TESIS DOCTORAL

por compendio de publicaciones

Biofilm formation by microorganisms of interest in food industries: intraspecies variability, biomarkers and control strategies based on non-thermal atmospheric plasma technologies

Formación de biopelículas por microorganismos de interés en la industria alimentaria: variabilidad intraespecífica, biomarcadores y estrategias para su control mediante tecnologías basadas en plasma atmosférico no térmico

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Summary

Microbial communities colonizing food processing environments in the form of biofilms have a major impact on food quality and safety. Therefore, the understanding of factors influencing the formation, ecology and architecture of biofilms from pathogenic and spoilage microorganisms is key for the development of novel biofilm control or removal strategies. In the present PhD Thesis, the intraspecific variability regarding biofilm formation ability and other phenotypic characteristics was examined in a wide collection of isolates from several microorganisms of interest in the food industry.

The importance of the general stress response regulator of Gram-negative bacteria RpoS on biofilm formation was assessed in nine field isolates of *Cronobacter sakazakii.* Their biofilm formation ability was screened in BHI and minimum media with different pH values and supplemented or not with the amino acids arginine, lysine and glutamic acid, resulting generally higher in buffered minimum media (pH 7.0) supplemented with lysine, despite the existing heterogeneity among the different strains. Biofilm formation was visualized by confocal laser scanning microscopy and scanning electron microscopy and was measured by spectrometric determination of the fixed crystal violet, with higher biofilm formation levels being obtained on stainless steel plates than on polystyrene. A lower ability to form biofilms was found for a strain with a loss-of-function mutation in the *rpoS* gene compared to the rest of the strains, which harboured a functional *rpoS*. The complementation of this strain with a functional *rpoS* gene increased it biofilm formation ability up to levels similar to the strains with a naturally functional *rpoS* gene after 24 h of incubation. However, the differences observed were reduced when a 48 h incubation was used. These results indicate that the loss of RpoS caused a delay in the development of mature biofilms, rather than a complete inhibition of biofilm production in *C. sakazakii*.

Also, the RpoS status and biofilm formation ability was evaluated in a collection of thirty-one extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* strains isolated from foods of animal origin and from human patients, and reference collection strains, together with their tolerance to food-associated stresses (heat, acid, non-thermal atmospheric plasma (NTAP) and UV-C light). Only minor differences were associated to the carriage of the ESBL genes bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV} and no loss-of-function mutations were found in the *rpoS* gene. The most relevant phenotypic differences among strains were observed for biofilm formation and heat

resistance, with food isolates being significantly more resistant to heat treatments at 58 ^{o}C for 1 and 2 min than clinical isolates. Also, the biofilm formation ability on stainless steel was significantly higher for the field isolates, both clinical- and food-related, than for the reference strains.

A collection of thirty-three *Pseudomonas* spp. isolates from food processing facilities was also investigated regarding their RpoS status, catalase activity, pigmentation on solid media, pyoverdine production, cellular hydrophobicity and biofilm formation on stainless steel, polystyrene and glass, in order to find biomarkers of strong biofilm production. The biofilm formation levels on stainless steel and polystyrene were significantly higher for the green-pigmented strains as compared to brown or not pigmented strains. Possible relations between pyoverdine production, colony pigmentation and iron availability were studied using an iron scavenger, 2,2-bipyridine, resulting in a decrease of 40 % in biofilm formation for the not pigmented strains. For most of the potential biomarkers studied, the phenotypic heterogeneity observed among strains was mainly dependent on the *Pseudomona*s species (*P. aeruginosa vs* other *Pseudomona*s species). However, the green colony pigmentation on solid media was identified as a potential biomarker of strong biofilm formation.

During this PhD thesis, two plasma-based biofilm control strategies were evaluated. The first one focussed on the development of anti-biofilm coatings that prevent bacterial adherence to the surfaces through physico-chemical modifications. Different coatings made of (3 aminopropyl)triethoxysilane (APTES), tetraethyl orthosilicate (TEOS) and acrylic acid (AA) were applied by Non-Equilibrium Atmospheric Plasma on stainless steel AISI 316. Their anti-biofilm activity was assessed against biofilms of *L. monocytogenes* CECT911 and *E. coli* CECT515 after incubation at 37 ^{o}C. The two best coatings, AP10+TE6 and AP10+AA6, that reduced *L. monocytogenes* biofilm production by 45 % and 74 %, respectively, when compared with the uncoated stainless steel, were selected for a further characterization together with a modification of those, AP10+SA6. The anti-biofilm activity was studied against two *L. monocytogenes* strains isolated from a meat industry and at lower incubation temperatures, more representative of food processing environments, obtaining the best results for the coating AP10+AA6, that reduced the biofilm formation by 90 % after incubation at 12 ^{o}C. The morphological and physico-chemical characterization of the selected coatings showed that the most anti-biofilm coating, AP10+AA6, presented the lower surface roughness and higher hydrophilicity, a characteristic that was enhanced by low temperature conditions, when the wettability of the strains was increased. Altogether, these results suggest the formation of a hydration layer that prevents the adherence of *L. monocytogenes* cells to the coated surface.

The characterization of the coating AP10+AA6 continued through the study of its anti-biofilm activity against multispecies biofilms containing *L. monocytogenes* (developed using indigenous microbiota from three different meat industries) and by assessing the disinfection effectiveness and biofilm evolution after sanitization with two food industry biocides, using culture-dependent and culture-independent approaches. A lower effectiveness of the coating against *L. monocytogenes* when inoculated in the multispecies biofilms developed for 7 days at 12 ^{o}C was observed, together with a partial and industry dependent control of the growth of the pathogen. The biofilm taxonomic composition was highly dependent on the industry, but it was not affected by the nature of the surface (uncoated *vs* coated stainless steel) and the artificial inoculation with *L. monocytogenes*. The biofilms formed after 7 days at 12 ^{o}C following the 15-min disinfection treatments showed reduced taxonomic diversity and, although sodium hypochlorite was slightly less effective than peracetic acid immediately after application, it caused a stronger growth control of the naturally present *L. monocytogenes* on the multispecies biofilms developed. This suggested that that a sanitization able to preserve interspecific competitive relationships between the members of the indigenous microbiota and *L. monocytogenes* might be more favourable for the long-term control of this pathogen in food processing facilities.

The second plasma-based technology evaluated in the current PhD Thesis was the use of plasma-activated water (PAW) as an alternative control strategy for *L. monocytogenes* inactivation. Different PAWs were generated through the activation of tap water using a surface dielectric barrier discharge (SDBD) plasma working at different conditions of discharge power (26 and 36 W) and activation time (5 and 30 min). The study of their physico-chemical composition (pH and levels of hydrogen peroxide, nitrates and nitrites) and antimicrobial efficacy against a cocktail of three *L. monocytogenes* strains on planktonic state allowed the identification of the best PAW generation conditions, i.e. 36 W of discharge power with 30 min of activation time (PAW HM30). The antimicrobial activity of this PAW against three-strain *L. monocytogenes* biofilms was lower than in the planktonic state, where 4.6 log reductions were achieved, but still able to obtain 1.9 and 1.8 log reductions after 15 min of exposure of biofilms formed on stainless steel and on polystyrene, respectively. The mechanisms of PAW inactivation were investigated through RNA-seq analysis of *L. monocytogenes* after its treatment with PAW HM30. The main transcriptomic changes affected carbon metabolism, some virulence genes and the general stress response, with several of the most overexpressed genes belonging to the cobalamin-dependent gene cluster, previously related to *L. monocytogenes* pathogenicity and its response to multiple stressors.

Resumen

Las comunidades microbianas que colonizan los ambientes de procesado de alimentos en forma de biopelículas tienen un gran impacto en la calidad y seguridad de los alimentos. Por lo tanto, el estudio de los factores que influyen en la formación, la ecología y la arquitectura de las biopelículas de microorganismos patógenos y alterantes es clave para el desarrollo de nuevas estrategias de control o eliminación de biopelículas. En la presente Tesis Doctoral se examinó la variabilidad intraespecífica en cuanto a la capacidad de formación de biopelículas y otras características fenotípicas en una amplia colección de aislados de varios microorganismos de interés en la industria alimentaria.

La importancia del regulador general de la respuesta a estrés de las bacterias Gram negativas, RpoS, en la formación de biopelículas se evaluó en nueve aislados de *Cronobacter sakazakii*. Su capacidad de formación de biopelículas se evaluó en BHI y medios mínimos con diferentes valores de pH y suplementados o no con los aminoácidos arginina, lisina y ácido glutámico, resultando ésta generalmente mayor en medios mínimos tamponados (pH 7,0) suplementados con lisina, a pesar de la heterogeneidad existente entre las diferentes cepas. La formación de biopelículas se visualizó mediante microscopía de barrido láser confocal y microscopía electrónica de barrido y se midió mediante determinación espectrométrica del cristal violeta fijado, mostrando niveles más altos de formación de biopelículas sobre acero inoxidable que sobre poliestireno. Se pudo apreciar una menor capacidad para formar biopelículas en una cepa con una mutación que resulta en la pérdida de función del gen *rpoS*, en comparación con el resto de las cepas estudiadas, que portan un gen *rpoS* funcional. La complementación de esta cepa con un gen *rpoS* funcional aumentó su capacidad de formación de biopelículas hasta niveles similares a los de las cepas con un gen *rpoS* funcional después de 24 h de incubación. Las diferencias observadas se redujeron cuando se utilizó una incubación de 48 h. Estos resultados indican que la pérdida del factor RpoS provoca un retraso en el desarrollo de biopelículas maduras, pero no una inhibición completa de la producción de biopelículas en *C. sakazakii*.

También se evaluó la funcionalidad del regulador RpoS y la capacidad de formación de biopelículas en una colección de treinta y una cepas de *Escherichia coli* productoras de β-lactamasas de espectro extendido (BLEE), aisladas de alimentos de origen animal y de pacientes humanos, y cepas de colección de referencia, junto con su tolerancia a condiciones de estrés comunes en la cadena alimentaria (calor, ácido, plasma atmosférico no térmico (PANT) y

luz UV-C). Solo se observaron diferencias menores asociadas a la presencia de los genes BLEE *bla*$_{TEM}$, *bla*$_{CTX-M}$ y *bla*$_{SHV}$, y no se encontraron mutaciones en el gen *rpoS* que conllevaran una pérdida de función. Las diferencias fenotípicas más relevantes se observaron para la formación de biopelículas y la resistencia al calor, siendo los aislados alimentarios significativamente más resistentes a los tratamientos térmicos a 58 ^{o}C durante 1 y 2 minutos que los aislados clínicos. Además, la capacidad de formación de biopelículas sobre acero inoxidable fue significativamente mayor para los aislados de campo, tanto de origen clínico como alimentario, que para las cepas de referencia.

Asimismo, se caracterizó una colección de treinta y tres cepas de *Pseudomonas* spp. aisladas de instalaciones de procesado de alimentos en cuanto a la funcionalidad del regulador RpoS, la actividad catalasa, la pigmentación en medio sólido, la producción de pioverdina, la hidrofobicidad celular y la formación de biopelículas sobre acero inoxidable, poliestireno y vidrio, con el fin de encontrar biomarcadores de fuerte producción de biopelículas. Los niveles de formación de biopelículas sobre acero inoxidable y poliestireno fueron significativamente más altos para las cepas con pigmentación verde en comparación con las cepas marrones o no pigmentadas. Las posibles relaciones entre la producción de pioverdina, la pigmentación de las colonias y la disponibilidad de hierro se estudiaron utilizando un secuestrador de hierro, 2,2-bipiridina, que provocó una disminución del 40 % en la formación de biopelículas por parte de las cepas no pigmentadas. Para la mayoría de los posibles biomarcadores estudiados, la heterogeneidad fenotípica observada entre las cepas dependía principalmente de la asignación taxonómica de la cepa de *Pseudomonas* (*P. aeruginosa* frente a otras especies de *Pseudomonas*). Sin embargo, la pigmentación verde de las colonias en medio sólido se identificó como un posible biomarcador de fuerte formación de biopelículas.

Durante esta Tesis Doctoral, se evaluaron dos estrategias de control de biopelículas basadas en la tecnología del plasma. La primera se centró en el desarrollo de recubrimientos anti-biopelícula que eviten la adherencia bacteriana a las superficies mediante modificaciones físico-químicas. Se aplicaron diferentes recubrimientos de (3 aminopropil)trietoxisilano (APTES), ortosilicato de tetraetilo (TEOS) y ácido acrílico (AA) mediante plasma atmosférico no equilibrado sobre acero inoxidable AISI 316. Se evaluó su actividad anti-biopelícula frente a biopelículas de *L. monocytogenes* CECT911 y *E. coli* CECT515 tras la incubación a 37 ^{o}C. Los dos mejores recubrimientos, AP10+TE6 y AP10+AA6, que redujeron la producción de biopelículas de *L. monocytogenes* en un 45 % y un 74 %, respectivamente, en comparación con el acero inoxidable sin recubrimiento, se seleccionaron para una caracterización más detallada junto con una modificación de esos recubrimientos, AP10+SA6. Se estudió la actividad anti-biopelícula

frente a dos cepas de *L. monocytogenes* aisladas de una industria cárnica y a temperaturas de incubación más bajas, representativas de ambientes de procesado de alimentos, obteniendo los mejores resultados para el recubrimiento AP10+AA6, que redujo la formación de biopelículas en un 90 %. después de la incubación a 12 ºC. La caracterización morfológica y físico-química de los recubrimientos seleccionados mostró que el recubrimiento más efectivo, AP10+AA6, presentaba una menor rugosidad superficial y mayor hidrofilicidad, característica que se vio favorecida por las condiciones de baja temperatura, cuando la humectabilidad de las cepas fue mayor. En conjunto, estos resultados sugieren la formación de una capa de hidratación que impide la adherencia de las células de *L. monocytogenes* a la superficie recubierta.

Se continuó con la caracterización del recubrimiento AP10+AA6 estudiando su actividad anti-biopelícula frente a biopelículas multiespecies que contienen *L. monocytogenes* (desarrolladas a partir de la microbiota autóctona de tres industrias cárnicas diferentes) y evaluando la eficacia desinfectante y evolución de las biopelículas tras la higienización con dos biocidas empleados en la industria alimentaria, utilizando enfoques dependientes e independientes del cultivo. Se observó una menor efectividad del recubrimiento frente a *L. monocytogenes* en los biofilms multiespecie desarrollados durante 7 días a 12 ºC, además de un control parcial, y dependiente de la industria, del crecimiento del patógeno. La composición taxonómica de la biopelícula dependió en gran medida de la industria, pero no se vio afectada por la naturaleza de la superficie (acero inoxidable sin revestimiento o con revestimiento) o por la inoculación artificial con *L. monocytogenes*. Las biopelículas formadas durante 7 días a 12 ºC después de los tratamientos de desinfección de 15 min mostraron una menor diversidad taxonómica y el hipoclorito sódico, aunque fue ligeramente menos efectivo que el ácido peracético inmediatamente después de la aplicación, permitió un mejor control del crecimiento de *L. monocytogenes* en las biopelículas multiespecies desarrolladas. Esto sugiere que una desinfección capaz de preservar las relaciones competitivas interespecíficas entre los miembros de la microbiota autóctona y *L. monocytogenes* podría ser más favorable para el control a largo plazo de este patógeno en las instalaciones de procesado de alimentos.

La segunda tecnología basada en plasma evaluada en la presente Tesis Doctoral fue el uso de agua activada por plasma (PAW) como estrategia de control alternativa para la inactivación de *L. monocytogenes*. Se generaron diferentes PAWs a través de la activación del agua del grifo utilizando un plasma de descarga de barrera dieléctrica superficial (SDBD) funcionando con diferentes condiciones de potencia de descarga (26 y 36 W) y tiempo de activación (5 y 30 min). El estudio de composición físico-química (pH y niveles de peróxido de hidrógeno, nitratos y nitritos) y eficacia antimicrobiana frente a un cóctel de tres cepas de *L. monocytogenes* en

estado planctónico permitió identificar las mejores condiciones de generación de PAW, que fueron 36 W de potencia de descarga con 30 min de tiempo de activación (PAW HM30). La actividad antimicrobiana de esta PAW frente a biopelículas de *L. monocytogenes* fue menor que en estado planctónico, donde se lograron reducciones logarítmicas de 4,6, pero aun así se pudieron obtener reducciones logarítmicas de 1,9 y 1,8 después de 15 minutos de exposición para las biopelículas formadas en acero inoxidable y en poliestireno, respectivamente. Los mecanismos de inactivación del PAW se investigaron mediante el análisis por ARN-seq de *L. monocytogenes* después de su tratamiento con PAW HM30. Los principales cambios en la expresión génica afectaron el metabolismo del carbono, algunos genes de virulencia y la respuesta general al estrés, perteneciendo varios de los genes más sobreexpresados al cluster de genes dependientes de la cobalamina, previamente relacionado con la patogenicidad de *L. monocytogenes* y su respuesta a múltiples condiciones de estrés.

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1. Introduction

Un biofilm o biopelícula puede definirse como una comunidad microbiana caracterizada por su adhesión a una superficie sólida y por la producción de una matriz polimérica extracelular en la que están embebidos los microorganismos asociados. Esta matriz proporciona protección a las células microbianas y contribuye a la captación de nutrientes, así como a la adhesión a la superficie en cuestión. La formación de biopelículas es un comportamiento social generalmente coordinado a través de sistemas de comunicación célula - célula, o sistemas de "quorum sensing". Dichos sistemas de "quorum sensing" detectan fluctuaciones en la densidad celular a través del reconocimiento de pequeñas moléculas de señalización secretadas, llamadas autoinductores, y responden regulando la expresión de funciones celulares especializadas entre las que se encuentran las responsables de la adhesión inicial a superficies y el subsiguiente crecimiento y maduración de la biopelícula. De particular relevancia resulta el hecho de que las células microbianas dentro de una biopelícula son significativamente más resistentes a diferentes tipos de intervenciones antimicrobianas, dirigidas a controlar su aparición, y que las biopelículas pueden actuar como un reservorio de microorganismos persistentes. Se puede encontrar información más detallada sobre la ecología de las biopelículas y el comportamiento microbiano dentro de las mismas en Flemming et al. (2016) y Nadell et al. (2016).

En la industria alimentaria, las superficies y los equipos se encuentran frecuentemente colonizados por microorganismos en forma de biopelículas. En la mayoría de las ocasiones, esto representa un desafío y una preocupación, ya que las biopelículas formadas por microorganismos alterantes y patógenos pueden servir como fuente de contaminación cruzada de los alimentos, reduciendo así la efectividad de las estrategias de conservación de alimentos y comprometiendo la calidad y seguridad de los mismos (Coughlan, Cotter, Hill, & Alvarez-Ordóñez, 2016). Por otro lado, las biopelículas formadas de manera controlada por microorganismos beneficiosos pueden representar una oportunidad, ya que pueden ser explotadas para aumentar el rendimiento de procesos fermentativos o para desarrollar aplicaciones biotecnológicas centradas en mejorar la calidad y seguridad de los alimentos (Berlanga & Guerrero, 2016).

A continuación, a modo de introducción de esta Tesis Doctoral, se incluye una adaptación de la publicación "Biopelículas y persistencia microbiana en la industria alimentaria" (https://doi.org/10.3989/arbor.2020.795n1002) realizada en la revista Arbor por P. Fernández-Gómez et al. (2020), ampliada con información relativa a aquellas estrategias de control de biopelículas empleadas en esta Tesis Doctoral: los recubrimientos anti-biopelícula y el agua activada por plasma.

Biopelículas y persistencia microbiana en la industria alimentaria

Biofilms and microbial persistence in the food industry

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1.5. Estrategias de modificación superficial

La modificación de los materiales utilizados en la industria alimentaria se ha revelado como una estrategia prometedora para prevenir la formación de biopelículas. Dado que la formación de una biopelícula implica como primer paso la unión o adhesión de células planctónicas a una superficie sólida, si dicha superficie se modifica en cierta medida, por ejemplo, alterando su morfología o propiedades físico-químicas (hidrofobicidad, hidrofilicidad, carga eléctrica, etc.), la adhesión microbiana y, en consecuencia, el crecimiento y la maduración de la biopelícula pueden ser controlados (Feng et al., 2014; Gkana, Doulgeraki, Chorianopoulos, & Nychas, 2017; Gomes, Deschamps, Briandet, & Mergulhão, 2018; Hsu, Fang, Borca-Tasciuc, Worobo, & Moraru, 2013; Huang, Chen, Nugen, & Goddard, 2016). Además, se han desarrollado recubrimientos superficiales que incorporan compuestos antimicrobianos que también han demostrado capacidad para prevenir la formación de biopelículas por varios patógenos transmitidos por los alimentos (Cossu, Si, Sun, & Nitin, 2017; Fialho et al., 2018;Kim et al., 2017).

En el siguiente apartado se profundiza en esta estrategia de control de biopelículas mediante recubrimientos aplicados por polimerización con plasma atmosférico no térmico.

1.5.1. Recubrimientos anti-biopelículas depositados por plasma

A diferencia de las técnicas de deposición convencionales (CVD, deposición química húmeda, plasma a alta/baja presión, etc.), la técnica de deposición mediante plasma-polimerización a baja temperatura (fría) presenta una serie de ventajas. Esta técnica permite una deposición seca, en la que se aplica un recubrimiento en una única etapa para conferir propiedades funcionales a la superficie diana. Además, se trata de una tecnología respetuosa con el medio ambiente, ya que no se producen productos químicos residuales, y económica, ya que el gasto de consumibles es relativamente bajo al aplicarse recubrimientos de espesores a escala nano o micro. Debido a que el proceso de deposición se realiza a presión atmosférica y a que la temperatura no supera los 100 ^{o}C, se evitan alteraciones no deseadas en las propiedades del sustrato. Finalmente, se puede llevar a cabo un control de las características de los recubrimientos obtenidos en función del tipo de precursor utilizado y de los parámetros del proceso (flujo de gas, potencia suministrada, etc.), lo que permite que se pueda mejorar la superficie del sustrato de manera específica manteniendo el resto de propiedades sin alterar (Sainz-García, Alba-Elías, Múgica-Vidal, & González-Marcos, 2017).

El plasma es el estado que alcanza un gas cuando se le aporta una cantidad de energía que logra ionizar sus moléculas y átomos. Es decir, el paso de la materia de estado gaseoso a estado de plasma se produce mediante una disociación de enlaces moleculares, acompañada de un

aumento o disminución de los electrones de los átomos, lo que da lugar a la formación de iones con carga positiva o negativa. En función de si se da o no un equilibrio térmico entre las partículas del plasma, se distingue el plasma térmico del frío. Un plasma frío o no equilibrado es aquel en el que la temperatura de los electrones (5000-10^5 ^oC) es mucho mayor que la de las partículas más pesadas (partículas neutras e iones), las cuales se encuentran a temperaturas próximas a la del ambiente (25-100 ^oC). De esta forma, la temperatura de un plasma frío se mantiene generalmente por debajo de los 100 ^oC, lo que permite que sea empleado en tratamientos superficiales sobre una gran variedad de materiales sin provocar su deterioro por un calentamiento excesivo (Múgica-Vidal, Alba-Elías, Sainz-García, & González-Marcos, 2017). La generación de plasma frío se puede llevar a cabo a presión atmosférica en un entorno abierto, es decir, sin requerir la utilización de sistemas de vacío. Estas características dotan a la tecnología de plasma atmosférico frío de una gran versatilidad, relativa simplicidad y bajo coste. Desde el punto de vista de su aplicación industrial, el plasma se ha convertido en una importante herramienta para llevar a cabo multitud de tratamientos superficiales (Sainz-García et al., 2017).

Una de estas aplicaciones es la de plasma-polimerización, que consiste en la deposición de recubrimientos finos utilizando monómeros en estado líquido como precursores a través de su exposición al flujo de plasma. La interacción entre el precursor y el plasma conduce a la fragmentación de las moléculas del precursor y a la formación de los grupos funcionales que determinan las propiedades del recubrimiento (Bhatt, Pulpytel, & Arefi-Khonsari, 2015). Los productos de las reacciones entre el precursor y el plasma, así como las especies precursoras y reactivas del plasma que no han reaccionado, caen sobre la superficie del sustrato donde se adsorben y tienen lugar reacciones superficiales. Como resultado, el material puede nuclearse formando grupos que se fusionan en núcleos más grandes, aumentando la rugosidad del recubrimiento. La formación de los grupos funcionales, las especies reactivas y los sitios de activación implicados en la polimerización por plasma, así como el crecimiento y las propiedades fisicoquímicas de los recubrimientos, dependen de muchos factores, como las características del sustrato, la composición química del precursor, el caudal del gas de trabajo y la potencia aplicada en la ionización (Duday et al., 2013; Ramamoorthy, Rahman, Mooney, MacElroy, & Dowling, 2009).

Entre los principales enfoques para la prevención de la formación de biopelículas se encuentran los recubrimientos (i) que contienen y liberan agentes biocidas, (ii) que inmovilizan agentes antimicrobianos y (iii) para la modificación físico-química de superficies (Múgica-Vidal et al., 2019) (**Figura 2**).

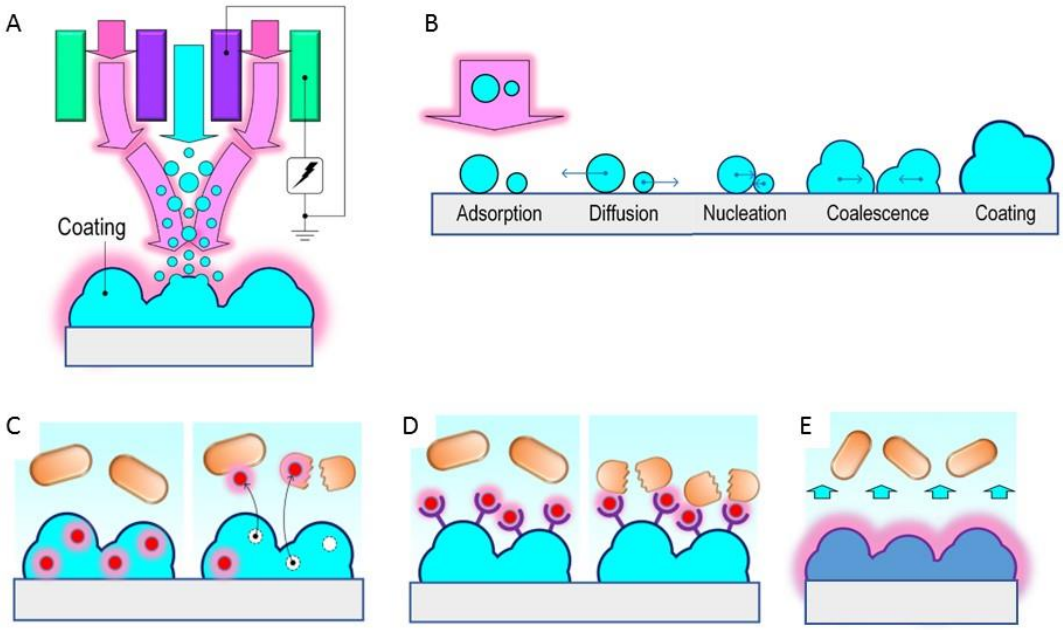

Figura 2. (A) Esquema del proceso de deposición de recubrimientos mediante un sistema de chorro de plasma atmosférico no equilibrado. (B) Interacciones superficiales de absorción, difusión, nucleación y coalescencia del material depositado. Actividad antimicrobiana de (C) recubrimientos compuestos que contienen y liberan agentes biocidas, (D) recubrimientos que inmovilizan agentes antimicrobianos en superficie y (D) recubrimientos que modifican las propiedades fisicoquímicas de las superficies. Adaptada de Múgica-Vidal et al. (2019).

1.5.1.1. Recubrimientos que contienen y liberan agentes biocidas

Uno de los enfoques para obtener superficies antibacterianas, limitando así la formación de biopelículas, implica la deposición de un recubrimiento compuesto. Este recubrimiento está formado por una matriz polimérica o inorgánica que contiene un agente biocida embebido que se libera cuando el recubrimiento entra en contacto con el medio circundante (Sardella, Palumbo, Camporeale, & Favia, 2016). Aunque se pueden utilizar muchos materiales diferentes como matriz del compuesto, los polímeros tienen la ventaja de mostrar una buena adherencia sobre las superficies y la capacidad de depositarse formando recubrimientos de bajo espesor sin alterar las propiedades mecánicas del sustrato. Es posible incluso controlar la velocidad de liberación del agente antibacteriano ajustando las características de estas capas poliméricas. Por lo tanto, se puede evitar una pérdida excesivamente rápida de la actividad antibacteriana de los recubrimientos debido a una velocidad de liberación demasiado rápida de los agentes antibacterianos (Vasilev, Griesser, & Griesser, 2011).

Deng et al. (2015) utilizaron un proceso de tres pasos para recubrir tejidos de tereftalato de polietileno (PET) mediante la incrustación de nanopartículas de plata entre dos capas de

organosilicio, que se depositaron a través de la polimerización por plasma atmosférico de tetrametildisiloxano (TMDSO). Estos autores, observaron que los recubrimientos mostraban actividad antimicrobiana frente a *Pseudomonas aeruginosa*, *Staphylococcus aureus* y *Candida albicans*. Su efectividad disminuyó a medida que aumentaba el espesor de la segunda capa de organosilicio (capa de barrera), sugierendo que el aumento de espesor provocaba una reducción en la cantidad de grietas y poros de la capa de barrera, lo que podría reducir la liberación de iones de plata de las nanopartículas al medio. Por lo tanto, esta característica podría utilizarse como una posible forma de controlar o modular la eficacia antimicrobiana de los recubrimientos y la durabilidad de su actividad antibacteriana.

Deng et al. (2014) emplearon un proceso de un solo paso para depositar recubrimientos de nanocompuestos en obleas de silicio mediante la generación de un chorro de plasma atmosférico (APPJ) en nitrógeno y alimentando el plasma con oxígeno, TMDSO y un polvo de nanopartículas de plata. Estos autores, observaron que las nanopartículas de plata metálica estaban incrustadas dentro de los recubrimientos, mientras que en la superficie de la película la plata se presentaba como AgO. Estos recubrimientos mostraron actividad antibacteriana frente a *E. coli* y *S. aureus*, siendo más efectivos frente al primer microorganismo. Liguori et al. (2016) también desarrollaron un proceso en un solo paso, utilizando un APPJ para depositar una matriz polimerizada por plasma de ácido poliacrílico con nanopartículas de plata incrustadas sobre sustratos de polietileno mediante la inyección de dos precursores: ácido acrílico para la matriz y una dispersión de nanopartículas de plata en etanol. Las pruebas de difusión en disco de agar frente a *E. coli* revelaron la formación de zonas de inhibición del crecimiento de esta bacteria, que se atribuyeró a los iones de plata liberados por la plata de la superficie de los recubrimientos o a través de las grietas que se observaron mediante microscopía electrónica de barrido.

Sin embargo, como apuntan Gupta & Xie (2018) en una revisión sobre las aplicaciones, toxicidad y normativa de las nanopartículas, estas pueden ser más tóxicas que las partículas de mayor tamaño por su mayor movilidad. Además, su liberación puede suponer un riesgo de contaminación ambiental. Dado que la toxicidad de los nanomateriales puede verse afectada por sus propiedades específicas y la información existente al respecto todavía es limitada, su regulación sigue siendo un desafío. Por ello, se están dedicando esfuerzos a clasificar los nanomateriales, así como a comprender y controlar sus riesgos potenciales. Por ejemplo, la Autoridad Europea de Seguridad Alimentaria (EFSA) ha desarrollado una guía sobre la evaluación de riesgos de la aplicación de la nanociencia y las nanotecnologías en la cadena de alimentos y piensos, que ofrece sugerencias sobre los recursos disponibles para la caracterización de nanomateriales y los parámetros clave que deben medirse (Scientific Committee et al., 2018).

Además, aunque la plata se utiliza como un biocida eficaz frente a bacterias en múltiples campos, incluidas las aplicaciones relacionadas con la biomedicina y los alimentos, su toxicidad para organismos superiores como los humanos es motivo de preocupación (Marambio-Jones & Hoek, 2010).

En lugar de usar plata u otras nanopartículas metálicas, es posible producir alternativas más seguras y respetuosas con el medio ambiente incorporando agentes antibacterianos orgánicos en la matriz. Los aceites esenciales naturales derivados de las plantas (Bazaka, Jacob, Chrzanowski, & Ostrikov, 2015) y las biomoléculas que son producidas naturalmente por los organismos vivos (p. ej., lisozima o nisina) se pueden emplear para proporcionar al recubrimiento compuestos con actividad antimicrobiana (Sardella et al., 2016). De hecho, se han desarrollado recubrimientos comestibles con eficacia antimicrobiana utilizando estas biomoléculas y aceites esenciales (Dhumal & Sarkar, 2018). Palumbo et al. (2015) utilizaron plasma a presión atmosférica generado en un equipo de descarga de barrera dieléctrica (Dielectric Barrier Discharge, DBD) para depositar recubrimientos de biocompuestos en obleas de silicio en un proceso de un solo paso. Estos recubrimientos consistían en una matriz orgánica obtenida por plasma-polimerización de etileno y con lisozima embebida. La liberación de la lisozima embebida en el agua y el mantenimiento de su actividad antibacterianatras su interacción con el plasma fue verificada mediante una prueba de agar frente a *Micrococcus lysodeikticus.*

1.5.1.2. Recubrimientos que inmovilizan agentes antimicrobianos

La cantidad del agente antimicrobiano activo que se libera de los recubrimientos cuando se utiliza el enfoque previamente expuesto debe controlarse para evitar problemas toxicológicos o sensoriales que podrían surgir como consecuencia de la migración de concentraciones antimicrobianas demasiado altas a los alimentos (Conte, Buonocore, Bevilacqua, Sinigaglia, & Nobile, 2006). Alternativamente, otro enfoque posible consiste en inmovilizar los agentes antimicrobianos en las superficies en contacto con los alimentos pero sin liberarlos. Así, se logra la actividad antimicrobiana frente a bacterias en contacto estrecho con la superficie evitando la migración de los agentes antimicrobianos al alimento (Conte et al., 2008). Varios autores han utilizado técnicas basadas en plasma para inmovilizar péptidos antimicrobianos (Duday et al., 2013), enzimas (Conte et al., 2008; Thallinger et al., 2015; Vartiainen, Rättö, & Paulussen, 2005) y polímeros (Tseng, Hsu, Wu, Hsueh, & Tu, 2009) en diferentes superficies.

Por ejemplo, Duday et al. (2013) utilizaron compuestos basados en organosilicio depositados por plasma-polimerización como capas reactivas para la inmovilización covalente de péptidos

antibacterianos de nisina sobre sustratos de acero inoxidable. Emplearon (3-Aminopropyl)trimethoxysilane (APTMS) como precursor en la polimerización, con el fin de generar grupos amino capaces de reaccionar con los grupos carboxilo de los péptidos, favoreciendo así su inmovilización, y dos tipos de equipos de plasma DBD atmosférico para comparar los efectos de ambos procesos: (i) DBD directo y (ii) DBD *afterglow*. Aunque los recubrimientos que se obtuvieron por el proceso DBD directo tenían mayor contenido de grupos amino, parecían ser inestables cuando se sumergieron en las soluciones peptídicas para la inmovilización covalente y mostraron baja actividad bactericida. Sin embargo, se obtuvo un compromiso más satisfactorio entre el contenido de grupos amino y la estabilidad de los recubrimientos mediante el uso del DBD *afterglow*. De esta forma, se lograron 3,6 reducciones logarítmicas de *Bacillus subtilis*. Además, las propiedades bactericidas de los recubrimientos aplicados con DBD *afterglow* se mantuvieron tras la aplicación de una serie de pruebas de envejecimiento y lavado.

De forma similar, Vartiainen et al. (2005) utilizaron plasma a presión atmosférica generado en un reactor DBD para activar recubrimientos de polipropileno mediante la creación de grupos amino y carboxilo para la inmovilización covalente de la enzima antimicrobiana glucosa oxidasa. Estos recubrimientos inhibieron completamente el crecimiento de *E. coli* y redujeron significativamente el de *B. subtilis*. Dado que la actividad antimicrobiana de la glucosa oxidasa se suele atribuir a la generación de H_2O_2, estos resultados concuerdan con la conocida menor resistencia de las bacterias Gram-negativas frente al peróxido de hidrógeno comparado con la de las bacterias Gram-positivas.

Tseng et al. (2009) fijaron agentes antimicrobianos, en este caso un oligómero o polímero de quitosano, en telas de nylon después de la activación por plasma atmosférico. Se utilizaron diferentes velocidades y números de pases para la activación de las diferentes muestras incluidas en el estudio. Las pruebas, que utilizaron *S. aureus* como microorganismo diana, sugirieron que la actividad antibacteriana de los tejidos generalmente tendía a ser mayor cuando se usaba una mayor velocidad y un mayor número de pases en la activación por plasma. Además, los tejidos que fueron modificados con el polímero de quitosano exhibieron una mayor efectividad antibacteriana que los modificados con el oligómero de quitosano.

1.5.1.3. Recubrimientos para la modificación físico-química de superficies

El plasma a presión atmosférica también se puede utilizar para la modificación de las propiedades físico-químicas de una superficie sin la deposición de ningún agente antimicrobiano, siendo un enfoque muy prometedor para combatir las biopelículas. Como se

mencionó anteriormente, la formación de biopelículas implica la adhesión de células planctónicas a una superficie. Entonces, si se modifica una superficie, por ejemplo, cambiando sus propiedades físico-químicas, se puede prevenir la adhesión bacteriana y, en consecuencia, el crecimiento y la maduración de la biopelícula (Li, 2016). A lo largo de los años se han ampliado los conocimientos sobre las propiedades físicas y químicas de los materiales, así como acerca de los factores clave que influyen en la adherencia celular. Como consecuencia, ha aumentado el interés en la modificación de las propiedades físico-químicas de las superficies para el desarrollo de enfoques destinados a prevenir la unión microbiana y la formación de biopelículas (Bazaka et al., 2015).

El óxido de polietileno (PEO) y el polietilenglicol (PEG) son polímeros comúnmente usados para este propósito debido a su capacidad para repeler las proteínas y, por lo tanto, reducir la adherencia bacteriana (Nisol, Poleunis, Bertrand, & Reniers, 2010). Aunque su mecanismo de acción no se comprende completamente, generalmente se cree que el comportamiento anti-biopelícula podría estar relacionado con la repulsión estérica y con la presencia de grupos funcionales hidrofílicos en la superficie de los polímeros. De hecho, estos grupos funcionales permiten la formación de una capa de agua cuando se encuentran en ambientes acuosos, evitando así el contacto directo entre los polímeros y las proteínas bacterianas que actúan como sitios receptores para la adhesión bacteriana y la colonización de la superficie (Dong, Jiang, Manolache, Wong, & Denes, 2007; Stallard, Solar, Biederman, & Dowling, 2016). Ponte et al. (2011, 2012) emplearon un sistema DBD para la deposición por plasma a presión atmosférica de revestimientos similares a PEO sobre sustratos de vidrio utilizando tetraetilenglicol dimetil éter (TEGDME) como precursor. Como reveló la caracterización química por espectroscopía de fotoelectrones de rayos X (XPS), al variar los parámetros de generación del plasma, fue posible lograr una alta retención de la estructura del monómero TEGDME en la superficie de los recubrimientos, que mostraron un contenido de éter (carácter PEO) del 70 % en el componente de carbono (es decir, en la señal C1s, que corresponde a las energías de enlace en el rango de 282-292 eV en los espectros XPS). Según la literatura, los recubrimientos con tal grado de carácter PEO no permiten la adhesión celular (Sardella, Gristina, Senesi, D'Agostino, & Favia, 2004). Aunque el potencial interés biológico y las aplicaciones de los recubrimientos obtenidos por estos autores son evidentes, en ninguno de estos dos estudios se realizaron ensayos antimicrobianos, sino que proporcionaron solo una caracterización de las propiedades físico-químicas de los recubrimientos producidos.

Venault et al. (2013) diseñaron un proceso de PEGilación inducida por plasma atmosférico en membranas de poli (tetrafluoroetileno) (ePTFE) expandido para mejorar su resistencia a la adsorción de proteínas y la unión bacteriana. Después de incubar las membranas de ePTFE en una solución de monómero de poli (etilenglicol) metil éter metacrilato (PEGMA) al 10 %, un tratamiento con plasma atmosférico indujo la copolimerización del ePTFE de la superficie y el monómero de PEGMA. Como revelaron las mediciones del ángulo de contacto con agua (WCA), se logró un cambio en la humectabilidad de las membranas. Mientras que las membranas de ePTFE sin recubrimiento eran hidrofóbicas (WCA de 105 ± 1°), las membranas PEGiladas obtenidas a partir de un tratamiento con plasma de 120 s eran muy hidrofílicas (WCA de 9 ± 1°). La caracterización química confirmó la PEGilación eficaz de las membranas de ePTFE y el análisis de la morfología de la superficie reveló una disminución de la porosidad de las membranas PEGiladas. A medida que aumentaba el tiempo de tratamiento con plasma de las membranas PEGiladas, disminuían la adsorción de proteínas y la unión bacteriana. Se concluyó que las membranas PEGiladas con tratamientos de plasma de al menos 60 s mostraban una reducida adsorción de fibrinógeno (en un 80 %) y una completa inhibición de la unión de *Staphylococcus epidermidis* y *E. coli*.

Stallard et al. (2012) evaluaron la adsorción de proteínas y adhesión bacteriana sobre la superficie de recubrimientos de siloxano depositados sobre silicio y titanio mediante el uso de un sistema APPJ. Se obtuvieron recubrimientos con características de humectabilidad que fueron desde superhidrofílicos a superhidrofóbicos mediante el uso de precursores con diferentes propiedades químicas, así como con diferentes gases, y ajustando parámetros de proceso como la velocidad de flujo del precursor y la distancia entre la fuente de plasma y el sustrato. En general, se observó que la adsorción de fibrinógeno y albúmina sérica bovina tendía a ser mayor en superficies hidrofóbicas que en superficies hidrofílicas. Sin embargo, los recubrimientos hidrofóbicos fluorados exhibieron una menor adsorción de proteínas que los recubrimientos hidrofílicos. Este hecho puede deberse a la presencia de grupos fluorocarbonados que provocan una disminución de la energía superficial, reduciendo así la interacción entre las proteínas y la superficie. Curiosamente, en este estudio, los niveles más bajos de adsorción de proteínas fueron exhibidos por los recubrimientos superhidrofóbicos, que se obtuvieron generando una nanotextura mediante la formación de características superficiales de entre 10 y 250 nm, provocando un aumento significativo de la rugosidad de las superficies. Los recubrimientos fluorados superhidrofóbicos depositados sobre titanio mostraron resistencia a la adhesión de *S. aureus*. Se concluyó que una combinación de una morfología nanotexturizada y una química de baja energía superficial (baja adhesión) creaba

una barrera contra la humectabilidad al atrapar aire en la morfología. Así, se redujo el área disponible para la difusión de proteínas desde un ambiente acuoso a la superficie, así como la fijación de bacterias.

Sarghini et al. (2011) recubrieron sustratos de acero inoxidable con cloruro de 3-(trimetoxisilil)-propildimetiloctadecilamonio (ODAMO) y butilamina a través de la deposición por plasma atmosférico en una cámara DBD de placas paralelas. En este caso, el precursor fue transportado e inyectado en el plasma en forma de aerosol por un flujo de nitrógeno o aire. Se analizó la influencia del precursor, el gas portador, la energía y el tiempo de deposición sobre las propiedades físico-químicas y la actividad anti-biofilm de los recubrimientos. Los recubrimientos obtenidos fueron lisos, delgados y, en la mayoría de los casos, muy hidrofílicos. Se identificó una fuerte influencia del gas portador en la humectabilidad de los recubrimientos de ODAMO, más hidrofóbicos cuando el precursor fue transportado por nitrógeno. La actividad antimicrobiana de las muestras se analizó frente a *E. coli*, observándose su eliminación en los recubrimientos basados en ODAMO obtenidos mediante el uso de aire como gas portador a cualquier potencia. Aunque los recubrimientos a base de butilamina dieron lugar a una menor proliferación bacteriana que el sustrato sin tratar, no fueron tan efectivos como los recubrimientos a base de ODAMO. De acuerdo con los resultados de la caracterización química, se concluyó que la actividad antibacteriana se debió a un compromiso entre dos factores: (i) un contenido sustancial de grupos amonio y (ii) la preservación de cadenas largas hidrocarbonadas provenientes de las moléculas del precursor, que se ve favorecida por el uso de niveles bajos de potencia.

Más recientemente, Hernández-Orta et al. (2018) utilizaron un reactor DBD operando a temperatura ambiente y presión atmosférica para inducir la polimerización y realizar una posterior cuaternización de recubrimientos de 4-vinilpiridina (4VP) sobre sustratos de polietileno de alta densidad. El sustrato de polietileno no mostró efecto anti-biofilm frente a *E. coli*, mientras que los recubrimientos cuaternizados sometidos a 6 y 12 ciclos de polimerización lograron una completa inactivación bacteriana. Estas propiedades bactericidas contra las bacterias Gram negativas se atribuyeron principalmente a la densidad de carga superficial relativamente alta de los recubrimientos, que provoca un efecto electrostático que desestabiliza la superficie de las bacterias y conduce a su destrucción.

1.6. El poder desinfectante del agua activada por plasma

Los líquidos activados por plasma se generan por la interacción del plasma atmosférico con líquidos y su potencial como agentes biocidas se han empezado a explorar en los últimos años,

siendo el agua activada por plasma (PAW, Plasma-Activated Water) el más comúnmente utilizado. Estas propiedades, que pueden llegar a persistir durante periodos de incluso un mes, se deben a la capacidad que presentan las especies químicas generadas en el plasma atmosférico no térmico (PANT), i. e. iones, positios y negativos, radicales libres, átomos y moléculas excitadas, de difundir y de interaccionar entre sí o con el agua, dando lugar a la formación de nuevas especies químicas (**Figura 3**). La presencia de especies reactivas del oxígeno (ROS) y del nitrógeno (RNS), incluyendo radicales hidroxilo, oxígeno singlete, anión superóxido, peróxido de hidrógeno, así como óxido nítrico y sus derivados formados con agua, tales como nitratos, nitritos y peroxinitritos, se ha puesto de manifiesto en diversos estudios (Choi et al., 2019; Khan & Kim, 2019; Xiang et al., 2019; Zhao et al., 2019, 2020). Además, el tratamiento de agua por PANT induce un aumento en su conductividad eléctrica y potencial de óxido-reducción y una reducción en su valor de pH, hasta valores próximos a 3 (Ma et al., 2015, 2016; Joshi et al., 2018; Xiang et al., 2020; Zhao et al., 2019, 2020; Machado-Moreira et al., 2021). Este interesante fenómeno supone un enfoque completamente novedoso en la aplicación del PANT, que consistiría en "activar" primero el agua y posteriormente utilizarla para el tratamiento de superficies. Es de destacar que el PAW proporciona una serie de ventajas adicionales frente al tratamiento directo con PANT, como son la facilidad de generación y aplicación, así como su capacidad para ser almacenada, lo que ha conducido a que diversos grupos de investigación estén explorando sus aplicaciones en la industria alimentaria. En este sentido, se ha demostrado que el PAW presenta un gran potencial como estrategia para mejorar la calidad microbiológica de los alimentos y descontaminar superficies de contacto.

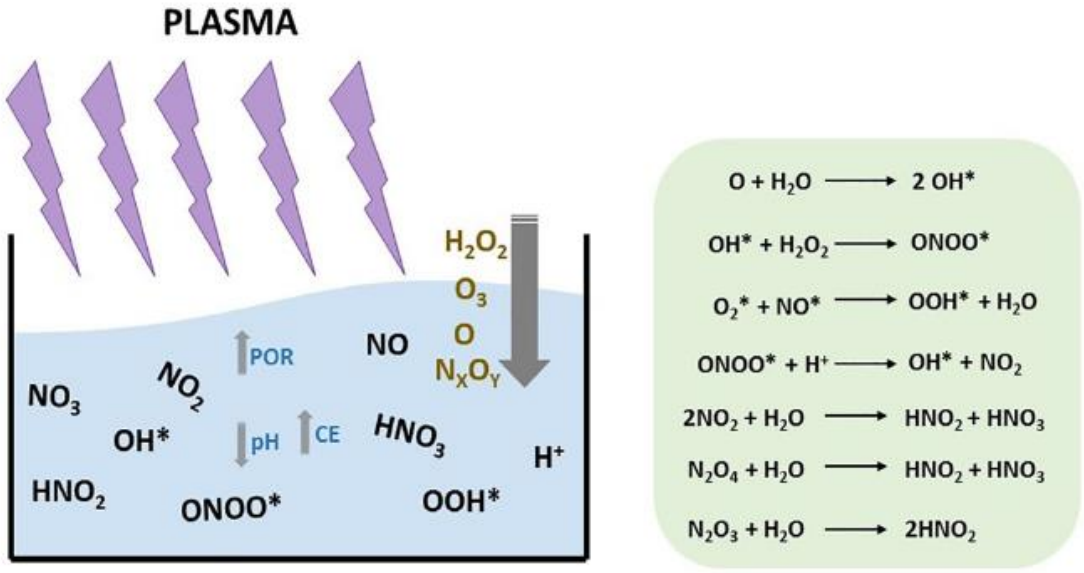

Figura 3. Difusión en el agua de especies químicas reactivas del plasma y formación de nuevas especies, generando agua activada por plasma (PAW). Aumento de la conductividad eléctrica (CE) y del potencial de óxido-reducción (POR) y descenso de pH (López, Fernández-Gómez, Prieto, Álvarez-Ordóñez & Oliveira, 2021).

1.6.1. Efecto de las condiciones de generación en la composición del PAW

Las condiciones de generación del PAW afectan directamente a su composición, dando lugar a PAWs con distintos niveles de especies reactivas de oxígeno y de nitrógeno (RONS) que por tanto serán más apropiadas para diferentes aplicaciones. Por ejemplo, mientras que las PAWs ricas en nitritos y nitratos son más adecuadas para su uso en agricultura, para estimular el crecimiento de plantas o la germinación de semillas, las PAWs con mayores concentraciones de especies reactivas del oxígeno son más apropiadas para aplicaciones terapéuticas, como terapias contra el cáncer, o para la inactivación de virus y bacterias, incluyendo las que se encuentran formando biopelículas (Ostrikov et al., 2020). La composición del PAW depende de múltiples factores, como la energía aplicada para la ionización, el gas utilizado, el volumen y la naturaleza de la solución activada, la configuración del electrodo y la distancia existente entre éste y la superficie del líquido, así como el tiempo de activación (Mai-Prochnow et al., 2021; Zhao, Patange, Sun, & Tiwari, 2020).

Se distinguen tres tipos de interacción plasma-líquido: (i) descargas del plasma en fase gaseosa sobre la superficie de la solución acuosa, (ii) descargas multifase del plasma, con el plasma aplicado en burbujas o en fase gaseosa pero mezclado con gotas de agua y (iii) descarga directa del plasma en la solución acuosa. Estos tipos de generación implican diferentes procesos de

difusión de las especies reactivas al líquido y por tanto las reaciones químicas desencadenadas y la composición final resultante son diferentes (Vanraes & Bogaerts, 2018). Las principales configuraciones de electrodo de los sistemas de plasma atmosférico frío utilizadas en la generación de PAW son los sistemas DBD, el chorro de plasma (plasma jet), la descarga de chispa (plasma spark), la descarga luminiscente (plasma glow) y el plasma de microondas (microwave plasma) (Lu et al., 2016). Diferentes estudios sugieren que en las PAWs obtenidas mediante descarga de chispa y DBD predominan el peróxido de hidrógeno y los nitratos, mientras que las generadas con plasma de microondas y descarga luminiscente muestran mayores concentraciones de nitritos y nitratos (Lu, Boehm, Bourke, Patrick, & Cullen, 2017; Niquet et al., 2018). Como se indicó anteriormente, el gas utilizado en la generación de plasma también influye en la composición del PAW, siendo más abundantes las especies reactivas del nitrógeno cuando se utilizan gases con una baja relación $O_2/(O_2+N_2)$ y las especies reactivas del oxígeno cuando se emplean aquellos con una elevada relación $O_2/(O_2+N_2)$ (Girard et al., 2018). Además, la interacción plasma-líquido multifase permite aumentar las interacciones de las especies reactivas generadas en el plasma con el líquido resultando en una mayor actividad del PAW. La modelización de sistemas de burbujeo de plasma parece indicar que el aumento de la transferencia de masa entre fases se relaciona con un incremento de la concentración final de RONS en el PAW bajo los mismos parámetros de descarga(Wright et al., 2019).

1.6.2. Mecanismos de inactivación del PAW

A pesar de que la capacidad del PAW para inactivar a un amplio rango de microorganismos se describe en un número cada vez mayor de publicaciones, los mecanismos responsables de del efecto letal no están completamente definidos, especialmente en el caso su actividad anti-biopelícula. No obstante, los principales mecanismos propuestos se basan en una combinación del bajo pH característico del PAW y el estrés oxidativo (Mai-Prochnow et al., 2021; Zhao, Patange, Sun, & Tiwari, 2020).

Inicialmente las RONS primarias y secundarias generadas en el PAW, que dan lugar a la reducción del pH y a un incremento de la conductividad eléctrica y del potencial de oxido-reducción, provocan estrés físico y oxidativo en las células microbianas. Algunas especies reactivas del oxígeno, como el radical hidroxilo o el ozono, y el peróxido de hidrógeno, pueden causar la peroxidación de los lípidos de la membrana y dañar la estructura de peptidoglicano de la pared celular bacteriana (Joshi et al., 2011; Yusupov et al., 2013). Además, las proteínas de la membrana también pueden verse oxidadas por la exposición a PAW, observándose un descenso del potencial de membrana y la pérdida de la integridad de la misma (Tian et al., 2015). De

hecho, se ha descrito un aumento de la permeabilidad de la membrana positivamente relacionado con el tiempo de exposición a PAW (Xiang et al., 2018).

Estos daños en la integridad de la membrana favorecen la liberación de iones, ácidos nucleicos y proteínas del citoplasma al exterior (Zhang et al., 2013). Del mismo modo, los protones libres y RONS presentes en el PAW pueden fluir hacia el interior de las células provocando estrés oxidativo y un descenso del pH intracelular, causando un gran impacto en las actividades metabólicas bacterianas debido a su dependencia del pH (Zhang et al., 2016). Algunos autores incluso sugieren que estos efectos podrían observarse en ausencia de daños severos en las envolturas celulares gracias a la capacidad de ciertas especies reactiva de difundir libremente a través de la membrana (Boehm, Curtin, Cullen, & Bourke, 2017). De hecho, se ha propuesto que el mecanismo de acción podría ser ligeramente distinto en bacterias Gram-positivas, con una capa de peptidoglicano más gruesa, y en bacterias Gram-negativas, cuya membrana externa adicional aporta resistencia frente a otros agentes antimicrobianos (Mai-Prochnow et al., 2016).

Múltiples estudios han descrito una menor efectividad del PAW frente a biopelículas que ante células en estado plantónico. Entre las posibles explicaciones para este efecto protector se ha propuesto la presencia de la matriz de sustancias poliméricas extracelulares (EPS), la mayor densidad celular en su interior, el estado fisiológico de las células sésiles y la heterogeneidad de las subpoblaciones presentes (Mai-Prochnow et al., 2021). Para alcanzar las bacterias presentes en el interior de las biopelículas, las especies reactivas del PAW tendrían que difundir a través de la matriz de EPS y los canales de agua, lo que ralentizaría su transporte y daría lugar, no solo a gradientes de concentración, sino que además provocaría que las especies de vida corta se transformaran en otras más estables. Sin embargo, los estudios disponibles hasta el momento acerca de la difusión y el efecto del PAW en la estructura de las biopelículas no son concluyentes (Chen, Su, & Liang, 2017; Hathaway et al., 2019; Hozák et al., 2018). También se ha propuesto que las RONS presentes en el PAW podrían degradar la matriz de la biopelícula liberando las bacterias presentes en el interior (Khosravian, Bogaerts, Huygh, Yusupov, & Neyts, 2014). Además, ciertos estudios de transcriptómica han observado una regulación negativa de genes relacionados con virulencia y quorum sensing en biopelículas tratadas con PAW (Li et al., 2019).

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2. Justification and objectives

Biofilms represent a source of cross-contamination by spoilage and pathogenic microorganisms in the food industry. In food processing environments, bacterial persistence may emerge through the development of these surface-associated microbial communities. Their self-produced extracellular polymeric matrix provides shelter to the inner cells, and contributes to the recruitment of nutrients and to the adhesion to surfaces. The increased tolerance to environmental stress conditions, including cleaning and disinfection agents, of biofilm-associated cells can result in the persistence of pathogenic and spoilage bacteria in equipment and food-contact surfaces, which might cause recurrent food product contamination events. All of this leads to economic losses to the producers and increased health risks for the consumers, highlighting the importance of controlling biofilm formation in food industries.

In the last decades, research efforts have focused on understanding the biotic and abiotic factors that influence the formation and maturation of biofilms in order to develop and validate new biofilm control strategies. Most of these studies are based on *in vitro* models using monospecies biofilms of domesticated strains from the most relevant foodborne pathogens, although some dual- and multi-species biofilm models have been also described. However, the knowledge regarding the ecology and structure of biofilms developed on the surfaces and equipment in real food processing settings is still limited. Also, the molecular determinants responsible for the intraspecies variability regarding biofilm formation ability and tolerance to food-associated stresses is also not fully understood yet.

The increased availability of tools for assessing the complexity of these microbial communities, based on culture-independent sequencing of DNA environmental samples, is already starting to revolutionize the study of biofilms in the food industry. A major challenge for the scientific community is to develop new tools capable of preventing biofilm formation or removing already existing biofilms while avoiding the emergence of microbial resistance phenotypes. Multiple approaches are being explored and, in the last few years, different applications based on the use of non thermal atmospheric plasma technologies have gained great attention. Among these novel technologies, apart from the direct exposure to plasma, plasma-based techniques for surface modification and coating deposition are also a promising approach. Likewise, liquids activated with plasma show a potent antimicrobial activity and have been proposed as a possible alternative to chemical disinfectants in the food industry, showing also potential for their application in the biomedical and agricultural sectors. Moreover, a possible solution to ensure the removal of biofilms formed by hazardous microorganisms that colonize and persist in food processing environments could be the intelligent combination of one or various of these novel biocontrol strategies in synergy with other conventional disinfection methodologies.

The general objective of this PhD Thesis was to study, for several microorganisms of interest in the food industry, the intraspecific variability regarding biofilm formation ability and its association with other phenotypic characteristics of the strains, including RpoS status, production of pigments, cellular hydrophobicity, antibiotic resistance or tolerance to food-related stresses; and to evaluate the effectiveness of two plasma-based biofilm control strategies, i.e. anti-biofilm coatings which modify the physico-chemical properties of materials commonly used in the food industry, applied by non-equilibrium atmospheric plasma-polymerization, and plasma-activated water as an alternative to conventional chemical disinfectants.

The specific objectives of this PhD Thesis are the following:

- To evaluate the influence of the alternative sigma factor RpoS in the ability to form biofilms of a number of field isolates of *Cronobacter sakazakii*, with known status in relation to their RpoS functionality.
- To assess the biofilm formation ability, colony pigmentation, pyoverdine production, RpoS functionality and cellular hydrophobicity of a collection of *Pseudomonas* spp. strains isolated from food processing facilities, in order to identify biomarkers of strong biofilm formation, which could be used in the future for the development of reliable, easy and rapid methods of detection of persistent strains.
- To evaluate the biofilm formation ability as well as the tolerance to heat, acid pH, non-thermal atmospheric plasma and UV-C light of a collection of ESBL-producing *E. coli* strains, isolated from foods of animal origin and from human patients, in order to find associations between biofilm formation ability, resistance to food-associated stresses, RpoS status, carriage of different ESBL encoding genes, and isolation source.
- To develop through non-equilibrium atmospheric plasma anti-biofilm coatings on stainless steel that modify the surface properties, and to thoroughly characterize their morphology, physico-chemical properties and anti-biofilm activity against complex poly-microbial biofilms formed from indigenous microbiota found in meat processing environments.
- To study the capacity of plasma activated water (PAW) to eliminate *Listeria monocytogenes* biofilms on stainless steel and polystyrene, and the mechanisms of *L. monocytogenes* inactivation by, and response to, PAW by testing the antimicrobial activity of defined chemical solutions that mimic some PAW composition parameters and the transcriptomic response of a *L. monocytogenes* strain to PAW through RNA-seq based gene expression analysis.

3. Results

Chapter I

The role of the general stress response regulator RpoS in *Cronobacter sakazakii* biofilm formation

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Chapter II

Biofilm formation ability and tolerance to food-associated stresses among ESBL-producing *Escherichia coli* strains from foods of animal origin and human patients

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Abstract

The dissemination of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* continues to be an important health concern, with the food production chain as a suggested reservoir for this group of bacteria. In this study, a collection of 31 strains, including ESBL-producing *E. coli* strains isolated from foods of animal origin and from human patients and reference collection strains, was evaluated regarding their biofilm formation ability, tolerance to food-associated stresses (heat, acid, non-thermal atmospheric plasma (NTAP) and UV-C light) and RpoS status. The most relevant phenotypic differences among strains were observed for biofilm formation and heat resistance, and they were found related to the source of isolation of the strains (clinical *vs* food *vs* reference strains), or to the sequence type (ST131 *vs* other STs), while only minor differences were related to the occurrence of the ESBL genes bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV}. The biofilm formation ability on stainless steel was significantly higher for the field isolates, both clinical- and food-related, than for the reference strains. Also, food isolates were significantly more resistant to heat treatments at 58 ºC for 1 and 2 min than clinical isolates. Minor differences among strain categories were observed for their tolerance to NTAP and UV-C radiation. Some polymorphisms were detected in the *rpoS* gene sequences, but loss-of-function mutations were not found in any case, with the clustering of strains being mainly based on their sequence type.

Chapter III

Heterogeneity in biofilm formation and identification of biomarkers of strong biofilm formation among field isolates of *Pseudomonas* spp.

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Chapter IV

Development and characterization of anti-biofilm coatings applied by Non-Equilibrium Atmospheric Plasma on stainless steel

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Chapter V

Formation and evolution of multispecies biofilms containing *Listeria monocytogenes* and indigenous meat industry microbiota on a plasma-polymerized anti-biofilm coating applied on stainless steel: impact of different chemical disinfection treatments

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Abstract

Biofilms are considered an important source of microbial contamination in the food industry and they can contribute to the persistence of foodborne pathogens like *Listeria monocytogenes*. In previously published works (Fernández-Gómez et al., 2022; Muro-Fraguas et al., 2021) we described the development and characterization of an anti-biofilm coating applied with an atmospheric-pressure plasma jet system on AISI 316 stainless steel that allowed a 90 % reduction of biofilm formation by *L. monocytogenes* upon incubation at 12 ^{o}C for up to 12 days. In the present study, its anti-biofilm activity against multispecies biofilms containing *L. monocytogenes* (developed using indigenous microbiota from three different meat industries) was evaluated using culture-dependent and culture-independent approaches. Also, the disinfection effectiveness and biofilm evolution after sanitization with two food industry biocides were assessed. A reduced anti-biofilm activity of the coating against *L. monocytogenes* when grown on multispecies biofilms developed for 7 days at 12 ^{o}C with the indigenous microbiota isolated from meat processing environments was observed. In addition, the resulting taxonomic composition of the biofilms formed was highly dependent on the industry, although it was not affected by the artificial inoculation with *L. monocytogenes* and the nature of the surface (uncoated *vs* coated stainless steel). The growth of *L. monocytogenes* was partially controlled in biofilms developed when this pathogen was artificially inoculated, being the magnitude of this effect lower for the industry with the lowest taxonomy richness and diversity. A 15-min disinfection treatments of the biofilms with either sodium hypochlorite or peracetic acid at 0.5 % resulted in total viable and *L. monocytogenes* counts below the limit of detection in most cases. However, the subsequent incubation of the sanitized plates for another 7 days at 12 ^{o}C led to biofilms with similar cell concentrations as before sanitization, although they showed lower bacterial richness and alpha diversity but higher beta diversity. Even though sodium hypochlorite was slightly less effective than peracetic acid immediately after application, it caused a stronger growth control of the naturally present *L. monocytogenes* on the multispecies biofilms developed. This finding suggests that sanitation strategies that avoid the complete removal of competing members of the microbiota might be more favourable for the long-term control of *L. monocytogenes* in food processing facilities.

Chapter VI

Susceptibility and transcriptomic response to plasma activated water of *Listeria monocytogenes* planktonic and sessile cells

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Abstract

Listeria monocytogenes is a foodborne pathogen that, due to its biocide tolerance and biofilm-mediated ability to persist in industrial environments, continues to be a serious concern in the food industry that demands the development of novel control strategies. In the present study, plasma activated water (PAW) was evaluated as an alternative control strategy for *L. monocytogenes* inactivation, both in planktonic state and in biofilms. PAW was generated from tap water using a surface dielectric barrier discharge (SDBD) plasma working at different conditions of discharge power (26 and 36 W) and activation time (5 and 30 min). The influence of these generation conditions on PAW physico-chemical composition (pH and levels of hydrogen peroxide, nitrates and nitrites) and antimicrobial efficacy against a cocktail of three *L. monocytogenes* strains on planktonic state was firstly evaluated. The greatest *L. monocytogenes* inactivation and concentration of reactive species and the lowest pH were achieved under the most intense PAW generation conditions (PAW HM30, generated at 36 W of discharge power with 30 min of activation time). PAW HM30 was subsequently used to study its biofilm removal capacity and mode of action. A lower antimicrobial activity was observed for the three-strain *L. monocytogenes* biofilms, with 15-min treatments achieving 4.6 log reductions for planktonic cells but only 1.9 and 1.8 log reductions for biofilms formed on polystyrene and stainless steel, respectively. The influence of the reactive species and pH on PAW inactivation efficacy was evaluated by assessing the antimicrobial activity of chemical solutions that mimic PAW HM30 composition against *L. monocytogenes* in planktonic state with an exposure time of 30 min. Additionally, a transcriptomic analysis of the response of *L. monocytogenes* ULE1265 to PAW showed a general remodelling of carbon metabolism, with a strong upregulation of the cobalamin-dependent gene cluster (CDGC), and the differential expression of some genes related to the general stress response, controlled by the alternative sigma factor SigB, and to virulence. Overall, our results show the potential of PAW as an alternative to traditional chemical disinfectants in the food industry and contribute to the understanding of its mechanisms of action.

4. Discussion

Biofilm mediated colonization of surfaces and equipment by spoilage or pathogenic microorganisms is an important concern in the food industry since biofilms can function as a source for cross-contamination of food products and can reduce the efficacy of cleaning and disinfection protocols, compromising food safety and quality (Alvarez-Ordóñez, Coughlan, Briandet, & Cotter, 2019; Coughlan, Cotter, Hill, & Alvarez-Ordóñez, 2016; Larsen et al., 2014). Therefore, great research efforts are being made to develop new strategies to remove biofilms from industrial settings or to prevent their formation. These alternative approaches must be more effective than the currently available ones as well as more economic and sustainable. A better understanding of the factors modulating biofilm formation and the bacterial response to environmental stresses prevailing in food processing can contribute to the development of novel biofilm control approaches.

In this PhD Thesis, the biofilm formation ability of a large collection of strains from various microorganisms of importance in the food industry (i.e., *Cronobacter sakazakii*, *Pseudomonas* spp., *Escherichia coli*, *Listeria monocytogenes*), including both pathogenic and spoilage bacteria, was evaluated on polystyrene and/or stainless steel. In addition, other phenotypical characteristics of interest, such as the RpoS status, production of pigments, cellular hydrophobicity, antibiotic resistance and tolerance to food-related stresses, were also monitored to find associations between biofilm formation and other cellular features, and to identify potential biomarkers of strong biofilm formation. Afterwards, two plasma-based biofilm control strategies were investigated. The first one was focused on the prevention of biofilm formation on industrial stainless steel surfaces through the application of a plasma-polymerized anti-biofilm coating that modifies the physico-chemical properties of the surface. The second one consisted on the use of plasma activated water (PAW) as an alternative to conventional disinfectants against mature biofilms of *Listeria monocytogenes*.

4.1. Inter- and intra-species variability in biofilm formation

Along the PhD Thesis, the biofilm formation ability of a collection of 10 *Cronobacter sakazakii*, 31 *Escherichia coli*, 33 *Pseudomonas* spp. and 3 *Listeria monocytogenes* strains isolated from different sources was evaluated on polystyrene and/or on stainless steel. Comparing the results obtained when the same culture conditions were used, i.e. BHI broth as culture media and an incubation time of 24 h at 37 ^{o}C (**Figure 1**), a high inter-and intra-species variability was observed regarding biofilm formation, and, in general, *Pseudomonas* spp. strains showed higher biofilm formation levels. Thus, the observed mean and SD values of $OD_{595\ nm}$ for biofilms formed on polystyrene were of 1.82±2.57 for *Pseudomonas* spp., 0.31±0.22 for *E. coli* and 0.11±0.08 for

C. sakazakii. Similarly, when biofilm formation was evaluated on stainless steel, only for *Pseudomonas* spp. and *E. coli*, these taxa showed mean values of $OD_{595\ nm}$ of 1.81±2.03 and 0.36±0.28, respectively. The observed generally higher biofilm formation by the *Pseudomonas* spp. strains compared to the *E. coli*, *C. sakazakii* and *L. monocytogenes* ones is in agreement with the well-known strong ability to form biofilms shown by the microorganisms from the *Pseudomonas* genus (Meliani & Bensoltane, 2015; Møretrø & Langsrud, 2017). In this case, it might also be related to the source of isolation since all the *Pseudomonas* spp. strains were isolated from food processing environments while the *C. sakazakii* strains were clinical isolates and the *E. coli* strains were either isolated from clinical specimens or food products. Additionally, as described in Chapter 5, when the indigenous microbiota from meat industries was used to develop biofilms on stainless steel, the relative abundance of the *Pseudomonas* genus was higher in the biofilms formed after 7 days at 12 ºC than on the initial inocula, which indicates that *Pseudomonas* is one of the members of the microbiota of the industries with the best biofilm formation capacity.

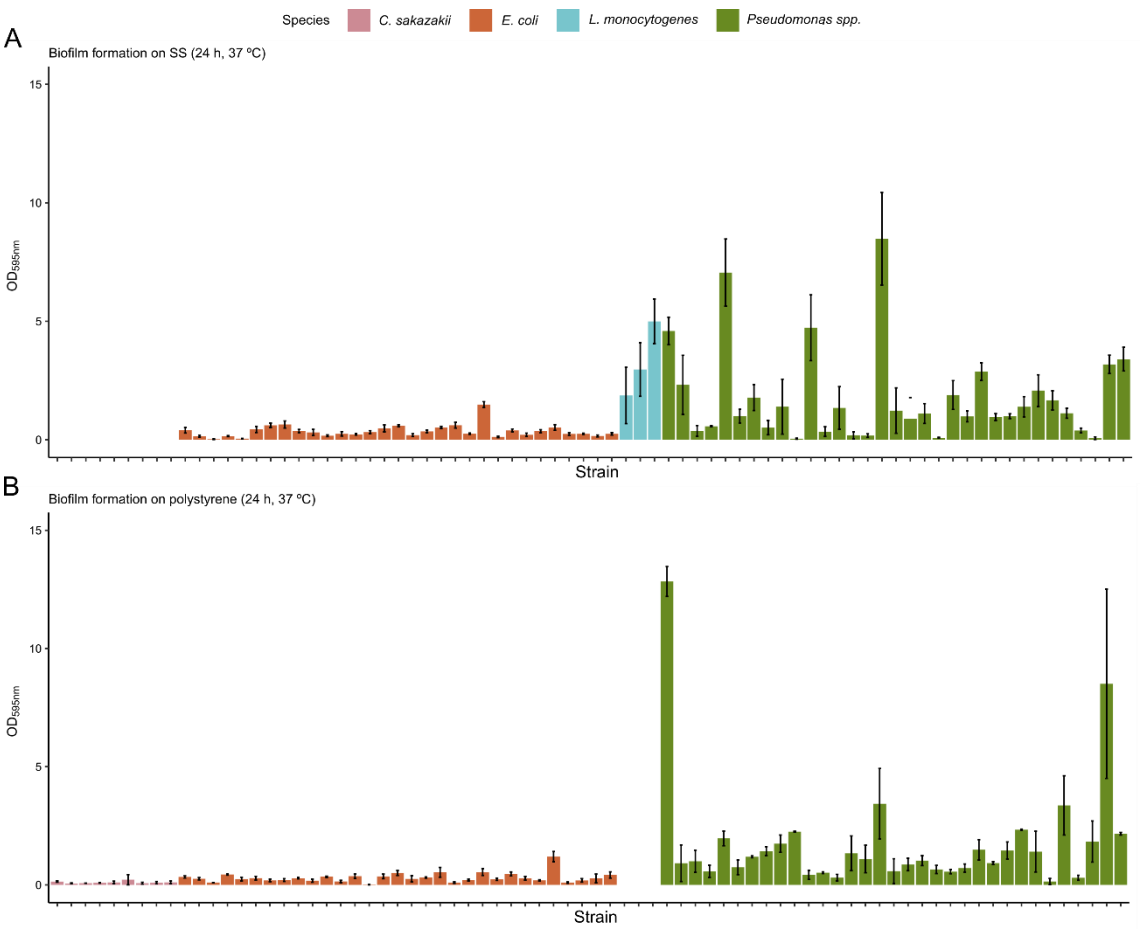

Figure 1. Biofilm formation by *C. sakazakii*, *E. coli, Pseudomonas* spp. and *L. monocytogenes* on (A) stainless steel and (B) polystyrene in BHI broth after incubation at 37 ºC for 24 h.

The influence of the surface nature (stainless steel *vs* polystyrene) on the microorganisms' ability to develop biofilms was variable depending on the genus. In the case of the *Pseudomonas* spp. collection, even though only small differences were detected between the mean values of $OD_{595\,nm}$, the biofilm formation ability of individual strains was different based on the surface type, as shown in **Figure 1**. However, biofilm formation by *E. coli* and *C. sakazakii* strains was barely influenced by the nature of the surface. *Pseudomonas* spp. strains presented the highest intra-species biofilm formation variability, showing a coefficient of variation of 1.12 on stainless steel and 1.55 on polystyrene and most of the strains were classified as moderate (41.9 % of the strains on stainless steel and 38.7 % of the strains on polystyrene) or strong biofilm producers (35.5 and 54.8 % on stainless steel and polystyrene, respectively). Lower intra-species variability was observed for *E. coli* strains, with a coefficient of variation of 0.77 on stainless steel and 0.72 on polystyrene, and most of the strains classified as weak (36.4 %) and moderate (51.5 %) biofilm producers on stainless steel, and as moderate (45.5 %) and strong (33.3 %) biofilm producers on polystyrene. *C. sakazakii* strains showed also a low intraspecies variability, similar to that of *E. coli*, with a coefficient of variation of 0.77 on polystyrene and all of the strains classified as no biofilm producers (66.7 %) or weak biofilm producers (33.3 %).

The effect of multiple culture conditions in the biofilm formation ability of the strains was studied for the *C. sakazakii* collection, including different growth media (brain heart infusion (BHI) and minimum medium (MM)), pH values (media non-buffered or buffered to pH 7.0) and the supplementation with amino acids (arginine, lysine and glutamic acid). Despite the observed intra-species variability, biofilm formation levels were higher in MM, especially when it was buffered at pH 7.0, than in BHI broth, indicating that low-nutrient conditions favour biofilm development while the natural acidification in the non-buffered media had the opposite effect, as previously described (Ariafar, Buzrul, & Akçelik, 2016; Tack, Nimmegeers, Akkermans, Hashem, & Van Impe, 2017). The supplementation of the culture media with arginine, lysine or glutamic acid barely affected biofilm formation and differences were only noticeable in buffered MM, which suggest that the variations are caused by the availability of amino acids and not by the activation of the homeostatic amino acid dependent decarboxylase systems, as these are only activated under low pH conditions (Richard & Foster, 2004). Subsequently, the biofilm formation ability on the two surface materials (stainless steel and polystyrene) was assessed using the buffered (pH 7.0) MM supplemented with lysine as culture medium and a generally higher biofilm formation was found on stainless steel than on polystyrene. This observation was not easily comparable to findings in the literature due to the differences in the strains and

methodology used and the lack of consensus in results (Iversen, Lane, & Forsythe, 2004; Kim, Ryu, & Beuchat, 2006).

The biofilm formation of the 3 *L. monocytogenes* strains included in this PhD Thesis for the optimization of anti-biofilm coatings was only evaluated on stainless steel, showing a mean $OD_{595\ nm}$ of 2.44±1.57 (**Figure 1A**). This high biofilm formation levels are due to the intentional selection of strong biofilm forming strains aimed at better observing the potential anti-biofilm activity of the tested developed coatings. Also, it is important to highlight that the biofilm formation ability by the two strains isolated from meat processing environments, with mean values of $OD_{595\ nm}$ of 2.97±1.13 and 5.00±0.94, was higher than that of the reference strain from the Spanish Type Culture Collection (CECT), which showed a mean $OD_{595\ nm}$ of 1.87±1.19.

4.2. Diversity in functionality and role in biofilm formation of the general stress response regulator RpoS

In Gram-negative bacteria, the alternative sigma factor RpoS, encoded by the *rpoS* gene, regulates the general stress response of stationary-phase cells (Battesti, Majdalani, & Gottesman, 2010; Weber, Polen, Heuveling, Wendisch, & Hengge, 2005; Wong et al., 2017). Moreover, the *rpoS* gene has been also shown to play a major role in the establishment of mature biofilms in *E. coli* through shifts in global gene expression that affect not only the response to stresses but also energy metabolism and motility (Ito et al., 2008; Ren et al., 2004). It has been described, for *E. coli* and *Salmonella* spp., that the chromosomic region where the *rpoS* gene is located is highly polymorphic, which leads to high phenotypic diversity among field isolates (Ferenci, 2003; Larsen et al., 2014; Robbe-Saule, Algorta, Rouilhac, & Norel, 2003; Saxer et al., 2014). In this PhD Thesis, the influence of *rpoS* gene polymorphisms on biofilm formation ability was evaluated for the field isolates of *Pseudomonas* spp., *E. coli* and *C. sakazakii*.

Regarding the *C. sakazakii* strains, the highly polymorphic nature of this gene was previously described by Álvarez-Ordóñez et al.(2012) and, for the 9 *C. sakazakii* natural isolates included in this PhD Thesis, 7 single nucleotide polymorphism (SNP) positions and 5 alleles were described. While 4 of the strains presented single substitutions on the amino acid sequence that did not affect the biofilm formation ability, one strain showed a 843-bp deletion including the first 174 bp of the *rpoS* open reading frame. This loss-of-function mutation resulted in reduced stress tolerance, as shown in Álvarez-Ordóñez et al.(2012), and, as described in this PhD Thesis, in lower biofilm formation levels.

The lower biofilm production observed both on stainless steel and polystyrene for the *C. sakazakii* strain that presented the loss-of-function mutation in the *rpoS* gene compared to the rest of tested strains, was confirmed through confocal laser scanning microscopy (CLSM) on glass-bottom 96-well plates. These findings are in agreement with the previously described importance of the alternative sigma factor RpoS in *E. coli* biofilm formation (Adnan et al., 2017; Álvarez-Ordóñez et al., 2013; Battesti et al., 2010; Ito et al., 2008; Ito, May, Taniuchi, Kawata, & Okabe, 2009). To further characterize the influence of the lack of a functional *rpoS* gene on *C. sakazakii* biofilm formation, a *C. sakazakii* complemented strain constitutively expressing a functional *rpoS* gene, previously constructed and described by Álvarez-Ordoñez et al. (2012), was introduced in the study. This complementation resulted in an enhanced biofilm formation after 24 h of incubation, on all the different tested abiotic surfaces, up to levels similar to those of *C. sakazakii* strains with a functional *rpoS* gene. However, after 48 h of incubation, the differences in biofilm formation between the *C. sakazakii* strain with the loss-of-function mutation in the *rpoS* gene and its complemented counterpart were no longer statistically significant. Additionally, the study of the biofilm architecture by CLSM and scanning electron microscopy (SEM) showed a similar scenario, with a more similar biofilm architecture between both strains after 48 h of incubation. These results seem to indicate that the lack of RpoS caused a delay in the development of mature biofilms, rather than a complete inhibition of biofilm production in *C. sakazakii*. Nevertheless, the universality of that conclusion should be confirmed using other *C. sakazakii* strains and further investigation is needed regarding the genetic mechanisms responsible for the observed delay in biofilm formation.

On the contrary, no association between *rpoS* polymorphisms and biofilm formation ability were observed on the tested *Pseudomonas* spp. and *E. coli* strain collections. In the case of the *Pseudomonas* spp. strain collection, the sequencing of the *rpoS* gene showed low variability among the analysed strains, with 31 single-nucleotide polymorphism (SNP) positions and 10 identified alleles that only resulted in changes in the amino acid sequence of the RpoS protein in 2 strains. One of these strains presented a frameshifting insertion and a premature stop codon at the end of the coding sequence while the other strain showed a substitution that resulted in a neutral change in the amino acid sequence. Even though the importance of RpoS has been previously described for multiple *Pseudomonas* species (Bouillet, Ba, Houot, Iobbi-Nivol, & Bordi, 2019; Liu, Xu, Zhu, Du, & Sun, 2019; Schuster, Hawkins, Harwood, & Greenberg, 2004; Suh et al., 1999), no clear clustering based on biofilm formation ability was observed (**Figure 2**).

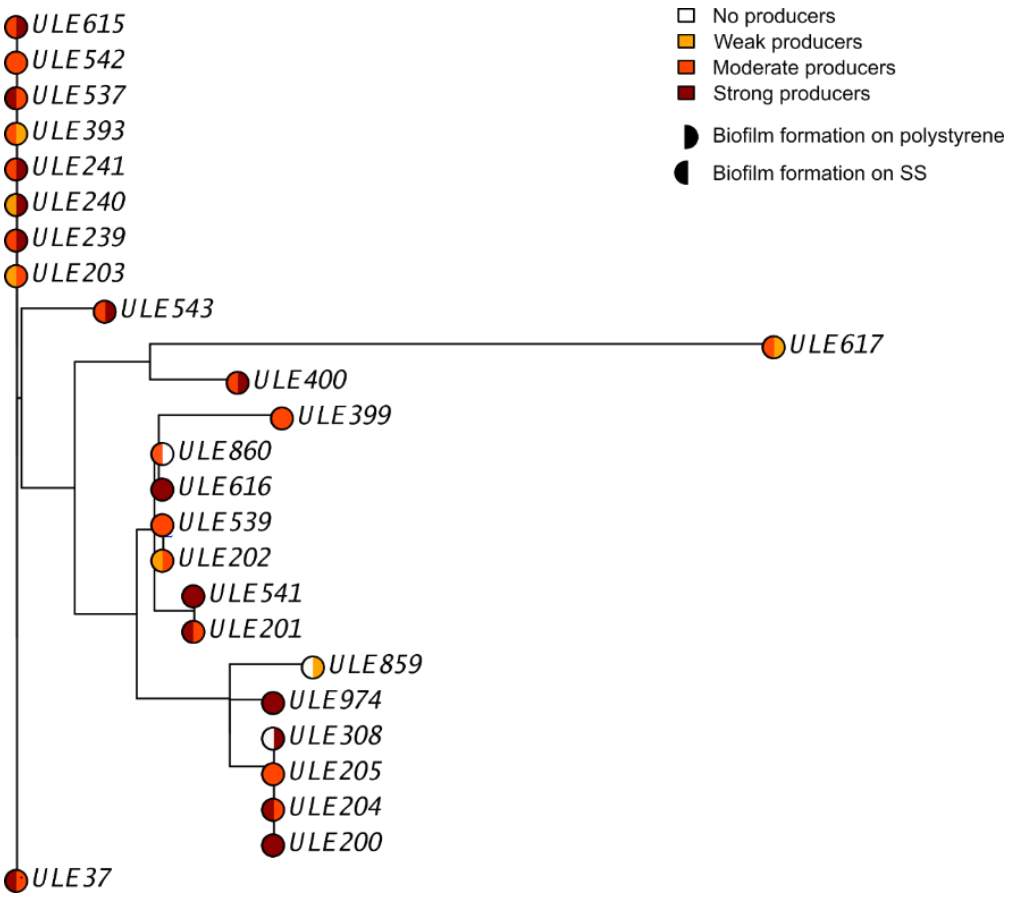

Figure 2. Rooted neighbour-joining phylogenetic tree of the *rpoS* gene sequences from the *P. aeruginosa* strains constructed by R studio software.

Similarly, the genetic diversity in the *rpoS* gene sequence for the studied *E. coli* strains was also low, with 11 SNP positions and 10 alleles being identified. These nucleotide polymorphisms resulted in 2 non-conservative changes in the amino acid sequence of 2 *E. coli* strains, respectively. However, no clear associations between *rpoS* sequence polymorphisms and biofilm formation ability were observed either (**Figure 3**).

Overall, out of the 74 strains included in this PhD Thesis, only one strain was found to have a compromised functionality of RpoS, due to a premature stop codon. The study of this strain, which belongs to *C. sakazakii*, confirmed the importance of the alternative sigma factor RpoS in biofilm formation, with the lack of a functional RpoS being associated with a delay in the development of mature biofilms but not to a complete inhibition of biofilm production. The relevance of RpoS for mature biofilm formation has been previously demonstrated in *E. coli* and *Pseudomonas*, but these observations could not be confirmed in this PhD Thesis due to the absence of strains from these species with compromised RpoS functionality.

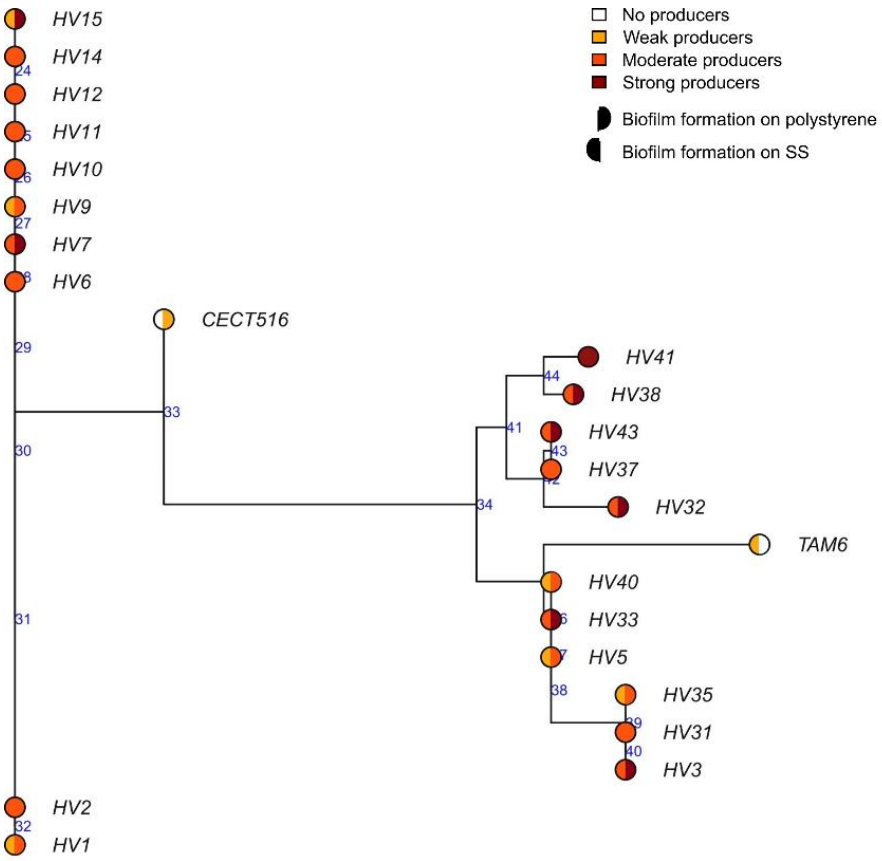

Figure 3. Rooted neighbour-joining phylogenetic tree of the *rpoS* gene sequences from the *E. coli* strains constructed by R studio software.

4.3. Evaluation of potential relations between biofilm formation ability, tolerance to food-stressors and resistance to β-lactam antibiotics in *E. coli*

Sigma factor transcriptional regulators, including RpoS in Gram-negative bacteria, have been proposed as a possible molecular mechanism responsible for an association between antibiotic resistance and tolerance to food-associated stress conditions (Liao et al., 2020). In this PhD Thesis, the collection of 31 *E. coli* strains, including 27 extended-spectrum β-lactamase (ESBL) producing strains, was screened regarding their RpoS status, biofilm formation ability and tolerance to different food-related stresses (heat, acid, non-thermal atmospheric plasma (NTAP) and UV-C light) in order to investigate the occurrence of associations between those phenotypes and the carriage of ESBL genes. ESBL-producing *Enterobacteriaceae* have become a major public health concern, since the production of these enzymes confers resistance to penicillins, first-, second-, third-, and fourth-generation cephalosporins, and to aztreonam, reducing the efficacy of many antibiotic therapies (Chong, Shimoda, & Shimono, 2018; Doi, Iovleva, & Bonomo, 2017; Kawamura et al., 2017). In fact, the continuous and worldwide dissemination of ESBL-producing *E. coli* has led to their inclusion on the World Health Organization (WHO) list of critical priority

pathogens for the discovery of new antibiotics in 2017 (WHO, 2017). The food production chain has been described as a possible transmission route of this group of bacteria from livestock to humans (Alegría et al., 2020; Bergšpica, Kaprou, Alexa, Prieto, & Alvarez-Ordóñez, 2020; Day et al., 2019; Kaesbohrer et al., 2019; Tekiner & Özpınar, 2016). The collection of *E. coli* strains evaluated in this PhD Thesis included 15 ESBL-producing strains of clinical origin, mainly from ST131, 12 ESBL-producing strains of food origin, from a wide range of STs different to ST131, and 4 reference strains.

The previously described polymorphisms in the *rpoS* gene were not associated with notably different biofilm formation abilities or resistance to food-related stresses, as already pointed out above. This is probably due to the absence of isolates in the collection with loss-of-function mutations in the *rpoS* gene. However, the constructed phylogenetic tree constructed with the nucleotide sequences showed a clustering of the *rpoS* sequences by sequence type of the strains, with isolates of clinical origin (mainly from ST131) clustering separately from isolates of food origin (from a range of other STs). The composition of the strain collection used is in agreement with the widely described predominance of ST131 among hospital-derived ESBL-producing *E. coli* (Chong et al., 2018; Nicolas-Chanoine, Bertrand, & Madec, 2014).

The biofilm formation ability on stainless steel of the *E. coli* strains included in this PhD Thesis was higher for the field isolates, both clinical- and food-related, than for the reference isolates, but no significant influence of the source of isolation was observed for the biofilms developed on polystyrene. These results could be attributed to the selection of strains with increased ability to form biofilms as a mean to survive the frequent sanitization protocols that are applied on hospitals and food processing plants. Additionally, some differences in biofilm formation were observed between groups of *E. coli* isolates based on the carriage of the ESBL genes bla_{TEM} and bla_{SHV}, with the presence of bla_{SHV} being associated with lower biofilm formation and the presence of bla_{TEM} with a higher biofilm development.

The phenotypic diversity regarding heat, acid, NTAP or UV-C light tolerance was limited and, again, most of the significant differences were related to the source of isolation and/or the sequence type while the carriage of the ESBL genes bla_{TEM}, $bla_{CTX\text{-}M}$ or bla_{SHV} showed minor associations with the strains´ stress tolerance. The strongest associations were found for heat resistance, with clinical isolates being more sensitive than food and reference strains. This higher tolerance of food isolates might be related to the common exposure to moderate thermal treatments in food production. Additionally, strains belonging to the ST131 and strains carrying the $bla_{CTX\text{-}M}$ gene were more sensitive to the heat treatments. The carriage of plasmids has been

usually linked to a fitness cost for the bacteria even though some studies have shown that certain *E. coli* lineages with ESBL-carrying plasmids have enhanced virulence while maintaining the fitness (Dimitriu et al., 2019; Ranjan et al., 2018; Schaufler et al., 2016).

The similar associations obtained in this PhD Thesis for clinical isolates and ST131 strains are likely due to the predominance of that ST among the clinical ESBL-producing *E. coli* strains tested. Likewise, in the *E. coli* strain collection studied in this PhD thesis, the $bla_{CTX\text{-}M}$ gene was detected in 93 % of the clinical isolates but only in 33 % of the food isolates, and these differential distribution of $bla_{CTX\text{-}M}$ carrying isolates among clinical and food *E. coli* isolates could influence the observed lower heat resistance of this group of strains. The presence of heat-resistant ESBL-producing *E. coli* strains has been previously described on the dairy industry and on isolates from hospital patients, highlighting their potential to survive heat-treatments and to act as a reservoir of plasmids encoding ESBL in those settings (Boll, Frimodt-Møller, Olesen, Krogfelt, & Struve, 2016; Marti et al., 2016). Regarding acid, NTAP and UV-C light tolerance, only minor differences among the studied strain categories were observed.

Altogether, in the *E. coli* strain collection evaluated in this PhD Thesis no strong associations were found between the carriage of the ESBL genes bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV} or the polymorphisms in the *rpoS* gene and the strains´ biofilm formation ability and resistance to food-related stresses such as heat, acid, NTAP and UV-C radiation. Instead, a notable influence of the source of isolation (food *vs* clinical *vs* reference), which was closely linked to the ST (ST131 *vs* other STs), was observed for biofilm formation ability and heat resistance. Biofilm formation levels were generally higher on field isolates (food and clinical) than on reference strains and clinical isolates were more sensitive to heat treatments than food-related and reference strains. These results contribute to the available knowledge on the role of the food chain in the spread of ESBL-producing *E. coli*.

4.4. Identification of biomarkers of biofilm formation in *Pseudomonas*

Apart from *rpoS* polymorphisms, various phenotypic characteristics that can influence biofilm formation (Banin, Vasil, & Greenberg, 2005; Hassett et al., 1996; Kang & Kirienko, 2017; Suh et al., 1999) were also monitored in the *Pseudomonas* spp. strain collection. Thus, cellular hydrophobicity, colony pigmentation, pyoverdine production and catalase activity were evaluated in the search for biomarkers of strong biofilm formation. Several *Pseudomonas* species, including the opportunistic pathogen *Pseudomonas aeruginosa*, are known to cause spoilage of chilled foods (Caldera et al., 2016; de Oliveira, Favarin, Luchese, & McIntosh, 2015; Raposo, Pérez, Faria, Ferrús, & Carrascosa, 2016) and there is a need for developing rapid tests

based on the monitoring of biomarkers that could be used for the early detection of persistent spoilage isolates, facilitating cleaning and disinfection strategy management in food processing environments.

The screening of catalase production by the *Pseudomonas* spp. strains was included as an indirect indicator or RpoS status, since a reduction in catalase production, with the subsequent increase in sensitivity to hydrogen peroxide, has been described in microorganisms with loss-of-function mutations in the *rpoS* gene (Suh et al., 1999). However, no clear associations between catalase activity and biofilm formation were found for the *Pseudomonas* spp. strain collection, which agrees with the previously described low variability in the *rpoS* amino acid sequences.

The colony pigmentation on solid media was evaluated as a possible biomarker since this feature has been previously proposed as an indicator of pathogenicity and, also, blue pigment production has been linked to higher biofilm production (Liu & Nizet, 2009; Rossi et al., 2018). Although a high inter- and intra-species variability in biofilm formation was observed in the *Pseudomonas* spp. collection, biofilm formation levels were significantly higher on the green-pigmented strains, both on stainless steel and polystyrene, but no statistical differences were observed for the biomass calculated from CLSM analyses. In order to test the possible influence of the different material surface properties (stainless steel *vs* polystyrene *vs* glass), the wettability of the strains was evaluated by an adhesion-to-hydrocarbon protocol (Hsu, Fang, Borca-Tasciuc, Worobo, & Moraru, 2013). Although cellular hydrophobicity has been previously linked to stronger biofilm formation in *Pseudomonas putida* (Baumgarten et al., 2012) and *P. aeruginosa* (Mirani et al., 2018), in this PhD Thesis no clear associations were observed. Nevertheless, strain cellular hydrophobicity was significantly higher for the *P. aeruginosa* isolates than for the ones belonging to other *Pseudomonas* species.

Considering the observed relation between biofilm formation and strain pigmentation on solid media, the production of pyoverdine, a siderophore involved in extracellular iron acquisition and responsible for *Pseudomonas* spp. green coloration (Hoegy, Mislin, & Schalk, 2014; Llamas et al., 2006), was investigated. However, no clear association between pyoverdine production and biofilm formation ability was observed. Instead, the most influential factor was the *Pseudomonas* species (*P. aeruginosa* vs other *Pseudomonas* species), with *P. aeruginosa* strains showing higher pyoverdine production. Remarkably, no statistically significant differences regarding pyoverdine production were found among the three pigmentation groups, even though higher values were in general obtained for green and brown strains than for not pigmented strains. While several researchers have shown a strong association between biofilm

formation and production of pyoverdine and other pigments in reference strains of *P. aeruginosa* and *Pseudomonas fluorescens* (Dave et al., 2020; Llamas, Imperi, Visca, & Lamont, 2014; Rossi et al., 2018), other studies using collections of field isolates suggest a strain-associated character for this relation (Kang, Turner, & Kirienko, 2018; Milojković et al., 2020). The temperature has also been shown to have an effect in these findings since higher biofilm biomass was previously observed on selective media at 15 ^{o}C for *P. fluorescens* and *Pseudomonas gessardii* strains with colored colonies (Quintieri et al., 2020).

The possible relation between colony pigmentation, siderophore production and biofilm formation was further evaluated through the study of the effect of iron availability on biofilm production on stainless steel. For this, various strains representative of each pigmentation category (not pigmented, brown and green) were selected to compare their biofilm formation ability in BHI broth supplemented with two concentrations of an iron scavenger, 2,2-bipyridine (22BP), and in non-supplemented BHI broth. A coordinated regulation of *Pseudomonas* iron-uptake and biofilm formation has been previously described (Llamas et al., 2014) and limiting iron availability through the addition of 22BP to the growth media has been related to reduced biofilm formation on several *Bacillus cereus* strains (Hayrapetyan, Muller, Tempelaars, Abee, & Groot, 2015), as well as on a reference strain of *P. aeruginosa* (O'May, Sanderson, Roddam, Kirov, & Reid, 2009). However, in the current study, biofilm production was not significantly affected by the addition of the iron scavenger 22BP, regardless of the strain pigmentation. These results are in agreement with the observations made by O'May et al. (2009) for some *P. aeruginosa* clinical isolates, which were unaffected by 22BP media supplementation.

Overall, the phenotypic heterogeneity observed for the majority of the potential biomarkers studied, including pyoverdine production, catalase activity and cellular hydrophobicity, was predominantly dependent on the *Pseudomona*s species rather than on the biofilm formation capacity. However, the green colony pigmentation on solid media has been identified as a potential biomarker of strong biofilm formation on stainless steel and polystyrene both in *P. aeruginosa* and *Pseudomonas* spp. These results need to be confirmed on a larger and more diverse collection of *Pseudomonas* field isolates under different culture conditions.

4.5. Towards novel biofilm control strategies: development and characterization of a plasma-polymerized anti-biofilm coating

Conventional methods of cleaning and disinfection applied in the food industry often show limited efficacy for the complete removal of mature biofilms. Biocides are less effective towards cells encased in biofilms than on planktonic state and, even if they are adequately used, the

presence of niches where microorganisms are exposed to sub-inhibitory concentrations can lead to the selection of resistant microorganisms (Alvarez-Ordóñez et al., 2019; Langsrud, Sidhu, Heir, & Holck, 2003; Larsen et al., 2014; Skowron et al., 2019). Additionally, the resident microbiota that colonizes food processing environments forms complex multispecies biofilms that frequently show higher resistance than their single species counterparts to disinfectants (Fagerlund et al., 2017; Li et al., 2021). These issues together with the potential environmental pollution and the consumers´ negative perception of chemical substances are leading research efforts towards the development of novel biofilm control strategies, including the prevention of biofilm formation by reducing the initial bacterial adhesion to surfaces (Gray et al., 2018; Mazaheri, Cervantes-Huamán, Bermúdez-Capdevila, Ripolles-Avila, & Rodríguez-Jerez, 2021). Among the different approaches available to achieve an anti-biofilm effect, the application of coatings that modify both the chemical and morphological characteristics of surfaces (Cao et al., 2018; Coughlan et al., 2016; Faure et al., 2012; Friedlander, Nir, Reches, & Shemesh, 2019; Zhong, Pang, Che, Wu, & Chen, 2013) was the one tested in this PhD Thesis.

For the optimization of anti-biofilm coatings, the biofilm formation levels by two strains of the major foodborne pathogenic microorganisms *E. coli* and *L. monocytogenes* were tested on a total of 20 coatings applied on stainless steel by NTAP-polymerization. Two coatings, AP10+AA6 and AP10+TE6, that consisted on a base coating of (3-aminopropyl)triethoxysilane (APTES) and a functional coating of acrylic acid (AA) or tetraethyl orthosilicate (TEOS), were selected for their anti-biofilm activity against *L. monocytogenes*, achieving a 45 and 74 % reduction, respectively, in biofilm formation at 37 ^{o}C for 24 h. After this initial screening, where biofilms were incubated for 24 h at 37 ^{o}C and biofilm development was assessed by spectrophotometric determination of the fixed crystal violet, these two selected coatings, together with a new coating (AP10+SA6), with the same base coating and a functional coating of succinic acid (SA), were characterised in detail in an extended experimental set up where a lower temperature of 12 ^{o}C with longer incubation times, of up to 12 days, were used as a closer representation of conditions found in food processing environments. Also, the anti-biofilm activity of the coatings was evaluated against two additional *L. monocytogenes* strains, isolated from a meat industry, and the results were confirmed by direct visualization of the biofilms through SEM. The highest anti-biofilm activity was obtained for the AP10+AA6 coating, which achieved a 90 % reduction in biofilm formation by the different *L. monocytogenes* strains after incubation at 12 ^{o}C for up to 12 days.

The mechanisms responsible for the observed anti-biofilm activity were investigated through the morphological and physico-chemical characterization of the three selected coatings by atomic force microscopy (AFM), SEM, X-ray photoelectron spectroscopy (XPS) and water contact

angle (WCA) measurement. The analysis of the surface topography showed that smoother surfaces, such as those of the coating AP10+AA6, might limit *L. monocytogenes* adhesion and biofilm formation. The APTES base coating not only provided mechanical resistance and adhesion to the surface but also created a smoother surface, reducing the occurrence of the characteristic grooves of stainless steel that potentially provide shelter and an increased contact area for microorganisms (Lorenzetti et al., 2015; Medilanski, Kaufmann, Wick, Wanner, & Harms, 2002; Mosquera-Fernández, Rodríguez-López, Cabo, & Balsa-Canto, 2014; Verran, Rowe, & Boyd, 2001; Wu, Zhang, Liu, Suo, & Li, 2018). The measurement of the wettability of the coatings showed the strong hydrophilic character of the most effective anti-biofilm coating (AP10+AA6), which presented the lowest WCA (18.74^{o}), while the uncoated stainless steel was more hydrophobic, with a WCA of 89.45^{o}. These observations suggested the formation of a hydration layer bound to the coating surface that would limit the interaction of bacterial proteins with the surface, reducing the initial bacterial adhesion necessary for biofilm development (Bazaka, Jacob, Chrzanowski, & Ostrikov, 2015; Oh et al., 2018; Peng, Song, & Fort, 2006; Sardella, Palumbo, Camporeale, & Favia, 2016; Yuan, Hays, Hardwidge, & Kim, 2017; Zheng et al., 2005). Additionally, the XPS chemical characterization revealeda relation between the abundance of oxygen polar groups (C-O, C=O, O-C=O) and the increased surface hydrophilicity. The influence of biofilm development conditions, at 37 or 12 ^{o}C, on the anti-biofilm activity of the coatings was associated to the described effect of growth temperature on bacterial hydrophobicity and surface attachment (Abdallah et al., 2019; Di Bonaventura et al., 2008; Lee, Hébraud, & Bernardi, 2017). In fact, *L. monocytogenes* strains showed higher cellular hydrophilicity at 12 ^{o}C, conditions at which the stronger anti-biofilm activity of the best coating AP10+AA6 was observed.

The promising results obtained with the coating AP10+AA6 against monospecies biofilms of *L. monocytogenes*, especially at lower temperatures, which would facilitate its implementation in the food industry, encouraged the additional characterization of this coating. Therefore, its anti-biofilm efficacy against complex multispecies biofilms containing *L. monocytogenes*, and after exposure to two common disinfectants (sodium hypochlorite and peracetic acid), was subsequently evaluated through culture-dependent and culture-independent approaches.

In Chapter 5 of this PhD Thesis, indigenous microbiota samples recovered from food-contact and non-food-contact surfaces on three meat processing plants were used to develop biofilms, with and without artificial inoculation with *L. monocytogenes*, on coated and on uncoated stainless steel plates incubated at 12 ^{o}C for 7 days. Similar biofilms, in bacterial load and taxonomic composition, were developed on coated and uncoated stainless steel, and a limited efficacy of

the coating against *L. monocytogenes* in the multispecies biofilms was observed, even though some anti- or pro-biofilm tendencies were found for the different industries. Culture-independent analyses showed a high similarity between the microbial community composition of biofilms formed on coated and uncoated stainless steel. Interestingly, an industry-dependent partial control of *L. monocytogenes*, both naturally present or artificially inoculated, on multispecies biofilms was observed, with this effect being less marked for the industry indigenous microbiota that presented the lowest taxonomy richness, diversity and initial microbial load. In addition to the differences in biofilm taxonomic composition amongindustries, important changes were detected from the initial industrial inocula to the biofilms developed on stainless steel after 7 days at 12 ^{o}C. While the most abundant genera in the industrial inocula before biofilm development were *Psychrobacter*, *Kocuria* and *Acinetobacter*, on the mature biofilms *Pseudomonas*, *Brochothrix*, *Carnobacterium* and *Vagococcus* were the predominant genera, suggesting their higher potential to colonize stainless steel surfaces under the studied conditions. These genera are among those more frequently dominating in meat processing environments and their cooperative, competitive or neutral interactions with *L. monocytogenes* have been previously described (Ammor, Tauveron, Dufour, & Chevallier, 2006; Bassey, Ye, Li, & Zhou, 2021; Daneshvar Alavi & Truelstrup Hansen, 2013; Hassan, Birt, & Frank, 2004; Lourenço, Machado, & Brito, 2011; Møretrø & Langsrud, 2017; Nilsson et al., 2005; Ripolles-Avila, Guitan-Santamaria, Pizarro-Giménez, Mazaheri, & Rodríguez-Jerez, 2022; Rodríguez-López, Saá-Ibusquiza, Mosquera-Fernández, & López-Cabo, 2015; Wiernasz et al., 2017).

The 15-min disinfection treatments with 0.5 % of sodium hypochlorite and peracetic acid resulted in important reductions of total viable counts and counts of *L. monocytogenes*, which reached levels below the limit of detection in almost all cases. A higher efficacy was found for peracetic acid under the conditions used in this PhD Thesis. However, the subsequent incubation of the sanitized plates with fresh BHI media for another 7 days at 12 ^{o}C led to biofilms with similar cell concentrations as before sanitization. Culture-independent analyses of the biofilm communities showed again the influence of the industry but not of the artificial inoculation with *L. monocytogenes* or the nature of the surface. The comparison with the taxonomical composition of biofilms before sanitization showed a reduction on the richness and alpha diversity of the communities as well as changes in the relative abundance of various genera, even though it was not possible to identify any general pattern of selection or inhibition. *L. monocytogenes* was detected on the multispecies biofilms after disinfection, as has been previously observed by other authors (Fagerlund et al., 2017; Pan, Breidt, & Kathariou, 2006). Nevertheless, the counts of *L. monocytogenes* naturally present in multispecies biofilms were

higher in biofilms treated with peracetic acid than in those treated with sodium hypochlorite, even when this latter disinfecting agent seemed to be less efficient immediately after application. Similarly, Ripolles-Avila et al. (2019) described a higher growth of *L. monocytogenes* after the complete elimination or drastic reduction of the indigenous microbiota from surfaces in a meat processing plant.

Altogether, these results show the need to continue improving the selected anti-biofilm coating and the importance of including multispecies biofilms that represent the microbiota found on the industrial environments when assessing the effectiveness of biofilm prevention or removal strategies. Besides, they suggest that a sanitization able to preserve interspecific competitive relationships between the members of the indigenous microbiota and *L. monocytogenes* might end up being more beneficial for the long-term persistence control of the pathogen in food processing facilities.

4.6. Towards novel biofilm control strategies: plasma activated water for the inactivation of *Listeria monocytogenes* in planktonic and biofilm state

The use of water activated by exposure to non-thermal atmospheric-pressure plasma (known as Plasma Activated Water, PAW) as an alternative to traditional sanitizers in the food industry has gained interest in the last few years, due to its advantages such as storage capacity, offsite generation, possibility for self-sanitation and reactivation, and sustainable production (Herianto et al., 2021; López et al., 2019; Zhao et al., 2020). Multiple generation parameters are known to influence PAW composition and antimicrobial efficacy, which is attributed to a synergistic effect between the low pH and the presence of reactive oxygen and nitrogen species (RONS) (Naítali, Kamgang-Youbi, Herry, Bellon-Fontaine, & Brisset, 2010; Zhou et al., 2018). Several studies have demonstrated the potential of PAW in the inactivation of microorganisms in planktonic state, but its efficacy against biofilms has been shown to be lower, with the protection offered by the matrix of extracellular polymeric substances (EPS) and the heterogenicity in the physiological cell state being some of the proposed reasons (Hozák et al., 2018; Mai-Prochnow et al., 2021; Smet et al., 2019; Zhao et al., 2020). In this PhD Thesis, PAW generation conditions were optimized based on its chemical composition and antimicrobial efficacy against *L. monocytogenes* in planktonic state. Afterwards, the efficacy of a selected PAW against *L. monocytogenes* biofilms formed on stainless steel and polystyrene was evaluated and its mechanisms of inactivation were investigated through the use of chemical solutions that mimic PAW composition and by RNA-seq based gene expression analyses.

The initial screening showed that higher plasma discharge powers and longer activation times resulted in lower pH values and higher RONS levels and antimicrobial activity against a three-strain *L. monocytogenes* cocktail in planktonic state. The high mode (power of 36 W) and 30 min activation time combination was selected as the best PAW generation condition, achieving 4.6±0.1 log reductions after 15-min PAW treatment. This PAW (HM30) showed a pH of 2.3±0.01 and a concentration of nitrates, nitrites and hydrogen peroxide of 32.35±5.55, 462.31±1.21 and 8.80±0.36 mg/L, respectively.

PAW HM30 was used to treat three-strain *L. monocytogenes* monospecies biofilms formed for 6 days at 12 ^{o}C. The observed microbial inactivation was lower than in planktonic state but it was still possible to achieve 1.9±0.1 and 1.8±0.2 log reductions in polystyrene and stainless steel, respectively, after a 15-min treatment. In planktonic state, a 30-min treatment with PAW HM30 allowed to reach a complete inactivation (below log 1.02 CFU/cm^2, the limit of detection of the plate counting method), while, for biofilms, the treatments time had to be extended to 60 min or more to achieve a complete inactivation. Also, a log-linear inactivation kinetic with estimated *D*-values of 11.3 and 11.2 min on stainless steel and polystyrene, respectively, was found for biofilm cells. The observed lower killing rate of PAW against biofilms compared to planktonic cells has been described by several authors, even though different PAW generation systems, strains and culture conditions were used (Mai-Prochnow et al., 2021; Smet et al., 2019).

In order to study the PAW mechanism of inactivation, the three-strain *L. monocytogenes* cocktail in planktonic state was exposed for 30 min to different chemical solutions that mimic PAW HM30 pH and composition in certain long-lived species (nitrates, nitrites and hydrogen peroxide), in order to evaluate their contribution to the antimicrobial activity. The acidified solution including the three studied RONS, at the same concentration as PAW HM30, was the most effective, followed by the solution just with adjusted pH 2.3, while barely any inactivation was observed for the individual solutions with nitrate, nitrite or hydrogen peroxide. However, the inactivation levels achieved with the chemical solutions were always lower than with PAW HM30, indicating that other reactive species might be playing a key role in PAW antimicrobial activity. These results do not completely align with the findings by other authors, since the low pH alone generally results is lower inactivation (Naítali et al., 2010; Zhou et al., 2018).

The changes in *L. monocytogenes* gene expression in response to PAW HM30 exposure were analysed by RNA-seq, both on biofilm and planktonic state. While a total of 399 differentially expressed genes (DEGs), both upregulated and downregulated, were identified for treated planktonic cells, a very low number (8) of DEGs, all of them upregulated, were detected for

biofilms cells due to the high variability between replicas. The transcriptomic response showed a general remodelling of carbon metabolism, that resulted in the up- and downregulation of multiple phosphoenolpyruvate (PEP)-dependent phosphotransferase systems (PTSs), responsible for specific carbohydrate transport (Stoll & Goebel, 2010). Also, a strong induction of the cobalamin-dependent gene cluster (CDGC), involved in ethanolamine and 1,2-propanediol metabolism and related to virulence and response to food associated stresses in *L. monocytogenes* (Anast, Bobik, & Schmitz-Esser, 2020; Fuchs, Eisenreich, Kern, & Dandekar, 2012) was observed, both in planktonic and biofilm cells. Additionally, the PAW treatment modified the expression of some genes involved in the general stress response, controlled by the alternative sigma factor SigB (Alvarez-Ordóñez, Broussolle, Colin, Nguyen-The, & Prieto, 2015; Liu et al., 2019), including the upregulation of a gene belonging to one of the main *L. monocytogenes* acid stress response systems, the glutamate decarboxylase (GAD) system (Arcari, Marie-Lucie, Guerreiro, Wu, & Conor P., 2020), which is likely linked to the low pH of PAW. Also, on PAW-treated planktonic cells, several DEGs, both up- and downregulated, were related to virulence. The induction of virulence-related genes has been previously described on RT-PCR studies when *L. monocytogenes* cells were exposed to low pH values (Cortes, Naditz, Anast, & Schmitz-Esser, 2020; Horlbog, Stevens, Stephan, & Guldimann, 2019). On the contrary, non termal atmospheric plasma and PAW treatments have been shown to downregulate the expression of virulence genes (Cui, Li, Abdel-Samie, Surendhiran, & Lin, 2021; Y. Li et al., 2019; Patange et al., 2019). Interestingly, most of the virulence DEGs identified in this PhD Thesis were related to flagellar functions, while the gene *inlA*, involved on cell invasion, presented a loss-of-function mutation characteristic of hypovirulent phenotypes (Alvarez-Molina et al., 2021; Ferreira da Silva et al., 2017). Despite the importance of RONS in PAW antimicrobial activity, no relevant changes in the expression of genes related to the oxidative stress response were detected. However, an upregulation of some genes associated with fatty acid metabolism was observed, which could indicate the occurrence of cell membrane modifications, as previously observed after the exposure of *L. monocytogenes* to other biocides (Casey et al., 2014). These results provide detailed information on the response of *L. monocytogenes* to PAW treatments, which, to our knowledge, has not yet been assessed by RNA-seq analysis. However, further studies are still needed in order to better understand the mechanisms responsible for PAW antimicrobial activity and to develop improved control strategies, given the observed potential of PAW as an alternative to traditional sanitizers applied in the food industry.

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5. Conclusions / Conclusiones

Conclusions

1. The results here obtained confirm the relevance of the alternative factor RpoS in the initial stages of biofilm formation by *C. sakazakii*. However, for *Pseudomonas* spp. and *E. coli*, the polymorphisms observed in the *rpoS* gene could not be related to changes in the strains´ biofilm formation capacity.

2. The characterization of a wide collection of *Pseudomonas* strains isolated from the food industry revealed their high ability to form biofilms, both on stainless steel and on polystyrene, and allowed the identification of green colony pigmentation on solid media as a biomarker of strong biofilm formation ability.

3. Although the phenotypic variability observed in the collection of *E. coli* strains was low, in general, the strains of food origin (belonging to different sequence types) showed greater resistance to heat treatments compared to the strains of clinical origin (mainly from sequence type 131); both groups showed a higher biofilm formation ability than the collection strains; and the strains that carried the genes bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M}$ did not show a characteristic ability to form biofilms or tolerate stress conditions.

4. The anti-biofilm coating applied on stainless steel by non-thermal atmospheric plasma polymerization, made of an APTES base and a functional layer of acrylic acid, reduced biofilm formation by *L. monocytogenes* by up to 90 %, due to its hydrophilic character and low roughness, presenting optimal activity at low temperatures. However, its effectiveness was reduced against multispecies biofilms formed from the indigenous microbiota from meat industry processing environments, which evidences the need for its improvement.

5. Treatments with two disinfectants commonly used in the food industry produced significant changes in the taxonomic composition of the multispecies biofilms developed on stainless steel, which influenced the persistence of *L. monocytogenes*. This indicates that *L. monocytogenes* persistence in food processing environments depends on interspecific competitive relationships.

6. Plasma-activated water was shown to be an effective alternative to the chemical agents currently used for the disinfection of food-contact surfaces, achieving, with short exposure times (30 minutes), an inactivation greater than 4 logarithmic units for both *L. monocytogenes* planktonic and biofilm cells.

7. The transcriptomic analysis, through RNA-seq, of *L. monocytogenes* after its treatment with plasma-activated water showed changes mainly in carbon metabolism, the expression of some virulence genes and the general stress response. It is worth noting that several of the most overexpressed genes belong to the cobalamin-dependent gene cluster, which has been previously related to *L. monocytogenes* pathogenicity and response to a wide range of stressors.

Conclusiones

1. Los resultados obtenidos en esta Tesis Doctoral han permitido confirmar la relevancia del factor alternativo RpoS en los estadíos iniciales de la formación de biopelículas por *C. sakazakii*. Sin embargo, para *Pseudomonas spp.* y *E. coli,* los polimorfismos observados en el gen *rpoS* no se pudieron relacionar con cambios en la capacidad de formación de biopelículas.

2. La caracterización de una amplia colección de cepas de *Pseudomonas* aisladas de la industria alimentaria puso de manifiesto su elevada capacidad para formar biopelículas, tanto en acero inoxidable como en poliestireno, y permitió identificar la pigmentación verde de las colonias en medio sólido como biomarcador de fuerte capacidad de formación de biopelículas.

3. Aunque la variabilidad fenotípica observada en la colección de cepas de *E. coli* fue reducida, en general, las cepas de origen alimentario (pertenecientes a distintas secuencias tipo) presentaron una mayor resistencia a los tratamientos térmicos en comparación con las cepas de origen clínico (mayoritariamente del ST 131); ambos grupos mostraron una mayor capacidad de formación de biopelículas que las cepas de colección; y las cepas potadoras de los genes de resistencia a antibióticos β-lactamicos bla_{SHV}, bla_{TEM} y $bla_{CTX\text{-}M}$ no presentaron una capacidad diferencial de formación de biopelículas o una tolerancia característica a las condiciones de estrés evaluadas.

4. El recubrimiento anti-biopelícula aplicado sobre acero inoxidable mediante polimerización por plasma atmosférico no térmico, compuesto por una base de APTES y una capa funcional de ácido acrílico, permitió reducir hasta en un 90 % la formación de biopelícula por *L. monocytogenes*, debido a sus características hidrofílicas y baja rugosidad, presentando actividad óptima a bajas temperaturas. Sin embargo, su efectividad se vio reducida frente a biopelículas multiespecie formados a partir de la microbiota autóctona de ambientes de procesado de industrias cárnicas, lo que indica que es necesario continuar trabajando en su mejora.

5. Los tratamientos con dos desinfectantes habitualmente empleados en la industria alimentaria produjeron modificaciones importantes en la composición taxonómica de los biopelículas multiespecie formados en acero inoxidable, que condicionaron la persistencia de *L. monocytogenes*, lo que indica que ésta depende de relaciones de competición interespecífica.

6. El agua activada por plasma resultó una alternativa eficaz a los agentes químicos actualmente utilizados para la desinfección de superficies en contacto con los alimentos, consiguiendo, con tiempos cortos de exposición (30 minutos), una inactivación superior a 4 unidades logarítmicas para células de *L. monocytogenes* tanto en estado planctónico como formando biopelículas.

7. El análisis transcriptómico mediante RNA-seq de *L. monocytogenes* tras su tratamiento con agua activada por plasma mostró cambios principalmente en el metabolismo del carbono, la expresión de algunos genes de virulencia y la respuesta general a estrés. Cabe destacar que varios de los genes más sobreexpresados pertenecen al cluster de genes dependientes de cobalamina, que ha sido previamente relacionado con la patogenicidad y respuesta a distintas condiciones de estrés de *L. monocytogenes*.