Enzymatic dispersal of dual-species biofilms carrying *Listeria monocytogenes* and other associated food industry bacteria

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Abstract

Enzyme-based treatments could therefore be used to complement regular cleaning processes. Most studies using enzymes as anti-biofilms strategy are focused on their outcome in mono-species biofilms. Nevertheless, in real environments mixed biofilms are prevalent. In this work, seven types of dual-species biofilms were selected to serve as targets for enzymatic treatments carrying different environmental strains of *L. monocytogenes* and accompanying bacteria isolated from dairy, meat and seafood processing plants. The effectiveness of nine commercial enzymatic preparations, including pronase, cellulase, pectinase, DNase I, lysozyme, phospholipase, peroxidase, β-glucanase and chitinase, was evaluated. For this, residual attached viable cells of both *L. monocytogenes* and its partners were enumerated through swabbing and colony plate counting following the action of each enzyme. Moreover, Confocal Laser Scanning Microscopy (CLSM) images were analyzed pre and after enzymatic treatments in order to quantify changes in biofilm thickness, covered area and volume. The viable attached population of *L. monocytogenes* was almost unaffected by all of the enzymes here tested, being eliminated on average just the 90% of the initially attached population (around 1 Log_{10} cfu·cm^{-2} reduction). Nevertheless, some of the partner species (*Escherichia coli* and *Staphylococcus saprophyticus*) were sometimes protected from

enzymatic detachment when in dual-species biofilms, depending on the enzyme tested and the accompanying *L. monocytogenes* strain. CLSM images showed important changes in biofilm covered area and volume after DNase I, pronase and pectinase treatments. These results demonstrate that enzymes can greatly weaken dual-species biofilms structure. Nevertheless, it cannot be disregarded that detached cells from these treatments would still be viable. Thus, a control of cell viability after an enzymatic procedure in the food industry must be always considered before designing an efficient disinfection treatment.

Keywords: *Listeria monocytogenes*, dual-species, biofilms, enzymatic cleaning, DNase, CLSM, food industry.

1. Introduction

Listeria monocytogenes is a food-borne pathogen ubiquitous in the environment. Its entrance into food processing plants can occur through many different routes, including raw materials and workers and if not eliminated, could finally attach to food contact surfaces (Møretrø & Langsrud, 2004). Once there, it may persist due to its ability to stand different environmental stresses and to form biofilms, among other factors (Magalhaes et al., 2017; Valderrama & Cutter, 2013). It is well known, however, that in natural environments bacteria rarely live in isolation. Indeed, multi-species biofilms are most likely to be the dominant lifestyle in environments such as food industry facilities, where they facilitate resilience to harsh conditions and provide shelter to certain microorganisms that would otherwise have little chance of surviving in these settings (Elias & Banin, 2012; Jahid & Ha, 2014; Little A.E.F. et al., 2008). The resident microbiota established in food processing plants, is able to adapt to the nutrients available, the growth-limiting physicochemical conditions and the disinfectants normally used during cleaning and disinfection (C&D) regimes (Bagge-Ravn et al., 2003; Flemming et al., 2016; Srey, Jahid, & Ha, 2013). Likewise, when present in those habitats, *L. monocytogenes* shares surfaces and shelters with other compatible bacteria, forming multi-species biofilms.

Despite the risk associated to the presence of *L. monocytogenes*-carrying biofilms in food industry, there is not specific protocol to eradicate them apart from the standard C&D routines, nor a definite prevention strategy. New alternatives in both processing

technologies and antimicrobials are being proposed to improve food safety and in this context, enzyme technology is certainly an option (Meireles, Borges, Giaouris, & Simões, 2016; Simões, Simões, & Vieira, 2010). Concerns about the increase in antibiotic resistance and environmental distribution of biocides and their derivates are among the problems to be solved. With respect to antimicrobial enzymes, the set of choices is wide and increasing (Thallinger, Prasetyo, Nyanhongo, & Guebitz, 2013). Most of the published procedures involving enzymes as antibiofilm agents have as target the integrity of the extracellular polymeric matrix (Boles & Horswill, 2011; Johansen, Falholt, & Gram, 1997; Kaplan et al., 2004; Lu & Collins, 2007; Orgaz et al., 2006) and there are different commercial enzymatic products used in food industry as cleaning agents and disinfectants (Augustin, Ali-Vehmas, & Atroshi, 2004). Enzymes whose substrates resemble the components of the matrix may degrade these to some extent (Johansen, Falholt, & Gram, 1997). Still, knowledge about matrix composition and structure (Flemming & Wingender, 2010; Harmsen, Lappann, Knøchel, & Molin, 2010; McCrate, Zhou, Reichhardt, & Cegelski, 2013) should guide the development of these products and procedures.

Nevertheless, most enzymatic approaches have only been tested for mono-species biofilm disruption and little is known on their effectiveness on multi-species communities such as those found in food industry (Marcato-Romain et al., 2012). In addition, there is a shortage of information on the changes induced by enzymatic cleaning on biofilm structure. The aim of this work was to evaluate the effectiveness of different enzymatic solutions to remove 7 dual-species biofilm models. These models were individual combinations of 5 environmental *L. monocytogenes* strains and accompanying bacteria isolated from the same *L. monocytogenes* positive surfaces. Namely, *Pseudomonas* spp., *Staphylococcus saprophyticus* and *Carnobacterium* spp. strains were selected as representatives of the Spanish meat sector, *S. saprophyticus* and *Escherichia coli* of seafood plants and a previously studied strain of *P. fluorescens* was selected as representative of the dairy sector. To evaluate enzymatic effectiveness, the remaining viable biofilm population after the treatments was measured. Biofilm structural changes (reduction of biovolume, covered area and biofilm thickness) were studied with Confocal Laser Scanning Microscopy (CLSM).

2. Material and Methods

2.1. Bacterial strains

All the strains used in this work are listed in Table 1. All of them were isolated from *L. monocytogenes* positive surfaces in food environments after C&D procedures (Rodríguez-López, Saá-Ibusquiza, Mosquera-Fernández, & López-Cabo, 2015).

To select the seven consortia, the most representative dual-species associations for each sector (*i.e*. the most frequently found *L. monocytogenes* strain and its most frequently associated partner isolated from the same *L. monocytogenes* positive sample) were chosen as models of dual-species biofilms. In the case of dairy consortia, as Rodríguez-López and coworkers did not find any *L. monocytogenes* positive surface in the sampled Spanish dairy industries, *L. monocytogenes* strains G1 and G2 were kindly provided by Dr. Luisa Brito (Technical University of Lisbon, Portugal) (Leite et al., 2006). *Pseudomonas fluorescens* B52 was isolated from refrigerated raw milk (Richardson & Te Whaiti, 1978). Working cultures of the strains were stored in Trypticase Soy Broth (TSB, Oxoid) with 15 % glycerol at -20 °C. 100 µl of working cultures were incubated overnight in 10 ml of TSB at 25 ºC and subcultured again in order to ensure a proper growth. From these, cells were harvested by centrifugation at 4000 × g for 10 min and washed twice in sterile TSB. OD_{600} values of each culture were adjusted to 0.1 by dilution with TSB, equivalent to a bacterial concentration of 10^8 CFU·mL^{-1}. These were diluted in TSB to be used as inoculum, starting the cultures with a cell density of 10^3 CFU·mL^{-1}, both in mono-species and in dual-species cultures (in this case in a proportion 1:1).

2.2. Experimental system for biofilm development

Biofilms were developed at 25 ºC for 48 h in disposable 24-well cell culture plates (Thermo Fisher Scientific, Code number 144530) using 10 x 10 mm AISI 304 stainless steel coupons as substrata. Before use, the coupons were gently swabbed with a postsurgical toothbrush and soap solution (Vaxel, SORO laboratories, Spain), rinsed with distilled water, placed on a glass Petri dish (10 coupons per dish) (∅: 100 mm, 20 mm in height) and autoclaved (121 ºC/ 20 min.). Sterile coupons were then individually placed into each well and 1 mL of the corresponding bacterial suspension was added. In order to prevent evaporation, the whole system was wrapped in aluminum foil and incubated for 48 h under constant shaking at 125 rpm. A tray filled with water was located under the microplate. In this system, only the top face of the coupon was

considered for biofilm quantification, marking initially the reverse face, to keep its position downwards along the assay.

2.3. Enzyme solutions

The 9 enzymes tested are listed in Table 2. 1 mL aliquots of enzymatic stock solutions (1 mg/mL) prepared according to the manufacturer instructions were stored at -20 ºC. For assays, a working concentration of 0.1 mg/mL was prepared just before use. The working concentration was selected after having done previous assays using 1 mg/ml and 0.01 mg/ml without benefit observed for the higher concentration.

2.4. Enzymatic treatments

To evaluate the effect of each enzymatic treatment on biofilms, coupons from 48h cultures were withdrawn with tweezers and submerged into sterile saline solution (0.9% NaCl) to discard weakly adhered cells. Then, they were suspended vertically to drain off residual liquid and immersed into 24-well microplates previously filled with the corresponding enzymatic solution (1 mL per well). Treatments were applied for 1 hour at room temperature. Control samples were submerged 1 h into the same enzymatic solutions previously autoclaved (121ºC/ 15 min) for enzyme inactivation. After treatment, samples were rinsed and drained off as previously described. Enzyme effectiveness was expressed by calculating log reductions with respect to control coupons.

2.5. Cell recovery and counting

For cell recovery and counting, residual attached cells were strongly scratched from the top surface of the coupons with a sterile cotton swab according to the following sequence: first from left to right, then from top to bottom and then diagonally. The swab carrying the detached cells was then transferred into a 1.5 mL peptone water tube that was vigorously stirred on a vortexer (IKA® Vortex 3) to detach and break up cell aggregates. The resulting cellular suspension was serially diluted in peptone water and plated into various culture media according to the drop method (Hoben & Somasegaran, 1982). In dual biofilms, selective media (OXOID) were used for plating: PALCAM Agar for *L. monocytogenes*, *Pseudomonas* Agar Base (PAB) with CFC supplement, for

Pseudomonas, Mannitol Salt Agar (MSA) for *Staphylococcus* spp. and McConkey agar for *E. coli*. Colony counting was performed after 48 h plate incubation at 30 ºC. In the case of the consortium E1C3, *Carnobacterium* spp. counts were obtained by substracting of *L. monocytogenes* counts (obtained in PALCAM) from the total CFU obtained in general medium (TSA, Oxoid).

2.6. Confocal laser scanning microscopy (CLSM)

Enzymatic effect on biofilm structure was evaluated by CLSM using a FLUOVIEW® FV 1200 Laser Scanning Confocal Microscope (OLYMPUS). For CLSM observation, biofilms grown on stainless steel coupons were rinsed as previously described in section 2.4. and stained with LIVE/DEAD® biofilm viability kit (L10316, Life Technologies), including Syto 9, which labels all bacteria with intact membranes and propidium iodide, which only penetrates cells with damaged membranes. Thus, for image analysis, green corresponds to living cells and red to dead or damaged cells. For calculation, the total area of the coupon was scanned with a 10X objective in order to select two or three representative areas. Then, CLSM images of 0.2 x 0.2 mm were examined with a 60x oil immersion objective. Three-dimensional projections (Maximum Intensity Projection, MIP) were reconstructed from z-stacks using IMARIS® 7.6 software (Bitplane AG, Zúrich, Switzerland). To calculate biovolume using the MeasurementPro module of the above mentioned software, the whole image was segmented into channels that were analyzed to obtain the total volume occupied by cells (that is, green cells plus red cells). To calculate biovolume reduction, that of control coupons was taken as the 100% reference. To calculate the percentage of covered area using the software ImageJ, the images obtained with IMARIS were first transformed into binary system (*i.e.*, black and white) quantifying the black surface (*i.e.*, cells). Both, the total area and that occupied by dead or damaged cells were computed. The reduction of the covered area was obtained as a percentage, considering that of control coupons being 100%.

2.7. Statistical analysis

At least two independent experiments were performed and two or three coupons were sampled each time. Data were analyzed using Statgraphics Centurion software (Statistical Graphics Corporation, Rockville, Md., USA). ONE-way analysis of variance

(ANOVA) was carried out to determine whether samples were significantly different at a 95.0 % confidence level ($P < 0.05$).

3. Results

3.1. Ecological interactions in dual biofilms

Figure 1 shows the attached cell populations after 48 h culture of mono and dual-species biofilms with several strains of *L. monocytogenes* and the corresponding associated species. In total ten mono-species biofilms and seven dual-species consortia were studied (Table 1). *L. monocytogenes* counts in mono-species biofilms were on average 6-7 log CFU·cm^{-2}. A significant inhibitory effect of *L. monocytogenes* attachment was only observed when co-coculture with *Pseudomonas* spp. (Meat I) and *E. coli* (Seafood II). None of the secondary species in biofilms was significantly influenced by *L. monocytogenes*, though a little still significant inhibition of *S. saprophyticus* was observed in the Seafood I consortium.

3.2. Enzymatic L. monocytogenes detachment effectiveness on mono vs. dual biofilms

Table 3 shows values of enzymatic treatment effectiveness expressed as log reductions of *L. monocytogenes* viable attached cells, both in mono and in dual-species biofilms. On the overall, the association of *L. monocytogenes* with other microorganisms did not modify its susceptibility to the different enzymes used. Moreover, the effect of the enzymes over *L. monocytogenes* attached population was moderate, independently of the type of biofilm. After the enzymatic treatments, its population was reduced on average about 1 log (approximately 90% of the viable population).

3.3. Effect of the presence of l. monocytogenes on its partners detachment

Table 4 shows *P. fluorescens* B52, *S. saprophyticus* C2 and *E. coli* C4 log reductions in mono and dual-species biofilms with different strains of *L. monocytogenes*. Again, the enzymatic efficiency was moderate in all the biofilms tested. Nevertheless, the association effect was, in some cases, beneficial for these species. The association of *P. fluorescens* B52 with G1 and G2 (Dairy I and II, respectively) tends to moderately

reduce *P. fluorescens* B52 susceptibility to cellulase and chitinase treatments. Apart from that, the presence of *L. monocytogenes* G1 and G2 was neutral or slightly detrimental for *P. fluorescens* detachment. Enzymatic detachment of *S. saprophyticus* from biofilms was, in some cases, significantly reduced when associated with *L. monocytogenes* strains E1 and A1 (Meat II and Seafood I, respectively). Interestingly, the same pattern was observed when *E. coli* was associated with *L. monocytogenes* A1 in the Seafood II consortium, especially when these biofilms were treated with pronase and glucanase. No statistically significant differences were found between cell detachment in mono and dual-species biofilms in the cases of *Pseudomonas* spp. and *Carnobacterium* spp. (Table 4).

3.4. Structural changes caused by enzymatic treatment

For CLSM studies three out of seven consortia were selected, one from each sector. To choose these, those partners whose susceptibility to enzymes was modified due to the association effect with *L. monocytogenes, i. e. P. fluorescens* B52, *S. saprophyticus* and *E. coli* were selected. Figure 2 shows CLSM images of these three consortia (Dairy I, Meat II and Seafood II) before and after enzymatic treatments with DNase I, pronase and pectinase. Consortium Dairy I, much thicker than the others (12 *vs.* 6 and 6 µm) (Table 5) was nevertheless the most vulnerable. Indeed, DNase I reduced its biovolume and covered area by 99% and 90%, respectively. The remaining leftovers after this treatment were small scattered colonies and disaggregated cells, most of them either damaged or dead (Fig. 2).

On the biofilms of consortium Meat II, both pronase and DNase I had similar effects, leading to a significant reduction in biovolume and occupied area (Table 5). These parameters were practically unaffected by pectinase treatment, which nonetheless made most of the remaining cells appeared in red, that is, dead or damaged (Fig. 2). Besides, the residues were reorganized in aggregated structures, unseen in control images.

CLSM images of consortium Seafood II biofilms, showed a less compact structure than that of the other two, with small microcolonies (maximum thickness around 6 µm) and dispersed cells adhered along the surface (Fig. 2). Pectinase treatment was the most successful, yielding a 65% loss of both biovolume and occupied area, being the living cell population the most affected (Table 5). Indeed, many of the post-pectinase residual

cells again appeared in red (Fig. 2). Nevertheless, DNase I treatment had a very limited effect on the structure of this biofilm (Table 5).

4. Discussion

A couple of criteria were initially used in this study to select *L. monocytogenes* partners for dual-biofilms aimed as targets for dispersal enzymes. One was their isolation from the same surface as the *L. monocytogenes* counterpart. Second, the partner should not outcompete its *L. monocytogenes* counterpart in experimentally developed biofilms. The first criterium had however to be reconsidered when the 72 surface samples analyzed at four dairy plants, turned out to be all *L. monocytogenes* negative (Rodríguez-López, Saá-Ibusquiza, Mosquera-Fernández, & López-Cabo, 2015). Well known dairy strains of *L. monocytogenes* and *P. fluorescens*, a previously studied dual-biofilm (Puga, SanJose, & Orgaz, 2014) were therefore chosen as alternatives.

The second criterium was challenged by the ecological interactions experimentally observed in the Seafood consortia. Moderate antagonism against *L. monocytogenes* strains A1 and F1 in consortia A1C4 with *E. coli* and F1C1 with *Pseudomonas* spp. was observed (Fig. 1). One fact that influenced *S. saprophyticus* acceptance in Seafood consortia was that the same clone had been isolated from the meat sector (Rodríguez-López, Saá-Ibusquiza, Mosquera-Fernández, & López-Cabo, 2015). The species has besides public health interest as it is often involved in urinary tract infections (Raz, Colodner, & Kunin, 2005).

Sampling in the meat product plants yielded a presumptive *L. monocytogenes* antagonist, *Carnobacterium* spp., a lactic acid bacterium of which certain bacteriocin producing strains have been proposed as biopreservatives to outcompete pathogens in the seafood sector (Ghanbari, Jami, Domig, & Kneifel, 2013; Leisner J.J et al., 2007; Matamoros et al., 2009). This *Carnobacterium* spp. strain however kept a neutral interaction in biofilms with its *L. monocytogenes* counterpart, which was already observed by Rodríguez-López, Saá-Ibusquiza, Mosquera-Fernández, & López-Cabo, 2015. On the whole, of the five selected partners, only *S. saprophyticus* C2 was antagonized by *L. monocytogenes* A1 (Seafood I, Fig. 1) and of the five *L. monocytogenes* selected strains, two were moderately antagonized, in the chosen assay conditions (F1 in Meat I and A1 in Seafood II, Fig. 1). Seven types of dual-biofilms are certainly rather few, but may give a hint of the diversity of actual targets. Criteria for the

design of representative mixed biofilm models for food industry hygienic purposes are arguable, as actual communities tend to be complex and circumstantial and ecological interactions between their members may depend on strain, prevalent growth conditions, respective population densities, type of substrate and species distribution in the multi-species biofilm (Carpentier & Chassaing, 2004; Elias & Banin, 2012; Jahid & Ha, 2014; Puga, SanJose, & Orgaz, 2016).

4.1. Effect of enzymes on the viable population of dual-species biofilms carrying L. monocytogenes

When exposing both the mono- and dual species biofilms to the different enzymatic preparations, there were no statistically significant differences in *L. monocytogenes* log reductions, irrespective of the biofilm developed (Table 3). Nevertheless, the influence of *L. monocytogenes* on its partners cell dispersal was rather variable. Interestingly, the species that had showed growth antagonism when co-culture with *L. monocytogenes*, *E. coli* and *S. saprophyticus* (Fig. 1), happened to be the ones whose cells appeared to be more protected against enzymatic attack in mixed biofilms. Indeed, the association with *L. monocytogenes* reduced the detachment of *E. coli* cells with most of the enzymes tested (Table 4). In the case of *S. saprophyticus*, results were dependent on both enzyme tested and *L. monocytogenes* strain, but with a trend towards higher resistance to enzymatic attack associated to co-culture (Table 4). These data suggest that target points for these enzymes on *E. coli* and *S. saprophyticus* mono-species biofilms are not yet accessible in their dual-species biofilms.

In mono-species biofilms, poly-N-acetylglucosamine (PNAG) is believed to be one of the most important biofilm matrix component for the staphylococci (Izano et al. 2008). *E. coli* is also able to synthesize PNAG, in addition to cellulose, colanic acid, capsular polysaccharides and functional amyloid proteins (Barnhart & Chapman, 2006; Wang, Preston, & Romeo, 2004). In the case of *L. monocytogenes*, Harmsen, Lappann, Knøchel, & Molin (2010) suggested that DNA might interact with N-acetylglucosamine forming a PNAG-like polymer that support adhesion and biofilm formation. In dual-species biofilms, all of these polymers might interact in a different way and new bonds could block in some way target points for enzymatic attack. Moreover, these structures are rather dense and thick gels, whose interconnected polymer network would severely hinder diffusion of enzymes and their end reaction products, as previously modeled (Van Wey et al., 2012; Xavier et al., 2005). It is

important to highlight that the optimal temperature of action of the enzymes here tested ranged from 37ºC to 55ºC. To develop a treatment feasible in the food industry, all experiments were performed at room temperature. This fact would have probably reduced their action. Another important fact to be considered, is that dispersed cells could be still viable and therefore in order to avoid recontamination of food-contact surfaces, a combination of enzymes with disinfectants such as benzalkonium chloride will be necessary (Rodríguez-López, Puga, Orgaz, & Lopez-Cabo, 2017).

4.2. Effect of enzymes on the structure of dual-species biofilms carrying L. monocytogenes

When analyzed in isolation, results in terms of log reductions (Table 3), did not provide any clues about the most effective enzymes. Moreover, efficiency was rather poor (on average, 1 log reduction, Table 3). Nevertheless, when quantification of enzyme effectiveness was performed by CLSM image analysis, changes in biofilm covered area and volume greatly differed according to the enzyme and target biofilm employed (Table 5). Structural damages appear to be greater than expected based on the results of log reductions (Fig. 2; Table 5 *vs*. Table 3). It is well known that a part of the biofilm population enters a dormant state in which cells lost their culturability (Lewis, 2007). Thus, biofilms pre- and after enzymatic treatments would carry both viable and non-viable cells including those that are damaged after the treatment and dormant cells, the latter being unable to be detected by culture dependent methodologies. Therefore, results in terms of log reductions could be underestimating this part of the population.

For an enzyme to be effective, the location and accessibility of the target compounds are likely to be determining factors. Extracellular DNA (eDNA) has proven to be a critical component for *L. monocytogenes* biofilm formation and an essential polymer of the *L. monocytogenes* biofilm matrix (Combrouse et al., 2013; Harmsen, Lappann, Knøchel, & Molin, 2010). The CLSM results here obtained suggest that the amount and location of eDNA in dual biofilm matrices rely on *L. monocytogenes*'s partner. *P. fluorescens* seems to impart a widespread and accessible eDNA pattern (DNase attaining 99% biofilm volume reduction) whereas *E. coli* would provide a scarce and/or unattainable one (very low DNase effect) (Table 5, Fig. 2). Enzymatic methods for biofilm eDNA extraction reveal an intricate molecular interaction of this polymer with other EPS components (Wu & Xi, 2009). Therefore, eDNA seems to play an important role in stabilizing G1B52 and E1C2 matrices, whereas in A1C4 matrix, proteins and

polysaccharides could be more important in maintaining its structure (pronase and pectinase yielded a 67% and 65% biovolume reduction, respectively) (Table 5). Indeed, curli have been described as the major proteinaceous components of the extracellular matrix of the Enterobacteriaceae family (Barnhart & Chapman, 2006).

Pectinase achieved 65-70 % volume reduction on dual biofilms of *L. monocytogenes* with Gram-negative bacteria (*P. fluorescens* or *E. coli*) but not on those with *L. monocytogenes* and another Gram-positive (*S. saprophyticus*) (Table 5, Fig. 2). Pectinase is a pectolytic enzyme preparation produced by *Aspergillus niger* that contains a mixture of pectolytic enzymes, small amounts of hemicellulases and cellulases and proteolytic activity. This enzyme could therefore act on both Gram-positive and Gram-negative cell walls and Gram-negative matrix polysaccharides, such as cellulose (Hufnagel, Depas, & Chapman 2015). A good volume reduction was obtained with pronase on all the dual-biofilms (Table 5) probably due to the diversity of proteinases the preparation contains. Pronase has also non-specific chitinase activity (Kumar, Gowda, & Tharanathan, 2004; Orgaz, Neufeld, & SanJose, 2007), useful to degrade chitin (N-acetilglucosamine) and its deacetylated derivative chitosan, whose structures have components similar to those found in the biofilm EPS (Kives, Orgaz, & SanJose, 2006). All those activities and the diversity of proteins distributed in the matrix may perhaps explain the diversity in pronase effects on average biofilm thickness reduction (Table 5).

Few clues on the spatial distribution of *L. monocytogenes* cells in multi-species biofilms are available. In mixed biofilm with *L. monocytogenes* and *Pseudomonas* spp., this last one is usually considered the primary colonizer, the host, providing *L. monocytogenes* with a thick matrix protection (Hassan, Birt, & Frank, 2004). But *L. monocytogenes* is not just a passively engulfed guest; though it grows more slowly than *P. fluorescens*, its cells get positioned underneath (Puga, SanJose, & Orgaz, 2014). A similar pattern, in which bottom layers of *L. monocytogenes*/*E.coli* dual-biofilms were occupied by the former, was previously described (Almeida et al., 2011). Nevertheless, co-culture-induced structural changes in *L. monocytogenes*/*E. coli* biofilm matrix are likely to be different from those produced in the *L. monocytogenes*/*P. fluorescens* system. That is at least suggested by their respective patterns of susceptibility, particularly when treated with DNase I and pronase (Table 5).

Probably the interactions between co-cultured species make the mixed biofilm matrix different in components and polymer junctions with respect to mono-species biofilms

and that may be the cause for their frequently reported higher resistance to antimicrobials (Burmølle, Ren, Bjarnsholt, & Sørensen, 2014; Ibusquiza, Herrera, Vazquez-Sanchez, & Cabo, 2012; Jahid & Ha, 2014; Puga, SanJose, & Orgaz, 2016), mechanical disintegration (Simões, Simões, & Vieira, 2009), desiccation (Alavi & Hansen, 2013) or enzymatic attack, as observed here. How these interactions transform the physical chemistry of the biofilms is still to be understood.

5. Conclusions

L. monocytogenes strains were equally susceptible to enzymatic attack whether in mono- or in dual-species young biofilms. The effect of the association however was beneficial for some of *L. monocytogenes* partners such as *E. coli* and *S. saprophyticus*. In terms of viable attached cell log reductions, the use of enzymes for the treatment of dual-species biofilms did not achieve good results. However, CLSM images showed significant structural damage after enzymatic treatment of these biofilms with DNase I, pronase and pectinase. Moreover, use of different enzymes yielded very different changes in biofilm structure, depending on the dual-species biofilm treated. Therefore, enzymes could be an interesting tool for weakening the structure of *L. monocytogenes* carrying biofilms likely to exist on the surfaces of food processing plants, always in combination with a disinfection treatment. Moreover, checking the action of different enzymes on biofilms could be regarded as a rough way of sensing and probing their external or accessible structure.

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Figure captions

Figure 1. 48 h-attached population of *L. monocytogenes* (bared bars), and its corresponding partner (lined bars) in mono (white) and dual-species (black and grey) biofilms. For each strain, asterisks denote significant differences between mono- and dual-species conditions (P<0.05).

Figure 2. CLSM zenital images of biofilms formed by consortia Dairy I, Meat II and Seafood II, before and after the enzymatic treatments. Coupons were stained with Live-Dead Kit (*i.e.* in green, live cells and in red, dead cells). For each consortium, left rows show live and dead cells (L+D). Right rows, only dead cells (D). The images covered an area of 0.12 x 0.12 mm. (Scale bar: 20μm).

Table 1. Selected consortia of *L. monocytogenes* strains and partner strains.

Table 2. Enzymes used in treatments against mono-species and dual-species biofilms-carrying *Listeria monocytogenes.*

Table 3. Enzymatic efficiency expressed as Logreduction of attached *L. monocytogenes* cells in the target mono-species (in total, 5) and dual-species biofilms (in total, 7 consortia). Values correspond to the average ± standard deviation (n=3).

Table 4. Enzymatic efficiency expressed as Log reduction of attached *P. fluorescens* B52, *S. saprophyticus* C2 and *E. coli* C4 cells in the target mono-species and dual-species biofilms. Results correspond to the average ± standard deviation (n=3).

Table 5. Biofilm structural parameters variation after enzymatic treatments of three consortia.

Table 2. Enzymes used in treatments against monospecies and dual species biofilms-carrying *Listeria monocytogenes*

Enzyme	*Origin*	*Catalog*
Pronase	*Streptomyces griseus*	11459643001, Roche
Cellulase	*Aspergillus niger*	C1184-5KU, Sigma
Pectinase	*Aspergillus niger*	1789-10G, Sigma
DNase I	Bovine pancreas	DN 25, Sigma
Lysozyme	Chicken egg white	62971-10G-F, Sigma
Phospholipase	*Thermomyces lanuginosus*	L3295-50ML, Sigma
Peroxidase	Horseradish	P8250-5KU, Sigma
β-Glucanase	*Trichoderma longibrachiatum*	G4423-100G, Sigma
Chitinase	*Streptomyces griseus*	C6137-5UN, Sigma

Table 3. Enzymatic efficiency expressed as Log reduction of attached *L. monocytogenes* cells in the target mono-species (in total, 5) and dual-species biofilms (in total, 7 consortia). Values correspond to the average ± standard deviation (n=3).

Enzyme	Dairy sector				Meat sector					Seafood sector		
	G1	***G1B52***	***G2***	***G2B52***	***F1***	***F1C1***	***E1***	***E1C2***	***E1C3***	***A1***	***A1C2***	***A1C4***
Pronase	0.9 ± 0.2	1.6 ± 0.4*	1.2 ± 0.7	1.6 ± 0.5	1.4 ± 0.4	1.3 ± 0.5	1.0 ± 0.4	1.2 ± 0.4	0.6 ± 0.4	1.0 ± 0.3	1.2 ± 0.3	1.0 ± 0.1
Cellulase	1.1 ± 0.7	1.3 ± 0.6	1.6 ± 0.2	1.0 ± 0.5	1.4 ± 0.5	1.4 ± 0.7	1.3 ± 0.4	1.4 ± 0.5	0.6 ± 0.4	1.0 ± 0.6	1.3 ± 0.5	1.0 ± 0.3
Pectinase	1.7 ± 0.1	1.3 ± 0.6	1.5 ± 0.4	1.4 ± 0.6	1.4 ± 0.5	1.3 ± 0.6	1.0 ± 0.3	1.5 ± 0.5	0.9 ± 0.3	0.9 ± 0.4	1.0 ± 0.3	0.9 ± 0.3
Dnase I	1.7 ± 0.6	1.1 ± 0.3	1.5 ± 0.3	1.5 ± 0.3	1.5 ± 0.4	1.4 ± 0.6	1.2 ± 0.4	1.2 ± 0.3	0.9 ± 0.4	0.9 ± 0.5	1.1 ± 0.4	0.9 ± 0.3
Lysozyme	1.2 ± 0.7	1.0 ± 0.5	1.2 ± 0.6	1.3 ± 0.3	1.2 ± 0.3	1.4 ± 0.4	1.6 ± 0.5	1.4 ± 0.3	0.8 ± 0.4*	1.1 ± 0.5	1.1 ± 0.6	0.8 ± 0.2
Phospholipase	1.0 ± 0.6	1.3 ± 0.8	1.3 ± 0.2	1.5 ± 0.2	1.6 ± 0.4	1.4 ± 0.6	0.9 ± 0.5	1.2 ± 0.6	0.8 ± 0.5	0.8 ± 0.3	0.8 ± 0.1	0.8 ± 0.3
Peroxidase	0.9 ± 0.3	1.3 ± 0.4	1.2 ± 0.6	1.0 ± 0.5	1.2 ± 0.2	1.3 ± 0.4	1.1 ± 0.3	1.2 ± 0.6	0.7 ± 0.6	1.2 ± 0.3	1.2 ± 0.4	1.0 ± 0.4
β-Glucanase	1.3 ± 0.5	1.2 ± 0.6	1.2 ± 0.7	1.2 ± 0.3	1.0 ± 0.4	0.8 ± 0.3	1.0 ± 0.6	1.3 ± 0.5	0.8 ± 0.7	0.6 ± 0.2	0.7 ± 0.3	1.0 ± 0.5
Chitinase	1.0 ± 0.2	1.1 ± 0.6	1.5 ± 0.4	0.9 ± 0.5	1.4 ± 0.4	1.6 ± 0.4	0.9 ± 0.4	1.4 ± 0.5	0.8 ± 0.5	1.3 ± 0.3	0.6 ± 0.1	1.0 ± 0.1

In rows, asterisks mean statistically significant differences between each pair mono-species/dual-species biofilms ($P<0.05$)

SD

4.30

	12.49
	10.82
	2.74
	11.48
	14.33
	8.36
	12.92
	3.73
SD	
	6.34
	11.08
	4.46
	5.86
	9.33
	8.72
	3.88
	6.04
	1.77

Table 4. Enzymatic efficiency expressed as Log reduction of attached *P. fluorescens* B52, *P. fluorescens* spp.C1, *S. saprophyticus* C2, *Carnobacterium* spp. C3 and *E. coli* C4 cells, in the target mono-species and dual-species biofilms. Results correspond to the average ± standard deviation (n=3).

Enzyme	***B52***	***G1B52***	***G2B52***	***C1***	***F1C1***	***C2***	***E1C2***	***A1C2***	***C3***	***E1C3***	***C4***	***A1C4***
Pronase	1.3 ± 0.3	1.8 ± 0.4	1.8 ± 0.4	1.4 ± 0.9	1.0 ± 0.3	1.0 ± 0.3	0.4 ± 0.1*	0.5 ± 0.3*	0.5 ± 0.3	1.1 ± 0.6	0.8 ± 0.1	0.4 ± 0.1*
Cellulase	1.8 ± 0.1	1.1 ± 0.5*	1.3 ± 0.2*	1.0 ± 0.5	1.0 ± 0.4	1.2 ± 0.3	0.9 ± 0.3	0.6 ± 0.1*	0.6 ± 0.4	1.5 ± 0.8	0.7 ± 0.2	0.6 ± 0.3
Pectinase	1.0 ± 0.4	1.2 ± 0.4	1.6 ± 0.5	1.5 ± 0.5	1.1 ± 0.2	1.4 ± 0.5	1.1 ± 0.3	0.7 ± 0.4*	1.5 ± 1.2	0.9 ± 0.6	0.6 ± 0.2	0.5 ± 0.1
DNaseI	1.1 ± 0.2	1.6 ± 0.4	1.8 ± 0.3*	1.2 ± 0.6	1.1 ± 0.2	1.0 ± 0.4	1.2 ± 0.2	0.6 ± 0.2*	0.9 ± 0.3	1.2 ± 0.7	0.9 ± 0.4	0.4 ± 0.4
Lysozyme	1.4 ± 0.6	1.1 ± 0.5	1.5 ± 0.5	1.2 ± 0.3	1.0 ± 0.4	1.0 ± 0.5	0.7 ± 0.4*	0.9 ± 0.3	0.5 ± 0.3	1.1 ± 0.9	0.6 ± 0.2	0.5 ± 0.3
Phospholipase	1.9 ± 0.3	1.4 ± 0.4	1.8 ± 0.4	1.2 ± 0.5	1.2 ± 0.4	0.9 ± 0.4	1.3 ± 0.6	0.5 ± 0.1*	0.5 ± 0.3	1.5 ± 0.7	0.6 ± 0.4	0.5 ± 0.2
Peroxidase	1.1 ± 0.2	1.7 ± 0.1*	1.5 ± 0.2	0.8 ± 0.4	0.6 ± 0.1	0.7 ± 0.6	0.7 ± 0.3	0.9 ± 0.4*	0.4 ± 0.2	1.6 ± 1.1	1.0 ± 0.4	0.5 ± 0.5
β-Glucanase	0.9 ± 0.4	1.3 ± 0.2	1.5 ± 0.3*	1.1 ± 0.5	0.7 ± 0.3	0.4 ± 0.1	0.9 ± 0.3	0.8 ± 0.3	0.7 ± 0.3	1.1 ± 0.6	0.9 ± 0.1	0.3 ± 0.3*
Chitinase	1.8 ± 0.6	1.5 ± 0.1*	1.9 ± 0.5	1.8 ± 0.7	1.2 ± 0.3	0.3 ± 0.0	0.9 ± 0.1*	0.4 ± 0.0*	1.1 ± 0.5	1.0 ± 0.6	1.0 ± 0.3	0.5 ± 0.3

In rows, asterisks mean statistically significant differences between each pair mono-species/dual-species biofilms ($P<0.05$)

Table 4. Enzymatic efficiency expressed as the % of eliminated attached *P. fluorescens* B52 cells in the target biofilms, monospecies and dual-species.

Enzyme	*Monocultured B52*	*Co-cultured G1B52*	*Co-cultured G2B52*	*Monocultured C2*	*Co-cultured E1C2*	*Co-cultured A1C2*	*Monocultured C4*	*Co-cultured A1C4*
	X ± SD	*X ± SD*	*X ± SD*	*X ± SD*	*X ± SD*	*X ± SD*	*X ± SD*	*X ± SD*
Pronase	97 ± 2	98 ± 2	99 ± 1	90 ± 5	62 ± 9*	76 ± 12*	85 ± 5	65 ± 10*
Cellulase	99 ± 0	89 ± 9*	97 ± 1*	94 ± 7	92 ± 2	77 ± 8*	81 ± 9	72 ± 15
Pectinase	95 ± 4	92 ± 7	96 ± 8	95 ± 4	90 ± 6	75 ± 12*	79 ± 8	73 ± 5
DNaseI	97 ± 1	96 ± 4	99 ± 0*	89 ± 7	93 ± 3	82 ± 14*	85 ± 14	66 ± 15
Lysozyme	97 ± 3	89 ± 9	96 ± 7	90 ± 9	76 ± 16*	86 ± 12	79 ± 8	71 ± 15
Phospholipase	99 ± 0	94 ± 8	99 ± 0	89 ± 6	91 ± 7	73 ± 5*	77 ± 11	73 ± 8
Peroxidase	93 ± 5	98 ± 1*	97 ± 3	85 ± 8	78 ± 11	92 ± 4*	88 ± 8	67 ± 19
β-Glucanase	91 ± 3	95 ± 2	98 ± 1*	74 ± 8	85 ± 11	82 ± 11	88 ± 5	69 ± 13*
Chitinase	99 ± 1	97 ± 1*	99 ± 0	72 ± 1	88 ± 4*	56 ± 1*	87 ± 8	76 ± 8

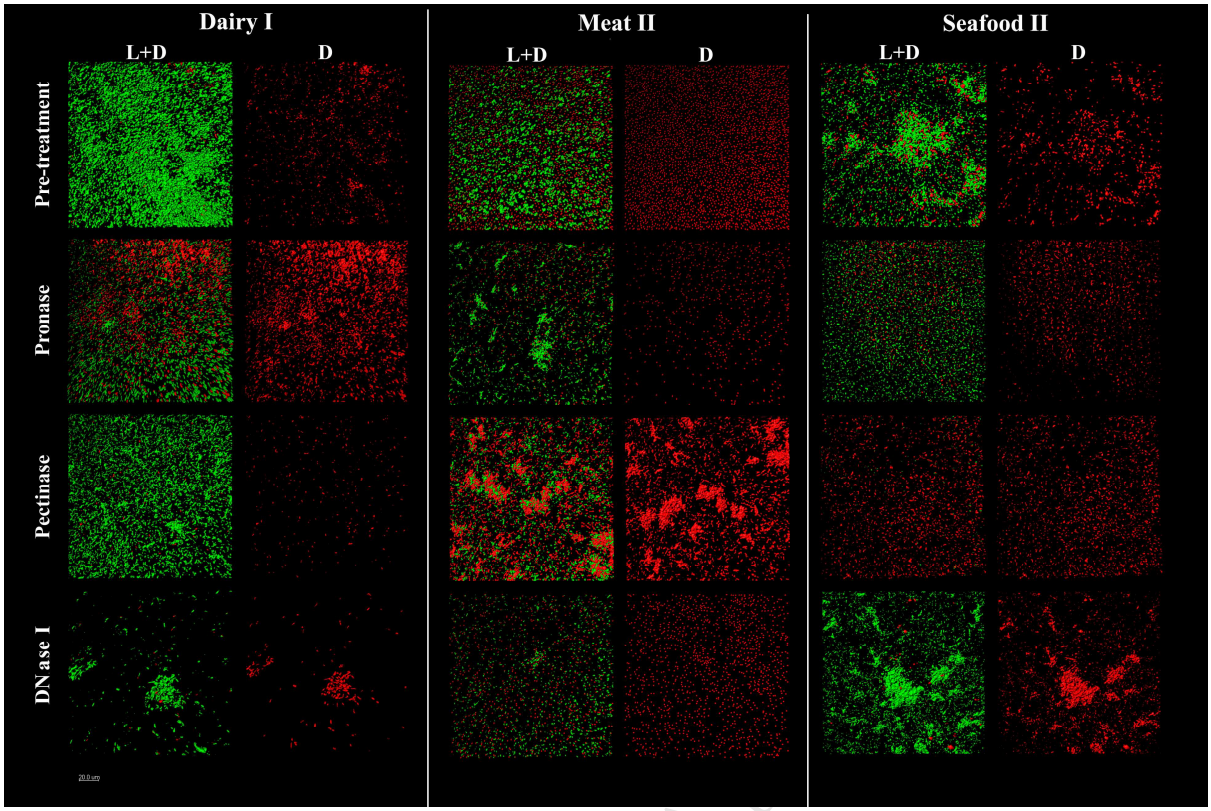
In rows, asterisks mean statistically significant differences between monocultured and co-cultured target biofilms (P<0.05)

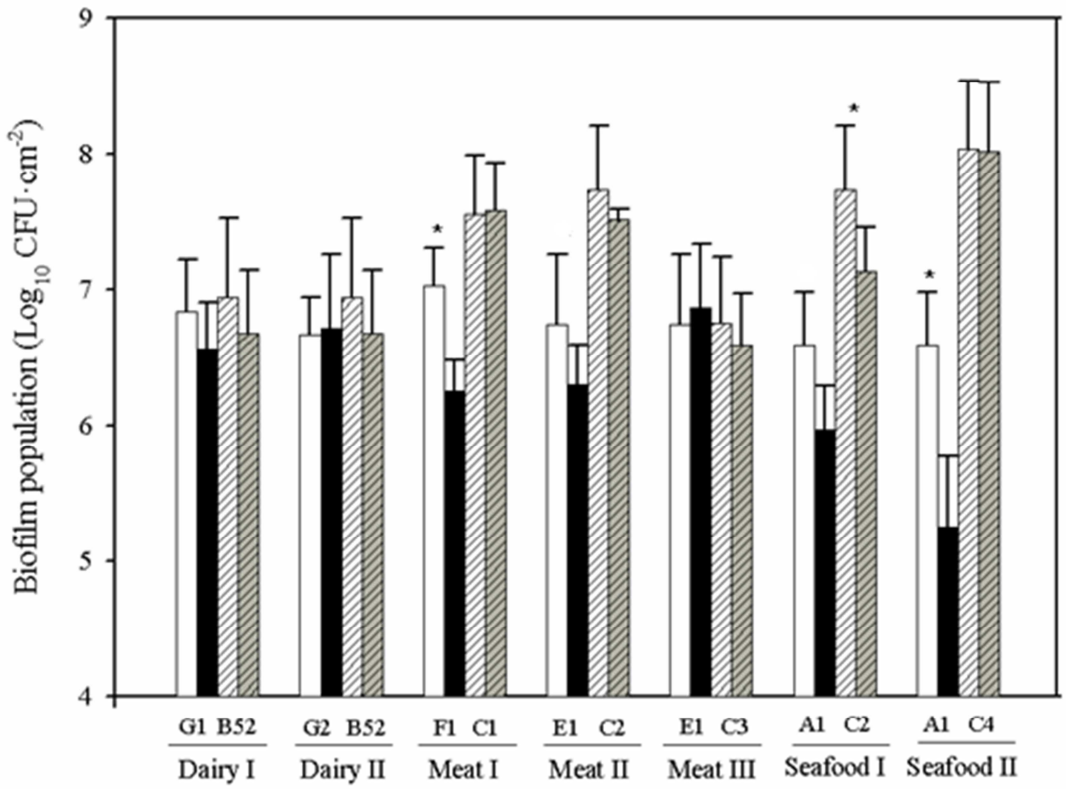
Table 1. Selected consortia of *L. monocytogenes* strains and partner strains

Partner strains	***L. monocytogenes* strains**				
	G1	**G2**	**F1**	**E1**	**A1**
Pseudomonas fluorescens B52	Dairy I	Dairy II			
Pseudomonas spp. C1			Meat I		
Staphylococcus saprophyticus C2				Meat II	Seafood I
Carnobacterium spp. C3				Meat III	
Escherchia coli C4					Seafood II

Table 5. Biofilm structrural parameters variation after enzymatic treatments of three consortia

Consortium	Sample	Biofilm thickness (µm)	Biofilm thickness reduction (%)	Covered area (%)			Covered area reduction (%)	Biovolume reduction (%)
				Total	Dead cells	Living cells	Total	
Dairy I	Control	12		39	4	35		
	Pronase	12	0	24	12	12	37	40
	Pectinase	10	17	25	1	24	36	70
	DNase I	8.5	29	4	2	2	90	99
Meat II	Control	6		21	13	8		
	Pronase	3	50	9	3	6	57	62
	Pectinase	6	0	24	17	7	0	12
	DNase I	4	33	8	6	2	67	73
Seafood II	Control	6		20	5	15		
	Pronase	1	83	11	4	7	45	67
	Pectinase	4	33	7	7	0	65	65
	DNase I	6	0	16	9	7	20	0




Biofilm population (Log10 CFU·cm-2)
9
8
7
6
5
4
*
*
*
G1 B52
G2 B52
F1 C1
E1 C2
E1 C3
A1 C2
A1 C4
Dairy I
Dairy II
Meat I
Meat II
Meat III
Seafood I
Seafood II

Highlights

- Some species were protected from enzymes in dual biofilms with *L. monocytogenes*
- Enzymes greatly affect dual-species biofilms structure and cellular integrity
- They can be a good alternative for weakening dual biofilms carrying *L. monocytogenes*