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# Environmental conditions and serotype affect *Listeria monocytogenes* susceptibility to phage treatment in a laboratory cheese model

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## ABSTRACT

*Listeria monocytogenes* can survive and grow in a variety of environments, including refrigeration, making it difficult to control and highlighting the importance of optimizing control strategies against this pathogen. *Listeria* phages are attractive biocontrol agents because phages bind to specific wall teichoic acids (WTA) on the bacterial cell wall, inhibiting pathogens without disrupting the normal microbiota or structure of the food. Common stresses found on dairy products can affect cell wall composition and structure and subsequently affect the efficiency of control strategies that target the cell wall. The goal of this study was to determine the effect of a range of pH and temperatures on the effectiveness of a commercial phage cocktail treatment against several strains of *L. monocytogenes* in a cheese matrix. We developed a laboratory-scale cheese model that was made at different pH, treated with phage, and then inoculated with *L. monocytogenes*. Cheeses were incubated at 6, 14, or 22°C for 14 d, and bacterial counts were determined on d 1, 7, and 14. Our data show that phage treatment has a limited ability to reduce *L. monocytogenes* counts at each temperature tested; however, it was more effective on specific strains of *L. monocytogenes* when cheese was stored at higher temperatures. More specifically, the average counts of *L. monocytogenes* on phage-treated cheese stored at 22°C were significantly lower than those on phage-treated cheese stored at 6 or 14°C. Similarly, phage treatment was significantly more effective at inhibiting *L. monocytogenes* on cheese made at higher pH (6 and 6.5) compared with counts on cheese made at pH 5.5, where *L. monocytogenes* did not grow. Furthermore, serotype was found to affect the susceptibility of *L. monocytogenes* to phage treatment; serotype 1/2 strains showed significantly higher susceptibility to phage treatment than serotype 4b strains. Overall, our results suggest the importance of considering the efficacy of phage under conditions (i.e., temperature and pH) specific to a given food matrix when applying interventions against this important foodborne pathogen.
**Key words:** bacteriophage, food safety, pH, temperature

## INTRODUCTION

*Listeria monocytogenes* is a gram-positive foodborne pathogen that can cause severe listeriosis in susceptible populations, such as immunocompromised individuals, pregnant women, newborns, and elderly adults (Jackson et al., 2018). This bacterium is the third leading cause of death related to foodborne illness in the United States (Barton Behravesh et al., 2011), and the Centers for Disease Control and Prevention (**CDC**) estimate that *L. monocytogenes* causes approximately 1,600 cases annually, including 260 deaths (Scallan et al., 2011; CDC, 2017). *Listeria monocytogenes* also causes disease in animals and can be isolated from both natural and food-processing environments (Nightingale et al., 2004).

Different serotypes of *L. monocytogenes* are associated with specific environments and disease cases (Orsi et al., 2011). The species consists of at least 4 lineages: I, II, III, and IV (Piffaretti et al., 1989; Rasmussen et al., 1995; Wiedmann et al., 1997; Roberts et al., 2006; Ward et al., 2008). Most *L. monocytogenes* isolates belong to lineages I and II, which contain serotypes more commonly associated with human clinical cases, including serotype 1/2a (lineage II) and serotypes 1/2b and 4b (lineage I) (Orsi et al., 2011). Lineage II strains are common in food products, widespread in natural and farm environments, and are less frequently isolated from human clinical cases (Kabuki et al., 2004; Manfreda et al., 2005). Most human listeriosis outbreaks are associated with lineage I isolates (Jeffers et al., 2001). Lineage III and IV strains are rare and predominantly isolated from animal sources (Wiedmann et al., 1997;

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Jeffers et al., 2001) and therefore, were not included among the strains used in this study.

Contamination with *L. monocytogenes* is a concern in dairy processing environments based on its ability to grow in a wide range of temperatures (0 to 45°C), pH (4.4 to 9.4), and at high salt concentrations (13–14 wt/vol%; Swaminathan et al., 2007). The ability of *L. monocytogenes* to rapidly adapt to changing environmental conditions enables it to survive harsh environments during food processing. Since 2006, the number of listeriosis outbreaks in the United States associated with cheese, two-thirds of which were Hispanic-style cheese, has increased (Jackson et al., 2018), which continues to raise concerns regarding *L. monocytogenes* contamination of fresh cheese and increases the need for precise intervention strategies. Soft-ripened or unripened cheese made from raw or improperly pasteurized milk is most commonly associated with disease outbreaks in cheese in the United States (Ibarra-Sánchez et al., 2017). Specifically, most dairy-associated foodborne outbreaks in the United States are linked to the Hispanic-style fresh cheese queso fresco (Ibarra-Sánchez et al., 2017). Hispanic-style fresh cheese is characterized by high water activity ($a_w$), low salt content, and near-neutral pH, which creates an ideal environment for survival and growth of several foodborne pathogens (Leggett et al., 2012), specifically *L. monocytogenes* (Uhlich et al., 2006; Ibarra-Sánchez et al., 2017).

One possible strategy to prevent problems associated with *L. monocytogenes* contamination in food and food-processing environments is the use of lytic bacteriophages or phages (Mahony et al., 2011). Phage are the most abundant organisms on earth, can be isolated from various environments, including food, and are components of the human gut microbiota (Clokie et al., 2011). Lytic phages infect bacteria, resulting in lysis of the bacterial cell. Phages recognize and kill specific target bacteria; therefore, phage-based interventions can target specific foodborne pathogens without disrupting the normal and beneficial microflora of foods (Goodridge and Bisha, 2011). Several phage-based preparations have been approved for food applications in the United States and Europe, such as ListShield (Intralytix, Baltimore, MD), Listex P-100 (PhageGuard/Micreos, Wageningen, the Netherlands), EcoShield (Intralytix), SalmoFresh (Intralytix), and Salmonelex (PhageGuard). Although some studies have shown that these phage preparations can decrease *L. monocytogenes* (Silva et al., 2014; Perera et al., 2015), *Escherichia coli* (Carter et al., 2012), and *Salmonella* (Sharma et al., 2015) numbers on produce, dairy, and meat products, whether they can reduce pathogen prevalence or outbreak incidence remains to be determined. Furthermore, the efficacy of phage has been shown to vary in different food matrices and under different environmental conditions (Guenther et al., 2009).

Given the unique conditions encountered in cheese, the objective of this study was to investigate the effect of environmental conditions (e.g., temperature and pH) on *L. monocytogenes* sensitivity to a commercial phage cocktail in a laboratory-scale cheese model. Effective and improved prevention of *L. monocytogenes*-related foodborne outbreaks and infections will require a thorough understanding of the effects of the environment on *L. monocytogenes* sensitivity to treatments to allow for appropriate development and application of control strategies.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

We obtained 4 recent cheese outbreak strains that encompass the most common *L. monocytogenes* serotypes (4b, 1/2a and 1/2b; Table 1). *Listeria monocytogenes* strain 10403S (serotype 1/2a) was used as a reference in this study. For all experiments, strains were streaked from frozen brain heart infusion (**BHI**; Difco, Becton Dickinson and Co., Sparks, MD) stocks, stored at −80°C in 15% glycerol, plated on a BHI agar plate, and then incubated at 37°C for 24 h. A single colony was subsequently inoculated into 5 mL of BHI broth in 16-mm tubes, followed by incubation at 37°C with shaking (230 rpm) for 16 h (Series 25 Incubator, New Brunswick Scientific, Edison, NJ). After 16 h, 50 μL of BHI culture was inoculated into 5 mL of fresh BHI broth and grown to an optical density (at 600 nm) of 1.0 at 30°C.

### Laboratory-Scale Cheese Model

We modified the method described by Van Tassell et al. (2015) to make approximately 10-g miniature cheeses in 6-well plates. Briefly, using aseptic technique, 600 mL of pasteurized, nonhomogenized whole milk (Trinity Valley Dairy, purchased at retail in 1-gallon containers) was warmed to 35°C. Then, $CaCl_2$ (Dairy Connection Inc., Madison, WI) was added to milk to a final concentration of 1 mg/mL and the milk was combined with 6 mL of rennet solution (90 μL of double-strength vegetable rennet, CHY-Max; Chr. Hansen, Milwaukee, WI) diluted in 5,910 μL of sterile water). To account for bacteria present and to ensure that no *L. monocytogenes* was in the milk before cheese was made, milk samples were plated on plate count agar (Difco, Becton Dickinson and Co.) and on *L. monocytogenes* plating

Table 1. *Listeria monocytogenes* strains used in this study

| FSL[1] ID | Previous ID | Outbreak | Source type | Source site | Serotype | Reference |
|---|---|---|---|---|---|---|
| FSL X1–0001 | — | Laboratory strain 10403S | Human | Skin lesion | 1/2a | Bishop and Hinrichs (1987) |
| FSL R9–5621 | 2021L-5324 | 2012 Ricotta cheese | Food | Cheese | 1/2a | CDC (2012) |
| FSL R9–5623 | 2013L-5223 | 2013 Semi-soft fresh-style cheese | Human | Placenta | 4b | CDC (2013) |
| FSL R9–5624 | 2014L-6028 | 2013 Queso fresco | Human | Blood | 1/2b | CDC (2014) |
| FSL R9–5625 | 2014L-6388 | 2014 Soft cheese | Human | Blood | 4b | CDC (2015) |

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medium (Difco, Becton Dickinson and Co.). After 48 h of incubation at 32°C for plate count agar and 24 h of incubation at 30°C for *L. monocytogenes* plating medium, colonies were counted using a Q Count Colony Counter (Advanced Instruments, Norwood, MA).

To make cheese at different pH, milk was acidified with the addition of vinegar (white, distilled) to reduce the pH from 6.5 to 6.0 or 5.5 before the addition of $CaCl_2$ and rennet. Although acetic (vinegar), citric, and lactic acids are commonly used when making fresh cheese (Hnosko et al., 2008), the textural properties of queso blanco-type cheese were rated higher by consumers for cheese made with acetic acid (Farkye et al., 1995; Hnosko et al., 2008). To ensure that the desired pH was achieved, the pH of the milk was measured using a surface pH meter (Mettler Toledo, Columbus, OH). The milk was then poured into "mini vats" and incubated at 35°C for 45 min in a water bath. The curds were cut using modified cheese harps. After cutting, curds were returned to the water bath and heated, and the temperature of the water bath was increased progressively until reaching 40°C (~30 min). Whey (60 mL) was removed and replaced with 60 mL of NaCl solution (0.16 g/mL). The curds were stirred gently with a plastic sterile loop and returned to the water bath for an additional 20-min incubation at 40°C. After incubation, the remaining whey was drained, and 15 g of curd was scooped into each well of a 6-well plate. Plates were covered and pressed with 30-mm caps for 2 h at room temperature. Cheese was subjected to analyses to determine pH and water activity (Aqualab Series 4TE, FF Instrumentation, Christchurch, New Zealand) throughout storage (Supplemental Table S1; https://doi.org/10.3168/jds.2019-16474). All samples were within the range of compositional values expected in commercial fresh cheese (Caro et al., 2014; Trmčić et al., 2017; Van Hekken et al., 2017; Holle et al., 2018).

### Growth of *L. monocytogenes* in the Cheese Model Containing a Commercial *Listeria*-Phage Cocktail

Immediately after cheese was made, phage was applied to the surface of cheese by evenly pipetting 200 μL of ListShield (Intralytix, Baltimore, MD), a US Food and Drug Administration–approved commercial *Listeria*-phage cocktail, consisting of a mixture of equal proportions of 6 lytic bacteriophages, specifically effective against *L. monocytogenes* serotypes, to cover the surface of the 10-g cheese for a target final concentration of approximately $8 \times 10^6$ plaque-forming units (**pfu**)/g or $9 \times 10^6$ pfu/cm$^2$ (the dose recommended by the manufacturer is between 1 and 4 mL/lb. of a working stock that has been diluted to $1 \times 10^9$ pfu/mL). Following 30 min of incubation at room temperature,

each of the cheese samples was then surface-inoculated by spotting 100 μL of 1 of the 5 selected *L. monocytogenes* strains at approximately $10^5$ cfu/g, with an additional uninoculated cheese control. Plates with 6 cheeses per plate were covered and incubated at 6, 14, or 22°C for 14 d, the typical shelf-life for a fresh cheese (Bermúdez-Aguirre and Barbosa-Canovas, 2010).

At 1, 7, or 14 d postinoculation, cheese was diluted 1/10 with PBS and homogenized using a Stomacher (Seward, Worthing, UK). Homogenates were then serially diluted and plated on modified Oxford agar (Difco, Becton Dickinson and Co.) using an Autoplate spiral plating system (Advanced Instruments.) for *L. monocytogenes* quantification. After 48 h of incubation at 30°C (Curtis et al., 1989), colonies were counted using a Q Count Colony Counter (Advanced Instruments). Experiments were performed in at least biological triplicates.

### Statistical Analysis

All statistical analyses were carried out in the R Statistical Programming Environment (R Core Team, 2015). We constructed individual linear mixed effects models for temperature and pH using the "lmer" function in the "lme4" package (Bates et al., 2015). For each model, the response was the number of *L. monocytognes* (log count; log cfu/g ) and random effects were (1) replicates and (2) plate nested within milk batch. Fixed effects were (1) temperature or pH, (2) day of incubation, (3) presence or absence of phage, (4) strain, (5) age of the milk (based on a 21-d code date), and (6) the aerobic plate counts (log cfu/mL; bacterial counts in the milk before cheese was made). We also included the interactions phage × temperature or pH, phage × strain, phage × day, and day × temperature or pH. Post hoc pairwise comparisons of means were performed using "lsmeans" (Lenth, 2016). The cut-off for significance was set at $P < 0.05$. Raw data and the R code used for statistical analyses are available on GitHub (https://github.com/lohenderson/Phage_Listeria_Analysis_Model/).

## RESULTS

### Temperature Affects Susceptibility of L. monocytogenes to Phage Treatment

To assess the effect of different cheese incubation temperatures on the ability of phage to reduce *L. monocytogenes* loads on cheese, the commercial phage cocktail was added to the surface of cheese made at pH 6.5 from pasteurized milk before surface inoculation of 5 different *L. monocytogenes* strains. We did not detect *L. monocytogenes* in the milk or uninoculated controls (data not shown). Counts of *L. monocytogenes* numbers were quantified at d 1, 7, and 14 for cheese incubated at 6, 14, or 22°C (Figure 1). A linear mixed effects model was used to specifically determine whether temperature, day of incubation, presence or absence of phage, and strain as well as interactions between temperature and presence of phage, temperature and day of incubation, and strain and presence of phage had significant effects on log-transformed bacterial numbers (Table 2). Figure 1 represents the actual observed data, and least squares means of estimated *L. monocytogenes* counts from the temperature model are shown across all strains in Figure 2 and calculated for each strain individually in Supplemental Figure S1 (https://doi.org/10.3168/jds.2019-16474).

Presence of phage had a significant effect ($P < 0.001$) on *L. monocytogenes* counts with a model-estimated effect size of −1.34 (Table 2), indicating a 1.34 log lower count in the presence of phage. This result indicates that phage significantly reduced *L. monocytogenes* counts in the model cheese, as evident by the data in Figure 1; for cheese incubated at 6°C, average *L. monocytogenes* counts (across strains and the 3 time points) were 6.33 and 7.13 log cfu/g for cheese with and without phage, respectively. Phage-treated cheese had consistently lower *L. monocytogenes* counts at d 1 compared with untreated cheese [0.76, 1.39, and 2.18 log cfu/g lower average counts (across the 5 strains tested) for 6, 14, and 22°C, respectively].

Not surprisingly, incubation day had significant effects on *L. monocytogenes* counts, with higher counts at d 7 and 14 compared with d 1 (Table 2). The significance of day is not surprising considering that *L. monocytogenes* grew over time (average counts across strains were 4.85 and 7.57 log cfu/g for d 1 and 14 at 6°C, respectively), consistent with previous observations that *L. monocytogenes* grows at refrigeration temperatures on soft cheeses at pH >5.9 (Genigeorgis et al., 1991; Silva et al., 2014).

Importantly, we found a significant interaction effect ($P = 0.006$) between phage and storage at 22°C with an effect size −0.79, indicating 0.79 log lower *L. monocytogenes* counts relative to phage treatment at 6°C. Although phage-treated cheeses had lower *L. monocytogenes* counts across temperatures, the difference between counts on treated and untreated cheese varied considerably by temperature; the smallest difference between phage-treated and untreated cheese for d 1 (0.49 log) was found for cheese incubated at 6°C, with higher corresponding differences of 0.86 and 1.80 log at 14 and 22°C, respectively (Figure 1). Importantly,

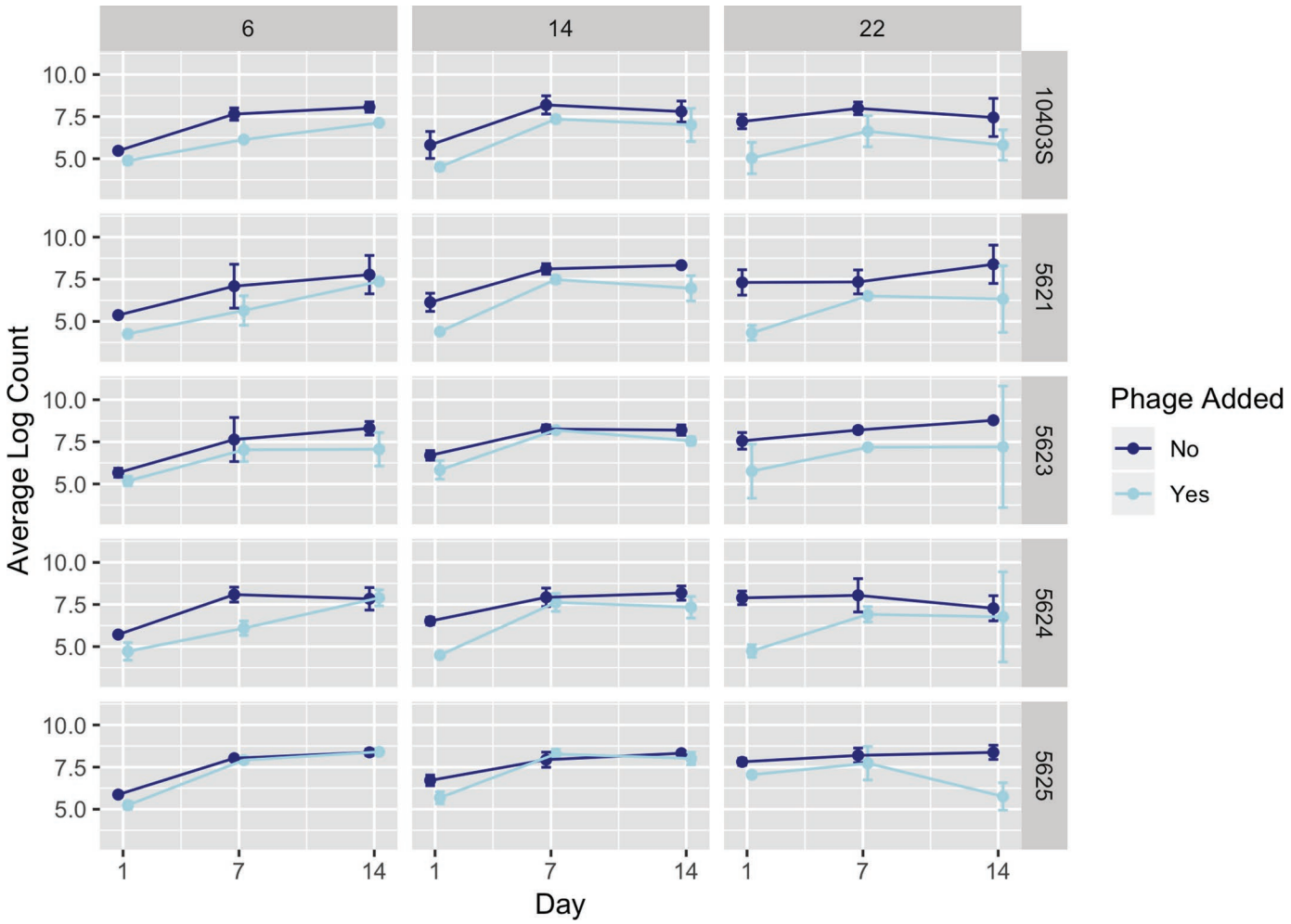


**Figure 1.** Average counts (log cfu/g) of *Listeria monocytogenes* in the presence (light blue) and absence (dark blue) of phage treatment in a laboratory-scale cheese model. Each cheese was inoculated with a single strain of *L. monocytogenes* (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, or FSL R9-5625) to a level of approximately 5 log cfu/g. These results represent the effect of temperature (6, 14, and 22°C) on the sensitivity of *L. monocytogenes* to a commercial phage cocktail. The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 d. All values are the arithmetic mean of 3 independent experiments, and error bars denote standard errors. For some data points, error bars are not visible because the standard error was too low to yield a visible error bar. All cheeses were made at pH 6.5.

however, growth of *L. monocytogenes* was still observed in phage-treated cheese (Figure 1) where, for several strains, the difference between *L. monocytogenes* counts on phage-treated and untreated cheese was <0.5 log by d 7 and 14 . For example, at 14°C, only strain 10403S (both d 7 and 14) and strain 5621 (only d 14) showed >0.5 log differences between phage-treated and untreated cheese. Interestingly, at higher temperatures (14 and 22°C), we found a few instances where d 14 *L. monocytogenes* counts in phage-treated cheese were lower than d 7 counts; this pattern was most pronounced for the serotype 4b strain 5625 (Figure 1).

We found a significant interaction effect between presence of phage and strain 5625, one of the two serotype 4b strains used here ($P < 0.001$; effect size of 0.68), indicating that this strain showed 0.68 log higher counts in the presence of phage compared with the reference strain 10403S. For strain 5623, the other serotype 4b strain used here, the interaction effect between strain and temperature did not meet the $P < 0.05$ cut-off ($P = 0.107$) but showed the second highest positive effect size (0.33) for the different strain × phage interaction analyses.

### *pH Affects Susceptibility of L. monocytogenes to Phage Treatment*

To assess the effect of different pH on phage inhibition of *L. monocytogenes* counts, the commercial phage cocktail was added to the surface of cheeses made at pH

**Table 2.** Model parameters for all fixed effects in the temperature model for *Listeria monocytogenes* counts

| Response variable and fixed effects | Level | Estimate | SE | *P*-value | Significance |
|---|---|---|---|---|---|
| *L. monocytogenes* count (log cfu/g) | | | | | |
| Temperature (°C) | 6 | Referent | | | |
| | 14 | 0.46 | 0.28 | 0.110 | |
| | 22 | 1.48 | 0.32 | <0.001 | *** |
| Phage[1] | N | Referent | | | |
| | Y | −1.34 | 0.27 | <0.001 | *** |
| Day | 1 | Referent | | | |
| | 7 | 1.57 | 0.26 | <0.001 | *** |
| | 14 | 2.35 | 0.26 | <0.001 | *** |
| Strain | 10403S | Referent | | | |
| | 5621 | −0.01 | 0.14 | 0.921 | |
| | 5623 | 0.37 | 0.14 | 0.011 | * |
| | 5624 | 0.22 | 0.14 | 0.131 | |
| | 5625 | 0.42 | 0.14 | 0.003 | ** |
| Milk age[2] | | 0.10 | 0.07 | 0.202 | |
| Milk apc[3] (log cfu/mL) | | −0.07 | 0.10 | 0.559 | |
| Temperature × Phage Y | 6 × Phage Y | Referent | | | |
| | 14 × Phage Y | −0.03 | 0.26 | 0.911 | |
| | 22 × Phage Y | −0.79 | 0.27 | 0.006 | ** |
| Phage Y × Day | Phage Y × 1 | Referent | | | |
| | Phage Y × 7 | 0.65 | 0.26 | 0.018 | * |
| | Phage Y × 14 | 0.48 | 0.27 | 0.085 | |
| Temperature × Day | 6 × Day 1 | Referent | | | |
| | 14 × Day 7 | 0.37 | 0.32 | 0.257 | |
| | 14 × Day 14 | −0.49 | 0.32 | 0.131 | |
| | 22 × Day 7 | −0.89 | 0.32 | 0.008 | ** |
| | 22 × Day14 | −1.84 | 0.34 | <0.001 | *** |
| Phage Y × Strain | Phage Y × Strain 10403S | Referent | | | |
| | Phage Y × Strain 5621 | −0.15 | 0.20 | 0.456 | |
| | Phage Y × Strain 5623 | 0.33 | 0.20 | 0.107 | |
| | Phage Y × Strain 5624 | −0.02 | 0.20 | 0.940 | |
| | Phage Y × Strain 5625 | 0.68 | 0.20 | <0.001 | *** |

[1]N denotes the absence of phage; Y denotes the presence of phage.
[2]Age of milk when cheese was made, based on a 21-d code date.
[3]Bacterial aerobic plate counts (apc; log cfu/mL) in milk before cheese was made.
***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

5.5, 6.0, and 6.5 before *L. monocytogenes* inoculation. The counts of *L. monocytogenes* were quantified after storage at 6°C for 1, 7, and 14 d. A linear mixed effects model was used to determine whether pH, day of incubation, presence or absence of phage, and strain, as well as interactions between pH and presence of phage and strain and presence of phage, and pH and day showed significant effects on log-transformed *L. monocytogenes* counts (Table 3). Data obtained (Figure 3) clearly show *L. monocytogenes* growth at pH 6.0 and 6.5, as well as a reduced effect of phage on *L. monocytogenes* counts at pH 5.5, relative to that at pH 6.0 and 6.5. Least squares means of estimated *L. monocytogenes* counts calculated from the pH model across strains (Figure 4) and for each strain individually (Supplemental Figure S2; https://doi.org/10.3168/jds.2019-16474) also showed no *L. monocytogenes* growth at pH 5.5, regardless of treatment.

Presence of phage had a significant effect ($P < 0.001$) with an effect size of −1.00, which indicates that *L. monocytogenes* counts were 1.00 log lower with phage treatment (Table 3). For cheese made at pH 6.0, average *L. monocytogenes* counts (across strains and time points) were 6.00 and 6.66 log cfu/g for cheese with and without phage, respectively. The average observed *L. monocytogenes* counts across strains (Figure 4) also indicated that phage-treated cheese had consistently lower *L. monocytogenes* counts (4.58 to 7.48 log cfu/g) on all days compared with untreated cheese (4.97 to 7.86 log cfu/g). Although phage-treated cheese had lower *L. monocytogenes* counts across pH, the difference between *L. monocytogenes* counts on treated and untreated cheese varied considerably by pH; for example, the smallest difference between phage-treated and untreated cheese for d 1 (0.23 log) was found for cheese made at pH 5.5, with numerically higher corresponding differences of 0.28 and 0.63 log for pH 6.0 and 6.5, respectively. Differences between *L. monocytogenes* counts on treated and untreated cheese made at pH 5.5 were minimal and ranged from 0.10 to 0.97 log,

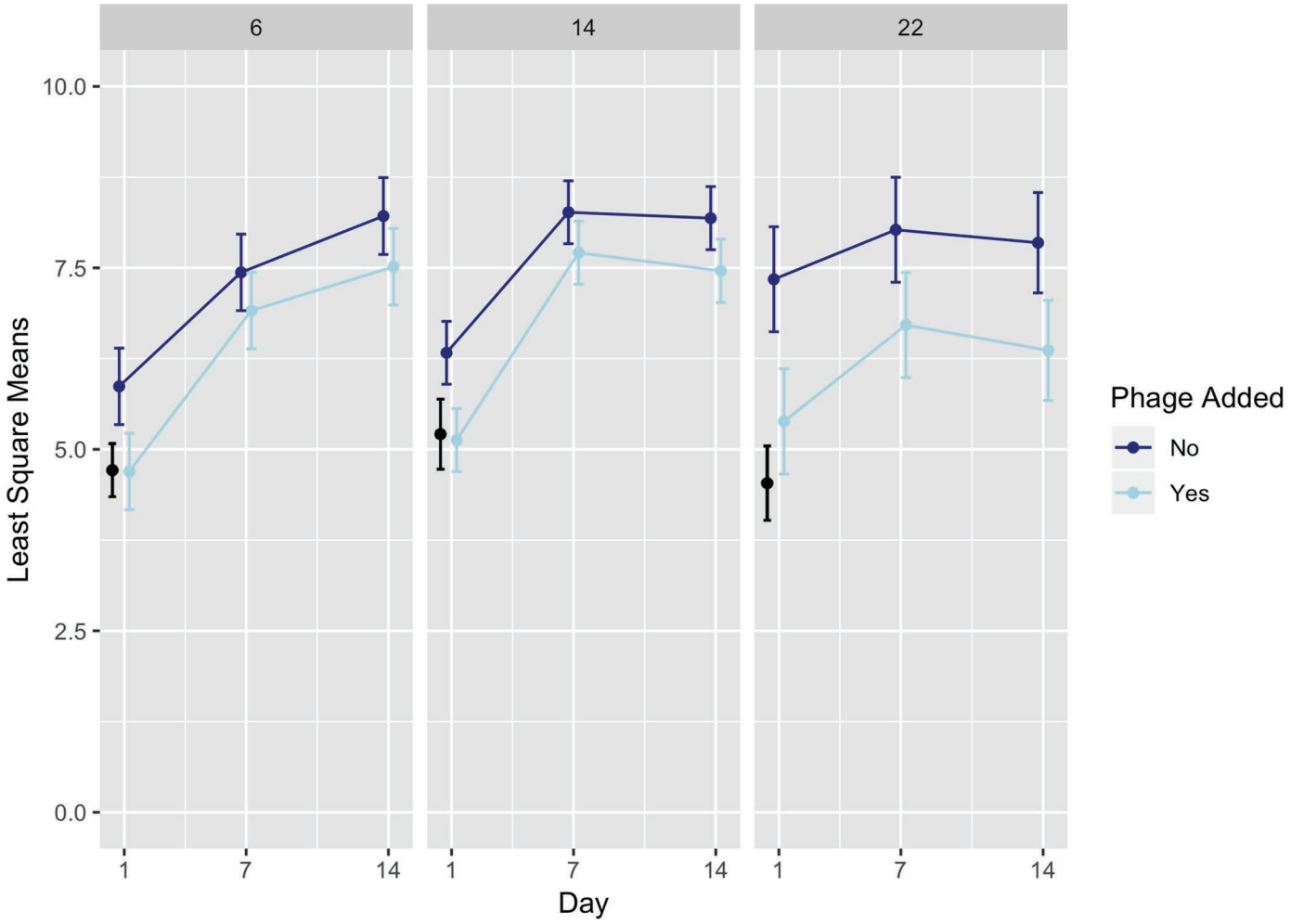


**Figure 2.** Least squares means of estimated numbers (log cfu/g) of *Listeria monocytogenes* calculated from the temperature model, based on data shown in Figure 1. Calculated initial (d 0) *L. monocytogenes* numbers based on the average inoculum level (~5 log cfu/g) are shown in black. Predicted numbers for cheese treated with phage are shown in light blue, and those for cheese without phage treatment are shown in dark blue. These results represent the predicted effect of temperature (6, 14, and 22°C) on the sensitivity of *L. monocytogenes* to a commercial phage cocktail across strains (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, and FSL R9-5625). The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 d. Error bars denote the 95% CI. All cheeses for these predictions were made at pH 6.5.

compared with 0.15 to 1.38 and 0.11 to 2.00 log for pH 6.0 and pH 6.5, respectively (Figure 3).

Similar to data obtained from the temperature model, d 7 and 14 also had significant effects on *L. monocytogenes* numbers with higher numbers at both days compared with the reference (i.e., d 1; Table 3). Again, the significance of day is not surprising considering that *L. monocytogenes* showed growth over time for cheese formulated at pH 6.0 and 6.5. For example, average counts across strains were 4.86 and 7.03 log cfu/g for d 1 and 14, respectively, on cheese made at pH 6.0. Similar to the observations for pH 6.5 cheese incubated at 6 and 14°C, pH 6.0 cheese incubated at 6°C also showed growth of *L. monocytogenes* for phage-treated cheese (Figure 3), with only an average 0.46 log difference between phage-treated and untreated pH 6.0 cheese at d 14 (Figure 4).

Finally, we found significant interaction effects between the presence of phage and strains 5623 and 5625, the two serotype 4b strains used here ($P = 0.011$; effect size of 0.43 and $P < 0.001$; effect size 0.66, respectively); this indicates that these strains showed 0.43 and 0.66 log cfu/g higher counts in the presence of phage compared with the reference strain 10403S. These findings are consistent with an initial plaque size screen of the 5 *L. monocytogenes* strains used here against the phage cocktail. Briefly, 2 independent replicates of a plaque assay on Luria Bertani medium buffered with 50 m*M*

**Table 3.** Model parameters for all fixed effects in the pH model for *Listeria monocytogenes* counts

| Response variable and fixed effects | Level | Estimate | SE | *P*-value | Significance |
|---|---|---|---|---|---|
| *L. monocytogenes* count (log cfu/g) | | | | | |
| pH | pH 6.5 | Referent | | | |
| | pH 6.0 | −0.27 | 0.22 | 0.245 | |
| | pH 5.5 | −0.44 | 0.37 | 0.288 | |
| Phage[1] | N | Referent | | | |
| | Y | −1.00 | 0.17 | <0.001 | *** |
| Day | 1 | Referent | | | |
| | 7 | 2.04 | 0.14 | <0.001 | *** |
| | 14 | 2.49 | 0.14 | <0.001 | *** |
| Strain | 10403S | Referent | | | |
| | 5621 | −0.06 | 0.12 | 0.618 | |
| | 5623 | 0.10 | 0.12 | 0.413 | |
| | 5624 | 0.22 | 0.12 | 0.066 | |
| | 5625 | 0.33 | 0.12 | 0.006 | ** |
| Milk age[2] | | 0.01 | 0.14 | 0.947 | |
| Milk apc[3] (log cfu/mL) | | 0.07 | 0.06 | 0.361 | |
| pH × Phage Y | pH 6.5 × Phage Y | Referent | | | |
| | pH 6.0 × Phage Y | 0.13 | 0.14 | 0.362 | |
| | pH 5.5 × Phage Y | 0.38 | 0.15 | 0.014 | * |
| Phage × Day | Phage Y × 1 | Referent | | | |
| | Phage Y × 7 | −0.30 | 0.14 | 0.044 | * |
| | Phage Y × 14 | 0.17 | 0.15 | 0.246 | |
| pH × Day | pH 6.5 × Day 1 | Referent | | | |
| | pH 6.0 × Day 7 | −0.39 | 0.17 | 0.031 | * |
| | pH 6.0 × Day 14 | −0.43 | 0.17 | 0.019 | * |
| | pH 5.5 × Day 7 | −2.00 | 0.17 | <0.001 | *** |
| | pH 5.5 × Day 14 | −2.77 | 0.19 | <0.001 | *** |
| Phage Y × Strain | Phage Y × Strain 10403S | Referent | | | |
| | Phage Y × Strain 5621 | −0.02 | 0.17 | 0.881 | |
| | Phage Y × Strain 5623 | 0.43 | 0.17 | 0.011 | * |
| | Phage Y × Strain 5624 | 0.15 | 0.17 | 0.376 | |
| | Phage Y × Strain 5625 | 0.66 | 0.17 | <0.001 | *** |

[1]N denotes the absence of phage; Y denotes the presence of phage.
[2]Age of milk when cheese was made, based on a 21-d code date.
[3]Bacterial aerobic plate counts (apc; log cfu/mL) in milk before cheese was made.
$^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

morpholinepropanesulfonic acid both showed weaker lysis of the 2 serotype 4b strains compared with the 3 other strains (Supplemental Table S2; https://doi.org/10.3168/jds.2019-16474).

## DISCUSSION

Although this study indicated that the commercial phage cocktail tested here, on average, reduced *L. monocytogenes* counts on cheese (with an overall effect size of around 1 log), we also showed that environmental conditions (especially temperature) and strain have significant effects on the efficacy of phage treatment in cheese. These findings have important implications as they (1) suggest that optimization of environmental conditions could be used to enhance the effectiveness of phage treatment, and (2) provide initial data that could be used in a formal risk assessment to quantify the effects of phage treatment with regard to reducing the risk of human listeriosis cases.

### Phage Reduction of *L. monocytogenes*

Although our data support that phage can significantly decrease *L. monocytogenes* counts in cheese, we also found that the effectiveness of phage seemed to be enhanced when cheese was stored at higher temperatures, and that considerable *L. monocytogenes* growth can occur even in the presence of the phage cocktail. Our data showing considerable *L. monocytogenes* growth on cheese treated with phage are consistent with a study by Soni et al. (2012), who found that although surface treatment of queso fresco with phage P100 (7.7 log pfu/cm$^2$) led to an initial decrease (reduced to an undetectable limit, <5 cfu/cm$^2$) of *L. monocytogenes* on cheese stored at 4°C, subsequent regrowth (back to inoculum level, approximately 4 log cfu/cm$^2$) of *L. monocytogenes* occurred by the end of 28 d of storage. Similarly, Silva et al. (2014) reported that when soft cheese was inoculated with approximately $10^5$ cfu/g of *L. monocytogenes* and treated with $8.3 \times 10^7$ pfu/g of phage

P100, there was an initial 2 log reduction (at 30 min after treatment); however, after 7 d of storage at 10°C, they found only a ~1 log reduction of *L. monocytogenes* numbers on phage-treated cheese compared with untreated controls. Studies in tryptic soy broth also showed regrowth of *L. monocytogenes* after 2 wk, even though phage treatment at $2 \times 10^9$ or $2 \times 10^{10}$ pfu/mL resulted in a 4 to 7 log reduction at different temperatures (4, 10, and 20°C) within the first 2 wk (Fister et al., 2016). Conversely, Carlton et al. (2005) reported complete eradication (below detection limit) and therefore no regrowth of *L. monocytogenes* on a ripened soft cheese stored at 14°C when the washing solution was inoculated with $2 \times 10^1$ cfu/cm$^2$ *L. monocytogenes* at the beginning of the ripening period, and $2 \times 10^6$ pfu/cm$^2$ P100 was subsequently applied to the washing/smearing solution during the rind washings (Carlton et al., 2005). The observation that our data did not show an initial reduction of the magnitude observed in the Soni et al. (2012) and Silva et al. (2014) studies or the undetectable level of *L. monocytogenes* reported by Carlton et al. (2005) could be due to differences in experimental set-up. For example, Silva et al. (2014) inoculated the cheese and homogenized the sample before phage treatment, which could increase the efficacy of phage treatment.

Overall, our data, as well as some previous data, indicate the potential for regrowth of *L. monocytogenes*

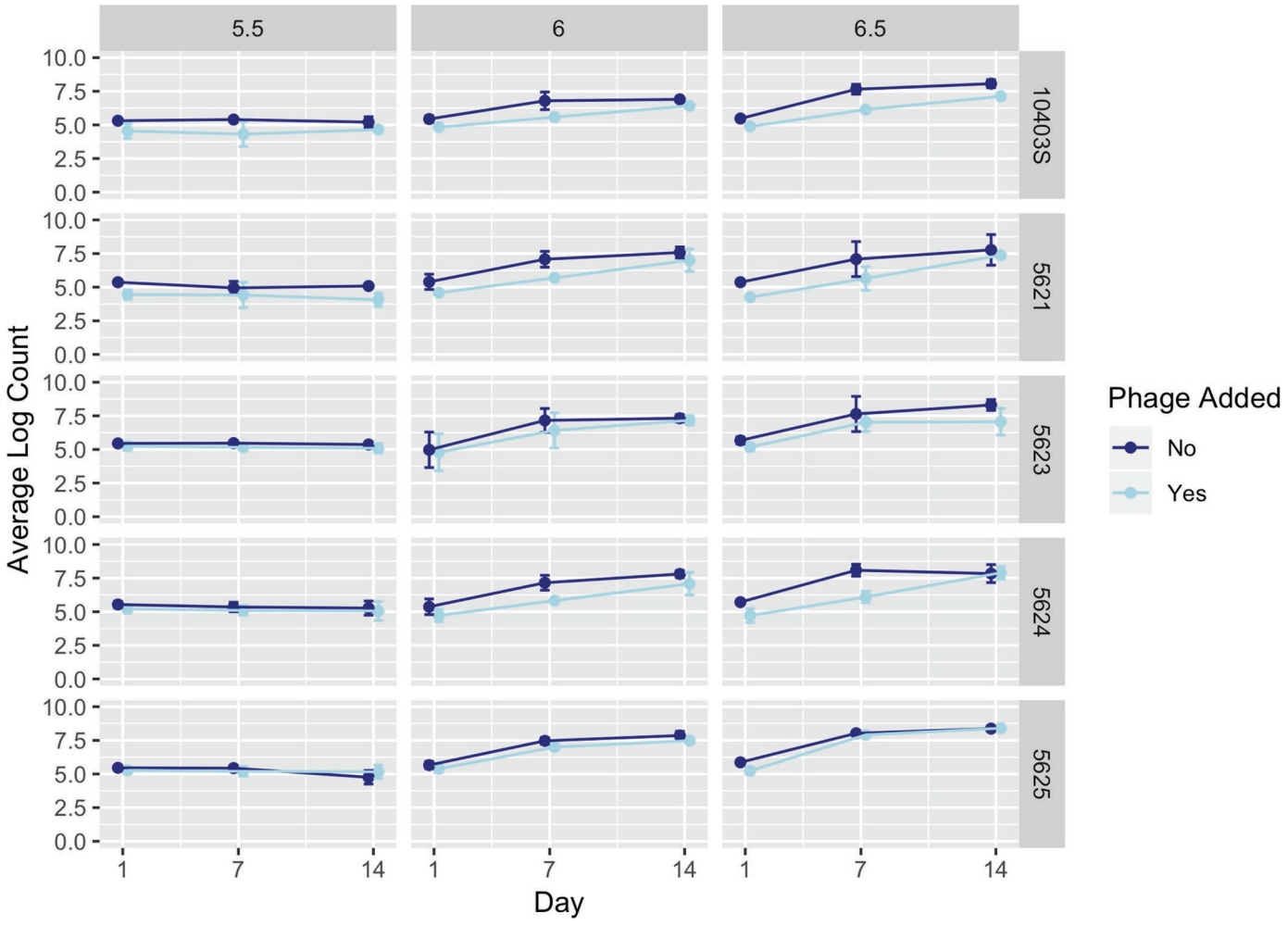


**Figure 3.** Average counts (log cfu/g) of *Listeria monocytogenes* in the presence (light blue) and absence (dark blue) of phage treatment in a laboratory-scale cheese model. Each cheese was inoculated with a single strain of *L. monocytogenes* (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, or FSL R9-5625) to a level of approximately 5 log cfu/g. These results represent the effect of pH (5.5, 6.0, and 6.5) on the sensitivity of *L. monocytogenes* to a commercial phage cocktail. The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 d. All values are the arithmetic mean of 3 independent experiments, and error bars denote standard errors. For some data points, error bars are not visible because the standard error was too low to yield a visible error bar. All cheeses for these predictions were stored at 6°C.

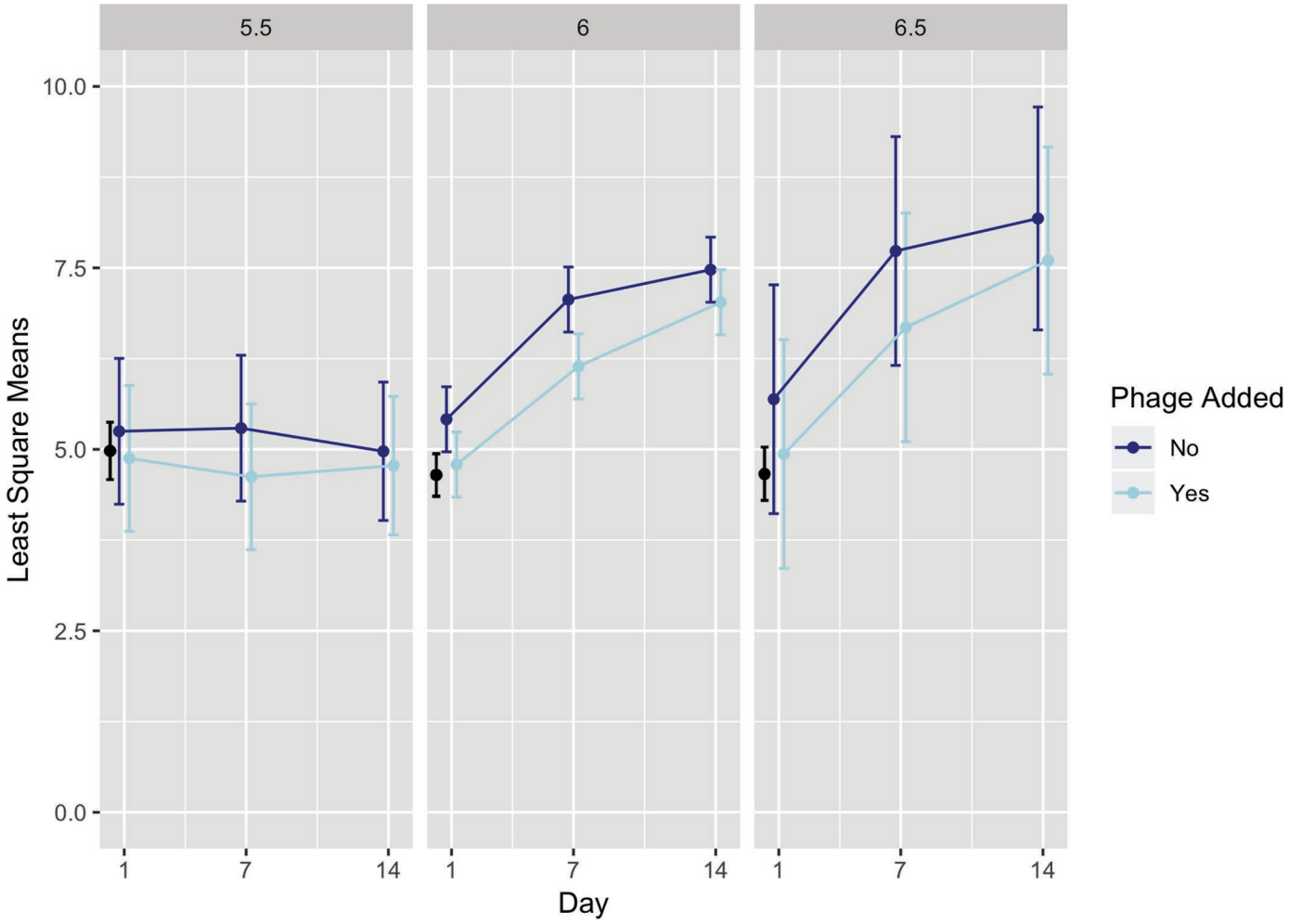


**Figure 4.** Least squares means of estimated *Listeria monocytogenes* numbers (log cfu/g) calculated from the pH model, which is based on data shown in Figure 3. Calculated initial (d 0) *L. monocytogenes* numbers based on the average inoculum level (~5 log cfu/g) are shown in black. Predicted numbers for cheese treated with phage are shown in light blue, and those for cheese without phage treatment are shown in dark blue. These results represent the predicted effect of pH (5.5, 6.0, and 6.5) on the sensitivity of *L. monocytogenes* to a commercial phage cocktail across strains (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, and FSL R9-5625). The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 d. Error bars denote the 95% CI. All cheeses for these predictions were stored at 6°C.

during long-term storage, even when phage treatment results in an initial *L. monocytogenes* reduction. Hence, studies over product shelf life are essential to appropriately evaluate the effectiveness of phage treatment; use of these data in risk assessments (which would consider, among other parameters, storage time before consumption) can then provide a more accurate assessment of the public health impact of phage applications. Several factors might explain the regrowth of *L. monocytogenes* after initial significant reduction, including, but not limited to, (1) emergence and subsequent growth of phage resistant mutants (e.g., as reported by Denes et al., 2015); (2) transient resistance of *L. monocytogenes* to phage cocktail; and (3) degradation of phage or binding of phage to food matrix components, inhibiting the ability of phage to infect *L. monocytogenes*.

Importantly, while we specifically found a significant interaction effect between phage treatment and cheese incubation at 22°C, storage or incubation at this temperature is neither feasible nor realistic for Hispanic-style fresh cheese because it can allow growth of molds and other spoilage or pathogenic microorganisms, which could reduce *L. monocytogenes* numbers due to competition. Consistent with our findings, Lee et al. (2017) reported that $10^6$ to $10^7$ pfu/mL of lytic phage (LMP7) inhibited *L. monocytogenes* growth in skim milk stored at 4, 10, and 30°C; however, phage treatment was most effective at 30°C, resulting in a nearly 1 log difference

between treated and untreated skim milk after 1 d compared with a <0.5 log difference at 10°C (Lee et al., 2017). Preliminary evidence for enhanced effectiveness of phage in foods incubated at higher temperatures has also been reported for nondairy foods. For example, Guenther and Loessner (2011) showed that *Listeria* phage A511 was able to reduce *L. monocytogenes* numbers on hot dogs, chocolate milk, and cabbage by 2.3 to 5 log cfu/cm$^2$ when stored at 6°C compared with untreated controls; at 20°C, phage was able to reduce *L. monocytogenes* numbers by 3.8 to 6.4 log cfu/cm$^2$.

Previous studies have shown that native bacteria present in the milk can grow on cheese, especially when stored at higher temperatures (Ostergaard et al., 2014; Tirloni et al., 2019) and subsequently outcompete *Listeria*, resulting in limited *L. monocytogenes* growth (Guillier et al., 2008; Mounier et al., 2008; Dalgaard and Mejlholm, 2019). However, our data show similar *L. monocytogenes* numbers at all temperatures in untreated cheese (Figures 1 and 2), suggesting that the enhanced effectiveness of phage-mediated *L. monocytogenes* reduction at higher temperatures is due to temperature affecting the phage–*Listeria* interaction and not due to an effect of the native bacteria on *Listeria*. Other possible explanations for greater phage-mediated *L. monocytogenes* reductions at higher temperatures include (1) enhanced sensitivity of metabolically more active *L. monocytogenes*, and (2) enhanced expression of phage receptors in *L. monocytogenes* grown at 22°C compared with lower temperatures (such as 6°C). Several studies have indicated that metabolically active bacteria are more effectively killed by phage (Chibani-Chennoufi et al., 2004; Clokie et al., 2011; Denes and Wiedmann, 2014). Prior studies have theorized that metabolically active bacteria are required for successful use of phage because these control strategies rely on host cell metabolism for phage replication and consequently bacterial cell lysis, leading to the release of progeny phage. Specific studies supporting this theory include a study by Fister et al. (2016), which found that conditions that reduced metabolism and growth rate of the host cell decreased the effectiveness of phage treatment. Previous studies also support that temperature can affect the physiological state of *L. monocytogenes* in a way that enhances the accessibility of phage receptors on the host. For example, previous studies have shown that phage adsorption of P100-like *Listeria* phage is more efficient at 30°C than at 37°C, potentially because of more accessible WTA receptors (Denes et al., 2015; Tokman et al., 2016). Finally, temperatures (and other environmental conditions) facilitating more rapid growth of *L. monocytogenes* will lead to larger *L. monocytogenes* populations, which will enhance the likelihood of phage-bacteria contact and lead to faster phage propagation, further enhancing the effectiveness of phage treatment. This mechanism could, in particular, contribute to the enhanced die-off seen here in at least one strain at d 14.

### Effectiveness of Phage

Although our data indicate that pH can play a role in the effectiveness of phage treatment, the predominant effect of pH observed here was a lack of *L. monocytogenes* growth on the laboratory-scale cheese formulated at pH 5.5, along with a reduced ability of phage to kill *L. monocytogenes* in cheese at that pH. The lack of *L. monocytogenes* growth at pH 5.5 is consistent with results from Genigeorgis et al. (1991), who reported that growth of *L. monocytogenes* was supported on various cheese types, characterized by higher pH (5.9 to 7.7), whereas cheese with low pH (4.9 to 5.7) did not support *L. monocytogenes* growth. Similarly, Guenther and Loessner (2011) reported that *L. monocytogenes* grew better on cheese when the pH increased above 6.0. Others have also shown that the acidity of the environment affects the efficiency of phage infection or the success of phage treatment. For example, Langlet et al. (2007) observed that phage MS2 formed 1.1 and 3.0 log pfu/mL fewer plaques on plaquing medium at pH 3.9 and 2.5, respectively, compared with plaquing medium at pH 6.7. Additionally, studies on different low-pH food matrices, such as hard cheese or apples, have reported little to no *L. monocytogenes* reduction in the presence of phage. For example, Perera et al. (2015) reported a 0.7 log reduction of *L. monocytogenes* when hard cheese (typically pH 5.1–5.4) samples were treated with $1 \times 10^8$ pfu/g of a commercial phage cocktail, whereas Silva et al. (2014) reported a 2 log reduction of *L. monocytogenes* on soft cheese (typically pH 6.2–6.5) treated with $8.3 \times 10^7$ pfu/g of phage P100. Furthermore, Oliveira et al. (2014) reported no significant difference in *L. monocytogenes* counts between control and phage-treated samples of fresh-cut apple slices (pH 3.76).

Importantly, our data confirm that the effectiveness of phage seems to be enhanced when cheese is made at a higher pH. Although we did not find a significant effect at pH 6.0 (relative to pH 6.5), cheese formulated at pH 6.0 showed the greatest phage-mediated reduction of *L. monocytogenes* numbers compared with untreated cheese. However, as observed with temperature, *L. monocytogenes* is still able to grow in the presence of the phage cocktail. Consistent with our findings, Silva et al. (2014) reported that in 2 types of Brazilian soft cheese, which have near neutral pH, bacteriophage P100 (applied at approximately $10^7$ pfu/g) initially

reduced *L. monocytogenes* counts by approximately 2 log compared with the untreated control at 30 min postinfection; however, the difference between treated and untreated was only 1 log after 7 d of storage. Guenther and Loessner (2011) also found that *Listeria* phage A511(approximately $10^8$ pfu/cm$^2$) was able to reduce *L. monocytogenes* inoculum levels below detectable levels (approximately $10^3$ cfu/cm$^2$) in cheese that had a pH of 7.6 after ripening.

Possible explanations for the enhanced effectiveness of phage-mediated *L. monocytogenes* reduction at higher pH (Figure 4) include (1) enhanced phage attachment to the host, and (2) increased sensitivity of *L. monocytogenes* not grown under multiple stress conditions. Reduced phage stability would also result in a decrease of overall efficacy of phage treatment; however, prior data suggest that reduced phage stability at the lower pH used here is not an issue; Fister et al. (2016) reported that phage P100 was stable when incubated in tryptic soy broth adjusted to a pH range of 4 to 10, and phage P100 numbers decreased by less than 0.5 log pfu/mL after 24 h and between 1 to 2 log pfu/mL after 1 mo, respectively. These data suggest that phage is stable under the pH conditions tested in our study and that the phage cocktail is likely able to remain effective over the shelf life of cheese. Besides phage stability, attachment of the phage to its bacterial host is essential to the success of phage treatment and can be affected by pH. Previous studies suggest that environmental factors can change *L. monocytogenes* cell envelope physiology, influencing the effectiveness of cell envelope–acting bactericidal treatments (Cotter and Hill, 2003). For example, a previous study showed that low pH changes the cell membrane composition, leading to tolerance of *Listeria* to other antilisterial compounds such as the cationic antimicrobial nisin (Verheul et al., 1997). Thus, we could hypothesize that a similar mechanism could contribute to an enhanced effect of phage (which also binds to cell envelope components) at higher pH.

### *L. monocytogenes* Serotype Affects Efficiency of the Phage Cocktail

In addition to the effects of environmental conditions, our data also indicate that serotype 4b strains had reduced sensitivity to the phage cocktail used, across pH and temperature conditions tested. These findings are consistent with a considerable body of work indicating that the host range of *Listeria* phage corresponds to host serotype (Wendlinger et al., 1996; Kim et al., 2008; Vongkamjan et al., 2012). Differences among *L. monocytogenes* serotypes can be attributed to the composition of their WTA, cell surface polysaccharides. For example, serotype 1/2 strains have terminal rhamnose and *N*-acetylglucosamine (GlcNAc) residues, whereas 4b strains are decorated with terminal glucose and galactose residues (Eugster et al., 2015). As an example of *Listeria*-phage host specificity patterns, Wendlinger et al. (1996) reported that *Listeria*-phage A118, a temperate Siphovirus, attacks 1/2 serotypes, and Siphoviral phage A500 primarily lyses serotype 4b, whereas the broad-host-range Myovirus phage A511 lyses most *L. monocytogenes* strains. Importantly, however, a putative CRISPR system has been identified in *Listeria*, which might defend against bacteriophage infection. Although locus I is conserved in both serotype 1/2a and 4b strains, locus II is only present in 4b strains. Furthermore, only locus II seems to be functional (Klumpp and Loessner, 2013). Although the phage cocktail used in this study included both *Siphoviridae* and *Myoviridae* phage, which should have a broad host range against *L. monocytogenes* serotypes, our data suggest that at least some phage in this cocktail could have reduced or limited ability to lyse either serotype 4b strains in general or the specific serotype 4b strains included here. Our findings are also consistent with a previous study indicating that at least some wild-type *L. monocytogenes* strains might be resistant to commercially used phage cocktails (Vongkamjan et al., 2012). In addition, or alternatively, the serotype 4b strains tested here might have a specific ability to adapt to the Hispanic-style fresh cheese environments, making them less susceptible to phage treatment in this food matrix. Overall, our findings, along with previous studies, support the importance of validating phage-based treatment strategies with both *L. monocytogenes* serotypes and environmental conditions relevant to a given application.

Our data suggest that pH, temperature, and serotype influence the effectiveness of phage treatment against *L. monocytogenes* in a laboratory-scale cheese model; however, the overall phage-dependent reduction was limited. Additionally, except for cheese made at pH 5.5, all treated samples allowed growth of *L. monocytogenes* after a 14-d incubation. Our results highlight a need for fine-tuning control strategies against *L. monocytogenes*. In comparison, the bacteriocin nisin can be added to cheese to control for *L. monocytogenes*, and it has been proven to reduce numbers in a dose-dependent way (Van Tassell et al., 2015). Whether environmental conditions affect the effectiveness of nisin treatment in cheese is the focus of our follow-up study. In addition to nisin, plant extracts such as ferulic acid have been used to inhibit the growth of *L. monocytogenes* on cheese, with some success (Van Tassell et al., 2015). Importantly, *L. monocytogenes* seems less likely to develop resistance to ferulic acid (Takahashi et al., 2015) than to nisin or

phage; therefore, a combined treatment approach could be an appropriate strategy to reduce *L. monocytogenes* numbers in a food matrix and decrease the likelihood of resistance, which could be effective regardless of environmental factors.

## CONCLUSIONS

Overall, our data suggest that phage-based control strategies can reduce *L. monocytogenes* populations in cheese, and that the effectiveness of these strategies is affected by *L. monocytogenes* strains and environmental conditions. Challenge studies, typically conducted by industry, provide only limited insights on the actual value of using phage-based control strategies either for public health or for an individual business that wants to reduce the risk of an outbreak or a recall associated with its products. Further work is needed to develop a formal risk assessment that can be used to predict the actual benefits of using phage-based control strategies to not only reduce the risk of contamination, but also decrease the number of human listeriosis cases, taking into account the expected distributions of storage temperatures and times between production and consumption, as well as the likelihood of contamination with different strain and serotypes. This is important because our data suggest that phage-based control strategies could have limited effect on reducing public health risks if, for example, cheese made at pH 6.5 is typically stored at 6°C or below and consumed after 14 d. In contrast, phage treatment could be substantial if cheese is typically stored at higher temperatures and consumed within a few days after production.

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