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1 **Title:** Listeriaphages and coagulin C23 act synergistically to kill *Listeria*
2 *monocytogenes* in milk under refrigeration conditions.
3
4 **Running Title:** Killing of *L. monocytogenes* in milk
5
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26 **Abstract**

27

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

47

48

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

51

52 **Highlights**

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

59

60

61 **1. INTRODUCTION**

62

63 *Listeria monocytogenes* is the causative agent of listeriosis, a foodborne disease
64 which affects the elderly, pregnant women, unborn and newborn babies and people with
65 weakened immune systems (Allerberger and Wagner, 2010). In healthy adults and
66 children, listeriosis causes few or no symptoms and may be mistaken for a mild viral
67 infection or flu which makes the incidence of listeriosis difficult to establish (Bortolussi,
68 2008). However, an increasing rate of this foodborne illness has been reported in
69 Europe in recent years related to a higher rate of listeriosis in people ≥ 65 years of age
70 (Goulet et al., 2008).

71 *L. monocytogenes* is a serious concern for the food industry as this pathogen can
72 grow at refrigeration temperatures commonly used to control pathogens in foods (4ºC to
73 10ºC) and tolerates high concentrations of salt and low pH (Ferreira et al., 2014). Many
74 categories of food have been related with listeriosis outbreaks: milk, soft cheeses and
75 other dairy products, sausages, smoked fish, salads, delicatessen and ready-to-eat
76 products (Garrido et al., 2010). The majority of detected outbreaks have been caused by
77 ready-to-eat meats and cheeses (Cartwright et al., 2013; Garrido et al., 2010). Although
78 *L. monocytogenes* is inactivated by thermal treatments used in processed food, post-
79 processing cross-contamination from equipment and environment may occur due to the
80 persistence of this pathogen in processing plants (Ferreira et al., 2014; Meloni et al.,
81 2014; Ortiz et al., 2014) as a result of the development of resistance to disinfectants and
82 its ability to form biofilms (Ferreira et al., 2014).

83 Listeriaphages have been proposed as new tools for *Listeria* biocontrol.
84 Bacteriophages or phages, the natural killers of bacteria, are widely distributed in all
85 environments, including food, and are presumed safe for humans, animals and plants.
86 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

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

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

179

180 **3. RESULTS**

181

182 **3.1 Coagulin C23 and the listeriaphages FWLLm1 and FWLLm3 act**
183 **synergistically to kill *L. monocytogenes* in broth.**

184 The potential synergistic effect of coagulin C23 and listeriaphages was assessed
185 in broth at 37ºC (Fig. 1). Combination of coagulin C23 and phage FWLLm1 decreased
186 the concentration of *L. monocytogenes* 2000/47 by 5.9 log units after 2 h of incubation
187 compared to the untreated control, while 3 and 4.8 log reductions were caused by
188 FWLLm1 and C23 alone, respectively (Fig. 1A). After two hours of incubation, re-
189 growth was observed in all samples. On the contrary, when coagulin C23 was combined
190 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

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

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

238

239 **4. DISCUSSION**

240

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

243 processed foods (Endersen et al., 2014). For reducing *L. monocytogenes* contamination
244 in food, there are already commercially available phage preparations against *L.*
245 *monocytogenes,* which have been approved by the US FDA as processing-aids (U.S.
246 FDA/CFSAN, 2007). On the other hand, the use of bacteriocins in food safety has been
247 addressed along the whole production chain, as well as their use as food
248 biopreservatives; authorized bacteriocin-containing products, including nisin and
249 pediocin PA1/AcH, are also marketed (Gálvez et al., 2010; Omar et al., 2013). Starter
250 and non-starter bacteriocin-producing strains have been successfully used to control *L.*
251 *monocytogenes* in fresh cheese (Coelho et al., 2014; Vera Pingitore et al., 2012). As
252 with any other potential food additive or processing aid, safety studies must be
253 conducted on a case-by case basis according to regulations in force in each country.
254 Moreover, the use of food biopreservatives must also be cost-effective. One way to
255 lower the effective concentrations without compromising activity is to use the
256 synergistic effects that are often observed between two antimicrobial agents that feature
257 different mechanisms of action or attack different targets. For example, combination of
258 nisin and polymyxin B allows a considerable reduction in the amount of nisin needed
259 for the effective inhibition of *Listeria* spp. (Naghmouchi et al., 2010).
260 In this work, we have assessed the combinations of two lytic listeriaphages and
261 an anti-*Listeria* bacteriocin to reduce or eliminate this foodborne pathogen under
262 laboratory conditions, i.e. optimal growth conditions, and in milk under refrigeration
263 conditions. Phages FWLLm1 and FWLLm3, and coagulin C23 alone were able to
264 reduce the amount of *L. monocytogenes* cells compared with the untreated control in
265 both broth at 37ºC and milk at 4ºC. However, the combination resulted in a higher
266 reduction or complete elimination of the viable counts, which suggests a synergistic
267 effect between the listeriaphages and coagulin C23. Enhanced killing activity by
268 combining phages and the bacteriocin nisin has been reported against *L. monocytogenes*

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

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

291 Another reason which may explain failure of bacteriocins and phages to inhibit
292 pathogen growth is the development of resistance (García et al., 2010). Bacterial
293 resistance to phage may be due to a number of mechanisms, including absence or
294 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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6. REFERENCES

345 Allende, A., Martínez, B., Selma, V., Gil, M.I., Suarez, J.E., Rodríguez, A., 2007.
346 Growth and bacteriocin production by lactic acid bacteria in vegetable broth and
347 their effectiveness at reducing *Listeria monocytogenes* in vitro and in fresh-cut
348 lettuce. Food Microbiol 24, 759-766.
349 Allerberger, F., Wagner, M., 2010. Listeriosis: a resurgent foodborne infection. Clin
350 Microbiol Infect 16, 16-23.
351 Arachchi G, Cruz CD, Dias-Wanigasekera BM, McIntyre L, Billington C, Hudson JA,
352 Flint SH, Mutukumira AN. 2013. Host range and in vitro lysis of *Listeria*
353 *monocytogenes* seafood isolates by bacteriophages. Food Sci Technol Int 20,
354 591-603.
355 Bergholz, T.M., Tang, S., Wiedmann, M., Boor, K.J., 2013. Nisin resistance of *Listeria*
356 *monocytogenes* is increased by exposure to salt stress and is mediated via LiaR.
357 Appl Environ Microbiol 79, 5682-5688.
358 Bigot, B., Lee, W.J., McIntyre, L., Wilson, T., Hudson, J.A., Billington, C., Heinemann,
359 J.A., 2011. Control of *Listeria monocytogenes* growth in a ready-to-eat poultry
360 product using a bacteriophage. Food Microbiol 28, 1448-1452.
361 Bortolussi, R., 2008. Listeriosis: a primer. Can Med Assoc J 179(8), 795-797.
362 Cartwright, E.J., Jackson, K.A., Johnson, S.D., Graves, L.M., Silk, B.J., Mahon, B.E.,
363 2013. Listeriosis outbreaks and associated food vehicles, United States, 1998-
364 2008. Emerg Infect Dis 19, 1-9; quiz 184.
365 Chibeu, A., Agius, L., Gao, A., Sabour, P.M., Kropinski, A.M., Balamurugan, S., 2013.
366 Efficacy of bacteriophage LISTEXP100 combined with chemical antimicrobials
367 in reducing *Listeria monocytogenes* in cooked turkey and roast beef. Int J Food
368 Microbiol 167, 208-214.

369 Coelho, M.C., Silva, C.C., Ribeiro, S.C., Dapkevicius, M.L., Rosa, H.J., 2014. Control
370 of *Listeria monocytogenes* in fresh cheese using protective lactic acid bacteria.
371 Int J Food Microbiol 191C, 53-59.
372 Davies, E., Bevis, H., Delves-Broughton, J., 1997. The use of the bacteriocin, nisin, as a
373 preservative in ricotta-type cheeses to control the food-borne pathogen *Listeria*
374 *monocytogenes*. Lett Appl Microbiol 24, 343-346.
375 Drider, D., Fimland, G., Héchard, Y., McMullen, L.M., Prévost, H., 2006. The
376 continuing story of class IIa bacteriocins. Microbiol Mol Biol Rev 70: 564-582.
377 Drider, D., Rebuffat, S., 2011. Prokaryotic antimicrobial peptides: from genes to
378 applications. Springer, USA.
379 Endersen, L., O'Mahony, J., Hill, C., Ross, R.P., McAuliffe, O., Coffey, A., 2014.
380 Phage therapy in the food industry. Annu Rev Food Sci Technol 5, 327-349.
381 Ferreira, V., Wiedmann, M., Teixeira, P., Stasiewicz, M.J., 2014. *Listeria*
382 *monocytogenes* persistence in food-associated environments: epidemiology,
383 strain characteristics, and implications for public health. J Food Prot 77, 150-
384 170.
385 Franklin, N.B., Cooksey, K.D., Getty, K.J., 2004. Inhibition of *Listeria monocytogenes*
386 on the surface of individually packaged hot dogs with a packaging film coating
387 containing nisin. J Food Prot 67, 480-485.
388 Gálvez, A., Abriouel, H., Benomar, N., Lucas, R., 2010. Microbial antagonists to food-
389 borne pathogens and biocontrol. Curr Opin Biotechnol 21, 142-148.
390 García, P., Madera, C., Martínez, B., Rodríguez, A., Evaristo Suárez, J., 2009.
391 Prevalence of bacteriophages infecting *Staphylococcus aureus* in dairy samples
392 and their potential as biocontrol agents. J Dairy Sci 92, 3019-3026.

393 García, P., Rodríguez, L., Rodríguez, A., Martínez, B., 2010. Food biopreservation:
394 promising strategies using bacteriocins, bacteriophages and endolysins. Trends
395 Food Sci Tech, 373-382.
396 Garrido, V., Vitas, A.I., García-Jalón, I., 2010. The problem of Listeriosis and ready-to-
397 eat products: prevalence and persistence, in: Menéndez-Vilas, A. (Ed.), Current
398 Research, Technology and Education Topics in Applied Microbiology and
399 Microbial Biotechnology Formatex, Badajoz, Spain, pp. 1182-1189.
400 Goulet, V., Hedberg, C., Le Monnier, A., de Valk, H., 2008. Increasing incidence of
401 listeriosis in France and other European countries. Emerg Infect Dis 14, 734-
402 740.
403 Guenther, S., Herzig, O., Fieseler, L., Klumpp, J., Loessner, M.J., 2012. Biocontrol of
404 *Salmonella* Typhimurium in RTE foods with the virulent bacteriophage FO1-E2.
405 Int J Food Microbiol 154, 66-72.
406 Guenther, S., Huwyler, D., Richard, S., Loessner, M.J., 2009. Virulent bacteriophage
407 for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. Appl
408 Environ Microbiol 75, 93-100.
409 Guenther, S., Loessner, M.J., 2011. Bacteriophage biocontrol of *Listeria monocytogenes*
410 on soft ripened white mold and red-smear cheeses. Bacteriophage 1, 94-100.
411 Hagens, S., Loessner, M.J., 2014. Phages of *Listeria* offer novel tools for diagnostics
412 and biocontrol. Front Microbiol 5, 159.
413 Hyman, P., Abedon, S.T., 2010. Bacteriophage host range and bacterial resistance. Adv
414 Appl Microbiol 70, 217-248.
415 Hyronimus, B., Le Marrec, C., Urdaci, M.C., 1998. Coagulin, a bacteriocin-like
416 inhibitory substance produced by *Bacillus coagulans* I4. J Appl Microbiol, 85,
417 42-50.

418 Kjos, M., Nes, I.F., Diep, D.B., 2011. Mechanisms of resistance to bacteriocins
419 targeting the mannose phosphotransferase system. Appl Environ Microbiol 77,
420 3335-3342.
421 Laursen, M.F., Bahl, M.I., Licht, T.R., Gram, L., Knudsen, G.M., 2014. A single
422 exposure to a sublethal pediocin concentration initiates a resistance-associated
423 temporal cell envelope and general stress response in *Listeria monocytogenes*.
424 Environ Microbiol. doi: 10.1111/1462-2920.12534.
425 Leverentz, B., Conway, W.S., Camp, M.J., Janisiewicz, W.J., Abuladze, T., Yang, M.,
426 Saftner, R., Sulakvelidze, A., 2003. Biocontrol of *Listeria monocytogenes* on
427 fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. Appl
428 Environ Microbiol 69, 4519-4526.
429 Martínez, B., Obeso, J.M., Rodríguez, A., García, P., 2008. Nisin-bacteriophage
430 crossresistance in *Staphylococcus aureus*. Int J Food Microbiol 122, 253-258.
431 Meloni, D., Consolati, S.G., Mazza, R., Mureddu, A., Fois, F., Piras, F., Mazzette, R.,
432 2014. Presence and molecular characterization of the major serovars of *Listeria*
433 *monocytogenes* in ten Sardinian fermented sausage processing plants. Meat Sci
434 97, 443-450.
435 Naghmouchi, K., Drider, D., Baah, J., and Teather, R.M., 2010. Nisin A and Polymyxin
436 B as Synergistic Inhibitors of Gram-positive and Gram-negative Bacteria.
437 Probiotics Antimicrob Proteins, 2(2):98-103.
438 O'Flaherty, S., Coffey, A., Meaney, W.J., Fitzgerald, G.F., Ross, R.P., 2005. Inhibition
439 of bacteriophage K proliferation on *Staphylococcus aureus* in raw bovine milk.
440 Lett Appl Microbiol 41, 274-279.
441 Omar, N., Abriouel, H., Fliss, I., Ferandez-Fuentes, M., Gálvez, A., & Drider, D., 2013.
442 Bacteriocins: Natural Weapons for Control of Food Pathogens. In A. Malik, E.

443 Grohmann & M. Alves (Eds.), Management of Microbial Resources in the
444 Environment, (pp. 471-494): Springer Netherlands.
445 Ortíz, S., López, V., Martínez-Suárez, J.V., 2014. Control of *Listeria monocytogenes*
446 contamination in an Iberian pork processing plant and selection of benzalkonium
447 chloride-resistant strains. Food Microbiol 39, 81-88.
448 Rilla-Villar, N., 2003. Aplicacion de Bacterias Lacticas productoras de Bacteriocinas en
449 Bioconservacion, PhD Thesis. Universidad de Santiago de Compostela, Spain.
450 Roces, C., Courtin, P., Kulakauskas, S., Rodríguez, A., Chapot-Chartier, M.P.,
451 Martínez, B., 2012. Isolation of *Lactococcus lactis* mutants simultaneously
452 resistant to the cell wall-active bacteriocin Lcn972, lysozyme, nisin, and
453 bacteriophage c2. Appl Environ Microbiol 78, 4157-4163.
454 Sesto, N., Touchon, M., Andrade, J.M., Kondo, J., Rocha, E.P., Arraiano, C.M.,
455 Archambaud, C., Westhof, E., Romby, P., Cossart, P., 2014. A PNPase
456 dependent CRISPR System in *Listeria*. PLoS Genet 10, e1004065.
457 Sim, J., Hood, D., Finnie, L., Wilson, M., Graham, C., Brett, M., Hudson, J.A., 2002.
458 Series of incidents of *Listeria monocytogenes* non-invasive febrile gastroenteritis
459 involving ready-to-eat meats. Lett Appl Microbiol 35, 409-413.
460 Sobrino-López, A., Martín-Belloso, O., 2008. Use of nisin and other bacteriocins for
461 preservation of dairy products. Int Dairy J 18, 329-343.
462 U.S. FDA/CFSAN, 2007. US FDA/CFSAN. 2007. Agency response letter: GRAS
463 Notice No. GRN 000218.
464 http://www.accessdata.fda.gov/scripts/fcn/gras:notices/701456A.PDF 2007.
465 Vadyvaloo, V., Arous, S., Gravesen, A., Hechard, Y., Chauhan-Haubrock, R., Hastings,
466 J.W., Rautenbach, M., 2004. Cell-surface alterations in class IIa bacteriocin-
467 resistant *Listeria monocytogenes* strains. Microbiology 150, 3025-3033.

468 Vera Pingitore, E., Todorov, S.D., Sesma, F., Franco, B.D., 2012. Application of
469 bacteriocinogenic *Enterococcus mundtii* CRL35 and *Enterococcus faecium*
470 ST88Ch in the control of *Listeria monocytogenes* in fresh Minas cheese. Food
471 Microbiol 32, 38-47.
472 Wan, J., Harmark, K., Davidson, B.E., Hillier, A.J., Gordon, J.B., Wilcock, A., Hickey,
473 M.W., Coventry, M.J., 1997. Inhibition of *Listeria monocytogenes* by piscicolin
474 126 in milk and Camembert cheese manufactured with a thermophilic starter. J
475 Appl Microbiol 82, 273-280.
476
477

478 **7. TABLES**

479

480 **Table 1.** Percentage of sensitive/resistant colonies isolated from milk samples treated

481 with each antimicrobial (n=10).

482

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

483

484

485 **8. FIGURES**

486

487 **Figure 1. Killing of *L. monocytogenes* 2000/47 at 37ºC in broth.** Samples were

488 inoculated with 5 × 10^6 CFU/ml of *L. monocytogenes* 2000/47 and incubated for 6 h at

489 37ºC without antimicrobials (control; ♦) or in the presence of A) bacteriophage

490 FWLLm1 (5 x 10^5 PFU/ml) (□), coagulin C23 (584 AU/ml) (▲), combination

491 FWLLm1+C23 (■); B) bacteriophage FWLLm3 (5 x 10^5 PFU/ml) (□), coagulin C23

492 (584 AU/ml) (▲), combination FWLLm3+C23 (■). Values are the means of two

493 independent experiments with standard deviation indicated by vertical bars.

494

495 **Figure 2. Killing of *L. monocytogenes* 2000/47 at 4ºC in Extended Shelf Life (ESL)**

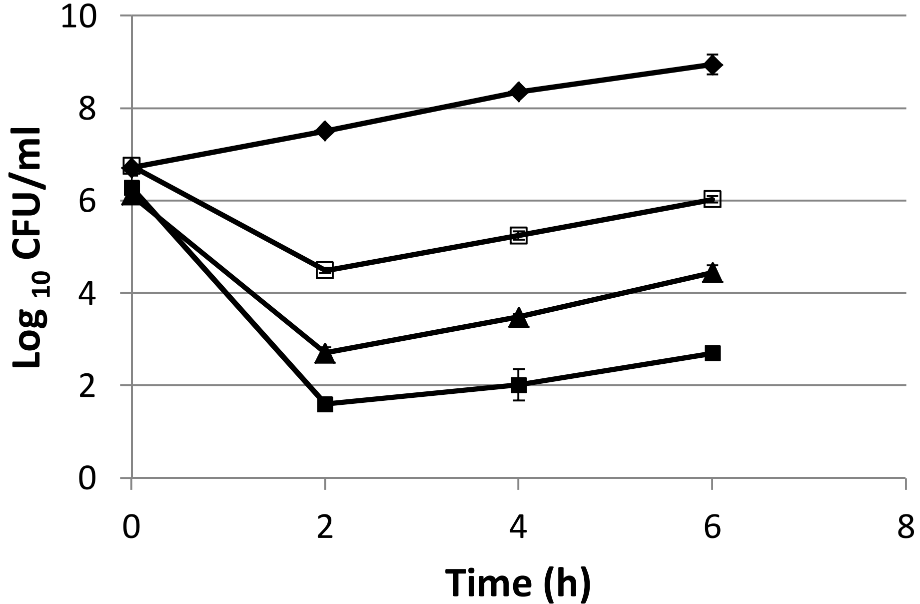
496 **milk.** Samples were inoculated with 5 × 10^4 CFU/ml of *L. monocytogenes* 2000/47 and

497 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

A



B

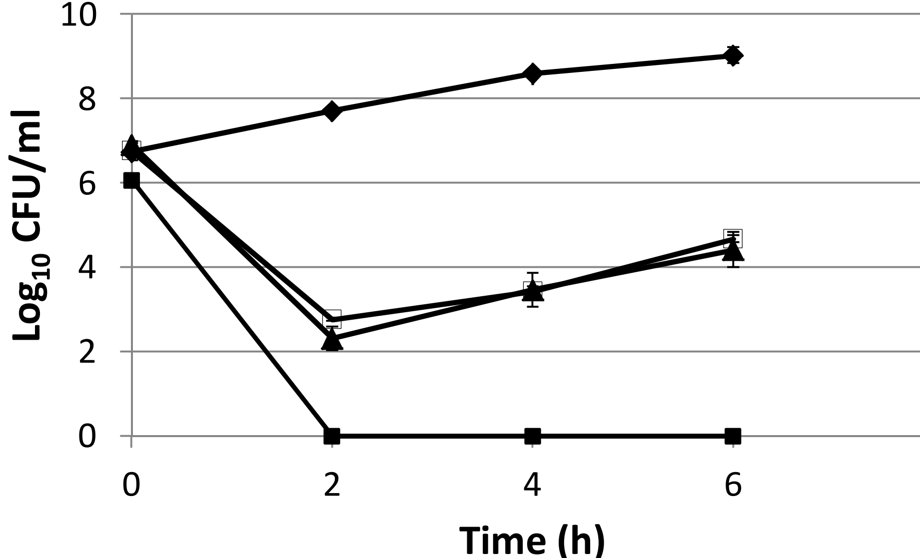
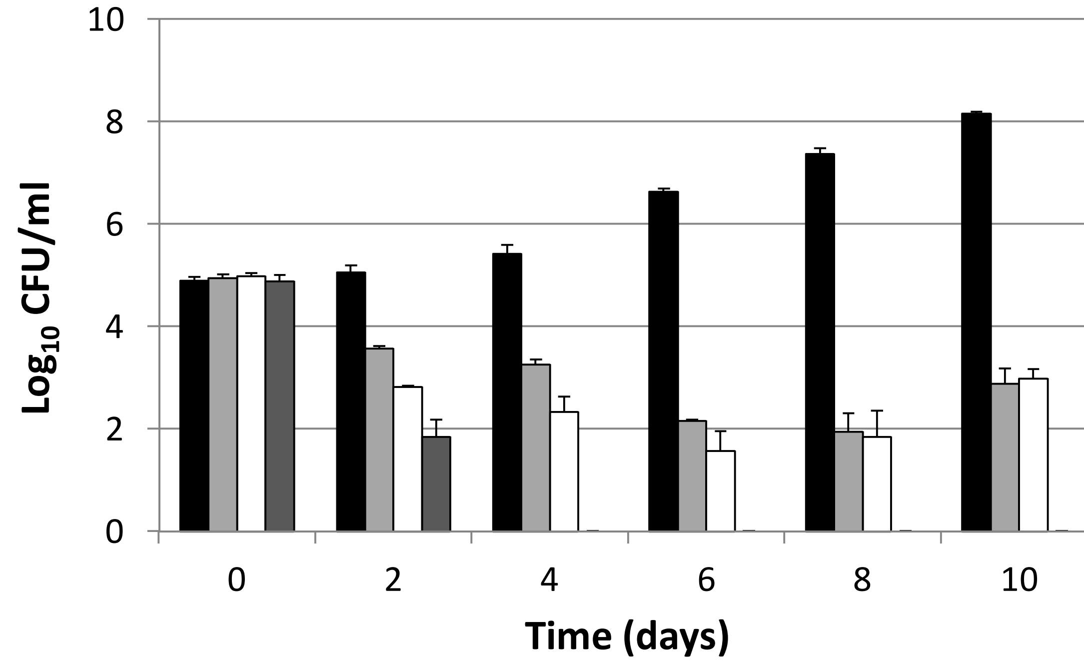


Figure 2

A



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