Coculture with Specific Bacteria Enhances Survival of *Lactobacillus plantarum* NC8, an Autoinducer-Regulated Bacteriocin Producer, in Olive Fermentations.

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Abstract

Bacteriocin production in *Lactobacillus plantarum* NC8 is activated by coculture with specific bacteriocin production-inducing bacterial strains. The system is further regulated by a three-component regulatory system involving a specific autoinducer peptide (PLNC8IF).We have used *L. plantarum* NC8 as a starter culture in Spanish-style green olive fermentations and examined the influence of coculturing in its survival. We found that *L. plantarum* NC8 greatly enhanced its growth and survival in the olive fermentations when coinoculated with two specific bacteriocin-production inducing strains, i.e. *Enterococcus faecium* 6T1a-20 and *Pediococcus pentosaceus* FBB63, when compared to singly-inoculated fermentations. In addition, a constitutive bacteriocin-producer NC8-derivative strain was used as a control in the olive fermentations and showed also better viability than the parental NC8 strain. Our results suggest the involvement of bacteriocin production in the viability enhancement found in both cases. We postulate that the presence of specific bacteria is recognized by *L. plantarum* NC8 as an environmental stimulus to switch a specific adaptive response on, most probably involving bacteriocin production. The design of novel bacteriocin-producing starter cultures for food fermentations should consider their constitutive *versus* regulated character.

1. Introduction

Bacteriocins are proteinaceous compounds produced by a wide range of bacteria exhibiting antimicrobial activity against a select range of other bacteria. Bacteriocin production by strains used as starter cultures for food fermentations has been largely proposed as one of their most desirable traits (Buckenhüskes, 1993; De Vuyst and Vandamme, 1994; Ray and Daeschel, 1994). This is most probably due to the enhanced competitive fitness which bacteriocin production confers to the producer strains (Dykes and Hastings, 1997; Riley, 1998). Many of the known bacteriocins are produced by food-grade lactic acid bacteria (LAB), offering the possibility of manipulating food microbial ecosystems in a deliberate fashion (Cotter et al., 2005). In previous reports, we showed that bacteriocin production helped *Lactobacillus plantarum* LPCO10 to survive and predominate in olive fermentations (Ruiz-Barba et al., 1994; Leal et al., 1998). Although bacteriocin production by the LPCO10 strain is constitutive, this is not the case in other LAB. Actually, bacteriocin production in many LAB is controlled by specific peptides called autoinducers (AIPs) *via* a three-component regulatory system which involves, in addition to the AIP, a histidine protein kinase and a response regulator, as part of a quorum sensing mechanism (Nes and Eijsink, 1999). We found that bacteriocin production in *L. plantarum* NC8 is activated by coculture with specific bacteriocin-production-inducing bacterial strains (Maldonado et al., 2003 and 2004a) and that this production is further regulated by a three-component regulatory system involving a specific autoinducer peptide, named PLNC8IF (Maldonado et al., 2004b). We have also shown that this regulatory system is working in liquid as well as in solid media, where bacteriocin production is apparently constitutive but, in fact, it is still regulated *via* quorum sensing (Maldonado et al., 2009).

The aim of this work was to find out whether we could enhance *L. plantarum* NC8 survival rate in olive fermentations by coculturing with bacteriocin production-inducing strains, therefore suggesting a role for bacteriocin production itself. For this, we inoculated the NC8 strain in combination with either of two different bacterial strains, i.e. *Enterococcus faecium* 6T1a-20 and *Pediococcus pentosaceus* FBB63, which had been found to specifically induce bacteriocin production in NC8 (Maldonado et al., 2004a). As a control, we used the NC8-derivative strain *L. plantarum* NC8(pSIG308). This strain is able to constitutively produce the specific autoinducer molecule PLNC8IF so that bacteriocins are also constitutively produced (Maldonado et al., 2004b). As we reasoned that salt could interfere with the regulatory mechanism for bacteriocin production in NC8 and somehow mask the results, we also set up salt-free fermentations in parallel. Viability of *L. plantarum* NC8 and its derivative strain in every fermentation condition was examined for *ca.* three months, which is the time Spanish-style green olive fermentation takes to be considered as completed under standard conditions (Garrido-Fernández et al., 1995).

2. Materials and Methods

2.1. Bacterial strains, culture media and growth conditions

L. plantarum NC8, kindly provided by Lars Axelsson (MATFORSK, Norwegian Food Research Institute, Osloveien, Norway), has been previously described to produce up to three inducible two-peptide bacteriocins named PLNC8αβ, PLNEF and PLNJK in response to cocultivation with specific Gram-positive bacteria (Maldonado et al., 2004a and 2004b). It was propagated in MRS broth (Oxoid, Basingstoke, Hampshire, England) at 30ºC without shaking. *L. plantarum* NC8(pSIG308) harbours a recombinant plasmid which renders

91 constitutive PLNC8IF production and, therefore, constitutive bacteriocin production
92 (Maldonado et al., 2004b). It was propagated at 30ºC without shaking in MRS containing
93 erythromycin (10 μg/ml). *E. faecium* 6T1a-20 (Rif^R) is a non-bacteriocin-producing mutant
94 derived from the enterocin I-producer *E. faecium* 6T1a, which was isolated from an olive
95 fermentation (Floriano et al., 1998). This strain induces bacteriocin production in NC8 by
96 cocultivation and it is resistant to NC8 bacteriocins (Maldonado et al., 2004a). It was
97 propagated at 30ºC without shaking in MRS plus rifampin (60 μg/ml). *P. pentosaceus* FBB63
98 (Rif^R) induces bacteriocin production in NC8 by cocultivation, and it is sensitive to NC8
99 bacteriocins (Maldonado et al., 2004a). It was propagated at 30ºC in MRS plus rifampin (60
100 μg/ml). Antibiotics were purchased from Fluka Chemie GmbH, Buchs, Switzerland. Prior to
101 use in the corresponding olive fermentations, all these bacterial strains were adapted to salt by
102 cultivating them twice in MRS broth containing 4% (w/v) NaCl. *L. pentosus* 128/2 was
103 propagated in MRS at 30 ºC without shaking and was used as the sensitive strain in
104 bacteriocin assays. This strain has been previously described as belonging to the *L. plantarum*
105 species, but it has been recently identified in our laboratory as *L. pentosus* according to the
106 molecular methods and criteria of Torriani et al. (2001).
107
108 *2.2. Olive fermentation set-up*
109
110 The traditional Spanish-style green olive brining procedure was followed (Garrido-
111 Fernández et al., 1995). Briefly, Hojiblanca var. green olives were aliquoted in 4.5-kg
112 portions and introduced in 8-liter polyethylene jars which were used as fermentors. A total of
113 20 fermentors were set up in this way. Olives were treated with 1.97% (w/v) NaOH for 6 h at
114 room temperature (*ca*. 20ºC), washed twice with tap water for 6 and 18 h consecutively, and
115 finally covered with *ca*. 3 liters of brine (11% NaCl, w/v; standard fermentations) or tap water

(salt-free fermentations). After 8 h, fermentors were inoculated in duplicates with the corresponding single or combined bacterial strains so that final concentration was *ca*. 10^6 CFU/ml of each strain. Bacterial strain combinations in the fermentors were as follows: i) *L. plantarum* NC8, single culture; ii) *L. plantarum* NC8 plus *E. faecium* 6T1a-20 (Rif^R); iii) *L. plantarum* NC8 plus *P. pentosaceus* FBB63 (Rif^R); iv) *L. plantarum* NC8(pSIG308), single culture; v) uninoculated control fermentations. Fermentors were left at room temperature and samples were taken twice a week and then weekly for the first 35 days of fermentation, and less frequently as fermentation progressed and stabilised (see Fig. 1). Samples were examined for microbial, physical and chemical progress for almost three months.

2.3. Microbiological, physical and chemical analyses

For the microbiological analysis, samples were serially diluted in sterile 0.1% peptone water and plated onto the different selective media using a WASP2 Spiral Plater (Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK). *L. plantarum* NC8 was enumerated in MRS-azide (0.02 % [w/v] sodium azide) at 30ºC. For this, all of the isolated colonies from the MRS-azide agar plates containing from 30 to 200 isolates, were previously numerated. Subsequently, ten of them were selected from each appropriate plate according to the ten first numbers appearing when running the List Randomizer programme at the True Random Number Service web page (www.random.org/lists/) and subjected to identification. This was finally carried out by colony and microscopic appearance, bacteriocin production and molecular identification as described below. *L. plantarum* NC8(pSIG308) was enumerated in MRS-azide containing erythromycin, at 30ºC. *E. faecium* 6T1a-20 was enumerated at 42ºC in Slanetz & Bartley medium (Oxoid) containing rifampin. *P. pentosaceus* FBB63 was enumerated in MRS-azide containing rifampin, at 30ºC. Titratable acidity, combined acidity

and pH were measured using a Metrohm 670 Titroprocessor (Herisau, Switzerland). Values of the acidity parameters were expressed as % (w/v) lactic acid. Salt concentration was determined by titration with $AgNO_3$ and expressed as % (w/v) NaCl.

2.4. Bacteriocin assays

Bacteriocin production of selected *L. plantarum*-like colonies isolated in MRS-azide at each sampling point was tested by overlaying these colonies with 4-ml MRS soft agar inoculated with *ca*. 10^5 CFU/ml *L. pentosus* 128/2, which was used as the indicator strain for plantaricin NC8. Bacteriocin activity of olive fermentation supernatants was assayed by the spot-on-lawn method, using *L. pentosus* 128/2 as the indicator strain.

2.5. Molecular identification of bacterial strains and species

Total DNA from selected colonies was extracted by the small-scale fast chloroform method as previously described (Ruiz-Barba et al., 2005). Primers used in PCR were synthesised by MWG Biotech (Ebersberg, Germany). *Taq* DNA polymerase (Promega, Madison, Wi) and a Gene Amp PCR System 2400 Thermal cycler (Perkin Elmer Co., Norwalk, Conn.) were used for the PCR amplifications. Identification of *L. plantarum* NC8 colonies was carried out by checking the presence of plantaricin NC8 structural genes, i.e. *plNC8A* and *plNC8B*. For this, PCR analysis was performed as previously described using the primer pair NC8-7/NC8-10, which amplifies a 217-bp chromosomal DNA fragment from *L. plantarum* NC8 (Maldonado et al., 2004b). Identification of colonies belonging to the *L. plantarum*, *L. pentosus,* and *Lactobacillus paraplantarum* species was carried out using the PCR method and criteria described by Torriani et al. (2001).

3. Results

3.1. Growth and survival of L. plantarum NC8 are enhanced by cocultivation with bacteriocin production-inducing bacterial strains

Growth curves of *L. plantarum* NC8 in the different olive fermentations are shown in Fig. 1. In virtually all of the conditions, maximal growth and survival rate of *L. plantarum* NC8 along the fermentation time were obtained when this strain was coinoculated with either *E. faecium* 6T1a-20 or *P. pentosaceus* FBB63 (Fig. 1 A and B). Actually, in the only case that *L. plantarum* NC8 performed in a similar way as in the singly inoculated fermentations (Fig. 1 A, NC8+PP-1), growth of the bacteriocin inducing strain (*P. pentosaceus* FBB63) was also very poor (Fig. 2 A, PP-1). Growth of *L. plantarum* NC8 was also influenced by the salt content of the fermentation medium, so that this strain performed better and more consistently in the salt-free fermentations. Cocultures also benefited from the lack of salt, so that *L. plantarum* NC8 could extent its presence for more than three months in these fermentations.

The constitutive bacteriocin-producing strain *L. plantarum* NC8(pSIG308) clearly performed better than NC8 both in the standard olive fermentations (Fig. 1 A) as well as in the salt-free fermentations (Fig. 1 B). However, growth curves of cocultivated NC8 strain showed higher population numbers than those shown by the singly-inoculated *L. plantarum* NC8(pSIG308) in virtually all cases (Fig. 1 A and B).

Along with the inoculated strains, spontaneous lactic acid bacteria (mainly lactobacilli and pediococci) grew during the fermentation (data not shown). This spontaneous microflora took over the inoculated *L. plantarum* NC8 when this strain lowered its numbers and/or disappeared from the fermentations. Finally, a strong decrease in the inoculants viability

could be observed in all the standard olive fermentations (Fig. 1A). This has been observed before and it is considered as part of an adaptation period which usually extends for the first week of fermentation (Ruiz-Barba et al., 1994; Garrido-Fernández et al., 1995). Very high initial pH values (Fig. S1) together with the high salt concentration of the brines (still not equilibrated) posse a handicap to the development of the inoculated strains compared to the salt-free fermentations.

3.2. E. faecium 6T1a-20 and P. pentosaceus FBB63, used to induce bacteriocin production by L. plantarum NC8, were able to grow in the olive fermentations, but to a limited extent

Both bacterial strains used to induce bacteriocin production by *L. plantarum* NC8 were able to grow in the olive fermentations (Fig. 2). However, none of them could be detected after the first three weeks of fermentation. Differences, though, were observed in the behaviour of each strain depending on the nature of the particular fermentation. *E. faecium* 6T1a-20 appeared to be more adapted to thrive in the olive brines, most probably reflecting its olive fermentation origin (Fig. 2). In contrast, *P. pentosaceus* FBB63 survived longer in salt-free olive fermentations, although results were not homogeneous (Fig. 2).

3.3. Physical and chemical parameters of the fermentations

Evolution of pH values was quite homogeneous inside each of the two groups of inoculated fermentations, i.e. standard and salt-free ones (see Fig. S1 A and C in the supplementary material). In contrast, pH drop in the standard fermentations was delayed by two to three weeks when compared to salt-free fermentations. Values around pH 4.0 were achieved in all of the fermentors after 3 months (Fig. S1 A and C). The standard olive

fermentations achieved an averaged pH value of 4.11, with a standard deviation (sd) of 0.054, while salt-free fermentations reached pH 4.19 in average, with a sd of 0.141. In the uninoculated control fermentations, the evolution of pH values was quite similar to those described above for the standard fermentations (Fig. S1 E). However, in the uninoculated salt-free fermentations pH drop was less marked and never achieved pH values below 5.3 (Fig. S1 E).

Titratable acidity, expressed as lactic acid, evolved quite similarly in the standard olive fermentations, achieving values ranging from 0.75 to 0.85% (sd 0.043)(Fig. S1 B). In contrast, in salt-free fermentations, titratable acidity evolved faster and achieved higher values, ranging from 0.95 to 1.10% (sd 0.117)(Fig. S1 D), except for one case (NC8+EF-2) which achieved a value of just 0.73% after 3 months (Fig. S1 D). Uninoculated standard control fermentations achieved values of titratable acidity quite similar to those of the inoculated ones, while the uninoculated salt-free fermentations rendered less than 50% of the averaged value obtained with the inoculated salt-free ones (Fig. S1 F).

In the standard fermentations, averaged NaCl concentration was 5.35% (w/v; sd 0.171) at equilibrium, which was reached into the first week of fermentation (not shown).

4. Discussion

Our results show that growth and survival of *L. plantarum* NC8 in olive fermentations is remarkably enhanced when it grows together with specific bacterial strains. These strains, *E. faecium* 6T1a-20 and *P. pentosaceus* FBB63, are two among those which had previously been described as having the ability to induce bacteriocin production by *L. plantarum* NC8 through coculturing (Maldonado et al., 2004a). This strongly suggests a link between both observations. A question immediately appears: whether this growth and viability

241 enhancement is actually due to bacteriocin production. The fact that *L. plantarum*
242 NC8(pSIG308), a constitutive bacteriocin producer, also displays an enhanced viability
243 profile in comparison to the parental NC8 strain indicates that bacteriocin production is most
244 probably involved. Bacteriocin activity, however, could not be detected in the fermentation
245 liquids (data not shown). This result is not surprising given the complexity of the olive
246 fermentation regarding both chemical composition and microflora. More specifically, apart
247 from other unspecific matrices, bacteriocins are being attached to the specific receptors in the
248 sensitive strains as they are produced along the fermentation, so that not many spare
249 bacteriocin molecules are available for further detection when sampling the fermentation
250 liquid. In a previous report, we showed that bacteriocin activity in olive fermentations
251 inoculated with the constitutive plantaricin S-producer *L. plantarum* LPCO10 was detected at
252 very low titre (200 BU/ml) only after concentration of the olive brines by 20 times (Ruiz-
253 Barba et al., 1994). Even so, we could demonstrate that bacteriocin production played a role
254 in LPCO10 predominance in the olive fermentations when compared to a non-bacteriocin-
255 producing mutant strain (Ruiz-Barba et al., 1994). Other authors could not provide any
256 evidence for bacteriocin activity in the food systems studied, but indeed attributed the
257 inhibitory effect observed to the presence of bacteriocin production in them (Schillinger et al.,
258 1991; Foegeding et al., 1992)
259
260 Although viability enhancement was observed in both cases, a significant difference
261 was noticed between cocultured *L. plantarum* NC8 and the singly inoculated, constitutive
262 bacteriocin-producing *L. plantarum* NC8(pSIG308) growth curves (Fig. 1 A and B). In all but
263 one case, coculture with the specific bacteriocin-production inducing strains allowed the
264 parental NC8 strain to achieve higher maximum population numbers and persistence in the
265 olive fermentations than its singly-inoculated derivative NC8(pSIG308). In the single case

266 when this was not the rule, it was shown that the inducing strain, i.e. *P. pentosaceus* FBB63,
267 actually performed very poorly also (Fig. 2A, PP-1). Most probably, this strain did not reach
268 enough numbers as to fully induce bacteriocin production in NC8. In the past, we had
269 observed that bacteriocin production by NC8 after coculture with the inducing strains was
270 always much higher in titre than that by the constitutive bacteriocin-producer NC8(pSIG308)
271 strain. For example, bacteriocin activity of a cell-free supernatant (CFS) of the coculture *L.*
272 *plantarum* NC8/*L. lactis* MG1363 was 1,280 BU/ml, while the CFS of singly inoculated *L.*
273 *plantarum* NC8(pSIG308) in the same experiment and using the same indicator strain was
274 640 BU/ml (Maldonado et al., 2004b). Although the actual reasons behind this difference in
275 bacteriocin production are currently unknown, this observation supports the hypothesis that
276 higher bacteriocin production by *L. plantarum* NC8 provides this strain with higher survival
277 rate in the olive fermentations. A question can be raised regarding the limited viability of the
278 inducing strains in the olive fermentations. In fact, none of them could be detected after the
279 first three weeks of fermentation (Fig. 2). However, our results are consistent with the
280 observation that heat-killed inducing cells retain their ability to induce bacteriocin production
281 when cocultured with *L. plantarum* NC8 (Maldonado et al., 2004a). Therefore, viable
282 inducing cells are not strictly necessary for the induction of bacteriocin production by *L.*
283 *plantarum* NC8. We have previously shown that cell-to-cell contact between *L. plantarum*
284 NC8 and the inducing bacteria is necessary for bacteriocin production (Maldonado et al.,
285 2004a). Therefore, the primary mechanism by which *L. plantarum* NC8 senses either its own
286 autoinducing molecule PLNC8IF or the presence of the inducing strains might be different
287 and thus differently affected by the fermentation conditions.
288
289 The best survival rates for *L. plantarum* NC8 were obtained in salt-free fermentations
290 instead of in the standard ones. This result could indicate that this strain is not well adapted to

291 standard olive fermentations, but also that salt could affect bacteriocin production by this
292 strain so that its competitiveness is reduced. Actually, it has been reported that salt can reduce
293 bacteriocin production in other AIP-regulated systems, most probably by negatively
294 influencing the binding of the corresponding AIP to its cognate receptor, i.e. its specific
295 histidine protein kinase. This is the case of Enterocins A and B, produced by *E. faecium*
296 CTC492 (Nilsen et al., 1998), and also Curvacin A, produced by *Lactobacillus curvatus*
297 LTH1174 (Verluyten et al., 2004). In contrast, bacteriocin production has been reported to be
298 enhanced by salt in some LAB which produce bacteriocins in a constitutive manner. This is
299 the case for plantaricin S, a bacteriocin constitutively produced by *L. plantarum* LPCO10
300 (Jiménez Díaz et al., 1993). This strain was actually isolated from an olive fermentation brine
301 and its maximum bacteriocin activity is achieved at NaCl concentrations *ca*. 2.5% (w/v) in the
302 culture medium (Leal Sánchez et al., 2002). Also, Uguen et al. (1999) communicated that
303 production of lacticin 481, a lantibiotic bacteriocin produced by *Lactococcus lactis* CNRZ481
304 isolated from raw milk (Piard et al., 1992), increased with NaCl concentrations ranging from
305 0.2 to 0.4 M (1.16 to 2.32 % [w/v]).
306
307 In conclusion, the use of mixed starter cultures involving selected bacteriocin-inducing
308 strains and coculture-induced bacteriocin-producing strains such as *L. plantarum* NC8
309 provides a mean to enhance the viability of the last in food fermentation systems. We
310 postulate that the presence of specific bacteria is recognized by strains such as *L. plantarum*
311 NC8 as an environmental stimulus to switch a specific adaptive response on, most probably
312 involving bacteriocin production. Finally, in our opinion, the design and application of
313 bacteriocin-producing starter cultures for food fermentations should take into account not
314 only a specific spectrum of activity but also the constitutive *versus* regulated nature of the
315 bacteriocins involved, how this regulation takes place (uncovering the molecular mechanisms

316 involved), and whether or not bacteriocin production significatively enhances the starters
317 performance in the specific fermentations where they are meant to be applied.
318
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329
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Legends to Figures

Figure 1. Growth of *Lactobacillus plantarum* NC8 in the standard (A) and salt-free (B) olive fermentations. Symbol keys: NC8-1 and NC8-2, growth of *L. plantarum* NC8 when inoculated as a pure culture; NC8+EF-1 and NC8+EF-2, growth of *L. plantarum* NC8 when coinoculated with *Enterococcus faecium* 6T1a-20; NC8+PP-1 and NC8+PP-2, growth of *L. plantarum* NC8 when coinoculated with *Pediococcus pentosaceus* FBB63; NC8/308-1 and NC8/308-2, growth of *L. plantarum* NC8(pSIG308) when inoculated as a pure culture.

Figure 2. Growth of the strains used as bacteriocin-production inducers which were coinoculated with *L. plantarum* NC8 in the standard (A) and salt-free (B) olive fermentations. Symbol keys: EF-1 and EF-2, growth of *E. faecium* 6T1a-20; PP-1 and PP-2, growth of *P. pentosaceus* FBB63.

Figure

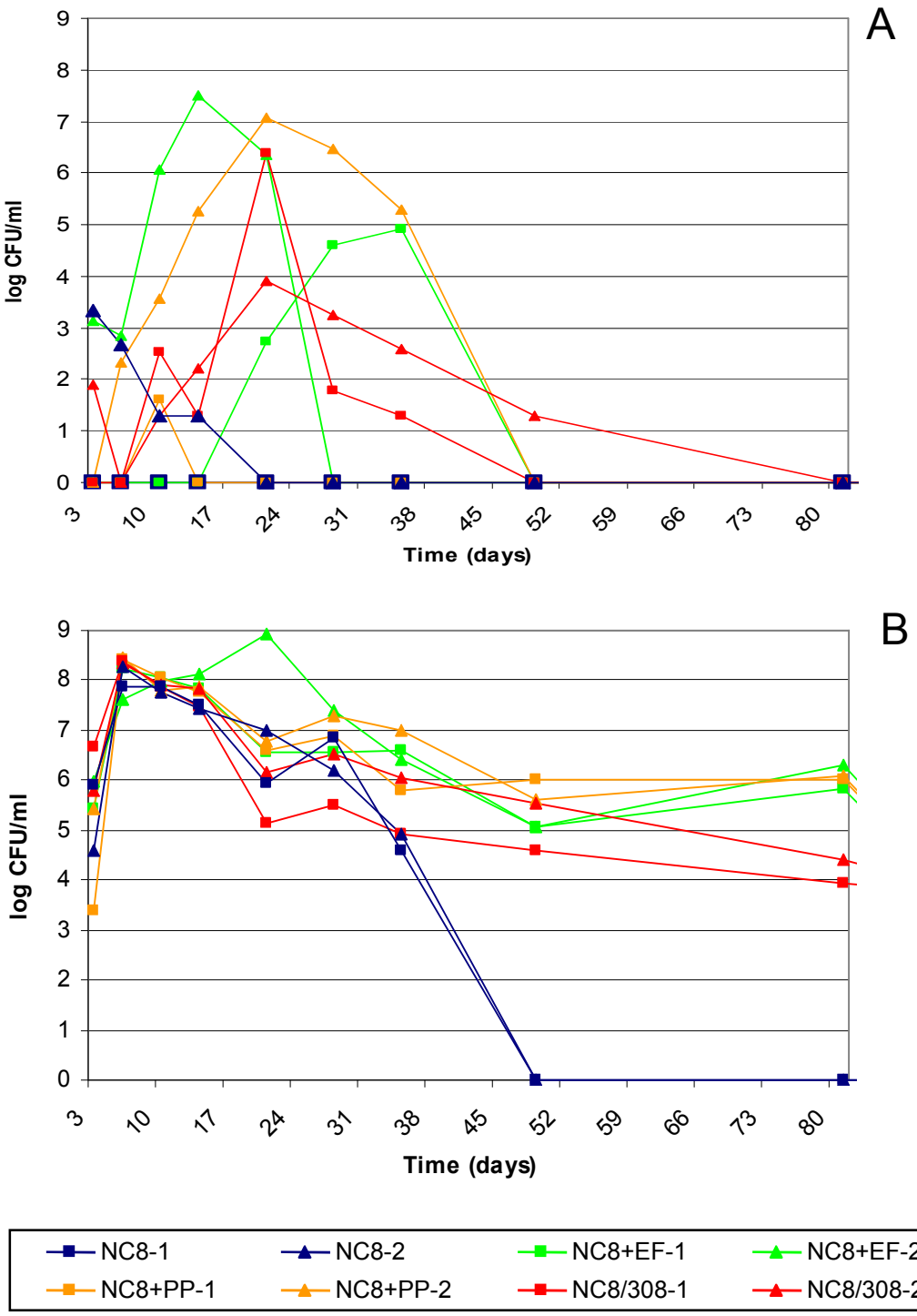

Figure 1, Ruiz-Barba, Caballero-Guerrero, Maldonado-Barragán, and Jiménez-Díaz

Figure

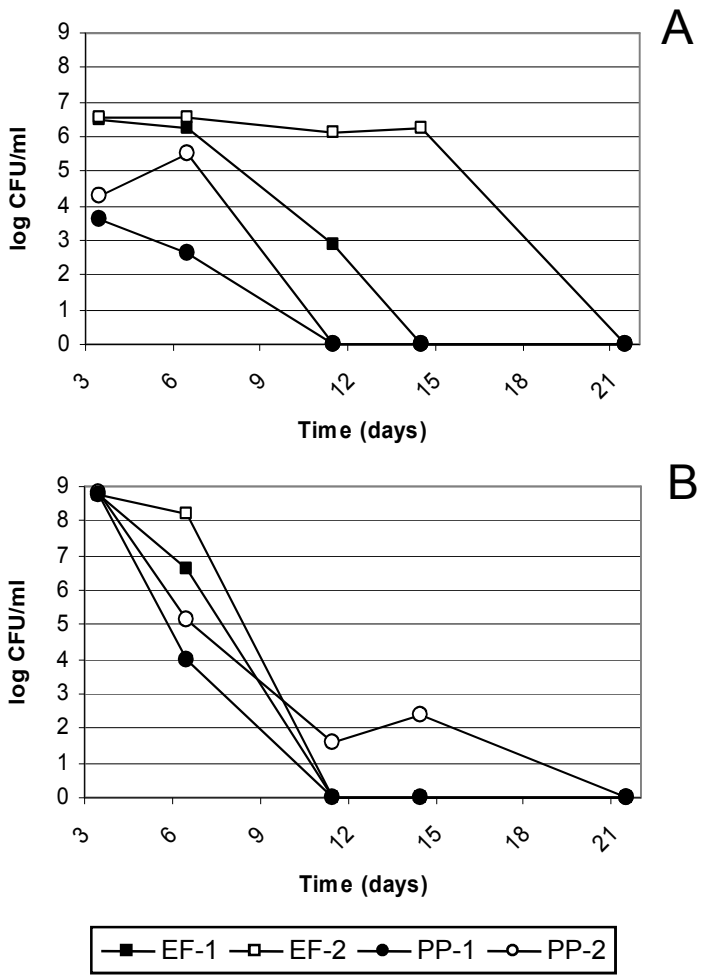

Figure 2, Ruiz-Barba, Caballero-Guerrero, Maldonado-Barragán, and Jiménez-Díaz

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