Evidence for the industrial application of bacteriophages to control *Listeria monocytogenes* in leafy greens

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Summary
The use of bacteriophages has provided a promising option for the control of *Listeria monocytogenes* (Lm) in fresh produce at a lab scale. However, there is a lack of knowledge on the effectiveness of bacteriophages in industrial settings. This project will validate the application of bacteriophages as a post-process treatment in baby spinach under industrial-scale operating procedures. The project will be able to demonstrate if there are benefits of the use of this biocontrol treatment despite the variability of the quality of raw material by seasonality and abusive storage conditions. Additionally, a cost-benefit analysis of the industrial application will be calculated. The effectiveness of bacteriophages will be confirmed by the absence of Lm/*Listeria* spp. compared with untreated spinach while preserving the quality of the product.

Objectives
1. To verify in an industrial setting the effectiveness of bacteriophages controlling *Listeria monocytogenes* (Lm) growth while preserving the quality of leafy greens affected by seasonality.
2. To confirm the beneficial impact of bacteriophages controlling Lm growth at abusive temperatures and the interaction with the natural microbiota.
3. To estimate the cost-benefits of the industrial application of bacteriophages as a post-process treatment to control Lm.

Methods
The methodology for the enumeration and detection of Lm/*Listeria* spp. has been optimized. *Listeria* spp. will be enumerated by filtration through sterile cellulose nitrate filters using a vacuum filtration system. Detection of Lm will be performed following the ISO standard method (EN ISO 11290-1) (**Figure 1**). Positive enrichment samples will be streaked onto two selective media: ALOA/OCLA Listeria agar and Modified Oxford *Listeria* Selective Agar (MOX). Presumptive positive isolates will be confirmed by PCR. Enumerations of bacteriophages will be carried out by the double agar plate technique using *L. innocua* as the host strain. Quality changes will be measured by sensory evaluation (**Figure 2**) and digital images using image analysis. Headspace gas composition (% O_2 + CO_2) related to the respiration rate of treated and untreated spinach will be measured.

Results to Date
The enumeration protocol to lower the detection limit was optimized by increasing the sample size using filtration as a concentration step, and a set of experiments were performed using different sample sizes. The optimized protocol for the enumeration of *Listeria* spp. that will be followed includes the recovery of bacteria from baby spinach (25 g) in BPW (225 mL) using a stomacher at a low speed for 30 s (**Figure 3**). An aliquot (25 mL) is filtered to increase the sample size. The optimized protocol was tested under lab-scale conditions, and the results showed that bacteriophages reduced the presence of Lm artificially inoculated in baby spinach by 0.7 log cfu/g (**Table 1**). The first application of bacteriophages was performed in the fresh-cut processing plant (**Figure 4**).

Benefits to the Industry
If the effectiveness of the use of bacteriophages in controlling Lm is confirmed, industry will benefit from the application of bacteriophages as a food safety preventive tool to be implemented in their facilities. This effective control treatment will protect consumers and help the industry to preserve its brand reputation, and consumers will be willing to pay the incremental cost. The specific outcomes are: i) Demonstrate the anti-*listeria* effectiveness of bacteriophage-treated baby spinach over different seasons; ii) Verify that the use of bacteriophages controls/reduces the growth of Lm/*Listeria* spp. in baby spinach during storage conditions along the supply chain, including abusive temperatures; iii) Elucidate the interactions of bacteriophages, native microbiota, and Lm growth; and iv) Cost-benefit knowledge for the industrial application of bacteriophages.

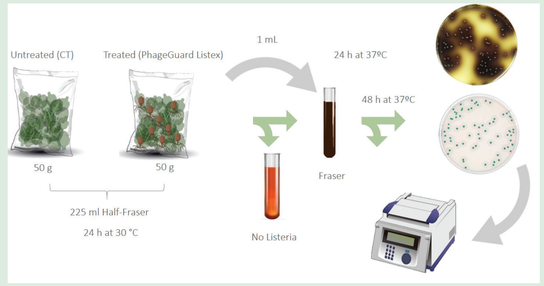

Figure 1. Protocol for detection of the presence of *Listeria monocytogenes* in baby spinach

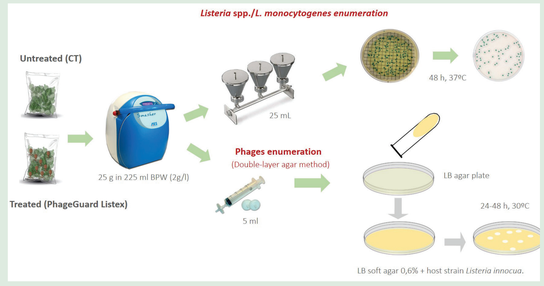

Figure 3. Protocol optimized for enumeration of *Listeria* spp. and bacteriophages in baby spinach

Figure 2. Sensory evaluation of baby spinach

Figure 4. Application of bacteriophages to baby spinach in a commercial processing line

	L. monocytogenes CFU/g	Reduction of *L. monocytogenes**
Untreated (Control)	3.72±0.4	0.73±0.4
Treated (PhageGuard Listex)	2.99±0.4	

Table 1. Levels of *Listeria monocytogenes* in untreated (CT) and treated (PhageGuard Listex) baby spinach