Bacteriophage biocontrol to fight *Listeria* outbreaks in seafood

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ABSTRACT

Listeria monocytogenes is a well-known pathogen responsible for the severe foodborne disease listeriosis. The control of *L. monocytogenes* occurrence in seafood products and seafood processing environments is an important challenge for the seafood industry and the public health sector. However, bacteriophage biocontrol shows great potential to be used as safety control measure in seafood. This review provides an update on *Listeria*-specific bacteriophages, focusing on their application as a safe and natural strategy to prevent *L. monocytogenes* contamination and growth in seafood products and seafood processing environments. Furthermore, the main properties required from bacteriophages intended to be used as biocontrol tools are summarized and emerging strategies to overcome the current limitations are considered. Also, major aspects relevant for bacteriophage production at industrial scale, their access to the market, as well as the current regulatory status of bacteriophage-based solutions for *Listeria* biocontrol are discussed.

1. Introduction

Listeria monocytogenes is a well-known pathogen responsible for listeriosis, one of the most serious foodborne diseases. Listeriosis cases are generally asymptomatic but could also manifest as febrile gastroenteritis, meningitis, encephalitis, septicemia or lead to preterm birth and spontaneous abortion (Gray and Killinger, 1966; Swaminathan and Gerner-Smidt, 2007). The latest European Centre for Disease Prevention and Control (ECDC) and European Food Safety Authority (EFSA) annual report on zoonoses reported 2549 confirmed cases of listeriosis in the European Union (EU) in 2018, with 229 related deaths (case-fatality rate of 15.6%) (ECDC/EFSA, 2019a) in at-risk-groups such as elderly people, pregnant women, newborns and immunocompromised patients (Muñoz et al., 2012; Madjunkov et al., 2017). The increasing trend in the number of confirmed cases during recent years (2014–2018) is of major concern for the public health sector and the food industry, and it underlines the need to improve the control of this pathogen in foods, responsible for ca. 99% of listeriosis cases (Allen et al.,2016).

Seafood products have been associated with an increased risk of human listeriosis, being the consumption of 'fish and fishery products', as well as 'crustaceans, shellfish, molluscs and products thereof' the second and fourth cause, respectively, in the list of strongly-evidenced outbreaks that occurred in the EU during the period 2010–2017 (ECDC/EFSA, 2019a). Different seafood products have been implicated in listeriosis outbreaks over time, including raw fish, gravad (sugar-salted), marinated and other minimally processed fish products, as well as Ready-To-Eat (RTE) seafood products (Table 1). Among the 13 described serotypes of *L. monocytogenes*, the serotypes 1/2a, 1/2b and 4b are involved in more than 95% of listeriosis cases (Orsi et al., 2011) and the serotypes 4b and 1/2a are the most frequently isolated ones from seafood (Momtaz and Yadollahi, 2013; Gillesberg Lassen et al., 2016).

Different studies have documented the presence of *L. monocytogenes* in fresh and minimally processed as well as RTE seafood products from many countries during the last decades (Table 2). This pathogen can reach the seafood products via contaminated raw materials or by cross-contamination during different steps of seafood processing (Nakari et al., 2014; Skowron et al., 2018). In addition, its biofilm-forming ability on food-contact surfaces, equipment, floors and drains, together with its high tolerance to disinfectants (Aase et al., 2000; Ortiz et al., 2016), are determining factors in the persistence of these bacteria, even for years, in food processing facilities. Furthermore, *L. monocytogenes* is able to survive and grow at a wide range of temperature (0.4–45 °C) and pH (4.7–9.2), high acidic solutions, high salt concentrations and under osmotic pressures (Gray and Killinger, 1966; Vermeulen et al., 2007; Ribeiro et al., 2014; Orsi and Wiedmann, 2016; Zoz et al., 2017). These unique capabilities of *L. monocytogenes* make it very difficult to eliminate this pathogen from seafood processing equipment and environments, therefore increasing the contamination risk of the final product. This is particularly important for RTE seafood prod-

Table 1
Seafood products associated with listeriosis outbreaks in different countries.

Seafood product	Nº of listeriosis cases (deaths)	Country	References
Cold-smoked salmon, Trout, Gravad salmon	9	Denmark	(ECDC/EFSA, 2019b) [a]
	6	Estonia	
	2	Finland	
	1	France	
	4	Sweden	
Crabmeat	61	UK	(Elson et al., 2019) [b]
Cold-smoked salmon	6	Denmark	(EFSA/ECDC, 2018) [c]
	5	Germany	
	1	France	
Cold-smoked salmon	6	Denmark	(Schjørring et al., 2017)
Cold smoked salmon, trout and halibut, Gravad fish	20 (7)	Denmark	(Gillesberg Lassen et al., 2016) [a]
Marinated herring cutlet	8 (1)	Germany	Aichinger (2010)
Vacuum-packed rainbow trout and salmon	9 (2)	Sweden	(Tham et al., 2000)
Vacuum packed fish	10 (4)	Finland	(Hatakka et al., 2000)

[a] Multi-country outbreak, with 5 deaths reported.
[b] Two outbreaks included.
[c] Multi-country outbreak, with 4 deaths reported.

ucts, as they do not require further treatment or cooking between production and consumption.

The control of *L. monocytogenes* is, therefore, an important challenge for the seafood industry and the public health sector. Innovative strategies, such as bacteriophage-based applications, could contribute to a reduction in the occurrence of *L. monocytogenes* in the food-processing environment and the final food product, helping to ensure the safety of seafood globally.

2. Bacteriophage biocontrol

Bacteriophages, or phages, are viruses able to specifically infect and replicate within target bacteria. They are the most abundant organisms on Earth with an estimated total number of 10^{32} phages (Hanlon, 2007) and play major ecological roles (Weinbauer, 2004; Ofir and Sorek, 2018; Batinovic et al., 2019). Bacteriophages are present in all natural environments in coexistence with their bacterial hosts (Batinovic et al., 2019). As in natural environments, bacteriophages are often consumed by humans as natural microbiota present in a wide variety of foods, including seafood products (Kennedy et al., 1986; Croci et al., 2000; Allwood et al., 2004; Atterbury et al., 2005).

Bacteriophages were independently discovered by William Twort in 1915 and Felix d'Herelle in 1917, the latter of which was also the first one to consider the therapeutic potential of these "bacteria eaters". They were used for therapeutic purposes in the pre-antibiotic era, but upon the development of antibiotics in the 1940s the medical use of bacteriophage was abandoned in western countries (Ofir and Sorek, 2018). However, bacteriophage therapy remained an active research topic in different parts of the former Soviet Union and Poland. In recent years, with the advent of biotechnology and the alarming emergence of antibiotic resistant bacteria, bacteriophages are being considered as potential antimicrobials for the treatment of bacterial diseases in humans, animals and plants (Jamal et al., 2019). Moreover, the use of the so-called virulent bacteriophages targeting specific foodborne

Table 2
Some studies on *L. monocytogenes* prevalence in seafood products.

Country	Year	Seafood product	Prevalence %	References
EU [a]	2018 [b]	RTE fish products	2.7 (198/7294)	ECDC/EFSA (2019b)
	2017 [c]		5.3 (435/8177)	
	2016 [c]		4.7 (155/3325)	
China	2016	Fresh seafood	1.4 (4/287)	(Zhang et al., 2019)
	2016	Fishes	2.6 (61/2328)	(Li et al., 2019)
		Shellfish	2.1 (19/898)	
India	2015–2018	Marine finfish	1.8 (97/5389)	(Basha et al., 2019)
Poland	2014–2016	Fresh fish	17.6 (18/102)	Wieczorek and Osek (2017)
Latvia	2014	Fresh fish	3.0 (1/31)	(Terentjeva et al., 2015)
Thailand	2013–2014	Smoked fish	18.4 (28/152)	(Vongkamjan et al., 2017)
		Raw seafood	2.7 (3/111)	
Iran	2011–2012	Fresh fish	11.4 (12/105)	(Fallah et al., 2013)
		Smoked fish	32.3 (10/31)	
	2010–2011	Fresh fish	7.7 (17/220)	Momtaz and Yadollahi (2013)
		Shellfish	2.5 (1/40)	
Canada	2010	RTE fish products	5 (2/40)	(Kovačević et al., 2012)
EEUU	2010	Fresh and frozen fish	4.3 (3/70)	(Wang et al., 2011)
		Shellfish	2.6 (1/38)	
Finland	2010	Vacuum-packed cold-smoked and gravad fish products	16 (20/126)	(Nakari et al., 2014)
Sweden	2010	Smoked fish	9.4 (32/340)	(Lambertz et al., 2012)
		Gravad fish	14.0 (28/200)	
Estonia	2008–2010	Fresh and frozen fish	2.8 (28/317)	(Kramarenko et al., 2013)
		RTE fish products	5.4 (112/2075)	
		Smoked fish	7.3 (41/563)	
Italy	2007–2009	Smoked fish	34.1 (45/132)	(Pinto et al., 2010)
	2003–2008	Salmon	23.8 (5/21)	(Di Ciccio et al., 2012)
Spain	2007–2009	Smoked fish	1.4 (7/509)	(Domenech et al., 2012)
	2003–2005	Smoked fish	25.0 (10/40)	(Garrido et al., 2009).

Table 2 (*Continued*)

Country	Year	Seafood product	Prevalence %	References
Australia	2006–2007	Shellfish (raw)	4.5 (22/488)	(Pagadala et al., 2012)
		Shellfish (cooked)	0.2 (1/624)	
Turkey	2006–2007	Fresh fish	2.0 (1/50)	(Siriken et al., 2013)
		Shellfish	2.0 (1/50)	
Belgium	2005–2007	Smoked fish	56.9 (33/58)	(Uyttendaele et al., 2009)

[a] Between 18 and 20 reporting Member States (MS) of the European Union (EU).
[b] 18 reporting MS of the EU, with Netherlands, Germany and Poland reporting major prevalence.
[c] 20 reporting MS of the EU.

pathogens in foods is also increasingly accepted as a natural and green food safety strategy usually termed "bacteriophage biocontrol" (Moye et al., 2018; O'Sullivan et al., 2019).

The use of bacteriophages as a food safety strategy is desirable since they are highly specific towards the pathogen of concern, they are harmless to plants, animals and humans, and they do not affect the existing commensal microbiota of the host, food and/or the environment, or alter food properties (Harada et al., 2018). Moreover, bacteriophages are self-replicating and self-limiting, meaning that they do only proliferate if there is a suitable host present (Abedon and Thomas-Abedon, 2010).

Bacteriophages are small viruses, ranging in size from 24 to 400 nm (Jamal et al., 2019), with a proteinaceous capsid (head) that encapsulates their genetic material (single or double stranded RNA or DNA) and/or a tail attached to the capsid through a connector. Receptor-binding proteins (i.e. fibers and spikes) at the end of the tail, are responsible for recognizing specific receptors on the host bacterial membrane (Harada et al., 2018). More than 96% of bacteriophages belong to the order *Caudovirales* with an icosahedral capsid containing double stranded DNA and a tail (Sharma et al., 2017). They have been classified into three families, namely: *Myoviridae* (long and contractile tail), *Siphoviridae* (long and non-contractile tail) and *Podoviridae* (extremely short and non-contractile tail) (Ackermann, 2007; Nobrega et al., 2018). The rest of bacteriophages are cubic, filamentous or pleomorphic (Ackermann, 2007).

According to their life cycle, bacteriophages have been classified into virulent or temperate. Virulent bacteriophages displaying lytic life cycles are able to take over the metabolic machinery of the host after the injection of their own genetic material, eventually leading to the lysis and death of the bacterial cell and the release of the bacteriophage progeny capable of infecting new hosts and repeating the lytic cycle (Batinovic et al., 2019). In contrast, temperate bacteriophages display a lysogenic life cycle involving the integration of their genetic material into the bacterial chromosome (prophage) and its subsequent passive replication as a part of the bacterial genome (Wernicki et al., 2017; Batinovic et al., 2019). Temperate bacteriophages can switch to the lytic cycle, either spontaneously, or in response to cellular internal or external triggers of activation (Sharma et al., 2017; O'Sullivan et al., 2019).

Integration of temperate bacteriophages into the host genome renders the bacterial cell resistant to superinfection by the same type of temperate bacteriophage. This phenomenon, called immunity or homoimmunity (Fogg et al., 2010), is the main reason why only virulent bacteriophages are suitable for biocontrol applications.

3. *Listeria* specific bacteriophages as biocontrol agents

3.1. *Listeria* bacteriophages

Listeria specific bacteriophages, also called listeriaphages, have been isolated mainly from environmental and other non-food sources, including faeces, wastewater, abattoir effluents, soil, farms and sewages (Kim et al., 2008; Schmuki et al., 2012; Ganegama Arachchi et al., 2013a; Denes et al., 2014; Lee et al., 2017; Vongkamjan et al., 2017). Furthermore, *Listeria* bacteriophages have also been successfully isolated from different food products, including seafood products, in studies conducted by our research group (unpublished data).

More than 500 *Listeria* bacteriophages have been identified. However, most of them have been reported as temperate bacteriophages and, therefore, discarded for biocontrol purposes (Hagens and Loessner, 2014). Only few virulent bacteriophages, with potential for *Listeria* biocontrol, have been fully characterized at molecular and genomic level so far (Roy et al., 1993; Carlton et al., 2005; Hagens and Loessner, 2010; Schmuki et al., 2012; Zhou et al., 2020). All virulent listeriaphages have been reported to be able to infect the major *L. monocytogenes* serotypes (1/2a, 1/2b, 1/2c, 4a, 4 ab, 4b, 4c, 4d, 4e) as well as *Listeria innocua* serotypes 5, 6a and 6b, whereas no listeriaphage able to lyse *L. monocytogenes* serotypes 3a, 3b, 3c or *Listeria grayii* have been found (Hagens and Loessner, 2014).

All described listeriaphages belong to the order *Caudovirales* and have been classified within three groups (Hagens and Loessner, 2014). The first group comprises listeriaphages of the *Myoviridae* family, with large genomes of about 140 kb and a broad host range, such as P100, A511, LiMN4L, LiMN4p, and LiMN17 (Carlton et al., 2005; Klumpp et al., 2008; Ganegama Arachchi et al., 2013a). The second group includes listeriaphages belonging to the *Siphoviridae* family with genome sizes of approximately 35–40 kb, such as P35 and P40, all of which are temperate. The last group involves also *Siphoviridae* bacteriophages, with a larger genome of ca. 70 kb, like the lytic phage P70 (Dorscht et al., 2009; Schmuki et al., 2012).

As previously stated, bacteriophages are host-specific, able to infect specific species or even few strains within a single species. Some listeriaphages are an exception to this rule as they can infect strains of different species within the entire *Listeria* genus (Aprea et al., 2018).

3.2. Requirements for biocontrol *Listeria* bacteriophages

Listeria specific bacteriophages must fulfil several requirements to be used as biocontrol agents. The most important criteria are based on their specificity, efficacy, stability and safety (Kakasis and Panitsa, 2019). These properties are mainly characterized by features such as the lytic spectra and other technological properties, such as one step growth curve and whole genome sequencing.

Specificity: Broad lytic spectra. Confirmation of the lytic activity of a bacteriophage is the most common and often the first characterization test and it helps to determine if further analysis is required. Lytic activity should be assessed against a panel of wild-type *L. monocytogenes* with different serotypes and origins as well as other non-*Listeria monocytogenes* strains reflecting the environment under study. Ideally, these *Listeria* strains should be well characterized in order to obtain a panel as diverse as possible. Broad lytic spectra listeriaphages and those capable of lysing bacterial strains that are less susceptible to a wide variety of bacteriophages are highly desirable. Achieving a broad host range or lytic spectrum with a single bacteriophage is very difficult, since bacteriophages are highly specific to a limited range of strains of the pathogen of concern (Harada et al., 2018; Romero-Calle et al., 2019). Thus, the use of bacteriophage cocktails has been proposed to obtain a broader specificity range (Romero-Calle et al., 2019). In these cocktails, the presence of bacteriophages targeting different

host receptors reduces the potential for emergence of bacteriophage resistant bacteria (Nobrega et al., 2015; Aprea et al., 2018).

Efficacy: One step growth curve. Candidate bacteriophages should also have high target pathogen clearance rates, displaying short latent period (time from bacteriophage entry into the bacteria until the first progeny is released), large burst size (number of newly synthesized bacteriophage particles from an infected bacterium) (Abedon et al., 2001; Merabishvili et al., 2018; Sinha et al., 2018; Jariah and Hakim, 2019) and short rise period (time over which a simultaneously infected population of bacteria lyse). The characterization of their single step growth curves allows the determination of these parameters essential to identify the listeriaphages that cause a faster lysis of the pathogen.

Stability under production, storage and application conditions. Listeria bacteriophages should be able to be produced at large commercial scale by using, if possible, prophage-free propagation hosts without virulence or antibiotic resistance markers (Nirmal Kumar et al., 2012). Moreover, they should remain stable and infective from their production time until they reach target *L. monocytogenes* within the seafood product and/or processing environment. Therefore, they should be stable in a wide range of pH values, temperatures, NaCl concentrations, etc.

Safety: Biocontrol listeriaphages should also be safe for human health and for release into the environment. As stated before, temperate bacteriophages should be avoided for biocontrol applications, being virulent bacteriophages the most suitable option for this purpose. Since the specific mechanism of action of each bacteriophage lies within their genome sequence, an exhaustive analysis of the whole genome of listeriaphage candidates must be performed prior to their use as biocontrol tools. This would allow to ensure that the bacteriophage is strictly virulent, lacking any genes involved in lysogeny (i.e. integrase encoding genes), as well as any loci encoding for toxins or antibiotic resistance markers (Fernández et al., 2019).

Sequence analyses have evidenced that *Listeria* strains are very prone to contain integrated prophages within their genome, except for *Listeria ivanovii* (Klumpp and Loessner, 2013; Vu et al., 2019). The presence of prophages has been confirmed in several other strains, e.g., phage A118 in *L. monocytogenes* WSLC 1118 (Loessner et al., 2000), PSA in *L. monocytogenes* ScottA (Briers et al., 2011), A500, A006, B025 and B054 (Dorscht et al., 2009; Kuenne et al., 2013). Hence, for propagation purposes, the use of prophage free host is required. In addition, non-pathogenic hosts for bacteriophage propagation should be prioritized to avoid any potential impurity from pathogens in further purification steps (Hagens and Loessner, 2010).

Humans and animals consume bacteriophages in their daily diets, and, since they consist only of proteins and nucleic acids, degraded bacteriophages would present no toxicological risk. In this sense, a study of the *Escherichia coli* phage T4 intake performed with human volunteers, detected no adverse events (Bruttin and Bru, 2005). Likewise, the results of a dose oral toxicity study performed in rats with listeriaphage P100, concluded that the use of this bacteriophage posed no risk to health at all (Carlton et al., 2005).

Finally, no cross-reactivity or negative impact would be expected on other microorganisms present in the habitat of application (e.g. food, gastrointestinal or environmental microbiome) after the application of *Listeria*-specific bacteriophages as biocontrol measure (Mai et al., 2010; Bueno et al., 2012; Hong et al., 2016; Dissanayake et al., 2019; Richards et al., 2019).

4. *Listeria* specific bacteriophages as a seafood safety strategy

4.1. Application of Listeria bacteriophages in seafood

As outlined in Table 3, various studies have shown the effectiveness of different *Listeria* specific bacteriophages to control *L. monocyto-*

genes in fresh and RTE seafood products (Guenther et al., 2009; Soni and Nannapaneni, 2010a; Soni et al., 2010, 2014; Perera et al., 2015; Baños et al., 2016; EFSA (BIOHAZ), 2016; Axelsson et al., 2020; Zhou et al., 2020).

Regarding the applications on raw fresh fish, the commercial preparation Listex™ P100 (monophage preparation consisting of the single P100 bacteriophage targeting *Listeria*), added at a MOI (multiplicity of infection: ratio between bacteriophage dose and *L. monocytogenes* load) of 10^3, induced 1.5 log reduction in raw cat fish, which remained consistent after 10 days of storage at 4 °C (Soni et al., 2010). In another study, Baños et al. (2016) treated raw hake at MOI of 10^4 and observed between 1.2 and 2.0 log reduction after 1 and 7 days of storage at 4 °C, respectively. Smaller reductions of only 1 log were found in raw salmon fillets treated under the same conditions (Baños et al., 2016), suggesting a clear effect of the seafood product properties (e.g. composition) on the effectiveness of Listex™ application. Slightly greater reduction of 1.4 log were found also in raw salmon one day after adding Listex™ P100 at a higher MOI of 10^6 (Soni and Nannapaneni, 2010a). In a very recent study *L. monocytogenes* inoculated raw salmon was treated with another single bacteriophage, named SH3-3, at a MOI of 10^3, finding a significant reduction of more than 4 log after 72 h of storage at 4 °C (Zhou et al., 2020). This fact confirms that bacteriophage(s) intrinsic properties (e.g. lytic spectra) directly affect treatment effectiveness.

Regarding the effectiveness of *Listeria* bacteriophages on RTE seafood products, some studies carried out with contaminated smoked salmon showed variable success (Table 3). In particular, for low inoculation levels of 1.2–2 log CFU/g the application of Listex™ P100 at a MOI of 10^7 -10^8 was observed to significantly reduce *L. monocytogenes* growth during storage, reporting differences in *Listeria* counts by up to 5 log among treated and non-treated samples after 28 days refrigerated storage (EFSA (BIOHAZ), 2016). However, applications of the same P100 bacteriophage at lower MOI of 10^5 and 10^4 yielded between 1 and 2.5 log reduction on contaminated smoked salmon after 30 days storage (Guenther et al., 2009; Soni et al., 2014; Baños et al., 2016), indicating the impact of process-related parameters (e.g. MOI ratio, mode of application, etc.) in the success of bacteriophage treatment. In fact, high MOI ratios of around five have been recommended for adequate *L. monocytogenes* reductions using Listex™ P100 in foods (Montañez-Izquierdo et al., 2012).

It is also worth pointing out that an enhanced effectiveness of Listex P100 has been reported when used in combination with other antimicrobials, such as enterocin or nisin, on the surface of fresh and RTE seafood products (Soni et al., 2014; Baños et al., 2016). Moreover, different inactivation results were found by some of these studies directly related to the *L. monocytogenes* strain tested (Guenther et al., 2009; Soni et al., 2010; EFSA (BIOHAZ), 2016) as well as to the initial *L. monocytogenes* contamination levels (EFSA (BIOHAZ), 2016a,b).

In conclusion, the effectiveness of *Listeria* bacteriophages to reduce *L. monocytogenes* loads in seafood is determined by a combination of the following parameters: (i) specific bacteriophage-related factors (e.g. monophage vs. cocktail, lysis spectra, infection kinetics parameters etc.); bacteria-related factors (e.g. *L. monocytogenes* strain and serotype, load, etc.); seafood product-related factors (e.g. composition, surface morphology with/without fissures, etc.); and, processing-related factors (e.g. ratio between bacteriophage dose and *L. monocytogenes* load (MOI), application mode, etc.).

4.2. Application of Listeria bacteriophages in biofilms and processing environments

Elimination of *Listeria* biofilms from processing equipment, seafood-contact surfaces, floors and drains is also a huge challenge in

Table 3

Studies of direct *Listeria* bacteriophages application against *L. monocytogenes* onto seafood products.

Seafood	Bacteriophage	Contamination	Treatment [a]	*Listeria monocytogenes* reduction	Reference
Raw catfish	Listex™ P100	**Mixture *Lm*:** EGD (ser. 1/2a) Scott A (ser. 4b) 10^4 CFU/g	**P100:** 2×10^7 PFU/g Spread on surface Storage: 10 d at 4 °C/10 °C	**4° C:** 1.5 log CFU/g (30 min–10 d) **10 °C:** 1.7 log CFU/g (30 min) 2.5 log CFU/g(10d) No regrowth after 10d of storage	(Soni et al., 2010)
Raw hake	Listex™ P100 and/or enterocin AS-48 [a]	**Mixture *Lm*:** Five strains 10^3 CFU/cm^2	**P100:** 2.3×10^7 PFU/cm^2 **AS-48:** 50 mg/mL Automated spray Storage: 7 d at 4 °C	**P100:** 1.2–2.0 log CFU/cm^2 (1 d and 7 d) **P100 + AS-48:** 2.0 log CFU/cm^2 from 1 d of storage	(Baños et al., 2016)
Raw salmon				**P100:** 0.85–1.06 log CFU/cm^2 (1 d and 7 d) **P100 + AS-48:** 2.0 log CFU/cm^2 from 2 d of storage	
Raw salmon fillets	Listex™ P100	**Mixture *Lm*:** EGD (ser. 1/2a) Scott A (ser. 4b) 10^2 CFU/g	**P100:** 10^8 PFU/g Spread on surface Storage: 10 d at 4 °C	1.4 log CFU/g (1 d) >2 logs CFU/g (10 d)	Soni and Nannapaneni (2010a)
Raw salmon	Phage vB-LmoM-SH3-3	***Lm*:** LM008 10^5 CFU/g	**SH3-3:** 10^8 PFU/g Spread on surface Storage: 72 h at 4 °C	2.67 log CFU/g (24 h) >4 log CFU/g (72 h)	(Zhou et al., 2020)
Smoked salmon	Listex™ P100 and/or Nisin	**Mixture *Lm*:** V7 (ser. 1/2a), EGD (ser. 1/2a), F4393 (ser. 4b), F5069 (ser. 4b), ATCC 43257 (ser. 4b)	**P100:** 10^8 PFU/g **Nisin:** 500 ppm Storage: 24 h at 4 °C Vacuum packaging bag	**P100:** 2.5 log CFU/cm^2 (24 h) **P100 + nisin:** 3.5 log CFU/cm^2 (24 h) (below detectable levels)	(Soni et al., 2014)
Smoked Salmon (SS)	Listex™ P100	***Lm*:** WSLC 1001 (ser.1/2c) 10^3 CFU/g	**P100:** 3×10^8 PFU/g, Spread on surface Storage: 6 d at 6 °C	**SS:** ~1 log CFU/g (6 h–6 d) **MS** > 2 log CFU/g (6 h and 6 d)	(Guenther et al., 2009)
Mixed seafood (MS)	A511	**Mixture *Lm*:** WSLC 1001 (ser. 1/2c) and Scott A (ser.4b). 10^3 CFU/g	**A511:** 3×10^8 PFU/g Spread on surface Storage: 6 d at 6 °C	**SS:** 0.8–2.2 logs CFU/g (6 h–6 d) depended on tested strain **MS**: ~2 log CFU/g (6 h) >2 log CFU/g (6 d)	

Table 3 (*Continued*)

Seafood	Bacteriophage	Contamination	Treatment [a]	*Listeria monocytogenes* reduction	Reference
Smoked salmon	Listex™ P100 and/or enterocin AS-48 [a]	**Mixture *Lm*:** Five strains 10^3 CFU/cm^2	**P100:** 2.3×10^7 PFU/cm^2 **AS-48:** 50 mg/mL Automated spray Storage: 30 d at 4 °C	**P100:** 0.85 log CFU/cm^2 (1d) 1.58 log CFU/cm^2 (30 d) **P100 + AS-48:** 2.0 log CFU/cm^2 from 1 to 15 d; Slight regrowth 15 d–30 d	(Baños et al., 2016)
Smoked salmon	ListShield™ (LIST-36, LMSP-25, LMTA-34, LMTA-57, LMTA-94 and LMTA-148)	**Mixture *Lm*:** Lm320 (ser. 4b) Lm68 (ser. 1/2b) Lm82 (ser. 1/2a) 10^3 CFU/g	**ListShield™ cocktail:** 9×10^5 PFU/g 2×10^6 PFU/g Spray gun Storage: 24 h at 4 °C	9×10^5 PFU/g: 0.4 log CFU/g (24 h) 2×10^6 PFU/g: 1 log CFU/g (24 h)	(Perera et al., 2015)
		***Lm*:** Lm376 2×10^3 CFU/g	**ListShield™ cocktail:** 1.5×10^6 PFU/g Spray gun Storage: 24 h at 4 °C	Below detectable levels (24h)	
Smoked salmon	Listex™ P100	***L.m*:** 215TJ01 **A)** 3×10 CFU/g **B)** 10^2 CFU/g	**P100:** 10^9 PFU/cm^2 Spread on surface Storage: 28 d (1/3 at 4 °C and 2/3 at 8 °C) Vacuum-packaging/Sealing	A) 4 log CFU/g (28 d) B) 3 log CFU/g (28 d)	(EFSA (BIOHAZ), 2016)
		***L.m*:** 71SDV1 **A)** 10 CFU/g **B)** 2 CFU/g		A) 5 log CFU/g (28 d) B) 4 log CFU/g (28 d)	
Rakfisk (salted and fermented fish)	Listex™ P100	**Mixture *Lm*:** MF2132, MF3508, MF3509, MF3511 and MF3512 10^4–10^5 CFU/g	Before ripening process **P100:** 10^8 PFU/cm^2 Spread on surface Storage: 91 d at 7 °C	1 log CFU/g (0 d–91 d)	(Axelsson et al., 2020)
			After ripening process **P100:** 10^8-10^9 PFU/piece Spread on surface Storage: 5 d at 8 °C	10^8 PFU/piece: 0.6 log CFU/g (1 d) 0.3 log CFU/g (5 d) 10^9 PFU/piece: 1 log CFU/g (1 d) 0.6 log CFU/g (5 d)	

Lm: *L. monocytogenes* strain/strains individually tested; **Mixture *Lm*:** mixture of *L. monocytogenes* strains; **MS:** This category included cooked and chilled cocktail of shrimp, mussels, and calamari; **d:** day/days; Spreading (including 'spot inoculation').

*Bacteriocin extracted from *Enterococcus faecalis* UGRA10.

[a] Treatment was added from 10 min to 1 h after products contamination with *L. monocytogenes*.

seafood safety since they are a significant source of contamination in the seafood production chain, threatening the safety of the final products.

Only few studies have analyzed the effectiveness of bacteriophages to eradicate *L. monocytogenes* biofilms at different maturity levels, ranging from 24 h to 2 weeks, on food processing surfaces and/or environments (Table 4).

Listeria bacteriophages H387, H387-A, and 2671 were shown to disrupt biofilms on polypropylene and stainless-steel surfaces, reducing *L. monocytogenes* by more than 3 log (Roy et al., 1993). Listeriaphages LiMN4L, LiMN4p, and LiMN17 were also used against biofilms of *L. monocytogenes* seafood isolates grown on stainless-steel and stainless-steel surfaces coated with fish protein preparations, inducing more

than 3 log reduction with both single phage and cocktail application (Ganegama Arachchi et al., 2013b). The cocktail ListShield™ induced lower inactivation of *L. monocytogenes* biofilm of 2 log on stainless-steel surfaces and of only 1 log reduction on a rubber surface (Sadekuzzaman et al., 2017). Similarly, a 1 log reduction was observed by Gutiérrez et al. (2017) on stainless-steel coupon surfaces treated with the cocktail ListShield™ for 4 h. This study also showed that the Listex™ P100 commercial preparation was only able to infect 7 out of 11 tested *Listeria* strains. Longer treatment with Listex™ P100 for 24 h at room temperature showed higher reductions of about 5 log on biofilms of *L. monocytogenes* cocktails grown on stainless-steel coupon surfaces (Soni and Nannapaneni, 2010b; Montañez-

Table 4
Studies of direct *Listeria* bacteriophages application against *L. monocytogenes* biofilms.

Surface	Bacteriophage	Contamination	Treatment	*L. monocytogenes* reduction	Reference
Stainless-steel polypropylene	H387, H387-A, 2671	**Lm:** 10401(ser. 4) and 8427	**Single phage and cocktail:** >3.5 × 10^8 PFU/mL Dipping	>3 log CFU/ml	(Roy et al., 1993)
Stainless-steel Stainless-steel coated with fish protein	LiMN4L, LiMN4p, LiMN17	**Lm:** 19CO9, 19DO3 and 19EO3 10^8 CFU/ml Biofilm: 5 h/ 7 d at 15 °C	**Single phage and cocktail:** 10^9 PFU/mL Dipping using microplates Incubation: 1 h at 15 °C	>3 log CFU/mL (1 h) with single phage and cocktail	(Ganegama Arachchi et al., 2013b)
Rubber coupon	ListShield™, (LIST-36, LMSP-25, LMTA-34, LMTA-57, LMTA-94 and LMTA-148)	**Lm:** ATCC 19113, ATCC 19115 and ATCC 13932 Biofilm: 72 h at 30 °C	**ListShield™:** 10^8 PFU/mL Incubation: 2 h at 10 °C and 30 °C	>1 log PFU/mL (2 h)	(Sadekuzzaman et al., 2017)
Stainless-steel coupon				>1.9 log PFU/mL (2 h)	
Stainless-steel coupon	ListShield™ Listex™ P100	**Lm:** Eleven strains 10^6 CFU/well Biofilm: 72 h at 12 °C	**ListShield™:** 10^7 PFU/well **P100:** 10^9 PFU/well Incubation: 4 h at 12 °C	**ListShield™ and P100** 0.3–4.9 log PFU/well in the most of strains	(Gutiérrez et al., 2017)
Stainless-steel coupon	Listex™ P100	**Lm:** CCUG 15526 10^3 CFU/ml Biofilm: 72 h at rt	**P100:** 10^5, 10^6, 10^7 or 10^8 PFU/mL Incubation: 48 h at rt	5.29 log CFU/cm^2 (24 h) Below detected levels (48 h)	(Montañez-Izquierdo et al., 2012)
Stainless-steel coupon	Listex™ P100	**Mixture Lm:** Five strains (ser. 1/2a and 4b) 10^8 CFU/cm^2 Spotting onto pieces Biofilm: 2 d and 7 d	**P100:** 10^9 PFU/mL Dipping using microplates Incubation: 24 h rt	3.5 log CFU/cm^2 (24 h in biofilms of 2 d) 5.4 log CFU/cm^2 (24 h in biofilms of 7 d)	Soni and Nannapaneni (2010b)
Stainless-steel wafers	Listex™ P100	**Mixture Lm:** Scott A, NCTC 7979, NCTC 10887, NCTC 10527 and DSA 25 Biofilm: 4 d at 30 °C	**P100:** 10^8 PFU/mL Incubation: 24 h at 20 °C	Complete elimination of biofilm	(Iacumin et al., 2016)
Stainless-steel surface with different grooves	Listex™ P100	**Mixture Lm:** LF 38 (ser. 1/2a), LF 36 (1/2b), LF 29 (4e) 10^7 CFU/ml Biofilm: 14 d using food residues (MAPCH, CE and SS) [a]	**P100:** 10^9 PFU/mL Spraying Incubation: 20 min at rt	**0.2 mm grooves:** >3 log CFU/ml **5 mm grooves:** 1.4 log CFU/ml	(Chaitiemwong et al., 2014)

Lm: *L. monocytogenes* strain/strains individually tested. **rt:** room temperatureE.

[a] **MAPCH:** Modified atmosphere–packed cooked ham; **CE:** chopped endives; **SS:** vacuum-packed smoked salmon. **d:** day/days.

Izquierdo et al., 2012). In a later work, the same treatment with Listex™ P100 (for 24 h at room temperature) produced the complete elimination of *L. monocytogenes* biofilm on stainless-steel wafers (Iacumin et al., 2016). However, this prolonged treatment application, is not practical for a food processing surface (Gray et al., 2018

). Moreover, Chaitiemwong et al. (2014) showed a decrease in the Listex™ P100 efficacy when treated surfaces presented crevice features and food residues, observing a maximum of 1.4 log reduction.

In conclusion, different parameters, including (i) specific bacteriophage(s)-related factors (e.g. monophage vs. cocktail, lysis spectra, in-

fection kinetics parameters, etc.); (ii) biofilm-related factors (e.g. structure and composition); (iii) bacteria-related factors (e.g. *L. monocytogenes* strain and serotype, metabolic state of the *Listeria* cells within the biofilm); (iv) extracellular matrix-related factors (e.g. presence of food components and nutrients); and (v) process-related factors (e.g. mode of application, treatment time and temperature), can affect the effectiveness of bacteriophages to disrupt *Listeria* biofilms. Further research considering multi-species biofilms as well as combination with other decontamination/disinfection methods, such as steps to disrupt biofilm or the removal of organic matter, is needed to better assess the potential of bacteriophage treatment to remove biofilms in processing environments.

5. Current limitations and strategies to overcome

5.1. Development of bacteriophage-resistance

It is well known that bacteria can protect themselves against bacteriophage infection through different mechanisms. The most relevant bacteriophage-resistance mechanisms aim to prevent host recognition and bacteriophage adsorption to the bacterial host and include the spontaneous mutations within receptor genes, the modification or loss of bacterial surface receptors (e.g. lipopolysaccharides, pili and flagella), the production of physical barriers hiding receptors (e.g. extracellular matrix or capsules), or the production of competitive inhibitors (Hyman and Abedon, 2010; Labrie et al., 2010; Rostøl and Marraffini, 2019). Other bacterial resistance mechanisms can act during bacteriophage replication within the host cell. These include the horizontal acquisition of restriction-modification systems, or the development of adaptive immunity systems by interfering clustered regularly interspaced short palindromic repeats (CRISPRs) or CRISPR-associated (cas) sequences, resulting in cleavage or degradation of the injected bacteriophage DNA (Hyman and Abedon, 2010; Labrie et al., 2010; Rostøl and Marraffini, 2019). Resistance mechanisms can be transmitted from resistant to sensitive bacteria through the transduction of bacterial DNA via bacteriophages, leading to the development of bacteriophage-resistant mutants (Aprea et al., 2018; Rostøl and Marraffini, 2019).

Rapid emergence and/or selection of bacteriophage-resistant *L. monocytogenes* may represent a concern associated with the use of listeriaphages as biocontrol agent. It has been shown the presence of bacteriophage-resistant *L. monocytogenes* isolates from samples taken from food processing facilities (Vongkamjan et al., 2013; Fister et al., 2016a, 2016b). However, very little is known about the bacteriophage-resistant mutant strains of *L. monocytogenes*. Fister and others associated the 2.7% of bacteriophage resistant *L. monocytogenes* strains (13 out 486) isolated from 5 out of 59 dairy processing facilities with the use of one bacteriophage as control agent in the facilities and the subsequent phage receptor modifications in *Listeria* mutants (Fister et al., 2016a). Concerning food-related *L. monocytogenes* isolates, some studies found no evidence of resistant mutant strains among isolates recovered from different food products after their bacteriophage treatment (Carlton et al., 2005; Chibeu et al., 2013; Fister et al., 2016a; Guenther et al., 2009; Guenther and Loessner, 2011; Kim et al., 2008). Guenther and Loessner (2011) observed phage resistance in 30% (3 out of 10) of the clones of one *L. monocytogenes* strain re-isolated from bacteriophage treated cheeses. However, no further clarification of the developed resistance mechanism was provided. Denes et al. (2014) isolated and sequenced 69 spontaneous mutant strains of *L. monocytogenes* that were shown to resist bacteriophage infection through mechanisms of adsorption inhibition (i.e., alterations of the cell surface that affect phage attachment). None of the bacteriophage-resistant mutant strains from this study developed post-adsorption resistance mechanisms, such as CRISPR-mediated bacteriophage immunity (Denes et al., 2014). However, restriction modification sys-

tems enforcing bacteriophage resistance were identified by another study in specific *L. monocytogenes* strains, although virulent bacteriophages might have reduced susceptibility to these systems than temperate bacteriophages (Strydom and Witthuhn, 2015).

It is worth noting that the bacteriophage-resistance acquisition could have substantial secondary costs for bacteria (Koskella and Brockhurst, 2014). For example, since bacteriophage receptors present on the bacterial surface often act as virulence factors, bacteriophage-resistant mutants arising through receptors loss or modification are generally less virulent than non-resistant strains (León and Bastías, 2015; Scanlan et al., 2015).

Although development of bacteriophage-resistance is one of the major concerns for bacteriophage biocontrol, it is also the most frequent mechanism driving the so-called bacteriophage-bacteria coevolution (reciprocal selection for resistance and infectivity), that involves bacteriophage adaptation to overcome host defense mechanisms and allow successful infection (Labrie et al., 2010; Koskella and Brockhurst, 2014; Rostøl and Marraffini, 2019). Furthermore, the adaptation to a specific host can both increase infectivity and reduce the likelihood of subsequent host resistance evolution (Scanlan et al., 2015).

There are other different strategies that can be used to minimize the likelihood of resistance formation. The main relevant strategy to avoid bacteriophage-resistance is the formulation of bacteriophage cocktails, since the activity of different bacteriophages (e.g. with different receptors on the surface of bacteria) combined against the same bacterial host, reduces significantly the possibility of bacteria developing resistance against more than one phage infection system simultaneously (Nobrega et al., 2015; Aprea et al., 2018). Moreover, it has been shown that the acquisition of resistance to one bacteriophage of the cocktail sometimes results in enhanced infection by other phages (Fernández et al., 2019). Moreover, bacteriophage cocktails could be modified to include novel bacteriophages targeting bacteria that have developed resistance against a previously used bacteriophage. Finally, the use of bacteriophages in combination with other antimicrobials, such as endolysins, could also be an alternative to overcome bacteriophage-resistance development (Strydom and Witthuhn, 2015).

5.2. High specificity and narrow lytic spectrum

A major hurdle in the use of bacteriophages for biocontrol is their high host specificity, being almost impossible to target all the strains within a species with a single bacteriophage (Chen et al., 2019). Therefore, the application of multiple bacteriophages within a cocktail compared to a single bacteriophage preparation may be superior, not only in reducing the risk of emerging resistant bacteria, but also in terms of providing broader coverage of the target pathogen. As proposed by several authors, a mixture of different and complementary bacteriophages is an option to broaden the limited lytic spectrum of a single bacteriophage (Hyman, 2019; Kakasis and Panitsa, 2019; Romero-Calle et al., 2019). As previously mentioned, bacteriophages with broad lytic spectra and those capable of lysing bacterial strains that are less susceptible to a wide variety of bacteriophages are highly desirable in cocktails preparations (Janež and Loc-Carrillo, 2013). However, the effectiveness of bacteriophages in cocktails need also to be tested to evidence additive, synergetic or even undesirable antagonistic interactions among them (Merabishvili et al., 2018).

Even with the combination of complementary bacteriophages it is difficult to cover all targeted bacterial strains or species. One strategy to overcome this limitation is the coevolution of bacteriophages with different host bacterial strains, in a so-called bacteriophage adaptation or training, which can be easily performed by serial passages of the bacteriophages on different propagation strains (Merabishvili et al., 2018). Other strategies have explored the antimicrobial synergy be-

tween bacteriophages and other natural antimicrobials and/or endolysins (Moye et al., 2018).

To specifically address this point, different authors have proposed the genetic manipulation of bacteriophage genomes (e.g. modifying the receptor-binding proteins of bacteriophages) as a way to shape bacteriophages into safe and efficient biocontrol agents (Barbu et al., 2016; Pires et al., 2016; Moye et al., 2018; Dunne et al., 2019). By means of genetic engineering the efficacy of bacteriophages could be improved not only broadening their lytic spectra, but also by removing self-inhibition mechanisms, overexpressing genes such as holins or incorporating new genes such as anti-CRISPR genes (Huss and Raman, 2020). Bacteriophages have also huge potential as immobilized components in food packages, which could be a successful strategy for bacterial growth biocontrol during food product storage (Anany et al., 2011; Lone et al., 2016).

5.3. Consumer acceptance

Bacteriophage biocontrol offers a natural, green and specific antimicrobial approach for improving seafood safety. However, one major concern is the acceptability of this biocontrol approach for producers and consumers. In fact, the final consumer may be disinclined to purchase foods processed with "viruses" and, therefore, may react negatively to bacteriophage food applications. Therefore, in order to increase bacteriophage biocontrol acceptability, it would be essential to provide education on the safety, efficacy and ubiquity of bacteriophages to both the final users (seafood processors) as well as the consumers. In a recent study of our group (unpublished data), consumers appeared to be willing to pay more for safer bacteriophage-treated food products after the advantages of this biocontrol solution were explained to them. Although the results are preliminary, acceptance among both producers and consumers was high, and these results represent a step forward in the application of bacteriophages for biocontrol of foodborne pathogens within the farm-to-fork process.

6. *Listeria* bacteriophages: from lab to market

6.1. Production at industrial scale

Under laboratory conditions bacteriophages are grown on a small scale (5–50 mL) or as plate lysates (Clokie and Kropinski, 2009). For laboratory use such crude lysates are commonly only minimally processed. Bacterial debris are removed by centrifugation, the cleared supernatants are filter sterilized and then stored at refrigeration temperatures in the medium used to grow the bacterial host. But some laboratory experiments, like electron microscopy, require pure and highly concentrated phage preparations (Ackermann, 2009). Also,. food, medical and veterinary applications require more elaborate purification steps (Merabishvili et al., 2009; Gill and Hyman, 2010; Van Belleghem et al., 2017; Hietala et al., 2019). To this end, bacteriophages were concentrated by polyethylene glycol precipitation (Yamamoto et al., 1970) followed by ultracentrifugation in a $CsCl_2$ gradient (Boulanger, 2009). A milder method based on anion-exchange chromatography using CIM® monolith columns was reported to produce pure phage preparations with high titers (Kramberger et al., 2010; Smrekar et al., 2011; Adriaenssens et al., 2012). However, neither of these two methods can easily be scaled up to purify bacteriophages in quantities relevant for a commercial production (i.e. more than 20 L).

Currently, a single bacteriophage (monophage) is fermented in batch cultures, which are preferred over continuous fermentation processes as they require less dedicated equipment and allow easy process control. For each monophage and its host, optimal bacterial growth conditions (medium, temperature, aeration and agitation) have to be formulated. After bacteriophage amplification the phage

lysates are purified with multistep protocols involving filtration, tangential flow-filtration, buffer exchange and sterile filtration steps, as described in the GRAS Notices No. 198, 218 and 528 (FDA, 2014, 2007, 2006). Stringent quality control measures will then ensure the identity, potency and sterility of the monophage preparation.

6.2. Commercial products and their regulatory status

In order to receive a product license for food safety applications, bacteriophage products have to meet specific criteria defined by administrative bodies (Sulakvelidze and Pasternack, 2014). Currently, only few countries have developed legislation concerning the application of bacteriophages as antimicrobial agents in food, food manufacturing and ready-to-eat food products (Moye et al., 2018). For example, the US FDA grants a GRAS-status ("Generally Recognized As Safe") to a bacteriophage-containing commercial product for a defined application, when the manufacturer is able to show that the active substance of his product is safe to the consumer in the intended amounts of use (non-toxic, not allergenic and does not contain any known virulence factors). The manufacturing process has to be safe and the intended application has to provide a benefit for the consumer. A granted GRAS-status allows a manufacturer to sell his product on the US market.

In 2006 the US FDA granted a GRAS status to a commercial product called Listex™ P100 to be used as an antimicrobial to control *L. monocytogenes* during cheese ripening, GRAS Notices No. 198 (FDA, 2006). In 2007 Listex™ P100 was additionally granted a GRAS-status as antimicrobial to control *L. monocytogenes* in food in general, including meat and poultry products, GRAS Notices No. 218 (FDA, 2007). Currently, this product is marketed as Phage Guard Listex™ P100 by the Dutch company Micreos B.V. as antimicrobial to control *L. monocytogenes* in food products when applied at levels of up to 10^9 PFU per gram of food. Phage Guard Listex™ P100 is a monophage preparation with a six months shelf-life, containing the well characterized phage P100 (Carlton et al., 2005). The phage is grown on a non-pathogenic *L. innocua* strain (ATCC33090, DSM, 20649) and its titer in the concentrated product is 10^{11} PFU/mL.

A second product, ListShield™ has been approved by the US FDA for *Listeria* biocontrol. It is sold by the US company Intralytix, GRAS Notices No. 528 (FDA, 2014). ListShield™ contains six bacteriophages that were isolated from the environment and characterized by a variety of methods, including electron microscopy, pulse-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) and genome sequence analysis. The phages are grown on four distinct *L. monocytogenes* hosts, which are the proprietary strains of the company. ListShield™ contains 10^{10} PFU/mL and is intended for use on food surfaces of such categories as fish and shellfish, fresh and processed fruits and vegetables and cheese, when applied at levels of up to 10^8 PFU per gram of food. The product shelf-life is 12 months.

Relevant health authorities of other countries such as Switzerland, Israel, Canada, Australia, New Zealand or Brazil, have also approved listeriaphage applications on foods. However, in the European Union there is currently no clear path regarding how to register a bacteriophage product. The EFSA Panel on Biological Hazards European Food Safety has published three Scientific Opinions related to the use of bacteriophages. The first one included general information about bacteriophages and their role as decontaminants, focusing on efficacy and reporting a few concerns (EFSA and BIOHAZ, 2009). The second one was published in response to Listex™ and focused on the safety of this product alone (EFSA and BIOHAZ, 2012). It was not until the third example that safety and efficacy of a product was accepted for several food commodities (EFSA and BIOHAZ, 2016a,b). Nevertheless, the European Commission did not agree on a regulatory path for bacteriophages in food production and relegated the responsibility back to the individual member states (European Commission, 2018).

6.3. Industrial application

Although two commercial products for *Listeria* biocontrol have been on the US market for several years, their implementation in the industrial setting is not trivial.

First, the load of *L. monocytogenes* on or in food in general is low. To ensure that every bacterial cell is destroyed, large numbers of bacteriophages have to be homogeneously distributed on or in the food. According to the data shown in Table 3, 10^7 to 10^8 PFU/g of food are required for a successful application, thus approximately 100 mL to 1 L of a bacteriophage product with a concentration of 10^{11} PFU/mL would be required to treat 1 ton of fish. Traditional preservatives like nisin or lauric arginate are used in comparable amounts, 500 g/ton or 200 g/ton, respectively.

Second, the application of a bacteriophage product initially requires extra time, extra equipment and an extra processing step in the food production workflow under conditions that allow the application of a liquid and that support the activity of the added bacteriophages. Nevertheless, a bacteriophage application could lead to a significant reduction of the number of batches which do not pass the microbial quality control, thus reduce food waste and improve food safety, justifying such a onetime investment.

Third, also the cost of a bacteriophage application might be a limiting factor in highly competitive markets. However, these costs might be counterbalanced by the unique opportunity to actively fight *Listeria monocytogenes* in food and the food production environment, using a natural and potent bacteriophage product.

7. Conclusions

Despite the great progress made to improve seafood safety, *L. monocytogenes* continues to be found in different raw, minimally processed and RTE seafood products, which have been implicated in numerous outbreaks of listeriosis in recent years.

The biological properties of lytic bacteriophages as well as available data on the effectiveness of *Listeria* specific bacteriophages on seafood products and seafood processing environments, make bacteriophage biocontrol a promising strategy for seafood safety applications. The use of *Listeria* bacteriophages could contribute as an additional tool in a multi-hurdle approach in order to safely reduce the occurrence and growth of *L. monocytogenes* in seafood and, therefore, prevent the incidence of seafood-related listeriosis outbreaks. Moreover, as natural and green innovative technology, bacteriophage biocontrol is prone to be accepted by the consumer who is increasingly reluctant to the use of chemical antibacterial substances.

Overall, the future of *Listeria* bacteriophages is positive, but some challenges remain before their application in seafood production can be considered routine. *Listeria* specific bacteriophages should be carefully selected to suit the requirements on their specificity, efficacy, stability and safety, as presented above. Among the greatest barriers to overtake are the limited lysis spectra of most of them and the emergence of bacteriophage-resistant bacteria. The formulation of *Listeria* specific bacteriophage cocktails could overcome both limitations and obtain a broader range specificity and, at the same time, minimize the likelihood of resistance development. Further research at semi-industrial and industrial level (challenge tests) is essential to assess the efficacy of new future (even near future) *Listeria* bacteriophage cocktails, as well as the potential development of resistant bacteria, during seafood storage (shelf life).

CRediT authorship contribution statement

Amaia Lasagabaster: Conceptualization, Data curation, Investigation, Investigation related to chapter 1–5, Writing - original draft, Writing - review & editing, Supervision. **Elisa Jiménez:** Conceptualization, Data curation, Investigation, Investigation related to chapter 1–5, Writing - original draft, Writing - review & editing. **Tatiana Lehnherr:** Data curation, Investigation, Investigation related to chapter 6, Writing - original draft, Writing - review & editing. **Katherine Miranda-Cadena:** Data curation, Investigation, Investigation related to chapter 1–5, Writing - original draft, Writing - review & editing. **Hansjörg Lehnherr:** Conceptualization, Data curation, Investigation, Investigation related to chapter 6, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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