Tolerance development in *Listeria monocytogenes-Escherichia coli* dual-species biofilms after sublethal exposures to pronase-benzalkonium chloride combined treatments.

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Published in final form: Food Microbiology (2017) 67:58-66

DOI: 10.1016/j.fm.2017.06.002

Abstract

This study was designed to assess the effects that sublethal exposures to pronase (PRN) and benzalkonium chloride (BAC) combined treatments have on *Listeria monocytogenes-Escherichia coli* dual-species biofilms grown on stainless steel in terms of tolerance development (TD) to these compounds. Additionally, fluorescence microscopy was used to observe the changes of the biofilm structure. PRN-BAC exposure was carried out using three different approaches and TD was evaluated treating biofilms with a final 100 µg/ml PRN followed by 50 µg/ml BAC combined treatment. Results showed that exposure to PRN-BAC significantly decreased the number of adhered *L. monocytogenes* ($P < 0.05$), while *E. coli* counts remained generally unaltered. It was also demonstrated that the incorporation of recovery periods during sublethal exposures increased the tolerance of both species of the mixed biofilm to the final PRN-BAC treatment. Moreover, control biofilms became more resistant to PRN-BAC if longer incubation periods were used. Regardless of the treatment used, log reduction values were generally lower in *L. monocytogenes* compared to *E. coli*. Additionally, microscopy images showed an altered morphology produced by sublethal PRN-BAC in exposed *L. monocytogenes-E. coli* dual-species biofilms compared to control samples. Results also demonstrated that *L. monocytogenes-E. coli* dual-species biofilms are able to develop tolerance to PRN-BAC combined treatments depending on way they have been previously exposed. Moreover, they suggest that the generation of bacterial tolerance should be included as a parameter for sanitation procedures design.

Keywords

Benzalkonium chloride; Biofilm; Enzyme; *Escherichia coli;* Fluorescence microscopy; *Listeria monocytogenes*; Pronase; Tolerance

Highlights

- *L. monocytogenes-E. coli* biofilms tolerance development to PRN-BAC was assessed.
- Sublethal exposure to PRN-BAC plus recovery periods made biofilms more resistant.
- Longer incubation periods also decreased the efficacy of PRN-BAC treatments.

1. Introduction

Listeria monocytogenes and *Escherichia coli* are foodborne pathogens currently considered as an issue of major concern in food safety due to their incidence and morbidity and mortality rates among the population (EFSA, 2015). In food related environments, these pathogens are normally present adhered to surfaces forming part of complex multispecies biofilms (Rodríguez-López et al., 2015). These associations give rise to the establishment of inter-species interactions that can provoke an increased resistance to chemical disinfectants (Holah et al., 2002; Saá Ibusquiza et al., 2012a; van der Veen and Abee, 2011).

In biofilms, such resistance has been proved to be of a multifactorial nature, however, one of the main reasons is the presence of a robust extracellular polymeric matrix (Abdallah et al., 2014). This structure, constituted by proteins, polysaccharides and extracellular DNA (eDNA) (Flemming and Wingender, 2010), acts as a protective permeability barrier hindering antimicrobials diffusion (Abdallah et al., 2014) and therefore creating a bactericidal concentration gradient into the biofilm (Allison, 2003; Flemming et al., 2007). Hence, cells especially those located in the deepest layers of the biofilms, would be exposed to sublethal concentrations of antimicrobials that would provoke an environmental pressure on the biofilm population and the subsequent selection for bactericide-tolerant phenotypes (Abdallah et al., 2014).

Enzymatic biofilm matrix breakdown previous to disinfectant application is nowadays considered an environmentally friendly alternative for both pathogen control and reduction of the amount of chemicals used. An extensive review on the application of enzymes in the food industry has been recently published (Meireles et al., 2016).

In particular, hydrolytic enzymes have been proposed as efficient agents to remove biofilms (Augustin et al., 2004; Orgaz et al., 2007; Simões et al., 2010). Especially in mature biofilms, they can be useful to remove from the surface the matrix of biofilms and to facilitate penetration of cleaning and disinfection agents (Abdallah et al., 2014). Among them, proteolytic enzymes have been proved to be effective against biofilms formed by pathogenic bacteria present in food industry premises (Augustin et al., 2004; Molobela et al., 2010).

In this line of research, previous studies carried out in our laboratory have demonstrated that the combined application of pronase (PRN) with benzalkonium chloride (BAC) is able to remove dual species biofilms of *L. monocytogenes* formed on stainless steel (Rodríguez-López et al., 2017).

Tolerance development (TD) after sublethal exposure to BAC and other quaternary ammonium compounds (QACs) has extensively reported in planktonic cells of *L. monocytogenes* (Jiang et al., 2016; Romanova et al., 2006; Saá Ibusquiza et al., 2012b; To et al., 2002) and *E. coli* (Bore et al., 2007; Moen et al., 2012). However, a significantly smaller number of studies involving TD after BAC sublethal exposure in mixed-species biofilms is currently found in the literature and, to the best of authors' knowledge, no studies were found assessing the tolerance to enzyme-BAC combined strategies. The only evidence found to BAC tolerance development in mixed-species biofilms was the study performed by Machado, Lopes, Sousa, & Pereira (2012). They demonstrated that after five sublethal exposures to BAC carried out every 24 h, *Pseudomonas aeruginosa-E. coli* biofilms grown in polystyrene presented a higher biomass content compared with unexposed samples. Nevertheless, the number of adhered cells was similar in both series.

One of the main advantages of applying enzymes is that they do not pose a selective pressure on bacteria (Meireles et al., 2016). However, if combined with biocides such as BAC, it seems to be important to consider both agents for a correct TD evaluation.

The main aim of this work was to assess the TD to PRN-BAC treatments after sublethal exposure to these compounds in *L. monocytogenes-E. coli* dual-species biofilms grown on SS coupons. The exposures were carried out following three different approaches based on the concentrations of the antimicrobials used, the intervals of dosage and the duration of the whole cycle. These approaches intend to mimic different conditions of exposures to the combined enzyme-BAC treatment that could occur in food industries with different programmes of hygienic control.

2. Materials and methods

2.1. Bacterial strains

Dual-species biofilms were formed by *L. monocytogenes* A1 and *Escherichia coli* A14, both isolated in a previous survey performed among pre-sanitised surfaces in a fish processing plant (Rodríguez-López et al., 2015).

Stock cultures of all strains were maintained at – 80 ºC in brain-heart infusion broth (BHI; Biolife, Italy) containing 50 % glycerol 1:1 (v/v) mixed. Working cultures were kept at -20 ºC in Trypticase Soy Broth (TSB, Cultimed, Barcelona) containing 50 % glycerol 1:1 (v/v) mixed.

131 *2.2. Set-up of dual-species biofilms*

132 A 100 µl of working cultures of *L. monocytogenes* or *E. coli* were cultured overnight in
133 5 ml sterile TSB at 37 ºC and subcultured and incubated overnight in order to ensure a
134 proper growth.

135 Inocula preparation was performed as described previously (Rodríguez-López et al.,
136 2017). Briefly, Abs_{700} of each culture was adjusted to 0.1 ± 0.001 in sterile phosphate
137 buffer saline (PBS) using a 3000 Series scanning spectrophotometer (Cecil Instruments,
138 Cambridge, England) corresponding to a bacterial concentration of about 10^8 CFU/ml.
139 Adjusted cultures were 1:10^4 diluted in sterile mTSB (TSB supplemented with 2.5 g/l
140 glucose (Vorquímica, S.L., Vigo, Spain) and 0.6 % yeast extract (Cultimed, Barcelona,
141 Spain) to obtain a final concentration of about 10^4 CFU/ml. Then, equal volumes of
142 these adjusted cultures were mixed to obtain the inoculum for dual-species biofilms.

143 Biofilms were grown on 10x10x1 mm AISI 316 stainless steel (SS) coupons (Comevisa,
144 Vigo, Spain). Coupon pre-treatment included individual washing with industrial soap
145 (Sutter Wash, Sutter Ibérica, S.A., Madrid, Spain), rinsing with tap water, a final rinse
146 with deionised water and autoclaving at 121 ºC for 20 min. After this, coupons were
147 individually placed into a 24 flat-bottomed well plate and each well was inoculated with
148 1 ml of the inoculum. Plates were incubated in a humidified atmosphere at 25 ºC
149 statically for 2 h for initial adhesion and then in constant shaking at 100 rpm at 25 ºC
150 until 24 h.

151 Before any assay was performed, samples (SS coupons) were aseptically collected and
152 briefly immersed in 1 ml sterile PBS to remove loosely attached cells.

153

154 *2.3. Adhered viable culturable cells (AVC) quantification*

155 Since studies dealing with antimicrobial testing against biofilms, the quantification of
156 biocidal activities is mostly performed by quantifying the number of adhered viable and
157 culturable cells (AVC) after a given treatment, in this work AVC was referred to this
158 particular cellular pool.

159 After PBS washing, AVC were collected from three different samples (SS coupons).
160 The surface of each sample was thoroughly swabbed using two sterile cotton swabs
161 moistened in buffered peptone water (BPW, Cultimed, Barcelona). In a previous assay,
162 absence of cells after swabbing was determined staining swabbed samples with Syto9
163 (Life Technologies, Eugene, OR, USA) and visualised under a Leica DM 6000
164 epifluorescence microscope (Leica, Wetzlar, Germany) (data not shown). Swabs
165 belonging to the same sample were pooled together in 2 ml of BPW and vortexed
166 vigorously for 1 min in order to release cells. Suspensions were serially diluted in BPW
167 and spread onto agar plates. *Listeria*-PALCAM (Liofilchem, Roseto degli Abruzzi,
168 Italy) was used for *L. monocytogenes* selection whereas HiCrome^{TM} Coliform Agar
169 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 5 µg/ml of vancomycin and
170 cefsulodine (Sigma-Aldrich) was used for *E. coli*. Plates were incubated at 37 ºC for 24-
171 48 h and results were expressed as the mean in log CFU/cm^2 or log CFU/cm^2 reduction
172 depending on the assay. The accepted limit of detection was 25 CFU in the plate of the
173 lowest dilution corresponding to 1.70 log CFU/cm^2 (Sutton, 2011).

174

175

176

177 *2.4. Microscopy assays*

178 In all cases, for microscopy assays, three different samples (SS coupons) were used.
179 After PBS washing, coupons were stained using LIVE/DEAD® BacLight^{TM} bacterial
180 viability kit (Life Technologies, Eugene, OR, USA) that allows distinguishing total cells
181 with undamaged membranes (green fluorescence) and damaged membranes (red
182 fluorescence). Staining solution was prepared mixing 0.25 µl of propidium iodide and
183 0.75 µl of Syto9 in 1 ml of filter sterilised distilled water. Fifty microlitres of this
184 solution was poured onto each coupon for 15 min contact time. Subsequently, staining
185 solution was carefully removed and samples were washed three times by immersion in 1
186 ml of sterile milliQ water for 30 s. Coupons were then air dried and visualised under a
187 Leica DM 6000 epifluorescence microscope using a 40x objective and 10x ocular
188 lenses. In each sample, a randomly chosen field was considered as start point to acquire
189 12 bit images using a Leica DFC365 FX camera. Each image set was composed by
190 three 5 x 5 mosaics each one covering a total surface of 1.92×10^6 µm^2. Image analysis
191 was then performed using the Integrated Morphometry Analysis (IMA) module of
192 Metamorph MMAF software (Molecular Devices, Sunnyvale, CA, USA) in order to
193 determine the occupied area (OA) by undamaged (green) cells. Results were expressed
194 in mm^2.

195

196 *2.5. Enzymes, BAC and neutralising solutions preparation*

197 Pronase (PRN, from *Streptomyces griseus*, Roche, Mannheim, Germany) stock solution
198 was prepared at concentration 2000 µg/ml dissolved in 0.1 M Tris-HCl (Sigma Aldrich)
199 buffer at pH = 7.5 ± 0.2. After preparation, stock solution was filter sterilised through a

0.2 µm pore diameter syringe filter (Sartorius, Göttingen, Germany) and kept at -20 ºC until use.

Benzalkonium chloride (BAC, Guinama, Alboraya, Spain) stock solution was prepared at concentration 4000 µg/ml diluting the commercial solution in sterile distilled water. The solution was kept at 4 ºC until use.

Neutralising solution to stop BAC biocidal effects had the following composition per litre: 10 ml of a 34 g/l KH_2PO_4 solution adjusted to pH = 7.2 with $NaOH_{(aq)}$, 3 g soy lecithin, 5 g $Na_2S_2O_3$, 1 g L-histidine, 30 ml Tween 80 and adjusted with distilled water. This solution was autoclaved at 121 ºC for 20 min and kept at 4 ºC until use.

2.6. Experimental design

A two-phase experimental procedure was designed to evaluate the tolerance development (TD) in dual-species *L. monocytogenes-E. coli* biofilms to the application of PRN-BAC combined treatments.

2.6.1. Phase 1: sublethal exposures

In this phase, 24 h *L. monocytogenes-E. coli* biofilms were exposed to a different number of consecutive PRN-BAC sublethal exposures with or without recovery periods as schematised in Fig. 1. Sublethal concentrations were determined in previous assays (data not shown). Both PRN and BAC stock solutions were diluted in mTSB at concentrations for sublethal exposure (Fig. 1). Additionally, a negative control series in which PRN and BAC solutions used were substituted by equal volumes of sterile

deionised water was included in each experiment. Thus, the experimental approaches and concentrations used were as follows:

2.6.1.1. Experimental approach 1: Short term PRN-BAC exposure

In this approach, samples were subjected to two consecutive PRN-BAC exposures. At 24 h, the bulk phase of the samples was carefully pipetted out and wells were re-filled with 1 ml mTSB containing PRN to a final concentration of 50 µg/ml PRN for 1 h contact time without agitation at room temperature. Then, 1.5 ml mTSB containing BAC to a final concentration of 25 µg/ml was poured into each well and plates were placed back at 25 ºC/100 rpm. At 48 h, bulk phase was newly removed and biofilms were exposed to 1 ml mTSB containing PRN to a final concentration of 100 µg/ml PRN, let to dwell 1 h statically at room temperature. Then, 1.5 ml mTSB containing BAC to a final concentration of 50 µg/ml was added to each sample and plates were placed back at 25 ºC/100 rpm until next step. Negative controls were subjected to the same exposure scheme but the volumes of PRN and BAC used were substituted by equal volumes of sterile deionised water (Fig. 1).

In this approach, TD quantification carried out as described below, was determined at 72 h (Fig. 1).

243 *2.6.1.2. Experimental approach 2: Short term PRN-BAC exposure with recovery*
244 *periods*

245 In this approach, exposure to treatments was carried out as described for approach 1 but
246 after every PRN-BAC exposure the bulk phase was pipetted out and replenished by 1
247 ml plain mTSB (i.e. at 48 and 96 h), and incubated at 25 ºC/100 rpm for 24 h (Fig. 1).
248 As in the previous approach, controls were subjected to the same exposure scheme but
249 PRN and BAC solutions used were again substituted by equal volumes of sterile
250 deionised water (Fig. 1).

251 In this second case, TD quantification to PRN-BAC as described below was carried out
252 at 120 h (Fig. 1).

253

254 *2.6.1.3. Experimental approach 3: Long term PRN-BAC exposure*

255 In this third approach, bulk phases of the samples were pipetted out and wells were re-
256 filled with 1 ml mTSB containing PRN to a final concentration of 50 µg/ml followed by
257 the addition of 1.5 ml mTSB containing BAC to a final concentration of 25 µg/ml at 24,
258 48, 72, 96 and 168 h (Fig. 1). As performed in the previous approaches, for negative
259 controls PRN and BAC volumes used were substituted by equal volumes of sterile
260 deionised water (Fig. 1).

261 At 190 h, final treatment and quantification of the TD to PRN-BAC, as described
262 below, was carried out (Fig.1).

263

264 *2.6.2. Phase 2: Quantification of tolerance development (TD) in L. monocytogenes-E.*
265 *coli biofilms to PRN-BAC combined treatments*

266 After growth, exposed and control dual-species biofilms were evaluated by assessing
267 the state of the biofilm and the TD after a final exposure to a PRN-BAC treatment.

268 Biofilm condition was assessed by quantifying the number of AVC and the OA by
269 undamaged (green) cells on the SS coupons in both experimental series (see sections
270 2.3. and 2.4., respectively). Results were expressed in log CFU/cm^2 and in mm^2 for
271 AVC and OA, respectively

272 TD was quantified by comparing the AVC and OA values of the dual-species samples
273 previously exposed to sublethal PRN-BAC concentrations with those obtained in
274 controls after a final PRN-BAC treatment. Specifically, 3 new coupons per series were
275 transferred to a new well and treated again with 1 ml of a 100 µg/ml PRN solution in
276 0.1 mM Tris-HCl for 1 h contact time at room temperature without agitation. Then, 1.5
277 ml of a 50 µg/ml BAC solution in deionised water was added and let to dwell statically
278 for 10 min at room temperature. To stop BAC effects, samples were newly transferred
279 to a well containing 1 ml of sterile neutraliser and immersed for 10 s at room
280 temperature. After neutralisation, coupons were processed for AVC and microscopy
281 assays as described above (sections 2.3. and 2.4.).

282

283 *2.7. Statistical analysis*

284 An independent-samples Student's *t* test was performed to determine differences
285 between values using Microsoft Excel 2016. Significance was expressed at the 95 %
286 confidence level ($\alpha = 0.05$) or greater.

3. Results

3.1. Approach 1: short term exposure

Comparison between the condition of *L. monocytogenes*-*E. coli* dual-species biofilm formed without any exposure to PRN-BAC and after two consecutive exposures to double-fold increasing PRN-BAC concentrations was carried out according to experimental approach 1 (Fig. 1). Obtained results showed that the number of *L. monocytogenes* adhered in exposed biofilms was significantly lower comparing with that obtained in the controls (3.95 ± 0.48 and 6.37 ± 0.46 log CFU/cm^2, respectively) (Fig. 2A). No significant differences were obtained in the number of *E. coli* in unexposed and exposed biofilms (Fig. 2A).

The final treatment with PRN-BAC decreased the number of cells in all experimental series assayed about 1.5-2.0 log CFU/cm^2 (Fig. 2B). However, when the resistance of exposed and control biofilms was compared in terms of log reduction of the number of adhered cells of both species, no significant differences ($P > 0.05$) were obtained (Fig. 3). Taken together, these results seem to indicate that *L. monocytogenes*-*E. coli* dual-species biofilms did not acquire any tolerance to PRN-BAC treatment after exposure following approach 1.

Microscopic images showed no significance in OA values by undamaged (green) cells between control biofilms (0.84 ± 0.06 mm^2) compared to PRN-BAC exposed samples (0.70 ± 0.16 mm^2) (Fig. 4). Last PRN-BAC treatment applied to the samples obtained with approach 1 had visible effects on control and exposed biofilms regarding the 2D morphology of the biofilm, however, no significant differences were observed in terms of OA values (Fig. 4).

310 *3.2. Approach 2: short term exposure with recovery periods*

311 This second approach was specifically designed to check whether *L. monocytogenes-E.*
312 *coli* dual-species biofilms were able to develop tolerance to PRN-BAC treatments when
313 incorporating 24 h-recovery periods after each of the 2 exposures scheduled in the
314 approach 1 using fresh mTSB (Fig. 1).

315 No significant differences ($P > 0.05$) were obtained in AVC values of control samples
316 neither in *L. monocytogenes* nor in *E. coli* (6.87 ± 0.08 and 7.72 ± 0.30 log CFU/cm^2,
317 respectively) compared to those obtained in controls of approach 1 (Fig. 2A). This
318 indicated that the inclusion of recovery periods in between exposures did not produce a
319 higher number of cells to be attached to the surface. On the other hand, AVC outcomes
320 in exposed samples were lower in both species compared to those obtained in approach
321 1 (3.11 ± 0.16 CFU/cm^2 for *L. monocytogenes* and 6.53 ± 0.18 CFU/cm^2 for *E. coli*)
322 (Fig. 2A).

323 Tolerance development to PRN-BAC of the dual-species biofilms of approach 2 was
324 then assessed in terms of log reduction and compared to results obtained in approach 1.
325 Outcomes demonstrated that the recovery periods included caused a drop in the log
326 reduction values in both unexposed (control) and exposed samples (Fig. 3). Considering
327 that AVC values before final PRN-BAC treatment between approaches 1 and 2 were not
328 significantly different in neither species (Fig. 2), that meant that the overall resistance of
329 the cells of *L. monocytogenes-E. coli* biofilms to the PRN-BAC treatment was higher,
330 even in the case of controls (Fig. 3). Indeed, whereas the log reduction values obtained
331 in approach 1 ranged from 1.56 ± 0.25 to 2.34 ± 0.50 log CFU/cm^2, using this second
332 exposure schedule the variation among log reduction outcomes was from 0.13 ± 0.08 to
333 1.06 ± 0.23 log CFU/cm^2 (Fig. 3). This increased resistance was observed in both

334 species, even though *L. monocytogenes* presented bigger differences than *E. coli* if
335 values between approaches are compared (Fig. 3). Surprisingly, a higher resistance to
336 PRN-BAC treatments was also observed in control biofilms respecting to those values
337 obtained in exposed biofilms of approach 1 (Fig. 3).

338

339 *3.2. Approach 3: long term exposure*

340 In order to check if longer incubation times with the sublethal treatments would
341 influence the final resistance of the biofilm, a third experimental approach consisting in
342 five consecutive exposures to sublethal PRN-BAC concentrations was used (Fig. 1).

343 Before final PRN-BAC treatment, control samples did not present significance in *E. coli*
344 (7.85 ± 0.26 log CFU/cm^2) respecting to previous approaches whereas in *L.*
345 *monocytogenes* the AVC value was significantly higher (7.65 ± 0.02 log CFU/cm^2)
346 compared to AVC outcomes obtained in previous approaches (Fig. 2A). In exposed
347 biofilms, AVC values were significantly ($P < 0.05$) higher than those of approach 2
348 (3.63 ± 0.05 and 8.10 ± 0.59 log CFU/cm^2 for *L. monocytogenes* and *E. coli*,
349 respectively) (Fig. 2A).

350 TD quantification demonstrated the lowest log reductions among control samples in *L.*
351 *monocytogenes* (0.23 ± 0.12 log CFU/cm^2) even presenting negative values (i.e. a slight
352 AVC increase) in *E. coli* (-0.25 ± 0.01 log CFU/cm^2) (Fig. 3). Among exposed samples,
353 the log reductions were higher than those of approach 2 but significantly lower than
354 those of approach 1 (Fig. 3), with final AVC values of 2.61 ± 0.63 and 7.10 ± 0.43 log
355 CFU/cm^2 in *L. monocytogenes* and *E. coli*, respectively (Fig. 2).

Analysis of microscopy images for OA determination before final PRN-BAC treatment, showed no significance between series yielding values of 0.87 ± 0.18 and 0.77 ± 0.12 mm^2 for control and exposed samples, respectively (Fig. 5). Besides, images corresponding to control samples presented a cloudy-like structures stained in green that were not visible among the exposed biofilms. After final PRN-BAC treatment, OA value in exposed samples (0.54 ± 0.09 mm^2) was significantly lower than controls (0.75 ± 0.25 mm^2). This effect was also visible among images where a lower green signal was obtained whereas the proportion of red-stained cells rised (Fig. 5).

4. Discussion

In the present study, the capability of *L. monocytogenes*-*E. coli* biofilms to grow and develop tolerance to PRN-BAC combined treatments after sublethal exposure following three different approaches was determined. Each individual approach was intended to assess the effects after three feasible situations related to the sanitation practise in the food industry. Thus, approaches 1 and 2 simulated procedures in which treatments are applied using increasing concentrations daily or every other day for biofilm removal. On the other hand, approach 3 intended to emulate the effects of one-week daily cleaning and disinfection routines using the same concentrations of PRN and BAC.

Before any antimicrobial treatment can be applied, its efficacy against a particular target must be previously tested. Despite this, to date, no standardised methods for antibiofilm testing are available (Malone et al., 2016) and none of them considers TD as a parameter, basic to determine the appropriate dosage to apply for proper cleaning and disinfection, avoiding the generation of antimicrobial tolerances.

379 Results showed that the number of *L. monocytogenes* AVC decreased after sublethal
380 exposure to PRN-BAC regardless of the exposure scheme used (Fig. 2A). Contrarily, in
381 *E. coli* the presence of PRN-BAC did not affect the number of AVC on SS coupons in
382 approaches 1 and 3 (Fig. 2A). In approach 2, the number of AVC of *E. coli* in exposed
383 samples was slightly lower compared to control samples but the differences between
384 series were smaller than those obtained in *L. monocytogenes* (Fig. 2A). These results
385 were not surprising considering that Gram-negatives are generally more resistant to
386 QACs than Gram-positives (Mcdonnell and Russell, 1999) and were in accordance with
387 results obtained by Machado, Lopes, Sousa, & Pereira (2012) who observed that the
388 number of adhered cells in 6-day old dual-species biofilms of *Pseudomonas*
389 *aeruginosa-E. coli* was not affected by presence of 328.5 µg/ml BAC in the culture
390 medium.

391 Obtained results showed that the acquired tolerance to PRN-BAC by *L. monocytogenes-*
392 *E. coli* dual-species biofilms is the result of the following two interdependent factors: i)
393 the effects produced by the cultural features (i. e. total incubation time and the way in
394 which biofilms were exposed to PRN and BAC) of each experimental approach giving
395 rise to a different *L. monocytogenes-E. coli* dual-species biofilms, and ii) the specific
396 physiological effects provoked by the sublethal PRN-BAC exposures.

397 Regarding the first comparison, significant higher values of log reduction were obtained
398 in the dual-species biofilms subjected to short-term approach (approach 1) compared to
399 those obtained when short-term with recovery periods and long-term exposures
400 (approaches 2 and 3, respectively) were applied (Fig. 3). In other words, biofilms grown
401 in approach 1 were much less tolerant to PRN-BAC. This fact, together with the
402 absence of significance between AVC values in all the experimental approaches before

403 and after the application of the last PRN-BAC treatment, pointed out that longer
404 incubation times (approaches 2 and 3) and the incorporation of recovery periods
405 (approach 2) gave rise to structures with higher PRN-BAC tolerance.

406 The TD in terms of the number of AVC that resist the last PRN-BAC treatment after
407 sublethal exposure was only observed in approach 2, in which significant differences ($P < 0.05$)
408 in the log reduction between control and exposed dual-species biofilms were
409 observed in both strains.

410 Bacterial recovery after sublethal injured has been reported to contribute to adaptation
411 and hardening of cells after stresses (Wesche et al., 2009). Among foodborne pathogens,
412 this recovery period is rather short; around 1 to 5 hours at 25-37 ºC in rich broth (Wu,
413 2008). Thus, in biofilms, these factors can promote the appearance of persistent strains
414 in food-related environments (Simões et al., 2006). Considering this, it is logical to
415 think that the incorporation of recovery periods in approach 2, allowed injured bacteria
416 to repair damaged structures, and develop stress-induced strategies to prepare the cells
417 for further external aggressions.

418 Membrane alterations together with the expression of BAC-induced efflux pumps, have
419 been previously reported by several authors as the main cause of BAC-sensitivity
420 reduction both in *L. monocytogenes* (Aase et al., 2000; Bisbiroulas et al., 2011;
421 Romanova et al., 2006; To et al., 2002) and *E. coli* (Bore et al., 2007; Langsrud et al.,
422 2004; Moen et al., 2012). Following these ideas, the recovery periods in approach 2
423 could have permitted the surviving subpopulation after PRN-BAC exposure to undergo
424 mechanisms for membrane damage repair, as previously observed in *L. monocytogenes*
425 after the application of sanitisers (Donnelly, 2002).

Additionally, these periods could have allowed cells in the bulk phase to have enough time to repair possible damaged cellular structures and express chromosome and plasmid-encoded efflux pumps, therefore causing the extrusion of BAC outside of the cell. With this regard, Tamburro et al., (2015) observed an alteration in the gene expression pattern concomitant with a higher tolerance to BAC in *L. monocytogenes* after exposure to 10 µg/ml BAC, highlighting a significant increase in the expression of *mdr*L (efflux pump) and *sig*B (transcription factor) genes. Similar phenomena have also been reported in *E. coli* after BAC sublethal exposure (Bore et al., 2007). This increased tolerance is even more relevant if we consider that these pumps can be effective against other molecules therefore promoting BAC-induced multidrug resistance (Aase et al., 2000; Rakic-Martinez et al., 2011)..

Furthermore, the recovery periods in approach 2 could have permitted to the exposed biofilm samples to undertake species spatial reorganisation after PRN-BAC contact and that this increased the overall protection of the structure. With this regard, observation in previous studies published, point out that species cross-protective effects depending on spatial distribution in mixed biofilms after antimicrobial exposure, significantly affects the overall biofilm tolerance (Lee et al., 2014; Leriche et al., 2003). Such cross-protection would be of the weakest by the most resistant species as previously stated (Lee et al., 2014). So, if this was the case, *L. monocytogenes* would be protected by *E. coli* since, generally, the latter is more resistant to BAC than Gram-positives (Mcdonnell and Russell, 1999).

Nevertheless, the specific effect of the recovery periods has not been previously described in biofilms. In planktonic cells of *Pseudomonas* spp., it was previously observed that the adaptive resistance acquired after short term exposure to 200 µg/ml of

BAC was rapidly lost after overnight incubation in absence of the disinfectant (Langsrud and Sundheim, 1997). Interestingly, the authors also demonstrated that the level of tolerance of *Pseudomonas* spp. to the application of BAC and BAC-EDTA was conditioned by the treatment applied during the sublethal exposure.

Besides, sublethal exposures to PRN-BAC would probably determine important specific biological modifications in the final L. *monocytogenes-E. coli* dual-species biofilm. In fact, previous studies have demonstrated that the presence of BAC during biofilm formation by *E. coli* gives rise to a denser matrix richer in proteins and polysaccharides (Machado et al., 2012). In this latter case, the possible presence of high amounts of protective colanic acid would make the matrix barrier to be very difficult to penetrate (Flemming and Wingender, 2010; Sutherland, 2001).

The way in which all these biological processes takes place in industrial settings, is directly related with the way in which sanitation procedures are applied. Insufficient rinsing is one of the most common causes of presence of sublethal amounts of biocides among surfaces treated with QACs in the food industry (Møretrø et al., 2017). This fact together with the time in-between scheduled cleaning and disinfection protocols applied in a processing plant, would permit bacteria to recover from biocide injury eventually boosting their resistance to chemicals as demonstrated by the results obtained.

5. Conclusions

Outcomes in this work have demonstrated that the manner in which *L. monocytogenes-E. coli* biofilms are exposed to sublethal concentrations of PRN-BAC influences the subsequent resistance to this combined treatment. Firstly, it was observed that recovery

periods lead to a selection of a resistant subpopulation compared to unexposed samples. In addition to this, it was also observed that longer incubation times also influenced the resistance of the biofilm. Therefore, for biofilm eradication, a thorough optimisation not only of the right amounts of antimicrobial compounds utilised but also a proper time scheduling would be necessary prior to the application of any sanitation procedure in order to obtain proper bactericidal effects while avoiding the selection of resistant variants.

Acknowledgments

This research was financially supported by the Spanish Ministerio de Economía y Competitividad (ENZYMONO, AGL2010-22212-C02-02). P. Rodríguez-López acknowledges the financial support from the FPI programme (Grant number: BES-2011-050544). All authors acknowledge S. Rodríguez and T. Blanco (laboratory technicians, IIM-CSIC) for their help throughout all the study.

All results and conclusions presented in this article are part of P. Rodríguez-López PhD dissertation in the framework of the microbiology doctorate programme of the Autonomous University of Barcelona.

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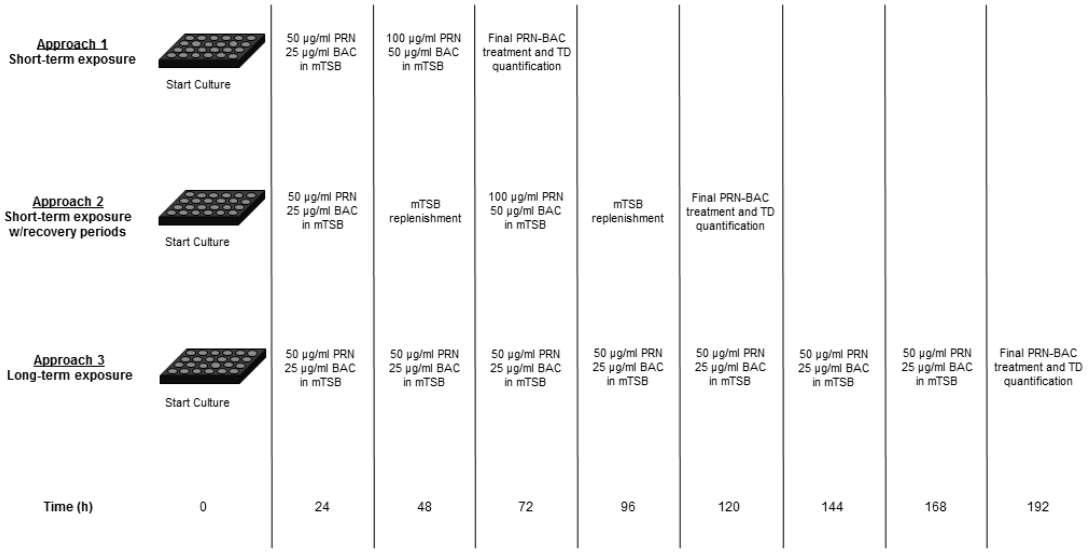

Figure 1. Experimental approaches followed in exposed samples. At each experimental time, bulk phase was removed and solutions of pronase (PRN) and benzalkonium chloride (BAC) were applied at room temperature at the indicated concentrations. For final PRN-BAC treatment a 100 µg/ml PRN solution in 0.1 mM Tris-HCl followed by the addition of a 50 µg/ml BAC solution was used and tolerance development (TD) quantification was performed as described in the text. In all cases, control samples were cultured and treated the same way substituting PRN and BAC solutions by equal volumes of sterile deionised water. (See text for further details)

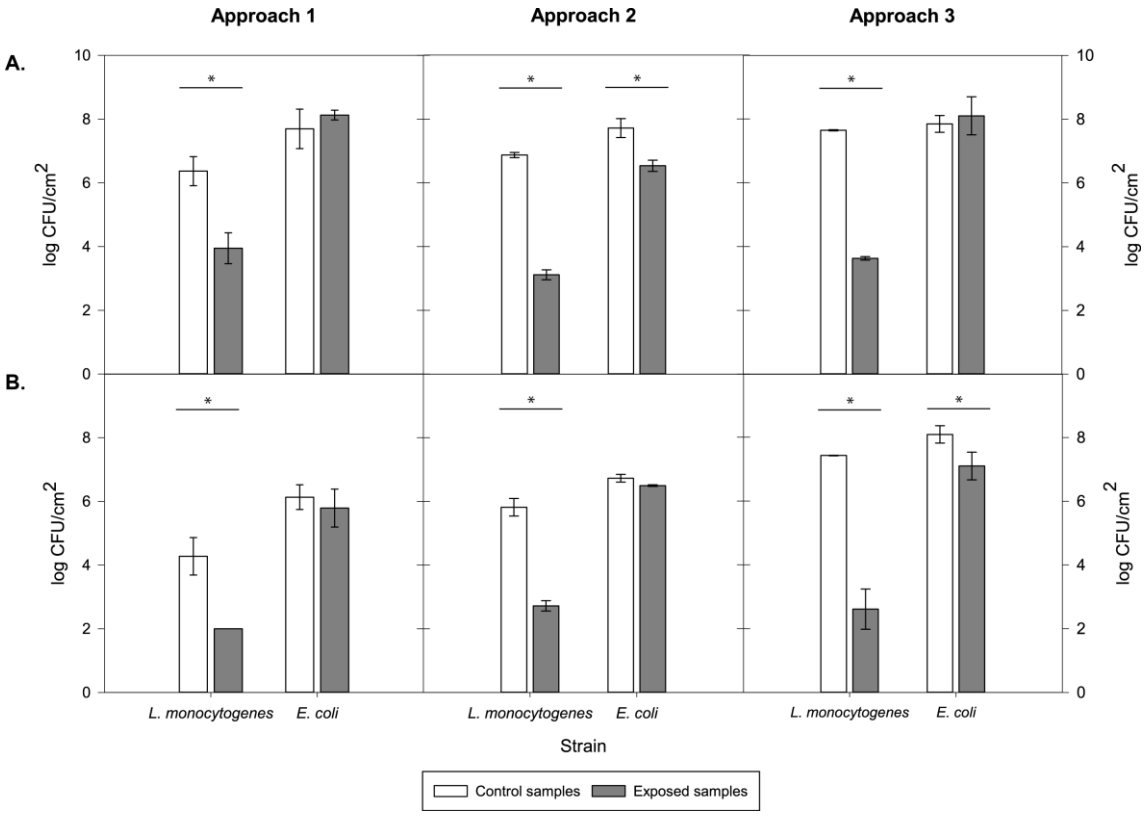

Figure 2. Adhered viable culturable cells (AVC) counts obtained in all experimental approaches A) before final and B) after final 100 µg/ml pronase followed by 50 µg/ml benzalkonium chloride combined treatment. Exposed samples refer to those subjected to a previous PRN-BAC exposure whereas in control samples biocides solutions volumes were replaced with water (see section 2.6. for further detail). Asterisks indicate statistical significance ($P < 0.05$).

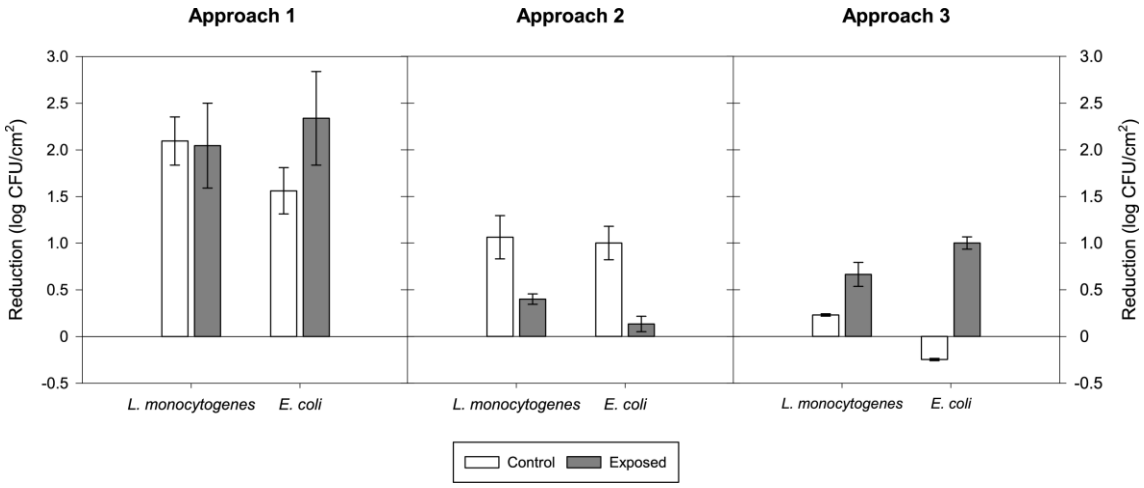

Figure 3. Log reductions obtained in all experimental approaches after the application of a final 100 µg/ml pronase (PRN) followed by 50 µg/ml benzalkonium chloride combined (BAC) treatment. Exposed samples refer to those subjected to a previous PRN-BAC exposure whereas in control samples biocides solutions volumes were replaced with water (see section 2.6. for further detail). In approach 3, the negative reduction in *E. coli* refers to a slight increase in the number of adhered viable cultivable cells recovered after final PRN-BAC treatment. Asterisks indicate statistical significance ($P < 0.05$).

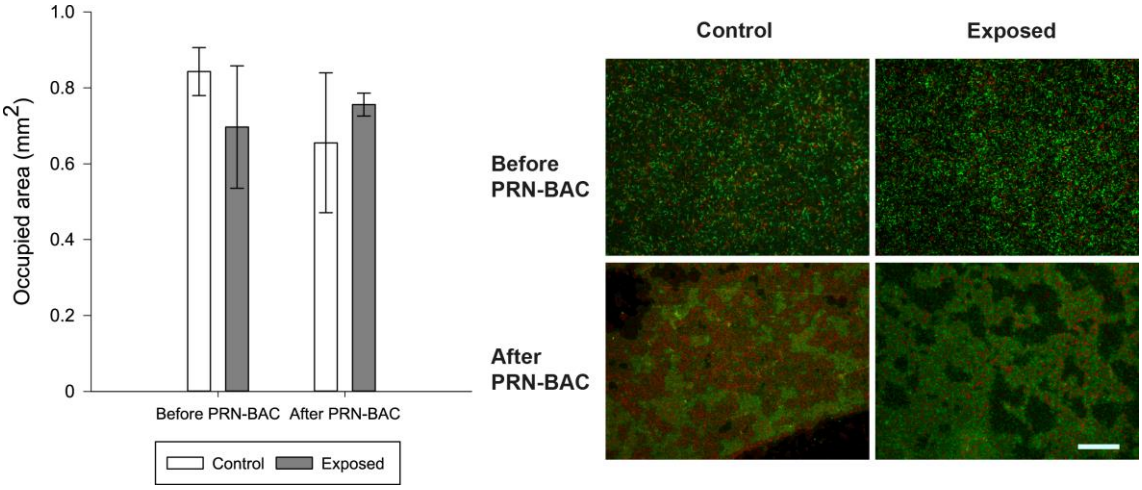

Figure 4. Values of occupied area obtained after analysis of 25-field mosaics of approach 1 before and after the application of pronase (PRN)-benzalkonium chloride (BAC) final treatment. Exposed samples were subjected to a previous PRN-BAC exposure whereas control samples biocide solution volumes were replaced with water (see section 2.6.1.1. for further detail). Scale bar = 50 µm.

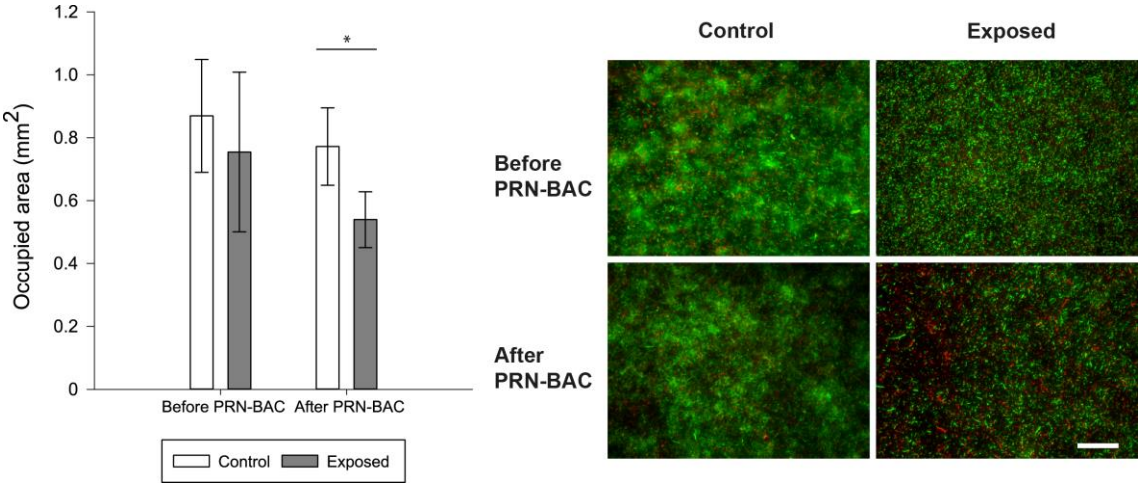

Figure 5. Values of occupied area obtained after analysis of 25-field mosaics of approach 3 before and after the application of pronase (PRN)-benzalkonium chloride (BAC) final treatment. Exposed samples were subjected to a previous PRN-BAC exposure whereas control samples biocide solution volumes were replaced with water (see section 2.6.1.3. for further detail). Asterisk indicates statistical significance ($P < 0.05$). Scale bar = 50 µm.