TESIS DE LA UNIVERSIDAD DE ZARAGOZA
2022
160

Daniel Berdejo Martínez

Aislamiento y caracterización de variantes genéticas bacterianas resistentes como herramienta de estudio para la mejora de la conservación de alimentos.

Director/es
Garcia Gonzalo, Diego
Pagán Tomás, Rafael

ISSN 2254-7606

Prensas de la Universidad
Universidad Zaragoza

ISSN 2254-7606

Universidad Zaragoza
1542

Tesis Doctoral

AISLAMIENTO Y CARACTERIZACIÓN DE VARIANTES GENÉTICAS BACTERIANAS RESISTENTES COMO HERRAMIENTA DE ESTUDIO PARA LA MEJORA DE LA CONSERVACIÓN DE ALIMENTOS.

Autor

Daniel Berdejo Martínez

Director/es

Garcia Gonzalo, Diego
Pagán Tomás, Rafael

UNIVERSIDAD DE ZARAGOZA
Escuela de Doctorado

Programa de Doctorado en Calidad, Seguridad y Tecnología de los Alimentos

2021

FACULTAD DE VETERINARIA

DEPARTAMENTO DE PRODUCCIÓN ANIMAL Y CIENCIA DE LOS ALIMENTOS

Aislamiento y caracterización de variantes genéticas bacterianas resistentes como herramienta de estudio para la mejora de la conservación de alimentos

Isolation and characterisation of resistant bacterial genetic variants as a research tool for the improvement of food preservation

Memoria para optar al grado de Doctor por la Universidad de Zaragoza presentada por:

Daniel Berdejo Martínez

Directores:

Dr. Diego García Gonzalo

Dr. Rafael Pagán Tomás

Abril, 2021

D. Rafael Pagán Tomás y D. Diego García Gonzalo, Catedrático y Profesor Titular de Universidad y, respectivamente, del Área de Tecnología de los Alimentos del Departamento de Producción Animal y Ciencia de los Alimentos de la Facultad de Veterinaria de la Universidad de Zaragoza,

Certifican:

Que la Tesis Doctoral titulada "Aislamiento y caracterización de variantes genéticas bacterianas resistentes como herramienta de estudio para la mejora de la conservación de alimentos", de la que es autor Daniel Berdejo Martínez, ha sido realizada bajo su dirección, su contenido corresponde con el Proyecto de Tesis aprobado en su momento y cumple las condiciones requeridas para optar al grado de Doctor por la Universidad de Zaragoza.

En Zaragoza, a 26 de abril de 2021

Fdo.: Dr. Rafael Pagán Tomás

Fdo.: Dr. Diego García Gonzalo

La realización de esta Tesis Doctoral ha sido posible gracias a una ayuda para la Formación de Profesorado Universitario (FPU, convocatoria 2015) concedida por el Ministerio de Educación, Cultura y Deporte (FPU15/02703), así como al apoyo y financiación del grupo de investigación de referencia A03_20R-Nuevas tecnologías de procesado de los alimentos de la Universidad de Zaragoza, al Instituto Agroalimentario de Aragón (IA2), y a la concesión de los siguientes proyectos:

- Proyecto PGC2018-093789-B-I00 "Generación de variantes microbianas resistentes (mutantes y esporos superlatentes) y desarrollo de estrategias que favorezcan su eliminación y/o prevención en alimentos". Agencia Estatal de Investigación (2019/2021). Investigador principal: Diego García Gonzalo, Rafael Pagán Tomás.
- Proyecto AGL2015-69565-P "Búsqueda de estrategias de conservación de alimentos que aseguren la inactivación de los microorganismos más resistentes (esporos y/o mutantes anormalmente resistentes)". MINECO (2016/2020). Investigador principal: Santiago Condón Usón.

Tesis Doctoral como compendio de publicaciones

La Tesis Doctoral titulada "Aislamiento y caracterización de variantes genéticas bacterianas resistentes como herramienta de estudio para la mejora de la conservación de alimentos" de la que es autor Daniel Berdejo Martínez se presenta como un compendio de las siguientes publicaciones que se distribuyen en la introducción, material y métodos y resultados:

Berdejo, D., Pagán, E., García-Gonzalo, D., Pagán, R. (2019). Review: Exploiting the synergism among physical and chemical processes for improving food safety. *Current Opinion in Food Science*, 30, 14-20. doi: 10.1016/j.cofs.2018.08.004.

Berdejo, D., Pagán, E., Merino, N., García-Gonzalo, D., Pagán, R. (Aceptado). Evolution assays for the isolation of mutant bacteria resistant to natural antimicrobials. Marciane, M. (Ed.), *Detection and enumeration of bacteria, yeast, viruses, and protozoan in foods and freshwater*. Springer Nature, 233 Spring Street, New York, NY 10013, U.S.A.

Berdejo, D., Chueca, B., Pagán, E., Renzoni, A., Kelley, W., Pagán, R., García-Gonzalo, D. (2019). Sub-inhibitory doses of individual constituents of essential oils can select for *Staphylococcus aureus* resistant mutants. *Molecules,* 24, 170. doi: 10.3390/molecules24010170.

Berdejo, D., Merino, N., Pagán, E., García-Gonzalo, D., Pagán, R. (2020). Genetic variants and phenotypic characteristics of *Salmonella* Typhimurium-resistant mutants after exposure to carvacrol. *Microorganisms*, 8, 937. doi: 10.3390/microorganisms8060937.

Berdejo, D., Pagán, E., Merino, N., Pagán, R., García-Gonzalo, D. (2020). Incubation with a complex orange essential oil leads to evolved mutants with increased resistance and tolerance. *Pharmaceuticals*, 13, 239. doi:10.3390/ph13090239.

Berdejo, D., Pagán, E., Merino, N., García-Gonzalo, D., Pagán, R. (Enviado). Emerging mutant populations of *Listeria monocytogenes* EGD-e under selective pressure of *Thymbra capitata* essential oil question its use in food preservation. *Food Research International*.

Berdejo, D., Pagán, E., Merino, N., Botello-Morte, L., García-Gonzalo, D., Pagán, R. (Enviado). *Salmonella enterica* genetic variants isolated after lethal treatment with *Thymbra capitata* essential oil show increased resistance in milk. *International Journal of Food Microbiology*.

Pagán, E., **Berdejo, D.**, Espina, L., García-Gonzalo, D., Pagán, R. (2018). Antimicrobial activity of suspensions and nanoemulsions of citral in combination with heat or pulsed electric fields. *Letters in Applied Microbiology,* 66, 63-70. doi:10.1111/lam.12815.

Merino, N.*, **Berdejo, D.***, Bento, R., Salman, H., Lanz, M., Maggi, F., Sánchez-Gómez, S., García-Gonzalo, D., Pagán, R. (2019). Antimicrobial efficacy of *Thymbra capitata* (L.) Cav. essential oil loaded in self-assembled zein nanoparticles in combination with heat. *Industrial Crops & Products*, 133: 98-104. doi: 10.1016/j.indcrop.2019.03.003.

López-Miranda, S., **Berdejo, D.**, Pagán, E., García-Gonzalo, D., Pagán, R. (2020). Modified cyclodextrin type and dehydration methods exert a significant effect on the antimicrobial activity of encapsulated carvacrol and thymol. *Journal of the Science of Food and Agriculture*. doi: 10.1002/jsfa.11017.

Índice

ÍNDICE

Resumen ……..…….…….……..……1

Abstract ……..……..……6

1. Introducción ....…….……...……11

1. La conservación de alimentos……13

1) Métodos de inhibición del crecimiento microbiano …….……13

2) Métodos de inactivación microbiana ……...……14

3) Toxiinfecciones alimentarias……15

2. Estudio de los mecanismos de inhibición e inactivación de los métodos de conservación..……..…….……....16

1) Estudio de daños a nivel celular……..……...…….…….17

2) Técnicas ómicas ...…….……...19

a) Estudios genómicos……..….20

b) Estudios transcriptómicos ..…….……..…….……..21

c) Estudios de proteómica y metabolómica……..……..……...23

3) Estudio de poblaciones resistentes bacterianas……..……...……24

a) Estudio de fenómenos de adaptación……..……..……..…...25

b) Estudio de variantes genéticas…….…..26

i. Técnicas de secuenciación del genoma……..…..27

ii. Ensayos de evolución frente a métodos de inhibición bacteriana ...…….…….……..….29

iii. Ensayos de evolución frente a métodos de inactivación bacteriana ……....30

3. Aceites esenciales (AE) y sus constituyentes individuales (CI)…….31

4. Procesos combinados…….....34

1) Manuscrito I. Exploiting the synergism among physical and chemical processes for improving food safety……..……..……..37

2. Objetivos ..……...…….……..47

1. Justificación de los trabajos…….……..……..……..49

2. Objetivos ....……..…….……55

3. Material y métodos…….…….55

1. Ensayos de evolución para la obtención y caracterización de variantes genéticas resistentes a nivel fenotípico y genotípico……..…….....58

2. Estudios de emulsificación y encapsulación de AE y CI para la mejora de su estabilidad y solubilidad en agua……..……...…..…**59**
3. Manucrito II. Evolution assays for the isolation of mutant bacteria resistant to natural antimicrobials……..…….**61**

4. Resultados ……...……....**81**

1. Manuscrito III. Sub-inhibitory doses of individual constituents of essential oils can select for *Staphylococcus aureus* resistant mutants…….….**83**
2. Manuscrito IV. Genetic variants and phenotypic characteristics of *Salmonella* Typhimurium-resistant mutants after exposure to carvacrol…..**107**
3. Manuscrito V. Incubation with a complex orange essential oil leads to evolved mutants with increased resistance and tolerance……...…..**137**
4. Manuscrito VI. Emerging mutant populations of *Listeria monocytogenes* EGD-e under selective pressure of *Thymbra capitata* essential oil question its use in food preservation……..…..**159**
5. Manuscrito VII. *Salmonella enterica* genetic variants isolated after lethal treatment with *Thymbra capitata* essential oil show increased resistance in milk....……... ……..…**193**
6. Manuscrito VIII. Antimicrobial activity of suspensions and nanoemulsions of citral in combination with heat or pulsed electric fields……..….**231**
7. Manuscrito IX. Antimicrobial efficacy of *Thymbra capitata* (L.) Cav. essential oil loaded in self-assembled zein nanoparticles in combination with heat…….……..**243**
8. Manuscrito X. Modified cyclodextrin type and dehydration methods exert a significant effect on the antimicrobial activity of encapsulated carvacrol and thymol..…….…….……**253**

5. Discusión general..…….…….....**265**

1. Puesta a punto de los ensayos de evolución para el aislamiento variantes genéticas resistentes…….……**267**
2. Efecto mutagénico por la exposición a AE y CI…….……**269**
3. Caracterización fenotípica de las variantes genéticas resistentes….……..…**271**
    1) Resistencia y tolerancia directa frente a AE y CI……..…….…….**271**
    2) Resistencia cruzada frente a otras tecnologías de conservación…….**275**
    3) Resistencia cruzada frente a antibióticos…….…….…….….**277**

4. Caracterización genotípica de las variantes resistentes e identificación de las modificaciones genéticas responsables de los cambios observados en su fenotipo...…….…...……...…**280**
5. Evaluación de la resistencia de las variantes genéticas en alimentos modelo para explorar su repercusión en la industria agroalimentaria……..**285**
6. Preparación de nanoemulsiones y encapsulados de AE y CI para facilitar y potenciar su empleo como conservantes alimentarios y biocidas……...**287**

6. Conclusiones……..…….……...…**293**

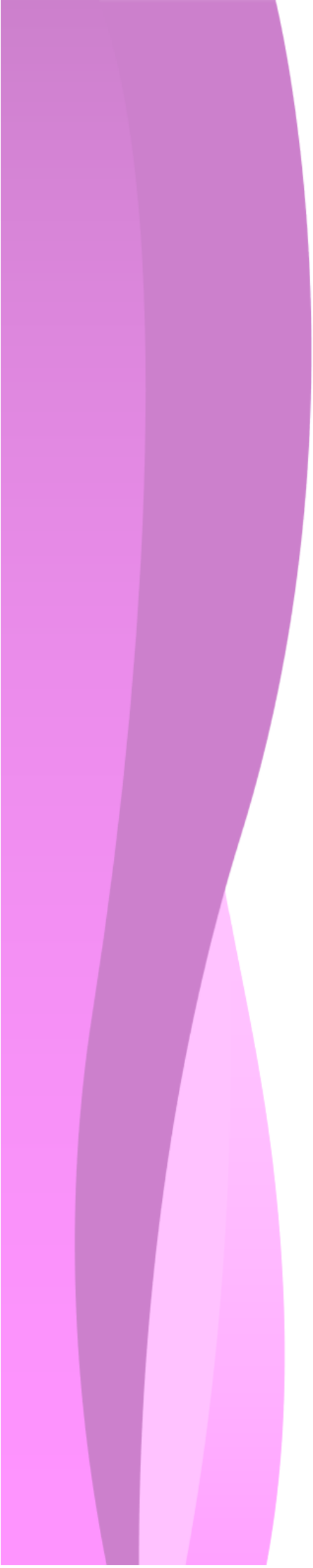
Conclusions ……..……..**298**

7. Bibliografía……...…….…....**303**

8. Anexos……..…….……..…….…….....**323**

1. Factor de impacto de las revistas y áreas temáticas……**325**
2. Aceptación Manuscrito II……...**327**

Resumen



Resumen

La mejora e implementación de los métodos de conservación de los alimentos en la industria agroalimentaria precisa del conocimiento detallado de sus mecanismos de inhibición e inactivación microbiana. La aparición de cepas bacterianas resistentes en la cadena alimentaria puede comprometer la inocuidad y estabilidad de los alimentos. Sin embargo, a su vez, también pueden utilizarse como herramienta de estudio. La descripción de las bases fisiológicas responsables de la resistencia y de los mecanismos de respuesta celular permite aportar conocimiento para el diseño de estrategias de conservación más efectivas y eficientes.

Por tanto, el principal objetivo de esta tesis doctoral fue la obtención de variantes genéticas bacterianas resistentes, y su caracterización fenotípica y genotípica, como estrategia para estudiar los mecanismos de resistencia celular frente al uso de antimicrobianos y otros métodos de conservación de los alimentos. Concretamente, se ha abordado por primera vez el aislamiento de variantes genéticas resistentes de *Staphylococcus aureus*, *Salmonella enterica* Typhimurium y *Listeria monocytogenes* mediante el uso de aceites esenciales (AE) y sus constituyentes individuales (CI), con el fin de conocer las bases de sus propiedades antimicrobianas, así como profundizar en la repercusión de la aparición de estas variantes en la cadena alimentaria.

En primer lugar, se realizó la puesta a punto de la metodología para la realización de ensayos de evolución, mediante la exposición prolongada a concentraciones subinhibitorias o la aplicación de tratamientos letales, logrando el aislamiento de variantes genéticas bacterianas frente a AE y CI. La determinación de la concentración mínima inhibitoria (CMI) y la concentración mínima bactericida (CMB) de las variantes aisladas y su comparación con las respectivas cepas parentales, demostraron la aparición de variantes resistentes y tolerantes frente a AE y CI tras los ensayos de evolución mediante ambos protocolos:

- *S. aureus* frente a carvacrol, citral, óxido de limoneno y AE de naranja mediante dosis subinhibitorias.
- *S.* Typhimurium frente a carvacrol, mediante dosis subinhibitorias y tratamientos letales, y frente a AE de tomillo mediante tratamientos letales.
- *L. monocytogenes* frente a AE de tomillo mediante dosis subinhibitorias y tratamientos letales.

No obstante, la aparición de variantes genéticas resistentes no habría sido provocada por un aumento de la frecuencia mutagénica tras la utilización de AE y CI, sino probablemente por la presión selectiva ejercida sobre las poblaciones bacterianas que permitió seleccionar aquellas variantes con mayor resistencia.

La caracterización fenotípica mediante el estudio de cinéticas de crecimiento en presencia de los AE y CI y la obtención de curvas de supervivencia tras tratamientos letales, permitió explicar cómo estas variantes resistentes emergerían a lo largo de los ensayos de evolución. Por un lado, debido a una mejor adaptación y mayor velocidad de crecimiento en los ensayos a dosis subinhibitorias y, por otro lado, a causa de una mayor supervivencia bacteriana en los ensayos de tratamientos letales.

Además de presentar una mayor supervivencia frente a los agentes de selección, algunas de las variantes resistentes revelaron el desarrollo de resistencia cruzada frente a otras tecnologías de conservación como los tratamientos térmicos, o frente a antibióticos como aminoglucósidos, betalactámicos, quinolonas y tetraciclinas.

La secuenciación genómica de las variantes genéticas resistentes permitió identificar las mutaciones seleccionadas a través de los ensayos de evolución frente a los AE y CI. Estas modificaciones genéticas fueron localizadas en reguladores transcripcionales relacionados con la respuesta celular al estrés oxidativo (*soxR yfhP*), enzimas relacionadas con la síntesis y reparación de las membranas celulares (*accA*, lmo1647), proteínas receptoras y de transporte de membrana (*fepA*, *nirC*, *trkA*) y diversas enzimas metabólicas (*aroC*, *hepT*, *nirB*), entre otras. Cabe destacar que las variaciones genéticas localizadas en *soxR* revelaron ser una de las principales causas del aumento de resistencia y tolerancia frente a los AE y CI, así como de la resistencia cruzada frente a antibióticos. De esta manera, se confirma el importante papel del estrés oxidativo en el mecanismo de inhibición e inactivación de estos antimicrobianos y se señala a las envolturas celulares como una de las principales estructuras diana de los AE y CI, así como de las diferentes tecnologías de conservación de los alimentos frente a las que se observó resistencia cruzada.

Por otro lado, el incremento de resistencia y tolerancia frente a AE se mantuvo e incluso fue mayor en un alimento modelo, leche desnatada, revelando el riesgo microbiológico que puede suponer la aparición de variantes resistentes en la cadena alimentaria.

De forma complementaria al objetivo principal, se evaluó el empleo de técnicas de emulsificación y encapsulación con el fin de facilitar y potenciar el empleo de AE y CI como conservantes alimentarios o biocidas. La preparación de nanoemulsiones, con tween 80 y etanol, y encapsulaciones, con zeínas y ciclodextrinas, permitió mejorar la estabilidad y la hidrosolubilidad de los AE y CI manteniendo, e incluso en algunos casos, mejorando sus propiedades antimicrobianas cuando se aplicaron de forma única o combinada con otras tecnologías de conservación, como los tratamientos térmicos o los pulsos eléctricos de alto voltaje.

El desarrollo de esta Tesis Doctoral supone la puesta a punto de herramientas para el aislamiento y caracterización de variantes genéticas bacterianas resistentes frente a métodos de conservación de alimentos. Estos estudios, además de evidenciar la aparición de variantes resistentes que pueden llegar a suponer un riesgo alimentario, permiten describir estructuras y rutas metabólicas relacionadas con la resistencia celular y, de tal modo, aportan información sobre los mecanismos de inhibición e inactivación bacteriana, lo que resulta imprescindible para mejorar las estrategias de conservación de los alimentos. En esta Tesis Doctoral se ha abordado el estudio de antimicrobianos naturales, concretamente de AE y sus CI, que pueden ser utilizados individualmente o en procesos combinados en la conservación de alimentos. Los principales resultados de este trabajo se han recogido en un capítulo de libro y 9 artículos científicos, uno de ellos de revisión bibliográfica.

El empleo de AE y CI a diferentes concentraciones facilitó el desarrollo de protocolos de aislamiento de variantes resistentes tanto mediante métodos de inhibición como de inactivación microbiana, de forma que pueden ser fácilmente implementados para el estudio de otros métodos de conservación. En este sentido, el diseño experimental puesto a punto en esta tesis doctoral permite dar continuidad a esta línea de investigación en mi grupo de investigación, habiéndose ya iniciado el aislamiento y caracterización de variantes genéticas bacterianas resistentes frente a otros métodos de conservación de los alimentos como el calor, las altas presiones hidrostáticas o los pulsos eléctricos de alto voltaje, etc., y de este modo, profundizando en sus mecanismos de inhibición o inactivación y resistencia bacteriana, así como en las implicaciones que pudieran ocasionar en el ámbito de la Salud Pública.

Abstract

The improvement and implementation of food preservation methods in the agri-food industry requires a deep knowledge of their microbial inhibition and inactivation mechanisms. The emergence of resistant bacterial strains in the food chain might compromise food safety and stability. However, those strains can also be used as a research tool. The description of the physiological bases responsible for resistance and cellular response mechanisms might provide knowledge for the design of more effective and efficient preservation strategies.

Therefore, the main objective of this PhD thesis was to isolate resistant bacterial genetic variants, and to chacterize their phenotype and genotype, as a strategy to study the mechanisms of cellular resistance against antimicrobials compounds and other food preservation methods. Specifically, the isolation of resistant genetic variants has been achieved for the first time in *Staphylococcus aureus*, *Salmonella enterica* Typhimurium and *Listeria monocytogenes* using essential oils (EOs) and their individual constituents (ICs), in order to understand the basis of their antimicrobial properties, as well as to study the impact of the emergence of those variants in the food chain.

Firstly, the methodology for evolution assays was carried out by prolonged exposure to subinhibitory concentrations or the application of lethal treatments, achieving the isolation of bacterial genetic variants against EOs and ICs. The determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the isolated variants and their comparison with their corresponding parental strains showed the occurrence of resistant and tolerant variants against EOs and ICs after the evolution assays using both protocols:

- *S. aureus* against carvacrol, citral, limonene oxide and orange EO by subinhibitory doses.
- *S.* Typhimurium against carvacrol by subinhibitory doses and lethal treatments, and against thyme EO by lethal treatments.
- *L. monocytogenes* against thyme EO by subinhibitory doses and lethal treatments.

However, the emergence of resistant genetic variants would not have been triggered by an increase in mutagenic frequency by EOs and ICs, but probably by the selective pressure exerted on the bacterial populations that allowed the selection of those variants with higher resistance.

Phenotypic characterisation by studying growth kinetics in the presence of EOs and ICs and by obtaining survival curves after lethal treatments allowed to explain how these resistant variants would emerge throughout the evolution assays. On the one hand, because of a better adaptation and higher growth rate in presence of subinhibitory doses and, on the other hand, due to a higher bacterial survival in presence of lethal doses.

In addition to higher survival against the selection agents, some of the resistant variants revealed the development of cross-resistance against other preservation technologies such as heat treatments, or against antibiotics such as aminoglycosides, beta-lactams, quinolones and tetracyclines.

Genomic sequencing of the resistant genetic variants allowed the identification of mutations selected during evolution assays in presence of EOs and ICs. Those genetic modifications were located in transcriptional regulators related to the cellular response to oxidative stress (*soxR*, *yfhP*); enzymes related to the synthesis and repair of cell membranes (*accA*, lmo1647); receptor and membrane transport proteins (*fepA*, *nirC*, *trkA*); and several metabolic enzymes (*aroC*, *hepT*, *nirB*), among others. It should be noted that genetic variations located in *soxR* were one of the main causes of increased resistance and tolerance to EOs and ICs, as well as cross-resistance to antibiotics. These results confirm the important role of oxidative stress in the mechanism of inhibition and inactivation by these antimicrobials compounds and point to cell envelopes as one of the main target structures of EOs and ICs, as well as of the different food preservation technologies against which cross-resistance was observed.

On the other hand, the increase in resistance and tolerance to EOs was maintained and even increased in a model food, skimmed milk, revealing the potential microbiological risk due to the emergence of those variants in the food chain.

In addition to the main objective, the use of emulsification and encapsulation techniques was evaluated in order to facilitate and improve the use of EOs and ICs as food preservatives or biocides. The preparation of nanoemulsions, with tween 80 and ethanol, and encapsulations, with zeins and cyclodextrins, improved the stability and water solubility of EOs and ICs while maintaining, and in some cases even improving, their antimicrobial properties when they were applied alone or in combination with other preservation technologies, such as heat treatments or pulsed electric fields.

The development of this PhD thesis involves the design and improvement of the required tools for the isolation and characterization of bacterial genetic variants resistant to food preservation methods. In addition to highlight that the appearance of resistant variants might pose a food risk, these studies allow to describe structures and metabolic pathways related to bacterial resistance and, consequently, to provide information on the mechanisms of bacterial inhibition and inactivation, which is essential for improving food preservation strategies. This PhD thesis has addressed the study of natural antimicrobials, specifically EOs and their ICs, which can be used individually or in combined processes in food preservation. The main results of this work have been compiled in a book chapter and 9 scientific articles, one of which is a scientific review.

The use of EOs and ICs at different concentrations allowed the development of resistant variant isolation protocols for both inhibition and inactivation preservation methods, so that they can be easily applied for the study of other food preservation methods. Thus, the experimental design developed in this PhD thesis allows the continuance of this research line in my research group, having initiated the isolation and characterization of bacterial genetic variants resistant to other food preservation methods such as heat, high hydrostatic pressure or pulsed electric fields, etc., and thus, providing knowledge about their mechanisms of inhibition or inactivation and bacterial resistance, as well as the implications they could pose in the field of public health.

1. Introducción

1. Introducción

1.1. La conservación de alimentos

La conservación de alimentos ha sido una de las principales preocupaciones de la sociedad para su subsistencia. A medida que el hombre ha evolucionado, los métodos de conservación también lo han hecho. Desde el descubrimiento del fuego, que permitía el cocinado de los alimentos, pasando por la desecación, la fermentación o la salazón, entre otros, el desarrollo de nuevas técnicas de conservación ha permitido prolongar la vida útil de los alimentos, aunque, en sus inicios, se desconocía el papel de éstas en el retraso del deterioro de los alimentos, así como de las enfermedades de transmisión alimentaria. Fue más tarde, con el descubrimiento de los microorganismos, cuando se comenzó a comprender el efecto de estas técnicas en la estabilidad y la seguridad de los alimentos.

Actualmente, la continua investigación en el ámbito de la higiene y la tecnología alimentaria fomenta la mejora de los métodos de conservación, con el objetivo de diseñar estrategias más seguras y eficientes que permitan alargar el periodo de vida útil de los alimentos (Abdel-Aziz, Asker, Keera, & Mahmoud, 2016). Estos métodos se pueden clasificar en dos grupos dependiendo de su objetivo. Por un lado, se encuentran los métodos que persiguen la inactivación de los microorganismos patógenos y alterantes presentes en el alimento (Mañas & Pagán, 2005) y, por otro lado, aquellos que buscan la inhibición o retraso de la proliferación microbiana para evitar concentraciones perjudiciales en el momento de su consumo (Montville & Matthews, 2013). No obstante, cabe destacar que las actuales estrategias en la industria se basan en la combinación de varios métodos de conservación, enmarcados en ambos grupos, con el propósito de controlar los diversos riesgos microbiológicos que presentan los alimentos (Erkmen & Bozoglu, 2016).

1.1.1. Métodos de inhibición del crecimiento microbiano

La refrigeración, normalmente junto a atmósferas modificadas, es una de las tecnologías más empleadas como método de inhibición del crecimiento microbiano para alargar el periodo de vida útil de los alimentos, sin apenas modificar su calidad. Sin embargo, precisa de su utilización de manera continuada (cadena del frío) y la estabilidad conseguida en el alimento es limitada, ya que, si bien consigue ralentizar la proliferación de la mayoría de los microorganismos, no logra en algunos casos, como sucede en los

microorganismos psicrótrofos, su completa inhibición. Existen otros métodos de conservación más efectivos como son la salazón, la desecación o la acidificación y/o fermentación, aunque su empleo conlleva la modificación de las propiedades sensoriales del alimento: sabores salados, ácidos, nuevos aromas, entre otros; o la congelación, pero esta tecnología provoca daños celulares en el producto que conducen a la alteración de su textura.

La adición de aditivos como conservantes alimentarios, tales como sorbatos, sulfitos o nitratos/nitritos también ocupan un importante lugar en la actualidad. Sin embargo, el empleo de estos conservantes de síntesis química está cada vez más restringido debido a los efectos alergénicos, tóxicos y carcinogénicos que pueden presentar estos compuestos, o sus derivados, en base a las concentraciones empleadas y a los tiempos de exposición, además de un fuerte y creciente rechazo social por parte de los consumidores (Carocho, Barreiro, Morales, & Ferreira, 2014). Por esta razón, se ha promovido el empleo de otros antimicrobianos de origen natural (Quinto et al., 2019) y, de hecho, algunos compuestos de origen microbiano como las bacteriocinas (natamicina) o de origen animal como la lisozima, se encuentran ya autorizados para su uso en la conservación de alimentos. Otros antimicrobianos naturales de origen vegetal, que han suscitado gran interés, son los aceites esenciales (AE) y sus constituyentes individuales (CI), debido al amplio espectro de acción y su elevada actividad antimicrobiana (Bhavaniramya, Vishnupriya, Al-Aboody, Vijayakumar, & Baskaran, 2019). En este sentido, algunas investigaciones persiguen su empleo en los envases alimentarios para prolongar la vida útil de los alimentos. No obstante, su uso en la industria alimentaria todavía se encuentra limitado por las modificaciones organolépticas que ocasionan en los alimentos a las dosis requeridas para su empleo de forma efectiva, así como por su inestabilidad frente a agentes oxidantes y su baja solubilidad en medios acuosos.

1.1.2. Métodos de inactivación microbiana

Los tratamientos térmicos, desde los más suaves como la termización, hasta los más intensos como la esterilización, pasando por la pasteurización, son el método de inactivación por excelencia más ampliamente utilizados en la industria agroalimentaria. El efecto del calor sobre múltiples dianas esenciales en las células microbianas, como son las envolturas celulares, el DNA, el RNA, las proteínas, los ribosomas, y otras enzimas, así como la generación de especies reactivas de oxígeno (ROS), respalda su elevada

efectividad para el control microbiano en la conservación de alimentos. No obstante, esta tecnología, y principalmente a altas intensidades, conlleva la alteración de los atributos del alimento, como es la modificación organoléptica: color, aroma, sabor, textura, etc., así como la pérdida de sus propiedades nutricionales y funcionales.

Por esta razón, la industria ha puesto a punto otras tecnologías no térmicas (Knorr et al., 2011; Zhang, Wang, Zeng, Han, & Brennan, 2019), como por ejemplo las altas presiones hidrostáticas (APH), empleadas en la conservación de productos cárnicos, pesqueros y bebidas; la luz ultravioleta, principalmente de longitud de onda tipo C (UV-C), utilizada para pasterizar alimentos líquidos, agua de consumo o de lavado, así como para la descontaminación superficial de algunos alimentos; o las radiaciones ionizantes (RI), cuyo uso se encuentra muy restringido en la Unión Europea, a productos muy determinados y a bajas dosis, pero que en otros países son ampliamente utilizados. A pesar de que estas tecnologías emergentes permiten la obtención de productos procesados de mayor calidad sensorial y nutricional, la estabilidad y salubridad de estos alimentos son notablemente inferiores a los obtenidos por los tratamientos térmicos. Otras tecnologías no térmicas como los pulsos eléctricos de alto voltaje (PEAV), los ultrasonidos (US), el plasma frío, el agua electrolizada, la luz pulsada y el ozono (O_3), se encuentran en estudio por su elevada actividad bactericida. De hecho, algunas de ellas ya se emplean para la desinfección de superficies, maquinarias o útiles empleados en la producción de alimentos, aunque precisan de adaptación y escalado para su uso de forma directa en la conservación de alimentos a nivel industrial.

1.1.3. Toxiinfecciones alimentarias

Pese a la continua mejora de los métodos de conservación actuales, así como de la implementación de nuevas estrategias para la producción de alimentos más seguros, las enfermedades transmitidas por los alimentos constituyen aún uno de los principales retos de salud pública. En este sentido, las toxiinfecciones alimentarias, aquellas originadas por la presencia y proliferación de microorganismos patógenos, así como por las toxinas sintetizadas derivadas de su metabolismo, suponen la principal causa de enfermedad de transmisión alimentaria. Durante el año 2018, se notificaron un total de 5.146 brotes en la Unión Europea relacionados con el consumo de alimentos y agua, que provocaron un total de 48.365 casos clínicos, 4.588 hospitalizaciones y 40 muertes. El 57% de estos brotes fueron causados por la presencia de bacterias patógenas, seguido por las toxinas

bacterianas, responsables del 24%, principalmente sintetizadas por *Bacillus cereus*, *Clostridium botulinum* y *Staphylococcus aureus.* Dentro de las toxiinfecciones alimentarias destacan *Salmonella enterica*, agente causal del 30% de los brotes, y *Listeria monocytogenes,* responsable de más del 50% de las muertes notificadas en el 2018 en la Unión Europea. En este sentido, aunque la mayor tasa de prevalencia de *S. enterica* se observó en carne de pollo, la mayoría de casos clínicos fueron relacionados con el consumo de huevos y ovoproductos, seguido de productos de panadería. Respecto a *L. monocytogenes*, la mayor tasa de prevalencia fue detectada en pescados y productos pesqueros, pero fue el consumo de verduras, zumos y derivados contaminados, la causa mayoritaria de los casos de toxiinfección alimentaria notificados (EFSA & ECDC, 2019). Esta tendencia se ha visto mantenida, e incluso ligeramente aumentada, en el último informe publicado sobre las zoonosis en la Unión Europea durante el año 2019: 49.463 casos de enfermedad, 3.859 hospitalizaciones y 60 muertes. *S. enterica* fue el principal agente causal de los brotes alimentarios notificados en el 2019 (18%), pero en menor medida que durante el año 2018, la mayoría de ellos relacionados con el consumo de huevos y ovoproductos, seguido de productos cárnicos y derivados, principalmente de aves. Por otro lado, *L. monocytogenes* mostró una tendencia creciente, como en los últimos 4 años, siendo causante de 349 casos de toxinfecciones alimentarias y responsable de más del 50% de las muertes notificadas, principalmente debido al consumo de carne y derivados cárnicos listos para consumo (EFSA & ECDC, 2021).

Estos datos respaldan que los métodos actuales de conservación, junto a las prácticas higiénicas aplicadas en los sistemas de producción, no son capaces de asegurar completamente la salubridad de los alimentos, y que, por lo tanto, todavía es necesario, entre otros, mejorar e implementar nuevas estrategias de conservación en la industria agroalimentaria para garantizar la inocuidad de los alimentos.

1.2. Estudio de los mecanismos de inhibición e inactivación de los métodos de conservación

Profundizar en los modos de acción de los métodos de conservación, así como conocer los mecanismos de resistencia bacteriana frente a los mismos, es fundamental para comprender por qué los tratamientos aplicados llegan a ser ineficaces frente a ciertas poblaciones bacterianas, las cuales son capaces de resistir a tratamientos letales o de crecer en condiciones no favorables. Además, identificar las estructuras celulares o rutas

metabólicas diana de las tecnologías y conservantes empleados puede aportar información relevante para el diseño de estrategias de conservación de alimentos más seguras y eficientes. Para este propósito, se han puesto a punto 1) estudios que evalúan los diversos daños celulares causados por los métodos de conservación, 2) técnicas "ómicas" que permiten profundizar en la respuesta celular de las bacterias y 3) estudios de poblaciones resistentes que revelan las estructuras y rutas metabólicas claves en la resistencia bacteriana.

1.2.1. Estudio de daños a nivel celular

Desde un punto de vista práctico, los métodos de conservación persiguen dañar componentes celulares esenciales de forma irreversible para lograr la inactivación bacteriana, o alterar ciertos mecanismos y estructuras que imposibiliten su proliferación. Por ello, los primeros avances que permitieron profundizar en estos mecanismos se basaron en el estudio de los daños celulares. Debido a la complejidad que supone, en algunos casos, evaluar los daños en células muertas, estas investigaciones habitualmente se focalizan en las células supervivientes que han sido dañadas subletalmente a causa del tratamiento aplicado, es decir, aquellas que mantienen su viabilidad, pero que precisan reparar sus estructuras afectadas como paso previo a su multiplicación (Wesche, Gurtler, Marks, & Ryser, 2009). En este sentido, las técnicas utilizadas para evaluar el daño subletal causado por una tecnología de conservación son muy variadas, debido a las múltiples estructuras a estudiar tras el tratamiento, así como la diversidad de posibles daños producidos incluso en la misma estructura.

Las envolturas celulares han sido objeto de estudio frente a prácticamente todos los métodos de conservación empleados o con potencial uso en la industria agroalimentaria, ya que son la primera barrera física de defensa celular y poseen múltiples funciones esenciales para la célula: conservar la estructura celular, regular el transporte, participar en la generación de energía a través de la cadena de transporte de electrones y actuar como elemento sensor frente a múltiples estímulos externos para el mantenimiento de la homeostasis celular, entre otras. Es por ello que también se han diseñado múltiples métodos para evaluar los daños en esta estructura, como su observación directa mediante microscopía electrónica, la determinación de su integridad y funcionalidad con el empleo de sondas fluorescentes, o la evaluación de pérdida de material intracelular (García-Gonzalo & Pagán, 2016). Una de las técnicas más empleadas en este sentido ha sido la recuperación de los microorganismos supervivientes en medios selectivos: la adición de

sales biliares o cloruro sódico al medio impide el crecimiento de los supervivientes dañados en su membrana externa (en Gram-negativas) o membrana citoplasmática, respectivamente (Mackey, 2000). La diferencia del recuento celular en estos medios en comparación con un medio nutritivo (no selectivo), permite estimar el número o porcentaje de la población que se encuentra dañada en las envolturas celulares y, así, conocer la implicación de esta estructura en la resistencia frente al tratamiento aplicado. No obstante, otras técnicas como la citometría de flujo combinada con el uso de fluorocromos específicos, la espectroscopía infrarroja transformada de Fourier, la microscopía de fuerza atómica, y la determinación del potencial zeta, han cobrado relevancia en la actualidad debido a su rapidez y precisión para evaluar la integridad y funcionalidad de las membranas celulares (Booyens & Thantsha, 2014; Bravo-Ferrada et al., 2015).

De igual modo, se han desarrollado otras técnicas de análisis para la evaluación del daño en el resto de estructuras esenciales de la célula que pueden ser diana de los tratamientos de conservación. Por ejemplo, los estudios de frecuencia mutagénica son empleados para evaluar el daño producido en el DNA bacteriano. En este sentido, la secuenciación genómica permite cuantificar el número de mutaciones inducidas por el tratamiento de forma precisa y, de tal modo, valorar el daño en el DNA (Nishant, Singh, & Alani, 2009). No obstante, existen métodos menos costosos que permiten realizar una estimación de la tasa de mutación como la determinación de generación de auxotrofías a través de la siembra en medio mínimos, como el test de Ames (Mortelmans & Zeiger, 2000), o el desarrollo de resistencias frente a antibióticos debido a mutaciones en genes diana, como por ejemplo la resistencia a la rifampicina causada por mutaciones en el gen *rpoB* (Garibyan et al., 2003). Por otro lado, la determinación del número de integridad (RIN) mediante microelectroforesis capilar permite evaluar la degradación del RNA para conocer el alcance del daño en esta estructura (Jahn, Charkowski, & Willis, 2008). En lo que respecta al daño en ribosomas, proteínas y enzimas su evaluación puede llegar a ser muy compleja, siendo necesario en la mayoría de casos su aislamiento, y su posterior estudio de estructura y funcionalidad *in vitro* mediante técnicas calorimétricas, espectrofotométricas, espectrofluorimétricas, inmunoquímicas y ensayos enzimáticos.

Los daños celulares, en ocasiones, no sólo se localizan en una única estructura, ya que algunas tecnologías o compuestos provocan daño en múltiples dianas celulares; por ejemplo, la generación de especies reactivas de oxígeno (ROS) inducida por algunos

tratamientos de conservación como el calor. Estos compuestos, entre lo que se incluyen los radicales anión superóxido ($O_2^{\bullet-}$), los peróxidos de hidrógeno (H_2O_2) y los radicales hidroxilo ($OH^{\bullet}$), son altamente inestables y reactivos, y actúan sobre diversas estructuras de la célula. Es por ello que algunas técnicas evalúan de forma indirecta el daño en la célula cuantificando el agente causal. En este caso, el empleo de fluorocromos como el 2',7'- dicloro-dihidro-fluoresceína diacetato (H_2DCFDA), el dihidroetidio (DHE) y la hidroxifenil fluoresceína (HPF), permiten cuantificar la generación de ROS a causa del tratamiento de conservación y, de tal modo, relacionarlo con los daños estructurales y la inactivación celular (Marcén, Ruiz, Serrano, Condón, & Mañas, 2017).

Por tanto, el estudio de los mecanismos de inhibición e inactivación a través del estudio de los daños celulares puede ser muy costoso y lento, ya que precisa de la puesta a punto de diversas técnicas para evaluar el daño en cada una de las estructuras y mecanismos de respuesta microbianos. Además, la información que proporciona este tipo de estudios puede ser limitada, ya que es probable que haya daños celulares que todavía no se han podido identificar con las técnicas de análisis actuales.

1.2.2. Técnicas "ómicas"

Los avances en herramientas ópticas, informáticas, computacionales y estadísticas han posibilitado el tratamiento y análisis masivo de datos y, consecuentemente, el estudio simultáneo de millones de moléculas y sus interacciones en los sistemas biológicos. De este modo, estamos asistiendo a un gran desarrollo de las técnicas "ómicas" (Fig.1.1), que estudian la totalidad de diferentes entidades, como los genes (genómica), el transcriptoma (transcriptómica) las proteínas (proteómica) y los metabolitos (metabolómica).

Estas técnicas han permitido, entre otros, la secuenciación y caracterización de la mayoría de los genomas bacterianos. Por otro lado, el desarrollo e implementación de herramientas de ingeniería genética y tecnología del DNA recombinante, han permitido la manipulación y edición de la mayoría de los genomas bacterianos de manera eficiente, cuyo empleo se ha focalizado en el estudio de la función de los genes a nivel celular, también llamado genómica funcional. En primera instancia, se pretendía conocer qué genes eran esenciales, codificantes o reguladores, y, a grandes rasgos, en qué función celular se encontraban implicados: crecimiento celular, metabolismo, movilidad, estructural, etc., pero, posteriormente, estos estudios fueron evolucionando y siendo más precisos, llegando a describir las funciones de forma detallada de la mayoría de los genes.

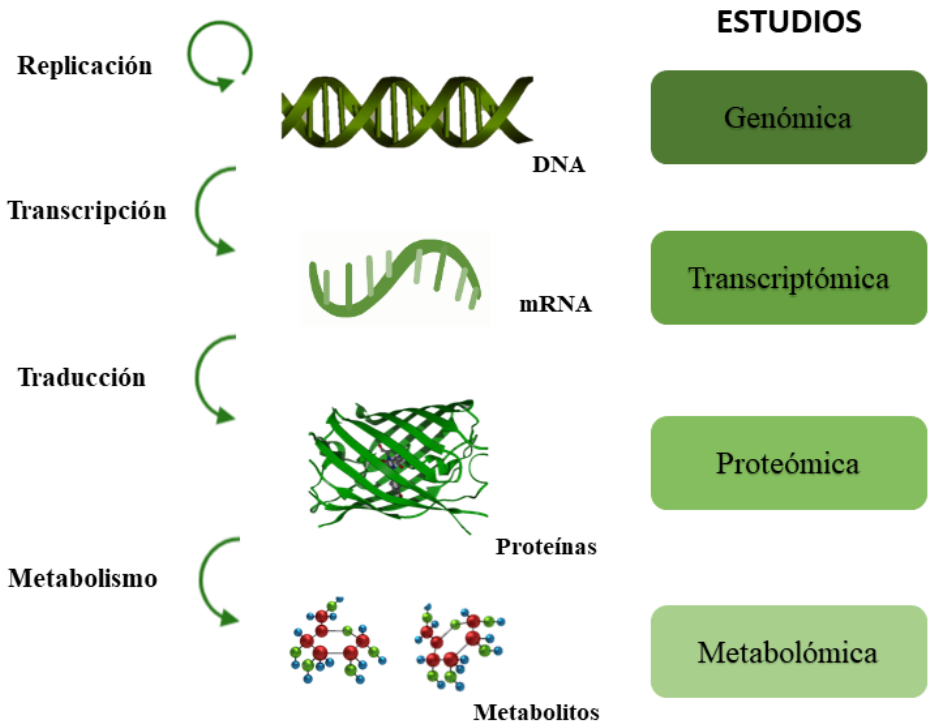


Figura 1.1. Representación esquemática de algunos campos de estudio de las ciencias "ómicas".

Sin embargo, todavía se desconoce la función de gran parte de los genes identificados. En el campo de la ciencia de los alimentos, el uso de técnicas "ómicas" podría ampliar el conocimiento sobre el papel de diferentes genes en la respuesta bacteriana celular frente a los métodos de conservación de alimentos y, de tal modo, profundizar en sus mecanismos de inhibición e inactivación.

a) Estudios genómicos

La genómica se dedica al estudio del DNA presente en una célula, es decir, de su genoma. La implementación de las técnicas de secuenciación durante la última década ha permitido la descripción de la mayoría de los genomas bacterianos de interés en la industria agroalimentaria. De este modo, se ha facilitado el empleo de herramientas de biología molecular para la edición genética que permite profundizar en la genómica funcional. Una de las técnicas más empleadas ha sido la creación de librerías de genes deletéreos de las bacterias de interés en la industria agroalimentaria. Estas librerías se construyen manipulando genéticamente la bacteria e inactivando los genes no esenciales de la célula (knock-out). Cada cepa de esta librería presenta la pérdida de función de un único gen, con el fin de conocer su papel en la célula. Así, se consigue un batería de cepas de la misma especie bacteriana que presenta una deleción ("knock-out") en cada uno de

los genes no esenciales de su genoma: en *Escherichia coli* (Baba et al., 2006), en *L. monocytogenes* (Cao, Bitar, & Marquis, 2007) en *S. enterica* (Porwollik et al., 2014), en *Cronobacter sakazakii* (Alvarez-Ordonez et al., 2014) o en *S. aureus* (Yajjala, Widhelm, Endres, Fey, & Bayles, 2016). Siguiendo esta metodología también se han creado otros tipos de librerías, por ejemplo, las basadas en la complementación genética o el empleo de promotores inducibles y reprimibles para modular la expresión de los genes (De Mey, Maertens, Lequeux, Soetaert, & Vandamme, 2007). De tal modo, el estudio del fenotipo de cada una de las cepas construidas, por ejemplo de la resistencia frente a los métodos de conservación, y su comparación con la cepa parental, permite conocer si el gen en cuestión está implicado en la respuesta celular frente a dicha tecnología o conservante (Chueca, Pagán, & García-Gonzalo, 2014a, 2014b; Chueca, Perez-Saez, Pagan, & Garcia-Gonzalo, 2017; Huertas et al., 2015; Patange et al., 2019).

No obstante, uno de los principales inconvenientes de esta metodología es la cantidad de genes que pueden estar implicados en la resistencia intrínseca de la célula. De modo que, aunque la mayoría de librerías ya están construidas y son de fácil acceso, el estudio fenotípico de todas las cepas frente a varias tecnologías de conservación a diferentes intensidades de tratamiento conllevaría una gran carga de trabajo, tiempo y dinero. Por esta razón, el estudio de estas librerías normalmente suele focalizarse en la utilización de un número limitado de cepas, aquellas cuyos genes han suscitado de forma previa interés para su evaluación. Por otro lado, esta técnica sólo permite evaluar el efecto de los genes de forma aislada, y no del conjunto de toda una respuesta celular, lo que puede complicar la comprensión de su papel en la resistencia bacteriana.

b) Estudios transcriptómicos

La transcriptómica se encarga del estudio y cuantificación de todas las moléculas de RNA, tanto del mensajero como del no codificante, presente en una célula en un momento determinado (transcriptoma). Los análisis de expresión génica también han sido ampliamente utilizados para estudiar los mecanismos de resistencia microbiana frente a los métodos de conservación de los alimentos, y de este modo, sus mecanismos de inhibición e inactivación. Identificar qué genes se encuentran sobreexpresados y reprimidos durante la respuesta celular frente a un tratamiento de conservación, permite evaluar su implicación en la resistencia bacteriana y, consecuentemente, determinar las estructuras o vías metabólicas dianas del tratamiento. Este estudio se lleva a cabo

mediante el análisis del transcriptoma, tras la aplicación de los tratamientos de conservación que inducen una respuesta bacteriana relacionada con sus mecanismos de resistencia (Lamas et al., 2019).

De forma previa al desarrollo de los estudios transcriptómicos, el estudio de expresión génica se llevaba a cabo mediante la construcción de librerías basadas en fusiones transcripcionales: construcciones genéticas formadas por el promotor del gen de interés junto a un gen reportero, que se introducen en el genoma de la célula. Se denomina gen reportero al gen estructural codificante de una proteína cuya síntesis pueda ser cuantificada de forma sencilla y, de tal forma, permita medir la actividad transcripcional de los promotores diana con el objetivo de evidenciar indirectamente la expresión del gen en estudio mediante la detección de la activación de dicho gen. Entre los genes reporteros más utilizados se encuentran el gen *lacZ*, codificante de la enzima β-galactosidasa (Fried, Lassak, & Jung, 2012); el gen *luxA*, que da lugar a una enzima luciferasa (Uliczka et al., 2011); y el gen *gfp,* que codifica la proteína fluorescente verde (GFP) (Wons, Koscielniak, Szadkowska, & Sektas, 2018).

Actualmente, el estudio de expresión génica se realiza a través de análisis transcriptómicos llevados a cabo mediante técnicas como los chips o *microarrays* de DNA, la secuenciación de RNA (RNA-seq) y, más recientemente, la secuenciación de RNA de células individuales (scRNA-seq). La tecnología de *microarrays* de DNA consta de un soporte sólido donde se localizan las diferentes sondas que hibridan con las moléculas diana de la muestra, en este caso el DNA complementario (cDNA) obtenido a partir del RNA bacteriano total presente en la célula. La hibridación entre la sonda y el cDNA produce una emisión de fluorescencia, que permite detectar y cuantificar el RNA total de una manera individualizada. Cabe destacar que esta medida es relativa, normalmente se emplea como control la misma población bacteriana no tratada, de modo que se puede conocer el aumento o disminución de la expresión de cada uno de los genes producida por el método de conservación aplicado. No obstante, esta técnica suele emplearse como cribado, puesto que es capaz de evaluar muchos genes, pero de forma poco precisa y robusta. Es por ello que, habitualmente, se lleva a cabo una validación de los resultados de los *microarrays* mediante la reacción en cadena de la polimerasa cuantitativa con transcriptasa inversa (qRT-PCR) de los genes de interés. Esta técnica se basa en una PCR cuantitativa a partir del cDNA obtenido del RNA total para estimar la cantidad del RNA específico de estudio. De este modo, muchos autores han profundizado

en la respuesta celular bacteriana frente a métodos actuales o potenciales en la conservación de los alimentos: tratamientos térmicos (Guernec, Robichaud-Rincon, & Saucier, 2013), estrés ácido y osmótico (Chakraborty & Kenney, 2018), APH (Bowman, Bittencourt, & Ross, 2008), PEAV (Chueca, Pagán, & García-Gonzalo, 2015) y antimicrobianos naturales (Chueca et al., 2017).

Las técnicas de secuenciación de nueva generación ("next-generation sequencing", NGS), están permitiendo la implementación de técnicas como RNA-seq en bacterias (Croucher & Thomson, 2010; Poulsen & Vinther, 2018). El RNA-seq se basa en la secuenciación masiva del cDNA obtenido del RNA total de la población bacteriana. Así, el número de veces que aparece la secuencia de cada gen reflejaría su expresión en la bacteria. A través de esta técnica se han revelado las vías de adaptación bacteriana frente a diversas condiciones de estrés: agentes oxidantes (Liu, Omar, Abrahante, Nagaraja, & Vidovic, 2020), baja actividad de agua (Crucello, Furtado, Chaves, & Sant'Ana, 2019); así como la respuesta celular frente a diversas tecnologías y conservante alimentarios como las APH (Duru et al., 2021) o los AE (Yang et al., 2020; Zhao et al., 2018). Debido a que esta tecnología y los *microarrays* se emplean en poblaciones bacterianas, no se pueden detectar las variaciones en la expresión génica entre las diferentes células que las conforman. Por ello, a partir de esta técnica, surgió scRNA-seq, que proporciona los perfiles de expresión a nivel individual. Actualmente, ya se encuentra puesta a punto para el estudio del transcriptoma bacteriano, como en *S. enterica* (Imdahl, Vafadarnejad, Homberger, Saliba, & Vogel, 2020), aunque todavía no ha sido empleada en estudios de conservación de los alimentos.

c) Estudios de proteómica y metabolómica

Otras técnicas para el estudio global de las proteínas (proteómica) y los metabolitos (metabolómica) presentes en una célula, también pueden utilizarse para abordar la descripción de los mecanismos de inhibición e inactivación de los métodos de conservación. No obstante, la implantación de estas técnicas en el estudio de la resistencia bacteriana frente a las tecnologías o compuestos antimicrobianos de la industria alimentaria es bastante reducida.

Dentro de la proteómica, en lo que respecta al estudio de respuesta celular, destaca la proteómica de expresión. Su fundamento se basa en evaluar el producto final global de la expresión génica, es decir, las proteínas sintetizadas. Cabe señalar que, en el estudio

del transcriptoma, la expresión de los genes se infiere indirectamente a partir de la cantidad del producto intermedio, es decir, del RNA mensajero. La expresión diferencial entre la muestra tratada y el control, permite conocer la respuesta celular al estrés expuesto y, de tal modo, el modo de acción del tratamiento aplicado. Recientemente, se han publicado estudios sobre la respuesta proteómica frente al estrés oxidativo (Karash, Liyanage, Qassab, Lay, & Kwon, 2017; Noster et al., 2019), a los PEAV (Liu et al., 2019), a las APH (Tamber, 2018) y frente a antimicrobianos naturales como AE y CI (Barbosa et al., 2020; Qi et al., 2020).

Por otro lado, el estudio de los metabolitos como reflejo de los procesos celulares mediante técnicas de metabolómica, ha dado lugar a un mayor conocimiento de los mecanismos de respuesta celular frente al calor (Kim et al., 2020; Ye et al., 2012), a las APH (Kimura, Inaoka, & Yamamoto, 2018), y a antimicrobianos naturales de origen vegetal (Chen et al., 2020; Tang et al., 2021).

1.2.3. Estudio de poblaciones resistentes bacterianas

Los estudios de cinética de inactivación han permitido describir las desviaciones de la linealidad en las gráficas de supervivencia, observándose la aparición de hombros y colas, especialmente en los tratamientos térmicos, de PEAV y APH. Dejando de lado las cuestiones metodológicas, una explicación a estos fenómenos reside en la presencia de subpoblaciones bacterianas con diferentes grados de resistencia, que pondrían en cuestión la efectividad de las condiciones aplicadas en los distintos tratamientos (Abee & Wouters, 1999; Wesche et al., 2009). Desde un enfoque de ciencia básica, el estudio de estas poblaciones y del desarrollo de resistencias frente a los métodos de conservación, puede ayudar a la compresión de los mecanismos de resistencia bacterianos, así como profundizar en los modos de acción de las tecnologías y conservantes empleados, o con potencial uso, en la conservación de alimentos (Abee, Koomen, Metselaar, Zwietering, & den Besten, 2016; Alvarez-Ordóñez, Broussolle, Colin, Nguyen-The, & Prieto, 2015). Desde un punto de vista aplicado, la presencia de subpoblaciones microbianas anormalmente resistentes amenazaría la inocuidad de los alimentos tratados y, de tal modo, respaldaría la necesidad de realizar estudios que contemplen este riesgo microbiológico en el diseño de estrategias de conservación. Para este propósito es necesario diferenciar dos tipos de resistencias: por un lado, los fenómenos de adaptación con carácter temporal y, por otro lado, la aparición de variantes genéticas estables.

a) Estudio de fenómenos de adaptación temporal

Los microorganismos modulan su respuesta celular en base a los estímulos externos para adaptarse al medio en el que se encuentran. En este sentido, las condiciones adversas inducen una respuesta celular que permite la habituación del microrganismo a su entorno. Los fenómenos de adaptación en términos de resistencia surgen como consecuencia de esta respuesta celular: los agentes estresantes externos inducen la activación de los mecanismos de resistencia de la bacteria que le proporcionan una mayor capacidad de sobrevivir o crecer en situaciones no favorables. Las respuestas al estrés suelen estar mediadas por la activación de determinados reguladores de la respuesta que controlan la expresión de conjuntos específicos de genes y orquestan respuestas muy complejas que implican la inducción de enzimas celulares, la síntesis de proteínas que protegen y/o reparan las proteínas celulares y el DNA, y la modulación de la composición y las propiedades físicas de las envolturas celulares (Alvarez-Ordóñez et al., 2015). Un buen ejemplo es la regulación del factor sigma 32 (σ^H, RpoH) en *E. coli*, activado por el estrés térmico, y que induce la síntesis de proteínas de choque térmico ("heat shock proteins", HSP): chaperonas, proteasas y enzimas reparadoras del DNA, entre otras, que proporciona un aumento de resistencia a los tratamientos térmicos, y también frente APH y agentes ácidos y oxidantes (Li & Gänzle, 2016). No obstante, cabe destacar que esta respuesta es temporal y el incremento de resistencia, por lo tanto, tiende a desaparecer cuando el agente estresante cesa o la célula se divide. De este modo, el aumento de resistencia no se transmitiría genéticamente ni a nivel horizontal en la población bacteriana, ni a nivel vertical hacia generaciones posteriores.

Por otro lado, debido a la heterogeneidad fisiológica en la población bacteriana, también pueden observarse fenómenos de resistencia entras las distintas subpoblaciones, isogénicas, pero en distinto estadío fisiológico. En ese sentido, se encuentran las células "*persisters*", aquellas células que cambian a un estado de latencia, incrementado su resistencia, pero paralizando su crecimiento celular.

El estudio de los fenómenos de adaptación ha permitido conocer la respuesta celular frente a diversos estreses inducidos por los métodos de conservación de alimentos que suponen un aumento de la resistencia bacteriana. Concretamente, se ha elucidado qué genes regulan la respuesta frente a los diversos estreses, como el térmico, el ácido y el oxidativo, las modificaciones inducidas a nivel celular en las envolturas celulares, los sistemas de homeostasis y reparación celular, síntesis de proteínas y enzimas de

protección, etc., y la repercusión en la resistencia de las bacterias patógenas sobre las tecnologías y conservantes alimentarios (Alvarez-Ordóñez et al., 2015; Begley & Hill, 2015). Cabe reseñar que, aunque en sus inicios estos estudios se focalizaban en conocer qué estreses conducían a un aumento de resistencia directa o cruzada frente a las métodos de conservación de alimentos (McMahon et al., 2000; Miller, Bayles, & Eblen, 2000; Pagán, Condon, & Sala, 1997), actualmente la evaluación de los fenómenos de resistencia se apoyan en análisis del transcriptoma y estudios de funcionalidad de las estructuras celulares, para profundizar en la expresión génica y conocer las causas a nivel fisiológico de la adaptación y aumento de la resistencia bacteriana (Burgess et al., 2016; Li & Gänzle, 2016; Zhang, Bai, & Bowman, 2019).

b) Estudio de variantes genéticas

En los últimos años se ha demostrado que las poblaciones microbianas no son isogénicas, sino que presentan una heterogeneidad genotípica que ha sido relacionada con aumentos de resistencia frente al estrés (Ryall, Eydallin, & Ferenci, 2012). La existencia de subpoblaciones con capacidad de sobrevivir y multiplicarse en diferentes condiciones, incrementaría la eficacia biológica ("fitness") de la población de un modo global, facilitando su supervivencia en un amplio rango de condiciones ambientales (Magdanova & Golyasnaya, 2013). De tal modo, en función de las condiciones ambientales, aquellas subpoblaciones cuyo genotipo les proporcione una mejor adaptación, es decir una mayor eficacia biológica, serán seleccionadas sobre el resto de subpoblaciones. Así, se producirá la fijación de estas mutaciones en la población microbiana (Ferenci, 2008). En este contexto, cabe tener en cuenta que, en comparación con los fenómenos de adaptación, el aumento de resistencia se debe a modificaciones genéticas estables en el genoma que, aunque de forma general no tienen relevancia en la transferencia horizontal, se transfieren de forma vertical a sus posteriores generaciones.

La presencia de variantes genéticas resistentes afecta directamente a la conservación de alimentos. La aparición de cepas resistentes en cualquier punto de la cadena alimentaria podría incluso provocar la dominancia de estas poblaciones en el alimento, por su mejor supervivencia frente a las medidas higiénicas empleadas para el control microbiológico, y su multiplicación en las materias primas y/o en el propio alimento (Abee et al., 2016). Por tanto, se considera esencial el estudio la aparición de variantes genéticas resistentes que puedan sobrevivir a tratamientos letales o crecer en condiciones

no favorables, conocer su repercusión en la inocuidad y salubridad de los alimentos, y considerar este riesgo microbiológico en el diseño de estrategias de conservación de los alimentos.

Sin embargo, la aparición de estas variantes resistentes también puede suponer una herramienta de estudio para conocer los mecanismos de resistencia bacterianos, así como de los modos de acción de las tecnologías de conservación de alimentos. Las técnicas de secuenciación actuales permiten identificar con precisión todas las modificaciones genéticas presentes en estas variantes, de modo que, conociendo la función de los genes mutados, se puede profundizar en el origen del incremento de resistencia.

i. Técnicas de secuenciación del genoma

El estudio de las variantes genéticas resistentes se basa en la genómica comparativa, que como su nombre indica, se basa en la comparación de la secuencia del DNA de dos o más microorganismos con el objetivo de conocer las causas genéticas del cambio en su fenotipo. Estos análisis actualmente son posibles gracias a los grandes avances realizados en el campo de la secuenciación genómica con la entrada de las tecnologías de NGS, que permiten de forma rápida, y cada vez más económica, conocer con exactitud la secuencia genética completa de cualquier microorganismo.

En este sentido, se dispone de diversas tecnologías de secuenciación de genoma como Roche/454, Illumina/Solexa, SOLiD, Ion Torrent, PacBio, Nanopore (Metzker, 2010). No obstante, para estos estudios de genómica comparativa que buscan identificar cualquier tipo de mutación a lo largo del genoma, desde cambios estructurales ("structural variants", SV) como inversiones y translocaciones, hasta modificaciones genéticas como pequeñas inserciones y deleciones (InDels) o variaciones de un solo nucleótido ("single nucleotide variant", SNV), se recomienda el uso de tecnologías de secuenciación con alta exactitud y fidelidad como la tecnología Illumina (Fig. 1.2), en vez de otras tecnologías de secuenciación de lecturas más largas como PacBio o Nanopore (De Maio et al., 2019).

La secuenciación mediante la tecnología Illumina se basa en la segmentación del DNA genómico bacteriano en pequeños fragmentos de un tamaño de aproximadamente de entre 75-200 pb. A estos fragmentos se les inserta una secuencia de referencia para su codificación y adaptadores a los extremos que les permite su adhesión a un soporte sólido. Posteriormente, se amplifican y agrupan en "clusters" para amplificar la señal, y se procede a la secuenciación de cada una de las lecturas mediante ciclos de terminación

reversible. Ésta se basa en emplear nucleótidos marcados cuya unión a la secuencia genómica produce una emisión de energía; en función de la longitud de onda de esta emisión se indica la base nitrogenada hibridada (adenina, citosina, guanina o timina). Tras obtener las lecturas, o bien se ensamblan entre sí para generar una secuencia consenso "novo assembly", o bien, si se dispone de un genoma de referencia con el cual se quiere comparar, se puede realizar un alineamiento y mapeado de las lecturas sobre él (Metzker, 2010).

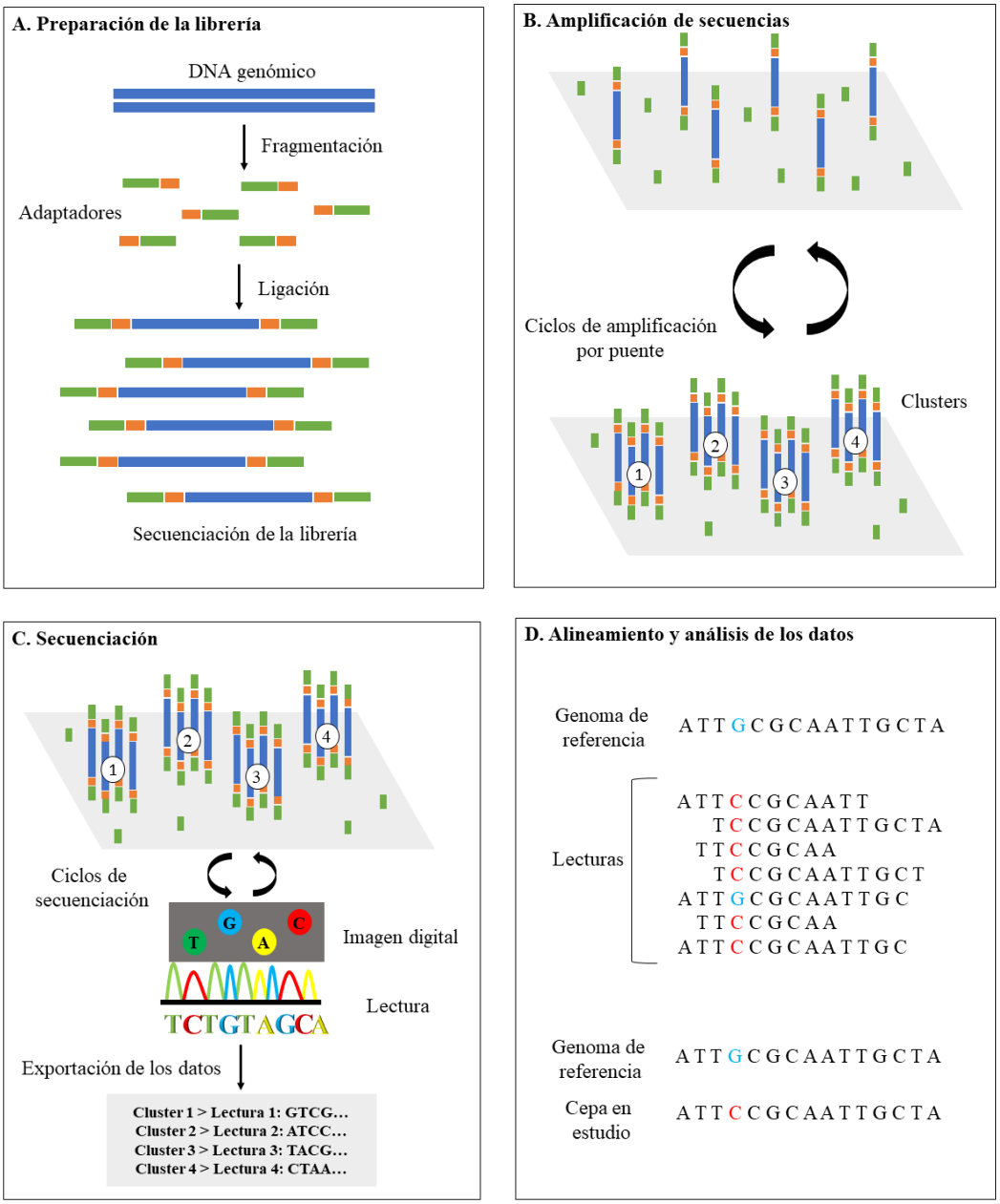


Figura 1.2. Fases de la secuenciación por Illumina: preparación de la librería (A), amplificación de secuencias (B), secuenciación (C), alineamiento y análisis de datos (D).

Aunque el estudio de resistencias se podría llevar a cabo a partir de variantes genéticas resistentes aisladas de muestras de la industria agroalimentaria, y de hecho en ocasiones se hace a través de análisis filogenéticos (Mercer et al., 2015), conocer las causas de su fenotipo puede ser muy complicado y laborioso debido a las múltiples modificaciones genéticas que se pueden llegar a identificar. Es por ello que la mayoría de autores han optado por la realización de ensayos de evolución que, bajo presión selectiva, favorecen la selección y aislamiento de variantes genéticas resistentes de una bacteria origen, cuyo genoma está completamente secuenciado y las funciones de sus genes ampliamente descritas y evidenciadas. De tal modo, se facilita la identificación de las mutaciones responsables del incremento de resistencia en las variantes genéticas aisladas, la interpretación del papel del gen mutado en la respuesta celular y la repercusión de la modificación genética a nivel fisiológico.

ii. Ensayos de evolución frente a métodos de inhibición bacteriana

Los ensayos de evolución mediante el empleo de antimicrobianos bacteriostáticos se basan en la exposición prolongada a dosis subletales del compuesto durante el crecimiento celular de la población bacteriana de forma cíclica. El objetivo es permitir la aparición de variantes genéticas resistentes en la población bacteriana que presente una mejor adaptación al agente antimicrobiano para su crecimiento celular. De tal modo, a lo largo de los ciclos del ensayo de evolución las variantes genéticas con mayor capacidad de desarrollarse en las condiciones impuestas por la presión selectiva del antimicrobiano irán creciendo y dominando sobre el resto de subpoblaciones, facilitando así su aislamiento para su posterior caracterización fenotípica y genotípica.

Los primeros avances que se han realizado en el estudio de cepas resistentes frente a compuestos antimicrobianos se han llevado cabo en el campo de los antibióticos, uno de los principales retos sanitarios a nivel mundial (Årdal et al., 2019; Peterson & Kaur, 2018). En este sentido, la aparición de variantes genéticas debida a mutaciones espontáneas, o inducidas, ha sido ampliamente estudiada en la generación de resistencia frente a antibióticos; es por ello que existen varios protocolos descritos en este campo para evaluar el desarrollo de resistencias genotípicas frente a antibióticos a través de su empleo continuado a concentraciones subletales (Andersson & Hughes, 2014; Kohanski, DePristo, & Collins, 2010) o letales (Levin-Reisman et al., 2017).

En lo que respecta a la conservación de alimentos, a través de la exposición frente a condiciones adversas o conservantes antimicrobianos, se ha comenzado a progresar en los mecanismos de resistencia y adaptación bacteriana frente a los métodos de inhibición. En este sentido, existen estudios sobre la identificación y caracterización de variantes genéticas resistentes frente a medios ácidos (Metselaar, den Besten, Abee, Moezelaar, & Zwietering, 2013), choques osmóticos (Guo, Winkler, & Kao, 2017) o antimicrobianos naturales, como la nisina (Collins, Curtis, Cotter, Hill, & Ross, 2010; Kawada-Matsuo et al., 2020) o bacteriocinas (Kumariya et al., 2019). No obstante, aunque el estudio de los mecanismos de resistencia bacteriana haya adquirido un papel relevante en el campo de los antibióticos, son escasas las investigaciones centradas en profundizar sobre los mecanismos de inhibición de los conservantes, o potenciales conservantes alimentarios, a través de ensayos de evolución.

Por el contrario, otros antimicrobianos naturales de origen vegetal, como los AE y sus CI, sí que han sido ampliamente testados y hasta la fecha se han considerado a este respecto seguros, entre otros motivos porque se aceptaba que su empleo no conduce al desarrollo de resistencias directas (de Souza, 2016). Una de las hipótesis que sustentaría esta presunción es la capacidad antioxidante de estos compuestos a bajas dosis (Hashemi, Khorram, & Sohrabi, 2017) que neutralizaría las ROS, una de las principales causas del daño al DNA (Sakai, Nakanishi, Yoshiyama, & Maki, 2006). De este modo, se reduciría la tasa mutagénica de las bacterias (Hammer, Carson, & Riley, 2008), evitando así la aparición de variantes genéticas resistentes. De hecho, esta es una de las principales razones por las que, a lo largo de los últimos años, varios autores han propuestos los AE y los CI como potencial alternativa a los antibióticos en la lucha contra la aparición y diseminación de resistencias bacterianas frente a los antibióticos (Mittal, Rana, & Jaitak, 2019; Yap, Yiap, Ping, & Lim, 2014). En este sentido, en la actualidad, la mayoría de estudios respaldan el uso de AE y su CI como antimicrobianos seguros para el control de bacteria patógenas en la conservación de alimentos (Pandey, Kumar, Singh, Tripathi, & Bajpai, 2017).

iii. Ensayos de evolución frente a métodos de inactivación bacteriana

Los ensayos de evolución mediante métodos de inactivación se basan en la aplicación de tratamientos letales, habitualmente cortos pero a altas intensidades, de forma cíclica a la población bacteriana. El objetivo de este ensayo es permitir la aparición

de variantes genéticas a lo largo del ensayo de evolución y, a través de los tratamientos letales, seleccionar y aislar aquellas variantes cuyas mutaciones induzcan una mayor resistencia a la tecnología en cuestión. De este modo, los tratamientos letales inactivarían las subpoblaciones sensibles, mientras que las variantes genéticas resistentes sobrevivirían y podrían ser recuperadas para su caracterización fenotípica y genotípica.

A partir de aquellos supervivientes responsables de los fenómenos de "cola" de las curvas de supervivencia tras la aplicación de tratamientos letales, se han conseguido aislar variantes genéticas resistentes frente a tecnologías de conservación de los alimentos como el calor (Gayán, Cambré, Michiels, & Aertsen, 2016; Van Boeijen, Francke, Moezelaar, Abee, & Zwietering, 2011), las APH (Liu, Ream, Joerger, Liu, & Wang, 2011; Sanz-Puig et al., 2019; Vanlint et al., 2011), los PEAV (Sagarzazu, Cebrian, Pagan, Condon, & Manas, 2013; Sanz-Puig et al., 2019) o la radiación UV-C (Alvarez-Molina et al., 2020; Selveshwari, Lele, & Dey, 2021). De tal modo, estos estudios, además de evidenciar la aparición de variantes genéticas resistentes que pueden llegar a suponer un riesgo alimentario frente a las tecnologías de conservación, la caracterización de estas cepas ha permitido conocer qué mutaciones producen un aumento de resistencia, qué genes y mecanismos de resistencia juegan un papel importante en la supervivencia bacteriana frente a la tecnología utilizada en la selección, y se han ampliado los conocimientos actuales sobre los modos de acción de estas tecnologías.

Cabe destacar que los ensayos de evolución mediante tratamientos de inactivación pueden también llevarse a cabo frente a compuestos antimicrobianos empleando dosis letales. Sin embargo, no existen evidencias sobre la aparición de variantes resistentes frente a conservantes alimentarios tras la aplicación de tratamientos letales.

1.3. Aceites esenciales (AE) y sus constituyentes individuales (CI)

Los AE son líquidos aromáticos aceitosos que se obtienen de diferentes partes de las plantas como flores, brotes, semillas, hojas, corteza, hierbas, madera, frutos, o raíces, y que a lo largo de la historia se han empleado para una amplia variedad de propósitos debido a sus múltiples propiedades antioxidantes, aromáticas, antimicrobianas, etc. (Hammer, Carson, & Riley, 1999; Jones, 1996). En particular, la actividad antimicrobiana de aceites y extractos de plantas ha constituido la base de muchas aplicaciones tradicionales, incluyendo la conservación de alimentos crudos y procesados, productos farmacéuticos, medicina alternativa y terapias naturales (Lis-Balchin & Deans, 1997).

Muchos de ellos se encuentran clasificados como sustancias generalmente conocidas como seguras (GRAS, *Generally Recognized As Safe* en inglés) e incluso algunos de sus componentes están legalmente registrados como aditivos en Europa y Estados Unidos, de forma que su empleo está permitido en los alimentos y pueden ser utilizados como conservantes alimenticios (Tajkarimi, Ibrahim, & Cliver, 2010). En este sentido, debido a su elevada actividad antimicrobiana y su abundancia en el medio ambiente destacan algunos AE de plantas como el romero (*Rosmarinus officinalis*), el orégano (*Origanum vulgare*) y el tomillo (*Thymbra capitata*), o de cáscaras de frutos cítricos como la naranja (*Citrus sinensis*) o el limón (*Citrus limon*).

Los AE son una mezcla de compuestos orgánicos de bajo peso molecular como los terpenoides (derivados del isopreno), que pueden variar en función de la especie vegetal, del momento y localización geográfica de la recolección, del órgano de la planta y del método de extracción usado (Calo, Crandall, O'Bryan, & Ricke, 2015). Es por ello que la utilización de estos AE puede entrañar dificultades debido a la gran variabilidad en su composición y concentración, por lo que algunos estudios optan por la evaluación de aquellos CI que muestren mayor actividad antimicrobiana, tales como el carvacrol, el citral o el óxido de limoneno (Fig. 1.3).

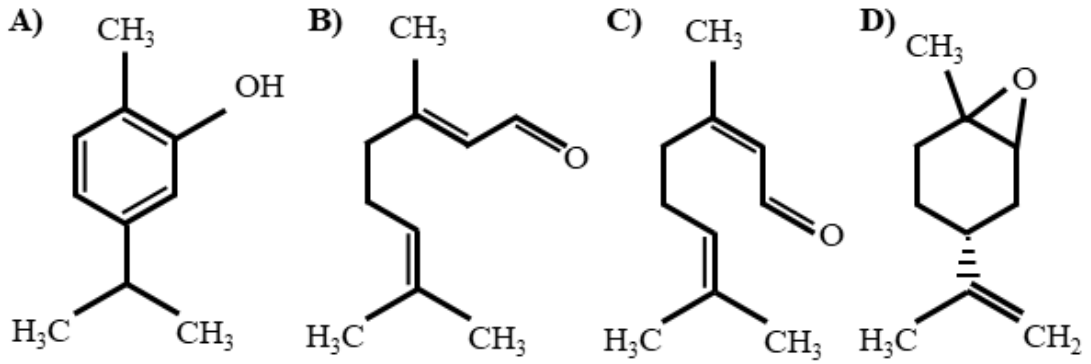


Figura 1.3. Estructura química de carvacrol (A), geranial (B), neral (C) y óxido de limoneno (D).

- El carvacrol (2-metil-5-(1-metiletil) fenol), isómero del timol, es un fenol monoterpenoide que proviene de las hojas de diferentes especies de plantas como el orégano (*Origanum vulgare*), el tomillo (*Thymus vulgaris*) o el romero (*Rosmarinus officinalis*) (Burt, Vlielander, Haagsman, & Veldhuizen, 2005; Kiskó & Roller, 2005).

- El citral es una mezcla de los isómeros geranial y neral. Este compuesto es un aldehído monoterpenoide acíclico insaturado que se encuentra en las hojas y frutos de

diferentes especies de plantas como el mirto (*Myrtus claraensis*), la albahaca (*Ocimum basilicum*) y la citronela (*Cymbopogon citratus*) (Fisher & Phillips, 2006).

- El óxido de limoneno es un CI monotepernoide extraído principalmente del epicarpio de frutas cítricas como la naranja (*Citrus sinensis*), el limón (*Citrus limon*) y la mandarina (*Citrus reticulata*) (Espina et al., 2011).

Sin embargo, a pesar del amplio espectro de acción y la elevada actividad antimicrobiana que poseen los AE y CI (Bhavaniramya et al., 2019), su uso en la conservación de alimentos se encuentra, a día de hoy, limitado por los siguientes inconvenientes:

a) Las potentes propiedades organolépticas que poseen estos antimicrobianos naturales, a las concentraciones requeridas para su empleo de forma efectiva, provocan la alteración del sabor y aroma del alimento y, de este modo, el rechazo por parte del consumidor.
b) Los AE y CI son compuestos lipofílicos poco solubles en agua. Por esta razón, su uso en alimentos líquidos con gran contenido en agua (como los zumos o la leche) se ve limitado al punto de solubilidad, además de requerir una agitación vigorosa para lograr una dispersión homogénea.
c) Estos compuestos son volátiles y altamente inestables frente agentes externos, como la luz o el oxígeno, de forma que su alteración podría conducir a una pérdida de sus propiedades antimicrobianas.

Así, en primer lugar, sería necesario reducir las concentraciones necesarias de AE y CI para evitar la alteración sensorial de los alimentos, sin que ello suponga un riesgo alimentario. Por esta razón, profundizar en los mecanismos de inhibición e inactivación de estos antimicrobianos naturales permitiría el diseño de estrategias de conservación de alimentos más efectivas con AE y CI que, a concentraciones más bajas, asegurarían la salubridad de los alimentos. En este sentido, y pese a que la mayoría de autores descartaban la posibilidad de aislar variantes genéticas resistentes frente a estos antimicrobianos naturales, nuestro grupo de investigación ha logrado aislar cepas resistentes de *Escherichia coli* tras ensayos de evolución en presencia de carvacrol, citral y óxido de limoneno (Chueca, Berdejo, Gomes-Neto, Pagán, & García-Gonzalo, 2016; Chueca et al., 2018), iniciando una importante línea de investigación en el campo del estudio de los mecanismos de resistencia microbiana frente a estos compuestos antimicrobianos.

Por otro lado, con el fin de solventar los problemas de solubilidad y de inestabilidad de los AE y CI, numerosos autores han planteado la utilización de técnicas de emulsificación y encapsulación que faciliten su hidrosolubilidad y protejan al compuesto encapsulado frente a los agentes externos. En este sentido, destaca el empleo de surfactantes como el polisorbato 80 (tween 80) y el etanol en la preparación de emulsiones de los AE y CI (Komaiko & McClements, 2016), o el uso de zeínas y ciclodextrinas para su encapsulación (Maes, Bouquillon, & Fauconnier, 2019). No obstante, aunque varios estudios han evaluado la estabilidad y protección de estas preparaciones de los antimicrobianos naturales, no se conoce en profundidad la repercusión sobre las propiedades antimicrobianas de estos compuestos emulsionados o encapsulados, empleados tanto de forma individual como en combinación con otras tecnologías o barreras de conservación de alimentos.

1.4. Procesos combinados

La generación de conocimiento sobre los mecanismos de inhibición e inactivación de las tecnologías y conservantes empleados, o con potencial uso, en la industria alimentaria, favorecen la implementación de nuevas estrategias más efectivas para la conservación de los alimentos. Una de esas estrategias es el diseño inteligente de procesos combinados, cuyo fundamento reside en la teoría de barreras (Leistner & Gorris, 1995). Los procesos combinados en la conservación de alimentos se basan en la combinación deliberada de dos o más métodos de conservación de forma simultánea o sucesiva, con el objetivo de reducir la intensidad del tratamiento aplicado, y de este modo sus efectos adversos sobre la calidad sensorial y nutricional de los alimentos, manteniendo y/o mejorando la estabilidad y salubridad del alimento (Sala, 1995).

El diseño inteligente de procesos combinados persigue obtener efectos sinérgicos entre métodos de conservación para la inactivación microbiana. Estas sinergias pueden aparecer porque:

a) los métodos a combinar actúan sobre las mismas estructuras y logran incrementar el daño hasta niveles que no son reparables y que, por lo tanto, conducen a la muerte celular, o porque,

b) uno de los métodos potencia la actividad del resto, por ejemplo, los "electroporos" provocados por los PEAV en las envolturas microbianas que favorecen la entrada de

compuestos antimicrobianos al interior celular y, de este modo, facilitan su acción intracelular.

Conforme se profundiza en el modo de acción de los métodos de conservación sobre los microorganismos, el diseño de procesos combinados es más efectivo y eficiente, asegurando la inocuidad de los alimentos y reduciendo las alteraciones indeseables del producto.

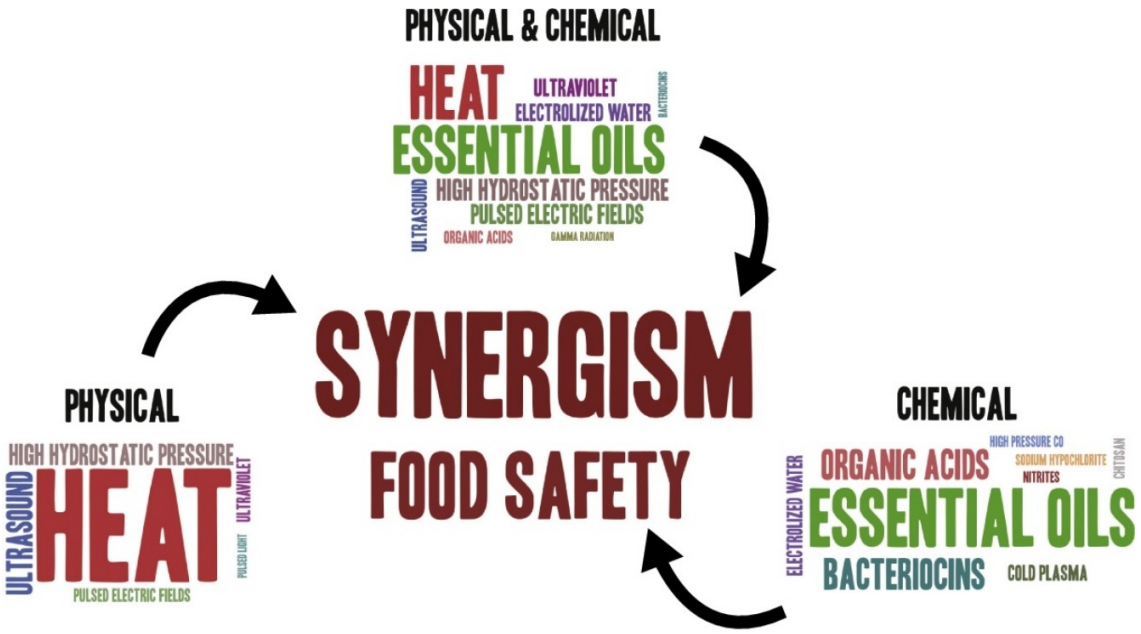
Actualmente, el diseño de procesos combinados es una de las estrategias más ampliamente utilizadas en la industria agroalimentaria. La mayoría de productos comercializados se encuentran sometidos a varios métodos de conservación que actúan de forma aditiva o sinérgica para asegurar su inocuidad. Sin embargo, algunos de estos procesos combinados, aunque son efectivos, han sido diseñados bajo ensayos de prueba y error y, por ello, ciertos fundamentos mecanísticos causantes de su efecto sinérgico no son totalmente explicables a día de hoy. En este sentido, se observa que nuestro conocimiento todavía es limitado y se requieren más estudios que eluciden de forma precisa los mecanismos de inhibición e inactivación microbiana de la mayoría de los métodos de conservación de los alimentos.

A continuación, se muestra una revisión bibliografía que ofrece una visión general de la información publicada recientemente sobre los efectos sinérgicos que aparecen en la combinación de tecnologías físicas y/o químicas de conservación de los alimentos.

Manuscrito I. Exploiting the synergism among physical and chemical processes for improving food safety

Berdejo, D., Pagán, E., García-Gonzalo, D., Pagán, R. (2019)

Current Opinion in Food Science, 30, 14-20.
doi: 10.1016/j.cofs.2018.08.004.



Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Food Science

Exploiting the synergism among physical and chemical processes for improving food safety

Daniel Berdejo, Elisa Pagán, Diego García-Gonzalo and Rafael Pagán

This article provides an overview of recent published information on the subject of synergistic lethal effects that emerge from the combination of physical and/or chemical processes applied to enhance food safety.Despite important recent advances in non-thermal technologies, the greatest synergistic lethal effects emerge from combining them with traditional, relatively mild heat treatments. The combined application of antimicrobials has shown that their main constituents interact effectively, and great synergistic effects have been described with the capacity of either inhibiting or inactivating pathogens. Moreover, natural antimicrobials are more effective when pathogens are previously damaged sublethally by the application of physical technologies. Such combinations allow for a considerable reduction of treatment intensity and costs, along with a noticeable improvement in food quality and safety.

Address
Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain

Corresponding author: Pagán, Rafael (pagan@unizar.es)

Current Opinion in Food Science 2018, **30**:14–20

This review comes from a themed issue on **Food safety**

Edited by **Marciane Magnani**

https://doi.org/10.1016/j.cofs.2018.08.004

Introduction

In the food safety sector, a successful synergism arises when two or more processes (hurdles) are applied simultaneously and/or successively, and the bacteriostatic or bactericidal effect obtained against pathogens is greater than that obtained by the hurdles applied separately (Figure 1). Valuable synergisms have been described and analysed in the last two decades [1••,2], ever since Leistner and Gorris [3] described the "hurdle theory". The present paper provides an overview of recent literature on the occurrence of synergisms when physical and/or chemical hurdles are combined in order to be applied as a tool for designing more effective treatments to improve food safety.

Synergisms resulting from the combination of physical technologies

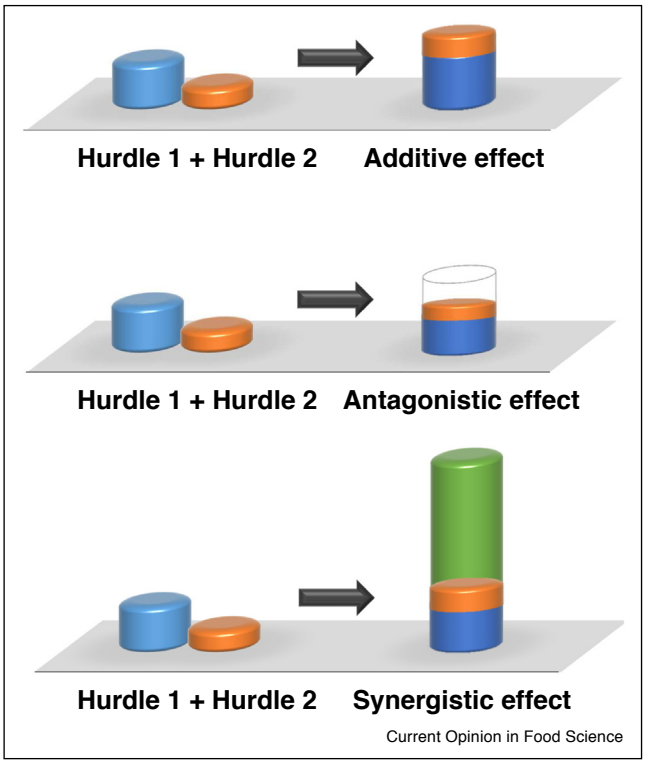
Thermal inactivation has played a major role in food preservation; nevertheless, the adverse effects of high-intensity heat treatments on food quality have led researchers to develop alternative non-thermal technologies. Among them, High Hydrostatic Pressure (HHP), Pulsed Electric Fields (PEF), ultraviolet light (UV), ultrasound (US), and Pulsed Light (PL) have attracted great interest in view of their capability to inactivate microorganisms without altering the organoleptic and nutritional properties of foods. HHP, like heat treatments, affects several kinds of cell structure, causing disruption of cell envelopes and protein denaturation with the purpose of inducing cell death. The other four non-thermal technologies are mainly focused on one target: PEF and US cause damages in cell envelopes, while UV and PL induce cellular lesions in genomic DNA. However, the high resistance displayed by certain pathogenic microorganisms under specific environmental conditions is restricting those technologies' use. Thus, the search for new, complex processes that combine different technologies in order to guarantee food safety and improve quality and shelf-life continues to represent a major challenge.

Despite advances in non-thermal technologies, most of the proposed synergisms continue to combine them with the application of mild heat (Figure 2). The combination of HHP with heat is one of the most effective known synergisms. According to Ates *et al.* [4], >6-$\log_s$ of *Listeria monocytogenes* were inactivated when HHP (625 MPa) and heat (40 °C) were applied to model soup. A great synergism was also observed against bacterial spores: while HHP (600 MPa) caused the inactivation of only 0.5-logs of *Bacillus cereus* spores in beef slurry, it succeeded in inactivating 4.9-logs in combination with heat (70 °C) [5].

PEF achieves a higher lethality when applied together with mild heat. Katiyo *et al.* [6] demonstrated that PEF (35 kV/cm) at 50 °C inactivated >5-logs of *Escherichia coli* and *Salmonella* Enteritidis in apple juice.

Mild heat also enhanced UV lethality. UV-C (10.6 kJ/m^2) at 45–50 °C caused 6-logs reduction of *E. coli* in juice

Figure 1



Lethal effect obtained by the combination of two different food preservation processes (hurdle). Additive effect: An effect in which two hurdles used in combination produce a total effect the same as the sum of their individual effects; Antagonistic effect: An effect arising between two hurdles that produces an effect smaller than the sum of their individual effects. Synergistic effect: An effect arising between two hurdles that produces an effect greater than the sum of their individual effects (green area).

carrot-orange blend after 15 min, compared to single UV-C treatment (2.5-logs) [7]. Gayan *et al.* [8•] observed a 20–70% increase in the lethality of foodborne pathogens (*E. coli*, *Salmonella* Typhimurium, *L. monocytogenes* and *Staphylococcus aureus*) in several liquid foods, such as vegetable and chicken broth or orange and apple juice, thanks to the combination of UV with heat. This was brought about thanks to DNA damage caused by reactive oxygen species.

An increased lethality was also achieved by the simultaneous application of US and mild temperatures, called thermosonication (TS). Interesting synergisms after TS (20–24 kHz/400–750 W/50–58 °C) were demonstrated in juices, reducing 5-logs of *E. coli*, as recommended by the FDA [9,10]. Li *et al.* [11•] were able to ascertain that sublethal injuries caused by heat in the membrane of *S. aureus* were the underlying cause.

In addition, synergistic effects were observed among non-thermal technologies, such as HHP and UV applied successively. Pretreatment with UV light (0.82–8.45 J/cm^2) prior to HHP (300–600 MPa) led to relevant synergisms against pathogens (*L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *S.* Typhimurium) in apple juice, achieving >6-logs at the maximum treatment intensity [12].

However, a consecutive treatment of US (20 kHz/600 W) and PL (0.0175 J/mL) against *E. coli* and *S.* Enteritidis in apple juice only resulted in an additive effect [13].

The successive combination of more than two physical technologies also displayed interesting results. A synergism was observed when applying HHP, US, and heat, in that order: a 5min treatment (350 MPa/560 W/40 °C) achieved 5.85-logs of *E. coli* inactivation [14]. A greater rate of inactivation is thereby achieved thanks to the microbial sublethal damages caused by the first treatments.

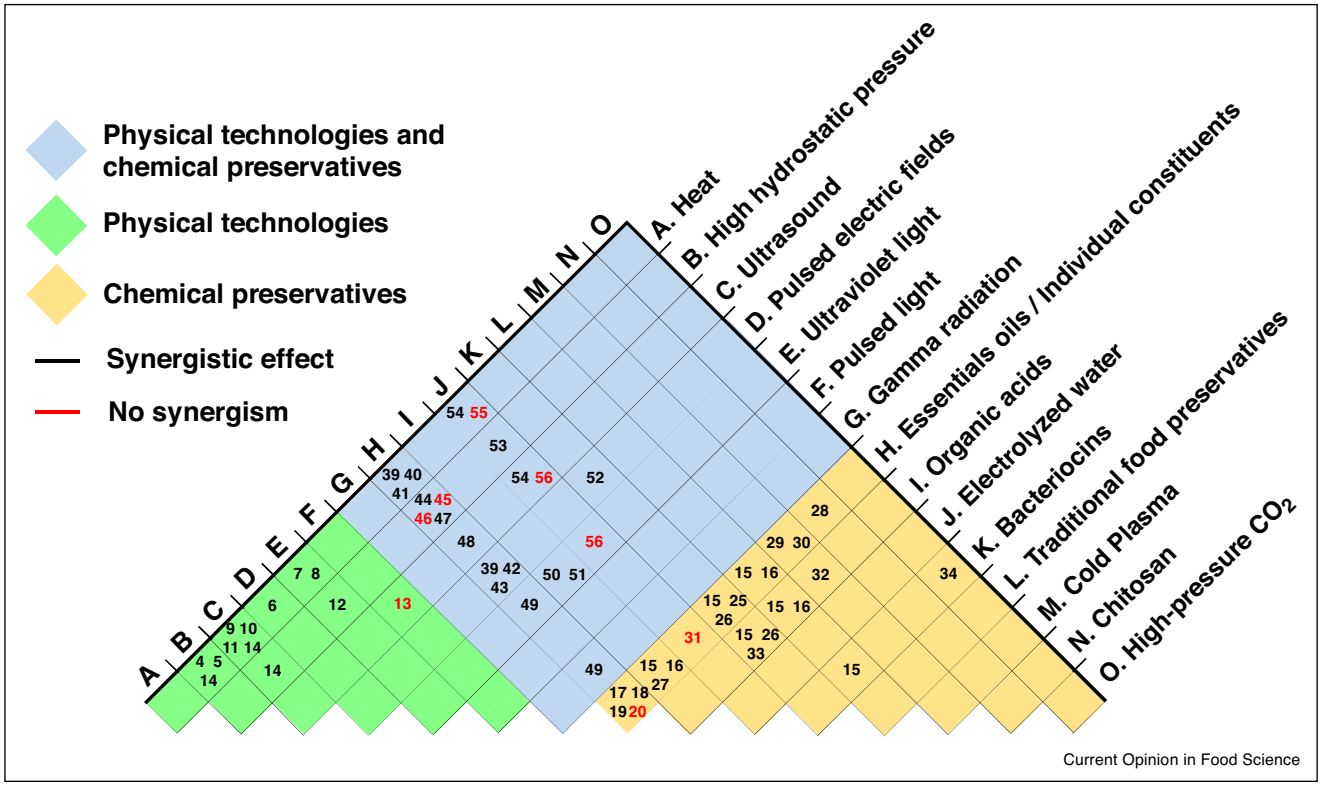
Synergisms resulting from the combination of antimicrobial compounds

Many synergisms resulting from the combination of several chemical compounds are currently being described (Figure 2). Widespread consumer rejection of synthetic compounds have led to an increase in this type of investigation. Certain studies thus describe the combination of traditional food preservatives, such as nitrites [15] or sodium hypochlorite [16], with natural compounds and organic acids in order to decrease the doses applied.

Research in natural antimicrobials is being particularly encouraged, since they are attracting worldwide interest as an alternative to synthetic preservatives. Among them, plant essential oils (EOs) and their individual constituents (ICs) are being extensively studied; their negative effects on organoleptic properties nevertheless restrict their use in foods to a certain extent. Recent studies have observed that combinations among EOs/ICs lead to synergisms that permit the reduction of doses applied. According to Barbosa *et al.* [17], antimicrobial properties of oregano and rosemary EOs were enhanced by their simultaneous application: apart from a bacteriostatic effect, a greater bactericidal effect against pathogens such as *L. monocytogenes*, *E. coli* and *S.* Enteritidis was ascertained in broth and leafy vegetables. Other authors reported similar synergistic effects between *Cymbopogon citratus* and *Allium cepa* EOs at low doses in spinach and lettuce in order to avoid consumer rejection of foodstuffs [18]. With the purpose of reducing EO doses, certain authors have calculated the optimal ratio among different EOs in order to achieve the greatest antimicrobial effect with the lowest amount of EOs [19].

Not all combinations of natural antimicrobials resulted in a synergistic bactericidal or bacteriostatic effect: the combination of mustard and cinnamon EO [20] only led to additive effects. Thus, the effect achieved by

Figure 2



Compilation of scientific publications of combined treatments classified based on the synergistic effects among physical technologies and/or chemical preservatives. The letters in the axes indicate the preservation method studied and the numbers showed in the table correspond to the scientific publications displayed as references in the present review.

combining EOs depends on the specific compounds, their mechanisms of inactivation, and the food matrix. Furthermore, synergistic effects were described when EOs were added to edible coating and films (active packaging) with modified atmosphere packaging for purposes of food preservation [21,22••,23,24].

Enhanced antimicrobial effects were also detected when combining EOs with other preservatives such as nisin in milk [25,26], organic acids in leafy vegetables [16,27], chitosan in laboratory broth [28] or cold nitrogen plasma applied to eggshell or lettuce [29,30]. Conversely, only a negligible bactericidal effect was observed when combining EOs with electrolyzed water (EW) against *E. coli* in nutrient broth medium [31]. Cold plasma also developed a strong synergism when applied in combination with organic acids and SDS in red chicory against *E. coli* and *L. monocytogenes* [32].

Other natural antimicrobials synthesized by microorganisms, such as nisin, displayed interesting bacteriostatic and bactericidal effects in combination with citric acid against *Cronobacter sakazaki* and *E. coli* [33]. Bacteriostatic synergistic effects resulting from the combination of nisin, lactobionic acid, and thymol against *L. monocytogenes* were also observed [26]. The magnitude of the synergism depended on the target bacteria, mainly because of divergent characteristics of envelopes in Gram-positive and Gram-negative bacteria. For instance, combined treatments of nisin and high-pressure carbon dioxide caused a higher inactivation of *S. aureus* than of *E. coli* [34•].

These studies not only describe the potential of treatments combining different chemical compounds for purposes of food preservation, but also assist researchers in identifying the main constituents that determine the efficacy of each antimicrobial substance, and the mechanism of interaction that justifies each synergism. In this regard, efficient food preservation strategies can be designed by coordinating molecular biology techniques with computer analysis in order to identify target structures, involved metabolic pathways, and microbial response to treatments [35,36]. In contrast with physical technologies, however, the lethal activity of antimicrobial compounds is highly influenced by the amount of bacterial concentration present in the foodstuffs [37]: when interpreting results, this must be taken into account.

Synergisms resulting from the combination of physical technologies and antimicrobial compounds

The search for new preservation processes that guarantee food safety and improve food quality has led research into combining physical technologies with food preservatives, particularly with natural antimicrobial compounds (Figure 2). The synergism observed is brought about by alterations of microbial cell envelopes via physical technologies, which facilitate the access of antimicrobials to the target [38].

The notable bactericidal effect of EOs/ICs in combination with mild heat has been widely demonstrated [39•] against planktonic and sessile cells that usually present a high resistance to common disinfectants [40]. Cell membrane disruption and loss of cell membrane potential are considered to be the main causes of bacterial inactivation by heat and ICs [41].

The combination of EOs/ICs with non-thermal technologies also results in synergistic lethal effects. Wang *et al.* [42•] observed a synergism between PEF and carvacrol against *S. aureus*, even when carvacrol was added to the recovery agar medium. This effect was associated with cell permeabilization by PEF, allowing the access of carvacrol to genomic DNA. An improved antimicrobial activity of EOs/ICs combined with PEF has been described for mandarin and cauliflower infusion (5%) against *S.* Typhimurium at 20 kV/cm, or citral (0.2 μL/mL) against *E. coli* (30 kV/cm) [43]. Nevertheless, under the same experimental conditions, EOs applied with heat were more effective than EOs with PEF [39•].

HHP has been combined with EOs, leading to divergent results. Sanz-Puig *et al.* [44] reported a synergistic effect against *S.* Typhimurium when HHP (200 MPa) was combined with cauliflower or mandarin infusion. However, *L. monocytogenes* inactivation by HHP (200–300 MPa) was not improved when combined with thyme extract [45]. Thus, a synergistic, additive, or antagonistic effect of HHP lethal activity can be observed in combined processes depending on the antimicrobial compounds applied, their mode of action, and dosage [46]. Moreover, de Carvalho *et al.* [47] demonstrated that the addition of *Mentha piperita* EO in the form of nanoemulsions improves the lethality of mild heat, PEF, or HHP treatments against *E. coli* O157:H7 in tropical fruit juices.

Successful synergism between physical and chemical processes is sometimes due to the bactericidal effect of the physical technology and the subsequent bacteriostatic effect of the antimicrobial during food shelf-life. For example, whilst US (130 W/20 kHz) reduced the initial microbial contamination in skim milk, citrus extract delayed growth of survivors [48]. Moreover, the application of gamma radiation at low doses (0.5–1 kGy), followed by the application of small amounts of oregano or lemongrass EO with citrus extract and lactic acid, was effective in controlling food pathogens in cauliflower such as *L. monocytogenes* and *E. coli* [49]. Those authors also tested the application of UV-C (5–10 kJ/m^2) in combination with the same compounds, and obtained a less effective but nonetheless promising alternative treatment against *E. coli*.

Apart from EOs/ICs, other natural preservatives such as organic acids and bacteriocins have been tested in combination with physical food preservation technologies. Thus, a synergy between UV-A and gallic or lactic acid inactivated 4.7-logs of *E. coli*, whereas the individual treatments, applied separately, reduced <1-log of initial population [50]. According to Wang *et al.* [51], once gallic acid reaches the cytoplasm, it induces the formation of reactive oxygen species (ROS) together with UV-A, thereby resulting in lethal oxidative damage. With regard to bacteriocins, the combination of nisin with PEF (30 kV/cm) caused up to 3.7-log$_{10}$ or 1.8-logs reduction of exponential- and stationary-phase *E. coli* cells, respectively [52].

One of the chemicals that is attracting great interest in combined treatments is EW. Synergies between EW and HHP were observed in the inactivation of *B. cereus* spores: although separate treatment with EW (44 mg/L of available chlorine) or HHP (500 MPa) inactivated only <1-logs, a combined treatment reached a 4-logs reduction [53]. Likewise, Luo and Oh [54] reported a considerable lethal effect by combining EW (28–30 mg/L) with heat (60 °C) and US (40 kHz/400 W), thereby inactivating ca. 4.5-logs of *S.* Typhimurium and *L. monocytogenes* in 5 min. In that case, the damages on the cell membrane caused by heat and US might facilitate the access of EW, thereby enhancing its lethality. However, the lethal effect of EW (4 mg/L of free available chlorine) on *E. coli* and *L. monocytogenes* was not influenced by heat: bactericidal activity was similar at 20 and 50 °C [55]. Likewise, the simultaneous application of EW with US or UV did not display any relevant synergism against *L. monocytogenes* [56].

The above-described synergistic lethal effects between physical and/or chemical treatments are of great value and might help decrease doses of antimicrobials or reduce treatment intensity, thereby preventing undesirable effects on food quality while ensuring food safety.

Conclusions

This review has summarized some of the most relevant recent publications in the field of combined treatments that associate physical and/or chemical processes for purposes of food preservation. Hundreds of papers related to this topic have been published over the last two years, reflecting the food industry's interest in providing

consumers with safe food of the highest quality. Such strategies are considered successful when they can take advantage of synergistic effects against food pathogens. Among the evaluated hurdles, heat and EOs showed the greatest synergistic effects when applied simultaneously. Moreover, it is noteworthy that, although most studies focus on non-thermal technologies for food preservation, some of those technologies' most interesting lethal effects are currently being obtained by combining them with traditional mild heat treatments. The high potential of the latter resides in their ability to cause sublethal damages in bacterial survivors that enhance the action of other preservation technologies. Thus, the described synergisms allowed an overall reduction of treatment intensities or doses, which helped maintain organoleptic and nutritional food properties. Nevertheless, combinations of non-thermal technologies with preservatives, such as nisin or EW, have also displayed notable synergisms that deserve further evaluation as promising alternatives to traditional treatments in the food industry.

Conflict of interest statement

Nothing declared.

Acknowledgements

This study received financial support from Spanish Ministry of Economy, Industry and Competitiveness (Project AGL2015-69565-P), from FEDER, the European Social Fund, from the Aragonese Office of Science, Technology and University Research (Number A03-17R), and from the Spanish Ministry of Sports, Culture and Education: the latter provided D. Berdejo and E. Pagán with grants to carry out this investigation.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. •• Khan I, Tango CN, Miskeen S, Lee BH, Oh D-H: **Hurdle technology: a novel approach for enhanced food quality and safety – a review**. *Food Control* 2017, **73**:1426-1444.

In this review, physical technologies and chemical preservatives currently applied or of potential use for purposes of food preservation are extensively discussed in an easily comprehensible, well-structured overview that comprises a great number of significant scientific publications.

2. Singh S, Shalini R: **Effect of hurdle technology in food preservation: a review**. *Crit Rev Food Sci Nutr* 2016, **56**:641-649.

3. Leistner L, Gorris LGM: **Food preservation by hurdle technology**. *Trends Food Sci Technol* 1995, **6**:41-46.

4. Ates MB, Rode TM, Skipnes D, Lekang O-I: **Modeling of *Listeria monocytogenes* inactivation by combined high-pressure and mild-temperature treatments in model soup**. *Eur Food Res Technol* 2016, **242**:279-287.

5. Evelyn, Silva FVM: **Modeling the inactivation of psychrotrophic *Bacillus cereus* spores in beef slurry by 600 MPa HPP combined, with 38–70 degrees C: comparing with thermal processing and estimating the energy requirements**. *Food Bioprod Process* 2016, **99**:179-187.

6. Katiyo W, Yang R, Zhao W: **Effects of combined pulsed electric fields and mild temperature pasteurization on microbial inactivation and physicochemical properties of cloudy red apple juice (*Malus pumila* Niedzwetzkyana (Dieck))**. *J Food Saf* 2017, **37**.

7. Garcia Carrillo M, Ferrario M, Guerrero S: **Study of the inactivation of some microorganisms in turbid carrot-orange juice blend processed by ultraviolet light assisted by mild heat treatment**. *J Food Eng* 2017, **212**:213-225.

8. • Gayan E, Jesus Serrano M, Alvarez I, Condon S: **Modeling optimal process conditions for UV-heat inactivation of foodborne pathogens in liquid foods**. *Food Microbiol* 2016, **60**:13-20.

This paper shows that the combined treatment of ultraviolet light and heat delivers truly interesting results in the inactivation of pathogenic bacteria in liquid foods. To quantify the synergism observed between UV and heat, the authors carried out a modeling of the experimental results with the purpose of optimizing the combined treatment to achieve the greatest synergistic effects.

9. Pokhrel PR, Bermudez-Aguirre D, Martinez-Flores HE, Garnica-Romo MG, Sablani S, Tang J, Barbosa-Canovas GV: **Combined effect of ultrasound and mild temperatures on the inactivation of *E. coli* in fresh carrot juice and changes on its physicochemical characteristics**. *J Food Sci* 2017, **82**:2343-2350.

10. Garud SR, Priyanka BS, Negi PS, Rastogi NK: **Effect of thermosonication on bacterial count in artificially inoculated model system and natural microflora of sugarcane juice**. *J Food Process Preserv* 2017, **41**.

11. • Li J, Suo Y, Liao X, Ahn J, Liu D, Chen S, Ye X, Ding T: **Analysis of *Staphylococcus aureus* cell viability, sublethal injury and death induced by synergistic combination of ultrasound and mild heat**. *Ultrason Sonochem* 2017, **39**:101-110.

This study demonstrates the synergistic effect between ultrasound and heat in inactivation treatments against*S. aureus*. The authors performed microbiological assays in selective medium, staining with fluorochromes, and cytometry along with microscopy analysis: they found that the amount of sublethal damage in the cell membrane is one of the main factors in the synergism between ultrasounds and heat.

12. Shahbaz HM, Yoo S, Seo B, Ghafoor K, Kim JU, Lee D-U, Park J: **Combination of TiO_2-UV photocatalysis and high hydrostatic pressure to inactivate bacterial pathogens and yeast in commercial apple juice**. *Food Bioprocess Tech* 2016, **9**:182-190.

13. Ferrario M, Guerrero S: **Effect of a continuous flow-through pulsed light system combined with ultrasound on microbial survivability, color and sensory shelf life of apple juice**. *Innov Food Sci Emerg Technol* 2016, **34**:214-224.

14. Zhu J, Wang Y, Li X, Li B, Liu S, Chang N, Jie D, Ning C, Gao H, Meng X: **Combined effect of ultrasound, heat, and pressure on *Escherichia coli* O157:H7, polyphenol oxidase activity, and anthocyanins in blueberry (*Vaccinium corymbosum*) juice**. *Ultrason Sonochem* 2017, **37**:251-259.

15. Ghabraie M, Khanh Dang V, Huq T, Khan A, Lacroix M: **Antilisterial effects of antibacterial formulations containing essential oils, nisin, nitrite and organic acid salts in a sausage model**. *J Food Sci Tech Mys* 2016, **53**:2625-2633.

16. Poimenidou SV, Bikouli VC, Gardeli C, Mitsi C, Tarantilis PA, Nychas GJ, Skandamis PN: **Effect of single or combined chemical and natural antimicrobial interventions on *Escherichia coli* O157:H7, total microbiota and color of packaged spinach and lettuce**. *Int J Food Microbiol* 2016, **220**:6-18.

17. Barbosa Id M, da Costa Medeiros JA, Rima de Oliveira KA, Gomes-Neto NJ, Tavares JF, Magnani M, de Souza EL: **Efficacy of the combined application of oregano and rosemary essential oils for the control of *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* Enteritidis in leafy vegetables**. *Food Control* 2016, **59**:468-477.

18. Ortega-Ramirez LA, Silva-Espinoza BA, Vargas-Arispuro I, Gonzalez-Aguilar GA, Cruz-Valenzuela MR, Nazzaro F, Ayala-Zavala JF: **Combination of *Cymbopogon citratus* and *Allium cepa* essential oils increased antibacterial activity in leafy vegetables**. *J Sci Food Agric* 2017, **97**:2166-2173.

19. Ouedrhiri W, Balouiri M, Bouhdid S, Moja S, Chandi FO, Taleb M, Greche H: **Mixture design of *Origanum compactum*, *Origanum majorana* and *Thymus serpyllum* essential oils: optimization of their antibacterial effect**. *Ind Crops Prod* 2016, **89**:1-9.

20. Clemente I, Aznar M, Silva F, Nerin C: **Antimicrobial properties and mode of action of mustard and cinnamon essential oils and their combination against foodborne bacteria**. *Innov Food Sci Emerg Technol* 2016, **36**:26-33.

21. Krepker M, Shemesh R, Poleg YD, Kashi Y, Vaxman A, Segal E: **Active food packaging films with synergistic antimicrobial activity**. *Food Control* 2017, **76**:117-126.

22. •• Kapetanakou AE, Skandamis PN: **Applications of active packaging for increasing microbial stability in foods: natural volatile antimicrobial compounds**. *Curr Opin Food Sci* 2016, **12**:1-12.
This review summarizes the latest advances in the use of natural compounds for the development of active packaging in order to enhance the microbial stability, improve the safety, and prolong the shelf life of foodstuffs. It provides an overview of a great number of successful experiments in active packaging with the use of natural compounds, and points out this strategy's great potential as a method for food preservation.

23. Atares L, Chiralt A: **Essential oils as additives in biodegradable films and coatings for active food packaging**. *Trends Food Sci Technol* 2016, **48**:51-62.

24. Ahmed I, Lin H, Zou L, Brody AL, Li Z, Qazi IM, Pavase TR, Lv L: **A comprehensive review on the application of active packaging technologies to muscle foods**. *Food control* 2017, **82**:163-178.

25. Zhao X, Shi C, Meng R, Liu Z, Huang Y, Zhao Z, Guo N: **Effect of nisin and perilla oil combination against *Listeria monocytogenes* and *Staphylococcus aureus* in milk**. *J Food Sci Tech Mys* 2016, **53**:2644-2653.

26. Chen H, Zhong Q: **Lactobionic acid enhances the synergistic effect of nisin and thymol against *Listeria monocytogenes* Scott A in tryptic soy broth and milk**. *Int J Food Microbiol* 2017, **260**:36-41.

27. Chai C, Lee S, Kim J, S-W Oh: **Synergistic antimicrobial effects of organic acids in combination with carvacrol against *Shigella sonnei***. *J Food Saf* 2016, **36**:360-366.

28. Marangon CA, Martins VCA, Leite PMF, Santos DA, Nitschke M, Plepis AMG: **Chitosan/gelatin/copaiba oil emulsion formulation and its potential on controlling the growth of pathogenic bacteria**. *Ind Crops Prod* 2017, **99**:163-171.

29. Cui H, Ma C, Li C, Lin L: **Enhancing the antibacterial activity of thyme oil against *Salmonella* on eggshell by plasma-assisted process**. *Food Control* 2016, **70**:183-190.

30. Cui H, Ma C, Lin L: **Synergetic antibacterial efficacy of cold nitrogen plasma and clove oil against *Escherichia coli* O157: H7 biofilms on lettuce**. *Food Control* 2016, **66**:8-16.

31. Sow LC, Tirtawinata F, Yang H, Shao Q, Wang S: **Carvacrol nanoemulsion combined with acid electrolysed water to inactivate bacteria, yeast in vitro and native microflora on shredded cabbages**. *Food Control* 2017, **76**:88-95.

32. Trevisani M, Berardinelli A, Cevoli C, Cecchini M, Ragni L, Pasquali F: **Effects of sanitizing treatments with atmospheric cold plasma, SDS and lactic acid on verotoxin-producing *Escherichia coli* and *Listeria monocytogenes* in red chicory (radicchio)**. *Food Control* 2017, **78**:138-143.

33. Campion A, Morrissey R, Field D, Cotter PD, Hill C, Ross RP: **Use of enhanced nisin derivatives in combination with food-grade oils or citric acid to control *Cronobacter sakazakii* and *Escherichia coli* O157:H7**. *Food Microbiol* 2017, **65**:254-263.

34. • Li H, Xu Z, Zhao F, Wang Y, Liao X: **Synergetic effects of high-pressure carbon dioxide and nisin on the inactivation of *Escherichia coli* and *Staphylococcus aureus***. *Innov Food Sci Emerg Technol* 2016, **33**:180-186.
This study demonstrated the notable synergistic effects of combined treatments of high-pressure carbon dioxide and nisin, applied simultaneously against*S. aureus*. Conversely, only a slight effect against *E. coli* was observed. According to the authors, the outer membrane of Gram-negative bacteria counteracts the synergism between those two technologies, which had proved successful against *S. aureus* (which is Gram-positive).

35. Chueca B, Perez-Saez E, Pagan R, Garcia-Gonzalo D: **Global transcriptional response of *Escherichia coli* MG1655 cells exposed to the oxygenated monoterpenes citral and carvacrol**. *Int J Food Microbiol* 2017, **257**:49-57.

36. Chueca B, Renzoni A, Berdejo D, Pagan R, Kelley WL, Garcia-Gonzalo D: **Whole-genome sequencing and genetic analysis reveal novel stress responses to individual constituents of essential oils in *Escherichia coli***. *Appl Environ Microbiol* 2018, **84**.

37. Somolinos M, Garcia D, Condon S, Mackey B, Pagan R: **Inactivation of *Escherichia coli* by citral**. *J Appl Microbiol* 2010, **108**:1928-1939.

38. Chueca B, Pagán R, García-Gonzalo D: **Transcriptomic analysis of *Escherichia coli* MG1655 cells exposed to pulsed electric fields**. *Innov Food Sci Emerg Technol* 2015, **29**:78-86.

39. • Pagan E, Berdejo D, Espina L, Garcia-Gonzalo D: **Antimicrobial activity of suspensions and nanoemulsions of citral in combination with heat or pulsed electric fields**. *Lett Appl Microbiol* 2018, **66**:63-70.
This study describes the improvement of the antimicrobial properties of citral in form of a nanoemulsion when it is applied simultaneously with heat and pulsed electric fields. The results suggest that the use of nanoemulsions of natural compounds could maintain or even increase the strong synergistic effects already achieved by antimicrobials in conjunction with physical technologies.

40. Espina L, Berdejo D, Alfonso P, Garcia-Gonzalo D, Pagan R: **Potential use of carvacrol and citral to inactivate biofilm cells and eliminate biofouling**. *Food Control* 2017, **82**:256-265.

41. Kim S-S, D-H Kang: **Combination treatment of ohmic heating with various essential oil components for inactivation of food-borne pathogens in buffered peptone water and salsa**. *Food Control* 2017, **80**:29-36.

42. • Wang L-H, Wang M-S, Zeng X-A, Zhang Z-H, Gong D-M, Huang Y-B: **Membrane destruction and DNA binding of *Staphylococcus aureus* cells induced by carvacrol and its combined effect with a pulsed electric field**. *J Agric Food Chem* 2016, **64**:6355-6363.
The results reported in this paper demonstrate the potential of pulsed electric fields to enhance the bactericidal effect of carvacrol against*S. aureus*. The authors show that carvacrol exerts its effect via two mechanisms: it increases the membrane permeability also brought about by pulsed electric fields, and binds directly to genomic DNA.

43. Sanz-Puig M, Santos-Carvalho L, Cunha LM, Consuelo Pina-Perez M, Martinez A, Rodrigo D: **Effect of pulsed electric fields (PEF) combined with natural antimicrobial by-products against *S. Typhimurium***. *Innov Food Sci Emerg Technol* 2016, **37**:322-328.

44. Sanz-Puig M, Moreno P, Consuelo Pina-Perez M, Rodrigo D, Martinez A: **Combined effect of high hydrostatic pressure (HHP) and antimicrobial from agro-industrial by-products against *S. Typhimurium***. *LWT – Food Sci Technol* 2017, **77**:126-133.

45. Bleoanca I, Saje K, Mihalcea L, Oniciuc E-A, Smole-Mozina S, Nicolau AI, Borda D: **Contribution of high pressure and thyme extract to control *Listeria monocytogenes* in fresh cheese – a hurdle approach**. *Innov Food Sci Emerg Technol* 2016, **38**:7-14.

46. Li H, Ganzle M: **Effect of hydrostatic pressure and antimicrobials on survival of *Listeria monocytogenes* and enterohaemorrhagic *Escherichia coli* in beef**. *Innov Food Sci Emerg Technol* 2016, **38**:321-327.

47. de Carvalho RJ, de Souza GT, Pagán E, García-Gonzalo D, Magnani M, Pagán R: **Nanoemulsions of *Mentha piperita* L. essential oil in combination with mild heat, pulsed electric fields (PEF) and high hydrostatic pressure (HHP) as an alternative to inactivate *Escherichia coli* O157: H7 in fruit juices**. *Innov Food Sci Emerg Technol* 2018, **48**:219-227.

48. Bevilacqua A, Speranza B, Iorio MC, Loi M, Sinigaglia M, Corbo MR: **UN-INACTIVATION of foodborne bacteria: screening in distilled water and combination with citrus extract in skim milk**. *LWT – Food Sci Technol* 2016, **70**:135-141.

49. Tawema P, Han J, Vu KD, Salmieri S, Lacroix M: **Antimicrobial effects of combined UV-C or gamma radiation with natural antimicrobial formulations against *Listeria monocytogenes*, *Escherichia coli* O157: H7, and total yeasts/molds in fresh cut cauliflower**. *LWT – Food Sci Technol* 2016, **65**:451-456.

50. de Oliveira EF, Cossu A, Tikekar RV, Nitin N: **Enhanced antimicrobial activity based on a synergistic combination of sublethal levels of stresses induced by UV-A light and organic acids**. *Appl Environ Microbiol* 2017, **83**.

51. Wang Q, de Oliveira EF, Alborzi S, Bastarrachea LJ, Tikekar RV: **On mechanism behind UV-A light enhanced antibacterial activity of gallic acid and propyl gallate against *Escherichia coli* O157: H7**. *Sci Rep* 2017, **7**:8325.

52. Novickij V, Staneviciene R, Grainys A, Luksa J, Badokas K, Krivorotova T, Sereikaite J, Novickij J, Serviene E: **Electroporation-assisted inactivation of *Escherichia coli* using nisin-loaded pectin nanoparticles**. *Innov Food Sci Emerg Technol* 2016, **38**:98-104.

53. Wang L, Xia Q, Li Y: **Synergistic effects of high pressure processing and slightly acidic electrolysed water on the inactivation of *Bacillus cereus* spores**. *Int J Food Sci Technol* 2017, **52**:2429-2435.

54. Luo K, D-H Oh: **Inactivation kinetics of *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium on fresh-cut bell pepper treated with slightly acidic electrolyzed water combined with ultrasound and mild heat**. *Food Microbiol* 2016, **53**:165-171.

55. Liu Q, Tan CSC, Yang H, Wang S: **Treatment with low-concentration acidic electrolysed water combined with mild heat to sanitise fresh organic broccoli (*Brassica oleracea*)**. *LWT – Food Sci Technol* 2017, **79**:594-600.

56. Miks-Krajnik M, Feng LXJ, Bang WS, H-G Yuk: **Inactivation of *Listeria monocytogenes* and natural microbiota on raw salmon fillets using acidic electrolyzed water, ultraviolet light or/and ultrasounds**. *Food Control* 2017, **74**:54-60.

2. Objetivos

Justificación de los trabajos

Uno de los primeros pasos de esta Tesis Doctoral consistió en conocer cuáles eran las técnicas y herramientas actuales de estudio de los mecanismos de inhibición e inactivación microbiana, así como las principales estrategias de conservación de los alimentos, entre las que destaca el diseño de procesos combinados (**Manuscrito I**). A través de esta revisión bibliográfica se observó que, aunque los procesos combinados pueden llegar a ser muy efectivos, en muchos casos carecen de base científica y son el fruto de ensayos de prueba y error.

La implementación de nuevas herramientas en el campo de la biología molecular ofrece nuevas estrategias para abordar una mejor descripción de los fenómenos que conducen a la inhibición o inactivación bacteriana por tecnologías de conservación de los alimentos. En este sentido, el estudio de cepas resistentes permite conocer la respuesta celular frente a los métodos de conservación y, de tal modo, profundizar en sus mecanismos de inhibición e inactivación bacteriana. Por esta razón, se llevó a cabo la puesta a punto de ensayos de evolución, inicialmente frente a antimicrobianos naturales, por su potencial como alternativa a los conservantes de síntesis química, mediante la exposición prolongada a concentraciones subinhibitorias o la aplicación de tratamientos letales, para el aislamiento de variantes genéticas bacterianas resistentes (**Manuscrito II**). En este sentido, el empleo de CI y AE a diferentes concentraciones facilitó el desarrollo de protocolos tanto para métodos de inhibición como de inactivación microbiana, de forma que pueden ser fácilmente implementados para el estudio de otros métodos de conservación.

En primer lugar, se quiso demostrar si, al igual que se había observado previamente en *E. coli*, era posible la aparición y el aislamiento de variantes genéticas resistentes en bacterias Gram-positivas a través de la exposición a dosis subinhibitorias de CI de forma prolongada (**Manuscrito III**). A continuación, se evaluó si la aparición de estas variantes resistentes estaba condicionada a la forma de empleo de los CI como agentes bacteriostáticos o bactericidas. Para ello, se llevaron a cabo ensayos de evolución mediante tratamientos letales y se procedió a su comparación con las variantes genéticas de la misma bacteria y frente al mismo CI obtenidas frente a dosis subinhibitorias, en este caso en una nueva especie Gram-negativa, *Salmonella enterica* Typhimurium (**Manuscrito IV**).

Los CI no se encuentran de forma individual y pura en la naturaleza, sino que estos metabolitos de origen vegetal coexisten en los AE de las plantas con decenas de otros CI. Por esta razón, el siguiente objetivo fue determinar si, al igual que se había observado frente a CI puros, era posible la aparición de variantes genéticas resistentes frente a AE complejos, a pesar de que no se habían descrito previamente y se consideraba poco probable debido a su complejidad composicional y, por tanto, a los múltiples mecanismos de inhibición e inactivación que pueden presentar (**Manuscrito V**).

Tras confirmar la aparición de variantes genéticas resistentes frente a AE, se realizó una aproximación del riesgo microbiológico que podrían suponer en la conservación de alimentos, tanto de variantes bacterianas Gram-positivas (**Manuscrito VI**) como Gram-negativas (**Manuscrito VII**).

Por otro lado, el uso de AE y CI en la conservación de alimentos se encuentra limitado debido a la baja solubilidad en medios acuosos y la inestabilidad frente a agentes externos como la luz y el oxígeno. Con el objetivo de facilitar y potenciar su empleo como conservantes alimentarios o biocidas, en primer lugar, se evaluó la estabilidad y solubilidad de CI mediante la preparación de nanoemulsiones por el método de inversión de fases con tween 80 y etanol, así como su impacto sobre sus propiedades antimicrobianas (**Manuscrito VIII**). A continuación, se evaluó el método de encapsulación mediante zeínas para AE y su empleo como antimicrobiano de forma individual y combinado con calor (**Manuscrito IX**). Por último, se estudió la encapsulación mediante ciclodextrinas y la obtención de encapsulados en forma sólida (polvo) mediante liofilización y secado por pulverización, así como su repercusión sobre la actividad antimicrobiana del CI (**Manuscrito X**).

2. Objetivos

El principal objetivo de esta tesis doctoral es la obtención, bajo presión selectiva, de variantes genéticas bacterianas resistentes, y su caracterización fenotípica y genotípica, como estrategia para estudiar los mecanismos de resistencia celular frente al uso de antimicrobianos y otros métodos de conservación de los alimentos. Concretamente, se propone abordar el aislamiento de variantes genéticas resistentes de *Staphylococcus. aureus*, *Salmonella enterica* Typhimurium y *Listeria monocytogenes* mediante el uso de aceites esenciales (AE) y sus constituyentes individuales (CI), con el fin de potenciar sus propiedades antimicrobianas como conservantes o biocidas, así como profundizar en la repercusión de la aparición de estas variantes en la cadena alimentaria. Para la consecución de este objetivo general, se establecieron los siguientes objetivos parciales:

1. Puesta a punto de los ensayos de evolución para la obtención de variantes resistentes mediante exposición a AE y CI tanto a concentraciones subinhibitorias como a dosis letales.
2. Determinación de la frecuencia mutagénica bacteriana bajo la exposición prolongada a los AE y CI.
3. Caracterización fenotípica de las variantes genéticas resistentes aisladas:
    3.1. Estudio de la resistencia y tolerancia a los antimicrobianos empleados en los ensayos de evolución, mediante la determinación de la concentración mínima inhibitoria y bactericida, el estudio de cinéticas de crecimiento bajo concentraciones subinhibitorias y la obtención de curvas de supervivencia frente a tratamientos letales.
    3.2. Estudio de la resistencia cruzada frente a otras tecnologías de conservación de alimentos como los tratamientos térmicos y los pulsos eléctricos de alto voltaje.
    3.3. Estudio de la resistencia cruzada frente a antibióticos como aminoglucósidos, betalactámicos, quinolonas y tetraciclinas.
4. Caracterización genotípica de las variantes resistentes e identificación de las modificaciones genéticas responsables de los cambios observados en su fenotipo.
5. Evaluación de la resistencia de las variantes genéticas en alimentos modelo para explorar su repercusión en la industria agroalimentaria.

De forma complementaria, en el marco de esta tesis doctoral se ha explorado el empleo de surfactantes, como polisorbato 80, y encapsulantes, como zeínas y ciclodextrinas, en la preparación de emulsiones y encapsulados de AE y CI con el objetivo de facilitar y potenciar su empleo como conservantes alimentarios, así como biocidas en sistemas de limpieza y desinfección. Para la consecución de este segundo objetivo, se plantearon los siguientes objetivos parciales:

1. Caracterización de las emulsiones y encapsulados de AE y CI, así como determinación de su estabilidad.
2. Estudio de las propiedades antimicrobianas de las emulsiones y encapsulados y su comparación con el uso de AE y CI en forma libre (en suspensión).
3. Valoración de la utilización de emulsiones y encapsulados de AE y CI en procesos combinados con otras tecnologías de conservación de alimentos como los tratamientos térmicos o los pulsos eléctricos de alto voltaje.

3. Material y Métodos

3. Material y Métodos

Puesto que los materiales y métodos se encuentran ampliamente detallados en cada uno de los manuscritos presentados en la presente Tesis Doctoral, en este apartado se presentan de forma esquemática los diseños experimentales que han permitido el desarrollo y la consecución de los objetivos previamente planteados.

A continuación, se muestra el diseño experimental del objetivo principal de las tesis:

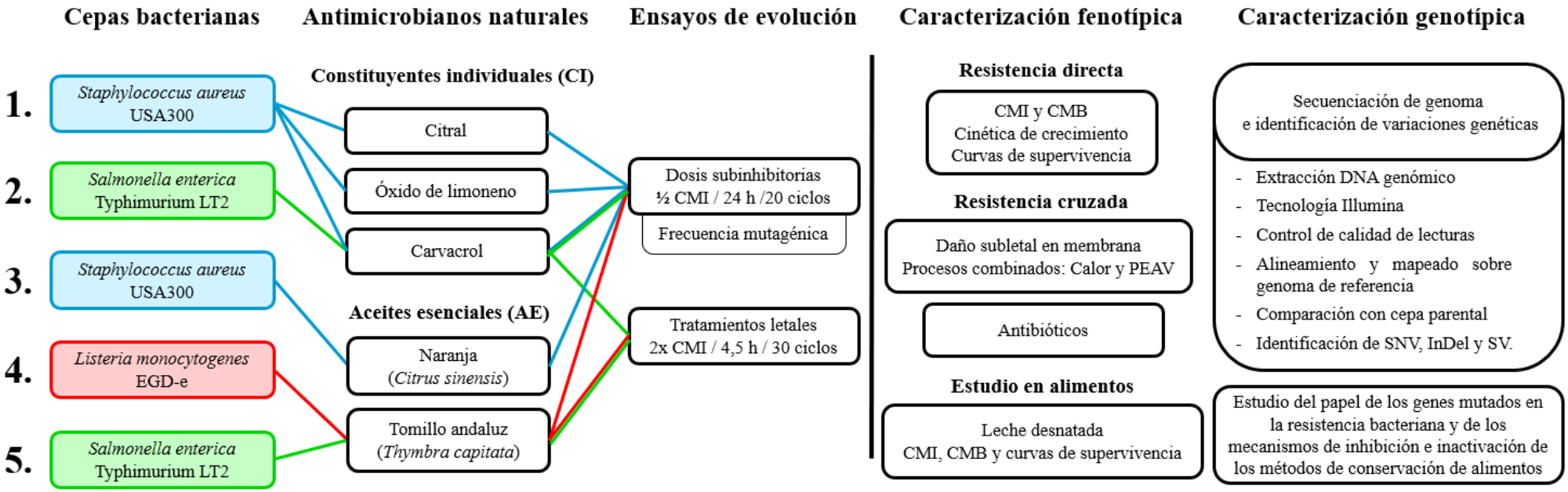
A) Aislamiento y caracterización de variantes genéticas bacterianas resistentes a nivel fenotípico y genotípico, en base a los 5 manuscritos que conforman esta sección de la Tesis Doctoral.

Asimismo, se expone el diseño experimental del objetivo complementario:

B) Estudios de emulsificación y encapsulación de AE y CI para la mejora de su estabilidad y solubilidad en agua con el fin de potenciar y facilitar su uso como conservante antimicrobiano y biocida, relacionado con los 3 manuscritos que conforman esta sección de la Tesis Doctoral.

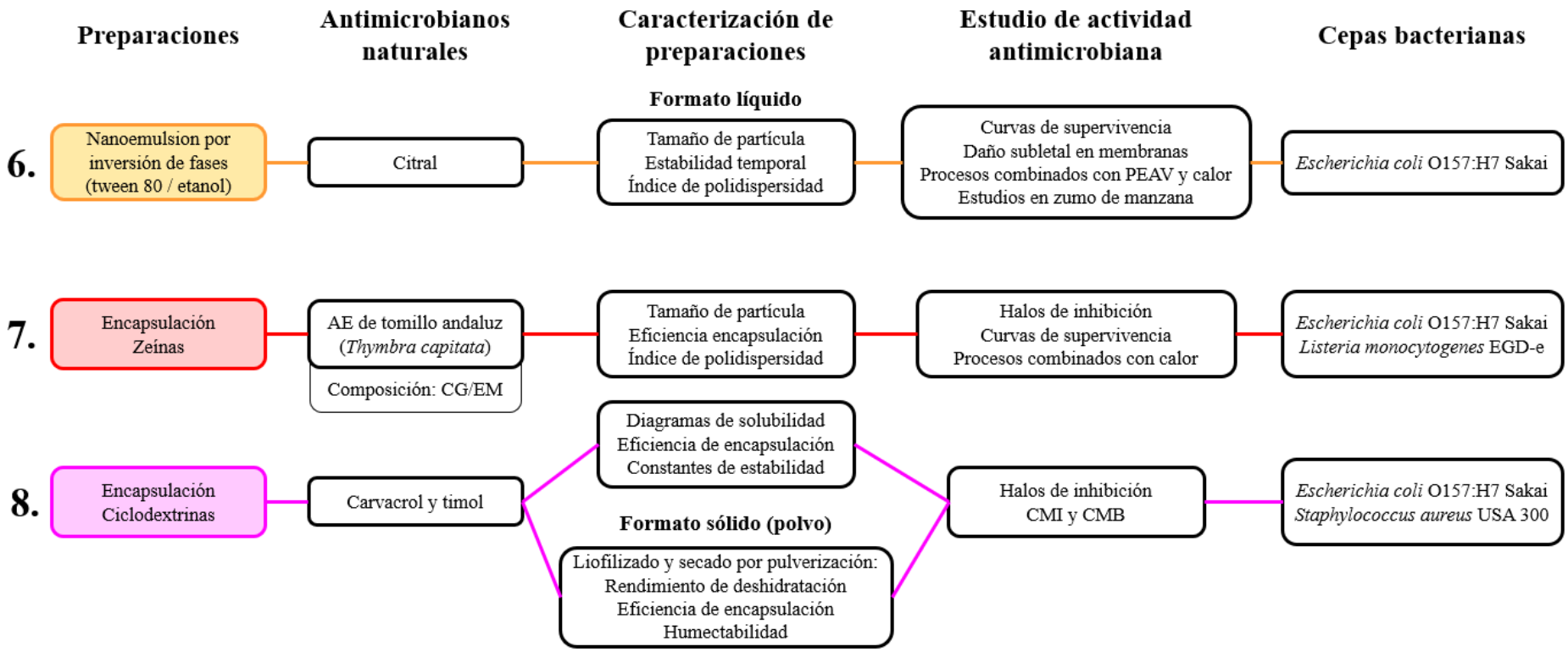
En último lugar, debido a la relevancia de los ensayos de evolución en esta Tesis Doctoral, se adjuntan los protocolos puestos a punto para la obtención de variantes genéticas resistentes, concretamente frente a AE y CI, mediante la exposición prolongada a dosis subinhibitorias y tras tratamientos letales, recogidos en un capítulo de libro (Manuscrito II).

A) Ensayos de evolución para la obtención y caracterización de variantes genéticas resistentes a nivel fenotípico y genotípico.



CMI: concentración mínima inhibitoria, CMB: concentración mínima bactericida, PEAV: Pulsos eléctricos de alto voltaje, SNV: Single nucleotide variant (variante de nucleótido único), InDel: Insertions and deletions (inserciones y deleciones), SV: Structural variant (variante estructural).

B) Estudios de emulsificación y encapsulación de AE y CI para la mejora de su estabilidad y solubilidad en agua.



CG/EM: Cromatografía de gases acoplada a espectrometría de masas, PEAV: Pulsos eléctricos de alto voltaje, CMI: concentración mínima inhibitoria, CMB: concentración mínima bactericida.

Manuscrito II. Evolution assays for the isolation of mutant bacteria resistant to natural antimicrobials

Berdejo, D., Pagán, E., Merino, N., García-Gonzalo, D., Pagán, R.

Marciane, M. (Ed.), *Detection and enumeration of bacteria, yeast, viruses, and protozoan in foods and freshwater*. Springer Nature.

Evolution assays for the isolation of mutant bacteria resistant to natural antimicrobials

Daniel Berdejo, Elisa Pagán, Natalia Merino, Diego García-Gonzalo and Rafael Pagán*

Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain

Running Head: Isolation of resistant mutant bacteria

*Corresponding author: Rafael Pagán Tomás, DVM PhD

Address: Tecnología de los Alimentos, Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Miguel Servet 177, 50013, Zaragoza, Spain.

Phone number: 34-976-762675

Fax number: 34-976-761590

E-mail: pagan@unizar.es

Abstract

Natural antimicrobial compounds have been proposed as a promising alternative to current preservation treatments for minimally processed foods. However, the currently required doses are too high, which leads to sensory alteration due to their strong organoleptic properties. For this reason, further research is still needed regarding their mode of action in order to optimize their antibacterial properties. In this regard, it has become useful to deliberately obtain resistant mutant strains in order to study the underlying mechanisms of antimicrobial resistance. Two different evolution assay protocols have been designed for the obtention of mutant strains with increased resistance against natural antimicrobials: cyclic exposure to prolonged sub-inhibitory doses, and cyclic exposure to short lethal treatments. The phenotypic and genotypic characterization of the evolved strains will provide knowledge about cellular response and resistance mechanisms against antimicrobial compounds, which will help to optimize their use as preservatives in the food industry, or as cleaning and disinfection treatments.

Key words Antimicrobial resistance, Mutagenesis, Evolution assay, Natural antimicrobial compounds, Essential oils, Individual constituents, Bacteria, Whole genome sequencing

1. Introduction

Thermal inactivation is the main technology used in the industry as a preservation method to ensure food safety and stability. However, new consumer trends have encouraged the search for preservation methods that manage to maintain the nutritional and sensory properties of food while ensuring microbial safety and stability. In this regard, natural antimicrobial compounds such as essential oils (EOs) and their individual constituents (ICs) have been proposed as a promising alternative to current preservation treatments for minimally processed foods ***(1, 2)***. These natural compounds have been extensively studied, and have been shown to possess excellent antimicrobial properties against food-related pathogens ***(3, 4)***. Moreover, most of them are generally recognized as safe (GRAS) by the U. S. Food and Drug Administration. However, the doses required to use them as a single method of food preservation are too high, since their strong organoleptic properties might cause sensory alteration ***(5, 6)***. One of the devised solutions is to apply them in combination with other antimicrobial compounds or food preservation technologies to achieve synergistic lethal effects ***(7)***, thereby reducing treatment dose and intensity while avoiding the alteration of treated food ***(8, 9)***. Nevertheless, the design of effective combined treatments for microbial control in foods also requires a thorough understanding of their mechanisms of action on bacteria: for this reason, further research is still needed in order to optimize their antibacterial properties.

In recent years, several investigations have focused on the study of microbial genotypic resistance with the purpose of obtaining a better understanding of the mechanisms of cellular response to antimicrobials. Antibiotic resistance studies are an outstanding example thereof ***(10)***. In fact, once the success derived from studying resistant mutant strains was observed, several authors devised laboratory evolution assays designed to obtain resistant strains that would allow for more in-depth studies ***(11)***. The first evolution

assays were based on the cyclic exposure of bacterial cell populations to prolonged sub-inhibitory doses ***(12)***. The obtention of mutant strains thus became a useful tool for the study of resistance to antibiotics. Nevertheless, this technique was initially discarded in favour of the study of bacterial behaviour against natural antimicrobials, due to the antioxidant properties of the latter ***(13)***, which reduced mutagenic frequency and would therefore prevent the occurrence of mutant strains ***(14)***. However, recent studies have reported the emergence of resistant strains to natural antimicrobials: against ICs (carvacrol, citral, and limonene oxide) in *Escherichia coli* ***(15)***, *Staphylococcus aureus* ***(16)***, *Listeria monocytogenes* (unpublished results), and *Salmonella* Typhimurium ***(17),*** and against EOs (*Citrus sinensis*) in *S. aureus* ***(18)*** by cyclic exposure to prolonged sub-inhibitory doses. In addition, whole genome sequencing of those strains allowed to identify the mutations responsible for the increased resistance ***(16, 17, 18, 19)***.

Evolution assays were adapted to lethal treatments in order to investigate resistant strains obtained from survivors to high antibiotic concentrations ***(20)***. This technique was likewise employed in other research areas such as food preservation, and new protocols were designed with the purpose of obtaining resistant mutant strains from the tails of survival curves after the application of lethal treatments. In this way, resistant strains were also obtained under physical food preservation technologies, such as heat ***(21)*** and high hydrostatic pressure ***(22)***. Evolution assays have thus also been adapted to the isolation of resistant mutant strains following the application of lethal doses of natural antimicrobial compounds ***(17)***.

In this chapter we describe the methodology required to perform evolution assays with natural antimicrobials by two different protocols: a) by cyclic exposure to prolonged sub-inhibitory doses, and b) by cyclic exposure to short lethal treatments. Our aim is to explain how to obtain and characterize mutant resistant strains against natural antimicrobial

compounds. The phenotypic and genotypic study of these strains will allow for a better understanding of the mechanisms of bacterial resistance and, consequently, lead to a more profound knowledge of the mechanisms of antimicrobial action displayed by natural antimicrobial compounds. This information might lead to the design of new and more effective food preservation strategies in the industry.

2. Materials

2.1. Evolution assay by cyclic exposure to prolonged sub-inhibitory doses

1. Cryovial of the bacterial strain to study.
2. Culturing tools: micropipettes, pipette tips, plastic 1.5 mL-tubes, Petri dishes (90 mm), inoculation loops, and L-shaped spreaders.
3. Growth media: tryptic soya agar and broth supplemented with 0.6% yeast extract (or any other nutritive agar and broth).
4. Natural antimicrobial compound (essential oils, individual constituents, natural extracts…).
5. Phosphate Buffer Saline (PBS).
6. Glass test tubes and caps.
7. Vortex.
8. Incubator with orbital shaker.
9. Cryovials.

2.2. Evolution assay by cyclic exposure to short lethal treatments

1. Cryovial of the bacterial strain to study.
2. Culturing tools: micropipettes, pipette tips, plastic 1.5 mL-tubes, Petri dishes (90 mm), inoculation loops, and L-shaped spreaders.

3. Growth media: tryptic soya agar and broth supplemented with 0.6% yeast extract (or any other nutritive agar and broth).
4. Natural antimicrobial compound (essential oils, individual constituents, natural extracts…).
5. Phosphate Buffer Saline (PBS).
6. Glass test tubes, glass flasks (250 mL) and caps.
7. Vortex.
8. Centrifuge.
9. Incubator with orbital shaker.
10. Cryovials.

3. Methods

Prior to either of the two evolution assay protocols, it is necessary to obtain a working bacterial culture of the wild type strain (WT) from which evolution assays will be triggered, as well as to determine the minimum inhibitory concentration (MIC) of the antimicrobial that is being tested. MIC is established as the lowest concentration of the antimicrobial compound capable of inhibiting bacterial growth. The MIC value will be used later on to perform the evolution assays.

The following steps (1-4) to obtain the **initial working bacterial culture** are common to both evolution protocols:

1. From a cryovial of WT, inoculate and streak over the agar plates surface with an inoculation loop to obtain individual colonies (*see* **Note 1**).
2. Incubate the agar plates for 24 h at 37 ºC (*see* **Note 2**).
3. Inoculate a single colony in 5 mL of growth broth in test tube (*see* **Note 3**).

4. Incubate overnight at 37 ºC and 130 rpm until a stationary phase culture is obtained (*see* **Note 4**).

It is then necessary to carry out **MIC determination** of the tested natural antimicrobial:

5. Prepare 5 mL test tubes of growth broth with increasing concentrations of the natural antimicrobial, and shake vigorously by vortex (*see* **Note 5**).
6. Inoculate the test tubes with a stationary phase culture at an initial concentration of $1x10^5$ CFU/mL (colony forming units/mL).
7. Prepare positive control tubes with 5 mL of growth broth inoculated at $1x10^5$ CFU/mL without antimicrobial, and negative control tubes with 5 mL of growth broth non-inoculated with the natural antimicrobial.
8. Incubate all the test tubes for 24 h at 37 ºC and 130 rpm.
9. Observe the turbidity of the growth broth. If the growth medium is cloudy, this means that bacteria have grown, whereas no turbidity indicates that the concentration of the antimicrobial is sufficient to inhibit bacterial growth (*see* **Note 6**).
10. The lowest concentration that has inhibited bacterial growth is established as the MIC.

Use 0.5x of the MIC to carry out the evolution assay by cyclic exposure to prolonged sub-inhibitory doses, and 2x the MIC to conduct the evolution assay by cyclic exposure to short lethal treatments. Figure 1 shows the scheme of the two protocols of evolution assays designed to obtain mutant strains resistant to natural antimicrobial compounds.

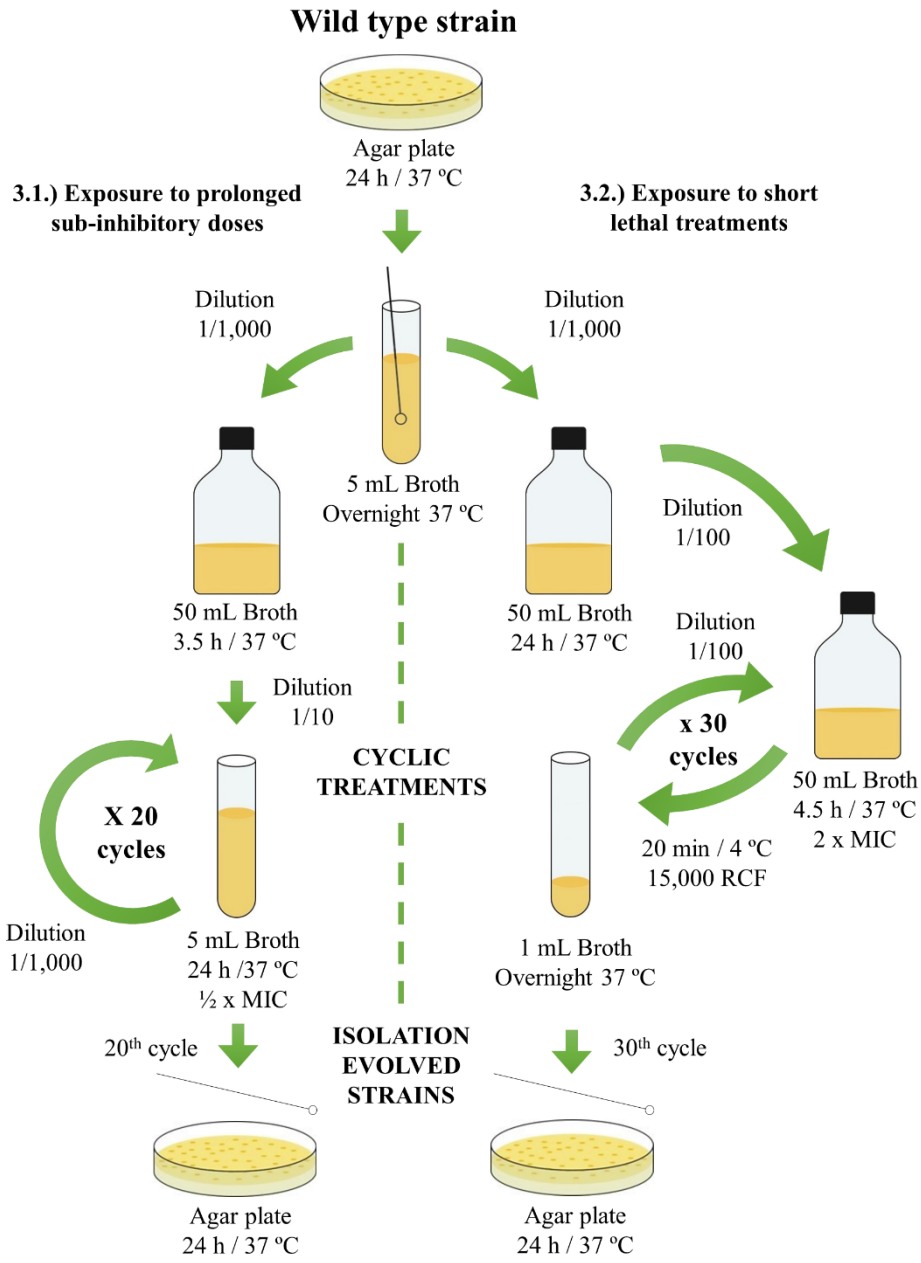


Figure 1. Diagram of evolution assay 3.1.) by cyclic exposure to prolonged sub-inhibitory doses and 3.2.) by cyclic exposure to short lethal treatments.

3.1. Evolution assay by cyclic exposure to prolonged sub-inhibitory doses

This protocol is based on the application of constant stress to the bacterial population at low concentration. The aim is to allow the occurrence of mutations in the bacterial population that improve its growth fitness in the presence of the antimicrobial agent. Thus, if such mutations occur, the agent's selective presence will facilitate the emergence

of such strains in contrast with the WT as well as with other mutants whose mutation is not related to resistance to the agent, thereby allowing the isolation of strains that are resistant to the selected antimicrobial compound (Figure 2).

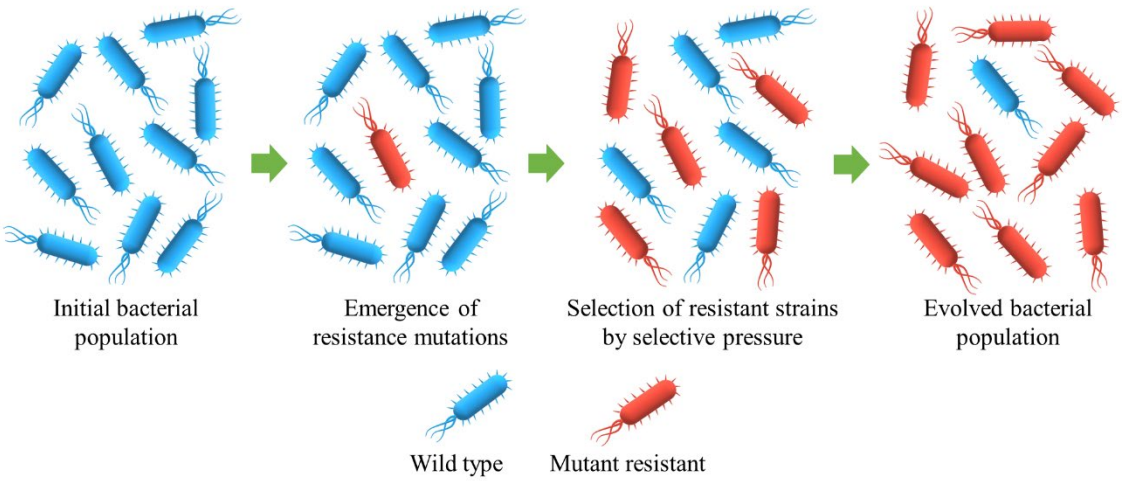


Figure 2. Selection of resistant mutant strains by cyclic exposure to prolonged sub-inhibitory doses.

To carry out the evolution assay by cyclic exposure to prolonged sub-inhibitory doses, the following steps should be taken (continue from step 4):

5. Dilute working bacterial culture of WT 1:1,000 into 50 mL growth broth and incubate for 3.5-4 h at 37 ºC and 130 rpm until an exponential phase culture is obtained (*see* **Note 7**).
6. Once grown, inoculate 5 mL growth broth in test tubes at an initial concentration of 10^6 CFU/mL in the presence of 0.5x of the MIC of the antimicrobial compound.
7. Incubate for 24 h at 37 ºC and 130 rpm until stationary phase is reached (*see* **Note 4**).
8. Return to step 6. After 20 cycles, continue with step 9 (*see* **Note 8**).
9. After the 20th cycle, dilute the bacterial culture in PBS, and inoculate and spread on agar plates to obtain individual colonies.

10. Incubate the agar plates for 24 h at 37 ºC (*see* **Note 2**).
11. After the incubation on agar plates, select several colonies and store them in cryovials (*see* **Note 9**).

3.2. Evolution assay by cyclic exposure to short lethal treatments

This protocol is based on the application of short cyclic treatments to the bacterial population at high concentration. Its goal is to provoke the emergence of mutations associated with resistance to lethal treatments of the antimicrobial agent. Evolved bacteria that have suffered mutations implying increased resistance will survive lethal treatments based on the resistance of the WT. In this way, the lethal treatments applied will inactivate the most sensitive cells, while sparing the mutant strains that are resistant to antimicrobial compounds (Figure 3).

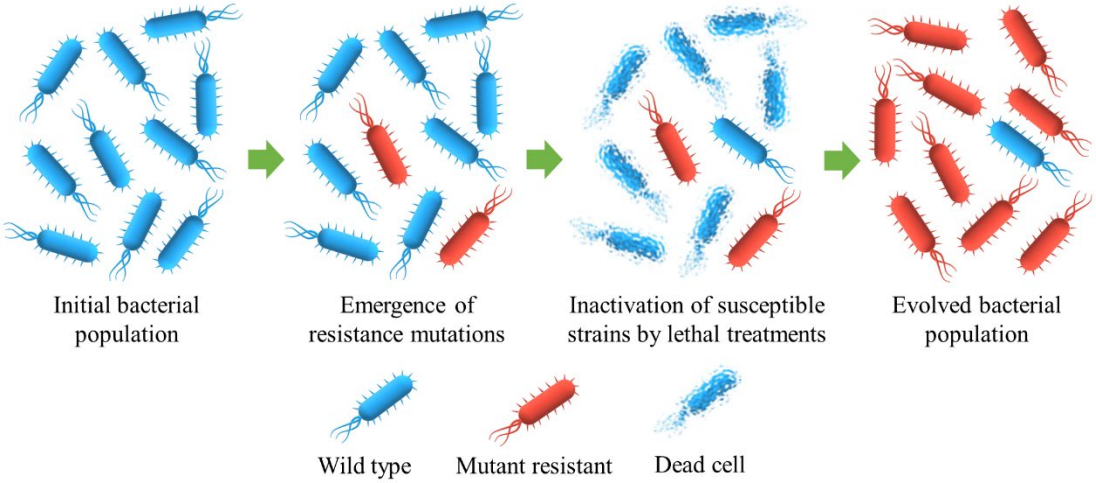


Figure 3. Selection of resistant mutant strains by cyclic exposure to short lethal treatments.

To carry out the evolution assay by cyclic exposure to short lethal treatments, the following steps should be taken (continue from step 4):

5. Dilute working bacterial culture of WT 1:1,000 into 50 mL growth broth and incubate for 24 h at 37 ºC and 130 rpm until a stationary phase culture is obtained (*see* **Note 4**).
6. Once incubated, dilute stationary phase culture 1:100 (initial concentration of 10^7 CFU/mL) in glass flasks containing 50 mL growth broth in presence of 2x the MIC of the antimicrobial compound.
7. Apply the treatment while maintaining the flask at 37 ºC for 4.5 h (*see* **Note 10**).
8. Centrifuge the treated cells for 20 min at 15,000 RCF and 4 ºC, wash twice with fresh growth broth and resuspend in 1 mL of growth broth in test tube.
12. Incubate test tubes overnight at 37 ºC and 130 rpm until stationary phase is reached (*see* **Note 4**).
13. Return to step 6. After 30 cycles, continue with step 14 (*see* **Note 8**).
14. After the 30^{th} cycle, dilute the bacterial culture in PBS, and inoculate and spread on agar plates to obtain individual colonies.
15. Incubate the agar plates for 24 h at 37 ºC (*see* **Note 2**).
16. After the incubation on agar plates, select several evolved colonies and store them in cryovials (*see* **Note 9**).

Finally, the isolated strains obtained either by cyclic exposure to prolonged sub-inhibitory doses or by short lethal treatments must be phenotypically and genotypically characterized. The WT must be used as control to evaluate the resistance of the evolved strains, and, likewise, in order to carry out the comparisons among the genomes with the purpose of finding the mutations that have occurred during evolution assays responsible for the increased resistance.

On the one hand, it is recommended to first evaluate the resistance of the evolved strains against the natural antimicrobial used in the evolution assay at both bacteriostatic and

bactericidal concentrations. For this purpose, MIC and minimum bactericidal concentration (MBC) can be determined, survival curves to lethal treatments can be obtained, and results can be compared with the WT. These methodologies are explained by Berdejo et al. ***(16)***. Increased resistance may also occur against other natural antimicrobials not used in the evolution assay ***(15)***; thus the same methodology can be used to test other antimicrobials. According to recent results in evolved strains, an antibiotic susceptibility test should be performed due to the fact that increased cross-resistance to antibiotics has been observed ***(17)***, and these compounds probably have similar mechanisms of action. In addition, resistant strains isolated by natural antimicrobials have also demonstrated increased resistance to other food preservation technologies such as heat or pulsed electric fields ***(15)***. Such results will provide more information on the behaviour of the evolved strains, as well as on the direct resistance and cross-resistance which will have emerged in the evolution assays between natural antimicrobials, antibiotics, and food preservation technologies.

On the other hand, whole genome sequencing (WGS) of WT and of the evolved resistant strains, followed by comparison between them, will allow a determination of the genetic modifications that cause the increase in resistance ***(16, 17)***. These results will thus provide knowledge regarding cellular response and resistance mechanisms (cellular targets, repair systems, etc.) against the antimicrobial compound, which might help to optimize their use as preservatives in the food industry, or as cleaning and disinfection treatments.

4. Notes

1. For evolution studies it is recommended to always use the same cryovial, or original strain, to avoid the occurrence and accumulation of random mutations in the WT, which make it more difficult to study genotypic resistance.

2. Incubation temperature and time should be modified according to the optimal growth conditions of the bacteria.
3. Check the size and shape of the colonies, and verify the homogeneity in the agar plate to avoid microbial contamination.
4. To obtain a stationary phase culture, incubation temperature and time should be modified according to the optimal growth conditions of the bacteria.
5. The concentration range should be adjusted based on the natural antimicrobial's bacteriostatic activity and on the resistance of the bacteria under study. Based on our experience, the range for ICs with high antimicrobial activity, such as carvacrol or thymol, or EOs, such as oregano or thyme EO, lies between 50 and 300 µL/L (with intervals of 50 µL/L). The range used for other less active compounds such as limonene oxide or citrus EOs, such as orange or lemon EO, is from 500 to 2,000 µL/L (with intervals of 100 µL/L).
6. To obtain an objective measurement, it is recommended to read the optical density at 595 nm (OD_{595}). 10% of the OD_{595} value of the positive control has been established as the lower limit to consider that a bacterial strain was grown ***(11)***.
7. To obtain an exponential phase culture, incubation temperature and time should be modified according to the optimal growth conditions of the bacteria.
8. Depending on the mutation frequency of the bacteria, the natural antimicrobial used, and treatment conditions, the evolution assay should be prolonged until increased resistance is observed. For this reason, it is recommended to perform an antimicrobial resistance test every 3 cycles to detect the emergence of resistant strains.

9. The population may be genotypically heterogeneous depending on the number of evolutionary cycles: for this reason it is recommended to evaluate several colonies in order to select the one that is most resistant.
10. Time and temperature of the lethal treatment can be modified according to the susceptibility of the WT to the antimicrobial used. This treatment's design seeks to inactivate a large part of the bacterial population (> 5 $\log_{10}$ cycles of reduction), while nevertheless allowing for the recovery of surviving cells before proceeding to the evolution assay.

References

1. de Souza EL, da Cruz Almeida ET, de Sousa Guedes JP (2016) The potential of the incorporation of essential oils and their individual constituents to improve microbial safety in juices: a review. Compr Rev Food Sci F 15(4):753-772. doi:10.1111/1541-4337.12208.
2. Bhavaniramya S, Vishnupriya S, Al-Aboody MS, Vijayakumar R, Baskaran D (2019) Role of essential oils in food safety: Antimicrobial and antioxidant applications. Grain Oil Sci Technol 2 (2):49-55. doi:10.1016/j.gaost.2019.03.001.
3. Calo JR, Crandall PG, O'Bryan CA, Ricke SC (2015) Essential oils as antimicrobials in food systems - a review. Food Control 54:111-119. doi:10.1016/j.foodcont.2014.12.040.
4. Pandey AK, Kumar P, Singh P, Tripathi NN, Bajpai VK (2017) Essential oils: sources of antimicrobials and food preservatives. Front Microbiol 7:2161. doi:10.3389/fmicb.2016.02161.
5. Mani-López E, Lorenzo-Leal AC, Palou, E, López-Malo, A (2017) Principles of sensory evaluation in foods containing essential oil. In: Hashemi SMB, Mousavi Khaneghah A, de Souza Sant'Ana A (eds) Essential oils in food processing. Wiley, New York. doi:10.1002/9781119149392.ch10.
6. Mahato N, Sharma K, Koteswararao R, Sinha M, Baral E, Cho MH (2019) Citrus essential oils: extraction, authentication and application in food preservation. Crit Rev Food Sci Nutr 59 (4):611-625. doi:10.1080/10408398.2017.1384716.
7. Berdejo D, Pagán E, García-Gonzalo D, Pagán R (2019) Exploiting the synergism among physical and chemical processes for improving food safety. Curr Opin Food Sci 30:14-20. doi:10.1016/j.cofs.2018.08.004.
8. de Carvalho RJ, de Souza GT, Pagán E, García-Gonzalo D, Magnani M, Pagán R (2018) Nanoemulsions of *Mentha piperita* L. essential oil in combination with mild heat, pulsed electric fields (PEF) and high hydrostatic pressure (HHP) as an alternative to inactivate *Escherichia coli* O157: H7 in fruit juices. Innov Food Sci Emerg Technol 48:219-227. doi:10.1016/j.ifset.2018.07.004.
9. Espina L, Condon S, Pagán R, García-Gonzalo D (2014) Synergistic effect of orange essential oil or (+)-limonene with heat treatments to inactivate *Escherichia coli* O157:H7 in orange juice at lower intensities while maintaining hedonic acceptability. Food Bioprocess Techn 7(2):471-481. doi:10.1007/s11947-013-1076-x.
10. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ (2007) A common mechanism of cellular death induced by bactericidal antibiotics. Cell 130(5):797-810. doi:10.1016/j.cell.2007.06.049.
11. Kohanski MA, DePristo MA, Collins JJ (2010) Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Mol Cell 37(3):311-320. doi:10.1016/j.molcel.2010.01.003.
12. Andersson DI, Hughes D (2014) Microbiological effects of sublethal levels of antibiotics. Nat Rev Microbiol 12(7):465-478. doi:10.1038/nrmicro3270.
13. Hashemi SMB, Khorram SB, Sohrabi M (2017) Antioxidant activity of essential oils in foods. In: Hashemi SMB, Mousavi Khaneghah A, de Souza Sant'Ana A (ed) Essential oils in food processing. Wiley, New York.. doi:10.1002/9781119149392.ch8.
14. Leite de Souza E (2016) The effects of sublethal doses of essential oils and their constituents on antimicrobial susceptibility and antibiotic resistance among food-related bacteria: a review. Trends Food Sci Technol 56:1-12. doi:10.1016/j.tifs.2016.07.012.
15. Chueca B, Berdejo D, Gomes-Neto NJ, Pagán R, García-Gonzalo D (2016) Emergence of hyper-resistant *Escherichia coli* MG1655 derivative strains after applying

sub-Inhibitory doses of individual constituents of essential oils. Front Microbiol 7:273-273. doi:10.3389/fmicb.2016.00273.
16. Berdejo D, Chueca B, Pagan E, Renzoni A, Kelley WL, Pagan R, Garcia-Gonzalo D (2019) Sub-inhibitory doses of individual constituents of essential oils can select for *Staphylococcus aureus* resistant mutants. Molecules 24(1):170. doi:10.3390/molecules24010170.
17. Berdejo D, Merino N, Pagán E, García-Gonzalo D, Pagán R (2020) Genetic variants and phenotypic characteristics of *Salmonella* Typhimurium-resistant mutants after exposure to carvacrol. Microorganisms 8(6):937. doi:10.3390/microorganisms8060937.
18. Berdejo D, Pagán E, Merino N, Pagán R, García-Gonzalo D (2020) Incubation with a complex orange essential oil leads to evolved mutants with increased resistance and tolerance. Pharmaceuticals 13(9):239. doi:10.3390/ph13090239.
19. Chueca B, Renzoni A, Berdejo D, Pagan R, Kelley WL, Garcia-Gonzalo D (2018) Whole-genome sequencing and genetic analysis reveal novel stress responses to individual constituents of essential oils in *Escherichia coli*. Appl Environ Microbiol 84(7). doi:10.1128/aem.02538-17.
20. Levin-Reisman I, Ronin I, Gefen O, Braniss I, Shoresh N, Balaban NQ (2017) Antibiotic tolerance facilitates the evolution of resistance. Science 355(6327):826-830. doi:10.1126/science.aaj2191.
21. Gayán E, Cambré A, Michiels CW, Aertsen A (2016) Stress-induced evolution of heat resistance and resuscitation speed in *Escherichia coli* O157:H7 ATCC 43888. Appl Environ Microbiol 82(22):6656-6663. doi:10.1128/AEM.02027-16.
22. Vanlint D, Mitchell R, Bailey E, Meersman F, McMillan PF, Michiels CW, Aertsen A (2011) Rapid acquisition of gigapascal-high-pressure resistance by *Escherichia coli*. MBio 2(1):e00130-00110. doi:10.1128/mBio.00130-10.

4. Resultados

Manuscrito III. Sub-inhibitory doses of individual constituents of essential oils can select for *Staphylococcus aureus* resistant mutants

Berdejo, D., Chueca, B., Pagán, E., Renzoni, A., Kelley, W., Pagán, R., García-Gonzalo, D. (2019)

Molecules, 24, 170. doi:10.3390/molecules24010170.

molecules

MDPI

Article

Sub-Inhibitory Doses of Individual Constituents of Essential Oils Can Select for *Staphylococcus aureus* Resistant Mutants

Daniel Berdejo [1], Beatriz Chueca [1], Elisa Pagán [1], Adriana Renzoni [2], William L. Kelley [3], Rafael Pagán [1] and Diego Garcia-Gonzalo [1,*]

1 Tecnología de los Alimentos, Instituto Agroalimentario de Aragón-IA2 (CITA-Universidad de Zaragoza), 930 50059 Zaragoza, Spain; berdejo@unizar.es (D.B.); bchuecaomella@gmail.com (B.C.); epagan@unizar.es (E.P.); pagan@unizar.es (R.P.)

2 Service of Infectious Diseases, Department of Medical Specialties, University Hospital of Geneva, 1205 Geneva, Switzerland; Adriana.Renzoni@unige.ch

3 Department of Microbiology and Molecular Medicine, University Hospital and Medical School of Geneva, 1205 Geneva, Switzerland; william.kelley@unige.ch

* Correspondence: diego.garcia@unizar.es; Tel.: +34-876-554246

Academic Editor: Francesca Mancianti
Received: 13 December 2018; Accepted: 27 December 2018; Published: 4 January 2019

Abstract: Increased bacterial resistance to food preservation technologies represents a risk for food safety and shelf-life. The use of natural antimicrobials, such as essential oils (EOs) and their individual constituents (ICs), has been proposed to avoid the generation of antimicrobial resistance. However, prolonged application of ICs might conceivably lead to the emergence of resistant strains. Hence, this study was aimed toward applying sub-inhibitory doses of the ICs carvacrol, citral, and (+)-limonene oxide to *Staphylococcus aureus* USA300, in order to evaluate the emergence of resistant strains and to identify the genetic modifications responsible for their increased resistance. Three stable-resistant strains, CAR (from cultures with carvacrol), CIT (from cultures with citral), and OXLIM (from cultures with (+)-limonene oxide) were isolated, showing an increased resistance against the ICs and a higher tolerance to lethal treatments by ICs or heat. Whole-genome sequencing revealed in CAR a large deletion in a region that contained genes encoding transcriptional regulators and metabolic enzymes. CIT showed a single missense mutation in *aroC* (N187K), which encodes for chorismate synthase; and in OXLIM a missense mutation was detected in *rpoB* (A862V), which encodes for RNA polymerase subunit beta. This study provides a first detailed insight into the mechanisms of action and *S. aureus* resistance arising from exposure to carvacrol, citral, and (+)-limonene oxide.

Keywords: whole genome sequencing; genotypic resistance; carvacrol; citral; (+)-limonene oxide

1. Introduction

Emergence of stable resistant strains during food processing can compromise food safety and shelf-life. Development of stable bacterial resistance is based on genetic modifications caused by mutations (i.e., genotypic resistance) [1] that produces variations in cellular responses to stress and damage [2]. Mutated microorganisms with an elevated resistance and/or tolerance might survive food preservation treatments that were previously considered sufficient as a control measure for spoiling. Thus, in recent years many efforts have been carried out in the search for new food preservatives capable of avoiding the generation of antimicrobial resistance.

Many authors have proposed the use of natural compounds, such as essential oils (EOs) and their individual constituents (ICs) as food preservatives. Carvacrol, citral, and (+)-limonene oxide are monoterpenes generally recognized as safe (GRAS), whose antimicrobial properties have been

demonstrated [3] and their mechanisms of microbial inactivation, mainly targeting cell envelopes, have been extensively studied [4–6]. However, the complete picture of the effects of these compounds over the bacterial cells is not fully understood.

Although it has been generally accepted that EOs and ICs do not have mutagenic properties [7,8], hyper-resistant strains of *Escherichia coli* have been isolated after bacterial exposure to carvacrol, citral, and (+)-limonene oxide during growth [9]. Rather than increasing the mutation rate, the presence of these compounds would create a selective pressure which leads to the emergence of mutant strains displaying resistance [9]. Due to the relevance of those results, it is convenient to determine the effect of exposure to sub-inhibitory concentrations of these natural compounds in the isolation of resistant mutants in pathogenic bacteria, such as the Gram-positive *Staphylococcus aureus* or *Listeria monocytogenes*. In addition, whole genome sequencing (WGS) of mutant strains would allow the identification of the precise genetic modifications in comparison to the wild type (WT) strain. This information might assist in the description of the mechanism of cell response to these antimicrobial compounds, providing valuable information for the design of more suitable food preservation methods. Accordingly, WGS of a resistant *E. coli* strain isolated from an evolution experiment in the presence of carvacrol revealed a relevant role of *soxR* in bacterial tolerance against the same IC used for selection, i.e., carvacrol (direct-tolerance), and other ICs, such as citral and (+)-limonene oxide (cross-tolerance) [10].

Thus, the aims of this study were (a) to isolate stable mutant resistant strains of *Staphylococcus aureus* USA300 by applying sub-inhibitory doses of carvacrol, citral, and (+)-limonene oxide during bacterial growth; (b) to characterize their resistance and survival against different food preservation technologies; and (c) to identify the genetic modification(s) associated with their increase of resistance and tolerance.

2. Results

2.1. Isolation of S. aureus USA300 Derivative Strains with Increased Resistance to ICs

In order to carry out the selection of stable-resistant strains by exposing *S. aureus* USA 300 to sub-inhibitory concentrations of ICs during bacterial growth, the minimum inhibitory concentration (MIC) of WT strain against carvacrol, citral, and (+)-limonene oxide was first determined (Table 1). Thus, 50 μL/L of carvacrol, 75 μL/L of citral, or 375 μL/L of (+)-limonene oxide ($1/2 \times$ MIC) were added to growth media for strain isolation. After a 10-day evolution experiment, six colonies from each culture were randomly selected, isolated, and re-cultured without any ICs. Hereinafter, the strains isolated after carvacrol exposure were referred to as CAR, those isolated after citral exposure as CIT, and after (+)-limonene oxide as OXLIM. The *S. aureus* USA300 WT strain was used as a reference to evaluate variations on the resistance of derivative strains by the disk diffusion assay and the MIC determination. The six colonies selected from each culture showed similar levels of resistance against the three ICs ($p > 0.05$; data not shown). Therefore, one colony from each culture was selected for further analyses. As shown in Table 1, derivative strains showed an increased resistance ($p \leq 0.05$) against the IC used in the evolution experiment (i.e., direct-resistance). The CAR strain showed an MIC against carvacrol of 150 μL/L, which represents an increase of 50% compared to WT. Similarly, CIT and OXLIM strains showed 65 and 100% increases in MIC against citral and (+)-limonene oxide, respectively. These increases in resistance were also observed using a disk diffusion assay (Table S1).

Additional analysis revealed that the CAR strain showed similar MIC levels against citral and (+)-limonene oxide compared with WT, whereas CIT and OXLIM showed cross-resistance against the other ICs tested (Table 1).

Table 1. Minimum inhibitory concentration (MIC; μL/L) of *Staphylococcus aureus* USA300 wild type (WT) and its derivative strains isolated: CAR, CIT, and OXLIM. Each value represents the MIC of the six colonies isolated by each IC. Increases in MIC with regard to WT are shaded.

	WT	CAR	CIT	OXLIM
Carvacrol	100	150	150	150
Citral	150	150	250	200
(+)-Limonene oxide	750	750	1000	1500

2.2. Effect of ICs on Mutation Frequency during Bacterial Growth

The role of ICs in the emergence of resistance was evaluated by the determination of mutation rates, specifically mutations leading to resistance against rifampicin [11]. We evaluated the mutation frequency during bacterial growth without (control) and with $^1/_2 \times$ MIC of each IC or with norfloxacin at 2000 mg/L. As shown in Figure 1, the WT strain showed a spontaneous frequency of rifampicin-resistant mutants over 100×10^{-9} during bacterial growth in absence of ICs. The incubation of WT strain in the presence of norfloxacin increased the emergence of rifampicin-resistant mutants 3-fold. However, no significant differences ($p > 0.05$) were observed in the mutation rate in the presence of citral or (+)-limonene oxide. Moreover, we observed that the mutation frequency was reduced in presence of carvacrol, with a mutation rate of approx. 60×10^{-9} ($p < 0.05$). Collectively, we conclude that the exposure of USA300 to ICs did not alter the mutation rate under our experimental conditions.

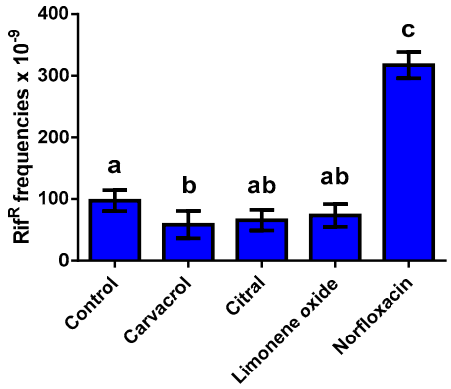


Figure 1. Mutagenesis frequency in *S. aureus* USA300 grown in broth without (control) and with carvacrol (50 μL/L), citral (75 μL/L), (+)-limonene oxide (375 μL/L), and norfloxacin (2000 mg/L). Mutagenesis frequency was expressed as rifampicin-resistant cells in the total microbial population. Data are means ± standard deviations (error bars) obtained from five independent experiments. Letters over the bars represent statistically significant differences; different lower-case letters above the bars represent statistically different values ($p \leq 0.05$), while results with the same letter show no significant difference ($p > 0.05$).

2.3. Evaluation of Derivative Strains against Lethal Treatments

After determination of the increased resistance of derivative strains, their tolerance against lethal treatments with ICs, heat, and pulsed electric fields (PEF) was determined. First, we evaluated the death of WT and derivative strains caused by the same IC used in their selection protocol (i.e., direct-tolerance, Figure 2). Survival at neutral and acid pH was evaluated. As shown in Figure 2A, CAR was more tolerant against a carvacrol lethal treatment than WT strain at pH 4.0: after 20 min, inactivation of WT cells was 3 $\log_{10}$ cycles higher than that of CAR cells. Differences in inactivation by carvacrol at pH 7.0 between WT and CAR were less relevant, showing a difference of 1 $\log_{10}$ cycles of inactivation only after 20 min of treatment.

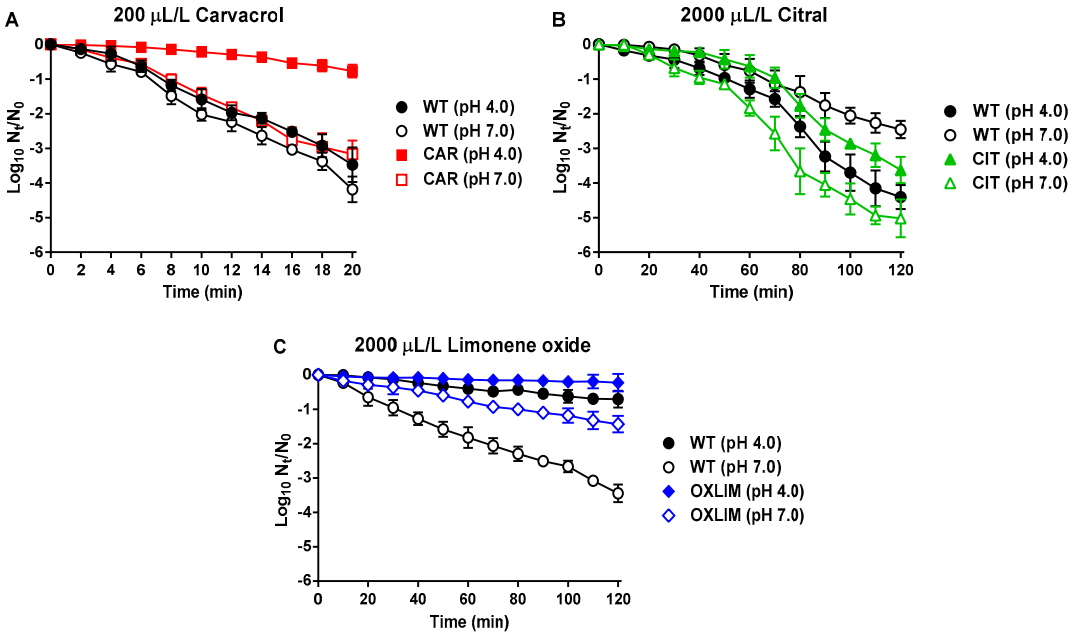


Figure 2. Inactivation of *Staphylococcus aureus* USA300 wild type (WT, ●,○) and its derivative strains; CAR (■,□) using 200 µL/L carvacrol (**A**), CIT (▲,△) using 2000 µL/L citral (**B**), and OXLIM (◆,◇) using 2000 µL/L (+)-limonene oxide (**C**) treatment, at pH 4.0 (black symbols) and pH 7.0 (white symbols). All treatments were performed at room temperature except (+)-limonene oxide, which were carried out at 37 °C. Data are means ± standard deviations (error bars) obtained from at least three independents experiments.

Although a slight increase in survival of CIT cells was detected after citral treatments at pH 4.0, a higher inactivation of CIT cells was determined at pH 7.0 in comparison to WT cells (Figure 2B): after 120 min with citral at pH 7.0, a microbial reduction of 2.5 $\log_{10}$ cycles of initial population of WT was achieved in contrast to 5 $\log_{10}$ cycles of CIT cells. While survival to carvacrol and citral treatments was evaluated at room temperature, higher temperature was needed to cause bacterial inactivation with (+)-limonene oxide. Therefore, the temperature was raised to 37 °C to carry out the lethal treatments of (+)-limonene oxide. A higher survival of OXLIM was demonstrated after (+)-limonene oxide treatment at pH 7.0, but not at pH 4.0 (Figure 2C), as compared to WT survival under the same conditions. An increase in tolerance with regard to WT against the IC used for selection was higher for CAR at pH 4.0 than for CIT at pH 7.0.

Cross-tolerance of derivative strains against physical food preservation technologies, such as heat and PEF treatments, was also evaluated. Figure 3 shows inactivation of WT and the derivative strains after a heat treatment at 60 °C in a buffer of pH 4.0 (Figure 3A) or 7.0 (Figure 3B). No significant differences were detected among the survival of WT, CIT, and OXLIM after heat treatments at pH 4.0 (Figure 3A). Only CAR showed a significantly higher survival than WT ($p \leq 0.05$) under these conditions: while inactivation of CAR after 8 min of heat treatment was approx. 3 $\log_{10}$ units, initial population of WT, CIT, and OXLIM was reduced in approx. 4 $\log_{10}$ units. With regard to heat treatments at pH 7.0, no differences were found between WT and CIT ($p > 0.05$), with a population reduction of 3.6 $\log_{10}$ units after 8 min (Figure 3B). However, in comparison to WT, an increased survival of CAR and OXLIM after the same treatment was detected ($p \leq 0.05$), with 2.5 and 2.9 $\log_{10}$ units of inactivation, respectively.

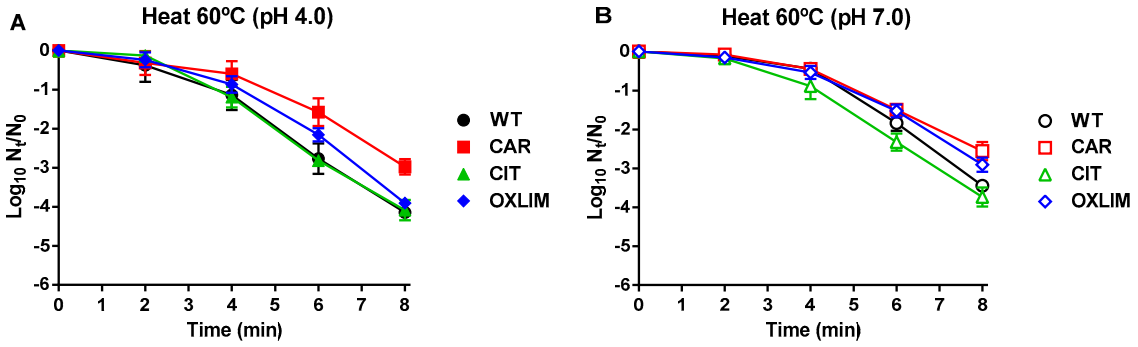


Figure 3. Inactivation of *Staphylococcus aureus* USA300 wild type (●,○) and its derivative strains: CAR (■,□), CIT (▲,△), and OXLIM (◆,◇) using heat treatments (60 °C) at pH 4.0 (A, black symbols) and pH 7.0 (B, white symbols). Data are means ± standard deviations (error bars) obtained from at least three independents experiments.

Figure 4 represents inactivation of WT and derivative strains caused by PEF treatments of 25 kV/cm, at pH 4.0 (Figure 4A) and 7.0 (Figure 4B). PEF treatments at pH 4.0 for 60 µs or longer caused approx. 3 $\log_{10}$ cycles of inactivation of WT, being more effective than at pH 7.0. Derivative strains showed a similar survival to PEF treatments ($p > 0.05$) in comparison to WT at both pH, indicating the absence cross-tolerance to PEF of the derivative strains.

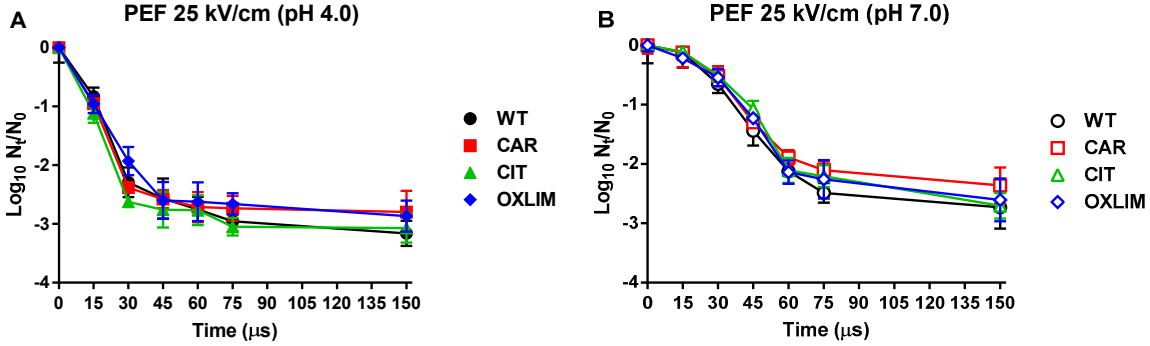


Figure 4. Inactivation of *Staphylococcus aureus* USA300 wild type (●,○) and its derivative strains: CAR (■,□), CIT (▲,△), and OXLIM (◆,◇) using pulsed electric fields treatments (PEF: 25 kV/cm, 1 Hz, 3 µs/pulse) at pH 4.0 (**A**, black symbols) and pH 7.0 (**B**, white symbols). Data are means ± standard deviations (error bars) obtained from at least three independents experiments.

Considering these results on survival to lethal treatments, CAR was shown to be the most tolerant strain. Therefore, this strain was selected to determine the occurrence of sublethal injuries in the cytoplasmic membrane after these treatments, in order to determine whether the increased survival was related to an increased intrinsic cell tolerance or an improved damage-repair system. CAR and WT were treated with carvacrol or heat at pH 4.0 (Figure 5), the pH at which the greater differences in tolerance between both strains were observed. Treated cells were plated in non-selective and selective media with NaCl added. The latter medium would avoid the growth of damaged cells in the cytoplasmic membrane and only permit the growth of intact cells [12]. Results from carvacrol (Figure 5A) and heat (Figure 5B) treatments revealed that although microbial counts were higher for CAR than for WT in the non-selective recovery medium, no significant differences ($p > 0.05$) were observed when samples were recovered in the selective medium.

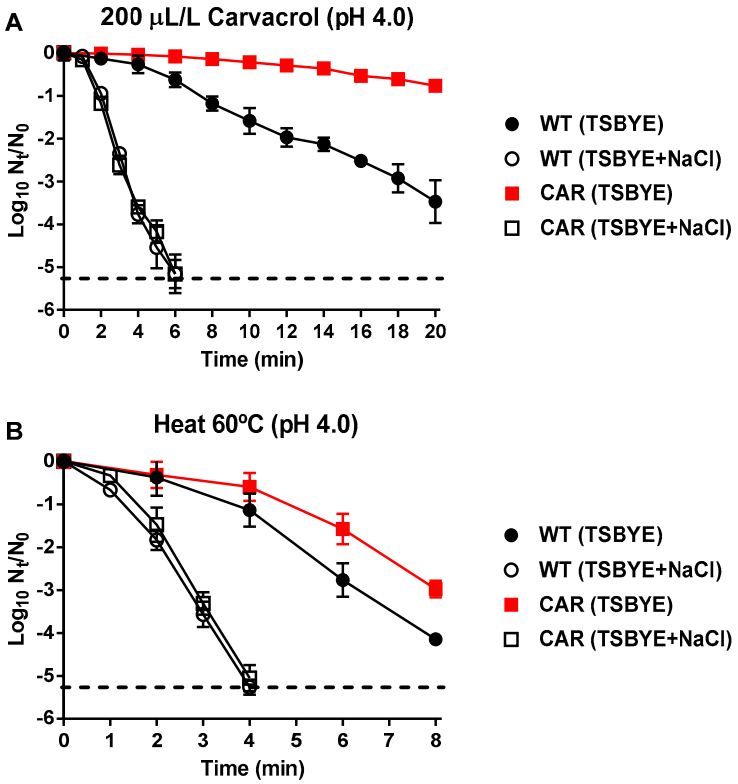


Figure 5. Inactivation of *Staphylococcus aureus* USA300 wild type (●,○) and CAR derivative strain (■,□) using carvacrol (200 µL/L; **A**) and heat treatments (60 °C; **B**) at pH 4.0. The treated samples were recovered in TSBYE without (black symbols) and with 14% of NaCl (semi-transparent symbols), used as selective agent to detect sublethal damage. Data are means ± standard deviations (error bars) obtained from at least three independents experiments. Discontinuous line shows the detection limit.

2.4. Genomic Sequencing of WT and Derivative Strains

In order to identify metabolic pathways and/or key structures involved in the increased resistance and survival of the derivative strains, WGS of these strains was conducted. Since WT, CAR, CIT, and OXLIM are derivatives of *S. aureus* USA300 (NCBI accession: NC_007793.1), this strain was used as a reference to evaluate genome coverage and facilitate contig assembly. The number of reads in whole-genomic sequencing were 17,311,886, 17,263,646, 13,788,036, and 16,852,412-bp paired-end reads for WT, CAR, CIT, and OXLIM, respectively, mapping the reference genome. Mapping covered 15,853,227, 16,399,422, 13,081,387, and 16,147,625 bases in WT, CAR, CIT, and OXLIM, resulting in 91.6%, 95.0%, 94.9%, and 95.8% of *S. aureus* USA300 genome coverage (2,872,769 bases). Since we sequenced the *S. aureus* USA300 strain that was the isogenic precursor of CAR, CIT, and OXLIM strains, differences were only extracted between our laboratory strain (WT) and ICs derivatives.

After assembling the genomes of the four strains, computer analysis revealed six mutations in CAR (3 single nucleotide polymorphisms (SNPs), 2 insertions or deletions (InDels), and 1 large deletion), 4 in CIT (3 SNPs and 1 InDels), and 7 in OXLIM (5 SNPs and 2 InDels) compared to WT strain. In order to confirm these genetic differences, specific primers (Table S2) were designed to examine the region of each mutation using PCR amplification using gDNA and Sanger sequencing of the amplified fragments. The confirmed mutations are depicted on circular schematic maps (Figure 6).

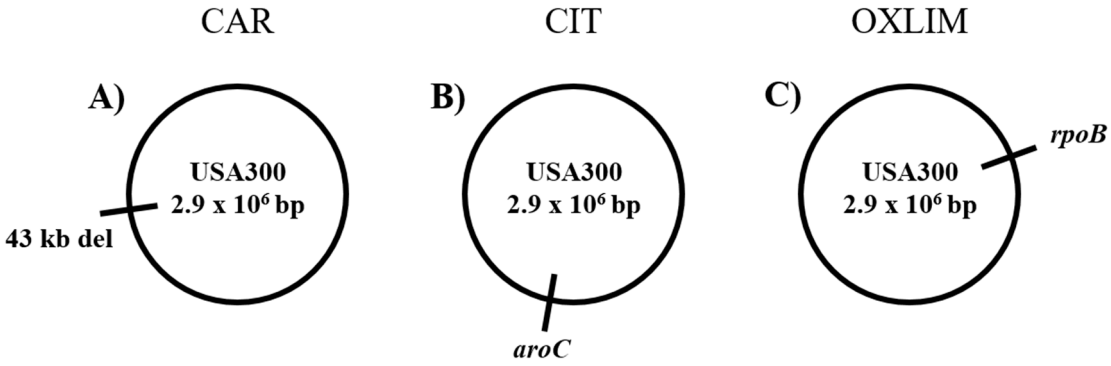


Figure 6. Genomic maps of the *Staphylococcus aureus* USA300 derivative strains from carvacrol (CAR; **A**), citral (CIT; **B**), and (+)-limonene oxide (OXLIM; **C**) chromosomes.

First, only the large deletion in CAR was confirmed (Table 2), while the other mutations were rejected after PCR confirmation. This large deletion was located from 2,084,675 to 2,127,700 bp (genome position) with a size of 43,025 bp. Consequently, 65 genes were deleted in CAR (Table S3).

Table 2. Mutations of *Staphylococcus aureus* USA300 derivative strains verified by Sanger sequencing.

Genome Position	Strain	Mutation	Gene	Locus tag	Description	Change
2,084,675–2,127,700	CAR	Large deletion	Δ (*hlb1–int3*)	SAUSA300_RS10505-SAUSA300_RS10835	Deletion of 65 genes	-
1,524,394	CIT	SNP (A→T)	*aroC*	SAUSA300_RS07395	Chorismate synthase	N187K
588,138	OXLIM	SNP (C→T)	*rpoB*	SAUSA300_RS02820	DNA-directed RNA polymerase subunit beta	A862V

With regard to CIT and OXLIM, only one SNP in each strain was verified (Table 2), and the remaining genetic differences detected using computer analysis of WGS were rejected. SNP verified in CIT was located in genome position 1,524,394 bp, changing adenine to thymine (transversion) in the *aroC* gene, resulting in a missense mutation AroC N187K. In OXLIM a SNP was detected in *rpoB* (genome position 588,138 bp), in which cytosine was replaced by a thymine (transition). Conceptual translation predicted a missense mutation RpoB A862V.

3. Discussion

The use of EOs and their ICs as food preservatives has been proposed due to their antimicrobial properties [3,7]. In addition, recent studies have investigated the use of these compounds as therapeutics for infectious diseases to avoid the emergence of resistant bacteria against antibiotics [13,14]. Although several studies have discarded the induction of stable bacterial resistance by the application of EOs and ICs [8,15–17], a recent study has demonstrated the emergence of hyper-resistant strains after exposure to carvacrol, citral, and (+)-limonene oxide in a Gram-negative bacteria, *E. coli* MG1655 [9]. Following the same protocol, in this study we have isolated mutant strains with increased resistance and tolerance of a Gram-positive bacteria, *S. aureus* USA300, after the exposure during bacterial growth in presence of ICs at sub-inhibitory concentrations (Table 1).

It is generally acknowledged that bacterial exposure to sub-lethal levels of antibiotics leads to emergence of antibiotic resistant strains; for example, fluoroquinolones and β-lactams increase the mutagenesis rate, thereby accelerating the generation of resistant strains [18]. In agreement with previous studies on ICs and EOs in *E. coli* MG1655 [9] and *S. aureus* ATCC 25923 [19], carvacrol, citral, and (+)-limonene oxide did not increase mutation frequency in *S. aureus* USA300 (Figure 1). As expected, nor increased the mutation frequency under the same growth conditions (Figure 1). Sublethal antibiotic treatments increase intracellular reactive oxygen species (ROS) production that damages DNA and activates SOS response and RecA activity [1,20]. This mechanism leads to bacterial multidrug

resistance via induced mutagenesis [18]. Although ROS are also involved in the mechanism of bacterial death by carvacrol, citral, and (+)-limonene oxide [4,5], a lower mutagenesis was induced by these antimicrobial compounds. Since these natural compounds also display strong antioxidant properties [21], they might alleviate oxidative stress, thereby protecting cells from DNA damage, which would decrease the mutagenesis rate.

After selecting one mutant strain from each evolution experiment (one with each IC), further sensitivity experiments against food preservation treatments were carried out. There are two bacterial strategies to survive under the presence of antimicrobials compounds as a function on the compound concentration and treatment duration: resistance and tolerance. While resistance is defined as the capability of bacteria to grow in the presence of an antimicrobial compound for a prolonged time, tolerance is a property of bacterial cells to survive to a high concentration of antimicrobial agent for a short time treatment [22]. Bacterial resistance is usually evaluated through bacteriostatic tests, such as MIC test, whereas tolerance determination requires an evaluation of survival against lethal treatments [23].

First, we demonstrated that the three strains isolated through the selective pressure of ICs had increased their MIC compared to WT. In addition, these strains did not only show a higher resistance against the ICs used in its selection, but also CIT and OXLIM displayed cross-resistance against the other ICs used in the selection protocol (Table 1). Next, we evaluated the survival of these resistant strains after lethal conditions (i.e., tolerance) with ICs and physical treatments (such as heat and PEF). As previously demonstrated in *S. aureus* [24,25], carvacrol and citral showed a strong bactericidal activity at room temperature against *S. aureus* USA300, causing a 4 $\log_{10}$ reduction of the initial population after 20 min at pH 4.0 with carvacrol and after 120 min at pH 7.0 with citral (Figure 2A,B). CAR and OXLIM showed an increased tolerance to lethal treatments of carvacrol and (+)-limonene oxide, respectively, compared to WT (Figure 2A,C). On the other hand, although CIT displayed an increased resistance in MIC, it was less tolerant than the WT strain to a lethal treatment using citral at pH 7 (Figure 2B).

Regarding cross-tolerance, inactivation caused using heat and PEF treatments is shown in Figure 3; Figure 4 respectively. On the one hand, CAR showed an increase of heat tolerance compared to WT at both treatment pHs, whereas OXLIM only displayed a rise in tolerance against heat treatments at pH 7.0. No significant differences ($p > 0.05$) were observed in microbial counts after heat treatments between WT and CIT (Figure 3). Even though heat treatments lead to DNA and RNA damage, ribosome destabilization, and enzyme and other proteins denaturation, among others, it is acknowledged that the principal structure affected by heat treatments is the cell envelope [12,26,27]. Hence, it is expected that mutations of CAR and OXLIM are involved in tolerance of the cell membrane or its reparation system. On the other hand, the cytoplasmic membrane is the main target of PEF treatments, causing pores in its surface (electroporation), and consequently, causing bacterial inactivation [28–30]. Although ICs and PEF treatments mainly target the cell envelopes, derivative strains (CAR, CIT, and OXLIM) showed a tolerance against PEF treatment similar to WT (Figure 4). It is likely that ICs and PEF target different structures and/or metabolic pathways in the cell envelopes, as previously shown by the absence of synergistic lethal effects in combined processes with ICs and PEF [31,32].

Our results indicate that mechanisms of resistance against ICs are different from those involved tolerance to ICs. According to Brauner, Fridman, Gefen, and Balaban [22], the phenomena of tolerance and resistance are mechanistically distinct and assumed to be unrelated. Recent studies have reported that antibiotic tolerance precedes and facilitates the evolution of resistance because tolerance enhances the chances for resistance mutations to disseminate in the bacterial population [33,34]. However, the resistant strains obtained in this study (CAR, CIT, and OXLIM) were not always tolerant to ICs. Further studies are necessary to evaluate the appearance of resistance and tolerance to ICs.

With the aim of discerning whether the increased survival of derivative strains was related to an increased intrinsic tolerance of the cell or to a better recovery from damages caused by heat or ICs, the occurrence of sublethally injured cells in the cytoplasmic membrane was evaluated. Evaluation of

sublethal damage was performed in the CAR strain after a carvacrol or a heat lethal treatment at pH 4.0, the conditions under the highest differences of tolerance were observed between WT and CAR. Similar survival curves were obtained after recovery of carvacrol- or heat-treated cells of CAR and WT in the selective media (Figure 5), indicating that the cytoplasmic membrane of both strains had the same intrinsic tolerance against heat and carvacrol [12]. Consequently, the higher microbial counts of treated CAR cells in a non-selective medium with regard to WT (Figure 5) would be due to an increased ability of CAR to repair the sublethal damages in its cytoplasmic membrane caused by heat or carvacrol.

Thus, in order to identify the molecular changes involved in the higher tolerance/resistance of the derivative strains, whole genome sequencing of WT, CAR, CIT, and OXLIM was carried out. First, the only genetic modification in CAR (Table 2) was a large deletion with a size of 43,025 bp, where 65 genes were involved. Most of these genes are involved in phage cycles, DNA replication or code for transcriptional regulators and enzymes involved in energy production and conversion [35]. The genes within the deletion must be non-essential or compensated for by redundant functions provided by genes elsewhere in the genome. Therefore, one or more of these deleted genes were the cause of the increase of carvacrol-resistance and carvacrol- and heat-tolerance of CAR. According to Chueca et al. [36], 61 genes in *E. coli* were upregulated during a carvacrol treatment, coding genes for DNA-binding transcriptional regulator and genes related with phage shock response. In addition, transcription of DNA-binding protein (H-NS and the 50S ribosomal proteins L7/L12) was downregulated by thymol, an IC similar to carvacrol, leading to bacterial DNA stability and inhibition of transcription as a protective mechanism [37]. Thus, deletion of several genes involved in different metabolic processes in CAR, such as coding genes of DNA-binding protein, replication protein DnaD, or Xenobiotic Response Element family transcriptional regulator, among others, could be involved in the increased resistance/tolerance of CAR strain against carvacrol or heat. Further research is needed in order to characterize the genes of this region and their role in the mechanisms of bacterial resistance and tolerance to ICs.

Second, in CIT only, an SNP was confirmed in genome position 1,524,394 bp, which involved the gene *aroC* (Table 2). This gene encodes for chorismate synthase that catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate to chorismate as a last step in the shikimate pathway, involved in synthesis of aromatic aminoacids [38]. For this reason, according to Foulongne et al. [39], loss of function of *aroC* would lead to a bacterial inability to grow in culture unless aromatic amino acids are provided. Moreover, chorismate is also a precursor of vitamin K and folate. The menaquinone, also known as vitamin K_2, plays an important role in the electron transport chain and is required for bacterial respiration, where ROS are produced. Thus, if chorismate is not synthesized, the amount of ROS would be reduced [40]. Since ROS are involved in mechanism bacterial death via carvacrol, citral, and limonene [4,5], decrease of ROS production would result in a lower sensitivity against these compounds. However, our CIT strain did not show small colony variants or gentamicin resistance (data not shown), as demonstrated by Wakeman et al. [41] in a mutant strain of *S. aureus* generated with a defect in electron transport.

Chorismate synthase has been extensively studied in order to develop broad-spectrum antimicrobial compounds because the design of appropriate inhibitors for this enzyme could be a plausible way to block multiple pathways essential for the survival of microorganism [42]. In addition, mutants in genes involved in the shikimate pathway, such as *aroC*, have shown an increased susceptibility to the action of some antimicrobial agents, such as ovotransferrin or EDTA, due to defects in cell wall and outer membrane integrity [43]. In CIT, modification of cell envelopes because of the observed SNP in *aroC* might also increase resistance against citral. Even though some studies have related mutations of *aroC* to alterations in cell envelopes, and their implication in the bacterial resistance, no previous research has reported increased resistance against antimicrobial compounds.

Lastly, in the OXLIM strain, one SNP was detected in *rpoB* gene at 588,138 bp genome position (Table 2). This gene encodes for the RNA polymerase β subunit and it is one of the minimal essential genes that has been extensively used for the accurate identification of *Staphylococcus* isolates and for the development of phylogenetic analysis of *S. aureus* [44]. Since rifampicin targets RpoB, mutations located in *rpoB* are closely associated with rifampicin resistance in *S. aureus* [45,46]. However, OXLIM did not show an increased resistance to rifampicin. *rpoB* mutations have also been identified as one of the major contributors to emergence of increased resistance to vancomycin in *S. aureus,* and especially the conversion to vancomycin-intermediate *S. aureus* (VISA) from heterogeneous VISA [47].

Such valuable information increases our knowledge of the mechanisms of bacterial inactivation via carvacrol, citral, and (+)-limonene oxide derived from EOs. Even though this research has elucidated the role of some genes in the resistance and tolerance of *S. aureus* against carvacrol, citral, and (+)-limonene oxide, once the whole genome of resistant strains are available, deeper genetic studies are required to acquire further knowledge about the inactivation mechanisms of these natural antimicrobials' compounds.

4. Materials and Methods

4.1. Microorganisms and Growth Conditions

The FPR3757 strain of *S. aureus* USA300 methicillin resistant was provided by Prof. Kolter laboratory (Harvard Medical School, Boston, MA, USA). This strain was isolated from an outbreak in USA [48]. Although, to the best of our knowledge, no food poisoning due to this specific strain has been reported, methicillin-resistant *Staphylococcus aureus* (MRSA) has been commonly isolated from retail meat, with a potential for widespread dissemination in the population [49].

Throughout this investigation, the cultures were kept at –80 °C in cryovials with glycerol. To prepare the broth subcultures one single colony from a plate was inoculated in a test tube with 5 mL of sterile tryptone soya broth (Oxoid, Basingstoke, Hampshire, England) with 0.6% yeast extract added (Oxoid, Basingstoke, Hampshire, England; TSBYE). The inoculated tubes were incubated overnight in aerobic conditions at 37 °C (Selecta Incudigit, Barcelona, Spain) to obtain bacterial subcultures. Two hundred and fifty (250) milliliter Erlenmeyer flasks containing 50 mL of TSBYE were inoculated with these subcultures to a final concentration of 10^5 colony forming units (CFU)/mL. To reach the stationary growth phase (2×10^9 CFU/mL approx.) bacterial cultures were incubated for 24 h under agitation (130 rpm) at 37 °C (Selecta Rotabit, Barcelona, Spain).

4.2. Determination of Minimum Inhibitory Concentration (MIC)

MIC against *S. aureus* USA300 and its derivative strains was determined for citral (95%; Sigma-Aldrich, Steinheim, Westphalia, Germany), carvacrol (95%; Sigma-Aldrich, Steinheim, Westphalia, Germany), and (+)-limonene oxide (97%; Sigma-Aldrich, Steinheim, Westphalia, Germany) with an initial concentration of 10^5 CFU/mL using the tube dilution method [50,51]. Tested concentrations were: 50, 100, 150, 200, and 250 µL/L of carvacrol or citral; and 500, 750, 1000, 1250, and 1500 µL/L of (+)-limonene oxide. In these experiments, positive controls containing TSBYE inoculated at 10^5 CFU/mL without ICs, and negative controls containing TSBYE inoculated at 10^5 CFU/mL with 1500 µL/L of each IC were prepared. Tubes were incubated at 37 °C for 24 h and 130 rpm. MIC was determined as the lowest concentration of IC in the presence of which bacteria showed no visible growth [50].

4.3. Isolation of Derivative Strains by Applying Sub-Inhibitory Doses of ICs During Bacterial Growth

The selection of derivative strains of *S. aureus* USA300 was carried out following the procedure described by Kohanski, DePristo, and Collins [1] for bactericidal antibiotics. A *S. aureus* culture grown for 12 h at 37 °C was diluted 1:10,000 in a 250 mL-flask with 50 mL TSBYE and grown at 37 °C and 130 rpm for 3.5 h. After incubation, this culture was diluted 1:3 into TSBYE containing sub-inhibitory

concentrations (1/2 × MIC) of carvacrol, citral or (+)-limonene oxide. Five (5) milliliters of these diluted cultures were grown in tubes at 37 °C and 130 rpm for 24 h. Each day, cells were diluted 1:1000 in a tube containing 1/2 × MIC concentration of the IC and 5 mL TSBYE and grown at 37 °C and 130 rpm for 24 h. After 10 days, 0.1 mL-samples of the culture were serially diluted in phosphate buffer saline (PBS) and pour-plated in tryptone soya agar (Oxoid, Basingstoke, Hampshire, England) with 0.6% yeast extract added (Oxoid, Basingstoke, Hampshire, England.; TSAYE) to select six colonies. Then, these colonies were grown in TSBYE, and MICs were determined in order to evaluate the direct-resistance against the IC used in the selection process and cross-resistance against other ICs, and to verify the occurrence of stable genotypic modifications.

Complete genomic sequences of the *Staphylococcus aureus* CAR, CIT, and OXLIM strains have been deposited in the NCBI database under the accession numbers CP029030.1, CP029031.1, and CP029032.1, respectively.

4.4. Mutagenesis Frequency Evaluation

The mutagenic effect of each IC was determined by calculating the rate of mutants resistant to rifampicin because of point mutations in the *rpoB* gene [11]. Overnight culture of *S. aureus* USA300 was diluted 1:10,000 into 50 mL TSBYE and incubated at 37 °C and 130 rpm for 3.5 h. Afterwards, this culture was diluted 1:3 in tubes containing 1/2 × MIC of carvacrol, citral, and (+)-limonene oxide and 20 mL fresh TSBYE. This assay was also carried out with norfloxacin (Sigma-Aldrich, Steinheim, Westphalia, Germany) at 2000 mg/L concentration as a positive control. These suspensions were grown at 37 °C and 130 rpm for 24 h (2×10^9 CFU/mL approx. for all suspensions). Samples of the culture were serially diluted in PBS and pour-plated on TSAYE in the presence and absence of 100 mg/L rifampicin (Sigma-Aldrich, Steinheim, Westphalia, Germany). Plates were incubated at 37 °C for 48 h and colonies were counted. Mutation rates were calculated by dividing the number of colonies present in rifampicin plates (mutation events) by the number of colonies present in plates without antibiotic [52].

4.5. Evaluation of Increased Bacterial Tolerance

Prior to the treatments, cultures of WT and the derivative strains were centrifuged for 5 min at 6000× *g* and resuspended in McIlvaine citrate-phosphate buffer of pH 7.0 or 4.0 at a final concentration of 2×10^7 CFU/mL. These pH values were chosen as representative of neutral and acid conditions.

4.5.1. Lethal ICs Treatments

Lethal ICs treatments were carried out in cultures resuspended in 10 mL buffer at both pHs added with 200 µL/L of carvacrol, 2000 µL/L of citral, or 2000 µL/L of (+)-limonene oxide. Treatments were applied at room temperature, except (+)-limonene oxide treatments that were performed at 37 °C. Samples were taken every 2 min up to 20 min at carvacrol treatments and every 10 min up to 120 min at citral and (+)-limonene oxide treatments.

4.5.2. Lethal Heat treatments

Heat treatments were carried out in an incubator (FX Incubator, mod. ZE/FX, Zeulab, Zaragoza, Spain) at 60 °C, and temperature was monitored with a thermocouple (Ahlborn, mod. Almemo 2450, Holzkirchen, Germany). Samples were taken every 2 min up to 8 min.

4.5.3. Lethal PEF Treatments

The PEF equipment (EPULSUS®-PM1-10, Energy Pulse System, Lisbon, Portugal) used in this investigation is a Marx generator that can apply monopolar square waveform pulses with a frequency up to 200 Hz, as described by Saldaña et al. [53]. The actual voltage, current, and pulse duration were

measured using a high voltage probe (Tektronix, P6015A, Wilsonville, OR, USA) and a current probe (Stangenes Industries Inc., Palo Alto, CA, USA), respectively, connected to an oscilloscope (Tektronix, TDS 220, Wilsonville, OR, USA). As a batch treatment chamber, a cylindrical plastic tube closed with two polished stainless-steel electrodes was used. The distance between electrodes was 0.25 cm, and the electrode area was 2.01 cm^2. The temperature of the treatment medium was measured and always kept under 30 °C in all experiments performed.

With a sterile syringe, 0.5 mL of the microbial suspension in McIlvaine buffer at pH 7.0 or 4.0 was placed in the batch treatment chamber. Samples were treated at 25 kV/cm with monopolar square pulses of 3 µs (3.75 kJ/kg per pulse) at a repetition rate of 1 Hz, for 15, 30, 45, 60, 75, and 150 µs.

4.6. Counting of Viable and Sublethally Injured Cells

Treated samples were appropriately diluted in PBS, and plated onto TSAYE (non-selective medium). Plates were incubated for 24 h at 37 °C. After incubation, the colonies were counted with an improved image analysis automatic colony counter (Protos, Analytical Measuring Systems, Cambridge, United Kingdom). In order to detect sublethal damage at the cytoplasmic membrane [12], some of the treated samples were also plated in the selective medium TSAYE added with 14% NaCl (Panreac, Barcelona, Spain). This concentration of NaCl was the maximum non-inhibitory concentrations for untreated cells as previously determined. Plates with selective media were incubated for 48 h at 37 °C. Bacterial inactivation was calculated by the difference in $\log_{10}$ counts before and after the lethal treatments. The proportion of sublethally injured cells was expressed as the difference in $\log_{10}$ counts determined in the non-selective and in the selective media.

4.7. Genome Sequencing and SNP Analysis

From an overnight culture of WT and derivative strains of *S. aureus* USA300, genomic DNA (gDNA) was extracted using a gDNA kit for extraction and purification (GeneJET Genomic DNA, Thermo Scientific, Waltham, MA, USA). Solexa technology was used to sequence gDNA of the four strains on an Illumina genome analyzer Hi-Seq 2500 instrument (Illumina; Fasteris, SA, Geneva, Switzerland). The quality control filtered paired-end reads (17.3 million 100-bp) were mapped on the *S. aureus* USA300 genome sequence (NCBI accession NC_007793.1) using a Burrows–Wheeler Alignment (BWA) tool [54] giving a raw coverage depth of approximately 650-fold. Mapping covered 91.6%, 95.0%, 94.9%, and 95.8% of *S. aureus* USA300 for our WT, CAR, CIT, and OXLIM strains, respectively. The generated consensus of the four strains sequences were then compared to detect single nucleotide polymorphisms (SNPs) and insertions or deletions (InDels) difference using BWA [54] together with the CLC workbench and SamTools [55] (Software can be downloaded from http://maq.sourceforge.net and http://samtools.sourceforge.net.). All detected SNPs and InDels were tested by PCR and Sanger sequencing.

4.8. Statistical Analysis

Results for MIC determination, mutation frequency, disk diffusion assay, and lethal treatments were obtained from at least three independent experiments carried out on different working days with different microbial cultures. These results were represented as the mean ± standard deviation, using GraphPad PRISM® program (GraphPad Software, Inc., San Diego, CA, USA). Data were analyzed and submitted to comparison of averages using analysis of variance (ANOVA) followed by *post-hoc* Tukey test and *t*-tests with GraphPad PRISM®. Differences were considered significant if $p \leq 0.05$.

5. Conclusions

Although the presence of ICs during *S. aureus* growth decreases mutation frequency, stable resistant strains might emerge during extended food preservation treatments with ICs or EOs, representing a risk for food safety and shelf-life. Since the presence of strains with increased resistance and tolerance might represent a challenge for the design of food preservation processes, further

research is required in order to determine whether the emergence of resistant strains in the presence of ICs and EOs is a general phenomenon. In addition, the consequences of the appearance of these mutants and strategies to avoid their emergence warrants further study. WGS of mutant strains allow the identification of stable genetic changes likely involved in their increased resistance and tolerance against ICs and heat treatments. These results lay the groundwork for future detailed genetic studies and demonstrate the power of deep sequencing methods for dissection of cellular responses to essential oils and their constituent components and for the design of future strategies for food preservation.

Supplementary Materials: The following are available online at http://www.mdpi.com/1420-3049/24/1/170/s1, **Table S1.** Zones of growth inhibition (mm) showing antibacterial activity for carvacrol (1 µL), citral (2.5 µL), and (+)-limonene oxide (30 µL) against *Staphylococcus aureus* USA300 wild type and its derivative strains isolated: CAR, CIT, and OXLIM. Each value represents the mean diameter of the inhibition halo ± standard deviation of the six colonies isolated by each IC. Increases in MIC with regard to WT are shaded. **Table S2.** Primers used for verification. **Table S3.** Large deletion of *Staphylococcus aureus* CAR derivative strain.

Author Contributions: conceptualization, D.B., B.C., W.L.K., R.P., and D.G.-G.; methodology, D.B., B.C., W.L.K., R.P., and D.G.-G; validation, D.B., B.C., and E.P.; formal analysis, D.B. and B.C.; investigation, D.B. and B.C.; writing—original draft preparation, D.B., W.L.K., R.P., and D.G.-G.; writing—review and editing, D.B., A.R., W.L.K., and D.G.-G.; visualization, D.B. and D.G.-G.; supervision, W.L.K., R.P., and D.G.-G.; project administration, R.P. and D.G.-G.; funding acquisition, R.P. and D.G.-G.

Funding: This research was funded by Ministerio de Economía y Competitividad, Gobierno de España (Grant AGL2015–69565-P), co-financed by FEDER funds (MINECO/FEDER/UE), by European Social Fund, and by Departamento de Innovación, Investigación y Universidad, Gobierno de Aragón. Ministerio de Educación, Cultura y Deporte also provided D. Berdejo, B. Chueca and E. Pagán with scholarships (FPU15/02703, FPU12/00170 and FPU17/02441, respectively) to carry out this investigation. Funding to pay the Open Access publication charges for this article was provided by Grant AGL2015–69565-P.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Kohanski, M.A.; DePristo, M.A.; Collins, J.J. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Mol. Cell* **2010**, *37*, 311–320. [CrossRef] [PubMed]
2. Foster, P.L. Stress-induced mutagenesis in bacteria. *Crit. Rev. Biochem. Mol. Biol.* **2007**, *42*, 373–397. [CrossRef] [PubMed]
3. de Souza, E.L.; da Cruz Almeida, E.T.; de Sousa Guedes, J.P. The potential of the incorporation of essential oils and their individual constituents to improve microbial safety in juices: A review. *Compr. Rev. Food Sci.* **2016**, *15*, 753–772. [CrossRef]
4. Chueca, B.; Pagán, R.; García-Gonzalo, D. Oxygenated monoterpenes citral and carvacrol cause oxidative damage in Escherichia coli without the involvement of tricarboxylic acid cycle and Fenton reaction. *Int. J. Food Microbiol.* **2014**, *189*, 126–131. [CrossRef]
5. Chueca, B.; Pagán, R.; García-Gonzalo, D. Differential mechanism of Escherichia coli inactivation by (+)-limonene as a function of cell physiological state and drug's concentration. *PLoS ONE* **2014**, *9*, e94072. [CrossRef]
6. Ait-Ouazzou, A.; Espina, L.; Gelaw, T.K.; de Lamo-Castellvi, S.; Pagan, R.; Garcia-Gonzalo, D. New insights in mechanisms of bacterial inactivation by carvacrol. *J. Appl. Microbiol.* **2013**, *114*, 173–185. [CrossRef] [PubMed]
7. Burt, S. Essential oils: Their antibacterial properties and potential applications in foods—a review. *Int. J. Food Microbiol.* **2004**, *94*, 223–253. [CrossRef] [PubMed]
8. Leite de Souza, E. The effects of sublethal doses of essential oils and their constituents on antimicrobial susceptibility and antibiotic resistance among food-related bacteria: A review. *Trends Food Sci. Technol.* **2016**, *56*, 1–12. [CrossRef]
9. Chueca, B.; Berdejo, D.; Gomes-Neto, N.J.; Pagan, R.; Garcia-Gonzalo, D. Emergence of hyper-resistant *Escherichia coli* MG1655 derivative strains after applying sub-inhibitory doses of individual constituents of essential oils. *Front. Microbiol.* **2016**, *7*, 273. [CrossRef] [PubMed]

10. Chueca, B.; Renzoni, A.; Berdejo, D.; Pagan, R.; Kelley, W.L.; Garcia-Gonzalo, D. Whole-genome sequencing and genetic analysis reveal novel stress responses to individual constituents of essential oils in *Escherichia coli*. *Appl. Environ. Microbiol.* **2018**, *84*, e02538. [CrossRef] [PubMed]
11. Gutierrez, A.; Laureti, L.; Crussard, S.; Abida, H.; Rodriguez-Rojas, A.; Blazquez, J.; Baharoglu, Z.; Mazel, D.; Darfeuille, F.; Vogel, J.; et al. β-lactam antibiotics promote bacterial mutagenesis via an RpoS-mediated reduction in replication fidelity. *Nat. Commun.* **2013**, *4*, 1610. [CrossRef] [PubMed]
12. Mackey, B.M. Injured bacteria. In *The Microbiological Safety and Quality of Food*; Lund, M., Baird-Par-ker, T.C., Gould, G.W., Eds.; Aspen Publisher: Gaithersburg, MD, USA, 2000; Volume I, pp. 315–341.
13. Yap, P.S.X.; Yiap, B.C.; Ping, H.C.; Lim, S.H.E. Essential oils, a new horizon in combating bacterial antibiotic resistance. *Open. Microbiol. J.* **2014**, *8*, 6–14. [CrossRef] [PubMed]
14. Chávez-González, M.L.; Rodríguez-Herrera, R.; Aguilar, C.N. Essential oils: A natural alternative to combat antibiotics resistance. In *Antibiotic Resistance*; Rai, M., Ed.; Elsevier Academic Press: London Wall, London, UK, 2016; pp. 227–237.
15. Silva da Luz, I.; Gomes Neto, N.J.; Tavares, A.G.; Nunes, P.C.; Magnani, M.; de Souza, E.L. Lack of induction of direct protection or cross-protection in *Staphylococcus aureus* by sublethal concentrations of *Origanum vulgare* L. essential oil and carvacrol in a meat-based medium. *Arch. Microbiol.* **2013**, *195*, 587–593. [CrossRef] [PubMed]
16. Apolonio, J.; Faleiro, M.L.; Miguel, M.G.; Neto, L. No induction of antimicrobial resistance in *Staphylococcus aureus* and *Listeria monocytogenes* during continuous exposure to eugenol and citral. *FEMS Microbiol. Lett.* **2014**, *354*, 92–101. [CrossRef] [PubMed]
17. Tavares, A.G.; Monte, D.F.; Albuquerque Ados, R.; Sampaio, F.C.; Magnani, M.; Siqueira Junior, J.P.; Souza, E.L. Habituation of enterotoxigenic *Staphylococcus aureus* to *Origanum vulgare* L. essential oil does not induce direct-tolerance and cross-tolerance to salts and organic acids. *Braz. J. Microbiol.* **2015**, *46*, 835–840. [CrossRef]
18. Andersson, D.I.; Hughes, D. Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol.* **2014**, *12*, 465–478. [CrossRef] [PubMed]
19. Hammer, K.A.; Carson, C.F.; Riley, T.V. Frequencies of resistance to *Melaleuca alternifolia* (tea tree) oil and rifampicin in *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis*. *Int. J. Antimicrob. Agents* **2008**, *32*, 170–173. [CrossRef]
20. Kohanski, M.A.; Dwyer, D.J.; Hayete, B.; Lawrence, C.A.; Collins, J.J. A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **2007**, *130*, 797–810. [CrossRef]
21. Brewer, M.S. Natural antioxidants: Sources, compounds, mechanisms of action, and potential applications. *Compr. Rev. Food Sci. Food Saf.* **2011**, *10*, 221–247. [CrossRef]
22. Brauner, A.; Fridman, O.; Gefen, O.; Balaban, N.Q. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat. Rev. Microbiol.* **2016**, *14*, 320–330. [CrossRef]
23. Fridman, O.; Goldberg, A.; Ronin, I.; Shoresh, N.; Balaban, N.Q. Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature* **2014**, *513*, 418–421. [CrossRef] [PubMed]
24. Aiemsaard, J.; Aiumlamai, S.; Aromdee, C.; Taweechaisupapong, S.; Khunkitti, W. The effect of lemongrass oil and its major components on clinical isolate mastitis pathogens and their mechanisms of action on *Staphylococcus aureus* DMST 4745. *Res. Vet. Sci.* **2011**, *91*, e31–e37. [CrossRef] [PubMed]
25. Espina, L.; Berdejo, D.; Alfonso, P.; García-Gonzalo, D.; Pagán, R. Potential use of carvacrol and citral to inactivate biofilm cells and eliminate biofouling. *Food Control* **2017**, *82*, 256–265. [CrossRef]
26. Nguyen, H.T.; Corry, J.E.; Miles, C.A. Heat resistance and mechanism of heat inactivation in thermophilic campylobacters. *Appl. Environ. Microbiol.* **2006**, *72*, 908–913. [CrossRef]
27. Mackey, B.M.; Miles, C.A.; Parsons, S.E.; Seymour, D.A. Thermal denaturation of whole cells and cell components of *Escherichia coli* examined by differential scanning calorimetry. *J. Gen. Microbiol.* **1991**, *137*, 2361–2374. [CrossRef] [PubMed]
28. Mañas, P.; Pagán, R. Microbial inactivation by new technologies of food preservation. *J. Appl. Microbiol.* **2005**, *98*, 1387–1399. [CrossRef] [PubMed]
29. Chueca, B.; Pagán, R.; García-Gonzalo, D. Transcriptomic analysis of *Escherichia coli* MG1655 cells exposed to pulsed electric fields. *Innov. Food Sci. Emerg. Technol.* **2015**, *29*, 78–86. [CrossRef]

30. Garcia, D.; Manas, P.; Gomez, N.; Raso, J.; Pagan, R. Biosynthetic requirements for the repair of sublethal membrane damage in *Escherichia coli* cells after pulsed electric fields. *J. Appl. Microbiol.* **2006**, *100*, 428–435. [CrossRef]
31. Espina, L.; Gelaw, T.K.; de Lamo-Castellvi, S.; Pagan, R.; Garcia-Gonzalo, D. Mechanism of bacterial inactivation by (+)-limonene and its potential use in food preservation combined processes. *PLoS ONE* **2013**, *8*, e56769. [CrossRef]
32. Somolinos, M.; Garcia, D.; Condon, S.; Mackey, B.; Pagan, R. Inactivation of *Escherichia coli* by citral. *J. Appl. Microbiol.* **2010**, *108*, 1928–1939. [CrossRef]
33. Lewis, K.; Shan, Y. Why tolerance invites resistance. *Science* **2017**, *355*, 796. [CrossRef] [PubMed]
34. Levin-Reisman, I.; Ronin, I.; Gefen, O.; Braniss, I.; Shoresh, N.; Balaban, N.Q. Antibiotic tolerance facilitates the evolution of resistance. *Science* **2017**, *355*, 826–830. [CrossRef] [PubMed]
35. Fuchs, S.; Mehlan, H.; Bernhardt, J.; Hennig, A.; Michalik, S.; Surmann, K.; Pané-Farré, J.; Giese, A.; Weiss, S.; Backert, L.; et al. AureoWiki—The repository of the *Staphylococcus aureus* research and annotation community. *Int. J. Med. Microbiol.* **2017**, *308*, 558–568. [CrossRef] [PubMed]
36. Chueca, B.; Perez-Saez, E.; Pagan, R.; Garcia-Gonzalo, D. Global transcriptional response of *Escherichia coli* MG1655 cells exposed to the oxygenated monoterpenes citral and carvacrol. *Int. J. Food Microbiol.* **2017**, *257*, 49–57. [CrossRef] [PubMed]
37. Nazzaro, F.; Fratianni, F.; De Martino, L.; Coppola, R.; De Feo, V. Effect of essential oils on pathogenic bacteria. *Pharmaceuticals* **2013**, *6*, 1451–1474. [CrossRef] [PubMed]
38. Horsburgh, M.J.; Foster, T.J.; Barth, P.T.; Coggins, J.R. Chorismate synthase from *Staphylococcus aureus*. *Microbiology* **1996**, *142*, 2943–2950. [CrossRef] [PubMed]
39. Foulongne, V.; Walravens, K.; Bourg, G.; Boschiroli, M.L.; Godfroid, J.; Ramuz, M.; O'Callaghan, D. Aromatic compound-dependent *Brucella suis* is attenuated in both cultured cells and mouse models. *Infect. Immun.* **2001**, *69*, 547–550. [CrossRef]
40. Proctor, R.A.; von Eiff, C.; Kahl, B.C.; Becker, K.; McNamara, P.; Herrmann, M.; Peters, G. Small colony variants: A pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat. Rev. Microbiol.* **2006**, *4*, 295–305. [CrossRef]
41. Wakeman, C.A.; Hammer, N.D.; Stauff, D.L.; Attia, A.S.; Anzaldi, L.L.; Dikalov, S.I.; Calcutt, M.W.; Skaar, E.P. Menaquinone biosynthesis potentiates haem toxicity in *Staphylococcus aureus*. *Mol. Microbiol.* **2012**, *86*, 1376–1392. [CrossRef]
42. Pitchandi, P.; Hopper, W.; Rao, R. Comprehensive database of chorismate synthase enzyme from shikimate pathway in pathogenic bacteria. *BMC Pharmacol. Toxicol.* **2013**, *14*, 29. [CrossRef]
43. Sebkova, A.; Karasova, D.; Crhanova, M.; Budinska, E.; Rychlik, I. *aro* mutations in *Salmonella enterica* cause defects in cell wall and outer membrane integrity. *J. Bacteriol.* **2008**, *190*, 3155–3160. [CrossRef]
44. Seong, W.J.; Kim, J.H.; Kwon, H.J. Comparison of complete *rpoB* gene sequence typing and multi-locus sequence typing for phylogenetic analysis of *Staphylococcus aureus*. *J. Gen. Appl. Microbiol.* **2013**, *59*, 335–343. [CrossRef] [PubMed]
45. Zhou, W.; Shan, W.; Ma, X.; Chang, W.; Zhou, X.; Lu, H.; Dai, Y. Molecular characterization of rifampicin-resistant *Staphylococcus aureus* isolates in a Chinese teaching hospital from Anhui, China. *BMC Microbiol.* **2012**, *12*, 240. [CrossRef]
46. Aubry-Damon, H.; Soussy, C.J.; Courvalin, P. Characterization of mutations in the *rpoB* gene that confer rifampin resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **1998**, *42*, 2590–2594. [CrossRef] [PubMed]
47. Watanabe, Y.; Cui, L.; Katayama, Y.; Kozue, K.; Hiramatsu, K. Impact of *rpoB* mutations on reduced vancomycin susceptibility in *Staphylococcus aureus*. *J. Clin. Microbiol.* **2011**, *49*, 2680–2684. [CrossRef] [PubMed]
48. Kreiswirth, B.; Kornblum, J.; Arbeit, R.D.; Eisner, W.; Maslow, J.N.; McGeer, A.; Low, D.E.; Novick, R.P. Evidence for a clonal origin of methicillin resistance in *Staphylococcus aureus*. *Science* **1993**, *259*, 227–230. [CrossRef] [PubMed]
49. Kluytmans, J.A.J.W. Methicillin-resistant *Staphylococcus aureus* in food products: Cause for concern or case for complacency? *Clin. Microbiol. Infect.* **2010**, *16*, 11–15. [CrossRef]

50. Patel, B.A.; Cockerill, F.R.; Bradford, P.A.; Eliopoulos, G.M.; Hindler, J.A.; Jenkins, S.G. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. In *M07-A9 Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically Approved Standard*, 10th ed.; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2015; Volume 35, number 2.
51. Rota, C.; Carraminana, J.J.; Burillo, J.; Herrera, A. In vitro antimicrobial activity of essential oils from aromatic plants against selected foodborne pathogens. *J. Food Prot.* **2004**, *67*, 1252–1256. [CrossRef]
52. Rosche, W.A.; Foster, P.L. Determining mutation rates in bacterial populations. *Methods (San Diego, Calif.)* **2000**, *20*, 4–17. [CrossRef]
53. Saldaña, G.; Cebrian, G.; Abenoza, M.; Sanchez-Gimeno, C.; Alvarez, I.; Raso, J. Assessing the efficacy of PEF treatments for improving polyphenol extraction during red wine vinifications. *Innov. Food Sci. Emerg. Technol.* **2017**, *39*, 179–187. [CrossRef]
54. Li, H.; Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **2010**, *26*, 589–595. [CrossRef] [PubMed]
55. Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N.; Marth, G.; Abecasis, G.; Durbin, R.; Proc, G.P.D. The sequence alignment/map format and SAMtools. *Bioinformatics* **2009**, *25*, 2078–2079. [CrossRef] [PubMed]

Sample Availability: Samples of the compounds are not available from the authors.

SUPPLEMENTAL MATERIAL

Disk diffusion assay

The agar disc diffusion method was used as preliminary assay for testing the antibacterial effect of the ICs against *S. aureus* USA300 and its derivative strains. 0.5 mL from a 10^7 CFU/mL bacterial suspension was spread on the surface of TSAYE plates. Sterile filter paper disks (Whatman Grade 1, 6 mm diameter) were individually impregnated with 1, 2.5 and 30 µL of carvacrol, citral and (+)-limonene oxide, respectively, dissolved in 3% absolute ethanol (Sigma-Aldrich) and then placed on the previously inoculated agar plates. These volumes were previously determined to produce an inhibition halo measurable (data not shown). Plates were kept at 4 °C for 2 h to allow for the diffusion of ICs, and then incubated at 37 °C for 18 - 24 h. The diameters of the resulting zones of inhibition were measured with a Vernier digital micrometre (accuracy: 0.01 mm) including the diameter of the paper disk. Disks impregnated with 3% ethanol caused no inhibition halos.

Table S1. Zones of growth inhibition (mm) showing antibacterial activity for carvacrol (1 µL), citral (2.5 µL) and (+)-limonene oxide (30 µL) against *Staphylococcus aureus* USA300 wild type and its derivative strains isolated: CAR, CIT and OXLIM. Each value represents the mean diameter of the inhibition halo ± standard deviation of the six colonies isolated by each IC. Increases in MIC with regard to WT are shaded.

	WT	CAR	CIT	OXLIM
Carvacrol	20.49 ± 0.31	14.79 ± 0.61*	17.12 ± 0.60*	16.21 ± 0.49*
Citral	25.73 ± 0.37	26.01 ± 0.49^{ns}	19.14 ± 0.21*	19.77 ± 0.53*
(+)-Limonene oxide	16.04 ± 0.54	16.12 ± 0.38^{ns}	14.34 ± 0.67*	11.18 ± 0.29*

^{ns} not significantly different from wild type (p>0.05).
* significantly different from wild type (p≤0.05).

Table S2. Primers used for verification.

Verification	Sequence F (5'→ 3')	Sequence R (5'→ 3')
Deleted 43 kb region in CAR	CTGACAATAGTGCCAAAGCCG	TGGGGTGTACATTCCACACA
	GGGACCCATTAGGGACTCCA	GGCCGAGTACAGGTGTTTGA
Flaking regions of 43 kb deletion in CAR	CTGACAATAGTGCCAAAGCCG	GGCCGAGTACAGGTGTTTGA
***aroC* SNP in CIT**	TCGCATTGCTTGTGCGATAC	TTGCAGTCGGTGCCTTATGT
***rpoB* SNP in OXLIM**	TGGTGCAGAAGTAAAAGATGGAGA	TCAAGAACGATACCGCCAGC

Table S3. Large deletion of *Staphylococcus aureus* CAR derivative strain.

Locus tag	Gene	Product	Locus tag	Gene	Product
SAUSA300_RS10505	*hlb-1*	phosphodiesterase	SAUSA300_RS10680		hypothetical protein
SAUSA300_RS10510		hypothetical protein	SAUSA300_RS10685		DUF1381 domain-containing protein
SAUSA300_RS10515		hypothetical protein	SAUSA300_RS10690		hypothetical protein
SAUSA300_RS10520		hypothetical protein	SAUSA300_RS10695	*dut*	dUTP pyrophosphatase
SAUSA300_RS10525	*scn*	staphylococcal complement inhibitor	SAUSA300_RS10700		hypothetical protein
SAUSA300_RS10530	*chp*	chemotaxis inhibitory protein	SAUSA300_RS10705		DUF1024 domain-containing protein
SAUSA300_RS10535		peptidoglycan hydrolase	SAUSA300_RS10710		hypothetical protein
SAUSA300_RS10540	*sak*	staphylokinase	SAUSA300_RS10715		hypothetical protein
SAUSA300_RS10545	*ami*	amidase	SAUSA300_RS10720		hypothetical protein
SAUSA300_RS10550		phage holin	SAUSA300_RS10725	*rusA*	Holliday junction DNA helicase
SAUSA300_RS10555		hypothetical protein	SAUSA300_RS10730		hypothetical protein
SAUSA300_RS10565		DUF2951 domain-containing protein	SAUSA300_RS10735	*dnaD 2*	replication protein DnaD
SAUSA300_RS10570		hypothetical protein	SAUSA300_RS10740	*ssb2*	single-stranded DNA-binding protein
SAUSA300_RS10575		hypothetical protein	SAUSA300_RS10745		MBL fold metallo-hydrolase
SAUSA300_RS10580		hypothetical protein	SAUSA300_RS10750	*recT*	recombinase
SAUSA300_RS10585		phage tail protein	SAUSA300_RS10755		ATPase
SAUSA300_RS10590		phage tail tape measure protein	SAUSA300_RS10760		DUF1108 domain-containing protein
SAUSA300_RS10600		hypothetical protein	SAUSA300_RS10765		hypothetical protein
SAUSA300_RS10605		phage tail protein	SAUSA300_RS10770		DUF1270 domain-containing protein
SAUSA300_RS10610		hypothetical protein	SAUSA300_RS10775		DUF771 domain-containing protein
SAUSA300_RS10615		hypothetical protein	SAUSA300_RS10780		hypothetical protein
SAUSA300_RS10620		phage head-tail adapter protein	SAUSA300_RS10785		oxidoreductase
SAUSA300_RS10625		phage head-tail adapter protein	SAUSA300_RS10790		hypothetical protein
SAUSA300_RS10630		hypothetical protein	SAUSA300_RS10795		DUF2829 domain-containing protein
SAUSA300_RS10635		phage major capsid protein	SAUSA300_RS10800		XRE family transcriptional regulator
SAUSA300_RS10640		Clp protease ClpP	SAUSA300_RS10805		transcriptional regulator
SAUSA300_RS10645		phage portal protein	SAUSA300_RS10810		XRE family transcriptional regulator
SAUSA300_RS10650		terminase large subunit	SAUSA300_RS10815		ATP-dependent helicase
SAUSA300_RS10655		hypothetical protein	SAUSA300_RS10820		hypothetical protein
SAUSA300_RS10660		HNH endonuclease	SAUSA300_RS10825		hypothetical protein
SAUSA300_RS10665		hypothetical protein	SAUSA300_RS10830		toxin MazF
SAUSA300_RS10670		hypothetical protein	SAUSA300_RS10835	*int3*	site-specific integrase
SAUSA300_RS10675		transcriptional activator RinB			

Manuscrito IV. Genetic variants and phenotypic characteristics of *Salmonella* Typhimurium-resistant mutants after exposure to carvacrol

Berdejo, D., Merino, N., Pagán, E., García-Gonzalo, D., Pagán, R. (2020)

Microorganisms, 8, 937. doi: 10.3390/microorganisms8060937.

microorganisms

MDPI

Article

Genetic Variants and Phenotypic Characteristics of *Salmonella* Typhimurium-Resistant Mutants after Exposure to Carvacrol

Daniel Berdejo, Natalia Merino, Elisa Pagán, Diego García-Gonzalo and Rafael Pagán *

Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), 50013 Zaragoza, Spain; berdejo@unizar.es (D.B.); merino@unizar.es (N.M.); epagan@unizar.es (E.P.); Diego.Garcia@unizar.es (D.G.-G.)
* Correspondence: pagan@unizar.es; Tel.: +34-976762675

Received: 26 May 2020; Accepted: 19 June 2020; Published: 22 June 2020

Abstract: The emergence of antimicrobial resistance has raised questions about the safety of essential oils and their individual constituents as food preservatives and as disinfection agents. Further research is required to understand how and under what conditions stable genotypic resistance might occur in food pathogens. Evolution experiments on *Salmonella* Typhimurium cyclically exposed to sublethal and lethal doses of carvacrol permitted the isolation of SeSCar and SeLCar strains, respectively. Both evolved strains showed a significant increase in carvacrol resistance, assessed by minimum inhibitory and bactericidal concentrations, the study of growth kinetics in the presence of carvacrol, and the evaluation of survival under lethal conditions. Moreover, antibiotic susceptibility tests revealed a development of SeLCar resistance to a wide range of antibiotics. Whole genome sequencing allowed the identification of single nucleotide variations in transcriptional regulators of oxidative stress-response: *yfhP* in SeSCar and *soxR* in SeLCar, which could be responsible for the increased resistance by improving the response to carvacrol and preventing its accumulation inside the cell. This study demonstrates the emergence of S. Typhimurium-resistant mutants against carvacrol, which might pose a risk to food safety and should therefore be considered in the design of food preservation strategies, or of cleaning and disinfection treatments.

Keywords: antimicrobial resistance; evolution assays; carvacrol; *Salmonella* Typhimurium; genotypic resistance; antibiotics; whole-genome sequencing; resistant mutants

1. Introduction

One of the greatest challenges to global health over the last two decades has been the emergence of antimicrobial resistance (AMR) and its spread in the environment [1,2]. This concern has shifted from clinical setups to further areas, raising questions about the safety of natural preservatives, such as essential oils (EOs) and individual constituents (ICs), as food preservatives or as cleaning and disinfection agents. Carvacrol is an IC mainly extracted from EO of *Origanum vulgare*, *Thymus vulgaris* and *Thymbra capitata* [3,4], generally recognized as safe (GRAS) by the U.S. Food and Drug Administration [5]. This monoterpenoid phenol has demonstrated outstanding antimicrobial properties [6–8] and is therefore regarded as one of the most promising ICs as a food preservative or as a cleaning and disinfection agent [9].

Since high antioxidant activity of EOs and ICs at low doses [10] would normally reduce the mutation frequency of treated bacteria [11,12], it has been commonly accepted that these compounds do not induce mutations that could lead to AMR [13]. In this regard, previous studies of evolution assays in *Salmonella* did not observe increased resistance after exposure to subinhibitory amounts of *Origanum*

vulgare and carvacrol [14]. However, Chueca, Berdejo, Gomes-Neto, Pagán and García-Gonzalo [11] and Berdejo, et al. [15] described for the first time the emergence of mutations in bacterial populations after prolonged cyclic exposure to subinhibitory doses of these compounds, thereby resulting in increased bacterial resistance. The identification of the genetic modifications in those resistant mutants led to a better understanding of the bacterial response against ICs and, consequently, of their mechanisms of action [15,16]. Interesting results were obtained in carvacrol evolution treatments in *Escherichia coli*: a mutation in the *soxR* gene was marked as responsible for a significant increase in resistance not only to carvacrol, but also to other ICs and even to antibiotics [11,16]. Moreover, a recent study reported the emergence of resistant strains of *E. coli* O23:H52 against carvacrol and oregano by cyclic exposure to subinhibitory doses [17]. In this regard, it is unknown whether the increased resistance observed in *E. coli* [11] or in *Staphylococcus aureus* [15] might also occur in one of the food pathogens most involved in food outbreaks, *Salmonella* spp., and whether the mutagenesis might follow a general pattern, or rather a specific one, as a function of the type of microorganism, of the bacteriostatic agent, or of treatment conditions.

Moreover, several studies have isolated resistant mutants from the tail of survival curves after cyclic lethal treatments with physical agents, such as heat [18] and high hydrostatic pressure [19], or with chemical agents, such as antibiotics [20]. However, it is unknown whether the application of lethal doses of EOs or ICs might favor the emergence of resistant mutants, as observed at sublethal doses, thus posing a risk to food safety.

For these reasons, further research is still needed in order to describe the occurrence of AMR under sublethal or lethal carvacrol concentrations, paving the way for further in-depth exploration of carvacrol's mechanisms of action. This knowledge would contribute towards enhancing the antimicrobial properties of carvacrol as a single agent or in combined processes [15] with other antimicrobial agents [21] or with physical treatments [22].

This study therefore seeks (a) to isolate mutant-resistant strains of *S. enterica* Typhimurium under two different protocols of carvacrol evolution assays: cyclic exposure to prolonged sublethal treatments, and cyclic exposure to short lethal treatments; (b) to describe the resistance of the isolated strains against carvacrol and antibiotics; (c) to identify the mutations involved in the observed bacterial resistance.

2. Materials and Methods

2.1. Microorganisms and Growth Conditions

Salmonella enterica subsp. *enterica* serovar Typhimurium LT2 (SeWT) was provided by the Spanish Type Culture Collection (CECT 722). Isolated in the 1940s, it is one of the principal strains used in cellular and molecular biology studies of *Salmonella* since its genome was completely sequenced in 2001 [23]. For this reason, we selected this strain to carry out our study of genetic evolution under selective pressure from carvacrol.

Throughout this investigation, the strain was kept in cryovials at −80 °C with glycerol (20% v/v), from which plates of tryptone soya agar (Oxoid, Basingstoke, England) with 0.6% yeast extract (Oxoid; TSAYE) were prepared on a weekly basis. To prepare the working bacterial cultures, test tubes containing 5 mL of tryptone soya broth (Oxoid,) with 0.6% yeast extract (TBSYE) were inoculated with one colony and then incubated aerobically on an orbital shaker (130 rpm; Heidolph Vibramax 100, Schwaback, Germany) for 12 h at 37 °C (Incubig, Selecta, Barcelona, Spain). Subsequently, flasks containing 10 mL of fresh TSBYE were inoculated with 2 µL of the resulting subculture to achieve an initial concentration of 10^6 colony forming units per mL (CFU/mL), and incubated for 24 h at 37 °C and 130 rpm until the stationary growth phase was reached (5×10^9 CFU/mL approximately). The bacterial concentration of the cultures was verified by spreading them on TSAYE plates. We applied the same protocol to obtain the working bacterial cultures of the isolated strains that resulted from the evolution experiments with carvacrol in this study.

2.2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentration determination was performed by inoculating selected strains in test tubes with 5 mL of mueller hinton broth cation (Sigma-Aldrich; MHB) adjusted to achieve an initial concentration of 5×10^5 CFU/mL in the presence of different concentrations of carvacrol: from 50 up to 500 µL/L, and incubated at 37 °C for 24 h and 130 rpm. Once the tubes were incubated, MIC was determined as the lowest concentration of the antimicrobial compound that was capable of avoiding bacterial growth. To objectively determine bacterial growth, the optical density was read at 595 nm (OD_{595}) using a microplate reader (Genios, Tecan, Männedorf, Switzerland). An amount of 10% of the OD_{595} measure of the positive control was established as the lower limit to consider that bacterial strain was grown [24]. Following the method described by Friedman et al., [25], a vigorous shaking by vortex (Genius 3, Ika, Königswinter, Germany) was used to prepare carvacrol dispersions in MHB, avoiding the use of solvents for their possible detriment in the antibacterial activity. Positive control tubes with 5 mL MHB inoculated at 5×10^5 CFU/mL without ICs, and negative control tubes with 5 mL MHB inoculated at the same concentration with 1000 µL/L of carvacrol, were also prepared in every experiment. This protocol was adapted from standard methods for antimicrobial susceptibility tests [26].

The minimum bactericidal concentration (MBC) of carvacrol was evaluated in parallel to the MIC test. From the test tubes employed in the MIC determination after incubation, 100 µL aliquot of each tube was spread onto mueller hinton agar cation-adjusted (Sigma-Aldrich; MHA) plates and incubated at 37 °C for 24 h. Colonies were counted and the lowest concentration of carvacrol that killed ≥ 99.9% of the initial bacterial concentration (5×10^5 CFU/mL) was defined as the MBC end point [27]. The same positive and negative controls as the MIC test were employed in this experiment. The MBC of evolved strains were compared to that of SeWT to assess the increased resistance to carvacrol.

2.3. Carvacrol Evolution Assays

The use of ICs in food preservation can lead either to the inhibition of bacterial growth or to bacterial inactivation, depending on IC concentration. Then, to obtain resistant *Salmonella* strains against carvacrol, two different protocols were followed in order to simulate bacteriostatic and bactericidal conditions: (a) cyclic exposure to prolonged sublethal treatments, and (b) cyclic exposure to short lethal treatments (Figure 1).

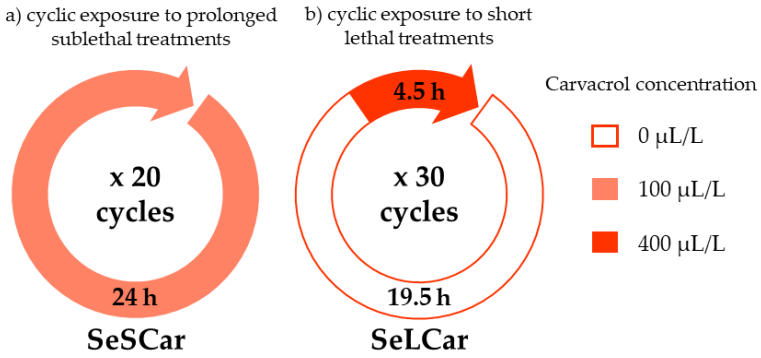


Figure 1. Schematic for the experimental protocols of carvacrol evolution assays: (**a**) cyclic exposure to prolonged sublethal treatments (SeSCar) and (**b**) cyclic exposure to short lethal treatments (SeLCar).

(a) The first protocol was based on the isolation of strains by prolonged exposure to a subinhibitory concentration of carvacrol during the growth phase of bacteria (Figure 2). *Salmonella* wild-type strain (SeWT) was grown on TSAYE plates for 24 h at 37 °C. A single colony was inoculated in 5 mL TSBYE and incubated under agitation for 12 h at 37 °C. This preculture was diluted 1:1000 into 50 mL TSBYE and incubated for 3.5 h to obtain an exponential phase culture. From this culture, 5 mL TSBYE were inoculated at an initial bacterial concentration of 10^6 CFU/mL in the presence of 100 µL/L of carvacrol

(1/2 of MIC for SeWT). The bacterial concentration of the cultures was verified by spreading them on TSAYE plates. This bacterial suspension was incubated 24 h/37 °C/130 rpm and, once the stationary phase was reached, the same step was repeated: the previous culture was diluted (10^6 CFU/mL) in 5 mL TSBYE with 100 µL/L of carvacrol (≥ 98%; Sigma-Aldrich) and incubated 24 h/37 °C/130 rpm. This procedure was repeated 20 times. After the 20th step, an aliquot was diluted in phosphate-buffered saline (Sigma-Aldrich, Steinheim, Westphalia, Germany; PBS) and spread on TSAYE plates (without carvacrol). After the incubation period, 5 colonies (SeSCar$_{1\text{-}5}$) were randomly selected to carry out phenotypic and genotypic characterization. This methodology was adapted from Kohanski, et al. [24] and Andersson and Hughes [28]. This approach mimes the use of carvacrol together with other natural substances for preventation purposes.

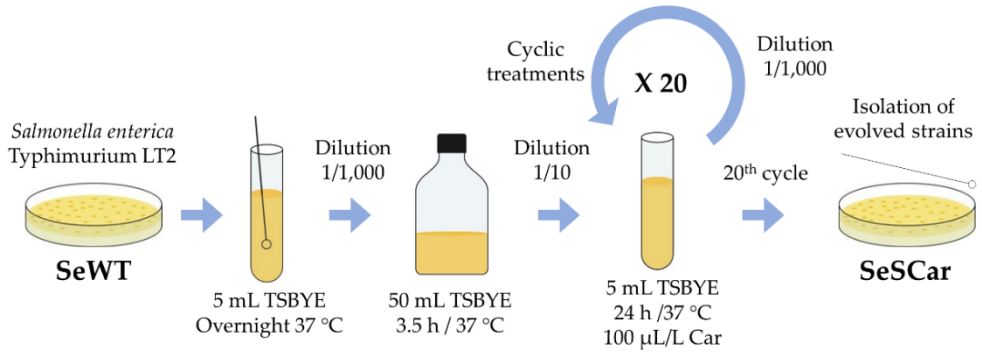


Figure 2. Diagram of evolution assay by prolonged sublethal treatments.

(b) The second protocol was based on the isolation of strains by recovering surviving cells after short-term lethal treatments with carvacrol (Figure 3). For this purpose, a stationary phase culture of SeWT was diluted 1:100 in 50 mL fresh TSBYE with 400 µL/L of carvacrol (2 × MIC for SeWT) for 4.50 h at 37 °C. Subsequently, treated cells were centrifuged for 20 min at 15,000 RCF, washed twice with TSBYE, resuspended in 1 mL TSBYE and incubated overnight at 37 °C. This procedure was repeated 30 times. After the 30th step, an aliquot was diluted in PBS and spread on TSAYE plates (without carvacrol), from which 5 colonies (SeLCar$_{1\text{-}5}$) were randomly selected to carry out phenotypic and genotypic characterization. This methodology was adapted from Levin-Reisman, Ronin, Gefen, Braniss, Shoresh and Balaban [20]. This approach simulates a single treatment after a *Salmonella* contamination.

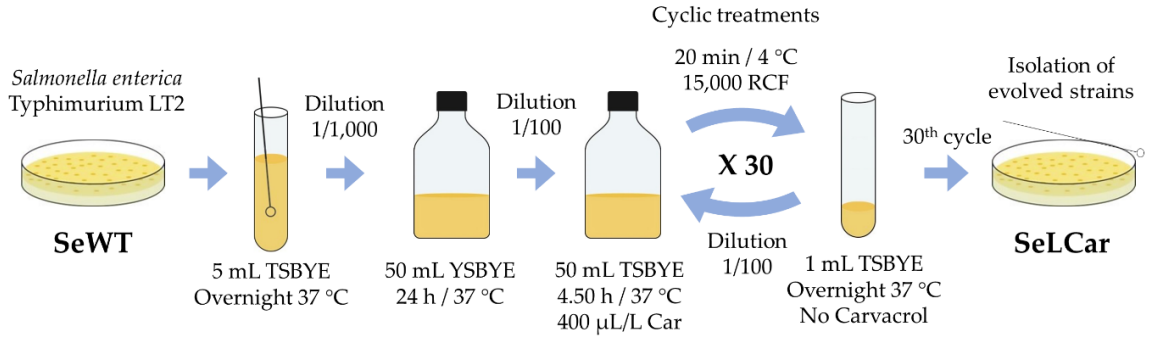


Figure 3. Diagram of evolution assay by short lethal treatments.

Once the 5 strains isolated by each evolution assay, SeSCar$_{1\text{-}5}$ and SeLCar$_{1\text{-}5}$, were obtained, the first approach to evaluate their resistance was to determine the MIC and the MBC of carvacrol and to compare with those of the SeWT.

2.4. Growth Curves in Presence of Carvacrol

In order to more deeply study the behavior of the isolated strains against carvacrol, the growth kinetics of SeWT and of evolved strains were evaluated in TSBYE at different concentrations of carvacrol.

First, carvacrol was added at different concentrations in tubes with 5 mL of TSBYE. Based on the results obtained in the MIC assay, the concentration range of carvacrol used was 0–150 µL/L for SeWT, 0–250 µL/L for SeSCar and 0–350 µL/L for SeLCar. Due to the hydrophobicity of carvacrol, it was necessary to apply vigorous agitation in the vortex to get a uniform suspension. Once the IC was added, test tubes were inoculated with the microbial culture at an initial concentration of 5×10^5 CFU/mL and incubated at 37 °C and 130 rpm for 24 h. Every hour, OD_{595} of the test tubes was measured by a microplate reader. The experiment was prolonged for more than 24 h at high carvacrol concentrations until reaching the stationary growth phase. A positive control (without antimicrobial added) and a negative control (without microbial culture added) were incorporated in all the assays. The values of OD_{595} obtained during the experiment was subtracted from the initial OD_{595} (at time 0), corresponding to the absorbance caused by the growth medium. Bacterial growth curves based on OD_{595} of SeWT, SeSCar, and SeLCar were graphically displayed and modelled by a modified Gompertz equation [29]:

$$y = A \exp\{-exp[\ (u_m\ e\ /A\ )(\lambda - t) + 1\ ]\} \quad (1)$$

where y: OD_{595}; t: time (h); A: maximum value reached (OD_{595} max); μ_m: maximum specific growth rate (h^{-1}); λ: lag time (h).

A least-squares adjustment was carried out to build the model and obtain A, μm and λ values using the GraphPrism®program (GraphPad Software, Inc., San Diego, CA, USA). The adjustment's goodness of fit was evaluated using standard error, R^2 and R^2 adjusted values, and the root mean square error (*RMSE*).

2.5. Survival Curves in Presence of Carvacrol

The resistance of SeWT and of the evolved strains against carvacrol was also evaluated with lethal treatments. In these cases, the treatment medium we used was citrate–phosphate buffer or "McIlvaine buffer", prepared from citric acid monohydrate (Panreac) and disodium hydrogen phosphate (Panreac), adjusted to pH 4.0 and pH 7.0. These pH values were chosen as representative of neutral and acid conditions. The treatment was carried out in 10 mL McIlvaine buffer previously tempered at 25 °C, to which carvacrol was added at a concentration of 150 µL/L and then vigorously agitated to obtain a homogeneous dispersion of the IC. This concentration was selected based on preliminary experiments using 100–300 µL/L of carvacrol against SeWT strain (results not shown), in order to apply a treatment that would achieve 5 $\log_{10}$ cycles of inactivation and whose inactivation kinetics would permit comparison with the resistance of the evolved strains. Once carvacrol was added, stationary phase culture was centrifuged for 5 min at 6000 RCF in a microcentrifuge (Mini Spin, Eppendorf, Hamburg, Germany) and resuspended in the treatment medium. Test tubes were then inoculated at 10^7 CFU/mL, thus initiating the lethal carvacrol treatment. Total treatment time was set to 30 min, during which aliquots were obtained every 5 min. These samples were diluted in PBS and subsequently spread on TSAYE plates. After plate incubation (24 h/ 37 °C), the count of survival cells was carried out in an automatic plate counter by image analysis (Analytical Measuring Systems, Protos, Cambridge, United Kingdom). Once survival curves of SeWT and evolved strains were obtained, inactivation kinetics were compared in order to evaluate the increase in resistance of SeSCar and SeLCar against carvacrol.

2.6. Antibiotic Susceptibility Test

Agar disk diffusion assay was used to test antimicrobial susceptibility according to CLSI [27,30]. First, bacterial suspension was spread on MHA plates and, after 5 min at room temperature, blank disks (Ø: 6.0 mm) (Thermo Scientific™ Oxoid™ Anti-microbial Susceptibility Disk Dispenser, ST6090, Waltham, MA, USA) were placed on the surface of plates and individually impregnated with 10 µL of

each antibiotic: 30 µg kanamycin sulfate, 30 µg tetracycline, 30 µg chloramphenicol, 400 µg nalidixic acid sodium, 50 µg rifampicin, 20 µg norfloxacin, 250 µg novobiocin sodium, 10 µg trimethoprim, 10 µg ampicillin, and 150 µg cephalexin (Sigma-Aldrich). These plates were incubated at 37 °C for 18–24 h, after which the diameters of the resulting inhibition zones were measured (paper disks included). We selected the range of antibiotics in order to cover different cellular targets that could be related to carvacrol resistance.

2.7. Whole Genome Sequencing (WGS) and Identification of Genetic Variations

Genomic DNA (gDNA) was extracted using a gDNA kit (DNeasy kit, Qiagen, Hilden, Germany) for extraction and purification of SeWT and the evolved strains. Illumina technology was used to carry out whole genome sequencing (WGS) on NextSeq equipment at mid-output flow, with a total of 2 × 150 cycles (Illumina; Fasteris, SA, Geneva, Switzerland). Subsequently, quality control was performed with FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) evaluating reading quality (Q_{30}), sequence length, presence of adapters, and overrepresented and duplicated sequences. The quality-control-filtered paired-end reads were mapped on the reference genome sequence (National Center for Biotechnology Information; NCBI accession: NC_003197.2): *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2, complete genome [23], using a Burrows–Wheeler Alignment (BWA) Tool [31] and Samtools software [32] (sources: http://bio-bwa.sourceforge.net/ and http://www.htslib.org/). A raw-coverage 150-fold depth was achieved for the three strains. Then, Samtools was applied to remove potential PCR duplicates according to reading positions on the reference genome; the resulting BAM files were then further processed using LoFreq-Star (source: http://csb5.github.io/lofreq/) to correct mapping errors and insert the quality values. Finally, single nucleotide variants (SNVs) and short insertion and deletions (InDels) were detected using LoFreq-Star, and toolbox snpEff (source: http://snpeff.sourceforge.net/) was employed to identify involved genes and to predict functional effect variations [33]. Coverage was further analysed by the Integrative Genomics Viewer (IGV; Broad Institute, source: https://software.broadinstitute.org/software/igv/) in order to find structural variations (SVs). Although mapping was carried out against the reference genome, SNVs, InDels, and SVs were identified between SeWT and isolated strains to ascertain the kind of mutations that had occurred during the evolution treatments. Finally, specific primers (Table S1) were designed with the "Primer designing tool" of NCBI to carry out PCR amplifications, as well as Sanger sequencings to verify the mutations detected in the WGS. Sanger sequencing reads were aligned and compared using the software Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The resulting genome sequences were deposited in the Sequence Read Archive (SRA) of NCBI (BioProject ID: PRJNA634825). The accession numbers of the samples are SAMN15009803 (SeWT), SAMN15009804 (SeSCar), SAMN15009805 (SeLCar). Additionally, Table S2 summarizes the genomic background of *S.* Typhimurium LT2.

2.8. Statistical Analysis

All phenotypic characterization results were obtained from at least 3 independent experiments carried out on different working days with different bacterial cultures. MIC and MBC data correspond to the results obtained from 5 different assays. Growth curve parameters, lethal treatment graphics, and antibiotic susceptibility tests are displayed as the mean ± standard deviation, using the GraphPrism®program. Data were analyzed and submitted to comparison of averages using analysis of variance (ANOVA), followed by post-hoc Tukey test and t-tests with GraphPrism®, and differences were considered significant if $p \leq 0.05$.

3. Results and Discussion

3.1. Isolation of Resistant Strains Obtained by Selective Pressure of Carvacrol

Two different protocols were followed to obtain resistant *Salmonella* strains against carvacrol: (a) cyclic exposure to prolonged sublethal treatments, and (b) cyclic exposure to short lethal treatments. From each evolution experiment, five colonies, (a) $SeSCar_{1\text{-}5}$ and (b) $SeLCar_{1\text{-}5}$, were randomly isolated after 20 and 30 cycles, respectively. Subsequently, phenotypic and genotypic characterization were performed to determine whether the carvacrol evolution assays allowed for the emergence of stable resistant bacterial strains.

The resistance of $SeSCar_{1\text{-}5}$ and $SeLCar_{1\text{-}5}$ against carvacrol was determined by assaying the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) (Table 1). The results of the evolved strains were compared with those of SeWT in order to assess increased resistance to carvacrol.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of carvacrol for *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT) and evolved strains: $SeSCar_{1\text{-}5}$ (5 strains selected by cyclic exposure to prolonged sublethal treatments of carvacrol) and $SeLCar_{1\text{-}5}$ (5 strains selected by cyclic exposure to short lethal treatments of carvacrol).

Strains	MIC (μL/L)	MBC (μL/L)
SeWT	200	200
$SeSCar_{1\text{-}5}$	300	300
$SeLCar_{1\text{-}5}$	400	400

Each value represents the result of 5 different experiments carried out for every strain tested, with different bacterial cultures and on different working days.

On the one hand, the bacteriostatic effect of carvacrol on *S. enterica* strains (SeWT, $SeSCar_{1\text{-}5}$ and $SeLCar_{1\text{-}5}$.) was evaluated by MIC determination (Table 1). The data of the five isolated colonies from the same evolution experiment were grouped in the same row, since the MIC results displayed the same values ($p < 0.05$). The MIC results demonstrated the strong antibacterial activity of carvacrol against *S.* Typhimurium. Similar MIC values have been obtained in other studies against *Salmonella* strains. The MIC determined by Mith, et al. [34] was 125 μL/L for *S.* Typhimurium CDC 6516-60 (ATCC 14028), and 188 μL/L for *S.* Typhimurium S0584 (isolated from pig carcass). Lu and Wu [35] obtained a MIC of 205 μL/L carvacrol against another *S.* Typhimurium strain.

As detailed in Table 1, both isolated mutants showed an increase in the MIC of carvacrol, from 200 μL/L against SeWT to 300 μL/L against $SeSCar_{1\text{-}5}$, and to 400 μL/L against $SeLCar_{1\text{-}5}$. This corresponds to 50% and 100% increased resistance after the carvacrol evolution treatments. Chueca, Berdejo, Gomes-Neto, Pagán and García-Gonzalo [11] and Berdejo, Chueca, Pagan, Renzoni, Kelley, Pagan and Garcia-Gonzalo [15] also observed an increase in resistance to carvacrol in the strains evolved by exposure to subinhibitory doses: a 300% increase of the MIC against *Escherichia coli* MG1655, and 50% against *Staphylococcus aureus* USA300, respectively. The MIC for $SeSCar_{1\text{-}5}$, evolved in the presence of subinhibitory doses, and was lower than that of $SeLCar_{1\text{-}5}$ evolved by lethal doses. However, there are no previous reports on the MIC determination of carvacrol for strains evolved by cyclic exposure to lethal doses.

The bactericidal effect of carvacrol was explored by MBC determination. As in the MIC test, the MBC values for the evolved strains obtained with the same protocol were identical ($p < 0.05$) and are consequently grouped in Table 1. The MBCs of carvacrol were the same as the MIC values for SeWT (200 μL/L) and for the evolved strains: 300 μL/L for $SeSCar_{1\text{-}5}$ and 400 μL/L for $SeLCar_{1\text{-}5}$. Similar MIC and MBC values have been associated with the strong bactericidal activity of carvacrol even at low concentrations. For instance, no differences between MIC and MBC of carvacrol were detected against *Escherichia coli* O157:H7; both concentrations reached values of 200 μL/L [22]. However, Lu and

Wu [35] and Mith et al. [34] observed different MBC and MIC values of carvacrol for each tested *S.* Typhimurium strain: between 200 and 400 µL/L and between 125 and 375 µL/L, respectively. Therefore, divergence among MIC and MBC values would be more due to strain-to-strain variation than to bactericidal activity of the antimicrobial compound.

Regarding the comparison of evolved strains with SeWT, MBC increased by 50% in $SeSCar_{1\text{-}5}$ and 100% in $SeLCar_{1\text{-}5}$. As shown by the MIC test, SeLCar exhibited a greater resistance than SeSCar: this could be due to the protocol applied in the corresponding evolution experiment, which applied a bactericidal concentration (400 µL/L). Nevertheless, there are no previous studies on the MBC assessment of carvacrol for evolved strains of any microorganism. These results reveal that evolved strains show a higher resistance to carvacrol, and resistance varies as a function of the method of evolution. In this regard, lethal treatments seem to lead to the emergence of more resistant strains than using sublethal doses.

The protocol we followed in the evolution assays with sublethal doses of carvacrol had been employed in previous studies on *E. coli* [11] and *S. aureus* [15]. In both studies, the evolved strains revealed increased resistance to carvacrol, and even cross-resistance to other ICs and antibiotics. In contrast, Gomes-Neto, et al. [36] did not observe the emergence of resistant strains of *S.* Typhimurium against *Rosmarinus officinalis* L. EO and 1,8-cineole in an evolution assay with subinhibitory doses but increasing the concentration in the course of the experiment. Regarding evolution assays with lethal treatments of EOs and ICs, to the best of our knowledge, no previous studies have evaluated the appearance of AMR: neither in *Salmonella* spp., nor in any other microorganism against these natural compounds. MIC and MBC results revealed that the resistance of all the colonies coming from the same evolution lineage displayed the same degree of resistance to carvacrol. These results suggest that all isolated colonies were identical, and that the bacterial cultures obtained from the evolution treatments were probably homogeneous. We therefore pursued the remainder of our research with one of the five strains selected from each of the evolution protocols: SeSCar (obtained by cyclic exposure to prolonged sublethal doses) and SeLCar (obtained by cyclic exposure to short lethal treatments).

3.2. Growth Kinetics under Carvacrol Stress

The growth kinetics in the presence of carvacrol were evaluated in order to describe in depth the behavior of the evolved strains against that IC. Figure 4 displays the growth curves of SeWT, SeSCar, and SeLCar modelled by modified Gompertz equation (Equation (1)) in the presence of varying concentrations of carvacrol (from 0 to 350 µL/L). The standard error, R^2 and R^2 adjusted values and the root mean square error (*RMSE*) supported a good least-squares adjustment (Table S3). In agreement with the MIC results, concentrations higher than 150 µL/L did not allow the growth of SeWT (Figure 4A); neither did those higher than 250 µL/L for SeSCar (Figure 4B), nor those higher than 350 µL/L for SeLCar (Figure 4C).

As can be observed in Figure 4, all strains showed an extended lag phase and a decrease in the maximum growth rate as the concentration of carvacrol was increased. However, this effect was more pronounced for SeWT than for the evolved strains. For instance, SeWT could not reach the stationary growth phase at 24 h in the presence of 150 µL/L carvacrol, whereas the evolved strains reached the stationary phase under the same conditions before 20 h. The parameters of the modified Gompertz equation: A (maximum OD_{595}), μ_m (maximum specific growth rate) and λ (lag time), for the three strains and under all the conditions tested, are provided in Table 2.

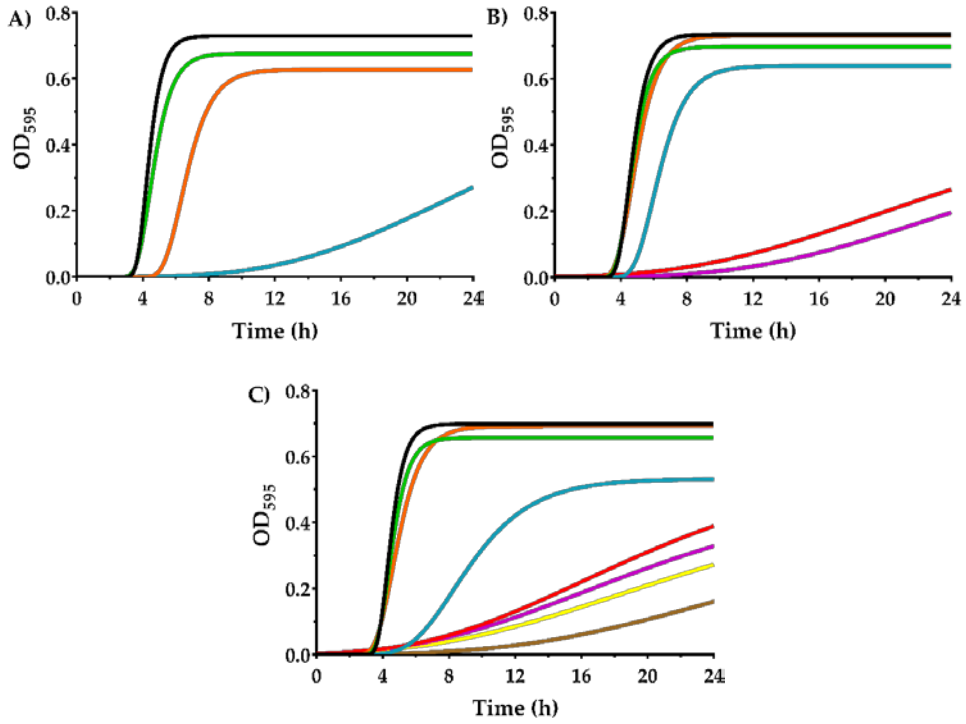


Figure 4. Growth curves of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (**A**); SeWT) and evolved strains: SeSCar (**B**); by cyclic exposure to prolonged sublethal treatments of carvacrol) and SeLCar (**C**); by cyclic exposure to short lethal treatments of carvacrol), in the absence (—) and presence of 50 (—), 100 (—), 150 (—), 200 (—), 250 (—), 300 (—) and 350 µL/L (—) of carvacrol, modelled using the modified Gompertz equation (Equation (1)).

As can be seen, maximum OD_{595} slightly decreased as the concentration of carvacrol in the growth medium increased. However, no statistically significant differences ($p \geq 0.05$) were observed between SeWT and the evolved strains at carvacrol concentrations below the MIC of SeWT (<200 µL/L). Regarding the maximum growth rate, a strong decrease thereof was noted in the three strains as the carvacrol concentration increased. This growth parameter also showed significant differences ($p < 0.05$) between SeWT and the evolved strains when carvacrol was added to the medium: at 150 µL/L, the maximum growth rate of SeWT was 0.024 OD_{595}/h, while SeSCar and SeLCar reached values of 0.262 and 0.088 OD_{595}/h, respectively. The lag phase was prolonged by the presence of carvacrol in the growth medium for the three strains ($p < 0.05$), but this effect was more pronounced in SeWT. The lag time of SeWT was longer than those of the evolved strains at all tested carvacrol concentrations above 50 µL/L. For instance, the lag time in SeWT at 150 µL/L was 12.8 h, which was 7 h and 6 h longer than in SeSCar and SeLCar, respectively.

Comparing the evolved strains, even though the MIC and MBC results revealed a greater resistance of SeLCar, the growth curves at low carvacrol concentrations (100–150 µL/L) displayed a higher growth rate of SeSCar. This improved adaptation of SeSCar to low doses of carvacrol is probably the consequence of the protocol followed in the evolution experiments, since the concentration used to obtain SeSCar was 100 µL/L.

The effect of the presence of EOs and ICs on bacterial growth has been previously studied. According to Braschi, et al. [37], a slower growth rate and a higher lag phase was observed in *Listeria monocytogenes* as the concentration of carvacrol in the medium increased. Similar results were obtained by Melo, et al. [38] in *E. coli* and *S. aureus* against *Ocimum gratissimum* L. EO: a reduced growth rate and a lag phase delay were observed at higher EO concentrations. Nevertheless, to the best of our knowledge, no previous studies have shown the influence of the presence of any EO or IC on the growth parameters of resistant mutants obtained by cyclic exposure to the same inhibitory agents.

The high growth rate and the short lag phase observed in the growth curve of SeSCar at 100 µL/L of carvacrol, compared to that of SeWT, reveals that the evolved strain could have emerged in the evolution assays by subinhibitory doses. In addition, the growth kinetics of the evolved strains compared with SeWT support not only the possibility of the emergence of resistant strains, but also a better growth fitness in the presence of carvacrol.

Table 2. A (maximum OD_{595}), μ_m (maximum specific growth rate) and λ (lag time) parameters of the modified Gompertz model obtained from growth curves of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT) and evolved strains: SeSCar (cyclic exposure to prolonged sublethal treatments of carvacrol) and SeLCar (by cyclic exposure to short lethal treatments of carvacrol), at different concentrations of carvacrol.

$A(OD_{595})$		Strains		
		SeWT	**SeSCar**	**SeLCar**
Carvacrol (µL/L)	**0**	0.729 ± 0.023 ^{a}	0.734 ± 0.041 ^{a}	0.698 ± 0.012 ^{a}
	50	0.676 ± 0.059 ^{a}	0.697 ± 0.012 ^{a}	0.657 ± 0.008 ^{abx}
	100	0.629 ± 0.051 ^{a}	0.731 ± 0.026 ^{a}	0.693 ± 0.046 ^{a}
	150	0.651 ± 0.013 ^{a}	0.638 ± 0.062 ^{ab}	0.530 ± 0.059 ^{bc}
	200		0.520 ± 0.026 ^{b}	0.602 ± 0.020 ^{abc}
	250		0.515 ± 0.104 ^{b}	0.547 ± 0.102 ^{bc}
	300			0.512 ± 0.041 ^{c}
	350			0.495 ± 0.044 ^{c}
$\mu_m(OD_{595}/h)$		Strains		
		SeWT	**SeSCar**	**SeLCar**
Carvacrol (µL/L)	**0**	0.480 ±0.023 ^{a}	0.410 ± 0.029 ^{a}	0.453 ± 0.013 ^{a}
	50	0.325 ± 0.024 ^{b}	0.351 ± 0.025 ^{b}	0.367 ± 0.047 ^{b}
	100	0.212 ± 0.022 ^{c}	0.302 ± 0.006 ^{bc*}	0.260 ± 0.026 ^{c}
	150	0.024 ± 0.001 ^{d}	0.262 ± 0.030 ^{c*}	0.088 ± 0.023 ^{d*†}
	200		0.017 ± 0.003 ^{d}	0.023 ± 0.003 ^{e}
	250		0.017 ± 0.003 ^{d}	0.022 ± 0.007 ^{e}
	300			0.017 ± 0.002 ^{e}
	350			0.014 ±0.003 ^{e}
λ(h)		Strains		
		SeWT	**SeSCar**	**SeLCar**
Carvacrol (µL/L)	**0**	3.635 ± 0.104 ^{a}	3.806 ± 0.078 ^{a}	3.714 ± 0.069 ^{a}
	50	3.644 ± 0.134 ^{a}	3.732 ± 0.119 ^{a}	3.675 ± 0.067 ^{ab}
	100	5.189 ± 0.087 ^{b}	3.748 ± 0.159 ^{a*}	3.591 ± 0.057 ^{a*}
	150	12.810 ± 0.848 ^{c}	5.209 ± 0.882 ^{a*}	6.051 ± 0.274 ^{bc*}
	200		8.499 ± 1.906 ^{b}	6.471 ± 0.924 ^{c}
	250		12.253 ± 1.485 ^{c}	7.494 ± 1.775 ^{c}
	300			7.350 ± 0.167 ^{c}
	350			12.653 ± 0.341 ^{d}

Each value represents the mean ± standard deviation from 3 independent experiments. Different superscript letters represent statistically significant differences ($p \leq 0.05$) among the means of the same column. * Significantly different from SeWT ($p \leq 0.05$). † Significantly different from SeSCar ($p \leq 0.05$).

3.3. Evaluation of Cell Survival against Carvacrol

Lethal treatments at 150 µL/L of carvacrol were applied to SeWT and the evolved strains at pH 4.0 and pH 7.0 (Figure 5), within the normal pH range of food.

As can be seen in Figure 5, only 150 µL/L of carvacrol were needed to reduce 5 $\log_{10}$ cycles of SeWT in 15 min at acidic pH, and in 20 min at neutral pH. This assay corroborates the strong bactericidal properties of carvacrol at low concentrations against *S.* Typhimurium, even after short treatments. Previous studies have also observed a great effectiveness of carvacrol against *S.* Typhimurium strains:

Chung, Cho and Rhee [21] reported a reduction of 2 $\log_{10}$ cycles in 5 min of treatment at 2 mM concentration of carvacrol (approx. 300 µL/L). A greater inactivation was reached by Mattson, Johny, Amalaradjou, More, Schreiber, Patel and Venkitanarayanan [8]: 7 $\log_{10}$ cycles of reduction of *S.* Typhimurium was achieved in just 1 min of treatment but using higher concentrations, e.g., 2500 µL/L of carvacrol.

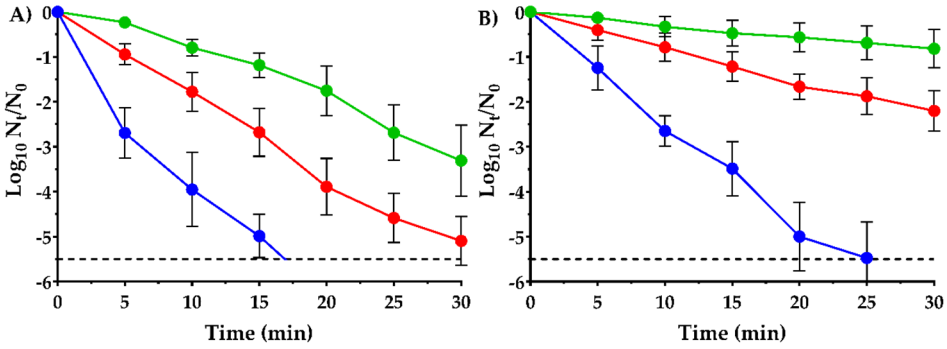


Figure 5. Survival curves of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (•); SeWT) and evolved strains: SeSCar (•); by cyclic exposure to prolonged sublethal treatments of carvacrol) and SeLCar (•); by cyclic exposure to short lethal treatments of carvacrol), after 150 µL/L carvacrol treatment at pH 4.0 (**a**) and pH 7.0 (**b**). Data are means ± standard deviations (error bars) obtained from at least 3 independent experiments. The dashed line represents the detection limit (−5.5 $\log_{10}$ N_t/N_0).

Additionally, comparing the survival curves at pH 4.0 (Figure 5A) with those at pH 7.0 (Figure 5B), a greater bactericidal activity of carvacrol was observed in acid medium than in neutral medium against all three strains. For instance, 5.5 $\log_{10}$ cycles of reduction of SeWT were reached at 17 min in acid pH, whereas up to 25 min were required in neutral pH. The hurdle effect between carvacrol and acid pH was already observed in two previous studies against *E. coli* [7,22]. In addition, EOs are more hydrophobic at acid pH, and therefore might interact better with the lipid bilayer of the cell membrane, thereby achieving cell injury or inactivation [39].

At acid pH, the evolved strains SeSCar and SeLCar showed a higher survival to the lethal treatment with carvacrol than SeWT. While over 5.5 $\log_{10}$ cycles of SeWT inactivation were achieved after 20 min of treatment, only 1.8 $\log_{10}$ cycles of SeSCar and 3.9 $\log_{10}$ cycles of SeLCar were inactivated within the same period. Similar results were obtained at neutral pH (Figure 5B): a greater resistance of SeSCar and SeLCar was also observed after lethal treatments at pH 7.0 compared to SeWT. For instance, whereas only 0.8 $\log_{10}$ cycles of SeSCar and 2.2 $\log_{10}$ cycles of SeLCar were inactivated after 30 min of treatment, 5.5 $\log_{10}$ cycles of reduction of SeWT were achieved within the same period. Comparing the evolved strains with one another, SeSCar displayed a greater survival rate than SeLCar at both pHs: after 30 min of treatment, SeLCar was inactivated 1.8 $\log_{10}$ cycles more than SeScar at acidic pH, and 1.4 $\log_{10}$ cycles more at neutral pH. A previous study by Chueca, Berdejo, Gomes-Neto, Pagán and García-Gonzalo [11] showed that evolution experiments on *E. coli* with subinhibitory doses of carvacrol resulted in strains that were even resistant to lethal treatments. A subsequent study with these strains revealed that combined treatments of carvacrol and heat were required to achieve comparable cell inactivation of mutant strains at low treatment intensities [40]. Strains of *S. aureus* likewise increased their resistance to lethal carvacrol treatments due to the improved bacterial repair systems in mutant strains isolated in evolution experiments at subinhibitory doses [15].

Similarly to the MIC, MBC and growth curve results discussed previously, the survival curves confirm the emergence of resistant strains of *S.* Typhimurium to carvacrol, not only after cyclic exposure to prolonged treatments at low doses, to which the bacteria can adapt, but also after cyclic exposure to short lethal treatments. However, contrary to what was expected considering the evolution protocols

we followed, SeSCar, obtained under the presence of a subinhibitory concentration of carvacrol, showed a greater survival rate than SeLCar under lethal treatments of carvacrol at both acid and neutral pH.

This is the first study that proves that the application of carvacrol, either under prolonged periods at low doses or with short repeated lethal treatments, allows the emergence of resistant strains. Previous researchers have also observed an increased resistance after a prolonged exposure to subinhibitory doses of carvacrol in other bacteria, but not after lethal treatments. In addition, the development of AMR in *Salmonella* spp. against natural antimicrobials had not been previously reported. These resistant mutants could grow at inhibitory doses or survive lethal carvacrol treatments, which would compromise food safety. In this regard, the emergence of resistant strains should be taken into account in the design of food preservation strategies to ensure consumer health.

3.4. Study of Antibiotic Susceptibility

As the last step in our phenotypic characterization, an antibiotic susceptibility test was conducted in order to ascertain whether any cross resistance with antibiotics could be detected. First, a preliminary control experiment was performed under the conditions shown in "Table 2A, zone diameter interpretative standards for *Enterobacteriaceae*"of CLSI [30] to assess the antibiotic resistance of SeWT (data not shown). The results demonstrated that antibiotic inhibition halos of SeWT were within the "intermediate range" according to CLSI [30], except against tetracycline and chloramphenicol, where the inhibition was higher ("susceptible"). Antibiotic concentration was subsequently increased to achieve larger halos (> 15.0 mm), and thus to increase analysis sensitivity (except for novobiocin and ampicillin, which were limited by their solubility).

Table 3 reports the inhibition halos of SeWT and of the evolved strains (Ø: 6.0 mm, included) against kanamycin, tetracycline, chloramphenicol, nalidixic acid, rifampicin, norfloxacin, novobiocin, trimethoprim, ampicillin, and cephalexin. No significant differences ($p \geq 0.05$) in antibiotic resistance were observed between SeWT and SeSCar by agar disk diffusion assay. However, it must be noted that Chueca, Renzoni, Berdejo, Pagan, Kelley and Garcia-Gonzalo [16] found an increased antibiotic resistance in mutant strains of *E. coli* evolved with subinhibitory doses of carvacrol, citral, and limonene oxide. In contrast, against all antibiotics tested except kanamycin and cephalexin, SeLCar exhibited an increased resistance compared to the SeWT strain ($p < 0.05$). In this regard, mutations in SeLCar are likely to trigger a general mechanism of bacterial response to antimicrobial compounds due to its broad spectrum of cross-resistance against antibiotics.

Table 3. Zones of growth inhibition for agar disk diffusion assays of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT) and evolved strains: SeSCar (by cyclic exposure to prolonged sublethal treatments of carvacrol) and SeLCar (by cyclic exposure to short lethal treatments of carvacrol) against antibiotics: 30 µg kanamycin sulfate, 30 µg tetracycline, 30 µg chloramphenicol, 400 µg nalidixic acid sodium, 50 µg rifampicin, 20 µg norfloxacin, 250 µg novobiocin sodium, 10 µg trimethoprim, 10 µg ampicillin, and 150 µg cephalexin.

Antibiotics		**Strains**		
Antibiotic	**Cell target**	**SeWT**	**SeSCar**	**SeLCar**
Kanamycin	Ribosome	15.20 ± 1.40	18.65 ± 0.86	16.14 ± 1.45
Tetracycline	Ribosome	25.80 ± 0.80	27.96 ± 1.28	21.54 ± 1.51 *
Chloramphenicol	Ribosome	26.10 ± 1.37	25.19 ± 1.68	18.57 ± 0.38 *
Nalidixic acid	DNA synthesis	30.08 ± 1.22	34.68 ± 2.20	22.70 ± 0.81 *
Rifampicin	RNA synthesis	17.59 ± 0.23	17.43 ± 1.06	14.84 ± 0.52 *
Norfloxacin	DNA synthesis	26.43 ± 1.03	28.31 ± 1.72	20.57 ± 0.59 *
Novobiocin	DNA synthesis	13.63 ± 0.40	14.01 ± 0.36	9.36 ± 0.27 *
Trimethoprim	Thymidine synthesis pathway	27.82 ± 1.10	29.60 ± 0.90	22.81 ± 0.78 *
Ampicillin	Cell wall	14.12 ± 0.17	14.00 ± 0.72	9.25 ± 0.58 *
Cephalexin	Cell wall	22.36 ± 0.40	23.76 ± 0.97	23.73 ± 0.66

Each value represents the mean diameter of the inhibition halo ± standard deviation (mm) from three independent experiments (Ø: 6.0 mm, included). * Significantly different from SeWT ($p \leq 0.05$).

These results demonstrate that emerging mutants can not only develop direct resistance against the IC applied in the evolution treatments (carvacrol in this case), but also cross-resistance to a wide range of antibiotics. Therefore, these results highlight the relevance of the genetic variations present in SeLCar for the development of AMR, which emerged through carvacrol evolution experiments but led to general antimicrobial resistance.

3.5. Detection of Genetic Variations in Evolved Strains

WGS was conducted on SeWT and on the evolved strains SeScar and SeLCar in order to find out which genetic variations were associated with increased resistance to carvacrol in SeSCar and SeLCar, and to antibiotics in SeLCar. A total of 3.65, 4.23 and 4.04 million of 150 bp-reads were obtained for SeWT, SeSCar and SeLCar, respectively. The average quality of the reads was 33.07, 33.05 and 33.01, and the percentage of reads above Q_{30} was 86.58 %, 86.99 % and 86.32 % for SeWT, SeSCar, and SeLCar, respectively. The quality-control-filtered paired-end reads were mapped at 98.12 %, 98.35 % and 97.94%, respectively, on the reference genome sequence (NCBI accession: NC_003197.2): *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 [23]. The reference genome was sufficiently covered to allow the detection of genetic variations among the strains studied; a 150-fold coverage depth was achieved for all three strains.

The genetic variations between the reference genome and SeWT were analyzed in order to discard those mutations as the cause of the increased resistance to carvacrol in the evolved strains. In this regard, a large deletion of 1179 bp was located from 4122,950 to 4124,130 bp, and several SNVs and InDels were identified (Table S4).

Although the sequences were mapped to the reference genome sequence, this study focused on the genetic variations between SeWT and the evolved strains (Figure 6). In this sense, knowledge of mutated genes and their relationship with the increased resistance in the evolved strains would allow us to find out the cell response mechanisms of *S.* Typhimurium against carvacrol. Genomic comparison of the strains revealed six SNVs and one insertion in SeSCar (Table 4), and five SNVs and one insertion in SeLCar (Table 5), with respect to SeWT.

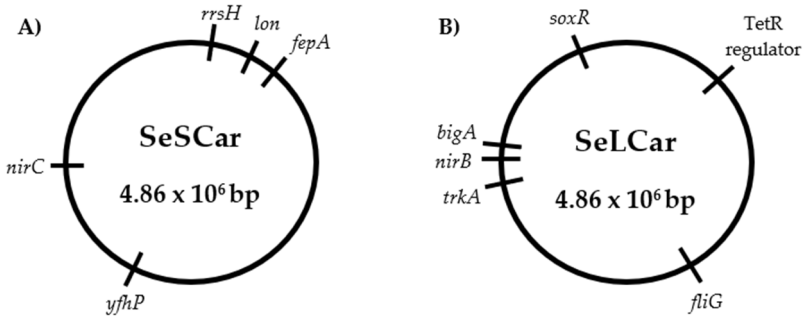


Figure 6. Genomic maps of the *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 evolved strains by cyclic exposure to prolonged sublethal treatments (SeSCar; (**A**)) and to short lethal treatments (SeLCar; (**B**)) of carvacrol.

Table 4. Mutations of SeSCar (strain evolved by cyclic exposure to prolonged sublethal treatments of carvacrol) in comparison with *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT), verified by Sanger sequencing. Single nucleotide variation (SNV), insertion (Ins) and deletion (Del).

Genome Position	Gene	Locus Tag	Mutation *	Change	Information
290,313	*rrsH*	STM0249	SNV: G1124A	No coding	RNA 16S ribosomal
290,319	*rrsH*	STM0249	SNV: C1130T	No coding	RNA 16S ribosomal
290,718	*rrsH*	STM0249	SNV: A1529C	No coding	RNA 16S ribosomal
506,753	*lon*	STM0450	SNV: G1211A	Gly404Asp	Protease
643,922	*fepA*	STM0585	Ins: + TTTGCA 107	No coding	Membrane receptor protein
2683,182	*yfhP*	STM2544	SNV: C245A	Ala82Glu	HTH IscR transcriptional regulator
3626,869	*nirC*	STM3476	SNV: T215C	Val72Ala	Membrane transport protein (Nitrite transport)

* Position with respect to the start of the coding region.

Table 5. Mutations of SeLCar (strain evolved by cyclic exposure to short lethal treatments of carvacrol) in comparison with *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT), verified by Sanger sequencing. Single nucleotide variation (SNV), insertion (Ins) and deletion (Del).

Genome Position	Gene	Locus Tag	Mutation *	Change	Information
638,192	-	638.192	Ins: + 17bp 522	Frame shift	TetR family transcriptional regulator
2058,821	*fliG*	STM1970	SNV: A611G	Asn204Ser	Flagellar protein
3581,011	*trkA*	STM3409	SNV: G1234A	Ala412Thr	Potassium transport regulating protein
3623,749	*nirB*	STM3474	SNV: T227C	Val76Ala	Large subunit nitrite reductase
3629,699	*bigA*	STM3478	SNV: C525T	Silent mutation (Ser175)	Putative surface-exposed virulence protein BigA
4504,453	*soxR*	STM4266	SNV: C58T	Arg20Cys	Redox sensitive transcriptional regulator SoxR

* Position with respect to the start of the coding region.

3.5.1. Identification of Genetic Variations in SeScar

As detailed in Table 4, the genetic variations that occurred in SeSCar via cyclic and prolonged exposure to subinhibitory doses of carvacrol were detected. In addition, the genes involved in the mutations, as well the coding proteins, were identified in order to understand the cause of the increased resistance observed.

Firstly, three SNVs were detected at positions 1121, 1130 and 1529 bp in the *rrsH* gene, a ribosomal RNA (rRNA) operon. The 16S rRNA ribosomal is the RNA component of the 30S small subunit of the ribosome and, consequently, is involved in protein synthesis. The 16S rRNA gene sequence has become the most widely used marker gene for profiling bacterial communities due to its hypervariability [41]; the mutations in SeSCar were indeed located at the variable regions 7 and 9 of the 16S rRNA. It is quite common to find mutations in this region of the genome, and they are probably not related to the increased resistance to carvacrol.

The insertion of six nucleotides observed in the *fepA* gene, which encodes an outer membrane receptor protein involved in the uptake of enterobactin (iron siderophore), was also discarded as a cause of the increased resistance against carvacrol, since this mutation was located between the ribosome binding site (RBS) and the promoter (−10 recognition region): thus, no codifying or transcriptional regions were altered.

Another SNV was detected at position 1211 bp in the *lon* gene, causing the substitution of the amino acid glycine (Gly) by aspartic acid (Asp). The *lon* gene encodes an ATP-dependent protease (Lon proteases) that regulates the selective degradation of dysfunctional proteins and short-lived regulatory proteins. Several studies have shown that Lon protease is a stress-induced protein essential to cellular homeostasis and cell survival; it mediates protein quality control and metabolic regulation [42]. Genetic variations in *lon* would lead to a change in the efficiency of maintaining cellular homeostasis and, consequently, to an increased resistance to carvacrol. In addition, mutations in *lon* have been associated with antibiotic resistance: not directly providing intrinsic resistance, but increasing genetic instability and enhancing genetic evolution towards it [43,44]. Perhaps the genetic variation in *lon* occurred in a previous step that was necessary for the rest of the mutations involved in the bacterial response against carvacrol to occur. In addition, according to Song [45], the mutation in *lon* could play an important role in the virulence of *S.* Typhimurium: adhesion to and invasion of epithelial cells, motility and replication in macrophages.

A similar hypothesis can help to explain the SNV present in the *nirC* gene: a missense mutation was located at 215 bp, resulting in a change from valine (Val) to alanine (Ala). This gene encodes the nitrite transporter NirC, an integral membrane protein which mediates the passage of the nitrite (NO_2^-) and nitrate (NO_3^-) anions across the cytoplasmic membrane [46]. The accumulation of nitrites inside the cells could be harmful to bacteria when reduced to nitric oxide (NO), since it causes genomic alterations by deamination of the DNA [47]. Therefore, based on the function of *nirC*, this mutation does not appear to be directly responsible for the increased resistance to carvacrol in SeSCar. Perhaps an increase in enzyme efficiency could reduce the oxidative damage to bacteria induced by carvacrol. However, it is likely that if the *nirC* mutation has produced a disruption in the regulation of nitrite and nitrate anions, an increase in mutagenesis is the result. In this respect, the probability of the emergence of resistant strains in the evolution treatments would have been increased by the *nirC* mutation.

Finally, a transversion from guanine to thymine was identified in the *yfhP* gene, resulting in a change in the predicted translation from alanine (Ala) to glutamic acid (Glu). This gene regulates the transcription of several operons and genes involved in the biogenesis of Fe-S clusters and Fe-S-containing proteins. Multiple Fe-S cluster assembly pathways are present in bacteria to carry out basal, stress-responsive, and enzyme-specific cluster assembly [48]. Previous transcriptional and proteogenomic studies on *S.* Typhimurium showed a high expression of *yfhP* to chlorine treatments [49] and to hydrogen peroxide [50], suggesting its important role in cellular responses to oxidative stress. According to Chueca, Pagán and García-Gonzalo [6], carvacrol promotes endogenous generation of reactive oxygen species (ROS) and, hence, *yfhP* is probably involved in one of the response pathways to the oxidative stress caused by carvacrol. In view of the extensive literature on *yfhP* and its putative role in cellular responses to oxidative agents, this mutation is probably the main cause of increased resistance to carvacrol observed in SeSCar. However, neither this mutation nor the others observed in this strain would be related to antibiotic resistance.

3.5.2. Identification of Genetic Variations in SeLcar

Genetic variations detected in the SeLcar strain, evolved under short lethal treatments of carvacrol, are summarized in Table 5. In addition, we analyzed protein coding and their functions to determine the origin of the strain's resistance to carvacrol.

Firstly, a SNV was detected at the position 1234 bp of the *trkA* gene, leading to a substitution of an alanine (Ala) by a threonine (Thr). This gene encodes the TrkA protein, an essential subunit of the transmembrane protein of potassium transport systems (K^+), which plays an important role in homeostasis, in cell turgor, and in adaptation to osmotic conditions. Moreover, K+ transporters are critical to the pathogenesis of *Salmonella* in mice and chicks and are involved in multiple virulence characteristics *in vitro*, including protein secretion, motility and invasion of epithelial cells [51]. However, according to Knöppel, et al. [52], this mutation probably occurs as a result of adaptation to the laboratory growth medium, and not as a mechanism of resistance to antimicrobials. A SNV

was detected at position 525 bp of the codifying region of the *bigA* gene, which results in a putative surface-exposed virulence protein BigA. Despite the close relationship observed between virulence factors and antibiotic resistance [53], this mutation was also discarded as a cause of the increased resistance, because it produced no change at the protein level (silent mutation).

In the *fliG* gene, a missense mutation was detected that produced the substitution of an asparagine (Asn) by the amino acid serine (Ser) in the position 204 aa of the FliG protein. The FliG protein forms the C-ring together with the FliN and FliM protein, a complex located at the base of the basal body of the flagellum. FliG is the most involved C-ring protein in the generation of the force necessary for flagellar mobility. Li, et al. [54] reported a down-regulation of *fliG* in *Aeromonas hydrophila* exposed to chlortetracycline. Perhaps a partial or total loss of function of *fliG* caused by the mutation could be associated with the increased resistance. However, to the best of our knowledge, no other studies have related *fliG* with AMR.

The *nirB* gene was also mutated in SeLCar at position 227 bp, resulting in the substitution of a valine (Val) by an alanine (Ala). This gene encodes the large subunit of the enzyme nitrite reductase NirBD, which transforms intracellular nitrite (NO_2^-) into ammonium cation (NH_4^+) and nitrogen (N_2), avoiding its transformation into nitric oxide (NO) and, consequently, DNA damage [47]. As discussed above, increased enzyme efficiency could perhaps reduce oxidative damage, but if the mutation caused the alteration of this enzyme, this could lead to an increase in intracellular nitric oxide (NO), thereby resulting in a high mutation rate which would lead, in turn, to the emergence of resistant strains.

A frame shift mutation was identified by an insertion of 17 bp in the STM0580 gene, which encodes a TetR family transcriptional regulator (TFR), probably leading to a loss of protein function. TFRs are widely associated with antibiotic resistance and the regulation of genes encoding small-molecule exporters. However, TFRs play a much broader role, controlling genes involved in the metabolism, antibiotic production, quorum sensing, and many other aspects of prokaryotic physiology [55]. Abouzeed et al. [56] reported that the inactivation of this regulator resulted in an increase in the expression of *ramA* and the AcrAB efflux pump, conferring an increased resistance, not only to tetracycline, but also to a wide range of antibiotics. In addition, an evolved strain of *E. coli* also presented a SNV in *acrR*, a TFR-encoding gene, and showed an increased resistance to carvacrol and antibiotics, but in this case, it was obtained under subinhibitory doses of limonene oxide [11]. Al-Mnaser [17] also observed a mutation in a gene related to antibiotic resistance, *marR*, in a resistant *E. coli* strain isolated by subinhibitory doses, but antibiotic susceptibility was not tested. In this regard, the STM0580 gene is probably related to the AMR previously observed, and also to antibiotics and perhaps to carvacrol; however, the lack of precise information regarding this gene in *S.* Typhimurium makes it difficult to know more about its implication in the resistance against carvacrol.

Finally, a transition from cytosine to thymine was observed at position 58 bp in the *soxR* gene. Consequently, the translation would be modified from arginine to cysteine at position 20 aa, specifically in the DNA-binding-domain of the SoxR protein. This gene codes the redox-sensitive transcriptional regulator SoxR, which regulates the expression of the regulon involved in defence against redox-cycling drugs [57] and in response to nitric oxide [58]. In the presence of compounds that generate oxidative stress, the 2Fe-2S group is oxidized and acquires the capability to activate the transcription of the *soxS* gene [59]. SoxS is also a transcription factor that activates the expression of more than 100 genes of the SoxRS regulon, providing cellular defense against oxidative stress [60]. The regulon SoxRS has been extensively studied and its function in the resistance to oxidizing agents and antibiotics extensively described; however, only few studies have pointed out its important role against ICs or EOs [16,61]. The main strategy of the SoxRS regulon is to minimize intracellular drug concentration through mechanisms that impede their entry, chemically modify them, or pump them out [57]. This cellular response is likely to be activated against carvacrol, which would explain the increase in resistance of SeLCar. A missense mutation of *soxR* (Asp137Tyr) was also identified in a strain of *E. coli* evolved in the presence of subinhibitory doses of carvacrol [16]. That strain, as well as SeLCar, showed an increased resistance not only to carvacrol, but also to a wide range of antibiotics [11,16].

Koutsolioutsou, et al. [62] also identified a mutation in the soxRS regulon, providing resistance against oxidant agents and multiple antibiotics. On the one hand, these results reveal that *soxR* is a key mechanism in the cellular response to carvacrol and to several antibiotics, and supports the assumption that genetic variations of this gene may occur during evolution experiments, allowing the emergence of resistant strains. On the other hand, these data suggest that oxidative stress is strongly involved in the *Salmonella* response to carvacrol as occurs in *E. coli* [6,63], leading to an excretion of carvacrol to avoid its increase on an intracellular level. A recent proteomic study in *Salmonella* also supports that oxidative stress could be related with the cell response to carvacrol and *Origanum vulgare* EO: a differential expression of superoxide dismutase, chaperones and molecular proteases, DNA-binding protein H-NS and other stress-related proteins associated with cellular biosynthesis processes, was observed [64]. Moreover, this mutation could affect the virulence of the strain since SoxS is a positive regulator of key pathogenesis genes and promotes intracellular replication and virulence of *S.* Typhimurium [65].

In summary, both mutations identified as the main cause of increased resistance, *yfhP* in SeSCar and *soxR* in SeLCar, imply that oxidative stress might be one of the main inducers of cellular response to carvacrol. Both genes are transcriptional regulators of oxidative stress-response, the relevance of which in the defense against oxidizing agents has been previously demonstrated, as well as against antibiotics, in the case of *soxR*. In this regard, the SNVs observed in *yfhP* and *soxR* would modify the regulation of cellular response to carvacrol, resulting in increased AMR. These results highlight the likely relevance of oxidative stress-response in the cell defense to carvacrol in *Salmonella.* In addition, all the genetic variations of both strains were located in the genome, not in mobile genetics elements, such as plasmids, transposons, etc., so they would be considered hereditable mutations, and the increased resistance would be considered stable.

As described by Mao, et al. [66], evolution assays exert a selective pressure on bacteria population, which facilitates the isolation of the most resistant mutants. Those strains that show a better growth fitness in the presence of the antimicrobial agent [28] or survive lethal treatments [20] will emerge above the rest of the bacterial population. However, those mutations that occur spontaneously during bacterial growth because of replication errors can be overselected [67]; moreover, several studies support the assumption that such mutations might be induced by the treatment, even as part of the cellular response to stress [68], such as the SOS system [69]. In addition, Jee, et al. [70] and Massey and Buckling [71] argue that increased mutations would occur in specific sites or regions as an adaptive response to environmental conditions. Jinks-Robertson and Bhagwat [72] and Hudson, et al. [73] explain that mutagenesis would tend to occur in the most transcriptionally active genes during cellular response to treatments. According to these studies, AMR might not only emerge randomly and spontaneously in the course of carvacrol treatments, but the latter would also induce specific mutations provoked by the stress that improves bacterial survival. This hypothesis would support the assumption that the mutations in SeSCar and SeLCar identified herein are related to key mechanisms in the bacterial response to oxidative stress activated by carvacrol. However, depending of the mutations that occurred during the evolution assays, the behaviour of the evolved strains was different. Comparing both evolved strains, the mutations identified in the SeSCar led to a greater increase in survival against lethal carvacrol treatments, while the genetic modifications detected in the SeLCar provided an improved fitness for growth in the presence of carvacrol, as well as an increased resistance to antibiotics. Unknown phenomena of epistasis may nevertheless also occur, thereby leading to increased resistance. In addition, regardless of whether certain mutations are induced by the treatment or not, the emergence of resistant strains would be more likely and, consequently, could pose a risk to food safety that remains unexplored.

4. Conclusions

By cyclic exposure to prolonged sublethal treatments as well as short lethal treatments, the carvacrol evolution experiments herein described enabled the selection of strains of *S. enterica* Typhimurium that were resistant against carvacrol: SeSCar (resulting from prolonged sublethal treatments), and

SeLCar (resulting from short lethal treatments). SeLCar also developed resistance to a wide range of antibiotics, such as tetracyclines, quinolones, aminoglycosides, and beta-lactams. The occurrence of stable resistance against carvacrol, which is a common constituent of many EOs recommended as food preservatives or disinfectant agents, could pose a risk to food safety. In this regard, further research is required in order to determine whether the emergence of resistant strains is dependent on the environmental conditions, the specific antimicrobial used, or it is a general phenomenon that should be considered in the design of food preservation strategies to ensure consumer health.

In this study, we adopted a novel approach to understand the antimicrobial action mechanisms of carvacrol. Whole genome sequencing (WGS) of SeSCar and SeLCar revealed the genetic variations responsible for those strains' increased resistance to carvacrol. Considering the mutated genes that are involved in cellular defense, *yfhP* in SeSCar and *soxR* in SeLCar, we conclude that carvacrol treatments probably induce an oxidative stress response in bacteria that activates resistance mechanisms in which homeostasis plays an essential role. Furthermore, based on the mutations found, the development of resistance may be linked to variations in the virulence of *S.* Typhimurium.

While we have presented a detailed analysis suggesting the genomic causes of the observed increased resistance based on previously available data, it is certainly possible that additional genes and pathways are involved and await discovery. Therefore, further research is required to completely understand the mode of action of carvacrol on bacteria in order to enhance its antimicrobial properties as a food preservative, or as a cleaning and disinfection agent.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/2076-2607/8/6/937/s1. Table S1: Primers used for PCR amplification and Sanger sequencing to verify the mutations in SeSCar and SeLCar. Table S2: Genomic background of *S.* Thypimurium LT2: a) antibiotic resistant genes and b) pathogenicity islands. Table S3: A (maximum OD_{595}), μ_m (maximum specific growth rate; h^{-1}) and λ (lag time; h) values and error standard of the modified Gompertz models of SeWT, SeSCar and SeLCar and the goodness of the fit: R^2 and adjusted R^2 values and the root mean square error (*RMSE*). Table S4: Genetic variations detected by whole genome sequencing (WGS) between SeWT and the reference genome of *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 (NCBI accession: NC_003197.2).

Author Contributions: Conceptualization, methodology and formal analysis, D.B., D.G.-G. and R.P.; investigation, D.B., N.M., and E.P.; resources, D.G.-G. and R.P.; data curation, D.B.; writing—original draft preparation, D.B., N.M., E.P., D.G.-G. and R.P.; writing—review and editing, D.B., D.G.-G. and R.P.; project administration and funding acquisition, D.G.-G. and R.P. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financially supported by Agencia Estatal de Investigación (Spain) (Project No. PGC2018-093789-B-I00), by the European Social Fund, and by the Aragonese Office of Science, Technology and University Research.

Acknowledgments: The Spanish Ministry of Sports, Culture and Education provided D. Berdejo and E. Pagán with a grant to carry out this investigation. The authors thank S. Hanks for his collaboration on the English-language revision of this manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Årdal, C.; Balasegaram, M.; Laxminarayan, R.; McAdams, D.; Outterson, K.; Rex, J.H.; Sumpradit, N. Antibiotic development - economic, regulatory and societal challenges. *Nat. Rev. Microbiol.* **2019**, *18*, 267–274. [CrossRef]
2. Peterson, E.; Kaur, P. Antibiotic Resistance Mechanisms in Bacteria: Relationships Between Resistance Determinants of Antibiotic Producers, Environmental Bacteria, and Clinical Pathogens. *Front. Microbiol.* **2018**, *9*. [CrossRef]
3. De Vincenzi, M.; Stammati, A.; De Vincenzi, A.; Silano, M. Constituents of aromatic plants: Carvacrol. *Fitoterapia* **2004**, *75*, 801–804. [CrossRef]
4. Merino, N.; Berdejo, D.; Bento, R.; Salman, H.; Lanz, M.; Maggi, F.; Sánchez-Gómez, S.; García-Gonzalo, D.; Pagán, R. Antimicrobial efficacy of *Thymbra capitata* (L.) Cav. essential oil loaded in self-assembled zein nanoparticles in combination with heat. *Ind. Crop. Prod.* **2019**, *133*, 98–104. [CrossRef]

5. Food and Drug Administration (FDA). Hazard analysis and critical control point (HACCP). In *Procedures for the Safe and Sanitary Processing and Importing of Juice*; Food and Drug Administration: Silver Spring, MD, USA, 2001; Volume 63, pp. 20450–20486.
6. Chueca, B.; Pagán, R.; García-Gonzalo, D. Oxygenated monoterpenes citral and carvacrol cause oxidative damage in *Escherichia coli* without the involvement of tricarboxylic acid cycle and Fenton reaction. *Int. J. Food Microbiol.* **2014**, *189*, 126–131. [CrossRef] [PubMed]
7. Ait-Ouazzou, A.; Espina, L.; Gelaw, T.K.; de Lamo-Castellvi, S.; Pagan, R.; Garcia-Gonzalo, D. New insights in mechanisms of bacterial inactivation by carvacrol. *J. Appl. Microbiol.* **2013**, *114*, 173–185. [CrossRef] [PubMed]
8. Mattson, T.E.; Johny, A.K.; Amalaradjou, M.A.; More, K.; Schreiber, D.T.; Patel, J.; Venkitanarayanan, K. Inactivation of *Salmonella* spp. on tomatoes by plant molecules. *Int. J. Food Microbiol.* **2011**, *144*, 464–468. [CrossRef] [PubMed]
9. Espina, L.; Berdejo, D.; Alfonso, P.; García-Gonzalo, D.; Pagán, R. Potential use of carvacrol and citral to inactivate biofilm cells and eliminate biofouling. *Food Control* **2017**, *82*, 256–265. [CrossRef]
10. Hashemi, S.M.B.; Khorram, S.B.; Sohrabi, M. Antioxidant Activity of Essential Oils in Foods. In *Essential Oils in Food Processing*; Hashemi, S.M.B., Khaneghah, A.M., Sant'Ana, A.S., Eds.; John Wiley & Sons Ltd.: Hoboken, NJ, USA, 2017; pp. 247–265.
11. Chueca, B.; Berdejo, D.; Gomes-Neto, N.J.; Pagán, R.; García-Gonzalo, D. Emergence of Hyper-Resistant *Escherichia coli* MG1655 Derivative Strains after Applying Sub-Inhibitory Doses of Individual Constituents of Essential Oils. *Front. Microbiol.* **2016**, *7*, 273. [CrossRef]
12. Hammer, K.A.; Carson, C.F.; Riley, T.V. Frequencies of resistance to *Melaleuca alternifolia* (tea tree) oil and rifampicin in *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis*. *Int. J. Antimicrob. Agents* **2008**, *32*, 170–173. [CrossRef]
13. Leite de Souza, E. The effects of sublethal doses of essential oils and their constituents on antimicrobial susceptibility and antibiotic resistance among food-related bacteria: A review. *Trends Food Sci. Technol.* **2016**, *56*, 1–12. [CrossRef]
14. Luz Ida, S.; Gomes Neto, N.J.; Tavares, A.G.; Nunes, P.C.; Magnani, M.; de Souza, E.L. Evidence for lack of acquisition of tolerance in *Salmonella enterica* serovar Typhimurium ATCC 14028 after exposure to subinhibitory amounts of *Origanum vulgare* L. essential oil and carvacrol. *Appl. Environ. Microbiol.* **2012**, *78*, 5021–5024. [CrossRef] [PubMed]
15. Berdejo, D.; Chueca, B.; Pagan, E.; Renzoni, A.; Kelley, W.L.; Pagan, R.; Garcia-Gonzalo, D. Sub-Inhibitory Doses of Individual Constituents of Essential Oils Can Select for *Staphylococcus aureus* Resistant Mutants. *Molecules* **2019**, *24*, 170. [CrossRef] [PubMed]
16. Chueca, B.; Renzoni, A.; Berdejo, D.; Pagan, R.; Kelley, W.L.; Garcia-Gonzalo, D. Whole-Genome Sequencing and Genetic Analysis Reveal Novel Stress Responses to Individual Constituents of Essential Oils in *Escherichia coli*. *Appl. Environ. Microbiol.* **2018**, *84*. [CrossRef] [PubMed]
17. Al-Mnaser, A.A.; Woodward, M.J. Sub-lethal Concentrations of Phytochemicals (Carvacrol and Oregano) Select for Reduced Susceptibility Mutants of *Escherichia coli* O23:H52. *Pol. J. Microbiol.* **2020**, *69*, 121. [CrossRef]
18. Gayán, E.; Cambré, A.; Michiels, C.W.; Aertsen, A. Stress-Induced Evolution of Heat Resistance and Resuscitation Speed in *Escherichia coli* O157:H7 ATCC 43888. *Appl. Environ. Microbiol.* **2016**, *82*, 6656–6663. [CrossRef]
19. Vanlint, D.; Mitchell, R.; Bailey, E.; Meersman, F.; McMillan, P.F.; Michiels, C.W.; Aertsen, A. Rapid Acquisition of Gigapascal-High-Pressure Resistance by *Escherichia coli*. *mBio* **2011**, *2*, e00130-10. [CrossRef]
20. Levin-Reisman, I.; Ronin, I.; Gefen, O.; Braniss, I.; Shoresh, N.; Balaban, N.Q. Antibiotic tolerance facilitates the evolution of resistance. *Science* **2017**, *355*, 826–830. [CrossRef]
21. Chung, D.; Cho, T.J.; Rhee, M.S. Citrus fruit extracts with carvacrol and thymol eliminated 7-log acid-adapted *Escherichia coli* 0157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes*: A potential of effective natural antibacterial agents. *Food Res. Int.* **2018**, *107*, 578–588. [CrossRef]
22. Ait-Ouazzou, A.; Cherrat, L.; Espina, L.; Lorán, S.; Rota, C.; Pagán, R. The antimicrobial activity of hydrophobic essential oil constituents acting alone or in combined processes of food preservation. *Innov. Food Sci. Emerg. Technol.* **2011**, *12*, 320–329. [CrossRef]

23. McClelland, M.; Sanderson, K.E.; Spieth, J.; Clifton, S.W.; Latreille, P.; Courtney, L.; Porwollik, S.; Ali, J.; Dante, M.; Du, F.; et al. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* **2001**, *413*, 852–856. [CrossRef] [PubMed]
24. Kohanski, M.A.; DePristo, M.A.; Collins, J.J. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Mol. Cell* **2010**, *37*, 311–320. [CrossRef] [PubMed]
25. Friedman, M.; Henika, P.R.; Mandrell, R.E. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J. Food. Prot.* **2002**, *65*, 1545–1560. [CrossRef] [PubMed]
26. Clinical and Laboratory Standards Institute (CLSI). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 10th ed.; CLSI document M07-A10; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2015.
27. Lallemand, E.A.; Lacroix, M.Z.; Toutain, P.-L.; Boullier, S.; Ferran, A.A.; Bousquet-Melou, A. In vitro Degradation of Antimicrobials during Use of Broth Microdilution Method Can Increase the Measured Minimal Inhibitory and Minimal Bactericidal Concentrations. *Front. Microbiol.* **2016**, *7*, 2051. [CrossRef]
28. Andersson, D.I.; Hughes, D. Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol.* **2014**, *12*, 465–478. [CrossRef] [PubMed]
29. Zwietering, M.H.; Jongenburger, I.; Rombouts, F.M.; van't Riet, K. Modeling of the bacterial growth curve. *Appl. Environ. Microbiol.* **1990**, *56*, 1875–1881. [CrossRef]
30. Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Susceptibility Testing*; CLSI Document M100-S24; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2014.
31. Li, H.; Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **2010**, *26*, 589–595. [CrossRef]
32. Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N.; Marth, G.; Abecasis, G.; Durbin, R. The sequence alignment/map format and SAMtools. *Bioinformatics* **2009**, *25*, 2078–2079. [CrossRef]
33. Cingolani, P.; Platts, A.; Wang, L.L.; Coon, M.; Nguyen, T.; Wang, L.; Land, S.J.; Lu, X.; Ruden, D.M. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. *Fly* **2012**, *6*, 80–92. [CrossRef]
34. Mith, H.; Dure, R.; Delcenserie, V.; Zhiri, A.; Daube, G.; Clinquart, A. Antimicrobial activities of commercial essential oils and their components against food-borne pathogens and food spoilage bacteria. *Food. Sci. Nutr.* **2014**, *2*, 403–416. [CrossRef]
35. Lu, Y.; Wu, C. Reduction of *Salmonella enterica* contamination on grape tomatoes by washing with thyme oil, thymol, and carvacrol as compared with chlorine treatment. *J. Food Prot.* **2010**, *73*, 2270–2275. [CrossRef] [PubMed]
36. Gomes-Neto, N.J.; Luz, I.S.; Franco, O.L.; Magnani, M.; Souza, E.L. Tolerance evaluation in *Salmonella enterica* serovar Typhimurium challenged with sublethal amounts of *Rosmarinus officinalis* L. essential oil or 1,8-cineole in meat model. *Int. J. Food Sci. Tech.* **2014**, *49*, 1912–1917. [CrossRef]
37. Braschi, G.; Serrazanetti, D.I.; Siroli, L.; Patrignani, F.; De Angelis, M.; Lanciotti, R. Gene expression responses of *Listeria monocytogenes* Scott A exposed to sub-lethal concentrations of natural antimicrobials. *Int. J. Food Microbiol.* **2018**, *286*, 170–178. [CrossRef] [PubMed]
38. Melo, R.S.; Azevedo, A.M.A.; Pereira, A.M.G.; Rocha, R.R.; Cavalcante, R.M.B.; Matos, M.N.C.; Lopes, P.H.R.; Gomes, G.A.; Rodrigues, T.H.S.; dos Santos, H.S.; et al. Chemical Composition and Antimicrobial Effectiveness of *Ocimum gratissimum* L. Essential Oil Against Multidrug-Resistant Isolates of *Staphylococcus aureus* and *Escherichia coli*. *Molecules* **2019**, *24*, 3864. [CrossRef]
39. Burt, S. Essential oils: Their antibacterial properties and potential applications in foods—a review. *Int. J. Food Microbiol.* **2004**, *94*, 223–253. [CrossRef]
40. Gayan, E.; Geens, E.; Berdejo, D.; Garcia-Gonzalo, D.; Pagan, R.; Aertsen, A.; Michiels, C.W. Combination of mild heat and plant essential oil constituents to inactivate resistant variants of *Escherichia coli* in buffer and in coconut water. *Food Microbiol.* **2020**, *87*, 103388. [CrossRef]
41. Yang, B.; Wang, Y.; Qian, P.-Y. Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. *BMC Bioinform.* **2016**, *17*, 135. [CrossRef]
42. Lee, I.; Suzuki, C.K. Functional mechanics of the ATP-dependent Lon protease- lessons from endogenous protein and synthetic peptide substrates. *Biochim. Biophys. Acta: Proteins Proteom.* **2008**, *1784*, 727–735. [CrossRef]

43. Nicoloff, H.; Andersson, D.I. Lon protease inactivation, or translocation of the *lon* gene, potentiate bacterial evolution to antibiotic resistance. *Mol. Microbiol.* **2013**, *90*, 1233–1248. [CrossRef]
44. Nicoloff, H.; Perreten, V.; Levy, S.B. Increased genome instability in *Escherichia coli lon* mutants: Relation to emergence of multiple-antibiotic-resistant (Mar) mutants caused by insertion sequence elements and large tandem genomic amplifications. *Antimicrob. Agents Chemother.* **2007**, *51*, 1293–1303. [CrossRef]
45. Song, X.; Zhang, H.; Ma, S.; Song, Y.; Lv, R.; Liu, X.; Yang, B.; Huang, D.; Liu, B.; Jiang, L. Transcriptome analysis of virulence gene regulation by the ATP-dependent Lon protease in *Salmonella* Typhimurium. *Future Microbiol.* **2019**, *14*, 1109–1122. [CrossRef] [PubMed]
46. Rycovska-Blume, A.; Lu, W.; Andrade, S.; Fendler, K.; Einsle, O. Structural and Functional Studies of NirC from *Salmonella* Typhimurium. *Methods Enzymol.* **2015**, *556*, 475–497. [CrossRef]
47. Wink, D.A.; Kasprzak, K.S.; Maragos, C.M.; Elespuru, R.K.; Misra, M.; Dunams, T.M.; Cebula, T.A.; Koch, W.H.; Andrews, A.W.; Allen, J.S.; et al. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* **1991**, *254*, 1001–1003. [CrossRef]
48. Ayala-Castro, C.; Saini, A.; Outten, F.W. Fe-S cluster assembly pathways in bacteria. *Microbiol. Mol. Biol. Rev.* **2008**, *72*, 110–125. [CrossRef] [PubMed]
49. Wang, S.; Phillippy, A.M.; Deng, K.; Rui, X.; Li, Z.; Tortorello, M.L.; Zhang, W. Transcriptomic responses of *Salmonella enterica* serovars Enteritidis and Typhimurium to chlorine-based oxidative stress. *Appl. Environ. Microbiol.* **2010**, *76*, 5013–5024. [CrossRef]
50. Karash, S.; Liyanage, R.; Qassab, A.; Lay, J.O.; Kwon, Y.M. A Comprehensive Assessment of the Genetic Determinants in *Salmonella* Typhimurium for Resistance to Hydrogen Peroxide Using Proteogenomics. *Sci. Rep.* **2017**, *7*, 17073. [CrossRef] [PubMed]
51. Liu, Y.; Kim Ho, K.; Su, J.; Gong, H.; Chang, A.C.; Lu, S. Potassium transport of *Salmonella* is important for type III secretion and pathogenesis. *Microbiology* **2013**, *159 Pt 8*, 1705–1719. [CrossRef] [PubMed]
52. Knöppel, A.; Knopp, M.; Albrecht, L.M.; Lundin, E.; Lustig, U.; Näsvall, J.; Andersson, D.I. Genetic Adaptation to Growth Under Laboratory Conditions in *Escherichia coli* and *Salmonella enterica*. *Front. Microbiol.* **2018**, *9*, 756. [CrossRef]
53. Schroeder, M.; Brooks, B.D.; Brooks, A.E. The Complex Relationship between Virulence and Antibiotic Resistance. *Genes* **2017**, *8*, 39. [CrossRef]
54. Li, W.X.; Ali, F.; Cai, Q.L.; Yao, Z.J.; Sun, L.N.; Lin, W.X.; Lin, X.M. Reprint of: Quantitative proteomic analysis reveals that chemotaxis is involved in chlortetracycline resistance of *Aeromonas hydrophila*. *J. Proteom.* **2018**, *180*, 138–146. [CrossRef]
55. Cuthbertson, L.; Nodwell, J.R. The TetR family of regulators. *Microbiol. Mol. Biol. Rev.* **2013**, *77*, 440–475. [CrossRef] [PubMed]
56. Abouzeed, Y.M.; Baucheron, S.; Cloeckaert, A. *ramR* Mutations Involved in Efflux-Mediated Multidrug Resistance in *Salmonella enterica* Serovar Typhimurium. *Antimicrob. Agents Chemother.* **2008**, *52*, 2428–2434. [CrossRef] [PubMed]
57. Gu, M.; Imlay, J.A. The SoxRS response of *Escherichia coli* is directly activated by redox-cycling drugs rather than by superoxide. *Mol. Microbiol.* **2011**, *79*, 1136–1150. [CrossRef] [PubMed]
58. Demple, B.; Ding, H.; Jorgensen, M. Escherichia coli SoxR protein: Sensor/transducer of oxidative stress and nitric oxide. In *Methods Enzymol*; Sies, H., Packer, L., Eds.; Academic Press: New York, NY, USA, 2002; Volume 348, pp. 355–364.
59. Ding, H.; Hidalgo, E.; Demple, B. The redox state of the [2Fe-2S] clusters in SoxR protein regulates its activity as a transcription factor. *J. Biol. Chem.* **1996**, *271*, 33173–33175. [CrossRef] [PubMed]
60. Pomposiello, P.J.; Bennik, M.H.; Demple, B. Genome-wide transcriptional profiling of the *Escherichia coli* responses to superoxide stress and sodium salicylate. *J. Bacteriol.* **2001**, *183*, 3890–3902. [CrossRef] [PubMed]
61. Sheng, L.N.; Rasco, B.; Zhu, M.J. Cinnamon Oil Inhibits Shiga Toxin Type 2 Phage Induction and Shiga Toxin Type 2 Production in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* **2016**, *82*, 6531–6540. [CrossRef]
62. Koutsolioutsou, A.; Martins, E.A.; White, D.G.; Levy, S.B.; Demple, B. A soxRS-constitutive mutation contributing to antibiotic resistance in a clinical isolate of *Salmonella enterica* (Serovar typhimurium). *Antimicrob. Agents Chemother.* **2001**, *45*, 38–43. [CrossRef]
63. Yuan, W.; Jing Seng, Z.; Singh Kohli, G.; Yang, L.; Yuk, H.G. Stress Resistance Development and Genome-Wide Transcriptional Response of Escherichia coli O157:H7 Adapted to Sublethal Thymol, Carvacrol, and trans-Cinnamaldehyde. *Appl. Environ. Microbiol.* **2018**, *84*, e01616-18. [CrossRef]

64. Barbosa, L.N.; Alves, F.C.B.; Andrade, B.F.M.T.; Albano, M.; Rall, V.L.M.; Fernandes, A.A.H.; Buzalaf, M.A.R.; Leite, A.d.L.; de Pontes, L.G.; dos Santos, L.D.; et al. Proteomic analysis and antibacterial resistance mechanisms of *Salmonella* Enteritidis submitted to the inhibitory effect of *Origanum vulgare* essential oil, thymol and carvacrol. *J. Proteom.* **2020**, *214*, 103625. [CrossRef]
65. Wang, P.; Zhang, H.; Liu, Y.; Lv, R.; Liu, X.; Song, X.; Wang, J.; Jiang, L. SoxS is a positive regulator of key pathogenesis genes and promotes intracellular replication and virulence of *Salmonella* Typhimurium. *Microb. Pathog.* **2020**, *139*, 103925. [CrossRef]
66. Mao, E.F.; Lane, L.; Lee, J.; Miller, J.H. Proliferation of mutators in a cell population. *J. Bacteriol.* **1997**, *179*, 417–422. [CrossRef]
67. Drake, J.W. A constant rate of spontaneous mutation in DNA-based microbes. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 7160–7164. [CrossRef] [PubMed]
68. Foster, P.L. Stress-induced mutagenesis in bacteria. *Crit. Rev. Biochem. Mol. Biol.* **2007**, *42*, 373–397. [CrossRef] [PubMed]
69. Schlacher, K.; Goodman, M.F. Lessons from 50 years of SOS DNA-damage-induced mutagenesis. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 587–594. [CrossRef] [PubMed]
70. Jee, J.; Rasouly, A.; Shamovsky, I.; Akivis, Y.; Steinman, S.R.; Mishra, B.; Nudler, E. Rates and mechanisms of bacterial mutagenesis from maximum-depth sequencing. *Nature* **2016**, *534*, 693–696. [CrossRef]
71. Massey, R.C.; Buckling, A. Environmental regulation of mutation rates at specific sites. *Trends Microbiol.* **2002**, *10*, 580–584. [CrossRef]
72. Jinks-Robertson, S.; Bhagwat, A.S. Transcription-associated mutagenesis. *Annu. Rev. Genet.* **2014**, *48*, 341–359. [CrossRef]
73. Hudson, R.E.; Bergthorsson, U.; Ochman, H. Transcription increases multiple spontaneous point mutations in *Salmonella enterica*. *Nucleic Acids Res.* **2003**, *31*, 4517–4522. [CrossRef]

Supplemental material

Table S1. Primers used for PCR amplification and Sanger sequencing to verify the mutations of evolved strains: SeSCar (by cyclic exposure to prolonged sublethal treatments of carvacrol) and SeLCar (by cyclic exposure to short lethal treatments of carvacrol),

SeSCar mutations	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
rrsH	AAGAAGCACCGGCTAACTCC	CCTTTGATTCGTTTCCGGGC
lon	GCGCGTAGCAAGGTCAAAAA	ACCGTCAGTTCGCCTTTCTT
fepA	GGCATGGTGCGGATGATTTC	CGCCAGTGATGTAGACCCAG
yfhP	TTCATCCTTCAGACGACCGC	GTCAATATCGCACGGAACG
nirC	CAGCCAACAAAGAGGCAGTG	ATAAACGCCAGCAGACACCA

SeLCar mutations	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
TetR regulator	ATCGTTGAGTAGCGAGACGG	TCGATCCATTACCGATGCCA
fliG	CAACGCCAACGAATACCTGC	GACATAGGTATCCTCGCCGC
trkA	GCGCCAAGAAAGTGATGGTG	ACCCACTGCCAAATCCACAA
nirB	AAAAGCCTGACAATTCCGCC	CGCCCATGCTTTCGATCTTG
bigA	CGCAAAACTCTCACCGACCT	GTTCAGCGTTTTACCGTCCG
soxR	CTCGGTCGTTCGTAGCTCAA	GTCTAACTCTTCGCGCCACT

Table S2. A (maximum OD_{595}), μ_m (maximum specific growth rate; h^{-1}) and λ (lag time; h) values and error standard of the modified Gompertz model obtained from 3 independently growth curves of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT) (A) and evolved strains: SeSCar (B; by cyclic exposure to prolonged sublethal treatments of carvacrol) and SeLCar (C; by cyclic exposure to short lethal treatments of carvacrol), at different concentrations of carvacrol. The goodness of the fit is shown by R^2 and adjusted R^2 values and the root mean square error (*RMSE*).

A) SeWT

Carvacrol	Values			Standard error			Goodness of fit		
(μL/L)	*A*	μ_{max}	λ	*A*	μ_{max}	λ	R^2	Adj. R^2	*RMSE*
0	0.7174	0.4642	3.576	0.0163	0.1491	0.248	0.9348	0.9285	0.0663
0	0.7565	0.4687	3.574	0.0119	0.1005	0.175	0.9680	0.9650	0.0483
0	0.7145	0.5069	3.755	0.0150	0.1361	0.192	0.9454	0.9402	0.0613
50	0.7117	0.3141	3.503	0.0239	0.1197	0.467	0.8756	0.8637	0.0940
50	0.7080	0.3523	3.660	0.0177	0.1014	0.313	0.9298	0.9231	0.0703
50	0.6070	0.3090	3.770	0.0098	0.0570	0.200	0.9701	0.9672	0.0389
100	0.6665	0.2116	5.279	0.0124	0.0348	0.282	0.9753	0.9730	0.0438
100	0.6502	0.1912	5.182	0.0133	0.0327	0.317	0.9697	0.9668	0.0463
100	0.5708	0.2344	5.105	0.0152	0.0663	0.379	0.9397	0.9340	0.0564
150	0.6429	0.0236	12.080	0.0305	0.0022	0.785	0.9719	0.9695	0.0309
150	0.6660	0.0242	12.610	0.0196	0.0014	0.473	0.9894	0.9885	0.0192
150	0.6431	0.0253	13.740	0.0314	0.0026	0.787	0.9680	0.9653	0.0321

B) SeSCar

Carvacrol	Values			Standard error			Godness of fit		
(μL/L)	*A*	μ_{max}	λ	*A*	μ_{max}	λ	R^2	Adj. R^2	*RMSE*
0	0.7584	0.4321	3.826	0.0092	0.0616	0.140	0.9832	0.9816	0.0368
0	0.6869	0.4195	3.873	0.0304	0.2237	0.499	0.8053	0.7868	0.1228
0	0.7559	0.3776	3.720	0.0171	0.0973	0.282	0.9403	0.9346	0.0678
50	0.6893	0.3474	3.866	0.0196	0.1144	0.366	0.9154	0.9073	0.0775
50	0.7099	0.3282	3.640	0.0222	0.1154	0.413	0.8954	0.8854	0.0876
50	0.6906	0.3787	3.691	0.0098	0.0633	0.164	0.9749	0.9725	0.0392
100	0.7091	0.2958	3.653	0.0062	0.0278	0.123	0.9911	0.9902	0.0242
100	0.7235	0.3016	3.659	0.0198	0.0883	0.383	0.9209	0.9134	0.0769
100	0.7601	0.3072	3.931	0.0170	0.0729	0.324	0.9486	0.9437	0.0653
150	0.5753	0.2336	6.227	0.0105	0.0434	0.250	0.9780	0.9759	0.0373
150	0.6400	0.2927	4.665	0.0134	0.0664	0.270	0.9599	0.9561	0.0512
150	0.6998	0.2590	4.736	0.0083	0.0300	0.171	0.9877	0.9865	0.0307
200	0.5247	0.0179	6.560	0.0549	0.0021	1.254	0.9132	0.9053	0.0386
200	0.4917	0.0142	8.567	0.0393	0.0011	0.822	0.9600	0.9564	0.0204
200	0.5439	0.0203	10.370	0.0293	0.0015	0.638	0.9752	0.9729	0.0204
250	0.4508	0.0190	13.860	0.0329	0.0024	0.862	0.9501	0.9456	0.0230
250	0.6351	0.0139	11.970	0.0768	0.0008	0.681	0.9712	0.9686	0.0165
250	0.4577	0.0179	10.930	0.0242	0.0013	0.631	0.9746	0.9723	0.0173

C) SeLCar

Carvacrol (μL/L)	Values			Standard error			Godness of fit		
	A	μ_{max}	λ	*A*	μ_{max}	λ	R^2	Adj. R^2	*RMSE*
0	0.6850	0.4468	3.634	0.0094	0.0826	0.142	0.9754	0.9731	0.0384
0	0.7017	0.4447	3.758	0.0103	0.0789	0.149	0.9736	0.9711	0.0416
0	0.7078	0.4684	3.750	0.0105	0.0861	0.145	0.9723	0.9696	0.0427
50	0.6647	0.4112	3.612	0.0161	0.1312	0.262	0.9284	0.9216	0.0654
50	0.6495	0.3180	3.668	0.0038	0.0213	0.074	0.9958	0.9954	0.0151
50	0.6570	0.3720	3.746	0.0120	0.0800	0.206	0.9598	0.9560	0.0481
100	0.6404	0.2889	3.612	0.0156	0.0788	0.328	0.9324	0.9259	0.0615
100	0.7275	0.2374	3.527	0.0064	0.0197	0.139	0.9919	0.9911	0.0241
100	0.7105	0.2528	3.634	0.0096	0.0338	0.205	0.9801	0.9782	0.0366
150	0.4898	0.0718	6.324	0.0188	0.0129	0.640	0.9462	0.9413	0.0485
150	0.5975	0.0789	6.054	0.0202	0.0114	0.561	0.9603	0.9567	0.0490
150	0.5034	0.1147	5.776	0.0104	0.0167	0.344	0.9746	0.9723	0.0342
200	0.6030	0.0198	5.405	0.0279	0.0010	0.558	0.9810	0.9793	0.0193
200	0.5818	0.0249	6.972	0.0180	0.0009	0.368	0.9908	0.9900	0.0152
200	0.6212	0.0254	7.036	0.0220	0.0011	0.425	0.9875	0.9863	0.0180
250	0.4497	0.0266	6.115	0.0297	0.0026	0.769	0.9573	0.9535	0.0320
250	0.6529	0.0140	9.497	0.0731	0.0009	0.725	0.9666	0.9636	0.0191
250	0.5375	0.0240	6.870	0.0262	0.0014	0.577	0.9769	0.9748	0.0229
300	0.4733	0.0188	7.425	0.0401	0.0019	1.020	0.9382	0.9326	0.0318
300	0.5541	0.0143	7.466	0.0435	0.0010	0.752	0.9669	0.9639	0.0197
300	0.5077	0.0169	7.158	0.0329	0.0012	0.765	0.9651	0.9619	0.0221
350	0.4467	0.0175	12.860	0.0360	0.0022	0.931	0.9463	0.9415	0.0240
350	0.5054	0.0136	12.840	0.0665	0.0014	0.875	0.9414	0.9361	0.0217
350	0.5336	0.0114	12.260	0.1156	0.0011	1.201	0.9227	0.9157	0.0228

Table S3. Genetic variations detected by whole genome sequencing (WGS) between SeWT and the reference genome of *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 (NCBI accession: NC_003197.2). Single nucleotide variation (SNV), insertion (Ins) and deletion (Del).

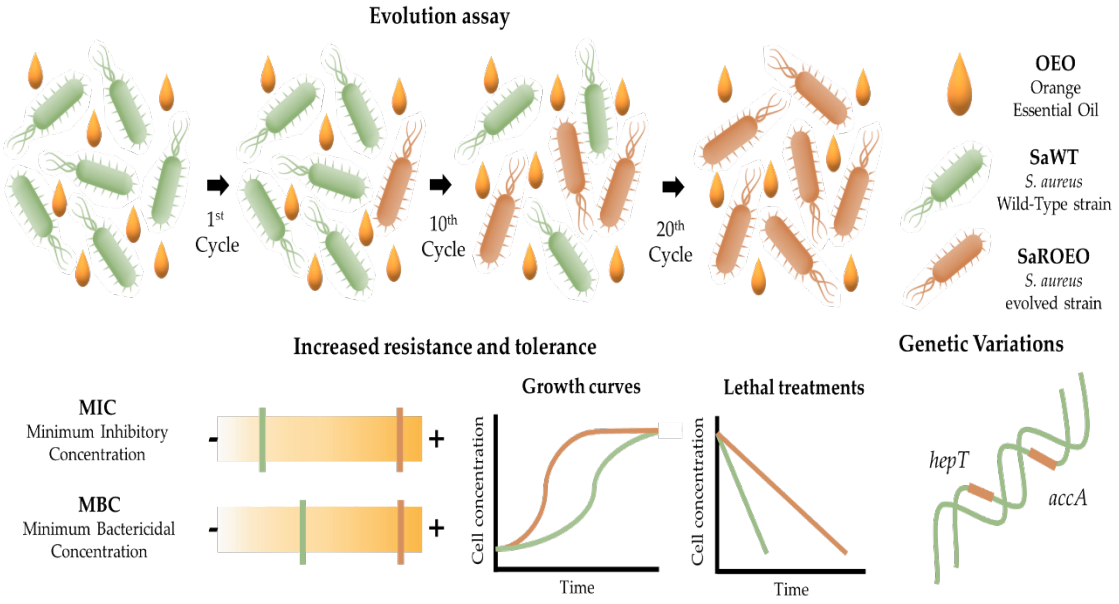
Genome position	Genes	Locus tag	Mutation*	Change	Information
290,718	*rrsH*	STM0249	SNV: C1529A	No coding	RNA 16S ribosomal
364,623	*crl*	STM0319	Del: -T 104	Frame shift	Sigma factor-binding protein
416,555	*prpR*	STM0367	SNV: C1159T	Leu387Phe	Operon regulator
453,939	*brnQ*	STM0399	SNV: C681T	Silent mutation (Tyr227)	Branched-chain amino acid transport system carrier protein
509,118	*cypD*	STM0452	SNV: T450A	Asp150Glu	Peptidylprolyl isomerase
608,859	*fimH*	STM0547	SNV: G182C	Gly61Ala	Adhesin
1,205,933	Intergenic *wraB - ycdF*	STM1119 STM1120	SNV: G →A	No coding	-
1,778,104	*ycjF*	STM1684	SNV: T821C	Leu274Pro	UPF0283 membrane protein
1,841,398	-	STM1747	SNV: G98A	Arg33Gln	Hypothetical protein
1,849,642	*hnr*	STM1753	SNV: T305G	Val102Gly	Regulator of RpoS
3,469,143	*dacB*	STM3300	SNV: C483T	Silent mutation (Ser161)	Transpeptidase
3,673,628	*malQ*	STM3513	SNV: T287G	Leu96Arg	4-Alpha-glucanotransferase
3,675,952	*malP*	STM3514	Del: -GCCGCCTG 358	Frame shift	Alpha-1,4 phosphorylase
3,819,815	-	STM3633	SNV: T562C	Silent mutation (Leu188)	LacI family transcriptional regulator
4,122,937	*gppA*	STM3913	SNV: G385T	Gly129Cys	Pyrophosphatase
4,122,950	*gppA* *rhlB*	STM3913 STM3914	Del: -1,179 pb	Knock-out (*gppA*, *rhlB*)	Pyrophosphatase ATP-dependent RNA helicase RhlB
4,291,432	*yiiQ*	STM4082	SNV: G323A	Stop-gain	Hypothetical protein
4,294,693	*glpK*	STM4086	SNV: G1171A	Asp391Asn	Glycerol kinase
4,697,694	*treB*	STM4454	Ins: + A 543	Frame shift	Pseudogene (trehalose metabolism)

*Position respect to the start of the coding region.

Manuscrito V. Incubation with a complex orange essential oil leads to evolved mutants with increased resistance and tolerance

Berdejo, D., Pagán, E., Merino, N., Pagán, R., García-Gonzalo, D. (2020)

Pharmaceuticals, 13, 239. doi:10.3390/ph13090239.



pharmaceuticals

MDPI

Article

Incubation with a Complex Orange Essential Oil Leads to Evolved Mutants with Increased Resistance and Tolerance

Daniel Berdejo, Elisa Pagán, Natalia Merino, Rafael Pagán and Diego García-Gonzalo *

Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), C/Miguel Servet, 177, 50013 Zaragoza, Spain; berdejo@unizar.es (D.B.); epagan@unizar.es (E.P.); merino@unizar.es (N.M.); pagan@unizar.es (R.P.)

* Correspondence: Diego.Garcia@unizar.es; Tel.: +34-976762675

Received: 25 August 2020; Accepted: 7 September 2020; Published: 9 September 2020

Abstract: Emergence of strains with increased resistance/tolerance to natural antimicrobials was evidenced after cyclic exposure to carvacrol, citral, and (+)-limonene oxide. However, no previous studies have reported the development of resistance and tolerance to complex essential oils (EOs). This study seeks to evaluate the occurrence of *Staphylococcus aureus* strains resistant and tolerant to a complex orange essential oil (OEO) after prolonged cyclic treatments at low concentrations. Phenotypic characterization of evolved strains revealed an increase of minimum inhibitory and bactericidal concentration for OEO, a better growth fitness in presence of OEO, and an enhanced survival to lethal treatments, compared to wild-type strain. However, no significant differences ($p > 0.05$) in cross-resistance to antibiotics were observed. Mutations in *hepT* and *accA* in evolved strains highlight the important role of oxidative stress in the cell response to OEO, as well as the relevance of the cell membrane in the cell response to these natural antimicrobials. This study demonstrates the emergence of *S. aureus* strains that are resistant and tolerant to EO (*Citrus sinensis*). This phenomenon should be taken into account to assure the efficacy of natural antimicrobials in the design of food preservation strategies, in cleaning and disinfection protocols, and in clinical applications against resistant bacteria.

Keywords: complex orange essential oil; *Staphylococcus aureus*; genotypic resistance; mutagenesis frequency; whole genome sequencing; minimum inhibitory and bactericidal concentrations; growth kinetics; antibiotic susceptibility

1. Introduction

Essential oils (EOs) and their individual constituents (ICs) have been proposed as food preservatives [1] and as disinfection agents due to their antimicrobial properties [2] and better social acceptance as compared to chemically synthesized compounds [3]. Moreover, since it is commonly accepted that these natural antimicrobials, they do not induce mutations that could lead to antimicrobial resistance (AMR) or tolerance [4]; they have also been studied for the treatment of bacterial infections in order to prevent the emergence of resistances to antibiotics [5–7]. Several authors have reported that exposure to ICs or EOs during bacterial growth maintains or even reduces the mutation rate [8–10]. This fact could be explained due to the antioxidant activity of EOs and ICs at low doses [11], which decrease the formation of reactive oxygen species (ROS) and, consequently, bacterial mutagenesis [12]. However, recent studies have demonstrated that cyclic exposure to ICs can lead to the emergence of strains that are resistant and tolerant to carvacrol, citral, or (+)-limonene oxide, since random mutations occurring in the bacterial population can provide a greater degree of fitness or survival than wild-type strain [9,10,13]. In addition, some of

these strains showed increased resistance and tolerance to other natural antimicrobials, to antibiotics, and even to other methods of food preservation or disinfection such as heat or pulsed electric fields [9]. According to Balaban, et al. [14], resistance is the ability of bacteria to replicate in the presence of an antimicrobial, usually at low doses for long periods of time, while tolerance is the bacterial capacity to survive at lethal doses of the antimicrobial.

Complex essential oil of sweet orange (OEO) is one of the most widely used EOs on an industrial level [15], widely employed in different fields such as food, cosmetics, pharmaceutics, and agrochemicals [16–18]. This EO is obtained from the peels of *Citrus sinensis* (L.) by cold pressing and is composed of more than 20 ICs, including limonene (>85%), myrcene, α-pinene, and sabinene [19–21]. The excellent antimicrobial properties of OEO can be attributed to that complex composition of ICs, both because of the functional groups that each IC presents and because of the synergism that occurs between them [21,22]. Several authors point out that cell envelopes are one of the most important bacterial targets of ICs and therefore of EOs, along with the internal damage caused by the accumulation of ROS when the antimicrobial is applied at high doses [23–25]. The emergence of EO-resistant or EO-tolerant strains has hitherto been ruled out due to the multitude of antimicrobial action mechanisms that EOs can exert on bacteria in view of their great complexity and compositional variety [26]. In order to develop resistance mechanisms to complex EOs, bacteria would have to mutate genes involved in multiple structures or metabolic pathways. Actually, no studies have evidenced as yet the emergence of EO-resistant or EO-tolerant strains through evolution assays [27–29].

In genomic studies of strains evolved in the presence of ICs [9,10,13], several mutations were related to general cell response mechanisms. For instance, mutations in *soxR*, a redox-sensitive transcriptional regulator, were related to carvacrol resistance and tolerance in *Escherichia coli* [30] and *Salmonella enterica* [13]. Evolved strains of *Staphylococcus aureus* likewise displayed an increased resistance and tolerance to carvacrol: not due to improved intrinsic antimicrobial resistance, but rather to a better repair of cellular damage [10]. Hence, if these mutations appeared in the presence of complex EOs, they would probably enhance general resistance and tolerance mechanisms, or provide an improved system for repairing cell damage, thereby leading to the emergence of resistances or tolerances to EOs. Moreover, the identification of genetic variations responsible for increased resistance or tolerance would allow for a better grasp of the mechanisms of action of natural antimicrobials (as yet not completely understood), and thus facilitate the design of more effective IC and EO treatments, as well as combating the emergence of AMR.

Therefore, the objectives of this study are a) to determine the emergence of *Staphylococcus aureus* strains resistant and tolerant to a complex essential oil of orange (*Citrus sinensis*) by evolution assays, b) to evaluate the direct resistance and tolerance of the evolved strains against orange essential oil, as well as cross-resistance to antibiotics, and c) to identify genetic modifications occurring during the evolution assay which lead to increased resistance/tolerance.

2. Results

2.1. Isolation of Resistant Strains by Evolution Assay with OEO

After evolution assay (20 days or steps), five colonies from a plate were randomly selected, namely, $SaROEO_{1–5}$ (i.e., $SaROEO_1$, $SaROEO_2$, $SaROEO_3$, $SaROEO_4$, and $SaROEO_5$), to carry out phenotypic characterization and to evaluate the emergence of resistant strains. Firstly, the antimicrobial resistance and tolerance of SaWT and $SaROEO_{1–5}$ against OEO was evaluated by testing minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), respectively (Table 1).

Since MIC and MBC values for the 5 isolated colonies were similar ($p > 0.05$), results are shown for the group $SaROEO_{1–5}$. MIC results revealed a >200% increased resistance of $SaROEO_{1–5}$ to OEO in comparison with SaWT. An OEO concentration of 1500 µL/L was enough to inhibit growth of SaWT, while $SaROEO_{1–5}$ could still grow in the presence of 5000 µL/L of OEO. Similarly, MBC data demonstrated the increased tolerance of the evolved strains to OEO: MBC was increased >100%,

from 2500 µL/L for SaWT to >5000 µL/L for SaROEO$_{1–5}$. It was not possible to determine MIC and MBC values above 5000 µL/L for SaROEO$_{1–5}$ due to OEO solubility problems and the high resistance and tolerance displayed by those evolved strains.

Table 1. Minimum inhibitory concentration (MIC; µL/L) and minimum bactericidal concentration (MBC; µL/L) of orange essential oil (OEO) for *Staphylococcus aureus* USA300 (SaWT) and evolved strain (SaROEO$_{1–5}$, 5 isolated strains). Each value represents the result of 5 different experiments carried out for every strain tested, with different bacterial cultures and on different working days.

Strains	Carvacrol Concentration (µL/L)												
SaWT	0	250	500	750	1000	1250	1500	1750	2000	2500	3000	4000	5000
SaROEO$_{1–5}$	0	250	500	750	1000	1250	1500	1750	2000	2500	3000	4000	5000

■ Cell growth; ■ Neither cell growth nor inactivation; ■ Cell inactivation.

MIC and MBC results revealed that all the colonies of the evolution assay displayed the same degree of resistance and tolerance to OEO. These results suggest that all isolated colonies were identical, and that the bacterial cultures obtained from the evolution assay were probably homogeneous. We therefore selected one of the five evolved strains for further experiments, from here onward referred to as SaROEO.

2.2. SaROEO Showed a Greater Fitness than SaWT in Presence of OEO

In order to further study the resistance of SaROEO, growth kinetics in tryptone soya broth (TSBYE) were studied at different concentrations of OEO. First, growth curves were obtained in absence and presence of OEO for SaWT and SaROEO, and modelled (Figure 1) by modified Gompertz equation (Equation (1)). In agreement with MIC results, OEO concentrations higher than or equal to 1500 µL/L did not allow the growth of SaWT, while SaROEO could reach the stationary phase in presence of 5000 µL/L OEO at 18 h of growth (Figure 1).

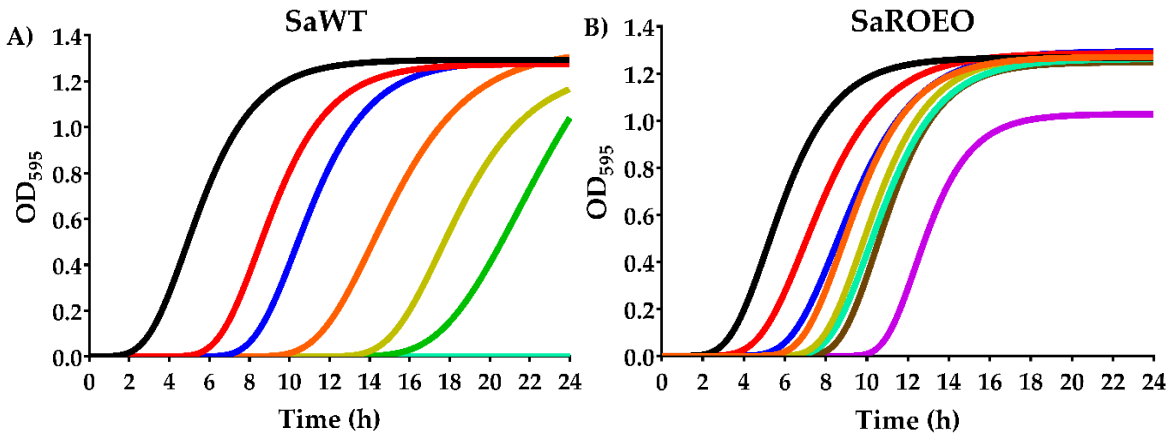


Figure 1. Growth curves of *Staphylococcus aureus* USA300 (**A**; SaWT) and evolved strain (**B**; SaROEO) in the absence (-) and presence of 250 (-), 500 (-), 750 (-), 1000 (-), 1250 (-), 1500 (-), 2000 (-), 5000 (-) of orange essential oil (OEO), modelled using the modified Gompertz equation (Equation (1)).

Table 2 summarizes the parameters of the modified Gompertz equation: A (maximum OD$_{595}$), μ_m (maximum specific growth rate) and λ (lag phase time), for both strains under all the conditions tested. The standard error, R^2 and R^2 adjusted values, and the root mean square error (*RMSE*) supported a good least-squares adjustment for both strains under all the concentrations tested (Table S1). Firstly, the growth parameters revealed that the presence of OEO slows down the microbial growth of both strains. Specifically, as the concentration of OEO was increased, μ_m and A were slightly reduced ($p \leq 0.05$), and λ was intensely prolonged ($p \leq 0.05$). Comparing the evolved strain with SaWT, with regard to A, a similar behaviour ($p > 0.05$) was shown at OEO concentrations below the MIC of

SaWT (≤1500 µL/L), hovering A values around 1.19−1.32 OD_{595}. No significant differences ($p > 0.05$) were observed in the μ_m between SaWT and SaROEO at OEO concentrations of 500 µL/L or lower. At higher concentrations, SaROEO showed a higher μ_m, than SaWT ($p \leq 0.05$). For instance, the μ_m of SaWT was reduced from 0.248 OD_{595}/h (without OEO) to 0.167 OD_{595}/h in presence of 1250 µL/L of OEO, while growth rate of SaROEO was not modified ($p > 0.05$) at this concentration (0.240 OD_{595}/h) compared to strains evolved in the absence of OEO (control).

Table 2. A (maximum OD_{595}), μ_m (maximum specific growth rate) and λ (lag phase time) parameters of the modified Gompertz model obtained from growth curves of *Staphylococcus aureus* USA300 (SaWT) and evolved strain (SaROEO), at different concentrations of orange essential oil (OEO). Each value represents the mean ± standard deviation from 3 independent experiments.

OEO (µL/L)	A (OD_{595})		μ_m (OD_{595}/h)		λ (h)	
	SaWT	SaROEO	SaWT	SaROEO	SaWT	SaROEO
0	1.294 ± 0.012 ^{a}	1.268 ± 0.010 ^{A}	0.248 ± 0.019 ^{a}	0.254 ± 0.018 ^{A}	2.902 ± 0.228 ^{a}	3.319 ± 0.191 ^{A}
250	1.276 ± 0.014 ^{a}	1.287 ± 0.013 ^{A}	0.238 ± 0.015 ^{a}	0.217 ± 0.014 ^{AB}	6.468 ± 0.215 ^{b}	4.776 ± 0.215 ^{B*}
500	1.293 ± 0.013 ^{a}	1.295 ± 0.019 ^{A}	0.229 ± 0.011 ^{a}	0.218 ± 0.015 ^{AB}	8.239 ± 0.162 ^{c}	6.311 ± 0.251 ^{C*}
750	1.324 ± 0.030 ^{ab}	1.271 ± 0.018 ^{A}	0.181 ± 0.008 ^{b}	0.244 ± 0.021 ^{AB*}	11.410 ± 0.170 ^{d}	6.876 ± 0.271 ^{D*}
1000	1.262 ± 0.032 ^{ab}	1.280 ± 0.018 ^{A}	0.183 ± 0.006 ^{b}	0.251 ± 0.020 ^{A*}	15.000 ± 0.099 ^{e}	7.884 ± 0.243 ^{E*}
1250	1.185 ± 0.038 ^{b}	1.280 ± 0.017 ^{A}	0.167 ± 0.008 ^{b}	0.240 ± 0.017 ^{AB*}	17.430 ± 0.149 ^{f}	7.761 ± 0.226 ^{F*}
1500	/	1.272 ± 0.019 ^{A}	/	0.210 ± 0.014 ^{AB}	/	8.062 ± 0.237 ^{G}
1750	/	1.254 ± 0.019 ^{AB}	/	0.237 ± 0.019 ^{AB}	/	8.447 ± 0.236 ^{H}
2000	/	1.246 ± 0.018 ^{ABC}	/	0.244 ± 0.019 ^{AB}	/	8.563 ± 0.225 ^{I}
2500	/	1.212 ± 0.023 ^{BC}	/	0.250 ± 0.019 ^{A}	/	9.482 ± 0.240 ^{J}
3000	/	1.198 ± 0.019 ^{C}	/	0.227 ± 0.017 ^{AB}	/	9.516 ± 0.204 ^{K}
4000	/	1.112 ± 0.020 ^{D}	/	0.208 ± 0.015 ^{AB}	/	10.300 ± 0.196 ^{L}
5000	/	1.052 ± 0.014 ^{E}	/	0.193 ± 0.010 ^{B}	/	10.850 ± 0.139 ^{M}

Different superscript letters represent statistically significant differences ($p \leq 0.05$) among the means of the same column; * Significantly different from SaWT ($p \leq 0.05$); /: no growth.

The major differences between SaWT and SaROEO were found in λ at all the tested OEO concentrations, but more prominently at high concentrations; the lag phase lasted a total of 17.4 h for SaWT at 1250 µL/L, thus 10 h longer than the SaROEO lag phase at the same OEO concentration.

2.3. Higher Survival of SaROEO after OEO Treatments at both pH 7.0 and 4.0

In order to further evaluate the tolerance of SaROEO, survival curves were obtained after lethal treatment with 2000 µL/L of OEO at pH 7.0 and pH 4.0 and compared to those of SaWT (Figure 2). These pH values were chosen as representative of neutral and acid conditions within the usual pH range of foods [31].

At neutral pH, significant differences ($p \leq 0.05$) were observed between SaWT and SaROEO inactivation after lethal treatments of OEO. While SaWT showed a bacterial reduction of 1.5 $\log_{10}$ cycles after 32 h of treatment at pH 7.0, only 0.6 $\log_{10}$ cycles of SaROEO were inactivated (Figure 2A). At pH 4.0 (Figure 2B), the inactivation reached was greater in both strains than at neutral pH. Similarly, SaROEO also exhibited a higher survival to the lethal treatment at acid pH compared to SaWT. For instance, after 9 h of treatment at pH 4.0, more than five $\log_{10}$ cycles of SaWT population were inactivated, whereas just over three $\log_{10}$ cycles of inactivation were achieved for SaROEO.

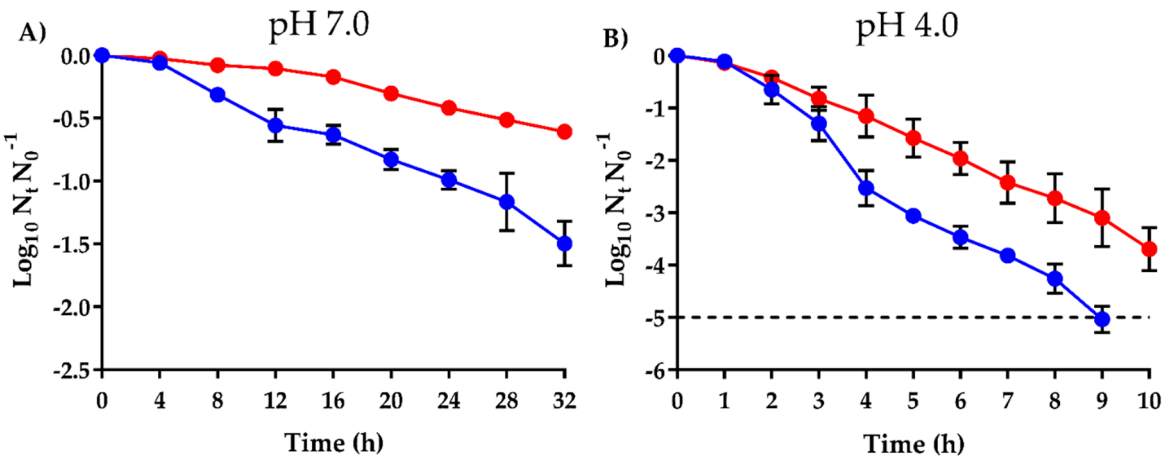


Figure 2. Survival curves of *Staphylococcus aureus* USA300 (•; SaWT) and evolved strain (•; SaROEO), after 2000 µL/L orange essential oil (OEO) treatment at pH 7.0 (**A**) and pH 4.0 (**B**) at 37 °C. Data are means ± standard deviations (error bars) obtained from at least 3 independents experiments. Dashed line represents the detection limit (−5.0 log_{10}).

2.4. SaROEO Displayed an Antibiotic Resistance Similar to SaWT

Finally, a disk diffusion test was carried out to evaluate cross-resistance against antibiotics. Table 3 presents the inhibition halos of SaWT and SaROEO against tetracycline, chloramphenicol, nalidixic acid, rifampicin, norfloxacin, novobiocin, trimethoprim, and cephalexin.

Table 3. Zones of growth inhibition (mm) for agar disk diffusion assays of *Staphylococcus aureus* USA300 (SaWT) and evolved strains (SaROEO) against antibiotics: 30 µg tetracycline, 30 µg chloramphenicol, 400 µg nalidixic acid sodium, 50 µg rifampicin, 60 µg norfloxacin, 50 µg novobiocin sodium, 10 µg trimethoprim and 150 µg cephalexin. Each value represents the mean diameter of the inhibition halo ± standard deviation from three independent experiments.

Antibiotics	Strains	
	SaWT	SaROEO
Tetracycline	28.07 ± 1.11	28.93 ± 2.03
Chloramphenicol	22.23 ± 1.32	23.37 ± 1.25
Nalidixic acid	15.88 ± 1.24	15.45 ± 1.27
Rifampicin	30.80 ± 0.67	30.51 ± 0.26
Norfloxacin	11.60 ± 0.31	12.02 ± 0.73
Novobiocin	27.38 ± 1.24	28.39 ± 1.56
Trimethoprim	22.18 ± 1.05	20.39 ± 1.20
Cephalexin	14.45 ± 0.72	13.39 ± 1.23

Inhibition halos larger than 15 mm were obtained for the antibiotics tested in order to be able to evaluate variations in the resistance of SaROEO compared to SaWT. It should be noted that *S. aureus* USA300 is a methicillin-resistant strain. Susceptibility assay revealed that none of the tested antibiotics featured significant differences ($p > 0.05$) between SaWT and SaROEO in terms of cross-resistance. Thus, the mutations that occurred during the evolution assay would not be related with resistance against a wide range of antibiotics: tetracyclines, quinolones, and aminoglycosides.

2.5. OEO does not Induce an Increased Mutagenesis

The mutation frequency was determined for SaWT in the absence or in the presence of OEO (at the same concentration used in the evolution assay, i.e., 1/2 MIC) to evaluate whether this complex EO could increase the mutation rate, which, in turn, would facilitate the emergence of genotypic resistances [32]. As shown in Figure 3, SaWT displayed a spontaneous frequency of rifampicin-resistant mutants over 60×10^{-9} during bacterial growth in absence of the OEO (control). Similar results were obtained when OEO and carvacrol were added to growth medium. A *t*-test revealed no significant differences ($p > 0.05$) among the control, the OEO at 750 µL/L, and carvacrol at 50 µL/L.

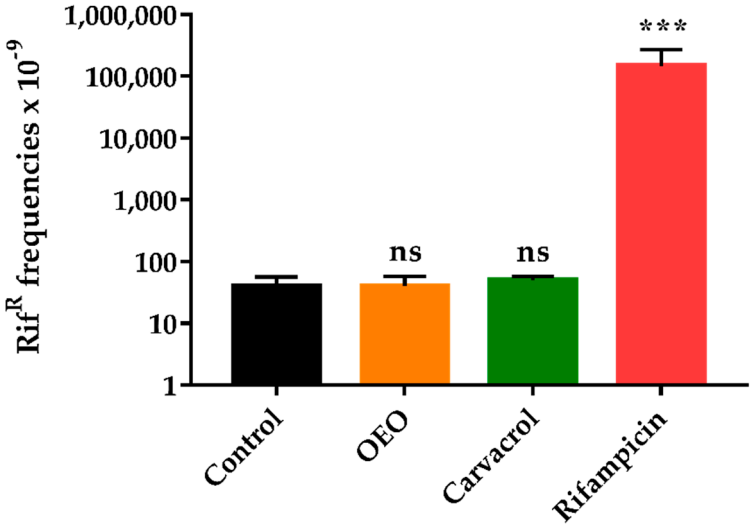


Figure 3. Mutagenesis frequency in *S. aureus* USA300 grown in broth (control, ■) and with orange essential oil (OEO; 750 µL/L; ■), carvacrol (50 µL/L, ■) and rifampicin (0.01 mg/L, ■). Mutagenesis frequency was expressed as rifampicin-resistant cells in the total microbial population. Data are means ± standard deviations (error bars) obtained from five independent experiments. ns: no statistically significant differences ($p > 0.05$); ***: statistically significant differences ($p \leq 0.001$), in comparison with control.

On the contrary, the presence of the rifampicin at 0.01 mg/L (1/2 MIC for SaWT) in the growth medium led to a mutation frequency around 150×10^{-6}; thus 1000 times higher compared to control or when natural compounds (OEO or carvacrol) were added.

2.6. Four Missense Mutations Identified in SaROEO

Whole genome sequencing (WGS) was performed on SaROEO and compared to SaWT genome in order to identify the mutations causing the increased resistance to OEO that occurred during the evolution assay. A total of 17.31 and 4.19 million of 150 bp-reads were obtained for SaWT and SaROEO, respectively. From those reads, 90.52% and the 88.05% displayed a Phred quality score above 30. The quality-control-filtered paired-end reads were mapped at 91.60% and 96.44%, respectively, on the reference genome sequence of *S. aureus* USA300 (NCBI accession: NC_007793.1). The reference genome was sufficiently covered to allow the detection of genetic variations between the strains studied; an at least 100-fold coverage depth was achieved for both strains. This study focused on the genetic variations between SaWT and SaROEO in order to identify the mutations which occurred during the evolution assay. After WGS, Sanger sequencing verified a total of four single nucleotide variations (SNVs) in the comparison of genomic sequence of SaROEO with that SaWT (Figure 4).

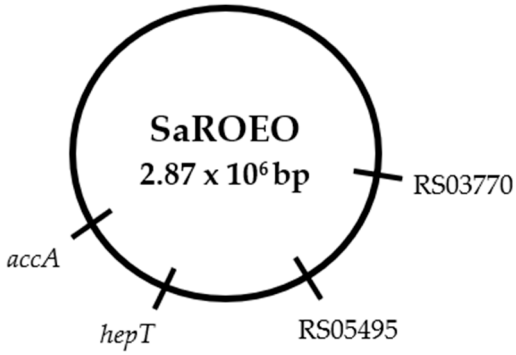


Figure 4. Genomic map of evolved strain (SaROEO) in comparison with *Staphylococcus aureus* USA300 (SaWT).

In addition, these mutations were also confirmed by Sanger sequencing in all the strains isolated in the evolution assay ($SaROEO_{1–5}$), supporting the supposition of homogeneity of the bacterial population at the end of the evolution assay.

Table 4 summarizes the genes involved in the mutations, as well the proteins coded in order to ascertain the cause of the increased resistance and tolerance to OEO observed in SaROEO.

Table 4. Mutations of evolved strain (SaROEO) in comparison with *Staphylococcus aureus* USA300 (SaWT). Single nucleotide variation (SNV).

Genome Position	Gene	Locus Tag [a] (Old Locus Tag)	Mutation [b]	Change	Information
776,659	-	RS03770 (0702)	SNV: A993T	Glu331Asp	Allophanate hydrolase
1,118,342	-	RS05495 (1021)	SNV: T26G	Ile9Ser	Hypothetical protein (DUF2129 domain containing protein)
1,526,963	*hepT*	RS07410 (1359)	SNV: C272T	Thr91Ile	heptaprenyl diphosphate synthase subunit II
1,808,243	*accA*	RS08985 (1646)	SNV: C481T	Pro161Ser	Acetyl-CoA carboxylase carboxyl transferase subunit alpha

[a] The gene locus tag corresponds to SAUSA300_RSXXXXX. Also, old locus tag is provided SAUSA300_XXXX; [b] Position respect to the start of the coding region.

The four mutations detected in SaROEO were the following:

(1) A SNV was detected at position 993 bp in the SAUSA300_RS03770 locus resulting in a change of glutamic acid by asparagine in the enzyme allophanate hydrolase at position 331 amino acid.
(2) A transversion from thymine to guanine was found at position 26 bp in SAUSA300_RS05495 locus coding a hypothetical protein in *S. aureus* USA300.
(3) A replacement of cytosine by thymine was observed at position 272 bp in the *hepT* gene. This missense mutation resulted in a protein modification in the position 91 amino acid, from threonine to isoleucine, in the heptaprenyl diphosphate synthase subunit II.
(4) A transition from cytosine to thymine was detected at position 481 bp in the *accA* gene. This mutation led to a protein change in the position 161 of proline to serine in the acetyl-CoA carboxylase carboxyl transferase subunit alpha.

3. Discussion

Cyclic exposure to prolonged subinhibitory doses of OEO enabled the selection of evolved strains of *S. aureus*: SaROEO$_{1–5.}$ MIC and MBC results revealed the increased resistance and tolerance to the OEO of *S. aureus* after the evolution assays. All five isolated strains SaROEO$_{1–5}$ showed a >200% increased resistance to OEO in comparison with SaWT (Table 1). Similarly, a >100% increase in MBC of the isolated strains revealed their higher tolerance compared to SaWT. It should be noted that the phenotypic characterization was performed with fresh cultures grown in the absence of OEO, thereby supporting the supposition that the increase in resistance and tolerance is stable and based on genetic modifications in the evolved strain. In addition, pending sequencing results, the same increase in resistance and tolerance to OEO displayed by the five isolated colonies (SaROEO$_{1–5}$) would imply that the same mutations were fixed in the microbial population, and therefore the culture obtained after the evolution assay would be homogeneous. Despite the development of resistance in *S. aureus* against antibiotics [33] and against other antimicrobial compounds such as peptides [34], no resistance against EO has been previously described to the best of our knowledge. These results provided the first evidence that the evolution protocol had led to the occurrence of resistant strains against a complex EO. Following the same evolution assay for 10 days against ICs, Berdejo, et al. [10] reported an increase in MIC values in *S. aureus* USA300 from 50% to 100% against carvacrol, citral, and (+)-limonene oxide, compared to wild-type strain. An increase in direct resistance to ICs has also been observed in Gram-negative bacteria after evolution assays; similar increases have also been observed in *S. enterica* (between 50% and 100%), and even greater in *E. coli* (up to 300%) in comparison with wild-type strain.

The study of growth kinetics in the presence of the OEO revealed significant differences ($p < 0.05$) in growth fitness between SaWT and SaROEO (Figure 1). Increases in OEO concentration led to a decrease in the maximum value of OD_{595} (A), which means that the bacterial concentration reached was lower when OEO concentration was higher. The addition of OEO to the growth medium also caused a reduction of the maximum specific growth rate (μ_m), and a longer lag phase time (λ). These results support the assumption that both strains grow more slowly in the presence of the OEO, and that they need a longer time to adapt to the environment as the OEO concentration increases. It is likely that EO is disturbing the cell membrane integrity and increasing the membrane permeability of both strains, thereby leading to a prolonged adaptation and lag phase time [35]. Comparing SaROEO to SaWT, no significant differences ($p < 0.05$) were observed when strains were grown in absence of OEO. However, SaROEO showed a divergent growth behavior in presence of OEO: its μ_m was greater and its λshorter than for SaWT, and the differences became more pronounced as the concentration of OEO increased. These results not only support the assumption that SaROEO can grow at higher concentrations, but also indicate that its growth rate at low concentrations is much more pronounced than that of SaWT. In regard to growth kinetics of evolved strains in presence of natural antimicrobials, previous studies have also observed differences in growth models compared to wild-type strain, mainly in growth rate and lag phase. *S. enterica* carvacrol-resistant mutants exhibited a maximum specific growth rate 10-fold higher and a lag phase 7 h shorter than wild-type strain in presence of carvacrol at 150 µL/L [13]. Although differences were observed in both parameters, SaROEO results revealed a greater relevance of lag phase in the bacterial adaptation to OEO than in the adaptation of *S. enterica* to carvacrol. One of the main bacterial targets of EO is the cell membrane [35]; it is likely that mutations in SaROEO are related to the cell envelopes, which allow for a faster adaptation to OEO and consequently a decrease in lag phase time. These results would thus explain why mutations of SaROEO were fixed in the bacterial population after evolution assay (750 µL/L OEO). In addition, the emergence of resistant mutant strains could pose a risk in food preservation, because bacterial growth at low doses of OEO might be underestimated.

Survival curves obtained after OEO lethal treatments also showed an increased tolerance of SaROEO at both neutral (Figure 2A) and acid pH (Figure 2B) compared to SaWT. For instance, inactivation of SaWT was 2 $\log_{10}$ cycles higher than that of SaROEO after 9 h of OEO lethal treatment at pH 4.0. Similarly, IC-evolved strains of *E. coli* [9], *S. aureus* [10], and *S. enterica* [13] also showed an increase in direct tolerance. Previous studies have reported that EO could serve as an effective

disinfectant agent to inactivate *S. aureus* [36] or to eradicate resistant forms, such as biofilms [36,37]. However, the presence of resistant strains might diminish the efficacy of disinfectants at the doses previously established for these purposes; the survival of such strains would pose a microbiological risk.

Development of mutant resistance after evolution assays with ICs indicated the occurrence of cross-resistance to antibiotics [13,30]. Carvacrol resistance developed by strains of *E. coli* [9] and *S. enterica* [13] likewise increased their resistance to tetracyclines, quinolones, aminoglycosides, and β-lactams. Those strains were mutated in genes related to stress response and to transcriptional regulators of sensor redox-cycling drugs, such as *soxR*, which are also involved in common bacterial resistance to all antibiotics tested [13,30]. However, in our study, SaROEO and SaWT (Table 3) displayed a susceptibility similar ($p > 0.05$) to that of all the tested antibiotics: tetracyclines, quinolones, and aminoglycosides. It should be noted that *S. aureus* USA300 is a methicillin-resistant strain. It is thus likely that the mutations which occurred in SaROEO during the evolution assay would not be related to general mechanisms of bacterial resistance, but rather to a specific resistance to OEO. In this regard, the clinical use of EO to combat bacterial infections and prevent AMR needs to be reconsidered [6,38].

Previous studies have demonstrated that EOs and ICs do not increase the frequency of mutation in bacteria [7,10]. Decreased mutation rates have actually been observed compared to controls, and they have been attributed to the antioxidant capacity of these natural compounds [11]. In accordance with our results, previous studies have reported a low mutation rate of *S. aureus* when it is exposed to natural compounds during growth, both in the presence of ICs [10] and EOs [8]. Our results, in accordance with those studies, show a mutagenesis frequency of SaWT that is similar both in absence or in presence of OEO at 1/2 MIC concentration (Figure 3). In contrast to antibiotics [32], the emergence of *S. aureus* mutants would not be caused directly by OEO, but because of the selective pressure of OEO on the growing bacterial population. In other words, OEO would select spontaneous and random mutants that emerge during evolution assay, and which display a better growth fitness than SaWT in presence of OEO. Finally, the selective pressure exerted by OEO would lead to the fixation of those growth-enhancing mutations in the bacterial population.

WGS and Sanger sequencing revealed four mutations in SaROEO in comparison to SaWT (Table 4). Given these mutations arose during the evolution assay, it is likely that some or all contribute to the cause of the increased resistance and tolerance observed in the phenotypic characterization of SaROEO. Consequently, this increase in resistance/tolerance allowed it to be isolated after the evolution assay at subinhibitory doses of OEO. Notably, these mutations were also verified in the five isolated strains ($SaROEO_{1–5}$) stemming from the same evolution lineage, thereby demonstrating that at the end of the evolution assay the bacterial population was homogeneous, i.e., mutations had been fixed in the bacterial culture.

A missense mutation was found in SAUSA300_RS03770 locus, whose product is the allophanate hydrolase. This enzyme catalyzes the hydrolysis of allophanate to ammonium (NH_4^+) and carbon dioxide (CO_2) [39], and belongs to the amidase signature family, a large group of hydrolytic enzymes that catalyse the hydrolysis of amide bonds (CO-NH_2) [40]. In prokaryotes, this enzyme has only been related to bacterial metabolism [41,42]. However, Juttukonda, et al. [43] reported that allophanate synthase was related to resistance in *Acinetobacter baumannii* against calprotectin, a protein with chelating properties on divalent zinc and manganese metal ions. To the best of our knowledge, however, no studies have related the antimicrobial effect of EOs to the presence of those divalent metal ions.

A SNV was located in SAUSA300_RS05495 locus that encodes the DUF2129 domain. This is an uncharacterized domain found in various hypothetical prokaryotic proteins whose function has not been determined in vivo. Structural modelling suggests that this domain may bind nucleic acids [44]. However, due to the lack of information regarding this locus's function, it is unknown how this mutation can influence the behavior of SaROEO.

The *hepT* gene mutation led to a change from threonine to isoleucine in the heptaprenyl diphosphate synthase subunit II. This enzyme is involved in the synthesis of menaquinone, also known as vitamin

K_2 [45]. It serves as a key electron transporter in many types of bacteria and is required for bacterial respiration where ROS are produced. According to Chueca, et al. [24,46], ROS are involved in bacterial death by several ICs, such as carvacrol, citral, and limonene: in fact, the latter is the main IC present in OEO. It is likely that the alteration of this metabolic pathway would cause a decrease in ROS accumulation, consequently resulting in a greater degree of survival to OEO. Evolved strains with increased resistance and tolerance to carvacrol also showed mutations in transcriptional regulators induced by oxidative stress, such as *soxR* and *yfhP*, in *E. coli* [30] and *S. enterica* [10]. Oxidative stress was also induced by treatment with complex EO in *Klebsiella pneumoniae* [47]. These results highlight the relevance of oxidative stress in the cell response to natural antimicrobials. Although SaROEO did not show increased cross-resistance to any antibiotics, Berti, et al. [48] reported that a SNV in the *hepT* gene was associated with a slight resistance increase and a pronounced increased tolerance to daptomycin.

Finally, a SNV was detected in *accA* in SaROEO. This gene codifies acetyl-CoA carboxylase carboxyl transferase subunit alpha, an enzyme involved in the malonyl-CoA biosynthesis pathway, which takes part in the formation of fatty acids [49]. Because fatty acids are constituents of membrane building phospholipids, this pathway is essential for bacterial growth [50]. In fact, carboxyl transferase subunits have been shown to be targets for antibiotic development [51]. According to Meades, et al. [52], cinnamon EO inhibits the carboxyltransferase subunit of *E. coli*, which partially explains that EO's antibacterial activity. Moreover, as is well known, cell membranes are the first barrier and one of the main structures affected by natural antimicrobials, which alter their permeability and disrupt their integrity [35,47]. It is likely that the mutation of *accA* could result in improved cell membrane formation and/or repair in presence of OEO. This hypothesis would explain the increase of the resistance and tolerance of SaROEO, as well as its better adaptation to OEO in the growth curves. These findings support the relevance of fatty acid synthesis in the cell response to complex OEO.

Briefly, the two mutations identified in *hepT* and *accA* could explain the increased resistance and tolerance observed in the phenotypic characterization of SaROEO. These results highlight the likely relevance of oxidative stress response in the cell defense against OEO, along with the important role played by the membrane in the resistance and tolerance to it. It should be noted that the genetic variations described were located in the genome, not in mobile genetic elements such as plasmids, transposons, etc. Such mutations would thus be considered hereditable, leading to stable increased resistance and tolerance. Once the emergence of resistant strains against complex EO has been demonstrated, we recommend to follow Sullivan, et al. [53] and Tyers, et al. [54] in combining different antimicrobials with various cell targets in order to avoid the occurrence of resistant strains while improving their antimicrobial properties. For instance, ICs from OEO, such as limonene, myrcene, and sabinene, can be used in combination with first-line tuberculostatic antibiotics for the treatment of resistant *Mycobacterium tuberculosis*.

It should be noted that the emergence of strains resistant and tolerant to OEO could be due to the fact that its ICs have the same cell targets. To avoid the emergence of resistant and tolerant strains, further research is thus required to better understand the mode of action of natural antimicrobials in order to design optimal treatments that combine EOs and ICs with different functional groups, or even with other kinds of antimicrobials.

4. Materials and Methods

4.1. Microorganisms and Growth Conditions

The wild-type microorganism was *S. aureus* USA300 methicillin-resistant strain (FPR3757 strain), provided by Prof. Kolter laboratory (Harvard Medical School, Boston, MA, USA).

OEO used in this investigation was kindly provided by Indulleida S.A. (Lleida, Spain). This commercial EO was prepared using a mixture of different *Citrus sinensis* varieties ('Washington Navel', 'Navelate', 'Navelina', 'Salustiana', and 'Valencia Late') by cold press system extraction.

The peels of fresh fruits were cold-pressed, the EO was separated from the crude extract by centrifugation, and stored in the dark in sealed glass vials at 4 °C until use. The composition of this batch of OEO (95.1% limonene, 2.0% myrcene, 0.7% α-pinene, 0.7% sabinene, and 1.5% other compounds) was previously analyzed by Bento, et al. [20].

Throughout this investigation, the strain was kept in cryovials at −80 °C with glycerol (20% *v*/*v*), from which plates of tryptone soya agar (Oxoid, Basingstoke, England) with 0.6% yeast extract (Oxoid; TSAYE) were prepared on a weekly basis. To prepare the working bacterial cultures, test tubes containing 5 mL of tryptone soya broth (Oxoid) with 0.6% yeast extract (TSBYE) were inoculated with one colony and then incubated aerobically on an orbital shaker (130 rpm; Heidolph Vibramax 100, Schwaback, Germany) for 12 h at 37 °C (Incubig, Selecta, Barcelona, Spain). Subsequently, flasks containing 10 mL of fresh TSBYE were inoculated with the resulting subculture to an initial concentration of 10^6 colony forming units per mL (CFU/mL) and incubated for 24 h at 37 °C and 130 rpm until the stationary growth phase was reached (2×10^9 CFU/mL approximately).

4.2. Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) of OEO was determined by inoculating the bacteria in test tubes with 5 mL of Mueller–Hinton broth cation adjusted (Sigma-Aldrich, Steinheim, Westphalia, Germany; MHB) at an initial concentration of 5×10^5 CFU/mL in presence of different concentrations of OEO: from 250 up to 5000 µL/L, and incubated at 37 °C for 24 h and 130 rpm. This protocol was adapted from standard methods for antimicrobial susceptibility tests [55]. A vigorous shaking by vortex (Genius 3, Ika, Königswinter, Germany) was used to prepare OEO dispersions in MHB, avoiding the use of solvents for their potentially detrimental effect on antibacterial activity. Positive control tubes with 5 mL MHB inoculated at 5×10^5 CFU/mL without OEO, and negative control tubes with 5 mL MHB were also prepared in each experiment. Once tubes were incubated, MIC was determined as the lowest concentration of the antimicrobial compound that was capable of avoiding bacterial growth. To objectively determine bacterial growth, optical density was read at 595 nm (OD_{595}) using a microplate reader (Genios, Tecan, Männedorf, Switzerland). The values of OD_{595} were subtracted from the negative control with the same concentration of the oil as the sample (without bacterial inoculation), corresponding to the absorbance caused by the growth medium and the oil. In total, 10% of the OD_{595} measure of the positive control was established as the lowest limit to consider that bacterial strain had been grown [12].

4.3. Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) of OEO was evaluated from the test tubes employed in the MIC determination after incubation. 100 µL aliquot of each tube was spread onto Mueller–Hintonagar cation adjusted (Sigma-Aldrich; MHA) plates and incubated at 37 °C for 24 h. Colonies were counted and the lowest concentration of carvacrol that killed ≥99.9% of the initial bacterial concentration (5×10^5 CFU/mL) was defined as the MBC end point [56]. The same positive and negative MIC test controls were employed in this experiment.

4.4. Evolution Assay of OEO

The evolution assay was based on the isolation of strains by prolonged exposure to subinhibitory concentration of OEO during bacterial growth [9,10,13]. *S. aureus* wild-type strain (SaWT) was grown on TSAYE plates for 24 h at 37 °C. A single colony was inoculated in 5 mL TSBYE and incubated under agitation for 12 h at 37 °C. This preculture was diluted 1:1000 into 50 mL TSBYE and incubated for 5.0 h to obtain an exponential growth phase culture. From this culture, SaWT were inoculated at an initial bacterial concentration of 10^6 CFU/mL in 5 mL TSBYE with 750 µL/L of OEO (1/2 MIC). This bacterial suspension was incubated 24 h/37 °C/130 rpm and, once stationary phase was reached, the same dilution steps were repeated 20 times: the culture was inoculated (10^6 CFU/mL) in 5 mL TSBYE with 750 µL/L of OEO and incubated 24 h/37 °C/130 rpm. After the 20th step, an aliquot was diluted in

phosphate buffered saline (Sigma-Aldrich; PBS) and spread on TSAYE plates. After the incubation period, five evolved strains were randomly selected for phenotypic and genotypic characterization.

4.5. Growth Curves in Presence of OEO

Bacterial growth curves were obtained at different concentrations of OEO in TSBYE. Based on the results obtained in MIC assay, the concentration ranges of OEO used were 0–1500 µL/L for SaWT and 0–5000 µL/L for evolved strains. OEO was added in test tubes with 5 mL of TSBYE, vigorously vortexed, inoculated with the microbial culture (5×10^5 CFU/mL), and incubated at 37 °C and 130 rpm for 24 h. During the culture incubation, OD_{595} of the test tubes was measured every hour in a microplate reader. A positive control (without antimicrobial added) and a negative control (without microbial culture added) were incorporated in all the assays. The values of OD_{595} obtained during the experiment were subtracted from the initial OD_{595} (at time 0), corresponding to the absorbance caused by the growth medium. Bacterial growth curves based on OD_{595} were graphically displayed and modelled by modified Gompertz equation (Equation (1)) [57].

$$y = A \exp\{-exp[\ (\mu_m\ e/A\ )(\lambda - t) + 1\ ]\} \quad (1)$$

where y: OD_{595}; t: time (h); A: maximum OD_{595} value reached; μ_m: maximum specific growth rate (h^{-1}); λ: lag phase time (h).

A least-squares adjustment was carried out to build the model and obtain A, μ_m, and λ values using Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). The adjustment's goodness of fit was evaluated using standard error, R^2 and R^2 adjusted values, and the root mean square error (RMSE).

4.6. Survival Curves after Lethal OEO Treatments

Tolerance to OEO was studied by evaluating bacterial survival after lethal treatments. The treatment medium was citrate-phosphate McIlvaine buffer, prepared from citric acid monohydrate (Panreac) and disodium hydrogen phosphate (Panreac), adjusted to pH 7.0 and pH 4.0. These pH values were chosen as representative of neutral and acid conditions within the normal pH range of food. The treatment was carried out in 10 mL McIlvaine buffer at 37 °C, to which OEO was added at a concentration of 2000 µL/L and then vigorously vortexed. Stationary phase culture was centrifuged for 5 min at 6000 RCF in a microcentrifuge (Mini Spin, Eppendorf, Hamburg, Germany) and resuspended in the treatment medium at 10^7 CFU/mL in order to initiate the lethal OEO treatment. During treatment, aliquots were obtained at established times. Those samples were adequately diluted in PBS and spread on TSAYE plates. After plates incubation (24 h/37 °C), colonies were counted by an automatic plate counter (Analytical Measuring Systems, Protos, Cambridge, United Kingdom). The increase in tolerance to OEO was evaluated by comparison of inactivation kinetics (i.e., survival curves) between SaWT and its evolved strains.

4.7. Antibiotic Susceptibility Test

Agar disk diffusion assay was used to test antimicrobial susceptibility according to the Clinical and Laboratory Standards Institute [55,58]. First, bacterial suspension was spread on MHA plates and, after 5 min at room temperature, blank disks (Thermo Scientific™ Oxoid™ Anti-microbial Susceptibility Disk Dispenser, ST6090, Waltham, MA, USA) were placed on the surface of plates and individually impregnated with 10 µL of each antibiotic: 30 µg tetracycline, 30 µg chloramphenicol, 400 µg nalidixic acid sodium, 50 µg rifampicin, 60 µg norfloxacin, 50 µg novobiocin sodium, 10 µg trimethoprim, and 150 µg cephalexin (Sigma-Aldrich). Plates were incubated at 37 °C for 16–18 h, after which the diameters of the resulting inhibition zones were measured (paper disks included).

4.8. Mutagenesis Frequency Evaluation

The mutagenic effect of OEO was determined by calculating the rate of mutants resistant to rifampicin due to point mutations in the *rpoB* gene [10,59]. Overnight culture of SaWT was diluted 1:10,000 into 50 mL TSBYE and incubated at 37 °C and 130 rpm for 24 h. This culture was then diluted 1:3 in tubes containing 10 mL TSBYE with 750 µL/L of OEO (same concentration used in the evolution assay) and without OEO. This experiment was also carried out with carvacrol (Sigma-Aldrich) at 50 µL/L as control of natural compounds from previous study [10] and with rifampicin (Sigma-Aldrich) at 0.01 mg/L concentration as a positive control of mutagenesis. Those suspensions were grown at 37 °C and 130 rpm for 24 h (ca. 2×10^9 CFU/mL). Samples of the culture were serially diluted in PBS and pour-plated on TSAYE in the presence and absence of 100 mg/L rifampicin (Sigma-Aldrich). Plates were incubated at 37 °C for 48 h and colonies were counted. Mutation rates were calculated by dividing the number of colonies present in rifampicin plates (mutation events) by the number of colonies present in plates without antibiotic [60].

4.9. Whole Genome Sequencing (WGS) and Identification of Genetic Variations

Genomic DNA (gDNA) was extracted using a gDNA kit (DNeasy kit, Qiagen, Hilden, Germany) from bacterial strains. Illumina technology was used to carry out whole genome sequencing (WGS) on NextSeq equipment at mid output flow, with a total of 2 × 150 cycles (Illumina; Fasteris, SA, Geneva, Switzerland). Subsequently, quality control was performed with FastQC software (version 0.11.9, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) evaluating the quality of the reads (Q_{30}), sequence length, presence of adapters, and overrepresented and duplicated sequences. The quality control-filtered paired-end reads were mapped on the reference genome sequence (NCBI accession: NC_007793.1): *S. aureus* subsp. *aureus* USA300_FPR3757 [61], using a Burrows–Wheeler Alignment (BWA) tool (version 0.7.5a) [62] and Samtools software (version 1.2) [63]. A raw 100-fold depth coverage was achieved for both strains. Samtools was then applied to remove potential PCR duplicates according to reading positions on the reference genome; the resulting BAM files were then further processed using LoFreq-Star (version 2.1.1, source: http://csb5.github.io/lofreq/) to correct mapping errors and insert the quality values. Finally, single nucleotide variants (SNV) and short insertions (Ins) and deletions (Dels) were detected using LoFreq-Star, and toolbox snpEff (source: http;//snpeff.sourceforge.net/) was employed to identify involved genes and to predict functional effect variations [64]. Coverage was further analyzed by the Integrative Genomics Viewer (IGV; Broad Institute, version 2.8.9, source: https://software.broadinstitute.org/software/igv/) in order to find structural variations (SVs). Although mapping was carried out against the reference genome, SNVs, Ins, Dels, and SVs were identified between our wild type and evolved strains to determine the mutations which had occurred during the evolution assay. The resulting genome sequences were deposited in the Sequence Read Archive (SRA) of NCBI (Bioproject ID: PRJNA657166). The accession numbers of the samples are SAMN15817977 (SaWT) and SAMN15817978 (SaROEO). Finally, specific primers (Table S2) were designed to carry out PCR amplifications for Sanger sequencing to verify the mutations detected in the WGS.

4.10. Statistical Analysis

All phenotypic characterization results (MIC and MBC determination, growth curves, lethal treatments, and antibiotic susceptibility test) were obtained from at least three independent experiments carried out on different working days with different bacterial cultures. Growth curve parameters, lethal treatment graphics, and antibiotic susceptibility tests are displayed as the mean ± standard deviation, using Prism 5.0. Data were analyzed and submitted to comparison of averages using analysis of variance (ANOVA), followed by post-hoc Tukey test and *t*-tests with Prism 5.0, and differences were considered significant if $p \leq 0.05$.

5. Conclusions

This study demonstrates for the first time the emergence of resistant and tolerant strains of *S. aureus* against a complex essential oil (EO) (*Citrus sinensis*). Prolonged exposition of *S. aureus* to low doses of sweet orange EO (OEO) led to the emergence of resistant strains (SaROEO). SaROEO displayed an increase of >200% in resistance and >100% in tolerance to OEO, compared to SaWT, by MIC and MBC determination, respectively. Moreover, SaROEO showed a better growth fitness in presence of OEO and a greater degree of survival to lethal treatments at both acid and neutral pH. WGS of SaROEO allow us to identify the genetic variations that occurred during the evolution assay responsible for that strain's increased resistance to OEO. Among the four mutations verified by Sanger sequencing, two were located in the genes *hepT* and *accA*. These genes highlight the important role of oxidative stress in the cell response to complex EO, as well as the relevance of the cell membrane in the resistance and tolerance against these natural antimicrobials. Nevertheless, it is certainly possible that mutations located in genes codifying other mechanisms and structures would give rise to yet undiscovered resistances similar to these.

These results highlight the great importance of taking these resistances into account, since evolved strains could represent a microbiological risk due to their ability to grow and survive under conditions established for their corresponding wild-type strains. Consequently, in order to ensure the efficacy of natural antimicrobials, the emergence of resistant strains should be taken into account in the design of food preservation strategies, or in cleaning and disinfection protocols. In this regard, further research will be fundamental in defining how such strains resistant and tolerant to natural antimicrobials emerge. Likewise, it is key to better understand the mechanisms of bacterial inactivation of EOs and ICs in order to enhance their antimicrobial properties as a food preservative, as cleaning and disinfection agents, or even in their potential clinical use against multi-drug resistant bacteria.

Supplementary Materials: The following are available online at http://www.mdpi.com/1424-8247/13/9/239/s1. Table S1: A (maximum OD595), μm (maximum specific growth rate; h-1) and λ (lag time; h) values and error standard of the modified Gompertz model obtained from 3 independently growth curves of *Staphylococcus aureus* subsp. *aureus* USA300_FPR3757 (SaWT) (A) and SaROEO at different concentrations of OEO, Table S2: Primers used for PCR amplification and Sanger sequencing to verify the mutations in SaROEO.

Author Contributions: Conceptualization, methodology and formal analysis, D.B., R.P. and D.G.-G.; investigation, D.B., E.P. and N.M; resources, R.P. and D.G.-G.; data curation, D.B.; writing—original draft preparation, D.B., E.P., N.M., R.P. and D.G.-G.; writing—review and editing, D.B., R.P. and D.G.-G.; project administration and funding acquisition, R.P. and D.G.-G. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financially supported by Agencia Estatal de Investigación (Spain) (Project No. PGC2018-093789-B-I00), by the European Social Fund, and by the Aragonese Office of Science, Technology and University Research.

Acknowledgments: The Spanish Ministry of Sports, Culture and Education and the Department of Science, Technology and University Education of the Government of Aragón (Spain) provided D. Berdejo, E. Pagán and N. Merino with grants to carry out this research. The authors thank S. Hanks for his collaboration on the English-language revision of this manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Quinto, E.J.; Caro, I.; Villalobos-Delgado, L.H.; Mateo, J.; De-Mateo-Silleras, B.; Redondo-Del-Río, M.P. Food Safety through Natural Antimicrobials. *Antibiotics* **2019**, *8*, 208. [CrossRef] [PubMed]
2. Espina, L.; Berdejo, D.; Alfonso, P.; García-Gonzalo, D.; Pagán, R. Potential use of carvacrol and citral to inactivate biofilm cells and eliminate biofouling. *Food Control* **2017**, *82*, 256–265. [CrossRef]
3. Carocho, M.; Barreiro, M.F.; Morales, P.; Ferreira, I.C.F.R. Adding molecules to food, pros and cons: A review on synthetic and natural food additives. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 377–399. [CrossRef]

4. Leite de Souza, E. The effects of sublethal doses of essential oils and their constituents on antimicrobial susceptibility and antibiotic resistance among food-related bacteria: A review. *Trends Food Sci. Technol.* **2016**, *56*, 1–12. [CrossRef]
5. Vasireddy, L.; Bingle, L.E.H.; Davies, M.S. Antimicrobial activity of essential oils against multidrug-resistant clinical isolates of the *Burkholderia cepacia* complex. *PLoS ONE* **2018**, *13*, e0201835. [CrossRef] [PubMed]
6. Yap, P.S.X.; Yiap, B.C.; Ping, H.C.; Lim, S.H.E. Essential oils, a new horizon in combating bacterial antibiotic resistance. *Open Microbiol. J.* **2014**, *8*, 6–14. [CrossRef] [PubMed]
7. Ambrosio, C.M.S.; Ikeda, N.Y.; Miano, A.C.; Saldaña, E.; Moreno, A.M.; Stashenko, E.; Contreras-Castillo, C.J.; Da Gloria, E.M. Unraveling the selective antibacterial activity and chemical composition of citrus essential oils. *Sci. Rep.* **2019**, *9*, 17719. [CrossRef]
8. Hammer, K.A.; Carson, C.F.; Riley, T.V. Frequencies of resistance to *Melaleuca alternifolia* (tea tree) oil and rifampicin in *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis*. *Int. J. Antimicrob. Agents* **2008**, *32*, 170–173. [CrossRef]
9. Chueca, B.; Berdejo, D.; Gomes-Neto, N.J.; Pagán, R.; García-Gonzalo, D. Emergence of hyper-resistant *Escherichia coli* MG1655 derivative strains after applying sub-inhibitory doses of individual constituents of essential oils. *Front. Microbiol.* **2016**, *7*, 273. [CrossRef]
10. Berdejo, D.; Chueca, B.; Pagan, E.; Renzoni, A.; Kelley, W.L.; Pagan, R.; Garcia-Gonzalo, D. Sub-inhibitory doses of individual constituents of essential oils can select for *Staphylococcus aureus* resistant mutants. *Molecules* **2019**, *24*, 170. [CrossRef]
11. Hashemi, S.M.B.; Khorram, S.B.; Sohrabi, M. Antioxidant activity of essential oils in foods. In *Essential Oils in Food Processing*; Hashemi, S.M.B., Khaneghah, A.M., de Souza Sant'Ana, A., Eds.; Wiley Blackwell: New Hoboken, NJ, USA, 2017; pp. 247–265.
12. Kohanski, M.A.; DePristo, M.A.; Collins, J.J. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Mol. Cell* **2010**, *37*, 311–320. [CrossRef] [PubMed]
13. Berdejo, D.; Merino, N.; Pagán, E.; García-Gonzalo, D.; Pagán, R. Genetic variants and phenotypic characteristics of *Salmonella* Typhimurium-resistant mutants after exposure to carvacrol. *Microorganisms* **2020**, *8*, 937. [CrossRef] [PubMed]
14. Balaban, N.Q.; Helaine, S.; Lewis, K.; Ackermann, M.; Aldridge, B.; Andersson, D.I.; Brynildsen, M.P.; Bumann, D.; Camilli, A.; Collins, J.J.; et al. Definitions and guidelines for research on antibiotic persistence. *Nat. Rev. Microbiol.* **2019**, *17*, 441–448. [CrossRef] [PubMed]
15. Lubbe, A.; Verpoorte, R. Cultivation of medicinal and aromatic plants for specialty industrial materials. *Ind. Crop. Prod.* **2011**, *34*, 785–801. [CrossRef]
16. Cherrat, L.; Espina, L.; Bakkali, M.; Pagán, R.; Laglaoui, A. Chemical composition, antioxidant and antimicrobial properties of *Mentha pulegium*, *Lavandula stoechas* and *Satureja calamintha* Scheele essential oils and an evaluation of their bactericidal effect in combined processes. *Innov. Food Sci. Emerg. Technol.* **2014**, *22*, 221–229. [CrossRef]
17. Isman, M.B. Bridging the gap: Moving botanical insecticides from the laboratory to the farm. *Ind. Crop. Prod.* **2017**, *110*, 10–14. [CrossRef]
18. McClements, D.J. Nanoemulsion-based oral delivery systems for lipophilic bioactive components: Nutraceuticals and pharmaceuticals. *Ther. Deliv.* **2013**, *4*, 841–857. [CrossRef]
19. Verzera, A.; Trozzi, A.; Dugo, G.; Di Bella, G.; Cotroneo, A. Biological lemon and sweet orange essential oil composition. *Flavour Fragr. J.* **2004**, *19*, 544–548. [CrossRef]
20. Bento, R.; Pagán, E.; Berdejo, D.; de Carvalho, R.J.; García-Embid, S.; Maggi, F.; Magnani, M.; de Souza, E.L.; García-Gonzalo, D.; Pagán, R. Chitosan nanoemulsions of cold-pressed orange essential oil to preserve fruit juices. *Int. J. Food Microbiol.* **2020**, *331*, 108786. [CrossRef]
21. Espina, L.; Somolinos, M.; Lorán, S.; Conchello, P.; García, D.; Pagán, R. Chemical composition of commercial citrus fruit essential oils and evaluation of their antimicrobial activity acting alone or in combined processes. *Food Control* **2011**, *22*, 896–902. [CrossRef]
22. O'Bryan, C.A.; Crandall, P.G.; Chalova, V.I.; Ricke, S.C. Orange essential oils antimicrobial activities against *Salmonella* spp. *J. Food Sci.* **2008**, *73*, M264–M267. [CrossRef] [PubMed]
23. Espina, L.; Gelaw, T.K.; de Lamo-Castellvi, S.; Pagan, R.; Garcia-Gonzalo, D. Mechanism of bacterial inactivation by (+)-limonene and its potential use in food preservation combined processes. *PLoS ONE* **2013**, *8*, e56769. [CrossRef] [PubMed]

24. Chueca, B.; Pagán, R.; García-Gonzalo, D. Differential mechanism of *Escherichia coli* inactivation by (+)-limonene as a function of cell physiological state and drug's concentration. *PLoS ONE* **2014**, *9*, e94072. [CrossRef] [PubMed]
25. Wang, C.-Y.; Chen, Y.-W.; Hou, C.-Y. Antioxidant and antibacterial activity of seven predominant terpenoids. *Int. J. Food Prop.* **2019**, *22*, 230–238. [CrossRef]
26. Lingan, K. A review on major constituents of various essential oils and its application. *J. Transl. Med.* **2018**, *08*, 201. [CrossRef]
27. Gomes Neto, N.J.; Luz Ida, S.; Tavares, A.G.; Honorio, V.G.; Magnani, M.; de Souza, E.L. *Rosmarinus officinalis* L. essential oil and its majority compound 1,8-cineole at sublethal amounts induce no direct and cross protection in *Staphylococcus aureus* ATCC 6538. *Foodborne Pathog. Dis.* **2012**, *9*, 1071–1076. [CrossRef]
28. Gomes-Neto, N.J.; Luz, I.S.; Franco, O.L.; Magnani, M.; Souza, E.L. Tolerance evaluation in *Salmonella enterica* serovar Typhimurium challenged with sublethal amounts of *Rosmarinus officinalis* L. essential oil or 1,8-cineole in meat model. *Int. J. Food Sci. Tech.* **2014**, *49*, 1912–1917. [CrossRef]
29. Silva da Luz, I.; Gomes Neto, N.J.; Tavares, A.G.; Nunes, P.C.; Magnani, M.; de Souza, E.L. Lack of induction of direct protection or cross-protection in *Staphylococcus aureus* by sublethal concentrations of *Origanum vulgare* L. essential oil and carvacrol in a meat-based medium. *Arch. Microbiol.* **2013**, *195*, 587–593. [CrossRef]
30. Chueca, B.; Renzoni, A.; Berdejo, D.; Pagan, R.; Kelley, W.L.; Garcia-Gonzalo, D. Whole-genome sequencing and genetic analysis reveal novel stress responses to individual constituents of essential oils in *Escherichia coli*. *Appl. Environ. Microbiol.* **2018**, *84*, e02538-17. [CrossRef]
31. Bridges, M.A.; Mattice, M.R. Over two thousand estimations of the pH of representative foods. *J. Dig. Dis.* **1939**, *6*, 440–449. [CrossRef]
32. Blázquez, J. Hypermutation as a factor contributing to the acquisition of antimicrobial resistance. *Clin. Infect. Dis.* **2003**, *37*, 1201–1209. [PubMed]
33. Chambers, H.F.; Deleo, F.R. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat. Rev. Microbiol.* **2009**, *7*, 629–641. [CrossRef] [PubMed]
34. Kubicek-Sutherland, J.Z.; Lofton, H.; Vestergaard, M.; Hjort, K.; Ingmer, H.; Andersson, D.I. Antimicrobial peptide exposure selects for *Staphylococcus aureus* resistance to human defence peptides. *J. Antimicrob. Chemother.* **2017**, *72*, 115–127. [CrossRef] [PubMed]
35. Bouyahya, A.; Abrini, J.; Dakka, N.; Bakri, Y. Essential oils of *Origanum compactum* increase membrane permeability, disturb cell membrane integrity, and suppress quorum-sensing phenotype in bacteria. *J. Pharm. Anal.* **2019**, *9*, 301–311. [CrossRef] [PubMed]
36. Vázquez-Sánchez, D.; Cabo, M.L.; Rodríguez-Herrera, J.J. Antimicrobial activity of essential oils against *Staphylococcus aureus* biofilms. *Food Sci. Technol. Int.* **2015**, *21*, 559–570. [CrossRef]
37. Kavanaugh, N.L.; Ribbeck, K. Selected antimicrobial essential oils eradicate *Pseudomonas* spp. and *Staphylococcus aureus* biofilms. *Appl. Environ. Microbiol.* **2012**, *78*, 4057–4061. [CrossRef]
38. Ragno, R.; Papa, R.; Patsilinakos, A.; Vrenna, G.; Garzoli, S.; Tuccio, V.; Fiscarelli, E.; Selan, L.; Artini, M. Essential oils against bacterial isolates from cystic fibrosis patients by means of antimicrobial and unsupervised machine learning approaches. *Sci. Rep.* **2020**, *10*, 2653. [CrossRef]
39. Fan, C.; Li, Z.; Yin, H.; Xiang, S. Structure and function of allophanate hydrolase. *J. Biol. Chem.* **2013**, *288*, 21422–21432. [CrossRef]
40. Lin, Y.; St Maurice, M. The structure of allophanate hydrolase from *Granulibacter bethesdensis* provides insights into substrate specificity in the amidase signature family. *Biochemistry (Moscow)* **2013**, *52*, 690–700. [CrossRef]
41. Shapir, N.; Sadowsky, M.J.; Wackett, L.P. Purification and characterization of allophanate hydrolase (AtzF) from *Pseudomonas* sp. strain ADP. *J. Bacteriol.* **2005**, *187*, 3731–3738. [CrossRef]
42. Cheng, G.; Shapir, N.; Sadowsky, M.J.; Wackett, L.P. Allophanate hydrolase, not urease, functions in bacterial cyanuric acid metabolism. *Appl. Environ. Microbiol.* **2005**, *71*, 4437–4445. [CrossRef] [PubMed]
43. Juttukonda, L.J.; Chazin, W.J.; Skaar, E.P. *Acinetobacter baumannii* coordinates urea metabolism with metal import to resist host-mediated metal limitation. *MBio* **2016**, *7*, e01475-16. [CrossRef] [PubMed]
44. Lu, S.; Wang, J.; Chitsaz, F.; Derbyshire, M.K.; Geer, R.C.; Gonzales, N.R.; Gwadz, M.; Hurwitz, D.I.; Marchler, G.H.; Song, J.S.; et al. CDD/SPARCLE: The conserved domain database in 2020. *Nucleic Acids Res.* **2020**, *48*, D265–D268. [CrossRef] [PubMed]

45. Desai, J.; Liu, Y.-L.; Wei, H.; Liu, W.; Ko, T.-P.; Guo, R.-T.; Oldfield, E. Structure, function, and inhibition of *Staphylococcus aureus* heptaprenyl diphosphate synthase. *ChemMedChem* **2016**, *11*, 1915–1923. [CrossRef] [PubMed]
46. Chueca, B.; Pagán, R.; García-Gonzalo, D. Oxygenated monoterpenes citral and carvacrol cause oxidative damage in *Escherichia coli* without the involvement of tricarboxylic acid cycle and Fenton reaction. *Int. J. Food Microbiol.* **2014**, *189*, 126–131. [CrossRef] [PubMed]
47. Yang, S.-K.; Yusoff, K.; Thomas, W.; Akseer, R.; Alhosani, M.S.; Abushelaibi, A.; Lim, S.-H.-E.; Lai, K.-S. Lavender essential oil induces oxidative stress which modifies the bacterial membrane permeability of carbapenemase producing *Klebsiella pneumoniae*. *Sci. Rep.* **2020**, *10*, 819. [CrossRef] [PubMed]
48. Berti, A.D.; Shukla, N.; Rottier, A.D.; McCrone, J.S.; Turner, H.M.; Monk, I.R.; Baines, S.L.; Howden, B.P.; Proctor, R.A.; Rose, W.E. Daptomycin selects for genetic and phenotypic adaptations leading to antibiotic tolerance in MRSA. *J. Antimicrob. Chemother.* **2018**, *73*, 2030–2033. [CrossRef] [PubMed]
49. Davis, M.S.; Solbiati, J.; Cronan, J.E., Jr. Overproduction of acetyl-CoA carboxylase activity increases the rate of fatty acid biosynthesis in *Escherichia coli*. *J. Biol. Chem.* **2000**, *275*, 28593–28598. [CrossRef] [PubMed]
50. Neidhardt, F.C.; Curtiss, R. *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 1st ed.; American Society for Microbiology: Washington DC, USA, 1988; Volume 2, pp. 463–464.
51. Freiberg, C.; Brunner, N.A.; Schiffer, G.; Lampe, T.; Pohlmann, J.; Brands, M.; Raabe, M.; Häbich, D.; Ziegelbauer, K. Identification and characterization of the first class of potent bacterial acetyl-coa carboxylase inhibitors with antibacterial activity. *J. Biol. Chem.* **2004**, *279*, 26066–26073. [CrossRef]
52. Meades, G., Jr.; Henken, R.L.; Waldrop, G.L.; Rahman, M.M.; Gilman, S.D.; Kamatou, G.P.; Viljoen, A.M.; Gibbons, S. Constituents of cinnamon inhibit bacterial acetyl CoA carboxylase. *Planta Med.* **2010**, *76*, 1570–1575. [CrossRef]
53. Sullivan, G.J.; Delgado, N.N.; Maharjan, R.; Cain, A.K. How antibiotics work together: Molecular mechanisms behind combination therapy. *Curr. Opin. Microbiol.* **2020**, *57*, 31–40. [CrossRef] [PubMed]
54. Tyers, M.; Wright, G.D. Drug combinations: A strategy to extend the life of antibiotics in the 21st century. *Nat. Rev. Microbiol.* **2019**, *17*, 141–155. [CrossRef] [PubMed]
55. CLSI. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically Approved Standard—Tenth Edition*; CLSI Doc. M07-A10; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2015.
56. Lallemand, E.A.; Lacroix, M.Z.; Toutain, P.-L.; Boullier, S.; Ferran, A.A.; Bousquet-Melou, A. In vitro degradation of antimicrobials during use of broth microdilution method can increase the measured minimal inhibitory and minimal bactericidal concentrations. *Front. Microbiol.* **2016**, *7*, 2051. [CrossRef] [PubMed]
57. Zwietering, M.H.; Jongenburger, I.; Rombouts, F.M.; van't Riet, K. Modeling of the bacterial growth curve. *Appl. Environ. Microbiol.* **1990**, *56*, 1875–1881. [CrossRef]
58. CLSI. *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-fourth Informational Supplement*; CLSI Doc. M100-S24; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2014.
59. Aubry-Damon, H.; Soussy, C.J.; Courvalin, P. Characterization of mutations in the *rpoB* gene that confer rifampin resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **1998**, *42*, 2590–2594. [CrossRef]
60. Rosche, W.A.; Foster, P.L. Determining mutation rates in bacterial populations. *Methods* **2000**, *20*, 4–17. [CrossRef]
61. Diep, B.A.; Gill, S.R.; Chang, R.F.; Phan, T.H.; Chen, J.H.; Davidson, M.G.; Lin, F.; Lin, J.; Carleton, H.A.; Mongodin, E.F.; et al. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. *Lancet (Lond. Engl.)* **2006**, *367*, 731–739. [CrossRef]
62. Li, H.; Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics (Oxf. Engl.)* **2010**, *26*, 589–595. [CrossRef]
63. Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N.; Marth, G.; Abecasis, G.; Durbin, R. The sequence alignment/map format and SAMtools. *Bioinformatics (Oxf. Engl.)* **2009**, *25*, 2078–2079. [CrossRef]
64. Cingolani, P.; Platts, A.; Wang le, L.; Coon, M.; Nguyen, T.; Wang, L.; Land, S.J.; Lu, X.; Ruden, D.M. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. *Fly (Austin)* **2012**, *6*, 80–92. [CrossRef]

Supplemental material

Table S1. A (maximum OD_{595}), μ_m (maximum specific growth rate; h^{-1}) and λ (lag time; h) values and error standard of the modified Gompertz model obtained from 3 independently growth curves of *Staphylococcus aureus* subsp. *aureus* USA300_FPR3757 (SaWT) (A) and SaROEO at different concentrations of OEO. The goodness of the fit is shown by R^2 and adjusted R^2 values and the root mean square error (*RMSE*).

A) SaWT

OEO (µL/L)	Values			Standard error			Goodness of fit		
	A	μ_{max}	λ	A	μ_{max}	λ	R^2	Adj. R^2	*RMSE*
0	1.294	0.248	2.902	0.012	0.019	0.228	0.9841	0.9832	0.0550
250	1.276	0.238	6.468	0.014	0.015	0.215	0.9897	0.9891	0.0517
500	1.293	0.229	8.239	0.013	0.011	0.162	0.9942	0.9938	0.0407
750	1.324	0.181	11.410	0.030	0.008	0.170	0.9920	0.9916	0.0470
1000	1.262	0.183	15.000	0.032	0.006	0.099	0.9964	0.9961	0.0251
1250	1.185	0.161	17.430	0.038	0.008	0.149	0.9893	0.9887	0.0295

B) SaROEO

OEO (µL/L)	Values			Standard error			Goodness of fit		
	A	μ_{max}	λ	A	μ_{max}	λ	R^2	Adj. R^2	*RMSE*
0	1.268	0.254	3.319	0.010	0.018	0.191	0.9888	0.9881	0.0465
250	1.287	0.217	4.776	0.013	0.014	0.215	0.9895	0.9889	0.0494
500	1.295	0.218	6.311	0.019	0.015	0.251	0.9871	0.9863	0.0576
750	1.271	0.244	6.876	0.018	0.021	0.271	0.9835	0.9825	0.0660
1000	1.280	0.2518	7.884	0.018	0.020	0.243	0.9862	0.9854	0.0614
1250	1.280	0.240	7.761	0.017	0.017	0.226	0.9888	0.9881	0.0555
1500	1.272	0.210	8.062	0.019	0.014	0.237	0.9882	0.9875	0.0564
1750	1.254	0.237	8.447	0.019	0.019	0.236	0.9858	0.9850	0.0622
2000	1.246	0.244	8.563	0.018	0.019	0.225	0.9850	0.9842	0.0636
2500	1.212	0.250	9.482	0.023	0.019	0.240	0.9848	0.9839	0.0624
3000	1.198	0.227	9.516	0.019	0.017	0.204	0.9868	0.9860	0.0581
4000	1.112	0.208	10.300	0.020	0.015	0.196	0.9893	0.9887	0.0485
5000	1.052	0.193	10.850	0.014	0.010	0.139	0.9953	0.9950	0.0316

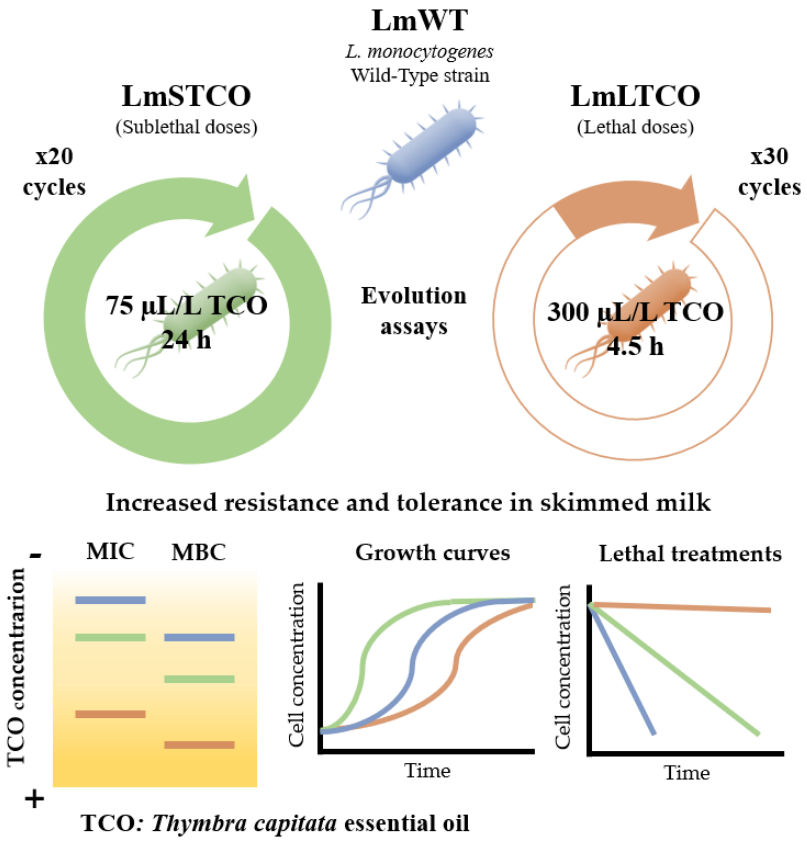
Table S2. Primers used for PCR amplification and Sanger sequencing to verify the mutations in SaROEO.

SaROEO mutations	Forward primer (5' → 3')	Reverse primer (5' → 3')
SAUSA300_RS03770	ATCGCATGGGGATGATGCTT	TGGGCTACAACTCATATTTGCTT
SAUSA300_RS05495	GCACCGACTAGTACCGCATT	GTACAGCCCCCTCAAGCATT
hepT	AAATCACTTCCGACCGGCTT	CGTAGTTTTACAGGGGGCGT
accA	ACCGCCAAGTGGTTCAGAAA	TTGCGCGTTTGCAAGAAAGA

Manuscrito VI. Emerging mutant populations of *Listeria monocytogenes* EGD-e under selective pressure of *Thymbra capitata* essential oil question its use in food preservation

Berdejo, D., Pagán, E., Merino, N., García-Gonzalo, D., Pagán, R.

Food Research International.



Title: Emerging mutant populations of *Listeria monocytogenes* EGD-e under selective pressure of *Thymbra capitata* essential oil question its use in food preservation

Author names: Daniel Berdejo, Elisa Pagán, Natalia Merino, Diego García-Gonzalo and Rafael Pagán*

Affiliation: Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain

***Corresponding author**: Dr. Rafael Pagán Tomás
E-mail: pagan@unizar.es
Address: Dpto. PACA. Facultad de Veterinaria. Universidad de Zaragoza.
C/ Miguel Servet, 177, 50013, Zaragoza, Spain.
Phone number: 34-976-762675 **Fax. number:** 34-976-761590

Highlights

T. capitata EO treatments lead to the emergence of resistant strains.
Stable mutations in *L. monocytogenes* are the cause of the increased resistance.
Mutation in *plsC* gene is involved in increased resistance to *T. capitata* EO.
Strains isolated by *T. capitata* EO also show cross-resistance to antibiotics.
Evolved strains show increased resistance and tolerance in skimmed milk.

Abstract

Due to their excellent antimicrobial properties, essential oils (EO) have been proposed as potential preservatives for certain kinds of foods, such as dairy products. However, the occurrence of pathogenic populations that are resistant to EOs could pose a health risk. This report seeks to assess the emergence of resistant populations in *Listeria monocytogenes* EGD-e growth at 37 °C under selective pressure of *Thymbra capitata* EO (TCO), to characterise their resistance in laboratory media, and to identify their genotypic changes, as well as to evaluate the resistance in skimmed milk. TCO cyclic treatment allowed the isolation of two *L. monocytogenes* EGD-e resistant strains against the EO: LmSTCO by sublethal doses (75 µL/L TCO) and LmLTCO by lethal doses (300 µL/L TCO) after 20 and 30 cycles, respectively. Both strains displayed an increase of the minimum inhibitory and bactericidal concentration against TCO and a higher survival rate after lethal treatments than the wild-type strain (LmWT). Growth kinetics revealed a better adaptation of LmSTCO in presence of TCO, while LmLTCO grew more slowly compared to LmWT, even in the absence of the antimicrobial. Moreover, a slight increase in cross-resistance to antibiotics was observed: LmSTCO to β-lactams and LmLTCO to a series of broad-spectrum antibiotics. The genomic study revealed one sole nucleotide change in LmSTCO located in *plsC* gene codifying an enzyme involved in the production of phosphatidic acid, a precursor in cell membrane synthesis. Five genetic variations were found in LmLTCO: among them, the deletion of an ATP-synthesis system involved in slowing bacterial growth. Inhibition and inactivation assays in skimmed milk confirmed the increased resistance of both strains, thereby indicating a safety risk in case these strains emerge in the food chain. These results strongly suggest that the occurrence of such resistances should be taken into account in order to ensure the efficacy of natural antimicrobials in the design of food preservation strategies.

Keywords

Listeria monocytogenes, *Thymbra capitata* essential oil, Evolution assays, Genotypic resistance and tolerance, Skimmed milk.

1. Introduction

Natural antimicrobials are emerging as an alternative to chemically synthesized food preservatives, which are subject to greater restrictions and are increasingly rejected by consumers (Carocho, Barreiro, Morales, & Ferreira, 2014). Essential oils (EOs) and their individual constituents (ICs) have demonstrated excellent antimicrobial and antioxidant properties (Faleiro & Miguel, 2020) that point toward their potential use in the food industry (Quinto et al., 2019). Their current use in food preservation is nevertheless limited by some drawbacks. Due to the strong organoleptic properties of EOs and ICs, the current required doses lead to an undesirable change of taste and smell in most foods, leading to their rejection by the consumer (Espina, García-Gonzalo, & Pagán, 2014). Many studies thus focus on understanding their antimicrobial activity in order to improve it and thereby reduce the doses required in food preservation (Falleh, Ben Jemaa, Saada, & Ksouri, 2020). On the other hand, the increase in antimicrobial resistance (AMR), mainly against antibiotics (Peterson & Kaur, 2018), has called into question the long-term effectiveness of EOs and ICs, and raised the question whether resistance to natural antimicrobials could also appear. The antioxidant activity of EOs and ICs at low doses (Hashemi, Khorram, & Sohrabi, 2017) has been associated with a low mutagenic rate in bacteria (Chueca, Berdejo, Gomes-Neto, Pagán, & García-Gonzalo, 2016; Hammer, Carson, & Riley, 2008) through the neutralisation of reactive oxygen species (ROS), one of the main causes of DNA damage (Sakai, Nakanishi, Yoshiyama & Maki, 2006). This, in turn, suggested that the occurrence of mutations caused by the application of EOs and ICs would be unlikely (Leite de Souza, 2016). Recent studies have shown, however, that IC treatments can lead to the emergence of resistant and tolerant strains. Prolonged evolution assays at sublethal and lethal doses demonstrated the emergence of resistant and tolerant strains against ICs, such as carvacrol, citral and limonene oxide, in food pathogens: *Escherichia coli* (Chueca et al., 2016), *Salmonella enterica* (Berdejo, Merino, Pagán, García-Gonzalo, & Pagán, 2020) and *Staphylococcus aureus* (Berdejo et al., 2019). It should be noted that "resistance" is the ability of bacteria to replicate and not just survive in the presence of a drug, whereas "tolerance" is the general ability of a population to survive longer treatments (Balaban et al., 2019).

The development of resistance against EOs had been discarded in view of their great complexity and compositional variety and, therefore, to the multitude of antimicrobial action mechanisms that their ICs can exert on bacteria (Lingan, 2018). For instance,

Thymbra capitata EO is one of the EOs with the best antimicrobial properties and is composed of more than 28 different ICs (Gagliano Candela, Maggi, Lazzara, Rosselli, & Bruno, 2019). Nevertheless, the development of resistance and tolerance against a complex EO, *Citrus sinensis,* has recently been observed in *S. aureus* (Berdejo, Pagán, Merino, Pagán, & García-Gonzalo, 2020), and even some of these evolved strains not only showed resistance to natural antimicrobials, but also developed cross-resistance to a wide range of antibiotics (Berdejo, Merino, et al., 2020; Chueca et al., 2018). These results would support the assumption that the mutations selected by the selective pressure exerted by EOs and ICs may also be associated with resistance to antibiotics.

The specific conditions under which resistance to natural antimicrobials can occur are unknown, as well as whether it may also occur in other foodborne pathogens, such as *Listeria monocytogenes*. However, elucidating which mutations occur and which stress response or metabolic pathways are affected would allow us to gain a better understanding of the mechanisms of cellular response to natural antimicrobials, thereby revealing their main modes of action and leading to safer, more efficient food preservation strategies.

We therefore carried out this study with the following goals: a) to assess whether the use of *Thymbra capitata* EO applied at sublethal or lethal doses could lead to the emergence of resistant or tolerant strains in *Listeria monocytogenes* EGD-e, b) to describe their direct resistance and tolerance to TCO, as well as cross-resistance to antibiotics, c) to identify their genetic changes in comparison to wild-type and d) to assess the magnitude of the increased resistance and tolerance in skimmed milk.

2. Materials and Methods

2.1. Microorganisms, growth conditions, and reagents

We selected *L. monocytogenes* EGD-e for our study of genetic evolution because this strain has been completely sequenced and characterized in detail (Glaser et al., 2001; Toledo-Arana et al., 2009). *L. monocytogenes* EGD-e was kindly provided by Prof. Chakraborty (Institute for Medical Microbiology, Giessen, Germany). Throughout this investigation, the strain was kept in cryovials at -80 ºC with glycerol (20% v/v), from which plates of tryptone soya agar (Oxoid, Basingstoke, England) with 0.6% yeast extract (Oxoid; TSAYE) were prepared on a weekly basis. To prepare the working bacterial cultures, test tubes containing 5 mL of tryptone soya broth (Oxoid) with 0.6% yeast extract (TSBYE) were inoculated with one colony and then incubated aerobically

overnight on an orbital shaker (130 rpm; Heidolph Vibramax 100, Schwaback, Germany) at 37 ºC (Incubig, Selecta, Barcelona, Spain). Subsequently, flasks containing 10 mL of fresh TSBYE were inoculated with the resulting subculture to achieve an initial concentration of 10^6 colony forming units per mL (CFU/mL), and incubated for 24 h at 37 ºC and 130 rpm until stationary growth phase was reached (2×10^9 CFU/mL approximately). We applied the same protocol to obtain the working bacterial cultures of the isolated strains that resulted from the evolution assays with *T. capitata* essential oil (TCO).

TCO was kindly provided by the TELIC Group (Barcelona, Spain). This EO was kept in the dark at 4 °C in sealed glass bottles. The composition of this batch of TCO was previously analysed by Merino et al. (2019): 73.8% carvacrol, 9.2% *p*-cymene, 5.3% γ-terpinene, 2.0% (*E*)-caryophyllene, and 9.7% other compounds.

UHT skimmed milk (Central Lechera Asturiana, Asturias, Spain) was purchased in supermarket; to ensure sterility, a new bottle was opened before each experiment.

2.2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC determination was performed according to CLSI (2015) with some modifications due to hydrophobicity of the EO. From the bacterial cultures, test tubes with 5 mL of cation-adjusted mueller hinton broth (MHB; Sigma-Aldrich) were inoculated in presence of different concentrations of TCO: from 0 to 500 µL/L, based on previous experiments (results not shown). Following the method described by Friedman, Henika & Mandrell (2002), a vigorous shaking by vortex was used to prepare TCO dispersions in MHB, avoiding the use of solvents for their possible detriment in the antibacterial activity. Once tubes were incubated for 24 h, MIC was determined as the lowest concentration of the antimicrobial compound that was capable to avoid bacterial growth. To determine objectively bacterial growth, the optical density was read at 595 nm (OD_{595}) using a microplate reader (Genios, Tecan, Männedorf, Switzerland). 10% of the OD_{595} measure of the positive control was established as the lower limit to consider that bacterial strain was grown (Kohanski, DePristo, and Collins, 2010). The minimum bactericidal concentration (MBC) of TCO was evaluated in parallel to MIC test. From the test tubes employed in the MIC determination after incubation, 100 µL aliquot of each tube was spread onto mueller hinton agar cation adjusted (Sigma-Aldrich; MHA) plates

and incubated at 37 °C for 48 h. Colonies were counted and the lowest concentration of TCO that killed ≥ 99.9% of the initial bacterial concentration ($5x10^5$ CFU/mL) was defined as the MBC end point

Additionally, MIC and MBC determinations were conducted in a food model, skimmed milk, following the same protocols applying a concentration range from 600 to 1,500 µL/L TCO. Since milk turbidity hinders a correct OD_{595} measurement, MIC was assessed by cell counting of the samples. An increase of 50% of the initial bacterial population (CFU/mL), i.e. one generation in bacterial growth, was established as the minimum to take bacterial growth into consideration. MBC determination in skimmed milk was conducted following the same protocol as in TSBYE.

2.3. TCO evolution assays

The use of EOs in food preservation can lead either to the inhibition of bacterial growth or to bacterial inactivation, depending on the concentration. To obtain mutant *L. monocytogenes* EGD-e strains against TCO, we followed two different protocols in order to simulate bacteriostatic and bactericidal conditions: a) cyclic exposure to prolonged sublethal treatments, and b) cyclic exposure to short lethal treatments.

The first protocol was based on the isolation of strains by prolonged exposure to a sub-inhibitory concentration of TCO during growth phase, applying a methodology adapted from Kohanski, DePristo, and Collins (2010) and Andersson and Hughes (2014). *L. monocytogenes* EGD-e wild-type strain (LmWT) was grown on TSAYE plates for 48 h at 37 ºC. A single colony was inoculated in 5 mL TSBYE and incubated under agitation for 16 h at 37 ºC. This preculture was diluted 1:1,000 into 50 mL TSBYE and incubated for 6 h to obtain an exponential phase culture. From that culture, 5 mL TSBYE were inoculated at an initial bacterial concentration of 10^6 CFU/mL in the presence of 75 µL/L of TCO (1/2x MIC for LmWT). This bacterial suspension was incubated 24 h/37 ºC/130 rpm and, once the stationary phase was reached, the same step was repeated: the previous culture was diluted (10^6 CFU/mL) in 5 mL TSBYE with 75 µL/L TCO and incubated 24 h/37 ºC/130 rpm. This procedure was repeated 20 times. An aliquot was then diluted in phosphate buffered saline (Sigma-Aldrich, Steinheim, Westphalia, Germany; PBS) and spread on TSAYE plates (without TCO). After the incubation period, 5 colonies ($LmSTCO_{1-5}$) were randomly selected to carry out phenotypic and genotypic characterization.

The second protocol was based on the isolation of strains by recovering survivors after lethal TCO treatments. This methodology was adapted from Levin-Reisman et al. (2017). A stationary phase culture of LmWT was diluted 1:100 in 50 mL fresh TSBYE with 300 µL/L of TCO (2x MIC for LmWT) for 4.50 h at 37 ºC. Treated cells were centrifuged for 20 min at 15,000 relative centrifuge force (RCF), washed twice with TSBYE, resuspended in 1 mL TSBYE, and incubated overnight at 37 ºC. This procedure was repeated 30 times. An aliquot was then diluted in PBS and spread on TSAYE plates (without TCO), from which 5 strains ($LmLTCO_{1-5}$) were randomly selected to carry out phenotypic and genotypic characterization.

Once the 5 strains had been isolated by each evolution assay, $LmSTCO_{1-5}$ and $LmLTCO_{1-5}$, were obtained; the first approach to evaluate their resistance and tolerance was to determine the MIC and the MBC of TCO and to compare it with those of LmWT.

2.4. Growth curves in presence of TCO

In order to study the behaviour of the isolated strains against TCO, the growth kinetics of LmWT and of evolved strains were evaluated in TSBYE in the presence of different concentrations of TCO following the protocol described by Berdejo, Pagán, et al. (2020): from 0 up to 300 µL/L TCO.

Bacterial growth curves based on OD_{595} of LmWT, LmSTCO, and LmLTCO were graphically displayed and modelled by a modified Gompertz equation (Zwietering, Jongenburger, Rombouts, & van 't Riet, 1990):

$$y = A\exp\{-\exp[(\mu_m e / A)(\lambda - t) + 1]\} \quad \text{(Equation 1)}$$

where y: OD_{595}; t: time (h); A: maximum value reached (OD_{595} max); μ_m: maximum specific growth rate (h^{-1}); λ: lag time (h).

A least-squares adjustment was carried out to build the model and obtain A, μm, and λ values using the Prism program (GraphPad Software, Inc., San Diego, USA). The experiment was prolonged for more than 24 h at high TCO concentrations until reaching stationary phase to allow an optimal adjustment to the growth curve. The adjustment's goodness of fit was evaluated using standard error, R^2 and R^2-adjusted values, and the root mean square error (RMSE).

2.5. Survival curves in the presence of TCO

The tolerance of LmWT and of the evolved strains against TCO was evaluated by lethal treatments following the protocol described by Berdejo, Pagán, et al. (2020). Treatments were performed in citrate-phosphate buffer, also called "McIlvaine buffer", at 25 °C with 150 µL/L TCO, at pH 4,0, and 200 µL/L TCO at pH 7,0. Those pH treatments were chosen as representative of neutral and acid conditions within the usual pH range of food. Treated samples were diluted in PBS and subsequently spread on TSAYE plates, which were incubated for 48 h at 37 ºC. Once survival curves of LmWT and evolved strains were obtained, inactivation kinetics were compared in order to evaluate the increase in tolerance of LmSTCO and LmLTCO against TCO. Next, following the same protocol, lethal treatments were performed in skimmed milk, at 1,600 µL/L TCO for 60 min, to assess the relevance of the increased tolerance of evolved strains in a food model.

2.7. Antibiotic susceptibility test

Agar disk diffusion assay was conducted to test antimicrobial susceptibility according to CLSI (CLSI, 2012, 2014). Following the suggestions for fastidious bacteria (CLSI, 2010), bacterial cultures were grown in MHB supplemented with 2.5% lysed horse blood (Sigma-Aldrich). Bacterial suspensions were then spread on MHA plates supplemented with 2.5% lysed horse blood and, after 5 min at room temperature, blank disks (Ø: 6.0 mm) (Thermo Scientific™ Oxoid™ Anti-microbial Susceptibility Disk Dispenser, ST6090, Waltham, MA, USA) were placed on the surface of plates and individually impregnated with the antibiotics: 30 µg kanamycin sulphate, 30 µg tetracycline, 30 µg chloramphenicol, 400 µg nalidixic acid sodium, 5 µg rifampicin, 30 µg norfloxacin, 150 µg novobiocin sodium, 10 µg trimethoprim, 10 µg ampicillin, and 150 µg cephalexin (Sigma-Aldrich). These plates were incubated at 37 ºC for 24 h, after which the diameters of the resulting inhibition zones were measured (paper disks included).

2.8. Statistical analysis

Each phenotypic characterization result was obtained from at least 3 independent experiments carried out on different working days with different bacterial cultures. MIC and MBC data correspond to the results obtained from 5 different assays. Growth curve parameters, lethal treatment graphics, and antibiotic susceptibility tests are displayed as the mean ± standard deviation, using the Prism program (GraphPad Software). Data were analysed and submitted to comparison of averages using analysis of variance (ANOVA), followed by *post-hoc Tukey* test and *t*-tests with Prism software, and differences were considered significant if $p \leq 0.05$.

2.9. Whole genome sequencing (WGS) and identification of mutations

From bacterial culture of LmWT and isolated strains: LmSTCO and LmTCO, genomic DNA (gDNA) was extracted using gDNA extraction and purification columns, following the protocol provided in the kit (GeneJET Genomic DNA, Thermo Scientific,Waltham, MA, USA). Illumina technology was used to carry out whole genome sequencing (WGS) of LmWT, LmSTCO, and LmLTCO, on NextSeq equipment at mid output flow, with a total of 2x150 cycles (Illumina; Fasteris, SA, Geneva, Switzerland). Quality control and genetic study was carried out as described by Berdejo, Pagán, et al. (2020). The quality-control-filtered paired-end reads were mapped on the reference genome sequence (National Center for Biotechnology Information; NCBI accession: NC_003210.1): *Listeria monocytogenes* EGD-e (Toledo-Arana et al., 2009). A total of 3.66, 4.31 and 4.55 million of 150 bp-reads were mapped for LmWT, LmSCar and LmLCar, respectively with an average Phred quality score of 33.07, 33.05 and 33.01. Single nucleotide variants (SNVs), short insertion (Ins), deletions (Del), and structural variations (SVs) were identified between LmWT and isolated strains to ascertain the kind of mutations that had occurred during the evolution treatments. The resulting genome sequences were deposited in the Sequence Read Archive (SRA) of NCBI (BioProject ID: PRJNA669703). The accession numbers of the samples are SAMN16457448 (LmWT), SAMN16457449 (LmSTCO), SAMN16457450 (LmLTCO). Finally, specific primers (Table S1) were designed to carry out PCR amplifications, as well as Sanger sequencings to verify the mutations detected in the WGS.

3. Results and Discussion

3.1. Emergence of resistant and tolerant strains by evolution assay with TCO

After carrying out the TCO evolution assays with *L. monocytogenes* EGD-e (LmWT) with two different protocols: cyclic exposure to a) prolonged sublethal doses, and b) short lethal treatments, we selected 5 colonies at random from each lineage, called $LmSTCO_{1-5}$ and $LmLTCO_{1-5}$ respectively. The evolved strains were kept and re-cultivated in absence of the EO to avoid a phenotypic adaption, and hence their behaviour can be associated with genotypic changes. The first assay with the purpose of assessing the emergence of resistant and tolerant strains was the phenotypical characterisation of $LmSTCO_{1-5}$ and $LmLTCO_{1-5}$ against TCO by MIC and MBC determinations, and their comparison with LmWT (Table 1). MIC and MBC results of the 5 colonies selected from the evolution assays were grouped in the same cell because they showed the same values for both parameters.

Table 1. Minimum inhibitory concentration (MIC; µL/L) and minimum bactericidal concentration (MBC; µL/L) of *Thymbra capitata* essential oil (TCO) for *Listeria monocytogenes* EGD-e (LmWT) and evolved strains: $LmSTCO_{1-5}$ (5 strains selected by cyclic exposure to prolonged sublethal doses) and $LmLTCO_{1-5}$ (5 strains selected by cyclic exposure to short lethal treatments). Each value represents the result of 5 different experiments carried out with different bacterial cultures and on different working days.

Strains	MIC (µL/L)	MBC (µL/L)
LmWT	150	200
$LmSTCO_{1-5}$	200	250
$LmLTCO_{1-5}$	300	400

Comparing evolved strains with LmWT, we observe that both strains exhibited higher MIC and MBC values against TCO. On the one hand, all $LmSTCO_{1-5}$ revealed an increase of 33% in MIC, from 150 to 200 µL/L, and of 25% in MBC, from 200 to 250 µL/L. On the other hand, $LmLTCO_{1-5}$ showed even higher resistance and tolerance than $LmSTCO_{1-5}$, reaching a MIC of 300 µL/L and a MBC of 400 µL/L, i.e., a 100% increase for both values compared to LmWT.

We thus observe that *L. monocytogenes* EGD-e evolved during cyclic exposure, both under sub-inhibitory doses and under lethal concentrations of TCO, resulting in an increase in resistance and tolerance against TCO. Previous studies of evolution assays did not evidence any change in bacterial susceptibility in *L. monocytogenes* against ICs such as eugenol and citral (Apolónio, Faleiro, Miguel, & Neto, 2014), or against EOs such as *Rosmanirus officinalis* (Gomes Neto, Luz, Honório, Tavares, & de Souza, 2012) or

Origanum vulgare (Luz, Neto, Tavares, Magnani, & de Souza, 2012). To the best of our knowledge, this is the first report to demonstrate that *L. monocytogenes* can evolve under EO treatments to the point of developing resistant strains. This is the first time that resistant and tolerant strains have been isolated under the application of sublethal and lethal treatments of a complex EO. Until now, increased resistance and tolerance to a complex EO had only been observed in *Staphylococcus aureus*, and only when the antimicrobial was applied at subinhibitory concentrations (Berdejo, Pagán, et al., 2020). These findings indicate that the use of TCO as a food preservative can permit the emergence of mutant subpopulations with either increased resistance and/or tolerance to EOs.

In addition, it is likely that the bacterial populations after the two evolution assays were homogeneous, since the five isolated colonies showed the same increase in MIC and MBC against TCO. These results would suggest that the mutations occurred during the evolution assays and were maintained due to selective TCO pressure before finally becoming fixed in the bacterial population. For this reason, we carried out phenotypic and genotypic characterization on only one of the 5 strains from each lineage, from here onward referred to as LmSTCO and LmLTCO. It should be noted that the temperatures employed during bacterial growth in both evolution assays may influence the mutagenic rate. In this regard, higher temperatures during incubation could favour the emergence of resistant genetic variants (Chu et al, 2018).

3.2. Better adaptation and growth of LmSTCO than LmWT in the presence of TCO

Growth kinetics studies were carried out in the presence of TCO to characterise the adaptation of evolved strains to the EO. By least-squares adjustment model, growth curves were obtained with excellent goodness of fit (Table S2). Figure 1 displays the growth curves modelled by Gompertz modified equation obtained from LmWT, LmSTCO, and LmLTCO in presence of varying concentrations of TCO: from 0 to 300 µL/L. As can be seen in Figure 1A, the presence of TCO in the growth medium mainly affected the lag phase, and, to a lesser extent, the growth rate of LmWT, as well as that of the evolved strains. Moreover, concentrations higher than 150 µL/L caused a decrease of the maximum bacteria concentrations achieved in the stationary phase of LmSTCO and LmLTCO.

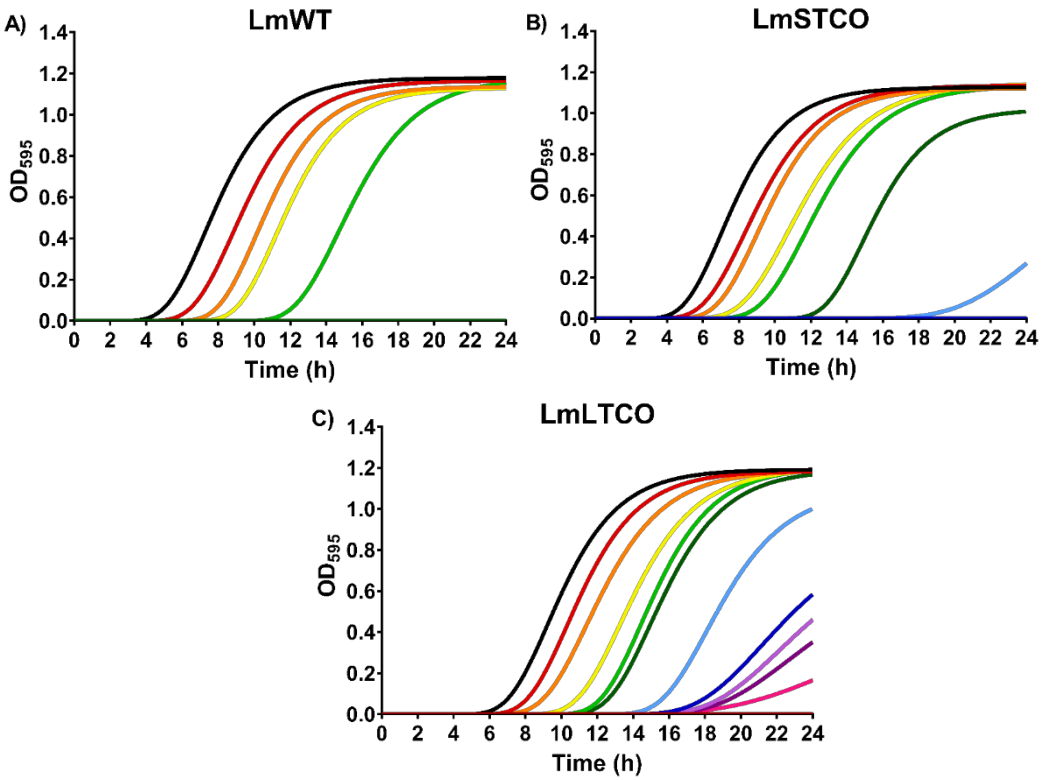


Figure 1. Growth curves of *Listeria monocytogenes* EGD-e wild type (A; LmWT) and evolved strains: LmSTCO (B; by cyclic exposure to prolonged sublethal doses) and LmLTCO (C; by cyclic exposure to short lethal treatments), in the absence (—) and presence of 50 (—), 75 (—), 100 (—), 125 (—), 150 (—), 175 (—), 200 (—),225(—), 250 (—), 275 (—) or 300 (—) of *Thymbra capitata* essential oil (TCO). Growth curves were modelled using the modified Gompertz equation (Eq. 1). Concentrations above the MIC were tested but they are not displayed to facilitate the visualization of the data.

Table 2 displays the values of the parameters A (maximum OD_{595}), μm (maximum specific growth rate) and λ (lag phase), obtained from the models of the three strains at all the tested concentrations. The A parameter revealed that there are no significant differences ($p>0.05$) in the bacterial concentration reached in the stationary phase for LmWT, LmSTCO, and LmLTCO when grown in the absence or in the presence of TCO up to a concentration of 125 µL/L. Nevertheless, there was a significant decrease ($p<0.05$) at concentrations above 125 µL/L: from 1.126 to 0.996 (OD_{595}) for LmSTCO at 175 µL/L TCO, and from 1.192 to 0.715 (OD_{595}) for LmLTCO at 275 µL/L TCO. Similar results were obtained for the μm parameter: LmWT showed no variation based on TCO concentration, and only the evolved strains showed a decrease in growth rate as EO concentration increased. The greatest differences were observed in the λ parameter both among the concentrations applied and between the evolved strains and LmWT. The three strains showed remarkable growth delay ($p<0.05$) as the TCO concentration increased. For instance, LmWT increased its lag phase from 5.149 h to 12.280 h when TCO was

added at a concentration of 125 µL/L. Comparing the evolved strains with LmWT, significant differences were observed in the lag phase at different concentrations of TCO *(p*<0.05). On the one hand, LmSTCO exhibited a growth behaviour similar to LmWT in absence of TCO; however, when TCO was added to medium, the lag phase of LmSTCO was shorter than that of LmWT (from 50 to 125 µL/L). On the other hand, the growth behaviour of LmLTCO was completely different from the other evolved strain. LmLTCO displayed a growth delay in presence of TCO compared to LmWT, but also in absence of the EO.

Previous studies on *L. monocytogenes* have also shown a slower growth rate and a higher lag phase as the concentration of thyme EO in the medium increased (Braschi et al., 2018). The EOs caused an alteration of cell membrane integrity and increased the membrane permeability of bacteria, thus prolonging adaptation and lag phase time (Bouyahya, Abrini, Dakka, & Bakri, 2019). *S. aureus* resistant to orange EO also exhibited a decrease in lag phase time in comparison with the wild-type strain (Berdejo, Pagán, et al., 2020), similarly to what we have observed regarding LmSTCO (Fig. 1B).

Table 2. A (maximum OD_{595}), μ_m (maximum specific growth rate) and λ (lag time) parameters of the modified Gompertz model obtained from growth curves of *Listeria monocytogenes* EGD-e (LmWT) and evolved strains: LmSTCO (by cyclic exposure to prolonged sublethal doses) and LmLTCO (by cyclic exposure to short lethal treatments), at 37 °C in TSBYE under continuous agitation (130 rpm) with different concentrations of *Thymbra capitata* essential oil (TCO).

A **(OD_{595})**		**Strains**		
		LmWT	**LmSTCO**	**LmLTCO**
TCO (µL/L)	**0**	1.178 ± 0.035[a]	1.126 ± 0.031[a]	1.192 ± 0.039[a]
	50	1.165 ± 0.029[a]	1.136 ± 0.030[a]	1.184 ± 0.040[a]
	75	1.136 ± 0.031[a]	1.122 ± 0.030[a]	1.189 ± 0.032[a]
	100	1.130 ± 0.033[a]	1.145 ± 0.039[a]	1.198 ± 0.043[a]
	125	1.180 ± 0.040[a]	1.137 ± 0.031[a]	1.205 ± 0.045[a]
	150		1.021 ± 0.034[b]	1.192 ± 0.038[a]†
	175		0.996 ± 0.021[b]	1.099 ± 0.023[a]†
	200			0.920 ± 0.029[b]
	225			0.851 ± 0.036[b]
	250			0.724 ± 0.039[c]
	275			0.715 ± 0.055[c]

μ_m (OD_{595}/h)		Strains		
		LmWT	**LmSTCO**	**LmLTCO**
TCO (µL/L)	**0**	0.203 ± 0.017^{a}	0.204 ± 0.013^{a}	0.199 ± 0.017^{a}
	50	0.194 ± 0.008^{a}	0.182 ± 0.009^{ab}	0.197 ± 0.015^{a}
	75	0.201 ± 0.010^{a}	0.189 ± 0.010^{ab}	0.183 ± 0.007^{ab}
	100	0.198 ± 0.010^{a}	0.167 ± 0.011^{b}	0.186 ± 0.010^{a}
	125	0.180 ± 0.007^{a}	0.172 ± 0.006^{b}	0.198 ± 0.011^{a}
	150		0.181 ± 0.007^{a}	0.190 ± 0.007^{a}
	175		0.075 ± 0.003^{c}	0.162 ± 0.007^{b}†
	200			0.095 ± 0.002^{c}
	225			0.083 ± 0.004^{cd}
	250			0.066 ± 0.009^{de}
	275			0.040 ± 0.004^{e}

λ (h)		Strains		
		LmWT	**LmSTCO**	**LmLTCO**
TCO (µL/L)	**0**	5.149 ± 0.261^{a}	4.984 ± 0.199^{a}	7.047 ± 0.298^{a}†
	50	6.624 ± 0.139^{b}	6.048 ± 0.177^{b}*	8.121 ± 0.262^{b}*†
	75	8.039 ± 0.165^{c}	6.832 ± 0.180^{c}*	9.020 ± 0.130^{c}*†
	100	9.110 ± 0.161^{d}	8.198 ± 0.244^{d}*	10.910 ± 0.182^{d}*†
	125	12.280 ± 0.124^{e}	9.266 ± 0.112^{e}*	12.220 ± 0.162^{e}†
	150		12.820 ± 0.107^{f}	12.652 ± 0.105^{e}
	175		20.430 ± 0.162^{g}	15.590 ± 0.133^{f}†
	200			17.648 ± 0.058^{g}
	225			18.392 ± 0.172^{gh}
	250			18.686 ± 0.481^{h}
	275			19.873 ± 0.513^{i}

Each value represents the mean ± standard deviation from 3 independent experiments. Different superscript letters represent statistically significant differences ($p<0.05$) among the means of the same column. *Significantly different from LmWT ($p<0.05$). †Significantly different from LmSTCO ($p<0.05$).

These results would explain how LmSTCO, the strain evolved by sub-inhibitory doses, could emerge during the evolution assays against LmWT. At the concentration used in the evolution cycles, 75 µL/L TCO, LmSTCO had a lag phase 1.2 h shorter than LmWT. This sub-population might therefore grow better than LmWT, to the point of taking over the culture. In regard to LmLTCO, the kinetics study revealed that its mutations have a fitness cost, and thereby lead to a growth delay in absence of TCO, even though this strain was more resistant and tolerant than LmWT in MIC and MBC

determination. No previous reports have shown that evolved strains with increased resistance and tolerance to natural antimicrobials grow slower than wild-type strain in the presence, and also in the absence, of EO as observed in LmLTCO. This phenomenon is probably caused by the evolution assay protocol we applied to select and isolate LmLTCO. These findings support the assumption that mutations fixed after the two evolution assays would be different and would behave differently.

3.3. No inactivation of LmLTCO at lethal TCO doses for LmWT

To further evaluate and compare the tolerance of the evolved strains with LmWT, lethal TCO treatments were carried out in citrate-phosphate buffer at pH 4.0 and 7.0. Figure 2 shows survival curves of LmWT, LmSTCO, and LmLTCO, after treatments with 150 µL/L TCO at pH 4.0 and 200 µL/L TCO at pH 7.0 for 30 min. Both treatments managed to inactivate more than 5 $\log_{10}$ cycles of LmWT, but not of the evolved strains.

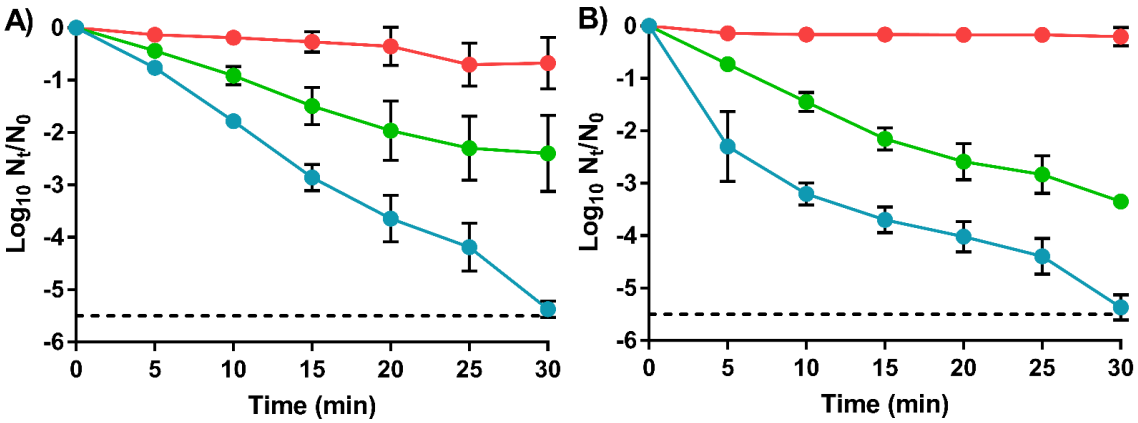


Figure 2. Survival curves of *Listeria monocytogenes* EGD-e wild type (●; LmWT) and evolved strains: LmSTCO (●; by cyclic exposure to prolonged sublethal doses) and LmLTCO (●; by cyclic exposure to short lethal treatments), after lethal treatment of *Thymbra capitata* essential oil (TCO) in citrate-phosphate buffer at pH 4.0 (150 µL/L TCO; A) and at pH 7.0 (200 µL/L TCO; B). Data are means ± standard deviations (error bars) obtained from at least 3 independent experiments. The dashed line represents the detection limit (-5.5 $\log_{10} N_t/N0$).

As can be seen in Figure 2A, only 2.5 and 0.5 $\log_{10}$ cycles of reduction were observed after 30 min of treatment for LmSTCO and LmTCO, respectively, at pH 4.0. Similar increased survival was noted in both evolved strains at neutral conditions. At pH 7.0, LmTCO was the most tolerant: no inactivation was observed in LmLTCO during the experiment, whereas LmSTCO reached a reduction of 3.3 $\log_{10}$ cycles after 30 min. Other studies also revealed an increased tolerance in *E. coli* (Chueca et al., 2016), *S. enterica* (Berdejo, Merino, et al., 2020), and *S. aureus* (Berdejo et al., 2019; Berdejo, Pagán, et al., 2020), after evolution assays with natural antimicrobials.

These data explain how LmLTCO could emerge more favourably along the evolution cycles than LmWT. The increased tolerance of the evolved strain allowed it to better survive lethal treatments than LmWT. It can thus be assumed that LmWT concentration would progressively decrease during the evolution assay until genetic variations of LmLTCO became fixed in the bacterial population.

3.4. Cross-resistance of evolved strains against antibiotics

We determined the susceptibility of LmWT and evolved strains to antibiotics to assess whether cross-resistance to other types of antimicrobials could also occur. Table 3 displays the inhibition halos of LmWT, LmSTCO and LmLTCO against several antibiotics: kanamycin, tetracycline, chloramphenicol, nalidixic acid, rifampicin, norfloxacin, novobiocin, trimethoprim, ampicillin, and cephalexin.

Table 3. Zones of growth inhibition for agar disk diffusion assays of *Listeria monocytogenes* EGD-e (LmWT) and evolved strains: LmSTCO (by cyclic exposure to prolonged sublethal doses) and LmLTCO (by cyclic exposure to short lethal treatments) against antibiotics: 30 µg kanamycin sulfate, 30 µg tetracycline, 30 µg chloramphenicol, 400 µg nalidixic acid sodium, 5 µg rifampicin, 30 µg norfloxacin, 150 µg novobiocin sodium, 10 µg trimethoprim, 10 µg ampicillin, and 150 µg cephalexin. Each value represents the mean diameter of the inhibition halo ± standard deviation (mm) from three independent experiments.

Antibiotics	Strains		
	LmWT	**LmSTCO**	**LmLTCO**
Kanamycin	22.86 ± 0.99	22.20 ± 0.97	19.27 ± 0.75*
Tetracycline	35.43 ± 0.76	35.70 ± 0.63	31.03 ± 0.83*
Chloramphenicol	24.28 ± 0.92	25.02 ± 0.67	22.40 ± 1.27
Nalidixic acid	21.62 ± 1.22	18.09 ± 2.34	19.88 ± 1.14
Rifampicin	33.91 ± 0.97	32.19 ± 1.64	32.36 ± 0.96
Norfloxacin	22.03 ± 1.16	24.74 ± 2.50	19.98 ± 1.21
Novobiocin	31.18 ± 0.41	33.20 ± 1.40	29.43 ± 0.41*
Trimethoprim	35.30 ± 1.05	33.56 ± 1.61	31.47 ± 1.68*
Ampicillin	20.28 ± 0.14	16.65 ± 0.44*	18.21 ± 0.61*
Cephalexin	21.97 ± 1.32	16.89 ± 0.86*	20.41 ± 0.47

* Significantly different from LmWT (p<0.05).

Limited information is provided in CLSI documents (CLSI, 2010, 2012) for testing *Listeria* strains, so concentrations were chosen and adjusted according to Yehia, Elkhadragy, Aljahani, and Alarjani (2020) and previous experiments (data not shown) to achieve inhibition halos higher than 20.0 mm of LmWT, and thus to enhance analysis sensitivity in the study of increased antibiotic resistance in the evolved strains.

LmSTCO only exhibited a significant (p<0.05) decrease of inhibition halos in comparison with LmWT for two antibiotics: ampicillin and cephalexin. Both antibiotics belong to β-lactams, whose main target is the inhibition of cell wall biosynthesis. These results suggest that the key to increased resistance and tolerance to the EO in LmSTCO lies in the cell envelope structure. On the other hand, in LmLTCO, an increase in resistance was observed for several kinds of antibiotics. Kanamycin, tetracycline, novobiocin, trimethoprim and ampicillin caused smaller inhibition halos in LmTCO than in LmWT. This decrease in antibiotic susceptibility could be associated with a general mechanism of defence against antimicrobial compounds. According to Pontes and Groisman (2019), a slow growth rate could lead to antibiotic resistance. It is likely that the delay of LmLTCO growth, previously observed in the absence of TCO, might lead to an increased resistance to a wide range of antimicrobials.

The antibiotic susceptibility tests evidence that strains resistant or tolerant to TCO could also develop a slight increase in cross-resistance to antibiotics. These results indicate that certain of the mechanisms of resistance to EOs could be involved in the response to antibiotics, and that cross-resistance between both types of antimicrobials may occur.

3.5. Genetic variations responsible for the increased resistance and tolerance to TCO

The identification of mutations of the evolved strains was carried out by comparing the genomes between LmWT (origin strain) LmSTCO, and LmLTCO (Figure 3) in order to identify the cause of the increased resistance and tolerance to TCO, as well as to antibiotics.

For this purpose, WGS was conducted and the reads were mapped onto reference genome: *L. monocytogenes* EGD-e (NCBI accession: NC_003210.1) (Toledo-Arana et al., 2009). Mutations in our LmWT were then identified with regard to the reference strain (Table S3) in order to discard them as the cause of increased resistance and tolerance, since they would also be found in the evolved strains. WGS revealed 1 SNV in LmSTCO (Table 4) and 5 genetic variations in LmLTCO: 2 SNVs, 1 Ins, 1 Del and 1 large deletion (Table 5). All mutations observed in WGS were verified by Sanger sequencing.

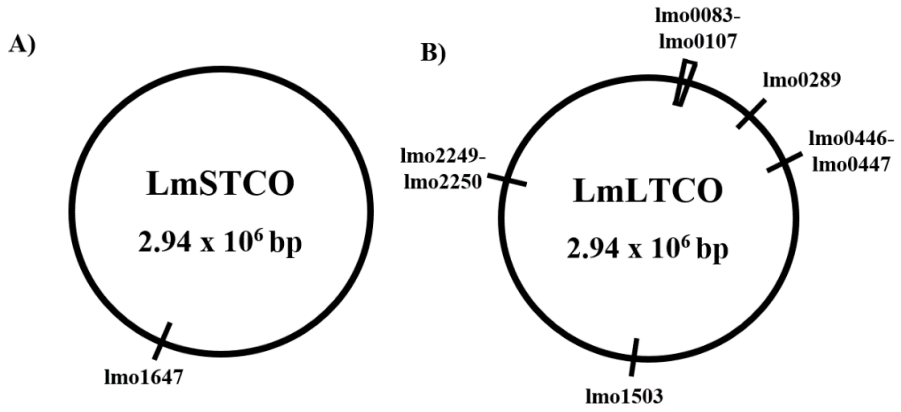


Figure 3. Genomic maps of the *Listeria monocytogenes* EGD-e evolved strains by cyclic exposure to prolonged sublethal doses (LmSTCO; A) and to short lethal treatments (LmLTCO; B) of *Thymbra capitata* essential oil (TCO).

The only LmSTCO mutation was found in *plsC* gene (lmo1647), a replacement of a cytosine by an alanine at position 17. This SNV led to a change from alanine to glutamic acid in the enzyme 1-acylglycerol-3-phosphate O acyltransferase, in the amino acid 6. This enzyme, PlsC, is located in the cell membrane and is involved in the biosynthesis of phosphatidic acid, the central precursor of membrane phospholipids, from acyl-ACP (Yao & Rock, 2013). For this reason, PlsC plays an essential role in the synthesis and repair of cell envelopes in Gram-positive bacteria (Geiger, Sohlenkamp, & López-Lara, 2010). Although there are no data on this gene in relation to resistance to natural antimicrobials, several authors have observed its involvement in antibiotic resistance. According to Sutterlin, Zhang, and Silhavy (2014), the overexpression of *plsC* increases phosphatidic acid, and consequently leads to a protective effect against vancomycin.

Table 4. Mutations of LmSTCO (strain evolved by cyclic exposure to prolonged sublethal doses of *Thymbra capitata* essential oil) in comparison with *Listeria monocytogenes* EGD-e (LmWT), verified by Sanger sequencing. Single nucleotide variation (SNV).

Genome position	Locus tag	Mutation	Change	Information
1,696,296	lmo1647	SNV: C17A*	Ala6Glu	1-acylglycerol-3-phosphate O acyltransferase

***** Position respect to the start of the coding region.

In another study, Li et al. (2015) isolated *Acinetobacter baumanii* strains from evolution assay with tigecycline, and observed that the cause of increased resistance was a mutation in *plsC.* In both studies, the increased resistance occurred against antibiotics targeting the bacterial membrane, as we observed in our study (ampicillin and cephalexin, table 3). Thus, it can be seen that the mutation of the *plsC* gene is responsible for the

selection of LmSTCO and highlights the capital importance of the cell envelope in the bacterial defence response to natural antimicrobials.

Regarding LmLTCO, the 2 SNVs we found were located in two intergenic areas: between lmo0446 and lmo0447, and between lmo2249 and lmo2250, respectively. Neither of the two mutations was present in either coding or regulatory zones; thus, their involvement in the increased resistance of LmLTCO strain to TCO can be discarded. WGS also detected 1 insertion of an alanine in lmo0289 and 1 deletion of an alanine in lmo1503. These two genes code hypothetical proteins whose functions have not been evidenced *in vivo*. Further studies would be necessary to describe their function in *L. monocytogenes* EGD-e, and to ascertain their potential role in bacterial defence against TCO and antibiotics.

Table 5. Mutations of LmLTCO (strain evolved by cyclic exposure to short lethal treatments of *Thymbra capitata* essential oil) in comparison with *Listeria monocytogenes* EGD-e (LmWT), verified by Sanger sequencing. Single nucleotide variation (SNV), insertion (Ins) and deletion (Del).

Genome position	Locus tag	Mutation	Change	Information
86,323-114,201	lmo0083-lmo0107	Large deletion	25 genes deleted	Table S4
313,664	lmo0289	Del: -A13*	Thr5* Frame shift	Hypothetical protein
479,717	lmo0446-lmo0447	SNV: C by T	Intergenic region	Non-coding DNA No regulatory region
1,532,296	lmo1503	Ins: +A105*	Gly36* Frame shift	Hypothetical protein
2,339,533	lmo2249-lmo2250	SNV: T by C	Intergenic region	Non-coding DNA No regulatory region

* Position respect to the start of the coding region.

Finally, 1 large deletion was detected in LmLTCO removing an amount of 25 genes from lmo0083 to lmo0107, coding of MerR family transcriptional regulator, oxidoreductase, 6 subunits of ATP synthases, 3 subunits of mannose transporter, 2 transcriptional regulators, NADH oxidase, chitinase B and ABC transporter (Table S4). In this regard, it was observed that most of the deleted genes have a function in carbohydrate and sugar catabolism, as well as in the synthesis of ATP and energy production. Their loss could explain the results obtained in the phenotypic characterization of LmLTCO. The reduction in energy production would explain the growth delay observed in the growth curves both in the absence and in the presence of varying TCO concentrations, as well as the higher generic resistance and tolerance to

antibiotics (Græsbøll, Nielsen, Toft, & Christiansen, 2014; Pontes & Groisman, 2019), and probably to TCO.

It should be noted that the deletion of the rest of the genes, such as the MerR family regulator related to the response to heavy metals, oxidative stress and antibiotics, or other transcriptional regulators, as well as the other mutations detected, could also mean a variation in the resistance and tolerance of LmLTCO. Moreover, although our evolution assays have resulted in the selection of these resistant strains, it does not mean that every evolution assay will lead to the isolation of the same genetic variants.

3.6. Evolved strains show increased resistance and tolerance to TCO in skimmed milk

A phenotypic characterization of LmSTCO and LmLTCO in a food model was carried out to assess whether these strains could pose a microbiological risk in case they reach the food chain. Their resistance and tolerance of the evolved strain to TCO was determined in skimmed milk as a food matrix because EOs and ICs have been proposed as food preservatives for dairy products due to their antimicrobial, anti-oxidant, and functional properties (Mishra et al., 2020). In this regard, *T. capitata* is one of the most widely studied EOs in milk preservation due to its excellent antimicrobial properties (Ben Jemaa et al., 2017, 2018). Moreover, we chose skimmed milk to avoid the effect of fat on the antimicrobial activity of TCO and, thus, to achieve the greatest sensitivity in our experiment and to ease the observation of the differences in resistance and tolerance between evolved strain and LmWT.

Table 6 shows the MICs and MBCs of TCO for LmWT, LmSTCO, and LmLTCO in skimmed milk. The concentration required to inhibit and to inactivate the growth of the three tested strains was around 4 or 5 times higher than in TSBYE. As in TSBYE, LmWT was the most susceptible strain in skimmed milk, MIC and MBC of 900 µL/L TCO. LmLTCO exhibited the highest MIC and MBC values of TCO: 1,200 µL/L and 1,300 µL/L, respectively, followed by LmSTCO: 1,000 µL/L and 1,200 µL/L. Nevertheless, the increase in resistance and tolerance in skimmed milk against TCO was proportionally lower than in TSBYE. It is therefore likely that certain milk components interfere with the antimicrobial activity of EO and/or modify the resistance of LmWT and evolved strains.

Table 6. Minimum inhibitory concentration (MIC; µL/L) and minimum bactericidal concentration (MBC; µL/L) of *Thymbra capitata* essential oil (TCO) for *Listeria monocytogenes* EGD-e (LmWT) and evolved strains: LmSTCO (by cyclic exposure to prolonged sublethal doses) and LmLTCO (by cyclic exposure to short lethal treatments) in skimmed milk. Each value represents the result of 5 different experiments carried out with different bacterial cultures and on different working days.

Strains	MIC (µL/L)	MBC (µL/L)
LmWT	900	900
LmSTCO	1,000	1,200
LmLTCO	1,200	1,300

For the inactivation treatments, the concentration was increased to 1,600 µL/L TCO because the concentration previously used in McIlvaine buffer was not capable of inactivating any of the 3 strains. As mentioned above, this is due to the protective effect that skimmed milk components may exert. Similarly to lethal treatments in McIlvaine buffer, Figure 4 revealed a higher survival of evolved strains to TCO inactivation treatments compared to LmWT. LmLTCO showed the highest tolerance: less than 1 $\log_{10}$ cycles of inactivation were achieved after 30 min treatment, whereas LmSTCO and LmWT reached a bacterial reduction of 3.6 and 5.5 $\log_{10}$ cycles, respectively.

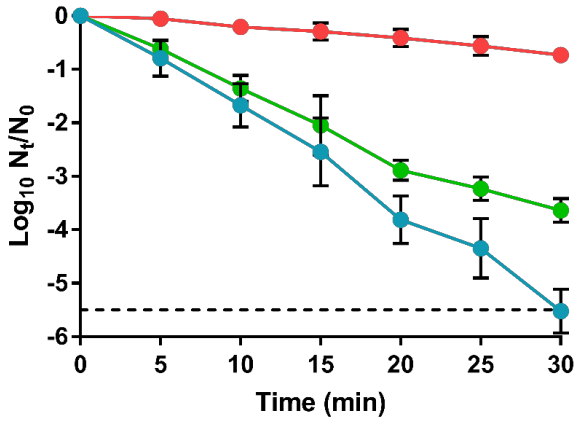


Figure 4. Survival curves of *Listeria monocytogenes* EGD-e wild type (●; LmWT) and evolved strains: LmSTCO (●; by cyclic exposure to prolonged sublethal doses) and LmLTCO (●; by cyclic exposure to short lethal treatments), after lethal treatment of 1,600 µL/L *Thymbra capitata* essential oil (TCO) in skimmed milk at 25 °C. Data are means ± standard deviations (error bars) obtained from at least 3 independent experiments. The dashed line represents the detection limit (-5.5 $\log_{10} N_t/N_0$).

Several authors highlight natural antimicrobials as an effective preservation method for dairy products; no previous studies have evaluated the food safety risk if mutant strains appear. Our results suggest, however, that the contamination of the food chain with these strains could pose a microbiological risk, since the increases in resistance and tolerance to TCO were also observed in food matrices such as skimmed milk.

4. Conclusions

This study evidences the emergence of resistant (LmSTCO) and tolerant (LmLTCO) strains of *L. monocytogenes* EGD-e against a complex essential oil: specifically, *Thymus capitata* (TCO). LmSTCO showed a better adaptation to TCO by decreasing its lag phase when growing in the presence of the antimicrobial. The increased tolerance of LmLTCO allowed it to emerge against wild-type strain LmWT, despite the fact that this had a fitness cost in the presence as well as in the absence of TCO. In addition, both strains developed a slight increased cross-resistance to antibiotics: LmSTCO to β-lactams antibiotics, and LmLTCO to a wide range of broad-spectrum antibiotics. These findings support the relevance of knowing the mechanisms of action of natural antimicrobials as alternative or in combination with antibiotics to combat the multi-drug resistant bacteria.

In LmSTCO, the genomic study identified the mutation in the *plsC* gene, coding an enzyme involved in the biosynthesis of phosphatidic acid, which was responsible for increasing resistance and tolerance to TCO, as well as to β-lactams. A total of 5 genetic changes were found in LmLTCO; among them, the deletion of an ATP synthesis system and energy production which produced a fitness cost and slowed down bacteria growth. This study adds to the knowledge about the mechanism of action of natural antimicrobials; however, it is still necessary to conduct further research with the aim of designing more efficient and safe food preservation strategies.

Our study has likewise shown a large increase in resistance of the evolved strains against TCO compared to the wild-type strain in a food matrix: skimmed milk.

Overall, these results indicate that it would be necessary to consider the emergence of mutant sub-populations in the design of food preservation strategies, since they could represent a microbiological risk due to their ability to grow and survive under conditions established for their corresponding wild-type strains. Further studies are required to understand how resistant strains could appear in the food chain, and to ascertain the real risk to food safety they might represent.

Acknowledgments

This study was financially supported by Agencia Estatal de Investigación (Spain) (Project No. PGC2018-093789-B-I00), by the European Social Fund, and by the Aragonese Office of Science, Technology and University Research. The Spanish Ministry of Sports, Culture and Education provided D. Berdejo and E. Pagán with a grant to carry out this investigation. The authors thank S. Hanks for his collaboration on the English-language revision of this manuscript.

Credit Author Statement

Daniel Berdejo: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing. **Elisa Pagán**: Investigation, Validation. **Natalia Merino**: Investigation, Validation **Diego García-Gonzalo**: Conceptualization, Funding acquisition, Resources, Supervision, Writing. **Rafael Pagán**: Conceptualization, Funding acquisition, Resources, Supervision, Writing.

References

Andersson, D. I., & Hughes, D. (2014). Microbiological effects of sublethal levels of antibiotics. *Nature Reviews: Microbiology, 12* (7), 465-478. https://doi.org/10.1038/nrmicro3270.

Apolónio, J., Faleiro, M. L., Miguel, M. G., & Neto, L. (2014). No induction of antimicrobial resistance in *Staphylococcus aureus* and *Listeria monocytogenes* during continuous exposure to eugenol and citral. *FEMS Microbiology Letters, 354* (2), 92-101. https://doir.org/10.1111/1574-6968.12440.

Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., Brynildsen, M. P., Bumann, D., Camilli, A., Collins, J. J., Dehio, C., Fortune, S., Ghigo, J.-M., Hardt, W.-D., Harms, A., Heinemann, M., Hung, D. T., Jenal, U., Levin, B. R., Michiels, J., Storz, G., Tan, M.-W., Tenson, T., Van Melderen, L., & Zinkernagel, A. (2019). Definitions and guidelines for research on antibiotic persistence. *Nature Reviews Microbiology, 17* (7), 441-448. https://doir.org/10.1038/s41579-019-0196-3.

Ben Jemaa, M., Falleh, H., Neves, M. A., Isoda, H., Nakajima, M., & Ksouri, R. (2017). Quality preservation of deliberately contaminated milk using thyme free and nanoemulsified essential oils. *Food Chemistry, 217*, 726-734. https://doi.org/10.1016/j.foodchem.2016.09.030

Ben Jemaa, M., Falleh, H., Saada, M., Oueslati, M., Snoussi, M., & Ksouri, R. (2018). *Thymus capitatus* essential oil ameliorates pasteurization efficiency. *Journal of Food Science and Technology, 55* (9), 3446-3452. https://doir.org/10.1007/s13197-018-3261-4.

Berdejo, D., Chueca, B., Pagan, E., Renzoni, A., Kelley, W. L., Pagan, R., & Garcia-Gonzalo, D. (2019). Sub-inhibitory doses of individual constituents of essential oils can select for *Staphylococcus aureus* resistant mutants. *Molecules, 24* (1). https://doir.org/10.3390/molecules24010170.

Berdejo, D., Merino, N., Pagán, E., García-Gonzalo, D., & Pagán, R. (2020). Genetic variants and phenotypic characteristics of *Salmonella* Typhimurium-resistant mutants after exposure to carvacrol. *Microorganisms, 8* (6). https://doir.org/10.3390/microorganisms8060937.

Berdejo, D., Pagán, E., Merino, N., Pagán, R., & García-Gonzalo, D. (2020). Incubation with a complex orange essential oil leads to evolved mutants with increased resistance and tolerance. *Pharmaceuticals (Basel), 13* (9). https://doir.org/10.3390/ph13090239.

Bouyahya, A., Abrini, J., Dakka, N., & Bakri, Y. (2019). Essential oils of *Origanum compactum* increase membrane permeability, disturb cell membrane integrity, and suppress quorum-sensing phenotype in bacteria. *Journal of Pharmaceutical Analysis, 9* (5), 301-311. https://doi.org/10.1016/j.jpha.2019.03.001.

Braschi, G., Serrazanetti, D. I., Siroli, L., Patrignani, F., De Angelis, M., & Lanciotti, R. (2018). Gene expression responses of *Listeria monocytogenes* Scott A exposed to sub-lethal concentrations of natural antimicrobials. *International Journal of Food Microbiology, 286*, 170-178. https://doi.org/10.1016/j.ijfoodmicro.2018.07.026.

Carocho, M., Barreiro, M. F., Morales, P., & Ferreira, I. C. F. R. (2014). Adding molecules to food, pros and cons: a review on synthetic and natural food additives. *13* (4), 377-399. https://doir.org/10.1111/1541-4337.12065.

Chu, X.L., Zhang, B.W., Zhang, Q.G., Zhu, B.R., Lin, K., & Zhang, D.Y. (2018). Temperature responses of mutation rate and mutational spectrum in an Escherichia coli strain and the correlation with metabolic rate. BMC evolutionary biology, 18 (1), 126-126.

Chueca, B., Berdejo, D., Gomes-Neto, N. J., Pagán, R., & García-Gonzalo, D. (2016). Emergence of hyper-resistant *Escherichia coli* MG1655 derivative strains after applying sub-Inhibitory doses of individual constituents of essential oils. *Frontiers in Microbiology, 7*, 273-273. https://doi.org/10.3389/fmicb.2016.00273.

Chueca, B., Renzoni, A., Berdejo, D., Pagan, R., Kelley, W. L., & Garcia-Gonzalo, D. (2018). Whole-genome sequencing and genetic analysis reveal novel stress responses to individual constituents of essential oils in *Escherichia coli*. *Applied and Environmental Microbiology, 84* (7). https://doir.org/10.1128/aem.02538-17.

CLSI. (2010). Methods for dilution antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria; approved standard - second edition. CLSI document M45-A2.

CLSI. (2012). Performance standards for antimicrobial disk susceptibility tests; approved standard - Eleventh edition. In (Vol. CLSI document M02-A11). Wayne, PA: Clinical and laboratory standards institute. CLSI document M02-A11

CLSI. (2014). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth INformational Supplement. CLSI Document M100-S24.

CLSI. (2015). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard - Tenth Edition. CLSI document M07-A10.

Espina, L., García-Gonzalo, D., & Pagán, R. (2014). Impact of essential oils on the taste acceptance of tomato juice, vegetable soup, or poultry burgers. *Journal of Food Science, 79* (8), S1575-1583. https://doir.org/10.1111/1750-3841.12529.

Faleiro, M. L., & Miguel, G. (2020). Antimicrobial and antioxidant activities of natural compounds: enhance the safety and quality of food. *Foods (Basel, Switzerland), 9* (9), 1145. https://doir.org/10.3390/foods9091145.

Falleh, H., Ben Jemaa, M., Saada, M., & Ksouri, R. (2020). Essential oils: a promising eco-friendly food preservative. *Food Chemistry, 330*, 127268. https://doi.org/10.1016/j.foodchem.2020.127268.

Friedman, M., Henika, P.R. & Mandrell, R.E. (2002). Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *Journal of Food Protection*, 65, 1545–1560. https://doi.org/10.4315/0362-028X-65.10.1545.

Gagliano Candela, R., Maggi, F., Lazzara, G., Rosselli, S., & Bruno, M. (2019). The essential oil of *Thymbra capitata* and its application as a biocide on stone and derived surfaces. *Plants (Basel, Switzerland), 8* (9), 300. https://doir.org/10.3390/plants8090300.

Geiger, O., Sohlenkamp, C., & López-Lara, I. M. (2010). Formation of bacterial membrane lipids: pathways, enzymes, reactions. In K. N. Timmis (Ed.), *Handbook of Hydrocarbon and Lipid Microbiology* (pp. 395-407). Berlin, Heidelberg: Springer Berlin Heidelberg. https://doir.org/10.1007/978-3-540-77587-4-27.

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., Berche, P., Bloecker, H., Brandt, P., Chakraborty, T., Charbit, A., Chetouani, F., Couvé, E., de Daruvar, A., Dehoux, P., Domann, E., Domínguez-Bernal, G., Duchaud, E., Durant, L., Dussurget, O., Entian, K. D., Fsihi, H., García-del Portillo, F., Garrido, P., Gautier, L., Goebel, W., Gómez-López, N., Hain, T., Hauf, J., Jackson, D., Jones,

L. M., Kaerst, U., Kreft, J., Kuhn, M., Kunst, F., Kurapkat, G., Madueno, E., Maitournam, A., Vicente, J. M., Ng, E., Nedjari, H., Nordsiek, G., Novella, S., de Pablos, B., Pérez-Diaz, J. C., Purcell, R., Remmel, B., Rose, M., Schlueter, T., Simoes, N., Tierrez, A., Vázquez-Boland, J. A., Voss, H., Wehland, J., & Cossart, P. (2001). Comparative genomics of *Listeria* species. *Science, 294* (5543), 849-852. https://doir.org/ 10.1126/science.1063447.

Gomes Neto, N. J., Luz, I. S., Honório, W. G., Tavares, A. G., & de Souza, E. L. (2012). *Rosmarinus officinalis* L. essential oil and the related compound 1,8-cineole do not induce direct or cross-protection in *Listeria monocytogenes* ATCC 7644 cultivated in meat broth. *Canadian Journal of Microbiology, 58* (8), 973-981. https://doir.org/10.1139/w2012-070.

Græsbøll, K., Nielsen, S. S., Toft, N., & Christiansen, L. E. (2014). How fitness reduced, antimicrobial resistant bacteria survive and spread: a multiple pig - multiple bacterial strain model. *PLoS ONE, 9* (7), e100458. https://doir.org/10.1371/journal.pone.0100458.

Hammer, K. A., Carson, C. F., & Riley, T. V. (2008). Frequencies of resistance to *Melaleuca alternifolia* (tea tree) oil and rifampicin in *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis*. *International Journal of Antimicrobial Agents, 32* (2), 170-173. https://doir.org/10.1016/j.ijantimicag.2008.03.013.

Hashemi, S. M. B., Khorram, S. B., & Sohrabi, M. (2017). Antioxidant activity of essential oils in foods. Hashemi (Ed.), *Essential Oils in Food Processing* (pp. 247-265).

Kohanski, M. A., DePristo, M. A., & Collins, J. J. (2010). Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Molecular Cell, 37* (3), 311-320. https://doi.org/10.1016/j.molcel.2010.01.003.

Leite de Souza, E. (2016). The effects of sublethal doses of essential oils and their constituents on antimicrobial susceptibility and antibiotic resistance among food-related bacteria: a review. *Trends in Food Science & Technology, 56*, 1-12. https://doir.org/10.1016/j.tifs.2016.07.012.

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., & Balaban, N. Q. (2017). Antibiotic tolerance facilitates the evolution of resistance. *Science, 355* (6327), 826. https://doir.org/10.1126/science.aaj2191.

Li, X., Liu, L., Ji, J., Chen, Q., Hua, X., Jiang, Y., Feng, Y., & Yu, Y. (2015). Tigecycline resistance in *Acinetobacter baumannii* mediated by frameshift mutation in *plsC*, encoding 1-acyl-sn-glycerol-3-phosphate acyltransferase. *European Journal of Clinical Microbiology & Infectious Diseases, 34* (3), 625-631. https://doir.org/10.1007/s10096-014-2272-y.

Lingan, K. (2018). A review on major constituents of various essential oils and its application. *Journal of Translational Medicine, 08*. https://doir.org/10.4172/2161-1025.1000201.

Luz, I. d. S., Neto, N. J. G., Tavares, A. G., Magnani, M., & de Souza, E. L. (2012). Exposure of *Listeria monocytogenes* to sublethal amounts of *Origanum vulgare* L. essential oil or carvacrol in a food-based medium does not induce direct or cross protection. *Food Research International, 48* (2), 667-672. https://doi.org/10.1016/j.foodres.2012.05.026.

Merino, N., Berdejo, D., Bento, R., Salman, H., Lanz, M., Maggi, F., Sánchez-Gómez, S., García-Gonzalo, D., & Pagán, R. (2019). Antimicrobial efficacy of *Thymbra capitata* (L.) Cav. essential oil loaded

in self-assembled zein nanoparticles in combination with heat. *Industrial Crops and Products, 133*, 98-104. https://doi.org/10.1016/j.indcrop.2019.03.003.

Mishra, A. P., Devkota, H. P., Nigam, M., Adetunji, C. O., Srivastava, N., Saklani, S., Shukla, I., Azmi, L., Shariati, M. A., Melo Coutinho, H. D., & Mousavi Khaneghah, A. (2020). Combination of essential oils in dairy products: A review of their functions and potential benefits. *LWT-Food Science and Technology, 133*, 110116. https://doi.org/10.1016/j.lwt.2020.110116.

Peterson, E., & Kaur, P. (2018). Antibiotic resistance mechanisms in bacteria: relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. *9* (2928). https://doir.org/10.3389/fmicb.2018.02928.

Pontes, M. H., & Groisman, E. A. (2019). Slow growth determines nonheritable antibiotic resistance in *Salmonella enterica*. *Science Signaling, 12* (592), eaax3938. https://doir.org/10.1126/scisignal.aax3938.

Quinto, E. J., Caro, I., Villalobos-Delgado, L. H., Mateo, J., De-Mateo-Silleras, B., & Redondo-Del-Río, M. P. (2019). Food safety through natural antimicrobials. *Antibiotics, 8* (4), 208. https://doir.org/10.3390/antibiotics8040208.

Sakai, A., Nakanishi, M., Yoshiyama, K. & Maki, H. (2006). Impact of reactive oxygen species on spontaneous mutagenesis in *Escherichia coli*. *Genes Cells*, 11 (7), 767-778. https://doi.org/10.1111/j.1365-2443.2006.00982.x.

Sutterlin, H. A., Zhang, S., & Silhavy, T. J. (2014). Accumulation of phosphatidic acid increases vancomycin resistance in *Escherichia coli*. *Journal of Bacteriology, 196* (18), 3214-3220. https://doir.org/10.1128/JB.01876-14.

Toledo-Arana, A., Dussurget, O., Nikitas, G., Sesto, N., Guet-Revillet, H., Balestrino, D., Loh, E., Gripenland, J., Tiensuu, T., Vaitkevicius, K., Barthelemy, M., Vergassola, M., Nahori, M.-A., Soubigou, G., Régnault, B., Coppée, J.-Y., Lecuit, M., Johansson, J., & Cossart, P. (2009). The *Listeria* transcriptional landscape from saprophytism to virulence. *Nature, 459* (7249), 950-956. https://doir.org/10.1038/nature08080.

Xue, J., Davidson, P. M., & Zhong, Q. (2017). Inhibition of *Escherichia coli* O157:H7 and *Listeria monocytognes* growth in milk and cantaloupe juice by thymol nanoemulsions prepared with gelatin and lecithin. *Food Control, 73*, 1499-1506. https://doi.org/10.1016/j.foodcont.2016.11.015.

Yao, J., & Rock, C. O. (2013). Phosphatidic acid synthesis in bacteria. *Biochimica et Biophysica Acta, 1831* (3), 495-502. https://doir.org/10.1016/j.bbalip.2012.08.018.

Yehia, H. M., Elkhadragy, M. F., Aljahani, A. H., & Alarjani, K. M. (2020). Prevalence and antibiotic resistance of *Listeria monocytogenes* in camel meat. *Bioscience Reports, 40* (6), BSR20201062. https://doir.org/10.1042/BSR20201062.

Zwietering, M. H., Jongenburger, I., Rombouts, F. M., & van 't Riet, K. (1990). Modeling of the bacterial growth curve. *Applied and Environmental Microbiology, 56* (6), 1875-1881. https://doi.org/10.1128/AEM.56.6.1875-1881.1990.

Supplemental material

Table S1. Primers used for PCR amplification and Sanger sequencing to verify the mutations of evolved strains: LmSTCO (by cyclic exposure to prolonged sublethal doses of *Thymbra capitata* essential oil (TCO)) and LmLTCO (by cyclic exposure to short lethal treatments of TCO).

LmSTCO mutations	Forward primer (5' → 3')	Reverse primer (5' → 3')
Lmo1647	CCCGCTAAAATAAGCGCGTC	GCACTCGGTCCAGGGTTATC

LmLTCO mutations	Forward primer (5' → 3')	Reverse primer (5' → 3')
Big deletion	ACCCCCAACATTTGCGATAG	TAATAACCCCTGACCGCGAC
	CGTTTTTCCTGCACCAGTCG	CACGAGAGCATCGCTGGAAT
lmo0289	TGAGCATTGCCGATGAAGGT	ACCTTGCGCCATTAGTTCGT
lmo0446-lmo0447	CGCAAGGTACAGGAACAAGC	CGTTCCCATGTAATGCTCCG
lmo1503	TCACTTATCGCTACGCCGAC	CCGCAGTCTCTGAAGAAGCA
lmo2249-lmo2250	TGGAACAGCTCACCGTGTTA	CGGTGGATAGTCAGCGGAAA

Table S2. A (maximum OD_{595}), μ_m (maximum specific growth rate; h^{-1}) and λ (lag time; h) values and error standard of the modified Gompertz model (Eq. 1) obtained from 3 independently growth curves of (A) *Listeria monocytogenes* EGD-e (LmWT) and evolved strains: (B) LmSTCO (by cyclic exposure to prolonged sublethal doses), and (C) LmLTCO (by cyclic exposure to short lethal treatments) at different concentrations of *Thymbra capitata* essential oil (TCO). The goodness of the fit is shown by R^2 and adjusted R^2 values and the root mean square error (*RMSE*).

A) LmWT

TCO (µL/L)	Values			Standard error			Goodness of fit		
	A	μ_{max}	λ	A	μ_{max}	λ	R^2	Adj. R^2	*RMSE*
0	1.178	0.203	5.149	0.035	0.019	0.261	0.9846	0.9837	0.0557
50	1.165	0.194	6.624	0.029	0.015	0.139	0.9961	0.9959	0.0287
75	1.136	0.201	8.039	0.031	0.011	0.165	0.9940	0.9937	0.0357
100	1.130	0.198	9.110	0.034	0.008	0.161	0.9932	0.9928	0.0386
125	1.180	0.180	12.280	0.040	0.008	0.124	0.9955	0.9952	0.0313

B) LmSTCO

TCO (µL/L)	Values			Standard error			Goodness of fit		
	A	μ_{max}	λ	A	μ_{max}	λ	R^2	Adj. R^2	*RMSE*
0	1.126	0.204	4.984	0.031	0.013	0.199	0.9906	0.9900	0.0416
50	1.136	0.182	6.048	0.030	0.009	0.177	0.9938	0.9934	0.0349
75	1.122	0.189	6.832	0.030	0.010	0.180	0.9935	0.9931	0.0361
100	1.145	0.167	8.198	0.039	0.011	0.244	0.9885	0.9878	0.0492
125	1.137	0.172	9.266	0.031	0.006	0.112	0.9970	0.9968	0.0254
150	1.021	0.181	12.820	0.034	0.007	0.107	0.9962	0.9960	0.0255
175	0.996	0.075	20.430	0.021	0.003	0.162	0.9936	0.9930	0.0185

C) LmLTCO

TCO (µL/L)	Values			Standard error			Goodness of fit		
	A	μ_{max}	λ	A	μ_{max}	λ	R^2	Adj. R^2	*RMSE*
0	1.192	0.199	7.047	0.039	0.017	0.298	0.9820	0.9809	0.0632
50	1.184	0.197	8.121	0.040	0.015	0.262	0.9855	0.9847	0.0578
75	1.189	0.183	9.020	0.032	0.007	0.130	0.9961	0.9958	0.0303
100	1.198	0.186	10.910	0.043	0.010	0.182	0.9905	0.9899	0.0475
125	1.205	0.198	12.220	0.045	0.011	0.162	0.9920	0.9915	0.0437
150	1.192	0.190	12.652	0.038	0.007	0.105	0.9966	0.9964	0.0274
175	1.099	0.162	15.590	0.023	0.007	0.133	0.9928	0.9923	0.0295
200	0.920	0.095	17.648	0.029	0.002	0.058	0.9985	0.9984	0.0279
225	0.851	0.083	18.392	0.036	0.004	0.172	0.9912	0.9907	0.0199
250	0.724	0.066	18.686	0.039	0.009	0.481	0.9401	0.9369	0.0486
275	0.715	0.040	19.873	0.055	0.004	0.513	0.9519	0.9493	0.0346

Table S3. Genetic variations detected by whole genome sequencing (WGS) between LmWT and the reference genome of *Listeria monocytogenes* EGD-e (NCBI accession: NC_003210.1). Single nucleotide variation (SNV).

Genome position	Locus tag	Mutation*	Change	Information
264,578	lmo0247	SNV: G147T	Silent mutation (Gly49)	Hypothetical protein
435,968	lmo0412-lmo0413	SNV: G by T	Non-coding region	Hypothetical protein - hypothetical protein
966,277	lmo0929	SNV: A33G	Silent mutation (Leu11)	Sortase
1,374,715	lmo1349	SNV: A597G	Silent mutation (Leu199)	Glycine dehydrogenase subunit 1
1,442,124	lmo1412-lmo1413	SNV: C by A	Non-coding region	Modulates DNA topology – peptidoglycan binding protein
2,229,938	lmo2144	SNV: G-11A	Regulatory region	GntR family transcriptional regulator
2,943,565	lmo2855 - lmo2856	SNV: C by A	Regulatory region	Ribonuclease P – 50S ribosomal protein L34

*Position respect to the start of the coding region.

Table S4. Large deletion of LmLTCO (evolved strain by cyclic exposure to short lethal treatments of *Thymbra capitata* essential oil (TCO)).

Locus gene	Function
lmo0083	MerR family transcriptional regulator
lmo0084	Oxidoreductase
lmo0085	Hypothetical protein
lmo0086	Hypothetical protein
lmo0087	Hypothetical protein
lmo0088	ATP synthase subunit C
lmo0089	ATP synthase subunit delta
lmo0090	ATP synthase F0F1 subunit alpha
lmo0091	ATP synthase F0F1 subunit gamma
lmo0092	ATP synthase F0F1 subunit beta
lmo0093	ATP synthase subunit epsilon
lmo0094	Hypothetical protein
lmo0095	Hypothetical protein
lmo0096	PTS mannose transporter subunit IIAB
lmo0097	PTS mannose transporter subunit IIC
lmo0098	PTS mannose transporter subunit IID
lmo0099	Hypothetical protein
lmo0100	Hypothetical protein
lmo0101	Transcriptional regulator
lmo0102	Hypothetical protein
lmo0103	NADH oxidase
lmo0104	Hypothetical protein
lmo0105	Chitinase B
lmo0106	Transcriptional regulator
lmo0107	ABC transporter ATP-binding protein

Manuscrito VII. *Salmonella enterica* genetic variants isolated after lethal treatment with *Thymbra capitata* essential oil show increased resistance in milk

Berdejo, D., Pagán, E., Merino, N., Botello-Morte, L., García-Gonzalo, D., Pagán, R.

International Journal of Food Microbiology.

Title: *Salmonella enterica* genetic variants isolated after lethal treatment with *Thymbra capitata* essential oil show increased resistance in milk

Author names: Daniel Berdejo, Elisa Pagán, Natalia Merino, Laura Botello-Morte, Rafael Pagán and Diego García-Gonzalo*.

Affiliation: Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain

***Corresponding author**: Dr. Diego García Gonzalo
E-mail: Diego.Garcia@unizar.es
Address: Dpto. PACA. Facultad de Veterinaria. Universidad de Zaragoza.
C/ Miguel Servet, 177, 50013, Zaragoza, Spain
Phone number: 34-876-554246 **Fax. number:** 34-976-761590

Abstract

The high prevalence of *Salmonella enterica* in milk poses a food risk of concern in the preservation of some dairy products, mainly those elaborated from raw milk. Essential oils (EOs) have been proposed as a promising food preservative for this kind of products due to their strong antimicrobial properties. Additionally, these natural antimicrobials have demonstrated to be effective against multi-drug resistant strains, so their use could also contribute to prevent the dissemination of antimicrobial resistances (AMR). However, recent evidences on the development of bacterial resistance under EOs treatments may call into question their use. This study seeks to assess the emergence of resistant genetic variants of *S. enterica* after cyclic exposure to lethal doses of *Thymbra capitata* EO, to evaluate the impact that it could have on milk preservation, to ascertain whether cross-resistance to antibiotics occurs and to identify the genetic variants responsible. Isolated strains (SeTCO) showed a two-fold increase in minimum inhibitory and bactericide concentrations (MIC and MBC) of TCO compared to wild-type strain (SeWT), as well as a greater adaptation and growth rate in the presence of the EO and a higher survival to TCO treatments in buffers of pH 4.0 and 7.0. The increased resistance of SeTCO was confirmed in skimmed milk: MIC and MBC were found to be more than double than those of SeWT and 300 µL/L TCO reduced only 1 $\log_{10}$ cycles of SeTCO's population while more than 5 $\log_{10}$ cycles of inactivation were achieved for SeWT. Moreover, SeTCO showed an increased cross-resistance against aminoglycosides, quinolones and tetracyclines. Whole genome sequencing revealed 5 mutations in SeTCO: 2 in virulence-related genes (*rfbV* and *rfbX*), 2 in genes related to adaptation to the growing medium (*trkA* and *glpK*), and 1 in a redox-sensitive transcriptional regulator (*soxR*). The phenotypic characterization of a constructed SeWT strain with mutant $soxR_{SeTCO}$, demonstrated that the mutation of *soxR* was the main cause of the increased resistance and tolerance observed in SeTCO against TCO and antibiotics. The emergence of resistant strains against EOs might jeopardize their use as food preservatives. For this reason, further studies are required to determine under which conditions these resistant strains could occur and to assess the food risk they may pose, as well as to ascertain their impact on the spread of AMR.

Keywords

Salmonella enterica, *Thymbra capitata* essential oil, Resistant strains, Skimmed milk, Genetics variants, *soxR* mutated.

1. Introduction

The dairy industry still faces important challenges in order to prevent food safety hazards, notwithstanding the continuous improvement of preservation methods for microbiological control. The presence and growth of pathogenic bacteria in raw milk and derived dairy products is one of the most worrying hazards (Boor et al., 2017). Currently, *Salmonella* spp. stands out among key pathogenic bacteria since it is highly prevalent in the food chain of milk production as well as in the manufacturing of dairy products (Sonnier et al., 2018). Recent food outbreaks caused by raw milk cheese consumption have been associated to the presence of *Salmonella* strains (Robinson et al., 2020; Ung et al., 2019).

Besides that, the huge increase of multidrug resistant (MDR) strains of *Salmonella* in the food industry represents a serious public health issues worldwide by the spread of antimicrobial resistances (AMR) in agri-food environments (McMillan et al., 2019). Recent studies have reported the high presence of resistance genes to a wide range of antibiotics along the milk production chain, mainly in *S. enterica* strains (Parry-Hanson Kunadu et al., 2018; Qamar et al., 2020). In fact, there are several reports of serious foodborne outbreaks of MDR *Salmonella* strains associated to the consumption of milk and dairy products (Olsen et al., 2004; Plumb et al., 2019).

Essential oils (EOs) and their individual constituents (ICs) were found to be promising agents in dairy products as food preservative due to their bio-preservative, antioxidant, antimicrobial activities (Mishra et al., 2020). These natural compounds have been extensively studied and shown great antimicrobial properties against food-related pathogens (Calo et al., 2015; Pandey et al., 2017). Some EOs, such as *Thymbra capitata*, have demonstrated high antimicrobial activity even against bacterial forms of resistance such as biofilms (Gagliano Candela et al., 2019), including those formed by *S. enterica* (Karampoula et al., 2016). Furthermore, EOs are also proposed as a possible alternative to antibiotics in the treatment of infectious diseases in order to overcome the generation and dissemination of resistance (Mittal et al., 2019; Yap et al., 2014), so their use could also be effective against MDR strains in food preservation.

Nevertheless, several studies support that the use of ICs can lead to the emergence of resistant and tolerant strains against natural antimicrobials, considering “resistance” as the ability of bacteria to grow in the presence of the antimicrobial, whereas “tolerance” as their ability to survive against lethal doses (Balaban et al., 2019). The application of cyclic treatments of ICs, such as carvacrol, citral or limonene oxide, allowed the selection

of genetic variants in microbial populations of *Escherichia coli* (Chueca et al., 2016; Chueca et al., 2018), *Staphylococcus aureus* (Berdejo et al., 2019) and *S. enterica* (Berdejo, Merino, et al., 2020) with increased direct-resistance to the same ICs used for their selection. In addition, some of these strains also developed increased cross-resistance to a wide range of antibiotics, supporting that those mutations contribute to non-specific bacterial resistance against antimicrobials (Berdejo, Merino, et al., 2020; Chueca et al., 2018). Due to the compositional complexity of EOs (up to dozens different ICs) and, consequently, the diverse mechanisms of antimicrobial action of their ICs (Lingan, 2018), the use of complex EOs has been considered a safe alternative to prevent the emergence of antimicrobial resistance. However, a recent study also evidenced the emergence of resistant and tolerant strains of *S. aureus* against a complex *Citrus sinensis* EO after prolonged exposure to sub-inhibitory doses of EO (Berdejo, Pagán, et al., 2020).

To the best of our knowledge, there is no evidence of the emergence of Gram-negative resistant strains by their exposure to complex EOs. Moreover, the risk posed by the emergence of these strains in food preservation has not yet been assessed. For those reasons, this study seeks a) to assess the emergence of resistant mutants of *S. enterica* due to the use of a complex EO, *Thymbra capitata*, b) to evaluate the magnitude of the increased resistance in skimmed milk as food model c) to study the occurrence of cross-resistance to antibiotics in resistant mutants and d) to identify the genetic modifications responsible for the increase in bacterial resistance.

2. Material and Methods

2.1. Microorganisms, growth conditions and reagents

Salmonella enterica subsp. *enterica* serovar Typhimurium LT2 (SeWT) was provided by the Spanish Type Culture Collection (CECT 722). The XTL298 strain, which contains *tetA-sacB* in the arabinose operon for strain construction, was kindly provided by Donald L. Court (National Cancer Institute at Frederick, USA).

T. capitata essential oil (TCO) was kindly provided by the TELIC Group (Barcelona, Spain). This EO was kept in the dark and under refrigeration temperature in sealed glass bottles. The composition of this batch of TCO was previously analysed by Merino et al. (2019): 73.8% carvacrol, 9.2% p-cymene, 5.3% γ-terpinene, 2.0% (E)-caryophyllene and 9.7% other compounds. Sterile skimmed milk (Central Lechera Asturiana, Asturias,

Spain) was purchased in a supermarket and a new bottle was aseptically opened before each experiment.

Throughout this investigation, the strain was kept in cryovials at -80 °C with glycerol (20% v/v), from which plates of tryptone soya agar (Oxoid, Basingstoke, United Kingdom) with 0.6% yeast extract (Oxoid; TSAYE) were inoculated on a weekly basis. To prepare the working bacterial cultures, test tubes containing 5 mL of tryptone soya broth (Oxoid) with 0.6% yeast extract (TSBYE) were inoculated with one colony and then incubated aerobically overnight on an orbital shaker (130 rpm; Heidolph Vibramax 100, Schwaback, Germany) at 37 °C (Incubig, Selecta, Barcelona, Spain). Subsequently, flasks containing 10 mL of fresh TSBYE were inoculated with the resulting subculture to achieve an initial concentration of 10^6 colony forming units per mL (CFU/mL), and incubated for 24 h at 37 °C and 130 rpm until the stationary growth phase was reached ($5x10^9$ CFU/mL approximately).

2.2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC determination was performed according to CLSI (2015) with some modifications due to hydrophobicity of TCO. From an exponential culture (0.5 McFarland) of the bacterial strains, 5 mL of TSBYE were inoculated 1:100 achieving an initial concentration of 5×10^5 CFU/mL in the presence of different concentrations of TCO: (50 - 600 µL/L). Once the tubes were incubated at 37 °C for 24 h and 130 rpm, MIC was determined as the lowest concentration of the antimicrobial compound that was capable of avoiding bacterial growth. To objectively determine bacterial growth, the optical density was read at 595 nm (OD_{595}) using a microplate reader (Genios, Tecan, Männedorf, Switzerland). The 10% of the OD_{595} measure of the positive control was established as the lower limit to consider that bacterial strain had grown (Kohanski et al., 2010). Following the method described by Friedman et al. (2002), a vigorous shaking by vortex (Genius 3, Ika, Königswinter, Germany) was used to prepare TCO dispersions in TSBYE, avoiding the use of solvents for their possible detriment in the antibacterial activity. Positive control tubes with 5 mL TSBYE inoculated without TCO, and negative control tubes with 5 mL TSBYE non-inoculated were also prepared in every experiment.

The minimum bactericidal concentration (MBC) of TCO was evaluated in parallel to the MIC test. From the test tubes employed in the MIC determination after incubation, 100 µL aliquot of each tube was spread onto TSYBE plates and incubated at 37 °C for 24

h. Colonies were counted and the lowest concentration of TCO that killed ≥ 99.9% of the initial bacterial concentration was defined as the MBC end point (Lallemand et al., 2016).

Additionally, MIC and MBC determinations were conducted in a food model, skimmed milk, following the same protocols using a concentration range from 200 to 1,800 µL/L TCO. It should be noted that in MIC determination, samples from every test tube were aliquoted and spread onto TSAYE plates to determine cell concentration because the milk turbidity avoids a correct OD_{595} measurement. An increase of 50% of the initial bacterial population (CFU/mL) was established as the limit for considering the bacterial growth.

2.3. TCO evolution assay

The evolution assay was based on the isolation of strains by recovering surviving cells after lethal treatments with TCO. This methodology was adapted from Levin-Reisman et al. (2017). A stationary phase culture of SeWT was diluted 1:100 in 50 mL fresh TSBYE with 500 µL/L of TCO (2 x MIC for SeWT) for 4.50 h at 37 °C to achieve an inactivation higher than 5 log_{10} cycles. Subsequently, treated cells were centrifuged for 20 min at 15,000 relative centrifuge force (RCF) (Heraeus Megafuge 1.0R, Thermo Electron LED GrnbH, Langenselbold Germany), washed twice with TSBYE, resuspended in 1 mL TSBYE and incubated overnight at 37 °C. This procedure was repeated 30 times. After the 30th step, an aliquot was diluted in PBS and spread on TSAYE plates (without TCO), from which 5 colonies ($SeTCO_{1-5}$) were randomly selected to carry out phenotypic and genotypic characterization.

2.4. Growth curves in presence of TCO

First, TCO was added at different concentrations in tubes with 5 mL of TSBYE: from 0 up to 600 µL/L TCO. Test tubes were inoculated with the microbial culture at an initial concentration of $5x10^5$ CFU/mL and incubated at 37 °C and 130 rpm for 24 h. Every hour, OD_{595} of the test tubes was measured by a microplate reader. A positive control (without antimicrobial added) and a negative control (without microbial culture added) were incorporated in all the assays. The initial OD_{595} (at time 0) was subtracted from values of OD_{595} obtained during the experiment, corresponding to the absorbance caused by the growth medium. Bacterial growth curves based on OD_{595} of SeWT and SeTCO were graphically displayed and modelled by a modified Gompertz equation (Eq. 1) (Zwietering et al., 1990).

$$y = A \exp\{-\exp[(\mu_m e / A) * (\lambda - t) + 1]\} \quad \text{(Eq. 1)}$$

where y: OD_{595}; t: time (h); A: maximum value reached (OD_{595} max); μ_m: maximum specific growth rate (h^{-1}); λ: lag time (h).

A least-squares adjustment was carried out to build the model and obtain A, μ_m and λ values using Prism software (GraphPad Software, Inc., San Diego, USA). The experiment was prolonged for more than 24 h at high TCO concentrations until reaching the stationary phase to allow fitting of the growth curve. The adjustment's goodness of fit was evaluated using standard error, R^2 and R^2-adjusted values, and the root mean square error (*RMSE*). In addition, secondary models of Gompertz's parameters were built based on TCO concentration by least-squares adjustment and subsequently tertiary growth models were obtained to estimate growth rate based on TCO concentration and incubation time.

2.5. Survival curves against TCO

The treatment medium used in the lethal treatments was citrate-phosphate buffer (McIlvaine buffer), prepared from citric acid monohydrate (Panreac) and disodium hydrogen phosphate (Panreac), adjusted to pH 4.0 and pH 7.0. These pH values were chosen as representative of neutral and acid conditions within the normal pH range of food. Stationary phase culture was centrifuged for 5 min at 6,000 RCF in a microcentrifuge (Mini Spin, Eppendorf, Hamburg, Germany) and resuspended in the treatment medium. The treatment was carried out in 10 mL McIlvaine buffer previously tempered at 25 °C with 150 µL/L of TCO. Once TCO was dispersed, test tubes were then inoculated at 10^7 CFU/mL. Aliquots were obtained every 5 min and subsequently were diluted in PBS and spread on TSAYE plates. After plate incubation (24 h/ 37 °C), colonies were counted in an automatic plate counter by image analysis (Analytical Measuring Systems, Protos, Cambridge, United Kingdom).

Following this same protocol, lethal treatments were performed in TSBYE and skimmed milk, with 300 µL/L and 1,500 µL/L TCO respectively for 30 min.

2.6. Antibiotic susceptibility test

Kirby-Bauer disk diffusion test was conducted to test antimicrobial susceptibility according to CLSI (2012, 2014). Several antibiotics with different modes of action were chosen in order to cover different cellular targets that could be related to TCO resistance:

400 µg nalidixic acid sodium, 20 µg norfloxacin, 250 µg novobiocin sodium, 10 µg trimethoprim, 50 µg rifampicin, 30 µg chloramphenicol, 30 µg kanamycin sulphate, 30 µg tetracycline, 10 µg ampicillin, and 150 µg cephalexin (Sigma-Aldrich). First, stationary phase culture was spread on cation adjusted Mueller Hinton agar plates (MHA; Sigma-Aldrich) and, after 5 min at room temperature, blank disks (Ø: 6.0 mm; Thermo Scientific™ blank anti-microbial susceptibility disk, Fisher Scientific, UK) were placed on the surface of plates and individually impregnated with each antibiotic. These plates were incubated at 37 °C for 18-24 h, after which the diameters of the resulting inhibition zones were measured (paper disks included).

2.7. Statistical analysis

All phenotypic characterization results were obtained from at least 3 independent experiments carried out on different working days with different bacterial cultures. Growth curve parameters, lethal treatment graphics and antibiotic susceptibility and motility tests are displayed as the mean ± standard deviation, using the Prism software. Data were analysed and submitted to comparison of averages using analysis of variance (ANOVA), followed by post-hoc Tukey test and t-tests with Prism software, and differences were considered significant if $p \leq 0.05$.

2.8. Whole genome sequencing (WGS) and identification of mutations

Illumina technology was used to carry out WGS of SeWT and the genetic variant, on NextSeq equipment at mid output flow, with a total of 2x 150 cycles (Illumina; Fasteris, SA, Geneva, Switzerland). Subsequently, quality control was performed with FastQC software evaluating reading quality (Q_{30}), sequence length, presence of adapters, and overrepresented and duplicated sequences. A total of 3.65 and 3.77 million of 150 pb-reads were obtained for SeWT and SeTCO, which a Phred quality score of 33.07 and 33.13, and 86.58 % and 86.88% of reads above Q_{30}, respectively. The quality-control-filtered paired-end reads were mapped at 98.12% and 98.35% on the reference genome sequence (NCBI accession: NC_003197.2): *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2, complete genome (McClelland et al., 2001), using a Burrows–Wheeler Alignment (BWA) Tool (Li and Durbin, 2010) and Samtools software (Li et al., 2009) (sources: http://bio-bwa.sourceforge.net/ and http://www.htslib.org/). A coverage 150-fold depth was achieved for both strains. Then, Samtools was applied to remove potential PCR duplicates according to reading positions

on the reference genome; the resulting BAM files were then further processed using LoFreq-Star (source: http://csb5.github.io/lofreq/) to correct mapping errors and insert the quality values. Finally, single nucleotide variants (SNVs) and short insertion and deletions (InDels) were detected using LoFreq-Star, and toolbox snpEff (source: http;//snpeff.sourceforge.net/) was employed to identify involved genes and to predict functional effect variations (Cingolani et al., 2012). Coverage was further analysed by the Integrative Genomics Viewer (IGV; Broad Institute, source: https://software.broadinstitute.org/software/igv/) in order to find structural variations (SVs). Although mapping was carried out against the reference genome, SNVs, InDels, and SVs were identified between SeWT and isolated strains to ascertain the kind of mutations that had occurred during the evolution treatments. The resulting genome sequences were deposited in the Sequence Read Archive (SRA) of NCBI (BioProject ID: PRJNAPRJNA634825). The accession numbers of the samples are SAMN15009803 (SeWT) and SAMN17313477 (SeTCO). Finally, specific primers (Table S1) were designed to carry out PCR amplifications, as well as Sanger sequencing of PCR products to verify the mutations detected in the WGS.

2.9. Mutated gene replacement

Red recombinase technology was used to perform the mutated gene replacement in SeWT in order to isolate the mutation detected in SeTCO. *tetA-sacB* cassette from XTL298 strain *(Li et al., 2013)* was used as the PCR template to generate DNA fragments for primary integration into chromosomal near to mutated gene ($soxR_{SeTCO}$) using specific primers containing 50 bp homologous sequences (Table S2). Plasmid *pKD46*, encoding the λ red recombinase genes behind the *araBAD* promoter, was used to enable the chromosomal integrations via double-crossover recombination, as previously described by Datsenko and Wanner (2000). To replace wild-type allele with gene variant in SeWT using intrachromosomal recombination, we first marked wild-type allele with *tetA-sacB* cassette and verified by colony PCR and Sanger sequencing. Then the *tetA–sacB* cassette of this strain was replaced intrachromosomally by gene variant with the aid of λ-red recombinase. For this purpose, 50 mL Luria Broth (LB) with 100 mg/L ampicillin were inoculated from bacterial cultures and incubated at 30 °C with shaking at 130 rpm. After 2 h, arabinose was added at 4 g/L and cultures were re-incubated under the same conditions until the optical density at 590 nm (OD_{590}) of the cultures reached 0.5. Then, cultures were subsequently centrifuged (5 min, 6,000 RCF, 4 °C), the supernatant was

discarded, and the remaining cell pellet was resuspended in 25 ml of 4 °C ultrapure water. This wash cycle was repeated three times and, finally, the supernatant was discarded and resuspended in 500 µL ultrapure water. For electroporation, 40 µL of competent cells were mixed with 100-200 ng of DNA. Following electroporation, cells were transferred to a sterile test tube containing 1 mL LB and incubated at 30 °C for 3 h. Cells were then plated on LB agar containing tetracycline (20 µg/mL) to select clones by antibiotic resistance during primary integration, and on LB agar containing tetracycline (20 µg/mL) and sucrose (1.5% w/v), to isolate clones by loss of antibiotic resistance and sucrose insensitivity during secondary integration. PCR and Sanger sequencing were used to verify positive clones.

3. Results

3.1. Isolation of mutants with increased resistance and tolerance to TCO

Five colonies were randomly selected after TCO evolution assay in *S. enterica*: $SeTCO_{1-5}$. Then, MIC and MBC determinations of SeWT and evolved strains ($SeTCO_{1-5}$) against TCO were performed to compare their resistances and tolerances (Table 1).

Table 1. Minimum inhibitory concentration (MIC; µL/L) and minimum bactericidal concentration (MBC; µL/L) of TCO for *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT) and evolved strains ($SeTCO_{1-5}$). Each value represents the result of 5 different experiments carried out with different bacterial cultures and on different working days.

Strains	MIC (µL/L)	MBC (µL/L)
SeWT	250	300
$SeTCO_{1-5}$	600	600

Evolved strains showed identical values among them, with two-fold increase resistance (as measured by MIC) and tolerance (as measured by MBC) against TCO after the evolution assay. While SeWT was inhibited at 250 µL/L TCO and inactivated at 300 µL/L TCO, $SeTCO_{1-5}$ showed values of MIC and MBC of 600 µL/L TCO. Since the 5 strains isolated from the evolution lineage displayed the same degree of resistance and tolerance to TCO, we considered that the bacterial population was homogeneous and we continue the research on only one of the 5 strains, from here onward referred to as SeTCO.

3.2. Evolved strain grows faster than SeWT in the presence of TCO

Growth kinetics studies were carried out in the presence of TCO to characterise the adaptation of evolved strains to the EO, and subsequently modelled by Gompertz equation (Eq. 1) by least-squares adjustment with excellent goodness of fit (Table S3).

Table 2. A (maximum OD_{595}), μ_m (maximum specific growth rate) and λ (lag time) parameters of the modified Gompertz model obtained from growth curves of for *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT) and evolved strain (SeTCO), at different concentrations of TCO.

A (OD_{595})		Strains	
		SeWT	**SeLTCO**
TCO (µL/L)	**0**	1.159 ± 0.009[a]	1.156 ± 0.006[a]
	50	1.171 ± 0.014[a]	1.156 ± 0.010[a]
	100	1.162 ± 0.019[a]	1.157 ± 0.025[a]
	150	1.174 ± 0.073[a]	1.168 ± 0.014[a]
	200	0.716 ± 0.037[b]	1.158 ± 0.035[a]*
	250		1.146 ± 0.018[a]
	300		1.161 ± 0.026[a]
	350		1.160 ± 0.034[a]
	400		1.101 ± 0.018[a]
	450		1.101 ± 0.026[a]
	500		0.792 ± 0.017[b]
	550		0.518 ± 0.028[c]

μ_m (OD_{595}/h)		Strains	
		SeWT	**SeLTCO**
TCO (µL/L)	**0**	0.287 ± 0.021[a]	0.288 ± 0.016[a]
	50	0.152 ± 0.008[b]	0.201 ± 0.011[b]*
	100	0.114 ± 0.005[c]	0.156 ± 0.016[c]*
	150	0.091 ± 0.009[cd]	0.156 ± 0.009[c]*
	200	0.076 ± 0.003[d]	0.147 ± 0.017[c]*
	250		0.164 ± 0.009[c]
	300		0.157 ± 0.010[c]
	350		0.161 ± 0.011[c]
	400		0.179 ± 0.007[bc]
	450		0.178 ± 0.009[bc]
	500		0.109 ± 0.005[d]
	550		0.103 ± 0.014[d]

λ (h)		Strains	
		SeWT	**SeLTCO**
TCO (µL/L)	**0**	3.423 ± 0.170[a]	3.391 ± 0.121[a]
	50	4.641 ± 0.243[b]	3.961 ± 0.185[ab]*
	100	5.828 ± 0.245[c]	4.581 ± 0.439[bc]*
	150	7.001 ± 0.555[d]	5.222 ± 0.241[c]*
	200	14.160 ± 0.154[e]	6.309 ± 0.518[d]*
	250		9.190 ± 0.201[e]
	300		10.158 ± 0.230[f]
	350		12.561 ± 0.225[g]
	400		13.650 ± 0.118[h]
	450		14.767 ± 0.147[i]
	500		17.961 ±0.148[j]
	550		18.352 ± 0.324[j]

Each value represents the mean ± standard deviation from 3 independent experiments. Different superscript letters represent statistically significant differences (p<0.05) among the means of the same column. *Significantly different from SeWT (p<0.05).

Table 2 displays the values of the parameters A (maximum OD_{595}), μ_m (maximum specific growth rate) and λ (lag phase), obtained from the models of SeWT and SeTCO at all the tested concentrations.

Additionally, secondary models were obtained for parameters A, μ_m and λ based on TCO concentration in SeWT (Eq. 2, 3, 4) and SeTCO (Eq. 5, 6, 7).

$$A_{SeWT} = 1.19 - (\mathrm{C_{TCO}}/240.68)\text{^}4.73 \quad \text{(Eq. 2)}$$

$$\mu_{m_{SeWT}} = 0.29 - 0.0038 * \mathrm{C_{TCO}} + 2.71 * 10^{-5} * \mathrm{C_{TCO}^2} - 6.54 * 10^{-8} * \mathrm{C_{TCO}^3} \quad \text{(Eq. 3)}$$

$$\lambda_{SeWT} = 2.61 \exp(0.0081 * \mathrm{C_{TCO}}) \quad \text{(Eq. 4)}$$

$$A_{SeTCO} = 1.17 - (\mathrm{C_{TCO}}/586.42)\text{^}7.14 \quad \text{(Eq. 5)}$$

$$\mu_{m_{SeTCO}} = 0.29 - 0.0017 * \mathrm{C_{TCO}} + 5.03 * 10^{-6} * \mathrm{C_{TCO}^2} - 5.51 * 10^{-9} * \mathrm{C_{TCO}^3} \quad \text{(Eq. 6)}$$

$$\lambda_{SeTCO} = 3.35 \exp(0.0033 * \mathrm{C_{TCO}}) \quad \text{(Eq. 7)}$$

where C_{TCO}: *Thymbra capitata* EO concentration; A: maximum value reached (OD_{595} max); μ_m: maximum specific growth rate (h^{-1}); λ: lag time (h).

Finally, growth models, integrating secondary models in Gompertz equation, were built for SeWT (Fig. 1A) and SeTCO (Fig. 1B) in order to estimate the growth (OD_{595}) depending on TCO concentration and incubation time. The surface graph shows the independent variables TCO concentration (µL/L) and incubation time (h) on the x-axis and z-axis, respectively, and as dependent variable on the y-axis the OD_{595} of the culture.

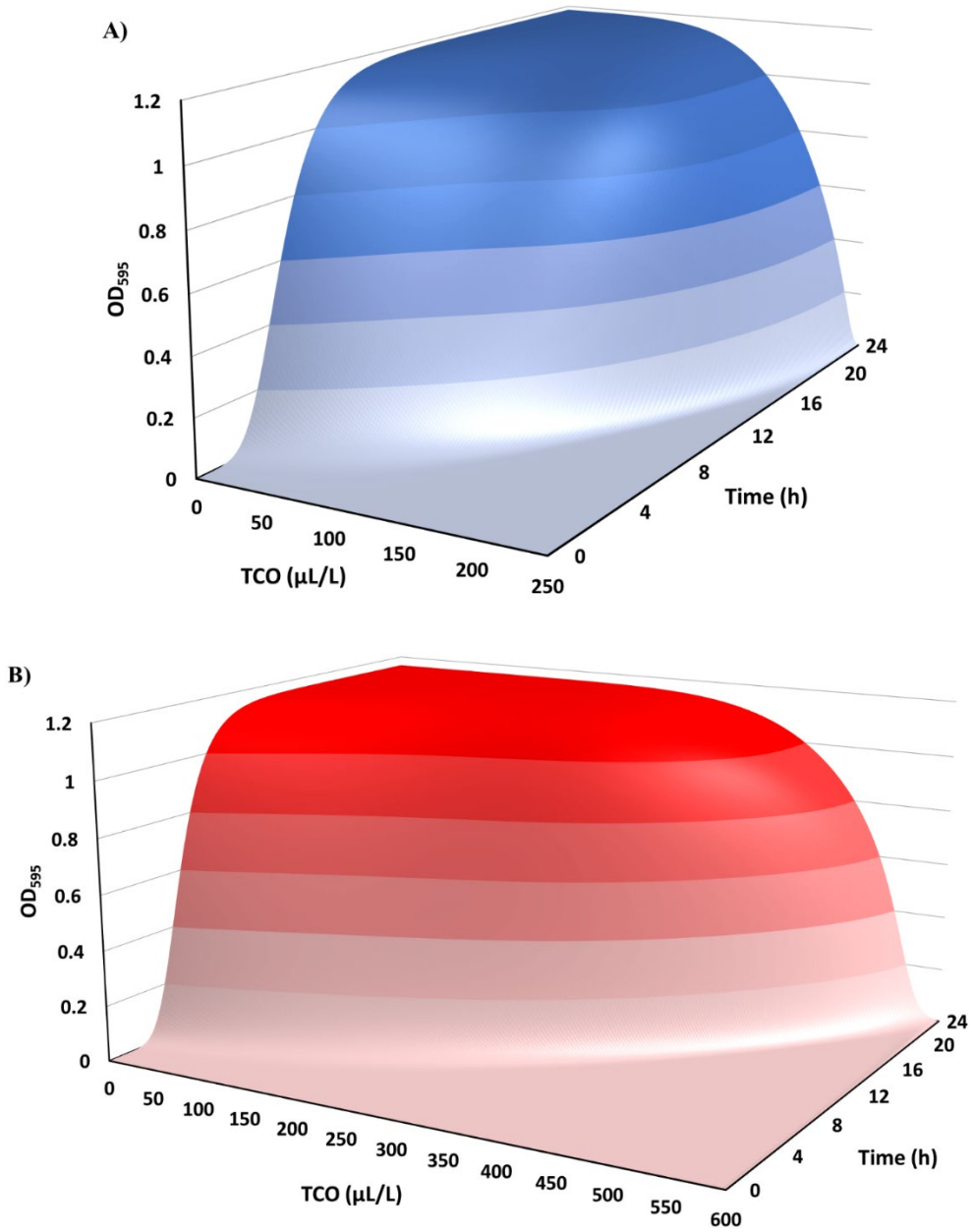


Figure 1. Growth models of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT; A) and evolved strain (SeTCO; B) at difference concentrations of TCO, modelled using the modified Gompertz equation (Eq. 1).

On the one hand, as we can see in the Figure 1, the growth kinetics of both strains revealed that as TCO concentration increased, A and μ_m parameters decreased considerably while λ became longer. This means that the presence of TCO decreases the cell growth rate, prolongs the lag phase and, at high concentrations, decreases the cell concentration in stationary phase. On the other hand, comparing SeTCO to SeWT, the behaviour of both strains was similar in the absence of TCO, but when the EO is added SeTCO exhibits a better adaption to TCO than SeWT observed in the lag phase, as well

a higher growth rate. In the presence of 200 µL/L TCO, the evolved strain showed 8 h shorter lag phase and up to twice the growth rate of the SeWT (Table 2).

3.3. Lethal TCO treatments to SeWT are not effective against the evolved strain.

The tolerance of SeTCO was further evaluated against lethal TCO treatments by comparing its inactivation kinetics with those of SeWT. Figure 2 shows the survival curves of SeWT and SeTCO after TCO lethal treatments in McIlvaine buffer at pH 4.0 (Fig. 2A) and 7.0 (Fig. 2B), as well in TSBYE (Fig. 2C) which is the growth medium used to carry out the evolution assay.

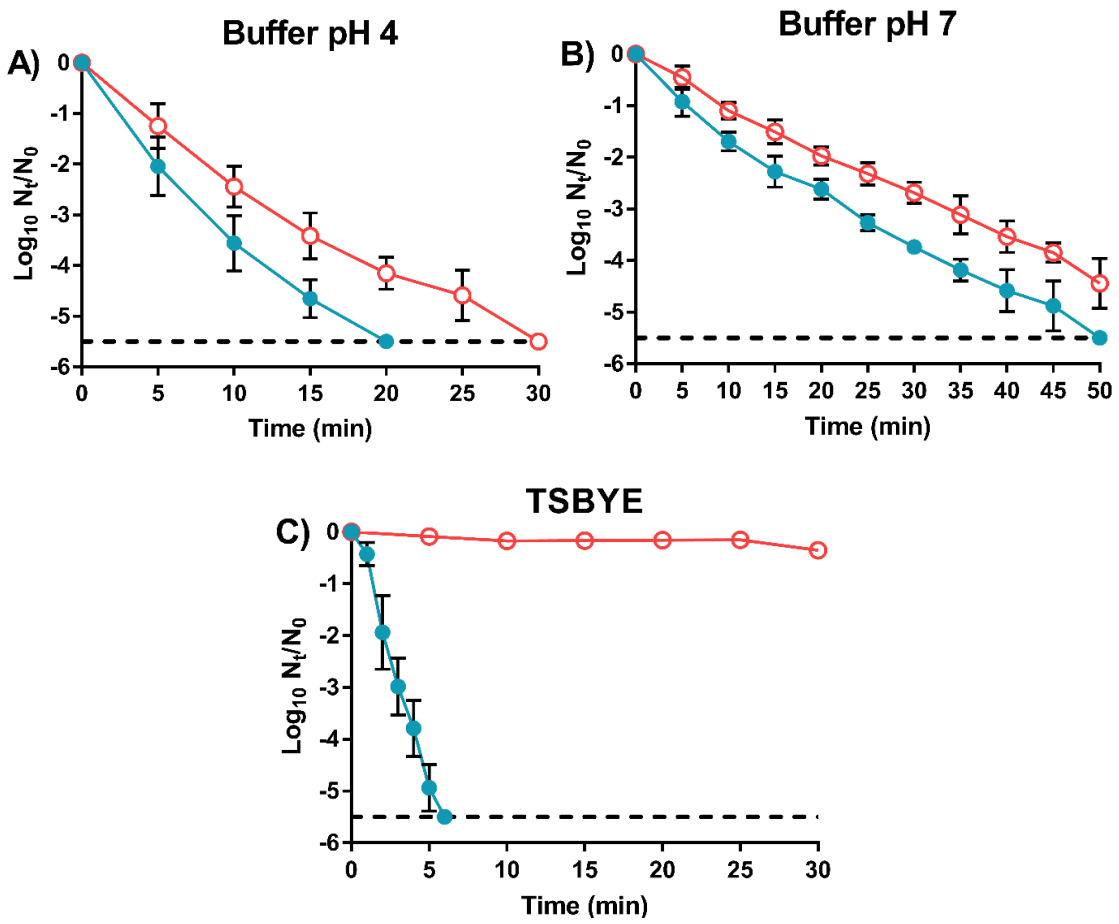


Figure 2. Survival curves of *Salmonella enterica* Typhimurium wild type (●; SeWT) and evolved strain (○; SeTCO) after 150 µL/L TCO treatment in phosphate-citrate buffer at pH 4.0 (A) and pH 7.0 (B) or 300 µL/L TCO treatment in TSBYE (C). Data are means ± standard deviations (error bars) obtained from at least 3 independent experiments. The dashed line represents the detection limit (-5.5 $\log_{10} N_t/N_0$).

The evolved strain showed higher tolerance than SeWT in McIlvaine at both acid and neutral pH. As can be seen in figure 2A, at pH 4.0 inactivation of SeTCO reaches 5.5 $\log_{10}$ cycles after 30 min of treatment, 10 min later than SeWT. Similarly, the lethal

treatment at pH 7.0 (Fig. 2B) revealed more than 1 $\log_{10}$ cycle of inactivation in SeWT compared to the evolved strain after 25 min of treatment. Moreover, the lethal treatments in TSBYE revealed a large difference in tolerance between both strains (Fig 2C): while SeWT showed an inactivation of more than 5 $\log_{10}$ cycles in 5 min of treatment, only 0.3 $\log_{10}$ cycles of population reduction was observed for SeTCO after 30 min.

3.4. Evolved strain as a risk in milk preservation by EOs

MIC and MBC were determined and lethal treatments were also performed in skimmed milk in order to assess the microbiological risk of appearance of resistant strains in the use of natural antimicrobials as food preservatives. Table 3 shows the MIC and MBC of TCO in skimmed milk for SeWT and SeTCO.

Table 3. Minimum inhibitory concentration (MIC; µL/L) and minimum bactericidal concentration (MBC; µL/L) of TCO for *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT) and evolved strain (SeTCO) in skimmed milk. Each value represents the result of 5 different experiments carried out with different bacterial cultures and on different working days.

Strains	MIC (µL/L)	MBC (µL/L)
SeWT	700	700
SeTCO	1600	1800

The concentrations of TCO to inhibit or inactivate both strains were more than double those observed in TSBYE. Likewise, SeTCO also showed an increased resistance and tolerance in skimmed milk in comparison with SeWT. MIC and MBC for SeTCO were 1,600 and 1,800 µL/L TCO, while SeWT was inhibited and inactivated with 700 µL/L.

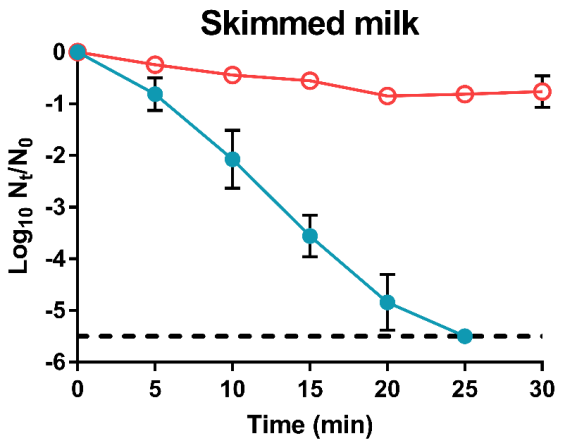


Figure 3. Survival curves of *Salmonella enterica* Typhimurium wild type (●; SeWT) and evolved strain (○; SeTCO) after 1,500 µL/L TCO in skimmed milk. Data are means ± standard deviations (error bars) obtained from at least 3 independent experiments. The dashed line represents the detection limit (-5.5 $\log_{10} N_t/N_0$).

Lethal treatments with 1500 µL/L TCO were also carried out to compare the tolerance of both strains (Fig. 3). As demonstrated in TSBYE, SeWT was more susceptible to TCO than SeTCO: after 25 min of treatment more than 5.5 log_{10} cycles of the initial population of SeWT inactivated while only 0.5 log_{10} cycles of SeTCO cells were reduced.

3.5. Emergence of TCO resistance leads to cross resistance to antibiotics

Kirby-Bauer disk diffusion test was carried out to assess the cross-resistance of evolved strains against antibiotics. Table 4 shows the zones of growth inhibition (mm) of nalidixic acid, norfloxacin, novobiocin, trimethoprim, rifampicin, chloramphenicol, kanamycin, tetracycline, ampicillin and cephalexin against SeWT and SeTCO.

Table 4. Zones of growth inhibition for agar disk diffusion assays of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT) and evolved strain (SeTCO) against antibiotics: 400 µg nalidixic acid sodium, 20 µg norfloxacin, 250 µg novobiocin sodium, 10 µg trimethoprim, 50 µg rifampicin, 30 µg chloramphenicol, 30 µg kanamycin sulfate, 30 µg tetracycline, 10 µg ampicillin, and 150 µg cephalexin. Each value represents the mean diameter of the inhibition halo ± standard deviation from three independent experiments.

Cell target	Antibiotic	Strains	
		SeWT	SeTCO
DNA replication	Nalidixic acid	30.08 ± 1.22	23.32 ± 0.76*
	Trimethoprim	27.82 ± 1.10	20.34 ± 0.43*
	Norfloxacin	26.43 ± 1.03	19.68 ± 0.23*
	Novobiocin	13.63 ± 0.40	8.21 ± 0.17*
RNA synthesis	Rifampicin	17.59 ± 0.23	15.34 ± 0.45*
Protein synthesis	Chloramphenicol	26.10 ± 1.37	22.17 ± 0.26*
	Tetracycline	25.80 ± 0.80	20.72 ± 1.80*
	Kanamycin	15.20 ± 1.40	17.62 ± 1.23
Cell wall synthesis	Cephalexin	22.36 ± 0.40	23.15 ± 0.69
	Ampicillin	14.12 ± 0.17	13.49 ± 0.39

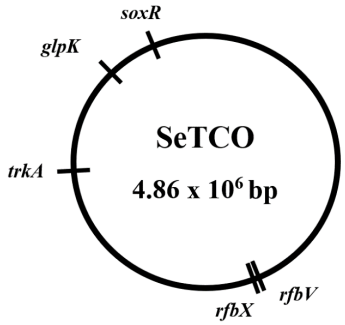
* Significantly different from SeWT (p<0.05).

Inhibition halos revealed cross-resistance against several antibiotics after the evolution assay. SeTCO exhibited smaller halos than SeWT (p<0.05) for tetracycline, chloramphenicol, both quinolone antibiotics (nalidixic acid and norfloxacin), novobiocin and trimethoprim. However, neither kanamycin nor the two β-lactams tested, ampicillin and cephalexin, showed significant differences (p>0.05) in inhibition halos between the SeWT and the evolved strain.

3.6. Genetic variations in the resistant strain to antimicrobials

Genetic differences between the reference genome (NC_003197.2) and our lab strain SeWT were identified to discard them as consequence of the evolution assay, since they were already present at the beginning of the evolution assay (Table S4). The genomic comparison of SeWT and SeTCO allowed the identification of mutations fixed throughout the evolution assay with TCO (Fig. 4), causing the increased resistance and tolerance against TCO, as cross-resistance against antibiotics. Sequencing data revealed 5 mutations in SeTCO: 1 deletion and 4 SNVs (Table 5). Sanger sequencing results verified the mutations in the 5 colonies isolated from the evolution assay, $SeTCO_{1-5}$, demonstrating the homogeneity of the bacterial culture after the evolution assay.

Figure 4. Genomic map of SeTCO: *Salmonella enterica* Typhimurium evolved strains by cyclic short lethal treatments of TCO.



WGS revealed the following genotypic changes in SeTCO:

a) 2 mutations in the *rfb* O antigen gene cluster (Fitzgerald et al., 2003). On the one hand, a deletion of an adenine was observed in position 178 of *rfbV*, which codifies an abequosyltransferase. On the other hand, a SNV was detected in *rfbX*, a change of cytosine by guanine that led to a replacement of proline by alanine in the position 381 of the transmembrane protein.

b) 2 SNVs in SeTCO genome: a transversion from guanine to thymine was detected in position 619 of *trkA,* which codifies an essential subunit of the transmembrane protein of potassium transport systems (K^+); and a change of guanine by adenine located in *glpK* encoding a glycerol kinase.

c) and a missense mutation located in *soxR* gene, a redox-sensitive transcriptional activator: a change of guanine by thymine resulting in an amino acid substitution of arginine by leucine at position 20, specifically in the DNA binding-domain of the SoxR protein.

Table 5. Mutations of SeTCO in comparison with SeWT verified by Sanger sequencing. Single nucleotide variation (SNV) and deletion (Del).

Genome position	Gene	Locus tag	Mutation*	Change	Information
2,168,086	*rfbV*	STM2087	Del: -A 531	Frameshift (178)	Abequosyltransferase
2,168,774	*rfbX*	STM2088	SNV: C1141G	Pro381Ala	Putative O-antigen transferase
3,580,396	*trkA*	STM3409	SNV: G619T	Asp207Tyr	Trk system potassium transport protein TrkA
4,295,798	*glpK*	STM4086	SNV: G66A	Met22Ile	Glycerol kinase
4,504,454	*soxR*	STM4266	SNV: G59T	Arg20Leu	Redox sensitive transcriptional regulator SoxR

***** Position respect to the start of the coding region.

After identifying the genetic modifications in SeTCO, $SeWT_{soxR}$ was constructed by replacing *soxR* in SeWT by the $soxR_{SeTCO}$ variant from SeTCO to determine the contribution of this unique mutation to the phenotype of the evolved strain. On the one hand, MIC and MBC results revealed a similar resistance and tolerance than SeTCO: MIC and MBC of 600 µL/L TCO. Similarly, MIC and MBC of $SeWT_{soxR}$ in skimmed milk showed the same values than SeTCO: 1,600 and 1,800 µL/L TCO respectively. On the other hand, antibiotic susceptibility test showed that $SeWT_{soxR}$ also developed cross-resistance to the same antibiotics as SeTCO (data not shown). No statistically significant differences ($p>0.05$) were observed between the inhibition halos obtained for SeTCO and $SeWT_{soxR}$.

4. Discussion

Several studies have evidenced that the exposure to ICs, such as carvacrol, citral and limonene oxide, could allow the occurrence of resistant strains in food pathogen populations as *E. coli* (Chueca et al., 2016), *S. aureus* (Berdejo et al., 2019) and *S. enterica* (Berdejo, Merino, et al., 2020). Later, despite the compositional complexity of EOs and their different mechanisms of action, Berdejo, Pagán, et al. (2020) also observed the emergence of resistant strains in a Gram-positive bacterium, *S. aureus* against *Citrus sinensis* EO, thus revealing the possibility that resistance to EOs may also occur.

In this regard, five strains of *S. enterica* were randomly isolated after an evolution assay with a complex EO (TCO), namely $SeTCO_{1-5}$. Evolved strains were re-cultivated in absence of TCO to conduct its phenotypic characterization avoiding a temporary

adaption. So, all changes of behaviour, resistance and tolerance observed in this study are permanent and caused by genetics modifications. Phenotypic characterization of SeTCO and SeWT against TCO by MIC and MBC determination, growth kinetics studies in the presence of the EO and survival curves, evidenced the large stable increase in resistance and tolerance of the evolved strain compared to the wild-type strain (origin of the evolution assay).

MIC and MBC results revealed an increase of resistance and tolerance to TCO in SeTCO in comparison with SeWT (Table 1). Previous studies in *S. enterica* did not observe an increase in resistance or tolerance after adaptation phases to natural antimicrobials of up to 72 h, both to ICs, such as cineole and carvacrol (Luz Ida et al., 2012), and to EOs, such as *Rosmarinus officinalis* or *Origanum vulgare* (Gomes-Neto et al., 2014; Monte et al., 2014). In this regard, this is the first study that evidenced the emergence of evolved resistant and tolerant strains against a complex EO in a Gram-negative bacterium.

Growth kinetics demonstrated that both strains grow similarly in the absence of TCO, but SeTCO is better adapted to the presence of TCO than SeWT at all the concentrations tested, with shorter lag phase and higher growth rate (Table 2). These results support that mutations in the evolved strains responsible of the better adaptation to the natural antimicrobial do not come at a cost of growth fitness in the absence of TCO, as previously observed in *S. enterica* against carvacrol (Berdejo, Merino, et al., 2020). In this regard, growth kinetics in the presence of TCO (Fig. 1) would explain how the emergence of this genetic variant could pose a risk in food preservation when these natural antimicrobials are used as bacteriostatic agent: the use of low doses to inhibit or slow down the growth of SeWT would be ineffective for genetic variants such as SeTCO.

Survival of derivative strain treated with TCO in acid or neutral buffer or in TSBYE was higher than SeWT (Fig 2). Notably, the tolerance of SeTCO was higher in the nutrient-rich medium TSBYE than in citrate-phosphate buffers. The fact that the evolution assay was conducted in TSBYE has probably led to the selection of other mutations providing a higher tolerance in this medium. In addition, the increase in tolerance of SeTCO observed in TSBYE would explain its emergence during the evolution cycles: the increased survival of SeTCO after every lethal treatment allowed this genetic variant to become fixed in the microbial population while SeWT concentration was progressively decreasing. In this sense, these results also highlight the

microbiological risk that the emergence of these strains would pose since they would be able to survive lethal treatments.

EOs and ICs have been extensively studied in the preservation of dairy products due to their antimicrobial properties, among others (Mishra et al., 2020). TCO have demonstrated its great antimicrobial properties in milk, alone (Ben Jemaa et al., 2017) or combined with heat treatments (Ben Jemaa et al., 2018), however no previous studies have assessed the food safety risk if resistant bacterial population have emerged. For this reason, skimmed milk was chosen as food model to characterise the resistance and tolerance of the evolved strain against TCO. In this regard, the MIC and MBC of TCO in the evolved strain and SeWT were determined and lethal treatments were conducted in the food model. It is noteworthy that for both strains the MIC and MBC values were higher and it was necessary to increase the TCO concentration in the lethal treatments in milk, compared to growth media and treatments buffer, since milk carbohydrates and proteins might interact with the EO and reduce its antimicrobial properties (Mishra et al., 2020). As observed in TSBYE, MIC and MBC values in milk also revealed twice the resistance and tolerance for SeTCO compared to SeWT (Table 3); and those lethal treatments which inactivated more than 5 $\log_{10}$ cycles of SeWT population in milk, were ineffective against the evolved strain (Fig. 3). In this regard, our results suggest that if these strains emerge in the food industry could grow and survive to treatments designed for wild-type strains. Nonetheless, it is required to further investigate how those resistant populations may appear in food products and their impact on food preservation in order to evaluate whether they may represent a real food risk.

Kirby-Bauer disk diffusion test revealed that SeTCO also increased cross-resistance against quinolones, tetracyclines, and aminoglycosides, with the exception of kanamycin (Table 4). However, both β-lactams tested showed the same activity against SeWT and the evolved strain (p>0.05). It is likely that the mutations selected by the evolutionary assay with TCO provide general antimicrobial resistance and are therefore also responsible for the development of cross-resistance to antibiotics. Berdejo, Merino, et al. (2020) reported that evolved strains of *S. enterica* in the presence of sub-inhibitory doses of carvacrol, the main IC of TCO, maintained the same susceptibility to antibiotics than wild-type strain, whereas evolution assays with lethal treatments of carvacrol led to derivative strains with cross-resistance to a wide range of antibiotics, such as tetracycline or rifampicin. Regarding complex EOs, *S. aureus* evolved with sub-inhibitory orange EO doses (Berdejo, Pagán, et al., 2020) or *S. enterica* adapted to *Origanum vulgare* EO did

not show variations in antibiotic resistance. The fact that mechanisms of bacterial resistance to EOs and antibiotics may be interrelated supports the importance of assessing the occurrence of resistance to natural antimicrobials in its use as alternative to antibiotics against MDR strains. Nonetheless, further studies are required to find out under which conditions cross-resistance may occur.

Genetic analysis revealed 5 mutations in $SeTCO_{1-5}$ after evolution assay in comparison with SeWT (Fig. 5): 1 deletion in *rfbV* and 4 SNVs in *rfbX, trkA, glpK* and *soxR* (Table 4). Both mutations in *rfb* O antigen gene cluster are located in transferases involved in the lipopolysaccharides O-antigen biosynthesis (Liu et al., 1996; Liu et al., 1995), which have an important role in bacterial virulence (Liu et al., 2014). In the field of antibiotics, the close relationship between antimicrobial resistance and virulence has been extensively studied, nevertheless, the correlation between them can be either positive or negative (Cepas and Soto, 2020). However, there are no data about the relation between the resistances to natural antimicrobials and the virulence of the mutant strains.

According to Knöppel et al. (2018), the mutations in genes involved in metabolic pathways, *trkA* and *glpK*, usually occur as consequence of adaptation to the medium growth. In fact, Berdejo, Merino, et al. (2020) also observed a mutation in *trkA* after evolution assay with carvacrol in *S. enterica*. In this regard, these mutations were probably selected because a nutrient medium as TSBYE was employed in the evolution assay and not due to the antimicrobial TCO.

The last SNV was detected in *soxR* which codifies a redox-sensitive transcriptional regulator involved in the defence against redox-cycling drugs (Gu and Imlay, 2011). Its oxidized form activates the transcription of the *soxS* gene that modulates the expression of more than 100 genes of the *sox*RS regulon to provide cellular defence against oxidative stress (Pomposiello et al., 2001). The main role of the *sox*RS regulon is to minimize intracellular drug concentration through mechanisms that impede their entry, chemically modify them, or pump them out (Gu and Imlay, 2011). This bacterial response has been described against oxidizing agents and antibiotics, and more recently, have linked it to the bacterial response against ICs or EOs (Chueca et al., 2018; Sheng et al., 2016). In addition, a recent proteomic study in *S. enterica* observed a differential expression of stress-related proteins, such as superoxide dismutase, when the bacterium was exposed to EO of *Origanum vulgare* supporting that oxidative stress would be related with the cell response to complex EOs (Barbosa et al., 2020). Previous studies in *E. coli* (Chueca et al., 2018) and *S. enterica* (Berdejo, Merino, et al., 2020) reported that *soxR* was mutated

after cyclic treatments of carvacrol, the main IC of TCO. In agreement with results in Table 4, both studies observed a general increase of resistance against a wide range of antibiotics, supporting that the mutation in *soxR* plays an important role in the decrease of the antibiotic susceptibility via the regulation of the multidrug efflux pumps (Du et al., 2018; Kumar et al., 2013). It should be noted that the mutation in *S. enterica* evolved with carvacrol (SeTCar; (Berdejo, Merino, et al., 2020)) was located at a different nucleotide but in the same codon than SeTCO: a replacement of arginine by cysteine. In fact, although both strains, SeTCar and SeTCO, showed increased cross-resistance to most antibiotics, their resistance against cephalexin was not modified. Thus, mutations in *soxR* may enhance the pump out of drugs with intracellular targets, such as quinolones, tetracyclines and aminoglycosides. However, it is likely that the susceptibility to β-lactams has been maintained since they act on the synthesis of the peptidoglycan wall, in the periplasmic space.

Determination of MIC and MBC to TCO for $SeWT_{soxR}$ in TSBYE and skimmed milk revealed that *soxR* mutation was the main cause of the increased resistance and tolerance of SeTCO against TCO. These findings highlight *soxR* as a key mechanism in the cellular response to TCO, induced by the oxidative stress caused by EOs (Chueca et al., 2014). Mutations in this gene could lead to the development of resistance by an increase in efflux of intracellular antimicrobials. Since $soxR_{SeTCO}$ mutation (codon 20) is located in the DNA binding-domain of SoxR, it is likely that the its stability or affinitity has been increased and, consequently, the expression of the *sox*RS regulon may have been modified. In addition, the genotypic study of a *S.* Typhimurium variant with increased resistance against carvacrol isolated by Berdejo, Merino, et al. (2020), SeTCar, also revealed a SNV in *soxR*, specifically in the same codon that SeTCO. These results support the great relevance of *soxR* in the resistance mechanisms against ICs and EOs in *S.* Typhimurium.

Furthermore, the increased cross-resistance to antibiotics of $SeWT_{soxR}$ demonstrates the important role of *soxR*, not only against natural antimicrobials, but also against antibiotics. In this regard, these results support that bacterial resistance mechanisms to EOs and antibiotics may be interrelated, so the occurrence of cross-resistance should be considered in the use of EOs as an alternative to antibiotics against MDR strains.

5. Conclusions

Cyclic exposure to lethal doses of *Thymbra capitata* essential oil (TCO) led to the emergence of resistant and tolerant strains in *Salmonella enterica* bacterial population (SeTCO). SeTCO revealed increased of MIC and MBC, greater growth rate in the presence of TCO, as well as higher survival compared to the wild-type strain (SeWT). The increased resistance and tolerance of SeTCO was confirmed in skimmed milk calling into question the efficacy of essential oils (EOs) as food preservatives if resistant and tolerant strains emerge. In this regard, further studies are required to define under which conditions these resistant strains could appear and to assess the food risk they may pose.

Furthermore, SeTCO also showed an increased resistance against several antibiotics: quinolones, tetracyclines and aminoglycosides. The fact that mechanisms of bacterial resistance to EOs and antibiotics may be interrelated supports the importance of deeper understand of how these resistances emerge by the use of natural antimicrobials in its use as alternative to antibiotics against multidrug resistant strains.

Genetic analysis detected 5 mutations in SeTCO: 2 in virulence-related genes, 2 in genes encoding metabolic enzymes probably selected due to the growth medium used in the evolution assay and 1 in *soxR*. Among them, *soxR* variation was the main contribution to the increased resistance and tolerance observed in SeTCO against TCO in laboratory media and in the food model, as well against antibiotics, probably enhancing the pump out of antimicrobial compounds outside the cell and limiting the antimicrobial effect of the EO. In this sense, this study provides relevant information on the mechanisms of action of EOs in the bacterial cell, supporting that these natural antimicrobials could also cause severe intracellular damages.

Acknowledgments

This study was financially supported by Agencia Estatal de Investigación (Spain) (Project No. PGC2018-093789-B-I00), by the European Social Fund, and by the Aragonese Office of Science, Technology and University Research. The Spanish Ministry of Sports, Culture and Education provided D. Berdejo and E. Pagán with a grant to carry out this investigation. The authors thank Elisa Gayan for her help on strain construction and S. Hanks for his collaboration on the English-language revision of this manuscript.

Author contributions

Daniel Berdejo: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing. **Elisa Pagán**: Investigation, Validation. **Natalia Merino**: Investigation, Validation. **Laura Botello-Morte**: Investigation, Validation. **Rafael Pagán**: Conceptualization, Funding acquisition, Resources, Supervision, Writing. **Diego García-Gonzalo**: Conceptualization, Funding acquisition, Resources, Supervision, Writing.

Declarations of interest

Declarations of interest: none

References

Balaban, N.Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D.I., Brynildsen, M.P., Bumann, D., Camilli, A., Collins, J.J., Dehio, C., Fortune, S., Ghigo, J.-M., Hardt, W.-D., Harms, A., Heinemann, M., Hung, D.T., Jenal, U., Levin, B.R., Michiels, J., Storz, G., Tan, M.-W., Tenson, T., Van Melderen, L., Zinkernagel, A., 2019. Definitions and guidelines for research on antibiotic persistence. Nature Reviews Microbiology 17, 441-448.

Barbosa, L.N., Alves, F.C.B., Andrade, B., Albano, M., Rall, V.L.M., Fernandes, A.A.H., Buzalaf, M.A.R., Leite, A.L., de Pontes, L.G., Dos Santos, L.D., Fernandes Junior, A., 2020. Proteomic analysis and antibacterial resistance mechanisms of Salmonella Enteritidis submitted to the inhibitory effect of Origanum vulgare essential oil, thymol and carvacrol. Journal of Proteomics 214, 103625.

Ben Jemaa, M., Falleh, H., Neves, M.A., Isoda, H., Nakajima, M., Ksouri, R., 2017. Quality preservation of deliberately contaminated milk using thyme free and nanoemulsified essential oils. Food Chemistry 217, 726-734.

Ben Jemaa, M., Falleh, H., Saada, M., Oueslati, M., Snoussi, M., Ksouri, R., 2018. Thymus capitatus essential oil ameliorates pasteurization efficiency. Journal of Food Science and Technology 55, 3446-3452.

Berdejo, D., Chueca, B., Pagan, E., Renzoni, A., Kelley, W.L., Pagan, R., Garcia-Gonzalo, D., 2019. Sub-Inhibitory Doses of Individual Constituents of Essential Oils Can Select for *Staphylococcus aureus* Resistant Mutants. Molecules 24.

Berdejo, D., Merino, N., Pagán, E., García-Gonzalo, D., Pagán, R., 2020. Genetic Variants and Phenotypic Characteristics of *Salmonella* Typhimurium-Resistant Mutants after Exposure to Carvacrol. Microorganisms 8.

Berdejo, D., Pagán, E., Merino, N., Pagán, R., García-Gonzalo, D., 2020. Incubation with a Complex Orange Essential Oil Leads to Evolved Mutants with Increased Resistance and Tolerance. Pharmaceuticals (Basel, Switzerland) 13.

Boor, K.J., Wiedmann, M., Murphy, S., Alcaine, S., 2017. A 100-Year Review: Microbiology and safety of milk handling. Journal of Dairy Science 100, 9933-9951.

Calo, J.R., Crandall, P.G., O'Bryan, C.A., Ricke, S.C., 2015. Essential oils as antimicrobials in food systems – A review. Food Control 54, 111-119.

Cepas, V., Soto, S.M., 2020. Relationship between Virulence and Resistance among Gram-Negative Bacteria. 9, 719.

Chueca, B., Berdejo, D., Gomes-Neto, N.J., Pagán, R., García-Gonzalo, D., 2016. Emergence of Hyper-Resistant *Escherichia coli* MG1655 Derivative Strains after Applying Sub-Inhibitory Doses of Individual Constituents of Essential Oils. Frontiers in Microbiology 7, 273-273.

Chueca, B., Pagán, R., García-Gonzalo, D., 2014. Oxygenated monoterpenes citral and carvacrol cause oxidative damage in *Escherichia coli* without the involvement of tricarboxylic acid cycle and Fenton reaction. International Journal of Food Microbiology 189, 126-131.

Chueca, B., Renzoni, A., Berdejo, D., Pagan, R., Kelley, W.L., Garcia-Gonzalo, D., 2018. Whole-Genome Sequencing and Genetic Analysis Reveal Novel Stress Responses to Individual Constituents of Essential Oils in *Escherichia coli*. Applied and Environmental Microbiology 84.

Cingolani, P., Platts, A., Wang le, L., Coon, M., Nguyen, T., Wang, L., Land, S.J., Lu, X., Ruden, D.M., 2012. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. Fly 6, 80-92.

CLSI, 2012. Performance standards for antimicrobial disk susceptibility tests; approved standard - Eleventh edition. Clinical and laboratory standards institute, Wayne, PA.

CLSI, 2014. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth INformational Supplement. CLSI Document M100-S24.

CLSI, 2015. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard - Tenth Edition. CLSI document M07-A10.

Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proceedings of the National Academy of Sciences 97, 6640.

Du, D., Wang-Kan, X., Neuberger, A., van Veen, H.W., Pos, K.M., Piddock, L.J.V., Luisi, B.F., 2018. Multidrug efflux pumps: structure, function and regulation. Nature Reviews Microbiology 16, 523-539.

Fitzgerald, C., Sherwood, R., Gheesling, L.L., Brenner, F.W., Fields, P.I., 2003. Molecular analysis of the rfb O antigen gene cluster of Salmonella enterica serogroup O:6,14 and development of a serogroup-specific PCR assay. Applied and Environmental Microbiology 69, 6099-6105.

Friedman, M., Henika, P.R., Mandrell, R.E., 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. Journal of Food Protection 65, 1545-1560.

Gagliano Candela, R., Maggi, F., Lazzara, G., Rosselli, S., Bruno, M., 2019. The Essential Oil of Thymbra capitata and its Application as A Biocide on Stone and Derived Surfaces. Plants (Basel, Switzerland) 8, 300.

Gomes-Neto, N.J., Luz, I.S., Franco, O.L., Magnani, M., Souza, E.L., 2014. Tolerance evaluation in *Salmonella enterica* serovar Typhimurium challenged with sublethal amounts of *Rosmarinus officinalis* L. essential oil or 1,8-cineole in meat model. International Journal of Food Science & Technology 49, 1912-1917.

Gu, M., Imlay, J.A., 2011. The SoxRS response of *Escherichia coli* is directly activated by redox-cycling drugs rather than by superoxide. Molecular Microbiology 79, 1136-1150.

Karampoula, F., Giaouris, E., Deschamps, J., Doulgeraki, A.I., Nychas, G.J., Dubois-Brissonnet, F., 2016. Hydrosol of Thymbra capitata Is a Highly Efficient Biocide against Salmonella enterica Serovar Typhimurium Biofilms. Applied and Environmental Microbiology 82, 5309-5319.

Knöppel, A., Knopp, M., Albrecht, L.M., Lundin, E., Lustig, U., Näsvall, J., Andersson, D.I., 2018. Genetic Adaptation to Growth Under Laboratory Conditions in *Escherichia coli* and *Salmonella enterica*. Frontiers in Microbiology 9, 756-756.

Kohanski, M.A., DePristo, M.A., Collins, J.J., 2010. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Molecular Cell 37, 311-320.

Kumar, S., Mukherjee, M.M., Varela, M.F., 2013. Modulation of Bacterial Multidrug Resistance Efflux Pumps of the Major Facilitator Superfamily. International Journal of Bacteriology 2013, 204141.

Lallemand, E.A., Lacroix, M.Z., Toutain, P.-L., Boullier, S., Ferran, A.A., Bousquet-Melou, A., 2016. In vitro Degradation of Antimicrobials during Use of Broth Microdilution Method Can Increase the Measured Minimal Inhibitory and Minimal Bactericidal Concentrations. Frontiers in Microbiology 7, 2051-2051.

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., Balaban, N.Q., 2017. Antibiotic tolerance facilitates the evolution of resistance. Science 355, 826.

Li, H., Durbin, R., 2010. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26, 589-595.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25, 2078-2079.

Li, X.-T., Thomason, L.C., Sawitzke, J.A., Costantino, N., Court, D.L., 2013. Positive and negative selection using the tetA-sacB cassette: recombineering and P1 transduction in Escherichia coli. Nucleic Acids Research 41, e204-e204.

Liu, B., Knirel, Y.A., Feng, L., Perepelov, A.V., Senchenkova, S.y.N., Reeves, P.R., Wang, L., 2014. Structural diversity in SalmonellaO antigens and its genetic basis. FEMS Microbiology Reviews 38, 56-89.

Liu, D., Cole, R.A., Reeves, P.R., 1996. An O-antigen processing function for Wzx (RfbX): a promising candidate for O-unit flippase. Journal of Bacteriology 178, 2102-2107.

Liu, D., Lindqvist, L., Reeves, P.R., 1995. Transferases of O-antigen biosynthesis in Salmonella enterica: dideoxyhexosyltransferases of groups B and C2 and acetyltransferase of group C2. Journal of Bacteriology 177, 4084-4088.

Luz Ida, S., Gomes Neto, N.J., Tavares, A.G., Nunes, P.C., Magnani, M., de Souza, E.L., 2012. Evidence for lack of acquisition of tolerance in Salmonella enterica serovar Typhimurium ATCC 14028 after exposure to subinhibitory amounts of Origanum vulgare L. essential oil and carvacrol. Applied and Environmental Microbiology 78, 5021-5024.

McClelland, M., Sanderson, K.E., Spieth, J., Clifton, S.W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W., Stoneking, T., Nhan, M., Waterston, R., Wilson, R.K., 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature 413, 852-856.

McMillan, E.A., Gupta, S.K., Williams, L.E., Jové, T., Hiott, L.M., Woodley, T.A., Barrett, J.B., Jackson, C.R., Wasilenko, J.L., Simmons, M., Tillman, G.E., McClelland, M., Frye, J.G., 2019. Antimicrobial Resistance Genes, Cassettes, and Plasmids Present in Salmonella enterica Associated With United States Food Animals. Frontiers in Microbiology 10, 832-832.

Merino, N., Berdejo, D., Bento, R., Salman, H., Lanz, M., Maggi, F., Sánchez-Gómez, S., García-Gonzalo, D., Pagán, R., 2019. Antimicrobial efficacy of *Thymbra capitata* (L.) Cav. essential oil loaded in self-assembled zein nanoparticles in combination with heat. Industrial Crops and Products 133, 98-104.

Mishra, A.P., Devkota, H.P., Nigam, M., Adetunji, C.O., Srivastava, N., Saklani, S., Shukla, I., Azmi, L., Shariati, M.A., Melo Coutinho, H.D., Mousavi Khaneghah, A., 2020. Combination of essential oils in dairy products: A review of their functions and potential benefits. LWT 133, 110116.

Mittal, R.P., Rana, A., Jaitak, V., 2019. Essential Oils: An Impending Substitute of Synthetic Antimicrobial Agents to Overcome Antimicrobial Resistance. Current Drug Targets 20, 605-624.

Monte, D.F., Tavares, A.G., Albuquerque, A.R., Sampaio, F.C., Oliveira, T.C., Franco, O.L., Souza, E.L., Magnani, M., 2014. Tolerance response of multidrug-resistant Salmonella enterica strains to habituation to Origanum vulgare L. essential oil. Frontiers in Microbiology 5, 721.

Olsen, S.J., Ying, M., Davis, M.F., Deasy, M., Holland, B., Iampietro, L., Baysinger, C.M., Sassano, F., Polk, L.D., Gormley, B., Hung, M.J., Pilot, K., Orsini, M., Van Duyne, S., Rankin, S., Genese, C., Bresnitz, E.A., Smucker, J., Moll, M., Sobel, J., 2004. Multidrug-resistant Salmonella Typhimurium infection from milk contaminated after pasteurization. Emerging Infectious Diseases 10, 932-935.

Pandey, A.K., Kumar, P., Singh, P., Tripathi, N.N., Bajpai, V.K., 2017. Essential Oils: Sources of Antimicrobials and Food Preservatives. 7.

Parry-Hanson Kunadu, A., Holmes, M., Miller, E.L., Grant, A.J., 2018. Microbiological quality and antimicrobial resistance characterization of Salmonella spp. in fresh milk value chains in Ghana. International Journal of Food Microbiology 277, 41-49.

Plumb, I.D., Schwensohn, C.A., Gieraltowski, L., Tecle, S., Schneider, Z.D., Freiman, J., Cote, A., Noveroske, D., Kolsin, J., Brandenburg, J., Chen, J.C., Tagg, K.A., White, P.B., Shah, H.J., Watkins, L.K.F., Wise, M.E., Friedman, C.R., 2019. Outbreak of Salmonella Newport Infections with Decreased Susceptibility to Azithromycin Linked to Beef Obtained in the United States and Soft Cheese Obtained in Mexico — United States, 2018–2019. Morbidity and Mortality Weekly Report. Centers for Disease Control and Prevention, 713–717.

Pomposiello, P.J., Bennik, M.H., Demple, B., 2001. Genome-wide transcriptional profiling of the *Escherichia coli* responses to superoxide stress and sodium salicylate. Journal of Bacteriology 183, 3890-3902.

Qamar, A., Ismail, T., Akhtar, S., 2020. Prevalence and antibiotic resistance of Salmonella spp. in South Punjab-Pakistan. PLoS ONE 15, e0232382.

Robinson, E., Travanut, M., Fabre, L., Larréché, S., Ramelli, L., Pascal, L., Guinard, A., Vincent, N., Calba, C., Meurice, L., Le Thien, M.A., Fourgere, E., Jones, G., Fournet, N., Smith-Palmer, A., Brown, D., Le Hello, S., Pardos de la Gandara, M., Weill, F.X., Jourdan-Da Silva, N., 2020. Outbreak of Salmonella Newport associated with internationally distributed raw goats' milk cheese, France, 2018. Epidemiology and Infection 148, e180.

Sheng, L.N., Rasco, B., Zhu, M.J., 2016. Cinnamon Oil Inhibits Shiga Toxin Type 2 Phage Induction and Shiga Toxin Type 2 Production in *Escherichia coli* O157:H7. Applied and Environmental Microbiology 82, 6531-6540.

Sonnier, J.L., Karns, J.S., Lombard, J.E., Kopral, C.A., Haley, B.J., Kim, S.W., Van Kessel, J.A.S., 2018. Prevalence of Salmonella enterica, Listeria monocytogenes, and pathogenic Escherichia coli in bulk tank milk and milk filters from US dairy operations in the National Animal Health Monitoring System Dairy 2014 study. Journal of Dairy Science 101, 1943-1956.

Ung, A., Baidjoe, A.Y., Van Cauteren, D., Fawal, N., Fabre, L., Guerrisi, C., Danis, K., Morand, A., Donguy, M.P., Lucas, E., Rossignol, L., Lefèvre, S., Vignaud, M.L., Cadel-Six, S., Lailler, R., Jourdan-Da Silva, N., Le Hello, S., 2019. Disentangling a complex nationwide Salmonella Dublin outbreak associated with raw-milk cheese consumption, France, 2015 to 2016. Euro Surveillance 24.

Yap, P.S.X., Yiap, B.C., Ping, H.C., Lim, S.H.E., 2014. Essential oils, a new horizon in combating bacterial antibiotic resistance. The Open Microbiology Journal 8, 6-14.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., van 't Riet, K., 1990. Modeling of the bacterial growth curve. Applied and Environmental Microbiology 56, 1875-1881.

Supplemental material

Table S1. Primers used for PCR amplification and Sanger sequencing to verify the mutations in SeTCO.

SeTCO mutations	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
rfbV	GCCATAACATGCCATAGCCA	GCAGTGATGATGCTCTTGCG
rfbX	TGGCCCAAGGTTAACGCTATT	TCAAAGGCTAACTCCTGCTGA
trkA	ACACTCCAAACCGTATCGCC	TCGCGGACATGATATTGGCT
glpK	TGTAATCTTCCAGGCCGTCG	TTTGGCCCCAAAATGTTCGC
soxR	CTCGGTCGTTCGTAGCTCAA	GTCTAACTCTTCGCGCCACT

Table S2. Primers used for *soxR* replacement in $SeWT_{soxR}$ construction.

soxR replacement	**Primer sequence**
Forward (5′→3′)	CATGCACTCGGCCATCGGGCTGAGCTCACCTAAAACTAAA GCGCCGCTAA**TCCTAATTTTTGTTGACACTCTATC**
Reverse (5′→3′)	ATCAGCGGGATAGAGCGAAAGACAAAGACCGGAAACAAACT AAAGCGCCC**ATCAAAGGGAAAACTGTCCATATGC**

*Underlined sequence indicates homologous region for targeting and boldface indicates ***tetA/sacB.***

Table S3. A (maximum OD_{595}), μ_m (maximum specific growth rate; h^{-1}) and λ (lag time; h) values and standard error of the modified Gompertz model obtained from 3 independently growth curves of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT) (A) and SeTCO (B) at different concentrations of TCO. The goodness of the fit is shown by R^2 and adjusted R^2 values and the root mean square error (*RMSE*).

A) SeWT

TCO (µL/L)	Values			Standard error			Goodness of fit		
	A	μ_{max}	λ	A	μ_{max}	λ	R^2	Adj. R^2	*RMSE*
0	1.159	0.287	3.423	0.009	0.021	0.170	0.989	0.988	0.042
50	1.171	0.152	4.641	0.014	0.008	0.243	0.988	0.989	0.044
100	1.162	0.114	5.828	0.019	0.005	0.245	0.992	0.991	0.037
150	1.174	0.091	7.001	0.073	0.009	0.555	0.964	0.962	0.076
200	0.716	0.076	14.160	0.037	0.003	0.154	0.992	0.992	0.019

B) SeTCO

TEO (µL/L)	Values			Standard error			Goodness of fit		
	A	μ_{max}	λ	A	μ_{max}	λ	R^2	Adj. R^2	*RMSE*
0	1.156	0.288	3.391	0.006	0.016	0.121	0.994	0.994	0.030
50	1.156	0.201	3.961	0.010	0.011	0.185	0.991	0.991	0.039
100	1.157	0.156	4.581	0.025	0.016	0.439	0.966	0.964	0.080
150	1.168	0.156	5.222	0.014	0.009	0.241	0.989	0.989	0.044
200	1.158	0.147	6.309	0.035	0.017	0.518	0.955	0.952	0.090
250	1.146	0.164	9.190	0.018	0.009	0.201	0.991	0.990	0.045
300	1.161	0.157	10.158	0.026	0.010	0.230	0.987	0.986	0.053
350	1.160	0.161	12.561	0.034	0.011	0.225	0.986	0.985	0.054
400	1.101	0.179	13.650	0.018	0.007	0.118	0.996	0.995	0.029
450	1.101	0.178	14.767	0.026	0.009	0.147	0.992	0.992	0.036
500	0.792	0.109	17.961	0.017	0.005	0.148	0.991	0.990	0.022
550	0.518	0.103	18.352	0.028	0.014	0.324	0.949	0.946	0.040

Table S4. Genetic variations detected by whole genome sequencing (WGS) between SeWT and the reference genome of *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 (NCBI accession: NC_003197.2). Single nucleotide variation (SNV), insertion (Ins) and deletion (Del). *Position respect to the start of the coding region.

Genome position	Genes	Locus tag	Mutation*	Change	Information
290,718	*rrsH*	STM0249	SNV: C1529A	No coding	RNA 16S ribosomal
364,623	*crl*	STM0319	Del: -T104	Frame shift	Sigma factor-binding protein
416,555	*prpR*	STM0367	SNV: C1159T	Leu387Phe	Operon regulator
453,939	*brnQ*	STM0399	SNV: C681T	Silent mutation (Tyr227)	Branched-chain amino acid transport system carrier protein
509,118	*cypD*	STM0452	SNV: T450A	Asp150Glu	Peptidylprolyl isomerase
608,859	*fimH*	STM0547	SNV: G182C	Gly61Ala	Adhesin
1,205,933	Intergenic *wraB - ycdF*	STM1119 STM1120	SNV: GA	No coding	-
1,778,104	*ycjF*	STM1684	SNV: T821C	Leu274Pro	UPF0283 membrane protein
1,841,398	-	STM1747	SNV: G98A	Arg33Gln	Hypothetical protein
1,849,642	*hnr*	STM1753	SNV: T305G	Val102Gly	Regulator of RpoS
3,469,143	*dacB*	STM3300	SNV: C483T	Silent mutation (Ser161)	Transpeptidase
3,673,628	*malQ*	STM3513	SNV: T287G	Leu96Arg	4-Alpha-glucanotransferase
3,675,952	*malP*	STM3514	Del: -GCCGCCTG 358	Frame shift	Alpha-1,4 phosphorylase
3,819,815	-	STM3633	SNV: T562C	Silent mutation (Leu188)	LacI family transcriptional regulator
4,122,937	*gppA*	STM3913	SNV: G385T	Gly129Cys	Pyrophosphatase
4,122,950	*gppA* *rhlB*	STM3913 STM3914	Del: -1,179 pb	Knock-out (*gppA*, *rhlB*)	Pyrophosphatase ATP-dependent RNA helicase RhlB
4,291,432	*yiiQ*	STM4082	SNV: G323A	Stop-gain	Hypothetical protein
4,294,693	*glpK*	STM4086	SNV: G1171A	Asp391Asn	Glycerol kinase
4,697,694	*treB*	STM4454	Ins: + A543	Frame shift	Pseudogene (trehalose metabolism)

Table S5. Zones of growth inhibition for agar disk diffusion assays of SeWTsoxR, constructed by replacing *soxR* in SeWT by the *soxR* variant from SeTCO, against antibiotics: 400 µg nalidixic acid sodium, 20 µg norfloxacin, 250 µg novobiocin sodium, 10 µg trimethoprim, 50 µg rifampicin, 30 µg chloramphenicol, 30 µg kanamycin sulfate, 30 µg tetracycline, 10 µg ampicillin, and 150 µg cephalexin. Each value represents the mean diameter of the inhibition halo ± standard deviation from three independent experiments.

Cell target	**Antibiotic**	**Strains**
		SeWT$_{soxR}$
DNA replication	Nalidixic acid	23.13 ± 1.54*
	Trimethoprim	21.02 ± 0.73*
	Norfloxacin	18.98 ± 0.82*
	Novobiocin	9.62 ± 1.04*
RNA synthesis	Rifampicin	15.37 ± 0.61*
Protein synthesis	Chloramphenicol	21.66 ± 0.67*
	Tetracycline	20.96 ± 1.27*
	Kanamycin	16.75 ± 1.18
Cell wall synthesis	Cephalexin	23.08 ± 0.98
	Ampicillin	14.17 ± 0.51

* Significantly different from SeWT (p<0.05).

Manuscrito VIII. Antimicrobial activity of suspensions and nanoemulsions of citral in combination with heat or pulsed electric fields

Pagán, E., Berdejo, D., Espina, L., García-Gonzalo, D., Pagán, R. (2018)

Letters in Applied Microbiology, 66, 63-70. doi:10.1111/lam.12815.

Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Antimicrobial activity of suspensions and nanoemulsions of citral in combination with heat or pulsed electric fields

E. Pagán, D. Berdejo, L. Espina, D. García-Gonzalo and R. Pagán

Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain

Significance and Impact of the Study: The exploration of different delivery systems of antimicrobial compounds such as citral in aqueous food products aids in the establishment of successful combined treatments for food preservation. While at room temperature, citral in form of a nanoemulsion shows a higher antimicrobial activity; its combination with heat would imply a partial loss of the outstanding synergistic lethal effect achieved when added in suspension form. Therefore, the most suitable procedure to magnify the synergism between heat and citral when processing juices would merely require an intense homogenization step prior to the combined treatment.

Keywords
antimicrobials, cell injury, food preservation, nonthermal processes, thermal processes.

Correspondence
Rafael Pagán, Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain.
E-mail: pagan@unizar.es

2017/1784: received 7 September 2017, revised 19 October 2017 and accepted 19 October 2017

doi:10.1111/lam.12815

Abstract

The application of essential oils in form of nanoemulsions has been proposed as a method to improve their solubility in aqueous solutions, and hence their antimicrobial activity. The objective of this study was to evaluate the antimicrobial activity of citral, applied directly or in combined treatments with heat or pulsed electric fields (PEF), as a function of the inoculation procedure assayed: (i) a simple, vigorous shaking method by vortex agitation (suspension of citral; s-citral) or (ii) the previous preparation of nanoemulsions by the emulsion phase inversion (EPI) method (nanoemulsion of citral; n-citral). n-Citral was more effective in either inhibiting or inactivating *Escherichia coli* O157:H7 Sakai than s-citral. However, when combined with heat, a greater synergistic effect was observed with s-citral rather than with n-citral, either in lab media (pH 7·0 and 4·0) or apple juice. For instance, while almost 5 $\log_{10}$ cell cycles were inactivated in apple juice after 15 min at 53°C in the presence of 0·1 μl ml^{-1} of s-citral, the use of n-citral required 30 min. The use of nanoemulsions did not modify the slight synergism observed when citral and mild PEF were combined (150 μs, 30 kV cm^{-1}).

Introduction

The design of successful food preservation processes relies on the establishment of those treatment conditions which guarantee the innocuity of the product with minimal detriment of nutritional and sensory parameters. A way of achieving such effective treatment conditions is through the combination of antimicrobial agents and physical methods, following the hurdle theory proposed by Leistner and Gorris (1995). Over the last few decades, essential oils (EOs) extracted from aromatic and medicinal plants or citrus fruits, as well as their individual constituents, have been tested as antimicrobial agents in combination with heat or pulsed electric field pulses (PEF). In many cases, remarkable synergistic effects in the lethality of these combinations allowed to decrease treatment temperatures and/or antimicrobial doses, or to potentiate the inactivation achieved by PEF (Corbo *et al.* 2009; Espina *et al.* 2010, 2012; de Souza *et al.* 2016). Among EO constituents, citral has displayed a broad-spectrum antimicrobial activity and has been shown to be one of the most effective antimicrobials applied either directly or in combined treatments. For instance, very low doses of citral (0·018–0·2 μl ml^{-1}) in combination with heat were capable of inactivating 5 $\log_{10}$ cell cycles of

Escherichia coli O157:H7, showing promising results for the preservation of apple juice (Espina *et al.* 2010).

Nevertheless, many authors observe that the hydrophobicity of EOs may hamper their homogenous dispersion in aqueous food products (Maswal and Dar 2014; Piorkowski and McClements 2014). As an effective approach to improve the dispersion of EOs into food products and minimize the phase separation, the formation of food-grade emulsions using low-energy preparation methods is a field of great interest (Komaiko and McClements 2016). Procedures such as the emulsion phase inversion (EPI) method generate metastable oil-in-water nanoemulsions are simple to implement, and no expensive equipment is required.

While the effect of emulsification on the antimicrobial activity of EOs has been studied in depth (Donsi *et al.* 2011; Maswal and Dar 2014; Moghimi *et al.* 2016; Zhang *et al.* 2017), few studies have evaluated their influence when applied in combination with other treatments such as heat or PEF. In this regard, to the best of our knowledge, there are no documented studies on the behaviour of EPI nanoemulsions of citral under heat or PEF treatments.

The first objective of this study was to evaluate the antimicrobial activity of citral against *E. coli* O157:H7 Sakai, applied directly or in combined treatments with heat or PEF, as a function of the citral preparation procedure used: (i) a simple vigorous shaking method by vortex agitation (suspension of citral; s-citral), and (ii) the previous preparation of nanoemulsions by the EPI method (nanoemulsion of citral; n-citral). As a second objective, the combination of heat with s- and n-citral to inactivate *E. coli* O157:H7 Sakai was assayed in apple juice.

Results and discussion

Droplet size and stability of nanoemulsions

Nanoemulsions of citral were prepared by the EPI method and characterized during a period of storage under refrigeration. As shown in Table 1, droplet size remained below 200 nm during the 4 months of storage. On the other hand, there were no significant differences ($P > 0{\cdot}05$) among survival curves of *E. coli* O157:H7 Sakai obtained in the presence of 0·6 μl ml^{-1} at pH 4·0, either from different nanoemulsion preparations and different storage times, which indicates that the EPI method assayed allows the obtention of reproducible and stable nanoemulsions of citral. Figure 1a shows the mean values and the standard deviation of nine survival curves corresponding to different emulsions and storage times. To the best of our knowledge, there are no documented studies on the production and characterization of nanoemulsions of citral using the EPI method. The stability of nanoemulsions of D-limonene obtained with the same methodology has already been shown by Zhang *et al.* (2014) and Mate *et al.* (2016) for 6 months.

Effect of citral as a suspension or nanoemulsion on antimicrobial activity

The use of citral in the form of a nanoemulsion decreased the MIC from 0·8 (s-citral) to 0·7 (n-citral) μl ml^{-1} ($P < 0{\cdot}05$) against *E. coli* O157:H7 Sakai. This result reveals that n-citral can inhibit microbial growth more efficiently than s-citral. Nevertheless, this increase in the antibacterial efficacy of citral after its emulsification was much lower than that observed for other antimicrobials in previous works (Komaiko and McClements 2016). For example, Moghimi *et al.* (2016) demonstrated a fourfold reduction of the MIC value with a nanoemulsion of sage oil.

Moreover, as shown in Fig. 1, n-citral was also more effective than s-citral in the inactivation of *E. coli* O157:H7 Sakai in both pH 4·0 and 7·0 treatment media. While the kinetics of inactivation obtained with s-citral showed a prolonged shoulder followed by a rapid decrease, those obtained with n-citral were approximately linear. The greater antimicrobial activity of EOs in the form of nanoemulsions has been associated with their increased polarity, thanks to the coating of the surfactants that reduces surface tension of the oil droplets (Piorkowski and McClements 2014). Thus, the emulsification of hydrophobic substances might reduce their immiscibility in aqueous solutions, making them readily dispersible in the treatment media. In this regard, Moghimi *et al.* (2016) proved that conversion of sage oil into a nanoemulsion improved its antibacterial activity by enhancing its ability to promote the destruction of bacterial cell membranes.

Therefore, the preparation of a nanoemulsion of citral seems to be the best option in treatments applied at room temperature. In addition, the nanoemulsion would also provide the chemical stability required for prolonged inhibitory or bactericidal treatments (Maswal and Dar 2014).

Table 1 Droplet size and polydispersity index (PDI) of nanoemulsions of citral stored under refrigeration. Data represent the mean ± standard error of the mean of at least three independent experiments

Storage time (months)	Droplet size (nm)	PDI
0	161 ± 5	0·096 ± 0·012
1	160 ± 2	0·159 ± 0·011
4	191 ± 0	0·291 ± 0·005

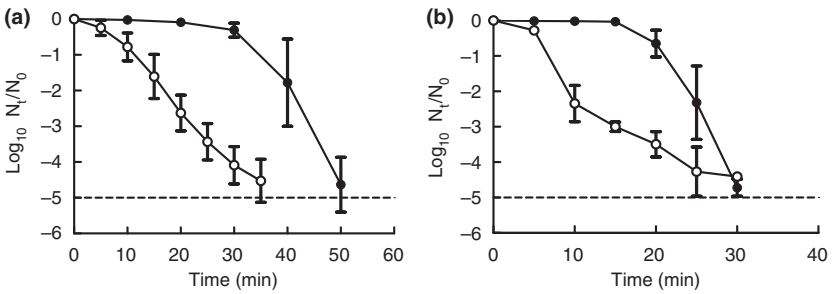


Figure 1 Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration: 3×10^7 CFU per ml) after exposure to s-citral (●) or n-citral (○) in buffer of pH 4·0 (a) (0·6 μl ml^{-1}) and 7·0 (b) (0·4 μl ml^{-1}) at room temperature and recovered in TSAYE. Data represent the mean ± standard error of the mean (error bars) of at least three independent experiments. The dotted line represents the detection limit.

On the other hand, the comparison of survival curves shown in Fig. 1a and b confirms the exceptional higher resistance of *E. coli* O157:H7 Sakai in acid than in neutral pH, already described by Somolinos *et al.* (2010), and shows that this phenomenon also occurs when citral is applied as a nanoemulsion.

Effect of citral as a suspension or nanoemulsion on microbial inactivation in combined treatments

The synergism observed when combining heat or PEF with EOs has been directly related to the detection of injured cells in the cytoplasmic and outer membranes of Gram-negative bacteria after the application of physical technologies as a single agent (Mackey 2000; Arroyo *et al.* 2010; Somolinos *et al.* 2010; Espina *et al.* 2012). In those studies, heat and PEF treatments were applied, and survivors were recovered in nonselective and selective media (Fig. 2). Results obtained in the nonselective medium show that heat and PEF treatments acting as single agents inactivated <1 $\log_{10}$ cell cycle. Based on the differences in the $\log_{10}$ cycles of inactivation achieved when comparing the nonselective with selective media, heat treatments at pH 4·0 (Fig. 2a) injured more survivors in the outer than in the cytoplasmic membrane ($P < 0{\cdot}05$), whereas PEF treatments (Fig. 2c) did the opposite. Under these treatment conditions, at least 90% of survivors were injured and susceptible to a citral attack during the combined treatments.

Regarding the combination of heat and citral, a remarkable synergism was observed: almost 5 $\log_{10}$ cells cycles of inactivation were achieved at both pH as a function of the citral addition procedure (s- or n-citral) and the antimicrobial concentration. In contrast with the results shown when citral was acting as a single agent (Fig. 1), the addition of s-citral was to some extent more effective than n-citral either at pH 7·0 or at pH 4·0, showing the greatest difference in the presence of 0·1 μl ml^{-1} at pH 7·0 ($P < 0{\cdot}05$) (Fig. 2b). Thus, maintaining the synergism when using n-citral would require higher concentrations of the hydrophobic compound to achieve the desired level of microbial inactivation. It should be noted that the high levels of inactivation achieved with the combined treatment might correspond to the tail of the survival curves. As a consequence, greater differences in the antimicrobial efficacy between s- and n-citral might be expected at lower concentrations or shorter treatment times of the combined processes. Further experiments should be performed to explain this unexpected inversion of the compared activity of s-citral and n-citral when increasing the treatment temperature up to 53°C. Possible hypotheses to consider include the increased solubility of s-citral under mild thermal treatments, providing the optimum dispersion in the treatment medium, and a greater availability of s-citral to interact with microbial cells in the short time span of the treatment in comparison with n-citral, which might be partially retained by the surfactant.

Nevertheless, these results differ from those obtained with the EPI method, using propylene glycol instead of ethanol as co-surfactant, to obtain nanoemulsions of D-limonene for the inactivation of *Listeria monocytogenes* (Mate *et al.* 2016). Therefore, it should be considered that the result of using nanoemulsions in combined treatments with heat is likely to vary as a function of the type or complexity of the antimicrobial molecules and/or the micro-organism investigated.

According to Arroyo *et al.* (2010), the detection of sublethal injury, specifically on the outer membrane of Gram-negative bacteria after PEF, is the key when identifying treatment conditions under which PEF may act synergistically with citral. Thus, the scarce presence of sublethally injured cells on the outer membrane of *E. coli* 0157:H7 Sakai after PEF (Fig. 2c and d) might justify the limited synergism observed with s-citral. To the best of our knowledge, the bacterial inactivation of PEF treatments with EPI nanoemulsions has never been previously tested. In the present study, no significant differences

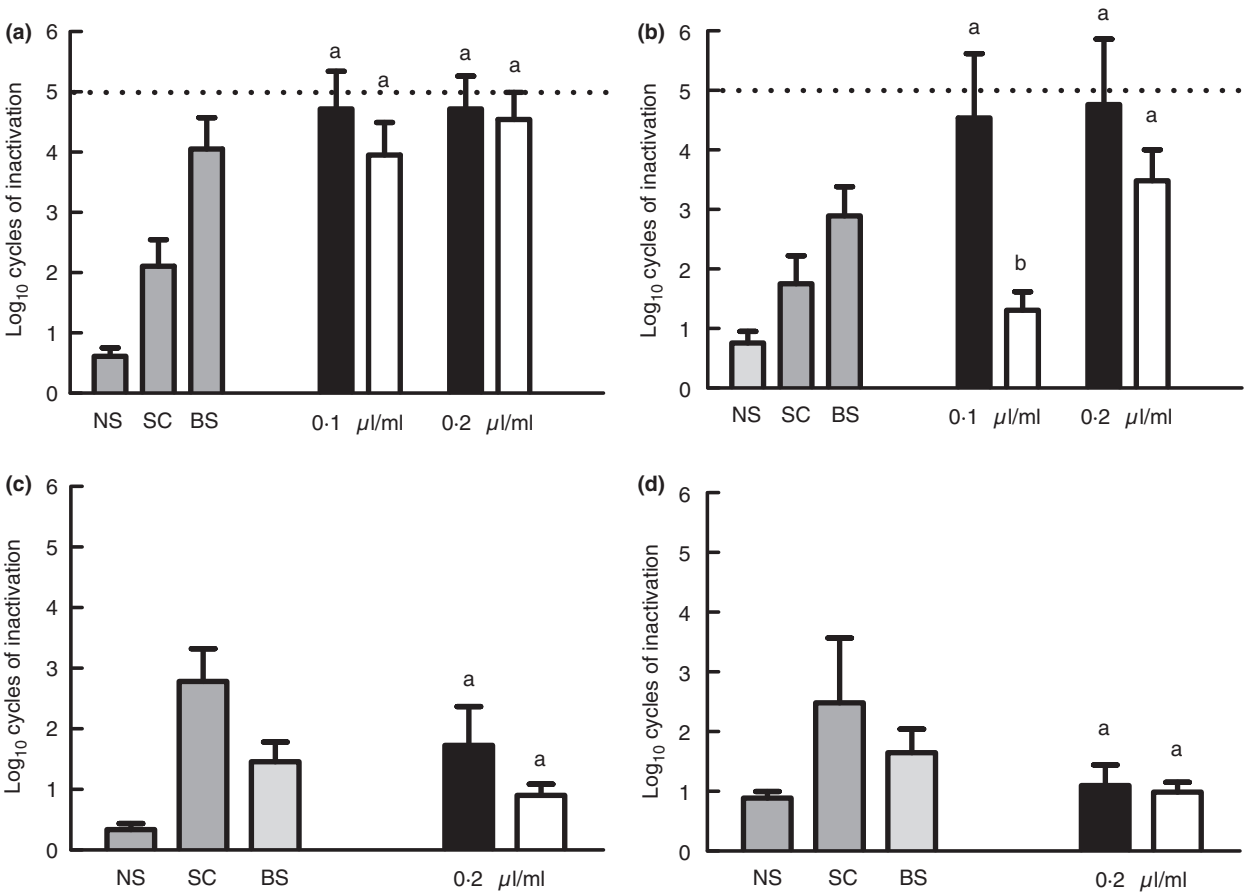


Figure 2 Cycles of inactivation of *Escherichia coli* O157:H7 Sakai (initial concentration: 3×10^7 CFU per ml) after a heat treatment at 53°C for 15 min (a and b) and after a PEAV treatment at 30 kV cm^{-1} for 150 μs (50 pulses of 3 μs) (c and d) in buffers of pH 4·0 (a and c) and 7·0 (b and d), and recovered in TSAYE (grey bars; NS), TSAYE-SC (grey bars; SC), TSAYE-BS (grey bars; BS), or recovered in TSAYE after combined treatment with heat and s-citral (black bar) or n-citral (white bar) (0·1 and 0·2 μl ml^{-1}). Data represent the mean ± standard error of the mean (error bars) of at least three independent experiments. Statistical differences (Student's *t*-test, $P < 0{\cdot}05$) among combined treatments is represented by different superscript letters. The dotted line represents the detection limit.

were found between the efficacy of the combined treatments using PEF and s- or n-citral, and no worsening or improvement of the overall lethality of the combined treatment was observed when emulsifying citral prior to its incorporation. Further experiments combining s-citral or n-citral with PEF applied at higher temperatures could be conducted to further explore the effect of emulsification on each one of these physical preservation treatments.

Effect of citral in the form of suspension or nanoemulsion on microbial inactivation by combined treatments applied to apple juice

In order to validate in a food model the results obtained with citral in form of suspension or nanoemulsion in lab media, apple juice was contaminated with *E. coli* O157:H7 Sakai and treated with a combined treatment of mild heat and citral (s- and n-citral) (Fig. 3). Again, the combined treatment with s- or n-citral was more effective than the use of mild heat as a single agent, showing a remarkable synergistic effect. In addition, the main conclusion obtained in laboratory media was confirmed in apple juice: s-citral was more effective than n-citral when applied at mild temperature at any treatment time. For instance, while almost 5 $\log_{10}$ cell cycles were inactivated in apple juice after 15 min at 53°C in the presence of 0·1 μl ml^{-1} of s-citral, the use of n-citral required doubling the treatment time. Thus, the vigorous agitation in vortex method for suspending citral, which might simulate the action of the actual industrial homogenizers employed as a previous stage in the pasteurization process of liquid foods, seems to be sufficient to disperse the oil correctly and favour its antimicrobial action in apple

juice, providing enough stability at least during the short duration of the combined treatment. In this regard, the treatment time required to comply with FDA regulation (FDA 2001), which recommended that juices should be hygienized reaching 5 $\log_{10}$ reductions (99·999%) of pathogens of concern such as *E. coli* O157:H7, would be approx. two times shorter when using s- than n-citral. Nevertheless, it should be highlighted that citral in the form of nanoemulsion also attained the 5 $\log_{10}$ reductions of the pathogen. Thus, if its use represented any advantage—for instance, for the purpose of limiting the modification of the flavour in food due to high EO concentrations in comparison with nonencapsulated ones—then it would be interesting to reconsider its use in the development of combined treatments for food preservation. Further studies are required in order to evaluate the influence of this and other encapsulation methods on the efficacy of combined processes with heat, PEF or other successful emerging technologies, such as high hydrostatic pressure, and other EOs and EO constituents.

Materials and methods

Bacterial strain and cultures

Escherichia coli O157:H7 Sakai *stx* $1A^-$/*stx* $2A^-$ was kindly provided by Kyu-Tae Chang (The National Primate Research Center, KRIBB, Ochang, South Korea). This strain was isolated from an outbreak involving white radish sprout (Michino *et al.* 1999). During this investigation, the cultures were maintained and kept frozen at −80°C in cryovials. Broth subcultures were prepared by inoculating one single colony from a plate into a test tube containing 5 ml of sterile tryptic soy broth (Biolife, Milan, Italy) with 0·6% yeast extract added (Biolife) (TSBYE). After inoculation, the tubes were incubated overnight at 37°C. Along with these subcultures, 250-ml Erlenmeyer flasks containing 50 mL of TSBYE were inoculated to a final concentration of 10^4 cells per ml. These flasks were incubated under agitation (130 rev min^{-1}; Selecta, mod. Rotabit, Barcelona, Spain) at the appropriate temperature until the stationary growth phase was reached (24 ± 2 h).

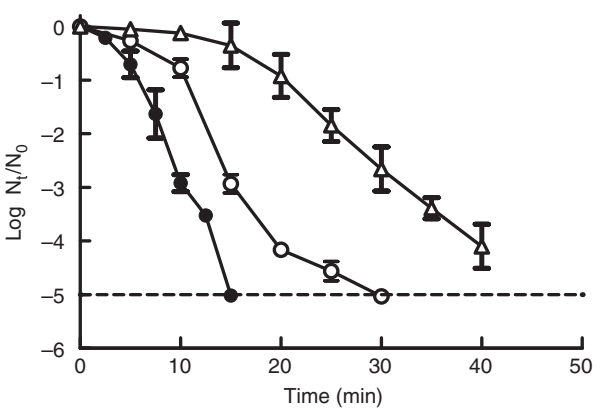


Figure 3 Survival curves of Escherichia coli O157:H7 Sakai (initial concentration: 10^7 CFU per ml) to a heat treatment at 53°C in apple juice (▲), and a combined treatment of heat and s-citral (●) or n-citral (○) (0·1 μl ml^{-1}) and recovered in TSAYE. Data represent the mean ± standard error of the mean (error bars) of at least three independent experiments. The dotted line represents the detection limit.

Antimicrobial and direct addition procedure

Citral was obtained from Sigma Aldrich Chemie (Steinheim, Germany). Following the procedure described by Friedman *et al.* (2002), a vigorous shaking method was used to prepare citral suspensions (s-citral) in the treatment media: citrate-phosphate buffer (McIlvaine's buffer) at pH 7·0 and pH 4·0 (Dawson *et al.* 1974) and apple juice.

Preparation of nanoemulsions

The preparation of nanoemulsions of citral (n-citral) was based on the catastrophic phase inversion method (Zhang *et al.* 2014, 2017), also known as the emulsion phase inversion (EPI) method. The aqueous phase was prepared by mixing 1·5 ml of ethanol (Sigma) with 40·5 ml of sterile distilled water. The oily phase was prepared by mixing 3 ml of Tween 80 (Panreac, Barcelona, Spain) with 5 ml of citral. Nanoemulsions were prepared from a mixture of oily phase by slowly adding aqueous phase with gentle magnetic agitation. The addition rate of aqueous phase was kept constant at approximately 1·0 ml min^{-1}. A water-in-oil (W/O) emulsion with a high oil-to-water ratio was formed, and then increasing amounts of water were added to the system with continuous stirring. The amount of water added to a W/O emulsion was progressively increased, until a phase inversion occurred and an oil-in-water (O/W) emulsion was formed. Final concentration of citral in the nanoemulsion was 587 mmol l^{-1}.

Droplet size and stability of nanoemulsions

The emulsion droplet size and size distribution (polydispersity index (PDI)) was determined using a particle size analyser (Brookhaven, 90 Plus, New York, NY). Droplet size was analysed using a dynamic light scattering (DLS) technique. Prior to all the experiments, the nanoemulsion formulations were diluted with water to eliminate the multiple scattering effects. Emulsion droplet size was estimated by an average of three measurements and is presented as the mean diameter of volume distribution.

Droplet size was evaluated after fresh preparation, and then after 1 month of storage under refrigeration. The reproducibility of the protocol for preparing nanoemulsions and their stability during 30 days was also evaluated by comparing the survival curves of *E. coli* O157:H7 Sakai in the presence of 0·6 μl ml^{-1} of n-citral at pH 4·0, as described below.

Evaluation of the antimicrobial activity of citral

Citral (s-citral and n-citral) was evaluated to determine the minimum inhibitory concentration (MIC) and to obtain survival curves against *E. coli* O157:H7 Sakai.

Regarding the MIC, tubes containing 5 ml of TSBYE and different concentrations of citral (0·5–1 μl ml^{-1}) were inoculated to a final concentration of 10^5 cells per ml. Negative control (without micro-organisms), positive control (without citral) and diluent control (the amount of ethanol corresponding to the maximum n-citral concentration assayed −1 μl ml^{-1}) were also prepared. After 24 h of incubation at the appropriate temperature in a shaking thermostatic incubator (Bunsen, mod. BTG, Madrid, Spain), survivors were enumerated, as described below. The MIC was the lowest concentration of citral at which bacteria failed to grow, showing counts equal to the initial concentration.

Moreover, the antimicrobial properties of s-citral and n-citral were evaluated by determining survival curves in treatment media of different pH. Cells from stationary-phase cultures were added at final concentrations of 10^7 cells per ml to buffers with citral (0·6 μl ml^{-1} at pH 4·0 and 0·4 μl ml^{-1} at pH 7·0). Buffer pH was not modified as a consequence of adding antimicrobial compounds. Antimicrobial compound treatments were carried out at room temperature (23 ± 2°C). Samples were taken at preset intervals and survivors were enumerated, as described below. Previous experiments showed that untreated cells of *E. coli* O157:H7 Sakai at concentrations of 10^7 cells per ml were insensitive to incubation at pH 7·0 or 4·0 for 1 h at room temperature (data not shown).

Evaluation of microbial inactivation by heat and heat combined with citral

For the preparation of heat-treated samples, micro-organisms were resuspended at a concentration of 10^7 cells per ml in treatment media thermostated at 53 ± 0·2°C (FX Incubator, A.F. Ingeniería S. L., Valencia, Spain). Buffer of pH 7·0 and 4·0, as well as the same treatment media with s-citral or n-citral to a final concentration of 0·1 and 0·2 μl ml^{-1} were used. Antimicrobials were added once the treatment media were thermostated, and prior to microbial inoculation. The actual temperature was controlled with a thermocouple wire introduced in a 0·9 ml buffer test tube inside the incubator. After 15 min at 53°C, samples were taken, immediately placed on ice, and survivors and sublethally injured cells were evaluated, as explained below.

Following the same methodology, heat treatments were also carried out in apple juice (Don Simón, Murcia, España), as well as in the presence of s-citral and n-citral (0·1 μl ml^{-1}). Samples were collected at preset intervals and survivors were evaluated to obtain survival curves.

Evaluation of microbial inactivation by PEF and PEF combined with citral

Pulsed electric fields treatments were carried out using a ScandiNova equipment (Modulator PG, ScandiNova, Uppsala, Sweden), described by Saldaña *et al.* (2010).

Before treatments, micro-organisms were centrifuged at 6000***g*** for 5 min and resuspended at a concentration of 10^7 cells per ml in citrate–phosphate buffer of pH 7·0 and 4·0 (electrical conductivity was adjusted to 1 mS cm^{-1}), as well as in the same treatment media with s-citral or n-citral to a final concentration of 0·2 μl ml^{-1}.Then, 0·5 ml of the microbial suspensions was placed into the treatment chamber with a sterile syringe. Exponential waveform pulses at an electrical field strength of 30 kV cm^{-1} and a pulse repetition rate of 1 Hz were used in this study. The specific energy input of each pulse 2·7 kJ kg^{-1}. Cell suspensions were treated for 50 pulses (pulse width 3 μs). Experiments started at room temperature (23 ± 2°C). In all experiments, the temperature of the samples after the application of 50 pulses was lower than 35°C. After treatment, samples were taken, and survivors and sublethally injured cells were evaluated, as explained below.

Counts of viable cells

After treatments, samples were adequately diluted in 0·1% w/v peptone water (Biolife). Subsequently, 0·1 ml samples were pour-plated onto Tryptic Soy Agar (Biolife) with 0·6% Yeast Extract added (Biolife) (TSAYE). Plates were incubated for 24 h at 37°C. Previous experiments showed that longer incubation times did not influence the surviving cell counts. After incubation, colonies were counted with an improved image analyser automatic counter (Protos; Analytical Measuring Systems, Cambridge, UK), as previously described (Condón *et al.* 1996).

Detection of sublethal injury

In order to determine bacterial cell injury, treated samples were also plated onto TSAYE with 4% sodium chloride

(Panreac) added (TSAYE-SC) and onto TSAYE with 0·25% bile salts (Oxoid, Hampshire, United Kingdom) added (TSAYE-BS) in order to evaluate cytoplasmic membrane damage and outer membrane damage respectively (Mackey 2000). These levels of sodium chloride and bile salts were previously determined as the maximum noninhibitory concentrations for native cells (data not shown). Samples recovered in selective media were incubated for 48 h. Previous experiments showed that longer incubation times did not influence survival counts.

The proportion of sublethally injured cells was estimated by the difference in the number of $\log_{10}$ cycles of colony forming units (CFU) obtained after plating treated cells in the nonselective (TSAYE) and selective (TSAYE-SC) media.

Data analyses

The error bars in the figures indicate the mean ± standard deviations from the data obtained from at least three independent experiments. All analyses were performed with GraphPad PRISM® software (GraphPad Software, Inc., San Diego, CA). Unpaired *t*-Student and one-way ANOVA tests were performed to test statistically significant differences among two or more groups respectively ($P = 0{\cdot}05$).

Acknowledgements

This study was financially supported by MINECO (Ministerio de Economía, Industria y Competitividad, Project No. AGL2015-69565-P), by FEDER, the European Social Fund, and by the Aragonese Office of Science, Technology and University Research.

Conflict of Interest

No conflict of interest declared.

References

Arroyo, C., Somolinos, M., Cebrián, G., Condon, S. and Pagán, R. (2010) Pulsed electric fields cause sublethal injuries in the outer membrane of *Enterobacter sakazakii* facilitating the antimicrobial activity of citral. *Lett Appl Microbiol* **51**, 525–531.

Condón, S., Palop, A., Raso, J. and Sala, F.J. (1996) Influence of the incubation temperature after heat treatment upon the estimated heat resistance values of spores of *Bacillus subtilis*. *Lett Appl Microbiol* **22**, 149–152.

Corbo, M.R., Bevilacqua, A., Campaniello, D., D'Amato, D., Speranza, B. and Sinigaglia, M. (2009) Prolonging microbial shelf life of foods through the use of natural compounds and non-thermal approaches – a review. *Int J Food Sci Technol* **44**, 223–241.

Dawson, R.M.C., Elliot, D.C., Elliot, W.H. and Jones, K.M. (1974) *Data for Biochemical Research*. Oxford: Clarendon Press.

Donsi, F., Annuziata, M., Sessa, M. and Ferrari, G. (2011) Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *LWT-Food Sci Technol* **44**, 1908–1914.

Espina, L., Somolinos, M., Pagán, R. and García, D. (2010) Effect of citral on the thermal inactivation of *Escherichia coli* O157:H7 in citrate phosphate buffer and apple juice. *J Food Prot* **73**, 2189–2196.

Espina, L., Somolinos, M., Ouazzou, A.A., Condon, S., García-Gonzalo, D. and Pagán, R. (2012) Inactivation of *Escherichia coli* O157:H7 in fruit juices by combined treatments of citrus fruit essential oils and heat. *Int J Food Microbiol* **159**, 9–16.

FDA (2001) Hazard analysis and critical control point (HACCP): procedures for the safe and sanitary processing and importing of juice. *Fed Regist* **66**, 6137–6202.

Friedman, M., Henika, P.R. and Mandrell, R.E. (2002) Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J Food Prot* **65**, 1545–1560.

Komaiko, J.S. and McClements, D.J. (2016) Formation of food-grade nanoemulsions using low-energy preparation methods: a review of available methods. *Com Rev Food Sci Food Safe* **15**, 331–352.

Leistner, L. and Gorris, L.G.M. (1995) Food preservation by hurdle technology. *Trends Food Sci Technol* **6**, 41–46.

Mackey, B.M. (2000) Injured bacteria. In *The Microbiological Safety and Quality of Food*, vol. **I**, ed. Lund, M., Baird-Parker, T.C. and Gould, G.W. pp. 315–341. Gaithersburg, Maryland: Aspen Publishing.

Maswal, M. and Dar, A.A. (2014) Formulation challenges in encapsulation and delivery of citral for improved food quality. *Food Hydrocolloids* **37**, 182–195.

Mate, J., Periago, P.M. and Palop, A. (2016) When nanoemulsified, D-limonene reduces *Listeria monocytogenes* heat resistance about one hundred times. *Food Control* **59**, 824–828.

Michino, H., Araki, K., Minami, S., Takaya, S., Sakai, N., Miyazaki, M., Ono, A. and Yanagawa, H. (1999) Massive outbreak of *Escherichia coli* O157: H7 infection in schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts. *Am J Epidemiol* **150**, 787–796.

Moghimi, R., Aliahmadi, A., McClements, D.J. and Rafati, H. (2016) Investigations of the effectiveness of nanoemulsions from sage oil as antibacterial agents on some food borne pathogens. *LWT-Food Sci Technol* **71**, 69–76.

Piorkowski, D.T. and McClements, D.J. (2014) Beverage emulsions: recent developments in formulation, production, and applications. *Food Hydrocolloids* **42**, 5–41.

Saldaña, G., Puértolas, E., Condón, S., Álvarez, I. and Raso, J. (2010) Modeling inactivation kinetics and occurrence of sublethal injury of a pulsed electric field-resistant strain of *Escherichia coli* and *Salmonella* Typhimurium in media of different pH. *Innov Food Sci Emerg Technol* **11**, 290–298.

Somolinos, M., García, D., Condón, S., Mackey, B. and Pagán, R. (2010) Inactivation of *Escherichia coli* by citral. *J Appl Microbiol* **108**, 1928–1939.

de Souza, E.L., Almeida, E.T.D. and Guedes, J.P.D. (2016) The potential of the incorporation of essential oils and their individual constituents to improve microbial safety in juices: a review. *Comp Rev Food Sci Food Safe* **15**, 753–772.

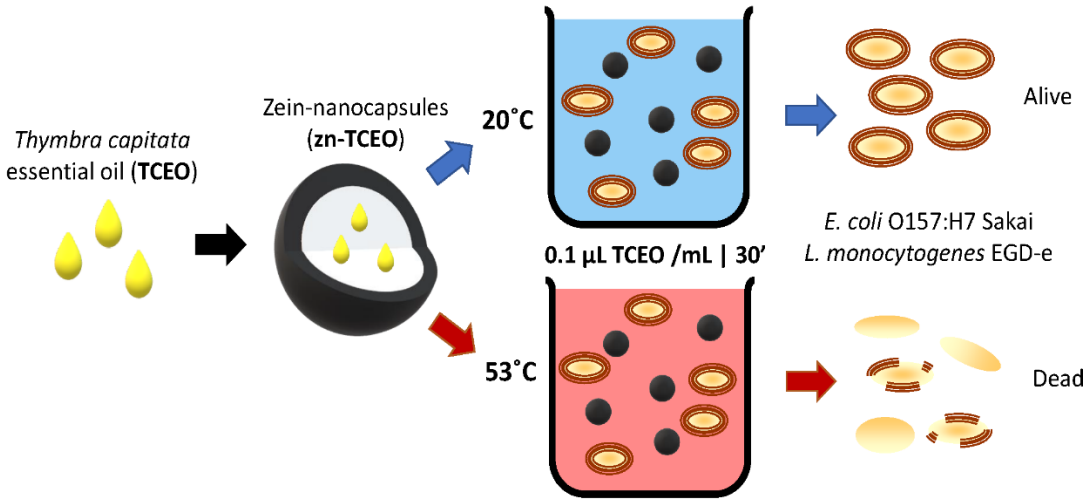
Zhang, Z.J., Vriesekoop, F., Yuan, Q.P. and Liang, H. (2014) Effects of nisin on the antimicrobial activity of D-limonene and its nanoemulsion. *Food Chem* **150**, 307–312.

Zhang, S.J., Zhang, M., Fang, Z.X. and Liu, Y.P. (2017) Preparation and characterization of blended cloves/cinnamon essential oil nanoemulsions. *LWT-Food Sci Technol* **75**, 316–322.

Manuscrito IX. Antimicrobial efficacy of *Thymbra capitata* (L.) Cav. essential oil loaded in self-assembled zein nanoparticles in combination with heat

Merino, N.*, Berdejo, D.*, Bento, R., Salman, H., Lanz, M., Maggi, F., Sánchez-Gómez, S., García-Gonzalo, D., Pagán, R. (2019)

Industrial Crops & Products, 133: 98-104.
doi: 10.1016/j.indcrop.2019.03.003.



Industrial Crops & Products 133 (2019) 98–104

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Antimicrobial efficacy of *Thymbra capitata* (L.) Cav. essential oil loaded in self-assembled zein nanoparticles in combination with heat

Natalia Merino[a,1], Daniel Berdejo[a,1], Roberta Bento[a], Hesham Salman[b], María Lanz[b], Filippo Maggi[c], Susana Sánchez-Gómez[b], Diego García-Gonzalo[a], Rafael Pagán[a,*]

[a] Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain
[b] Bionanoplus SL, Noain, Spain
[c] School of Pharmacy, University of Camerino, Camerino, Italy

ARTICLE INFO

Keywords:
Thymbra capitata essential oil
Chemical composition
Nanocapsules
Zeins
Heat
Synergism

ABSTRACT

This study reports on the chemical composition of *Thymbra capitata* essential oil (TCEO) and its antimicrobial activity when applied with heat either as a suspension (s-TCEO) or loaded in self-assembled zein nanoparticles (zn-TCEO). Zein, a plant protein isolated from corn and maize, is proposed as natural, and biocompatible carrier. TCEO composition was analysed by GC–MS, and 35 components were identified. Carvacrol, a monoterpenoid, was the major constituent (73.8%). zn-TCEO were prepared under low shear conditions and characterized according to droplet size (< 180 nm) and encapsulation efficiency (77.8%). The two TCEO formulations (s-TCEO and zn-TCEO) were compared in terms of antibacterial activity against *Escherichia coli* O157:H7 Sakai and *Listeria monocytogenes* EGD-e. The zn-TCEO displayed a greater bacteriostatic activity than s-TCEO, probably due to their improved dispersion in the growth media. However, zn-TCEO exerted a lower bactericidal activity than s-TCEO, probably due to the EO progressive release. The combination of TCEO and heat (53 °C) exerted valuable synergistic lethal effects, causing the death of up to 5 $\log_{10}$ cycles of both microorganisms. The effectiveness of zn-TCEO was especially improved at pH 4.0. Therefore, the application of this new delivery system, designed to encapsulate and protect EOs, and ensure their controlled release, might represent an advantageous alternative for food, cosmetic or pharmaceutical industries to improve the efficacy of higienization processes or surface cleaning and disinfection procedures when combined with mild heat.

1. Introduction

Plant essential oils (EOs) are natural compounds extracted mainly from aromatic and medicinal plants. Due to their antimicrobial properties, they have been frequently recommended as biocides, preservatives or for cleaning and disinfection procedures (Burt, 2004; Souza et al., 2016). However, their chemical instability due to oxidation, high reactivity, and hydrophobicity thwarts any attempt to incorporate them directly into cleaning solutions, cosmetic products, or food beverages (Donsì and Ferrari, 2016). New delivery systems have therefore been designed in order to protect their chemical properties and ensure their controlled release (Prakash et al., 2018).

Active encapsulation has mainly been used to protect bioactive compounds from adverse environmental factors, to enhance solubility of poorly soluble actives and to grant them certain specific properties, such as sustained or controlled release. Unfortunately, most of these promising nanoencapsulation methods require expensive equipment and present difficulties in the scale-up phase (spray drying, freeze-drying, etc.). Recent developments in the preparation of nanoparticles have been marked by a series of emerging issues: the requirement for less toxic reagents, a simplification of the procedure with the purpose of allowing economic scale-up, and optimization to improve yield and entrapment efficacy (Reis et al., 2006).

Thymbra capitata (L.) Cav. (syn. *Thymus capitatus* (L.) Cav., Lamiaceae) is an aromatic herb that grows in the Mediterranean area and produces an essential oil (TCEO). Thanks to its recognized antimicrobial and antioxidant properties, it can be used as a preservative for foodstuffs and formulations (Delgado-Adámez et al., 2017; Falcó et al., 2018; Neves et al., 2017).

Several natural polymers such as zein (Weissmueller et al., 2016),

* Corresponding author at: Dpto. PACA, Facultad de Veterinaria, Universidad de Zaragoza, C/ Miguel Servet, 177, 50013, Zaragoza, Spain.
E-mail address: pagan@unizar.es (R. Pagán).
[1] These authors contributed equally to the work.

https://doi.org/10.1016/j.indcrop.2019.03.003
Received 22 December 2018; Received in revised form 27 February 2019; Accepted 1 March 2019

casein (Peñalva et al., 2018), or chitosan (Yuan et al., 2016) have been proposed as natural, and biocompatible carriers for different actives. Zein is a plant protein isolated from corn or maize belonging to a family of prolamines which are composed of hydrophobic aminoacids (Salman et al., 2013). In this context, the use of zein in conjunction with a controlled self-assembly method for encapsulation of TCEO would allow us to develop a carrier for this natural, lowcost, biocompatible and GRAS (generally recognized as safe) EO (Patel and Velikov, 2014).

Most of the studies that propose the encapsulation of EOs describe how their progressive release from nanocapsules can help inhibit microbial growth by developing a prolonged bacteriostatic activity over time (Chouhan et al., 2017). However, when a bactericidal effect is required in a short treatment, as tends to occur during higienization or surface cleaning and disinfection, the control of the liberation of EOs might entail a limitation, since the doses required to exert a bactericidal effect are not easily achieved.

In this regard, remarkable synergistic lethal effects have been described when EO suspensions (Espina et al., 2010; Guevara et al., 2015; Raybaudi-Massilia et al., 2009) or nanoemulsions (de Carvalho et al., 2018; Mate et al., 2016; Pagán et al., 2018) are applied in combination with physical processes, for instance mild heat treatments (50–60 °C). Such combined treatments have been proposed not only as alternatives to traditional fruit juice preservation methods, but also as a good means of eradicating biofilms of *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus* from plastic surfaces (Espina et al., 2017). The described synergism has enabled the reduction of treatment temperatures and/or antimicrobial doses: in view of the strong flavor of most EOs, this can represent an enormous advantage.

As far as we can ascertain, the behavior of zein nanocapsules under heat treatments or of the antimicrobial properties of encapsulated EOs with zeins in combination with heat against pathogenic microorganisms has not been previously studied.

This research was therefore carried out in order to (i) evaluate the chemical composition of TCEO and its effectiveness *in vitro* on growth and survival of two pathogenic bacteria: *E. coli* O157:H7 Sakai and *L. monocytogenes* EGD-e; ii) obtain and characterize novel self-assembled zein nanocapsules loaded with TCEO (zn-TCEO) for TCEO protection and progressive release, and iii) to assess the antimicrobial efficacy of zn-TCEO as a single hurdle or in combination with heat as a function of treatment medium pH.

2. Material and methods

2.1. Thymbra capitata essential oil (TCEO)

One-hundred-percent pure and natural TCEO was obtained by hydrodistillation of the flower heads of *Thymbra capitata*. TCEO was kindly provided by the TELIC Group (Barcelona, Spain). Once received, the EO was kept at room temperature, in the dark, in sealed glass vials until used.

Following the method described by Friedman et al. (2002), we applied a vigorous shaking procedure to prepare TCEO suspensions (s-TCEO) in McIlvaine buffer at pH 7.0 and at pH 4.0.

2.2. Chemical analysis of Thymbra capitata essential oil

α-Pinene, camphene, β-pinene, 1-octen-3-ol, myrcene, α-phellandrene, δ-3-carene, *p*-cymene, limonene, 1,8-cineole, (*E*)-β-ocimene, γ-terpinene, terpinolene, linalool, borneol, terpinen-4-ol, α-terpineol, thymol, carvacrol, (*E*)-caryophyllene, α-humulene, and caryophyllene oxide were purchased from Sigma-Aldrich (Milan, Italy) and used for peak assignment. A mix of *n*-alkanes ranging from octane (C_8) to triacontane (C_{30}) (Supelco, Bellefonte, CA, USA) was used to calculate the temperature-programmed retention index (RI).

An Agilent 6890 N gas chromatograph coupled to a single quadrupole 5973 N mass spectrometer (Agilent, CA, USA) was used for the analysis of TCEO. Components were separated on apolar HP-5 MS (30 m × 0.25 mm i.d., 0.1 μm f.t.; Agilent) made up of 5% phenylmethylpolysiloxane. Oven was programmed as follows: 5 min at 60 °C raised at 4 °C/min up to 220 °C, then 11 °C/min up to 280 °C, held for 15 min. The temperature of injector and detector (single quadrupole) was set to 280 °C. Helium (99.99%) was the carrier gas with a flow of 1 mL/min. TCEO was diluted in *n*-hexane (6 μL in 594 μL of solvent) and injected (2 μL) in split mode (1:50 ratio) into the GC–MS system. The spectra were acquired in full scan (29–400 *m/z*) using the electron-impact (EI, 70 eV) mode.

The software applications used for peak assignment as well as the adopted identification criteria were the same as those reported by Benelli et al. (2018). Semi-quantification of EO components was obtained by peak area normalisation, taking the same response factor for all volatile components into account.

2.3. Encapsulation of TCEO with zeins (zn-TCEO)

The TCEO-loaded zein nanocapsules were prepared according to self-assembly methods, avoiding the use of volatile organic solvent (Salman et al., 2013). We obtained the oil phase by mixing TCEO, surfactants (Tween 20; Panreac, Madrid, Spain), cosurfactants (propanediol, Dupont Tate & Lyle Bioproducts, TN, USA; propylene glycol and denatured alcohol, Guinama, Valencia, Spain), zein plasticizers (Oleic acid, Panreac, Madrid, Spain), and zein (Flo Chemical Corporation, MA, USA) under magnetic stirring. The surfactant-oil mixture was subsequently added into water under continuous agitation to form the nanocapsules.

2.4. Characterization, stability and encapsulation efficiency of zn-TCEO

We measured droplet size distribution and the polydispersity index of the zn-TCEO with dynamic light scattering (DLS) analysis in a particle size analyzer (90S Particle Size Analyzer, Brookhaven Instruments, NY, USA).

Encapsulation efficiency, which corresponds to the percentage of encapsulated TCEO, was determined by carvacrol quantification via an analytical HPLC method on an Agilent 1260 infinity system equipped with a quaternary pump, an auto-sampler with high sensitivity cell, a thermostatized column compartment, and a diode array detector. UV spectra were collected at 220 and 278 nm. Instrument control, data collection, and data processing were carried out with Agilent OpenLab CDS software. The column was a Zorbax SB-C18 (250 x 4.6 mm, 5 μm; Agilent). The mobile phase was an isocratic combination of acetonitrile ACN:H_2O (Merck, Darmstadt, Germany) (50:50) with a flow rate of 2 mL/min. Injection volume for standard solutions and all samples was 10 μL.

In order to separate free from encapsulated TCEO, a 100 kDa Amicon was used (Merck Millipore, Darmstadt, Germany). After centrifugation, the filtrate was collected and quantified. Encapsulation efficiency (EE) was calculated as follows:

$$EE\ (\%) = \frac{Active\ concentration\ in\ formulation - Active\ concentration\ in\ filtrate}{Active\ concentration\ in\ formulation} \times 100 \quad (1)$$

To ensure the stability of the zn-TCEO, they were subjected to 3 heating-cooling cycles (1 h at 70 °C and 1 h at 4 °C) and characterized according to their size and encapsulation efficiency as describe above.

2.5. Micro-organisms and growth conditions

E. coli O157:H7 Sakai *stx1A/stx2 A*$^-$ was kindly provided by The National Primate Research Center, KRIBB, Ochang, South Korea (Prof. Kyu-Tae Chang), a strain isolated from an outbreak associated with

white radish sprout (Michino et al., 1999), and genetically modified thereafter in order to remove Shiga toxin genes. *L. monocytogenes* EGD-e was kindly provided by the Institute for Medical Microbiology in Giessen, Germany (Prof. Chakraborty). Culture preparation and growth conditions were the same as those reported by Luis-Villaroya et al. (2015).

2.6. Evaluation of the antimicrobial properties of s-TCEO and zn-TCEO

A modified filter paper disc agar diffusion technique (Meena and Sethi, 1994) was applied in order to screen antimicrobial activity of s-TCEO and of zn-TCEO against *E. coli* O157:H7 Sakai and against *L. monocytogenes* EGD-e. We applied filter paper disks (Whatman No. 1; diameter: 6 mm) containing 20 µL of s-TCEO or of zn-TCEO diluted in sterile tryptone soya broth (Oxoid, Hampshire, England) with 0.6% yeast extract added (Oxoid) (TSBYE) (TCEO final concentration: 0.5%) to the surface of agar plates of tryptone soya agar (Oxoid) supplemented with 0.6% yeast extract (TSAYE) that had been previously seeded by spreading one sterile hyssop impregnated with a stationary phase culture. For a period of 24 h, the plates were incubated at the appropriate temperature (37 °C). The diameter of the resulting zone of partial inhibition was measured in mm.

Moreover, we evaluated the antimicrobial properties of s-TCEO and of zn-TCEO by ascertaining the degree of bacterial inactivation as a function of treatment medium pH. For this purpose we added cells from stationary-phase cultures at final concentrations of 3×10^7 CFU/mL to buffers (pH 7.0 and 4.0), both with and without s-TCEO and zn-TCEO (TCEO final concentration: 0.2 µL/mL). Buffer pH was not altered by the addition of antimicrobial compounds. We applied antimicrobial compound treatments for 20 min at 20 °C. Samples were taken after intervals of 5, 10, 15, and 20 min, and survivors were counted as described below.

2.7. Measurement of cell inactivation by heat treatment alone, and by heat treatments combined with s-TCEO or zn-TCEO

Heat treatments and combined treatments were carried out at 53 °C in an incubator (FX Incubator, mod. ZE/FX, Zeulab, Zaragoza, Spain). To monitor heating temparature, a thermocouple was used (Ahlborn, mod. Almemo 2450, Holzkirchen, Germany). Treatment temperatures were chosen on the basis of preliminary results (data not shown). In order to match previously published data (Espina et al., 2010; Luis-Villaroya et al., 2015; Pagán et al., 2018), the chosen initial bacterial concentration was approximately 3×10^7 CFU/mL. As treatment media, we used a sterile McIlvaine buffer of pH 7.0 and 4.0, as well as the same media with s-TCEO or zn-TCEO added (TCEO final concentration: 0.1 and 0.2 µL/mL). Samples were taken and survivors were counted.

2.8. Counts of viable cells

Samples were diluted after treatment in 0.1% w/v peptone water (Oxoid). Subsequently, 0.1-mL samples were pour-plated onto a recovery medium (TSAYE). Plates were incubated at 37 °C for 24 h (*E. coli* O157:H7), or for 48 h (*L. monocytogenes* EGD-e). An image analyzer automatic counter (Protos; Analytical Measuring Systems, Cambridge, United Kingdom) was used in order to count the CFUs. Inactivation was expressed in terms of the extent of reduction in $\log_{10}$ counts (CFU) after each type of treatment.

2.9. Statistical analysis

Results were obtained from at least three independent experiments carried out on separate working days with different microbial cultures in order to evaluate the disk diffusion assay and the efficacy of lethal treatments. Results were represented as the mean ± standard deviation using the PRISM® program (GraphPad Software, Inc., San Diego, USA). Data were analyzed and submitted to comparison of averages via ANOVA followed by a *post-hoc* Tukey test and *t*-tests with GraphPad PRISM®. Differences were considered significant if $P < 0.05$.

Table 1
Chemical composition of the essential oil of Thymbra capitata.

No	Component[a]	RI[b]	RI Lit.[c]	%[d]	ID[e]
1	α-thujene	921	924	0.4 ± 0.1	RI,MS
2	α-pinene	927	932	0.9 ± 0.2	Std,RI,MS
3	camphene	940	946	0.1 ± 0.0	Std,RI,MS
4	β-pinene	969	974	0.1 ± 0.0	Std,RI,MS
5	1-octen-3-ol	977	974	0.1 ± 0.0	Std,RI,MS
6	3-octanone	987	979	Tr[f]	RI,MS
7	myrcene	990	988	0.9 ± 0.1	Std,RI,MS
8	3-octanol	999	988	Tr	RI,MS
9	α-phellandrene	1003	1003	0.1 ± 0.0	Std,RI,MS
10	δ-3-carene	1008	1008	Tr	Std,RI,MS
11	α-terpinene	1014	1014	1.0 ± 0.2	RI,MS
12	*p*-cymene	1022	1020	9.2 ± 1.7	Std,RI,MS
13	limonene	1026	1024	0.1 ± 0.0	Std,RI,MS
14	β-phellandrene	1026	1025	0.2 ± 0.0	RI,MS
15	1,8-cineole	1028	1026	0.1 ± 0.0	Std,RI,MS
16	(*E*)-β-ocimene	1047	1044	Tr	Std,RI,MS
17	γ-terpinene	1056	1054	5.3 ± 1.1	Std,RI,MS
18	*cis*-sabinene hydrate	1065	1065	0.1 ± 0.0	RI,MS
19	terpinolene	1085	1086	0.1 ± 0.0	Std,RI,MS
20	*p*-cymenene	1087	1089	Tr	RI,MS
21	*trans*-sabinene hydrate	1096	1098	Tr	RI,MS
22	linalool	1100	1095	0.9 ± 0.2	Std,RI,MS
23	borneol	1160	1165	0.3 ± 0.0	Std,RI,MS
24	terpinen-4-ol	1173	1174	0.5 ± 0.1	Std,RI,MS
25	*p*-cymen-8-ol	1184	1179	Tr	RI,MS
26	α-terpineol	1188	1186	0.1 ± 0.0	Std,RI,MS
27	carvacrol, methyl ether	1243	1241	0.1 ± 0.0	RI,MS
28	thymol	1295	1289	1.7 ± 0.3	Std,RI,MS
29	carvacrol	1306	1298	73.8 ± 4.2	Std,RI,MS
30	carvacrol acetate	1372	1370	0.1 ± 0.0	RI,MS
31	(*E*)-caryophyllene	1409	1417	2.0 ± 0.4	Std,RI,MS
32	α-humulene	1443	1452	Tr	Std,RI,MS
33	β-bisabolene	1505	1505	0.1 ± 0.0	RI,MS
34	(*E*)-α-bisabolene	1540	1540	Tr	RI,MS
35	caryophyllene oxide	1571	1583	0.1 ± 0.0	Std,RI,MS
	Total identified (%)			98.6 ± 0.4	
	Grouped compounds (%)				
	Monoterpene hydrocarbons			18.6 ± 0.9	
	Oxygenated monoterpenes			77.5 ± 2.8	
	Sesquiterpene hydrocarbons			2.2 ± 0.5	
	Oxygenated sesquiterpenes			0.1 ± 0.0	
	Others			0.2 ± 0.0	

[a] Compounds are listed in order of their elution from a HP-5MS column.
[b] Linear retention index on HP-5MS column, experimentally determined using homologous series of C_8-C_{30} alkanes.
[c] Linear retention index taken from Adams (2007) or NIST 17 MS data base.
[d] Relative percentage values are means of two determinations ± SD.
[e] Identification methods: std, based on comparison with authentic compounds; MS, based on comparison with WILEY, ADAMS, FFNSC2 and NIST 17 MS databases; RI, based on comparison of calculated RI with those reported in ADAMS, FFNSC 2 and NIST 08.
[f] Tr, % below 0.1%.

3. Results and discussion

3.1. Chemical composition of Thymbra capitata essential oil (TCEO)

Qualitative and quantitative analysis of the TCEO is summarized in Table 1. Thirty-five volatile components were identified, representing 98.6% of all detected constituents. The components were grouped into main four classes: monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes.

As shown in Table 1, TCEO contained mostly oxygenated monoterpenes, which accounted for 77.5% of its composition. Carvacrol was

the component present in the greatest amount (73.8%). Apart from carvacrol, 4 constituents were present in concentrations greater than 1%: *p*-cymene (9.2%), γ-terpinene (5.2%), (*E*)-caryophyllene (2%) and thymol (1.7%), as previously observed in most of *Thymus* species (Lemos et al., 2017; Trindade et al., 2018). The total sum of these components represented 93% of the TCEO composition.

These results are similar to those described for the EO of *T. capitatus* (a synonym of *T. capitata*) obtained from three different locations in Tunisia (Jendouba, Haouaria and Ain Tounine) (Bounatirou et al., 2007), from Badajoz (Spain) (Delgado-Adámez et al., 2017) and from Sicily (Italy) (Ramos et al., 2017), in which carvacrol was the predominant component, followeb by *p*-cymene, γ-terpinene and *β*-caryophyllene.

3.2. Characterization of self-assembled zein nanoparticles loaded with TCEO

The design of the oil-loaded nanocapsules consisted in modifying the concentrations and the type of excipients. For this purpose, different surfactants and co-surfactants were added to TCEO and were mixed in presence of different concentrations of zein. Several formulations were pre-selected (those with an encapsulation efficiency [EE] greater than 70%) and subjected to three heating-cooling cycles (1 h at 70 °C and 1 h at 4 °C). The final formulation (namely zn-TCEO) was selected for its stability even after the stress conditions. DLS spectra showed low polydispersed particles, with a particle size of 180 nm (Fig.1). As summarized in Table 2, it contained 0.5% of TCEO, and its particle size and its EE remained stable (less than 6% variation).

A similarly high EE using zein as vehicle (close to 80%) has been previously described (Bilenler et al., 2015; Wu et al., 2012), reaching values between 60–90%. However, those studies involved solvent evaporation steps that impede scale-up and industrialization of the nanoparticles. The nanoparticles described in the present study present the advantages of conventional systems, while avoiding the drawbacks of industrial manufacturing. Moreover, they are based on the use of a clear, odorless, non-toxic, biodegradable and water-insoluble vegetable protein, isolated from corn or maize, and compatible with food, cosmetic and pharmaceutical products (Salman et al., 2013).

Table 2
Relevant characteristics of zein nanoparticles loaded with *Thymbra capitata* (L.) Cav. (zn-TCEO).

		zn-TCEO
Active concentration (%)		0.5
Zein concentration (%)		0.25
Characteristics before stress condition	**Size (nm)**	180
	PDI [1]	0.250
	EE [2] **(%)**	77.8
Characteristics after stress condition	**Size (nm)**	190
	PDI [1]	0.250
	EE [2] **(%)**	77.8

[1] Polydispersity index; [2] Encapsulation efficiency.

3.3. Evaluation of the antimicrobial properties of s-TCEO and zn-TCEO

After having prepared and characterized the self-assembled zein nanoparticles loaded with TCEO, the present study's goal was to evaluate and compare the bacteriostatic and bactericidal effectiveness of s-TCEO and zn-TCEO.

Preliminary screening of the *in vitro* bacteriostatic activity of s-TCEO and zn-TCEO was carried out against one Gram-negative and one Gram-positive pathogenic bacterium, respectively, namely *E. coli* O157:H7 Sakai and *L. monocytogenes* EGD-e, applying a modified version of filter paper disc agar diffusion technique. In this regard, it should be noted that the TCEO concentration in both preparations was much lower (0.5%) than that usually employed when this technique is carried out with pure EOs; thus, the obtained results are not directly comparable with previous works. Table 3 summarizes the antibacterial activity of the two TCEO preparations under study (s-TCEO and zn-TCEO). Despite their low concentration, both EO preparations were partially active against growth of both microorganisms. Inhibitory halos were clearly defined, but isolated colonies grew randomly inside them; therefore, the inhibitory halos were only partial. As a result, the use of TCEO in the form of loaded nanoparticles of zeins displayed a greater diffusion through the growth media and an increased partial inhibition halo from 12.1 (s-TCEO) to 41.7 (zn-TCEO) mm ($P < 0.05$) against *E. coli* O157:H7 Sakai and from 10.1 (s-TCEO) to 72.3 (zn-TCEO) mm ($P < 0.05$) against *L. monocytogenes* EGD-e (Table 3). These results

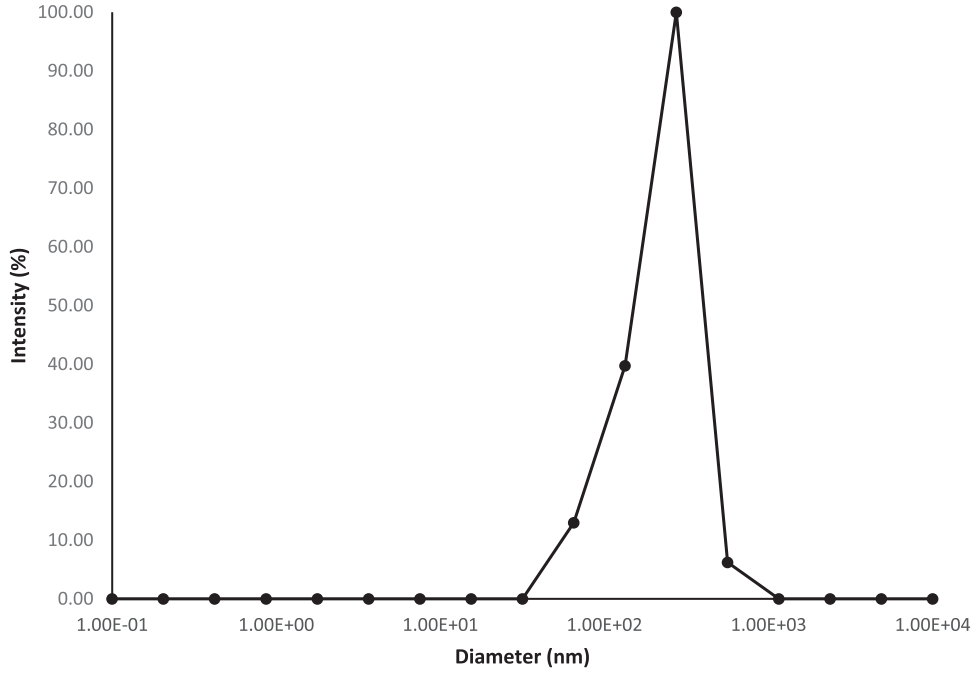


Fig. 1. DLS graph of *Thymbra capitata* essential oil-loaded zein nanocapsules (zn-TCEO).

Table 3
Zones of partial growth inhibition (mm) for a suspension of *Thymbra capitata* (L.) Cav. essential oil (s-TCEO) and *T. capitata* loaded in self-assembled zein nanoparticles (zn-TCEO) against *Escherichia coli* O157:H7 Sakai and *Listeria monocytogenes* EGD-e; disk diameter 6.0 mm.

Microorganism	s-TCEO	zn-TCEO
E. coli O157:H7 Sakai	12.1 ± 1,3	41.7 ± 3.4
L. monocytogenes EGD-e	10.1 ± 1.5	72.3 ± 2.9

indicate that zn-TCEO can inhibit microbial growth more effectively than s-TCEO. The improved bacteriostatic activity of zn-TCEO might be associated with its higher stability and improved dispersion throughout the hydrophilic growth media. Similar results were observed when preparing TCEO nanoemulsion formulations (Ben Jemaa et al., 2018; Benjemaa et al., 2018). The comparison of these results with those obtained by Ait-Ouazzou et al. (2011), where a carvacrol inhibition halo against *E. coli* O157:H7 and *L. monocytogenes* EGDe was ascertained, allows us to conclude that the greater proportion of the antimicrobial activity of TCEO is due to its high carvacrol content (73.8%).

The encapsulation of EOs has proven to be a good practice when one is seeking to maintain or even enhance their bacteriostatic activity (Donsì and Ferrari, 2016). However, this practice has the drawback that EO is only gradually released (Hu et al., 2018). Thus, when a rapid bioavailability of EOs is required, as is the case in the course of classical inactivating procedures (higienization, surface disinfection, cleaning protocols, etc.), encapsulation and prolonged release of EOs may limit their antimicrobial activity.

As shown in Fig. 2, the measurement of cell inactivation after exposure to 0.2 µL/mL of s-TCEO and zn-TCEO in buffer of pH 7.0 and 4.0 at room temperature for a short treatment time (up to 20 min) indicated that zn-TCEO displayed a lower antimicrobial activity compared with s-TCEO. Whereas the inactivation of 5 $\log_{10}$ cycles of *L. monocytogenes* EGD-e in buffer of pH 4.0 using s-TCEO required 10 min, no significant inactivation ($P < 0.05$) was observed in the presence of zn-TCEO after 20 min (Fig. 2B). In fact, under the treatment conditions investigated, the encapsulated EO did not exert any significant degree of inactivation against *E. coli* O157:H7 Sakai or *L. monocytogenes* EGD-e. This might be associated with the sustained release of TCEO from zein nanocapsules. In fact, our research group has determined that the release of TCEO from the nanocapsules at pH 7.0 starts at 4 h (data not shown).

This EO concentration (0.2 µL/mL) was established for comparative purposes based on previous results (Ait-Ouazzou et al., 2011, 2013). Thus, *E. coli* O157:H7 Sakai displayed a greater sensitivity to s-TCEO when the treatment was carried out in buffer at pH 7.0 than at pH 4.0 (Fig. 2A). Whereas the 20-min. treatment at pH 7.0 achieved 2.3 $\log_{10}$ cycles of reduction of the cell population, at pH 4.0 only 1.7 $\log_{10}$ cycles were inactivated. This effect of pH is not common, although it has already been observed in treatments with citral against *E. coli* BJ4 (Somolinos et al., 2010). On the contrary, in two studies that evaluated the antimicrobial activity of carvacrol against *E. coli* O157: H7 (phage type 34) (Ait-Ouazzou et al., 2011) and *E. coli* BJ4 (Ait-Ouazzou et al., 2013), a greater sensitivity to the compound was observed at pH 4.0, which indicates that microbial resistance to carvacrol under different treatment medium pH is dependent on the strain that is being studied.

Regarding results on *L. monocytogenes* EGD-e, the latter microorganism was more sensitive at pH 4.0 than at pH 7.0 (Fig. 2B). Whereas at pH 4.0 the treatment caused the inactivation of 4.7 $\log_{10}$ cell cycles after 7 min of treatment, at pH 7.0 the degree of inactivation was less than 1 $\log_{10}$ cycle after a 20 min-treatment. These results are in agreement with several studies conducted with carvacrol against *L. monocytogenes* EGD-e (Ait-Ouazzou et al., 2011, 2013). This may be due to the fact that at acidic pH, the EOs and their individual constituents display a higher hydrophobicity and, consequently, interact better with cell envelopes (Burt, 2004).

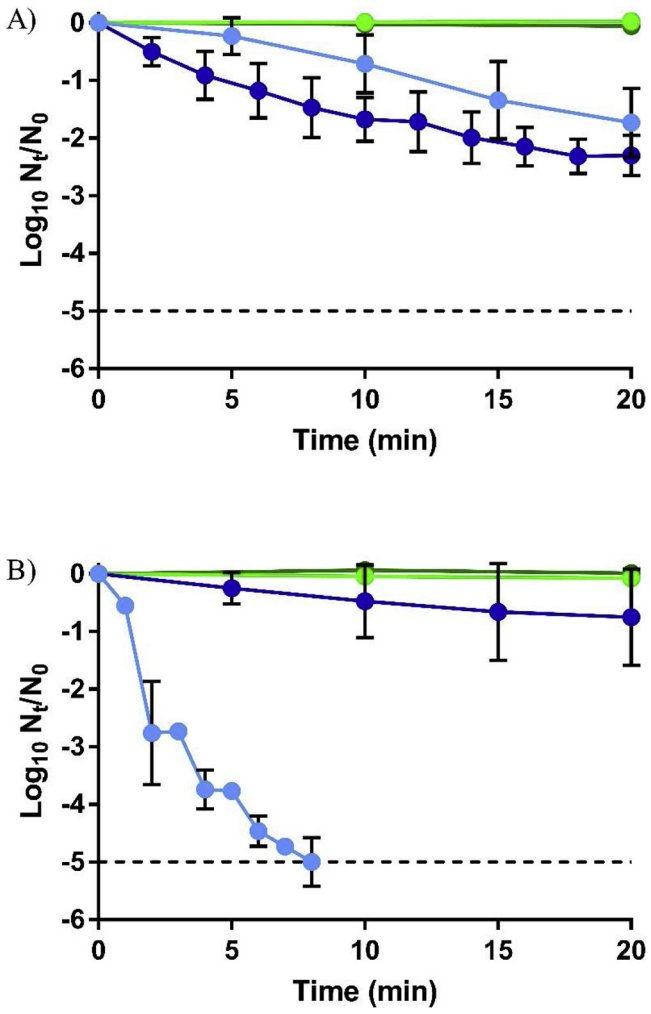
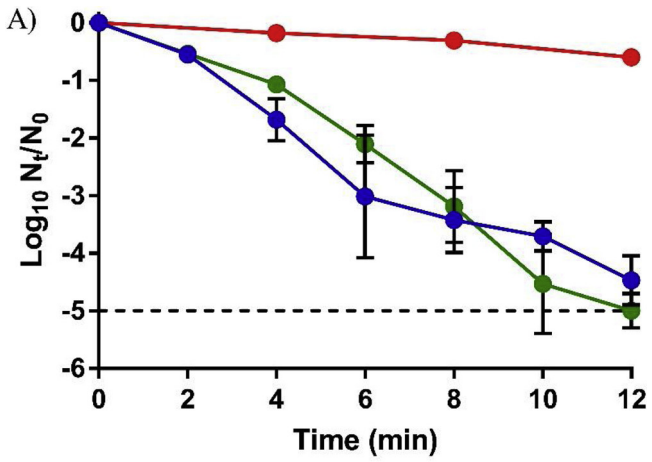


Fig. 2. Survival curves of *Escherichia coli* O157:H7 Sakai (A) and *Listeria monocytogenes* EGDe (B) (initial concentration: 3×10^7 CFU/mL) after exposure to s-TCEO (●, ●) or zn-TCEO (●, ●) (0.2 µL/mL of TCEO) in buffer of pH 7.0 (●, ●) and 4.0 (●, ●) at room temperature. Data represent the mean ± standard deviation (error bars) of at least three independent experiments. The dotted line represents the detection limit.

3.4. Synergistic effect of heat and s-TCEO and zn-TCEO

On the basis of the hurdle theory introduced by Leistner and Gorris (1995), many researchers have observed synergistic lethal effects when heat is applied in combination with novel chemical preservatives, such as EOs, and have recommended their use for the improvement of food preservation, product higienization or in order to enhance the effectiveness of cleaning and disinfectant methods (Espina et al., 2017; Guevara et al., 2015; Mate et al., 2016; Pagán et al., 2018). These treatments tend to be very brief (sec to min). It is unknown whether the use of zein nanocapsules would hamper the synergism between heat and EOs described above.

Fig. 3 shows the survival curves of *E. coli* O157:H7 Sakai after a combined treatment at 53 °C for 12 min in the presence of s-TCEO or zn-TCEO (0.1 µL/mL of TCEO) in buffers of pH 7.0 and 4.0. As can be observed in the figure, the inactivation of *E. coli* O157:H7 Sakai via the combined treatment was more rapid than the inactivation achieved through heat treatment. An outstanding synergism was observed between heat and either s-TCEO or zn-TCEO: while heat or TCEO acting separately scarcely caused the inactivation of < 0.5 $\log_{10}$ cycles of *E. coli* O157:H7 Sakai at pH 7.0 or 4.0 after 12- and 10 min-treatments, respectively, their simultaneous use caused the inactivation of up to 5



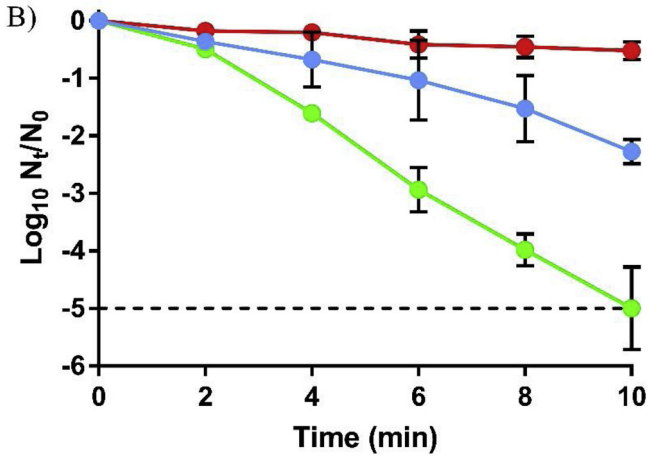
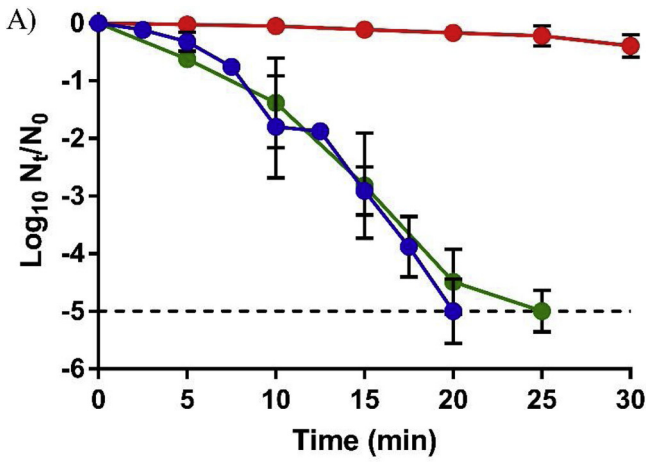


Fig. 3. Survival curves of *Escherichia coli* O157:H7 Sakai (initial concentration: 3×10^7 CFU/mL) after a heat treatment at 53 °C for 15 min in buffers of pH 7.0 (A) and 4.0 (B) (●), or after combined treatment with heat and s-TCEO ●, ●) or zn-TCEO (●, ●) (0.1 μL/mL of TCEO). Data represent the mean ± standard deviation (error bars) of at least three independent experiments. The dotted line represents the detection limit.



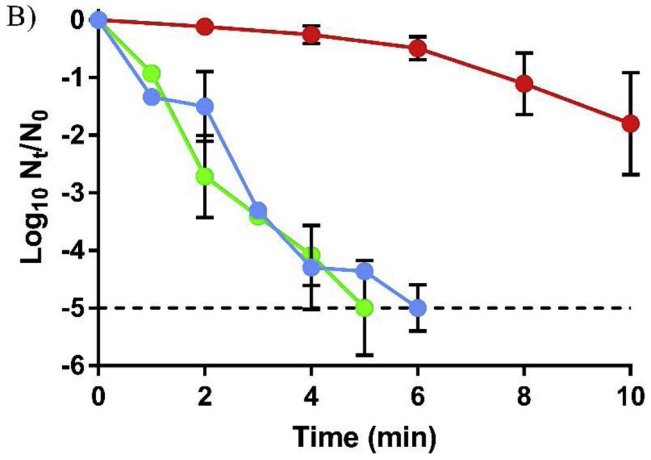


Fig. 4. Survival curves of L. *monocytogenes* EGD-e (initial concentration: 3×10^7 CFU/mL) after a heat treatment at 53 °C for 15 min in buffers of pH 7.0 (A) and 4.0 (B) (●), or after combined treatment with heat and s-TCEO (●, ●) or zn-TCEO (●, ●) (0.1 μL/mL of TCEO). Data represent the mean ± standard deviation (error bars) of at least three independent experiments. The dotted line represents the detection limit.

$\log_{10}$ cycles as a function of EO preparation and treatment medium pH. Whereas no significant differences ($P > 0.05$) in lethality were observed when combining heat and s-TCEO or zn-TCEO at pH 7.0 (Fig. 3A), the combination of heat and zn-TCEO was more effective at pH 4.0 (Fig. 3B). The unexpected results obtained at pH 4.0 pointed toward encapsulation with zeins as the most efficient way to enhance synergism between heat and TCEO. These results may be related to cell sensitization caused by zeins at acidic pH. In this regard, since the isoelectric point of zein is 6.8, under low pH conditions (i.e. pH 4.0) the surface of the zein nanoparticles is positively charged. This phenomenon would enhance the interaction of TCEO-loaded zein nanocapsules with the negatively charged outer membrane of *E. coli*, thereby enhancing the antimicrobial activity of TCEO, and especially of its main constituent, carvacrol.

On the other hand, at pH 7.0, a further experiment was performed to determine the concentration of TCEO loaded in nanocapulses of zeins which is required to reach the degree of lethality observed when combining heat and s-TCEO: no significant differences ($P > 0.05$) were observed when using 0.1 μL/mL of s-TCEO and 0.2 μL/mL of zn-TCEO in combination with mild heat (data not shown). Thus, considering that the use of the nanocapsules is a preferable choice in view of the previously described advantages regarding protection and improved distribution of EOs, the implementation of nanocapsules would require a double concentration of the selected EO.

Regarding the *L. monocytogenes* EGD-e results, Fig. 4 shows the survival curves at 53 °C in the presence of s-TCEO or zn-TCEO (0.1 μL/mL of TCEO) in buffers of pH 7.0 and 4.0. As one can observe in the figure, the extent of inactivation achieved thanks to combined treatment always exceeded the amount of inactivation obtained by heat treatment at either pH 7.0 or 4.0, which indicates a synergism between both hurdles against *L. monocytogenes* EGD-e. Moreover, unlike the results obtained with *E. coli* O157:H7 Sakai, the synergism achieved against *L. monocytogenes* EGD-e was the same using TCEO in the form of a suspension or loaded in nanocapsules of zeins, either at pH 7.0 (Fig. 4A) or 4.0 (Fig. 4B). According to these results, no interference of treatment medium pH or of TCEO preparation was observed when the combined treatment was applied against *L. monocytogenes* EGD-e, probably due to this bacterium's lack of outer membrane. Thus, the use of mild temperatures might contribute to accelerate the release of TCEO, increasing its bioavailability to interact against the bacterial population.

4. Conclusions

The use of self-assembled zein nanoparticles to encapsulate EOs might represent an alternative preferable to the use of EOs in suspension, not only in order to overcome their high volatility and to improve EO dispersion in hydrophilic solutions, but also in order to enhance their antimicrobial activity. Moreover, the use of a non-toxic, biocompatible, natural polymer isolated from corn or maize (zein) for EO nanoencapsulation, combined with a method that is simple and easy to scale up, permits it to be used in many different applications of interest for food, cosmetic or pharmaceutical industries.

The bacteriostatic activity of TCEO-loaded zein nanoparticles was greater than that of unprotected EO solutions. In terms of bactericidal activity, the combination of mild heat with TCEO nanoparticles displayed a great synergism, especially against *E. coli* O157:H7 Sakai at low pH. These synergistic effects hold promise for the improvement of alternative higienization processes, as well as for cleaning and disinfection procedures.

Acknowledgements

This study was financially supported by MINECO (Spanish Ministry of Economy and Competitiveness, Project No. AGL2015-69565-P), by the European project SKHINCAPS – "SKin Healthcare by Innovative NanoCAPsuleS" (H2020 - 685909), by FEDER, by the European Social Fund, and by the Aragonese Office of Science, Technology and University Research.

References

Adams, R.P., 2007. Identification of essential oil components by gas chromatography/mass spectrometry. Allured Publishing Corporation, Carol Stream.

Ait-Ouazzou, A., Cherrat, L., Espina, L., Lorán, S., Rota, C., Pagán, R., 2011. The antimicrobial activity of hydrophobic essential oil constituents acting alone or in combined processes of food preservation. Innov. Food Sci. Emerg. Technol. 12, 320–329. https://doi.org/10.1016/j.ifset.2011.04.004.

Ait-Ouazzou, A., Espina, L., Gelaw, T.K., de Lamo-Castellvi, S., Pagan, R., García-Gonzalo, D., 2013. New insights in mechanisms of bacterial inactivation by carvacrol. J. Appl. Microbiol. 114, 173–185. https://doi.org/10.1111/jam.12028.

Ben Jemaa, M., Falleh, H., Serairi, R., Neves, M.A., Snoussi, M., Isoda, H., Nakajima, M., Ksouri, R., 2018. Nanoencapsulated *Thymus capitatus* essential oil as natural preservative. Innov. Food Sci. Emerg. Technol. 45, 92–97. https://doi.org/10.1016/j.ifset.2017.08.017.

Benelli, G., Pavela, R., Cianfaglione, K., Nagy, D.U., Canale, A., Maggi, F., 2018. Evaluation of two invasive plant invaders in Europe *(Solidago canadensis* and *Solidago gigantea*) as possible sources of botanical insecticides. J. Pest Sci. https://doi.org/10.1007/s10340-018-1034-5.

Benjemaa, M., Neves, M.A., Falleh, H., Isoda, H., Ksouri, R., Nakajima, M., 2018. Nanoencapsulation of *Thymus capitatus*essential oil: Formulation process, physical stability characterization and antibacterial efficiency monitoring. Ind. Crops Prod. 113, 414–421. https://doi.org/10.1016/j.indcrop.2018.01.062.

Bilenler, T., Gokbulut, I., Sislioglu, K., Karabulut, I., 2015. Antioxidant and antimicrobial properties of thyme essential oil encapsulated in zein particles. Flavour Frag. J. 30, 392–398. https://doi.org/10.1002/ffj.3254.

Bounatirou, S., Smiti, S., Miguel, M.G., Faleiro, L., Rejeb, M.N., Neffati, M., Costa, M.M., Figueiredo, A.C., Barroso, J.G., Pedro, L.G., 2007. Chemical composition, antioxidant and antibacterial activities of the essential oils isolated from Tunisian *Thymus capitatus* Hoff. et Link. Food Chem. 105, 146–155. https://doi.org/10.1016/j.foodchem.2007.03.059.

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods: a review. Int. J. Food Microbiol. 94, 223–253. https://doi.org/10.1016/j.ijfoodmicro.2004.03.022.

Chouhan, S., Sharma, K., Guleria, S., 2017. Antimicrobial activity of some essential oils-present status and future perspectives. Medicines. 4, 58. https://doi.org/10.3390/medicines4030058.

de Carvalho, R.J., de Souza, G.T., Pagán, E., García-Gonzalo, D., Magnani, M., Pagán, R., 2018. Nanoemulsions of *Mentha piperita*L. essential oil in combination with mild heat, pulsed electric fields (PEF) and high hydrostatic pressure (HHP) as an alternative to inactivate*Escherichia coli* O157: H7 in fruit juices. Innov. Food Sci. Emerg. Technol. 48, 219–227. https://doi.org/10.1016/j.ifset.2018.07.004.

Delgado-Adámez, J., Garrido, M., Bote, M.E., Fuentes-Pérez, M.C., Espino, J., Martín-Vertedor, D., 2017. Chemical composition and bioactivity of essential oils from flower and fruit of *Thymbra capitata* and *Thymus* species. J. Food Sci. Technol. 54, 1857–1865. https://doi.org/10.1007/s13197-017-2617-5.

Donsì, F., Ferrari, G., 2016. Essential oil nanoemulsions as antimicrobial agents in food. J. Biotechnol. 233, 106–120. https://doi.org/10.1016/j.jbiotec.2016.07.005.

Espina, L., Somolinos, M., Pagán, R., García-Gonzalo, D., 2010. Effect of citral on the thermal inactivation of *Escherichia coli* O157:H7 in citrate phosphate buffer and apple juice. J. Food Prot. 73, 2189–2196. https://doi.org/10.4315/0362-028X-73.12.2189.

Espina, L., Berdejo, D., Alfonso, P., García-Gonzalo, D., Pagán, R., 2017. Potential use of carvacrol and citral to inactivate biofilm cells and eliminate biofouling. Food Control 82, 256–265. https://doi.org/10.1016/j.foodcont.2017.07.007.

Falcó, I., Verdeguer, M., Aznar, R., Sánchez, G., Randazzo, W., 2018. Sanitizing food contact surfaces by the use of essential oils. Innov. Food Sci. Emerg. Technol. https://doi.org/10.1016/j.ifset.2018.02.013.

Friedman, M., Henika, P.R., Mandrell, R.E., 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. J. Food Prot. 65, 1545–1560. https://doi.org/10.4315/0362-028X-65.10.1545.

Guevara, L., Antolinos, V., Palop, A., Periago, P.M., 2015. Impact of moderate heat, carvacrol, and thymol treatments on the viability, injury, and stress response of Listeria monocytogenes. Biomed Res. Int. https://doi.org/10.1155/2015/548930.

Hu, J., Zhang, Y., Xiao, Z., Wang, X., 2018. Preparation and properties of cinnamon-thyme-ginger composite essential oil nanocapsules. Ind. Crops Prod. 122, 85–92. https://doi.org/10.1016/j.indcrop.2018.05.058.

Leistner, L., Gorris, L.G.M., 1995. Food preservation by hurdle technology. Trends Food Sci. Tech. 6, 41–46. https://doi.org/10.1016/S0924-2244(00)88941-4.

Lemos, M.F., Lemos, M.F., Pacheco, H.P., Guimarães, A.C., Fronza, M., Endringer, D.C., Scherer, R., 2017. Seasonal variation affects the composition and antibacterial and antioxidant activities of Thymus vulgaris. Ind. Crops Prod. 95, 543–548 https://doi.org/10.1016.

Luis-Villaroya, A., Espina, L., Garcia-Gonzalo, D., Bayarri, S., Perez, C., Pagan, R., 2015. Bioactive properties of a propolis-based dietary supplement and its use in combination with mild heat for apple juice preservation. Int. J. Food Microbiol. 205, 90–97. https://doi.org/10.1016/j.ijfoodmicro.2015.03.020.

Mate, J., Periago, P.M., Palop, A., 2016. When nanoemulsified, D-limonene reduces *Listeria monocytogenes* heat resistance about one hundred times. Food Control 59, 824–828. https://doi.org/10.1016/j.foodcont.2015.07.020.

Meena, M.R., Sethi, V., 1994. Antimicrobial activity of essential oils from spices. J. Food Sci. Technol. 31, 68–70.

Michino, H., Araki, K., Minami, S., Takaya, S., Sakai, N., Miyazaki, M., Ono, A., Yanagawa, H., 1999. Massive outbreak of *Escherichia coli* O157:H7 infection in schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts. Am. J. Epidemiol. 150, 787–796. https://doi.org/10.1093/oxfordjournals.aje.a010082.

Neves, A., Marto, J., Duarte, A., Gonçalves, L.M., Pinto, P., Figueiredo, A.C., Ribeiro, H.M., 2017. Characterization of Portuguese *Thymbra capitata*, *Thymus caespititius* and *Myrtus communis* essential oils in topical formulations. Flavour Frag. J. 32, 392–402. https://doi.org/10.1002/ffj.3393.

Pagán, E., Berdejo, D., Espina, L., García-Gonzalo, D., Pagán, R., 2018. Antimicrobial activity of suspensions and nanoemulsions of citral in combination with heat or pulsed electric fields. Lett. Appl. Microbiol. 66, 63–70. https://doi.org/10.1111/lam.12815.

Patel, A.R., Velikov, K.P., 2014. Zein as a source of functional colloidal nano- and microstructures. Curr. Opin. Colloid Interface Sci. 19, 450–458. https://doi.org/10.1016/j.cocis.2014.08.001.

Peñalva, R., Morales, J., González-Navarro, C., Larrañeta, E., Quincoces, G., Peñuelas, I., Irache, J., 2018. Increased oral bioavailability of resveratrol by its encapsulation in casein nanoparticles. Int. J. Mol. Sci. 19, 2816. https://doi.org/10.3390/ijms19092816.

Prakash, B., Kujur, A., Yadav, A., Kumar, A., Singh, P.P., Dubey, N.K., 2018. Nanoencapsulation: an efficient technology to boost the antimicrobial potential of plant essential oils in food system. Food Control 89, 1–11. https://doi.org/10.1016/j.foodcont.2018.01.018.

Ramos, J.C., Andrade de Meneses, D., Pereira de Vasconcelos, A., Alves Piauilino, C., de Castro Almeida, F.R., Marco Napoli, E., Ruberto, G., Machado de Araújo, D.A., 2017. Essential oil composition and antinociceptive activity of Thymus capitatus. Pharm. Biol. 55, 782–786. https://doi.org/10.1080/13880209.2017.1279672.

Raybaudi-Massilia, R.M., Mosqueda-Melgar, J., Soliva-Fortuny, R., Martín-Belloso, O., 2009. Control of pathogenic and spoilage microorganisms in fresh-cut fruits and fruit juices by traditional and alternative natural antimicrobials. Compr. Rev. Food Sci. Food Saf. 8, 157–180. https://doi.org/10.1111/j.1541-4337.2009.00076.x.

Reis, C.P., Neufeld, R.J., Ribeiro, A.J., Veiga, F., 2006. Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. Nanomed. 2, 8–21. https://doi.org/10.1016/j.nano.2005.12.003.

Salman, H., Azcárate, I.G., Catalán, I.E., 2013. Nanoparticles Comprising a Vegetable Hydrophobic Protein and a Water Miscible Non-volatile Organic Solvent and Uses Thereof. Bionanoplus, S.L., Spain. .

Somolinos, M., Garcia, D., Condon, S., Mackey, B., Pagan, R., 2010. Inactivation of *Escherichia coli* by citral. J. Appl. Microbiol. 108, 1928–1939. https://doi.org/10.1111/j.1365-2672.2009.04597.x.

Souza, E.L., Almeida, E.T., Guedes, J.P., 2016. The potential of the incorporation of essential oils and their individual constituents to improve microbial safety in juices: a review. Compr. Rev. Food Sci. F. 15, 753–772. https://doi.org/10.1111/1541-4337.12208.

Trindade, H., Pedro, L.G., Figueiredo, A.C., Barroso, J.G., 2018. Chemotypes and terpene synthase genes in *Thymus* genus: state of the art. Ind. Crops Prod. Ind. Crops Prod. 124, 530–547. https://doi.org/10.1016/j.indcrop.2018.08.021.

Weissmueller, N.T., Lu, H.D., Hurley, A., Prud'homme, R.K., 2016. Nanocarriers from GRAS zein proteins to encapsulate hydrophobic actives. Biomacromolecules 17, 3828–3837. https://doi.org/10.1021/acs.biomac.6b01440.

Wu, Y., Luo, Y., Wang, Q., 2012. Antioxidant and antimicrobial properties of essential oils encapsulated in zein nanoparticles prepared by liquid–liquid dispersion method. LWT-Food Sci. Technol. 48, 283–290. https://doi.org/10.1016/j.lwt.2012.03.027.

Yuan, G., Chen, X., Li, D., 2016. Chitosan films and coatings containing essential oils: the antioxidant and antimicrobial activity, and application in food systems. Food Res. Int. 89, 117–128. https://doi.org/10.1016/j.foodres.2016.10.004.

Manuscrito X. Modified cyclodextrin type and dehydration methods exert a significant effect on the antimicrobial activity of encapsulated carvacrol and thymol

López-Miranda, S., Berdejo, D., Pagán, E., García-Gonzalo, D., Pagán, R. (2020)

Journal of the Science of Food and Agriculture. doi: 10.1002/jsfa.11017.

Research Article

Resultados SCI

Received: 30 July 2020 Revised: 17 November 2020 Accepted article published: 14 December 2020 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.11017

Modified cyclodextrin type and dehydration methods exert a significant effect on the antimicrobial activity of encapsulated carvacrol and thymol

Santiago López-Miranda,[a*] Daniel Berdejo,[b] Elisa Pagán,[b] Diego García-Gonzalo[b] and Rafael Pagán[b]

Abstract

BACKGROUND: The antimicrobial activity of essential oils and their constituents has led to increasing interest in using them as natural preservative agents. However, their high sensitivity to light and oxygen, their volatility and their low aqueous solubility are all obstacles to their application in the food, cosmetic or pharmaceutical industries. Encapsulation in cyclodextrins (CDs) is a solution for the application of such essential oils.

RESULTS: The complexation of carvacrol and thymol with hydroxypropyl (HP)-α-, HP-β- and HP-γ-CD, the behavior of the solid complexes prepared by freeze-drying and spray-drying methods and the antibacterial activity of solid complexes were studied. K_c values of HP-α- and HP-γ-CD complexes with carvacrol (118.4 and 365.7 L mol^{-1}) and thymol (112.5 and 239.7 L mol^{-1}) were far lower than those observed for HP-β-CD complexes with carvacrol (2268.2 L mol^{-1}) and thymol (881.6 L mol^{-1}). The lower stability of HP-α- and HP-γ-CD complexes increased the release of compounds, thereby affecting the antimicrobial activity of carvacrol and thymol to a lesser extent than complexation with HP-β-CD, normally used in the encapsulation of carvacrol and thymol. HP-β-CD encapsulation of carvacrol and thymol markedly reduced their antimicrobial activity. The freeze-drying method barely affected the antimicrobial activity of carvacrol and thymol after encapsulation, while spray drying could be considered for the production of solid complexes in combination with the appropriate CD.

CONCLUSIONS: It was thus demonstrated that HP-α- and HP-γ-CD are very suitable alternatives for the encapsulation of carvacrol and thymol with the purpose of preserving their bacteriostatic and bactericidal activities.

Keywords: carvacrol; thymol; cyclodextrin; antimicrobial; spray drying; freeze drying

INTRODUCTION

In recent years, the food industry has profoundly innovated in terms of food preservation processes and food safety. Essential oils (EOs), as well as their constituents, have been shown to be powerful and natural antimicrobial agents against a large number of pathogens.[1] These antibacterial agents are extracted from natural sources, and are generally regarded as healthy, environmentally friendly and safe,[2] which makes them attractive to consumers.[3] However, food industrial application of these compounds faces challenges that need to be solved, such as their limited aqueous solubility as well as their high volatility and instability against external agents such as light and oxygen, all of which render difficult a controlled release of such compounds and reduce their antimicrobial efficacy.[4]

One of the techniques most widely used to address these limitations has been encapsulation, comprising a wide and varied range of procedures such as freeze drying, spray drying, coacervation, gelation, precipitation and nanoemulsion,[4,5] as well as a wide range of encapsulating agents such as maltodextrins, gums, starch, chitosan, proteins and zeins.[4–7]

Among all the encapsulation alternatives for EOs and their components, the use of molecular encapsulation in cyclodextrins (CDs) stands out. CDs are cyclic oligomers that are widely used and recommended for industrial use as protectors for light-sensitive, oxygen-sensitive and heat-sensitive compounds, solubilizers

* Correspondence to: S López-Miranda, Department of Food Technology and Nutrition, Molecular Recognition and Encapsulation (REM) Group, UCAM Universidad Católica de Murcia, Avenida de los Jerónimos 135, 30107 Guadalupe, Murcia, Spain, E-mail: *slmiranda@ucam.edu*

a *Department of Food Technology and Nutrition, Molecular Recognition and Encapsulation (REM) Group, UCAM Universidad Católica de Murcia, Murcia, Spain*

b *Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain*

of dyes and vitamins, flavor stabilizers, suppressants of flavors and unpleasant flavors, and regulators of controlled release of food additives and drugs.[8]

Several authors have previously studied the effect of encapsulation in CDs on the solubility, stability, controlled release and bioactive properties, particularly the antimicrobial activity, of various EOs such as clove and oregano,[9] coriander,[10] thyme,[11] cinnamon,[3] basil and tarragon,[12] *Mentha* × *villosa* Hudson,[13] guava leaf[14] and pepper,[15] as well as some of their main components with high antimicrobial potential such as carvacrol and thymol.[16–20] However, despite a wide range of existing literature, there is no consensus as to whether the encapsulation of these compounds in CDs positively or negatively affects their biological properties, especially their antimicrobial activity.[21] Although encapsulation in CDs increases the aqueous solubility of EOs and terpenes, the stability of the resulting complex can affect the degree of release of the active compounds as well as their biological activity.

In addition, the majority of previous studies almost exclusively focused on the use of β-CD and hydroxypropyl (HP)-β-CD, with almost no references to the encapsulation of EOs or their components with modified HP-α-CD or HP-γ-CD. In a recent review of the characterization of volatile compounds encapsulated in CDs, Kfoury *et al.*[22] provided more than 300 values of complexation constants with a series of CDs, but not with HP-α- and HP-γ-CD. Rakmai *et al.*[23] report 26 technological applications of complexed EOs in CDs, 25 of which are focused on encapsulation with β-CD or HP-β-CD. On the other hand, Lima *et al.*[24] published almost 150 references, of which only two consider the complexation of terpenes in HP-γ-CD and none in HP-α-CD. This preference for β-CD and HP-β-CD is due to the greater compatibility of their hydrophobic cavity with most terpenes and oils with a reduced molecular weight (200–800 g mol^{-1}),[25] which provides complexes with high stability. However, complexes with high stability can reduce compound release from CDs and limit their functional properties. It is therefore of great interest to study the ability to modulate the biological activity of antimicrobial compounds by complexing with other CDs, especially with modified ones such us HP-α- and HP-γ-CD, in order to obtain complexes with lower stability constants than those provided by the most widely used β-CD and HP-β-CD.

Solid-state CD complexes are another key aspect for food application, because solid state improves handling and stability and allows for standardized dosage of active compounds. The most frequently used methods for obtaining solid complexes of EOs are those that do not require high temperatures, such as freeze drying, kneading and precipitation.[13,15,16,17,25] However, the use of the spray-drying method, despite being easy to perform on an industrial scale,[5] is less widespread in the dehydration of EO complexes with CDs due to its high process temperature. Therefore, it is key to advance the study of interactions between CD type and solid complex dehydration methods in order to modulate the biological activity of the encapsulated compounds for industrial applications.

The aims of the study reported here were, on the one hand, to study the effect that the stability of CD complexes has on the antimicrobial activity of carvacrol and thymol, introducing, in addition to the well-known HP-β-CD, the modified HP-α- and HP-γ-CD, the behavior of which has not been previously described in the literature. On the other hand, the effect of the method of preparing solid complexes, via spray drying as well as via freeze drying, was determined for the physical and antimicrobial properties of those complexes.

MATERIALS AND METHODS

Reagents and standards

Carvacrol (98% purity) and thymol (99% purity) were purchased from TCI Europe NV (Zwijndrecht, Belgium). The HP-α-, HP-β- and HP-γ-CD were purchased from Winplus International Limited (Ningbo, China). High-performance liquid chromatography (HPLC) reagents acetonitrile and water were purchased from JT Baker (Deventer, The Netherlands). Other chemical reagents used were of analytical grade.

Complexation and phase solubility diagrams

The complexation of carvacrol and thymol in CDs was carried out using phase solubility diagrams according to the method described by Higuchi and Connors with some modifications.[26] Excess amounts of carvacrol and thymol were added to 50 mL of aqueous solutions in concentrations increasing from 0 to 50 mmol L^{-1} for HP-α-, HP-β- and HP-γ-CD, and were continuously stirred during 24 h at 20 °C in the dark. After 24 h, solutions were filtered using 0.45 µm cellulose acetate membrane filters (Chromafil Macherey-Nagel, Düren, Germany) for HPLC assays. Phase solubility diagrams were made in triplicate.

The complexation constant K_c between carvacrol and thymol and each type of CD was calculated from the slope of the phase solubility diagram and the solubility of the compound aqueous solution (S_0) using Eqn (1):

$$K_c\left(\mathrm{L\,mol^{-1}}\right)=\frac{\mathrm{Slope}}{S_0(1-\mathrm{slope})} \tag{1}$$

Complexation efficiency (CE) is the ratio between the concentration of dissolved complex and the concentration of free CDs. It is independent of S_0, and was calculated from the slope of the phase solubility profiles using Eqn (2):

$$\mathrm{CE}\,(\%)=\frac{\mathrm{Slope}}{(1-\mathrm{slope})}\times 100 \tag{2}$$

The molar ratio of drug to CD (D:C) was calculated using the CE value with Eqn (3):

$$\mathrm{D:C}=1:\left(1+\frac{1}{\mathrm{CE}}\right) \tag{3}$$

The solubilization potential (S_t/S_0) was calculated as the relation between compound solubility at 50 mmol L^{-1} CD concentration (S_t) and compound aqueous solubility (S_0).

Solid complexes by freeze drying and spray drying

Solid complexes were obtained by dehydrating a volume of 100 mL with a molar proportion of 50:25 (CD:compound) for HP-α-CD and HP-γ-CD and 50:40 (CD:compound) for HP-β-CD. These molar proportions were selected according to the solubilization potential of the different CDs in order to ensure that all of the active compound was complexed. Solutions were continuously stirred during 24 h at 20 °C in the dark. After 24 h, the solutions containing the complexes were either freeze-dried or spray-dried.

Solutions of carvacrol and thymol complexes were freeze-dried using a Christ Alpha 1-2 LD Plus (Martin Christ, Osterode am Harz, Germany) freeze dryer at −48 °C during 3 days. Solutions of carvacrol and thymol complexes were spray-dried using a Buchi B-290 device (Flawil, Switzerland). The spray drier configuration was:

inlet air temperature, 170 °C; outlet air temperature, 68 °C; inlet air flow, 35 $m^3\ h^{-1}$; pump flow, 5 mL min^{-1}; compressed air caudal, 360 L h^{-1}. The recovered freeze-dried and spray-dried solid complexes were stored in an airtight glass container for subsequent analysis and characterization. For carvacrol and thymol HPLC quantification, solid complexes were dissolved in distilled water and filtered using 0.45 µm cellulose acetate membrane filters (Chromafil Macherey-Nagel, Düren, Germany).

The dehydration yield (DY) was calculated using the following equation:

$$\mathrm{DY}\,(\%) = \frac{\text{Solid complexes obtained}\,(\mathrm{g})}{\text{Total solids in solution}\,(\mathrm{g})} \times 100 \qquad (4)$$

The encapsulation efficiency (EE) was calculated using the following equation:

$$\mathrm{EE}\,(\%) = \frac{\text{Total compound encapsulated in solid complex}\,(\mathrm{mg})}{\text{Initial total compound in solution}\,(\mathrm{mg})} \times 100 \qquad (5)$$

The drug load (DL) was calculated using the following equation:

$$\mathrm{DL}\,(\mathrm{mg\,g^{-1}}) = \frac{\text{Total compound encapsulated in solid complex}\,(\mathrm{mg})}{\text{Total solid complexes}\,(\mathrm{g})} \qquad (6)$$

Wettability is defined as the time taken by a solid to completely submerge in a humid medium. The analysis was carried out by gently dropping 500 mg of powder on the surface of 100 mL of distilled water at 20 °C.

EE, DL and wettability were measured in triplicate for each sample for purposes of statistical analysis.

Carvacrol and thymol determination using HPLC

Carvacrol and thymol were quantified using HPLC analysis with an Agilent Technologies model 1200 (Agilent, Santa Clara, California, USA) equipped with a diode array detector set at 280 nm, injecting 20 µL of filtered complexes. Separations were carried out on an endcapped (5 µm) HPLC Cartridge 250-4 LiChospher 100 RP-18. The column temperature was set to 30 °C, and the flow rate was 0.7 mL min^{-1}. The mobile phase was water with 0.5% of acetic acid (A) *versus* acetonitrile (B), for a total running time of 7 min and a constant proportion of 20% (A) and 80% (B). Time retentions were 4.3 min for carvacrol and 4.1 min for thymol. The data were processed using Agilent ChemStation software.

Particle size and shape

Shape and structural characteristics of the solid complexes were studied using field emission scanning electron microscopy (SEM) images. Uncoated samples were examined using a MERLIN™ VP COMPACT microscope (Carl Zeiss Microscopy, Oberkochen, Germany). Images detailing morphology were obtained using an SE2 detector under an accelerating voltage of 1 kV.

Antibacterial activity

Bacteria and growth conditions

Escherichia coli O157:H7 Sakai/*stx1A*/*stx2A* was kindly provided by Kyu-Tae Chang.[27] FPR3757 strain of methicillin-resistant *Staphylococcus aureus* USA300 was provided by Prof. Kolter (Harvard Medical School, Boston, MA, USA). This strain was isolated from an outbreak in the USA.[28]

Throughout this investigation, both strains were kept at −80 °C in cryovials with glycerol. To prepare the broth subcultures, one single colony from a tryptone soya agar plate supplemented with 0.6% yeast extract (TSAYE; Oxoid, Basingstoke, UK) was inoculated in a test tube with 5 mL of sterile tryptone soya broth with 0.6% yeast extract added (TSBYE; Oxoid). The inoculated tubes were incubated overnight in aerobic conditions at 37 °C (Selecta Incudigit, Barcelona, Spain) to obtain bacterial subcultures. Erlenmeyer flasks (250 mL) containing 50 mL of TSBYE were inoculated with those subcultures to a final concentration of 10^5 colony forming units (CFU) mL^{-1}. To reach the stationary growth phase (*ca* 2×10^9 CFU mL^{-1}), bacterial cultures were incubated for 24 h under agitation (130 rpm) at 37 °C (Selecta Rotabit).

Disk diffusion test

A modified disk diffusion test was applied in order to screen the antimicrobial activity of carvacrol and thymol encapsulated in HP-α-, HP-β- and HP-γ-CD against *E. coli* O157:H7 Sakai and *S. aureus* USA300.[29] Filter paper disks (Whatman No. 1; diameter: 6 mm) containing 600 µg of encapsulated active compound were applied on TSAYE plates that had been previously inoculated with a diluted culture (0.5 McFarland) and spread on the surface. For a period of 24 h, the plates were incubated at the appropriate temperature (37 °C), and the resulting zone of partial inhibition was observed.

Minimum inhibitory and bactericidal concentrations

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of carvacrol and thymol, free and encapsulated in HP-α, HP-β and HP-γ-CD, were evaluated against *E. coli* O157:H7 Sakai and *S. aureus* USA300 with the purpose of studying the effect of encapsulation on the antimicrobial properties of those two compounds following a protocol adapted from CLSI (2015). Test tubes containing 5 mL of Mueller Hinton broth (MHB; Oxoid) with a diluted (10^5 CFU mL^{-1}) stationary phase culture were inoculated with CD-encapsulated thymol and carvacrol at various concentrations (from 150 to 1000 mg L^{-1} at intervals of 50 mg L^{-1}). Subsequently, test tubes were incubated at 37 °C for 24 h in a shaking thermostatic incubator (Bunsen, model BTG, Madrid, Spain) at 130 rpm. Positive controls containing MHB with microbial cultures at 10^5 CFU mL^{-1} without carvacrol and thymol, as well as negative controls containing MHB with encapsulated carvacrol and thymol without bacteria were also prepared. MIC was determined as the lowest concentration of carvacrol or thymol in the presence of which bacteria showed no visible growth. Additionally, test tubes were aliquoted and spread onto Mueller Hinton agar plates (MHA; Oxoid), and, after incubation (37 °C/24 h), survivors were counted with an improved image analyzer automatic counter (Protos, Analytical Measuring Systems, Cambridge, UK). The MBC was set as the minimum concentration of the compound that inactivated more than 3 $\log_{10}$ cycles (<10^2 CFU mL^{-1}).

RESULTS AND DISCUSSION

Complexation of carvacrol and thymol in HP-α-, HP-β- and HP-γ-CD

The phase diagrams of carvacrol and thymol encapsulated in HP-α-, HP-β- and HP-γ-CD are shown in Fig. 1. The phase solubility diagrams showed an A_L type profile, for both compounds and for all CDs, indicating that water-soluble complexes had been formed. In

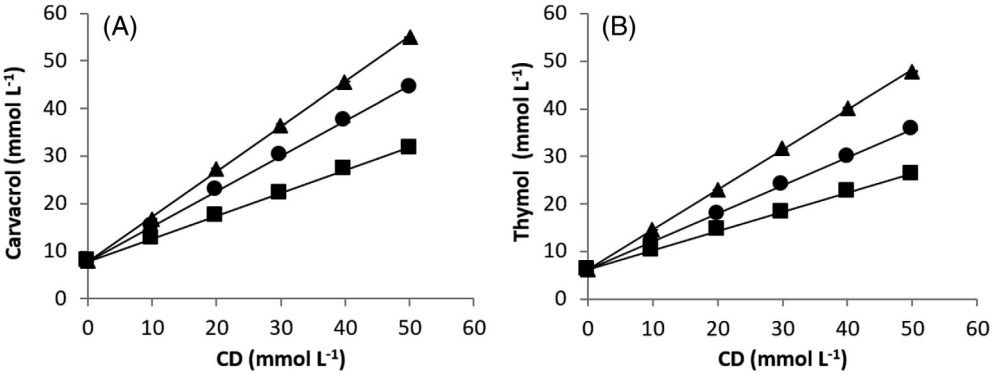


Figure 1. Phase solubility diagrams of (A) carvacrol and (B) thymol with HP-α-CD (■), HP-β-CD (▲) and HP-γ-CD (●) in aqueous solution.

all cases studied, the slope of the solubility diagrams was lower than 1, indicating that the stoichiometry of the complexes was 1:1, whereby each molecule of carvacrol or thymol enters into one molecule of CD.[26] The same A_L diagram was previously observed for carvacrol and thymol encapsulated in HP-β-CD by Kamimura *et al.*[16] and Kfoury *et al.*[18] thereby suggesting that this 1:1 stoichiometry is common for these compounds.[30] To the best of our knowledge, these are the first published results concerning carvacrol and thymol encapsulation with HP-α- and HP-γ-CD.

From the information plotted in the phase diagrams, various parameters that characterize the complexation of these compounds in CDs were calculated. Table 1 presents K_c, CE, D:C and S_t/S_0 of HP-α-, HP-β- and HP-γ-CD complexes.

K_c was calculated by using linear regression analysis from the phase solubility diagrams according to Eqn (1). One can observe that the highest values of K_c were obtained for HP-β-CD. The K_c values for carvacrol and thymol encapsulated in HP-β-CD were 2268.2 and 881.6 L mol^{-1}, respectively. These values are similar to those obtained by Kfoury *et al.*[18] K_c values of carvacrol and thymol complexes with HP-α- and HP-γ-CD were significantly lower ($P < 0.05$) than those obtained for HP-β-CD. K_c values for HP-α-CD were 118.5 and 112.5 L mol^{-1}, and for HP-γ-CD they were 365.7 and 237.7 L mol^{-1} for carvacrol and thymol, respectively. K_c values lay, in general, within the range 200 to 2000 L mol^{-1}, which are usual values for aromatic compounds.[30] No K_c values for HP-α- and HP-γ-CD have been described in previous literature, neither for these compounds nor for others of a similar nature. In fact, the vast majority of the available literature almost exclusively focuses on studying the interactions of various molecules with β-CD and HP-β-CD, which have been cited as the most suitable for the encapsulation of compounds of an aromatic nature.[18,20,22,23]

The stability of a CD complex depends on an adequate inclusion of the host molecule inside the hydrophobic cavity of the CD molecule. Based on the classification proposed by Carrier *et al.*,[31] the stability of carvacrol and thymol complexes with HP-α- and HP-γ-CD could be classified as very weak (0–-500 L mol^{-1}), while that of complexes with HP-β-CD would be between weak (500–1000 L mol^{-1}) and moderate (1000–-5000 L mol^{-1}). The K_c values obtained in this study suggest that the size of HP-α-CD could be too small for carvacrol and thymol to be able to complete their encapsulation within the hydrophobic cavity. On the other hand, the cavity size of HP-γ-CD could be too large, thereby causing carvacrol and thymol molecules to fail to establish a sufficient amount of stable interactions with hydrophobic cavity atoms. In previous studies carried out with these and similar molecules, it has been observed that K_c values with native CDs have been generally higher for β-CD than for α- and γ-CD.[12,18,22] Obtaining complexes with a different stability as a result of using different CDs could represent an advantage in controlling and modulating the release of biologically active compounds.

K_c indicates complexation strength or complex stability, and it is normally used to compare the affinity of any guest molecule with different CD types. However, in order to analyze the solubilizing effect of CDs, CE is more suitable because it is independent of S_0. CE represents the ratio between complex and free CD concentrations, and, for 1:1 complexes, it can be calculated from the slope of the phase solubility diagram with Eqn (2).[32] In addition, CE can be used to calculate D:C, which indicates the ratio between

Table 1. Aqueous solubility (S_0), complexation constant (K_c), complexation efficiency (CE), molar ratio (D:C) and solubility ratio (S_t/S_0) at 50 mmol L^{-1} of CDs for carvacrol and thymol for HP-α-CD, HP-β-CD and HP-γ-CD

Compound	CD	S_0 (mmol L^{-1})	K_c (L mol^{-1})	CE (%)	Molar ratio (D:C)	S_t/S_0
Carvacrol	HP-α-CD	7.80 ± 0.19	118.4 ± 4.8^a	92.4 ± 3.8^a	1:2.1 ± 0.04^c	4.07 ± 0.14^a
	HP-β-CD		2268.2 ± 184.2^c	1769.2 ± 143.7^c	1:1.1 ± 0.00^a	7.05 ± 0.18^c
	HP-γ-CD		365.7 ± 13.7^b	285.2 ± 10.7^b	1:1.4 ± 0.01^b	5.73 ± 016^b
Thymol	HP-α-CD	6.10 ± 0.08	112.5 ± 3.1^a	68.6 ± 1.9^a	1:2.5 ± 0.04^c	4.30 ± 0.12^a
	HP-β-CD		881.6 ± 6.6^c	537.8 ± 4.0^c	1:1.2 ± 0.00^a	7.85 ± 0.01^c
	HP-γ-CD		239.7 ± 2.0^b	146.2 ± 1.2^b	1:1.7 ± 0.01^b	5.85 ± 0.00^b

In each column, statistical difference between means of HP-α-, HP-β- and HP-γ-CD for each compound is shown ($P < 0.05$) (a–c).
Values represent means of triplicate determination (± standard deviation).

the number of CD-forming complexes and the total number of CDs in solution (Eqn (3)).

The obtained values of CE and molar ratio are presented in Table 1. The highest CE value was obtained for the carvacrol–HP-β-CD complex (1769.2%) followed by the thymol–HP-β-CD complex (537.8%). These values are similar to those obtained by other authors for the same compounds and CDs.[18] Significantly lower values ($P < 0.05$) were observed for carvacrol and thymol complexes with HP-γ-CD (285.2% and 146.2%, respectively). The lowest CE values were displayed by carvacrol and thymol complexes with HP-α-CD (92.4% and 68.6%, respectively). In general, the CE values obtained in this study were higher than those reported by other authors for different types of compounds and CDs.[32] CE values close to or even greater than 100% mean that there was a higher proportion of CD-forming soluble complexes than of free CDs, which indicates that CDs were being used effectively to solubilize the guest molecule by forming soluble complexes. Almost all of the dissolved HP-β- and HP-γ-CD molecules were complexed with a carvacrol molecule, as shown by the 1:1.1 and 1:1.4 molar ratios, respectively (Table 1). The behavior of thymol complexes was similar, with a molar ratio of 1:1.2 and 1:1.7 for HP-β- and HP-γ-CD, respectively. HP-α-CD complexes displayed a lower molar ratio than HP-β- and HP-γ-CD, indicating that two molecules of HP-α-CD in solution were each forming soluble complexes (1:2.1 and 1:2.5 for carvacrol and thymol, respectively).

The solubilization potential (S_t/S_0) was comparable for carvacrol and thymol, although slightly higher values were obtained for thymol than for carvacrol, mainly due to the lower S_0 value of thymol (Table 1), as suggested by previous studies.[18] At a CD concentration of 50 mmol L^{-1}, the solubility of carvacrol and thymol increased more than 7-fold with HP-β-CD and almost 6-fold with HP-γ-CD, which indicates the high solubilization potential not only of HP-β-CD but also of HP-γ-CD. HP-α-CD showed a significantly ($P < 0.05$) lower solubilization potential, increasing the aqueous solubility 4.07- and 4.30-fold for carvacrol and thymol, respectively.

Characterization of solid complexes

Table 2 presents the characterization parameters of the solid complexes of carvacrol and thymol with HP-α-, HP-β- and HP-γ-CD dehydrated by freeze drying or spray drying. DY (grams of solid complex recovered with respect to the initial grams of soluble solids, Eqn (4)) was higher for freeze drying than for spray drying. On average, freeze drying afforded a recovery yield of 93% in contrast with 80% for spray drying. Despite the lower values for spray drying relative to freeze drying, the DY of spray drying of 80% was over 50%, which is the minimum recommended recovery rate for adequate dehydration process efficiency.[33] This fact indicates that conditions of temperature, inlet air flow and atomization level were adequate for obtaining solid complexes by spray drying. On the other hand, a slightly increasing trend was observed in the DY: HP-α-CD < HP-β-CD < HP-γ-CD. This increasing trend could be due to the fact that the preparation of solid complexes was carried out with the same initial CD concentration of 50 mmol L^{-1}, which means that the initial amount of soluble solids was increasing, HP-α-CD < HP-β-CD < HP-γ-CD, thereby resulting in a more efficient recovery of dehydrated complexes.[34]

EE (Eqn (5)) represents the amount of active matter that has been recovered after the dehydration process, indicating whether significant compound losses occur during the dehydration process. For all the solid complexes obtained, EE was significantly higher ($P < 0.05$) for freeze drying than for spray drying. On average, the EE for freeze drying was 93.5%. These values were similar to those obtained by Kfoury *et al.*[30] for thymol encapsulated in HP-β-CD, and slightly higher than those reported by Kamimura *et al.*[16] for carvacrol likewise encapsulated in HP-β-CD, as well as by Hill *et al.*[3] for aromatic compounds that were similar although encapsulated in other CDs. The low-temperature conditions of the freeze-drying process were key in limiting aromatic compound losses during the process. On the other hand, the EE of spray drying ranged from 64.9% to 88.8% for carvacrol, and from 70.2% to 79.8% for thymol.

Regarding the effect of CD type on EE, no statistically significant differences ($P > 0.05$) were observed among freeze-dried dehydrated complexes, except between thymol complexes with HP-α-CD and with HP-β-CD. When complexes were dehydrated by spray drying, EE was higher for HP-α-CD and lower for HP-γ-CD, with HP-β-CD in an intermediate position. A high concentration of soluble solids, as occurred with HP-γ-CD, could increase the solution viscosity prior to being sprayed, which can negatively affect the retention of encapsulated compounds.[35] Kfoury *et al.* also observed a reduction in EE for native CDs with the trend α-CD > β-CD > γ-CD.[12]

In relation to the amount of compound that can be encapsulated per gram of complex (DL, Eqn (6)), the freeze-drying process showed higher values than spray drying, with statistically significant differences ($P < 0.05$) for all solid complexes studied. Regarding the effect of CD type, HP-β-CD had the highest DL value for

Table 2. Dehydration yield (DY), encapsulation efficiency (EE), drug load (DL) and wettability of solid complexes obtained by freeze drying (FD) and spray drying (SD) for carvacrol and thymol encapsulated with HP-α-CD, HP-β-CD and HP-γ-CD

		DY (%)		EE (%)			DL (mg g^{-1})			Wettability (s)		
Compound	CD	FD	SD	FD	SD	SS	FD	SD	SS	FD	SD	SS
Carvacrol	HP-α-CD	92.3	77.6	96.5 ± 0.2^{a}	88.8 ± 3.2^{b}	**	59.0 ± 0.1^{b}	53.6 ± 1.9^{b}	**	$<1^{a}$	22.3 ± 1.5^{a}	**
	HP-β-CD	89.8	79.5	98.1 ± 1.7^{a}	81.6 ± 7.7^{b}	*	72.5 ± 1.2^{c}	59.4 ± 5.6^{b}	*	$<1^{a}$	53.3 ± 5.5^{a}	**
	HP-γ-CD	94.1	81.9	95.7 ± 1.7^{a}	64.9 ± 3.5^{a}	**	40.9 ± 0.7^{a}	28.1 ± 1.5^{a}	**	$<1^{a}$	145.3 ± 27^{b}	**
Thymol	HP-α-CD	90.0	77.5	88.3 ± 1.6^{a}	79.8 ± 2.0^{c}	**	54.8 ± 1.0^{b}	49.2 ± 1.2^{b}	**	$<1^{a}$	24.3 ± 4.5^{a}	**
	HP-β-CD	92.3	79.6	99.2 ± 0.1^{b}	75.2 ± 0.5^{b}	**	73.07 ± 0.1^{c}	54.5 ± 0.4^{c}	**	$<1^{a}$	67.3 ± 4.0^{b}	**
	HP-γ-CD	97.7	85.0	94.3 ± 5.7^{ab}	70.2 ± 1.0^{a}	**	39.5 ± 2.4^{a}	29.9 ± 0.4^{a}	**	$<1^{a}$	156.7 ± 28.4^{c}	**

In each column, the statistical difference between means of HP-α-, HP-β- and HP-γ-CD for each compound is shown ($P < 0.05$) (a–c).
Statistical significance (SS) between FD and SD: **$P < 0.01$; *$P < 0,05$; ns, not significant.
Values represent means of triplicate determination (± standard deviation).

both carvacrol and thymol, and for both the freeze-drying and spray-drying methods. From a toxicological and economic point of view, increasing DL can be advantageous for the bulk formulation and bioavailability of active material.[36] However, it should be noted that when complexes were prepared with HP-α-CD and spray-dried, the lower compound:CD molar ratio and the adequate EE enabled HP-α-CD to have a DL close to that of HP-β-CD, with no statistical differences ($P > 0.05$) between these CDs for carvacrol solid complexes.

One of the physical characteristics of solid complexes that was most affected by the dehydration method was wettability (Table 2). This parameter is affected by various factors such as particle size, water content and crystalline structure. All solid complexes dehydrated by freeze drying had a wettability time of under 1 s, without any observable difference between CDs or compounds. However, spray-dried complexes had a much higher wettability time. The difference in wettability behavior between dehydration methods is probably related to the solubilization capacity associated with the different crystal and particle structures that result from using a specific procedure. Figure 2 shows the SEM images of the particles obtained by freeze drying (Figs 2(A) and (B)) and spray drying (Figs 2(C) and (D)) for the solid complexes of carvacrol with HP-β-CD. These images were representative of the other solid complexes obtained, regardless of CD type or encapsulated compound. One can observe that the particles of the freeze-dried solid complexes were composed of crystals with angular, sharp shapes and straight edges. Similar shapes were obtained by Tao *et al.*[25] for thymol and β-CD complexes. Conversely, solid complexes dehydrated by the spray-drying method showed the typical wrinkled globular structure of particles obtained through this procedure.[37] The capacity of water to interact with the crystalline structure of freeze-dried complexes could be closely related to the short wettability times, which cause an instantaneous dissolution of the lyophilized solid complexes in water. On the other hand, the use of high temperatures in the spray-drying process allows for a semipermeable membrane to be formed on the surface of the particles, which could slow down the powder wettability.[4]

Antimicrobial activity of solid complexes

Antimicrobial activity of the solid complexes was initially monitored using the inhibition halo technique, for *E. coli* O157:H7 Sakai as a representative Gram-negative bacterium and for *S. aureus* USA300 as a representative Gram-positive type. This technique has been previously used to evaluate the antimicrobial activity of EOs encapsulated in CDs.[9,10] In view of their high EE and DL, freeze-dried solid complexes were selected to carry out a preliminary evaluation of the antimicrobial activity of carvacrol and thymol complexed in modified CDs. Table 3 presents the results of the inhibition halos of freeze-dried solid complexes of carvacrol and thymol encapsulated with HP-α-, HP-β- and HP-γ-CD against *E. coli* O157:H7 Sakai and against *S. aureus* USA300. The use of this basic technique revealed the relevance of CD type for the antimicrobial activity of carvacrol and thymol. As evident from Table 3, inhibition halos for both *E. coli* O157:H7 Sakai and *S. aureus* USA300 were significantly higher ($P < 0.05$) for both HP-α-CD and HP-γ-CD than for HP-β-CD. In order to analyze the interactions between the microorganisms and the complexes in further detail, we carried out MIC and MBC determinations.

Table 4 presents the MIC and MBC values for *E. coli* O157:H7 Sakai and for *S. aureus* USA300 of carvacrol and thymol free and encapsulated in HP-α-, HP-β- and HP-γ-CD. MIC values of free

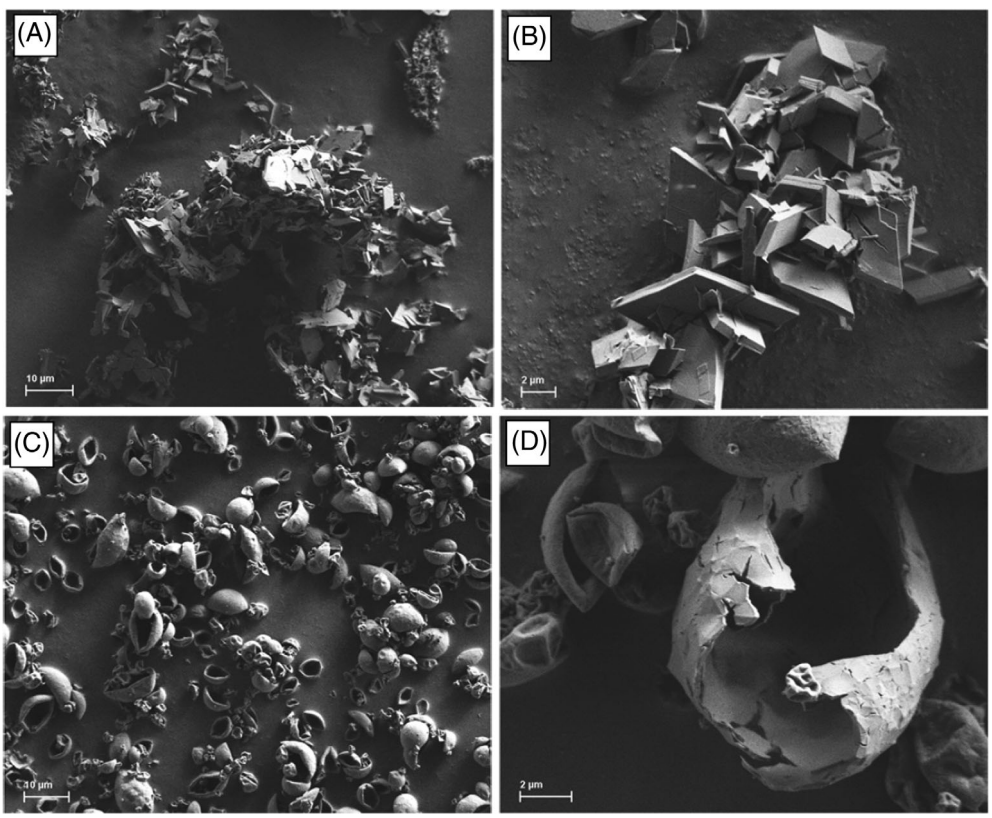


Figure 2. SEM images of (A, B) freeze-dried and (C, D) spray-dried complexes of carvacrol in HP-β-CD.

Table 3. Inhibition halos for carvacrol and thymol against *E. coli* O157:H7 Sakai and *S. aureus* USA300 encapsulated in HP-α-, HP-β- and HP-γ-CD and dehydrated by freeze-drying (disk diameter: 6.0 mm)

Compound	Encapsulation	Inhibition halo (mm)	
		E. coli	*S. aureus*
Carvacrol	HP-α-CD	12.7 ± 0.4[b]	12.0 ± 0.6[c]
	HP-β-CD	6.4 ± 0.4[a]	7.1 ± 0.5[a]
	HP-γ-CD	11.8 ± 0.4[b]	10.4 ± 0.1[b]
Thymol	HP-α-CD	13.7 ± 0.3[c]	13.8 ± 0.8[c]
	HP-β-CD	8.9 ± 0.5[a]	8.2 ± 0.1[a]
	HP-γ-CD	11.3 ± 0.1[b]	11.8 ± 0.5[b]

In each column, the statistical difference between means of HP-α-, HP-β- and HP-γ-CD for each compound is shown ($P < 0.05$) (a–c).
Values represent means of triplicate determination (± standard deviation).

compounds ranged from 150 to 200 mg L^{-1} and MBC values from 200 to 250 mg L^{-1}. These values are similar to those previously reported in the literature,[38–41] but lower than those of some other previous research works.[16,17,20] When carvacrol and thymol were encapsulated, antimicrobial activity was reduced to a greater or lesser extent as a function of the type of CD used for encapsulation. For freeze-dried complexes, carvacrol and thymol encapsulation in HP-α-CD and HP-γ-CD maintained the antimicrobial activity against *E. coli* O157:H7 Sakai and *S. aureus* USA300 very close to that exhibited by the free compounds, increasing MIC and MBC values only by 50 mg L^{-1} for most cases studied. However, when both compounds were encapsulated in HP-β-CD, antimicrobial activity was clearly reduced. The freeze-dried complexes presented MIC values four and two times higher than free carvacrol and thymol, respectively. Regarding bactericidal activity, the MBC values of HP-β-CD freeze-dried complexes were four and three times higher than those of free compounds, exceeding, in some cases, the maximum tested concentration of 1000 mg L^{-1}.

Many authors have previously studied the effect of CD encapsulation on the biological properties, mainly the antimicrobial and antioxidant activity, of EOs and/or their components. However, conclusive results cannot be reached. In contrast with our results, Kamimura *et al.*[16] and Santos *et al.*[17] observed a significant increase in the antimicrobial activity of carvacrol encapsulated in HP-β-CD and β-CD against *E. coli* K12 and *S. typhimurium* LT2 with respect to the free compound, although encapsulation conversely reduced antioxidant capacity. Rodríguez-López *et al.*[20] also concluded that encapsulation in HP-β-CD improved the antimicrobial capacity of carvacrol and thymol against *E. coli* CECT 943 and *S. aureus* CECT 239. Similar conclusions have likewise been reached by other authors.[3,14] On the other hand, Anaya-Castro *et al.*[9] observed that encapsulation of Mexican oregano EO in β-CD did not significantly affect its antimicrobial activity, and Dima *et al.*[10] reported a decrease in the antimicrobial and antioxidant capacity of coriander EO encapsulated in β-CD with respect to free EO. Regarding other biological features such as antifungal activity, del Toro-Sánchez *et al.*[11] observed that the encapsulation of thyme EO in β-CD reduced its antifungal capacity, and Kfoury *et al.*[42] determined that the antifungal activity of phenylpropanoids was reduced when encapsulated in HP-β-CD. This disparity in the results of previous studies has already been indicated by Kfoury *et al.*, who concluded that the effect of encapsulation in CDs on the biological activity of aromatic compounds can present effects that do not always go in the same direction.[21] One of the reasons that could explain this disparity in results may be related to the use of different methodologies for the characterization of antimicrobial activity of free compounds, which can lead to a wide range of reported MIC and MBC values. Based on previous work, the methodology used in this study only applied vigorous shaking as a method to ensure sufficient dispersion of the terpene in the culture medium.[43] Using this methodology, values of MIC and MBC obtained in this study were in accord with those reported in previous studies.[38–41] However, when additional actions were carried out to disperse the terpenes in the culture medium, such as the use of surfactants, the values of MIC and MBC of the terpenes in the free state were higher.[16,17,20] As a result, those authors observed a significant increase in the antimicrobial activity of encapsulated compounds, while those who reported lower MIC and MBC values for free compounds, as concluded in this study, affirmed that efficiency was maintained or decreased.[9,10]

Taking into account the method used for antimicrobial activity determination in this study, this research demonstrated that encapsulation in CDs reduces, to a greater or lesser extent, the antimicrobial capacity of carvacrol and thymol, proving that the stability of the complexes, defined as the K_c values and dependent on the type of CD, can modulate these compounds' antimicrobial activity. The highest K_c value, which was determined for the

Table 4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of carvacrol and thymol, free or encapsulated in HP-α-CD, HP-β-CD and HP-γ-CD using the freeze-drying (FD) and spray-drying (SD) methods, against *E. coli* O157:H7 Sakai and *S. aureus* USA300

Compound	Encapsulation	MIC (mg L^{-1})				MBC (mg L^{-1})			
		E. coli		*S. aureus*		*E. coli*		*S. aureus*	
		FD	SD	FD	SD	FD	SD	FD	SD
Carvacrol	Free	150		200		200		250	
	HP-α-CD	200	600	250	600	250	900	450	900
	HP-β-CD	700	>1000	700	>1000	>1000	>1000	>1000	>1000
	HP-γ-CD	250	250	250	300	250	500	450	600
Thymol	Free	150		150		250		250	
	HP-α-CD	200	500	200	500	250	500	350	800
	HP-β-CD	350	750	400	700	650	800	900	>1000
	HP-γ-CD	200	300	200	300	300	350	450	600

carvacrol–HP-β-CD complex (2268.1 L mol^{-1}; Table 1), indicates a greater stability of carvacrol–HP-β-CD but a lower amount of release of the antimicrobial compound. As a consequence, the antimicrobial activity of such compounds would decrease, as demonstrated by the highest MIC and MBC values obtained for the freeze-dried HP-β-CD complexes. Furthermore, it was determined that encapsulation of carvacrol and thymol in HP-α- and HP-γ-CD, not previously studied in the literature, provided a lower K_c value than HP-β-CD (Table 1), thereby allowing a more efficient release of the compounds while scarcely affecting their antibacterial activity.

Regarding the effect of the type of dehydration method used to obtain solid complexes, it was observed that spray drying reduced the antimicrobial activity of carvacrol and thymol in relation to the antimicrobial activity of freeze-dried complexes. The MIC and MBC values (Table 4) of HP-α-CD complexes were, on average, 160% and 147% higher for spray-dried than for freeze-dried complexes, respectively. For carvacrol–HP-β-CD complexes, the MIC values increased from 700 mg L^{-1} (freeze-dried) to over 1000 mg L^{-1} (spray-dried) for both bacteria, whereas for thymol–HP-β-CD complexes the MIC values were, on average, 95% higher for spray-dried than for freeze-dried complexes. The reduction in antimicrobial activity of spray-dried complexes may be related to the high temperatures to which such complexes are subjected during the dehydration process, thereby negatively affecting the antimicrobial capacity of the antibacterial compounds,[44] as well as to the lower ease of water dissolution of spray-dried complexes as shown by their degree of wettability (Table 2), which could hamper the interaction of antimicrobial compounds with bacteria. However, HP-γ-CD has been shown to better protect the antimicrobial capacity of carvacrol and thymol after spray drying than HP-α-CD or HP-β-CD. In the case of HP-γ-CD spray-dried complexes, the loss of antimicrobial activity resulted in an increase of MIC values lying between 0 and 50 mg L^{-1} for carvacrol against *E. coli* O157:H7 Sakai and against *S. aureus* USA300, respectively, and corresponding to 100 mg L^{-1} for thymol against both microorganisms. On the one hand, the K_c values of 365.7 and 239.7 L mol^{-1} for carvacrol and thymol with HP-γ-CD (Table 1) may be high enough to protect the compounds during the dehydration process, but, on the other hand, these K_c values were still sufficiently low to allow for fast release of the compound when it was rehydrated. In addition, the S_t/S_0 values of HP-γ-CD were significantly higher than those of HP-α-CD, allowing for better water dissolution of antimicrobial compounds and thus improving their antimicrobial capacity.

CONCLUSIONS

This research shows that modified HP-α-CD and HP-γ-CD represent very suitable alternatives for carvacrol and thymol encapsulation, since they afford complexes with a lower K_c value and release the compounds more readily, thus leading to bacteriostatic and bactericidal activities significantly higher than those for the complexes with HP-β-CD that are normally used for the encapsulation of these compounds. In this regard, it was observed that the HP-β-CD encapsulation of carvacrol and thymol markedly reduces their antimicrobial activity. Therefore, in order to consider the use of HP-β-CD for the encapsulation of this type of compound, it would be necessary to study mechanisms that activate the release of the host molecule from the inner cavity of the CD: procedures such as combined treatments with changes in pH and/or temperature. On the other hand, freeze drying is the preferable alternative to obtain solid complexes with the purpose of maintaining the antimicrobial activity of carvacrol and thymol after encapsulation, although the spray-drying method, mainly combined with HP-γ-CD, allows for obtaining solid complexes that maintain an antimicrobial activity of a level comparable to that displayed by compounds in a free state.

ACKNOWLEDGEMENTS

This study was financially supported by MINECO (Spanish Ministry of Economy and Competitiveness, project no. PGC2018-093789-B-I00), by the European Social Fund and by the Aragonese Office of Science, Technology and University Research.

CONFLICT OF INTEREST

The authors declare that this research was carried out in the absence of commercial or financial relationships that could be construed as a possible conflict of interest.

REFERENCES

1 Rivera J, Crandall PG, O'Bryan CA and Ricke SC, Essential oils as antimicrobials in food systems – a review. *Food Control* **54**:111–119 (2015). https://doi.org/10.1016/j.foodcont.2014.12.040.
2 Ju J, Chen X, Xie Y, Yu H, Gou Y, Cheng Y *et al.*, Application of essential oils as a sustained release preparation in food packaging. *Trends Food Sci Technol* **92**:22–32 (2019). https://doi.org/10.1016/j.tifs.2019.08.005.
3 Hill LE, Gomes C and Taylor TM, Characterization of beta-cyclodextrin inclusion complexes containing oils (*trans*-cinnamaldehyde, eugenol, cinnamon bark, and clove bud extracts) for antimicrobial delivery applications. *LWT–Food Sci Technol* **51**:86–93 (2013). https://doi.org/10.1016/j.lwt.2012.11.011.
4 Alvarenga D, de Barros RV and Vilela S, Microencapsulation of essential oils using spray drying technology, in *Microencapsulation and Microspheres for Food Applications*, ed. by Sagis LMC. Academic Press, Cambridge, MA, pp. 235–231 (2015). https://doi.org/10.1016/B978-0-12-800350-3.00013-3.
5 el Asbahani A, Miladi K, Badri W, Sala M, Aït Addi EH, Casabianca H *et al.*, Essential oils: from extraction to encapsulation. *Int J Pharm* **483**:220–243 (2015). https://doi.org/10.1016/j.ijpharm.2014.12.069.
6 Hasheminejad N, Khodaiyan F and Safari M, Improving the antifungal activity of clove essential oil encapsulated by chitosan nanoparticles. *Food Chem* **275**:113–122 (2019). https://doi.org/10.1016/j.foodchem.2018.09.085.
7 Merino N, Berdejo D, Bento R, Salman H, Lanz M, Maggi F *et al.*, Antimicrobial efficacy of *Thymbra capitata* (L.) Cav. essential oil loaded in self-assembled zein nanoparticles in combination with heat. *Ind Crop Prod* **113**:98–104 (2019). https://doi.org/10.1016/j.indcrop.2019.03.003.
8 Astray G, Gonzalez-Barreiro C, Mejuto JC, Rial-Otero R and Simal-Gándara J, A review on the use of cyclodextrins in foods. *Food Hydrocolloids* **23**:1631–1640 (2009). https://doi.org/10.1016/j.foodhyd.2009.01.001.
9 Anaya-Castro MA, Ayala-Zavala JF, Muñoz-Castellanos L, Hernández-Ochoa L, Peydecastaing J and Durrieu V, β-Cylodextrin inclusion complexes containing clove (*Eugenia caryophyllata*) and Mexican oregano (*Lippia berlandieri*) essential oils: preparation, physicochemical and antimicrobial characterization. *Food Packag Shelf* **14**:96–101 (2017). https://doi.org/10.1016/j.fpsl.2017.09.002.
10 Dima C, Cotarlet M, Tiberius B, Hahrim G, Alexe P and Dima S, Encapsulation of coriander essential oil in beta-cyclodextrin: antioxidant and antimicrobial properties evaluation. *Rom Biotechnol Lett* **19**:9128–9140 (2014) http://e-repository.org/rbl/vol.19/iss.2/4.pdf.
11 del Toro-Sánchez CL, Ayala-Zavala JF, Machi L, Santacruz H, Villegas-Ochoa MA, Álvarez-Parrilla E *et al.*, Controlled release of antifungical volatiles of thyme essential oil from β-cyclodextrin capsules. *J Inclusion Phenom Macrocyclic Chem* **67**:431–441 (2010) https://link.springer.com/article/10.1007/s10847-009-9726-3.
12 Kfourly M, Auezova L, Ruellan S, Greige-Gerges H and Fourmentin S, Complexation of estragole as pure compound and as main

component of basil and tarragon essential oils with cyclodextrins. *Carbohydr Polym* **118**:156–164 (2015). https://doi.org/10.1016/j.carbpol.2014.10.073.
13 Martins AP, Craveiro AA, Machado MIL, Raffin FN, Moura TF, Novák CS *et al*., Preparation and characterization of *Mentha x Villosa* Hudons oil-*β*-cyclodextrin complex. *J Thermal Anal* **88**:363–371 (2007). https://link.springer.com/article/10.1007/s10973-005-7407-z.
14 Rakmai J, Cheirsilp B, Mejuto JC, Simal-Gándara J and Torrado-Agrasar A, Antioxidant and antimicrobial properties of encapsulated guava leaf oil in hydroxypropyl-beta-cyclodextrin. *Ind Crop Prod* **111**:219–225 (2018).
15 Suprani C, Gonçalves S, Diirr L, Oliveira JC, Fontes P, Moreira D *et al*., *β*-Cyclodextrin inclusion complexes with essential oils: obtention, characterization, antimicrobial activity and potential application for food preservative sachets. *Food Res Int* **119**:499–509 (2019). https://doi.org/10.1016/j.foodres.2019.01.016.
16 Kamimura JA, Santos EH, Hill LE and Gomes CL, Antimicrobial and antioxidant activities of carvacrol microencapsulated in hydroxypropyl-beta-cyclodextrin. *LWT–Food Sci Technol* **57**:701–709 (2014). https://doi.org/10.1016/j.lwt.2014.02.014.
17 Santos EH, Kamimura JA, Hill LE and Gomes CL, Characterization of carvacrol beta-cyclodextrin inclusion complexes as delivery systems for antibacterial and antioxidant applications. *LWT–Food Sci Technol* **60**:583–592 (2015). https://doi.org/10.1016/j.lwt.2014.08.046.
18 Kfoury M, Landy D, Ruellan S, Auezova L, Greige-Gerges H and Fourmentin S, Determination of formation constants and structural characterization of cyclodextrin inclusion complexes with two phenolic isomers: carvacrol and thymol. *J Org Chem* **12**:29–42 (2016) https://www.beilstein-journals.org/bjoc/articles/12/5.
19 Rodríguez-López MI, Mercader-Ros MT, López-Miranda S, Pellicer JA, Pérez-Garrido A, Pérez-Sánchez H *et al*., Thorough characterization and stability of HP-*β*-cyclodextrin thymol inclusion complexes prepared by microwave technology: a required approach to a successful application in food. *J Sci Food Agr* **99**:1322–1333 (2018). https://doi.org/10.1002/jsfa.9307.
20 Rodríguez-López MA, Mercader-Ros MT, Pellicer JA, Gómez-López V, Martínez-Romero D, Núñez-Delicado E *et al*., Evaluation of monoterpene-cyclodextrin complexes as bacterial growth effective hurdles. *Food Control* **108**:106814 (2020). https://doi.org/10.1016/j.foodcont.2019.106814.
21 Kfourly M, Auezova L, Greige-Gerges H and Fourmentin S, Cyclodextrin for essential oils applications. I cyclodextrin application, in *Cyclodextrin Applications in Medicine, Food, Environment and Liquid Crystals*, ed. by Fourmentin S, Crini S and Lichtfouse E. Springer, Berlin, pp. 81–123 (2018). https://doi.org/10.1007/978-3-319-76162-6_4.
22 Kfoury M, Landy D and Fourmentin S, Characterization of cyclodextrin/volatile inclusion complexes: a review. *Molecules* **23**:1204 (2018). https://doi.org/10.3390/molecules23051204.
23 Rakmai J, Cheirsilp B, Cid A, Torrado-Agrasar A, Mejuto JC and Simal-Gandara J, Encapsulation of essential oils by cyclodextrins: characterization and evaluation, in *Cyclodextrin: A Versatile Ingredient*, ed. by Aroa P and Dhingra N. IntechOpen, London, pp. 236–290 (2018). https://doi.org/10.5772/intechopen.73589.
24 Lima P, Lucchese A, Araújo-Filho H, Menezes P, Araújo A, Quintans-Júnior L *et al*., Inclusion of terpenes in cyclodextrins: preparation, characterization and pharmacological approaches. *Carbohydr Polym* **151**:965–987 (2016). https://doi.org/10.1016/j.carbpol.2016.06.040.
25 Tao F, Hill LE, Peng Y and Gomes CL, Synthesis and characterization of *β*-cyclodextrin inclusion complexes of thymol and thyme oil for antimicrobial delivery applications. *LWT–Food Sci Technol* **59**:247–255 (2014). https://doi.org/10.1016/j.lwt.2014.05.037.
26 Higuchi T and Connors KA, Phase solubility techniques. *Adv Anal Chem Instrum* **4**:56–63 (1965).
27 Kim SH, Lee SR, Kim KS, Ko A, Kim E, Kim YH *et al*., Shiga toxin a subunit mutant of *Escherichia coli* O157:H7 releases outer membrane vesicles containing the B-pentameric complex. *FEMS Immunol Med Microbiol* **58**:412–420 (2010). https://doi.org/10.1111/j.1574-695X.2010.00654.x.
28 Kreiswirth B, Kornblum J, Arbeit RD, Eisner W, Maslow JN, McGeer A *et al*., Evidence for a clonal origin of methicillin resistance in *Staphylococcus aureus*. *Science* **259**:227–230 (1993). https://doi.org/10.1126/science.8093647.
29 Meena MR and Sethi V, Antimicrobial activity of essential oils from spices. *J Food Sci Technol Mysore* **31**:68–70 (1994).
30 Kfoury M, Auezova L, Fourmentin S and Greige-Gerges H, Investigation of monoterpenes complexation with hydroxipropyl-*β*-cyclodextrin. *J Inclusion Phenom Macrocyclic Chem* **80**:51–60 (2014) https://link.springer.com/article/10.1007/s10847-014-0385-7.
31 Carrier RL, Miller LA and Ahmed I, The utility of cyclodextrins for enhancing oral bioavailability. *J Control Release* **123**:78–99 (2007). https://doi.org/10.1016/j.jconrel.2007.07.018.
32 Loftsson T, Hreinsdóttir D and Másson M, The complexation efficiency. *J Inclusion Phenom Macrocyclic Chem* **57**:545–552 (2007) https://link.springer.com/article/10.1007/s10847-006-9247-2.
33 Fang Z and Bhandari B, Effect of spray drying and storage on the stability of bay berry polyphenols. *Food Chem* **129**:1139–1147 (2011). https://doi.org/10.1016/j.foodchem.2011.05.093.
34 Tontul I and Topuz A, Spray-drying of fruit and vegetable juices: effect of drying conditions on the product yield and physical properties. *Trends Food Sci Technol* **63**:91–102 (2017). https://doi.org/10.1016/j.tifs.2017.03.009.
35 de Barros RV, Borges SV and Botrel DA, Gum Arabic/starch/maltodextrin/inulin as wall materials on the microencapsulation of rosemary essential oil. *Carbohydr Polym* **101**:524–532 (2014). https://doi.org/10.1016/j.carbpol.2013.09.083.
36 Loftsson T, Hreinsdottir D and Masson M, Evaluation of cyclodextrin solubilization of drugs. *Int J Pharm* **302**:18–28 (2005). https://doi.org/10.1016/j.ijpharm.2005.05.042.
37 Wang S, Shi Y and Han L, Development and evaluation of microencapsulated peony seed oil prepared by spray drying: oxidative stability and its release behavior during in-vitro digestion. *J Food Eng* **231**:1–9 (2018). https://doi.org/10.1016/j.jfoodeng.2018.03.007.
38 Ait-Ouazzou A, Cherrat L, Espina L, Lorán S, Rota C and Pagán R, The antimicrobial activity of hydrophobic essential oil constituents acting alone or in combined processes of food preservation. *Innov Food Sci Emerg Technol* **12**:320–329 (2011). https://doi.org/10.1016/j.ifset.2011.04.004.
39 Espina L, Pagán R, López D and García-Gonzalo D, Individual constituents from essential oils inhibit biofilm mass production by multidrug resistant *Staphylococcus aureus*. *Molecules* **20**:11357–11372 (2015). https://doi.org/10.3390/molecules200611357.
40 Chueca B, Berdejo D, Gomes-Neto NJ, Pagán R and García-Gonzalo D, Emergence of hyper-resistant *Escherichia coli* MG1655 derivative strains after applying sub-inhibitory doses of individual constituents of essential oils. *Front Microbiol* **2016**:273 (2016). https://doi.org/10.3389/fmicb.2016.00273.
41 García-Salinas S, Elizondo-Castillo H, Arruebo M, Mendoza G and Irusta S, Evaluation of the antimicrobial activity and cytotoxicity of different components of natural origin present in essential oils. *Molecules* **23**:1–18 (2018). https://doi.org/10.3390/molecules23061399.
42 Kfoury M, Sahraoui A, Bourbon N, Laruelle F, Fontaine J, Auezova L *et al*., Solubility, photostability and antifungal activity of phenylpropanoids encapsulated in cyclodextrins. *Food Chem* **196**:518–525 (2016). https://doi.org/10.1016/j.foodchem.2015.09.078.
43 Friedman M, Henika PR and Mandrell RE, Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J Food Prot* **65**:1545–1560 (2002). https://doi.org/10.4315/0362-028X-65.10.1545.
44 dos Santos R, da Silva RA, Corso MP and Canan C, Essential oils microencapsulated obtained by spray drying: a review. *J Essent Oil Res* **31**:457–473 (2019). https://doi.org/10.1080/10412905.2019.1612788.

5. Discusión general

5. Discusión general

Aunque la discusión de los resultados se ha realizado en profundidad en cada uno de los manuscritos presentados, esta sección pretende ofrecer una visión global de toda la investigación realizada a lo largo de la Tesis Doctoral. La discusión se presenta dividida en 6 secciones que se corresponden con cada uno de los objetivos planteados: 1) Puesta a punto de los ensayos de evolución para el aislamiento de variantes genéticas resistentes; 2) Efecto mutagénico por la exposición a AE y CI; 3) Caracterización fenotípica de las variantes genéticas resistentes; 4) Caracterización genotípica de las variantes resistentes e identificación de las modificaciones genéticas responsables de los cambios observados en su fenotipo; 5) Evaluación de la resistencia de las variantes genéticas en alimentos modelo para explorar su repercusión en la industria agroalimentaria; y 6) Preparación de nanoemulsiones y encapsulados de AE y CI para facilitar y potenciar su empleo como conservantes alimentarios y biocidas.

5.1. Puesta a punto de los ensayos de evolución para el aislamiento variantes genéticas resistentes

El estudio de variantes genéticas bacterianas ha supuesto una herramienta muy útil y eficaz en la descripción de la aparición de resistencias frente a antibióticos, así como para elucidar sus mecanismos de acción. Es por ello que existen varios protocolos descritos en este campo para evaluar el desarrollo de resistencias basadas en cambios genotípicos a través de su empleo continuado a concentraciones subletales (Andersson & Hughes, 2014; Kohanski, DePristo, & Collins, 2010) o letales (Levin-Reisman et al., 2017). De tal modo, se ha propuesto la realización de este tipo de estudios para profundizar en los mecanismos de inhibición e inactivación bacteriana frente a métodos de conservación de alimentos (Collins, Curtis, Cotter, Hill, & Ross, 2010; Gayán, Cambré, Michiels, & Aertsen, 2016; Guo, Winkler, & Kao, 2017; Kawada-Matsuo et al., 2020; Kumariya et al., 2019; Liu, Ream, Joerger, Liu, & Wang, 2011; Metselaar, den Besten, Abee, Moezelaar, & Zwietering, 2013; Sagarzazu, Cebrian, Pagan, Condon, & Manas, 2013; Sanz-Puig et al., 2019; Selveshwari, Lele, & Dey, 2021; Van Boeijen, Francke, Moezelaar, Abee, & Zwietering, 2011; Vanlint et al., 2011).

En el campo de la tecnología de los alimentos los métodos de conservación se pueden clasificar en dos grupos: los métodos que persiguen la inhibición o retraso de la proliferación microbiana de alterantes y patógenos (Montville & Matthews, 2013) y, por

otro lado, aquellos que buscan la inactivación de los microorganismos presentes en el alimento (Mañas & Pagán, 2005). Únicamente el uso de conservantes químicos permite abordar al mismo tiempo ambas estrategias, al depender su actividad bacteriostática o bactericida de la concentración de antimicrobiano utilizada. Es por ello que, para la puesta a punto de la metodología requerida para llevar a cabo ensayos de evolución se optó por el uso de antimicrobianos naturales, concretamente de AE y CI, dado que, hasta la fecha, los trabajos descritos en la bibliografía habían planteado la duda de que el uso de estas sustancias naturales pudiera favorecer el aislamiento y selección de variantes genéticas resistentes (de Souza, 2016). Una vez implementada la metodología con estas sustancias, puede ser fácilmente adaptada para el estudio de otros métodos de conservación. En este sentido, se diseñaron protocolos de ensayos de evolución A) mediante la exposición prolongada a concentraciones subinhibitorias (Fig. 5.1A) o B) tras la aplicación de tratamientos letales (Fig. 5.1B) de dichos antimicrobianos naturales, con el objetivo de crear herramientas para el aislamiento de variantes genéticas resistentes cuyo estudio permita profundizar en los mecanismos de inhibición e inactivación de los métodos de conservación (**Manuscrito II**).

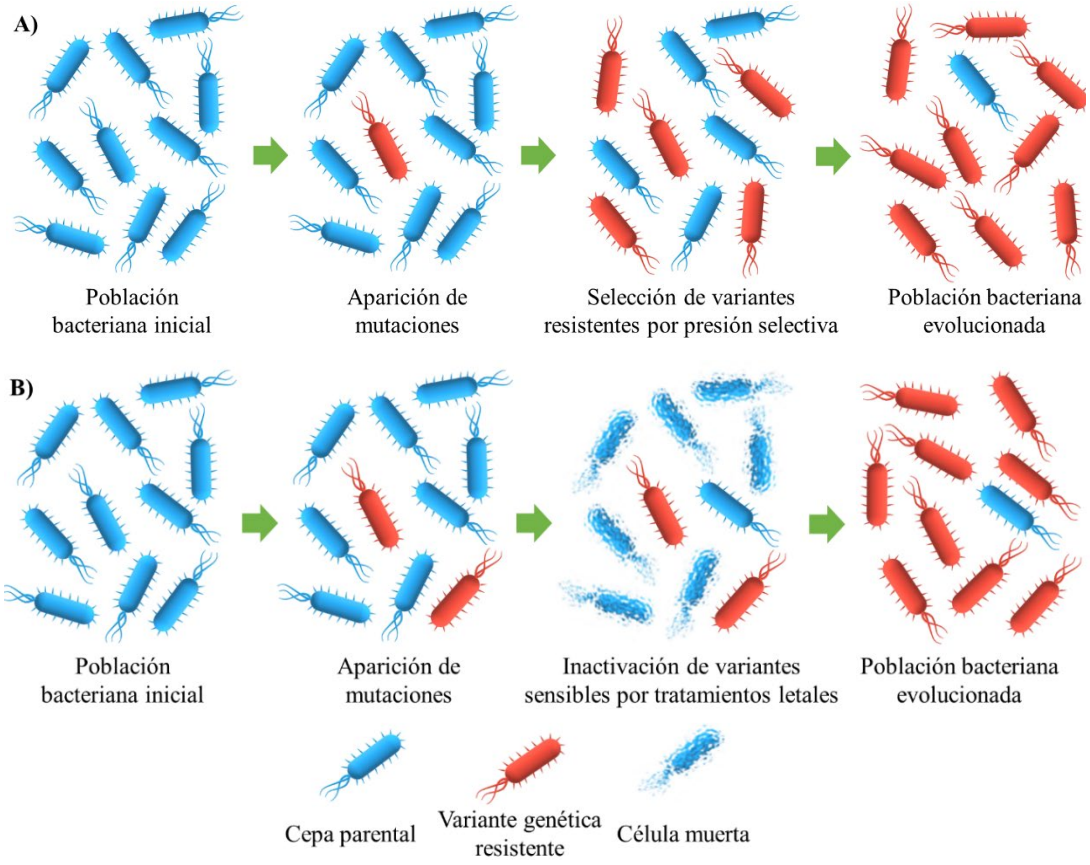


Figura 5.1. Esquemas de los ensayos de evolución mediante la exposición prolongada a concentraciones subinhibitorias o B) tras la aplicación de tratamientos letales.

A través de esta metodología se aislaron numerosas variantes genéticas resistentes (Tabla. 5.1) que fueron posteriormente caracterizadas fenotípica y genotípicamente, como se discute en los apartados 5.3 y 5.4.

Tabla 5.1. Variantes genéticas resistentes aisladas A) mediante la exposición prolongada a concentraciones subinhibitorias o B) tras la aplicación de tratamientos letales.

Manuscrito	Cepa bacteriana	Compuesto antimicrobiano	Ensayo de evolución	Variante genética resistente
III	*Staphylococcus aureus* USA300	Carvacrol	A)	SaCar
		Citral	A)	SaCit
		Óxido de limoneno	A)	SaOxLim
V		AE de naranja	A)	SaROEO
IV	*Salmonella enterica* Typhimurium LT2	Carvacrol	A)	SeSCar
		Carvacrol	B)	SeLCar
VII		AE de tomillo	B)	SeTCO
VI	*Listeria monocytogenes* EGD-e	AE de tomillo	A)	LmSTCO
		AE de tomillo	B)	LmLTCO

5.2. Efecto mutagénico por la exposición a AE y CI

Actualmente, se conoce que la exposición bacteriana a concentraciones subletales de antibióticos induce el desarrollo de resistencias, por ejemplo, frente a fluoroquinolonas y β-lactámicos. Los tratamientos con antibióticos aumentan la producción de especies reactivas de oxígeno (ROS) a nivel intracelular que dañan el DNA y activan la respuesta SOS y el sistema RecA de reparación del daño al DNA (Kohanski et al., 2010; Kohanski, Dwyer, Hayete, Lawrence, & Collins, 2007), incrementado la frecuencia mutagénica y, acelerando así, la heterogeneidad en la población y la aparición de variantes genéticas resistentes (Andersson & Hughes, 2014). Por tanto, una vez obtenidas variantes resistentes tras los ensayos de evolución frente a AE y CI, nos preguntamos si esas variantes habían sido inducidas por el uso de estos compuestos o, por el contrario, habían sido fruto de un proceso de selección natural basado en mutaciones aleatorias que emergen por la presión selectiva ejercida por estos compuestos.

La determinación de la frecuencia mutagénica, a través del estudio de la generación de resistencia frente a rifampicina por mutaciones espontáneas en el gen *rpoB* (Gutierrez et al., 2013), reveló que tanto la exposición a CI, como el carvacrol, citral y el óxido de

limoneno (**Manuscrito III**), como a AE, como el de naranja (**Manuscrito V**), a dosis subinhibitorias, no incrementó la frecuencia mutagénica en *S. aureus* (Fig. 5.2).

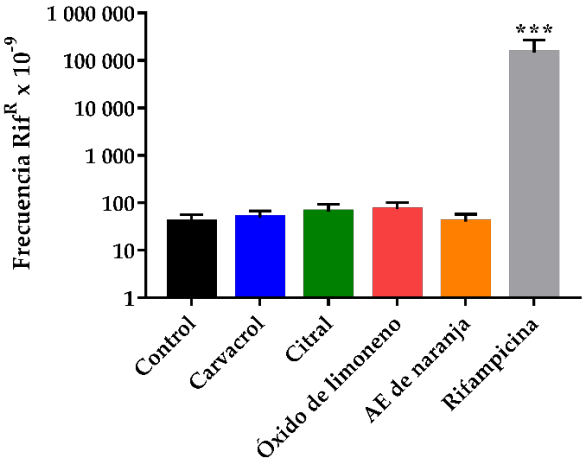


Figura 5.2. Frecuencia mutagénica en S. *aureus* crecido en ausencia (■) y presencia de carvacrol (50 µL/L; ■), citral (75 µL/L; ■), óxido de limoneno (375 µL/L; ■), AE de naranja (750 µL/L; ■) y rifampicina (0.01 mg/L; ■), expresada en celulares resistentes a rifampicina en la población total bacteriana. ***: diferencias estadísticamente significativas ($p \leq 0.001$) con respecto al control.

De acuerdo a nuestros resultados, estudios anteriores también habían observado una baja tasa de mutación en *S. aureus* ATCC 25923 tras la exposición a AE del árbol de té (*Maleluca alternifolia*) durante el crecimiento bacteriano (Hammer, Carson, & Riley, 2008). Otros resultados en *E. coli* MG1655 (Chueca, Berdejo, Gomes-Neto, Pagán, & García-Gonzalo, 2016) también demostraron que la exposición a carvacrol, citral y óxido de limoneno no aumentaba la frecuencia de mutación con respecto al control (sin CI).

Aunque las ROS también están implicadas en el mecanismo de muerte bacteriana por el carvacrol, el citral y el óxido de limoneno (Chueca, Pagán, & García-Gonzalo, 2014a, 2014b), estos compuestos antimicrobianos indujeron una menor mutagénesis. Este hecho se ha atribuido a las propiedades antioxidantes de estos compuestos naturales a bajas concentraciones (Brewer, 2011; Hashemi, Khorram, & Sohrabi, 2017), que son capaces de aliviar el estrés oxidativo a través de la neutralización de ROS, una de las principales causas de daños en el DNA (Sakai, Nakanishi, Yoshiyama, & Maki, 2006), lo que conduciría a una disminución de la frecuencia de mutagénesis. No obstante, cabe destacar que estos antimicrobianos naturales a altas concentraciones también se han relacionado con un aumento del estrés oxidativo celular (Chueca et al., 2014b).

De tal modo, al contrario que con los antibióticos (Blázquez, 2003), la aparición de variantes resistentes de *S. aureus* no sería inducida directamente por la exposición al AE de naranja, sino por la presión selectiva que ejerce la presencia de antimicrobianos sobre

la población bacteriana durante su crecimiento. En otras palabras, el AE favorecería el crecimiento de la subpoblación de variantes genéticas que surgen a causa de mutaciones espontáneas y que muestran una mejor aptitud de crecimiento frente a la cepa parental (en presencia del AE), provocando un aumento progresivo de su presencia, y causando, en última instancia, la fijación de esas mutaciones en la población bacteriana.

5.3. Caracterización fenotípica de las variantes genéticas resistentes

Tras el aislamiento de las variantes bacterianas a través de los ensayos de evolución, se llevó a cabo su caracterización fenotípica y su comparación con la cepa parental para evaluar la variación de:

a) resistencia y tolerancia frente a los AE y CI usados en los ensayos de evolución,

b) resistencia cruzada frente a otras tecnologías de conservación, y

c) resistencia cruzada frente antibióticos.

A este respecto cabe destacar que, en el campo de los antimicrobianos, y más concretamente en el de los antibióticos, se diferencian dos tipos de respuesta bacteriana relacionadas con el concepto de resistencia. Mientras que el término "**resistencia**" se define como la capacidad de las bacterias para crecer en presencia de un compuesto antimicrobiano, se denomina "**tolerancia**" a la capacidad de las células bacterianas para sobrevivir a concentraciones letales del antimicrobiano (Balaban et al., 2019; Brauner, Fridman, Gefen, & Balaban, 2016). La resistencia bacteriana suele evaluarse mediante pruebas bacteriostáticas, como la prueba de CMI, mientras que la determinación de la tolerancia requiere una evaluación de la supervivencia frente a concentraciones letales, como la CMB (Fridman, Goldberg, Ronin, Shoresh, & Balaban, 2014). No obstante, cabe destacar que en el campo de la conservación de alimentos no se utilizan los términos resistencia y tolerancia con dicha connotación y, es por ello, que para el estudio de la supervivencia frente a tratamientos letales como el calor o los PEAV se utiliza el término resistencia cruzada.

5.3.1. *Resistencia y tolerancia directa frente a AE y CI*

En primer lugar, para confirmar el incremento de resistencia y tolerancia frente a los antimicrobianos naturales empleados en los ensayos de evolución, se determinaron la

CMI y CMB de las variantes bacterianas aisladas y se compararon con sus respectivas cepas parentales (Tabla 5.3).

Tabla 5.2. Concentración mínima inhibitoria (CMI; µL/L) y concentración mínima bactericida (CMB; µL/L) de AE y CI frente a las cepas parentales (WT) y sus respectivas variantes genéticas obtenidas tras los ensayos de evolución A) mediante la exposición prolongada a concentraciones subinhibitorias o B) tras la aplicación de tratamientos letales.

Manuscrito		**Compuesto antimicrobiano**	**Cepa parental (WT)**		**Variante genética**	
			CMI	**CMB**	**CMI**	**CMB**
III	SaCar	Carvacrol	100	-	150	-
	SaCit	Citral	150	-	250	-
	SaOxLim	Óxido de limoneno	750	-	1500	-
IV	SeSCar	Carvacrol	200	200	300	300
	SeLCar	Carvacrol	200	200	400	400
V	SaROEO	AE de naranja	1500	2500	>5000	>5000
VI	LmSTCO	AE de tomillo	150	200	200	250
	LmLTCO	AE de tomillo	150	200	300	400
VII	SeTCO	AE de tomillo	250	300	600	600

Los resultados demostraron que todas las variantes bacterianas aisladas independientemente de su especie, del compuesto antimicrobiano al que fueron expuestas o del protocolo de ensayo de evolución seguido para su aislamiento, incrementaron su resistencia y tolerancia directa frente al compuesto usado en el ensayo de evolución. La única excepción fue la variante de *S. aureus* aislada por exposición a concentraciones subinhibitorias de citral, que mostró una mayor sensibilidad frente a tratamientos letales de citral que la cepa parental (**Manuscrito III**). De tal modo, estos resultados lograron evidenciar por primera vez:

a) la aparición de variantes resistentes y tolerantes frente a CI en bacterias Gram-positivas (**Manuscrito III**),

b) el aislamiento de variantes resistentes y tolerantes frente a CI tras la aplicación de tratamientos letales (**Manuscrito IV**), y

c) el aislamiento de variantes resistentes y tolerantes frente a AE complejos, tanto en bacterias Gram-positivas (**Manuscrito V, VI**) como en Gram-negativas (**Manuscrito VII**), mediante ambos protocolos de evolución diseñados en la presente Tesis Doctoral (**Manuscrito II**).

Pese a que múltiples estudios han evaluado el desarrollo de resistencias y tolerancias directas frente a estos antimicrobianos naturales, e incluso cruzada frente a antibióticos, en bacterias como *S. aureus* (Apolonio, Faleiro, Miguel, & Neto, 2014; Gomes Neto, Luz Ida, et al., 2012; Luz et al., 2013; Thomsen, Hammer, Riley, Van Belkum, & Carson, 2013), *S.* Typhimurium (Gomes-Neto, Luz, Franco, Magnani, & Souza, 2014; Luz Ida et al., 2012) o *L. monocytogenes* (Apolonio et al., 2014; Gomes Neto, Luz, Honório, Tavares, & de Souza, 2012; Luz, Neto, Tavares, Magnani, & de Souza, 2012), ninguno de ellos observó la aparición de variantes resistentes, o en algunos casos solo variaciones menores. De tal modo, las evidencias científicas hasta el momento respaldaban que este fenómeno de resistencia no se producía frente a CI y AE (de Souza, 2016). Sin embargo, nuestro grupo de investigación consiguió, por primera vez, aislar variantes bacterianas resistentes frente a CI, como carvacrol, citral y óxido de limoneno en *E. coli* (Chueca et al., 2016), demostrando que la exposición continuada frente a estos compuestos puede dar lugar a la aparición de resistencias.

A continuación, con el fin de profundizar en el fenotipo de las variantes resistentes se llevaron a cabo:

a) estudios de cinética de crecimiento en ausencia y presencia de los AE y CI para explorar en mayor detalle el desarrollo de resistencia, y

b) tratamientos letales para la obtención de curvas de supervivencia que describieran en mayor profundidad el incremento de tolerancia.

Los estudios de cinética de crecimiento de las variantes genéticas bacterianas en presencia de los antimicrobianos naturales revelaron una reducción de la fase de latencia de la curva de crecimiento en comparación con la respectiva cepa parental, evidenciando así una mejor adaptación al antimicrobiano previa al comienzo de la replicación celular; y/o un aumento de la tasa máxima de crecimiento que indica una mayor velocidad de multiplicación celular durante la fase exponencial de crecimiento. De tal modo, las variantes resistentes no solo se mostraron capaces de crecer a concentraciones del antimicrobiano superiores a la cepa parental, sino también de crecer más rápido a concentraciones subinhibitorias. Además, estos resultados explicaron cómo las variantes genéticas bacterianas se convirtieron en la población mayoritaria del cultivo microbiano en los ensayos de evolución bajo dosis subinhibitorias de los AE y CI, ya que su mayor velocidad de crecimiento les permitía aumentar su proporción con respecto a la cepa

parental a lo largo de los ciclos evolutivos, favoreciendo su posterior aislamiento y estudio.

Las curvas de supervivencia de las variantes genéticas bacterianas, obtenidas tras la aplicación de tratamientos letales con los antimicrobianos naturales, revelaron un aumento de la supervivencia microbiana con respecto a la cepa parental, tanto a pH neutro (7,0) como a pH ácido (4,0), excepto SaCit. Estos resultados también demostraron que la mayor supervivencia de las variantes genéticas bacterianas sobre la cepa parental, condujo a la predominancia del cultivo bacteriano a lo largo de los ciclos del ensayo de evolución mediante tratamientos letales, y su posterior aislamiento y estudio.

Estos resultados respaldan la hipótesis de que los mecanismos de resistencia y tolerancia frente a los antimicrobianos naturales, pese a que pueden estar relacionados, son diferentes, ya que el incremento de resistencia y tolerancia de las variantes aisladas no es en todos los casos de la misma magnitud, e incluso en alguna variante como SaCIT, son opuestos. A este respecto, cabe destacar que la mayoría de variantes aisladas, pese a que el objetivo inicial de los ensayos era aislar cepas resistentes por exposición a concentraciones subinhibitorias o tolerantes tras tratamientos letales, mostraron un incremento tanto de resistencia como de tolerancia, independientemente del protocolo de ensayo de evolución. De hecho, en algunas variantes como las obtenidas en *S.* Typhimurium frente a carvacrol, SeSCar (aislada por exposición a concentraciones subinhibitorias) reveló una mayor tolerancia que SeLCar (aislada tras tratamientos letales), mientras que SeLCar mostró una mayor resistencia que SeSCar (Fig. 5.3).

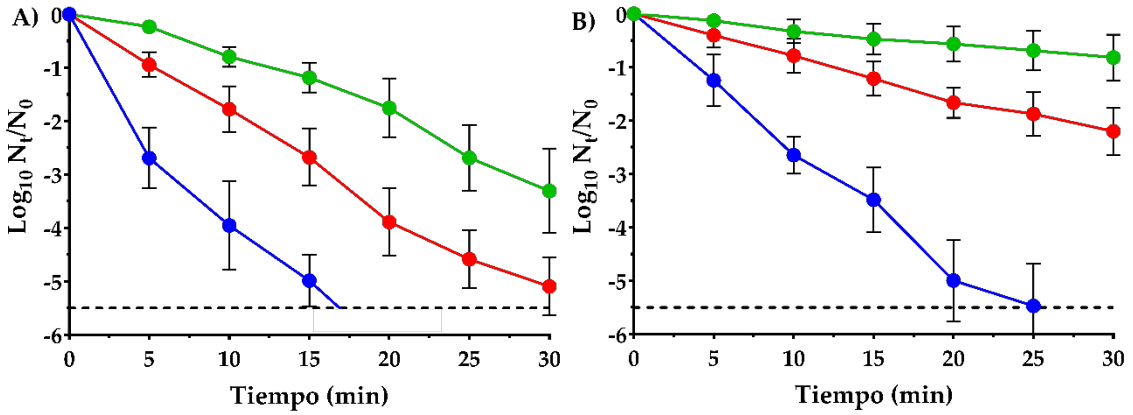


Figura 5.3. Curvas de supervivencia de *S.* Typhimurium de la cepa parental (●; SeWT) y de las variantes genéticas aisladas: SeSCar (●) y SeLCar (●), tras tratamientos con 150 µL/L de carvacrol a pH 4,0 (A) y pH 7,0 (B). Los datos representan las medias ± desviaciones estándar (barras de error) obtenidas de al menos 3 experimentos independientes. La línea discontinua representa el límite de detección (-5,5 $\log_{10} N_t/N_0$).

Según Brauner et al. (2016), los fenómenos de tolerancia y resistencia son mecánicamente distintos y se supone que no están relacionados. Sin embargo, otros estudios recientes han informado de que la tolerancia a los antibióticos precede y facilita la evolución de la resistencia porque la tolerancia aumenta las posibilidades de que las mutaciones difundan en la población bacteriana (Levin-Reisman et al., 2017; Lewis & Shan, 2017). En este sentido, se precisan más estudios respecto a AE y CI, puesto que alguna de las variantes aisladas reveló un incremento de resistencia, pero no de tolerancia.

5.3.2. *Resistencia cruzada frente a otras tecnologías de conservación*

Una vez confirmado el aumento de la resistencia y la tolerancia directa frente a los antimicrobianos naturales empleados en los ensayos de evolución, se planteó la cuestión de si estas variantes genéticas podían haber desarrollado también resistencia cruzada frente a otros métodos de conservación. En este sentido, se llevaron a cabo tratamientos letales térmicos (tecnología representante de un mecanismo de inactivación microbiana complejo, que afecta a la mayor parte de las estructuras celulares y rutas metabólicas (Cebrián, Condón, & Mañas, 2017)), y de PEAV (tecnología representante de un mecanismo de inactivación microbiana más simple, que afecta fundamentalmente a las envolturas celulares a través del fenómeno de la electroporación (García, Mañas, Gómez, Raso, & Pagán, 2006; Somolinos, Garcia, Manas, Condon, & Pagan, 2008) con las variantes resistentes de *S. aureus*: SaCar, SaCit y SaOxLim. Estos estudios permitieron comparar las curvas de supervivencia de las 3 cepas mutantes con las respectivas de la cepa parental para evaluar el desarrollo de resistencias cruzadas (**Manuscrito III**).

Los resultados revelaron que no todas las variantes respondían de igual manera a las tecnologías de conservación de alimentos. Por un lado, frente a los tratamientos térmicos, mientras que SaCAR mostró un aumento de resistencia cruzada tanto a pH 4,0 como a pH 7,0, SaOxLim sólo reveló una mayor supervivencia a pH neutro, y SaCit no mostró diferencias con respecto la cepa parental. Por otro lado, ninguna de las variantes aisladas desarrolló resistencia cruzada a los PEAV, ni a pH ácido ni a pH neutro (Fig. 5.4). Un estudio previo sobre ensayos de evolución en *E. coli* frente a carvacrol, citral y óxido de limoneno, también observó en todas sus variantes aisladas un desarrollo de resistencia cruzada frente a los tratamientos térmicos, aunque de distinta magnitud entre ellas, mientras que la resistencia frente a PEAV fue similar a la cepa parental (Chueca et al., 2016).

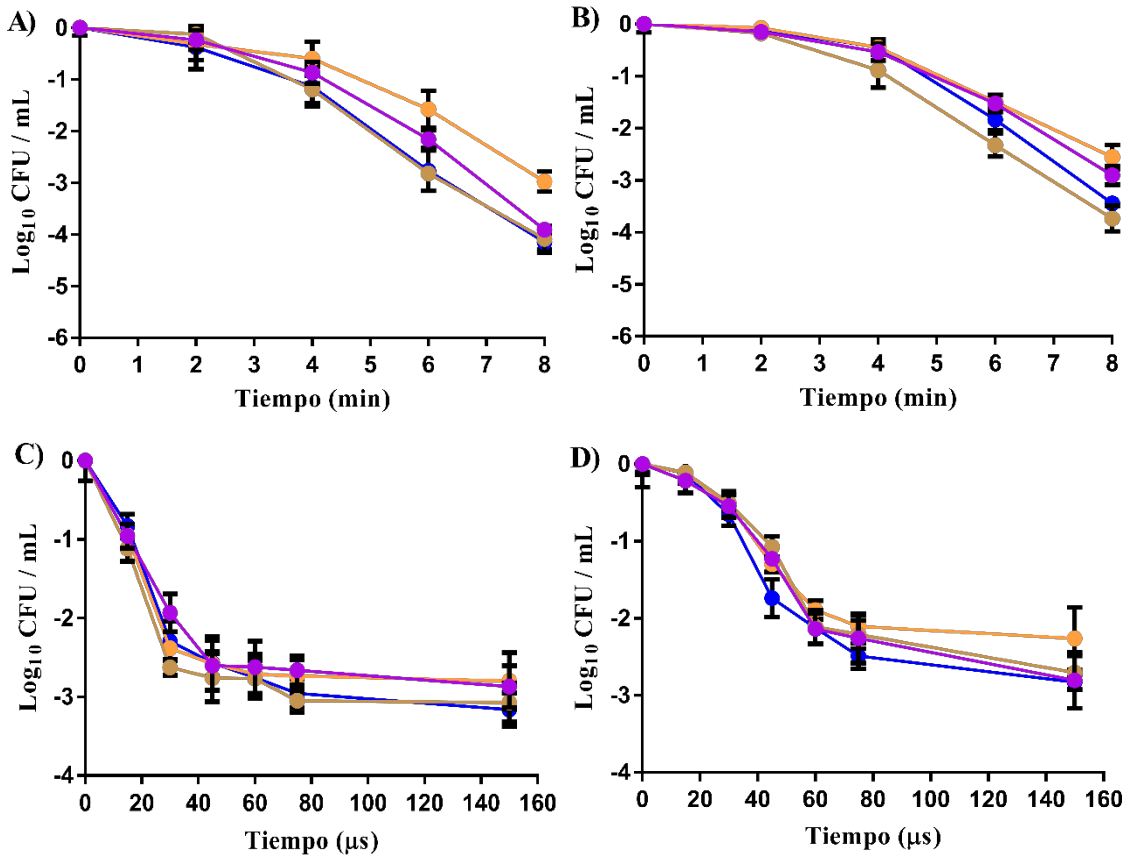


Figura 5.4. Curvas de supervivencia de S. *aureus* de la cepa parental (SaWT; ●) y de las variantes genéticas aisladas: SaCar (●), SaCit (●), y SaOxLim (●), tras tratamientos letales de calor (A, B; 60 ºC) y PEAV (C, D; 25 kV/cm, 1Hz, 3 µs/ pulse) a pH 4,0 (A, C) y pH 7,0 (B, D). Los datos representan las medias ± desviaciones estándar (barras de error) obtenidas de al menos 3 experimentos independientes.

Los tratamientos térmicos provocan daños en el DNA y en el RNA, la desestabilización de ribosomas, la desnaturalización de enzimas y otras proteínas, etc., pero la mayoría de autores señalan como principal estructura afectada las envolturas celulares (Mackey, 2000; Mackey, Miles, Parsons, & Seymour, 1991; Nguyen, Corry, & Miles, 2006). Por esta razón, se realizó un estudio del daño subletal de la membrana celular tras los tratamientos térmicos en la variante más resistente, SaCar, que reveló una mejora de su reparación de membrana tras los tratamientos de inactivación en lugar de un aumento de su resistencia intrínseca (Fig. 5.5). En este sentido, es esperable que las mutaciones de SaCar estén relacionadas con enzimas implicadas en la formación y/o reparación de la envoltura celular.

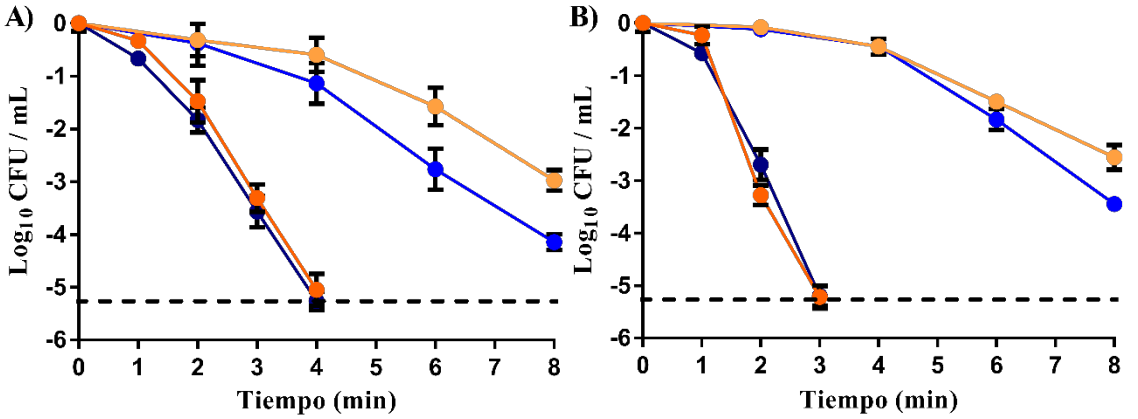


Figura 5.5. Curvas de supervivencia de S. *aureus* de la cepa parental (SaWT; ●, ●) y de la variante genéticas aislada SaCar (●, ●), tras tratamientos letales de calor (60 ºC) a pH 4,0 (A) y pH 7,0 (B) recuperadas en TSBYE sin (●,●) y con NaCl al 14% (●,●). Los datos representan las medias ± desviaciones estándar (barras de error) obtenidas de al menos 3 experimentos independientes. La línea discontinua representa el límite de detección (-5,2 $\log_{10} N_t/N_0$).

Por otro lado, la membrana citoplasmática es la principal estructura afectada de los tratamientos PEAV, causando poros (electroporación) y, como consecuencia, su inactivación (Chueca, Pagán, & García-Gonzalo, 2015; García et al., 2006; Mañas & Pagán, 2005). En este caso, aunque los tratamientos de CI y PEAV se dirigen principalmente a las envolturas celulares, las variantes aisladas mostraron una resistencia similar a la cepa parental. Probablemente, este fenómeno esté causado porque los CI y los PEAV actúan sobre diferentes dianas y/o vías metabólicas de las envolturas celulares, como se ha evidenciado previamente por la ausencia de efectos letales sinérgicos en procesos combinados con CI y PEF (Espina, Gelaw, de Lamo-Castellvi, Pagan, & Garcia-Gonzalo, 2013; Somolinos, García, Condón, Mackey, & Pagán, 2010).

De tal modo, estos datos reflejaron que es posible que la aparición de resistencias y tolerancias frente a los antimicrobianos conduzca a la aparición de resistencias cruzadas frente a otras tecnologías de conservación. Es probable que la aparición de resistencias cruzadas esté condicionada por la variante genética seleccionada a lo largo de los ensayos de evolución y, por tanto, sea dependiente de cada variante aislada. No obstante, cabe destacar que cuanta mayor similitud exista entre las estructuras dianas y los mecanismos de inhibición e inactivación bacteriana entre los métodos de conservación, mayor será la probabilidad de observar resistencias cruzadas.

5.3.3. *Resistencia cruzada frente a antibióticos*

A continuación, se llevó a cabo la determinación de la susceptibilidad frente a antibióticos mediante el test de Kirby-Bauer para evaluar si la aparición de resistencia

frente a AE y CI conllevaba el desarrollo de resistencia cruzada frente a antibióticos (Tabla 5.3).

Tabla 5.3. Esquema de resistencias cruzadas frente a antibióticos: ácido nalidíxico (NAL), norfloxacina (NOR), novobiocina (NVB), trimetoprima (TMP), rifampicina (RIF), cloranfenicol (CHL), kanamicina (KAN), tetraciclina (TCY), ampicilina (AMP) y cefalexina (LEX), de las variantes genéticas aisladas en comparación con sus respectivas cepas parentales.

Diana celular	**Antibiótico**	**Variantes genéticas resistentes**					
		SeSCar	**SeLCar**	**SaROEO**	**LmSTCO**	**LmLTCO**	**SeTCO**
Replicación de DNA	NAL	X	✓	X	X	X	✓
	NOR	X	✓	X	X	X	✓
	NVB	X	✓	X	X	✓	✓
	TMP	X	✓	X	X	✓	✓
Síntesis de RNA	RIF	X	✓	X	X	X	✓
Síntesis de proteínas	CHL	X	✓	X	X	X	✓
	KAN	X	X	X	X	✓	✓
	TCY	X	✓	X	X	✓	✓
Síntesis de envolturas celulares	AMP	X	✓	X	✓	✓	X
	LEX	X	X	X	✓	X	X

X: No hay diferencias estadísticamente significativas con respecto a la cepa parental ($p>0.05$).
✓: Diferencia estadísticamente significativas con respecto a la cepa parental ($p\leq0.05$).

Los halos de inhibición frente a antibióticos revelaron que no todas las variantes aisladas se comportaban de la misma manera, ni tampoco cada una de ellas frente a los distintos antibióticos. Por ejemplo, respecto a las cepas de *S.* Typhimurium resistentes a carvacrol, SeSCar (aislada por dosis subinhibitorias) no mostró resistencia cruzada frente a los antibióticos mientras que SeLCar (aislada por tratamientos letales) incrementó su resistencia frente a ácido nalidíxico, ampicilina, cloranfenicol, norfloxacina, novobiocina, rifampicina, tetraciclina y trimetoprima, pero no frente a cefalexina y kanamicina (**Manuscrito IV**). SeTCO también exhibió un incremento de resistencia general frente a aminocumarinas, aminoglucósidos, cloranfenicol, rifampicina, quinolonas y tetraciclinas, excepto frente a los betalactámicos (**Manuscrito VI**). En este sentido, es probable que las mutaciones de SeLCAR y SeTCO afecten a mecanismos generales de resistencia que, de forma no específica, desencadenen una mayor resistencia frente a la mayoría de

compuestos antimicrobianos. Por otro lado, SaROEO no mostró aumento de resistencia frente a ninguno de los grupos de antibióticos testados: aminocumarinas, aminoglucósidos, betalactámicos, cloranfenicol, rifampicina, quinolonas y tetraciclinas (**Manuscrito V**). En este caso, las mutaciones responsables del aumento de resistencia frente al AE de naranja en esta variante no estarían relacionadas con la respuesta celular a los antibióticos, al menos frente a los evaluados. En cuanto a las variantes de *L. monocytogenes,* mientras que LmSTCO sólo incrementó su resistencia frente a los betalactámicos, LmLTCO desarrolló una leve resistencia cruzada frente a ampicilina, kanamicina, novobiocina, tetraciclina y trimetoprima **(Manuscrito VII)**. Estos resultados sugieren que la clave del aumento de la resistencia y la tolerancia al AE en LmSTCO, así como frente a los betalactámicos, reside en alguna mutación relacionada con las envolturas celulares, mientras que la menor tasa de crecimiento y una mayor fase de latencia de LmTCO podría justificar el aumento de resistencia frente a antibióticos. (Pontes & Groisman, 2019)

Estudios previos en variantes genéticas de *E. coli* resistentes a CI evidenciaron también el desarrollo de resistencia cruzada frente a todos los antibióticos testados excepto a la rifampicina (Chueca et al., 2018). Este fenómeno de resistencia cruzada frente a antibióticos no sólo se ha observado tras el uso de antimicrobianos naturales, sino que otros autores han demostrado su aislamiento tras el uso de tecnologías no térmicas como la radiación UV-C y el plasma atmosférico frío aplicados de forma cíclica (Alvarez-Molina et al., 2020), o la exposición a biocidas de forma prolongada, dando lugar a cepas resistentes frente a antibióticos (Oniciuc et al., 2019).

El enorme aumento de cepas multirresistentes en la industria alimentaria representa un grave problema de salud pública en todo el mundo, siendo uno de los puntos clave en la propagación de las resistencias antimicrobianas (Bergspica, Kaprou, Alexa, Prieto-Maradona, & Alvarez-Ordonez, 2020; McMillan et al., 2019). Estudios recientes han informado de la elevada presencia de genes de resistencia a una amplia gama de antibióticos en la cadena de producción, por ejemplo, en la industria láctea (Parry-Hanson Kunadu, Holmes, Miller, & Grant, 2018; Qamar, Ismail, & Akhtar, 2020).

Por otro lado, se dispone de escasos datos sobre la contribución de las bacterias resistentes a antibióticos a la carga global de las enfermedades transmitidas por los alimentos. En este sentido, Brown et al. (2017) mostraron que *Salmonella* multirresistente (MDR) se aisló en el 29% (14/48) de los brotes alimentarios procedentes de animales

terrestres y en el 8% (3/40) de los brotes alimentarios procedentes de productos vegetales durante el periodo 2003-2012 en Estados Unidos. Además, *Salmonella enterica* serovar Heidelberg multirresistente se ha asociado con numerosos brotes de enfermedades humanas transmitidas por los alimentos debido al consumo de aves de corral y sus derivados (Bearson, Bearson, Looft, Cai, & Shippy, 2017). Y más recientemente, Xiang et al. (2020) han investigado un brote de salmonelosis causado por *S.* Typhimurium multirressistente en China revelando que estas cepas del brote también portaban un gran número de genes de virulencia. Estos recientes brotes causados por *S. enterica* multirresistente ponen de manifiesto la necesidad de mejorar las estrategias de prevención y control de las bacterias patógenas resistentes frente a antibióticos.

En este sentido, los AE y CI han demostrado ser eficaces contra las cepas multirresistentes (Ragno et al., 2020) y, por esta razón, su uso en la industria agroalimentaria podría contribuir a prevenir la diseminación de las resistencias antimicrobianas (Mittal, Rana, & Jaitak, 2019; Yap, Yiap, Ping, & Lim, 2014). Sin embargo, los resultados anteriormente descritos revelan que los antimicrobianos naturales también pueden dar lugar a resistencias cruzadas frente a antibióticos. En este sentido, el hecho de que los mecanismos de resistencia bacteriana a los AE y a los antibióticos puedan estar interrelacionados, cuestionarían su efectividad en la lucha para prevenir la aparición y diseminación de estas resistencias. No obstante, se requieren más estudios para conocer bajo qué condiciones puede producirse la resistencia cruzada frente a antibióticos, así como para profundizar en la repercusión y el alcance de estas variantes resistentes en la industria agroalimentaria y en la salud pública.

5.4. Caracterización genotípica de las variantes resistentes e identificación de las modificaciones genéticas responsables de los cambios observados en su fenotipo

Tras haber caracterizado fenotípicamente las variantes genéticas resistentes, se prosiguió con la caracterización genotípica con el objetivo de identificar las modificaciones genéticas aisladas responsables del aumento de resistencia y tolerancia frente a los antimicrobianos naturales, así como de la resistencia cruzada frente a otros métodos de conservación y antibióticos (**Manuscritos III, IV,V,VI y VII**). De tal modo, la secuenciación genómica de las variantes resistentes y su comparación con la cepa parental (cepa origen de los ensayos de evolución) permitió la identificación de los SNV,

Ins, Dels y SV, que fueron verificados mediante PCR y secuenciación de Sanger (Fig. 5.6), así como de los genes afectados y su proceso biológico (Fig. 5.7).

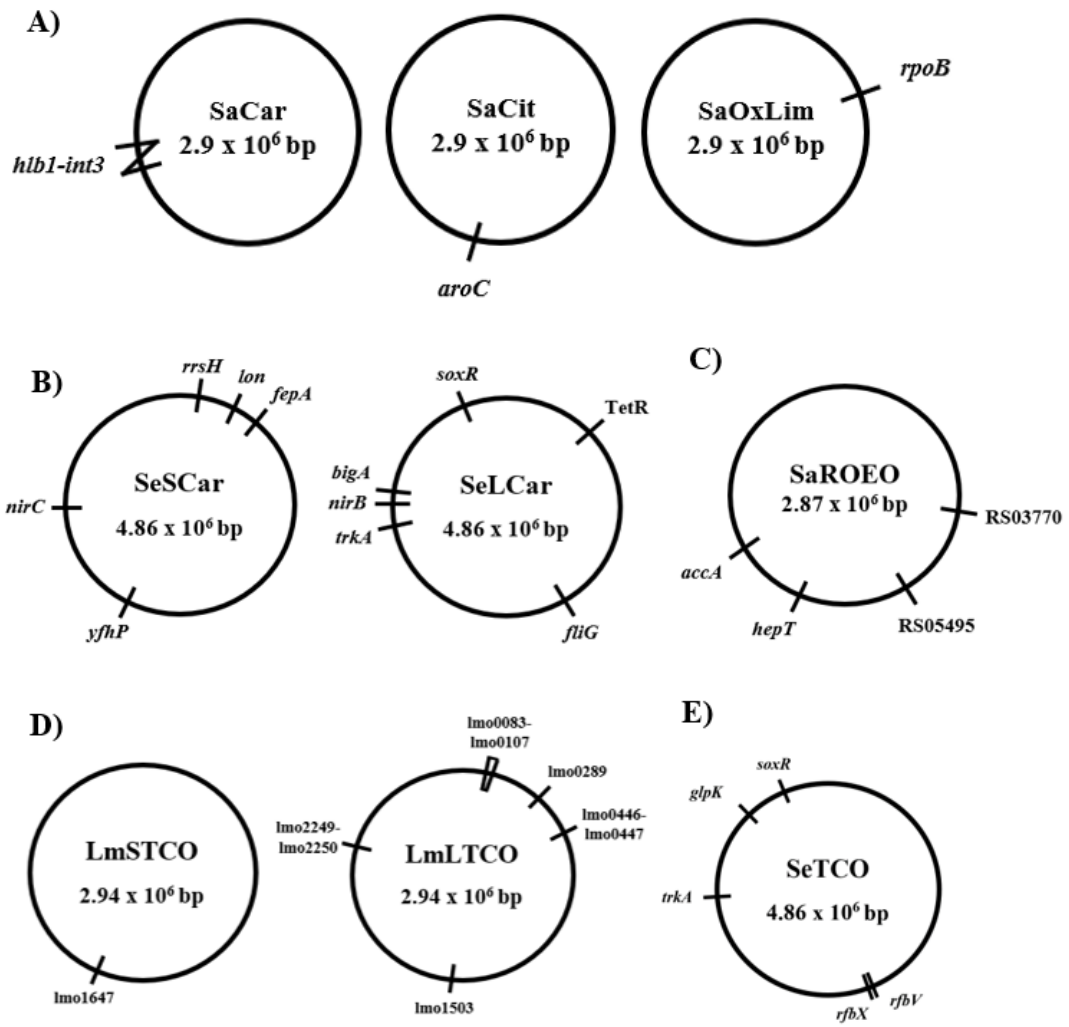
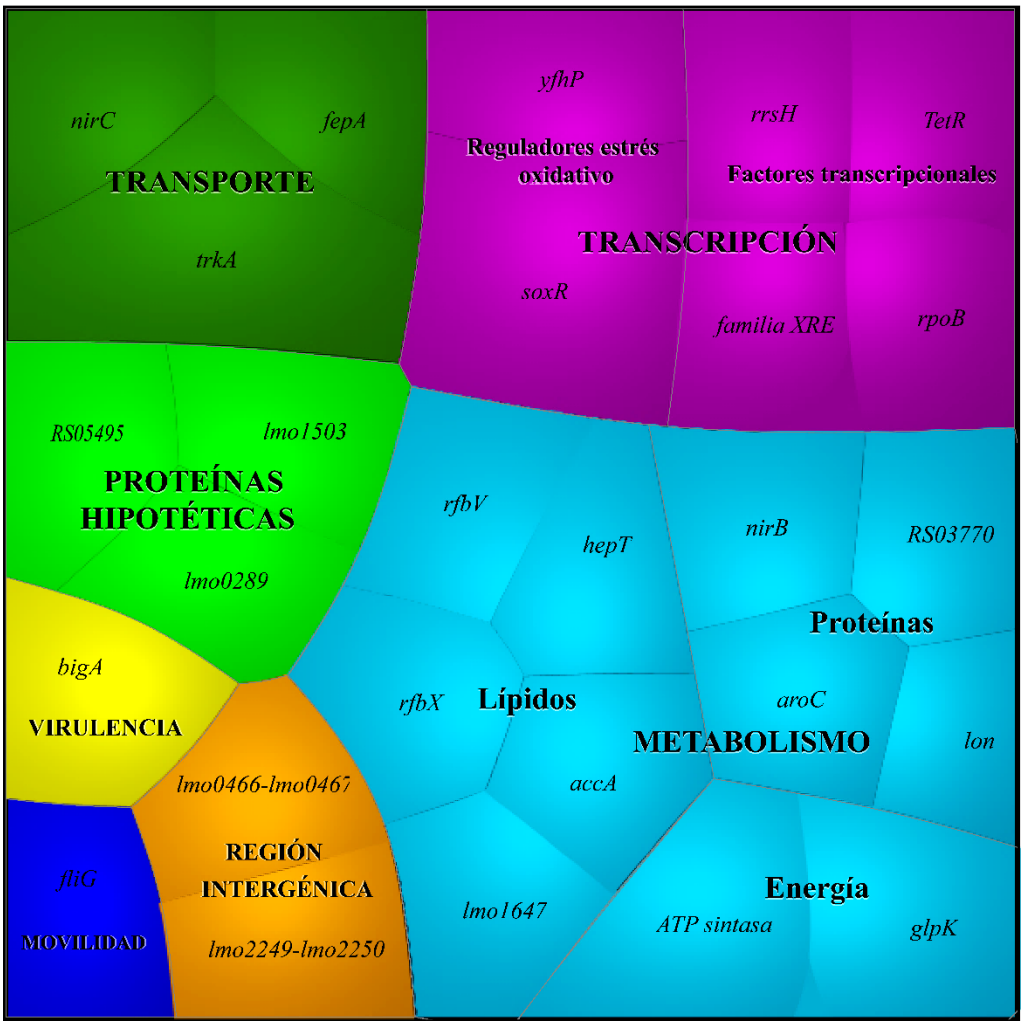


Figura 5.6. Mapas genéticos de las variantes resistentes aisladas en los **Manuscritos III** (A)**, IV** (B)**, V** (C)**, VI** (D) **y VII** (E)**.**

Una de las limitaciones de este tipo de estudios es que se encuentran condicionados por el conocimiento previo existente sobre la función de los diferentes genes que componen el genoma bacteriano. De tal modo, aquellos genes mutados cuya función es desconocida o codifican proteínas hipotéticas cuyo papel no ha sido evidenciado *in vivo*, limitan la interpretación que se puede extraer sobre los mecanismos de resistencia. Por este motivo, normalmente se emplean bacterias modelo ampliamente caracterizadas a nivel genómico; no obstante, en estos casos tampoco la función de todos sus genes ha sido ya descrita. En este sentido, no ha sido posible determinar la repercusión de la mutación de algunos de los genes, como SAUSA300_RS05495, lom0289 y lmo1503, entre otros, sobre el papel del gen, ya sea regulador o codificante.



Figuras 5.7. Diagrama de Voronoi que representa los genes mutados de las variantes genéticas resistentes agrupados en base a su proceso biológico: metabolismo (●), movilidad (●), transcripción (●), transporte (●) y virulencia (●), así como las mutaciones en regiones intergénicas (●) o en genes codificantes de proteínas hipotéticas (●).

Por otro lado, algunas de las modificaciones genéticas detectadas en genes implicados en rutas metabólicas microbianas, como por ejemplo en *trKA* y *glpK,* según Knöppel et al. (2018) suelen producirse como consecuencia de la adaptación al medio de crecimiento durante los ensayos de evolución, en este caso al TSBYE, por lo que tampoco parece probable que estén relacionados directamente con los mecanismos de resistencia microbiana frente a los métodos de conservación de alimentos.

Entre las modificaciones genéticas detectadas en las variantes resistentes destacan el elevado número de mutaciones que se encuentran en genes relacionados con las envolturas celulares, tanto codificantes de proteínas receptoras y de transporte localizadas en la membrana (*fepA*, *nirC*) como de enzimas relacionadas con la síntesis y reparación de las membranas celulares (*accA*, lmo1647). Las membranas celulares son la primera

barrera física de resistencia de las bacterias, así como una de las principales estructuras dañadas por los antimicrobianos naturales, que alteran su permeabilidad y su integridad (Bouyahya, Abrini, Dakka, & Bakri, 2019; Yang et al., 2020). De tal modo, es probable que las modificaciones genéticas localizadas en genes cuya traducción de lugar a proteínas estructurales de la membrana, receptoras, de transporte o reguladoras de la homeostasis celular situadas en la membrana, así como de enzimas implicadas en la síntesis o reparación de las envolturas celulares, conlleve la modificación de la resistencia y/o tolerancia frente a los AE y CI. De este modo, estos resultados respaldan el importante papel de las envolturas celulares en la resistencia y tolerancia frente a estos antimicrobianos naturales y, consecuentemente, que los mecanismos de inhibición e inactivación de los CI y AE se focalizan, en gran parte, en las envolturas celulares.

Otros de los genes más comúnmente afectados por las modificaciones genéticas, y que revelaron su importante papel en la resistencia y tolerancia de las variantes genéticas frente a CI y AE, fueron los reguladores transcripcionales relacionados con la respuesta al estrés oxidativo, como *yfhP y soxR.* Por un lado, varios estudios han relacionado una elevada expresión de *yfhP* como respuesta frente a agentes oxidantes, como el peróxido de hidrógeno (Karash, Liyanage, Qassab, Lay, & Kwon, 2017). Según Chueca et al. (2014b), los CI provocan la generación endógena de especies reactivas de oxígeno (ROS) y, por tanto, *yfhP* estaría implicado en una de las vías de respuesta al estrés oxidativo inducido por los CI. Por otro lado, *soxR* codifica un regulador transcripcional sensible al potencial redox que participa en la defensa frente a antibióticos del ciclo redox (Gu & Imlay, 2011). Su forma oxidada activa la transcripción del gen *soxS* que modula la expresión de más de 100 genes del regulón *soxRS* para proporcionar defensa celular contra el estrés oxidativo (Pomposiello, Bennik, & Demple, 2001). La función principal del regulón *soxRS* es minimizar la concentración intracelular de fármacos a través de mecanismos que impiden su entrada, los modifican químicamente o los expulsan del interior celular (Gu & Imlay, 2011). Esta respuesta bacteriana se ha descrito frente a agentes oxidantes y antibióticos, y más recientemente, se ha relacionado con la respuesta frente a CI o AE (Chueca et al., 2018; Sheng, Rasco, & Zhu, 2016). Por otro lado, el hecho de que dos ensayos de evolución independientes con antimicrobianos naturales hayan seleccionado mutaciones en *soxR*, más concretamente en su codón 20 en *S.* Typhimurium, apuntaba a que este gen tenía un papel fundamental en la respuesta celular frente a AE y CI, y respaldaba la aparición de variaciones genéticas de este gen durante

los ensayos de evolución, permitiendo la aparición de cepas resistentes. Por esta razón, se llevó a cabo la construcción de la cepa SeWT$_{soxR,}$ mediante la sustitución de *soxR* en SeWT por la variante *soxR*$_{SeTCO}$ (**Manuscrito VII**). Así, su caracterización fenotípica confirmó que la modificación genética en *soxR* era la principal causa del incremento de resistencia y tolerancia observada frente al AE, tanto en medios de laboratorio como en alimentos, así como frente a los antibióticos. Estos resultados ponen de manifiesto que *soxR* es un mecanismo clave en la respuesta celular a los AE inducida por el estrés oxidativo. Dado que la mutación se localiza en el dominio de unión al DNA de la proteína SoxR, es probable que se haya alterado la afinidad o la estabilidad con las secuencias reguladoras de *soxS* y, en consecuencia, se haya modificado la expresión del regulón *soxRS*. De este modo, la expulsión de los antimicrobianos al exterior de la célula se habría visto potenciada, evitando su acumulación a nivel intracelular.

Estos resultados aportan conocimiento sobre los mecanismos de inhibición e inactivación de los AE y CI sobre las bacterias y señalan al estrés oxidativo como uno de los mecanismos de acción de estos compuestos antimicrobianos y a las envolturas celulares como una de las principales estructuras diana de los tratamientos. De tal modo, la aplicación de estos protocolos de los ensayos de evolución permitirá en un futuro contribuir, a través de esta vía, a la descripción de los mecanismos de inhibición e inactivación de otros métodos de conservación de los alimentos.

Por otra parte, el origen y la aparición de variantes genéticas resistentes todavía se encuentra bajo discusión por el mundo científico. Según Mao, Lane, Lee, and Miller (1997), los ensayos de evolución ejercen una presión selectiva sobre la población heterogénea bacteriana, que facilita el aislamiento de las variantes genéticas más resistentes. Aquellas cepas que muestren una mejor aptitud de crecimiento en presencia del agente antimicrobiano (Andersson & Hughes, 2014) o que sobrevivan a los tratamientos letales (Levin-Reisman et al., 2017) emergerán por encima del resto de subpoblaciones bacterianas. Sin embargo, aquellas mutaciones que se producen de forma espontánea durante el crecimiento bacteriano debido a errores de replicación también pueden ser seleccionadas (Drake, 1991). Varios estudios respaldan la hipótesis de que tales mutaciones podrían ser inducidas por el tratamiento, incluso como parte de la respuesta celular al estrés (Foster, 2007), como el sistema SOS (Schlacher & Goodman, 2007). En este sentido, Jee et al. (2016) y Massey and Buckling (2002) sostienen que el aumento de mutaciones se produciría en sitios o regiones específicas como respuesta

adaptativa a las condiciones ambientales. Este fenómeno podría darse según Jinks-Robertson and Bhagwat (2014) y Hudson, Bergthorsson, and Ochman (2003), debido a que las mutaciones tenderían a producirse en los genes más activos desde el punto de vista de la transcripción durante la respuesta celular a los tratamientos. Según estos estudios, las resistencias no sólo podrían surgir de forma aleatoria y espontánea en el curso de los tratamientos con AE y CI, sino que también induciría mutaciones específicas provocadas por el estrés que mejora la supervivencia bacteriana.

Estas hipótesis apoyarían el hecho de que las modificaciones genéticas detectadas en las variantes resistentes estén relacionadas con mecanismos clave en la respuesta bacteriana frente a los AE y CI, principalmente las relacionadas con el estrés oxidativo y las envolturas celulares. En este sentido, tales afirmaciones respaldarían que la repetición de los ensayos de evolución pudiera dar lugar a distintas variantes genéticas resistentes, pero, probablemente, las modificaciones genéticas podrían tener lugar en los mismos genes o distintos genes, pero cuya función estuviera implicada en los mismos mecanismos de resistencia celular. Todo ello es dependiente de los diversos mecanismos de resistencia celular frente a AE y CI que puedan llegar a estar presentes en las bacterias, de los cuales, muchos permanecen desconocidos. Por esta razón, se requieren más investigaciones que permitan abordar el estudio de las resistencias bacterianas, con el fin de conocer los mecanismos de inhibición e inactivación para mejorar y potenciar el uso de los AE y CI como conservantes antimicrobianos y biocidas.

5.5. Evaluación de la resistencia de las variantes genéticas en alimentos modelo para explorar su repercusión en la industria agroalimentaria

Una vez caracterizado el genotipo de las variantes genéticas resistentes frente a los antimicrobianos naturales, se decidió explorar el riesgo que podría suponer la aparición de estas variantes resistentes en la cadena alimentaria. Para ello, se determinó la resistencia y la tolerancia de las variantes aisladas frente a un AE en un alimento modelo: leche desnatada. Los AE y CI han sido ampliamente testados en la conservación de productos lácteos debido a sus propiedades antimicrobianas, antioxidantes y funcionales (Mishra et al., 2020). En este sentido, el AE de tomillo es uno de los más estudiados en la conservación de la leche debido a sus excelentes propiedades antimicrobianas, empleado solo (Ben Jemaa et al., 2017) o combinado con tratamientos térmicos (Ben Jemaa et al., 2018). Además, se escogió leche desnatada para evitar el efecto inhibidor de

la grasa sobre la actividad antimicrobiana del AE y así facilitar la observación de diferencias de resistencias con las cepas parentales.

Los resultados de la determinación de la CMI y CMB, así como las curvas de supervivencia obtenidas en leche desnatada frente al AE de tomillo, confirmaron que variantes genéticas tanto de bacterias Gram-positivas, concretamente *L. monocytogenes* (**Manuscrito VI**), como Gram-negativas, *S.* Typhimurium (**Manuscrito VII**), habían desarrollado también un incremento de resistencia y tolerancia de igual magnitud, e incluso mayor, en el alimento (Figura 5.8). En este sentido, estos resultados sugieren que si estas cepas emergen en la industria alimentaria podrían crecer en condiciones no favorables y sobrevivir a tratamientos de conservación de alimentos diseñados para las cepas de tipo salvaje, comprometiendo así la inocuidad de los alimentos.

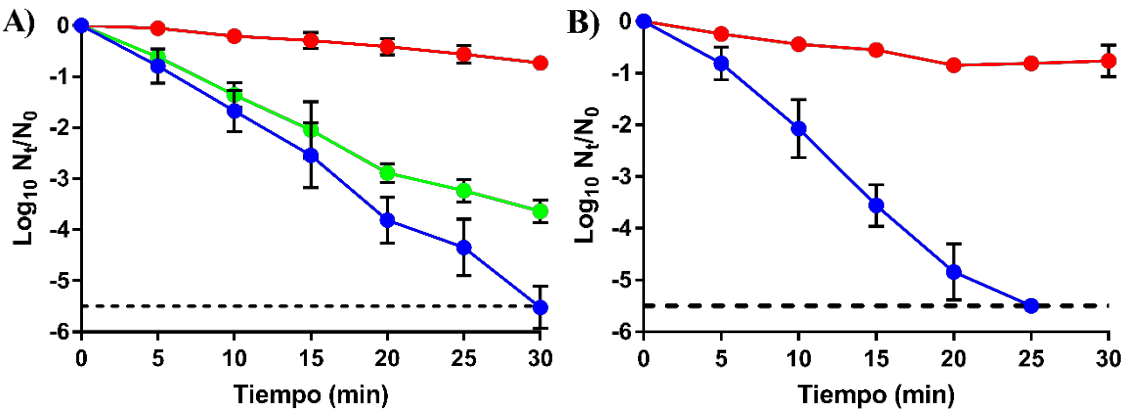


Figura 5.8. Curvas de supervivencia de *L. monocytogenes* (A) y *S.* Typhimurium (B) tras tratamientos de inactivación de aceite esencial de tomillo a 1600 µL/L y 1500 µL/L respectivamente, en leche desnatada, de las cepas parentales (SeWT y LmWT;●) y de las variantes genéticas aisladas mediante la exposición prolongada a dosis subinhibitorias (LmSTCO; ●) o tras tratamientos letales (LmLTCO y SeTCO; ●). Los datos representan las medias ± desviaciones estándar (barras de error) obtenidas de al menos 3 experimentos independientes. La línea discontinua representa el límite de detección (-5,5 $\log_{10} N_t/N_0$).

La utilización de AE y CI como conservantes antimicrobianos está ampliamente respalda por estudios científicos que defienden su elevada eficacia en la conservación de alimentos (Bhavaniramya, Vishnupriya, Al-Aboody, Vijayakumar, & Baskaran, 2019; Burt, 2004; Calo, Crandall, O'Bryan, & Ricke, 2015; Pandey, Kumar, Singh, Tripathi, & Bajpai, 2017), como por ejemplo en zumos (de Souza, da Cruz Almeida, & de Sousa Guedes, 2016), quesos (Gouvea, Rosenthal, & Ferreira, 2017; Ritota & Manzi, 2020) y otros productos lácteos (Mishra et al., 2020). Sin embargo, a día de hoy, no existen estudios que valoren la aparición de estas variantes resistentes ni su repercusión en la conservación de los alimentos. Por esta razón, es necesario seguir investigando para

conocer si estas variantes resistentes pueden aparecer en los sistemas de producción y conservación de los alimentos, así como realizar evaluaciones de riesgo que valoren este peligro en la seguridad alimentaria. En este sentido, es recomendable tener en cuentas estos fenómenos de resistencia en el diseño de estrategias de conservación de modo que, en caso de que surjan estas variantes resistentes, las tecnologías y antimicrobianos empleados sean suficientes para controlar el riesgo alimentario. Del mismo modo, se plantea la duda de si los microorganismos resistentes a antibióticos, que actualmente están alcanzando la cadena alimentaria por multitud de vías (materias primas de origen animal y vegetal, ingredientes, agua, etc.), podrían también comprometer la seguridad de los actuales procesos de conservación de alimentos, al compartir, en algunos casos, mecanismos de adquisición de resistencia frente a métodos de conservación de alimentos.

5.6. Preparación de nanoemulsiones y encapsulados de AE y CI para facilitar y potenciar su empleo como conservantes alimentarios y biocidas

El uso de AE y CI en la conservación de alimentos se encuentra actualmente limitado, además de por sus fuertes propiedades organolépticas, por su carácter lipofílico que limita su solubilidad y su homogeneidad en medios acuosos, y por su alta volatilidad e inestabilidad frente agentes externos, como la luz o el oxígeno, que conducen a una pérdida de su actividad antimicrobiana. En este sentido, en la última parte de esta Tesis Doctoral, se ha querido abordar esta problemática con la preparación de nanoemulsiones y encapsulaciones de AE y CI para potenciar y facilitar su empleo en la conservación de alimentos, así como en sistemas de limpieza y desinfección.

En primer lugar, se testó la preparación de nanoemulsiones utilizando métodos de baja energía, concretamente mediante el método de inversión de fases, para mejorar la solubilidad y dispersión de los AE y CI en los productos alimentarios acuosos (Komaiko & McClements, 2016). Las nanoemulsiones de citral, preparadas mediante el método de inversión de fases con polisorbato 80 (tween 80) y etanol (**Manuscrito VIII**), fueron estables durante al menos 4 meses desde su preparación, manteniendo un tamaño de partícula menor de 200 nm. No obstante, según Zhang, Vriesekoop, Yuan, and Liang (2014) y Maté, Periago, and Palop (2016), siguiendo las misma metodología con otro CI, limoneno, se pueden alcanzar hasta 6 meses de estabilidad. La determinación de la CMI y las curvas de supervivencia frente a *Escherichia coli* O157:H7 Sakai, revelaron un incremento de la actividad bacteriostática y bactericida del citral en su forma emulsionada

con respecto a su empleo de forma libre. Sin embargo, el incremento de las propiedades antimicrobianas fue inferior que a las observadas con otros CI y AE en trabajos previos (Komaiko & McClements, 2016; Moghimi, Aliahmadi, McClements, & Rafati, 2016; Moghimi, Ghaderi, Rafati, Aliahmadi, & McClements, 2016). Además, las nanoemulsiones de citral mostraron un efecto sinérgico elevado en combinación con tratamientos térmicos de baja intensidad (53 ºC/15 min), contrariamente a lo observado por Maté et al. (2016), lo que permitió el diseño de procesos combinados efectivos para el tratamientos de zumo de manzana, inactivando 5 ciclos $\log_{10}$ de la población de *E. coli* en menos de 30 min a una temperatura de 53 °C. Sin embargo, el efecto sinérgico observado con PEAV fue muy limitado debido al escaso daño ocasionado en la membrana externa (Arroyo, Somolinos, Cebrián, Condón, & Pagán, 2010) que resulta de gran relevancia en los procesos combinados con citral.

A continuación, se prosiguió la investigación con la preparación de encapsulados, ya que permiten solubilizar los AE y CI, proteger los compuestos activos de agentes externos y facilitar su liberación de forma controlada. Este estudio se realizó con la colaboración de personal con experiencia en este campo, como la Dra. Susana Sánchez de la empresa Bionanoplus S.L., e investigadores como el Dr. Santiago López-Miranda de la Universidad Católica San Antonio de Murcia, que nos proporcionaron los compuestos encapsulados que fueron testados como conservantes antimicrobianos.

Por un lado, uno de los polímeros más utilizados para la encapsulación es la zeína, una proteína vegetal aislada del maíz que permite la generación de estructuras coloidales funcionales biodegradables y autorizadas para su uso en los alimentos. Esta proteína, debido a que está compuesta por aminoácidos hidrofóbicos, proporciona una excelente barrera para los compuestos funcionales encapsulados mejorando su estabilidad y reduciendo su degradación (Patel & Velikov, 2014). Por estas razones, se seleccionó inicialmente la zeína para abordar la encapsulación de los AE y evaluar su repercusión sobre las propiedades antimicrobianas, con el objetivo de valorar su uso en la conservación de alimentos. El AE de tomillo encapsulado en zeínas (**Manuscrito IX**) mostró una mayor actividad bacteriostática frente *E. coli* O157:H7 Sakai y *L. monocytogenes* EGD-e con respecto a su empleo en forma libre, mientras que su actividad bactericida bajo tratamientos letales cortos se vio reducida en su forma encapsulada frente a ambas bacterias, tanto a pH ácido (4,0) como neutro (7,0). De tal modo, la encapsulación de AE demostró ser una buena alternativa cuando se busca mantener o incluso potenciar

su actividad bacteriostática (Donsì, Annunziata, Sessa, & Ferrari, 2011). Sin embargo, esta preparación tiene el inconveniente de que el AE se libera de forma gradual (Hu, Zhang, Xiao, & Wang, 2018). Por tanto, cuando se requiere de una rápida biodisponibilidad de los AE, como en los tratamientos de inactivación o en los sistemas de limpieza y desinfección, la liberación limitada de los AE encapsulados, puede dar lugar a una reducción de su actividad antimicrobiana. No obstante, el AE de tomillo reveló una fuerte sinergia con el calor (53 ºC) alcanzado una inactivación similar en su forma encapsulada que en su forma libre (Fig.). En ese sentido, el uso de temperaturas suaves podría contribuir a acelerar la liberación del AE, aumentando su biodisponibilidad para actuar sobre la población bacteriana.

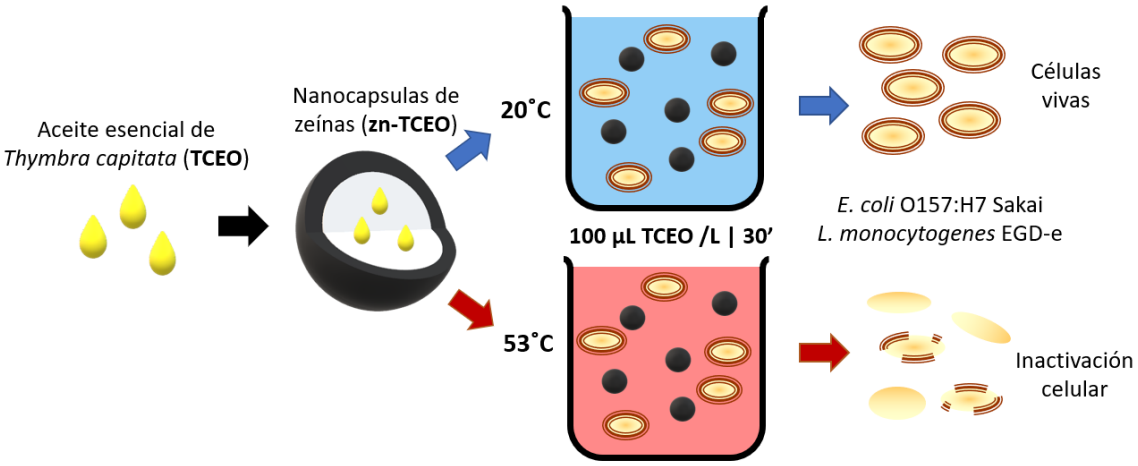


Figura 5.9. Representación esquemática de la encapsulación de aceite esencial de *Thymbra capitata* en zeínas, su liberación y su potencial antimicrobiano en combinación con calor.

Otra de las alternativas de encapsulación de los AE abordada en esta Tesis Doctoral es la encapsulación molecular en ciclodextrinas (CD). Las CDs son oligómeros cíclicos ampliamente utilizados a nivel industrial como protectores de compuestos sensibles a la luz, al oxígeno y al calor, solubilizantes de colorantes y vitaminas, y reguladores de la liberación controlada de aditivos alimentarios y fármacos (Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero, & Simal-Gándara, 2009), y cuyo empleo con AE y CI ha comenzado recientemente a testarse (Anaya-Castro et al., 2017). La encapsulación con hidroxipropil-ciclodextrinas modificadas (HP-CD) de carvacrol y timol (**Manuscrito X**) reveló una alta capacidad de complejación, una elevada estabilidad y un aumento de la solubilidad de CI en medios acuosos. En este sentido, las HP-β-CD mostraron una encapsulación más eficiente y estable con respecto a los encapsulados de HP-α-CD y HP-γ-CD. Por esta razón, normalmente los estudios publicados sobre encapsulación con ciclodextrinas en el

campo de conservación de alimentos (Astray et al., 2009), y más concretamente de AE, se focalizan en la utilización de las HP-β-CD (Anaya-Castro et al., 2017; Dima, 2014). Sin embargo, las encapsulaciones en HP-α-CD y HP-γ-CD mostraron una mayor actividad bacteriostática y bactericida que en HP-β-CD. Este fenómeno se relacionó con su menor estabilidad y, consecuentemente, con una mayor liberación del agente antimicrobiano. Por otro lado, respecto al método de obtención de los encapsulados en forma sólida, independientemente del tipo de encapsulantes empleados, la liofilización demostró un mejor mantenimiento de las propiedades antimicrobianas que el secado por pulverización (Figura XXXXX). Según Veiga, Aparecida Da Silva-Buzanello, Corso, and Canan (2019), la reducción de la actividad antimicrobiana de los complejos secados por pulverización puede estar relacionada con las altas temperaturas a las que se someten dichos complejos durante el proceso de deshidratación, lo que afecta negativamente la capacidad antimicrobiana de los compuestos antibacterianos.

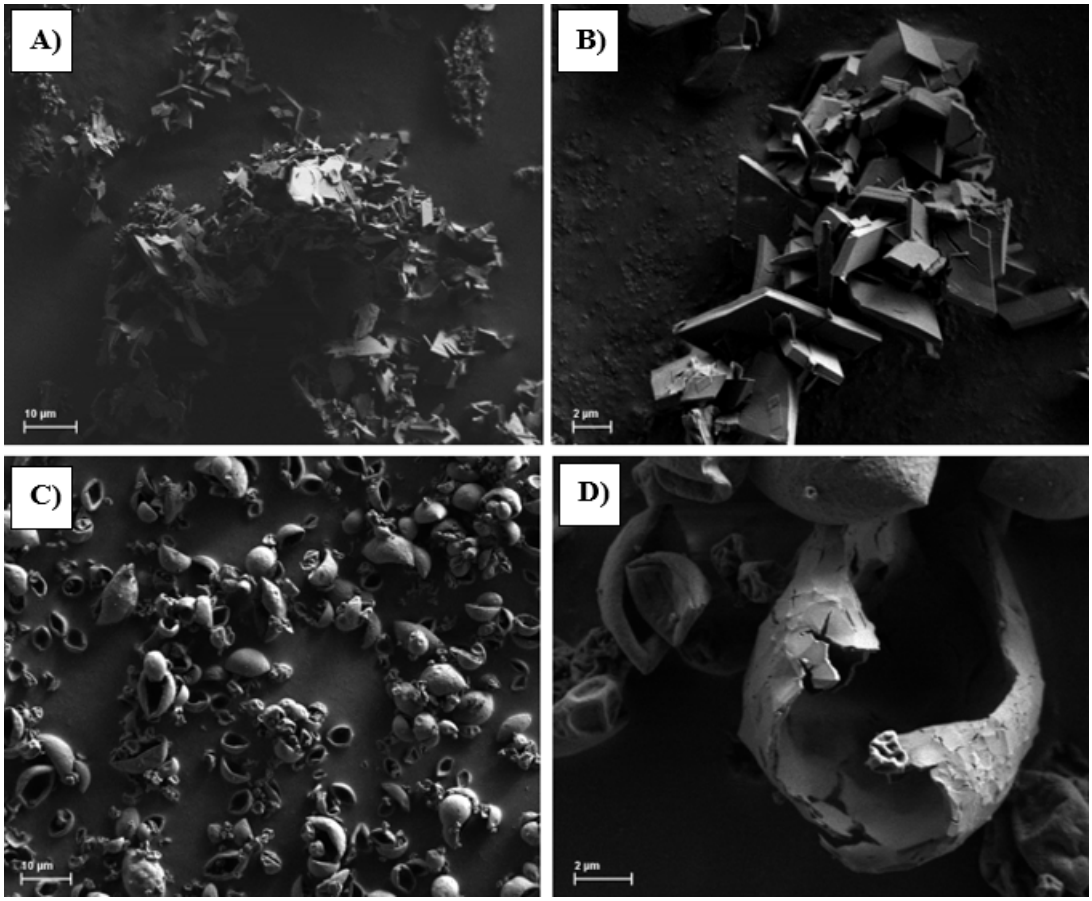


Figura 5.10. Imágenes de microscopio electrónico de barrido de emisión de campo (FESEM) de los encapsulados de carvacrol en ciclodextrinas liofilizados (A y B) y secados por pulverización (C y D).

De este modo, la elaboración de emulsiones y encapsulados permitió prolongar la estabilidad de los AE y CI a lo largo del tiempo, evitando su degradación por agentes externos como la luz y el oxígeno, y mejorando su solubilidad en soluciones acuosas, manteniendo, e incluso en algunos casos mejorando, sus propiedades antimicrobianas cuando se aplicaron de forma única o combinada con otras tecnologías de conservación, como los tratamientos térmicos o los pulsos eléctricos de alto voltaje. En este sentido, aunque aún se requieren más estudios para profundizar en la estabilidad y propiedades de las emulsiones y encapsulaciones de AE y CI, se observa un gran potencial en estas herramientas para facilitar la utilización de estos compuestos antimicrobianos naturales en la conservación de alimentos o, como biocidas, en sistemas de limpieza y desinfección.

6. Conclusiones

6. Conclusiones

En base a los objetivos planteados en la presente Tesis Doctoral, los resultados obtenidos respecto a la puesta a punto de nuevas herramientas para el estudio de los mecanismos de resistencia celular frente al uso de antimicrobianos y otros métodos de conservación de alimentos han dado lugar a las siguientes conclusiones:

1. Los ensayos de evolución llevados a cabo mediante la exposición prolongada a CI a concentraciones subinhibitorias durante el crecimiento bacteriano permitieron evidenciar el aislamiento de variantes genéticas resistentes en bacterias Gram-negativas:

   - *Salmonella* Typhimurium aislada frente a carvacrol (SeSCar),

   y, por primera vez, en bacterias Gram-positivas, concretamente en:

   - *Staphylococcus aureus* aislada frente a carvacrol (SaCar), citral (SaCit) y óxido de limoneno (SaOxLim).

2. Los ensayos de evolución basados en la aplicación de tratamientos letales con CI permitieron, por primera vez, el aislamiento de variantes genéticas bacterianas resistentes, concretamente en:

   - *S.* Typhimurium aislada tras tratamientos letales de carvacrol (SeLCar).

3. Ambos protocolos de ensayo de evolución permitieron el aislamiento de variantes genéticas resistentes en bacterias Gram-positivas y Gram-negativas, por primera vez, frente a AE complejos:

   - *S. aureus* aislada por exposición a concentraciones subinhibitorias de AE de naranja (SaROEO).
   - *S.* Typhimurium aislada tras tratamientos letales de AE de tomillo (SeTCO).
   - *Listeria monocytogenes* aislada por exposición a concentraciones subinhibitorias (LmSTCO) o tras tratamientos letales (LmLTCO) de AE de tomillo.

4. La aparición de variantes genéticas resistentes no fue causada por un aumento de la frecuencia mutagénica tras la aplicación de AE y CI, como sí se produce frente a algunos antibióticos como norfloxacina y rifampicina. En este sentido, el empleo de estos antimicrobianos naturales no induciría a la generación de variantes resistentes,

pero sí ejercería una presión selectiva que facilitaría su ventaja evolutiva y posterior selección y dominancia sobre el resto de subpoblaciones sensibles.

5. Los estudios de cinética de crecimiento de las variantes genéticas bacterianas en presencia de AE y CI revelaron su menor fase de latencia y/o mayor velocidad de crecimiento. Además, estos resultados explican cómo las variantes genéticas bacterianas emergen en los ensayos de evolución bajo dosis subinhibitorias de los AE y CI ya que su proporción aumentaría con respecto a la cepa parental, debido a su menor fase de latencia y/o mayor velocidad de crecimiento, a lo largo de los ciclos evolutivos y, de este modo, se facilitaría su posterior aislamiento y estudio.

6. Las curvas de supervivencia de las variantes genéticas bacterianas, procedentes tanto de ensayos de evolución a concentraciones subinhibitorias como de tratamientos letales, revelaron un aumento de la tolerancia microbiana con respecto a la cepa parental. Estos resultados también demuestran cómo la mayor supervivencia de las variantes resistentes conduciría a un aumento progresivo de su proporción con respecto a la cepa parental, a lo largo de los ciclos del ensayo de evolución mediante tratamientos letales, lo que permite su posterior aislamiento y estudio.

7. Algunas de las variantes aisladas por AE y CI mostraron resistencia cruzada frente a otras tecnologías de conservación de alimentos. SaCar mostró un incremento de resistencia cruzada frente al calor, causado por una mejora de la reparación del daño en su membrana citoplasmática y no por un aumento de su resistencia intrínseca.

8. Del mismo modo, algunas de las variantes aisladas mostraron resistencia cruzada frente a antibióticos de uso común en medicina. SeLCar y SeTCO exhibieron un aumento de resistencia global principalmente frente a tetraciclinas, aminoglucósidos y quinolonas, pero no frente a betalactámicos. Por otro lado, LmSTCO aumentó su resistencia frente a betalactámicos y LmLTCO frente a antibióticos con distintas estructuras diana.

9. La secuenciación genómica de las variantes genéticas resistentes permitió la identificación de mutaciones localizadas en genes codificantes de reguladores transcripcionales relacionados con la respuesta celular al estrés oxidativo (*soxR, yfhP*), enzimas relacionadas con la síntesis y reparación de las membranas celulares (*accA*, lmo1647), proteínas receptoras y de transporte de membrana (*fepA*, *nirC*, *trkA*) y diversas enzimas relacionadas con el metabolismo celular (*aroC, hepT*, *nirB*),

entre otras. Los SNVs en *soxR* revelaron ser una de las principales causas del aumento de resistencia y tolerancia frente a los AE y CI, así como de la resistencia cruzada frente a antibióticos, en las variantes de *S.* Typhimurium. De este modo, estos resultados respaldarían que el estrés oxidativo se encuentra implicado en el mecanismo de inhibición e inactivación de los AE y CI y apuntarían a las envolturas celulares como una de las principales estructuras diana de estos antimicrobianos, así como de las diferentes tecnologías de conservación frente a las que se observó resistencia cruzada.

10. El aumento de resistencia y tolerancia de las variantes genéticas aisladas frente a AE se mantuvo e incluso fue mayor en un alimento modelo, leche desnatada, revelando el potencial riesgo microbiológico por la aparición de estas variantes en la cadena alimentaria.

En relación al objetivo de facilitar y potenciar el empleo de AE y CI como conservantes alimentarios o biocidas mediante la preparación de emulsiones y encapsulados que eviten su degradación y mejoren su solubilidad, se obtuvo la siguiente conclusión:

11. Tanto las nanoemulsiones por inversión de fases con tween 80 y etanol (tamaño de partícula < 200 nm), como las encapsulaciones en zeínas y ciclodextrinas (HP-CD), dieron lugar a preparaciones de AE y CI estables en el tiempo y solubles en agua:
    - Las nanoemulsiones de citral mostraron un incremento de la actividad bacteriostática y bactericida con respecto al CI en su forma libre, y un elevado efecto sinérgico con tratamientos térmicos suaves en zumo de manzana.
    - El AE de tomillo encapsulado en zeínas mostró una mayor actividad bacteriostática que en su forma libre, mientras que su actividad bactericida en tratamientos cortos se vio reducida. Por otro lado, se observó una fuerte sinergia con el calor alcanzando una inactivación similar en su forma encapsulada o libre.
    - El estudio de complejación y estabilidad de CI con HP-CD mostró una encapsulación más eficiente y estable con HP-β-CD. Sin embargo, la menor estabilidad de los encapsulados HP-α-CD y HP-γ-CD se relacionó con una mayor liberación de los CI y, por tanto, con una mayor actividad antimicrobiana. Por otro lado, la liofilización fue la técnica de elección para la preparación de encapsulados en formato polvo frente al secado por pulverización, al verse afectada en menor medida la actividad antimicrobiana de los CI.

Conclusions

Based on the objectives of this PhD thesis, the results obtained with regard to the development of new tools for the study of the mechanisms of cellular resistance against antimicrobials compounds and other methods of food preservation have led to the following conclusions:

1. Evolution assays carried out by prolonged exposure to IC at subinhibitory concentrations during bacterial growth enabled the isolation of resistant genetic variants in Gram-negative bacteria:

   - *Salmonella* Typhimurium isolated in presence of carvacrol (SeSCar),

   and, for the first time, in Gram-positive bacteria, specifically in:

   - *Staphylococcus aureus* isolated in presence of carvacrol (SaCar), citral (SaCit) and limonene oxide (SaOxLim).

2. Evolution assays based on the application of cyclic short lethal treatments with IC allowed for the first time the isolation of resistant bacterial genetic variants, specifically in:

   - *S.* Typhimurium isolated after lethal carvacrol treatments (SeLCar).

3. Both evolution assay protocols evidenced the isolation of resistant genetic variants in Gram-positive and Gram-negative bacteria for the first time against complex EOs:

   - *S. aureus* in presence of orange EO at subinhibitory concentrations (SaROEO).
   - *S.* Typhimurium isolated after lethal thyme EO treatments (SeTCO).
   - *Listeria monocytogenes* isolated in presence of thyme EO at subinhibitory concentrations (LmSTCO) or lethal treatments (LmLTCO).

4. The emergence of resistant genetic variants was not triggered by an increase in mutagenic frequency of EOs and ICs, as demonstrated for some antibiotics such as norfloxacin and rifampicin. Thus, the use of these natural antimicrobials would not induce the generation of resistant variants, indeed it would rather exert a selective pressure that would facilitate their evolution and subsequent selection and dominance over the less resistant subpopulations.

5. Growth kinetics of bacterial genetic variants in the presence of EOs and ICs revealed their shorter latency phase and/or higher growth rate. Furthermore, these results

might explain the emergence of bacterial genetic variants in the evolution assays under subinhibitory doses of EOs and ICs: their numbers would increase with regard to the parental strain, due to their shorter latency phase and/or higher growth rate, throughout the evolution cycles. The highest concentration of resistant variants with regard to parental strain would facilitate their isolation and subsequent studies.

6. Survival curves of resistant variants, from both evolution assays at subinhibitory concentrations and lethal treatments, revealed their increase in tolerance compared to parental strain. These results also demonstrate that the increased survival of resistant variants would lead to a progressive rise in their concentration with regard to parental strain throughout cycles of lethal treatments, allowing their isolation and subsequent studies.

7. Some of the resistant variants showed cross-resistance to other food preservation technologies. SaCar exhibited increased cross-resistance to heat, not by an increase in its intrinsic heat resistance, but by an enhanced repair of heat damage in the cell membrane.

8. Similarly, some of the isolated variants showed cross-resistance to antibiotics commonly used in medicine. SeLCar and SeTCO showed increased resistance to most of the antibiotics tested, mainly tetracyclines, aminoglycosides and quinolones, but not to beta-lactams. On the other hand, LmSTCO revealed an increased resistance to beta-lactams, and LmLTCO to several antibiotics with different target structures.

9. Genomic sequencing of the resistant genetic variants allowed the identification of mutations located in genes encoding transcriptional regulators related to the cellular response to oxidative stress (*soxR*, *yfhP*); enzymes related to synthesis and repair of cell membrane (*accA*, lmo1647); receptor and membrane transport proteins (*fepA*, *nirC*, *trkA*); and several enzymes related to cell metabolism (*aroC*, *hepT*, *nirB*), among others. SNVs in *soxR* were found to be one of the main causes of increased resistance and tolerance to EOs and ICs, as well as cross-resistance to antibiotics, in *S.* Typhimurium variants. These results would support that oxidative stress is involved in the mechanism of inhibition and inactivation of EOs and ICs and would point to cell envelopes as one of the main target structures of these antimicrobial, as well as of the different food preservation technologies against which cross-resistance was observed.

10. The increased resistance and tolerance of the isolated genetic variants to EOs was maintained and even increased in a model food, skimmed milk, revealing the potential microbiological risk that might be caused by the emergence of these variants in the food chain.

With regard to the objective of facilitating and improving the use of EOs and ICs as food preservatives or biocides by the preparation of emulsions and encapsulations that prevent their degradation and improve their solubility, the following conclusion was achieved:

11. Both nanoemulsions by phase inversion with tween 80 and ethanol (particle size < 200 nm) and encapsulations in zeins and cyclodextrins (HP-CD) resulted in time-stable and water-soluble EOs and ICs preparations:

    - Citral nanoemulsions showed increased bacteriostatic and bactericidal activity compared to IC in its free form, and a high synergistic effect with mild heat treatments in apple juice.

    - Thyme EO encapsulated in zeins showed higher bacteriostatic activity than in its free form, while its bactericidal activity in short lethal treatments was reduced. On the other hand, a great synergy with heat was observed, reaching a similar inactivation both in its encapsulated and free form.

    - The complexation and stability studies of IC with HP-CD showed a more efficient and stable encapsulation with HP-β-CD. However, the lower stability of HP-α-CD and HP-γ-CD complexes was related to a higher release of the ICs and, therefore, to a greater antimicrobial activity. On the other hand, freeze-drying was a better technique than spray-drying for the preparation of powdered encapsulates, since the antimicrobial activity of the ICs was less affected by freeze-drying.

7. Bibliografía

7. Bibliografía

Abdel-Aziz, S. M., Asker, M. M. S., Keera, A. A., & Mahmoud, M. G. (2016). Microbial food spoilage: control strategies for shelf life extension. In N. Garg, S. M. Abdel-Aziz & A. Aeron (Eds.), *Microbes in Food and Health* (pp. 239-264). Springer International Publishing.

Abee, T., Koomen, J., Metselaar, K. I., Zwietering, M. H., & den Besten, H. M. (2016). Impact of pathogen population heterogeneity and stress-resistant variants on food safety. *Annual review of food science and technology, 7*, 439-456.

Abee, T., & Wouters, J. A. (1999). Microbial stress response in minimal processing. *International Journal of Food Microbiology, 50* (1), 65-91.

Alvarez-Molina, A., de Toro, M., Ruiz, L., Lopez, M., Prieto, M., & Alvarez-Ordonez, A. (2020). Selection for antimicrobial resistance in foodborne pathogens through exposure to UV light and nonthermal atmospheric plasma decontamination techniques. *Applied and Environmental Microbiology, 86* (9), 12.

Alvarez-Ordonez, A., Begley, M., Clifford, T., Deasy, T., Collins, B., & Hill, C. (2014). Transposon mutagenesis reveals genes involved in osmotic stress and drying in *Cronobacter sakazakii*. *Food Research International, 55*, 45-54.

Alvarez-Ordóñez, A., Broussolle, V., Colin, P., Nguyen-The, C., & Prieto, M. (2015). The adaptive response of bacterial food-borne pathogens in the environment, host and food: Implications for food safety. *International Journal of Food Microbiology, 213*, 99-109.

Anaya-Castro, M. A., Ayala-Zavala, J. F., Muñoz-Castellanos, L., Hernández-Ochoa, L., Peydecastaing, J., & Durrieu, V. (2017). β-Cyclodextrin inclusion complexes containing clove (*Eugenia caryophyllata*) and Mexican oregano (*Lippia berlandieri*) essential oils: preparation, physicochemical and antimicrobial characterization. *Food Packaging and Shelf Life, 14*, 96-101.

Andersson, D. I., & Hughes, D. (2014). Microbiological effects of sublethal levels of antibiotics. *Nature Reviews: Microbiology, 12* (7), 465-478.

Apolonio, J., Faleiro, M. L., Miguel, M. G., & Neto, L. (2014). No induction of antimicrobial resistance in *Staphylococcus aureus* and *Listeria monocytogenes* during continuous exposure to eugenol and citral. *FEMS Microbiology Letters, 354* (2), 92-101.

Årdal, C., Balasegaram, M., Laxminarayan, R., McAdams, D., Outterson, K., Rex, J. H., & Sumpradit, N. (2019). Antibiotic development - economic, regulatory and societal challenges. *Nature Reviews: Microbiology, 18*, 267-274.

Arroyo, C., Somolinos, M., Cebrián, G., Condón, S., & Pagán, R. (2010). Pulsed electric fields cause sublethal injuries in the outer membrane of *Enterobacter sakazakii* facilitating the antimicrobial activity of citral. *Letters in Applied Microbiology, 51* (5), 525-531.

Astray, G., Gonzalez-Barreiro, C., Mejuto, J. C., Rial-Otero, R., & Simal-Gándara, J. (2009). A review on the use of cyclodextrins in foods. *Food Hydrocolloids, 23* (7), 1631-1640.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., & Mori, H. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular Systems Biology, 2* (1), 2006.0008.

Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., Brynildsen, M. P., Bumann, D., Camilli, A., Collins, J. J., Dehio, C., Fortune, S., Ghigo, J.-M., Hardt, W.-D., Harms, A., Heinemann, M., Hung, D. T., Jenal, U., Levin, B. R., Michiels, J., Storz, G., Tan, M.-W., Tenson, T., Van Melderen, L., & Zinkernagel, A. (2019). Definitions and guidelines for research on antibiotic persistence. *Nature Reviews Microbiology, 17* (7), 441-448.

Barbosa, L. N., Alves, F. C. B., Andrade, B., Albano, M., Rall, V. L. M., Fernandes, A. A. H., Buzalaf, M. A. R., Leite, A. L., de Pontes, L. G., Dos Santos, L. D., & Fernandes Junior, A. (2020). Proteomic analysis and antibacterial resistance mechanisms of *Salmonella* Enteritidis submitted to the inhibitory effect of *Origanum vulgare* essential oil, thymol and carvacrol. *Journal of Proteomics, 214*, 103625.

Bearson, B. L., Bearson, S. M. D., Looft, T., Cai, G., & Shippy, D. C. (2017). Characterization of a multidrug-resistant *Salmonella enterica* Serovar Heidelberg outbreak strain in commercial turkeys: colonization, transmission, and host transcriptional response. *Frontiers in Veteterinary Science, 4*, 156.

Begley, M., & Hill, C. (2015). Stress adaptation in foodborne pathogens. *Annual review of food science and technology, 6* (1), 191-210.

Ben Jemaa, M., Falleh, H., Neves, M. A., Isoda, H., Nakajima, M., & Ksouri, R. (2017). Quality preservation of deliberately contaminated milk using thyme free and nanoemulsified essential oils. *Food Chemistry, 217*, 726-734.

Ben Jemaa, M., Falleh, H., Saada, M., Oueslati, M., Snoussi, M., & Ksouri, R. (2018). *Thymus capitatus* essential oil ameliorates pasteurization efficiency. *Journal of Food Science and Technology, 55* (9), 3446-3452.

Bergspica, I., Kaprou, G., Alexa, E. A., Prieto-Maradona, M., & Alvarez-Ordonez, A. (2020). Identification of risk factors and hotspots of antibiotic resistance along the food chain using next-generation sequencing. *EFSA Journal, 18*, 8.

Bhavaniramya, S., Vishnupriya, S., Al-Aboody, M. S., Vijayakumar, R., & Baskaran, D. (2019). Role of essential oils in food safety: antimicrobial and antioxidant applications. *Grain & Oil Science and Technology, 2* (2), 49-55.

Blázquez, J. (2003). Hypermutation as a factor contributing to the acquisition of antimicrobial resistance. *Clinical Infectious Diseases, 37* (9), 1201-1209.

Booyens, J., & Thantsha, M. S. (2014). Fourier transform infra-red spectroscopy and flow cytometric assessment of the antibacterial mechanism of action of aqueous extract of garlic (*Allium sativum*) against selected probiotic Bifidobacterium strains. *BMC complementary and alternative medicine, 14*, 289-289.

Bouyahya, A., Abrini, J., Dakka, N., & Bakri, Y. (2019). Essential oils of *Origanum compactum* increase membrane permeability, disturb cell membrane integrity, and suppress quorum-sensing phenotype in bacteria. *Journal of pharmaceutical analysis, 9* (5), 301-311.

Bowman, J. P., Bittencourt, C. R., & Ross, T. (2008). Differential gene expression of *Listeria monocytogenes* during high hydrostatic pressure processing. *Microbiology, 154* (2), 462-475.

Brauner, A., Fridman, O., Gefen, O., & Balaban, N. Q. (2016). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nature Reviews Microbiology, 14* (5), 320-330.

Bravo-Ferrada, B. M., Gonçalves, S., Semorile, L., Santos, N. C., Tymczyszyn, E. E., & Hollmann, A. (2015). Study of surface damage on cell envelope assessed by AFM and flow cytometry of *Lactobacillus plantarum* exposed to ethanol and dehydration. *Journal of Applied Microbiology, 118* (6), 1409-1417.

Brewer, M. S. (2011). Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. *Comprehensive Reviews in Food Science and Food Safety, 10* (4), 221-247.

Brown, A. C., Grass, J. E., Richardson, L. C., Nisler, A. L., Bicknese, A. S., & Gould, L. H. (2017). Antimicrobial resistance in *Salmonella* that caused foodborne disease outbreaks: United States, 2003-2012. *Epidemiology & Infection, 145* (4), 766-774.

Burgess, C. M., Gianotti, A., Gruzdev, N., Holah, J., Knøchel, S., Lehner, A., Margas, E., Esser, S. S., Sela, S., & Tresse, O. (2016). The response of foodborne pathogens to osmotic and desiccation stresses in the food chain. *International Journal of Food Microbiology, 221*, 37-53.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods - a review. *International Journal of Food Microbiology, 94* (3), 223-253.

Burt, S. A., Vlielander, R., Haagsman, H. P., & Veldhuizen, E. J. (2005). Increase in activity of essential oil components carvacrol and thymol against Escherichia coli O157: H7 by addition of food stabilizers. *Journal of Food Protection, 68* (5), 919-926.

Calo, J. R., Crandall, P. G., O'Bryan, C. A., & Ricke, S. C. (2015). Essential oils as antimicrobials in food systems - a review. *Food Control, 54*, 111-119.

Cao, M., Bitar, A. P., & Marquis, H. (2007). A *mariner*-Based Transposition System for *Listeria monocytogenes*. *Applied and Environmental Microbiology, 73* (8), 2758.

Carocho, M., Barreiro, M. F., Morales, P., & Ferreira, I. C. F. R. (2014). Adding molecules to food, pros and cons: a review on synthetic and natural food additives. *13* (4), 377-399.

Cebrián, G., Condón, S., & Mañas, P. (2017). Physiology of the inactivation of vegetative bacteria by thermal treatments: mode of action, influence of environmental factors and inactivation kinetics. *Foods (Basel, Switzerland), 6* (12), 107.

Chakraborty, S., & Kenney, L. J. (2018). A new role of OmpR in acid and osmotic stress in *Salmonella* and *E. coli*. *Frontiers in Microbiology, 9* (2656).

Chen, J., Tang, C., Zhang, R., Ye, S., Zhao, Z., Huang, Y., Xu, X., Lan, W., & Yang, D. (2020). Metabolomics analysis to evaluate the antibacterial activity of the essential oil from the leaves of *Cinnamomum camphora* (Linn.) Presl. *Journal of ethnopharmacology, 253*, 112652.

Chueca, B., Berdejo, D., Gomes-Neto, N. J., Pagán, R., & García-Gonzalo, D. (2016). Emergence of hyper-resistant *Escherichia coli* MG1655 derivative strains after applying sub-inhibitory doses of individual constituents of essential oils. *Frontiers in Microbiology, 7*, 273-273.

Chueca, B., Pagán, R., & García-Gonzalo, D. (2014a). Differential mechanism of *Escherichia coli* inactivation by (+)-limonene as a function of cell physiological state and drug's concentration. *PLOS ONE, 9* (4), e94072.

Chueca, B., Pagán, R., & García-Gonzalo, D. (2014b). Oxygenated monoterpenes citral and carvacrol cause oxidative damage in *Escherichia coli* without the involvement of tricarboxylic acid cycle and Fenton reaction. *International Journal of Food Microbiology, 189*, 126-131.

Chueca, B., Pagán, R., & García-Gonzalo, D. (2015). Transcriptomic analysis of *Escherichia coli* MG1655 cells exposed to pulsed electric fields. *Innovative Food Science & Emerging Technologies, 29*, 78-86.

Chueca, B., Perez-Saez, E., Pagan, R., & Garcia-Gonzalo, D. (2017). Global transcriptional response of *Escherichia coli* MG1655 cells exposed to the oxygenated monoterpenes citral and carvacrol. *International Journal of Food Microbiology, 257*, 49-57.

Chueca, B., Renzoni, A., Berdejo, D., Pagan, R., Kelley, W. L., & Garcia-Gonzalo, D. (2018). Whole-genome sequencing and genetic analysis reveal novel stress responses to individual constituents of essential oils in *Escherichia coli*. *Applied and Environmental Microbiology, 84* (7).

Collins, B., Curtis, N., Cotter, P. D., Hill, C., & Ross, R. P. (2010). The ABC transporter AnrAB contributes to the innate resistance of *Listeria monocytogenes* to nisin, bacitracin, and various beta-lactam antibiotics. *Antimicrobial Agents & Chemotherapy, 54* (10), 4416-4423.

Croucher, N. J., & Thomson, N. R. (2010). Studying bacterial transcriptomes using RNA-seq. *Current Opinion in Microbiology, 13* (5), 619-624.

Crucello, A., Furtado, M. M., Chaves, M. D. R., & Sant'Ana, A. S. (2019). Transcriptome sequencing reveals genes and adaptation pathways in *Salmonella* Typhimurium inoculated in four low water activity foods. *Food Microbiology, 82*, 426-435.

De Maio, N., Shaw, L. P., Hubbard, A., George, S., Sanderson, N. D., Swann, J., Wick, R., AbuOun, M., Stubberfield, E., Hoosdally, S. J., Crook, D. W., Peto, T. E. A., Sheppard, A. E., Bailey, M. J., Read, D. S., Anjum, M. F., Walker, A. S. & Stoesser, N. (2019). Comparison of long-read sequencing technologies in the hybrid assembly of complex bacterial genomes. *Microbial genomics, 5* (9).

De Mey, M., Maertens, J., Lequeux, G. J., Soetaert, W. K., & Vandamme, E. J. (2007). Construction and model-based analysis of a promoter library for *E. coli*: an indispensable tool for metabolic engineering. *BMC biotechnology, 7* (1), 34.

de Souza, E. L. (2016). The effects of sublethal doses of essential oils and their constituents on antimicrobial susceptibility and antibiotic resistance among food-related bacteria: a review. *Trends in Food Science & Technology, 56*, 1-12.

de Souza, E. L., da Cruz Almeida, E. T., & de Sousa Guedes, J. P. (2016). The potential of the incorporation of essential oils and their individual constituents to improve microbial safety in juices: a review. *Comprehensive Reviews in Food Science and Food Safety, 15* (4), 753-772.

Dima, C., Cotarlet, M., Tiberius, B., Hahrim, G., Alexe, P., Dima, S. (2014). Encapsulation of coriander essential oil in beta-cyclodextrin: antioxidant and antimicrobial properties evaluation. *Romanian Biotechnological Letters, 19* (2), 9128-9140.

Donsì, F., Annunziata, M., Sessa, M., & Ferrari, G. (2011). Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *LWT - Food Science and Technology, 44* (9), 1908-1914.

Drake, J. W. (1991). A constant rate of spontaneous mutation in DNA-based microbes. *Proceedings of the National Academy of Sciences of the United States of America, 88* (16), 7160.

Duru, I. C., Bucur, F. I., Andreevskaya, M., Nikparvar, B., Ylinen, A., Grigore-Gurgu, L., Rode, T. M., Crauwels, P., Laine, P., Paulin, L., Løvdal, T., Riedel, C. U., Bar, N., Borda, D., Nicolau, A. I., & Auvinen, P. (2021). High-pressure processing-induced transcriptome response during recovery of *Listeria monocytogenes*. *BMC Genomics, 22* (1), 117.

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control). (2019). The European Union One Health 2018 zoonoses report. *EFSA Journal*, 17 (12), 5926.

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control). (2021). The European Union One Health 2019 zoonoses report. *EFSA Journal*, 19 (2), 6406.

Erkmen, O., & Bozoglu, T. F. (2016). Food preservation by combination of techniques (hurdle technology). In O. Erkmen and T.F. Bozoglu (Eds.), *Food microbiology: Principles into practice* (pp. 166-180). John Wiley & Sons.

Espina, L., Gelaw, T. K., de Lamo-Castellvi, S., Pagan, R., & García-Gonzalo, D. (2013). Mechanism of bacterial inactivation by (+)-limonene and its potential use in food preservation combined processes. *PLOS ONE, 8* (2), e56769.

Espina, L., Somolinos, M., Lorán, S., Conchello, P., García, D., & Pagán, R. (2011). Chemical composition of commercial citrus fruit essential oils and evaluation of their antimicrobial activity acting alone or in combined processes. *Food Control, 22* (6), 896-902.

Ferenci, T. (2008). The spread of a beneficial mutation in experimental bacterial populations: the influence of the environment and genotype on the fixation of *rpoS* mutations. *Heredity, 100* (5), 446-452.

Fisher, K., & Phillips, C. A. (2006). The effect of lemon, orange and bergamot essential oils and their components on the survival of *Campylobacter jejuni*, *Escherichia coli* O157, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* in vitro and in food systems. *Journal of Applied Microbiology, 101* (6), 1232-1240.

Foster, P. L. (2007). Stress-induced mutagenesis in bacteria. *Critical reviews in biochemistry and molecular biology, 42* (5), 373-397.

Fridman, O., Goldberg, A., Ronin, I., Shoresh, N., & Balaban, N. Q. (2014). Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature, 513* (7518), 418-421.

Fried, L., Lassak, J., & Jung, K. (2012). A comprehensive toolbox for the rapid construction of *lacZ* fusion reporters. *Journal of Microbiological Methods, 91* (3), 537-543.

García-Gonzalo, D., & Pagán, R. (2016). Detection of electroporation in microbial cells: techniques and procedures. In D. Miklavcic (Ed.), *Handbook of electroporation*. Springer International Publishing.

García, D., Mañas, P., Gómez, N., Raso, J., & Pagán, R. (2006). Biosynthetic requirements for the repair of sublethal membrane damage in *Escherichia coli* cells after pulsed electric fields. *Journal of Applied Microbiology, 100* (3), 428-435.

Garibyan, L., Huang, T., Kim, M., Wolff, E., Nguyen, A., Nguyen, T., Diep, A., Hu, K., Iverson, A., Yang, H., & Miller, J. H. (2003). Use of the *rpoB* gene to determine the specificity of base substitution mutations on the *Escherichia coli* chromosome. *DNA Repair, 2* (5), 593-608.

Gayán, E., Cambré, A., Michiels, C. W., & Aertsen, A. (2016). Stress-induced evolution of heat resistance and resuscitation speed in *Escherichia coli* O157:H7 ATCC 43888. *Applied and Environmental Microbiology, 82* (22), 6656-6663.

Gomes-Neto, N. J., Luz, I. S., Franco, O. L., Magnani, M., & Souza, E. L. (2014). Tolerance evaluation in *Salmonella enterica* serovar Typhimurium challenged with sublethal amounts of *Rosmarinus officinalis* L. essential oil or 1,8-cineole in meat model. *International Journal of Food Science & Technology, 49* (8), 1912-1917.

Gomes Neto, N. J., Luz Ida, S., Tavares, A. G., Honorio, V. G., Magnani, M., & de Souza, E. L. (2012). *Rosmarinus officinalis* L. essential oil and its majority compound 1,8-cineole at sublethal amounts induce no direct and cross protection in *Staphylococcus aureus* ATCC 6538. *Foodborne Pathogens and Disease, 9* (12), 1071-1076.

Gomes Neto, N. J., Luz, I. S., Honório, W. G., Tavares, A. G., & de Souza, E. L. (2012). *Rosmarinus officinalis* L. essential oil and the related compound 1,8-cineole do not induce direct or cross-protection in *Listeria monocytogenes* ATCC 7644 cultivated in meat broth. *Can J Microbiol, 58* (8), 973-981.

Gouvea, F. S., Rosenthal, A., & Ferreira, E. H. (2017). Plant extract and essential oils added as antimicrobials to cheeses: a review. *Ciencia Rural*, *47*, 01-09.

Gu, M., & Imlay, J. A. (2011). The SoxRS response of *Escherichia coli* is directly activated by redox-cycling drugs rather than by superoxide. *Molecular Microbiology, 79* (5), 1136-1150.

Guernec, A., Robichaud-Rincon, P., & Saucier, L. (2013). Whole-genome transcriptional analysis of *Escherichia coli* during heat inactivation processes related to industrial cooking. *Applied and Environmental Microbiology, 79* (16), 4940.

Guo, Y., Winkler, J., & Kao, K. C. (2017). Insights on osmotic tolerance mechanisms in *Escherichia coli* gained from an *rpoC* mutation. *Bioengineering, 4* (3), 61.

Gutierrez, A., Laureti, L., Crussard, S., Abida, H., Rodriguez-Rojas, A., Blazquez, J., Baharoglu, Z., Mazel, D., Darfeuille, F., Vogel, J., & Matic, I. (2013). β-lactam antibiotics promote bacterial mutagenesis via an RpoS-mediated reduction in replication fidelity. *Nature Communications, 4*, 1610.

Hammer, K. A., Carson, C., & Riley, T. (1999). Antimicrobial activity of essential oils and other plant extracts. *Journal of Applied Microbiology, 86* (6), 985-990.

Hammer, K. A., Carson, C. F., & Riley, T. V. (2008). Frequencies of resistance to *Melaleuca alternifolia* (tea tree) oil and rifampicin in *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis*. *International Journal of Antimicrobial Agents, 32* (2), 170-173.

Hashemi, S. M. B., Khorram, S. B., & Sohrabi, M. (2017). Antioxidant activity of essential oils in foods. In S. M. B. Hashemi, A. M. Khaneghah & A. d. S. Sant'Ana (Eds.), *Essential oils in food processing* (pp. 247-265). Wiley Blackwell.

Hu, J., Zhang, Y., Xiao, Z., & Wang, X. (2018). Preparation and properties of cinnamon-thyme-ginger composite essential oil nanocapsules. *Industrial Crops and Products, 122*, 85-92.

Hudson, R. E., Bergthorsson, U., & Ochman, H. (2003). Transcription increases multiple spontaneous point mutations in *Salmonella enterica*. *Nucleic acids research, 31* (15), 4517-4522.

Huertas, J. P., Alvarez-Ordonez, A., Morrissey, R., Ros-Chumillas, M., Esteban, M. D., Mate, J., Palop, A., & Hill, C. (2015). Heat resistance of *Cronobacter sakazakii* DPC 6529 and its behavior in reconstituted powdered infant formula. *Food Research International, 69*, 401-409.

Imdahl, F., Vafadarnejad, E., Homberger, C., Saliba, A.-E., & Vogel, J. (2020). Single-cell RNA-sequencing reports growth-condition-specific global transcriptomes of individual bacteria. *Nature Microbiology, 5* (10), 1202-1206.

Jahn, C. E., Charkowski, A. O., & Willis, D. K. (2008). Evaluation of isolation methods and RNA integrity for bacterial RNA quantitation. *Journal of Microbiological Methods, 75* (2), 318-324.

Jee, J., Rasouly, A., Shamovsky, I., Akivis, Y., Steinman, S. R., Mishra, B., & Nudler, E. (2016). Rates and mechanisms of bacterial mutagenesis from maximum-depth sequencing. *Nature, 534* (7609), 693-696.

Jinks-Robertson, S., & Bhagwat, A. S. (2014). Transcription-associated mutagenesis. *Annual Review of Genetics, 48*, 341-359.

Jones, F. A. (1996). Herbs-useful plants. Their role in history and today. *European journal of gastroenterology & hepatology, 8* (12), 1227-1231.

Karash, S., Liyanage, R., Qassab, A., Lay, J. O., & Kwon, Y. M. (2017). A comprehensive assessment of the genetic determinants in *Salmonella* Typhimurium for resistance to hydrogen peroxide using proteogenomics. *Scientific Reports, 7* (1), 17073.

Kawada-Matsuo, M., Watanabe, A., Arii, K., Oogai, Y., Noguchi, K., Miyawaki, S., Hayashi, T., & Komatsuzawa, H. (2020). *Staphylococcus aureus* virulence affected by an alternative nisin a resistance mechanism. *Applied and Environmental Microbiology, 86* (8), 14.

Kim, S., Kim, Y., Suh, D. H., Lee, C. H., Yoo, S. M., Lee, S. Y., & Yoon, S. H. (2020). Heat-responsive and time-resolved transcriptome and metabolome analyses of *Escherichia coli* uncover thermo-tolerant mechanisms. *Scientific Reports, 10* (1), 17715-17715.

Kimura, K., Inaoka, T., & Yamamoto, K. (2018). Metabolome analysis of *Escherichia coli* ATCC25922 cells treated with high hydrostatic pressure at 400 and 600 MPa. *Journal of bioscience and bioengineering, 126* (5), 611-616.

Kiskó, G., & Roller, S. (2005). Carvacrol and p-cymene inactivate Escherichia coli O157: H7 in apple juice. *BMC microbiology, 5* (1), 1.

Knöppel, A., Knopp, M., Albrecht, L. M., Lundin, E., Lustig, U., Näsvall, J., & Andersson, D. I. (2018). Genetic adaptation to growth under laboratory conditions in *Escherichia coli* and *Salmonella enterica*. *Frontiers in Microbiology, 9*, 756-756.

Knorr, D., Froehling, A., Jaeger, H., Reineke, K., Schlueter, O., & Schoessler, K. (2011). Emerging technologies in food processing. *Annual review of food science and technology, 2* (1), 203-235.

Kohanski, M. A., DePristo, M. A., & Collins, J. J. (2010). Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Molecular cell, 37* (3), 311-320.

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., & Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell, 130* (5), 797-810.

Komaiko, J. S., & McClements, D. J. (2016). Formation of food-grade nanoemulsions using low-energy preparation methods: a review of available methods. *Comprehensive Reviews in Food Science and Food Safety, 15* (2), 331-352.

Kumariya, R., Garsa, A. K., Rajput, Y. S., Sood, S. K., Akhtar, N., & Patel, S. (2019). Bacteriocins: classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. *Microbial Pathogenesis, 128*, 171-177.

Lamas, A., Regal, P., Vázquez, B., Miranda, J. M., Franco, C. M., & Cepeda, A. (2019). Transcriptomics: a powerful tool to evaluate the behavior of foodborne pathogens in the food production chain. *Food Research International, 125*, 108543.

Leistner, L., & Gorris, L. G. M. (1995). Food preservation by hurdle technology. *Trends in Food Science & Technology, 6* (2), 41-46.

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., & Balaban, N. Q. (2017). Antibiotic tolerance facilitates the evolution of resistance. *Science, 355* (6327), 826-830.

Lewis, K., & Shan, Y. (2017). Why tolerance invites resistance. *Science, 355* (6327), 796-796.

Li, H., & Gänzle, M. (2016). Some like iIt hot: heat resistance of *Escherichia coli* in food. *Frontiers in Microbiology, 1763* (7).

Lis-Balchin, M., & Deans, S. (1997). Bioactivity of selected plant essential oils against *Listeria monocytogenes*. *Journal of Applied Microbiology, 82* (6), 759-762.

Liu, X., Omar, M., Abrahante, J. E., Nagaraja, K. V., & Vidovic, S. (2020). Insights into the oxidative stress response of *Salmonella enterica* serovar Enteritidis revealed by the next generation sequencing approach. *Antioxidants, 9* (9), 849.

Liu, Y., Ream, A., Joerger, R. D., Liu, J., & Wang, Y. (2011). Gene expression profiling of a pressure-tolerant *Listeria monocytogenes* Scott A *ctsR* deletion mutant. *Journal of Industrial Microbiology and Biotechnology, 38* (9), 1523-1533.

Liu, Z., Zhao, L., Zhang, Q., Huo, N., Shi, X., Li, L., Jia, L., Lu, Y., Peng, Y., & Song, Y. (2019). Proteomics-based mechanistic investigation of *Escherichia coli* inactivation by pulsed electric field. *Frontiers in Microbiology, 10* (2644).

Luz Ida, S., Gomes Neto, N. J., Tavares, A. G., Nunes, P. C., Magnani, M., & de Souza, E. L. (2012). Evidence for lack of acquisition of tolerance in *Salmonella enterica* serovar Typhimurium ATCC 14028 after exposure to subinhibitory amounts of *Origanum vulgare* L. essential oil and carvacrol. *Applied and Environmental Microbiology, 78* (14), 5021-5024.

Luz, I. d. S., Gomes Neto, N. J., Tavares, A. G., Nunes, P. C., Magnani, M., & de Souza, E. L. (2013). Lack of induction of direct protection or cross-protection in *Staphylococcus aureus* by sublethal concentrations of *Origanum vulgare* L. essential oil and carvacrol in a meat-based medium. *Archives of Microbiology, 195* (8), 587-593.

Luz, I. d. S., Neto, N. J. G., Tavares, A. G., Magnani, M., & de Souza, E. L. (2012). Exposure of *Listeria monocytogenes* to sublethal amounts of *Origanum vulgare* L. essential oil or carvacrol in a food-based medium does not induce direct or cross protection. *Food Research International, 48* (2), 667-672.

Mackey, B. M. (2000). Injured bacteria. In B. Lund, A. C. Baird-Parker & G. W. Gould (Eds.), *The microbiological safety and quality of food* (Vol. I, pp. 315–341). Aspen Publisher.

Mackey, B. M., Miles, C. A., Parsons, S. E., & Seymour, D. A. (1991). Thermal denaturation of whole cells and cell components of *Escherichia coli* examined by differential scanning calorimetry. *Journal of General Microbiology, 137* (10), 2361-2374.

Maes, C., Bouquillon, S., & Fauconnier, M. L. (2019). Encapsulation of essential oils for the development of biosourced pesticides with controlled release: a review. *Molecules, 24* (14), 2539.

Magdanova, L. A., & Golyasnaya, N. V. (2013). Heterogeneity as an adaptive trait of microbial populations. *Microbiology, 82* (1), 1-10.

Mañas, P., & Pagán, R. (2005). Microbial inactivation by new technologies of food preservation. *Journal of Applied Microbiology, 98* (6), 1387-1399.

Mao, E. F., Lane, L., Lee, J., & Miller, J. H. (1997). Proliferation of mutators in A cell population. *Journal of Bacteriology, 179* (2), 417-422.

Marcén, M., Ruiz, V., Serrano, M. J., Condón, S., & Mañas, P. (2017). Oxidative stress in *E. coli* cells upon exposure to heat treatments. *International Journal of Food Microbiology, 241*, 198-205.

Massey, R. C., & Buckling, A. (2002). Environmental regulation of mutation rates at specific sites. *Trends in Microbiology, 10* (12), 580-584.

Maté, J., Periago, P. M., & Palop, A. (2016). When nanoemulsified, d-limonene reduces *Listeria monocytogenes* heat resistance about one hundred times. *Food Control, 59*, 824-828.

McMahon, C. M. M., Byrne, C. M., Sheridan, J. J., McDowell, D. A., Blair, I. S., & Hegarty, T. (2000). The effect of culture growth phase on induction of the heat shock response in *Yersinia enterocolitica* and *Listeria monocytogenes*. *Journal of Applied Microbiology, 89* (2), 198-206.

McMillan, E. A., Gupta, S. K., Williams, L. E., Jové, T., Hiott, L. M., Woodley, T. A., Barrett, J. B., Jackson, C. R., Wasilenko, J. L., Simmons, M., Tillman, G. E., McClelland, M., & Frye, J. G. (2019). Antimicrobial resistance genes, cassettes, and plasmids present in *Salmonella enterica* associated with United States food animals. *Frontiers in Microbiology, 10*, 832-832.

Mercer, R. G., Zheng, J., Garcia-Hernandez, R., Ruan, L., Gänzle, M. G., & McMullen, L. M. (2015). Genetic determinants of heat resistance in *Escherichia coli*. *Frontiers in Microbiology, 6*, 932-932.

Metselaar, K. I., den Besten, H. M., Abee, T., Moezelaar, R., & Zwietering, M. H. (2013). Isolation and quantification of highly acid resistant variants of *Listeria monocytogenes*. *International Journal of Food Microbiology, 166* (3), 508-514.

Metzker, M. L. (2010). Sequencing technologies - the next generation. *Nature Reviews Genetics, 11* (1), 31-46.

Miller, A. J., Bayles, D. O., & Eblen, B. S. (2000). Cold shock induction of thermal sensitivity in *Listeria monocytogenes*. *Applied and Environmental Microbiology, 66* (10), 4345-4350.

Mishra, A. P., Devkota, H. P., Nigam, M., Adetunji, C. O., Srivastava, N., Saklani, S., Shukla, I., Azmi, L., Shariati, M. A., Melo Coutinho, H. D., & Mousavi Khaneghah, A. (2020). Combination of essential oils in dairy products: a review of their functions and potential benefits. *LWT - Food Science and Technology, 133*, 110116.

Mittal, R. P., Rana, A., & Jaitak, V. (2019). Essential oils: an impending substitute of synthetic antimicrobial agents to overcome antimicrobial resistance. *Current drug targets, 20* (6), 605-624.

Moghimi, R., Aliahmadi, A., McClements, D. J., & Rafati, H. (2016). Investigations of the effectiveness of nanoemulsions from sage oil as antibacterial agents on some food borne pathogens. *LWT - Food Science and Technology, 71*, 69-76.

Moghimi, R., Ghaderi, L., Rafati, H., Aliahmadi, A., & McClements, D. J. (2016). Superior antibacterial activity of nanoemulsion of *Thymus daenensis* essential oil against *E. coli*. *Food Chemistry, 194*, 410-415.

Montville, T. J., & Matthews, K. R. (2013). Physiology, growth, and inhibition of microbes in foods. In M. P. Doyle & R. L. Buchanan (Eds.), *Food Microbiology*. American Society of Microbiology.

Mortelmans, K., & Zeiger, E. (2000). The Ames *Salmonella*/microsome mutagenicity assay. *Mutation research, 455* (1-2), 29-60.

Nguyen, H. T., Corry, J. E., & Miles, C. A. (2006). Heat resistance and mechanism of heat inactivation in thermophilic campylobacters. *Applied and Environment Microbiology, 72* (1), 908-913.

Nishant, K. T., Singh, N. D., & Alani, E. (2009). Genomic mutation rates: what high-throughput methods can tell us. *BioEssays : news and reviews in molecular, cellular and developmental biology, 31* (9), 912-920.

Noster, J., Chao, T.-C., Sander, N., Schulte, M., Reuter, T., Hansmeier, N., & Hensel, M. (2019). Proteomics of intracellular *Salmonella enterica* reveals roles of *Salmonella* pathogenicity island 2 in metabolism and antioxidant defense. *PLOS Pathogens, 15* (4), e1007741-e1007741.

Oniciuc, E. A., Likotrafiti, E., Alvarez-Molina, A., Prieto, M., Lopez, M., & Alvarez-Ordonez, A. (2019). Food processing as a risk factor for antimicrobial resistance spread along the food chain. *Current Opinion in Food Science, 30*, 21-26.

Pagán, R., Condon, S., & Sala, F. J. (1997). Effects of several factors on the heat-shock-induced thermotolerance of *Listeria monocytogenes*. *Applied and Environment Microbiology, 63* (8), 3225-3232.

Pandey, A. K., Kumar, P., Singh, P., Tripathi, N. N., & Bajpai, V. K. (2017). Essential oils: sources of antimicrobials and food preservatives. *Frontiers in Microbiology, 7* (2161).

Parry-Hanson Kunadu, A., Holmes, M., Miller, E. L., & Grant, A. J. (2018). Microbiological quality and antimicrobial resistance characterization of *Salmonella* spp. in fresh milk value chains in Ghana. *International Journal of Food Microbiology, 277*, 41-49.

Patange, A., O'Byrne, C., Boehm, D., Cullen, P. J., Keener, K., & Bourke, P. (2019). The effect of atmospheric cold plasma on bacterial stress responses and virulence using *Listeria monocytogenes* knockout mutants. *Frontiers in Microbiology, 10*, 2841-2841.

Patel, A. R., & Velikov, K. P. (2014). Zein as a source of functional colloidal nano- and microstructures. *Current Opinion in Colloid & Interface Science, 19* (5), 450-458.

Peterson, E., & Kaur, P. (2018). Antibiotic resistance mechanisms in bacteria: relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. *Frontiers in Microbiology, 9* (2928).

Pomposiello, P. J., Bennik, M. H., & Demple, B. (2001). Genome-wide transcriptional profiling of the *Escherichia coli* responses to superoxide stress and sodium salicylate. *Journal of Bacteriology, 183* (13), 3890-3902.

Pontes, M. H., & Groisman, E. A. (2019). Slow growth determines nonheritable antibiotic resistance in *Salmonella enterica*. *Science Signaling, 12* (592).

Porwollik, S., Santiviago, C. A., Cheng, P., Long, F., Desai, P., Fredlund, J., Srikumar, S., Silva, C. A., Chu, W., Chen, X., Canals, R., Reynolds, M. M., Bogomolnaya, L., Shields, C., Cui, P., Guo, J., Zheng, Y., Endicott-Yazdani, T., Yang, H.-J., Maple, A., Ragoza, Y., Blondel, C. J., Valenzuela, C., Andrews-Polymenis, H., & McClelland, M. (2014). Defined single-gene and multi-gene deletion mutant collections in *Salmonella enterica* sv Typhimurium. *PLOS ONE, 9* (7), e99820.

Poulsen, L. D., & Vinther, J. (2018). RNA-Seq for bacterial gene expression. *Current Protocols in Nucleic Acid Chemistry, 73* (1), e55.

Qamar, A., Ismail, T., & Akhtar, S. (2020). Prevalence and antibiotic resistance of *Salmonella* spp. in South Punjab-Pakistan. *PLOS ONE, 15* (11), e0232382.

Qi, Y., Zhao, W., Wang, T., Pei, F., Yue, M., Li, F., Liu, X., Wang, X., & Li, H. (2020). Proteomic analysis of the antimicrobial effects of sublethal concentrations of thymol on

Salmonella enterica serovar Typhimurium. *Applied Microbiology and Biotechnology, 104* (8), 3493-3505.

Quinto, E. J., Caro, I., Villalobos-Delgado, L. H., Mateo, J., De-Mateo-Silleras, B., & Redondo-Del-Río, M. P. (2019). Food safety through natural antimicrobials. *Antibiotics, 8* (4), 208.

Ragno, R., Papa, R., Patsilinakos, A., Vrenna, G., Garzoli, S., Tuccio, V., Fiscarelli, E., Selan, L., & Artini, M. (2020). Essential oils against bacterial isolates from cystic fibrosis patients by means of antimicrobial and unsupervised machine learning approaches. *Scientific Reports, 10* (1), 2653.

Ritota, M., & Manzi, P. (2020). Natural preservatives from plant in cheese making. *Animals, 10* (4), 749.

Ryall, B., Eydallin, G., & Ferenci, T. (2012). Culture history and population heterogeneity as determinants of bacterial adaptation: the adaptomics of a single environmental transition. *Microbiology and molecular biology reviews, 76* (3), 597-625.

Sagarzazu, N., Cebrian, G., Pagan, R., Condon, S., & Manas, P. (2013). Emergence of pulsed electric fields resistance in *Salmonella enterica* serovar Typhimurium SL1344. *International Journal of Food Microbiology, 166* (2), 219-225.

Sakai, A., Nakanishi, M., Yoshiyama, K., & Maki, H. (2006). Impact of reactive oxygen species on spontaneous mutagenesis in *Escherichia coli*. *Genes Cells, 11* (7), 767-778.

Sala, F. J. (1995). Food preservation through combined processes. *Microbiologia, 11* (1), 23-32.

Sanz-Puig, M., Velázquez-Moreira, A., Torres, C., Guerrero-Beltrán, J. Á., Cunha, L. M., Martinez, A., & Rodrigo, D. (2019). Resistance changes in *Salmonella enterica* serovar Typhimurium treated by high hydrostatic pressure and pulsed electric fields and assessment of virulence changes by using *Caenorhabditis elegans* as a test organism. *Innovative Food Science & Emerging Technologies, 51*, 51-56.

Schlacher, K., & Goodman, M. F. (2007). Lessons from 50 years of SOS DNA-damage-induced mutagenesis. *Nature Reviews Molecular Cell Biology, 8* (7), 587-594.

Selveshwari, S., Lele, K., & Dey, S. (2021). Genomic signatures of UV resistance evolution in *Escherichia coli* depend on the growth phase during exposure. *Journal of Evolutionary Biology, 00*, 1-15.

Sheng, L. N., Rasco, B., & Zhu, M. J. (2016). Cinnamon oil inhibits Shiga toxin type 2 phage induction and Shiga toxin type 2 production in *Escherichia coli* O157:H7. *Applied and Environmental Microbiology, 82* (22), 6531-6540.

Somolinos, M., García, D., Condón, S., Mackey, B., & Pagán, R. (2010). Inactivation of *Escherichia coli* by citral. *108* (6), 1928-1939.

Somolinos, M., Garcia, D., Mañas, P., Condon, S., & Pagan, R. (2008). Effect of environmental factors and cell physiological state on pulsed electric fields resistance and repair capacity of various strains of *Escherichia coli*. *International Journal of Food Microbiology, 124* (3), 260-267.

Tajkarimi, M. M., Ibrahim, S. A., & Cliver, D. O. (2010). Antimicrobial herb and spice compounds in food. *Food Control, 21* (9), 1199-1218.

Tamber, S. (2018). Population-wide survey of *Salmonella enterica* response to high-pressure processing reveals a diversity of responses and tolerance mechanisms. *Applied and Environmental Microbiology, 84* (2), e01673-01617.

Tang, C., Chen, J., Zhou, Y., Ding, P., He, G., Zhang, L., Zhao, Z., & Yang, D. (2021). Exploring antimicrobial mechanism of essential oil of *Amomum villosum* Lour through metabolomics based on gas chromatography-mass spectrometry in methicillin-resistant *Staphylococcus aureus*. *Microbiological Research, 242*, 126608.

Thomsen, N. A., Hammer, K. A., Riley, T. V., Van Belkum, A., & Carson, C. F. (2013). Effect of habituation to tea tree (*Melaleuca alternifolia*) oil on the subsequent susceptibility of *Staphylococcus* spp. to antimicrobials, triclosan, tea tree oil, terpinen-4-ol and carvacrol. *International Journal of Antimicrobial Agents, 41* (4), 343-351.

Uliczka, F., Pisano, F., Kochut, A., Opitz, W., Herbst, K., Stolz, T., & Dersch, P. (2011). Monitoring of gene expression in bacteria during infections using an adaptable set of bioluminescent, fluorescent and colorigenic fusion vectors. *PLOS ONE, 6* (6), e20425.

Van Boeijen, I. K. H., Francke, C., Moezelaar, R., Abee, T., & Zwietering, M. H. (2011). Isolation of highly heat-resistant *Listeria monocytogenes* variants by use of a cinetic modeling-based sampling scheme. *Applied and Environmental Microbiology, 77* (8), 2617.

Vanlint, D., Mitchell, R., Bailey, E., Meersman, F., McMillan, P. F., Michiels, C. W., & Aertsen, A. (2011). Rapid acquisition of gigapascal-high-pressure resistance by *Escherichia coli*. *MBio, 2* (1), e00130-00110.

Veiga, R. D. S. D., Aparecida Da Silva-Buzanello, R., Corso, M. P., & Canan, C. (2019). Essential oils microencapsulated obtained by spray drying: a review. *Journal of Essential Oil Research, 31* (6), 457-473.

Wesche, A. M., Gurtler, J. B., Marks, B. P., & Ryser, E. T. (2009). Stress, sublethal injury, resuscitation, and virulence of bacterial foodborne pathogens. *Journal of Food Protection, 72* (5), 1121-1138.

Wons, E., Koscielniak, D., Szadkowska, M., & Sektas, M. (2018). Evaluation of GFP reporter utility for analysis of transcriptional slippage during gene expression. *Microbial Cell Factories, 17* (1), 150.

Xiang, Y., Li, F., Dong, N., Tian, S., Zhang, H., Du, X., Zhou, X., Xu, X., Yang, H., Xie, J., Yang, C., Liu, H., Qiu, S., Song, H., & Sun, Y. (2020). Investigation of a salmonellosis outbreak caused by multidrug resistant *Salmonella* Typhimurium in China. *Frontiers in Microbiology, 11* (801).

Yajjala, V. K., Widhelm, T. J., Endres, J. L., Fey, P. D., & Bayles, K. W. (2016). Generation of a transposon mutant library in *Staphylococcus aureus* and *Staphylococcus epidermidis* using bursa aurealis. *Methods in Molecular Biology, 1373*, 103-110.

Yang, S.-K., Yusoff, K., Thomas, W., Akseer, R., Alhosani, M. S., Abushelaibi, A., Lim, S.-H.-E., & Lai, K.-S. (2020). Lavender essential oil induces oxidative stress which modifies the bacterial membrane permeability of carbapenemase producing *Klebsiella pneumoniae*. *Scientific Reports, 10* (1), 819.

Yang, Y., Chen, Y., Zhang, G., Sun, J., Guo, L., Jiang, M., Ou, B., Zhang, W., & Si, H. (2020). Transcriptomic analysis of *Staphylococcus aureus* under the stress condition caused by *Litsea cubeba* L. essential oil via RNA sequencing. *Frontiers in Microbiology, 11* (1693).

Yap, P. S. X., Yiap, B. C., Ping, H. C., & Lim, S. H. E. (2014). Essential oils, a new horizon in combating bacterial antibiotic resistance. *The open microbiology journal, 8*, 6-14.

Ye, Y., Zhang, L., Hao, F., Zhang, J., Wang, Y., & Tang, H. (2012). Global metabolomic responses of *Escherichia coli* to heat stress. *Journal of Proteome Research, 11* (4), 2559-2566.

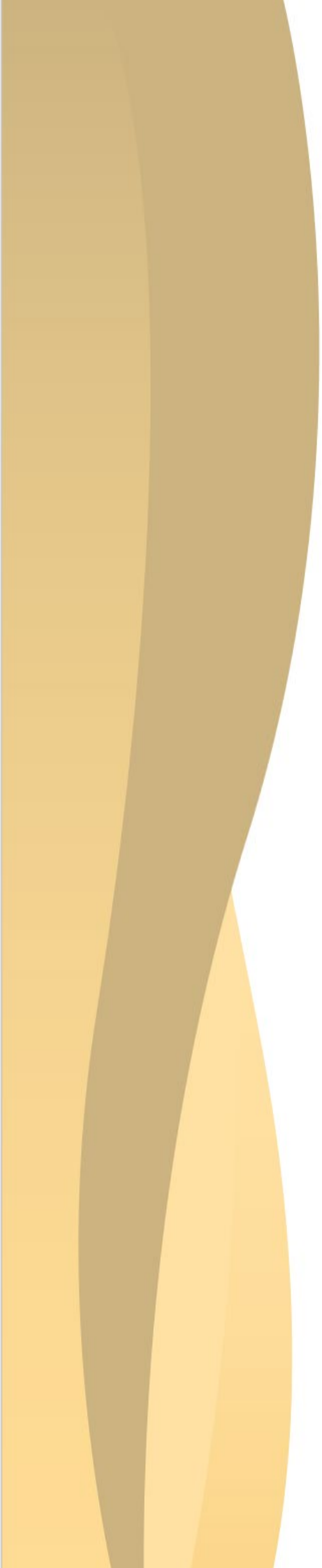
Zhang, D. L., Bai, Y. L., & Bowman, J. P. (2019). Impact of combined acidic and hyperosmotic shock conditions on the proteome of *Listeria monocytogenes* ATCC 19115 in a time-course study. *Journal of Food Quality, 2019*, 3075028.

Zhang, Z.-H., Wang, L.-H., Zeng, X.-A., Han, Z., & Brennan, C. S. (2019). Non-thermal technologies and its current and future application in the food industry: a review. *International Journal of Food Science & Technology, 54* (1), 1-13.

Zhang, Z., Vriesekoop, F., Yuan, Q., & Liang, H. (2014). Effects of nisin on the antimicrobial activity of d-limonene and its nanoemulsion. *Food Chemistry, 150*, 307-312.

Zhao, X., Liu, Z., Liu, Z., Meng, R., Shi, C., Chen, X., Bu, X., & Guo, N. (2018). Phenotype and RNA-seq-based transcriptome profiling of *Staphylococcus aureus* biofilms in response to tea tree oil. *Microbial Pathogenesis, 123*, 304-313.

Anexos



Factor de impacto de las revistas y áreas temáticas

Manuscrito I: Berdejo, D., Pagán, E., García-Gonzalo, D., Pagán, R. (2019). Review: Exploiting the synergism among physical and chemical processes for improving food safety. *Current Opinion in Food Science*, 30, 14-20. doi: 10.1016/j.cofs.2018.08.004.

Factor de impacto (2019): 4.577 (Food Science & Technology).

Manuscrito II: Berdejo, D., Pagán, E., Merino, N., García-Gonzalo, D., Pagán, R. (Aceptado). Evolution assays for the isolation of mutant bacteria resistant to natural antimicrobials. Marciane, M. (Ed.), *Detection and enumeration of bacteria, yeast, viruses, and protozoan in foods and freshwater*. Springer Nature, 233 Spring Street, New York, NY 10013, U.S.A.

Manuscrito III: Berdejo, D., Chueca, B., Pagán, E., Renzoni, A., Kelley, W., Pagán, R., García-Gonzalo, D. (2019). Sub-inhibitory doses of individual constituents of essential oils can select for *Staphylococcus aureus* resistant mutants. *Molecules,* 24, 170. doi: 10.3390/molecules24010170.

Factor de impacto (2019): 3.267 (Chemistry, multidisciplinary).

Manuscrito IV: Berdejo, D., Merino, N., Pagán, E., García-Gonzalo, D., Pagán, R. (2020). Genetic variants and phenotypic characteristics of *Salmonella* Typhimurium-resistant mutants after exposure to carvacrol. *Microorganisms*, 8, 937. doi: 10.3390/microorganisms8060937.

Factor de impacto (2019): 4.152 (Microbiology).

Manuscrito V: Berdejo, D., Pagán, E., Merino, N., Pagán, R., García-Gonzalo, D. (2020). Incubation with a complex orange essential oil leads to evolved mutants with increased resistance and tolerance. *Pharmaceuticals*, 13, 239. doi:10.3390/ph13090239.

Factor de impacto (2019): 4.286 (Pharmacology & Pharmacy).

Manuscrito VI: Berdejo, D., Pagán, E., Merino, N., García-Gonzalo, D., Pagán, R. (Enviado). Emerging mutant populations of *Listeria monocytogenes* EGD-e under selective pressure of *Thymbra capitata* essential oil question its use in food preservation. *Food Research International*.

Factor de impacto (2019): 4.972 (Food Science & Technology).

Manuscrito VII: Berdejo, D., Pagán, E., Merino, N., Botello-Morte, L., García-Gonzalo, D., Pagán, R. (Enviado). *Salmonella enterica* genetic variants isolated after lethal treatment with *Thymbra capitata* essential oil show increased resistance in milk. *International Journal of Food Microbiology*.

Factor de impacto (2019): 4.187 (Food Science & Technology).

Los siguientes tres trabajos fueron publicados en coautoría, de los cuales se ha presentado la renuncia expresa de los coautores no doctores tal como se indica en el artículo 20 "Tesis como compendio de publicaciones " del Reglamento sobre Tesis Doctorales de la Universidad de Zaragoza. La contribución del doctorando recae, principalmente, en la parte experimental sobre la evaluación de las propiedades antimicrobianas de los aceites esenciales y sus constituyentes individuales en su forma libre, emulsionada y encapsulada, aplicados de forma individual o combinado con otras tecnologías de conservación, así como su participación en la revisión bibliográfica, análisis y discusión de resultados, redacción y revisión de los artículos:

Manuscrito VIII: Pagán, E., Berdejo, D., Espina, L., García-Gonzalo, D., Pagán, R. (2018). Antimicrobial activity of suspensions and nanoemulsions of citral in combination with heat or pulsed electric fields. *Letters in Applied Microbiology,* 66, 63-70. doi:10.1111/lam.12815.

Factor de impacto (2018): 1.805 (Food Science & Technology).

Manuscrito IX: Merino, N.*, Berdejo, D.*, Bento, R., Salman, H., Lanz, M., Maggi, F., Sánchez-Gómez, S., García-Gonzalo, D., Pagán, R. (2019). Antimicrobial efficacy of *Thymbra capitata* (L.) Cav. essential oil loaded in self-assembled zein nanoparticles in combination with heat. *Industrial Crops & Products*, 133: 98-104. doi: 10.1016/j.indcrop.2019.03.003.

* Los autores han contribuido por igual en el estudio.

Factor de impacto (2019): 4.244 (Agronomy).

Manuscrito X: López-Miranda, S., Berdejo, D., Pagán, E., García-Gonzalo, D., Pagán, R. (2020). Modified cyclodextrin type and dehydration methods exert a significant effect on the antimicrobial activity of encapsulated carvacrol and thymol. *Journal of the Science of Food and Agriculture*. doi: 10.1002/jsfa.11017.

Factor de impacto (2019): 2.614 (Agriculture, Multidisciplinary).

Publishing Agreement

for Contributions in Collected Works

SPRINGER NATURE

This Publishing Agreement (this "**Agreement**") has been approved by and entered into between
Berdejo, Daniel;
Pagán, Elisa;
Merino, Natalia;
García-Gonzalo, Diego (0000-0002-7629-8101);
Pagán, Rafael (0000-0002-0238-6328).

Addresses: Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), Zaragoza, Spain]
(the "**Author**")
whereas, in the event that the Author is more than one person, ***Rafael Pagán Tomás*** serves as corresponding author (the "**Corresponding Author**")

on the one part and

Springer Science+Business Media, LLC
233 Spring Street, New York, NY 10013, U.S.A.
(the "**Publisher**")

on the other part;
together hereinafter referred to as the "**Parties**".

The Publisher intends to publish the Author's contribution in a collected work provisionally entitled:
Detection and Enumeration of Bacteria, Yeast, Viruses, and Protozoan in Foods and Freshwater
(the "**Work**")
edited by:
Marciane Magnani
(the "**Editor**")

The Publisher intends to publish the Work under the imprint **Humana Press**.
The Work may be published in the book series **Methods and Protocols in Food Science.**

§1 Contracting authors

When the Author is more than one person then, unless otherwise indicated in this Agreement or agreed in writing by the Publisher: (a) the expression "Author" as used in this Agreement will apply collectively for all such persons (each a "**co-author**"); (b) the Corresponding Author hereby warrants and represents that all co-authors of the contribution have expressly agreed that the Corresponding Author has full right, power and authority to sign this Agreement on their behalf, that the Corresponding Author is entitled to act on their behalf, and that they shall be bound by the Corresponding Author, with respect to all matters, responsibilities, notices and communications related to this Agreement; the Corresponding Author shall obtain authorisations and make them available to the Publisher on request; and (c) each co-author is jointly and severally responsible for the Author's obligations under this Agreement which apply to each co-author individually and to the co-authors collectively and the Publisher shall not be bound by any separate agreement or legal relationship as between the co-authors.

§2 Subject of the Agreement

2.1 The Author will prepare a contribution provisionally entitled:
Evolution assays for the isolation of mutant bacteria resistant to natural antimicrobials

The expression "**Contribution**" as used in this Agreement means the contribution as identified above, and includes without limitation all related material delivered to the Publisher by or on behalf of the Author whatever its media and form (including text, graphical elements, tables, videos and/or links) in all versions and editions in whole or in part.

2.2 The Contribution may contain links (e.g. frames or in-line links) to media enhancements (e.g. additional documents, tables, diagrams, charts, graphics, illustrations, animations, pictures, videos and/or software) or to social or functional enhancements, complementing the Contribution, which are provided on the Author's own website or on a third party website or repository (e.g. maintained by an institution) subject always to the Author providing to the Editor, at the latest at the delivery date of the manuscript for the Contribution, an accurate description of each media enhancement and its respective website or repository, including its/their owner, nature and the URL. The Publisher is entitled to reject the inclusion of, or suspend, or delete links to all or any individual media enhancements.

2.3 In the event that an index is deemed necessary, the Author shall assist the Editor in its preparation (e.g. by suggesting index terms), if requested by the Editor.

§ 3 Rights Granted

3.1 The Author hereby grants to the Publisher the perpetual, sole and exclusive, world-wide, transferable, sub-licensable and unlimited right to publish, produce, copy, distribute, communicate, display publicly, sell, rent and/or otherwise make available the Contribution in any language, in any versions or editions in any and all forms and/or media of expression (including without limitation in connection with any and all end-user devices), whether now known or developed in the future, in each case with the right to grant further time-limited or permanent rights. The above rights are granted in relation to the Contribution as a whole or any part and with or in relation to any other works.
Without limitation, the above grant includes: (a) the right to edit, alter, adapt, adjust and prepare derivative works; (b) all advertising and marketing rights including without limitation in relation to social media; (c) rights for any training, educational and/or instructional purposes; and (d) the right to add and/or remove links or combinations with other media/works.
The Author hereby grants to the Publisher the right to create, use and/or license and/or sub-license content data or metadata of any kind in relation to the Contribution or parts thereof (including abstracts and summaries) without restriction.
The Publisher also has the right to commission completion of the Contribution in accordance with the Clause "**Author's Responsibilities – Delivery and Acceptance of the Manuscript**" and of an updated version of the Contribution for new editions of the Work in accordance with the Clause "**New Editions**".

3.2 The copyright in the Contribution shall be vested in the name of the **Author**. The Author has asserted their right(s) to be identified as the originator of the Contribution in all editions and versions, published in all forms and media. The Author agrees that all editing, alterations or amendments to the Contribution made by or on behalf of the Publisher or its licensees for the purpose of fulfilling this Agreement or as otherwise allowed by the above rights shall not require the approval of the Author and will not infringe the Author's "moral rights" (or any equivalent rights). This includes changes made in the course of dealing with retractions or other legal issues.

§ 4 Self-Archiving and Re-Use

4.1 **Self-Archiving:** The Publisher permits the Rights Holder to archive the Contribution in accordance with the Publisher's guidelines, the current version of which is set out in the **Appendix "Author's Self-Archiving Guidelines"**.

4.2 **Re-Use:** The Publisher permits the Author to copy, distribute or otherwise re-use the Contribution, without the requirement to seek specific prior written permission from the Publisher, in accordance with the Publisher's guidelines, the current version of which is set out in the **Appendix "Author's Re-Use Rights"**.

§ 5 The Publisher's Responsibilities

5.1 Subject always to the other provisions of this Clause below, the Publisher will undertake the production, publication and distribution of the Contribution and the Work in print and/or electronic form at its own expense and risk within a reasonable time after acceptance of the Work unless the Publisher is prevented from or delayed in doing so due to any circumstances beyond its reasonable control. The Publisher shall have the entire control of such production, publication and distribution determined in its sole discretion in relation to any and all editions and versions of the Contribution and the Work, including in respect of all the following matters:
(a) distribution channels, including determination of markets;
(b) determination of the range and functions of electronic formats and/or the number of print copies produced;
(c) publication and distribution of the Contribution, the Work, or parts thereof as individual content elements, in accordance with market demand or other factors;
(d) determination of layout and style as well as the standards for production;
(e) setting or altering the list-price, and allowing for deviations from the list-price (if permitted under applicable jurisdiction);
(f) promotion and marketing as the Publisher considers most appropriate to optimise sales.

5.2 All rights, title and interest, including all intellectual property or related rights in the typography, design and/or look-and-feel of the Contribution shall remain the exclusive property of and are reserved to the Publisher. All illustrations and any other material or tangible or intangible property prepared at the expense of the Publisher including any marketing materials remain, as between the Parties, the exclusive property of the Publisher. The provisions of this sub-clause shall continue to apply notwithstanding any termination of, and/or any reversion of rights in the Contribution to the Author, under this Agreement.

5.3 Without prejudice to the Publisher's termination and other rights hereunder including under the Clause "**The Author's Responsibilities**", it is agreed and acknowledged by the Parties that nothing in this Agreement shall constitute an undertaking on the part of the Publisher to publish the Contribution unless and until: (i) any and all issues in relation to the Work (including all necessary revisions, consents and permissions) raised by the Publisher have been resolved to the Publisher's satisfaction, and (ii) the Publisher has given written notice of acceptance in writing of the final manuscript of the entire Work to the Editor. If following (i) and (ii) above the Publisher has not published the Contribution in any form within a reasonable period and the Author has given written notice to the Publisher requiring it to publish within a further reasonable period and the Publisher has failed to publish in any form, then the Author may terminate this Agreement by one month's written notice to the Publisher and all rights granted by the Author to the Publisher under this Agreement shall revert to the Author (subject to the provisions regarding any third party rights under any subsisting licence or sub-licence in accordance with the Clause "**Termination**").
The Author may also give such written notice requiring publication on the same terms as above if the Publisher has published the Contribution but subsequently ceases publishing the Contribution in all forms so that it is no longer available.
This shall be the Author's sole right and remedy in relation to such non-publication and is subject always to the Author's continuing obligations hereunder including the Clause "**Warranty**".

§ 6 The Author's Responsibilities

6.1 Delivery and Acceptance of the Manuscript

6.1.1 The Author shall deliver the Contribution to the Editor (or, if requested by the Publisher, to the Publisher) on or before **January 2021** (the "**Delivery Date**") electronically in the Publisher's standard requested format or in such other form as may be agreed in writing with the Publisher.

The Author shall retain a duplicate copy of the Contribution. The Contribution shall be in a form acceptable to the Publisher (acting reasonably) and in line with the instructions contained in the Publisher's guidelines as provided to the Author by the Publisher. The Author shall provide at the same time, or earlier if the Publisher reasonably requests, any editorial, publicity or other information (and in such form or format) reasonably required by the Publisher. The Publisher may exercise such additional quality control of the manuscript as it may decide at its sole discretion including through the use of plagiarism checking systems and/or peer-review by internal or external reviewers of its choice. If the Publisher decides at its sole discretion that the final manuscript does not conform in quality, content, structure, level or form to the stated requirements of the Publisher, the Publisher shall be entitled to terminate this Agreement in accordance with the provisions of this Clause.

6.1.2 The Author must inform the Publisher at the latest on the Delivery Date if the sequence of the naming of any co-authors entering into this Agreement shall be changed. If there are any changes in the authorship (e.g. a co-author joining or leaving), then the Publisher must be notified by the Author in writing immediately and the Parties will amend this Agreement accordingly. The Publisher shall have no obligation to consider publication under this Agreement in the absence of such agreed amendment.

6.1.3 If the Author fails to deliver the Contribution by the Delivery Date, or within any extension period given by the Publisher, at its sole discretion, in accordance with the provisions of this Clause above, or if the Author, or in the case of co-authors having entered into this Agreement, any co-author, dies or becomes incapacitated or otherwise incapable of performing the Author's obligations under this Agreement, the Publisher shall be entitled to either:
(a) elect to continue to perform this Agreement in accordance with its terms and the Publisher may commission an appropriate and competent person (who, in the case of co-authors having entered into this Agreement, may be a co-author) to complete the Contribution; or
(b) terminate this Agreement with immediate effect by written notice to the Author or the Author's successors, in which case all rights granted by the Author to the Publisher under this Agreement shall revert to the Author/Author's successors (subject to the provisions of the Clause "**Termination**").

6.1.4 The Author agrees, at the request of the Publisher, to execute all documents and do all things reasonably required by the Publisher in order to confer to the Publisher all rights intended to be granted under this Agreement.

6.1.5 The Author warrants that the Contribution is original except for any excerpts from other works including pre-published illustrations, tables, animations, text quotations, photographs, diagrams, graphs or maps, and whether reproduced from print or electronic or other sources ("**Third Party Material**") and that any such Third Party Material is in the public domain (or otherwise unprotected by copyright/other rights) or has been included with written permission from or on behalf of the rights holder (and if requested in a form prescribed or approved by the Publisher) at the Author's expense unless otherwise agreed in writing, or is otherwise used in accordance with applicable law. On request from the Publisher, the Author shall in writing indicate the precise sources of these excerpts and their location in the manuscript. The Author shall also retain the written permissions and make them available to the Publisher on request.

6.2 Approval for Publishing

6.2.1 The Author shall proofread the page proofs for the Contribution provided by or on behalf of the Publisher, including checking the illustrations as well as any media, social or functional enhancements and give approval for publishing, if and when requested by the Publisher. The Author's approval for publishing is deemed to have been given if the Author does not respond within a reasonable period of time (as determined by the Publisher) after receiving the proofs. The Publisher shall not be required to send a second set of corrected proofs unless specifically requested by the Author in writing but in any event no further amendments may be made or requested by the Author.
In the event of co-authors having entered into this Agreement the Publisher shall send the page proofs to the Corresponding Author only and all persons entering into this Agreement as Author agree that the Corresponding Author shall correct and approve the page proofs on their behalf.

6.2.2 If the Author makes changes other than correcting typographical errors, the Author shall bear all the Publisher's costs of such alterations to proofs including without limitation to alterations to pictorial illustrations. The Publisher shall have the right to charge and invoice these costs plus value added or similar taxes (if applicable) through its affiliated company Springer Nature Customer Service Center GmbH or Springer Nature Customer Service Center LLC, respectively, to the Author, payable within 14 days of receipt of the invoice.

§ 7 Co-operation

Without prejudice to the warranties and representations given by the Author in this Agreement, the Author shall cooperate fully with the Editor and the Publisher in relation to any legal action that might arise from the publication or intended publication of the Contribution and the Author shall give the Publisher access at reasonable times to any relevant accounts, documents and records within the power or control of the Author.

§ 8 Warranty

8.1 The Author warrants and represents that:
(a) the Author has full right, power and authority to enter into and perform its obligations under this Agreement; and
(b) the Author is the sole legal owner of (and/or has been fully authorised by any additional rights owner to grant) the rights licensed in the Clause "**Rights Granted**" and use of the Contribution shall in no way whatever infringe or violate any intellectual property or related rights (including any copyright, database right, moral right or trademark right) or any other right or interest of any third party subject only to the provisions in the Clause "**The Author's Responsibilities**" regarding Third Party Material (as defined above); and
(c) the Contribution shall not contain anything that may cause religious or racial hatred or encourage terrorism or unlawful acts or be defamatory (or contain malicious falsehoods), or be otherwise actionable, including, but not limited to, any action related to any injury resulting from the use of any practice or formula disclosed in the Contribution and all of the purported facts contained in the Contribution are according to the current body of science and understanding true and accurate; and

(d) there is no obligation of confidentiality owed in respect of any contents of the Contribution to any third party and the Contribution shall not contain anything which infringes or violates any trade secret, right of privacy or publicity or any other personal or human right or the processing or publication of which could breach applicable data protection law and that informed consent to publish has been obtained for all research or other featured participants; and

(e) the Contribution has not been previously licensed, published or exploited and use of the Contribution shall not infringe or violate any contract, express or implied, to which the Author, or any co-author, who had entered into this Agreement, is a party and any academic institution, employer or other body in which work recorded in the Contribution was created or carried out has authorised and approved such work and its publication.

8.2 The Author warrants and represents that the Author, and each co-author who has entered into this Agreement, shall at all times comply in full with:
(a) all applicable anti-bribery and corruption laws; and
(b) all applicable data protection and electronic privacy and marketing laws and regulations; and
(c) the Publisher's ethic rules (available at https://www.springernature.com/gp/authors), as may be updated by the Publisher at any time in its sole discretion. The Publisher shall notify the Author in the event of material changes by email or other written means
(the "**Applicable Laws**").
If the Author is in material breach of any of the Applicable Laws or otherwise in material breach of accepted ethical standards in research and scholarship, or becomes the subject of any comprehensive or selective sanctions issued in any applicable jurisdiction (e.g. being subject to the OFAC sanctions list) or if, in the opinion of the Publisher, at any time any act, allegation or conduct of or about the Author prejudices the production or successful exploitation of the Contribution and the Work or brings the name and/or reputation of the Publisher or the Work into disrepute, or is likely to do so, then the Publisher may terminate this Agreement in accordance with the Clause "**Termination**".

8.3 The Publisher reserves the right to amend and/or require the Author to amend the Contribution at any time to remove any actual or potential breach of the above warranties and representations or otherwise unlawful part(s) which the Publisher or its internal or external legal advisers identify at any time. Any such amendment or removal shall not affect the warranties and representations given by the Author in this Agreement.

§ 9 Author's Discount and Electronic Access

9.1 The Author, or each co-author, is entitled to purchase for their personal use the Work and other books published by the Publisher at a discount of 40% off the list price, for as long as there is a contractual arrangement between the Author and the Publisher and subject to any applicable book price law or regulation. The copies must be ordered from the affiliated entity of the Publisher (Springer Nature Customer Service Center GmbH or Springer Nature Customer Service Center LLC, respectively). Resale of such copies is not permitted.

9.2 The Publisher shall provide the electronic final published version of the Work to the Author, provided that the Author has included their e-mail address in the manuscript of the Contribution.

§ 10 Consideration

10.1 The Parties agree that the Publisher's agreement to its contractual obligations in this Agreement in respect of its efforts in considering publishing and promoting the Contribution and the Work is good and valuable consideration for the rights granted and obligations undertaken by the Author under this Agreement, the receipt, validity and sufficiency of which is hereby acknowledged by the Author.
The Parties expressly agree that no royalty, remuneration, licence fee, costs or other moneys whatsoever shall be payable to the Author, subject to the following provisions of this Clause.

10.2 The Publisher and the Author each have the right to authorise collective management organisations ("**CMOs**") of their choice to manage some of their rights. Reprographic and other collectively managed rights in the Contribution ("**Collective Rights**") have been or may be licensed on a non-exclusive basis by each of the Publisher and the Author to their respective CMOs to administer the Collective Rights under their reprographic and other collective licensing schemes ("**Collective Licences**"). Notwithstanding the other provisions of this Clause, the Publisher and the Author shall each receive and retain their share of revenue from use of the Contribution under Collective Licences from, and in accordance with the distribution terms of their respective CMOs. To the fullest extent permitted by law, any such revenue is the sole property of the Publisher and the Author respectively and, if applicable, the registration and taxation of that revenue is the sole responsibility of the respective recipient party. The Publisher and the Author shall cooperate as necessary in the event of any change to the licensing arrangements set out in this Clause.

§ 11 New Editions

11.1 The Publisher has the sole right to determine whether to publish any subsequent edition of the Work containing an updated version of the Contribution, but only after reasonable consultation with the Author. Once notified by the Publisher that an update of the Contribution is deemed necessary, the Author agrees to deliver an updated manuscript in accordance with the terms of the Clause "**The Author's Responsibilities**" and the other relevant provisions of this Agreement, together with the material for any new illustrations and any other supporting content including media enhancements, within a reasonable period of time (as determined by the Publisher) after such notification. Substantial changes in the nature or size of the Contribution require the written approval of the Publisher at its sole discretion. The terms of this Agreement shall apply to any new edition of the Work that is published under this "**New Editions**" Clause.

11.2 If the Author, for whatever reason, is unwilling, unable or fails (including as a result of death or incapacity) to submit an updated manuscript that meets the terms of this Agreement within the above stated period, then the Publisher is entitled to revise, update and publish the content of the existing edition or to designate one or more individuals (which, where co-authors have entered into this Agreement, may be one or more of the co-authors) to prepare this and any future editions provided that the new editions shall not contain anything that is a derogatory use of the Author's work that demonstrably damages the Author's scientific reputation. In such case, the Author shall not participate in preparing any subsequent editions. The Author agrees that the Publisher shall be entitled but not obliged to continue to use the name of Author on any new editions of the Work together with the names of the person or persons who contributed to the new editions. Should the Author or the Author's successors object to such continuing use then they must notify the Publisher in writing when first contacted by the Publisher in connection with any new edition.

§12 Termination

12.1 In addition to the specific rights of termination set out in the Clause "**The Publisher's Responsibilities**" and the Clause "**The Author's Responsibilities**", either Party shall be entitled to terminate this Agreement forthwith by notice in writing to the other Party if the other Party commits a material breach of the terms of the Agreement which cannot be remedied or, if such breach can be remedied, fails to remedy such breach within 45 days of being given written notice to do so.

12.2 Termination of this Agreement, howsoever caused, shall not affect:
(a) any subsisting rights of any third party under any licence or sub-licence validly granted by the Publisher prior to termination and the Publisher shall be entitled to retain its share of any sum payable by any third party under any such licence or sub-licence;
(b) except where stated otherwise in this Agreement, any claim which either Party may have against the other for damages or otherwise in respect of any rights or liabilities arising prior to the date of termination;
(c) the Publisher's right to continue to sell any copies of the Work which are in its power, possession or control as at the date of expiry or termination of this Agreement for a period of 6 months on a non-exclusive basis.

§13 General Provisions

13.1 This Agreement, and the documents referred to within it, constitute the entire agreement between the Parties with respect to the subject matter hereof and supersede any previous agreements, warranties, representations, undertakings or understandings. Each Party acknowledges that it is not relying on, and shall have no remedies in respect of, any undertakings, representations, warranties, promises or assurances that are not set forth in this Agreement. Nothing in this Agreement shall exclude any liability for or remedy in respect of fraud, including fraudulent misrepresentation. This Agreement may be modified or amended only by agreement of the Parties in writing. For the purposes of modifying or amending this Agreement, "in writing" requires either a document written and signed by both the Parties or an electronic confirmation by both the Parties with DocuSign or a similar e-signature solution. Any notice of termination and/or reversion and, where applicable, any preceding notices (including any requesting remediable action under the Clause "**Termination**") must be provided in writing and delivered by post, courier or personal delivery addressed to the physical address of the relevant Party as set out at the beginning of this Agreement or any replacement address notified to the other Party for this purpose. All such notices shall become effective upon receipt by the other Party. Receipt is deemed to have taken place five working days after the respective notice was sent by post or left at the address by courier or personal delivery. If the Publisher is the terminating Party the notice need only be provided to the address of the Corresponding Author. If the Author is the terminating Party a copy of the notice must also be sent to the Publisher's Legal Department located at Heidelberger Platz 3, 14197 Berlin, Germany.

13.2 Nothing contained in this Agreement shall constitute or shall be construed as constituting a partnership, joint venture or contract of employment between the Publisher and the Author. No Party may assign this Agreement to third parties but the Publisher may assign this Agreement or the rights received hereunder to its affiliated companies. In this Agreement, any words following the terms "include", "including", "in particular", "for example", "e.g." or any similar expression shall be construed as illustrative and shall not limit the sense of the words preceding those terms.

13.3 If any difference shall arise between the Author and the Publisher concerning the meaning of this Agreement or the rights and liabilities of the Parties, the Parties shall engage in good faith discussions to attempt to seek a mutually satisfactory resolution of the dispute. This Agreement shall be governed by, and shall be construed in accordance with, the laws of *New York State*. The courts of *competent jurisdiction in New York, N.Y.* shall have the exclusive jurisdiction.

13.4 A person who is not a party to this Agreement (other than an affiliate of the Publisher) has no right to enforce any terms or conditions of this Agreement. This Agreement shall be binding upon and inure to the benefit of the successors and assigns of the Publisher. If one or more provisions of this Agreement are held to be unenforceable (in whole or in part) under applicable law, each such provision shall be deemed excluded from this Agreement and the balance of the Agreement shall remain valid and enforceable but shall be interpreted as if that provision were so excluded. If one or more provisions are so excluded under this Clause then the Parties shall negotiate in good faith to agree an enforceable replacement provision that, to the greatest extent possible under applicable law, achieves the Parties' original commercial intention.

The Corresponding Author signs this Agreement on behalf of any and all co-authors.

Signature of Corresponding Author: **Date:**

…… …….

For internal use only:
Order Number: 89069976
GPU/PD/PS: 2/28/191
Book_Contributor_Non-OA_Normal_EN (Short Non-RG) - 05/2019

Appendix "Author's Self-Archiving Rights"

The Publisher acknowledges that the Author retains rights to archive the Contribution but only subject to and in accordance with the following provisions:

1. **Pre-Print:**
   A "**Pre-print**" is defined as the Author´s version of the Contribution submitted to the Publisher but before any peer-review or any other editorial work by or on behalf of the Publisher has taken place.
   The Author may make available the Pre-print of the Contribution for personal and private reading purposes only on any of:
   (a) the Author's own personal, self-maintained website over which the Author has sole operational control; and/or
   (b) a legally compliant, non-commercial pre-print server, such as but not limited to arXiv, bioRxiv and RePEc; provided always that once the "Version of Record" (as defined below) of the Contribution has been published by or on behalf of the Publisher, the Author shall immediately ensure that any Pre-print made available above shall contain a link to the Version of Record and the following acknowledgement:
   "This is a pre-print of the following chapter: [author of the chapter], [chapter title], published in [book title], edited by [editor of the book], [year of publication], [publisher (as it appears on the cover of the book)] reproduced with permission of [publisher (as it appears on the copyright page of the book)]. The final authenticated version is available online at: http://dx.doi.org/[insert DOI]".
2. **Author's Accepted Manuscript:**
   The "Author's Accepted Manuscript" ("**AAM**") is defined as the version of the Contribution following any peer-review and acceptance, but prior to copyediting and typesetting, by or on behalf of the Publisher.
   The Author may make available the AAM of the Contribution on any of: (a) the Author's own, personal, self-maintained website over which the Author has sole operational control; and/or (b) the Author's employer's internal website or their academic institution or funder´s repository; provided that in each case the respective part of the AAM is not made publicly available until after the Embargo Period.
   The "**Embargo Period**" is a period ending twenty-four (24) months from the first publication of the "Version of Record" (as defined below) of the Contribution by or on behalf of the Publisher.
   The Author must ensure that any part of the AAM made available contains the following:
   "Users may only view, print, copy, download and text- and data-mine the content, for the purposes of academic research. The content may not be (re-)published verbatim in whole or in part or used for commercial purposes. Users must ensure that the author's moral rights as well as any third parties' rights to the content or parts of the content are not compromised."
   These terms shall also be applicable to the Author.
   Once the Version of Record (as defined below) of the Contribution has been published by or on behalf of the Publisher the Author shall immediately ensure that any part of the AAM made available shall contain a link to the Version of Record and the following acknowledgement:
   "This is an Author Accepted Manuscript version of the following chapter: [author of the chapter], [chapter title], published in [book title], edited by [editor of the book], [year of publication], [publisher (as it appears on the cover of the book)] reproduced with permission of [publisher (as it appears on the copyright page of the book)]. The final authenticated version is available online at: http://dx.doi.org/[insert DOI]".
3. **Version of Record:**
   The "**Version of Record**" is defined as the final version of the Contribution as originally published, and as may be subsequently amended following publication in a contractually compliant manner, by or on behalf of the Publisher.
4. Any linking, collection or aggregation of self-archived Contributions from the same Work is strictly prohibited.

Appendix "A*uthor's Re-Use Rights*"

1. The Publisher acknowledges that the Author retains the ability to copy, distribute or otherwise re-use the Contribution, without the requirement to seek specific prior written permission from the Publisher, ("**Re-Use**") subject to and in accordance with the following provisions:
   (a) Re-Use of the Contribution or any part of it is permitted in a new edition of the Work or in a new monograph or new textbook written by the same Author provided that in each case the new work is published by the Publisher under a publishing agreement with the Publisher; and
   (b) Re-Use of the Version of Record (as defined below) of the Contribution or any part of it is permitted in a thesis written by the same Author, and the Author is entitled to make a copy of the thesis containing content of the Contribution available in a repository of the Author's academic institution; and
   (c) any other Re-Use of the Contribution in a new book, book chapter, proceedings or journal article, whether published by the Publisher or by any third party, is limited to three figures (including tables) or single text extracts of less than 400 words; and
   (d) any further Re-Use of the Contribution is permitted only to the extent and in so far as is reasonably necessary: (i) to share the Contribution as a whole to no more than 10 research colleagues engaged by the same institution or employer as the Author for each colleague's personal and private use only; (ii) for classroom teaching use by the Author in their respective academic institution provided that this does not permit inclusion of any of the Contribution in course packs for sale or wider distribution to any students, institutions or other persons nor any other form of commercial or systematic exploitation; or (iii) for the Author to use all or parts of the Contribution in the further development of the Author's scientific and/or academic career, for private use and research or within a strictly limited circulation which does not allow the Contribution to become publicly accessible nor prejudice sales of, or the exploitation of the Publisher's rights in, the Contribution (e.g. attaching a copy of the Contribution to a job or grant application).
2. Any Re-Use must be based on the Version of Record only, and provided the original source of publication is cited according to current citation standards. The "**Version of Record**" is defined as the final version of the Contribution as originally published, and as may be subsequently amended following publication in a contractually compliant manner, by or on behalf of the Publisher.
3. In each case where the Author has Re-Use rights or the Publisher grants specific use rights to the Author according to the above provisions, this shall be subject always to the Author obtaining at the Author's sole responsibility, cost and expense the prior consent of any co-author(s) and/or any relevant third party.
4. Any linking, collection or aggregation of re-used Contributions from the same Work is strictly prohibited.