Tesis Doctoral

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CARACTERIZACIÓN DE LOS BIOFILMS FORMADOS POR *Lentilactobacillus parabuchneri* PRINCIPAL PRODUCTOR DE HISTAMINA EN QUESOS

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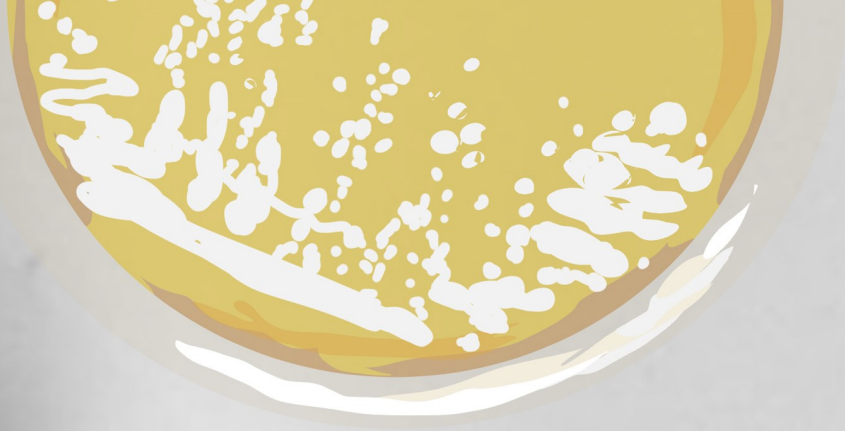

CSIC
Consejo Superior de Investigaciones Científicas

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Caracterización de los biofilms formados por *L. parabuchneri*, principal productor de histamina en quesos

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Esta Tesis Doctoral se ha realizado en el Instituto de Productos Lácteos de Asturias (IPLA-CSIC)

Ilustraciones de la portada y contraportada: gel de agarosa y placa con colonias de *L. parabuchneri*, Juan Gregorio Hauciartz y Agustina Sarquis

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Caracterización de los biofilms formados por *Lentilactobacillus parabuchneri*, principal productor de histamina en quesos	Inglés: Characterization of biofilms formed by *Lentilactobacillus parabuchneri*, the main histamine producer in cheeses

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RESUMEN (en español)

Las bacterias lácticas, son un grupo heterogéneo de microorganismos que juegan un papel determinante en el desarrollo y características finales de productos fermentados. Forman parte de nuestra alimentación a través de los alimentos fermentados que consumimos y se consideran seguras. Sin embargo, algunas cepas o especies son capaces de producir aminas biógenas (AB), tras la descarboxilación de determinados aminoácidos. Estos compuestos acumulados en los alimentos pueden provocar reacciones adversas en los consumidores y las autoridades sanitarias recomiendan la reducción o eliminación de AB en los alimentos. La histamina se sintetiza tras la descarboxilación enzimática de la histidina y es una las AB más frecuentes en productos lácteos, donde puede alcanzar concentraciones elevadas causando reacciones tóxicas en los consumidores. *L. parabuchneri* es el principal productor de histamina en quesos y trabajos previos revelaron la capacidad de algunas de sus cepas para formar biofilms.

Los biofilms son comunidades de microorganismos que se adhieren a una superficie y sobreviven inmersos en una matriz extracelular, constituyendo posibles focos de contaminación en la industria alimentaria.

El objetivo general de esta Tesis Doctoral fue investigar los factores y mecanismos moleculares implicados en la formación de biofilms de *L. parabuchneri* productores de histamina.

Inicialmente, se analizó la capacidad para formar biofilms de 25 cepas de *L. parabuchneri*. Las cepas fueron clasificadas como fuertes, moderadas o débiles formadoras de biofilm en función de la biomasa formada y se seleccionaron dos cepas de cada grupo para estudios posteriores.

En las seis cepas seleccionadas se analizó la influencia de distintos factores abióticos, relacionados con la elaboración de productos lácteos, en la formación de biofilms. Temperaturas bajas, pH neutro y la presencia de cloruro de sodio disminuyen la formación de biofilm.

Mientras que respuesta a los diferentes azúcares ensayados resultó dependiente de cepa.

El tipo de superficie es otro factor determinante en la formación de biofilms, se analizó la capacidad de adhesión de las cepas seleccionadas a las superficies más habituales en el sector alimentario: madera, acero inoxidable, goma y plástico. Siendo la madera el material que favorece una mayor adhesión.

Se analizó la composición de la matriz extracelular. Los estudios se realizaron a tiempo final aplicando diferentes enzimas y a tiempo real durante 48h mediante técnicas innovadoras de espectroscopia (IR-ATR). Identificando proteínas, ácidos nucleicos, ácido láctico, y exopolisacáridos (EPS) como los componentes mayoritarios de la matriz observándose en las cepas previamente identificadas como fuertes productoras de biofilm un notable incremento de proteínas y EPS en su matriz.

Para identificar los elementos genéticos implicados en la formación de biofilm, se secuenció y analizó el genoma de las 6 cepas seleccionadas. Solo en las cepas fuertes formadoras, se identificó un *cluster*, que mostró similitud con genes implicados en la formación de *pili*, según el modelo *sortase-mediated pilus*, y que por tanto podía estar relacionado con la capacidad de adhesión a superficies. La aplicación de técnicas de secuenciación como PacBio confirmó que este *cluster* se localiza en un plásmido de 33,4 kb, lo cual, facilitaría su transmisión a otras cepas, transfiriéndoles la habilidad de adhesión. La funcionalidad del *cluster* se comprobó mediante transformación heteróloga en *L. cremoris* NZ9000 comprobándose la capacidad de adhesión conferida mediante microscopia y midiendo la biomasa del biofilm.

Finalmente, se evaluaron diferentes métodos de prevención y eliminación de biofilms algunos convencionales disponibles en el mercado y nuevas alternativas bio-sostenibles basadas en la aplicación de bacteriocinas, nisina A y AS-48. De las 12 cepas evaluadas, la nisina A previno la formación de biofilm de 8 cepas y la AS-48 de 11 cepas.

RESUMEN (en Inglés)

Lactic acid bacteria represent a diverse group of microorganisms with a crucial role in the characteristics and final attributes of fermented products. These organisms are integral to our dietary intake through the consumption of fermented foods and are

recognized as safe. However, certain strains or species have the ability to produce biogenic amines (BA) via the decarboxylation of specific amino acids. The accumulation of these compounds in foods can cause adverse reactions in consumers, prompting regulatory agencies to advise the reduction or elimination of BA in food products. Of these compounds, histamine, is synthesized through the enzymatic decarboxylation of histidine, and is the BA most prevalent found in dairy products, occasionally reaching concentrations with toxic responses in consumers. *L. parabuchneri* has been identified as a the main responsible of histamine accumulation in cheeses, and previous studies revealed the ability of some strains to form biofilms.

Biofilms, are microbial communities adhering to surfaces and embedded within an extracellular matrix, could serve as potential reservoirs of contamination in the food industry. The aim of this Doctoral Thesis is to investigate the factors and molecular mechanisms involve in biofilm formation of histamine-producing *L. parabuchneri*.

The biofilm-forming ability of 25 *L. parabuchneri* strains was initially evaluated, and categorized as strong, moderate, or weak biofilm formers based on biomass accumulation. Two strains from each category were chosen for subsequent analyses. The influence of various abiotic factors, relevant to dairy product manufacturing, on biofilm formation was examined across these six selected strains. Lower temperatures, neutral pH, and the presence of sodium chloride decreased biofilm development, while presence of sugar is dependent strain.

Surface type emerged as another crucial factor of biofilm formation. Adhesion capacity to common surfaces in food industry—wood, stainless steel, rubber, and plastic—was analyzed. Wood promoted the highest degree of adhesion.

Analysis of the extracellular matrix composition revealed proteins, nucleic acids, lactic acid, and exopolysaccharides (EPS) as predominant components. Notably, strains identified as robust biofilm formers exhibited a substantial increase in protein and EPS content.

Genetic elements contributing to biofilm formation were investigated through genome sequencing of the six selected strains. A cluster resembling genes implicated in pili formation, specifically following the sortase-mediated pilus model, was identified

exclusively in strong biofilm-forming strains. This cluster was confirmed to be located on a 33.4 kb plasmid through PacBio sequencing, potentially facilitating its transfer to other strains, thereby conferring adhesion capability. The functionality of this cluster was validated through heterologous transformation into *L. cremoris* NZ9000, confirming its role in adhesion ability.

Finally, for biofilm prevention and elimination conventional methods were evaluated, and innovative sustainable strategies, as bacteriocins. Among the 12 strains tested, nisin A exhibited preventive effects in 67% strains biofilms, while AS-48 demonstrated efficacy against biofilm development in 92% strains.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN _____

A mi familia,

especialmente a mi tía Martita y a Grace

Y a Busi, obvio...

AGRADECIMIENTOS

Una persona empieza a escribir un relato mucho antes del momento de sentarse a escribir dice Casciari y esta tesis no es la excepción. Creo que empezó a escribirse el día que desde Buenos Aires, dije SI a cruzar el charco…

Y pienso que por eso es tan difícil enfrentarse a la hoja en blanco, inclusive para los agradecimientos. ¿cómo unas líneas pueden resumir todo lo vivido estos años?, ¿cómo una hoja A4 puede ser suficiente para agradecer a quienes me han acompañado en este proceso?…

Nada de lo que escriba va a ser suficiente para agradecer a todos aquellos que se han dedicado a ayudarme, a hacer de este objetivo parte de sus anhelos por al menos 5 minutos o por el simple hecho de empatizar con el.

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A los almuerzos del día a día con sonrisa y empatía. A los mates del domingo entre charlas, llantos y risas. A las caminatas y las amistades *para siempre*.

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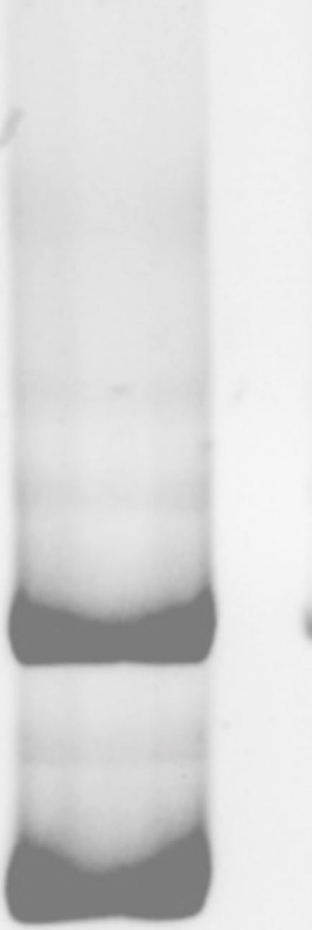
Y a la ciencia, por ser hermosa, atrapante y complicada.



INDICE

INDICE

LISTA DE FIGURAS

Nota: todas las figuras han sido creadas con Biorender.co

ABREVIATURAS

AB Amina/s biógena/s

ADN Ácido desoxirribonucleico

ARN Ácido ribonucleico

ATR *Attenuated total reflactance* **(**Reflectancia total atenuada)

BAL Bacterias del ácido láctico

CFS *Cell free supernatant* (Sobrenadantes libres de células)

CV Cristal violeta

EFSA *European Food Safety Authority* (Autoridad Europea de Seguridad Alimentaria)

EPS Exopolisacáridos

FDA *Food and Drug Administration* (Agencia Americana de Medicamentos y Alimentación)

HDC Histidina descarboxilasa

IR *Infrared spectroscopy* **(**Espectroscopía infrarroja)

LA Lactic acid **(**Ácido láctico)

LDA *Linear discriminant analysis* (análisis discriminante lineal)

LVs *Latent variables* (Variables ocultas)

MIC *Minimum inhibitory concentration* (Concentración mínima inhibitoria)

NA *Nucleic acids* (Ácidos nucleicos)

PCR *Polymerase chain reaction* (Reacción en cadena de la Polimerasa)

PCA *Principal component analysis* (Análisis de componentes principales)

PIA/PNGA *Polysaccharide intracellular adhesion*/*Poly-N-Acetylglucosamine* (Polisacárido de adhesión intercelular/Poli-N-acetilglucosamina)

PLS *Partial least squares* (Regresión de mínimos cuadrados parciales)

PS/A Adhesina de antígeno capsular no tengo claro que el nombre esté bien

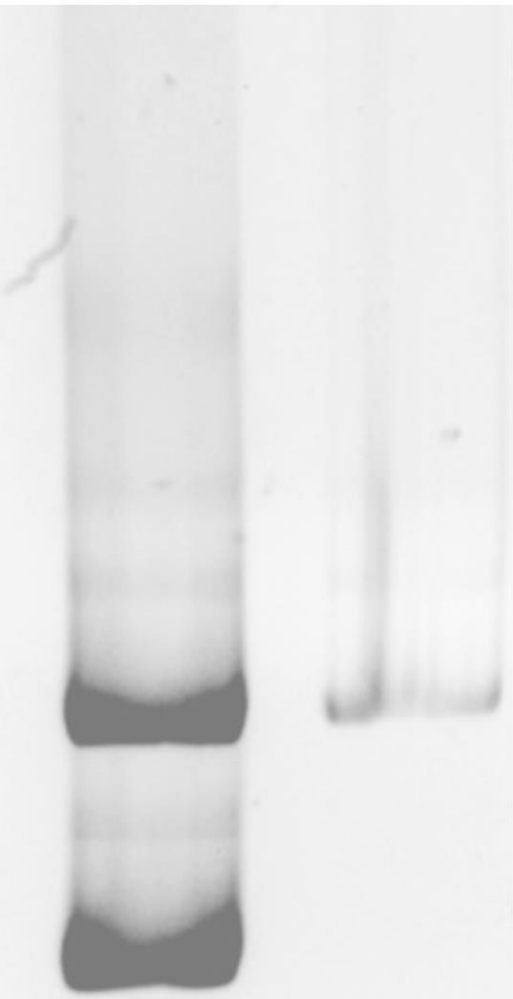
PSL-DA *Partial least squares discriminant analysis* (Análisis discriminante de mínimos cuadrados parciales)

qPCR Quantitative Polymerase chian reaction **(**PCR cuantitativa a tiempo real)

QS *Quroum sensing*

SEM *Scanning electron microscopy* (Microscopía electrónica de Barrido

Ufc Unidades formadoras de colonia



INTRODUCCION

1. INTRODUCCION

1.1. BACTERIAS DEL ACIDO LÁCTICO (BAL)

Las bacterias del ácido láctico (BAL) son un grupo diverso de bacterias Gram-positivas clasificadas en el filo *Firmicutes*, clase *Bacilli*, orden *Lactobacillales*, familia *Lactobacillaceae*, que se caracterizan por la producción de ácido láctico como producto mayoritario del catabolismo de los azúcares (Zheng et al., 2020). Las BAL también se caracterizan por ser inmóviles, no esporuladas, sin pigmentación e incapaces de reducir nitratos. Son anaerobias aerotolerantes o microaerófilas, aunque algunas BAL son anaerobias estrictas (Bintsis, 2018; Carr et al., 2002). La gran mayoría de las bacterias que conforman este grupo carecen de catalasas, sin embargo, poseen enzimas antioxidantes como peroxidasas y superóxido dismutasas que les permiten protegerse del estrés oxidativo y mantener su integridad celular en condiciones desfavorables (Ladero and Sánchez, 2017).

En general, presentan requerimientos nutricionales complejos, puesto que muchas de ellas no son capaces de sintetizar determinados aminoácidos o vitaminas. Esto se debe a que, se encuentran en hábitats ricos en nutrientes como la superficie de las plantas, o formando parte de la microbiota normal de seres vivos como el hombre y otros animales (Carr et al., 2002). En estos casos, se ha observado que las BAL pueden conferir beneficios para la salud del hospedador, mejorando la digestibilidad de ciertos alimentos, disminuyendo la colonización por patógenos, y actuando como coadyuvantes inmunológicos (Lebeer et al., 2008), motivos por los cuales han adquirido gran relevancia como potenciales probióticos en la industria alimentaria.

Pero sin duda, las BAL juegan un papel relevante en la fermentación de alimentos, donde participan en la transformación de la materia prima, leche, mosto, vegetales o carnes, en un producto con una mayor vida útil y con nuevas características organolépticas (Wang et al., 2021).

Según el papel que las BAL desempeñan en la fermentación de alimentos, se pueden dividir en dos grandes grupos. Aquellas que se utilizan como cultivos iniciadores o "starters" y que son responsables del inicio de la fermentación (Johansen, 2018). En el caso de los productos lácteos los iniciadores participan en la transformación de la lactosa en lactato y de este modo acidifican la materia prima haciendo posible la interacción entre los glóbulos de caseína para la formación de la cuajada. Y aquellas que

participan como cultivos adjuntos o secundarios, también conocidas como bacterias lácticas no "starter", y que están involucradas en el proceso de maduración y en el desarrollo del aroma, sabor y textura (Bintsis, 2018).

Streptococcus thermophilus, *Lactobacillus helveticus*, *Leuconostoc* spp., *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus* y *Limosilactobacillus fermentum* son algunas de las especies comúnmente utilizadas como cultivos iniciadores, siendo sin duda *Lactococcus lactis* y *Lactococcus cremoris* las especies predominantes en la mayoría de los cultivos iniciadores (Gürsoy and Türkmen, 2018; Iyer et al., 2010).

Como cultivos secundarios se utilizan principalmente lactobacilos heterofermentativos, es decir aquellos que producen, -además de ácido láctico-, etanol y CO_2 como productos del metabolismo de los azúcares. Entre las principales especies utilizadas podemos mencionar *Lacticaseibacillus casei*, *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Latilactobacillus curvatus*, *L. fermentum*, y *L. delbrueckii* (Johansen, 2018). Y dependiendo del tipo de producto, puede haber otras bacterias, levaduras y mohos tales como *Brevibacterium linens*, *Propionibacterium freudenreichii*, *Corynebacterium spp*, *Staphylococcus spp*, *Micrococcus spp*, *Geotrichum candidum*, *Debaryomyces hansenii*, *Penicillium roqueforti*, *Penicillium camemberti*, los cuales pueden tener un papel relevante para la obtención de un producto final con las características organolépticas deseadas (Briggiler-Marcó et al., 2007; Gürsoy and Türkmen, 2018).

El consumo diario durante siglos de alimentos fermentados con BAL en su matriz, ha hecho que sean consideradas en general, como seguras por las autoridades sanitarias europeas (European Food Safety Authority, EFSA). Sin embargo, ciertas BAL realizan actividades metabólicas que dan lugar a la acumulación de compuestos tóxicos que podrían comprometer la salud del consumidor.

1.1.1. *L. parabuchneri*

El género *Lentilactobacillus* fue descrito como tal en 2020 por Zheng et al., (2020). Son bacterias Gram-positivas, con forma de bastón, catalasa negativas y heterofermentativas, pertenecientes a la familia *Lactobacillaceae*. Las bacterias de este género se caracterizan por un crecimiento lento, el cual determinó su denominación.

Hasta su reciente reclasificación en 2020, este género se había considerado como una especie dentro del género *Lactobacillus*, el cual había sido propuesto en 1901 por Beijerinck y comprendía, 261 especies extremadamente diversas a nivel fenotípico, ecológico y genotípico. El estudio taxonómico del género *Lactobacillus*, incluyendo entre otros parámetros la filogenia del genoma central y la identidad de aminoácidos promedio, determinó la reclasificación del mismo en 25 nuevos géneros, entre los que se encuentra *Lentilactobacillus* (Zheng et al., 2020).

L. parabuchneri es una de las quince especies del género *Lentilactobacillus* y esta especie se caracteriza por presentar un amplio rango de temperaturas de crecimiento entre 10 y 40ºC, con un tamaño medio de su genoma de 2.61 Mbp. Las especies de este nuevo género estaban previamente clasificadas como *Lactobacillus ferintoshensis*, *Lactobacillus frigidus, Lactobacillus buchneri*, y *Lactobacillus parabuchneri* y se han aislado de diferentes ambientes: saliva, ensilados y cerveza. En productos lácteos, *L. parabuchneri* puede formar parte del cultivo secundario y estar presente en la microbiota láctea al final del periodo de maduración en algunas variedades de quesos como Mozzarella, Emmental, Cabrales y Parmesano (Ascone et al., 2017; Fröhlich-Wyder et al., 2013). Si bien *L. parabuchneri* podría tener un papel importante como cultivo secundario debido a la producción de CO_2, que en algunos tipos de quesos determina la formación de sus agujeros característicos, en otros casos, su actividad metabólica determina la formación de grietas no deseadas, que reducen la calidad del queso. Pero sin duda, al margen de su papel en la calidad final del producto, *L. parabuchneri* ha sido descrito como el principal responsable de la acumulación de histamina en quesos, comprometiendo la seguridad del producto final (Ascone et al., 2017; Maria Diaz et al., 2016d; Wechsler et al., 2021).

1.2. AMINAS BIÓGENAS: Histamina

Las aminas biógenas (AB) son compuestos orgánicos nitrogenados, de bajo peso molecular y con actividad biológica, que se producen como resultado de la actividad metabólica de animales, plantas y microorganismos (Maria Diaz et al., 2016; Linares et al., 2011). Algunas AB desempeñan importantes funciones como neurotransmisores, hormonas y reguladores del crecimiento celular (EFSA, 2011; Ladero et al., 2010). En el caso de las bacterias, la biosíntesis de AB se ha propuesto como un mecanismo de

resistencia al estrés ácido y como fuente de energía (Barbieri et al., 2019; Marcobal et al., 2012). Estos compuestos que intervienen en procesos tan diversos, se pueden acumular en altas concentraciones en determinados alimentos debido a la actividad microbiana y pueden provocar efectos adversos en la salud del consumidor, lo que representa un riesgo en términos de seguridad alimentaria (EFSA, 2011; Ladero et al., 2010; Ruiz-Capillas and Herrero, 2019).

La histamina se sintetiza mediante la descarboxilación del aminoácido histidina; reacción que está catalizada por la histidina descarboxilasa (HDC) (EC 4.1.22) (Figura 1). La histamina cumple un rol fundamental como hormona local y neurotransmisor en el cuerpo humano, ya que, interactúa con receptores específicos en células diana y modula diversas funciones como la secreción de ácido gástrico, el ritmo circadiano, la atención y la cognición (Chaidoutis et al., 2019). Asimismo, la histamina tiene un papel importante en la respuesta inmune y en procesos inflamatorios, a través de la unión a receptores específicos que activan respuestas vasculares y bronquiales, pudiendo desencadenar la reacción alérgica (EFSA, 2011; Ladero et al., 2010).

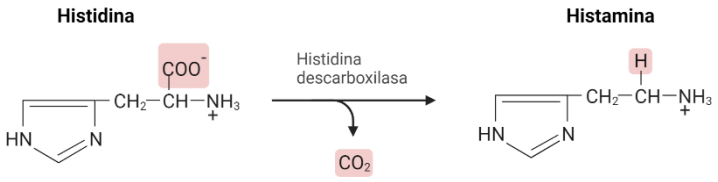


Figura 1. Biosíntesis de histamina a partir del aminoácido histidina. Creada por Biorender.com

Teniendo en cuenta todas estas funciones, es determinante regular la concentración de histamina en el torrente sanguíneo y por este motivo, el cuerpo humano cuenta con enzimas responsables de su detoxificación, localizadas principalmente en el epitelio intestinal. De hecho, niveles elevados de histamina pueden provocar diferentes síntomas tales como: migrañas, urticaria, diarrea y problemas respiratorios, los cuales se conocen como intoxicación por histamina (Ladero et al., 2010; Ruiz-Capillas and Herrero, 2019) que pueden ser debidos al consumo de alimentos con elevadas concentraciones de histamina o a un descenso en la actividad de las enzimas responsables de su detoxificación.

La intoxicación por histamina asociada a alimentos se denominó inicialmente como escombroidosis por su relación con la ingesta de pescados de la familia *Scombridae*, en

los que se observó una acumulación de histamina debido a la actividad metabólica de bacterias contaminantes (Linares et al., 2016). Diferentes estudios han demostrado que los productos fermentados, y especialmente los quesos, pueden acumular concentraciones elevadas de histamina (EFSA, 2011), siendo en este caso las BAL las principales productoras de esta AB (Maria Diaz et al., 2016d; Río et al., 2020).

Por todo lo expuesto, para favorecer la seguridad alimentaria y preservar así la salud de los consumidores, resulta fundamental evitar, controlar y/o minimizar la acumulación de histamina en alimentos fermentados, siendo la reducción de la presencia de microorganismos productores de histamina en dichos alimentos una de las estrategias propuestas.

Tal como se ha indicado anteriormente, *L. parabuchneri* ha sido identificada como la especie principalmente responsable de la producción y acumulación de histamina en productos lácteos (Berthoud et al., 2017; Maria Diaz et al., 2016d; Fröhlich-Wyder et al., 2015; Wüthrich et al., 2017). La acumulación de histamina requiere además de la presencia del aminoácido sustrato y de las condiciones ambientales adecuadas que permitan tanto la expresión de los genes implicados, como la actividad de la histidina descarboxilasa. El gen que codifica este enzima, *hdcA*, forma parte de un cluster junto a dos genes mas: un gen que codifica el transportador, responsable del intercambio histidina/histamina y un tercer gen, *hdcB* necesario para la activación del gen *hdcA* (Martín et al., 2005; Trip et al., 2011). La caracterización y localización de este cluster ha sido importante para determinar la posible transferencia de esta capacidad de unas cepas a otras. Mientras que en algunas cepas de ambiente vinícola como *Lentilactobacillus hilgardii* (Lucas et al., 2005; Zheng et al., 2020) este cluster se ha localizado en un plásmido, en los principales productores asociados a productos lácteos, como *L. parabuchneri*, el cluster es de localización cromosómica (Wüthrich et al., 2017). En *L. parabuchneri* la presencia de este cluster y la capacidad de producir histamina, había sido considerado como una característica de la especie (Ferrario et al., 2014; Yokoi et al., 2011), sin embargo trabajos más actuales determinaron la ausencia de este cluster en algunas cepas, definiéndose como una característica cepa dependiente (Gumustop and Ortakci, 2022).

Además, trabajos previos de nuestro grupo (Maria Diaz et al., 2016a) demostraron que algunas cepas de *L. parabuchneri* productoras de histamina tienen la capacidad de

formar biofilms. La formación de biofilms por parte de *L. parabuchneri* supone un problema para la industria láctea, ya que estos biofilms constituirían un reservorio de microorganismos productores de histamina que podrían contaminar el alimento y de este modo, favorecer la presencia de histamina en el producto final. Sin embargo, aún se desconoce la naturaleza exacta de estos biofilms y las condiciones que favorecen su formación.

1.3. BIOFILMS

Los biofilm son comunidades microbianas heterogéneas y dinámicas, que se adhieren firmemente a una superficie abiótica o biótica. Fueron descritos por primera vez por William Costerton en 1978, quien afirmó que las bacterias se adhieren a las superficies disponibles con lo que denominó un "glicocálix" (matriz extracelular) (Costerton et al., 1978), estableciendo un nuevo paradigma en la microbiología.

El biofilm es considerado como la forma predominante de vida microbiana. El 80-99% de los microorganismos del planeta se encuentran en este estado, debido a su alta capacidad de colonizar nuevas superficies y a la gran tolerancia frente al estrés medioambiental que otorga a los microorganismos que lo componen (Patiño Bello et al., 2018).

Los biofilms están formados por microorganismos de una o varias especies, embebidos en una matriz extracelular producida por ellos mismos que es fundamental para el desarrollo y el mantenimiento del biofilm. En dicha estructura compleja se encuentran diversas subpoblaciones celulares: células en crecimiento activo (metabólicamente activas), células que otorgan resistencia ("persistidores") y células metabólicamente menos activas (células viables en estado latente) (Rapacka-Zdonczyk et al., 2021) (Figura 2). Además, la matriz puede estar formada por exopolisacáridos (EPS), proteínas, ácidos nucleicos (ADN y ARN extracelulares), lípidos, lipopolisacáridos y otros biopolímeros, que pueden llegar a constituir hasta el 90% de la masa de un biofilm (Flemming et al., 2022). Las sustancias poliméricas extracelulares son responsable de la adhesión irreversible de las células bacterianas a las superficies y las protegen de diferentes tipos de estrés, como antibióticos, agentes antimicrobianos, oxidación y detergentes (Fahs et al., 2014; Yin et al., 2019). Estos componentes otorgan además diversas características al biofilm, puesto que por ejemplo, el ADN extracelular permite

la adhesión microbiana, es fuente de carbono, nitrógeno y fosfato y cumple un rol fundamental en la transferencia de genes y reparación de ADN dañado, proporcionando un reservorio genético para las bacterias presentes que podrían tomar material genético y recombinarlo con fragmentos de ADN mediante transformación natural (Flemming et al., 2022). La estructura química de los EPS varía de acuerdo al tipo de organismo y las condiciones ambientales presentes (Momba et al., 2000; Muro et al., 2012). Además, protegen a las moléculas señalizadoras del *quorom sensing (QS)* y a las enzimas y metabolitos extracelulares, favoreciendo de este modo la comunicación célula-célula.

La matriz está formada por canales que permiten la llegada de agua y nutrientes a todas las células, pudiendo distinguir dos zonas: la base, que actúa de soporte, y la zona donde se desarrollan las colonias de bacterias. Las células del biofilm se organizan y distribuyen en ambas zonas lo que implica distintos roles y necesidades metabólicas. Aunque, a simple vista, una capa de biofilm puede parecer uniforme, en realidad suele ser irregular y heterogénea en la mayoría de los casos. Además, la morfología del biofilm puede variar dependiendo de las condiciones en las que crece y se desarrolla. Dicho de otra forma, la apariencia y estructura del biofilm pueden ser muy diferente, dependiendo no solo de los microorganismos que lo conforman, sino también del entorno en el que se encuentra (Flemming et al., 2022).

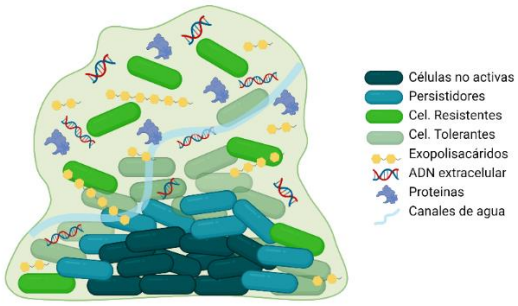


Figura 2. Esquema de la estructura de un biofilm bacteriano y sus componentes. Un biofilm se caracteriza por un entorno heterogéneo y la presencia de una variedad de subpoblaciones. La estructura de un biofilm está compuesta por células metabólicamente activas (tanto resistentes como tolerantes) y células no activas (células viables latentes y persistidores), así como una matriz que contiene entre otros componentes, exopolisacáridos, ADN extracelular, proteínas. Creado con Biorender.com.

1.3.1. Formación de biofilm

El proceso de formación de un biofilm y su posterior desarrollo es un proceso dinámico que se divide en cinco fases: adsorción o adhesión reversible, adhesión, formación y desarrollo de la matriz, maduración y propagación.

1. Adsorción o acondicionamiento de la superficie: en esta primera fase, las células planctónicas se adhieren a la superficie de forma reversible, mediante apéndices bacterianos, como fimbrias, flagelos y/o *pili* (Gupta et al., 2016; Joo and Otto, 2012; Veerachamy et al., 2014). Durante este proceso, moléculas orgánicas (nutrientes, productos del metabolismo de los microorganismos y residuos orgánicos) se fijan a la superficie, neutralizan su carga y facilitan el acercamiento de las bacterias (González, 2016; Sharma et al., 2023). Esta etapa de adhesión puede verse afectada por diversos factores externos, que pueden ser de naturaleza física como la temperatura, la rugosidad de la superficie, topografía y la tensión superficial; o química como la naturaleza del material de la superficie, la composición del medio en el que se desarrolla el biofilm, el pH o el oxígeno disponible, entre otros (Kierek-Pearson and Karatan, 2005).

2. Adhesión: en esta fase se produce la agregación bacteriana y la adhesión irreversible (Olanbiwoninu and Popoola, 2023). Las bacterias utilizan las fimbrias, flagelos o *pili* para establecer una unión activa. Durante este proceso, los organismos producen sustancias poliméricas extracelulares que interactúan con los materiales de la superficie y ligandos específicos, lo que les permite, adherirse de manera segura y formar una estructura estable (Sharma et al., 2023).

Al igual que en la etapa anterior, factores externos como, el oxígeno, el agua, el pH o la concentración de nutrientes pueden afectar la adhesión y consecuentemente la formación del biofilm (Kierek-Pearson and Karatan, 2005).

3. Formación y desarrollo de la matriz: en esta etapa de multiplicación y división celular, se forma una matriz externa de moléculas organizadas en múltiples capas, en donde se forman microcolonias y comienzan a producir EPS y proteínas que permiten mantener adheridas las células a las superficies y que actúan como un sistema de agregación e intercambio de nutrientes (Liu et al., 2023; Sharma et al., 2023). En la medida en que se acumulan más nutrientes, más bacterias van colonizando la superficie y una vez adheridas, comienzan a dividirse y a extenderse alrededor del sitio de unión, formando de este modo una microcolonia y aumentando el volumen del sistema (Costerton et al., 1999).

A medida que colonizan la superficie, las bacterias comienzan a producir más EPS y proteínas que otorgan una formación tridimensional al biofilm (Liu et al., 2023; Rubuli, 2001). Esta matriz de sustancias poliméricas extracelulares proporciona nutrientes y

constituye una red de interconexión entre las células que con el tiempo se vuelve más densa, y da como resultado un sistema irreversible. La composición de esta matriz y de los polisacáridos que la componen, varía dependiendo tanto de las bacterias que conforman el biofilm como de las condiciones ambientales en las que se encuentren (nutrientes disponibles, humedad, pH) (Flemming et al., 2022).

4. Maduración: mientras continua la producción de EPS y hay disponibilidad de nutrientes, las células que componen el biofilm continúan reproduciéndose y comienzan a adherirse otras bacterias que colonizan la superficie y contribuyen a la heterogeneidad del biofilm, formándose una estructura tridimensional más compleja (Milho et al., 2021). Así, se van formando diferentes capas, pudiendo las más internas llegar a ser anaerobias, dando lugar a un biofilm con canales de comunicación propios y con una distribución bacteriana especifica (González, 2016). El pH interno, la concentración de oxígeno, las fuentes de carbono y la osmolaridad son factores internos que también afectan la maduración del biofilm (Kierek-Pearson and Karatan, 2005).

5. Dispersión y Propagación: una vez que el biofilm ha alcanzado la madurez, algunas células individuales o fragmentos localizados en las capas externas de esta comunidad compleja y metabólicamente cooperativa, se desprenden debido a factores como la escasez de nutrientes y el estrés externo (Lu et al., 2022) y colonizan nuevas superficies (Figura 3), cerrando el ciclo de formación del biofilm (Donlan, 2002).

De este modo, el biofilm se presenta como una estrategia de supervivencia para las bacterias, puesto que brinda protección frente a las posibles agresiones del entorno, y a su vez cuenta con un sistema de canales que le permiten vincularse con el medio externo para intercambiar nutrientes y eliminar metabolitos de desecho.

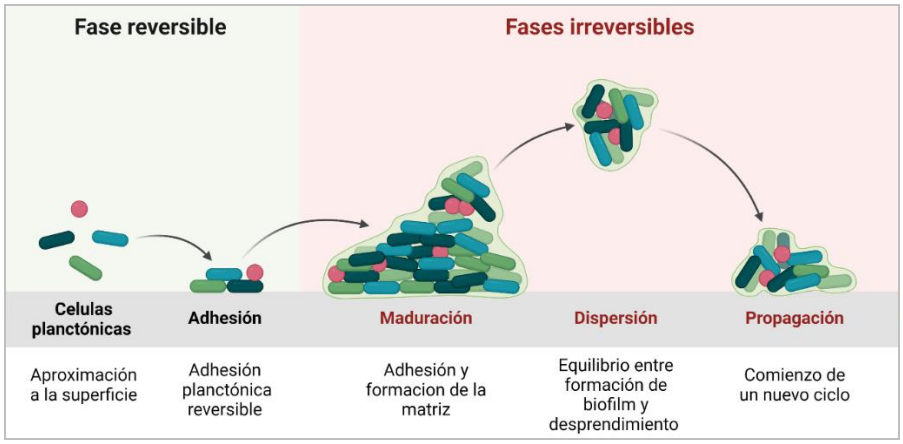


Figura 3. Ciclo de formación de biofilms. Creado con Biorender.com

Cabe destacar, que Sauer y col. (2022) (Sauer et al., 2022) expusieron que este modelo de 5 fases de formación de biofilms, no tiene en cuenta la amplia variedad de arquitecturas de biofilms observados en realidad, y propusieron un modelo de tres etapas de formación de biofilm: agregación y adhesión, crecimiento y acumulación y desagregación y desprendimiento, con el cual pretenden otorgar una visión más dinámica y amplia del desarrollo de biofilms.

1.3.2. Biofilms en la industria alimentaria

La formación de biofilms en la industria alimentaria compromete la seguridad y la calidad del producto final. Los biofilms se pueden formar en las superficies de trabajo, equipos, utensilios e instalaciones, convirtiéndose en focos de contaminación bacteriana difíciles de erradicar (Gupta et al., 2016; Lu et al., 2022). Es por ello que los biofilms constituyen una de las principales amenazas a lo largo de toda la cadena de producción de alimentos, siendo causantes de la propagación y transmisión de microorganismos patógenos que comprometen la seguridad alimentaria (Khatoon et al., 2018; Schirone et al., 2017). También son fuente de microorganismos alterantes que pueden comprometer las características organolépticas del producto final. Por tanto, como reservorios altamente resistentes de microorganismos patógenos y alterantes, los biofilms constituyen un riesgo para la salud pública y ocasionan grandes pérdidas económicas para las empresas (Galié et al., 2018; Schirone et al., 2017).

En la industria láctea, los biofilms se pueden formar a lo largo de todo el proceso de producción, desde la obtención y manipulación de la leche hasta las diferentes etapas de fabricación e incluso en el tratamiento de desechos (Mogha et al., 2014; Öner and Ölmez, 2011). Los nutrientes presentes en la leche, como las caseínas, constituyen un factor crítico en la adhesión bacteriana a las superficies de los equipos, ya que forman una capa adherente que proporciona un sustrato adecuado para que las bacterias se adhieran y formen biofilms. Los surfactantes naturales y fosfolípidos presentes en la leche también facilitan la adhesión bacteriana, incrementando el riesgo de formación de biofilms en los tanques de almacenamiento y a lo largo de las líneas de producción y pasteurización de la leche (Marchand et al., 2012).

Al mismo tiempo, los restos de leche que permanecen en las tuberías proporcionan una fuente de nutrientes que favorece la supervivencia de las bacterias termotolerantes y

termófilas que sobreviven a la pasteurización y que suelen colonizar las superficies de acero inoxidable formando biofilms (Wong, 1998). La naturaleza del material de las superficies de los equipamientos tiene sin duda un papel relevante en la capacidad de adhesión de las bacterias, siendo por ejemplo termodinámicamente favorable en goma y acero inoxidable 316 y 304, y menos favorable en vidrio y polimetilmetacrilato, todos ellos presentes y utilizados en la maquinaria de la industria láctea (Teixeira et al., 2005).

Actualmente, aunque se utilizan métodos convencionales para controlar el desarrollo de biofilm en los procesos industriales, estos enfoques suelen ser temporales y debido a la resistencia que presentan los biofilms bacterianos a los métodos de lavado y desinfección, no proporcionan soluciones duraderas (Galié et al., 2018).

1.3.3. Principales especies bacterianas presentes en los biofilms de la Industria Alimentaria

Como se mencionó previamente, los biofilms constituyen un desafío significativo en diversos ámbitos de la industria alimentaria, incluyendo la industria láctea.

La leche es una fuente de microorganismos con capacidad para formar biofilms. Estos se han aislado en tuberías, tanques de enfriamiento, utensilios, mesas de trabajo y camiones cisterna. El desarrollo de técnicas independientes de cultivo en los últimos años ha permitido conocer con más detalle la variedad de géneros y especies que constituyen el microbioma lácteo. Entre las bacterias patógenas encontradas en leche y productos lácteos las principales responsables de brotes asociados al consumo de lácteos son *Escherichia coli*, *Salmonella*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Stapylococcus aureus*. Sin embargo, los géneros bacterianos destacados por su capacidad para adherirse a las superficies en el sector lácteo, son: *Listeria*, *Staphylococcus*, *Bacillus*, *Pseudomonas y Lactobacillus* (Galié et al., 2018; Gutiérrez et al., 2012).

Listeria monocytogenes: Las bacterias pertenecientes al género *Listeria* son bacilos Gram-positivos, no esporulados ni ramificados y que agrupan en la actualidad a más de 21 especies diferentes. *L. monocytogenes* tiene la capacidad de formar biofilms en los equipos utilizado en la industria láctea, como superficies de plástico, acero inoxidable y policarbonato, así como en otros materiales que se encuentran en contacto con los alimentos, lo que las convierte en una especie capaz de sobrevivir y crecer en los diferentes microambientes que pueden encontrarse en distintos puntos de la cadena

alimentaria (Coelho et al., 2014; Olanbiwoninu and Popoola, 2023; Poimenidou et al., 2023).

La formación de biofilms de *L. monocytogenes* en la industria láctea está influenciada por diversas condiciones, como la temperatura (Di Bonaventura et al., 2008; Moltz and Martin, 2005) y la naturaleza de la superficie de adhesión (hidrofobicidad) (Midelet et al., 2006). Generalmente, se estudió su formación a 37 ± 2°C, temperatura óptima de crecimiento de *L. monocytogenes*. Sin embargo, Di Bonaventura et al. (Di Bonaventura et al., 2008) analizaron la formación de biofilms de 44 aislamientos de *L. monocytogenes* en diferentes superficies a cuatro temperaturas (4, 12, 22 y 37°C). Observaron una organización compleja de los biofilms *de L. monocytogenes* a 22 y 37°C en términos de número de células y EPS producidos, mientras que se observó una biopelícula básica compuesta de agrupaciones dispersas de células y pocos EPS a 4 y 12°C. Los autores sugirieron que estos resultados se debieron a un crecimiento reducido de las bacterias. Además, a 4 y 12°C pudo formar biofilms más robustos en vidrio en comparación con el acero inoxidable y poliestireno (más hidrofóbicos).

Otros autores analizaron la formación de biofilms a temperaturas similares a las de los refrigeradores, como Bonsaglia et al. (Bonsaglia et al., 2014),y observaron la formación de biofilms a 4°C en diferentes superficies, con niveles más altos de biofilms en acero inoxidable y vidrio en comparación con el poliestireno. La capacidad de *L. monocytogenes* para producir biofilms a temperaturas bajas utilizadas durante el procesamiento y almacenamiento de alimentos aumenta la probabilidad de contaminación (Colagiorgi et al., 2017).

También se observaron diferentes arquitecturas de biofilms entre cepas de *L. monocytogenes* (Guilbaud et al., 2015; Reis-Teixeira et al., 2017). Se observaron dos estructuras diferentes en términos de volumen, espesor medio y rugosidad, que iban desde multiláminas planas hasta estructuras complejas similares a panal de abeja. Entre las estructuras observadas, el morfotipo similar a panal de abeja fue el dominante, caracterizado por capas de células cohesivas, distribuidas de manera heterogénea, decoradas con huecos vacíos y bolsas localizadas que contenían células muertas y ADN extracelular (eDNA) (Guilbaud et al., 2015). Se ha descrito que en los biofilms de *L. monocytogenes* cuya arquitectura es de panal de abejas, las células que se adhieren inicialmente presentan un menor tamaño en comparación con las células planctónicas de

forma de bastón. Durante la formación del biofilm, las células se elongan y forman microcolonias (Wang et al., 2022).

Staphylococcus aureus: las bacterias del género *Staphylococcus* son Gram-positivas, no esporuladas, inmóviles y anaerobias facultativas. Su patogenicidad en alimentos está principalmente asociada a su capacidad para producir toxinas (Carrascosa et al., 2021). *S. aureus* es un patógeno oportunista humano, principalmente debido a su capacidad de producir enterotoxinas a temperaturas que van de los 10 a los 46°C. Los biofilms de *Staphylococcus* pueden desarrollarse en las membranas mucosas y la piel de los manipuladores de alimentos, a partir del cual pueden alcanzar el alimento. Una vez en la matriz alimentaria son capaces de producir sus enterotoxinas generando un gran problema en la industria alimentaria, ya que las enterotoxinas estafilocócicas son resistentes a condiciones adversas como el calor (Giaouris et al., 2015).

S. aureus puede formar biofilms tanto en superficies bióticas como abióticas a lo largo de toda la cadena de producción de alimentos. El crecimiento de los biofilms de *S. aureus* se ve incluso favorecido por diversos métodos de procesamiento presentes en la industria alimentaria, como temperaturas subóptimas para su crecimiento, una desinfección inadecuada o matrices con baja actividad de agua como alimentos con altas concentraciones de sal o glucosa (Galié et al., 2018).

El biofilm de *S. aureus* se caracteriza por una matriz extracelular compuesta principalmente por un exopolisacárido llamado poli-N-acetilglucosamina (PNAG) o adhesina intercelular (PIA). Además del EPS, esta matriz contiene también proteínas procedentes de las propias bacterias y ADN extracelular (eDNA) procedente de la lisis de las bacterias muertas. La presencia de proteínas y eDNA fortalece la estructura del biofilm y promueve la adhesión bacteriana (Avila-Novoa et al., 2018).

Cabe destacar que la composición de la matriz del biofilm estafilocócico, puede variar según el entorno en el que este se desarrolle (Avila-Novoa et al., 2018) y el tratamiento de remoción del biofilm es diferente según la composición de la matriz. Algunas opciones para esta remoción son hidrolasas glicosídicas (como Dispersin B) y proteasas (como la proteasa K) (Fagerlund et al., 2017; Kaplan, 2010).

Bacillus cereus*:* Las bacterias del género *Bacillus* son bacilos Gram-positivos esporulados, de crecimiento aerobio y en ocasiones anaerobio facultativo. Diferentes especies pertenecientes a este género pueden encontrarse en las plantas de

procesamiento de productos lácteos y actuar como fuente de contaminación y re-contaminación del producto (Srey et al., 2013). Debido a su capacidad para formar esporas, logran sobrevivir a los tratamientos con altas temperaturas y algunas especies como *Bacillus cereus*, son capaces de formar biofilms (Olanbiwoninu and Popoola, 2023). Se ha observado la formación de biofilms de *B. cereus* en superficies de acero de los tanques de almacenamiento y en cintas transportadoras que entran en contacto con los alimentos (Christison et al., 2007).

Los biofilms de *B. cereus* suelen estar asociados con otros microorganismos a lo largo de las líneas de procesamiento de alimentos (Majed et al., 2016). Esta asociación se favorece debido a su compleja matriz de exopolisacáridos, proteínas y ADN extracelular, que son necesarios para su adhesión en diferentes superficies como el vidrio (Vilain et al., 2009). La adhesión inicial de *B. cereus* en las superficies de fabricación de alimentos causa un efecto de acondicionamiento, facilitando la rápida adhesión de otras especies bacterianas, que de otra manera serían removidas por el flujo de agua, corrientes de leche u otros mecanismos físicos presentes en estas industrias (Marchand et al., 2012). *B. cereus* es comúnmente encontrado en fábricas de productos lácteos y sus biofilms se encuentran principalmente en la interfaz aire-líquido (Fagerlund et al., 2014). Algunas cepas de esta especie también son capaces de desarrollar biofilms en superficies sumergidas, como tanques y tuberías de acero inoxidable (Hayrapetyan et al., 2015b). Tanto en la interfaz aire-líquido como en condiciones sumergidas, la motilidad flagelar está involucrada en el desarrollo del biofilm (Hayrapetyan et al., 2015a).

***Pseudomonas spp**.:* son bacterias Gram-negativas, aeróbias y móviles, con forma de bacilos rectos o ligeramente curvados (Carrascosa et al., 2021). Por su capacidad de crecer en condiciones de refrigeración y su potente actividad proteolítica y lipolítica, es uno de los alterantes más frecuentes en productos lácteos y es capaz de formar biofilms. Las enzimas proteolíticas y lipolíticas termorresistentes producidos por *Pseudomonas spp*. causan el deterioro de la leche, afectando tanto a su calidad sensorial como a la vida útil de los productos. Estas enzimas toleran la pasteurización (72ºC 15 seg) y tratamientos de ultra alta temperatura (149ºC 10 seg) y son activas a temperaturas de refrigeración (4-8ºC) (Young et al., 2022).

Los biofilms formados por *Pseudomonas spp.* se caracterizan por contener una abundante cantidad de EPS, lo cual les permite adherirse a todo tipo de superficies de

acero inoxidable (Olanbiwoninu and Popoola, 2023). Además, estos biofilms adquieren una estructura que es similar a la forma de un hongo. En las primeras etapas del desarrollo del biofilm, las células forman microcolonias que se agrupan como los tallos del hongo y en las etapas de biofilm maduro, las subpoblaciones móviles forman una estructura denominada cápsula encima de los tallos, alcanzando finalmente la forma de un hongo (Mann and Wozniak, 2012).

1.4. FACTORES IMPLICADOS EN LA FORMACIÓN DEL BIOFILM

En un proceso complejo como es la formación de biofilms, son múltiples los factores que influyen en su desarrollo, tales como la cepa microbiana implicada (capacidad de adhesión, de secreción de EPS), la presencia de más de una especie, la hidrofobicidad y material de la superficie, las condiciones ambientales (pH, temperatura, tiempo de incubación, presencia de oxígeno, medio/disponibilidad de nutrientes), etc. (Govaert et al., 2018). De todos los factores que afectan el desarrollo de los biofilms, Nazar y col. (Nazar C, 2007) consideran que los tres principales son: la temperatura, el pH y la actividad de agua (a_w). Cuando las comunidades bacterianas capaces de formar biofilm se encuentran en condiciones adecuadas para su desarrollo, se multiplican con mayor facilidad, permitiendo la formación y el crecimiento de biofilms más robustos (Kierek-Pearson and Karatan, 2005; O'Toole and Kolter, 1998).

En cuanto a los microorganismos implicados, la formación de biofilms requiere de la expresión coordinada de múltiples genes y la interacción entre diferentes componentes celulares, así como en muchos casos la actividad coordinada de los diferentes microorganismos, mediada por un sistema de *quorum sensing* (Dickschat, 2010). No todas las bacterias tienen la capacidad de formar biofilms, ya que se requiere la presencia de genes implicados en este proceso (Tolker-nielsen, 2015). Los genes requeridos para la formación de biofilms codifican proteínas que están involucradas en la adhesión bacteriana a la superficie, en la producción de matriz extracelular y en la formación de estructuras tridimensionales (Abee et al., 2011; Rather et al., 2021; Tolker-nielsen, 2015). La presencia y la expresión de estos genes son determinantes para conferir a una bacteria la capacidad de formar biofilms. El estudio de los genes involucrados en este proceso es fundamental para comprender los mecanismos moleculares que subyacen al mismo y para desarrollar estrategias dirigidas a prevenir o controlar la formación de biofilms.

1.4.1. Genes implicados en la formación y desarrollo de biofilms

El desarrollo de la genómica ha permitido la identificación de genes relacionados con la formación de biofilms en diferentes especies, aunque no siempre se tienen evidencias funcionales de la participación de esos genes en estos procesos. A continuación se indican aquellos genes en los que se ha determinado un papel específico en alguna de las diferentes fases de formación de biofilms.

1.4.1.1. Genes implicados en las etapas de adhesión

Cluster Pilus tipo *SpaCBA*

Este cluster está implicado en la primera etapa de la formación de un biofilm, ya que permite la formación de *pili* cuya función principal es favorecer la adhesión de las bacterias a las superficies y ha sido identificado tanto en bacterias Gram positivas (Krishnan et al., 2016), tales como *Streptococcus salivarius, Enterococcus faecalis, Enterococcus faecium, B. cererus*, *L. lactis, L. rhamnosus*, entre otras.

El modelo de pilus mediado por sortasa -tipo SpaA – fue encontrado por primera vez en el patógeno gram positivo *Corynebacterium diptheriae*. Se caracterizó como un *cluster* formado por 4 genes localizados en el genoma bacteriano, que incluye tres genes que codifican para distintos tipos de pilinas: pilina de punta, pilina basal y pilina mayor, y un cuarto gen que codifica para una sortasa específica (Figura 4). Además, dependiendo del microorganismo en el que se encuentre, dicho cluster puede presentar variaciones en el número de sortasas, en la organización de los genes y en la presencia de transposones (Krishnan et al., 2016).

El análisis de la estructura del *pilus* en *C. diptheriae* reveló que las pilinas están unidas entre sí por enlaces covalentes mediados por sortasa. La pilina de punta se encuentra en el extremo del *pilus*, la pilina basal se localiza en la base del eje y ayuda a anclar la estructura del *pilus* a la pared celular y la pilina mayor forma el eje del *pilus*, es decir, múltiples copias de la pilina mayor dan lugar a la formación de ese eje siguiendo una disposición cabeza-cola (Krishnan et al., 2016).

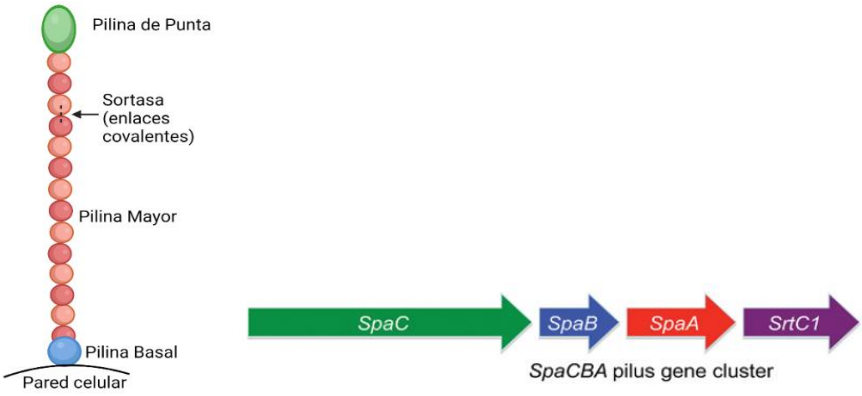


Figura 4. Estructura del *pilus* formado por el *cluster pilus* tipo *SpaCBA*. Creado con Biorender.com

Operón *icaABCD*

Los biofilms de *Staphylococcus* spp, en la primera etapa de formación, requieren que las células se adhieran a una superficie a través de un polisacárido/adhesina de antígeno capsular (PS/A). El operón de adhesión intercelular – *ica*- es responsable de la síntesis de PS/A y está formado por los genes *icaA*, *icaB*, *icaC* y *icaD*, así como por el gen regulador *icaR* (Ibrahim et al., 2022). Este operón también está implicado en la etapa de maduración del biofilm estafilocócico, ya que induce la producción de adhesina intercelular de polisacárido (PIA) que forma múltiples capas de la matriz extracelular, contribuyendo a la formación y estructura del biofilm.

El gen *icaA* codifica la enzima N-acetilglucosaminiltransferasa involucrada en la síntesis de N-acetilglucosamina y el gen *icaD* contribuye a su expresión y actividad, lo que conduce a la expresión fenotípica del polisacárido capsular (Gad et al., 2009).

Gen *clsA*

En los biofilms de *Listeria monocytogenes* 15G01, un gen relevante es *clsA*, que codifica la enzima cardiolipina sintetasa, que contribuye a la síntesis de cardiolipina, que puede influir en la adhesión y agregación celular. La ausencia de cardiolipina puede afectar la incorporación o unión de proteínas específicas, como flagelos, a los polos celulares, lo que resulta en una disminución de la motilidad y posiblemente una alteración en la formación de biofilms (Djordjevic et al., 2002).

1.4.1.2. Genes implicados en la maduración del biofilm

Operón *TapA*

En *B. subtilis* (Arnaouteli et al., 2021) describieron que el operón *tapA*, codifica tres proteínas: TapA, SipW y TasA (Figura 5).

Arnaouteli y col. (2021) han descrito a TapA como una proteína de ensamblaje y anclaje de TasA. Es decir, la presencia de TapA en el biofilm contribuye a la polimerización de las fibras de TasA y por ende a la formación de una matriz extracelular cohesionada y estructurada.

TasA es esencial para mantener la integridad estructural del biofilm y sus fibras estimulan la expresión de genes de movilidad y regulan la expresión de subpoblaciones de células, contribuyendo a la propagación de colonias en superficies (Steinberg et al., 2020).

La función de TapA está relacionada con la de TasA y ambas proteínas deben ser expresadas simultáneamente (Arnaouteli et al., 2021).

SipW es una peptidasa de señal tipo I que actúa sobre TasA y TapA para que realicen su función. Además, el dominio de SipW que se encuentra dentro de la célula, activa la expresión de los genes *epsA-O* necesarios para la producción de exopolisacáridos (Terra et al., 2012).

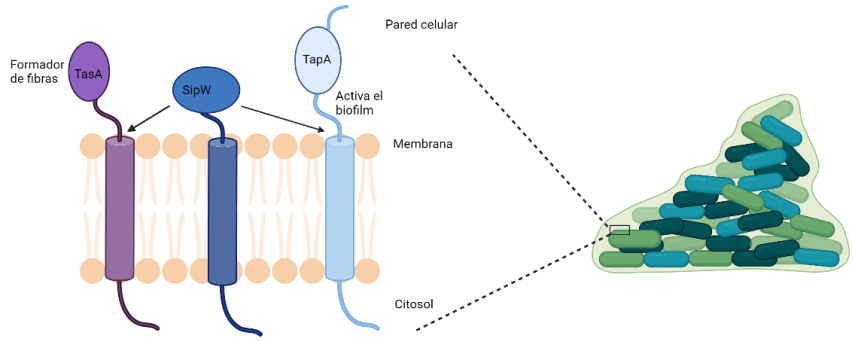


Figura 5. Estructura Sistema TapA y sus componentes proteicos incrustados en la membrana celular. SipW (azul oscuro) corta post-traduccionalmente TapA (celeste) y TasA (púrpura) en la superficie celular con las flechas que representan la ubicación del corte. Creado con Biorender.com

Gen *BslA*

En los biofilms de *B. subtilis* este gen codifica la BslA localizada en la periferia del biofilm. Tiene dos funciones: una relacionadas con la hidrofobicidad del biofilm y otra con su arquitectura.

La función de hidrofobicidad de BslA consiste en formar un revestimiento hidrofóbico en la periferia del biofilm, que repele el agua y ayuda a mantener la integridad de la estructura, lo cual es fundamental para resistir condiciones ambientales fluctuantes.

En cuanto a la función de arquitectura, BslA trabaja con otros componentes de la matriz, como las fibras TasA y el EPS, para generar complejidad estructural en el biofilm. Esta función determina la arquitectura del biofilm y sus propiedades físicas y mecánicas, como la porosidad, la elasticidad y la fuerza de adhesión, que afectan su función y supervivencia. Cabe destacar que en mutantes que carecen de *BslA* la producción de fibras TasA y EPS no se altera, pero la estructura del biofilm se ve comprometida, lo que indica que *BslA* es esencial en la arquitectura del biofilm.

Operón *icaABCD*

Como se mencionó previamente los biofilms de *Staphylococcus* spp., luego de la adhesión inicial, crecen formando múltiples capas que induce la producción de polisacáridos - PIA (Ibrahim et al., 2022). El operón *ica* es responsable de la síntesis de PIA, que permite a las bacterias a adherirse entre sí y a la superficie, contribuyendo a la formación y estructura del biofilm.

Gen *rocD,* y gen *gudB*

Además, en la maduración de los biofilms de *S. aureus*, se han identificado varios genes involucrados en diferentes vías metabólicas y procesos de señalización que regulan el crecimiento y desarrollo del biofilm (Nassar et al., 2021):

El gen *rocD*, codifica la enzima ornitina-oxoácido transaminasa, que cataliza la conversión de ornitina a glutamato 5-semialdehído, y ambos metabolitos están implicados en el metabolismo de glutamato y arginina (Hassanov et al., 2018).

Otro gen relevante en *S. aureus* es *gudB,* que codifica la enzima glutamato deshidrogenasa, involucrada en el metabolismo del glutamato, glutamina y amoníaco (Hassanov et al., 2018).

Aunque se requieren más estudios para dilucidar los efectos/funciones que ambos genes, *rocD* y *gudB* tienen sobre los biofilm, es evidente que están involucrados en rutas metabólicas que juegan roles importantes en el desarrollo del biofilm, lo que sugiere que interferir con su síntesis y función podría tener efectos perjudiciales en el biofilm de *S. aureus* (Nassar et al., 2021).

Gen *lafA, mgtB, uvrB*

En los biofilms de *L. monocytogenes* 15G01, el gen *lafA* parece estar implicado en la etapa temprana de maduración de biofilms, ya que codifica una glicosiltransferasa necesaria para el desarrollo de la membrana y si bien se desconoce exactamente cómo influye, se observó que la inserción de un transposón en este gen causaba una reducción en la formación de biofilms (Nowak et al., 2017).

Además, se identificaron genes relacionados con el transporte de nutrientes, como el gen *mgtB* que codifica una ATPasa que transporta Mg^{2+} al interior celular y se ha demostrado que la falta de magnesio induce la formación de biofilms (Djordjevic et al., 2002).

Otro gen identificado que se asocia a la formación de biofilms es *uvrB*. Forma parte de un complejo enzimático que media en la reparación de ADN y se activa en respuesta al daño en el ADN, ha sido vinculado a la formación de biofilms en diversas especies bacterianas, incluyendo *L. monocytogenes* (Djordjevic et al., 2002).

Conocer y analizar los genes implicados en la formación y desarrollo de los biofilms puede ser una herramienta clave para comprender aún más su estructura, funcionalidad y posibles modos de prevención y eliminación.

1.5. MECANISMOS DE PREVENCIÓN Y ELIMINACIÓN DE BIOFILMS

Para eliminar y controlar la formación de biofilms en la industria láctea, se utilizan generalmente métodos de limpieza convencional de los equipos, los cuales se basan en el uso de detergentes y desinfectantes. Sin embargo se sabe que no siempre son efectivos debido a la gran resistencia de los biofilms bacterianos (Flint et al., 2020; Sharma et al., 2017; Vishwakarma, 2020).

1.5.1. Limpieza y desinfección

La fermentación de la leche y la elaboración de productos lácteos requiere el desarrollo de métodos eficientes de limpieza y desinfección que permitan que un proceso que se realiza en condiciones no-estériles y partiendo de una matriz rica en microorganismos y nutrientes (carbohidratos, proteínas, grasa) (Roy et al., 2020), no se vea alterada por el desarrollo incontrolado de microorganismos patógenos y alterantes. En muchos casos, dichos métodos van especialmente dirigidos a la eliminación de biofilms como

reservorios de estos microorganismos (Srey et al., 2013; Vishwakarma, 2020; Zhao et al., 2017). Así, el desarrollo de estrategias de limpieza en la industria láctea se basa en la aplicación de agentes enzimáticos y quelantes que logren penetrar en la matriz del biofilm y provoquen su eliminación o en la aplicación de compuestos que inhiban la comunicación entre las bacterias (*quorum sensing*) y por lo tanto impidan la formación de biofilms (Anand et al., 2014).

Otro método ampliamente utilizado en la industria alimentaria es el método CIP (clean-in-place), que se define como la limpieza de equipos o circuitos de tuberías sin desmontaje o apertura del equipo y con poca o ninguna intervención manual. El proceso implica la pulverización de superficies con soluciones de limpieza (Thomas and Sathian, 2014). Sin embargo, se demostró que el método CIP no consigue eliminar completamente las bacterias adheridas al acero inoxidable (Bremer et al., 2006) y además, Jang y col. (2006), advirtieron que los biofilms de especies mixtas, (Jang et al., 2006) son capaces de sobrevivir aún más a los procedimientos de limpieza y desinfección, y pueden representar un reservorio de contaminación de los alimentos, llevando a su consiguiente deterioro y riesgo para la seguridad alimentaria (Lindsay et al., 2002).

Por lo expuesto anteriormente, podríamos decir que actualmente los métodos de lavado y desinfección no son lo suficientemente eficientes para el control y eliminación de biofilms. Además, otro de los desafíos a los que se enfrenta el sector lácteo no es solo el desarrollo de métodos de limpieza eficientes, sino que estos métodos deben ser sostenibles con el medio ambiente, lo que condiciona el empleo de determinados detergentes o agentes químicos.

1.5.2. Nanomateriales

Además del desarrollo de nuevos métodos de limpieza, en los últimos años, el uso de la nanotecnología ha surgido como una prometedora alternativa para tratar los biofilms. La nanotecnología se basa en el uso de moléculas en el rango de 1-1.000 nm (Smith et al., 2013), cuyo pequeño tamaño les permite penetrar en las capas del biofilm. La investigación en nanotecnología para el tratamiento de biofilms se ha centrado en dos áreas principales: el uso de nanopartículas con actividad antimicrobiana y el desarrollo de sistemas de administración de fármacos.

La primera categoría incluye el uso de partículas inorgánicas como plata, zinc, titanio, cobre y oro (Banerjee et al., 2020; Natan and Banin, 2017) y el desarrollo de nuevos materiales biofuncionales que impidan o reduzcan la adhesión celular y por lo tanto la formación de biofilms (Vishwakarma, 2020). Por otro lado, los sistemas de administración de fármacos basados en nanotecnología han demostrado permitir la liberación lenta y controlada de medicamentos. Se han desarrollado numerosos sistemas basados en diferentes compuestos como lípidos y polímeros (Malaekeh-Nikouei et al., 2020; Pinto et al., 2019).

La nanotecnología ofrece múltiples ventajas en comparación con los tratamientos tradicionales, incluyendo una mayor reactividad debido a una relación superficie-volumen más favorable. Además, la nanotecnología evita problemas como la degradación enzimática, la toxicidad y la liberación no específica (Ramasamy and Lee, 2016).

En la industria alimentaria, la utilización de nanomateriales, también conocidos como nanopartículas, está ampliamente extendida como bioconservantes. Estos materiales, exhiben propiedades únicas tales como liberar antimicrobianos y regular la humedad y el intercambio de aire, lo cual prolonga la vida útil de los productos (Onyeaka et al., 2022). Los nanomateriales antimicrobianos, como las nanopartículas de metales y óxidos metálicos, pueden generar especies reactivas del oxígeno que dañan las células bacterianas, previniendo la contaminación y deterioro de los alimentos. La aplicación de nanomateriales en la industria alimentaria representa una prometedora estrategia para mejorar la seguridad alimentaria (Marchianò et al., 2023; Onyeaka et al., 2022).

Sin embargo, a pesar de los beneficios que ofrecen las nanopartículas, aún se debe estudiar su interacción y toxicidad con el cuerpo humano, así como sobre los efectos a largo plazo, tales como la acumulación en tejidos y órganos (Tran et al., 2020). También es importante tener en cuenta que, al igual que con los antibióticos tradicionales, es posible que se desarrolle resistencia a las nanopartículas antimicrobianas.

1.5.3. Bacteriófagos

Los bacteriófagos, también conocidos como fagos, son virus que infectan bacterias y son las entidades biológicas más abundantes y diversas a nivel mundial. Generalmente, los fagos matan a su hospedador bacteriano al final del ciclo lítico, por lo que en la

actualidad son alternativas eficaces para el tratamiento de enfermedades bacterianas en humanos, animales y plantas (Jamal et al., 2019; Nagel et al., 2016) y puede utilizarse como una estrategia natural, efectiva y económica para garantizar la seguridad alimentaria (Agún et al., 2018; Moye et al., 2018; O'Sullivan et al., 2019).

Para ser un candidato potencial como antimicrobiano, un bacteriófago debe cumplir ciertos requisitos (Fernández et al., 2019). La condición más importante es que debe ser un bacteriófago virulento, ya que los bacteriófagos temperados, además de no provocar la lisis del hospedador, contribuyen a la transferencia horizontal de marcadores de virulencia y resistencia a los antibióticos. Otra propiedad deseable es que el bacteriófago exhiba un amplio rango de hospedador dentro de su especie objetivo (Kranjec et al., 2021). Además, para ser un agente *antifouling* o antiincrustante, las partículas del bacteriófago deben ser capaces de alcanzar e infectar a sus células huésped dentro de la estructura compleja del biofilm. Algunos bacteriófagos estafilocócicos poseen enzimas que degradan polisacáridos (depolimerasas) que pueden ayudar a eliminar la matriz extracelular de los biofilms (Gutiérrez et al., 2015). Además, las depolimerasas se pueden complementar con antibióticos o con otros antimicrobianos derivados de bacteriófagos como las proteínas líticas, las cuales también pueden ser utilizadas por si solas como poderosas herramientas antibacterianas capaces de degradar peptidoglicano (Kranjec et al., 2021).

Sin embargo, existen muchos factores a tener en cuenta, como las bacterias diana, la ruta de aplicación, el momento de administración de los fagos (profiláctica vs. terapéutica), el número de administraciones de fagos (única vs. repetida), el número de fagos utilizados (único vs. cóctel), la composición de los alimentos o la temperatura de almacenamiento, entre otros, ya que pueden implicar diferencias en los resultados en cuanto a los efectos en el biocontrol de patógenos en alimentos y animales (Mosimann et al., 2021; Vikram et al., 2020).

1.5.4. Utilización de bacteriocinas

Las bacteriocinas son péptidos antimicrobianos producidos por bacterias Gram-negativas y Gram-positivas, capaces de inhibir el crecimiento de otras bacterias y/o microorganismos. Por lo general, suelen ser secretadas al medio circundante, en el cual inhiben o eliminan las bacterias sensibles siguiendo diferentes mecanismos dependiendo

del tipo de bacteriocina: bien mediante la formación de poros en la membrana celular de las bacterias sensibles, como es el caso de la nisina, o mediante la interferencia con la síntesis de componentes celulares esenciales como por ejemplo ácidos nucleicos, proteínas o componentes de la pared celular (Darbandi et al., 2022; Kumariya et al., 2019).

En este contexto, las bacteriocinas se presentan como una opción interesante para ser utilizada como conservante natural en alimentos debido a las características físico-químicas que presentan. Desde el punto de vista tecnológico presentan ciertas ventajas: son termoestables, resistentes a pH ácido y son producidas por bacterias de grado alimentario, poseen la capacidad de inhibir microorganismos patógenos, evitan el deterioro alimentario y se degradan en el tracto gastrointestinal del consumidor por la acción de enzimas gástricas e intestinales (Cotter et al., 2005; López M. et al., 2008).

Actualmente, la industria alimentaria utiliza bacteriocinas producidas por BAL como antimicrobianos naturales para preservar el alimento e incrementar su vida útil. En la Unión Europea, la bacteriocina aprobada para su uso como conservante alimentario es la nisina (E 234) y en Estados Unidos también se utiliza la pediocina PA-1 (Mokoena, 2017; Wang et al., 2009).

Debido al potencial demostrado por estos péptidos bioactivos, se realizan múltiples investigaciones en busca de nuevas bacteriocinas que puedan utilizarse en alimentos. Entre los bioconservantes alimentarios más estudiados, podría mencionarse la bacteriocina cíclica catiónica enterocina AS-48, producida por *Enterococcus faecalis* (Maqueda et al., 2005). Esta bacteriocina exhibe un amplio espectro de actividad antimicrobiana frente a bacterias Gram-positivas, es eficaz contra bacterias patógenas y alterantes en diversas categorías de alimentos (Cobo Molinos et al., 2008; Muñoz et al., 2007), reduce la concentración de AB en productos pesqueros (Ananou et al., 2014) y es activa en un amplio rango de temperaturas y pH.

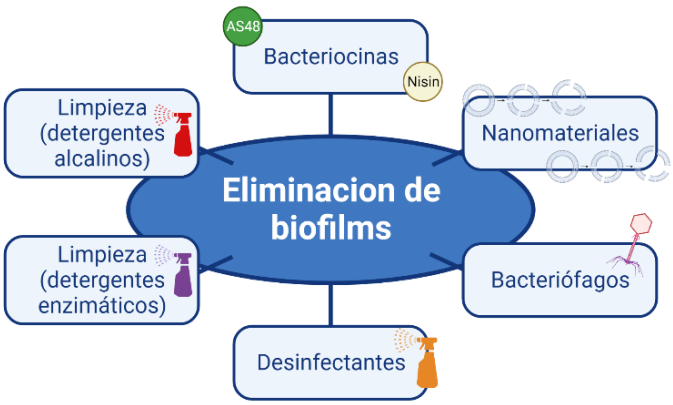
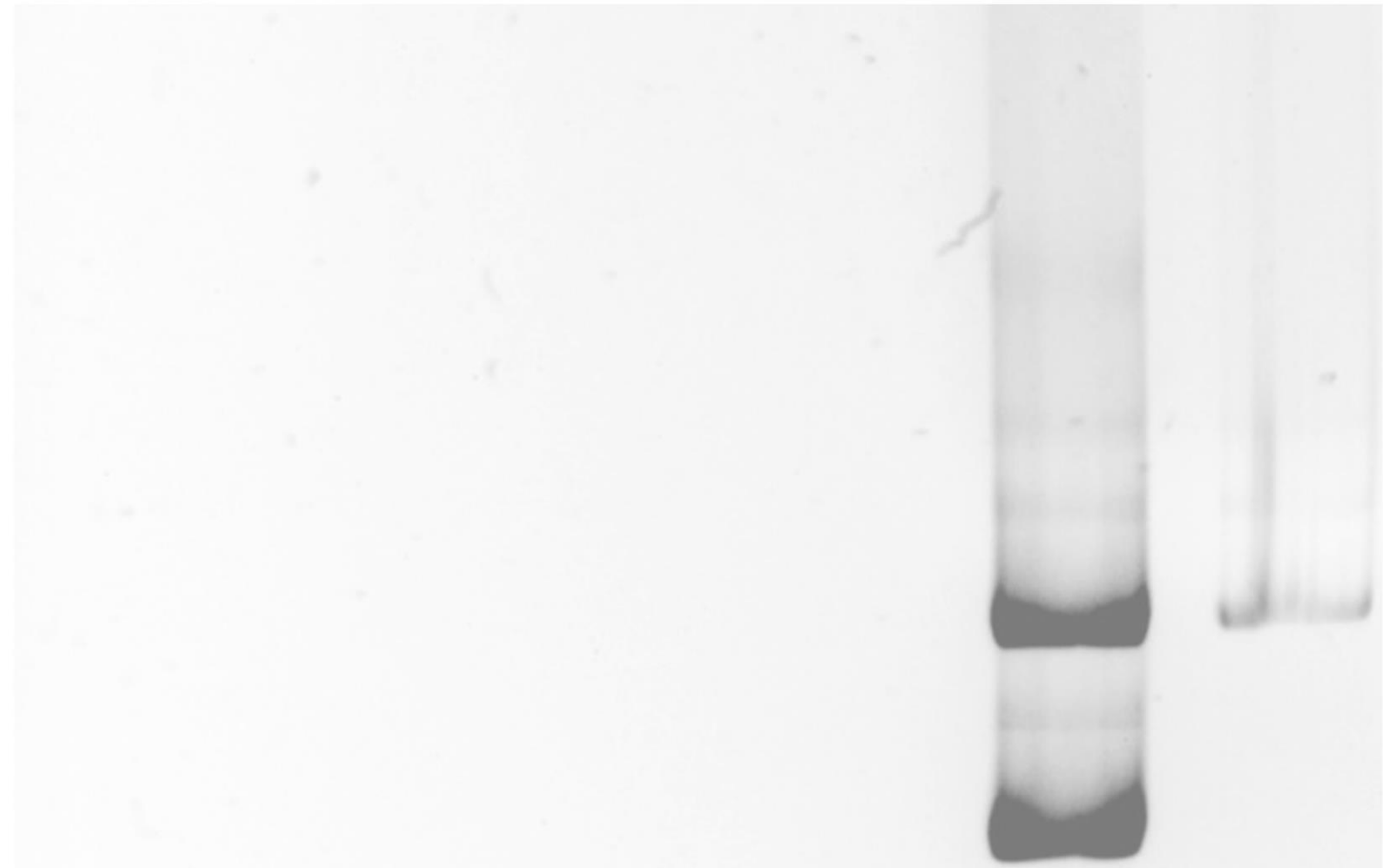


Figura 6. Métodos actuales utilizados para la eliminación y prevención de biofilms. Creada con Biorender.

En conclusión, los biofilms bacterianos representan un desafío significativo en la industria alimentaria debido a su capacidad de adaptación y resistencia a los métodos convencionales de eliminación. Para abordar este problema, es fundamental investigar en profundidad los factores abióticos, como la temperatura, la acidez y el tipo de superficie, que influyen en la formación de biofilms. Además, es crucial comprender los factores moleculares, como los genes implicados en el desarrollo del biofilm, para obtener una visión completa de los mecanismos subyacentes.

Al identificar y comprender estos factores, se podrían desarrollar estrategias efectivas para prevenir y controlar la formación de biofilms en la industria láctea y alimentaria. Estos hallazgos no solo contribuirán a mejorar la calidad y seguridad de los productos alimenticios, sino que también tendrán un impacto positivo en la eficiencia y rentabilidad de las operaciones de procesamiento.

En consecuencia, esta Tesis se propone investigar y analizar los factores abióticos y moleculares que influyen en la formación de biofilms de cepas de *L. parabuchneri* productoras de histamina y aisladas de quesos. Se pretende que los resultados obtenidos proporcionen una base sólida para el desarrollo de estrategias de prevención y control más efectivas, lo que beneficiará tanto a la industria como a los consumidores al garantizar la producción de alimentos más seguros y mejor calidad.



OBJETIVOS

2. OBJETIVOS

Como se ha expuesto previamente en la introducción, los biofilms son comunidades de microorganismos que permanecen adheridos a una superficie y embebidos en una matriz extracelular. Se encuentran presentes habitualmente en la industria alimentaria y pueden contaminar los alimentos al adherirse a los equipos utilizados. Al mismo tiempo, dependiendo de las especies que conformen el biofilm, pueden causar el deterioro del alimento tanto de sus características organolépticas como nutricionales, con las consecuentes pérdidas económicas para la industria e incluso pueden ser responsables de intoxicaciones alimentarias.

L. parabuchneri es una bacteria heterofermentativa del ácido láctico que suele estar presente en productos lácteos fermentados, y se ha identificado como la principal causante de la acumulación de concentraciones elevadas de histamina en quesos. El consumo de quesos con altas concentraciones de esta amina biogénica puede tener efectos toxicológicos en la salud en los consumidores, suponiendo un problema primordial de seguridad alimentaria.

Se ha determinado que algunas cepas de *L. parabuchneri* producen biofilms en las distintas superficies de los equipos utilizados en la industria láctea. Estos biofilms son muy resistentes a los procedimientos de limpieza y desinfección, y actúan como reservorios de bacterias productoras de histamina. Por lo tanto, los quesos se contaminan durante los procesos de elaboración y al ser consumidos, pueden contener concentraciones de histamina indeseables para el consumo humano.

En el marco de la problemática expuesta, el objetivo principal de esta tesis fue investigar los factores y mecanismos moleculares implicados en la formación de biofilms de *L. parabuchneri*.

Para llevar a cabo este objetivo, se establecieron los siguientes objetivos específicos:

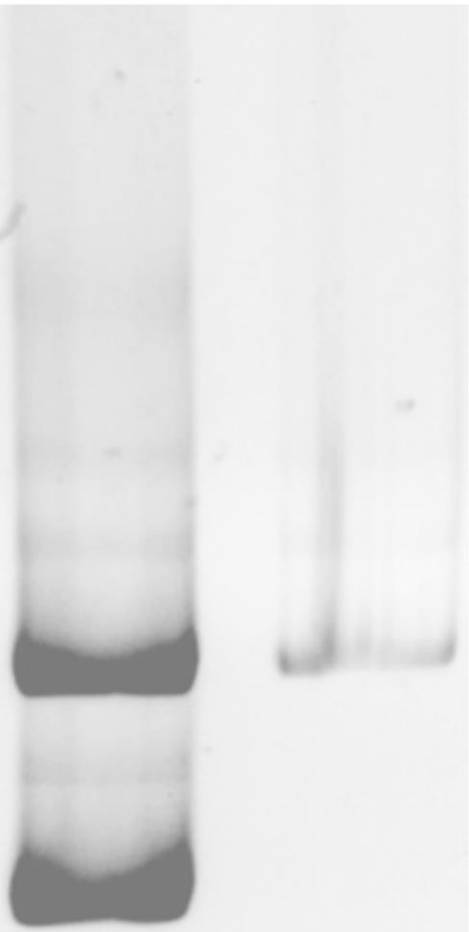
Objetivo 1: Cuantificar la capacidad de formación de biofilms de las cepas de *L. parabuchneri* disponibles en la colección del IPLA.

Objetivo 2: Analizar la influencia de distintos factores abióticos en la formación de biofilms: materiales de superficies, fuentes de carbono, pH, temperatura.

Objetivo 3: Estudiar la composición de la matriz extracelular de los biofilms de *L. parabuchneri* con distintas capacidades de adhesión.

- **Objetivo 4:** Identificar y localizar los elementos genéticos implicados en la formación de biofilms: secuenciación y anotación de genomas.
- **Objetivo 5:** Determinar la funcionalidad del clúster de adhesión.
- **Objetivo 6:** Analizar, evaluar y proponer métodos biosostenibles para la prevención y eliminación de los biofilms formados por bacterias del ácido láctico productoras de aminas biógenas.

Con la realización de estos objetivos, se espera contribuir al conocimiento sobre la formación de biofilms en *L. parabuchneri* y, en última instancia, contribuir a la prevención de la contaminación de alimentos con histamina en la industria alimentaria.



RESULTADOS

3. RESULTADOS

CAPITULO 1

Objetivo 1. Cuantificar la capacidad de formación de biofilms de las cepas de *L. parabuchneri* disponibles en la colección del IPLA.

Objetivo 2. Analizar la influencia de distintos factores abióticos en la formación de biofilms: materiales de superficie, fuentes de carbono, pH, temperatura.

Como se ha descrito en la introducción, *L. parabuchneri* es el principal responsable de la acumulación de histamina en quesos y al mismo tiempo, se había descrito la capacidad de algunas de sus cepas para formar biofilms.

L. parabuchneri ha sido asociada con diferentes nichos ecológicos: quesos, ensilados, saliva, cerveza. Por este motivo, el primer objetivo del proyecto de esta Tesis Doctoral fue determinar si la capacidad de formar biofilms era una característica general en las cepas de esta especie. Se determinó la capacidad de formar biofilms en diferentes cepas de *L. parabuchneri* aisladas de distintos quesos y presentes en la colección del IPLA y se procedió a su clasificación como fuertes, moderadas y débiles formadoras

Posteriormente se analizó la influencia de diferentes factores externos y/o ambientales, relacionados con el proceso de elaboración de productos lácteos en su capacidad para formar biofilms.

Los resultados obtenidos para estos dos objetivos de la tesis se encuentran en el Artículo 1, presentado a continuación:

> **Artículo 1: Agustina Sarquis**, Diellza Bajrami, Boris Mizaikoff, Victor Ladero*, Miguel A. Alvarez and Maria Fernandez (2023). Characterization of the biofilms formed by histamine-producing *L. parabuchneri* strains in the dairy environment. *Foods* 12, 1503. https://doi.org/10.3390/foods12071503

foods

Article

Characterization of the Biofilms Formed by Histamine-Producing *L. parabuchneri* Strains in the Dairy Environment

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Abstract: *L. parabuchneri*, a lactic acid bacterium, is largely responsible for the production and accumulation of histamine, a toxic biogenic amine, in cheese. *L. parabuchneri* strains can form biofilms on the surface of industry equipment. Since they are resistant to cleaning and disinfection, they may act as reservoirs of histamine-producing contaminants in cheese. The aim of this study was to investigate the biofilm-producing capacity of *L. parabuchneri* strains. Using the crystal violet technique, the strains were first categorized as weak, moderate or strong biofilm producers. Analysis of their biofilm matrices revealed them to be mainly composed of proteins. Two strains of each category were then selected to analyze the influence on the biofilm-forming capacity of temperature, pH, carbon source, NaCl concentration and surface material (i.e., focusing on those used in the dairy industry). In general, low temperature (8 °C), high NaCl concentrations (2–3% *w*/*v*) and neutral pH (pH 6) prevented biofilm formation. All strains were found to adhere easily to beech wood. These findings increase knowledge of the biofilm-forming capacity of histamine-producing *L. parabuchneri* strains and how their formation may be prevented for improving food safety.

Keywords: biogenic amines; histamine; *L. parabuchneri*; biofilms

1. Introduction

A biofilm is a community of bacterial cells enclosed in a self-produced polymeric matrix adhered to an inert or living surface [1–3]. The composition of the biofilm matrix is variable but contains a combination of bacterial cells, proteins, enzymes, polysaccharides, lipids and nucleic acids [4,5]. Biofilms provide a protective structure for bacteria, allowing them to grow in hostile environments [1,6]. Since they can form on both living and nonliving surfaces they can cause serious problems for different industries, leading to reduced product quality and economic losses [7–9]. Bacteria growing in biofilms are more resistant to stress factors, such as temperature, pH and disinfectants, than are their planktonic counterparts [10]. Generally, biofilms grow in damp places where they have access to nutrients [11,12]. In the dairy industry, the main source of nutrients for bacteria is left-over product on equipment; the biofilms that grow then cause the microbial contamination of the next batch of product [13,14].

Foodborne diseases remain a major threat to public health and the economy. Indeed, food intoxication is among the most important causes of mortality and morbidity [15],

with approximately 42,000 deaths and 600 million cases of illness each year [8,16]. The great majority of problems are of bacterial origin, with contamination of food during manufacturing and preconsumption storage, the usual routes of bacterial entry. In the food industry, most, but not all, biofilms are formed by nonpathogenic bacteria [17]. The resistance of biofilms to chemical/physical cleaning and sanitizing procedures renders those that contain pathogens a definite health hazard [8,11]; certainly, biofilms that act as reservoirs of biogenic amine (BA)-producing bacteria pose a food safety problem [18].

BAs are compounds of low molecular weight, organic and nitrogen-containing that are synthesized by the enzymatic decarboxylation of specific amino acids [19,20]. In many organisms, they have important biological functions [21,22]. However, in foodstuffs they can accumulate in large quantities (especially fermented foods and beverages) owing to the metabolism of certain microorganisms, and this can cause health problems [23,24]. In fermented dairy products, BAs are mainly formed by certain lactic acid bacteria (LAB) present in the starter culture or introduced as contaminants during manufacturing [25].

One of the most dangerous BAs in dairy products is histamine; the ingestion of cheese with high concentrations of histamine can lead to toxic neurological, gastrointestinal and respiratory effects [26–28]. Indeed, after fish, the food most commonly at the root of histamine poisoning is cheese. Problems have been recorded after the consumption of different types of cheese, including Swiss and Cheddar [29].

L. parabuchneri has been identified as the species largely responsible for histamine production and accumulation in dairy products [30]. It is part of the non-starter culture microbiota and has been found in numerous cheese varieties, including Caciocavallo Pugliese, Spanish farmhouse cheese, Parmigiano Reggiano, Camembert, Gouda-type cheese, Pecorino Crotonese, Cheddar, Emmental and Swiss goat milk cheese [30,31]. Some *L. parabuchneri* strains influence the organoleptic characteristics of cheese, such as eye formation, during the later stages of ripening [32]. However, most of the strains studied so far have been found to produce histamine as well [33–35]. Moreover, *L. parabuchneri* has consistently been shown present on different dairy machinery surfaces, resulting in contaminated milk and cheese, the latter eventually containing high concentrations of histamine [31,36]. Diaz et al. [37] reported some histamine-producing strains of *L. parabuchneri* to form biofilms, which explains the apparent ease which they persist in dairy facilities. However, little is known about the nature of these biofilms or of the conditions that favor their formation.
The aim of the present work was to investigate biofilm formation by different *L. parabuchneri* strains, to determine the composition of the biofilm matrices produced, and to examine the influence on biofilm formation of temperature, pH, NaCl concentration, carbon source and surface type.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Table 1 lists the 25 *L. parabuchneri* strains used in this study the type strain plus 24 strains of dairy origin. All were cultured in MRS broth (Oxoid, Basingstoke, Hampshire, UK). Unless otherwise indicated, incubation proceeded at 37°C under anaerobic

conditions (10% H2, 10% CO2 and 80% N2) in a Mac 1000 anaerobic workstation (Don Whitley Scientific, Shipley, UK) with the temperature and gas concentration under automatic control [38]. To analyze the influence of the carbon source on biofilm formation, however, the medium employed was MRS broth without dextrose (US Biological, Salem, MA, USA), supplemented with glucose, galactose or lactose as required (2% w/v). The strains were pre-grown in this medium before analyzing their biofilm forming capacity. To study the effect of NaCl concentration on biofilm formation, MRS broth was supplemented with 1%, 2%, or 3% w/v NaCl.

Table 1. *L. parabuchneri* strains used in this study.

Strain	Origin	Reference
B301	Emmental	[39]
DSM 5987	Cheese	DSMZ-German Collection of Microorganisms
IPLA11117	Zamorano	[18]
IPLA11122	Emmental	[37]
IPLA11123	Emmental	Molecular Microbiology Group IPLA-CSIC
IPLA11125	Emmental	[37]
IPLA11126	Emmental	[37]
IPLA11129	Emmental	[37]
IPLA11131	Emmental	[37]
IPLA11132	Emmental	[37]
IPLA11133	Emmental	Molecular Microbiology Group IPLA-CSIC
IPLA11137	Emmental	Molecular Microbiology Group IPLA-CSIC
IPLA11150	Cabrales	[18]
IPLA11151	Cabrales	[18]
IPLA11152	Zamorano	[18]
IPLA15003	Mozzarella	Molecular Microbiology Group IPLA-CSIC
IPLA15005	Mozzarella	[18]
IPLA15006	Mozzarella	Molecular Microbiology Group IPLA-CSIC
IPLA15007	Mozzarella	Molecular Microbiology Group IPLA-CSIC
IPLA15008	Mozzarella	Molecular Microbiology Group IPLA-CSIC
IPLA15009	Mozzarella	[18]
IPLA15010	Mozzarella	Molecular Microbiology Group IPLA-CSIC
IPLA15012	Mozzarella	[18]
St2A	Zamorano	[40]
DSM 5707T	Human saliva (type strain)	DSMZ-German Collection of Microorganisms

IPLA-CSIC: Instituto de Productos Lácteos de Asturias—Consejo Superior de Investigaciones Científicas. DSMZ: German Collection of Microorganisms and Cell Cultures GmbH.

2.2 Biofilm Formation Capacity of L. parabuchneri Strains

Although the capability to form biofilm was previously studied in some of the strains examined in this work, (indicated with a reference in Table 1), to compare results and categorize them, they were included in the present study. The *L. parabuchneri* strains were initially categorized in terms of their ability to form biofilms on polystyrene using the crystal violet method [41]. Briefly, overnight cultures in MRS broth were diluted to a concentration of 10^6 CFU/mL. A total of 200 µL were then inoculated into the wells of a 96-well (round-bottomed) microtiter plate (Nunc MicroWell Plates with a Nunclon Delta Surface) (preparing at least 3 biological and 2 technological replicates for all experiments, with experiments performed in at least triplicate). As a negative control, sterile medium was used.

These plates were routinely incubated at 37°C for 48 h. However, to analyze the influence of time and temperature on biofilm formation, they were incubated for 12, 24, 48, 72 and 96 h, and at 8, 12 and 24 °C for 2, 7, 14 and 30 days, respectively. To examine the influence of an acidic environment, the strains were incubated in MRS broth at pH 4.7 (and at pH 6 as a control) under standard time and temperature conditions. The biomass of the biofilm detected using the crystal violet technique was quantified as described by Diaz et al. [18], with slight modifications. After incubation the supernatant was removed and the wells washed twice with 200 µL of PBS buffer to eliminate nonadherent cells. All the wells were then air-dried for 30 min at room temperature in a CRUMAir 9005-FL laminar flow cabinet (CRUMA, Barcelona, Spain). The biofilms formed were stained with 250 µL of 0.5% (*w*/*v*) crystal violet diluted with distilled, sterilized water (dH_2O) for 30 min at room temperature. The nonbound dye was eliminated and the wells washed 3 times with 200 µL of dH_2O. Finally, the bound dye was solubilized with 250 µL of acetic acid (33% *v*/*v*) and the absorbance measured at 595 nm using a Benchmark Plus microplate spectrophotometer (BioRad, Hercules, CA, USA). The biomass of the biofilm formed under each set of conditions was expressed as the mean of the results for the biological and technical replicates. The ability to produce biofilm was expressed using cut-off values [42,43]. The mean of the optical density (OD) SD for the 3 replicates was calculated for each strain. The mean for the negative controls, noninoculated media from the same batch, including biological and technical replicates (ODnc) was used to determine the cut-off values used to define the different categories of biofilm producers (ODc). These threshold values were calculated as the ODnc value plus 3 SDs. This allowed the strains to be classified into different categories:

- $ODc < OD \leq 2 \times ODc$ = weak producer
- $2 \times ODc < OD \leq 4 \times ODc$ = moderate producer
- $OD > 4 \times ODc$ = strong producer.

The OD value is the average value of biological and technical replicates for each strain.

2.3 Biochemical Composition of Biofilm Matrices: Dispersal Assays

Bacterial biofilms were prepared in 96-well microtiter plates as described above. After 48 h of incubation, nonbound cells were removed and the wells washed once with PBS buffer. The adhered biofilm matrix was then treated as previously described [44,45], adding 200 µL per well of different enzyme suspensions: DNAseI (100 µg/mL in 150 mM NaCl; 1 mM $CaCl_2$) to degrade extracellular DNA (eDNA); proteinase K (100 µg/mL) and trypsin (100 µg/mL [in 20 mM Tris-HCl pH 7.5; 100 mM NaCl]) to degrade proteins; RNAse (10 mg/mL [in 5 mM of $MgCl_2$]) to degrade RNA; and 10 mM sodium periodate ($NaIO_4$ [in 50 mM sodium acetate buffer pH 4.1]) to degrade exopolysaccharides (EPSs). Any biofilm dispersal (with the accompanying loss of biomass) indicated the presence of the corresponding substrate in the matrix. Control wells were filled with PBS buffer without enzymes.

All plates were incubated at 37 °C for 24 h. The enzyme mixture was then removed and the biofilms washed once with PBS, dried and stained with 0.5% crystal violet as described above. Biofilm dispersal was assessed by measuring the absorbance at

595 nm. Three biological replicates were made for each sample, and each experiment repeated at least 3 times.

2.4 Bacterial Adherence to Different Surfaces

Bacterial adherence to different surfaces was examined using 1 cm^2 'coupons' of each material. These included food-grade stainless steel (type AISI 304), beech wood, rubber and food-grade plastic, i.e., the most typical surfaces found in the dairy industry [10,46–48]. All were cleaned and sterilized in an autoclave prior to use. 100 µL of each strain suspension (10^9 CFU/mL) were then added to a tube containing a sterile coupon in 10 mL of MRS. For each material, a tube without cells was used as negative control. All tubes were incubated at 37 °C for 48 h. Any nonadhered cells were then removed by rinsing the coupon twice with 2 mL of PBS buffer. The biofilm produced on 1 of the faces of the coupon was then removed with a sterilized swab± and immersed in 1 mL of PBS. The number of adhered bacteria was determined by plating serial dilutions on MRS [18]. All experiments were performed with 3 replicates for each strain and material, employing independent cultures. The results were expressed as $\log_{10}$ CFU (mean ± SD of the replicates).

2.5 Scanning Electron Microscopy Images

Biofilm formation on different surfaces was observed as previously described [41] with some modifications. Briefly, the strains were incubated for 48 h at 37 °C in tubes with 10 mL of MRS medium containing the above-described coupons (1x1 cm^2). After incubation, the coupons were cleaned twice with PBS buffer and fixed in 2.5% glutaraldehyde (Sigma–Aldrich, Munich, Germany) in PBS for 16 h at room temperature. The fixed biofilms were then dehydrated using a graded series of acetone solutions (50–100% *v*/*v*), dried with argon, coated with platinum (using a SCD 005 sputter coater) and observed using a dual-beam FIB/SEM system (Quanta 3D FEG, FEI Company, Eindhoven, NL, USA).

2.6 Data Analysis

Biofilm formation at different times, temperatures and on different surfaces was compared by 2-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test. The Student *t* test were used to compare the effect of pH, NaCl concentration and carbon source, on biofilm formation. The same test was used to compare the effect of the different enzyme treatments on the biofilm matrix. All calculations were performed using SPSS Statistics v.15.0 software. Significance, unless otherwise indicated, was set at $p < 0.05$.

3. Results

3.1 Biofilm Formation by L. parabuchneri Strains

The crystal violet technique categorized the strains as either weak, moderate or strong biofilm producers (Figure 1). Two strains belonging to each category, *L. parabuchneri* IPLA11117 and 11122 (weak producers), 11125 and 11129 (moderate producers) and 11150 and 11151 (strong producers), were selected for further work.

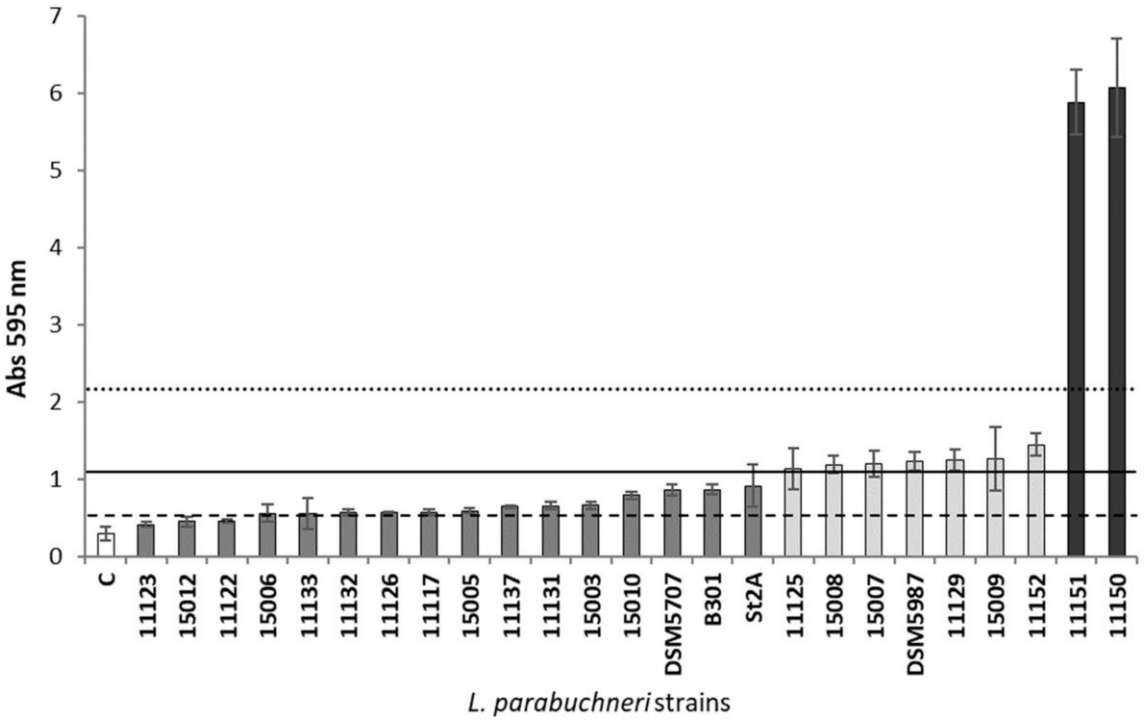

Figure 1. Biofilm-producing capacity on polystyrene of the histamine producing *L. parabuchneri* strains. The strains were incubated at 37 ◦C for 48 h. Data represent means ± SD (error bars) of at least three independent experiments. Shading of the same color indicates no significant difference. The producer/nonproducer cut-off (weak producer) is signaled by the dashed line (ODc), the solid line is 2 × ODc (moderate producer) and the dotted line 4 × ODc (strong producer).

3.2 Biochemical Composition of Biofilms

L. parabuchneri IPLA11150 and IPLA11151, classified as strong biofilm producers, were chosen to produce biofilms for matrix analyses. Assaying biofilm dispersal is the main method used to infer the components involved in biofilm matrix. Mature biofilms were treated with proteinase K, trypsin, DNAse I, RNAse or $NaIO_4$ (and PBS buffer as a negative control) as previously described [45,49,50]. After 48 h, the biofilms formed in microwell plates were subjected to each of the aforementioned treatments for 24 h (Figure 2). For both the IPLA11150 and IPLA11151 strains, the proteinase K treatment had the greatest effect in terms of the dispersal of the biofilm, most probably due to its broader spectrum of cleavage. Exposure to trypsin (another protease) also led to a significant dispersal, although less strong. For IPLA11151, DNAse I only slightly dispersed the biofilms. No significant effect was observed for the RNAse treatment. In contrast, the $NaIO_4$ treatment led to a significant increase in biofilm biomass formation for both strains.

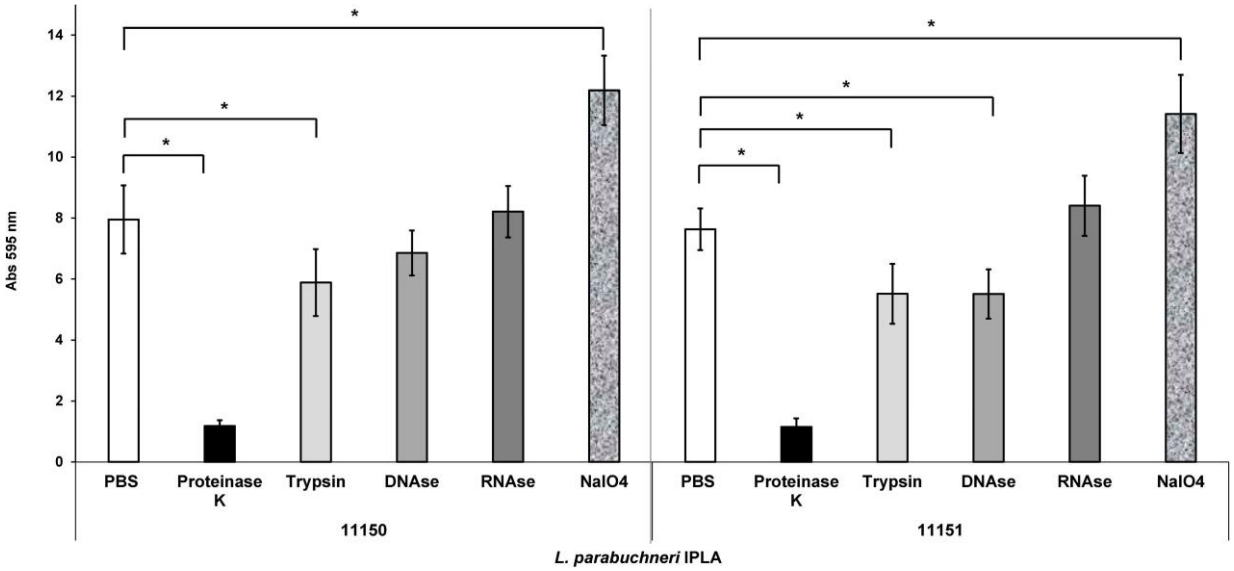

Figure 2. Effect of proteinase K, trypsin, DNAse I, RNAse and $NaIO_4$ on biofilms produced by the strong biofilm producers *L. parabuchneri* IPLA11150 and IPLA11151. The strains were incubated for 48 h at 37 ˚C and subjected to treatments with the stated agents for 24 h. Data represent means ± SD (error bars) of three experiments. Values marked with * differ significantly.

3.3 Biofilm Formation after Different Incubation Times

Biofilm formation by the selected strains was analyzed at different incubation times. For most of the assayed strains, biofilm biomass was at a maximum after 48 h of incubation (Figure 3). The exceptions were the moderate biofilm-producing strain IPLA11125 and the weak biofilm producer IPLA11122, which reached a maximum biofilm biomass after 72 h and 96 h of incubation, respectively. However, after 48 h, both had reached biomass values within the range of their established biofilm-producer categories, and the increment observed after 72 or 96 h did not reach the threshold required to change category. Consequently, the incubation time was fixed at 48 h for all assays. It is noteworthy that *L. parabuchneri* IPLA11151 and 11150 produced biofilms after just 12 h of incubation.

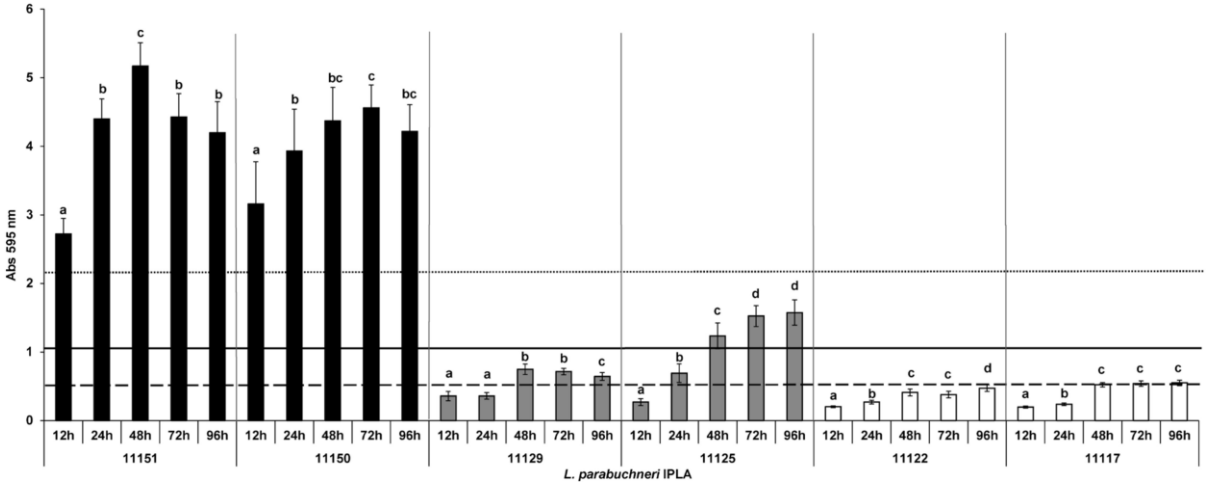

Figure 3. Biofilm-producing capacity on polystyrene at different times. The strains were incubated at 37 °C for 12, 24, 48, 72 and 96 h. Data represent means ± SD (error bars) of three experiments. For each strain, values marked with the same letter are not significantly different ($p > 0.05$ according to the Bonferroni post-hoc test). The producer/nonproducer cut-off (weak producer) is signaled by the dashed line (ODc), the solid line is 2 × ODc (moderate producer) and the dotted line 4 × ODc (strong producer).

3.4 Biofilm Formation at Colder Temperatures

The influence of cold temperatures on biofilm formation was examined given the importance of refrigeration for dairy products. Since refrigeration reduces microbial growth, the incubation times were extended (Figure 4). In general, all the analyzed strains showed a reduction in their ability to form biofilms at 8 °C. None of them, not even the strong producers IPLA11150 and IPLA11151, produced biofilm at this temperature, even after 30 days of incubation. Indeed, significant differences ($p < 0.001$) were seen between mean biofilm biomass formation after 30 days at 8°, 12° and 24 °C for *L. parabuchneri* IPLA11151 (0.28 ± 0.19; 2.20 ± 0.11; 3.79 ± 0.31, respectively), IPLA11150 (0.29 ± 0.02; 2.40 ± 0.23; 4.44 ± 0.51) and IPLA11125 (0.19 ± *0.01*; 0.37 ± 0.06; 0.43 ± 0.04). For the other three strains, biofilm formation after 30 days of incubation at 8 °C differed significantly with that seen at 12 °C and 24 °C ($p < 0.001$): for *L. parabuchneri* IPLA11129 0.18 ± 0.01 compared to 0.28 ± 0.02 and 0.29 ± 0.03, respectively; for IPLA11122 (a weak biofilm producer) 0.18 ± 0.01 compared to 0.28 ± 0.06 and 0.27 ± 0.03, respectively; and for IPLA11117 0.21 ± 0.05 compared to 0.44 ± 0.12 and 0.38 ± 0.07, respectively. In general, biofilm formation was clearly greater at 24 °C than at lower temperatures.

Interestingly, strong biofilm-producing strains reached their category threshold value even at 12 °C. At this temperature they needed seven days to reach maximum production, while they did so after only two days at 24 °C. The moderate and weak biofilm-producing strains showed a reduction in biofilm-forming capacity at the intermediate temperatures assayed. *L. parabuchneri* IPLA11125 and IPLA11129 were not even able to reach the minimum absorbance value for the category to which they belonged (moderate).

Although, the ability of *L. parabuchneri* to produce biofilms at colder temperatures was strain dependent, the strong biofilm producers formed notable biofilm biomass at 12 °C and 24 °C, a result of some importance given the temperatures associated with cheesemaking, especially during ripening [51–53].

3.5 Biofilm Formation in an Acidic Environment

Milk acidification is an important phenomenon during the manufacture of fermented dairy products. The effect of an acidic pH (4.7) on biofilm formation in MRS broth was therefore analyzed for all six selected strains, comparing the results to those obtained at pH 6 (the normal pH of MRS). The biofilm biomass increased very significantly during cultivation in the acidic broth (Figure 5). The largest increment almost double the biofilm biomass measured as OD was recorded for the strong biofilm producers. However, *L. parabuchneri* IPLA11125, a moderate producer, also behaved like a strong biofilm producer at the acidic pH (Figure 5). A significant increase in biofilm biomass was also observed for the other moderate producer IPLA11129, although not as for IPLA11125. In contrast, the weak biofilm-producing strains showed a reduced capacity to form biofilms at pH 4.7.

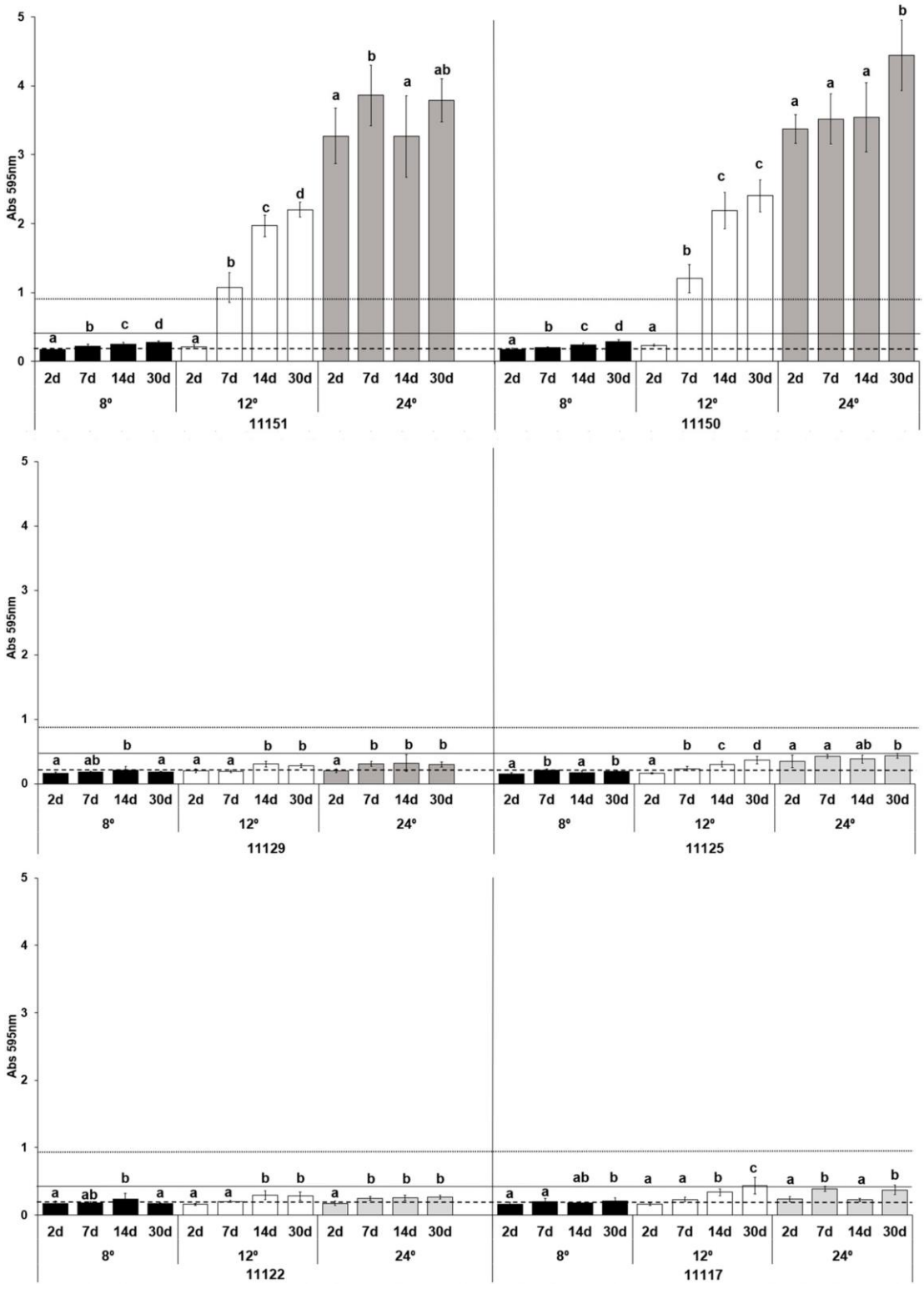

Figure 4. Biofilm-producing capacity on polystyrene of the histamine-producing *L. parabuchneri* strains incubated at 8, 12 and 24 ˚C. The strains were incubated for 2, 7, 14 and 30 days. Data represent means ± SD (error bars) of three experiments. Values marked with the same letter do not differ significantly ($p > 0.05$ according to the Bonferroni post-hoc test). The producer/nonproducer cut-off (weak producer) is signaled by the dashed line (ODc), the solid line is 2 × ODc (moderate producer) and the dotted line 4 × ODc (strong producer).

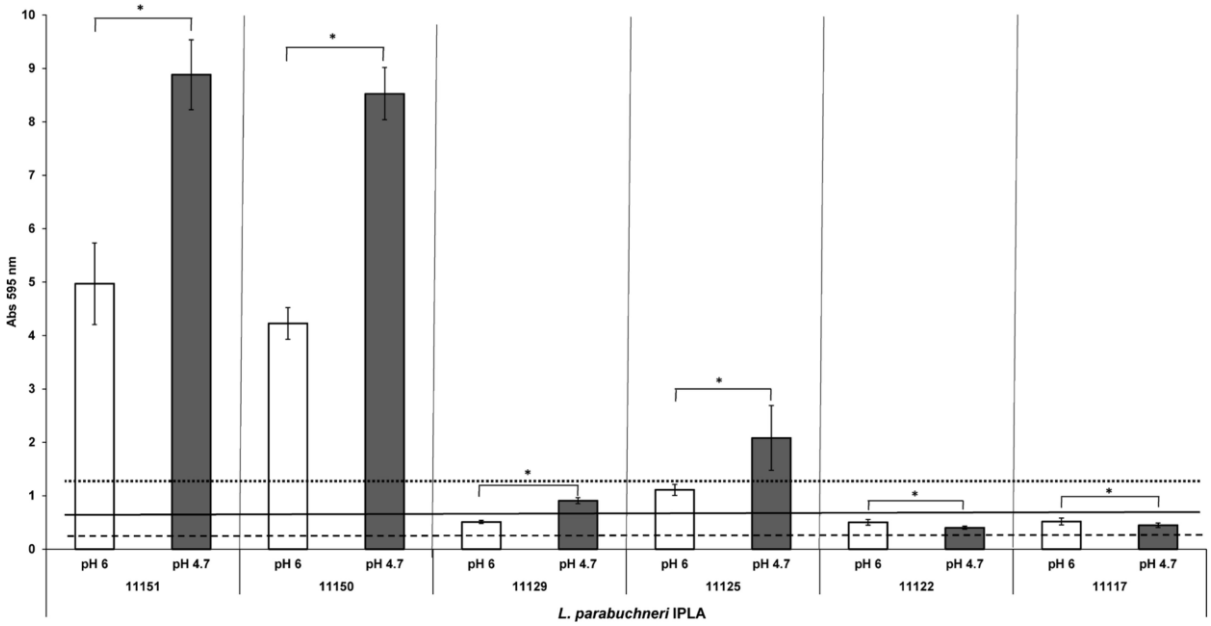

Figure 5. Biofilm-producing capacity on polystyrene of the biogenic amine-producing *Lentilactobacillus* strains incubated with MRS at pH 4.7 and 6. The strains were incubated for 48 h at 37 °C. Data represent means ± SD (error bars) of three experiments. Values marked with * differ significantly. The producer/nonproducer cut-off (weak producer) is signaled by the dashed line (ODc), the solid line is 2 × ODc (moderate producer) and the dotted line 4 × ODc (strong producer).

3.6 Influence of Carbon Source on Biofilm Formation

The most abundant sugar in the dairy environment is lactose, which is metabolized to glucose and galactose by β-galactosidases. The influence of these three sugar sources on biofilm formation by the six selected *L. parabuchneri* strains was studied (Figure 6). For the strong producers, biofilm formation was greatest in the presence of glucose (standard condition). With lactose and galactose, their production of biofilm biomass was less strong, although they still returned biomass values exceeding the threshold value for strong biofilm producers. Both moderate producers showed an increase in biofilm formation in the presence of galactose. However, when lactose was the carbon source, IPLA11125 showed an increase in biofilm biomass formation, while for IPLA11129, production was close to that of a weak biofilm producer. For the weak producer IPLA11122, biofilm formation increased with lactose and galactose in comparison with glucose, while for IPLA1117, it increased only with galactose. However, in both cases, the production values were still those of weak producers, or indeed, under the threshold for recognition as a biofilm producer.

3.7 Effect of NaCl on Biofilm Formation

During cheesemaking, NaCl is added in variable quantities at different times, either directly or by immersion in brine. The effect of different NaCl concentrations on the biofilm formation capacity of the six *L. parabuchneri* strains was therefore analyzed (Figure 7). This capacity was reduced in all the analyzed strains as the NaCl concentration increased. For the strong producers, the inhibitory effect was greater on IPLA11151 than on IPLA11150 (not seen until a salt concentration of 2% was tested). The moderate producers were also inhibited, more so *L. parabuchneri* IPLA11125 than IPLA11129 (no reduction recorded until 3% NaCl was tested). At the concentrations tested, NaCl had a small effect on the weak producer IPLA11117 and no significant effect on the other weak producer IPLA11122.

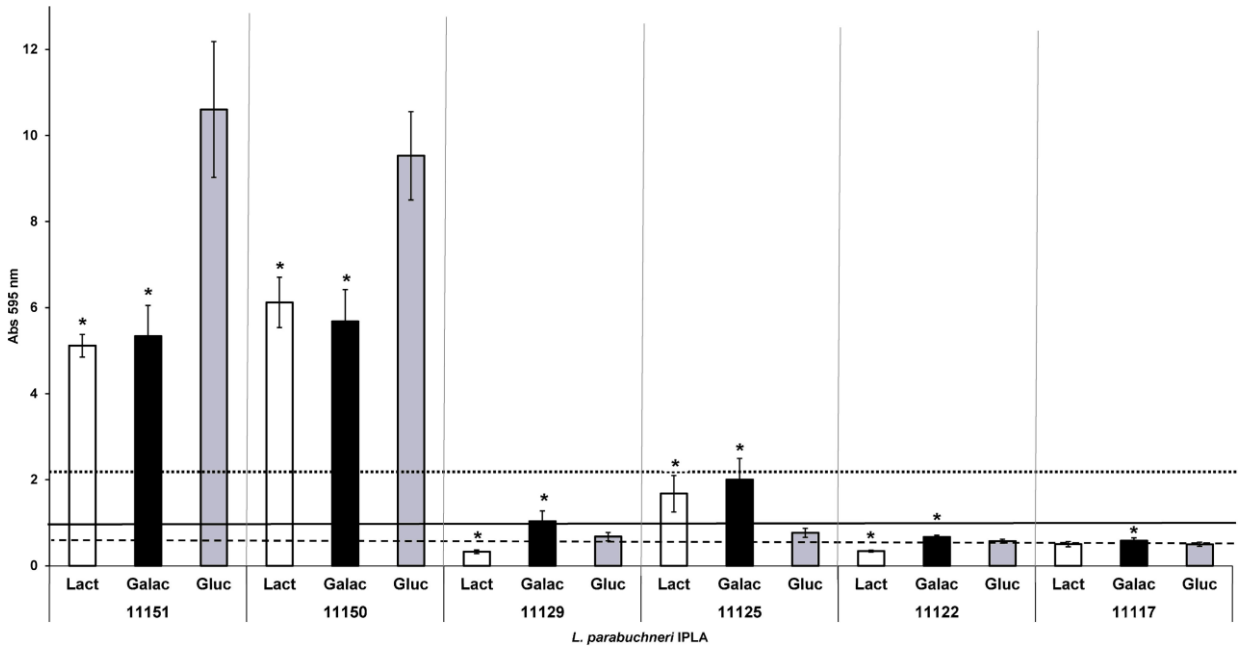

Figure 6. Biofilm-producing capacity on polystyrene of the histamine-producing *L. parabuchneri* strains incubated with MRS supplemented with different carbon sources. The strains were incubated for 48 h at 37 °C. Data represent means ± SD (error bars) of three experiments. Values marked with * differ significantly, considering glucose as the control. The producer/nonproducer cut-off (weak producer) is signaled by the dashed line (ODc), the solid line is 2 × ODc (moderate producer) and the dotted line 4 × ODc (strong producer). Lact: Lactose; Galac: Galactose; Gluc: Glucose.

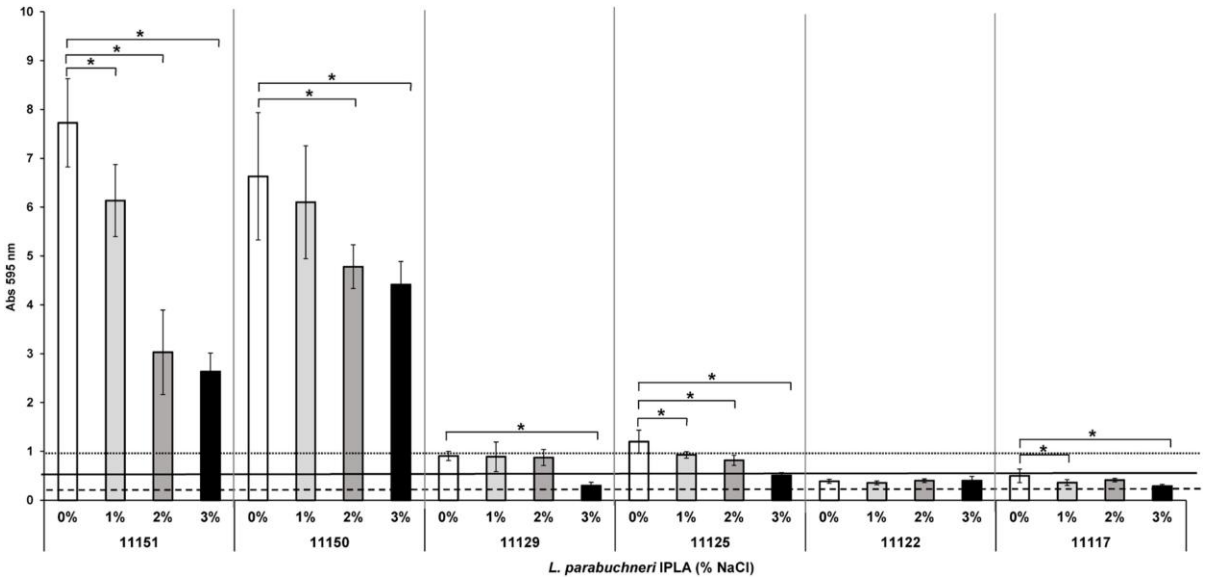

Figure 7. Effect of NaCl concentration (in % p/v) on biofilm-producing capacity on polystyrene of the histamine-producing *L. parabuchneri* strains. The strains were incubated for 48 h at 37 ◦C. Data represent means ± SD (error bars) of three experiments. Values marked with * differ significantly. The producer/nonproducer cut-off (weak producer) is signaled by the dashed line (ODc), the solid line is 2 × ODc (moderate producer) and the dotted line 4 × ODc (strong producer).

3.8 Bacterial Adherence to Stainless Steel, Food-Grade Plastic, Beech Wood and Rubber

All six tested strains adhered to all the surfaces tested (adhesion capacity was expressed via the number of viable cells adhered to the surfaces [CFU/mL cm^2]). Apart from

the control polystyrene, beech wood was the material to which all the strains adhered best. The strong producer *L. parabuchneri* IPLA11151 adhered similarly well to all the surfaces studied, suggesting it poses a risk of forming biofilms throughout the cheese production process. The other strong biofilm producer, IPLA11150, adhered similarly well to beech wood and polystyrene, but log-reduced values were recorded for the other surfaces. The moderate producer IPLA11129 showed similar adhesion values for polystyrene, beech wood and plastic and slightly reduced values for stainless steel and rubber. Surprisingly, IPLA11125 (a moderate producer), IPLA11122 and IPLA11117 (weak producers) returned strong adhesion values for all surfaces, but especially for polystyrene and beech wood, with values similar to those recorded for the strong producers (Table 2). Thus, biofilm formation depends on more than cell adhesion capacity alone.

Table 2. Viable cells of *L. parabuchneri* strains adhered to surface material coupons after 48 h of incubation at 37 °C.

	L. parabuchneri IPLA					
Material/Strain	**11151**	**11150**	**11129**	**11125**	**11122**	**11117**
Polystyrene	6.79 ± 0.74	6.68 ± 1.12	6.45 ± 1.18	6.93 ± 0.74	7.08 ± 0.76	6.90 ± 0.87
Stainless Steel	6.22 ± 1.03^{ac}	$4.07 \pm 0.99^{bc*}$	$4.29 \pm 0.83^{bc*}$	$4.6 \pm 0.32^{abc*}$	$5.05 \pm 1.15^{ab*}$	$3.18 \pm 1.29^{bc*}$
Plastic	5.37 ± 0.93^{a}	$4.10 \pm 0.68^{ab*}$	5.17 ± 0.69^{a}	$4.85 \pm 0.64^{ab*}$	$4.97 \pm 0.65^{ab*}$	$3.52 \pm 1.24^{b*}$
Beech Wood	6.11 ± 0.37	5.53 ± 0.93	6.40 ± 0.55	6.01 ± 0.48	6.13 ± 0.20	5.49 ± 1.39
Rubber	5.58 ± 0.71^{a}	$4.32 \pm 1.45^{ab*}$	$4.53 \pm 0.43^{ab*}$	$5.21 \pm 0.43^{a*}$	$4.46 \pm 0.86^{ab*}$	$3.40 \pm 1.12^{b*}$

Data are expressed as CFU mL^{-1} cm^2 and represent the mean SD of three experiments. Significant differences, as determined by ANOVA ($p < 0.05$ according to Bonferroni post-hoc test), in adhesion between the strains for each material are labeled with different letters. * denotes significant differences within strains with respect to the polystyrene control.

Overall, no significant differences were seen between the tested strains with respect to adhesion to polystyrene and beech wood, confirming that these materials facilitate cell attachment. However, with respect to stainless steel, cell adhesion was higher for IPLA11151, IPLA11125 and IPLA11122. The value recorded for IPLA11117, in contrast, was half that recorded for IPLA11151. The strong producer IPLA11151 and the moderate produced IPLA11129 showed the highest adherence values with respect to plastic. Finally, for rubber, the highest adhesion values were returned by the strong producer IPLA11151 and the moderate producer IPLA11125, again showing that factors other than cell attachment are involved in biofilm formation.

3.1 SEM Analysis of Biofilm Formation on Different Surfaces

Scanning electron microscopy (SEM) photomicrographs were made of the biofilms formed by the strong producer IPLA11151, the moderate producer IPLA11125 and the weak producer IPLA11122. Images of the coupons were taken after 48 h of incubation in MRS medium (Figure 8). The images show the visual characteristics of each *L. parabuchneri* strain and its ability to attach to each of the surfaces, which have different roughness and permeability properties, etc. The stainless steel and plastic surfaces were clearly smoother than the beech wood and rubber surfaces (adhesion was more easily achieved on these rougher and more hydrophobic surfaces). SEM is frequently used to observe biofilm spatial structure and to detect the presence of extracellular polymeric substances [54]. In the present work, some cells produced such polymers around themselves after 48 h of incubation, and some cells were elongated. This indicates that the biofilms had progressed beyond the first stages of attachment.

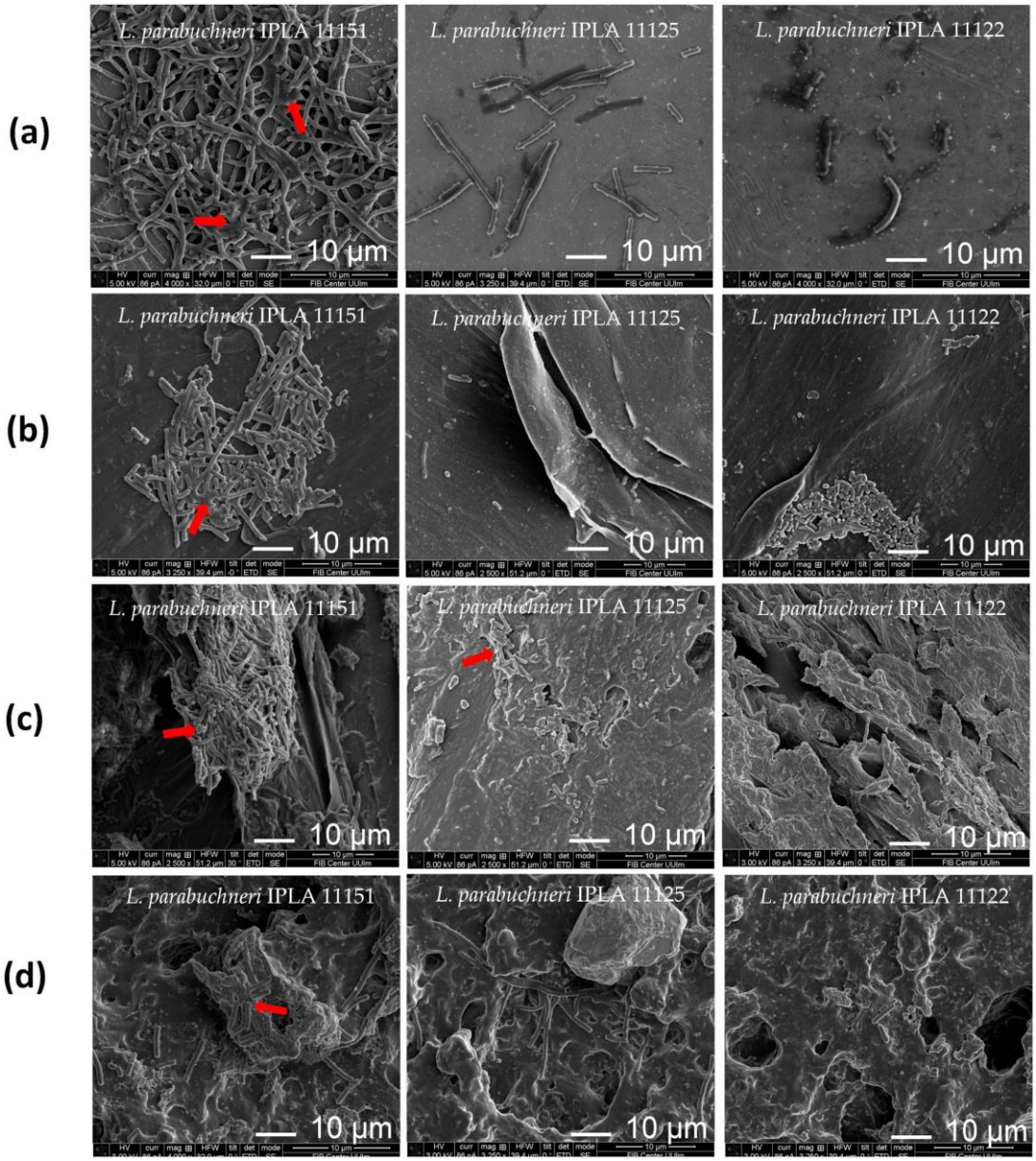

Figure 8. Scanning electron microscopy images of *L. parabuchneri* strain biofilms grown at 37 ˚C for 48 h on different surfaces: (**a**) stainless steel; (**b**) food-grade plastic; (**c**) beech wood; and (**d**) rubber. Scale bars (10 µm) are shown on the photomicrographs. Red arrows indicate extracellular polymeric substances in the matrix.

Interestingly, EPSs provide mechanical stability to biofilms and help in the development of three-dimensional spatial structures that influence functional properties, such as resistance to antimicrobial agents and cleaning treatments [4]. In the present work, EPS production was greater in the strong producer IPLA11151 than in either of the other two strains tested. This was especially true with respect to the stainless steel, plastic and rubber coupons (Figure 8).

4. Discussion

In the food industry, microbial biofilms are of great concern given their connection with food safety and quality [2,12,13,54]. The great majority of foodborne diseases are of bacterial origin, with the contamination of food during manufacturing and preconsumption storage the usual routes of bacterial entry and development. *L. parabuchneri* is known to be largely responsible for the production and accumulation of histamine in cheese [30]. Histamine is one of the most toxic of BAs [23,28] and the only one for which a legal limit has been established, although only for certain foods [23,55]. In fact, there is no specific regulation regarding its concentration in dairy products, not even for cheese, in which it can reach high concentrations [56]. Reducing the presence of *L. parabuchneri* in cheese is, however, recommended [25,37]. Some histamine-producing strains of *L. parabuchneri* are also known to produce biofilms in the dairy environment [18,37], which could act as reservoirs of food- contaminating microorganisms that subsequently cause the accumulation of histamine at undesirable concentrations [18,31]. This phenomenon is especially problematic with respect to cheeses that are processed post-ripening for market presentation (cut, sliced or grated). Biofilms can form on the surface of the equipment used [57], from which histamine-producing bacteria can contaminate the cheese being processed [58].

Significant strain-specific differences in biofilm formation have been observed for well-known biofilm-forming species such as *S. aureus* and *Salmonella* spp. [59]. The present work examined and compared the biofilm-forming capacity of 24 histamine-producing *L. parabuchneri* strains of dairy origin (plus the type of strain included as a reference). All were able to form biofilms and were classified as either strong, moderate or weak producers based on their ability to produce a biofilm on polystyrene. More than one-third of the analyzed strains were classified as moderate or strong producers (Figure 1). This would give these strains an advantage in colonizing surfaces at dairy facilities and represents a threat to food safety. Two strains from each category were selected for further study: *L. parabuchneri* IPLA11150 and 11151 as strong producers, IPLA11125 and 11129 as moderate producers and IPLA11117 and 11122 as weak producers.

Biofilm dispersal assays are the method most commonly used to determine the com- ponents involved in biofilm matrix. A reduction in biofilm biomass was seen for the strong biofilm producers, IPLA11151 and 11150, after treatment with proteinase K and trypsin. These proteases recognize different protein substrates, suggesting that the biofilm matrix is composed mainly of proteins (Figure 2). In addition, the biomass of the biofilm produced by IPLA11151 was reduced by treatment with DNAse I, suggesting that eDNA is also present in its biofilm matrix. In some food spoilage *Lactobacillus plantarum* strains, the biofilm matrix was also shown to be mainly formed by protein and eDNA, although differences in the importance of both compounds between strains were also observed [60]. However, no effect was observed for either IPLA11150 or IPLA11151 after treatment with RNAse, suggesting RNA to be absent from their biofilms. $NaIO_4$ was used as a dispersal agent to test if EPSs were present, but its use actually led to an increase in biomass. This might be due to the effect of $NaIO_4$ on EPSs that are chemically identical in structure, but that have differences in terms of the acetates O-linked to succinate, or the acetylation levels of amino groups [61,62]. In biofilms, polysaccharides may be segregated or associated with other molecular species such as DNA, proteins and lipids, with which they can interact [63]. As a consequence, the depolymerization of EPSs in response to $NaIO_4$ varies depending on a biofilm's composition. Dakheel et al. [44] reported that $NaIO_4$ could show strong to weak biofilm dispersal capacity, revealing different patterns of interaction between EPSs and proteins. Sager et al. [64] showed that $NaIO_4$ had a stimulating influence on established biofilms of *Pasteurella pneumotropica*, an effect similar to that

seen in the present work, indicating the presence of EPSs associated with other components that affect its biofilm dispersing capacity. However, with the techniques used in the present work, it is difficult to know what the precise involvement of EPSs in the biofilm matrix of *L. parabuchneri* may be. The biofilm producing capacity of microbes is influenced by factors such as the attachment surface, the temperature, the presence of other species, and nutrient avail- ability, etc. [13,65,66]. New materials that might prevent microbial growth are being explored [67,68], including the use of safe natural antimicrobial coating surfaces [69], but there remain many that are difficult to clean. The design requirements of equipment can also render effective cleaning and disinfection difficult [36]. In the present work, the adherence of the tested strains to materials commonly used in the dairy industry (and traditional facilities)—stainless steel, beech wood, food-grade plastic and rubber—was investigated. In other biofilm-forming species, such as *L. monocytogenes*, the surface material was found to be a major factor affecting biofilm production and with variations seen between strains [70]. The present histamine-producing strains of *L. parabuchneri* were all able to form biofilms on all the surfaces tested. The categorization of the strains as strong, moderate or weak produces (as determined on polystyrene using the crystal violet technique) was not upheld on the plastic and rubber surfaces, for which no significant differences in the numbers of adhered cells were seen (Table 2).

SEM images showed the beech wood to be the material on which the biofilms reached the greatest biomass (Table 2; Figure 8). On this material, even the weak biofilm producers were able to adhere in numbers similar to those recorded for the two strains classified as strong producers. This might be related to the roughness of this material, which was easily observable in the images (Figure 8). Although beech wood is not often used in large industrial facilities, it is still used for shelving in some traditional settings, e.g., where ripening occurs in environments such as natural caves. Rubber is a critical material in the industry, usually found at the connections between pipes and storage tanks; the capacity of histamine-producing *L. parabuchneri* strains to adhere to this material is a problem given the difficulties in its cleaning. In fact, this was one of the main localizations in which *L. parabuchneri* was located in some dairy facilities [36]. Stainless steel would seem to be the most appropriate material to use in the dairy industry since adhesion to this surface was apparently more difficult for all the analyzed strains, with the exception of the strong producer IPLA11151. It is also easier to clean and disinfect stainless steel than any of the other materials assayed, and it can better resist more extreme or abrasive treatments for biofilm elimination. Anyhow, the fact that some strains, such as *L. parabuchneri* IPLA11151 shown a greater adhesion capability indicates the need to maintain and maximize cleaning procedures to avoid the risk of contamination.

The SEM images revealed differences in roughness and porosity of the examined surfaces. Beech wood and rubber were the most porous materials explaining the enhanced cell adhesion values (i.e., especially beech wood). After 48 h of incubation, cells of all the three analyzed strains (IPLA11151, 11125 and 11122 [strong, moderate and weak producer, respectively]) were adhering to all the surfaces. During biofilm formation, cells elongate to connect adjacent microcolonies and to produce EPSs [44,71]. In the present work, the strong producer IPLA11151 yielded longer cells and larger amounts of EPSs. Indeed, the EPSs in biofilms plays a critical role in providing mechanical stability and in the formation of 3D spatial structures [72]. These structures aid in providing functional properties such as the ability to resist antimicrobial agents and cleaning treatments [43].

One of the most important factors affecting bacterial attachment to a given surface is the temperature of the environment. High temperatures make the surface of bacteria more hydrophobic, facilitating their tight binding to surfaces [10]. In the

present work, cold temperatures reduced the capacity to form biofilms for all the tested strains (Figure 4). At 8 °C (refrigerator temperature), biofilm production over 30 days was most reduced for the ostensibly strong producers. The reduction effect on biofilm formation at reduced temperatures seems to be a general effect, as observed in other foodborne pathogens or spoilage bacteria [60,73,74], that could be related to a lower growth rate. Keeping production and storage temperature as low as possible could therefore improve food safety. In the manufacture of cheese this is not always possible [28,31], but it could still be important during post-ripening processing and storage. In the case of medium and weak biofilm producer strains, the reduction in incubation temperature has a prevention effect, making longer incubation times necessary, up to 30 days, to get closer to the absorbance values obtained at optimal temperature. However, in the case of strong biofilm producer strains, they reach the threshold value at 48 h at 24 °C and in seven days at 12 °C. It should be remembered that *L. parabuchneri* can still produce histamine at low temperatures [75], but in a temperature-dependent manner. Thus, by reducing the risk of biofilm formation and of histamine accumulation, maintaining a low temperature would have a doubly positive effect in terms of food safety.

pH also influences the ability of *L. parabuchneri* to produce biofilms. When incubated at pH 4.7, the moderate and strong producers returned greater biofilm biomass values than when incubated at pH 6 (Figure 5). Unfortunately, in the dairy environment, where the pH is usually low due to the production of lactate during lactose fermentation, the ability of *L. parabuchneri* to form biofilms would be favored. In *E. coli* acidic pH (5.5) also enhances the ability to form biofilm, although this effect was temperature dependent, at optimal growth temperature the acidic pH enhances the biofilm formation, while at restrictive temperature the biofilm formation in acidic conditions was reduced [76]. Probably this reduction effect was linked to a lower growth rate in nonoptimal environmental conditions. Biofilms offer resistance to acid stress, and some LAB are reported to survive acidic environments because of their ability to form biofilms [42,77,78].

In the dairy environment, the most abundant carbon source is lactose, the main sugar in milk. However, as soon as manufacturing begins, lactose is catabolized to glucose and galactose by the action of β-glucosidases. In the present work, (Figure 6) the strong producers made more biofilm in the presence of glucose, while for the moderate and weak producers, galactose favored greater production. In a dairy isolate of *Staphylococcus epidermidis*, glucose also favors biofilm formation, enhancing their three-dimensional structure in comparison with lactose. This effect was associated with an increase in EPSs formation, although it was not similar for all the tested strains. Thus, biofilm production with respect to carbon source would seem to be strain dependent. This might be related to the ability to consume the different sugar moieties or a hierarchical preference for consumption, as shown for other LAB species [79]. Carbon source could also influence the formation of EPSs. In some dairy isolated lactobacilli, glucose increased the formation of EPSs [80], which could explain the higher biomass observed, but in the *L. parabuchneri* strains studied in this work, we could not assess the presence and influence of EPSs in the biofilm matrix (Figure 2).

Although increased salinity has been shown to increase EPSs and biofilm formation in some bacterial species [81], in *L. parabuchneri* it had an antimicrobial effect (Figure 7). A clear reduction in biofilm biomass was observed for all the tested strains as the NaCl concentration of the medium increased (Figure 7). Thus, NaCl could be used to reduce the presence of biofilms. However, there is a consumer demand for foods with less salt, given the latter's potentially harmful effects on health [82,83]. Unfortunately, this has led producers to reduce the

presence of NaCl during cheesemaking, and this has been shownto increase the risk of histamine accumulation [84].

5. Conclusions

As far as we know, this is the first report of the influence of technological factors on biofilm formation by histamine-producing *L. parabuchneri* strains isolated from cheese. The attachment of *L. parabuchneri* cells to surfaces varies between strains, and some of them were able to form strong biofilms, mainly composed of proteins and eDNA. An acidic environment, concomitant to fermented dairy products, was found to promote biofilm production by the moderate and strong biofilm-producing strains. Biofilm formation was shown to be reduced as incubation temperature is reduced. However, the strong biofilm producers, showed resistance to acidic pHs and capacity to form biofilm at cold temperatures (12 ˚C), together with their ability to form biofilm in stainless steel surfaces and in rubber, mainly present in pipes and tube connections, constitute a safety risk threat. Refrigeration could be an important preventive measure to reduce the risk of biofilm formation, but it should be maintained over time. The addition of salt was also shown to reduce the ability to form biofilm. The best combination of environmental factors, low temperature and adequate salt concentration, needs to be maintained during cheese production to prevent biofilms formed by *L. parabuchneri*. This, plus using methods of biofilm elimination, may be the best strategy for reducing the presence of histamine in cheese and other foods.

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CAPITULO 2

Objetivo 3. Estudio de la composición de la matriz extracelular de los biofilms de *L. parabuchneri*.

Conocer la composición de un biofilm y sus cambios a lo largo del tiempo, son determinantes para poder proponer estrategias de eliminación que sean compatibles con la industria alimentaria y sostenibles con el medio ambiente.

Para lograr este objetivo, en colaboración con la Universidad de Ulm se aplicaron las técnicas de reflectancia total atenuada (ATR) y espectroscopía infrarroja (IR), llamada IR-ATR, combinadas para estudiar la composición de los biofilms durante el periodo de formación.

La aplicación de estas técnicas permitió un análisis "*in vivo*" de la composición del biofilm, desde la primera etapa de formación y adhesión de células planctónicas (t=0) hasta obtener el biofilm maduro (48 h). Se analizaron las seis cepas de *L. parabuchneri* con distinta capacidad para formar biofilms y se monitorearon los cambios en la composición de la matriz extracelular y otros parámetros como la presencia del oxígeno.

Los resultados de IR mostraron cambios significativos en amidas, lactato, ácidos nucleicos y sustancias poliméricas extracelulares. Además, la aplicación de dos modelos diferentes para el análisis de los datos obtenidos: análisis de los componentes principales (PCA) y análisis discriminante de mínimos cuadrados parciales (PLS-DA), facilitaron la determinación y clasificación de las cepas de *L. parabuchneri*, en base a la composición y capacidad de producción de biofilm.

Los resultados obtenidos se presentan en el siguiente manuscrito (enviado para su evaluación y publicación):

> **Artículo 2:** Diellza Bajrami**, Agustina Sarquis**, Victor M. Ladero, María Fernández, Boris Mizaikoff. Rapid discrimination of *L. parabuchneri* biofilms via in situ infrared spectroscopy.

Rapid discrimination of *L. parabuchneri* biofilms via in situ infrared spectroscopy

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Abstract

Microbial contamination in food industry is a source of foodborne illnesses and biofilm-related diseases. In particular, biogenic amines (BAs) accumulated in fermented foods via lactic acid bacterial activity exert toxic effects on human health. Among these, biofilms of histamine-producer *L. parabuchneri* strains adherent at food processing equipment surfaces can cause food spoilage and poisoning. Understanding the chain of contamination is closely related to elucidating molecular mechanisms of biofilm formation. In the present study, an innovative approach using integrated chemical sensing technologies is demonstrated to fundamentally understand the temporal behavior of biofilms at the molecular level by combining mid-infrared (MIR) spectroscopy and fluorescence sensing strategies. Using these concepts, the biofilm forming capacity of six cheese-isolated *L. parabuchneri* strains (IPLA 11151, 11150, 11129, 11125, 11122 and 11117) was examined. The cut-off values for the biofilm production ability of each strain was quantified using crystal violet (CV) assays. Real-time infrared attenuated total reflection spectroscopy (IR-ATR) combined with fluorescence quenching oxygen sensing provides insight into distinct molecular mechanisms for each strain. IR spectra showed significant changes in characteristic bands of amides, lactate, nucleic acids, and extracellular polymeric substances (i.e., lipopolysaccharides, phospholipids, phosphodiester, peptidoglycan, etc.), which are major contributors to biofilm maturation involved in the initial adhesion processes. Chemometric methods including principal component analysis and partial least square-discriminant analysis facilitated the rapid determination and classification of cheese isolated *L. parabuchneri* strains unambiguously differentiating the IR signatures based on their ability to produce biofilm. All biofilms were morphologically characterized by confocal laser scanning microscopy on relevant industrial equipment surfaces. In summary, this innovative approach combining MIR spectroscopy with luminescence sensing enables real-time insight into the molecular composition and formation of biofilms of *L. parabuchneri*.

***Keywords*:** *L. parabuchneri*, cheese-isolated strains, biofilm formation, extracellular polymeric substances, infrared attenuated total reflection spectroscopy, luminescence sensing, multivariate data analysis/classification, principal component analysis, partial least square-discriminant analysis

1. Introduction

Biogenic amines (BAs) are nitrogenous compounds naturally generated by plants, animals, and microorganisms, produced by the decarboxylation of amino acids that can accumulate in dairy products [1,2]. High concentrations of BAs in food can cause intoxication in consumers with symptoms such as headache, nausea, diarrhoea, urticaria, tachycardia, and blood pressure disorders [3,4]. Consequently, there is general consensus regarding the minimization of BAs in foods [5,6].

Lactic acid bacteria (LAB), which are Gram-positive bacteria present in fermented dairy products are responsible for the fermentation process of food products [7]. However, certain LABs are the main producers of BAs and are responsible for their accumulation in fermented dairy food [8–10] One of the BA that has the greatest relevance in terms of food safety is histamine [11], which is the result of decarboxylation of the amino acid histidine [12]. Several species of LAB have been identified as potential histamine producers due to the presence of the HDC cluster, which is composed of three genes that allow the decarboxylation of histidine to histamine and increase the accumulation of this compound in fermented dairy products [13,14]. However, among them *L. parabuchneri*, a Gram-positive heterofermentative LAB species has been identified as the main responsible for histamine accumulation at elevated concentrations [10,15]. *L. parabuchneri* strains containing the HDC cluster, reach the dairy products from milk as part of the starter cultures or may contaminate these commodities during production [16].Namely, histamine-producing microorganisms such as *L. parabuchneri* form parts of starter cultures that may contaminate cheese during its production [17], and during post-ripening manufacture processes like slicing, grating, cutting, etc., which affects the presence of histamine-producing strains thereby increasing the probability of food spoilage and contamination [18].

L. parabuchneri has been identified in different cheese varieties such as Caciocavallo Pugliese, Spanish farmhouse cheese, Parmigiano Reggiano, Camembert, Gouda-type cheese, Pecorino Crotonese, Cheddar, Emmental [15] and it has been reported that some strains of this species are able to produce sessile communities that can survive on surfaces of dairy industrial equipment [15,19]. These sessile associations are also termed biofilms [20,21]. Biofilms are a sizeable challenge in dairy industries, as they bind to a variety of surfaces including stainless steel, plastic, rubber, glass, polypropylene, and even food products [22,23]. Consequently, they can cause damage

to industrial equipment and food contamination [21–24]. Biofilms formed as a result of spoilage or presence of pathogenic bacteria act as a source of cross-contamination in fermented products and consequently reduce the effectiveness of food processing strategies, which is why they are a major concern in food quality and safety [25].

The biofilm composition along with its characteristics and dynamics of the extracellular matrix affect the development, maturation, and life cycle of the biofilm [26,27]. Certain components of the biofilm matrix such as extracellular polymeric substances (EPS) preserve biofilm structure and functionality by keeping the cells together and protecting the community by providing a flexible and adaptable structure of multicellular microbial life against hostile environments [28,30]. As a result, extracellular polymeric components are the main contributors to the adhesion of biofilms in food industry surfaces adding to the resistance against pH, temperature, pressure, and sanitization changes [31,32]. Most chemical and functional studies on extracellular polymeric substances (EPS) are conducted on bacteria present in clinical environments, however, the associated microorganisms are not commonly responsible for biofilms in nature or in industrial environments [26]. Studies on Gram-negative pathogens have shown that biofilm matrices including proteins, exopolysaccharides, amyloid fibers, and extracellular DNA (eDNA) form strong networks that are stress-resistant to environmental changes [33].Hence, understanding the composition and extracellular matrix of *L. parabuchneri* species and their biofilm formation capacity is a relevant strategy, as an expanded knowledge on the matrix composition, architecture, functionality, stability, and dynamics of bacterial biofilms mediate effective measures toward their prevention and elimination in food industries.

A range of analytical techniques are available for investigating bacterial biofilms including various microscopies, fluorescence imaging, molecular genetic studies, proteomics, biochemical assays, microarray analysis, mass spectrometry, electrochemical assay, etc. [34–36]. The present state-of-progress in the field of bioanalytical methodology emphasizes the need for in situ examination of biological samples [37]. Most analytical methods address biofilms in static systems, even though dynamic flow-through conditions effectively replicate real-world scenarios [38–40]. Infrared attenuated total reflection (IR-ATR) spectroscopy has recently gained importance as a non-destructive method for obtaining molecular information on biofilm processes, metabolic and chemical reactions, and variations at different stages of bacterial biofilm growth [41,42]. Comprehensive studies demonstrated that IR-ATR

offers yet unprecedented insight on the relationship between spectral parameters and structural changes of major biomolecules including but not limited to lipids and proteins, and their interactions with water [43] IR-ATR spectroscopy using an integrated flow-through cell is a versatile analytical technique for evaluating microbial activity at real-world conditions close to real-time observing living bacteria, cells and colonies [44], and for monitoring biofilm development [41,45,46]. In the present study, the matrix composition of the sample was analyzed via IR-ATR spectroscopy using appropriate total internal reflection waveguides [47,48]. The chemical characteristics of the biofilm attached to the ATR waveguide surface were studied at different stages of biofilm formation.

In comparison to planktonic cells (i.e., individual microbial cells behaving as free-floating entities), biofilms show an increased antibiotic tolerance. Extracellular polymeric substances (EPS) serve as a quasi-hydrogel-like matrix encasing cells within the biofilm leading to adherence at surfaces [49,50]. Real-time IR-ATR techniques facilitate the analysis of microbial cells and the involved EPS components via characteristic IR spectral signatures characteristic for the biofilm matrix when attaching at a suitable ATR waveguide surface [21,51]. IR spectral data are essentially 'molecular fingerprints' of microbial biofilms entailing signatures of the molecules that are part of the matrix, which renders in situ IR-ATR spectroscopy an excellent tool for quantitative molecular studies [52]. In addition, IR-ATR spectroscopy may be performed in a continuous monitoring mode spectroscopically following the entire process of biofilm production via *L. parabuchneri* from the first reversible stage to the late stages of maturation and dispersion [41,53] without interfering with biofilm proliferation. Furthermore, molecular-level variations in the composition of extracellular matrices among distinct isolated strains were classified via label-free in situ IR spectroscopy. This study herein focuses on real-time monitoring of food spoiling *L. parabuchneri* strains with varying biofilm production capacities during the observation of cyclic changes of molecular mechanisms and temporal adhesion behavior at continuous nutrient flow. Chemometrics techniques have been extensively applied in microorganism discrimination and biofilm analysis. Multivariate exploratory techniques such as principal component analysis (PCA) [54] and partial least squares linear discriminant analysis (PLS-DA) are among the most commonly applied statistical methods for evaluating IR spectroscopic data. PCA and PLS are based on an orthogonal transformation process converting a set of observations represented as variables into an

Eigenvector-based coordinate system. In this transformed data space, a new set of uncorrelated variables (a.k.a., principal components) are orthogonally arranged such that they capture the maximum variance present within the original data, thereby effectively reducing the dimensionality [54,55]. Discriminant analysis (DA) methods are widely applied to model experimental data blocks and for facilitating classifications [56,57]. Partial least squares discriminant analysis (PLS-DA) simultaneously provides the aforementioned dimensional reduction and discrimination based on the relation of instrumental responses to the binary-coded block of sample classes [58]. If needed, more advanced nonlinear algorithms derived from machine learning and artificial intelligence including but not limited to supported vector machines(SVMs), etc. may be used. SVMs handle nonlinear classification tasks using kernel functions to transform the original feature space into a higher-dimensional one making data points potentially linearly separable [58–60]. However, during the present study it was determined that PCA as an unsupervised method for dimensionality reduction and feature extraction, and PLS-DA as a supervised method explicitly designed for classification tasks were sufficient [61,62].

The main aim of the present study was to establish real-time methods for understanding the molecular behavior , the chemical development and significant differences in biofilm matrix composition during all stages of the biofilm growth process for six individual *L. parabuchneri* strains isolated from different types of cheese and with different capacity for biofilm production via advanced in-situ IR-ATR spectroscopy. Understanding the matrix composition during biofilm formation and the factors influencing the adhesion properties are crucial for a the fundamental understanding of biofilm lifecycles facilitating strategies for preventing biofilm formation. Discriminatory statistical models enabled close to real-time classification of *L. parabuchneri* isolated strains based on their IR-ATR signatures, which are essentially ‘molecular fingerprints’ based on the biochemical composition and biofilm formation ability. Combining IR-ATR spectroscopy with luminescence-based oxygen sensors provides unprecedented insight onmetabolic changes further substantiating the differences observed during multivariate classification.

2. **Materials and methods**

2.1. Bacterial strains and culture conditions

L. parabuchneri IPLA 11151, 11150, 11129, 11125, 11122 and 11117 strains isolated from different types of cheese were provided by the Dairy Research Institute (IPLA-CSIC, Asturias, Spain) from a collection of strains with different biofilm production capacity [22]. All *Lentilactobacillus* selected strains used were routinely cultured in MRS broth (Oxoid, Basingstoke, Hampshire, UK) and were incubated at 37°C under anaerobic conditions on a workstation (Forming gas - 95% N_2+ 5% H_2) and then incubated for 24 h. The strains were kept at -80°C in MRS with 10% (w/v) sterile glycerol.

2.2. Quantification of biofilm production on polystyrene

The biofilm-forming ability of six selected strains on polystyrene surfaces was studied. Although this ability had been previously assessed [20,22], this study was included to ensure accurate comparison and categorization of results. The biomass of biofilm formation was analyzed using the crystal violet staining (CV) assay described by Kubota et al. [63] with some modifications. For this purpose, overnight cultures (in MRS medium) of each strain were diluted to approximately 10^6 CFU/mL (colony forming units per milliliter) and inoculated (200 µl) in a round-bottomed polystyrene 96-well microtiter plate (Thermo Fischer Scientific). The negative control was neat sterile medium present in 8 wells of the same plate. The microtiter plate was incubated at 37°C, and after 48 h the supernatant was removed, and the wells were washed twice with PBS buffer to remove non-adherent cells. Then, the plate was dried at room temperature in a laminar flow cabinet (CRUMAir 9005-FL) for 30 min and the biofilms present were stained with 250 µl of CV (0.5% w/v) in distilled water for 30 min at room temperature. After that, the non-bound dye was removed followed by 3-times washing with 300 µl of distilled water; the bound dye was extracted with acetic acid (33%) and the absorbance was analyzed at 595 nm using a Benchmark Plus microplate spectrophotometer (BioRad).

The capability of biofilm formation was expressed using cut-off values following the procedure defined by Extremina et al. [64]. The average optical density (ODc) and standard deviation (SD) of three replicates were calculated for each strain. To distinguish between biofilm producers and non-producers, the cut-off values for defining different categories of biofilm producers were established as the mean ODnc value, which represents the mean optical density (OD) of the non-inoculated media

(negative control). ODc is calculated as the mean of ODnc plus three times the standard deviation (SD) of the negative control ODnc: ODc = ODnc+(3*SD). The strains were then classified into the following categories: ODc < OD ≤ 2 x ODc = weak biofilm producer; 2 x ODc < OD ≤ 4 x ODc = moderate biofilm producer; OD > 4 x ODc = strong biofilm producer [18,41].

2.3. Analysis by CLSM microscopy

For the six histamine-producing *L. parabuchneri* strains, confocal laser scanning microscopy (CLSM) analyses were performed as described by Fernandez et al. [65] with some modifications. Biofilms were grown on eight-well polystyrene plates (ibidi, USA). Each well was inoculated with overnight cultures diluted to approximately a cell suspension containing 10^6 CFU/mL in MRS broth. After 48 h of biofilm growing at 37°C, to examine the attachment and ability to produce biofilm of each strain, the planktonic phase was removed, and wells were rinsed with sterilized PBS buffer to removed non-adherent cells. The preformed biofilms were stained with SYTO 9, which dyes live cells, and propidium iodide, which dyes dead cells and extracellular DNA (eDNA), using the LIVE/DEAD BacLight kit (Invitrogen AG, Basel, Switzerland). Finally, all *L. parabuchneri* samples were studied under CLSM (DMi8; Leica Microsystems) with a 63xoil objective.

2.4. Planktonic state of L. parabuchneri strains

For the first IR-ATR experiment, 24 h overnights of the six studied strains were grown at standard conditions (37°C) in 10 mL of MRS broth. The tubes were centrifugated, the supernatant was removed, the pellet was resuspended in fresh MRS medium, and the optical density was measured at 600 nm using a UV-VIS spectrophotometer (Specord S600; Analytik Jena AG, Germany). The bacterial concentration of the sub-cultures reached an OD_{600} of approximately 0.8-1.5 depending on the overnight culture strain. The measurements were collected using an FT-IR spectrometer (Alpha I; Bruker Optik GmBH, Ettlingen, Germany) combined with a single-bounce diamond ATR assembly (Platinum ATR; Bruker Optik GmBH, Ettlingen, Germany). After a background spectrum of dried MRS medium was recorded, the spectra of each planktonic *L. parabuchneri* strain were taken. The IR spectrum of the planktonic *L. parabuchneri* strain was collected during 60 min of the drying process under environmental conditions. The IR-spectra obtained from planktonic cells of different *L. parabuchneri*

strains were analyzed, and important molecular signatures including amides type I and II, proteins, lactic acid, phospholipids, nucleic acids, and polysaccharides were investigated. Additionally, the influence of the nutrient source was investigated comparing metabolized media and fresh MRS media. Metabolized media are a nutritive source that undergoes biochemical changes via nutrient-deprived cells in the substratum or via cell lysis induced by nutrient starvation. MRS metabolized medium used for planktonic analysis was obtained from the remaining portion after centrifugation of the overnight culture, whereby the pellet was collected and resuspended in fresh nutritive media. The supernatant (i.e., metabolized MRS) was recorded as a dry background prior to recording IR spectra of planktonic cells.

2.5. IR-ATR flow system for biofilm analysis

The IR-ATR flow cell assembly used for biofilm monitoring studies combined a multireflection ATR assembly with a Tensor II Fourier transform infrared (FT-IR) spectrometer (Bruker Optics, Ettlingen, Germany) equipped with a DTGS detector and a six-reflection ATR ZnSe crystal (dimensions of approximately 72 mm × 10 mm × 6 mm at the top side) [66]. A custom-made flow cell was designed and fabricated by the machinery shop at Ulm University from polyether ether ketone (PEEK) including mounts for optical fibers facilitating contact-less oxygen measurements via fluorescent dyes quenched in the presence of molecular oxygen immobilized in between the IR-active hot spots at the ATR crystal surface (i.e., within the biofilm) [41,66]. The cell volume was approx. 1.75 mL. The assembly was equipped with a peristaltic pump (Watson Marlow, Alitea, Sweden) and silicon tubing connected via Luer-lock connectors. During biofilm monitoring IR-spectra were recorded in the range 4000–400 cm^{-1} at a spectral resolution of 2 cm^{-1} with 100 scans averaged for each spectrum. The flow cell is mounted into the sample multi reflection compartment chamber of infrared spectrometer. Prior to in situ measurements, the ATR waveguide surface was cleaned in a UV-light chamber. The flow cell assembly was then cleaned with ethanol and rinsed with sterilized water for 30 min. The spectrum of deionized water was used as the background spectrum prior to the introduction of MRS into the flow system. The sterilized MRS medium was flushed through the system for 2 h with a delay of 5 min between measurements at a flow rate of 0.7 mL/min, which resulted in a residence time of 150 s within the flow-cell. After recording the MRS conditioning film background to minimize the water contributions to the overall spectra, the MRS medium was replaced

by bacterial solution (OD_{600}=0.8-1.5) for 2 h at a flow rate of 0.78 mL/min with 10 min delay between measurements. This period is optimized as the time required to initiate the early attachment of *L. parabuchneri* biofilms to the ZnSe waveguide [41,66]. After 2 h of initial attachment, the sterile MRS medium was pumped again through the IR-ATR flow-cell assembly for 24-48 h at a flow rate of 0.5 mL/min. Fresh MRS medium washed out free cells that were not attached and delivered nutrients to microorganisms. Recording of spectra and all spectral calculations were performed using the OPUS 8.1 software package (Bruker Optics, Ettlingen, Germany). Baseline correction was performed for all obtained IR spectra. For further processing, the IR spectra were analyzed using the EssentialFTIR software package (Operant LLC, Madison, USA) and the OriginLab 2019b software package (OriginLab Corp., Massachusetts, USA). The IR-ATR long-term biofilm monitoring experiments were performed at 22 ± 1°C in an air-conditioned laboratory.

2.6. Fluorescence sensing for tracing metabolic oxygen concentrations

To investigate the location of evanescent field hot spots at the surface of the ATR element, discrete PSCB polymer spots were deposited, and their absorbance signature facilitated visualizing the active sensing regions. The ZnSe crystal was mounted in a customized horizontal flow cell with the top plate removed facilitating polymer deposition at a periodic distance of 3 mm between individual spots. By plotting the integrated peak values (IPVs) of the eight evaluated spectral regions at marked positions at the ATR crystal surface vs. distance from the in-coupling facet of the crystal, areas for the immobilization of fluorescent dyes at the IR waveguide surface in between the active hot spots (i.e., without contributing IR signatures by the dye) were determined [67]. Thus deposited oxygen sensor spots at the ZnSe waveguide surface were interrogated via optical fibers mounted into the lid of the IR-ATR flow-cell assembly for non-invasively monitoring changes in oxygen concentration within the biofilm [67]. The optical oxygen sensors were obtained from PyroScience GmbH (Aachen, Germany); preliminary tests ensured that the oxygen sensor readings are independent of the flow rate and provided excellent long-term stability, which is crucial for long-term biofilm experiments [66].

2.7. Multivariate data analysis

PLS Toolbox 8.7 (PLS Toolbox 6.5, Eigenvector Research Inc., Wenatchee, WA, USA) was used to generate statistical results in combination with the MATLAB software R2018b (The MathWorks, Inc., Natick, MA, USA). Principal component analysis (PCA) and partial least squares linear discriminant analysis (PLS-DA) were applied to the spectra of each dataset obtained for long-term monitoring of strains of *L. parabuchneri* biofilm formation. The selected statistical models were suitable for the discrimination of *L. parabuchneri* strains based on IR spectral signatures. The spectral region of 1700–900 cm^{-1} was determined as the most suitable spectral identification range for discriminating *L. parabuchneri* species. Cross-validation was performed to determine the optimal number of principal components (PC) or latent variables (LV). Commonly, a range of data pre-processing steps are used to exclude variability that is not representative of the target analytes from individual biofilm samples and for numerically preparing IR data for modelling. In all models described herein, weighted least squares automatic baseline removal (AWLS, order = 2) has been applied for obtaining mean-centered spectral data to improve the signal-to-noise ratio (SNR) and to adjust the baseline. Besides mean centering, 1st derivative Savitzky-Golay filtering (filter parameters: order = 2, window: 9 pt.) was used increasing the robustness of the multivariate classification model [58,68]. For establishing the classifications and predictions models, 120 IR spectra (i.e., 20 for each isolated-strain) were considered capturing the molecular contribution of *L. parabuchneri* planktonic cells during the drying process for a period of up to 1 h. During 48 h of real-time monitoring of biofilm formation, 288 IR spectra were collected (i.e., 48 IR spectra for each of the studied strains; 1 spectrum per hour). The chemometric models were based on selecting 2-3 latent variables (LVs) capturing the majority of variance within the data sets using the root mean square error of calibration (RMSEC) and the root mean square error of cross-validation (RMSECV) for selecting the optimum number.

3. Results and discussion

3.1. Biofilm formation on polystyrene

The six selected strains of *L. parabuchneri* isolated from different type of cheese were analyzed for their ability to produce biofilm on polystyrene plates under tested conditions. For all studied strains, the biofilm biomass was maximal after 48 h at 37°C (Fig. 1). As was described before, the mean ± SD of the absorbance of the negative

control was used to calculate the cut-off values and the biofilm production strength for each strain was analyzed. The bar graph in Fig. 1 for the OD (mean ± SD) confirmed that *L. parabuchneri* IPLA11117 and 11122 are weak biofilm producers (less than ODc x 2), while *L. parabuchneri* IPLA11125 and 11129 acted as moderate producers and *L. parabuchneri* IPLA11150 and 11151 are stronger biofilm producers with an optical density (OD) higher than ODc x 4.

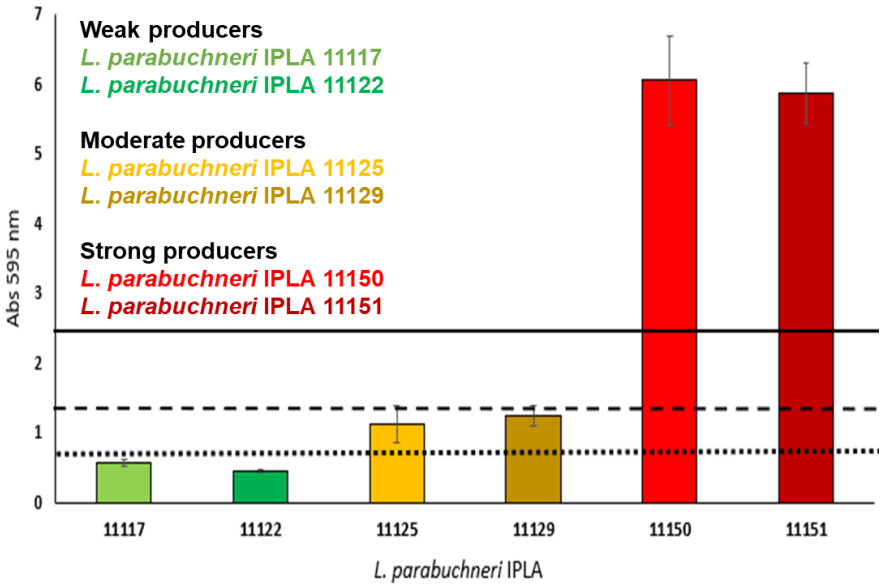

Fig. 1. Biofilm-forming ability of the histamine-producing *L. parabuchneri* on polystyrene. The strains were incubated for 48h at 37°C in MRS medium. Data represent the mean ± SD (error bars) of three experiments. (·····) Cut-off line (ODc); (- -) 2 x ODc; (—) 4 x ODc.

Biofilm growth is governed by a number of physical, chemical and biological parameters including energy, surface functionality, bacterial orientation, temperature, pressure, etc., which are local environmental variables contributing to bacterial adhesion. The secretion of polysaccharide molecules, intercellular adhesion polymers and the presence of divalent cations also play an important role for bonding in between cells. The presence and expression of relevant genes (i.e., genes related to surface proteins or the excretion of products such as polysaccharides, etc.) has also been associated with cellular adhesion. The genomic diversity within *L. parabuchneri* specie has been shown by Gumustop et al. [69] suggesting that genomic changes may also contribute differences in adhesion in between the studied strains, which is a subject of future studies.

3.2. CLSM characterization

CLSM analyses were performed to observe the attachment and biofilm formation of six selected *L. parabuchneri* strains at polystyrene surfaces as a complementary method to validate the in situ IR-ATR spectroscopy technique, which is extensively used for the discrimination and classification of different bacterial biofilms [70]. To do so, biofilms were grown at standard conditions, stained with the LIVE/DEAD Baclight kit and studied by CLSM.

The CLSM images revealed differences in biofilm production abilities at the polystyrene surface for each *L. parabuchneri* strain. The low attachment of bacterial cell and minimal biofilm structure for *L. parabuchneri* IPLA11117 and 11122 (Fig. 2(a, b), respectively) indicated weak biofilm producers. On the contrary, *L. parabuchneri* IPLA11125 and 11129 (Fig. 2 c and d) presented increased cell attachment and organized biofilms that almost cover the entire surface suggesting a moderate ability to produce biofilms. Finally, well-organized biofilms that completely covered the surface with layers of extracellular matrix were evident protecting the structure for *L. parabuchneri* IPLA11150 (Fig. 2(e)) and 11151 (Fig. 2(f)) considered strong biofilm producers.

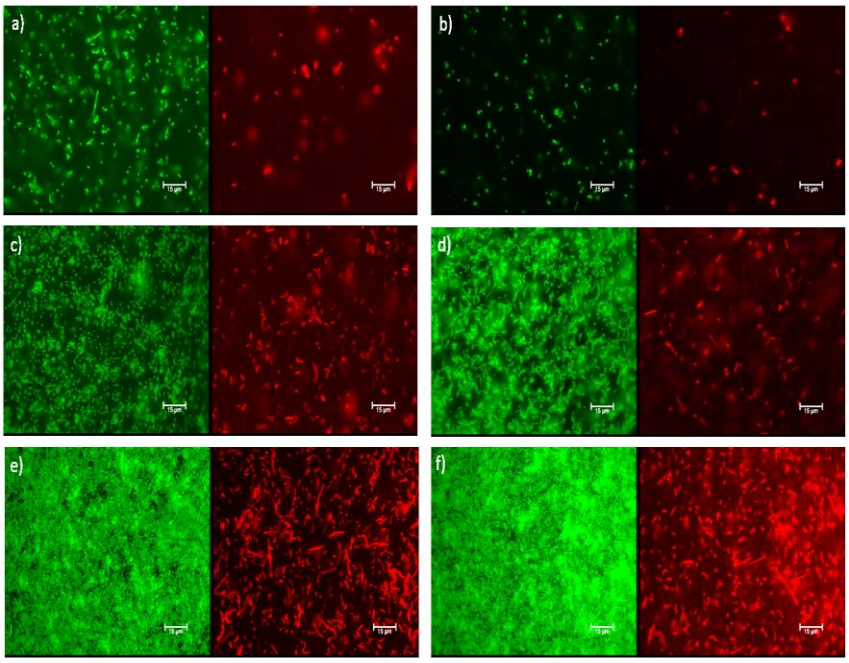

Fig. 2. Confocal laser scanning micrographs of six strains of *L. parabuchneri* biofilms grown on polystyrene**.** Biofilms were grown for 48h at 37°C, after which the planktonic phase was removed, and samples were stained with the LIVE/DEAD BacLight staining kit. Intact cells stained green and dead cells stained red. The images correspond to stained biofilm samples of *L. parabuchneri* weak biofilm producers IPLA 11117 (a) and 11122 (b), *L. parabuchneri* IPLA

11125 (c), and 11129 (d) moderate producers, and IPLA 11150 (e) and 11151 (f) strong biofilm producers.

The CLSM results regarding the observation of biomass formation by the analyzed strains are in line with those obtained with Cristal Violet technique and with the classification previously done in a wider study of the biofilm forming ability of *L. parabuchneri* strains [22]. The classification by CV method regarding the biofilm producing ability of *L. parabuchneri* strains into weak, moderate, and strong producers is similar to the present observations by CLSM.

3.3. Monitoring the oxygen concentration during biofilm development

Synchronous oxygen measurements via sensors integrated into the IR-ATR flow system facilitated the evaluation of nascent *L. parabuchneri* biofilms after initial microbial attachment during the introduction of sterile MRS media. To understand biofilm composition, in addition to IR spectra it is essential to assess living biomass and vital parameters such as oxygen. Infrared spectroscopy combined with fluorescence-based sensing schemes has been used to simultaneously trace molecular changes and metabolic oxygen [41].. As described previously, *L. parabuchneri* is a gram-positive bacterium proliferating in anaerobic environments [71,72]. The primary consideration was monitoring oxygen level changes during the entire flow experiment for 48 h to ensure that microaerophilic conditions were maintained inside the IR-ATR flow-cell (Fig. 3(a); oxygen decrease during biofilm growth of cheese-isolated *L. parabuchneri* strains during 48 h of monitoring is exemplarily shown). Oxygen levels may be subject to significant variations based on the biofilm thickness (see oxygen concentration gradient during 48 h of *L. parabuchneri* biofilm formation in Fig. 3 b). Reduced values in oxygen concentration from 4 to 0.9 mg/L were observed during controlled biofilm growth indicating low metabolic oxygen levels related to the reproduction of microorganisms with an associated increase in biofilm thickness [66,73]. Evidently, the initial phase of *L. parabuchneri* attachment at the waveguide surface promotes microaerophilic conditions via the consumption of available oxygen inside the flow cell. The EPS layer increases in thickness and the diffusion of oxygen and nutrients to the attached film is limited [74]. The attachment of bacterial cells also occurs at the immobilized fluorescence sensor spots providing real-time information on the oxygen concentration at the surface. Temporal changes of oxygen consumption (see Fig. 3 a)

indicate low oxygen availability during biofilm formation processes almost in real-time. This phenomenon was noted even for weak biofilm producer strains such as *L. parabuchneri* IPLA 11122 and 11117, whereby the reduction in oxygen levels appears proportional to the thickness of the associated biofilm indicating a microaerophilic setting inside the IR-ATR flow-cell that effectively promotes bacterial growth.

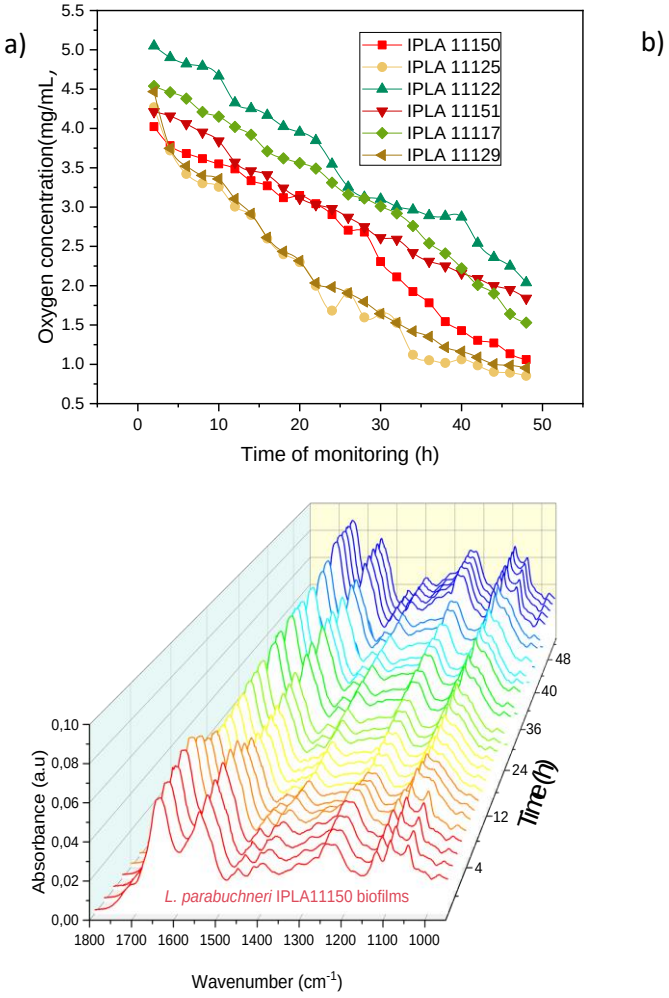

Fig. 3. a) Concentration profiles for oxygen during bacterial attachment of six cheese-isolated *L. parabuchneri* IPLA strains onto the ZnSe surface and **b)** Associated exemplary IR-ATR spectra recorded during real-time monitoring of *L. parabuchneri* IPLA11150 biofilms within a period 48 h.

The decrease in the dissolved oxygen concentration before and after inoculation determined using a fluorescence microsensor indicated that even small levels of oxygen were present in the anaerobic environment of *L. parabuchneri* biofilms. The maximal oxygen values obtained via the optical microsensor for normal *L. parabuchneri* cultivation indicate a gradual decrease in metabolic oxygen levels from 5 mg L^{-1}[66]. The influence of microaerophilic conditions on biofilm formation was investigated by

tracking the levels of oxygen inside the IR-ATR flow-system via additional oxygen microsensors. Resulting, the analyzed strains exhibited comparable oxygen levels and bacterial respiration patterns confirming a consistent behavior similar to the *L. parabuchneri* DSMZ 5987 strain [66]. Metabolic regulation by terminal oxidases in a microaerobic environment correlates with low cellular oxygen consumption, which occurs during the first stage of biofilm formation. The reduced values of oxygen close to the ATR waveguide surface facilitated understanding on the facultative metabolism of *L. parabuchneri* and its relation to cellular behavior and proliferation ability [75]. The accumulation of biogenic amines in cheese is primarily influenced by factors that favor the development of biogenic amine producing bacteria in fermented food including low oxygen content. Hence, the growth of species such as histamine-producing *L. parabuchneri*, which are traditionally classified as oxygen-tolerant anaerobes is facilitated [16,66]. At the surface of the ZnSe ATR waveguide, the initial attachment of the biofilm was evidenced by a continuous increase in bands associated with relevant biofilm constituents (Fig. 3 b). Notably, signatures of amides (I and II), nucleic acids, and EPS were substantially increasing with time. The consistently modest changes in amide III signatures and nucleic acid levels suggest that vibrational signatures of membrane proteins, fatty acid chains, and ribosomes - which are essential components of bacterial cells - are predominantly responsible for these spectral contributions. . The bottom layer of biofilm mass is accumulated in close proximity to the waveguide surface encased within rich extracellular polymeric components owing to the continuous maturation process. The horizontal spread of biofilms with a limited oxygen gradient required for microaerophilic conditions is necessary for facilitating the growth of facultative *L. parabuchneri*. The molecular changes related to the biochemical mechanisms of *L. parabuchneri* histamine-producer strains are further discussed below.

3.4. Planktonic cells analyzed via IR-ATR spectroscopy

Prior to biofilm studies planktonic cells of each *L. parabuchneri* strain were investigated by IR-ATR spectroscopy. The bacterial strains were harvested during the stationary growth phase from the same overnight culture, which was later used for in situ biofilm monitoring. A suspension of *L. parabuchneri* was drop-casted onto a diamond ATR crystal and dried for 15 min at ambient air. The IR spectra were collected during the entire drying process for 1 h. The spectrum of dry *L. parabuchneri* was

recorded every 10 min for a period of 1 h. Prior to recording spectra of planktonic *L. parabuchneri*, the MRS medium was drop-casted onto the diamond ATR crystal, evaporated, and a background spectrum was collected. Exemplary spectra of *L. parabuchneri* planktonic cells analyzed during the drying process for 1 h are shown in Fig. 4 encompassing the spectral features for proteins, amides, lactic acid, nucleic acids, phospholipids, and polysaccharides. These characteristic absorption bands are assigned predominantly to cell walls consisting of a rigid peptidoglycan framework, capsular lipids, and polysaccharides [76].

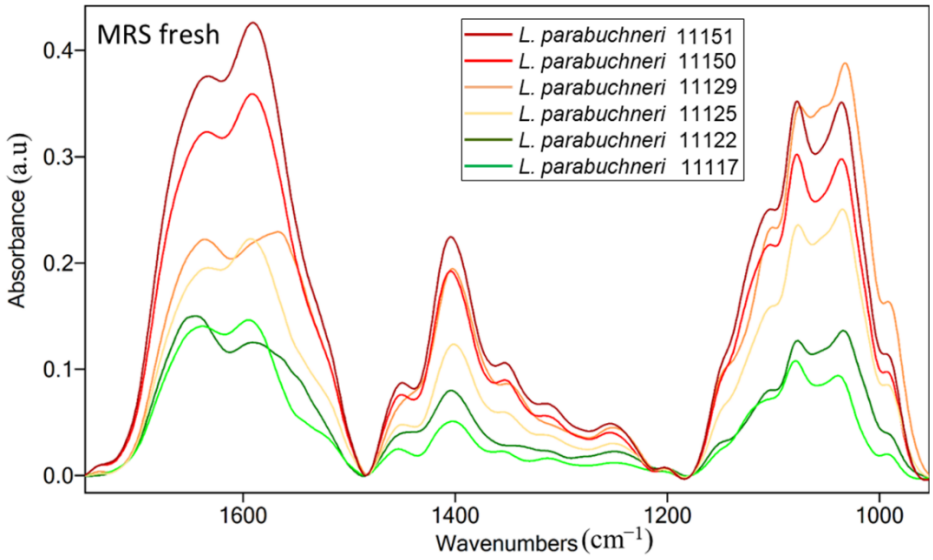

Fig. 4. Exemplary IR-ATR spectra of planktonic *L. parabuchneri* strains in fresh MRS media after solvent evaporation. All spectra were baseline corrected.

The spectral features of the polysaccharides were mainly localized in the region of 1200-900 cm^{-1}. The amide bands indicated the presence of cell proteins [77]. The amide I band is caused by N-H stretching vibrations and vibrations of the peptide linkage (1650-1660 cm^{-1}) [78]. The distinct band at approximately 1540 cm^{-1} is attributed to the out-of-phase combination vibrations of the NH in-plane bending and CN stretching motion. Terminal C-H bending vibrations in fatty acids were observed at 1396/1389 cm^{-1}, whereas P-O-C groups in phospholipids and P-O-P groups in pyrophosphates in the regions 965 and 915 cm^{-1} were only weakly absorbed [41]. The attachment of bacterial cells from different strains at the waveguide surface revealed minute differences in the representative bands (i.e., amide I, amide II, lactate, nucleic acids, amide III and exopolysaccharides (EPSs)) corresponding to strain performance (Fig. 4). According to recent studies [22], the biofilm-forming capacity examination and comparison of 24

histamine-producing *L. parabuchneri* strains of dairy origin showed that *L. parabuchneri* strains were able to form biofilms at polystyrene surfaces, which were already classified based on their biofilm-forming capacity. *L. parabuchneri* IPLA11150 and 11151 were classified as strong producers, IPLA11125 and 11129 as moderate producers and IPLA11117 and 11122 as weak biofilm producers. Indeed, these results have been confirmed with the IR spectroscopic investigations. As expected, the highest absorption intensity was noted for *L. parabuchneri* IPLA 11151 and 11150, followed by IPLA 11129, 11125 and the lowest intensity for *L. parabuchneri* IPLA 11122 and 11117. The EPS content of planktonic *L. parabuchneri* IPLA 11129 was as high as that of IPLA 11151 and 11150 strains. The comparison of the planktonic cell spectra recorded with a fresh MRS background versus a metabolized MRS background revealed slight changes in the molecular composition of bacterial cells. The highest EPS content was observed for *L. parabuchneri* IPLA 11150 and IPLA 11129, whereas the protein content derived from changes in amide I and amide II bands were derived for IPLA 11151 and IPLA 11125 bacterial strains. The IR signature for nucleic acids, amide III, and lactate information revealed a synchronized gradual increase following the order of strong, moderate, and weak bacterial strains. (see Fig. S1 in SM). The IR signature of the respective *L. parabuchneri* strains recorded from the background of metabolized MRS media (rest after centrifugation of overnight culture) remained similar to that recorded using fresh MRS broth as a background prior to deposition of the bacterial suspension on top of the ATR crystal. During the drying process, spectra were recorded every 5 min providing spectral information in regions comprising the major bands for microbial identification. The results show that the IR measurements of six *L. parabuchneri* strains had different band intensities and peak locations. The IR patterns characteristic for planktonic cells indicate the differentiation via composition of cell wall membrane, proteins and carbohydrates content.

3.5. Real-time IR-ATR monitoring of L. parabuchneri biofilms

Long-term monitoring of biofilm formation at flow conditions reveals cyclical fluctuations in the biofilm lifecycle via IR spectroscopy in molecular detail [41]. In the present study, 48 h real-time monitoring of *L. parabuchneri* biofilms was performed comparing the differences in relative intensities of characteristic IR features. The spectral range of 1700-900 cm^{-1} was determined as most informative for the

identification of *L. parabuchneri* strain biofilms (see Table 1). The infrared spectroscopic response reveals distinguishable stages of biofilm formation evaluated within three distinct infrared spectral regions for the analysis of *L. parabuchneri* biofilms (Fig. 5(a, b)).

First region: amide I and amide II in the range of 1700-1550 cm^{-1}. The most prominent changes in the protein regime are related to the amide content [39,79]. The amide I band at 1635 cm^{-1} reveals spectral information on $_{\nu}$C=O stretching vibrations (70–85%) and $_{\nu}$C–N vibrations (10–20%) [79]. These bands are related to peptides and protein chains within the bacterial cell membrane [40]. In previous studies, this band was also attributed to α-helices as constituents of secondary protein structures [78,80].

The amide II band at 1551 cm^{-1} is attributed to the combination of $_{\delta}$N−H vibrations and υC-N stretching vibrations from peptide groups [41,81]. The kinetics of *L. parabuchneri* attached to the waveguide surface were predominantly monitored based on the increase of the amide II band intensities during bacterial growth. This band yields biomass quantity [82] due to the fact that proteins are reported as the most abundant molecular species in microbial cells [83]. The obtained IR-ATR spectra showed that the most pronounced amide I and amide II bands were characteristic for the biofilm-producing strains *L. parabuchneri* IPLA11151 and 11150, which are strong biofilm-producers. These bands are indicative of the highly proteinaceous nature of the biofilm matrix with an increase in intensity after 48 h of biofilm formation. Previous studies of the extracellular matrix composition using enzymatic treatment in *L. parabuchneri* strong biofilm-producer strains showed that the biofilms were mainly composed of proteinaceous material [22], thus confirming the results obtained herein via IR-ATR spectroscopy. Other *L. parabuchneri* strains isolated from grated Emmental cheese [18] have similar amide I bands assigned to α-helical structures and β-pleated sheet structures [84]. The lowest content was observed for *L. parabuchneri* IPLA 11125, 11122 and 11117, as expected. The metabolic activity of *L. parabuchneri* strains leads to the accumulation of histamine [85,86]. Histamine is a heterocyclic diamine that exhibits amine N-H stretching and bending vibrations of the N-H bond within the amine group (NH_2), and amine C-N stretching associated with the stretching vibration of the C-N bond within the amine group (NH_2) [87]. The pronounced bending vibration of the NH_2 side chain groups at 1626 cm^{-1} of *L. parabuchneri* inoculated in MRS medium are related to histamine production. These vibrational bands contribute to amide I and II signals indicating biogenic amine species within the biofilms of the *L. parabuchneri*

strains due to the fact that histamine is a BA produced by decarboxylation of the amino acid histidine [88].

Second region: lactic acid, amide III and nucleic acid in the range of 1460-1220 cm^{-1}. In correlation with biofilm growth, the acidity of the microbial media inside the flow cell increases over time, as does lactic acid production in the continuous process of *L. parabuchneri* biofilm formation [89]. *L. parabuchneri* IPLA strains isolated from different types of cheeses have the ability to produce biofilms by adhering to different substrates and interfaces and increase their survival under acidic stress [90]. The appearance of bands at 1456 and 1399 cm^{-1} are indicatory of lactic acid production [91]. The obtained spectral information relates to the bending vibrations (scissoring) of the acyl CH_2 groups in lipids [92], and the band at 1399 cm^{-1} arises from the COO^- symmetric stretching of amino acid side chains and fatty acids [41,81]. The conversion of lactate into lactic acid was higher for *L. parabuchneri* IPLA 11125, 11150, and 11151 (Fig. 5(a, b)). The slowly increasing signals at 1257 and 1237 cm^{-1} are indicative of high amounts of nucleic acids [93]. The biomass of *L. parabuchneri* IPLA11151 promotes that the extracellular DNA (eDNA) present within the biofilm matrix yields an increased amount of nucleic acids. Enzymatic treatment studies previously conducted by Sarquis et al., revealed the presence of extracellular DNA (eDNA) in strain 11151[22]. These finding are consistent with the results obtained herein using IR-ATR analysis (see Fig. 5(a)), whereby the nucleic acid peak increased for IPLA 11151 vs. other strains after 24 h of biofilm formation. These finding are consistent with the results obtained herein using IR-ATR analysis (see Fig. 5(a)), whereby the nucleic acid peak increased for 11151 vs. other strains after 24 h of biofilm formation. The increase in biomass of IPLA11151 yields an increased amount of nucleic acids in the form of extracellular DNA (eDNA). These bands are associated with the PO_2^- asymmetric and symmetric stretching vibrations of phosphodiester bonds in nucleic acids [79,94]. Nucleic acids are also characterized via the >C═O, >C═N, and >C═C< stretching vibrations of the DNA or RNA heterocyclic base structures [84].

Structural differences related to the secondary structure of proteins are better resolved in the spectral range 1350–1200 cm^{-1}, which is characteristic for amide III bands resulting from a combination of in-plane $_{\delta}$N−H and $_{\nu}$C−N vibrational modes that are highly sensitive to secondary folding structures [41].The advantage of evaluating the peak at 1216 cm^{-1} is that there is no spectral interference by water. Hence, even though the signal of the amide III bands is ∼5-10-fold less intense than that of the amide I bands,

the amide III region is still of substantial interest for evaluating the protein secondary structure. A higher intensity of the amide III band was observed for *L. parabuchneri* IPLA 11151 strain after 24 h of biofilm formation, while for 48 h old biofilms nearly the same amide III intensity was noticed for *L. parabuchneri* IPLA 11125 strain (Fig. 5(b) and Fig. 6(d)).

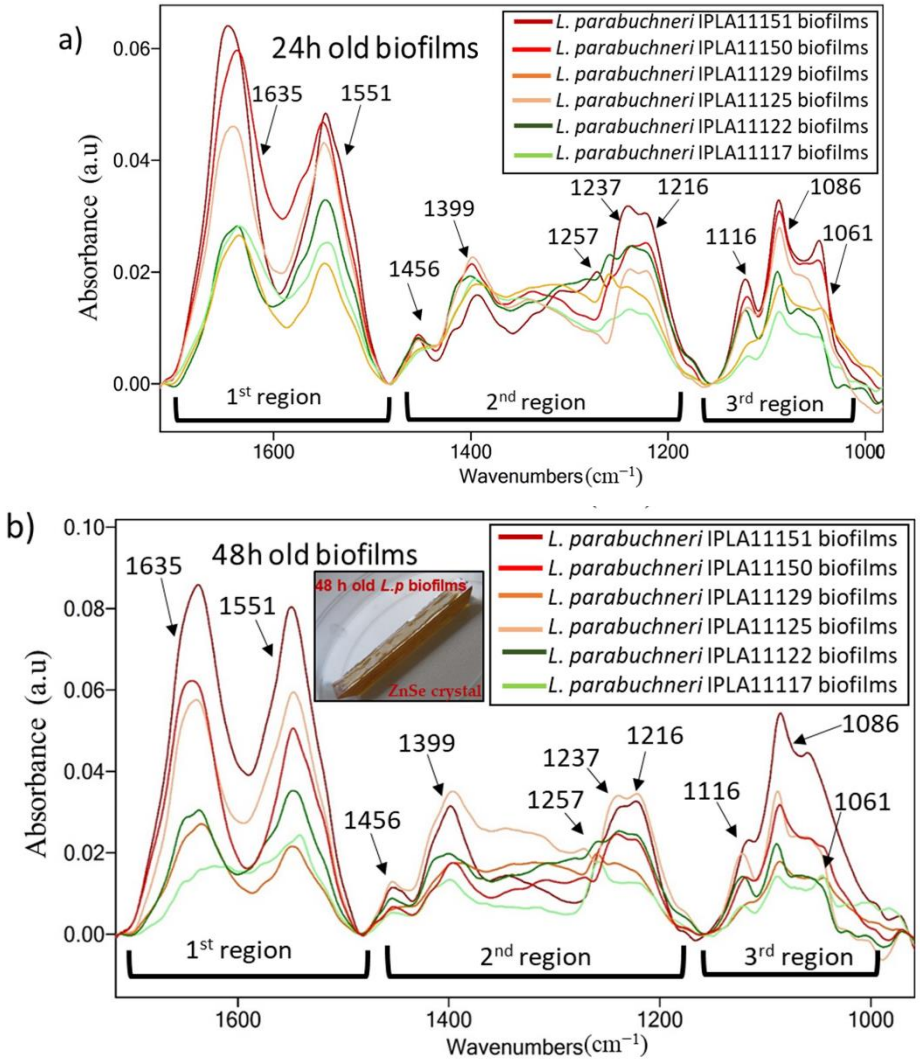

Fig. 5. IR-ATR spectra of *L. parabuchneri* IPLA strains after **a)** 24 h of biofilm formation and **b)** 48 h of biofilm formation.

Third region: EPS, phosphate group and carbohydrate in the range 1200-976 cm^{-1}. The bands at 1116 and 1086 cm^{-1} include a combination of C–O, C–C, C–O–C, P–O–C, and other functional groups [95]. The main cellular components related to the assigned spectral features are membranes, nucleotides, ribosomes, storage inclusions and peptidoglycan. The bacterial composition evaluated via IR-ATR shows the correlation with changes in the physiological state of *L. parabuchneri* analyzed during the growth of sessile bacteria and sustained by extracellular polymeric substances produced. The IR

band at and 1061 cm^{-1} is related to the C-O-C symmetric stretching of polysaccharides [83] as a peak alteration of C−O stretching coupled with C−O bending of the C−OH of carbohydrates (including glucose, fructose, glycogen, etc.) [79]. In the case of microbial biofilms, this band is predominantly considered a so-called 'biofilm indicator' revealing the presence of EPSs as main constituents of the biofilm extracellular matrix [41].The peak at 968-970 cm^{-1} reveals spectral information on C−C vibrations of the DNA backbone (C−O and C−C stretching (υ_sC−O, υ_sC−C)) related to phosphodiesters and ribose [79,84]. The IR spectra shown in Fig, 4. were obtained at the same time increments for each analyzed strain. The biofilm formation ability is a key factor characterized by changes indicated via biomass markers including proteins, lipids, nucleic acids, and extracellular polymeric matrices responsible for the accumulation of sessile cells at the waveguide surface [96]. *L. parabuchneri* moderate and weak biofilm producers resulted in fewer structural changes vs. strong biofilm producing strains. The highest EPS content after 48 h of biofilm formation was observed for *L. parabuchneri* IPLA 11151, which is the strongest biofilm-producing strain in the present study (Fig. 5(b)). It is noteworthy that during previous studies using dispersal of biofilm with $NaIO_4$ conclusive results on the presence of EPSs within the biofilm of *L. parabuchneri* IPLA 11151 were not obtainable [22]. Treatment with $NaIO_4$, only provides information on the nature of EPSs and their modifications [97,98].IR-ATR spectroscopy exclusively detects the in situ presence of EPSs within the biofilm enabling long-term observations of biofilm evolution without the need for any dispersal. The other strong biofilm-producing strain *L. parabuchneri* IPLA 11150 has a polysaccharide composition similar to that of the moderate biofilm-producing strain *L. parabuchneri* IPLA11125 (Fig. 6(b, d)). The weak biofilm producers *L. parabuchneri* IPLA 11122 and IPLA 11117 revealed similar adhesion activity vs. moderate *L. parabuchneri* IPLA 11129 (Fig. 6(c, e, f).

Table 1. Infrared band assignment for *L. parabuchneri* cells and biofilms [41].

Spectral region	**Wavenumber (cm^{-1})**	**Functional group assignment**
1st region: Protein content	**1700-1630**	Stretching C=O coupled with N-H δH2O from membrane lipids and fatty acids (amide I band) [41,99]
	1578-1476	δN-H bending and υC-N stretching in proteins (amide II band) [100]

2nd region: Nucleic acid	**1468-1455**	δCH2, δ_aCH3 bending from membrane lipids [101]
	1465-1293	υ_sCOO- of lactic acid and fatty acid chains [91]
	1350-1200	τCH2, ρCH2, υC-N coupled with δN-H (amide III band) [93]
3rd region: Polysaccharides	**1280-1175**	vibrations of -COOH and C-O-H; Double bond stretching >P=O of general phosphoryl groups and phosphodiester of nucleic acids [93]
	1225	Stretching of P=O in phosphates [41]
	1116	Symmetric stretching υ_sC-OH, υC-O of carbohydrates [41]
	1086	Stretching of P=O in phosphates, $\upsilon_s PO^{2-}$[41,102]
	1038-989	Stretching of P=O of phosphodiester, phosphorylated proteins, phospholipids and polyphosphate products [93]
	976	Symmetric stretching vibration of phosphoryl groups (υC- C, υP-O-P) [101]

Histamine-producing microorganisms in fermented food products associated with *L. parabuchneri* species have strong biofilm-forming capacity [103,104], and are therefore hypothesized to be associated with an organized multilayered structure of extracellular polymeric substances (EPS). The EPS content reflected by the IR spectroscopic information revealed distinct differences among the tested strains. During extended observation periods, *L. parabuchneri* IPLA 11151 showed intense bands at 1138–998 cm^{-1} including IR signals arising from symmetric stretching vibrations resulting from $_\upsilon$C–O ring vibrations, $_\upsilon$C–OH υC–O–C stretching modes, and $_\upsilon$P–O–C groups [41] (Fig. 6(a)).

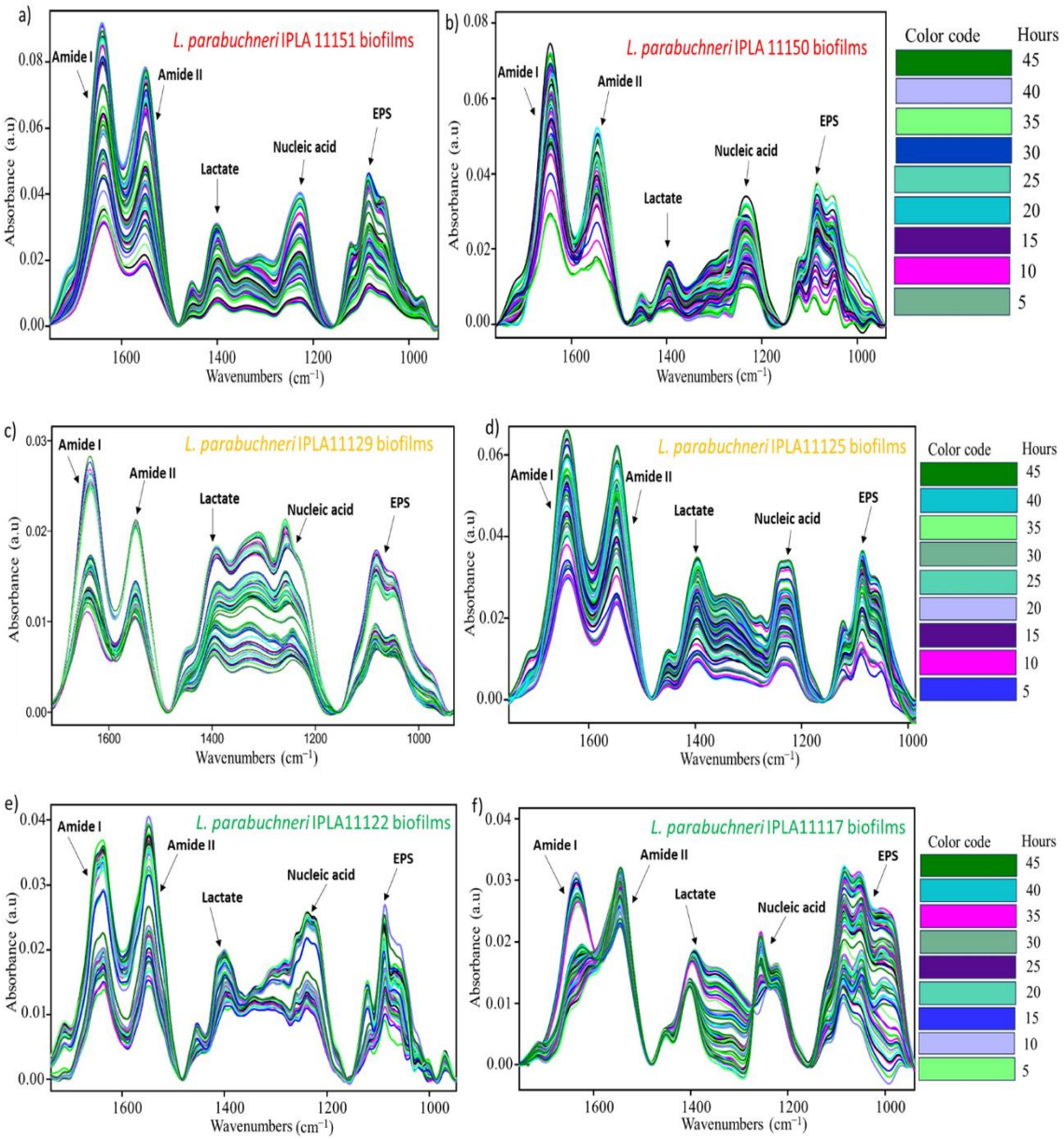

Fig. 6. IR-ATR spectra recorded during formation of 48-hour-old biofilms of six different *L. parabuchneri* strains.: **a)** *L. parabuchneri* IPLA 11151, **b)** *L. parabuchneri* IPLA 11150, **c)** *L. parabuchneri* IPLA 11129, **d)** *L. parabuchneri* IPLA 11125, **e)** *L. parabuchneri* IPLA11122, and **f)** *L. parabuchneri* IPLA 11117. The color code refers to specific infrared spectra acquired at various temporal intervals during the monitoring process within the IR-ATR flow system.

During the initial 48 hours of incubation in the ATR flow-cell assembly, the diverse isolated strains exhibited molecular fluctuations in biofilm content monitored in real-time conditions. The observed adhesion kinetics indicate potential changes in culture conditions, growth phase properties and bacterial species inoculated into the IR-ATR flow system. All vibrational signatures were found to increase after 48 h of growth for *L. parabuchneri* biofilms. The band at 1399 cm^{-1} corresponds to the symmetric stretching of the carboxylate ion, and the band at 1257 cm^{-1} is associated with the

stretching vibrations of C–O–C for esters as functional groups [41]. The corresponding IR signatures verified that polysaccharides with O-acetyl groups were richly present within the *L. parabuchneri* biofilms of each IPLA strain. A weak lactate band at 1455 cm^{-1} (C–H bending of CH_2 group) [96] resulting from lipopolysaccharides in the extracellular matrix was detected. As the biofilm matures, the identical band is increased consistently over time. The resulting IR spectra obtained for the six *L. parabuchneri* IPLA strains indicated a progressive accumulation of polysaccharides in the EPS matrix for all isolates proving strong adherence of the microbial biofilm at the ZnSe waveguide surface (Fig. 6).

3.5.1. Evolution of Amide, LA, NA and EPS bands in early-stage biofilms

Integrated peak areas revealing the periodic changes for amide I and amide II bands (1725.973 - 1490.221 cm^{-1}) are shown in Fig. 7(a). It is evident that as a function of time the fluctuating behavior of the bands related to proteins of the cell wall membrane changes during early-stage biofilm formation. The steady increase for *L. parabuchneri* IPLA 11150 and 11151 after 7 h of biofilm formation was attributed to the rapid attachment of microbial biomass during the first few hours of biofilm monitoring. The moderate biofilm producer *L. parabuchneri* IPLA 11129 showed a constant increase as a function of time. A similar behavior was observed for the weak biofilm producer *L. parabuchneri* IPLA 11117. In the case of the cheese-isolated strain *L. parabuchneri* IPLA 11125, bacterial colonization at the ATR waveguide surface was comparable to the strong biofilm producer IPLA 11150.

A discriminant IR study of lactic acid bacteria (LAB) revealed the vibrational characteristics of individual strains in the optimal growth conditions under continuous nutrient flow [105]. The fermentation process of LAB during the first hours of the biofilm monitoring process is associated with the microbial activity [106]. The evaluation of IR bands related to the levels of lactic acid (LA) and nucleic acid (NA) in the region 1475.290 –1167.242 cm^{-1} supports the hypothesis that during early-stage biofilm formation there is a constant production of nucleic acid and lactate even after 24 h (Fig. 7(b)). A distinct indicator of bacterial attachment and biofilm growth associated with lactic acid production is the temporal change of the peak areas related to lactate production [41]. The level of nucleic acids - as represented by the amide III peak at 1273 cm^{-1} - changed with strain performance. The weak biofilm producer *L. parabuchneri* IPLA 11117 showed an almost constant behavior at an integrated peak

value (IPV) of 1.9±0.03 until 12 h of monitoring, which then increased to 2.3. Similarly, *L. parabuchneri* IPLA 11150 - a strong biofilm producer - exhibited periodic microbial colonization at the waveguide surface, whereas *L. parabuchneri* IPLA 11151 and 11125 exhibited a decrease after 12 h of monitoring. The remaining constant microbial activity is analogous for *L. parabuchneri* IPLA 11129 and 11122 with an IPV of 4.0±0.2.

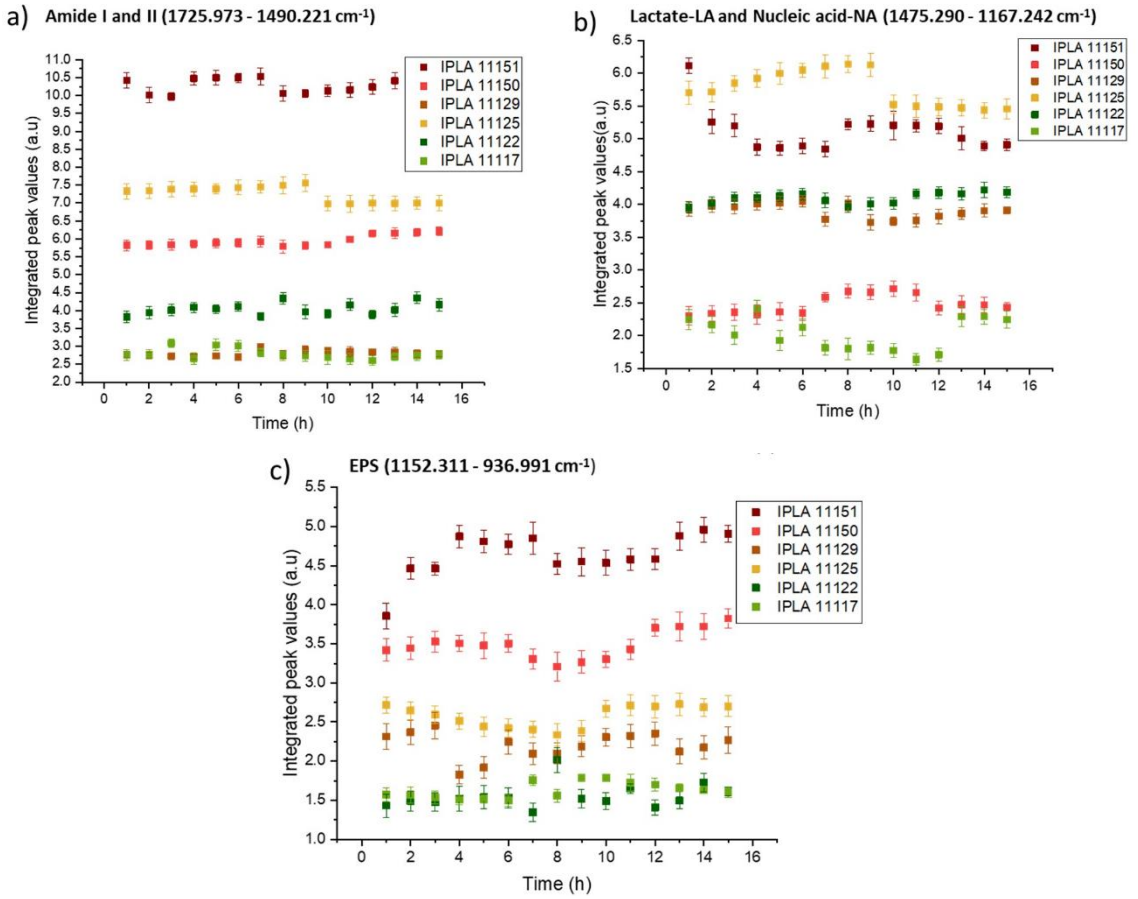

Fig. 7. Integrated peak values (IPVs) of characteristic IRE bands for *L. parabuchneri* early-stage biofilms as a function of time: **a)** Amide I and II (1725.973–1490.221 cm^{-1}), **b)** Lactate and nucleic acid (1475.290–1167.242 cm^{-1}), and **c)** EPS (1152.311–936.991 cm^{-1}). Error bars represent the standard deviation of IPVs extracted from three independent in situ IR-ATR experiments investigating *L. parabuchneri* biofilm formation.

The periodic changes in integrated peak area for EPS (1121-997 cm^{-1}) is a distinct indicator of bacterial biofilm growth and only observed, if *L. parabuchneri* cells are inoculated into the IR-ATR flow assembly and biofilms are formed during the first 4 h [41,107]. EPS levels significantly increased as a function of time corresponding to elevated protein levels, as observed during the analysis of *L. parabuchneri* IPLA 11150 (Fig. 7(c)). The EPS content increased continuously, and the derived bacterial adhesion followed the order of strains according to their biofilm-producing ability. Accordingly,

the highest microbial coverage was hierarchically assigned to strong, moderate and weak biofilm producers with lower IPV values.

During the initial attachment and accumulation of microbial biomass, a steady increase in the IR absorption signal characteristic for bacteria was evident. Simultaneously, as the surface coverage near the penetration depth expanded, bacteria established a strong biofilm structure embedded into an EPS matrix. The development of microbial biofilm is a defining feature of bacterial colonization onto the crystal surface. A sterile culture medium is used to promote monolayer biofilm formation, during which the intensity of all vibrational bands steadily rises over the first few hours of observation. The increase of polysaccharide band indicates not only cell division but also an excessive production of extracellular polysaccharides [44,53]. During the measurements, different adhesion kinetics were observed indicating potential changes in culture conditions, growth phase, strain performance and number of bacteria inoculated into the flow system. Screening of *L. parabuchneri* strains isolated from cheese requires the classification and discrimination of dairy BA-producing bacteria at different levels of microbial contamination. Herein, we show that IR spectroscopy during biofilm formation reveals significant changes of the bacterial metabolism and the associated molecular composition of the biofilm. However, strains with different ability to develop biofilms could not be immediately classified. Hence, multivariate data evaluation techniques were introduced facilitating the classification of biofilm-forming microbial strains including *L. parabuchneri* biofilm-producing species isolated from cheese.

3.6. Classification of biofilms via principal component analysis

IR spectroscopy in combination with multivariate data analysis provides a versatile strategy for the rapid identification of bacterial species. Unsupervised methods aim to explore the inherent patterns and structures within the data without the use of predefined labels or categories. These methods are used to identify clusters or groups of similar data points, discover hidden correlations, reduce the dimensionality of the data and extract meaningful features [106]. Principal component analysis (PCA) is primarily used for reducing the dimensionality of the data by identifying the most important features or variables and determining the directions (i.e., principal components) of maximum variance within the data. PCA is an adaptive data analysis technique commonly used to interpret large datasets such as spectral data. Its application is primarily descriptive rather than deductive, meaning it helps identify patterns and

relationships within the data [54,108]. Molecular changes during biofilm formation are exhibited by spectral variations, which needs to be extracted from the associated complex patterns, here, in the mid-infrared spectral regime. Planktonic *L. parabuchneri* cells were classified from the six selected strains grown at standard conditions while drying on top of the ATR waveguide surface. The sample numbers refer to the number of 120 IR spectra recorded during drying process for 1 hour at environmental conditions; a single-bounce diamond ATR assembly was used for the IR measurements of *L. parabuchneri* planktonic cells. IR-data from measurements of *L. parabuchneri* planktonic cells IPLA 11151, 11150, 11129, 11125, 11122 and 11117 strains analyzed during the drying process for 1 h classifying the microbial strains into six different categories. In Fig. 8, the first principal component is plotted against the samples which are representative of IR spectra recorded over time already allowing to classify the classes of six IPLA *L. parabuchneri* strains. The IR spectral matrix for chemometric evaluation of the sample number refers to the individual planktonic cell spectral data for each of the *L. parabuchneri* isolated strains. The two-dimensional scatter plot of the degree of variation captured by the first principal component and the 120 IR spectra (sample number, 20 spectra for each individual strain for a drying process of 1 h) is shown in Fig. 8. Each data point represents a recorded IR spectrum in the dataset revealing the underlying patterns and relationships between them. The sample/score plot in Fig. 8 correlates with the data of the scores plot based on first two principal components, which account for 92.14% of the total variance in the *L. parabuchneri* biofilms dataset (Fig. S2 in SM). Firstly, IR datasets of *L. parabuchneri* IPA 11150 biofilms were considered for sample classification according to IPLA 11151, 11129, 11125, 11122 and 11117. The total number of samples consists of 288 *L. parabuchneri* biofilms spectra recorded in real-time inside the IR-ATR flow assembly for a monitoring period of 48 hours.

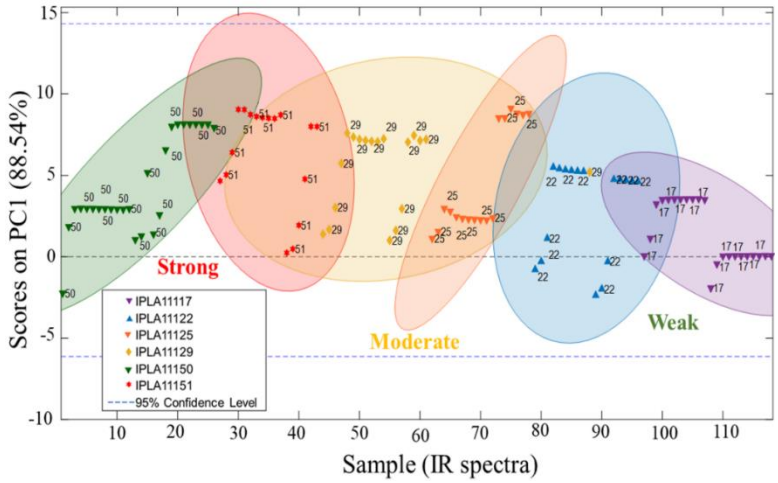

Fig. 8. Samples/scores of IR datasets modeling six strains with different abilities to produce biofilm (strong, moderate and weak producers) of *L. parabuchneri* planktonic cells. The confidence ellipse indicates a 95 % level of certainty.

The obtained classification results confirmed that 98.5% of the total variance in the dataset can be explained with only 2 PCs (88.54% from PC1 and 9.96% from PC2) indicating that the IR-spectra collected from planktonic cells already reveal significant molecular differences between the analyzed strains. The separation may in part be explained by genetic differences and associated DNA expression during the cheese isolation process [18].

PCA was performed using the original IR-ATR spectra of the biofilms over a period of 48 h. Furthermore, effects of biofilm ageing on the IR response was investigated. Hence, another PCA model was built based on a comparison of the spectra associated with matured biofilms formed by three selected categories of *L. parabuchneri* strains (see Fig. S2 in SM) revealing three distinct clusters owing to *L. parabuchneri* strong biofilm producers. Their biofilms apparently attach faster to the waveguide surface and establish a continuous biofilm with time via EPS expression. The explosion in such large datasets by means of principal components (PCs) has been presented as linear combinations of the (centered) original variables. However, the variables (biofilms of respective microbial strains) did not show a strong relationship with each other, especially when it came to early-stage biofilms, as there was no clear distinction between them. Until 36 h of biofilm formation strong, moderate and weak biofilm-producing strains did not reveal significant differences in biofilm composition using simple PCA, which then led to establishing a more refined model based on partial least squares regression (PLS) combined with linear discriminant analysis (LDA).

3.7. Classification of biofilms via partial least squares discriminant analysis

Supervised classification using partial least squares-discriminant analysis (PLS-DA) revealed an unambiguous classification of the investigated bacterial strains based on two latent variables (LVs). PLS-DA aims at determining latent variables that not only explain the maximum variance within the dataset but also maximize the separation between predefined classes or groups. By considering the class labels during the transformation, PLS-DA can effectively distinguish between the variance of different groups (i.e., between-group variation) vs. the variance within each group (i.e., within-group variation). Hence, PLS-DA is ideally suited to solve classification problems aiming at discriminating between different groups based on their features. The variance (%) indicated in Fig. 9 represents the percentage of variance accounted for in LV1 and LV2. Evidently, all strains could be individually classified based on the variance in the IR spectra using only two latent variables. Long-term monitoring (48 h) of *L. parabuchneri* biofilms for the six investigated strains showed that bacterial cultures result in significantly different molecular behavior affected by each strain biofilm-forming ability unambiguously grouping them into distinctly separated classes based only on their IR spectra. In order to achieve a robust model for the biofilm-producing capability of the six cheese-isolated *L. parabuchneri* strains, the sample size consist of maturated biofilms collected after every 1 hour of in situ IR-ATR monitoring. The total number of samples are 288 *L. parabuchneri* biofilms spectra recorded in real-time inside the IR-ATR flow assembly for a 48 h monitoring period. The 48 infrared spectra of biofilms recorded each hour were divided into six groups based on the behavior of individual microbial strains in the training model, consisting of 288 samples. These samples accounted for 24% of the total number of samples (1200 IR spectra recorded for all the monitoring studies), respectively. The two selected LVs cover 94.53% of the total variance with excellent predictive performance. The PLS-LDA model is shown in Fig. 9 and classifies the bacterial strains based on their differences in molecular composition of the biofilms reflected in the associated IR-ATR spectra.

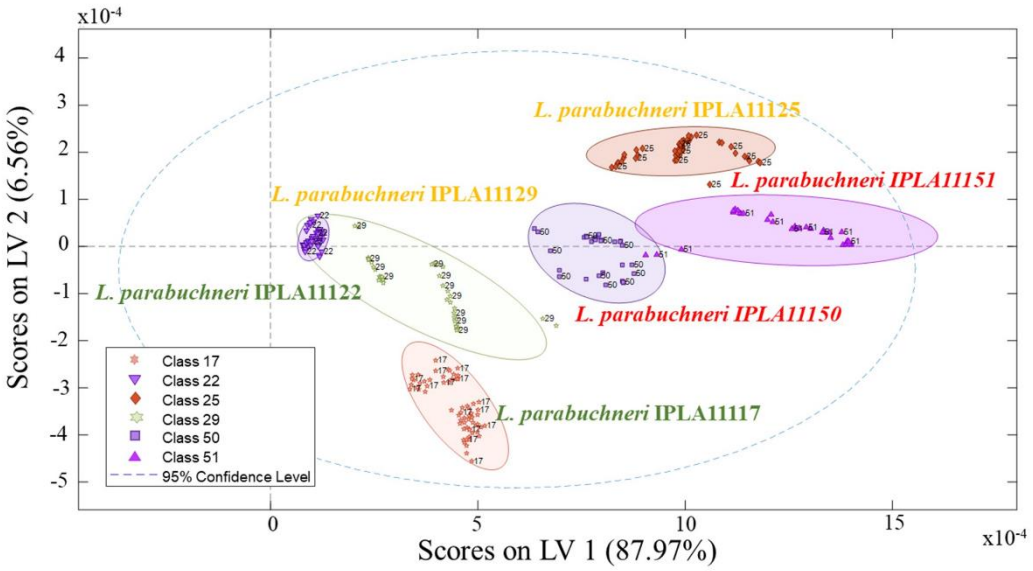

Fig. 9. Score plot after PLS-DA classification of *L. parabuchneri* histamine-producing strains after 48 h of biofilm formation. The six classes are well separated based on two LVs derived from the IR-ATR datasets. Biofilm-producing samples are well classified using only two latent variables (LVs) based on the evaluated infrared spectral dataset obtained via infrared attenuated total reflection (IR-ATR) spectroscopy. The confidence ellipse includes a 95% confidence level.

The molecular changes are elaborately discussed in Table 1 and illustrated in Fig. 6 via the characteristic infrared bands of the biofilm signatures in the range 1700-900 cm^{-1}, which is ideally suited for the identification of *L. parabuchneri* biofilms. A detailed analysis of the obtained IR-ATR spectra for 48 h of biofilm monitoring processes verified the associated molecular changes reflected in the vibrational modes of the amide I, amide II, NA, and EPS region. More precisely, an increase in protein content was observed for *L. parabuchneri* IPLA 11151, 11150 and 11125 strains as the peaks for amide I (1635 $cm^{-1)}$ and amide II (1551 cm^{-1}) increased over time. These findings are consistent with previous studies that have reported higher protein content for stronger biofilm producers such as *L. parabuchneri* IPLA 11151 and 11150 [22]. In addition, the signals of nucleic acids and amide III at 1350–1200 cm^{-1}, polysaccharides, phospholipids, and phosphodiesters in the range 1200-997 cm^{-1} were characterized by lower absorbance intensities for the bacterial strains *L. parabuchneri* IPLA 11129, 11122 and 11117, which are moderate and weak biofilm producers, respectively.

Fig. 10 shows the supervised PLS-DA sample class plot for each individual bacterial strain related to individual predicted values. Similarly, the corresponding samples/scores graph of the PLS model for *L. parabuchneri* strains after 48 h of biofilm monitoring indicated that the predicted values correlate with the strain classification (Fig. S3).

While all training samples have been classified correctly by the discriminant analysis, the variation captured via the IR spectral information collected on the biofilms formed in real-time enables the accumulation of large datasets, which may be instantaneously assigned to a specific strain based on the molecular differences. The 'among-group-differences' are significantly larger vs. the 'within-group-differences' such that *L. parabuchneri* biofilms may be unambiguously classified using the established PLS-DA model. The clear discrimination between the strain sample isolates regarding biofilm-forming capacity after 48 h of *in-situ* real-time monitoring via IR-ATR spectroscopy confirms the ability of this technology for label-free, non-destructive and long-term classification of biofilm-forming microbial species.

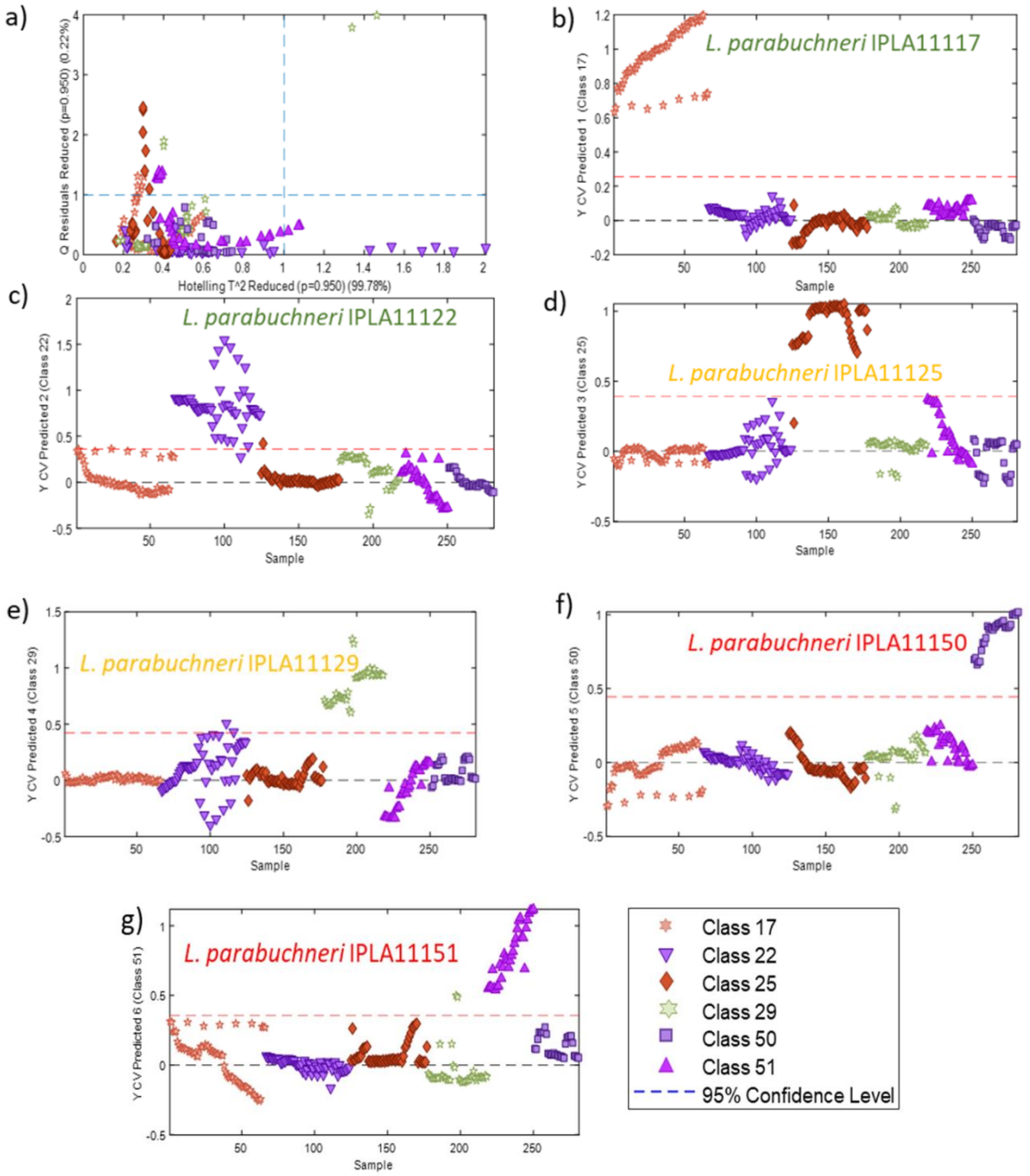

Fig. 10. PLS-DA classification for each investigated *L. parabuchneri* strain vs. other biofilm-forming strains; **a)** The Hotelling's $T^{\wedge 2}$ statistic plots with the reduced values (p=0.950) explaining 99.78% of the total variance for **b)** *L. parabuchneri* IPLA 11117, **c)** *L. parabuchneri* IPLA 11122, **d)** *L. parabuchneri* IPLA 11125, **e)** *L. parabuchneri* IPLA 11129, **f)** *L. parabuchneri* IPLA 11150 and **g)** *L. parabuchneri* IPLA 11151. The confidence ellipse indicates a 95% confidence level.

The six isolated bacterial strains shared common boundaries within the samples that were used to train the PLS-DA model [59]. Fig. 10 displays the origin of the prediction for unknown biofilm-producing strains based on cross validation. The model was found to be highly reliable in determining the source of each class in the datasets that were used to train it. This was demonstrated by a perfect match between the identification of each individual class and its source. Table 2 provides the corresponding statistical values of constructed PLS-DA model which establish the quality of classification scheme.

Table 2. Calibration and classification statistical outputs of PLS-DA model based on IR-ATR spectra of *L. parabuchneri* 48 h old biofilms.

	Analyte	**RMSEC**	**RMSECV**	**R^2 Cal**	**R^2 CV**
IR-ATR spectroscopic Datasets	Biofilm producing strains (Num. of LVs: 3, 98.46% cumulative variance captured)				
	L. parabuchneri 11117	0.163	0.184	0.9502	0.9431
	L. parabuchneri 11122	0.248	0.260	0.9815	0.9753
	L. parabuchneri 11125	0.173	0.242	0.9630	0.9572
	L. parabuchneri 11129	0.094	0.223	0.9957	0.9941
	L. parabuchneri 11150	0.216	0.240	0.9428	0.9269
	L. parabuchneri 11151	0.099	0.235	0.9860	0.9752

[*] *RMSEC* Root Mean Square Classification Error; *RMSECV* Root Mean Square Error of Cross-Validation; *R^2Cal* Coefficient of Determination for Calibration; *R^2CV* Coefficient of Determination for Cross-Validation.

The comparison of 48 h old biofilms of *L. parabuchneri* strains revealed three distinct classes based on two LVs in the PLS-DA model (Fig. 11(a)) covering 89.46%of the total variance. *L. parabuchneri* IPLA 11151 and 11150 were classified as strong biofilm producers, *L. parabuchneri* IPLA 11129 and 11125 were classified as moderate biofilm producers and *L. parabuchneri* IPLA 11122 and 11117 as weak biofilm producers. Fig. 11a illustrates the biofilm-producing strains which were clearly separated using 2 LVs capturing 89.46% of the total variance. By estimating the loadings of LV1 and LV2 shown in Fig. 11(b) it is evident which wavelength regimes in the IR spectra provide the

most characteristic signatures of *L. parabuchneri* biofilms. The loadings of both LV1 and LV2 exhibit prominent peaks at approximately 1061 cm^{-1} clearly indicate the presence of carbohydrates in the EPS matrix, indicating mature biofilms [109]. Additionally, loadings indicate that the dominating vibrational signature changes occur in the amide I and amide II region [41]. The LV1 score readily explains 81.46% of the total variance and the LV2 score explains 7.48% with corresponding R^2 values for the classes of strong, moderate and week biofilm-producers confirming a predictive accuracy ranging from 0.9428 to 0.9957. The data of the respective three classes are well-separated from each other suggesting reduced correlation with the amount of biofilm biomass attached to waveguide surface.

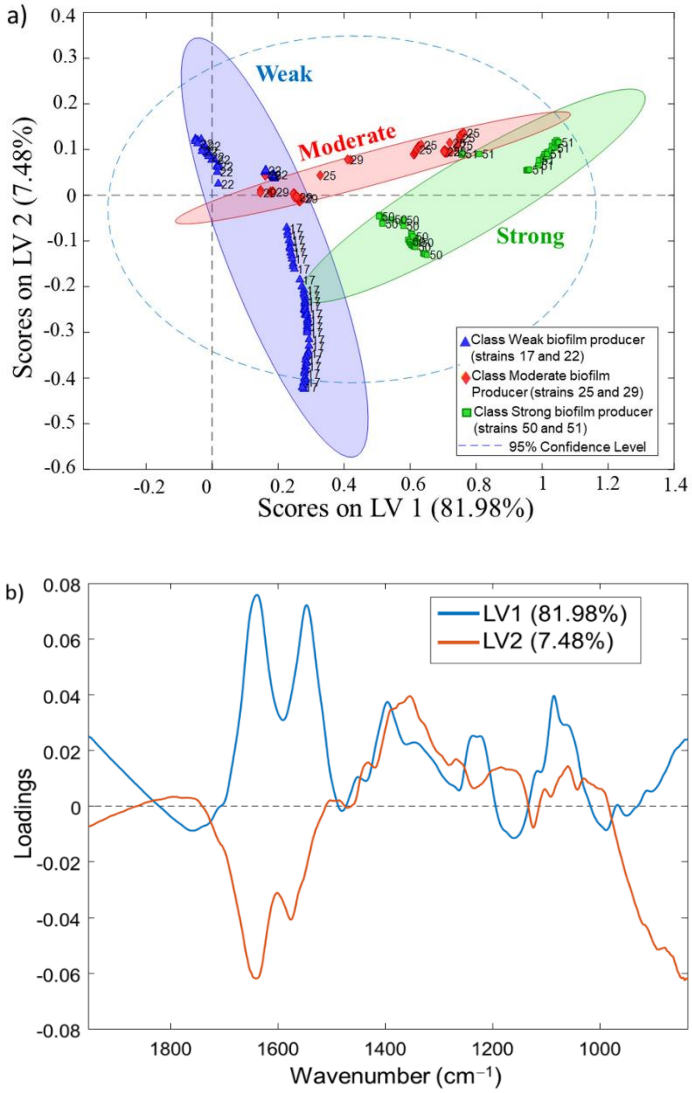

Fig. 11. a) PLS-DA model of *L. parabuchneri* biofilms classified into three clusters: weak, moderate, and strong biofilm-producing strains, and **b)** loadings for first latent variable (LV1) and second latent variable (LV2) indicating IR spectral regions of relevance. The confidence ellipse indicates a 95% confidence level.

The distribution of the predicted values is based on the Bayesian theorem [110] for the probability to identify the non-parametric trends during biofilm evolution over continuous nutrient flow. The Bayesian statistics estimate the probability that a sample may be a member of a certain class given the predicted value [111]. The prediction probability results for two LVs are obtained from the PLS-DA model comparing the biofilms produced by 6 *L. parabuchneri* strains via IR-ATR spectroscopy. Evidently, a precise classification model is developed using the IR signature of microbial biofilms from six strains, which discriminates according to the occurring molecular differences categorizing strong, moderate and weak biofilm-producing abilities only via indicative IR signatures. Early-stage biofilms formed inside the flow-system at the surface of the ATR waveguide within 24 hours of monitoring yielding IR-spectra that captured the biofilm composition and structural changes related to each of the six strains (Fig. S4(b) in SM). By employing Hotelling's T^2 Reduced values (p=0.950) (Fig. S5 in SM) a substantial reduction in the distribution of selected variables was achieved. The differences in means among the multivariate data of 24 h old biofilms were tested, attributing to the variable selection based on biofilm-forming capacity. This projection in the model enabled to measure the variation within each sample in the model, indicating how far each biofilm sample from the six microbial strains deviated from the model's center. Given a microbial biofilm sample, the probability of belonging to class 1 was calculated as follows:

$$\text{probability (class 1)} = \frac{P(y,1)}{[P(y,1)+P(y,2)]} \qquad (1)$$

where y is the predicted value from the used PLS-DA model of the sample analyzed; P(y,1) is the probability that this sample is a member of class 1 given the value of y and P(y,2) is the probability that this sample is a member of class 2 given the value of y. Hence, a sample with a predicted value at the threshold had a 50% probability of belonging to either class [61,110,111]. In the presented model of biofilm-producing species (Fig. 11(a)), some of the moderate producers *L. parabuchneri* IPLA 11129 biofilms cannot be unambiguously classified from the other weak biofilm-producing species marked by a small overlap of confidence ellipses, so the probability of belonging to the class of weak biofilm producers is 50%. When three LVs will be applied to the model, surely all biofilm samples could be completely classified, and the three separated classes were distinguishable. The classification and prediction results for the PLS model using two LVs shown in Fig. 11 classify all biofilms of *L. parabuchneri*

strains based on their biofilm-producing ability. This classification obtained by real-time IR-ATR measurements under hydrated conditions, resembles the most accurate classification for *in situ* monitoring of *L. parabuchneri* biofilms. To classify the performance of the biofilm-producing analyzed strains, the PLS-DA model was also constructed for only the first 24 h of IR-ATR monitoring extracted datasets (Fig. S4(a) in SM). Owing to two latent variables (LVs) derived from the PLS-DA model, 98.52% of total variance is explained. The IR-data related to early stage biofilms (i.e., not-mature) formed inside the flow-system, precisely on top of ATR waveguide surface, have no significant differences in the biofilm composition within the biofilm-producing capacity (Fig. S4(b) in SM). The phenomenon of confidence ellipse overlapping for the moderate and weak biofilm-producing strains, verifies that the best data explanation does not come from the 24 h of biofilm monitoring set, although there is a huge reduction in the number of selected variables within the model expressed by Hotelling's T^ 2 Reduced (p=0.950) with 99.63% of total variance (Fig. S(5) in SM).

Eventually, it is noted that a more distinct differentiation based on latent variable LV1 and LV2 for the discrimination of 48 h old biofilms has been obtained. This is reflected in the loadings (LV1 by 81.98% and LV2 by 7.48% of total variance) acquired for the strong peaks at 1700-900 cm^{-1}, which are attributed to the characteristic spectrum of *L. parabuchneri* biofilms. These findings suggest that PLS-DA with only two latent variables allows differentiating strong vs. moderate and weak biofilm-producing species. In situ IR-ATR spectroscopy has been used to thoroughly investigate the composition of the EPS matrix, which serves as the primary component for the spectroscopic identification of biofilms [112]. The most prominent changes of molecular vibrations for the functional groups C═O, N–H and C–O as distinct indicators of biofilms with the main biomolecular components such as lipids, proteins, nucleic acids, EPS including polysaccharides, phosphoryl groups, polyphosphate and phosphodiester products. Loadings in Fig. 11(b) confirmed that the higher absorbance intensity originates from proteins (1700-1550 cm^{-1}) and exopolysaccharides (1200-997 cm^{-1}). These components are identified as the fundamental structural constituents of biofilm extracellular matrices [22].

The constructed PLS-DA model facilitates a distinguished classification of *L. parabuchneri* microbial isolates based on their different biofilm-forming capacities. The differentiation of strong vs. moderate and weak biofilm producers *L. parabuchneri* strains as a result of their molecular changes and differences in IR signatures was for the

first time confirmed via in situ, real-time IR-ATR spectroscopy. In view of the constructed multivariate strategy, 98.46% of the total variance captured with only three latent variables (LVs) established PLS-DA as the most outstanding chemometric model to differentiate cheese-isolated histamine producing strains. The unique spectral characteristics of biofilms are accordingly related to the compositional changes of *L. parabuchneri* biofilm-producing species. The implemented chemometric strategy accomplished the rapid determination and classification of *L. parabuchneri* biofilm-producing species.

4. Conclusions

The present study contributes to the identification of the molecular composition and chemical properties of biofilms produced by six cheese--isolated *L. parabuchneri* histamine-producing strains associated with the production of toxic compounds and spoilage of fermented food in the dairy industry.

Using infrared spectroscopic techniques based on real-time IR-ATR spectroscopy for in situ monitoring of the biofilm matrix has facilitated comprehensive understanding on all stages of biofilm formation and maturation at extended time scales. This approach has enabled the detection and in situ analysis of significant changes occurring throughout biofilm development. Multivariate data evaluation strategies and data classification algorithms were used to achieve a rapid, efficient and comprehensive discrimination of microbial biofilm contaminations. Furthermore, IR-ATR offered a deep study of the molecular mechanisms involved in *L. parabuchneri* biofilm growth attributed to infrared spectral characteristics (proteins, nucleic acids, exopolysaccharides) and correlated with simultaneously tracing metabolic oxygen concentrations.

The microbiological results from biofilm quantification assay and CLSM microscopical imaging, verified the classification of the six *L. parabuchneri* strains as weak, moderate, and strong biofilm producers. Although viability assays showed that there is a slight difference in performance of each *L. parabuchneri* strain, it is essential to investigate and understand the biofilm matrix composition. This involves observing key variables between histamine-producing *L. parabuchneri* strains including but not limited to biofilm biomass and the presence of intact and dead cells using CLSM microscopy.

In terms of metabolic changes, IR studies indicated that all tested strains of *L. parabuchneri* exhibited reduced oxygen levels during biofilm development, creating

microaerophilic conditions conducive to biofilm proliferation. The initial attachment of each *L. parabuchneri* strain to the ZnSe waveguide surface caused distinct IR fluctuations in the intensities of biofilm-relevant bands. Stronger biofilm-producing strains displayed more pronounced molecular changes compared to moderate and weak biofilm producers, although there were minor differences in representative bands. Further analysis of the IR-ATR spectra over a 48-hour period of biofilm monitoring confirmed that the molecular structural components, varied among the strains, influenced by their biofilm-forming ability. Three LVs in the constructed PLS-DA model effectively grouped the biofilm samples into distinct classes. The first two LVs, which accounted for 88.94% of the total variance, revealed that stronger biofilm producers (IPLA 11151 and 11150) exhibited pronounced biofilm growth and molecular changes by anticipating the key infrared bands of the analyzed molecular components including membrane proteins (such as amide I and II) and cyclic fluctuations of EPSs within the biofilm matrix with a significant increase as a function of time. In summary, this study showed the unique applicability of in situ IR-ATR spectroscopy and a rapidly constructed PLS-DA model to comprehensively investigate the chemical composition of the biofilm matrix of *L. parabuchneri* strains, and successfully classified them based on their biofilm-forming capacities. These findings may further contribute to the development of novel strategies for the prevention and elimination of biofilms by understanding the differences in the chemical composition of individual biofilm-forming strains and their biomass enrichment.

CRediT authorship contribution statement

Diellza Bajrami and **Agustina Sarquis** contributed equally to this work. Diellza Bajrami and Agustina Sarquis: Conceptualization, Methodology, Formal analysis, Data curation, Visualization, Writing – original draft and editing.

Victor Ladero: Writing – review and editing, Investigation, Supervision, Validation.

María Fernández: Methodology, Scientific discussions, Writing – review and editing,

Boris Mizaikoff: Supervision, Coordination, Funding acquisition, Review and editing, Resources.

Declaration of competing interest

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

Data availability

Data will be available from the corresponding author upon reasonable request.

Acknowledgments

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Supplementary material: Rapid discrimination of *L. parabuchneri* biofilms via in situ infrared spectroscopy

Number of figures:5
Number of pages:4

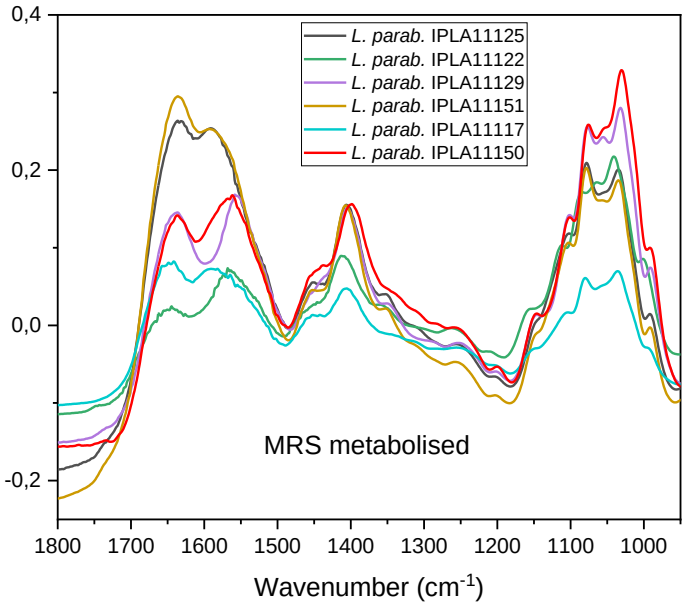


Fig. S1. IR-ATR spectra of planktonic *L. parabuchneri* IPLA strains in metabolized MRS media.

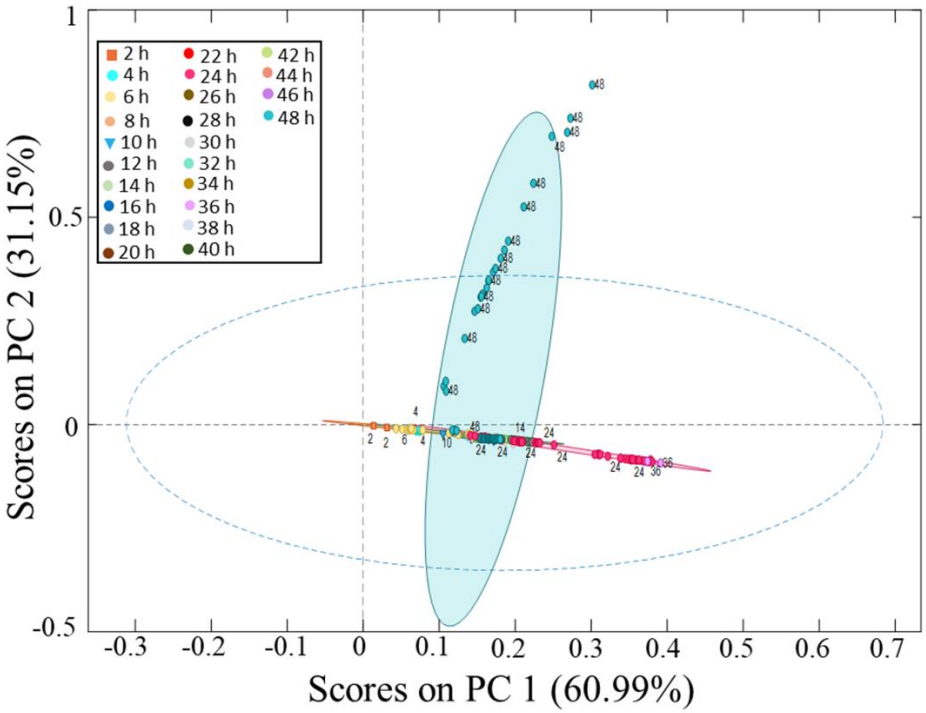


Fig. S2. PCA analysis of the IPLA isolated strains' IR spectra classified as a matter of monitoring time (hours) and the maturation age. The explained variance by 2PCs was 92.14%. Numbers written represent the clusters for the hours of IR-ATR flow monitoring process.

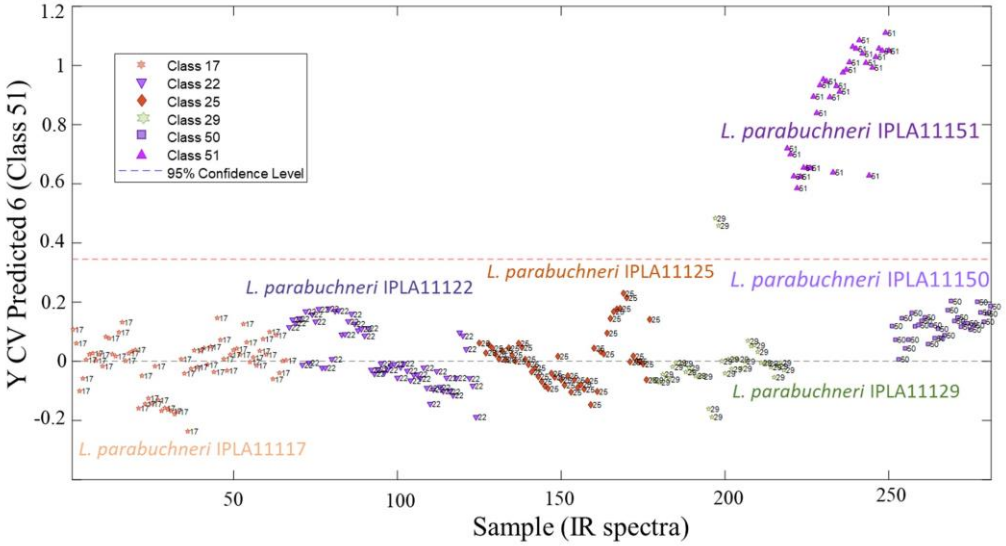
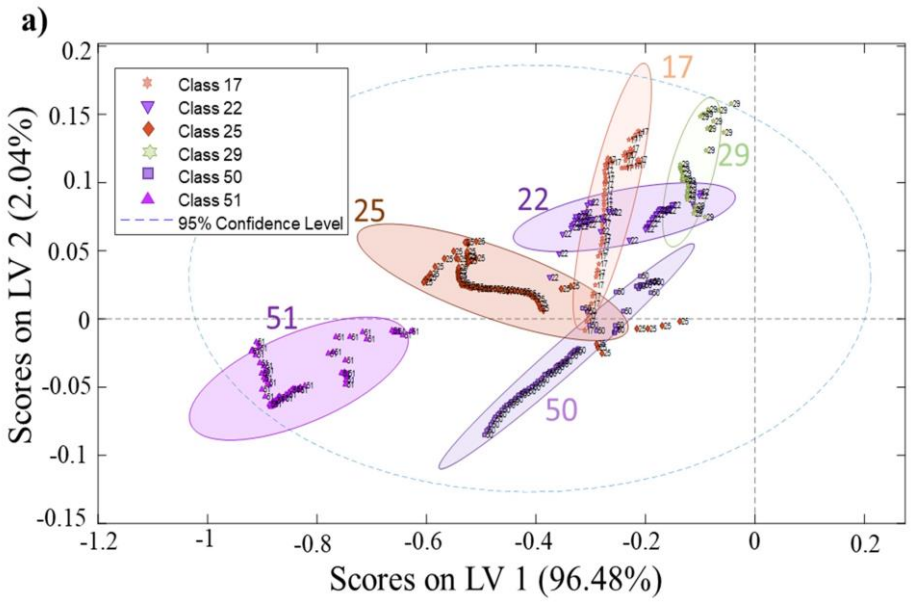


Fig. S3. PLS-DA model of Samples/Scores for *L. parabuchneri* IPLA strains after 48 h of biofilm monitoring. The predicted values of samples are related to the strain classes.



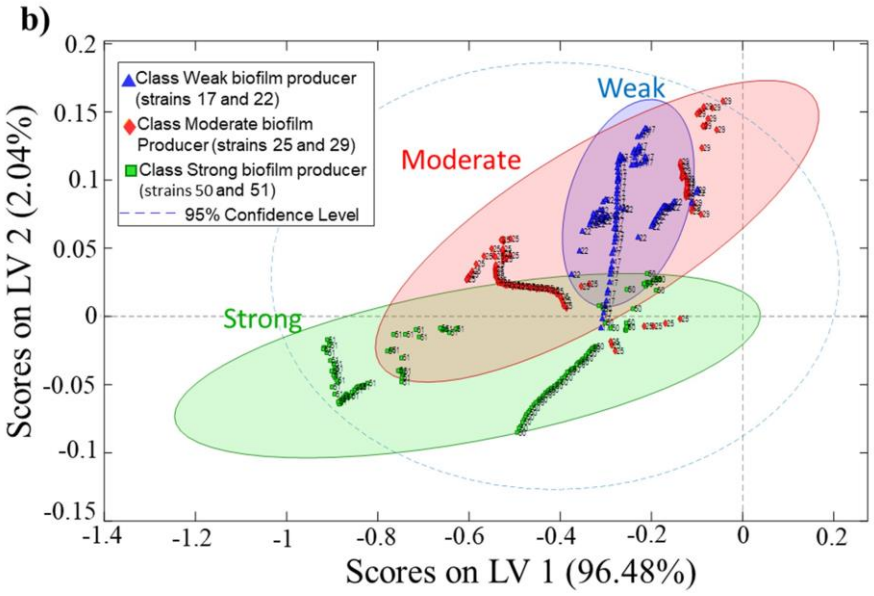


Fig. S4. The PLS-DA model constructed for the classification performance of *L. parabuchneri* biofilms after 24 h of monitoring for **a)** each isolated strains and **b)** biofilm producing classes.

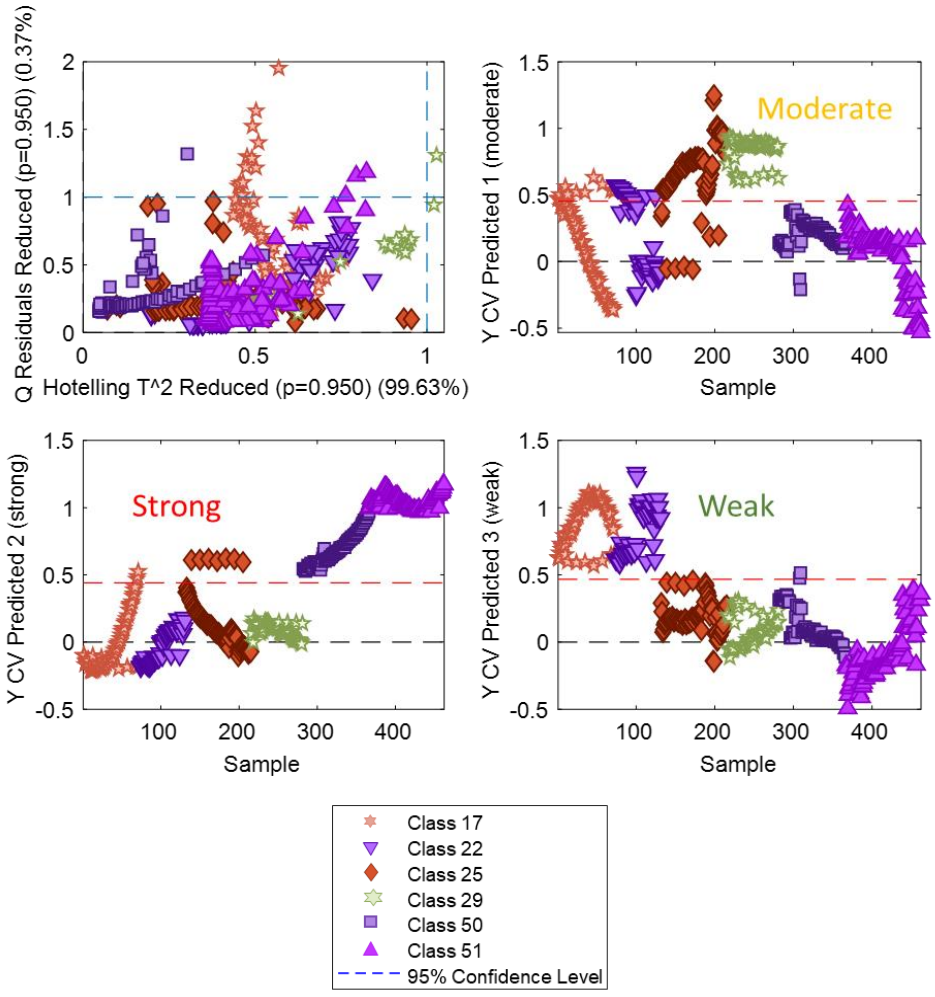


Fig. S5. The values of Hotelling's T^ 2 Reduced (p=0.950) with the 99.63% of total variance showing huge reduction in the number of selected variables compared to using the model with the full dataset, based in biofilm forming capacity variable selection.

CAPITULO 3

Objetivo 4. Identificar y localizar los elementos genéticos implicados en la formación de biofilms: secuenciación y anotación de genomas.

Objetivo 5. Determinar la funcionalidad del clúster de adhesión.

Para alcanzar estos dos objetivos, en primer lugar, se secuenció y analizó el genoma de las 6 cepas de *L. parabuchneri* en las que se había determinado diferentes capacidades de formación de biofilms (fuertes, moderadas y débiles). Sin embargo, únicamente en las cepas formadoras de biofilm fuerte, se identificó un *cluster* formado por cuatro genes, que presentaba homología con genes implicados en la formación de *pili*, siguiendo el modelo *sortase-mediated pilus*, y que por tanto podría estar relacionado con la capacidad de adhesión a superficies.

Para comprobar la funcionalidad del *cluster* identificado se procedió a su clonación en cepas en *Lactococcus lactis* NZ9000. Se escogió este huésped por la posibilidad de su manipulación genética además de no formar biofilm. Los resultados obtenidos permitieron comprobar la funcionalidad e implicación en la adhesión del clúster identificado. Además, mediante secuenciación con PacBio, se determinó la localización plasmídica del clúster lo cual podría facilitar su transmisión a otras cepas, transfiriéndoles la habilidad de adhesión a superficies.

Los resultados obtenidos se presentan en el siguiente manuscrito (enviado para su evaluación y publicación):

> **Artículo 3: Agustina Sarquis**, Víctor Ladero, María Díaz, Esther Sánchez-Llana, María Fernández, Miguel A. Alvarez. The gene cluster associated with strong biofilm-formation capacity by histamine-producing *L. parabuchneri* encodes a sortase-mediated pilus and is located on a plasmid.

The gene cluster associated with strong biofilm-formation capacity by histamine-producing *L. parabuchneri* encodes a sortase-mediated pilus and is located on a plasmid

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Highlights

- The genomes of histamine- and biofilm-producing *L. parabuchneri* strains were compared
- Only strong biofilm-producers harbour a pilus-encoding gene cluster (PGC)
- Heterologous PGC expression reflects its role in adhesion and biofilm formation
- The PGC is located on a plasmid and is flanked by insertion sequences
- Strong biofilm-forming capacity could be transferred by horizontal gene transfer

Abstract

Histamine is a biogenic amine synthesized through the enzymatic decarboxylation of the amino acid histidine. It can accumulate at high concentrations in foods through the metabolism of certain bacteria, sometimes leading to adverse reactions in consumers. In cheese, histamine can accumulate at toxic levels; *L. parabuchneri* has been identified the major cause of this problem. Previous studies have shown some *L. parabuchneri* strains to form biofilms on different surfaces, posing a contamination risk during cheese production, particularly for cheeses that are processed post-ripening (e.g., grating or slicing).

Biofilms are communities of microorganisms embedded in an extracellular matrix that adhere to surfaces. In the food industry they can be commonly found on the surfaces of processing equipment. The food contamination they cause can result in economic losses and even foodborne illness depending on the species involved. The aim of the present work was to identify the genes of *L. parabuchneri* involved in biofilm formation, and to determine their function. The genomes of six strains with different biofilm-production capacities (strong, moderate and weak) were sequenced and analysed. The histidine decarboxylase (*HDC*) gene cluster involved in histamine production was confirmed present in all strains. Additionally, a cluster of four genes (8 kb), similar to those involved in sortase-mediated pilus formation, was identified in the strong biofilm-producers, suggesting it to have a role in surface adhesion. Cloning and heterologous expression in *Lactococcus cremoris* NZ9000 confirmed its functionality and involvement in adhesion and, therefore, in biofilm formation. PacBio sequencing showed this cluster to be located on a 33.4 kb plasmid, which might increase its chances of horizontal transmission. These findings provide insight into the genetic factors associated with biofilm formation in histamine-producing *L. parabuchneri*, and into the risks associated with this bacterium in cheese production.

Keywords: Food safety, biogenic amines, lactic acid bacteria, dairy, cell adhesion, fimbria

1. Introduction

Histamine is a biogenic amine (BA) synthesized via the decarboxylation of the amino acid histidine. It can be found in certain foods and beverages as a result of microbial activity, and can cause adverse health effects when consumed in large amounts (Ladero et al., 2010; Ruiz-Capillas and Herrero, 2019; Silla Santos, 1996). The consumption of foods with high concentrations of histamine is particularly dangerous for individuals who are sensitive to it, or whose histamine metabolism is impaired (Ladero et al., 2010; Sahu et al., 2015). Those who are affected may experience neurological (headaches, dizziness, migraines), gastrointestinal (diarrhoea, abdominal pain), and respiratory symptoms (difficulty breathing, asthma-like reactions) (del Rio et al., 2017; Maintz and Novak, 2007). In addition, *in vitro* studies have shown histamine to be cytotoxic (Linares et al., 2016), and that this cytotoxicity is increased by the presence of another common BA, tyramine (del Rio et al., 2017).

Some of the highest food concentrations of histamine develop in cheese (EFSA, 2011; Ladero et al., 2009; Şanli and Şenel, 2015). Its production in this food is attributed to the histidine-decarboxylase activity of certain bacteria, mainly lactobacilli (Berthoud et al., 2017; Diaz et al., 2015; Fröhlich-Wyder et al., 2013; Linares et al., 2012). *L. parabuchneri* has been identified by independent culture methods as the species largely to blame for this problem (Maria Diaz et al., 2016d; O'Sullivan et al., 2015). Several histamine-producing *L. parabuchneri* strains have been isolated from different types of cheese (Ascone et al., 2017; Maria Diaz et al., 2016a; Wüthrich et al., 2017).

Some histamine-producing *L. parabuchneri* strains form biofilms on the surface of dairy equipment - a problem of notable technological importance to the dairy industry (Maria Diaz et al., 2016a). Biofilms are complex communities of microorganisms, coated in a protective matrix of extracellular polymeric substances (Deo et al., 2019; Donlan et al., 2004), that adhere to different surfaces (Faille et al., 2014; Stoodley et al., 2002). They are highly resistant to cleaning and sanitizing procedures, making them difficult to control (Galié et al., 2018; Goetz et al., 2022; Toushik et al., 2020). Certainly, biofilms of histamine-producing *L. parabuchneri* on dairy equipment surfaces can contaminate cheese during its production, especially when processing ripened cheeses (grating and slicing, etc.) (Ladero et al., 2009).

Biofilm formation by *L. parabuchneri* is influenced by different factors, including temperature, pH and nutrient availability (Sarquis et al., 2023). Our group has reported strong biofilm-forming capacity to be strain-dependent (Maria Diaz et al., 2016).

However, the genetic factors that contribute to this capacity in this species' strains remains largely unexplored. Although there have been studies comparing the genomes of *L. parabuchneri* strains (Gumustop and Ortakci, 2022; Wüthrich et al., 2017), none has examined the specific genes influencing their biofilm-forming abilities. The aim of the present work was to sequence, analyse and compare the genomes of six histamine-producing *L. parabuchneri* strains with different biofilm production capacities (strong, moderate and weak) in order to identify genes that might play a critical role in biofilm formation. Additionally, the functional properties of these genes were examined. To our knowledge, this is the first report of the genetic factors involved in biofilm-formation capacity in histamine-producing *L. parabuchneri* strains.

2. Materials and Methods

2.1 Bacteria, plasmids and culture conditions

The bacterial strains and plasmid vectors used in the present work are summarized in Table 1. *Escherichia coli* DH10B was grown in Lysogeny Broth (LB) with shaking at 37°C. *Lactococcus cremoris* NZ9000 was grown statically at 32°C in M17 medium (Oxoid, Basingstoke, UK) supplemented with 0.5% (w/v) glucose (GM17). Agar (2% w/v) was added when required. The growth medium was also supplemented with antibiotics for the selection of transformants and plasmid maintenance (kanamycin 10 µg ml^{-1} for *E. coli*, and erythromycin 5 µg ml^{-1} for *L. lactis*). *L. parabuchneri* was grown in MRS (Oxoid) under anaerobic conditions at 37°C (Sarquis et al., 2023).

2.2 DNA manipulation

L. parabuchneri IPLA 11150 genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations, with the modification of adding mutanolysin (Sigma-Aldrich, Germany) with the lysozyme in the first lysis step.

PCR products were separated by electrophoresis on 0.8% agarose gels, stained with EZ-vision (VWR, Madrid, Spain), and visualized using a UV transilluminator. PCR products and DNA restriction fragments were purified using the ATP Gel/PCR Extraction Kit (ATP Biotech, Taipei, Taiwan) following the manufacturer’s recommendations.

Genetic construct in *L. cremoris* IPLA1301 was produced using *E. coli* DH10B as an intermediate host. Plasmid DNA was isolated from *E. coli* by the alkaline lysis method

(Sambrook JD; Russell WD, 2001). Ligations were performed using 5 U/µl T4 DNA ligase (Thermo Fisher Scientific, Barcelona, Spain) following the manufacturer's recommendations. Electrocompetent cells of *E. coli* DH10B were prepared as previously reported (Sambrook JD; Russell WD, 2001), as were electrocompetent *L. cremoris* cells (Holo and Nes, 1989). Electroporation was performed in a Bio-Rad pulser apparatus (Bio-Rad, Barcelona, Spain) following standard protocols for *E. coli* and *L. cremoris*. Plasmids constructed in these species were checked by Sanger sequencing (performed at Macrogen, Madrid, Spain).

PCR amplifications were performed using the DreamTaq PCR Kit (Thermo Scientific, Barcelona, Spain), following the manufacturer's instructions. Extracted DNA was resuspended in Dream Taq Buffer containing dNTPs, oligonucleotide primer, DreamTaq DNA polymerase (thermostable DNA polymerase), and H_2O. Table 1 shows the sequences of the primers designed (performed using Clone Manager Software [Sci Ed Software. Westminster, CO, USA]). PCR amplification was performed as follows: initial denaturation at 95°C for 5 min, followed by DNA amplification over 35 cycles consisting of a denaturation step at 95°C for 30 s, annealing at 55°C for 30 s, and an extension step at 72°C for 1 min (except for the last cycle in which the extension step lasted 10 min).

The Expand Long PCR Kit (Roche, Vienna, Austria) was used to amplify the gene cluster of interest. The total genomic DNA of *L. parabuchneri* IPLA 11150 was then added to a mixture of dNTPs, plus the primer Lpbsort 2 (Table 1) (the primer binds at identical inverted repeats flanking the cluster; Figure S1), Expand Long Template PCR Buffer 3, Expand Long Template enzyme mixture (which contains thermostable Taq DNA polymerase) and H_2O. Buffer 3 was chosen following the above manufacturer's instructions, taking into account the size of the sequence to amplify. The amplification protocol consisted of an initial denaturation step at 94°C for 2 min, followed by 10 cycles of denaturation, annealing, and extension. Each cycle involved denaturation at 94°C for 10 s, annealing at 60°C for 30 s, and extension at 68°C for 5 min. This was followed by 25 cycles with a denaturation step at 94ºC for 15 s, annealing at 60ºC for 30 s, and an elongation step at 68ºC for 5 min 20 s; the last cycle included an extension step of 7 min. After amplification, the PCR products were purified and digested with *EcoR*I at internal sites located near both ends (Fig. S1).

2.3 Plasmid construction

To construct pIPLA1300, the pUK21 vector digested with *Eco*RI was ligated with a fragment containing a gene cluster of interest obtained by Long Expand PCR (see 2.2). This product was then purified, digested with *Eco*RI, and treated with alkaline phosphatase (Fig. S1). Transformants of *E. coli* DH10B were selected (whitish-blue colonies) on LB plates supplemented with the kanamycin, 20 mg/mL 5-bromo-4-chloro-3-indolyl-ßD-galactopyranoside (X-Gal) (Apollo Scientific, Stockport, UK), and 0.5 M isopropyl-ß-D-thiogalactopyranoside (Apollo Scientific).

The restriction fragment *Bam*H-*Xho*I from pIPLA1300 was ligated with pIL253, digested with *Bgl*II and *Sal*I, and used to transform *L. cremoris* NZ9000. Transformants were randomly picked from GM17 agar plates containing erythromycin. The colonies with the desired construct were selected by specific PCR, using Sor-F and Sor-R primers (Table 1) which target the sortase gene of the cluster. The template used was total DNA obtained by phenol-chloroform extraction from selected bacterial colonies. PCR-positive colonies underwent additional PCR using Tip-F/R, Bpili-F/Mpili-R and Mpili-F/Sort-R primers (Table 1) to verify the integrity of the gene cluster. This was followed by plasmid purification and Sanger sequencing (performed at Macrogen) to confirm the absence of mutations in the transformant constructs.

2.4 Illumina sequencing

Total DNA from the six *L. parabuchneri* strains with different abilities to produce biofilm (Table 1) was extracted using the DNeasy Blood and Tissue Kit (Qiagen). A genomic library consisting of fragments with a size of 0.5 kbp was created and sequenced using a HiSeq 1000 System sequencer (Illumina) (performed at GATC Biotech, Munich, Germany) employing 150 paired-end sequencing. Quality-filtered reads were assembled using SPAdes v.3.13.0 software (Bankevich et al., 2012).
Annotation was performed using the Prokaryotic Genomes Annotation Pipeline (PGAP) (Tatusova et al., 2016) on the NCBI server. Annotation for relevant genes was improved by BLAST analyses.

2.5 DNA sequence comparisons

To identify genes that might influence biofilm production, a comparative analysis of those present in the genomes of the six *L. parabuchneri* strains (Table 1), including the

core and accessory genome, was performed using Roary v.3.11.2 software (Page et al., 2015). The cluster tree generated was visualized using Phandango v.1.3.0 software (Hadfield et al., 2018).

2.6. Determination of relative gene copy numbers by real time quantitative PCR (qPCR)

To determine the location (plasmid or chromosome) of the pilus gene cluster (PGC), the relative copy number (RCN) of the cluster genes in relation to others genes known to be located on the chromosome was calculated (Garay-Novillo et al., 2019) using the following formula (Lee et al., 2006):

$$RCN= (1+E_{pcrReference})^{CtpcrReference}/(1+E_{pcrTaget})^{CtpcrTarget}$$

where E is the amplification efficiency of each primer pair, and CtpcrReference and CtpcrTarget the threshold cycle values obtained for the corresponding amplicons. The reference genes were two chromosomal genes, i.e., *EF-Tuf* and *GADPH*, and the target genes were two pilus genes, i.e., *tip pilin* and *basal pilin*. *E* values for the reference ($E_{pcrReference}$) and target ($E_{pcrTarget}$) reactions were empirically calculated for each qPCR primer pair using the equation $E=10^{-1/slope}$ -1, as previously described (Martín et al., 2010). The primers ET-Tuf-F/ET-Tuf-R for the reference *EF-Tuf* gene (locus_tag=A7B51_RS11550), GADPH-F/GADPH-R for the reference *GADPH* gene (locus_tag=A7B51_RS02800), Lpb50_piliF/Lpb50_piliR for the target *Tip pilin* gene (locus_tag=A7322_RS12235), and Lpb50BasalF/Lpb50BasalR for the target *basal pilin* gene (locus_tag=A7322_RS12240), were designed specifically for their tasks (Table 1). Experiments were performed in triplicate, and mean values used to calculate RCN values (Garay-Novillo et al., 2019; Martín et al., 2010).

2.7 PacBio sequencing

To identify the plasmids in *L. parabuchneri* IPLA 11150, the PacBio RS II platform was used to sequence the strain's genome (performed at FISABIO, Valencia, Spain). PacBio RS II allowed high-quality, long-read sequence data to be obtained, providing more information regarding the location of the cluster of interest. Total DNA was extracted as described above (see 2.2). The nucleotide sequence of plasmid pIPLA1302 (Table 1) was deposited in the NCBI database under accession number OR237555.

2.8 Biofilm formation on polystyrene

The ability of *L. cremoris* to form biofilms on polystyrene surfaces was investigated using the crystal violet method as previously described (Sarquis et al., 2023), but modifying the incubation conditions to 32°C for 48h.

2.9. Scanning electron microscopy

Scanning electron microscopy (SEM) images of *L. cremoris* IPLA10001 and IPLA10002 were obtained following a previously described procedure (Sarquis et al., 2023). Briefly, the strains were incubated for 48 h at 32°C in tubes with 10 mL of MRS medium containing 1 cm^2 stainless steel coupons (type AISI 304 – a typical stainless steel used in the dairy industry). After incubation, the coupons were cleaned twice with PBS buffer and fixed in 2.5% glutaraldehyde in PBS for 16 h at room temperature. The fixed biofilms were dehydrated using a graded series of acetone solutions (50–100% v/v), dried with argon, coated with platinum using a SCD 005 sputter coater, and observed using a dual-beam FIB/SEM system (Quanta 3D FEG, FEI Company, Hillsboro, OR, USA).

3. Results

3.1 Sequencing and comparison of the genomes of the six histamine-producing *L. parabuchneri* strains of different biofilm-forming capacity

The six strains of different biofilm-forming capacity were: *L. parabuchneri* IPLA11151 and 11150 (strong biofilm-producers), *L. parabuchneri* IPLA 11129 and 11125 (moderate producers), and *L. parabuchneri* IPLA 11122 and 11117 (weak biofilm-producers). Total DNA was extracted from all of them and sequenced by Illumina. The genome sequences were deposited in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA320351) under the accession numbers SAMN19969485, SAMN04992858, SAMN19969475, SAMN19969455, SAMN04932665, and SAMN05150101, respectively.

Table S1 shows the general genomic information obtained for the six strains. Whole-genome assembly revealed draft genome sizes ranging from 2,561,595 to 2,769,427 bp. To identify those genes that were unique to the strong biofilm-producers, pangenome analysis was performed using Roary software; this identified a total 2976 different genes. The core genome was composed of 2186 genes, 382 of which were shared

between two or more strains; 408 others were present in one strain only. The strains showed a variable number of unique genes ranging from 24 in IPLA 11151 (strong biofilm-producer) up to 196 in IPLA 11117 (weak biofilm-producer) (Table 2). In addition, the strains IPLA 11151 and 11150 (strong biofilm-producing strains) had 164 genes in common that differed to those seen in the other strains.

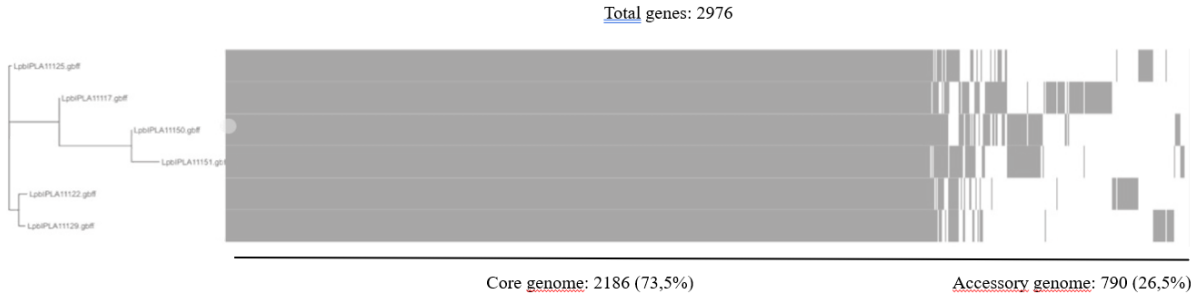

Figure 1. Pangenome analysis (using Roary software) of the six histamine-producing *L. parabuchneri* strains of different biofilm-producing capacity. The cluster tree based on 2976 genes was produced using Phandango software.

A cluster tree based on the pangenome results was constructed (Fig. 1), revealing two major groups, one including strains IPLA 11151, 11150, 11117 and 11125, and the other IPLA 11122 and 11129.

3.2 Identification of relevant genes in the histamine-producing *L. parabuchneri* strains

As expected, the *HDC* gene cluster involved in histamine production was detected in all six strains (Table S2). Genes possibly related to biofilm formation were also found in all six strains. A separate group of genes present in the strong biofilm-producers (*L. parabuchneri* IPLA 11150 and 11151) was also identified (listed in Table S2). Interestingly, a cluster of four genes similar to those involved in the production of pili was also identified only in the strong biofilm-producers. This gene cluster was similar to that coding for a sortase-mediated pilus (SpaCBA) in *L. rhamnosus* GG and LMS2-1 (Krishnan et al., 2016) (Fig. 2), which might be involved in facilitating adhesion to surfaces (Krishnan et al., 2016). The latter authors called it the "pilus gene cluster" (PGS), a designation followed in the present work.

The SpaCBA model cluster comprises four genes, although variations in the organization and number have sometimes been observed in some species (Krishnan et al., 2016). Three of these genes encode two ancillary pilins (the *tip pilin* and *basal pilin*

genes) and a major pilin (the *major pilin* gene), while the fourth codes for sortase (*sortase* gene). Conserved motifs for pilus assembly in the SpaCBA pilins of *L. rhamnosus* were found in the *L. parabuchneri* PGC, supporting the idea that it codes for a similar pilus structure of similar function. The four genes of these pilus model are typically located in the genome as a cluster. Interestingly, transposon elements are often observed in the vicinity, and might be involved in horizontal gene transfer. It has been suggested that the SpaCBA cluster in *L. rhamnosus* GG was acquired via horizontal gene transfer from *L. casei* since it is surrounded by insertion sequences (Krishnan et al., 2016). Similarly, the PGC in *L. parabuchneri* IPLA11150 and IPLA11151 was surrounded by several IS elements, suggesting horizontal acquisition.

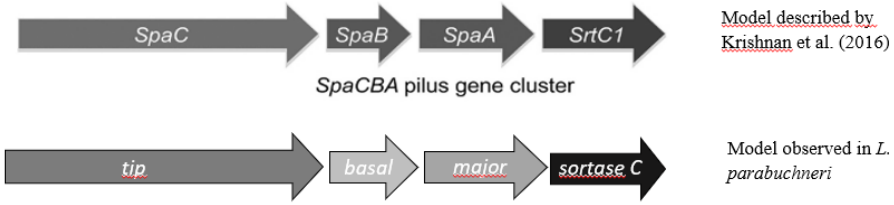

Figure 2. Diagram of the pilus gene cluster in the sortase-mediated pili (SpaCBA) model, and of the cluster observed in *L. parabuchneri*. The detected cluster is composed of four genes that code for three types of pilin: *tip*, *basal*, and *major*. These pilins are connected to each other by covalent links (sortase C-mediated). The major pilins form the shaft of the pilus in a head-to-tail arrangement. The tip pilin is typically found at the tip of the pilus, and projects an adhesion domain. The basal pilin is often located at the base of the pilus shaft, aiding in anchoring it to the cell wall via a housekeeping sortase. Tip pilin (SpaC), and to a lesser extent the basal pilin (SpaB), are also found sporadically throughout the SpaCBA pilus backbone.

3.3 Presence of similar PGCs in other lactobacilli genomes held in databases

Blast analysis was performed to determine whether the genomes of the other 34 *L. parabuchneri* strains genomes available in the NCBI databases harbour this PGC cluster. Clusters were identified in another eight strains with a similarity of 96.7 to 99.46% (Table S3).

The latter analysis was then extended to other lactobacilli. Blast analysis revealed the presence of a similar PGC cluster in 22 strains with similarity ranging from 93.18 to 97.36% (Table S4). Among these 22 strains were two of *Levilactobacillus brevis*, four of *Lactiplantibacillus plantarum*, and 16 of *Lactobacillus delbrueckii* (Table S4).

The plasmid or chromosomal location of the PGC cluster has not been determined in most of the lactobacilli strains that carry it, although it has been reported in the chromosome of four strains of *L. delbrueckii* subsp. *lactis* (ME-797, KCTC 3035,

MAG, and NWC_1_2; see Table S4) and in *L. brevis* SRCM101174. However, in *L. plantarum* LZ227 and *L. brevis* SRCM101174, the putative PGC cluster was found in a plasmid (Table S4).

3.4 Relative copy number of the PGC of *L. parabuchneri* IPLA11150 and 11151, as determined by qPCR

Since in some lactobacilli the cluster was located in a plasmid, checks were made to determine whether the same was true for the *L. parabuchneri* strong biofilm-producer strains IPLA 11150 and IPLA11151. A qPCR assay was developed to determine the RCN of the PGC, using two primer pairs specific for the analysed cluster (Lpb50_pili F/R; Lpb50_Basal F/R) and two pairs for two genes located on the chromosome (GADPH F/R; ET-Tuf- F/R) (Table 1).

Using total DNA from IPLA 11150 and IPLA 11151 as a template, the determined RCN value for the target genes of the PGC was greater than one for both strains (Table 3). This indicates there to be more than one copy of these genes per cell. It is possible that the genes are located on a plasmid with a low copy number since the average RCN was <10 for both strains.

The above assay was repeated using plasmid DNA extracts from IPLA 11150 and IPLA 11151 as a template instead of total DNA. In this case the RCN values were higher - over 50 - for both strains, providing further evidence that the PGC is located on a plasmid.

3.5 Identification of the *L. parabuchneri* IPLA11150 plasmid harbouring the PGC

Agarose gel analysis of plasmid DNA extracts from both strong biofilm-producers (IPLA 11150 IPLA 11151) revealed the presence of several plasmids (data not shown). To determine the plasmid location of the PGC, and to characterize the corresponding plasmid, the genome of *L. parabuchneri* IPLA11150 was sequenced using PacBio long-read sequencing technology. The *de novo* assembly of the PacBio reads resulted in six contigs, the largest representing the chromosome (2,599,083 bp) and the other five, smaller contigs, i.e., pLpb50_p51 (51,909 bp), pLpb50_p35 (35,740 bp), pLpb50_p33 (33,452 bp), pLpb50_p11 (11,142 bp) and pLpb50_p03 (3,876 bp) representing plasmids. The PGC was located in contig pLpb50_p33 (Figure 3), which was

subsequently named plasmid pIPLA1302 (Table 1). This contained 47 open reading frames (ORFs), 28 of which were assigned putative functions (Table S5). Interestingly, several genes that might be involved in plasmid mobility (including a *mobA*-like gene, which would encode the MobA protein) and horizontal gene transfer (11 genes coding for transposon or insertion sequences) were found. These could have a potential role in facilitating the transmission of the PGC to other strains, conferring upon the recipients the ability to adhere to surfaces.

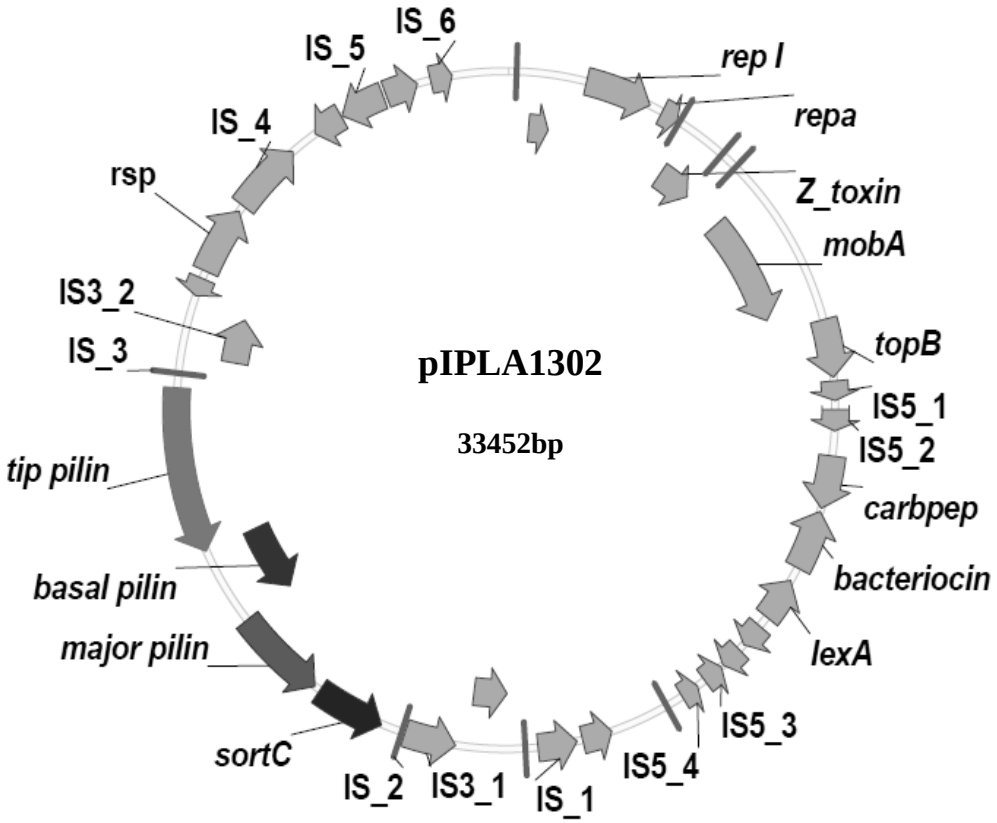

Figure 3. Gene map of pIPLA1302. Arrows of different colour represent the PGC, which is composed of four genes: the *tip pilin*, *basal pilin*, *major pilin*, and *sortase C* genes. IS: insertion sequences. *rep* I and *repa* are similar to genes for plasmid replication, *Z_toxin* is similar to a toxin-antitoxin system gene, *mobA* is similar to a mobility and transfer gene, *topB* is similar to a DNA structural gene, *carbpep* is similar to a carboxypeptidase-encoding gene, *bacteriocin* is similar to a class III bacteriocin-encoding gene, and *lexA* is similar to DNA repair regulatory gene.

3.7 Heterologous expression of the *L. parabuchneri* IPLA 11150 PGC in *L. cremoris* NZ9000

The pIPLA1301 plasmid, which contains the PGC, was constructed as indicated in Material and Methods (Figure S1) and used to transform *L. cremoris* NZ9000 to obtain the *L. cremoris* IPLA1301 strain. The crystal violet technique was then used to compare

the biofilm-forming capacities of *L. cremoris* IPLA1301 and *L. cremoris* IPLA-pIL253, which carries the empty vector (pIL253) (providing a control). The biomass of biofilm formed revealed that the strain containing the PGC, *L. cremoris* IPLA1301, showed significant increased biofilm-formation capacity, nearly doubling the values recorded for the control (Fig. 4). In fact, the ODc cut-off values (Sarquis et al., 2023) classified *L. cremoris* IPLA1301 as a moderate biofilm-producer, and *L. cremoris* IPLA-pIL253 as a weak producer, suggesting that the presence of the PGC considerably increases biofilm production capacity (Fig. 4).

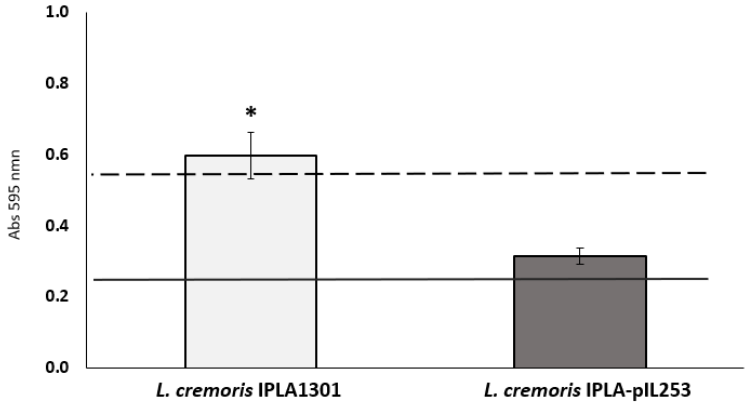

Figure 4. Biofilm-producing capacity on polystyrene of *Lactococcus cremoris* IPLA1301. The strains were incubated at 32ºC for 48 h. Data represent means ± SD (error bars) of at least three independent experiments. Values marked with * differ significantly ($p<0.01$, Student *t* test). The producer/non-producer cut-off (weak producer) is indicated by the dashed line (ODc); the solid line is 2 x ODc (moderate producer). ODc=OD control medium.

Given that *L. parabuchneri* IPLA 11150 forms biofilms on stainless steel, tests were performed to see whether *L. cremoris* IPLA1301 could do the same. SEM images confirmed its ability to attach to the stainless steel coupons provided. Notably, the images revealed *L. cremoris* IPLA1301 harbouring the PGC to show a significantly greater capacity to attach to the coupons than the control *L. cremoris* IPLA-pIL253, with notably larger groups of cells recorded (Fig. 5).

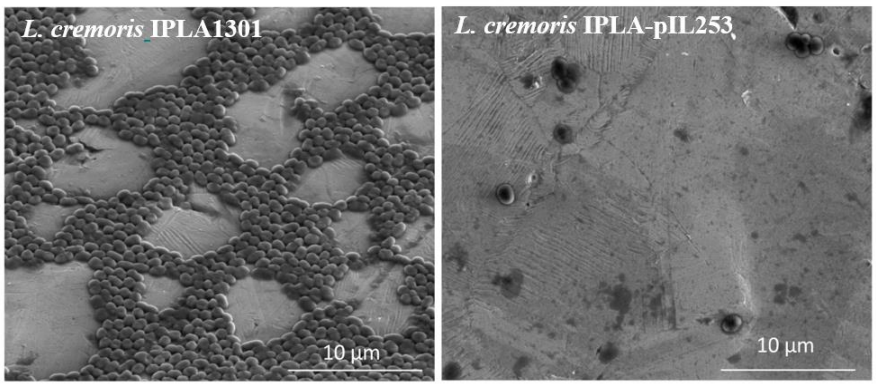

Figure 5. **Scanning electron microscopy images**. *L. cremoris* IPLA1301 and *L. cremoris* IPLA-pIL253 attached to the surface of stainless-steel coupons. 10 µm scale bars are shown on the photomicrographs.

4. Discussion

Histamine is one of the most toxic BAs, and is that most frequently associated with food-borne intoxications (Ladero et al., 2010; Visciano et al., 2020). High concentrations of histamine can commonly be found in cheese (EFSA, 2011; Maurer et al., 2016; Ruiz-Capillas and Herrero, 2019). Avoiding the associated health problems requires measures that prevent food becoming contaminated with histamine-producing bacteria. Biofilms are a particularly problematic source of microbial contamination in the dairy industry, particularly those associated with *L. parabuchneri*, which bears the greatest responsibility for histamine accumulation in cheese (Maria Diaz et al., 2016a). The species produces biofilms on the surfaces of equipment (Ascone et al., 2017; Maria Diaz et al., 2016; Sarquis et al., 2023) including processing equipment and pipelines, leading to the contamination of dairy products and an increased risk of them accumulating histamine (Berthoud et al., 2017; Fröhlich-Wyder et al., 2015, 2013). Unfortunately, biofilms are highly resistant to cleaning and sanitizing procedures, making them difficult to control (Galié et al., 2018; Goetz et al., 2022; Toushik et al., 2020). Moreover, they can protect the cells embedded in them from environmental stressors (Flint et al., 2020; Yin et al., 2019), including antimicrobial treatments. Some authors (Azmi et al., 2019; Luther et al., 2018) have suggested that some bacterial strains may have genetic traits that enable them to form more robust and persistent biofilms, which would further complicate control efforts.

The genome analysis results obtained in the present work indicate that the six strains examined share 73% of their core genes, including the *HDC* gene cluster involved in histamine biosynthesis (Maria Diaz et al., 2016d) (Table S2). Genes unique to particular

strains and that might contribute to specific characteristics or adaptations were also detected, as were accessory genes that might provide additional functions. Interestingly, *L. parabuchneri* IPLA 11150 and 11151 - the strong biofilm-producer strains - had 164 genes (5.5%) in common that were not present in the remaining studied strains (Table S2). Among these genes was a group of four showing strong similarity to genes of the SpaCBA model (the *tip, basal and major pilin* and *sortase* genes) (Krishnan et al., 2016). This group of four genes was designated the pilus gene cluster (PGC).

Pili or fimbriae are long filamentous structures employed by most pathogenic bacteria to establish contacts and mediate interactions with the host. These components are involved in adhesion and are crucial for bacterial colonization (Hendrickx et al., 2011; Proft and Baker, 2009; Scott and Zähner, 2006). Sortase-mediated pili have been identified in several species and strains of Gram-positive pathogens, as well as in commensal gut bacteria in which they are essential for adherence and colonization (Kuhn, 2019). Variation in the number of associated genes, pilins and pilin-specific sortases have been reported, as well as in pilus architecture (Krishnan et al., 2016). Surface piliation is now recognized as a niche-adaptation factor (Ellison et al., 2022; Hendrickx et al., 2011). The SpaCBA model for the structure of pili in *L. rhamnosus* GG is reported to have an important role in adherence, biofilm formation and host signalling (Rasinkangas et al., 2020). The heterologous expression of the PGC of *L. parabuchneri* IPLA 11150 in *L. cremoris* NZ9000 (Figs. 4 and 5) showed it to be involved in the ability of *L. parabuchneri* to adhere to stainless steel surfaces and to render it a strong biofilm-producer.

The PGCs in different bacteria are often found near transposon elements (Krishnan et al., 2016), perhaps indicating they were acquired by horizontal transfer. The present results suggest that the four genes found in the *L. parabuchneri* strong biofilm-producers follow the SpaCBA model for pilus biosynthesis. They appear to be organized in a manner similar to the SpaCBA and SpaFED described for *L. rhamnosus* and *L. casei* (*tip – basal – major – sortase C*) (Fig. 2) and to be flanked by two pairs of identical and divergently orientated insertion sequences (Fig. 3, Fig. S1).

Although it has been suggested that these Spa-like PGCs are located in the chromosome of Gram-positive bacteria (Krishnan et al., 2016), some authors indicate that they are sometimes located on a plasmid, as is the case of *L. casei* LOCK0919 (Aleksandrzak-Piekarczyk et al., 2016). In fact, the present BLAST analysis revealed some PGCs similar to those present in *L. parabuchneri, L. brevis*, *L. delbrueckii* and *L. plantarum*

strains, to be located on plasmids. In the present work, the plasmid location of the PGC in *L. parabuchneri* IPLA 11150 and IPLA 11151 was first suggested by the relative copy numbers obtained in qPCR assays (around six times that of chromosome-located genes). Indeed, this became even clearer when the same qPCR assay was repeated using a plasmid DNA extract (Table 3), and was confirmed by PacBio sequencing. This is important since plasmids are prominent drivers of horizontal gene transfer in prokaryotes, enabling the dissemination of ecologically crucial accessory traits among phylogenetically divergent bacterial taxa (Bottery, 2022). *L. parabuchneri* IPLA 11150 harbours the PGC in a plasmid of 33 kb (Fig. 3) that contains several ORFs with similarities to transposases and insertion sequences. This indicates a remarkable degree of genetic plasticity, as well as the potential ability to undergo horizontal gene transfer. Other genes found on the same plasmid with similarities to mobilization genes included *mob*A, which is potentially involved in plasmid mobility and transfer (Garcillán-Barcia et al., 2019). It is important to highlight that the presence of the PGC on a plasmid increases the possibility of the capacity to adhere to surfaces, and consequently the possibility of forming biofilms being passed on to other histamine-producing *L. parabuchneri* strains. Indeed, other species that produce histamine or other toxic BAs (which could have technological consequences for the dairy industry), and even pathogenic species, might acquire this capacity in the same way (Grohmann et al., 2003; Krishnan et al., 2016; Rasinkangas et al., 2020).

5. Conclusions

Comparative analysis of the genomes of six histamine-producing strains of *L. parabuchneri* showing different biofilm production capacity, allowed the identification of a four-gene cluster with strong similarities to the sortase-mediated pilus model (SpaCBA model). The involvement of this cluster in the adherence to surfaces, including stainless steel, was confirmed by heterologous expression in *L. cremoris* NZ9000. In addition, in *L. parabuchneri* IPLA11150, the PGC was located on a 33kb plasmid (pIPLA1302) with several genes that might be involved in its horizontal transfer. These results provide valuable information for developing strategies to control biofilm formation by histamine-producing *L. parabuchneri* strains in the dairy industry, with the final purpose of preventing the accumulation of histamine in cheese.

6. Authors' contributions

Conceptualization, V.L. and M.A.A.; methodology, A.S., V.L., M.D., E.S.L., and M.A.A.; writing-original draft preparation, A.S.; review and editing, A.S., V.L., and M.A.A.; supervision, V.L., M.F. and M.A.A; Funding acquisition M.F. and M.A.A. All authors have read and agreed to the published version of the manuscript.

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9. Conflicts of interest

None to declare.

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11. Tables. Table 1. Bacterial strains, constructs and plasmids used in the present study.

Strains	**Relevant genotype, description, or properties**	**Reference or source**
Escherichia coli DH10B	F–, *mcrA* Δ(*mrr-hsdRMS-mcrBC*), ϕ80*lacZ*ΔM15, Δ*lacX74*, *recA1*, *endA1*, *araD139*, Δ (*ara-leu*)7697, *galU*, *galK*, λ– *rpsL*(Str^R), *nupG*	(Durfee et al., 2008)
Lactococcus cremoris NZ9000	*L. cremoris* MG1363: *nisRK*; plasmid-free	(Kuipers et al., 1998)
L. parabuchneri IPLA 11117, IPLA11122.	*Hdc* cluster, weak biofilm-producers.	(Maria Diaz et al., 2016)
L. parabuchneri IPLA 11125, IPLA11129.	*Hdc* cluster, moderate biofilm-producers.	(Maria Diaz et al., 2016a)
L. parabuchneri IPLA 11150, IPLA11151.	*Hdc* cluster, strong biofilm-producers.	(Maria Diaz et al., 2016)
E. coli IPLA10000	*E. coli* DH10B with plasmid pIPLA1300 carrying the *pilus* genes *tip pilin, basal pilin, major pilin, and sortC* - cloned; Km^r	This work
L. cremoris IPLA-pIL253	*L. cremoris* NZ9000 with pIL253 cloning vector; Em^r	This work
L. cremoris IPLA1301	*L. cremoris* NZ9000 harbouring plasmid pIPLA1301 with the *pilus* genes *tip pilin, basal pilin, major pilin, and sortC*; Em^r	This work
Plasmids		
pUK21	General cloning vector; Km^r	(Jeffrey and Joachim, 1991)
pIPLA1300	pUK21, *E. coli* plasmid with the pilus genes *tip pilin, basal pilin, major pilin, and sortC*- cloned; Km^r	This work
pIL253	High copy-number cloning vector for *Lactococcus*; Em^r	(Simon and Chopin, 1987)
pIPLA1301	pIL253 plasmid with the *pilus* genes *tip pilin, basal pilin, major pilin, and sortC*- cloned; Em^r	This work
pIPLA1302	*L. parabuchneri* plasmid containing the pilus genes -*tip pilin, basal pilin, major pilin, and sortC*	This work
Primers	**Sequence 5'-3'**	**Function**
Lpbsort 2	GCGGCTGATTCGCCTTGTTTATATAG	Amplification of the PGC cluster to

		clone it.
Tip-F	GATCTAATTCGTGATACATGGGC	*tip pilin* amplification (F). Cloning verification
Tip-R	TACACCTCCCATCTACGACGGG	*tip pilin* amplification (R) Cloning verification
Bpili-F	GTTACGTTGATAAAGGTGGTGGC	*basal pilin* amplification (F) Cloning verification
Mpili-R	CAGCTTGTTGAGTAACATCTGTAGG	*major pilin* amplification (R) Cloning verification
Mpili-F	TACAGATATAACTTCTACCCCG	*major pilin* amplification (F) Cloning verification
Sort-R	TTAATGGCCTCTACATAGAAGGG	*sortase C* amplification (R) Cloning verification
Sor-F	ACTGTTGGAGTGATTATCGCATTATA TCCC	*sortase C* amplification (F) Verification the presence of the PGC cluster
Sor-R	AACGTGATACAATCCTCCTGGC	*sortase C* amplification (R) Verification the presence of the PGC cluster
Lpb50_piliF	ATGGGCTGCAACTTTAGGTGAG	*tip pilin* to determine copy number (F)
Lpb50_piliR	CCAGTAGTTTCATGCGCTGTTC	*tip pilin* to determine copy number (R)
Lpb50_BasalF	TGGGTAACTTCCTCAGATCC	*basal pilin* to determine copy number (F)
Lpb50_BasalR	CCTTTGGCTGACTGAACTTCAC	*basal pilin* to determine copy number (R)
ET-TUF-F	TGGATGGTGCTATTCTGGTTGT	*ET-TUF* control chromosomal gene, to determine copy number (F)
ET-TUF-R	GCAAGCAAGATATGTTCACGAGTT	*ET-TUF* control chromosomal gene, to determine copy number (R)
GADPH-F	AACCGTACTGCCAGTGCTAACA	*GADPH* control chromosomal gene, to determine copy number (F)
GADPH-R	TGCAAGATCAGGGATAACCAAA	*GADPH* control chromosomal gene, to determine copy number (R)

Km^r, kanamycin resistant; Em^r, erythromycin resistant; -F, forward; -R, reverse.

Table 2. Unique and accessory genes found in the *L. parabuchneri* strains analysed. Unique genes are present in only one strain, while accessory genes are present in some, but not all the analysed strains.

Strain	Unique genes	Accessory genes
***L. parabuchneri* IPLA11117**	196	594
***L. parabuchneri* IPLA11122**	79	711
***L. parabuchneri* IPLA11125**	49	741
***L. parabuchneri* IPLA11129**	69	721
***L. parabuchneri* IPLA11150**	31	759
***L. parabuchneri* IPLA11151**	24	766

Table 3. qPCR results for the genomic DNA and plasmid DNA of *L. parabuchneri* IPLA11150 and 11151 strains.

Total DNA	**PGC genes**	**Amplification efficiency**	**Chromosomal genes**	**Amplification efficiency**	**Relative copy number (RCN)**
L. parabuchneri IPLA11150	*tip pili*	0.98	*ET TUF*	0.98	6.28 ± 1.66
	basal pilin	1.00	*GADPH*	1.01	9.56 ± 1.42
L. parabuchneri IPLA11151	*tip pili*	0.98	*ET TUF*	0.98	12.32 ± 1.42
	basal pilin	1.00	*GADPH*	1.01	6.74 ± 2.08
Plasmid DNA	**PGC genes**	**Amplification efficiency**	**Chromosomal genes**	**Amplification efficiency**	**Relative copy number (RCN)**
L. parabuchneri IPLA11150	*tip pili*	0.98	*ET TUF*	0.98	72.32 ± 33.4
	basal pilin	1.00	*GADPH*	1.01	88.71 ± 23.52
L. parabuchneri IPLA11151	*tip pili*	0.98	*ET TUF*	0.98	56.66 ± 22.14
	basal pilin	1.00	*GADPH*	1.01	44.29 ± 26.27

Suplementary Material The gene cluster associated with strong biofilm-formation capacity by histamine-producing *L. parabuchneri* encodes a sortase-mediated pilus and is located on a plasmid

Table S1. Genome features of the histamine-producing *L. parabuchneri* strains analysed in this work.

***L. parabuchneri* strains**	**Genome length (bp)**	**GC content (%)**	**Nº of contigs**	**Largest contig (bp)**	**N50 (bp)**	**Protein coding genes (CDS)**	**tRNAs**	**rRNAs**
IPLA11151	2,658,873	43.3	68	457,949	181,536	2,414	60	8
IPLA11150	2,689,337	43.3	74	726,833	181,610	2,474	58	9
IPLA11129	2,581,419	43.5	36	651,916	253,657	2,323	60	11
IPLA11125	2,581,796	43.5	32	647,647	200,485	2,322	58	11
IPLA11122	2,561,595	43.5	24	527,792	214,172	2,349	58	10
IPLA11117	2,769,427	43.2	67	676,120	177,745	2,550	58	9

Table S2. Annotation and localization (*locus_tag*) of the main features related to the safety and biofilm formation properties seen in the genomes of the histamine-producing *L. parabuchneri* strains analysed in this work.

Features

HDC genes cluster		***L. parabuchneri* IPLA11151**	***L. parabuchneri* IPLA11150**	***L. parabuchneri* IPLA11129**	***L. parabuchneri* IPLA11125**	***L. parabuchneri* IPLA11122**	***L. parabuchneri* IPLA11117**
Gene annotation	**Gene**	**Locus_tag**	**Locus_tag**	**Locus_tag**	**Locus_tag**	**Locus_tag**	**Locus_tag**
Basic amino acid/polyamine antiporter	*hdc C*	KVB92_RS09340	A7322_RS00285	KVB90_RS04480	KVB91_RS07940	A7B51_RS09970	A8O18_RS01770
Histidine decarboxylase, pyruvoyl type	*hdc A*	KVB92_RS09345	A7322_RS00290	KVB90_RS04475	KVB91_RS07945	A7B51_RS09965	A8O18_RS01765
DUF5449 family protein	*hdc B*	KVB92_RS09350	A7322_RS00295	KVB90_RS04470	KVB91_RS07950	A7B51_RS09960	A8O18_RS01760
Histidine--tRNA ligase	*his S*	KVB92_RS09355	A7322_RS00300	KVB90_RS04465	KVB91_RS07955	A7B51_RS09955	A8O18_RS01755
Sortase-mediated pilus gene cluster (SpaCBA-type)	**Gene**	***L. parabuchneri* IPLA11151**	***L. parabuchneri* IPLA11150**	***L. parabuchneri* IPLA11129**	***L. parabuchneri* IPLA11125**	***L. parabuchneri* IPLA11122**	***L. parabuchneri* IPLA11117**
Gene annotation	**Gene**	**Locus_tag**	**Locus_tag**	**Locus_tag**	**Locus_tag**	**Locus_tag**	**Locus_tag**
VWA domain-containing protein		KVB92_RS12360	A7322_RS08065	-	-	-	-
LPXTG cell wall anchor domain-containing protein		KVB92_RS12365	A7322_RS08070	-	-	-	-
SpaH/EbpB family LPXTG-anchored major pilin		KVB92_RS12370	A7322_RS08075	-	-	-	-
Class C sortase		KVB92_RS12375	A7322_RS08080	-	-	-	-
Genes involved in exopolysaccharide production		***L. parabuchneri* IPLA11151**	***L. parabuchneri* IPLA11150**	***L. parabuchneri* IPLA11129**	***L. parabuchneri* IPLA11125**	***L. parabuchneri* IPLA11122**	***L. parabuchneri* IPLA11117**
Gene annotation	**Gene**	**Locus_tag**	**Locus_tag**	**Locus_tag**	**Locus_tag**	**Locus_tag**	**Locus_tag**
GtrA family protein	-	KVB92_RS10540	A7322_RS00960	KVB90_RS08995	KVB91_RS09610	A7B51_RS11970	A8O18_RS01415
TIM barrel protein (a glucolytic enzyme)	-	KVB92_RS06150	A7322_RS10635	KVB90_RS11810	KVB91_RS03665	A7B51_RS07920	A8O18_RS10870
UDP-N-acetylglucosamine 2-epimerase (polysaccharide synthesis)	*wecB*	KVB92_RS02475	A7322_RS06650	KVB90_RS05960	KVB91_RS05995	A7B51_RS07480	A8018_RS07750
bifunctional lysyphosphatidylglycerol flippase/synthetase MprF	*mprF*	KVB92_RS04110	A7322_RS02235	KVB90_RS00270	KVB91_RS00245	A7B51_RS03135	A8018_RS02305
galactokinase	-	KVB92_RS04115	A7322_RS02240	KVB90_RS00275	KVB91_RS00250	A7B51_RS03130	A8018_RS02310
UDP-glucose 4-epimerase GalE	*galE*	KVB92_RS04120	A7322_RS02245	KVB90_RS00280	KVB91_RS00255	A7B51_RS03125	A8018_RS02315
UDP-glucose--hexose-1-phosphate uridylyltransferase	-	KVB92_RS04125	A7322_RS02250	KVB90_RS00285	KVB91_RS00260	A7B51_RS03120	A8018_RS02320
D-2-hydroxyacid dehydrogenase	-	KVB92_RS04130	A7322_RS02255	KVB90_RS00290	KVB91_RS00265	A7B51_RS03115	A8018_RS02325
sugar phosphate isomerase/epimerase	-	KVB92_RS05260	A7322 RS08760	KVB90_RS08410	KVB91_RS06580	A7B51_RS08905	A8O18_RS08775
L-ribulose-5-phosphate 4-epimerase	-	KVB92_RS05380	A7322_RS08640	KVB90_RS08290	KVB91_RS06460	A7B51_RS08785	A8O18_RS08655
aldose 1-epimerase family protein	-	KVB92_RS06380	A7322_RS10410	KVB90_RS04630	KVB91_RS03435	A7B51_RS07695	A8O18_RS00810
ribulose-phosphate 3-epimerase	*rpe*	KVB92_RS01830	A7322_RS03825	KVB90_RS01530	KVB91_RS01505	A7B51_RS10665	A8O18_RS03560
UDP-N-acetylglucosamine 2-epimerase (non-hydrolysing)	-	KVB92_RS02475	A7322_RS06650	KVB90_RS05960	KVB91_RS05995	A7B51_RS07480	A8O18_RS07750

[a] Bagel software was used to predict putative bacteriocin clusters in the genomes; only the locus_tag corresponding to the genes coding for bacteriocin peptides is indicated.

Table S3. *L. parabuchneri* strains with the pilus gene cluster.

Microorganism (contig)	Sequence ID	Contig Length	Q Cover (%)	Percentage identity (%)	Location
L. parabuchneri FAM 23169 (FAM23169_scf0047)	NZ_VBSV01000047.1	7311	100	99.46	ND
L. parabuchneri FAM23169 (FAM23169_scf_0070)	NZ_MSBD01000070.1	7311	100	99.46	ND
L. parabuchneri FAM23280 (FAM23280_scf_0032)	NZ_MSBG01000032.1	17168	98	96.99	ND
L. parabuchneri FAM23281 (FAM23281_scf_0048)	NZ_MSBH01000048.1	17257	98	96.99	ND
L. parabuchneri FAM23279 (FAM23279_scf_0052)	NZ_MSBF01000052.1	17261	98	96.99	ND
L. parabuchneri FAM23282 (FAM23282_scf_0029)	NZ_MSBI01000029.1	8065	98	96.97	ND
L. parabuchneri FAM21834 (FAM21834_scf_0082)	NZ_MSAV01000082.1	9362	97	96.76	ND
L. parabuchneri DSM 15352 (NODE_36)	NZ_JQBJ01000023.1	8917	88	97.33	ND

ND: Not determined.

Table S4. Strains from other lactobacilli with the pilus gene cluster.

Strain (contig)	Sequence ID	Length	Q Cover (%)	Percentage identity (%)	Location
Levilactobacillus brevis 30.8.38	NZ_JAJHVU010000076.1	7831	99	96.66	ND
Levilactobacillus brevis SRCM101174 plasmid pLB1174-2	NZ_CP021480.1	50390	99	96.63	Plasmid
Lactobacillus delbrueckii ME-783	NZ_BNHQ01000058.1	10670	79	95.77	ND
Lactobacillus delbrueckii subsp. *lactis* NBRC 3376	NZ_BJLK01000048.1	6882	79	95.76	ND
Lactobacillus delbrueckii subsp. *lactis* NCIMB 8183 (NCIMB8183-i1-1_scf33)	NZ_JAJNTQ010000033.1	8580	79	95.76	ND
Lactobacillus delbrueckii DSM 20073 (20073-i1-1_scf36)	NZ_JAJNTX010000036.1	8579	79	95.76	ND
Lactobacillus delbrueckii subsp. *Lactis* NCIMB 8964 (NCIMB8964-i11_scf33)	NZ_JAJNTO010000033.1	8580	79	95.76	ND

Lactobacillus delbrueckii subsp. *Lactis* DSM 20076 (DSM20076-i1-1_scf38)	NZ_JAJNTW010000038.1	8586	79	95.74	ND
Lactobacillus delbrueckii ME-797	NZ_BNID01000059.1	100068	79	95.33	Chromosome
Lactobacillus delbrueckii subsp. *bulgaricus* PB2003 (044-T3-4)	NZ_AEAT01000117.1	8989	79	95.33	ND
Lactobacillus delbrueckii subsp. *lactis* CIP110109 (CIP110109-i1-1_scf41)	NZ_JAJNUK010000041.1	13094	74	95.18	ND
Lactobacillus delbrueckii subsp. *lactis* DSM 20355 (DSM20355-i1-1_scf37)	NZ_JAJNTV010000037.1	8737	79	95.88	ND
Lactobacillus delbrueckii subsp. *lactis* KCTC 3035	NZ_CP018156.1	1972735	79	95.88	Chromosome
Lactobacillus delbrueckii ME-796	NZ_BNIC01000060.1	8458	69	95.97	ND
Lactobacillus delbrueckii ME-791	NZ_BNHY01000067.1	8458	69	95.97	ND
Lactobacillus delbrueckii ME-801	NZ_BNIH01000056.1	6174	63	96.23	ND
Lactobacillus delbrueckii subsp. *lactis* NWC_1_2	CP029250.1	2250954	75	93.18	Chromosome
Lactobacillus delbrueckii subsp. *lactis* MAG (MAG_rmk202_ldel)	CP046131.1	2166765	79	93.81	Chromosome
Lactiplantibacillus plantarum WS1.1	NZ_WWDA010000056.1	8280	88	96.30	ND
Lactiplantibacillus plantarum R62 (R62_1.clean_(paired)_contig_16)	NZ_JAJCUS010000016.1	17995	88	96.15	ND
Lactiplantibacillus plantarum R95 (R95_1.clean_(paired)_contig_100)	NZ_JAJCUP010000100.1	11303	88	96.15	ND
Lactobacillus plantarum LZ227 plasmid LZ227p5	CP015862.1	38212	88	97.36	Plasmid

ND: Not determined.

CAPITULO 4

Objetivo 6. Analizar, evaluar y proponer métodos bio-sostenibles para la prevención y eliminación de los biofilms formados por bacterias del ácido láctico productoras de aminas biógenas.

Tras la caracterización de los biofilms formados por distintas cepas de *L. parabuchneri* aislados de quesos, se analizó el efecto de detergentes (enzimáticos o alcalinos) y desinfectantes utilizados en las distintas superficies de la industria láctea. Se comprobó su eficiencia en diferentes superficies (plástico, goma, acero inoxidable y madera) para las cepas fuertes formadoras de biofilm, *L. parabuchneri* IPLA 11150 y 11151. Resultando todos ellos eficientes en las condiciones ensayadas.

Con el fin de proponer nuevas estrategias dirigidas a reducir el uso de detergentes y desinfectantes sintéticos, se estudió la capacidad de inhibición bacteriana y prevención de biofilms de BAL productoras de AB, mediante la aplicación de productos bio-sostenibles, como las bacteriocinas: nisina A y AS-48.

En el presente capítulo, se exponen los resultados obtenidos a los ensayos de comprobación de la acción de detergentes y desinfectantes, y también se incluyen 1 artículo y 1 manuscrito referidos a la aplicación de bacteriocinas en la prevención y eliminación de los biofilms formados por las cepas de *L. parabuchneri* productoras de histamina.

> **Artículo 4:** Luis Alberto Villarreal, Víctor Ladero, **Agustina Sarquis**, Beatriz Martinez, Beatriz del Rio, and Miguel A. Álvarez. Bacteriocins against lactic acid bacteria responsible for the accumulation of biogenic amines in cheese: Nisin A showed the broadest antimicrobial spectrum and even prevented the formation of biofilms. (Manuscrito preparado).
>
> **Artículo 5: Agustina Sarquis**, Luis Alberto Villarreal, Víctor Ladero, Mercedes Maqueda, Beatriz del Rio, and Miguel A. Álvarez. Enterocin AS-48 inhibits the growth and biofilm formation of lactic acid bacteria responsible for the accumulation of biogenic amines in cheese. (Artículo publicado).

Evaluación de detergentes y desinfectantes de la industria láctea frente a los biofilms formados por *L. parabuchneri* productores de histamina

La limpieza de las tuberías, de las estaciones de ordeño, de los tanques de almacenamiento, cisternas, superficies y útiles de trabajo relacionados con el tratamiento de la leche, su transformación y elaboración de productos lácteos, implican la eliminación de todos los residuos inorgánicos, principalmente carbonato cálcico y orgánicos tanto grasos como proteicos asociados a un producto tan rico y complejo como es la leche y sus derivados. Además, es necesario reducir o eliminar microorganismos alterantes o patógenos que en muchos casos pueden quedar protegidos mediante la formación de biofilms. Esto supone un reto adicional para el diseño de los sistemas de limpieza en la industria láctea donde en muchos casos, estos se deben implementar con sistemas de limpieza “in situ”, conocidos por sus siglas en inglés “cleaning in place” CIP.

Todo esto, requiere un enfoque multidisciplinar que debe cubrir diferentes aspectos que implican: el diseño higiénico de las instalaciones, el desarrollo de protocolos adecuados de limpieza y desinfección dirigidos a la eliminación de compuestos, microrganismos y en algunos casos, también los biofilms por estos formados contando con la necesaria monitorización de todo el proceso y sin olvidar que el producto final es un alimento que debe llegar al consumidor.

Productos desincrustantes, antiespumantes, detergentes alcalinos, clorados, en algunos casos espumantes que pueden incorporan también tensoactivos son los más habituales. El uso de detergentes enzimáticos ampliamente aplicados en otros sectores con resultados muy favorables tiene a día de hoy una aplicación reducida en la industria alimentaria, restringida a aplicaciones concretas como como son la eliminación de biofilms o alérgenos y la limpieza de membranas (Delhalle et al., 2020).

Combinaciones de detergentes de diferentes tipos: alcalino, alcalino-clorados con compuestos oxidantes o enzimáticos junto con biocidas o combinaciones de estos, han demostrado su eficacia frente a la suciedad de origen proteico y frente a diferentes tipos de biofilms. Los protocolos de limpieza optimizan también variables como temperatura, agitación, concentración y tiempo en función de la naturaleza y el tipo de superficie a tratar combinando en algunos casos métodos de choque con otros protocolos preventivos. Y en muchos casos en función de las superficies, la limpieza con

detergentes se combina con la aplicación posterior de desinfectantes de distinta naturaleza: ácido peracético, trialquilaminas, cloro, amonios cuaternarios y alcohol.

El objetivo de este capítulo era evaluar la eficacia de algunos de los tratamientos disponibles en el mercado frente a los biofilms formados por diferentes cepas de *L. parabuchneri* productoras de histamina.

Para abordar este trabajo se solicitaron los productos y protocolos de limpieza y desinfección que se emplean en la industria láctea y se adaptaron a los volúmenes de trabajo en el laboratorio.

Materiales y Métodos

Bacterias y condiciones de cultivo

Se seleccionaron dos cepas de *L. parabuchneri*, IPLA 11151 y 11150, que se caracterizan por su alta capacidad de producción de biofilms (M Diaz et al., 2016; Sarquis et al., 2023). Las cepas de *L. parabuchneri* se incubaron en medio MRS (Oxoid, Basingstoke, Hampshire, UK) a 37ºC en condiciones de anaerobiosis (gas anaerobio - 95% N2+ 5% H2) durante 24 h.

Formación de biofilms en superficies de distintos materiales

En este trabajo, las superficies ensayadas se presentaron en forma de cupones de 1 cm^2, y se seleccionaron los materiales que más frecuentemente se encuentran en la industria láctea y específicamente en la producción de quesos: acero inoxidable (AISI 304), madera de haya, plástico alimentario y goma o caucho (Sarquis et al., 2023). Previamente, los cupones se limpiaron con jabón y agua destilada estéril y fueron esterilizados en autoclave. Luego, se añadieron a tubos estériles con 10 mL de MRS y posteriormente se inoculó el cultivo en 10^6 UFC/mL de las cepas de *L. parabuchneri* y se incubaron por 48 hs a 37 ºC en condiciones normales de crecimiento de dichas bacterias. Tras el tiempo de incubación, los cupones fueron extraídos y se les aplicó los correspondientes métodos de lavado y desinfección. Estos ensayos se realizaron por triplicado técnico y biológico.

Tratamiento de limpieza y desinfección

Se seleccionaron tres tipos de detergentes que se comercializan para eliminar biofilms en la industria láctea (Tabla 1).

Se realizaron los tratamientos de limpieza y desinfección siguiendo los protocolos recomendados por las casas comerciales y detallados en la Tabla 1 y se evaluó la efectividad de estos tratamientos en la eliminación de los biofilms formados sobre las diferentes superficies previamente mencionadas, acero inoxidable, madera de haya, plástico y goma.

Tabla 1. Tratamientos utilizados.

Nombre del Tratamiento	Tipo de Detergente	Nombre comercial	Tratamiento: concentración en % y temperatura
D1	Alcalino	VIXFILM	5% a 50^{0}C
D2	Alcalino clorado	VIXCLOR	5% a 50^{0}C
D3	Neutro con enzimas	ENZIVIX30	3% a 50^{0}C

En un tubo estéril con medio MRS se inocularon 10^6 UFC/mL de cada uno de los cultivos seleccionados para este estudio: *L. parabuchneri* IPLA 11151 y 11150. En cada uno de estos tubos se incluyó un cupón de (1x1 cm^2) de los materiales a estudiar y se incubaron tal como se indicó en el apartado anterior.

Finalizado el tiempo de incubación, se retiró el medio y se lavó el material ensayado con agua destilada estéril para eliminar las células débilmente adheridas. A continuación, durante quince minutos se realizó el tratamiento con detergente, como se indica en la Tabla 1. Se incluyó como control para cada una de las cepas y materiales una muestra a la que no le aplicó el tratamiento con detergente sino únicamente PBS.

Una vez eliminado el detergente, la mitad de las muestras fueron tratadas con un desinfectante, un biocida en este caso ácido peracético espumante al 1,5 % (Asepbact) que se utiliza para superficies alimentarias. Las muestras se trataron durante 15 minutos, a temperatura ambiente, y al 3% con este compuesto comercial y a continuación, se realizaron dos lavados con agua destilada estéril.

De todas las muestras se recogieron las células adheridas con un hisopo estéril, se realizaron diluciones y se sembraron en placas de MRS que se incubaron a 37 ºC en anaerobiosis durante 48 h para determinar las células bacterianas viables.

Resultados

El efecto de los detergentes ensayados de forma aislada o en combinación con el tratamiento de desinfección sobre los biofilms formados en diferentes superficies, se recogen en las Figuras 1 y 2.

En el caso de la cepa *L. parabuchneri* 11150 (Figura 1), ninguno de los tres detergentes aplicados funcionó en todas las superficies. El D3 que incluía enzimas en su composición resultó el más efectivo de los tres ensayados en cuanto al número de superficies sobre las que era activo. Aunque sobre madera no pareció tener ningún efecto. El D1, un detergente alcalino, resultó el menos efectivo para *L. parabuchneri* IPLA 11150, puesto que solo logra eliminar el biofilm en goma y en las otras superficies solo se observó una ligera reducción del biofilm formado.

Es interesante destacar que, en todos los casos, la combinación de detergente con desinfectante permitió la eliminación de los biofilms formados por la cepa *L. parabuchneri* IPLA 11150 en todas las superficies ensayadas, excepto en el caso del tratamiento con D1 donde se observó una importante reducción del biofilm formado sobre madera pero no una eliminación completa.

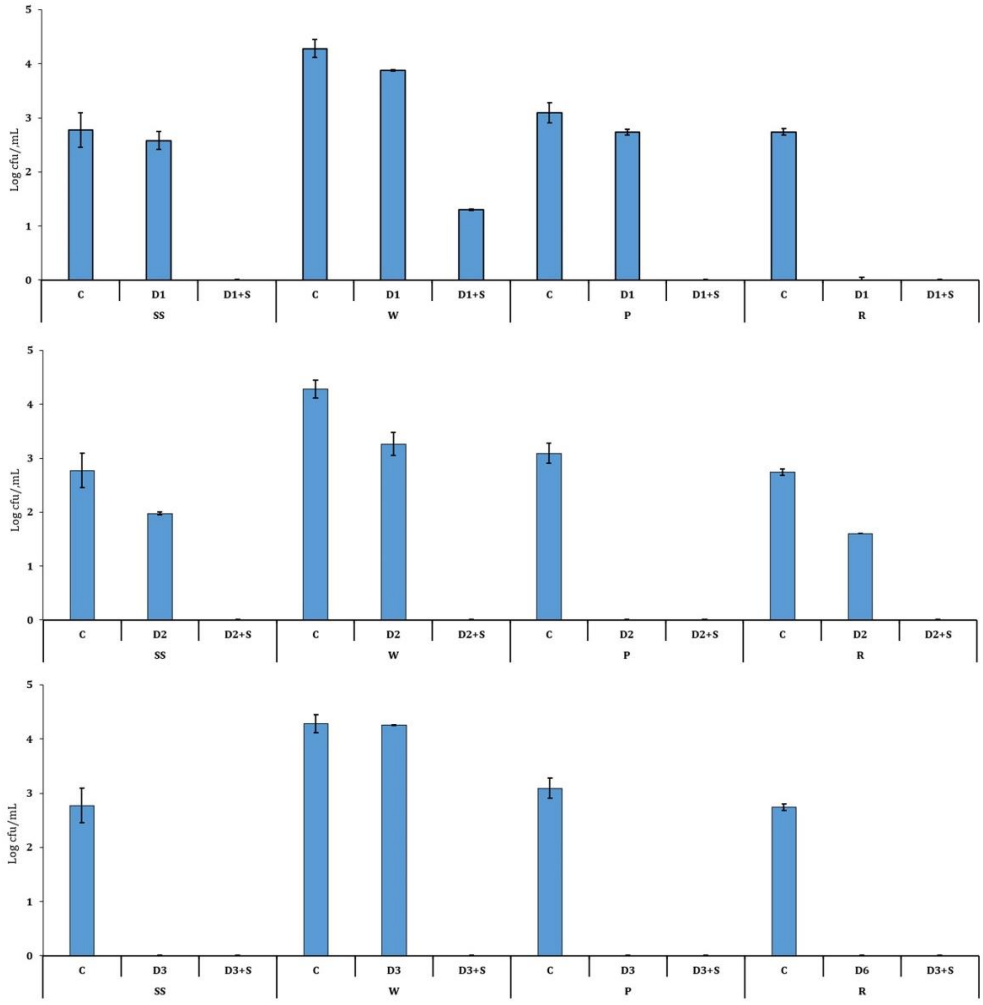

Figura 1. Efecto del detergente y de la combinación de detergente y desinfectante en la eliminación de los biofilms formados por la cepa *L. parabuchneri* IPLA11150 en diferentes superficies. Se representan las células viables frente al tratamiento con tres tipos de detergentes D1, D2 y D3 y tras la combinación con desinfectante D+S. Los ensayos se realizaron en cuatro superficies (SS: acero inoxidable; W: madera; P: plástico; R: goma). Como control (C) se incluye un tratamiento con PBS sin tratamiento de detergente, ni desinfectante.

Los resultados obtenidos para la cepa *L. parabuchneri* IPLA 11151 se muestran en la Figura 2. Los tres detergentes se comportaron del mismo modo en todas las superficies ensayadas. Es decir, los tres fueron efectivos frente a los biofilms formados en acero inoxidable y goma pero ninguno fue efectivo frente a los biofilms formados en madera y plástico.

La acción secuencial de detergente y desinfectante resultó más exitosa en el caso de los detergentes D2 y D3 pero en el caso del detergente D1, el tratamiento consecutivo de detergente y desinfectante aunque reduce los biofilms formados, especialmente en el caso de madera, no elimina por completo estas biopelículas.

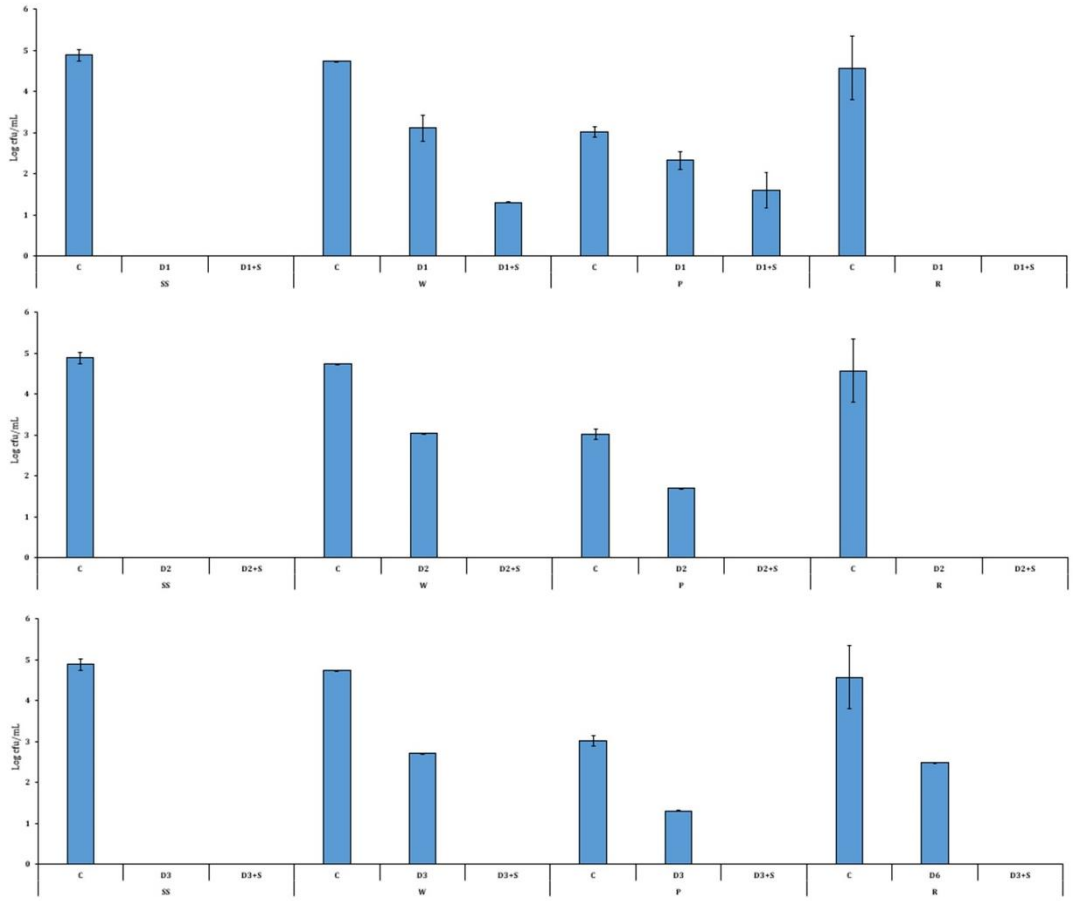

Figura 2. Efecto del detergente y de la combinación de detergente y desinfectante en la eliminación de los biofilms formados por la cepa *L. parabuchneri* IPLA 11151 en diferentes superficies. Se representan las células viables frente a tres tipos de detergentes D1, D2 y D3 y tras la combinación con desinfectante D+S en cuatro superficies (SS: acero inoxidable; W: madera; P: plástico; R: goma). Como control (C) se incluye un tratamiento con PBS sin tratamiento de detergente, ni desinfectante.

Los resultados obtenidos sugieren que la eficacia de los tratamientos no solo está condicionada por el tipo de superficie sino también por la cepa formadora de biofilms y evidencian la importancia del estudio de la estructura y composición de la matriz del biofilm que puede ser determinante para el éxito de la estrategia de limpieza y desinfección aplicada. Algunos autores han demostrado también que la aplicación indiscriminada de determinados tratamientos puede provocar la aparición de cepas tolerantes a los productos químicos utilizados para la limpieza y desinfección (Fagerlund et al., 2017) o favorecer la selección y propagación de bacterias patógenas resistentes a biocidas (Dancer, 2014; Vergnano, 2015). Todo ello determina la necesidad de desarrollar nuevas alternativas efectivas frente a la eliminación de biofilms que

puedan ser sostenibles con el medio ambiente y reduzcan la posible aparición de resistencias microbianas.

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Bacteriocins against lactic acid bacteria responsible for the accumulation of biogenic amines in cheese: Nisin A showed the broadest antimicrobial spectrum and even prevented the formation of biofilms

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Abstract

Cheese is one of the foods in which toxic concentrations of biogenic amines (BA) can be reached, mainly as consequence of the decarboxylative metabolism of certain Lactic Acid Bacteria (LAB). To maintain the food safety of cheese, it is necessary to develop strategies that, in a specific and environmentally friendly way, prevent the growth of such BA-producing LAB and BA accumulation. Some natural compounds with a great potential as food biopreservatives are bacteriocins produced by LAB. This work was aimed to evaluate the antimicrobial potential of seven bacteriocin-containing cell-free supernatants (CFS) of LAB against forty-eight strains belonging to the LAB species mainly responsible for the accumulation of the most relevant BA in cheese i.e. histamine, tyramine and putrescine. The CFS tested were coagulin A-CFS, enterocin A-CFS, enterocin P-CFS, lacticin 481-CFS, nisin A-CFS, nisin Z-CFS and plantaricin A-CFS. The susceptibility of BA-producing strains to bacteriocin-containing CFS was strain-dependent. The histamine-producing species showing the broadest spectrum of sensitivity were *L. parabuchneri* and *Pediococcus parvulus*. Moreover, *Enterococcus faecium* was the tyramine-producing species showing the broadest spectrum of sensitivity towards CFS, and *Enterococcus faecalis* and *Enterococcus hirae* were among the most sensitive putrescine producer species. In general, Nisin A- and nisin Z-CFS were the ones showing the broadest antimicrobial spectrum, being active against 31 and 27 out of the 48 BA-producing strains, respectively. Moreover, commercial nisin A prevented the biofilm formation in 67% of the BA-producing, biofilm-forming LAB strains. This study remarks the potential of bacteriocins against BA-producing LAB and supports the use of nisin A as a food grade biopreservative to control BA-producing LAB in cheese and thereby to reduce BA accumulation.

Keywords: Food Safety; Histamine; Tyramine; Putrescine

1. Introduction

Biogenic amines (BA) are nitrogenous organic compounds synthetized by most living beings (plants, animals and microorganisms), which play important roles in many of their physiological activities (del Rio et al., 2018). The biosynthesis of BA involves the enzymatic decarboxylation of certain amino acids. For example, histamine and tyramine are produced by the decarboxylation of histidine and tyrosine, respectively. Therefore, the metabolism of microorganisms present in food with the corresponding amino acid-decarboxylase activity is responsible of the accumulation of high concentrations of BA (Linares et al., 2012). Abundant scientific evidence recognizes that the consumption of foods with high concentration of BA can have harmful effects on human health, the severity of which can be greater or lesser depending on the BA -or their combination- and the dose ingested (Ladero, Calles-Enriquez, et al., 2010). Due to their toxicity, their presence in foods must be avoided (Ruiz-Capillas & Herrero, 2019).

Cheese is one of the fermented foods in which the highest concentrations of toxic BA such as histamine, tyramine and putrescine have been found (European Food and Safety Authority (EFSA), 2011; Redruello et al., 2013), even exceeding 1,000 mg kg^{-1} (Diaz, Ladero, del Rio, et al., 2016). The microorganisms responsible of BA accumulation in cheese are mainly species that belong to the group of Lactic Acid Bacteria (LAB), which form part of the normal microbiota of milk and cheeses, such as the ones belonging to the genera *Enterococcus, Lactococcus, Streptococcus*, and the former genera *Lactobacilli* (Linares et al 2012). Regarding histamine, the main responsible for its accumulation in cheese is *L. parabuchneri* (Berthoud et al., 2017; Diaz, del Rio, et al., 2016; Diaz, Ladero, Redruello, et al., 2016). Other LAB species isolated from cheese such as *Limosilactobacillus reuteri* (Diaz, Ladero, Redruello, et al., 2016) and *Limosilactobacillus vaginalis* (Diaz et al., 2015) have been also found to be histamine-producers. The most abundant tyramine producers in cheese are species belonging to the *Enterococcus* genus, such as *Enterococcus hirae*, *Enterococcus durans*, *Enterococcus faecalis* and *Enterococcus faecium* among others (Ladero et al., 2017). In these three later species the capacity to produce tyramine has been defined as a species-level trait (Ladero, Fernández, et al., 2012). Moreover, *E. faecalis* has been identified as the main responsible for the accumulation of tyramine at high concentrations in cheese (Ladero, Fernández, et al., 2010; O'Sullivan et al., 2015). In addition, some strains of different

lactobacilli species have also been described as tyramine producers, such as *Levilactobacillus brevis* or *Latilactobacillus curvatus* (Ladero et al., 2015), being predominant in some cheeses (Ladero, Fernández, et al., 2010). Similarly, different species of LAB has been identified as putrescine producers in this food. It is noteworthy, that some of the aforementioned tyramine producers have the capacity to also produce putrescine such as *E. faecalis* -a species-level trait for this species (Ladero, Fernández, et al., 2012)-, *E. hirae*, *L. brevis* and *L. curvatus* (Ladero, Cañedo, et al., 2012) . In addition, some strains of *Lactococcus cremoris* and *Lactococcus lactis* isolated from cheeses, have been described as putrescine-producers (Ladero, Rattray, et al., 2011), which is an important issue since these two taxa are the major starter cultures used in cheese production.

Importantly, we found that many of those BA-producing LAB species are able to form biofilms on stainless steel (Diaz, del Rio, et al., 2016; Diaz, Ladero, del Rio, et al., 2016), which is the most common material used in the industrial equipment involved in the production and processing of cheese. Biofilms of BA-producing LAB are a cause of concern as they pose a risk to the food safety of cheeses; biofilms act as reservoir of BA-producing LAB that can contaminate cheeses during their manufacturing and post-ripening processing and has as a final consequence in the accumulation of high amounts of BA in the final product (Diaz, del Rio, et al., 2016; Diaz, Ladero, del Rio, et al., 2016). Moreover, the biofilm growth mode increases the microbial resistance to diverse environmental challenges such as disinfectants and cleaning products used in sanitation routines of dairy plants (Diaz, del Rio, et al., 2016; Diaz, Ladero, del Rio, et al., 2016). Therefore, the prevention of biofilm formation is of great importance to food industries in general and specifically to cheese factories (Vishwakarma, 2020).

A major concern is that the BA-producing LAB mentioned above, belong to LAB species that also play an essential role in the fermentation process and are responsible for the organoleptic characteristics of the final product. This fact is crucial for designing strategies aimed to prevent the presence of BA-producing LAB and avoid the accumulation of BA in cheese; the measures taken must specifically prevent the presence and growth of BA-producing LAB, without affecting other microorganisms which are essential for the production process (Linares et al., 2012). The use of bacteriocins could be a useful strategy towards the selective elimination of BA-

producing LAB during cheese manufacturing and processing. Bacteriocins, are small ribosomally synthesized peptides with antimicrobial activity, produced by bacteria. Those produced by LAB are of great interest for food industry, since they are considered to be food grade natural compounds safe for consumption, with valuable technological properties (i.e. heat stability, lack of toxicity, no influence in flavour) (Alvarez-Sieiro et al., 2016). Bacteriocins have traditionally been used as food preservatives to improve shell-life, directly added as an ingredient, such as nisin or pediocin PA-1, or by the inclusion of a bacteriocin-producing LAB strain as part of the starter culture used to lead the fermentation process (Daba & Elkhateeb, 2020). In dairy products, the addition of bacteriocins have been shown to inhibit the growth of pathogenic bacteria such as *Listeria monocytogenes* (García-Díez & Saraiva, 2021), or spoilage bacteria such as coliforms (Fernández et al., 2014) or *Clostridium tyrobutyricum* (Ávila et al., 2020). Theses bioactive compounds have been also included into packaging films to control the growth of pathogenic bacteria in which their gradual release into the food matrix allowed for an increased shell-life of products (Guerra et al., 2005). However, there are a few studies that have examined the antimicrobial effect of bacteriocins-produced by LAB or their cell-free supernatants (CFS) towards BA-producing LAB and their capability to prevent the formation of their biofilms.

In this work, we have examined the susceptibility spectrum of histamine-, tyramine- and putrescine-producing LAB strains mainly isolated from dairy foods, against seven bacteriocin-containing CFS produced by four different dairy LAB species. In addition, the potential of commercial nisin A - the bacteriocin which showed the broadest antimicrobial spectrum in this study - to prevent the formation of biofilms of BA-producing LAB was assessed.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

The bacteriocin-producing bacterial strains used in this work as well as the bacteriocins produced by each of them, are shown in Table 1. They belong to the species *Lactiplantibacillus paraplantarum* (1), *Enterococcus faecium* (2), *Lactococcus lactis* subsp. *lactis* (3), and *Lactiplantibacillus plantarum* (1). Table 2 shows the forty-eight BA-producing bacterial strains used in this work: 16 histamine-producers

(*Lentilactobacillus hilgardii* [1], *L. parabuchneri* [6], *Limosilactobacillus reuteri* [1], *Limosilactobacillus vaginalis* [5], *Pediococcus parvulus* [1], and *Streptococcus thermophilus* [2]); 10 tyramine-producers (*Enterococcus durans* [5], *Enterococcus faecium* [4], and *Latilactobacillus curvatus* [1]); 11 tyramine- and putrescine-producers (*Enterococcus faecalis* [5], *Enterococcus hirae* [1], *Levilactobacillus brevis* [3], and *Latilactobacillus curvatus* [2]); and 11 putrescine-producers (*Levilactobacillus brevis* [1], *Lactococcus cremoris* [5], and *Lactococcus lactis* subsp. *lactis* [5]).

These strains were previously isolated from dairy, wine or human sources. The indicator strain used to determine the antimicrobial spectrum of bacteriocins was *Latilactobacillus sakei* CECT 906^T (Colección Española de Cultivos Tipo [CECT], Spain), a bacterium determined in preliminary works to be sensitive to the bacteriocins used in this study (data not shown).

Enterococci, lactococci and streptococci were grown in M17 (Oxoid, Basingstoke, UK) supplemented with 5 g L^{-1} glucose (GM17). Lactobacilli were grown in Man, Rogosa and Sharpe (MRS) medium (Oxoid). For the determination of the minimum inhibitory concentration (MIC) values, enterococci and lactococci were grown in Iso-Sensitest medium (IST, Oxoid), and lactobacilli and pediococci in LSM media (90% IST, 10% MRS) (Flórez et al., 2016). Enterococci, lactococci, pediococci and *L. sakei* CECT 906^T were grown at 32ºC, the other lactobacilli at 37ºC, and the streptococci at 42 ºC. Enterococci, lactococci and lactobacilli were incubated without aeration; pediococci and streptococci were incubated under anaerobic conditions (10% H_2, 10% CO_2 and 80% N_2) in a Mac500 chamber (Down Whitley Scientific, Bradford, UK).

2.2. Preparation of bacteriocin-containing cell-free supernatants (CFS)

Fifty mL of cultures of the bacteriocin-producing strains were grown at the corresponding temperature for 24 h. Bacteriocin-containing CFS (namely coagulin A-CFS, enterocin A-CFS, enterocin P-CFS, lacticin 481-CFS, nisin A-CFS, nisin Z-CFS or plantaricin A-CFS) were collected after centrifugation at 10,000 x *g* during 10 min, neutralized to pH 7.0 ± 0.2 with NaOH, and sterilized by filtration through a 0.20 μm pore diameter membrane (Millipore, Bedford, MA, USA). Supernatants were stored at -20ºC until used.

2.3. Antimicrobial spectrum of bacteriocin-containing CFS

The antimicrobial activity of bacteriocin-containing supernatants against BA-producing LAB was determined using the agar-well diffusion assay essentially as described by (Schillinger & Lücke, 1989) with some modifications. Briefly, MRS or GM17 agar (12 g L^{-1}) plates inoculated with 10^5 cfu mL^{-1} of an overnight culture of the corresponding BA-producing strain were prepared (the indicator strain *L. sakei* CECT 906^T was used as a positive control of the antimicrobial activity of the bacteriocin-containing CFS). Five mm-diameter wells were then excavated in the agar and 30 µL of the bacteriocin-containing supernatants added to each. The plates were then incubated at the corresponding temperature for 48 h, and the diameter of any inhibitory halo around the wells measured. An inhibitory zone of >13 mm in diameter was deemed to indicate strong inhibition, one between 8 and 13 mm to indicate moderate inhibition, and one measuring <8 mm to indicate no inhibition. Tests were performed at least in duplicate.

2.4. Prevention of biofilm formation by nisin A

The potential of commercial nisin A (Sigma-Aldrich, Madrid, Spain) to prevent biofilm formation was examined using the strongest biofilm-forming strains of the examined species -as reported by (Diaz, Ladero, del Rio, et al., 2016)- i.e., *Enterococcus durans* IPLA 655, *Enterococcus faecalis* 15a, *Enterococcus faecalis* 52C, *Enterococcus faecium* spp_R, *Enterococcus hirae* 268, *Levilactobacillus brevis* CECT 3811, *Latilactobacillus curvatus* VI16, *L. parabuchneri* IPLA 11150, *Limosilactobacillus reuteri* IPLA 11078, *Limosilactobacillus vaginalis* IPLA 11068, *Lactococcus cremoris* CECT 8666 and *Lactococcus lactis* subsp. *lactis* 1AA59.

96-well microtiter plates (Nunc Micro Well Plates with a Nunclon Delta Surface, [Thermo Fisher Scientific, Waltham, MA, USA]) were prepared with nisin A (0.60 µM) or sterilized water as a negative control. After allowing the wells to air-dry, 200 µL of the BA-producing strains (10^6 CFU mL^{-1}) were introduced and incubated for 48 h under standard conditions to allow for biofilm formation. The cells were then stained with crystal violet (CV) following the protocol of (Diaz, Ladero, del Rio, et al., 2016) with some modifications. Briefly, each well was cleaned twice with 200 µl of phosphate buffered saline to remove any non-adhered cells. Then, after 30 min drying in a flow cabinet, 250 µL of CV (5 g L^{-1} in distilled water) were added. After 30 min, any biofilm present was rinsed three times with 200 µL distilled water. Finally, the bound dye was solubilized in 250 µL of acetic acid (330 ml L^{-1}) and the absorbance at 595 nm

measured using a Benchmark Plus microplate spectrophotometer (BioRad, Hercules, CA, USA).

2.5. Statistical analysis

Means ± standard deviations were calculated (from at least three independent replicates) and compared using the Student t-test of the SigmaPlot version 15.0 software. Significance was set at $p<0.05$.

3. Results and Discussion

3.1. Sensitivity of BA-producing LAB to bacteriocin-containing CFS

Table 3 shows that the susceptibility of BA-producing strains to bacteriocin-containing CFS was strain-dependent. Below it is described the sensitivity of histamine-, tyramine-, or putrescine-producing strains towards CFS.

3.1.1. Sensitivity of histamine-producing strains

P. parvulus was the histamine-producing species showing the broadest spectrum of sensitivity towards the CFS, since it was inhibited by all the bacteriocin-containing CFS tested (excepting the enterocin A-CSF). This specie was strongly inhibited (inhibitory halos >13 mm in diameter) by coagulin A-, enterocin P-, nisin A-, and nisin Z-CFS, and moderately inhibited (inhibitory halos between 8 and 13 mm in diameter) by lacticin 481-and plantaricin C-CFS. The following most sensitive specie was *L. parabuchneri*; all the strains belonging to this species were strongly inhibited by the action of enterocin P-, nisin A- and nisin Z-CFS, while they were resistant to coagulin A-, enterocin A-, lacticin 481-, and plantaricin C-CFS (excepting IPLA 15009 that was moderately inhibited by the later CFS). Moreover, *L. hilgardii* 321 as well as *L. reuteri* IPLA 11078 (the only strains of these species tested in this study) were only sensitive to nisin A-CFS (strong inhibition) and to nisin Z-CFS (moderate inhibition). Similarly, all the *L. vaginalis* strains were exclusively sensitive towards nisin A- and nisin Z-CFS. An exception was the strain IPLA 11054, which was resistant to all the CFS tested in this study. Finally, *S. thermophilus* was the most resistant histamine-producing species, since the two strains tested were only inhibited (although strongly) by plantaricin C-CFS; the strain CHCC 1524 was also strongly inhibited by lacticin 481-CFS.

The CFS that exhibited the broadest antibacterial spectrum against the 16 histamine-producing strains tested were those containing nisin A and nisin Z. Both CFS had a similar antimicrobial spectrum as previously described by (de Vos et al., 1993). Nisin A- and nisin Z-CFS inhibited the growth of 87% of the strains, which included all the ones belonging to the species *L. hilgardii*, *L. parabuchneri*, *L. reuteri*, *L. vaginalis* (excepting the strain IPLA 11054), and *P. parvulus*. Moreover, 1 strain was sensitive to coagulin A-CFS, 8 strains to enterocin P-CFS (all strains belonging to the *L. parabuchneri* species, and *P. parvulus* 276), 2 to lacticin 481-CFS, and 4 to plantaricin C-CFS. All the strains were resistant to the action of enterocin A-CFS.

A very few studies have examined the antimicrobial effect of CFS enriched in bacteriocins towards the population of histamine-producing LAB and their effect on the modulation of BA-accumulation. The sensitivity of *L. parabuchneri* to nisin found in this study was in agreement with previous studies performed by (Joosten & Nuñez, 1996), which found a panel of histamine-producing *L. buchneri* strains to be sensitive to a CSF of the nisin-producing *L. lactis* subsp. *lactis* ESI 561 strain. Moreover, (Tabanelli et al., 2014) showed that the co-culture of some *L. lactis* subsp. *lactis* strains producing either nisin Z or lacticin 481, in combination with histamine-producing *S. thermophilus*, induced the death or inhibit the growth of the strain, and also reduced histamine accumulation.

3.1.2. Sensitivity of tyramine-producing strains

E. faecium was tyramine-producing species showing the broadest sensitivity spectrum towards the CFS, as it was inhibited by four out the seven bacteriocin-containing CFS tested: coagulin A-CFS (strong inhibition), enterocin P-CFS (moderate inhibition), and nisin A-, and nisin Z-CFS (some strains showed strong inhibition and others a moderate inhibition). All the strains showed a similar inhibitory pattern, excepting *E. faecium* spp_R, which was more sensitive as it was also moderately inhibited by enterocin A- and lacticin 481-CFS. *L. curvatus* and *E. hirae* were sensitive to 3 out of the 7 CSF tested; both were inhibited by coagulin A-CSF (excepting the strains *L. curvatus* VI16 that was resistant). In addition, *L. curvatus* was strongly inhibited by nisin A- and nisin Z-CFS (VI16 was moderately inhibited), and *E. hirae* by enterocin A- and enterocin P-CSF (moderate inhibition). The species *E. durans* and *E. faecalis* showed a narrow sensitivity spectrum. Most of the strains belonging to those species were strongly

inhibited by coagulin A-CFS, and some strains (e.g. *E. durans* IPLA 655 and *E. faecalis* 52C) were also moderately inhibited by nisin A- and nisin Z-CFS. The less susceptible species was *L. brevis;* it also showed a narrow susceptibility pattern since it was only inhibited by enterocin P- and nisin A-CFS (moderate inhibition).

The coagulin A-CFS showed the broadest antibacterial spectrum against the 21 tyramine-producing strains tested (including those that also produced putrescine) among all the CFS. It inhibited the growth of 81% of tyramine-producing strains, which comprised all the strains belonging to the species *E. durans*, *E. faecium*, *L. curvatus* (excepting the tyramine- and putrescine-producer *L. curvatus* VI16), *E. faecalis*, and *E. hirae* 268 (the only strain of this species tested). Nisin A-CFS also showed a broad antibacterial spectrum, since it affected the growth of 71% of the tyramine-producing strains, which comprised all the strains belonging to *E. faecium*, *L. curvatus*, and *L. brevis* species, and some strains belonging to the *E. durans* species (e.g. IPLA 665) and *E. faecalis* (e.g. 52C). Nisin Z-CFS showed a narrower antibacterial spectrum compared to nisin A-CFS, despite nisin Z has been described to have a higher solubility and diffusivity than nisin A (Gharsallaoui et al., 2016). Nisin Z inhibited the growth of 52% of the strains, and it had no effect towards *L. brevis* species. Enterocin P-CFS had antibacterial effects against 43% of the strains, which comprised all the strains belonging to the species *E. faecium*, *E. hirae* 268 (the only strain of this species tested), and *L. brevis,* and the *L. curvatus* VI14 strain. Tyramine-producing strains were highly resistant to the action of enterocin A- and lacticin 481-CFS; only *E. faecium* spp_R and *E. hirae* 268 were sensitive to enterocin A-CSF (*E. faecium* spp_R was also sensitive to lacticin 481-CFS). All the tyramine-producing strains were resistant to plantaricin C-CFS.

Some studies have described the susceptibility of certain tyramine-producing LAB species to CFS of bacteriocin-producing LAB. For instance, (Joosten & Nuñez, 1996) found the CFS of the nisin-producing *L. lactis* subsp. *lactis* ESI 561 strain, to inhibit the growth of a great number of tyramine-producing *L. brevis* strains. Also (Purevdorj et al., 2021) found that both nisin-producing *Lactococcus lactis* strains and their CFS inhibited the growth of tyramine-producing LAB strains belonging to the species *Lb. curvatus*, *Lb. brevis* and *Lb. plantarum*, and besides inhibited the tyramine production. Other studies described that the co-culture of some *L. lactis* subsp. *lactis* strains

producing either nisin Z or lacticin 481, in combination with tyramine-producing *E. faecalis* strains, significantly reduced the growth and BA production of the amino biogenic strain (Tabanelli et al., 2014).

3.1.3. Sensitivity of putrescine-producing strains

E. faecalis and E. hirae were among putrescine-producing species showing the broadest spectrum of sensitivity towards the CFS. As described above in *section 3.1.2*, all the strains of the *E. faecalis* species were strongly inhibited by coagulin A-CFS; additionally, one strain (BA64) was moderately inhibited by both nisin A- and nisin Z-CFS, and other (52C) by nisin A-CFS (moderate inhibition). *E. hirae* was inhibited by 3 out the 7 bacteriocin-containing CFS tested, i.e. coagulin A-CFS (strong inhibition), and enterocin A-, and enterocin P-CFS (moderate inhibition).

L. brevis and *L. curvatus* species were both sensitive to nisin A-CFS: all the strains of *L. brevis* were moderately inhibited while *L. curvatus* strains showed a strong inhibition. Additionally, all the strains of *L. brevis* were moderately inhibited by enterocin P-CFS (the strain 3824 was also inhibited by nisin Z-CFS), and all the strains of *L. curvatus* to nisin Z-CFS (the strain VI14 was also moderately inhibited by coagulin A-CFS). Interestingly, the putrescine-producing lactococci were highly resistant to the bacteriocin-containing CFS; only two *L. cremoris* strains (3AA9 and 3AA23) were moderately inhibited by nisin A- and nisin Z-CFS. All the *L. lactis* subsp. *lactis* strains were resistant to the action of all the bacteriocin containing-CSF tested.

Nisin A-CFS was the CFS showing the broadest antibacterial spectrum against the 22 putrescine-producing strains tested (including those that also produced tyramine). It inhibited the growth of 45% of the putrescine-producing strains, comprising all the strains belonging to the species *L. brevis*, and *L. curvatus*, and only two *L. cremoris* strains (i.e. 3AA9 and 2AA23). Coagulin A-CFS inhibited the growth of 32% of the strains, including all the *E. faecalis* strains, *E. hirae* 268, and one *L. curvatus* strain (VI14). Interestingly, it only affected the growth of strains producing both tyramine and putrescine, but it had no effect on the strains that only produced putrescine, such as the ones belonging to the *L. cremoris* and *L. lactis* species. Enterocin P-CFS affected the growth of 6 strains, which included *E. hirae* 268, all the strains belonging to the *L. brevis* species, and one *L. curvatus* strain (VI14). Nisin Z-CFS showed a narrower

antibacterial spectrum compared to nisin A-CFS (it inhibited 6 strains); it differed from the later in that it had much lower effects towards *L. brevis* species. Enterocin A-CFS had a very narrow antibacterial spectrum, since it only inhibited the growth of one strain, *E. hirae* 268. Regarding lacticin 481-CFS and plantaricin C-CFS, they had no effect towards any of the putrescine-producing strains tested.

The capability of coagulin A-CFS (and at lesser extent nisin A- and nisin Z- CFS) to inhibit the growth of *E. faecalis* is technologically relevant since putrescine biosynthesis was found to be a species-level trait of *E. faecalis* (Ladero, Fernández, et al., 2012) whose presence has been largely associated to the accumulation of putrescine -in addition to tyramine- in cheese (Ladero, Fernández, et al., 2012); the presence of *Enterococcus* with putrescine (and tyramine) producing capability may be avoided by means the use of such bacteriocins during cheese manufacturing. It is important to highlight the absence of sensitivity of putrescine-producing lactococci against all the CFS tested. It should be remembered that *L. lactis* and *L. cremoris* are widely used as primary starter cultures in the manufacturing of cheese. Since putrescine production in lactococci is a strain-dependent trait (Ladero, Rattray, et al., 2011), it is advisable to take this characteristic into account and select non-putrescine producing starters.

3.2. Prevention of biofilm formation by nisin A

Since nisin A-CFS was de supernatant with the broadest spectrum of action towards BA-producing LAB, we further assess the capacity of commercial nisin A to prevent the formation of biofilms of those bacteria, which were previously reported to form strong biofilms (Diaz, del Rio, et al., 2016; Diaz, Ladero, del Rio, et al., 2016). Nisin A significantly inhibited biofilm formation in 8 out the 12 examined strains (Fig. 1). The capacity of this bacteriocin to reduce biofilm formation ranged from 84% in *L. vaginalis* IPLA 11068 (Fig. 1J) to 27% in *E. faecalis* 52C (Fig. 1C).

Importantly, nisin A prevented biofilm formation of all BA-producing strains belonging to genera of the taxon formerly known as *Lactobacillus* such as the histamine-producers *L. parabuchneri* IPLA 11150 (Fig. 1H), *L. reuteri* IPLA 11078 (Fig. 1I) and *L. vaginalis* IPLA 11068 (Fig. 1J). The prevention of biofilm formation of *L. parabuchneri* IPLA 11150 is of technological relevance for dairy industry and the food safety of cheeses. *L. parabuchneri*, despite it is commonly found in the microbiota of cheese

(Berthoud et al., 2017; Diaz, Ladero, Redruello, et al., 2016), it is an undesirable species in the cheese industry that negatively affects the food safety of cheese and whose presence must be avoided. In addition to have been identified as the major responsible for the accumulation of histamine in cheeses (Ascone et al., 2017; Diaz, Ladero, Redruello, et al., 2016), it can adhere to and form biofilms on materials (polystyrene and stainless steel) commonly used in the machinery used in cheese making and processing, which increases its potential to contaminate this food (Diaz, del Rio, et al., 2016). *L. parabuchneri* is also capable of surviving the milk pasteurization process (Ladero, Sánchez-Llana, et al., 2011) and even growth and accumulate histamine in cheese at the refrigeration temperatures set during storage (Diaz et al., 2018). The antimicrobial effect of nisin A on the growth of this species and the biofilms it forms, would greatly contribute to reducing the presence of this species in cheeses and the accumulation of histamine. Nisin A also prevented the biofilm formation of *L. reuteri* IPLA 11078 and *L. vaginalis* IPLA 11068 (Fig. 1I and 1J, respectively), which are other two known histamine-producing, biofilm-forming species that have been previously isolated from cheeses (Diaz et al., 2015). Although the frequency with which these species appears in cheeses is quite low (Diaz et al., 2015; Diaz, Ladero, Redruello, et al., 2016), their reduction by the effect of nisin A would also contribute to reduce the total pull of histamine-producing LAB in cheese and eventually, the total amount of histamine.

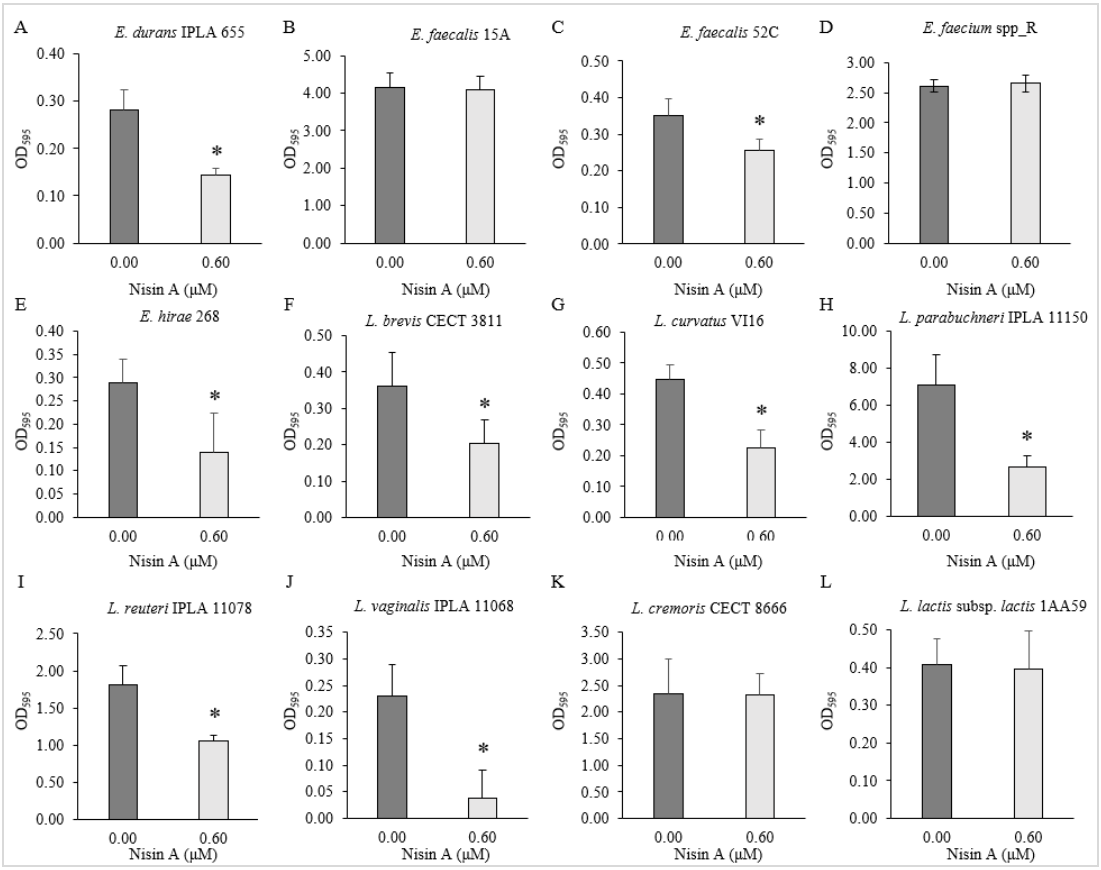

Fig. 1. The bacteriocin nisin A prevents the formation of biofilms by BA-producing LAB strains. Data represent the mean of at least three biological replicates. Vertical bars represent standard deviations. *p<0.05 compared to control cultures.

Moreover, nisin A prevented the formation of biofilms of tyramine- and putrescine-producer lactobacilli such as *L. brevis* CECT 3811, *L. curvatus* VI16 (Fig. 1F and 1G, respectively) and, also enterococci, in which prevented the formation of biofilms in 3 out of the 5 enterococcal strains tested, i.e. the tyramine-producing *E. durans* IPLA 655 (Fig. 1A), and the tyramine- and putrescine-producing *E. faecalis* 52C (Fig. 1C) and *E. hirae* 268 (Fig. 1E). In contraposition, other two enterococcal strains, the tyramine- and putrescine-producing *E. faecalis* 15A (Fig. 1B), and the tyramine-producing *E. faecium* spp (Fig. 1D) were resistant to the action of nisin A. Since the enterococcal species are the main responsible for the accumulation of tyramine and putrescine at elevated concentrations in cheese (Ladero, Cañedo, et al., 2012; Ladero, Fernández, et al., 2010), the reduction of biofilms of such tyramine- and putrescine- producers, would finally reduce the accumulation of those BA in cheese. In fact, previous studies showed that there is a correlation between the levels of tyramine- or putrescine- producer bacteria with the final amount of those BA in the final product (Ladero, Cañedo, et al., 2012; Ladero, Fernández, et al., 2010). Moreover, if the population of tyramine-producers do

not exceed a certain threshold value, the concentration of tyramine may not reach values of concern for the consumer´s health (Ladero, Martínez, et al., 2010). The two putrescine-producing lactococcal strains tested, *L. cremoris* CECT 8666 (Fig. 1K) and *L. lactis* subsp. *lactis* 1AA59 (Fig. 1L) were resistant to the action of nisin A. To prevent the formation of biofilms of putrescine producing lactococci, other bacteriocins that inhibit the growth of these species could be tested as anti-biofilm forming agents, such as lactococin A (Holo et al., 1991) or lactococcin Z (Ishibashi et al., 2015). Nonetheless, if the final objective pursued is to avoid the accumulation of BA in cheeses, it is essential to correctly choose non-putrescine producing lactococci as part of the starter cultures (Ladero, Cañedo, et al., 2012; Ladero, Rattray, et al., 2011).

4. Conclusions

All the LAB species that have the greatest impact in the accumulation of BA in cheese, were susceptible to one or more CFS tested (excepting *L. lactis*) and their susceptibility spectrum was strain-dependent. The CFS containing either nisin A or nisin Z, were the ones showing the broadest antimicrobial spectrum. Moreover, commercial nisin A prevented the formation of biofilms of a variety of strong biofilm-former strains. Among the latter, it is worth noting that some of such strains belong to LAB species whose decarboxylating metabolic activity contribute to a greater extent to BA accumulation in cheese, i.e. *L. parabuchneri* (histamine) and *E. faecalis* (tyramine and putrescine). These results highlight the significant impact of nisin A in preventing biofilm formation of histamine-producing lactobacilli and tyramine- and putrescine-producing enterococci, which may considerably reduce or even eliminate the hazard that poses the persistence of such microorganisms in cheese manufacturing and processing equipment.

To date, no single bacteriocin has been described that can inhibit the growth and biofilm formation of all BA-producing LAB species. The results of this study suggest that the use in dairy fermented products of a rational combination of bacteriocins (as biopreservatives) -or bacteriocinogenic LAB (as bioprotective cultures)- posing different antimicrobial spectrum against BA-producing LAB, could be a useful strategy for increasing the safety of cheeses by reducing the presence of those microorganisms and preventing the accumulation toxic amounts of histamine, tyramine and putrescine in the final product.

5. Authors' contributions

Concept, M.A.; methodology, L.V., V.L., A.S., B.M., and B.d.R.; writing-original draft preparation, V.L., B.d.R.; writing, review and editing, B.d.R., and M.A.; funding acquisition, M.A. All authors have read and agreed to the published version of the manuscript.

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8. Conflicts of Interest

None.

9. References

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Tables.

Table 1. Bacteriocins used in this study. The producer microorganisms and their isolation source are indicated

Bacteriocin	Bacteriocin classification[1]	Producer strain	Source of isolation	Reference
Coagulin A	Class IIa[4]	*Lactiplantibacillus paraplantarum* IPLA C23	Cheese	(Allende et al., 2007), IPLA[5]
Enterocin A	Class IIa	*Enterococcus faecium* MMRA	Dairy beverage	(Rehaiem et al., 2010)
Enterocin P	Class IIa	*Enterococcus faecium* SP-17	Cheese	(Portilla-Vázquez et al., 2016)
Lacticin 481	Class Ia, type II[3]	*Lactococcus lactis* subsp. *lactis* IPLA C270	Cheese	(Böttiger et al., 2009), IPLA
Nisin A	Class Ia, type I[2]	*Lactococcus lactis* subsp. *lactis* 3AA28	Cheese	(Alegría et al., 2010)
Nisin Z	Class Ia, type I	*Lactococcus lactis* subsp. *lactis* 2BB9	Cheese	(Alegría et al., 2010)
Plantaricin C	Class Ia, type II	*Lactiplantibacillus plantarum* LL441	Cheese	(Gonzalez et al., 1994)

[1] Bacteriocin classification based on (Alvarez-Sieiro et al., 2016)
[2] Class Ia, type I: small posttranslationally modified peptide, lanthipeptide (LanBC-modified)
[3] Class Ia, type II: small posttranslationally modified peptide, lanthipeptide (LanM-modified)
[4] Class IIa: unmodified peptide (less than 10 kDa), pediocin-like bacteriocin
[5] IPLA: Instituto de Productos Lácteos de Asturias (Dairy Research Institute) Culture Collection, Spain

Table 2. BA-producing microorganisms used in this study.

BA-producing LAB	Origin	Reference/Source
Histamine-producing strains		
Lentilactobacillus hilgardii 321	Wine	(Landete et al., 2005)
L. parabuchneri DSM 5707^T	Human saliva	DSMZ[4]
L. parabuchneri IPLA 11117	Cheese	(Diaz, Ladero, Del Rio, et al., 2016), IPLA
L. parabuchneri IPLA 11122	Cheese	(Diaz et al., 2018), IPLA
L. parabuchneri IPLA 11150	Cheese	(Diaz, Ladero, Del Rio, et al., 2016), IPLA
L. parabuchneri IPLA 15009	Cheese	(Diaz, Ladero, Del Rio, et al., 2016), IPLA
L. parabuchneri st2A	Cheese	(Molenaar et al., 1993)
Limosilactobacillus reuteri IPLA 11078	Cheese	(Diaz, Ladero, Redruello, et al., 2016), IPLA
Limosilactobacillus vaginalis IPLA 11050	Cheese	(Diaz et al., 2015), IPLA
Limosilactobacillus vaginalis IPLA 11054	Cheese	(Diaz et al., 2015), IPLA
Limosilactobacillus vaginalis IPLA 11068	Cheese	(Diaz et al., 2015), IPLA
Limosilactobacillus vaginalis IPLA 11070	Cheese	(Diaz et al., 2015), IPLA
Limosilactobacillus vaginalis IPLA 11144	Cheese	(Diaz et al., 2015), IPLA
Pediococcus parvulus 276	Wine	(Landete et al., 2005)
Streptococcus thermophilus CHCC 1524	Dairy	(Calles-Enríquez et al., 2010), CHCC[5]
Streptococcus thermophilus CHCC 6483	Dairy	(Calles-Enríquez et al., 2010)
Tyramine-producing strains		
Enterococcus durans CECT 411^T	Powder milk	CECT[1]
Enterococcus durans IPLA 655	Cheese	(Linares et al., 2012), IPLA[2]
Enterococcus durans AA46	Cheese	(Ladero, Fernández, et al., 2012)
Enterococcus durans IPLA 1567	Cheese	(Ladero et al., 2010)
Enterococcus durans Ace	Cheese	(Ladero, Fernández, et al., 2012)
Enterococcus faecium 11149	Cheese	(Diaz, Ladero, Del Rio, et al., 2016)
Enterococcus faecium AM	Cheese	(Ladero, Fernández, et al., 2012)

Enterococcus faecium B1	Cheese	(Ladero, Fernández, et al., 2012)
Enterococcus faecium spp_R	Yogurt	(Ladero, Fernández, et al., 2012)
Latilactobacillus curvatus PO8	Cheese	(López & Mayo, 1997)
Tyramine- and putrescine- producing strains		
Enterococcus faecalis 15a	Cheese	(Ladero, Fernández, et al., 2012)
Enterococcus faecalis BA64	Cheese	(Ladero, Fernández, et al., 2012)
Enterococcus faecalis 23a	Cheese	(Ladero, Fernández, et al., 2012)
Enterococcus faecalis 52C	Cheese	(Ladero, Fernández, et al., 2012)
Enterococcus faecalis V63	Cheese	(Ladero, Fernández, et al., 2012)
Enterococcus hirae 268	Dairy	(Ladero, Cañedo, et al., 2012)
Levilactobacillus brevis CECT 3810	Dairy	(Romano et al., 2014), CECT
Levilactobacillus brevis CECT 3811	Dairy	(Romano et al., 2014), CECT
Levilactobacillus brevis C311	Dairy	(Romano et al., 2014), CECT
Latilactobacillus curvatus VI14	Dairy	(Ladero, Cañedo, et al., 2012), IPLA
Latilactobacillus curvatus VI16	Dairy	(Ladero, Cañedo, et al., 2012), IPLA
Putrescine-producing strains		
Levilactobacillus brevis CECT 3824	Dairy	(Romano et al., 2014), CECT
Lactococcus cremoris CECT 8666	Cheese	(Linares et al., 2015), IPLA
Lactococcus cremoris 2A22	Cheese	(Ladero et al., 2011), IPLA
Lactococcus cremoris 3AA9	Cheese	(Ladero et al., 2011), IPLA
Lactococcus cremoris 3AA23	Cheese	(Linares et al., 2013), IPLA
Lactococcus cremoris 2A5	Cheese	(Ladero et al., 2011), IPLA
Lactococcus lactis subsp. *lactis* 1AA59	Cheese	(Ladero et al., 2011), IPLA
Lactococcus lactis subsp. *lactis* L39	Cheese	(Ladero et al., 2011), IPLA
Lactococcus lactis subsp. *lactis* 1A38	Cheese	(Ladero et al., 2011), IPLA
Lactococcus lactis subsp. *lactis* T3/33	Cheese	(Ladero et al., 2011), IPLA
Lactococcus lactis subsp. *lactis* 2BA40	Cheese	(Ladero et al., 2011), IPLA

[1] CECT: Spanish Type Culture Collection, Spain
[2] IPLA: Instituto de Productos Lácteos de Asturias (Dairy Research Institute) Culture Collection, Spain
[3] ATCC: American Type Culture Collection, US
[4] DSMZ: German Collection of Microorganisms and Cell Cultures, Germany
[5] CHCC: Christian Hansen Culture Collection, Denmark

Table 3. Susceptibility of BA-producing LAB and the indicator strain *Latilactobacillus sakei* CECT 906^{T} to bacteriocin-containing CFS

	Bacteriocin-containing cell free supernatants						
BA-producing LAB	**Coagulin A**	**Enterocin A**	**Enterocin P**	**Lacticin 481**	**Nisin A**	**Nisin Z**	**Plantaricin C**
Histamine-producing strains							
L. hilgardii 321	−	−	−	−	++	+	−
L. parabuchneri DSM 5707^{T}	−	−	++	−	++	++	−
L. parabuchneri IPLA 11117	−	−	++	−	++	++	−
L. parabuchneri IPLA 11122	−	−	++	−	++	++	−
L. parabuchneri IPLA 11150	−	−	++	−	++	++	−
L. parabuchneri IPLA 15009	−	−	++	−	++	++	+
L. parabuchneri st2A	−	−	++	−	++	++	−
L. reuteri IPLA 11078	−	−	−	−	++	+	−
L. vaginalis IPLA 11050	−	−	−	−	+	++	−
L. vaginalis IPLA 11054	−	−	−	−	−	−	−
L. vaginalis IPLA 11068	−	−	+	−	++	++	−
L. vaginalis IPLA 11070	−	−	−	−	+	+	−
L. vaginalis IPLA	−	−	−	−	+	+	−

11144							
P. parvulus 276	++	−	++	+	++	++	+
S. thermophilus CHCC 1524	−	−	−	++	−	−	++
S. thermophilus CHCC 6483	−	−	−	−	−	−	++
Tyramine-producing strains							
E. durans CECT 411^{T}	+	−	−	−	−	−	−
E. durans IPLA 655	++	−	−	−	+	+	−
E. durans AA46	+	−	−	−	−	−	−
E. durans IPLA 1567	++	−	−	−	+	+	−
E. durans Ace	++	−	−	−	+	+	−
E. faecium 11149	++	−	+	−	++	++	−
E. faecium AM	++	−	+	−	+	+	−
E. faecium B1	++	−	+	−	+	+	−
E. faecium spp_R	++	+	+	+	++	+	−
L. curvatus PO8	++	−	−	−	++	++	−
Tyramine- and putrescine-producing strains							
E. faecalis 15a	+	−	−	−	−	−	−
E. faecalis BA64	++	−	−	−	+	+	−
E. faecalis 23a	++	−	−	−	−	−	−
E. faecalis 52C	++	−	−	−	+	−	−
E. faecalis V63	++	−	−	−	−	−	−
E. hirae 268	++	+	+	−	−	−	−
L. brevis CECT 3810	−	−	+	−	+	−	−

L. brevis CECT 3811	–	–	+	–	+	–	–
L. brevis C311	–	–	+	–	+	–	–
L. curvatus VI14	+	–	+	–	++	++	–
L. curvatus VI16	–	–	–	–	++	+	–
Putrescine-producing strains							
L. brevis CECT 3824	–	–	+	–	++	+	–
L. cremoris CECT 8666	–	–	–	–	–	–	–
L. cremoris 2A22	–	–	–	–	–	–	–
L. cremoris 3AA9	–	–	–	–	+	+	–
L. cremoris 3AA23	–	–	–	–	+	+	–
L. cremoris 2A5	–	–	–	–	–	–	–
L. lactis subsp. *lactis* 1AA59	–	–	–	–	–	–	–
L. lactis subsp. *lactis* L39	–	–	–	–	–	–	–
L. lactis subsp. *lactis* 1A38	–	–	–	–	–	–	–
L. lactis subsp. *lactis* T3/33	–	–	–	–	–	–	–
L. lactis subsp. *lactis* 2BA40	–	–	–	–	–	–	–
Indicator strain							
Latilactobacillus sakei CECT 906^{T}	++	++	++	++	++	++	++

++, Inhibitory zone > 13 mm; +, Inhibitory zone 8–13 mm; –, No inhibition

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Enterocin AS-48 inhibits the growth of - and biofilm formation by- lactic acid bacteria responsible for the accumulation of biogenic amines in cheese

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Abstract

Cheese is a fermented food in which toxic concentrations of biogenic amines (BA) may accumulate, mainly as a result of the metabolism of certain lactic acid bacteria (LAB); the formation of these compounds needs to be limited. This work reports the antimicrobial potential of bacteriocin AS-48 against the LAB largely responsible for the accumulation of the BA histamine, tyramine and putrescine in cheese. Most of the 50 analyzed BA-producing LAB strains were sensitive to AS-48. The bacteriocin also prevented biofilm formation of 92% of the BA-producing strains tested. This result is of great technological importance since, in cheese production, biofilms are an important source of contamination by BA-producing LAB. This study supports the potential use of AS-48 as a biopreservative for controlling BA-producing LAB in fermented food.

Keywords: Bacteriocin;. Food Safety, Dairy Foods; Histamine; Tyramine; Putrescine

1. Introduction

The metabolic activity of certain microorganisms that may be present in foods and beverages can lead to their accumulation of toxic metabolites, including biogenic amines (BA). The latter are produced by the enzymatic decarboxylation of certain amino acids. The most important BA in terms of food safety are histamine, tyramine and putrescine (Ladero, Calles-Enríquez, Fernández & Alvarez, 2010a, Ruiz-Capillas & Herrero, 2019). Histamine and tyramine are produced by the decarboxylation of the amino acids histidine and tyrosine, respectively. Putrescine is produced from arginine, but in addition to its decarboxylation, deimination is necessary.

BA mainly accumulate in fish and fish products, in beverages, and in fermented foods. In fermented foods, certain types of cheeses had been reported with accumulation of

high concentration of BA. The main producers of BA in fish and fish derivatives are usually Gram-negative bacteria. Their appearance can be prevented by good hygiene and preservation practices (Alvarez & Moreno-Arribas, 2014). For cheese products, however, which may have long maturation periods, the solution is more complicated; despite many microorganisms including yeast and Gram-negative enterobacteria seem able to accumulate BA in cheese (Barbieri et al., 2019; Gardini et al., 2006; Linares et al., 2011)the main BA producers associated with these foods are Gram-positive lactic acid bacteria (LAB), i.e., the very microorganisms responsible for fermentation and madurarion of cheese and development of organoleptic properties (Linares, Martín, Ladero, Alvarez & Fernández, 2011). In cheese, such organisms may include *L. parabuchneri*, the main producer of histamine (Diaz et al., 2016). Other LAB include strains of *Pediococcus parvulus* (Møller, Ücok & Rattray, 2020) and *Streptococcus thermophilus* (Calles-Enríquez et al., 2010), which also produce histamine, and members of the genus *Enterococcus*, which are also the main producers of tyramine. In fact, tyramine biosynthesis is a species-level characteristic of *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans* (Ladero et al., 2012b). Tyramine-producing strains of *Levilactobacillus brevis* and *Lactilactobacillus curvatus* have also been described (Linares et al., 2012a). Lactobacilli required in cheese production have also been reported to produce putrescine, although enterococci are largely responsible for its accumulation (Ladero, Fernández, Cuesta & Alvarez, 2010b). Indeed, putrescine synthesis is a species-level trait of *Enterococcus faecalis* (Ladero et al., 2012b), as it is of certain strains of *Lactococcus lactis* subsp. *lactis* and *Lactococcus cremoris* (Ladero et al., 2011), yet both these latter taxa are major starters used in cheese production. Evidently, measures are required that block the accumulation of BA by these organisms without inhibiting their roles in fermentation and the development of sensorial properties.

A further concern is that most BA-producing bacteria are able to form biofilms on dairy industry surfaces and equipment (Diaz et al., 2016a, b). These can be strongly resistant to cleaning and disinfection routines, and act as sources of contamination (Diaz et al., 2016a, b). Even the instruments used in the post-ripening processing (slicing, grating etc.) of cheeses can be important source of contamination by BA-producing bacteria (Ladero, Fernández & Alvarez, 2009), which will be finally responsible for the accumulation of BA in cheese.

One strategy deserving of evaluation as a means of reducing BA accumulation in fermented foods such as cheese is the use of bacteriocins. These natural alternatives to chemical preservatives are antimicrobial peptides produced by bacteria (which may undergo post-translational modification by enzymes) that are exported into the extracellular medium (Cotter, Hill & Ross, 2005). The bacteriocins produced by LAB are particularly interesting given the long history of safe use of certain types of these bacteria, and their 'Generally Regarded as Safe (GRAS)' and 'Qualified Presumption of Safety (QPS)' status (Alvarez-Sieiro, Montalbán-López, Mu & Kuipers, 2016). A bacteriocin with great potential as a food preservative is the circular cationic enterocin known as AS-48. It has a broad spectrum of antimicrobial activity (especially against Gram-positive bacteria), is resistant to a wide range of temperature and pH, and is sensitive to digestive proteases (Galvez, Maqueda, Valdivia, Quesada & Montoya, 1986; Samyn et al., 1994). In fact, AS-48 has been shown effective against pathogenic and spoilage bacteria in different types of food (Ananou et al., 2010; Cobo Molinos et al., 2008; Grande et al., 2006, 2007; Muñoz et al., 2007), as well as in reducing the concentration of BA in fish (Ananou et al., 2014).

The present work reports the antimicrobial activity of bacteriocin AS-48 against the bacteria mainly responsible for the accumulation of histamine, tyramine and putrescine in cheese, and its ability to prevent the formation of biofilms by these bacteria. The results suggest that AS-48 could be used to prevent the accumulation of BA in cheese.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Table 1 shows the 50 BA-producing strains examined in this work: *Enterococcus durans* (5 strains), *Enterococcus faecalis* (6), *Enterococcus faecium* (4), *Enterococcus hirae* (1), *Levilactobacillus brevis* (5), *Latilactobacillus curvatus* (3), *Lentilactobacillus hilgardii* (1), *L. parabuchneri* (6), *Limosilactobacillus reuteri* (1), *Limosilactobacillus vaginalis* (5), *Lactococcus cremoris* (5), *Lactococcus lactis* subsp. *lactis,* (5) *Pediococcus parvulus* (1), and *Streptococcus thermophilus* (2). All these strains had previously been isolated from food sources (dairy and wine), except for *Enterococcus faecalis* V583 (from human blood), *Levilactobacillus brevis* ATCC 367 (from silage), and the type strain *L. parabuchneri* DSM 5707^{T} (from human saliva). The indicator

strain used to determine the antimicrobial spectrum of AS-48 was *Latilactobacillus sakei* CECT 906^T (Colección Española de Cultivos Tipo [CECT], Spain), a bacterium determined in preliminary work to be sensitive to AS-48 (data not shown).

Enterococci, lactococci and streptococci were grown in M17 (Oxoid, Basingstoke, UK) supplemented with 5 g/l glucose (GM17), while lactobacilli and pediococci were grown in Man, Rogosa and Sharpe (MRS) medium (Oxoid). For the determination of minimum inhibitory concentrations (MIC), enterococci, lactococci and streptococci were grown in Iso-Sensitest medium (IST, Oxoid), and lactobacilli and pediococci in LSM medium (90% IST, 10% MRS). Enterococci, lactococci, pediococci and *Lb. sakei* CECT 906^T were grown at 32ºC, the other lactobacilli at 37ºC, and the streptococci at 42ºC. Enterococci, lactococci and lactobacilli were incubated without aeration; pediococci and streptococci were incubated under anaerobic conditions (10% H_2, 10% CO_2 and 80% N_2) in a Mac500 chamber (Down Whitley Scientific, Bradford, UK).

2.2. Production and purification of AS-48

Bacteriocin AS-48 was produced and purified as described by Perales-Adán et al. (2018) and initially held in lyophilized form before resuspension in dimethyl sulphoxide (Sigma-Aldrich, Spain). The final preparations were stored at 4ºC until use.

2.3. Antimicrobial spectrum of AS-48

The antimicrobial activity of AS-48 against BA-producing LAB was determined using agar-well diffusion assays as described by Schillinger & Lücke (1989) with some modifications. Briefly, MRS or GM17 agar (12 g/l) plates inoculated with 10^5 cfu/ml of an overnight grown culture of the corresponding BA-producing strain (the indicator strain *Lb. sakei* CECT 906^T was used as a positive control of the antimicrobial activity of AS-48). Five mm-diameter wells were then excavated in the agar and 30 μl of AS-48 (6.63 μM) added to each. The plates were then incubated at the corresponding temperature for 48 h, and the diameter of any inhibitory zone around the wells measured. An inhibitory zone of >13 mm in diameter was deemed to indicate strong inhibition, one between 8 and 13 mm to indicate moderate inhibition, and one measuring <8 mm to indicate no inhibition. Tests were performed at least in duplicate.

2.4. Determination of AS-48 minimum inhibitory concentration values

Minimum inhibitory concentration (MIC) values for AS-48 were determined using the broth microdilution method based on ISO IDF Standard 10932 (International Organization for Standardization, 2010). BA-producing LAB were grown in the corresponding solid media (IST or LSM supplemented with 20 g/l agar). Isolated colonies were then used to prepare suspensions with a turbidity similar to McFarland standard one (OD_{600nm}=0.1-0.2; approximately 10^8 cfu/ml) in an NaCl solution (8.5 g/l). The inoculated saline suspensions were diluted 1:1000 in liquid media (IST or LSM) to reach an approximate inoculum size of 10^5 cfu/ml. 96-well polystyrene round bottom microwell plates were then prepared (100 µl per well) with serial two-fold dilutions of AS-48 (diluted in IST or LSM liquid media; the concentrations assayed ranged from 1.66 µM to 3.16 pM), plus 100 µl of the diluted bacterial inocula. Each plate had a positive control (with bacterial inoculum but without AS-48) and negative control (IST or LSM liquid media, with no bacterial inoculum or AS-48). In addition, a positive control for the antimicrobial activity of AS-48 was included in each assay using the indicator strain *Lb. sakei* CECT 906^T as the bacterial inoculum. The prepared plates were incubated for 24, 48 and >96 h under standard conditions for the growth of each bacterial species (either 37º or 32ºC and in aerobic or anaerobic conditions as required). MIC values were recorded as the weakest dilution that inhibited bacterial growth (no growth observed). All assays were performed at least three times with biological replicates.

2.5. Prevention of biofilm formation by AS-48

The potential of AS-48 to prevent biofilm formation was examined using the strongest biofilm-producing strains of the examined species (as reported by Diaz et al. [2016b]), i.e., *Enterococcus durans* IPLA 655, *Enterococcus faecalis* 15a, *Enterococcus faecalis* 52C, *Enterococcus faecium* spp, *Enterococcus hirae* 268, *Levilactobacillus brevis* CECT 3811, *Latilactobacillus curvatus* VI16, *L. parabuchneri* IPLA 11150, *Limosilactobacillus reuteri* IPLA 11078, *Limosilactobacillus vaginalis* IPLA 11068, *Lactococcus cremoris* CECT 8666 and *Lactococcus lactis* subsp. *lactis* 1AA59. 96-well microtiter plates (Nunc MicroWell Plates with a Nunclon Delta Surface [Thermo Fisher Scientific, Waltham, MA, USA]) were prepared with AS-48 (1.75 or 3.5 µM) or

sterilized water (negative controls). After allowing the wells to air-dry, 200 µl of the BA-producing strains (10^6 CFU/ml) were introduced and incubated for 48 h under standard conditions to allow for biofilm formation. The cells were then stained with crystal violet (CV) following the protocol of Diaz et al. (2016a) with some modifications. Briefly, each well was cleaned twice with 200 µl of PBS to remove any non-adhered cells. Then, after 30 min drying in a flow cabinet, 250 µl of CV (5 g/l in distilled water) were added. After 30 min, any biofilm present was rinsed three times with 200 µl distilled water. Finally, the bound dye was solubilized in 250 µl of acetic acid (330 ml/l) and the absorbance at 595 nm measured using a Benchmark Plus microplate spectrophotometer (BioRad, Hercules, CA, USA).

2.6. Statistical analysis

Means ± standard deviations were calculated (from at least three independent replicates) and compared using the Student t-test (SigmaPlot v.15.0 software). Significance was set at $P<0.05$.

3. Results and Discussion

3.1. Antimicrobial spectrum of AS-48

Table 2 shows that susceptibility to AS-48 was strain-dependent. The inhibitory halos associated with enterococcal strains were between 8 and 13 mm in diameter (moderate inhibition). In general, *Lb. brevis* was strongly inhibited, except for *Lb. brevis* CECT 3811 which was moderately inhibited, *Lb. curvatus* VI16 and PO8 which were completely inhibited, and *Lb. curvatus* VI14 which was moderately inhibited. Tests with *Lb. hilgardii* 321 and the *Lb. parabuchneri* strains all exhibited a zone of inhibition of >13 mm diameter, indicating them to be strongly inhibited. *Lb. reuteri* and *Lb. vaginalis* were the least sensitive strains, while *Lb. vaginalis* IPLA 11068 was strongly inhibited, *Lb. reuteri* IPLA 11078 was moderately inhibited, as were *Lb. vaginalis* IPLA 11050 and 11070, and *Lb. vaginalis* IPLA 11054 and 11144 were not inhibited at all.

The *L. cremoris* and *L. lactis* subsp. *lactis* strains examined were moderately inhibited. *L. lactis* subsp. *lactis* 2BA40 was strongly inhibited, as were *P. parvulus* 276 and *S. thermophilus* CHCC 1524. Finally, *S. thermophilus* CHCC 6483 was moderately inhibited.

The inhibitory spectrum found in this work was in accordance with previous reports, in which AS-48 showed antibacterial activity against strains of enterococcal species e.g. *E. faecalis*, *E. faecium*, *E. durans* (Galvez et al., 1989), and also towards other LAB such as lactobacilli, lactococci and pediococci (Burgos et al., 2014). Given the lack of works with purified bacteriocins, more studies have to be performed to be able to compare the effectiveness and antimicrobial spectrum of AS-48 against BA-producing LAB.

3.2. Determination of the AS-48 minimum inhibitory concentrations

Table 2 also shows the MIC values for AS-48 with respect to the examined strains. Again, the values recorded were strain-dependent.

With respect to *E. durans*, the MIC of AS-48 was independent of the incubation time. For *E. durans* CECT 411^{T} and AA46, the lowest MIC was 414.46 nM, while for the remaining *E. durans* strains it was 1.66 µM. The MIC of AS-48 with respect to *E. faecalis* was also independent of the incubation time, with a value of 414.46 nM for all the species strains except for *E. faecalis* 15a (828.91 nM). For all the tested *E. faecium* strains, the MIC was 414.46 nM, with the exception of *E. faecium* spp (828.91 nM). *E. hirae* 268, the only taxon of the genus to be associated with a MIC dependent on the incubation period, increasing from 414.46 nM to 828.91 nM after 96 h of treatment.

No bacterial growth was recorded for *Lb. brevis* after 24 h, but *Lb. brevis* 3810, 3811 and C311 continued to slightly resist the bacteriocin after 96 h of incubation (MIC 25.30 pM, 6.32 pM and 25.30 pM respectively). No *Lb. curvatus* strain grew in the presence of AS-48, except for *Lb. curvatus* VI14 (MIC 25.30 pM after 96 h), as did *Lb. hilgardii* 321 (MIC 404.74 pM after 48 h, and 809.48 pM after 96 h). *Lb. parabuchneri* IPLA 11150 was the least sensitive of its species' strains, with a MIC of 25.90 nM after 96 h. With respect to *Lb. reuteri* 11078, the MIC increased with incubation time to 51.81 nM, 103.61 nM and 414.46 nM at 24, 48 and 96 h, respectively. The *Lb. vaginalis* strains showed some resistance after 96 h (MIC 1.66 µM), with the exception of *Lb. vaginalis* 11050 (828.91 nM).

For the *L. cremoris* strains, MIC values of 414.46-828.91 nM were recorded, as they were for the *L. lactis* subsp. *lactis* strains. The inhibition of growth of putrescine-producing *L. lactis* and *L. cremoris* would be desirable for dairy industry as means of

preventing the accumulation of this BA in cheeses. Since these are the LAB species most widely used as primary starter in cheese manufacturing, it would be necessary to carry out more technological studies to determine the best application procedures for AS-48 during the cheese-making process to improve the safety and quality of this food. The eventual lysis of starter cultures caused by AS-48, would release intracellular enzymes to the cheese matrix that could potentially improve flavour development and accelerate cheese ripening, similarly as it was previously shown for other bacteriocins such as lacticin 481 (O´sullivan, Ross & Hill, 2003).

Finally, the growth of *P. parvulus* 276 was inhibited at all tested AS-48 concentrations and incubation times, while for *S. thermophilus* a MIC of 414.46 nM was recorded for all incubation times. Thus, most of the BA-producing LAB strains analyzed (Table 1) were sensitive to low concentrations of AS-48, even after 96 h of incubation. Some studies suggest this bacteriocin may have a more pronounced effect at acidic pH (Muñoz et al., 2007), which might enhance its inhibitory effect in dairy fermentations. Other studies also indicate AS-48 and nisin to act in a synergistic manner (Perales-Adán et al., 2018), which might provide greater protection against the development of BA-producing bacteria. The combination of AS-48 with other hurdles, could also potentiate the protective action of AS-48. For instance, Anaoun et al. (2014) observed a significant reduction of histamine-and tyramine- producing LAB and decrease in the accumulation of AB, in sardines treated with AS-48 and packaged under vacuum or modified atmosphere during storage.

3.3. Prevention of biofilm formation by AS-48

The bacteriocin significantly inhibited biofilm formation in 11 out the 12 examined strains, even at the lowest concentration tested (1.75 µM) (Fig. 1). The only strain that produced biofilm in the presence of AS-48 was *E. durans* IPLA 655 (Fig. 1B). Indeed, the capacity of AS-48 to reduce biofilm formation was remarkable: by 100% in *E. faecium* spp, *E. faecalis* 52C, *Lb. vaginalis* IPLA 11068 and *Lb. reuteri* IPLA 11068 (compared to control cultures), by 96% in *L. lactis* subsp. *lactis* 1AA59, and by 65% in *Lb. curvatus* VI16. Importantly, AS-48 prevented biofilm formation by *Lb. parabuchneri*, the main histamine-producer in cheese (Ascone et al., 2017; Diaz et al., 2016; Wechsler et al., 2021). The biofilms this species can produce on food processing

equipment represent a source of contamination for post-ripening-processed cheeses (Diaz et al., 2016a). It should be noted that, for the sensitive strains, no significant difference was seen in terms of biofilm production between the two concentrations of AS-48 tested (Fig. 1). Thus, even with a concentration of just 1.75 µM, this bacteriocin can prevent biofilm formation by histamine-producing *Lb. parabuchneri*.

Our data did not show a correlation between the inhibition of bacterial growth caused by AS-48 and the prevention of the formation of their biofilms. For instance, *Lb. curvatus* VI16, which growth was the most sensitive of all the biofilm-forming strains (MIC value <3.16 pM), showed a lower reduction of biofilm formation (by 65%) compared to other strains more resistant to AS-48, e.g. *E. faecium* spp or *E. faecalis* 15a (MIC values 828.91 nM), in which AS-48 caused a reduction of 100% of biofilm formation. The difference in the effectiveness of AS-48 in preventing the formation of biofilms may also depend on the structure and extracellular matrix of different biofilms, which might affect the access of the bacteriocin to the cell membrane where it acts (Burgos, Pulido, Aguayo, Gálvez & Lucas, 2014; Maqueda et al., 2005).

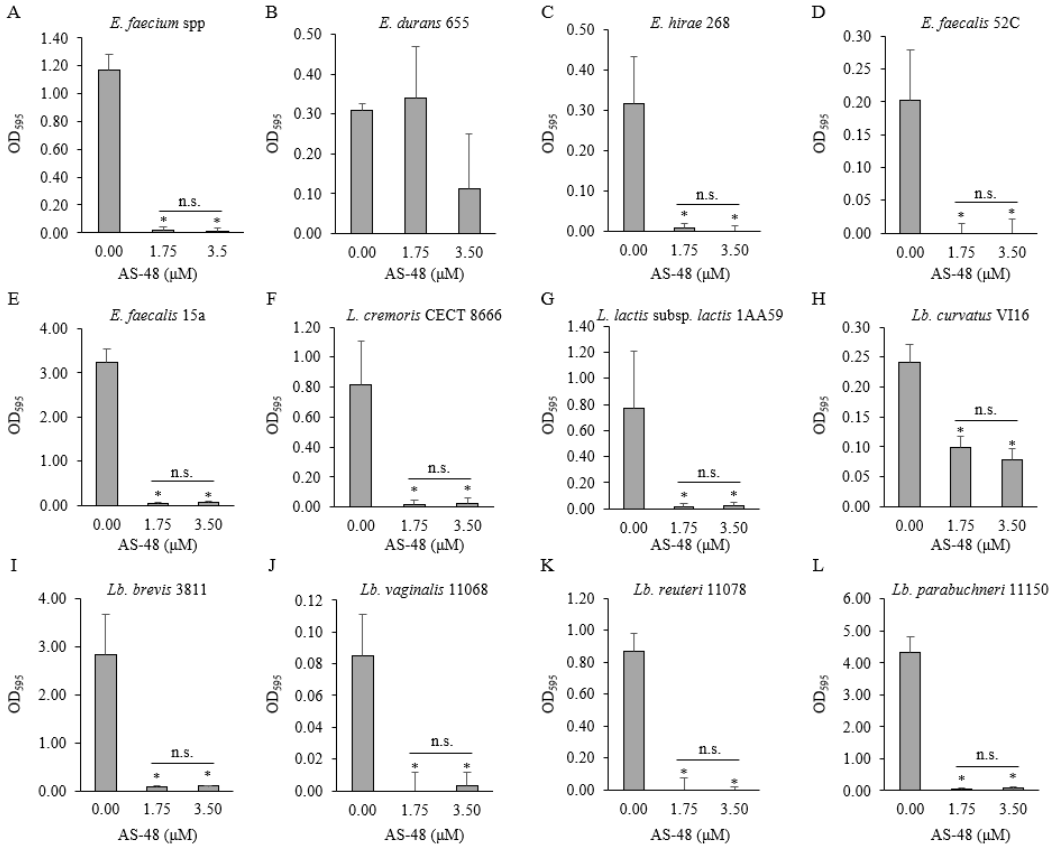

Fig. 1. The bacteriocin AS-48 prevents the formation of biofilms by BA-producing LAB strains. Polystyrene 96-wells microtiter plates were prepared with AS-48 at 0, 1.75 or 3.50 µM,

and the wells were allowed to air-dry. Twelve selected BA-producing LAB strains were allowed to form biofilms in the AS-48-treated wells under appropriate conditions for 48 h. Biofilm biomass values were determined using the crystal violet staining method, determining the final optical density at 595 nm (OD_{595}). Data represent the mean of at least three biological replicates. Vertical bars represent standard deviations. *P<0.05 compared to control cultures; n.s. not significant.

4. Conclusions

AS-48 effectively inhibited the growth of most of the BA producing-strains analyzed. While the power of this inhibition was strain-dependent, only two strains (both of the same species), i.e., *Lb. vaginalis* IPLA 11054 and 11144, were resistant to its action. In general, lactobacillal strains were more sensitive than enterococcal or lactococcal strains. Importantly, *Lb. parabuchneri*, the main producer of histamine in cheese, was strongly inhibited.

AS-48 was very effective in preventing the formation of biofilms, even by the strongest biofilm-producers tested. In fact, AS-48 inhibited - and at a very low concentration (1.75 µM) - the development of biofilm by almost all the BA-producing species tested (92%). A suitable strategy to completely remove all BA producers in cheese would be combining AS-48 with other bacteriocins with different structures and mode of actions, seeking for a synergistic antimicrobial effect. However, AS-48 completely inhibited the formation of biofilms of *Lb. parabuchneri*, which -given the importance of this species- could be of particular technological importance in preventing the accumulation of histamine in cheese. Moreover, AS-48 could be very useful by itself as an inhibitor of growth and biofilm formation by the main producers of histamine, tyramine and putrescine in cheese.

5. Authors' contributions

Concept, M.A.; methodology, B.d.R., L.V., V.L., M.M., and A.S.; writing—original draft preparation, B.d.R., M.A. and A.S.; writing—review and editing, B.d.R., V.L., M.M., A.S., and M.A.; funding acquisition, M.A. All authors have read and agreed to the published version of the manuscript.

6. Acknowledgments

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7. Ethical Guidelines

Ethics approval was not required for this research.

8. Funding

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9. Conflicts of interest

None to declare

10. Tables

Table 1. BA-producing microorganisms used in this study, isolation source, biogenic amine produced, and references.

Strains	Origin	BA-produced	Reference/Source
Enterococci			
E. durans CECT 411^{T}	Powder milk	Tyramine	CECT[1]
E. durans IPLA 655	Cheese	Tyramine	(Linares et al., 2012b), IPLA[2]
E. durans AA46	Cheese	Tyramine	(Ladero et al., 2012b)
E. durans IPLA 1567	Cheese	Tyramine	(Ladero, Martinez, Martin, Fernandez & Alvarez, 2010b)
E. durans Ace	Cheese	Tyramine	(Ladero et al., 2012b)
E. faecalis V583	Human blood	Tyramine and putrescine	(Ladero et al., 2012b)
E. faecalis 15a	Cheese	Tyramine and putrescine	(Ladero et al., 2012b)
E. faecalis BA64	Cheese	Tyramine and putrescine	(Ladero et al., 2012b)
E. faecalis 23a	Cheese	Tyramine and putrescine	(Ladero et al., 2012b)
E. faecalis 52C	Cheese	Tyramine and putrescine	(Ladero et al., 2012b)
E. faecalis V63	Cheese	Tyramine and putrescine	(Ladero et al., 2012b)

E. faecium 11149	Cheese	Tyramine	(Diaz et al., 2016b)
E. faecium AM	Cheese	Tyramine	(Ladero et al., 2012b)
E. faecium B1	Cheese	Tyramine	(Ladero et al., 2012b)
E. faecium spp	Yogurt	Tyramine	(Ladero et al., 2012b)
E. hirae 268	Dairy	Tyramine and putrescine	(Ladero et al., 2012a)
Lactobacilli			
Levilactobacillus brevis CECT 3810	Dairy	Tyramine and putrescine	(Romano, Ladero, Alvarez & Lucas, 2014), CECT
Lb. brevis CECT 3811	Dairy	Tyramine and putrescine	(Romano et al., 2014), CECT
Lb. brevis C311	Dairy	Tyramine and putrescine	(Romano et al., 2014), CECT
Lb. brevis CECT 3824	Dairy	Putrescine	(Romano et al., 2014), CECT
Lb. brevis ATCC 367	Silage	Tyramine and putrescine	(Romano et al., 2014), ATCC[3]
Latilactobacillus curvatus VI14	Dairy	Tyramine and putrescine	(Ladero et al., 2012a), IPLA
Lb. curvatus VI16	Dairy	Tyramine and putrescine	(Ladero et al., 2012a), IPLA
Lb. curvatus PO8	Cheese	Tyramine	(López & Mayo, 1997)
Lentilactobacillus hilgardii 321	Wine	Histamine	(Landete, Ferrer & Pardo, 2005)
L. parabuchneri DSM 5707^{T}	Human saliva	Histamine	DSMZ[4]
Lb. parabuchneri IPLA 11122	Cheese	Histamine	(Diaz et al., 2018), IPLA
Lb. parabuchneri IPLA 11150	Cheese	Histamine	(Diaz et al., 2016b), IPLA
Lb. parabuchneri IPLA 15009	Cheese	Histamine	(Diaz et al., 2016b), IPLA
Lb. parabuchneri IPLA 11117	Cheese	Histamine	(Diaz et al., 2016b), IPLA
Lb. parabuchneri st2A	Cheese	Histamine	(Molenaar, Bosscher, Ten Brink, Driessen & Konings, 1993)
Limosilactobacillus reuteri IPLA 11078	Cheese	Histamine	(Diaz et al., 2016b), IPLA
Limosilactobacillus vaginalis IPLA 11050	Cheese	Histamine	(Diaz et al., 2015), IPLA
Lb. vaginalis IPLA 11054	Cheese	Histamine	(Diaz et al., 2015), IPLA
Lb. vaginalis IPLA 11070	Cheese	Histamine	(Diaz et al., 2015), IPLA
Lb. vaginalis IPLA 11068	Cheese	Histamine	(Diaz et al., 2015), IPLA
Lb. vaginalis IPLA 11144	Cheese	Histamine	(Diaz et al., 2015), IPLA
Lactococci			
Lactococcus cremoris	Cheese	Putrescine	(Linares et al., 2015), IPLA

CECT 8666			
L. cremoris 2A22	Cheese	Putrescine	(Ladero et al., 2011), IPLA
L. cremoris 3AA9	Cheese	Putrescine	(Ladero et al., 2011), IPLA
L. cremoris 3AA23	Cheese	Putrescine	(Linares et al., 2013), IPLA
L. cremoris 2A5	Cheese	Putrescine	(Ladero et al., 2011), IPLA
Lactococcus lactis subsp. *lactis* 1AA59	Cheese	Putrescine	(Ladero et al., 2011), IPLA
L. lactis subsp. *lactis* L39	Cheese	Putrescine	(Ladero et al., 2011), IPLA
L. lactis subsp. *lactis* 1A38	Cheese	Putrescine	(Ladero et al., 2011), IPLA
L. lactis subsp. *lactis* T3/33	Cheese	Putrescine	(Ladero et al., 2011), IPLA
L. lactis subsp. *lactis* 2BA40	Cheese	Putrescine	(Ladero et al., 2011), IPLA
Pediococci			
Pediococcus parvulus 276	Wine	Histamine	(Landete et al., 2005)
Streptococci			
Streptococcus thermophilus CHCC 1524	Dairy	Histamine	(Calles-Enríquez et al., 2010), CHCC[5]
S. thermophilus CHCC 6483	Dairy	Histamine	(Calles-Enríquez et al., 2010)

[1]CECT: Spanish Type Culture Collection, Spain
[2]IPLA: Instituto de Productos Lácteos de Asturias (Dairy Research Institute) Culture Collection, Spain
[3]ATCC: American Type Culture Collection, US
[4]DSMZ: German Collection of Microorganisms and Cell Cultures, Germany
[5]CHCC: Christian Hansen Culture Collection, Denmark

Table 2. Antimicrobial spectrum and minimum inhibitory concentration (MIC) of purified AS-48 against BA-producing LAB. For the MIC assays, plates were incubated for 24, 48 and >96 h under the optimum conditions for each bacterial species (37ºC or 32ºC, aerobic or anaerobic, as required).

BA-producing LAB	**Antimicrobial spectrum** AS-48 (6.63 µM)	**AS-48 MIC values**		
		24 h	48 h	>96 h
Enterococci				
E. durans CECT 411^T	+	414.46 nM	414.46 nM	414.46 nM
E. durans IPLA 655	+	1.66 µM	1.66 µM	1.66 µM
E. durans AA46	+	414.46 nM	414.46 nM	414.46 nM
E. durans IPLA 1567	+	1.66 µM	1.66 µM	1.66 µM
E. durans Ace	+	1.66 µM	1.66 µM	1.66 µM
E. faecalis V583	+	414.46 nM	414.46 nM	414.46 nM
E. faecalis 15a	+	828.91 nM	828.91 nM	828.91 nM
E. faecalis BA64	+	414.46 nM	414.46 nM	414.46 nM
E. faecalis 23a	+	414.46 nM	414.46 nM	414.46 nM
E. faecalis 52C	+	414.46 nM	414.46 nM	414.46 nM

E. faecalis V63	+	414.46 nM	414.46 nM	414.46 nM
E. faecium 11149	+	414.46 nM	414.46 nM	414.46 nM
E. faecium AM	+	414.46 nM	414.46 nM	414.46 nM
E. faecium B1	+	414.46 nM	414.46 nM	414.46 nM
E. faecium spp	+	828.91 nM	828.91 nM	828.91 nM
E. hirae 268	+	414.46 nM	414.46 nM	828.91 nM
Lactobacilli				
Lb. brevis CECT 3810	++	Sensitive	12.65 pM	25.30 pM
Lb. brevis CECT 3811	+	Sensitive	Sensitive	6.32 pM
Lb. brevis C311	++	Sensitive	25.30 pM	25.30 pM
Lb. brevis CECT 3824	++	Sensitive	Sensitive	Sensitive
Lb. brevis ATCC 367	++	Sensitive	Sensitive	Sensitive
Lb. curvatus VI14	+	Sensitive	Sensitive	25.30 pM
Lb. curvatus VI16	++	N.D.	Sensitive	Sensitive
Lb. curvatus PO8	++	N.D.	Sensitive	Sensitive
Lb. hilgardii 321	++	N.D.	404.74 pM	809.48 pM
Lb. parabuchneri DSM 5707^{T}	++	50.59 pM	809.48 pM	1.62 nM
Lb. parabuchneri IPLA 11122	++	6.32 pM	50.59 pM	404.74 pM
Lb. parabuchneri IPLA 11150	++	1.62 nM	6.48 nM	25.90 nM
Lb. parabuchneri IPLA 15009	++	Sensitive	Sensitive	809.48 pM
Lb. parabuchneri IPLA 11117	++	Sensitive	809.48 pM	809.48 pM
Lb. parabuchneri st2A	++	Sensitive	Sensitive	Sensitive
Lb. reuteri IPLA 11078	+	51.81 nM	103.61 nM	414.46 nM
Lb. vaginalis IPLA 11050	+	828.91 nM	828.91 nM	828.91 nM
Lb. vaginalis IPLA 11054	–	1.66 µM	1.66 µM	1.66 µM
Lb. vaginalis IPLA 11070	+	828.91 nM	828.91 nM	1.66 µM
Lb. vaginalis IPLA 11068	++	828.91 nM	828.91 nM	1.66 µM
Lb. vaginalis IPLA 11144	–	828.91 nM	1.66 µM	1.66 µM
Lactococci				
L. cremoris CECT 8666	+	414.46 nM	414.46 nM	414.46 nM
L. cremoris 2A22	+	414.46 nM	414.46 nM	414.46 nM
L. cremoris 3AA9	+	828.91 nM	828.91 nM	828.91 nM
L. cremoris 3AA23	+	828.91 nM	828.91 nM	828.91 nM
L. cremoris 2A5	+	414.46 nM	828.91 nM	828.91 nM
L. lactis subsp. *lactis* 1AA59	+	414.46 nM	414.46 nM	828.91 nM
L. lactis subsp. *lactis* L39	+	414.46 nM	414.46 nM	414.46 nM
L. lactis subsp. *lactis* 1A38	+	828.91 nM	828.91 nM	828.91 nM
L. lactis subsp. *lactis* T3/33	+	414.46 nM	414.46 nM	414.46 nM
L. lactis subsp. *lactis* 2BA40	++	414.46 nM	414.46 nM	414.46 nM
Pediococci				
P. parvulus 276	++	N.D.	Sensitive	Sensitive
Streptococci				
S. thermophilus CHCC 1524	++	414.46 nM	414.46 nM	414.46 nM

S. thermophilus CHCC 6483	+	414.46 nM	414.46 nM	414.46 nM

++, Inhibitory halo >13 mm diam.; +, Inhibitory halo 8–13 mm diam.; –, No inhibition (<8mm diam.). N.D.: not determined due to the absence of growth in the positive control without AS-48, inoculated with the corresponding bacterial strain. Sensitive: no bacterial growth when treated with any of the AS-48 concentrations assayed (lowest concentration tested: 3.16 pM).

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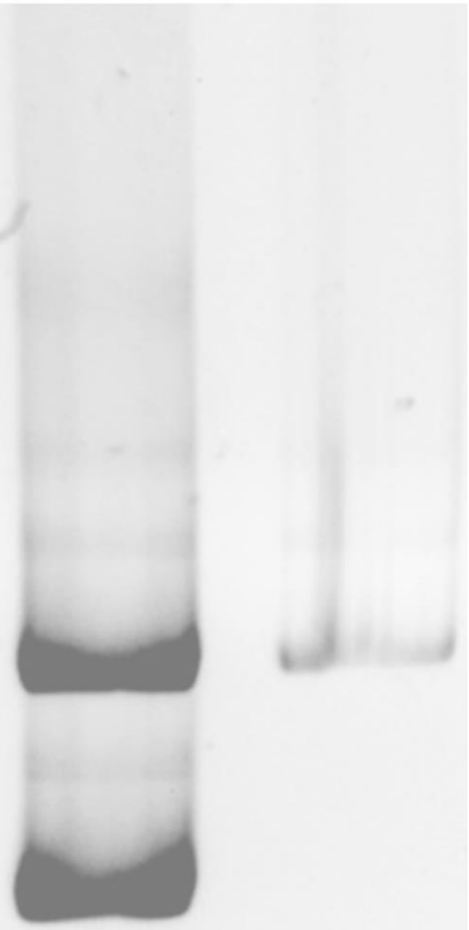


DISCUSION

4. DISCUSION

Esta sección se ha dividido en apartados considerando los objetivos planteados y los cuatro capítulos en los que se divide esta Tesis Doctoral.

En el contexto de la industria láctea, trabajos previos realizados por Diaz y col. (2016) han identificado a *L. parabuchneri* como el principal responsable de la producción y acumulación de histamina en quesos (Maria Diaz et al., 2016d; O'Sullivan et al., 2015). La histamina es una amina biógena tóxica (Ladero et al., 2010; Maintz and Novak, 2007) cuya presencia en niveles indeseables en el alimento representa un riesgo para la salud de los consumidores. Si bien no existe una regulación específica respecto a los niveles aceptables de histamina en productos lácteos, se recomienda reducir la presencia de microorganismos productores de esta AB (Debeer et al., 2021; Ladero et al., 2010). Por lo tanto, en el caso del queso es importante evitar la presencia de *L. parabuchneri*. Además, se ha observado que ciertas cepas de *L. parabuchneri* productoras de histamina, son capaces de formar biofilms en las superficies de los equipamientos utilizados en la industria láctea, lo cual contribuiría a la contaminación del queso y por ende a la acumulación de histamina en el producto final (Ascone et al., 2017; Maria Diaz et al., 2016b), con el riesgo de provocar efectos indeseados en la salud del consumidor y las consecuentes pérdidas económicas. Cabe destacar, que este fenómeno es especialmente problemático en los quesos que se procesan después de la maduración para su presentación en el mercado, como los quesos rayados y loncheados, dado que se pueden formar biofilms en los equipos empleados para su procesamiento, los cuales actúan como reservorios de bacterias productoras de histamina, que contaminan el queso provocando que se acumule dicha AB en el producto final (Galié et al., 2018; Ladero et al., 2009).

Capítulo 1:

Objetivo 1: **Cuantificar la capacidad de formación de biofilms de las cepas de *L. parabuchneri* disponibles en la colección del IPLA.**

En el presente trabajo se analizó la capacidad de formación de biofilms de 24 cepas de *L. parabuchneri* productoras de histamina, aisladas de diversos quesos (Emmental, Mozzarella y Cabrales), además de la cepa tipo (*L. parabuchneri* DSM5707T) aislada

de saliva humana y utilizada como referencia. Todas las cepas formaron biofilms en poliestireno, aunque con distinta capacidad (fuerte, moderada y débil). Nueve cepas fueron clasificadas como moderadas o fuertes formadoras y son las que representan una mayor amenaza para la seguridad alimentaria. Para analizar, las condiciones que podrían favorecer la formación de biofilms se seleccionaron dos cepas de cada tipo: *L. parabuchneri* IPLA11150 y 11151 como fuertes formadoras, IPLA11125 y 11129 como moderadas e IPLA11122 y 11117 como débiles.

Objetivo 2: **Analizar la influencia de distintos factores abióticos en la formación de biofilms: materiales de superficie, fuentes de carbono, pH, temperatura.**

La capacidad de los microorganismos para formar biofilms está influenciada por diversos factores, tales como el material de la superficie, la temperatura, el pH del medio, la disponibilidad de nutrientes y la presencia de otras especies, entre otros (Flint et al., 2020; Liu et al., 2015; Vishwakarma, 2020). En el primer capítulo de esta tesis se han analizado qué factores favorecen o no la formación de biofilms de *L. parabuchneri*.

En primer lugar, se estudió su capacidad para adherirse a las superficies más utilizadas en la industria láctea: acero inoxidable, plástico alimentario, goma y madera de haya. Se comprobó, mediante recuentos, que las seis cepas seleccionadas, eran capaces de adherirse a todas las superficies ensayadas (Articulo 1, Tabla 2). Sin embargo, la clasificación de las cepas como fuertes, moderadas o débiles determinada en poliestireno mediante la técnica de cristal violeta, no se observó mediante los recuentos en las distintas superficies, ya que en el poliestireno y la madera no se observaron diferencias significativas entre las seis cepas. Además, estos materiales fueron los que más facilitaron la adhesión celular. En el acero inoxidable, la adhesión fue mayor para *L. parabuchneri* IPLA11151, IPLA11125 e IPLA11122, en cambio, la adhesión de *L. parabuchneri* IPLA11117 fue la mitad de la registrada para IPLA11151. En plástico, IPLA11151 (fuerte) e IPLA11129 (moderada) mostraron valores de adhesión más altos que las demás. Por último, en goma, los valores de adhesión más elevados fueron obtenidos por IPLA11151 y IPLA11125, lo que demuestra que otros factores además de la adhesión celular estarían involucrados en la formación del biofilm.

Además, se observaron mediante microscopía electrónica (SEM) los biofilms formados por las cepas IPLA11151, 11125 y 11122 en las distintas superficies (Articulo 1, Figura

8), sugiriendo que la madera de haya es el material en el que los biofilms alcanzan un volumen mayor, incluso en las cepas moderadas y débiles formadoras (IPLA11125 y IPLA11122). Esto podría estar relacionado a la rugosidad y porosidad del material que, como se observa en la Figura 8 (Artículo 1), es muy elevada. Aunque la madera no se utiliza con frecuencia en las grandes instalaciones industriales, se sigue empleando por ejemplo para estanterías en producciones artesanales, donde la maduración se produce en cuevas naturales. La goma, otro material con alta porosidad, si es un material más utilizado en la industria, ya que suele encontrarse en las conexiones entre tuberías y depósitos de almacenamiento, lo que dificulta su limpieza. Por lo tanto, la capacidad de las cepas de *L. parabuchneri* para adherirse a este material supone un problema. De hecho, ésta fue una de las principales localizaciones en las que se encontró *L. parabuchneri* en algunas instalaciones lácteas (Maurer et al., 2016). Además, en las imágenes de SEM se observó que IPLA11151 (fuerte) presentó células más largas y mayores cantidades de EPS. Como se ha expuesto, durante la formación de biofilm las células se alargan y producen EPS (Bridier et al., 2010; Dakheel et al., 2016), que proporciona estabilidad a la estructura (Olszewska and Diez-Gonzalez, 2021) y resistencia a agentes antimicrobianos y tratamientos de limpieza (Bajrami et al., 2022b).

Los resultados obtenidos indican que el acero inoxidable sería el material más apropiado para su uso en la industria láctea, no solo por la dificultad de adhesión que presentó para las cepas analizadas, con excepción de IPLA11151, sino también por la facilidad para su limpieza y desinfección, y la resistencia a tratamientos abrasivos necesarios para eliminar biofilms. Sin embargo, el hecho de que algunas cepas, como *L. parabuchneri* IPLA11151, hayan mostrado una mayor capacidad de adhesión indica la necesidad de mantener y maximizar los procedimientos de limpieza para evitar el riesgo de contaminación de equipamientos de acero inoxidable.

Uno de los factores más importantes implicados en la adhesión bacteriana es la temperatura del entorno. En general, las altas temperaturas aumentan la hidrofobicidad de la superficie celular, facilitando la adhesión (Karaca et al., 2019). En el presente trabajo, se observó que las bajas temperaturas (8°C) redujeron la capacidad de todas las cepas ensayadas de formar biofilms (Articulo 1, Figura 4). La producción de biofilms después de 30 días de incubación a 8ºC, se redujo en las cepas fuertes formadoras (IPLA11150 y 11151) respecto a 12 y 24ºC. Dicho efecto de reducción de biofilms a temperaturas más bajas, se ha observado en otros microorganismos patógenos

transmitidos por alimentos (Bajrami et al., 2022a; Kadam et al., 2013; Ma et al., 2019), lo que podría estar relacionado con una menor tasa de crecimiento. En el presente trabajo, las cepas fuertes productoras, lograron formar biofilms robustos a 24ºC tras 48 horas y a 12ºC tras siete días de incubación. En el caso de las cepas de *L. parabuchneri* moderadas y débiles, la reducción en la temperatura de incubación (8°C) tuvo un efecto preventivo, aunque después de tiempos prolongados de incubación (30 días a 12° y 24°C) lograron desarrollar biofilms. Es importante resaltar que estudios previos determinaron que *L. parabuchneri* es capaz de producir histamina inclusive a temperaturas por debajo de la temperatura óptima de crecimiento (Diaz et al., 2018), en tiempos prolongados de incubación. Por lo tanto, mantener la temperatura de producción y almacenamiento lo más baja posible es una medida eficaz para mejorar la seguridad alimentaria, aunque en la fabricación de queso no siempre es posible (Ascone et al., 2017; Şanli and Şenel, 2015).

Respecto al efecto del pH, se observó que tanto en las fuertes formadoras como en las moderadas aumentó la biomasa de los biofilms al incubarse a pH ácido (4.7) en comparación con un pH más neutro (6.0). Por lo tanto, la habilidad de las cepas de *L. parabuchneri* para formar biofilms se vería favorecida en matrices lácteas, donde generalmente el pH es ácido debido a la producción de lactato durante la fermentación de la lactosa. De hecho, se ha demostrado que algunas BAL sobreviven en entornos ácidos debido a su capacidad para formar biofilms (Extremina et al., 2011; Yin et al., 2019).

La principal fuente de carbono en el ambiente lácteo es la lactosa, el azúcar de la leche que durante la fermentación se cataboliza a galactosa y glucosa por acción de las β-galactosidasas. Es por ello que analizamos la capacidad de formar biofilms en presencia de los tres azucares mencionados. Los resultados obtenidos mostraron que *L. parabuchneri* IPLA11150 y 11151 (fuertes) formaron más biofilm en presencia de glucosa, mientras *L. parabuchneri* IPLA11129 y 11125 (moderadas) aumentaron su producción de biofilm en presencia de galactosa. En otras especies como *Staphylococcus epidermis*, la glucosa favorece la formación de biofilms en comparación a la lactosa, y dicho efecto se atribuye al incremento en la formación de EPS; Yuksekdag y col. (2008) también demostraron que en algunas BAL la glucosa favorece la producción de EPS y consecuentemente incrementa la biomasa del biofilm (Yuksekdag and Aslim, 2008). Por lo tanto, la producción de biofilms en relación con la

fuente de carbono parece ser dependiente de cepa y podría estar relacionado con la capacidad para consumir los diferentes azúcares o con una preferencia jerárquica de su consumo, como se ha observado en otras BAL (del Rio et al., 2015).

Por último, se estudió el efecto de distintas concentraciones de cloruro de sodio en la formación de biofilm, puesto que se ha demostrado que el incremento de la sal incrementa la producción de EPS y por tanto la formación de biofilms en algunas especies (Shi et al., 2017). En *L. parabuchneri* se observó un efecto antimicrobiano, es decir a mayor concentración de sal, menor fue la formación de biofilms de las cepas analizadas. Lo que sugiere que el cloruro de sodio podría utilizarse para reducir la formación de biofilms de *L. parabuchneri*. Sin embargo, hoy en día los consumidores demandan alimentos más saludables, reducidos en sal, debido a los efectos nocivos que la misma produce sobre la salud (Grimes et al., 2009; Kim et al., 2012), lo que implica reducir la presencia de NaCl durante la elaboración de quesos, lo que aumenta el riesgo de acumular microorganismos productores de histamina (Møller et al., 2020).

Se determinaron los principales componentes de la matriz extracelular de los biofilms de las cepas de *L. parabuchneri* fuertes formadoras utilizando métodos enzimáticos u otros agentes de dispersión y se observó, que la matriz de los biofilms se reducía por acción de la proteinasa K y la tripsina, lo cual sugiere que la matriz estaría compuesta principalmente por proteínas. Además, la matriz del biofilm producido por IPLA11151 se redujo por tratamiento con DNAsa I, lo que sugiere que el ADN extracelular seria otro de los componentes presentes en su matriz. No se observó ningún efecto en los biofilms por el tratamiento con RNasa, lo que sugiere que el ARN no sería uno de los componentes de la matriz. A su vez, para analizar la presencia de exopolisacáridos en la matriz, se utilizó el agente dispersante metaperiodato de sodio ($NaIO_4$), pero con esta técnica, no se pudo comprobar la presencia de los EPS en la matriz del biofilm de *L. parabuchneri*.

Capitulo 2:

Objetivo 3: **Estudiar la composición de la matriz extracelular de los biofilms de *L. parabuchneri*.**

Para el análisis de la composición de los biofilms de *L. parabuchneri*, se utilizó una técnica innovadora de espectroscopía IR-ATR, y se continuó trabajando con las seis

cepas seleccionadas. Esta técnica, combina la espectroscopía infrarroja (IR) y la reflectancia total atenuada (ATR), llamada IR-ATR por sus siglas en inglés. La ATR se utiliza para analizar la composición química de muestras, y la IR se basa en el análisis de la interacción entre la radiación infrarroja y las moléculas presentes en la muestra. Combinar ambas técnicas, permite realizar análisis químicos de forma rápida y precisa (Bajrami et al., 2022b). Para el presente trabajo, IR-ATR demostró ser una técnica altamente eficaz para monitorear y analizar en tiempo real los cambios en la matriz de los biofilms de *L. parabuchneri*.

En primer lugar, se observó la composición de las células planctónicas de las seis cepas de *L. parabuchneri*. Los resultados revelaron variaciones en las bandas espectrales correspondientes a proteínas, amidas, ácido láctico, ácidos nucleicos, fosfolípidos y polisacáridos. Aunque se observaron diferencias mínimas en las bandas espectrales, se notó una mayor intensidad de las bandas en las cepas IPLA 11151 y 11150 (fuertes formadoras de biofilms), seguidas de IPLA 11129 y 11125 (moderadas) y IPLA11122 y 11117 (débiles) (Figura 4, Artículo 2), es decir, se correspondió la intensidad de las bandas con la clasificación realizada mediante la técnica del CV. Podría decirse que a mayor capacidad de adhesión de las células planctónicas, mayor intensidad de las bandas espectrales obtenidas.

Sin embargo, el análisis de las células planctónicas no es suficiente para determinar la composición de la matriz extracelular, por lo tanto, se observó la formación de biofilms a tiempos prolongados (48 h).

Además, se observaron los niveles de oxígeno dentro de la celda de IR-ATR (Figura 3a, Artículo 2). A medida que el espesor del biofilm aumentaba, el oxígeno disminuía y este efecto fue similar en todas las cepas, como se había observado previamente en *L. parabuchneri* DSMZ 5987 (Bajrami et al., 2022b). Borriello y colaboradores (2014) indicaron que esto se debe a que la capa de EPS aumenta el volumen de la estructura y consecuentemente, limita la difusión de oxígeno y nutrientes en el interior del biofilm (Borriello et al., 2004), lo cual en el caso de *L. parabuchneri* es una ventaja mantener las condiciones de anaerobiosis debido a que son cepas que crecen en condiciones anaerobias facultativas (Zheng et al., 2020).

El análisis espectral de la composición de los biofilms de *L. parabuchneri* se dividió en tres regiones principales: amida I y amida II (1700-1500cm^{-1}), ácido láctico, amida III y

ácidos nucleicos (1460-1220cm^{-1}), y carbohidratos y EPS (1350-1200 cm^{-1}) (Gieroba et al., 2020) (Figura 5a, b, Articulo 2).

Se observó una mayor intensidad de las bandas de amida I y II en las cepas fuertes formadoras de biofilms, lo que sugiere mayor contenido de proteínas, (Hlaing et al., 2017; Kong and Yu, 2007) y péptidos (Stenclova et al., 2019) en la matriz extracelular.

La presencia de ácido láctico en la matriz extracelular varió entre las cepas. En el Artículo 1 observamos que la presencia de pH ácido en el medio y el desarrollo de biofilm eran cepa dependiente, y que *L. parabuchneri* IPLA11125, 11150 y 11151 formaban más biofilms en ambientes ácidos. En este analisis, la presencia de ácido láctico en la matriz fue mayor en las mismas cepas (Figura 5a, b, Articulo 2), lo cual podría indicar cierta relación entre el biofilm de *L. parabuchneri* y la acidez, ya sea que el biofilm se forme en un entorno ácido o que la propia matriz genere un entorno ácido, por el propio metabolismo de las cepas, como en este caso sería mediante la producción de ácido láctico.

Otro hallazgo interesante fue la presencia de ácidos nucleicos (1257 y 1237 cm^{-1}) (Ojeda et al., 2008), especialmente en la matriz de *L. parabuchneri* IPLA 11151 donde se observó un aumento. Dicho incremento, se asoció con la presencia de ADN extracelular en la matriz, que se sugirió previamente mediante tratamiento enzimático en el Articulo 1 (Figura 1, Artículo 1). Los resultados obtenidos aquí (Figura 5A, Articulo 2), donde se observó un aumento del pico de ácido nucleico para esta cepa en comparación con las demás después de 24 h de formación del biofilm, confirma lo expuesto previamente.

En las bandas de amida III (1350-1200 cm^{-1}) (Bajrami et al., 2022b), se observó una mayor intensidad para *L. parabuchneri* IPLA 11151 después de 24 horas, y una intensidad similar para la cepa 11125 tras 48 horas (Figura 5b y 6d, Articulo 2), las bandas de amida III se relacionan con la presencia de proteínas y componentes celulares como nucleoides y ribosomas, indicando actividad celular (Tabla S1, Articulo 2). Además, en la región correspondiente a los carbohidratos y EPS, se observó que el contenido de EPS aumentó continuamente. El contenido de EPS más alto después de 48 horas, se observó en *L. parabuchneri* IPLA 11151, fuerte formadora (Figura 5b, Articulo 2). *L. parabuchneri* IPLA 11150, también mostró una composición de polisacáridos elevada, similar a la moderada productora, *L. parabuchneri* IPLA 11125 (Figura 6b y d,

Articulo 2), mientras que *L. parabuchneri* IPLA 11129, 11122 y 11117 mostraron una actividad de EPS similar entre sí, y menor a las demás (Figura 6c, e y f, Articulo 2). En estudios anteriores (Figura 1, Articulo 1), para evaluar la presencia de EPS, se utilizó el agente dispersante $NaIO_4$ y no se obtuvieron resultados concluyentes (Sarquis et al., 2023). El tratamiento con $NaIO_4$ solo proporciona información sobre la naturaleza de los exopolisacáridos y sus modificaciones en un tiempo puntual (Dakheel et al., 2016; Sager et al., 2015), mientras que con espectroscopia de IR-ATR se indica la presencia de EPS sin generar la dispersión, permitiendo observar la evolución del biofilm y sus componentes en tiempos prolongados. Por lo tanto, se observó las variaciones en el contenido de EPS en todas las cepas ensayadas, siendo las fuertes formadoras quienes lo presentaron en mayor proporción en su matriz extracelular, reforzando la teoría del rol fundamental de los EPS en los biofilms bacterianos (Shi et al., 2017). Además, la banda de EPS se podría considerar un "indicador de biofilm" puesto que la presencia de EPS se considera el principal constituyente de la matriz extracelular del biofilm (Bajrami et al., 2022b; Hossain et al., 2023).

Por otro lado, las Figuras 7a, Articulo 2, muestra que las bandas de amida I y amida II a lo largo del tiempo para cada cepa estudiada, presentaron leves modificaciones. Se aprecian ligeros aumentos en *L. parabuchneri* IPLA 11150 y 11122, una leve disminución de 11125, *L. parabuchneri* IPLA 11151 y 11129 se mostraron prácticamente constantes. En estudios realizados por Bajrami y col. (2022) se observó que en *L. parabuchneri* DSM 5987, las variaciones obtenidas en el contenido de amida I se incrementaba notablemente, y el de amida II presentaba ligeros aumentos, al igual que 11150 (fuerte) y 11122 (débil). En el Articulo 1 se definió a DSM 5987 como moderada formadora de biofilm, por lo tanto, el contenido de amidas sería cepa dependiente.

El análisis de las bandas IR de los niveles de ácido láctico (LA) y ácidos nucleicos (NA) en un monitoreo menor a 24 horas, mostró una producción prácticamente constante y cepa dependiente (Figura 7b). En *L. parabuchneri* IPLA 11117 se observó un comportamiento casi constante, que posteriormente aumentó ligeramente. De manera similar, *L. parabuchneri* IPLA 11150, fuerte productora, exhibió un leve incremento, mientras que *L. parabuchneri* IPLA 11151 y 11125 mostraron una disminución y *L. parabuchneri* IPLA 11129 y 11122 fueron constantes. Los ligeros aumentos obtenidos

por la moderada formadora de biofilm *L. parabuchneri* DSM5987 respaldan la teoría de los niveles de LA y NA son cepa dependientes (Bajrami et al., 2022b).

Como se mencionó previamente, en las seis cepas de *L. parabuchneri*, los niveles de EPS aumentaron significativamente en función del tiempo correspondiéndose con la clasificación de fuertes, moderadas y débiles formadoras (Figura 7c, Articulo 2). El contenido de EPS aumentó continuamente y siguió el orden de las cepas según su capacidad de producción de biofilm. En el desarrollo del biofilm, el aumento de esta banda indica un incremento en la producción de polisacáridos y EPS y división celular en la matriz (Humbert and Quilès, 2011; Kang et al., 2006).

La aplicación de análisis multivariados, como el análisis de componentes principales (PCA) y el *análisis discriminante de mínimos cuadrados parciales* (PLS-DA), permitió una clasificación efectiva de las cepas según su composición y capacidad de formación de biofilms.

Mediante el PCA se observó una clara separación de los datos de acuerdo a la composición de las células planctónicas (Figura 8, Articulo 2), y por ende, reveló que solo dos componentes principales pueden explicar la variabilidad en los datos. A su vez, las diferencias significativas en la composición de las células planctónicas podrían fundamentarse en la capacidad de adhesión de las cepas, la cual podría deberse a la diversidad genética de las cepas (Maria Diaz et al., 2016a; Gumustop and Ortakci, 2022) y la presencia de ciertos genes que podrían otorgar mayor capacidad de adhesión (como se expone en el Artículo 3 del presente trabajo).

El PLS-DA permitió clasificar las cepas en función de su composición y capacidad de formación de biofilms. La separación en diferentes grupos tras 48 h de monitoreo de la composición de la matriz se logró utilizando este modelo (Figura 9 del Artículo 2).
El análisis de los datos permitió verificar los cambios en las regiones de amida I, amida II, ácidos nucleicos y EPS. Se observó un aumento en el contenido de proteínas para las cepas *L. parabuchneri* IPLA 11151, 11150 y 11125 a medida que los picos de amida I y II aumentaban en el tiempo. Estos hallazgos son consistentes con los resultados obtenidos en el Artículo 1 de la presente Tesis Doctoral, que han informado un mayor contenido de proteínas para las fuertes formadoras de biofilms, 11151 y 11150 (Sarquis et al., 2023). Además, las señales de ácidos nucleicos y amida III, polisacáridos y

fosfolípidos se vieron disminuidos para las cepas *L. parabuchneri* IPLA 11129, 11122 y 11117, que son productores de biofilms moderados y débiles, respectivamente.

El modelo PLS-DA utilizando tres variables latentes (LVs), al comparar los biofilms de *L. parabuchneri* tras 48 h de desarrollo, reveló la existencia de tres grupos distintos, con un 98.46% de la variabilidad (Figura 11a). Estos tres grupos fueron similares a los obtenidos mediante la técnica de cristal violeta descrito en el Artículo 1: *L. parabuchneri* IPLA 11151 y 11150 (fuertes), 11129 y 11125 (moderadas) y 11122 y 11117 (débiles), confirmando la veracidad de los resultados (Sarquis et al., 2023). Otro dato interesante es que las principales diferencias observadas en la composición de los biofilm de *L. parabuchneri*: ocurrieron en las regiones de amida I y amida II (Figura 11b).

También se construyó un modelo PLS-DA utilizando solo las primeras 24 h de formación de biofilms (Figura S4a). Los biofilms de 24 h, es decir, no maduros y no estables, no presentaron diferencias significativas en la composición (Figura S4b). La *superposición* de los datos entre las cepas moderadas y débiles productoras, confirma que para analizar los biofilms formados por cepas de *L. parabuchneri* es necesario por lo menos 48 h de incubación, como previamente se había observado en el Articulo 1 (Figura 3).

Para concluir, podríamos decir que se logró una diferenciación clara de la composición de los biofilms de *L. parabuchneri* en tiempo real. Se confirmó que los principales componentes de la matriz extracelular de los biofilms formados por *L. parabuchneri* IPLA11150 y 11151 son los exopolisacáridos y las proteínas. La presencia de ambos componentes en la matriz extracelular es de suma importancia para comprender la formación y estabilidad de los biofilms de estas cepas. Puesto que, como se expuso anteriormente, los EPS cumplen un papel fundamental en la arquitectura y cohesión de los biofilms. Su naturaleza polimérica y su capacidad para formar una matriz tridimensional proporcionan una estructura que protege las células de factores estresantes (Flemming et al., 2022; Yin et al., 2019). Esta confirmación refuerza la importancia de los EPS en la formación y la integridad del biofilm, lo cual se correlaciona con los biofilms fuertes de las cepas *L. parabuchneri* IPLA11150 y 11151.

Además, la identificación de proteínas en la matriz extracelular sugiere una interacción compleja con el entorno, ya que, las proteínas cumplen múltiples roles en la formación

del biofilm, incluyendo funciones de movilidad, adhesión, comunicación celular y respuesta a estrés (Carrascosa et al., 2021; Sharma et al., 2023). Estos resultados se complementan con los obtenidos mediante tratamientos enzimáticos en el Artículo 1, sugiriendo una posible relación entre la presencia de ciertas proteínas y su contribución a la formación del biofilm y con el Articulo 3 en el que se define la presencia de un cluster de genes que permite la síntesis de un pili en las cepas fuertes formadoras, para el cual son necesarias proteínas, específicamente las identificadas por las bandas espectrales de amidas I y II.

Por otro lado, el hecho de que los exopolisacáridos y proteínas sean los componentes predominantes permite suponer que existe cierta interdependencia y cooperación entre estos dos elementos para la formación de los fuertes biofilms de IPLA11150 y 11151, al igual que ocurre en los biofilms de *Vibrio cholerae* donde los EPS generan una estructura viscoelástica que evita el colapso de la matriz, al mismo tiempo que las proteínas, desempeñan su papel en la adhesión célula-célula y en la unión del biofilm a las superficies (Berk et al., 2012; Fong et al., 2017).

Finalmente, utilizando el modelo PLS-DA fue posible determinar y analizar la composición de la matriz de los biofilms de *L. parabuchneri* y establecer una notable discriminación entre las cepas en relación con su composición y la capacidad de formar biofilms. La técnica de espectroscopía de IR-ATR resultó una herramienta eficiente para la clasificación no destructiva y a largo plazo de especies microbianas formadoras de biofilms.

Capítulo 3:

Objetivo 4: **Identificar y localizar los elementos genéticos implicados en la formación de biofilms: secuenciación y anotación de genomas.**

Objetivo 5: **Determinar la funcionalidad del clúster de adhesión.**

En el presente apartado de esta Tesis doctoral, se analizaron factores genéticos que pudiesen influir en la formación de biofilms de cepas de *L. parabuchneri*, productoras de histamina y aisladas de quesos.

Para lograr dichos objetivos, se secuenciaron y compararon los genomas de las seis cepas seleccionadas. Los resultados indicaron que el 73% de los genes eran compartidos entre las seis cepas estudiadas, incluyendo el cluster de genes *HDC*, necesario para la síntesis de histamina (Maria Diaz et al., 2016d) (Tabla S2, Articulo 3). *L. parabuchneri*, IPLA11150 e IPLA11151 (fuertes formadoras de biofilm) presentaron 164 genes en común (5.5%) distintos a las demás cepas en estudio (Tabla S2, Articulo 3). Entre estos genes se encontró un *cluster* de cuatro genes que mostró similitudes con genes de un modelo de pili mediado por sortasa: organizándose de manera similar a los sistemas SpaCBA y SpaFED descritos para *L. rhamnosus* y *L. casei* (genes *tip – basal – major – sortC)* (Fig. 2, Articulo 3) (Krishnan et al., 2016). A este grupo de genes se le designó con el nombre de *pilus gene cluster* (PGC).

Como se describió previamente, los pili o fimbrias son estructuras filamentosas utilizadas por las bacterias para interactuar con el huésped, establecer el contacto célula-célula y participar en la adhesión a superficies (Hendrickx et al., 2011; Proft and Baker, 2009; Scott and Zähner, 2006). El modelo de pili mediado por sortasa, se identificó en múltiples cepas y especies de patógenos Gram-positivos, así como en bacterias comensales de la microbiota intestinal en donde es esencial para la adherencia y colonización (Kuhn, 2019). Este modelo puede presentar variaciones en las distintas especies bacterianas, tanto en el número de genes asociados, como en las pilinas que forman, las sortasas específicas que unen las pilinas así como en la estructura del pili final (Krishnan et al., 2016). Las bacterias que poseen pili en su superficie han desarrollado esta característica como una estrategia para adaptarse y sobrevivir en un entorno específico (Ellison et al., 2022; Hendrickx et al., 2011). Además, el modelo de SpaCBA para la formación del pili en *Lactobacillus rhamnosus* GG se ha descrito que cumple un rol fundamental en la adherencia, en la formación de biofilm y señalización del huésped (Rasinkangas et al., 2020). La expresión heteróloga del *cluster* PGC de *L. parabuchneri* IPLA11150 en *L. cremoris* NZ9000 (Fig. 4 y 5, Articulo 3), demostró su implicación en la capacidad de *L. parabuchneri* de adherirse a superficies de poliestireno y acero inoxidable, lo que le permitiría formar biofilms fuertes.

Además, se ha descrito que los genes PGC, presentes en diferentes bacterias, generalmente se encuentran cerca de trasposasas (Krishnan et al., 2016), por lo que probablemente fueron adquiridos por transferencia horizontal. Los resultados obtenidos en el presente trabajo muestran que en *L. parabuchneri* IPLA11150 este *cluster* está

flanqueado por dos secuencias de inserción idénticas y orientadas divergentemente (Fig. 3, Fig. S1, Articulo 3). A pesar de que Krishnan y col. (2016) sugirieron que el modelo de pilus mediado por sortasa se localizaba en el cromosoma de bacterias Gram-positivas (Krishnan et al., 2016), algunos autores indicaron que en ciertos casos se localiza en un plásmido como en *L. casei* LOCK0919 (Aleksandrzak-Piekarczyk et al., 2016). De hecho, el análisis BLAST realizado en este trabajo indicó que algunos PGC similares a los presentes en las cepas de *L. parabuchneri*, *L. brevis*, *L. delbrueckii* y *L. plantarum* se localizaban en plásmidos. La localización plasmídica del PGC en *L. parabuchneri* IPLA 11150 y 11151 fue sugerida inicialmente mediante un ensayo de qPCR en el que se comparó el número de copias estimado de genes de conocida localización cromosómica y los de PGC, partiendo de ADN genómico. Este ensayo se repitió bajo las mismas condiciones partiendo de ADN plasmídico y la diferencia en el número de copias estimado fue mucho mayor. Finalmente, se confirmó mediante secuenciación PacBio que en *L. parabuchneri* IPLA 11150 el PGC se localiza en un plásmido de 33 kb (Figura 3, Articulo 3) que posee varios ORF similares a transposasas y secuencias de inserción. Esto indica mayor plasticidad genética y la posibilidad de transferencia horizontal de estos genes. También en el mismo plásmido se encontraron otros genes con similitudes a genes que permiten la movilidad como *mobA*, que podría estar involucrado en la transferencia de plásmidos (Garcillán-Barcia et al., 2019). Es importante destacar que la presencia del PGC en un plásmido es un dato de gran relevancia ya que los plásmidos favorecen la transferencia horizontal de genes entre bacterias, permitiendo la diseminación de características accesorias ecológicamente fundamentales (Bottery, 2022) y en este caso, la transmisión horizontal del PGC, aumenta las posibilidades de que la capacidad de adherirse a superficies y de formar biofilms, sea transferida a otras cepas de *L. parabuchneri* productoras de histamina. Incluso podría transferirse a otras especies bacterianas productoras de histamina o de otras aminas biógenas o podría ser adquirida por especies patógenas, con todas las complicaciones que esto podría suponer para la industria láctea (Grohmann et al., 2003; Krishnan et al., 2016; Rasinkangas et al., 2020).

Capítulo 4:

Objetivo 6: **Analizar, evaluar y proponer métodos bio-sostenibles para la prevención y eliminación de los biofilms formados por bacterias del ácido láctico productoras de aminas biógenas.**

En el último apartado se han analizado métodos de prevención y eliminación de biofilms formados por BAL productoras de aminas biógenas, entre las que se incluyen *L. parabuchneri* IPLA11150 y 11151.

Inicialmente, se analizó la eficacia de los métodos de limpieza y desinfección utilizados en la industria láctea para la eliminación de los biofilms fuertes de *L. parabuchneri* IPLA 11150 y 11151 en distintas superficies como acero inoxidable, madera de haya, plástico y goma. Si bien los métodos utilizados fueron eficientes para su eliminación, estudios previos han demostrado que el uso de biocidas presenta múltiples desventajas como el desarrollo de tolerancia y resistencia bacteriana a los productos químicos utilizados (Fagerlund et al., 2017; Pezzuto et al., 2016), disminución de la eficiencia en presencia de biofilms mixtos (Singh and Anand, 2022), y también se ha demostrado que los biocidas pueden favorecer la propagación de patógenos resistentes a antibióticos y productos químicos, cuando no se utilizan adecuadamente (Dancer, 2014; Vergnano, 2015). Por los motivos expuestos, es necesario desarrollar nuevas estrategias de prevención y eliminación de biofilms bacterianos utilizando sustancias biodegradables, más naturales y sostenibles, en concordancia con el cuidado del medio ambiente.

Si bien, el enfoque principal de esta Tesis Doctoral reside en el análisis de las BAL productoras de histamina, se llevó a cabo una investigación exhaustiva sobre el potencial antimicrobiano de bacteriocinas frente a cepas productoras de AB en alimentos. Esto se debe a que las BAL suelen ser productoras de diversas AB (tiramina, histamina y putrescina) en las matrices lácteas estudiadas (Linares et al., 2011). En este sentido, se ha analizado el potencial de bacteriocinas para prevenir la formación de biofilms de los principales productores de AB en queso. Concretamente, en el Articulo 4, se evaluó el potencial antimicrobiano de bacteriocinas producidas por BAL, y específicamente la capacidad de la Nisina A de prevenir la formación de biofilm. En el Artículo 5 se determinó el potencial de la bacteriocina AS-48 producida por distintas cepas de *Enterococcus faecalis* algunas aisladas de quesos como *E. faecalis* UGRA10 (Grande Burgos et al., 2014).

En primer lugar, se evaluó la sensibilidad de las cepas de BAL productoras de AB a los sobrenadantes libres de células (CFS) con distintas bacteriocinas (Coagulin A, Enterocin A, Enterocin P, Lacticin 481, Nisin A, Nisin Z y Plantaricin C), lo cual reveló una respuesta variable y cepa dependiente. Las distintas cepas productoras de histamina, tiramina y/o putrescina presentaron diferentes grados de sensibilidad a las bacteriocinas.

En las cepas productoras de histamina, se observó una variabilidad en la respuesta: todas las cepas de *L. parabuchneri* analizadas presentaron sensibilidad a las bacteriocinas enterocina P, nisina A y P, siendo prácticamente las más sensibles de este grupo, a excepción de *P. parvulus* que demostró ser aún más sensible, mostrando inhibición frente a más tipos de bacteriocinas. Las cepas *L. hilgardii* 321 y *L. reuteri* IPLA 11078 solo resultaron sensibles a nisina A y nisina Z y entre las cepas de *L. vaginalis*, se observaron respuestas variadas, siendo IPLA 11054 resistente a todas las bacteriocinas analizadas. En general, la nisina A y nisina Z mostraron el espectro antibacteriano más amplio, inhibiendo el crecimiento del 87% de las cepas productoras de histamina.

Sin embargo, en el Articulo 5 se determinó la capacidad de inhibición que la AS-48 tenía sobre las cepas productoras de histamina de *L. parabuchneri*, *L. hilgardii*, *L. vaginalis*, *L. reuteri*, *P. parvulus* y *S. thermophilus*, que en general indicaron una fuerte o moderada inhibición. Aunque, *L. reuteri* y *L. vaginalis* fueron menos sensibles, especialmente *L. vaginalis* IPLA 11054 y 11144 que no fueron inhibidos en absoluto. La AS-48 inhibió el crecimiento del 88% de las cepas productoras de histamina.

A diferencia de los productores de histamina, las cepas productoras de tiramina fueron más sensibles a la bacteriocina coagulina A, inhibiendo el crecimiento del 81% de las cepas. Aunque también se observaron respuestas variadas entre cepas: *E. faecium* mostró una alta sensibilidad, en comparación con *E. durans* y *L. curvatus*. En contraste, *L. brevis* –productora de tiramina y putrescina- presentó una menor susceptibilidad, siendo inhibida únicamente por enterocina P y nisina A. Similar a la coagulina A, la nisina A y nisina Z también presentaron efectos significativos, mientras que la enterocina A tuvo un espectro más limitado. Por otro lado, la acción de la AS-48 sobre el 100% de las cepas productoras de tiramina o tiramina y putrescina, entre las que se incluyen enterococos, cepas de *L. brevis* y *curvatus*, fueron moderada o fuertemente inhibidas.

Las cepas productoras de putrescina fueron más resistentes que las productoras de histamina. Las cepas de *L. brevis* y *L. curvatus* mostraron sensibilidad a la nisina A, mientras que nisina Z tuvo un efecto más limitado. Nisina A demostró el espectro antibacteriano más amplio, inhibiendo el crecimiento del 45% de las cepas y la coagulina A también tuvo un efecto significativo. Sin embargo, a comparación de los productores de histamina, el efecto de las bacteriocinas frente a los productores de putrescina presentaron espectros más restringidos. Por último, frente a la AS-48, el 100% de las cepas productoras de putrescina fueron inhibidas, *Lc. cremoris* y *Lc. lactis* subsp. *lactis*, fueron moderadamente inhibidas, y dos de ellas fueron fuertemente inhibidas *Lc. lactis* subsp. *lactis* 2BA40 y *L. brevis* CECT 3824.

En el Articulo 5, la AS-48 mostró una inhibición del 96% sobre el total de las cepas ensayadas, por lo cual, se analizó la concentración mínima inhibitoria (CMI) de las cepas a distintos tiempos de incubación (24, 48 y >96 h, a temperaturas estándares), obteniéndose valores cepa-dependiente:

Las BAL productoras de histamina como *L. hilgardii* 321 demostraron ser ligeramente más resistente a la AS-48 y dependiente del tiempo de incubación. *L. parabuchneri* IPLA 11150, fue la menos sensible de las cepas de su especie y las cepas de *L. vaginalis* mostraron cierta resistencia después de 96 h, a diferencia de *P. parvulus* 276 que fue inhibido en todas las concentraciones y en todos los tiempos de incubación probados, mientras que para *S. thermophilus* se registró una misma CIM para todos los tiempos de incubación.

Las cepas productoras de tiramina o tiramina y putrescina, *E. durans, E. faecalis*, *E. faecium* presentaron una CIM independiente del tiempo de incubación, a diferencia de las productoras de histamina. *E. hirae* 268, fue el único taxón del género asociado a una CIM dependiente del período de incubación, aumentando después de 96 h de tratamiento. Por otro lado, no se registró crecimiento bacteriano para *L. brevis* tras 24 h, aunque *L. brevis* 3810, 3811 y C311 mostraron una ligera resistencia después de 96 h de incubación. Las cepas de *L. curvatus* fueron muy sensibles puesto que ninguna creció en presencia de AS-48, excepto *L. curvatus* VI14 (después de 96 h).

Finalmente, para las cepas productoras de putrescina de *Lc. cremoris*, se registraron valores de CIM similares a las cepas de *Lc. lactis* subsp. *lactis* e independientes del

tiempo de incubación y *L. brevis* productora de putrescina fue sensible a la acción de la bacteriocina.

En resumen, si bien la capacidad inhibitoria de las bacteriocinas fue cepa dependiente, las bacteriocinas que mostraron mayor inhibición fueron la nisina A (Tabla 2, Articulo 4) con un 65% de inhibición del total de cepas y la AS-48 con un 96% de inhibición, incluso a bajas concentraciones y después de 96 h de incubación (Tabla 2, Artículo 5).

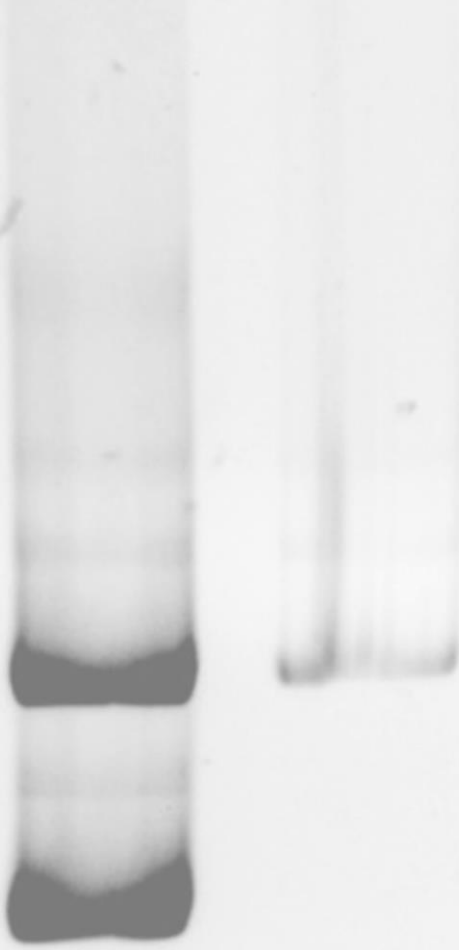
Posteriormente, se analizó la capacidad de estas bacteriocinas (AS-48 y nisina A) de prevenir la formación de biofilms de las bacterias productoras de AB y para ello se seleccionaron las cepas de cada especie clasificadas previamente como fuertes formadoras de biofilm (Maria Diaz et al., 2016b).

La nisina A fue capaz de prevenir la formación de biofilm de un 67% de las cepas (Fig. 1, Articulo 4) en cambio, la AS-48 inhibió significativamente la formación de biofilm de 92% de las cepas examinadas, incluso a la concentración más baja probada (1.75 μM). De hecho, para las cepas sensibles, no se observaron diferencias significativas en entre las dos concentraciones de AS-48 ensayadas (Fig. 1, Articulo 5). La única cepa que produjo biofilm en presencia de AS-48 fue la cepa productora de tiramina, *E. durans* IPLA 655 (Fig. 1B, Articulo 5), cuya producción fue significativamente reducida por la acción de la nisina A (Fig. 1A, Articulo 4). Se demostró que la nisina A inhibió significativamente la formación de biofilms con reducciones que oscilaron entre el 27% y el 84% y previno la formación de biofilms en cepas de *L. parabuchneri*, *L. reuteri* y *L. vaginalis*, productoras de histamina. Además, la nisina A previno la formación de biofilms en cepas de *Enteroccocus* productores de tiramina y putrescina, lo que podría contribuir a reducir la acumulación de estas aminas en productos lácteos. La diferencia en la eficacia de las bacteriocinas puede depender de la estructura y la matriz extracelular de diferentes biofilms, lo que podría afectar el acceso de la bacteriocina a la membrana celular donde actúa (Grande Burgos et al., 2014; Maqueda et al., 2005). Algunos estudios sugieren que la AS-48 puede tener un efecto más pronunciado a pH ácido (Muñoz et al., 2007), lo que podría mejorar su efecto inhibitorio en las fermentaciones lácticas. Otros estudios también indican que AS-48 y la nisina actúan de manera sinérgica (Perales-Adán et al., 2018), lo que podría proporcionar una mayor protección contra el desarrollo de bacterias productoras de AB.

También, es importante destacar que ambas bacteriocinas AS-48 y nisina A, fueron capaces de prevenir la formación de biofilm por parte de *L. parabuchneri* IPLA11150, el principal productor de histamina en el queso (Ascone et al., 2017; Maria Diaz et al., 2016d; Wechsler et al., 2021). Como se ha mencionado previamente, el control de los biofilms formados por *L. parabuchneri* es fundamental para preservar la seguridad alimentaria en los productos lácteos, puesto que los biofilms de esta especie pueden producirse en equipos de procesamiento de alimentos y representa una fuente de contaminación específicamente para los quesos procesados después de la maduración (Maria Diaz et al., 2016a).

En resumen, estos estudios sugieren que tanto la nisina A como la AS-48 tiene un amplio espectro de acción contra cepas productoras de AB y puede prevenir eficazmente la formación de biofilms en muchas de estas cepas.

La implementación de estrategias bio-sostenibles como el uso de bacteriocinas, representaría una herramienta muy valiosa para la prevención de biofilms, la acumulación de AB y la contaminación bacteriana en alimentos. Considerándose una herramienta prometedora para la industria alimentaria. No obstante, se requieren investigaciones adicionales para optimizar los métodos de aplicación de bacteriocinas y garantizar su eficacia y seguridad en alimentos.



CONCLUSIONES

5. CONCLUSIONES

1. Se clasificaron 24 cepas de *L. parabuchneri* aisladas de queso y productoras de histamina, y la cepa tipo, de acuerdo a la capacidad de formar biofilm en fuertes, moderadas y débiles formadoras.

2. Se determinaron los componentes de la matriz extracelular de las cepas *L. parabuchneri* fuertes formadoras de biofilm utilizando métodos enzimáticos o sustancias degradativas de la matriz. Las proteínas podrían ser componentes clave en la matriz extracelular del biofilm, y en el caso de *L. parabuchneri* IPLA 11151 también el ADN extracelular.

3. Se estableció la influencia de factores abióticos de los biofilms formados por cepas de *L. parabuchneri* productoras de histamina con distintas capacidades de formar biofilm. Estableciéndose que 48 h de incubación, el pH ácido, la ausencia de sal y la madera, son factores claves que favorecen la adhesión y desarrollo de los biofilms, mientras que la presencia de azúcares es cepa dependiente.

4. Se analizó la composición de la matriz extracelular del biofilm de seis cepas de *L. parabuchneri* en tiempo real, utilizando técnicas de espectroscopía novedosas (IR-ATR) y se complementaron con los resultados obtenidos por otro tipo de técnicas (cristal violeta y microscopía confocal).

5. Se analizaron las variaciones de la composición de amidas I y II, ácidos nucleicos, acido láctico y EPS, en cepas con distintas capacidades de producción de biofilm, desde el primer estadio de adhesión (células planctónicas) hasta el desarrollo del biofilm maduro, utilizando distintas herramientas para el análisis de los datos (PCA y PLS-DA).

6. Se estudiaron y confirmaron cambios moleculares en la composición de la matriz de las distintas cepas de *L. parabuchneri*, evidenciándose que los componentes de la matriz del biofilm de las fuertes formadoras (IPLA11150 y 11151) sería proteínas y EPS y en IPLA11151 también ADN extracelular.

7. se secuenciaron y analizaron seis cepas de *L. parabuchneri* con distintas capacidades de formar biofilm y se encontró un clúster de 4 genes presente solo en las fuertes formadoras.

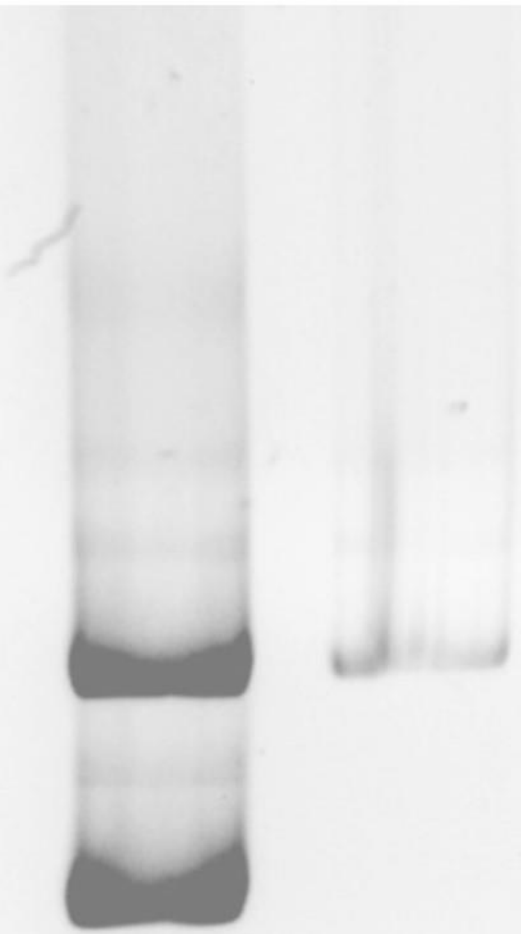
8. Se confirmó la funcionalidad del clúster en la capacidad de adhesión a superficies mediante expresión heteróloga en *Lc. cremoris* NZ9000, utilizando la técnica del cristal violeta y observando los biofilms formados mediante microscopia electrónica.

9. Se confirmó la localización del PGC en un plásmido de 33 kb en *L. parabuchneri* IPLA 11150, rodeado de secuencias de inserción y trasposasas que podrían facilitar la transmisión de estos genes a otras cepas y especies bacterianas y por ende la habilidad de adhesión.

10. Se determinó la eficiencia de métodos químicos de limpieza y desinfección, para la eliminación de biofilms de *L. parabuchneri* IPLA 11150 y 11151 fuertes formadoras en distintas superficies.

11. Se analizó la capacidad antimicrobiana de distintas bacteriocinas producidas por BAL frente a cepas presentes en el entorno lácteo y productoras de AB (histamina, tiramina y putrescina), y la capacidad de prevención de la formación de biofilm de BAL fuertes formadoras de biofilm utilizando la bacteriocina más eficiente, la bacteriocina nisina A.

12. Se estableció la capacidad de inhibición de la bacteriocina AS-48 frente a BAL productoras de AB y se analizó la capacidad de prevenir la formación de biofilm de las BAL productoras de AB fuertes formadoras de biofilm utilizando este método más bio-sostenible.



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