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Cold Stress and Presence of *Pseudomonas fluorescens* Affect *Listeria monocytogenes* Biofilm Structure and Response to Chitosan

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Abstract

Life in biofilms (BF) provides microorganisms with protection against different adverse conditions and agents. In food industry, as they can host and transfer to food both pathogenic and spoilage microorganism, they have to be constantly kept under control. Many hygienic practices and disinfectants aim at preventing and/or destroying BF, and chitosan has a promising future in this respect. *Listeria monocytogenes* (*Lm*) is a dangerous foodborne pathogen that can live in BF and survive many restrictive conditions used to preserve foods, such as refrigeration. In this work, nine Lm strains, persistently or sporadically isolated from a meat processing plant, were cultured at 20°C and 4°C to obtain mature BF either in isolation or with *Pseudomonas fluorescens* (*Pf*), both species being simultaneously inoculated at similar low population levels. *Pf* was more compatible with the persistent Lm strains than with the rest, enhancing or maintaining their viable counts in the corresponding dual species BF. All dual species BF formed at 4°C were much thinner than those formed at 20°C, but contained more cells per cm3 of BF biomass. Chitosan damage was observed both as reduction of *Lm* viable cells and by confocal laser scanning microscopy (CLSM) with Live/Dead stains. In *Lm* monospecies BF, 1 h chitosan exposure reduced viable counts between 3 and 6 Log when cultured at 20°C and 2-4 Log when at 4°C. Both temperature of BF formation and Lm strain affected their susceptibility to chitosan in dual species BF. CLSM showed focalized chitosan injuries in binary BF, particularly in those with persistent *Lm* strains.

Keywords: Chitosan; *Listeria monocytogenes*; *Pseudomonas fluorescens*; Mixed biofilms; Low temperature; CLSM; Food safety; Biofilm structure

Introduction

Listeria monocytogenes (*Lm*) emerged as a "new" human pathogen in the 1980s, with several high profiles food-associated listeriosis outbreaks. The case fatality rate is now in Europe of 15.6% but is still close to 30% in other locations. The EU notification rate was in 2013 of 0.44 per 100,000 populations, which represented an 8.6% increase compared with 2012 [1]. In the US, listeriosis accounted for approximately 28% of the deaths and the highest hospitalization rate (91%) caused by known food-borne infections [2]. Transmission through contaminated food has been conclusively demonstrated to account for 99% of both outbreak and sporadic cases of listeriosis. Food types most often associated to listeriosis are ready-to-eat (RTE) foods (processed products that can be consumed directly as sold, without a previous hygienization step) that can support the growth of *Lm*: soft cheeses, marinated and smoked fish, deli salads and certain deli meat products, apart from uncooked or undercooked foods. Norms regarding *Lm* tolerance in retail foods are among the most strict ones in the microbiological food safety field.

A biofilm (BF) can be defined as a sessile community of microbial cells irreversibly associated with a surface and embedded in a matrix of extracellular polymeric substances (EPS) that they produce themselves; those cells display an altered phenotype with respect to gene expression, protein production and growth. Among phenotypic differences are loss of mobility appendages, production and secretion of EPS, adaptation to different degrees of anaerobiosis (depending on the cell's depth inside the matrix), higher ability to destroy active oxygen molecular species and much slower growth rate than their free, planktonic counterparts. It has been estimated that 80% or more of the world's microbial mass live in BF. In the food context, BF formation is a problem for food safety and quality, since it may harbour pathogens, such as *Lm* [2] and also spoilage organisms in food processing plants and even transfer them directly into hygienized foods. Besides, life in BF confers its dwellers an increased resistance to many chemical or biological hostile agents [3,4].

Though monospecies BF have been for long the most frequent experimental form of BF, multispecies BF are far more common in everyday life; they are in fact considered to be the prevalent form of life in natural and artificial environments [5]. Sustained coexistence in the same BF implies compatibility between species and a cooperative or at least neutral ecological interaction between them. Mixed BF provide even more protection than monospecies BF, so there is increasing interest to identify at least the most common of the actual partners in the BF formed on certain raw foods or food industry processing surfaces, to reproduce them in model BF, to use as targets to test the efficiency of new hygienization agents such as chitosan [6].

To identify the most common real BF partners, repeated samplings of the same sites are usually performed, to discard sporadic contaminants. Microbial strains repeatedly isolated from the same industrial surfaces along extended periods of time (several months or even years) are called persistent. Ortiz et al. [7] have collected a set of persistent and another set of non-persistent *Lm* strains from an Iberian pig slaughterhouse and meat processing plant and have tried to identify differences between them. Though bacterial persistence is not thought to be a phenotypic trait [8] it is presumably based on selective

advantages to withstand the stress conditions prevailing at a given location. Some of those advantages are probably very specific for the site, whereas others are likely to be of a more general character. Some authors however claim differential gene expression in certain persistent *Lm* strains [9].

We have previously studied some of the strains of Ortiz collection and have observed that persistent *Lm* strains recover more quickly than non-persistent ones from the damages caused on BF by chitosan exposure [10]. Our interest in chitosan as cleaning and disinfection agent relies on previous encouraging antibiofilm results [11] and good sustainability perspectives. In this work, we have extended those studies to dual species *Lm/Pf* BF and low temperature conditions, as both mixed BF and refrigeration are realistic issues in food industry.

Materials and Methods

Bacterial strains

Pseudomonas fluorescens ATCC 948TM (*Pf*), the reference *Listeria monocytogenes* (*Lm*) strain, Lm Scott A (serotype 4b, lineage I), and nine *Lm* environmental strains isolated from an Iberian pig slaughterhouse and processing plant by Ortiz et al. [7] were used as biofilm (BF) former organisms. The latter were recovered over a period of three years, identified and characterized by serotyping and PFGE (pulsed field gel electrophoresis) by the same authors. Six of these *Lm* strains were classified as persistent (their name is followed by a p, such as 1p) as they were repeatedly sampled over a large timespan (from 1 to 3 years). The other three were considered non persistent ones (Table 1). All were stored at -20°C in Tryptone Soya Broth (TSB) (OXOID, Basingstoke, UK) supplemented with 15% glycerol. Preinocula were obtained after overnight culture in TSB/20°C. Cells were harvested by centrifugation at 4000×g for 10 min, washed twice in sterile TSB and their OD_{600} adjusted in order to reach 10^3 CFU/ml of each bacterium after inoculation, both in single and binary cultures.

Strain name	PFGE type	Serotype	Lineage	Persistency
1p	S1	1/2a	II	+
2p	S2	1/2a	II	+
4p	S4-1	1/2b	I	+
5p	S5	1/2c	II	+
10p	S10-1	1/2a	II	+
17p	S17-1	1/2b	I	+
6	S6	1/2a	II	
11	S11	1/2a	II	
18	S18	1/2b	I	

Table 1: *Listeria monocytogenes* strains. Environmental *Listeria monocytogenes* strains, selected from those isolated by Ortiz et al. [7] from a slaughterhouse and meat processing plant.

Experimental system

BF were developed on single-use 22 × 22mm thin, borosilicate commercial microscope glass coverslips. As described in Orgaz et al., [11] 16 coverslips were held vertically by marginal insertion into the narrow radial slits of a Teflon carousel platform (6.6 cm diameter). The platform and its lid were assembled by an axial metallic rod for handling and placed into a 600 ml beaker. The whole system, i.e. coverslips, carousel and the covered 600 ml beaker, were heat-sterilized as a unit, before aseptically introducing 60 ml of inoculated TSB. For dual species BF, containing *Pf* and one of the ten *Lm* strains afore mentioned, both bacteria were inoculated at the same level (103 CFU/ml). *Lm* monospecies BF were used as controls. Incubation was carried out at 20°C/48 h or 4°C/20 d, in a rotating shaker at 80 rpm. Under these conditions, BF growth occupied about 70% of the coverslip's surface.

Antimicrobial testing

Chitosan with ≥ 75% deacetylation degree was purchased from SIGMA ALDRICH (St. Louis, Mo., USA). A 1% (w/v) chitosan solution was prepared in 1% (v/v) acetic acid for testing its antibiofilm properties. For chitosan treatment of the BF, the coverslips were aseptically extracted from the carousel platform with sterile tweezers and washed by dipping in sterile NaCl (0.9% w/v), in order to eliminate weakly attached cells. They were individually immersed into Falcon test tubes containing 15 ml of the sterile chitosan cleaning solution, for 1h at room temperature. After treatment, the washing step with 0.9% NaCl was repeated before cell recovery and counting.

Cell recovery and counting

For cell recovery and counting of residual BF dwelling cells, loosely attached cells were removed by swabbing both coverslip faces. The rest were then transferred into 1.5 ml of peptone water in tubes that were vigorously stirred in a vortex to break up cell aggregates, diluted in peptone water and plated into the indicated culture media according to the drop method [12]. In dual BF, selective media (OXOID) were used for plating: PALCAM Agar Base for *Lm* and *Pseudomonas* Agar Base (PAB) for *Pseudomonas*. In monospecies BF, general medium Tryptone Soya Agar (TSA, OXOID) was used. Colonies were counted after 48h incubation at 37°C or 30°C, for *Lm* or *Pf*, respectively. Untreated control samples were processed for cell recovery and counting as described before, and considered as controls having 100% of attached cells. Chitosan efficiency was expressed as Log reduction of *Lm* attached cells.

Confocal laser scanning microscopy (CLSM)

The effect of chitosan on BF structure and cell damaged was evaluated by CLSM. Images were obtained using a FLUOVIEW® FV 1200 Laser Scanning Confocal Microscope (OLYMPUS, Tokyo, Japan). For CLSM observation, BF developed in glass coverlips were rinsed with sterile 0.9% NaCl before being stained with LIVE/DEAD® viability kit (L10316, LIFE TECHNOLOGIES, Madrid, Spain), including SYTO 9, which labels all bacteria in a population, both those with intact membranes and those with damaged ones and propidium iodide, which only penetrates in cells with damaged membranes. Thus, for image analysis, green corresponds to living cells and red to dead or damaged cells. For image analysis, firstly the total area of the coupon was observed with a 2X objective. Then, selected representative areas within a region were observed with a 10X objective and finally, the 60X immersion objective was used to obtain 0.12 × 0.12 mm images. Three-dimensional projections (Maximum Intensity Projection, MIP) were reconstructed from z-stacks using IMARIS® 7.6 software (BITPLANE AG, Zurich, Switzerland).

Statistical analysis

At least two independent experiments were performed and four coupons were sampled each time, in total n=8. Data were analyzed using ONE-way ANOVA, STATGRAPHICS PLUS 5.0 software (STATISTICAL GRAPHICS CORPORATION, Rockville, Md., USA). Mean comparisons were carried out to determine significant differences at a 95.0% confidence level ($p<0.05$).

Results and Discussion

Temperature and *Pf* effect on viable biofilm *Lm* counts

When BF were cultured at 20°C with *Lm* as a single species, both persistent and non-persistent strains attained similar cell densities after 48h incubation (Table 2). When cultured at 4°C however, 10-fold longer times were needed for *Lm* to reach comparable counts. Cell densities attained by persistent and sporadic strains in BF at both temperatures were almost the same. Scott A strain also formed BF with similar cell densities as the food industry environmental *Lm* strains used.

Lm strain	20° C - 48 hours		4° C - 20 days	
	Pure *Lm* biofilms	Binary biofilms *Lm*+*Pf*	Pure *Lm* biofilms	Binary biofilms *Lm*+*Pf*
	X ± SD	X ± SD	X ± SD	X ± SD
1p	5.89 ± 0.52	6.62 ± 0.62*	5.36 ± 0.39	5.71 ± 0.33
2p	6.26 ± 0.37	6.24 ± 0.62	5.79 ± 0.25	5.61 ± 0.37
4p	6.25 ± 0.28	7.23 ± 0.33*	5.31 ± 0.35	6.22 ± 0.55*
5p	6.21 ± 0.42	6.15 ± 0.37	5.71 ± 0.33	5.8 ± 0.38
10p	5.87 ± 0.44	6.5 ± 0.56*	5.49 ± 0.55	5.13 ± 0.30
17p	6.14 ± 0.51	5.94 ± 0.55	5.78 ± 0.23	5.15 ± 0.40*
6	6.22 ± 0.39	5.48 ± 0.72*	4.98 ± 0.53	4.15 ± 0.48*
11	6.27 ± 0.12	5.36 ± 0.27*	5.02 ± 0.36	3.42 ± 0.20*
18	6.17 ± 0.16	5.32 ± 0.13*	5.29 ± 0.14	4.17 ± 0.53*
Scott A	6.21 ± 0.51	6.9 ± 0.25*	4.98 ± 0.40	4.29 ± 0.32*

Table 2: Effect of *Pseudomonas fluorescens* presence on the amount (Log_{10}) of *Lm* in biofilms from cultures at 20 and 4°C. Figures from binary cultures with asterisk are statistically different ($p<0.05$) from their respective pure culture control (n=8).

No differences associable to serotype or lineage (Table 1) were noticed. Some authors have tried to associate BF forming ability with persistence without conclusive results; culture conditions may be critical in this respect [10,13].

The effect of adding *Pf* on BF *Lm* counts, when both species started the culture with the same inoculum size, as in this case, depended on *Lm* strain and temperature (Table 2). The interaction's outcome was moderately negative (in most cases, less than 1 Log) on *Lm* BF counts of all non-persistent strains, regardless of culture temperature. On those BF with persistent *Lm* strains, however, *Pf* had a variable effect, depending on temperature and each *Lm* particular strain (Table 2). Scott A counts in binary BF increased with respect to those in pure BF at 20°C but not at 4°C; an interaction pattern close to that of the persistent strains.

According to these results, *Lm* strains matter regarding cooperation between these two species in BF. These sporadic *Lm* strains, seemed to be as a rule less compatible with *Pf* in BF than the persistent ones. It could be reminded that all these environmental isolates came from a meat product plant where various *Pseudomonas* are likely to be more frequent than *Lm*. Sasahara and Zottola [14] first studied *Pseudomonas* (fragi) influence on *Listeria* being incorporated into BF, in a pioneer work published in 1993. Using a scanning electron microscopy technique, they described a positive influence of Pseudomonas on Listeria attachment, highlighting the role of *Pseudomonas* as a primary colonizer, critical to bind *Lm* to surfaces in significant numbers. Many studies on the ecological relationships in BF between *Lm* and other bacteria have kept assuming that, though *Lm* can form its own BF, though thin and sparse [15]. Though experimental conditions are difficult to compare, not all bacteria and not even all *Pseudomonas* tested, positively contribute to *Lm* attachment [16-18]. Besides strain differences, aspects such as the absolute and relative size of the respective species inocula and the sequence of access to the substratum surface, are likely to influence the interaction's outcome.

Temperature effect on dual species BF structure

Figure 3 shows the tridimensional structure of the dual species BF formed at 20°C (warm) and 4°C (cold) of *Pf* with three *Lm* strains, persistent 10p, non-persistent 6 and ScottA. The images of control BF, before chitosan exposure, are shown in the first and third columns. Though there was not a large difference in average viable cells of *Lm* per square centimeter of the coupons supporting binary BF formed at 4 or 20°C (Table 2), all-cell densities per cubic cm of the microcolonies (as seen by CLSM) were much higher at 4°C (not shown) since BF formed under refrigeration were much thinner (Figure 3) with a much smaller matrix volume per cell [17]. Thickness values of cold binary BF were 16, 38 and 54% of the respective values in warm ones for strains 10p, 6 and Scott A.

It should be reminded that *Pseudomonas* grows more quickly and produces much more matrix in these conditions than *Lm* [17] so, much or may be most of the dual species BF thickness, is probably due to *Pf*. The reasons behind the thinner matrix of the cold *Pf*/*Lm* BF are still unknown, but cold stress modulates specifically the expression of some genes involved in BF formation in *Pf* [19] and *Lm* [20,21].

Figure 3 also shows different patterns of surface coverage of the binary BF, depending on temperature and *Lm* strain involved. At 20°C, *Pf* with all the three tested *Lm* strains gave rise to an even, homogeneous coverage. At 4°C that was only the case of the persistent *Lm* strain (10p); the others provided a dense but patchy coverage as if, in the process of BF formation, cell adhesion had been slower or more impaired than adhered cell multiplication.

Presence and distribution of dead or severely damaged cells also differed among the control dual species BF (Figure 3). Those with the persistent *Lm* strain 10p showed a significant amount of dead cells across the thickness of both the warm and cold BF. Warm BF controls with the non-persistent *Lm* strain 6 showed fewer, homogeneously dispersed dead cells, but in cold ones, dead cells appeared predominantly in the void spaces not covered by micro colonies.

The underlying mechanisms for death or damage of these (*Pf* or *Lm*) cells are probably different for the two types of *Lm* strains. Let us remember that non persistent *Lm* populations in BF were not enhanced by *Pf* co culture; may be those easily damaged cells, excluded to the BF surface or in void spaces, could be Lm cells that failed to be sheltered or protected in the *Pf* matrix.

Chitosan effect on Lm and *Lm/Pf* biofilms: viable cells

The results on *Lm* of 1 h exposure of warm BF to 1% chitosan, expressed as log reductions of *Lm* viable counts, are shown in Figure 1. In warm monospecies BF, inactivation efficiency ranged between 3 and 6 log; variations in susceptibility depended on *Lm* strain, but could not be associated to serotype, lineage or persistent character. Two of the *Lm* strains, the persistent 17p and the non-persistent 6, were clearly more susceptible than average in those warm monospecies BF (over 5 log reduction). Cold *Lm* monospecies BF, were in general less susceptible to chitosan's treatment than warm ones (Figure 2). Inactivation efficiency ranged between 2 and 4 log (3 on average). 17p and number 6 strains were also the more susceptible strains in both cold and warm *Lm* monospecies BF.

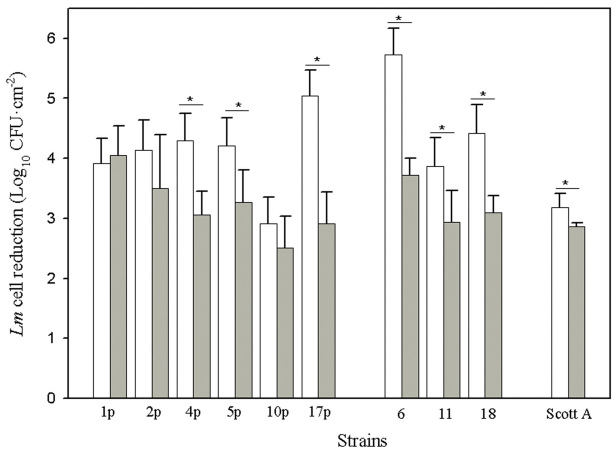

Figure 1: Chitosan effect against warm biofilms. Log reduction of Lm viable cells in pure (white bars) and binary (gray bars) 48 h/ 20°C biofilms after their exposure to 1% chitosan for 1h. Asterisks mark statistical differences ($p<0.05$) between pure and binary figures (n=8).

Variations in chitosan susceptibility of *Lm* in BF related to *Pf* presence and temperature were observed. In general, in warm BF, *Lm* was less susceptible to chitosan in dual species than in mono species BF (Figure 1); actually, *Pf*'s company protected from chitosan seven out of the ten *Lm* tested species (up to 2 log). Scott A was also less susceptible to chitosan in binary BF, either cold or warm, than in single species BF. Our results on *Lm* protection against chitosan obtained with warm binary BF coincide with what has been published by many authors on the increased resistance to sanitizers in mixed BF [22,23], particularly in the case of *Lm* and quaternary ammonium compounds. *Lm* has several mechanisms that improve the ability of this microorganism to adapt and survive at low temperatures [20]. Modifications in surface hydrophobicity and membrane fatty acid composition, for instance, may affect biocide penetration. Our results on chitosan susceptibility of *Lm* in cold BF (Figure 2) were in several ways, unexpected. For one thing, all environmental food industry *Lm* strains were less chitosan susceptible in cold mono species BF than in warm ones. Besides, in contrast with what was observed in warm BF, coculture with *Pf* increased chitosan susceptibility of several *Lm* strains in cold binary BF. More precisely, four *Lm* strains, all persistent ones, became more chitosan sensitive (in cold binary BF than in cold single species BF), three became less sensitive (including Scott A) and another three showed no difference in sensitivity.

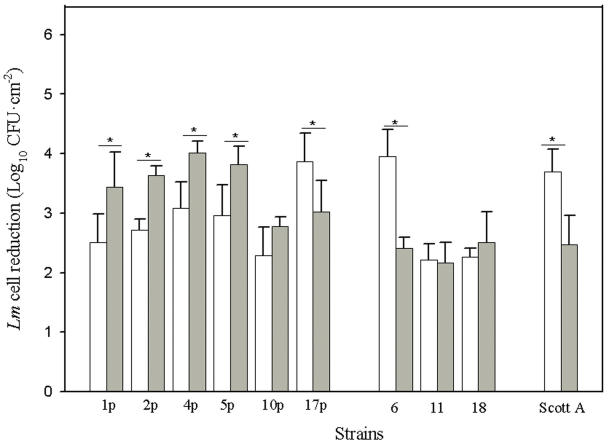

Figure 2: Chitosan effect against cold biofilms. Log reduction of *Lm* viable cells in pure (white bars) and binary (gray bars) 20 d/4°C biofilms after their exposure to 1% chitosan for 1h. Asterisks mark statistical differences ($p<0.05$) between pure and binary figures (n=8).

It seems therefore that low temperatures determine a particular mode of growth for *Pf* and *Lm* (may be not the same for all *Lm*), possibly another kind of interaction between the two species and in turn, a different BF structure, resulting in a different response to chitosan action. It is obvious that BF development at low temperature deserves much further study, for food safety reasons.

Changes on biofilm´s structure induced by chitosan

The effect of chitosan treatment was also studied by confocal microscopy. Figure 3 shows at relatively large scale (60X) the structure and the amount and distribution of dead and live cells (of either *Lm* or *Pf*) in cold or warm binary BF formed by *Pf* and three *Lm* strains (persistent 10p, non-persistent 6 or Scott A) before and after exposure to chitosan. Apart from the persistence trait, the two environmental strains chosen to obtain images had their own peculiarities. p10 BF were among the more resistant to chitosan (less than 3 log reduction) irrespective of temperature and *Pf* company. In contrast with that, strain 6 BF were much more susceptible to chitosan than average and *Pf* company substantially protected *Lm* in binary BF, irrespective of the temperature at which they were formed. As chitosan targets, binary BF with strain p10 or 6 can therefore be presumed to have relatively different properties.

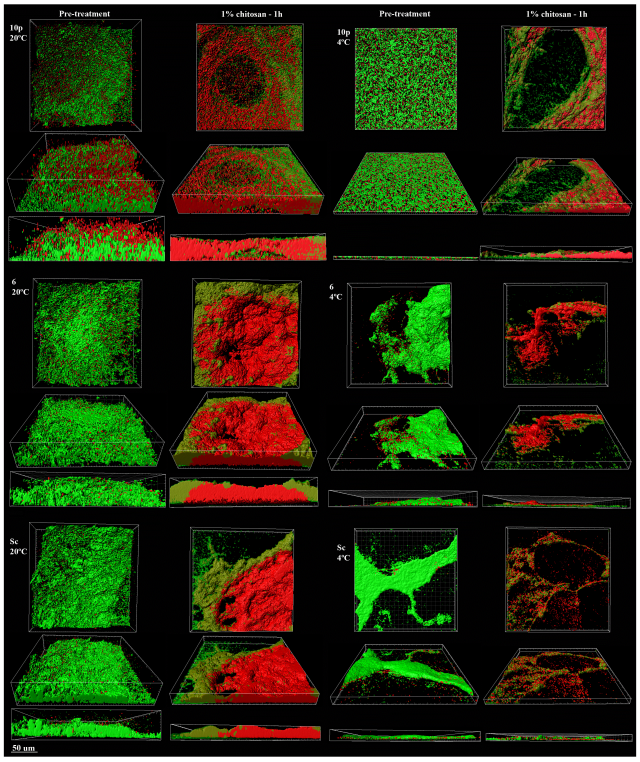

Figure 3: Structural changes in binary biofilms due to culture temperature and chitosan treatment. CLSM zenital, sagital and x-y sections images of binary biofilms formed after 48 h/20°C (left) or 20 days/4°C (right) by *Lm* strains 10p, 6 or Scott A, in presence of *Pf*, before and after 1h exposure to 1% chitosan. BF were stained with Live-Dead Kit; i.e. live cells in green (*Lm* and *Pf*); damaged or dead cells, in red (*Lm* and *Pf*).

The effect of chitosan on BF biovolume was much smaller than on viable cells, never above 50% of the total biomass and sometimes negligible (not shown). It gave rise however to CLSM-visible "injuries" in BF structure that differed depending on *Lm* strain (Figure 3). On both warm and cold BF carrying the persistent *Lm* strain 10p, typical injuries appeared as rather large perforations or holes, from which cells had been detached. Injuries caused by chitosan on the warm binary BF of non-persistent *Lm* strain 6 (Figure 3) were also localized, but instead of large holes, they were just small cavities or pits.

As a matter of fact, 1% chitosan in 1% acetic acid is a well dispersed, but rather viscous preparation. Low diffusion speed may favour a focalized reaction process, possibly not just a direct action of chitosan but a cooperative set of events, whose effects could slowly expand during exposure time. Local degradation of chitosan could be caused by enzymes produced by BF embedded cells, giving rise to chitooligosaccharides, sometimes more active against microorganisms than the large MW molecules [6]. Chitosan can be hydrolyzed unspecifically by many different enzymes, including proteases [24,25] and certainly also by chitinases, which have been reported to be produced by, or elicited in, *Listeria* and other bacteria [26-28]. The differences in morphological damages at the microcolony level (holes, pits, etc.) could be based on the amount and type of extracellular enzymes degrading chitosan produced by the different strains. Hollows in different types of BF occurring by not biocide-related mechanisms of "seeding dispersal", have been appraised by Kaplan [29]. Young [30] suggested that chitosan could open pores on vegetal polygalacturonic layers by sequestering the Ca that stabilizes its gel structure; that could also break the polymeric network of the BF matrix.

In conclusion, regarding environmental strains of *Lm* in dual species BF with *Pf*, it could be said that BF formed at 4°C were remarkably thinner, more compact and more cell dense than those formed at 20°C. The ecological interaction outcome between the two species was, for *Lm*, neutral or positive in the case of persistent strains and negative (about one Log) for non-persistent ones. Both the pattern of species interaction and the temperature-dependent mode of growth seem to have a relevant role on the effects of chitosan exposure, which were more significant on viable *Lm* cells than on BF biomass.

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