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Antimicrobial effects of sanitizers against planktonic and sessile *Listeria monocytogenes* cells according to the growth phase

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Abstract

This study was designed to investigate the individual or combined effects of sanitizers on survival of planktonic or sessile *Listeria monocytogenes* cells at different phase of growth. The sanitizers tested included: (i) acetic acid (pH 5.0), (ii) NaOH (pH 12.0), (iii) 10% Na_2SO_4, (iv) 10% Na_2SO_4 and acetic acid (pH 5.0), (v) 10% Na_2SO_4 and NaOH (pH 12.0), (vi) a quaternary ammonium (20 ppm) and (vii) glyceryl monolaurate (75 ppm). Results revealed a great efficacy of alkaline treatments on both sessile and planktonic cells with a slightly higher resistance of 6 h biofilms. Quaternary ammonium appeared very effective in killing more than 98% of cells, but a resistance of 7 days biofilm was observed. Other sanitizers did not succeed in inhibiting totally the pathogen but acted in a similar way on both sessile and planktonic cells. Renewing the medium or not do not seem to be the major cause of a resistance emergence.

Keywords: *Listeria monocytogenes*; Sanitizer; Planktonic; Biofilm; Mortality

1. Introduction

The presence of *Listeria monocytogenes* in food processing plants is often considered as an important source of (re)contamination of foodstuffs and surfaces especially when this microorganism is present as a biofilm [1,2]. This Gram-positive foodborne pathogen is commonly found in the environment and has been isolated from an extensive range of food products such as cheese or meat [3,4]. Numerous disinfecting tests were performed in order to prevent this ubiquitous pathogen from developing in food processing plants [5,6]. Most of these tests have been performed on planktonic cells, but studies made in this area have indicated that adherent bacteria (sessile cells) could be more resistant to disinfecting agents than their planktonic counterparts [7,8].

Adhesion and colonization of surfaces lead to important modifications in cell physiology [9,10]. A more important resistance to several stresses and disinfecting agents has been reported [11,12], which may be explained in part by phenotypic [13] and proteomic [14] variations. The potential for biofilm development by *L. monocytogenes* on various kinds of surfaces is well documented [15–19] and several studies have focused on the increased resistance of sessile *L. monocytogenes* cells to higher concentrations of sanitizing agents such as quaternary ammonium compounds and products containing chlorine or iodine compared to planktonic cells [20,21]. Nevertheless few studies have been carried out on both the resistance displayed by sessile and planktonic cells and the age of these cells.

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The aim of this study was to test the resistance at different phases of growth of *L. monocytogenes* LO28 cells, in culture or adhering to stainless steel with several physical or chemical agents. The renewing or not of the medium was also investigated to know if a new nutrient supply could affect the biofilm formation of the strain and its resistance to sanitizing agents.

2. Materials and methods

2.1. Bacterial strain, media and culture conditions

L. monocytogenes LO28 was a clinical isolate of serotype 1/2c kindly provided by Dr. P. Cossard (Institut Pasteur, Paris). Cultures were performed on MCDB 202 (Molecular Cellular Development of Biology 202, CryoBiosystem, L'Aigle, France) a chemically defined medium adjusted to pH 7.3 and supplemented with 1% (w/v) yeast nitrogen base without amino-acids (Difco, Pont-de-Claix, France) and 3.6 g of glucose per litre (Carlo-Erba, Val de Reuil, France).

The strain was grown overnight (18 h) on BHI (Difco) slopes at 37 °C to inoculate a 20 ml pre-culture of MCDB 202. The pre-culture was incubated for 24 h at 20 °C in a waterbath (Aquatron, Infors, HT Switzerland) with orbital agitation (150 rpm) and used to inoculate a 100 ml culture to an OD_{600} of 0.1 (approximately 10^7 cfu ml^{-1}) measured on a Shimadzu UV160A spectrophotometer (Roucaire, Courtaboeuf, France). The culture was incubated at 20 °C until cells reached exponential (15 h) or stationary phase (40 h).

2.2. Biofilm formation

The surface used was AISI 304 stainless steel (Goodfellow, Cambridge Science Park, UK) cut into 3×1.5 cm rectangular coupons. Before each experiment, coupons were soaked 10 min with a 2% TFD4 detergent solution (Franklab, Saint Quentin en Yvelines, France) in hot tap water, rinsed 5×5 min with hot tap water, 5×5 min with distilled water and then autoclaved for 15 min at 120 °C.

Cells issued from cultures were harvested by centrifugation at 7000*g* for 10 min (MR22i, Jouan, Nantes, France) and suspended in their own supernatant to obtain an OD_{600} between 0.6 and 0.7. Seven ml of this bacterial suspension were poured into a Petri dish (55 mm diameter, Prolabo, Fontenay-sous-Bois, France) containing a coupon and stored at 20 °C, coupons were placed flat into the Petri dishes allowing the biofilm growth only on one side. The medium was renewed after 2 h, and then every 24 h. Treatments with sanitizers were made 6 h, 1 and 7 days after initial adhesion. Experiments were also conducted for 7 days but without renewing the culture medium (only after the first 2 h of adhesion).

2.3. Scanning electron microscopy

Coupons were washed 2×1 min in a Petri dish with 2×35 ml of sterile TS on a rotating table (Belly Dancer, Bio-Rad, Ivry-sur-Seine, France) to remove non-adherent cells, and sessile cells were fixed with a solution of 3% glutaraldehyde (Electron Microscopy Science, Washington, USA) in 0.2 M cacodylate buffered at pH 7.4 (Electron Microscopy Science) and rinsed in the same buffer. After postfixation for 1 h with osmic acid vapors (Electron Microscopy Science), cells were dehydrated using a graded ethanol series (Prolabo) and then a graded acetone series (Prolabo) as previously described [14]. Coupons were coated with gold in an Emscope SC500 (Elexience, Verrières le Buisson, France) and observed with a Philips SEM 505 (FEI, Eindhoven, Holland) scanning electron microscope (SEM).

2.4. Treatments and cell enumeration

Seven treatments (I–VII) were employed, each solution was made from MCDB 202 and filtered at 0.2 μm (Nalgene, Hereford, UK). Treatment I: pH adjusted to 5.0 with acetic acid 96% (Carlo-Erba). Treatment II: pH adjusted to 12.0 with NaOH 5 N (Prolabo). Treatment III: 10% of sodium sulphate (Prolabo). Treatment IV: 10% of sodium sulphate and pH adjusted to 5.0. Treatment V: 10% of sodium sulphate and pH adjusted to 12.0. Treatment VI: 50 ml of MCDB 202 were supplemented with 100 μl from a 1% stock solution of a quaternary ammonium (QAC), benzyldimethyl-tetradecylammonium chloride (BDTA, Sigma, l'Isle d'Abeau Chesnes, France) to obtain a final concentration of 20 ppm. Treatment VII: 50 ml of MCDB 202 were completed with 100 μl from a 3.75% stock solution of monolaurin (1-monolauroyl-rac-glycerol, Sigma) in absolute ethanol to obtain a final concentration of 75 ppm.

Concerning planktonic cells, 10 ml of stationary or mid-log phase cell cultures were centrifuged at 20 °C (5000 rpm) for 15 min. Resulting pellets were suspended in 10 ml of a treatment solution (or sterile culture medium for control) for 30 min at 20 °C in a rotating waterbath. After treatment, bacterial suspensions were centrifuged (5000*g*, 15 min, 20 °C), pellets were homogenized in 10 ml of Tryptone-salt (TS) and cells were enumerated.

For sessile cells, the medium was removed after 6 h, 1 or 7 days of adhesion, and 7 ml of a treatment solution (or sterile medium culture for control) were softly poured onto coupons for 30 min at 20 °C. Non-adherent cells were removed as described previously (Section 2.3). Sessile cells were detached from coupons in a sterile bottle containing 10 ml of sterile TS with a Vibracell

sonication bath (Deltasonic, Meaux, France) for 3 min at 50 KHz.

Viable and cultivable cells were enumerated by serial dilution (in TS) and plated with a spiral plater (Interscience, Saint Nom-La-Breteche, France) on Tryptic Soy Agar (Difco). Results obtained after 24 h of incubation at 37 °C were expressed as cells ml^{-1} and cells cm^{-2} for planktonic and sessile cell enumerations, respectively. Each experiment was performed in triplicate (three repetitions for each data in final).

2.5. Statistical analysis

Results were analysed using the SAS-STAT software (Statistical Analysis System release 6.12, CA, USA). Data were first expressed as percentage of mortality in comparison with controls, and then transformed according to the formula $\arcsin^{1/2}$ (expressed as degrees) to obtain a normal distribution; then an analysis of variance was realised on these data with a level of probability of $p < 0.05$. Statistical tests were performed simultaneously on each growth phase inside a given treatment, and on each treatment for a given growth phase so as to detect significant differences resulting from the treatment applied or from the growth phase.

3. Results

3.1. Controls for sanitizer treatments and cell enumeration

Final pH of supplemented MCDB 202 was 7.1. Addition of quaternary ammonium or monolaurin resulted in a final pH of 7.13. Addition of Na_2SO_4 to supplemented MCDB 202 slightly modified the pH at 7.35 and at 11.83 with the presence of NaOH (5 N) and did not affect significantly pH of acetic acid solution (5.08).

For the controls of mid-log and stationary phase planktonic cells, a mean value of 8.8×10^8 and 2.3×10^9 cells ml^{-1} was obtained, respectively. After 6 h of adhesion, 3.4×10^7 cells per cm^2 were counted, 2.7×10^7 cells per cm^2 after 1-day incubation and 2.3×10^8 or 4.3×10^6 cells per cm^2 after 7-days incubation with daily renewed medium or not, respectively. The first three steps of biofilm development chosen to carry out the treatments are illustrated by SEM in Fig. 1. All controls were not at the same growth level and for the same log reduction, the number of living cells remaining after sanitizing was not the same in all cases especially in terms of hygiene norms. Thus, to allow comparison between the experiments, the results were presented as percentage of cell mortality in comparison with their own control.

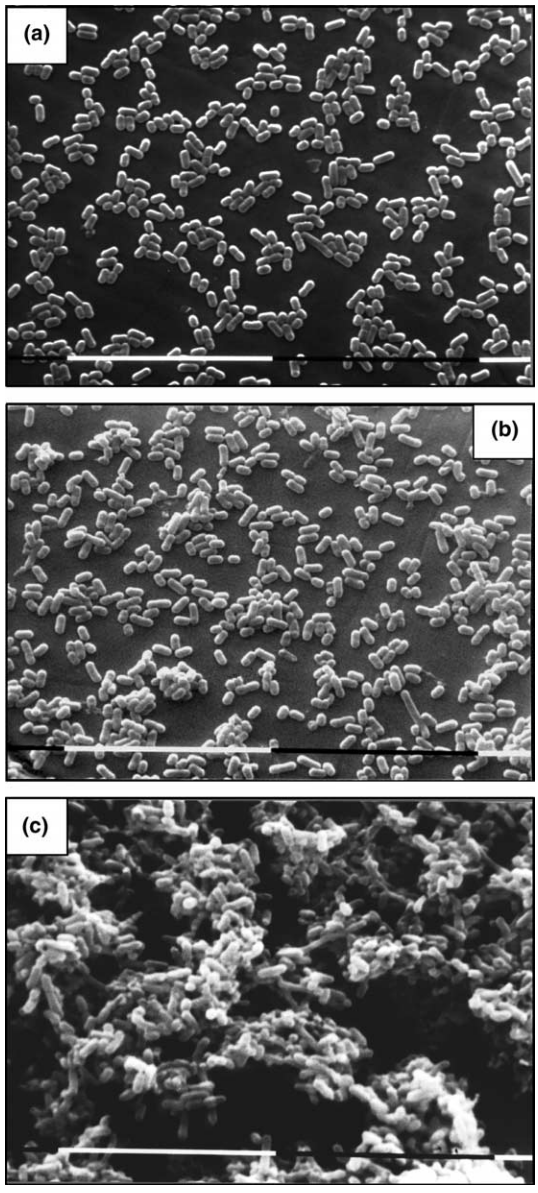

Fig. 1. SEM observations of *L. monocytogenes* LO28 biofilm formation at 20 °C on stainless steel at 6 h (a), 1 day (b), and 7 days with daily renewed medium (c). Bar, 10 μm.

3.2. Efficiency of pH variation on cell mortality

After 30 min acidification with acetic acid, the percentage of mortality was the lowest in comparison with all treatments tested (Fig. 2), and never exceeded 23%. No significant differences were observed between planktonic cells and 1 or 7 days biofilms without renewed medium (Fig. 3). Only a slight difference existed between these previous conditions of growth and 6 h and 7 days biofilms without renewing medium that appeared completely resistant to pH 5.0 acetic acid treatment.

NaOH (5 N) generated a high percentage of mortality in both planktonic and sessile cells. All modes of growth studied showed a similar susceptibility to alkaline

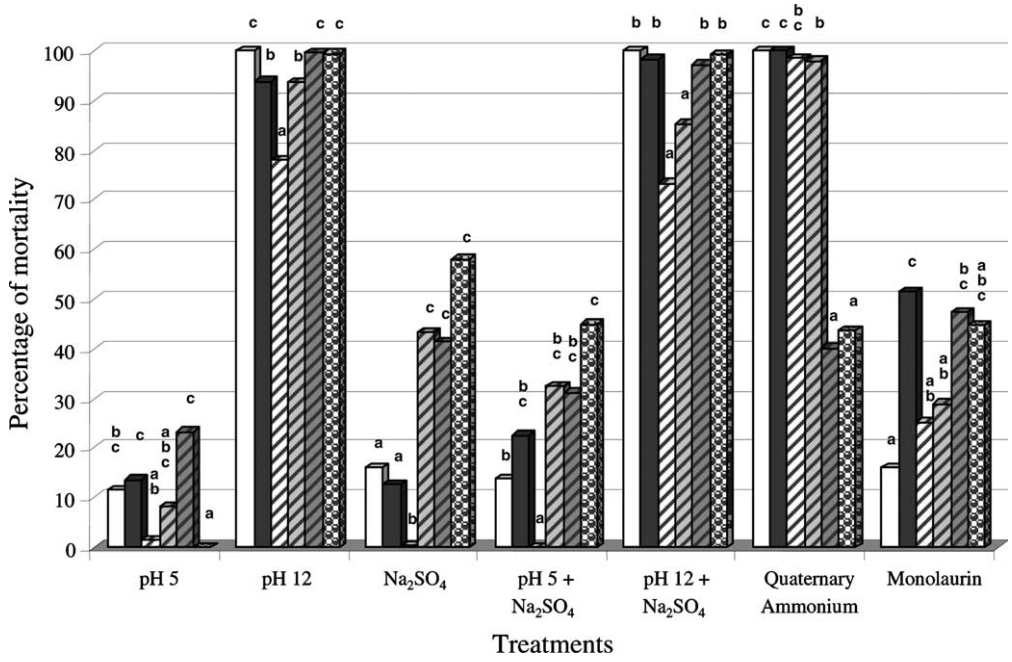

Fig. 2. Percentage of mortality of planktonic and sessile *L. monocytogenes* LO28 cells according to the sanitizer treatment. Planktonic cells in exponential (□) or stationary (■) phase of growth, sessile cells after different times of adhesion to stainless steel: (▨) 6 h, (▨) 1 day, (▨) 7 days, (▨) 7 days without renewing medium. For each treatment tested, bars having the same letters are not significantly different ($p > 0.05$).

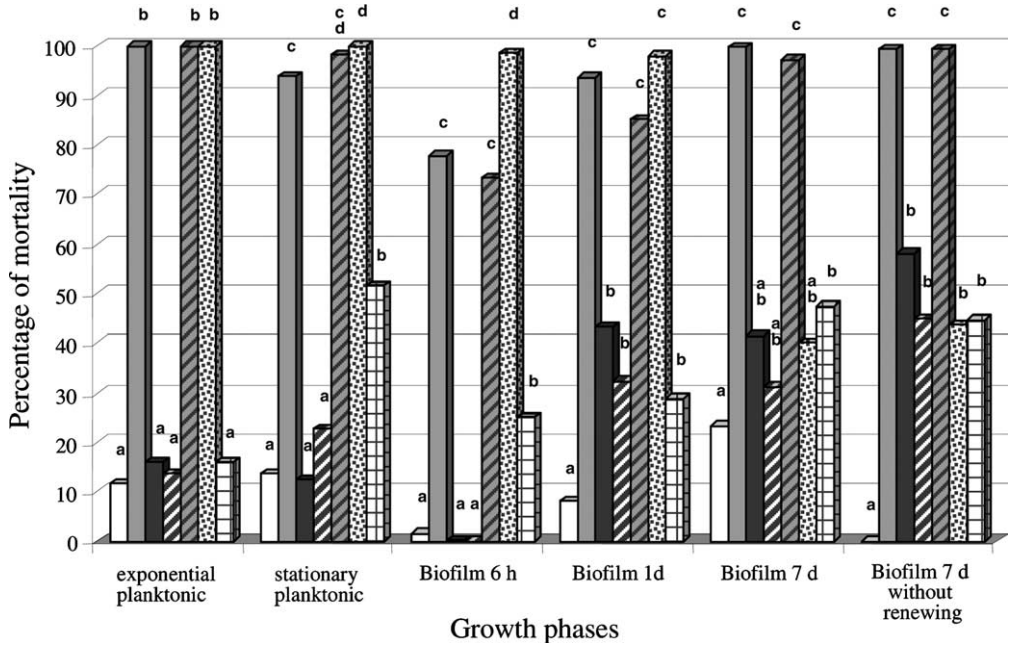

Fig. 3. Percentage of mortality of planktonic and sessile *L. monocytogenes* LO28 cells according to the growth phase. (□) pH 5 with acetic acid, (▨) pH 12 with NaOH; (■) 10% Na_2SO_4, (▨) pH 5 and 10% Na_2SO_4, (▨) pH 12 and 10% Na_2SO_4, (▨) 20 ppm quaternary ammonium and (▨) 75 ppm monolaurin. For each growth stage, bars having the same letters are not significantly different ($p > 0.05$).

treatment with more than 93% of mortality except 6 h biofilms which appeared more resistant with less than 80% of cell death. In comparison with the other treatments tested, increasing pH to 12 with NaOH was the most efficient treatment to eliminate *L. monocytogenes* whatever its mode and phase of growth.

3.3. Effect of adding chemicals on cell mortality

Responses of *L. monocytogenes* cells to Na_2SO_4 addition showed a wide diversity according to the mode of growth studied. Indeed young biofilms (6 h) were particularly resistant showing no decrease in cell population. On the other hand, a significant difference with these biofilms was observed with planktonic cells with more than 10% of mortality (either in a stationary or exponential state), but the stronger sensitivity was demonstrated for older biofilms aged 1 or 7 days (Fig. 2). However, the action of Na_2SO_4 remained limited whatever the mode of growth, never exceeding 60% of mortality.

Addition of BDTA (Fig. 2) revealed no significant variations in sensitivity between planktonic and young adhered cells (6 h and 1 day of adhesion). A stronger resistance appeared when biofilms get older (7 days). Fig. 3 illustrates the great efficiency of this compound on planktonic cells and young adhered cells with cell mortality greater than 98%. On the contrary, the action of this QAC on older biofilms never led to a decrease of cell population greater than 45%.

Treatment with monolaurin revealed a higher efficacy on stationary planktonic cells than on exponential cells. Indeed, no significant differences could be observed between each biofilm stage and planktonic and sessile cells. Monolaurin did not show a great efficiency on cells whatever the growth stage studied never exceeding a 50% reduction of cell population (Fig. 2).

3.4. Combinations of 10% Na_2SO_4 with pH variation

Addition of 10% Na_2SO_4 with acetic acid (pH 5) resulted in a stronger resistance of young biofilms in comparison to planktonic cells while older biofilms appeared more sensitive. Therefore cell mortality remained low and never exceeded 50% of the cell population (Fig. 3). The efficiency of this combination could be attributed mostly to Na_2SO_4. Indeed, the results show the same pattern as those obtained with Na_2SO_4 solely whatever the mode of growth studied and no significant differences were observed between these two treatments.

Increasing the pH up to 12.0 in the presence of 10% Na_2SO_4 appeared particularly efficient, with a reduction of more than 70% of cell population for both planktonic and sessile cells. Nevertheless, such a combination showed the same pattern as those obtained with NaOH solely with a stronger resistance of young biofilms and demonstrated that no significant differences could be obtained between these two treatments for the growth stages tested.

3.5. Effect of renewing the medium on cell mortality

The daily renewing or not-renewing of the medium had no effect on cell sensitivity in 7 days biofilms for all sanitizing agents tested, except for the pH 5 treatment for which a significant difference appeared (Fig. 2).

4. Discussion

Numerous authors have demonstrated that sessile bacteria resist in a better way to several physicochemical stresses such as antibacterial agents [22] or pH variations [23]. Thus, ubiquitous pathogens such as *L. monocytogenes*, able to form biofilm on numerous surfaces, may represent a potential risk of infection/contamination in hospitals and food processing environments. The aim of this study was to investigate the effects of several sanitizing agents against planktonic and sessile cells of *L. monocytogenes* LO28 at different times of growth to evaluate the capacity of this pathogen to survive and consequently to represent a contamination risk.

The method described in this paper to obtain biofilms appeared to be quite reproducible and allowed a great rate of adhesion of *Listeria* cells. Indeed, up to 2.3×10^8 cells per cm^2 were obtained after 7 days of adhesion. Therefore, renewing the medium had a positive impact on the adhesion, whereas without renewing only 4.3×10^6 cells adhered to the substrate, probably because of detachment phenomena of cellular aggregates. Such biofilm formation protocol could thus represent a good model to study the behaviour of *Listeria* sessile cells and their resistance to several sanitizers.

L. monocytogenes is well known to develop under a broad range of pH [24,25] and it is not surprising to observe a poor cell mortality whatever the stage tested when pH is reduced to 5 by addition of acetic acid. Such treatment does not have a different impact on sessile or planktonic cells, and only biofilms without renewing medium appeared more resistant. This may be explained by the acid production of *L. monocytogenes* from glucose since in our growth condition, pH of the medium rapidly decreased up to 5.4 (data not shown). This pre-acidification may encourage an acid tolerance phenomenon, as previously described [26], leading to a better resistance of cells from non-renewed biofilms. On the contrary, alkaline solution had a great impact on the survival of *Listeria* cells whatever the growth stage studied with more than 90% of cell mortality except for 6 h biofilms (80%). Alkaline solutions as NaOH are generally used in detergents to eliminate carbonised sediment, oil or grease [27]. They facilitate protein denaturation, fat saponification and have a bactericidal activity causing damage to the outer membrane, ribosomes, proteins and DNA [28]. *Listeria* was already shown to be very sensitive to alkaline treatments. Indeed, the lag time of five strains cultivated at 20 °C in a planktonic mode of growth was considerably increased in a medium adjusted with NaOH to pH 10.5 and 11 [5]. Nevertheless, 6 h biofilms were more resistant, and two

hypotheses may explain this phenomenon: (i) renewing the medium after 2 h of adhesion, by increasing the pH, decrease the amplitude of the pH 12 treatment which becomes less important than for other growth stages studied; (ii) an adaptation of the cellular physiology of adhering cells in the first steps of adhesion may have induced a higher resistance. Such adaptation could then explain the increased resistance of 6 h biofilms in contact with 10% Na_2SO_4. Indeed, in the presence of these saline conditions, older biofilms appeared less resistant than their planktonic counterparts and younger biofilms. But another answer for a higher cell survival in young biofilms could be given by the fact that Na_2SO_4 may weaken the biofilm structure and then increase detachment of sessile cells. Indeed SEM results of this study (Fig. 1), strengthened by previous SEM observations [16], revealed that 6 h adhered cells did not form a three dimensional structure at 20 °C on stainless steel, and only single adhering cells appeared. This result may explain the higher resistance of young adhering cells. The combination of Na_2SO_4 with a pH variation do not show a synergetic effect. Indeed cells submitted to alkaline or acid treatment, both with Na_2SO_4, present respectively the same pattern as cells in the presence of NaOH (5 N) or Na_2SO_4 alone.

Increasing pH revealed a great efficacy on biofilm grown on clean stainless steel surfaces while low pH did not induce a large cellular death. Such stresses are mainly based on a wide variation of pH to disrupt membrane porosity, metabolic activity, and ionic flux in order to affect microbial growth [29]. In disinfection processes, a large variety of chemical compounds have been used, among which are QAC. Application of 20 ppm of BDTA successfully inactivate planktonic cells, as described by Mustapha et al. [30], and young biofilms aged 6 h and 1 day within 30 min whereas older biofilms develop a resistance phenomenon to this sanitizer. Such resistance has already been demonstrated with other QAC [31,32] and require to increase the bactericidal concentration to successfully disinfect *L. monocytogenes* biofilms aged of more than 48 h. These agents especially dedicated to Gram-positive bacteria are hydrophilic cationic molecules. Since the bacterial surface is hydrophilic and negatively charged, QAC could absorb and penetrate the wall so as to disrupt the cytoplasmic membrane of planktonic cells. But concerning biofilms, adhered bacteria act as a "shield" and reduce the accessibility of sessile cells present in the bottom of the biofilm. Moreover, QAC are confronted to glycocalyx development by sessile cells. This matrix may act as a polyanionic barrier functioning as an ion-exchange resin capable of binding a very large number of molecules [33] hampering the access of the disinfectant to the cell membrane as described for *E. coli* [34]. Such hypothesis could explain the higher resistance acquired by 7 days biofilms, which have already developed a tri-dimensional structure unlike younger biofilms. Finally, deep layers of 7 days biofilms could be constituted of slowly growing bacteria, at the stage of dormancy likely to be less metabolically active because of the poor access of nutrients and oxygen. Such embedded bacteria are known to express a dramatically different physiology in terms of gene and protein expression [9,14,35] that may lead to an increased resistance to different sanitizers.

Another agent usually present in disinfection processes is monolaurin, a non-ionic medium chain lauric acid ($C_{12:0}$). The bactericidal effects of such compound are primarily observed on membranes [36] and it acts as a disrupter of the cell permeability and nutrient transport [37]. In our study, this monoester had a more important impact on stationary cells than on exponential cells in the planktonic mode of growth. Nevertheless, this compound does not eliminate more than 50% of cell population. Such results agree with the partial but incomplete inactivation of planktonic and sessile cells of *L. monocytogenes* after 25 min of contact with 50 or 100 μg/ml of monolaurin [38] and with the absence of inhibition up to 400 μg/ml when adhered to catfish fillets [39]. Monolaurin bactericidal activity appears higher in association with lower pH [36], probably due to an increased susceptibility of *L. monocytogenes* at lower pH for this compound. Such results are in accordance with ours, showing a better sensitivity of stationary cells in supplemented MCDB 202, a non-buffered medium.

The medium used plays also a non-negligible role in the resistance as numerous sanitizers may interact with several components (organic matter...) and exhibit changes in their efficiency from a medium to another [40]. In this work, renewing the medium increased the sensibility of sessile cells when an acid treatment was applied, certainly because of the wider decrease of pH when the medium is renewed. In all the other sanitizing solutions tested, no significant differences appeared when the medium was renewed or not, showing that for these tests the resistance response is non-medium-dependent.

Under our experimental conditions, alkalisation with NaOH and quaternary ammonium were the best sanitizers to inhibit *L. monocytogenes*. Nevertheless, biofilm formation could lead to an increased resistance of sessile cells to certain sanitizers (such as QAC) especially when this biofilm is aged of a few days. Such results may be of high concern for food processing plants demonstrating that biofilm development must be prevented to avoid hazardous contamination by remaining pathogen cells.

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