The cost and benefit of quorum sensing controlled bacteriocin production in *Lactobacillus plantarum*

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Running title: Quorum sensing and Bacteriocins

Abstract

Bacteria eliminate competitors via 'chemical warfare' with bacteriocins. Some species appear to adjust bacteriocin production conditionally in response to the social environment. We tested whether variation in the cost and benefit of producing bacteriocins could explain such conditional behaviour, in the bacteria *Lactobacillus plantarum*. We found that: (1) bacterial bacteriocin production could be upregulated by either the addition of a synthetic autoinducer peptide (PLNC8IF; signalling molecule), or by a plasmid which constitutively encodes for the production of this peptide; (2) bacteriocin production is costly, leading to reduced growth when grown in poor and, to a lesser extent, in rich media; (3) bacteriocin production provides a fitness advantage, when grown in competition with sensitive strains; (4) the fitness benefits provided by bacteriocin production is greater at higher cell densities. These results show how the costs and benefits of upregulating bacteriocin production can depend upon abiotic and biotic conditions.

Keywords: Bacteriocins, Quorum sensing, Costs, Benefits, Fitness

51 **1. INTRODUCTION**

52 The growth and success of many bacteria, both free living and pathogenic, often depends on
53 their ability to eliminate competitors via 'chemical warfare' with bacteriocins (Cotter, Hill, &
54 Ross, 2005; Riley & Wertz, 2002). Bacteriocins are small peptides that possess antimicrobial
55 activity against other bacteria, but against which the producing cell has a specific immunity
56 mechanism. Both theoretical and empirical studies have suggested that the production of
57 bacteriocins is a social trait, because the benefits of eliminating competitors are shared with
58 clone mates that also possess immunity (Brown & Buckling, 2008; Bucci, Nadell, & Xavier,
59 2011; Chao & Levin, 1991; Czárán & Hoekstra, 2003; Doekes, de Boer, & Hermsen, 2019;
60 Frank, 1994; Gardner, West, & Buckling, 2004; Hawlena, Bashey, & Lively, 2010; Inglis,
61 Gardner, Cornelis, & Buckling, 2009; Kerr, Riley, Feldman, & Bohannan, 2002; Libberton,
62 Horsburgh, & Brockhurst, 2015; Mavridou, Gonzalez, Kim, West, & Foster, 2018; Waite &
63 Curtis, 2008; West, Griffin, Gardner, & Diggle, 2006).

64 Bacteria appear to adjust bacteriocin production conditionally in response to local
65 conditions, and especially the social environment (Bhattacharya, Pak, & Bashey, 2018;
66 Gonzalez & Mavridou, 2019; Majeed, Gillor, Kerr, & Riley, 2011; Maldonado, Ruiz-Barba,
67 & Jiménez-Díaz, 2004a). In *Escherichia coli*, bacteriocin production is increased in response
68 to the presence of bacteriocins produced by competing strains and clone mates (Gonzalez,
69 Sabnis, Foster, & Mavridou, 2018; Majeed et al., 2011; Mavridou et al., 2018). In
70 *Lactobacillus plantarum* and *Lactobacillus gasseri*, bacteriocin production is controlled by a
71 'quorum sensing' signalling system that causes production to be activated when cells are at
72 higher densities and in the presence of competing bacteria from different species (Maldonado,
73 Jiménez-Díaz, & Ruiz-Barba, 2004b; Maldonado-Barragán, Ruiz-Barba, & Jiménez-Díaz,
74 2009; Maldonado-Barragán, Caballero-Guerrero, Lucena-Padrós, & Ruiz-Barba, 2013;
75 Maldonado-Barragán, Caballero-Guerrero, Martín, Ruiz-Barba, & Rodríguez, 2016).

76 Evolutionary theory suggests that a potential explanation for these conditional shifts in
77 bacteriocin production is that the relative costs and benefits of bacteriocin production depend
78 upon the social environment (Gardner et al., 2004). For example, producing bacteriocins is
79 predicted to become less beneficial when the density of the competing strains is low (Gardner
80 et al., 2004). However, we know relatively little about how the costs and benefits of
81 bacteriocin production depend upon social conditions (Gonzalez et al., 2018; Gonzalez &
82 Mavridou, 2019; Inglis et al., 2009).

83 We examined the cost and benefit of producing bacteriocins, and how this varied with
84 social conditions, in *Lactobacillus plantarum* NC8 (Figure 1a). This strain regulates
85 bacteriocin production by quorum sensing, leading to bacteriocin production being activated
86 at high cell densities in solid medium, but not in liquid medium (Maldonado et al., 2004b;
87 Maldonado-Barragán et al., 2009). Mechanistically, bacteriocin production in *L. plantarum*
88 NC8 is activated by co-culturing with other gram-positive bacteria (inducer bacteria), and
89 requires cell-to-cell contact with the inducer bacteria (Maldonado, Ruiz-Barba & Jiménez-
90 Díaz, 2003; Maldonado et al., 2004a; Maldonado et al., 2004b). This activates the expression
91 of the operon *plNC8IF-plNC8HK-pln*D encoding a three-component regulatory system
92 (TCRS) formed by an autoinducer peptide (PLNC8IF), a histidine protein kinase (PLNC8HK)
93 and a response regulator (PlnD), which is indispensable for bacteriocin production by NC8
94 and is thought to be involved in quorum sensing (Maldonado et al., 2004b; Maldonado-
95 Barragán et al., 2009; Figure 1b). Thus, co-culture of NC8 with inducer strains, the external
96 addition of PLNC8IF to NC8 cultures, or the constitutive expression of PLNC8IF in NC8,
97 activates the transcription (via the TCRS) of the genes encoding three two-peptide Class IIb
98 bacteriocins, plantaricin NC8 (PLNC8α and PLNC8β), plantaricin EF (plnE and plnF) and
99 plantaricin JK (plnJ and plnK) (Maldonado et al., 2004b).

100 We can experimentally manipulate bacteriocin production in the *L. plantarum* NC8

101 system in a number of ways. As described above, the wild type NC8 strain only produces
102 bacteriocins under certain conditions. Alternatively, even in absence of inducing bacteria, we
103 can add autoinducer peptide PLNC8IF to activate both PLNC8IF synthesis (autoinduction)
104 and bacteriocin production (Maldonado et al., 2004b; Maldonado et al., 2013). Finally, we
105 can use a recombinant strain NC8:pSIG308, which produces PLNC8IF constitutively – when
106 this PLNC8IF reaches a sufficient concentration it will activate bacteriocin production
107 (Maldonado et al., 2004b).

108 Our specific aims were to test: (1) whether our experimental and genetic
109 manipulations influence bacteriocin production; (2) whether bacteriocin production is costly;
110 (3) the fitness benefits of bacteriocin production when competing against susceptible strains;
111 (4) whether the fitness benefits of bacteriocin production are greater at higher cell densities.

112

113 **2. METHODS**

114 ***(a) Bacterial strains and growth media***

115 We used eight strains in this study, whose main features are summarized in Table 1: (1)
116 *Lactobacillus plantarum* NC8, a wild-type conditional bacteriocin-producer (Bac^C) strain that
117 produces QS-regulated bacteriocins on solid medium, but requires co-culture with certain
118 specific inducer strains or the external addition of the autoinducer peptide PLNC8IF to
119 produce bacteriocins in liquid cultures (Maldonado et al., 2004b). Bacteriocins produced
120 belong to the two-peptide ClassIIb bacteriocins, which are released from cells in their active
121 mature form by a dedicated ABC transporter, without cell lysis (Cotter et al., 2005).

122 (2) *L. plantarum* NC8:pSIG308 (Bac^+), is a derivative strain of NC8 which carries the
123 plasmid pSIG308 (Maldonado et al., 2004a). This plasmid harbours the gene *plNC8IF,* which
124 encodes the autoinducer peptide PLNC8IF, cloned behind the constitutive lactococcal
125 promoter P53. In contrast to the wild type strain (NC8), NC8:pSIG308 produces PLNC8IF at

126 sufficient amounts to activate QS-regulated bacteriocins (Bac^{+}) and is resistant to
127 erythromycin (20 µg/ml).
128 (3) *L. pentosus* 128/2-Rif (NI/S): a Rifampicin resistant (30 µg/ml) derivative strain of *L.*
129 *pentosus* 128/2. This strain is sensitive (S) to bacteriocins produced by *L. plantarum* NC8 but
130 does not induces (NI) bacteriocin production in NC8.
131 (4) *Lactococcus lactis* MG1363-Rif (I/R): a Rifampicin resistant (30 µg/ml) derivative strain
132 of *L. lactis* MG1363. This strain is resistant (R) to bacteriocins produced by *L. plantarum*
133 NC8 and induces (I) bacteriocin production in NC8.
134 (5) *Pediococcus pentosaceus* FBB63-Rif (I/S): a Rifampicin resistant (30 µg/ml) derivative of
135 *P. pentosaceous* FBB63. This strain is sensitive (S) to bacteriocins produced by *L. plantarum*
136 NC8 and induces (I) bacteriocin production in NC8.
137 We obtained the strains 128/2-Rif, MG1363-Rif and FBB63-Rif in this study by sequential
138 selection of the wild-type strains 128/2, MG1363 and FBB63 (Table 1), in increasing
139 concentrations of Rifampicin (from 1 to 50µg/ml). Unless indicated, we grew all strains at
140 30ºC in de Man, Rogosa and Sharpe broth medium (MRS; Oxoid Ltd, Basingstoke,
141 Hampshire, UK) or in MRS agar plates (1.5% w/v). We froze aliquots of each strain at -80ºC
142 in MRS plus 20% glycerol (v/v).
143 For differential selection of bacteria on solid medium, we used MRS-BPB agar (MRS plus
144 0.01% w/v Bromophenol Blue; Sigma-Aldrich), MRS-Rif (MRS plus 30µg/ml Rifampicin)
145 and MRS-Ery (MRS plus 100µg/ml Erythromycin). In MRS-BPB we could easily
146 differentiate NC8 (*L. plantarum*) from 128/2-Rif (*L. pentosus*), MG1363-Rif (*L. lactis*) and
147 FBB63 (*P. pentosaceus*) since the pH change produced during fermentation of these Lactic
148 Acid Bacteria changes the colour of accumulated BPB (pH indicator), being yellow at pH 3.0
149 and blue at pH 5.0 (Lee & Lee, 2008).
150 For testing the growth of lactobacilli under nutrient limitation, we developed a minimal

151 medium (MM) containing K2HPO4 2g L^{-1}, Sodium acetate monohydrate 5g L^{-1}, MnSO4.H2O
152 0.0152g L^{-1} and Tween 80 0.5 ml L^{-1}. The MM was supplemented with 5g L^{-1} Yeast Extract
153 (MM+YE) as nitrogen source.

154 ***(b) Bacteriocin Inducing Experiments***

155 The native PLNC8IF was shown to induce bacteriocin production and its proper synthesis in
156 *L. plantarum* NC8 through the activation of TCRS (PLNC8IF-PLNC8HK-PLND)
157 (Maldonado et al., 2004b). In this study, we deduced the amino acid sequence of the
158 autoinducer peptide PLNC8IF (KTKTISLMSGLQVPHAFTKLLKALGGHH) from its
159 encoding gene *pLNC8IF* (accession number: AF522077; Maldonado et al., 2004b), and
160 ordered the chemical synthesis of the peptide (GenScript USA Inc.,NJ). We dissolved one
161 milligram of the peptide (> 95% of purity) in 1 ml of Hypure Water (GE Healthcare) and
162 serially diluted to obtain concentrations ranging from 1 to 10^{-12} mg ml^{-1}. To test the ability to
163 induce bacteriocin production in NC8, we added ten microliters of each PLNC8IF dilution to
164 990 µl of MRS containing ca.10^8 colony forming units per milliliter (CFU/ml) of NC8, thus
165 obtaining final PLNC8IF concentrations ranging from 3 to 3 10^{-12} mM. In control cultures
166 (NC8 without PLNC8IF) we added ten microliters of MRS instead of PLNC8IF. The inducing
167 activity of synthetic PLNC8IF was compared with that of *L. lactis* MG1363-Rif, whose
168 parental strain, *L. lactis* MG1363 (I/R), is able to induce bacteriocin production in NC8
169 trough co-culture (Maldonado et al., 2003; Maldonado et al., 2004a). Specifically, we added
170 ten microliters of a 16-h old culture of MG1363-Rif to NC8 cultures (prepared as described
171 above), giving a final concentration of ca. 10^7 CFU/ml. In parallel, we compared both the
172 inducing activity of PLNC8IF and MG1363-Rif with the inducing activity of native
173 PLNC8IF, which is endogenously produced by plasmid pSIG308 in the recombinant strain
174 NC8:pSIG308 (Bac^+). For this, we inoculated NC8:pSIG308 in the same conditions as
175 described above for their parental strain NC8. Cultures were done in triplicate and incubated

176 at 30ºC for seven hours. Bacteriocin activity of cell-free supernatants was quantified as
177 described below. To test for antimicrobial activity of PLNC8IF we carried out serial dilutions
178 of the peptide in MRS medium as described above, and then quantified bacteriocin activity.

179 ***(c) Bacteriocin quantification***

180 For quantification of bacteriocin activity in cell-free supernatants (CFSs) we used a microtiter
181 plate assay, as described previously (Maldonado-Barragán et al., 2013), using *L. pentosus*
182 128/2-Rif as the indicator strain. We define one bacteriocin unit (BU) as the amount of active
183 CFS that inhibited the growth of the indicator strain by 50%, using as a reference the turbidity
184 of control cultures without CFS added. This was expressed as the reciprocal of the highest
185 dilution exhibiting 50% inhibition of the indicator strain per milliliter (BU/ml). Bacteriocin
186 activity per cell (BU/cell) was calculated by dividing bacteriocin activity (BU/ml) by the
187 number of cells (CFU/ml) at a given point. Before analyses, we log transformed bacteriocin
188 activity (log_{10}BU/ml) and number of cells (log_{10}CFU/ml).

189 ***(d) Growth curves***

190 We determined the growth curves of NC8 (Bac^{C}) (with and without the addition of the
191 autoinducer PLNC8IF) and NC8:pSIG308 (Bac^{+}) in MRS medium. For this, we diluted 16-h
192 old cultures in MRS until an optical density (measured at 600nm; OD600nm) of 0.01. We
193 measured the OD600nm in a spectrophotometer (SpectraMax M2; Molecular devices) and
194 recorded with the software SoftMaxPro (Molecular devices). For each strain we prepared six
195 independent cultures, and for each culture we deposited 195 µl per well in a 96-wells
196 microtiter plate. In the case of NC8 plus PLNC8IF, we added 5 µl of PLNC8IF per well to
197 reach a final concentration of 0.3 mM, while in the other cultures (NC8 without PLNC8IF and
198 NC8: pSIG308) we added 5 µl of MRS. We used non-inoculated MRS as the blank control.
199 We then incubated plates at 30ºC for 48 hours, in a Synergy 2 Multi-Mode Reader (BioTek),
200 measuring OD600nm at 15 min intervals. We recorded OD600nm data and maximum growth

201 rate (Vmax), and analyzed with the "Gen5 Data Analysis Software" (BioTek).

202 We carried out the same experiment under nutrient limitation, using MM+YE as culture
203 media. For this, we washed (two times) 16-h old cultures of each strain (NC8 and
204 NC8:pSIG308) and diluted cultures in MM+YE until an OD600nm of 0.01. We prepared and
205 incubated the 96-wells plate in the same conditions we described above, using MM+YE as the
206 blank. For each strain (NC8, NC8+PLNC8IF and NC8:pSIG308), we obtained six growth
207 curves in each culture media (MM+YE and MRS).

208 ***(e) Competition assays***

209 *(e.1) General protocol*

210 We did competition assays to measure the fitness cost/benefits of producing bacteriocins,
211 rather than estimating the metabolic costs/benefits directly. Before each competition
212 experiment, we thawed frozen stocks (-80ºC) of each strain used, inoculated in MRS broth
213 and incubated at 30ºC for 16 hours without agitation. Later, we diluted each culture separately
214 in MRS to reach an OD600nm of 0.01 (ca. 1 x 10^7 CFU/ml) in order to ensure similar
215 numbers of bacteria per milliliter. Finally we mixed the competing strains (at a proportion of
216 50%) to reach a final titre of ca. 1x10^5 CFU/ml and incubated a 30ºC for 24 h without
217 agitation. We determined the CFU/ml for each strain in the mix before (initial point at 0-h)
218 and after 24-h incubation at 30ºC (final point). To calculate CFU/ml, we diluted the cultures
219 in saline (NaCl 0.85% w/v) and spread appropriate dilutions on MRS-BPB, MRS-Rif or
220 MRS-Ery agar plates. Bacteriocin activity in mixed cultures was quantified as described
221 above.

222 *(e.2) Costs/Benefits of bacteriocin production triggered by PLNC8IF*

223 We measured the relative fitness (*w*) of NC8 (Bac^C) in competition with 128/2-Rif (NI/S),
224 with and without the addition of the autoinducer peptide PLNC8IF. We distributed aliquots of
225 990 µl from mixed cultures into 1-ml tubes and then we added 10µl of PLNC8IF to reach

226 final concentrations of 0.003, 0.03, 0.3 and 3 millimolar (mM). In control experiments (mixed
227 cultures without PLNC8IF) we added 10µl of MRS. Each treatment was repeated between 8
228 and 11 times, thus obtaining a total of 46 independent replicates, distributed as follows:
229 control experiments (n=11); treatment with PLNC8IF: 0.003mM (n=8), 0.03 mM (n=11), 0.3
230 mM (n=8), 3 mM (n=8).

231 We also measured the relative fitness (*w*) of NC8:pSIG308 (Bac^{+}; produces PLNC8IF and
232 therefore bacteriocins) against 128/2-Rif (NI/S), without the external addition of synthetic
233 PLNC8IF. We carried out competition experiments as described above, independently
234 replicating each treatment 16 times (n=16). This competition experiment allowed us to test the
235 fitness consequences of constitutively producing PLNC8IF, compared with our previous
236 experiment, which examined the consequences of adding synthetic PLNC8IF

237 *(e.3) Costs/Benefits of bacteriocin production triggered by inducer strains*

238 We measured the relative fitness (*w*) of NC8 (Bac^{C}) in competition with either FBB63-Rif
239 (I/S) or MG1363-Rif (I/R). Both these two strains induce bacteriocin production in NC8, but
240 one is sensitive (FBB63-Rif), and the other is resistant (MG1363-Rif) to such bacteriocins.
241 We replicated each competition 7 times (n=7).

242 *(e.4) Density and fitness*

243 We tested if the relative fitness benefit of producing bacteriocins depends upon cell density,
244 by conducting competition experiments between NC8 (Bac^{C}) and 128/2-Rif (NI/S) across a
245 range of cell densities (10^{2}, 10^{4}, 10^{6} and 10^{8} CFU/ml). We induced bacteriocin production
246 artificially, with the addition of PLNC8IF (0.3 mM final concentration), so that bacteriocins
247 would produce even at low densities (Darch, West, Winzer, & Diggle, 2012). We mixed the
248 competing strains at equal starting frequencies (50% each) and serially diluted to obtain
249 mixed cultures at different starting cell densities (10^{2}, 10^{4}, 10^{6} and 10^{2} CFU/ml). After
250 incubation at 30ºC for 4 h, we calculated the growth of NC8 to that of the competitor strain by

251 determining the CFU/ml for each strain at 0 h (initial) and after 4-h incubation (final). CFS
252 were quantified for bacteriocin activity as described above. We repeated each treatment five
253 times, obtaining four-five independent replicates of each cell density as follows: 10^2 (n=4),
254 10^4(n=5), 10^6 (n=5) and 10^8 (n=5).

255 ***(i) Statistical analyses***

256 We measured the relative fitness of a given strain (w) by the equation $w = p_2 (1-p_1)/p_1(1-p_2)$,
257 where p_1 is the initial proportion of the strain, and p_2 is the proportion after growth (Ross-
258 Gillespie, Gardner, West, & Griffin, 2007). The value of w signifies whether the strain
259 increases (w>1), decreases (w<1) or stays at the same (w=1) frequency. We obtained the same
260 conclusions when repeating our analyses, logging the raw values of w before calculating the
261 mean for each independent replicate, to test for any biases arising from within replicate
262 variation in w (Jiricny et al., 2010). We used standard general linear models (GLMs)
263 implemented in IBM SPSS statistics (v. 22). The curves shown are regression lines fitted to
264 the raw data.

265

266 **RESULTS**

267 ***(a) Inducing Bacteriocin production***

268 Bacteriocin production was increased by both the constitutive expression of PLNC8IF by the
269 plasmid pSIG308 (NC8:pSIG308, constitutive producer) and the addition of cells of an
270 inducing strain, *L. lactis* MG1363-Rif (I/R) (Figure 2a; $F_{1,7} = 394.3$, $p<0.01$). We were also
271 able to induce bacteriocin production by addition of synthetic peptide PLNC8IF, with larger
272 amounts of peptide leading to greater bacteriocin production (ANOVA: $F_{1,13} = 222.3$,
273 $p<0.001$, $r^2=0.942$; Figure 2b).

274

275 ***(b) Cost of producing bacteriocins***

276 We upregulated bacteriocin production in two ways, by the addition of synthetic PLNC8IF,
277 and by the constitutively expressed plasmid pSIG308. In a minimal medium supplemented
278 with yeast extract (MM+YE), we found that upregulating bacteriocin production led to
279 reduced growth in the exponential phase of growth (Figure 3a), with a decreased maximum
280 growth rate (Vmax) (ANOVA: $F_{1,16} = 13.77$, $p = 0.002$, $R^2 = 0.463$; Figure 3b), and a lower
281 final cell density ($F_{1,16} = 8.18$, $p=0.012$; no significant difference in growth between the
282 PLNC8IF and pSIG308 manipulations: $F_{1,10} = 1.70$, $p=0.22$) (Figure 3a&c) .
283 In a nutrient rich media, de Man, Rogosa and Sharpe (MRS), we found that
284 upregulating bacteriocin production led to a reduced growth in the exponential phase of
285 growth (Figure 3d), with a decreased Vmax with the addition of PLNC8IF (NC8+PLNC8IF;
286 ANOVA: $F_{1,10} = 17.45$, $p=0.002$) but not constitutive expression of PLNC8IF
287 (NC8:pSIG308; ANOVA: $F_{1,10} = 0.30$, $p =0.59$) (Figure 3e). There was no influence on final
288 cell density (ANOVA: $F_{1,16} = 0.27$, $p=0.61$; no significant difference in growth between the
289 PLNC8IF and pSIG308 manipulations: $F_{1,10} = 1.05$, $p=0.33$) (Figure 3d&f).
290
291 ***(c) Fitness Benefit of Producing Bacteriocins***
292 We tested whether bacteriocin production conferred a benefit to the producing strain, when
293 competing with *L. pentosus* 128/2-Rif (NI/S) a strain which is sensitive to bacteriocins
294 produced by NC8. We upregulated bacteriocin production in two ways: (i) both the
295 constitutive expression of PLNC8IF by the plasmid pSIG308 (constitutive producer); and (ii)
296 addition of synthetic PLNC8IF.
297 In both cases, we found that increased bacteriocin provided a fitness benefit when in
298 competition with *L. pentosus* 128/2-Rif (NI/S). The constitutive producer (NC8:pSIG308;
299 Bac^+) produced more bacteriocin per cell (Figure 4a; ANOVA: $F_{1,26} = 119.95$, $p < 0.001$), and
300 had a higher relative fitness than the wild type NC8 (Bac^C) strain (Figure 4b; ANOVA: $F_{1,32} =$

301 52.36, $p < 0.001$), which only produces bacteriocins conditionally. The addition of synthetic
302 PLNC8IF also led to increased bacteriocin production per cell (Figure 4c; ANOVA: $F_{1,44}$ =
303 688.95, $p < 0.001$; $R^2 = 0.940$), and that increased bacteriocin production led to an increase in
304 fitness until a maximum after which the fitness benefits level off (Figure 4b; ANOVA: $F_{2,43}$
305 = 174.70, $p < 0.001$; $R^2 = 0.890$).

306

307 ***(d) Fitness benefit and cost of activating bacteriocin production after cell-to-cell contact***
308 ***with inducer strains***

309 We also tested the relative benefit of QS controlled bacteriocin production by competing our
310 conditional bacteriocin producing strain (NC8) against two strains which both induce
311 bacteriocin production, but where one strain is sensitive to the bacteriocins produced by NC8
312 (FBB63-Rif; I/S), and the other strain is resistant (MG1363-Rif; I/R). Consistent with a net
313 benefit of bacteriocin production originating from the killing competitors, we found that our
314 conditional bacteriocin producing strain (NC8) had a higher fitness than the sensitive strain
315 (FBB63-Rif; $t_6 = 58.10$, $p < 0.001$) (Figure 5). In contrast, the fitness of the conditional
316 bacteriocin producing strain (NC8) was slightly lower than the resistant strain (MG1363-Rif;
317 $t_6 = -2.69$, $p = 0.036$) (Figure 5). Although the fitness difference versus the resistant strain is
318 small, and there are other genetic differences between the lines, this result is consistent with a
319 cost of bacteriocin production, in a situation where there is no benefit.

320

321 ***(e) Density assays***

322 We then tested whether the benefits of bacteriocin production were greater at higher cell
323 densities. We added synthetic PLNC8IF to activate bacteriocin production in the conditional
324 producing strain (NC8; Bac^C), when grown in competition with a sensitive non-inducing
325 strain (128/2-Rif; NI/S), at a range of different starting densities. At higher starting densities,

326 the relative fitness of the bacteriocin producing PLNC8IF strain was greater (ANOVA: $F_{1,17}$ =
327 79.51, p < 0.001, R^2 = 0.824; Figure 6b). Although we found that bacteriocin production was
328 induced by the addition of synthetic PLNC8IF, this result could be confounded by cell
329 density, which also positively correlates with bacteriocin production (ANOVA: $F_{1,18}$ =
330 164.92, p < 0.001, R^2 = 0.902; Figure 6a). Although in a multiple regression, the relative
331 fitness of the bacteriocin producing strain was greater both when they produced more
332 bacteriocins ($F_{1,16}$ = 17.64, p=0.001), and at higher cell densities ($F_{1,16}$ = 5.59, p=0.031).
333

334 **DISCUSSION**

335 We examined the social cost and benefit of *quorum* sensing (QS) controlled bacteriocin
336 production in the bacteria *L. plantarum* NC8. We found that: (1) bacteriocin production could
337 be induced by the addition of a synthetic PLNC8IF peptide, or by a plasmid which
338 constitutively encodes for the production of this peptide (Figure 2); (2) bacteriocin production
339 is costly, leading to reduced growth when grown in poor and, to a lesser extent, in rich media
340 (Figure 3), and when in competition with resistant bacteria (Figure 6); (3) bacteriocin
341 production provides a fitness advantage, when grown in competition with sensitive strains
342 (Figures 4-6); (4) the fitness benefits provided by bacteriocin production is greater at higher
343 cell densities (Figure 6).
344

345 Quorum sensing and the benefits of bacteriocin production

346 It is frequently assumed that bacteria use quorum sensing to conditionally switch on certain
347 behaviours when they are relatively more beneficial. In particular, it is thought that quorum
348 sensing is used to turn on cooperative behaviours at high densities, when they would provide
349 a greater benefit (Brown & Johnstone, 2001; West, Winzer, Gardner, & Diggle, 2012).
350 However, there are few studies that have tested this assumption (Darch et al., 2012;

351 Koschwanez, Foster, & Murray, 2013). We have shown here, consistent with quorum sensing
352 theory, that quorum sensing controlled bacteriocin production provides a greater benefit at
353 higher cell densities (Figure 7).

354 The benefits of producing QS-regulated bacteriocins could be greater at high densities
355 because: (a) greater cell-to-cell contact with inducer bacteria can increase bacteriocin
356 effectivity; (b) bacteriocins could reach sensitive strains at a greater rate; (c) the benefits of
357 killing competitors, via reduced resource competition, could be greater; (d) the benefits of
358 producing bacteriocins and killing the sensitive strain, freeing-up resources, could be more
359 likely to be shared with clone mates. A potential problem with our density manipulation is
360 that it also altered bacteriocin production (Figure 6a). Although our results suggest that both
361 bacteriocin production and density have an influence, it would be useful to develop a density
362 manipulation that did not have any other potentially confounded influences. In addition, it is
363 also important to note that initial frequency of bacteriocin producer relative to the sensitive
364 strain can influence the relative benefit of producing bacteriocins (Gardner et al. 2004; Inglis
365 et al., 2009). Our experiments were carried out at intermediate frequencies (each strain at
366 50%), where bacteriocins are expected to confer the greatest fitness advantage from killing
367 competitors, by reducing competition for both the bacteriocin producing cells and their
368 relatives (Gardner et al., 2004; Inglis et al., 2009).

369

370 The cost of producing bacteriocins

371 We found that producing bacteriocins reduced growth rate, in both poor and rich media, and
372 reduced final cell density in poor, but not rich media (Figure 3). This is consistent with
373 previous results finding that the cost of traits are reduced or eliminated when the resources for
374 producing them are not limited (Sexton & Schuster, 2017; Xavier, Kim, & Foster, 2011).
375 When growing alone either in poor or in rich medium, NC8 produce little or no bacteriocins,

376 thus avoiding the cost of producing bacteriocins when not required (no benefit of killing
377 competitors). In addition, we found that in rich medium the relative fitness of NC8 depend on
378 their social environment, which is determined by the resistance/sensitivity of the social
379 partner (competing strain) to bacteriocins produced by NC8.

380

381 Conditional bacteriocin production

382 In NC8, conditional bacteriocin production could be seen as a mechanism directed to save
383 metabolic costs when there is less benefit to their production. In absence of competitors, the
384 QS mechanism is not active which avoids costly bacteriocin production when there is little
385 benefit. In contrast, when a competing strain is sufficiently common, such that it would be
386 beneficial to eliminate them with bacteriocins, and there are sufficient NC8 to produce those
387 bacteriocins, their production is upregulated (Figure 1a). In nature, there could be selection to
388 detect "sensitive" competitors or to avoid being detected – although there is no evidence yet
389 for this, it could help explain the existence of non-inducer strains that are resistant to
390 bacteriocins. Mechanistically, the co-culture with certain specific strains (inducer bacteria)
391 belonging to different species led to the activation of the QS system in NC8, activating
392 production of the autoinducer PLNC8IF and therefore production of bacteriocins (Figure 1b)
393 (Maldonado et al., 2004a; Maldonado et al., 2004b). Thus, in NC8, bacteriocin production
394 depends on first instance on the identity of the interacting partner (Maldonado et al., 2004b),
395 and second, on the ability to reach a QS driven by the autoinducer peptide PLNC8IF (Figure
396 1a) (Maldonado et al., 2004a; Maldonado-Barragán et al., 2009). This implies that quorum
397 sensing information is used not only for signalling among self-cells, but also integrated with
398 the detection of specific cues produced by other genotypes (for detecting evolutionary
399 competition; Cornforth & Foster (2013).

400

401 Conclusions

402 We have demonstrated that bacteriocin production can be costly, but that it can provide a
403 benefit when competing against susceptible strains. Furthermore, that the benefits are greater
404 at higher cell densities. These results provide an explanation for why bacteriocin production
405 should be controlled by quorum sensing, and preferentially turned on at higher cell densities.
406 More generally, we build upon previous results, which emphasise the advantage of
407 conditionally adjusting bacteriocin production, in response to the social environment (Gardner
408 et al., 2004; Gonzalez et al., 2018; Gonzalez & Mavridou, 2019; Majeed et al., 2011;
409 Mavridou et al., 2018).

410

411 **Acknowledgments**

412 We thank Dr. John B. Bruce and Dr. Melanie Ghoul for discussion and support in the
413 laboratory, and comments on the manuscript. We thank two anonymous reviewers and Dr.
414 Christoph Haag for comments on the manuscript. We thank to the Spanish National Research
415 Council (CSIC) and Instituto de la Grasa-CSIC for the administrative management of the
416 Andalucia Talent Hub program and providing access to the bacterial strains used in this work.
417 This study was funded by the Andalucía Talent Hub Program launched by the Andalusian
418 Knowledge Agency, co-funded by the European Union's Seventh Framework Program, Marie
419 Skłodowska-Curie actions (COFUND – Grant Agreement nº 291780) and the Ministry of
420 Economy, Innovation, Science and Employment of the Junta de Andalucía.

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540

541 **FIGURE LEGENDS**

542

543 **Figure 1. (a) Conditional Bacteriocin Production in L. plantarum NC8 depends upon the**
544 **social environment.** Bacteriocin upregulation requires both a signal of sufficient clonemate
545 density (assessed via QS) and sufficient density of unrelated competitors (assessed via cell-to-
546 cell contact). **(b) Model of Regulation of Bacteriocin Production in Lactobacillus**
547 **plantarum NC8.** The cell-to-cell contact with inducer bacteria activates the expression
548 (through a hitherto unknown mechanism) of the operon plNC8IFHK-plnD encoding a three-
549 component regulatory system, formed by an autoinducer peptide (PLNC8IF) a histidine
550 protein kinase (PLNC8HK) and a response regulator (PlnD). As consequence of this

551 interaction, the autoinducer peptide PLNC8IF is produced. Once PLNC8IF reaches the
552 threshold concentration (Quorum sensing) is sensed by their corresponding histidine kinase
553 PLNC8HK, which activates the response regulator PlnD through phosphorylation. Active-
554 PlnD induces the expression of the genes involved in bacteriocin production in NC8 as well
555 as the proper TCRS (autoinduction).
556
557 **Figure 2. Validating strains.** (a) Bacteriocin activity of the conditional bacteriocin producer
558 *L. plantarum* NC8 (Bac^C) when grown as single culture (NC8) or in co-culture with the
559 inducer bacteria *L. lactis* MG1363-Rif (I/R) (NC8+MG1363), and its derivative strain
560 NC8:pSIG308 (Bac^+) that constitutively produces the autoinducer peptide (PLNC8IF). Both
561 the co-culture with *L. lactis* and the production of PLNC8IF driven by plasmid pSIG308 led
562 to increased bacteriocin production. (b) The addition of larger amounts of synthetic PLNC8IF
563 peptide leads to greater bacteriocin production in *L. plantarum* NC8. Error bars were not
564 displayed because they are too small to be seen.
565
566 **Figure 3. Bacteriocin production is costly.** Growth curves, maximum growth rates (Vmax)
567 and final cell densities in nutrient poor (MM+YE) media (a, b and c) and nutrient rich (MRS)
568 media (d, e and f). Data are shown for the conditional bacteriocin producer strain producing
569 negligible bacteriocins (*L. plantarum* NC8; Bac^C), and two strains producing appreciable
570 bacteriocins: NC8 with added PLNC8IF autoinducer (NC8+PLNC8IF; Bac^+) and the
571 constitutive PLNC8IF producer (NC8:pSIG308; Bac^+). OD_{600nm} measurements are means for
572 six replicates. The addition of PLNC8IF peptide and constitutive PLNC8IF production both
573 led to reduced growth in nutrient poor media, but not in nutrient rich media, although there
574 was a trend to decreased maximum growth rate at the exponential phase of growth in both

575 culture media. Error bars, which represent standard errors, were not displayed in figure 3d
576 because they are too small to be seen.
577
578 **Figure 4. Benefit of Bacteriocin Production.** Bacteriocin activity (a) and relative fitness (b)
579 of the conditional bacteriocin producer (NC8; Bac^{C}) and the constitutive PLNC8IF producer
580 (NC8:pSIG308; Bac^{+}) when in competition with *L. pentosus* 128/2-Rif (sensitive strain;
581 NI/S). The constitutive producer both produced more bacteriocins, and had a higher relative
582 fitness, when compared with the conditional producer. Bacteriocin activity (c) and relative
583 fitness (d) of the conditional bacteriocin producer *L. plantarum* NC8 (Bac^{C}), when in
584 competition with *L. pentosus* 128/2-Rif (sensitive strain; NI/S) after the initial addition of
585 increasing concentrations of the autoinducer peptide PLNC8IF. The addition of PLNC8IF
586 induced bacteriocin production in NC8, increasing its fitness against the sensitive strain
587 128/2-Rif, although this increase in fitness approached an asymptote or even peaked. Relative
588 fitness (*w*) was estimated relative to the sensitive strain, and plotted on a log scale.
589 Bacteriocin activity was expressed as bacteriocin units per cell (BU/cell). mM, millimolar.
590 The curves are regression lines fitted to the raw data.
591
592 **Figure 5. The Cost and Benefit of naturally induced bacteriocin production.** The relative
593 fitness of the conditional bacteriocin producing strain NC8 (Bac^{C}) is shown when grown with
594 inducing strains that are: (a) sensitive (FBB63-Rif; I/S); and (b) resistant (MG1363-Rif; I/R)
595 to the bacteriocins produced by NC8. Relative fitness (*w*) was plotted on log scale.
596
597 **Figure 6. Density-dependent fitness benefits of responding to QS.** Bacteriocin activity (a)
598 and relative fitness (b) of the conditional bacteriocin producer *L. plantarum* NC8 (Bac^{C}),
599 when in competition with the sensitive *L. pentosus* 128/2-Rif strain (NI/S) at different starting

cell densities (from 10^2 to 10^8 cells per millilitre). The fitness benefit of adding signal (PLNC8IF), and therefore inducing QS and bacteriocin production in the conditional producer *L. plantarum* NC8 was greater at higher population densities. The dashed lines indicate if the conditional producer NC8 increases (w>1), decreases (w<1) or remains at the same frequency (w=1).

611 **Table 1.** Bacterial strains used in this study

Strains	Characteristics	Bacteriocin Production	†Resistant (R) /Sensitive (S)	‡Inducer (I) /Non-inducer (NI)	Reference
Lactobacillus plantarum NC8*	Inducible plantaricins (PLNC8αβ, PlnEF and PlnJK) producer	Conditional (Bac^{C})	R	NI	Shrago, Chassy and Dobrogosz (1986) Maldonado et al. (2003; 2004b)
L. plantarum NC8:pSIG308	Derivative of NC8; Produces PLNC8IF constitutively from plasmid pSIG308. Erm^{R}	Constitutive (Bac^{+})	R	I	Maldonado et al. (2004b)
Lactobacillus pentosus 128/2	Indicator strain for bacteriocin activity	Negative (Bac^{-})	S	NI	Maldonado et al. (2003)
L. pentosus 128/2-Rif	Derivative of *L. pentosus* 128/2; Rif^{R}	Negative (Bac^{-})	S	NI	This work
Lactococcus lactis MG1363	Indicator strain for bacteriocin activity	Negative (Bac^{-})	R	I	Maldonado et al. (2003)
L. lactis MG1363-Rif	Derivative of *L. lactis* MG1363; Rif^{R}	Negative (Bac^{-})	R	I	This work
Pediococcus pentosaceus FBB63	Indicator strain for bacteriocin activity	Negative (Bac^{-})	S	I	Maldonado et al. (2003)
P. pentosaceus FBB63-Rif	Derivative of *P. pentosaceus* FBB63; Rif^{R}	Negative (Bac^{-})	S	I	This work

612

613 * Kindly provided by Lars Axelsson from MATFORSK, Norwegian Food Research Institute, Osloveien, Norway.

614 † Erm^{R}: resistant to erythromycin; Rif^{R}: resistant to rifampicin;

615 ‡ Resistant or Sensitive to bacteriocins produced by *L. plantarum* NC8

616 § Ability to induce bacteriocin production in co-culture with *L. plantarum* NC8

617 FIGURE 1

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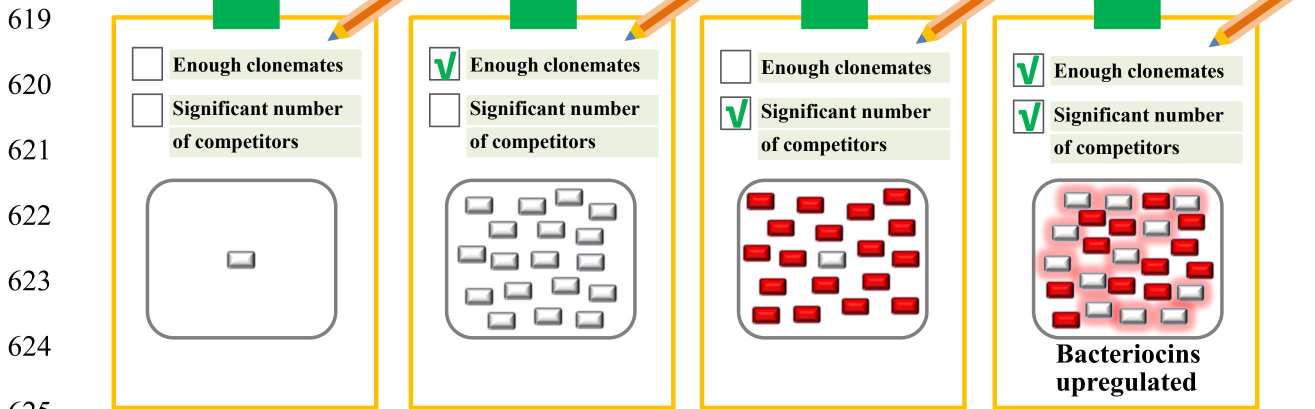

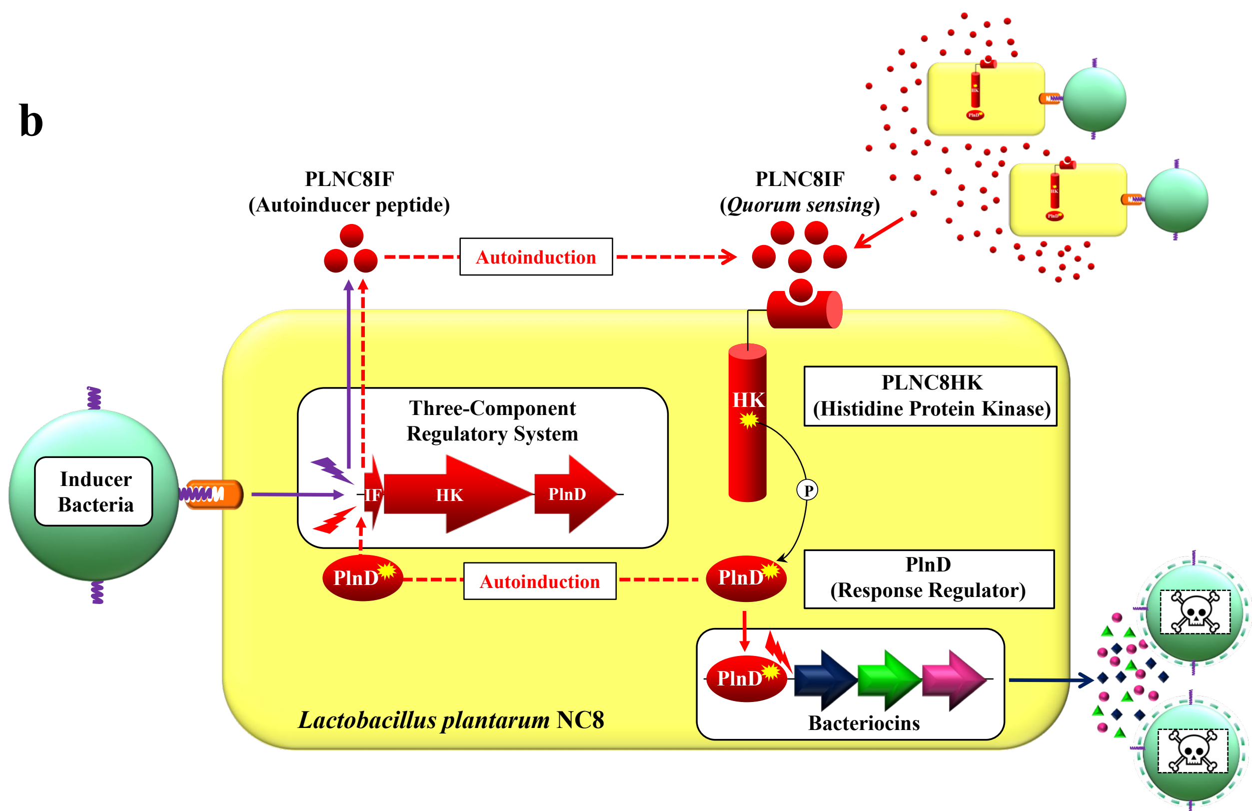

645 **FIGURE 2**

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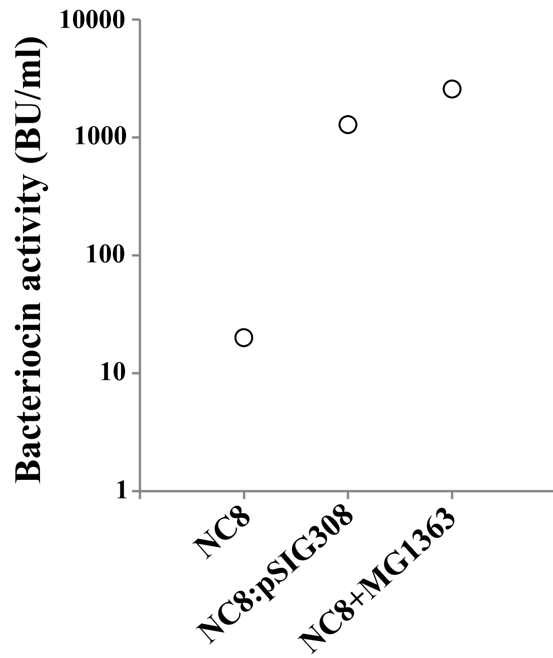

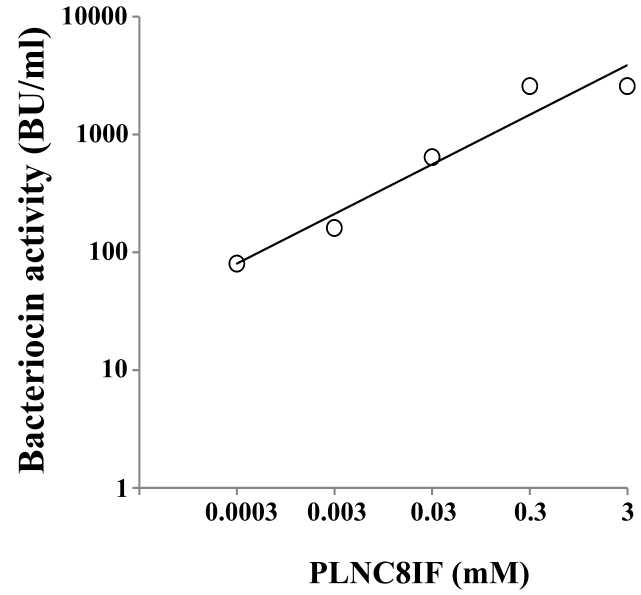

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673 **FIGURE 3**

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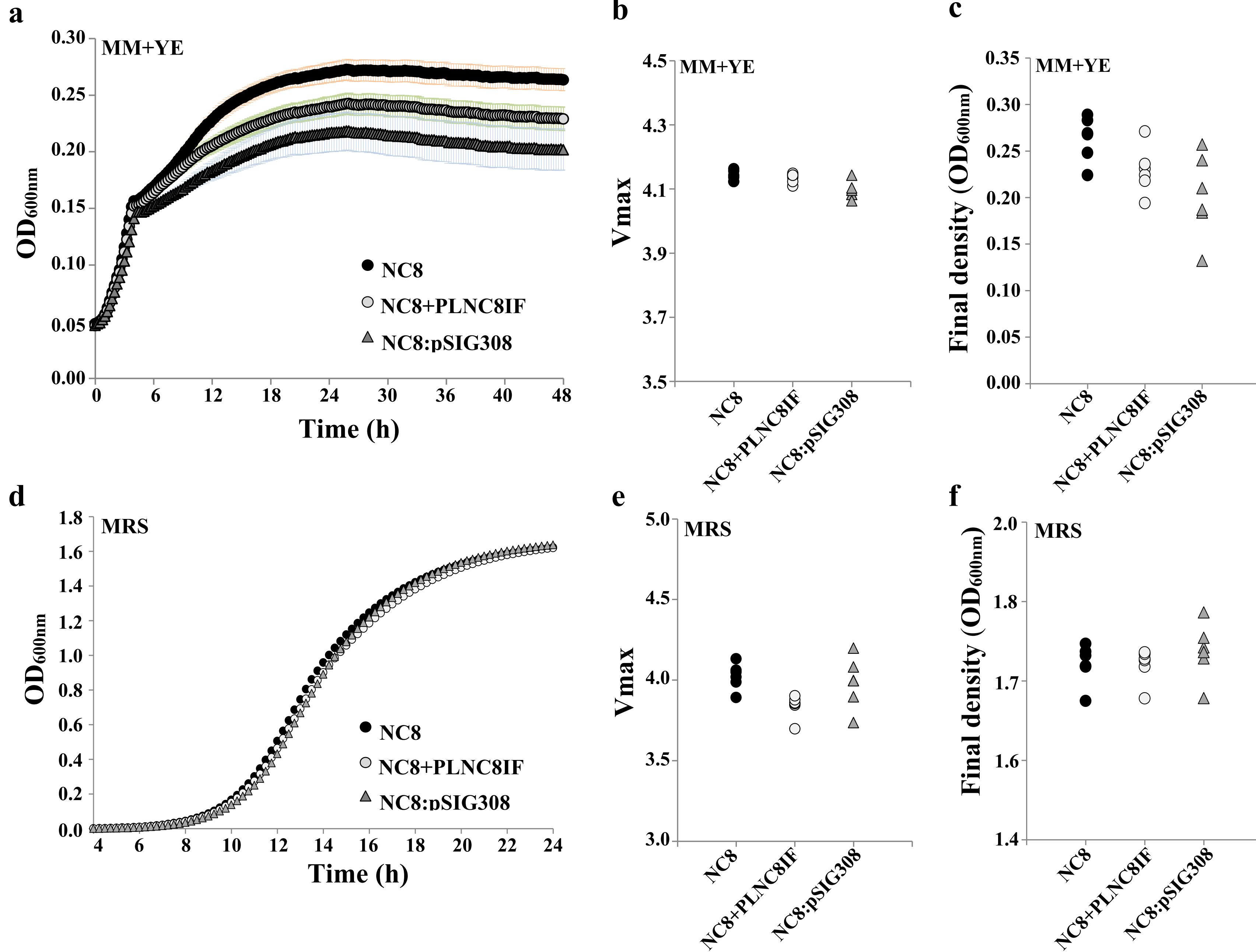

676 **FIGURE 4**

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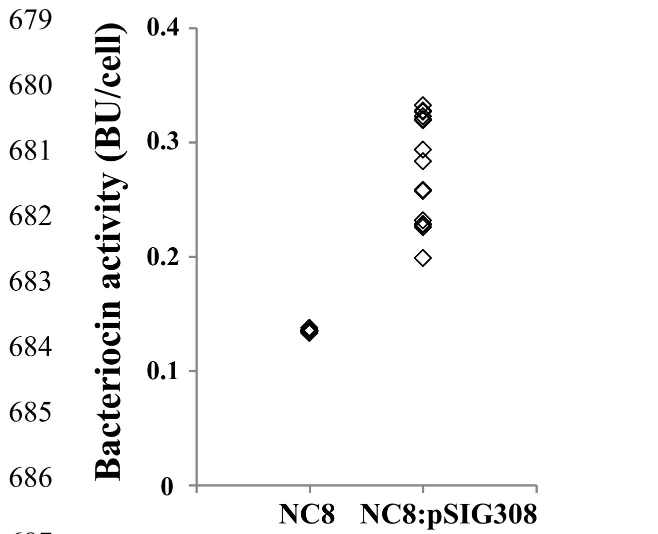

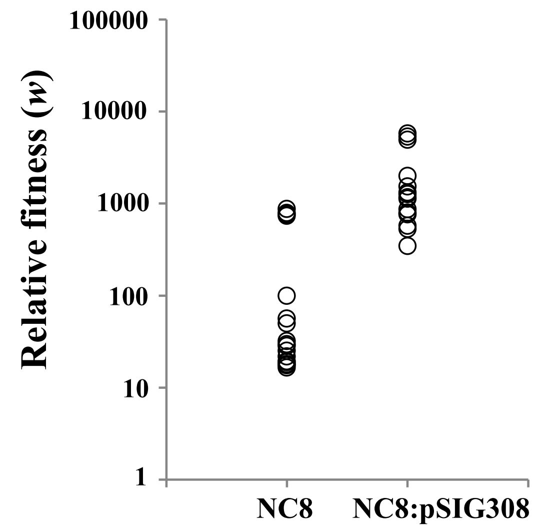

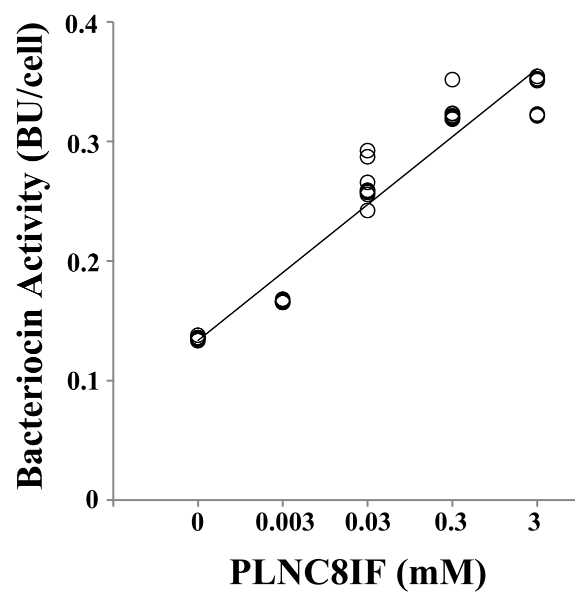

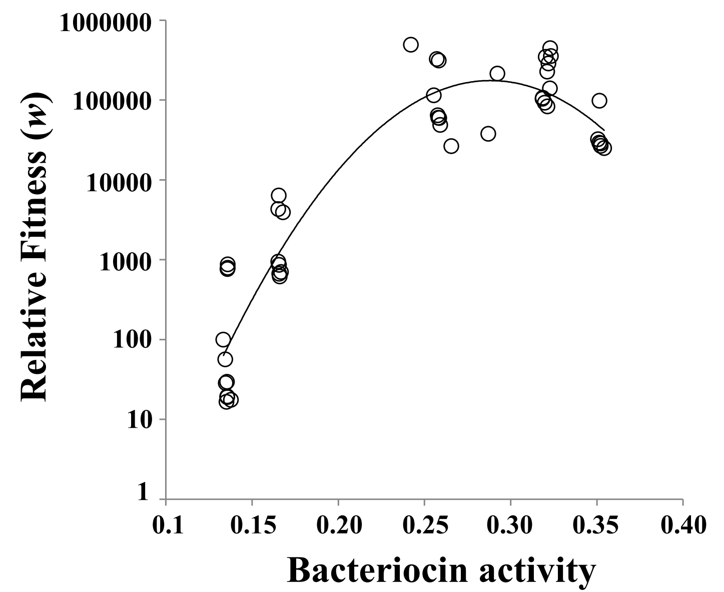

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704 **FIGURE 5**

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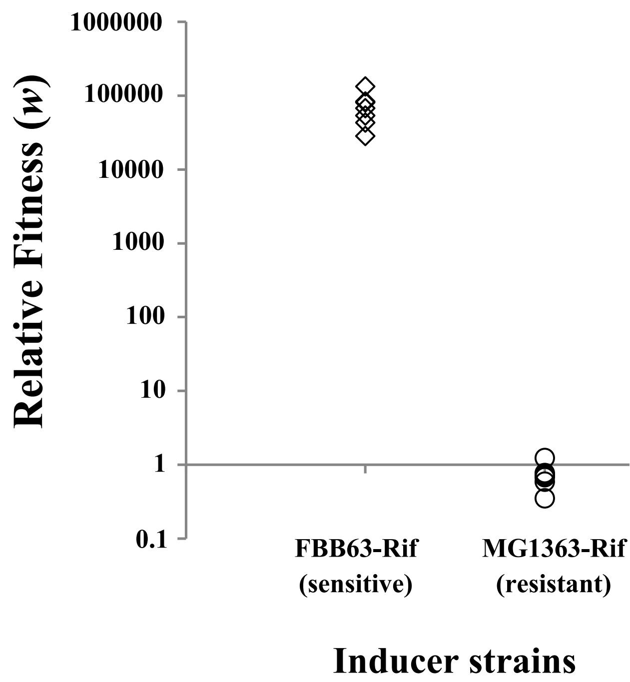

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732 **FIGURE 6**

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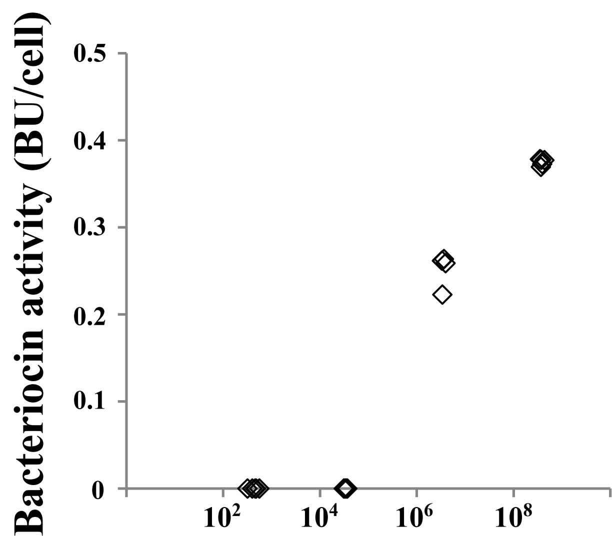

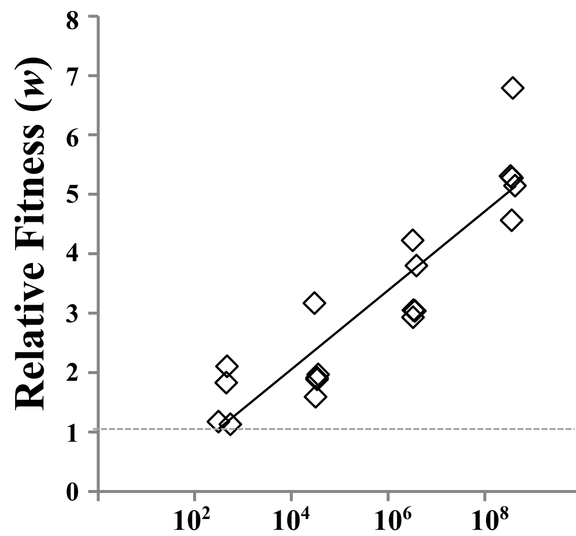

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746 **Cell Density (CFU/ml)**

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