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

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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: (a) 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; (b) bacteriocin production is costly, leading to reduced growth when grown in poor and, to a lesser extent, in rich media; (c) bacteriocin production provides a fitness advantage, when grown in competition with sensitive strains; and (d) the fitness benefits provided by bacteriocin production are 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, benefits, costs, fitness, quorum sensing

1 | INTRODUCTION

The growth and success of many bacteria, both free living and pathogenic, often depends on their ability to eliminate competitors via 'chemical warfare' with bacteriocins (Cotter, Hill, & Ross, 2005; Granato, Meiller-Legrand, & Foster, 2019; Riley & Wertz, 2002). Bacteriocins are small peptides that possess antimicrobial activity against other bacteria, but against which the producing cell has a specific immunity mechanism. Both theoretical and empirical studies have suggested that the production of bacteriocins is a social trait, because the benefits of eliminating competitors are shared with clone mates that also possess immunity (Brown & Buckling, 2008; Bucci, Nadell, & Xavier, 2011; Chao & Levin, 1981; Czárán & Hoekstra, 2003; Doekes, de Boer, & Hermsen, 2019; Frank, 1994; Gardner, West, & Buckling, 2004; Hawlena, Bashey, & Lively, 2010; Inglis, Gardner, Cornelis, & Buckling, 2009; Kerr, Riley, Feldman, & Bohannan, 2002; Libberton, Horsburgh, & Brockhurst, 2015; Mavridou, Gonzalez, Kim, West, & Foster, 2018; Waite & Curtis, 2008; West, Griffin, Gardner, & Diggle, 2006).

Bacteria appear to adjust bacteriocin production conditionally in response to local conditions and especially the social environment (Bhattacharya, Pak, & Bashey, 2018; Gonzalez & Mavridou, 2019; Majeed, Gillor, Kerr, & Riley, 2011; Maldonado, Ruiz-Barba, & Jiménez-Díaz, 2004; Mavridou et al, 2018). In *Escherichia coli*, bacteriocin production is increased in response to the presence of bacteriocins produced by competing strains and clone mates (Gonzalez, Sabnis, Foster, & Mavridou, 2018; Majeed et al., 2011; Mavridou et al., 2018). In *Lactobacillus plantarum* and *Lactobacillus gasseri*, bacteriocin production is controlled by a 'quorum sensing' signalling system that causes production to be activated when cells are at higher densities and in the presence of competing bacteria from different species (Maldonado, Jiménez-Díaz, & Ruiz-Barba,

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2004; Maldonado-Barragán, Caballero-Guerrero, Lucena-Padrós, & Ruiz-Barba, 2013; Maldonado-Barragán, Caballero-Guerrero, Martín, Ruiz-Barba, & Rodríguez, 2016; Maldonado-Barragán, Ruiz-Barba, & Jiménez-Díaz, 2009). Evolutionary theory suggests that a potential explanation for these conditional shifts in bacteriocin production is that the relative costs and benefits of bacteriocin production depend upon the social environment (Gardner et al., 2004). For example, producing bacteriocins is predicted to become

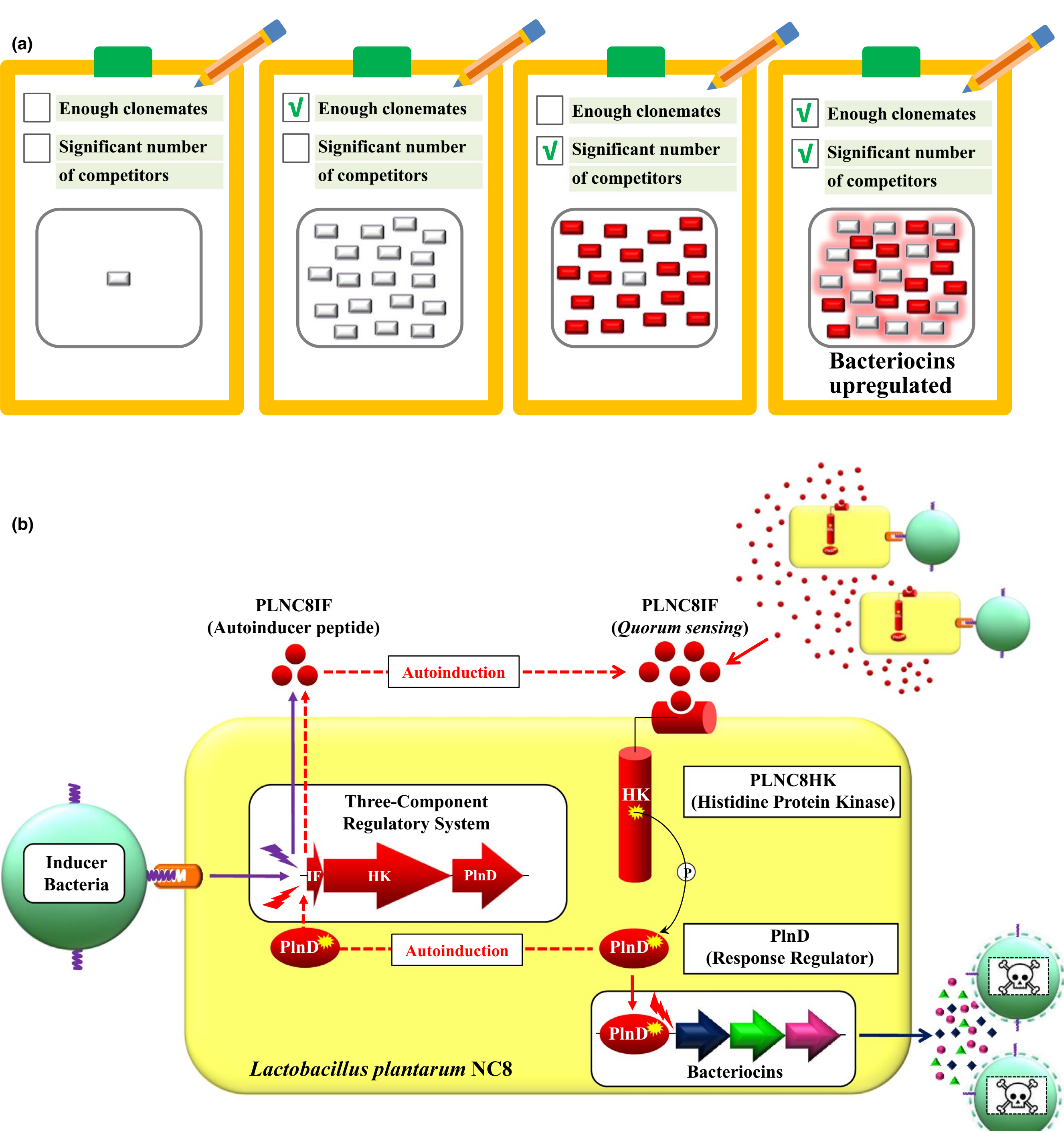

FIGURE 1 (a) Conditional bacteriocin production in L. plantarum NC8 depends upon the social environment. Bacteriocin upregulation requires both a signal of sufficient clonemate density (assessed via QS) and sufficient density of unrelated competitors (assessed via cell-to-cell contact). (b) Model of regulation of bacteriocin production in Lactobacillus plantarum NC8. The cell-to-cell contact with inducer bacteria activates the expression (through a hitherto unknown mechanism) of the operon plNC8IFHK-plnD encoding a three-component regulatory system, formed by an autoinducer peptide (PLNC8IF) a histidine protein kinase (PLNC8HK) and a response regulator (PlnD). As consequence of this interaction, the autoinducer peptide PLNC8IF is produced. Once PLNC8IF reaches the threshold, concentration (quorum sensing) is sensed by their corresponding histidine kinase PLNC8HK, which activates the response regulator PlnD through phosphorylation. Active-PlnD induces the expression of the genes involved in bacteriocin production in NC8 as well as the proper TCRS (autoinduction)

less beneficial when the density of the competing strains is low (Gardner et al., 2004). However, we know relatively little about how the costs and benefits of bacteriocin production depend upon social conditions (Gonzalez & Mavridou, 2019; Gonzalez et al., 2018; Inglis et al., 2009).

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

We can experimentally manipulate bacteriocin production in the *L. plantarum* NC8 system in a number of ways. As described above, the wild-type NC8 strain only produces bacteriocins under certain conditions. Alternatively, even in the absence of inducing bacteria, we can add autoinducer peptide PLNC8IF to activate both PLNC8IF synthesis (autoinduction) and bacteriocin production (Maldonado, Jiménez-Díaz, et al., 2004; Maldonado-Barragán et al., 2013). Finally, we can use a recombinant strain NC8:pSIG308, which produces PLNC8IF constitutively—when this PLNC8IF reaches a sufficient concentration, it will activate bacteriocin production (Maldonado, Jiménez-Díaz, et al., 2004).

Our specific aims were to test: (a) whether our experimental and genetic manipulations influence bacteriocin production; (b) whether bacteriocin production is costly; (c) the fitness benefits of bacteriocin production when competing against susceptible strains; and (d) whether the fitness benefits of bacteriocin production are greater at higher cell densities.

2 | METHODS

2.1 | Bacterial strains and growth media

We used eight strains in this study, whose main features are summarized in Table 1: (a) *Lactobacillus plantarum* NC8, a wild-type conditional bacteriocin producer (Bac^{C}) strain that produces QS-regulated bacteriocins on solid medium, but requires co-culture with certain specific inducer strains or the external addition of the autoinducer peptide PLNC8IF to produce bacteriocins in liquid cultures

TABLE 1 Bacterial strains used in this study

Strains	Characteristics[a]	Bacteriocin production	Resistant (R)/sensitive (S)[b]	Inducer (I)/noninducer (NI)[c]	Reference
Lactobacillus plantarum NC8[d]	Inducible plantaricins (PLNC8 αβ, PlnEF and PlnJK) producer	Conditional (Bac^{c})	R	NI	Shrago, Chassy, and Dobrogosz (1986) Maldonado et al. (2003), Maldonado, Jiménez-Díaz, et al. (2004)
L. plantarum NC8:pSIG308	Derivative of NC8; produces PLNC8IF constitutively from plasmid pSIG308. Erm^{R}	Constitutive (Bac^{+})	R	I	Maldonado, Jiménez-Díaz, et al. (2004)
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

[a]Erm^{R}: resistant to erythromycin; Rif^{R}: resistant to rifampicin.
[b]Resistant or sensitive to bacteriocins produced by *L. plantarum* NC8.
[c]Ability to induce bacteriocin production in co-culture with *L. plantarum* NC8.
[d]Kindly provided by Lars Axelsson from MATFORSK, Norwegian Food Research Institute, Osloveien, Norway.

(Maldonado, Jiménez-Díaz, et al., 2004). Bacteriocins produced belong to the two-peptide ClassIIb bacteriocins, which are released from cells in their active mature form by a dedicated ABC transporter, without cell lysis (Cotter et al., 2005).

(b) *L. plantarum* NC8:pSIG308 (Bac^+) is a derivative strain of NC8 which carries the plasmid pSIG308 (Maldonado, Ruiz-Barba, et al., 2004). This plasmid harbours the gene *plNC8IF*, which encodes the autoinducer peptide PLNC8IF, cloned behind the constitutive lactococcal promoter P53. In contrast to the wild-type strain (NC8), NC8:pSIG308 produces PLNC8IF at sufficient amounts to activate QS-regulated bacteriocins (Bac^+) and is resistant to erythromycin (20 µg/ml).

(c) *L. pentosus* 128/2-Rif (NI/S): a rifampicin-resistant (30 µg/ml) derivative strain of *L. pentosus* 128/2. This strain is sensitive (S) to bacteriocins produced by *L. plantarum* NC8 but does not induces (NI) bacteriocin production in NC8.

(d) *Lactococcus lactis* MG1363-Rif (I/R): a rifampicin-resistant (30 µg/ml) derivative strain of *L. lactis* MG1363. This strain is resistant (R) to bacteriocins produced by *L. plantarum* NC8 and induces (I) bacteriocin production in NC8.

(e) *Pediococcus pentosaceus* FBB63-Rif (I/S): a rifampicin-resistant (30 µg/ml) derivative of *P. pentosaceous* FBB63. This strain is sensitive (S) to bacteriocins produced by *L. plantarum* NC8 and induces (I) bacteriocin production in NC8.

We obtained the strains 128/2-Rif, MG1363-Rif and FBB63-Rif in this study by sequential selection of the wild-type strains 128/2, MG1363 and FBB63 (Table 1), in increasing concentrations of rifampicin (from 1 to 50 µg/ml). Unless indicated, we grew all strains at 30°C in de Man, Rogosa and Sharpe broth medium (MRS; Oxoid Ltd) or in MRS agar plates (1.5% w/v). We froze aliquots of each strain at −80°C in MRS plus 20% glycerol (v/v).

For differential selection of bacteria on solid medium, we used MRS-BPB agar (MRS plus 0.01% w/v bromophenol blue; Sigma-Aldrich), MRS-Rif (MRS plus 30 µg/ml rifampicin) and MRS-Ery (MRS plus 100 µg/ml erythromycin). In MRS-BPB, we could easily differentiate NC8 (*L. plantarum*) from 128/2-Rif (*L. pentosus*), MG1363-Rif (*L. lactis*) and FBB63 (*P. pentosaceus*) since the pH change produced during fermentation of these lactic acid bacteria changes the colour of accumulated BPB (pH indicator), being yellow at pH 3.0 and blue at pH 5.0 (Lee & Lee, 2008).

For testing the growth of lactobacilli under nutrient limitation, we developed a minimal medium (MM) containing K2HPO4 2 g/L, sodium acetate monohydrate 5 g/L, MnSO4.H2O 0.0152 g/L and Tween 80 0.5 ml/L. The MM was supplemented with 5 g/L yeast extract (MM + YE) as nitrogen source.

2.2 | Bacteriocin inducing experiments

The native PLNC8IF was shown to induce bacteriocin production and its proper synthesis in *L. plantarum* NC8 through the activation of TCRS (PLNC8IF-PLNC8HK-PLND) (Maldonado, Jiménez-Díaz, et al., 2004). In this study, we deduced the amino acid sequence of the autoinducer peptide PLNC8IF (KTKTISLMSGLQVPHAFTKLLKALGGHH) from its encoding gene *pLNC8IF* (accession number: AF522077; Maldonado, Jiménez-Díaz, et al., 2004) and ordered the chemical synthesis of the peptide (GenScript USA Inc.). We dissolved one milligram of the peptide (> 95% of purity) in 1 ml of HyPure Water (GE Healthcare) and serially diluted to obtain concentrations ranging from 1 to 10^{-12} mg/ml. To test the ability to induce bacteriocin production in NC8, we added ten microlitres of each PLNC8IF dilution to 990 µl of MRS containing ca.10^8 colony-forming units per millilitre (CFU/ml) of NC8, thus obtaining final PLNC8IF concentrations ranging from 3 to 3 10^{-12} mM. In control cultures (NC8 without PLNC8IF), we added ten microlitres of MRS instead of PLNC8IF. The inducing activity of synthetic PLNC8IF was compared with that of *L. lactis* MG1363-Rif, whose parental strain, *L. lactis* MG1363 (I/R), is able to induce bacteriocin production in NC8 trough co-culture (Maldonado et al., 2003; Maldonado, Ruiz-Barba, et al., 2004). Specifically, we added ten microlitres of a 16-hr old culture of MG1363-Rif to NC8 cultures (prepared as described above), giving a final concentration of ca. 10^7 CFU/ml. In parallel, we compared both the inducing activity of PLNC8IF and MG1363-Rif with the inducing activity of native PLNC8IF, which is endogenously produced by plasmid pSIG308 in the recombinant strain NC8:pSIG308 (Bac^+). For this, we inoculated NC8:pSIG308 in the same conditions as described above for their parental strain NC8. Cultures were done in triplicate and incubated at 30°C for 7 hr. Bacteriocin activity of cell-free supernatants was quantified as described below. To test for antimicrobial activity of PLNC8IF, we carried out serial dilutions of the peptide in MRS medium as described above and then quantified bacteriocin activity.

2.3 | Bacteriocin quantification

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

2.4 | Growth curves

We determined the growth curves of NC8 (Bac^C) (with and without the addition of the autoinducer PLNC8IF) and NC8:pSIG308 (Bac^+) in MRS medium. For this, we diluted 16-hr old cultures in MRS until an optical density (measured at 600 nm; OD600 nm) of 0.01. We measured the OD600 nm in a spectrophotometer (SpectraMax M2; Molecular devices) and recorded with the software SoftMaxPro (Molecular devices). For each strain, we prepared six independent cultures, and for each culture, we deposited 195 µl per well in a 96-well microtitre plate. In the case of NC8 plus PLNC8IF, we added 5 µl of

PLNC8IF per well to reach a final concentration of 0.3 mM, whereas in the other cultures (NC8 without PLNC8IF and NC8: pSIG308), we added 5 µl of MRS. We used noninoculated MRS as the blank control. We then incubated plates at 30°C for 48 hr, in a Synergy 2 Multi-Mode Reader (BioTek), measuring OD600 nm at 15-min intervals. We recorded OD600 nm data and maximum growth rate (Vmax), and analysed with the 'Gen5 Data Analysis Software' (BioTek).

We carried out the same experiment under nutrient limitation, using MM + YE as culture media. For this, we washed (two times) 16-hr old cultures of each strain (NC8 and NC8:pSIG308) and diluted cultures in MM + YE until an OD600 nm of 0.01. We prepared and incubated the 96-well plate in the same conditions we described above, using MM + YE as the blank. For each strain (NC8, NC8 + PLNC8IF and NC8:pSIG308), we obtained six growth curves in each culture media (MM + YE and MRS).

2.5 | Competition assays

2.5.1 | General protocol

We did competition assays to measure the fitness cost/benefits of producing bacteriocins, rather than estimating the metabolic costs/benefits directly. Before each competition experiment, we thawed frozen stocks (−80°C) of each strain used, inoculated in MRS broth and incubated at 30°C for 16 hr without agitation. Later, we diluted each culture separately in MRS to reach an OD600 nm of 0.01 (ca. 1×10^7 CFU/ml) in order to ensure similar numbers of bacteria per millilitre. Finally we mixed the competing strains (at a proportion of 50%) to reach a final titre of ca. 1×10^5 CFU/ml and incubated a 30°C for 24 hr without agitation. We determined the CFU/ml for each strain in the mix before (initial point at 0-hr) and after 24-hr incubation at 30°C (final point). To calculate CFU/ml, we diluted the cultures in saline (NaCl 0.85% w/v) and spread appropriate dilutions on MRS-BPB, MRS-Rif or MRS-Ery agar plates. Bacteriocin activity in mixed cultures was quantified as described above.

2.5.2 | Costs/Benefits of bacteriocin production triggered by PLNC8IF

We measured the relative fitness (w) of NC8 (Bac^C) in competition with 128/2-Rif (NI/S), with and without the addition of the autoinducer peptide PLNC8IF. We distributed aliquots of 990 µl from mixed cultures into 1-ml tubes and then we added 10 µl of PLNC8IF to reach final concentrations of 0.003, 0.03, 0.3 and 3 millimolar (mM). In control experiments (mixed cultures without PLNC8IF), we added 10 µl of MRS. Each treatment was repeated between 8 and 11 times, thus obtaining a total of 46 independent replicates, distributed as follows: control experiments (n = 11); treatment with PLNC8IF: 0.003 mM (n = 8), 0.03 mM (n = 11), 0.3 mM (n = 8), 3 mM (n = 8).

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

2.5.3 | Costs/Benefits of bacteriocin production triggered by inducer strains

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

2.5.4 | Density and fitness

We tested whether the relative fitness benefit of producing bacteriocins depends upon cell density, by conducting competition experiments between NC8 (Bac^C) and 128/2-Rif (NI/S) across a range of cell densities (10^2, 10^4, 10^6 and 10^8 CFU/ml). We induced bacteriocin production artificially, with the addition of PLNC8IF (0.3 mM final concentration), so that bacteriocins would produce even at low densities (Darch, West, Winzer, & Diggle, 2012). We mixed the competing strains at equal starting frequencies (50% each) and serially diluted to obtain mixed cultures at different starting cell densities (10^2, 10^4, 10^6 and 10^2 CFU/ml). After incubation at 30°C for 4 hr, we calculated the growth of NC8 to that of the competitor strain by determining the CFU/ml for each strain at 0 hr (initial) and after 4-hr incubation (final). CFSs were quantified for bacteriocin activity as described above. We repeated each treatment five times, obtaining four-five independent replicates of each cell density as follows: 10^2 (n = 4), 10^4(n = 5), 10^6 (n = 5) and 10^8 (n = 5).

2.6 | Statistical analyses

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

3 | RESULTS

3.1 | Inducing Bacteriocin production

Bacteriocin production was increased by both the constitutive expression of PLNC8IF by the plasmid pSIG308 (NC8:pSIG308,

constitutive producer) and the addition of cells of an inducing strain, *L. lactis* MG1363-Rif (I/R) (Figure 2a; $F_{1,7}$ = 394.3, p < .01). We were also able to induce bacteriocin production by the addition of synthetic peptide PLNC8IF, with larger amounts of peptide leading to greater bacteriocin production (ANOVA: $F_{1,13}$ = 222.3, p < .001, r^2 = 0.942; Figure 2b).

3.2 | Cost of producing bacteriocins

We upregulated bacteriocin production in two ways, by the addition of synthetic PLNC8IF and by the constitutively expressed plasmid pSIG308. In a minimal medium supplemented with yeast extract (MM + YE), we found that upregulating bacteriocin production led to reduced growth in the exponential phase of growth (Figure 3a), with a decreased maximum growth rate (Vmax) (ANOVA: $F_{1,16}$ = 13.77, p = .002, R^2 = 0.463; Figure 3b) and a lower final cell density ($F_{1,16}$ = 8.18, p = .012; no significant difference in growth between the PLNC8IF and pSIG308 manipulations: $F_{1,10}$ = 1.70, p = .22) (Figure 3a,c).

In a nutrient-rich media, de Man, Rogosa and Sharpe (MRS), we found that upregulating bacteriocin production led to a reduced growth in the exponential phase of growth (Figure 3d), with a decreased Vmax with the addition of PLNC8IF (NC8 + PLNC8IF; ANOVA: $F_{1,10}$ = 17.45, p = .002) but not constitutive expression of PLNC8IF (NC8:pSIG308; ANOVA: $F_{1,10}$ = 0.30, p = .59) (Figure 3e). There was no influence on final cell density (ANOVA: $F_{1,16}$ = 0.27, p = .61; no significant difference in growth between the PLNC8IF and pSIG308 manipulations: $F_{1,10}$ = 1.05, p = .33) (Figure 3d,f).

3.3 | Fitness benefit of producing bacteriocins

We tested whether bacteriocin production conferred a benefit to the producing strain, when competing with *L. pentosus* 128/2-Rif (NI/S) a strain which is sensitive to bacteriocins produced by NC8. We upregulated bacteriocin production in two ways: (a) both the constitutive expression of PLNC8IF by the plasmid pSIG308 (constitutive producer); and (b) the addition of synthetic PLNC8IF.

In both cases, we found that increased bacteriocin provided a fitness benefit when in competition with *L. pentosus* 128/2-Rif (NI/S). The constitutive producer (NC8:pSIG308; Bac$^+$) produced more bacteriocin per cell (Figure 4a; ANOVA: $F_{1,26}$ = 119.95, p < .001) and had a higher relative fitness than the wild-type NC8 (Bac^C) strain (Figure 4b; ANOVA: $F_{1,32}$ = 52.36, p < .001), which only produces bacteriocins conditionally. The addition of synthetic PLNC8IF also led to increased bacteriocin production per cell (Figure 4c; ANOVA: $F_{1,44}$ = 688.95, p < .001; R^2 = 0.940) and that increased bacteriocin production led to an increase in fitness until a maximum after which the fitness benefits level off (Figure 4b; ANOVA: $F_{2,43}$ = 174.70, p < .001; R^2 = 0.890).

3.4 | Fitness benefit and cost of activating bacteriocin production after cell-to-cell contact with inducer strains

We also tested the relative benefit of QS-controlled bacteriocin production by competing our conditional bacteriocin producing strain (NC8) against two strains which both induce bacteriocin production, but where one strain is sensitive to the bacteriocins produced by NC8 (FBB63-Rif; I/S), and the other strain is resistant (MG1363-Rif; I/R). Consistent with a net benefit of bacteriocin production originating from the killing competitors, we found that our conditional bacteriocin producing strain (NC8) had a higher fitness than the sensitive strain (FBB63-Rif; t_6 = 58.10, p < .001) (Figure 5). In contrast, the fitness of the conditional bacteriocin producing strain (NC8) was slightly lower than the resistant strain (MG1363-Rif; t_6 = −2.69,

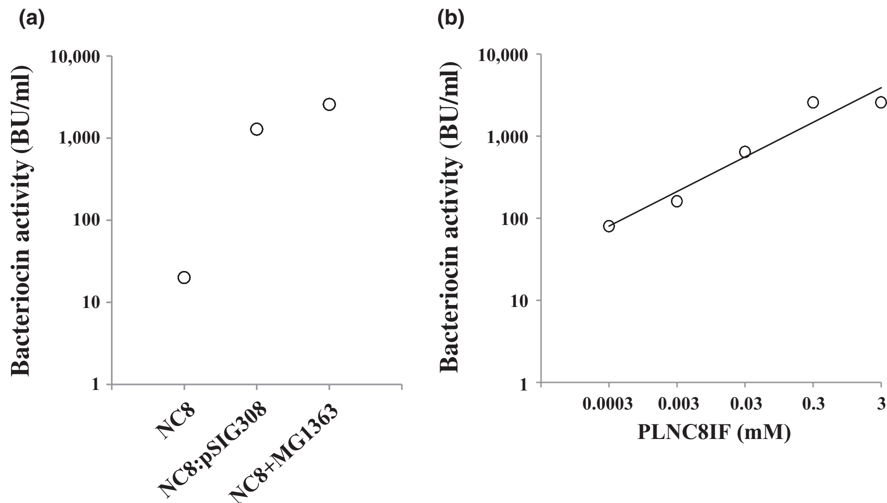

FIGURE 2 Validating strains. (a) Bacteriocin activity of the conditional bacteriocin producer *L. plantarum* NC8 (Bac^C) when grown as single culture (NC8) or in co-culture with the inducer bacteria *L. lactis* MG1363-Rif (I/R) (NC8 + MG1363), and its derivative strain NC8:pSIG308 (Bac$^+$) that constitutively produces the autoinducer peptide (PLNC8IF). Both the co-culture with *L. lactis* and the production of PLNC8IF driven by plasmid pSIG308 led to increased bacteriocin production. (b) The addition of larger amounts of synthetic PLNC8IF peptide leads to greater bacteriocin production in *L. plantarum* NC8. Error bars were not displayed because they are too small to be seen

$p = .036$) (Figure 5). Although the fitness difference versus the resistant strain is small, and there are other genetic differences between the lines, this result is consistent with a cost of bacteriocin production, in a situation where there is no benefit.

3.5 | Density assays

We then tested whether the benefits of bacteriocin production were greater at higher cell densities. We added synthetic PLNC8IF to activate bacteriocin production in the conditional producing strain (NC8; Bac^C), when grown in competition with a sensitive noninducing strain (128/2-Rif; NI/S), at a range of different starting densities. At higher starting densities, the relative fitness of the bacteriocin producing PLNC8IF strain was greater (ANOVA: $F_{1,17} = 79.51$, $p < .001$, $R^2 = 0.824$; Figure 6b). Although we found that bacteriocin production was induced by the addition of synthetic PLNC8IF, this result could be confounded by cell density, which also positively correlates with bacteriocin production (ANOVA: $F_{1,18} = 164.92$, $p < .001$, $R^2 = 0.902$; Figure 6a). Although in a multiple regression, the relative fitness of the bacteriocin producing strain was greater both when they produced more bacteriocins ($F_{1,16} = 17.64$, $p = .001$) and at higher cell densities ($F_{1,16} = 5.59$, $p = .031$).

4 | DISCUSSION

We examined the social cost and benefit of *quorum* sensing (QS)-controlled bacteriocin production in the bacteria *L. plantarum* NC8.

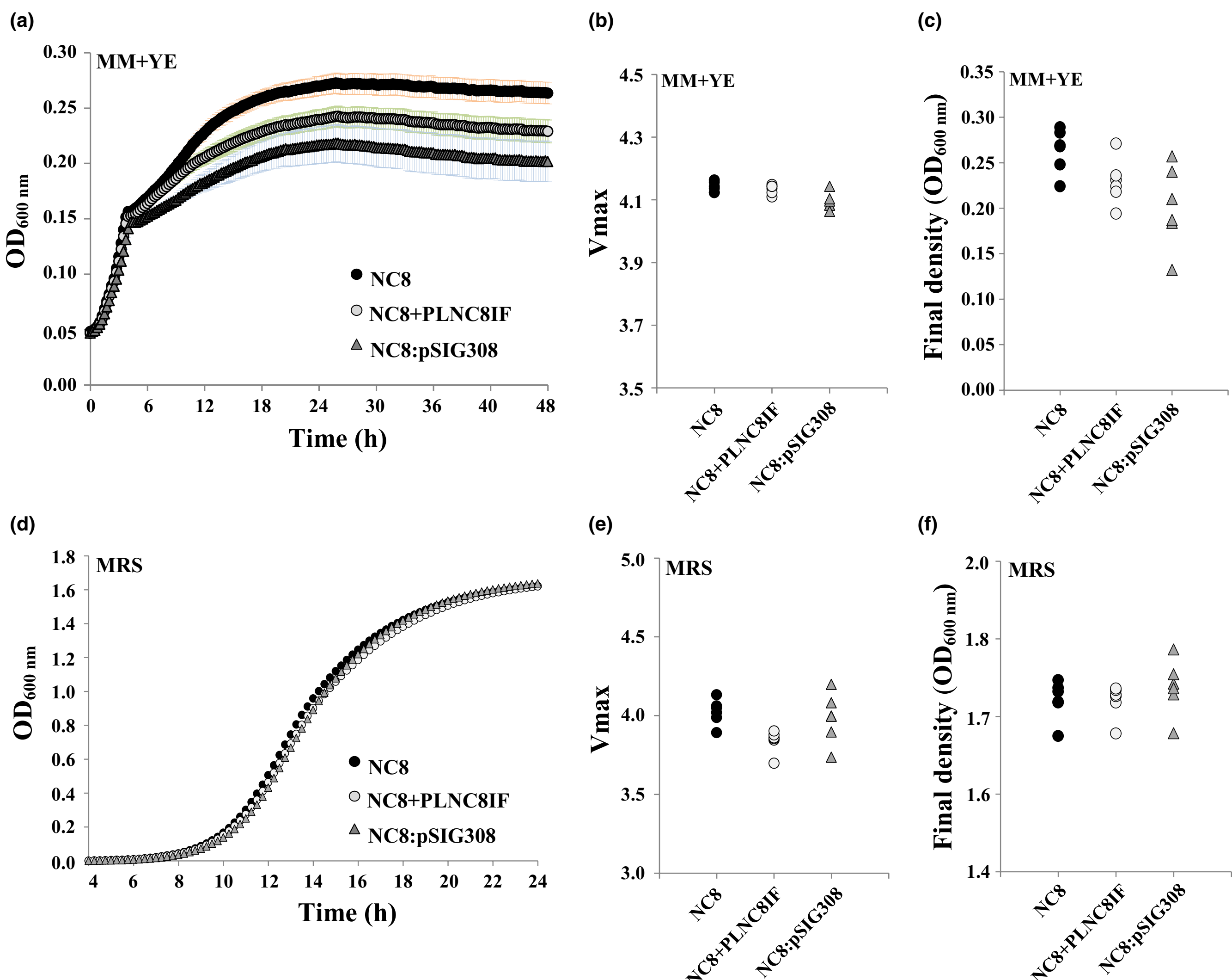

FIGURE 3 Bacteriocin production is costly. Growth curves, maximum growth rates (Vmax) and final cell densities in nutrient poor (MM + YE) media (a, b and c) and nutrient-rich (MRS) media (d, e and f). Data are shown for the conditional bacteriocin producer strain producing negligible bacteriocins (*L. plantarum* NC8; Bac^C), and two strains producing appreciable bacteriocins: NC8 with added PLNC8IF autoinducer (NC8 + PLNC8IF; Bac^+) and the constitutive PLNC8IF producer (NC8:pSIG308; Bac^+). OD_{600nm} measurements are means for six replicates. The addition of PLNC8IF peptide and constitutive PLNC8IF production both led to reduced growth in nutrient poor media, but not in nutrient-rich media, although there was a trend to decreased maximum growth rate at the exponential phase of growth in both culture media. Error bars, which represent standard errors, are not displayed in Figure 3d because they are too small to be seen

We found that: (a) bacteriocin production could be induced by the addition of a synthetic PLNC8IF peptide or by a plasmid which constitutively encodes for the production of this peptide (Figure 2); (b) bacteriocin production is costly, leading to reduced growth when grown in poor and, to a lesser extent, in rich media (Figure 3), and when in competition with resistant bacteria (Figure 5); (c) bacteriocin production provides a fitness advantage, when grown in competition with sensitive strains (Figures 4–6); and (d) the fitness benefits provided by bacteriocin production are greater at higher cell densities (Figure 6).

4.1 | Quorum sensing and the benefits of bacteriocin production

It is frequently assumed that bacteria use quorum sensing to conditionally switch on certain behaviours when they are relatively more beneficial. In particular, it is thought that quorum sensing is used to turn on cooperative behaviours at high densities, when they would provide a greater benefit (Brown & Johnstone, 2001; West, Winzer, Gardner, & Diggle, 2012). However, there are few studies that have tested this assumption (Darch et al., 2012; Koschwanez, Foster, & Murray, 2013). We have shown here, consistent with quorum sensing theory, that quorum sensing-controlled bacteriocin production provides a greater benefit at higher cell densities (Figure 6).

The benefits of producing QS-regulated bacteriocins could be greater at high densities because: (a) greater cell-to-cell contact with inducer bacteria can increase bacteriocin effectivity; (b) bacteriocins could reach sensitive strains at a greater rate; (c) the benefits of killing competitors, via reduced resource competition, could be greater; and (d) the benefits of producing bacteriocins and killing the sensitive strain, freeing-up resources, could be more likely to be shared with clone mates. A potential problem with our density manipulation is that it also altered bacteriocin production (Figure 6a). Although our results suggest that both bacteriocin production and density have an influence, it would be useful to develop a density manipulation that did not have any other potentially confounded influences. In addition, it is also important to note that initial frequency of bacteriocin producer relative to the sensitive strain can influence

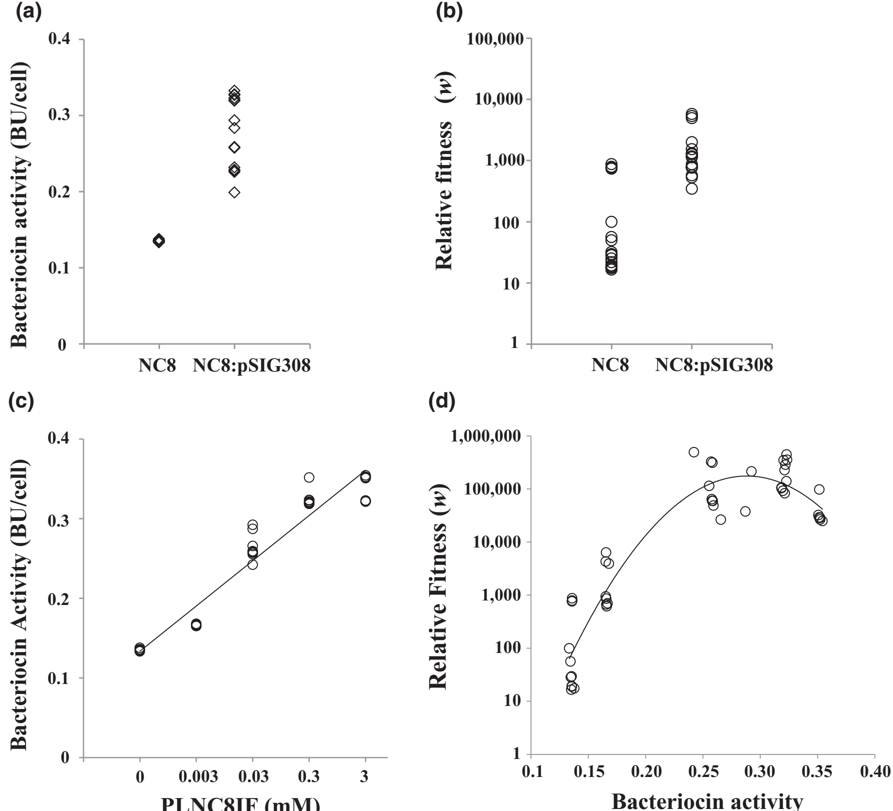

FIGURE 4 Benefit of bacteriocin production. Bacteriocin activity (a) and relative fitness (b) of the conditional bacteriocin producer (NC8; Bac^{C}) and the constitutive PLNC8IF producer (NC8:pSIG308; Bac^{+}) when in competition with *L. pentosus* 128/2-Rif (sensitive strain; NI/S). The constitutive producer both produced more bacteriocins and had a higher relative fitness, when compared with the conditional producer. Bacteriocin activity (c) and relative fitness (d) of the conditional bacteriocin producer *L. plantarum* NC8 (Bac^{C}), when in competition with *L. pentosus* 128/2-Rif (sensitive strain; NI/S) after the initial addition of increasing concentrations of the autoinducer peptide PLNC8IF. The addition of PLNC8IF induced bacteriocin production in NC8, increasing its fitness against the sensitive strain 128/2-Rif, although this increase in fitness approached an asymptote or even peaked. Relative fitness (*w*) was estimated relative to the sensitive strain and plotted on a log scale. Bacteriocin activity was expressed as bacteriocin units per cell (BU/cell). mM, millimolar. The curves are regression lines fitted to the raw data

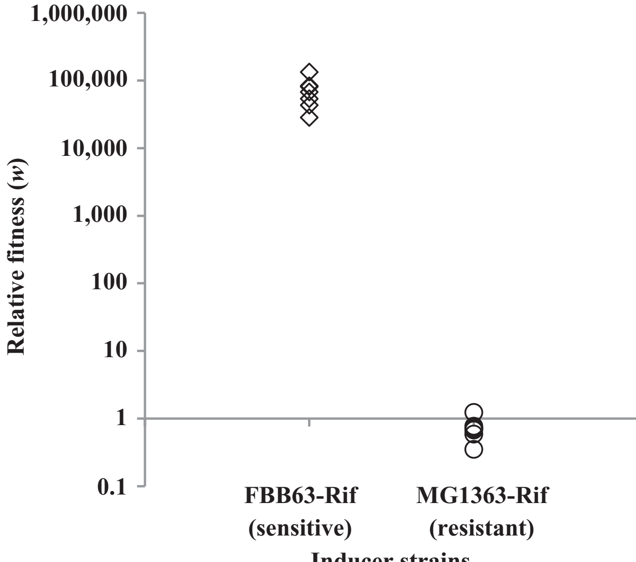

FIGURE 5 The cost and benefit of naturally induced bacteriocin production. The relative fitness of the conditional bacteriocin producing strain NC8 (Bac^C) is shown when grown with inducing strains that are (a) sensitive (FBB63-Rif; I/S) and (b) resistant (MG1363-Rif; I/R) to the bacteriocins produced by NC8. Relative fitness (*w*) was plotted on log scale

the relative benefit of producing bacteriocins (Gardner et al., 2004; Inglis et al., 2009). Our experiments were carried out at intermediate frequencies (each strain at 50%), where bacteriocins are expected to confer the greatest fitness advantage from killing competitors, by reducing competition for both the bacteriocin producing cells and their relatives (Gardner et al., 2004; Inglis et al., 2009).

4.2 | The cost of producing bacteriocins

We found that producing bacteriocins reduced growth rate, in both poor and rich media, and reduced final cell density in poor, but not rich media (Figure 3). This is consistent with previous results finding that the cost of traits is reduced or eliminated when the resources for producing them are not limited (Sexton & Schuster, 2017; Xavier, Kim, & Foster, 2011). When growing alone either in poor or in rich medium, NC8 produce little or no bacteriocins, thus avoiding the cost of producing bacteriocins when not required (no benefit of killing competitors). In addition, we found that in rich medium, the relative fitness of NC8 depends on their social environment, which is determined by the resistance/sensitivity of the social partner (competing strain) to bacteriocins produced by NC8.

4.3 | Conditional bacteriocin production

In NC8, conditional bacteriocin production could be seen as a mechanism directed to save metabolic costs when there is less benefit to their production. In the absence of competitors, the QS mechanism is not active which avoids costly bacteriocin production when there is little benefit. In contrast, when a competing strain is sufficiently common, such that it would be beneficial to eliminate them with bacteriocins, and there are sufficient NC8 to produce those bacteriocins, their production is upregulated (Figure 1a). In nature, there could be selection to detect 'sensitive' competitors or to avoid being detected—although there is no evidence yet for this, it could help explain the existence of noninducer strains that are resistant to bacteriocins. Mechanistically, the co-culture with certain specific strains (inducer bacteria) belonging to different species led to the activation of the QS system in NC8, activating production of the autoinducer PLNC8IF and therefore production of bacteriocins (Figure 1b) (Maldonado, Jiménez-Díaz, et al., 2004; Maldonado, Ruiz-Barba, et al., 2004). Thus, in NC8, bacteriocin production depends on first instance on the identity of the interacting partner (Maldonado, Jiménez-Díaz, et al., 2004), and second, on the ability to reach a QS driven by the autoinducer peptide PLNC8IF (Figure 1a) (Maldonado, Ruiz-Barba, et al., 2004; Maldonado-Barragán et al., 2009). This

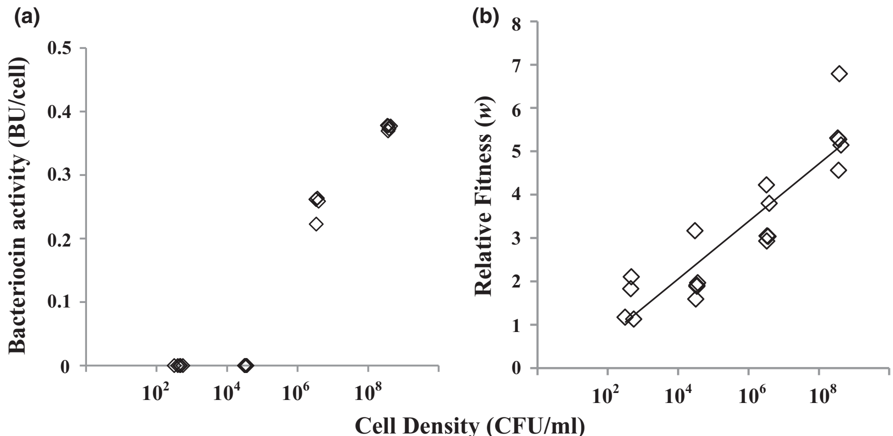

FIGURE 6 Density-dependent fitness benefits of responding to QS. Bacteriocin activity (a) and relative fitness (b) of the conditional bacteriocin producer *L. plantarum* NC8 (Bac^C), when in competition with the sensitive *L. pentosus* 128/2-Rif strain (NI/S) at different starting cell densities (from 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 whether the conditional producer NC8 increases (w > 1), decreases (w < 1) or remains at the same frequency (w = 1)

implies that quorum sensing information is used not only for signalling among self-cells, but also integrated with the detection of specific cues produced by other genotypes (for detecting evolutionary competition; Cornforth and Foster (2013).

5 | CONCLUSIONS

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

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