicity has led to many studies that have identified virulence factors for each stage during infection, describing the entire infection process of *L. monocytogenes* (Cossart and Toledo-Arana 2008).

Some of the studies in Table 1 also reported strains with virulence factors (1, 6, 10, and 13). The presence of *inlA*, *inlC*, and *inlJ* was determined in studies 1 and 10 by a multiplex PCR method (Liu and others 2007). All but 1 strain in study 1 ($n = 97$) and all the strains in study 10 ($n = 18$) tested positive for these virulence markers. The first gene sequenced for *L. monocytogenes hlyA* with known virulence association (Cossart 2007) was reported in 100% of the strains tested in study 13 (Table 1). Furthermore, listeriolysin S-positive strains were identified in study 1 (Table 1) by targeting *llsX* with a PCR method (Clayton and others 2011). Twelve of 97 strains were positive for *llsX* and 5 (5/12) belonged in epidemic clone I. Strains belonging in epidemic clone I were associated with major listeriosis outbreaks in different countries and described as a "cosmopolitan clonal group" associated with virulence (Chen and Knabel 2007). Although these results do not explicitly confirm virulence, they do indicate the potential of these strains to cause infection. The differentiation between virulent and avirulent *L. monocytogenes* strains is critical and more studies should include descriptions of the virulence potential of strains isolated from the food industry. In fact, a risk assessment by the combined efforts of the FDA and USDA's Food Safety and Inspection Service (FSIS) included the varied virulence found among *L. monocytogenes* strains and concluded that the risk might significantly be lower due to the presence of avirulent strains (Whiting 2003).

Despite the genetic diversity seen between strains of *L. monocytogenes*, the pan genome (comparison of 16 strains representing all serotypes) of the species have been described as very stable, containing a high percentage conserved genes. Diversity in the pan-genome of the 16 *L. monocytogenes* strains were found to be influenced by prophage-related genes (Kuenne and others 2013). Although lysogeny in *Listeria* strains is very common (Loessner and Rees 2005), prophage genes are unevenly distributed among serotypes. Some strains are completely free or do not have

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complete prophages such as WSCL 1001 and WSCL 1042 (Klumpp and Loessner, 2013; Klumpp and others 2014), as well as F2365 (Nelson and others 2004), L312 (Chatterjee and others 2006), and SLCC 2376 (Haase and others 2011) as found by Kuenne and others (2013). These particular strains belong to serotypes 1/2a, 4b, and 4c.

An interesting study recently showed the influence of a prophage in a critical step in the infection cycle of *L. monocytogenes*, where excision of the prophage from the *comK* gene activates the gene, expressing the ComK protein. In turn, this protein activates the Com system that is necessary for escape of the bacterial cell from the vacuole formed during phagocytosis (Rabinovich and others 2012). This is not the only function attributed to the prophage interrupting the *comK* gene. In some *L. monocytogenes* strains, a particular prophage in the *comK* gene might also have a role in persistence of these strains in processing facilities (Verghese and others 2011). This is somewhat contradictory to the review of *L. monocytogenes* persistence in processing facilities by Carpentier and Cerf (2011), who concluded that this species does not have unique abilities which facilitates persistence. The influence of phages on the genetic diversity and regulation of genes in *L. monocytogenes* is an interesting field of research with very little information and many questions.

L. monocytogenes bacteriophage biocontrol

Despite strict regulatory policies, *L. monocytogenes* still has a major detrimental influence in the food industry, causing food product recalls and disease outbreaks. In a recent review, Carpentier and Cerf (2011) discussed reasons why *Listeria* cells persist in processing facilities, maintaining that these pathogens possess no unique abilities to adapt to these environments, apart from their ability to grow at very low temperatures. They concluded that difficulty in controlling these bacteria are due to inability to identify and destroy harborage sites. It is clear that control measures in quality control systems and the implementation of these practices are not sufficient to manage these bacteria inside processing facilities. Preservation methods (chemical or physical) used for food products are also not sufficient to control growth after initial contamination. Additional methods are, therefore, needed to control these bacteria. The use of bacteriophages to aid in these problems is now often discussed and numerous reviews regarding biocontrol of bacteria in the food industry have been published. Here we will discuss the properties of bacteriophages and possible applications with the results of studies describing the effects of bacteriophage treatments on *L. monocytogenes* in food products.

Bacteriophages are natural bactericidal agents

The complete destruction of bacterial cells infected by lytic phages is one of the advantages over antibiotics, which are sometimes only bacteriostatic, such as tetracycline (Loc-Carrillo and Abedon 2011). Bacteriophage treatment of pathogenic or spoilage bacteria in the food industry have high potential, because some sanitizers are also bacteriostatic. Bacteriophages are a better control option compared to chemical food preservatives as they are natural enemies of bacteria. In addition, their specificity regarding targeted bacterial strains adds to the potential use in food products.

Since 2003, 11 studies have described the effect of bacteriophages on *L. monocytogenes* in food products (Table 2). All of these studies reported some success where the bacteriophage treatment reduced the *L. monocytogenes* counts under various circumstances. It is not certain if complete eradication of the bacteria in the food matrix has been accomplished, because enrichment culturing is not used to determine the *Listeria* counts after phage treatment. Carlton and others (2005) did report no regrowth of *L. monocytogenes* on cheese after 21 d after bacteriophage treatment but this is the only case where the results were confirmed by selective enrichment and subsequent plating of the product samples. In a few biocontrol studies, the bacteria counts dropped below levels of detection with direct plating, but rose again after prolonged incubation (10 to 22 d) (Guenther and others 2009; Bigot and others 2011; Guenther and Loessner 2011; Rossi and others 2011). Determination of *L. monocytogenes* concentration in biofilms with direct epifluorescence microscopy showed higher survival rates after bacteriophage treatment compared to when the concentrations were determined with conventional methods (Montañez-Izquierdo and others 2012). Nonetheless, even without complete eradication and different testing methods, bacteriophage treatment has a significant influence on *L. monocytogenes* in food products.

Host–phage interaction

Bacteriophages have to physically attach to their target bacteria before infection can take place. Bacteria, however, adapt very readily to their environments, which can result in structural changes of the cell membranes. These changes can influence the attachment of phages and, therefore, also the effectiveness of the phage treatment. A recent review reported on the phage-host interactions regarding responses of bacterial hosts to their environment (Denes and Wiedmann 2014). The bacterial condition in a food matrix or food-processing facility is discussed with specific reference to productivity of infection in host cells that are in a stationary or lag phase. The production of new virons would be much less in these cells compared to hosts cells in the exponential phase, because the amount of nucleic acid available in the host will have a direct influence on the amount of virons produced (Bouvier and Maurice 2011).

The food matrix will have an influence on the phage–host interaction, which in turn will impact the success of a bacteriophage treatment. Food products that have been tested with *L. monocytogenes* biocontrol include fresh fruit and fruit juice (Leverentz and others 2003; 2004; Oliveira and others 2014; Hong and others 2015), poultry products (Guenther and others 2009; Bigot and others 2011; Chibeu and others 2013), dairy products (Guenther and others 2009; Guenther and Loessner 2011; Soni and others 2012), fresh sausage (Rossi and others 2011), as well as RTE seafood, vegetables, and hot dogs (Guenther and others 2009). Greater reduction of *L. monocytogenes* in chocolate milk and mozzarella cheese brine was achieved compared to solid food products (Guenther and others 2009). This is probably due to unrestricted passive diffusion of phages in a liquid matrix, which is not possible on the surface of solid food products. Bacteria on the surface of food products such as chicken breast or cheese are more difficult to reach, and Guenther and others (2009) concluded that it was the limited diffusion and, therefore, lack of contact between phages and bacteria that reduced efficacy on solid food products.

The acidity of the environment also has an effect on the success of phage treatment. In apple fruit juice, no significant differences between control and phage-treated samples were recorded, whereas pear and melon juice samples had a reduction in *L. monocytogenes* counts. Similar results were seen when fresh-cut fruit slices inoculated with *L. monocytogenes* were treated with bacteriophages (Leverentz and others 2003; Oliveira and others 2014). No effect was observed on apple slices, whereas phage treatment on other fruit such as melons and pears reduced *L. monocytogenes* counts. In all cases, the pH of the apple juice or slices (pH of 4.4

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Table 2–Bacteriophage biocontrol studies of *L. monocytogenes.*

Bacteriophages	Matrix	Reference
Phage LM-103[a], phage LMP-102[a], nisin	Fresh-cut red delicious apples fresh-cut honeydew melons	Leverentz and others (2003)
LMP-102[a]	Honeydew melon tissue	Leverentz and others (2004)
12 Phages isolated from 4 plants	Isolated from turkey processing plants and host ranges tested on BHI plates (spot tested)	Kim and others (2008)
A511, P100	RTE food products	Guenther and others (2009)
FWLLm1	BHI broth, RTE chicken breast	Bigot and others (2011)
A511	Soft-ripened cheese	Guenther and Loessner (2011)
P100[b]	Brazilian fresh sausage	Rossi and others (2011)
P100[b]	Biofilms on stainless steel surfaces	Montañez-Izquierdo and others (2012)
P100[b], LAE, PL-SD	Queso fresco cheese	Soni and others (2012)
P100[b], PL, SD	Cooked turkey, roast beef	Chibeu and others (2013)
P100[b]	Fresh-cut fruit, fruit juices	Oliveira and others (2014)
LMP-102	Fresh-cut melon	Hong and others (2015)

[a] Intralytix, Inc. (Baltimore, Md., U.S.A.).
[b] LISTEX™ (USA).
LAE, lauric arginate; PL, potassium lactate; SD, sodium diacetate.

or less) were lower than the other fruits. Phage titers have also been shown to decline rapidly in the acidic environment of apple juice (pH 3.7) when compared to melon and pear juice (pH 4.6 to 5.9; Oliveira and others 2014).

Bacteriophage treatment

One advantage in bacteriophage therapy is a once-off application or treatment, the theory being that the phages will multiply sufficiently to eradicate all target bacteria. This is, however, not possible in an environment such as food-processing facilities and food products, where there are low contamination levels, especially if the strains have a wide genetic make-up as seen in *L. monocytogenes*.

Studies with food products have shown that the first treatment of phages with the highest titer had the biggest influence on bacterial cells and that repeated doses did not have continuous bactericidal effects. Also, with or without repeated doses, the bacteria are reported to increase in numbers, although not as high as in control samples (Bigot and others 2011; Guenther and Loessner 2011; Soni and others 2012; Chibeu and others 2013). This is not always the case as no regrowth (after 8 d) of *L. monocytogenes* in melon juice or on cheese was reported after bacteriophage treatment (Carlton and others 2005; Oliveira and others 2014).

In all cases, the titer of the bacteriophages are a deciding factor in the success of the treatment. Some of the highest reductions in *L. monocytogenes* counts were reported when at least 10^8 pfu/unit phages were applied to the food products (Leverentz and others 2004; Guenther and others 2009; Guenther and Loessner 2011). *L. monocytogenes* counts on fresh melon tissue had a 6.7 log unit decrease when a phage treatment (10^8 pfu/mL) was added 1 h before bacterial inoculation (Leverentz and others 2004). In the liquid products (chocolate milk and mozzarella cheese brine) tested by Guenther and others (2009), bacterial counts dropped below levels of detection after a 10^8 pfu/g bacteriophage treatment, whereas the control samples reached between 10^4 and 10^5 cfu/mL. In the same study, the bacterial counts on solid food products (hot dogs, sliced turkey meat, smoked salmon, seafood, sliced cabbage, and lettuce leaves) were reduced up to 5 log units. In addition, when different concentrations of phage treatments were tested, the higher concentration always had a greater effect (Guenther and others 2009; Bigot and others 2011; Guenther and Loessner 2011). Even when bacteriophages were used to target *L. monocytogenes* biofilms on stainless steel coupons, the higher concentrations had a greater reduction in bacterial cells (Montañez-Izquierdo and others 2012).

Combination treatments

A few studies have reported the effect of bacteriophage treatment in combination with chemicals such as nisin, a bacteriocin that has generally regarded as safe (GRAS) status (FDA 2001). Leverentz and others (2003) recorded a reduction of 5.7 log units of *L. monocytogenes* on melon slices with a phage treatment in combination with nisin. Nisin creates nonselective pores in the bacterial plasma membrane which can possibly aid the bacteriophage since bacterial cells with weakened membranes are more susceptible to infection and lysis. As nisin is more active at a lower pH, it is potentially a good addition to bacteriophage treatments of acidic food products such as fruit or fruit juices. Especially, as bacteriophage treatment in fruit juices with higher acidities have not been found to be as successful when compared to fruit juice with a higher pH (Leverentz and others 2003; Oliveira and others 2014).

Addition of lauric arginate (LAE) resulted in a greater decrease in *L. monocytogenes* levels in cheese compared to a bacteriophage treatment, although a regrowth of the bacteria was detected at 4 °C. The addition of potassium lactate-sodium diacetate (PL-SD) to samples with either LAE or a bacteriophage treatment prevented the regrowth of *L. monocytogenes*, although PL-SD alone had no effect on the bacteria concentration (Soni and others 2012). Chibeu and others (2013) tested the effect of bacteriophages on *L. monocytogenes* on cooked turkey and roast beef samples, treated during processing with PL and PL-SD, respectively. Only roast beef samples with bacteriophage and PL-SD treatments did not have bacterial cell regrowth after 28 d at 4 °C. Addition of PL-SD has been found to be listeriostatic rather than listeriocidal (Vogel and others 2006), but it increased the effect of the bacteriophage treatment on cheese and beef samples, indicating the potential in using a combination of bacteriophages and other antimicrobials.

L. monocytogenes biocontrol in food processing facilities

The presence and persistence of *Listeria* in food-processing facilities is a major problem as it is one of the main sources of contamination of food products. The potential use of bacteriophages as biosanitation agents, as part of hurdle technology, has been suggested but not investigated specifically for *L. monocytogenes* (Mahony and others 2011). These pathogens readily form

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biofilms on stainless steel and other surfaces, and this is believed to be one of the reasons why these bacteria can be so persistent in processing facilities, as the biofilms can protect the cells from sanitizers in harborage sites (Orgaz and others 2013). Targeting mature biofilms is essential in removing resident strains from processing facilities, as this is a source of contamination of *L. monocytogenes*. The close proximity of the cells in a biofilm should provide bacteriophages easy access to cells for secondary infection; however, biofilms are surrounded by extracellular polymeric substance (EPS) consisting of bacterial polysaccharides, which protects the cells from antimicrobial agents (Chan and Abedon 2015). Some bacteriophages have polysacharases or polysaccharide lyases that can degrade the EPS and provide entrance to the cells in the biofilm. Montañez-Izquierdo and others (2012) found that the rate of biofilm degradation is dependent on the physiological state of the cells, rather than the EPS barrier. Either way, bacteriophage P100 (Listex^{TM}) have been used to destroy listerial biofilms on stainless steel coupons by significantly reducing the cell counts up to 5.4 log/cm^2 (Soni and Nannapaneni 2010).

L. monocytogenes resistance to bacteriophages

One of the main reasons for the popularity in bacteriophage research is the resistance that bacteria have developed against available antibiotics. In the food industry resistance to sanitizers is also frequently discussed and bacteriophages are thought to induce resistance at a slower rate, as these viruses are the natural predators of bacteria. There are, however, ways in which bacteria can avoid lyses by phages including adsorption reduction, postinfection blocks of the virus, and abortive infections where both the bacterial cell and the bacteriophage die (Hyman and Abedon 2010).

Restriction modification (RM) systems that play a role in phage resistance have been identified in specific *L. monocytogenes* strains. First, a specific RM system has only been identified in strains from epidemic clone II and contains a restriction endonuclease that is expressed at 30 °C and down-regulated at higher temperatures. This system enforces phage resistance in *L. monocytogenes* strains at 30 °C or less, but not at 37 °C (Kim and Kathariou 2009; Kim and others 2012). Two RM systems, which are more widespread, have been identified in *L. monocytogenes* strains from lineage I, II, and III. Evidence that these RM systems can defend the bacterial cell against phage infection is given by Lee and others (2012). In addition, the genomic content of lytic, as well as lysogenic phages were checked for the presence of recognition sites for these RM systems, and it seems that lytic phages might have reduced susceptibility to these RM systems.

Some biocontrol studies have tested the development of resistant *L. monocytogenes* strains to bacteriophages used to treat the bacteria in food products (Carlton and others 2005; Guenther and others 2009; Guenther and Loessner 2011; Chibeu and others 2013). Strains recovered from bacteriophage-treated samples were infected again with the same bacteriophages and checked for resistance to phages. Only clones (3/10) of one *L. monocytogenes* strain were resistant to secondary bacteriophage infection (Guenther and others 2009). In all other cases no resistance was detected to bacteriophages and the bacteria were always destroyed. However, a recent study in Austria found resistance of *L. monocytogenes* strains to Listex^{TM} P100 (2.7%; n = 486) (Fister and others 2015). The strains were isolated over a period of 15 y from 59 dairy processing facilities, some of which used Listex^{TM} P100 as a control agent for *L. monocytogenes*. Resistance of *Listeria* strains to the bacteriophage were associated with the use of Listex^{TM} P100 in the facilities, indicating adaptation of the strains to this specific phage.

Detection of phage-resistant strains after bacteriophage treatment in facilities (Fister and others 2015) is troubling and should serve as an early warning sign about the consequences for using phages. However, strategic application of bacteriophages can aid in preventing the development of bacterial resistance, and availability of current technology enables the monitoring of developing resistance in bacteria. Bacteriophage treatments that include multiple virus strains have been a suggestion to prevent or slow the development of resistant strains and this will also aid in targeting a more genetically variable population as one could find in a processing facility (Sulakvelidze 2013). Using bacteriophages in combination with other antimicrobials as an additional hurdle might also slow resistance. Another option is to use bacteriophage treatments as emergency measures and not routinely (Fister and others 2015). Meaden and Koskella (2013) give a review on the risk of using bacteriophages in natural environments, with a focus on the development of bacterial resistance to phages. Given the possibility of inducing resistance with the use of bacteriophages, future applications should be carefully approached to prevent mistakes similar to when bacteriophages were first discovered.

Choosing a phage

To date, many listeria phages have been identified, although most were isolated during typing studies and, therefore, not characterized for biocontrol application. Klumpp and Loessner (2013) give an updated review of the different listeria phages and their unique characteristics. Most phages are temperate belonging to the family siphoviridae, although there are exceptions. This certainly has implications for biocontrol intentions as temperate phages can integrate into the host DNA and change the genomic makeup. Also, many listeria phages have been found to be capable of generalized transduction during which small pieces of the bacterial host DNA is packaged into the phage head during assembly (Hodgson 2000). Phages intended for biocontrol should, therefore, be obligate lytic with no option for exchange of genetic material.

All evidence indicates that bacteriophages are harmless to humans (Hagens and Loessner 2010) and bacteriophage studies in humans and rats have revealed no adverse effects in test subjects (Bruttin and others 2005; Carlton and others 2005). Although legislation regarding the use of bacteriophages is still unclear, the European Commission stated that bacteriophages should be considered as food additives or as substances used for reducing surface contamination when used on food products (Andreoletti and others 2009). There are, in fact, a few ways in which phage-based products can be regulated; these are described by Sulakvelidze (2013). Hagens and Loessner (2010) listed specific attributes, which phages for food applications should possess, including being issued GRAS status by the U.S. FDA. The first phage product that was approved by the FDA was ListShield^{TM} (formerly known as LMP-102), which was used in several biocontrol studies (Leverentz and others 2003, 2004; Hong and others 2015). Only a month later, another *Listeria*-targeted phage product, Listex^{TM}, was awarded GRAS status by the U.S. FDA.

Apart from the genetic requirements and regulatory approvals for phage-based biocontrol products, there are also the challenges of manufacturing a high-titer product and application of this product in a food-processing environment or food product which will have a significant reduction, if not a complete eradication of the targeted bacteria. Although almost all biocontrol studies have not tested for complete eradication of *L. monocytogenes* after bacteriophage treatment, they do report significant reduction in

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contamination levels. A risk assessment from the FDA and USDA's FSIS found that even a ten-fold reduction in *L. monocytogenes* levels will have a significant effect on the mortality rate of listeriosis (Whiting 2003). If the same results can be accomplished in the processing facilities, bacteriophage treatments will certainly have a massive effect on the safety of food products. However, even in the controlled experiments there are many factors influencing the success of bacteriophage treatment, such as bacterial contamination load and diversity of targeted strains, or rather lack of diversity. In the *L. monocytogenes* biocontrol studies, only one *L. monocytogenes* strain was tested (Leverentz and others 2003, 2004; Bigot and others 2011) or in some cases 2 strains (Guenther and others 2009; Guenther and Loessner 2011). This does not represent a diverse bacterial population and certainly not cells that are under stress, which would be the case in the processing environment.

Use of bacteriophage endolysins to control *L. monocytogenes*

Bacteriophages use lysins or endolysins to degrade the peptidoglycan layer of the bacterial cell wall to facilitate DNA injection, as well as release of newly formed virons. These lysins comprise a diverse group of enzymes that can hydrolyze the peptidoglycan bonds and include amidases, endopeptidases, glycosidases, and carboxypeptidases. The lysins produced by bacteriophages that target Gram-positive bacteria such as *L. monocytogenes* also have cell wall binding domains that strengthen the affinity of the substrate to the enzyme (Callewaert and others 2011). Phage lysins can lyse peptidoglycan of Gram-positive bacteria from the outside, and can therefore be used as antimicrobials. However, research regarding biocontrol of *L. monocytogenes* with lysins in food products is not as frequently published as direct bacteriophage treatments. One study did test a lysin, LysZ5, against *L. monocytogenes* in soy milk. The bacteria were reduced by 4 log units after only 3 h at 4 °C. In addition, the lysins were active against *L. innocua* and *L. welshimeri*, but not *Staphylococcus aureus* and *Enterococcus feacalis* (Zhang and others 2012). Other endolysins from *Listeria* phages have been characterized but their lytic ability has not been shown in food-related matrixes (Zimmer and others 2003; Klumpp and others 2008; Dorscht and others 2009).

Conclusion

L. monocytogenes continues to be a problem for the food industry despite numerous regulations. Persistence of these pathogens in the processing environment, as well as retail shops, is a major concern and regular sanitizers are not always able to kill the bacteria, as seen by the prevalence studies discussed in this review. Additional methods for controlling *L. monocytogenes,* such as treatment with bacteriophages, are needed to aid food manufacturers in meeting the strict regulations for food products. Reductions of *L. monocytogenes* in food products through bacteriophage treatments have been reported. There are, however, many factors that influence the success of these treatments, such as the food matrix itself and the bacteriophages used in the treatment. Liquid food products are treated more successfully, probably due to dispersal of the phages and treatment with higher phage titers (10^8 pfu/mL) always have better results. Only a few studies reported complete eradication of the bacteria, although only one used enrichment culturing to determine bacterial counts after bacteriophage treatment. The reductions are, however, significant and will have an effect on the risk that is presented by *L. monocytogenes*.

Factors that will have a collective influence on the success of a bacteriophage include genetic diversity and distribution throughout a food processing facility or in a food product. In addition, these cells will probably not be in exponential phase or protected by biofilms or the food matrix. No reports of *in situ* bacteriophage biocontrol of *L. monocytogenes* have so far been published, but it can be expected to be less successful than *in vitro* experiments, since *L. monocytogenes* can vary genetically in the environment. The specificity of bacteriophages can be a disadvantage in this case and a phage with a broad host range or a cocktail of phages such as the product ListShield™ is needed to target environmental *L. monocytogenes* strains. Biocontrol with endolysins might also be more successful in treating a more genetically variable population. Evaluation of the *Listeria* population in a specific food processing facility might be necessary before a bacteriophage treatment is applied.

Based on results from the surveillance and biocontrol studies discussed in this review, we have concluded that phage treatments have the potential to positively impact the food industry. However, strategic implementation of bacteriophages or bacteriophage-derived products is necessary for the successful and sustainable control of L. monocytogenes, with the monitoring of resistant strains. To be successful, bacteriophage treatments should also be implemented as part of an existing quality control system in hurdle technology. Finally, the use of bacteriophages in the food industry may also aid in development of therapeutic phage products.

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