Biofilms of *Listeria monocytogenes* Produced at 12°C either in Pure Culture or in Co-Culture with *Pseudomonas aeruginosa* Showed Reduced Susceptibility to Sanitizers

António Lourenço, Henrique Machado, and Luisa Brito

Abstract: The biofilm-forming ability of 21 *Listeria monocytogenes* isolates, previously pulsotyped and corresponding to 16 strains, from different origins was evaluated using the Calgary Biofilm Device®, at 37 °C. Biofilms of 4 selected strains were also produced either on pure cultures or on co-cultures with *Pseudomonas aeruginosa* (PAO1), at 12 °C and at 37 °C. For these biofilms, the minimum biofilm eradication concentrations (MBECs) of 4 commercial dairy sanitizers (1 alkyl amine acetate based—T99, 2 chlorine based—T66 and DD, and 1 phosphoric acid based—BP) were determined. *Listeria monocytogenes* biofilms grown, either at 37 °C or 12 °C, were able to achieve similar cell densities by using different incubation periods (24 h and 7 d, respectively). In co-culture biofilms, *P. aeruginosa* was the dominant species, either at 37 °C or at 12 °C, representing 99% of a total biofilm population of 6 to 7 log CFU/peg. Co-culture biofilms were generally less susceptible than *L. monocytogenes* pure cultures. More interestingly, the biofilms produced at 12 °C were usually less susceptible to the sanitizers than when produced at 37 °C. Single or co-culture biofilms of *L. monocytogenes* and PAO1, particularly produced at 12 °C, retrieved MBEC values for agents T99 and BP that were, at times, above the maximum in-use recommended concentrations for these agents. The results presented here reinforce the importance of the temperature used for biofilm formation, when susceptibility to sanitizers is being assessed.

Keywords: biofilms, Calgary Biofilm Device, *Listeria monocytogenes*, low temperature, sanitizers

Practical Application: Since most food plants have cold wet growth niches in production and storage areas, susceptibility testing should be performed on biofilms produced at refrigeration temperatures. Moreover, the efficiency of the sanitizers used in food industries should be performed on mixed culture biofilms, since in field conditions these will predominate. The results presented here highlight the importance of the temperature used for biofilm formation, when susceptibility to disinfectants is being assessed, as biofilms produced at lower temperature were less susceptible to sanitizers.

Journal of Food Science A Publication of the Institute of Food Technologists

Introduction

Listeria monocytogenes is a foodborne pathogen that may cause severe human and animal disease. Listeriosis occurs primarily in pregnant woman, newborn infants, elderly, and immunocompromised. It has a high-mortality rate from 16% to 38% (Mitjà and others 2009). The ability to grow under a wide range of environmental stresses such as extreme pH values, high salt concentrations and low temperatures as well as the ability to form biofilms make this bacterium difficult to eradicate on food processing facilities. Inadequate cleaning and disinfecting procedures on food processing environments may lead to the emergence of persistent strains resulting in cross-contamination of food products.

According to Donlan and others (2002), biofilms are defined as a microbial sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances (EPSs) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription. The biofilm-forming ability of bacteria has been assessed by several techniques, which include biofilm visualization and biofilm quantification. Some of these methods have been reviewed by Branda and others (2005).

Biofilm quantification can be evaluated by direct viable counts of the cells recovered from the biofilm, or indirectly by staining. The disruption of the biofilm by sonication, vortexing, or scraping may lead to biofilm clumps difficult to dissociate into single-cell for plating, nevertheless, the conventional staining methods will not provide information about cell viability (Pettit and others 2005). The Calgary Biofilm Device® (CBD) is a system that allows, with a simple batch culture technique, to reliably grow 96 equivalent biofilms at a time, in an area of about 110 mm^2 each. The 96 identical pegs are inserted into the wells of a microtiter plate with

MS 20100919 Submitted 8/12/2010, Accepted 11/15/2010. *Authors are with CBAA, Laboratório de Microbiologia, Inst. Superior de Agronomia, Technical Univ. of Lisbon, Lisbon, Portugal. Direct inquiries to author Brito (E-mail: lbrito@isa.utl.pt).*

doi: 10.1111/j.1750-3841.2010.02011.x

growth medium and incubated in an orbital shaker allowing the development of biofilms for testing an array of antimicrobial compounds with varying concentrations (Ceri and others 1999). These tests are usually performed with biofilms produced at temperatures favoring growth of the cultures, such as 25 °C (Ayebah and others 2005). However, in much of the food industry, the portions of the processing environment most likely to culture positive for *Listeria* spp. are at cold temperatures. For this reason, susceptibility testing to disinfectant should be performed on biofilms produced at refrigeration temperatures.

Previous work has shown that neither the resistance nor the adaptation of planktonic cells to the sanitizers seems to be the main reason for the existence of persistent *L. monocytogenes* strains in traditional cheese dairies (Lourenço and others 2009).

In this work, the ability of *L. monocytogenes* strains to form biofilms and the sanitizer susceptibility of biofilms produced at 37 °C and at 12 °C, either in pure cultures or in co-cultures with *Pseudomonas aeruginosa*, were evaluated. To our knowledge, this is the first report on data from susceptibility testing of *L. monocytogenes* biofilms produced at low temperature.

Materials and Methods

Characterization of the isolates

Twenty-one *L. monocytogenes* isolates corresponding to 16 strains and 1 reference strain of *P. aeruginosa* (PAO1) were used in this study (Table 1). The *L. monocytogenes* isolates included 3 culture strains obtained from CECT (Colleción Española de Cultivos Tipo) and CIP (Collection de l'Institut Pasteur); CECT936 (serovar 1/2b), CECT4032 (serovar 4b), and CIP104794 (= NCTC7973) (serovar 1/2a). Twelve of the 21 *L. monocytogenes* isolates were selected from 6 traditional cheese dairies (A, B, C, D, E, and F) producers of soft ewe's cheese, according to their origin (milk, cheese, or dairy environment) (Table 1). The procedures for the collection, identification, and characterization of these isolates by serotyping, multiplex polymerase chain reaction, and pulsed field gel electrophoresis subtyping were previously described (Leite and others 2006; Neves and others 2008).

Preparation of the inocula

In order to prepare the inocula, isolates from cryogenic collection (–80 °C) were struck onto TSA-YE (30 g/L tryptone soya agar [Oxoid, Hampshire, UK], 6 g/L yeast extract [Oxoid]) and incubated overnight at 37 °C. The purity of the cultures was confirmed by streaking isolated colonies onto the chromogenic selective medium CHROMagar™ Listeria (ASEPT, Laval, France) and onto horse blood agar (HBA): Nutritive agar (Oxoid, Hampshire, England) overlayed with blood agar (Oxoid) containing 5% (v/v) defibrinated horse blood (Probiológica, Belas, Portugal).

For each isolate, bacterial colonies were collected from TSA-YE plates and suspended into tryptone soya broth (TSB) (Biokar Diagnostics, Beauvais, France) in order to achieve bacterial concentration of 1×10^7 CFU m/L. The determination of the bacterial concentration was made using a calibration curve relating A_{600nm} to CFU/mL. The concentration of the inocula was always confirmed by plating adequate decimal dilutions onto TSA-YE. The inocula suspensions were carefully vortexed to eliminate possible bacterial clumps. The co-culture inocula were prepared by mixing suspensions at a ratio of 1:1 in order to maintain a total bacterial concentration of 1×10^7CFU m/L.

Disinfectant agents used

Table 2 presents the characteristics of the 4 sanitizers (2 alkaline/chlorine based—DD and T66, 1 acidic—BP, and 1 alkyl amine acetate based—T99) used in this study. The experimental conditions (exposure time and temperature) used for the minimum biofilm eradication concentration (MBEC) determination were chosen according to the product's datasheet and are also presented on Table 2.

In the cheese dairies where the dairy isolates were collected, the sanitation and disinfection procedures consisted of the manual usage of a tensio-active agent for debris and grease removal, followed by manual disinfection with a chlorine-based agent (DD or T66). Agent DD and agent T66 were dissolved, or diluted in water, at about 50 °C, or at room temperature, respectively, and sprayed on surfaces, equipments, and utensils. Once a week, the chlorine

Table 1–Characterization of the isolates.

Isolate CBISA[a]	Species	Serovar/ Serogroup	Pulsotype[b]	Origin	Cheese dairy
3001	*L. monocytogenes*	4b	31	Reference strain serovar 4b (=CECT4032[c])	–
3004	*L. monocytogenes*	1/2b	34	Reference strain serovar 1/2b (=CECT936[c])	–
3007	*L. monocytogenes*	1/2a	36	Reference strain serovar 1/2a (=CIP104 794[d])	
3096	*L. monocytogenes*	4b	6	Cheese	C
3107	*L. monocytogenes*	4b	6	Dairy environment	D
3119	*L. monocytogenes*	1/2b	3	Cheese	B
3130	*L. monocytogenes*	1/2a	1	Cheese	B
3169	*L. monocytogenes*	4b	10	Cheese	F
3172	*L. monocytogenes*	4b	6	Dairy equipment	A
3176	*L. monocytogenes*	4b	9	Cheese	E
3183	*L. monocytogenes*	4b	7	Bulk milk	A
3188	*L. monocytogenes*	4b	7	Dairy equipment	A
3192	*L. monocytogenes*	4b	8	Dairy equipment	D
3219	*L. monocytogenes*	4b	7	Cheese	E
3845	*L. monocytogenes*	4b	20	Human	–
3846	*L. monocytogenes*	4b	21	Human	–
3849	*L. monocytogenes*	1/2b	5	Human	–
3851	*L. monocytogenes*	1/2b	4	Human	–
3880	*L. monocytogenes*	1/2c-3c	2	Cheese	E
3999	*L. monocytogenes*	4b-4d-4e	37	Lettuce and tomato salad	–
4009	*L. monocytogenes*	4b-4d-4e	37	Lettuce and tomato salad	–
4076	*P. aeruginosa*	–	–	Strain PAO1–IBB[e]	–

[a]CBISA—Colecção de Bactérias do Instituto Superior de Agronomia, ISA/UTL. [b]From Leite and others 2006 and Neves and others 2008. [c]CECT—Collección Española de Cultivos Tipo. [d]CIP—Collection de l' Institut Pasteur. [e]IBB—Inst. for Biotechnology and Bioengineering, IST/UTL.
Pulsotype 6—isolates with this pulsotype were collected during 1 y from cheese dairies A, C, and D (Leite and others 2006).

disinfectant was replaced by T99. At the parlors, clean in place systems were used. Twice a day, after milking, the equipment was rinsed with cold water and then a chlorine-based agent (DD or T66) was used. Once a week, instead of the chlorine disinfectant, agent BP was used.

For the evaluation of the MBEC, stock solutions of each sanitizer were prepared just before use by completely diluting or dissolving each agent in sterile Ringer's solution.

Evaluation of biofilm-forming ability using the CBD

The biofilm-forming ability of the strains was evaluated using the CBD with an incubation period of 24 h, at 37 °C. Four *L. monocytogenes* strains were further selected according to their origin (dairy environment, human, and cheese), serovar (4b, 1/2b, and 1/2c) including a persistent strain (3107). These strains were also evaluated on their biofilm-forming ability at 12 °C for 7 d. Co-culture biofilms with each of these 4 strains and *P. aeruginosa* were produced in both growth conditions.

The CBD was inoculated with 150 μL of inoculum in each well, sealed with Parafilm®, and placed on an orbital shaker set to 150 revolutions per minute, in a humidified atmosphere, at 37 °C (Agitorb 200IC, Aralab, Lisbon, Portugal) or at 12 °C (Minitron A172, HT infors, Bottmingen, Switzerland). After the CBD incubation, the pegs on the lid were rinsed in 200 μL Ringer's solution in a microtiter plate. The quantification of the formed biofilm was then achieved by removal (with flamed pliers) of 3 pegs from the lid of the CBD of each culture and immersion into 200 μL Ringer's solution in a microtiter plate. This plate was then transferred onto the tray of a sonicator (P-Selecta, Barcelona, Spain), and the pegs were sonicated for 1 min to detach the cells. The resultant suspension was decimal diluted and triplicate plated onto TSA-YE.

Suspensions resulting from co-culture biofilms were additionally plated, in triplicate, onto PALCAM (Biokar Diagnostics) which by inhibiting the growth of *P. aeruginosa* allowed the estimation of this bacterial population by the difference between the total count on TSA-YE and the *L. monocytogenes* population on PALCAM. Plates were incubated at 37 °C, during 24 h and 48 h, for TSA-YE and PALCAM, respectively, before counting the colonies.

Determination of the MBEC

The MBEC of the 4 disinfectant agents was determined for the 5 strains (the *P. aeruginosa* and the 4 *L. monocytogenes* strains) either in pure cultures or in co-cultures of *P. aeruginosa* strain with each of the *L. monocytogenes* strains. The determination of the MBEC of each disinfectant was performed in triplicate for the biofilms (either in pure culture or in co-culture) produced at 37 °C and at 12 °C.

After biofilm growth and rising as previously described, the CBD lid was transferred to a microtiter plate containing serial 2-fold dilutions of each disinfectant agent in Ringer solution in a final volume of 200 μL. After the contact time and temperature with the respective disinfectant agent (Table 2), the CBD lid was rinsed twice, as described previously, for 90 s each time. The lid was then transferred to a recover microtiter plate containing 200 μL of TSB in each well. This recover plate was sonicated, as described before, in order to disrupt biofilms from the surface of the pegs. The recovery plate was then incubated for 24 h at 37 °C, and the wells were visually checked for turbidity.

The MBEC was defined as the lowest concentration of the disinfectant agent required to eradicate the biofilm of a selected isolate after a recovery period of 24 h at 37 °C.

Interpretation of the results and data analysis

In biofilm production data, the homogeneity of variance was confirmed and ANOVA of the log CFU values was carried out using least significant differences *post hoc* multiple comparison tests by running the program Statistica, version 6, from Statsoft, Tulsa, Okla., U.S.A. Those values whose probability of occurrence was greater than 95% ($P < 0.05$) were considered as significant values.

In MBEC assays, a result was considered positive, thus presenting microbial growth, when at least 2 of 3 replicates presented visible growth. When visible growth was detected only in one or in none of the 3 replicates, the result was considered negative.

Results and Discussion

Evaluation of biofilm-forming ability using the CBD

In order to identify the sonication time necessary and sufficient to promote the detachment of biofilms, several sonication times

Table 2–Information according to product's safety datasheet on disinfectant agents and contact time and temperature used for MBEC determination.

			Manufacturer's recommendations			Conditions used for MBEC determination	
Agent (manufacturer)	Properties	pH	Concentration	Time min	Temperature	Time min	Temperature (°C)
Topax 99–T99 (Henkel Ecolab)	Detergent/disinfectant (5% to 15% alkyl amine acetate)	9 (1%, 20 °C)	2% to 3 % (v/v)= 19800 to 29700 μg/mL	10 to 20	Room temperature	20	Room temperature
			0.5% to 1% (v/v)= 5000 to 9900 μg/mL	More than 20			
Topax 66–T66 (Henkel Ecolab)	Liquid alkaline-/chlorine-based detergent/disinfectant	11.8 (1%)	2% to 5% (v/v)= 22600 to 56500 μg/mL	15	Rinse previously with water (40 to 60 °C)	15	37
Basochlor DD–DD (Langlois)	Powder alkaline/chlorine-based detergent/disinfectant	12.4 ± 0.2	0.5% to 2% (w/v)= 5000 to 20000 μg/mL	10 to 15	70 to 80 °C (greasy material) 40 to 50 °C (not greasy material)	15	50
Basotop–BP (Langlois)	Acid disinfectant (phosphoric acid)	1.7 ± 0.2 (1%)	0.5% to 1% (v/v) = 5700 to 11500 μg/mL	10 to 15	50 to 70 °C	15	60

were tested (30 s, 1, 2, 5, 6, 10, and 15 min) using 3 *L. monocytogenes* strains (3001, 3077, and 3130). The highest values of CFU per peg were obtained after 1 min of sonication (data not shown). This sonication time was then selected for the removal of biofilms from the pegs.

The resulting viable count data from biofilm-forming ability assessment, at 37 °C, were transformed to log CFU/peg (Figure 1). The results ranged from 5.3 to 6.4 log CFU/peg for *L. monocytogenes*. *Pseudomonas aeruginosa* displayed significantly higher count data (7.5 log CFU/peg).

Pseudomonas aeruginosa PAO1 presented a biofilm-forming ability significantly higher than *L. monocytogenes* isolates. For *L. monocytogenes*, it was possible to establish 4 statistically homogeneous groups ($P > 0.05$). Strain 3007 exhibited biofilm-forming ability significantly lower than the other strains. Within the other *L. monocytogenes* isolates, it was also possible to establish 3 statistically homogeneous groups ($P > 0.05$). A group with a lower biofilm-forming ability, an intermediate group, both with 4 strains, and a group with higher biofilm-forming ability that included isolates (3096, 3172) of the persistent strain used (Figure 1 and Table 1).

In agreement with the report by Kalmokoff and others (2001), in the present work, the method used for evaluation of biofilm-forming ability suggested the lack of association between biofilm-forming ability and the serovar of the strains. When compared the phylogenetic group of the isolates with its corresponding biofilm-forming ability, it was not possible to establish a tendency. In fact, the groups identified as having a poor biofilm-forming ability included isolates of the 3 tested serovars (1/2a, 1/2c, and 4b). Serovars 1/2a, 1/2b, and 4b were also identified within the group having a good biofilm-forming ability (Figure 1 and Table 1).

In contrast with other methods, such as cristal violet staining (Djordjevic and others 2002; Borucki and others 2003), the CBD determinations are based on direct quantification of viable cells which gives a more useful information for setting sanitization operation procedures.

Co-cultures of *L. monocytogenes* and *P. aeruginosa* at 12 °C

Four *L. monocytogenes* strains were selected based on different serovar, origin, and biofilm-forming ability to be used for culture at 12 °C and also for co-culture with *P. aeruginosa*, at 37 and 12 °C. When grown in single culture at 12 °C (Figure 2), isolate 3849 showed a significantly lower ($P < 0.05$) biofilm-forming ability compared not only to *P. aeruginosa* but also to the other *L. monocytogenes* strains. Strain 3880, although not significantly different from the other 2 *L. monocytogenes* strains, was also not significantly different from *P. aeruginosa*, thus presenting an intermediate biofilm-forming ability.

A careful comparison of the strains' ability to form biofilms at 37 °C and 12 °C must be made since the incubation time was different (24 h and 7 d, respectively). Despite these differences, no significantly different biofilm production ability (log values of CFU per peg) was obtained at both temperatures for all strains. Although not quantified in this work, it is known that the amount of the biofilm also depends on the amount of polysaccharide in the matrix, as well as on its composition that are dependent on incubation time and temperature (Zameer and others 2010). This fact can account for differences in biofilm susceptibility and reinforces the importance of using biofilms produced at low temperatures in disinfectant testing.

Figure 3 represents the mean values of log CFU/peg obtained for co-culture biofilms of *L. monocytogenes* and *P. aeruginosa* produced at 37 °C and at 12 °C. Among the *Listeria* strains, no significant differences ($P > 0.05$) were detected either for the co-cultures grown at 37 °C or at 12 °C, except for the co-culture M3 (with the lower biofilm producer *L. monocytogenes* 3849) at 37 ° C (Figure 3).

In co-cultures, either at 37 °C or at 12 °C, the total log CFU/peg was not significantly different from the *P. aeruginosa* log CFU/peg (Figure 3). In fact, *P. aeruginosa* was the dominant species and *L. monocytogenes* represented an average of 1% of the total biofilm although the 2 species were in initial equal concentrations in the inocula. These results are consistent with those from Fatemi and Frank (1999) in which *L. monocytogenes* represented only 0.01% of the total co-culture biofilm of *L. monocytogenes* and *Pseudomonas* sp. Nevertheless, the same order of magnitude of *L. monocytogenes* cells was obtained in both studies (5 log CFU/cm^2).

Determination of the MBEC

In addition to biofilm-forming ability evaluation, the CBD allows a rapid screening of several isolates to determine the MBEC. Biofilms of 4 *L. monocytogenes* strains and a *P. aeruginosa* strain as well as the respective co-culture biofilms, produced at 37 °C and at 12 °C, were challenged against 4 commercial sanitizers under conditions similar to those recommended by the agent's manufacturers (Table 2). The MBEC values are presented in Table 3.

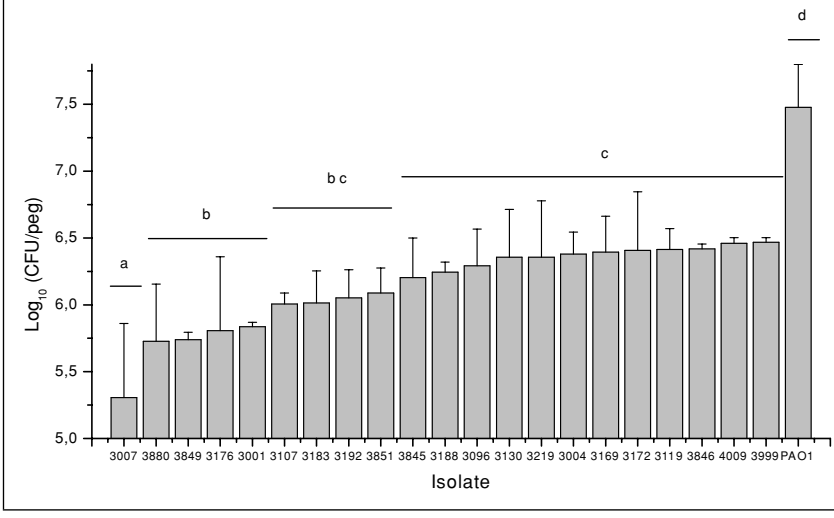

Figure 1–Biofilm-forming ability of *Listeria monocytogenes* isolates, using the Calgary Biofilm Device® (CBD), after 24 h of biofilm growth at 37 °C. Medium values of log CFU/peg ± standard deviation (SD) (error bars). The lines over the bars group strains with no significant differences among them. Equal letters mean no significant differences between count values. Different letters mean that count values are statistically different ($P < 0.05$).

For biofilms produced at 37 °C, the highest value of MBEC of T99 (30720 μg/mL) was recorded for the *P. aeruginosa* biofilm. At the same temperature of growth, the low biofilm forming strain 3880 (Figure 1) registered the lowest MBEC value (1920 μg/mL) among the *L. monocytogenes* strains. This effect was no longer recorded when the biofilm was produced at 12 °C for 7 d (MBEC of T99 = 30720 μg/mL) (Table 3). In fact, during growth at 12 °C, this *L. monocytogenes* strain presented an intermediate biofilm-forming ability between *L. monocytogenes* strains and the higher biofilm-forming strain of *P. aeruginosa*. These results suggest that the kinetics of biofilm formation and maturation may be strain dependent, but differences among strains will be reduced or even eliminated with extended incubation times. This fact also reinforces the importance of prompt and proper sanitization of industrial facilities.

In general, at 12 °C, the MBEC values of T99 obtained for the co-culture biofilms were among the values obtained for the pure cultures of *L. monocytogenes* and *P. aeruginosa* (Table 3). Moreover, the MBEC values of T99 for *P. aeruginosa* at 37 °C and at 12 °C, as well as for strain 3880 and the co-culture M1 produced at 12 °C (30.7 mg/mL) were above the recommended in-use concentrations for this agent (29.7 mg/mL) (Table 2 and 3).

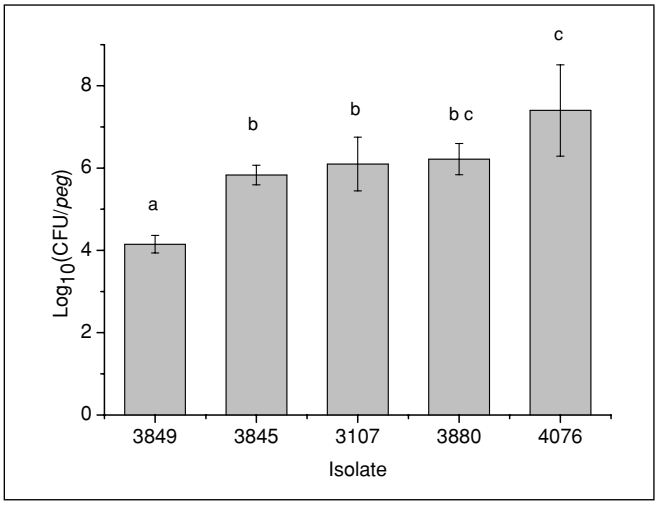

Figure 2–Means values of log CFU/peg ± SD (error bars) corresponding to biofilms of *Pseudomonas aeruginosa* PAO1 and of 4 *L. monocytogenes* strains, respectively, after 7 d of biofilm growth at 12 °C. Equal letters mean no significant differences between count values. Different letters mean that count values are statistically different ($P < 0.05$).

For the biofilms produced at 37 °C, the sanitizer agent T66 presented MBEC values very homogeneous for the *L. monocytogenes* strains. These values were lower than for the *P. aeruginosa* strain and the co-culture biofilms. Nevertheless, when biofilm growth was performed at 12 °C, the *L. monocytogenes* strains presented the same MBEC value as *P. aeruginosa* and more remarkable, the co-culture biofilms presented higher MBEC values, although below the in-use concentrations recommended for this agent.

For the chlorine-based agent DD, the pure cultures grown at 37 °C presented higher MBEC values compared with the cultures grown at 12 °C, however, the obtained values were always below the in-use recommended concentrations. With this sanitizer, after incubation, the recovered suspensions of co-culture biofilms, which had been previously exposed to the higher concentrations of this sanitizer, presented a turbidity characteristic of pure cultures of *L. monocytogenes*. In contrast, the recovered suspensions, which had been exposed to lower concentrations of this sanitizer, presented a turbidity characteristic of pure cultures of *P. aeruginosa*. Nevertheless, when Gram staining of these suspensions was performed, both species were identified. It is possible that *L. monocytogenes* will be preferably present in the inner layers of the biofilm, becoming this way more protected of the sanitizer action, whereas *P. aeruginosa* will be preferably present on the outer layers of the biofilm. The fact that *L. monocytogenes* is a facultative anaerobe and *P. aeruginosa* is an obligate aerobe may reinforce this hypothetical species disposition within the biofilm. This will be further investigated.

For agent BP, MBEC for biofilms produced at 37 °C were within the recommend range of in-use concentrations (Table 2 and 3). However, the biofilms produced at 12 °C by pure cultures of strains 3107, 3880, PAO1, and the co-culture M4 (3880 + PAO1) presented MBEC values higher than the recommended concentrations for this agent.

Generally, co-culture biofilms were less susceptible to biocides than the respective pure culture biofilms. These results agree with the findings of Norwood and Gilmour (2000), which by using co-cultures of *L. monocytogenes*, *P. fragi*, and *Staphylococcus xylosus*

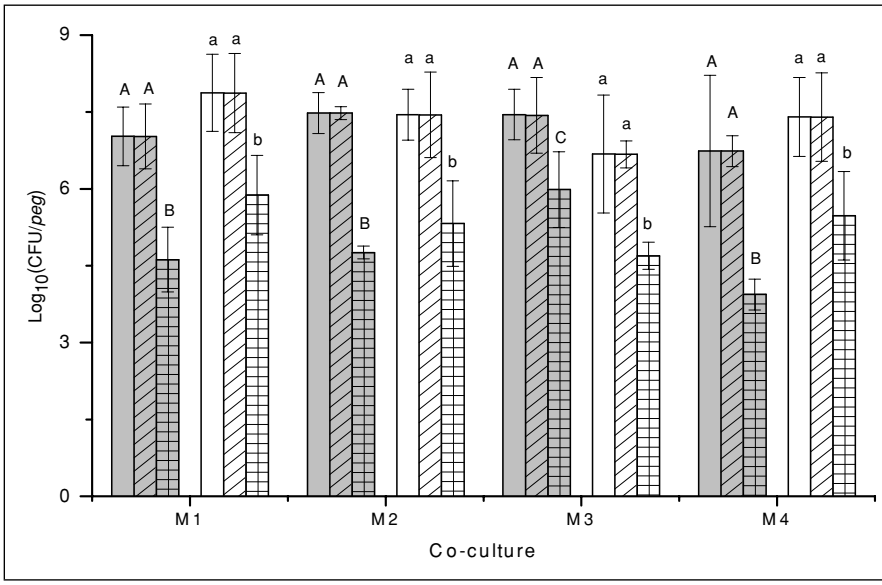

Figure 3–Means values of log CFU/peg ± SD (error bars) corresponding to co-culture biofilms, of each of the 4 *L. monocytogenes* strains with *P. aeruginosa* PAO1, formed at 37 °C for 24 h (grey) and 12 °C for 7 d (white). *M1 = 3107 + PAO1; M2 = 3845 + PAO1; M3 = 3849 + PAO1; M4 = 3880 + PAO1.* ▩—Total CFU per peg (37 °C); ▨— *P. aeruginosa* (37 °C); ▦—*L. monocytogenes* (37 °C); □—Total CFU per peg (12 °C); ▨—*P. aeruginosa* (12 °C); ▦—*L. monocytogenes* (12 °C). Upper case equal letters refer to statically undifferentiated values of CFU per peg for biofilms grown at 37 °C. Lower case letters refer to statistically undifferentiated values of CFU per peg for biofilms grown at 12 °C. Different letters refer to statistically different values of CFU per peg ($P < 0.05$).

Table 3–MBEC values of 4 sanitizer agents for biofilms produced at 37 °C and at 12 °C, of 4 *L. monocytogenes* strains, 1 *P. aeruginosa* (PAO1), and the respective co-culture biofilms.

		Strain/co-culture								
		3107	3845	3849	3880	PAO1	M1	M2	M3	M4
Biofilm formation	Disinfectant agent based on	MBEC (μg/mL)								
37 °C, 24 h	Alkyl amine acetate (T99)	7680	3840	7680	1920	30720	15360	7680	3840	15360
	Liquid alkaline/chlorine (T66)	50	50	25	50	400	200	1600	400	800
	Powder alkaline/chlorine (DD)	625	1250	312.5	312.5	2500	2500	2500	10000	1250
	Phosphoric acid (BP)	1000	4000	1000	4000	8000	4000	8000	2000	4000
12 °C, 7 d	Alkyl amine acetate (T99)	15360	15360	15360	30720	30720	30720	15360	7680	3840
	Liquid alkaline/chlorine (T66)	400	400	100	400	400	1600	6400	6400	6400
	Powder alkaline/chlorine (DD)	156.3	156.3	78.1	312.5	1250	1250	20000	10000	20000
	Phosphoric acid (BP)	16000	4000	1000	16000	32000	4000	1000	1000	32000

M1 = 3107 + PAO1; M2 = 3845 + PAO1; M3 = 3849 + PAO1; M4 = 3880 + PAO1.

also observed a protective effect of the mixed culture biofilms when compared with the pure culture biofilms. This effect is pointed out by Carpentier and Cerf (1993) as the result of higher numbers of bacteria and higher amounts of EPSs. In fact, although in this work, EPS production was not evaluated, the number of total viable bacteria (CFU) present in the co-culture biofilms was always at least more than 1 log higher than *L. monocytogenes* pure culture biofilms (Figure 1–3).

Conclusion

The study of biofilm-forming ability of contaminant microorganisms and especially pathogens, on equipments and other surfaces of food industries, is useful for the development of effective hygienization routines, and ultimately to ensure food safety. Ineffective sanitization operations may allow biofilms to develop and strains to persist for long periods that may prompt the occurrence of recurrent contaminations.

In field conditions, mixed cultures biofilms will predominate. Likewise, since most food plants have cold wet growth niches in production and storage areas, susceptibility testing should be performed on biofilms produced at refrigeration temperatures. Notwithstanding, to our knowledge, this is the first study on biofilm susceptibility to commercial sanitizers performed on biofilms produced at low temperature.

In a previous study with the same sanitizers used in this work, the results from susceptibility testing of *L. monocytogenes* strains suggested that except for agent BP (in the presence of organic matter), the other in-use sanitizers were bactericidal for persistent and nonpersistent strains at in-use concentrations (Lourenço and others 2009). In the present study with *L. monocytogenes* biofilms, in pure culture as in co-culture with *P. aeruginosa*, isolates of the persistent strain were included in the group of good biofilm formers. Biofilms of *L. monocytogenes*, *P. aeruginosa,* and co-culture biofilms, particularly produced at 12 °C, retrieved MBEC values for agents T99 and BP that were, at times, above the maximum in-use recommended concentrations for these agents. We did not observe this with the chlorine-based agents (DD and T66). In fact, agent T66 was found the most effective sanitizer for the eradication of these biofilms, even produced at the refrigeration temperature (12 °C).

The MBEC data presented here, although not intended for direct extrapolation of the concentrations to be used in field, show that biofilms produced at 12 °C were generally less susceptible than the biofilms produced at 37 °C, despite cell population was similar in both conditions used. The obtained results highlight the importance of the temperature used for biofilm formation, when susceptibility to disinfectants is being evaluated.

Acknowledgments

The authors thank Dr. Arsénio Fialho (IBB, IST/UTL) for strain *P. aeruginosa* PAO1 used in this study and João Madanelo (ANCOSE) for the tested sanitizer agents and for helpful information regarding hygiene routines at the dairies. We also thank Professor Santos Oliveira (FCT/UNL) for his relevant suggestions regarding the study of biofilms formed at low temperatures. The financial support of Fundação para a Ciência e Tecnologia (FCT) (Project PPCDT/SAU-ESP/56243/2004) is gratefully acknowledged.

References

Ayebah B, Hung Y-C, Frank JF. 2005. Enhancing the bactericidal effect of electrolyzed water on *Listeria monocytogenes* biofilms formed on stainless steel. J Food Prot 68(7):1375–80.

Borucki MK, Peppin JD, White W, Loge F, Call DR. 2003. Variation in biofilm formation among strains of *Listeria monocytogenes*. Appl Environ Microbiol 69(12):7336–42.

Branda SS, Vik A, Friedman L, Kolter R. 2005. Biofilms: the matrix revisited. Trends Microbiol 13(1):20–6.

Carpentier B, Cerf O. 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. J Appl Bacteriol 75(6):499–511.

Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. 1999. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol 37(6):1771–6.

Djordjevic D, Wiedmann M, McLandsborough LA. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl Envir Microbiol 68(6):2950–8.

Donlan RM, Costerton JW. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15(2):167–93.

Fatemi P, Frank JF. 1999. Inactivation of *Listeria monocytogenes*/*Pseudomonas* biofilms by peracid sanitizers. J Food Prot 62(7):761–5.

Kalmokoff ML, Austin JW, Wan X-D, Sanders G, Banerjee S, Farber JM. 2001. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. J Appl Microbiol 91(4):725–34.

Leite P, Rodrigues R, Ferreira MASS, Ribeiro G, Jaquet C, Martin P, Brito L. 2006. Comparative characterization of *Listeria monocytogenes* isolated from Portuguese farmhouse ewe's cheese and from humans. Int J Food Microbiol 106(2):111–21.

Lourenço A, Neves E, Brito L. 2009. Susceptibility of *Listeria monocytogenes* from traditional cheese-dairies to in-use sanitizers. Food Control 20:585–9.

Mitjà O, Pigrau C, Ruiz I, Vidal X, Almirante B, Planes A, Molina I, Rodríguez D, Pahissa A. 2009. Predictors of mortality and impact of aminoglycosides on outcome in listeriosis in a retrospective cohort study. J Antimicrob Chemoth 64(2):416–23.

Neves E, Lourenço A, Silva AC, Coutinho R, Brito L. 2008. Pulsed-field gel electrophoresis (PFGE) analysis of *Listeria monocytogenes* isolates from different sources and geographical origins and representative of the twelve serovars. Syst Appl Microbiol 31(5):387–92.

Norwood DE, Gilmour A. 2000. The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in steady-state multispecies biofilme. J Appl Microbiol 88(3):512–20

Pettit RK, Weber CA, Kean MJ, Hoffmann H, Pettit GR, Tan R, Franks KS, Horton ML. 2005. Microplate alamar blue assay for *Staphylococcus epidermidis* biofilm susceptibility testing. Antimicrob Agents Chemother 49(7):2612–7.

Zameer F, Gopal1 S, Krohne G, Kreft J. 2010. Development of a biofilm model for *Listeria monocytogenes* EGD-e. World J Microbiol Biotech 26(6):1143–7.