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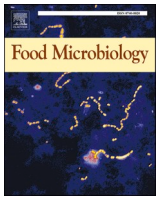

Cross-contamination of mature *Listeria monocytogenes* biofilms from stainless steel surfaces to chicken broth before and after the application of chlorinated alkaline and enzymatic detergents

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ABSTRACT

The objectives of this study were, firstly, to compare a conventional (*i.e.*, chlorinated alkaline) versus an alternative (chlorinated alkaline plus enzymatic) treatment effectivity for the elimination of biofilms from different *L. monocytogenes* strains (CECT 5672, CECT 935, S2-bac and EDG-e). Secondly, to evaluate the cross-contamination to chicken broth from non-treated and treated biofilms formed on stainless steel surfaces. Results showed that all *L. monocytogenes* strains were able to adhere and develop biofilms at approximately the same growth levels (≈5.82 log CFU/cm^2). When non-treated biofilms were put into contact with the model food, obtained an average transference rate of potential global cross-contamination of 20.4%. Biofilms treated with the chlorinated alkaline detergent obtained transference rates similar to non-treated biofilms as a high number of residual cells (*i.e.*, around 4 to 5 Log CFU/cm^2) were present on the surface, except for EDG-e strain on which transference rate diminished to 0.45%, which was related to the protective matrix. Contrarily, the alternative treatment was shown to not produce cross-contamination to the chicken broth due to its high effectivity for biofilm control (<0.50% of transference) except for CECT 935 strain that had a different behavior. Therefore, changing to more intense cleaning treatments in the processing environments can reduce risk of cross-contamination.

1. Introduction

Microbial biofilms, which can contain and transmit pathogenic and spoilage microorganisms, are considered one of the greatest challenges for the food industry nowadays (Fagerlund et al., 2021). This is because biofilms survive and consequently remain on industrial surfaces after the regular cleaning and disinfection procedures are applied (Ripolles-Avila et al., 2020). *Listeria monocytogenes* is among the pathogens with the greatest impact in the food sector due to its ability to form biofilms and establish ecological niches (Mazaheri et al., 2021). The formation of these structures facilitates adaptation to the food processing environment and therefore promotes the persistence of the bacteria. One affected type of industry is meat processing plants, where *L. monocytogenes* can be introduced from raw material (*e.g.*, cattle) and subsequently contaminate the processing environment (Lakicevic et al., 2015). The meat processing plant is one of the affected type of industry as *Listeria* spp. Presence is favored by the environmental conditions (Rothrock et al., 2019). The pathogen can be found in different parts of the factories such as walls, floors, carts, tool cabinets, drains, and door handles, among others (Bolocan et al., 2016; Ripolles-Avila et al., 2019b), and when established it can survive for a long time. In this regard, it has been suggested that pathogen persistence could be directly associated with particular phenotypic and genotypic traits, which may explain why some distinct subtypes persist in a specific ecological niche (Sun et al., 2021). Different studies have shown persistent *L. monocytogenes* contamination in food facilities for months or even decades (Ortiz et al., 2010; Zhang et al., 2021). This fact is highly important since the route most associated with the transfer of the pathogen to food products is through cross-contamination of industrial surfaces (Fagerlund et al., 2021; Ferreira et al., 2014; Giaouris et al., 2014; Ortiz et al., 2010; Zhang et al., 2021).

As has been intensively described, biofilms are microbial communities adhered to biotic or abiotic surfaces that are embedded on a self-produced matrix composed of extracellular components (*e.g.*, proteins,

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carbohydrates, lipids and eDNA) and are highly resistant to treatments (González-Rivas et al., 2018). This leads us to consider as crucial the sanitization of industrial surfaces by applying new methodologies that allow biofilm elimination. If these operations are ineffective, cross-contamination to products can be induced when conducting operations such as cutting and slicing (Lourenco et al., 2022). Nevertheless, the reduction of the costs of these operations and the time dedicated to them are priorities for the food industry, so cleaning and disinfection procedures are often combined in a single step with the use of chlorinated alkaline products. According to Ripolles-Avila et al. (2019a), the effectiveness of cleaning agents directly relates to the structure of the matrix and biofilm produced by different *L. monocytogenes* strains. Furthermore, when *L. monocytogenes* generates a robust matrix, chlorinated alkaline detergents' detachment effect is significantly reduced when compared with enzymatic detergents (Mazaheri et al., 2022; Ripolles-Avila et al., 2020). At formulation level, the difference between the two treatments derives from the inclusion of enzymes with the ability to destabilize the biofilm by directly disrupting the matrix containing, as described above, proteins, polysaccharides, lipids, extracellular DNA and other substances, thereby improving the efficacy of biofilm detachment (Stiefel et al., 2016). In this regard, the study of the nature of the biofilms present in the industrial reality can guide the choice of the type of enzymatic detergent to optimize targeted cleaning.

Moreover, it should be considered that *L. monocytogenes* persistence can also be related to the resistance of strains to disinfectants when microbial cells are exposed to sublethal or sub-inhibitory concentrations (Ortiz et al., 2014, 2016). This is because to control the pathogen, the food industry uses disinfectants as a first line of defense, and residues of these may remain present on industrial surfaces at sublethal concentrations after disinfection. Duze et al. (2021) indicates that this represents a threat to food safety and public health since it subjects *L. monocytogenes* to selection pressure, inducing tolerant strains. Consequently, if chlorinated alkaline detergents are ineffective in completely remove biofilms and parts of the structures remain on the surfaces (Ripolles-Avila et al., 2020), surviving and protected biofilm cells could be exposed to sublethal chlorine doses. In such cases, biocide resistance mechanisms are heightened and associated mechanisms of cross-resistance or co-resistance to antibiotics can be also stimulated (Capita and Alonso-Calleja, 2013).

For all the above reasons, procedures for biofilm elimination must be well designed and evaluated, and the capacity of surviving microbial cells to cross-contaminate food products thoroughly investigated. To this end, the objectives of the present study were: (1) To compare the effectiveness of chlorinated alkaline and enzymatic products for mature biofilms formed by different *L. monocytogenes* (*i.e.*, CECT 935, CECT 5672, S2-bac, EDG-e) strains; and (2) To evaluate the cross-contamination to chicken broth from non-treated and treated biofilms formed on stainless steel surfaces.

2. Material and methods

2.1. Strains and bacterial suspension

Four different L. *monocytogenes* strains were used in this study: 5672 and 935, belonging to serotype 4 b, obtained from the Spanish Type Culture collection (CECT, Paterna, Spain), and S2-bac and EDG-e, belonging to serotype 1/2a, isolated from an Iberian pig processing plant (Ortiz et al., 2014). All the strains were obtained as freeze-dried cultures and were recovered on Tryptic Soy Broth (TSB; Oxid, Madrid, Spain) with an incubation at 30 °C for 48 h. After this period, cells were cultured on Tryptic Soy Agar (TSA; Oxid, Madrid, Spain) and incubated at 37 °C for 24 h. Last, grown plates were kept at 4 °C for up to 1 month as working cultures. For each new experiment, a fresh culture was grown on TSA at 37 °C for 24 h and a new bacterial suspension was prepared. To do so, several isolated colonies from a specific *L. monocytogenes* strain were inoculated into TSYEB $_{gluc1\%+Nacl2\%}$ [*i.e.*, TSB-enriched with 0.3% w/v yeast extract (BD, Madrid, Spain), 1% w/v glucose (Biolife, Madrid, Spain), and 2% w/v sodium chloride (Panreac, Castellar del Vallès, Spain)] until reaching 0.2 McFarland Units, with a final approximate concentarion of 10^6 CFU/ml (Ripolles-Avila et al., 2018a). This was considered the bacterial suspension to conduct the mature biofilm formation (see section 2.3.).

2.2. Surfaces

AISI 316 2 B grade stainless steel coupons (2 cm in diameter and 1 mm thick) were used for the experiments. Prior to their use and according to UNE-EN 13697:2015 (AENOR, 2015), the surfaces were subjected to cleaning and disinfection procedures. First, a neutral detergent (ADIS Hygiene, Madrid, Spain) was employed to submerge the coupons for 1 h, subsequently rinsing them with runing tap water. Afterwards, the surfaces were disinfected with a solution of 70% isopropanol (Panreac Química, Castellar del Vallès, Spain) and air-dried in a laminar flow cabinet (PV-30/70, Telstar, Terrasa, Spain). Last, and with the objective of ensuring they were completely sterile, the surfaces were autoclaved at 121 °C for 15 min.

2.3. Biofilm formation

The surfaces were placed on sterile Petri dishes as a recipient to contain them and 30 μl of the prepared bacterial suspensions (see section 2.1) were subsequently inoculated onto the centre of each stainless steel coupon. They were immeidately introduced into a humidity chamber and incubated at 30 °C to force mature biofilm formation, as established by Fuster-Valls et al. (2008); Ripolles-Avila et al. (2018). Following the procedure proposed by Ripolles-Avila et al. (2018)for the development of an *in vitro* model to form mature *L. monocytogenes* biofilms, the inoculated surfaces were incubated for 7 days with a series of washings and renewal of nutrients at 48 h +24 h +24 h +72 h. For the washings, 3 ml of sterile distilled water in duplicate were introduced onto the surface with the objective of removing non-adhered cells. Afterwards, 30 μl of sterile $TSYEB_{gluc1\%+NaCl2\%}$ were added to the coupons to provide more nutrients and stimulate adhered bacteria to continue consolidating biofilm structure.

2.4. Biofilm elimination

After mature L. *monocytogenes* biofilms were formed, the surfaces were treated with two different treatments, a conventional one (*i.e.*, chlorinated alkaline) and an alternative one (*i.e.*, combination of a chlorinated alkaline followed by an enzymatic treatment). The chlorinated alkaline product consisted in a mixture of sodium hydroxide (8.5%) and sodium hypochlorite (6%) and was applied at 20 °C for 15 min with an in-use concentration of 1%. The enzymatic product, with a proprietary formulation, was composed of ethoxylated sodium lauryl, ether glycolate, amines, C12-14 (even numbered)-alkyldimethyl, *N*-oxides, anionic surfactants (<5%), non-ionic surfactants (<5%), proteases (<5%) and phenoxyethanol. Differently, this product was applied at 50 °C for 15 min, also with an in-use concentration of 1%. In-use concentrations were prepared in hard water and with dirt conditions, again according to the 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 distilled 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 their mixing 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.

For the application of the treatments, the surfaces were washed with 3 ml of sterile distilled water in duplicate with the objective of

eliminating non-attached cells and then placed in sterile flasks with 3 ml of the tested products (*i.e.*, either chlorinated alkaline for the conventional treatment, or first the chlorinated alkaline and then the enzymatic product for the alternative one). For this combined treatment, between the first treatment (*i.e.*, chlorinated alkaline) and the second treatment (*i.e.*, enzymatic), a washing to simulate rinsing was performed with 3 ml of sterile distilled water. Once the treatments were completed, the surfaces were also rinsed with 3 ml of sterile distilled water to remove chemical residues simulating industrial conditions and were used either for the evaluation of cross-contamination to chicken broth as food models or for treatment effectivity (see section 2.5. and 2.6., respectively).

2.5. Cross-contamination from non-treated and treated surfaces to chicken broth

Standardized commercial chicken broth (Knorr, Unilever, Spain) was used as a food model system to conduct this experiment. Non-treated (*i.e.*, coupons with mature *L. monocytogenes* biofilms) and treated surfaces (*i.e.*, coupons subjected to biofilm elimination and therefore suitable for evaluating possible cross-contamination after the cleaning stage) were included in the study design. In both cases, 3 ml of sterile distilled water were used to remove any non-attached cells and the surfaces were further introduced into sterile flasks containing 5 ml of the chicken broth. The surfaces were maintained on the food model for 5 min at 25 °C to promote cross-contamination, followed by quantification of both the cell remaining on the surfaces and potential cell transference to the chicken broth.

2.6. Quantification of viable cells

The cells remaining on surfaces after treatments (*i.e.*, treatment effectivity), after the contact with the food model (*i.e.*, cells that remained on surfaces and did not migrate) and transferred to the chicken broth (*i.e.*, cross-contamination) were quantified using the TEMPO system (bioMérieux, Marcy l'Etoile, France). For this, non-treated and treated coupons and the coupons that had been put into contact with the chicken broth were transferred to sterile flasks containing 3.5 g of glass beds and 10 ml of a neutralizer solution [1 g of tryptone (BD, Madrid, Spain), 8.5 g of NaCl (Panreac) and 30 g Tween 80 (Scharlab, Barcelona, Spain) for every 1000 ml of sterile distilled water in pH (7.0 ± 0.2)]. The samples were vortexed for 90 s at 40 Hz to remove adhered biofilm cells, and serial dilutions in Tryptone Saline Solution [TSS; 1 g of tryptone and 8.5 g of NaCl per liter in pH (7.0 ± 0.2)] were carried out, followed by quantification. The chicken broth that had been put into contact with the contaminated surfaces was also serially diluted in TSS. After that, and proceeding from distinct dilutions, 1 ml of each sample was introduced in a TEMPO vial containing culture medium previously hydrated with 3 ml of sterile distillated water. The vials were homogenized by vortex, transferred onto an enumeration card by the TEMPO filler and incubated at 30 °C for 48 h. A detection limit of 10 CFU/ml (*i.e.*, 0.50 log CFU/cm^2) was established since all *L. monocytogenes* biofilms produced in the coupons (*i.e.*, treated or not) were recovered by vortexing them with glass beads in 10 ml of neutralizer.

2.7. Statistical analysis

Each experiment was performed in triplicates on three independent days (n = 9) for each *L. monocytogenes* strain. The obtained bacterial counts were converted into decimal logarithmic values to almost match the assumption of a normal distribution. Calculations for the detachment percentage was done using the log values. Data were analyzed using STATISTICA 7.0.61.0. "T-Test" or ''One Way ANOVA″ with a posterior contrast with the Tukey Test, depending on the experiment being analyzed, was carried out to observe possible differences between each of the data obtained, considering statistically significant a $P < 0.05$.

Pathogen transference between surfaces on which biofilms were formed to the chicken broth was assessed. The transference rate was calculated as follows:

$$T\ (\%) = \frac{N2 * \left(\frac{V}{S}\right)}{N1} * 100$$

On where:

T: Transference rate.

N_1: Microbial count in CFU/cm^2 on source (*i.e.*, biofilm before the transfer).

N_2: Microbial count in CFU/ml on destination (*i.e.*, chicken broth after the transfer).

V: volume of the receptor source which is 5 ml.

S: area of the contact surface which is 3,14 cm.^{2}

3. Results and discussion

3.1. Biofilm formation of selected L. monocytogenes strains

The ability of different *L. monocytogenes* strains (*i.e.*, CECT 5672, CECT 935, S2-bac and EDG-e) to form mature biofilms was evaluated quantitatively to observe differences in cell growth. It was considered important to conduct this investigation as an initial study since not all *L. monocytogenes* strains are capable of forming biofilms on stainless steel surfaces with the same intensity (Dygico et al., 2020; Grudlewska-Buda et al., 2020). In the present study, the four strains were demonstrated the ability to adhere and develop biofilms at approximately the same growth levels (Table 1 – first column), which reinforces what has previously been demonstrated by other authors, which is the high capacity of *L. monocytogenes* to rapidly adhere to different food contact materials and produce robust biofilms (Ripolles-Avila et al., 2018b; Silva et al., 2008).

As can be observed, none of the strains showed significant differences ($P > 0.05$) at counts level for mature biofilm formation at 7 days of incubation. This finding is in concordance with other studies such as Mazaheri et al. (2022) and Ripolles-Avila et al. (2019a), with the exception of CECT 5672. This L. *monocytogenes* strain has been described in the above studies as a strong biofilm producer; however, in the present study, when compared to the other strains its biofilm forming capacity did not differ significantly ($P > 0.05$). The rest of the evaluated strains (*i.e.*, CECT 935, S2-bac and EDG-e) did not show differences in their biofilm formation capacity in comparison with these reference studies, irrespective as to which serotypes the strains belonged to. In this case, CECT 5672 and CECT 935 are serotype 4 b, which have been described as highly pathogenic (Martins and Leal Germano, 2011), and S2-bac and EDG-e pertain to serotype 1/2a, which is highly prevalent in food processing plants (Iannetti et al., 2016; Zhang et al., 2007). In this regard, although some authors have indicated that there may be a

Table 1
L. monocytogenes cell counts obtained after biofilm formation, cell counts obtained after the contact with the food model and calculated *L. monocytogenes* transferal rates. For the calculations, microbial counts (*i.e.*, not converted into logarithmic values) were used. Data show the means ± standard deviation (n = 9). A global mean for all strains was included in the row *L. monocytogenes* (n = 36).

Strain	Biofilm count Log (CFU/cm^2)	Cell transferred to food Log (CFU/ml)	Transference (%)
CECT 5672	5.89 ± 0.36^a	3.75 ± 0.37^a	0.78 ± 1.58^a
CECT 935	5.58 ± 0.36^a	3.80 ± 0.87^a	5.85 ± 0.73^b
S2-bac	6.00 ± 0.30^a	3.80 ± 0.37^a	1.60 ± 1.53^a
EDG-e	5.78 ± 0.27^a	5.48 ± 0.74^b	90.74 ± 4.39^c
L. monocytogenes	5.82 ± 0.35	4.22 ± 0.99	20.40 ± 1.60

$^{a\text{-}c}$ Values within a column lacking a common letter differ significantly ($P < 0.05$).

relation between the serotype to which the strain belongs and biofilm formation capacity, a direct relationship has not yet been found (Ripolles-Avila et al., 2019a).

Moreover, the EGD-e strain was included in the study because it is considered a model strain with a large body of biochemical, functional and genetic data available on it and its genome completely sequenced and annotated (Zameer et al., 2010). As reported in the present study, strain EDG-e did not differ significantly from the other evaluated strains ($P = 0.897$, $P = 0.671$, $P = 0.565$; respectively for CECT 5672, CECT935 and S2-bac), showing a similar behavior when conforming mature biofilms. Similarly, *L. monocytogenes* S2-bac was chosen because Mazaheri et al. (2020) demonstrated that this strain has higher resistance to enzymatic treatments, leading us to think that it may produce a biofilm structure with a more robust matrix which, consequently, would be more resistant to disruption. However, as demonstrated in the results obtained in the present study, no significant differences ($P > 0.05$) in terms of cell numbers were obtained between strains. To observe structure disposition and matrix production, another study should be carried out using microscopic techniques, as conducted by other researchers (Reis-Teixeira et al., 2017; Ripolles-Avila et al., 2018a; Rodríguez-Melcón et al., 2019a).

3.2. Cross-contamination of mature L. monocytogenes biofilms to chicken broth

L. monocytogenes adherence and survival on food contact surfaces has been extensively studied, even determining that certain materials can reduce the potential risk of cross-contamination in industrial, commercial and domestic environments (Wilks et al., 2006). However, the dynamics of cross-contamination and the possible transfer rates generated after putting a surface in contact with a food model have not been a study target of high interest so far. The results obtained in the present study, including initial biofilm cells on the surface, cells transferred to the food model and the transference rate are shown in Table 1. As can be observed, *L. monocytogenes* cells conforming the mature biofilms on stainless-steel surfaces can be transferred to liquid matrices of neutral pH (*i.e.*, chicken broth). Results are consistent with other authors such as Lin et al. (2005), who demonstrated the transfer from a commercial slicer to deli meats, correlating the degree of transfer with the number of cells inoculated on the slicer blade. Jiang et al. (2018) indicated that the transfer of *L. monocytogenes* may be greater when the surfaces where the biofilms are formed are smooth, as the generated structures are less protected by surface roughness helping to cover them. Nevertheless, in the present study, transference was demonstrated to be at different level rates depending on the strain. *L. monocytogenes.* EDG-e was the one with the highest cross-contamination transference rate, accounting for 90.74% as an average rate, a figure that was significantly different from the rest of the strains ($P < 0.05$ for CECT 5672, CECT935 and S2-bac) which presented average transfer rates of less than 6% in all cases. This result may be explained by the strain EDG-e generating a biofilm matrix in a lower proportion than strains CECT 5672, CECT 935 and S2bac. This would make cell transference after the contact with the food model significantly higher ($P < 0.05$) than for the rest of the strains due to a lower protection. Following the same argument, Ripolles-Avila et al. (2020) have indicated that the effectiveness of cleaning treatments depends directly on the robustness of the matrix generated by different *L. monocytogenes* strains when conforming biofilms, with strains with higher biofilm matrix production that do not contain enzymes in their formulation more resistant to treatments. This has been linked to the fact that each *L. monocytogenes* strain has its own biofilm production capacity and could differ in terms of the structure and matrix generated (Mazaheri et al., 2020; Ripolles-Avila et al., 2019a). Strains CECT 5672 and S2-bac did not present significant differences ($P = 0.25$) between them regarding transference rates, although they showed significant differences from CECT 935 ($P < 0.05$ for CECT 5672 and S2-bac), reinforcing the fact that each strain has its own behavior. These results would indicate that structure and matrix consolidation of *L. monocytogenes* biofilms on surfaces used in the food industry is a crucial factor to influence and spread cross-contamination. Moreover, bacterial transference can also be influenced by the biofilm stage of formation, maturation being the stage when most cells can be released. Wilks et al. (2006) demonstrated significantly higher transference rates for *L. monocytogenes* ST9 and ST87 on cantaloupe surfaces when biofilms were in their mature stage in comparison with either initial adhesion or dispersion stages, obtaining values of microbial migration of 5.34 ± 0.36 to 5.80 ± 0.32 Log CFU/cm^2, similar to those obtained for *L. monocytogenes* EDG-e in the present study.

It is also important to highlight that the transference percentages calculated from the individual strains may have been lower than expected considering the cell count obtained in the chicken broth (*i.e.*, values expressed in Table 1 as logarithms). This is because the calculations were done with the values derived from the microbial counts (*i.e.*, without converting them to logarithmic values). However, although the transference percentages may seem low, they are highly relevant. For example, strain S2-bac, with an approximate transference rate of 2%, is producing a migration of 1.6×10^4 *L. monocytogenes* cells to the chicken broth, which is not a negligible number as it is an insufficient risk reduction.

It was considered important to understand the impact at species level (*i.e.*, not considering the independent values of each strain) to know *L. monocytogenes* global behavior. For this reason, results were grouped globally to find the detectable L. monocytogenes biofilm transfer rate in the food industry, which would lead to an understanding of the potential risk of cross-contamination in food processing environments. Globally, the cross-contamination transfer rate from the biofilm contaminated surfaces to the chicken broth was established at 20.40% as an average rate. In this sense, EDG-e strain was the responsible for the overall transfer rate increasing with respect to the rest of the strains, which, as previously discussed, did not exceed 6% of transfer to the food model in any of the cases, which shows a variability between strains and, with it, different behaviors. This pattern of variability between *L. monocytogenes* strains has already been observed in other studies (Mazaheri et al., 2022; Ripolles-Avila et al., 2019a, 2020). This average rate of 20.40% as the global *L. monocytogenes* cell transfer poses a potential risk for the food safety as approximately 1.66×10^4 cells would directly migrate to the product in 5 min of contact when an initial amount of approximate 10^6 cells is found on a surface. In fact, this concentration is considered normal when mature biofilms are formed on laboratory (Ripolles-Avila et al., 2019a) and industrial (Ripolles-Avila et al., 2019b) settings. Although it is complicated to establish the infective dose of the pathogen as there is considerable variability across population subgroups and *L. monocytogenes* strains (Pouillot et al., 2015), the microbial load transferred to the food model would surpass 1000 cells. This migration is significant as the modelized food would permit the growth of the pathogen, incrementing therefore the cell numbers on the product.

This level of transfer would also be of concern since any spillage of cross-contaminated liquid food could quickly spread and recontaminate other industrial areas (Ivanek et al., 2004). Although it is true that the food industry applies cleaning and disinfection operations at the end of every day to prevent this from happening (Obe et al., 2020), the listericidal effect remains incomplete, consequently leaving cells that persist on surfaces and constantly generate biofilms (Zhang et al., 2021). In this case, any contamination level left at the end of the day can increase the risk of cross-contamination to food products by *L. monocytogenes* (Ivanek et al., 2004). For this reason, it was considered important to know how cleaning procedures affect the elimination of mature *L. monocytogenes* biofilms, which can be the cause of cross-contamination to the food model after treatments.

3.3. Effectivity of applied treatments for biofilm removal

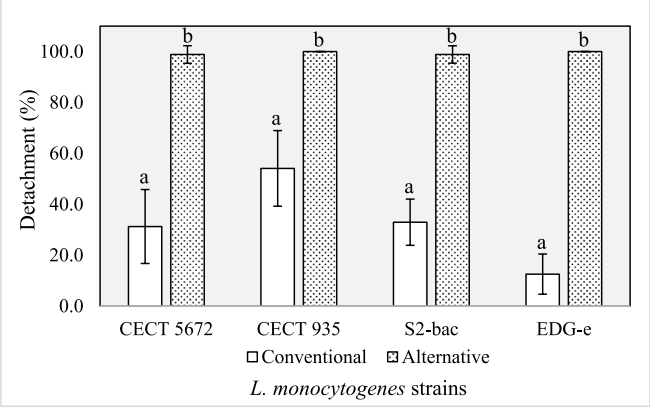
An important objective for the food industry is the elimination of

biofilms through cleaning and disinfection programs, which are established to prevent possible cross-contaminations to food products (González-Rivas et al., 2018). To understand the overall treatment effectiveness, the detachment percentages of the mature biofilms from the different *L. monocytogenes* strains after the application of conventional (*i.e.*, chlorinated alkaline) and alternative (*i.e.*, combination of chlorinated alkaline and enzymatic) treatments were calculated. As can be observed in Fig. 1, the effectiveness of the conventional treatment ranged between 12.55 and 54.09%, CECT 935 being the highest affected strain. These percentages imply a residual microbial load with the subsequent possibility of cross-contamination to food products or dispersal to other industrial surfaces. These findings are in concordance with those reported by Kim et al. (2018) and Ripolles-Avila et al. (2020), who have indicated that although a certain number of cells conforming the structures can be dispersed after the application of a conventional chlorinated alkaline detergent, the treatment is not completely effective. Rodríguez-Melcón et al. (2019b) also reported that using sodium hypochlorite as a disinfectant agent at a concentration equivalent to the minimum inhibitory concentration (MIC), established at 3500 ppm, or at higher doses, decreases cell biovolume up to a maximum of 90%. Such treatment ineffectiveness leaves residual *L. monocytogenes* cells on the surface that can continue to form biofilms, which may have been exposed to sublethal doses of the chlorinated agent. This can generate two potential problems, the first related to increases in MIC after being exposed to sublethal doses of chlorine (Bansal et al., 2018); and the second related to the appearance of viable but not cultivable populations (VBNC) in response to treatment with the disinfectant agent (Brauge et al., 2020). Part of this problem is because the use of a product that has a detergent and disinfectant effect in a single step means that the biofilm matrix is not completely destroyed, possibly leaving parts on the surface, protecting the cells of the deeper layers. Ripolles-Avila et al. (2020) showed Direct Epifluorescent Microscopy (DEM) images of different *L. monocytogenes* strains exposed to a chlorinated alkaline detergent, with intact parts of the matrix after treatment.

In the case of the alternative treatment, between 98.85 and 100% biofilm detachment was obtained in all cases. This treatment was capable of dispersing the mature structure and completely reducing the microbial load. In this case, the combined treatment (*i.e.*, alternative) enhanced detachment effectivity over the use of just the enzymatic product. In this regard, Mazaheri et al. (2020) and Mazaheri et al. (2022) indicated effectiveness of the same enzymatic product ranging from 68% to 99% depending on the *L. monocytogenes* strain evaluated, and of 95.73%–100% depending on the concentration of the product used for *L. monocytogenes* S2-bac, respectively for the first and second cited study. Nevertheless, such effectivity is improved in the present study because a combination treatment was implemented. In this case, chlorinated alkaline detergent application aims to remove organic residues from industrial surfaces (Fagerlund et al., 2020) and the enzymatic product application helps to destroy biofilm matrix and force cell dispersion. This is demonstrated in the study of Mazaheri et al. (2022), in which detachment activity of the chlorinated alkaline treatment was increased from 77% if applied alone to 100% if applied in combination with the enzymatic treatment for the same *L. monocytogenes* strains employed in the present study.

3.4. Cell transference from treated surfaces to chicken broth

When food contact surfaces are adequately cleaned and disinfected, the potential for cross-contamination from industrial surfaces to food products can be significantly reduced (Yang et al., 2017). However, it is important to evaluate the impact of the treatment on the subsequent transfer that may occur on industrial surfaces to observe how significant the application of the treatment under evaluation is for the prevention of cross-contamination. Table 2 and Table 3 show the results derived from the present study, including initial biofilm cells on the surface, cells transferred to the food model and the transference rate after the conventional (*i.e.*, chlorinated alkaline) and alternative (*i.e.*, chlorinated alkaline plus enzymatic) treatments were applied. As can be observed, after the application of the conventional treatment, a high number of residual cells (*i.e.,* around 4 to 5 Log CFU/cm^2) were present on the surface, except for CECT 935, which showed the highest detachment effectivity, as discussed in the previous section, and therefore the lowest number of adhered cells ($P < 0.05$). Gu et al. (2021) observed that there was a cross-contamination of different strains of *Salmonella enterica* to papayas when a sponge moistened with washing water that contained chlorine as a disinfectant was put in contact with the papaya, showing that this occurred when the dose of disinfectant was low and could not control the microbial load. Similarly, in the present study, the treatment was not completely effective, and the dose of the disinfectant used in the product (*i.e.*, chlorinated alkaline) was not able to control the *L. monocytogenes* cells. Moreover, the strain least affected by the chlorinated alkaline treatment and, consequently, the one that left a greater cell load adhered to the surface was EDG-e (Table 2), which presented significant differences from the rest of the strains ($P < 0.05$), a result that also coincides with data already presented regarding effectivity. No significant differences were found between strain CECT 5672 and S2-bac



[a-b] Values within a bar lacking a common letter differ significantly ($P < 0.05$). The statistical analysis was performed by strain comparing both treatments (*i.e.*, conventional *vs* alternative).

Fig. 1. Detachment percentage of different *Listeria monocytogenes* strains after the application of the conventional (*i.e.*, chlorinated alkaline) and the alternative (*i.e.*, combination of chlorinated alkaline and enzymatic) treatments. Each value corresponds to a mean of three replicates performed on three separate days (n = 9). The error bars represent the standard deviation.

Table 2
L. monocytogenes cell counts obtained after the application of chlorinated alkaline treatment, cell counts obtained after the contact with the food model once the treatment was applied and calculated *L. monocytogenes* transferal rates. For calculations, microbial counts (*i.e.*, not converted into logarithmic values) were used. Data show the means ± standard deviation (n = 9). A global mean for all strains was included in the row *L. monocytogenes* (n = 36).

Strain	Biofilm count Log (CFU/cm^2)	Cell transferred to food Log (CFU/ml)	Transference (%)
CECT 5672	4.12 ± 0.87^{b}	2.40 ± 0.88^{b}	3.51 ± 1.43^{c}
CECT 935	2.61 ± 0.85^{a}	0.96 ± 0.95^{ab}	3.04 ± 1.58^{c}
S2-bac	4.13 ± 0.57^{b}	2.16 ± 0.86^{a}	1.12 ± 13.03^{b}
EDG-e	5.13 ± 0.49^{c}	2.43 ± 1.11^{b}	0.45 ± 0.83^{a}
L. monocytogenes	3.96 ± 1.15	1.99 ± 1.10	1.02 ± 5.88

$^{a\text{-}c}$ Values within a column lacking a common letter differ significantly ($P < 0.05$).

Table 3
L. monocytogenes cell counts obtained after the application of alternative treatment, cell counts obtained after the contact with the food model once the treatment was applied and calculated *L. monocytogenes* transferal rates. For calculations, microbial counts (*i.e.*, not converted into logarithmic values) were used. Data show the means ± standard deviation (n = 9). A global mean for all strains was included in the row *L. monocytogenes* (n = 36).

Strain	Biofilm count Log (CFU/cm^2)	Cell transferred to food Log (CFU/ml)	Transference (%)
CECT 5672	0.56 ± 0.17^{a}	<0.50 ± 0.00^{a}	<0.50 ± 0.00^{a}
CECT 935	<0.50 ± 0.00^{a}	0.83 ± 0.66^{a}	163.00 ± 132.00^{b}
S2-bac	0.56 ± 0.17^{a}	<0.50 ± 0.00^{a}	<0.50 ± 0.00^{a}
EDG-e	<0.50 ± 0.00^{a}	<0.50 ± 0.00^{a}	<0.50 ± 0.00^{a}
L. monocytogenes	<0.50 ± 0.12	0.58 ± 0.35	116.00 ± 70.00

$^{a\text{-}c}$ Values within a column lacking a common letter differ significantly ($P < 0.05$).

($P = 1.00$) in terms of residual cell load. The transference rates obtained coincide approximately with those previously reported in reference to when biofilms were not exposed to any cleaning and disinfection treatment. When comparing specifically the transference percentage between strains, S2-bac demonstrated to have the second lowest transfer rate (*i.e.*, 1.12%) although with the highest variability. Such variability obtained is important in terms of treatments effectiveness since it can make that, under industrial conditions, a lesser disruption effect could be observed increasing therefore the transfer rates as the environment cannot be so controlled. Nevertheless, what is most surprising is the behavior of EDG-e, the strain least affected by the applied conventional treatment. In this case, EDG-e was the strain which, in the absence of the application of any treatment, was able to transfer to the food model around 90% of its cell content, the highest rate found. After the application of the chlorinated alkaline, the transfer rate dropped to less than 1%. This result could be explained by the same factor discussed in the previous section: the protective matrix (Mazaheri et al., 2020; Ripolles-Avila et al., 2019a). The EDG-e strain may not have had as much matrix coating the structure as the other strains and, because of this, cells were easily transferable. As treatment was applied, EDG-e may have become more resistant, rapidly producing a protective matrix that caused transference to decrease drastically. To corroborate this supposition, an in-depth study should be carried out on the affectation of *L. monocytogenes* biofilm matrix after treatments with chlorinated alkaline detergents.

After the application of the alternative treatment on the mature *L. monocytogenes* biofilms, the transference rate from contaminated surfaces to chicken broth significantly ($P < 0.05$) decreased to < 0.5% in CECT 9835 and EDG-e strains and to 0.56 Log CFU/cm^2 of residual cell counts on CECT 5672 and S2-bac strains. It can be observed that cross-contamination was not generated because the alternative treatment was completely effective and did not leave significant ($P < 0.05$) residual bacterial cells on the surface (Table 3). The use of enzymatic detergents has been indicated as an important strategy to decrease cross-contamination from surfaces to food products in processing environments (Mazaheri et al., 2020; Ripolles-Avila et al., 2020; Sadekuzzaman et al., 2015; Simões et al., 2010). However, the results of the present study demonstrate that cross-contamination is simply not produced when the evaluated alternative treatment is applied. From Table 3 it could apparently seem that strain CECT 935 have a higher number of cells transferred to food (*i.e.*, 0.83 ± 0.66 Log CFU/cm^2) that what there were on the surface after the alternative treatment (*i.e.*, <0.05 ± 0.00 Log CFU/cm^2), which can be explained through the variability as some residual cells could have remained on the surface after the alternative treatment. However, statistical analysis did not show significant differences ($P > 0.05$) between the biofilm count after the alternative treatment and the cells transferred to food. Moreover, *L. monocytogenes* CECT 935 has also shown variability in the results when the same alternative treatment has been applied (Mazaheri et al., 2022). This makes that the transfer rate to the food model was established at 163% for this strain, although with low levels of cell load transferred and high variability. On this regard, the combination of a chlorinated alkaline detergent followed by an enzymatic product application showed the highest effectivity, detaching and dispersing the biofilm *L. monocytogenes* cells. In their study of cross-contamination to apples, Sheng et al. (2020) point out that treatments that are capable of eliminating resident *L. monocytogenes* cells in washing solutions used for cleaning and disinfection are the ones that will have the highest effectivity in avoiding cross-contamination. However, however, if there are low residual loads, they could be transferred to the food matrix with a higher transfer rate, although the cell load would be lesser. For this reason, it must not be forgotten that the alternative treatment would be followed by a subsequent disinfection, which would optimize the treatment applied and the complete elimination of the microbial load by having dispersed the biofilm structure of *L. monocytogenes*.

4. Conclusions

The present work showed that mature *L. monocytogenes* biofilms formed on stainless-steel surfaces are easily transferred to chicken broth, although the transferal rates depended on the strain. Treatment detaching capacity is related to biofilm matrix disruption and cell release, which is why the combined treatment was shown to be more effective. The use of a chlorinated alkaline treatment to control *L. monocytogenes* biofilms could pose a potential risk of cross-contamination to food products as transference rates were demonstrated to be similar to those obtained when no treatment was applied to the mature *L. monocytogenes* biofilms. Differently, the proposed combined treatment using the chlorinated alkaline product followed by an enzymatic treatment showed no cross-contamination as no residual cells were adhered to the surface. Important efforts have previously been made to control biofilms in the food industry, but more research still needs to be conducted on the influence of cross-contamination to assure higher food safety level.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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