Title: Listeriaphages and coagulin C23 act synergistically to kill *Listeria monocytogenes* in milk under refrigeration conditions.

Running Title: Killing of *L. monocytogenes* in milk

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Abstract

Bacteriophages and bacteriocins are promising biocontrol tools in food. In this work, two *Listeria* bacteriophages, FWLLm1 and FWLLm3, were assessed in combination with the bacteriocin coagulin C23 to inhibit *Listeria monocytogenes*. Preliminary results under laboratory conditions demonstrated that both antimicrobials act synergistically when they were applied in suboptimal concentrations. The combined approach was further assessed in milk contaminated with 5×10^4 CFU/ml *L. monocytogenes* 2000/47 and stored at 4ºC for 10 days. When used alone, phage FWLLm1 added at 5×10^6 PFU/ml, FWLLm3 at 5×10^5 PFU/ml and coagulin C23 at 584 AU/ml kept *L. monocytogenes* 2000/47 counts lower than the untreated control throughout storage. However, when used in combination, inhibition was enhanced and in the presence of FWLLm1 and coagulin C23, *L. monocytogenes* 2000/47 counts were under the detection limits (less than 10 CFU/ml) from day 4 until the end of the experiment. Resistant mutants towards phages and coagulin C23 could be obtained, but cross-resistance was not detected. Mutants resistant to FWLLm3 and coagulin C23 were also recovered from surviving colonies after cold storage in milk which may explain the failure of this combination to inhibit *L. monocytogenes*. Remarkably, the fraction of resistant mutants isolated from the combined treatment was lower than that from each antimicrobial alone, suggesting that synergy between bacteriocins and phages could be due to a lower rate of resistance development and the absence of cross-resistance.

Keywords: Bacteriophage, *Listeria monocytogenes*, bacteriocin, synergism, biocontrol, milk, resistance.

Highlights

- The combined used of bacteriocins and bacteriophages synergistically inhibits *Listeria monocytogenes* in broth
- Both antimicrobials enhanced safety of milk under cold storage
- Synergy could be linked to a lower frequency of development of resistant mutants to each antimicrobial
- Cross-resistance was not detected

1. INTRODUCTION

Listeria monocytogenes is the causative agent of listeriosis, a foodborne disease which affects the elderly, pregnant women, unborn and newborn babies and people with weakened immune systems (Allerberger and Wagner, 2010). In healthy adults and children, listeriosis causes few or no symptoms and may be mistaken for a mild viral infection or flu which makes the incidence of listeriosis difficult to establish (Bortolussi, 2008). However, an increasing rate of this foodborne illness has been reported in Europe in recent years related to a higher rate of listeriosis in people ≥ 65 years of age (Goulet et al., 2008).

L. monocytogenes is a serious concern for the food industry as this pathogen can grow at refrigeration temperatures commonly used to control pathogens in foods (4ºC to 10ºC) and tolerates high concentrations of salt and low pH (Ferreira et al., 2014). Many categories of food have been related with listeriosis outbreaks: milk, soft cheeses and other dairy products, sausages, smoked fish, salads, delicatessen and ready-to-eat products (Garrido et al., 2010). The majority of detected outbreaks have been caused by ready-to-eat meats and cheeses (Cartwright et al., 2013; Garrido et al., 2010). Although *L. monocytogenes* is inactivated by thermal treatments used in processed food, post-processing cross-contamination from equipment and environment may occur due to the persistence of this pathogen in processing plants (Ferreira et al., 2014; Meloni et al., 2014; Ortiz et al., 2014) as a result of the development of resistance to disinfectants and its ability to form biofilms (Ferreira et al., 2014).

Listeriaphages have been proposed as new tools for *Listeria* biocontrol. Bacteriophages or phages, the natural killers of bacteria, are widely distributed in all environments, including food, and are presumed safe for humans, animals and plants. They have been successfully assessed as new tools to reduce levels of foodborne

87 pathogens and spoilage bacteria along the food chain. *Listeria* phages have been
88 isolated from several environments (sewage plants, silage, food processing
89 environments, lysogenic strains) and used for detection of *L. monocytogenes* in food as
90 well as for reducing its presence in food processing equipment and on ready-to-eat
91 products (García et al., 2010; Hagens and Loessner, 2014).

92 Another strategy to ensure food safety is the use of bacteriocins as natural
93 biopreservatives. Bacteriocins are ribosomally synthesized antimicrobial peptides or
94 proteins with bactericidal or bacteriostatic activity produced by bacteria (Drider and
95 Rebuffat, 2011). These antimicrobial peptides have been traditionally used as
96 biopreservatives to extend the shelf life of food products without compromising their
97 nutritional and organoleptic properties (Gálvez et al., 2010; García et al., 2010). In this
98 context, bacteriocins are regarded as an additional barrier to inhibit growth of
99 undesirable microorganisms (Omar et al., 2013) and have already been successfully
100 applied in several food systems to control the growth of *L. monocytogenes* (Davies et
101 al., 1997; Franklin et al., 2004; Wan et al., 1997).

102 In this work, two *Listeria* phages, FWLLm1 and FWLLm3, and one bacteriocin,
103 coagulin C23, were used in combination looking for a synergistic effect to reduce or
104 eliminate the presence of *L. monocytogenes* in milk. Coagulin C23 is produced by
105 *Lactobacillus paraplantarum* IPLA C23 and has been reported to have antimicrobial
106 activity against *L. monocytogenes* (Allende et al., 2007; Rilla-Villar, 2003). Coagulin
107 C23 is identical to coagulin A, a natural variant of the class IIa bacteriocin pediocin
108 PA1/AcH (Hyronimus et al., 1998). FWLLm1 and FWLLm3 are myoviruses isolated
109 from sheep faeces that infect strains of the species *L. monocytogenes* as well as other
110 species such as *Listeria ivanovii* and *Listeria welshimeri* (Bigot et al., 2011; Arachchi et
111 al., 2013). FWLLm1 was reported to immediately reduce 2.5 $\log_{10}$ CFU/cm^2 a *L.*
112 *monocytogenes* contamination after addition on the surface of vacuum-packed ready-to-

113 eat chicken breast roll (Bigot et al., 2011) suggesting its potential for biocontrol of this
114 pathogen in food.
115
116 **2. MATERIAL AND METHODS**
117
118 **2.1 Bacteria, phages, bacteriocin and growth conditions**
119 *L. monocytogenes* 2000/47 strain, which is a subtype that has caused listeriosis
120 sporadic cases and outbreaks in New Zealand for several years (Sim et al., 2002), was
121 used to contaminate milk samples and as host of phages for propagation. *Listeria* cells
122 were grown at 32ºC under static conditions or at 37°C with shaking in TSB (Triptone
123 Soy Broth, Scharlau, Barcelona, Spain) or TSB supplemented with 2% (w/v)
124 bacteriological agar (TSA). Phage FWLLm1 and FWLLm3 preparations were obtained
125 from 10 ml of *L. monocytogenes* 2000/47 which was infected with the phages at a ratio
126 1:1 (phage:bacteria). The infected cultures were then incubated for 3 h at 37ºC with
127 shaking. Concentrated coagulin C23 supernatants were obtained from *L. paraplantarum*
128 IPLA C23 cultures grown overnight at 32ºC in MRS broth (Scharlau, Spain). Ninety ml
129 of the supernatant were mixed with 10 ml trichloroacetic acid 100% and incubated 1 h
130 at 4ºC. After centrifugation, the supernatant was discarded and the dried pellet was
131 resuspended in 5 ml sodium phosphate buffer 50 mM pH 6.8 by shaking at room
132 temperature. The insoluble fraction was removed by centrifugation and the supernatant
133 was adjusted to pH 5.5-6.5 with NaOH and filter sterilized. Bacteriocin activity was
134 quantified by the agar diffusion test using 20 µl of 2-fold dilutions placed in wells made
135 on *L. monocytogenes* 2000/47 indicator plates. Arbitrary units (AU) were defined as the
136 inverse of the last dilution that gave a clear halo and expressed by ml. Coagulin C23
137 samples routinely contained 12,800 AU/ml.
138

139 **2.2 Challenge test in liquid medium**

140 Two ml TSB were inoculated with a colony of *L. monocytogenes* 2000/47 and
141 incubated overnight at 32ºC. Then, 10 ml TSB were inoculated at 1% with the overnight
142 culture and grown to an optical density OD_{600nm} of 0.1 followed by a 1/10 dilution to
143 obtain 5×10^6 CFU/ml. The culture was divided into 2 ml aliquots, each aliquot was
144 mixed with 1 ml of TSB containing 10 mM $Ca(NO_3)_2$, 10 mM $MgSO_4$ and the following
145 volumes of FWLLm1 (36 µl), FWLLm3 (820 µl), coagulin C23 (137 µl), respectively,
146 or a mix of phage and bacteriocin to get a final 1:10 phage:bacteria ratio and coagulin
147 C23 at 584 AU/ml final concentration. Samples were incubated at 32ºC and viable
148 counts were monitored every 2 h on TSA for a period of 6 h by serial dilutions of the
149 samples.

150

151 **2.3 Challenge test in milk under refrigeration conditions**

152 Ten ml of Extended Shelf Life milk (ESL) were inoculated with approximately 5
153 $\times 10^4$ CFU/ml of *L. monocytogenes* 2000/47 grown to $OD_{600nm} = 0.1$. Aliquots (2 ml)
154 were mixed with ESL milk (final volume of 1 ml) containing 10 mM $Ca(NO_3)_2$, 10 mM
155 $MgSO_4$ and the following volumes of FWLLm1(12.5 µl), FWLLm3 (25 µl), coagulin
156 C23 (137 µl) or a mix of phage and bacteriocin. FWLLm3 was added at ratio
157 phage:bacteria 10:1, FWLLm1 at 100:1 and coagulin C23 at 584 AU/ml final
158 concentration. Samples were incubated a 4ºC for 10 days. Viable counts were checked
159 every 24 h on TSA.

160

161 **2.4 Resistance development to coagulin C23 and listeriaphages**

162 Drops (10 µl) of coagulin C23 were placed onto a *L. monocytogenes* 2000/47
163 lawn and incubated 48 h at 32ºC. Putative coagulin C23 resistant colonies were picked
164 from the inhibition halos, grown overnight in TSB and tested against coagulin C23

using the agar diffusion test. *L. monocytogenes* resistant cells were grown for 5 additional overnight cultures in TSB in the absence of selective pressure to allow any putative phenotype-reversion of non-genetically altered strains. Then, cultures were tested again against coagulin C23. Cross-resistance to phages was tested by dropping phages FWLLm1 and FWLLm3 (5 µl) onto lawns of the C23-resistant cultures.

Bacteriophage-insensitive mutants (BIMs) were also obtained from *L. monocytogenes* 2000/47 using phage FWLLm3. One-hundred µl of a 1:10 diluted overnight culture of *L. monocytogenes* 2000/47 (10^7 CFU) were incubated with 100 µl of phage (10^8 PFU) for 10 min at 37ºC. Then, the mixture was poured onto a TSA plate and covered with 3 ml of 0.7% TSA. Plates were incubated during 16 h at 37ºC. Ten surviving colonies were picked up and grown in fresh TSB for 16 h at 37ºC. Bacteriophage susceptibility was tested by the drop assay. Cross-resistance development was tested by dropping coagulin C23 onto the lawns obtained from the surviving colonies.

3. RESULTS

3.1 Coagulin C23 and the listeriaphages FWLLm1 and FWLLm3 act synergistically to kill *L. monocytogenes* in broth.

The potential synergistic effect of coagulin C23 and listeriaphages was assessed in broth at 37ºC (Fig. 1). Combination of coagulin C23 and phage FWLLm1 decreased the concentration of *L. monocytogenes* 2000/47 by 5.9 log units after 2 h of incubation compared to the untreated control, while 3 and 4.8 log reductions were caused by FWLLm1 and C23 alone, respectively (Fig. 1A). After two hours of incubation, re-growth was observed in all samples. On the contrary, when coagulin C23 was combined with FWLLm3, *L. monocytogenes* 2000/47 was under detection limits after 2 h and no

191 re-growth occurred. FWLLm3 and C23 alone were able to reduce the initial
192 contamination by 4 and 4.4 log units, respectively (Fig. 1B) but growth resumed
193 afterwards.
194
195 **3.2. Coagulin C23 and listeriaphages FWLLm1 and FWLLm3 act synergistically**
196 **to kill *L. monocytogenes* in milk under storage conditions.**
197 The synergistic effect between listeriaphages and coagulin C23 was further
198 confirmed in milk inoculated with 10^4 CFU/ml of *L. monocytogenes* 2000/47 at 4ºC for
199 10 days of storage (Fig. 2). In the control cultures the *Listeria* strain steadily multiplied
200 up to 10^8 CFU/ml at the end of the experiment. Phage FWLLm1 progressively reduced
201 viable counts, reaching 10^2 CFU/ml (6 log units reduction) after 8 days of incubation. At
202 day 10 an increase of 1 log unit was observed (Fig. 2A). Phage FWLLm3 was able to
203 restrict the concentration to approximately 10^4 CFU/ml (4 log units reduction) until day
204 8, reaching 10^6 CFU/ml at the end of the experiment (Fig. 2B). When coagulin C23 was
205 used alone, a progressive reduction could be also observed reaching 10^2 CFU/ml in the
206 middle of the incubation period followed by re-growth. When phage FWLLm1 and
207 coagulin C23 were used in combination, a complete reduction of the viable counts
208 under the detection limits after 2 days of incubation at 4ºC and until the end of the
209 assessed period was observed (Fig. 2A). On the contrary, combination of FWLLm3 and
210 C23 led to a cell count reduction of 7.5 log units in day 4 followed by a 1.7 log units
211 increase at the end of the experiment (Fig. 2B).
212
213 **3.3 Assessment of resistance development towards coagulin C23 and listeriaphage**
214 **after antimicrobial challenges**
215 *L. monocytogenes* 2000/47 was exposed to the antimicrobials (coagulin C23 and
216 FWLLm3) individually and the resistant phenotypes of surviving colonies were

examined. Six resistant colonies were obtained from the inhibition halo produced by coagulin C23 onto a *L. monocytogenes* 2000/47 lawn. All these colonies were resistant to the bacteriocin as judged by the absence of inhibition halos by the agar spot test (data not shown). Furthermore, these mutants remained resistant to coagulin C23 after 5 days of consecutive culturing in broth without the bacteriocin, suggesting that the resistant phenotype was fairly stable. Likewise, five bacteriophage-insensitive mutants were also obtained from a plate containing *L. monocytogenes* 2000/47 and FWLLm3. Cross-resistance was assessed using both coagulin C23 and FWLLm3 resistant cells. All bacteriocin resistant cells were sensitive to FWLLm3 and all BIMs were sensitive to coagulin C23 (data not shown). Therefore, no cross-resistance was observed.

To determine if the bacterial re-growth observed in milk challenges could be due to the selection of resistant variants, resistance development was also assessed at the end of the incubation period (10 days) in those samples where re-growth was observed. Ten surviving colonies from FWLLm1, FWLLm3, C23 and FWLLm3+C23 counting plates were cultured in TSB to prepare lawns and drops of the corresponding antimicrobial were spotted. In case of colonies isolated from FWLLm1 plates, all of them were sensitive to this phage (Table 1) while half of the colonies isolated from FWLLm3 plates were phage-resistant. Similarly, 55% of the colonies isolated from C23 plates were resistant to the bacteriocin. When FWLLm3 was used in combination with C23, all the colonies were sensitive to the phage and only 40% were resistant to C23 (Table 1).

4. DISCUSSION

The potential of phages as biocontrol agents in food is supported by several studies that indicate an efficient reduction of pathogens levels in meat, fresh fruits, vegetables and

processed foods (Endersen et al., 2014). For reducing *L. monocytogenes* contamination in food, there are already commercially available phage preparations against *L. monocytogenes,* which have been approved by the US FDA as processing-aids (U.S. FDA/CFSAN, 2007). On the other hand, the use of bacteriocins in food safety has been addressed along the whole production chain, as well as their use as food biopreservatives; authorized bacteriocin-containing products, including nisin and pediocin PA1/AcH, are also marketed (Gálvez et al., 2010; Omar et al., 2013). Starter and non-starter bacteriocin-producing strains have been successfully used to control *L. monocytogenes* in fresh cheese (Coelho et al., 2014; Vera Pingitore et al., 2012). As with any other potential food additive or processing aid, safety studies must be conducted on a case-by case basis according to regulations in force in each country. Moreover, the use of food biopreservatives must also be cost-effective. One way to lower the effective concentrations without compromising activity is to use the synergistic effects that are often observed between two antimicrobial agents that feature different mechanisms of action or attack different targets. For example, combination of nisin and polymyxin B allows a considerable reduction in the amount of nisin needed for the effective inhibition of *Listeria* spp. (Naghmouchi et al., 2010).

In this work, we have assessed the combinations of two lytic listeriaphages and an anti-*Listeria* bacteriocin to reduce or eliminate this foodborne pathogen under laboratory conditions, i.e. optimal growth conditions, and in milk under refrigeration conditions. Phages FWLLm1 and FWLLm3, and coagulin C23 alone were able to reduce the amount of *L. monocytogenes* cells compared with the untreated control in both broth at 37ºC and milk at 4ºC. However, the combination resulted in a higher reduction or complete elimination of the viable counts, which suggests a synergistic effect between the listeriaphages and coagulin C23. Enhanced killing activity by combining phages and the bacteriocin nisin has been reported against *L. monocytogenes*

in artificially contaminated fresh-cut melons and apples (Leverentz et al., 2003) and against *S. aureus* in pasteurized milk (Martínez et al., 2008), supporting the potency of this approach in food biopreservation.

When keeping the same phage to bacteria ratio *in vitro,* the combination FWLLm3+C23 was more effective than FWLLm1+C23 in eliminating *L. monocytogenes* since re-growth was observed with the latter, while FWLLm3+C23 viable counts were under the detection limits after 2 h and onwards. Therefore, in order to improve the lytic ability of phage FWLLm1, challenge assays in milk were performed using a 100:1 phage:bacteria ratio. In these conditions, FWLLm1+C23 effectively inhibited *L. monocytogenes* while surviving cells were recovered in samples treated with FWLLm3+C23. Failure to completely inhibit bacterial growth has been reported in phage-treated solid food, which has been explained by the immobilization of the phage particles on the food surfaces and matrix, and their inability to reach the bacterial targets (Chibeu et al., 2013; Guenther et al., 2012; Guenther et al., 2009; Guenther and Loessner, 2011). Even when FWLLm1 was used to control *L. monocytogenes* in RTE chicken breast roll at 30ºC, re-growth was observed after 5 h of incubation (Bigot et al., 2011). Milk may also present barriers to phage and bacteriocin biocontrol. For example, milk proteins or fat globules may hamper contact between phages and their target cells, resulting in inactivation of the phages (García et al., 2009; O'Flaherty et al., 2005). In addition, the negative effect of milk fat on the antimicrobial potency of bacteriocins, caused by adsorption of these hydrophobic molecules onto fat globules has also been reported (Sobrino-López and Martín-Belloso, 2008).

Another reason which may explain failure of bacteriocins and phages to inhibit pathogen growth is the development of resistance (García et al., 2010). Bacterial resistance to phage may be due to a number of mechanisms, including absence or masking of phage receptors, prevention of injection of the phage genome into the host

295 cells, immunity to superinfection, and restriction-modification (RM) systems that
296 degrade phage DNA while the host DNA is protected by methylation (Hyman and
297 Abedon, 2010). Recently, a novel type of CRISPR system of phage resistance has been
298 described in *L. monocytogenes* (Sesto et al., 2014). The development of resistance
299 towards pediocin-like bacteriocins such as coagulin C23 is typically caused by a
300 decreased expression of the mannose phosphotransferase system and by an altered cell
301 surface (Drider et al., 2006; Kjos et al., 2011; Vadyvaloo et al., 2004). In *L.*
302 *monocytogenes,* exposure to sublethal concentrations of pediocin (Laursen et al., 2014)
303 or environmental stresses (Bergholz et al., 2013) promotes an adaptive response that
304 facilitates resistance development.

305 In this context, we explore resistance towards coagulin C23 and the
306 listeriaphages within the surviving colonies in our experiments as well as the risk of
307 cross-resistance. In the milk challenge with the phages alone, phage resistance was only
308 detected against FWLLm3 where 50% of the tested colonies were phage-insensitive
309 (Table 1). Thus, failure of inhibiting *L. monocytogenes* could be also explained by
310 interference with the food matrix and the inability of the phage to reach its target. On
311 the other hand, and in line with previous reports (Drider et al., 2006; Kjos et al., 2011),
312 resistant mutants to coagulin C23 were frequently isolated which would preclude the
313 use of this bacteriocin alone. Remarkably, the fraction of resistant mutants was lower
314 when the bacteriocin was combined with FWLLm3. Likewise, surviving colonies from
315 the combined FWLLm3+C23 treatment were all phage sensitive in contrast to the
316 higher frequency of phage resistant mutants when the phage was used alone. These
317 results support the notion that synergy between phages and bacteriocins could be
318 explained by a lower rate of resistance development. Nevertheless, further research is
319 needed to decipher the mechanisms involved.

No cross-resistance was observed between coagulin C23 and the listeriaphages. Phage-insensitive cells were sensitive to coagulin C23 and coagulin C23 resistant cells were sensitive to listeriaphages, which further ensures the efficacy of the combination. It seems that access to phage receptors was not hindered by the cell envelope changes involved in resistance to coagulin C23. Previous results for *S. aureus* showed that nisin-adapted cells seriously compromised bacteriophage activity (Martinez et al., 2008). Similarly, resistant-mutants to the bacteriocin lactococin 972 were not infected by lytic phage c2 (Roces et al., 2012).

Overall, we have demonstrated that the combination of listeriaphages and the bacteriocin coagulin C23 is more effective as a biopreservative in milk against *L. monocytogenes* under refrigeration conditions than each antimicrobial alone, and thus it could be a smart strategy to ensure milk safety during storage conditions.

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7. TABLES

Table 1. Percentage of sensitive/resistant colonies isolated from milk samples treated with each antimicrobial (n=10).

Antimicrobial	Sensitive (%)	Resistant (%)
FWLLm1	100	-
FWLLm3	50	50
Coagulin C23	45	55
FWLLm3+C23	60 (to C23)	40
	100 (to FWLLm3)	-

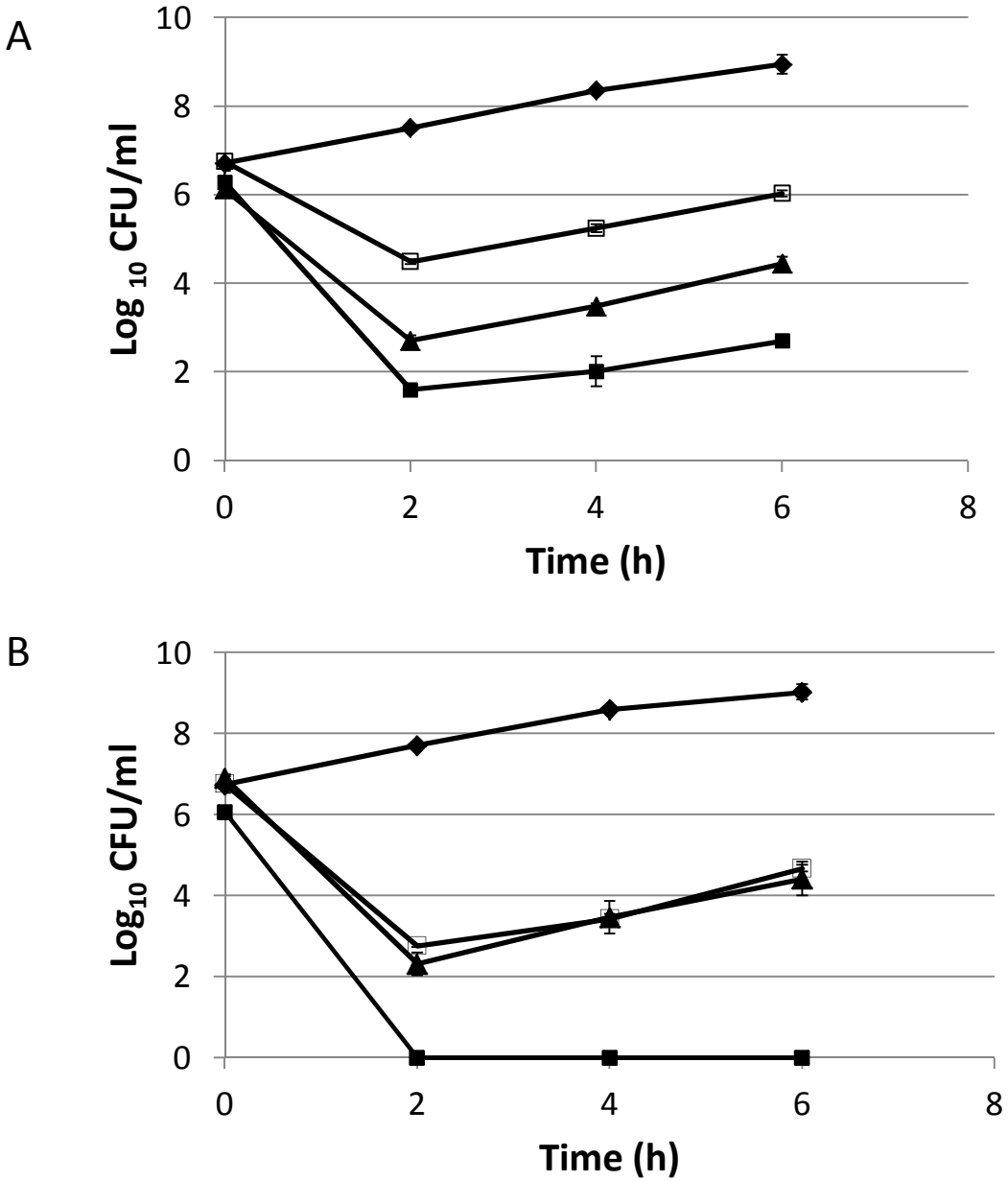
8. FIGURES

Figure 1. Killing of *L. monocytogenes* 2000/47 at 37ºC in broth. Samples were inoculated with 5×10^6 CFU/ml of *L. monocytogenes* 2000/47 and incubated for 6 h at 37ºC without antimicrobials (control; ♦) or in the presence of A) bacteriophage FWLLm1 (5×10^5 PFU/ml) (□), coagulin C23 (584 AU/ml) (▲), combination FWLLm1+C23 (■); B) bacteriophage FWLLm3 (5×10^5 PFU/ml) (□), coagulin C23 (584 AU/ml) (▲), combination FWLLm3+C23 (■). Values are the means of two independent experiments with standard deviation indicated by vertical bars.

Figure 2. Killing of *L. monocytogenes* 2000/47 at 4ºC in Extended Shelf Life (ESL) milk. Samples were inoculated with 5×10^4 CFU/ml of *L. monocytogenes* 2000/47 and incubated for 10 days at 4ºC without antimicrobials (control; black bars) or in the

498 presence of A) bacteriophage FWLLm1 (5 x 10^6 PFU/ml) (light grey bars), coagulin
499 C23 (584 AU/ml) (white bars), combination FWLLm1+C23 (dark grey bars); B)
500 bacteriophage FWLLm3 (5 x 10^5 PFU/ml) (light grey bars), coagulin C23 (584 AU/ml)
501 (white bars), combination FWLLm3+C23 (dark grey bars). Values are the means of two
502 independent experiments with standard deviation indicated by vertical bars.

Figure 1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10
8
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Time (h)
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Log10 CFU/ml
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2
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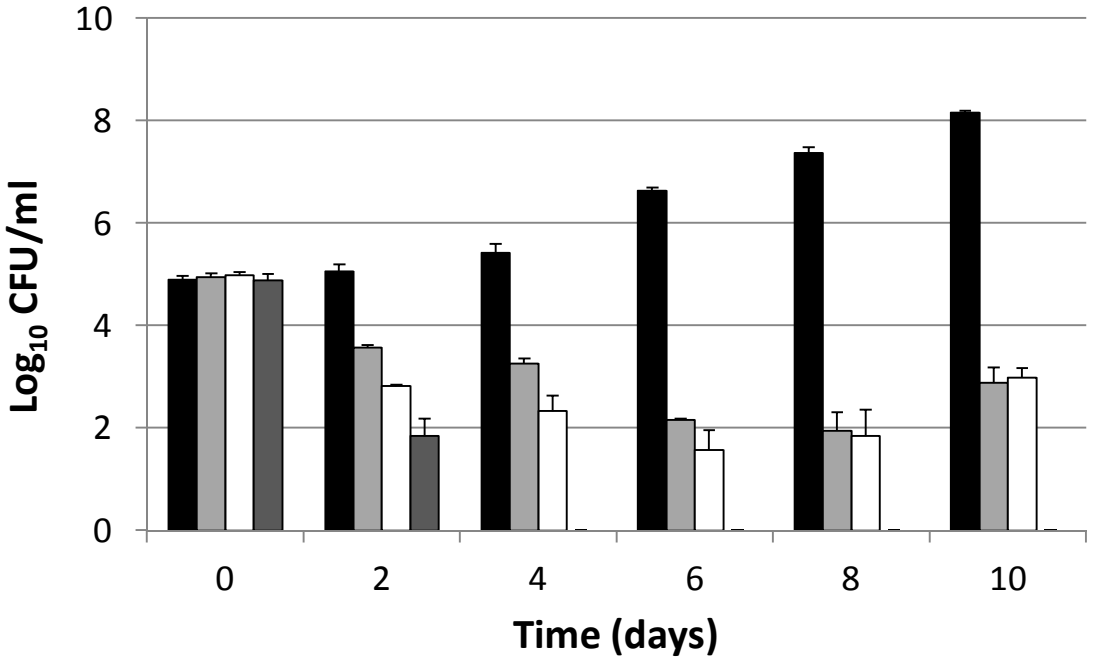
6
8
Time (h)

Figure 2

A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Log10 CFU/ml
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8
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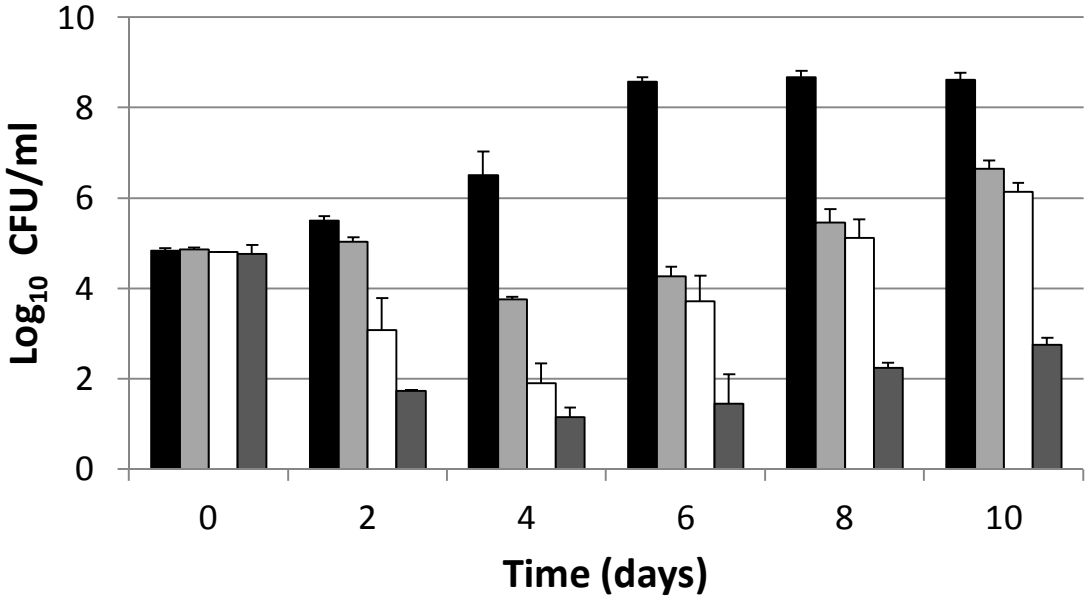
Time (days)

B


10
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Log10 CFU/ml
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Time (days)