Removal of *Listeria monocytogenes* dual-species biofilms using enzyme-benzalkonium chloride combined treatments

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Abstract

The effects of pronase (PRN), cellulase (CEL) or DNaseI alone or combined with benzalkonium chloride (BAC) against *Listeria monocytogenes*-carrying biofilms were assayed. Best removal activity against *L. monocytogenes-Escherichia coli* biofilms was obtained using DNaseI followed by PRN and CEL. Subsequently, a modified logistic model was used to quantify the combined effects of PRN or DNaseI with BAC. A better BAC performance after PRN compared to DNaseI eradicating *L. monocytogenes* was observed. In *E. coli* the effects were the opposite. Finally, effects of DNaseI and DNaseI-BAC treatments were compared against two different *L. monocytogenes*-carrying biofilms. DNaseI-BAC was more effective against *L. monocytogenes* when co-cultured with *E. coli*. Nonetheless, comparing the removal effects after BAC addition, these were higher in mixed-biofilms with *Pseudomonas fluorescens.* However, a high number of released viable cells were observed after combined treatments. These results open new perspectives of enzymes as an antibiofilm strategy for environmental pathogen control.

Keywords

Benzalkonium chloride; Biofilm; Disinfection; Enzymes; Fluorescence microscopy; *Listeria monocytogenes*

Introduction

In recent decades biofilms have been considered a major issue of concern in food processing related environments due to their relevance as a source of product contamination that results in product loss and contributes to food related illnesses caused by contaminated foodstuffs (Brooks & Flint 2008; Simões et al. 2010).

Listeria monocytogenes is a Gram-positive foodborne pathogen known to live as a saprophyte in environments rich in decaying plant material. This bacterium can cause listeriosis an invasive disease that primarily affects the elderly, newborns, pregnant women and immunocompromised individuals (Farber & Peterkin 1991; Swaminathan & Gerner-Smidt 2007) with symptoms that may include sepsis, meningitis and miscarriages (Vázquez-Boland et al. 2001; Freitag et al. 2009). The European Food Safety Authority reported an incidence of 0.52 cases per 100000 inhabitants of confirmed European *L. monocytogenes* infections, 30 % higher regarding previous published data (EFSA 2015). Moreover, this microorganism is well known to survive for long periods attached to food industry surfaces (Borucki et al. 2003; Carpentier & Cerf 2011) as part of multi-species sessile communities (Carpentier & Chassaing 2004; Rodríguez-López et al. 2015).

Chemicals disinfectants such as acids, peroxides, sodium hypochlorite and quaternary ammonium compounds (QACs) have been used extensively in industrial settings for disinfection and control of biofilms (da Silva & De Martinis 2013). Benzalkonium chloride (BAC) is considered one of the most used QACs due to its action upon bacterial membranes altering their structural integrity (Gerba 2015). Nevertheless, it has been extensively demonstrated that biofilms exhibit higher resistance/tolerance to BAC compared to planktonic cells both in Gram-positives such as *L. monocytogenes* (Saá

Ibusquiza et al. 2011; Nakamura et al. 2013) and in Gram-negatives such as *Escherichia coli* (Houari & Di Martino 2007) or *Pseudomonas* sp. (Giaouris et al. 2013).

In the last few years, enzymes have increasingly become a method used for biofilm control. These environmentally friendly compounds have been shown to both prevent the initial adhesion and remove formed structures (Johansen et al. 1997; Orgaz et al. 2006; Lequette et al. 2010; Cordeiro & Werner 2011) because of their dispersive effect on the sessile structures acting on target molecules present in the biofilm matrix (Giaouris et al. 2014; Kaplan 2014; Bridier et al. 2015). However, enzymes do not necessarily have bactericidal activity which makes them unsuitable to be used as a strategy for disinfection (Nguyen & Burrows 2014).

To overcome this, a feasible strategy to obtain both biofilm disinfection and removal would be the combination of an enzyme and BAC solution. Although enzyme-based cleaners and detergents have been proved to be effective for biofilm removal (Parkar et al. 2004; Vickery et al. 2004; Stiefel et al. 2016), to the best of the authors' knowledge, the only evidence found about an enzyme-BAC combination against biofilms was the study performed by Kaplan, (2009) who demonstrated that 24 h *Staphylococcus aureus* biofilms pre-treated with DNaseI were more sensitive to BAC and achieved about 4 log CFU reduction in the remaining adhered cells when compared to the non-pre-treated samples.

The main hypothesis of the present work was to utilise an approach using both enzyme treatment and subsequent disinfection using BAC on dual-species biofilms containing *L. monocytogenes* in an effort to facilitate the effect of the chemical disinfectant. For this purpose, classical plate counts as well as epifluorescence microscopy were used to assess the effects of the application of different enzyme solutions (pronase, cellulase or DNaseI)

on *L. monocytogenes-E. coli* fluorescent tagged biofilms grown on stainless steel coupons. Next, a comparative study to quantify the effectiveness of combining the enzymatic solutions with BAC against 48 h *L. monocytogenes-E. coli* biofilms was carried out. Finally, the more effective enzymatic solution against the elimination of *L. monocytogenes* from the mixed biofilms was applied alone and combined with BAC against *L. monocytogenes- E. coli* and *L. monocytogenes-Pseudomonas fluorescens* in order to quantify the influence of the species composition on the effectiveness of cleaning and disinfection method.

Methods

Bacterial strains

Listeria monocytogenes A1 and *Escherichia coli* A14 were isolated from a fish processing plant in a previous survey (Rodríguez-López et al. 2015). *Pseudomonas fluorescens* B52, a strong biofilm former and associated with milk and dairy products spoilage, was kindly provided by Dr. Carmen San José (Allison et al. 1998).

In all situations, stock cultures were kept at -80 ºC in Brain-Heart infusion broth (BHI; Biolife, Milan, Italy) containing 50% glycerol 1:1 (v/v) mixed. Work cultures were kept at -20 ºC in Tripticase Soy Broth (TSB; Cultimed, Barcelona, Spain) containing 50% glycerol 1:1 (v/v) mixed.

Construction of fluorescent-tagged stains

Genetic modification for constitutive expression of a fluorescent reporter of strains *L. monocytogenes* A1 and *E. coli* A14 was carried out in the laboratory of Prof. Colin Hill (School of Microbiology, University College Cork (UCC), Ireland).

Modification of L. monocytogenes

L. monocytogenes was modified for Green fluorescent protein (GFP) constitutive expression. Briefly, the fragment of pNF8 corresponding to the P*dlt*Ω*gfp-mut1* (Fortineau et al. 2000) was amplified with primers P*dlt* For-*Kpn*I and GFP pNF Rev-*Pst*I (Table 1) containing *Kpn*I and *Pst*I restriction sites, respectively, digested and cloned into pPL2 (Lauer et al. 2002) previously digested with *Kpn*I and *Pst*I and further treated with rAPid

Alkaline Phosphatase (Roche) to avoid religation. Ligation was performed using T4-ligase (Roche, Germany) in a PCR thermocycler as follows: 4 ºC for 5 h, 12 h ramp increasing 1 ºC/h, 16 ºC for 2 h and back to 4 ºC giving a plasmid of 7393 bp coded as pROLO1. The plasmid solution was dialysed in sterile deionised water on a 0.025 µm pore nitrocellulose filter (Millipore, Germany) for 30 min and then kept at -20 ºC until use. pROLO1 was then introduced into *E. coli* TOP10 cells (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions and cultured overnight in LB (Merck, Germany) + 10 µg/ml chloramphenicol (Cm; Sigma Aldrich, Saint Louis, MO) at 37 ºC. Plasmid extraction was then performed using a Gene JET Plasmid MiniPrep Kit (Thermo Fisher Scientific, Waltham, MA). P*dltΩgfp-mut1* integration was checked with primers pPL2 MCS-for and pPL2 MCS-rev (Table 1).

Electroporation was carried out by mixing 50 µl of electrocompetent cells prepared as previously described (Monk et al. 2008) with 2 µl of plasmid prep in 2 mm cuvettes using a BTX ECM 630 Generator (Harvard Apparatus, Holliston, MA). Conditions: field strength: 10 kV/cm; time constant: 5 ms; voltage: 2 kV; resistance: 400 Ω; capacitance: 25 µF. Cells were then resuspended in fresh sterile BHI + 0.5M Sucrose, incubated at 37 ºC for 1 h and then plated on BHI + 1,5 % agar + 10 µg/ml Cm and incubated at 37 ºC for 48 h. Colonies were picked and PCR was performed to check for plasmid integration using primers PL95 and PL102 (Bron et al. 2006) (Table 1). The resulting isolate was named *L. monocytogenes* A1-gfp.

Modification of E. coli

E. coli was modified for mCherry constitutive expression using the λ-red system (Serra-Moreno et al. 2006; Hillyar 2012). *E. coli* A14 electrocompetent cells prepared as

previously described (Gonzales et al. 2013) using 10 % glycerol for the final cell resuspension. Then, they were transformed with the thermosensitive plasmid pKOBEGA, analogue to pKOBEG (Chaveroche et al. 2000) in which *cat* gene has been substituted by bla_{amp} gene (Sutcliffe 1978). This plasmid also contains the genes *exo, bet* and *gam*, necessary for Red system-mediated recombination (Chaveroche et al. 2000). Electroporation was carried out in 2 mm cuvettes in a BTX ECM 630 Generator. Conditions: field strength: 10 kV/cm; time constant: 5 ms; voltage: 2.5 kV; resistance: 200 Ω; capacitance: 25 µF. Transformants were selected on LB agar + 50 µg/ml ampicillin (Amp; Sigma Aldrich, Saint Louis, MO) at 30 ºC for 24 h.

Then, *E. coli* A14 pKOBEGA electrocompetent cells were prepared as above and newly transformed with pMP7607 miniTn7 (Lagendijk et al. 2010) carrying the mCherry gene and a streptomycin (Sm) resistance gene. Transformants were selected onto LB agar + 50 µg/ml Amp + 50 µg/ml Sm (Sigma Aldrich, Saint Louis, MO) and incubated at 30 ºC for 24 h. Fifty randomly chosen transformants were picked and spread onto LB agar + 50 µg/ml Sm and incubated at 42 ºC. The resulting isolate was named *E. coli* A14-mChy.

To assess the correct fluorescent signal, ten randomly picked colonies of each modified strain were diluted in a drop of deionised water on a glass slide and visualized under the fluorescence microscope.

Biofilms setup

One hundred microlitres of work cultures was grown overnight at 37 ºC in 5 ml of BHI + 10 µg/ml Cm for *L. monocytogenes* A1-gfp and LB + 50 µg/ml Sm for *E. coli* A14-mChy and subcultured overnight so as to ensure a proper growth.

Inocula preparation was performed following a modification of a protocol previously described (Rodríguez-López et al. 2015). Briefly, cultures were adjusted to Abs_{700} = 0.1 ± 0.001 in sterile phosphate buffer saline (PBS) using a Cecil3000 scanning spectrophotometer (Cecil Instruments, Cambridge, England), corresponding to a concentration of about 10^8 CFU/ml. Adjusted cultures were further diluted in sterile mTSB (TSB supplemented with 2.5 g/l glucose (Vorquímica, S.L., Vigo, Spain) and 0.6 % yeast extract (Cultimed, Barcelona, Spain)) to a final concentration of about 10^4 CFU/ml. Then, equal volumes of these adjusted cultures were mixed to obtain the inoculum for dual-species biofilms.

Biofilms were grown on 10 x 10 x 1 mm AISI 316 stainless steel (SS) coupons (Comevisa, Vigo, Spain). Pre-treatment of coupons included individual washing with industrial soap (Sutter Wash, Sutter Ibérica, S.A., Madrid), rinsing with tap water, a final rinse with deionised water and autoclaved at 121 ºC for 20 min. Coupons were then placed individually into a 24 flat-bottomed well plate and each well was inoculated with 1 ml of the corresponding culture. Plates were incubated in a humidified atmosphere at 25 ºC statically for 2 h so as to allow initial adhesion, and then in constant shaking at 100 rpm.

Biofilm formation kinetics

Samples (SS coupons) were collected at 24, 36, 48, 72 and 100 h and briefly immersed in sterile PBS in order to remove loosely attached cells before any analysis was performed.

Determination of the number of adhered viable cells (AVC)

Three different coupons were scraped using two cotton swabs pre-moistened with buffered peptone water (BPW; Cultimed, Barcelona, Spain). The swabs were then placed in 2 ml of BPW vigorously vortexed for 1 min to resuspend cells. The cell suspensions were then serially diluted in BPW and spread in duplicates onto agar plates. *Listeria*-PALCAM (Liofilchem, Italy) was used to select *L. monocytogenes* and HiCrome^{TM} Coliform agar (Sigma Aldrich, Saint Louis, MO) with a supplement of 5 µg/ml of Vancomycin and Cefsulodine (Sigma Aldrich, Saint Louis, MO) for *E. coli* selection. Plates were incubated at 37 ºC for 24-48 h and results were expressed as the mean in log CFU/cm^2 of samples. The accepted limit of detection for this and all assays involving viable cell counts was at least 25 CFU in the plate of the lowest dilution corresponding to a total of 1.70 log CFU/cm^2 (Sutton 2011).

Epifluorescence microscopy visualisation

At each sampling time, three coupons were air dried avoiding as much as possible direct light exposure. Samples were then visualised under a Leica DM6000 epifluorescence microscope using a 40x objective and 10x ocular lenses. Microscope was equipped with filter cubes L5 (Excitation 480/40) for A1-gfp and TX2 (Excitation 560/40) for A14-mChy. Images were taken using a Leica DFC365 FX controlled with Metamorph MMAF software (Molecular Devices, Sunnyvale, CA camera from 10 representative fields).

Effect of enzymatic solutions on dual-species biofilms

Enzyme solutions were prepared at concentrations 200, 400, 700 and 1000 µg/ml. Pronase (PRN, from *Streptomyces griseus*, Roche) was dissolved in 0.1 M Tris-HCl (Sigma Aldrich) buffer at pH = 7.5 ± 0.2. Cellulase (CEL, from *Aspergillus niger*, Sigma Aldrich) was dissolved in 100 mM citrate (Sigma Aldrich) buffer at pH = 6.0 ± 0.1. Finally, DNaseI (from bovine pancreas, Sigma Aldrich) was dissolved in 10 mM Tris-HCl (pH = 7.5 ± 0.2) buffer also containing 2.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$. After preparation, all solutions were filter sterilised through a 0.2 µm syringe filter (Sartorius) and kept at -20 ºC until use.

The biofilm removal action of each enzymatic solution was evaluated against 24 h biofilms. Three coupons were washed as before and then placed in a clean well. One millilitre of each enzyme solution was added and allowed to act for 30 min at 37 ºC for PRN and 32 ºC for CEL and DNaseI. Negative controls were run in parallel by adding the corresponding buffer solution without enzyme. Solutions were then gently removed by pipetting and SS coupons were subsequently washed with 1 ml of sterile PBS in order to remove residual enzyme. Determination of remaining adhered cells and visualisation of coupons was performed as described above. Results were expressed as the reduction in log CFU/cm^2, calculated as the mean of each replica difference in log CFU/cm^2 before enzymatic and after enzymatic treatment. After this, the two most effective enzymes were used in the following experiments.

Effect of benzalkonium chloride combined with either PRN or DNaseI on L. monocytogenes-E. coli biofilms

Benzalkonium chloride solutions (BAC; Guinama, Alboraya, Spain) were prepared in sterile deionised water at concentrations 25, 50, 100, 250 and 500 µg/ml. Each solution was applied after 30 min treatment with 400 µg/ml of either PRN or DNaseI solution against 48 h *L. monocytogenes* A1-gfp-*E. coli* A14-mChy biofilms.

Fourteen different coupons washed with sterile PBS for loosely attached cells removal were used for each enzyme series: two for the negative controls (no treatment), two for enzymes treatment without BAC (only enzyme and deionised water were applied), and two for each BAC concentration after enzymatic treatment performed as described above. In this latter case, 1.5 ml of each BAC solution was added to each coupon for a 10 min contact time at room temperature. For negative controls, buffer without enzymes and deionised water were sequentially used. Coupons were then transferred to a new well and immersed for 30 s in 1 ml of a neutralising solution (composition per litre: 10 ml of a 34 g/l KH_2PO_4 buffer (pH = 7.2); soybean lecithin: 3 g; Tween 80: 30 ml; $Na_2S_2O_3$: 5 g; L-histidine: 1 g) at room temperature followed by a final 10 s wash by immersion with sterile PBS to remove any neutraliser residues.

Following its application, neutralising solution was serially diluted in BPW and spread in duplicate onto appropriate agar media to determine the number of released viable cells (RVC) after treatments. Outcomes were expressed as mean of log CFU/ml. Microscopic visualisation and determination of the remaining attached cells were performed as described above. In the latter case, results were expressed as percentage of biofilm removal with respect to the log CFU/cm^2 obtained in control samples.

Determination of BAC effect: Calculation of lethal dose 90 (LD_{90})

LD_{90}, defined as the dose of an antimicrobial required to achieve a 90 % kill of the initial bacterial population, was used as a parameter to determine the effect of BAC on dual-species biofilms. To assess this, a modified logistic model proposed by Cabo et al. (1999) was used. Logistic equations are widely recognised as suitable for describing dose-response kinetics (Knight & McKellar 2007; Murado & Vázquez 2007). Firstly, outcomes were obtained by fitting of the experimental data obtained in plate count assays, expressed in percentage of biofilm removal according to following equation [1] using the least-squares method (quasi-Newton) of the SOLVER tool of Microsoft Excel 2016:

$$BR = K\left(\frac{1}{1+0.11e^{r(LD_{90}-D)}} - \frac{1}{1+0.11e^{rLD_{90}}}\right) \qquad [1]$$

where BR = biofilm removal expressed in percentage; LD_{90} = dose of BAC that removes 90% of the initial adhered population; D = dose of BAC used; K = maximum percentage of biofilm removal (asymptote); and r = specific inhibition coefficient (dimensions: inverse of the dose).

Since the equation [1] modifies the resulting Dose/Response parameters by subtracting the intercept of the original logistic equation, results were further adjusted to obtain the new K value (K'):

$$BR_{max} = K' = \lim_{D\to\infty} BR \qquad [2]$$

Then, the real LD_{90} (RD_{90}) was determined according to a modification of an equation described previously (Murado et al. 2002):

$$RD_{90} = \frac{1}{r}\ln(9 + e^{rD}) \qquad [3]$$

Influence of L. monocytogenes accompanying species in the resistance to DNaseI-BAC treatments

Two different 48 h dual-species biofilms were used: *L. monocytogenes* A1-*E. coli* A14 and *L. monocytogenes* A1-*P. fluorescens* B52 to evaluate sequential DNaseI-BAC treatments.

400 µg/ml DNaseI + 100 µg/ml BAC treatments and plate count analysis for attached and released cells determination were performed as described above. For *P. fluorescens* selection Pseudomonas Agar Base (PAB; Liofilchem, Italy) supplemented with CFC supplement (Liofilchem, Italy) was used and incubated at 30 ºC for 48 h.

For microscopic visualisation, samples were stained using LIVE/DEAD Bacterial viability kit (Life Technologies) to distinguish total cells with undamaged membranes (green fluorescence) and damaged cells (red fluorescence). Staining solution was prepared by mixing 0.25 µl of Propidium iodide and 0.75 µl of Syto9 in 1 ml of filter sterilized deionised water. Fifty microlitres of this solution was then poured onto each coupon and allowed to dwell for 15 min in the dark. Coupons were then washed three times in 1 ml of sterile milliQ water, air dried and visualised under the epifluorescence microscope to obtain images of representative fields.

Statistical analysis

Experimental results were analysed for statistical significance using IBM SPSS Statistics 23. An independent-samples two-tailed Student's *t* test was performed to assess differences between species in the biofilm formation kinetics and the effects of BAC in RVC after PRN and DNaseI treatments. Differences among the effects of the different

enzymatic treatments and treatments' effects in different dual-species biofilms were determined using a one-way ANOVA with a *post hoc* Bonferroni test. In all cases, significance was expressed at the 95 % confidence level ($\alpha = 0.05$) or greater.

In RD_{90} determination, correlation coefficient (r^2) was calculated to quantify the discrepancy between the observed experimental values and those expected according with the model.

Results

L. monocytogenes-E. coli biofilm formation kinetics on AISI 316 stainless steel

Dual-species biofilm formation dynamics are depicted in Fig. 1. Plate count assays showed a significantly higher number of AVC in *E. coli* with respect to that obtained for *L. monocytogenes* at 24 and 100 hours of growth yielding differences of 3.11 and 2.63 log CFU/cm^2 respectively. No significance was observed among the values of the rest sampling times.

Microscopic images displayed in Fig. 2 showed a uniform distribution of *E. coli* and *L. monocytogenes* over the coupon. In spite of this uniform distribution, at 24 h *E. coli* presented about 3 log higher AVC counts compared to *L. monocytogenes* (Fig. 1). A tendency for aggregation was observed at 24 and 36 h yielding a final composite structure with both species intermingled therein (Fig. 2). From this point onward, the amount of cells increased and the biofilm developed a cloud-shape structure which was maintained in the last three sampling times (Fig. 2).

Effects of pronase, cellulase and DNaseI on the elimination of mixed biofilms formed by L. monocytogenes-E. coli

The effects of the application of PRN, CEL and DNaseI on the number of AVC of 24 h *L. monocytogenes – E. coli* biofilm were compared. Results were expressed in terms of log CFU/cm^2 reduction (Fig. 3).

In general terms, *L. monocytogenes* was more sensitive than *E. coli* to treatments used yielding higher log reductions in most of the concentrations and enzymes used with exception of DNaseI at 1000 µg/ml where *E. coli* log reductions were significantly higher

(Fig. 3). Comparing the effects of the enzymes, higher concentrations were required to achieve a comparable log reduction of AVC in *E. coli* being especially relevant in the case of PRN and CEL (Fig. 3).

In both species, maximum effects (about 2 log reduction) were obtained after the application of 400 µg/ml of DNaseI. In *L. monocytogenes*, log reduction value was significantly higher when treated with DNaseI as compared to treatments with PRN and CEL in 2 out of 4 concentrations tested (200 and 400 µg/ml) (Fig. 3). On the other hand, considering *E. coli* removal by DNaseI, significance was only observed after applying a 400 µg/ml solution (Fig. 3). In both species, application of higher concentrations of this enzyme resulted in a lower log reduction. In fact, biofilm removal decreased about 1.5 log CFU/cm^2 when the DNaseI concentration applied increased from 400 to 600 µg/ml.

The application of CEL resulted in lower log reductions in both species tested compared to outcomes obtained after treatment with DNaseI with exception of 1000 µg/ml against *L. monocytogenes* where CEL significantly performed better than DNaseI (Fig. 3).

Finally, results displayed a concentration-dependent increase in log reduction in both species when PRN was used with maximum log reductions at 1000 µg/ml of 1.17 ± 0.42 and 0.70 ± 0.31 log CFU/cm^2 for *L. monocytogenes* and *E. coli*, respectively (Fig. 3).

Combined effects of BAC and PRN or DNaseI solutions for 48 h L. monocytogenes – E. coli biofilm elimination

Maximum percentage of biofilm removal (K') and lethal doses 90 (RD_{90}) values for *L. monocytogenes* and *E. coli* were calculated according to equations [1] to [3] after sequential treatment with 400 µg/ml of either PRN or DNaseI followed by disinfection

with different concentrations of BAC. In these experiments 48 h biofilms were preferred to provide a more challenging scenario to enzyme-BAC treatments.

Results showed a satisfactory fitting of experimental data ($r^2 = 0.984$) and demonstrated a higher efficacy of both combined treatments removing *L. monocytogenes* with respect to *E. coli* as indicated by K' values (Fig. 4, Table 2). Whereas in the case of *L. monocytogenes* BAC performed better after DNaseI treatment compared to PRN, in *E. coli* RD_{90} values showed a higher effect of BAC after PRN treatment compared to DNaseI (Table 2).

Outcomes of RVC (*L. monocytogenes* and *E. coli*) demonstrated a high level of cell dispersion after the application of sequential enzyme-BAC treatments, with values ranging from about 3 to 5 log CFU/ml (Fig. 5). Student's *t* test showed significance ($P < 0.05$) between treatments at BAC concentrations of 25, 50 µg/ml in *L. monocytogenes* and 25 and 100 µg/ml in *E. coli*, with a general tendency to lower RVC values as the BAC concentration increased (Fig. 5). If only RVC values of *L. monocytogenes* are considered, is important to highlight that no viable cells were detected after ≥ 100 µg/ml BAC neither in PRN nor in DNaseI-treated samples (Fig. 5).

Role of the accompanying species (E. coli, P. fluorescens) in the adhesion and resistance of L. monocytogenes to DNaseI and DNaseI-BAC treatments in dual-species biofilms

Cell counts demonstrated that *L. monocytogenes* was able to achieve significant higher number of adhered cells in presence of *P. fluorescens* compared to co-culture with *E. coli* reaching values of 7.23 ± 0.04 and 5.48 ± 0.05 log CFU/cm^2, respectively (Figs. 6A, B).

The application of a 400 µg/ml DNaseI solution gave higher *L. monocytogenes* log reduction values in co-culture with *E. coli* (2.47 log CFU/cm^2) compared to that obtained in co-culture with *P. fluorescens* (0.58 log CFU/cm^2) (Fig. 6A, B). Combined treatments (400 µg/ml DNaseI + by 100 µg/ml BAC) also produced a significant reduction in *L. monocytogenes* compared to controls, being of 3.24 and 2.83 log CFU/cm^2 in co-culture with *E. coli* and *P. fluorescens*, respectively (Fig. 6A, B). Nevertheless, if only BAC effects on *L. monocytogenes* are considered, by comparing the log reductions of DNaseI alone and DNaseI-BAC treatments, these were higher in *L. monocytogenes-P. fluorescens* biofilms (2.55 log CFU/cm^2) compared to *L. monocytogenes-E. coli* samples (0.77 log CFU/cm^2) (Fig. 6A, B).

L. monocytogenes RVC after DNaseI-BAC treatment did not present significant differences comparing both dual-species biofilms (4.23 ± 0.41 log CFU/ml in *L. monocytogenes-P. fluorescens* and 3.65 ± 0.41 log CFU/ml in *L. monocytogenes-E. coli*). Notice that *E. coli* presented a significant higher number of RVC (6.22 ± 0.09 log CFU/ml) after DNaseI-BAC combined treatment compared with *P. fluorescens* and *L. monocytogenes* in both dual-species biofilms (Fig. 6C, D).

Microscopic analysis showed that both biofilms presented remarkable differences in their 2D-morphologies (Fig. 7). While *L. monocytogenes-E. coli* biofilms showed a reticular distribution in all biofilm, *L. monocytogenes-P. fluorescens* biofilms were characterised by the presence of microcolonies surrounded by small cell groups. These microcolonies presented a local accumulation of damaged cells compared to the rest of the sample, as observed by a higher red signal in the central part of the microcolony. The same microcolony formation tendency was also observed in our laboratory with other *L. monocytogenes* strains when co-cultured with *P. fluorescens* B52 (data not shown).

Sequential DNaseI-BAC treatments produced a significant increase of the red cell signal especially in *L. monocytogenes-E. coli* samples pointing out the BAC killing effects. Besides, noticeable structural changes were observed in samples of both dual-species biofilms, especially in *L. monocytogenes-P. fluorescens* biofilms in which the cellular groups surrounding the microcolonies were substituted by sparsely distributed cells (Fig. 7).

Discussion

Biofilm kinetics of the *L. monocytogenes* A1-gfp-*E. coli* A14-chy biofilm showed a typical biofilm fit-curve with minor fluctuations (Fig. 1). *E. coli* viable counts were significantly higher than *L. monocytogenes* at 24 and 100 h. Differences in AVC counts at 24 h could be attributed to a better initial adhesion of *E. coli* compared with *L. monocytogenes* as previously reported (Azevedo et al. 2014). However, AVC values of both species were equilibrated at 36, 48 and 72 hours (Fig. 1).

Microscopic images showed a uniform distribution of *E. coli* and *L. monocytogenes* over the coupon despite the differences up to 3 log present between these species at 24 h (Figs. 1, 2). Almeida et al. reported that in 48 h *L. monocytogenes-E. coli* biofilms grown on stainless steel and plastic, species presented this sort of uniform distribution with *E. coli* being present in higher numbers (Almeida et al. 2011). The fact that green fluorescence, corresponding to *L. monocytogenes* cells, was similar to red despite viable counts (Fig. 1), could have been caused in part because a fraction of this green signal was emitted by cells in the viable but non culturable (VBNC) state. Previous authors have observed that 24 h-old *L. monocytogenes* biofilms present a part of VBNC (Gião & Keevil 2014). In

such condition, GFP remains totally functional and fluoresces even though cells are not able to grow in solid media (Cho & Kim 1999; Lowder et al. 2000).

Enzymes have been previously used as a biofilm removal strategy due to their specificity and their low environmental impact (Cordeiro & Werner 2011; Thallinger et al. 2013; Meireles et al. 2016). In this work, comparison between the effects of cellulase (CEL), DNaseI and pronase (PRN) demonstrated a maximum effect of a 400 µg/ml of DNaseI solution reducing about 2 log CFU/cm^2 the number of AVC in *L. monocytogenes* and *E. coli* (Fig. 3). This reduction was followed by that produced by PRN and CEL, despite no broad differences were observed between these two (Fig. 3).

It has been reported that extracellular DNA (eDNA) is present in considerable amounts of the extracellular matrix and considered as a requisite for biofilm formation in *L. monocytogenes* (Harmsen et al. 2010) as well as in other Gram-positives (Qin et al. 2007; Vilain et al. 2009). Hence, DNaseI has been proposed as an antibiofilm enzyme cleaving eDNA and thus interfering in biofilm development. As an example, Harmsen et al. (2010) observed that 100 µg/ml DNaseI solution at 37 ºC, completely prevented *L. monocytogenes* EGDe biofilm formation if applied up to 24 h after strain inoculation and, from that point onwards, DNaseI antibiofilm capacity was reduced. In other Gram-positives such as *S. aureus*, 1 h contact time at 37 ºC of a 100 µg/ml DNaseI solution significantly reduced the biomass of 24 h biofilms grown on polystyrene plates (Izano et al. 2008). Despite this previously reported data, no complete removal with DNaseI was achieved among the experiments performed in this work. This could be due to the application of a more realistic time of action (30 min) or to the biofilm age, which could affect DNaseI biofilm removal activity (Harmsen et al. 2010). Experimental data also showed an inverted effect of DNaseI at concentrations higher that 400 µg/ml (i.e. higher doses produced a lower log reduction), both in *L. monocytogenes* and *E. coli* (Fig. 3).

Nguyen & Burrows (2014) demonstrated a similar enzymatic stimulatory effect on planktonic *L. monocytogenes* cells in which the more proteinase K present in the culture, the more stimulated its growth was. Focusing in our experimental approach, these effects in the number of cells in the planktonic state, could have had eventually provoked an upturn in the number of cells adhered to the biofilm detected in AVC assays.

Proteases have also been proved to be effective in removing biofilms. In this line, Nguyen & Burrows (2014) demonstrated that the addition of 100 µg/ml of proteinase K for 24 h is able to disperse 72 h *L. monocytogenes* biofilms grown on polystyrene up to undetectable levels. In *S. aureus* it has been recently reported that active proteases remove biofilms formed in polystyrene plates (Stiefel et al. 2016). However, PRN effects against *L. monocytogenes* were lower than expected compared with DNaseI considering the proteinaceous nature of *L. monocytogenes* biofilm matrix (Combrouse et al. 2013; Nguyen & Burrows 2014) even though it has been demonstrated that teichoic acids are also present (Brauge et al. 2016).

Previous investigations have reported that interspecies interactions that take place within multi-species biofilms significantly modify the matrix composition if compared with monocultures (Giaouris et al. 2015; Sanchez-Vizuete et al. 2015). This differential composition can affect, among others, the efficacy of enzymes as well as several antimicrobial compounds (Sanchez-Vizuete et al. 2015). In our particular case, the dominance of *E.coli* in 24 h biofilms (Fig. 1) could have given rise to a matrix with a higher polysaccharide content as proposed for most Gram-negative bacteria (Sutherland 2001). Nevertheless, CEL showed the lowest effects against *L. monocytogenes-E. coli* biofilms perhaps because polysaccharide constituents interacted among themselves and among other molecules present thus concealing enzyme targets or they simply lack on

glucose-glucose bonds susceptible to cleavage by CEL specific ß (1→4) endoglucanase activity.

Considering the aforementioned results, it is logical to think that the use of dual-species biofilms represents a more challenging environment for biofilm-degrading enzymes due to a higher matrix complexity. Thus, the idea of a combination of enzymes would be an interesting option to be considered for proper biofilm removal (Simões et al. 2010; Meireles et al. 2016) especially when dealing with Gram-negatives such as *Pseudomonas* sp. (Stiefel et al. 2016). Efficacy of enzymatic mixtures have been previously reported by Orgaz et al., (2006) using proteinase, cellulase, pectinesterase, pectin lyase and alginate lyase derived from fungal cultures against 24-hour-old *P. fluorescens* B52 biofilms on glass achieving removal values up to an 84 % of the total biomass.

In any case, enzymatic solutions show only dispersing-but-not-killing effect as previously reported (Nguyen & Burrows 2014). As a consequence, enzyme based disinfection may need to be performed in combination with biocides that are able to kill the cells avoiding the dispersion of live cells released from the biofilm (Meireles et al. 2016; Stiefel et al. 2016).

In food related premises, RVC could provoke a pathogen thus enhancing the formation of new reservoirs and increasing the probability of product contamination. Also, pathogens could be easily spread through rinse after disinfection via water or aerosols produced (Todd et al. 2009) or by means of typical cleaning tools such as sponges or wipes (Kusumaningrum et al. 2003). Therefore, controlling RVC after cleaning and disinfection treatments appears to be as an interesting topic to consider for further investigation.

Enzyme-BAC combined treatments showed a differential effect on *L. monocytogenes*-*E. coli* biofilms depending on the species. More specifically, BAC performed better against *L. monocytogenes* when preceded by DNaseI whereas removal of *E. coli* from the coupon was higher after PRN-BAC treatment (Table 2, Fig. 4). In *L. monocytogenes* the lower BAC RD_{90} values obtained after DNaseI treatment indicated that despite proteins are considered the main fraction in *L. monocytogenes* biofilm matrix (Combrouse et al. 2013), eDNA degradation by DNaseI provokes a higher decrease in *L. monocytogenes* AVC counts thus confirming the key role of eDNA to maintain already formed biofilms (Harmsen et al. 2010; Nguyen & Burrows 2014). This biofilm-dispersing capacity of DNaseI to facilitate BAC access into the biofilm is especially relevant in *L. monocytogenes*-carrying biofilms as this bacterium is usually located in the bottom layers (Almeida et al. 2011) . In *E. coli*, a better performance of BAC after enzymatic dwelling was also observed but to a lesser extent (Fig. 4). This can be attributed to its intrinsic higher resistance to QACs (Mcdonnell & Russell 1999; Augustin et al. 2004) and also because of the possible presence of protective colanic acid capsules (Miajlovic & Smith 2014).

It is important to remark the fact that BAC effects against 48 h samples were different depending on the species (Fig. 4) whereas in 24 h biofilms DNaseI was the most efficient enzyme in both species of the mixed biofilms (Fig. 3). This points out that the biofilm matrix varies its molecular composition along time. So, if proper enzyme-based biofilm cleaning strategies are intended to be designed it is important to determine the constituents (proteins, eDNA and polysaccharides) of the matrix of the target sessile community.

A release of live cells of both species is observed from biofilms after PRN-BAC or DNaseI-BAC treatments, especially at low BAC concentrations (Fig. 6). Pathogen dispersal after sanitation is a factor to take into account in cleaning and disinfection

methodologies (Cordeiro & Werner 2011; Nguyen & Burrows 2014). This fact can be minimised by using appropriate effective concentrations of disinfectants (e.g. BAC) after dispersing agents, enzymes in this particular case, to avoid dissemination of live cells in adjacent areas after biofilm removal.

Another important issue to be assessed in dual-species biofilms of *L. monocytogenes* is the role of the accompanying species. Significant differences were observed in the *L. monocytogenes* AVC counts, as well as in the effect of the enzyme and enzyme-BAC treatment depending on the accompanying bacterium (Fig. 6). Regarding the first, a higher number of *L. monocytogenes* A1 cells was attached to stainless steel after 48 h in presence of *P. fluorescens* respecting to *E. coli*, probably due to an entrapping of the *L. monocytogenes* into the polymeric matrix secreted by the *P. fluorescens*. Morphological features agreed with previously reported data in which *L. monocytogenes-E.coli* biofilms appeared as uniform layers (Almeida et al. 2011) whereas *L. monocytogenes-P. fluorescens* were characterised by local microcolony formation surrounded by smaller biofilm aggregates randomly distributed (Fig. 7) (Puga et al. 2014).

DNaseI produced a significant decrease of *L. monocytogenes* only in the mixed biofilm with presence of *E. coli*, probably because matrix composition differently affected its diffusion and effectiveness (Fig. 6) (Allison 2003). Nevertheless, the application of BAC against *L. monocytogenes* was more effective when co-cultured with *P. fluorescens* despite the latter is considered a strong biofilm former (Fig. 6) (Allison et al. 1998; Jackson et al. 2001).

In summary, in this work the effectiveness of treatments with an enzyme solution alone and combined with a BAC dose on *L. monocytogenes* dual-species biofilms was demonstrated. In addition to this, results demonstrated that the removal efficacy of a

combined enzyme-BAC treatment against mixed biofilms depends not only on the enzyme chosen but also on the biofilm species composition. Following this idea, for proper biofilm removal in food related surfaces as well as in others capable of harbour bacterial biofilms, customised treatments depending on the species composition should be considered when developing new cleaning and disinfection methodologies. This would be intended not only to impede biofilm formation but also to significantly remove already present structures while minimising the amount of live cells released.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Allison DG. 2003. The biofilm matrix. Biofouling. 19:139–50.

Allison DG, Ruiz B, Sanjose C, Jaspe A, Gilbert P. 1998. Extracellular products as mediators of the formation and detachment of Pseudomonas fluorescens biofilms. FEMS Microbiol Lett. 167:179–184.

Almeida C, Azevedo NF, Santos S, Keevil CW, Vieira MJ. 2011. Discriminating multi-species populations in biofilms with peptide nucleic acid fluorescence in situ hybridization (PNA FISH). PLoS One. 6:e14786.

Augustin M, Ali-Vehmas T, Atroshi F. 2004. Assessment of enzymatic cleaning agents and disinfectants against bacterial biofilms. J Pharm Pharm Sci. 7:55–64.

Azevedo AS, Almeida C, Melo LF, Azevedo NF. 2014. Interaction between atypical microorganisms and E. coli in catheter-associated urinary tract biofilms. Biofouling. 30:893–902.

Borucki MK, Peppin JD, White D, Loge F, Call DR. 2003. Variation in biofilm formation among strains of Listeria monocytogenes. Appl Environ Microbiol. 69:7336–7342.

Brauge T, Sadovskaya I, Faille C, Benezech T, Maes E, Guerardel Y, Midelet-Bourdin G. 2016. Teichoic acid is the major polysaccharide present in the Listeria monocytogenes biofilm matrix. FEMS Microbiol Lett. 363:fnv229.

Bridier A, Sanchez-Vizuete P, Guilbaud M, Piard JC, Naïtali M, Briandet R. 2015. Biofilm-associated persistence of food-borne pathogens. Food Microbiol. 45:167–178.

Bron PA, Monk IR, Corr SC, Hill C, Gahan CGM. 2006. Novel luciferase reporter system for in vitro and organ-specific monitoring of differential gene expression in Listeria

monocytogenes. Appl Environ Microbiol. 72:2876–84.

Brooks JD, Flint SH. 2008. Biofilms in the food industry: problems and potential solutions. Int J Food Sci Technol. 43:2163–2176.

Cabo ML, Murado MA, Gonzalez MP, Pastoriza L. 1999. A method for bacteriocin quantification. J Appl Microbiol. 87:907–914.

Carpentier B, Cerf O. 2011. Review - Persistence of Listeria monocytogenes in food industry equipment and premises. Int J Food Microbiol. 145:1–8.

Carpentier B, Chassaing D. 2004. Interactions in biofilms between Listeria monocytogenes and resident microorganisms from food industry premises. Int J Food Microbiol. 97:111–22.

Chaveroche MK, Ghigo JM, d'Enfert C. 2000. A rapid method for efficient gene replacement in the filamentous fungus Aspergillus nidulans. Nucleic Acids Res. 28:E97.

Cho J-C, Kim S-J. 1999. Viable, but non-culturable, state of a green fluorescence protein-tagged environmental isolate of Salmonella typhi in groundwater and pond water. FEMS Microbiol Lett. 170:257–264.

Combrouse T, Sadovskaya I, Faille C, Kol O, Guérardel Y, Midelet-Bourdin G. 2013. Quantification of the extracellular matrix of the Listeria monocytogenes biofilms of different phylogenic lineages with optimization of culture conditions. J Appl Microbiol. 114:1120–31.

Cordeiro AL, Werner C. 2011. Enzymes for Antifouling Strategies. J Adhes Sci Technol. 25:2317–2344.

EFSA. 2015. The European Union summary report on trends and sources of zoonoses,

zoonotic agents and food-borne outbreaks in 2014. EFSA J. 13:4329.

Farber JM, Peterkin PI. 1991. Listeria monocytogenes, a food-borne pathogen. Microbiol Rev. 55:476–511.

Fortineau N, Trieu-Cuot P, Gaillot O, Pellegrini E, Berche P, Gaillard J-L. 2000. Optimization of green fluorescent protein expression vectors for in vitro and in vivo detection of Listeria monocytogenes. Res Microbiol. 151:353–360.

Freitag NE, Port GC, Miner MD. 2009. Listeria monocytogenes - from saprophyte to intracellular pathogen. Nat Rev Microbiol. 7:623–8.

Gerba CP. 2015. Quaternary Ammonium Biocides: Efficacy in Application. Appl Environ Microbiol. 81:464–469.

Gião MS, Keevil CW. 2014. Listeria monocytogenes can form biofilms in tap water and enter into the viable but non-cultivable state. Microb Ecol. 67:603–11.

Giaouris E, Chorianopoulos N, Doulgeraki A, Nychas G-J. 2013. Co-culture with Listeria monocytogenes within a dual-species biofilm community strongly increases resistance of Pseudomonas putida to benzalkonium chloride. PLoS One. 8:e77276.

Giaouris E, Heir E, Desvaux M, Hébraud M, Møretrø T, Langsrud S, Doulgeraki A, Nychas G-J, Kačániová M, Czaczyk K, et al. 2015. Intra- and inter-species interactions within biofilms of important foodborne bacterial pathogens. Front Microbiol. 6:1–26.

Giaouris E, Heir E, Hébraud M, Chorianopoulos N, Langsrud S, Møretrø T, Habimana O, Desvaux M, Renier S, Nychas GJ. 2014. Attachment and biofilm formation by foodborne bacteria in meat processing environments: Causes, implications, role of bacterial interactions and control by alternative novel methods. Meat Sci. 97:289–309.

Gonzales MF, Brooks T, Pukatzki SU, Provenzano D. 2013. Rapid protocol for preparation of electrocompetent Escherichia coli and Vibrio cholerae. J Vis Exp. 50684:e50684.

Harmsen M, Lappann M, Knøchel S, Molin S. 2010. Role of extracellular DNA during biofilm formation by Listeria monocytogenes. Appl Environ Microbiol. 76:2271–9.

Hillyar CRT. 2012. Genetic recombination in bacteriophage lambda. Biosci Horizons. 5:1–7.

Houari A, Di Martino P. 2007. Effect of chlorhexidine and benzalkonium chloride on bacterial biofilm formation. Lett Appl Microbiol. 45:652–6.

Izano EA, Amarante MA, Kher WB, Kaplan JB. 2008. Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in Staphylococcus aureus and Staphylococcus epidermidis biofilms. Appl Environ Microbiol. 74:470–476.

Jackson G, Beyenal H, Rees WM, Lewandowski Z. 2001. Growing reproducible biofilms with respect to structure and viable cell counts. J Microbiol Methods. 47:1–10.

Johansen C, Falholt P, Gram L. 1997. Enzymatic removal and disinfection of bacterial biofilms. Appl Environ Microbiol. 63:3724–3728.

Kaplan JB. 2009. Therapeutic potential of biofilm-dispersing enzymes. Int J Artif Organs. 32:545–554.

Kaplan JB. 2014. Biofilm matrix-degrading enzymes. Methods Mol Biol. 1147:203–13.

Knight KP, McKellar RC. 2007. Influence of cinnamon and clove essential oils on the D- and z-values of Escherichia coli O157:H7 in apple cider. J Food Prot. 70:2089–94.

Kusumaningrum HD, Riboldi G, Hazeleger WC, Beumer RR. 2003. Survival of

foodborne pathogens on stainless steel surfaces and cross-contamination to foods. Int J Food Microbiol. 85:227–236.

Lagendijk EL, Validov S, Lamers GEM, de Weert S, Bloemberg G V. 2010. Genetic tools for tagging Gram-negative bacteria with mCherry for visualization in vitro and in natural habitats, biofilm and pathogenicity studies. FEMS Microbiol Lett. 305:81–90.

Lauer P, Yin M, Chow N, Loessner MJ, Portnoy A, Calendar R, Portnoy DA. 2002. Construction , Characterization , and Use of Two Listeria monocytogenes Site-Specific Phage Integration Vectors Construction , Characterization , and Use of Two Listeria monocytogenes Site-Specific Phage Integration Vectors. J Bacteriol. 184:4177–4186.

Lequette Y, Boels G, Clarisse M, Faille C. 2010. Using enzymes to remove biofilms of bacterial isolates sampled in the food-industry. Biofouling. 26:421–31.

Lowder M, Unge A, Maraha N, Jansson JK, Swiggett J, Oliver JD. 2000. Effect of Starvation and the Viable-but-Nonculturable State on Green Fluorescent Protein (GFP) Fluorescence in GFP-Tagged Pseudomonas fluorescens A506. Appl Environ Microbiol. 66:3160–3165.

Mcdonnell G, Russell AD. 1999. Antiseptics and disinfectants: Activity, action, and resistance. Clin Microbiol Rev. 12:147–179.

Meireles A, Borges A, Giaouris E, Simões M. 2016. The current knowledge on the application of anti-biofilm enzymes in the food industry. Food Res Int. 86:140–146.

Miajlovic H, Smith SG. 2014. Bacterial self-defence: How Escherichia coli evades serum killing. FEMS Microbiol Lett. 354:1–9.

Monk IR, Gahan CGM, Hill C. 2008. Tools for functional postgenomic analysis of listeria

monocytogenes. Appl Environ Microbiol. 74:3921–34.

Murado MA, González MP, Vázquez JA. 2002. Dose–response relationships: an overview, a generative model and its application to the verification of descriptive models. Enzyme Microb Technol. 31:439–455.

Murado MA, Vázquez JA. 2007. The notion of hormesis and the dose-response theory: A unified approach. J Theor Biol. 244:489–499.

Nakamura H, Takakura K-I, Sone Y, Itano Y, Nishikawa Y. 2013. Biofilm formation and resistance to benzalkonium chloride in Listeria monocytogenes isolated from a fish processing plant. J Food Prot. 76:1179–86.

Nguyen UT, Burrows LL. 2014. DNase I and proteinase K impair Listeria monocytogenes biofilm formation and induce dispersal of pre-existing biofilms. Int J Food Microbiol. 187:26–32.

Orgaz B, Kives J, Pedregosa AM, Monistrol IF, Laborda F, SanJose C. 2006. Bacterial biofilm removal using fungal enzymes. Enzyme Microb Technol. 40:51–56.

Parkar SG, Flint SH, Brooks JD. 2004. Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel. J Appl Microbiol. 96:110–116.

Puga CH, SanJose C, Orgaz B. 2014. Spatial distribution of Listeria monocytogenes and Pseudomonas fluorescens in mixed biofilms. In: Hambrick EC, editor. List monocytogenes Food Sources, Preval Manag Strateg. New York, NY: Nova Publishers; p. 115–132.

Qin Z, Ou Y, Yang L, Zhu Y, Tolker-Nielsen T, Molin S, Qu D. 2007. Role of autolysin-mediated DNA release in biofilm formation of Staphylococcus epidermidis.

Microbiology. 153:2083–2092.

Rodríguez-López P, Saá-Ibusquiza P, Mosquera-Fernández M, López-Cabo M. 2015. Listeria monocytogenes – carrying consortia in food industry. Composition, subtyping and numerical characterisation of mono-species biofilm dynamics on stainless steel. Int J Food Microbiol. 206:84–95.

Saá Ibusquiza P, Herrera JJR, Cabo ML. 2011. Resistance to benzalkonium chloride, peracetic acid and nisin during formation of mature biofilms by Listeria monocytogenes. Food Microbiol. 28:418–25.

Sanchez-Vizuete P, Orgaz B, Aymerich S, Le Coq D, Briandet R. 2015. Pathogens protection against the action of disinfectants in multispecies biofilms. Front Microbiol. 6:705.

Serra-Moreno R, Acosta S, Hernalsteens JP, Jofre J, Muniesa M. 2006. Use of the lambda Red recombinase system to produce recombinant prophages carrying antibiotic resistance genes. BMC Mol Biol. 7:31.

da Silva EP, De Martinis ECP. 2013. Current knowledge and perspectives on biofilm formation: the case of Listeria monocytogenes. Appl Microbiol Biotechnol. 97:957–68.

Simões M, Simões LC, Vieira MJ. 2010. A review of current and emergent biofilm control strategies. LWT - Food Sci Technol. 43:573–583.

Stiefel P, Mauerhofer S, Schneider J, Maniura-Weber K, Rosenberg U, Ren Q. 2016. Enzymes enhance biofilm removal efficiency of cleaners. Antimicrob Agents Chemother. 60:3647–3652.

Sutcliffe JG. 1978. Nucleotide sequence of the ampicillin resistance gene of Escherichia

coli plasmid pBR322. Proc Natl Acad Sci. 75:3737–3741.

Sutherland IW. 2001. Microbial polysaccharides from Gram-negative bacteria. Int Dairy J. 11:663–674.

Sutton S. 2011. Accuracy of plate counts. J Valid Technol. 17:42–46.

Swaminathan B, Gerner-Smidt P. 2007. The epidemiology of human listeriosis. Microbes Infect. 9:1236–1243.

Thallinger B, Prasetyo EN, Nyanhongo GS, Guebitz GM. 2013. Antimicrobial enzymes: an emerging strategy to fight microbes and microbial biofilms. Biotechnol J. 8:97–109.

Todd ECD, Greig JD, Bartleson CA, Michaels BS. 2009. Outbreaks Where Food Workers Have Been Implicated in the Spread of Foodborne Disease. Part 6. Survival of Pathogens in the Food Processing and Preparation environment. J Food Prot. 72:202–219.

Vázquez-Boland JA, Domínguez-Bernal G, González-Zorn B, Kreft J, Goebel W. 2001. Pathogenicity islands and virulence evolution in Listeria. Microbes Infect. 3:571–584.

Vickery K, Pajkos A, Cossart Y. 2004. Removal of biofilm from endoscopes: Evaluation of detergent efficiency. Am J Infect Control. 32:170–176.

Vilain S, Pretorius JM, Theron J, Brözel VS. 2009. DNA as an adhesin: Bacillus cereus requires extracellular DNA to form biofilms. Appl Environ Microbiol. 75:2861–8.

FIGURES

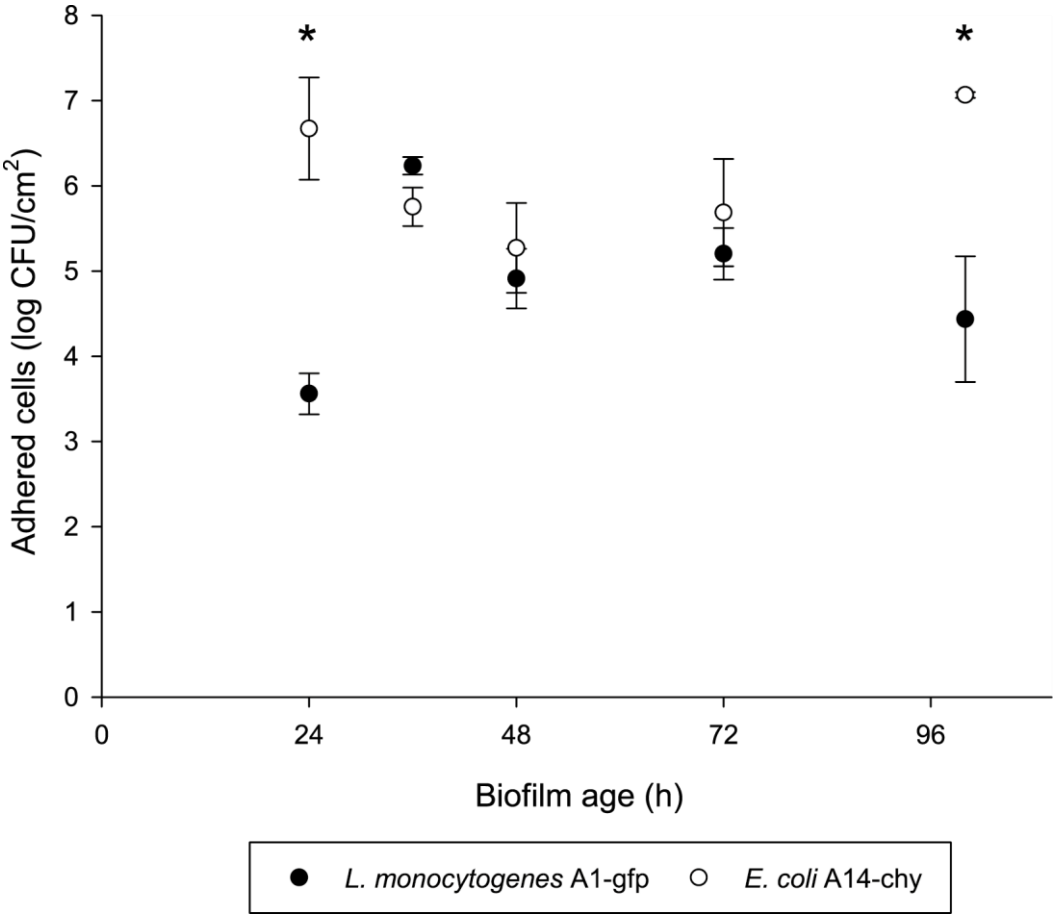

Figure 1. Growth dynamics of the *L. monocytogenes*-gfp-*E. coli*-mChy dual-species biofilm. Asterisks indicate statistically significant differences ($\alpha = 0.05$). Error bars = SD values of each sampling time dataset (n =3).

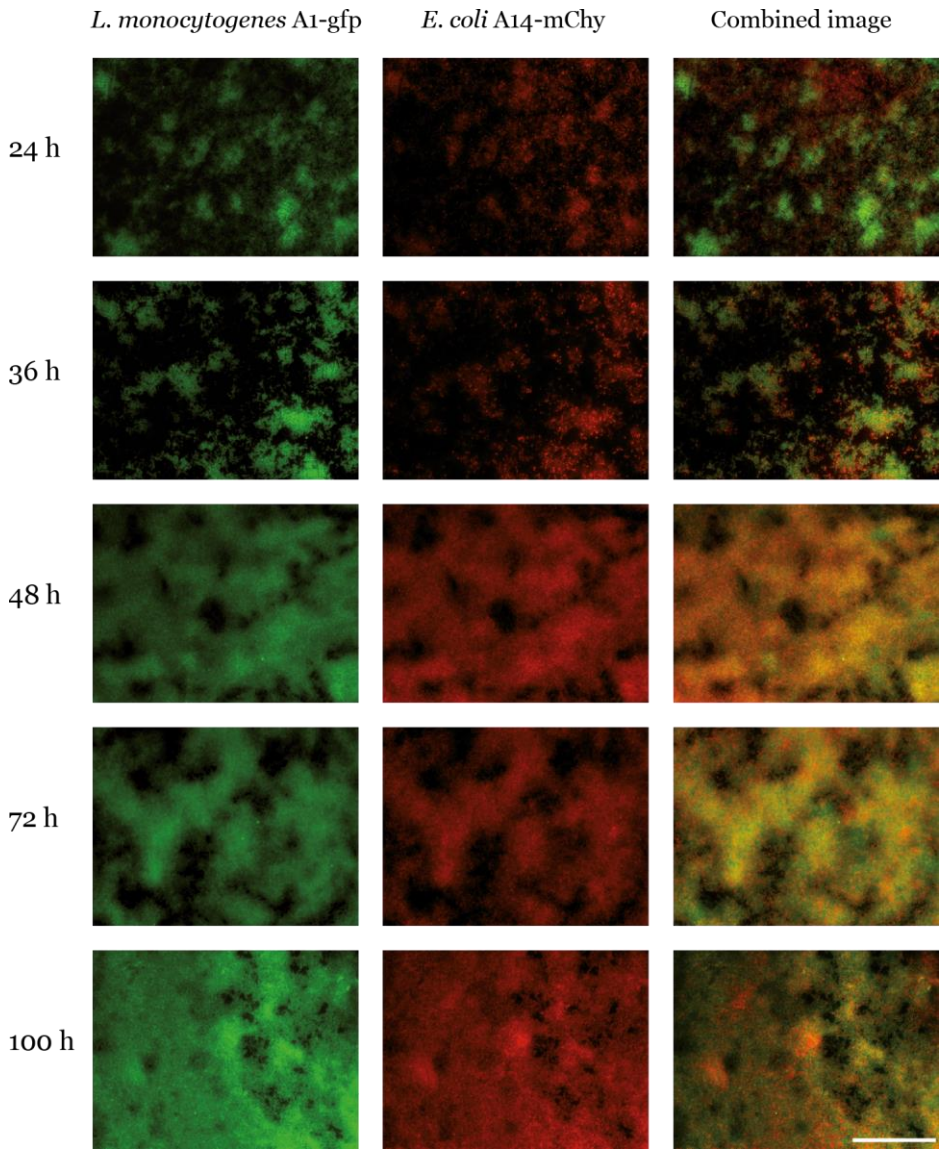

Figure 2. Fluorescence microscopy 40x-field images of *L. monocytogenes* A1-gfp, *E. coli* A14-mChy and combined fields in dual-species biofilm formation kinetics. Scale bar = 100 µm.

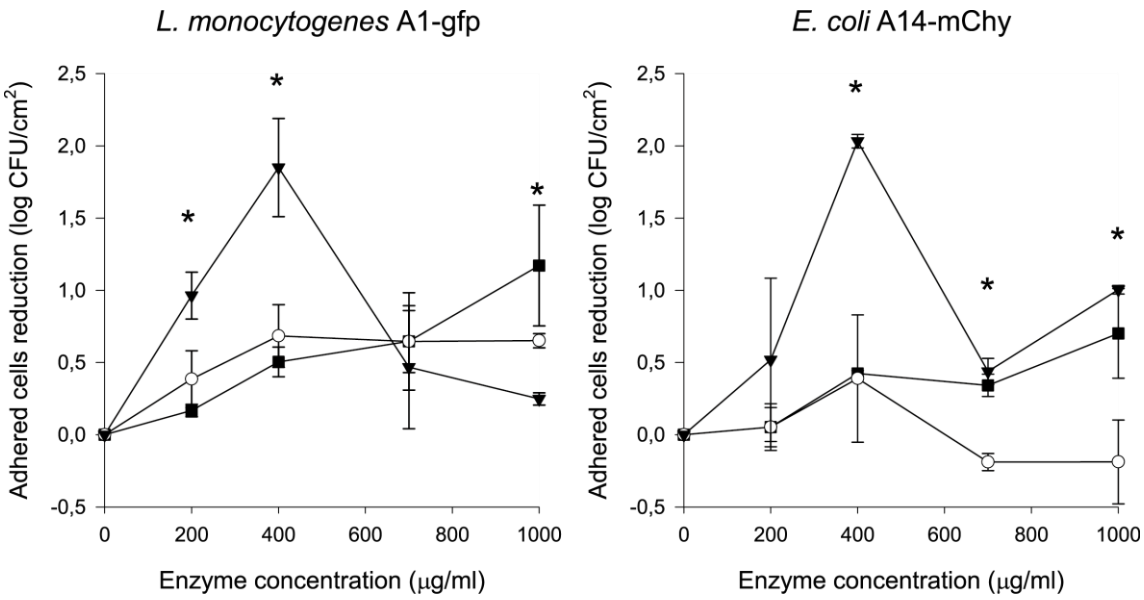

Figure 3. Logarithmic reductions of adhered cells obtained on 24 h *L. monocytogenes* A1-gfp-*E. coli* A14-mChy dual-species biofilms after an enzymatic treatment with PRN (■), CEL (○) or DNAseI (▼). Asterisks indicate statistically significant differences in any of the treatments at a given concentration. Error bars = SD of each dataset (n = 3).

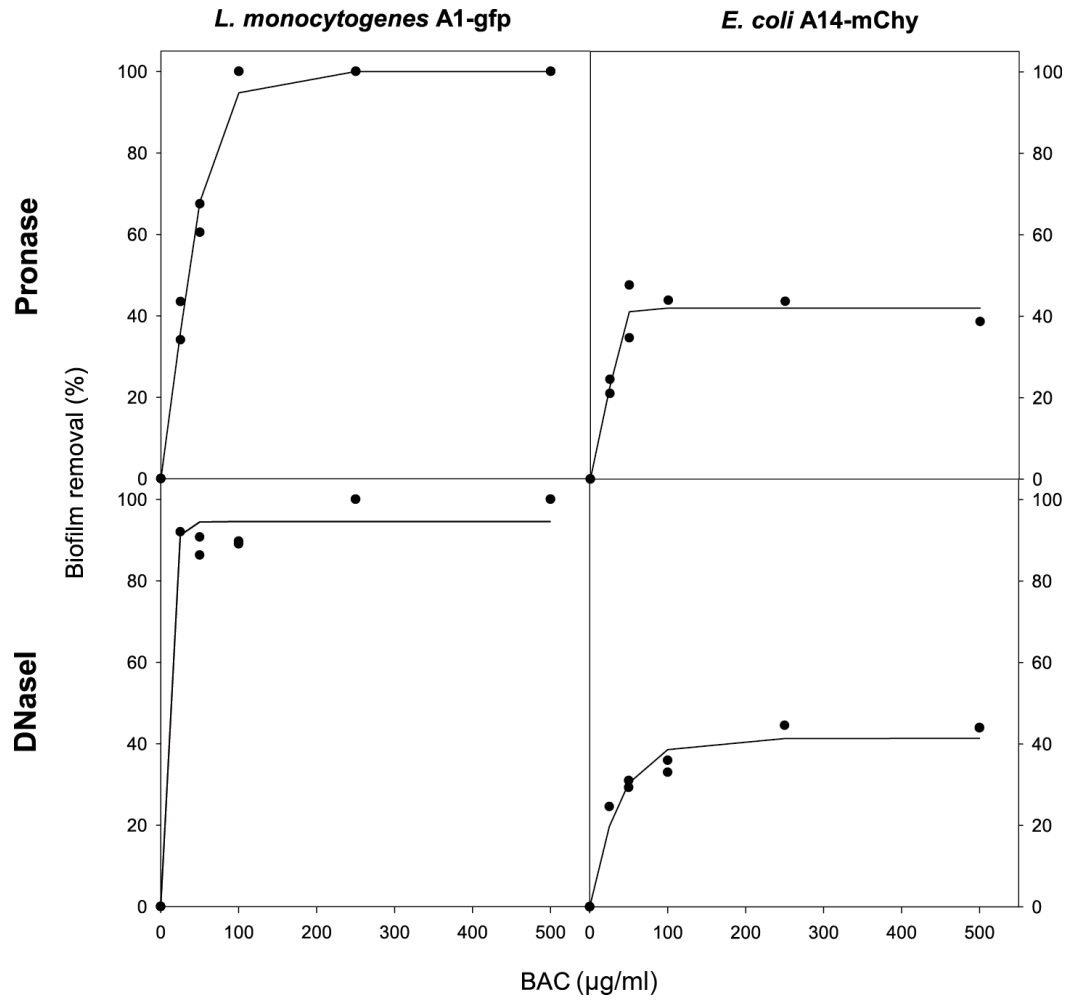

Figure 4. Lethal dose 90. Fit of biofilm removal values against *L. monocytogenes-E. coli* mixed biofilms obtained after the application of PRN-BAC or DNaseI-BAC treatments according to equation [1].

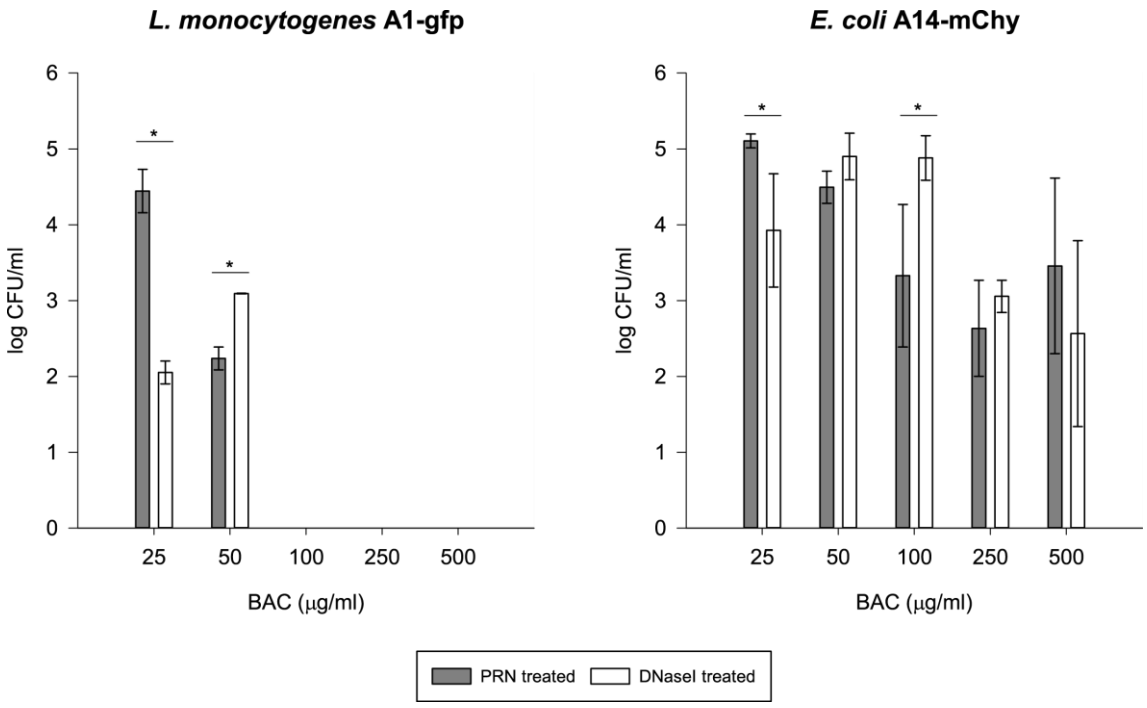

Figure 5. Released viable cells of *L. monocytogenes* (left) and *E. coli* (right) coming from 48 h dual-species biofilms after the application of different BAC solutions following a single dose of a 400 μg/ml solution of pronase (filled bars) or DNaseI (void bars). Error bars = SD of each sample set. Asterisks indicate statistically significant differences between enzymatic treatments at each BAC concentration ($\alpha = 0.05$).

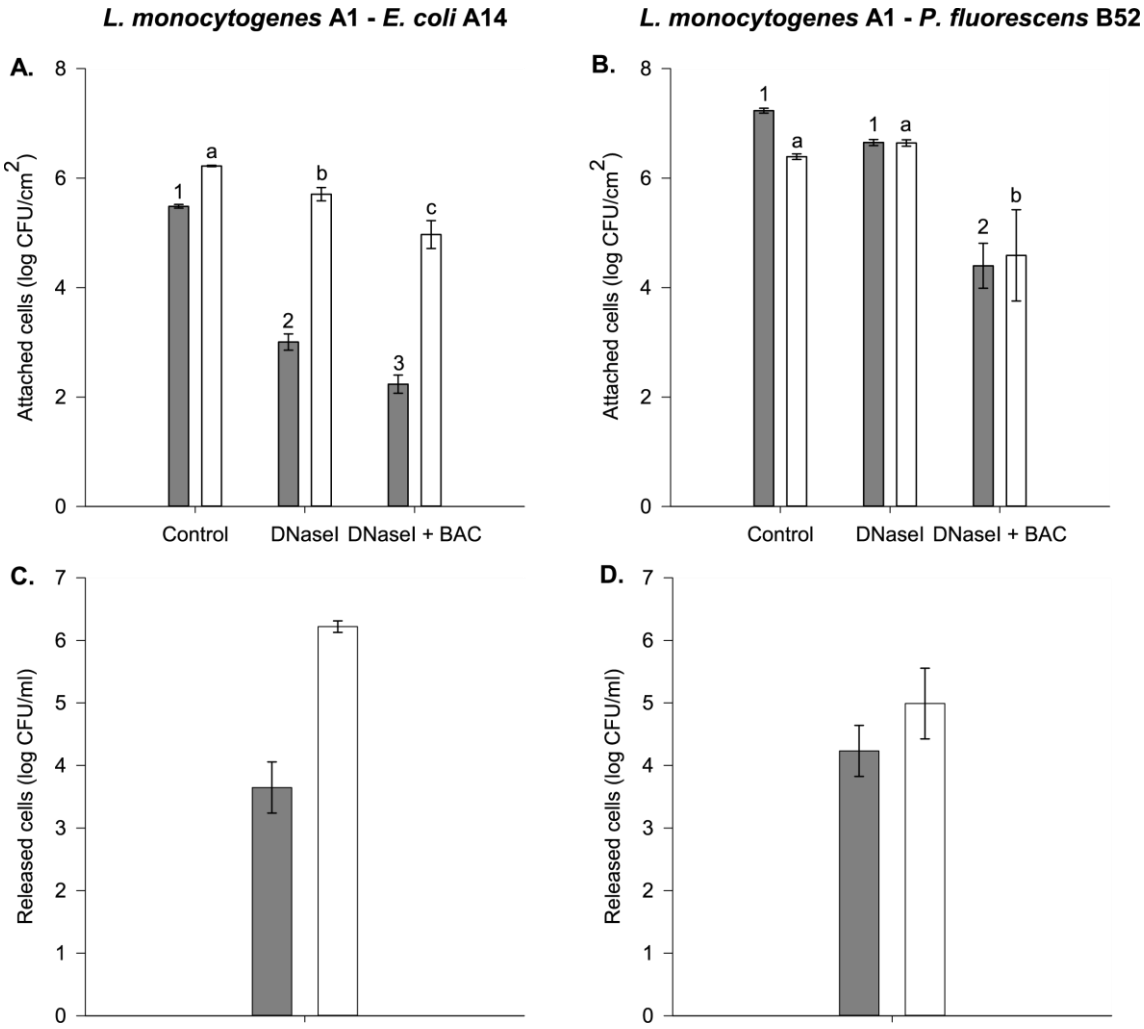

Figure 6. Sensitivity of 48 h *L. monocytogenes* A1 dual-species biofilms to the application of DNaseI and DNaseI-BAC. A, B: Number of viable attached cells of *L. monocytogenes* (filled bars) and of *E. coli* A14 (A) and *P. fluorescens* B52 (B) (void bars). For each species separately, bars with different number or letter indicate significant differences ($\alpha = 0.05$). C, D: Number of viable released cells after the DNaseI–BAC treatment. Error bars represent the standard deviation of each sample set (n = 3).

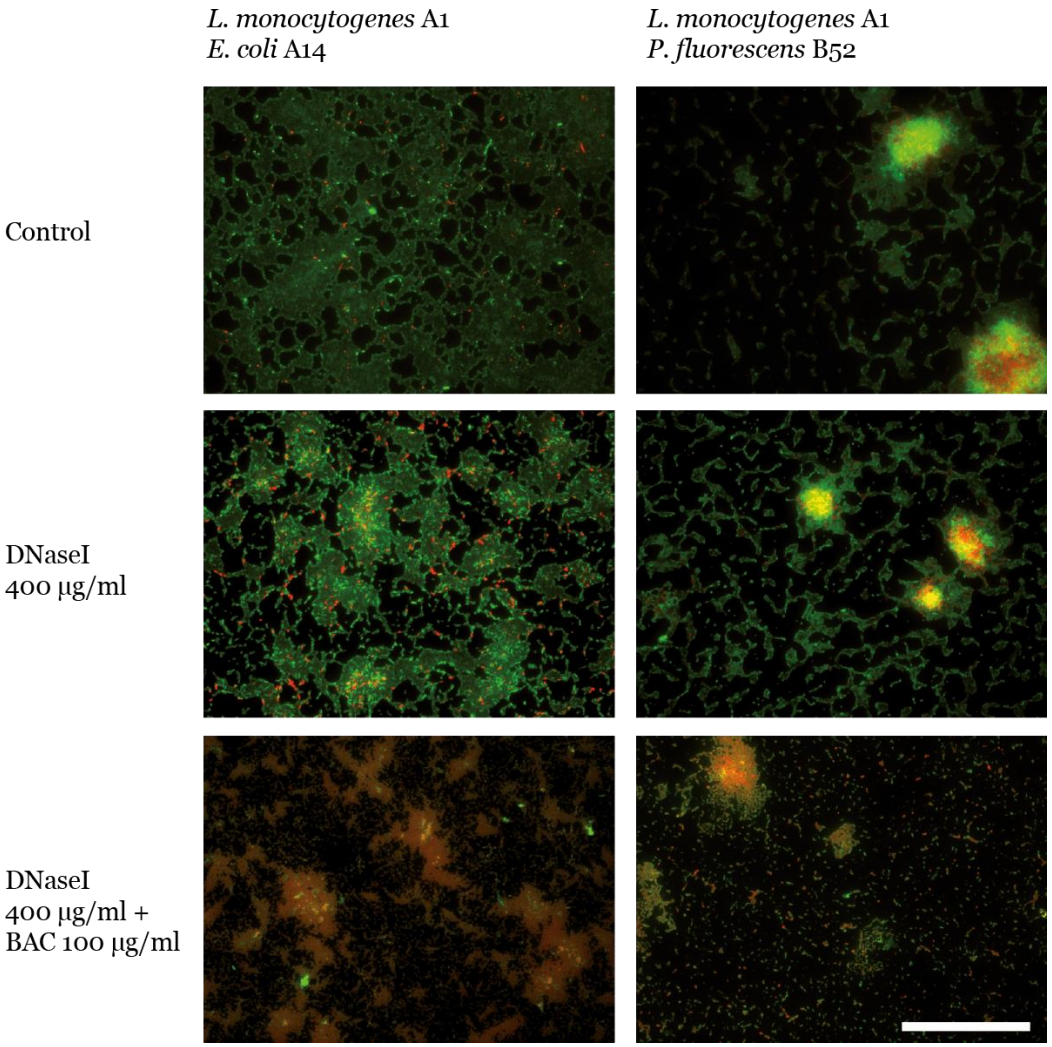

Figure 7. Fluorescence microscopy 40x-field images for comparison of the effects of DNaseI-BAC combined treatments in two different 48 h *L. monocytogenes* dual-species biofilms. Scale bar = 100 µm.

Primer	Sequence (5'→3')	Reference
P*dlt* For-*Kpn*I	TGGGTACCATTATACTCGTACCTAC	This study
GFP pNF Rev-*Pst*I	AAACTGCATTTATTTGTATAGTTCATCCATGCCA	This study
MCS for	GACGTCAATACGACTCACTATAGG	This study
pPL2 MCS-rev	GATAATAAGCGGATGAATGGCAG	This study
PL95	ACATAATCAGTCCAAAGTAGATGC	Bron et al. 2006
PL102	TATCAGACCTAACCCAAACCTTCC	Bron et al. 2006

For, forward; Rev, Reverse; Underlined, Restriction site

Table 1. Sequences of primers used in this work.

	L. monocytogenes A1-gfp		*E. coli* A14-chy	
	K' (%)	BAC RD_{90} (mg/Kg)	K' (%)	BAC RD_{90} (mg/Kg)
Pronase	100.00	82.28	42.06	38.90
DNaseI	94.59	16.74	41.39	82.10

Table 2. Parameters obtained after fitting biofilm removal experimental data to equations [1] to [3]. Maximum percentage of reduction (K') and real lethal dose 90 (RD_{90}) values obtained due to BAC action after a single application of 400 µg/ml solution of either PRN or DNaseI.