International Journal of Food Microbiology 381 (2022) 109888

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Removal of *Listeria monocytogenes* biofilms on stainless steel surfaces through conventional and alternative cleaning solutions

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ARTICLE INFO

Keywords:
Listeria monocytogenes
Biofilms
Cell detachment
Biocidal effectivity
Food safety

ABSTRACT

Conventional treatments are not effective enough to completely remove *Listeria monocytogenes* biofilms from surfaces, thus implying the presence of certain persistent bacterial forms. In this study, eleven treatments (*i.e.* two enzymatic agents applied at two different temperatures and concentrations, two alkaline cleaners and one acid detergent) were used to remove mature *L. monocytogenes* S2-bac biofilms. A combined treatment was then selected for its application to four different *L. monocytogenes* strains (*i.e.* CECT 5672, CECT 935, S2-bac, EDG-e). Effectivity of the treatments was evaluated quantitatively using TEMPO and qualitatively by direct epifluorescent microscopy (DEM). Bacterial detachment obtained after the application of acid, alkaline and chlorinated alkaline treatments were 6.03, 6.24 and 4.76 Log CFU/cm^2, respectively. Enzymatic treatments applied at 50 °C obtained the greatest detachment and biocidal activity. The results derived from the observation of the remaining biofilm structure by DEM proved that conventional treatments were unable to completely remove conformed structures with the potential risk this entails. Last, the application of a combined treatment using a chlorinated alkaline cleaner followed by an enzymatic treatment enhanced the dispersal of the bacterial cells from surfaces, thus consolidating this as a good option to recommend for the 5-step cleaning procedure.

1. Introduction

In the food and beverage industry, equipment surfaces are considered an important source of microbial contamination, associated over time with foodborne disease outbreaks and consequently impacting public health (Faille et al., 2018). Among the pathogens that stand out due to food contamination as a consequence of cross-contamination from industrial surfaces is *Listeria monocytogenes* (Churchill et al., 2019). In 2020, this pathogen produced a total of 780 reported cases of invasive listeriosis in Europe, presenting a mortality rate of 13 % (EFSA-ECDC, 2021), one of the highest rates among the zoonotic agents. Recent foodborne outbreaks have been related to *L. monocytogenes* presence in foods from distinct origins (*i.e.* vegetable and animal) such as the one linked to the consumption of enoki mushrooms (U.S. FDA/USDA/CDC, 2020), hard-boiled eggs (U.S. FDA/USDA/CDC, 2019) and Bologna sausages (Allam et al., 2018; Salama et al., 2018). Due to the non-decreasing trend of listeriosis cases and the fact that its control through the food chain is not enough to reduce its presence (EFSA-ECDC, 2021), questions are raised about *Listeria spp.* lifestyle in the food processing environment context and new ways to eliminate the pathogen are being sought (Zwirzitz et al., 2021).

There are thirteen different serotypes of *L. monocytogenes* (*i.e.* 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7), although most of the human diseases produced are associated with serotypes 1/2a, 1/2b, 1/2c and 4b (Maćkiw et al., 2020). Of these, serotype 4b is the one considered the most pathogenic and 1/2a the one that is most prevalent in the food industry environment (Laksanalamai et al., 2014; Lee et al., 2012). Therefore, it is important to include various L. *monocytogenes* serotypes in the studies concerned with its control to represent industrial reality. *L. monocytogenes* occurrence in the food industry has been related to the pathogen's ability to survive in a wide range of environments, such as in cold temperatures, with low oxygen, low pH or even a lack of nutrients (Sadekuzzaman et al., 2017). To do so, *L. monocytogenes* forms biofilms, biological structures which are considered an

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https://doi.org/10.1016/j.ijfoodmicro.2022.109888
Received 31 March 2022; Received in revised form 16 August 2022; Accepted 22 August 2022
Available online 26 August 2022

Table 1
Enzymatic and chemical cleaning detergents used in the treatments for the elimination of L. *monocytogenes* S2-bac mature biofilms.

Type of treatment	Cleaner	Composition	In-use temperature (°C)	In-use concentartion (%)
Enzymatic	Product A	Ethoxylated sodium lauryl ether glycolate Amines, C12–14 (even numbered)-alkyldimethyl, N-oxides Anionic surfactants (<5 %) Non-ionic surfactants (<5 %) Proteases (<5 %) Phenoxyethanol	20 and 50	1 and 3
	Product B	Nonylphenoxypoly (ethyleneoxy)ethanol (10 %) Protease (Savinase® <3 %) α-amylase (Termamyl® <3 %)	20 and 50	1 and 3
Conventional	Acid	Thyme oil (0.5 %) Cinnamon oil (0.5 %) Phosphoric acid (25–50 %) Amines, C12–14 (even numbered)-alkyldimethyl, N-oxides (1–5 %)	20	1
	Alkaline	Potassium hydroxide (<25 %) Sodium hydroxide (2–5 %)	40	1
	Chlorinated alkaline	Sodium hydroxide (8.5 %) Sodium hypochlorite (6 %)	20	1

assemblage of microbial cells adhered to surfaces, embedded in a matrix of extracellular polymeric substances that consists of polysaccharides, proteins and DNA (González-Rivas et al., 2018). This structure defines the main physiological processes in relation to their resistance and persistence (Mosquera-Fernández et al., 2016), which are important aspects for the development of control strategies for their elimination. Biofilm thickness and shape is directly related to antimicrobial diffusion, causing the cells that form the biofilms to increase their capacity to resist disinfectants, thus limiting their efficacy and the further elimination of these microbial communities (Torlak and Sert, 2013). This enables the bacteria to persist in food processing environments on locations that are not easy to clean using conventional cleaning solutions, such as cutting machines, smoking areas, and totes and cracks in the floors (Cripe and Losikoff, 2021), thereby considered a critical problem for the food industry (McEntire, 2018; Sadekuzzaman et al., 2017). It is also important to detect foodborne pathogens rapidly and efficiently to reduce the probability of a pathogen remaining on surfaces and so that suitable action can be undertaken (Ripolles-Avila et al., 2020). A good hygiene program of such actions must be applied in all food sectors (Ben Braïek et al., 2020). Thus, cleaning and disinfection processes are highly important for the food industry to achieve a minimum risk for the safety and quality of food products (Holah et al., 2014; Yang et al., 2016). As previously suggested by Waghmare and Annapure (2015), sanitation programs in the food industry are commonly based on chlorine, including sodium hypochlorite, since this is an effective oxidizing compound for microbial activities and more economical than other chemical products.

The greatest challenge for the cleaning and disinfection procedures applied in the food industry is to find biofilms in their mature state (Ripolles-Avila et al., 2020). After a biofilm is found in this state, the application of antimicrobials is not an effective solution to remove and eliminate the structure from the surface (Srey et al., 2013). Mazaheri et al. (2020) and Ripolles-Avila et al. (2020) recently demonstrated that aggressive treatments using a combination of enzymes need to be applied to eliminate mature *L. monocytogenes* biofilms. In this case, enzymatic action causes a disruption of the biofilm extracellular matrix and stimulates the release of microbial cells to facilitate their elimination by applying a disinfectant product (Pleszczyńska et al., 2017). Nevertheless, it becomes important to extend these types of studies and to compare regular products employed in the food industry. To this end, the general objective of this study was to compare different strategies to eliminate mature L. *monocytogenes* biofilms formed on stainless steel surfaces to find out the best cleaning methodology to apply. The first specific objective was to assess the effectiveness of eleven treatments used for mature L. *monocytogenes* biofilm elimination, modelling S2-bac strain as a reference according to our previous study (Mazaheri et al., 2020). The second specific objective was to evaluate a combined treatment to remove mature biofilms formed by four more L. *monocytogenes* strains (*i.e.* CECT 5672, CECT 935, S2-bac, EDG-e, from distinct serotypes and origins).

2. Materials and methods

2.1. Bacterial strains

L. monocytogenes strain S2-bac obtained from Ortiz et al. (2014) was selected for the evaluation and effectivity comparison of 11 different treatments on the basis of it showing the maximum matrix production and greatest resistance (Mazaheri et al., 2020). After comparison of the 11 treatments, two of the agents were chosen to remove mature biofilms of different L. *monocytogenes* strains [*i.e.* CECT 5672 and CECT 935, obtained from the Spanish Type Culture Collection (CECT, Paterna, Spain) and L. *monocytogenes* EDG-e, which was isolated from an Iberian pig processing plant (Ortiz et al., 2016)]. All the strains were obtained as freeze-dried cultures and recovered by culturing them in Tryptic Soy Broth (TSB; Oxid, Madrid, Spain) for 48 h at 30 °C. Suspensions were then cultured on Tryptic Soy Agar (TSA; Oxid, Madrid, Spain) and incubated at 37 °C for 24 h. Isolated colonies were used to prepare stock cultures on TSA, which were stored for up to 1 month at 4 °C.

2.2. Surfaces

Stainless steel coupons AISI 316 grade 2B (2 cm in diameter and 1 mm thick) were submited to a cleaning and disinfection procedure, according to European standard UNE-EN 13697:2015 (AENOR, 2015). First, the coupons were cleaned with detergent (ADIS Hygiene, Madrid, Spain) for at least 1 h, washed with running tap water, and further disinfected with 70 % isopropanol (Panreac Química, Castellar del Vallès, Spain). The surfaces were susequently dried in a laminar flow cabinet (PV-30/70, Telstar, Terrasa, Spain). Last, to complete the sterilization stage, the coupons were autoclaved at 121 °C for 15 min before their use.

2.3. Biofilm formation on surfaces

Several colonies obtained from the incubation of *L. monocytogenes* strains in TSA at 37 °C for 24 h were inoculated into TSYEB $_{gluc1\%+Nacl2\%}$ [*i.e.* TSB enriched with 0.3 % *w/v* yeast extract (BD, Madrid, Spain), 1 %

Table 2
Comparison between different cleaning treatments for the removal of *L. monocytogenes* S2-bac mature biofilms. Values correspond to the mean ± standard error (n = 9).

Treatment	Temperature (°C)	Concentration (%)	Reduction Log (CFU/cm^2)	Detachment (%)
Product A	20	1	3.23 ± 0.31^{cd}	52.41 ± 4.97^{cd}
		3	4.08 ± 0.55^{bc}	67.70 ± 8.23^{bc}
	50	1	6.24 ± 0.00^{a}	100 ± 0.00^{a}
		3	5.96 ± 0.28^{a}	95.73 ± 4.27^{a}
Product B	20	1	2.60 ± 0.39^{d}	42.24 ± 6.13^{d}
		3	3.42 ± 0.57^{cd}	55.58 ± 8.87^{cd}
	50	1	4.99 ± 0.28^{b}	80.07 ± 4.59^{b}
		3	6.10 ± 0.09^{a}	97.86 ± 1.42^{a}
Acid	20	1	6.03 ± 0.10^{a}	96.57 ± 1.72^{a}
Alkaline	40	1	6.24 ± 0.00^{a}	100 ± 0.00^{a}
Chlorinated alkaline	20	1	4.76 ± 0.73^{b}	77.45 ± 11.14^{b}

$^{a-d}$Means within a column without a common superscript differ significantly ($P < 0.05$).

w/v glucose (Biolife, Madrid, Spain), and 2 % w/v sodium chloride (Panreac, Castellar del Vallès, Spain)] until reaching 0.2 McFarland units, equivalent to approximetely 10^6 CFU/ml (Ripolles-Avila et al., 2018). The resulting microbial suspension was then used for the surface inoculation. For this, 30 µl were transferred to the middle of each stainless steel coupon and placed in sterile Petri dishes, which were inserted in a humidity chamber for biofilm formation (Fuster-Valls et al., 2008; Ripolles-Avila et al., 2018). The surfaces were incubated statically for seven days at 30 °C with washing and renewal of nutrients. For this procedure, the stainless steel coupons were washed twice with 3 ml of sterile distilled water to remove non-adhered bacterial cells, and then 30 µl of $TSYEB_{gluc1\%+NaCl2\%}$ were added to stimulate biofilm growth and maturation at 48 h + 24 h + 24 h + 72 h (Ripolles-Avila et al., 2018). After the renewal of nutrients was completed, the surfaces were again placed back in the humid chamber to complete the 7-day incubation period.

2.4. Cleaning agents

Two enzymatic products obtained from iTram Higiene (Vic, Spain) and three chemical products obtained from Proquimia (Vic, Spain) were used in this study (Table 1). The in-use concentrations of the agents were prepared by diluting them in hard water following international standard UNE-EN 13697:2015 (AENOR, 2015). Hard water was obtained by adding 3 ml of solution A [19.84 g of $MgCl_2$ (Sigma, Madrid, Spain) and 46.24 g of $CaCl_2$ (Sigma, Madrid, Spain) per 1000 ml of distilled water], 4 ml of solution B [35.02 g $NaHCO_3$ (PanReac Applichem, Madrid, Spain) per 1000 ml of distillate water] and 100 ml of interfering solution [1.5 g of bovine serum albumin (Sigma, Madrid, Spain) per 100 ml of distilled water]. Prior to being mixed to obtain the hard water, all these solutions were sterilized by a filter membrane (Millex-GP 0.22 µm, Merck, Barcelona, Spain) and then mixed with distilled water to obtain a final volume of 500 ml. After a week, the inoculated surfaces were rinsed twice with 3 ml of sterile distilled water to remove any unattached cells prior to subjecting them to the cleaning treatment. To evaluate the effect of treatments on biofilms, the coupons were placed in a sterile flask with 3 ml of the treatment solutions covering the surfaces (Mazaheri et al., 2020; Ripolles-Avila et al., 2020). All treatments were applied for 15 min but under different conditions (*i.e.* temperature and concentration), as summarised in Table 1, and according to the technical data sheet of the products.

2.5. Quantification of microbial cells by TEMPO

For biofilm cell recovery and quantification after treatment application, the surfaces were rinsed with 3 ml of sterile distilled water to remove the non-attached cells and the disinfectant residues. The biofilm cells were subsequently detached by vortexing the surfaces at 40 Hz for 90 s (Ripolles-Avila et al., 2019a). To do so, each coupon was introduced in a sterile flask containing glass beads and 10 ml of neutralizer solution [1 g of tryptone (BD, Madrid, Spain), 8.5 g of NaCl (Panreac, Castellar del Vallès, Spain) and 30 g Tween 80 (Scharlab, Barcelona, Spain) for every 1000 ml of sterile distilled water in pH (7.0 ± 0.2)]. In the case of the control surfaces, these were washed twice with 3 ml of sterile distilled water and placed directly in a sterile flask for vortexing with glass beads and 10 ml of neutralizer solution without the disinfection treatment.

The TEMPO system (bioMérieux, Marcy l'Etoile, France) was used to quantify the *L. monocytogenes* cells within the biofilms. The resulting suspension after biofilm cell recovery was decimally diluted in Tryptone Saline Solution [TSS; 1 g of tryptone and 8.5 g of NaCl per litre in pH (7.0 ± 0.2)]. Prior to the quantification, the TEMPO vials were hydrated with 3 ml of sterile distilled water and then, 1 ml of the dilution to be quantified added to them. The TEMPO vials were then vortexed to homogenize their content, transferred into an enumeration card by the TEMPO filler and incubated at 30 °C for 48 h.

2.6. Observation of resident L. monocytogenes biofilm communities by direct epifluorescent microscopy (DEM)

After the surfaces were subjected to the different treatments, the coupons were washed twice with 3 ml of sterile distilled water, further stained with 5 µl of Live/Dead BackLight bacterial viability kit (Molecular Probes, Oregon, USA) and incubated at 20–22 °C for 15 min. To differentiate between intact and damaged membranes, two fluorescent dyes, SYTO9 and propidium iodide (PI), were used. The first dye enters both live and dead bacterial cells and dyes them green, whereas PI penetrates only the cells with damaged membranes and reduced SYTO9 dye, producing a red color. All the stained surfaces were evaluated with an Olympus BX51/BX52 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a 100 W mercury lamp (USH-103OL, Olympus) and a dual-pass filter cube (U-M51004 F/*Re*-V2, Olympus), and coupled to a digital camera (DP73, Olympus). The biofilms were observed with a twenty magnification (20×) lens. The obtained images were analysed using the analySIS Auto 3.2 software (Soft Imaging System, Münster, Germany).

2.7. Evaluation of the biocide activity of the cleaning agents washing solution

The antimicrobial efficacy of the cleaning agents against L. *monocytogenes* was evaluated after the cleaning treatment was applied to the surfaces. The washing solution (*i.e.* remaining solution after a surface was treated) was filtered using a sterile membrane (MF-Millipore, Cellulose Mixed Esters, Hydrophilic; 0.45 µm, 25 mm). The membrane and filtration equipment was sterilized by autoclave at 121 °C for 15 min before use. The membrane was rinsed twice with 10 ml Buffered Peptone Water (BPW; Oxoid, Ltd., Basingstoke, United Kingdom) to remove any residue of the cleaning agents, placed in TSA and incubated at 30 °C for 48 h.

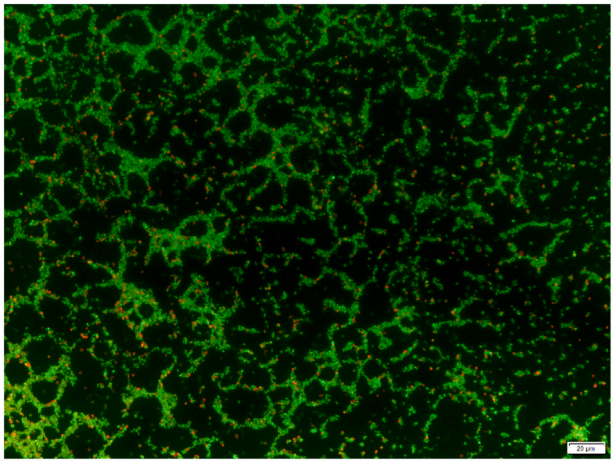

Fig. 1. Epifluorescence digital images of Live/Dead-stained mature *L. monocytogenes* S2-bac biofilms. Magnification 20×.

2.8. Calculations and statistical analysis

All the experiments were performed in triplicate on three different days ($n = 9$). The bacterial counts were converted into decimal logarithmic values to almost match the assumption of a normal distribution. Cell reduction after treatments was calculated by the differentiation between initial biofilm cell count and cell counts remaining on the surface after the treatments. Biocidal effect (*i.e.* dead cells) were calculated from the difference between the cells detached from the surface after the treatment and the ones present on the washing solution. The statistical analysis was performed using One Way ANOVA and the Fisher LSD Test with the STATISTICA 7.0.61.0 statistical software package. Statistical significance was defined as $P < 0.05$.

3. Results and discussion

3.1. Effect of eleven treatments on the removal of mature L. monocytogenes S2-bac biofilms

Considering the resistance profile of different strains when conducting experimental studies for their elimination is important for drawing conclusions that can be extrapolated to the microbial set. In the present study, *L. monocytogenes* S2-bac was chosen as a representative strain because it was the most resistant strain after the application of an enzymatic treatment in a previous study (Mazaheri et al., 2020). Comparative scientific studies are important to critically select the appropriate type of cleaning agent for any particular situation (Liikanen et al., 2002), one of them being the removal of mature biofilms. Table 2 shows the results obtained in terms of cell reduction and subsequent detachment after the different applied cleaning treatments. As can be observed, the treatments with the greatest effectiveness (*i.e.* highest detachment percentage) were the enzymatic treatments applied at 50 °C (except Product B at 1 % concentration) and the alkaline and acid treatments, which exerting a similar effect as shown by the non-significant differences ($P > 0.05$) obtained between them. First, the conventional detergents used in this study were classified into two different groups depending on the pH of application, thus finding the acid and alkaline detergents. The results derived from the present study demonstrated that both alkaline and acid treatments are highly effective in detaching mature L. *monocytogenes* biofilms. Alkaline detergents can denature proteins due to the action of hydroxyl ions, can saponify fats and, at high concentrations, can have a bactericidal action (Lelieveld, 2013). In this case, cell detachment from the surface after the alkaline treatment was applied could be related to the fact that extracellular L. *monocytogenes* biofilm matrices are composed mainly of proteins (Colagiorgi et al., 2016) and therefore the treatment would be promoting protein denaturation and matrix disruption. On the other hand, acid detergents act as descalers, favoring the elimination of mineral deposits (Fagerlund et al., 2020). In this case, the application of phosphoric acid as the acid treatment obtained a high cell detachment (*i.e.* 6.03 Log CFU/cm^2), which could be due to oxidative action of the product, increasing the concentration of hydrogen ions and affecting cell viability (Arias-Moliz et al., 2008; Hashim et al., 2020). Despite the effectiveness having been shown to be high in the present study, its transfer to industrial environments with certain amounts of residues could reduce its action of eliminating the biofilm matrix and structure. In this regard, Parkar et al. (2004) demonstrated differences between the effect of alkaline and acid cleaning agents on the biofilm matrix compared to enzymatic treatments.

As a green alternative for industrial surface cleaning, enzymatic detergents have been established as a viable option for the fight against biofilms in the food industry (Delhalle et al., 2020). In the present study, enzymatic treatments were applied at two different concentrations and temperatures to observe the effect with varying parameters. The results showed that Product A (1 % and 3 %) and Product B (3 %) applied at 50 °C obtained the highest detachment percentage, consolidating them as the most effective treatments as well as alkaline and acid treatments ($P > 0.05$). In this case, the substrate specificity of the enzymes can contribute to a higher efficiency for biofilm removal compared to alkaline and acidic cleaning agents due to the enzyme's capacity to disrupt and break up biofilm matrix (Fagerlund et al., 2020). Moreover, when Product A and Product B were applied at the recommended temperature (*i.e.* 50 °C) with the lowest concentration (*i.e.* 1 %), their effect differed significantly ($P < 0.05$), with Product A showing higher effectivity, even though this product contained just one type of enzyme. However, biofilm matrix is completely heterogeneous, and even more so if we take into consideration the fact that in food processing environments different microbial species coexist within the biofilm structure. To this effect, enzymatic formulations composed of mixtures of enzymes that attack different substrates to destabilize the matrix, including proteases, cellulases, polysaccharide depolymerases, alginate lyases, dispersing B and DNases, are more effective when applied at an industrial level (Bridier et al., 2015). In the present study, Product B was composed of different enzymes, such as α-amylase, protease and different essential oils including thyme and cinnamon oils, which can cause better outcomes in industrial experiments. Other authors have also reported the higher dispersal activity of proteases and amylases combined in the formulations of detergent for different food industries and uses (Guerrero-Navarro et al., 2022; Mitidieri et al., 2006).

Moreover, it has been indicated that the effectiveness of cleaning agents can depend on the structure and matrix produced by the different L. *monocytogenes* strains (Ripolles-Avila et al., 2018; Ripolles-Avila et al., 2019b). Moreover, according to Mazaheri et al. (2020), *L. monocytogenes* S2-bac generated a more robust matrix, and this could have been the reason why enzymatic treatment was more effective than a chlorinated alkaline product when assessing L. *monocytogenes* biofilm removal. *L. monocytogenes* S2-bac belongs to the serotype 1/2a, the serotype most frequently found in the food industry, as discussed in previous sections. Serotype 1/2a could generate more compact and robust structures when consolidating biofilms, which could also be the reason why this serotype is more widespread in processing plants (D'Arrigo et al., 2020).

Our cleaning tests showed that it is essential to use the correct concentrations of agents and the recommended temperatures, as also indicated by Parkar et al. (2004) and Guerrero-Navarro et al. (2022). In this last study, it was reported that enzymatic cleaning products in food processing plants are not always used according to recommendations, for example in cold storage rooms which are not able to reach 50 °C, leading to a decrease in the application temperature and concentrations failing to remove all surface cells, indicating that effectiveness is directly dependent on both parameters. To this effect, when the concentration of Product B increased to 3 % and was applied at 50 °C, the treatment was

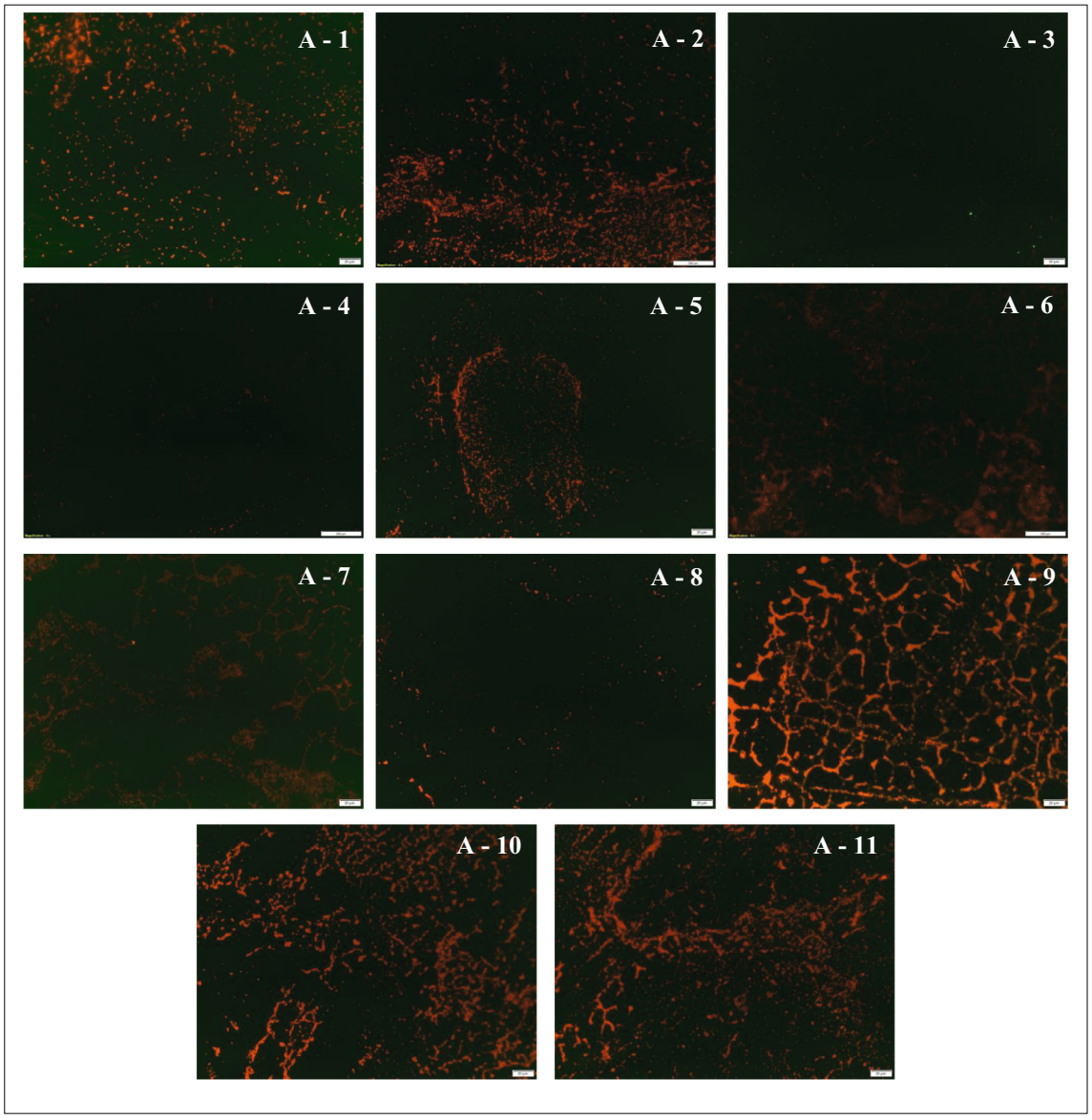

Fig. 2. Epifluorescence digital images of Live/Dead-stained mature *L. monocytogenes* S2-bac biofilms after the application of tested treatments: Product A 1 % - 20 °C (A-1); Product A 3 % - 20 °C (A-2); Product A 1 % - 50 °C (A.3), Product A 3 % - 50 °C (A-4); Product B 1 % - 20 °C (A-5), Product B 3 % - 20 °C (A-6); Product B 1 % - 50 °C (A-7), Product B 3 % - 50 °C (A-8); Acid (A-9), Alkaline (A-10), Chlorinated alkaline (A-11). Magnification 20×.

Table 3
Antimicrobial activity of the treatments applied for the elimination of L. *monocytogenes* S2-bac mature biofilms. Values correspond to the mean ± standard error (n = 9).

Treatment	Temperature (°C)	Concentration (%)	Dead cells (Log CFU/cm^2)
Product A	20	1	1.05 ± 0.27^{d}
		3	2.81 ± 0.60^{ef}
	50	1	5.00 ± 0.35^{ab}
		3	5.75 ± 0.26^{b}
Product B	20	1	0.00 ± 0.06^{cd}
		3	0.00 ± 0.37^{c}
	50	1	2.34 ± 0.17^{e}
		3	4.64 ± 0.54^{a}
Acid	20	1	5.52 ± 0.25^{ab}
Alkaline	40	1	5.26 ± 0.25^{ab}
Chlorinated alkaline	20	1	3.41 ± 0.45^{f}

$^{a\text{-}f}$Means within a column without a common superscript differ significantly ($P < 0.05$).

Table 4
Counts in Log CFU/cm^2 of *L. monocytogenes* cells that conformed the mature biofilms before and after the application of the combined treatment used for their elimination.

L. monocytognes strains	Control	Combined treatment
CECT 5672	7.05 ± 0.15^{a}	< 0.10 ± 0.00^{a}
S2-bac	6.87 ± 0.11^{ab}	< 0.10 ± 0.00^{a}
EDG-e	6.64 ± 0.15^{b}	< 0.10 ± 0.00^{a}
CECT 935	6.63 ± 0.14^{b}	0.17 ± 0.17^{a}

$^{a\text{-}b}$Means within a column without a common superscript differ significantly ($P < 0.05$).

1.04 Log CFU/cm^2 more effective in terms of cell detachment. All the enzymatic treatments, when applied at 20 °C, were significantly ($P < 0.05$) less effective than when applied at the highest temperature. The results also demonstrated that when applied at 3 %, all the enzymatic treatments were significantly ($P < 0.05$) more effective.

The application of any of the cleaning treatments proven to be highly

Table 5
Microbial dead cell counts (Log CFU/ml) obtained from the treatment washing solutions. Values correspond to the mean ± standard error ($n = 9$).

L. monocytognes strains	Cleaner	Dead cells (Log CFU/ ml)
CECT 5672	Chlorinated alkaline	4.27 ± 0.58^{b}
S2-bac		5.41 ± 0.17^{a}
EDG-e		2.89 ± 0.24^{c}
CECT 935		3.99 ± 0.46^{b}
CECT 5672	Product A	5.80 ± 0.25^{a}
S2-bac		5.76 ± 0.14^{a}
EDG-e		5.42 ± 0.22^{a}
CECT 935		5.28 ± 0.26^{a}

[a-c]Means within a column without a common superscript differ significantly ($P < 0.05$).

effective must be complemented with the application of a disinfection procedure since cleaning treatments remove a high percentage of microorganisms but cannot eliminate them completely (González-Rivas et al., 2018). Therefore, the effectiveness of the treatments applied in this study would increase with the application of the disinfection process (Ripolles-Avila et al., 2020). The only treatment that would not improve in terms of effectivity is the applied chlorinated alkaline detergent since it is a single-step cleaning and disinfection treatment. The results of the present study demonstrated that 22.55 % of L. *monocytogenes* S2-bac cells remained adhered on the surface after the application of the chlorinated alkaline treatment. The findings presented are in agreement with what has been reported by other authors such as Kim et al. (2018) and Ripolles-Avila et al. (2020), who demonstrated that chlorinated alkaline detergents can detach a certain number of cells that conform these structures, but that the treatments are not completely effective as they do not completely disperse the structure. By scanning electronic microscopy, Mendonca et al. (1994) showed that *L. monocytogenes* cells exposed to pH 9.00, 10.00, 11.00 and 12.00 did not leak constituents and did not change their cell structure, thus generating lower biocidal effect than other pHs. Chlorinated alkaline treatment is recommended in the 5 cleaning steps for areas where risk assessment concludes that the zone does not pose a potential risk. However, the microbial population that will resist treatment must be considered, consolidate again the structures, their acquired resistance and their capacity to migrate to other places of the food industry, thus posing a risk of re-contamination.

3.2. Impact on the structure and matrix of the treated L. monocytogenes S2-bac biofilms

The structure and viability of *L. monocytogenes* S2-bac biofilms were also investigated by direct epifluorescence microscopy (DEM) before and after the biofilms were subjected to cleaning treatments. Fig. 1 shows mature *L. monocytogenes* S2-bac biofilms obtained after a one-week incubation period at 30 °C. In this regard, the formation of biofilms can be determined from the organization of the cells from which they are formed, observed by DEM (Ripolles-Avila et al., 2018). As observed in the results, the biofilm had reached its maturity as cell distribution on the surface was in geometric shapes and covered a large part the surface, while leaving interstitial spaces that can be assumed to be water channels. The results are in concordance with what was reported by Mazaheri et al. (2020) and Ripolles-Avila et al. (2018), who demonstrated that *L. monocytogenes* conformed mature biofilms at one week of incubation. This also concurs with the findings of Centorame et al. (2017), who affirm that biofilms achieve a more complex organization and a higher density of attached cells after this minimum period of incubation. Moreover, conditioning in a humidity-saturated chamber led to the formation of mature biofilms, as also stated by Mai et al. (2006), since this is a primary determinant in the adhesion of microorganisms and a way to help them to distribute on the surface.

L. monocytogenes S2-bac cells and structure remaining on the surface after the application of the eleven treatments is shown in Fig. 2. When the mature biofilms were exposed to the enzymatic treatments, the remaining structure was made up of small, scattered colonies or disaggregated cells, most of which were either damaged or dead (Fig. 2 A-1; A-2; A-3; A-4; A-5; A-6; A-7; A-8). This result has been observed by other authors (Mazaheri et al., 2020; Ripolles-Avila et al., 2020). Qualitatively, the images coincide with the results obtained at a quantitative level. In this regard, it was observed that increasing the concentration of enzymes from 1 % to 3 % at the same temperature slight reduced the number of remaining biofilm cells on the surfaces (*e.g.*, at 20 °C of application; Fig. 2 A-1 and A-5 *vs* A-2 and A-6). However, when the temperature was increased to 50 °C, a complete disintegration of the mature biofilm structure and a distortion of the proportion of dead cells were observed (*e.g.,* at 3 %; Fig. 2 A-2 and A-6 *vs* A-4 and A-8). Each enzyme needs an optimal temperature to exhibit its maximum activity and, in the event that combinations of enzymes are used, it is important to establish the temperature closest to the optimal activity of each enzyme used. Fagerlund et al. (2020) reported that the optimum temperature for the application of enzymatic detergents is between 45 °C–55 °C. The most suitable temperature of the enzymatic treatments applied to exhibit the best cell detachment activity and a complete elimination of the biofilm structure was 50 °C, coinciding with Ripolles-Avila et al. (2019b), who applied a similar treatment for the elimination of *Salmonella enterica* and *Cronobacter sakazakii* biofilms.

Differently from what was obtained following the application of the enzymatic treatments, the rest of the applied treatments (*i.e.* chlorinated alkaline, alkaline and acid) did not disperse the structure of the biofilm, consequently leaving both microbial population and biofilm structure on the surface. Although a reddish color is observed in the whole structure on the images, the cells can be damaged rather than completely dead. In the case of the alkaline and acid treatments, a subsequent disinfection would be applied, so there may be a greater reduction in the microbial load. Moreover, in the food industry, all evaluated treatments would have also been accompanied with physical removal so this could also influence by potentiating the detachment effect. On the other hand, the application of the chlorinated alkaline treatment would not entail no other subsequent treatment, implying that the endured structure would remain on the surface. This can aggravate the state of hygiene, since by not completely eliminating the structure, *L. monocytogenes* cells could regenerate and re-start the formation of biofilms (Thomas and Sathian, 2014). In this regard, Mnif et al. (2020) demonstrated that after treating biofilms with alkaline and acid agents, the remaining adhered biofilm cells re-consolidated the structure and increased their resistance to chemical agents.

3.3. Biocidal effect of the eleven treatments on the detached L. monocytogenes S2-bac cells that conformed the biofilm

Table 3 shows the biocidal activity of each product, calculated from the solution where the surfaces were treated. The formulation used for the design of the detergents could also have biocidal effects when applied as treatments. For this reason, this was considered important to evaluate. The maximum antimicrobial activity found was for the treatment with Product A applied at 3 % and 50 °C, obtaining a microbial reduction of 5.75 log CFU/cm^2. This low microbial load observed in the treatment solution (*i.e.* implying greater effectiveness) could have two explanations: (i) the low count in the washing solution is derived from the fact that *L. monocytogenes* S2-bac remained on the surface because the treatment was not effective; (ii) could be related to the fact that the cells released from the surface and passed into the washing liquid were in a non-viable state (*i.e.* antimicrobial effect). It was demonstrated that it was the second case since both the quantification and DEM studies on the cells that remained after treatment on the surface showed that Product A was completely disintegrated and effective, indicating that this enzymatic product applied under these conditions has antimicrobial activity. However, and parallel to this, a subsequent disinfection should be applied to further improve the effectiveness of Product A. The factor

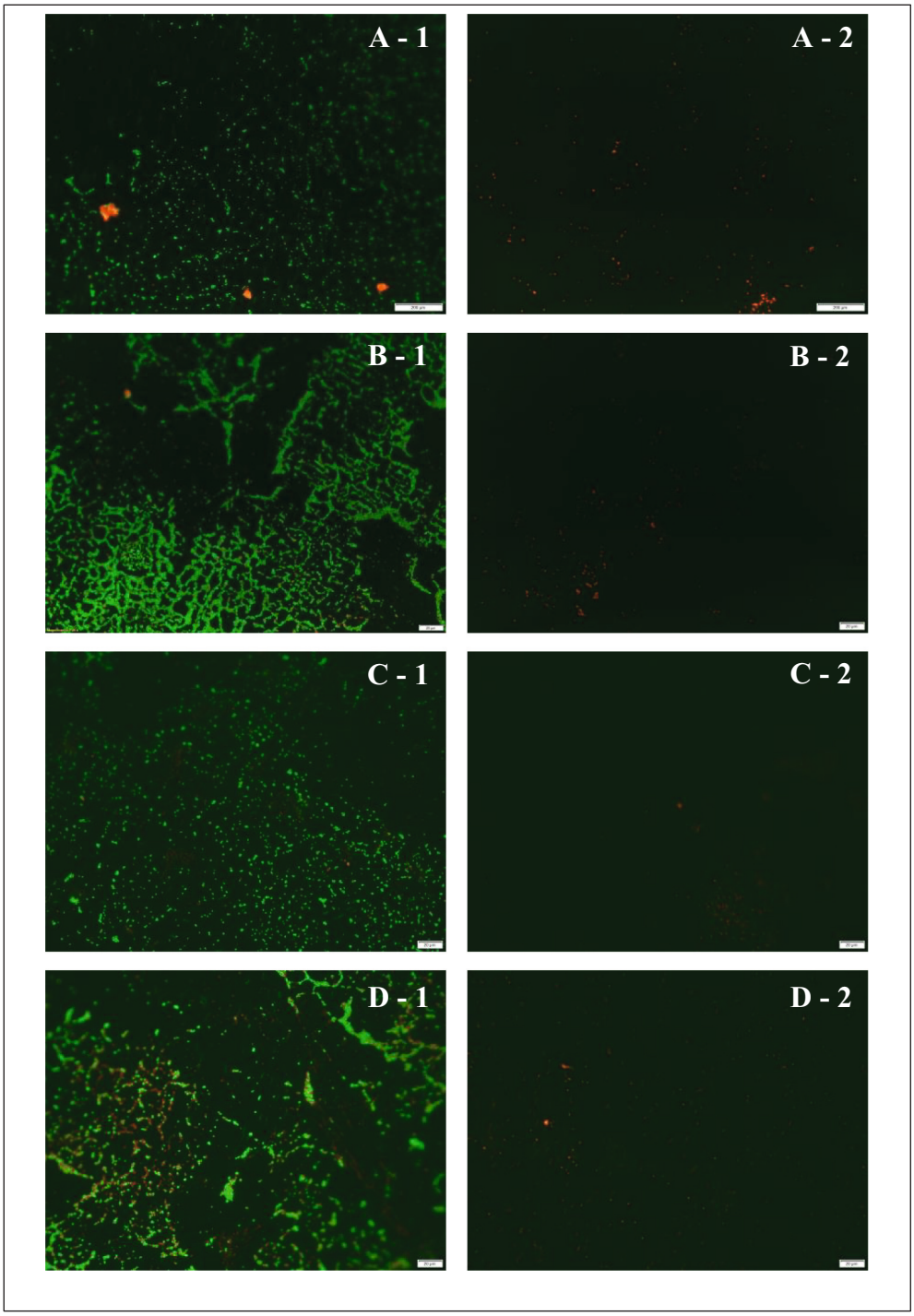

Fig. 3. Epifluorescence digital images of Live/Dead-stained mature *L. monocytogenes* biofilms before (1) and after (2) the application of the combined treatments (*i.e.* chlorinated alkaline 1 % + Product A 1 %) on four different strains: CECT 5672 (A); S2-bac (B); EDG-e (C); CECT 935 (D). Magnification 20×.

that reduced the antimicrobial action of Product A was the decrease in the treatment temperature (*i.e.* 20 °C), the importance of the optimal temperature application having been discussed in the previous two sections.

In addition, Product A treatment (*i.e.* 3 % at 50 °C) did not present significant differences ($P > 0.05$) on the exerted biocidal activity with the alkaline and acid treatments (Table 3). Other authors have reported similar logarithmic reductions when these treatments were applied to eliminate biofilms. For example, Taormina and Beuchat (2002) reported antibacterial effectiveness of an alkaline detergent of 5 or 6 Log CFU/ml reduction. Moreover, approximately 5 logs of dead cells were also obtained for the acidic cleaning agent, similar to what has been obtained by other authors (Fagerlund et al., 2020).

Surprisingly, one treatment that exerted one of the lowest biocidal activities was the chlorinated alkaline detergent (*i.e.* logarithmic reduction of 3.41 Log CFU/cm^2). In this case, biofilm removal from the surface was lower, which could also be the reason why the biocidal effectivity was lower (*i.e.* less microbial load was being released to the solution). It is also important to point out that the biocidal activity of alkaline and chlorinated alkaline, although not evaluated in the present study, can be influenced by the interaction with organic matter, subsequently adversely affecting the efficacy of many biocides such as sodium

hypochlorite, included in the formulation of the chlorinated alkaline detergent (Ramos et al., 2013).

3.4. Effectivity, disaggregation capacity and biocidal effect of the combined treatment on biofilms of different L. monocytogenes strains

The selected strains to conduct this study were L. *monocytogenes* CECT 5672, CECT 935, EDG-e and S2-bac, chosen based on assessing different serotypes (*i.e.* 4b and 1/2a). In this case, a comparison between an effective treatment and one that demonstrated lower removal capacity was included as part of the study with the objective of subjecting treatments to more strains. Hence, chlorinated alkaline (1 % at 20 °C) and Product A (1 % at 50 °C) were selected as treatments. Table 4 shows not only the effectivity of the applied treatments but also the biofilm formation capacity of each strain. Starting from this last point, *L. monocytogenes* CECT 5672 was the largest biofilm producer in conjunction with S2-bac. Strain CECT 5672 has already been reported as a high biofilm producer in comparative studies employing 17 different *L. monocytogenes* strains (Mazaheri et al., 2020; Ripolles-Avila et al., 2019a).

The results obtained after the application of combined treatments (*i.e.* chlorinated alkaline and Product A) for 30 min on each L. *monocytogenes* strain are also presented in Table 4. As described by Fagerlund et al. (2020), the application of chlorinated alkaline helps to remove organic matter from industrial surfaces. The posterior application of the enzymatic product increased the detachment activity of the chlorinated alkaline treatment alone from 77 % to 100 % in the majority of the strains (*i.e.* > 6 log reduction). This combination can therefore ensure an adequate level of cleaning and elimination of the cells detached from the biofilms. In this regard, a two-step cleaning with chlorinated alkaline and an enzymatic product produced the largest microbial cells reduction and could be a recommended treatment to substitute cleaning and disinfection in the same procedure (*i.e.* 5-step cleaning protocol). Fagerlund et al. (2020) evaluated the same combined treatment (*i.e.* chlorinated alkaline followed by an enzymatic based cleaner as the second step), giving a >3 log reduction in *L. monocytogenes* biofilms formed on stainless steel coupons. The difference in the effectivity in comparison with the results obtained could be related to the increase in the treatment temperature (*i.e.* 50 °C).

The antimicrobial efficacy of the combined treatment against the mature *L. monocytogenes* biofilms is shown in Table 5. In this case, the results are separated from each treatment because although applied in combination (*i.e.* first the chemical treatment and then the biological one), the remaining cleaning solution was independent (*i.e.* two different washing solutions rather than a mixed washing solution). Again, lower antimicrobial activity was found in the chlorinated alkaline treatment, which can be attributed to the fact that less microbial load was being released. With the application of the subsequent enzymatic treatment, the antimicrobial activity significantly increased.

3.5. Qualitative evaluation of the combined effect of chlorinated alkaline and enzymatic solutions for L. monocytogenes biofilm removal

Microscopic images showed how the two treatments combined heightened the biofilm removal effectivity (Fig. 3). The use of chlorinated alkaline and enzymatic treatments over fixed periods of 30 min showed almost complete removed the biofilm structures. As previously described by Mnif et al. (2020) and Ripolles-Avila et al. (2020), and also observed in the present study, chlorinated alkaline treatment was unable to completely disintegrate the biofilms structure, remaining on the surface with the potential consequences. One of the direct consequences if cells are not completely dead is that they can repair themselves from damages derived from treatments and consolidate again biofilms structures. Therefore, conventional cleaning and disinfection treatments are not considered a good weapon to remove and eliminate bacterial cells from the surfaces. Nevertheless, the application of the enzymatic product as a second treatment step resulted in the complete dispersion of the structure.

4. Conclusion

The application of conventional treatments such as alkaline and acid detergents for biofilm removal greatly reduce the cells conforming L. *monocytogenes* mature biofilms, with chlorinated alkaline detergent being more effective and therefore an option in terms of conventional treatments. However, the structure formed on the surfaces was not dispersed, with cells remaining on the stainless-steel coupons with the potential consequences in terms of cell reparation to be viable again and potentially re-contaminate other industrial surfaces. In contrast, applying enzymatic treatments had the two effects, high detachment capacity and high dispersion of the structure, thus being consolidated as the most effective treatment. Potentiating chemical-based cleaning detergents effectivity with enzymatic treatments could be an option to optimize treatments, so combined treatment could therefore be a good option to recommend when applying 5-step cleaning protocols.

Funding

This study was supported by Research Project grants RTI2018-098267-R-C32 from the Ministerio de Ciencia, Innovación y Universidades.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

The authors acknowledge Ms. Sarah Davies for the English grammar review and Dolors Busquets for her technical support. The authors alone are responsible for the content and writing of the paper.

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