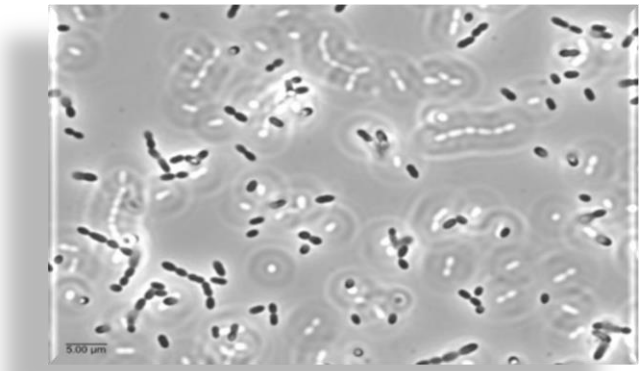
Universidad de Oviedo

Programa de Doctorado en Ingeniería Química, Ambiental y Bioalimentaria

Mejora de la supervivencia y aptitud tecnológica de cepas industriales de *Lactococcus lactis* mediante evolución adaptativa



MARÍA JESÚS LÓPEZ GONZÁLEZ

Tesis Doctoral

2021

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

CSIC
Consejo Superior de Investigaciones Científicas

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A mis padres, Menchu y Chuso, que dedicaron su vida en cuerpo y alma a sus hijas.

Mamá, espero que desde donde estés veas cumplir tu sueño.

Siempre en mi corazón

Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Mejora de la supervivencia y aptitud tecnológica de cepas industriales de *L. lactis* mediante evolución adaptativa	Inglés: Improving survival and technological performance of industrial strains of L. lactis by adaptive evolution

2.- Autor	
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RESUMEN (en español)

Lactococcus lactis es el componente mayoritario de los cultivos iniciadores mesófilos utilizados en la elaboración de productos lácteos fermentados. Durante la fermentación, *L. lactis* se enfrenta a diferentes condiciones de estrés que, en muchas ocasiones, tienen un impacto sobre la pared celular, una estructura celular esencial, cuya integridad es un factor determinante para garantizar la viabilidad. Resultados previos a esta Tesis Doctoral habían demostrado que la modulación de la pared celular en cepas de laboratorio de *L. lactis* daba lugar a cepas más robustas y resistentes a condiciones de estrés tecnológico. Estos resultados, unidos a la creciente demanda de productos fermentados con características organolépticas distintivas, han propiciado la búsqueda de nuevas estrategias para aumentar la diversidad de los cultivos iniciadores disponibles que sean robustos y que presenten nuevos fenotipos y funcionalidades.

En este contexto se ha desarrollado el trabajo de esta Tesis Doctoral fundamentado en la aplicación de la evolución adaptativa bajo condiciones de estrés sobre la pared celular, EA-CES, como estrategia para diversificar y mejorar la aptitud tecnológica de cepas industriales de *L. lactis* usando la bacteriocina Lcn972 como agente selectivo.

El proceso de EA-CES se aplicó a tres cepas acidificantes de *L. lactis* y cinco cepas productoras de nisina, una de ellas comercializada como cultivo protector. Se desarrolló en dos pasos: uno de adaptación, que implicó realizar cultivos en presencia de concentraciones crecientes de Lcn972 para seleccionar mutantes resistentes Lcn972R, y otro de estabilización que conllevó pases sucesivos en ausencia de presión selectiva, sin Lcn972, para fijar aquellas mutaciones que no interfiriesen negativamente con el crecimiento.

Se obtuvieron mutantes estables y resistentes a Lcn972 a partir de todas las cepas. Salvo los mutantes derivados del cultivo protector que perdieron el plásmido de la lactosa durante la adaptación a Lcn972, todos los demás mantuvieron los mismos parámetros de crecimiento en condiciones de laboratorio, así como la misma tasa de acidificación en leche. Por el contrario, sí se detectaron cambios significativos en las propiedades de superficie (hidrofobicidad), siendo algunos mutantes Lcn972R más hidrófobos que su cepa parental, en el grado de autolisis, que en algunos casos fue mayor que las cepas de origen, resistencia a estrés oxidativo, al tratamiento térmico

y/o a otros antimicrobianos de pared (lisozima, bacitracina y nisina). El perfil de susceptibilidad a la infección por bacteriófagos no se vio alterado, excepto en el caso de tres cepas productoras de nisina cuyos derivados Lcn972R fueron más sensibles a la infección. La producción de nisina tampoco se vio afectada, detectándose concentraciones similares tanto en medio de cultivo como en leche.

Por otro lado, mediante el análisis genómico preliminar de los mutantes Lcn972R se detectaron mutaciones puntuales en genes implicados mayoritariamente en 3 categorías funcionales: reguladores transcripcionales, estrés/detoxificación y envuelta celular. Las mutaciones más frecuentes se detectaron en los genes que codifican un módulo de detección y detoxificación de tipo Bce, YsaDCB/TCS-G, no caracterizado previamente en *L. lactis*. Los resultados mostraron que el transportador YsaDCB estaba implicado en la resistencia de *L. lactis* tanto a la Lcn972 como a la bacitracina. También se demostró que las mutaciones seleccionadas presentes en el gen *ysaB,* que codifica la permeasa del transportador, provocaban la activación constitutiva del sistema de dos componentes asociado TCS-G.

La obtención de diferentes fenotipos tanto entre mutantes derivados de la misma cepa de partida como derivados de distintas cepas confirma la plasticidad de *L. lactis* en su adaptación al estrés sobre la pared celular y avala el uso de la EA-CES para generar diversidad funcional en *L. lactis* sin hacer uso de la tecnología del ADN recombinante.

RESUMEN (en inglés)

Lactococcus lactis is the main component of the mesophilic starters used in the manufacture of several fermented milk products. During fermentation, *L. lactis* is exposed to several stressful conditions that usually have an impact on the cell wall, an essential cell structure whose integrity is a crucial factor to ensure cell viability. Previous results to this work had demonstrated that modulating the cell envelope of *L. lactis* laboratory strains led to resistance to technological relevant stresses. These results, together with the growing demand for fermented products with distinctive organoleptic characteristics, have encouraged the search for novel strategies to increase robutness and the diversity of available starter cultures with new phenotypes and technological properties.

Based on this knowledge, the main objetive of this study was to assess the feasibility of adaptive evolution under cell envelope stress, AE-CES, imposed by the cell wall-active bacteriocin Lcn972, as a strategy to diversify and enhance the performance of industrial *L. lactis* strains.

The AE-CES process was applied to three acidifying strains of *L. lactis* and five nisin-producing strains, one of which is sold as a protective culture. It was developed in two steps: an adaptation step, where *L. lactis* strains were exposed repeatedly to increasing concentrations of Lcn972 to select resistant mutants, Lcn972R, and a stabilization step, which involved successive transfers in the absence of selective pressure, without Lcn972, to fix mutations that would not interfere negatively with growth.

Stable Lcn972R mutants could be selected from all strains. All of them retained the main technological aptitudes in laboratory conditions (milk acidification and growth

parameters) except for the mutants derived from the protective culture that lost the lactose plasmid during adaptation to Lcn972. In contrast, significant changes were detected in surface properties (hydrophobicity), with some Lcn972R mutants being more hydrophobic than their parental strain, in the degree of autolysis, which was higher than wild types in some cases, resistance to oxidative stress, heat treatment and/or cross-resistance to other cell wall antimicrobials (lysozyme, bacitracin and nisin). Bacteriophage infection was not altered, except in the case of three nisin-producing strains whose Lcn972R derivatives were more sensitive to infection. Nisin production was unaltered, with similar concentrations detected in both broth and milk.

The preliminary genome analysis of the Lcn972R mutants revealed distinct and shared non-synonymous mutations depending on the parental strain and were found in genes involved in stress response, detoxification modules, cell envelope biogenesis and regulatory functions. The most frequent mutations were detected in genes coding for a previously uncharacterized Bce-type detection and detoxification module, YsaDCB/TCS-G in *L. lactis*. The results showed that the YsaDCB transporter was involved in the resistance of *L. lactis* to both Lcn972 and bacitracin. Selected mutations in the *ysaB* gene, which encodes the transporter permease, were also shown to cause constitutive activation of the associated two-component system TCS-G.

As a whole, obtaining both inter- and intra-strain-dependent phenotypes confirms the plasticity of *L. lactis* in its adaptation to cell envelope stress and supports the use of AE-CES as a feasible strategy to diversify industrial *L. lactis* strains without using recombinant DNA technology.

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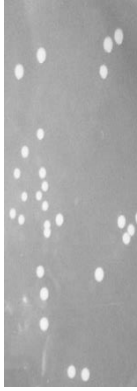

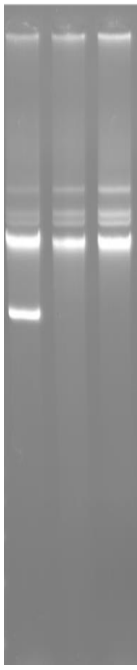
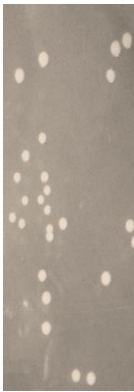

RESUMEN

Resumen

Lactococcus lactis es el componente mayoritario de los cultivos iniciadores mesófilos utilizados en la elaboración de productos lácteos fermentados. El éxito de una fermentación industrial depende en gran medida de la viabilidad y actividad metabólica de los cultivos microbianos utilizados. Por tanto, para mejorar la competitividad y optimizar el uso de nuestros recursos microbianos, se demandan cepas robustas que resistan las condiciones adversas, inherentes a estos procesos industriales. Durante el proceso fermentativo, *L. lactis* debe enfrentarse a diferentes condiciones de estrés que, en muchas ocasiones, tienen un impacto sobre la pared celular, una estructura celular esencial por lo que su integridad es un factor determinante para garantizar la viabilidad. De hecho, resultados previos a esta Tesis Doctoral habían demostrado que la modulación de la pared celular en cepas de laboratorio de *L. lactis* daba lugar a cepas más robustas y resistentes a condiciones de estrés tecnológico. Estos resultados, unidos a la creciente demanda de productos fermentados con características organolépticas distintivas, han propiciado la búsqueda de nuevas estrategias para aumentar la diversidad de los cultivos iniciadores disponibles que sean robustos y que presenten nuevos fenotipos y funcionalidades.

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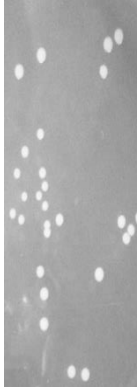
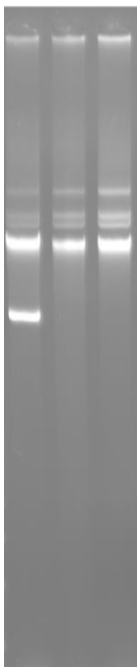
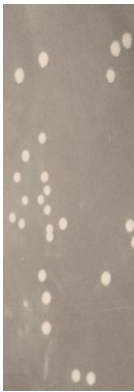
AGRADECIMIENTOS



Agradecimientos

Si me pidieran definir mi paso por la Tesis diría que ha sido como una montaña rusa unida a una carrera de obstáculos en la que además del vértigo, unido al placer tenía que ir sorteando continuamente piedras que, en más de una ocasión, se volvieron muros. Durante estos años, perdí a mi madre, casi pierdo a mi hermana, fui tía, me casé, me convertí en interina en educación, me operé, me diagnosticaron cáncer erróneamente, me operé de nuevo, llegó una pandemia, perdí a mi padre y, por fin, terminaré la Tesis casi a la par de la Comunión de mi sobrina. Ha sido un largo camino en el que he sido afortunada por todas las personas que siempre me han apoyado.

Las primeras líneas de este apartado se las dedico a mi Frente Mieres. Silvi, fuiste la persona que me empujó literalmente a vivir esta locura y la verdad no sé si darte las gracias o tirarte por un barranco, no lo tengo muy claro, jajaja. No, la verdad es que, si no hubiera sido por ti, jamás hubiera tenido esta oportunidad, así que para lo bueno y para lo malo eres como mi mentor, la persona que ha hecho posible no solo que esta aventura empezara, sino que nunca tirase la toalla en muchos momentos muy complicados. Has sido un gran apoyo emocional, la vocecita que siempre me recordó que luchando se consiguen los sueños; por todo ello, y mucho más que tú ya sabes, infinitas gracias. Evi, para mí has sido el otro pilar fundamental en todo este camino, la otra pata imprescindible para estabilizar un trinomio que se balanceó muchas veces, pero nunca cayó, siempre con esa dulce sonrisa que hace que los problemas no parezcan tan grandes. Lo cierto es que formamos un tándem indisoluble, “Frente Mieres”, con la moral de un alcoyano que, entre ataques de risa y de pánico, fue sorteando toda clase de obstáculos. Serán inolvidables los infinitos viajes Mieres – Villa - Mieres en un forito hiper-revolucionado, que en las cuestas empujábamos con la mente de los Picapiedra, un Hunday sin suspensión, que nos hacía volar en cada bache y una furgoneta sin aire, con las ventanas abiertas contra viento y marea. Nieve, granizo, niebla, viento, lluvia, sol. Aquellos viajes eran verdaderos sumideros de proyectos, “nenes tengo una idea”, de ataques de llorar de risa y también de echar fuego por la boca (más bien ponzoña jaja), un volcán de emociones que siempre terminaba con un viaje más. Así que, mil gracias porque ya se hace imposible entender la vida sin vosotras.

Mi primer contacto con el laboratorio fue con Ana B., ¡pobre, no sabía lo que se le venía encima!, en ese momento le cayó un tsunami de dudas y preguntas sin que pudiera ni respirar. Bea me dijo, pégate a ella para “¡hacer manos!” y aprender todo lo que haga...jajaja ¡qué frase aquella¡, y oye, yo que soy muy obediente, como una lapa

con mi libreta y mi bolígrafo apuntando hasta si estornudaba. A veces me decía, “ahora voy al baño” y ahí sí, ya me daba la cabeza para no ir detrás, jaja. ¡Cuánto aprendí contigo Ana! Qué fantástica maestra para una persona que no sabía ni lo que era “sembrar”, qué paciencia e increíble claridad para explicar todo, porque tenías que explicarme TODO, y qué fácil fue sentir esa confianza que me trasladaste con tú mirada. Pero no solo aprendí cosas que eran básicas, sino que lo que más me quedó grabado fue tu rigor a la hora de trabajar, y que he intentado trasladar a otros ámbitos de mi vida. Muchísimas gracias por todo tu apoyo tanto a nivel profesional como personal. Hubo algunos momentos realmente complicados a lo largo de estos años, y tú sólo con mirarme a los ojos supiste detectarlo y ofrecerme tu ayuda. Nunca lo olvidaré.

Mi vida en el laboratorio fue mucho más amena gracias a Ro. Estábamos solas ante el peligro en “el otro” lab, ahí desterradas, donde te mueres de frío y de calor, donde no te enteras de nada de lo que pasa en el mundo; ahí se generó un microcosmos que no cambiaría por nada. “Ay madre, Ro, ya la armé, madre mía no sé qué hice, ayyyyy…”. A ritmo de los 80 fuiste mi salvavidas permanente ante dudas y problemas miles. Me volví experta en apertura de tubos de tapa verde gracias a tus entrenamientos tipo Rocky Balboa. “Cuando vayas por los pasillos, llevas un tubo en el bolsillo de la bata y vas entrenando, hasta que no lo consigas, no coges más jajaja”, y lo conseguí, con la derecha, con la izquierda, con más de uno en la mano, con los ojos cerrados… jajaja. Cuántos truquetes me diste, Ro, cuántas horas de trabajo me ahorraste; muchas gracias por tu ayuda y por tantas veces que me has escuchado.

Gracias a Juan, Javi, Ester y María Jesús por tantas charlas, algunas para desahogar, otras para reír y desconectar unos minutillos y coger fuerzas de nuevo.

Al grupo Coffee Break, Su, Silvi, Evi, Lu, Ana B y allegados varios de estancias como Joanna, Seila, Silvia Mex y como no Flor, que nos enganchó a todos con sus risas. No no, no me olvido de Jorge, el hombre tranquilo de eterna paciencia y continua sonrisa, ¡mucho nos aguantaste! Espero que sepas que te tengo un cariño especial Jorge, no se me olvida tu presencia en el hospital consiguiendo sacarme más de una sonrisa en aquel momento tan complicado. Sois gente “guapa” como diría mi tía, guapa por dentro, la vida de alguna manera nos irá separando físicamente, pero no mentalmente, espero que nunca perdamos el contacto.

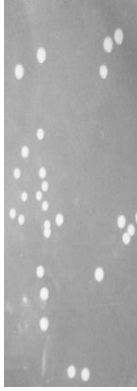
Por supuesto, quiero extender mis agradecimientos a todo el personal del IPLA que de diferente forma contribuyen a que la maquinaria siga funcionando. Especialmente a Clara, por su apoyo como tutora y a María, que siempre me dio ánimo

para no tirar la toalla y además apoyó muchos de los “líos” en los que constantemente me meto. siendo clave para poder visualizar el mundo de la investigación en los alumnos de secundaria y bachillerato. Muchas gracias, María, por impulsar con entusiasmo y sin reservas este tipo de acciones.

Dejo para el final a las personas más relevantes en todos estos años como doctoranda que son, evidentemente, AnaR y Bea. No tengo palabras para explicar todo lo que han significado para mí desde el primer día que apostaron por un reto que se adivinaba muy complicado, hasta el final, y es que nunca ha sido fácil. Ambas se han convertido en un referente para mí, tanto profesional como personalmente y espero alcanzar algún día su nivel humano ya que el profesional está dificilillo. Ha sido un aprendizaje impagable, no de microbiología que, por supuesto, también, aunque desgraciadamente cada día que pasa pienso que menos sé, sino de aspectos como empatía, humildad, profesionalidad, sentido de la responsabilidad, capacidad para decirte esa palabra que siempre te reconforta y nunca te hace sentir pequeñita y tantas cosas más. Ana, siempre me has empujado hacia adelante, me has escuchado y has dado consejos con ese tono de voz tan cariñoso, gracias por ser como eres y por haber creído en mí. Bea, lo que sé de biología es evidentemente gracias a ti, esa paciencia infinita dándome clases de conceptos básicos que otras personas ya tendrían claros desde el principio, pero lo más sorprendente es que nunca me hiciste sentir mal por ello. He tenido 3 momentos muy críticos en estos años; lo que no sabes es que en una ocasión me dirigí a tu despacho para decirte que ya no seguía, que no podía, pero cuando entré, me recibiste con un abrazo y un grito de alegría, aun sabiendo que no había hecho nada en todo ese tiempo…y arrancaste de nuevo con una energía que me arrastró sin dudar. Ahora sabes lo que ha significado para mí esa reacción tuya que has repetido en tantas ocasiones, he seguido literalmente GRACIAS A TI. No sé si te tocará otro reto de dirección de Tesis como este, espero que no, porque no puede ser bueno para tu salud. Por mi parte, lo que tengo claro es que no pude haber tenido más suerte, infinitas gracias, Bea por enseñarme lo que realmente significa la palabra empatía.

Y, por último, por supuesto, a mi familia que es la que siempre me ha aguantado todos los sinsabores. A Andrés, que como un día me dijo Bea, no tienes hijos, pero tienes una tesis, jejeje…; lo malo es que también lo tuvo que sufrir él y en este caso ha sufrido sólo lo malo y no lo bueno. Y, por último, a mi hermana y mi sobrina, mis princesas, que son mi sonrisa y mi motor para seguir adelante en cualquier aspecto de mi vida. Tati, necesitaría infinitas palabras para expresar lo que significas en mi vida, intento resumirlo en una frase: eres la razón de mi existencia, GRACIAS.

Agradecimientos



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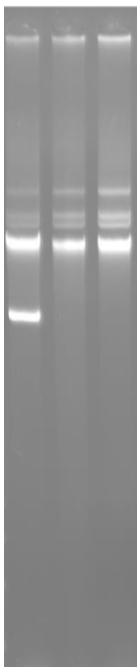

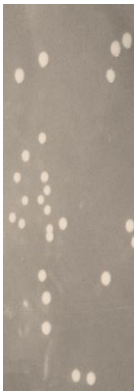

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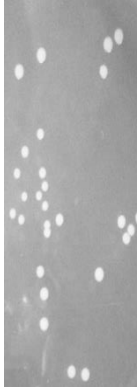

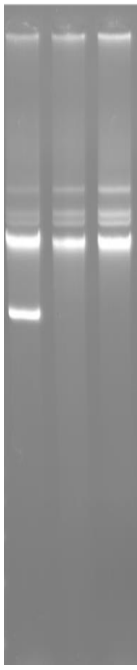
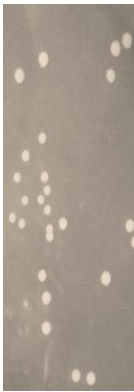

ÍNDICE DE FIGURAS Y TABLAS

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Índice de Figuras

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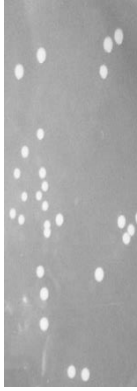
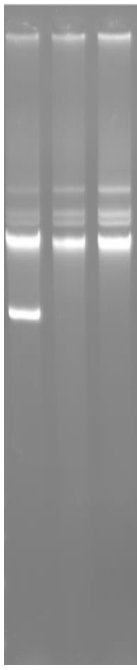
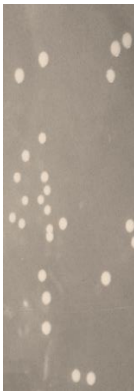


ABREVIATURAS

Abreviaturas

ABC: ATP Binding Cassette.

ADN: Ácido desoxirribonucleico.

AMPs: Péptidos antimicrobianos.

ARN: Ácido ribonucleico**.**

EA-CES: Evolución adaptativa bajo condiciones de estrés sobre la pared celular.

ALS: Acetolactato sintasa.

ATP: Adenosín trifosfato**.**

BAL: Bacterias lácticas.

CES: Cell Envelope Stress.

CIP: Cold-Induced Protein.

CM: Membrana citoplasmática.

CWPS: Polisacáridos de pared.

DOP: Denominación de Origen Protegida**.**

DVS: Direct-to-Vat-Set cultures.

ECF: Extracytoplasmic Function.

EFFCA: Asociación Europea de Cultivos Microbianos para Alimentación Animal y Humana.

EPS: Exopolisacárido

FAO: Organización de las Naciones Unidas para la Agricultura y la Alimentación.

FDA: Food and drug administration (Administración de Alimentos y Medicamentos).

GABA: Ácido γ-aminobutírico.

GRAS: Generally regarded as safe (Generalmente reconocido como seguro).

HK: Histidin quinasa.

HSPs: Heat Shock Proteins.

IGP: Indicación Geográfica Protegida.

IMFC: Inventory of Microbial Food Cultures.

Lcn972R: mutantes resistentes a la lactococina 972.

LDH: Lactato deshidrogenasa.

LDP: Leche descremada en polvo.

LEP: Leche entera en polvo.

LTA: Ácidos lipoteicoicos.

MP: Membrana plasmática.

NADH: Nicotinamida adenina dinucleótido en forma reducida.

NAD+: Nicotinamida adenina dinucleótido en forma oxidada.

NICE: Sistema de expresión inducible por nisina.

NSLAB: Bacterias lácticas no starters.

OCDE: Organización para la Cooperación y el Desarrollo Económicos.

OD: Densidad óptica

OMGs: Organismos genéticamente modificados.

PEP-PTS: Sistema fosfoenol-piruvato-fosfotransferasa.

PDH: Piruvato deshidrogenasa.

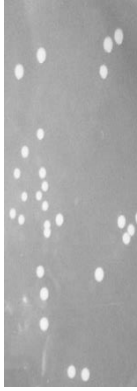
PG: Peptidoglicano.

RpoA: ARN polimerasa.

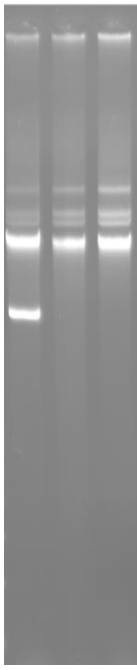
ROS: Especies reactivas del oxígeno.

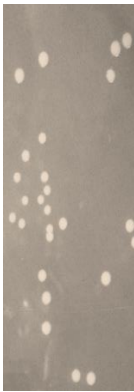
RR: Regulador de respuesta.

TCS: Two Component System.



INTRODUCCIÓN





Introducción

1 Fermentaciones lácteas

1.1 Contexto histórico

La fermentación es una de las formas de conservación de alimentos más antiguas que se conocen pues se remonta a miles de años a.C. Los procesos fermentativos ocurrían de forma empírica y descontrolada por la acción de microorganismos presentes de forma natural en la materia prima empleada en la elaboración de productos como el queso, la cerveza, el pan o el vino. Las primeras bebidas alcohólicas fermentadas derivadas de zumos de frutas datan del año 20.000 a.C., mientras que la elaboración de leches fermentadas se establece en torno al año 7.000 a.C. en Europa (Salque *et al*., 2013).

El queso forma parte de la dieta del hombre desde hace más de 8.000 años, siendo la elaboración del mismo uno de los ejemplos más antiguos de aplicación de procesos biotecnológicos en la elaboración de alimentos. El inicio de la producción de queso probablemente ocurrió de forma accidental por coagulación ácida de la leche al crecer, de forma espontánea, la microbiota láctica con la consiguiente producción de ácido, o por coagulación enzimática derivada del almacenaje de leche en bolsas fabricadas con estómagos de animales que contenían enzimas coagulantes. La conservación de la cuajada en vasijas habría provocado la separación del suero y cuajada, esta última con sabor agradable, que podía ser consumida fresca o conservada más tiempo siguiendo un proceso de maduración que daría lugar a cambios de aroma, sabor y textura, transformándose en un producto mucho más agradable desde un punto de vista sensorial (Rodríguez *et al*., 2014).

La transformación de leche en queso aportó a los primeros elaboradores una serie de ventajas: estabilidad durante el almacenamiento, facilidad de transporte y diversificación de la dieta. Todo ello favoreció la extensión de la elaboración de queso desde Oriente Medio a otras áreas geográficas: Egipto, Grecia y Roma, y tras la caída del Imperio Romano, a toda Europa (Fox y McSweeney, 2017).

No obstante, la falta de control del proceso fermentativo implicaba que la calidad del producto fermentado obtenido dependiese del tipo y número de microorganismos presentes, dando lugar a productos poco uniformes y, aleatoriamente, defectuosos. Con

el tiempo, la técnica *backslopping* consiguió reducir en gran medida el número de fermentaciones fallidas, la cual utiliza como inóculo una pequeña cantidad de un producto fermentado para comenzar una nueva fermentación, logrando de este modo añadir a la materia prima una mayor cantidad de los microorganismos mejor adaptados al proceso (Leroy y De Vuyst, 2004).

Las primeras bases científicas relacionadas con los procesos fermentativos datan del siglo XVII (1665-1683), cuando Hooke y Leeuwenhoek pusieron de manifiesto la existencia de organismos microscópicos implicados (Gest, 2004). Más adelante, ya a finales del siglo XIX, Sir Joseph Lister demostró que la fermentación láctica de la leche se conseguía únicamente con la presencia de un cultivo puro de un microorganismo llamado *Bacterium lactis*, que normalmente estaba presente en la misma (Santer, 2010).

Sin embargo, no fue hasta después de la Segunda Guerra Mundial cuando se empezaron a desarrollar técnicas biotecnológicas fiables gracias a los avances científicos relacionados con aspectos microbiológicos. Se impulsó así el diseño de nuevos procesos de fermentación que posibilitaron un mayor control y robustez de las condiciones óptimas para la industrialización y comercialización del producto fermentado, consiguiendo productos alimenticios con propiedades deseables, como una vida útil prolongada y buenas propiedades organolépticas (Smid y Hugenholtz, 2010). También existen ejemplos de procesos de fermentación que implican un aumento en el valor nutricional o digestibilidad de las materias primas alimenticias (Jägerstad *et al*., 2005). En general, cada vez son más las evidencias que vinculan el consumo de productos fermentados con determinados beneficios para la salud como la prevención de enfermedades cardiovasculares, trastornos gastrointestinales, reacciones alérgicas, etc. (Tamang *et al*., 2016). Como consecuencia de ello, existe un creciente interés en la mejora de los procesos fermentativos de alimentos a través del control de las relaciones existentes entre la diversidad de especies microbianas fermentadoras y la calidad y diferencia de las propiedades del producto final.

1.2 Importancia de los productos lácteos fermentados

Los alimentos fermentados constituyen, en la actualidad, alrededor del 30% de la dieta mundial, siendo una proporción muy significativa de la dieta de los países desarrollados y parte esencial de la base alimenticia en países en desarrollo, por lo que cada vez existe mayor variedad en el mercado, dependiendo de las diferentes culturas y

zonas geográficas existentes. Suponen, pues, una fuente de ingresos muy relevante dentro del mercado mundial de alimentos y bebidas (Shiferaw y Augustin, 2019).

El informe sobre Perspectivas Agrícolas 2017-2026 realizado por la Organización para la Cooperación y el Desarrollo Económicos (OCDE) y la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO) (OCDE-FAO, 2017) estima que, en el periodo de tiempo 2017-2026, el consumo mundial de productos lácteos frescos crecerá anualmente un 2.1%, y el de productos lácteos procesados [queso, mantequilla, leche entera en polvo (LEP), leche descremada en polvo (LDP)] crecerá un 1.7% anual. La dinámica del consumo diferirá considerablemente entre los países desarrollados y aquellos en desarrollo. Los países desarrollados consumen sobre todo productos lácteos procesados, y el consumo per cápita de queso crecerá a una tasa anual de un 0.9%, de mantequilla un 1.2%, de LEP un 1.6% y de LDP un 2.1%. En este contexto, la Unión Europea seguirá siendo el principal exportador de queso, con el 34% de las exportaciones mundiales previstas para 2026. No obstante, el contexto social producido por la pandemia COVID-19 implica que las previsiones realizadas por la OCDE-FAO para los próximos 10 años sean mucho más inciertas, amenazando principalmente a las cadenas de suministro de productos perecederos como la leche y los productos lácteos, aunque aún no existen datos suficientes para poder realizar una estimación adecuada (OCDE/FAO, 2020).

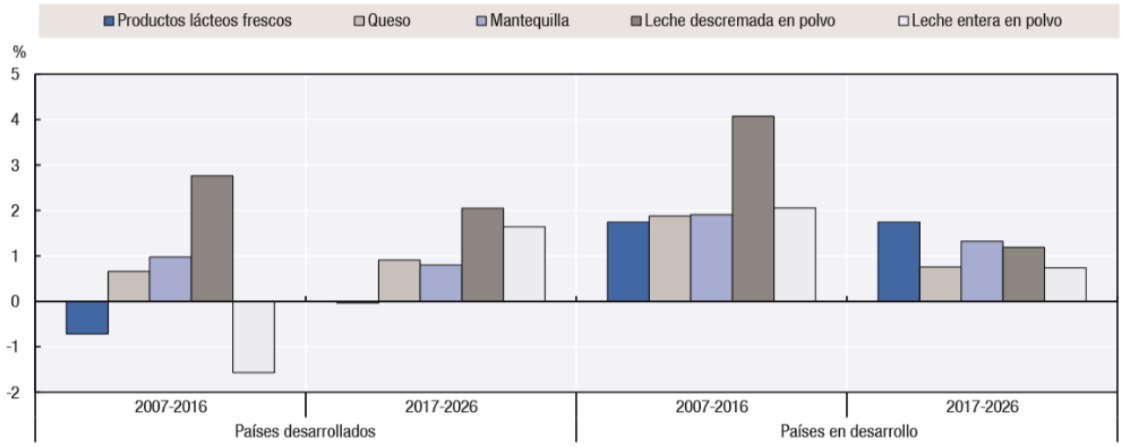


Figura 1. Tasas de crecimiento anual de consumo per cápita de productos lácteos. [Fuente: OCDE/FAO (2017)]

Por otro lado, el mismo informe pone de manifiesto que en los países desarrollados existe un cambio en las percepciones del sabor y en la preferencia por alimentos menos procesados por parte del consumidor, que se relacionan con las

evaluaciones más positivas de la grasa de la leche en términos de salud. También se espera que los países en desarrollo consuman más productos lácteos a medida que los ingresos crezcan, y las dietas se globalicen (OCDE/FAO, 2017).

Actualmente, el queso es el derivado lácteo más importante en términos de producción y consumo global, existiendo más de 2.000 variedades disponibles en todo el mundo y produciéndose más de 22 millones de toneladas al año. En Europa, la producción de queso supera los 10 millones de toneladas anuales, observándose en el año 2020 un incremento del 1.6% respecto del año anterior. Estos datos hacen que la Unión Europea se posicione como la primera productora de queso en el mundo representando, aproximadamente, el 50% de la producción mundial (European Commission, 2020; European Commission, 2021). En España, el consumo doméstico de derivados lácteos durante 2019 supuso un gasto superior a los 5,7 millones de euros, lo que implica un aumento del 1.3% del gasto respecto al año anterior. Al mismo tiempo, el consumo per cápita se situó en 35,14 kg-L/persona/año, lo que supone una reducción del 1,2% (Ministerio de Agricultura Pesca y Alimentación, 2020). Dentro de los derivados lácteos, las leches fermentadas y el queso son los que tienen mayor relevancia tanto en términos de gasto total (€) como de volumen consumido (kg o litros). El queso lidera el gasto, representando el 46,6% del total (2.694 millones de €), siendo el 22,6% el volumen consumido (aproximadamente 360 millones de kg) del total de derivados lácteos, mientras que las leches fermentadas suponen un 41,02% del volumen consumido (aproximadamente 665 millones de litros) y el 25% del gasto total (aproximadamente 1.459 millones de €) (Ministerio de Agricultura Pesca y Alimentación, 2020) (Figura 2). Respecto al consumo por persona y año, el de queso se sitúa en 7,8 kg y el de leches fermentadas en 14,42 kg (CMR GROUP, 2020). Respecto a la producción anual, cabe señalar que en 2019 se produjeron en España 442.200 toneladas de queso y 957.100 toneladas de yogures y otras leches fermentadas (InLac, 2021).

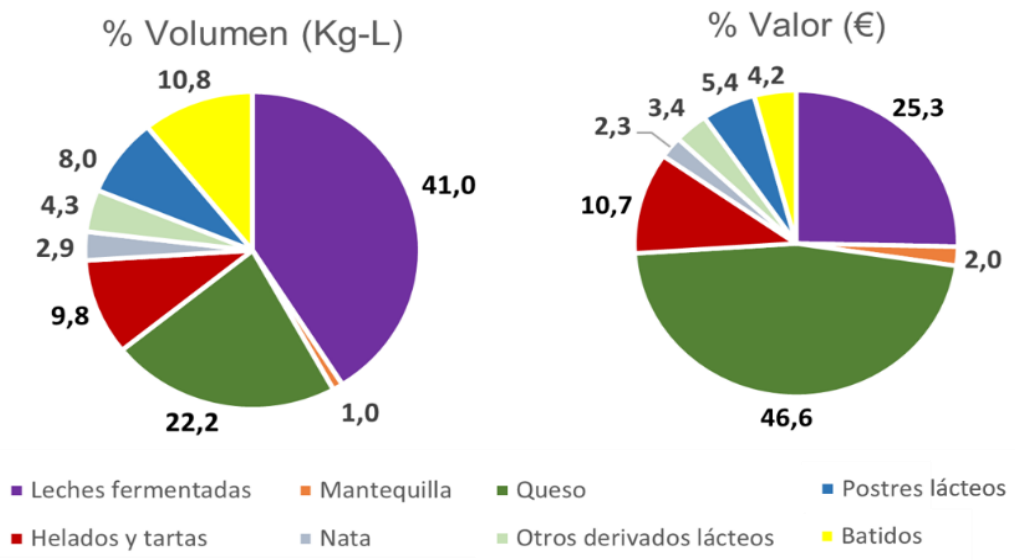


Figura 2. Importancia de los tipos de derivados lácteos en España. [Fuente: Ministerio de Agricultura Pesca y Alimentación (2020)]

En este contexto, se produce un aumento cada vez más pronunciado de la demanda del consumidor hacia una gama de productos más amplia, de mayor calidad nutricional y cualidades organolépticas mejoradas.

Es importante destacar que siguen existiendo áreas geográficas del sur de Europa donde se mantiene la elaboración de quesos artesanales con características propias y diferenciadas, dependientes del tipo de leche, los pastos, el clima y el método de elaboración empleado. A este respecto, los indicadores geográficos de calidad establecidos por la Unión Europea (Denominación de Origen Protegida -D.O.P.-, e Indicación Geográfica Protegida -I.G.P.) han supuesto un gran impulso para los quesos, con el consiguiente estímulo en la actividad económica en numerosas áreas geográficas.

2 Cultivos iniciadores lácticos

La Asociación Europea de Cultivos Microbianos para Alimentación Animal y Humana (EFFCA) propone la siguiente definición de cultivo iniciador: “bacterias vivas, levaduras o mohos usados en la producción de alimentos”. Son formulaciones que consisten en concentrados de una o más especies y/o cepas microbianas vivas y activas, que incluyen los componentes necesarios para su supervivencia, almacenamiento y estandarización que faciliten su aplicación en fermentaciones alimentarias (Bourdichon *et al*., 2012).

Hoy en día, la mayoría de los quesos y leches fermentadas se fabrican con iniciadores específicos que proporcionan condiciones de fermentación controladas, buscando nuevos atributos para satisfacer las demandas de los consumidores de productos fermentados estables, seguros y que promuevan la salud. Cabe señalar que la notable mejora de la calidad higiénica de la leche en los últimos años ha supuesto la disminución de los niveles de la microbiota láctica siendo esta, junto con la estandarización del proceso, una de las principales causas del uso habitual de fermentos comerciales.

Los componentes principales de los cultivos iniciadores de productos lácteos son las bacterias lácticas (BAL), aceptadas como especies bacterianas seguras para el consumo humano (Generally regarded as safe, GRAS). Constituyen un grupo heterogéneo de bacterias del filo Firmicutes, clase *Bacilli* y orden *Lactobacillales*. Se caracterizan por la producción de ácido láctico como producto único o principal a partir del metabolismo de la lactosa, lo que provoca un descenso de pH y la consecuente coagulación de la leche, generando además un ambiente hostil para el desarrollo de microorganismos indeseables. En los últimos años, se han atribuido a algunas cepas efectos beneficiosos para la salud adquiriendo especial relevancia como probióticos.

Las BAL son, en general, bacilos o cocos Gram-positivos, no esporulados, generalmente inmóviles y de bajo contenido en G+C, oxidasa y catalasa negativos, carecen de citocromos y no reducen los nitratos a nitritos. Son anaerobios facultativos aerotolerantes, que carecen del ciclo de Krebs, por lo que la generación de ATP ocurre mediante la fermentación de carbohidratos y compuestos relacionados, acoplada a la fosforilación a nivel de sustrato. Por otro lado, las BAL son ácido-tolerantes pudiendo sobrevivir en medios donde otras bacterias no podrían hacerlo (Carr *et al*., 2002). Debido a su adaptación a ambientes ricos en nutrientes, la mayoría de las BAL utilizadas como

cultivos iniciadores tienen una limitada capacidad biosintética y son microorganismos muy exigentes nutricionalmente. Para su multiplicación requieren de una fuente de carbono fermentable (azúcares), además de aminoácidos, vitaminas, ácidos grasos y otros factores de crecimiento. Además, presentan numerosas auxotrofías para un gran número de aminoácidos, entre 4 y 14 según la especie y la cepa (García-Cayuela *et al.*, 2016).

Dependiendo de que el producto final mayoritario de fermentación sea el ácido láctico (producción superior al 85%) o no, las BAL se clasifican en *homofermentadoras*, en el primer caso, o *heterofermentadoras* cuando además de ácido láctico también se producen otros metabolitos como etanol, ácido acético y dióxido de carbono. (Rodríguez *et al*., 2012). Además, bajo ciertas condiciones ambientales (limitación de la fuente de carbono, condiciones aeróbicas, etc) el metabolismo *homofermentativo* deriva hacia un metabolismo *ácido-mixto* que da lugar a varios metabolitos. Estos metabolitos incluyen varios compuestos aromáticos tales como acetaldehído, etanol y diacetilo (Puri *et al*., 2014).

En la tabla 1 se muestran las especies de BAL y otras bacterias comúnmente utilizadas en la producción de quesos.

2.1 Funciones principales de los cultivos iniciadores lácticos usados en la industria láctea

En general, el uso de cultivos iniciadores en la industria láctea, y concretamente en la elaboración del queso, permite estandarizar las características organolépticas del producto final, y consecuentemente su calidad, permitiendo tasas de acidificación constantes, particularmente relevantes para programar el tiempo de producción del queso (Johnson, 2017).

Las principales funciones de los fermentos lácticos están relacionadas con su actividad acidificante y proteolítica y con la producción de sustancias antimicrobianas y compuestos aromáticos que confieren al producto final una identidad diferencial frente a otros. Además, cabe resaltar otras funciones como la resistencia a bacteriófagos, especialmente interesante para evitar fermentaciones fallidas, y la actividad probiótica que, aunque no directamente relacionada con la fermentación de la leche, es demandada por la industria láctea debido a su efecto beneficioso en la salud del consumidor.

Tabla 1. Ejemplos de especies bacterianas que participan en la producción de quesos

Especie bacteriana	Características	Productos metabólicos	Productos fermentados
Homofermentadoras			
Lactococcus lactis subsp. *lactis* *L. lactis* subsp. *cremoris*	Acidificante	L(+)-lactato	Cheddar, Cottage, Feta, Gouda, Edam
L. lactis subsp. *lactis* biovar. *diacetylactis*	Contribución al aroma y producción de gas a partir de la fermentación del citrato	L(+)-lactato; diacetilo	Gouda, Edam, Camembert, Brie
Streptococcus thermophilus	Contribución al aroma y textura	L(+)-lactato; acetaldehído; diacetilo	Cheddar, Feta, Cottage Gruyere, Emmental, Jarlsberg, Grana, Mozzarella, Parmesano
Lactobacillus delbrueckii subsp. *bulgaricus / lactis*	Contribución al aroma y textura	D(-)-lactato; acetaldehído; diacetilo	Grana, Parmesano, Mozzarella
Lactobacillus helveticus	Contribución al sabor	D,L-lactato	Emmental, Jarlsberg, Grana, Parmesano
Heterofermentadoras			
Leuconostoc mesenteroides subsp. *cremoris*	Producción de gas y contribución al aroma	D(-)-lactato; diacetilo; etanol; CO_2	Cottage, Gouda, Edam, Camembert, Brie
Otras Bacterias			
Brevibacterium linens	Contribución al aroma, sabor y textura	Compuestos volátiles de azufre	Munster, Limburger, Brick
Propionibacterium freuderenreichii	Contribución al sabor y formación de “ojos”	Propionato; acetato; CO_2	Emmental, Gruyere

[Fuente: Modificada de Rodríguez *et al*., (2012)]

La actividad acidificante de los cultivos iniciadores proviene de la síntesis de ácido láctico fundamentalmente. Esta acidificación conlleva dos aspectos tecnológicos relevantes: por un lado, la coagulación de la leche que favorece la sinéresis de la cuajada (expulsión del suero) y mejora la actividad del cuajo y su retención en la cuajada promoviendo la conversión de las proteínas de la leche en péptidos y aminoácidos y, por

otro lado, la prevención y reducción del crecimiento de microorganismos patógenos y alterantes, lo que mejora las características de conservación del producto fermentado.

En ecosistemas complejos como la leche, la cantidad de péptidos y aminoácidos libres es relativamente baja, siendo la caseína la principal fuente de nitrógeno. Los cultivos iniciadores poseen, por tanto, un sistema proteolítico eficiente, constituido por proteinasas, sistemas de transporte de péptidos y peptidasas, que les permite obtener, a partir de la degradación enzimática de las proteínas de la leche, todos aquellos aminoácidos que no pueden sintetizar (Savijoki *et al*., 2006). Desde un punto de vista industrial, el sistema proteolítico de las BAL es importante por su contribución al desarrollo de las características nutricionales, reológicas y organolépticas de los productos lácteos fermentados (Leroy y De Vuyst, 2004) y para disminuir la presencia de péptidos hidrofóbicos involucrados en el desarrollo de amargor en el queso (Picon *et al*., 2005).

Los cultivos iniciadores producen, además, una amplia variedad de compuestos volátiles implicados en el desarrollo del aroma y sabor de los productos lácteos fermentados como el acetaldehído, acetoína, 2-butanona, 2,3-butanodiol y diacetilo. Cabe señalar que el acetaldehído es el principal componente del perfil aromático del yogur, y es producido fundamentalmente por *L. delbrueckii* subsp. *bulgaricus* a través de la ruta de conversión del aminoácido treonina a glicina en la que interviene el enzima treonina aldolasa (Ardö, 2006), mientras que el diacetilo es el principal responsable del aroma en queso fresco y mantequilla, y es producido a partir del citrato por cepas de *L. mesenteroides* y *L. lactis* subsp. *lactis* biovar. *diacetylactis* (Hugenholtz *et al*., 1993; Smid y Kleerebezem, 2014). La producción de CO_2 durante el metabolismo del citrato es responsable de la formación de "ojos" en los quesos de tipo holandés. Por otro lado, la producción de exopolisacáridos influye en la textura y viscosidad del producto fermentado, siendo especialmente importante su presencia en quesos de bajo contenido en grasa ya que mejoran la retención de agua, evitando que el queso se reseque (Bhaskaracharya y Shah, 2000; Perry *et al*., 1997).

Otra de las principales funciones de los cultivos iniciadores es la capacidad de producir sustancias antimicrobianas, que inhiben el crecimiento de microorganismos patógenos y alterantes, contribuyendo así a la seguridad y estabilidad del producto final. Los ácidos orgánicos (ya comentados anteriormente), el peróxido de hidrógeno y las bacteriocinas son los más representativos. La acción bactericida del peróxido de hidrógeno se relaciona con la fuerte acción oxidativa sobre las estructuras celulares al generar radicales hidroxi que causan peroxidación de los lípidos de la membrana

plasmática. Por ejemplo, en leche cruda, el peróxido de hidrógeno generado por las BAL puede reaccionar con el tiocianato presente en la leche para formar hipotiocianato y otros productos con actividad antimicrobiana que potencian el efecto bactericida del peróxido de hidrógeno consiguiendo prolongar así la vida útil de la leche (Kussendrager y van Hooijdonk, 2000).

Respecto a las bacteriocinas, cabe señalar que son péptidos antimicrobianos de síntesis ribosomal, generalmente catiónicos, con un marcado carácter anfipático y termoestables, que pueden mostrar un amplio espectro de inhibición contra bacterias Gram positivas como *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium tyrobutyricum* y *Bacillus cereus* (Cotter *et al*., 2005), lo que unido a otra serie de ventajas biotecnológicas (ausencia de toxicidad, no provocan cambios organolépticos, estabilidad en un amplio intervalo de pH, producción a gran escala, producción *in situ* y cepas multiproductoras) hace que sean una buena opción para ser usadas como bioconservantes en alimentos. Además, pueden jugar un papel relevante en procesos de maduración acelerada de quesos y en el control del desarrollo de la microbiota secundaria durante la maduración de alimentos fermentados (Martínez *et al*., 2014). Sin embargo, solamente dos (nisina y pediocina PA1) están aprobadas por la American Food and Drug Administration (FDA), como conservantes seguros para uso alimentario en diferentes países, (Daba y Elkhateeb, 2020). A modo de ejemplo, la nisina (producida por cepas de *L. lactis*) es utilizada en la industria láctea, fundamentalmente en la elaboración de quesos procesados, donde existe elevado riesgo de contaminación debido a los valores de pH $\geq$ 6.0, la actividad del agua y condiciones anaerobias generadas en el envase en el que se comercializan (Martínez *et al.*, 2014). La mayoría de las bacteriocinas pueden ser clasificadas en tres grandes clases, siendo la clase I (lantibióticos como la nisina) y la clase II (no modificadas post-traduccionalmente) las más estudiadas en su aplicación alimentaria (Martínez *et al*., 2016). Su actividad antimicrobiana se atribuye a la interacción con la membrana citoplasmática donde forman poros que anulan la permeabilidad selectiva de la misma, y/o a la inhibición de la síntesis de la pared celular (Héchard y Sahl, 2002).

2.2 Tipos de cultivos iniciadores utilizados por la industria láctea

Los cultivos iniciadores pueden ser clasificados según la función principal (*primarios* y *secundarios*); temperatura de crecimiento (mesófilos y termófilos) y composición (c*ultivos naturales de suero, cultivos naturales de leche, cultivos definidos mixtos* y *cultivos definidos múltiples*) (Hui y Evranuz, 2016; Rodríguez *et al*., 2014).

Según la función:

Primarios: responsables de la producción de ácido láctico a partir de la lactosa. A su vez, se clasifican en *mesófilos* (temperatura óptima en torno a 30ºC) y *termófilos* (temperatura óptima en torno a 42ºC) (Rodríguez *et al.*, 2014). Los cultivos primarios mesófilos pueden contener tanto, especies acidificantes (*L. lactis* subsp. *lactis* y/o *L. lactis* subsp. *cremoris*) como especies productoras de aroma y CO_2 que metabolizan el citrato (*L. lactis* subsp. *lactis* biovar *diacetylactis* y/o *Leuconostoc sp.*). Se usan en la producción de leches fermentadas, crema de mantequilla, y en una amplia variedad quesos como el Edam, Gouda, Cheddar, Camembert, etc. (Parente *et al*., 2017). Los cultivos primarios termófilos están constituidos por las especies *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis*, *L. acidophilus*, and *L. helveticus,* y son usados en la producción de yogur, quesos blandos como Mozzarella o quesos de pasta cocida como Emmental o Gruyère.

Secundarios: están involucrados en el proceso de maduración del queso y en el desarrollo y control de su aroma, sabor y textura, y constituidos por una microbiota compleja (Hui y Evranuz, 2016).

- Bacterias lácticas no starters (NSLAB): se desarrollan en todos los quesos ya sean producidos de forma industrial con leche pasteurizada o de forma artesanal con leche cruda. Se compone fundamentalmente de lactobacilos mesófilos heterofermentativos facultativos, involucrados en la liberación de péptidos y aminoácidos que contribuyen al sabor del queso, especialmente en aquellos que son madurados durante largos períodos de tiempo.
- Propionibacterias: representan la microbiota secundaria esencial de los quesos suizos. Su función principal es la conversión del lactato producido por las bacterias del cultivo iniciador en propionato, acetato (que intervienen en la producción de sabor), y dióxido de carbono, que será el responsable de la formación de los característicos “ojos” de este tipo de quesos.
- Corinebacterias: contribuyen a las características de aroma, sabor, textura y apariencia final, siendo responsables de la pigmentación anaranjada de algunos quesos. Los géneros más representativos son: *Brevibacterium*, *Arthrobacter*, *Corynebacterium*, *Microbacterium*.
- Levaduras y mohos: contribuyen al aroma, sabor y textura de determinados quesos. Entre las levaduras presentes en quesos destacan *Debaryomyces hansenii*, *Saccharomyces cerevisiae, Kluvveromyces marxianus o Pichia*

fermentans, mientras que *Penicillium roqueforti* está presente en los quesos azules (Roquefort, Cabrales, Stilton, etc), (Rodríguez *et al*., 2012).

Según la composición

Los cultivos naturales o artesanales (de suero y de leche) están constituidos por una mezcla indefinida de especies y cepas distintas, que se crecen diariamente en condiciones de temperatura y pH controladas, para favorecer la selección de las cepas mejor adaptadas.

- Los cultivos de suero se elaboran a partir de suero procedente de la fermentación previa de la leche, que se incuba a 45ºC hasta alcanzar pH 3,3. Estos fermentos se utilizan en la fabricación de quesos extraduros italianos (Parmigiano Reggiano y Grana Padano) y quesos de pasta hilada, donde predomina la especie *hL. helveticus*.
- Los cultivos naturales de leche se elaboran a partir de leche cruda (tratada a elevada temperatura (65ºC, 10 min) y posterior enfriamiento (37-45ºC), con objeto de seleccionar la microbiota deseada. Son utilizados en la fabricación de quesos artesanales italianos (Assiago Presato, Gorgonzola y Montasio), siendo *S. thermophilus*, en este caso, la especie dominante.

Los cultivos definidos (mixtos y múltiples) están constituidos por un número conocido de especies y cepas distintas, que se han seleccionado a partir de los cultivos naturales.

- Cultivos mixtos: incluyen varias cepas conocidas de distintas especies (acidificantes como *L. lactis* subsp. *lactis* y *L. lactis* subsp. *cremoris)* y fermentadoras de citrato *L. lactis* subsp. *lactis* biovar *diacetylactis* y/o *Leuconostoc*). Muestran una notable resistencia a la infección por bacteriófagos. Se usan habitualmente en la producción de diferentes variedades de quesos suizos y holandeses.
- Cultivos definidos: incluyen una (cultivos de cepa única) o varias cepas de una única especie (cultivos múltiples) bien caracterizadas. (Rodríguez *et al*., 2012). Aunque su comportamiento tecnológico es muy reproducible, presentan mayor riesgo frente la exposición a fagos. Es necesario, por tanto, recurrir a la rotación de cultivos definidos con diferentes perfiles de susceptibilidad a fagos. Se utilizan, por ejemplo, en la elaboración de queso Cheddar.

2.3 Producción y conservación de cultivos iniciadores utilizados por la industria láctea

El éxito de los procesos fermentativos en la industria láctea depende tanto de factores intrínsecos de las cepas usadas como cultivos iniciadores (estado fisiológico, aptitud metabólica, tolerancia a condiciones de estrés tecnológico y existencia de profagos en su genoma) como de factores extrínsecos relacionados con las condiciones de fabricación (presencia en la leche de residuos de antibióticos u otros antimicrobianos, por ejemplo, las bacteriocinas o bacteriófagos).

En este sentido, los avances producidos en los procesos de fermentación a gran escala implican la necesidad de optimizar la selección de cepas que permitan resultados óptimos en cuanto a densidad celular, actividad, estabilidad y composición, ya que por muy buenas que sean las aptitudes tecnológicas de una cepa, si no mantienen su viabilidad durante el proceso o no producen rendimientos adecuados de biomasa, no serían válidas para su comercialización.

Dada la gran cantidad de cepas disponibles para ser usadas en la industria láctea, el proceso de selección (figura 3) debe sistematizarse para ir reduciendo de forma progresiva el número de posibles “candidatos”, incluyendo un primer cribado donde se descartan las cepas que no son capaces de adaptarse a las condiciones de estrés impuestas en el proceso de fabricación del queso, seguido por la identificación de aquellas cepas productoras de determinados metabolitos deseados. Las cepas preseleccionadas deben someterse a la evaluación de los parámetros tecnológicos de interés, siendo seleccionadas aquellas que presenten mayor número de propiedades sin contemplar ningún otro posible rasgo negativo (de Melo *et al*., 2020).

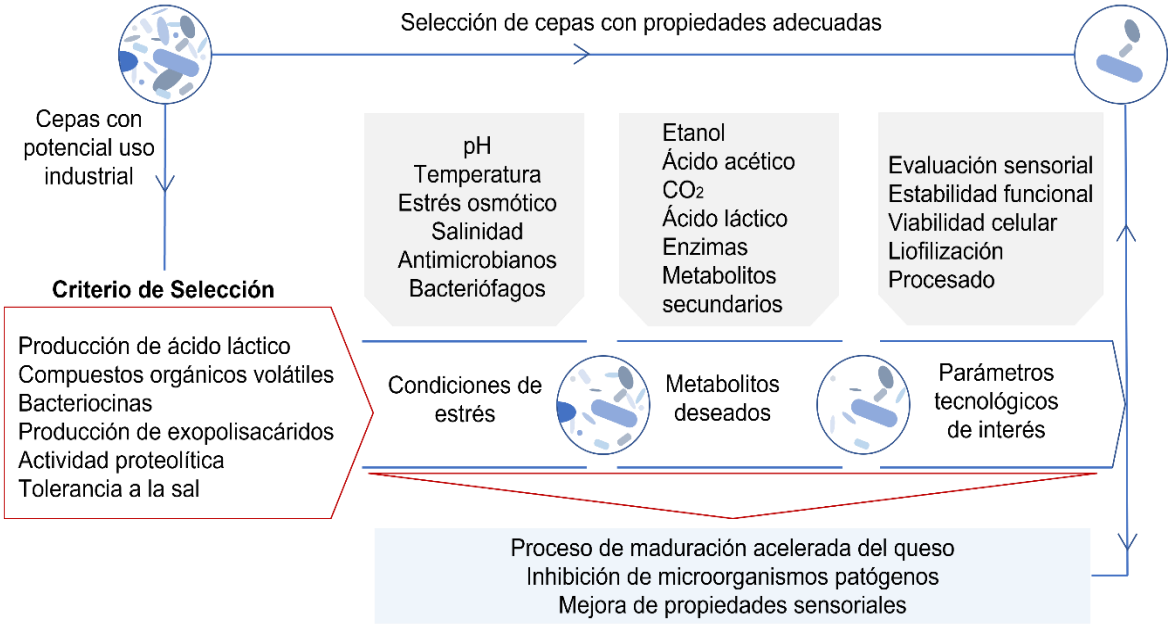


Figura 3. Representación esquemática del proceso de selección de cepas para uso industrial a gran escala en el proceso de elaboración del queso. [Fuente: este trabajo]

Un gran avance en la mejora de la producción industrial de biomasa es el aprovechamiento de la capacidad respiratoria de algunas BAL, como *L. lactis*, en presencia de oxígeno mediante la adición del grupo hemo al medio de cultivo, ya que se consiguen mayores rendimientos celulares y un aumento la supervivencia después del crecimiento. (Arioli *et al*., 2013; Pedersen *et al*., 2012).

Por otro lado, desde un punto de vista comercial, los cultivos iniciadores deben ser fáciles de usar, rentables en términos económicos, además de ser resistentes a los procesos de secado, envasado, almacenamiento y rehidratación, sin perder en ningún caso sus propiedades esenciales. En la actualidad, las cepas iniciadoras se comercializan en formato concentrado, ya sea congeladas o liofilizadas, presentando un elevado número de células viables, lo que permite la adición directa a la cuba de elaboración de queso (Direct-to-Vat-Set cultures; DVS). Esto conlleva, por un lado, la reducción de costes de transporte y almacenamiento y, por otro lado, evita las sucesivas transferencias que serían necesarias para alcanzar el volumen idóneo para inocular la leche destinada a la fermentación, con la consiguiente disminución del riesgo de contaminación tanto fágica como bacteriana (Hansen, 2002). No obstante, el empleo de este tipo de técnicas de conservación, especialmente en el caso de la congelación, origina un estrés celular que puede afectar a la actividad de los cultivos y a su capacidad de multiplicarse activamente en medios complejos como la leche.

3 Estrategias de mejora de cultivos iniciadores

El desarrollo de nuevos cultivos iniciadores puede llevarse a cabo a través de la inclusión de nuevos microorganismos aislados en ambientes naturales o mejorando las cepas que ya están en uso por medio de técnicas microbiológicas y genéticas modernas. La primera opción implica el acceso a repositorios de cepas microbianas, sujetas actualmente al Protocolo de Nagoya, y su cribado en busca de aquellas que presenten la combinación de características deseadas, lo que representa un elevado coste en tiempo de ejecución. En este sentido, aunque la aplicación de procesos altamente automatizados puede contrarrestar ese elevado coste del tiempo del cribado, no siempre es posible encontrar cepas con las propiedades deseadas y que además sobrevivan a las condiciones de producción industrial. Todo ello implica la necesidad de aplicar otras técnicas de mejora como la mutagénesis y la consiguiente selección de mutantes, estrategias de evolución adaptativa u otros métodos más novedosos basados en recombinación o edición genética, siempre y cuando estos sean compatibles con la legislación actual (Johansen, 2018).

En la figura 4 se muestran los pros y contras de cada una de estas técnicas y algunos resultados de su aplicación experimental en cepas de *L. lactis*.

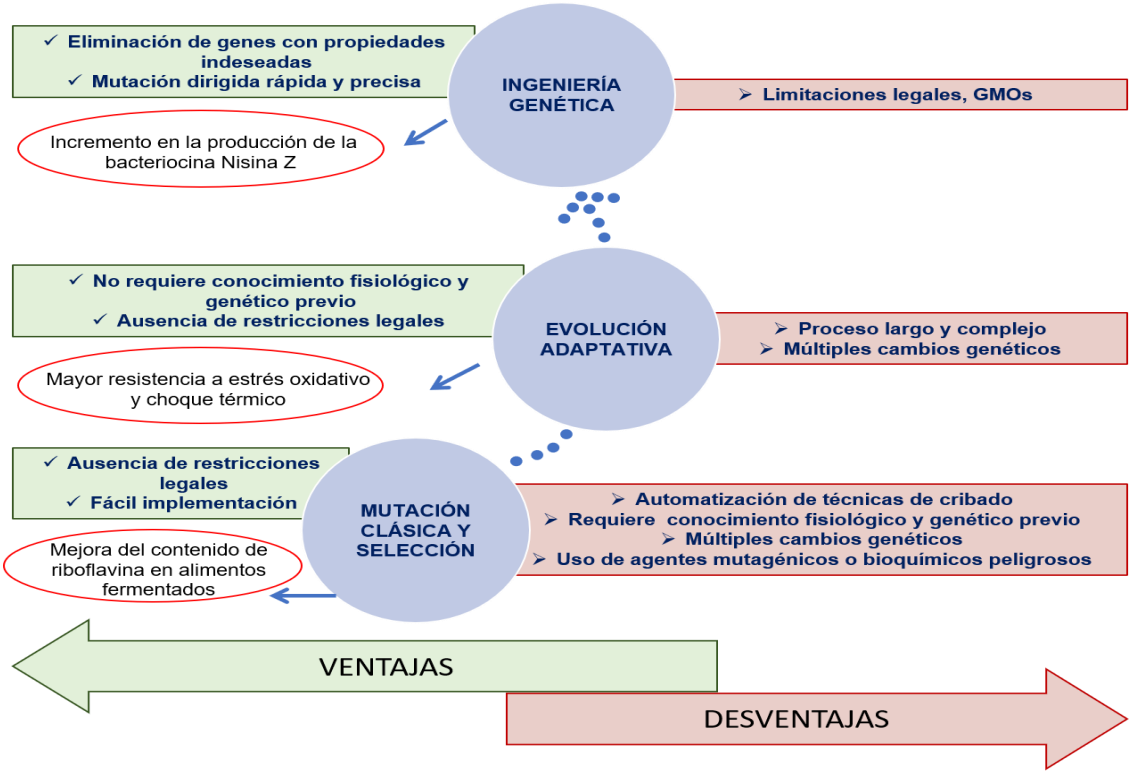


Figura 4. Principales ventajas y desventajas de las técnicas de mejora de cepas usadas en el desarrollo de cultivos iniciadores. [Fuente: López-González, *et al*., (2019)]

3.1 Mutación clásica y selección

Se ha comprobado experimentalmente que mediante selección natural se producen pérdidas de algunos genes o mutaciones que modifican las características fenotípicas de las cepas de origen. Un ejemplo característico es la capacidad que tienen las cepas de *L. lactis* aisladas de plantas de crecer en el ambiente lácteo, tras realizar cultivos sucesivos en leche, produciéndose la pérdida o sobreexpresión de aquellos genes que son necesarios o no (Bachmann *et al*., 2012).

La técnica más comúnmente aplicada para la mejora y diversificación de cultivos iniciadores consiste en la exposición a agentes mutagénicos que introducen mutaciones no dirigidas en el genoma bacteriano. Tras la mutagénesis, se realiza un cribado bajo presión selectiva, es decir, que permite la propagación de aquellos mutantes que hayan adquirido mutaciones con repercusión en los fenotipos deseados. Con esta estrategia se han seleccionado cepas con características diferenciales que repercuten en el sabor, aroma o textura del alimento fermentado, cepas super-productoras de vitaminas y, por supuesto, cepas resistentes a bacteriófagos, uno de los objetivos tradicionales de la industria de los cultivos iniciadores (Chen *et al*., 2017; Johansen, 2018).

Las ventajas de este método (figura 4) se asocian a cambios fenotípicos y genéticos relativamente simples, aunque permita la acumulación de mutaciones desconocidas, y a la ausencia de restricciones legales. Sin embargo, al igual que con la búsqueda de nuevas cepas, la selección de mutantes con las propiedades deseadas requiere técnicas de cribado automatizadas y un conocimiento profundo de los aspectos fisiológicos y genéticos del microorganismo para diseñar la estrategia de selección más adecuada. Además, conlleva el uso de agentes mutagénicos o bioquímicos peligrosos.

3.2 Evolución adaptativa

Cualquier organismo vivo tiene la capacidad de adaptarse a los cambios y sobrevivir en l.os diferentes ambientes en los que se puede desarrollar fruto, por un lado, de la selección evolutiva, y por otro, de la existencia de sistemas de detección de parámetros ambientales y de respuesta rápida que activan redes de regulación génica complejas. Este proceso de adaptación conlleva un gasto energético por lo que la supervivencia estará favorecida cuando exista un equilibrio coste/beneficio. Cuando el estímulo desaparece, algunas modificaciones pueden ser revertidas a su estado original y otras mutaciones se habrán adquirido de forma permanente, transmitiéndose a la progenie (Schimel *et al*., 2007).

La evolución adaptativa permite seleccionar fenotipos de interés simulando el proceso evolutivo natural bajo condiciones impuestas en el laboratorio. El proceso conlleva la realización de cultivos sucesivos durante varias generaciones bajo condiciones de presión selectiva. En función de la presión selectiva aplicada se obtienen mutantes adaptados con nuevos fenotipos de interés respecto a la cepa de referencia. Por ejemplo, tras la exposición continuada de *L. lactis* MG1363 a elevadas temperaturas, se eleva la producción de láctico acelerando la tasa de acidificación (Chen *et al*., 2015). Otros experimentos realizados *en L. lactis* apuntan, entre otros aspectos, a la obtención de un mutante de *L. lactis* NZ9000 con alta tolerancia al isobutanol (prometedor biocombustible de segunda generación), tras cultivar la cepa durante más de 60 días en presencia de concentraciones crecientes de este compuesto (Gupta *et al*., 2020). Las cepas de lactococos con alta tolerancia al isobutanol podrían ser utilizadas para la producción de este biocombustible a través del catabolismo de la valina (Priyadharshini *et al*., 2015). Cabe citar también la aplicación de la evolución adaptativa para mejorar la capacidad *de L. lactis* para producir manitol (Xiao *et al*., 2020).

La principal ventaja de esta técnica (figura 4), aparte de no existir restricciones legales de uso, es que no necesita un conocimiento genético y fisiológico específico del microorganismo usado, ya que la selección está dirigida por el parámetro aplicado (ácido, temperatura). En cambio, el proceso experimental es largo, complejo y puede conllevar múltiples cambios genéticos, en algunos casos no deseados (Derkx *et al*., 2014).

3.3 Ingeniería genética

Las estrategias de mejora anteriores no son dirigidas, ya que permiten la acumulación de mutaciones, independientemente de que participen o no en el fenotipo de interés. En este sentido, se han desarrollado numerosas técnicas de ingeniería o edición genética, es decir, de modificación intencionada de la dotación genética para generar mutantes de interés. Por ejemplo, se pueden obtener mutantes de *L. lactis* que dupliquen la producción de nisina Z frente a su cepa de origen pudiendo contribuir a la disminución de la contaminación bacteriana (Zhang *et al*., 2014).

En el caso de las BAL, existen en la actualidad una gran variedad de herramientas que permiten realizar cambios específicos en la secuencia de ADN, bien sea alterando o eliminando un solo nucleótido o miles de pares de bases del genoma. Además del uso más tradicional de plásmidos específicos, ya existen algunos ejemplos de herramientas

basadas en la tecnología CRISPR/Cas para ser utilizadas en BAL (Hidalgo-Cantabrana *et al.*, 2017).

Los principales beneficios de la ingeniería genética giran en torno a la reducción del tiempo empleado en la mejora de cepas y el aumento de precisión en las mutaciones en genes específicos y se dirigen, mayoritariamente, a la eliminación de genes responsables de características no deseadas, tales como resistencia a antibióticos o producción de aminas biógenas (toxinas), y a la rápida inserción de mutaciones en genes específicos (figura 4). Según la técnica empleada, las cepas mejoradas por ingeniería genética pueden ser consideradas como organismos genéticamente modificados (OMGs) y están sujetas a un proceso de evaluación y autorización antes de ser utilizadas (Reglamento CE No 1829/2003).

4 Lactococcus lactis

El género *Lactococcus* pertenece a la familia *Streptococcaceae* y se compone de bacterias de morfología cocoide, Gram positivas, aerotolerantes, catalasa negativas, no esporuladas, microaerófilas y mesófilas. Existen hasta 22 especies descritas, de las cuales son tres las que se encuentran contenidas en el último inventario de cultivos microbianos alimenticios (Inventory of Microbial Food Cultures, IMFC): *L. lactis (*subsp*. lactis y cremoris)*; *L. raffinolactis* y *L. piscium*, siendo *L. lactis* la especie mejor estudiada y más utilizada en la industria quesera como componente esencial de los cultivos iniciadores acidificantes mesófilos (Bourdichon *et al*., 2018). Cabe destacar que, recientemente, se ha elevado a la categoría de especie la subspecie *cremoris* (Li *et al*., 2019). En esta memoria se ha mantenido la distinción entre las subespecies *lactis* y *cremoris,* acorde a la literatura utilizada durante su redacción.

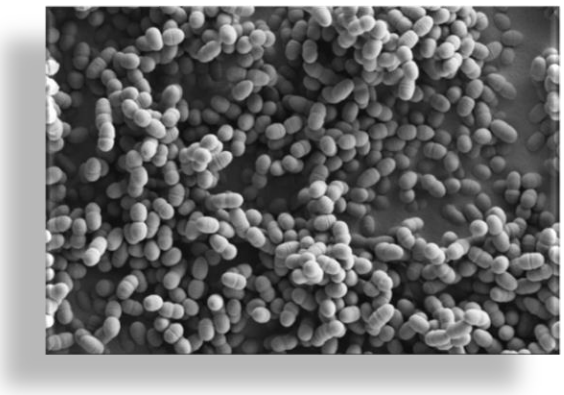


Figura 5. Microscopía electrónica de barrido de *L. lactis*. [Fuente: Foto tomada por Clara Roces]

Las subespecies *lactis* y *cremoris*, que presentan alrededor del 80% de similitud genómica, se diferencian fenotípicamente en función de la tolerancia salina, la temperatura máxima de crecimiento, la desaminación de la arginina y la producción de ácido γ-aminobutírico (GABA). Así, *L. lactis* subsp. *lactis* tiene la capacidad de crecer a 40ºC, en presencia de 4% de NaCl, a pH9.2, de degradar la arginina y de producir GABA, mientras que *L. lactis* subsp. *cremoris* no tiene estas características. No obstante, no siempre existe una estricta correlación entre fenotipo y genotipo siendo posible encontrar cepas de *L. lactis* con fenotipo *lactis* y genotipo *lactis* o cepas de *L. lactis* con fenotipo *lactis* y genotipo *cremoris* (Fernández *et al*., 2011; Laroute *et al*., 2017).

Aunque la mayoría de cepas con genotipo *cremoris* han sido aisladas de ambientes lácteos, se considera que el nicho original de *L. lactis* es principalmente vegetal (van Hylckama Vlieg *et al*., 2006). La adaptación de *L. lactis* al ambiente lácteo se ha caracterizado por la mutación de ciertas rutas enzimáticas, sobre todo las relacionadas con el metabolismo de aminoácidos y de carbohidratos complejos, como reflejo de su adaptación a la riqueza nutricional de la leche, llevando incluso a perder la capacidad de desarrollarse en otro hábitat diferente (Cavanagh *et al*., 2015).

La adaptación de las especies de lactococos al ambiente lácteo ha conducido al estado “domesticado” de la especie. De esta manera, *L. lactis* ha ido evolucionando en función de este entorno creado por el hombre, mostrando varias pautas remarcables de adaptación como son:

- Reducción genómica con pérdida de capacidades biosintéticas innecesarias en el ambiente lácteo.
- Adquisición de plásmidos que codifican funciones metabólicas importantes que les permite prosperar en ambientes lácteos. De hecho, la abundancia de plásmidos en estirpes de *L. lactis* de origen lácteo es una característica distintiva de las mismas (van Hylckama Vlieg *et al*., 2006). Los plásmidos codifican funciones de interés tecnológico al estar relacionadas con las respuestas de estrés, con funciones de transporte y metabolismo de lactosa, aprovechamiento del citrato, producción de bacteriocinas, resistencia a bacteriófagos y resistencia a antibióticos, entre otros (García-Cayuela *et al.*, 2016).

L. lactis ha sido la primera BAL cuyo genoma ha sido totalmente secuenciado (Bolotin *et al*., 2001). Gracias, entre otras cosas, al pequeño tamaño de su genoma (2.3 Mbp) y la facilidad de modificación genética, *L. lactis* ha servido como modelo para

estudios metabólicos y fisiológicos, así como para el diseño de herramientas de ingeniería genética, como vectores de clonación y sistemas de expresión [(por ejemplo, el sistema de expresión inducible por nisina NICE (Mierau y Kleerebezem, 2005)].

Todo ello, ha llevado a proponer a *L. lactis* como factoría celular (Song et al., 2017), además de su extendido uso como cultivo iniciador. *L. lactis* presenta algunas ventajas frente a otras factorías celulares tradicionales como *Escherichia coli*, *Bacillus subtilis* o *Saccharomyces cerevisiae*: es considerada especie segura, carece de lipopolisacárido y presenta un menor número de proteasas extracelulares (Song et al., 2017). En concreto, se ha evaluado la eficiencia de *L. lactis* para la producción de compuestos con fines tecnológicos y/o clínicos. Algunos ejemplos destacables son los siguientes: diversas proteínas recombinantes (enzimas), metabolitos aromáticos acetilados (diacetilo, acetaldehído y acetato) sintetizados como consecuencia de cambios en la ruta metabólica del proceso de fermentación de carbohidratos, metabolitos no alimentarios, como las vitaminas B11 y B12, y la producción de bacteriocinas, usadas principalmente como bioconservantes (Song *et al*., 2017). Investigaciones más recientes proponen a *L. lactis* como factoría celular para producir etanol como único producto de fermentación para emplearlo como biocombustible (Liu *et al*., 2016).

4.1 Características metabólicas de *L. lactis*

En el caso de la mayoría de las estirpes industriales de *L. lactis,* la lactosa se incorpora como azúcar fosforilado a través del sistema fosfoenol-piruvato-fosfotransferasa (PEP-PTS), siendo hidrolizada a glucosa y galactosa por la fosfo-β-galactosidasa (Hui y Evranuz, 2016).

L. lactis es una BAL homofermentadora que convierte el pirúvico mayoritariamente en láctico a través del enzima lactato deshidrogenasa (LDH). Sin embargo, dependiendo de las condiciones ambientales existentes tales como pH, concentración de carbohidratos, concentración de pirúvico, aerobiosis/anaerobiosis o niveles de $NADH/NAD^+$, se pueden activar diferentes enzimas que modifican los patrones de fermentación (Neves *et al*., 2005). En condiciones anaerobias y elevadas concentraciones de azúcar, prácticamente todo el azúcar se convierte en lactato (fermentación homoláctica) a partir del piruvato (glucolisis) (figura 6A). Sin embargo, en condiciones de aerobiosis, cuando la concentración de azúcar es menor o en presencia de otros azúcares diferentes a la glucosa o lactosa, puede producirse una fermentación ácido-mixta (formación de lactato, acético, acetoína, fórmico, etanol y CO_2) caracterizada

por relaciones de NADH/NAD$^+$ inferiores a las encontradas en la fermentación homoláctica (Gaudu *et al*., 2019).

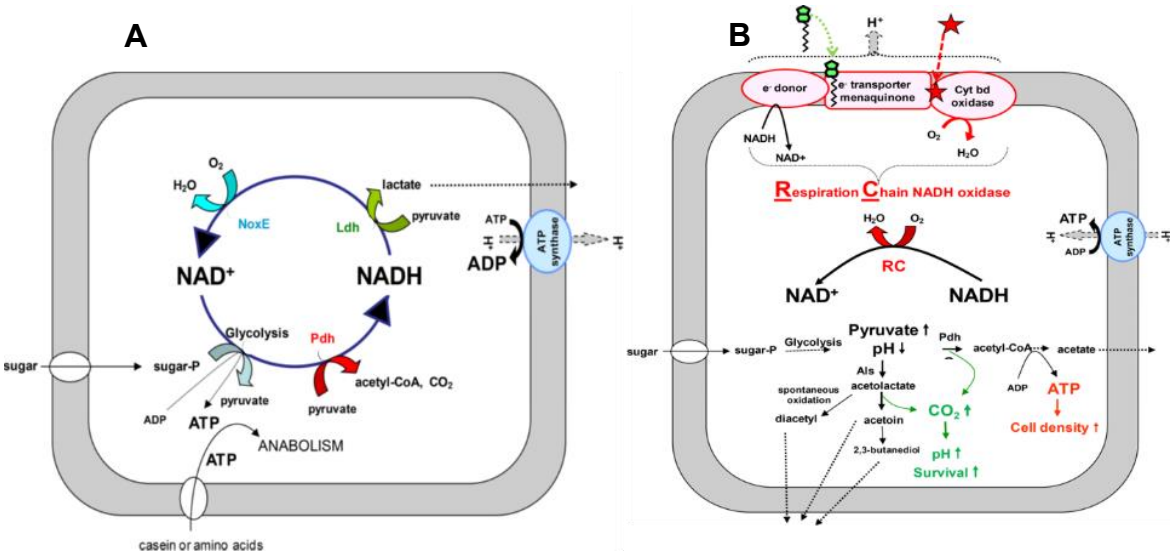


Figura 6. A. Metabolismo fermentativo de *L. lactis*. Tras la glicolisis se genera NADH que será oxidado a NAD$^+$ por la conversión de piruvato a lactato y en presencia de oxígeno, por la NADH oxidasa (NoxE) y la piruvato deshidrogenasa (Pdh). La ATPasa expulsa protones a expensas del ATP para evitar la acidificación debida a la glucólisis. **B.** Metabolismo respiratorio de *L. lactis*. La adición del grupo hemo (estrella roja) activa la citocromo oxidasa produciéndose la expulsión final de protones y la reducción del O_2. [Fuente: Gaudu *et al.*, (2019)]

L. lactis es capaz, no solo de fermentar azúcares, sino que en presencia externa de un grupo hemo, activa un metabolismo respiratorio (figura 6B) debido a su capacidad para formar una cadena de transporte de electrones, lo que implica mejoras en términos de crecimiento (puede llegar a duplicar la producción de biomasa celular), producción de metabolitos (aumenta en gran medida la producción de acetoína) y supervivencia bacteriana (debido al aumento de pH y consumo de oxígeno) (Arioli *et al*., 2013; Duwat *et al*., 2001; Gaudu *et al*., 2019). Esta cadena transportadora de electrones consta de un donante de electrones (NADH deshidrogenasa), componentes no proteicos (menaquinonas) que transfieren los electrones a las oxidorreductasas terminales (citocromo oxidasa), que a su vez incorporan el grupo hemo y usan oxígeno como aceptor de electrones. *L. lactis* carece de un ciclo de Krebs completo por lo que el NADH necesario para la cadena respiratoria se produce a través del catabolismo del carbono donde el azúcar se cataboliza a piruvato vía glicolisis con producción de ATP y NADH. El consumo de NADH por la cadena respiratoria hace que la actividad de la LDH disminuya acumulándose el ácido pirúvico que se disociará en piruvato y un protón, lo que provoca una disminución del pH interno. Para contrarrestar esta acidificación, el piruvato se convierte en acetolactato vía acetolactato sintasa (Als) y luego en acetoína, liberando

CO_2. También se produce diacetilo por oxidación espontánea del acetolactato. Se produce así un aumento de pH y una mejora en la supervivencia celular. Por otro lado, el piruvato se puede convertir en acetil-CoA, vía piruvato deshidrogenasa (PDH) proporcionando más NADH y CO_2, que se convertirá en acetato con producción de ATP, promoviendo una mayor densidad celular (Gaudu *et al*., 2019).

4.2 Respuesta al estrés

Durante la producción a gran escala del cultivo iniciador, su almacenamiento (congelación y/o liofilización) y distribución, así como durante la elaboración del queso, pueden darse situaciones de estrés tecnológico relacionadas con temperaturas extremas, acidez, condiciones osmóticas y presencia de antimicrobianos o bacteriófagos entre otras, haciendo necesaria la activación de respuestas generales y/o específicas para cada condición de estrés. Dada su relevancia, los mecanismos de respuesta a estrés de las BAL y, concretamente de *L. lactis,* han sido objeto de numerosos estudios (Papadimitriou *et al*., 2016). Entre las condiciones adversas más destacadas destacan:

- *Estrés ácido*: el descenso de pH provocado por la producción de ácido láctico afecta al correcto funcionamiento de la célula, pudiendo ocasionar la muerte celular. *L. lactis* presenta diferentes mecanismos para reducir la acidificación del citoplasma como la bomba de protones H^+-ATPasa, la ruta de la arginina deiminasa, o la descarboxilación de algunos compuestos, como por ejemplo aminoácidos.

- *Estrés oxidativo*: provocado por la presencia de especies reactivas del oxígeno (ROS: OH^-; O^{2-}; H_2O_2), bien sea por estar presentes en el ambiente o por haberse formado intracelularmente durante la reducción parcial del oxígeno a agua, afectando a nivel molecular y metabólico. Existen diferentes estrategias desarrolladas por *L. lactis* para reparar el daño ocasionado por las especies ROS. Como ejemplos se pueden mencionar el sistema NADH oxidasa-NADH peroxidasa que reduce el contenido de oxígeno formando H_2O_2, y es reducido a H_2O (Higuchi *et al*., 2000) o el enzima superóxido dismutasa que elimina el ión superóxido (Sanders *et al*., 1995). Además, se considera que su capacidad para respirar en presencia del grupo hemo es una defensa adicional frente al estrés oxidativo.

- *Estrés osmótico*: consecuencia del uso de sal (NaCl) en la elaboración de quesos, la cual actúa como conservante y modulador de producción de ácido, además de

aportar sabor al producto final. El estrés osmótico disminuye la turgencia de la célula bacteriana como resultado de la pérdida de H_2O. El mecanismo de respuesta más importante en *L. lactis* son los sistemas de transporte activo que modulan la concentración de solutos compatibles como la sacarosa, prolina, glicina o betaína (transportador ABC OpuA glicina-betaína), contribuyendo a mantener el equilibrio osmótico entre el interior y exterior de la célula.

- *Estrés térmico*: la exposición a temperaturas altas alcanzadas en el proceso de elaboración de varios tipos de quesos provoca, de forma general, la desnaturalización de macromoléculas. Como mecanismo de defensa, *L. lactis* sintetiza chaperonas como las proteínas HSPs (*Heat Shock Proteins*) o las proteínas inducidas por frío, CIP (*Cold-Induced Protein*) frente a la exposición a temperaturas elevadas y bajas, respectivamente.

4.2.1 Respuesta al daño sobre la pared celular

Una estructura celular clave para la supervivencia celular ante condiciones de estrés es la pared celular. Al ser una bacteria Gram-positiva, la pared celular de *L. lactis* (figura 7) se caracteriza por una capa gruesa de peptidoglicano que rodea la membrana citoplasmática y que, además de conferir una estructura reticular dinámica, sirve de anclaje para la unión de otros componentes de la pared celular como ácidos lipoteicoicos, proteínas y polisacáridos de pared (CWPS) (Chapot-Chartier & Kulakauskas, 2014).

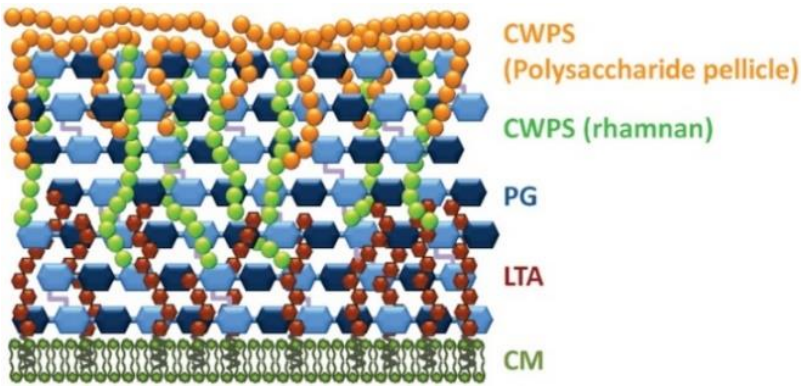


Figura 7. Representación esquemática de la pared celular en *L. lactis*. CWPS, polisacáridos de pared; PG, peptidoglicano; LTA, ácidos lipoteicoicos; CM, membrana citoplasmática. [Fuente: Martínez *et al*., (2020)]

La estructura dinámica de la pared celular hace posible que se produzcan cambios durante el crecimiento o como consecuencia a la exposición a estímulos externos, con el objetivo de mantener un equilibrio entre su rigidez y su plasticidad. De esta forma, la pared celular está implicada en diferentes funciones básicas para la supervivencia de la célula: mantiene su forma e integridad durante los procesos de crecimiento y división celular, resiste la presión osmótica interna del citoplasma y constituye la barrera de protección y comunicación entre la bacteria y su entorno. (Martínez *et al*., 2020).

Dada su relevancia en la supervivencia, las bacterias disponen de sistemas que vigilan la integridad de la pared celular para que, ante una situación de estrés, se active una respuesta que neutralice el daño causado y permita adaptarse a las nuevas condiciones (Martínez *et al*., 2020). Los sistemas de detección, junto con los mecanismos de defensa que son activados, constituye lo que se denomina la respuesta al daño de la pared celular o respuesta CES (Cell Envelope Stress) en la que participan generalmente, los sistemas de dos componentes (TCS, *Two Component System*) y los factores sigma de función extracelular (ECF, Extracytoplasmic Function). Estos sistemas se encargan de **detectar** los cambios en las condiciones ambientales y **transmitir** la información para que se desencadene la respuesta que repare y restablezca la funcionalidad de la pared celular (Jordan *et al*., 2008).

Los TCSs son los sistemas de transducción de señales más comúnmente encontrados en bacterias. Se basan en procesos de fosforilación/desfosforilación formados por dos proteínas independientes, una histidín quinasa (HK), normalmente integrada en la membrana, que actúa como sensor de estímulos, y un regulador de respuesta (RR), citosólico, que induce una respuesta. Cuando la HK detecta un estímulo determinado, se autofosforila y transfiere el grupo fosfato al RR que sufre un cambio conformacional, activándose e induciendo una respuesta acorde al estímulo (figura 8) que consiste, mayoritariamente, en el aumento de los niveles de transcripción de genes específicos, ya sea uniéndose al ADN o a algún componente de la maquinaria de transcripción (Laub y Goulian, 2007; Stock *et al*., 2000). Los dominios relacionados con las reacciones de fosforilación/desfosforilación en las HKs y los RRs están conservados; sin embargo, los dominios sensores (N-terminal en HK) y efectores (C-terminal en RR) son característicos de cada TCS y determinan su especificidad (Koretke *et al*., 2000).

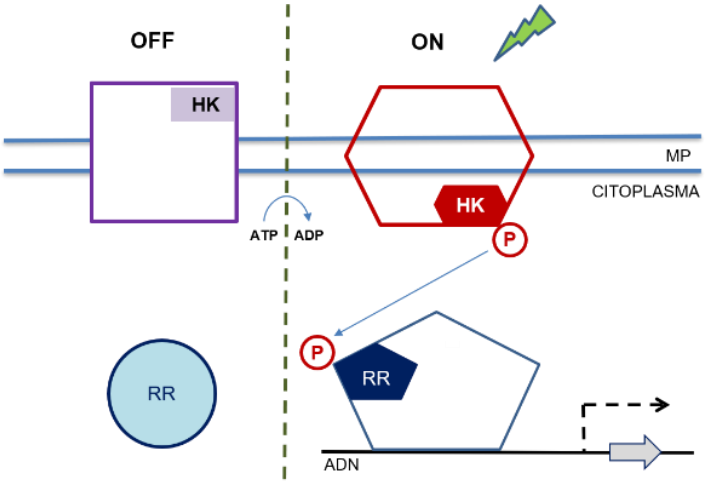


Figura 8. Esquema de los mecanismos de detección y respuesta al estrés mediados por TCS en bacterias. OFF: ausencia de estímulo. ON: situación de estrés o estímulo. HK: histidín kinasa. RR: regulador de respuesta. P: fosfato. MP: membrana plasmática. Flecha punteada: transcripción activada. [Fuente: este trabajo]

En *L. lactis* la respuesta CES está mediada por el TCS CesSR, ortólogo de LiaSR de *B. subtillis*, que detecta el daño sobre la pared celular y activa la transcripción de un conjunto de genes. Este sistema es activado por compuestos que interfieren con el ciclo del lípido II como vancomicina, bacitracina y la bacteriocina Lcn972 *(*Martinez *et al.,* 2008; Martínez *et al.*, 2007), pudiendo activarse también en condiciones de infección por fagos, síntesis de proteínas heterólogas y tratamiento con lisozima (Martínez *et al*., 2020).

El sistema CesSR consta del sensor HK CesS que, al no tener un dominio que detecte el estímulo, necesita de otras proteínas de membrana para su activación, y el RR CesR. Es importante mencionar que la proteína de membrana YjbB modula la actividad de la HK CesS actuando como inhibidor en ausencia de condiciones de estrés (figura 9).

CesSR regula la expresión de al menos 21 genes que en su mayoríacodifican proteínas de membrana de función desconocida y otras son proteínas reguladoras (Martínez *et al*., 2007). Entre dichos genes, tiene especial relevancia el operón *yth* (*llmg_2164-llmg_2163*) ya que podría estar involucrado en la modulación de la actividad de CesS, además de conferir resistencia a Lcn972 (Martínez *et al*., 2007; Roces *et al.*, 2009).

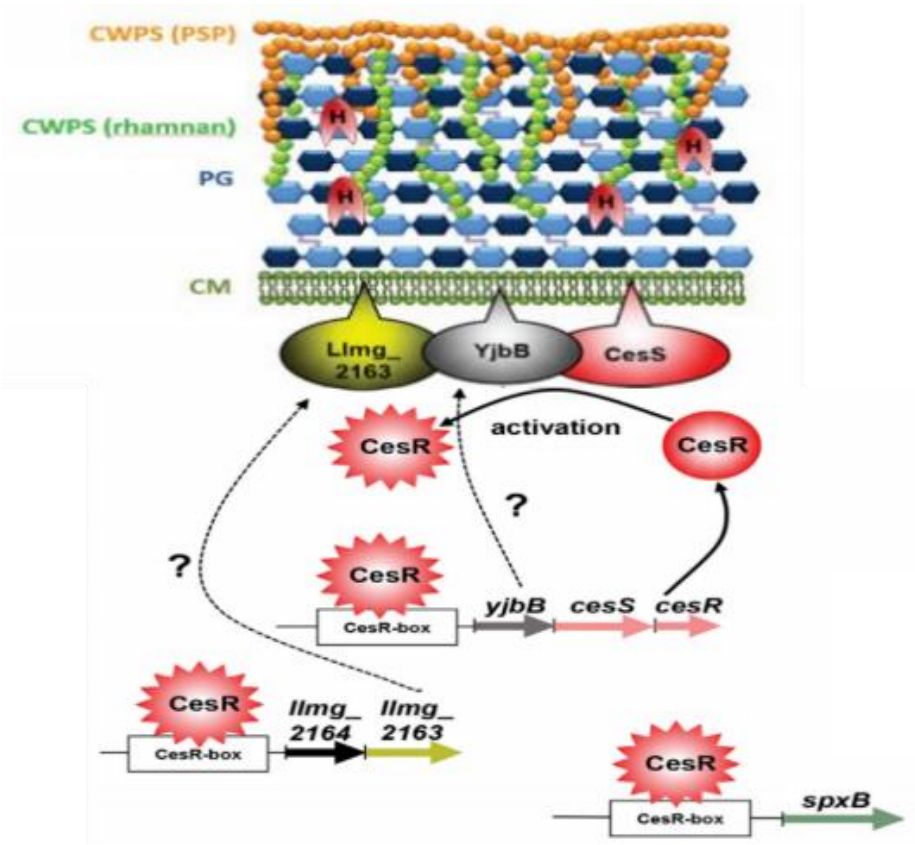


Figura 9. Respuesta al estrés de la pared celular en lactococos. El TCS CesSR (figuras rojas) se activa frente a una situación de estrés sobre la pared celular. El regulador de respuesta CesR es fosforilado a través de la HK CesS (flechas negras) induciendo, entre otros genes, el operón *yjbBcesSR*, el operón *llmg2164_2163* y *spxB*. Las proteínas de membrana, YjbB (en gris) y Llmg2163 (en amarillo) podrían modular la actividad de CesS (rayas negras punteadas). CWPS, polisacáridos de pared; PG, peptidoglicano; CM, membrana citoplasmática. [Fuente: Martínez *et al*., (2020)]

Otro de los genes regulados por CesSR es *spxB,* uno de los 7 parálogos *spx* identificados en *L. lactis* (Veiga *et al*., 2007). Las proteínas Spx se diferencian de otros reguladores transcripcionales en que no se unen al ADN, sino que interaccionan con la subunidad alfa de la ARN polimerasa (RpoA) determinando la unión de ésta a regiones promotoras específicas, regulando así la transcripción génica (Zuber, 2004). En *L. lactis* se ha demostrado que SpxB induce la transcripción de *oatA*, que codifica para la proteína OatA, responsable de la *O*-acetilación del peptidoglicano. De este modo, se confiere resistencia a lisozima y autolisinas (Veiga *et al*., 2007). Otro de los genes *spx*, *trmA*, también se ha relacionado con respuestas a estrés de pared celular (Tan *et al.*, 2008; Veiga *et al*., 2007), oxidativo (Turner *et al*., 2007) y térmico (Frees *et al*., 2001).

En algunas cepas de lactococos existe también otra ruta de detección y respuesta CES constituida por un complejo de transducción de señales tipo Bce, que consta de un

sistema TCS donde la HK carece de la capacidad para detectar estímulos, y un transportador ABC (ATP Binding Cassette). Las HKs detectan la actividad del transportador ABC (flux sensing) que, además de participar como mecanismo de resistencia, tiene la función de activar el TCS, siendo así el transportador el que actúa finalmente como sensor (Dintner *et al*., 2014; Koh *et al*., 2021).

Los transportadores ABC son responsables del transporte de diferentes tipos de sustancias a través de la membrana, en contra de un gradiente de concentración o potencial electroquímico, por lo que requieren consumo de energía que obtienen a través de la hidrólisis del ATP. Suelen estar compuestos por una permeasa (proteína integral de membrana) y por la proteína que une el ATP para poder hidrolizarlo y obtener así la energía necesaria. Participan en muchos procesos fisiológicos bacterianos, destacando por su papel en la resistencia a antibióticos (Dintner *et al*., 2014; Du *et al*., 2018).

Los transportadores ABC tipo Bce participan mayoritariamente en la resistencia a péptidos antimicrobianos. Se caracterizan por tener una permeasa de unos 650 aminoácidos (aa), que tiene 10 segmentos transmembrana, y un dominio extracelular de unos 200 aa. Los transportadores Bce pueden actuar sólo como sensores, sin participar en la resistencia; pueden conferir sólo resistencia, sin estar involucrados en el proceso de detección de la señal, o pueden tener ambas funciones actuando de sensores para activar la HK y confiriendo el fenotipo de resistencia al antimicrobiano correspondiente. Un ejemplo de este último caso es el sistema BceRS-BceAB, ampliamente caracterizado en *B. subtilis*, muy eficaz en la resistencia a bacitracina (figura 10) (Dintner *et al*., 2014; Gebhard, 2012; Revilla-Guarinos *et al*., 2020).

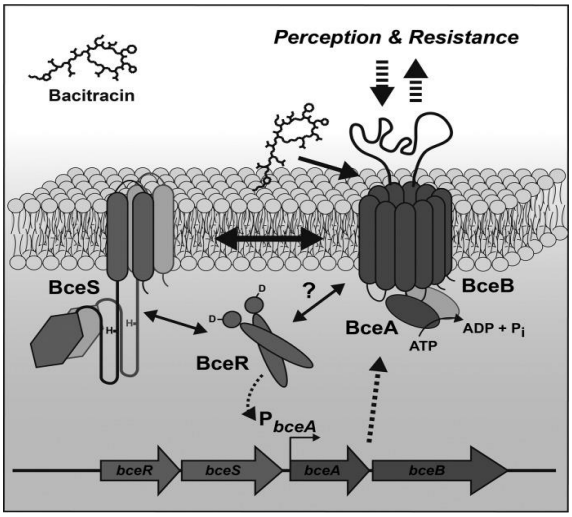


Figura 10. Modelo del sistema de resistencia a bacitracina **BceRS-BceAB** en *B. subtilis*. La HK BceS detecta la actividad del transportador BceAB lo que desencadena la activación del sistema

de dos componentes produciéndose la fosforilación del regulador de respuesta BceR que, a su vez, activa el promotor (P_{bceA}). Doble flecha, interacción entre proteínas; flechas punteadas, inducción y síntesis del transportador; interrogante, posible interacción entre BceR y el sistema formado por BceS y BceAB. [Fuente: Dintner *et al*., (2014)]

En esta Tesis Doctoral se pondrá de manifiesto la implicación de este tipo de transportadores ABC (BceAB) en la resistencia tanto a la bacitracina como a la bacteriocina Lcn972 (ambos antimicrobianos actúan sobre la pared bacteriana) en *L. lactis*. El sistema está formado por el sistema de dos componentes TCS-G, que incluye la HK KinG y el regulador de respuesta LlrG, y por el transportador YsaDCB, con la permeasa YsaB y la proteína de unión a ATP YsaC.

4.3 Lactococina 972 (Lcn972)

Se han identificado numerosas bacteriocinas producidas por *L. lactis* (Pérez *et al*., 2014). Entre ellas, Lcn972 es una bacteriocina no modificada post-traduccionalmente (Clase IId), de 66 aminoácidos, con una masa molecular de 7,5 kDa, catiónica, hidrofílica y de codificación plasmídica (plásmido pBL1 de 10.9 kpb) (Martinez *et al*., 1996; Sánchez *et al*., 2000). Su estructura en solución se caracteriza por presentar un plegamiento β-sandwich con 3 dobles láminas beta antiparalelas unidas (figura 11) (Turner *et al*., 2013). Es producida por algunas cepas de *L. lactis subsp. lactis* (Alegría *et al.*, 2010; Martínez *et al*., 1995). La cepa productora utilizada en esta Tesis Doctoral, *L. lactis subsp. lactis* IPLA972, fue identificada en el Instituto de Productos Lácteos de Asturias (IPLA-CSIC), durante el cribado de cepas de *L. lactis* productoras de bacteriocinas aisladas de quesos artesanales asturianos (Martínez *et al*., 1995).

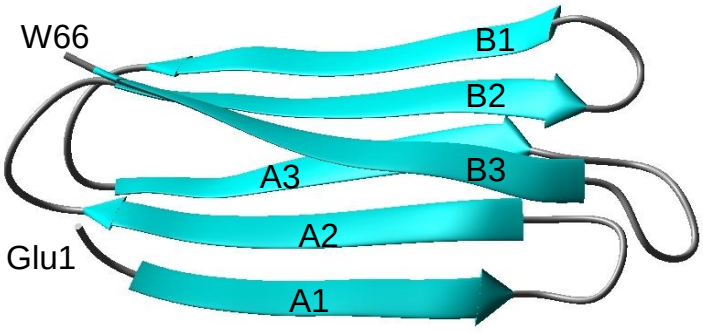


Figura 11. Estructura tridimensional de Lcn972. Glu1, extremo N-terminal; W66, extremo C-terminal (W, Trp). Las láminas β se presentan en forma de flechas planas numeradas. [Fuente: Turner *et al*., (2013)]

Lcn972 es una bacteriocina atípica que presenta diversas peculiaridades que se resumen en la figura 12. Una de las principales diferencias con otras bacteriocinas es su modo de acción. Lcn972 es la primera bacteriocina descrita de la clase II (no modificada post-traduccionalmente) cuya diana no es la membrana plasmática (Martinez *et al*., 1996). Su modo de acción se basa en la interacción con el lípido II, precursor de la síntesis del peptidoglicano, durante la formación del septo de división en células en crecimiento activo. Como consecuencia, las células aumentan de tamaño hasta que la relación superficie/volumen se vuelve insostenible y, finalmente mueren (Martinez *et al*., 2008; Martínez *et al*., 2000).

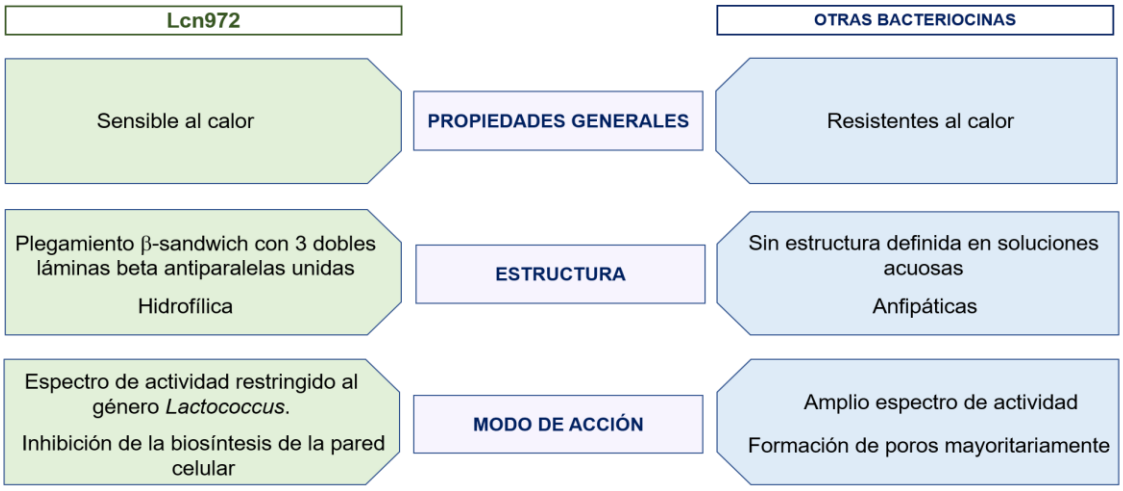
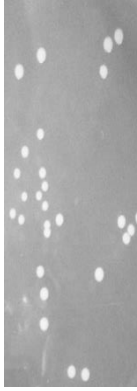
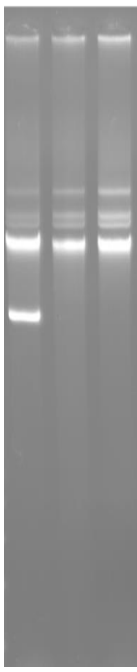
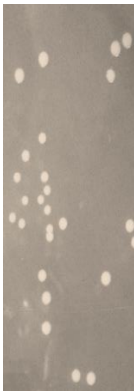


Figura 12. Principales aspectos diferenciales entre la Lcn972 y otras bacteriocinas producidas por BAL. [Fuente: este trabajo]

El hecho de que la bacteriocina Lcn972 tenga un espectro de actividad muy estrecho, dirigido exclusivamente al género *Lactococcus*, descarta su posible uso como conservante de alimentos. Sin embargo, ha sido una herramienta muy útil en otras aplicaciones biotecnológicas de interés tales como mecanismo de estabilización de plásmidos (Campelo *et al*., 2014), para el estudio de la respuesta CES en *Lactococcus* (Martínez *et al*., 2007; Roces *et al*., 2009) y como agente selectivo en experimentos de evolución adaptativa como los realizados en esta Tesis Doctoral (López-González *et al*., 2018 a,b).

ANTECEDENTES Y OBJETIVOS



Antecedentes y Objetivos

A pesar de la existencia de una gran diversidad fenotípica y genotípica en la especie *L. lactis*, no todas las cepas son capaces de resistir los procesos industriales ya que, aunque superen el proceso de selección, posteriormente deben ser producidas a gran escala y distribuidas. En ese sentido, no todas las cepas que se encuentran en la naturaleza reúnen las características adecuadas para ello. Además, la creciente demanda de una gama de productos lácteos más amplia, de mayor calidad nutricional y cualidades organolépticas mejoradas, ha impulsado la ampliación de la "cartera de cepas" por parte de las empresas productoras de cultivos iniciadores. Las nuevas cepas deben ser más robustas y capaces de tolerar los diversos ambientes que pueden suponer una situación de estrés y, además, presentar características específicas que las diferencien de las existentes como por ejemplo, tasas de acidificación elevadas, resistencia a bacteriófagos, producción de antimicrobianos o perfiles diferenciados de aroma y/o sabor (Rodríguez *et al*., 2012). Así pues, y dado que la biodiversidad de cepas disponibles para desarrollar nuevos cultivos iniciadores con diferentes aplicaciones es relativamente pequeña, resulta imprescindible el estudio de estrategias que diversifiquen y mejoren sus aptitudes tecnológicas (McAuliffe, 2018).

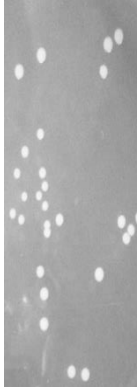
El éxito del proceso fermentativo depende de la viabilidad y actividad metabólica del cultivo iniciador y en este sentido es crucial la integridad de la pared celular, ya que es la primera línea de defensa. Ante situaciones de estrés se dispara la respuesta CES para neutralizar el daño causado y adaptarse a las nuevas condiciones (Martínez et al., 2020). Resultados previos obtenidos en nuestro laboratorio habían demostrado que la modulación de la pared celular en cepas de laboratorio daba lugar a cepas más robustas y resistentes a condiciones de estrés tecnológico. Por ejemplo, la exposición de la cepa *L. lactis* MG1614 a concentraciones crecientes de Lcn972 dio lugar a mutantes resistentes que presentaban fenotipos de interés industrial mejorados respecto a la cepa de partida, como mayor resistencia a la nisina, lisozima, e incluso a bacteriófagos (Roces *et al*., 2012).

En este contexto, el objetivo global de este proyecto de Tesis Doctoral ha sido estudiar la viabilidad de la evolución adaptativa bajo condiciones de estrés sobre la pared celular (EA-CES) como estrategia de mejora de cepas industriales de *L. lactis* y profundizar, de este modo, en el conocimiento de los mecanismos implicados en la respuesta CES de *L. lactis*. Para ello, se abordaron los siguientes objetivos:

Objetivo 1: Aislamiento de mutantes de cepas industriales de *L. lactis* de distinto origen, resistentes a Lcn972, para evaluar la viabilidad de la EA-CES. Este proceso implica el cultivo de *L. lactis* en concentraciones crecientes de Lcn972 para seleccionar mutantes resistentes (Lcn972R), seguido de pases sucesivos en ausencia de presión selectiva con objeto de fijar mutaciones que no interfieran negativamente en el crecimiento. La EA-CES se aplicó a tres cepas acidificantes y cinco cepas productoras de nisina, una de las cuales es comercializada como cultivo protector.

Objetivo 2: Caracterización fenotípica de los mutantes seleccionados (Lcn972R) para determinar el impacto de la EA-CES sobre propiedades tecnológicas relevantes (ej.: parámetros de acidificación en leche, resistencia a fagos, etc.), propiedades de la pared celular (ej.: hidrofobicidad) y resistencia cruzada a otros antimicrobianos.

Objetivo 3: Descripción del fundamento molecular de las mutaciones adquiridas para conocer los mecanismos mediante los cuales *L. lactis* responde al daño de la pared celular.



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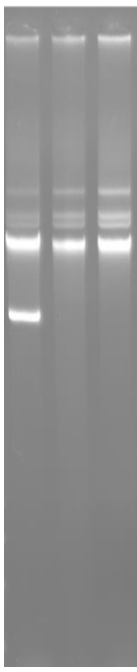

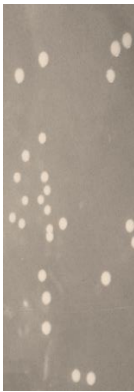

Resultados

Capítulo I.- La resistencia a la bacteriocina Lcn972 mejora la tolerancia de *Lactococcus lactis* IPLA947 al oxígeno sin comprometer su función como cultivo iniciador

En este capítulo se describen los resultados obtenidos tras haber sometido la cepa *L. lactis* IPLA947 a EA-CES. Esta cepa, previamente seleccionada como cepa acidificante para la elaboración de queso *Afuega'l Pitu*, presentaba problemas de acidificación en presencia de oxígeno, por lo que el estudio se centró en la búsqueda de mutantes resistentes al estrés oxidativo.

Se ha obtenido un mutante, *L. lactis* R5, con características mejoradas respecto a la cepa original: mayor resistencia en presencia de agua oxigenada, mejores parámetros de crecimiento y acidificación de la leche en condiciones aerobias y mayor producción de acetoína. Por otro lado, se mantuvieron los parámetros de acidificación en leche, el consumo de lactosa, la producción de ácidos orgánicos y de compuestos volátiles (excepto la acetoína ya mencionada) y la supervivencia durante el almacenamiento a bajas temperaturas y a la liofilización.

En conclusión, la EA-CES permitió la obtención de cepas con fenotipos más robustos tales como la resistencia frente al estrés oxidativo, sin comprometer los parámetros tecnológicos esenciales como cultivos iniciadores.

Artículo 1.- López-González, M.J., Campelo, A.B., Picón, A., Rodríguez, A., and Martínez, B. 2018. Resistance to bacteriocin Lcn972 improves oxygen tolerance of Lactococcus lactis IPLA947 without compromising its performance as a dairy starter. BMC Microbiology 18: 76. DOI: 10.1186/s12866-018-1222-8.

Contribución personal al trabajo

- Concepción y diseño de experimentos junto con el resto de co-autores.
- Realización de los ensayos experimentales.
- Análisis de datos.
- Redacción del manuscrito y elaboración de tablas y figuras.

López-González et al. BMC Microbiology (2018) 18:76
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RESEARCH ARTICLE Open Access

Resistance to bacteriocin Lcn972 improves oxygen tolerance of *Lactococcus lactis* IPLA947 without compromising its performance as a dairy starter

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Abstract

Background: *Lactococcus lactis* is the main component of the mesophilic starters used in cheese manufacture. The success of milk fermentation relies on the viability and metabolic activity of the starter bacteria. Therefore, robust strains able to withstand the harsh conditions encountered during cheese manufacture and starter production are demanded. In this work, we have applied adaptive evolution under cell envelope stress imposed by the cell wall active bacteriocin Lcn972 to evolve strains with more robust phenotypes.

Results: Consecutive exposure of the starter strain *L. lactis* IPLA947 to Lcn972 yielded a stable mutant, *L. lactis* R5, with enhanced survival when challenged with hydrogen peroxide. *L. lactis* R5 exhibited faster growth rates in aerobic fermentations in broth and was able to acidify milk to a lower pH in aerated milk cultures. The improved behavior of *L. lactis* R5 in the presence of oxygen did not translate into a better performance in the presence of heme (i.e. respiration metabolism) or into higher survival during storage at cold temperatures or after freeze-drying compared to the wild type *L. lactis* IPLA947. *L. lactis* R5 retained the same milk acidification rate and no changes in the consumption of lactose and production of organic acids were noticed. However, the profile of volatile compounds revealed a significant increase in 3-hydroxy-2-butanone (acetoin) in curds manufactured with *L. lactis* R5.

Conclusions: Based on our results, *L. lactis* R5 can be proposed as a suitable dairy starter with improved survival under oxidative stress and enhanced metabolic traits. The results support the notion that adaptive evolution under cell envelope stress might be useful to generate strain diversity within industrial *L. lactis* strains.

Keywords: Dairy starter, *Lactococcus lactis*, Bacteriocin, Oxidative stress, Adaptive evolution, Lcn972

Background

Lactic acid bacteria (LAB) are the main components of dairy starters and are used to produce a wide variety of fermented products such as cheese, butter, fermented milk, yogurt, etc., which are highly appreciated by consumers. Among LAB, *Lactococcus lactis* is the main component of the mesophilic starters used in cheese manufacture. The main function of *L. lactis* is to produce L (+) lactic acid from lactose leading to the pH decrease required for milk clotting. The enzymatic pool of *L. lactis* is further involved in the development of the flavor and texture of the final product [35]. For this reason, starter strains have been carefully selected according to their metabolic and technological properties along with the absence of undesirable traits (e.g. antibiotic resistance genes) [15]. Suitability for large scale production is also a major criterion in starter selection. A key step forward has been achieved by the introduction of a starter production strategy based on respiration (revised by [28]). Biomass yields and survival of *Lactococcus* and other LAB are greatly enhanced by aerobic growth in the presence of heme, i.e. under respiration metabolism where oxygen is reduced to water and protons are extruded [14, 30]. Otherwise, the presence of oxygen

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originates reactive oxygen species that compromise *L. lactis* growth [7]. Indeed, several studies have confirmed that dissolved oxygen, as well as the positive redox potential of milk, can seriously compromise the acidification rate of *L. lactis* in milk [16, 22].

Considering that the success of milk fermentation relies on the viability and metabolic activity of *L. lactis*, continuous efforts have been made to understand the physiology behind robust phenotypes of industrially relevant microorganisms (reviewed by [27]). The knowledge gained on *L. lactis* stress physiology has been translated, mostly by recombinant DNA technology, into engineered strains with superior traits. However, legal constraints and the negative opinion of consumers towards genetically modified organisms (GMOs) pose a barrier for further marketing and create demand for alternative strategies [12, 20].

Adaptive evolution is becoming a popular non-GMO strategy to enhance starter fitness and develop evolved strains with enhanced technological traits. During adaptive evolution, bacterial populations are confronted with a particular stressor for several generations to select for mutations that enhance fitness under those prevailing conditions [37]. Adaptive evolution has been recently applied, for example, to improve *L. lactis* tolerance to high temperature [8] and acid tolerance in *Lactobacillus casei* and *Leuconostoc mesenteroides* [17, 38]. Interestingly, a common occurrence in adaptive evolution experiments is the selection of additional phenotypes not directly related to the applied stress, and which may likely arise due to mutations in global regulators, metabolic adaptations and/or cross-protection phenomena [8, 27].

The aim of this work was to assess adaptive evolution under cell envelope stress as a means to enhance robustness of *L. lactis*. Cell envelope stress was applied by exposure to the anti-lactococcal bacteriocin Lcn972 that inhibits cell wall biosynthesis by specifically binding to the cell wall precursor lipid II, thereby triggering the cell envelope stress response in *L. lactis* [24, 25]. Previous work carried out with the laboratory strain *L. lactis* MG1614 (a derivative of the laboratory strain *L. lactis* MG1363) suggested that exposure to Lcn972 activated genes that contribute to survival to heat and acid stresses [31]. Moreover, Lcn972-resistant mutants of *L. lactis* MG1614 had a more densely packed peptidoglycan and increased resistance to nisin and lysozyme [32]. Based on these observations, we hypothesized that continuous exposure of *L. lactis* to Lcn972 might result in evolved strains with additional beneficial traits, besides becoming resistant to Lcn972. We have applied this strategy to the industrial strain *L. lactis* IPLA947 (CECT 5180), which is the main acidifying strain in a mixed-strain starter culture designed for the manufacture of the Afuega'l pitu cheese [3]. This cheese is an acid-coagulated cheese, made with pasteurized milk, in which coagulation relies heavily on the optimal activity of the added starter bacteria [10]. Because the initial levels of oxygen in milk may interfere with acidification by *L. lactis*, we have specifically studied if evolved strains could better withstand oxidative stress.

Methods

Bacterial strains and growth conditions

L. lactis IPLA947 [3] was incubated at 30 °C in M17 (Biokar Diagnostics, Beauvais, France) containing lactose (0.5%) as a carbon source. Batch cultures were carried out in standing 12-ml tubes filled in with 10 ml of M17 broth. Aerated cultures were grown in 50-ml Falcon tubes with culture volumes not exceeding 20 ml and under shaking at 250 rpm. When required, hemin (Sigma-Aldrich, Alcobendas, Spain) was added to M17 at 0.01 mg/ml to activate respiration. Growth parameters were calculated from, at least, two independent cultures started by inoculating pre-warmed M17 with overnight cultures at an optical density at 600 nm (OD_{600}) of 0.05. Growth rates (μ) and generation times (g) were calculated by linear regression of $\ln(OD_{600})$ versus time in the exponential phase and as $\ln(2)/\mu$, respectively. OD_{600} measurements were done in 1-cm cuvettes in a Biophotometer (Eppendorf, Hamburg, Germany) or followed in a Benchmark Plus Microplate spectrophotometer (BioRad Laboratories, Hercules, CA, USA), as indicated. Colony forming units per milliliter (CFU/ml) were determined by plating serial decimal dilutions made in Ringer solution on M17 agar plates (Merck, Darmstadt, Germany).

Adaptation to Lcn972 and susceptibility tests

Lcn972 was purified and quantified as described elsewhere [25] and stocks (320 μg/ml, 12,800 AU/ml) were kept at − 20 °C in 50 mM sodium phosphate buffer, pH 6.8. Adaptation was approached as previously described [32] with modifications. *L. lactis* IPLA947 was inoculated at 1% (*v*/v) from an overnight culture and was sequentially cultivated at 30 °C for 16 h in M17 (2 ml) with doubling Lcn972 concentrations ranging from 20 to 1280 AU/ml. Decimal dilutions from cultures grown with 1280 AU/ml were prepared in Ringer solution and plated on M17 agar to isolate single colonies. Selected colonies were consecutively grown in M17 for 100 generations in the absence of Lcn972. After colony purification, stabilized cultures were stored at − 80 °C in M17 broth supplemented with 10% glycerol for further characterization. Minimal inhibitory concentrations (MICs) were determined during the stabilization step (i.e. growth without Lcn972) by the broth microdilution method [24]. To do that, overnight cultures were first adjusted to an OD_{600} of 0.5 and then further diluted 1:1000 to prepare a cell suspension with a concentration

of 3×10^5 CFU/ml, which was subsequently used to inoculate microtiter plates, containing a gradient of Lcn972 concentrations.

Resistance to hydrogen peroxide

Survival to oxidative stress was determined according to Dijkstra et al. [13] with some modifications. Briefly, overnight M17 cultures were resuspended in 50 mM sodium phosphate buffer, pH 6.8, and exposed to 5 mM H_2O_2 for 2 h at 30 °C in a water bath. Decimal dilutions were plated on M17 agar plates. Untreated controls were handled equally but using sodium phosphate buffer instead of H_2O_2. Three independent cultures per strain were analyzed.

Phase-contrast microscopy

Aliquots of exponentially (4 h) and stationary (24 h) cultures were deposited on glass slides and observed with a DMi8 inverted microscope (Leica microsystems, Wetzlar, Germany). Cell length and width was measured in cells ($n = 32$) from four different fields per sample using the microscope built-in software LasX.

Survival during cold storage

Late exponentially growing cultures of *L. lactis* IPLA947 and R5 were used to inoculate 20 ml of M17 at OD_{600} 0.05. Subsequently, 10-ml aliquots were transferred to 12-ml tubes for batch incubation at 30 °C or to 50-ml tubes which were agitated at 250 rpm. Incubation proceeded for 14 h (early stationary phase) before storage at 4 °C. Samples were taken at weekly intervals and viability (CFU/ml) was determined by plating decimal dilutions on M17 agar plates. Two independent cultures per strain were analyzed.

Lyophilisation

Cells from late exponentially growing batch cultures (100 ml) of *L. lactis* IPLA947 and R5 were collected, washed with Ringer solution and resuspended in 10 ml of 11% reconstituted Difco skim milk (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were frozen at − 80 °C and lyophilized in a Virtis Freezemobile 12EL (VirTis, Gardiner, NY, USA) for 48 h. Lyophilized powder was homogenized by vigorous shaking and stored in 50-ml tubes at 4 °C.

Milk acidification and detection of lactose and organic acids

Milk cultures were carried out in commercially available homogenized UHT milk (CAPSA, Granda, Spain). Milk (50 ml) was inoculated at 2% (v/v) with M17 overnight cultures, which were previously washed twice with Ringer solution, and then incubated at 21 °C for 19 h. Acidification was monitored with a real-time pHmeter ORION™ Versa Star™ (Thermo Scientific Inc., Waltham, MA, USA), which recorded pH values every 15 min. As descriptors of the acidification curves, we use maximum acidification rates (Vm), defined as pH decrease (mU) per min, the time interval at which the maximum acidification rate was maintained (Tm), as well as the time in min (Te) to reach pH 4.6 [19]. Curves were performed in duplicate.

At the end of the incubation, samples (1 ml) were homogenized with 5 ml of 4.5 mM H_2SO_4 for 1 h at 37 °C under continuous shaking, centrifuged (16,000 x g, 15 min, 4 °C), and filtered (0.22 μm). Lactose and organic acids were determined by HPLC using an ICSep ICE-ION-300 ion-exchange column (mobile phase 0.0085 N H_2SO_4, operating temperature 65 °C, flow rate 0.4 ml min^{-1}). A 996 Photodiode Array Detector (Waters) for the determination of organic acids (detection wavelength 210 nm), and a Waters 410 differential refractometer for sugar determination (detection wavelength 280 nm) were used. For quantification, regression equations ($R^2 \geq 0.99$) were calculated by using different concentrations of the corresponding standards.

Detection of volatile compounds

L. lactis IPLA947 and R5 were activated by two successive transfers in 10% reconstituted skim milk prior to inoculation at 1% (v/v) in fresh semi-skim pasteurized milk supplemented with 1 ml l^{-1} of a 100 g l^{-1} $CaCl_2$ solution. They were grown in two independent experiments at 25 °C for 24 h in 100 ml glass flasks. Cultures were transferred, with the help of a sterile spatula, to centrifuge tubes and centrifuged at 12857 x g for 20 min in an Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany). Pellets were wrapped in aluminium foil, vacuum packed in HT3050 plastic bags (Cryovac Sealed Air Corporation, Milano, Italy) and kept at − 35 °C until further analysis. Duplicate curd samples (7 g) were homogenized in a mechanical grinder with 14 g of anhydrous Na_2SO_4 (Merck, Darmstadt, Germany) and 25 μl of an aqueous solution of 495 mg l^{-1} cyclohexanone (Sigma-Aldrich) added as internal standard. Volatile compounds were extracted by solid-phase microextraction (SPME) using a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) coated fibre (Supelco, Bellefonte, PA, USA). They were analyzed and identified by gas chromatography–mass spectrometry (GC–MS) (HP 6890-MSD HP 5973, Agilent, Palo Alto, CA, USA) as previously described [29].

Statistical analyses

Statistically significant differences were analyzed by Student t-test as implemented in Microsoft Excel 2010 (Microsoft Corporation). One-way analysis of variance was conducted with IBM SPSS Statistics (ver. 24.0.0.1) to compare cell length and width of IPLA947 and R5

cells from aerobic and batch cultures. A P value below 0.05 was considered statistically significant.

Results and discussion

We have explored exposure of a *L. lactis* dairy starter strain to the cell wall active bacteriocin Lcn972 as a means of obtaining evolved strains with robust phenotypes. The reasoning behind this was that resistance to Lcn972 in laboratory strains is accomplished through several mechanisms that, as a whole, contribute to enhanced survival under harsh conditions. Such mechanisms involved remodeling of the cell envelope towards a more densely packed peptidoglycan, the likely production of structural or surface polysaccharides and the activation of genes with protecting functions [1, 32, 33]. However, it was not possible to anticipate if similar results could be obtained with industrial or environmental *L. lactis* strains because they often differ phenotypically and genetically from laboratory strains [6, 18]. Therefore, as a proof of concept, we applied this strategy to the starter strain *L. lactis* IPLA947.

Selection of *L. lactis* IPLA947 derivatives resistant to Lcn972

The MIC of the bacteriocin Lcn972 for *L. lactis* IPLA947 was 20 AU/ml (Table 1). This value was similar to those described for other lactococcal strains isolated from commercial dairy starters and for the laboratory strain *L. lactis* MG1614 [23]. Adaptive evolution under cell envelope stress (CES) was conducted by a two-step process consisting of an adaptation step, which involved exposing the cultures to increasing amounts of Lcn972, and a subsequent stabilization step, in which bacterial cultures were grown for 100 generations in the absence of Lcn972. Adapted cultures were able to grow at bacteriocin concentrations of up to 1280 AU/ml, which is 64 times the initial MIC and near the immunity levels (1600 AU/ml) provided by the Lcn972 immunity proteins in Lcn972 producers [2]. Higher concentrations inhibited growth and fully dense M17 cultures (i.e. OD_{600} = 2.0–2.5) could not be reached in 16 h. The culture able to grow at 1280 AU/ml was serially diluted to isolate single colonies and twenty-two colonies were randomly selected. Their MICs ranged from > 160 AU/ml (9 clones), 160 AU/ml (12 clones) to 80 AU/ml (1 clone) (data not shown). Three resistant variants, with MICs over 160 AU/ml (R2A and R3A) and 80 AU/ml (R5A), were selected for further characterization. Partial sequencing of the 16S rDNA and RAPD-PCR profiles confirmed the identity of these clones and excluded unintentional contamination (data not shown).

After adaptation, a single colony from each resistant variant was consecutively grown in M17 in the absence of selective pressure, to confirm that the higher Lcn972 MICs shown by these evolved clones was not transitory, i.e. as a result of the induction of the CES response orchestrated by the two-component system CesSR [25]. Contrary to our previous observation with the laboratory strain *L. lactis* MG1614 [32], the resistant phenotype of the selected clones was not lost during stabilization and they retained the same Lcn972 MIC recorded right after the adaptation step ("RnA" strains) (Table 1). From each stabilized culture of R2A, R3A and R5A, a single colony was stored and named R2, R3 and R5, respectively. To assess possible fitness costs associated to the Lcn972 resistant phenotype, growth of *L. lactis* IPLA947 and its Lcn972R derivatives in M17 was monitored in a microtiter plate reader at 30 °C (Table 1). All the resistant strains had similar growth rates and no significant differences were noted ($P > 0.05$) compared to the wildtype strain. Therefore, stable Lcn972 resistant mutants derived from this industrial strain could be isolated and the putative mutations accumulated during adaptive evolution did not seem to pose a physiological burden.

Resistance to hydrogen peroxide

To initially address if the Lcn972R mutants had become resistant to oxidative stress, their viability was measured after being challenged with 5 mM H_2O_2 (Fig. 1). Under these conditions, *L. lactis* R2 behaved as the wildtype strain, while *L. lactis* R3 was the most sensitive with a viability loss of 0.4 Log CFU/ml. In contrast, the viability loss of mutant *L. lactis* R5 was significantly reduced by

Table 1 Properties of *L. lactis* IPLA947 and its Lcn972-resistant derivatives R2, R3 and R5

L. lactis	MIC Lcn972 (AU/ml)	Growth rate (μ) and generation time (g)[a]					
		Batch		Aeration[b]		Respiration	
		μ (h^{-1})	g(h)	μ (h^{-1})	g(h)	μ (h^{-1})	g(h)
947 (WT)	20	0.64 ± 0.10	1.10 ± 0.16	0.38 ± 0.02	1.81 ± 0.08	0.86 ± 0.01	0.81 ± 0.01
R2	> 160	0.60 ± 0.02	1.16 ± 0.05	0.43 ± 0.01*	1.62 ± 0.04	ND	ND
R3	> 160	0.63 ± 0.02	1.11 ± 0.03	0.44 ± 0.01*	1.59 ± 0.04	ND	ND
R5	80	0.63 ± 0.01	1.10 ± 0.02	0.50 ± 0.01**	1.38 ± 0.04*	0.84 ± 0.01	0.83 ± 0.01

[a]Two independent cultures were analysed. Results are expressed as means ± standard deviation
[b]Significantly different from the wildtype (WT) *L. lactis* IPLA947. (*) $P < 0.05$; (**) $P < 0.01$
ND: not determined

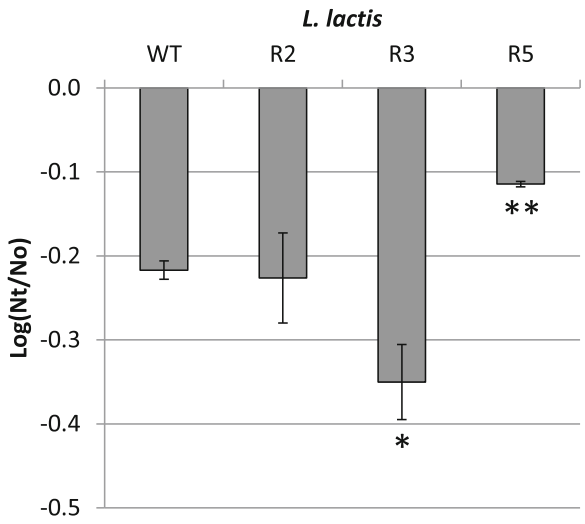

Fig. 1 Survival of *L. lactis* IPLA947 (WT) and its Lcn972R derivatives *L. lactis* R2, R3 and R5 after exposure to 5 mM H_2O_2 for 2 h at 30 °C. Nt: CFU/ml after treatment; No: CFU/ml control. Results are the average of three independent cultures. Error bars are standard deviations. (*) $P < 0.05$; (**) $P < 0.01$, significantly different from *L. lactis* IPLA947

50% ($P < 0.01$) compared to the wildtype strain, supporting the notion that *L. lactis* R5 coped with oxidative stress better after adaptive evolution under CES. These results anticipate the likely selection for different mutations within the population during adaptive evolution which is further supported by the different Lcn972 MICs (see Table 1).

Growth of Lcn972 resistant derivatives in the presence of oxygen

Resistance to oxidative stress was evaluated in aerated M17 cultures as well. Faster growth rates and reduced generation times (Table 1) were observed for all Lcn972R mutants over those of the wildtype strain. *L. lactis* R2 and R3 grew moderately faster ($P < 0.05$) than the wildtype strain but more slowly than *L. lactis* R5. In particular, the *L. lactis* R5 strain showed a 32% improvement in growth rate.

Prompted by this result and the higher resistance of *L. lactis* R5 to H_2O_2, this mutant was further characterized. The performance of *L. lactis* R5 under respiration conditions (i.e. in the presence of hemin) was compared to that of the wildtype *L. lactis* IPLA947. In agreement with literature reports [28], both *L. lactis* IPLA947 and R5 grew faster under respiration conditions than in aerated cultures, almost doubling the growth rate (Table 1). However, the enhanced growth of the evolved *L. lactis* R5 in aerated cultures did not translate into a better performance under respiration as no significant differences in growth rate were observed between R5 and IPLA947 (Table 1). It has been reported that respiring *lactococci* are under lower oxidative stress due to the elimination of oxygen by the respiration metabolism [30]. Therefore, it is possible that the enhanced tolerance of *L. lactis* R5 to oxidative stress is masked under respiration, i.e. the mutations acquired during adaptive evolution may not represent an advantage under respiration conditions.

An intriguing observation during these growth experiments was that, while in batch cultures *L. lactis* IPLA947 and R5 strains reached similar maximum OD_{600} values, in aerated cultures the evolved *L. lactis* R5 consistently reached higher OD_{600} than the wildtype strain (Fig. 2a). However, this increment in OD_{600} did not translate into higher CFU counts which were 0.6 Log units lower than the CFU counts of *L. lactis* IPLA947 (Fig. 2b). Discrepancies between OD_{600} values and CFU counts might be explained by changes in cell morphology, by the presence of longer chains which would underestimate cell counts as one chain would yield a single colony, or by a viable but non-culturable state [36]. Hence, new aerated cultures of *L. lactis* IPLA947 and R5 strains were started and samples were taken for microscopy observation during exponential (4 h) and stationary (24 h) growth. Two main morphological features were observed by phase-contrast microscopy (Fig. 3). On the one hand, *L. lactis* R5 formed longer chains than the wildtype strain in aerated cultures during the exponential phase (Fig. 3a, b). Also, R5 cells were both longer and thicker than those of the wildtype strain (Fig. 3c, d), particularly after prolonged incubation times. These differences were not observed in batch cultures (Fig. 3). According to these results, the lower CFU counts of *L. lactis* R5 seem to be due to the formation of longer chains and the higher OD_{600} values night be related to the increase in cell size. To further confirm the enhanced growth of *L. lactis* R5 in the presence of oxygen, aerated cultures of *L. lactis* IPLA947 and R5 were carried out in milk and the pH decrease was measured as an indication of metabolic activity. Milk was inoculated in duplicate and incubated at 30 °C with vigorous shaking. During the first 8 h, a similar trend was observed for both strains, but after 24 h, *L. lactis* R5 reached a significantly ($p < 0.05$) lower pH value than the wildtype strain (4.91 ± 0.04 vs 5.18 ± 0.02) (Fig. 4). This result confirmed that the evolved *L. lactis* R5 performed better than the wildtype strain in the presence of oxygen not only in M17 broth but also in milk. This enhanced trait could have important technological consequences because the presence of oxygen in milk due to milk handling in the factory may slow down the acidification rate [16, 22].

Survival during cold storage

We sought to determine if the better performance of *L. lactis* R5 strain during aerobic growth compared to the

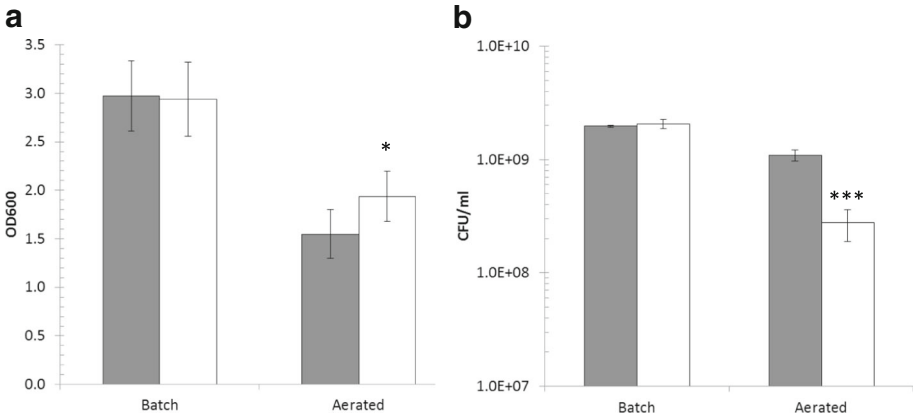

Fig. 2 Optical density (OD_{600}) (**a**) and CFU/ml (**b**) of batch and aerated cultures of *L. lactis* IPLA947 (grey bars) and the Lcn972R derivative *L. lactis* R5 (white bars) at the end of the exponential phase. Results are the average of two independent cultures. Error bars are standard deviations. (*) $P < 0.05$; (***) $P < 0.001$, significantly different from *L. lactis* IPLA947

wildtype strain also implied better viability upon storage at refrigeration temperatures. Thus, *L. lactis* IPLA947 and R5 strains were either incubated statically or shaken for 14 h prior to storage at 4 °C and samples were subsequently taken on a weekly basis to check their viability. The initial bacterial counts remained constant for 3 weeks and decreased substantially after 5 weeks of storage (Fig. 5a). Survival of aerated cultures was roughly 3 Log units higher compared to batch cultures ($P < 0.05$), which is in line with previous reports showing that fermentation

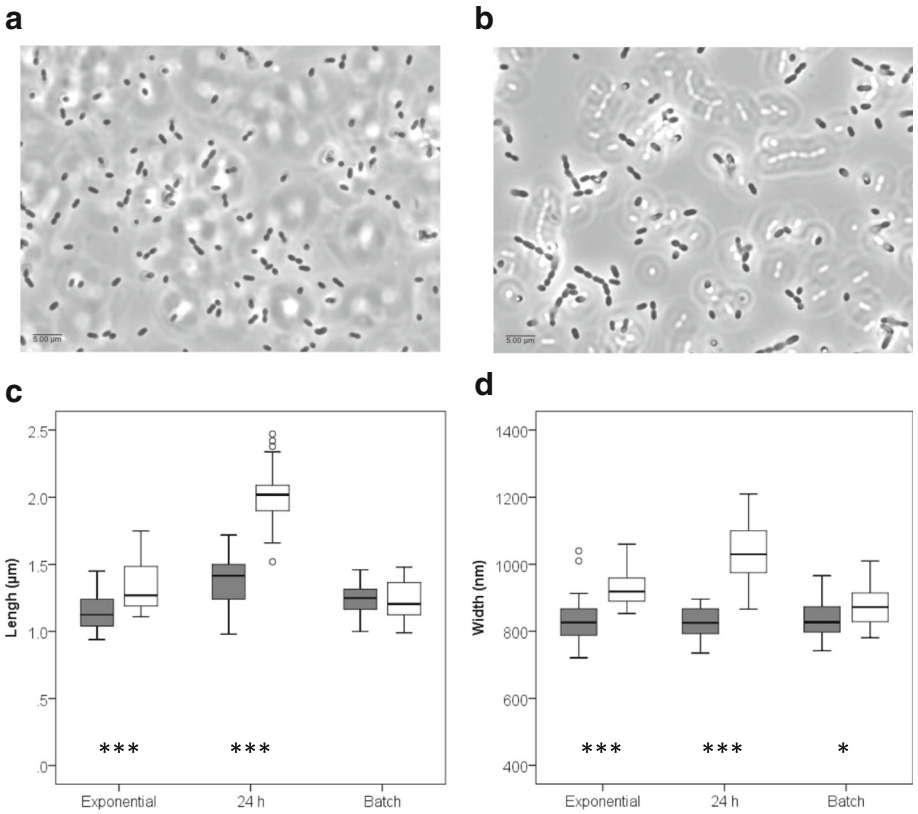

Fig. 3 Cell morphology of *L. lactis* IPLA947 and the Lcn972R derivative *L. lactis* R5. Phase contrast microscopy of *L. lactis* IPLA 947 (**a**) and *L. lactis* R5 (**b**) sampled during growth in the presence of oxygen. Length (**c**) and width (**d**) of cells of *L. lactis* IPLA947 (grey) and *L. lactis* R5 (white) from exponentially and stationary (24 h) aerated cultures and exponentially growing batch cultures. (*) $P < 0.05$; (***) $P < 0.001$, significantly different from *L. lactis* IPLA947

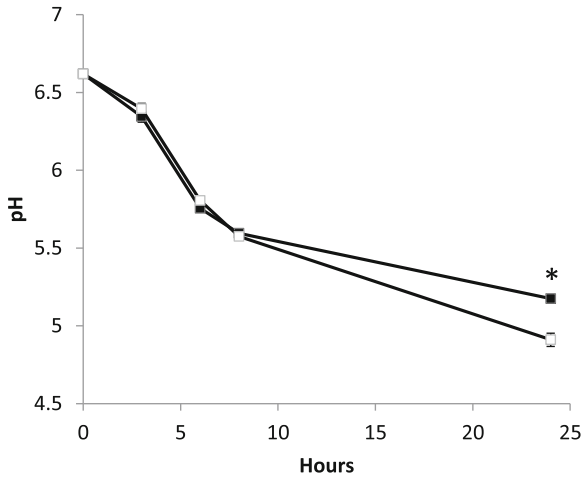

Fig. 4 Decrease of pH in *L. lactis* IPLA947 (black squares) and the Lcn972R mutant *L. lactis* R5 (white squares) in aerated cultures in milk. Results are the average of two independent cultures. Error bars are standard deviations. (*) $P < 0.05$, significantly different from *L. lactis* IPLA947

conditions may enhance *L. lactis* robustness and viability [13]. However, the R5 strain did not survive better than *L. lactis* IPLA947 ($P > 0.05$).

We also compared survival after freeze-drying and storage at 4 °C under air. Freeze-drying is a common method to preserve and deliver dairy starters, in which several factors including storage conditions are deemed relevant to maintain cell viability [4, 34]. For example, loss of membrane integrity due to oxidation of unsaturated fatty acids occurs during storage in the presence of oxygen, leading to reduced viability during storage [5]. As shown in Fig. 5b, the viability of *L. lactis* IPLA947 and R5 decreased during storage under air down to 3 Log units after 7 months but no differences were observed among them. Based on these results, the mutations acquired by *L. lactis* R5 seemed to protect it against exposure to oxygen during active growth but were not sufficient to guarantee better survival during storage.

Performance of *L. lactis* R5 in milk fermentation

Growth in milk of *L. lactis* R5 was followed to verify that no major technological traits were lost upon adaptation to Lcn972 and that the evolved *L. lactis* R5 strain would still be as suitable as a dairy starter as the parental strain *L. lactis* IPLA947. The acidification rate was tested at 21 °C, the temperature at which Afuega'l Pitu cheese is coagulated [10]. As shown in Table 2, no significant differences ($P > 0.05$) were observed in any of the descriptors of the acidification curves. Similar maximum acidification rates were observed in both strains. The time to reach pH 4.6, the isoelectric point of casein, was extended by an average of 45 min in *L. lactis* R5 but the data were not significantly different ($P > 0.05$) (Table 2). The metabolic profile was also similar. After 19 h of incubation, residual lactose and production of the major organic acids (lactic, acetic and formic acids) were similar in both cultures (Table 2). Nonetheless, *L. lactis* R5 seemed to consume lactose more efficiently and showed a slightly higher mixed-acid metabolism compared to the wildtype strain, although these differences were not statistically significant ($P > 0.05$).

In a separate experiment, the volatile profile of curds made with *L. lactis* IPLA 947 and R5 strains was determined. After 24 h of incubation, eight volatile compounds (three acids, one alcohol and four ketones)

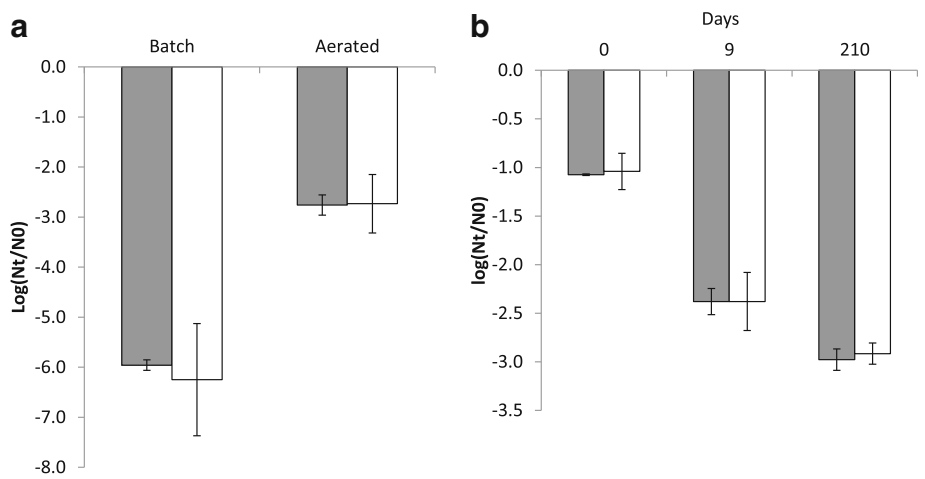

Fig. 5 Survival during cold storage of *L. lactis* IPLA947 (grey) and the Lcn972R derivative *L. lactis* R5 (white). (**a**) Viability of batch and aerated cultures in M17 broth stored for 5 weeks at 4 °C. (**b**) Viability after freeze-drying and stored at 4 °C under air. Results are the average of two independent experiments. Error bars are standard deviations

Table 2 Acidification and metabolites produced by *L. lactis* IPLA947 and the Lcn972 resistant mutant *L. lactis* R5 grown in milk

L. lactis strain	Acidification parameters[a]			Residual lactose (mM)	Organic acids (mM)		
	Vm (mpHUnits/min)	Tm (min)	Te (min)		Lactic	Acetic	Formic
947 (WT)	3.27 ± 0.04	150	960 ± 0	103.8 ± 6.5	61.8 ± 4.2	0.68 ± 0.07	0.27 ± 0.02
R5	3.32 ± 0.11	150	1005 ± 21	91.5 ± 4.9	52.4 ± 2.5	1.01 ± 0.02	0.24 ± 0.00

[a]*Vm* maximum acidification rate, *Tm* time interval at which the maximum acidification rate was maintained, *Te* time to reach pH 4.6. Results are expressed as means ± standard deviation of two independent experiments

were detected. Levels of volatile compounds for both strains were not significantly ($P < 0.05$) different, with the only exception of 3-hydroxy-2-butanone (acetoin), whose levels were almost twofold higher in curds produced by *L. lactis* R5 than in those produced by *L. lactis* IPLA947 (Fig. 6). Acetoin is synthesized from pyruvate by the enzyme acetolactate synthase when the NADH pool is low and the activity of lactate and pyruvate dehydrogenases is not optimal [26]. The increased levels of acetoin in *L. lactis* R5 curd suggest activation of the acetolactate synthase pathway, diverting pyruvate metabolism towards acetoin production. This pathway has been shown to be activated to cope with the presence of oxygen in both M17 broth and milk cultures [9, 21]. It seems plausible that, due to the stress imposed by Lcn972 during adaptive evolution, metabolic adaptations might have occurred as a strategy to produce additional energy and lower the stress level, as often described in the literature [27]. Therefore, mutations leading to the activation of the acetolactate synthase pathway might be behind the enhanced response of *L. lactis* R5 to oxidative stress. Moreover, acetoin, the reduction product of 2,3-butanodione (diacetyl), imparts a sour-milk aroma and together with diacetyl, which has buttery and vanilla notes, are key aroma components of many cheese varieties [11]. Hence, the incorporation of the evolved *L. lactis* R5 strain as part of the Afuega'l pitu cheese starter might improve cheese flavor. Nevertheless, Afuega'l pitu cheese pilot plant trials using pasteurized milk should be carried out to confirm the performance of the evolved strain under production conditions.

Conclusions

In this work, it has been shown that adaptive evolution of the industrial starter *L. lactis* IPLA947 under cell envelope stress may provide evolved strains with enhanced robustness phenotypes as exemplified by the higher tolerance to oxygen of *L. lactis* R5. Furthermore, adaptation to Lcn972 did not compromise essential technological parameters, such as milk acidification rate, and interesting metabolic changes were selected that might improve cheese flavour. Although it cannot be anticipated that every single *L. lactis* strain will evolve in a similar fashion as *L. lactis* IPLA947, our proposed strategy might be useful to generate strain diversity within industrial *L. lactis* strains, and could be adopted by the dairy industry in product development and market expansion.

The complexity of the stress-responsive regulatory networks that often overlap and provide cross-protection, together with additional metabolic adaptations, makes it difficult to predict and draw hypotheses on the putative mutations selected during adaptive evolution that are involved in resistance to the cell wall active Lcn972 and/or oxygen tolerance. Genome sequencing and transcriptional

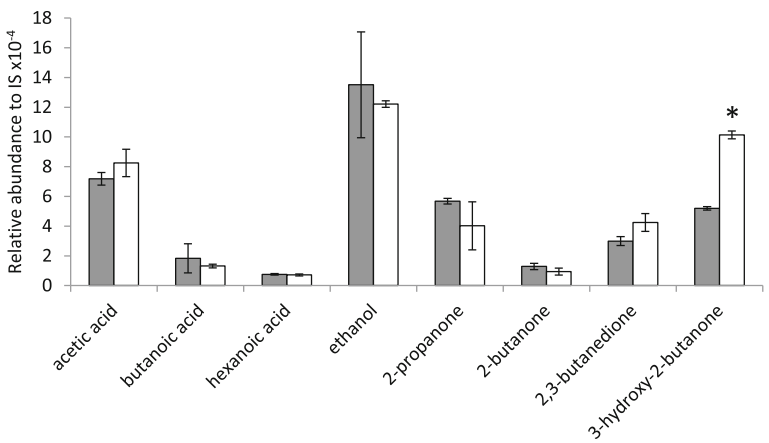

Fig. 6 Volatile compounds detected by GC-MS in milk cultures of *L. lactis* IPLA947 (grey bars) and the Lcn972R mutant *L. lactis* R5 (white bars). Results are the mean ± standard deviation of two independent cultures. IS, internal standard (*) $P < 0.05$, significantly different from *L. lactis* IPLA947

analyses will have to be approached in the near future to identify the molecular basis of the observed phenotypes and hopefully the results may contribute to a better understanding of the stress physiology of *L. lactis*.

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Availability of data and materials
All data generated or analyzed in this study are presented within this manuscript. All materials used in this study including raw data shall be available upon reasonable request.

Authors' contributions
AR and BM conceived and designed the study; MJLG, ABC, AP, and BM performed the experiments. All authors contributed to interpretation of the results and manuscript drafting. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Capítulo II.- La evolución adaptativa bajo estrés sobre la pared celular proporciona diversidad fenotípica en cepas industriales de *Lactococcus lactis*

El éxito obtenido en la aplicación de la EA-CES en la cepa IPLA947 (capítulo I) propició el uso de esta estrategia en una gama más amplia de cepas de *L. lactis* de carácter industrial. En este capítulo se utilizaron siete cepas de diferente origen, dos acidificantes empleadas en cultivos comerciales, una productora de nisina A usada como cultivo protector y cuatro productoras de nisina Z, seleccionadas de la colección IPLA-CSIC, que habían sido aisladas del queso *Afuega'l Pitu*.

En todos los casos se han obtenido mutantes resistentes a la Lcn972 con parámetros de crecimiento similares a los de la cepa de partida. En general, la acidificación de la leche no se vio comprometida, excepto en uno de los mutantes que perdió el plásmido de la lactosa, siendo inviable su crecimiento en leche. Además, se aislaron mutantes con cambios significativos en fenotipos con repercusión tecnológica que afectan a la superficie bacteriana (ej. hidrofobicidad), sensibilidad a fagos, resistencia cruzada a antimicrobianos de pared como la lisozima, bacitracina o vancomicina y al grado de autolisis, entre otros. Por otro lado, se abordó la secuenciación de su genoma y un análisis preliminar de las mutaciones seleccionadas mediante la EA-CES. Se detectaron principalmente mutaciones en genes relacionados con la respuesta a estrés, funciones reguladoras y de transporte, biogénesis de la pared celular y/o metabolismo nucleotídico. Este estudio genómico preliminar ha puesto de manifiesto la diversidad genética entre los mutantes caracterizados, reflejando los diferentes mecanismos de *L. lactis* para defenderse del daño de la pared celular.

En conclusión, este estudio ha permitido identificar ventajas y desventajas de la aplicación de la EA-CES, y poner de manifiesto su viabilidad como estrategia para introducir diversidad fenotípica y genotípica en cepas industriales y silvestres de *L. lactis*.

Artículo 2.- López-González, M.J., Escobedo, S., Rodríguez, A., Neves, A.R., Janzen, T., and Martínez, B. 2018. Adaptive Evolution of Industrial *Lactococcus lactis* Under Cell Envelope Stress Provides Phenotypic Diversity. Frontiers in Microbiology 9:2654. DOI: 10.3389/frnicb.2018.02654.

Contribución personal al trabajo

- Diseño del estudio junto con el resto de co-autores
- Experimentos de evolución adaptativa y caracterización fenotípica.
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Adaptive Evolution of Industrial *Lactococcus lactis* Under Cell Envelope Stress Provides Phenotypic Diversity

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Lactococcus lactis is widely used as a starter in the manufacture of cheese and fermented milk. Its main role is the production of lactic acid, but also contributes to the sensory attributes of cheese. Unfortunately, the diversity of suitable strains to be commercialized as dairy starters is limited. In this work, we have applied adaptive evolution under cell envelope stress (AE-CES) as means to provide evolved *L. lactis* strains with distinct physiological and metabolic traits. A total of seven strains, three of industrial origin and four wild nisin Z-producing *L. lactis*, were exposed to subinhibitory concentrations of Lcn972, a bacteriocin that triggers the cell envelope stress response in *L. lactis.* Stable Lcn972 resistant (Lcn972R) mutants were obtained from all of them and two mutants per strain were further characterized. Minimal inhibitory Lcn972 concentrations increased from 4- to 32-fold compared to their parental strains and the Lcn972R mutants retained similar growth parameters in broth. All the mutants acidified milk to a pH below 5.3 with the exception of one that lost the lactose plasmid during adaptation and was unable to grow in milk, and two others with slower acidification rates in milk. While in general phage susceptibility was unaltered, six mutants derived from three nisin Z producers became more sensitive to phage attack. Loss of a putative plasmid-encoded anti-phage mechanism appeared to be the reason for phage susceptibility. Otherwise, nisin production in milk was not compromised. Different inter- and intra-strain-dependent phenotypes were observed encompassing changes in cell surface hydrophobicity and in their autolytic profile with Lcn972R mutants being, generally, less autolytic. Resistance to other antimicrobials revealed cross-protection mainly to cell wall-active antimicrobials such as lysozyme, bacitracin, and vancomycin. Finally, distinct and shared non-synonymous mutations were detected in the draft genome of the Lcn972R mutants. Depending on the parental strain, mutations were found in genes involved in stress response, detoxification modules, cell envelope biogenesis and/or nucleotide metabolism. As a whole, the results emphasize the different strategies by which each strain becomes resistant to Lcn972 and supports the feasibility of AE-CES as a novel platform to introduce diversity within industrial *L. lactis* dairy starters.

Keywords: dairy starter, bacteriocin, stress, adaptive evolution, cell wall

INTRODUCTION

Dairy starters have been applied for the production of fermented dairy products more than a century ago, when the first dairy starter strains were isolated and intentionally added to milk. The main components of dairy starters are lactic acid bacteria (LAB) and, in particular, *Lactococcus lactis* is the most common acidifying strain used in the production of cheese. According to their composition, cheese starters are classified into undefined and defined starters. Undefined starters are complex mixtures of unknown composition, whereas defined starters are blends of well-characterized strains of one (single) or multiple (mixed) species (Rodríguez et al., 2012). These starter strains have been isolated and selected according to their technological properties, namely based on fast growth and acidification rate in milk, proteolytic activity and bacteriophage resistance. Other features such as the synthesis of aroma compounds, texturing agents and inhibitory compounds are also of interest (Derkx et al., 2014). Commercial defined starters are currently available in highly concentrated frozen and freeze-dried formats, ready to be added directly to vat milk to minimize the risk of starter contamination during handling and changes in starter composition in the factory. Overall, the use of starters results in reliable cheese quality and, most importantly, in a more consistent acidification rate that allows cheese making be conducted on a fixed time schedule (Johnson, 2017).

Changes in consumer preferences toward less additives and artificial ingredients in fermented products put pressure on companies engaged in the production of starters to expand their strain portfolio to satisfy these demands (Johansen et al., 2015). However, there is a general consensus that the biodiversity of available commercial starters is relatively small to develop defined starter blends for novel applications. Consequently, either new sources or novel strategies for strain development are required (McAuliffe, 2018). Starter improvement may be approached by knowledge-based screenings of large culture collections, looking for the desired combination of properties, or by evolving new phenotypes from existing starters. The former approach is limited by the fact that not all strains survive the conditions imposed by industrial production, whereas the latter may be applied to strains whose production at large-scale has been already optimized. The success of strain improvement based on natural selection and experimental evolution has been recently reviewed by Johansen (2018). Besides classical mutagenesis and standard or cutting-edge genome editing technologies, knowledge-based strategies to diversify may include positive selection under a particular condition. For example, to increase yogurt sweetness, growth of *Streptococcus thermophilus* mutants able to consume the galactose moiety of lactose and excrete the glucose moiety could be isolated. First, growth on galactose to select for galactose positive mutants was approached, followed by subsequent selection on the non-metabolized glucose analog 2-deoxyglucose (Sørensen et al., 2016). Additionally, adaptive evolution experiments that involved serial propagation under selective conditions have also been applied to LAB to evolve strains with specific phenotypes (see Bachmann et al., 2017 for a recent review). Examples of adaptive evolution in *L. lactis* include adaptation to high temperature (Chen et al., 2015), or adaptation of a plant isolate to grow in milk (Bachmann et al., 2012).

An alternative route to diversify *L. lactis* populations may be the use of bacteriocins in evolution experiments. Bacteriocins are bacterial ribosomally synthesized antimicrobial peptides. Those produced by LAB have received much attention due to their biotechnological potential as food biopreservatives, based on their potent inhibitory activity against foodborne pathogens and spoilage microorganisms. LAB bacteriocins encompass a large and diverse group of peptides with multiple structures that kill target bacteria by interfering with cell envelope functions, either by disruption of membrane permeability by pore formation and/or inhibition of cell wall synthesis (Álvarez-Sieiro et al., 2016). It has also been shown that bacteriocin resistant mutants (BacR) can be isolated under laboratory conditions. BacR mutants may display altered phenotypes from changes in cell wall constituents and membrane fluidity to alterations of carbon metabolism. The latter occurs namely by mutation or repression of bacteriocin receptors which are also involved in sugar uptake (Tessema et al., 2011; Bastos et al., 2015). However, most of these studies have focused on mutants of main foodborne pathogens such as *Listeria monocytogenes* or *Staphylococcus aureus*. Much less is known on the impact of bacteriocin resistance in industrially relevant bacteria such as *L. lactis*. Nisin resistance in *L. lactis* IL1403 has been linked to an increased D-alanylation of lipoteichoic acids and a thickened cell wall at the septum (Kramer et al., 2008). Transcriptomic studies further suggested that other mechanisms (e.g., ABC transporters and metabolic adaptations) may be also involved (Kramer et al., 2006). The phenotypic consequences of resistance to the lantibiotic lacticin 3147 have also been characterized in *L. lactis* IL1403 (Guinane et al., 2006). Resistance to other anti-lactococcal bacteriocins such as lactococcin G and LsbB has been associated with mutations of the enzyme undecaprenyl pyrophosphate phosphatase (UppP), involved in cell wall biosynthesis, and of a membrane metallopeptidase (YvjB), respectively (Kjos et al., 2014; Miljkovic et al., 2016). These proteins are suggested to be bacteriocin receptors required for antimicrobial activity.

Lactococcin 972 (Lcn972) is a non-pore forming bacteriocin only active against *Lactococcus* that inhibits cell wall biosynthesis at the septum by specifically binding to lipid II (Martínez et al., 2008). In line with this mode of action, Lcn972 triggers the cell envelope stress response in *Lactococcus* through the activation of the two component system CesSR (Martínez et al., 2007). Although we were not able to isolate spontaneous mutants after a single exposure to Lcn972, a stable resistant mutant of the laboratory strain *L. lactis* MG1614 could be selected by subculturing in the presence of increasing Lcn972 concentrations. The characterization of a single resistant mutant demonstrated that changes on the cell surface, along with chromosomal deletions and transcriptional gene activation mediated by insertion sequences, had occurred (Roces et al., 2012a,b). This mutant revealed cross-resistance to lysozyme and nisin and insensitivity to the bacteriophage c2 (Roces et al., 2012a). More recently, to assess if industrial strains are also prone to Lcn972 plasticity, we have applied the same procedure to the cheese starter *L. lactis* IPLA947. *L. lactis* R5, a Lcn972 resistant

mutant (Lcn972R) four times more resistant than *L. lactis* IPLA947, showed increased resistance to oxidative stress without compromising the acidification rate in milk (López-González et al., 2018).

These previous results encourage us to propose that Adaptive Evolution under Cell Envelope Stress (AE-CES), using Lcn972 as a stressor, might be an option to diversify industrial *L. lactis*, i.e., to introduce new phenotypes in existing dairy starters. Thus, the aim of this work was to apply AE-CES to seven *L. lactis* strains of different origins (industrial and raw milk cheese isolates), and assess the phenotypes of their Lcn972R mutants with special emphasis in milk growth, surface properties and stress survival. Both unique and common phenotypes were observed. Moreover, distinct point mutations could be detected in the draft genome sequence of thirteen Lcn972R mutants that revealed the heterogeneity of the different strategies of *L. lactis* to cope with the stress imposed by Lcn972.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Lactococcus lactis strains used in this work and their sources and properties are shown in **Table 1**. Additionally, *L. lactis* IPLA947 (Cárcoba et al., 2000), the acidifying strain of the *Afuega'l Pitu* cheese starter, and its Lcn972R mutant R5 (López-González et al., 2018) were also included for the phenotypic and genotypic tests. *L. lactis* was routinely grown at 30°C statically in LM17 (Biokar Diagnostics) that incorporates lactose at 0.5% (w/v) in its composition. For testing lactose fermentation, a basal broth was prepared with: (w/v) 0.5% tryptone (Oxoid), 0.3% meat extract (Biokar Diagnostics), and bromocresol purple dye (BCP) at 0.004% supplemented with either lactose (BCP-lac) or glucose (BCP-glc) at 0.5%. *L. lactis* MG1614 was grown in M17 (Oxoid) supplemented with glucose (GM17) at 0.5% and used as indicator for Lcn972 quantification. *Micrococcus luteus* NCIMB8166 was used as indicator for nisin quantification and was grown in Tryptic Soy broth (TSB, Biokar Diagnostics) at 37°C. Plates were prepared with agar at 2%. Frozen stocks were kept at −80°C in the presence of 10% glycerol. Working culture stocks were prepared from overnight (16–18 h) cultures started with a single colony. Glycerol was added to a final concentration of 10% and 40 μl-aliquots were stored at −80°C. Before each experiment, one aliquot was unfrozen and used to inoculate 4 ml of LM17. Growth curves were carried out in a Benchmark Plus Microplate spectrophotometer (Bio-Rad Laboratories). Growth was started by diluting overnight cultures in pre-warmed LM17 to an optical density at 600 nm (OD_{600}) of 0.05. Growth rate (μ) was calculated by linear regression of $\ln(OD_{600})$ versus time in the exponential phase in, at least, two independent experiments.

Minimal Inhibitory Concentration

Susceptibility to Lcn972 was determined by the broth microdilution method as previously described (Martínez et al., 2008). Purified Lcn972 stock was prepared in 50 mM sodium phosphate buffer, pH 6.8, with a specific activity of 34.6 AU/μg (12,800 AU/ml, 370 μg/ml) and was kept at −20°C. Twofold dilutions (100 μl) were made in broth and placed on microtiter plates. Wells were inoculated with 100 μl of an exponentially growing culture adjusted to OD_{600} of 0.05 and further diluted 1:100. Plates were incubated for 24 h at 30°C.

Adaptive Evolution Experiments

Adaptation to Lcn972 proceeded essentially as described by Roces et al. (2012a) and is depicted in **Figure 1A**. Cultures were started with a single colony in LM17. After overnight incubation at 30°C, they were used to inoculate at 1% (v/v) 10 ml of pre-warmed LM17 containing 10 AU/ml of Lcn972. After incubation at 30°C for 16 h, the same procedure was applied, doubling the concentration of Lcn972 in each round. A total of eight consecutive transfers were done into fresh LM17, starting at 10 AU/ml up to 1,280 AU/ml, with the exception of L98 that was stopped after 5 transfers at 160 AU/ml. Decimal dilutions of each culture grown at the highest Lcn972 concentration were spread on LM17 plates without Lcn972 to isolate single colonies. From *L. lactis* L81 and L62, 43, and 30 colonies were picked, respectively. For nisin producers, 16 colonies per strain were picked (**Figure 1B**). Each colony was transferred to 1-ml deepwell microtiter plates filled with LM17 and grew for 24 h at 30°C. These microtiter plates were replicated by inoculating at 1% (v/v) new LM17 deepwell plates that were incubated at 30°C for 24 h. After 10 serial transfers, the susceptibility to Lcn972 was checked. To do so, cells from each well were diluted 1:100 in the case of *L. lactis* L81 and L62 or 1:160 for nisin producers in fresh LM17 and 5 μl were used to inoculate 200 μl of LM17 plus Lcn972 at 320 AU/ml (L81 and L62) or 80 AU/ml (nisin producers). Two cultures per *L. lactis* strain able to grow in the presence of Lcn972 were streaked on a LM17 plate and a single colony was stored at −80°C.

Milk Acidification and Production of Lactic Acid

Skim milk powder (Difco) was reconstituted at 11% (w/v) with distilled water and treated at 100°C for 30 min. Overnight LM17 cultures were centrifuged and washed once with Ringer solution (Merck, Germany). Milk (10 ml) was inoculated at 3% (v/v) with the cell suspension and incubated at 30°C for 6 h. pH was measured with the pHmeter micropH 2001 (Crison, Spain). Acidification curves of L81 and L62 and their Lcn972 resistant mutants (Lcn972R) were started by inoculation of 40 ml of milk with the cell suspension at 2% (v/v) and pH was followed during the incubation at 30°C with the real-time pHmeter Orion^{TM} Versa^{TM} Start (Thermo Scientific Inc., United States) every 30 min for 20 h. Maximum acidification rate (V_m) and the time at which pH 4.6 was reached (Te) were used as acidification parameters as described by Kristo et al. (2003).

Lactic acid production was analyzed by HPLC using an ICSep-ICE-ION-300 column with 0.0085 N H_2SO_4 as mobile phase at flow rate of 0.4 ml/min and operating temperature of 65°C. Lactic acid was detected at 210 nm by a 996 photodiode array detector (Waters). Milk samples (1 ml) were deproteinized with 5 ml of 4.5 mM H_2SO_4 for 1 h at 37°C in a rotary shaker. After centrifugation (16,000 × *g*, 15 min, 4°C), supernatants were

TABLE 1 | Source and properties of *Lactococcus lactis* subsp. *lactis* strains used in this work.

Strain-code	Source and properties	Reference	Lcn972 MIC[1] (AU/ml)	Growth rate[2] (h^{-1})
L. lactis L81	Commercial mesophilic starter. Acidifying strain	CHCC collection	10	0.69 ± 0.10
L81-D1	Resistant to Lcn972	This work	80	0.83 ± 0.04*
L81-E2	Resistant to Lcn972	This work	320	0.79 ± 0.01
L. lactis L62	Commercial mesophilic starter. Acidifying strain	CHCC collection	10	0.84 ± 0.01
L62-C9	Resistant to Lcn972	This work	320	0.89 ± 0.04
L62-G9	Resistant to Lcn972	This work	80	0.80 ± 0.02
L. lactis L98	Bioprotective culture. Nisin A producer	CHCC collection	40	0.95 ± 0.01
L98-C1	Resistant to Lcn972	This work	160	0.90 ± 0.04*
L98-E2	Resistant to Lcn972	This work	160	0.81 ± 0.09*
L. lactis IPLA517	Raw milk cheese, nisin Z producer	Martínez et al., 1995	10	0.95 ± 0.01
517-B5	Resistant to Lcn972	This work	320	0.74 ± 0.06
517-C6	Resistant to Lcn972	This work	160	0.82 ± 0.16
L. lactis IPLA641	Raw milk cheese, nisin Z producer	Martínez et al., 1995	10	0.98 ± 0.09
641-C8	Resistant to Lcn972	This work	80	0.87 ± 0.09
641-D8	Resistant to Lcn972	This work	160	0.79 ± 0.01
L. lactis IPLA729	Raw milk cheese, nisin Z producer	Martínez et al., 1995	10	1.08 ± 0.04
729-D10	Resistant to Lcn972	This work	160	0.97 ± 0.00
729-F9	Resistant to Lcn972	This work	160	0.90 ± 0.03*
L. lactis IPLA1064	Raw milk cheese, nisin Z producer	Martínez et al., 1995	10	0.97 ± 0.00
1064-C11	Resistant to Lcn972	This work	160	0.81 ± 0.01
1064-E11	Resistant to Lcn972	This work	160	0.78

[1] *MIC, Minimal Inhibitory Concentration. AU, Arbitrary Units.*
[2] *Growth rate in LM17 broth at 30°C. Results are the mean ± standard deviation (n = 2).*
* *Statistically different ($p < 0.05$) compared to the equivalent wild-type strain.*

filtered through a 0.45 μm PTFE filter (VWR international). All the experiments were carried out with at least two independent cultures.

Nisin Production

Nisin was quantified by the agar well diffusion method. Plates were prepared by inoculating melted TSB agar 1.2% with 10^5 colony forming unit (CFU) per ml of *M. luteus* NCIMB8166. After solidification, wells (4 mm diameter) were made and filled with 20 μl of culture or milk supernatants obtained by centrifugation (15,400 × *g*, 15 min, 4°C). Plates were incubated at 37°C for 24 h to score inhibition halos. A calibration curve was prepared with pure nisin (a gift from Applin & Barret, United Kingdom) with concentrations from 5 to 25 μg/ml diluted in 0.05% acetic acid. Quantification was performed in two independent cultures.

Bacteriophage Susceptibility

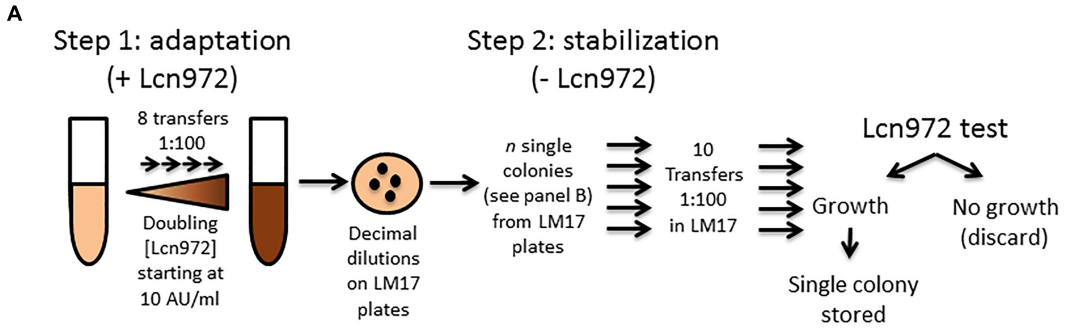
Different phages from the Chr. Hansen collection infecting *L. lactis* L81, L62, and L98 were tested by the double layer assay. Phage decimal dilutions prepared in LM17 were spotted (10 μl) onto LM17 plates containing 10 mM Ca^{++} and overlaid with soft LM17 agar (0.7%) inoculated with 100 μl of the *L. lactis* overnight culture. Clear halos and isolated lysis plaques were observed after overnight incubation at 30°C. When required, plaque forming units per ml (PFU/ml) were estimated after incubating 100 μl of the phage dilution with 100 μl of the bacterial culture for 10 min, then mixed with 8 ml of soft LM17 and poured on a LM17 plate. To identify phages infecting the *L. lactis* nisin producers IPLA517, IPLA641, IPLA729, and IPLA1064, a growth inhibition test in milk was carried out. Bacterial cultures were challenged with 62 phages at *ca.* 10^6 PFU/ml and the pH was monitored. When the pH of a control culture without added phage was at 5.0, the pH of the infected culture was recorded, and a phage was considered infective when the pH difference was more than 0.5.

Surface Properties and Autolysis

Cell surface hydrophobicity was measured by the Microbial-Adhesion To Solvent (MATS) protocol using hexadecane and stationary phase cells as described by Roces et al. (2012a). The degree of autolysis was determined according to Meyrand et al. (2007). Briefly, exponentially growing cells (OD_{600} = 0.3–0.5) were harvested, washed with 50 mM potassium phosphate buffer, pH 7.0, and resuspended in the same buffer supplemented with Triton X-100 at 0.05%. The OD_{600} of 300 μl-aliquots of the cell suspension was followed in the microtiter reader for 6 h at 30°C. Three biological replicates per strain were assayed. Cell suspensions without Triton X-100 were used as controls. The OD_{600} values were expressed as % of the initial OD_{600}. For comparison, values of $\%OD_{600}$ after 150 min of incubation were taken.

Survival to Heat and Acidic pH

Cells from overnight cultures were harvested by centrifugation (15,400 × *g*, 5 min). For the heat shock, cells from 200 μl-aliquots were washed with Ringer solution and diluted in 1 ml



B

L. lactis	Selected colonies from step 1 (*n*)[1]	Colonies able to grow in the presence of Lcn972 after step 2: 320 AU/ml	80 AU/ml
L81	43	8	
L62	30	5	
L98	16		16
IPLA517	16		16
IPLA641	16		14
IPLA729	16		14
IPLA1064	16		13

[1] Adaptation proceeded up to at 1,280 AU/ml of Lcn972 except for L98 that was stopped at 160 AU/ml

FIGURE 1 | Outline of the adaptive evolution experiment to isolate Lcn972R mutants. **(A)** During the adaptation step, *Lactococcus lactis* strains were subcultured in LM17 with increasing (twofold) Lcn972 concentrations. After this step, several colonies from the adapted cultures grown at the highest Lcn972 concentration were subcultured in the absence of Lcn972 (stabilization step). **(B)** Number of colonies subjected to the stabilization step and number of those which still retained resistance to Lcn972 after stabilization.

of the same solution (approximately 4–5 × 10^8 CFU/ml). Cell suspensions were divided into two 0.5 ml-aliquots, one was hold in a thermoblock (VWR international) at 50°C for 30 min and the other left at room temperature. All the tubes were transferred to ice and decimal dilutions immediately prepared in Ringer solution. CFU/ml counts were estimated after spotting (6×) 5 μl of each dilution on LM17 plates. The experiment was carried out with three biological replicates. For the acidic shock, cells from 1 ml were washed twice with 150 mM NaCl, and finally resuspended in 0.5 ml of 150 mM NaCl. Each cell suspension (100 μl) was diluted in 900 μl of 150 mM NaCl and in 150 mM NaCl adjusted to pH 2 with HCl. Samples were prepared in duplicate and incubated for 30 min at 30°C. After the incubation, the tubes were centrifuged and the pellet resuspended in 1 ml of phosphate buffered saline (PBS, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Decimal dilutions were prepared in PBS to neutralize the pH and CFU/ml calculated as described after the heat shock. Survival was determined as log(Nt/No) where Nt and No are CFU/ml of treated samples and non-treated controls, respectively. In both experiments, three independent cultures per strain were used.

Resistance to Antimicrobials

Decimal dilutions of exponentially growing cultures (OD_{600} = 0.3–0.5) were prepared in Ringer solution and spotted (5 μl) on LM17 plates supplemented with each antimicrobial at final concentrations of 1 μg/ml bacitracin, 0.1 mg/ml penicillin G, 0.4 μg/ml vancomycin and 0.5 mg/ml lysozyme from chicken egg white (all purchased from Sigma). LM17 plates supplemented with 5% NaCl were also prepared. Resistance to nisin (5 μg/ml) was also checked for all the starter strains and Lcn972R mutants but the nisin producers.

Plasmid Isolation and Plasmid Curation

Plasmids were isolated from overnight cultures according to O'sullivan and Klaenhammer (1993). For curing experiments, *L. lactis* IPLA517 was grown in LM17 at 37°C for 24 h in the presence of novobiocin at 2 μg/ml. These cultures were transferred daily to LM17 with increasing novobiocin concentrations (5, 10, and 15 μg/ml). Cultures grown at 15 μg/ml of novobiocin were spread on LM17 plates to isolate single colonies.

Identification of Non-synonymous Single Point Mutations

Draft genomes sequences were obtained at the sequencing facilities at Chr. Hansen. To perform full genome sequencing, DNA of the selected strains was isolated using the DNA DNeasy Blood and Tissue kit with the protocol for Gram-positive bacteria (Qiagen, Germany) and sequenced on the Illumina MiSeq platform with 2 × 250-bp paired-end sequencing (Illumina, United States). Sequencing reads were trimmed, analyzed, and

assembled using CLC Genomics Workbench 10.1.1 (Invitrogen). The assembled contigs were annotated by RASTtk (Brettin et al., 2015). Whole Genome Shotgun projects have been deposited at DDBJ/ENA/GenBank under the bioproject accession number PRJNA492214. Detection of single point mutations in the genomes of the Lcn972R mutants was performed with CLC Genomics Workbench 10.1.1 (Invitrogen). Variants located in mobile elements and prophages were disregarded. Moreover, BLASTN[1] was used to identify possible sequencing/assembling errors found in the wild type genome used as reference due to low coverage or low quality readings. Mutations found in plasmid-encoded genes which were also present in the chromosome (e.g., oligopeptide transport genes-*opp*) were not included in the analysis as the extra-copy may influence the outcome of the variant caller. Heatmap for representing the mutations found in the Lcn972R mutants was prepared with Heatmapper (Babicki et al., 2016). Hierarchical clustering was done by the average linkage method and distances were computed by the Spearman rank correlation. Functional analysis of the non-synonymous mutations detected in the Lcn972R strains was performed with GSEA_pro[2] as implemented in Genome2D[3]. The SIFT algorithm was used to predict if an amino acid substitution affects protein function[4].

Statistical Analysis

Results are reported as mean ± SD where appropriate. Differences between the wild type strains and their Lcn972R derivatives were assessed by one-tailed *t*-test as implemented in Microsoft Excel 2010 (2010 Microsoft Corporation). $p < 0.05$ was considered to be significant.

RESULTS

Adaptive Evolution Under Cell Envelope Stress (AE-CES) Applied to Industrial and Dairy Strains Selects for Stable *L. lactis* Mutants Resistant to Lcn972

To appreciate the value of AE-CES as means to evolve *L. lactis* and provide new phenotypes, this procedure was applied to seven wild type (WT) *L. lactis* subsp. *lactis* strains from different sources (**Table 1**). *L. lactis* L81 and L62 are used as acidifying strains in commercial starter blends. L98 is a nisin A producer, available as a bioprotective culture. The four nisin Z producers (IPLA517, IPLA641, IPLA729, and IPLA1064) were selected from the IPLA-CSIC laboratory collection. These strains were isolated from homemade raw milk cheeses made without starters in Northern Spain, and were chosen as representatives of each of the plasmid profiles found among the 23 isolates characterized by Martínez et al. (1995). They are genetically very closely related, according to their identical *Sma*I band pattern resolved by pulsed-field gel electrophoresis PFGE (our own unpublished results). Nonetheless, they were all subjected to AE-CES to determine if they generate similar outcomes.

The seven WT *L. lactis* strains were susceptible to Lcn972 with a MIC of 10 AU/ml and 40 AU/ml for L98 (**Table 1**). The AE-CES experiment involved two steps (see **Figure 1A**). In the first step, cultures were adapted to grow in increasing Lcn972 concentrations (adaptation step). All the cultures were able to grow up to 1,280 AU/ml (128× MIC) of Lcn972. The only exception was L98 that grew poorly at concentrations over 160 AU/ml (4× MIC). From each adapted culture, single colonies were picked and subjected to the stabilization step, consisting in ten serial transfers in LM17 without Lcn972. This step was carried out to enrich for the fittest clones. Then, their ability to grow in the presence of Lcn972 was tested Not all of them were able to grow (**Figure 1B**), suggesting that, in some cases, resistance to Lcn972 may be lost during the stabilization step. From two independent stabilized cultures per strain that retained the Lcn972R phenotype, a single colony was isolated, named after its parental strain, and stored for further characterization. Their Lcn972 MICs are shown in **Table 1**. MICs were increased from 4- to 32-fold. Most frequently, the MIC value was 160 AU/ml, while three mutants reached 320 AU/ml. It is important to note that the inoculum for the MIC determination differs from that used in the Lcn972 test. MIC plates were inoculated with approximately 1.5×10^5 CFU/ml from exponentially growing cultures, while the Lcn972 test was carried out with stationary phase cultures and inoculated with $3.0 - 6.0 \times 10^5$ CFU/ml. This difference in the inoculum may explain why Lcn972R mutants from *L. lactis* L81 and L62 had MICs below 320 AU/ml, the Lcn972 concentration used in the Lcn972 test. Moreover, the MICs were also below 1,280 AU/ml, the highest concentration used during adaptation. In this case, the transitory CesSR response must be activated during adaptation, which likely helps to cope with the stress and may allow growth at high Lcn972 concentrations.

In general, according to their growth rate in LM17 (**Table 1**), growth of the Lcn972R mutants was not hampered under laboratory conditions. *L. lactis* L81-D1 grew even faster than its parent (L81) ($p < 0.05$). On the contrary, *L. lactis* IPLA729-F9 and the two mutants from L98 were slower (**Table 1**). Furthermore, while the final OD_{600} in LM17 overnight cultures was similar to that of the parental strains (2.5–3.0) (data not shown), the mutants L98-C1 and L98-E2 reached a low OD_{600} compared to that of L98 (0.8 vs. 2.5, respectively). The bioprotective culture *L. lactis* L98 had a particular behavior. When streaked on LM17 plates, two colony phenotypes were always observed with an estimated frequency of 50%: a typical lactococcal white and smooth colony and a translucent and flat colony, which was later confirmed as lactose negative variants (**Supplementary Figure S1**). Adaptation was started with a lactose positive colony but it seems that during this step, only lactose negative variants were selected. In fact, lack of lactose fermentation and loss of a large plasmid band in L98-C1 and L98-E2 was confirmed by the absence of growth in BCP-lac (**Supplementary Figure S2C**) and plasmid isolation, respectively (**Supplementary Figure S2A**). Another mutant that lost one

[1] https://blast.ncbi.nlm.nih.gov/

[2] http://gseapro.molgenrug.nl/

[3] http://genome2d.molgenrug.nl/

[4] http://sift.bii.a-star.edu.sg/

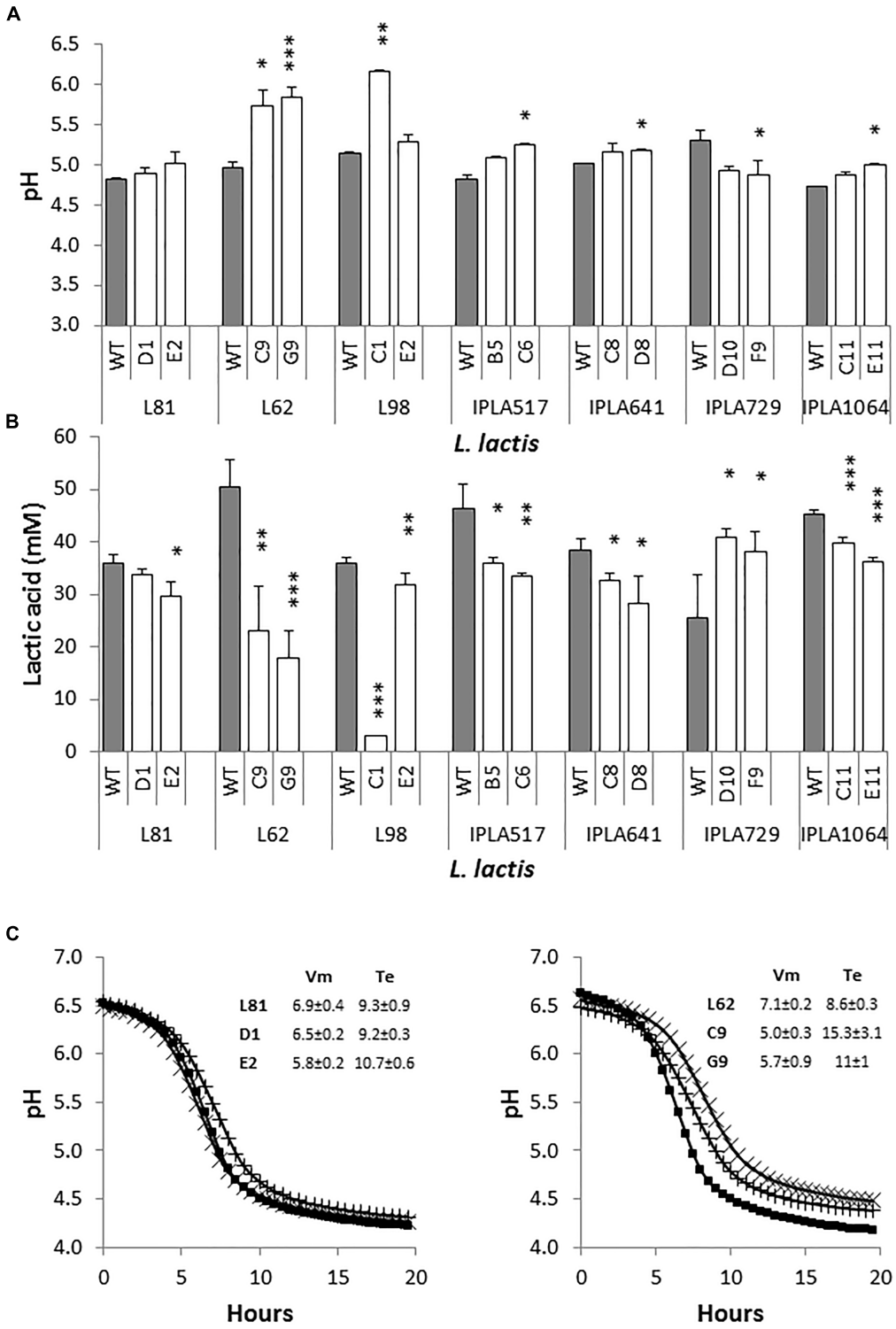


FIGURE 2 | Growth of *L. lactis* in pasteurized milk. pH **(A)** and production of lactic acid **(B)** of *L. lactis* wild-type (gray bars) and their Lcn972R mutants (white bars). Cultures were inoculated at 3% and incubated for 6 h at 30°C. **(C)** Acidification curves of *L. lactis* L81 (left panel) and *L. lactis* L62 (right panel) and their Lcn972R mutants in pasteurized milk. (■), wild type strains; (X), L81-D1 and L62-C9; (+), L81-E2 and L62-G9. The insert shows the acidification curve descriptors. Vm, maximum acidification rate (dpH/dt, mpH/min); Te, time to reach pH 4.6 (h). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ significantly different to the wild type strain.

plasmid was L62-C9 (**Supplementary Figure S2**) but in this case, lactose fermentation was not impaired (data not shown).

Technological Aptitude of Lcn972R Mutants in Milk

The first step in the characterization of the Lcn972R mutants selected after AE-CES was to assess whether dairy technological traits were retained or not. Accordingly, their ability to grow in pasteurized milk was compared to that of their WT counterparts. Similarly, nisin production was examined as well as bacteriophage resistance.

Growth and Lactic Acid Production in Milk

Starting with overnight cultures in LM17, pasteurized milk was inoculated at 3% (v/v) and pH as well as the production of lactic acid of milk cultures was measured after 6 h of incubation at 30°C (**Figure 2**). All strains acidified to a pH below a threshold of 5.3, regarded as a standard for a dairy starter under these experimental conditions (Cogan et al., 1997). The exception was the Lcn972R mutants of the starter *L. lactis* L62 and the lactose negative *L. lactis* L98-C1 which hardly acidified milk. In addition, the mutant L98-E2, that was shown to be also lactose negative in broth, lowered the pH as much as the WT *L. lactis* L98 ($p > 0.05$) (**Figure 2A**). This behavior may reflect the ability of this mutant to use other energy sources from milk.

Differences were observed in the production of lactic acid after 6 h of incubation (**Figure 2B**). In general, the amount of lactic acid generated by the Lcn972R mutants ranged from 70 to 90% of that produced by their parental strains with the exception of *L. lactis* IPLA729-D10 and F9 with higher levels compared to the WT IPLA729. *L. lactis* L62-C9 and L62-G9 produced less than 50% of the lactic acid levels of their WT strain. These results suggested a long lag phase in milk when these mutants were transferred from LM17 broth to milk and/or a slower acidification rate. To get a better picture of the acidification behavior of these mutants in milk, acidification was followed measuring the pH with a real-time pH meter for 20 h. As a reference, acidification curves for *L. lactis* L81 and its Lcn972R mutants were also performed (**Figure 2C**). In agreement with the pH and lactic acid concentration determined after 6 h of incubation (**Figures 2A,B**), *L. lactis* L81-D1 behaved as its WT, while L81-E2 was slightly slower ($p < 0.05$) in terms of maximum acidification rate (Vm) (**Figure 2C**). Similarly, the mutants L62-C9 and L62-G9 showed a slower maximum acidification rate ($p < 0.05$) and it took 7 and 2.5 h longer (Te), respectively, to reach pH 4.6 compared to *L. lactis* L62 (**Figure 2C**). Therefore, these two Lcn972R mutants had difficulties to grow in milk, were slower in lowering the pH and were not able to reach the same pH as *L. lactis* L62.

Nisin Production

All the nisin producers were able to synthesize nisin in LM17 broth (data not shown), but the production of nisin in milk was investigated as a key technological trait which should be retained by the evolved strains. As shown in **Figure 3**, no significant differences ($p > 0.05$) were observed with the Lcn972R mutants derived from the nisin Z producing strains isolated from raw milk cheeses. As expected, no detectable levels of nisin were measured in L98-C1 milk cultures, due to the absence of growth. On the contrary, the mutant L98-E2 did produce nisin, despite its lactose negative phenotype, although levels were slightly lower ($p < 0.05$) than the WT L98 (**Figure 3**).

Bacteriophage Resistance

Another technological trait of interest is bacteriophage resistance since bacteriophages are one of the leading causes of fermentation failures worldwide (Garneau and Moineau, 2011). Thus, the susceptibility of the Lcn972R mutants to phages infecting the WT strains was examined. Phages infecting the industrial strains L81, L62, and L98 still propagate and reach similar phage titers when plated on the Lcn972R mutants (data not shown). Since the phage susceptibility profile of the nisin Z producers was unknown, a growth inhibition assay was initially carried out to select phages able to infect the WT strains. *L. lactis* IPLA517, IPLA641, IPLA729, and IPLA1064 were screened against 62 lactococcal phages. Five phages, all belonging to the c2 family, strongly impaired acidification of all strains. Additionally, *L. lactis* IPLA729 was also inhibited by two extra c2 phages and *L. lactis* IPLA641 was partially inhibited by a 936 family phage and by an unclassified one (**Supplementary Table S1**). Two c2 phages, CHPC1130 and CHPC1183, were chosen to study further their infectivity on the Lcn972R mutants. Despite the effect observed in the growth inhibition assay, none or just a few lysis plaques were detected when plating undiluted lysates of CHPC1130 (1.5×10^{10} PFU/ml) and CHPC1183 (4.6×10^{9} PFU/ml) onto the WT strains IPLA517, IPLA641, and IPLA1064 (**Figure 4**). On the contrary, the phages did plaque on their Lcn972R mutants and lysis plaques were observed with titers up to 10^{7} PFU/ml (**Figure 4**). No changes in phage susceptibility were detected for *L. lactis* IPLA729 and its Lcn972R mutants.

We attempted to find a plausible explanation for the phage infectivity in the Lcn972R mutants. Since plasmid loss had been already observed for some Lcn972R mutants, we speculated that a putative plasmid-encoded phage resistance mechanism could have been lost during adaptation. To test this hypothesis, we attempted to compare the plasmid profiles of these strains and their Lcn972R mutants but it was not possible to clearly discriminate if any of the plasmids were lost (data not shown). As an alternative, we used novobiocin as a plasmid-curing agent to cure plasmids from *L. lactis* IPLA517. After several passages, ten single colonies were grown and tested against the phage CHPC1130. Lysis plaques were observed in all of them with titers even higher than those found on the Lcn972R mutant 517-B5 (**Figure 4C**). All the cured clones seem to have lost several large plasmids present in *L. lactis* IPLA517 (**Supplementary Figure S3**). Based on these results, a likely explanation for the phage susceptibility of the Lcn972R mutants is the loss of plasmid-encoded anti-phage mechanisms.

Surface Hydrophobicity and Autolytic Behavior of Lcn972R Mutants

A common theme in bacteriocin resistance is the existence of changes of the physicochemical properties of the bacterial surface

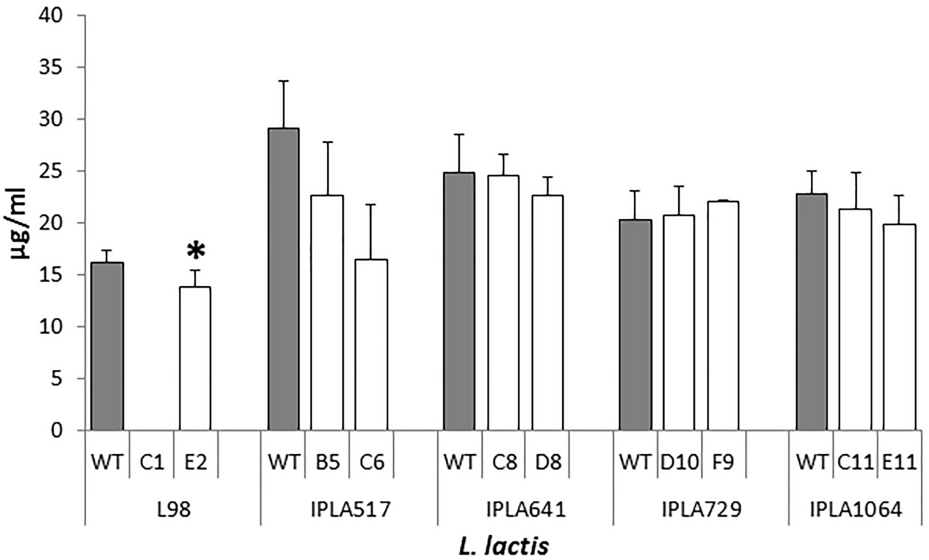


FIGURE 3 | Nisin production by *L. lactis* and their Lcn972R mutants (white bars) in pasteurized milk incubated at 30°C for 6 h. *, significantly different ($p < 0.05$) from the wild-type (gray bars).

to prevent or reduce binding of the antimicrobial peptide to the cell (Kramer et al., 2008; Bastos et al., 2015). Moreover, alterations on the bacterial surface may influence the activity of autolysins (Steen et al., 2005). In this context, we studied cell hydrophobicity and autolysis within the Lcn972R mutants. In these experiments, the starter *L. lactis* IPLA947 and its Lcn972 resistant derivative *L. lactis* R5 were also included.

Nine Lcn972R mutants changed their surface hydrophobicity with respect to the parental strains (**Figure 5A**). Within the group of nisin Z producers IPLA517, IPLA641, and IPLA1064 and the starter IPLA947, their Lcn972R mutants were more hydrophobic with values between 20 and 40%, as measured by their transfer to the hexadecane phase in the MATS test. A dramatic shift in surface hydrophobicity was noted for L62 whose Lcn972R mutants completely lost its hydrophobic character. Mutants from *L. lactis* L81, L98, and IPLA729 did not suffer any major changes in surface hydrophobicity.

As to their autolytic behavior, the Lcn972R mutants became less autolytic than their parental strains (**Figure 5B**) with two exceptions: *L. lactis* L81-E2 and *L. lactis* R5. In both Lcn972R mutants, down to 40% of the initial OD_{600} was recorded after 150 min in the presence of triton X-100 while 60% was recorded for the WT strains. The Lcn972R mutants from *L. lactis* L62 behave as the WT strain (**Figure 5B**). It is interesting to note that the two Lcn972R mutants from the same parental strain, e.g., *L. lactis* L81, behaved opposite to each other with regard to their autolytic activity (**Figure 5B**).

Cross-Resistance to Technological Stresses and Cell Wall Antimicrobials

Considering that Lcn972 inhibits cell wall biosynthesis and triggers the cell envelope stress response in *L. lactis*, it was anticipated that mutations leading to changes in cell wall structure or composition could have been selected for during adaptation. Because the cell wall is crucial for survival (Chapot-Chartier and Kulakauskas, 2014), we presumed that these possible alterations could make Lcn972R mutants to withstand better other stresses such as heat, low pH, and high osmotic pressure, all of them technological stresses encountered during cheese production. Besides, cross-resistance to other antimicrobials acting at the cell wall might arise, as previously observed with the laboratory strain *L. lactis* MG1614 that became resistant to lysozyme and nisin (Roces et al., 2012a). Therefore, the following experiments were designed to compare the response of the Lcn972R mutants to their parental strains.

Cross-Resistance to Heat and Low pH

A representative set of Lcn972 mutants, including those from the industrial *L. lactis* L81, L62, and L98, two nisin Z producers IPLA517 and IPLA641, and the dairy starter IPLA947 were exposed to 50°C and the viability was determined after 30 min (**Figure 6A**). Out of the eleven Lcn972R mutants, one, *L. lactis* R5, was more resistant to heat shock and three equally resistant to their wild types (L81-D1, IPLA517-B5, and IPLA517-C6). All the others became sensitized and lost around 1 log unit more than their wild types (**Figure 6A**). Once again, the two Lcn972R mutants from L81 and L62 behaved opposite to each other. On the contrary, none of the Lcn972R mutants became more sensitive to low pH. For all the strains tested, exposure to pH 2 led to a 4–4.5 log units decrease in viability (**Figure 6B**) and no significant differences ($p > 0.05$) were observed for any of the Lcn972R mutants and their parental strains.

Cross-Resistance to NaCl and Cell Wall Antimicrobials

Cross resistance was qualitatively evaluated by spotting decimal dilutions of exponentially growing cells on LM17 plates,

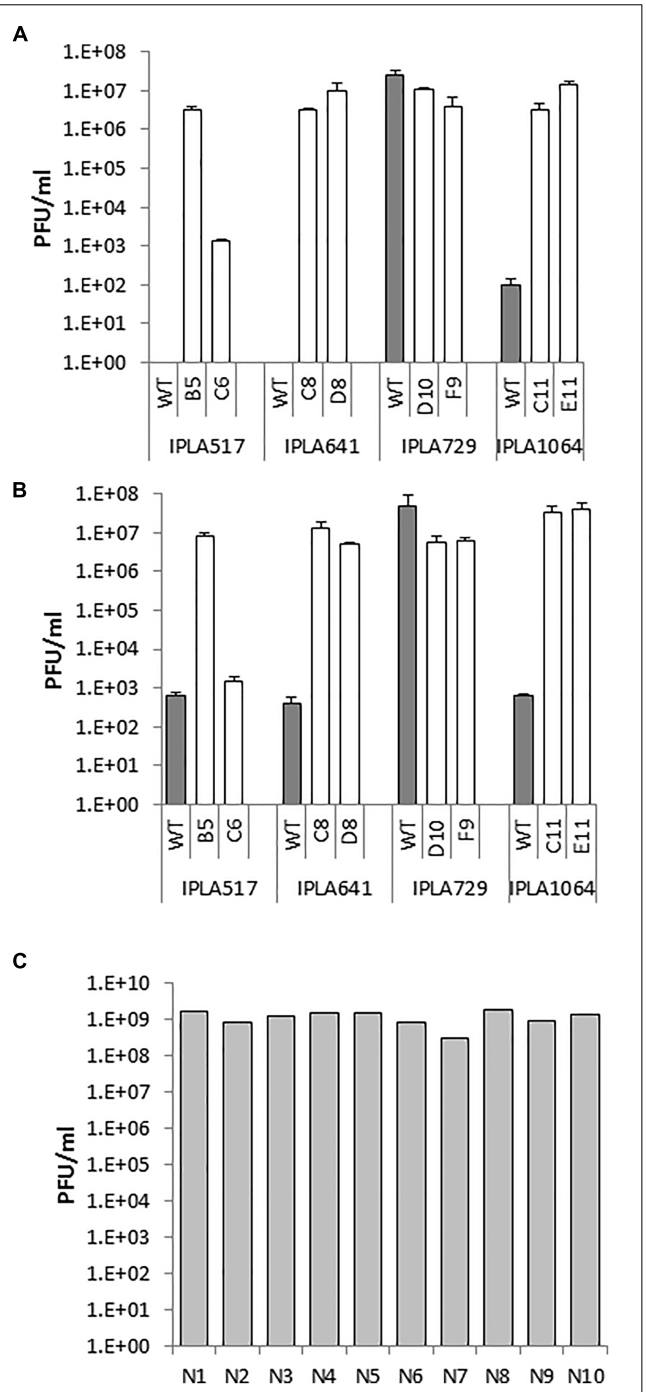


FIGURE 4 | Phage susceptibility of *L. lactis* IPLA517, IPLA641, IPLA729, and IPLA1064 (gray bars) and their Lcn972R mutants (white bars). Phage titer was determined by the double layer assay. **(A)** Phage CHPC1130 (1.5×10^{10} PFU/ml). **(B)** Phage CHPC1180 (4.6×10^{9} PFU/ml). **(C)** titers of phage CHPC1130 on novobiocin plasmid-cured derivatives (N1–N10) of *L. lactis* IPLA517.

supplemented with bacitracin, lysozyme, penicillin G and vancomycin at the concentrations indicated in **Table 2**. Growth under hyperosmotic conditions was assessed on LM17 plus NaCl at 5% (0.86 M). Resistance to nisin was only examined for non-nisin producers. As a general trend, half of the Lcn972R mutants became sensitive to penicillin G (60%) and to NaCl (46%). On the contrary, most of them (73%) were more resistant to lysozyme and to vancomycin (50%), while cross-resistance to bacitracin was observed in four cases only. Among the non-nisin producers, three out of five Lcn972R mutants showed cross-resistance to nisin. Taken as a whole, Lcn972R mutants from the same strain did not phenocopy each other and only those derived from *L. lactis* L98 and IPLA641 exhibited exactly the same phenotypes (**Table 2**).

Overview of Non-synonymous Mutations Within Lcn972R Mutants

To gain a preliminary insight into the genetic diversity introduced during evolution in the presence of Lcn972 and identify single mutations (and possible compensatory-mutations) selected during the adaptation-stabilization steps, draft genome sequences of the Lcn972R mutants from *L. lactis* L81, L62, L98, IPLA517, IPLA641, IPLA 1064 as well as the Lcn972R mutant from IPL947, *L. lactis* R5, were analyzed (**Figure 7** and (**Supplementary Table S2**).

Two to seven non-synonymous mutations were detected in the Lcn972R mutants within a total of 39 genes. Among them, 23 were predicted to affect protein function according to the SIFT algorithm or a frameshift and/or a stop codon was introduced (**Supplementary Table S2**). Noticeably, mutants clustered together according to the parental strain. Lcn972R *L. lactis* from the protective culture L98 and R5 displayed an independent set of mutated genes, while nisin Z producers clustered together and apart from the commercial starters *L. lactis* L81 and L62 (**Figure 7A**). Taken together, these results reinforce the notion that AE-CES may lead to different outputs depending of the genetic background of the parental strain.

Six main COGs categories were overrepresented in the gene set were non-synonymous mutations were detected (**Figure 7B**). Not surprisingly, some mutations were found in genes involved in cell envelope biogenesis (COG M) such as *murF* (D-Ala-D-Ala adding enzyme), *tagD1* (glycerol-3-phosphate cytidylyltransferase) and *pbp1* (penicillin binding protein 1A/1B) detected in IPLA517-C6, L98-C1 and E2, and L81-E2, respectively. Two genes *dltB* and *dltC* encoding functions involved in D-alanylation of the lipoteichoic acid (LTA) were also mutated in *L. lactis* IPLA1064-C11 and E11 and IPLA517-C6, respectively. Mutations in the genes *rgpCD*, coding for a putative ATP binding cassette (ABC) transporter likely involved in the synthesis of the polysaccharide pellicle (PSP), were also detected in the two Lcn972R mutants from *L. lactis* IPLA517.

Mutations in signal transduction mechanisms and transcriptional regulators were also found in several strains, anticipating a complex scenario at the transcriptional, and thus, phenotypic level. Namely, the two component system TCS-G accumulated mutations in the six Lcn972R mutants derived from the nisin Z producers. Other four mutants from the starters *L. lactis* L81 and L62 revealed mutations in *ysaBC* coding for a BceAB-like ABC transporter adjacent to TCS-G. Therefore,

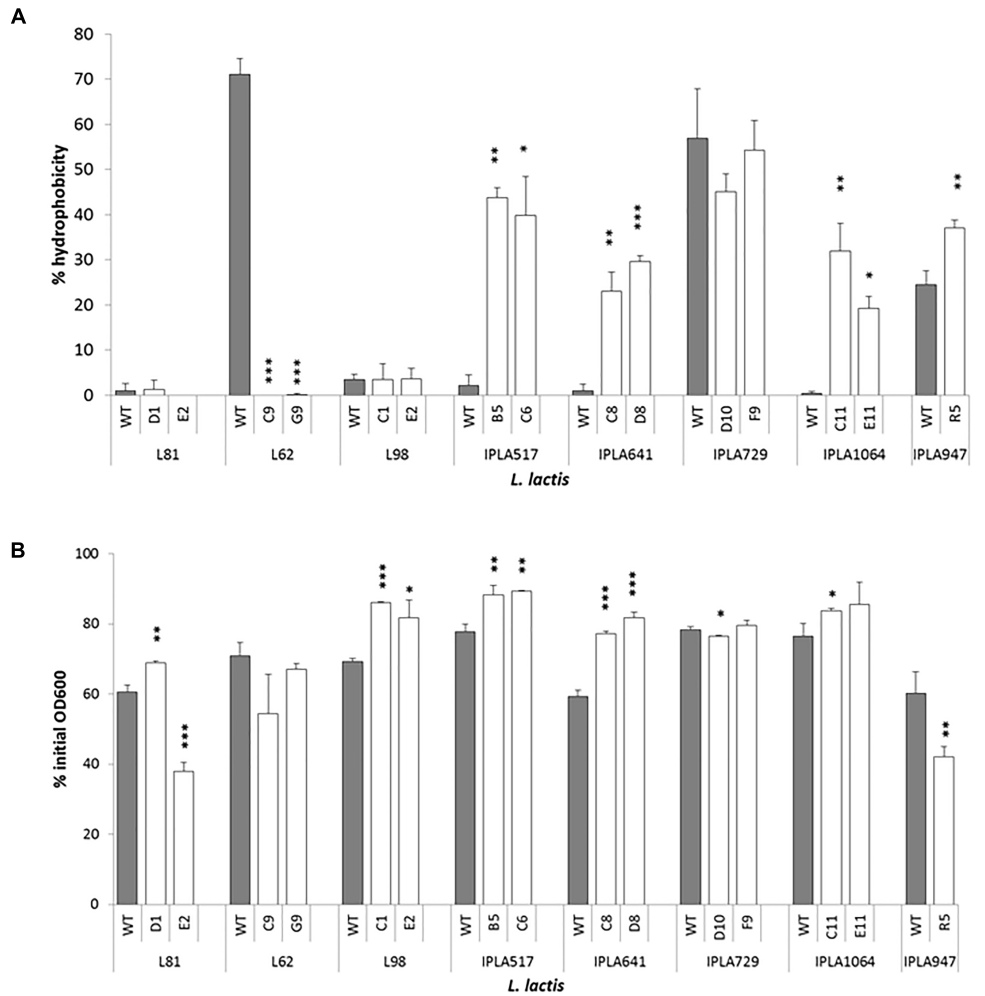


FIGURE 5 | Surface hydrophobicity **(A)** and autolysis **(B)** of *L. lactis* strains and their Lcn972R mutants. For autolysis, the OD_{600} was measured after 150 min of incubation of cell suspensions at 30°C. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ significantly different to the wild type strain.

the most frequent mutations among Lcn972R mutants were found in the BceAB-like TCS module which in a model organism such as *Bacillus subtilis* is involved in sensing and resistance to antimicrobial peptides (Ohki et al., 2003). Mutations in other stress-related genes were also found in *ftsH*, coding for the membrane protease FtsH involved in protein quality control and regulatory functions, and in *relA*, encoding the alarmone ppGpp synthetase. Nucleotide metabolism appeared to be also affected in some Lcn972R mutants. The uridylate kinase gene involved in pyrimidine metabolism (*pyrH*) was mutated in all the Lcn972R mutants from IPLA517, IPLA641, and IPLA1064 as well as the purine operon regulator *purR* in *L.lactis* L98-C1.

DISCUSSION

Lactic acid bacteria bacteriocins have been traditionally recognized as potent antimicrobials to be employed in food biopreservation. However, narrow-spectrum bacteriocins targeting beneficial bacteria (e.g., the dairy starter *L. lactis*) also have a niche of application in food biotechnology. For example, premature lysis of starter cells induced by the anti-lactococcal bacteriocins lactococcin A, B, and M facilitated the release of intracellular enzymes and accelerated cheese ripening (Morgan et al., 1997). In this work, we have made use of the narrow spectrum bacteriocin Lcn972 in evolution experiments with the aim of introducing diversity within *L. lactis* industrial starters. Our hypothesis was supported by our previous results on the characterization of Lcn972R mutants from the laboratory strain *L. lactis* MG1614 and the starter *L. lactis* IPLA947 but, in a way, this work was also inspired by the wealth of knowledge gained in the antibiotic field that clearly verified how low-level drug exposure generates genetic and phenotypic variability within pathogenic bacteria (Andersson and Hughes, 2014). On the other hand, evolution experiments have already been undertaken in *L. lactis* (see Bachmann et al., 2017 and references therein). These studies have demonstrated the genomic plasticity of *L. lactis* to fix beneficial mutations when exposed to particular conditions.

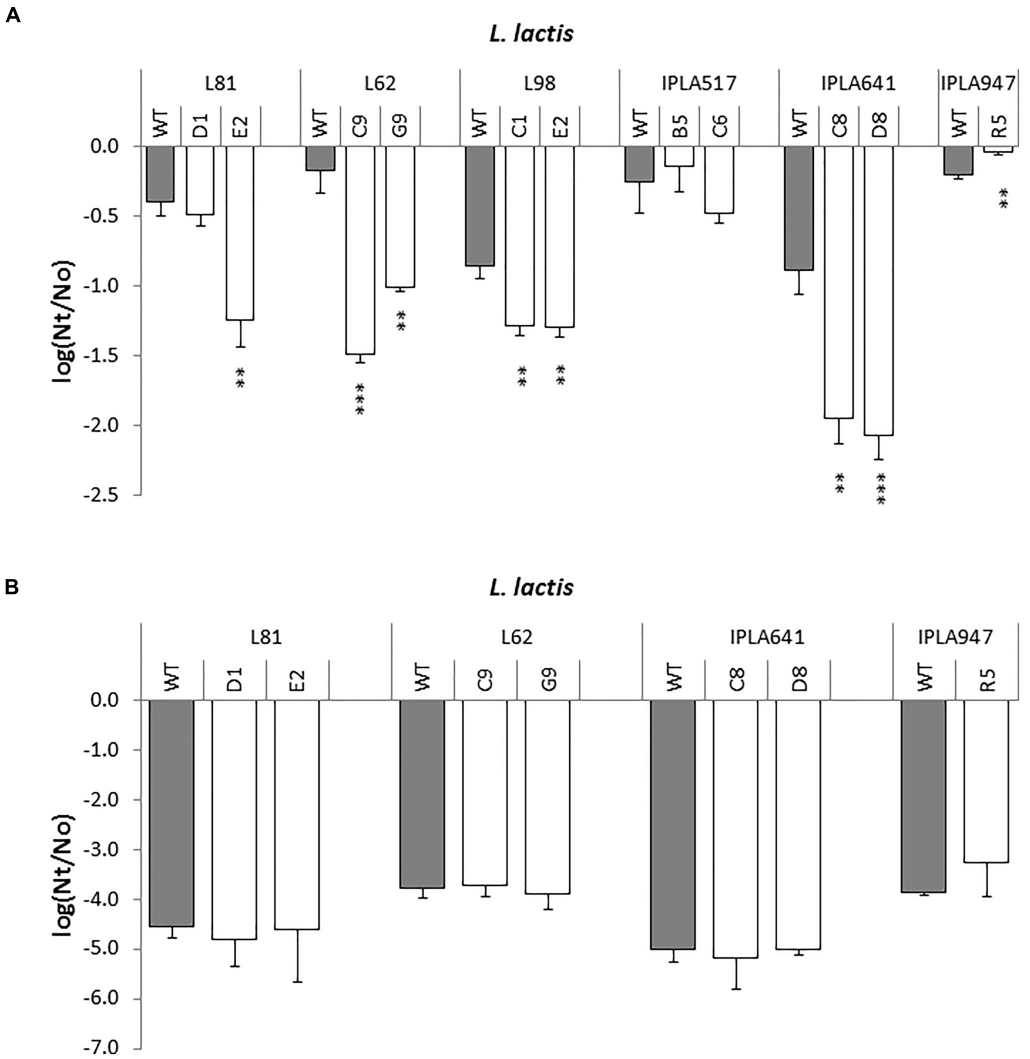


FIGURE 6 | Survival of *L. lactis* (gray bars) and their Lcn972R mutants (white bars) after exposure to heat **(A)** and low pH **(B)**. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different to the wild type strain.

However, most of these studies have been carried out as proof of concept studies, using laboratory strains that differ greatly from industrial or natural *L. lactis* strains (Cavanagh et al., 2015; Kelleher et al., 2017).

On this basis, we have applied AE-CES to seven *L. lactis* strains, including commercial and isolates from raw milk cheeses, to gather information about the influence of the strain background. It was possible to select Lcn972R mutants in all cases, supporting the idea that using Lcn972 in AE-CES experiments could be extended to virtually all Lcn972-sensitive *lactococci*. Moreover, in contrast to nisin resistant *Lactococcus*, that required the presence of nisin to retain their resistant phenotype (Kramer et al., 2006), Lcn972R mutants were stable, growth under laboratory conditions was not impaired and their resistance was not lost upon subcultivation. Two factors might have contributed to their stability. One is a possible high mutation rate in cells exposed to Lcn972. Lcn972 inhibits cell wall biosynthesis during cell division without pore formation (Martínez et al., 2008). So, cells are not instantly killed, increasing the chance to select mutations. Besides, it is known for several antibiotics, including cell wall antibiotics that mutation frequency increases when the SOS response is activated (Gutierrez et al., 2013). Although it has not been experimentally confirmed, Lcn972 seems to trigger the SOS response, as evidenced by the activation of prophages after Lcn972 treatment (Madera et al., 2009). A second factor that may have influenced the selection for stable mutants is the stabilization step after Lcn972 adaptation. Competition between clonal variants may have helped fixing beneficial compensatory mutations and the selection for fitter variants.

Aside from the physiological implications of using Lcn972 as a stressor in AE-CES, a key question was if this strategy would be compatible with the functionality of the dairy starters. The first issue we addressed was the technological

TABLE 2 | Cross-resistance to cell wall antimicrobials and NaCl of *L. lactis* mutants resistant to Lcn972.

L. lactis	Bacitracin[1] (1 μg/ml)	Lysozyme[1] (0.5 mg/ml)	Penicillin G[1] (0.1 μg/ml)	Vancomycin[1,2] (0.4 μg/ml)	Nisin[1,2] (5 μg/ml)	NaCl[1] (5%)
L81-D1	S	R	S	E	S	E
L81-E2	R	R	S	E	E	E
L62-C9	R	R	S	R	R	E
L62-G9	R	R	S	R	R	S
L98-C1	E	E	E	S	NA	E
L98-E2	E	E	E	S	NA	E
IPLA517-B5	E	R	S	E	NA	S
IPLA517-C6	E	R	S	R	NA	S
IPLA641-C8	S	R	S	R	NA	S
IPLA641-D8	S	R	S	R	NA	S
IPLA729-D10	S	R	E	E	NA	E
IPLA729-F9	E	R	E	ND	NA	S
IPLA1064-C11	S	R	S	R	NA	E
IPLA1064-E11	E	E	E	R	NA	E
IPLA947-R5	R	E	E	ND	R	S

[1]*S, E and R stand for more susceptible, equal and more resistant than the wild type counterpart. Susceptibility/resistance was determined by spotting decimal dilutions of cell suspensions on LM17 plates supplemented with the indicated concentrations.*
[2]*NA, not applicable; ND, not determined.*

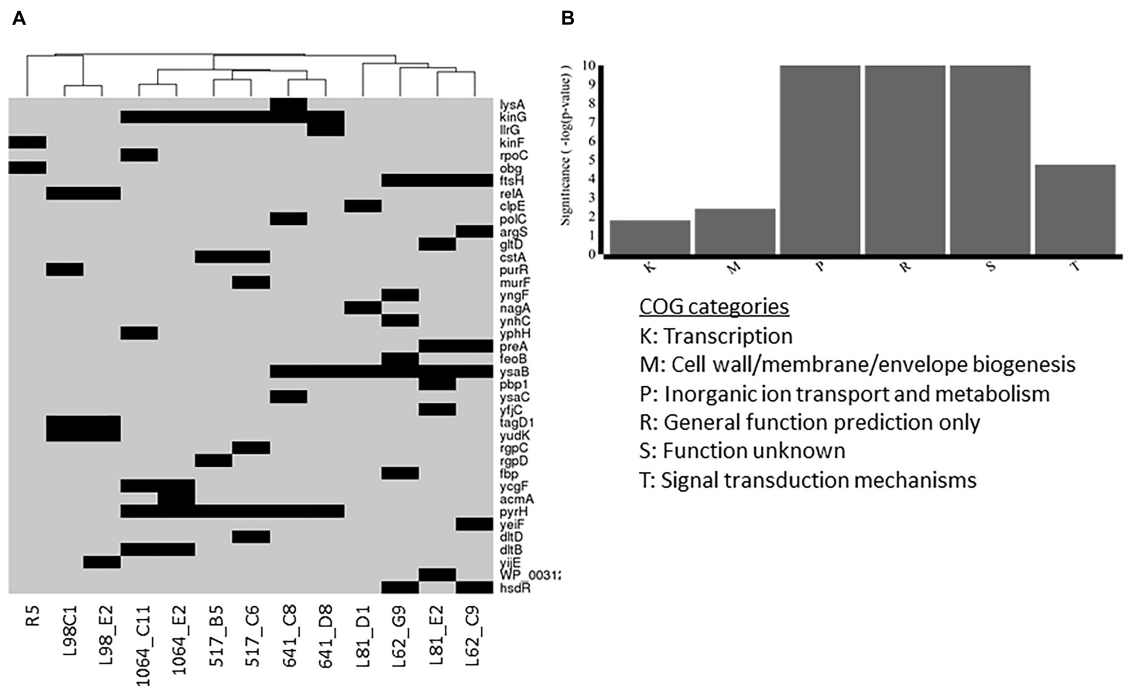


FIGURE 7 | Non-synonymous mutations present in Lcn972R *L. lactis* mutants **(A)** and functional categories overrepresented within the mutated genes **(B)**. Presence of mutations in **(A)** is represented by a black rectangle.

characterization of the Lcn972R mutants. In general, milk acidification was within the standards for dairy starters (Cogan et al., 1997) and, half of the mutants retained the same acidification rate as their corresponding WT. On the other hand, lactic acid levels were in most cases lower, suggesting a shift from homolactic to a more mixed-acid fermentation as a possible consequence of the adaptation to Lcn972. Whether this shift results in higher acetaldehyde or diacetyl levels or not remains to be investigated. In addition, nisin production levels were not altered with the exception of the lactose negative mutants L98-C1 and E2. Taken together, the results showed that evolved mutants that retained the wild type technological phenotypes may be recovered after AE-CES.

However, a major disadvantage encountered during AE-CES was the loss of plasmids in some strains. The plasmid complement of *Lactococci* is large and several relevant technological traits are plasmid-encoded, from lactose fermentation, proteolytic activity to bacteriophage resistance mechanisms (Ainsworth et al., 2014b). Two examples of the negative consequences of plasmid loss have been observed in this work. One was the loss of the lactose plasmid in the Lcn972R mutants from *L. lactis* L98 that occurred in spite of the presence of lactose as a carbon source during AE-CES. In the wild type *L. lactis* L98, the lactose plasmid appears to be intrinsically unstable, as described for other lactose plasmids such as pLP712 (Wegmann et al., 2012), and only lactose negative variants were selected during adaptation. The other example was the loss of a plasmid (or plasmids) encoding phage resistance mechanisms that occurred in *L. lactis* IPLA517 and, most likely, in the other nisin Z producing strains IPLA641 and IPLA1064. Presence of plasmid-encoded anti-phage mechanisms in the wild type strains is supported by the isolation of phage sensitive clones after plasmid curing using novobiocin, although chromosomal re-organization events might have occurred as well. Two scenarios may take place. A putative plasmid-encoded exopolysaccharide (EPS) could shield the phage receptor, as described for the lactococcal plasmid pCI658 that encodes the production of an EPS protecting *Lactococcus* from infection by phages phi712 and c2 (Forde and Fitzgerald, 2003). In our case, differences in the expression level of the EPS genes in LM17 and in milk may explain why no lytic plaques were observed when phages were plated on the WT strains, despite the inhibition observed in the growth inhibition test. Alternatively, loss of genes coding for abortive infection mechanisms (Abi) may also explain why Lcn972R mutants became phage sensitive and the lack of lysis plaques on otherwise "phage sensitive" *L. lactis*. Abi systems are altruistic phage resistant mechanisms, whereby the phage cycle is stopped and infected cells die before the phage ends its intracellular cycle (Labrie et al., 2010). Thus, progeny phages are not released, i.e., lysis plaques are not observed, and milk acidification is slowed down due to cell death. So far, searching draft genomes for putative Abi systems retrieved no relevant hits. On the contrary, a putative EPS gene cluster flanked by insertion sequences seems to be present in *L. lactis* IPLA517, IPLA641, and IPLA1064 and absent in their Lcn972R mutants and in the phage sensitive *L. lactis* IPLA729. Unfortunately, the identified contigs basically contain genes involved in EPS synthesis and the absence of any plasmid-related sequences next to the EPS genes precludes us from confirming the plasmidic nature of the EPS operon. Nevertheless, reads matching plasmid replication genes were also missing in the genomes of the Lcn972R mutants, further confirming plasmid loss in the mutants as already observed in plasmid preparations. Based on these examples, and considering that both lactose fermentation and phage resistance are key technological dairy traits, caution must be taken when applying AE-CES depending on the strains and their plasmid complement.

Further phenotypic characterization of the Lcn972R mutants, complemented by the preliminary overview of non-synonymous mutations, supports one of the outcomes of this study. That is both the inter- and intra-strain variability observed among the evolved mutants. In other words, the results emphasize the different solutions found by *L. lactis* to overcome the stress imposed by Lcn972. In spite of the relative low number of Lcn972R mutants characterized per strain (n = 2), different phenotypes were observed. This is the case, for instance, of the two *L. lactis* L81 Lcn972R mutants that behaved opposite to each other regarding autolysis and heat susceptibility. Moreover, as discussed below, the phenotypes selected after AE-CES also differed from those described for the laboratory strain *L. lactis* MG1614 (Roces et al., 2012a,b).

Changes that happened at the cell surface are exemplified by alterations on surface hydrophobicity and the autolytic behavior of the Lcn972R. Seven mutants increased their surface hydrophobicity, whereas L62-C9 and G9 lost completely the hydrophobic character of their WT. Interesting, the phage sensitive Lcn972R mutants derived from IPLA517, IPLA641, and IPLA1064 became hydrophobic, likely due to the loss of a putative hydrophilic EPS involved in phage resistance (see above). Autolysis was more uniform within Lcn972R mutants and, in general, evolved strains were less autolytic than the WT reference. These new surface properties may have a technological impact as well. It has been recently shown that the properties of *L. lactis* cell surface affects textural parameters of fermented milk (Tarazanova et al., 2018). Moreover, autolytic *L. lactis* may influence the development of cheese flavor (Lortal and Chapot-Chartier, 2005), opening an avenue for new applications of some of the evolved starters such as *L. lactis* L81-E2.

Considering that the cell wall is crucial for survival, one of our initial premises based on the mode of action of Lcn972 was that mutations leading to resistance to Lcn972 could have positive consequences on the survival to other stresses encountered during cheese manufacture. For example, extensive remodeling of the cell wall by increasing D-Asp amidation, *O*-acetylation, and *N*-deacetylation of the peptidoglycan promoted acid resistance in *L. lactis* (Hao et al., 2017; Cao et al., 2018). However, cross-protection to low pH was not observed within Lcn972R mutants. Furthermore, resistance to Lcn972 seems to be linked to a higher susceptibility to heat and NaCl (see **Figure 6** and **Table 2**). Therefore, AE-CES using Lcn972 does not necessarily evolve robust *L. lactis*, at least, for this group of strains and the studied phenotypes. In contrast, cross-resistance to other cell wall antimicrobials was found. Increased lysozyme tolerance and cross-protection to other bacteriocins appears to be a common theme among bacteriocin resistance mutants (see Bastos et al., 2015 and references therein), regardless the mode of action, i.e., pore-forming or cell wall inhibiting bacteriocins. Likewise, sensitivity to β-lactam antibiotics is often observed (Guinane et al., 2006; Roces et al., 2012a) and our results are in line with these reports.

The preliminary overview on the non-synonymous mutations detected in the Lcn972R mutants revealed, first of all, that selected mutations seem to be dictated by the background of the parental strain. Yet, distinct intra-strain mutation profiles were observed in agreement with the notion that "in an evolutionary trajectory, multiple solutions may lead to a fitness

increase" (Bachmann et al., 2017). These diversity may have been introduced in both adaptation and stabilization steps.

Mutations were preferentially found in genes involved in cell envelope biogenesis, active transport and regulatory functions. It should be noted that mutations that are predicted to affect protein function do not necessarily mean loss of function. Thus, the interpretation of the impact of a given mutation should be taken cautiously at this stage. With this in mind, it is fair to assume that the mutations found in the BceAB-like *ysaCB*-TCS-G module, the most frequent within Lcn972R mutants, may lead to activation of this detoxification module rather than to its inactivation and, thereby, provide resistance against bacitracin, vancomycin and Lcn972 as well. Mutations in the ABC permease component in *Streptococcus pneumoniae* led to increased transcription of the ABC transporter genes and resistance to vancoresmycin (Becker et al., 2009). Further studies are in progress to confirm if the same applies to our Lcn972R mutants.

Another frequent mutation was found in *pyrH*, encoding the UMP kinase involved in the last steps of the synthesis of RNA precursors. This mutation may have direct consequences on the architecture of the cell wall and turn cells resistant to cell wall antimicrobials as lysozyme and Lcn972. Recently, mutations in *pyrB*, coding for the aspartate carbamoyltransferase in pyrimidine metabolism, were frequently found in *L. lactis* resistance to lysozyme (Solopova et al., 2016). The authors claimed that limiting the availability of L-Asp for nucleotide biosynthesis results in a highly cross-linked and rigid peptidoglycan that interferes with lysozyme activity. Other mutations that may have an impact on surface properties are those found in the mutants from IPLA517 in the genes *rgpCD* located in the cell wall polysaccharide gene cluster. These genes putatively encoded the ABC transporter of the polysaccharide precursors and, when non-functional, the amount of the polysaccharide pellicle may be reduced, increasing surface hydrophobicity. This cell wall polysaccharide is a well-stablished receptor for several *L. lactis* phages of the 936 and P335 groups (Ainsworth et al., 2014a). Unfortunately, none of the phages able to infect the WT *L. lactis* IPLA517 belong to any of these phage groups and it could not be established if these mutations may render IPLA517 less prone to phage attack. Finally, absence of growth in milk of *L. lactis* L98-C1 could be linked to the mutation in *purR*. The inactivation of this transcriptional activator would potentially render the mutant unable of *de novo* synthesis of purines required for growth in milk, a substrate low in purine content (Kilstrup and Martinussen, 1998). Interestingly, mutations connected to purine nucleotide metabolism are often behind multi-stress resistance in *L. lactis* (Ryssel et al., 2014) which may explain the selection of such mutations during AE-CES.

CONCLUSION

In this work we have identified advantages and disadvantages of applying AE-CES in *L. lactis* and the consequences for its performance as a dairy starter. AE-CES appears as a feasible strategy to introduce phenotypic and genetic diversity in *L. lactis*, regardless of the strain origin. Evolved strains may retain similar technological traits as the strains they are derived from, while acquiring new ones. Plasmid loss was one of the main disadvantages that might be overcome by combining AE-CES with conditions for selection of the plasmid of interest or by screening a larger number of evolved clones. Although still preliminary, data gathered from draft genomes anticipates the likely selection for mutations activating detoxification modules and changes at the cell surface which may have practical implications in milk fermentations. The phenotypic and genetic characterization of the evolved strains has also emphasized the plasticity of *L. lactis* to give rise to new phenotypes, providing the versatility required for adaptive evolution to become an excellent tool in strain development programs.

DATA AVAILABILITY

Datasets are available on request. The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

ML-G ran the evolution experiments. ML-G, TJ, and BM performed the phenotypic characterization experiments. SE, TJ, and BM analyzed mutations. AR, AN, TJ, and BM conceived and designed the study. All authors were involved in analysis of the results and drafting the manuscript prior to submission. Tasks were supervised by AR and BM at IPLA-CSIC and by AN at Chr. Hansen A/S.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest Statement: AN and TJ are employees of Chr. Hansen A/S.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Capítulo III.- Mutaciones seleccionadas tras la exposición a la bacteriocina Lcn972 activan módulos de resistencia a antibióticos en *Lactococcus lactis*

El análisis genómico preliminar de los diferentes mutantes de *L. lactis* Lcn972R obtenidos mediante los experimentos de EA-CES descritos en esta Tesis Doctoral (capítulo II) mostró que muchos de ellos presentan mutaciones puntuales en un supuesto módulo de detección y detoxificación denominado YsaDCB-TCS-G de tipo Bce. Estos módulos están formados por un transportador tipo ABC (YsaDCB) asociado a un sistema de dos componentes (TCS-G) y, en general, son mecanismos de resistencia muy eficaces frente a péptidos antimicrobianos, incluyendo antibióticos de naturaleza peptídica como la bacitracina, péptidos de nuestro sistema inmunitario como las defensinas, o bacteriocinas como la nisina o la lactococina 972 (Lcn972).

En este capítulo se ha estudiado la función de este módulo de detección y detoxificación y el papel de las mutaciones en el gen *ysaB*, que codifica la permeasa del transportador YsaDCB, detectadas en tres mutantes de *L. lactis* Lcn972R, dos de los cuales eran además resistentes a bacitracina. En primer lugar, el análisis transcripcional mediante PCR cuantitativa demostró que los genes que codifican el transportador en los 3 mutantes estaban sobreexpresados. Posteriormente, estos genes se clonaron y expresaron en la cepa *L. lactis* NZ9000, que carece del transportador YsaDCB, lo que permitió confirmar su papel en la resistencia tanto a la bacitracina como a la Lcn972. El análisis de las versiones mutadas del gen *ysaB* permitió determinar que las mutaciones implicaban la activación permanente del sistema de dos componentes TCS-G y que la protección frente a la bacitracina y a Lcn972 ocurre, probablemente, a través de mecanismos diferentes.

En conclusión, el transportador ABC YsaDCB junto con el TCS TCS-G forman un módulo de detección y detoxificación que protege a *L. lactis* frente a la bacitracina y la Lcn972 y mutaciones en el gen de la permeasa provocan su activación.

Artículo 3.- Campelo, A.B., López-González, M.J., Escobedo, S., Janzen, T., Neves, A.R., Rodríguez, A. and Martínez, B. 2020. Bacteriocin-induced mutations activate antibiotic resistance modules in *Lactococcus lactis*. Frontiers in Microbiology. DOI: 10.3389/fmicb.2020.01805.

Contribución personal al trabajo

- Experimentación relacionada con el estudio de la resistencia a antimicrobianos.
- Análisis de resultados junto con el resto de co-autores.
- Elaboración de tablas y figuras junto con el resto de los co-autores.

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Mutations Selected After Exposure to Bacteriocin Lcn972 Activate a Bce-Like Bacitracin Resistance Module in *Lactococcus lactis*

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Resistance against antimicrobial peptides (AMPs) is often mediated by detoxification modules that rely on sensing the AMP through a BceAB-like ATP-binding cassette (ABC) transporter that subsequently activates a cognate two-component system (TCS) to mount the cell response. Here, the *Lactococcus lactis* ABC transporter YsaDCB is shown to constitute, together with TCS-G, a detoxification module that protects *L. lactis* against bacitracin and the bacteriocin Lcn972, both AMPs that inhibit cell wall biosynthesis. Initially, increased expression of *ysaDCB* was detected by RT-qPCR in three *L. lactis* resistant to Lcn972, two of which were also resistant to bacitracin. These mutants shared, among others, single-point mutations in *ysaB* coding for the putative Bce-like permease. These results led us to investigate the function of YsaDCB ABC-transporter and study the impact of these mutations. Expression *in trans* of *ysaDCB* in *L. lactis* NZ9000, a strain that lacks a functional detoxification module, enhanced resistance to both AMPs, demonstrating its role as a resistance factor in *L. lactis*. When the three different *ysaB* alleles from the mutants were expressed, all of them outperformed the wild-type transporter in resistance against Lcn972 but not against bacitracin, suggesting a distinct mode of protection against each AMP. Moreover, P_{ysaD} promoter fusions, designed to measure the activation of the detoxification module, revealed that the *ysaB* mutations unlock transcriptional control by TCS-G, resulting in constitutive expression of the *ysaDCB* operon. Finally, deletion of *ysaD* was also performed to get an insight into the function of this gene. *ysaD* encodes a secreted peptide and is part of the *ysaDCB* operon. YsaD appears to modulate signal relay between the ABC transporter and TCS-G, based on the different response of the P_{ysaD} promoter fusions when it is not present. Altogether, the results underscore the unique features of this lactococcal detoxification module that warrant further research to advance in our overall understanding of these important resistance factors in bacteria.

Keywords: cell wall, bacteriocin, antimicrobial peptides, ABC transporter, resistance, *Lactococcus lactis*

INTRODUCTION

ATP-binding cassette (ABC) transporters are bacterial efflux pumps that mediate transport across the membrane at the expenses of the energy liberated by the hydrolysis of ATP (Du et al., 2018). They are constituted by a transmembrane domain (TMD) with a substrate-binding pocket and the nucleotide-binding domain (NBD) that alternate between two structural conformations, inward-open and outward-open, to translocate substrates. ABC transporters are functionally diverse and participate in bacterial virulence, quorum sensing, nutrient uptake, or export of toxic molecules. In particular, ABC transporters are regarded as the fastest-acting and very effective resistance mechanisms to many antimicrobials (Du et al., 2018). Moreover, their role as sensors and information processors in signal relay is being increasingly recognized (Piepenbreier et al., 2017).

In Firmicutes, some ABC transporters confer resistance to antimicrobial peptides (AMPs) including peptide antibiotics (e.g., bacitracin), host antimicrobial peptides (e.g., LL-37 and hBD3 defensins), and bacteriocins (e.g., nisin). Based on their domain architecture and phylogenetic relationships, five groups have been established (Gebhard, 2012). Two of them are required for the export of newly synthesized AMPs (SunT and NisT type) and three are involved in resistance (LanFEG, BceAB, and BcrAB type). BceAB-type transporters are characterized by the NBD protein BceA and the permease BceB with 10 transmembrane helices (TMH) and a large extracellular loop (EC_L) between TMH-VII and VIII. Many BceAB-type transporters are genetically and functionally linked to two component systems (TCS), involved in signal transduction and gene regulation. Actually, the Bce-type permeases and the histidine kinases of the cognate TCS have coevolved toward functional AMP detoxification modules (Dintner et al., 2011). Moreover, the histidine kinases of these TCSs are intramembrane-sensing kinases that completely rely on the ABC transporter to sense the AMP (Bernard et al., 2007). One of the best-characterized detoxification modules is BceRS-BceAB involved in resistance of *Bacillus subtilis* to bacitracin. The BceAB transporter actively participates in bacitracin resistance, likely by freeing its target from the antibiotic grip (Kobras et al., 2020), and interacts directly with the histidine kinase BceS to form a sensory complex that controls the expression of *bceAB* (Dintner et al., 2014). This detoxification module responds to a novel signaling mechanism, so-called flux sensing, whereby monitoring the activity of the individual ABC transporters is the cue to activating the cognate TCS and triggering the cell response (Fritz et al., 2015).

The role of BceAB-type transporters in sensing, together with their role in detoxification, has prompted their classification into three main functional groups proposed by Revilla-Guarinos et al. (2014): (i) those with a dual function involved in sensing and resistance (e.g., BceAB, ABC09 of *Lactobacillus casei*); (ii) sensing transporters that trigger the response but are unable to confer resistance on their own (e.g., VraFG that activates the GraXSR TCS in *Staphylococcus aureus*); and (iii) standalone detoxification pumps which are often regulated by a nongenetically linked TCS (e.g., VraDEH of *S. aureus*).

Additional or accessory components within the ABC/TCS resistance modules may also be present and are thought to be involved in fine tuning both signaling and transport activities. The cytosolic protein GraX from the TCS GraXRS of *S. aureus* has been shown to interact with the histidine kinase GraS, and it is required for GraR-gene activation (Falord et al., 2012). Likewise, *vraH*, located downstream in the *vraDEH* operon, encodes a small membrane protein, which is required for high-resistance to daptomycin and gallidermin (Popella et al., 2016). Other protecting mechanisms may also cluster with ABC/TCS detoxification modules. In *Streptococcus agalactiae*, the module NsrFP/NsrKR involved in nisin resistance is complemented with the nisin resistance protein Nsr, a nisin-degrading protease (Khosa et al., 2013; Reiners et al., 2017).

In a previous work, we have carried out adaptive evolution experiments with several *Lactococcus lactis* strains, which are used worldwide as starters in milk fermentation (López-González et al., 2018). These strains were grown under the selective pressure exerted by the bacteriocin Lcn972, a class IId bacteriocin that binds to lipid II and inhibits cell wall biosynthesis, triggering the cell envelope stress response in *Lactococcus* (Martínez et al., 2007, 2008). The preliminary insight into the genome of these mutants resistant to Lcn972 revealed that, among others, non-synonymous mutations often occurred in a putative Bce-like detoxification module, comprised by an ABC transporter (YsaDCB) and an adjacent TCS (TCS-G) (López-González et al., 2018). Precisely, three of these mutants shared mutations in *ysaB*, coding for a BceB-like permease and two of them were also resistant to bacitracin. These observations prompted us to hypothesize that the YsaDCB ABC transporter might be part of a Bce-like resistance module in *L. lactis*. Therefore, we set out to determine the role of YsaDCB in resistance to bacitracin and Lcn972 and to investigate the consequences of the *ysaB* mutations. The results show that *L. lactis* YsaDCB is involved in both sensing and resistance to AMPs and that those mutations in *ysaB* may impair either function. Moreover, YsaDCB is shown to protect against bacitracin and Lcn972 by a distinct mode of action. Finally, the secreted peptide YsaD is proposed to modulate signal transduction between the ABC transporter and the histidine kinase of TCS-G.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

L. lactis strains used in this work and their main characteristics are listed in **Table 1**. The wild-type (WT) *L. lactis* L81 and L62 and their Lcn972R mutants L81-D1, L81-E2, and L62-G9 were grown at 30°C in M17 with lactose at 0.5% (LM17). For the plasmid-free laboratory strains *L. lactis* IL1403 and *L. lactis* NZ9000, glucose at 0.5% was used (GM17). Antibiotics chloramphenicol (Cm), erythromycin (Em), and tetracycline (Tet) were added at 5 μg/ml depending on the plasmid

TABLE 1 | Bacterial strains and plasmids used in this study.

Strain	Properties[a]	Reference
L. lactis* subsp. *lactis		
L81	WT strain, commercial starter culture	López-González et al. (2018)
L81-D1	Lcn972R from L81, *ysaB* mutation $F_{577}S$, $P_{605}T$	
L81-E2	Lcn972R from L81, *ysaB* mutation $F_{577}V$	
L62	WT strain, commercial starter culture	
L62-G9	Lcn972R from L62, *ysaB* mutation $I_{594}F$	
IL1403	Laboratory strain, plasmid free	Bolotin et al. (2001)
L. lactis* subsp. *cremoris		
NZ9000	Host for nisin-inducible gene expression; *ysaB* pseudogene	Kuipers et al. (1998)
Plasmid		
pUK200	Nisin-inducible expression vector. Cm^R	Wegmann et al. (1999)
pRCR	Promoter-probe vector, *mrfp* (mCherry). Cm^R	Mohedano et al. (2015)
pPTPL	Promoter-probe vector, *lacZ*. Tet^R	Burgess et al. (2004)
pILG	Based on pIL252. Em^R	Campelo et al. (2010)
pDCB_n	*ysaDCB* genes in pUK200, "n" stands for strain origin	This work
pCB_n	*ysaCB* genes in pUK200, "n" stands for strain origin	This work
pP_{ysaD}::*lacZ*	$ysaD_{IL}$ promoter fused to *lacZ* in pPTPL	This work
pRCR_P_{ysaD}::*mrfp*	$ysaD_{IL}$ promoter fused to *mrfp* in pRCR	This work
pIL_P_{ysaD}::*mrfp*	$ysaD_{IL}$ promoter fused to *mrfp* in pILG	This work

[a]*WT, wild-type; Cm, chloramphenicol; Tet, tetracycline; Em, erythromycin. L81-D1, L81-E2, and L62-G9 carry several mutations (López-González et al., 2018), but only those in ysaB are specified.*

(**Table 1**) and the same concentrations were used when two plasmids were present in the same lactococcal cell.

Antimicrobial Susceptibility Tests

Minimum inhibitory concentrations (MICs) were determined by the broth dilution method in microtiter plates as previously described (López-González et al., 2018). Two-fold dilutions of the antimicrobials to be tested (bacitracin, nisin, and vancomycin) were done in GM17 or LM17 broth depending on the lactococcal strain. Plates were inoculated with exponentially growing cells adjusted to an optical density at 600 nm (OD_{600}) of 0.05, and then further diluted 1/100. Susceptibility of the *L. lactis* NZ9000 clones carrying the expression plasmids pDCB_n and pCB_n (**Table 1**) was scored according to the inhibitory concentration able to inhibit growth by 50% (IC_{50}). IC_{50} was determined essentially as described by Reiners et al. (2017). IC_{50} plates were inoculated with overnight cultures adjusted to an OD_{600} of 0.1. For gene expression, 1 ng/ml of nisin was routinely added to both pre-cultures and IC_{50} determinations.

RNA Extraction and Analysis

Three independent cultures of *L. lactis* L81, *L. lactis* L62, and their Lcn972R mutants (**Table 1**) were grown on LM17 at 30°C until they reached an OD_{600} of 1.0, when RNAprotect Bacteria Reagent (Qiagen, Germany) was added. Total RNA was extracted using the Illustra RNAspin Mini Kit (GE Healthcare, UK) and treated with Turbo DNAse (Ambion) and SUPERase RNase Inhibitor (Ambion). RNA quality was checked by agarose gel electrophoresis, and its concentration was determined by absorbance at 260 nm in an Epoch microplate spectrophotometer (BioTek). One microgram of each RNA sample was used to generate cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA).

RT-qPCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Warrington, UK). Primers used for RT-qPCR are listed in **Supplementary Table 1** and were supplied by Macrogen (Korea). Amplification was carried out in 25 µl containing 2.5 µl of a 1:25 dilution of cDNA, 1× Power SYBR Green (Applied Biosystems), and each primer at a concentration of 0.2 µM. Each cDNA amplification was repeated in duplicate. After incubation at 95°C for 10 min, amplification proceeded with 40 cycles of 95°C for 15 s and 60°C for 1 min. Fold changes were calculated following the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and the reference gene was the elongation factor Tu *tuf*.

PCR reactions to identify transcription units were performed in a MyCycler Thermal Cycler (Bio-Rad Laboratories). Primers are listed in **Supplementary Table 1**. PCR reactions were carried out in a final volume of 25 µl containing 1 µl of cDNA, 1× Taq DNA Polymerase Master Mix Red (Ampliqon, Denmark) and each primer at a concentration of 0.2 µM. PCR amplification conditions were as follows: 1 cycle at 95°C for 4 min followed by 30 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s and a final cycle at 72°C for 7 min. PCR controls were carried with either RNA or genomic DNA. PCR products were analyzed by 2% agarose gel electrophoresis.

pDCB and pCB Expression Plasmids

All the primers required for plasmid construction are listed in **Supplementary Table 1**. Restriction enzymes and T4 ligase were supplied by Eurofins Genomics (Germany) and Fisher Scientific (Spain), respectively, and used according to the manufacturer's instructions. Commercial kits were used for purification of chromosomal DNA (GenElute Bacterial Genomic DNA Kit, Sigma, Spain), plasmids (High Pure Plasmid Isolation Kit, Roche, Germany), and DNA fragments using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK). The expression plasmids were based on pUK200, placing the *ysaDCB* and *ysaCB* genes under the control of the inducible nisin promoter to yield the plasmids pDCB_n and pCB_n (**Table 1**), tagged after the source of the *ysa* genes as pDCB_IL (IL1403), pDCB_L81, pDCB_D1, pDCB_E2, pDCB_G9, pCB_IL, and pCB_L81. The genes were amplified by PCR with Pwo SuperYield polymerase (Roche, Spain) using as template genomic DNA from *L. lactis* strains IL1403, L81, D1, E2, and G9 and annealing T of 58°C. PCR products

were digested with RcaI and BamHI in the case of the pDCB_n plasmids and with NcoI and BamHI for the pCB_n plasmids. After digestion, the DNA fragments were ligated into pUK200 cut with NcoI and BamHI. The resulting plasmids were established into *L. lactis* NZ9000 by electroporation and checked by DNA sequencing (Macrogen, Spain) to confirm proper cloning.

P_{ysaD} Reporter Plasmids

The β-galactosidase reporter plasmid pP_{ysaD}::*lacZ* was based on pPTPL (**Table 1**). The promoter P_{ysaD} was amplified by PCR using Pwo SuperYield polymerase (Roche, Spain) and the primers described in **Supplementary Table 1**. As template, genomic DNA from *L. lactis* IL1403 was used since the upstream *ysaD* DNA sequence was identical in *L. lactis* IL1403, L81, and L62. The PCR product and the vector were digested by EcoRI and XbaI, ligated, and electroporated into *L. lactis* NZ9000. Two fluorescent reporter plasmids harboring the *mrfp* gene coding for mCherry were constructed (**Table 1**). pRCR_P_{ysaD}::*mrfp* was based on pRCR (**Table 1**). The PCR-amplified P_{ysaD} promoter was digested with XbaI only to leave the 5' end blunt and ligated into SmaI-XbaI-digested pRCR. *Escherichia coli* DH10B (Invitrogen) was used as an intermediate host for cloning. The plasmid was introduced into *L. lactis* strains IL1403 and NZ9000 and the lactose positive *L. lactis* L81, L81-D1, and L81-E2 by electroporation. The BglII-SacI DNA fragment encompassing the P_{ysaD}::*mrpf* fusion from pRCR_P_{ysaD}::*mrfp* was excised and cloned into pILG, digested with the same restriction enzymes to yield pIL_P_{ysaD}::*mrfp* (**Table 1**). This plasmid carries an erythromycin resistance marker, which made possible to maintain the reporter plasmid together with pDCB_n and pCB_n expression plasmids (Cm^R) in the same cell. Correct cloning was confirmed by DNA sequencing (Macrogen, Spain).

Activity of the P_{ysaD} Promoter With the Reporter Plasmid pP_{ysaD}::*lacZ*

Fresh overnight cultures of *L. lactis* NZ9000 clones carrying the different expression plasmids pDCB_n and pCB_n and the reporter plasmid pP_{ysaD}::*lacZ* were diluted to OD_{600} of 0.05 in GM17/Cm/Tet and nisin at 1 ng/ml to ensure expression of the *ysa* genes. When the clones reached an OD_{600} of 0.2–0.3 (approximately 3 h at 30°C), 2 ml samples were taken and bacitracin was added at 5 and 1 μg/ml and incubation proceeded for 30 min. Cultures without bacitracin were taken as baseline controls. Cells were collected by centrifugation, and the β-galactosidase reaction was carried out at 30°C for 10 min as described elsewhere (Martínez et al., 2007). β-Galactosidase activity assays were carried out, at least, with three independent cultures.

Activity of the P_{ysaD} Promoter With the Reporter Plasmid pIL_P_{ysaD}::*mrfp*

L. lactis NZ9000 pDCB_n and pCB_n and the reporter plasmid pIL_P_{ysaD}::*mrfp* were inoculated in GM17/Cm/Em and 1 ng/ml nisin and grown as described for pP_{ysaD}::*LacZ*. After induction with bacitracin (1 and 5 μg/ml) or Lcn972 (80, 40, and 20 AU/ml), cells (1 ml) were washed with saline phosphate buffer (PBS), pH 7.3, and collected in half of the initial volume. Cell suspensions were kept in the dark for 3 h for mCherry maturation (Garay-Novillo et al., 2019). Fluorescence (F) of 20-μl aliquots was quantified in a 7500 Fast Real-Time PCR System (Applied Biosystems, Warrington, UK) using the built-in presence/absence protocol for detecting ROX, a fluorophore with similar excitation (Ex. 580 nm) and emission (Em. 605 nm) wavelengths as mCherry (Ex. 587 nm, Em. 612 nm). After blank correction (PBS background), raw fluorescence data was normalized by dividing F by the OD_{600} of the cell suspensions (200 μl) measured in a Benchmark Plus microplate spectrophotometer (Bio-Rad).

Statistical Analysis

When indicated, differences were assessed by one-tailed *t*-test as implemented in Microsoft Excel 2010 (2010 Microsoft Corporation) and $p < 0.05$ was considered to be significant.

RESULTS

Resistance of Lcn972R *L. lactis* Mutants to AMPs Other Than Lcn972

In our previous work, three Lcn972 resistant mutants (Lcn972R) L81-D1, L81-E2, and L62-G9 (**Table 1**) were isolated after continuous exposure of the wild-type (WT) strains *L. lactis* subsp. *lactis* L81 and *L. lactis* subsp. *lactis* L62 to subinhibitory concentrations of the cell wall-active bacteriocin Lcn972. The preliminary phenotypic characterization already suggested that the two mutants L81-E2 and L62-G9 displayed cross-resistance to bacitracin, while L81-D1 was more susceptible than its parent *L. lactis* L81 (López-González et al., 2018). Standard MIC determinations confirmed that bacitracin MICs for L81-E2 and L62-G9 increased by 8- and 4-fold, respectively, whereas the MIC for *L. lactis* L81-D1 was reduced 2-fold (**Table 2**). MICs of other antimicrobials such as nisin and vancomycin also increased 2- or 4-fold for some Lcn972R mutants (**Table 2**) in line with our previous results (López-González et al., 2018).

TABLE 2 | Minimum inhibitory concentrations (MICs) of several antimicrobial peptides for *L. lactis* strains.

L. lactis	MIC			
	Lcn972 (AU/ml)[a]	Bacitracin (μg/ml)	Vancomycin (μg/ml)	Nisin (μg/ml)[b]
L81 (WT)	10	8	0.5	1
L81-D1	80	4	0.5	2
L81-E2	320	64	0.5	4
L62 (WT)	10	8	0.5	2
L62-G9	80	32	1	4
IL1403	40	0.125	0.5	ND
NZ9000	20	0.5	0.5	ND

[a]*Taken from López-González et al. (2018).*
[b]*ND, not determined.*

Presence of a BceAB-Like Detoxification Module in *L. lactis*

Three Lcn972R mutants L81-D1, L81-E2, and L62-G9 carried, among others, non-synonymous mutations in *ysaB*, a gene coding for the permease of a putative ABC transporter (López-González et al., 2018). Inspection of the genome of the WT strains L81 and L62 (European Nucleotide Archive under accession number ENA: PRJNA 492214) and the laboratory strain *L. lactis* subsp. *lactis* IL1403 (GenBank AE005176.1) revealed that *ysaB* is flanked by *ysaC* and genes encoding the TCS-G (**Figure 1A**). YsaC contains the ATP-binding domain and YsaB shares the features of BceB-like permeases with 10 transmembrane helices (TMHs) and an extracellular loop (233 aa), according to TMpred[1] and Psort[2] predictions. All the *ysaB* mutations detected in the Lcn972R strains mapped in the C-terminus. Three mutations ($F_{577}V$, $F_{577}S$, and $I_{594}F$) were found in the predicted inside loop between TMH VIII and IX. The additional mutation $P_{605}T$ detected in *L. lactis* L81-D1 was positioned within TMH IX. The genes encoding the TCS-G with the response regulator LlrG and the intramembrane histidine kinase KinG are located downstream of *ysaB* (**Figure 1A**). This genetic arrangement is similar to that found for other BceAB-like ABC transporters which are genetically linked to regulatory elements such as TCSs and involved in resistance to AMPs (Dintner et al., 2011).

Additional genes (*ysaD* and *ysaA*) were also identified (**Figure 1A**). Homology and conserved domain searches identified YsaD (119 aa) as a member of TIGR01655 (uncharacterized protein YxeA), a family of small uncharacterized secreted proteins which are found exclusively in Gram-positive bacteria. YsaA (354 aa) is predicted to be an integral membrane protein with nine TMHs. It contains a VanZ domain present in glycopeptide antibiotic resistance proteins (pfam04892), denoting a possible role in resistance to AMPs.

The module YsaDCB/TCS-G is present in 41 out of the 192 complete *L. lactis* genomes deposited at NCBI (as of June 2020) according to BLASTN[3] searches, and it seems highly conserved (over 80% identity at the nucleotide level) within the two *L. lactis* subspecies *cremoris* and *lactis*. It is worth mentioning that, in the laboratory workhorse strain *L. lactis* MG1363 and its derivative *L. lactis* NZ9000, the ABC transporter appears to be nonfunctional due to a stop codon at position 310 that created a truncated YsaB permease of 103 residues. Otherwise, its TCS-G is complete. A detailed comparison at the protein level and accession numbers is included in **Supplementary Table 2**.

The Putative Detoxification Module YsaDCB/TCS-G in *L. lactis* Is Transcribed in Two Polycistronic mRNAs

The genes of the putative Ysa-TCS-G module are transcribed in two polycistronic mRNAs as determined by RT-PCR using primers annealing within the gene ends (**Figure 1B**).

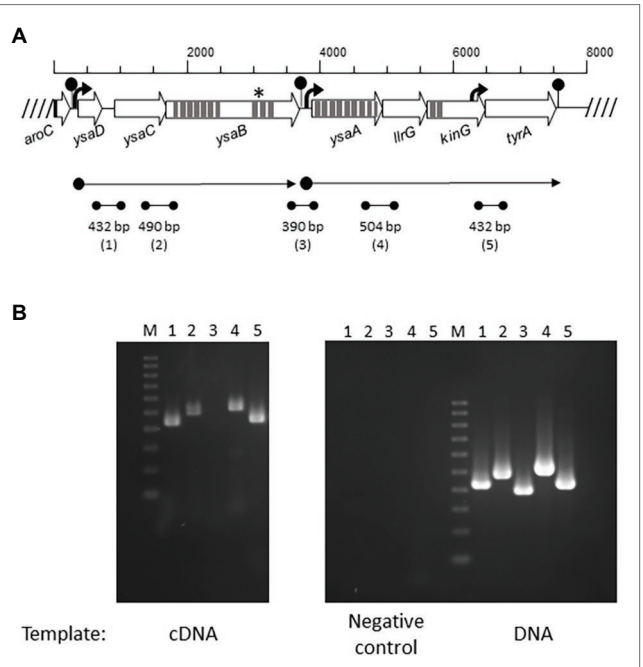

FIGURE 1 | Operon organization of the Ysa TCS-G module in *Lactococcus lactis*. **(A)** Overview of the *ysa* and flanking genes (open arrows). Promoters are depicted as black arrows and terminators as lollypops. Predicted transmembrane helices are shown as dark gray strips. Localization of *ysaB* mutations is marked with an asterisk (*). The two predicted mRNAs are displayed by dot-end arrows. Intergenic regions (1–5) amplified by RT-PCR are shown by dot-end lines. **(B)** RT-PCR amplified bands (1–5) using as template cDNA, RNA (negative control), and genomic DNA (positive control) from *L. lactis* L81.

Based on the amplification results, two mRNAs *ysaDCB* and *ysaA-llrG-kinG-tyrA* are synthesized. Consistent with this, two putative promoters could be identified upstream of *ysaD* (P*ysaD*) and *ysaA* (P*ysaA*), as well as three rho-independent terminators downstream of *aroC*, *ysaB*, and *tyrA* (**Figure 1B**). A positive PCR amplification of the intergenic region *kinG-tyrA* was detected, which could be due to the presence of a putative promoter at the 3' end of *kinG*, upstream of the forward primer used in RT-PCR (**Figure 1B**). The flanking genes *tyrA* and *aroC* coding for prephenate dehydrogenase and chorismate synthase, respectively, are involved in the biosynthesis of aromatic amino acids. Hence, it is unlikely that they participate in the transport and/or sensing functions of the YsaDCB/TCS-G module.

ysaDCB but Not TCS-G Genes Are Highly Expressed in Lcn972R *L. lactis* Mutants

Bearing in mind the role of BceAB-like transporters in resistance to AMPs, the expression levels of *ysaDCB*, *ysaA*, *llrG*, and *kinG* were determined in exponentially growing cultures of the *L. lactis* Lcn972R mutants by RT-qPCR. The expression of the TCS-G genes was not altered in any of the mutants. On the contrary, *ysaDCB* were upregulated in the Lcn972R mutants albeit to a different extent. Highest expression occurred in *L. lactis* L81-E2 (close to 20-fold), followed by *L. lactis*

[1]https://embnet.vital-it.ch/software/TMPRED_form.html
[2]https://www.psort.org/psortb/
[3]https://blast.ncbi.nlm.nih.gov/Blast.cgi

L62-G9 (up to 10-fold; **Figure 2**). In *L. lactis* L81-D1, which carries two *ysaB* mutations ($F_{577}S$ and $P_{605}T$) and is sensitive to bacitracin, the expression of *ysaDCB* was lower but still up to 5-fold compared to the WT *L. lactis* L81 (**Figure 2**). Closer inspection and resequencing of the PCR-amplified P_{ysaD} promoter in all these strains did not show any anomalies, which could explain the higher expression levels of the *ysaDCB* operon in the Lcn972R mutants.

Further confirmation of the increased expression of the *ysaDCB* genes in the Lcn972R *L. lactis* mutants was gathered by monitoring the functional expression of the fluorescent mCherry protein gene *mrfp* placed under the control of the P_{ysaD} promoter in the reporter plasmid pRCR_P_{ysaD}::*mrfp*. While hardly any fluorescence could be measured in both exponential and stationary phase cultures of *L. lactis* L81, fluorescence was 30-fold and 10-fold higher in *L. lactis* L81_E2 and *L. lactis* L81_D1 (**Supplementary Figure S1A**).

Of note, the expression levels of *ysaDCB* mirrored resistance to Lcn972 but not to bacitracin (**Table 2**). Despite their increased expression in *L. lactis* L81-D1, this mutant was more sensitive to bacitracin than the WT. Moreover, different susceptibility to bacitracin was also noticed within the laboratory strains *L. lactis* IL1403, with a complete YsaDCB transporter, and *L. lactis* NZ9000 that lacks a functional YsaB permease, being 4-fold more resistant to bacitracin than IL1403. These observations suggest that bacitracin resistance mechanisms other than YsaDCB are functional in *L. lactis*.

Mutated Versions of *ysaB* Provide Distinct Resistance Levels to Bacitracin and Lcn972

In order to confirm the suspected role of the YsaDCB transporter in bacitracin and Lcn972 resistance and assess the impact of the *ysaB* mutations, the WT and mutated *ysaB* genes were cloned under the inducible nisin promoter (plasmids pDCB_n) and expressed in the *L. lactis* NZ9000 background (*ysaB* deficient).

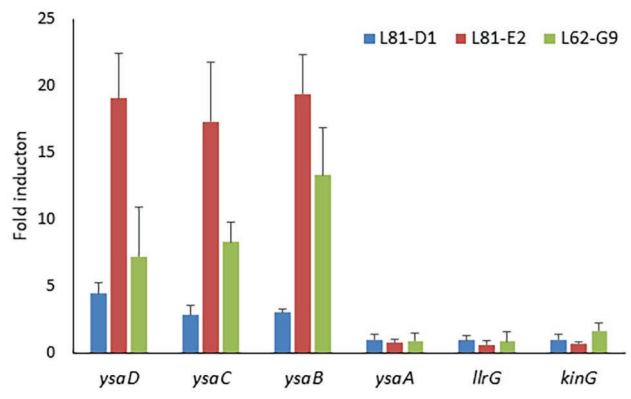

FIGURE 2 | Relative expression of Ysa and TCS-G genes in the *L. lactis* Lcn972R mutants determined by RT-qPCR. Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method using the WT strains *L. lactis* L81 (for L81-D1 and L81-E2) and *L. lactis* L62 for L62-G9 as reference. Average of three biological replicates and SD (error bars) are shown.

In this way, the level of protection provided by the ABC transporter itself and its mutated versions could be assessed and compared within the same genetic background, independently of their own regulation and the other mutations present in the Lcn972R mutants. The transporter genes of *L. lactis* IL1403 were also cloned (pDCB_IL) because they have been previously linked to nisin resistance (Kramer et al., 2006). Dose-response curves showed that expression of the WT genes *ysaDCB* of *L. lactis* L81 and *L. lactis* IL1403 conferred a 5-fold increase in the bacitracin and Lcn972 concentration required to inhibit growth by 50% (IC_{50}), as compared to the control with the empty vector pUK200 (**Figure 3**). Hence, the role of YsaDCB in resistance of *L. lactis* to both AMPs was confirmed.

In the case of the mutated alleles of *ysaB*, represented by the *ysaDCB* operons from L81-D1, L81-E2, and L62-G9 (the WT *ysaDCB* from *L. lactis* L62 is identical to L81), contribution to resistance varied depending on the AMP (**Figure 3**). In line with the MICs reported in **Table 2**, the double *ysaB* mutant transporter pDCB_D1 failed to protect against bacitracin as judged by the low IC_{50} values, similar to those with the control *L. lactis* pUK200. On the contrary, the transporters in pDCB_E2

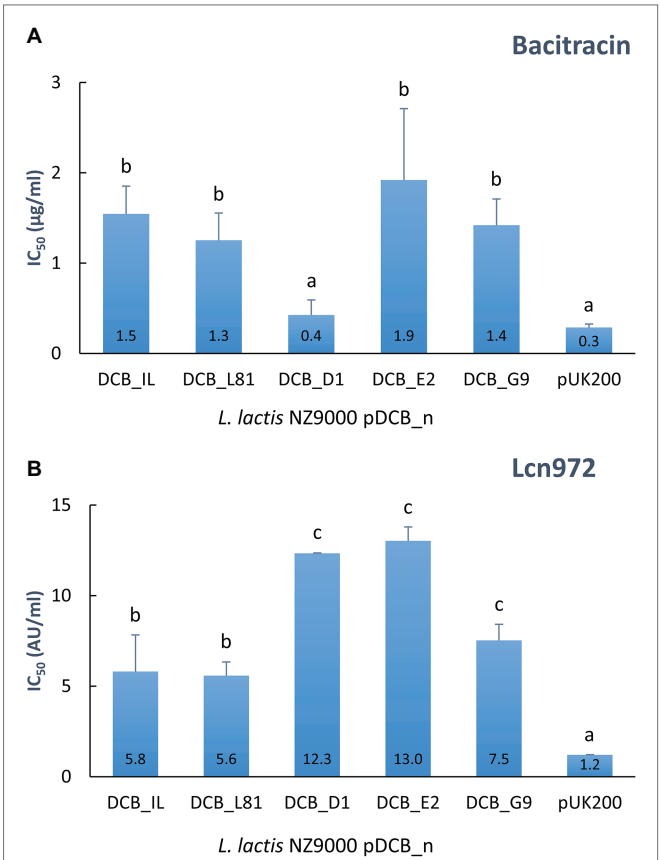

FIGURE 3 | Resistance to bacitracin **(A)** and Lcn972 **(B)** of *L. lactis* NZ9000 clones expressing the different versions of *ysaDCB* genes. Calculated IC_{50} values are displayed inside the bars. Average and SD from, at least, two independent IC_{50} determinations are shown. Values with different letters are significantly different ($p < 0.05$).

and pDCB_G9 provided similar levels of protection as the WT transporter. As for resistance to Lcn972, the scenario was different. Regardless of the *ysaB* allele, all the transporters boosted protection against Lcn972 over the WT genes ($p < 0.05$; **Figure 3** IC_{50}), with pDCB_G9 being less effective than pDCB_D1 and pDCB_E2 ($p < 0.05$). These results suggest that the mechanisms whereby YsaDCB protects *L. lactis* against bacitracin and Lcn972 might differ.

ysaB Mutations Trigger the P_{ysaD} Promoter in the Absence of Bacitracin

A mutagenesis study of the BceSR/BceAB resistance module has shown that single amino acid substitutions in the *B. subtilis* permease BceB may have different consequences in either signaling or resistance (Kallenberg et al., 2013). Moreover, the authors observed that those mutations that primarily affect the activity of the transporter tend to cluster in the C-terminal of the permease, which is the case with the *ysaB* mutations identified in the Lcn972R *L. lactis* mutants (see **Table 1**). Therefore, we next investigated if the mutations in *ysaB*, besides affecting resistance, could also alter onward signal transmission through the TCS-G and lead to induction of the *ysaDCB* operon upon detection of the stress. With this aim, bacitracin was chosen to measure P_{ysaD} promoter activity due to the different resistance levels provided by the *ysaB* alleles (see **Figure 3A**). We first confirmed that P_{ysaD} was silent (OFF-state) in *L. lactis* NZ9000 lacking *ysaB*, even in the presence of bacitracin. As shown in **Supplementary Figure S1B**, while mCherry fluorescence in *L. lactis* IL1403/pRCR_P_{ysaD}::*mrfp* increased proportionally to increasing bacitracin concentration, there was no response in *L. lactis* NZ9000/pRCR_P_{ysaD}::*mrfp*. Therefore, *L. lactis* NZ9000 was deemed as a suitable host to study the signaling ability of YsaDCB and the existence of any other mechanisms that could induce P_{ysaD} was ruled out.

To quantify the activity of the P_{ysaD} promoter in the *L. lactis* NZ9000 pDCB_n clones expressing the different *ysaB* alleles, the reporter plasmid pP_{ysaD}::*lacZ* was constructed in which a Tet resistance marker is present to help keeping both pDCB_n and reporter plasmids in *L. lactis* NZ9000. Although the β-galactosidase assay was not sensitive enough to detect activation of P_{ysaD} in the presence of pDCB_IL, the promoter was responsive to bacitracin when the *L. lactis* L81 *ysa* genes (pDCB_L81) were expressed (**Figure 4**). Hence, it was possible to compare signaling driven by the different *ysaB* alleles. Under non-inducing conditions, *L. lactis* NZ9000 expressing the mutated *ysaB* transporters exhibited a higher P_{ysaD} basal activity than that achieved with the WT transporter YsaDCB_L81 ($p < 0.05$). This basal activity was remarkably high in the presence of YsaDCB_E2 and YsaDCB_G9 transporters (**Figure 4**). Besides this apparent deregulation, the P_{ysaD} promoter was still induced by bacitracin, although the level of response was clearly diminished in relation to the WT transporter. In this latter case, β-galactosidase activity rose by 5-fold after the challenge with bacitracin at 5 μg/ml, whereas it only increased 1.2-fold in the presence of YsaDCB_D1 and 1.5-fold and in the presence of the other two mutated transporters.

The Secreted Peptide YsaD Seems to Modulate the Activity of the YsaDCB/TCS-G Module

The fact that *ysaD* forms an operon with *ysaCB* and that it is conserved within *L. lactis* raised the question whether this secreted peptide was somehow required for proper functioning of the YsaDCB/TCS-G module. Bearing in mind the dual role of the transporters in resistance and signal relay, we initially determined the IC_{50} values of bacitracin and Lcn972 for *L. lactis* NZ9000 expressing *ysaCB* from *L. lactis* IL1403 (pCB_IL) or from *L. lactis* L81 (pCB_L81) and compared to those expressing the whole cluster *ysaDCB* (**Table 3**). YsaD appears

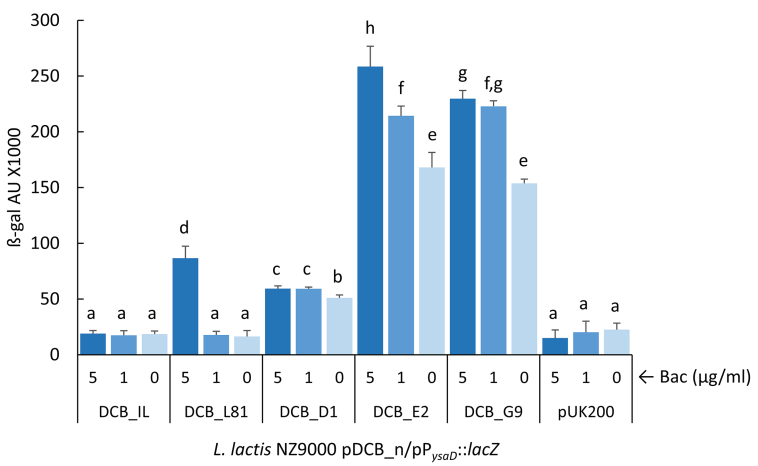

FIGURE 4 | Induction of P_{ysaD} by bacitracin. Exponentially growing *L. lactis* NZ9000 clones expressing the different versions of *ysaDCB* genes (pDCB_n) and carrying the reporter plasmid pP_{ysaD}::*lacZ* were challenged with varying concentrations of bacitracin (Bac) for 30 min. Average and SD from three independent experiments are shown. Values with different letters are significantly different ($p < 0.05$).

TABLE 3 | IC_{50} values[a] for *L. lactis* NZ9000 expressing *ysaCB*.

L. lactis NZ9000	Bacitracin (μg/ml)	Lcn972 (AU/ml)
pDCB_L81[b]	1.25 ± 0.30	5.58 ± 075
pCB_L81	2.41 ± 0.14*	6.63 ± 0.13
pDCB_IL[b]	1.55 ± 0.31	5.81 ± 2.02
pCB_IL	1.52 ± 0.23	4.45 ± 0.89
pUK200[b]	0.29 ± 0.01	1.21 ± 0.01

[a]*Average ± standard deviation of, at least, two independent biological replicates are shown.*
[b]*IC_{50} values are also shown in **Figure 3** but are included here for comparison.*
**p < 0.05, significant difference between pDCB_L81 vs. pCB_L81. For all clones, bacitracin and Lcn972 IC_{50} are increased in comparison with L. lactis NZ9000 with the empty vector pUK200.*

to be dispensable for resistance. All the clones were more resistant to bacitracin and Lcn972, compared to the control with the empty vector, and showed comparable bacitracin and Lcn972 IC_{50} values regardless the presence of *ysaD*. Only *L. lactis* NZ9000 pCB_L81 exhibited a 2-fold increase in the bacitracin IC_{50}, compared to pDCB_L81, suggesting a possible but marginal role of *ysaD* in resistance.

We also measured P_{ysaD} activity in the absence of *ysaD* to assess if YsaD could interfere with signal relay. Due to the low sensitivity of the *lacZ* reporter, unable to reveal induction of the P_{ysaD} promoter by the *L. lactis* IL1403 YsaDCB transporter, a more sensitive reporter plasmid pIL_P_{ysaD}::*mrfp*, based on the mCherry fluorescent protein, was used. This reporter was transferred to *L. lactis* NZ9000 pDCB_n and pCB_n clones. As shown in **Figure 5**, induction of P_{ysaD} by bacitracin in the presence of pDCB_IL could be demonstrated, albeit the response was roughly 10-fold lower than with pDCB_L81 (**Figure 5**). When the activity of P_{ysaD} was compared in the absence and presence of *ysaD* after the addition of bacitracin, the results were different depending on the transporter. The P_{ysaD} promoter was turned down in *L. lactis* NZ9000/pCB_L81, while it was strongly induced in *L. lactis* NZ9000/pCB_IL (**Figure 5**). This apparently incongruent behavior must rely on subtle differences within the L81 and IL1403 ABC transporters that could be important for the interaction with the *L. lactis* NZ9000 TCS-G (see section Discussion). Nonetheless, YsaD appears to modulate the activity of the YsaDCB/TCS-G module by altering signal relay. Finally, it is worth noting that attempts to show induction of P_{ysaD} by Lcn972 failed. All the reporter strains with either pDCB_n or pCB_n were exposed to Lcn972 at 80, 40, and 20 AU/ml, but fluorescence was never detected (data not shown).

DISCUSSION

The *L. lactis* YsaDCB ABC transporter is proposed to form a functional AMP detoxification module with the adjacent TCS-G, based on the genetic organization and the domain architecture of both the permease YsaB and the intramembrane-sensing histidine kinase KinG, two landmarks shared among AMP resistance modules in Firmicutes (Dintner et al., 2011; Gebhard, 2012). Accordingly, we have shown that increased expression of *ysaDCB* confers resistance against bacitracin and Lcn972 in *L. lactis* and that the module does not respond to bacitracin in the absence of a functional ABC transporter.

Previous studies have also linked this ABC transporter to nisin resistance in *L. lactis* (Kramer et al., 2006). Thereby, YsaDCB/TCS-G is able to provide resistance to cationic AMPs that differ in their mode of action. This is similar to other modules such as the staphylococcal GraXSR/VraFG and BraRS/BraDE reviewed by Kawada-Matsuo et al. (2011), whereas in *B. subtilis* the different Bce-like modules seem to be more specialized for bacitracin (BceSR/BceAB), nisin and other lipid II-binding lantibiotics (PsdRS/PsdAB) or cationic peptides such as the human defensin LL-37 (YxdJK/YxdLM-YxeA; Staron et al., 2011). The reason likely relies on the scope of the genes which are regulated by these resistance modules. Some modules only regulate the ABC transporter genes (e.g., BceSR/BceAB), while others (e.g., GraXSR/VraFG) include genes such as the *dlt* operon and *mprf* involved in D-alanylation of lipoteichoic acids and lysylination of phosphatidylglycerol, respectively, that

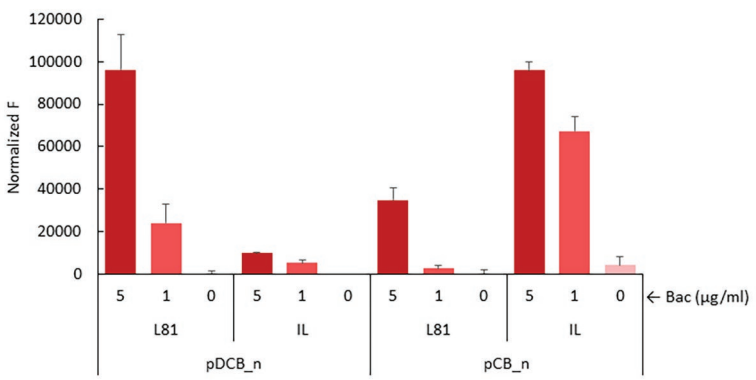

FIGURE 5 | Induction of P_{ysaD} in the presence of pDCB_n and pCB_n. Exponentially growing *L. lactis* NZ9000 clones expressing the full *ysaDCB* (DCB_n) operon or *ysaCB* (CB_n) lacking *ysaD* from *L. lactis* L81 (L81) or *L. lactis* IL1403 (IL) and the reporter plasmid pIL_P_{ysaD}::*mrfp* were challenged with bacitracin at 5 and 1 μg/ml for 30 min before mCherry fluorescence (F) was measured.

contribute to AMP resistance by decreasing the net negative charge of the bacterial surface. BraRS/BraDE and VirSR/VirAB also regulate the expression of other ABC transporters such as VraDEH in *S. aureus* and AnrAB in *Listeria monocytogenes*, respectively. These transporters likely function as a "hydrophobic vacuum cleaner," removing the AMPs out of their site of action (Collins et al., 2010; Hiron et al., 2011).

Our results have demonstrated that point mutations in *ysaB* activate the YsaDCB/TCS-G module. P_{ysaD} is active in the absence of bacitracin, when the mutated *ysaB* alleles are expressed in *L. lactis* NZ9000 and not in the case of the WT transporters. Gain-of-function mutations in the Bra(Nsa)SR/BraDE module have been mapped on *braS* or *braR* or on the TCS promoter in nisin-resistant *S. aureus* (Randall et al., 2018; Arii et al., 2019), but there are also examples in which mutations in the permease gene enhance resistance by inducing the expression of the ABC-transporter genes (Becker et al., 2009). In view of the current flux sensing model (Fritz et al., 2015), one possible explanation could be that the permease mutations reduce the activity of the YsaDCB transporter. This scenario would impose more pressure onto the system to increase the rate of *de novo* transporter synthesis and get more transporter molecules to relieve the stress. However, it is difficult to explain the constitutive expression in the absence of the AMP and the loss of responsiveness to AMP concentration, as observed with the mutated YsaDCB transporters. Moreover, since flux sensing relies on ATP hydrolysis, it would be clearly deleterious for the cells. Alternatively, the mutations in the YsaB permease could modify protein-protein interactions within the module, triggering activation of the histidine kinase, irrespectively of the activity of the YsaDCB transporter. Recently, it has been proposed that the ABC transporter BraDE, beyond its role in sensing and activation of BraSR regulon, also enables onward signal transduction through the histidine kinase BraS in some way, which is independent of ATP consumption (Randall et al., 2018). This role was proposed after observing that the nisin-resistant phenotype of a gain-of-function *braS* mutant lacking *braDE* could be complemented with an ATP-deficient *braDE* copy. In this scenario, it is conceivable that mutations in the permease could prompt activation of the TCS. However, further biochemical evidence is required to prove if this is the case of the *ysaB* mutations.

Increased expression of *ysaDCB*-D1 was not enough to confer resistance to bacitracin, whereas protection against Lcn972 was enhanced compared to the WT YsaDCB transporter. This takes us to speculate that YsaDCB may protect *L. lactis* by two different means. For bacitracin, it has been proposed that the *B. subtilis* BceAB transporter binds to the complex bacitracin-undecaprenyl pyrophosphate and releases the antibiotic from it, freeing the cell wall precursor to proceed with cell wall biosynthesis (Kobras et al., 2020). The double amino acid substitution $F_{577}S/P_{605}T$ in YsaB-D1 could make the ABC transporter less efficient for bacitracin release, and thus unable to protect against bacitracin. Kobras et al. (2020) also hypothesize that a similar target protection mechanism should also work for lipid II binding peptides such as mesarcidin and actagardine. However, YsaDCB-D1 is as effective as YsaDCB-E2 against Lcn972. Since YsaDCB-D1 is still competent to trigger activation of the module, we speculate that downstream activities (i.e., other functions regulated by the YsaDCB/TCS-G module) are responsible for resistance to Lcn972. In support of this hypothesis, motif-based searches, using the consensus binding sequence for BceR-like response regulators proposed by Dintner et al. (2011), detected a putative BceR-binding box in the promoter of the *dlt* genes in *L. lactis*. Moreover, D-alanylation of lipoteichoic acids is known to constitute an Lcn972 resistance factor (Roces et al., 2012).

The *L. lactis* BceAB-like transporter is equipped with an additional protein YsaD whose structural gene is co-transcribed with *ysaCB*. According to BlastP searches, YsaD belongs to the family TIGR01655 of small uncharacterized proteins with N-terminal signal sequences, conserved in Gram-positive organisms. These properties are shared with YxeA, in the *B. subtilis* module YxdJK/YxdLM-YxeA (Joseph et al., 2004), but its putative function has not been addressed so far. Our results suggest that these proteins may play an accessory role, modulating the activity of the transporter and/or governing signal transduction through protein-protein interactions within the module. Despite having an identical YsaD, removal of this protein had two opposite effects: a drop of signaling in the case of the transporter YsaDCB-L81 and boosting the otherwise poor ability of the *L. lactis* IL1403 transporter to activate *L. lactis* NZ9000 TCS-G (see **Figure 5**). Being aware that our experimental setup is not the best scenario to interrogate for fine tuning or regulatory feedback, simply because it relies on the formation of a heterologous ABC/TCS module, this discrepancy could be explained when specific YsaD-YsaB or YsaD-YsaB-KinG interactions regulate transport activity and that such interactions differ within these two transporters. Both YsaDCB-L81 and YsaDCB-IL are highly homologous with only one non-conservative amino acid substitution in the ATP-binding protein YsaC (E_{139} in L81 and K_{139} in IL1403), outside the conserved protein domains and unlikely to interfere with transport activity. The other one is in the YsaB permease (K_{621} in L81 and E_{621} in IL1403). This position is predicted to be located at the external linker between the last two TMHs IX and X. From our results and others (Kallenberg et al., 2013), it is known that single amino acid substitutions in the permease, namely, in the C-terminus, may severely alter signal transduction. Consistent with this, there is a 10-fold difference in the ability of the IL1403 and L81 transporters to trigger the P_{ysaD} promoter in response to bacitracin. ABC connectors or accessory proteins such as the cytosolic GraX and the small membrane protein VraH have already been described (Falord et al., 2012; Popella et al., 2016). Whether the same applies for a soluble and likely secreted peptide remains to be investigated. It is worth mentioning that in *L. lactis* there is an additional gene, *ysaA*, which is co-transcribed with the TCS-G gene and specifies for a transmembrane protein. YsaA, as yet unique to *L. lactis*, is likely to play a role in the transduction pathway too.

There was another result that will require further attention. Under the experimental conditions used in this work, we were unable to detect activation of P_{ysaD} by Lcn972, which is known

to bind to lipid II as many other Bce-like inducers. In a way, this was unexpected given that the gain-of-function mutations in *ysaB* were selected under the stress imposed by this bacteriocin (López-González et al., 2018). However, YsaDCB/TCS-G is likely regulating the *dlt* operon, which should protect against Lcn972 and, thereby, growth of gain-of-function mutants would be expedited. At this stage, it is difficult to propose a reasonable explanation for the lack of induction by Lcn972. We cannot disregard a host effect, i.e., that Lcn972 is unable to trigger the response in *L. lactis* NZ9000 but in other strain backgrounds, as recently observed with VirAB/VirRS in *L. monocytogenes* (Grubaugh et al., 2018; Jiang et al., 2019).

In conclusion, we have shown that YsaDCB/TCS-G participates in AMP resistance in *L. lactis* anticipating alternative mechanisms of resistance against different AMPs. Specific mutations which are easily selected under cell envelope stress have been shown to activate the module constitutively. Moreover, YsaDCB/TCS-G seems to be unique as to the intricate regulatory circuits due to the presence of additional proteins that modulate its activity, thereby warranting further research to understand these important resistance factors and their contribution to antimicrobial resistance. While for a nonpathogenic bacterium such as *L. lactis* antibiotic resistance may not be an issue of concern so far, this study opens new avenues for developing biotechnologically proficient strains able to withstand better the presence of bacteriocins or other cationic antimicrobials as lysozyme, currently in use as preservatives in food.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, and further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AR and BM conceived and designed the study. AC, ML-G, SE, and BM performed the experiments. AN and TJ contributed with strains and materials. AR and BM wrote the draft manuscript. All authors participated in the interpretation of the results and read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01805/full#supplementary-material.

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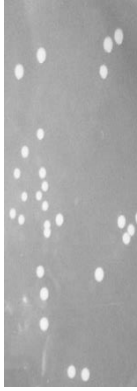
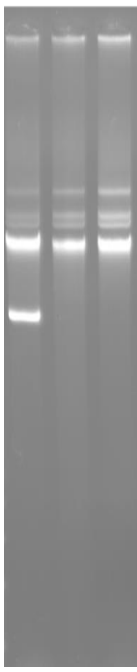
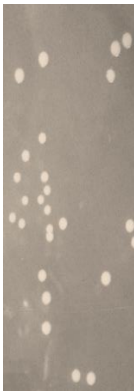
The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



DISCUSIÓN GENERAL





Discusión General

Los cultivos iniciadores mesófilos utilizados en la elaboración de quesos están constituidos fundamentalmente por cepas de *L. lactis* responsables del proceso de acidificación de la leche, e influyen además en muchas de las propiedades organolépticas del producto fermentado. La creciente necesidad de diversificar el mercado de este tipo de productos implica la demanda de cepas más robustas con propiedades mejoradas y capaces de tolerar condiciones adversas que, directa o indirectamente, tienen un impacto sobre la pared celular, la estructura más externa y crucial para la supervivencia de la célula.

Resultados previos del grupo DairySafe (IPLA-CSIC) habían puesto de manifiesto que la principal ruta de detección y respuesta al estrés de la pared celular de *L. lactis* está mediada por el sistema de dos componentes (TCS) CesSR (la ruta tipo Lia) que detecta el daño y activa la transcripción de un conjunto de genes, la mayoría de función desconocida (Martínez *et al*., 2007). Posteriormente, se demostró el papel de alguno de estos genes en la supervivencia de *L. lactis* a estreses de relevancia tecnológica (ácido, concentración de sal, bacteriófagos) (Roces *et al*., 2009). Además, se puso de manifiesto que la exposición de cepas de laboratorio (ej. *L. lactis* MG1614) a la bacteriocina Lcn972 activaba mecanismos de defensa relacionados con la modulación de la pared celular en *L. lactis*, tales como la capacidad para modificar la estructura del peptidoglicano, o la activación/inactivación de genes con función reguladora adicional, dando lugar a mutantes más resistentes a determinadas condiciones de estrés (Campelo *et al*., 2011; Roces *et al*., 2012a; Roces *et al*., 2012b). Estos resultados constituyeron el punto de partida de esta Tesis Doctoral en la que se ha evaluado la viabilidad de la evolución adaptativa bajo condiciones de estrés de la pared celular (EA-CES) como estrategia alternativa a la modificación genética, usando la bacteriocina Lcn972 como agente selectivo. El conocimiento de la fisiología de *L. lactis* ha permitido diseñar muchas estrategias basadas en ingeniería genética para la diversificación de los cultivos iniciadores, sin embargo, son rechazadas por gran parte de los consumidores (Derkx *et al*., 2014). Adicionalmente, el uso de esta bacteriocina, que actúa específicamente sobre lactococos, ha permitido profundizar en el conocimiento de los mecanismos de respuesta de *L. lactis* para detectar y responder al daño de la pared celular. En la literatura existen otros desarrollos biotecnológicos con bacteriocinas anti-lactococos de espectro estrecho. Por ejemplo, las lactococinas A, C y M inducen la lisis prematura de las células facilitando

la liberación de enzimas extracelulares que aceleran la maduración del queso (Morgan *et al*., 1997)

Por otro lado, también existen ejemplos de aplicación de la evolución adaptativa llevados a cabo con cepas de laboratorio de *L. lactis* que muestran la posibilidad de seleccionar diferentes mutaciones en función de las condiciones de presión selectiva utilizadas. Este es el caso de la obtención de mutantes resistentes a elevadas temperaturas o a condiciones de estrés oxidativo (Bachmann *et al*., 2017; Chen *et al*., 2015; Liu *et al*., 2021; Papiran y Hamedi, 2021). Además, también se han podido seleccionar otros fenotipos de interés que no estaban directamente relacionados con el agente estresor y que podrían deberse a fenómenos de resistencia cruzada (Chen *et al*., 2015; Roces *et al*., 2012a). Más recientemente, otras investigaciones también apuntan hacia la validez de esta estrategia para seleccionar cepas de BAL con mejores niveles de producción de bacteriocinas (Moreira *et al*., 2021; Papiran y Hamedi, 2021). Estos estudios demuestran la plasticidad de *L. lactis* para fijar mutaciones beneficiosas cuando se expone a determinadas condiciones. Sin embargo, no ha sido posible hasta ahora aportar resultados similares cuando dicha estrategia se aplica a cepas industriales, dadas las diferencias que estas cepas presentan respecto a las cepas de laboratorio (Cavanagh *et al*., 2015; Kelleher *et al*., 2017).

En este contexto, el primer objetivo de este trabajo fue aplicar la EA-CES a la cepa *L. lactis* IPLA947 como ensayo piloto para optimizar el proceso y decidir si era oportuno trasladarlo a otras cepas industriales de *L. lactis* (capítulo I). *L. lactis* IPLA 947 es la principal cepa acidificante del cultivo iniciador diseñado para la elaboración de queso *Afuega'l Pitu* (Cárcoba *et al*., 2000). Dado que esta cepa presentaba problemas de producción a gran escala y era sensible además a la presencia de oxígeno, el estudio se centró en la posible obtención de derivados resistentes al estrés oxidativo.

Los resultados mostraron que, aunque los tres mutantes resistentes estables seleccionados presentaban parámetros de crecimiento similares a los de la cepa original, cada uno mostraba un comportamiento diferente en condiciones de aerobiosis. En el caso concreto del mutante *L. lactis* R5, se observaron mayores tasas de crecimiento que en la cepa parental y en el resto de mutantes y, además, dicho mutante alcanzó una OD máxima superior. Por otro lado, en los experimentos realizados en presencia de agua oxigenada se observó una viabilidad de *L. lactis* R5 52.8% superior a la de la cepa parental. La presencia de oxígeno disuelto en la leche da lugar a especies reactivas que influyen negativamente en los parámetros de crecimiento de *L. lactis* (Cesselin *et al*., 2011; Jeanson *et al*., 2009; Larsen *et al*., 2015). Sin embargo, gracias a su metabolismo

respiratorio, el oxígeno se reduce a agua liberando protones en presencia del grupo hemo, lo que representa una ventaja en la producción de biomasa (Duwat *et al*., 2001; Pedersen *et al*., 2012; Rezaïki *et al*., 2004). En nuestro caso, tal como se esperaba, los ensayos realizados en presencia del grupo hemo pusieron de manifiesto la mejora del rendimiento en condiciones de agitación tanto en la cepa silvestre como en su derivado *L. lactis* R5. Sin embargo, no se observaron diferencias al comparar los parámetros de crecimiento de ambos. Como el metabolismo respiratorio conlleva la eliminación de oxígeno, es posible que las ventajas adquiridas por el mutante respecto al estrés oxidativo queden enmascaradas, no representando una ventaja cuando se activa dicho metabolismo.

Además, la caracterización fenotípica de *L. lactis* R5 puso de manifiesto que las ventajas adquiridas frente al estrés oxidativo no comprometían los parámetros tecnológicos esenciales como cultivo iniciador, ya que se mantenían las características metabólicas y las propiedades acidificantes en leche, además de la viabilidad a lo largo del tiempo durante el almacenamiento a 4ºC. Por lo tanto, todo apuntaba a que el uso de la EA-CES podría ser útil para diversificar las cepas de *L. lactis* disponibles, pudiendo ser algunas de ellas de interés para la industria láctea.

A la vista de los resultados obtenidos, se aplicó la EA-CES a otras 7 cepas de *L. lactis* subsp. *lactis* de diferente origen (capítulo II): tres cepas comerciales, dos de ellas acidificantes (L81 y L62) utilizadas como cultivo iniciador, una cepa productora de nisina A (L98) usada como cultivo protector, y cuatro cepas silvestres productoras de nisina Z (IPLA517- IPLA641- IPLA729 e IPLA1064) pertenecientes a la colección IPLA-CSIC que habían sido aisladas en el queso artesanal *Afuega´l Pitu* elaborado con leche cruda en Asturias. En todos los casos se obtuvieron mutantes resistentes a la Lcn972, lo que sugiere que el uso de esta bacteriocina como agente estresor en experimentos de EA-CES podría ser extensible a todos los lactococos sensibles a la misma. Todos los mutantes fueron estables y mantuvieron los parámetros de crecimiento en condiciones de laboratorio, a diferencia de lo que sucede con las cepas de lactococos resistentes a la nisina, que siguen necesitando de su presencia para mantener ese fenotipo de resistencia (Kramer *et al*., 2006). Esta estabilidad puede ser debida al paso de estabilización, posterior a la adaptación a la Lcn972, en el que se fijarían aquellas mutaciones más beneficiosas para la célula. Otra explicación podría estar basada en el modo de acción de la Lcn972 que inhibe la síntesis de la pared celular sin formar poros (Martinez *et al*., 2008). Esto permite que la célula no muera de forma instantánea, aumentando así la probabilidad de acumular un mayor número de mutaciones. Por otro

lado, se ha demostrado que la tasa de mutación es mayor cuando determinados antibióticos de pared activan la respuesta SOS (Gutierrez *et al*., 2013). Aunque no está experimentalmente demostrado, hay indicios de que esta respuesta SOS también podría desencadenarse en presencia de Lcn972, dado que se ha observado la activación de profagos después del tratamiento con esta bacteriocina (Madera *et al*., 2009).

Como ya se ha mencionado anteriormente, un aspecto clave en el estudio de la viabilidad de la EA-CES como estrategia de mejora y diversificación es que no pierdan las características funcionales del cultivo iniciador.

Para comprobar las características tecnológicas esenciales de las cepas se realizaron estudios relativos a los parámetros de crecimiento, la capacidad de acidificar la leche, la producción de nisina en las cepas correspondientes y la sensibilidad a bacteriófagos. De forma general, todos los mutantes resistentes mantuvieron sus parámetros de crecimiento y los niveles de producción de nisina respecto a la cepa parental y fueron capaces de acidificar la leche considerando los valores estándar aceptados para los cultivos iniciadores (Cogan *et al*., 1997). Por otro lado, se observó, en la mayoría de los casos, un descenso en los niveles de ácido láctico detectados, lo que podría indicar que el proceso de adaptación a la Lcn972 implicaría un cambio hacia un metabolismo fermentativo ácido-mixto. En el caso del mutante derivado de la cepa IPLA947, *L. lactis* R5, se detectaron niveles superiores de acetoína que los producidos por la correspondiente cepa parental, lo que parece indicar la activación de la ruta acetolactato sintasa que dirigiría el metabolismo del piruvato hacia la producción de acetoína. De este modo, se produciría la energía adicional necesaria para superar el estrés provocado por el proceso de adaptación a la Lcn972, tal y como como se ha descrito previamente frente a otras situaciones de estrés (Papadimitriou *et al*., 2016). De hecho, se ha demostrado que esta ruta metabólica se induce en presencia de oxígeno tanto en medio de cultivo como en leche (Cretenet *et al*., 2014; Larsen *et al*., 2016).

Cabe señalar que no en todos los casos analizados se mantuvieron las aptitudes tecnológicas de la cepa parenteral, aspecto que se ha asociado a la pérdida de plásmidos. Esta es, quizás, una de las principales desventajas de la EA-CES identificadas en este trabajo, ya que en *L. lactis* varias de estas funciones están codificadas en plásmidos como, por ejemplo, la fermentación de la lactosa, la actividad proteolítica o los mecanismos de resistencia a bacteriófagos (Ainsworth *et al*., 2014). Los dos mutantes derivados de la cepa productora de nisina L98 (L98-C1 y L98-E2) perdieron la capacidad de fermentar la lactosa *in vitro* y, concretamente L98-C1, no fue capaz de crecer ni producir nisina en leche. Este hecho se ha asociado a la inestabilidad del

plásmido de la lactosa en la cepa de partida, de modo que tan sólo se pudieron seleccionar variantes lactosa negativos durante el proceso de adaptación de la EA-CES. Existen otros ejemplos de la inestabilidad de otros plásmidos responsables del catabolismo de la lactosa como pLP712 (Wegmann *et al*., 2012).

La pérdida de plásmidos de relevancia tecnológica se observó también en los mutantes derivados de las cepas productoras de nisina Z, los cuales eran más sensibles a la infección por bacteriófagos que su cepa parental. En el caso de la cepa IPLA517 se demostró que la pérdida de plásmidos tras el tratamiento con novobiocina generaba cepas curadas con una mayor sensibilidad a la infección fágica. Se puso de manifiesto así la existencia de plásmidos que codifican mecanismos de resistencia frente a bacteriófagos en la cepa IPLA517, pudiendo suceder lo mismo en otras dos de las cepas productoras de nisina Z (IPLA641 e IPLA1064). Un resultado intrigante ha sido la ausencia de placas de lisis del fago CHPC1130 sobre las cepas originales, a pesar de que en los ensayos iniciales de sensibilidad realizados en leche, la presencia de este fago ralentizaba claramente la acidificación. Es posible que el plásmido implicado codifique un EPS que enmascare el receptor de este fago tipo c2, al igual que lo descrito para el plásmido pCI658 que codifica la producción de EPSs protegiendo a lactococos de la infección por los bacteriófagos phi712 y c2 (Forde y Fitzgerald, 2003). Dado que existen precedentes sobre el impacto que tienen las condiciones de cultivo en la producción y composición de EPSs (Sánchez *et al*., 2006), es factible que la producción del EPS sea diferente en LM17, el medio en el que se realizan los ensayos de plaqueo, que en leche, medio en el que se evaluó inicialmente la sensibilidad al fago. Alternativamente, el plásmido podría estar implicado en la síntesis de un sistema de infección abortiva Abi. Estos mecanismos provocan que la célula muera antes de que el fago termine su ciclo lítico y pueda liberar la progenie viral *(*Labrie *et al.*, 2010). En este caso, no sería posible observar placas de lisis en LM17, pero sí tendría lugar la ralentización de acidificación de la leche. Sin embargo, no se identificaron genes Abi en el genoma de las cepas originales. Por el contrario, sí se detectaron genes implicados en la síntesis de EPSs en las cepas IPLA517, IPLA641 e IPLA1064 que no estaban presentes en los mutantes derivados. Por esta razón, se cree que la sensibilidad de los mutantes al fago CHPC1130 es debida a la pérdida de un EPS protector.

Para conocer el grado de heterogeneidad de la EA-CES y el posible impacto de las mutaciones seleccionadas sobre otras propiedades relevantes, se llevó a cabo un estudio fenotípico más detallado de los mutantes Lcn972R, incluyendo también el mutante *L. lactis* R5 derivado de la cepa IPLA947.

Dado que, en general, la exposición a bacteriocinas provoca cambios en las propiedades físico-químicas de la envuelta celular, tanto en la membrana citoplasmática como en la pared celular (Bastos *et al*., 2015; Draper *et al*., 2015), los experimentos relacionados con las propiedades de superficie se centraron en el estudio de la hidrofobicidad y la capacidad autolítica. Salvo en 3 excepciones (*L. lactis* R5, L81-E2 e IPLA729-D10), las cepas sometidas a EA-CES mostraron menor actividad autolítica. Este resultado no es sorprendente teniendo en cuenta que el agente estresor utilizado inhibe la síntesis de la pared celular (Martinez *et al*., 2008; Martínez *et al*., 2000). Respecto a la hidrofobicidad, los resultados mostraron mayor variabilidad. Mientras que algunos mutantes perdieron completamente su carácter hidrofóbico, el resto mantuvieron o incluso aumentaron sus características hidrofóbicas. Se observó además que los 6 derivados que eran sensibles a fagos (IPLA517-B5 y C6; IPLA641-C8 y D8; IPLA1064-C11 y E11), mostraban al mismo tiempo un aumento de su carácter hidrofóbico, lo que podría estar relacionado con la pérdida de un EPS, posiblemente hidrofílico, involucrado en el mecanismo de resistencia a bacteriófagos. Tanto la autolisis como la hidrofobicidad pueden implicar cambios en el desarrollo del aroma, el sabor y la textura del producto fermentado (Lortal y Chapot-Chartier, 2005; Tarazanova *et al*., 2018), lo cual apoya el uso de la EA-CES como estrategia de diversificación.

También se evaluó la resistencia a estreses tales como el pH ácido o altas temperaturas, ya que cambios en la pared celular como el aumento del grado de la D-Asp amidación, *O*-acetilación y *N*-deacetilación del peptidoglicano favorece la resistencia a pH ácidos en *L. lactis* (Cao *et al*., 2018; Hao *et al*., 2017). Sin embargo, nuestros resultados no apuntan en la misma dirección y no se ha observado esta resistencia cruzada a pH ácido. Igualmente, los experimentos realizados en condiciones de estrés térmico o salino tampoco mostraron resistencia cruzada, siendo los mutantes Lcn972R más sensibles a estas condiciones que las cepas parentales. Tan sólo uno de los resistentes (*L. lactis* R5) aumentó su resistencia al calor respecto a la cepa original. La mayor sensibilidad de las cepas evolucionadas al pH ácido y/o temperatura constituye, junto con la pérdida de plásmidos, una de las desventajas de la EA-CES observadas en este trabajo y que requiere ser evaluada caso por caso. Estos costes adicionales son habituales durante el proceso evolutivo y difíciles de evitar (Kleerebezem *et al*., 2020).

Por el contrario, sí se detectaron resistencias cruzadas con antimicrobianos de pared. La mayoría de los mutantes presentaron resistencia a lisozima y, aproximadamente la mitad, a vancomicina, mientras que 3 de los 5 derivados, obtenidos a partir de las cepas no productoras de nisina, también mostraron resistencia cruzada a

nisina. Nuestros resultados son acordes con otros estudios que habían mostrado resistencia cruzada a lisozima en mutantes resistentes a otras bacteriocinas, independientemente de su modo de acción (Bastos *et al*., 2015; Roces *et al*., 2012). Por otro lado, 4 de los 15 mutantes estudiados, L81-E2, L62-C9, L62-G9 y *L. lactis* R5, fueron resistentes a bacitracina, un fenotipo que estudiamos posteriormente (capítulo III).

Otro de los objetivos marcados en esta Tesis ha sido el estudio preliminar de las mutaciones adquiridas durante el proceso de EA-CES que pudiesen explicar tanto la resistencia a Lcn972 como los otros fenotipos observados. Para ello, se secuenciaron los genomas de todos los mutantes seleccionados para identificar mutaciones puntuales que pudieran haber sido seleccionadas en el paso de adaptación a la Lcn972 (capítulos I y II). Cabe señalar, no obstante, que no fueron secuenciados los mutantes derivados de la cepa IPLA729 debido a su menor tasa de crecimiento y acidificación en leche. Aunque se observaron clústeres de similitud entre cepas, los perfiles de mutaciones variaban, no solo entre los mutantes de diferentes cepas, sino también entre los que se derivaban de la misma cepa de partida. Esta observación tiene lógica ya que en todo proceso evolutivo pueden existir múltiples soluciones que conduzcan a un mismo beneficio (Herwig Bachmann et al., 2017). Un ejemplo relevante es la mutación detectada en el gen *purR*, que está implicado en la regulación de la síntesis de purinas (Kilstrup y Martinussen, 1998). Este hecho podría explicar la ausencia de crecimiento en leche del derivado L98-C1, ya que sería incapaz de realizar la síntesis *de novo* de las purinas requeridas para el crecimiento en la misma. De hecho, el otro mutante (L98-E2), que no presenta esta mutación, y que también ha perdido el plásmido de la lactosa, sí es capaz de acidificar la leche.

El análisis genómico preliminar reveló que durante el proceso EA-CES se acumulaban frecuentemente mutaciones en algunos de los genes de un supuesto módulo de detección y detoxificación constituido por los genes *ysaDCB* que codificarían el transportador ABC (*ysaBC*) y *llrGkinG* del sistema de dos componentes asociado TCS-G (capítulo III). Este módulo, YsaDCB/TCS-G, conserva las características propias de un módulo de detección y detoxificación del tipo Bce, con una permeasa (YsaB) con 10 hélices transmembrana, un dominio extracelular entre las hélices 7 y 8, y una histidín kinasa (KinG) del tipo “sensor de membrana” (intramembrane-sensing) que carece de un dominio específico de detección extracelular (Gebhard, 2012; Revilla-Guarinos *et al*., 2014).

Trabajos previos realizados sobre el módulo de *B. subtilis* BceSR_AB mostraron que cambios puntuales en la secuencia aminoacídica de la permeasa del transportador

podían alterar tanto la resistencia a la bacitracina como la transducción de señales, es decir, la activación de la histidín quinasa (Kallenberg et al., 2013). De forma similar, en *Streptococcus pneumoniae,* también se han asociado mutaciones en la permeasa con un aumento de la transcripción de los genes del transportador ABC y mayor resistencia a la vancoresmicina (Becker *et al*., 2009). En nuestro caso, 3 de los 4 mutantes que mostraron resistencia a la bacitracina (L81-E2, L62-C9 y L62-G9) presentan mutaciones puntuales en *ysaB*. Por el contrario, el mutante L81-D1 era más sensible a la bacitracina que la cepa parental, a pesar de que también es portador de una versión del gen *ysaB* con dos cambios puntuales. Los resultados de RT-qPCR demostraron que, en efecto, los genes *ysaDCB* están sobreexpresados en los mutantes L81-D1, L81-E2, L62-G9, aunque el grado de inducción es menor en el caso de L81-D1.

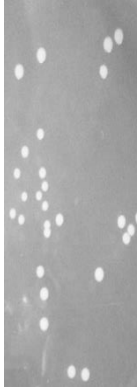
Los genes que codifican el transportador YsaDCB de las cepas parentales, así como las versiones con el gen *ysaB* mutado, fueron clonados bajo el control del promotor de la nisina (P_{nis}) y se expresaron en *L. lactis* NZ9000 para conocer su implicación en la resistencia a bacitracina y Lcn972. Los resultados confirmaron que este transportador confería resistencia a ambos antimicrobianos. Kramer et al. (2006) también habían identificado este transportador como uno de los mecanismos de resistencia a nisina. Por tanto, al contrario de lo que ocurre con el módulo BceRS_AB de *B. subtilis*, que confiere resistencia específicamente a bacitracina, el módulo equivalente en *L. lactis* parece tener un espectro más amplio como ocurre con otros módulos tipo Bce como GraXSR/VraFG y BraRS/BraDE de *S. aureus* (Kawada-Matsuo *et al*., 2011). Esto se debe a que la activación de estos módulos, además de inducir los genes del transportador ABC que actúan como mecanismo de detoxificación, desencadena también la inducción de otros mecanismos protectores como es el caso de los genes *dlt*. Estos genes modifican la estructura de los ácidos lipoteicoicos, disminuyendo la carga negativa de la superficie celular, lo que confiere mayor resistencia a péptidos antimicrobianos catiónicos (Peschel *et al*., 1999).

Al comparar el grado de protección que conferían los genes de los mutantes con el de los genes silvestres, se observó que todos conferían una resistencia a Lcn972 mayor que la proporcionada por los genes de las cepas parentales. Sin embargo, no ocurría lo mismo con la bacitracina frente a la cual, solo conferían protección los genes de los mutantes L81-E2 y L62-G9, mientras que los del mutante L81-D1 no eran activos. Estos resultados sugieren que la resistencia frente a bacitracina y Lcn972 ocurre mediante mecanismos distintos. La resistencia a bacitracina estaría asociada a la actividad del transportador *per se*, que no se vería alterada por las mutaciones presentes

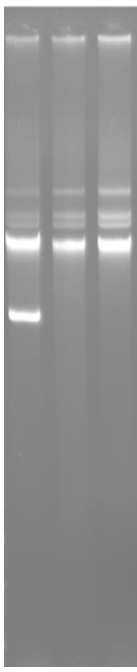
en L81-E2 y L62-G9, pero sí en el caso de la doble mutación presente en L81-D1. Por otro lado, la resistencia a Lcn972 estaría mediada por otros mecanismos de protección, como por ejemplo, la inducción de los genes *dlt*. De hecho, la D-alanización de los ácidos lipoteicoicos es un factor de resistencia frente a Lcn972 (Roces *et al*., 2012). Además, la secuencia consenso de unión de los reguladores transcripcionales tipo Bce ha sido detectada en la región promotora de los genes *dlt* en *L. lactis* (Dintner *et al*., 2011).

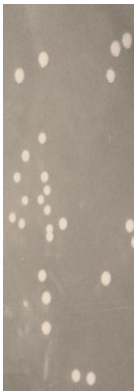
Los resultados obtenidos al estudiar la actividad del promotor del operón *ysaDCB* (P_{ysaD}) demostraron que las mutaciones en *ysaB* seleccionadas durante el proceso de EA-CES provocan la activación constitutiva del sistema de dos componentes TCS-G, que se encuentra activo incluso en ausencia del inductor, en este caso, bacitracina. En el caso del módulo BceRS_AB de *B. subtilis* los cambios conformacionales de la permeasa BceB, provocados por la presencia de bacitracina, constituyen la señal detectada por la histidín quinasa BceS que desencadena la respuesta (Dintner *et al*., 2011; Koh *et al*., 2021). Siguiendo este modelo, postulamos que las mutaciones adquiridas podrían interferir con las interacciones proteína-proteína entre la permeasa YsaB y KinG, de tal modo que KinG se mantendría permanentemente activa, aunque hasta ahora carecemos de evidencias experimentales que lo demuestren.

En este trabajo también se ha abordado el estudio de la función del gen *ysaD* que se co-transcribe con los genes *ysaB* y *ysaC*. La proteína YsaD es una proteína que se secreta y que tiene homología con la proteína YxeA del módulo YxdJK/YxdLM-YxeA de *B. subtilis* (Joseph *et al*., 2004), pero se desconoce su función. Nuestros resultados sugieren que el gen *ysaD* podría jugar un papel modulador en la transmisión de señales dentro del módulo YsaDCB/TCS-G, aunque los datos no son concluyentes pues se han obtenido resultados opuestos al estudiar su efecto sobre la activación del módulo. Mientras que la deleción de *ysaD* en el plásmido portador de los genes de la cepa L81 redujo drásticamente la activación del promotor P_{ysaD}, ocurrió lo contrario cuando los genes expresados eran los de la cepa IL1403. En este contexto, es necesario indicar que nuestro sistema experimental no es el más adecuado para estudiar las interacciones proteína-proteína ya que se basa en la formación de un módulo híbrido entre el transportador ABC, codificado en el plásmido, y el sistema de dos componentes TCS-G de la cepa *L. lactis* NZ9000. Además, el módulo de *L. lactis* presenta otras singularidades como la presencia de la proteína de membrana YsaA, cuyo gen se co-transcribe con *llrGkinG*, que también podría participar en la transducción de señales, de modo similar a lo observado con YsaD y otras proteínas accesorias como GraX y VraH presentes en otros módulos de detoxificación (Falord *et al*., 2012; Popella *et al*., 2016).



CONCLUSIONES





Conclusiones

1. La bacteriocina Lcn972 es una herramienta útil para realizar experimentos de evolución adaptativa con *Lactococcus lactis* ya que se han aislado mutantes resistentes Lcn972R a partir de cepas industriales y silvestres.

2. La evolución adaptativa bajo condiciones de estrés sobre la pared celular (EA-CES) permite obtener cepas de *L. lactis* con propiedades tecnológicas de interés como la resistencia a estrés oxidativo o cambios en la superficie celular sin comprometer parámetros tecnológicos esenciales para la utilización de dichas cepas como componentes de un cultivo iniciador/protector.

3. La pérdida de plásmidos es una de las principales desventajas de la EA-CES, pudiendo afectar a la resistencia a bacteriófagos y a la fermentación de la lactosa.

4. El perfil de las mutaciones seleccionadas varía en función del fondo genético de la cepa que se somete a EA-CES.

5. Las mutaciones más frecuentes ocurren en los genes que codifican un módulo de detección y resistencia a péptidos antimicrobianos constituido por el transportador ABC YsaDCB y el sistema de dos componentes TCS-G. Las mutaciones en el gen de la permeasa (*ysaB*)provocan la activación constitutiva del módulo.

6. El módulo YsaDCB/TCS-G de *L. lactis* participa en la resistencia a bacitracina y Lcn972 previsiblemente mediante mecanismos diferentes.

7. Se han identificado otras mutaciones en genes cuya caracterización funcional supondrá un avance en el conocimiento de la fisiología del estrés sobre la pared celular de *L. lactis*.

8. La plasticidad demostrada por *L. lactis* en su respuesta al estrés sobre la pared celular constituye un sólido apoyo de la validez de la EA-CES como estrategia de mejora y diversificación fenotípica y genotípica en cepas industriales y silvestres de *L. lactis*.

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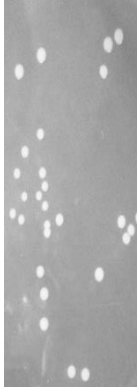

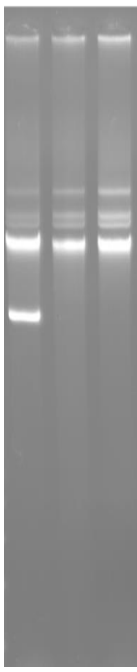
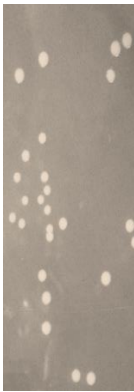

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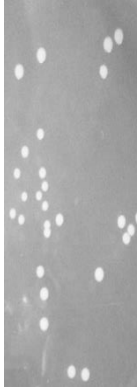
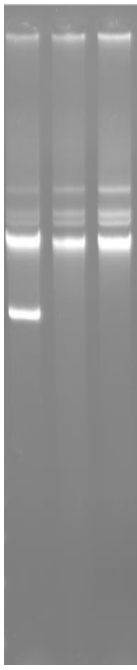
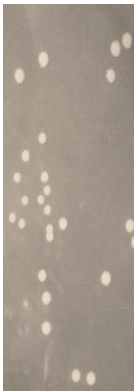
ANEXOS



Anexos

1 Factor de impacto de las publicaciones presentadas

El factor de impacto de cada publicación corresponde con el que figura en el "Journal Citation Report (JCR)" del año de publicación:

1. López-González, M.J., Campelo, A.B., Picón, A., Rodríguez, A., and Martínez, B. 2018. Resistance to bacteriocin Lcn972 improves oxygen tolerance of Lactococcus lactis IPLA947 without compromising its performance as a dairy starter. BMC Microbiology 18: 76. -article. DOI: 10.1186/s12866-018-1222-8

   Revista BMC Microbiology. Factor de Impacto (JCR 2018): 3,287 (Microbiology, Q2)

2. López-González, M.J., Escobedo, S., Rodríguez, A., Neves, A.R., Janzen, T., and Martínez, B. 2018. Adaptive evolution of industrial *Lactococcus lactis* under cell envelope stress provides phenotypic diversity. Frontiers in Microbiology 9:2654 I Journal-article. DOI: 10.3389/fmicb.2018.02654

   Revista Frontiers in Microbiology. Factor de Impacto (JCR 2018): 4.259 (Microbiology Q1)

3. Campelo, A.B., López-González, M.J., Escobedo, S., Janzen, T., Neves, A.R., Rodríguez, A. and Martínez, B. 2020. Bacteriocin-induced mutations activate antibiotic resistance modules in *Lactococcus lactis*. Frontiers in Microbiology. Journal-article. DOI: 10.3389/fmicb.2020.01805

   Revista Frontiers in Microbiology. Factor de Impacto (JCR 2020): 5.640 (Microbiology Q1)

2 Material suplementario del Capítulo II

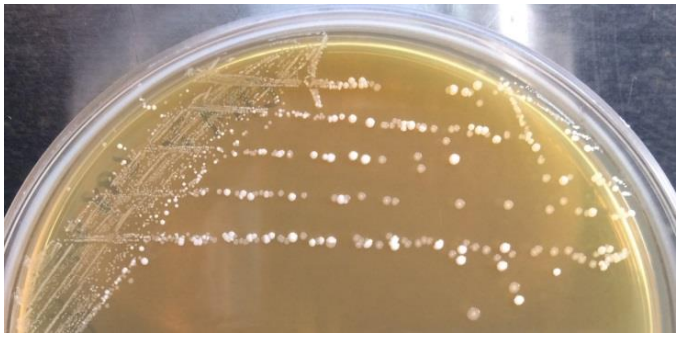


Figure S1. Colony morphology of *L. lactis* L98 streaked on a LM17 agar plate

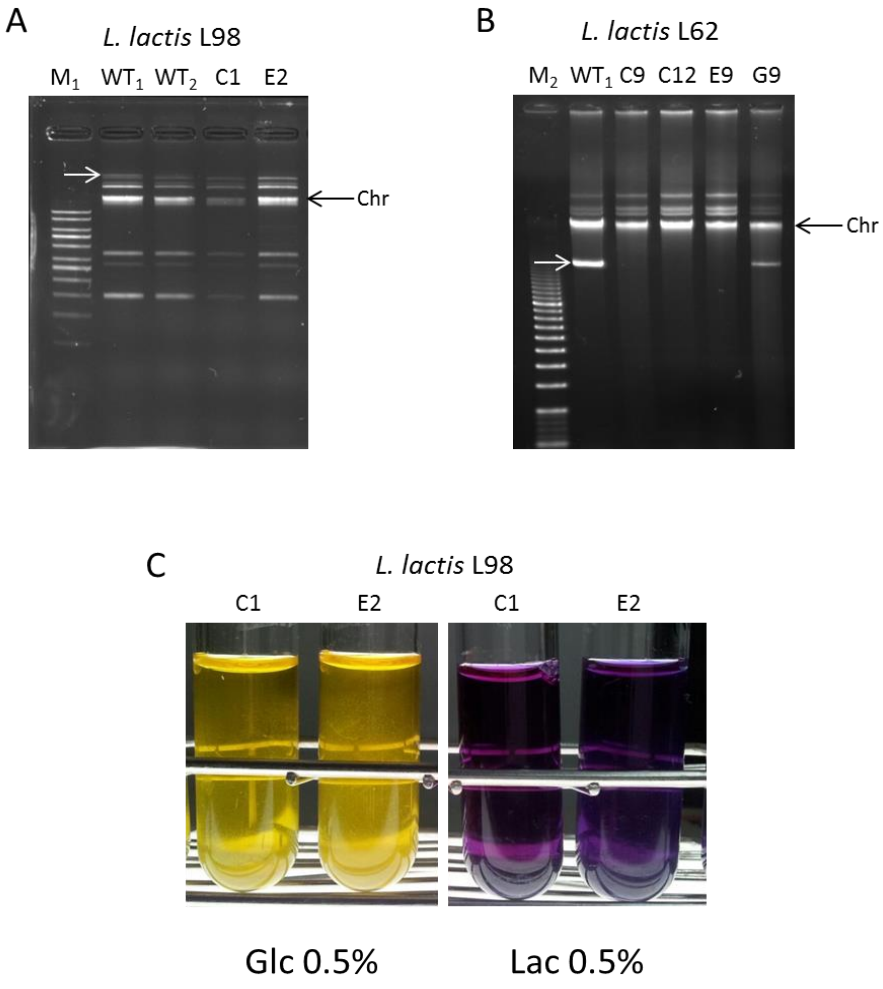


Figure S2. Plasmid profile of (A) *L. lactis* L98 (WT) and its Lcn972R mutants L98-C1 and -E2 and (B) *L. lactis* L62 (WT) and its Lcn972R mutants L62-C9, -C12,-E9 and -G9. Chr: Chromosomal DNA. M_1: DirectLoad 1 kb ladder (SIGMA). M_2: 0.5 kbp ladder (Bio-Rad). The white arrow points to the missing band. (C): Growth and acidification of *L. lactis* L98-C1 and L98-E2 in the basal broth BCP supplemented with glucose or lactose at 0.5%.

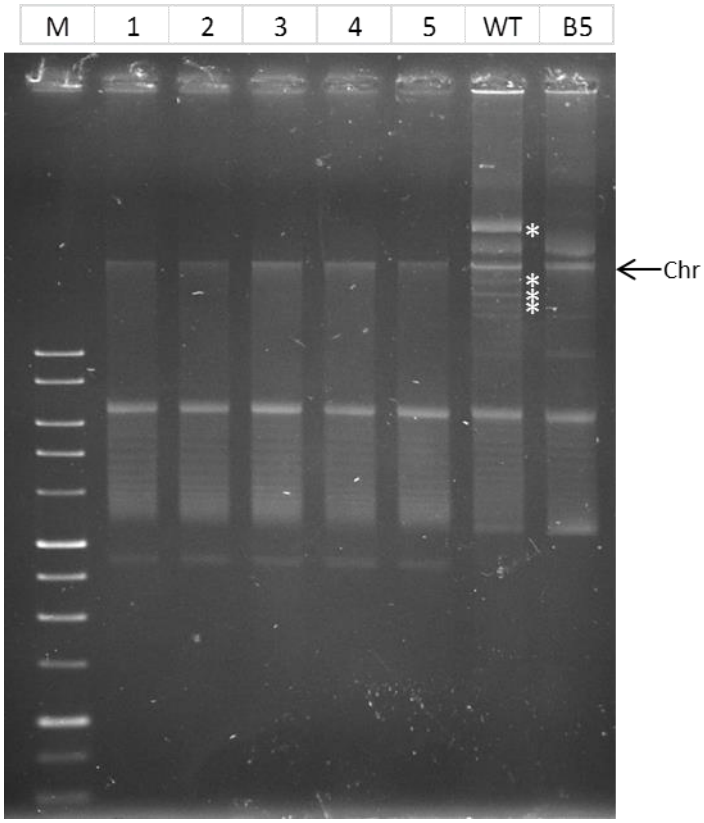


Figure S3. Plasmid profile of *L. lactis* IPLA517 (WT), its Lcn972R mutant IPLA517-B5 (B5) and five clones (N1 to N5) isolated after novobiocin treatment of *L. lactis* IPLA517. M: 0.5 kb ladder (Bio-Rad). Electrophoresis was carried out in 0.6% agarose gel in TAE buffer at 30 V for 6 h. White starts show plasmidic bands present in IPLA517 and absent in B5.

Anexos

Table S1. Inhibition of growth in milk of Lactococcus lactis strains in the presence of phages[1]

Phage	IPLA 517wt	IPLA 641wt	IPLA 729wt	IPLA 1064wt	Phage family
CHPC52	-	-	-	-	
CHPC116	-	-	-	-	
CHPC122	-	-	-	-	
CHPC129	-	-	-	-	
CHPC134	pos	pos	pos	pos	c2
CHPC142	-	-	-	-	
CHPC148	-	-	-	-	
CHPC209	-	-	-	-	
CHPC239	-	pos	-	-	unknown
CHPC324	-	-	-	-	
CHPC361	-	pos	-	-	936
CHPC367	-	-	-	-	
CHPC412	-	-	-	-	
CHPC646	-	-	-	-	
CHPC667	-	-	-	-	
CHPC670	-	-	-	-	
CHPC735	-	-	-	-	
CHPC763	-	-	-	-	
CHPC781	-	-	-	-	
CHPC836	-	-	-	-	
CHPC840	-	-	-	-	
CHPC882	-	-	-	-	
CHPC955	-	-	-	-	
CHPC956	-	-	-	-	
CHPC958	-	-	-	-	
CHPC959	-	-	-	-	
CHPC960	-	-	-	-	
CHPC961	-	-	-	-	
CHPC962	-	-	-	-	
CHPC963	-	-	-	-	
CHPC964	-	-	-	-	
CHPC965	-	-	-	-	
CHPC966	-	-	-	-	
CHPC967	-	-	-	-	
CHPC969	-	-	-	-	
CHPC971	-	-	-	-	
CHPC973	-	-	-	-	
CHPC974	-	-	-	-	
CHPC976	-	-	-	-	
CHPC989	-	-	-	-	
CHPC997	-	-	-	-	
CHPC1020	-	-	-	-	

Phage	IPLA 517wt	IPLA 641wt	IPLA 729wt	IPLA 1064wt	Phage family
CHPC1078	-	-	-	-	
CHPC1112	-	-	-	-	
CHPC1130	pos	pos	pos	pos	c2
CHPC1161	-	-	pos	-	c2
CHPC1166	-	-	-	-	
CHPC1170	-	-	-	-	
CHPC1182	-	-	pos	-	c2
CHPC1183	pos	pos	pos	pos	c2
CHPC1206	-	-	-	-	
CHPC1218	-	-	-	-	
CHPC1220	-	-	-	-	
CHPC1235	-	-	-	-	
CHPC1237	-	-	-	-	
CHPC1242	-	-	-	-	
CHPC1257	-	-	-	-	
CHPC1266	-	-	-	-	
CHPC1267	-	-	-	-	
CHPC1285	pos	pos	pos	pos	c2
CHPC1287	-	-	-	-	
CHPC1307	pos	pos	pos	pos	c2

[1] Bacterial cultures were challenged with 62 phages at ca. 106 PFU/ml and the pH was monitored. When the pH of a control culture without added phage was at 5.0, the pH of the infected culture was recorded, and a phage was considered infective when the pH difference was more than 0.5 (pos).

Table S2. Non-synonymous mutations found in Lcn972R mutants

			Lactococcus lactis													
			L81		L62		L98		IPLA517		IPLA641		IPLA1064		IPLA947	
Gene	**Annotation[1]**	**Sift[2]**	**D1**	**E2**	**C9**	**G9**	**C1**	**E2**	**B5**	**C6**	**C8**	**D8**	**C11**	**E11**	**R5**	
clpE	ATP-dependent protease	T	Gly246Ser													
nagA	N-acetylglucosamine-6-phosphate deacetylase	T	Ala105Val													
ysaB	ABC transporter permease	I	Pro605Thr/Phe577Ser	Phe577Val	Ile594Phe	Ile180Phe					Gly281Asp	Gly281Asp				
WP_003129615.1	Branched-chain amino acid transport system	fs		Phe98fs												
gltD	Glutamate synthase (NADPH/NADH) small chain	T		Ala21Thr												
yfjC	Acylphosphatase	T		Thr91Pro												
pbp1	Penicillin binding protein 1A/1B	I		Ala427Asp												
ftsH	Cell division protease FtsH	I		Val263Ile/ Asp343Tyr	Gly228Asp	Trp137*										
preA	Prenyl transferase	I		Asp102Tyr	Ala61Glu											
yeiF	Hypothetical protein	T			Val127Ile											
argS	Arginyl-tRNA synthetase	fs			Glu453Ala Ile452fs His451fs											
hsdR	Type I restriction endonuclease subunit R	T			Asp105Asn	Asp105Asn										
ynhC	Hypothetical protein	I				Ser299Ile										
yngF	Sugar ABC transporter permease	T				Ala87val										
feoB	Ferrous ion transport protein B	stop				Arg419*										
fbp	Fructose-1,6-bisphosphatase	T				Glu506Lys										
relA	ppGpp synthetase I	T/stop					Arg228Leu	Arg228*								
tagD1	Glycerol-3-phosphate cytidylyltransferase	stop					Glu46*	Glu46*								
yudK	Hypothetical protein	T					Met203Ile	Met203Ile								
purR	pur operon repressor	I					His30Tyr									
yijE	Hypothetical protein	I						Gln156Lys								
kinF	Sensor histidine kinase	T													Leu238Met	
obg	GTPase ObgE	T													Ser209Asn	
kinG	Sensor protein kinase	I							Ala108Asp	Ala108Asp	Ala107Val	Ala107Val	Val104Phe	Val104Phe		
pyrH	Uridylate kinase	I							Asn170Lys	Asn170Lys	Thr81Ile	Thr81Ile	Gly25Glu	Gly25Glu		
rgpD	Polysaccharide ABC transporter ATP-binding protein	Stop							Ser386*							
cstA	Carbon starvation protein	T							Ala468Thr	Ala468Thr						
rgpC	Polysaccharide ABC transporter permease	I								Arg167Ile						
murF	D-Ala-D-Ala adding enzyme	I								Ser430Ile						
dltD	D-alanine transfer protein	NA								Pro96Leu						
ysaC	ABC transporter ATP-binding protein	I									Ser146Leu					
polC	DNA polymerase III PolC	I									His1160Tyr					
lysA	Diaminopimelate decarboxylase	T									Val252Ile					
llrG	Two-component system regulator	T										Ala189Glu				
dltB	Peptidoglycan biosynthesis protein	I											Pro154Ala	Pro154Ala		
yphH	Hypothetical protein	NA											Ser126Pro			
rpoC	DNA-directed RNA polymerase subunit beta	I											Glu822Lys			
ycgF	Hypothetical protein	Stop											Gln135*	Gln135*		
acmA	Autolysin	fs												Ser240Ala Thr241Asn Ser267fs		

[1], According to KEEG (https://www.genome.jp/)

[2], Tolerant (T, white cells) or Intolerant (I, shaded cells) mutation according to SIFT: sorting Intolerant from tolerant (http://sift.bii.a-star.edu.sg/); NA, Not applicable.; *, a stop codon was introduced; fs, frameshifting,

3 Material suplementario del Capítulo III

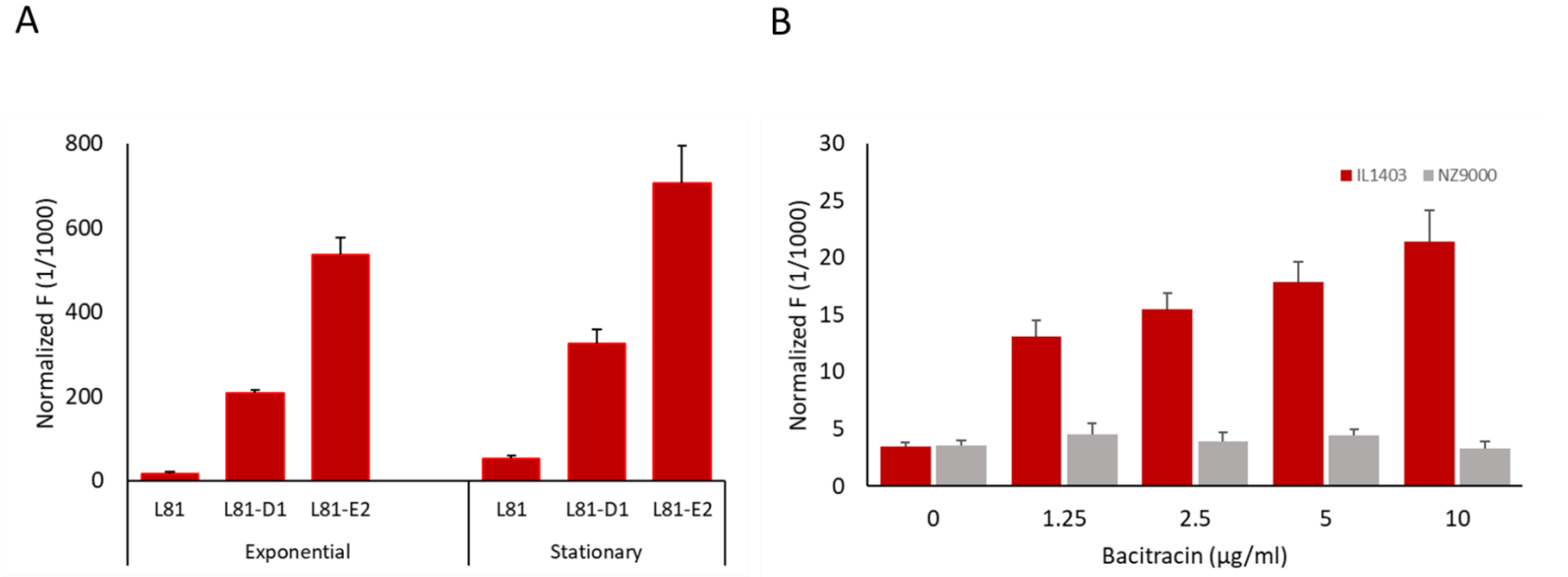


Figure S1. Activity of the P_{ysaD} promoter in *L. lactis* determined by the reporter plasmid pPRC_P_{ysaD}::*mrfp*. Detection of mCherry fluorescence (F) was recorded at 605 nm after excitation at a wavelength of 580 nm and normalized by the OD_{600} of the cell suspensions. A. Cells from *L. lactis* L81, L81-D1 and L81-E2 exponential and stationary phase cultures (only one biological replicate was analyzed, error bars are from technical replicates). B. *L. lactis* IL1403 (red bars) and NZ9000 (grey bars) after induction with bacitracin (X-axis). Average and standard deviation of three biological replicates is depicted.

Table S1. Primers used in this work

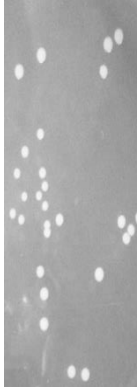
Primer	Sequence	Position and use
NcoIysaCB-F	GAGAAAACCATGGTACTCGAAGTAAAACATCTC	5' *ysaC,* cloning *ysaCB*
ysaDRca	GAAACATCATGAAAAAAATACTTTTTGGAA	5' *ysaD,* cloning *ysaDCB*
BamHIysaCB-R	GAGGCATATGGATCCCTTCTTTTTTATC	3' *ysaB,* cloning *ysaCB* and *ysaDCB*
ysaDPf	GGAATTCGTTACAAAAAGCGCTTGT	5' promoter *ysaD,* cloning in pPTPL
PysaDfPstI	CGCTGCAGCCAGTTACAAAAAGC	5' promoter *ysaD,* cloning in pRCR
ysaDPr	GCTCTAGATGATAAGTGCTATTATTCC	3' promoter *ysaD,* cloning in pPTPL and pRCR
ysaD-tF	TATGGCAGACCACAATCTCC	Intragenic *ysaD*, RT-qPCR, RT-PCR *ysaDC*
ysaD-R	GGGCTTCTTTAGGAACCTCTG	Intragenic *ysaD*, RT-qPCR
ysaC-qF	ACGTGCCTTGATTAGCCAAC	Intragenic *ysaC*, RT-qPCR
ysaC-qR	GGCTTGCAGCTAAACTTGAGTG	Intragenic *ysaC*, RT-qPCR
ysaB-qF	GCAAATGCTGTTGGTCTAGG	Intragenic *ysaB*, RT-qPCR
ysaB-qR	TGCTTCTCGGGTATAGTAGG	Intragenic *ysaB*, RT-qPCR
ysaA-qF	TCCTCCTGTGAGTGAAGTAG	Intragenic *ysaA*, RT-qPCR
ysaA-qR	TTAGAGCTGCAAGCCAAG	Intragenic *ysaA*, RT-qPCR
llrG-qF	ACCGCTATGAATCAAGGAGCTG	Intragenic *llrG,* RT-qPCR
llrG-qR	GCTCAATTTCTTCGCGTTCACC	Intragenic *llrG,* RT-qPCR, RT-PCR *ysaAllrG*
kinG-qF	CTTGCTGAGCGTGAAATGGAAG	Intragenic *kinG,* RT-qPCR
kinG-qR	TTCATCTGGTGCGACCAGAC	Intragenic *kinG,* RT-qPCR
Tuf-F	GGTAGTTGTCGAAGAATGGAGTGTGA	RT-qPCR reference
Tuf-R	TAAACCAGGTTCAATCACTCCACACA	RT-qPCR reference
ysaC-tR	TTCGCCTTCTTCTACACC	intragenic *ysaC*, RT-PCR *ysaDC*
ysaC-tF	TTCTTGGGAGCATCAGAG	intragenic *ysaC*, RT-PCR *ysaCB*
ysaB-tR	TGCTGTGGGACTAATGAC	intragenic *ysaB*, RT-PCR *ysaCB*
ysaB-tF	GCAACGAGTCGTGTTTAC	intragenic *ysaB*, RT-PCR ysaBA
ysaA-tR	GGCATAAGCGCAAATGAG	intragenic *ysaA*, RT-PCR ysaBA
ysaA-tF	GTGAGTGATACAGAAATGATAGC	intragenic *ysaA*, RT-PCR ysaA*llrG*
kinG-tF	GGGACGTCTGTGACAATAAC	Intragenic kinG, RT-PCR *kinGtyrA*
tyrA-tR	TATCTGCCGCCATTACTC	Intragenic *tyrA,* RT-PCR *kinGtyrA*

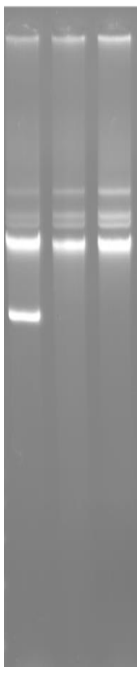
Tabla S2. Comparison of the Ysa and the two component system G proteins from *L. lactis* strains according to BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

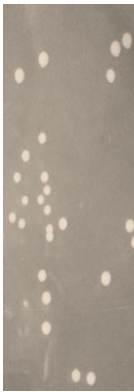
	L. lactis	Accesion	Identities	Positives	e value
YsaD (119 aa)	**L81/IL1403**	AAK05849.1	119/119(100%)	119/119(100%)	6,00E-84
	L81/NZ9000	ADJ60910.1	95/119(80%)	109/119(91%)	2,00E-66
	IL1403/NZ9000	AAK05849.1 / ADJ60910.1	95/119(80%)	109/119(91%)	2,00E-66
YsaC (259 aa)	**L81/IL1403**	AAK05848.1	257/259(99%)	258/259(99%)	0.0
	L81/NZ9000	ADJ60908.1	252/259(97%)	256/259(98%)	0.0
	IL1403/NZ9000	AAK05848.1 / ADJ60908.1	252/259(97%)	257/259(99%)	0.0
YsaB (667 aa)	**L81/IL1403**	AAK05847.1	665/667(99%)	666/667(99%)	0.0
	L81/NZ9000	Sin similitud			
	IL1403/NZ9000				

	L. lactis	Accesion	Identities	Positives	e value
YsaA (354 aa)	**L81/IL1403**	AAK05846.1	353/354(99%)	353/354(99%)	0.0
	L81/NZ9000	ADJ60907.1	317/354(90%)	337/354(95%)	0.0
	IL1403/NZ9000	AAK05846.1 / ADJ60907.1	318/354(90%)	338/354(95%)	0.0
LlrG (223 aa)	**L81/IL1403**	AAK05845.1	223/223(100%)	223/223(100%)	2E-164
	L81/NZ9000	ADJ60906.1	212/223(95%)	217/223(97%)	8E-157
	IL1403/NZ9000	AAK05845.1 / ADJ60906.1	212/223(95%)	217/223(97%)	8E-157
KinG (291 aa)	**L81/IL1403**	AAK05844.1	291/291(100%)	291/291(100%)	0.0
	L81/NZ9000	ADJ60905.1	257/291(88%)	278/291(95%)	0.0
	IL1403/NZ9000	AAK05844.1 / ADJ60905.1	257/291(88%)	278/291(95%)	0.0

DOCUMENTACIÓN







Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

FORMULARIO RESUMEN DE TESIS POR COMPENDIO

1.- Datos personales solicitante	
Apellidos: López González	Nombre: María Jesús

Curso de inicio de los estudios de doctorado	2014

	SI	NO
Acompaña acreditación por el Director de la Tesis de la aportación significativa del doctorando	X	
Acompaña memoria que incluye		
Introducción justificativa de la unidad temática y objetivos	X	
Copia completa de los trabajos *	X	
Resultados/discusión y conclusiones	X	
Informe con el factor de impacto de las publicaciones	X	
Se acompaña aceptación de todos y cada uno de los coautores a presentar el trabajo como tesis por compendio (Art. 32.4.b)	X	
Se acompaña renuncia de todos y cada uno de los coautores no doctores a presentar el trabajo como parte de otra tesis de compendio (Art. 32.4.c)		

* Ha de constar el nombre y adscripción del autor y de todos los coautores así como la referencia completa de la revista o editorial en la que los trabajos hayan sido publicados o aceptados en cuyo caso se aportará justificante de la aceptación por parte de la revista o editorial

FOR-MAT-VOA-033

Artículos, Capítulos, Trabajos

Trabajo, Artículo 1

Titulo (o título abreviado)	Resistance to bacteriocin Lcn972 improves oxygen tolerance of *Lactococcus lactis* IPLA947 without compromising its performance as a dairy starter
Fecha de publicación	20/07/2018
Fecha de aceptación	10/07/2018
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	JCR 2018. BMC Microbiology, 18: 76- Q2
Factor de impacto	3,287

Coautor2 X Doctor □ No doctor . Indique nombre y apellidos	Ana Belén Campelo
Coautor3 X Doctor □ No doctor . Indique nombre y apellidos	Antonia Picón
Coautor4 X Doctor □ No doctor . Indique nombre y apellidos	Ana Rodríguez
Coautor5 X Doctor □ No doctor . Indique nombre y apellidos	Beatriz Martínez

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Trabajo, Artículo 2

Titulo (o título abreviado)	Adaptive Evolution of Industrial Lactococcus lactis Under Cell Envelope Stress Provides Phenotypic Diversity
Fecha de publicación	05/11/2018
Fecha de aceptación	17/10/2018
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	JCR 2018. Frontiers in microbiology, 9:2654- Q1
Factor de impacto	4,259

Coautor2 X Doctor □ No doctor . Indique nombre y apellidos	Susana Escobedo
Coautor3 X Doctor □ No doctor . Indique nombre y apellidos	Ana Rodríguez
Coautor4 X Doctor □ No doctor . Indique nombre y apellidos	Ana Rute Neves
Coautor5 X Doctor □ No doctor . Indique nombre y apellidos	Thomas Janzen
Coautor6 X Doctor □ No doctor . Indique nombre y apellidos	Beatriz Martínez

Trabajo, Artículo 3

Titulo (o título abreviado)	Mutations Selected After Exposure to Bacteriocin Lcn972 Activate a Bce-Like Bacitracin Resistance Module in Lactococcus lactis
Fecha de publicación	13/08/2020
Fecha de aceptación	09/07/2020
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	JCR 2020. Frontiers in microbiology, 11:1805- Q1
Factor de impacto	5,64

Coautor1 X Doctor □ No doctor . Indique nombre y apellidos	Ana Belén Campelo
Coautor2 □ Doctor □ No doctor . Indique nombre y apellidos	María Jesús López González
Coautor3 X Doctor □ No doctor . Indique nombre y apellidos	Susana Escobedo
Coautor4 X Doctor □ No doctor . Indique nombre y apellidos	Thomas Janzen
Coautor5 X Doctor □ No doctor . Indique nombre y apellidos	Ana Rute Neves
Coautor6 X Doctor □ No doctor . Indique nombre y apellidos	Ana Rodríguez
Coautor7 X Doctor □ No doctor . Indique nombre y apellidos	Beatriz Martínez

En caso de compendio de un número de artículos superior a seis, se incorporarán hojas suplementarias conforme a este modelo