Dynamics of phage resistance in *Listeria monocytogenes* treated with individual and combined phages

Leticia Alejandra Orellana Galindo

Escuela Agrícola Panamericana, Zamorano
Honduras
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By:

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Resumen. El uso de bacteriófagos para combatir bacterias patógenas está siendo promovido en la industria de los alimentos. Uno de los problemas más críticos respecto a este tema es el desarrollo de resistencia. En este estudio se incubó por 60 horas *Listeria monocytogenes* 10403S con los bacteriófagos LP-048 y LP-125, aplicados de forma independiente y combinada (MIX). El comportamiento de *Listeria* infectada con los diferentes tratamientos fue descrito a través de cambios en su densidad óptica a 600 nm. Los resultados fueron clasificados en dos fases: crecimiento y re-crecimiento. Se sugiere que factores relacionados a "quorum sensing" pudieron afectar la tasa de absorción de los bacteriófagos. La emergencia de bacterias resistentes a la infección de los bacteriófagos ocurrió después de 21 horas para LP-048 y 27 horas para LP-125 y MIX. Este estudio demuestra la cercana relación entre los tratamientos LP-125 y MIX por lo que se sugiere que la aplicación combinada de LP-125 y LP-048 no es más eficiente que su aplicación independiente. Se propone que no ocurrió ningún costo en la aptitud de la bacteria debido a la mutación. Todos los tratamientos mostraron los mismos conteos de UFC/ml de células sobrevivientes después de siete horas de haber sido infectadas. Los porcentajes de susceptibilidad y resistencia no cambiaron durante la fase de re-crecimiento para los tratamientos LP-125 y MIX mientras que el tratamiento LP-04 mostró un efecto revertido en los porcentajes de células resistentes. Más investigación es necesaria para establecer los cambios genéticos de la bacteria y los bacteriófagos debido a su mutación.

Palabras clave: Aptitud, bacteriófago, células mutantes, resistencia, reversión, sobrevivientes**.**

Abstract. The use of bacteriophages against pathogenic bacteria is being promoted in the food industry. One of the most critical issues regarding this approach is the emergence of phage resistance. In this study, a prolonged incubation of *Listeria monocytogenes* 10403S and phages was performed for 60 hours, phages LP-048 and LP-125 were applied both independently and combined (MIX). The growth dynamics of the host was described through optical density measurements at 600 nm. Results were classified into two phases: growth and re-growth. It is suggested that quorum sensing factors may affect the phage adsorption rate. The emergence of phage-resistant cells occurred after 21 hours for LP-048 and 27 hours for both LP-125 and the MIX. This study demonstrates the close relationship between LP-125 and MIX, It is proposed that the combination of LP-125 and LP-048 is not more efficient than the use of them independently. It is suggested that no fitness cost occurred for mutation. All of the treatments showed the same CFU/ml counts of survivor cells after infection where the percentage of susceptible and resistant cells did not change during the re-growth stage in the LP-125 and MIX treatments. In contrast, LP-048 had a possible reversible effect over the resistant mutant cells. More research is needed to understand the effect in the genome of the bacteria and phage by the mutation.

Keywords: Bacteriophage, fitness cost, mutant cells, resistance, reversible, survivors.

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1. INTRODUCTION

The pathogenic bacteria, *Listeria monocytogenes*, is responsible of causing at least 1600 cases of infections in the United States every year. Pregnant women, the elderly, children, and those who are immunocompromised are the most vulnerable to *Listeria* poisoning (Scallan *et al.* 2011). There are at least 17 different species of *Listeria* identified; two of them, the aforementioned *L. monocytogenes* and *L. ivanovii*, have been recognized as pathogenic to humans and/or animals. Likewise, 13 serotypes or serovars of *Listeria* are known and have been studied. Research has revealed that serotypes 1/2a, 1/2b and 4b are responsible for 96% of human listeriosis cases in the world (Radoshevich and Cossart 2017).

Listeria monocytogenes is widely dispersed in the environment and transmitted to humans and animals through contaminated products. Inhibiting *L. monocytogenes* is one of the food industry's biggest concerns, being responsible for causing several recalls on products from grocery stores due to the possibility of contamination during their processing or storage (Strydom and Witthuhn 2015). Characteristics of *L. monocytogenes*, such as its rapid adaptability to high osmolarity, low temperatures and low pH, allow this bacterium to proliferate even in adverse conditions. Many of the barriers employed by the industry to limit bacterial growth are not very effective against *L. monocytogenes* (Wonderling *et al*. 2004).

New strategies have been developed to combat this pathogen. Among the variety of approaches, the one that is gaining special interest is the use of bacteriophages (henceforth referred to as phages) as biocontrol agents in ready-to-eat (RTE) products. Phages are viruses that infect and kill bacteria. These parasites are able to bind to the microbial surface, inject their genetic material and replicate inside the host bacterium, causing lysis to their host cell (Sulakvelidze *et al.* 2001). Phage tail fibers contain proteins that recognize a specific receptor on the bacteria´s cell wall surface. The bactericidal activity of bacteriophage was observed for first time in 1896 and identified as a virus capable of infecting bacteria in 1915. They started of being used as a treatment of bacterial diseases in humans and animals in 1919. It is thought that there are likely 10^{30} bacteriophages present in the biosphere (Chanishvili 2012).

The application of bacteriophages proposes an alternative to antibiotics. In addition, this application can be used as a bio-preservative and as an indicator in food products to identify the presence of pathogenic bacteria in food processing plants (García *et al*. 2008). Common methods of sanitization including chemicals that are non-specific against *Listeria* may result in less efficiency (Leverentz *et al.* 2003).

Recent research has shown the ability of bacteria to generate resistance against phage infection which occur through different defense mechanisms. It has influenced the evolution and virulence of several pathogens due to the rapid adaptation of bacteria to the environment through structural changes in their cellular envelope. Recently, it has been shown that *Listeria monocytogenes* 10403S generates resistance to phages LP-125, LP-048 and a mixture of both (Denes *et al.* 2014).

The success of using phage against bacteria, similar to the use of antibiotics, will depend on the ability of bacteria to develop resistance. Some researchers have suggested that the use of a cocktail of phages should delay the appearance of phage-resistant cells (Tanji *et al.* 2004). In this study, Optical Density (OD) measurements were used to describe *Listeria monocytogenes* 10403S growth in the presences of two phages applied independently and combined.

The objectives of this study were:

- To describe the growth of *Listeria monocytogenes* in the presence of bacteriophages LP-125, LP-048 and a mixture of both in different stages of growth.

- To determine the susceptibility of *Listeria monocytogenes* cells to bacteriophages LP-125 and LP-048 during different phases of the growth.

- To quantify survivor cell after phage infection.

2. MATERIALS AND METHODS

Phase I. Dynamics of growth.

Bacterial strain and bacteriophage. The bacterial strain used for this study was *Listeria monocytogenes* 10403S (Lineage II, serovar 1/2a). Two *Listeria* phages were used: LP-125 and LP-048, both belonging to the *Myoviridae* family. Phages were isolated from silage samples collected at two dairy farms in New York, United States, between August 2008 and July 2009 (Denes *et al*. 2014).

Host preparation. A frozen stock of *Listeria monocytogenes* 10403S, stored at -80 °C in BHI (Brain Heart Infusion) broth containing 15% glycerol was streaked on BHI agar, and incubated at 30 °C for 16 ± 2 hours to obtain isolated colonies. To inoculate overnight cultures, a single colony was selected and inoculated into a glass tube with 5 mL of LB-MOPS (Luria-Bertani medium buffered with 50 mM morpholinepropanesulfonic acid [MOPS] at a pH of 7.6) and incubated at 30 °C with shaking for 16 ± 2 hrs. One milliliter of the overnight growth was back-diluted into a flask with 50 mL of LB-MOPS, 50 µl of 1 M $MgCl_2$, 50 µl of 1 M $CaCl_2$, and 300 µl of 1 M Glucose. The flask was incubated with agitation at 30 °C. Its optical density was monitored at 600 nm (OD_{600}) every 30 minutes in a spectrophotometer until reaching a reading of 0.05.

Infection dynamics. After *L. monocytogenes* reached an OD_{600} of 0.05 in the flask, its content was divided into four test tubes and subjected to three different treatments with phage (LP-125, LP-048, and a cocktail of them called MIX). A control consisted of a culture of *L. monocytogenes* 10403S without phage infection. Bacteriophages were added (multiplicity of infection [MOI] of ~1.5), in the test tubes as follows: (A) For LP-125, 72 µl of phage suspension at a titer of 1.4×10^9 PFU/mL, 128 µl of SM (Saline Magnesium) buffer (2 g of $MgSO_4$, 5.8 g of NaCl, 5mL of 2% gelatin, 50 mL of 1M Tris-HCl [pH 7.5], and 945 mL of deionized (DI) water) were added to 5 mL of the host. (B) For LP-048, 26 µl of phage suspension at a titer of 4×10^9 PFU/mL, 128 µl of SM-buffer and 5 mL of the host. C) For the cocktail, (MIX), 13 µl of LP-125, 36 µl of LP-048 were added, 151 µl of SM-buffer and 5 mL of the host were added. D) As a control, 200 µl of SM buffer and 5 mL of *Listeria* from the flask were added to the tube. The infected tubes were incubated in a water bath at 30 °C with constant shaking. To observe its growth kinetics, its OD_{600} was monitored every 60 minutes for 60 hours.

Analysis of results. In order to analyze the growth dynamics of the three cultures, a completely randomized statistical design and a multiple comparison Tukey test were employed to compare the means of each treatment. The program used to carry out the

Analysis was the SAS Program, Version 9.4® to find if there was a statistical difference between the optical density values of each treatment evaluated during the first growth and the re-growth.

A statistical fit was employed due to the variation of the samples repetition between each treatment. Gaussian functions were applied to describe normal distributions in the graphs from the cultures (Molina Rodríguez 2015). The data analysis framework ROOT, a C++ based package from CERN (Brun and Rademakers 1996), was used to fit the OD_{600} distribution, in order to find the mean time where the highest values were obtained, i.e. where the cultures reach their highest population, and times where re-growth started to occur.

A one-dimensional fit to the OD_{600} distribution is used to obtain the mean time that will subsequently be used to compare three cultures. The data have been fitted using two Gaussian functions, denoted by the equation [1]. Where A is the normalization term (not showed in this study), t is the time, μ is the mean and σ is the standard deviation. Those functions were implemented using a C++ based package (Molina Rodríguez and Orellana 2018) which uses the histogram of the distribution to obtain the real values describing the behavior of data. In this case, the resulting two Gaussian functions have widths σ_1 and σ_2, means μ_1 and μ_2.

$$G(t, \mu, \sigma) = Ae^{\frac{(t-\mu)^2}{2\sigma^2}} \quad [1]$$

Phase II. Resistant colonies evaluation.

Survivor Cells. During the development of Phase I, three important phases were described: Growth, apparent equilibrium (flat-line), and after that an exponential phase (re-growth). Three samples were taken when the OD_{600} remained between the range of 0.006-0.008 (flat-line) approximately 4 hours post-infection. Two more samples were taken during the re-growth phase, while the OD_{600} reading reached 0.05-0.1 and 0.15-0.2. Each sample consisted of taking aliquots of 100 µl of each treatment, to dilute and spread them in petri dishes containing BHI agar as a culture medium. Plates were incubated at 30 °C for 16 ± 2 hours. Colonies from each treatment were counted and reported as CFU/mL.

Statistical analysis of results. Survivor counts were expressed in CFU/mL. A completely randomized design and a multiple comparison Tukey test to compare the means of every treatment to identify any difference between them using the program SAS, Version 9.4®.

Streak-Spot Assay (SSA). The susceptibility of mutant cells of *Listeria* to phage LP-125 and LP-048 was determined by performing the SSA on the mutant strains of the three cultures evaluated in this study and wild-type *Listeria monocytogenes* 10403S strain as a control. Ten Colonies from samples of flat-line and re-growth for each culture were randomly selected and dispersed in a line on BHI agar plates. Aliquots of 5 µl of each bacteriophage at a concentration of $1x10^8$ PFU were added on the lines of each colony (Figure 1). As a control, colonies of wild-type *Listeria monocytogenes* 10403S from a

frozen stock were evaluated against the two phages to confirm its susceptibility. They were then incubated at 30 °C for 16 ± 2 hours. The results were evaluated according to the presence or absence of plaques that indicate the susceptibility or resistance of the bacteria, respectively. The difference between resistant colonies in flat-line and re-growth was compared statistically.

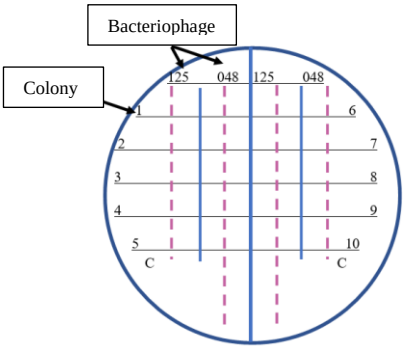

Figure 1. Label of the BHI plate to carry out the streak spot assay

Statistical analysis of results. For the Streak-spot assay, results are presented in percentage of susceptibility to each bacteriophage evaluated. A dependent samples statistical analysis was performed using the program SAS, Version 9.4® to find if there existed a difference between the percentage of cells resistant during the apparent equilibrium (flat-line) and re-growth.

3. RESULTS AND DISCUSSION

Infection dynamics.

This study describes the growth dynamics of *L. monocytogenes* 10403S with phages LP-125 and LP-048 applied independently and combined (MIX). Nine replicates were performed for the experiment. The experiment started at an OD_{600} reading of ~0.06, which then increased to a range of 0.15-0.2. After reaching a maximum value, the OD values decreased (due to cell fragmentation) until reaching an apparent equilibrium (flat-line). Prolonged incubation resulted in a new increase of the OD_{600} (re-growth) due to the emergence of phage resistant mutant cells. During re-growth, high variation in the plot dispersion was observed mainly in LP-125 and MIX cultures (Figure 2).

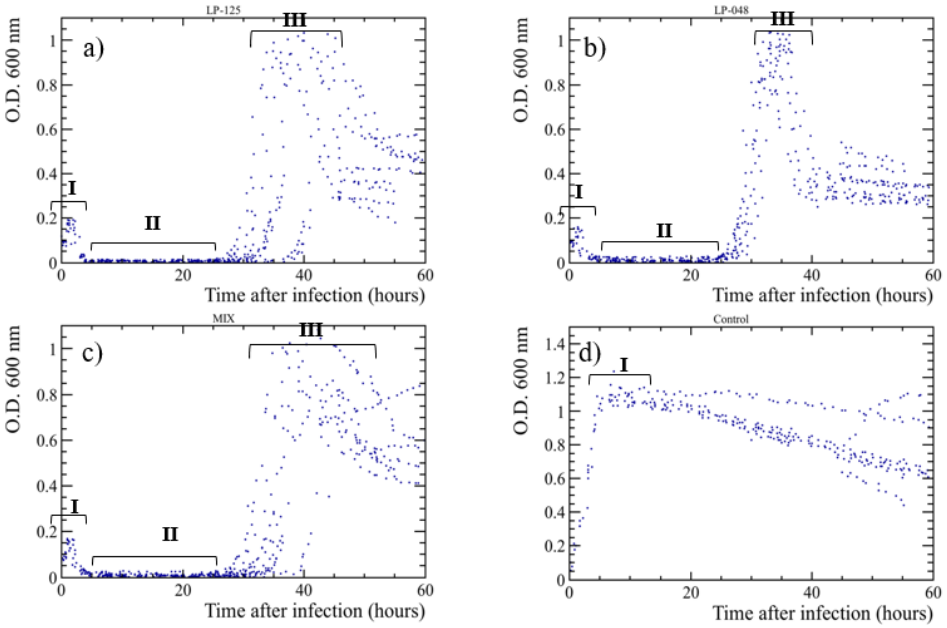

Figure 2. Dispersion plot of the changes in the OD600 readings as a function of time for the *Listeria monocytogenes* 10403S infected with phages. (**a)** LP-125**, (b)** LP-048, **(c)** MIX, and **(d)** Control sample with *Listeria monocytogenes* 10403S without phage. (**I**) Growth phase, (**II**) flat-line, and (**III**) re-growth.

To describe the growth kinetics of *L. monocytogenes* 10403S in the presence of two phages applied independently and combined, results were divided in two parts, growth and re-growth. The first part, growth, covers from the OD_{600} increasing after infection, reaching a maximum value, and then decreasing until reaching an apparent equilibrium (flat-line). The

second part, re-growth, includes the times where re-growth started to occur until its maximum OD_{600} reading.

Part I. Growth. While the control of the experiment (*Listeria monocytogenes* 10403S without phages) showed its maximum growth 5-7 hours after infection, the maximum growth of cultures with phages took place after a much shorter time period. This was due to phage reproduction via its lytic life cycle, which caused bacteria cell lysis and growth decline until reaching an apparent equilibrium (flat-line). All of the curves were adjusted with a Gaussian model and the parameters are summarized in Chart 1.

Chart 1. Summary of growth dynamic parameters of *Listeria monocytogenes* 10403S infected with phages applied independently and combined. μ represents the mean of the data and σ the standard deviation.

	Growth			**Re-growth**		
Phage treatment	**Maximum OD_{600}**	**Time (hours)**		**Maximum OD_{600}**	**Time (hours)**	
		μ	**σ**		**μ**	**σ**
LP-048	0.13 ± 0.02^{c}	1.2 ± 0.2	0.9 ± 0.1	1.01 ± 0.04^{a}	33.4 ± 0.3	3.1 ± 0.2
LP-125	0.17 ± 0.02^{a}	1.6 ± 0.2	1.0 ± 0.2	0.95 ± 0.03^{a}	35.2 ± 0.8	2.0 ± 0.7
MIX	0.14 ± 0.02^{b}	1.5 ± 0.3	1.1 ± 0.2	0.95 ± 0.14^{a}	47.2 ± 0.8	7.7 ± 0.5
Control	$1.07 \pm 0.01^{a\S}$					

[abc]Means with different letters in the same column represent statistical difference between the values of each part ($P < 0.05$).
§ The value of the control is compared with the maximum OD_{600} readings from re-growth.
Coefficient of Variation of the maximum OD_{600} was 6.21 for Growth and 7.47 for Re-growth.

The times observed between the treatments with phages applied independently and combined are more related between the treatment LP-125 and MIX, it is suggested that the host possess a higher concentration of receptors or a greater affinity to bind LP-125 than LP-048 when they are applied in combination (Schwartz 1976), this could be a possible explanation of the similar behavior of the MIX treatment with the LP-125. The maximum OD_{600} readings recorded represent the maximum growth of *Listeria monocytogenes* after being infected by the phages. Statistically difference was observed from each treatment, where LP-125 had the maximum value, followed by MIX and LP-048. Results from this part of the infection dynamics differ with the results obtained from Denes *et al.* in 2015, in which higher efficiency of LP-125 over LP-048 was demonstrated where LP-125 had advantage on some key parameters of the phage´s lytic cycle (absorption, eclipse period, latent period, and burst size).

Mathematical models used to studying the evolutionary dynamics of bacteria infected by bacteriophage suggest that the density of susceptible bacteria and phage affect the behavior of their interaction (Payne and Jansen 2003). The density of phage used by the treatments in this study overcome the density of *Listeria monocytogenes* 10403S cells (MOI ~1.5), in contrast with Denes *et al.* 2015, who used a higher concentration of the host than the phage (MOI of ~0.1). It is proposed that LP-125 reduce its adsorption rate due to the low concentration of its host. Studies have demonstrated that phage do not increase in number when the density of its host remains below of a critical density to phage replication (Wiggins and Alexander 1984). Quorum sensing is an intracellular communication among bacteria, where auto inducers are secreted and accumulated by bacteria as it is increasing in number (Kievit and Iglewski 2000). In this study it is suggested that this bacterium may possess phage receptors which are expressed in response to quorum factors which could explain the host cell density dependence to phage replication (Høyland-Kroghsbo *et al.* 2013).

Part II. Re-growth. After the apparent equilibrium phase, cell re-growth was observed for cultures infected with LP-048, LP-125, and MIX. Considering the normally distributed data and taking the left tail of the Gaussian curve (in order to reach a probability of 0.9994 with a confidence level = $1 - 2P(Z > -3.99)$) it is possible to affirm with 99.997% certainty that re-growth occurred at 21.031 hours, 27.22 hours, and 27.52 hours after infection respectively (Chart 2). LP-048 demonstrated the most rapid regrowth while MIX and LP-25 had closely related times.

Chart 2. *Listeria monocytogenes* 10403S re-growth starting time caused by increase of its OD_{600} values. It is considered to have 99.997% certainty assuming normally distributed data.

Phage Treatment	Time (hours)
LP-048	21.03
LP-125	27.22
MIX	27.52

In order to survive the attack of phage, bacteria have evolved diverse strategies of defense. Mutations in the target bacteria could lead to the loss of specific phage receptors. Denes *et al.* in 2015 hypothesized that LP-048 and LP-125 phages possess different mechanisms to bind to their host cells. The authors showed that *L. monocytogenes* 10403S resists phage infection of LP-125 and LP-048 through mechanisms of adsorption inhibition, where resistance to LP-125 occurs due to lacking terminal *N*-acetylglucosamine in the wall teichoic acid (WTA) of the mutant strains. The resistant strains to both phages have disruptive mutations in their rhamnose biosynthesis operon but still possess *N*-acetylglucosamine in their WTA.

Studies have shown that the use of a cocktail of phages with different bacterial receptors for binding, delays the appearance of phage-resistant cells of *E. coli* O157:H7 (Tanji *et al.* 2004). It is suggested that LP-125 and LP-048 may possess different mechanisms to bind its host cells due to the different times to develop resistance and attribute the high similitude between the MIX and the LP-125, observed in Chart 2, to a higher affinity of the host to bind the phage LP-125, a higher adsorption rate and faster replication of LP-125 proposed by Denes *et al.* 2015. It is suppose *Listeria* was more infected by LP-125 phage causing the parameters from the MIX were more related to this phage. The purpose of using a cocktail of phages as a biocontrol agent in the food industry is to minimize the possibility of the target bacteria generating resistance (FDA 2006). According to the information obtained from this study, the combination of LP-125 and LP-048 is not efficient to that aim.

Much of the research about bacteria–phage evolution has been carried out *in vitro*, little is known regarding natural populations. Guenther and Loessner (2011) showed that after 22 days phage doses did not affect bacterial viable counts of *Listeria monocytogenes* in soft ripened cheeses. Other studies have found no phage resistance development after 5 to 13 days in ready-to-eat products treated with *Listeria*-Phages (Gunther *et al* 2009). The development of phage resistance on foods appears to be more variable and harder to predict, but the occurrence seems to be slower compared to *in vitro* studies (Cairns *et al.* 2008).

Re-growth was observed in the three cultures when OD_{600} readings rose due to the growth of phage-resistant cells. Two Gaussian's curves were used to fit the data to determine the time in which the maximum OD_{600} reading occurred after re-growth. The maximum OD_{600} of the control was 1.0686 ± 0.0148. Comparing this result with the maximum OD_{600} readings the treatments had after regrowth, it is remarkable that these values are closely related and statistically no different (Chart 1). A possible explanation for this similarity could be that bacteria do not generate any fitness cost associated with the development of phage resistance. Some researchers have observed no fitness cost from bacteria-phage interaction experiments in nutrient-rich media but it has been detected in its natural environment (Gómez and Buckling 2011; Meaden *et al.* 2015). However, it is supposed that different rates of growth occurred from the staring times of regrowth to the times needed to reach the maximum OD_{600} values of each treatment. The time which *Listeria* last of reaching the maximum OD_{600} after regrowth were 12.37 hours for LP-048, 7.98 hours for LP-125, and 19.68 for MIX, more information is necessary to ensure this assumption.

One of the most critical issues for the food industry regarding phage treatments against pathogens is the possible emergence of phage resistance. However, an advantage of the use of bacteriophages is that whereas antibiotic-resistant strains persist in the environment, antagonistic phage populations and bacteria potentially coevolve (Escobar-Páramo *et al.* 2012). Investigations have demonstrated long-term arms races between the infectivity of phage and the resistance of its bacterial host (Buckling and Rainey 2002). Kunisaki and Tanji in 2010 suggested that the administration of a phage cocktail might cause the emergence of resistance from superinfection of mutant phages, which adapt to the environment. A potential solution for overcoming phage resistance is by the use of a combined treatment of phage with antibiotics (Escobar-Páramo *et al.* 2012; Bedi *et al.* 2009;

Ryan *et al.* 2012), although further investigation is needed to confirm phage-antibiotic synergy.

Bacterial survivor counts.

The experiment started with an average of 7.87 ± 0.053 Log CFU/mL of *Listeria monocytogenes* 10403S which then was infected with phage. When a bacterial population is exposed to an antagonistic phage population, evidence has shown that even phage infection occurs, a small population of bacteria survive due to the presence of spatial refuge for sensitive bacteria on the surface of the glass tube used during the experiment (Schrag and Mittler1996). Results confirm that *Listeria monocytogenes* 10403S could not be eliminated from the continuous culture by phages LP-048 and LP-125 which were applied either independently or combined (Chart 3). The count of *Listeria* cells obtained from the flat-line reflects the total number of survivor cells after infection; all of the treatments had the same effectiveness at reducing initial bacterial load. It is suggested that due to the low density of bacteria population during flat-line, when the density of a susceptible population of bacteria the rate of phage replication do not overcome the rate of phage loss and its population declined.

Chart 3. Cell counts of *Listeria monocytogenes* 10403S taken from flat-line and regrowth. Results are expressed in Log CFU/mL.

Treatment	Flat-line[NS]	Re-growth[NS]
LP-048	2.5 ± 0.3	7.8 ± 0.4
LP-125	1.8 ± 0.5	7.8 ± 0.7
MIX	1.8 ± 0.5	8.0 ± 0.7

[NS] No statistical difference.

Susceptibility of cells.

Streak-spot assays were performed during flat-line and re-growth. An estimated of 90 colonies were evaluated per phase and per treatment, to identify the percentage of each type of mutant *Listeria* cells (Chart 4). Results from the streak-spot assay showed four mutation types of *Listeria monocytogenes* 10403S cells classified according to their susceptibility (S) or resistance (R) against phage infection. Type 1: was susceptible (SS) to both phages, Type 2: was susceptible (S) for LP-125 and resistant (R) to LP048, Type 3: was susceptible (S) for LP-048 and resistant (R) to LP-125, and Type 4: resistant to both phages (RR).

The cultures treated with LP-048 and LP-125 showed a strong presence of two types of mutant *Listeria* cells, both had in common Type 4. The other types were those which present a remarkable percentage of resistance cells for the same phage of the treatment; Type 2 for LP-048 and Type 3 for LP125. The existence of Type 4 cells indicates that resistance to one phage may confer resistance to the other phage used. A reversible behavior was observed

during re-growth from LP-048 which changed its susceptibility and resistant percentage of cells. During the flat-line phase, the optical density of the cell population of *L. monocytogenes* 10403S cultures did not change much for a long time. Due to the stability of the low optical density in flat-line, it could be considered that all of the susceptible cells contributed to phage proliferation while the phage resistant cells accounted for bacterial proliferation, which allowed this equilibrium to be maintained (Kunisaki and Tanji 2010).

In order to evaluate the persistence of each type of *Listeria*, a paired sample statistical analysis was performed using SAS Program Version 9.4®. Results showed that for the treatments LP-125 and MIX, the percentage of cells observed during flat-line of each type of *Listeria* were the same as re-growth. In contrast, LP-048 presented a statistical difference for Type 1 and Type 3 of *Listeria* mutant cells between flat-line and re-growth (Chart 4). It is suggested that a cost associated with the development of resistance may allow the susceptible bacteria to overcome resistant-cells during re-growth due to a higher growth rate (Bowers *et al.* 1994). Phage-resistant cells which arise even after multiple generations (re-growth) and produce susceptible and resistant cells, could share the same genotypic background (Kunisaki & Tanji 2010). Due to some cells from the cultures exhibiting some susceptibility to the phage, there exists the possibility that phage growth was present in the cultures even after re-growth.

Chart 4. Phage susceptibility test of mutant cells of *Listeria monocytogenes* after infection of LP-125, LP-048 and its combination (MIX). The presence of four types of cells obtained from flat-line and re-growth were evaluated and compared between sampling phases. Susceptible cells are represented by (S) and resistant by (R).

Phage treatments	**Sampling phase**	**(S) LP-125 (S)LP-048 Type 1**	**(S)LP-125 (R)LP-048 Type 2**	**(R)LP-125 (S)LP-048 Type 3**	**(R)LP-125 (R)LP-048 Type 4**
LP-048	Flat-line	0%*****	4%	1%	94%*****
	Re-growth	7%*****	20%	0%	73%*****
LP-125	Flat-line	1%	0%	58%	41%
	Re-growth	2%	0%	59%	38%
MIX	Flat-line	0%	0%	0%	100%
	Re-growth	0%	1%	2%	97%

***** There is a statistical difference between the percentage in the flat-line and regrowth of the same type of cells.

4. CONCLUSIONS

- *Listeria monocytogenes* 10403S developed phage-resistance after 21.031, 27.22, and 27.52 hours for LP-048, LP-125, and MIX respectively. Results obtained from the MIX treatment were closely related with the LP-125. The MIX did not have any advantage in reducing the time of developing resistance

- Resistance to one phage may confer resistances to the other phage. The MIX treatment was more efficient to confer resistance to both. Reversible behavior of LP-048 may occurred during re-growth as represented by the different percentage of phage resistant and susceptible cells.

- The survivor cells counts after phage infection and after regrowth at the same optical density had no statistical difference in the treatments.

5. RECOMMENDATIONS

- Determine the alterations to DNA that are responsible for the altered phenotypes of the mutant strains and possible mutant phage.

- Carry out a similar experiment but comparing optical density measurements with CFU/mL.

- Extend this experiment to other strains of *Listeria monocytogenes* with suiChart phages.

- Compare the dynamics of *Listeria monocytogenes* 10403S infected with phages LP-125 and LP-048 *in vivo* with results obtained during this experiment.

- Evaluate the efficiency of different cocktail of phages to delay the appearance of phage resistant cells of *Listeria monocytogenes* 10403S.

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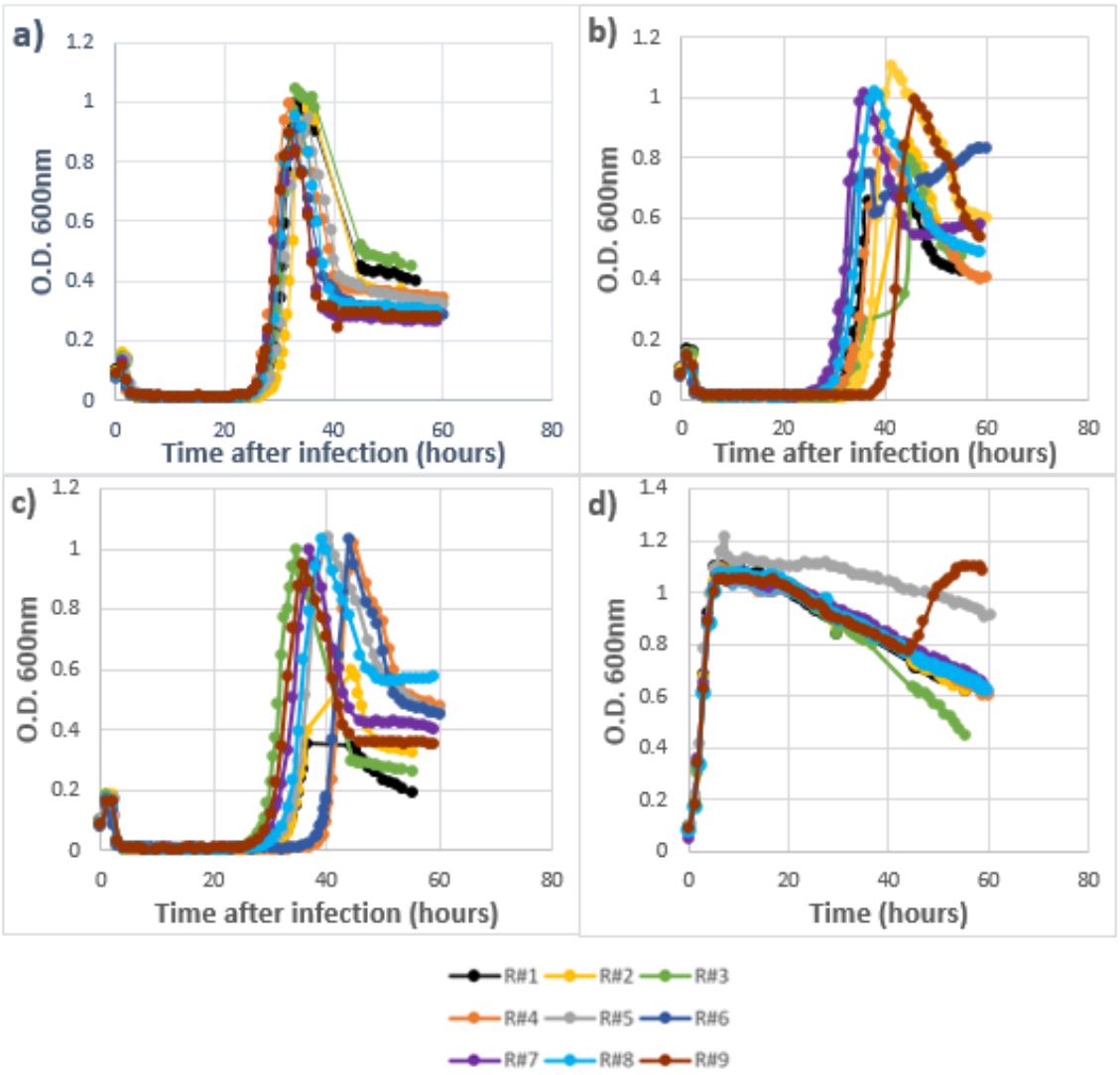
7. APPENDIX

Appendix 1. Raw data of OD_{600} measurements, which define the growth of *L. monocytogenes* 10403S infected with phage.



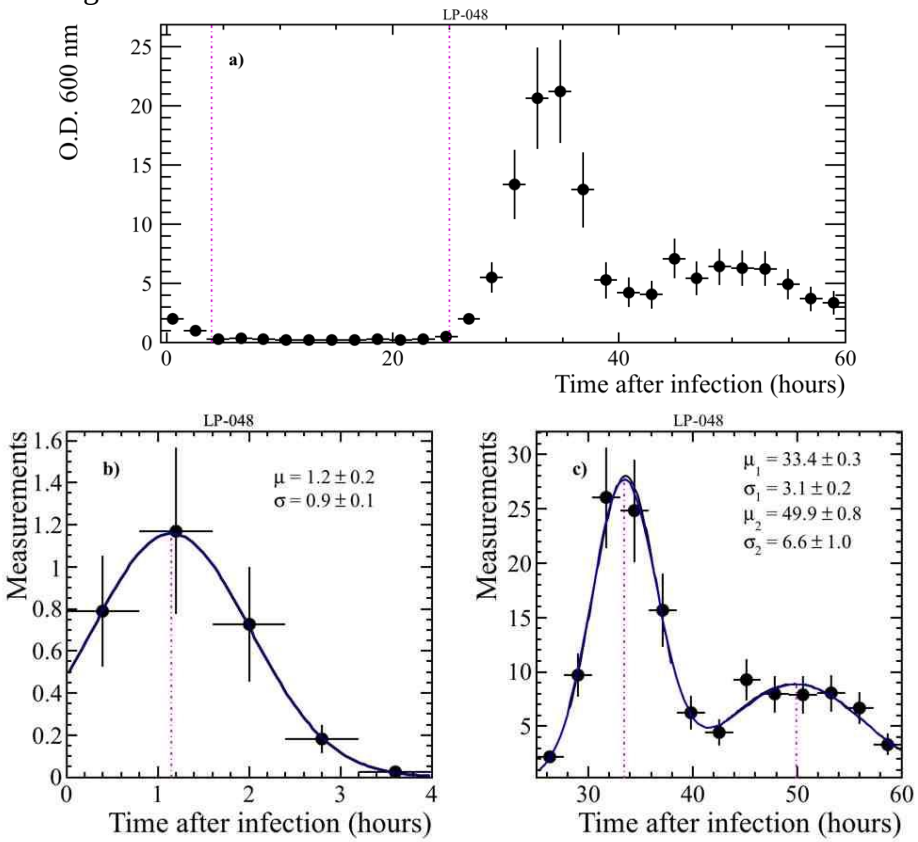
(a) LP-048**, (b)** MIX, **(c)** LP-125**,** and **(d)** Control. Data represent nine replicates per treatment.

Appendix 2. Results from paired sample statistical design applied to evaluate the persistence of phage resistant cells during flat-line and re-growth phases. Statistical analysis SAS Program Version 9.4® P < 0.05 indicate the existence of statistical difference.

Treatments	***LP-125(+) LP-048(+) Pr > |t|***	***LP-125(+) LP-048(-) Pr > |t|***	***LP-125(-) LP-048(+) Pr > |t|***	***LP-125(-) LP-048(-) Pr > |t|***
LP-048	0.0479*****	0.0636	0.3506	0.0482*****
LP-125	0.3506	.	0.7518	0.4198
MIX	.	0.3506	0.3506	0.1950

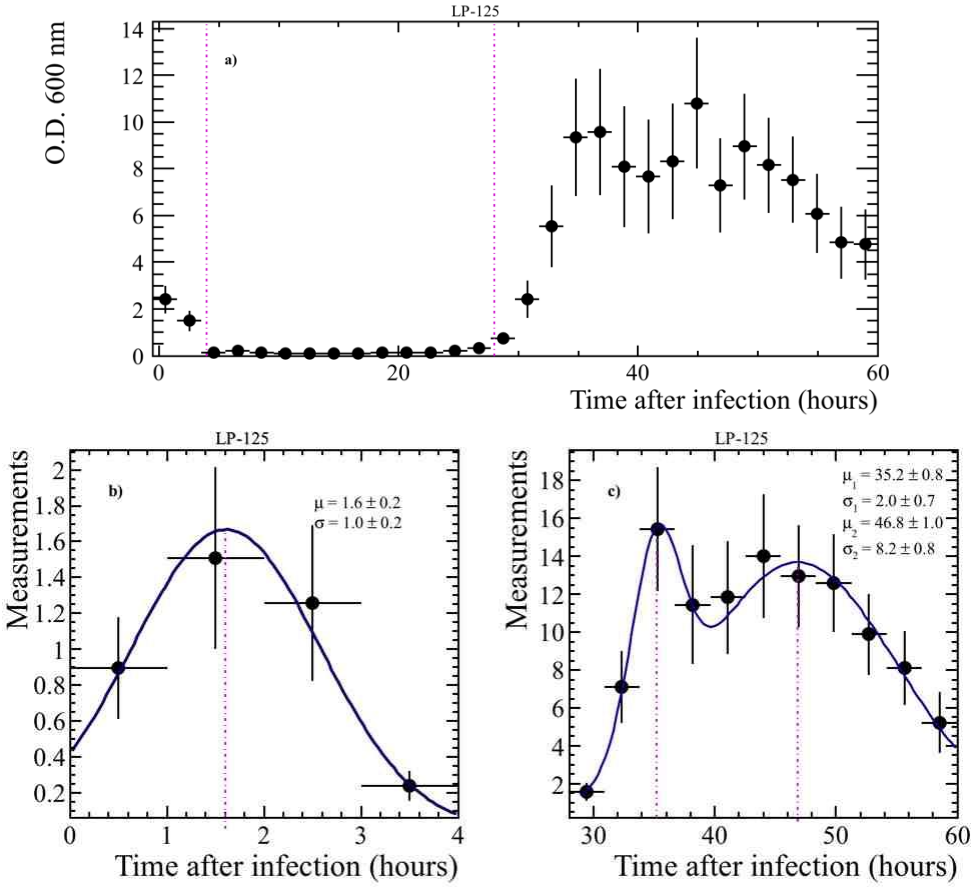
*****P < 0.05

Appendix 3. Curve of the growth of *Listeria monocytogenes* 10403S infected with LP-048 phage, assuming a normal distribution of the data.



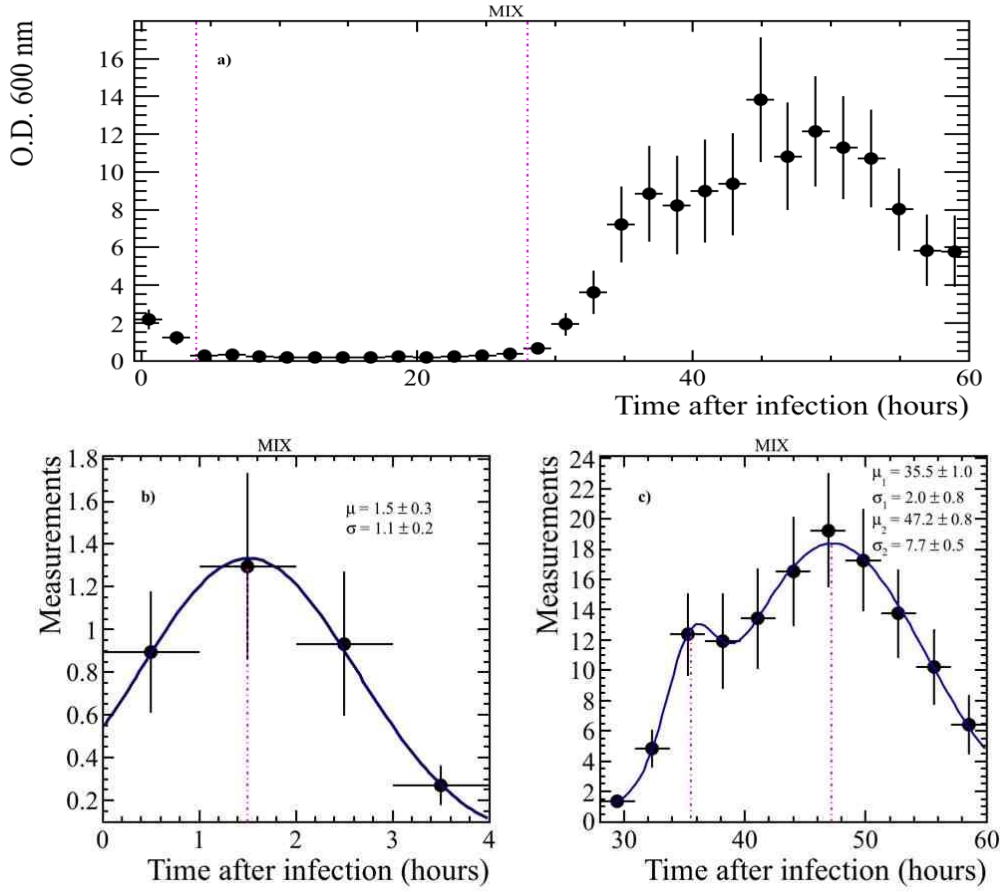
a) Raw data of *Listeria monocytogenes* 10403S infected with LP-048. **b)** Fitted distribution for the first 4 hours (Time ≤ 4 hours). **c)** Fitted distribution for time greater than 28 hours (Time ≥ 28 hours). Dots with error bars represent real data and blue line is the fitted function. Parameters for each fit function are shown in both b and c figures.

Appendix 4. Fitted curve of the growth of *Listeria monocytogenes* 10403S infected with LP-125 phage, assuming a normal distribution of the data.



a) Raw data of *Listeria monocytogenes* 10403S infected with LP-125 phage. **b)** Fitted distribution for the first 4 hours (Time ≤ 4 hours). **c)** Fitted distribution for time greater than 28 hours (Time ≥ 28 hours). Dots with error bars represent real data and blue line is the fitted function. Parameters for each fit function are shown in both b and c figures.

Appendix 5. Fitted curve of the growth of *Listeria monocytogenes* 10403S infected with two phages, LP-125 and LP048, assuming a normal distribution of the data. Fitted curve of MIX.



a) Raw data of *Listeria monocytogenes* 10403S infected with phages LP-048 and LP-125. **b)** Fitted distribution for the first 4 hours (Time ≤ 4 hours). **c)** Fitted distribution for time greater than 28 hours (Time ≥ 28 hours). Dots with error bars represent real data and blue line is the fitted function. Parameters for each fit function are shown in both b and c figures.