Universitat Autònoma de Barcelona

CONTROL OF LISTERIA MONOCYTOGENES BIOFILMS IN THE FOOD INDUSTRY: FIGHTING A RECURRING PROBLEM BY STUDYING ITS FORMATION AND ELIMINATION

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CONTROL OF *LISTERIA MONOCYTOGENES* BIOFILMS IN THE FOOD INDUSTRY: FIGHTING A RECURRING PROBLEM BY STUDYING ITS FORMATION AND ELIMINATION

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HACEN CONSTAR:

Que la memoria titulada "Control of *Listeria monocytogenes* biofilms in the food industry: Fighting a recurring problem by studying its formation and elimination", presentada por Tina Mazaheri para optar al grado de Doctor por la "Universitat Autònoma de Barcelona", ha sido realizada bajo su dirección y considerándola finalizada, autorizan su presentación para que sea juzgada por la comisión correspondiente.

Y para que conste a los efectos oportunos, firma el presente certificado en Bellaterra, a 21 de setiembre de 2022.

Carolina Ripollés Ávila José Juan Rodríguez Jerez

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SUMMARY

Foodborne diseases have been recognized, over time, not only a major public health problem worldwide, but also as a cause of reduced economic productivity. That is why one of the main concerns regarding food industrial development is food safety. Pathogenic microorganisms are capable of colonizing industrial surfaces through the formation of biofilms, promoting the generation of microbial reservoirs and enabling cross-contamination of the final product. To prevent industrial surfaces from being a constant source of contamination, it is of utmost importance to apply adequate cleaning and disinfection operations. However, the usual processes may be insufficient to eliminate and prevent the implantation of such biofilms.

The present work is included in the LISSA National Project (RTI2018-098267-R-C32), a project that focuses its objectives on two innovative strategies in the field of alternative antibiofilm applications. On the one hand, the potential of developing new materials for the food industry, and, secondly, the potential of harnessing biodiversity to discover and develop new natural biocides. Among the key microorganisms to be studied, the project focuses on the study of the foodborne pathogen with the highest mortality rate in Europe, *Listeria monocytogenes*. This microorganism is related to the formation of biofilms in processing environments, which makes its eradication a challenge as resistance to different elimination treatments applied in the food industry is conferred.

For this reason, in this doctoral thesis different studies were carried out focused on understanding the influence of treatments for *L. monocytogenes* biofilms control. In the first place, starting from a standard *in vitro* model for *L. monocytogenes* biofilms formation in its mature stage on stainless steel surfaces, it was proceeded to evaluate the efficacy of an enzymatic product for the detachment of biofilms. The results showed that the maximum reduction obtained was approximately 6.9 Log CFU/cm^2 for the CECT 5672 strain, reaching values of detachment effectiveness of mature biofilms between 85 and 99 % for the different *L. monocytogenes* strains. In addition, the results showed that the detachment of these structures may be directly related to the strain under study, and may be due to the fact that some strains are capable of generating biofilms with a more robust matrix, making their removal more difficult.

In a second study, eleven treatments were compared, including enzymatic agents applied at different temperatures and concentrations, alkaline and acid detergents, for the elimination of *L. monocytogenes* S2-bac, the strain that showed the greatest resistance in the previous study. Subsequently, two treatments were selected to combine them with each other and observe if their application could be an alternative for biofilms detachment. Results showed that *L. monocytogenes* cells conforming the biofilms diminished their counts after applying the acid, alkaline and chlorinated alkaline treatments on 6.03, 6.24 and 4.76 Log CFU/cm^2, respectively. The observation of the biofilms remaining structure on the surface by direct epifluorescence microscopy (DEM) demonstrated that conventional treatments were not able to completely eliminate the formed structures. On the contrary, the enzymatic treatments applied at 50ºC obtained the highest detachment and biocidal activity, although without reaching what was shown by the combined treatment, which improved cell dispersion and increased the effectiveness.

Thirdly, given that the main concern regarding biofilms consolidation in the food industry is cross-contamination to the product, it was considered necessary to evaluate the impact in terms of transference. For this reason, a comparison was made on the effectiveness of a conventional treatment (*i.e.* chlorinated alkaline) *versus* an alternative treatment (*i.e.* combined treatment evaluated in the previous stage of this thesis) for the elimination of biofilms formed by different *L. monocytogenes* strains, and their consequent cross-contamination to chicken broth was evaluated targeting untreated and treated biofilms. The results showed that, when the biofilms were not subjected to treatment, the transference rates obtained led to cross-contamination with a global value calculated, at the species level, of 20.4 %, which represents a potential risk for food safety, since approximately 1.66x10^4 cells would be migrating to the product. The conventional treatment transferred rates similarly to those obtained for the untreated biofilms, contrary to what was obtained after the application of the combined treatment, which proved not to produce cross-contamination to the chicken broth due to its high detachment effectiveness.

Afterwards, and derived from the importance of the presence of dirtiness on industrial surfaces due to its interaction with the elimination treatments, the impact of both the conventional and the combined treatment was evaluated. At the same time, the

regeneration capacity of cells conforming the structure was studied after the application of the selected treatments to observe if the remaining residual load was capable of consolidating the structure again after 24 hours. The alternative treatment was significantly ($P < 0.05$) more effective than the conventional one, but none of them completely eradicated the pathogen from the tested surfaces (*i.e.* with or without a preconditioning layer). Biofilm regeneration was found, achieving cell counts similar to controls (*i.e.* approximately 6.0 Log CFU/cm^2).

Finally, a study was carried out to determine the microbial contamination in different industrial surfaces of a meat industry, through the implementation of a sensor-based sampling system. Subsequently, an antibiofilm treatment was applied for several weeks to determine the reduction impact of the different microbial groups, focusing on *L. monocytogenes* detection after the application of such treatment. The results obtained showed two main groups of zones with greater and lesser degree of microbiological contamination, being the total aerobic counts the microbial group with the highest contribution. *L. monocytogenes* presence was detected on five different surfaces throughout the sampling. The applied antibiofilm treatment showed a reduction in all the microbial groups evaluated during the weeks in which it was implemented, compared to those weeks in which no disruptive treatment was applied.

RESUMEN

Las enfermedades de transmisión alimentaria han sido reconocidas, a lo largo del tiempo, no solo un problema importante de salud pública a nivel mundial, sino también como una causa de reducción de la productividad económica. Es por ello que una de las preocupaciones en cuanto al desarrollo industrial de los alimentos es la inocuidad alimentaria. Los microorganismos patógenos son capaces de colonizar las superficies de la industria mediante la formación de biofilms, fomentando la generación de reservorios microbianos y posibilitando la contaminación cruzada al producto final. Para evitar que las superficies industriales sean una fuente constante de contaminación, es de grasn importancia aplicar operaciones de limpieza y desinfección adecuadas. No obstante, los procesos habituales pueden ser insuficientes para eliminar y prevenir la implantación de dichos biofilms.

El presente trabajo se engloba dentro del Proyecto Nacional LISSA (RTI2018-098267-R-C32), un proyecto que centra sus objetivos en dos estrategias innovadoras en el campo de las aplicaciones antibiofilm alternativas. Por una parte, el potencial que tiene el desarrollo de nuevos materiales para la industria alimentaria, y, en segundo lugar, el potencial que tiene el aprovechamiento de la biodiversidad para descubrir y desarrollar nuevos biocidas naturales. De entre los microorganismos clave a estudiar, el proyecto se centra en el estudio del patógeno con mayor porcentaje de mortalidad por transmisión alimentaria a nivel europeo, *Listeria monocytogenes*. Este microorganismo se relaciona con la formación de biofilms en ambientes de procesado, lo que dificulta su erradicación al conferirle resistencia a distintos tratamientos de eliminación aplicados en la industria alimentaria.

Por ello, en esta tesis doctoral se realizaron diferentes estudios enfocados a conocer la influencia de tratamientos para el control de biofilms formados por *L. monocytogenes*. En primer lugar, al partir de un modelo estándar, a escala de laboratorio, para la formación de biofilms de *L. monocytogenes* en su etapa madura sobre superficies de acero inoxidable, se procedió a evaluar la eficacia de un producto enzimático sobre el desprendimiento de biofilms maduros del patógeno. Los resultados mostraron que la reducción máxima obtenida fue de, aproximadamente, 6,9 Log CFU/cm^2 para la cepa

CECT 5672, llegando a valores de efectividad de desprendimiento de biofilms maduros entre el 85 y el 99 % para las distintas cepas de *L. monocytogenes*. Además, los resultados mostraron que el desprendimiento de estas estructuras puede estar directamente relacionado con la cepa bajo estudio, pudiendo deberse a que algunas cepas son capaces de generar biofilms con una matriz más robusta, dificultando su eliminación.

En un segundo estudio se compararon once tratamientos, incluyendo agentes enzimáticos aplicados a temperaturas y concentraciones diferentes, detergentes alcalinos y ácidos, para la eliminación de *L. monocytogenes* S2-bac, cepa que resultó mostrar mayor resistencia en el anterior estudio. Posteriormente, se seleccionaros dos tratamientos para combinarlos entre ellos y observar si, su aplicación conjunta podría ser una alternativa para el desprendimiento de los biofilms generados. El desprendimiento de *L. monocytogenes* obtenido tras la aplicación de los tratamientos ácido, alcalino y alcalino clorado fue de 6,03, 6,24 y 4,76 Log UFC/cm^2, respectivamente. La observación por microscopia de epiluminiscencia directa (DEM) de la estructura del biofilm remanente en superficie demostró que los tratamientos convencionales no fueron capaces de eliminar completamente las estructuras conformadas. Por el contrario, los tratamientos enzimáticos aplicados a 50ºC obtuvieron el mayor desprendimiento y actividad biocida, aunque sin llegar a lo demostrado por el tratamiento combinado, el cual mejoró la dispersión celular y aumentó la efectividad.

En tercera instancia, dado que la principal preocupación en cuanto a la consolidación de biofilms en la industria alimentaria es la contaminación cruzada a producto, se consideró necesario evaluar el impacto en términos de transferencia. Por ello, se realizó una comparación de la efectividad de un tratamiento convencional (*i.e.* alcalino clorado) versus uno alternativo (*i.e.* tratamiento combinado evaluado en la anterior etapa de la presente tesis) para la eliminación de biofilms formados por distintas cepas de *L. monocytogenes*, y se evaluó la contaminación cruzada al caldo de pollo de biofilms no tratados y tratados con ambos tratamientos. Los resultados mostraron que, cuando los biofilms no eran sometidos a tratamiento, las tasas de transferencia obtenidas conllevaban una contaminación cruzada con un valor global calculado, a nivel de especie, del 20,4 %, lo que representa un riesgo potencial para la inocuidad alimentaria, ya que aproximadamente 1,66x10^4 células estarían migrando al producto. El tratamiento convencional obtuvo tasas de transferencia similares a las obtenidas para los biofilms no

tratados, contrariamente a lo obtenido tras la aplicación del tratamiento combinado, el cual demostró no producir contaminación cruzada al caldo de pollo debido a su alta efectividad de desprendimiento.

En cuarto lugar, y derivado de la importancia que tiene la presencia de residuos por su interacción con los tratamientos de eliminación, se procedió a evaluar el impacto tanto del tratamiento convencional como del combinado, ambos seleccionados en anteriores etapas de estudio. Paralelamente, se estudió la capacidad de regeneración de la estructura tras la aplicación de dichos tratamientos para observar si la carga residual remanente era capaz de consolidar de nuevo la estructura tras 24 horas. El tratamiento alternativo fue significativamente ($P < 0,05$) más eficaz que el convencional, pero ninguno de ellos erradicó por completo el microorganismo de las superficies evaluadas (con o sin preacondicionamiento). Se encontró regeneración de los biofilms, lográndose recuentos celulares similares a los controles (*i.e.* 6,0 Log UFC/cm^2 aproximadamente).

Finalmente, se realizó un estudio para la determinación de la contaminación microbiana en diferentes superficies industriales de una industria cárnica, mediante la implantación de un sistema de muestreo a base de sensores. Posteriormente, se aplicó un tratamiento antibiofilm durante diversas semanas para conocer el impacto de reducción de los distintos grupos microbianos, centrándose en la detección de *L. monocytogenes* después de la aplicación de dicho tratamiento. Los resultados obtenidos mostraron dos agrupaciones principales de zonas con mayor y menor grado de contaminación microbiológica, siendo los recuentos en microorganismos aerobios totales el grupo microbiano con mayor contribución. Se detectó la presencia de *L. monocytogenes* en cinco superficies diferentes a lo largo del muestreo. El tratamiento antibiofilm aplicado demostró una reducción en todos los grupos microbianos evaluados durante las semanas en que se implementó, en comparación con aquellas semanas en las que no se aplicó ningún tratamiento disruptivo.

RESUM

Les malalties de transmissió alimentària han estat reconegudes, al llarg del temps, no només com un problema important de salut pública a escala mundial, sinó també com una causa de reducció de la productivitat econòmica. És per això que una de les preocupacions pel que fa al desenvolupament industrial dels aliments és la innocuïtat alimentària. Els microorganismes patògens són capaços de colonitzar les superfícies de la indústria mitjançant la formació de biofilms, fomentant la generació de reservoris microbians i possibilitant la contaminació creuada al producte final. Per evitar que les superfícies industrials siguin una font constant de contaminació, és rellevant aplicar operacions de neteja i desinfecció adequades. No obstant això, els processos habituals poden ser insuficients per eliminar i prevenir la implantació dels biofilms.

El present treball s'engloba dins del Projecte Nacional LISSA (RTI2018-098267-R-C32), un projecte que centra els objectius en dues estratègies innovadores en el camp de les aplicacions antibiofilm alternatives. D'una banda, el potencial que té el desenvolupament de nous materials per a la indústria alimentària i, en segon lloc, el potencial que ofereix l'aprofitament de la biodiversitat per descobrir i desenvolupar nous biocides naturals. Entre els microorganismes clau a estudiar, el projecte es centra en l'estudi del patogen amb major percentatge de mortalitat per transmissió alimentària a escala europea, *Listeria monocytogenes*. Aquest microorganisme es relaciona amb la formació de biofilms en ambients de processament, cosa que dificulta la seva erradicació al conferir-li resistència a diferents tractaments d'eliminació aplicats a la indústria alimentària.

Per això, en aquesta tesi doctoral es van realitzar diferents estudis enfocats a conèixer la influència de tractaments per al control de biofilms formats per *L. monocytogenes*. En primer lloc, a partir d'un model estàndard, a escala de laboratori, per a la formació de biofilms de *L. monocytogenes* en la seva etapa madura sobre superfícies d'acer inoxidable, es va avaluar l'eficàcia d'un producte enzimàtic per l'eliminació de biofilms madurs del patogen. Els resultats van mostrar que la reducció màxima obtinguda va ser d'aproximadament 6,9 Log CFU/cm^2 per la soca CECT 5672, arribant a valors d'efectivitat de despreniment de biofilms madurs entre el 85 i el 99 % per a les diferents soques de *L. monocytogenes*. A més, els resultats van mostrar que el despreniment

d'aquestes estructures pot estar directament relacionat amb la soca sota estudi, ja que algunes d'aquestes soques són capaces de generar biofilms amb una matriu més robusta, dificultant-ne l'eliminació.

En un segon estudi es van comparar onze tractaments, incloent-hi agents enzimàtics aplicats a temperatures i concentracions diferents, detergents alcalins i àcids, per a l'eliminació de *L. monocytogenes* S2-bac, soca que va mostrar una major resistència a l'anterior estudi. Posteriorment, es van seleccionar dos tractaments per combinar-los entre ells i analitzar veure si, la seva aplicació, podria ser una alternativa per a l'eliminació dels biofilms generats. El despreniment de *L. monocytogenes* obtingut després de l'aplicació dels tractaments àcid, alcalí i alcalí clorat va ser de 6,03, 6,24 i 4,76 Log UFC/cm^2, respectivament. L'observació per microscòpia d'epiluminescència directa (DEM) de l'estructura del biofilm romanent en superfície va demostrar que els tractaments convencionals no eren capaços d'eliminar completament les estructures conformades. Pel contrari, els tractaments enzimàtics aplicats a 50ºC van obtenir el major despreniment i activitat biocida, encara que sense arribar al resultat obtingut pel tractament combinat, el qual va millorar la dispersió cel·lular i va augmentar l'efectivitat.

En tercer lloc, atès que la principal preocupació pel que fa a la consolidació de biofilms a la indústria alimentària és la contaminació creuada a producte, es va considerar necessari avaluar l'impacte en termes de transferència. Per això, es va realitzar una comparació de l'efectivitat d'un tractament convencional (*i.e.* alcalí clorat) versus un d'alternatiu (*i.e.* tractament combinat avaluat a l'anterior etapa de la present tesi) per a l'eliminació de biofilms formats per diferents soques de *L. monocytogenes*, i es va avaluar la contaminació creuada a brou de pollastre a partir de biofilms no tractats i tractats amb tots dos tractaments. Els resultats van mostrar que, quan els biofilms no eren sotmesos a tractament, les taxes de transferència obtingudes comportaven una contaminació creuada amb un valor global calculat, a nivell d'espècie, del 20,4 %, fet que representa un risc potencial per a la innocuïtat alimentària, ja que aproximadament 1,66x10^4 cèl·lules estarien migrant al producte. El tractament convencional va aconseguir taxes de transferència similars a les obtingudes per als biofilms no tractats, contràriament al que es va obtenir després de l'aplicació del tractament combinat, el qual va demostrar no produir contaminació creuada al brou de pollastre a causa de la seva alta efectivitat de despreniment.

En quart lloc, i derivat de la importància que té la presència de residus per la seva interacció amb els tractaments d'eliminació, es va avaluar l'impacte de tant el tractament convencional com del combinat, ambdós seleccionats en etapes d'estudis anteriors. Paral·lelament, es va estudiar la capacitat de regeneració de l'estructura després de l'aplicació d'aquests tractaments per observar si la càrrega residual romanent era capaç de tornar a consolidar l'estructura després de 24 hores. El tractament alternatiu va ser significativament ($P < 0,05$) més eficaç que el convencional, però cap d'ells va erradicar completament el microorganisme de les superfícies avaluades (amb precondicionament o sense). Es va trobar regeneració dels biofilms, aconseguint-se recomptes cel·lulars similars als controls (*i.e.* 6,0 Log UFC/cm^2 aproximadament).

Finalment, es va fer un estudi per a la determinació de la contaminació microbiana de diferents superfícies industrials d'una indústria càrnica, mitjançant la implantació d'un sistema de mostreig a base de sensors. Posteriorment, es va aplicar un tractament antibiofilm durant diverses setmanes per conèixer l'impacte de reducció dels diferents grups microbians, centrant-se en la detecció de *L. monocytogenes* després de l'aplicació del tractament. Els resultats obtinguts van mostrar dues agrupacions principals de zones amb major i menor grau de contaminació microbiològica, sent els recomptes en microorganismes aerobis totals el grup microbià amb més contribució. Es va detectar la presència de *L. monocytogenes* en cinc superfícies diferents al llarg del mostreig. El tractament antibiofilm aplicat va demostrar una reducció en tots els grups microbians avaluats durant les setmanes en què es va implementar, en comparació amb aquelles setmanes en què no es va aplicar cap tractament disruptiu.

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CHAPTER 1

Statement of the problem, objectives and working plan

Bacteria's ability to adhere to industrial surfaces and subsequently trigger the formation of biofilms has significant implications for the food industry, especially due to its consequences, both at the public health and economic levels (Khelissa et al., 2017; Vogeleer et al., 2014). Biofilms are described as microbial aggregations that grow on surfaces excreting different substances, such as polysaccharides, proteins or extracellular DNA, among others, which are included in an extracellular matrix (Donlan, 2002; Donlan & Costerton, 2002) . These structures allow pathogens to remain in the food industry for long periods of time, allowing some strains to adapt to harsh environments and develop different resistance mechanisms, thus increasing the chances of cross-contamination of food (Fox et al., 2011). The generated matrix confers protection to microorganisms against environmental factors such as desiccation, UV rays, salinity and treatments with disinfectant agents, which is why they are particularly difficult to eradicate (Speranza et al., 2016).

A microorganism that presents a relevant risk to public health, due to its high mortality rate among vulnerable population such as people with weak immune systems or the elderly, and its relationship with abortions, is *Listeria monocytogenes* (Mateus et al., 2013). This pathogen is capable of adhering, colonizing and forming biofilms on different surfaces used in the food industry, persisting for years in food industries, and causing cross-contamination between surfaces and food products (Camargo et al., 2017). The presence of *L. monocytogenes* in the food processing environment is challenging as it is one of the factors associated with foodborne diseases outbreaks.

An important point for the prevention of food microbiologically contaminated is maintaining high hygiene standards in the food processing environment, especially in areas such as surfaces, equipment and facilities (Spanu & Jordan, 2020). For this reason, cleaning and disinfection procedures are an important part of the prerequisites within the HACCP system. Keeping this system up-to-date, together with the continuous evaluation of biofilms presence in food processing plants, could help to provide clearer information on microbiological contamination as well as to develop biofilm-free processing systems. In this sense, the surveillance and control of cleaning and disinfection procedures, using traditional and/or rapid techniques, is the only way to verify their effectiveness, as well as to ensure adequate hygienic conditions for the food processing and handling (Bloomfield et al., 2017; Ripolles-Avila, Martínez-Garcia, et al., 2020). For that purpose,

researchers are focusing on finding innovative and effective methods to inhibit and control biofilm formation and development.

The development of experimental systems that effectively simulate the conditions of the food processing environment can certainly make a great contribution to the study of biofilms. For this reason, forming mature biofilms is considered crucial to understand how to reproduce and eliminate them, not only *in vitro*, but also in real conditions. Historically, there has been no treatment or chemical product that can ensure the complete elimination of *L. monocytogenes* biofilms in the food industry surfaces (Rodríguez-López et al., 2018). Therefore, different approaches have been employed, such as physical, chemical, and biological methods, which have major disadvantages, including low efficiency, inefficient contact of the surface with the adequate temperature, cost, safety, and regulatory issues. At this point, the search for new alternatives for their control and eradication, as well as the critical evaluation of treatments' effectiveness considering the conditions found in processing environments, is established as a real need for the food industry.

The specific objectives established within the present doctoral thesis were:

- To eliminate biofilms of distinct *L. monocytogenes* strains by the application of different strategies using *in vitro* models and conditions that can influence treatments effectivity.
- To evaluate the microbiological contamination of industrial surfaces, with a special approach to the detection of *L. monocytogenes*, and to apply an anti-biofilm treatment in real industrial conditions.

The specific objectives established within the present doctoral thesis were:

a) To conduct a critical literature review to comprehend whether the cleaning and disinfection systems applied nowadays in the food industry are effective for the elimination of *L. monocytogenes* biofilms, including therefore the understanding of biofilm formation and their involvement in cross-contamination in different types of food industries.

b) To protocolize the *in vitro* formation of biofilms formed by distinct *L. monocytogenes* strains, considering different conditions to simulate real food processing environments.
c) To adjust TEMPO as the quantitative method for determining biofilm cell content and treatments effectivity.
d) To adjust the Direct Epifluorescence Microscopy (DEM) as a methodology for the qualitative observation of biofilm structure consolidation and detachment/dispersion after treatments.
e) To evaluate the efficacy of different treatments used for the removal of mature *L. monocytogenes* biofilms, including conventional and alternative procedures.
f) To determine if combined treatments are an alternative to eliminate mature biofilms formed by different *L. monocytogenes* strains.
g) To adjust a membrane filtration method for understanding the biocidal capacity of the products applied.
h) To determine the cross-contamination transference from surfaces contaminated with *L. monocytogenes* biofilms to a food model system, after the application or not of different cleaning treatments.
i) To evaluate the effect of treatments on the removal of *L. monocytogenes* biofilms formed on preconditioned and non-preconditioned surfaces.
j) To investigate the regeneration capacity of cells conforming biofilms after the application of elimination treatments.
k) To evaluate the microbiological state of different surfaces of a meat industry and to potentially link their relation with the presence of *L. monocytogenes*.
l) To determine the effectivity of an antibiofilm treatment applied on the meat processing industry under study and the relation with *L. monocytogenes* presence.

To achieve all these specific objectives, the following studies, ordered by chapters, were conducted:

1. *Listeria monocytogenes* biofilms in the food industry: Is the current hygiene program sufficient to combat the persistence of the pathogen? (Chapter 2)
2. Quantitative and qualitative study on the effect of an enzymatic treatment on the removal of mature *Listeria monocytogenes* biofilms. (Chapter 3)

3. Conventional and alternative cleaning solutions to remove *Listeria monocytogenes* biofilms from stainless steel surfaces. (Chapter 4)
4. Chlorinated alkaline and enzymatic detergents for the removal of mature *Listeria monocytogenes* biofilms and cross-contamination to chicken broth. (Chapter 5)
5. Elimination of mature *Listeria monocytogenes* biofilms formed on preconditioned and non-preconditioned surfaces and structure regeneration after treatments application. (Chapter 6)
6. Evaluation of microbial counts of different surfaces of an Iberian pig processing plant and the application an antibiofilm treatment to control the contamination. (Chapter 7)

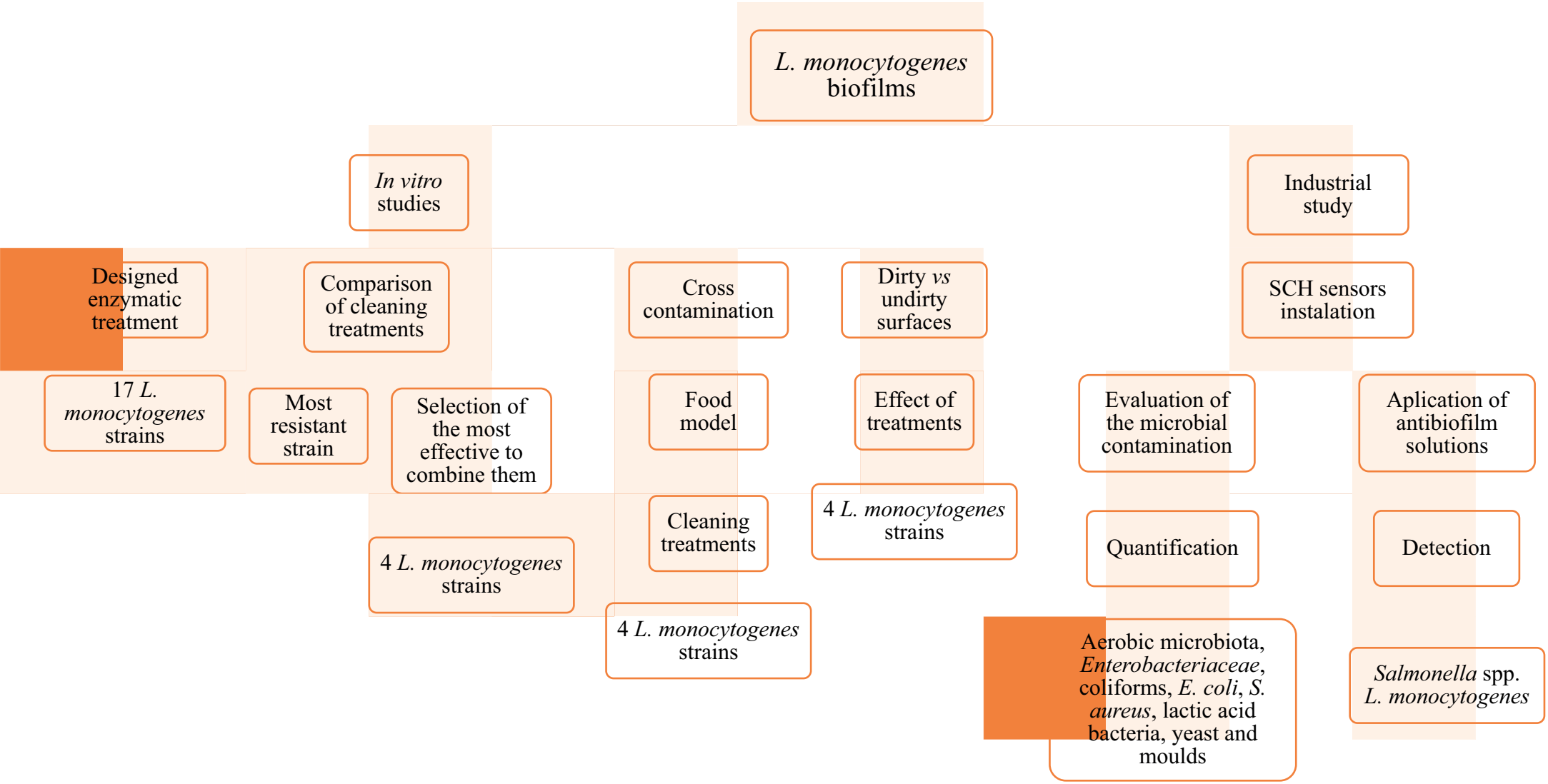

Figure 1. Diagram of research project plan followed through the study.

CHAPTER 2

Literature review

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1. Introduction

Foodborne diseases occur due to the ingestion of food contaminated by biological or chemical agents, consequently causing social, economic, and public health problems (Espinosa et al., 2014; WHO, 2022). In the latest report on the burden of foodborne diseases, it was estimated that 1 in 10 people in the world become ill after ingesting contaminated food and approximately 420,000 people die each year (WHO, 2015), a fact that requires the implementation of rigorous strategic prevention systems, control measures, and surveillance. For all these repercussions, which directly threaten public health and the world economy, it is important to invest in technologies that contribute to preventing foodborne diseases from occurring or to the early detection of threats in terms of food safety (Hoelzer et al., 2018). One of the most important prevention tools is the effective application of cleaning and disinfection methods to guarantee food safety (González-Rivas et al., 2018). In this regard, there is a high risk of contamination associated with a lack of procedures to ensure surfaces with a zero load of pathogenic microbiota, given the high probability that the product will come into contact with these areas during the handling process (Ripolles-Avila, Hascoët, et al., 2019). Among the most relevant pathogenic microorganisms at a European level, *Listeria monocytogenes* stands out for its high mortality rate of up to 15.6 % (EFSA-ECDC, 2019b). In recent years, a significantly increasing trend in the number of confirmed cases of *L. monocytogenes* in humans has been observed in the EU, up from 1883 confirmed cases in 2013 to 2549 in 2018, representing a notification rate of 0.47 cases per 100,000 inhabitants (EFSA-ECDC, 2019b). Furthermore, Dewey-Mattia et al., (2018) indicate the relevance of this pathogen, since it is one of the etiologic agents most involved in hospitalizations and deaths in the USA. Therefore, the control of this pathogen has become one of the targets of greatest interest to the food industry (Silva et al., 2020). Biofilms of *L. monocytogenes* on food contact surfaces have been identified as an important pathway for pathogenic persistence and subsequent product contamination (Nowak et al., 2017; Pažin et al., 2018; Rodríguez-Campos et al., 2019). To this effect, in-depth study of the nature, formation, detection, and elimination of biofilms on surfaces is of great importance due to their impact as a risk factor on outbreaks of foodborne diseases that affect public health (Jahid & Ha, 2012; Ripolles-Avila, Ríos-Castillo, & Rodríguez-Jerez, 2018). The complex nature of biofilms and the capacity of the cells that compose them to strongly fix on surfaces that are difficult to access make the action of the disinfectants that are currently

being applied less effective (González-Rivas et al., 2018). Another factor to consider is the ability of pathogenic microorganisms to generate resistance to current antimicrobial agents when cells form biofilms (Martínez-Suárez et al., 2016). For all these reasons, it must be considered whether the current cleaning and disinfection procedures applied in food industries of different types are effective, or whether new methodologies or strategies are needed to solve the problem.

The objective of the present study was to determine, by means of a literature survey, whether the cleaning and disinfection systems applied nowadays in the food industry are effective for the elimination of *L. monocytogenes* biofilms. To do so, biofilm formation and its involvement in cross-contamination will be discussed to further evaluate the cleaning and disinfection treatments applied in different types of food industries.

2. Importance of cross-contamination

Over time, inadequate food handling or cooking procedures, breakage of the cold chain, and cross-contamination have been identified as the main drivers of foodborne illnesses (WHO, 2006). Among these factors, cross-contamination has been highly involved in recent years, accounting for up to 91.7 % of cases (Londero et al., 2019). Microbial cross-contamination is the transfer of any microorganism from a contaminated biotic or abiotic matrix to an uncontaminated food product (Pérez-Rodrıíguez et al., 2008). This contamination can happen during any stage of food processing, making the application of control and prevention systems with a global perspective crucial in the food industry (Finn et al., 2013). While different raw and processed foods, such as inadequately pasteurized milk and ready-to-eat (RTE) products containing meat, eggs, and fish, have been identified as major sources of *L. monocytogenes* contamination (EFSA-ECDC, 2012), other food products have also been linked with the pathogen due to cross-contamination from industrial surfaces (**Table 1**). This is related to the ability of *L. monocytogenes* to adhere to and subsequently form biofilms on different food-processing equipment (Doijad et al., 2015; Melo et al., 2015). In this regard, equipment such slicing and grinder machines, cutting boards, knives, and tables have been identified as nutritive areas where the pathogen can easily grow due to being difficult to clean and disinfect (Møretrø & Langsrud, 2004). Moreover, other industrial surfaces such as floors and sinks have been highlighted as potential focuses of this pathogen and as an initial route point for *L.*

monocytogenes transfer to other surfaces (Ripolles-Avila, Hascoët, et al., 2019). It has been demonstrated that this foodborne pathogen can survive for long periods on industrial surfaces and can be transferred to food products, thus compromising its innocuity (Finn et al., 2013; Magalhães et al., 2016).

Table 1. Involvement of the environment and the industrial surfaces on the cross-contamination of food-products by *Listeria monocytogenes*.

Implicated food product	Type of industry	Country	Surface[x]	Reference
Pasteurized milk cheese	Cheese retailers and cheese processing plant	Canada	Knives, cutting boards, counters, cheese plates, packers, refrigerator handles, brine solution.	Gaulin et al., (2012)
Raw and cooked meat of blue crab	Meat processing plants	USA	Floor drain, raw crab cooler, receiving dock, gloves, table.	Pagadala et al., (2012)
Whole whitefish, whole salmon and salmon fillet	Smoked fish processing plant	USA	Floors, drains, cutting table, fork truck bars, carts, coolers, trash can, slicer.	Pagadala et al., (2012)
Cantaloupe	Cantaloupe farm and processing plant	USA	Cooler, truck, downstream equipment.	McCollum et al., (2013)
Ice cream	Ice cream facilities	USA	Floor, drain.	CDC, (2015)
Pecorino Romano PDO and ricotta salata cheese made from pasteurised or thermised sheep milk	Sheep's cheese making plants	Italy	Moulds, filters, floors, drains, tables, conveyor belts, shelves, washing machines.	Spanu et al., (2015)
Ricotta salata made from pasteurized sheep's milk	Semi-finished cheeses processing plant	Italy	Washing machine's brush, manhole, knife, cutting machine, table, floor, trolley shelf.	Acciari et al., (2016)
Raw pork pieces and minced meat samples	Open meat markets	China	Meat mincers, cutting tables and weighing scales.	Luo et al., (2017)
Raw pork	Meat retail market	China	Chopping boards and knives, the inner and outer surfaces of chest freezers, meat mincers, hands of people, floors and walls.	Li et al., (2018)
Chilled roasted pork meat	Minced meat factory	Spain	Oven cart, larding needles.	WHO, (2019)
Plastic-packaged RTE Meatballs	RTE meat production facility	Germany	Conveyor belts, pulleys, freezers, condensate lines or cable ducts, gullies.	Lüth et al., (2020)

[x]The type of material of which surfaces were made of was included in the search but was not found on the studies analyzed.

Industrial surfaces, then, are important microbial reservoirs that need to be controlled to avoid cross-contamination. There are different determining factors that can influence this phenomenon, such as the fact that when surfaces are dry the risk of cross-contamination occurring is reduced since the growth and survivability of bacteria decreases. However, cross-contamination can be enhanced when surfaces are wet (Carrasco et al., 2012). There are bacteria capable of withstanding prolonged dry conditions on surfaces (Ríos-Castillo

et al., 2020) . Different studies show that there are pathogens that remain viable on dry stainless-steel surfaces for long periods of time, depending on different factors such as the characteristics of a microorganism, the levels of contamination, and its surrounding environment (Fuster-valls et al., 2008; Ríos-Castillo et al., 2020). The persistence and resistance of pathogens such as *L. monocytogenes* to extreme environmental conditions is directly related to the ability of microorganisms to form biofilm (Lee et al., 2019).

3. Biofilms

Biofilms are defined as complex microbial communities, irreversibly attached to a biotic or abiotic surface and embedded in an extracellular component matrix (ECM) which exhibits an altered phenotype in relation to the rate of growth and gene transcription (Ripolles-Avila, Hascoët, et al., 2018). The ability of microorganisms to form biofilms is an adaptive and resistance strategy, which allows them to increase the availability of nutrients for their growth, facilitates the use of water, enables the transfer of genetic material, and what is most worrying for the food industry, gives them resistance to antimicrobial agents (González-Rivas et al., 2018). Consequently, routine cleaning and disinfection operations are often ineffective to remove and eliminate the microorganisms that make up these structures (Møretrø et al., 2013). Furthermore, they have been shown to be more resistant to high temperatures, low pH (Castro-Rosas & Escartín, 2005), desiccation, UV rays, and salinity, thus increasing their difficulty to be controlled (Speranza et al., 2016). This resistance facilitates the persistence of the microbial cells that make up the biofilms on food contact surfaces and equipment, constituting a continuous source of contamination (Yin et al., 2019). Hence, it is understandable how, according to the National Institute of Health (NIH) and the Center for Disease Control (CDC), biofilms are involved in more than 65 % of foodborne diseases. It is therefore important that, to increase their effectiveness, cleaning and disinfection procedures are designed according to the type of problem that is detected.

3.1. Initial attachment and development

Biofilm formation is a dynamic process that takes place sequentially and includes five main stages. Initial adhesion is the first stage of the biofilm formation process, and is a reversible and weak type of adhesion where planktonic microbial cells adhere to a surface

using physical forces and/or appendages such as pili, fimbria, or flagella (González-Rivas et al., 2018). The type of surface, the temperature, and the pressure can all modulate this adhesion phenomenon. The electrical charge of the cell surface, the Van der Waals forces, the hydrophobicity of the surface, the steric interactions, and electrostatic are also involved in this process (Tribedi & Sil, 2013). At this stage, adhesion is reversible until the microorganisms differentiate by triggering morphological changes. Cells can still detach and return to planktonic form when they are in the reversible adhesion phase (Stoodley et al., 2002). Different covalent and hydrophobic interactions occur (Kumar & Anand, 1998) during irreversible adhesion, the second stage of the biofilm formation process, which is when the cells permanently adhere to each other and the surface (Chmielewski & Frank, 2003). Fixation occurs due to the action of different microbial appendages (Donlan, 2002) and by ECM secretion (Flemming et al., 2007). In the third stage, the simultaneous production of ECM together with the accumulation and growth of attached microorganisms leads to the formation of microcolonies, where the bond between bacteria and the substrate is strengthened and the microbial colony stabilized (Donlan, 2002). Such accumulation stimulates the recruitment of planktonic cells from the surrounding environment through cell-to-cell communication, also named quorum sensing (Chmielewski & Frank, 2003). The fourth stage is when there is a mature biofilm due to the development of a highly organized ecosystem and a three-dimensional structure, which can be flat or mushroom-shaped (Chmielewski & Frank, 2003). During maturation, biofilms develop a rigid structure by means of the cellular production of extracellular polymeric substances (EPS) (Bogino et al., 2013). When using in vitro models to study pathogenic biofilms, it can take between seven (Ripolles-Avila, Hascoët, et al., 2018) and ten days or more (Stoodley et al., 2002) to obtain structural maturity, depending on the microorganism and the environmental conditions established. Biofilm maturation is reached when these structures are crossed by water channels or pores, which ensure both the exchange of nutrients and metabolites and eliminate bacterial waste (González-Rivas et al., 2018). It has been indicated that when evaluating the effectivity of a cleaning and disinfecting treatment on biofilms it is preferable to use in vitro models that reproduce the structures in this mature stage as this is when they present most resistance and so the results can be more representative of the industrial reality (Ripolles-Avila, Hascoët, et al., 2019; Ripolles-Avila, Ramos-Rubio, et al., 2020). Lastly, the fifth stage is related to dispersion, where sessile cells can return to their planktonic forms and transfer to the environment, once again able to colonize new surfaces (Sauer et al., 2002).

Detachment may be due to low nutrient conditions as a survival mechanism and may be genetically determined. Dispersal is important for microorganisms to escape unfavorable habitats and generate new niches (Percival et al., 2011).

In the specific case of *L. monocytogenes*, flagella play a predominant role, at least in the early stages of biofilm formation. In this regard, temperature regulates flagellation of *L. monocytogene*s cells (Todhanakasem & Young, 2008), so this is a factor that influences the process. The effect of temperature on *L. monocytogenes* biofilm formation has been extensively investigated, demonstrating that the pathogen is flagellated and motile at temperatures ≤ 30°C, and generally not flagellated and not motile at temperatures above 30°C (Gründling et al., 2004). Although flagellum-mediated attachment is a proven fact of initiation in biofilm formation (Lemon et al., 2007), *L. monocytogenes* can adhere to inert surfaces through a process of passive independent binding of flagella (Tresse et al., 2009). Tresse et al., (2007), have also reported a pH dependence for flagellation of *L. monocytogenes* and its consequences for cell adhesion. It has been shown that not all variables influence biofilm development to the same degree. For example, Poimenidou et al., (2016), determined that the impact of nutrient availability on *L. monocytogenes* biofilm formation on stainless steel surfaces is greater than the influence of temperature. Once *L. monocytogenes* is irreversibly attached to the surface its cell mobility and autolytic capacity is reduced, a phenotypic variation that has been indicated to enhance the ability of this opportunistic pathogen to colonize environments (Monk et al., 2004)

3.2. Extracellular component matrix (ECM)

ECM is highly hydrated since it incorporates large amounts of water within its structure, reaching up to 97 % of the whole biofilm matrix (Donlan, 2002) . In most biofilms, the microbial count represents less than 10 %, while the matrix can represent more than 90 % (Colagiorgi et al., 2016). The ECM gives biofilms their mechanical stability, mediates their adhesion to surfaces, and forms a cohesive three-dimensional polymeric network that temporarily interconnects and immobilizes biofilm cells (Flemming & Wingender, 2010). Due to the retention of extracellular enzymes, a versatile external digestive system is generated, sequestering dissolved nutrients from the aqueous phase and allowing them to be used as sources of nutrients and energy (González-Rivas et al., 2018). The matrix also acts as a recycling center, keeping all lysed cell components available. This includes

DNA, which can represent a gene pool for horizontal gene transfer (Flemming and Wingender, 2010). ECM can also serve as a source of nutrients, although some components of ECM are slowly biodegradable, and in fact complete degradation of all of their components requires a wide range of enzymes due to their complexity (Mazaheri et al., 2020). The matrix generally protects microorganisms against desiccation, oxidizing or charged biocides, some antibiotics, metal cations, and ultraviolet radiation (Flemming & Wingender, 2010). Among the components that make up the ECM, there are mainly polysaccharides, proteins, and eDNA (Jahid & Ha, 2012), in addition to various products from bacterial lysis in smaller quantities (Branda et al., 2005).

Polysaccharides are part of the extracellular matrix and perform various essential functions for the formation of biofilms, generally those associated with adhesion to surfaces and maintenance of structural integrity (Ryder et al., 2007). Furthermore, the proteins present in the extracellular matrix have functions that allow the growth of the biofilm and the survival of the housed cells through access to nutrients or the regulation of the integrity and stability of the biofilms. Proteins are involved in the formation and stabilization of the matrix polysaccharide network and constitute a link between the bacterial surface and the most glucidic components (Lasa & Penadés, 2006). Lastly, the eDNA is also an integral part of the biofilm matrix. It acts as an intercellular connector, as a surface adhesive, or even as an antimicrobial, causing cell lysis by chelating lipopolysaccharide stabilizing cations and the bacterial outer membrane (Whitchurch et al., 2002). The matrix of *L. monocytogenes* biofilms is mainly composed of proteins (Combrouse et al., 2013; Ripolles-Avila, Hascoët, et al., 2018). In fact, treating biofilms of *L. monocytogenes* with proteases has been shown to damage the development of these structures or to cause cell dispersion (Longhi et al., 2008). Regarding its content in polysaccharides, Brauge et al., (2016), demonstrate that Teichoic acids are the main components of the matrix. Colagiorgi et al., (2016) demonstrate that eDNA is a relevant structural component in the *L. monocytogenes* matrix, where it cooperates with polysaccharides and proteins, guaranteeing the integrity of the biofilm (Colagiorgi et al., 2016). Investigating the composition of these structures in macromolecules is of real importance since knowledge of them leads to the development of new alternative strategies for their elimination.

3.3. Mechanisms of resistance

The resistance acquired by the cells that conform the biofilms is attributed to the properties associated with the biofilm, which include reduced diffusion, physiological changes due to reduced growth rates, and the production of enzymes that degrade antimicrobial substances (Kumar & Anand, 1998). It is difficult to establish a single mechanism as the cause of resistance as, in fact, this is given by a combination of many of them. There are studies where it has been observed that disinfectants such as peracetic acid, mercuric chloride, and formaldehyde have been shown to have no effect on certain microorganisms when they are in the form of biofilms (Kumar & Anand, 1998). The explanation for the reduced efficacy of these agents against these communities is the incomplete penetration of the biocides through the matrix. It has also been determined that exposure of microorganisms to subinhibitory concentrations of quaternary ammonium compounds (QAC), which can happen when they are in a biofilm form, can lead to the selection of resistant microorganisms that can survive subsequent disinfection treatments applied with higher concentrations of the same compounds (Martínez-Suárez et al., 2016; Tezel & Pavlostathis, 2015). To this effect, Chambless et al., (2006) propose four possible mechanisms of resistance to biocidal agents of the cells present in the biofilms: (i) difficulty of biocides to penetrate into external areas of the biofilm; (ii) generation of a stress response by some microorganisms; (iii) alteration of the biofilm environment in response; and (iv) microbial resistance by phenotypic differentiation.

In the case of *L. monocytogenes*, it has been determined that the persistence of certain strains, even after cleaning and disinfection, may be related to subinhibitory exposure to disinfectants. This phenomenon can be explained not only by the acquisition of resistance mechanisms by *L. monocytogenes*, but also by the existence of niches or reservoirs in the environment not reached by disinfectants, and by the formation of biofilms and the consequent creation of protected microenvironments (Martínez-Suárez et al., 2016). For example, genotypic resistance to QAC by this pathogen has been described. Multiple reflux pumps have been characterized that confer some resistance to QACs. However, as previously commented, the dilution or inactivation of QACs in the environment due to an erroneous cleaning and disinfection protocol also has an influence. This resistance to QAC can end up contributing to its adaptation and environmental persistence (Møretrø & Langsrud, 2017).

4. *Listeria monocytogenes*

4.1. Generalities and characteristics

L. monocytogenes is a ubiquitous bacterium which has been isolated from soil, plants, silage, and water, particularly from food processing environments and especially in refrigerated premises, despite them being routinely cleaned and disinfected Ferreira et al., (2014), hence, they are responsible for numerous food outbreaks (Freitag et al., 2009). The pathogen is also a transitory resident of the intestinal tract in humans, with 2–10 % of the general population carrying the microorganism with no apparent health consequences (Buchanan et al., 2017). Its entry into food processing plants can occur through many different routes, from raw materials to contact with contaminated surfaces on equipment or generally in the facilities (Møretrø & Langsrud, 2004). *L. monocytogenes* is a rod-shaped, Gram-positive, catalase positive, facultative anaerobic, non-sporulating, psychotrophic mesophilic pathogen (Wilks et al., 2006). Its ability to survive temperatures between −0.4 to 50°C, pH from 4.6–9.5, low water activity up to 0.92, and high concentrations of salt (10–2 %) and sugar (39.4 % sucrose) Gandhi & Chikindas, (2007;) and Liu, (2006), contribute to its persistence in food processing environments, which implies a permanent risk of crosscontamination of products (Giaouris et al., 2015). Up to now, at least 13 distinct *L. monocytogenes* serotypes have been identified, although only serotypes 1/2a, 1/2b, 1/2c and 4b have been involved in 98 % of human listeriosis cases worldwide (Francisque et al., 2011). These 13 serotypes are grouped into 4 different lineages (I, II, III and IV), defined using molecular typing methods such as pulsed field gel electrophoresis (PFGE) (Orsi et al., 2011). It has been observed that different serotypes can generate different population structures and may have different abilities to combat environmental stress (Dunn et al., 2009). Regarding incidence, the majority of listeriosis cases are caused by *L. monocytogenes* strains belonging to serotypes 1/2a, 1/2b, and 4b, and to a lesser extent, 1/2c. Interestingly, isolates of serotype 1/2a are highly prevalent in food processing settings, compared to isolates of serotype 4b (Ortiz et al., 2010).

4.2. Recent food-related crises

L. monocytogenes causes a foodborne illness named listeriosis, which primarily affects pregnant women, neonates, the elderly, and immunocompromised individuals (Carpentier

& Cerf, 2011). Although *L. monocytogenes* is responsible for only 1 % of foodborne illness, its mortality rate is high, far exceeding that of other foodborne pathogens (Gómez et al., 2014). The first listeriosis food crisis occurred in Canada and was associated with the consumption of contaminated cabbage salad (Schlech et al., 1983). Since then, there have been various food crises related to this foodborne pathogen, standing out among them the largest listeriosis outbreak ever documented, which occurred in South Africa. Between January 2017 and May 2018, there were 1034 laboratory-confirmed cases of listeriosis, more than 400 (42 %) cases in newborns, and 204 associated deaths. The case-fatality rate in South Africa was estimated at 28.6 % and was comparable to other reported outbreaks of listeriosis worldwide (Salama et al., 2018). In 2018, 2549 confirmed cases of listeriosis in humans were reported in the EU. There has been a statistically significant upward trend in confirmed cases of listeriosis in the EU for the period 2012–2018, with a case fatality in the EU of 15.6 % (EFSA-ECDC, 2019b). Focusing on the crises caused by this pathogen in the EU in recent years, in 2018 an outbreak of listeriosis in a Hungarian factory was reported, linked to the production of frozen vegetables and affecting seven countries and a total of 47 people. This factory produced and exported to more than 100 countries (EFSA-ECDC, 2018b). Consequently, European Food Safety Authority (EFSA) recently published an evaluation of the risk posed by *L. monocytogenes* during the processing of frozen fruits and vegetables to enhance its control and avoid subsequent crises (Chemaly et al., 2020). Furthermore, the pathogen has been highly related to processed meat outbreaks (Gelbíčová et al., 2018). On this regard, in August 2019, the most important listeriosis outbreak in the history of Spain was recorded, affecting over 200 people, of whom three died and five cases resulted in miscarriages (WHO, 2019).

4.3. L. monocytogenes and its affinity for materials

L. monocytogenes has the ability to adhere to and from biofilms on industrial surfaces, especially where food residues accumulate (Colagiorgi et al., 2017). As previously discussed, this is a mechanism of potential resistance to antimicrobial agents, biocides, and heat (Cloete, 2003). The resistance of bound bacteria to biocides has been mainly associated with mechanical protection due to the synthesis of ECM and the surrounding nutrients, or with intrinsic physiological factors such as the adaptation of biofilm cells to stresses like acid, oxidative stress, and starvation, among many others (Pan et al., 2006).

A series of studies on materials commonly used in food facilities and premises have demonstrated the presence of *L. monocytogenes* Møretrø & Langsrud (2004), showing its capacity to adhere to and develop on polystyrene as a material employed to construct drains (Ripolles-Avila, Cervantes-Huaman, et al., 2019); polytetrafluoroethylene (PTFE) used in conveyor belts Chavant et al., (2002); stainless steel used for the majority of the equipment employed in the food industry Ripolles-Avila, Hascoët, et al., (2019); polyester used as a floor sealer Blackman & Frank, (1996); and rubber used in joints and glass Borucki et al., (2003), or glass and Teflon (Renier et al., 2011) . However, the degree to which *L. monocytogenes* adheres to these materials differs depending on each type.

5. Control strategies implemented in the food industry

Cleaning and disinfection are an essential part of the Hazard Analysis and Critical Control Points (HACCP) system. Auditing is also an important factor, guaranteeing a reduction in the risk and increasing food safety to provide a safe environment for the manufactured food products in the food industry (Fraqueza & Barreto, 2014; Holah et al., 2014). Sanitation programs have a different objective in food processing environments, among which the following stand out: the removal of visible soil (*i.e.* organic or inorganic) and allergens, which would be detrimental to the safety or organoleptic quality of subsequent production runs; and the elimination of microorganisms that may cause an alteration of the organoleptic characteristics or can pose a risk to public health (Holah et al., 2014).

5.1. Cleaning

In food processing industries, cleaning is based on the removal of residues and harmful microorganisms such as *L. monocytogenes* to protect food from contamination from surfaces, employing physical or chemical methods (Guerrero-Navarro et al., 2019; Holah & Childs, 2019). Another objective of the cleaning program is to ensure a clean environment for employees, and to prepare equipment and other industrial surfaces in the food area which are difficult to clean, with the aim of extending the product shelf-life and preventing future damage (Hofmann et al., 2018; Holah 2018). Effective cleaning must break or damage extracellular matrices of biofilms, so that later disinfectants can access the microbial viable cells (Simões et al., 2006). Cleaning programs are defined according to the type of dirt present and the type of food processing environment produced. This

operation is carried out by applying detergents, which are selected based on the type of product to being processed, the type of residue it generates, and the physic-chemical properties of the surfaces being cleaned (Troller, 1993). Knowing the type of dirt to be removed allows products, systems, and conditions to be chosen to optimize cleaning processes.

Cleaning products must have three important characteristics: chelating power, a degreaser, and a dispersant. The first refers to the ability to sequester minerals, preventing them from crystallizing, precipitating, and embedding in the materials on which it is being applied. The second relates to the ability to emulsify and disperse fats, and the third is the ability to break down dirt particles and keep them in suspension (Fontecha-Umaña, 2014). During the cleaning process, a proportion of the microorganisms present can be detached from food contact surfaces. However, some can be non-detached and if water and nutrients are present, during a period of time some microorganisms can adhere to the other surfaces to re-start the cycle of biofilm production. Subsequent disinfection must therefore be applied to remove all foodborne pathogens (Gram et al., 2007).

5.2. Disinfection

Disinfection is the procedure to eliminate the microorganisms completely or to reduce their number to an acceptable level using antimicrobial products, chemical agents, or thermal methodologies(Mcentire, 2018; Simões et al., 2010). This is an important step in the sanitization process as the presence of foodborne pathogens such as *L. monocytogenes* in food industries can be extremely harmful for public health (Ferreira et al., 2014; Pricope et al., 2013). Therefore, *L. monocytogenes* biofilms adhered in processing plants possess increased resistance to environmental conditions, making their removal more difficult (Rodríguez-Melcón, Capita, et al., 2019). For this reason, selecting the composition of a disinfectant, particularly the active biocidal substance or a combination of several of them, is also dependent on the extracellular matrix component of biofilms. For example, QACs such as benzalkonium chloride are cationic surfactants that act by disrupting lipid membrane bilayers and are effective against a number of pathogenic microorganisms, especially Gram-positive bacteria (Henriques et al., 2017).

5.3. Complementary alternative strategies

The development of sanitation processes is looking towards alternatives that do not enhance resistance or towards strategies that prevent irreversible adhesion to surfaces and the subsequent development of mature biofilms. The growing negative perception of consumers regarding chemical substances has pushed research towards different environmentally friendly alternatives (Gabriel et al., 2018). Among these are enzymes, bacteriophages, quorum sensing inhibitors, essential oils, and others (Galié et al., 2018). Enzymatic technology allows for a high degree of personalization in surface sanitization, and so depending on the composition of the biofilm matrix formed by the predominant microorganisms on the surfaces, specific strategies can be defined to optimize their effectiveness. However, its use must be optimized due to its high cost, which is achieved by adjusting the optimal temperature and pH conditions (Guerrero-Navarro et al., 2020). This allows the concentration of the enzymes to be reduced to a minimum, thus maintaining their effectiveness. In the case of bacteriophages, their antimicrobial action is specific against prokaryotic cells and harmless to humans, animals, and plants (Galié et al., 2018). However, other authors disagree on its total safety (Gutiérrez et al., 2016). The main limitation of phage treatments is their ability to access and attack bacterial cells within the biofilm due to its structure, which acts as a physical obstacle. However, some phages possess depolymerases, which improve the phage invasion and dispersion process through the biofilm under treatment (Parasion et al., 2014). Quorum sensing is a mechanism for regulating gene expression in response to cell population density. In biofilms, it regulates population density and all metabolic activity. This achieves better adaptation to the environment and greater resistance in hostile environments and disinfection processes (Blana et al., 2016). Its inhibition is therefore a preventive strategy focused on interrupting biofilm formation by controlling the stages of microbial microcolony formation (Coughlan et al., 2016; Ripolles-Avila and Rodríguez-Jerez, 2020). Essential oils are generally recognized as safe by the US Food and Drug Administration (FDA). Several studies have shown that they have strong antimicrobial and anti-biofilm activity against a variety of microorganisms (Cui et al., 2019; Hu et al., 2019). Lastly, another interesting approach to the microbiological control of surfaces is the use of microorganisms that may have the ability to compete with foodborne pathogens and thereby prevent their growth (Ripolles-Avila, Hascoët, et al., 2019). Even more interesting is the use of microbial species belonging to the resident microbiota of the food

industries, which is not only an interesting ecological alternative to explore, but also opens up a field of study with great future prospects (Hascoët et al., 2019).

In the specific case of biofilms formed by *L. monocytogenes*, various investigations have been carried out on complementary alternative strategies for their control. It is known that the composition of a biofilm formed by *L. monocytogenes* is mostly protein Colagiorgi et al., (2016) , which is why protease treatments have been used on surfaces and have been shown to trigger an alteration in the development of biofilms (Nguyen & Burrows, 2014). An example of a bacteriophage used is *Listeria* phage P100, which was produced to eliminate biofilms present in processed meat products and on food contact surfaces in processing industries (Montañez-Izquierdo et al., 2012). Gao et al., (2020) have investigated the anti-biofilm efficacy of Fingered Citron Essential Oil (FCEO) against *L. monocytogenes*. In this study, FCEO was found to exhibit strong anti-biofilm activity, inhibiting biofilm formation, eradicating preformed biofilm, and also causing cell death. Further studies are needed to determine the viability of *L. monocytogenes* in industrial conditions.

5.4. L. monocytogenes in the food industry: Its control

5.4.1. Dairy industry

The presence of *L. monocytogenes* in this type of industry is highly relevant given that the pathogen is able to grow at refrigeration temperature and there are certain processing steps that imply the use of low temperatures to preserve raw materials or processed ones, such as when milk is stored in tanks (4°C). The risk of contaminating dairy products such as cheese by *L. monocytogenes* is directly related to transfer from farm to dairy animals, unhygienic processes, poor pasteurization, and cross-contamination after heat treatment (McIntyre et al., 2015). Within the dairy industry, cheese is considered one of the products most frequently contaminated with *L. monocytogenes* (Aspri et al., 2017). According to Ramaswamy et al., (2007), blue-veined and molded cheeses like Brie, Camembert, Danish Blue, Stilton, and Gorgonzola possess highly nutritive sources and constitute a perfect environment for the growth of this pathogen. Different studies to control *L. monocytogenes* such as Ultra High- Pressure Homogenization (UHPH), pressurized jet water Mucchetti et al., (2008), ozone Morandi et al., (2009), and infrared light Bernini et al., (2015), have demonstrated that, in Gorgonzola rinds, these technologies are effective

in reducing *L. monocytogenes* levels by up to 2–3 Log. Treating these types of products with antimicrobials produced by lactic acid bacteria (LAB) or some yeasts such as bacteriocins, ethanol, and other organic acid has been proposed to prevent *L. monocytogenes* growth if cross-contamination of cheeses occurs (Silva et al., 2018). In this regard, LAB can be considered a non-chemical alternative in dairy products. Furthermore, a recent study conducted by Ripolles-Avila, Hascoët, et al., (2018) shows that *L. monocytogenes* takes seven days to form highly mature biofilms on industrial surfaces when a constant nutrient source is present in the system. This is why a cleaning program is supposed to remove most of the organic matter in food industry areas and the cleaning program should also focus on floors, walls, milking equipment, and difficult to clean areas (Holah et al., 2002). A well implemented cleaning program can help to displace milk deposits, dissolve milk proteins, emulsify fat, and aid the removal of dirt. In (Guerrero-Navarro et al., 2019), two commercial agents were used, one with chemical components and another which was based on a biological solution, the use of enzymes. The results showed that in the dairy industry, enzymatic cleaning agents obtained better results in terms of eliminating organic matter than chemical agents. In this regard, enzymatic products are eco-friendly, therefore not harmful to the environment, and help to reduce wastewater in dairy factories (Rodríguez-López & López-Cabo, 2017).

5.4.2. Meat processing industry

In the meat industry, raw meat and RTE products are considered an important vehicle for *L. monocytogenes* transmission (Fallah et al., 2012; Gohar et al., 2017). In addition to water and handlers, industrial surfaces are an important factor to control and prevent the cross-contamination of meat by *L. monocytogenes* (Hellström, 2011). As has been indicated, for the control of cross-contamination, the most important action is the cleaning and disinfection procedures of industrial surfaces. In this regard, Mazaheri et al., (2020), indicate that enzymatic cleaning treatments could remove mature *L. monocytogenes* isolated from stainless steel surfaces in an Iberian pig processing plant with an effectivity of 85–99 %. In the same line, Ripolles-Avila, Ramos-Rubio, et al., (2020) compare the effectivity of enzymatic treatment and chlorinated alkaline treatment for the elimination of mature *L. monocytogenes* biofilms from strains also isolated from the meat industry. The results showed a significantly higher effectivity of the enzymatic treatment, demonstrating that the inclusion of new perspectives is needed to combat these structures

in the food industry. Furthermore, some authors have begun to indicate that the total elimination of microorganisms present on industrial surfaces may not always be of interest. For example, in meat products such as certain fermented sausages, it may be desirable that LAB such as *Lactobacillus* spp. and *Leuconostoc* spp. remain on the surfaces to improve and facilitate the fermentation process (Møretrø & Langsrud, 2017). In a recent study, Ripolles-Avila, Hascoët, et al., (2019) observe that the complete elimination or a great reduction of the resident microbiota from the surfaces can enhance the growth of pathogens such as *L. monocytogenes*. This may be because this pathogen is a poor competitor and microorganisms such as LAB or aerobic mesophilic may impede its growth (Hascoët et al., 2019). It may also be due to the production of *L. monocytogenes* inhibitory substances by the resident microbiota. In addition, there are authors who demonstrate an interrelation between microorganisms of different species and pathogens such as *L. monocytogenes*, forming multispecies biofilms (Fagerlund et al., 2017; Ripolles-Avila, Hascoët, et al., 2019c).

5.4.3. Fish processing industry

Fish is another of the foods susceptible to *L. monocytogenes* contamination (EFSA-ECDC, 2019a). The microbial contamination of fish and seafood usually happens naturally from the environment during harvesting or occurs during handling and manufacturing in industry (Kleter, 2004; Shikongo-Nambabi et al., 2011). Most foodborne pathogens are not able to grow below 10°C, and the few that do will not grow under 4°C. Hence the risk of contamination in frozen fish is not extreme (Tatterson & Windsor, 2001). However, different microorganisms such as *Aeromonas* spp., *Plesiomonas* spp., *Clostridium botulinum*, *L. monocytogenes* and *Vibrio* spp. are mainly responsible for fish product spoilage or pathogenicity and can survive at chill temperatures (Tatterson & Windsor, 2001; Wekell et al., 1994). The foodborne pathogen *L. monocytogenes* is ubiquitous and has been found in natural environments such as water or food processing environments, hence it can enter into contact with fish or fish products (Møretrø & Langsrud, 2017). Distinct products have been identified as potential sources of *L. monocytogenes* exposure to humans (Holck et al., 2018). One of the products most involved is smoked salmon (Holck et al., 2018). Research on sanitization in fish factories has been carried out to optimize treatments and increase effectivity. According to Holck et al., (2018) and Mcleod et al., (2018), UV light could be an alternative for surface

decontamination given that it causes microbial inactivation through DNA damage. Chlorine and the products that produce chlorine include hydrogen peroxide and quaternary ammonium compound, two of the disinfectants commonly used in seafood plants (Brauge, Faille, et al., 2020; Duong, 2005). Chlorine and the products that produce chlorine include hydrogen peroxide and quaternary ammonium compound, two of the disinfectants commonly used in seafood plants (Brauge, Faille, et al., 2020; Duong, 2005). A recent study by Lasagabaster et al., (2020) proposes the use of bacteriophage as a green strategy to eliminate *L. monocytogenes* biofilms from processing equipment, thus improving seafood safety. Sadekuzzaman et al., (2017) explore treating *L. monocytogenes* with bacteriophage reduced biofilm cells on stainless-steel surfaces and rubber surfaces.

5.4.4. Chilled vegetable industry

As part of the modern lifestyle, consumers search for healthier, easier to prepare food to reduce preparation time. One of these products is frozen vegetables (De Roever, 1998). As previously indicated, *L. monocytogenes* is a psychotropic bacterium that can grow at refrigeration temperaturas and can be present in frozen vegetables (Gandhi & Chikindas, 2007). According to EFSA (Chemaly et al., 2020), in the EU (2015–2018) there was an outbreak of *L. monocytogenes* ST6 related to blanched frozen vegetables in several countries. Evidence of foodborne outbreaks shows that *L. monocytogenes* is the most relevant pathogen associated with this type of product. The food industry commonly uses chlorine compounds such as chlorhexidine and benzalkonium chloride as disinfectants. However, the resistance of some *L. monocytogenes* isolates to these compounds has been described by (Soumet et al., 2005). To this effect, Popowska et al., (2006) analyze a total of 96 identified *L. monocytogenes* strains of frozen foods and dairy products, with the aim of determining their susceptibility to benzalkonium chloride and chlorhexidine. In the case of benzalkonium chloride, 16 % of the strains were characterized by a reduced susceptibility of 2 to 4 times. For chlorhexidine, however, 82 % of the studied strains had a reduced susceptibility to the disinfectant of 2 to 4 times. Furthermore, Godinez-Oviedo et al (2015) characterize *L. monocytogenes* strains isolated from a frozen vegetable processing plant to determine the pathways of contamination of the pathogen. It was determined that the pathogen persistence spaces correspond mainly to those with contact with food, which therefore become an important source of cross contamination.

5.4.5. Ready-to-Eat (RTE) products industry

According to previous study by the FDA and the Food Safety and Inspection Service USDA & FDA, (2003) and Pouillot et al., (2015), some of the physical and chemical characteristics of RTE such as pH and water activity create a suitable environment for *L. monocytogenes* growth. In this regard, Cossu et al., (2016) show that an RTE sandwich factory had a high rate of contamination by *L. monocytogenes*. In this regard, the addition of some of the antimicrobial substances mentioned before could prevent *L. monocytogenes* growth (FDA, 2017). For example, some combination acidic substances such as sorbic acid and benzenic acid could prevent the growth *L. monocytogenes* in deli-type salads (Liewen & Marth, 1985; Vermeulen et al., 2007). The formulation of RTE products including a combination of natural antimicrobial substances has been demonstrated to be effective against *L. monocytogenes* growth during the shelf-life of these products (Scott et al., 2013).

Control of *L. monocytogenes* in RTE meat products, especially high-risk ones such as hot dogs and deli meats, is based on the use of intensive programs of environmental sanitation, thermal processing such as cooking or pasteurizing in the package, and the incorporation of antimicrobial agents as part of the ingredient formulation (*i.e.* nitrites, acetates, citrates, diacetates, lactates, and propionates) and sometimes as surface sprays (*i.e*. lauric arginate and essential oils). The irradiation of RTE products has been widely explored, and the technology has been considered by the FDA to be safe for use in meat and poultry. High hydrostatic pressure has also been evaluated for the control of *L. monocytogenes* in RTE meats with promising safety results, although quality parameters remain compromised. There is also biocontrol, which refers to the use of natural or controlled microorganisms or their antimicrobial products to extend the shelf life or improve the microbiological safety of food. In food, biocontrol is generally carried out by two groups of biological agents: bacteriophages or viruses that specifically infect bacteria and LAB (Chaves & Brashears, 2016). The Smoked Seafood Working Group (SSWG) has developed guidelines to minimize *L. monocytogenes* contamination of smoked seafood products. The SSWG have identified 5 elements required for a complete *Listeria* spp. control program, namely *Listeria* specific Good Manufacturing Practices

and sanitation procedures, employee training, environmental microbiological monitoring and testing, raw material controls, and temperature controls for finished products (Gall et al., 2004). Furthermore, Valenzuela-Martinez (2015) evaluate the use of vinegar against *L. monocytogenes* in RTE and poultry products stored at 4°C. The results showed that the vinegar-treated samples resulted in a growth of < 1 Log CFU/g over the shelf life (120 days). This research provides an alternative to guarantee the food safety of RTE meat products.

CHAPTER 3

Quantitative and qualitative study on the effect of an enzymatic treatment on the removal of mature *Listeria monocytogenes* biofilms

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1. Intoduction

L. monocytogenes is a Gram-positive bacteria responsible for a foodborne disease named listeriosis, considered as one of the deadliest diseases worldwide with a high mortality rate up to 30 % (EFSA-ECDC, 2018b; Mohammad Sadekuzzaman et al., 2017). It is an ubiquitous microorganism capable of contaminating a wide variety of food products when introduced into food-processing environments due to its hardy growth characteristics (Ripolles-Avila, Ríos-Castillo, Guerrero-Navarro, et al., 2018). Within the 13 different serotypes described, serotypes 1/2a, 1/2b and 4b are the most involved in human listeriosis. Serotype 4b strains account for most human cases, whereas most of the *L. monocytogenes* strains isolated from foods or food-processing plants belong to serotype 1/2a (Ortiz et al., 2010). The pathogen has been linked to important outbreaks during the past few years. Examples of these are the ones related to the consumption of ready-to-eat processed products such as chilled roasted pork meat (WHO, 2019), smoked salmon (EFSA-ECDC, 2018b), bologna sausages (Allam et al., 2018), frozen corn EFSA-ECDC, (2018a) or turkey meat (Gelbíčová et al., 2018). Contamination of food products by this pathogen often occur during food processing through cross-contamination from food contact surfaces that are in an unhygienic state (Ripolles-Avila, Cervantes-Huaman, et al., 2019)

The existence of persistent niches of *L. monocytogenes* in different industrial surfaces has been demonstrated, including walls, floors, sinks, conveyor belts and equipment, among many others (Ciccio et al., 2012; Lehto et al., 2011). These persistent bacterial niches are directly related to the presence of biofilms (González-Rivas et al., 2018). Biofilms are microbial aggregations adhered to an inert or living surface and embedded by an extracellular matrix (ECM) able to function as both protective barrier and structural scaffold (González-Machado et al., 2018; Häussler & Parsek, 2010). The ECM can represent more than 90 % of the total dry mass of a biofilm and is formed as a hydrated gel that contains a mixture of multiple compounds such as proteins, polysaccharides, eDNA and other polymeric substances (Flemming & Wingender, 2010). The generated structure makes biofilm-forming cells more easily accessible to nutrients, while being protected from disinfectants since their entry is prevented in the deeper layers (Paul Stoodley et al., 2013). Therefore, microbial cells can be exposed to sublethal biocide doses and acquire resistance to antimicrobials over time (Martínez-Suárez et al., 2016; Rodríguez-Melcón, Capita, et al., 2019). For those reasons, it is crucial to detect these

structures when assessing the hygienic state of the industrial environment and take relevant decisions for cleaning and disinfection procedures to eliminate them (Ripolles-Avila, Ríos-Castillo, & Rodríguez-Jerez, 2018b). Cleaning and disinfection programs in the food industry are based on first removing the non-desired residues such as organic matter, foreign bodies, chemicals and microorganisms (Gibson et al., 1999; Guerrero-Navarro et al., 2019). Second, disinfection is applied to eliminate the microbial contamination of the industrial surfaces and eradicate biofilms (Schlisselberg & Yaron, 2013). It has been reported that conventional cleaning and disinfection treatments are insufficient to eliminate biofilms (González-Rivas et al., 2018). In fact, the potential resistance to disinfectants that cells making up the biofilms can develop due to continued exposure to these antimicrobial agents is considered as a problem, so their continued use and potential inefficiency against biofilm removal justify the search for new strategies for *L. monocytogenes* control (Gray et al., 2018). In this regard, an alternative can be the use of enzymes to disrupt biofilm matrix and release cell content to then further employ disinfectants to eliminate the pathogen (Nahar et al., 2018). On this sense, enzymatic solutions for cleaning operations can be an ecofriendly, greener and safe alternative to be implemented on the food industry to control and remove pathogens (Rodríguez-López & López-Cabo, 2017). In particular, hydrolytic enzymes have been proposed as effective agents to remove biofilms from surfaces (Augustin et al., 2004; Orgaz et al., 2007; Simões et al., 2010). In this regard, Walker et al., (2007) indicated that when selecting enzymes for the cleaning solutions to implement in the food industry, it is important to understand the nature of the ECM. Therefore, the effectivity of each enzyme for microbial biofilm removal will depend on the ECM composition (Walker et al., 2007; Xavier et al., 2005). In addition, it will also depend on the complex microbial communities, the structure thickness and surface materials on where biofilms develop (Skowron et al., 2019). The main objective for this study was to investigate the effect of an enzymatic detergent on the detachment of mature *L. monocytogenes* biofilms, including both, reference and wild strains. To do so, biofilm forming ability and their removal efficacy were evaluated by quantifying the residual cells making up the structure after the application of the treatment, and also by a qualitative observation of the impact of the enzymatic detergent on the dispersal of the cells within the structure.

2. Materials and methods

2.1. Bacterial strains

A total of seventeen *L. monocytogenes* strains were used in this study, which were either collected from the Spanish Type Culture Collection (CECT, Paterna, Spain) or isolated from an Iberian pig processing plant (**Table 2**). Once obtained, the strains were freeze-dried to keep them as stock cultures. Prior to their use, the freeze-dried strains were rehydrated in 10 ml of Tryptic Soy Broth (TSB; Oxoid, Madrid, Spain) and incubated at 30°C for 48 hours. The suspension was then cultured on Tryptic Soy Agar (TSA; Oxid, Madrid, Spain) and incubated at 37°C for 24 hours.

Table 2. *Listeria monocytogenes* isolated strains used in this study.

Strains	Serotype	Origin[a]
5366	4b	CECT
5672	4b	CECT
935	4b	CECT
911	1/2c	CECT
5873	1/2a	CECT
A7	1/2a	López et al., (2008)
P12	1/2a	López et al., (2007)
R6	1/2a	López et al., (2013)
S1(R)	1/2a	Ortiz et al., (2014)
S1(S)	1/2a	Ortiz et al., (2014)
S2–1	1/2a	Ortiz et al., (2014)
S2-bac	1/2a	Ortiz et al., (2014)
4423	1/2a	Ortiz et al., (2016)
CDL69	1/2a	Ortiz et al., (2016)
EGD-e	1/2a	Ortiz et al., (2016)
S2-2	1/2a	Ortiz et al., (2016)
S10-1	2a	Ortiz et al., (2016)

[a]CECT (Spanish Type Culture Collection). From López et al., 2007, 2008, 2013 and Ortiz et al., 2014, 2016. The strains were isolated and collected during different studies in an Iberian pigs processing plant.

2.2 Surfaces

Stainless steel AISI 316 grade 2B coupons (2 cm in diameter and 1 mm thick) were used in this study. The surfaces were cleaned with a detergent (ADIS Hygiene, Madrid, Spain)

for at least 1 hour, washed with water, and disinfected with 70 % isopropanol (Panreac Química, Castellar del Vallès, Spain). The surfaces were subsequently dried in a laminar flow cabinet (PV-30/70, Telstar, Terrasa, Spain) according to European standard UNE-EN 13697:2015 (AENOR, 2015). Finally, surfaces were autoclaved at 121°C for 15 minutes before use to assure complete sterility (Ripolles-Avila, García-Hernández, et al., 2019).

2.3. Biofilm formation

L. monocytogens strains were grown in TSA at 37°C for 24 hours. Various of the colonies obtained in TSA were inoculated into TSYEB $_{\text{gluc 1 \% + NaCl 2 \%}}$, which consisted of TSB enriched with 0.3 % w/v yeast extract (BD, Madrid, Spain), 1 % w/v glucose (Biolife, Madrid, Spain), and 2 % w/v NaCl (Panreac, Castellar del Valles, Spain) until 0.2 MacFarland units were reached, as this is approximetely 10^6 CFU/ml, the microbial concentration established for biofilm formation assays (Ripolles-Avila, Hascoët, et al., 2018). Biofilm formation was initiated by aseptically placing 30 µl of the suspension of the different *L. monocytogenes* strains in the middle of each stainless-steel coupon. The surfaces were then placed in a sterile Petri dish and implanted in a humidity chamber maintained at saturated relative humidity to promote the formation of the structures (Fuster-Valls et al., 2008). The surfaces containing the bacterial suspension for the biofilm formation were incubated at 30°C for a total of a week, with washing and renewal of nutrients established at 48 hours + 24 hours + 24 hours + 72 hours (Ripolles-Avila, Hascoët, et al., 2018). For the nutrients renewal, the stainless steel coupons were first washed twice with 3 ml of sterile distillated water, and 30 µl of sterile TSYEB $_{\text{gluc 1 \% + NaCl 2 \%}}$ was added to the inoculated surfaces to increase *L. monocytogenes* growth and potentiate the formation of these structures. The surfaces were again placed in the humidity chamber to complete the incubation period.

2.4. Cleaning agents and treatment

The product used for the enzymatic cleaning (EnzyJet, iTram Higiene, Vic, Spain) was newly generated each time a treatment was applied. The solution contained ethoxylated sodium lauryl ether glycolate, N-oxide N,N-dimethyl-C12-C14-alkylamine, anionic surfactants (< 5 %), nonionic surfactants (< 5 %), enzymes including proteases (< 5 %),

and phenoxyethanol. A chlorinated alkali product (Kersia Iberica, Navarra, Spain) was used as a conventional cleaning treatment to compare it with the tested enzymatic detergent for the strain with the least removal effectivity. Both products were prepared to obtain a concentration of 1 % by diluting it in hard water following international standard UNE-EN 13697:2015. Hard water was obtained by mixing together 3 ml of solution (A), which consisted of 19.84 g of $MgCl_2$ (Sigma, Madrid, Spain) and 46.24 g of $CaCl_2$ (Sigma, Madrid, Spain) per 1000 ml of distillate water; 4 ml of solution (B), which consisted of 35.02 g $NaHCO_3$ (PanReac Applichem, Madrid, Spain) per 1000 ml of distillate water; and 100 ml of interfering solution, which consisted of 1.5 g of bovine serum albumin (Sigma, Madrid, Spain) per 100 ml of distilled water. All the solutions were then sterilized by membrane filter with (Millex-GP 0.22 μm, Merck, Barcelona, Spain) and mixed with distilled water to obtain a final volume of 500 ml. After the incubation period, the inoculated coupons were washed twice with 3 ml of sterile distilled water to remove unattached cells. The mature biofilm structure was then treated with the enzymatic detergent. To do so, the surfaces were placed on a sterile flask with 3 ml of the enzymatic solution covering the stainless steel and washed for 15 minutes at 50°C in a water bath with agitation. In the case of the chlorinated alkaline treatment, the procedure was the same with the exception of the treatment application which was set at 15 minutes at room temperature (20-22°C), following the instructions for use provided in the technical sheet of the product.

2.5. Biofilm cell recovery

After the applied enzymatic treatment, surfaces were washed with 3 ml of sterile distilled water to remove the residual detergent and placed on sterile flasks 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 positive controls, the inoculated coupons were washed twice with 3 ml of sterile distilled water to remove unattached cells and were further placed on sterile flask with 10 ml of the neutralizer solution. Once all samples (*i.e*. controls and enzymatic treated surfaces) were placed on the flasks, they were vortexed at 40 Hz for 90 seconds with the objective of detaching the remaining adhered cells from surfaces (Ripolles-Avila, Cervantes-Huaman, et al., 2019).

2.6. Quantification of the remaining cells within the biofilms

The TEMPO system (bioMérieux, Marcy l'Etoile, France) was employed to quantify the cells remaining within the structure. The resulting suspension after the microbial recovery was decimally diluted in Tryptone Saline Solution [TSS; 1 g of tryptone and 8.5 g of NaCl per liter in pH (7.0 ± 0.2)] and 1 ml of the corresponding dilution was transferred to a previously hydrated TEMPO vial with 3 ml of sterile distilled water. The vial was then vortexed to homogenize its content, transferred into an enumeration card and incubated at 30°C for 48 hours.

2.7. Qualitative evaluation of structure dispersal

Surfaces were washed with 3 ml of sterile distilled water and stained with 5 µl of Live/Dead BacLight bacterial viability kit (Molecular Probes, Oregon, USA). The surfaces were incubated in darkness at room temperature for 15 minutes. This viability kit uses a mixture of SYTO 9 green-fluorescent nucleic acid stain that shows the viable cells, and propidium iodide (PI) which shows dead or damaged cells in an intense fluorescent red. The stained surfaces were evaluated using 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 stained samples were observed with 20X objective and the obtained images were analyzed using the analySIS Auto 3.2 software (Soft Imaging System, Münster, Germany).

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. The equations used for the different calculations were:

(a) $Reduction = Log\left(\bar{x}\left(\frac{C1+C2+\cdots C9}{n}\right)\right) - Log\left(\bar{x}\left(\frac{T1+T2+\cdots T9}{n}\right)\right)$

(b) $Detcahment\ percentage = \frac{Log\left(\bar{x}\left(\frac{(C1-T1)+(C2-T2)+\cdots(C9-T9)}{n}\right)\right)}{Log\left(\bar{x}\left(\frac{C1+C2+\cdots C9}{n}\right)\right)} * 100$

The data from the experiments were analyzed using One Way ANOVA and Fisher LSD Test with the Statistica 7.0.61.0 statistical software package. A $P < 0.05$ was considered as statistically significant.

3. Results and discussion

3.1. Assessment of L. monocytogenes biofilm-forming capacity

Results showed that the seventeen strains of *L. monocytogenes* were able to adhere and subsequently form biofilms on stainless steel surfaces (**Table 3**), a fact that was in accordance with previous reports (Blackman & Frank, 1996; de Oliveira et al., 2010; Ripolles-Avila, Hascoët, et al., 2018). The biofilm-forming ability of *L. monocytogenes* allows it to persist in food processing environments and subsequently be transferred to food products (Martínez-Suárez et al., 2016). It was observed that some of the strains were better biofilm-formers than others, which was determined by the difference in the microbial counts of the viable cells that formed the biofilms, with a difference of 1.54 Log CFU/cm^2 between the major biofilm producer strain and the weakest one. In this regard, *L. monocytogenes* CDL69 and 5672 were the most producing strains, obtaining counts of between 7.30 ± 0.15 Log CFU/cm^2 and 7.21 ± 0.15 Log CFU/cm^2, respectively. This difference in the biofilm-forming capacity between strains has been reported by other authors such as Ripolles-Avila, Cervantes-Huaman, et al., (2019) and Ripolles-Avila, García-Hernández, et al., (2019), who also indicated that the CECT 5672 strain was the major producer. In contrast, *L. monocytogenes* 5366 was the strain with the lowest biofilm-forming capacity, although its count only differed significantly ($P < 0.05$) from the five strains with the highest biofilm production (*i.e.* CDL69, 5672, S1(R), S1(S) and S2-bac). This result is not in line with (Ripolles-Avila, Cervantes-Huaman, et al., 2019) and Ripolles-Avila, García-Hernández, et al., (2019), who demonstrated that *L. monocytogenes* 911 was the strain that produced less biofilm mass. However, neither strain (*i.e.* 5366 and 911) presented significant differences ($P < 0.05$), either in the present study or in the one mentioned on stainless-steel surfaces. It is important to understand the biofilm-forming capacity of different strains, some of them isolated from industrial environments, since the occurrence of these organized microbial communities in the food industry causes a constant microbial reservoir that constitutes a source of contamination to raw materials and processed food products (Winkelströter & De Martinis, 2015). In this sense, Lunden et al., (2000) showed that *L. monocytogenes* biofilms found sporadically in the food industry were thinner than those formed by strains that persist in food processing environments. This fact coincides with the results of the present study, in which the strains that have a major capacity to form biofilms were obtained from recurring isolates from the food industry. Serotypes are one of the most important tools

for strain differentiation, which is why studies have focused on associating serotypes with biofilm formation capacity (Orsi et al., 2011; Wang et al., 2017; Zoz et al., 2017). However, for this pathogen, the relationship between serotype and biofilm formation remains unsolved (Kadam et al., 2013).

Table 3. *Listeria monocytogenes* biofilm cell count (Log CFU/cm^2) ordered from the major biofilm producer to the lowest one. Values correspond to the mean ± standard error (n = 9).

***L. monocytognes* strains**	**Control**
CDL69	7.30 ± 0.15^{d}
5672	7.21 ± 0.15^{d}
S1(R)	6.85 ± 0.31^{bcd}
S1(S)	6.78 ± 0.21^{bcd}
S2-bac	6.82 ± 0.13^{cd}
EDG-e	6.40 ± 0.32^{abc}
S10-1	6.39 ± 0.28^{abc}
R6	6.33 ± 0.31^{abc}
S2-2	6.23 ± 0.18^{ab}
935	6.13 ± 0.25^{ab}
911	6.01 ± 0.19^{a}
5873	5.98 ± 0.35^{a}
P12	5.98 ± 0.22^{a}
A7	5.87 ± 0.22^{a}
4423	5.85 ± 0.28^{a}
S2-1	5.83 ± 0.39^{a}
5366	5.76 ± 0.26^{a}

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

Table 4 shows the relationship between serotypes and biofilm-forming ability. No significant differences between serotypes and *L. monocytogenes* biofilm-forming ability were found, hence it cannot be concluded whether there is a relationship between the serotype and biofilm-forming capacity. This may be because the distribution of the serotypes analyzed was not homogeneous, since 12 of them came from the food industry where this type of serotype 1/2a dominates (Ripolles-Avila, Cervantes-Huaman, et al., 2019). Nilsson et al., (2011), reported that out of a total of 95 *L. monocytogenes* strains with distinct origins and serotypes, 1/2a strains produced significantly more biofilm than the others, indicating that there is a correlation between biofilm formation and serotype, with 1/2a being the persistent food industry strain. In this case, most of the isolates

employed in the study were from this serotype (*i.e.* 1/2a); however, the relationship with the mature biofilm production model was not proven.

Table 4. Comparison of *Listeria monocytogenes* biofilm production among the four different serotypes employed in the study.

Comparison			Difference of means	*P* value
1/2a	vs	1/2c	0.314	0.270
1/2a	vs	2a	0.069	0.818
2a	vs	1/2c	0.384	0.333
4b	vs	1/2a	0.001	0.999
4b	vs	1/2c	0.314	0.331
4b	vs	2a	0.069	0.836

3.2. Biofilm elimination through the application of an enzymatic treatment

Results showed that biofilms of the different strains of *L. monocytogenes* significantly reduced ($P < 0.05$) their cellular load after the enzymatic treatment was applied (**Table 5**). This fact is in concordance with what has been reported in other studies, where the role of enzymes as dispersants of these structures has been indicated (Chen et al., 2018; Nahar et al., 2018; Puga et al., 2018). The final count of the remaining *L. monocytogenes* cells on the surface ranges from 0.07 Log CFU/cm^2 to 1.02 Log CFU/cm^2 between strains S10–1 and S2-bac, respectively. In fact, it was shown that the *L. monocytogenes* S10-1 strain was significantly ($P < 0.05$) higher compared to the rest of the strains, revealing that the structure it produced was the most sensitive to an enzyme treatment. No correlation was observed between the strains with the highest biofilm-producing capacity and least detachment, and the subsequent reduction of microbial load.

Table 5. Remaining *Listeria monocytogenes* cells within the residual biofilm structure (Log CFU/cm²) after the enzymatic treatment. Values correspond to the mean ± standard error (n = 9).

***L. monocytognes* strains**	**Treatment**
CDL69	0.67 ± 0.34^{ab}
5672	0.30 ± 0.23^{ab}
S1(R)	0.93 ± 0.38^{b}
S1(S)	0.26 ± 0.14^{ab}
S2-bac	2.04 ± 0.66^{c}
EDG-e	0.64 ± 0.22^{ab}
S10-1	0.07 ± 0.07^{a}
R6	0.40 ± 0.29^{ab}
S2-2	0.34 ± 0.22^{ab}
935	0.14 ± 0.09^{ab}
911	0.13 ± 0.13^{ab}
5873	0.21 ± 0.10^{ab}
P12	0.65 ± 0.28^{ab}
A7	0.30 ± 0.30^{ab}
4423	0.74 ± 0.36^{ab}
S2-1	0.65 ± 0.23^{ab}
5366	0.29 ± 0.15^{ab}

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

It has been indicated that the biofilms produced by *L. monocytogenes* mostly have a protein composition (Colagiorgi et al., 2016; Combrouse et al., 2013; Ripolles-Avila, Hascoët, et al., 2018). Hence, a protease treatment could induce cell dispersion (Longhi et al., 2008). It has also been indicated that a proteinase K treatment can prevent the future formation of biofilms (Nguyen & Burrows, 2014), revealing again the importance of proteins in the structure formed by *L. monocytogenes*. The composition of the enzyme product under study had a higher percentage of protease than the rest of the enzymes used, so this could be the reason why it exerted a high dispersant role for most of the strains. Apart from breaking up the matrix, the enzymatic treatment was also shown to reduce the surface cell count. This fact has also been observed by other authors such as Araújo et al., (2017), who demonstrated that a protease treatment causes a greater reduction of biofilm-forming *L. monocytogenes* cells compared to the treatment with lipase, β-glucanase and α-amylase. This result again reveals that the proteins within the biofilm matrix are an essential part of it and that directing the attack on their structures can lead to an increase in the effectiveness of the applied treatment. In another study conducted by Rodríguez-

López & López-Cabo, (2017) it was indicated that a biofilm treatment of *L. monocytogenes* with pronase and benzalkonium obtained reductions of between 3.9 and 6.3 Log CFU/cm^2. If these results and those of the present study are compared, it can be observed that the effectiveness is lower, since the maximum reduction obtained was approximately 6.9 Log CFU/cm^2 for the strain *L. monocytogenes* 5672.

The detachment percentage of the mature biofilms by the different *L. monocytogenes* strains after enzymatic treatment was calculated to visualize the overall effectiveness of the treatment. It was observed that effectiveness ranged between 85 % and 99 %, all high percentages that caused the dispersal of the mature structure and reduced the microbial load (**Figure 2**). Other authors have reported similar effectiveness percentages, indicating a reduction of *L. monocytogenes* viable cell counts in the dispersed biofilms of up to 90 % (Puga et al., 2018).

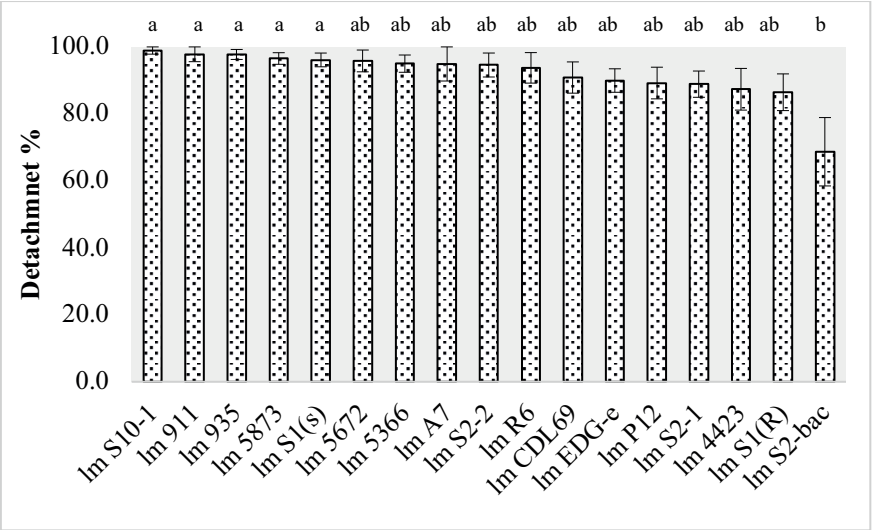

Figure 2. Detachment percentage of different *Listeria monocytogenes* (Lm) strains. Each value corresponds to a mean of three replicates performed on three separate days (n = 9). The error bars represent the standard error of the mean.
a-b indicates significant differences ($P < 0.05$).

The results obtained indicate that the effectiveness of the treatment could depend on the robustness of the matrix generated by the *L. monocytogenes* strains. To this effect, it could be that *L. monocytogenes* S2-bac was the most robust matrix producing strain and S10-1 the weakest. This may be associated with the fact that *L. monocytogenes* S10-1 obtained the lowest viable cell load adhered on the surface, while *L. monocytogenes* S2-bac was

the one that presented a higher cell count after treatment. This could be attributed to the consistency of the protective matrix. Hence, it has been indicated that the effectiveness of the detachment-promoting agents to disperse and eliminate cells conforming biofilms is highly dependent on the production of ECM by microorganisms (Xavier et al., 2005).

3.3. Evaluation of the chlorinated alkaline treatment for the removal of mature S2-bac L. monocytogenes strain

Once the evaluation of the enzymatic treatment effectiveness against the 17 *L. monocytogenes* strains was carried out, an assessment of a commonly used conventional treatment in the food industry (*i.e.* chlorinated alkaline detergent) was performed on the strain whose elimination was demonstrated to be the least affected. This was done with the objective of comparing the effectiveness obtained after the application of an alternative treatment with a conventional one, to observe if by applying this type of treatment in the food industry, it would have a greater impact on the removal of highly structured and robust mature *L. monocytogenes* biofilms. On this regard, S2-bac strain was chosen as a comparison model, since it was the one with the least efficiency after the enzymatic treatment application. **Table 6** shows the results obtained with the different cleaning treatments applied for the removal of S2-bac biofilms. As can be observed, although there are no significant differences ($P > 0.05$) in terms of the logarithmic reduction and percentage of detachment between the applied treatments (*i.e.* enzymatic and chlorinated alkaline), it can be observed how the enzymatic treatment was slightly more effective than the chlorinated alkaline detergent. This is in line with the results reported by Fernandes et al., (2015) on where, among different cleaning detergents procedures evaluated for the removal of *Enterococcus faecalis* and *Enterococcus faecium*, the chlorinated alkali did not present significant differences with other alternative cleaning procedures applied such as an anionic tensioactive cleaning or an anionic tensioactive cleaning + sanitization. However, if a disinfection after the application of the enzymatic treatment was added to the present study, the obtained removal percentage would increase more, so that the action of the alternative treatment would be enhanced and therefore more effective results would be obtained. This point could be considered as a subsequent step to improve the effectiveness of the applied alternative treatment, since the enzymes will have removed the ECM from the biofilm,

so that the cells will be more exposed to the action of a disinfectant (Nguyen & Burrows, 2014; Puga et al., 2018). In addition, the strategy based on the elimination of biofilms on industrial surfaces through the use of enzymatic treatments represents an ecological and facilities friendly alternative, since it does not generate corrosion problems and therefore damage to the industrial equipment materials, for which these can be used for longer.

Table 6. Comparison between enzymatic versus chlorinated alkaline treatment effectiveness for the removal of S2-bac *Listeria monocytogenes* mature biofilms. Values correspond to the mean ± standard error (n = 9).

***L. monocytognes* strain**	**Treatment**	**Log reduction (CFU/cm^2)**	**Detechment (%)**
S2-bac	Enzymatic	5.80 ± 0.54	85.06 ± 5.96
	Chlorinated alkaline	5.45 ± 0.92	76.37 ± 11.00

3.4. Biofilm detachment qualitatively observed by DEM

The organization of the cells that make up the microbial biofilms is a visual, interesting parameter that helps us to understand the ability of biofilms formation. Therefore, the use of DEM for their observation is considered as a good analytical technique (Ripolles-Avila, Hascoët, et al., 2018). It has been indicated that an arrangement of disaggregated cells signifies that a biofilm has not been formed, while if there is cell aggregation and a three-dimensional network it can be assumed that a biofilm with an organized, compact structure has been established on the surface (Chmielewski & Frank, 2003). The images of *L. monocytogenes* biofilms obtained by DEM are represented in **Figure 3**. All the *L. monocytogenes* strains employed in the study showed their capacity to produce mature biofilms with highly organized arrangements of honeycomb-like structures as the biofilm morphotype, as shown previously by other authors (Guilbaud et al., 2015; Marsh et al., 2003; Ripolles-Avila, Hascoët, et al., 2018). In concordance with Reis-Teixeira et al., (2017), a yellow coloration from the overlap of the dyes SYTO9 and PI was obtained for some of the *L. monocytogenes* strains (*i.e.* S1(S), S2-bac, R6, S2-2, 911, 5873, P12, A7) (**Figure 3 A-3** and **A-4**), suggesting cell lysis and the release of extracellular DNA (e-DNA). It has been demonstrated that e-DNA is a relevant structural component of the *L. monocytogenes* ECM matrix, cooperating with polysaccharides and proteins to ensure the integrity of the biofilm (Colagiorgi et al., 2016).

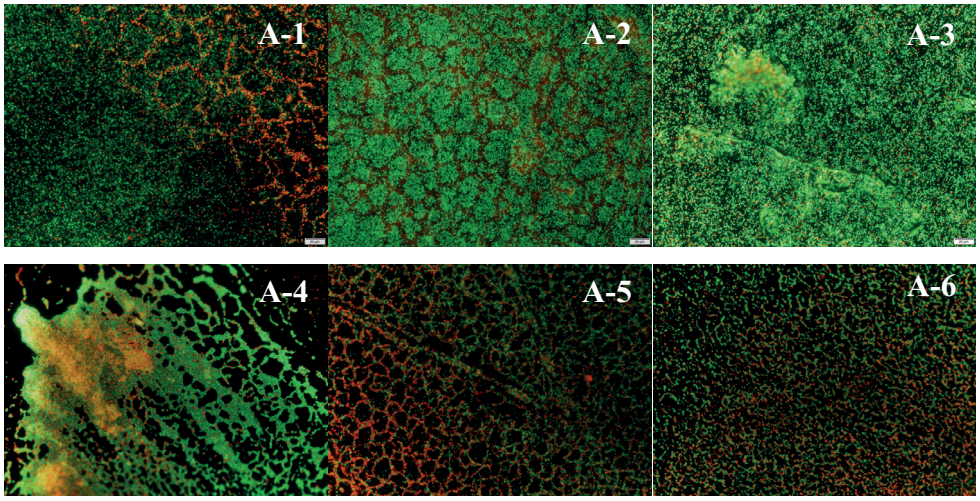

Figure 3. Epifluorescence digital images of Live/Dead stained mature biofilms of different *L. monocytogenes* strains classified from higher to lower biofilm-producing capacity: CDL69 (A-1), 5672 (A-2), S2-bac (A-3), 911 (A-4), 5366 (A-5) and S10-1 (A-6). Magnification 20X.

At a qualitative level, it was observed that once the enzymatic treatment was applied, there was an obvious reduction in the remaining microbial load on the surface (**Figure 4**). Coinciding with the results presented at a quantitative level, *L. monocytogenes* S10-1 was the strain that obtained a greater detachment, and the S2-bac strain the lowest. As can be observed, a complete dispersion of the mature biofilm structure was found for most cases. A clear example of this dispersion is *L. monocytogenes* CECT 5366, whose cells were dispersed and no defined structure was found on the surface after the treatment. However, for strains S2-bac and EDG-e, there were still cells and a certain structure attached to the surface. This could be attributed, again, to the fact that these strains may have produced a more robust matrix that protected them from the cleaning procedure. To this effect, it has been reported that each strain of *L. monocytogenes* has its own capacity for biofilm formation, which could be used to explain the results found in this study (Ripolles-Avila, Cervantes-Huaman, et al., 2019).

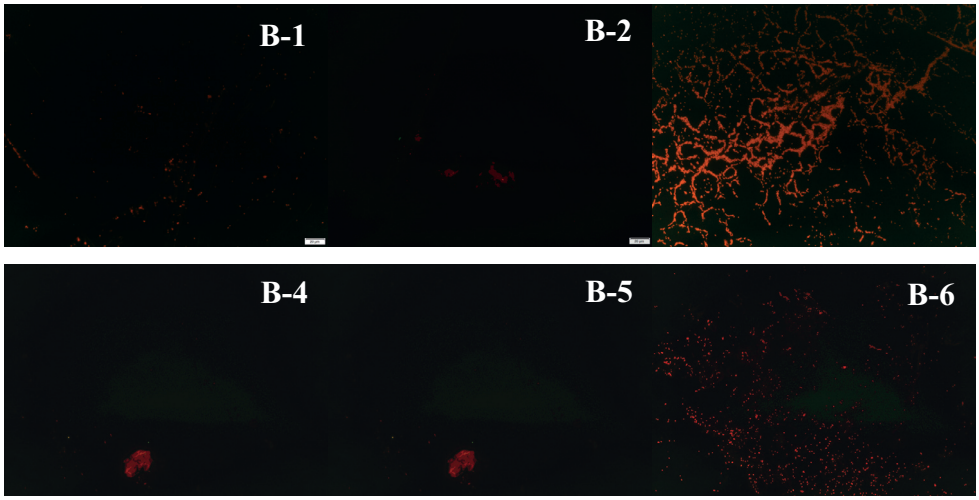

Figure 4. Epifluorescence digital images of Live/Dead-stained mature biofilms after the enzymatic treatment of different *L. monocytogenes* strains classified from higher to lower biofilm-producing capacity: CDL69 (B-1), 5672 (B-2), S2-bac (B-3), 911 (B-4), 5366 (B-5) and S10-1 (B-6). Magnification 20X.

CHAPTER 4

Conventional and alternative cleaning solutions to remove *Listeria monocytogenes* biofilms from stainless steel surfaces

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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., 2017). Among the pathogens that stand out due to food contamination as a consequence of cross-contamination from industrial surfaces is *L. 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, 2021b), 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 (FDA-CDC, 2020), hard-boiled eggs (FDA-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, 2021a), 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 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 & 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 & 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, Ramos-Rubio, 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 & 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, Ramos-Rubio, 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, Ramos-Rubio, 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. Material 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 hours at 30ºC. Suspensions were then cultured on Tryptic Soy Agar (TSA; Oxid, Madrid, Spain) and incubated at 37ºC for 24 hours. Isolated colonies were used to prepare stock cultures on TSA, which were stored for up to 1 month at 4ºC.

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 hour, 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 minutes 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 hours were inoculated into TSYEB $_{\text{gluc 1 \% + NaCl 2 \%}}$ [*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 NaCl (Panreac, Castellar del Vallès, Spain)] until reaching 0.2 McFarland units, equivalent to approximetely 10^6 CFU/ml (Ripolles-Avila, Hascoët, 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, Hascoët, 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 $_{\text{gluc 1 \% + NaCl 2 \%}}$ were added to stimulate biofilm growth and maturation at 48 hours + 24 hours + 24 hours + 72 hours (Ripolles-Avila, Hascoët, 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 7**). 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, Ramos-Rubio, et al., 2020). All treatments were applied for 15 minutes but under different conditions (*i.e.* temperature and concentration), as summarised in **Table 7**, and according to the technical data sheet of the products.

Table 7. 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 (ºC)	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 %) Thyme oil (0.5 %) Cinnamon oil (0.5 %)	20 and 50	1 and 3
Conventional	Acid	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

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 seconds (Ripolles-Avila, Cervantes-Huaman, et al., 2019). 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 30ºC for 48 hours.

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 20-22ºC for 15 minutes. 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 colour. 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 (20X) 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 minutes 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 hours.

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 8** 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 nonsignificant 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, 2014). 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.

Table 8. 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 ($P < 0.05$).

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, Hascoët, et al., 2018; Ripolles-Avila et al., 2019). 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 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 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, Ramos-Rubio, 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, Ramos-Rubio, 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. **Figure 5** 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, Hascoët, 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, Ríos-Castillo, & Rodríguez-Jerez, (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.

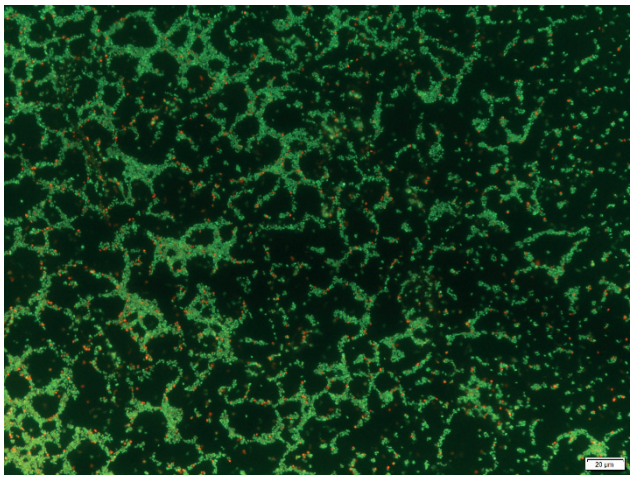

Figure 5. Epifluorescence digital images of Live/Dead-stained mature *L. monocytogenes* S2-bac biofilms. Magnification 20X.

L. monocytogenes S2-bac cells and structure remaining on the surface after the application of the eleven treatments is shown in **Figure 6**. 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 (**Figure 6 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, Ramos-Rubio, 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; **Figure 6 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 %; **Figure 6 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., (2019), 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 & 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.

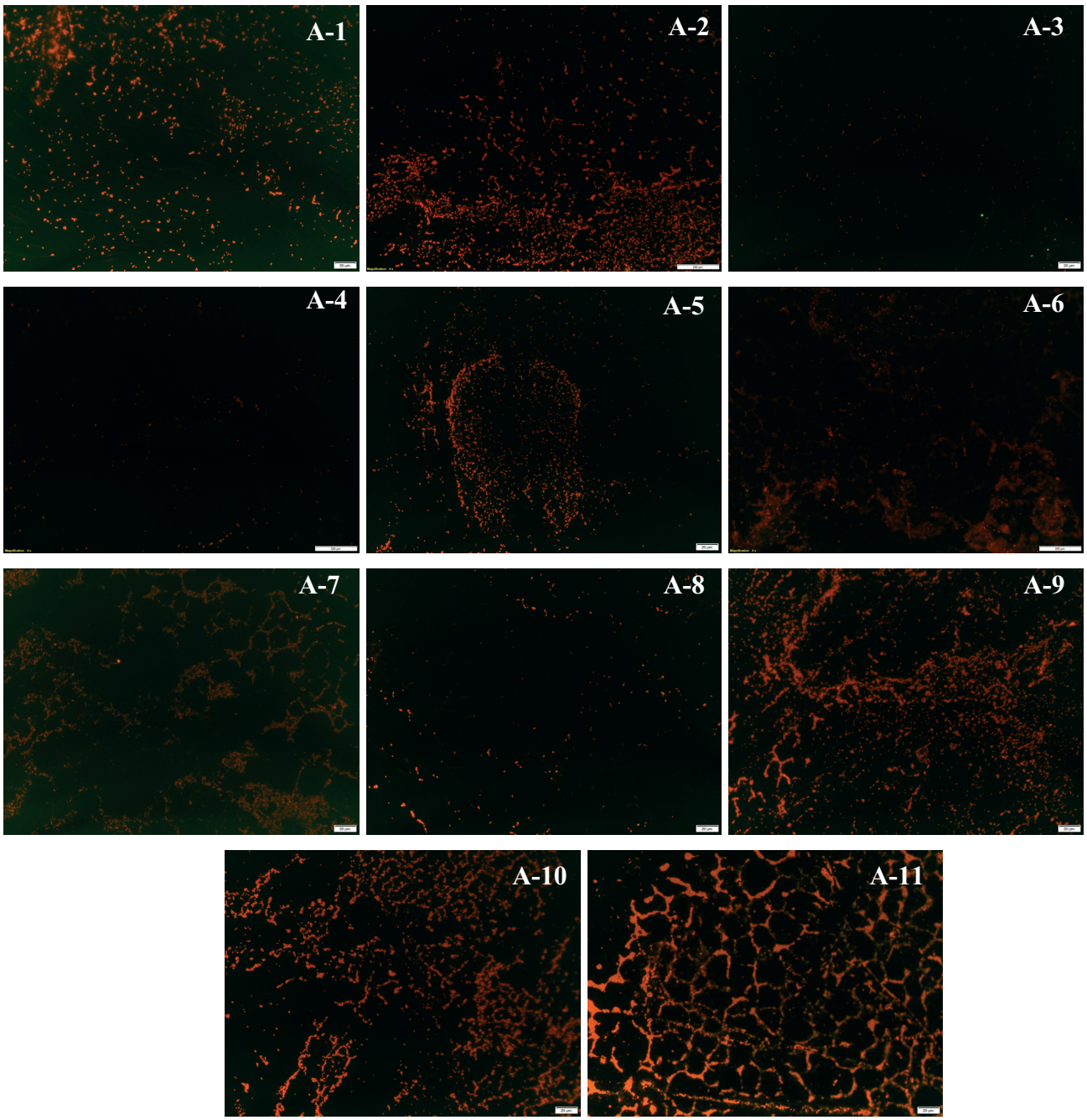

Figure 6. Epifluorescence digital images of Live/Dead-stained mature *L. monocytogenes* S2-bac biofilms after the application of tested treatments: Enzyjet 1 % - 20ºC (A-1); Enzyjet 3 % - 20ºC (A-2); Enzyjet 1 % - 50ºC (A.3), Enzyjet 3 % - 50ºC (A-4); Enzybac 1 % - 20ºC (A-5), Enzybac 3 % - 20ºC (A-6);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

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

Table 9 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 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 9**). Other authors have reported similar logarithmic reductions when these treatments were applied to eliminate biofilms. For example, Taormina & 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).

Table 9. 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 (ºC)	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-f] Means within a column without a common superscript differ significantly ($P < 0.05$).

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 10** 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, Cervantes-Huaman, et al., 2019)

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 10**. 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).

Table 10. 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}	0.00 ± 0.00^{a}
S2-bac	6.87 ± 0.11^{ab}	0.00 ± 0.00^{a}
EDG-e	6.64 ± 0.15^{b}	0.00 ± 0.00^{a}
CECT 935	6.63 ± 0.14^{b}	0.17 ± 0.17^{a}

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

The antimicrobial efficacy of the combined treatment against the mature *L. monocytogenes* biofilms is shown in **Table 11**. 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.

Table 11. 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 ($P < 0.05$).

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 (**Figure 7**). 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, Ramos-Rubio, 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.

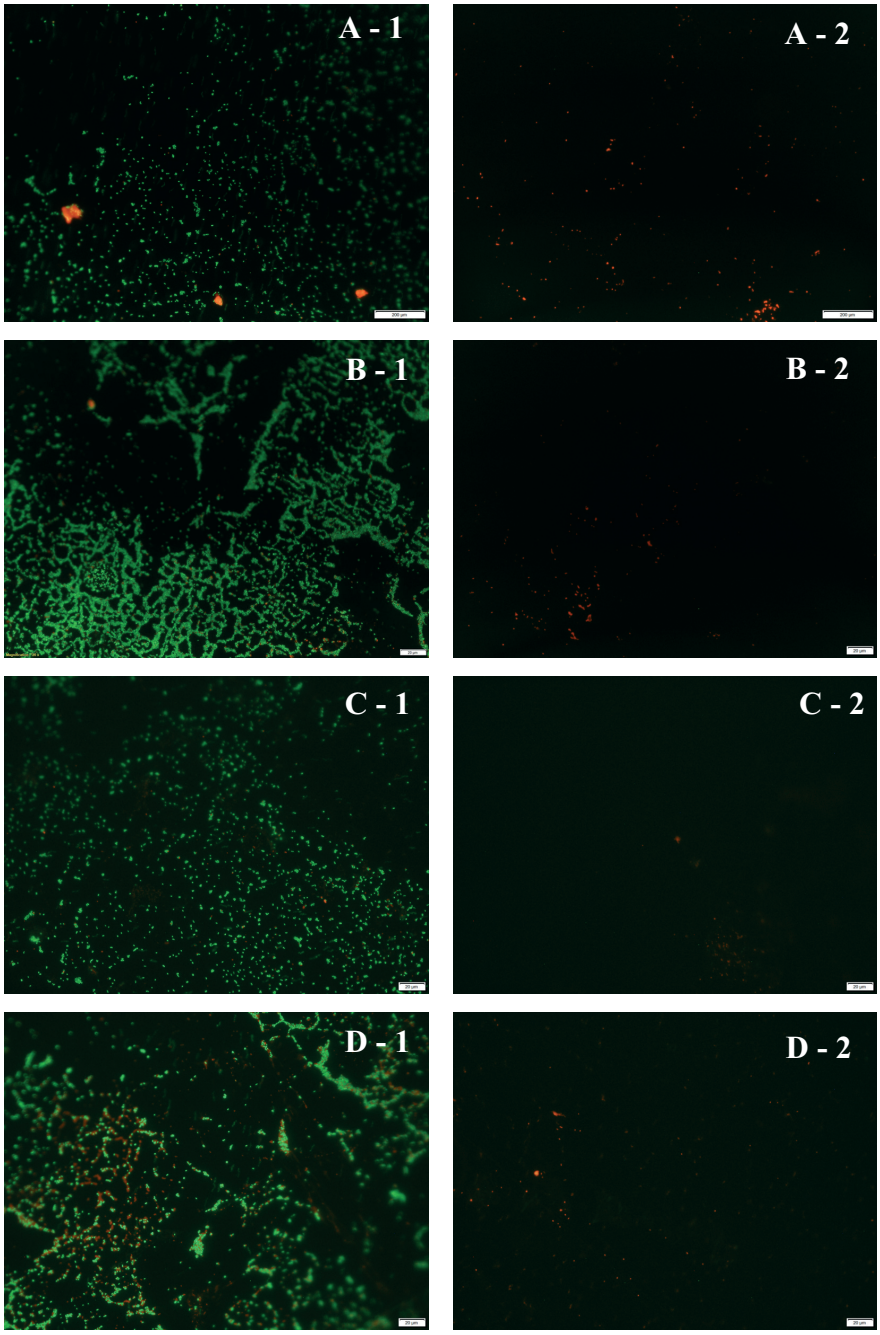

Figure 7. Epifluorescence digital images of Live/Dead-stained mature *L. monocytogenes* biofilms before (1) and after (2) the application of the combined treatments (*i.e.* Enzyjet 1 % + chlorinated alkaline 1 %) on four different strains: CECT 5672 (A); S2-bac (B); EDG-e (C); CECT 935 (D). Magnification 20X.

CHAPTER 5

Chlorinated alkaline and enzymatic detergents for the removal of mature *Listeria monocytogenes* biofilms and cross-contamination to chicken broth

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Mazaheri, T., Ripolles-Avila, C., Rodríguez-Jerez, J.J. (2022).

In vitro study of the 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.

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, Ramos-Rubio, et al., 2020). Among the pathogens with the greatest impact in the food sector due to its ability to form biofilms and establish ecological niches is *L. monocytogenes* (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 (*i.e.* cattle) and subsequently contaminate the processing environment (Lakicevic et al., 2015). Among these processing plants, one type of industry where pathogen prevalence has received little attention is conventional poultry processing plants, despite the environmental conditions also being favorable to the presence of *Listeria* spp. (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, Hascoët, et al., 2019a), 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).

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 (*i.e.* proteins, 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, since 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., (2019), 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, Ramos-Rubio, 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, Ramos-Rubio, 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 & 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 4b, 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 30ºC for 48 hours. After this period, cells were cultured on Tryptic Soy Agar (TSA; Oxid, Madrid, Spain) and incubated at 37ºC for 24 hours. Last, grown plates were kept at 4ºC for up to 1 month as working cultures. For each new experiment, a fresh culture was grown on TSA at 37ºC for 24 hours and a new bacterial suspension was prepared. To do so, several isolated colonies from a specific *L. monocytogenes* strain were inoculated into TSYEB $_{\text{gluc 1 \% + NaCl 2 \%}}$ [*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, Hascoët, et al., 2018). This was considered the bacterial suspension to conduct the mature biofilm formation (see section 2.3.).

2.2. Surfaces

AISI 316 2B 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 hour, 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 121ºC for 15 minutes.

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 30ºC to force mature biofilm formation, as established by Fuster-Valls et al., (2008); Ripolles-Avila, Hascoët, et al., (2018). Following the procedure proposed by Ripolles-Avila, Hascoët, 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 hours + 24 hours + 24 hours + 72 hours. 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 20ºC for 15 minutes with an in-use concentration of 1 %. The enzymatic product 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 50ºC for 15 minutes, 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 1,000 ml of distilled water], 4 ml of solution B [35.02 g $NaHCO_3$ (PanReac Applichem, Madrid, Spain) per 1,000 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 minutes at 25ºC 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 1,000 ml of sterile distilled water in pH (7.0 ± 0.2)]. The samples were vortexed for 90 seconds 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 30ºC for 48 hours.

2.7. Statistical analysis

Each experiment was performed in triplicates on three independent days (n = 9) for each *L. monocytogenes* strain. Data were analyzed using STATISTICA 7.0.61.0. "One Way ANOVA" with a posterior contrast with the Tukey Test 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:

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

On where:

T: Transference rate

N_1: Microbial count in CFU/cm^2 on destination

N_2: Microbial count in CFU/cm^2 on source

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 to be able to adhere and develop biofilms at approximately the same growth levels (**Figure 8**), 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, Ríos-Castillo, & Rodríguez-Jerez, 2018; . Silva et al., 2008).

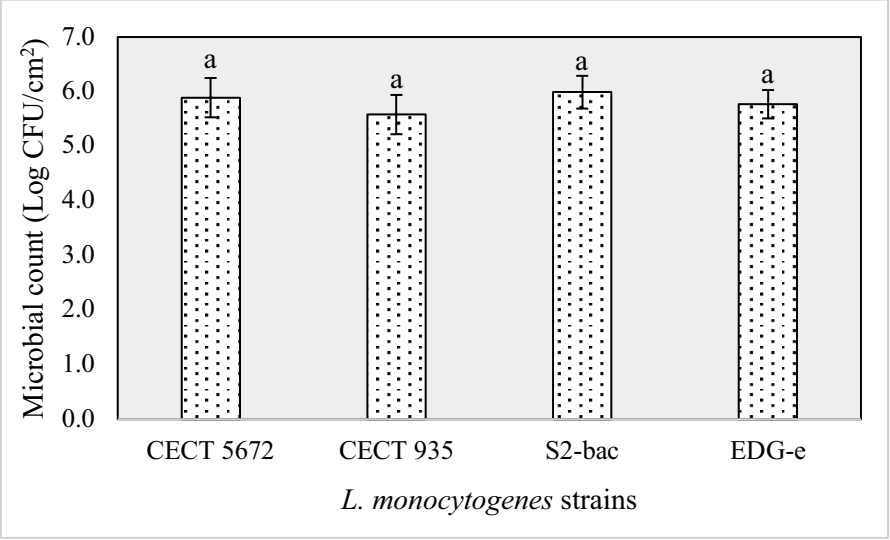

Figure 8. Total, count of cells conforming mature biofilms from different *L. monocytogenes* strains. Each column corresponds to an average of three repetitions performed on three separate days (n = 9). Error bars represent the standard deviation.
[a] Values within a column lacking a common letter differ significantly ($P < 0.05$).

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., (2019), 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 4b, which have been described as highly pathogenic (Martins & 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 relation between the serotype to which the strain belongs and biofilm formation capacity, a direct relationship has not yet been found (Ripolles-Avila, Cervantes-Huaman, et al., 2019).

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, Hascoët, et al., 2018; Rodríguez-Melcón, Alonso-Calleja, et al., 2019).

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 12**. 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 %, a figure that was significantly different from the rest of the strains ($P < 0.05$ for CECT 5672, CECT935 and S2-bac). 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, therefore making 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, Ramos-Rubio, 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, Cervantes-Huaman, et al., 2019). 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.

Table 12. *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 transferal to food Log (CFU/ml)	Transference (%)
CECT 5672	5.89 ± 0.36^a	3.75 ± 0.37^a	0.78^a
CECT 935	5.58 ± 0.36^a	3.80 ± 0.87^a	5.85^b
S2-bac	6.00 ± 0.30^a	3.80 ± 0.37^a	1.60^a
EDG-e	5.78 ± 0.27^a	5.48 ± 0.74^b	90.74^c
L. monocytogenes	5.82 ± 0.35	4.22 ± 0.99	20.40

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

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 12** 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 1.6x10^4 *L. monocytogenes* cells to the chicken broth, which is not a negligible number.

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 %, posing a potential risk for the food safety as approximately 1.66x10^4 cells would directly migrate to the product in 5 minutes of contact. Considering that an ineffective does of *L. monocytogenes* is fixed at 1,000 cells, the microbial load transferred to the food model would surpass this limit. 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 **Figure 9**, the effectiveness of the conventional treatment ranged between 69.35 to 99.72 %, 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, Ramos-Rubio, 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, Riesco-Peláez, et al., (2019) also reported that using sodium hypochlorite as a disinfectant agent at a concentration equivalent to the minimum inhibitory concentration (MIC), established at 3,500 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, Faille, 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, Ramos-Rubio, et al., (2020) showed DEM images of different *L. monocytogenes* strains exposed to a chlorinated alkaline detergent, with intact parts of the matrix after treatment.

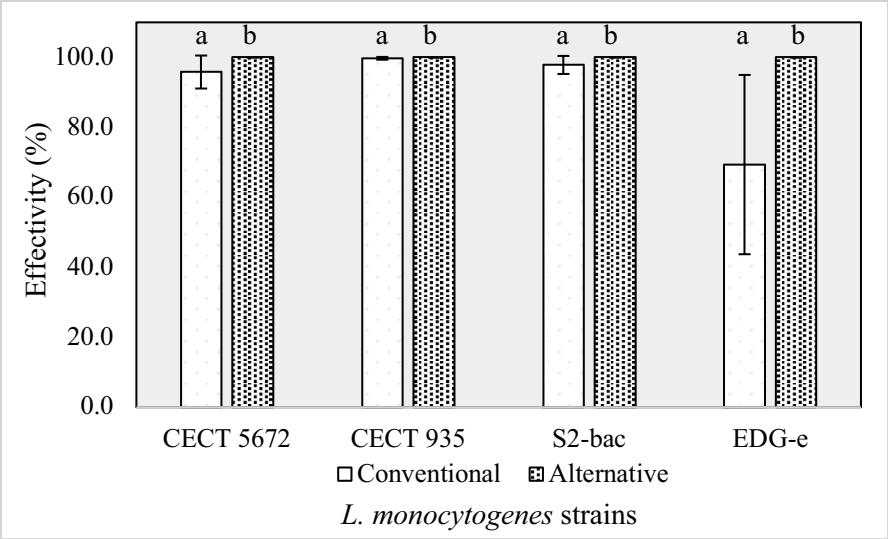

Figure 9. Detachment percentage of different *Listeria monocytogenes* strains after the application of the conventional (*i.e.* chlorinated alkaline) and the alternative (i.e. combination of chlorinated alkaline and enzymatic) treatments. Each value corresponds to a mean of three replicates performed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

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

In the case of the alternative treatment, 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 % to 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 13** and **Table 14** 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 13**), 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 ($P = 1.00$) 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. 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 1 %. This result could be explained by the same factor discussed in the previous section: the protective matrix (Mazaheri et al., 2020; Ripolles-Avila, Cervantes-Huaman, et al., 2019) 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.

Table 13. *L. monocytogene*s 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 transferal to food Log (CFU/ml)	Transference (%)
CECT 5672	4.12 ± 0.87^{b}	2.40 ± 0.88^{b}	3.51^{c}
CECT 935	2.61 ± 0.85^{a}	0.96 ± 0.95^{ab}	3.04^{c}
S2-bac	4.13 ± 0.57^{b}	2.16 ± 0.86^{a}	1.12^{b}
EDG-e	5.13 ± 0.49^{c}	2.43 ± 1.11^{b}	0.45^{a}
L. monocytogenes	3.96 ± 1.15	1.99 ± 1.10	1.02

[a-c] Values within a column lacking a common letter differ significantly ($P < 0.05$).

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 % in all cases, including CECT 5672 and S2-bac, with residual cell counts of 0.06 Log CFU/cm^2. 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 14**). 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, Ramos-Rubio, 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. The combination of a chlorinated alkaline detergent followed by an enzymatic product application showed complete effectivity, detaching and dispersing all 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.

Table 14. *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 transferal to food Log (CFU/ml)	Transference (%)
CECT 5672	0.06 ± 0.17^a	0.00 ± 0.00^a	0.00 ± 0.00^a
CECT 935	0.00 ± 0.00^a	0.33 ± 0.66^a	0.00 ± 0.00^a
S2-bac	0.06 ± 0.17^a	0.00 ± 0.00^a	0.00 ± 0.00^a
EDG-e	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
L. monocytogenes	0.00 ± 0.12	0.08 ± 0.35	0.00 ± 0.00

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

CHAPTER 6

Elimination of mature *Listeria monocytogenes* biofilms formed on clean and dirty surfaces and structure regeneration after treatments application

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Mazaheri, T., Ripolles-Avila, C., Rodríguez-Jerez, J.J. (2022). Elimination of mature *Listeria monocytogenes* biofilms formed on preconditioned and non-preconditioned surfaces after the application of cleaning treatments and their cell regeneration.

1. Introduction

Many foodborne diseases are caused by cross-contamination from industrial food contact surfaces, representing a challenge for the food industry from a food safety point of view (Gonzalez-Gonzalez et al., 2021). This fact is directly related to the presence of biofilms on these surfaces, implying a potential entry route of pathogens into food. These microbial communities are consolidated because nutrients and humidity that generate a prone microenvironment for their proliferation and adaptation can always be found on industrial surfaces (Pang et al., 2019).

Biofilms are complex structures produced by microorganisms that have a surrounding matrix for their protection (González-Rivas et al., 2018). Microorganisms adhere to a surface in an irreversible way and then initiate biofilm formation by starting to form microcolonies and expel extracellular components (*i.e.* mostly carbohydrates, proteins, eDNA and lipids) to generate the matrix that stabilizes and protects the community from different types of stress (*i.e.* low temperature, pH, nutrients, humidity, among others) (Colagiorgi et al., 2017). These structures allow bacteria to remain in the food industry for long periods of time, leading to the development of different resistance mechanisms and increasing the probability of cross-contamination to food (Wilks et al., 2006). Among all the pathogens considered as significant in terms of cross-contamination, *L. monocytogenes* is highlighted (Hua et al., 2019). This bacteria forms robust biofilms that are a challenge to eliminate from industrial surfaces (Ripolles-Avila, Cervantes-Huaman, et al., 2019). This means that surfaces in the food industry must be cleaned deeply to prevent cross-contamination, with these operations considered an essential part of the cleaning and disinfection program for the control of these structures within the food industry (Ripolles-Avila, Hascoët, et al., 2018). Among the usual cleaning and disinfection operations in this industry, the use of conventional methods such as alkaline and acid treatments followed by the application of disinfectants such as chlorine-based products, hydrogen peroxide, iodine, ozone or peracetic acid has been most common. However, in recent years, their ineffectiveness in the elimination of biofilms has been demonstrated (Fagerlund et al., 2020). If a biofilm matrix disruption is not accomplished, disinfectants cannot penetrate to the lowest zones of the biofilm structure (Aarnisalo et al., 2007), thus requiring the biocide to be at sublethal concentrations and influencing resistance phenomena (Capita & Alonso-Calleja, 2013). For all these reasons, in recent

years, the search for new sanitation alternatives for biofilm control has been encouraged, including the utilization of enzymatic detergents (Ripolles-Avila, Ramos-Rubio, et al., 2020). In this regard, these detergents have been indicated as environmentally friendly and can help reduce wastewater in factories (Mazaheri et al., 2020).

In food processing plants such as the meat industry a high presence of *L. monocytogenes* has been detected on dirty surfaces (*e.g*. cutting boards, conveyor belts, and containers) in comparison to clean surfaces, given that these hard-to-reach places are often difficult to keep clean and food residue and dirt tend to accumulate there (Carpentier & Cerf, 2011). Dirtiness can be divided into two different classes depending on the type of residues, be they organic (*i.e*. fats, sugars, and proteins) or inorganic residues (*i.e*. deposits of insoluble mineral salts). Exposure to thermal processes can stimulate fouling formation by the caramelization of sugars, the denaturation of proteins, and the carbonization of other organic material, which makes effective cleaning more difficult to achieve (Basso et al., 2017; Guerrero Navarro, 2017). Residues are considered as a nutrient substrate for the growth of microorganisms in the food industry (Paz-Méndez et al., 2017). To this effect, the cleaning procedures and their adequate application are highly important, improving microbial elimination and thus causing minor problems of antimicrobial resistance acquisition (Basso et al., 2017; Capita & Alonso-Calleja, 2013; Paz-Méndez et al., 2017). Alternative strategies for biofilm control in the food industry are being studied to optimize cleaning treatments, such as the use of enzymes to disrupt the biofilm matrix and to enhance disinfectant penetration to deep zones of the structures (Thallinger et al., 2013). Enzymes not only participate in the biochemical decomposition of the matrix, but they also inhibit quorum sensing signalling (*i.e*. the mechanism that regulates gene expression as a function of cell density), degrade adhesiveness cells from the surface and deactivate other enzymes necessary for microbial growth (Nahar et al., 2018).

This study aimed to evaluate the efficacy of a conventional method and an alternative cleaning method for the detachment of *L. monocytogenes* biofilms on preconditioned and non-preconditioned stainless-steel surfaces and to evaluate the survival and regeneration capacity of *L. monocytogenes* cells. To achieve this objective, preconditioned surfaces with dirt were designed and biofilm formation capacity under these conditions of different *L. monocytogenes* strains was also evaluated.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

Four *L. monocytogenes* strains were used in this study. They were collected either from the Spanish Type Culture Collection (CECT) or isolated from an Iberian pig processing plant (**Table 15**). Strains were selected based on the high probability of presence in food facilities (*i.e*. serotype 1/2a strains) or higher pathogenicity (*i.e*. serotype 4b strains), with the aim of making a comparative analysis of the reaction of different serotypes to different cleaning and disinfection processes. Strains were received as freeze-dried cultures and were activated in 10 ml of Tryptic Soy Broth (TSB; Oxoid, Madrid, Spain) at 30°C for 48 hours. The bacterial suspensions were cultured on Tryptic Soy Agar (TSA; Oxoid, Madrid, Spain) and incubated at 37°C for 24 hours. From this starting culture, colonies were isolated, reinoculated on TSA plates and incubated at 37°C for 24 hours. Last, the grown TSA plates were stored as stock cultures at a temperature of 4°C for a maximum period of one month.

Table 15. *L. monocytogenes* strains used in this study.

Strain	Serotype	Origin
5672	4b	CECT*
935	4b	CECT*
S2-bac	1/2a	Ortiz et al., (2014)
EDG-e	1/2a	(Ortiz et al., 2016)

*CECT (Spanish Type Culture Collection).

2.2. Surfaces

Stainless-steel coupons AISI 316 grade 2B, 2 cm in diameter and 1mm thick, cleaned, disinfected, and sterilized according to the European standard UNEEN 13697:2015 were used (AENOR, 2015). The surfaces were washed with a non-bactericidal detergent (ADIS Higiene, Madrid, Spain), disinfected by immersion in 70 % isopropanol (Panreac Química, Castellar del Vallès, Spain) for 15 minutes and air-dried inside a laminar flow cabinet (PV-30/70, Telstar, Terrasa, Spain). To ensure complete sterility, the surfaces were autoclaved at 12°C for 15 minutes.

2.3. Generation of preconditioned surfaces

A dirt conditioning layer was created on stainless-steel coupons to compare dirty and clean conditions for biofilm formation and cleaning effectivity. For this purpose and using a laminar flow cabinet, surfaces were placed in sterile Petri dishes and 50 µl of previously sterilized chicken broth (Frías Nutrición, Burgos, Spain) were dispersed throughout the coupons. Maintaining sterile conditions, the surfaces were then placed in an oven and dried at 50ºC for 24 hours. Once this time had elapsed, the surfaces were left at room temperature until inoculation (*i.e*. maximum 2 hours later).

2.4. Biofilm formation

For the monospecies biofilm formation, several colonies of the four *L. monocytogenes* strains were initially transferred onto TSA plates and incubated at 37ºC for 24 hours. The inoculum was then prepared by transferring several colonies of these cultures into 10 ml TSYEB $_{\text{gluc 1 \% + NaCl 2 \%}}$, which consisted of TSB with 1 % of glucose (Biolife, Madrid, Spain), 2 % of NaCl (Biolife, Madrid, Spain) and 0.3 % of yeast extract (BD, Madrid, Spain), until reaching a turbidity of 0.1-0.2 McFarland units, an approximate concentration of 10^6 CFU/ml. This was then used as the initial cell level for biofilm formation (Ripolles-Avila, Hascoët, et al., 2018). The amount of 30 µl of the suspension derived from each *L. monocytogenes* strain were then inoculated in the center of each stainless-steel coupon. To make the comparison between dirty and clean conditions, surfaces with the preconditioned dirt layer prepared as described in the previous section (*i.e.* dirty conditions) and sterile discs (*i.e.* clean conditions) were used. Last, the inoculated surfaces were placed into sterile Petri dishes for 72 hours at 30°C and put in a humid chamber to promote the formation of the structures (Fuster-Valls et al., 2008).

2.5. Cleaning and disinfection treatments

The first treatment consisted of a conventional product and a chlorinated alkaline detergent was evaluated. The second treatment was considered as an alternative solution and consisted in a combined treatment that included the combination of the previously used chlorinated alkaline product followed by an enzymatic detergent (*i.e*. enzymatic product A) (**Table 16**). All cleaning agents were diluted to 1 % in hard water, prepared

in accordance with UNE-EN 13697:2015 by mixing 3 ml of solution A [19.84 g of $MgCl_2$ (Sigma, Madrid, Spain) and 46.24g of $CaCl_2$ (Sigma, Madrid, Spain) in 1000 ml of distilled water], 4 ml of solution B [35.02 g $NaHCO_3$ (Panreac Applichem, Madrid, Spain) in 1000 ml of distilled water] and 100 ml of interfering solution [1.5 g bovine serum albumin (Sigma, Madrid, Spain) in 100 ml of distilled water], and brought to a volume of 1000 ml with sterile distilled water. All solutions were sterilized prior to mixing by membrane filtration (Millex- GP 0.22 μm, Merck, Barcelona, Spain).

Table 16. Cleaning agents employed in the present study.

Type of treatment	Cleaner	Composition	In-use temperature (°C)	In- use concentration (%)
Conventional	Chlorinated alkaline	Sodium hydroxide (8.5 %) Sodium hypochlorite (6 %)	20	1
Alternative	Chlorinated alkaline + Enzymatic product A	Sodium hydroxide (8.5 %) Sodium hypochlorite (6 %) Ethoxylated sodium lauryl ether glycolate Amines, C12-14 (even numbered)-alkyldimethyl, N-oxides Anionic surfactants (< 5 %) Non-ionic surfactants (< 5 %) Proteases (< 5 %) Phenoxyethanol	20 + 50	1

2.6. Application of the cleaning procedures: Conventional and alternative treatments

After the incubation period for promoting biofilm formation, the surfaces (*i.e.* preconditioned with dirt or without the preconditioned layer) were washed twice with 3 ml of sterile distilled water and placed in a sterile flask. Then, 3 ml of the corresponding cleaning product was introduced in the flask. The chlorinated alkaline treatment was applied for 15 minutes at 20°C. Once the treatment had finished, the surfaces were rinsed with 3 ml of sterile distilled water and the effect of the disinfectant was neutralized by placing the coupons in sterile flasks with 3.5 g of glass beads and neutralizer [1 g of tryptone (BD, Madrid, Spain)], 8.5 g of NaCl (Panreac, Castellar del Vallès, Spain), and 30 g of Tween 80 (Scharlab, Barcelona, Spain) per liter of distilled water (pH= 7.0 ± 0.2)] for subsequent quantification. At the same time and as mentioned, the first step of the

combined treatment was carried out with the same chlorinated alkaline detergent, for which the procedure was the same as described above. However, to continue with the combined treatment, the surfaces were placed in another sterile flask with 3 ml of an enzymatic product and brought to 50ºC (*i.e.* optimum temperature) in a bath for 15 minutes, with agitation (Mazaheri et al., 2020). Once the treatment was completed, the samples were rinsed with 3 ml of sterile distilled water and quantified as in the case of the treatment with only chlorinated alkaline (*i.e.* introduced in sterile flasks with glass beads and neutralizer). Control surfaces (*i.e*. biofilms formed on surfaces in both conditions and without applying any treatment) were included in the experiment. All surfaces were quantified using the TEMPO method (see section 2.8.).

2.7. Cell regeneration capacity of monospecies biofilms after the application of treatments

Following the cleaning treatments, some of the surfaces were evaluated to observe cell regeneration capacity to reconsolidate a biofilm structure. To do so, the treated surfaces (*i.e*. preconditioned with dirt or without the preconditioned layer, followed by the application of conventional or alternative treatments) were placed in sterile Petri dishes and 30 µl of TSYEB $_{\text{gluc 1 \% + NaCl 2 \%}}$ were added in the center of the stainless-steel coupons. This was done to promote the growth of cells that resisted the treatments and could once again induce biofilm formation. The surfaces were again placed in a humid chamber for 24 hours at 30ºC. After incubation, the surfaces were washed twice with 3 ml of sterile distilled water to discard any non-adhered cells that did not conform biofilm structure, and then placed in sterile flasks with glass beads and neutralizer for their quantification (see section 2.8.).

2.8. Quantification of biofilms by TEMPO

For all the assays carried out under the two conditions, the surfaces were quantified using the TEMPO system. For this, the prepared flasks containing the sample were vortexed for 90 seconds at a frequency of 40 Hz, with the aim of recovering the cells adhering to the surface by friction of the beads (Ripolles-Avila, Cervantes-Huaman, et al., 2019). A series of decimal dilutions were then performed with the aim of reducing the microbial load obtained after treatment and allowing interpretable results to be obtained. Quantification

was performed using the TEMPO quantitative system (bioMérieux, Marcy l'Etoile, France). To do so, the TEMPO vials were hydrated with 3 ml of sterile distilled water, then 1 ml of the sample to be analyzed was transferred and vortexed to ensure the homogenization of its content. Through the filling unit, the contents of the vials were then distributed on the cards composed of 48 wells with 3 different volumes, recorded in the system and incubated for 48 hours at 30ºC. After this period, the results were read using the TEMPO reader unit.

2.9. Statistical analysis

For all tests performed, triplicates were made on three independent days (n = 9) for each *L. monocytogenes* strain, condition, treatment and type of assay (*i.e.* treatment effectivity or biofilm regeneration capacity). The results were evaluated as decimal logarithmic values to assume a normal distribution. They were analyzed using the STATISTICA 7.0.61.0 software and "One Way ANOVA". "Two Way ANOVA" followed by the Tukey Test contrast were applied to observe possible differences between the data obtained, considering statistically significant a $P < 0.05$.

3. Results and discussion

3.1. L. monocytogenes biofilm formation on preconditioned and non-preconditioned surfaces

Biofilm formation capacity of different *L. monocytogenes* strains was evaluated on surfaces that contained a preconditioned dirty layer or not to simulate either possible food residues from the meat industry or completely cleaned and disinfected surfaces also in the food industry. The results obtained for the four *L. monocytogenes* strains are shown in **Table 17**. As can be observed, the pathogen was able to adhere and form biofilms in both conditions tested, although with different tendencies depending on the evaluated strain. It has been demonstrated that *L. monocytogenes* has the capacity to rapidly adhere to different food contact materials and produce robust biofilms (Ripolles-Avila, Hascoët, et al., 2018; S. Silva et al., 2008). Although different authors such as (Ortiz et al., 2014) and Skowron et al., (2018) have pointed out that biofilm formation capacity differs between *L. monocytogenes* strains, it was not possible to find a direct relationship between biofilm formation and the serotype to which the strain belongs (Ripolles-Avila, Cervantes-Huaman, et al., 2019). In the present study, serotypes 1/2a (*i.e.* S2-bac and EDG-e) and 4b (*i.e.* CECT 5672 and CECT 935) were used because of their high and continuous presence in food processing plants and their high pathogenicity, respectively. A comparison between strains to produce biofilms in this study could help to understand how the presence of organic matter can affect their formation. In this regard, the results of each specific strain did not show significant differences ($P = 0.232$) when compared between them for the non-preconditioned surfaces. Differently, strain CECT 5672 showed significant differences when conforming biofilms on the preconditioned surfaces with S2-bac and EDG-e strains ($P = 0.017$ and $P = 0.001$, respectively). This difference could be explained by the fact that, as some studies have shown, strain CECT 5672 is a high biofilm producer (Mazaheri et al., 2020; Ripolles-Avila, Cervantes-Huaman, et al., 2019), and effectively it was the only strain that the preconditioned layer did not influence in its biofilm consolidation ($P = 0.22$). In this regard, Hua et al., (2021) reported that *L. innocua* biofilms formed after 7 days on different preconditioned and non-preconditioned surfaces achieved cell counts at similar ranking levels between 6.8 and 7.3 Logs CFU/coupon. When comparing the influence of the non-preconditioned and the preconditioned surface on the biofilm formation of each strain, CECT 935, S2-bac and EDG-e biofilm counts significantly differed between conditions ($P < 0.05$), with cell

counts higher when residues were not present. To this effect, the biofilms structure on the preconditioned surfaces could have been weaker and more easily broken, and consequently released into the environment (Paz-Méndez et al., 2017). This would make this type of residue not support the growth of *L. monocytogenes* in such structures.

In addition to the evaluation of each strain under the two conditions (*i.e.* non-preconditioned and preconditioned surfaces), and to visualize in greater detail the biofilm consolidation capacity of different *L. monocytogenes* strains, the results were also grouped globally to understand the behavior at specie level. The results obtained at this level (*i.e.* including the four strains under study) are also shown in **Table 17**. As can be observed, no significant differences were obtained between the two conditions on comparing the whole group ($P < 0.05$). The accumulation of organic and inorganic residues in food processing environments can act as a conditioning layer, increasing nutrient levels and favoring the formation of biofilms. The results obtained in the present study do not show a clear difference at specie level, which is not in accordance with other studies that point out that residues stimulate the adhesion and subsequent generation of biofilms (Donlan & Costerton, 2002; Lorite et al., 2011).

Table 17. *L. monocytogenes* biofilm cell counts (Log CFU/cm^2) obtained after their formation on non-preconditioned and preconditioned surfaces with food residues for different strains. Values correspond to the mean ± standard deviation (n = 9) and the global result for the four strains (*L. monocytogenes*) with the global mean ± standard deviation (n = 36).

Strain	Non-preconditioned surface	Preconditioned surface
CECT 5672	6.75 ± 0.23^{Aa}	6.21 ± 0.59^{Aa}
CECT 935	6.41 ± 0.45^{Aa}	5.71 ± 0.32^{ABb}
S2-bac	6.57 ± 0.53^{Aa}	5.24 ± 0.52^{Bb}
EDG-e	6.78 ± 0.39^{Aa}	5.51 ± 0.37^{Bb}
L. monocytogenes	6.63 ± 0.42^{a}	5.71 ± 0.60^{a}

$^{A-B}$ means within a column without a common superscript differ significantly ($P < 0.05$).

3.2. Elimination of biofilms through the application of a conventional and an alternative treatment

The effectivity of two treatments for removing biofilms of distinct *L. monocytogenes* strains formed under two different conditions (*i.e.* non-preconditioned and preconditioned

surfaces) is presented in **Table 18**. After the application of the conventional treatment (*i.e.* chlorinated alkaline detergent), the results demonstrated that its effectivity is significantly higher on preconditioned than on non-preconditioned surfaces in all strains ($P < 0.05$) except the EDG-e ($P = 0.180$). Chlorinated alkaline detergent was applied as a conventional treatment as it is regularly used in the food industry to eliminate organic matter (*i.e.* residues) and microorganisms using chlorine (Fagerlund et al., 2020). This could be why the conventional treatment was more effective on the preconditioned surfaces than on the surfaces that did not have the presence of food residues. These results are in agreement with Hua et al., (2021), who demonstrated that the cleaning treatment was more effective to detach *L. innocua* biofilms when surfaces were preconditioned with apple juice residues than when surfaces had no residues. Moreover, treatment applied to biofilms formed on non-preconditioned surfaces demonstrated significant differences between strains CECT 5672 and CECT 935 ($P = 0.033$), even though they belonged to the same serotype (*i.e.* 4b). This could be due to CECT 5672 being the strain with the highest biofilm cell count, while CECT 935 was the lowest, followed by S2-bac. In this regard, S2-bac showed the highest biofilm cell reduction independently under the two conditions (*i.e.* preconditioned and non-preconditioned surfaces; 3.10 Log CFU/cm^2 and 2.66 Log CFU/cm^2, respectively). Therefore, each strain has its own behavior and resistance, which may depend on the generated structure and matrix in the formation of biofilms by each strain, as also indicated by (Ripolles-Avila, Ramos-Rubio, et al., 2020).

Table 18. Remaining *L. monocytogenes* cells within the residual biofilm structure (Log CFU/cm^2) after the application of conventional and alternative treatments on non-preconditioned and preconditioned stainless-steel surfaces. Values correspond to the mean ± standard deviation (n = 9).

Strain	Non-preconditioned surface		Preconditioned surface	
	Chlorinated alkaline	**Combined treatment**	**Chlorinated alkaline**	**Combined treatment**
CECT 5672	6.35 ± 0.18^{Cc}	0.19 ± 0.36^{Aa}	4.75 ± 0.28^{Bb}	0.17 ± 0.34^{Aa}
CECT 935	5.16 ± 1.08^{Bb}	0.46 ± 0.70^{ABa}	4.87 ± 0.67^{Bb}	0.11 ± 0.31^{Aa}
S2-bac	3.91 ± 0.95^{Ac}	0.18 ± 0.38^{Aa}	2.14 ± 1.31^{Ab}	0.00 ± 0.00^{Aa}
EDG-e	5.74 ± 0.89^{BCb}	1.40 ± 1.20^{Ba}	4.86 ± 0.23^{Bb}	1.36 ± 0.79^{Ba}
L. monocytogenes	5.29 ± 1.21^{a}	0.57 ± 0.76^{c}	4.19 ± 1.36^{b}	0.42 ± 0.72^{c}

$^{A-C}$ means within a column without a common superscript differ significantly ($P < 0.05$).
$^{a-c}$ means within a row without a common superscript differ significantly ($P < 0.05$).

Although when applying the conventional treatment there was a reduction in all cases compared to their controls, it was not as effective as the alternative one (*i.e.* combined treatment with chlorinated alkaline plus enzymatic). Overall, biofilm cell reduction after the application of this chlorinated alkaline treatment ranged between 0.40 to 3.40 Log CFU/cm^2. After applying the alternative treatment, significant differences ($P < 0.05$) were obtained in all strains used and conditions tested. However, no significant differences were obtained when each strain was compared with the preconditioned or non-preconditioned surfaces ($P > 0.05$). Its high effectivity could be linked not only to the action of the chlorinated alkaline detergent for the residue layer removal, but also to the capacity of the enzymes to disintegrate and destroy the biofilm structure for cell detachment and subsequent elimination (Araújo et al., 2017; Simões et al., 2010). In this case, the results demonstrated a cell reduction ranging between 4.15 to 6.39 Log CFU/cm^2, which is highly different from what was found for the chlorinated alkaline single treatment application. Significant differences between EDG-e and CECT 5672 ($P = 0.012$) were specifically found on the non-preconditioned surfaces and between EDG-e and the rest of the strains ($P < 0.001$ in all cases). This indicates that *L. monocytogenes* EDG-e was more resistant when applying the alternative treatment in conditions with the presence or not of residues, as higher cell counts adhered to the stainless-steel surfaces were obtained. One of the reasons for this could be that this strain produces a more robust biofilm matrix and is therefore more protected from stressors such as the application of a treatment (Ripolles-Avila, Ramos-Rubio, et al., 2020). Moreover, it should be noted that it belongs to serotype 1/2a, which is characterized by being more persistent in food processing environments (Ortiz et al., 2010). This explanation can also be suggested for the application of the conventional treatment for strain EDG-e since likewise it did not show greater detachment under the two tested conditions. Moreover, it should also be mentioned that S2-bac was the strain that showed the highest cell reduction after the application of the two treatments (*i.e.* conventional or alternative). These results are different from the ones reported by Mazaheri et al., (2020), who showed that after the application of an enzymatic treatment, S2-bac was the least affected strain. However, the conditions for biofilm formation were different, with the biofilms generated in 7 and 3 days as the incubation period in the cited and present studies, respectively. To this effect, S2-bac may need more incubation time to produce maximum mature structures and the treatment was less effective as a result.

If we observe the results at specie, the same trend is found as in the results obtained for the individual strains. The application of the combined treatment was 4.72 Logs and 3.77 Logs CFU/cm^2 higher than for the treatments where only chlorinated alkaline was applied for both non-preconditioned and preconditioned surfaces. Therefore, in any of the realities found in the food industry (*i.e*. presence or not of residues), the application of the combined treatment leads to a greater detachment and elimination of *L. monocytogenes* biofilms, as also suggested by Mazaheri et al., (2020) on non-preconditioned surfaces. Biofilm detachment percentage for the distinct *L. monocytogenes* strains was also calculated to evaluate the overall effectiveness of each treatment (**Figure 10**). It is important to note that organic matter influences the effectiveness of disinfection, which is why proper cleaning must be ensured to eliminate biofilms (Nyati et al., 2012; Waters & Hung, 2014). In the case of conventional treatment, the presence of organic matter does not seem to have a direct influence on the effectiveness of the treatment, showing no clear trend between strains and conditions (*i.e*. non-preconditioned or preconditioned), although a slightly higher effectiveness was observed on surfaces with a preconditioned dirty layer. This could be due to the interaction of the detergent with the organic matter, implying its destabilization and allowing a breakage of organic residues that lead to the detachment of the structure. The percentage detachment obtained after chlorinated alkaline treatment application varied between 60 % and 99 %, a very wide range to affirm that the treatment is completely effective for *L. monocytogenes* biofilm detachment from stainless-steel surfaces. Chlorinated alkaline detergents are designed to remove the organic matter present and this could be one of the reasons why biofilms do not completely detach. The organic substances released could also interfere with the activity of the chlorine and did not present greater antimicrobial activity (Nyati et al., 2012; Waters & Hung, 2014). It is also important to note that the number of biofilm cells remaining on the surface after the application of conventional treatments implies that *L. monocytogenes* cells are still found on the surface and may even form more resistant biofilms (Ripolles-Avila, Ramos-Rubio, et al., 2020), which can lead to cross-contamination until the next sanitization process is carried out.

Furthermore, the results showed that the effectiveness of the alternative treatment was higher in all cases, regardless of whether there was the presence of residues on the surfaces or not. In this case, an effectiveness range of 99 % to 100 % was found, demonstrating that the alternative treatment had a highly biofilm detaching capacity. As

previously discussed, this effectivity could be the sum of residue and matrix removal (Chen et al., 2018). Comparing the effectiveness found in the present study with others where single enzymatic detergents (*i.e.* not combined with other products) were applied, authors have reported effectiveness of a maximum of 90 % (Mazaheri et al., 2020; Puga et al., 2018), thus reinforcing the present findings.

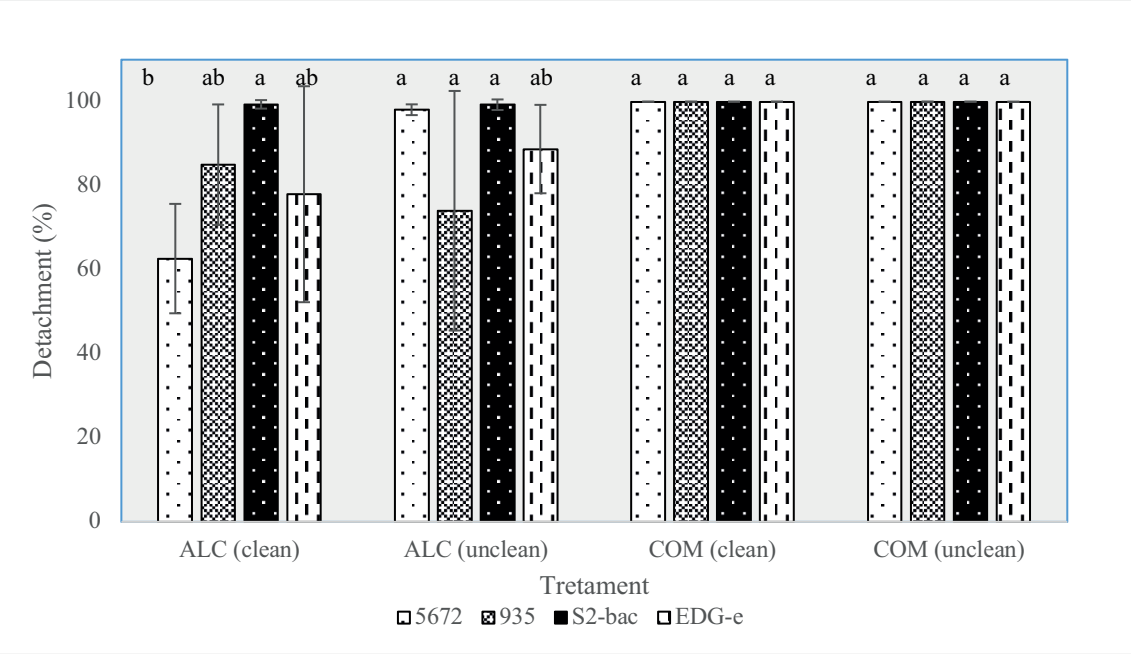

Figure 10. Percentage detachment of different *L. monocytogenes* strains after the application of conventional (ALC) and alternative (COM) treatments on non-preconditioned (clean) and preconditioned (unclean) stainless-steel surfaces. Each value corresponds to a mean of three replicates performed on three separate days (n = 9). The error bars represent the standard error of the mean.

Moreover, other authors have evaluated the application of the same treatments in the inverse direction to the present study (*i.e.* first the application of the enzymatic product and then the chlorinated alkaline detergent for also disinfecting) and effectiveness was also found to be around 90 % (Fagerlund et al., 2020). The increased effectiveness of the alternative treatment is due to the action of the enzymes that the product contains in the formulation. Authors such as Huang et al., (2014) have reported that after the application of various types of enzymes for biofilm removal, proteases were the enzymes with the highest biofilm structure removal. It should also be pointed out that effectiveness of the treatment could depend on the robustness of the matrix generated by *L. monocytogenes*,

as indicated in another study (Ripolles-Avila, Ramos-Rubio, et al., 2020), wherein strains such as EDG-e, which showed a lower effectiveness with respect to the other strains, could have generated a more robust matrix.

3.3. L. monocytogenes biofilm regeneration after the application of cleaning treatments

Survival is influenced by bacterial strain, temperature, time, humidity, nutrient availability, pH, presence of inhibitors, and the type of surface material, among many others (Allan et al., 2004). In addition, *L. monocytogenes* is a pathogen that is highly difficult to eradicate and has varying survival mechanisms and resistance to cleaning and disinfection methods used to date in the food industry (Somers & Lee Wong, 2004). For this reason, it was considered of interest to evaluate *L. monocytogenes* biofilm regeneration capacity 24 hours after the application of the conventional and alternative treatments. The results demonstrated that all *L. monocytogenes* strains were able to survive and regenerate biofilm structure under both conditions tested (*i.e.* non-preconditioned and preconditioned surfaces) and regardless of the treatment applied (*i.e.* conventional or alternative one) (**Table 19**). In general, biofilm regeneration after the application of the chlorinated alkaline detergent on non-preconditioned surfaces showed greater cell growth, obtaining counts similar to their controls. This tendency was also observed when comparing biofilm regeneration on the preconditioned surfaces, except for CECT 935 and S2bac *L. monocytogenes* strains, which showed lower cell counts compared to their controls. This situation is worrying considering that the controls needed 72 hours to obtain such cell levels. When comparing the cells remaining on the surface after the regeneration process in the two treatments (*i.e.* conventional and alternative), the results showed that biofilm regeneration was lower when the alternative treatment was applied, irrespective of the condition tested (*i.e.* non-preconditioned and preconditioned), although always reaching control values similar to when chlorinated alkaline detergent was applied. Moreover, the cells that survived the treatment and regenerated the biofilm structure may have been exposed to sublethal doses of the product, which increases the minimum inhibitory concentration (MIC) and thus increases their resistance, as found for chlorinated agents (Bansal et al., 2018). This is why it is important to improve biofilm removal technologies implemented in the food industry to reduce the number of cells found on surfaces after cleaning treatments, thereby reducing biofilm regeneration and optimizing cleaning cycles (Sanawar et al., 2018).

Table 19. *L. monocytogenes* cells counts (Log CFU/cm^2) after 24 hours of biofilm regeneration once the conventional and alternative treatments were applied on non-preconditioned and preconditioned stainless-steel surfaces. Values correspond to the mean ± standard deviation (n = 9).

Strain	Non-preconditioned surface		Preconditioned surface	
	Chlorinated alkaline	Combined treatment	Chlorinated alkaline	Combined treatment
CECT 5672	7.00 ± 0.23^{Bb}	2.92 ± 0.83^{Ba}	6.17 ± 0.60^{Bb}	2.63 ± 0.82^{Ba}
CECT 935	6.01 ± 0.48^{Ab}	2.56 ± 0.94^{Ba}	4.96 ± 0.93^{ABb}	2.14 ± 0.78^{ABa}
S2-bac	6.67 ± 0.42^{Bc}	0.57 ± 1.13^{Aa}	4.07 ± 1.10^{Ab}	1.26 ± 1.10^{Aa}
EDG-e	6.59 ± 0.56^{Bc}	3.50 ± 0.53^{Bb}	6.00 ± 0.63^{Bc}	2.65 ± 0.43^{Ba}
L. monocytogenes	6.57 ± 0.56^{a}	2.38 ± 1.42^{b}	5.37 ± 1.29^{a}	2.24 ± 0.98^{b}

$^{A-B}$ means within a column without a common superscript differ significantly ($P < 0.05$).
$^{a-c}$ means within a row without a common superscript differ significantly ($P < 0.05$).

In addition, and considering that *L. monocytogenes* strains are grouped by serotypes, serotypes 1/2a and 1/2b have been indicated to be stronger biofilm producers than serotype 4b (Keeney et al., 2018). After regeneration, the results in the present study do not correlate with this finding because CECT 5672 and CECT 935 (*i.e.* serotype 4b) presented greater cell growth and more resistance to the cleaning treatments applied regardless of the tested condition (*i.e.* non-preconditioned and preconditioned). *L. monocytogenes* EDG-e showed higher biofilm regeneration capacity after the application of the combined treatment, with a significantly higher capacity than the S2-bac strain ($P = 0.005$ and $P = 0.008$; respectively for non-preconditioned and preconditioned surfaces). Overall, the results obtained in the present study reveal a potential problem for the food industry, because nowadays the evaluated conventional treatment (*i.e.* chlorinated alkaline detergent) is often applied as a cleaning and disinfection procedure in a single step which, with the demonstrated efficacy, means that resistant *L. monocytogenes* cells survive and regenerate to form a robust biofilm structure. Within 24 hours, the pathogen was able to consolidate the structure with higher cell counts compared to the controls (*i.e.* biofilms formed at 72 hours), which leads to significant hygiene problems. This implies the need to use more aggressive treatments such as the alternative one to weaken and disperse the biofilm structure, leading to more safety and less risk (Bremer et al., 2006). In this regard, the results obtained with the combined treatment also showed biofilm

regeneration under both conditions, but it should be considered that a posterior disinfection would be carried out, thus leading to a higher effectivity of the whole treatment.

CHAPTER 7

Evaluation of microbial counts of different surfaces of an Iberian pig processing plant and the application an antibiofilm treatment to control the contamination

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Mazaheri, T., Ripolles-Avila, C., Rodríguez-Jerez, J.J. (2022). Microbial evaluation of industrial surfaces using surface sensors in an Iberian pork processing plant and application of an antibiofilm treatment to control contamination.

1. Introduction

The agri-food sector currently faces new challenges in terms of demands for higher quality, competitiveness and especially safety. To face these challenges, effective management of all the activities conducted in food industries is necessary. To this effect, management systems are presented as an effective and very useful tool that serves as a support for quality assurance and food safety (Fung et al., 2018). Among the most important factors to control, the prevention of cross-contamination through food contact surfaces is highlighted. In this regard, and with the goal of maintaining high levels of hygiene on both the industrial surfaces and in the environment, agri-food industries must consider the cleaning and disinfection system as key in the day-to-day operations of the factory. However, although these sanitation programs are designed to completely eliminate microorganisms adhered to industrial surfaces, in reality this goal is not achieved (Cobo-Díaz et al., 2021). To this effect, an entire microbial ecology can be found in each industry (Møretrø & Langsrud, 2017), meaning that food processing environments can be an important reservoir of different microorganisms. This microbial ecology occurs because there are microorganisms able to withstand cleaning and disinfection treatments, named residential microbiota. In the meat industry, species from the genera *Pseudomonas* spp., *Bacillus* spp., *Staphylococcus* spp., and *Aeromonas* spp., are found, as well as different *Enterobacteriaceae*, lactic acid bacteria (LAB) and yeasts and moulds (Hascoët et al., 2019; Møretrø & Langsrud, 2017).

Certain pathogens can also be found, such as *L. monocytogenes*, an opportunistic foodborne bacterium that causes the disease known as listeriosis (Amagliani et al., 2021; Quereda et al., 2021; Ranasinghe et al., 2021). It mainly affects the elderly, children, pregnant women, and people with depressed immune systems, causing sepsis, meningitis, and miscarriage (Colagiorgi et al., 2017). In the European Union in the decade 2012-2021, there was a marked increase in infections by this pathogen, associated especially with ready-to-eat products and milk (EFSA-ECDC, 2021b). Moreover, *L. monocytogenes* is one of the most relevant microorganisms related to surface sanitation and subsequent cross-contamination, because of its ability to form biofilms (Ripolles-Avila, Hascoët, et al., 2018). These are complex cell aggregates of one or more microbial species, which adhere to each other and/or to surfaces or interfaces in a matrix formed predominantly by extracellular polymers (González-Rivas et al., 2018). In these structures, it has been

observed that certain microbial interactions are developed, which could favour the growth and survival of microbial members of the biofilm community, as well as influence their virulence abilities (Peters et al., 2012). These interactions can be classified into cooperatives, where all species benefit in some way from the presence of others, either from the metabolism of certain substances beneficial for the growth of certain microorganisms, or from the elimination of metabolites which would slow their growth. Interactions can also be competitive, where there are limited nutrient sources or the production of compounds that suppress the growth of other species (Giaouris et al., 2015). The matrix protects the aggregates from the action of different antimicrobial substances and from cleaning and disinfection processes, thereby reducing their effectiveness (Mazaheri et al., 2020; Ripolles-Avila, Ramos-Rubio, et al., 2020). Different antibiofilm treatments are available in the market, one of which is based on hydrogen peroxide (H_2O_2), a strong oxidizing agent that has been demonstrated to damage bacterial DNA, proteins, and cellular membranes (Robbins et al., 2005) and has been reported as an effective disinfectant. However, understanding the impact of these antibiofilm treatments in real industrial conditions is necessary to determine their short and long-term effectiveness in terms of the resident microbial ecology.

In the food industry, different methods for sampling surfaces can be used to assess and control microbial contamination. To date, the techniques used have been based on two main ideas: friction, using swabs, sponges or similar, and direct contact, using for example contact plates (Brauge, Barre, et al., 2020). Luyckx et al., (2015) consider that friction methods are more sensitive than the other methods, while Brauge, Barre, et al., (2020) assures that the various methods do not present significant differences. However, the traditional methods may present some standardization problems when collecting samples from the relevant surfaces. To overcome this issue, hygiene surface sensors (SCH) have been designed to monitor microbiological contamination and offer an alternative to traditional sampling methods, and have been previously used in other studies (Hascoët et al., 2019; Ripolles-Avila, Hascoët, et al., 2019b).

The aim of the present study was to evaluate the microbiological contamination of different surfaces of an Iberian pig processing plant by implanting SCH sensors, and to apply an antibiofilm treatment to visualize its impact in an industrial scale study.

2. Material and methods

2.1. Processing plant and selection of sampling surfaces

Surfaces selected for the sampling and the location of the SCH sensors coincided with a previous study conducted by Ripolles-Avila, Hascoët, et al., (2019b). Samples came from two different industrial plants (*i.e.* A and B) belonging to the same company, which produces Iberian meat products. In Plant A, the raw and cured meat products are sliced and packaged ready for consumption, having come from plant B, a medium-sized industrial slaughterhouse with a slaughter capacity of 300 animals/day, and attached to a processing plant, where mainly cured meat products (*i.e.* hams, loins, fermented sausages such as *chorizo* and *salchichón*, among others) are made. **Table 20** summarizes the 13 different surfaces selected, which were chosen according as the points of the plants where high levels of microbial contamination had been previously detected by the company and where the presence of *L. monocytogenes* was detected (Ripolles-Avila, Hascoët, et al., 2019). SCH sensors were installed at these 13 points.

Table 20. Industrial surfaces where the SCH sensors were installed.

Processing plant	ID surface	Surface
A	1	Sump in the deboning room
	2	Slicer
	3	Sump in the slicing room
B	4	Floor of the carcasses airing room
	5	Storage cabinet for tools
	6	Floor of the work room
	7	Floor of the fresh meat carts cleaning room
	8	Floor of the cured meat carts cleaning room
	9	Slicing table
	10	Iberian sausage transportation carts
	11	Side of vacuum machine
	12	Floor of the heat treatment room
	13	Sink

* Plant A is where raw meat products and RTE are sliced and packaged. Plant B consists of a slaughterhouse and a processing room where cured meat products are prepared.

2.2. SCH sensors and sampling procedure

The sensors consisted of metal parts attached to a stainless-steel base via the action of neodymium magnets coated with an epoxy paint. The structures have three holes in which to place the sensors, enabling each sensor to be in the plant for three consecutive weeks before being collected for sampling. The sensors were fixed to the selected surfaces, which meant that they were contaminated and cleaned in the same way as the rest of the installations. Every week, one of the SCH sensors was extracted from the base structure using a sterile magnetic bar and aseptically placed in a sterile container. The samples were send to the laboratory where microbiological analysis was carried out.

2.3. Cell recovery from SCH

The first step was to recover microbial cells from the sensors. To do so, they were transferred into sterile containers containing 3.5 g of glass beads, adding 9 ml of peptone water (PW; bioMérieux, Marcy l'Etoile, France). The containers were shaken in a vortex for 1.5 minutes at 40 Hz of frequency, causing the attached cells to be dislodged from the SCH sensors, thereby making it possible to perform the corresponding microbiological quantifications and detections (Ripolles-Avila, Cervantes-Huaman, et al., 2019).

2.4. Microbiological analysis

For the quantification of microbial groups or species, the resulting suspension was decimally diluted in peptone water and transferred to different culture media, which differed depending on the microorganism to be quantified (*i.e.* total of seven different microbial groups). In the case of total aerobic mesophilic counts, the TEMPO system (TEMPO, bioMérieux, Marcy l'Etoile, France) was used. To this end, TEMPO vials were rehydrated with 3 mL of sterile distilled water, adding 1 mL of the sample's corresponding decimal solution. The inoculated vial was shaken to homogenize its content and then transferred to a reading card, which had 48 "wells" of three different volumes, using the filling unit. During incubation (*i.e.* 30ºC for 48 hours), microbial growth causes a change in the fluorescent signal of the medium, which is then detected by the TEMPO reading unit. Depending on the number and size of the positive wells, the system calculates the number of microorganisms present in the sample. For

enterobacteria, ISO 21528-2 standard was followed, cultivating the samples on Violet Red Bile Glucose Agar (VRBG Agar; Oxoid, Madrid, Spain) for 24 hours at 37ºC. For coliforms and *Escherichia coli*, ISO 4832 standard was followed, cultivating the samples in Compass ECC Agar (Oxoid, Madrid, Spain) for 24 hours at 37ºC. For *Staphylococcus* spp. coagulase positive determination, ISO 6888-3 standard was followed, using chromogenic Baird-Parker Agar with Rabbit Plasma Fibrinogen (BP-RPF Agar; Oxoid, Madrid, Spain) for 48 hours at 37ºC. For LAB, ISO 15214 standard was followed, cultivating the samples in the Man, Rogosa and Sharpe Agar medium (MRS Agar; Oxoid, Madrid, Spain) for 48 hours at 30ºC. Last, for the determination of yeasts and moulds, ISO 21527 standard was followed, using Sabouraud Agar (Oxoid, Madrid, Spain) and cultivating the samples for 5 days at 22ºC. After the incubation period had elapsed, microbial quantification was performed, and the calculations were made to express the results in CFU/cm^2.

In the case of *L. monocytogenes* and *Salmonella* spp. determination, detection procedures were carried out according to ISO 11290-1 and ISO 6579, respectively. In the case of *L. monocytogenes*, 1 ml of each of the recovered samples were transferred to 9 ml of Half Fraser broth (bioMérieux, Marcy l'Etoile, France) and incubated for 24 hours at 37ºC. The samples were then transferred to 9 ml of Fraser broth (bioMérieux, Marcy l'Etoile, France) and incubated again for 24 hours at 37°C. Last, and using a cultural loop, the incubated samples were transferred to ALOA Agar (bioMérieux, Marcy l'Etoile, France) and incubated for 24 hours at 37ºC. Assumedly positive colonies (*i.e.* positive β-glucosidase and phosphatidylinositol lipase activities) were confirmed by qPCR with an IQ-Check *Listeria monocytogenes* II PCR detection kit (Bioser, Barcelona, Spain). For *Salmonella* spp. detection, the recovered samples were incubated in the same container with peptone water for 24 hours at 37°C. Then, 0.1 ml of the pre-incubated sample was transferred to 9.9 ml of Rappaport-Vassiliadis broth (RVP, Oxoid, Madrid, Spain) and incubated for 24 hours at 42°C. Last, selective incubated samples were inoculated into Xylose Lysine Deoxycholate Agar (XLD Agar, Oxoid, Madrid, Spain) and incubated for 24 hours at 37°C to assess its presence.

2.5. Aplication of the antibiofilm treatment

The product selected was Betelene BF15 (Christeyns, Madrid, Spain), which contained hydrogen peroxide (≥ 10 % to ≤ 25 %), tetrasodium ethylenediaminetetraacetate (< 2.5 %), C12-14 alkyldimethyl amine N-oxide (< 2.5 %) and [[(phosphonomethyl)imino] bis[(ethylenenitrile)bis(methylene)]]tetrakisphosphonic acid (< 2.5 %). The product was diluted in water in a foam generating system to a final concentration of 3 % (w/w), applied for 15 minutes and then rinsed with water. Evaluated surfaces were the floor of the work room (*i.e.* surface 6) and the floor of the fresh meat cart cleaning room (*i.e.* surface 7). This aggressive antibiofilm treatment was applied every two weeks from November 2021 to April 2022 (*i.e.* from 16/11/2021 to 26/04/2022, alternating the treatment every fortnight).

2.6. Statistical analysis

The study had an approximate duration of 14 months, with samples taken over 46 weeks on the 13 points described, making a total of 598 samples analysed during the period. The results obtained from the microbiological counts were expressed in logarithmic units per square centimetre, while the results of the detection of pathogens were expressed as 0 or 1 depending on their absence or presence, respectively.

The obtained experimental data were used to make comparisons and observe trends via statistical analyses. The level of total microbial contamination among the different sampling areas was evaluated, performing an ANOVA with posterior Tukey contrast to find out if significant differences were present between the various sampling points within the same microbiological analysis, establishing $P < 0.05$ as significance level. At the same time, a classification by hierarchical clusters was conducted to distinguish the degree of contamination between areas. ANOVA with Tukey contrast was also used to determine significant differences in the points considered to be the most or least contaminated sites during the study.

3. Results and discussion

3.1. Global study of the microbiological contamination of the different sampling points

The evaluation of the contamination level of the surfaces was carried out by implementing SCH sensors in determinate locations of the food processing plant. The sensors were kept in place for the established period, reflecting the conditions of the industrial surfaces. Authors such as Ripolles-Avila, Hascoët, et al., (2019) and Hascoët et al., (2019) have demonstrated the representativeness of results using SCH, consequently considering the use of these sensors as an appropriate surface sampling system.

Table 21 shows the mean of the contamination levels, including all microbial groups and species evaluated, along with their standard deviation from the total samples analysed in each week of the study. Significant differences ($P < 0.05$) were observed between different sampling areas and the microbial counts in all parameters except for two, *E. coli* ($P = 0.523$) and coagulase positive *Staphylococcus* spp., since the microorganism was not detected on any surface during the study. Regarding the results derived from *E. coli*, previous studies have shown that dominant resident microbiota from meat industries does not affect its growth (Hascoët et al., 2019), although *Staphylococcus* species could favour its presence, which would justify its low presence in the study (Marouani-Gadri et al., 2009). Other authors also indicate that biofilms of LAB inhibit *E. coli* growth (N. N. Kim et al., 2019; Patel et al., 2021; Tan et al., 2017), although these inhibitory actions depend directly on particular species (Cisneros et al., 2021), with no clear relationship indicated in the present study because the three surfaces where counts of this microorganism were found coincided with the highest counts for LAB.

Moreover, no significant differences were obtained for *Enterobacteriaceae* counts ($P = 0.162$) from the different monitored areas, except on the sink (*i.e.* surface 13). Ripolles-Avila, Hascoët, et al., (2019) evaluated the same pig processing plant and surfaces in the period 2016-2018, and for *Enterobacteriaceae* counts found completely homogeneous contamination between sampled areas with no significant differences obtained ($P > 0.05$). Therefore, approximately five years later, only the sink had significantly increased its *Enterobacteriaceae* count level. Differently, when evaluating coliforms, a microbial group considered as faecal indicators and highly analysed on industrial surfaces (Reitter et al., 2021), variable contamination levels were found. Álvarez Gurrea, (2015) indicated

that an increase in microbial loads of *Enterobacteriaceae* and coliforms may be related to work accumulation or markedly increased movement of handlers in these areas. In this case, the surfaces that presented a higher coliforms contamination in comparison to the rest of the areas ($P < 0.05$) were the floor of the fresh meat cart cleaning room (*i.e.* surface 7), the side of the vacuum machine (*i.e.* surface 11) and the sink (*i.e.* surface 13), which coincide with surfaces that are continuously in use and also presented *Enterobacteriaceae* counts.

LAB and yeast and moulds were detected on all surfaces, although with variability among the different areas ($P < 0.05$). These types of microorganisms are markedly present in meat industries (Chevallier et al., 2006; Talon et al., 2007) due to the technological processes carried out and the type of products being processed, predominantly when they are cured and fermented (Gounadaki et al., 2008).This can be observed in **Table 21**, where the surface with the highest counts for LAB and yeast and moulds (*i.e.* 3.05 Log CFU/cm^2 and 3.57 Log CFU/cm^2, respectively) was the slicer (*i.e.* surface 2), which has direct contact with the fermented products.

Table 21. Global average of the microbial counts obtained from the different areas where the SCH sensors were installed as a sampling method throughout the 74 weeks of study in two Iberian pig processing plants (Plant A and B). The number of times that *Salmonella* spp. and *L. monocytogenes* were detected is also included. The data represents the mean in Log (CFU/cm^2) ± the standard error of the mean.

		Log (CFU/cm^2)							Absence/Presence	
Processing plant	**Surface**	**Aerobic count**	**Enterobacteria**	**Coliforms**	***E. coli***	**Coagulase positive *Staphylococcus***	**Lactic acid bacteria**	**Yeast and moulds**	***Salmonella* spp.**	***L. monocytogenes***
A	Sump in the deboning room	1.79±0.38^{abcd}	0.44±0.22^{a}	0.37±0.20^{ab}	0.00±0.00^{a}	0.00±0.00^{a}	1.60±0.41^{bc}	2.06±0.42^{cde}	0	0
	Slicer	3.30±0.28^{de}	0.04±0.05^{a}	0.05±0.06^{a}	0.00±0.00^{a}	0.00±0.00^{a}	3.05±0.38^{d}	3.57±0.28^{e}	0	0
	Sump in the slicing room	0.86±0.21^{ab}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.57±0.19^{abc}	0.47±0.13^{ab}	0	0
B	Floor of the carcasses airing room	3.48±0.49^{e}	0.61±0.26^{a}	0.51±0.20^{ab}	0.00±0.00^{a}	0.00±0.00^{a}	1.83±0.33^{cd}	2.82±0.42^{de}	0	0
	Storage cabinet for tools	2.08±0.41^{bcde}	0.43±0.19^{a}	0.31±0.13^{ab}	0.00±0.00^{a}	0.00±0.00^{a}	1.26±0.39^{abc}	2.01±0.42^{bcde}	0	1
	Floor of the work room	1.43±0.25^{abc}	0.07±0.05^{a}	0.07±0.03^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.63±0.16^{abc}	0.73±0.20^{abc}	0	1
	Floor of the fresh meat carts cleaning room	2.91±0.37^{cde}	0.80±0.28^{a}	0.76±0.23^{abc}	0.21±0.22^{a}	0.00±0.00^{a}	1.65±0.36^{cd}	2.66±0.46^{de}	0	0
	Floor of the cured meat carts cleaning room	2.09±0.35^{bcde}	0.27±0.16^{a}	0.23±0.13^{ab}	0.09±0.10^{a}	0.00±0.00^{a}	1.81±0.38^{cd}	1.93±0.38^{abcd}	0	4
	Slicing table	1.37±0.26^{abc}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.89±0.26^{abc}	0.75±0.23^{abc}	0	0
	Iberian sausage transportation carts	1.49±0.23^{abc}	0.06±0.04^{a}	0.04±0.04^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.92±0.23^{abc}	0.55±0.22^{abc}	0	0
	Side of vacuum machine	0.51±0.25^{a}	0.10±0.11^{a}	1.02±0.33^{bc}	0.00±0.00^{a}	0.00±0.00^{a}	0.22±0.17^{ab}	0.42±0.25^{a}	0	0
	Floor of the heat treatment room	0.73±0.17^{ab}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.13±0.04^{a}	0.59±0.20^{abc}	0	0
	Sink	3.14±0.54^{de}	2.01±0.51^{b}	1.47±0.34^{c}	0.07±0.05^{a}	0.00±0.00^{a}	1.68±0.43^{cd}	2.55±0.55^{de}	0	1

3.2. Contribution of all microbial agents involved

The microbiota found on industrial surfaces, which can influence the hygiene status of a final product, depend directly on the type of food industry (Maes et al., 2019). The contribution of each microbial group to the total contamination in the monitored meat processing industry is shown in **Figure 11**. Top of the list of agents involved are the mesophilic aerobes, accounting for 34.70 % of the total contamination, followed by yeasts and moulds with 29.01 %, with no significant differences ($P = 0.123$) observed between groups. These results coincide with those of other studies, where mesophilic aerobes are likewise the first contamination contributors (Barros et al., 2007; Ripolles-Avila, Hascoët, et al., 2019). Food industries generally include mesophilic counts in their routine evaluations of microbial contamination of food industrial surfaces as they are considered good hygiene indicators (Pedonese et al., 2020). However, there are few studies on the determination of resident mesophilic aerobes on surfaces in the food industry, especially ones that focus on isolates of these bacteria in food production environments. Therefore, extensive information on growth characteristics and survival of this microbial group in these environments is lacking, probably because these bacteria are of less relevance to food safety and quality than *Staphylococcus* spp., *Bacillus* spp., and LAB, among others (Møretrø & Langsrud, 2017). The third most contributing agent was lactic acid bacteria (*i.e.* 22.38 % of the total contamination and $P < 0.05$ among the rest of groups), which are a group of bacteria that is highly detected in environments where fermentation takes place (Gounadaki et al., 2008; Talon et al., 2007). The main reasons for the dominance of these agents are defined by the environmental and working characteristics of the company, and are especially related to the raw materials used and certain habits of different countries (Talon et al., 2007).

Moreover, the isolation of different dominant microbial groups on industrial surfaces has revealed a great heterogeneity and disparity in the results, depending on the industry. Some authors have determined that after cleaning and disinfection in meat processing environments, *Pseudomonas* spp., *Microbacterium* spp., and enterobacteria, such as *Serratia* spp. remain as resident microbiota at rates of 84 %, 11 % and 4 %, respectively (Brightwell et al., 2006; Gounadaki et al., 2008). The same authors indicated that most of the sampled areas were highly contaminated by spoilage microbiota, such as *Pseudomonas* spp. and enterobacteria, with the most contaminated surfaces being blades,

tables, and mincers. In contrast, other authors have observed that resident bacteria after cleaning and disinfection are lactic acid bacteria, *Staphylococcus* spp. and *Bacillus* spp., and *Yersinia* spp. (Hultman et al., 2015). In a comparison made between two different meat industries, Stellato et al., (2016) pointed out that *Brochothrix* spp., *Psychrobacter* spp., *Pseudomonas* spp., and *Acinetobacter* spp. were found in both, but as subdominant populations, and lactic acid bacteria, *Streptococcus* spp. and *Carnobacterium* spp. as dominant populations.

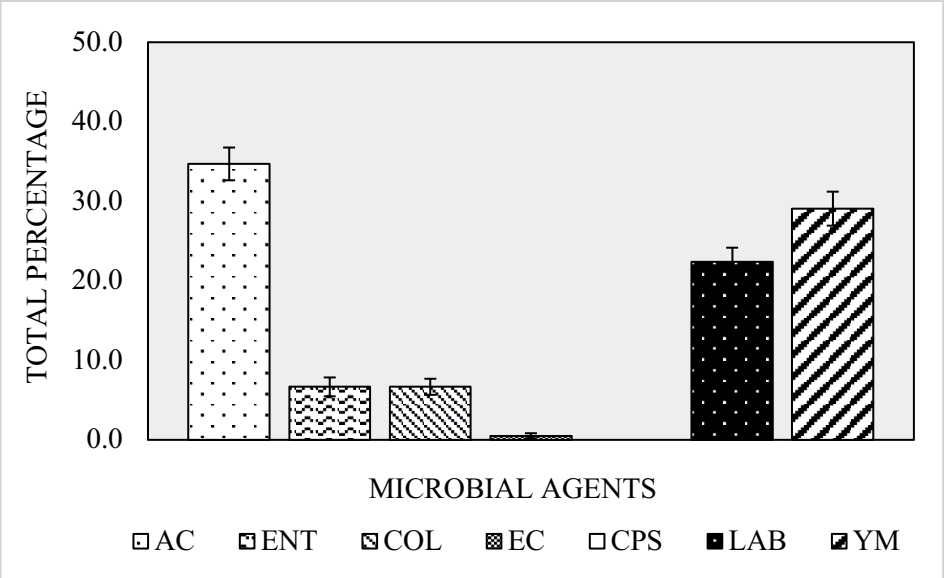

Figure 11. Contribution percentage of aerobic counts (AC), *Enterobacteriaceae* (ENT), coliforms (COL), *Escherichia coli* (EC), coagulase positive *Staphylococcus* (CPS), lactic acid bacteria (LAB) and yeast and moulds (YM) for the total microbiological contamination (n=1183 in total, n=169 for each microbial group) throughout the study. Error bars indicate standard error of the mean (SEM).

3.3. Classification of evaluated areas by greater and lesser degree of microbial contamination

To characterize the different working areas and classify them according to their degree of contamination, a hierarchical clusters analysis was performed using the aerobic counts obtained from the different surfaces as the major contributor agent (**Figure 12**). As can be observed, two differentiated conglomerates can be highlighted. First, the conglomerate that groups the areas at the bottom of the figure (*i.e.* the slicer, the floor of the carcass airing room, the floor of the fresh meat cart cleaning room and the sink), which can be

considered as the surfaces with the highest contamination since the highest counts were obtained from them. The group is made up of heterogeneous areas, ranging from floors, walls, and work surfaces. In this regard, Eisel et al., (1997) evaluated the microbial load of mesophilic aerobes, total coliforms and *E. coli* of various surfaces in contact with food, equipment, walls and floors, determining that the walls and floors were the most contaminated surfaces. Soils are an important source of contamination since resident microorganisms can be transferred to different areas of an industry on workers' shoes as they circulate within the establishment, so disseminating them. In fact, (Barros et al., 2007) indicated that the fresh product storage boxes were one of the points with the highest contamination of mesophilic aerobes and total coliforms, and it was observed that these had previously been in direct contact with floors, either those of the plant itself or those of the refrigeration chambers and the retail sales rooms. Drains and floors can provide a favorable environment for microbial growth and, consequently, are an important reservoir, as has been shown for *Pseudomonas* spp. and *Aeromonas* spp. (Hood & Zottola, 1997), *Salmonella* spp. (Rivera-Betancourt et al., 2004) and *L. monocytogenes* (Ciccio et al., 2012). Floors can be a direct source of spread, especially if cleaning is done with high pressure water, a practice that can spread contamination by suspending microorganisms in the air in small water droplets (Barros et al., 2007). For this reason, it is extremely important to design and apply the cleaning and disinfection operations in the food industry correctly.

The second conglomerate, containing the rest of the areas, was considered as the lowest contributor to contamination. Moreover, this second conglomerate can be observed to initially contain a total of three well-defined clusters. The statistical analysis performed, which can be extracted from **Table 21**, coincides with the results obtained in the hierarchical cluster analysis, where the areas farthest from each other showed significant differences ($P < 0.05$), as opposed to those closest to each other ($P > 0.05$), for example the floor of the work room (*i.e.* surface 6), the slicing table (*i.e.* surface 9) and the Iberian sausage transportation carts (*i.e.* surface 10), whose subsets of values were the same. As has been already mentioned, in the study conducted by Ripolles-Avila, Hascoët, et al., (2019), the same surfaces were evaluated as in the present study. In this case, the floor of the carcass airing room (*i.e.* surface 4) and the floor of the fresh meat cart cleaning room (*i.e.* surface 7) were also considered as surfaces with a greater degree of contamination, which would indicate that it a historically accumulating area of microorganisms.

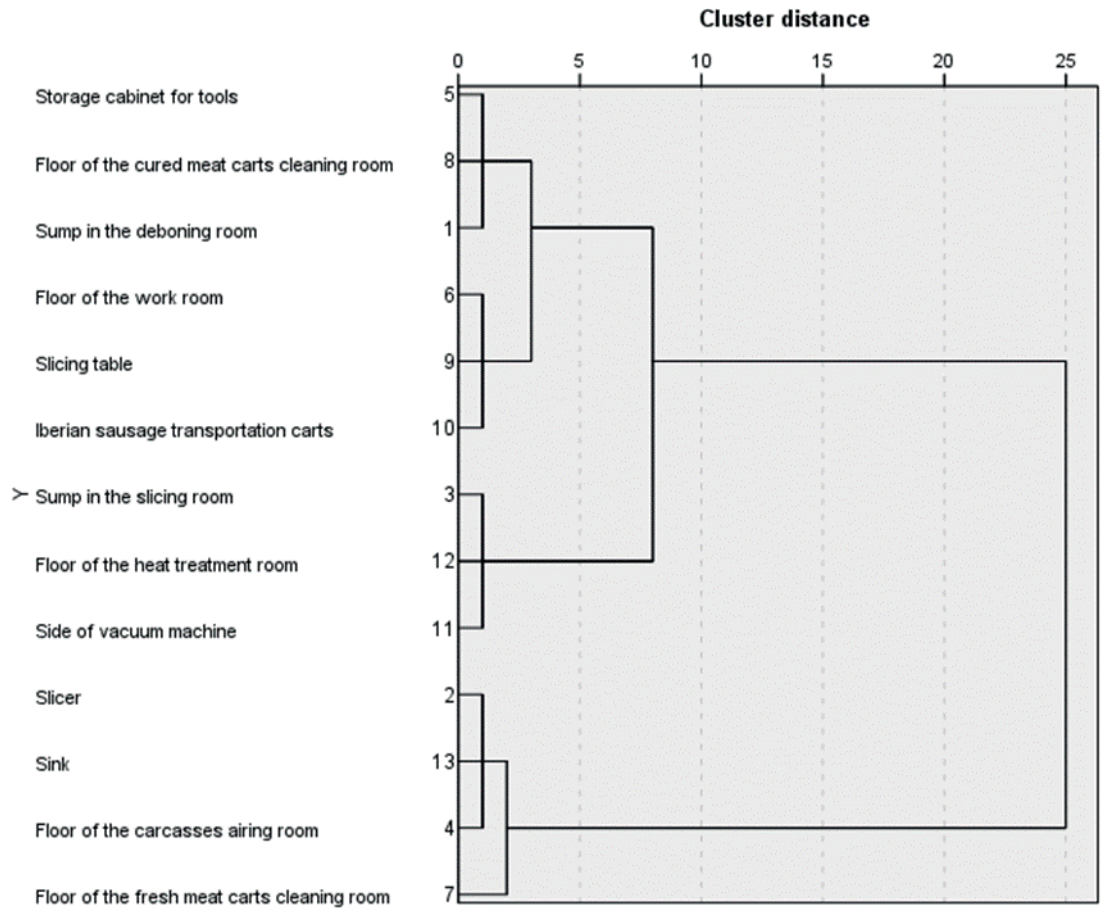

Figure 12. Recombination between the different sampling areas and their subsequent assignment in clusters by similarity based on the mesophilic aerobic counts from the total contamination.

Focusing on the areas with the greatest contamination, the four surfaces were further analysed to observe the percentage of microbial agents involved by surface, and to see if there were any correlations (**Figure 13**). Aerobic count was the maximum contributor of microbial contamination in all areas except for the slicer. However, no equal contribution of the different microbial groups was observed, which could be caused by the working characteristics of each area (Talon et al., 2007). A high percentage of lactic acid bacteria and yeasts and moulds were obtained for the slicer, which could be related to transfer of the cured and fermented food products to the work surface, where they are in constant contact. This trend could also be related to the sink, where higher percentages of enterobacteria and coliforms compared to other points were obtained. The sink is an area that has already been seen to provide a favourable environment for the development of these groups of bacteria (Barros et al., 2007). The surface that presented the greatest

percentage of aerobic counts when analysed by area was the floor of the carcass airing room. This fact could be attributed to its working mechanism. Immediately after the slaughtering process, the carcasses go to the airing room where they are stored until transported to the deboning room. The temperature of the carcass following slaughter ranges from between 30ºC on the surface to 40ºC inside the animal. It is recommended that this temperature decreases below 7ºC before 16 hours have passed post-death (Merai et al., 2019). To do so, the temperature in the chamber gradually decreases over time until the target temperature is reached. These temperature levels, the long duration, and the fact that there is a lot of movement in this area could be the causes of the high counts of mesophilic aerobic microorganisms.

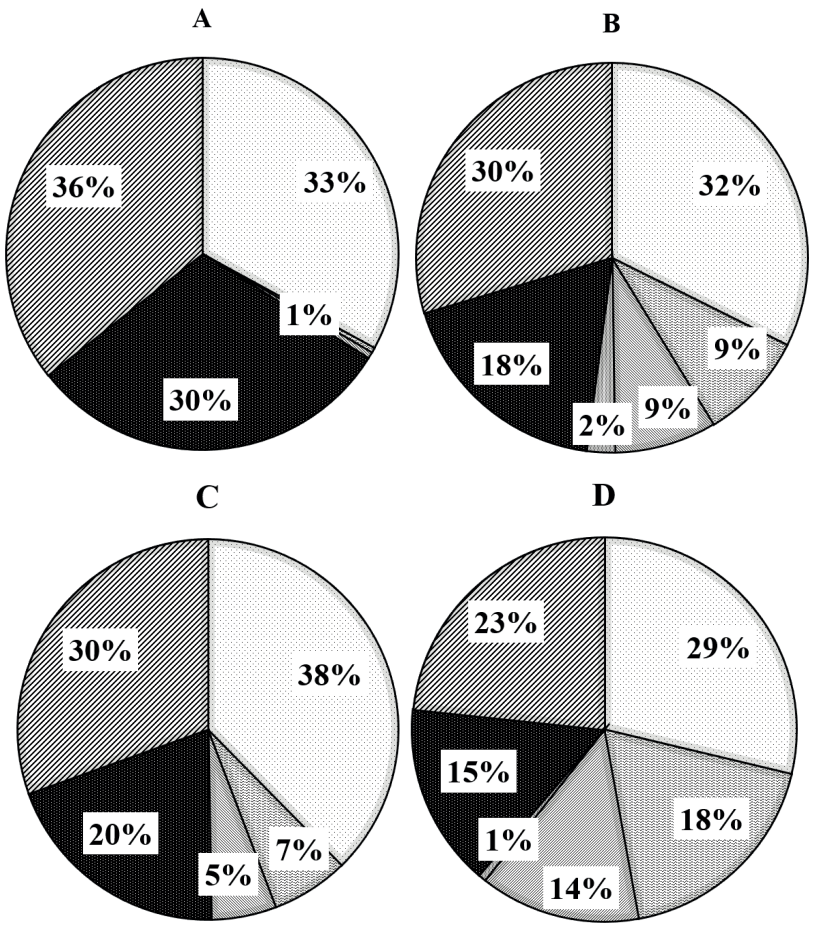

Figure 13. Contamination percentage of aerobic counts (AC), *Enterobacteriaceae* (ENT), coliforms (COL), *Escherichia coli* (EC), coagulase positive *Staphylococcus* (CPS), lactic acid bacteria (LAB) and yeast and moulds (YM) in the areas considered as the greatest contaminated surfaces: (A) Slicer, (B) Floor of the carcass airing room, (C) Floor of the fresh meat cart cleaning room, (D) Sink.

If a comparison between the surfaces considered as the greatest contaminated areas in terms of the aerobic counts obtained is made over the months of the study, no clear relation is observed between months except in a few cases (**Figure 14**). This would indicate that a high aerobic count obtained from one surface does not necessary imply the same for the rest of the greatest contaminated areas in the same month. This may suggest that a high count at a certain point and at a given time cannot significantly be spread to other areas of the factory with a persistent and noticeable effect at month level. It has been reported that workers' movements throughout the food plant causes contamination to be spread throughout the plant (Barros et al., 2007), but this can be a transitory issue if cleaning and disinfection procedures are correctly applied. From the results, it can also be suggested that the Iberian pig processing plant under evaluation is working effectively in this sense, preventing cross-contamination by employee footwear and clothing.

Moreover, the months when the highest aerobic counts were obtained in this study, which can be clearly observed from the average percentage calculated in **Figure 14**, were from June 21' to September 21' which are months when temperatures in Spain tend to be at their annual highest. Djekic et al., (2016) reported that when there are high temperatures of between 16ºC and 20ºC, higher levels of enterobacteria and *Staphylococcus* are detected, both on handlers' hands and on industrial surfaces. It has also been reported that at a higher level of precipitation, there are more elevated counts of different microorganisms on surfaces. Further, if we compare March 2021 and March 2022, one of the two months that coincide in the two years, some conclusions can be drawn. Over one year, the contamination on the slicer and on the floor of the carcass airing room was stable as no significant differences were obtained ($P > 0.05$), with an increase of 0.50 % and 2.90 %, respectively, from 2021 to 2022. In contrast, an increase in contamination was observed on the floor of the fresh meat cart cleaning room and on the sink, reaching 17.6 % and 18.98 %, respectively. Although at first glance it may seem that the increase could be significant, statistical analysis demonstrated non-significant differences ($P = 0.148$ and $P = 0.119$, respectively). The increase in contamination levels could be related to deficiencies in hygiene in the corresponding months or the cleaning system employed. Washing with high pressure water should be avoided, as it can favour the spread of microorganisms, which would be suspended in the air along with the small water droplets, causing a further critical point of recontamination (Barros et al., 2007).

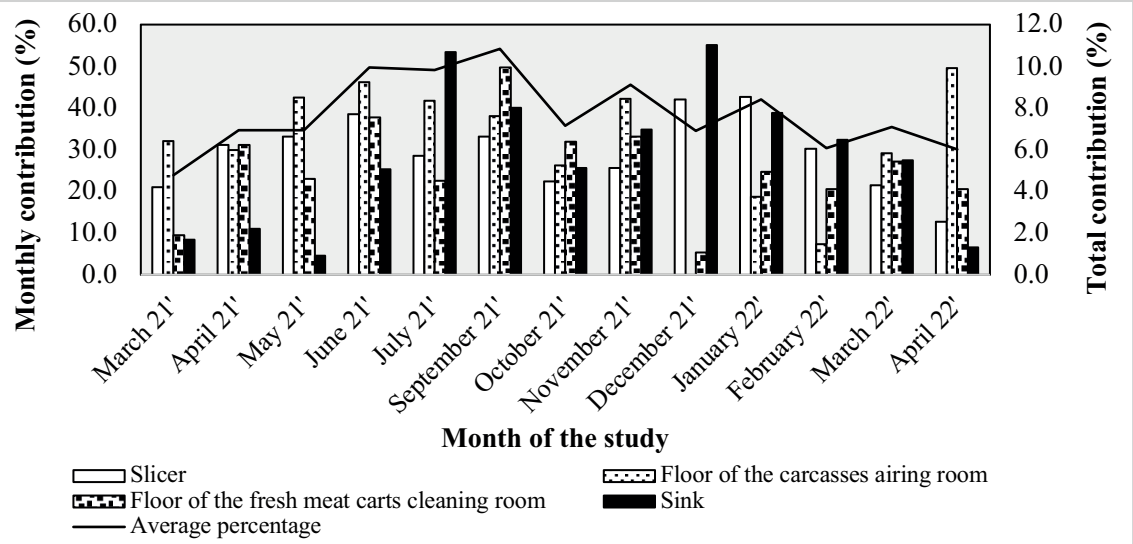

Figure 14. Comparison of the aerobic counts (AC) obtained monthly on the points considered as the greatest contaminated areas over the year of the study. A trend line indicating the average percentage of each month on the AC is included.

3.4. Detection of Listeria monocytogenes on the evaluated surfaces

Throughout the study, there were a total of five areas where *L. monocytogenes* was detected. **Figure 15** shows the counts of the different microorganisms analysed and their evolution over the months. Previous studies have indicated that there may be a correlation between aerobic counts and *L. monocytogenes* presence (Ripolles-Avila, Hascoët, et al., 2019). In this regard, it has been reported that when aerobic counts are between 2 and 4 Log (CFU/cm^2), the pathogen is not present. Three possibilities have been raised to explain this situation: (i) there may be microorganisms that inhibit *L. monocytogenes* growth, so when the aerobic count increases, the pathogen cannot grow due to an incompatibility with mixed growth; (ii) there might be microorganisms that promote *L. monocytogenes* growth, so the pathogen would be detected by increasing the total aerobic count, and if counts are low, the pathogen would not appear as promoters would be missing; (iii) the last possibility would be a combination of the previous two, where the pathogen could appear in high and low aerobic counts because inhibitors and promoters might coexist in the ecosystem as a whole.

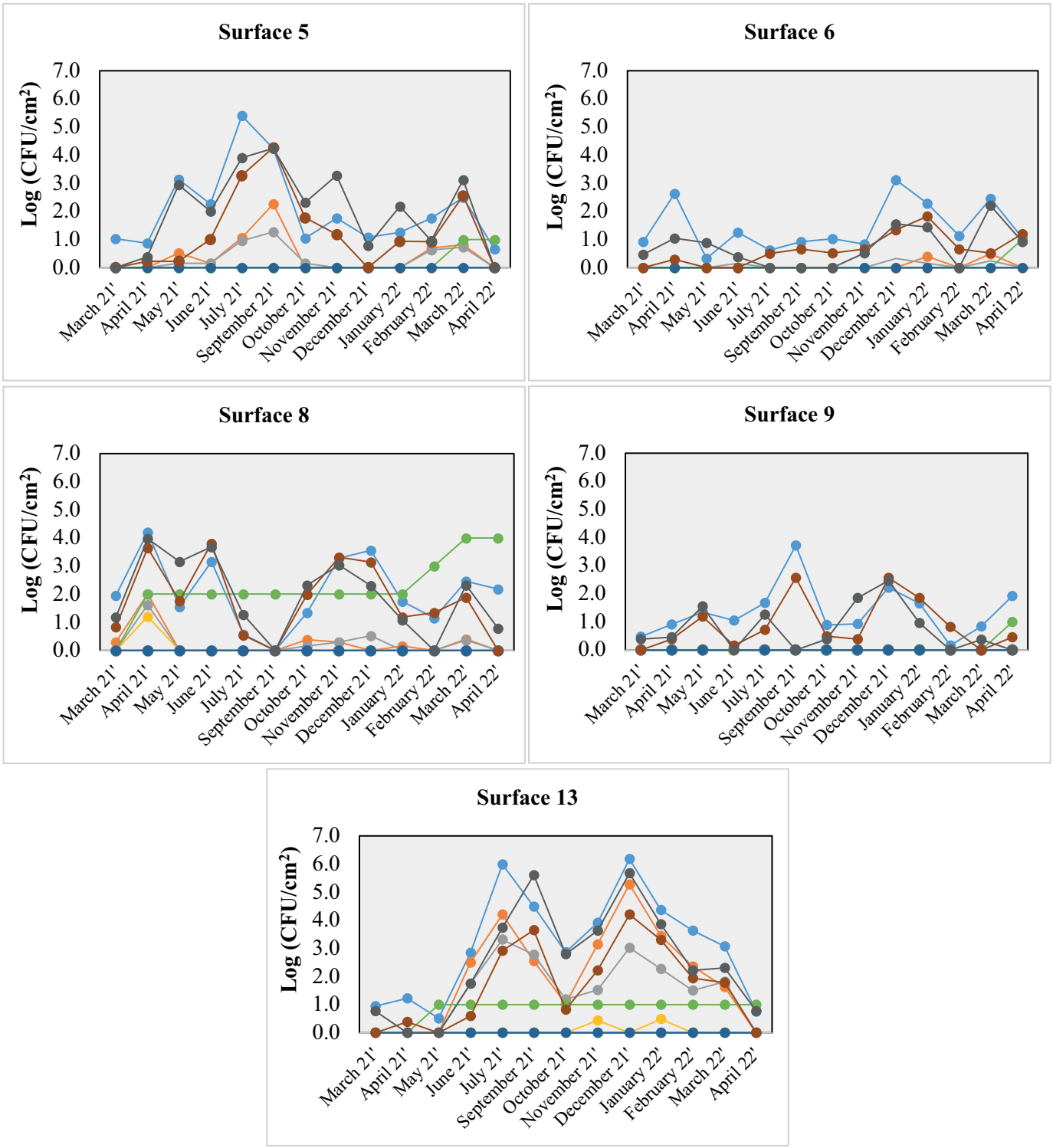

Figure 15. Monthly evolution of the microbial counts obtained from some of the surfaces evaluated in the meat industry under study. The graph shows both the Log count (CFU/cm^2) for aerobic count (—●—), *Enterobacteriaceae* (—●—), coliforms (—●—), *E. coli* (—●—), positive coagulase *Staphylococcus* (—●—), lactic acid bacteria (—●—), and yeasts and moulds (—●—), and the cumulative detection of *Salmonella* spp. (—●—) and *L. monocytogenes* (—●—), assuming that each presence equalled 1.

The results demonstrated that surfaces 5 (*i.e.* storage cabinet for tools) and 8 (*i.e.* floor of the cured meat cart cleaning room) obtained a total of three presences in April 2021, one on surface 5 and two on surface 8, and were associated with aerobic counts of 5.24, 6.23 and 5.94 Log (CFU/cm^2), respectively. Contrarily, on surfaces 9 (*i.e.* slicing table), 13 (*i.e.* sink) and 8 (*i.e.* floor of the cured meat cart cleaning room) in February and March 2022, pathogen presence corresponded to aerobic counts of 1.89, 0.00, 1.34, 1.76 Log (CFU/cm^2), respectively. Last, on surface 6 (*i.e.* floor of the work room) the presence of the pathogen was associated with values of 2.14 (CFU/cm^2) of mesophilic aerobes. Regarding the safety area hypothesis, in all cases except surface 6 it would be met, by mesophilic aerobic values either above or below the established limit (*i.e.* 2 to 4 Logs), following the theories of competitive inhibition, competitive stimulation and variable competition.

Moreover, a trend that is repeated in all detections is the concentration ratio between mesophilic aerobes, LAB and yeast and moulds. When the pathogen is present in a high count of mesophilic aerobes, there is a parallel increase in the concentration of the other two (*i.e.* LAB and yeast and moulds), and when mesophilic aerobes values are low, so are those of the other two. This could be related to a possible symbiotic interaction between the three microbial groups, thus favouring their growth. This possibility is indicated in other studies (Gounadaki et al., 2008; Talon et al., 2007), where a correlation between *L. monocytogenes* growth and high counts of these species is also observed. In contrast, other authors such as Kim et al., (2022) have observed that on food contact surfaces the presence of biofilms of certain LAB (*e.g. Lactobacillus sakei* and *Pediococcus pentosaceus*) produce adverse effects for the growth of different pathogens, among which *L. monocytogenes* is included. Bogéa et al., (2021) have also shown that certain LAB, such as *Bifidobacterium animalis* and *Lactobacillus plantarum*, have an inhibitory action on *L. monocytogenes*, delaying biofilm formation. Another factor that has been shown to favour *L. monocyotgenes* growth is the level of enterobacteria, where high counts stimulate its growth (Hascoët et al., 2019). In the case of the present study, this was not entirely fulfilled and when the pathogen was present, with enterobacteria levels very low or non-existent.

3.5. Application of the antibiofilm treatment

Surfaces 6 (*i.e*. floor of the work room) and 7 (*i.e.* floor of the fresh meat cart cleaning room) were selected because they are small rooms where it was easier to apply the treatment and control the impact on the microbial load in a more representative way. In addition, both areas have been determined as relevant either due to the presence of *L. monocytogenes* or because they had obtained high microbial counts in previous studies (Ripolles-Avila, Hascoët, et al., 2019). The results obtained on the effectiveness of the treatment applied every two weeks from November 2021 to April 2022 are presented in **Figure 16**. As can be observed, the aggressive antibiofilm treatment was able to reduce the microbial load in most of the weeks when the treatment was applied compared to the previous week (*i.e.* when the treatment was not applied). In general, a cyclical pattern of decrease and increase was observed depending on when the antibiofilm treatment was applied on both surfaces 6 and 7. For example, on surface 7, before starting to apply the aggressive antibiofilm treatment, high counts of mesophilic aerobes, LAB, and yeast and moulds were present on the monitored surfaces. Once applied, a decrease in the counts was observed the week after the treatment was applied, and likewise after applying the following treatment, going from an approximate aerobic count of 6 Log CFU/cm^2 to an approximate count of 0 following the two consecutive treatments applied within two weeks in a one-month study. Moreover, and notably, in most of the cases the treatment did not achieve the complete elimination of all microbial groups. It has been reported that an antibiofilm treatment that can meet this objective is the application of enzymatic treatments (Nahar, Mizan, et al., 2018; Yuan et al., 2021). Therefore, the evaluation of enzymes included in formulations for aggressive treatments of industrial surfaces would be extremely interesting to understand not only efficacy, but also microbial patterns that conform resident microbiota.

From a long-term perspective, if the data are grouped monthly, for surface 7 (data not shown) we can conclude that after the aggressive antibiofilm treatment was implemented, counts of all the microbial groups assessed were lower than in previous months (*i.e.* from March 2021 to October 2021). To date and to our knowledge, no studies have modelled

the impact of aggressive antibiofilm treatments in a real food industry in the short and long terms, so comparisons cannot be made.

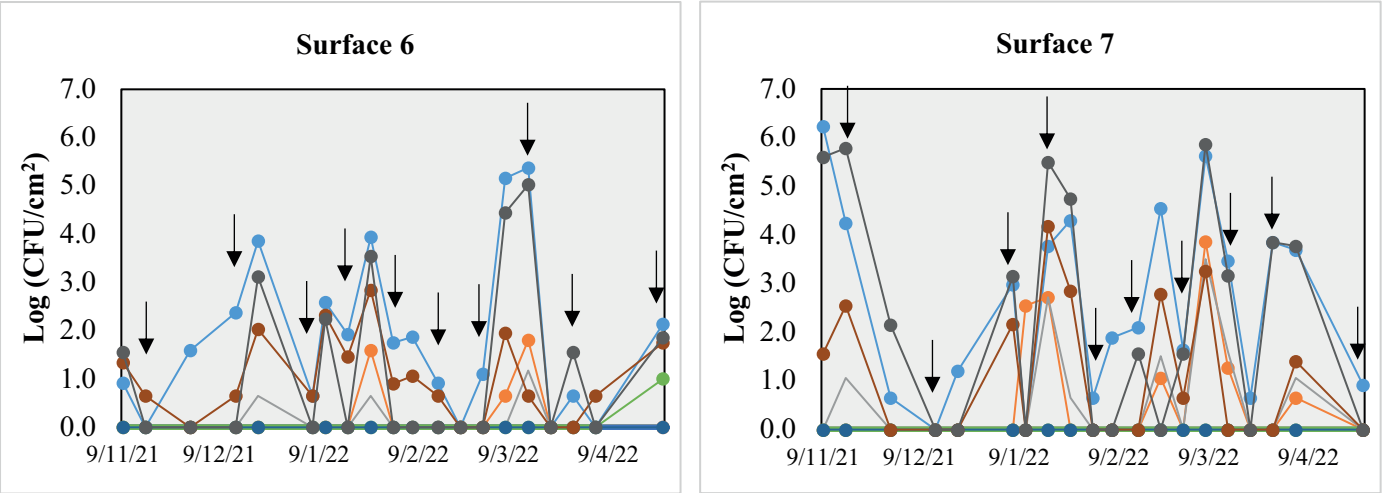

Figure 16. Weekly evolution of the microbial counts obtained from the monitored surfaces where the antibiofilm treatment was applied (*i.e.* floor of the work room - surface 6; and floor of the fresh meat cart cleaning room - surface 7). The graph shows both the Log count (CFU/cm^2) for aerobic count (—●—), *Enterobacteriaceae* (—●—), coliforms (—●—), *E. coli* (—●—), positive coagulase *Staphylococcus* (—●—), lactic acid bacteria (—●—), and yeasts and moulds (—●—), and the cumulative detection of *Salmonella* spp. (—●—) and *L. monocytogenes* (—●—), assuming that each presence equalled 1. Arrows indicate when the antibiofilm treatment was applied.

CHAPTER 8

General discussion

Public administrations, the different members of the food production chain and consumers are highly concerned about food safety. The microbiological contamination of foods, considered for a long time as one of the main triggers of foodborne illnesses, is more than an evident problem (Faille et al., 2017). One of the main routes of food products contamination is cross-contamination by biofilms residing on industrial surfaces (González-Rivas et al., 2018). That is why there is a constant microbiological evaluation of such surfaces focused on understanding their hygienic situation (Møretrø & Langsrud, 2017). In addition to this food-induced contamination there is the obvious effect of climate change. Historically, the official statistics of Spain reflect a clear seasonality in terms of occurrence of product contamination. In fact, between the years 2012 and 2020, the critical months were those comprised between May and October, with the peaks of greatest intensity in the months of July and August (Martín et al., 2012). According to these authors, the number of outbreaks in summer doubled those detected in winter. Therefore, an increase in the average temperature will directly imply a greater risk of transmission of pathogens with an uncontrolled origin. Surfaces can be one of the most important reservoirs of pathogens such as *L. monocytogenes* or *Salmonella* spp. In this situation, specific monitoring and control is essential, both for surface contamination itself and for better management of pathogen elimination systems. These systems must be based on cleaning and disinfection aimed at their control.

For these reasons, the studies that accompany this thesis try to contrast and simulate several important aspects for the food industry and for the health of potential consumers. On the one hand, focused on *L. monocytogenes* as one of the most relevant pathogens today (EFSA-ECDC, 2017), an enzymatic treatment was applied for the elimination of mature biofilms formed by different *L. monocytogenes* strains. For that purpose, a model system for the *in vitro* formation of mature biofilms was used in order to simulate what happens in the industrial environment. The use of biofilm formation models in their mature stage is absolutely crucial to understand how to reproduce them on a laboratory scale, but at the same time in a way that reflects the industrial reality. (Ripolles-Avila, Hascoët, et al., (2018) demonstrated that the best initial incubation period was the one established after 48 hours, compared to 24 hours or 72 hours. The pathogen needs at least this time to irreversibly bind to the surface and initiate structure formation (Moltz & Martin, 2005; Norwood & Gilmour, 1999). This fact is truly concerning at the industrial level when production stops at the weekend. In this period, *L. monocytogenes* could begin

to consolidate the biofilm. On the study of Ripolles-Avila, Hascoët, et al., (2018), it was also shown that the maximum growth obtained under the different *in vitro* conditions tested was after one week of incubation, with different washes and renewal of nutrients (*i.e.* 48 hours + 24 hours + 24 hours + 72 hours, with 7.25 Log CFU/cm^2 and a cell survival rate of 94.47 %). Those results coincided with the ones reported in our comparison of the biofilm formation of different *L. monocytogenes* strains before the enzymatic treatment was applied. Nevertheless, strains showed different growth levels and structure generation. In all cases, a structure was also observed, which included certain indicators of biofilm maturity, such as a three-dimensional network of superimposed cells, with interstitial voids, similar to the honeycomb-like morphotypes already described for *L. monocytogenes* (Guilbaud et al., 2015; Marsh et al., 2003). Our results, in conjunction with the ones reported by (Ripolles-Avila, Hascoët, et al., 2018), suggest that the way of working at an industrial level, with weekend halts and incomplete intermediate washings or cleanings, can allow optimal conditions for the formation of biofilms by this pathogen. If, in addition, the cleaning is not adequate to eliminate the three-dimensional structures created, we can estimate that the risk of *L. monocytogenes* presence will be very significant. Perhaps for these reasons we have shifted from doubting whether this microorganism was or not pathogenic in 1995 (FAO, 1995) to be confirmed as one of the pathogens with highest risk in 2022. In this regard, many cleaners and disinfectants, as they are used in the food industry, are ineffective against biofilm removal. Amongst them, the use of enzymatic detergents should be highlighted, which have been shown to improve the efficacy of biofilm detachment from surfaces (Stiefel et al., 2016). After the application of the enzymatic treatment in the first study, results showed that the maximum obtained reduction was approximately 6.9 Log CFU/cm^2 for the strain CECT 5672, with a mature biofilm removal rate between 85 to 99 % for the different *L. monocytogenes* strains. These results coincide with those obtained in other studies included in the present thesis, especially when different enzymatic treatments with distinct concentrations of enzymes and application temperatures were compared, inasmuch as others reported by authors such as Puga et al., (2018) that accomplished biofilm dispersion of 90 %. Detachment of mature biofilms may be directly related to the strain of *L. monocytogenes* in question, which could be due to the fact that certain strains can generate structures with a more robust matrix (Ripolles-Avila, Cervantes-Huaman, et al., 2019), making their detachment more difficult as also reported by (Ripolles-Avila, Ramos-Rubio, et al., 2020).

After the initial evaluation of a modelized enzymatic treatment on different *L. monocytogenes* strains, the strain with the highest resistance to the treatment was selected and the elimination of mature biofilms with other different treatments was compared. Conventional treatments are not effective enough to completely remove *L. monocytogenes* biofilms from surfaces, thus implying the presence of certain persistent bacterial forms. That is why the development of alternatives that replace or complement their traditional use in the food industry has become a current need (Srey et al. 2013). To that end, 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, the strain with the highest resistance. 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). The 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. The observation of the remaining biofilm structure by DEM proved that conventional treatments were unable to completely remove conformed structures, with the potential risk that this entails. Enzymatic treatments applied at 50ºC produced the greatest detachment and biocidal activity, which is in agreement with the findings previously reported by other authors (Araújo et al., 2017; Lequette et al., 2010; Molobela et al., 2010). It is important to note that improving cleaning products can lead to reduced disinfection needs. In this sense, the inclusion of antibiofilm enzymes in detergents will break the matrix of these structures, releasing the microorganisms that compose them. Once removed, the surfaces will not need intense disinfectant treatments, which will reduce the high consumption of water and energy linked to these industrial processes. It is precisely the ability of these enzymes to break and disintegrate the produced structure that makes the microorganisms more vulnerable to subsequent disinfection (Ripolles-Avila, Ríos-Castillo, & Rodríguez-Jerez, 2018) . However, it is interesting to remark that the detachment efficacy of mature *L. monocytogenes* biofilms obtained in the present study could reflect situations closer to reality, once applied at an industrial level. The generalization of these treatments in an industrial study, as done in the last part of the present doctoral thesis, could reinforce the results obtained, as well as verify if the model is robust and representative of the industrial environment when these structures are present in their state of maturity. Finally, 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.

Biofilms in the food industry constitute a serious economic and public health problem, the latter being crucial (Galié et al., 2018). This is due to the possibility of cross-contamination with the final product, especially when biofilms harbor pathogenic microorganisms inside. For this reason, it was considered highly important to conduct a study to determine the cell transference rate of *L. monocytogenes* biofilms, as well as to examine how it behaves once elimination treatments have been applied for their dispersion. Results showed that all *L. monocytogenes* strains were able to adhere and develop biofilms at approximate the same growth levels (≈ 5.82 Log CFU/cm^2). In this case, slightly lower biofilm cell numbers were obtained in comparison with others reported by (Ripolles-Avila, Cervantes-Huaman, et al., 2019). When non-treated biofilms were put in contact during 5 min with the model food (*i.e.* chicken broth), the obtained transference rates demonstrated a potential cross-contamination with a calculated global value (*i.e.* at specie level) of 20.4 %, posing a potential food safety risk as approximately 1.66x10^4 cells would be migrating to the product. Nevertheless, this directly depends on the strain, being EDG-e the one with the highest cross-contamination potential. EDG-e is considered a model strain as a large body of biochemical, functional and genetic data is available, with its genome completely sequenced and annotated (Zameer et al., 2010). A possible explanation for what has been obtained could be associated with a possible lower production of biofilm matrix and therefore less protection and higher cell dispersion (Ripolles-Avila, Cervantes-Huaman, et al., 2019). Chlorinated alkaline treatment demonstrated a detachment of 69.35 to 99.72 %, being CECT 935 the highest affected strain, in comparison with the alternative treatment (*i.e.* chlorinated alkaline plus enzymatic treatment), which demonstrated 100 % effectivity in all cases. Biofilms treated with the chlorinated alkaline detergent produced similar transference rates to those of the 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. This also implies a potential risk, as a high percentage of pathogenic cells would be apparently migrating to the final product when the surface should be free of microorganisms. On the contrary, the alternative treatment demonstrated not to produce cross-contamination to the chicken broth due to its high effectivity for biofilm elimination. Therefore, moving to more intense cleaning treatments in the

processing environments can effectively mitigate risks of *L. monocytogenes* cross-contamination associated with industrial surfaces.

On the last *in vitro* experimental study, four different *L. monocytogenes* strains belonging to serotypes 1/2a and 4b were used to determine biofilm formation capacity on preconditioned (*i.e.* containing a layer of dirt) and non-preconditioned (*i.e.* hygienic) surfaces. Conventional (*i.e.* chlorinated alkaline) and alternative (*i.e.* chlorinated alkaline plus enzymatic) treatments were applied to observe their effectivity in the simulated models. This study was performed due to the fact that, over time, it has been determined that fouling in the food industry can influence the consolidation of biofilms and reduce the effectiveness of cleaning and disinfection procedures (Guerrero-Navarro et al., 2020). Biofilm regeneration after the application of treatments was also evaluated to understand the potential risks associated with the non-complete elimination of the structures. Results demonstrated that *L. monocytogenes* formed biofilms in both conditions tested, although their growth depended on the strain, showing higher formation on non-preconditioned surfaces than on preconditioned surfaces (*i.e.* 6.63 ± 0.42 and 5.71 ± 0.60 Log CFU/cm^2 as a mean value for all strains, respectively). The alternative treatment was significantly ($P < 0.05$) more effective than the conventional one but neither of them completely eradicated the microorganism from the preconditioned and non-preconditioned surfaces. Its high effectivity could be linked not only to the action of the chlorinated alkaline detergent for the residue layer removal, but also to the capacity of the enzymes to disintegrate and destroy the biofilm structure for cell detachment and subsequent elimination (Araújo et al., 2017; Simões et al., 2010). After 24 hours from the application of both treatments, biofilm was regenerated to similar counts as the controls. Moreover, the cells that survived the treatment and regenerated the biofilm structure may have been exposed to sublethal doses of the product, which increases the minimum inhibitory concentration (MIC) and thus increases their resistance, as found for chlorinated agents (Bansal et al., 2018). The non-complete elimination of a biofilm structure poses a risk since part of it remains on the surface, increasing the chance of cells regrowing and producing cross-contamination. This is why it is pertinent to improve biofilm removal technologies implemented in the food industry, with a view to reduce the number of cells found on surfaces after applying cleaning treatments, thereby reducing biofilm regeneration and optimizing cleaning cycles (Sanawar et al., 2018).

Finally, this thesis is also accompanied with an industrial study, which consisted on the evaluation of the resident microbiota in industrial surfaces through an integrated sampling system, which allows to obtain results that reflect a closer reality, together with the application of an antibiofilm treatment for the elimination of biofilms at an industrial scale. The choice of the sampling method to be used in the food industry is usually based on traditional indirect methodologies, although fast, cheap and easy-to-apply methods in the field must be sought nowadays (Branck et al., 2017; Martinon et al., 2012). It has been shown that the chosen sampling method has a great effect when obtaining microorganisms residing on the surface, and that this will depend on a number of factors, among which are: the properties of the target microorganism, the sampling site, the detachment method and the methodology to recover the microorganisms (Montañez, 2013; Moore & Griffith, 2002; Abel Guillermo Ríos-Castillo et al., 2021). The sampling should be designed for a specific industrial environment, since, at the moment, it is not possible to use an optimal universal methodology (Møretrø & Langsrud, 2017). Innovative methods are needed to obtain more representative results of the industrial environment, in order to better control microbiological contamination, which can come from equipment and work surfaces in the food industry (Ismaïl et al., 2013; Abel Guillermo Ríos-Castillo et al., 2021). In the present study it has been demonstrated that the integration of SCH sensors, as constitutive parts of the surface and, consequently, being direct test points, have helped to determine different microbial populations residing on industrial surfaces in a more realistic way. The use of this surface control system has been described in meat facilities, allowing to evidence the existence of interactions between microbial groups (Ripolles-Avila, García-Hernández, et al., 2019) . In fact, detecting the presence of *L. monocytogenes* in facilities was more likely with a high total microbial load - greater than 4 Log CFU/cm^2 or lower than 2 Log CFU/cm^2. For this reason, this system can give us more information and can allow an adequate intervention in situations that are expected to be critical. In this study, therefore, a methodology for obtaining indirect samples, such as swabs or contact plates, has not been used, largely due to the aforementioned justification but also because there is no scientific evidence that guarantees a complete recovery of the cells that form biofilms (Fontecha-Umaña, 2014). Such complete recovery is a necessity on an industrial scale, because part of the sample may be otherwise lost and thus the results could be underestimated. In our study, there is no sample loss, because the evaluation of the sensor is complete, therefore developing effective strategies to guarantee high hygiene standards and ensuring a complete

evaluation of what happens on surfaces, especially when pathogens may be present on them. Results showed two large groups with different levels of contamination determined from the mesophilic aerobic counts, which were the microbial group with the most considerable presence in the samples, coinciding with what was reported by (Ripolles-Avila, Hascoët, et al., 2019). Surfaces with a higher level of contamination could be a source of cross-contamination and reservoirs for the growth of certain pathogens, such as *L. monocytogenes*, whose presence was detected on five different surfaces over the months of the study. Moreover, a commercial antibiofilm product was applied on the floors of the work room and the fresh meat cart cleaning room (*i.e.* surfaces 6 and 7, respectively) to observe the impact of the treatment at real industrial scale. The aggressive antibiofilm treatment demonstrated a reduction in all microbial groups assessed over the weeks when the treatment was implemented, in comparison with when no disruptive treatment was applied. The results coincide with those reported by (Guerrero-Navarro et al., 2022), on which the efficacy of the enzymatic treatment in removing fouling at 50ºC was comparable to that of the clean-in-place method, with alkaline–acid cleaning performed at 80ºC. In the referenced study, microbiological analysis showed that the cleaning treatments guaranteed adequate hygienic conditions of the dairy products manufactured. The enzymatic treatment fulfills food industry objectives, saving water and energy during washing by reducing chemical product use (Guerrero-Navarro et al., 2020). Considering that enzymatic cleaning is biodegradable after use and that its economic cost is competitive when compared to chemical cleaning, it represents a viable alternative to the chemical cleaning of biofilm removal of the food industry.

CHAPTER 9
CONCLUSIONS

1. The enzymatic treatment applied was able to disperse and detach mature biofilms produced by 17 different strains of *L. monocytogenes* on stainless-steel surfaces. Pathogen removal from the biofilm is related to the action of the enzymes on the produced matrix, which can directly depend on the inherent ability of each *L. monocytogenes* strain to produce the structures.

2. Effectiveness of the treatment can be dependent on the robustness of the matrix produced by the different strains. However, it was neither possible to relate biofilm-forming ability with the robustness of the generated matrix, nor to establish a relationship between biofilm forming ability and serotype. Therefore, further research with an increased number of strains under study is necessary.

3. The application of conventional treatments for biofilm removal, such as alkaline and acid detergents, greatly reduced the cells conforming *L. monocytogenes* mature biofilms. However, the structure formed on the surfaces was not dispersed, with cells remaining on the stainless-steel coupons. This could have consequences in terms of potential risk of cell reparation, reacquisition of viability and re-contamination of other industrial surfaces. In contrast, applying enzymatic treatments had the two effects, namely high detachment capacity and high dispersion of the structure, thus being consolidated as the most effective treatment.

4. Potentiating the effectivity of chemical-based cleaning detergents by combining them with enzymatic treatments could be an option to optimize such detergents. Therefore, the combined treatment (*i.e.* chlorinated alkaline detergent plus enzymatic treatment) could be a suitable option to be recommended when applying 5-step cleaning protocols.

5. Mature *L. monocytogenes* biofilms formed on stainless-steel surfaces are easily transferred to chicken broth, although the transference rates depend on the strain.

6. The use of a chlorinated alkaline treatment to control *L. monocytogenes* biofilms could pose a potential risk of cross-contamination to food products, since transference rates demonstrated to be similar to those obtained when no treatment

was applied to the mature *L. monocytogenes* biofilms. By contrast, the proposed combined treatment using the chlorinated alkaline product followed by an enzymatic treatment did not show any cross-contamination, as no residual cells remained adhered to the surface.

7. Biofilm formation of *L. monocytogenes* on preconditioned and non-preconditioned surfaces differed, observing a lower cell growth when non-hygienic conditions were fomented. Moreover, it was not possible to establish a direct relationship between the serotype of the studied strains and the response to the different conditions evaluated.

8. The evaluated cleaning procedures were effective in reducing bacterial cell numbers, although they could not completely eliminate them. Overall effectiveness showed that the conventional treatment (*i.e.* chlorinated alkaline) had less detachment capacity than the alternative treatment (*i.e.* chlorinated alkaline plus enzymatic treatment) under both conditions tested (*i.e.* preconditioned and non-preconditioned surfaces).

9. After being exposed to the different treatments, *L. monocytogenes* was able to regenerate biofilms with an incubation period of 24 hours, reaching cell values similar to those obtained for the controls (*i.e.* non-treated surfaces). It is suggested that this study shall be continued so as to determine if the combined treatment is equally effective for mature biofilms (*i.e.* higher incubation periods), and also to evaluate the survival at different incubation times in order to visualize the growth rate for biofilm formation and to observe potential changes in biofilm matrix production after the application of the different treatments.

10. Contamination levels in the processing plants vary depending on the raw materials, the activities conducted and the climate. The use of SCH surface sensors has demonstrated its effectiveness, enabling us to optimally obtain samples for further processing to evaluate the resident microbiota and to determine the hygienic state of the surfaces. Areas with a higher degree of contamination were determined and were linked to the climatic and working conditions of those points.

11. The surfaces on which *L. monocytogenes* was detected were related to the predominance of certain resident microbiota in the meat plants. Knowing the microbiological species resident in processing plants and the interaction that they have with pathogens could help to improve the cleaning and disinfection processes, thereby enhancing their performance and ensuring food quality and safety for the final products.

12. Finally, the application of aggressive antibiofilm treatments allows for a more effective control of the resident microbiota on industrial surfaces. It is suggested that this study is continued through the application of an enzymatic treatment in industrial conditions. Based on the results obtained *in vitro*, the application of the evaluated enzymatic treatment or the proposed combined treatment (*i.e.* chlorinated alkaline plus enzymatic treatment) would be an interesting ecological strategy to apply as a shock treatment on open surfaces in the food industry for the elimination of mature *L. monocytogenes* biofilms.

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