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Programa de Doctorado: Biociencias y Ciencias Agroalimentarias

Título de la tesis (español e inglés): Mejora de la calidad y seguridad alimentaria de envasados de aceitunas de mesa con DOP *Aloreña de Málaga.*

Improvement of the food quality and safety of PDO *Aloreña de Málaga* table olive packaging.

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TITULO: *Mejora de la calidad y seguridad alimentaria de envasados de aceitunas de mesa con DOP Aloreña de Málaga*

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TESIS DOCTORAL

MEJORA DE LA CALIDAD Y SEGURIDAD ALIMENTARIA DE ENVASADOS DE ACEITUNAS DE MESA CON DOP *ALOREÑA DE MÁLAGA*

LICENCIADA VERÓNICA ROMERO GIL

UNIVERSIDAD DE CÓRDOBA

DEPARTAMENTO DE BROMATOLOGÍA Y TECNOLOGÍA DE LOS ALIMENTOS

CURSO ACADÉMICO 2017/2018

D. FRANCISCO NOÉ ARROYO LÓPEZ, D. FRANCISCO RODRÍGUEZ GÓMEZ, D. PEDRO GARCÍA GARCÍA DOCTORES DEL DEPARTAMENTO DE BIOTECNOLOGÍA DE LOS ALIMENTOS DEL INSTITUTO DE LA GRASA, CSIC Y DÑA. ROSA MARÍA GARCÍA GIMENO DOCTORA DEL DEPARTAMENTO DE BROMATOLOGÍA Y TECNOLOGÍA DE LOS ALIMENTOS DE LA UNIVERSIDAD DE CÓRDOBA:

CERTIFICAN:

Que el trabajo presentado por la Licenciada Verónica Romero Gil con el título: **"Mejora de la calidad y seguridad alimentaria de envasados de aceitunas de mesa con DOP *Aloreña de Málaga*"** se ha desarrollado bajo nuestra dirección y tutela, durante los años 2013-2017, considerando que reúne los requisitos necesarios para optar al grado de Doctor por la Universidad de Córdoba.

Y para que así conste, expedimos el presente certificado en Córdoba, a 11 de Diciembre de 2017.

Firma del/de los director/es

Fdo.: Dr. Francisco Noé Arroyo López

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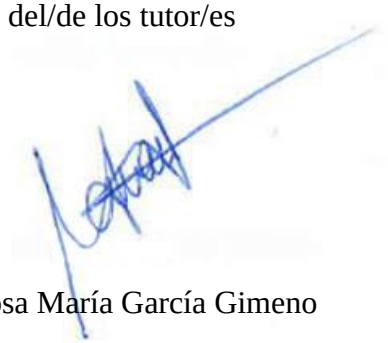


Fdo.: Dra. Rosa María García Gimeno

TÍTULO DE LA TESIS: Mejora de la calidad y seguridad alimentaria de envasados de aceitunas de mesa con DOP *Aloreña de Málaga*

DOCTORANDO/A: Dña. Verónica Romero Gil

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La presente tesis doctoral se enmarca dentro del programa de formación de doctores en empresa (convocatoria 2013) del campus de Excelencia Internacional Agroalimentario *ceia3* y del proyecto motriz de la Junta de Andalucia (convocatoria 2011, AGR-7755) titulado "*Control microbiológico de las poblaciones de enterobacterias en envasados de aceitunas de mesa con DOP Aloreña de Málaga*" (acrónimo PrediAlo). Todo el trabajo de laboratorio ha sido llevado a cabo íntegramente en las instalaciones del Departamento de Biotecnología de Alimentos del Instituto de la Grasa (CSIC, Sevilla) bajo la supervisión de los directores de tesis, mientras que la toma de muestras se ha llevado a cabo en diferentes industrias de la provincia de Málaga que forman parte de la Asociación de Aderezadores Aceitunas Aloreña de Málaga. Se trata, por tanto, de un claro ejemplo de investigación aplicada al sector industrial y cuya finalidad es la formación en el tejido productivo de personas cualificadas y de alto rigor científico que puedan llevar a cabo actividades de I+D+i.

La presente Tesis surge de la necesidad por parte del sector de mejorar la estabilidad, calidad y seguridad alimentaria de los envasados de estas preparaciones de aceitunas de mesa que al ser bastante inestables limitan sus ventas al entorno de la zona de producción (Valle del Guadalhorce, provincia de Málaga). Sin embargo, su expansión y, por tanto, el crecimiento del sector requeriría ampliar su área de comercialización a otros nichos de mercado más alejados. Por ello, es necesario suministrar al tejido productivo información actualizada sobre la vida de mercado de

los envases, la preparación de nuevas formulaciones de envasado, así como documentar la seguridad microbiológica de los usualmente utilizados.

Los trabajos desarrollados durante la Tesis Doctoral han implicado un enfoque multidisciplinar a lo largo de los cuatro años de duración (2013-2017), utilizando técnicas y herramientas de biología molecular y genotipado, microbiología predictiva, análisis sensorial, análisis multivariante y química analítica, a lo que hay que sumar la dificultad de trabajar con patógenos alimentarios del grupo 2 (*Listeria, Salmonella, Escherichia* y *Staphylococcus*). En todo momento la doctoranda ha mostrado una alta capacidad para el aprendizaje de las técnicas utilizadas, capacidad de iniciativa, trabajo en equipo y aptitud para la obtención y análisis de resultados.

Los resultados que se incluyen en la Tesis representan un resumen de una serie de trabajos científicos que se presentan como un compendio de publicaciones de acuerdo con la normativa reguladora de los estudios de doctorado en la Universidad de Córdoba, y que se ha dividido en las siguientes secciones:

> Primera sección. Incluye estudios encaminados a determinar el tiempo de vida de mercado de envasados comerciales de aceitunas de mesa con DOP Aloreña de Málaga y las principales causas por la que se produce la alteración de los mismos. En esta sección se incluyen dos trabajos científicos, una ya aceptado en el LWT Food Sci. Techonol. (2016: 70, 252-260: *Lactobacillus pentosus is the dominant species in spoilt packaged Aloreña de Málaga table olives*) y otro en proceso de revisión en el J. Food Sci. Agric. (*Shelf life of traditional seasoned Aloreña de Málaga table olives based on their packages and fruits characteristics*)

> Segunda sección. Las investigaciones se han dirigido a valorar la aplicación de nuevos conservantes en los envasados de aceitunas Aloreña de Málaga como alternativa a sorbatos y benzoatos utilizados tradicionalmente. Además, se estudia la interacción de estos nuevos conservantes con diferentes compuestos presentes en los envasados como son los compuestos fenólicos y el cloruro de sodio. En esta sección se incluyen 3 trabajos científicos todos aceptados en revistas SCI de alto índice de impacto como Food Microbiology (2016: Vol 57, 71-80: *Effect of zinc formulations, sodium chloride, and hydroxytyrosol on the growth/no-growth boundaries of table olive related yeasts*), Food Microbiology (2016: Vol 54, 72-79: *Susceptibility and resistance of lactic acid bacteria and yeasts against preservatives with potential application in table olives*) y Frontiers in Microbiology (2016: Vol 7, Art 1370: *In silico logistic model*

for table olive related microorganisms as a function of sodium metabisulphite, cinnamaldehyde, pH, and type of acidifying agent).

> Tercera sección. Se incluyen estudios destinados al conocimiento de la seguridad microbiológica de los envasados de aceitunas Aloreña de Málaga para lo que se han inoculado en salmueras y envasados diferentes patógenos para constatar su inhibición y, así, poder garantizar la seguridad microbiológica del producto final. En esta sección se incluye un trabajo aceptado en el Food Microbiol. (2016: 59, 104-111: *Survival of foodborne pathogens in natural cracked olive brines*) y otro en proceso de revisión en el Int J Food Microbiol (*Foodborne pathogen survival in commercial Aloreña de Málaga table olive packaging*).

Por todo ello, se autoriza la presentación de la tesis doctoral.

Sevilla, 30 de Noviembre de 2017

Firma del/de los director/es

Fdo.: Dr. Francisco Noé Arroyo López. **Fdo.: Dr. Francisco Rodríguez Gómez**

Fdo.: Dr. Pedro García García

La realización de la tesis doctoral ha sido posible gracias al programa de doctores en empresas del campus de excelencia internacional agroalimentario (ceiA3), cofinanciada por ceiA3, el Ministerio de Educación Cultura y Deporte y el Banco Santander. Esta tesis se encuentra enmarcada dentro del proyecto de excelencia motriz de la Junta de Andalucía (PEJA11-AGR-7755) titulado "*Control microbiológico de las poblaciones de enterobacterias en envasados de aceitunas de mesa con DOP Aloreña de Málaga*" (acrónimo PrediAlo) y del convenio firmado con la Asociación de Aderezadores de Aceituna de Mesa de la Comarca Natural del Valle del Guadalhorce.

TABLA DE CONTENIDOS

RESUMEN/SUMMARY

La *Aloreña de Málaga* fue la primera aceituna de mesa en obtener el sello de calidad de Denominación de Origen Protegida (DOP) en España (BOJA nº215, 2009). Se trata de una aceituna de mesa autóctona del Valle del Guadalhorce, muy apreciada en la costa del sol (provincia de Málaga) donde tienen lugar la mayor parte de sus ventas. Esto es debido, en gran parte, a la gran inestabilidad que presentan los envasados de ciertos tipos de elaboraciones como las verdes frescas y tradicionales, lo que dificulta que puedan ser comercializados en lugares alejados de su zona de producción. Por lo tanto, aumentar la vida de mercado de sus envasados es, actualmente, una prioridad para las empresas. Además, el sector también desea conocer el efecto que el uso de diferentes condiciones de envasado (pH, sal y conservantes) podría tener sobre la calidad y seguridad microbiológica del producto final.

Por tanto, la presente Tesis Doctoral se orienta al estudio de los envasados de aceitunas de mesa con DOP *Aloreña de Málaga* con el objetivo de mejorar la calidad y seguridad microbiológica de los mismos. Está dividida en tres secciones. En la primera de ellas se realiza un estudio fisicoquímico, microbiológico y sensorial de los envasados para determinar su vida de mercado y actuales causas de alteración. En la segunda, se evalúa *in silico*, mediante la aplicación de modelos matemáticos (dosis respuesta y logístico/probabilístico), el empleo de diferentes conservantes (sales de zinc, metabisulfito de sodio, cinamaldehido, ácido pirúvico, ácido fumárico y natamicina) como alternativas a los habituales sorbatos y benzoatos, con la finalidad de mejorar la estabilidad y calidad de los productos mediante la inhibición del desarrollo microbiano. En la tercera sección, se evalúa la seguridad microbiológica de los envasados mediante la inoculación artificial con patógenos alimentarios (*Listeria monocytogenes, Escherichia coli, Staphylococcus aureus* y *Salmonella enterica*), determinando su supervivencia y su relación con la presencia de ciertos compuestos inhibidores presentes en el fruto.

El estudio de la vida comercial de los envasados ha demostrado que, actualmente, el crecimiento de bacterias ácido lácticas es la principal causa de alteración microbiológica. A los 2 meses de envasado se observa la presencia de una alteración caracterizada por la aparición de salmueras turbias y viscosas, así como la presencia en los frutos de manchas blanquecinas. Durante el envasado también ocurre una evidente disminución, confirmada instrumental y sensorialmente, de la textura y de la tonalidad verde de los frutos.

Esta alteración se relaciona con un genotipo específico de la especie *Lactobacillus pentosus* (O-A-2). El análisis de valoración sensorial realizado por un panel de consumidores (n=35) tanto de las aceitunas como de la apariencia de los envasados durante un periodo de 8 meses, ha puesto de manifiesto que la valoración inicial de los envases (garrafas PET 1.6L) es mayor que la de las aceitunas. Sin embargo, con el transcurso del tiempo, los primeros van obteniendo puntuaciones más bajas mientras los frutos las mantienen.

Se ha utilizado un modelo logístico/probabilístico para determinar el efecto anti-fúngico de diversas sales de zinc ($ZnCl_2$ y $ZnSO_4$) sobre el crecimiento en medio de laboratorio de diferentes especies de levaduras aisladas de aceitunas y su interacción con el cloruro de sodio e hidroxitirosol (compuesto fenólico presente en las salmueras de envasado). Los datos muestran que el cloruro de sodio, a las concentraciones utilizadas, no tiene efecto alguno sobre el crecimiento de las levaduras, mientras que el hidroxitirosol ejerce, cuando se encuentra a elevadas concentraciones, un efecto antagónico sobre las sales de zinc. Las interfaces de crecimiento/no crecimiento obtenidas indican que la inhibición del cóctel de levaduras puede obtenerse con un contenido de Zn en el rango de 4 – 5 mM, obtenido a partir de $ZnCl_2$.

Cuando se aplica un modelo de dosis respuesta para determinar la susceptibilidad y resistencia de las principales especies de bacterias ácido lácticas y levaduras aisladas de aceitunas de mesa frente al metabisulfito de sodio, cinamaldehido, ácido pirúvico, ácido fumárico y natamicina, se observa que, para dos de estos conservantes, metabisulfito de sodio y cinamaldehido, existe un gran efecto inhibidor. En el caso del metabisulfito de sodio, la concentración mínima inhibitoria para las poblaciones de bacterias ácido lácticas es de 50 mg/L y para las levaduras de 772 mg/L. Para el cinamaldehido, la concentración mínima inhibitoria para bacterias ácido lácticas es de 1.060 mg/L y para las levaduras de 125 mg/L. El ácido pirúvico, que también posee un importante efecto inhibidor frente a las bacterias ácido lácticas y levaduras (3.211 y 3.038 mg/L, respectivamente) podría, además, representar una alternativa para la acidificación de las salmueras de envasado.

El efecto inhibidor del metabisulfito de sodio y cinamaldehido así como la influencia que el pH y el tipo de agente acidificante (HCl o ácido pirúvico) tienen sobre las poblaciones de bacterias acido lácticas, levaduras y enterobacterias ha sido investigado también mediante un modelo logístico/probabilístico. Los resultados muestran que el pH tiene una gran

influencia sobre el efecto inhibidor del metabisulfito de sodio (especialmente por debajo de pH 4,0), pero no así sobre el cinamaldehido, cuyo efecto fue independiente del pH. La acidificación con HCl da lugar a mayores efectos inhibidores que cuando se emplea ácido pirúvico. El crecimiento de las poblaciones de enterobacterias se inhibe siempre a pH ≤ 4,0, incluso en ausencia de conservantes.

Finalmente, los estudios de inoculación de patógenos en salmueras y envasados de diferentes tipos de elaboraciones de aceitunas *Aloreña de Málaga*, muestran el gran poder inhibidor que tienen algunos de los componentes de las aceitunas. La muerte de los patógenos es más rápida en las salmueras (24 h) que en los envasados con aceitunas (48 h). También influye sobre la supervivencia de los mismos el tipo de presentación de *Aloreña de Málaga* estudiado. La inhibición es más lenta en los envasados de aceitunas verdes frescas ecológicas, que no utilizan conservantes. En las salmueras, *E. coli* resulta ser el patógeno más resistente, mientras que en los envasados es *S. aureus*. El análisis multivariante realizado muestra que la supervivencia de los patógenos se relaciona con la presencia de ciertos compuestos fenólicos (Ty, Hy-4-glucósido, EDA, HyEDA), la concentración de azúcares y conservantes en las salmueras de envasado, cuyos valores, a su vez, dependen del tipo de elaboración. Estos datos confirman, una vez más, que las salmueras de aceitunas constituyen un hábitat muy adverso para los patógenos alimentarios y justifican la seguridad microbiológica que ha ofrecido tradicionalmente este producto.

The *Aloreña de Málaga* was the first table olive speciality to obtain the Protected Designation of Origin (PDO) in Spain (BOJA nº215, 2009). Its production is located in Guadalhorce Valley and is highly appreciated in the Costa del Sol (Malaga, Spain), where it is mainly commercialised. The restriction of its delivery is due, at least in part, to the commercial packaging instability, which prevents its distribution to markets far from the production area. Thus, increasing the shelf life of packaging is nowadays a priority for companies. Moreover, the sector also wishes information on the effect that the different packaging conditions (pH, salt, and preservatives) may have on the quality and microbiological safety of the final products.

This Doctoral Thesis is focused on the study of the packaging conditions of *Aloreña de Málaga* table olives to improve the quality and microbiological safety of their commercial products. It is divided into three sections. In the first, a physicochemical, microbiological and sensory study of commercial packaging is carried out to determine its shelf life and possible causes of spoilage. In the second, the use of different preservatives (zinc salts, sodium metabisulfite, cinnamaldehyde, pyruvic acid, fumaric acid, and natamycin) as alternatives for the substitution of sorbates and benzoates is evaluated, using mathematical models. In the last section, the microbiological safety of the final products is evaluated using challenge tests. They consisted of inoculating the commercial packaging with foodborne pathogens (*Listeria monocytogenes, Escherichia coli, Staphylococcus aureus*, and *Salmonella enterica*) for determining the influence of specific inhibitory compounds from the fruits on their survival.

The study of the packaging shelf life has shown that, nowadays, the primary cause of alteration is the growth of lactic acid bacteria. This way, after two months of packaging, spoilage, characterised by the appearance of turbid and viscous brines as well as fruits with whitish spots, was observed. During the packaging, there was also an evident decrease, confirmed by instrumental and sensory techniques, of the texture and the green colour of the fruits. This spoilage was related to a specific genotype of the species *Lactobacillus pentosus* (O-A-2). The sensory analysis carried out by a panel of consumers (n=35) of both the olives and the packaging appearance along eight months, showed that the initial score for containers (1.6 L PET bottles) was higher than for fruits. However, as time progressed, the packaging scores decreased while those for the fruits remained constant.

A logistic/probabilistic model was used to determine the anti-fungal effect of different zinc salts ($ZnCl_2$ and $ZnSO_4$) on the growth of diverse yeast species isolated from table olive processing and their interaction with sodium chloride and hydroxytyrosol (phenolic compound present in olives). The data showed that sodium chloride, at the concentrations assayed, did not affect yeast growth, while hydroxytyrosol exerted an antagonistic effect, at high concentrations, when was used simultaneously with zinc salts. The growth/non-growth boundaries obtained showed that the inhibition of the yeast cocktail was achieved with 4-5 mM zinc, using $ZnCl_2$.

The susceptibility and resistance to different preservatives (sodium metabisulfite, cinnamaldehyde, pyruvic acid, fumaric acid, and natamycin) on the growth of the main species of lactic acid bacteria and yeasts isolated from table olive processing were studied using a dose-response model. Two of these preservatives (sodium metabisulfite and cinnamaldehyde) showed the highest inhibitory effects. In the case of sodium metabisulfite, the minimum inhibitory concentrations for lactic acid bacteria and yeasts were obtained at a concentration of 50 and 772 mg/L, respectively. For cinnamaldehyde, the minimum inhibitory concentration was 1,060 mg/L for lactic acid bacteria and 125 mg/L for yeasts. The pyruvic acid, which also showed an important effect against lactic acid bacteria and yeasts (3,211 and 3,038 mg/L, respectively), may also represent an alternative for the acidification of packaging brines.

The inhibitory effect of sodium metabisulfite and cinnamaldehyde, in combination with pH levels and type of acidifying agent (HCl/pyruvic acid) on the growth of lactic acid bacteria, yeasts and *Enterobacteriaceae* populations, was also investigated using a logistic/probabilistic model. The results showed that pH had a considerable influence on the inhibitory effect of sodium metabisulfite (particularly at pH below 4.0) but not on cinnamaldehyde, whose effect was pH-independent. Acidification with HCl always led to higher inhibitory effects than pyruvic acid. The growth of *Enterobacteriaceae* population was always inhibited at $pH \leq 4.0$, even in the absence of preservatives.

Finally, the inoculation of foodborne pathogens in the packaging brines of different types of *Aloreña de Málaga* table olives always showed faster death rates (24 h) than in commercial packaging (48 h). The type of preparation of *Aloreña de Málaga* also influenced the survival of the foodborne pathogens. Inhibition was slower in the packaging of organic

fresh green olives, without preservatives. In brines, *E. coli* proved to be the most resistant pathogen in brines, while *S. aureus* had higher survival in the commercial packaging. The multivariate analysis showed that the persistence of pathogens was related to the presence of certain phenolic compounds (Ty, Hy-4-glucoside, EDA, and HyEDA), the concentration of sugars, and preservative content. The levels of them in brine, except the last one, depended, in turn, on the type of elaboration. These data confirm that the prevailing environment in packaged fruits represents a very adverse habitat for the survival of foodborne pathogens, and justify the traditional microbiological safety offered by the olive products.

LISTADO DE ABREVIATURAS Y SÍMBOLOS

a. C.	Antes de Cristo
ASEMESA	Asociación de Exportadores e Industriales de Aceitunas de Mesa
BAL	Bácterias Ácido Lácticas
BOE	Boletín Oficial Español
BOJA	Boletín Oficial de la Junta Andalucía
CIN	Cinamaldehido
CO_2	Dióxido de carbono
COI	Comité Oleícola Internacional
d. C.	Después de Cristo
DO	Densidad Óptica
DOP	Denominación de Origen Protegida
DOUE	Diario de la Unión Europea
ESYRCE	Encuesta sobre superficies y rendimientos de cultivos
EDA	Forma dialdehídica descarboximetilada del ácido elenólico
h	Horas
H^+	Protón
ha	Hectáreas
HCl	Ácido clorhídrico
Hy	Hidroxitirosol
HyEDA	EDA unido a hidroxitirosol
Hy-4-glucósido	Hidroxitirosol 4 glucósido
ISO	Organización Internacional de Normalización
kg	kilogramos
L	Litros
MET	Metabisulfito de sodio
mg	Miligramo
Mg^{2+}	Magnesio
MIC	Concentración Mínima Inhibitoria
mM	Milimolar
MPa	Megapascal
NaCl	Cloruro de sodio
NIC	Concentración no inhibidoria
O_2	Oxígeno
p	Probabilidad de crecimiento
PET	Tereftalato de polietileno
PIB	Producto interior bruto
RD	Real Decreto
t	toneladas
T^a	Temperaura
Ty	Tirosol
var	Variedad
Zn	Zinc
$ZnCl_2$	Cloruro de zinc
$ZnSO_2$	Sulfato de zinc
ºC	Centígrados
€	Euros
%	Tanto por ciento

MARCO DE LA TESIS

La presente tesis doctoral se enmarca dentro del programa de formación de doctores en empresa (convocatoria 2013) del campus de excelencia internacional agroalimentario *ceia3* de las Universidades de Córdoba, Cádiz, Huelva, Almería y Jaén, y del proyecto motriz de la Junta de Andalucía (convocatoria 2011, AGR-7755) titulado "*Control microbiológico de las poblaciones de enterobacterias en envasados de aceitunas de mesa con DOP Aloreña de Málaga*" (acrónimo PrediAlo). Los fondos económicos necesarios para la contratación por parte de la empresa de la doctoranda se pudieron conseguir mediante un convenio firmado entre el *ceia3* y la Asociación de Aderezadores de Aceituna de Mesa de la Comarca Natural del Valle del Guadalhorce. El material fungible y equipamiento necesario para la realización de las actividades experimentales se financiaron gracias a los recursos propios del proyecto PrediAlo. De este modo, todo el trabajo de laboratorio ha sido llevado a cabo íntegramente en las instalaciones del Departamento de Biotecnología de Alimentos del Instituto de la Grasa (CSIC, Sevilla), mientras que la toma de muestras se ha llevado a cabo en diferentes industrias de la provincia de Málaga que forman parte de la Asociación de Aderezadores. Se trata, por tanto, de un claro ejemplo de investigación aplicada al sector industrial y cuya finalidad es la formación en el tejido productivo de personas cualificadas y de alto rigor científico que puedan llevar a cabo actividades de I+D+i.

La aceituna de mesa con DOP *Aloreña de Málaga* fue la primera aceituna de mesa de España en obtener este sello de calidad por orden de 26 de Octubre del 2009 de la Consejería de Agricultura y Pesca de la Junta de Andalucía (BOJA nº 215, 2009), reconocimiento que fue, posteriormente, homologado por la Unión Europea (DOUE 2012). La presente Tesis surge de la necesidad por parte del sector de mejorar la estabilidad, calidad y seguridad alimentaria de los envasados de estas preparaciones de aceitunas de mesa, que son bastante inestables, lo que limita sus ventas sólo al entorno de la zona de producción (Valle del Guadalhorce, provincia de Málaga). Sin embargo, su expansión requeriría llevar su comercialización a otros nichos de mercado más alejados. La presente Tesis Doctoral profundiza en los estudios iniciados por el grupo de investigación PAIDI AGR-125 del Instituto de la Grasa con el trabajo "*Conservación y envasado de aceitunas de mesa aliñadas Manzanilla-Aloreña: Diseño de modelos matemáticos para el crecimiento e inhibición de las poblaciones de microorganismos*", presentado en el 2007 por el Dr. Francisco Noé Arroyo López (director de la doctoranda), para la defensa de su propia Tesis doctoral. En la misma se inició la caracterización físico-química y microbiológica de los procesos de elaboración de la

aceituna de mesa *Aloreña de Málaga,* suministrando al sector procedimientos y conocimientos para abordar las principales problemáticas que tenían las industrias en ese momento, entre las que destacaba claramente la inestabilidad de los envasados, debido al desarrollo de levaduras y la respiración celular del fruto. Una década después, se hace necesario una puesta a punto para profundizar aún más en el conocimiento de los procesos, aplicando nuevas metodologías y valorando la utilización de otros conservantes. Con ello se pretende suministrar al tejido productivo información actualizada sobre la vida de mercado de los envases y los frutos, preparación de nuevas formulaciones, así como documentar la seguridad microbiológica de los envases. Para ello, se ha dividido la tesis doctoral en las siguientes secciones:

> Primera sección. Capítulos 1 y 2. Incluye estudios encaminados a determinar el tiempo de vida de mercado de envasados comerciales de aceitunas de mesa con DOP *Aloreña de Málaga* y las principales causas por la que se produce la alteración de los mismos.

> Segunda sección. Capítulos 3, 4 y 5. Se valora la aplicación de nuevos conservantes en los envasados de aceitunas *Aloreña de Málaga* como alternativa a sorbatos y benzoatos. Además, se estudia la interacción de estos nuevos conservantes con diferentes compuestos presentes en los envasados como son los compuestos fenólicos y el cloruro de sodio.

> Tercera sección. Capítulos 6 y 7. Incluye estudios de desafío o *challege tests* consistentes en la inoculación artificial de salmueras y envasados de aceitunas *Aloreña de Málaga* con diferentes patógenos para constatar su inhibición y, así, poder garantizar la seguridad microbiológica del producto final.

Los trabajos de la Tesis Doctoral han implicado un enfoque multidisciplinar a lo largo de los cuatro años de estudio (2013-2017), utilizando técnicas y herramientas de biología molecular y genotipado, microbiología predictiva, análisis sensorial, análisis multivariante y química analítica, a lo que hay que sumar la dificultad del trabajo con patógenos alimentarios del grupo 2 (*Listeria, Salmonella, Escherichia* y *Staphylococcus*). Los resultados que se muestran a continuación representan un resumen de una serie de trabajos científicos que se presentan como un compendio de publicaciones de acuerdo con la normativa reguladora de los estudios de doctorado en la Universidad de Córdoba. Las principales conclusiones derivadas de esta tesis, que se exponen al final del manuscrito, sin duda ayudarán al sector de

la *Aloreña de Málaga*, y al de la aceituna de mesa en general, al desarrollo de envasados con una mayor calidad del producto, estabilidad y seguridad microbiológica. Por lo tanto, se pretende que el presente manuscrito constituya una fuente de información relevante para la comunidad científica, el sector industrial y las autoridades sanitarias en materia de calidad y seguridad alimentaria, siendo la base, a su vez, para el desarrollo de futuros estudios científicos, trabajos de fin de máster y Tesis Doctorales.

Finalmente, mencionar la presión que el sector de la aceituna de mesa está sufriendo por parte de los mercados para bajar los niveles de sodio en sus productos, por lo que existe una necesidad en las empresas de reducir los contenidos de NaCl que actualmente se utiliza en el proceso de transformación y envasado. Además, específicamente en el caso de la *Aloreña de Málaga*, las industrias están muy interesadas en poder envasar a valores de pH más altos. En la actual legislación española sobre aceitunas de mesa (Real Decreto 679/2016), se da una mayor libertad a las empresas en cuanto a niveles de pH, sal y acidez libre a los que poder envasar que en el anterior reglamento del 2001. Sin embargo, actualmente, la legislación en vigor especifica claramente que la empresa es la responsable de garantizar la seguridad microbiológica del producto final y aportar estudios que así lo demuestren frente a las autoridades competentes. La presente Tesis Doctoral pretende suministrar este tipo de información al sector para los diferentes tipos de elaboraciones de aceitunas de mesa *Aloreña de Málaga.*

1. INTRODUCCIÓN

1.1. GENERALIDADES SOBRE LA ACEITUNA DE MESA

El olivo es por excelencia el árbol del Mediterráneo y su zona de cultivo lo marca el límite de este clima, denominado en ocasiones "*clima del olivo*". Hablamos de una especie longeva, siempre verde y de lento crecimiento, que resiste sequías y fuertes vientos, pero no las bajas temperaturas (<12ºC). Por lo tanto, el olivo silvestre o acebuche (*Olea europea*) es un árbol muy común en el cercano oriente y en todo el entorno mediterráneo (Figura 1). Su cultivo data de las culturas fenicia, asiria, judía, egipcia y griega, originándose hace más de 6.000 años en Oriente Medio. Posteriormente, la difusión del mismo se realizó de Oriente a Occidente a través de las dos orillas del Mediterráneo. En este proceso, parece ser que los primeros olivicultores de cada zona seleccionaron de sus bosques de acebuche los individuos más sobresalientes por su productividad, oleosidad, tamaño de fruto y adaptación al medio.

Figura 1. Actual zona de crecimiento del olivar en la cuenca mediterránea

La influencia que el olivo tiene en la cultura, religión y dieta mediterránea es enorme. Los embalsamadores del antiguo Egipto, hace ya más de 3.200 años, adornaban coquetamente las cabezas de las momias con guirnaldas formadas por ramas de olivo. También hay frescos del palacio de Cnossos (Creta) datados hace más de 3.600 años que representan un toro sagrado ante un olivo. Los primeros documentos escritos que constatan la presencia del olivo son unas tablillas micénicas en barro, procedentes del reinado del rey Minos (2.500 a. C.) que dan testimonio de la importancia del aceite de oliva y la aceituna para la economía cretense.

El cultivo del olivo fue introducido en España durante la dominación marítima de los fenicios (1.050 a. C.), pero no se desarrolló en extensiones notorias hasta la llegada de Scipio (212 a. C.) y el poder de Roma (45 a. C.). Los árabes introdujeron sus propias variedades de olivo en el sur de España, ampliaron su cultivo e influenciaron en la utilización de los vocablos que han llegado hasta nuestros días como aceituna, aceite, o almazara. Con el descubrimiento de América (1492), el cultivo del olivo se extendió más allá de los confines del Mediterráneo, sobre todo en Sudamérica. Los primeros olivos se llevaron a las Indias Occidentales desde el puerto de Sevilla.

La aceituna de mesa es el fruto del olivo cultivado (*Olea europaea* var. *sativa*) que se caracteriza por ser una drupa carnosa que contiene un componente amargo (oleuropeina), un bajo contenido en azúcares (2.6 - 6%) y un elevado contenido en grasa (12 - 30%), según estado de madurez y variedad. Estas características hacen que la aceituna no pueda ser consumida directamente del árbol y tenga que someterse a determinados procesos, en función de la variedad o región, para hacerla comestible (Garrido-Fernández y col., 1997). Existen documentos que explican la elaboración de aceitunas de mesa desde el siglo I de nuestra era. De hecho, Columela en el año 42 d. C., en su obra *De Re Rustica* indica diversas maneras de cómo "*adobar aceitunas*" (Columela, 42), mientras que Plinio el viejo, en su monumental *Naturalis historia*, describe también detalladamente la elaboración y el procesado de diferentes tipos de alimentos, entre ellos la aceituna de mesa (Plinio, 77).

Según el Consejo Oleícola Internacional (COI, 2004): "*la aceituna de mesa es el producto preparado a partir de frutos sanos de variedades de olivo cultivado que han alcanzado un grado de maduración apropiado para su procesamiento y que han sido elegidas por producir frutos cuyo volumen, forma, proporción de pulpa respecto al hueso, delicadeza de la pulpa, sabor, firmeza y facilidad para separarse del hueso los hacen particularmente aptos para la elaboración; sometidos a tratamientos para eliminar el amargor natural y conservados mediante fermentación natural o tratamiento térmico, con o sin conservantes, envasados con o sin líquido de gobierno*". Comparado con otros vegetales fermentados, la producción de aceitunas de mesa solo representa el 2% de la producción mundial de este tipo de alimentos (Figura 2). España es el primer país productor de aceitunas de mesa del mundo, seguido muy de cera de otros países como Egipto, Turquía, Argelia y Marruecos (todos ellos de la cuenca mediterránea). La producción media mundial de aceitunas de mesa durante las

últimas cinco campañas de recogida (2012-2017) ascendió a un total de 2.620.600 t/año, de las cuales 542.300 se produjeron en España, es decir, un 21% del total (COI, 2017). Este sector se caracteriza por ser uno de los más importantes de la industria agroalimentaria española. Según la encuesta sobre superficies y rendimientos de cultivos de 2015, España cuenta con 2.605.252 ha olivar, de las que 75.080 ha (2,83%) se dedican a la aceituna de mesa. Dichas hectáreas se concentran principalmente en Andalucía y Extremadura, sumando entre las dos comunidades más del 99% de las plantaciones destinadas a aceituna de mesa (ESYRCE, 2015). Esta actividad se estima que genera 8.000 empleos directos, más de seis millones de jornales por la recolección y el cultivo del olivo, a los que hay que añadir los originados por las empresas y fábricas auxiliares como las de vidrio, hojalata, cartonaje, maquinaria, transportes, etc. Esto supone el 27% del empleo generado por el sector nacional de conservas y preparados de productos vegetales, participando con un 22% en el valor nacional de este sector y aportando al PIB alrededor de 1.100 millones de euros. En el año 2016, las exportaciones españolas de aceitunas de mesa alcanzaron la cifra de 332.319 t (peso neto escurrido), cuyo valor ascendió a unos 760 millones de euros (ASEMESA, 2017). Además, comparativamente, la industria agroalimentaria de la aceituna de mesa genera más riqueza económica y empleo en términos relativos, que la industria del aceite de oliva (Garcia-Brenes, 2007).

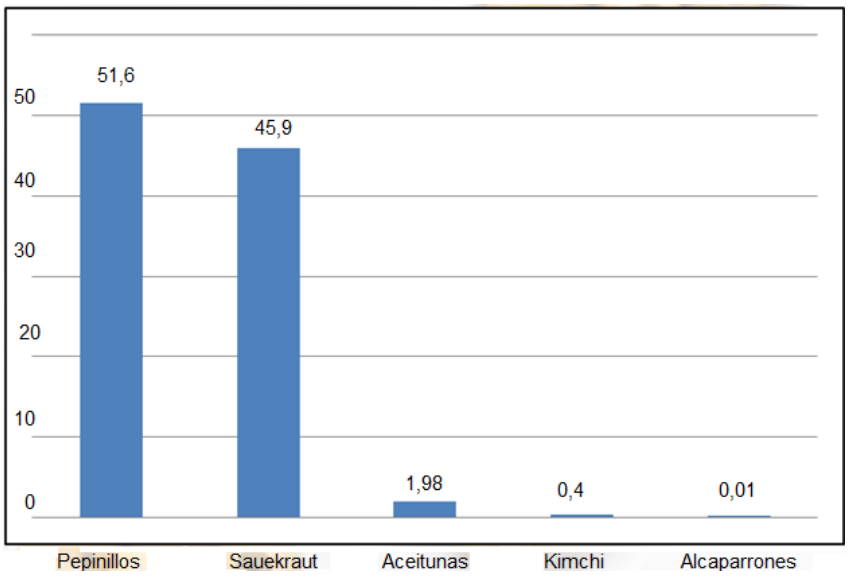


Figura 2. Porcentajes de producción mundial de diferentes tipos de vegetales fermentados. La cifra asciende a un valor total de 126.14 millones de t/año. Fuente: elaboración propia.

La norma de estándares de calidad comercial del COI (2004) indica que, dependiendo de la forma de eliminar el amargor de las aceitunas se tendrán las siguientes preparaciones comerciales:

·**Aceitunas aderezadas**: *"aceitunas verdes, de color cambiante o negras sometidas a un tratamiento alcalino y acondicionadas en salmuera, donde sufren una fermentación total o parcial, conservadas con o sin acidificantes."*

·**Aceitunas al natural***: "aceitunas verdes, de color cambiante o negras tratadas directamente con una salmuera, donde sufren una fermentación total o parcial, y conservadas con o sin acidificantes."*

·**Aceitunas deshidratadas y/o arrugadas***:* "*aceitunas verdes, de color cambiante o negras, sometidas o no a un ligero tratamiento alcalino, conservadas en salmuera o parcialmente deshidratadas con sal seca y/o aplicando calor o cualquier otro proceso tecnológico*".

·**Aceitunas ennegrecidas por oxidación***:* "*aceitunas verdes o de color cambiante conservadas en salmuera, fermentadas o no, ennegrecidas por oxidación en medio alcalino y conservadas en recipientes herméticos mediante esterilización térmica. Su coloración negra es uniforme*".

Las principales variedades de aceitunas empleadas en España para el consumo de mesa son Manzanilla, Hojiblanca, Cacereña, Gordal, Verdial, Morona y *Aloreña de Málaga*. Cada una de estas variedades tiene unas características intrínsecas diferenciales que las hacen adecuadas para tipos específicos de presentaciones comerciales.

La aceituna de mesa es un alimento básico en la dieta mediterránea y sigue presente a diario en las rutinas gastronómicas de un gran número de países ya sea como desayuno (Oriente Medio), aperitivo o ingrediente culinario. Su versatilidad y propiedades nutricionales la hacen perfecta para la elaboración de cualquier plato. Desde el punto de vista nutricional, tienen un aporte energético de 150 kilocalorías por cada 100 gramos, dependiendo de la variedad. Dichas calorías las aporta principalmente su contenido en grasa, de la cual aproximadamente el 82% es monoinsaturada (principalmente ácido oleico). Además, contribuyen a cubrir la cantidad diaria recomendada de fibra, aportan hidratos de carbono y

proteínas. Contienen también minerales esenciales como el calcio, hierro, potasio, magnesio, fósforo y yodo y su concentración en polifenoles y provitaminas A y E la dotan de propiedades antioxidantes que además de evitar el envejecimiento prematuro de la piel, protegen de los rayos UVA y estimulan la microcirculación cutánea (Arroyo-López y col., 2016). Por todo ello, los expertos aconsejan tomar 25 gramos de aceituna al día, que sustituyen a media ración de grasa.

Actualmente, en España el sector de la aceituna de mesa tiene que resolver diferentes retos entre los que destacan: i) la problemática nutricional derivada de su alto contenido en sodio y grasa, ii) baja penetración en mercados emergentes como el asiático, iii) la bajada de su ingesta per cápita al ser desplazado por otros aperitivos de nueva aparición, y iv) tratamientos y gestión de vertidos. Además, el sector está sufriendo la presión por terceros países en los que los costes de producción son muchos menores. Por ello, tanto las asociaciones españolas de productores de aceitunas de mesa como el sector científico español, han puesto su punto de mira en revitalizar el sector. De este modo se están realizando campañas publicitarias tanto en países de Europa del Este como en el mercado asiático, además, de dotar económicamente proyectos científicos con el objetivo de mejorar la calidad, seguridad, las características nutricionales y el valor añadido del producto final. Dentro de estos proyectos destacan la elaboración de una base de datos de la composición nutricional de las diferentes elaboraciones y presentaciones de las aceitunas de mesa, gracias al cual se está realizado un etiquetado nutricional más completo, adecuándose a los requerimientos de los diversos países de destino. Otros trabajos científicos se están focalizando en objetivos diversos, como la utilización de sales alternativas al cloruro de sodio, el estudio de la presencia de compuestos beneficiosos para la salud como son compuestos del tipo fenólico y triterpénico y la aplicación de microorganismos con características multifuncionales en las fermentaciones de aceitunas, todo ello para posibilitar en un futuro que las aceitunas de mesa sean declaradas como alimento saludable por organismos públicos de control alimentario.

1.2. CALIDAD Y SEGURIDAD ALIMENTARIA EN LA ACEITUNA DE MESA

La norma comercial del COI aplicable a la aceituna de mesa (COI, 2004) estipula los límites de pH, sal y acidez libre que los diferentes tipos de elaboraciones de aceitunas de mesa deben tener para garantizar la seguridad y estabilidad microbiológica del producto envasado.

En la norma también se incluye las condiciones mínimas que deben tener los tratamientos térmicos de pasteurización y esterilización para garantizar la buena conservación de los envasados, y se especifica claramente que: "*las aceitunas no deberán contener microorganismos patógenos o contaminantes capaces de reproducirse en el producto o almacenamiento, así como cualquier metabolito que se derive de su crecimiento y que pueda suponer un riesgo para la salud"*.

Por el contrario, la nueva reglamentación española RD 679/2016 por el que se establece la norma de calidad de las aceitunas de mesa, hace referencia exclusivamente a las características de los productos terminados en cuanto a la presencia de defectos (frutos blandos, molestado, presencia de pedúnculos, defectos de relleno, etc.), pasando a ser responsabilidad de la empresa garantizar la seguridad microbiológica del producto. Por ello, en dicha reglamentación, ya no se especifica los límites fisicoquímicos de los productos terminados ni las condiciones mínimas de los tratamientos térmicos para impedir alteraciones de origen microbiológico en los envasados.

La aceituna de mesa es un vegetal fermentado en el que las BAL y levaduras juegan un papel muy importante en la calidad y seguridad del producto final, dotándolo además de unas adecuadas características organolépticas. Las fermentaciones de aceitunas de mesa se caracterizan por las altas concentraciones de cloruro de sodio y la elevada acidez que se origina durante el proceso. Las BAL son las principales responsables del consumo de azúcares y producción de ácido láctico, lo que lleva a una bajada de pH, que junto con la producción de bacteriocinas, aseguran la estabilidad y seguridad microbiológica del producto fermentado. *Lactobacillus pentosus* y *Lactobacillus plantarum* son las especies primordialmente responsables de llevar a cabo la fermentación ácido-láctica de las aceitunas de mesa (Hurtado y col., 2012). Sin embargo, en ciertos tipos de elaboraciones, sin tratamiento alcalino, la presencia de ciertos compuestos inhibidores (polifenoles) retrasa o inhibe el crecimiento de las BAL y, en este caso, las levaduras son los microorganismos predominantes al inicio de la fermentación. Entre ellas se encuentran diferentes especies de los géneros *Wickerhamomyces, Pichia, Candida, Saccharomyces* y *Debaryomyces*. Estos microorganismos eucarióticos pueden jugar diferentes aspectos positivos durante la fermentación, favoreciendo el crecimiento de la BAL, formación de biofilms, producción de aromas y sabores agradables o actividad β-glucosidasa (Arroyo-López y col., 2012). Sin

embargo, cuando la secuencia de microorganismos no es la adecuada y se desarrollan otros ajenos a los de un proceso normal de fermentación, se producen distintos tipos de alteraciones.

Las principales, según el origen y fase del proceso en que suceden, son las siguientes:

Butírica. Se debe al desarrollo de distintas especies de *Clostridium* durante las primeras fases de la fermentación. El ácido butírico que se produce altera el sabor, pudiéndose evitar su formación manteniendo un nivel adecuado de sal (nunca menor de 5 %) y siguiendo buenas prácticas higiénicas de elaboración.

Zapatería. Producida por el desarrollo de bacterias propiónicas y *Clostridium*. Se origina durante la conservación cuando el valor de pH no se mantiene por debajo de 4,3 unidades y la concentración de sal es baja. Se identifican en la salmuera por la presencia de una serie de compuestos volátiles que producen aromas anómalos a la fermentación. Se evita incrementando la concentración de sal (>8,0%) para inhibir el desarrollo de los microorganismos responsables y, de esta forma, estabilizar el valor de pH durante la conservación.

Alambrado. Se produce por la formación de hendiduras en el exterior de las aceitunas y huecos internos en la pulpa (Figura 3). A veces, la formación de gas produce también vejigas o ampollas bajo la piel. Se evita ajustando el valor del pH inicial. El problema es originado en ciertas ocasiones por el crecimiento de levaduras y bacilos gran negativos productores de CO_2 o por el propio proceso de respiración del fruto.

Ablandamiento. Debido a un desarrollo excesivo de microorganismos con actividad pectinolítica y proteolítica: bacilos, levaduras y mohos. Se previene evitando el desarrollo de los mismos, especialmente durante la conservación, y limitando la presencia excesiva de O_2. En ocasiones, esta alteración también se debe a un exceso de adición de ácidos que dañan la estructura del fruto.

Consumo de láctico. En presencia de O_2 ciertas especies de levaduras y mohos son capaces de consumir el ácido láctico producido por las BAL originando una subida del pH que puede comprometer la seguridad microbiológica del producto. El desarrollo de estas levaduras y

mohos en la superficie de los fermentadores da lugar a las conocidas natas o velos que hay que eliminar lo antes posible.

Enturbiamiento, oscurecimiento y pardeamiento de salmueras. Se debe al crecimiento de microorganismos que producen un aumento de la turbidez de las salmueras, lo cual es especialmente negativo en los envasados. El oscurecimiento por su parte está ligado a la oxidación de ciertos compuestos fenólicos que da lugar a tonalidades marrones. Esta alteración es especialmente negativa en los envasados y supone una pérdida de calidad en el producto final. Se evita usando un producto bien fermentado y ajustando un valor de pH inferior a 3,3 unidades en el envasado, o bien pasteurizando.

Sedimento y gas. Se da en el producto envasado cuando no se mantiene estable, bien por desarrollo de diversos tipos de bacterias o levaduras cuando hay restos de materia fermentable en las aceitunas, o bien por desarrollo de las bacterias propiónicas que consumen ácido láctico. La producción de gas puede dar lugar a un abombamiento y derrame de líquido en el producto envasado. Se evita, como en el caso anterior, usando un producto bien fermentado y ajustando un valor de pH inferior a 3,3 unidades en el envasado, o bien pasteurizando.

Figura 3. Ejemplo de aceitunas alambradas con fisuras en el interior del fruto.

Aunque, en general, la aceituna de mesa se considera un alimento seguro, y numerosos trabajos muestran la inhibición de diversas especies patógenas durante el proceso de elaboración de aceitunas de mesa (para una revisión sobre el tema ver Medina-Pradas &

Arroyo-López, 2015), unas malas prácticas de elaboración puede suponer un riesgo sanitario para los consumidores. De hecho, aunque pocas, han existido algunas alertas de intoxicaciones alimentarias debido al crecimiento de microorganismos patógenos en aceitunas. Su presencia se relaciona siempre con fallos en los procesos de elaboración, bien porque no se alcance un valor de pH inferior a 4,3 unidades para aceitunas verdes o debido a que la esterilización fuera deficiente para aceitunas negras. Ello refuerza la necesidad de mantener siempre unas condiciones higiénico-sanitarias estrictas y seguir las normas establecidas por la legislación. Entre los principales peligros de origen microbiano en aceitunas de mesa se pueden encontrar:

- Producción de micotoxinas. Ciertas especies de mohos de los géneros *Aspergillus* y *Penicillium* pueden producir metabolitos secundarios tóxicos conocidos generalmente como micotoxinas (aflotoxinas y ocratoxinas). Las mismas pueden producir alergias, irritaciones, depresión del sistema inmunológico y se consideran potencialmente cancerígenas. El desarrollo de estos microorganismos y sus toxinas se favorece en aceitunas de mesa por la presencia de O_2 pudiendo formar natas en la parte superior de los fermentadores que hay que eliminar periódicamente lo más rápidamente posible.

- Producción de aminas biógenas. Ciertas especies de levaduras y BAL encontradas en aceitunas de mesa han mostrado la capacidad de producir aminas biógenas a través de un proceso de descarboxilación de los aminoácidos precursores correspondientes. Elevadas concentraciones de estos compuestos pueden producir dolores de cabeza y enrojecimiento de la piel. En aceitunas de mesa se ha encontrado la presencia de tiramina, cadaverina y putrescina, relacionándose estos compuestos con el crecimiento de los microorganismos que producen la zapatería (García-García y col., 2001).

- Botulismo. Es el principal peligro sanitario y su aparición se debe al desarrollo de *Clostridium botulinum* y la producción de la toxina botulínica. El desarrollo de las esporas de *Clostridium* ocurre cuando el pH es superior a 4,6 unidades y suele estar ligado a unas malas prácticas de esterilización del producto en condiciones de anaerobiosis. Se ha informado de intoxicaciones de este tipo en diferentes países como Estados Unidos, Francia e Italia con muertes asociadas (Medina-Pradas & Arroyo-López, 2015).

- Presencia de *Listeria, Staphylococcus* y *Pseudomonas*. Aunque la aceituna de mesa no es el hábitat más idóneo para el desarrollo de estos microorganismos, diversos trabajos han mostrado la presencia de estos microorganismos en fermentaciones y envasados de aceitunas. El problema puede venir en el caso de una hipotética ingesta de aceitunas contaminadas con los mismos, que, aun inicialmente en baja proporción, podrían luego reproducirse en el aparato digestivo humano produciendo fiebre, dolores musculares y vómitos. Altas concentraciones de sal y bajos niveles de pH, así como la presencia de compuestos fenólicos (HyEDA, EDA, Hy) provocan su inhibición (Media-Pradas & Arroyo-López, 2015).

Como puede apreciarse, la mayoría de estas alteraciones e intoxicaciones tienen posibles soluciones tecnológicas. Sin embargo, muchas de ellas podrían evitarse utilizando un cultivo iniciador al inicio de la fermentación, ya que este dotaría a la salmuera y a las aceitunas de las condiciones físico-químicas óptimas (bajada de pH, disminución en la producción de CO_2 durante la fermentación, buena capacidad de producción de ácido láctico, etc.). De esta manera se evitaría el desarrollo de posibles microorganismos alterantes o patógenos gracias a la producción de compuestos antimicrobianos o, simplemente, por un consumo exhaustivo de la materia fermentable (Arroyo-López y col., 2017). También la utilización de conservantes y un correcto tratamiento térmico de los envasados puede ayudar a estabilizar el producto final.

1.3. MICROBIOLOGÍA PREDICTIVA

La microbiología de los alimentos es una rama de la ecología microbiana cuya finalidad es estudiar la presencia y comportamiento de los microorganismos en alimentos. Diversas especies de bacterias, levaduras y hongos están presentes en los alimentos en los que pueden actuar como microorganismos beneficiosos o perjudiciales. La microbiología predictiva surge probablemente en 1922 con la aparición del primer modelo que describe la inactivación térmica de esporas de *C. botulinum* tipo A (Esty & Meyer, 1922). Es un área multidisciplinar donde microbiólogos, estadísticos, químicos y tecnólogos interactúan entre sí. Esta disciplina utiliza modelos matemáticos para predecir el crecimiento o inactivación de los microorganismos en los alimentos en función de los factores ambientales (Tª, pH, sal o conservantes) (McKellar & Lu, 2004). Su aplicación en la industria ayuda a la obtención de alimentos seguros al determinar las condiciones de envasado que impiden el crecimiento de organismos patógenos, al mismo tiempo que reducen las pérdidas económicas provocadas por

alimentos alterados. Al predecir el comportamiento de los microorganismos en los alimentos, se puede determinar, por tanto, la vida de mercado de un producto. Cualquier modelo es una simplificación que representa los complejos procesos bioquímicos que controlan el crecimiento de los microorganismos. Las variables de entrada de un modelo deben reducirse hasta un número razonable, que además puedan ser fácilmente medidas en el alimento. El modelo debe ser, a la vez, lo suficientemente complejo para aportar una predicción útil del comportamiento microbiano, pero lo bastante simple como para poder ser utilizado.

La elaboración de un modelo predictivo ha de ir precedida de una correcta planificación del experimento a realizar. Son muchas las cuestiones que se han de aclarar antes de comenzar a trabajar, entre las que destacan: i) determinar los parámetros biológicos a modelar, ii) conocer las variables ambientales a estudiar y sus intervalos, iii) establecer el sustrato o medio de cultivo sobre el que realizar el modelo y en el que posteriormente se va a validar, iv) elegir el diseño matemático que mejor se adapte a los objetivos perseguidos y contemple un menor número de experimentos, y v) cuando se trabaje con varios microorganismos, seleccionar siempre como diana aquel que presenta una mayor resistencia frente a factores adversos, o bien, utilizar un cóctel con varias especies. La Figura 4 muestra las diferentes etapas que contempla el desarrollo de un modelo predictivo en alimentos.

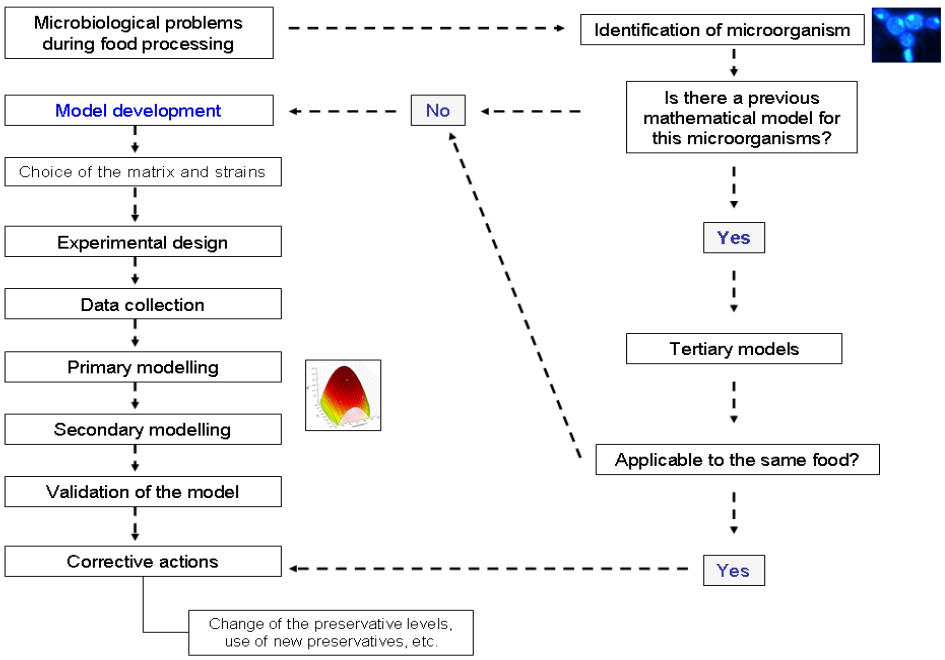


Figura 4. Etapas para el desarrollo de un modelo predictivo en alimentos. Cortesía de Arroyo-López y col. (2012)

Recientemente, esta disciplina ha comenzado a utilizarse con asiduidad en aceitunas de mesa a través de diferentes trabajos y modelos realizados principalmente por investigadores españoles y griegos (para una completa revisión ver Arroyo-López y col., 2010). A continuación se detallan los tipos de modelos predictivos que se han utilizado en la presente tesis doctoral:

Modelos logísticos/probabilísticos. Este tipo de modelos se utiliza para determinar las regiones de crecimiento/no crecimiento de los microorganismos en función de las variables ambientales o conservantes. En otras palabras, determinan, con una probabilidad fijada previamente, la región donde un microorganismo es capaz de crecer y en la que no. Los modelos probabilísticos incorporan datos binarios de crecimiento (crece/no crece) los cuales son procesados mediante una regresión logística que relaciona la probabilidad de crecimiento (*p*) con la de no crecimiento (*1-p*). La gran ventaja de la utilización de estos modelos es que pueden ser automatizados para obtener la respuesta del microorganismo mediante medidas de DO, permitiendo testear un alto número de variables y niveles con una menor carga de trabajo. Una importante característica de estos modelos es que también se puede configurar el nivel de probabilidad con el que se quiere trabajar, que estará relacionada con el riesgo a asumir. Por lo tanto, estos modelos tienen una aplicación directa en la formulación de condiciones de envasado que inhibían el crecimiento de los microorganismos, optimizando los niveles mínimos de conservantes que han de utilizarse y determinando la interacción entre ellos.

Modelos de dosis respuesta. Lambert & Pearson (2000) desarrollaron un método muy simple para determinar la concentración mínima inhibitoria (MIC) y la concentración a la que ya empieza a observase un efecto inhibidor (NIC) para un microorganismo en presencia de un determinado compuesto inhibidor mediante medidas de la DO. El procedimiento relaciona el área bajo las curvas de DO/tiempo con el grado de inhibición observado, utilizando para ello el cociente entre la respuesta del microorganismo a diferentes concentraciones del compuesto inhibidor (test) frente a la que presenta en ausencia del inhibidor (control). Conforme aumenta la concentración del compuesto inhibidor, disminuye el crecimiento del microorganismo, lo cual queda acreditado por una reducción en su área de crecimiento. Esta respuesta se puede ajustar mediante la ecuación modificada de Gompertz adaptada para inhibición (Lambert & Pearson, 2000). La respuesta del microorganismo frente al compuesto inhibidor

posteriormente es dividida en 3 secciones: i) puntos correspondientes a concentraciones hasta el valor NIC (concentraciones del compuesto en el que no se observa un efecto inhibidor), ii) puntos correspondientes entre el valor NIC y MIC (dentro de este rango de concentraciones el efecto inhibidor va progresivamente incrementándose), y iii) puntos por encima del valor MIC (donde ya no se observa crecimiento del microorganismo).

1.4. ESTUDIOS DE VIDA COMERCIAL Y SENSORIAL

La vida útil de un alimento es el periodo de tiempo que transcurre entre la producción o envasado del producto y el momento en el cual el alimento pierde sus cualidades físico-químicas y organolépticas. Este parámetro viene determinado por cada empresa y está relacionado con las condiciones de conservación del alimento que deben indicarse en el etiquetado. Por lo tanto, la vida útil depende tanto de las propias características del alimento como de las técnicas de conservación aplicadas al mismo. Los parámetros de calidad vienen determinados por diferentes aspectos: organolépticos, nutricionales, higiénicos, microbiológicos, etc. Así, determinados procesos (físicos, químicos o microbiológicos) pueden provocar el deterioro del producto y modificar la vida útil del mismo.

Hemos de distinguir también entre fecha de caducidad y fecha de consumo preferente. La fecha de caducidad hace referencia al momento a partir del cual un alimento ya no es apto para su consumo porque podría ser perjudicial para la salud. Por el contrario, la fecha de consumo preferente hace referencia a cuando las propiedades físico-químicas y organolépticas del producto empiezan a modificarse y pueden ser percibidas de forma negativa por el consumidor.

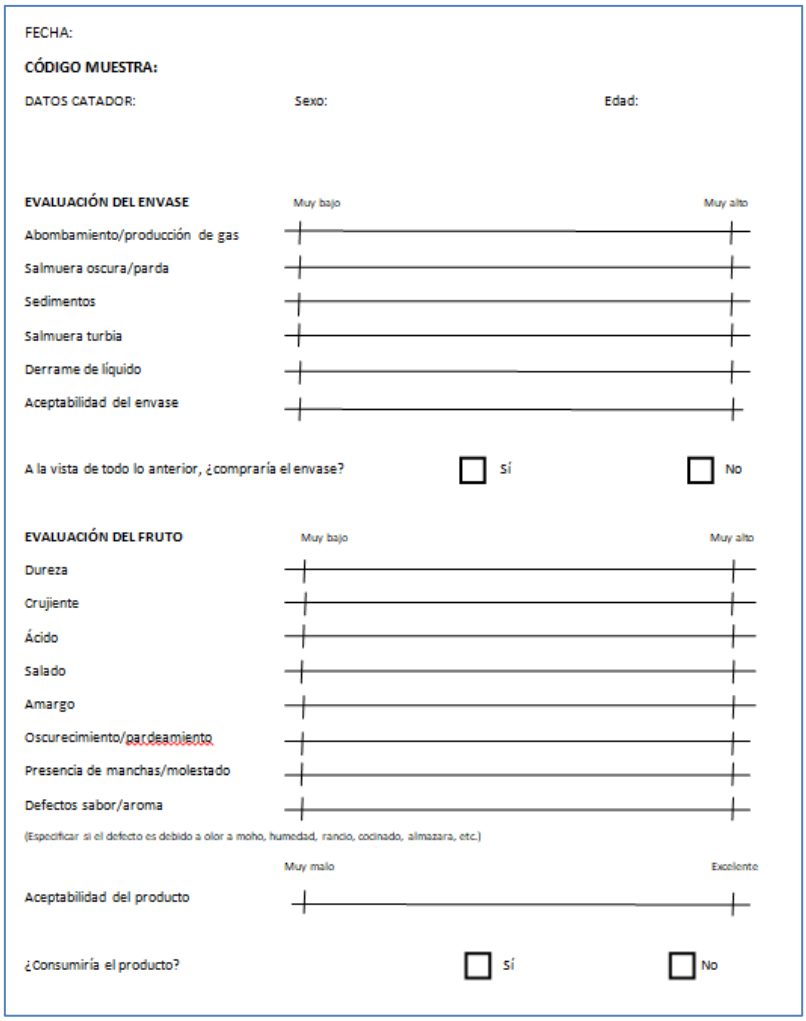
Uno de los métodos que se utiliza para estimar la vida útil sensorial de los alimentos es el método de supervivencia que se basa en la opinión del consumidor. Este método se basa fundamentalmente en conocer la actitud del consumidor hacia el producto, haciendo un test sensorial sobre si consumiría o no el producto. Para ello, sólo se requiere disponer de muestras almacenadas a lo largo del tiempo y muestras recién fabricadas de un mismo producto. Con estos estudios sensoriales las empresas aseguran que la vida útil estimada está acorde con los parámetros de calidad percibidos por el consumidor como claves en los productos, evitando posibles rechazos y cumpliendo con lo que el consumidor espera encontrar en el punto de venta.

La evaluación de los alimentos a través de los sentidos es una función primaria del hombre. Este, desde su infancia y de forma más o menos consciente, acepta o rechaza los alimentos de acuerdo con las sensaciones que experimenta al observarlos y/o ingerirlos. Este aspecto de la calidad global de los alimentos es lo que se denomina calidad sensorial. La evaluación de esta calidad se lleva a cabo mediante una disciplina científica, el análisis sensorial, cuyo instrumento de medida es el propio ser humano. El análisis sensorial se define como el "*examen de las propiedades organolépticas de un producto por los órganos de los sentidos*". Estas propiedades organolépticas son la apariencia, el flavor (olor/aroma, sabores básicos y sensaciones trigeminales) y la textura. La apariencia se define como todos los atributos visibles de un alimento y constituye un elemento absolutamente fundamental para su selección. La primera impresión que se recibe siempre es la visual y determina en gran medida, la decisión de aceptación o rechazo de un alimento (también del envase). Dentro de la apariencia se establecen tres grandes categorías de atributos: las propiedades ópticas (color, brillo, translucidez), la forma física (forma, tamaño y textura visual) y el modo de presentación. Se define el flavor como la combinación compleja de sensaciones olfativas (olor y aroma), gustativas (sabores básicos) y trigeminales percibidas durante la degustación. Finalmente, la textura es la propiedad de los alimentos detectada por los sentidos del tacto, la vista y el oído y que se manifiesta cuando el alimento sufre una deformación. Es muy importante notar que la textura no puede ser percibida si el alimento no ha sido deformado, es decir probado.

Para que los resultados aportados por este tipo de análisis sean exactos y precisos, se requiere unas condiciones de trabajo muy estrictas que afectan al grupo de personas que realizan el análisis (catadores), a su coordinador, (jefe de panel), al lugar donde se desarrolla la evaluación (sala de cata y sala de reunión), a la zona de preparación de las muestras (laboratorio y cocina) y a las condiciones de realización del propio análisis (horario, número de análisis, recipientes de degustación, técnica de cata o degustación y muestras). Por ello, para realizarlo de forma adecuada, existen normas internacionales de referencia (ISO) donde se establecen las condiciones necesarias para llevarlo a cabo. Para el análisis sensorial de la aceituna de mesa existe, además, una normativa específica establecida por el COI, que está basada en las normas genéricas ISO (COI, 2011).

En el caso específico de la aceituna de mesa *Aloreña de Málaga*, por sus características especiales, se creó una normativa específica para su caracterización sensorial en la que se establecen los criterios necesarios para valorar las características de apariencia, textura y flavor de la aceituna en sus tres formas de preparación: verdes, tradicionales y curadas, haciendo referencia al procedimiento de cata, los criterios de aceptación o rechazo de las aceitunas para la DOP, el programa de tratamiento de datos específico para la DOP y el programa de formación del panel analítico (Galán-Soldevilla & Ruiz Pérez-Cacho, 2012; Galán-Soldevilla y col., 2013).

En la presente tesis doctoral se ha tenido en cuenta toda esta información, pero a la vez se ha diseñado una hoja de valoración estructurada que permitiera también evaluar la vida comercial tanto de las aceitunas como de la apariencia de los envasados (Figura 5). Se trata de una hoja de valoración pensada para ser utilizada no por un panel de experto, sino de consumidores (>30), en la que se han incluido atributos que valoran la apariencia, flavor y textura de frutos y envases.


FECHA:

CÓDIGO MUESTRA:

DATOS CATADOR: Sexo: Edad:

EVALUACIÓN DEL ENVASE Muy bajo Muy alto

Abombamiento/producción de gas

Salmuera oscura/parda

Sedimentos

Salmuera turbia

Derrame de líquido

Aceptabilidad del envase

A la vista de todo lo anterior, ¿compraría el envase? Sí No

EVALUACIÓN DEL FRUTO Muy bajo Muy alto

Dureza

Crujiente

Ácido

Salado

Amargo

Oscurecimiento/pardeamiento

Presencia de manchas/molestado

Defectos sabor/aroma

(Especificar si el defecto es debido a olor a moho, humedad, rancio, cocinado, almazara, etc.)

Muy malo Excelente

Aceptabilidad del producto

¿Consumiría el producto? Sí No

Figura 5. Hoja de valoración de la vida comercial de envasados y aceitunas Aloreña de Málaga utilizada en la presente tesis doctoral.

Arroyo-López y col. (2005) estudiaron por primera vez los cambios fisicoquímicos y microbiológicos en el envasado de aceitunas de mesa *Aloreña-Málaga* en garrafas PET de 1,2 L. La vida comercial de los mismos fue baja (<2 meses) al observarse crecimiento microbiano, desaparición de los conservantes y pardeamiento de los frutos. Otro nuevo estudio sobre la inestabilidad de los envasados de este tipo de aceitunas confirmó la corta vida de mercado de algunas presentaciones, que no superaron los 7 días en el caso de aceitunas verdes frescas (Arroyo-Lopez y col., 2009). El estudio técnico llevado a cabo por Abelló Linde (2011) muestra que la vida comercial de estas aceitunas en atmósfera modificada no supera los 4 meses. Posteriormente, Bautista-Gallego y col. (2011) evaluaron la utilización de $ZnCl_2$ como conservante en el envasado de aceitunas *Aloreña de Málaga*, encontrando una fuerte inhibición de las poblaciones de microorganismos y mejorando el perfil organoléptico

de los frutos, alargando de este modo la vida de mercado de los envases hasta el final del periodo estudiado (3 meses). A la vista de los resultados anteriores, es necesario profundizar en los métodos de análisis de la vida comercial de los envasados así como diseñar nuevas estrategias para aumentarla.

1.5. LA ACEITUNA DE MESA ALOREÑA DE MÁLAGA

En 1746 Juan Antonio de Estrada no duda en decir que Álora tiene «*afamadas aceitunas*». En 1770 Cristóbal Medina Conde afirma rotundamente que es «*la mejor aceituna de España*». Posteriormente, Pascual Madoz, especifica en su diccionario que Álora posee: «*aceitunas verdes muy celebradas*» y Antonio Botello en 1913 no duda en decir que Álora toma renombre por sus famosas aceitunas Manzanilla verde. En definitiva, las anteriores opiniones dan cuenta, en primer lugar, de la antigüedad del olivar en Álora y, en segundo lugar, de la fama y comercialización de sus productos que le han permitido obtener la primera DOP de España (BOJA nº 215, 2009). Este distintivo de calidad le fue otorgado gracias a sus características diferenciales con respecto al resto de aceitunas de mesa, que se suman al hecho de ser un producto artesano, de apreciadas características organolépticas y gran valor nutricional.

La aceituna *Aloreña de Málaga* es autóctona del Valle del Guadalhorce, situado en el suroeste de la provincia de Málaga, englobando a un total de 19 municipios (Tabla 1). Algunos de estos municipios se engloban en lugares de alto valor medioambiental como el parque natural Sierra de las Nieves o el desfiladero de los Gaitanes. La comarca natural del valle del Guadalhorce se rodea de un extenso perímetro de montañas que la protegen de los vientos continentales al mismo tiempo que está ligeramente sometida a la influencia marina por la desembocadura del río que le da nombre. Como consecuencia se crea un microclima muy peculiar que permite el desarrollo de gran diversidad de cultivos, entre ellos el olivar con un total de 23.210 ha distribuido aproximadamente en 1.339 fincas, presentando este una gran vecería entre diferentes campañas de recogida. Se trata de plantaciones centenarias, en la mayoría de los casos de un solo pie injertados sobre acebuche, con amplios marcos de plantación, ya que cuando estos olivos fueron plantados se practicaba la asociación de cultivos, normalmente localizados en las zonas marginales y con elevadas pendientes. Son estas condiciones orográficas y climatológicas las que influyen decisivamente en las

características finales del producto, muy diferenciado del resto de aceitunas de mesa y fuertemente vinculado a su historia, sus gentes y su tradición.

Tabla 1. Superficie total, agraria útil y de olivar de los distintos municipios que integran la comarca natural del Guadalhorce.

Municipios	Superficie total (ha)	Superficie agraria útil (ha)	Superficie olivar (ha)
Alhaurín de la Torre	8.270	1.901	211
Alhaurín el Grande	7.310	1.356	634
Almogía	16.290	7.203	2.407
Álora	16.960	6.983	2.904
Alozaina	3.390	2.701	1.595
Ardales	10.650	4.360	1.185
Burgo (El)	11.840	4.239	1.567
Carratraca	2.240	436	53
Cártama	10.510	3.280	349
Casarabonela	11.370	4.965	1.353
Coín	12.740	4.339	757
Guaro	2.240	873	645
Málaga (capital)	39.510	8.205	2.612
Monda	5.770	1.460	519
Pizarra	6.360	2.811	135
Ronda	39.760	33.165	4.072
Tolox	9.440	6.279	1.010
Valle de Abdalajís	2.120	1.921	304
Yunquera	5.520	1.175	897
TOTAL	222.290	97.653	23.210

Los últimos datos de que dispone el Consejo Regulador de la DOP *Aloreña de Málaga* (2016), muestran que el sector tiene una producción media anual de 3.500 t, con una estimación de volumen de negocio entre 12-14 millones de €. El número de empresas acreditadas que elaboran este tipo de especialidad de aceituna de mesa es en la actualidad de 23, dando empleo aproximadamente a 4.000 familias incluyendo también las actividades relacionadas con la recolección, gestión del olivar e industrias auxiliares. Un producto con un sello de calidad como es la DOP es altamente apreciado en mercados internacionales, y la *Aloreña de Málaga* ya ha sido vendida en países como Alemania, Francia, Holanda, Irlanda, Italia, Polonia, Reino Unido, Japón y República Dominicana, aunque la mayor parte de sus ventas se realiza en la provincia de Málaga y sobre todo en la costa del sol.

La aceituna de mesa *Aloreña de Málaga* se caracteriza y diferencia de otros tipos de aceitunas por:

• Relación pulpa-hueso; posee una gran pulpa frente a un hueso muy pequeño, esta cualidad junto con su homogeneidad, textura crujiente y firmeza de la pulpa reciben una magnifica valoración en los paneles de cata.

• Hueso flotante; que permite que la pulpa se despegue fácilmente del hueso. Tradicionalmente, los agricultores de la zona para determinar el momento óptimo de la recolección cortaban la aceituna por el ecuador y si las dos mitades podían despegarse fácilmente indicaba que se podía dar comienzo la campaña de recogida (Figura 6). Este es un atributo valorado muy positivamente en la industria de elaboración de aceituna de mesa.

• Presenta una baja concentración en oleuropeína, por lo que no necesita ser tratada con álcali para eliminarle el amargor.

• Presenta una textura porosa, lo que permite que tome los aliños que se utilizan durante la elaboración con mucha facilidad.

Figura 6. Corte realizado sobre una aceituna de mesa Aloreña de Málaga para determinar el momento óptimo de su recolección.

Siglos de tradición arropan la elaboración de la aceituna *Aloreña de Málaga*, desde su recolección hasta su aderezo. El proceso de elaboración sigue siendo aún muy artesanal, no estando mecanizado, y comienza con la recogida manual de los frutos y su entrada en la fábrica, lo cual suele ser a mediados del mes de Septiembre. La recolección se realiza cuando adquieren su mayor tamaño y antes del envero. Al ser un fruto muy sensible a los golpes se

debe recolectar a mano mediante el sistema de ordeño, así se van seleccionando las aceitunas de mayor calibre y mejor aspecto que se sitúan en cajas de plástico (20 kg).

La aceituna *Aloreña de Málaga* se enmarca dentro de las aceitunas naturales (sin tratamiento con álcali), y en concreto: i) por el grado de maduración son "aceitunas verdes", ii) por la preparación comercial se trata de "especialidades", iii) por la presentación: "aceitunas machacadas o partidas", y iv) por los defectos y tolerancias que presenten se clasifican en categoría extra" (suprema) y "categoría primera" (superior). El reglamento y pliego de condiciones de la DOP (BOJA nº51, 2015) distingue tres elaboraciones, dependiendo del grado de fermentación de las aceitunas y características físico-químicas y organolépticas de los frutos:

i) Aceitunas Aloreñas Verdes Frescas. Una vez las aceitunas han sido recolectadas, se clasifican, lavan y se procede al machacado o partido de los frutos. Posteriormente, se colocan los frutos en bombonas (220L) para su endulzado con una salmuera entre 7 y 11% NaCl. Las bombonas se pueden conservar bajo sombrajos o nave cubierta, o bien en cámaras frigoríficas (máximo 8ºC), donde podrán permanecer mientras no cambien las características que definen esta forma de preparación. La fermentación que sufren los frutos en este proceso es prácticamente nula ya que la conservación en frío ralentiza cualquier cambio, manteniendo los frutos gran parte de las características de la materia prima. Durante esta fase las aceitunas deben mantener el color verde y su textura crujiente. Una vez que se considera que se han endulzado lo suficiente, lo cual se consigue en un mínimo de 3 días, se sacan de las bombonas para continuar la preparación, añadiéndose los aliños típicos que le dan su característico aroma y sabor (ajo, tomillo, hinojo y pimiento) en una proporción que suele ir del 1-3% en el momento del envasado. El pliego de condiciones también permite la utilización de especias u oleorresinas basadas en esos mismos productos naturales. La salmuera madre en envasado se añade a una concentración tal que en el equilibrio esté en torno al 5-6% NaCl. En el envasado está permitida la adición de diferentes conservantes y aditivos entre los que destaca el sorbato de potasio, ácido cítrico y benzoato de sodio. Para alargar el periodo de comercialización, se puede someter a algún tipo de tratamiento, como puede ser la pasteurización o el envasado al vacío con atmósfera modificada, siempre que el producto final conserve sus características organolépticas y físicas originarias. Un tratamiento térmico excesivo de los frutos supone una pérdida de calidad del producto, al degradarse su textura, pérdida del apreciado color verde y

la aparición de sabor a cocinado (López-López y Garrido-Fernández, 2010), por lo que no es muy habitual su realización.

Las aceitunas verdes frescas se caracterizan por presentar una coloración verde clara, con un olor a fruta verde y a hierba muy agradables que sugieren su frescor y cercana recolección en el tiempo. Así mismo, se nota la presencia de los aliños característicos de su elaboración. Como sabores básicos mencionar que el amargor es la nota característica, así como en ocasiones se puede notar la presencia del salado según las características de su aderezo. La astringencia y el picor son también descriptores que aparecen. Las características físico-químicas que caracterizan a esta forma de preparación de aceitunas son pH comprendido entre 4,2-4,3 y acidez libre entre 0,3-1,0%.

ii) Aceituna Aloreña de Málaga tradicional. En esta preparación las aceitunas se parten y se colocan en bombonas (220L) bajos sombrajos con una salmuera de 11% NaCl por un periodo mínimo de 20 días. Estas aceitunas se comercializan según demanda y no alcanzan las mejores condiciones de calidad hasta bien iniciada la primavera. En este caso se produce una fermentación parcial o total (según el tiempo y temperatura de conservación de las bombonas) por levaduras en una primera etapa y por BAL en una segunda (Arroyo-López, 2007). Posteriormente, se envasan de la misma forma que las aceitunas verdes frescas.

En esta elaboración los frutos presentan una coloración verde-amarillo pajizo, no presentando en esta ocasión un verde intenso. Su olor sugiere a la fruta fresca y a los aliños propios de su aderezo, no percibiéndose las notas a hierba fresca propias de las aceitunas verdes frescas. De su textura, mencionar que se trata de una aceituna menos firme, pero que sigue manteniendo sus propiedades en cuanto a lo crujiente, buena separación de la carne con respecto al hueso y a la presencia de piel. De sabor ligeramente amargo es una aceituna menos astringente y de picor menos apreciable que las verdes frescas. Las características físico-químicas que se especifican para esta preparación son un pH que oscila entre 4,0 y 4,3 unidades y una acidez libre entre 0,4 y 1,5%.

iii) Aceituna Aloreña de Málaga curada. En este caso los frutos no se parten antes de la colocación en salmuera sino que se introducen directamente en grandes fermentadores (16.000 L de volumen) empleándose una salmuera con una concentración que varía entre el 6,0 y 7,5% NaCl junto con una concentración de ácido acético que puede variar entre el 0,5%

y el 0,8%. En esta forma de preparación, se suele añadir el ácido acético con el fin de facilitar la fermentación natural de las aceitunas y asegurar su buena conservación. Las aceitunas pueden conservarse en estas condiciones durante periodos prolongados de tiempo, siendo el tiempo mínimo de permanencia en fermentadores de 90 días, lo cual garantiza una fermentación total del proceso, llevado a cabo fundamentalmente por levaduras y BAL (Arroyo-López, 2007). Posteriormente, se produce el machacado, aliñado y envasado de los frutos como en las elaboraciones anteriores.

La aceituna curada se caracteriza por presentar una coloración amarilla-marrón, con un olor a fruta madura y a hierba fresca. Se nota la presencia de los aliños y de notas lácticas, características de su elaboración y del proceso de fermentación. De textura menos firme y crujiente, presentan una buena separación de la carne con respecto al hueso, así como se manifiestan restos de la presencia de piel tras su masticación. De sabor ácido, pierde su amargor, resultando picantes tras su degustación. Las condiciones físico-químicas que caracterizan este tipo de producto oscilan en un pH entre 3,3 y 3,8 y una acidez libre entre 1,5 y 3,0%.

1.5.1. Inestabilidad de envasados

Por las propias características del proceso de elaboración, la aceituna de mesa *Aloreña de Málaga* presenta una gran inestabilidad en los envasados. Aunque un proceso de pasteurización podría asegurar su buena conservación, no es muy recomendable realizarlo ya que: i) produce una pérdida del color verde de las aceitunas, el cual es un atributo altamente apreciado en este tipo de elaboración, ii) origina la aparición de notas de sabor a cocinado en los frutos, y iii) conlleva una pérdida apreciable de la textura y dureza de las aceitunas (López-López & Garrido-Fernández, 2010). Por estos motivos, el tratamiento térmico no es habitual realizarlo entre las principales empresas del sector. Por el contrario, las industrias optan por la estabilización química de los envases, sobre todo mediante la adición de sorbato de potasio y benzoato de sodio. Sin embargo, estos conservantes presentan varios inconvenientes, entre los que cabe destacar: i) ambos son solubles en la grasa con un coeficiente de reparto mayor que en la salmuera, por lo que terminan acumulándose en la pulpa de las aceitunas y desapareciendo del líquido de gobierno (Brenes y col., 2004), ii) su efecto inhibidor depende del pH del medio, iii) pueden alterar el sabor de los frutos y provocar rechazo en algunos consumidores, iv) tienden a oscurecer las salmueras y frutos, y

v) debido al ajuste de los niveles de sodio que están efectuando las industrias, en muchas ocasiones la eficacia de los mismos es limitada al bajar sus concentraciones.

Por todo lo anteriormente expuesto, es conveniente el estudio de otros conservantes y técnicas que puedan ser utilizados en sustitución, o en aplicación conjunta, con las sales de sorbato y benzoato. La aplicación de altas presiones en envasado de aceitunas *Aloreña de Málaga* fue valorada por Abriouel y col. (2014) en combinación con antimicrobianos naturales y aceites esenciales; aunque se conseguía un incremento de la estabilidad microbiológica del envasado, la aplicación de altas presiones (400 MPa) produce una considerable pérdida de textura en los frutos. También se ha valorado el envasado en seco con atmósfera modificada (nitrógeno), los resultados muestran que no se consiguió tampoco la estabilización físico-química y microbiológica del producto final, sufriendo los envases hinchamiento y abombamiento (Abelló Linde, 2011).

En estudios previos, se había observado que las principales causas de alteración de los envasados eran debido a factores como la propia respiración celular del fruto y el crecimiento principalmente de las poblaciones de levaduras, favorecido por la alta presencia de nutrientes en los envasados (Arroyo-López y col., 2005; Arroyo-López y col., 2009). Esto daba lugar al enturbiamiento de las salmueras, la producción de gas y el posterior abombamiento y derrame de líquido de los envases que eran de material PET. Por lo tanto, no se alcanza una completa estabilización físico-química y microbiológica del producto. Como consecuencia de todos estos trabajos, el sector modificó sus condiciones de envasado incrementando la concentración de conservantes (sorbato y benzoato) hasta los máximos legales permitidos (1.000 mg/L expresado como ácido sórbico o benzoico en pulpa) para inhibir el crecimiento de levaduras, lo que ha supuesto la aparición de otros tipos de alteraciones no descritas con anterioridad y que se muestran en la presente Tesis Doctoral.

Otro gran problema que tienen los envasados de las aceitunas *Aloreña de Málaga* es la pérdida del color verde de los frutos y el pardeamiento de las salmueras. Estos procesos están originados por la degradación de los compuestos clorofílicos a feofitinas debido, bien a la sustitución del Mg^{2+} central en el anillo de la clorofila por H^{+} en un medio ácido, o por la rotura de las aceitunas liberando la enzima clorofilasa (Gallardo-Guerrero y col. 2013). Este proceso se ve más favorecido a medida que desciende el pH, por lo que el sector, canalizado

por el Consejo Regulador de la DOP *Aloreña de Málaga*, demanda la posibilidad de envasar a pH superiores a 4,6, lo cual podría suponer un elevado riesgo microbiológico. En línea con esta petición, la presente Tesis Doctoral desarrolla estudios de inoculación de patógenos en salmueras de *Aloreña de Málaga* a diferentes niveles de pH para estudiar la supervivencia y seguridad microbiológica del producto final y obtener suficiente información científica que de soporte a futuras alegaciones.

También es conocido que existe una estrecha correlación entre la composición fenólica, la actividad de la enzima polifenoloxidasa y el pardeamiento de las aceitunas, debido a que la misma cataliza la hidrolisis de los monofenoles a orto-difenoles y su posterior oxidación a orto-diquinonas (Sciancalepore & Longone, 1984). En muchas ocasiones, la pérdida del color verde de los frutos determina el fin de la vida de mercado de los envases, por lo que es muy importante de determinar la cinética de degradación de este atributo.

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2. HIPÓTESIS Y OBJETIVOS DE TRABAJO

La presente Tesis Doctoral está centrada en el estudio de envasados de aceitunas de mesa con DOP *Aloreña de Málaga* para la mejora de su calidad y seguridad microbiológica. Para este fin, se parte de las siguientes hipótesis de trabajo:

i) Las actuales formulaciones de envasado que utilizan las empresas con altas concentraciones de conservantes (sorbato y benzoato) dan lugar a otros tipos de alteraciones que no aparecían con anterioridad y que pueden tener un origen microbiano.

ii) Existen otros conservantes que podrían aplicarse en el envasado de aceitunas de mesa, y específicamente en *Aloreña de Málaga*, mejorando la calidad del producto final y favoreciendo su estabilidad microbiológica.

iii) Al igual que otros tipos de elaboraciones de aceitunas de mesa, los envasados de *Aloreña de Málaga* representan un medio adverso para el desarrollo de patógenos alimentarios, por lo que constituyen un alimento seguro para el consumidor.

Para confirmar estas hipótesis de trabajo, se han planteado los siguientes objetivos específicos:

1) Estudio fisicoquímico, microbiológico y sensorial de envasados obtenidos en diferentes industrias del sector para determinar su vida de mercado y causas de alteración. Todo ello se realiza con la finalidad de conocer el punto de partida en el que se encuentra la industria en lo referente a calidad y seguridad microbiológica de los envasados. Objetivo abordado en las siguientes publicaciones:

Capítulo 1. *Lactobacillus pentosus* is the dominant species in spoilt packaged *Aloreña de Málaga* table olives. LWT – Food Science and Technology 70 (2016) 252-260.

Capítulo 2. Shelf life of traditional seasoned *Aloreña de Málaga* table olives based on their packages and fruits characteristic.

2) Evaluar la utilización de otros conservantes como alternativas a los sorbatos y benzoatos en el envasado de aceitunas *Aloreña de Málaga*, determinando su acción sobre los principales grupos microbianos presentes en los envasados: levaduras, BAL y enterobacterias. Objetivo abordado en las siguientes publicaciones:

Capítulo 3. Effect of zinc formulations, sodium chloride, and hydroxytyrosol on the growth/no-growth boundaries of table olive related yeast. Food Microbiology 57 (2016) 71-80.

Capítulo 4. Susceptibility and resistance of lactic acid bacteria and yeasts against preservatives with potential application in table olives. Food Microbiology 54 (2016) 72-79.

Capítulo 5. *In silico* logistic model for table olive related microorganisms as a function of sodium metabisulphite, cinnamaldehyde, pH, and type of acidifying agent. Frontiers in Microbiology 7 (2016) 1370.

3) Determinar la supervivencia de microorganismos patógenos y su relación con la presencia de determinados compuestos inhibidores en los diferentes tipos de envasados de *Aloreña de Málaga*. También conocer la influencia que sobre la inhibición de su crecimiento tiene las condiciones físico-químicas (pH y concentración en NaCl) y el uso de conservantes. Objetivo abordado en las siguientes publicaciones:

Capítulo 6. Survival of foodborne pathogens in natural cracked olivebrines. Food Microbiology 59 (2016) 104-111.

Capítulo 7. Foodborne pathogen survival in comercial *Aloreña de Málaga* table olive packaging.

3. RESULTADOS

3.1 SECCIÓN I

3.1.1 CAPÍTULO 1:

Lactobacillus pentosus is the dominant species in spoilt packaged *Aloreña de Málaga* table olives.

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Lactobacillus pentosus is the dominant species in spoilt packaged *Aloreña de Málaga* table olives

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ABSTRACT

The present study focused on investigating a peculiar spoilage of traditional *Aloreña de Málaga* table olives characterized by the formation of whitish and soft regions on the olive surface. To determine its causes, the main microbiological and physicochemical changes in 50 commercial packages were monitored until the alteration in appearance (63 days). Colour and firmness of fruits deteriorated progressively in the packaged olives during storage. Sugar in brines (7.5 g/L) remained stable during the first month and then decreased gradually (5.0 g/L) while, in parallel, the lactic acid bacteria population and titratable acidity increased. After two months of storage, evidence of spoilage was noticed, coinciding with the maximal lactic acid bacteria populations in fruit (>6.5 $\log_{10}$ CFU/g). The spoilage affected ~25% of the fruits within packages. The microbial species detected in the product after packaging were *Enterobacter gergoviae* and *Lactobacillus plantarum* among bacteria, and *Candida tropicalis*, *Candida parapsilosis*, and *Lodderomyces elongisporus* among the yeasts while *Lactobacillus pentosus* was the dominant species in the spoilt packages. A specific biotype of *L. pentosus* was only detected in the damaged fruits. Further studies will be made to confirm the association between the spoilage and the presence of this *L. pentosus* biotype.

1. Introduction

Table olives have a great importance in the diet and culture of many Mediterranean countries. Green Spanish-style, Greek naturally black and ripe Californian styles are the most popular commercial preparations (Garrido-Fernández, Fernández-Díez, & Adams, 1997). However, in the last years, consumers are purchasing more traditional and natural homemade seasoned olives.

Aloreña de Málaga table olive is a traditional green olive preparation from Guadalhorce Valley (Málaga, Spain) with a Protected Designation of Origin (PDO) recognized by the European Union (DOUE, 2012). This olive variety has unique features, related to the production area, which make them quite different from others: its fruits are characterized by an excellent flesh-to-stone ratio, a green–yellow colour, a crispy firmness, and a peculiar mild bitter taste. Due to its low-to-moderate concentrations of oleuropein, the processing does not include alkaline debittering (López-López & Garrido-Fernández, 2006). The manufacturing process is carried out by small and medium enterprises placed in, or very close to, the region of production. For the elaboration of traditional PDO *Aloreña de Málaga*, fruits are cracked after harvesting, and brined in a 10–11% NaCl solution for at least 20 days. Then, the olives are seasoned with pepper, fennel, thyme and garlic, and packaged according to demand (López-López & Garrido-Fernández, 2006).

The stabilization of packaged natural cracked green olives is difficult due to the high residual sugar content and to the subsequent risk of post-fermentation by microorganisms, causing gas formation and top package leakages (Arroyo-López et al., 2009; Arroyo-López et al., 2005). For preventing these problems and extend the shelf life, nowadays, the industries use a combination of different preservatives (sorbate, benzoate, citric, acetic and lactic acid). The goal of all these preservatives is primarily the inhibition of yeasts but not particular attention is played to lactic acid bacteria (LAB) also common in these products (Abriouel, Benomar, Gálvez, & Pérez Pulido, 2014; Arroyo-López et al., 2005; Arroyo-López, Durán-Quintana, Ruiz-Barba, Querol, & Garrido-Fernández, 2006;

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Arroyo-López et al., 2009; Bautista-Gallego, Arroyo-López, Romero-Gil, Rodríguez-Gómez, & Garrido-Fernández, 2011;). Albeit the role played by LAB species during olive fermentation is positive producing lactic acid through sugar consumption and the consequent pH decrease (Hurtado, Requant, Bordons, & Rozes, 2012), their presence during packaging could jeopardize its stability (Johanningsmeier & McFeeters, 2013; Montaño, Higinio-Sánchez, Casado, Beato, & de Castro, 2013; Pothakos, Devlieghere, Villani, Björkroth, & Ercolini, 2015). The pasteurization of seasoned olives is not an option due to its negative effect on the typical green colour, the development of cooked taste, and weird off-flavours from condiments. Other technologies like high hydrostatic pressure (Abriouel et al., 2014), application of ozone (Arroyo-López, Duran-Quintana, & Garrido-Fernandez, 2006), the use of zinc chloride (Bautista-Gallego et al., 2011), or sorbic and benzoic acids (Alves, Esteves, & Quintas, 2015) have also been studied, although a complete stabilization of the final product has not been fully achieved yet.

The aim of this work was to investigate the cause of a new spoilage, which appears during storage of these cracked seasoned olives. Particularly, molecular techniques (DNA-fingerprinting and sequencing) and bioinformatics analysis were used to identify the microbial groups present in the spoilt product. The results of this study may be useful for the development of new integral preservation methods for improving the stability, quality and safety of seasoned olives in general.

2. Material and methods

2.1. Sampling of commercially packaged olives

This survey was carried out with *Aloreña de Málaga* fruits previously fermented in brine for 20 days (the traditional style) without the addition of any starter culture, and then packaged by an unique industry in the Guadalhorce Valley (Málaga, Spain). Polyethylene terephthalate (PET) packages (1.6 L volume) were filled with 0.9 kg of olives, 16 g of seasoning material (a mixture of diced garlic, pepper strips, and small pieces of fennel, and thyme) and 0.7 L of cover brine (5.5% NaCl, 0.3% citric acid, 0.2% potassium sorbate, 0.1% sodium benzoate, 0.1% ascorbic acid and 0.08% lactic acid, expressed as g/100 mL). Packages were kept at room temperature (23 ± 2 °C) and the lot (a total of 50 commercial packages) sampled (in duplicate and without replacement) at 1, 3, 7, 10, 14, 21, 28, 42 and 63 days. At this last time, spoilage was noticed in all packages of the lot causing the end of the study. To determine the frequency of fruits affected by the alteration, approximately 27% of the olives included in duplicated packages (n = 98 from a total of 360 fruits) were removed (54 and 44, respectively) photographed, and classified (0, absence of alteration; 1, presence of alteration). As described below, 10 lactobacilli were isolated from fruits with alteration, while other 10 were obtained from unaltered fruits.

2.2. Physicochemical and microbiological analyses

The analyses of olive brine for pH, titratable acidity, combined acidity, and NaCl were carried out using the routine methods described by Garrido-Fernández et al. (1997). Firmness and surface colour of fruits followed methods described elsewhere (Bautista-Gallego et al., 2011), determining the CIE parameters: L^* (lightness), a^* (freshness, negative values indicate green while positive values are related to red tones), and b^* (negative values indicate blue and positive values associated to yellowish). The darkness of brine packing (B_d) was estimated according to Montaño, Sánchez-Gómez, and Rejano (1988). Individual reducing sugars (glucose,

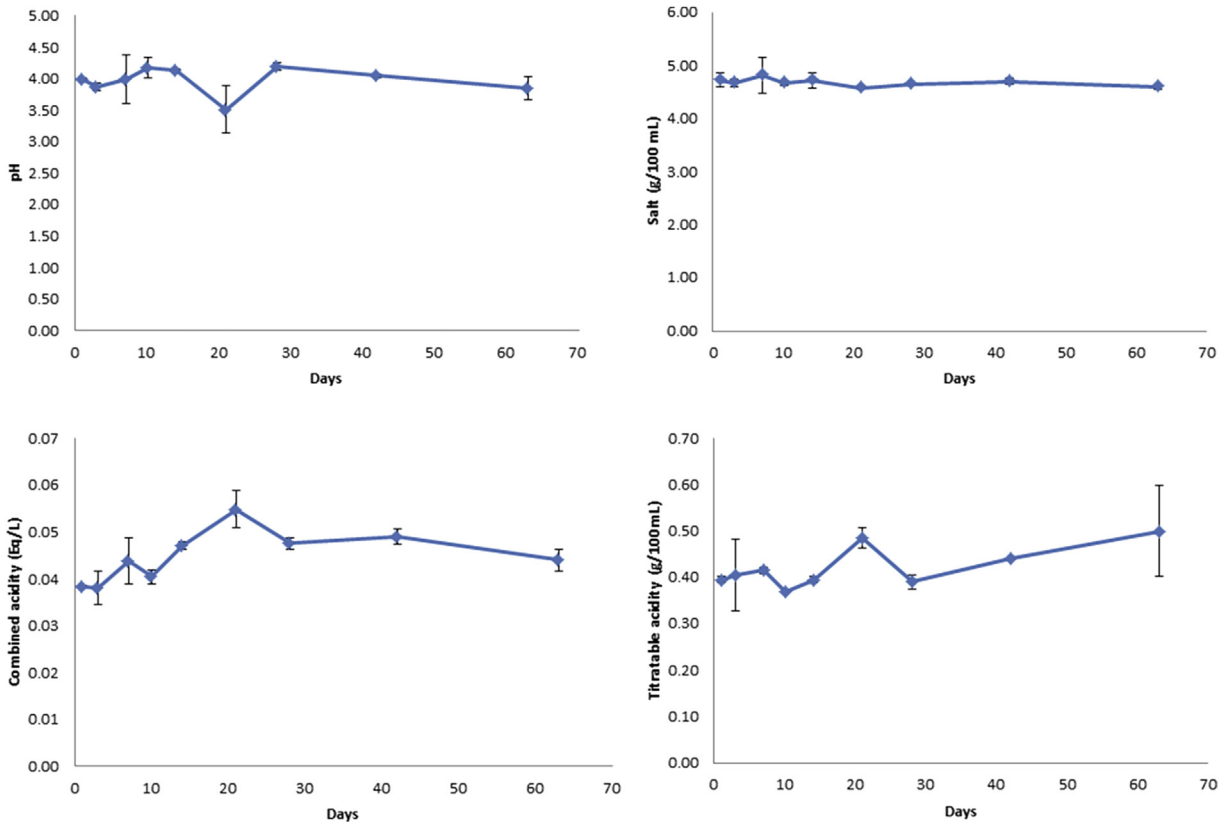

Fig. 1. Evolution of the main physicochemical parameters of brines (pH, salt, combined and titratable acidity) during shelf life (0–63 days) of traditional PDO *Aloreña de Málaga* table olives. Mean and standard deviations were obtained from measurements made in duplicated packages (n = 2).

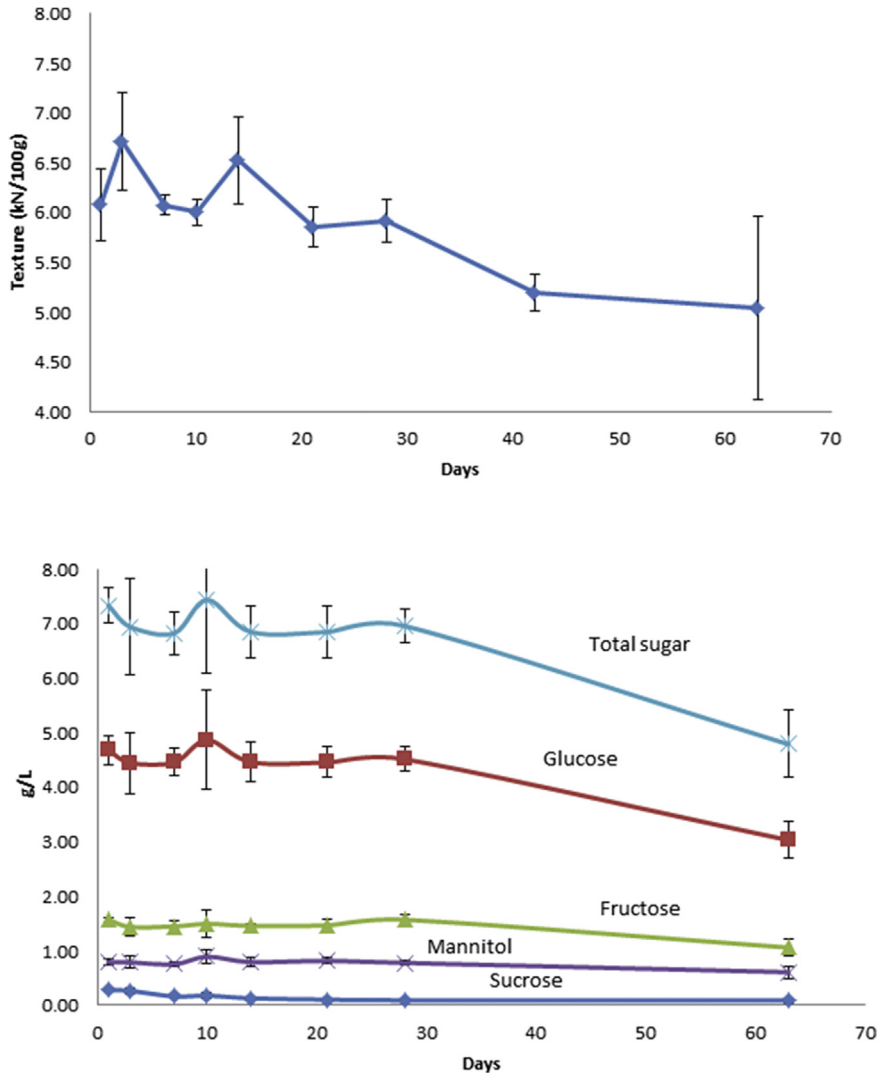

Fig. 2. Evolution of the firmness of fruits and sugar content in brine (total and individual) during shelf life (0–63 days) of traditional PDO *Aloreña de Málaga* table olives. Mean and standard deviations were obtained from measurements made in duplicated packages (n = 2).

fructose, sucrose and mannitol) were determined by HPLC according to the methods developed by Sánchez, De Castro, Rejano, and Montaño (2000).

For the isolation of the *Enterobacteriaceae*, yeasts and LAB populations in both brine and olive samples, a culture-dependent approach was used according to methods described by Rodríguez-Gómez et al. (2015). Counts were expressed as $\log_{10}$ CFU/mL for brines or $\log_{10}$ CFU/g for olives.

2.3. Characterization and identification of microbial populations

A total of 20 yeast and 65 LAB isolates were obtained from brines or olives of commercially packaged olives at different sampling times as well as from fruits with visible spoilage evidence at the end of the experiment. Isolates were labelled with different letters as a function of their origin (B, brine; O, non-spoilt olives; A, altered fruits) and sampling time (I, initial 1 day; M, middle 28 days; F, final 63 days). Two isolates of *Enterobacteriaceae* were also obtained from fruits just after packaging and directly destined for sequencing analysis. The yeast and LAB isolates were genotypically characterized by DNA-based typing techniques such as RAPD-PCR and rep-PCR with primer M13 and GTG_5, respectively (Gevers, Huys, & Swings, 2001; Tofalo et al., 2009). The resulting fingerprints were digitally captured and analysed with the BioNumerics 6.6 software package (Applied Maths, Kortrijk, Belgium). The similarity between digitalized profiles was calculated using the Pearson product–moment correlation coefficient. Dendrograms were obtained using the UPGMA clustering algorithm. *Candida boidinii* TOMC-Y5, *Wickerhamomyces anomalus* TOMC-Y2 and TOMC-Y20, *Saccharomyces cerevisiae* TOMC-Y4 and TOMC-Y30, *Pichia galeiformis* TOMC-Y8 and TOMC-Y27 for the yeasts, and *Lactobacillus pentosus* TOMC-LAB2, TOMC-LAB3 and TOMC-LAB4, *Lactobacillus plantarum* TOMC-LAB8 and TOMC-LAB9 for the LAB, were used as internal control to determine the reproducibility of the techniques. All these microorganisms were obtained from the Table Olive Microorganisms Collection (Instituto de la Grasa, Seville, Spain). A reproducibility of 80.5% for yeasts (RAPD-PCR), and 85.1% for bacteria (rep-PCR) was obtained (data not shown). Then, one representative isolate from different clusters obtained above cut-points was selected for molecular identification using sequencing of D1/D2 domains of the 26S rDNA gene with primers NL1 and NL4 for yeasts (Kurtzman & Robnett, 1998) and small-subunit 16 rRNA gene with universal primers 27F and 1492R for bacteria (Barrangou, Yoon, Breidt, Fleming, & Klaenhammer, 2002). Percentage of

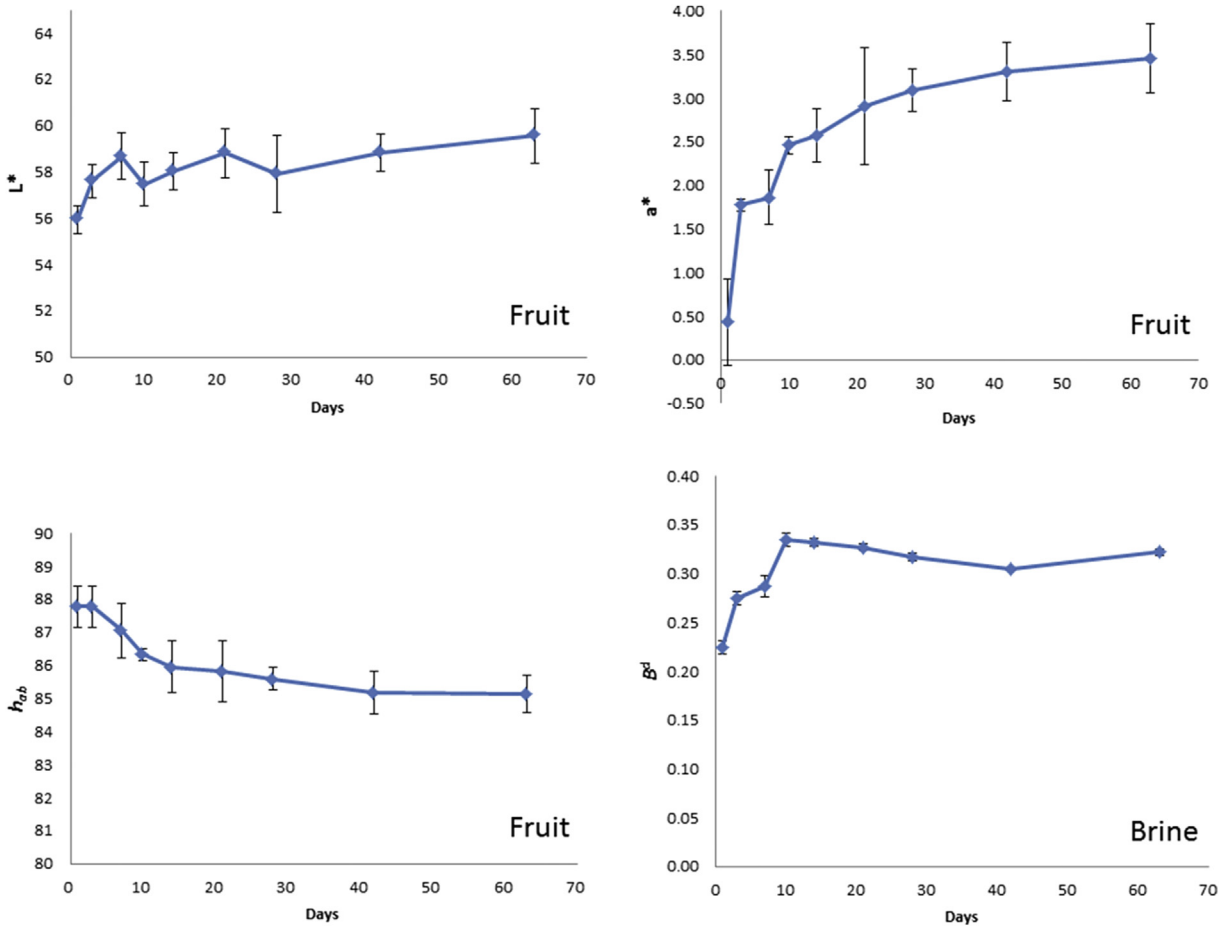

Fig. 3. Evolution of the colour parameters of fruits (L^*, luminosity; a^*, green-fresh; h_{ab}, hue angle) and brines (B_d, brine darkness) during shelf life (0–63 days) of traditional PDO *Aloreña de Málaga* table olives. Mean and standard deviations were obtained from measurements made in duplicated packages (n = 2).

identity with available sequences obtained from NCBI GenBank database was deduced from Blast analysis. For discrimination between *L. pentosus* and *L. plantarum* species, multiplex PCR assay based on *recA* gene was used (Torriani, Felis, & Dellaglio, 2001).

2.4. Statistical data analysis

Graph, mean and standard deviations for the different physicochemical and microbiological parameters were obtained from duplicated packages at each sample time using the One-way ANOVA module of Statistica 7.1 software package (Statsoft Inc., Tulsa, USA).

3. Results

3.1. Physicochemical and microbiological changes during packaged olive storage

Salt (~4.75 g/100 mL) and pH (~4.0) values were kept quite stable during the study (63 days), without significant differences among sampling times, while the combined and titratable acidity showed a slightly increasing trend (Fig. 1). Firmness slightly decreased from an initial 6.0 value to a final 5.0 kN/100 g after two months (Fig. 2), showing a high variability in measurements which did not allow establishing significant statistical differences. The initial total sugar content in brines was ~7.5 g/L and was composed of glucose (4.8 g/L), fructose (1.5 g/L), mannitol (0.8 g/L) and sucrose (0.4 g/L). Total sugar concentrations significantly decreased throughout shelf life and reached ~5.0 g/L after two months, being glucose the only sugar whose level decreased (Fig. 2), because, as usual, the simple sugars are the first to be consumed by microorganisms. The L* colour parameter significantly increased ($p < 0.05$) from an initial 56 to final 59 value at the end of storage. The a^* colour parameter also rose from 0.5 to 3.5, which means a significant loss of green colour. Hue angle (h_{ab}) decreased from 88° (initial) to 85° (final), indicating the browning of fruits. The packing brine's was also browning progressively (B_d increased from the initial 0.25 to final 0.35) (Fig. 3). Therefore, the main physicochemical changes that compromised the stability and quality of the product were: loss of green colour in fruits, a decrease of firmness, browning of brines and glucose reduction. From Figs. 1–3 can be deduced that the 20th day was critical in the evolution of many of the physicochemical parameters.

Enterobacteriaceae were only found on olives after packaging (1st day) at very low population levels (<2.2 $\log_{10}$ CFU/g). The sequencing of small-subunit 16 rRNA gene of two isolates obtained from fruits showed that both belonged to the same species, *Enterobacter gergoviae* (Table 1). Yeasts and LAB were the main microbial groups detected in both brines and fruits during shelf life, although with completely different evolutions. Changes in yeasts in both brines and olives during storage were quite similar (Fig. 4). They were found at population levels around 3.2 $\log_{10}$ CFU in both brines (per mL) and fruits (per g); but, after the first week of storage had a sharp decline, and they were not detected from the 10th day

Table 1
Microbial isolates obtained from different clusters of the DNA-fingerprinting analysis from PDO *Aloreña de Málaga* table olive packages and subjected to further molecular identification.

Microbial group	Isolate reference	Cluster number	[a]Matching nucleotides/identity%	[b]Closest relative species	[c]Identification based in *recA* gene product (bp)
LAB	O-M-6	I	765 bp/99%	*Lactobacillus plantarum*|gi|758840967|CP010528.1	218 bp/*Lactobacillus pentosus*
	O-A-5	I	446 bp/100%	*Lactobacillus plantarum*|gi|746590652|KM657203.1	218 bp/*Lactobacillus pentosus*
	O-A-2	II	796 bp/100%	*Lactobacillus plantarum*|gi|675277874|KJ917253.1	218 bp/*Lactobacillus pentosus*
	B-F-5	III	1000 bp/99%	*Lactobacillus plantarum*|gi|749389291|CP005942.2	218 bp/*Lactobacillus pentosus*
	O-A-7	III	346 bp/99%	*Lactobacillus plantarum*|gi|675277874|KJ917253.1	218 bp/*Lactobacillus pentosus*
	B-M-9	IV	974 bp/100%	*Lactobacillus plantarum*|gi|749389291|CP005942.2	218 bp/*Lactobacillus pentosus*
	B-I-4	V	783 bp/100%	*Lactobacillus plantarum*|gi|732665802|KM577184.1	318 bp/*Lactobacillus plantarum*
	O-I-3	VI	989 bp/99%	*Lactobacillus plantarum*|gi|731188889|KM507561.1	318 bp/*Lactobacillus plantarum*
Enterobacteriaceae	F-I-1		647 bp/100%	*Enterobacter gergoviae*|gi|404211719|JX567313.1	
	F-I-2		461 bp/99%	*Enterobacter gergoviae*|gi|359803192|AB682278.1	
Yeast	B3	I	526 bp/100%	*Lodderomyces elongisporus*|gi|588284414|KF935228.1	
	O7	II	552 bp/100%	*Candida tropicalis*|gi|736603388|KP064125.1	
	O2	III	544 bp/100%	*Candida tropicalis*|gi|736603388|KP064125.1	
	O4	IV	537 bp/99%	*Candida tropicalis*|gi|657234362|KF359928.1	
	B1	V	557 bp/99%	*Candida tropicalis*|gi|170676492|EU543680.1	
	B2	VI	541 bp/99%	*Candida tropicalis*|gi|736603388|KP064125.1	
	O8	VII	543 bp/100%	*Candida parapsilosis*|gi|672941186|KJ817165.1	
	B6	VIII	546 bp/100%	*Candida parapsilosis*|gi|672941186|KJ817165.1	
	B5	IX	547 bp/100%	*Lodderomyces elongisporus*|gi|588284414|KF935228.1	

[a] Sequence identity of the D1/D2 domains of the 26S ribosomal gene for yeasts, and small-subunit 16S rRNA gene for bacteria.
[b] Closest related species found in the NCBI GenBank database with their respective accession numbers.
[c] Final identification for lactobacilli deduced from multiplex PCR assay based on *recA* gene (Torriani et al., 2001).

onwards (Fig. 4, upper panels). Apparently, the presence of preservatives (sorbate and benzoate) resulted in a reduction in the numbers of these microorganisms after approximately 10 days. The behaviour of LAB was completely different compared to yeasts (Fig. 4, lower panels). After packaging their population levels were 4.1 $\log_{10}$ CFU/mL (brine) and 2.3 $\log_{10}$ CFU/g (fruits), which were stable during the first 15 days. However, after this time, the population increased progressively and reached after two months levels close to 6.5 $\log_{10}$ CFU/mL (brine) or 6.8 $\log_{10}$ CFU/g (olives), significantly ($p < 0.05$) higher than those found initially in the case of fruits samples. It must notice the differences sometimes observed between replicated measurements, as usual in natural and cracked samples obtained from different packages of the lot. This high variability did not allow establishing significant statistical differences in certain sampling times.

3.2. Evidence of spoilage

At the 63rd day of study, all packages of the lot showed clear evidence of spoilage that affected the fruits' product appearance. This coincided with the LAB maximum population and forced the termination of the study. The spoilage (Fig. 5) affected to 25.6 ± 0.84% of fruits ($n = 98$) within a package. The appearance of the spoilage (whitish and soft olive parts close to the cracked border) was associated with the presence of a more viscous, browning and mucous brine (personal observation).

3.3. DNA-fingerprinting and identification of microbial populations

The dendrogram generated with the pattern profiles of the 65 lactobacilli isolates obtained from commercially packaged olives (Fig. 6) showed six major biotypes that were differentiated below reproducibility of the technique (85.1%). In practice, all isolates obtained from the initial sampling point formed two groups were clearly separated from the rest (cluster V and VI) sharing a 62.1% similarity between them. Sequencing analysis of small-subunit 16 rRNA gene and further confirmation by multiplex PCR assay based on *recA* gene (amplification 318 bp), allowed identification of isolates B-I-4 and O-I-3 (belonging to clusters V and VI, respectively) as *L. plantarum* species (Table 1). Both DNA fingerprint profiles were not found later and at middle and final sampling points, in either brines or olives, while other four different genotypes were noted (clusters I, II, III and IV). Representative isolates of these groups were subjected, as in the previous case, to molecular identification; but, in this instance, the study of *recA* gene (amplification 218 bp) allowed assignation of isolates O-M-6, O-A-5, O-A-2, B-F-5, O-A-7 and B-M-9 to *L. pentosus* species (see Table 1 or Fig. 6 for assignation to clusters). Therefore, the strains of *L. plantarum* found after packaging were displaced by the rest of genotypes of *L. pentosus* throughout shelf life. A total of 10 lactobacilli were also obtained exclusively from fruits with visible evidence of spoilage. These 10 isolates (labelled with the letter A in Fig. 6) were distributed in 3 biotypes: I (6 isolates), II (3 isolates) and III (1 isolate), all of them belonging to *L. pentosus* species. In clusters, I and III were also present other isolates obtained from the middle, and final samplings in both brines and non-altered fruits: but, on the contrary, all isolates obtained from cluster II were exclusively related to spoilt fruits.

The dendrogram generated using the patterns profile of the 20 yeast isolates obtained after packaging (then this group of microorganism disappeared) (Fig. 7) differentiated a total of 9 major groups below reproducibility of the technique (80.5%). Hence, there is a greater number of biotypes in yeast population compared to that of LAB. The clusters with a larger number of isolates were I, IV, VI and VIII, with three isolates each one. As in the previous case, isolates from different clusters were randomly selected for further identification by sequencing of D1/D2 domains of 26S gene and Blast analysis. *Candida tropicalis* was the predominant species in clusters II, III, IV, V and VI (a total of 10 isolates), while *Candida parapsilosis* was present in clusters VII and VIII (6 isolates) and *Lodderomyces elongisporus* in clusters I and IX (4 isolates). The percentage of identity with previously published sequences in NCBI GenBank database was always high (>99%) (see Table 1).

4. Discussion

The shelf life of traditional packaged *Aloreña de Málaga* table olives for a period similar to that noticed in this work was

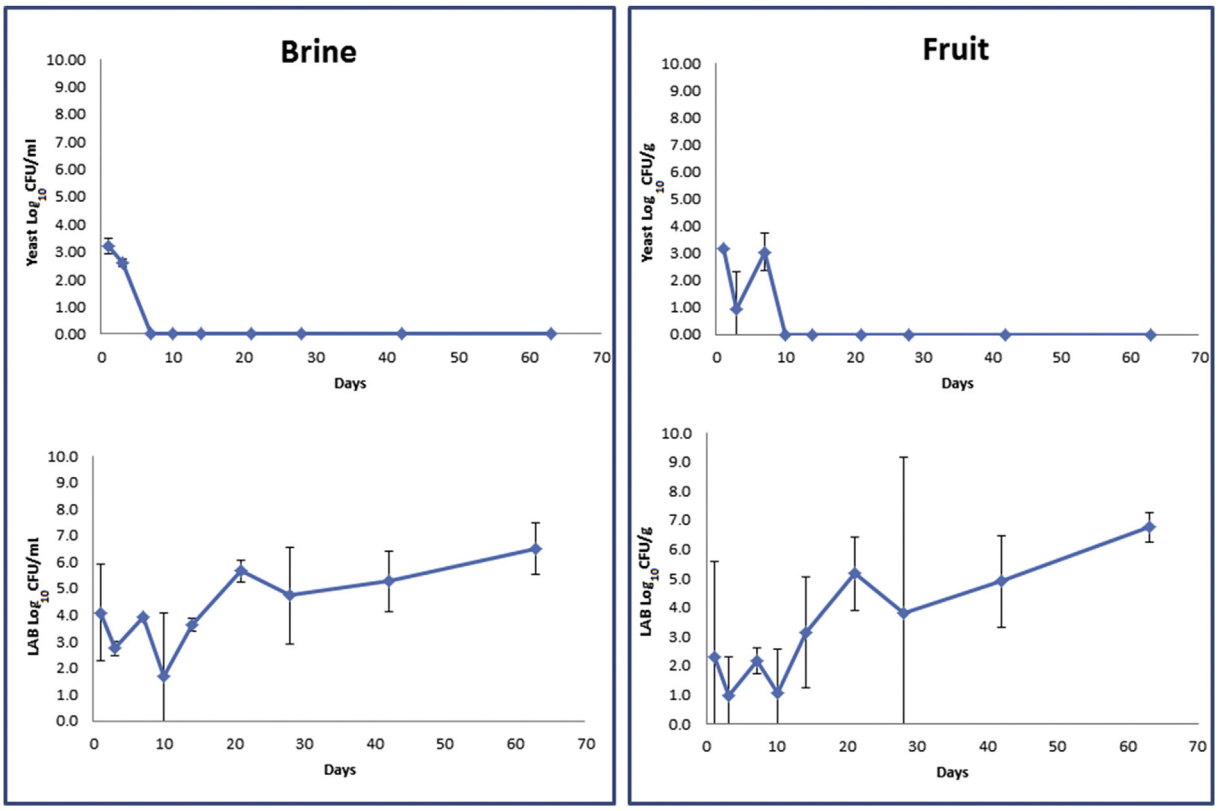

Fig. 4. Lactic acid bacteria and yeast counts in both brines and fruits during shelf life (0–63 days) of traditional PDO *Aloreña de Málaga* table olives. Mean and standard deviations were obtained from measurements made in duplicated packages (n = 2).

determined in previous studies (Arroyo-López et al., 2005, 2009, Arroyo-López, Duran-Quintana, & Garrido-Fernandez, 2006).

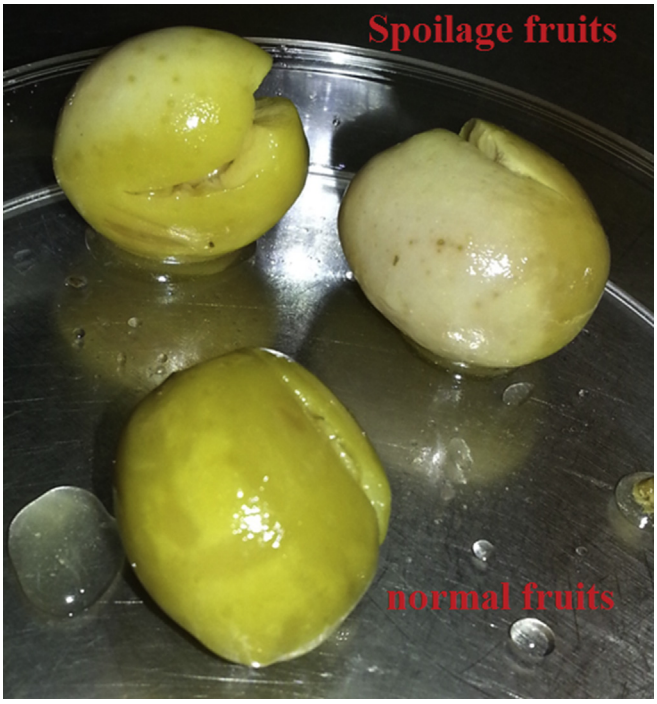

Fig. 5. An example of fruits with spoilage evidence in traditional PDO *Aloreña de Málaga* table olives. The presence of this spoilage (63rd day) in all packages marked the end of the shelf life experiment.

However, during the last decade, the industry has considerably modified the conditions of the packaging brine using new combinations and higher concentrations of preservatives compared to the past. The more stringent packaging conditions have prevented the gas production and container swelling spoilages (related to yeast growth), but on the contrary, have favoured apparition of a new type of spoilage not described before characterized by the formation of whitish and soft regions on the olive surface.

The use of the new packaging conditions has also altered the physicochemical characteristics of the packages. Respect to previous studies, now NaCl concentration is lower, the fruit firmness has decreased, but the titratable acidity, L^*, a^* and sugar concentration have increased (Arroyo-López et al., 2005, 2009, Arroyo-López, Duran-Quintana, & 2006). Alves et al. (2015) also have reported a considerable residual sugar concentration and an increase in the a^* colour parameter during storage of cracked green table olives from Maçanilha cultivar in the presence of sorbic and benzoic acids. A loss of fresh appearance, together with the presence of high levels of reducing sugars, limit considerably the shelf life of packaged cracked olives (Alves et al., 2015; Arroyo-López et al., 2005). However, darkening of brines in the present study was lower than those reported by Alves et al. (2015) for Maçanilha green cracked olives.

In this study, *Enterobacteriaceae* were only detected at the first sampling time (1 day after packaging) and at low population levels. Therefore, they are not related to the spoilage. Their strong inhibition was possibly due to the low pH (~4.0) prevailing during all the shelf life period. The disappearance of these microorganisms during the first week after olive packaging has also been reported by other authors (Arroyo-López et al., 2005, 2009; Alves et al., 2015; Bautista-Gallego et al., 2011). The species found, *E. gergoviae*, has

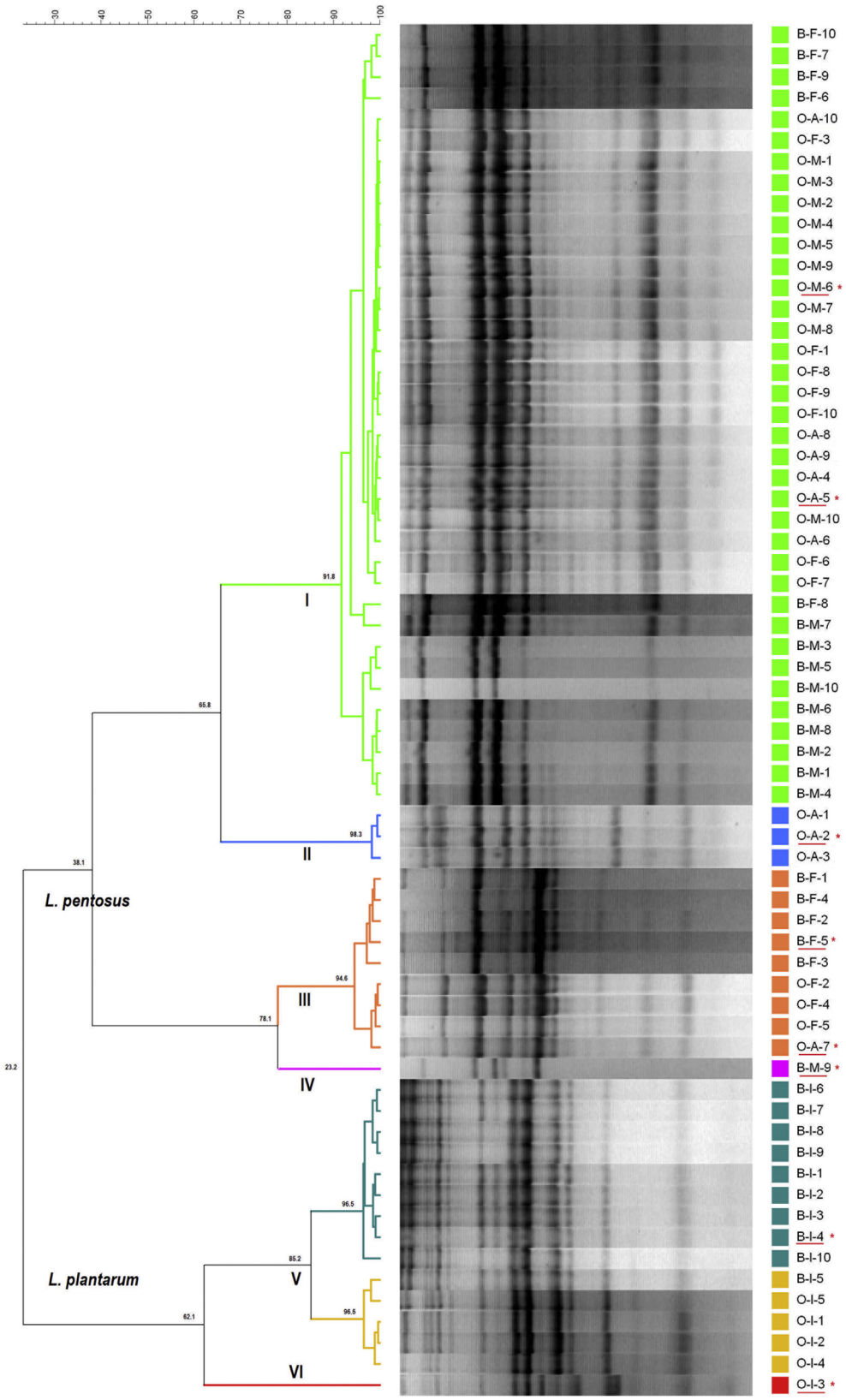

Fig. 6. Dendrogram generated after cluster analysis of the digitalized rep-PCR fingerprints with a GTG_5 primer of 65 lactobacilli isolates. They were obtained from brines (B) or olives (O) at the initial sampling time (I, 1 day), middle (M, 28 days) and end shelf life (F, 63 days) as well as in fruits with evidence of spoilage (A). 1–10 is the number of the isolate, obtained from the different sampling periods. Clustering parameters: 0.5% optimization and 0.0% curve smoothing. Isolates selected for sequencing from different clusters below 85.1% similarity (reproducibility of technique) are marked with an asterisk (*).

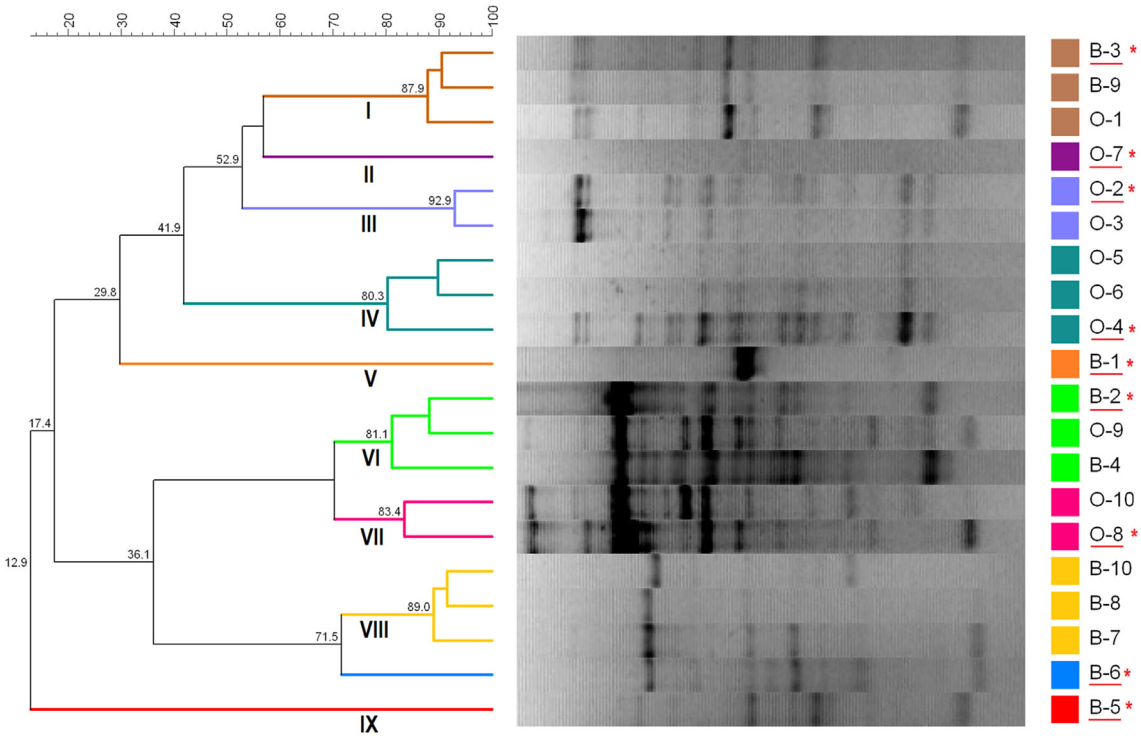

Fig. 7. Dendrogram generated after cluster analysis of the digitalized RAPD-PCR fingerprints with an M13 primer of 20 yeast isolates obtained from brines (B, 1–10) or olives (O, 1–10) after packaging (0 days). Clustering parameters: 0.5% optimization and 0.0% curve smoothing. Isolates selected for sequencing from different clusters below 80.5% similarity (reproducibility of technique) are marked with an asterisk (*).

been related to different human diseases outbreaks (Batt & Tortorello, 2014) and has also been isolated from diverse vegetables marketed in Spain and Germany (Falomir, Rico, & Gozalbo, 2013; Schwaiger, Helmke, Hölzel, & Bauer, 2011) but, as far as we know, this is the first time that has been isolated from table olives. *E. gergoviae* presence in packaged *Aloreña de Málaga* reinforces the need for maintaining low pH levels in this brine mixture, to ensure the final product safety. Also, industry should wait for at least 24 h before bringing the packages to market to ensure the inhibition of this species.

Another difference found in this work with respect to the previous finding is related to yeast populations. In the past, the yeast population during storage of *Aloreña de Málaga* olives was reduced in only 1.5 $\log_{10}$ cycle (from 5.0 to 3.5 $\log_{10}$ CFU/mL) after 67 days (Arroyo-López et al., 2005). However, in other cases, a marked yeast increase was even noticed reaching population levels up to 6 $\log_{10}$ CFU/mL; they were mainly caused by the presence of *Saccharomyces cerevisiae, Issatchenkia occidentalis, Geotrichum candidum, Zygosaccharomyces bailli, Candida diddensiae* and *Candida holmii* species (Arroyo-López, Duran-Quintana, & Garrido-Fernandez, 2006, Arroyo-López, Durán-Quintana, Ruiz-Barba, et al., 2006, 2009; Bautista-Gallego et al., 2011). In the presence of sorbic and benzoic acids, Alves et al. (2015) reported a reduction of the yeast population (from approximately 4 to 2 $\log_{10}$ CFU/mL) in packed cracked green table olives from Maçanilha cultivar. However, in this study, there was a faster and complete inhibition of the yeast population from the 10th day onward due to the higher concentrations of potassium sorbate and sodium benzoate currently applied in packaging, which have a strong inhibitory effect on table olive related yeasts (Arroyo-López, Bautista-Gallego, Durán-Quintana, & Garrido-Fernández, 2008). The species found initially were *L. elongisporus, C. parapsilosis* and especially *C. tropicalis*, the latter strongly associated with table olive processing (Arroyo-López et al., 2012). However, *L. elongisporus* (synonym *Saccharomyces elongisporus*) is frequently related to soft drinks and concentrated juices (Kurtzman, Fell, & Boekhout, 2011). Because of phenotypic similarities, *L. elongisporus* had been considered the teleomorph state of *C. parapsilosis*. In any case, there is a close taxonomic relationship among *C. tropicalis, L. elongisporus* and *C. parapsilosis* species (Kurtzman et al., 2011).

The presence of higher sugar concentrations, lower salt contents, and yeast inhibition has favoured in this survey the growth of LAB. The survival of LAB during storage of diverse green table olives is well documented (Blana, Polymeneas, Tassou, & Panagou, 2016; Casado et al., 2011). Because of the high identity value (>99%) shared between *L. plantarum* and *L. pentosus* in 16S ribosomal DNA sequences (Collins et al., 1991), all selected isolates for sequencing were identified in the first step as *L. plantarum* species (see Table 1). However, multiplex PCR assay based on *recA* gene permitted the discrimination between isolates belonging to *L. plantarum* and *L. pentosus*, which identified *L. plantarum* as the species dominant after packaging, being displaced by *L. pentosus* in the middle and final storage times. This way, *L. plantarum* was not present when the alteration was noticed. Blana et al. (2016) also found a better adaptation of *L. pentosus* than *L. plantarum* to survive under olive packaging conditions of Halkidiki variety. Both species have already been identified using molecular methods from packaged *Aloreña de Málaga* table olives by Bautista-Gallego et al. (2011). A specific biotype (II) of *L. pentosus* was present only in the spoilt fruits (and possible a general overpopulation of the species) and could be associated with the spoilage detected at the end of storage. Their presence could also be linked to the "ropy" and the browning aspect of the surrounding brine, possibly due to the production of extracellular polysaccharides, although this point should be proved in further studies. Diverse LAB species have been characterized to metabolize organic acids and preservatives into undesirable

compounds in foods and cause clouding and spoilage in the packaged low acidified vegetables, meats and foods in general (Johanningsmeier & McFeeters, 2013; Pothakos et al., 2015). In particular, Pothakos et al. (2015) mention the importance of the LAB characterization at strain level in the spoilt product, because significant distinction among biotypes is substantiated by studies determining spoilage potential as a strain-specific trait. In this context, Montaño et al. (2013) identified a strain of *L. pentosus* (L6) from table olive processing, with the ability to remove sorbate from the medium and compromise the packed product stability.

5. Conclusions

The present study reports for the first time the presence of a new type of spoilage affecting fruit quality and stability of directly brined table olives. *Enterobacteriaceae* and yeasts were absent during most of the storage time and were unlikely to be associated with the spoilage. The NaCl, sugar content and yeast inhibition in brine may have favoured the dynamic of the LAB population, which changed throughout shelf life from being predominantly *L. plantarum* (after packaging) to *L. pentosus* (at the end of the study). Therefore, the alteration, apparently, was caused by an increasing higher population of LAB, which overpopulation coincided with the product spoilage (after 2 months packaging) and, particularly, with the presence of a specific biotype (II) of *L. pentosus* isolated from spoilt olives. However, more controlled experiments are required to confirm the association between this microorganism and the unique spoilage observed in this study, as well as the origin of these organisms (spices, fermentation process, industrial equipment, etc.). These results emphasise the need for developing more appropriate preservatives and packaging conditions for this presentation and green directly brined olives in general.

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3.1.2 CAPÍTULO 2:

Shelf life of traditional seasoned *Aloreña de Málaga* table olives based on their packages and fruits characteristics.

Shelf life of traditional seasoned *Aloreña de Málaga* table olives based on their packages and fruits characteristics

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Running Title: Changes in *Aloreña de Málaga* packaging

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Abstract

This work studies the physicochemical, microbiological and sensory evolution of industrial *Aloreña de Málaga* packaging during 259 days. Results showed that the commercial products were free of *Enterobacteriaceae*, the initial yeast population was progressively inhibited, and only lactic acid bacteria grew during shelf life. Among physicochemical characteristics, pH decreased, titratable acidity (and lactic acid) was formed while citric acid and mannitol (as well as total sugar in specific cases) were consumed. These changes produced gradual olive texture degradation and green colour fading along packaging. Multivariate analysis showed that the packaged olives with shelf life between 6 and 42 days enjoyed the highest acceptance while, after the 74th day, the products were progressively losing acceptability, which was mainly evident at the 131st day of packaging (the buying willingness was 50%). Thus, a complete microbiological stabilisation would require the use of alternative preservatives, since thermal treatment are not convenient for the sensory preservation of the product characteristics.

Keywords: *Aloreña de Málaga*; lactic acid bacteria; packaging evolution; sensory evaluation.

1. Introduction

Aloreña de Málaga processing is a genuine expression of the use of olive fruits for human consumption. In fact, its elaboration presents a great similarity with the procedures described by Columela in its book "De Re Rustica" (Columela, 42)[1]. The product may be included in the "Trade Standard Applying to Table Olives" (IOC, 2004)[2], within the "Trade preparations" section, under the epigraph "Specialities". Such sub-section includes olives different from the usual industrial presentations, which are prepared following particular recipes and, therefore, are characteristics of a specific region and, usually, reach relatively low productions. This way, *Aloreña de Málaga* olives are cracked before brining and try to preserve their natural initial green colour as much as possible. The product received the first olive Protected Designation of Origin (PDO) in Spain. Fruits packaged in fresh (olives storage in cold rooms) and traditional (fermented in brine for at least 20 days) designations are the most appreciated. The definitions of the different presentations, as well as the properties and characteristics that may fulfill the product, are established in the legal documents (DOUE, 2012)[3]. They are valid for their usual expected shelf life and distribution area, close to the production region (Guadalhorce Valley, Málaga, Spain). As the recognition of the product quality increases and the areas of commercialisation are expanding, more extended shelf life is required.

A first study of the instability of the fresh or traditional packaged "seasoned" *Aloreña de Málaga* olives showed that their short shelf life (<7 days for the fresh green designation) was caused by the activity of organisms (yeasts) present in the packaging brine, which increased after washing and the seasoning material incorporation (Arroyo-Lopez et al., 2009)[4]. In fact, after packaging, there was a considerable load of lactic acid bacteria (LAB) and yeasts in the product. They, despite the preservative (sodium

sorbate), usually grew and caused diverse changes in the physicochemical characteristics (titratable acidity increased while pH decreased) which, eventually, lead to swelling spoilage, although the product changes (due to low pH and high titratable acidity) did not represent any health risky. As a result of the study, major emphasis was paid to the inhibition of yeasts. However, the excess of LAB may also lead to certain specific cases of spoilage like the peculiar alteration of traditional *Aloreña de Málaga* olives, characterised by the production of whitish and soft regions on the olive surface, which appearance coincided with the maximum population of these organisms (Romero-Gil et al., 2016)[5]. The dominant species found in the affected packages was a specific genotype of *Lactobacillus pentosus* (even in the olive biofilm) while the microbiota in the non-affected ones was composed of diverse microbial populations of other organisms such as *Lactobacillus plantarum,* among the bacteria, and *Candida tropicalis*, *Candida parapsilosis*, and *Lodderomyces elongisporus*, among yeasts (Romero-Gil et al., 2016)[5].

Hence, the product changes under the current industrial packaging conditions require a revision to provide the basis for a further extension of its shelf life, which is still the primary bottleneck for increasing the production of this speciality. Thus, the aim of this work was a survey of the microbiological and physicochemical changes that seasoned *Aloreña de Málaga* table olives undergo at the current industrial packaging conditions. The study should identify the weakness and develop new strategies to improve the final product quality and shelf life. The work also includes a first tentative study of the sensory profile and consumers' opinion evolution as the product shelf life progressed.

2. Material and methods

2.1. Commercial packaging

This work has been carried out with commercial seasoned packaged cracked olives of the *Aloreña de Málaga* cultivar prepared using fruits previously stored in brine for 20 days (the traditional style, according to the PDO). Packaging from two different industries (denominated as COP and TOL) were used to evaluate the effect of the factory and olive history (different conditions prevailing in the storage/fermentation phase). The following concentrations of chemicals, habitually used by the companies, were used for packaging: 55 g L^{-1} NaCl, 3 g L^{-1} citric acid, 2 g L^{-1} potassium sorbate, 1 g L^{-1} sodium benzoate, 1 g L^{-1} ascorbic acid, and 0.8 g L^{-1} lactic acid, expressed as weight/volume. Pet packages (1.6 L total volume) were filled with 900 g of olives, 16 g of seasoning material (a mixture of garlic dices, pepper strips, and small pieces of fennel, and thyme) and 0.7 L of cover brine. Packages were maintained at room temperature (23± 2 °C). At preselected time intervals (0, 6, 20, 42, 74, and 131days) two containers were removed and used for sensory analysis and determination of packaged characteristics. In contrast, the physiochemical and microbiological control was extended up to 158 and 259 days.

2.2. Physicochemical analysis

The analysis of pH, titratable acidity, combined acidity, and NaCl in the cover brine of the packages were performed using the routine methods described by Garrido-Fernández et al. (1997)[6]. Individual reducing sugars (glucose, fructose, sucrose and mannitol), organic acids (acetic, lactic, and citric) and ethanol were determined by HPLC according to the methods developed by Sánchez et al. (2000)[7].

Firmness was measured objectively using a Kramer shear compression cell coupled to an Instron Universal Testing Machine (Canton, MA). The cross-head speed

was 200 mm/min. The firmness of the olives was expressed as the mean of 10 measurements, each of which was performed on two cracked, pitted fruit. Shear compression force was expressed as kN/100 g pitted olives.

Surface colour analyses were performed on olives using a BYK-Gardner Model 9000 Color-view spectrophotometer, provided with an illuminant C at 10º and equipped with computer software to calculate the CIE coordinates: L^* (lightness), a^* (negative values indicate green while positive values are related to red tones), and b^* (negative values indicate blue and positive values associated to yellowish). Interference by stray light was minimized by covering samples with a box, which had a matt black interior. The data for each measurement were the mean of 10 olives.

2.3. Microbiological analyses

Brine samples and their decimal dilutions were plated onto the specific selective media described below using a Spiral System model dwScientific (Don Whitley Scientific Limited, England). Subsequently, the plates were counted using a Flash & Go (IUL, Barcelona, Spain) image analysis system, and the results were expressed as $\log_{10}$ CFU mL^{-1}. *Enterobacteriaceae* were counted on VRBD (Crystal-violet Neutral-Red bile glucose)-agar (Merck, Darmstadt, Germany), lactic acid bacteria (LAB) on MRS (de Man, Rogosa and Sharpe)-agar (Oxoid, Basingstoke, UK) with 0.2 g L^{-1} sodium azide (Sigma, St. Luis, USA), yeasts on YM (yeast-malt-peptone-glucose medium)-agar (Difco™, Becton and Dickinson Company, Sparks, MD, USA) supplemented with oxytetracycline and gentamicin sulphate as selective agents for yeasts, and finally total aerobic mesophilic microorganisms (AM) on Plate-Count-Agar (PCA, Oxoid). The plates were incubated at 30 ºC for 24 (*Enterobacteriaceae* and AM*)* or 48 (LAB and yeasts) hours. The four microbial groups were also studied on fruits. In this case, two

olives were pitted, weighted (approximately 10 g) and placed in 25 mL of sterile saline solution (9 g L^{-1} NaCl) for homogenization during 2 minutes in a stomacher model Seward 400 (Seward Medical Ltd, West Sussex, England). Then, the resulting suspension or appropriate dilutions was spread on the selective media described above. Counts were expressed as $\log_{10}$ CFU g^{-1}.

2.4. Evaluation of packages and fruits

For the sensory analysis, there was agreement between the experts from the industry and Instituto de la Grasa (IG-CSIC) that neither the evaluation sheet of the International Olive Council (IOC, 2011)[8] nor that developed by Galan-Soldevilla et al. (2013)[9] for *Aloreña de Málaga* olives, which contains an excessively large number of attributes and was intended for being used by a specifically trained panel, were appropriate for the objectives of this research. Then, as a first task, a simple and easily interpretable evaluation sheet, which could be properly used in the consumers' tests, was developed. It was prepared bearing in mind all the previous works but also the special commercialization characteristics of these products (packages and olives) and consisted of two different sections. The first included the attributes that could be appreciated on the packages (swelling and brine leakage) or observed without opening them (colour of brines and turbidity); it ended with a global scoring of the package appearance and a question on the overall package acceptability, which answers were restricted to only two options: yes or not. A second part included specific descriptors related to *Aloreña de Málaga* fruits (browning, appreciation of external damages, and any kind of defects) and the attributes considered by the sheet IOC (2011)[8] for the green Spanish-style olive classification (acid, salty, bitter, hardness, crunchiness); it also ended with a global evaluation of the olive quality and acceptability. As in the case of

packages, there were only two possible answers, yes (the olives characteristics were good enough as to be purchased) or not (they were of poor quality).

The consumers' panel was chosen among the personal staff of Instituto de la Grasa and was composed of 35 members (16 men and 19 women), selected because of their compromise with the sensory evaluation in general and their habitual consumption of table olives. The number of participants is above the minimum number accepted for consumer test (30 consumers) and represents a still manageable group when used successively along the shelf life, as required in this study. The alternative method of testing samples with different shelf life periods simultaneously was discarded because of the possibility that the necessary cold storage would not guarantee appropriate stabilisation. Before the tests, people were informed on the aim of the study, the sensory peculiarities of traditional *Aloreña de Málaga* olives, and performed several informal tests to be accustomed to the product. The samples were presented to consumers in blue glass, according to the recommendations of the standard COI/T.20/Doc.No 5 (Glass for oil tasting) (IOC, 1987)[10], always coded with three digits also randomly chosen, and in random order concerning their origins. Only two samples were analysed in each session. After the first sample test, the consumers were requested to cleanse the palate with tap water before to proceed with the second one. All the descriptors were evaluated on an unstructured scale which ranged from 1 to 11, in which 1 was associated with the complete absence of the attribute and 11 to its presence in the highest intensity. The consumers were asked to mark on the scale the intensity of each attribute perceived. The sheets were read by the panel leader with 0.1 cm precision, except for buying predisposition based on packages appearance or olive quality which answers (yes or not) were coded as 1 or 0.

2.5. Statistical analysis

Data were plot using Sigmaplot v. 13 (Systat Software, Inc. Germany) and the changes, when appropriate and after testing several options, fit by a linear model. Comparison between sensory data from the two packaging industries over time was made by a nested (time within origin) ANOVA. Significant models were those that provided better estimation than just the means at $p<0.05$. The significance of the individual explanatory variables was also tested at $p<0.05$. The estimation of shelf life according to the acceptation or not of samples by consumers (based on the appearance of packages or quality of fruits) was made by the parametric survival models usually applied to the estimation of the time at which an event occurs based on a Weibull distribution. Then, the time at which quartile 25%, median (50%), and quartile 75% were estimated based on the model fitted. Sensory scores were also subjected to Principal Components Analysis (PCA) to relate attributes among them and with treatments over time. All the statistical analysis were performed by using XLSTAT v. 2017 (Addinsoft, Paris, France).

3. Results

3.1 Changes in the microbial populations during shelf life

The *Enterobacteriaceae* were never detected in any package, but AM was always present during shelf life in both brine and fruits, and their populations showed a chaotic evolution (Fig. 1 and 2, panel A). The lab was absent for around 25 days and then grew sharply to reach population levels between 4 and 6 $\log_{10}$ CFU/mL. In general, their average counts in packages from TOL industry were lower (but not significantly different) than in COP industry. The trends in brine and fruits were similar (Fig. 1 and 2, panel B). The counts of yeasts decreased progressively during the first period of shelf life (Fig. 1 and 2, panel C). Overall, their average counts in brine or olives from TOL

were also lower (but not significantly different) than those from COP. As a result, yeasts in packages from TOL also fell below the detection limit earlier than in COP. The long permanence of yeast in brine or olives, despite the use of preservatives, was not expected and may represent a spoilage risk during the first period of shelf life if their metabolisms were not conveniently reduced.

Modelling AM and LAB populations was not attempted due to their limited changes (in the first case) or their apparent variability (in both groups) which will make unrealistic any prediction in either brine or fruits. However, in yeasts, the initial populations were similar in both industries, but the decrease rate was practically linear in both matrixes (brine or fruits) and faster in the packages from TOL industry (Table 1). Also, the fit was always better for counts in brine.

3.2 Changes in the physicochemical parameters of brine during shelf life

The changes in LAB population had their reflection on the evolution of pH and titratable acidity. Overall, the change in pH was remarkable and moved from 4.65-4.75 down to ~4.1 (Fig. 3, panel A). The linear decrease was modelled by a linear kinetic and was slightly faster in the olives from COP industry (Table 1). The pH trend (regardless of industry) was due to the simultaneous increase in titratable acidity, produced by the LAB during the first 140 days, which reached a maximum concentration of ~0.6 g L^{-1}. The further decrease in pH might have been caused by the formed acid diffusion into the flesh when its production ceased. The hypothesis is also supported by the absence of any further change in acidity after reaching the equilibrium (Fig. 3, panel B). The combined acidity, in general, showed a slightly increasing trend, possibly caused by the lactic acid produced (Fig. 3, panel C). On the contrary, the salt content, after a fist salt leakage from fruits into the brine, was kept constant at approximately 45 g L^{-1} in COP

and 6 g L^{-1} in TOL, with remarkable differences between the two industries (Fig. 3, panel D).

As expected, the primary metabolite formed during packaging was lactic acid (Fig. 4), which was produced in variable rates (considerable variability between the packages during this period) and particularly along the final shelf life phase. However, this lactic acid increment was not reflected as titratable acidity. The lack of correlation might have been caused by the probable incorporation of at least part of the lactic acid formed as combined acidity, which also increased slightly (Fig. 3, panel C). However, in the *Aloreña de Málaga* olive packages are also present other acids as well as ethanol. In this case, the initial citric acid added at packaging progressively decreased, acetic acid showed a slight increase, likely due to the presence of heterofermentative LAB strains, while ethanol concentration remained quite stable, indicating that the yeasts, although present, had a limited activity (Fig. 4).

The amount of total sugar at the beginning of packaging was not abundant (1-2 g L^{-1}), being glucose the main component in both TOL (highest concentration) and COP (Fig. 5). Only in the brines of COP total sugars were utterly exhausted at the end of the shelf life (mainly because of the glucose and mannitol use). In the packaging from TOL industry, only mannitol was, apparently, consumed during packaging.

3.3. Changes in the physicochemical parameters of olive fruits during shelf life

The most affected characteristic during packaging was texture. Its instrumental value decreased from 7 to about 3.5 kN/100 g pitted olives (Fig. 6, panel A). A linear model could be adjusted in this case. According to Fig. 5 and Table 1, the olives from both industries had similar textures and were degraded at the same rate.

With regard to fruit colour (Fig. 6, panels B and C), *L** (luminance) values from TOL olives were lower than those from COP, although followed similar trends over time. The changes may be summarized as a marked initial increased, possibly due to the impact of the low pH and high acidity of the fresh brine used at packaging, followed by a slight non-significant decreasing tendency. However, the values of *a** had similar changes regardless of the industry, with the *a** value increase being linked to the LAB growth (from the 40^{th} day onward), with the subsequent production of titratable acidity and pH decrease. The phase of linear increase followed a simple linear model. The parameters indicated a similar rate of change irrespective of the industry (Table 1), with a loss of the fruits' green appearance.

3.4. Evaluation of fruits and packages

At selected intervals, the samples were offer to the consumers for evaluation of packages and fruits. Results (Tables 2 and 3) were then subjected to nested (time within type of industry) ANOVA analysis (Table 4). The p-values for the overall model indicated that the following attributes were not significant at $p>0.05$: brine turbidity, brine leakage, overall defects of fruits, and overall acceptability of fruits. That is, the average values given by the consumers to the containers (first two parameters) and fruits (last two) were similar regardless of the industry and the sampling period and, consequently they did not significantly change throughout the shelf life.

Also, the industry was not significant for any of the attributes related to the container appearance, but swelling, brine browning, and overall acceptability showed significant changes over time. After removing the variable industry, the model for these containers' attributes, led to right predictions (Fig. 7). Swelling had a slight non-significant decrease during the first part of the shelf life but a progressive increase after

the 74th day (Fig 7A). On the contrary, brine browning always showed an increasing trend which was more evident after the 74th day and, notably, at the 133rd day which scores were significantly different from those with 0-42 day shelf life containers (Fig. 7B). Finally, overall acceptability showed an always decreasing trend, which was more rapid after the 74th-day shelf life (Fig 7C).

In the case of fruits, within the significant models, the industry did not have significant ($p>0.05$) effect on browning, bruises, bitter, and crunchiness (Table 4) but all of them depended on time. The scores for fruits' browning and bruises had abnormal high initial scores, due to a possible confusion on the initial evaluation, which was later corrected since the following scores were lower and relatively similar over time. Thus, in practice, these attributes may be considered stable during the shelf life period studied (Fig. 7, D and E). Concerning bitterness, possibly there was an initial diffusion, due to its water solubility, followed by a certain re-equilibrium and a final decrease probably related to the oleuropein hydrolysis (Fig. 7f). Crunchiness (Fig. 7G) and hardness (Fig. 8D) always increased significantly after packaging. The only attribute which depended on the industry was salty (Table 4), because of the different levels habitually used by them, as commented above; the scores for the olives from TOL were always higher than those from COP (Fig. 7h).

The models of only two attributes (acid, and hardness) depended on the two explanatory variables of the ANOVA (industry and packaging time) (Table 4 and Fig. 8). The olives from TOL had higher scores than those from COP for both parameters. The acid scores showed an initial descending trend followed by an increased during the last part of the shelf life while hardness had an evolution similar to crunchiness (Fig 8).

The predisposition of consumer (proportion who would buy or not the products), based on either their container external appearance or the sensory characteristics of fruits were arranged in a table similar to those used in the parametric survival curve. The Weibull model was fit, and their parameters and quartiles obtained. The table of quartiles allow the estimation of the shelf life according to the proportions of dissatisfied consumers able to be assumed. Based on packages the model estimated a median of 153 days for COP industry; that is, it should be expected that after 153 days, 50% of the consumers will be satisfy with the quality of the product. However, if one wish to reduce the rejection risk to the first quartile (25%) the shelf life of the product should be reduced to only 39 days. On the contrary, after 252 (days) (3^{rd} quartile) only 25% consumers will buy the product. The same study estimated a shorter shelf life for TOL than for COP, which were 30, 149 and 527 days for 1^{rst} quartile, median, and 3^{rd} quartile, respectively. In any case, no model was able to be fit in case of predisposition to buy based on the evaluation of fruits.

A multivariate study of the sensory data (Fig. 9) showed that the fresh packaged olives, regardless of the industry, received similar and particular scores. They were characterised by its high values in bitter, bruises and olive browning, although the association of the two last ones should not be real since bruises were produced before packaging. Also, fruits after the 7^{th}, 40^{th}, and mainly the 133^{rd} packaging days were progressively different from the original ones and were associated with brine browning and turbidity. In general, the olives received the greatest acceptance at intermediate packaging days (6 to 42), regardless of the origin. Their positions were linked to low scores in swelling, pack leakage, defects, acid, and salty and also, but less strongly, to hardness, crunchiness, package acceptance and olive acceptance.

4. Discussion

A survey carried out one decade ago with *Aloreña de Málaga* packaging showed that the leading cause of instability, noticed by swelling and brine leakage, were diverse. In the fresh packaging, the problem was caused by the carbon dioxide produced by the fruits' respiration. However, in the traditional presentations, the spoilage was caused by the gas formed by the natural microbiota, mainly yeasts, which counts markedly increased during shelf life (Arroyo-López et al., 2009)[4]. The microbial load on the packaged olives was due to a carryover effect, which caused a progressive accumulation of microorganisms from the previous storage/fermentation, the packaging operations, and the natural ingredients added at packaging (Arroyo-López et al., 2009)[4]. The initial microbiota in *Aloreña de Málaga* olives may depend on the type of storage. Yeasts seemed to be the most predominant populations when the olives are stored in cold rooms (*Saccharomyces cerevisiae* and *Candida* cf. *apicola),* while LAB counts increased towards the end of the storage in plastic drums and large-scale vessels at ambient temperatures (Abriouel et al., 2011)[11]. Among the LAB, Abriouel et al. (2012)[12] characterised a total of 144 isolates from small medium enterprises. They included lactobacilli (82%), *Leuconostoc* (10%), and pedioccocci (8%) with *Lactobacillus pentosus, Pediococcus parvulus,* and *Leuconostoc pseudomesenteroides* being the only species identified. However, they were not clonally related and exhibited a considerable degree of genomic diversity for lactobacilli and *Leuconostoc*.

Diverse treatments have been applied to promote the retention of freshness and diminish the level of microorganisms during storage/fermentation of *Aloreña de Málaga* fruits (Arroyo-Lopez et al., 2008)[13]. The use of ascorbic acid (AA) (up to 15 g L^{-1}) reduced the de-greening rate. On the contrary, the addition of sodium metabisulphite (SM) (1.5 g L^{-1}) partially inhibited the LAB population at intermediate concentrations of AA (7.5 g L^{-1}) and the highest NaCl level (70 g L^{-1}) but did not affect

yeast growth. Therefore, a modified storage/fermentation using maximum levels of AA and SM could improve olive colour and reduced the carryover LAB population into packaged products.

The combined use of sorbate and benzoate is allowed in table olives (IOC, 2004)[2] but their minimum inhibitory concentrations (MIC) or the possibly synergy had been scarcely studied. Arroyo-López et al. (2008)[14] was the first to investigate their specific effects on a cocktail of the main species of yeasts usually found in *Aloreña de Málaga* olives. At pH 4.0, the average level for this speciality, the MIC for sorbic and benzoic acids were 3.31 and 10.0 mM, respectively. However, no synergy between them was found. The study was useful for fixing the appropriate level of preservatives, which at the current packaging conditions used in this work, not only prevented the yeast growth but caused their disappearance, although not as faster as desirable. However, such conditions were not strong enough for preventing, after a short lag phase, the rapid growth of LAB during shelf life. To notice that the behaviours of LAB and yeasts, in brine or on fruits, were opposed, indicating certain competitiveness between both groups of microorganisms. This similarity between both matrices could be due to the easy exchange of components between the brine and the cracked olives. Conversely, the high variability found in the microbial changes indicates that the packaging conditions do not promote a homogeneous behaviour but particular answers, depending on package. Then, the preservation conditions used do not represent a robust procedure and requires reconsideration, including the olive history.

From a safety point of view, the pH decrease and the formation of lactic acid (about 0.6 g L^{-1} brine), as observed in this study, is interesting and helps to improve the antifungal activity of the preservatives and, subsequently, yeasts disappearance. However, these changes also may produce a progressive decrease of texture and the

greenish appearance of the olives as demonstrated by Brenes et al. (1994)[15] and Gallardo Guerrero et al. (2013)[16], respectively, with the changes related to the colour being particularly important for *Aloreña de Málaga* olives. However, the green colour fading should not be associated exclusively with the packaging shelf life itself since the main changes on this attribute occurred during the first storage month, although its degradation continues at lower rates along the entire storage/fermentation and the packaging periods (Gallardo-Guerrero et al. (2013)[16]. In parallel, there is an accumulation of the brown pigments derived from not only the polyphenol oxidation but from the degradation of chlorophylls (Gallardo-Guerrero et al., 2013)[16]. The increase observed in the *a** values are then strongly negatively, or positively, correlated to the overall green, or brownish pigments, respectively (Gallardo-Guerrero et al., 2013)[16].

The changes in the LAB population caused limit troubles on the olives aspect in this work, as deduced from the sensory evaluation along the shelf life. However, an excessive LAB presence may represent a potential risk of spoilage like that produced by the particular phenotype of *L. pentosus* who caused damages on the olive flesh (Romero-Gil et al., 2016)[5]. Subsequently, although not harmful for health and of reduced influence on organoleptic characteristics, LAB presence should also be controlled. Also, the use of some antioxidant (e.g. erythorbic acid, usually added to *Aloreña de Málaga* at packaging), may promote the microbial growth and firmness deterioration (Gallardo-Guerrero et al., 2013)[16]. Therefore, this work confirms new sources of instability in *Aloreña de Málaga* packaging and focus now the interest on reducing the LAB growth, although the objective is quite tricky because of the ability of this organism to use various olive compounds as a nutrient source like mannitol, as observed in this work, or even polyphenol glucosides.

With regard to the sensory characteristics, the number of attributes evaluated was lower than those proposed by Galan-Soldevilla et al. (2013)[9] but higher than those proposed by the IOC (2011)[8]. In fact, our evaluation sheet not only included those already present in the IOC Evaluation Sheet (IOC, 2011)[8] but a few additional ones. In general, the evaluation sheet proved to be adequate, although led to sporadic abnormal scores at the first sampling times (browning or bruises), probably by an incorrect application to *Aloreña de Málaga* of similar quality criteria than to green Spanish-style. However, apart from these, not abnormal attributes' changes were observed during shelf life. The lack of significance of the model for overall fruits' acceptability means that, regardless of the other attributes, the fruits were similarly acceptable during the whole shelf life studied. This behaviour also is in agreement with the proportion of consumers who would buy the product, which remained substantially the same during the shelf life, regardless of the production industry. The level of buying predisposition based on the container appearance was even higher, indicating that this criterion may not be in agreement with that of olive fruits when establishing the product shelf life.

Even in case of ANOVA significant models, the fruits from both industries received statistically similar scores in several attributes (swelling, browning, overall container acceptability, browning, bruises, bitter, and crunchiness), although most of them showed significant changes during shelf life. The initial slow decrease of bitter scores was due to the diffusion of phenols into the brine while the sharp decrease can be related to their hydrolysis (mainly oleuropein) by some of the LAB strains present at the end of the shelf life. The increase in crunchiness and hardness after packaging may be due to the absorption of Ca by the olive flesh. Salty was the only attribute which depended exclusively on the industry because of the use of different salt concentration

during the storage/fermentation phase since, at packaging, both companies use similar brine concentrations.

Only the scores of two attributes (acid and hardness) depended simultaneously on the previous storage/fermentation and packaging conditions. The different levels of acid between the two industries are due to the different acidification level in their respective storage/fermentation solutions. The first changes in acid may be due to its initial equilibrium of acid between fruits (which apparently had higher contents than those required after packaging), but the next increment in acid taste should be caused by the acid produced by the LAB. The initial increase in hardness was possible due to Ca absorption by the flesh; however, once the absorption is finished, the stability of this attribute may be due to the absence of additional sources of Ca. The final decrease in hardness at the end of shelf life may be linked to the effect of the lactic acid produced and the subsequent pH decrease.

In general, the proportion of consumers showing a predisposition to buying the product was above the median, except at 74 days for olives from TOL. Since the estimations of the shelf life could be obtained only from answers based on the package appearance, no estimations of the shelf life of the packaged olives can be deduced. Therefore, the first impact of the product on consumers is related to the packages' appearance (absence of swelling or leakage) which, in turn, is the result of a low yeast activity; apparently, they, although present, had a limit metabolic activity and produced only a reduced level of gas. Evidently, the current packaging conditions cause strong yeast inhibition but scarce LAB growth control. Consequently, the current shelf life of *Aloreña de Málaga* table olives is quite longer than that observed in previous surveys (Arroyo-López et al., 2009)[4] but the predisposition of the consumer to use them decrease progressively after approximately 50 days of putting them on the market.

Furthermore, since the consumers who will buy the product, based on fruits quality, decreased after 149-153 packaging period, above 150 days only 50% consumer will like them. This evolution, although, less sensitive to health risks, may also lead to economic losses, product deterioration (Romero-Gil et al., 2016)[5] and, in the end, consumers' and dealers' distrust on the product.

5. Conclusions

This survey has demonstrated that the current packaging conditions of *Aloreña de Málaga* table olives, despite its apparent assumed stability, allow important changes in diverse product's attributes. In fact, a marked microbial activity mainly related to the LAB presence and growth during the whole shelf life was detected. Such bioactivity caused changes in pH, titratable acidity, texture and the greenish appearance during shelf life but hardly influenced the external appearance of the packages and their sensory characteristics for 42 days. Later, a progressive decrease in the appreciation of the products by the consumer was observed although they had a limited impact on product consumers' refusal. Furthermore, after 134 days shelf life, the products still received consumers support above the median, and, at the 144th day, they still showed an approximate 50% buying predisposition. However, results pointed out on the convenience of modifying the current packaging conditions so that the LAB bioactivity should also be controlled not only along the current shelf life but for more extended periods to allow further market expansion.

Acknowledgements

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Table 1. Modelling the changes of the yeast counts, pH, texture and *a** colour parameter during shelf life.

Parameter/industry	Matrix	Intersection (a)	Slope (death rate) (b)	Fit (R^2)
Yeast counts				
COP	Brine	3.7 (±0.3)[1]	-0.029 (±0.006)[2]	0.73
COP	Fruits	3.9 (±0.5)	-0.031 (±0.009)	0.58
TOL	Brine	3.1 (±0.2)	-0.038 (±0.005)	0.88
TOL	Fruits	3.4 (±0.5)	-0.084 (±0.024)	0.68
pH changes				
COP	Brine	4.71(±0.04)[3]	-0.003 (±0.000)[4]	0.78
TOL	Brine	4.60 (±0.03)	-0.002 (±0.000)	0.84
Texture				
COP	Fruits	7.18 (±0.18)[5]	0.024(±0.02)[6]	0.91
TOL	Fruits	7.36 (±0.21)	0.024(±0.02	0.89
*a**				
COP	Fruits	5.24 (±0.24)[7]	0.010 (±0.001)[8]	0.76
TOL	Fruits	5.24 (±0.17)	0.012 (0.001)	0.84

Notes: [1]($\log_{10}$CFU mL^{-1} or g^{-1}); [2]($\log_{10}$ CFU mL^{-1}(or g^{-1})·$days^{-1}$); [3] pH units; [4] pH units·$days^{-1}$; [5]kN; [6]kN·$days^{-1}$; [7]*a** units; [8]*a**units·$days^{-1}$

Table 2. Scores received by the different attributes of packages and olives from COP industry as well as their proportion of acceptance over shelf life.

	Packaging time					
	(0 days)	(6 days)	(20 days)	(42 days)	(74 days)	131 days)
Package container						
Swelling	1.25 (0.35)	0.66 (0.20)	0.91 (0.24)	0.62 (0.16)	1.21 (0.36)	1.80 (0.48)
Brine browning	2.53 (0.34)	2.50 (0.31)	2.59 (0.34)	2.53 (0.36)	3.52 (0.45)	3.98 (0.40)
Brine turbidity	3.48 (0.39)	2.91 (0.37)	2.84 (0.39)	3.42 (0.40)	3.94 (0.41)	4.19 (0.44)
Brine leakage	1.28 (0.41)	0.53 (0.15)	0.68 (0.22)	0.33 (0.05)	1.58 (0.48)	1.15 (0.40)
Acceptability	8.11 (0.27)	7.80 (0.40)	7.82 (0.35)	8.18 (0.32)	6.88 (0.51)	6.46 (0.47)
Buying predisposition (%)	97%	94%	97%	94%	84%	81%
Fruits						
Browning	6.37 (0.32)	2.42 (0.33)	1.97 (0.28)	2.06 (0.30)	2.85 (0.36)	2.63 (0.52)
Bruises	6.77 (0.27)	2.11 (0.32)	2.03 (0.34)	1.99 (0.32)	1.87 (0.29)	2.24 (0.44)
Acid	3.65 (0.37)	2.89 (0.35)	2.53 (0.32)	2.55 (0.29)	3.31 (0.30)	2.82 (0.55)
Salty	4.08 (0.35)	4.33 (0.41)	3.31 (0.36)	3.35 (0.33)	4.25 (0.36)	3.97 (0.78)
Bitter	5.04 (0.37)	3.88 (0.37)	3.89 (0.36)	4.07 (0.39)	4.33 (0.40)	3.07 (0.60)
Hardness	2.32 (0.31)	5.11 (0.35)	5.25 (0.39)	5.54 (0.37)	5.51 (0.33)	4.60 (0.90)
Crunchiness	1.79 (0.32)	6.39 (0.35)	5.66 (0.33)	6.05 (0.35)	5.64 (0.39)	5.10 (0.99)
Defects	1.89 (0.30)	1.52 (0.34)	1.92 (0.40)	2.18 (0.42)	2.18 (0.44)	2.41 (0.47)
Acceptability	5.72 (0.44)	7.14 (0.88)	5.72 (0.42)	5.38 (0.40)	5.18 (0.35)	5.40 (1.05)
Buying predisposition (%)	66%	74%	77%	64%	72%	69%

Table 3. Scores received by the different of packages and olives from TOL industry as well as their proportion of acceptance over shelf life.

	Packaging time					
	(0 days)	(6 days)	(20 days)	(42 days)	(74 days)	131 days)
Package						
Swelling	1.29 (0.37)	0.81 (0.21)	0.90 (0.23)	0.88 (0.26)	0.99 (0.23)	1.53 (0.39)
Brine browning	2.89 (0.36)	2.96 (0.33)	3.34 (0.35)	3.21 (0.42)	3.26 (0.40)	4.22 (0.52)
Brine turbidity	3.04 (0.40)	2.71 (0.38)	4.46 (0.36)	3.30 (0.38)	2.84 (0.33)	3.77 (0.42)
Brine leakage	1.93 (0.53)	0.54 (0.14)	0.71 (0.24)	0.35 (0.06)	0.95 (0.34)	2.48 (0.62)
Acceptability	7.85 (0.35)	7.70 (0.42)	7.10 (0.42)	7.89 (0.40)	7.06 (0.49)	6.05 (0.49)
Buying predisposition (%)	94%	97%	94%	94%	87%	77%
Fruits						
Browning	6.28 (0.33)	2.26 (0.30)	2.68 (0.33)	2.10 (0.30)	2.63 (0.29)	3.06 (0.36)
Bruises	6.41 (0.33)	2.18 (0.28)	2.31 (0.30)	2.02 (0.32)	2.26 (0.34)	2.28 (0.33)
Acid	3.83 (0.37)	3.58 (0.40)	3.11 (0.32)	2.95 (0.33)	3.60 (0.35)	3.64 (0.44)
Salty	5.19 (0.44)	4.82 (0.41)	4.61 (0.32)	5.04 (0.36)	5.76 (0.42)	5.10 (0.43)
Bitter	5.36 (0.34)	4.51 (0.45)	3.67 (0.35)	4.05 (0.36)	5.23 (0.39)	3.23 (0.42)
Hardness	3.10 (0.35)	5.40 (0.37)	5.21 (0.35)	5.65 (0.40)	5.78 (0.35)	5.37 (0.44)
Crunchiness	2.60 (0.40)	5.98 (0.40)	5.36 (0.38)	8.74 (2.62)	6.12 (0.37)	6.02 (0.39)
Defects	3.15 (0.50)	2.12 (0.48)	2.12 (0.44)	1.94 (0.40)	2.53 (0.52)	2.49 (0.44)
Acceptability	5.12 (0.40)	5.92 (0.42)	5.65 (0.37)	5.67 (0.38)	4.32 (0.34)	5.81 (0.40)
Buying predisposition (%)	53%	67%	76%	71%	42%	76%

Table 4. Summary of the ANOVA (nested model) parameters for either the complete model and each of the explanatory variables when applied to the scores assigned to the attributes according to containers and fruits.

Fit parameter	Attributes related to the container appearance					Attributes related to fruits								
	Swelling	Brine browning	Brine turbidity	Brine leakage	Overall acceptability	Browning	Bruises	Acid	Salty	Bitter	Hardness	Crunchiness	Overall defects	Overall acceptability
R^2	0.922	0.911	0.683	0.773	0.968	0.986	0.995	0.935	0.930	0.927	0.978	0.909	0.640	0.771
F	9.868	8.480	1.795	2.841	24.816	58.736	164.397	12.015	11.090	10.619	36.465	8.333	1.484	2.807
Pr > F	0.012	0.017	0.269	0.136	0.001	0.000	< 0.0001	0.008	0.009	0.010	0.001	0.017	0.341	0.139
Industry	0.008*	6.356	1.463	0.739	5.015	0.516	0.452	24.457	51.015	3.011	6.853	2.311	3.176	1.639
	0.934**	0.053	0.280	0.429	0.075	0.505	0.531	0.004	0.001	0.143	0.047	0.189	0.135	0.257
Time (days)	11.841*	8.905	1.862	3.262	28.777	70.380	197.186	9.527	3.105	12.141	42.387	9.537	1.145	3.041
	0.008**	0.016	0.256	0.110	0.001	0.000	< 0.0001	0.014	0.120	0.008	0.000	0.014	0.443	0.124

Notes: The first three parameters refer to the complete model; * F values for the explanatory variable according to attributes;** p-values for the explanatory variables according to attributes.

Figure Legends

Figure 1. Changes in the AM, LAB and yeasts populations in brine over time during shelf life, according to industry.

Figure 2. Changes in the AM, LAB and yeasts populations in olives over time during shelf life, according to industry.

Figure 3. Changes in pH, titratable acidity, combined acidity and salt content over time during shelf life, according to industry.

Figure 4. Changes in the lactic acid, acetic acid, citric acid and ethanol over time during shelf life, according to industry.

Figure 5. Changes in the total sugars, sucrose, glucose, fructose, and mannitol over time during shelf life, according to industry.

Figure 6. Changes in the texture, CIE L^* and a^* colour parameters over time during shelf life, according to industry.

Figure 7. Predictions based on the nested (time within origin) ANOVA for significant model based on only one attribute (shelf life time or industry).

Figure 8. Predictions based on the nested (time within origin) ANOVA for acid and hardness as a function of the industry and shelf life time.

Figure 9. Relationship, according to multivariate (biplot) analysis, among the scores of the attributes evaluated in containers and fruits, shelf life periods, and industry.

Figure 1

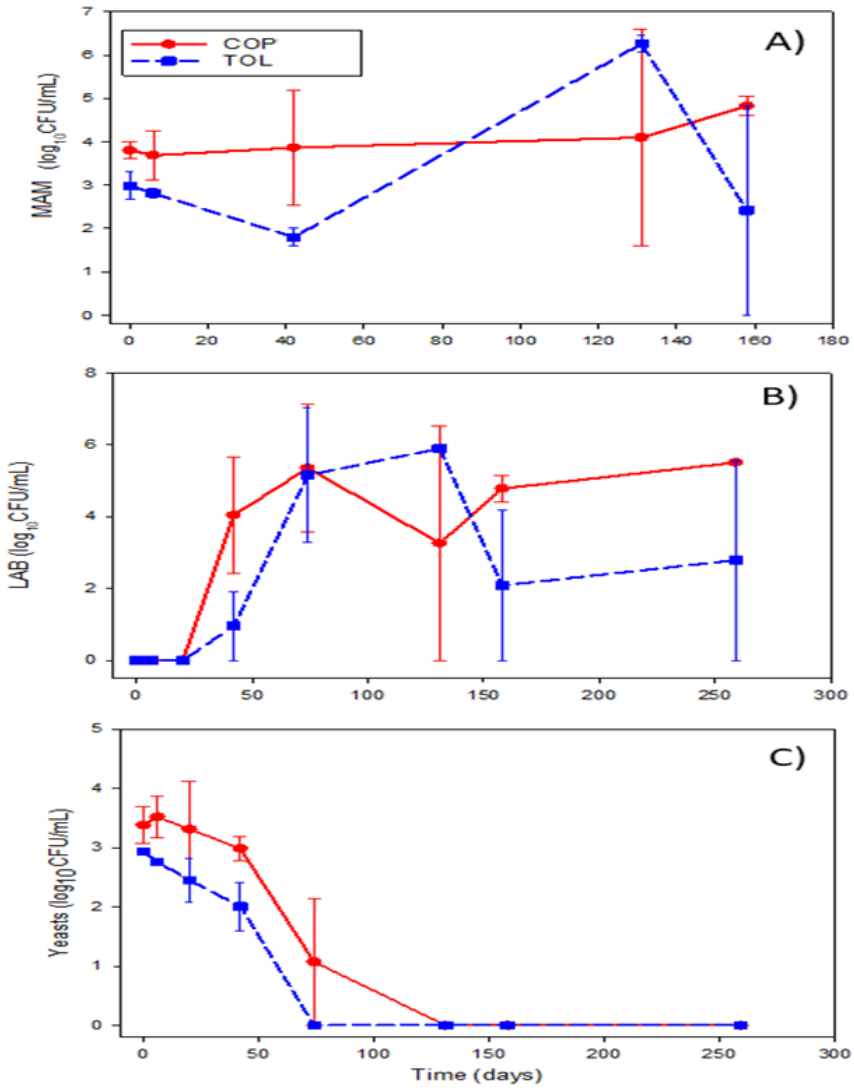

A)
COP
TOL
MAM (log10CFU/mL)
B)
LAB (log10CFU/mL)
C)
Yeasts (log10CFU/mL)
Time (days)

Figure 2

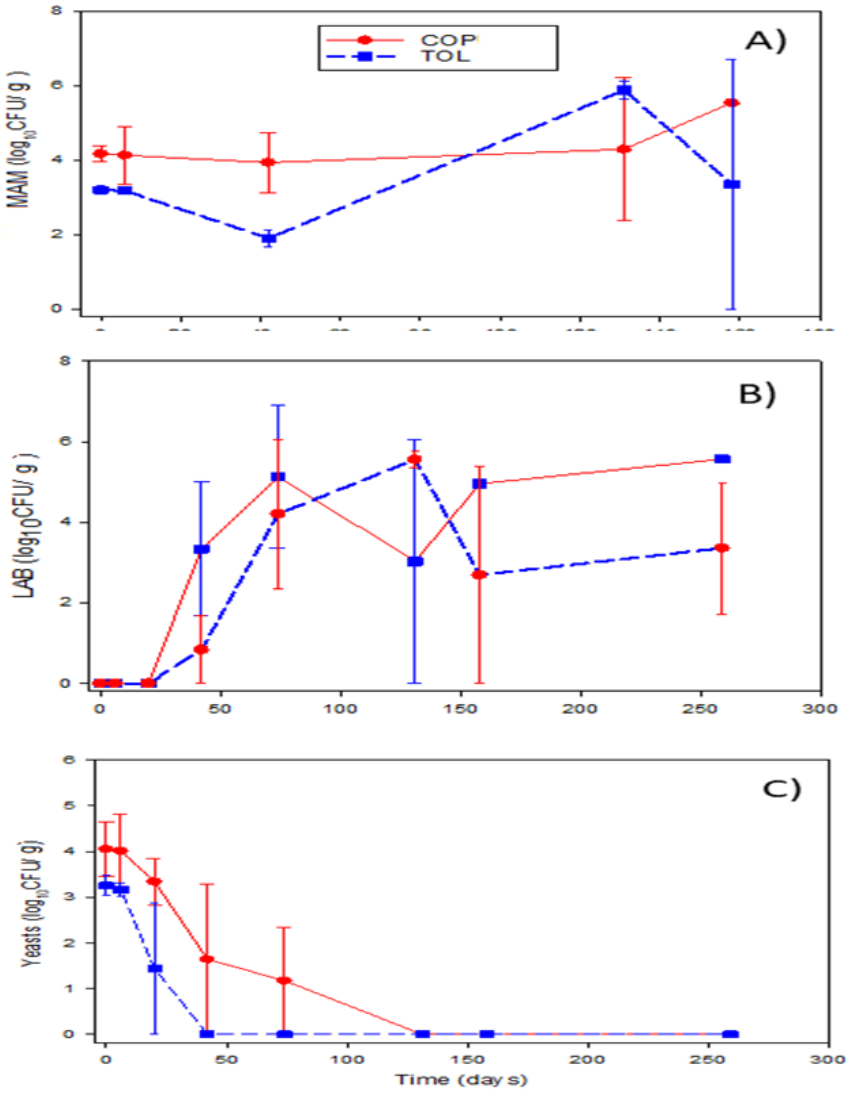

COP
TOL
A)
MAM (log10CFU/g)
B)
LAB (log10CFU/g)
C)
Yeasts (log10CFU/g)
Time (days)

Figure 3

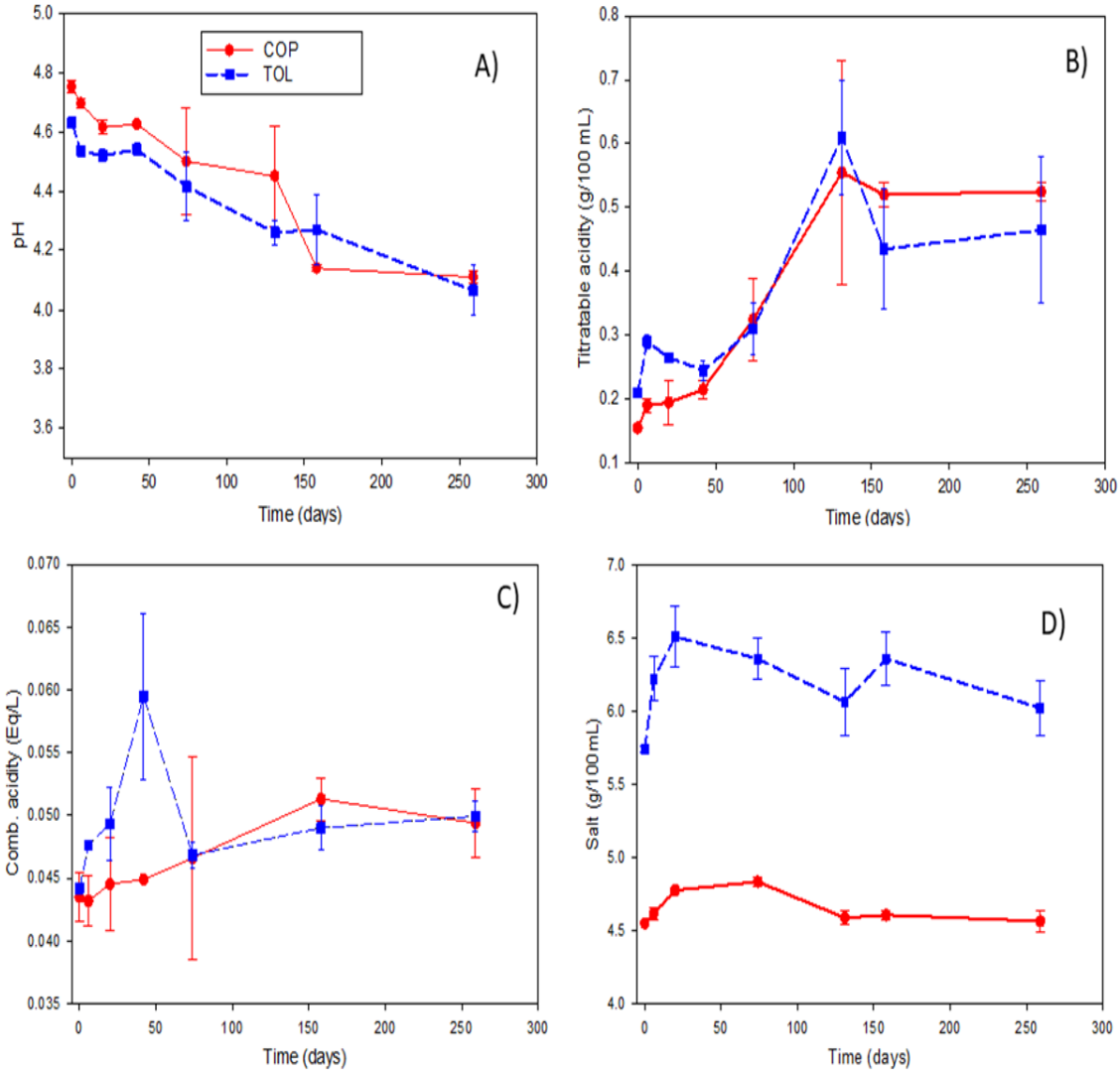

A)
COP
TOL
pH
Time (days)
B)
Titratable acidity (g/100 mL)
Time (days)
C)
Comb. acidity (Eq/L)
Time (days)
D)
Salt (g/100mL)
Time (days)

Figure 4

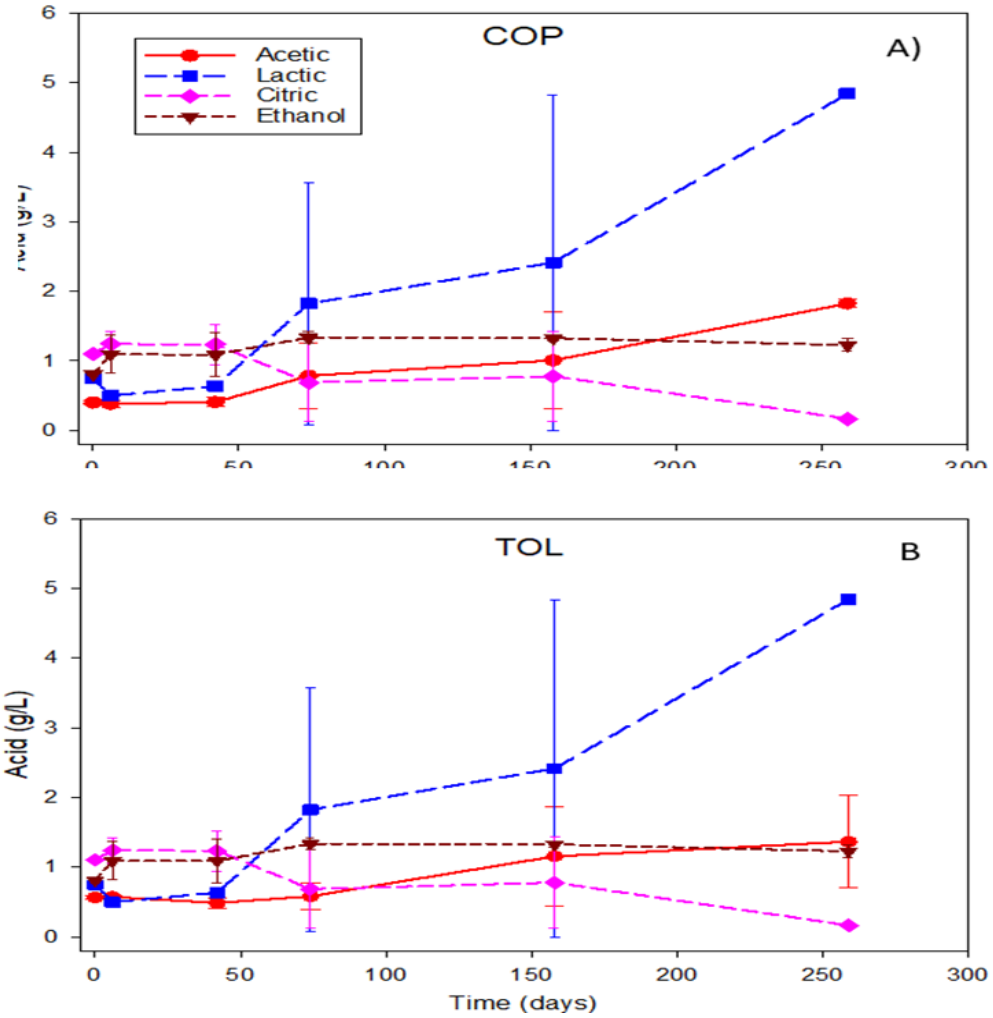

COP
A)
Acetic
Lactic
Citric
Ethanol
TOL
B
Acid (g/L)
Time (days)
0
50
100
150
200
250
300
1
2
3
4
5
6

Figure 5

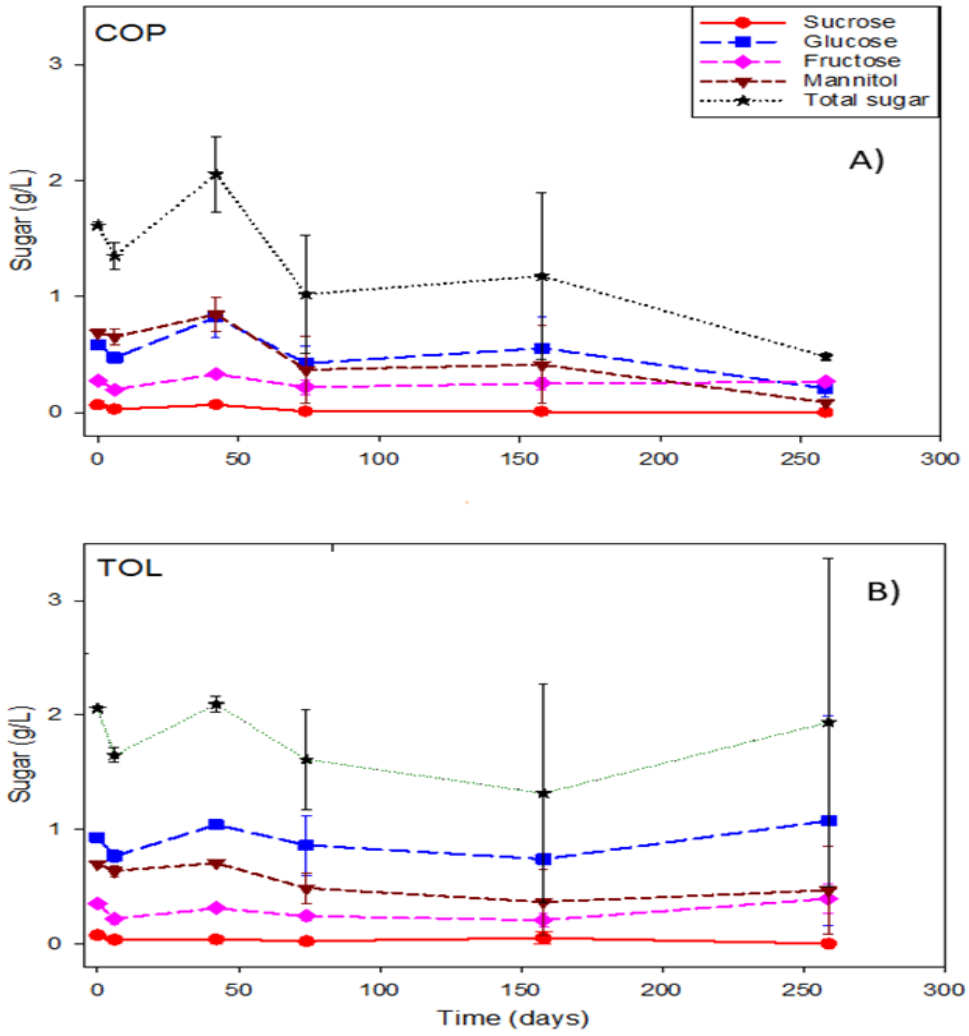

COP
Sucrose
Glucose
Fructose
Mannitol
Total sugar
A)
Sugar (g/L)
0
1
2
3
0
50
100
150
200
250
300
TOL
B)
Sugar (g/L)
Time (days)

Figure 6

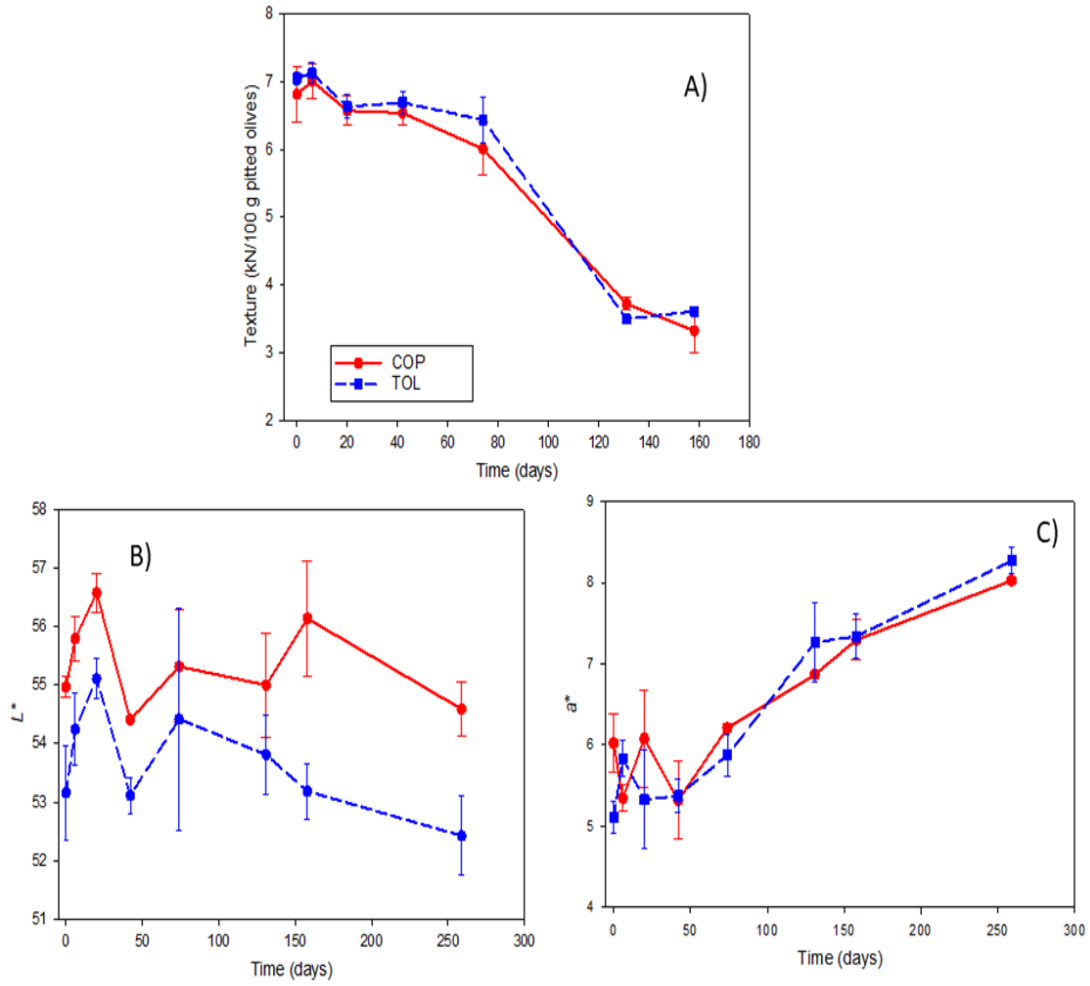

A)
Texture (kN/100 g pitted olives)
Time (days)
COP
TOL
B)
L*
Time (days)
C)
a*
Time (days)

Figure 7

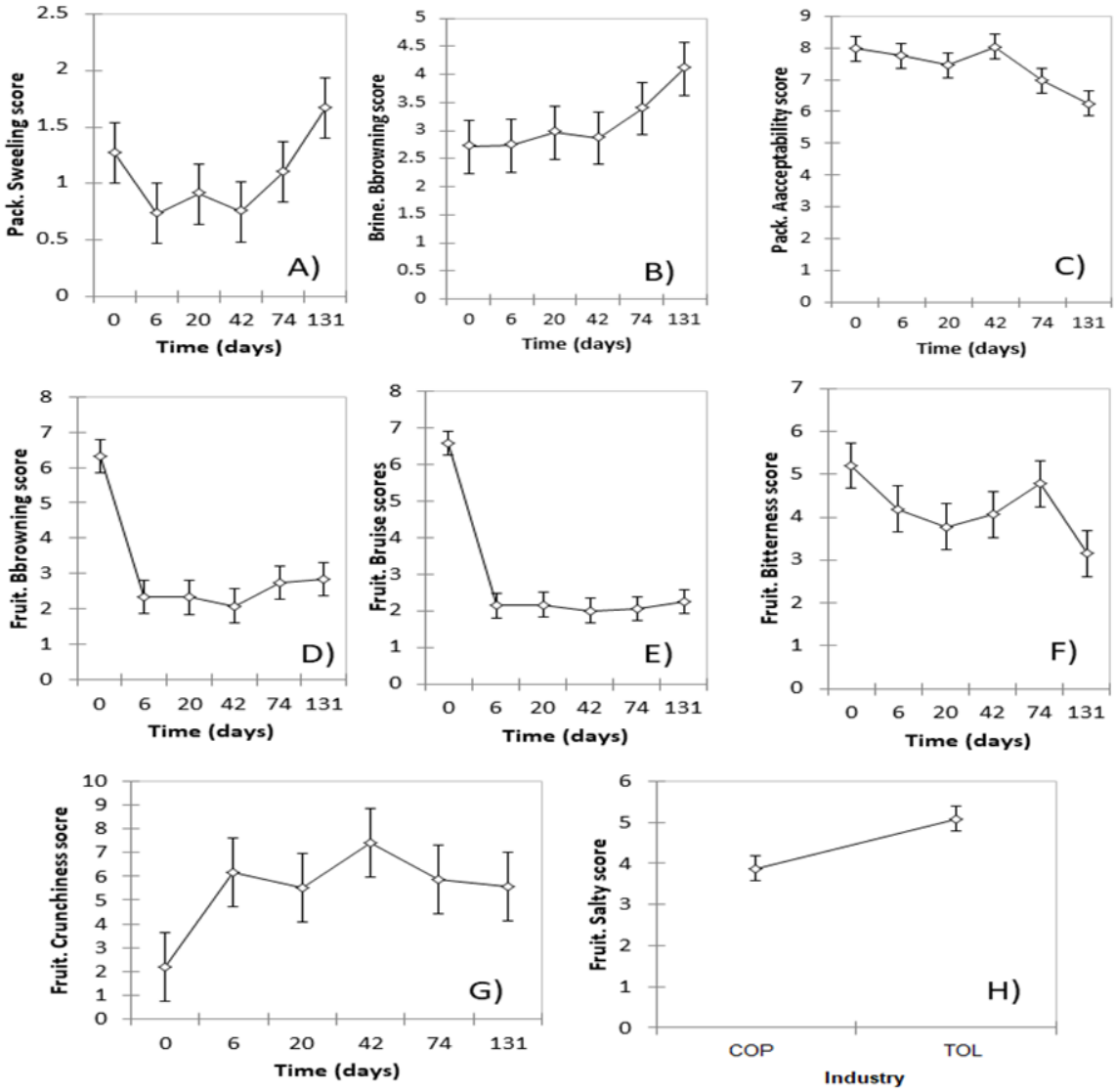

Pack. Sweeling score
A)
Time (days)
Brine. Bbrowning score
B)
Time (days)
Pack. Aacceptability score
C)
Time (days)
Fruit. Bbrowning score
D)
Time (days)
Fruit. Bruise scores
E)
Time (days)
Fruit. Bitterness score
F)
Time (days)
Fruit. Crunchiness socre
G)
Time (days)
Fruit. Salty score
H)
COP
TOL
Industry

Figure 8

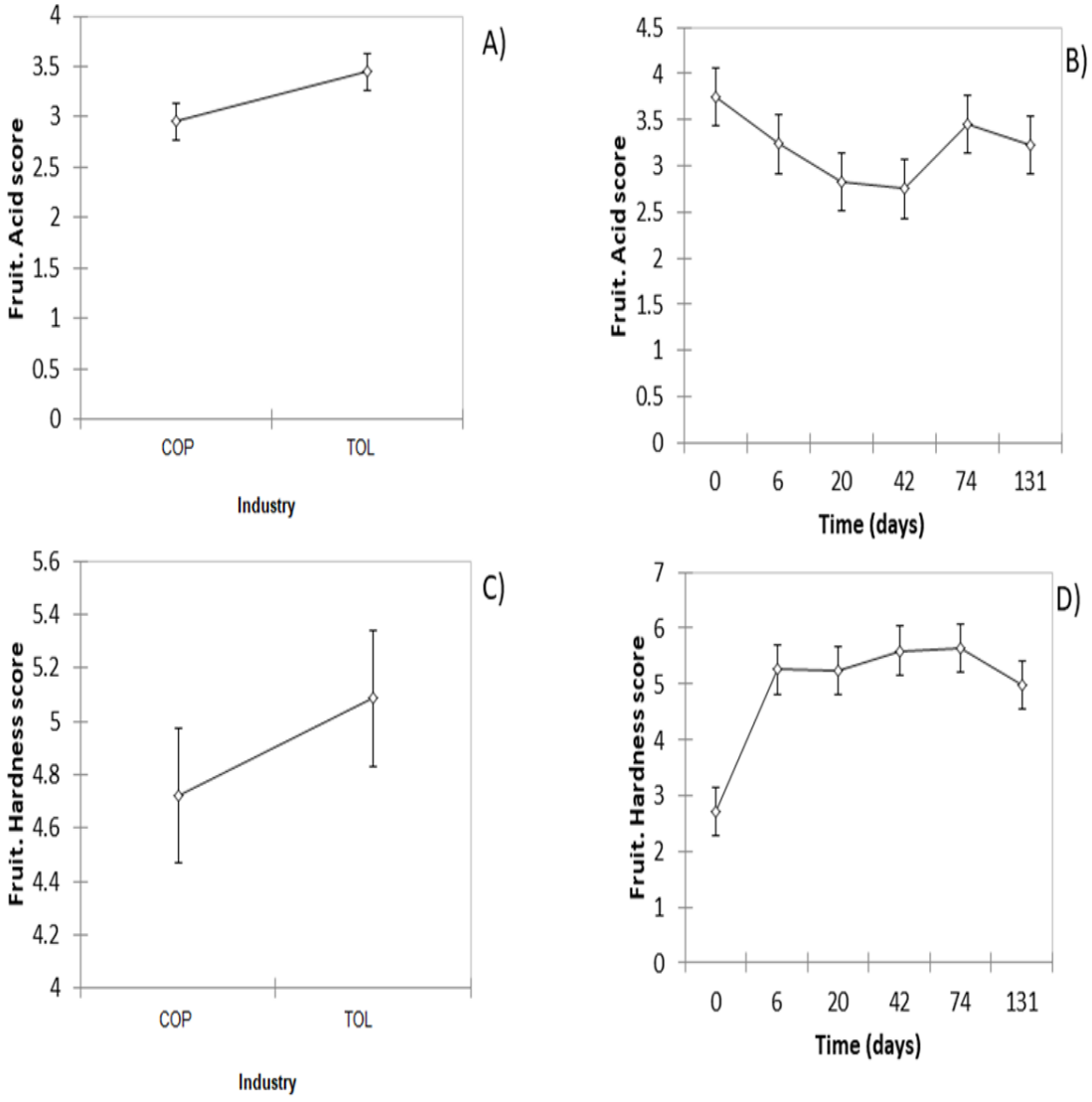
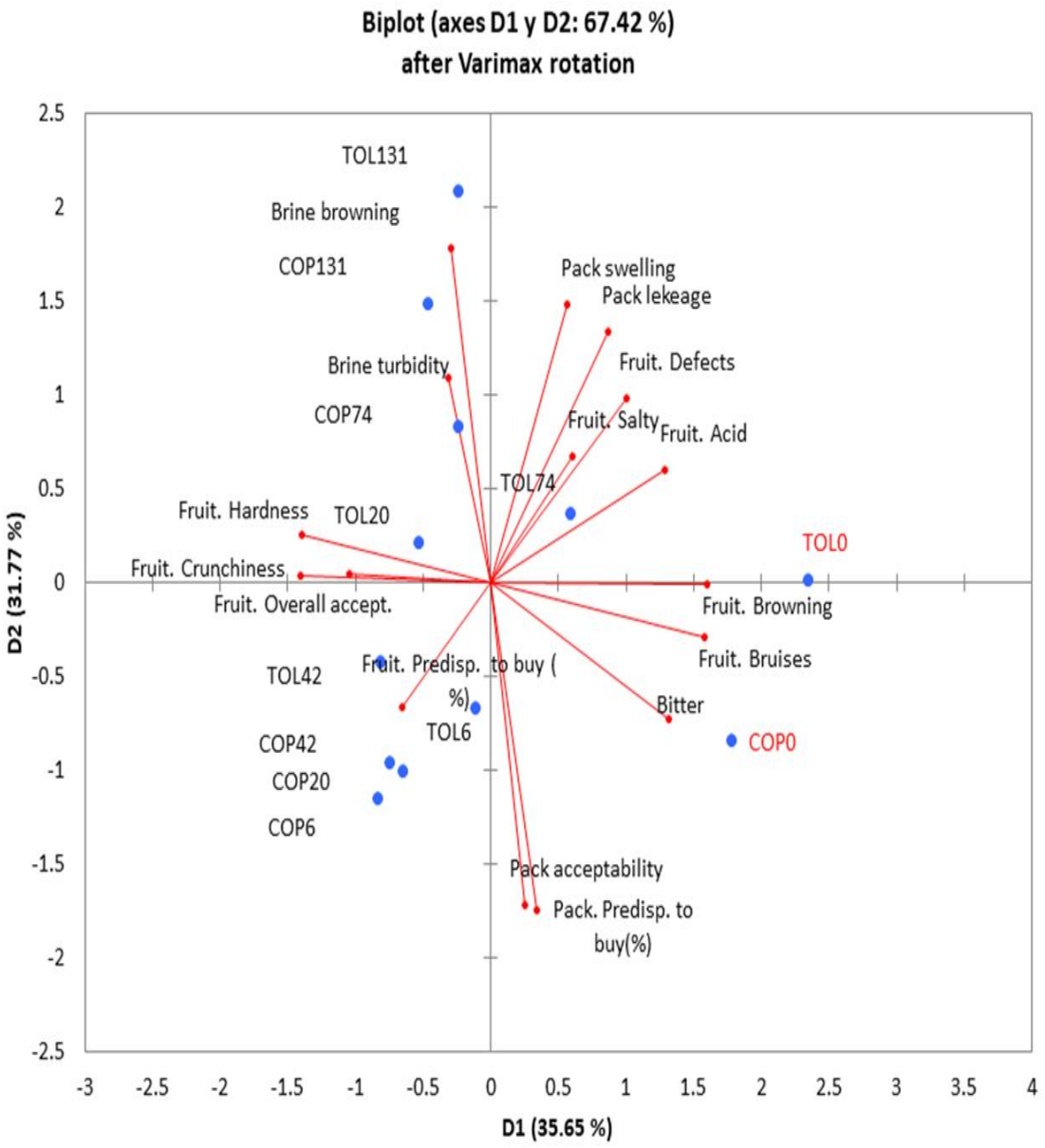

A)
Fruit. Acid score
4
3.5
3
2.5
2
1.5
1
0.5
0
COP
TOL
Industry
B)
Fruit. Acid score
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
6
20
42
74
131
Time (days)
C)
Fruit. Hardness score
5.6
5.4
5.2
5
4.8
4.6
4.4
4.2
4
COP
TOL
Industry
D)
Fruit. Hardness score
7
6
5
4
3
2
1
0
0
6
20
42
74
131
Time (days)

Figure 9


Biplot (axes D1 y D2: 67.42 %)
after Varimax rotation
TOL131
Brine browning
COP131
Brine turbidity
COP74
TOL74
Fruit. Hardness
TOL20
Fruit. Crunchiness
Fruit. Overall accept.
TOL42
COP42
COP20
COP6
Fruit. Predisp. to buy (%)
TOL6
Pack swelling
Pack lekeage
Fruit. Defects
Fruit. Salty
Fruit. Acid
TOL0
Fruit. Browning
Fruit. Bruises
Bitter
COP0
Pack acceptability
Pack. Predisp. to buy(%)
D2 (31.77 %)
D1 (35.65 %)

3.2 SECCIÓN II

3.2.1 CAPÍTULO 3:

Effect of zinc formulations, sodium chloride, and hydroxytyrosol on the growth/no-growth boundaries of table olive related yeasts.

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Effect of zinc formulations, sodium chloride, and hydroxytyrosol on the growth/no-growth boundaries of table olive related yeasts

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ABSTRACT

This study uses a mathematical approach to assessing the inhibitory effect of Zn^{2+} (0–10 mM, obtained from $ZnCl_2$ and $ZnSO_4$) in presence of NaCl (0–8%) and hydroxytyrosol (0–2588 mg/L), on a yeast cocktail formed by species *Pichia galeiformis, Pichia kudriavzevii, Pichia manshurica* and *Candida thaimueangensis* obtained from spoilt green olive packages. The logistic/probabilistic models were built in laboratory medium using a total of 1980 responses (1188 for NaCl and 792 for hydroxytyrosol). $ZnCl_2$ showed significantly higher inhibitory effect than $ZnSO_4$ in the presence of both NaCl ($p < 0.033$) and hydroxytyrosol ($p < 0.009$). NaCl did not interfere the effect of Zn^{2+} while hydroxytyrosol, at high levels, had a slight antagonistic effect. According to models, Zn^{2+} inhibits ($p = 0.01$) the yeast cocktail in the range 4.5–5.0 mM for $ZnCl_2$, or 8.5–9.5 mM for $ZnSO_4$. Therefore, this work confirms the fungicidal activity of zinc compounds (mainly $ZnCl_2$) in synthetic medium, and also shows that the loss of zinc effectiveness in real green Spanish-style olive packaging is not due to the presence of NaCl or hydroxytyrosol, two of the most abundant chemical compounds in the product.

1. Introduction

The production of table olives (nowadays >2.5 million tons/season) represents an important economic resource for producing countries (Spain, Turkey, Egypt, Italy and Greek among the most important). Green Spanish-style constitutes the most popular table olive presentation in the markets (Garrido-Fernández et al., 1997). Lactic acid bacteria (LAB) and yeasts have an important role during processing of this specific type of table olive elaboration (Arroyo-López et al., 2012a; Hurtado et al., 2012). In particular, yeasts can act as desirable microorganisms (due to both technological and probiotic characteristics) but also may cause the product spoilage through production of CO_2, unwanted odors/flavors, the consumption of lactic acid, the softening of fruits, and the clouding of olive brines (Arroyo-López et al., 2012a). These drawbacks are particularly relevant when a residual sugar concentration (or nutrients in general) is present in the packaged product. Usually, product stabilization, in absence of thermal treatment, requires preservatives to control microbial growth. However, the usual chemical additives currently used by industry (sorbic and benzoic acids or their respective salts) may produce diverse negative effects such as brine browning, development of off-flavour or accumulation in the oily phase and other tissue components of fruits (Brenes et al., 2004; Garrido-Fernández et al., 1997). This absorption reduces their level in brines and consequently the efficacy for the product preservation. Sorbic and benzoic acids proved to be effective in laboratory medium to control the growth of a native yeast cocktail isolated from seasoned cracked Manzanilla-Aloreña olives (Arroyo-López et al., 2008). However, in real olive packaging, sorbic contents decreased with time causing progressive product instability (Arroyo-López et al., 2009; Bautista-Gallego et al., 2013). Similar problems can also occur in non-thermally treated partially fermented (cured) green Spanish-style olives (Garrido-Fernández et al., 1997). These circumstances make the investigation of new alternative packaging procedures and preservatives a priority for non-pasteurized table olive presentations.

Microorganisms need zinc (Zn) for the proper functioning of their metabolism due to the element participation as cofactor of diverse enzymes in numerous biological reactions. Nevertheless, above a threshold concentration, it may act as cytotoxic due to its competition with other metal ions for intracellular transport

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proteins or the active sites of enzymes (Walker, 2004). Above such levels, Zn cation could be used as preservative agent in food processing provided its addition be in agreement with the conditions required for the mineral fortification of products. This possibility is supported by previous studies which have shown that $ZnCl_2$, in laboratory medium, has a strong inhibitory effect against a considerable number of table olive related yeasts (Bautista-Gallego et al., 2012). Also, in cracked (directly brined) Aloreña de Málaga table olives, the presence of $ZnCl_2$ led to a more marked reduction in the yeast population during shelf life than the traditional preservatives (Bautista-Gallego et al., 2011). However, the inhibitory effect of $ZnCl_2$ was not observed in green Spanish-style Manzanilla olive packaging (Bautista-Gallego et al., 2015). Apparently, the species of yeasts present in this product could be resistant to Zn, or alternatively, a potential interference of the chemical compounds present in the presentation could be responsible for the activity decrease. NaCl and hydroxytyrosol (Hy) are among the most abundant chemical compounds present in this type of olive preparation (Blekas et al., 2002; Garrido-Fernández et al., 1997; Romero et al., 2004). Disclosing the causes of the different inhibitory behaviour of Zn in laboratory medium and in real green olive packaging has then an unquestionable scientific interest.

In this survey, the yeast population present in green Spanish-style table olive plastic pouches with evidence of spoilage were isolated and molecularly identified. Then, the effect of Zn (from $ZnCl_2$ and ZnSO4), and the possible interference with NaCl and Hy, on a cocktail formed with the most representative isolated species, was evaluated using a probabilistic/logistic model. Predictive microbiology is a valuable tool to describe quantitatively the response of microorganisms as a function of environmental variables or preservatives (McMeekin et al., 1993). By using this mathematical approach, it is possible: i) to determine the sensitivity of native yeasts to the assayed chemical compounds, evaluating the applicability of Zn as a new preservative in olive packaging, ii) to study the potential interference of NaCl and Hy on the inhibitory effect of the diverse Zn formulations, and iii) to estimate the corresponding growth/no growth (G/NG) boundaries of microorganisms.

2. Material and methods

2.1. Yeast isolation, characterization, and identification

A total of 20 yeast isolates were obtained from diverse plastic pouches of commercial Manzanilla green Spanish-style table olives stuffed with red paper paste and with spoilage signs (clouding of brines and swelling). Firstly, yeast isolates were genotypically characterized by RAPD-PCR with primer M13 (Tofalo et al., 2009). The resulting fingerprints were digitally captured and analysed with the BioNumerics 6.6 software package (Applied Maths, Kortrijk, Belgium). The similarity among digitalized profiles was calculated using the Pearson product–moment correlation coefficient. Dendrogram was obtained using the Unweighted Pair Group Method with the Arithmetic Average (UPGMA) clustering algorithm. *Candida boidinii* TOMC-Y5 was used as internal control to determine the reproducibility of the technique. Then, one representative isolate from different clusters was selected for molecular identification, which was achieved using an RFLP analysis of the 5.8S-ITS rDNA region (Esteve-Zarzoso et al., 1999) and the sequencing of D1/D2 domains of the 26S rDNA gene (Kurtzman and Robnett, 1998).

2.2. Yeast cocktail preparation

Yeast cocktail for modelling purposes was prepared using representative isolates obtained from the different clusters of the dendrogram: two isolates (MBY-14 and MBY-19) were ramdoly chosen from different sub-clusters of cluster I, which was the most numerous, one from cluster II (isolate MBY-5) and another from cluster III (isolate MBY-11).

The four mentioned yeast isolates were inoculated separately into 5 mL of a Yeast–Malt–peptone–glucose broth medium (YM, Difco™, Becton and Dickinson Company, Sparks, USA) and incubated at 28 °C for 48 h. Then, tubes were centrifuged at 9000 *g* for 15 min and the pellets re-suspended separately in 5 mL of sterile peptone water (0.1%, wt/vol). To form the yeast cocktail, 3 mL of each culture were mixed at the same proportion to reach a final volume of 12 mL with a population level of approximately 7 $\log_{10}$ CFU/mL, which was confirmed by surface spread on YM agar plates. Therefore, the composition of the cocktail was formed by approximately 25% of each strain (MBY-14, MBY-19, MBY-5 and MBY-11).

2.3. Growth media and experimental design

The basal media selected for all experiments consisted of YM broth set at pH 4.0 by citric acid addition. This value mimics the pH conditions usually found during packaging of Spanish-style green table olives. Subsequently, the media was conditioned with different concentrations of NaCl (Panreac ITW Companies, Castellar del Valles, Barcelona, Spain), $ZnCl_2$ (VWR International, Leuven, Belgium), $ZnSO_4$ (Panreac), and Hy (Extrasynthèse, Genay, France), according to treatments shown in Table 1. All reagents had >99% purity. Hy, $ZnCl_2$ and $ZnSO_4$ levels were prepared from different stock mother solutions (51,768 mg/L for Hy, 200 mM for $ZnCl_2$ and $ZnSO_4$). The Hy content was determined by HPLC at the end of the experiment to assess the stability of the phenolic compound (Romero et al., 2004). The experimental design consisted of 4 full-factorial combinations of the levels of the following couple of chemicals: $ZnCl_2$–NaCl, $ZnSO_4$–NaCl, $ZnCl_2$–Hy, and $ZnSO_4$–Hy. Six replicates of each treatment were run in parallel.

2.4. Optical density measurements

Yeast growth was recorded in a Bioscreen C automated spectrophotometer (Labsystem, Helsinki, Finland) with a wideband filter (420–580 nm) set at 28 °C for 7 days. Measurements were taken every 2 h after a pre-shaking of 10 s to avoid cell sedimentation. The wells of the micro-plates were filled with 0.02 mL of inoculum and 0.35 mL of the basal media (modified according to the experimental design), always reaching an initial optical density (OD) of approximately 0.2 units (initial inoculum level of 2×10^6 CFU/mL). This inoculum level is similar to the populations found in the packaged of Spanish-style and natural green olives (Arroyo-López et al., 2006; López-Lopez et al., 2004) or that used by Lambert and Pearson (2000) or Vermeulen et al. (2007) for the estimation of the G/NG interfaces of LAB describing the influence of pH in acidified sauces. The inoculum was always above the detection limit of the apparatus, which was determined by comparison with a previously established calibration curve (data not shown). Un-inoculated wells for each experimental series were included in the micro-plate to determine, and subtract, the noise signal due to possible chemical oxidation process. For each well, growth (coded as 1) was assumed when the OD increase with respect to the initial OD (after subtraction of the noise signal) was higher than 0.1; no-growth (coded as 0) was recorded when the initial OD remained stable or the increase was <0.1. Responses for each replicate were recorded independently, and the whole matrix was subjected to statistical analysis. After concluding the experiments, randomly selected wells (which included both growth and no-growth

Table 1
Quantitative and dummy variables tested in the present study with their respective levels.

Variable	Units	Type	Levels
NaCl (S)	% (g/100 mL)	Quantitative	0, 1, 2, 3, 4, 5, 6, 7, 8
Hydroxytyrosol (Hy)[a]	mg/L	Quantitative	0, 324, 647, 1294, 1941, 2588
Zinc concentration (Zn)[b]	mM	Quantitative	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10
Type of zinc formulation (T)	Coded	Dummy	0 ($ZnCl_2$); 1 ($ZnSO_4$)

[a] Hy content was quantified by HPLC at the end of the experiment from triplicate measurements.
[b] Zn concentration was expressed as the concentration of the divalent cation [Zn^{++}] obtained from $ZnCl_2$ or $ZnSO_4$ salts.

samples, representing 5% of the total cases) were spread on YM agar plates and their counts were estimated to corroborate G/NG assumption.

2.5. Logistic model development

A logistic regression model links the probability of occurrence of a conditional event (Y), which depends on a vector (x) of explanatory variables. The quantity, $p(x) = E(Y/x)$ represents the conditional mean of y (in this case growth probability) given x (environmental factors) when the logistic distribution is used. It takes the expression:

$$\text{Logit}\,(p) = \text{Ln}[p(x)/(1 - p(x))] = \beta_0 + \ldots + \beta_n x_n,$$

where $\beta_0 + \ldots + \beta_i$ are the intercept and the coefficients of the polynomial function and x_i ($i = 1 \ldots n$) are the environmental variables (Hosmer and Lemeshow, 2000). The model may also include quadratic and interaction terms.

The predicted survival probability as a function of the independent variables, deduced from the logistic regression, is as follows:

$$p(x_i \ldots x_n) = \exp\,(\text{logit}\,(p))/(1 + \exp(\text{logit}(p)))$$

According to the number of independent variables, a surface, two dimensions probability curves (given particular values of the rest), and the G/NG interfaces for selected probabilities can be deduced (Arroyo-López et al., 2012b).

The logistic regression model was fitted to the G/NG data obtained from the diverse treatments (combinations of type of Zn salts with NaCl or Hy at different levels), using XLSTAT v. 2015.4.01.20116 (Addinsoft, Paris, France). The initial models assumed were:

For NaCl: $\text{Logit}(p) = \text{Intercept} + [S] + [Zn] + T + [S]\cdot[Zn] + [S]\cdot T + [Zn]\cdot T + [S]\cdot[Zn]\cdot T$

For Hy: $\text{Logit}(p) = \text{Intercept} + [Hy] + [Zn] + T + [Hy]\cdot[Zn] + [Hy]\cdot T + [Zn]\cdot T + [Hy]\cdot[Zn]\cdot T$

where S and Hy stand for the NaCl (%, g/100 mL) and hydroxytyrosol contents (mg/L), Zn is the concentration of Zn^{++} (mM), and T is the type of Zn salt assayed (introduced as categorical dummy variable, $ZnCl_2$ or $ZnSO_4$). Therefore, in addition to the individual variables, the models included the following two ([S]·[Zn], [Hy]·[Zn], [S]·T, [Hy]·T, [Zn]·T) and three-way ([S]·[Zn]·T, [Hy]·[Zn]·T) interactions.

For the selection of variables, the stepwise backward option, with 0.05 and 0.10 p-values to enter and remove respectively, was used. The number of maximum allowable runs was set to 100 and the tolerance to 0.0001. Validation was achieved with 300 (NaCl) and 400 (Hy) cases, randomly selected and not used for the model building (XLSTAT v. 2015.4.01.20116) (Addinsoft, Paris, France). The use in the model of a dummy variable for the qualitative factor $ZnCl_2$ and $ZnSO_4$ allows a direct statistical comparison between the effects of each Zn salt type.

The log-likehood statistic was used to evaluate the significant contribution of each term in the model to the response (G/NG data). To verify the overall fit, the log-likehood, score, Wald test, overall hit rate, sensitivity and specificity as well as the Cox and Snell, the McFadden, and the Nagelkerke R-squares were used (Hosmer and Lemeshow, 2000).

The interpretation of the model was based on the multiplicative form of logit (p) which establishes that changes in the odds ratio are expressed by exp(b), which means the change in the odds ratio caused by one unit change in the variable of interest when the others remain at the same level. A value of 1 ($e^0 = 1$) indicates that the variable under study does not cause any effect on the odds ratio, values <1 reduce it while values >1 increase it (Hosmer and Lemeshow, 2000). The predicted growth probabilities for the NaCl or Hy and Zn^{2+} combinations and the G/NG boundaries for the diverse probabilities ($p = 0.01$, 0,05 and 0.10) were deduced from the expression $\text{Ln}[p/(1\text{-}p)] = \text{logit}\,(p)$ (Arroyo-López et al., 2012b; Dang et al., 2010).

3. Results

3.1. Characterization and identification of the yeast population isolated from table olive packaging

The dendrogram (Fig. 1), generated from the RAPD-PCR pattern profiles of 20 yeast isolates obtained from the plastic pouches of Manzanilla olives with evidence of spoilage, revealed the presence of three major groups for a cut point of 84.2% (reproducibility of the technique using *C. boidinii* TOMC-Y5 as an internal control). The number of isolates was 15, 2 and 3 for clusters I, II, and III, respectively. Isolates MBY14 and MBY-19 from cluster I (which had different sub-clusters), MBY-5 from cluster II and MBY-11 from cluster III were randomly selected for further identification.

The identification (Table 2), after the RFLP analysis of the 5.8-ITS region and the sequencing of the D1/D2 domains of the 26S rDNA gene of the selected yeast isolates, showed three different banding profiles for the PCR-amplified products of the 5.8S-ITS region, with variations in length ranging from 425 to 500 bp. The profile comparisons of these isolates with the Yeast-id database (www.yeast-id.org) and published papers (Esteve-Zarzoso et al., 1999) allowed identifying unequivocally the yeast isolates as *Pichia galeiformis*/*Pichia manshurica* (MBY-14 and MBY-19), *Pichia kudriavzevii* (MBY-5) and *Candida thaimueangensis* (MBY-11). The study of the sequence alignments of the D1/D2 domains of the 26S rDNA gene of these isolates confirmed the previous identifications based on the restriction analyses, but differed between *P. galeiformis* (MBY-14) and *P. manshurica* (MBY-19). The percentage of identity with previous sequences deposited in the NCBI GenBank data library was always high, ranging from 99 to 100% (see Table 2).

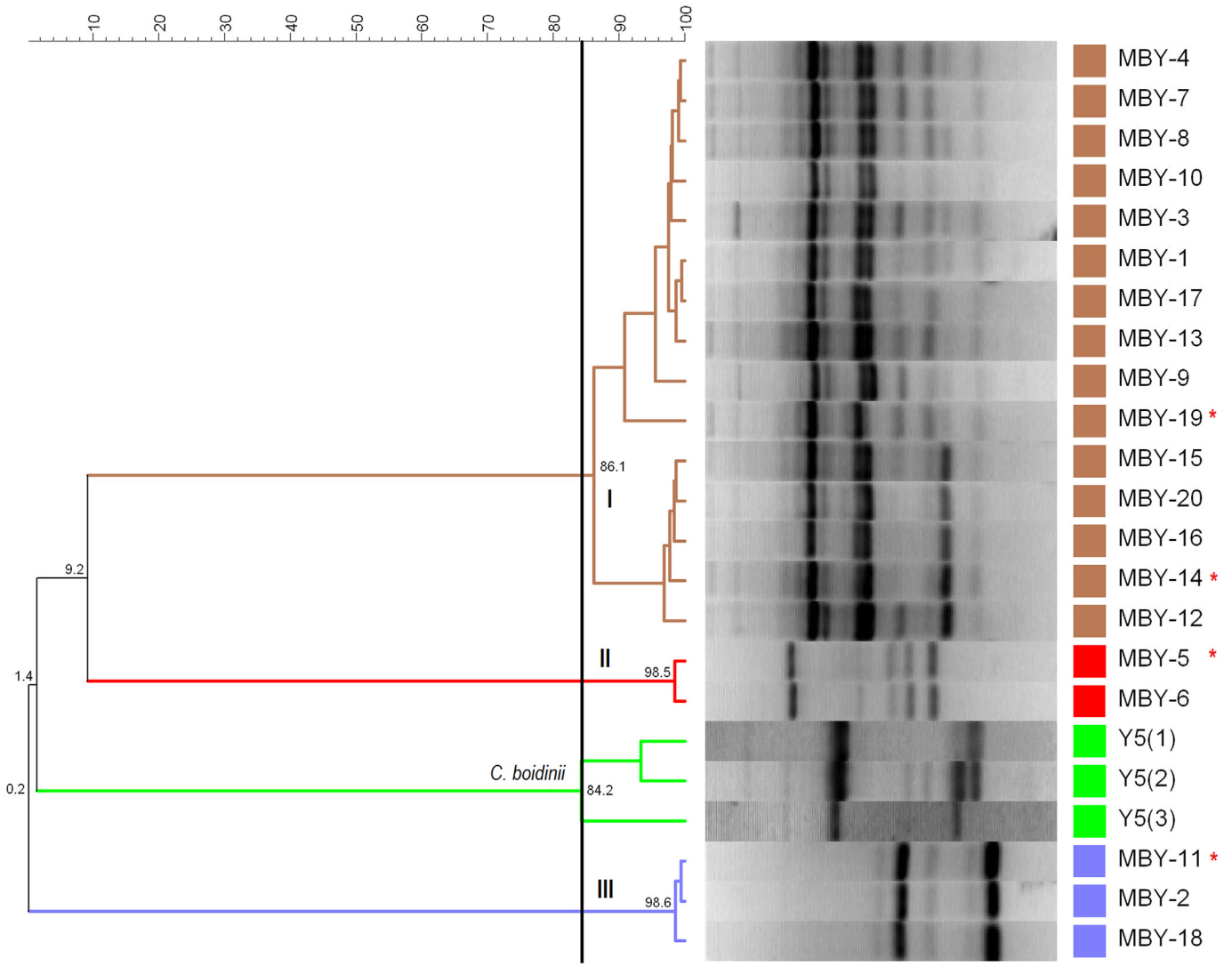

Fig. 1. Dendrogram generated after cluster analysis of the digitalized RAPD-PCR fingerprints with primer M13 of 20 yeast isolates obtained from the commercial packaging of Green Spanish style Manzanilla table olives stuffed with red pepper streams. Isolates selected for further identification are marked with an asterisk while clusters above the reproducibility technique (84.2%) are identified by Roman numbers.

Table 2
RFLP analysis of the 5.8-ITS region and sequencing of the D1/D2 domains of 26 genes for the representative olive yeast isolates obtained from the different clusters of the dendrogram.

Yeast isolate	RFLP 5.8 ITS-region (bp)				Sequencing D1/D2 domains 26S	
	ITS PCR product	*Cfo*I	*Hae*III	*Hinf*I	Identity% (matching nucleotides)	Closest related species
MBY-5	500	200 + 190 + 80	375 + 90	220 + 150 + 70	100% (488/488)	*Pichia kudriavzevii* (AB916580.1)[a]
MBY-11	425	175 + 100 + 90	300 + 125	230 + 200 + 70	100% (531/531)	*Candida thaimueangensis* (FJ432617.1)
MBY-14	450	220 + 100 + 80	325 + 90	220 + 200 + 70	100% (528/528)	*Pichia galeiformis* (KF442633.1)
MBY-19	450	220 + 100 + 80	325 + 90	220 + 200 + 70	99% (426/428)	*Pichia manshurica* (AB916578.1)

[a] Closest accession number found in the NCBI gene bank for the representative olive yeast isolates.

3.2. Logistic models for the yeast cocktail

In the case of NaCl, the total number of cases analysed was 1188 (594 at each level of the dummy variable), with a distribution of G/NG data of 606/582. For the experiments with Hy, the number of cases analysed was 792 (396 at each level of the dummy variable), with a distribution of G/NG data of 440/352. The assessment of the response was based on the OD increase (>0.1) with respect to the initial OD value after subtraction of the noise signal, which practically remained unchanged during all experimental time. Consequently, chemical oxidation processes leading to changes in OD were not noticed. The appropriateness of this assumption was corroborated by spreading randomly selected wells onto YM plates and comparing counts at the end of the experiment with those found after inoculation. For all randomly selected wells, the G/NG assumption was satisfactorily confirmed (data now shown).

The probabilistic models for both NaCl and Hy fitted the data satisfactorily, as upheld by the following statistical tests: McFadden's rho-squared, 0.857 (NaCl) and 0.895 (Hy); Nagelkerke's R^2, 0.927 (NaCl) and 0.948 (Hy); Cox & Snell R^2, 0.695 (NaCl) and 0.709 (Hy). In addition, the −2log(likehood) chi-square was 1050 ($p < 0.0001$) (NaCl) and 606 ($p < 0.0001$) (Hy). The goodness of fit was also assessed by the overall hit (accuracy) to the data used in model development and validation, which indicate an almost perfect segregation between G/NG treatments (Table 3). The sensitivity (true growth rate) was 96% (NaCl) and 98% (Hy) while the specificity (true no-growth rate) was 95% (NaCl) and 99% (Hy). Furthermore, the predictions obtained for 700 validation cases (300 for NaCl and 400 for Hy) led also to high sensitivity (92% for NaCl, 92% for Hy) and specificity (92% for NaCl, 97% for Hy). Therefore, it can be stated that models achieved an adequate segregation between G/NG data and can be considered appropriate for representing the G/NG events as a function of the levels of Zn^{2+}, NaCl, and Hy concentrations, according to the type of Zn formulation. The statistical analysis also allowed the comparison among Zn salt types (dummy variable), which was significant in the NaCl ($\chi^2 = 4.526$, gl = 1, $p = 0.033$) and Hy ($\chi^2 = 6.760$, gl = 1, $p = 0.009$) experiments, with the $ZnCl_2$ being the most efficient formulation.

The reduced models (variables selected by backward stepwise) (Table 4) were chosen because, albeit the improvement in degree of freedom was limited, the full models did not lead to better either goodness of fit or accuracy, sensitivity or specificity. In both models (for NaCl and Hy), $ZnCl_2$ was the reference category (0) for dummy variable. The interpretation of the coefficients in the logit equation can be made in terms of their odds ratio ($e^{\beta x}$) but in models with interactions or dummy variables their meanings are not straightforward. In these cases, the graphical presentation of the functions is more convenient because the relevant effects of variables and interactions are then clearly displayed.

3.3. Graphical representation of the models

The models for logit (p), according to NaCl, Hy, and Zn salt type can be deduced from the retained coefficients (Table 4). For both NaCl and Hy, the equation for growth probabilities in treatments with $ZnCl_2$ is composed of those terms without involving any Zn salt (reference), whilst those with $ZnSO_4$ is constructed by adding to the previous ones those terms including this formulation in parenthesis. Thus, the logit (p) equations would be:

Table 3
The model hits for $ZnCl_2$ and $ZnSO_4$ in the presence of NaCl and Hy.

Samples for model building in presence of NaCl				
From/to	*0*	*1*	Total	% correct
0	416	19	435	95.63%
1	24	429	453	94.70%
Total	440	448	888	95.16%
Samples for model validation				
From\to	*0*	*1*	Total	% correct
0	131	13	144	90.97%
1	11	145	156	92.95%
Total	142	158	300	92.00%
Samples for model building in presence of Hy				
From\to	*0*	*1*	Total	% correct
0	176	5	181	97.24%
1	1	210	211	99.53%
Total	177	215	392	98.47%
Samples for model validation				
From\to	*0*	*1*	Total	% correct
0	170	19	189	89.95%
1	5	206	211	97.63%
Total	175	225	400	94.00%

NaCl vs $ZnCl_2$: $\text{Logit}(p) = 26.184 - 0.268[S] - 6.332[Zn] + 0.061[S]\cdot[Zn]$

NaCl vs $ZnSO_4$: $\text{Logit}(p) = 9.203 + 0.201[S] - 1.661[Zn] - 0.025[S]\cdot[Zn]$

Hy vs $ZnCl_2$: $\text{Logit}(p) = 18.387 - 0.004[Hy] - 5.159[Zn] - 0.001[Hy]\cdot[Zn]$

Hy vs $ZnSO_4$: $\text{Logit}(p) = 33.759 - 0.008[Hy] - 4.580[Zn] + 1.066[Hy]\cdot[Zn]$

The effects of NaCl and Hy on the growth probability (y-axis) of the yeast cocktail in presence of Zn compounds, in a 2D graph, was obtained by using the x-axis for Zn concentrations [Zn^{2+}], and assigning selected values to NaCl (0, 4 and 8%) and Hy (0, 1290 and 2588 mg/L). For this purpose, the equations for the probability as a function of $ZnCl_2$ and $ZnSO_4$ were:

NaCl vs $ZnCl_2$: $p(\text{growth}) = 1/(1 + \exp\text{-}(26.184 - 0.268[NaCl] - 6.332[Zn] + 0.061[NaCl]\cdot[Zn]))$

NaCl vs $ZnSO_4$: $p(\text{growth}) = 1/(1 + \exp\text{-}(9.203 + 0.201[NaCl] - 1.661[Zn] - 0.025[NaCl]\cdot[Zn]))$

Hy vs $ZnCl_2$: $p(\text{growth}) = 1/(1 + \exp\text{-}(18.387 - 0.004[Hy] - 5.159[Zn] - 0.001[Hy]\cdot[Zn]))$

Hy vs $ZnSO_4$: $p(\text{growth}) = 1/(1 + \exp\text{-}(33.759 - 0.008[Hy] - 4.580^*[Zn] + 1.066^*[Hy]\cdot[Zn]))$

Their graphic presentation showed that $ZnCl_2$ had a stronger inhibitory power than $ZnSO_4$, because the probability curves and inhibitory concentrations of the former were associated, regardless of the other components, to lower concentration of Zn (Fig. 2). The increasing concentrations (0, 4, 8%) of NaCl (Fig. 2, panels A and B), had not practical influence on $ZnCl_2$ inhibition because their curves overlapp and was only slightly appreciable on $ZnSO_4$ (curves according to concentrations distinguishable). The interaction of Hy with $ZnCl_2$ (Fig. 2, panel C) was appreciable and more pronounced with $ZnSO_4$ (Fig. 2, panel D); in both cases, the phenolic compound enhanced and decrease inhibition (curves for 0, 1290 and 2588 mg/L Hy displaced) at high and low probabilities, respectively. The range from non-inhibitory (~2 mM Zn) to minimum inhibition concentration (~9.5 mM Zn) of the mixture NaCl·$ZnSO_4$ was quite large and wider than for any other combination. Hence, on the one hand, there are marked differences between the inhibitory effects of the two types of Zn formulations and, on the other, the presence of NaCl and Hy have very slight or low effect, respectively, on the potential Zn inhibitory activity against the cocktail of olive yeasts tested, but both had higher influences when using $ZnSO_4$ as Zn formulation.

Another way to disclose the relationships between Zn salts and NaCl or Hy is by plotting the G/NG interfaces for the respective combinations at different growth probabilities (0.01, 0.05, and 0.10) (Fig. 3). Such interfaces are, in practice, straight lines in the presence of NaCl, situated at lower concentrations (4–5 mM) for $ZnCl_2$ and shifted to higher levels (7–9 mM) for $ZnSO_4$ (Fig. 3). The sharp decrease in growth probability as the $ZnCl_2$ increases is reflected in this graph as a reduced separation between the interfaces for $p = 0.10$, 0.05 and 0.01 (Fig. 3, panel A). On the contrary, the slower progressive inhibition found for the combination $ZnSO_4$ *vs.* NaCl and the slightly more pronounced effect on growth probability were presented as a wider interval between the curves for the diverse probabilities (Fig. 3, panel B). Also, the small effect of NaCl

Table 4
Retained coefficients of the logistic regression and odds ratios for NaCl and Hy models.

Estimated coefficient	Value	SD	χ^2 Wald	p> χ^2	Odds ratio	Lower CL	Upper CL
Model for interaction with NaCl							
Intercept	26.184	7.599	11.874	0.001			
[S]	−0.268	0.103	6.828	0.009	0.765	0.626	0.935
[Zn]	−6.332	1.892	11.200	0.001	0.002	0.000	0.073
($ZnSO_4$)	−16.918	7.982	4.526	0.033	0.000	0.000	0.263
[S]·[Zn]	0.061	0.026	5.744	0.017	1.063	1.011	1.118
[S]·($ZnSO_4$)	0.469	0.132	12.533	0.000	1.598	1.233	2.071
[Zn]·($ZnSO_4$)	4.672	1.935	5.831	0.016	106.944	2.411	4743.631
[S]·[Zn]·($ZnSO_4$)	−0.087	0.029	9.214	0.002	0.917	0.867	0.970
Model for interaction with Hy							
Intercept	18.387	5.811	10.011	0.002			
[Hy]	−0.004	0.002	4.802	0.028	0.996	0.992	1.000
[Zn]	−5.159	1.136	20.606	0.000	0.066	0.001	0.053
($ZnSO_4$)	15.372	5.912	6.670	0.009	47424483.981	43.998	†
[Hy]·[Zn]	0.001	0.001	4.506	0.034	1.001	1.000	1.002
[Hy]·($ZnSO_4$)	−0.004	0.002	4.478	0.034	0.996	0.992	1.002
[Zn]·($ZnSO_4$)	0.579	0.833	0.483	0.487	1.785	0.349	9.134

Notes: Hy, hydroxytyrosol; S, NaCl; SD, standard deviation. † excessively high to be shown.

concentration is reflected as a very slight inclination with respect to the NaCl axis that was a little bit higher in the case of $ZnSO_4$. For the Hy model, the presence of progressive concentrations of this phenolic compound caused greater separation between the probability curves (required more Zn concentrations as p-values decreased) and also displaced the respective curves to the right (Fig. 3, panels C and D), indicating a possibly interference (reduction in the inhibitory effect) of this compound at higher concentrations, regardless of the Zn salt type. Thereby, for a selected growth probability of 0.01, the inhibition of the yeast cocktail at 0–8 g/100 mL NaCl would be obtained with approximately 5.0 mM Zn^{2+} (from $ZnCl_2$), or 8.5 mM Zn^{2+} (from $ZnSO_4$). For the same probability, to get inhibition at 0 mg/L Hy, 4.5 mM ($ZnCl_2$) or 8.5 mM ($ZnSO_4$) of Zn^{2+} are necessary, but at 2500 mg/L Hy, slightly higher concentrations, 5.4 mM ($ZnCl_2$) and 9.5 mM ($ZnSO_4$) of Zn^{2+}, are required.

4. Discussion

There are several studies concerning the molecular identification of the yeasts species present during table olive packaging because their presence during storage may jeopardize the stability of the finished products (Argyri et al., 2015; Arroyo-López et al., 2012a; Panagou, 2004). In this regard, Doulgeraki et al. (2012) detected the presence of *Pichia membranifaciens, Wickerhamomyces anomalus, Pichia fermentans* and *Saccharomyces* sp. in packaged of natural black Conservolea olives, Arroyo-López et al. (2006) identified *Candida diddensiae*, *Candida holmii*, *Saccharomyces cerevisiae*, *Issatchenkia occidentalis*, and *Geotrichum candidum* in seasoned cracked green Manzanilla-Aloreña olives, and Bautista-Gallego et al. (2015) reported the presence of *P. membranifaciens* during packaging of green Spanish-style olives. However, to our knowledge, this is the first time that the species identified in this work (*P. galeiformis, P. manshurica, P. kudriavzevii* and *C. thaimueangensis*) have been related to the packaging of this last table olive elaboration, although their presence is fairly usual during the fermentation process (Arroyo-López et al., 2012a; Lucena-Padrós et al., 2014; Nisiotou et al., 2010; Tofalo et al., 2013).

This survey tries to elucidate the interactions of Zn with diverse components of olive matrix and evaluate the applicability of Zn salts as a new preservative to control the growth of yeasts during table olive packaging, as alternative to classic sorbic and benzoic acids. To this aim, the yeast species previously identified were combined to form a cocktail that mimicked the yeast microbiota in real packages and, therefore, the effects on it were representative of the antifungal activity of the diverse formulations of Zn and their interactions with NaCl and Hy. The use of a microbial cocktail rather than individual species is a convenient and faster way of checking the overall effects that inhibitory compounds could have against a specific microbial group. This way, the G/NG boundaries will be obtained for the most resistant strain of the cocktail. This strategy has been successfully used in food microbiology to estimate the overall response of microorganisms as a function of storage conditions or preservatives (Arroyo-López et al., 2012b; Leong et al., 2014).

Ratkowsky and Ross (1995) were the first researchers modelling the bacterial G/NG interfaces. Years later, Presser et al. (1998) and Lanciotti et al. (2001) used the visible increase of turbidity to deduce the growth limits of *Escherichia coli, Bacillus cereus, Staphylococus aureus* and *Salmonella enteridities*. The application of the logistic/probabilistic models (based on OD) to determine the G/NG boundaries of both bacteria and yeasts, as a function of diverse environmental variables such as temperature, pH, a_w, organic acids or preservatives, is rather habitual in predictive microbiology (Arroyo-López et al., 2012b; Astoreca et al., 2012; Boziaris et al., 2006; Tabanelli et al., 2014; Valero et al., 2010). Also, the use of tubidimetry led to the estimation of accurate and reproducible minimum and non-inhibitory concentrations (Lambert and Pearson, 2000). That is, determination of the G/NG interfaces of spoilage and pathogen microorganisms, based on OD, at selected probabilities levels has become a common practice for establishing conditions to avoid economic losses and food outbreak illness, respectively. Furthermore, the logistic model has also been used for the estimation of the G/NG interface of *Byssochlamys fulva* and *Byssochlamys nivea* fungus over time in terms of colony dimeter (Panagou et al., 2010).

Massana and Baranyi (2000) observed that inoculum levels caused dramatic changes in the probability of growth at the boundary with minor changes in pH and NaCl as well as in the localization of the interface. The use of absorbance data (*vs.* viable counts) for the estimation of some kinetic parameters (specific growth and lag times) led to some differences when using Exponential, Gompertz and Logistic models but accurate estimations were obtained from individual absorbance growth curves when applying other equations (Dalgaard and Koutsoumanis, 2001). The effect of inoculum size (and the acid adaptation) on the G/NG interfaces of *E. coli* O157:H7 and *Listeria monocytogenes* was modelled by Skandamis et al. (2007) and Koutsoumanis and Sofos (2005). It

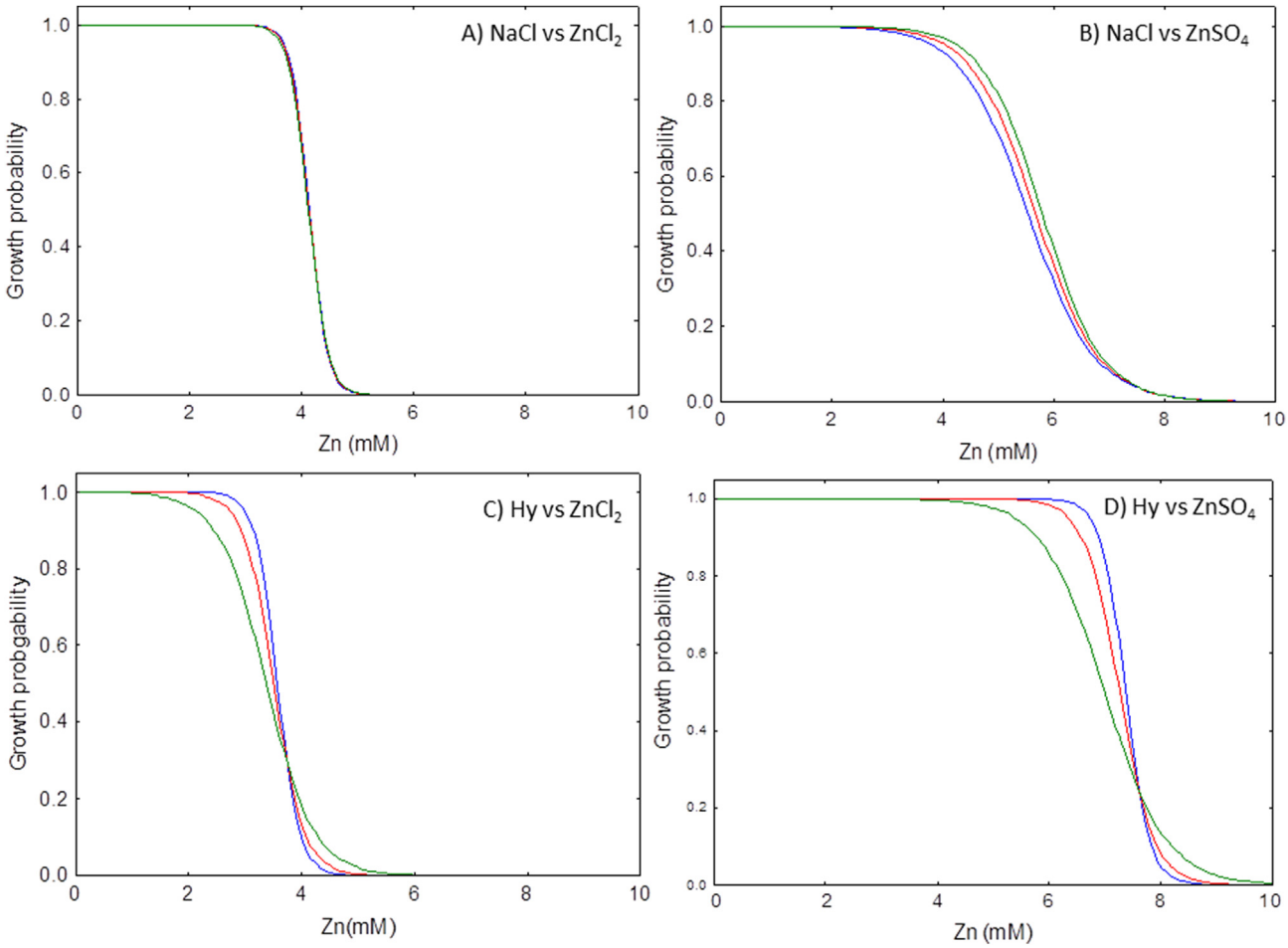

Fig. 2. Growth probability of the yeast cocktail as a function of the Zn concentration (mM), according to Zn salt type ($ZnCl_2$ or $ZnSO_4$), at selected NaCl (0 blue, 4% red, and 8% green curves) and Hy (0 blue, 1290 red, and 2588 green curves) concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

has been demonstrated that such effect is directly related to the microbial number and not due to other suggested phenomenom (Bidlas et al., 2008). Usually, the higher the initial inoculum the lower (more stric conditions) were the minimum pH and a_w values permiting growth (Skandamis et al., 2007). The inocum size used in this work was always similar and around the highest values found in packaged olives (Arroyo-López et al., 2006; López-Lopez et al., 2004). Therefore, the assays would led to the estimation of the stricter conditions for the yeast cocktail inhibition in the synthetic medium and might provide the apparent safer packaging conditions in the presence of NaCl and Hy. To our knowledge, this is the first probabilistic model built for diverse Zn formulations in presence of NaCl and Hy using yeasts as target microorganisms.

Hy is a potent antioxidant found in olive products. Polymerization of Hy (and caffeic acid) is considered responsible for the formation of the black colour of ripe olives (Brenes et al., 1992) and the browning caused by mechanical harvesting in green olives (Segovia Bravo et al., 2009). Apparently, this phenolic compound could be oxidised in an aqueous medium when present at low concentrations; however, its stability increases considerably at concentrations higher than 25 mg/L (Zafra-Gómez et al., 2011). In olive brines, the concentration of Hy during fermentation remained high and fairly stable due to the low pH (4.0) prevailing during processing (Arroyo-López et al., 2007; Brenes et al., 1995; Charoemprasert and Mitchell, 2012). Also, the use of non-inoculated wells as internal controls containing Hy in combination with NaCl and Zn, as well as the determination of the Hy content by HPLC at the end of the experiment, confirm the high stability of this compound under the assayed conditions. This phenolic compound has antimicrobial activity against bacteria (Cicerale et al., 2012; Ruiz-Barba et al., 1993) and yeasts (Zoric et al., 2013). The dialdehydic form of decarboxymethyl elenolic acid linked to Hy has the strongest antimicrobial activities among the inhibitory compounds found in table olives (Medina et al., 2007). However, in this work a concentration around 2500 mg/L was not enough to inhibit the growth of olive yeasts in synthetic media, which is in agreement with the results found by Medina et al. (2007) who did not find an inhibitory effect of Hy against the species *S. cerevisiae*.

Dang et al. (2010) reported abrupt changes of growth probabilities around the transition zones (between the G/NG regions), indicating that minor variations in environmental conditions near de G/NG boundaries can cause a significant impact. A similar situation has been found in other publications with strong inhibitory compounds like sorbate (Arroyo-López et al., 2008). These abrupt changes are also observed when estimating the NIC and MIC concentrations of preservatives (Lambert and Pearson, 2000). In this work, this effect was observed with $ZnCl_2$ in presence of NaCl, but the changes were slightly more gradual for $ZnSO_4$ and in the presence of Hy. Apparently, there is a critical threshold above which

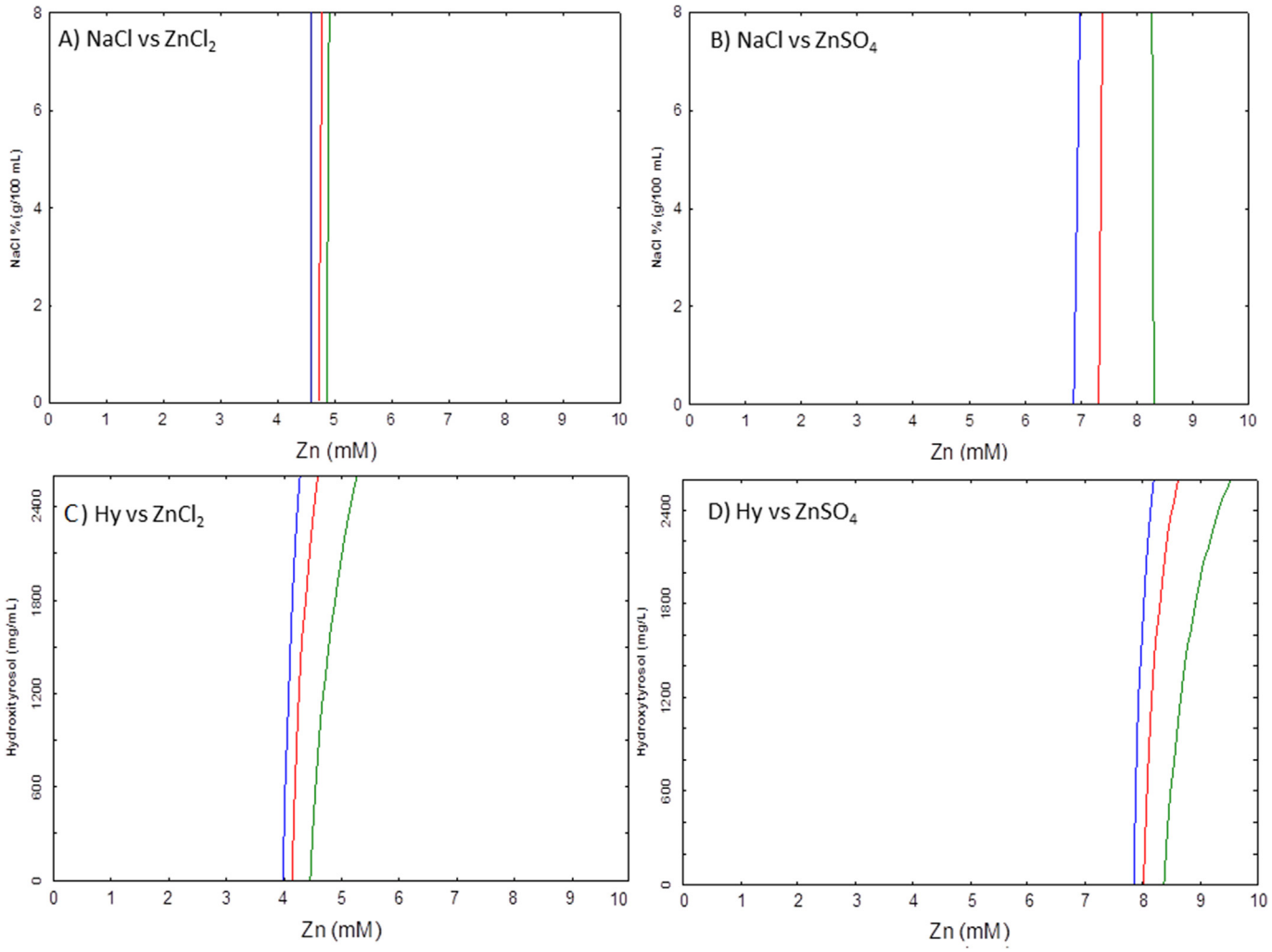

Fig. 3. Yeast cocktail growth/no-growth boundaries (bi-dimensional graph) as a function of the Zn concentration (mM) (obtained from $ZnCl_2$ or $ZnSO_4$), in the presence of NaCl or Hy, according to selected probabilities ($p = 0.01$ green, 0.05 red and 0.10 blue curves). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the suvival of the microorganism is seriously compromised and the precise estimations of low growth probabilities would require detailed studies around the interfaces.

There are only a few quantitative studies devoted to determine the resistance of microorganisms to diverse Zn levels and the interference of other compounds on this activity. Babich and Stotzky (1978) studied the toxicity of Zn^{2+} and its interaction with progressive NaCl concentrations with various fungi, bacteria and coliphage species. These authors found that a 10 mM concentration of Zn^{2+} completely inhibited the mycelial growth of *Rhizoctonia solani* and reduced the growth of *Fusarium solani, Cunninghamella echinulata, Aspergillus niger* and *Trichoderma viride*. They also reported that Zn toxicity for diverse microorganisms decreased with increasing concentrations of NaCl. The decline was attributed to a probable reduction in the levels of Zn^{2+} caused by the formation of Zn–Cl species, which could be less effective towards microbes than the free divalent form. Moreover, *A. niger* tolerated higher concentrations of Zn in the presence of NaCl at 37 °C than at 25 °C (Babich and Stotzky, 1978). However, this effect was not observed in the present study. In general, the response of yeasts to increasing $ZnCl_2$ concentrations is variable, even within strains of the same species (Bautista-Gallego et al., 2012). Walker (2004) already reported that diverse yeast strains could have different metal requirements. Furthermore, the degree of toxicity depends on the metal, its concentration, bioavailability (related to Zn formulation), and strain.

The use of Zn acetate, chloride, citrate, gluconate, lactate, oxide, carbonate, and sulphate are permitted in the European Union to fortify foods by the Directive 2002/46/CE. In the USA, the same compounds are allowed and are considered as GRASS (ODS, 2011). Therfore, the addition of Zn salts to table olives is supported by the current food legislation. A concentration of approximately 2 mM of Zn^{2+} (formulated as $ZnCl_2$) inhibited, in a laboratory medium, the yeast growth of 22 yeast strains of the genera *Saccharomyces, Wickerhamomyces, Debaryomyces, Issatchenkia, Candida, Pichia, Kluyveromyces* and *Torulaspora*, all of them isolated from diverse table olive processing; furthermore, concentrations above 3.6 mM of Zn produced a clear reduction in the initial inoculum population (Bautista-Gallego et al., 2012). Thus, such $ZnCl_2$ levels could be used in table olive, and other fermented vegetable, packagings to improve the preservation (by inhibiting the yeast micro-flora) and, simultaneously, lead to healthy products fortified in Zn. However, in real table olive packaging, the inhibitory effect of Zn was not as efficient as expected from *in vitro* experiments. In this way, Zn^{2+} concentrations of up to 7.3 mM (formulated as $ZnCl_2$) did not guarantee the complete inhibition of yeast growth in cracked table

olive packaging, but caused a significant reduction with respect to the usual packaging conditions (0.12 g/100 mL potassium sorbate) (Bautista-Gallego et al., 2011). Olives packed with 5.5 mM of Zn^{2+} (from $ZnCl_2$) showed an improved sensory profile as determined by panel test (Bautista-Gallego et al., 2011). In all these studies, a final concentration of 10–20 mg of Zn per 100 g of olive flesh was obtained. These results show that validation studies should be carried out in real food matrixes because Zn could have unexpected behaviours or be trapped by diverse food components or chemicals (additives) used in packaging (Nicola et al., 2009; Walker, 2004).

5. Conclusions

This study has confirmed, using a logistic/probabilistic model, that Zn has a strong *in vitro* inhibitory action (mainly $ZnCl_2$) against yeasts isolated from olive packaging. The G/NG interfaces obtained in the present study show that at Zn concentration of about 5.0 mM, and the NaCl and Hy levels normally found in table olive packaging, should inhibit yeast growth when formulated as $ZnCl_2$, although the effect may not be reached in practice (Bautista-Gallego et al., 2015). The models show that the presence of NaCl and Hy in these products are not resposible for this Zn inhibitory effect lessening. Therefore, other conditions inherent to this presentation or compounds present in olive packaging may interfere with the expected Zn effect.

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3.2.2 CAPÍTULO 4:

Susceptibility and resistance of lactic acid bacteria and yeasts against preservatives with potential application in table olives.

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Susceptibility and resistance of lactic acid bacteria and yeasts against preservatives with potential application in table olives

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ABSTRACT

In the present study, a dose-response model was used to investigate the susceptibility (NIC) and resistance (MIC) of the lactic acid bacteria and yeast populations with respect to five chemical preservatives (fumaric and pyruvic acids, cinnamaldehyde, sodium metabisulphite and natamycin) with potential application in table olives. Results were compared with respect to potassium sorbate, a well-known preservative habitually used in olive packaging. Sodium metabisulphite was the most efficient preservative to control lactic acid bacteria growth (MIC, 50 ppm), followed by cinnamaldehyde (1060 ppm) while pyruvic acid required higher concentrations (3211 ppm). Natamycin (25 ppm) was highly efficient against yeasts, followed by cinnamaldehyde (125 ppm), potassium sorbate (553 ppm), sodium metabisulphite (772 ppm) and pyruvic acid (3038 ppm). Fumaric acid, in the range assayed (0–2000 ppm), did not show any inhibitory effect against these two microbial groups. This survey presents for the first time a comparative study of the efficiency of potential preservatives to control the growth of table olive related microorganisms. Further studies should be performed to validate their effects and interactions in the food matrix.

1. Introduction

Worldwide table olive production reached 2,595,500 tons in 2014/2015 season (IOC, 2015). The elaboration of this fermented food is mainly related to the Mediterranean basin, but there are also important production regions in Australia, South-America and USA. The most popular processing styles are: i) green Spanish-style (olives debittered by alkaline treatment), ii) natural (directly brined) olives, and iii) Californian style (olives darkened by oxidation in an alkaline medium) (Garrido-Fernández et al., 1997).

Yeasts (mainly from *Saccharomyces*, *Candida*, *Debaryomyces* and *Pichia* genera), and lactic acid bacteria (LAB) (belonging especially to *Lactobacillus* genera) have an essential role during processing of table olives determining quality, flavour and safety of final products. Both microbial groups can coexist during fermentation and they are responsible for diverse favourable effects such as sugar consumption, production of lactic acid, bacteriocins, killer factors and desirable volatile compounds, among others (Arroyo-López et al., 2012a; Hurtado et al., 2012). However, their uncontrolled presence during packaging may cause product spoilage due to the production of CO_2, swollen containers, softening of fruits, and clouding of brines. Hence, the microbiological stabilization of the final products during the commercialization period is critical.

Due to its high pH (close to neutrality), ripe olives require sterilization while Spanish-style and natural olives are fermented products that may be preserved by different methods (physico-chemical characteristics, modified atmosphere, vacuum or pasteurization) (Garrido-Fernández et al., 1997). However, the thermal treatments may cause undesirable changes in the traditional flavour of several presentations, particularly seasoned (alkali treated or natural) olives which, thus, should be stabilized by the use of preservatives (Arroyo-López et al., 2009). Currently, the only two preservatives permitted in table olives, according to the Table Standard Applying to Table Olives (IOC, 2004) are benzoic and sorbic acids (or their respective salts) at maximum doses of 1000 ppm (wt/wt flesh) for benzoic and 500 ppm for sorbic acid, or 1000 ppm for their combination. However, these chemical compounds have some drawbacks such as i) accumulation in the olive (flesh) fat, with the subsequent limitation of their effects in the brines, ii) development of undesirable sensorial notes for

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consumers, iii) browning of fruits, and iv) degradation by microorganisms (Garrido-Fernández et al., 1997; Arroyo-López et al., 2005). As a result, the table olive sector is demanding research for obtaining more appropriate preservatives.

Predictive microbiology uses mathematical models to describe quantitatively the response of microorganisms as a function of environmental variables or preservatives (McMeekin et al., 1993). One of the most common methods used for the estimation of the effect of an inhibitory compound is the calculus of its NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration) values with a progressive inhibitory effect as the concentrations move from the NIC to the MIC. As shorter is the range between both points, the stronger is the inhibitory effect (Lambert, 2001; Chorianopoulos et al., 2006). The method developed can be easily automatized using optical density (OD) measurements. This technique has been used for testing the growth response of *Salmonella typhimurium* in the presence of natural and synthetic antimicrobials (Guillier et al., 2007), the effect of lemon extract on foodborne microorganisms (Conte et al., 2007) or the antifungal activity of fatty acids and their monoglycerides against *Fusarium* spp. in a laboratory medium (Altieri et al., 2009). In table olives, the same methodology has been used to study the effects of diverse chloride salts on *Lactobacillus pentosus* and *Saccharomyces cerevisiae* growth (Bautista-Gallego et al., 2008), modelling the inhibitory effect of $ZnCl_2$ on table olive related yeasts (Bautista-Gallego et al., 2012), or testing the effect of salt (NaCl) on table olive related microorganisms (Romero Gil et al., 2013; Bonatsou et al., 2015). Hence, this technique has been widely used and validated to investigate the efficiency of diverse compounds for controlling the microorganisms involved in table olive packaging.

In the present survey, we use statistical modelling techniques (dose-response model) to quantify the individual effects of five chemical compounds (fumaric and pyruvic acids, sodium metabisulphite, natamycin and cinnamaldehyde) to prevent the growth of yeasts and LAB species related to table olive packaging. Results were compared with those obtained for potassium sorbate, a preservative habitually used for the stabilization of packaged olives. Data obtained could provide clues for producing safer and more stable olive presentations when thermal treatments are non-viable. Also, it may also be helpful for supporting possible changes in their legal status in table olives.

2. Material and methods

2.1. Microorganisms and cocktail preparation

A total of 10 LAB and 8 yeast strains, representing the yeast and LAB species usually found in table olive processing, were used in the present study (Table 1). All of them were previously identified by molecular methods (data not shown) and belong to the Table Olive Microorganisms Collection (TOMC) of Instituto de la Grasa (CSIC, Seville). The use of a microbial cocktail instead individual species is a convenient and faster way of checking the overall susceptibility/sensibility that a particular compound could have against a specific microbial group. This way, the NIC and MIC values will be obtained for the most resistant species or strain of the cocktail. This strategy has been successfully used in food microbiology to estimate the overall response of the yeast and bacteria populations as a function of storage conditions or preservatives (Arroyo-López et al., 2012b; Leong et al., 2014). Inoculum were prepared by inoculating one single colony of each strain into 5 mL of a YM broth medium (Difco™, Becton and Dickinson Company, Sparks, USA) for yeasts; or 5 mL of a MRS broth medium (de Man, Rogosa and Sharpe) (Oxoid, Cambridge, UK) for LAB. After 48 h of incubation at 30 °C, 1 mL from each tube was centrifuged at 9000× *g* for 10 min, the pellets were washed with sterile saline solution (9 g/L), centrifuged and re-suspended again in 0.5 mL of a sterile saline solution to obtain a concentration of about 7 $\log_{10}$ CFU/mL for yeasts and 8 $\log_{10}$ CFU/mL in the case of LAB, which was confirmed by surface spread on appropriate media. These microorganism suspensions were mixed and the same proportions, obtaining one cocktail for yeasts and other for LAB, and then used to inoculate the different experiments as described below.

Table 1
Yeasts and lactic acid bacteria species and strains used to prepare the microbial cocktails.

Microbial cocktail	Strains
LAB	*Lactobacillus pentosus TOMC-LAB2*
	Lactobacillus pentosus TOMC-LAB3
	Lactobacillus pentosus TOMC-LAB4
	Lactobacillus pentosus TOMC-LAB5
	Lactobacillus pentosus TOMC-LAB6
	Lactobacillus plantarum TOMC-LAB8
	Lactobacillus plantarum TOMC-LAB9
	Lactobacillus paraplantarum 271
	Pediococcus pentosaceus E11
	Pediococcus pentosaceus P56
Yeasts	*Candida diddensiae TOMC-Y1*
	Issatchenkia occidentalis TOMC-Y3
	Saccharomyces cerevisiae TOMC-Y4
	Debaryomyces hansenii TOMC-Y25
	Pichia membranifaciens TOMC-Y31
	Candida boidinii TOMC-Y47
	Candida tropicalis TOMC-Y72
	Lodderomyces elonsgisporus TOMC-Y73

2.2. Modelling the inhibitory effects of preservatives

Growth was monitored in a Bioscreen C automated spectrophotometer (Labsystem, Helsinki, Finland) with a wideband filter (420–580 nm). Measurements were taken every 2 h after a pre-shaking of 5 s for 7 days. The wells of the microplate were filled with 20 μL of inoculum and 330 μL of medium (according to treatment as described below), always reaching an initial OD of approximately 0.2 (inoculum level above 6 $\log_{10}$ CFU/mL). The inocula were always above the detection limit of the apparatus, which was determined by comparison with a previously established calibration curve. Uninoculated wells for each experimental series were also included in the microplate to determine, and consequently subtract, the noise signal.

Sterilized YM or MRS broth were modified with 5% NaCl and adjusted to pH 4.0 by citric acid addition (mother stock solution 30%) to mimic industrial packaging conditions. Based in our experience and bibliography, this pH level is usually found in real table olive packaging (Arroyo-López et al., 2009; Blana et al., 2016), and therefore, appropriate for a first selection of the preservatives with the highest inhibitory effects. The basal media were supplemented with the different chemical compounds and concentrations shown in Table 2. The use of a well-known, standardized synthetic laboratory medium to carry out the experiments was preferred because, in the olive matrix, the presence of diverse components released by fruits such as polyphenols, organic acids, etc., may mask the real inhibitory effect of preservatives.

The basis of the technique used for estimating the NIC and MIC values of the assayed microbial cocktails for preservatives was the comparison of the area under the OD/time curve of a positive control (absence of preservative, optimal conditions) with the areas of the tests (presence of preservative, increasing inhibitory conditions). As the amount of inhibitor in the well increases, the effect on

Table 2
Type of preservatives and concentrations (ppm) assayed in the present study for the modification of basal YM (yeasts) and MRS (lactic acid bacteria) broth laboratory medium.

Preservatives	Concentrations (ppm)
Pyruvic acid	0, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000
Fumaric acid	0, 10, 25, 50, 75, 100, 150, 250, 500, 1000, 1500, 2000
Sodium metabisulphite	0, 10, 25, 50, 75, 100, 150, 250, 500, 1000, 1500, 2000
Potassium sorbate	0, 10, 25, 50, 75, 100, 150, 250, 500, 1000, 1500, 2000
Natamycin	0, 2, 4, 6, 8, 10, 12, 16, 18, 20, 25, 30
Cinnamaldehyde	0, 20, 50, 100, 150, 200, 250, 500, 750, 1000, 1250, 1500

the growth of the organism also increases. This effect on growth is manifested by a reduction in the area under the OD/time curve relative to the positive control at any specified time. The areas under the OD/time curves were calculated by integration using OriginPro 7.5 software (OriginLab Corporation, Northampton, USA). The relative amount of growth for each preservative concentration, denoted as the fractional area (*Fa*), was obtained using the ratios of the test area ($area_{test}$) to that of the positive control of the microbial cocktails ($area_{cont}$), according to the following formula:

$$Fa = (area_{test})/(area_{cont})$$

The plot of the *Fa* versus the natural logarithm (ln) of the preservative concentration produced a sigmoid-shape curve that could be well-fitted with a reparameterized modified Gompertz function for decay (Bonatsou et al., 2015), which had the following expression:

$$y = \exp\Big(- \Big(x \Big/ (ln(MIC)\Big/\exp(- (ln(ln(NIC)/ln(MIC))/2.71828)))\Big)\hat{}(- 2.71828/(ln(ln(NIC)/ln(MIC)))))$$

where y is the dependent variable (*Fa*), x is the independent variable (ln preservative concentration, ppm), MIC is the minimum preservative concentration (ppm) above which growth is not observed, and NIC is the preservative concentration (ppm) above which an inhibitory effect begin to be observed. These parameters were obtained by non-linear regression procedure, minimizing the sum of squares of the difference between the experimental data and the fitted model, i.e., loss function (observed-predicted)^2. This task was accomplished using the non-linear module of the Statistica 7.1 software package (StatSoft Inc, Tulsa, OK, USA) and its Quasi-Newton option. Fit adequacy was checked by the proportion of variance explained by the model (R^2) with respect to the experimental data.

2.3. Statistical data analysis

Significant differences among NIC and MIC values for preservatives were checked by one-way ANOVA using Statistica 7.1 software (Statsoft Inc., Tulsa, USA). Post-hoc comparisons were performed using the least significant difference (LSD) test. Data were obtained from four independent experiments.

3. Results

To determine the individual effect of five different chemical preservatives with potential application in table olive processing (pyruvic and fumaric acids, sodium metabisulphite, natamycin and cinnamaldehyde) and comparison with another currently used by the industrial sector (potassium sorbate), a total 47,040 raw data belonging to 560 OD growth curves (280 for the LAB and other 280 for the yeasts) were obtained in an automated spectrophotometer and then modelled. The addition of fumaric acid did not show any inhibitory effect within the concentration range tested (0–2000 ppm) for either LAB or yeast. Potassium sorbate and natamycin did not affect LAB growth, and *Fa* was kept constant around 1.0 value, regardless of their concentrations. However, for the rest of chemical compounds, there was a clear *Fa* decrease as concentrations were greater. Thereby, a dose-response model was properly fitted in the case of inhibition, with an R^2 usually above 0.922 (data not shown).

Fig. 1 shows two examples of the reparameterized Gompertz equation for decay fitted to the experimental data, for both yeast (upper panel) and LAB (lower panel) as a function of the ln sodium metabisulphite and cinnamaldehyde concentrations (ppm), respectively. The fit followed a typical sigmoid decay function, which could be divided into three sections: i) a first section corresponding to preservative concentrations below the NIC (concentrations at which no effect of the inhibitor was observed and *Fa* was around 1), ii) concentrations between NIC and MIC values (within which growth inhibition progressively occurred and the *Fa* decreased), and iii) a third section above MIC (where no growth relative to the control was recorded, and *Fa* was close to 0).

Table 3 shows the NIC and MIC values individually obtained for the preservatives with an inhibitory effect on the growth of the yeast and LAB cocktails. Values are the average of four experiments for each microbial cocktail and preservative, performed and fitted independently. The NIC value, related to susceptibility of microorganism to the specific chemical compound, was widespread among preservatives and ranged from 6 ppm (natamycin in the case of yeasts) to 2713 ppm (pyruvic acid in the case of LAB), while the MIC value, related to the resistance of the microorganism to the preservative, ranged from 25 ppm (natamycin in the case of yeasts) to 3211 ppm (pyruvic acid in the case of LAB). According to values shown in Table 3, only pyruvic acid, sodium metabisulphite and cinnamaldehyde showed inhibitory effects on both LAB and yeast populations. Among them, pyruvic acid was the preservative with the lowest inhibitory effects (the highest NIC and MIC values), whilst sodium metabisulphite and cinnamaldehyde were the compounds with the highest inhibitory effects for both LAB and yeasts, respectively. Statistically significant differences were found among preservatives within the same microbial cocktail (LAB or yeast) according to the LSD posthoc comparison test.

According to Fig. 2, which shows the concentration range where the progressive inhibitory effect of preservatives (from NIC to MIC) was noticed for microorganisms, the microbial behaviour depended on the preservative assayed. As shorter is the range between both values, the stronger is the inhibitory effect for the chemical compound. Sodium metabisulphite for LAB, and cinnamaldehyde and natamycin for yeasts, were extremely toxic for cells, with a very narrow inhibitory range, while this range was wider for the rest of

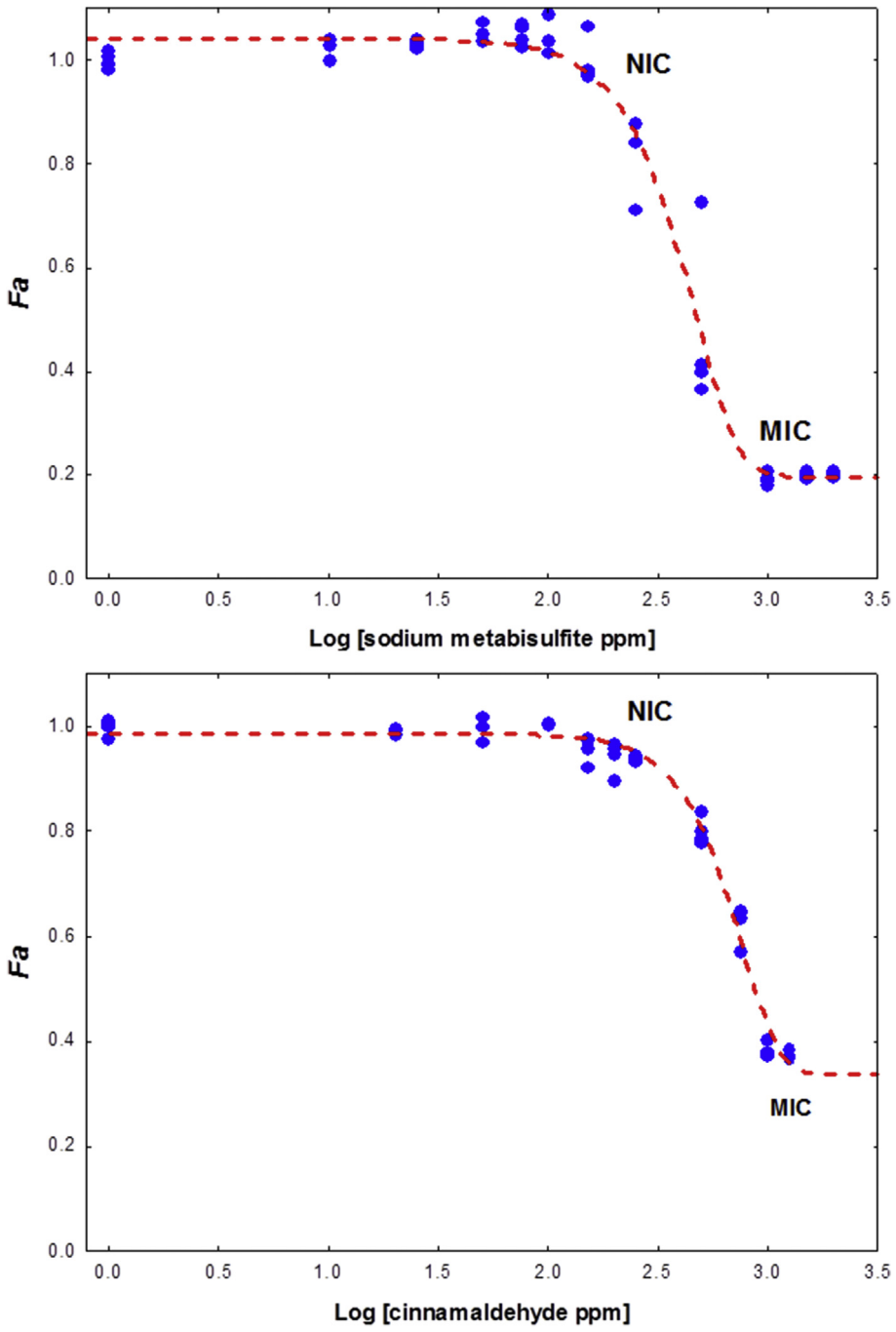

Fig. 1. Fit of the reparameterized Gompertz equation for decay (see Materials and methods) to the fractional areas (*Fa*) of the yeast (upper panel) and lactic acid bacteria (lower panel) populations as a function of ln (ppm) of sodium metabisulphite and cinnamaldehyde, respectively, for the estimation of NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration) values. Parameters for each preservative were the average of four independent experiments.

preservatives, especially for pyruvic acid in the case of yeasts.

The ANOVA analysis carried out with the NIC, and MIC values obtained for the LAB and yeast populations (Fig. 3) showed that, effectively for both microbial groups, pyruvic acid showed the lowest inhibitory effect without significant differences between yeasts and LAB. The preservative with the highest inhibitory effect on LAB was sodium metabisulphite followed by cinnamaldehyde (with significant differences between them), whilst the preservative with the highest inhibitory effect on yeasts was natamycin, followed by cinnamaldehyde (without significant differences between them). Sodium metabisulphite had a very similar effect than potassium sorbate on yeasts while this later preservative did not show any inhibitory effect against bacteria.

4. Discussion

The control of spoilage microorganisms is one of the most important aspects in food preservation. Many of the food preservatives habitually used by industry for this purpose are weak acids, such as sorbic, benzoic, propionic, acetic and sulphite (Piper, 2011). Weak acids are widely used in low-pH foods, where its inhibitory power increases. Therefore, they could have direct

Table 3
NIC and MIC (ppm) values obtained for the preservatives assayed in this work against the lactic acid bacteria and yeasts cocktails. Mean and standard deviation (in parentheses) values were obtained from four independent experiments (n = 4).

	LAB		Yeasts	
Preservative	NIC	MIC	NIC	MIC
Pyruvic acid	2713.97 (54.50)[a]	3210.99 (42.52)[a]	2050.81 (134.25)[d]	3037.63 (105.16)[d]
Fumaric acid	*	*	*	*
Sodium metabisulphite	49.00 (0.00)[b]	50.07 (0.09)[b]	296.08 (85.16)[c]	771.89 (172.77)[c]
Potassium sorbate	*	*	150.41 (15.58)[a]	552.98 (58.15)[b]
Natamycin	*	*	6.49 (0.99)[b]	24.59 (2.76)[a]
Cinnamaldehyde	382.85 (23.62)[c]	1060.18 (66.77)[c]	124.00 (0.00)[a]	125.00 (0.00)[a]

*It was not observed a reduction of the *Fa* (value close to 1) within the range of concentrations assayed. Values followed by different superscript letters, within the same column, are significantly different according to the LSD posthoc comparison test.

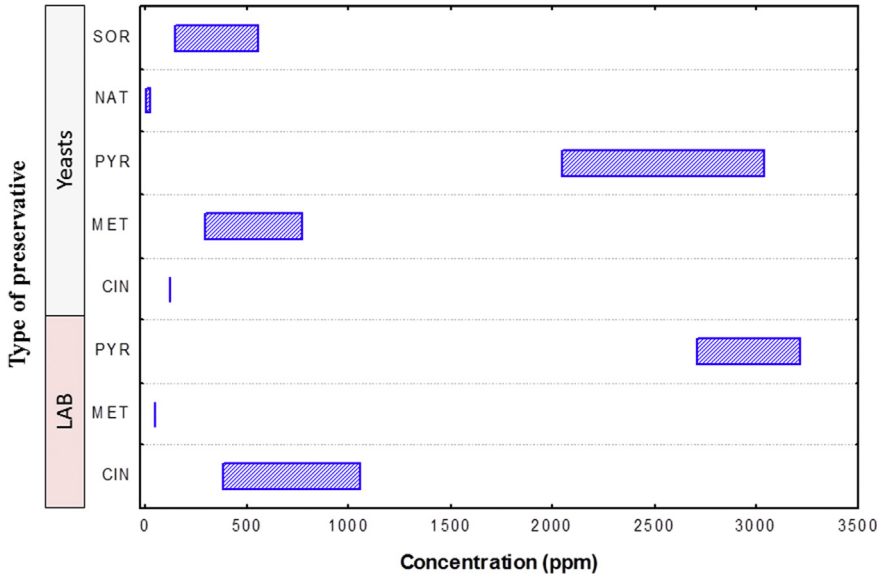

Fig. 2. NIC to MIC interval for the LAB and yeast cocktails as a function of the preservative concentrations. CIN, MET, SOR, NAT and PYR stand for cinnamaldehyde, sodium metabisulphite, potassium sorbate, natamycin and pyruvic acid, respectively. Parameters for each compound were the average of four independent experiments.

application in table olive packaging, albeit the experience on their effects on table olive related microorganisms is scarce. This work attempts to determine, using a dose-response model, the influence of different preservatives to control the growth of LAB and yeasts isolated from table olive processing. This type of modelling has proved to be appropriated to obtain the NIC and MIC values of diverse chemical compounds against table olive related microorganisms (Bautista-Gallego et al., 2008, 2012; Romero-Gil et al., 2013; Bonatsou et al., 2015).

The effect of potassium sorbate on the main olive yeast species has already been studied in several occasions, using a probabilistic model for the determination of the growth/no growth interfaces in combination with other additives (Arroyo-López et al., 2007a, b, 2008b). Its use in table olive packaging is accepted regardless of legislation (CODEX, EU or Spanish Government), provided the maximum dose allowed (500 ppm of sorbic acid in pulp) is not exceeded. In previous works, a concentration of 300 ppm of potassium sorbate together with 5–6% NaCl at pH 4.0 was enough to inhibit *S. cerevisiae* and *Issatchenkia occidentalis* growth (Arroyo-López et al., 2007a, b). However, scarce information is available for bacteria. Arroyo-López et al. (2005) showed that a concentration of 175 ppm of potassium sorbate was not enough to inhibit LAB growth in real olive packaging. The effect of the influence of this weak acid on microorganisms is strongly related to the pH of the medium. Data obtained in this study show that sorbate in the range assayed (0–2000 ppm), did not have any inhibitory effect against LAB at pH 4.0 but, on the contrary, exerted a clear inhibitory effect against a cocktail formed by a considerable number of yeast species, with a MIC value of 553 ppm (413 ppm expressed as sorbic acid). The comparison of its efficiency with respect to other new potential preservatives could be of interest for the proper selection of an adequate alternative.

Fumaric acid is an unsaturated dicarboxylic acid with low water solubility and a strong acid taste; however, its combination with flavouring compounds may intensify the aftertaste of a flavour. The use of this acid in food as either acidifying agent or microbial inhibitor is rather usual (Davidson et al., 2005). Particularly, it has been efficient against LAB for the preservation of acidified cucumbers (Pérez-Díaz, 2011). Due to the rather similarity between cucumbers and table olives, fumaric acid could have application for preventing spoilage by LAB in vegetable products. However, due to the lack of effectiveness noticed in the present study for this compound, which did not exert inhibitory effect in the range tested for either LAB or yeasts, no further discussion on its role as preservative in table olives is pertinent.

According to the General Standard for Food Additives (Codex Alimentarius, 2015) the use of metabisulphite is permitted for the products included in the Food Category num. 04.2.2.3 (which includes table olives). The recently issued Codex Standard for Table Olives (Codex Stan 66-1891, rev 2013) also refers to this Standard in the section related to food additives. However, according to Directive (CE) No 1333/2008 (European Parliament and

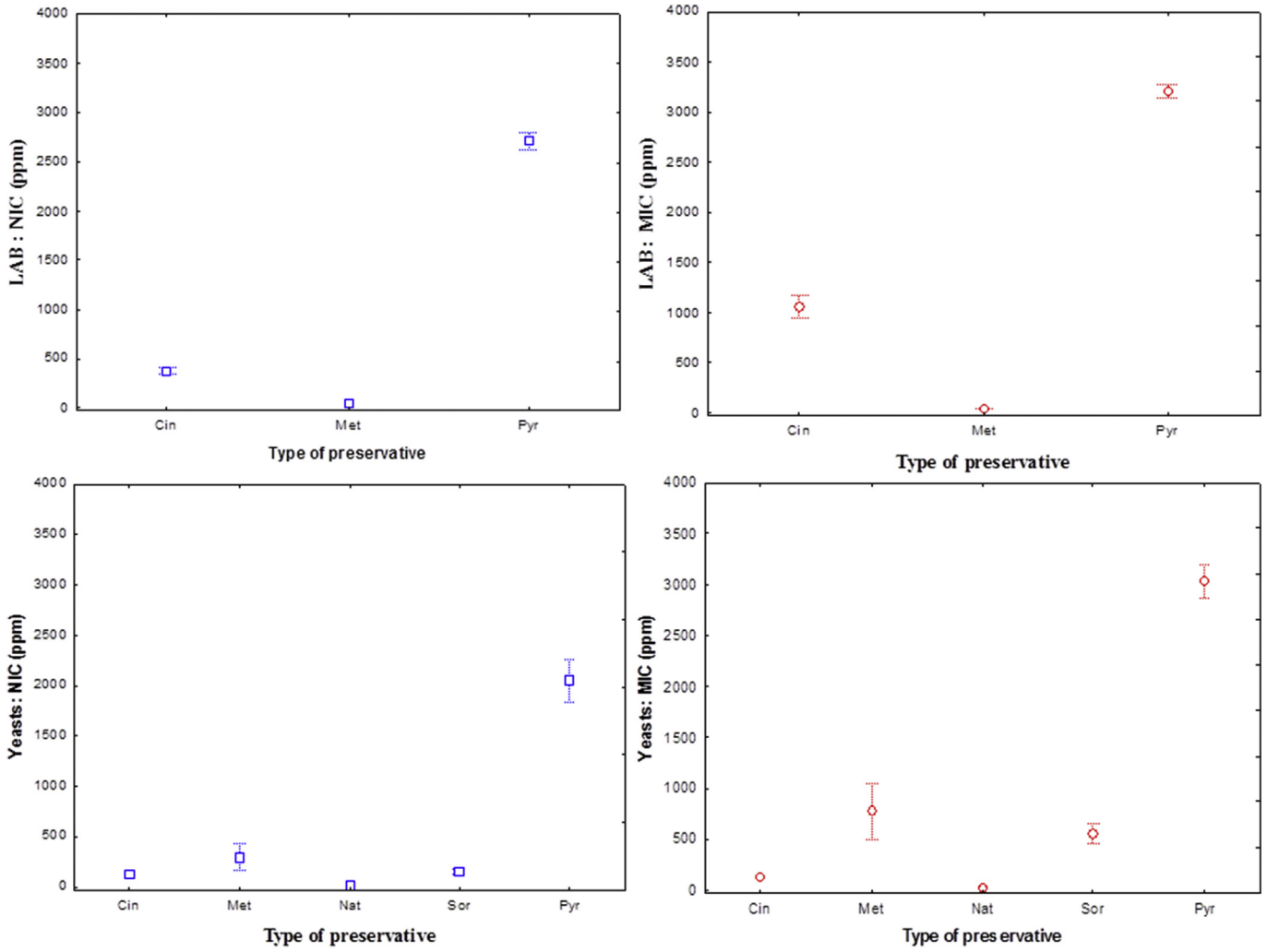

Fig. 3. Graphical representation of the one-way ANOVA for the NIC and MIC (ppm) parameters as a function of the different preservatives (categorical variable) and microbial cocktail. CIN, MET, SOR, NAT and PYR stand for cinnamaldehyde, sodium metabisulphite, potassium sorbate, natamycin and pyruvic acid, respectively. Parameters for each chemical compound were the average of four independent experiments.

Council, 2014), which follows a similar scheme and criterion that the Food Additive Standards issued by the Codex, the metabisulphite, although allowed for products in the food category num. 04.2.2 (which include olives), is explicitly excluded for table olives and yellow peppers in brine. Apparently, the re-introduction of this additive in the Standard issued by the Codex (trv. 2006) has not implied the subsequent rectification in the European Directive, in spite of the diverse modifications suffered in the last years. The Spanish legislation (Ministerio de la Presidencia, 2001) does not permit either the use of metabisulphite due to its submission in this aspect to the EU regulation on additives. However, metabisulphite was traditionally used in table olives until its temporary prohibition in the Food Additive Standards issued by the Codex, which also caused its elimination from the Directive (CE) No 1333/2008 (European Parliament and Council, 2014) and from the Trade Standard for Table Olive (COI, 2004). However, after the re-inclusion of the metabisulphite use in Codex Stan 192-1995, rev 2006) neither of these legislative organisms has modified the metabisulphite status accordingly. Nowadays, the discrepancies between the EU legislation and Codex may lead to disputes and insecurity in the international table olive trade. Thus, studies on the inhibitory effects on table olive related microorganisms are necessary to help legislators on the homogenization of standards. Besides, its use in table olives would be convenient due to its antioxidant (browning prevention) and inhibitory effects on the microbial populations (Arroyo-Lopez et al., 2008a; Echevarria et al., 2010). Furthermore, sodium metabisulphite may also remain as a result of its use as antioxidant during postharvest treatments (Segovia-Bravo et al., 2010) and this carry over effect should also be considered. In the present study, this compound has shown to have a moderate inhibitory effect in laboratory medium against yeast (MIC value 772 ppm) and especially against LAB (MIC value 50 ppm) cocktails. However, a concentration of 1500 ppm was not enough to inhibit LAB and yeast populations in real olive fermentations for two months, albeit showed a higher inhibitory effect than ascorbic acid (Echevarria et al., 2010). Taking into consideration these results, probably the metabisulphite levels necessary to inhibit LAB growth could be compatible with olive packaging. On the contrary, the higher doses necessary to control yeast growth may cause allergic reactions and headache in sensitive persons to this preservative. In the specific case of table olives, its residue would be below the 100 mg/kg flesh (expressed as sulphur dioxide) as established in the Codex Stan 192-1995 rev. 2014 (Codex Alimentarius, 2015). At this level, any possible health effect would be markedly reduced for most consumers.

Natamycin is a preservative used in diverse dairy products (Thomas and Delves-Broughton, 2003; Gallo et al., 2006). The first tentative of use in table olives was reported by Mahjoub and

Bullerman (1986) to control the mould growth and the production of aflatoxin. Natamycin has also shown good behaviour for the prevention of mould growth on the surface of natural black Greek-style fermenting olives at 100 ppm (Hondrodimou et al., 2011). Recently, Arroyo-López et al. (2012b) found natamycin very efficient (12–30 ppm) against table olive related yeasts at NaCl concentrations around 4.5%, albeit the presence of citric acid (and low pH levels in general) decreased its effect. Hence, the use of natamycin in table olives could be promising to control yeast and mould growth. However, according to data obtained in this work, this preservative did not exert any inhibitory effect against LAB at levels assayed. The EFSA Panel for Food Additives and Nutrient Sources for Foods has revised its application as preservative and has concluded that natamycin is very poorly absorbed in the gastrointestinal tract. Hence, its intake hardly can induce antimicrobial resistance and there is an appropriate margin of safety for its current application (EFSA, 2009). This position opens the possibility of natamycin utilization in other foods, provided its use could be adequately supported. In this context, this work has showed its usefulness in controlling the yeast population.

Pyruvic acid was first patented for its preservative properties by Ernst et al. (1979) to stabilize high moisture food products without refrigeration. The use of pyruvic acid with natural colorants may improve their stabilities at acidic pH and presence of ascorbic acid (Ojwang and Awika, 2008). Pyruvic (and acetaldehyde)-bound sulphur dioxide produced inhibition against wine LAB at concentration of 5 ppm, albeit the LAB finally degraded such compounds, suggesting that sulphur dioxide -bound pyruvic acid could have a bacteriostatic effect rather than bactericidal action (Wells and Osborne, 2011). Pyruvate was effective for lowering lipid oxidation in high-oxygen meat packages; then, its use in table olive might also have an favourable antioxidant effect on olive fat due to the high proportion of oil in the processed fruits and the adverse environmental condition (e.g. high storage temperature) during transportation or shelf live (Ramathan et al., 2011). Moreover, pyruvic acid has a low pK_a value (2.39), a circumstance that also validates its use for acidification purposes in table olives. The inhibitory effect was very similar for both LAB and yeasts populations, although the concentrations required were relatively high; its MIC values were 3211 and 3037 ppm, respectively.

Recently, cinnamaldehyde was applied to stabilize acidified cucumbers that were adequately preserved free of yeasts (Pérez-Díaz, 2011). The presence of essential oils is common in seasoned table olives due to the usual addition to them of garlic, rosemary, or extracts. However, one of the leading causes of instability in these products is the yeast growth (Arroyo-López et al., 2012a). Considering the efficient inhibition of yeast in cucumbers, testing cinnamaldehyde against the microorganisms (mainly yeasts) present in table olives may be interesting, especially for the development of table olives with other flavours. This compound is obtained from the cinnamon bark. The mechanism of the bactericidal action of cinnamaldehyde against *Listeria monocytogenes*, possible inhibition of glucose uptake and utilization and effects on membrane permeability, was suggested by Gill and Holley (2004). This compound had both antimicrobial and antioxidant activities when applied to meat, thus preventing microbial spoilage and lipid oxidation (Naveena et al., 2013). Cinnamaldehyde has been reported to show a potential inhibitory effect on methicillin-resistant *Staphylococcus aureus* biofilm-related to infections (Jia et al., 2011). Recently, cinnamaldehyde has been suggested as a useful compound for the control of *Escherichia coli* at refrigeration temperature (Visvalingam and Holley, 2012). Data obtained in this work show that this organic compound was effective to control microorganisms, but its effect was microbial group dependent, with a higher inhibitory effect on yeast (125 ppm) than for LAB (1060 ppm).

5. Conclusions

In summary, the results obtained in this work show that three preservatives (sodium metabisulphite, pyruvic acid and cinnamaldehyde) had a broad inhibitory effect against the growth of both LAB and/or yeasts and may have application in table olive packaging, whilst traditional preservative (potassium sorbate) only showed inhibitory effect against yeasts. Further studies should be performed to determine the possible interaction of these compounds with food matrixes and their influence on the organoleptic profile of final products, which could be especially relevant in the case of the essential oils (cinnamaldehyde).

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3.2.3 CAPÍTULO 5:

In silico logistic model for table olive related microorganisms as a function of sodium metabisulphite, cinnamaldehyde, pH and type of acidifying agent.

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In silico Logistic Model for Table Olive Related Microorganisms As a Function of Sodium Metabisulphite, Cinnamaldehyde, pH, and Type of Acidifying Agent

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A probabilistic/logistic model, based on binary data (growth/no growth), was used to assess the effects of sodium metabisulphite (SM) and cinnamaldehyde (CIN; 0–1000 mg/L) against the main microbial groups found in table olive environment [lactic acid bacteria (LAB), yeasts, and *Enterobacteriaceae*], according to pH (range 3.5–5.0), and type of acidifying agent (HCl or pyruvic acid). The inhibitory effect of SM depended on the pH while that of CIN was scarcely influenced by it (except for LAB). LAB were more sensitive to SM, while yeasts were to CIN. The use of pyruvic acid for correction of pH always produced a reduction (compared to HCl) of the inhibitory power of both preservatives. The *in silico* models for HCl showed that, at pH 4.0, and growth probability 0.01, the LAB population might be inhibited by the presence in the medium of 150 mg/L SM or 1000 mg/L CIN, while in the case of yeasts, 450 mg/L SM, or 150 mg/L CIN are required. No growth of *Enterobacteriaceae* was observed at this (or lower) pH level. The results obtained may contribute to the stabilization of non-thermally treated table olive packaging.

Keywords: olive packaging, predictive microbiology, preservatives, sulphites, cinnamaldehyde

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INTRODUCTION

The olive tree is well adapted to the Mediterranean climate and its products (table olives and olive oil) are basic elements of the culture and diet of many countries (Spain, Turkey, Egypt, Greek, or Italy) around the basin. Nowadays, the worldwide production of table olives exceeds 2.6 million tons/year according to the last final balance for the crop year 2013–2014 (IOOC, 2015), with green Spanish-style (olives debittered by alkaline treatment), natural (directly brined) olives, and Californian style (olives darkened by oxidation in an alkaline medium) as the main preparation types (Garrido-Fernández et al., 1997). Uncontrolled growth of microorganisms during olive packaging may cause product spoilage due to the production of CO_2, swollen containers, softening of fruits, clouding of brines, or consumption of lactic acid in aerobic conditions. Hence, the microbiological stabilization of the final products during the commercialization period is critical, and there is a need to improve the knowledge of the factors required to achieve it (Arroyo-López et al., 2009).

Due to its high pH (close to neutrality), ripe olive packaging requires sterilization while Spanish-style, and natural olives are fermented products that may be preserved by different methods (modified atmosphere, vacuum, use of preservatives, pasteurization, or by their physicochemical characteristics) (Garrido-Fernández et al., 1997). Nowadays, the thermal treatment is widely applied; however, it may cause undesirable changes in the traditional flavor of several presentations, particularly seasoned fruits which should, then, be stabilized by preservatives (Arroyo-López et al., 2009). Currently, the preservatives and the levels permitted for addition in table olives vary according to legislations. In the Trade Standards Applying to Table Olives (IOOC, 2004), benzoic, and sorbic acids (or their respective salts) are the only preservatives permitted at maximum doses of 1000 mg/kg (benzoic) and 500 mg/kg (sorbic acid) or 1000 mg/kg for their combination. In the EU, both preservatives are also allowed but at levels of 1000 mg/kg (sorbic) and 500 mg/kg (benzoic), expressed as acids (Regulation EU 1129/2011). Finally, the CODEX [1]Standard for Table Olives (STAN 66-1981 rev 2013), and CODE[2]X Standard for Food Additives (STAN 192-1995 rev 2014) permit the addition of higher levels of benzoic (2000 mg/kg) and sorbic (1000 mg/kg) acids, as well as the use of sulphites (metabisulphite, sulfur dioxide, or bisulphite) at a maximum dose of 100 mg/kg flesh in the final product. Hence, there are evident discrepancies between the EU legislation and CODEX concerning the levels and preservatives allowed in table olives. Such differences may lead to disputes and insecurity in the international table olive commercial trading. Therefore, studies on the inhibitory effects of preservatives on table olive related microorganisms are necessary to assist legislators on the homogenization of standards.

The use of sorbic and benzoic acids in table olive packaging could also have some drawbacks. Among the most important are: (i) accumulation in the olive (flesh) fat, with the subsequent limitation of their effects in the brines, (ii) development of undesirable sensorial notes for consumers, (iii) browning of fruits, and (iv) degradation by microorganisms (Garrido-Fernández et al., 1997; Arroyo-López et al., 2005). As a result, the table olive sector is demanding research for obtaining more appropriate preservatives. Particularly, sulphites, which, in addition to their proved antimicrobial activity, may also produce an important, and persistent antioxidant effect for preventing browning (Arroyo-López et al., 2008; Echevarria et al., 2010; Segovia-Bravo et al., 2010). In this content, the contribution of Juneja and Friedman (2008) on the effect of carvacrol and cinnamaldehyde (CIN) for facilitating the thermal destruction of *Escherichia coli* O157:H7 in raw ground beef, and the results obtained by Taboada-Rodríguez et al. (2013) on sodium metabisulphite (SM), potassium sorbate, and dimethyl dicarbonate are particularly pertinent.

In a previous study, the individual inhibitory effects of diverse preservatives on lactic acid bacteria (LAB) and yeasts cocktails isolated from table olives, at fixed levels of pH (4.0) and salt (5%), were tested *in vitro* using a dose-response model (Romero-Gil et al., 2016). Among the diverse compounds assayed, SM, CIN, and pyruvic acid (PYR) showed the higher perspective of application in table olives. The present work represents a further step in this research, using a probabilistic/logistic model to determine the influence of pH and type of acidifying agent (HCl or PYR) on the inhibitory effects of SM and CIN, also expanding the work to the *Enterobacteriaceae* population. Predictive microbiology is a useful tool to describe the response of microorganisms as a function of environmental variables quantitatively (McMeekin et al., 1993). By using this mathematical approach, it is possible to determine: (i) the more sensitive microbial gropup to preservative and type of acidifying agent, (ii) the compound with the highest inhibitory effects on microorganisms, and (iii) the growth/no growth (G/NG) boundaries of microorganisms as a function of the preservative, pH levels, and type of acidifying agent.

MATERIALS AND METHODS

Microorganisms and Cocktail Preparation

A total of 24 strains belonging to different LAB, yeasts, and *Enterobacteriaceae* species were used in the present study. Many of the LAB and yeast strains were previously isolated from diverse table olive trade preparations and identified by molecular methods (data not shown). Many of them belong to the Table Olive Microorganisms Collection (TOMC) of Instituto de la Grasa (CSIC, Seville), while the *Enterobacteriaceae* strains were kindly supplied by Dr. Antonio Valero Díaz (University of Córdoba, Spain), and purchased from the CECT (Spanish Type Culture Collection, University of Valencia, Spain). Their references and origin are shown in **Table 1**. Inoculum were prepared by introducing one single colony of each strain into 5 ml of a YM broth medium (Difco™, Becton, and Dickinson Company, Sparks, USA) for yeasts, 5 ml of a MRS broth medium (de Man, Rogosa, and Sharpe; Oxoid, Cambridge, UK) for LAB, or 5 ml of VRBD (Crystal-violet Neutral-Red bile glucose) broth medium (Merck, Darmstadt, Germany) for *Enterobacteriaceae*. After 48 h of incubation at 30°C, 1 ml from each tube was centrifuged at 9000 × *g* for 10 min, the pellets were washed with sterile saline solution (9 g/L), centrifuged and re-suspended again in 0.5 mL of a sterile saline solution to obtain a concentration of about 7 $\log_{10}$ CFU/mL for yeasts and 8 $\log_{10}$ CFU/mL in the case of LAB and *Enterobacteriaceae*, which were confirmed by enumeration on appropriate media. The microorganism suspensions belonging to each group were gently mixed in the same proportions, obtaining three different cocktails (yeast, LAB, and *Enterobacteriaceae*), which were then used to inoculate the media described below.

Growth Media and Data Collection

Sterilized YM, MRS, or VRBD broth were modified with 5% NaCl and adjusted to different pH levels (3.5, 4.0, 4.5, and 5.0) by HCl (37% purity, Applichem Panreac, Damstadt, Germany), or PYR (99% purity, Merck, Damstadt, Germany) additions. The three basal media were then individually supplemented

[1]CODEX Stand, 66-1982. (2013). General Standard for Table Olives. *Rev* 2013. pp 9.

[2]CODEX Stand, 192-1995. (2014). General Standard for Food Additives. Rev 2014. *pp* 264–265.

TABLE 1 | Microbial strains used in the present study for preparation of the LAB, yeasts, and *Enterobacteriaceae* cocktails.

Microbial group	Species	Strain	Origin
LAB	*Lactobacillus pentosus*	TOMC-LAB2	Spanish-style green olive fermentations Gordal variety (Spain)
		TOMC-LAB3	Spanish-style green olive fermentations Gordal variety (Spain)
		TOMC-LAB4	Spanish-style green olive fermentations Hojiblanca variety (Spain)
		TOMC-LAB5	Spanish-style green olive fermentations Gordal variety (Spain)
		TOMC-LAB6	Spanish-style green olive fermentations Manzanilla variety (Spain)
	Lactobacillus plantarum	NC8	Grass silage (Norway)
		TOMC-LAB9	Directly brined olive fermentations Gordal variety (Spain)
	Lactobacillus paraplantarum	TOMC-LAB12	Green Spanish-style olive fermentations (Spain)
	Pediococcus pentosaceus	P56	Fermented food. University of Valencia (Spain)
		FBB-63	Fermented food. Michigan State University (United States)
Yeast	*Candida diddensiae*	TOMC-Y1	Spoilage of directly brined green olive packaging (Spain)
	Issatchenkia occidentalis	TOMC-Y3	Spoilage of directly brined green olives packaging (Spain)
	Saccharomyces cerevisiae	TOMC-Y4	Spoilage of directly brined green olives packaging (Spain)
	Debaryomyces hansenii	TOMC-Y25	Directly brined green olive fermentations Manzanilla-Aloreña variety (Spain)
	Pichia membranifaciens	TOMC-Y31	Directly brined green olive fermentations Manzanilla-Aloreña variety (Spain)
	Candida boidinii	TOMC-Y47	Spoilage of directly brined green olive packaging (Spain)
	Candida tropicalis	TOMC-Y72	Spoilage of directly brined green olive packaging (Spain)
	Lodderomyces elongisporus	TOMC-Y73	Spoilage of directly brined green olive packaging (Spain)
Enterobacteriaceae	*Escherichia coli*	CECT 405	American Cyanamid Co. (United States)
		CECT 4267*	Human feces, stool from outbreak of hemorrhagic colitis (United States)
		CECT 4782*	Human stool from outbreak of hemorrhagic colitis (United States)
	Salmonella enterica subsp. *enterica*	CECT 443	Food poisoning (United Kingdom)
		CECT 556	Water, Albufera lake (Spain)
		CECT 4396	Human gastroenteritis (Denmark)

**E. coli serotype O157:H7.*

Excluding the Enterobacteriaceae strains, all microorganisms were also previously used by Romero-Gil et al. (2016) for testing the inhibitory effects of diverse preservatives by using a dose-response model.

with SM (Applichem Panreac) and trans-CIN (Sigma-Aldrich, St Luis, USA) at 0, 25, 50, 100, 250, 500, and 1000 mg/L. The experimental design consisted in a full-factorial design with 56 different treatments for SM and other 56 for CIN (7 levels for preservative*4 levels of pH*2 types of acidifying agents) with 7 replicates by treatment in the case of LAB and yeasts. For *Enterobacteriaceae,* 7 replicates were performed for experiments with HCl, while 3 replicates were carried out in the case of PYR. The design was individually executed for each microbial cocktail, making a total of 2128 treatments.

Growth was monitored in a Bioscreen C automated spectrophotometer (Labsystem, Helsinki, Finland) with a wideband filter (420–580 nm). Measurements were taken every 2 h after a pre-shaking of 5 s for 7 days, making a total of 178,752 raw data to be analyzed. The wells of the microplate were filled with 20 μL of inoculum and 330 μL of medium (according to treatment as described above), always reaching an initial OD of approximately 0.2 (inoculum level above 6 $\log_{10}$ CFU/mL). The inocula were always above the detection limit of the apparatus, which was determined by comparison with a previously established calibration curve. Uninoculated wells for each experimental series were also included in the microplate to determine, and subsequently, subtract the noise signal. For each well, growth (coded as 1) was assumed when the OD increase on the initial OD (after subtraction of the noise signal) was higher than 0.1; no-growth (coded as 0) was recorded when the initial OD remained stable or the increase was <0.1. Thus, only a binary data (0 or 1) is possible for each assay. Responses from each replicate were recorded independently, and the whole matrix was subjected to statistical analysis. After concluding the experiments, randomly selected wells (which included both growth and no-growth samples, representing 1% of the total cases) were spread on YM, MRS, or VRBD agar plates and their counts were estimated to corroborate G/NG assumption (data not shown).

The *In silico* Probabilistic/Logistic Model

A logistic regression model links the probability of occurrence of a conditional event (Y), which depends on a vector (x) of explanatory variables. The quantity, $p(x) = E(Y/x)$ represents the conditional mean of y (in this case growth probability) given x (environmental factors) when the logistic distribution is used. In its simplest form, it takes the expression:

$$\text{logit}(p) = \ln[p(x)/(1 - p(x))] = \beta_0 + \ldots + \beta_n x_n$$

where $\beta_0 + \cdots + \beta_i$ are the intercept and the coefficients of the polynomial function and x_i ($i = 1 \ldots n$) are the environmental variables.

The predicted survival probability as a function of the independent variables, deduced from the logistic regression, is as follows:

$$p\,(x_i \ldots x_n) = \exp\,(\text{logit}\,(p))/(1 + \exp\,(\text{logit}\,(p)))$$

According to the number of independent variables, two dimension probability curves (given particular values of another variables), and the G/NG interfaces for selected probabilities can be deduced. The logistic regression model was fit to the binary data (G/NG) obtained from the different treatments (combinations of type of preservative, pH, acidifying agent, and microbial cocktail), using XLSTAT software package (2015.4.01.20116, Addinsoft, Paris, France). The initial model assumed was:

$$\text{logit}\,(\hat{p}) = \text{Intercept} + \text{pH} + [\text{P}] + \text{A} + \text{pH}\cdot[\text{P}] + \text{pH}\cdot\text{A} + [\text{P}]\cdot\text{A} + \text{pH}\cdot[\text{P}]\cdot\text{A}$$

where pH stands for the $-\log_{10}[\text{H}+]$ in the medium, P for the different preservatives assayed (SM and CIN), and A for the type of acidifying agent (HCl or PYR). For building the equations, the model using HCl for the pH correction was the reference, and its equation just consisted of the sum of all the terms (preserving their signs) which does not include A-PYR. The model for treatments with pH corrected with PYR was built by adding to the previous ones the terms including A-PYR (but without this indication, which was used just as a label).

For the selection of variables, the stepwise backward option, with 0.05, and 0.10 *p*-values to enter and remove, respectively, was used. The number of maximum allowable runs was set to 100 and the tolerance to 0.0001. Validation was achieved with a total of 575 cases (275 for SM and 300 for CIN), randomly selected and not used for the model building (XLSTAT software).

The log-likelihood statistic was used to evaluate the significant contribution of each term in the model to the response (G/NG data). To verify the overall fit, the McFadden, the Cox and Snell, and the Nagelkerke R-squares were used. The null hypothesis was tested by −2log(likelihood), Score, and Wald and Hosmer-Lemeshow statistics. Also, overall hit rate, sensitivity, and sensibility were also estimated (Hosmer and Lemeshow, 2000).

The interpretation of the model is usually based on the multiplicative form of logit (*p*) which establishes that changes in the odds ratio are expressed by exp(b), which means the change in the odds ratio caused by one unit change in the variable of interest when the others remain at the same level. A value of 1 ($e^0 = 1$) indicates that the variable under study does not cause any effect on the odds ratio, values <1 reduce it while values >1 increase it (Hosmer and Lemeshow, 2000). The predicted growth probabilities for the preservative concentration, pH, and acidifying agent combinations according to the microbial cocktail, and their corresponding G/NG boundaries for diverse probabilities ($p = 0.15$, 0.10, 0.05, and 0.01) were deduced from the expression $\ln[p/(1-p)] = \text{logit}(p)$.

RESULTS

Logistic Models for the Lab Cocktail

A total of 559 cases were used to build the model for LAB (292 for SM and 267 for CIN), with a distribution of G/NG data of 169/123 for SM, and 209/58 for CIN. The probabilistic model for both preservatives fit the data satisfactorily as demonstrated by values obtained from the diverse tests (see Table S1 in Supplementary Material). The goodness of fit was also assessed by the overall hit (accuracy) to the data used in model development and validation, which indicate an almost perfect segregation between G/NG treatments for both SM and CIN (Table S2, Supplementary Material). The specificity (true no-growth rate) were 97 (SM) and 98% (CIN) while the sensitivity (true growth rate) were 96 (SM) and 100% (CIN). Furthermore, the predictions obtained for the 225 validation cases (100 for SM and 125 for CIN) also led to high values of specificity (90 for SM and 95% for CIN) and sensitivity (97 for SM and 97% for CIN). Therefore, it can be stated that LAB models achieved an adequate segregation between G/NG data and can be considered appropriate for representing the G/NG events of this microbial group as a function of the levels of SM, CIN, pH, and type of acidifying agent.

For both preservatives and types of acids used for pH correction, the models for the logit (*p*) of LAB population can be easily deduced from the coefficients (Table S3, Supplementary Material). These equations were:

$$\begin{aligned}
\text{For SM} - \text{HCl}: \ \text{logit(p)} &= -9.983 + 3.024^{*}\text{pH} - 0.077^{*}[\text{SM}] \\
&\quad + 0.011^{*}\text{pH}^{*}[\text{SM}] \\
\text{For SM} - \text{PYR}: \ \text{logit(p)} &= -64.880 + 17.290^{*}\text{pH} + 0.024^{*} \\
&\quad [\text{SM}] - 0.006^{*}\text{pH}^{*}[\text{SM}] \\
\text{For CIN} - \text{HCl}: \ \text{logit(p)} &= 24.325 - 4.305^{*}\text{pH} \\
&\quad - 0.125^{*}[\text{CIN}] + 0.029^{*}\text{pH}^{*}[\text{CIN}] \\
\text{For CIN} - \text{PYR}: \ \text{logit(p)} &= -59.190 + 16.199^{*}\text{pH} + 0.019^{*} \\
&\quad [\text{CIN}] - 0.007^{*}\text{pH}^{*}[\text{CIN}]
\end{aligned}$$

The growth probability (y-axis) of the LAB cocktail in the presence of SM or CIN, in a 2D graph, can be deduced by using the preservative concentrations as the x-axis and assigning selected values to pH (5.0, 4.5, 4.0, and 3.5). Using HCl as an acidifying agent, the growth probability graph shows a strong inhibitory effect of SM on LAB growth, and also that the inhibition increases as the pH levels decrease (curves shifted to left; **Figure 1A** left). This way, at pH 5.0, 500 mg/L of SM inhibited LAB cocktail but, at pH 3.5, the concentration required was around 150 mg/L. The CIN had, in general, a lower inhibitory power that SM for this microbial group, and was also markedly influenced by pH (**Figure 1B** left); in fact, LAB were able to grow at any CIN concentrations at pH 5.0 and 4.5, and only were inhibited at pH 4.0 or lower. At pH 3.5 the inhibitory effect of CIN began at 200 mg/L, but was necessary 600 mg/L of CIN to achieve a complete inhibition of the LAB cocktail.

Also, by plotting pH levels *vs.* preservative concentrations, the G/NG interfaces at different growth probabilities (0.15, 0.10, 0.05, and 0.01) can be visualized, and the graph is particularly useful

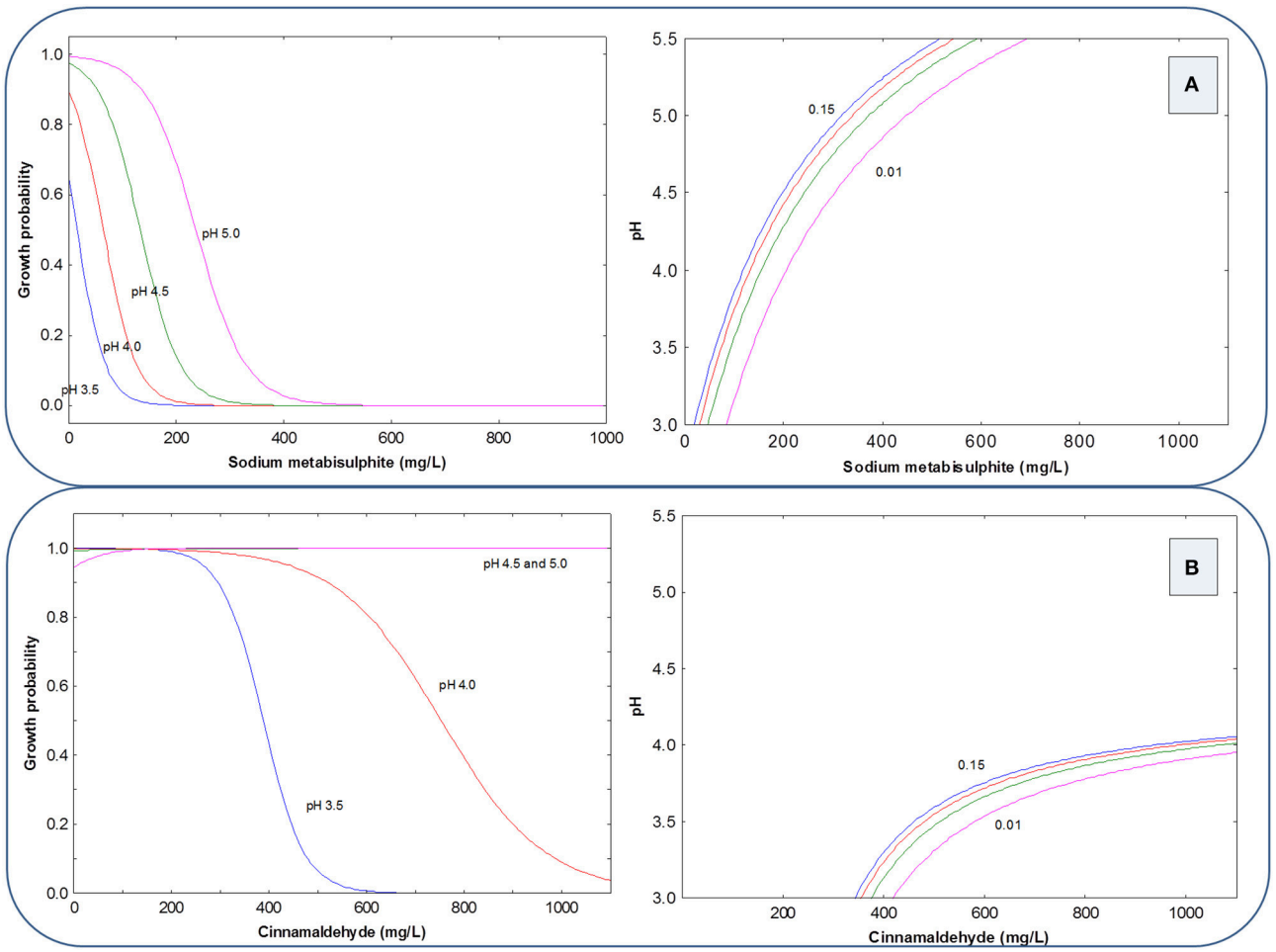

FIGURE 1 | Growth probability and G/NG interfaces of the LAB cocktail as a function of SM (A) and CIN (B) concentrations in a pH range from 3.5 to 5.0, using HCl as an acidifying agent.

for selecting the appropriate combination to achieve inhibition at a predetermined probability. The LAB G/NG interfaces for SM and CIN, using HCl as acidifying agent, shows the considerable effect of the correction of pH with HCl on the inhibitory power of both preservatives, especially on CIN (curves with a reduced inhibition region, always below pH 4.0; **Figures 1A,B**, right). Thus, at pH 4.0 (usual pH value in olive packaging conditions) and 0.01 growth probability (or 0.99 inhibition), LAB control requires about 150 mg/L of SM but around 1000 mg/L of CIN. In general, the G/NG interfaces are useful to deduce the different combinations of preservatives, and pH levels that potentially can control the growth of the LAB cocktail at selected growth probabilities, depending on the risk to be assumed by the operator.

When the LAB cocktail was inhibited with the same preservatives but using PYR for the pH correction, the 2D graphical representation of growth probability showed that regardless of the type of preservative assayed, growth was prevented only at pH below 3.5 (**Figures 2A,B**, left). The same conclusion is deduced from the respective G/NG interfaces (**Figures 2A,B**, right). Then, the pH correction with PYR markedly reduces the inhibitory effects of both SM and CIN since the inhibition observed at pH = 3.5 may be due just to the extremely low pH value reached.

Logistic Models for the Yeast Cocktail

For this eukaryotic microorganisms, 584 cases were analyzed, 292 for SM and other 292 for CIN, with a distribution of G/NG data of 238/54 for SM, and 156/136 for CIN. The probabilistic models for both preservatives fit the experimental data satisfactorily, as upheld by the diverse statistical tests (Table S1 in Supplementary Material). The goodness of fit was also assessed by the overall hit (accuracy) to the data used in model development and validation, which indicate an almost perfect segregation between G/NG treatments (Table S2 in Supplementary Material). The specificity was 96 (SM) and 98% (CIN) while the sensitivity was 100 (SM) and 97% (CIN). Furthermore, the predictions obtained for 200 validation cases (100 for SM and other 100 for CIN) also led to high specificity (100 for SM, 98% for CIN) and sensibility (99 for SM, 98% for CIN). Therefore, it can be stated that models

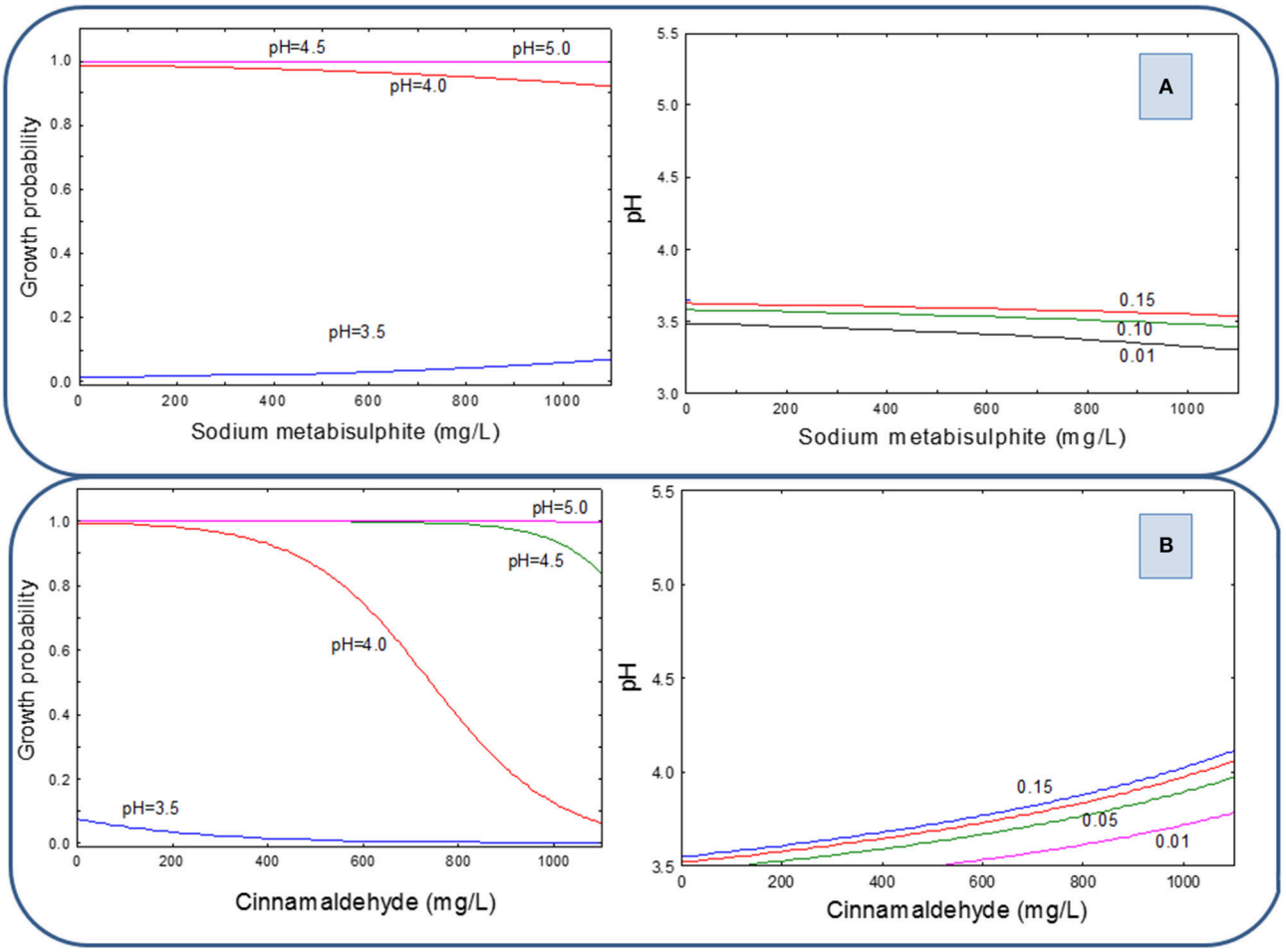

FIGURE 2 | Growth probability and G/NG interfaces of the LAB cocktail as a function of SM (A) and CIN (B) concentrations in a pH range from 3.5 to 5.0, using PYR as an acidifying agent.

achieved an adequate segregation between G/NG data and can be considered appropriate for representing the G/NG yeast events as a function of the levels of SM, CIN, pH, and type of acidifying agent.

The models for logit (p) of yeasts for both preservatives can be deduced from the respective coefficients (Table S4 in Supplementary Material) as already explained for LAB. The equations for the models, according to preservative and acid used for the pH correction were:

$$\text{For SM} - \text{HCl}: \ \text{logit(p)} = -26.199 + 8.915^{*}\text{pH} - 0.063^{*}[\text{SM}] + 0.009^{*}\text{pH}^{*}[\text{SM}]$$

$$\text{For SM} - \text{PYR}: \ \text{logit(p)} = 23.446 - 3.417^{*}\text{pH} - 0.039^{*}[\text{SM}] + 0.006^{*}\text{pH}^{*}[\text{SM}]$$

$$\text{For CIN} - \text{HCl}: \ \text{logit(p)} = 4.507 + 0.431^{*}\text{pH} - 0.062^{*}[\text{CIN}] - 0.001^{*}\text{pH}^{*}[\text{CIN}]$$

$$\text{For CIN} - \text{PYR}: \ \text{logit(p)} = 9.130 - 0.496^{*}\text{pH} - 0.050^{*}[\text{CIN}] + 0.003^{*}\text{pH}^{*}[\text{CIN}]$$

As in LAB, the graphical representation of these equations shows that for HCl, the inhibitory effect of SM against the yeast cocktail was strongly dependent on the pH levels (**Figure 3A**, left). In fact, at pH 5.0, there was always (within the range of concentrations assayed) yeast growth, and at pH 4.5, the growth probability decrease begins around 400 mg/L and reaches the total inhibition at above 900 mg/L. Interestingly, at pH 4.0, the inhibition begins at 200 mg/L and the growth was totally inhibited at approximately 600 mg/L SM (**Figure 3A** left). On the contrary, there was not observed any influence of pH on the inhibitory power of CIN. In fact, the curves for pH from 3.5 to 5.0 overlapped. Therefore, the total inhibition of the yeast cocktail can be achieved, independently of the pH values, at (or above) 200 mg/L of CIN (**Figure 3B** left). The G/NG interfaces for the yeast cocktail at different growth probabilities (0.15, 0.10, 0.05, and 0.01) show the above-mention interactions in the complete experimental region (ranges of both preservative and pH). The effect of pH is only observed on SM. At high pH are required high concentrations of SM to control the yeast cocktail (**Figure 3A** right) while, on the contrary, the G/NG interfaces for CIN are pH

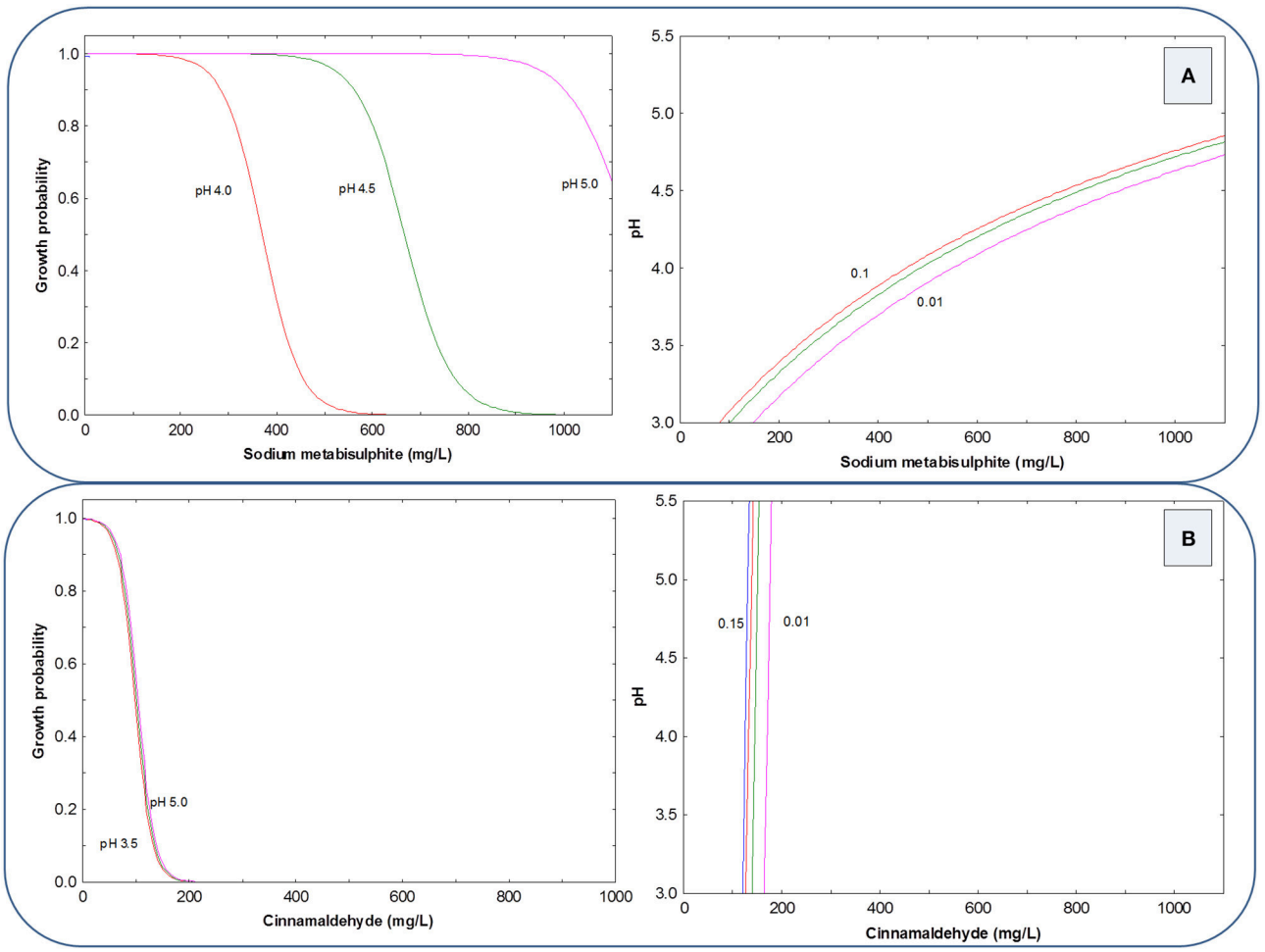

FIGURE 3 | Growth probability and G/NG interfaces of the yeast cocktail as a function of SM (A) and CIN (B) concentrations in a pH range from 3.5 to 5.0, using HCl as acidifying agent.

independent, and practically perpendicular lines to the pH-axis (**Figure 3B** right).

When the pH correction was achieved with PYR (**Figure 4**), the concentrations of SM required for yeast inhibition was higher; in fact, the inhibition only begins at concentrations above 500 mg/L and was completely obtained at levels higher than 1000 mg/L at pH values of 3.5 and 4.0. At higher pH values, it would not be possible to reach complete inhibition even with the highest used preservative proportions of SM (**Figure 4A** left). Then, the experimental region for the G/NG interfaces at $p = 0.01$ is reduced to the region above 900 mg/L SM and pH below 4.0 (**Figure 4A** right). On the other hand, CIN in the presence of PYR reached full inhibition at concentrations above 300 mg/L solution, regardless of pH value (**Figure 4B**).

Logistic Models for the *Enterobacteriaceae* Cocktail

For this gram-negative bacteria, a total of 410 cases were analyzed, (205 for SM and 205 for CIN), with a distribution of G/NG data of 80/125 for SM and 47/158 for CIN. The probabilistic models for preservatives fit the data satisfactorily, as supported by the statistical tests applied to the model fit (Table S1 in Supplementary Material). The goodness of fit was also assessed by the overall hit (accuracy) to the data used in model development and validation (Table S2 in Supplementary Material), which indicate an almost perfect segregation between G/NG treatments. This way, the specificity was 99 (SM) and 100% (CIN) while the sensitivity was 100 (SM) and 100% (CIN). Furthermore, the predictions obtained for the 150 validation cases (75 for SM and 75 for CIN) also led to 100% high specificity and sensitivity, regardless of preservative assayed. Therefore, the models achieved an adequate segregation between G/NG data and they are appropriate for representing the G/NG *Enterobacteriaceae* interfaces as a function of the levels of SM, CIN, pH, and type of acid.

As in the other microbial groups, the models for logit (p) for *Enterobacteriaceae* population was deduced from the estimated coefficients (Table S5 in Supplementary Material) by applying

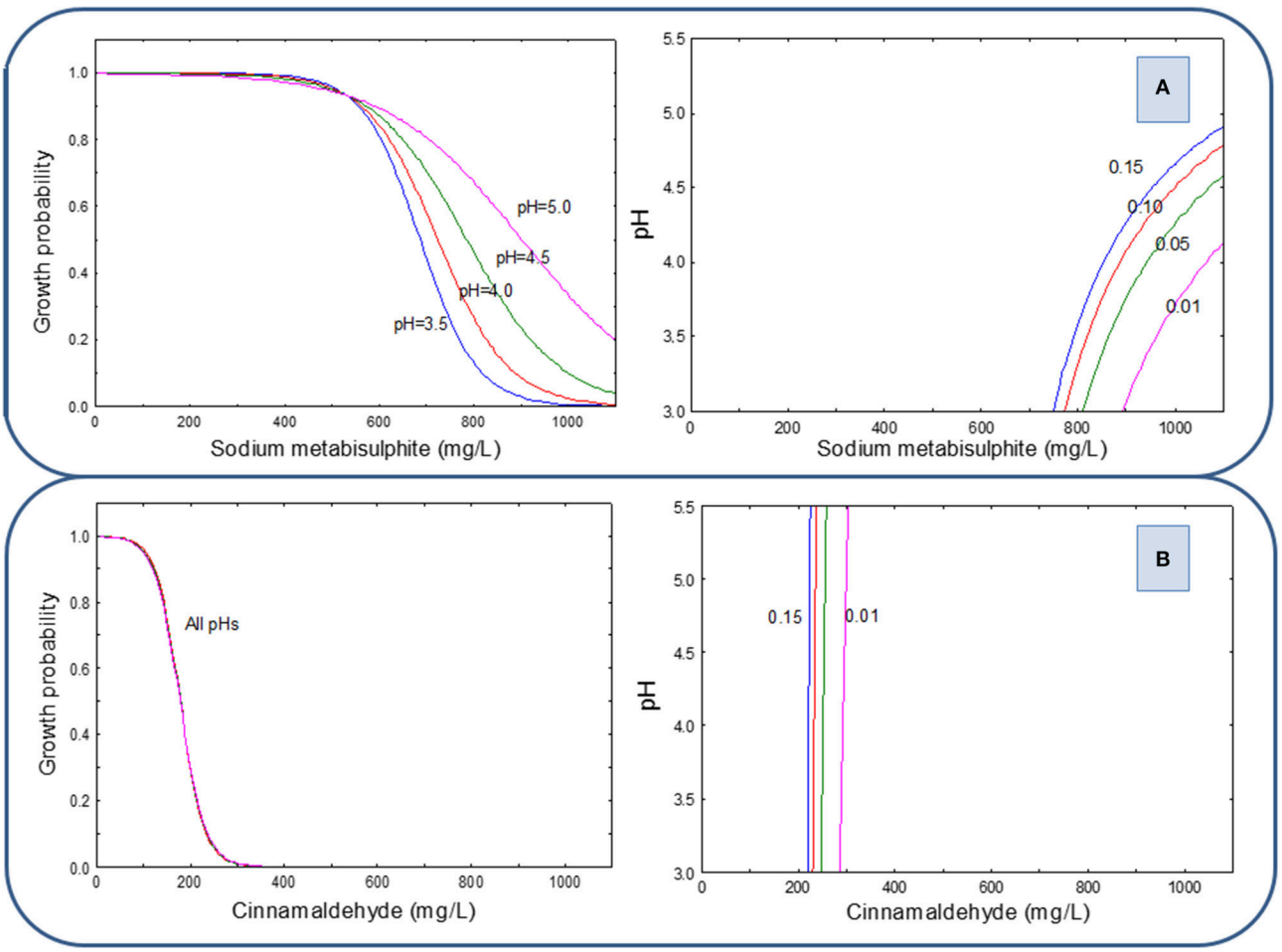

FIGURE 4 | Growth probability and G/NG interfaces of the yeast cocktail as a function of SM (A) and CIN (B) concentrations in a pH range from 3.5 to 5.0, using PYR as an acidifying agent.

the same methodology previously described. The following equations were obtained:

$$\text{For SM} - \text{HCl}: \ \text{logit(p)} = -342.118 + 77.307^{*}\text{pH} + 0.426^{*}[\text{SM}] - 0.102^{*}\text{pH}^{*}[\text{SM}]$$

$$\text{For SM} - \text{PYR}: \ \text{logit(p)} = -58.143 + 13.729^{*}\text{pH} + 0.041^{*}[\text{SM}] - 0.010^{*}\text{pH}^{*}[\text{SM}]$$

$$\text{For CIN} - \text{HCl}: \ \text{logit(p)} = -91.283 + 21.968^{*}\text{pH} + 0.410^{*}[\text{CIN}] - 0.114^{*}\text{pH}^{*}[\text{CIN}]$$

$$\text{For CIN} - \text{PYR}: \ \text{logit(p)} = -41.204 + 9.383^{*}\text{pH} + 0.103^{*}[\text{CIN}] - 0.027^{*}\text{pH}^{*}[\text{CIN}]$$

The graphical representation of the growth probability using HCl as acidifying agent shows the great importance that pH had on the growth of this microbial group (**Figure 5A** left). *Enterobacteriaceae* were not able to grow below pH 4.0 (even in the absence of preservatives) but the increasing levels of pH required higher concentrations of SM (curves shifted to the right). At pH 5.0, it was necessary to add approximately 600 mg/L of SM for the total inhibition of *Enterobacteriaceae* population, while it was necessary the presence of only 350 mg/L SM at pH 4.5 (**Figure 5A** left). The effect of pH was scarcely noticed in the case of CIN in which the growth probability curves for 4.5, and 5.0 pH values were fairly close, and the total inhibition at both pH values was obtained with lower CIN concentrations, approximately 150 mg/L (**Figure 5B** left). The G/NG interfaces at different growth probabilities (**Figures 5A,B** right) confirm that higher concentrations of SM are necessary to control *Enterobacteriaceae* cocktail at higher pHs while the CIN inhibition is less influenced by the pH which interaction was limited to values below 200 mg/L concentration.

As in the previous microbial groups, the pH correction with PYR caused a marked diminution of the inhibitory effect of SM, which only achieved almost complete inhibition below a pH value of 4.0 and total at 3.5, regardless of the preservative concentration assayed (**Figure 6A** left). As a result, at pH 4.0, the G/NG interfaces were fairly horizontal with a slight downward curvature (lower pH values) for high SM contents. On the

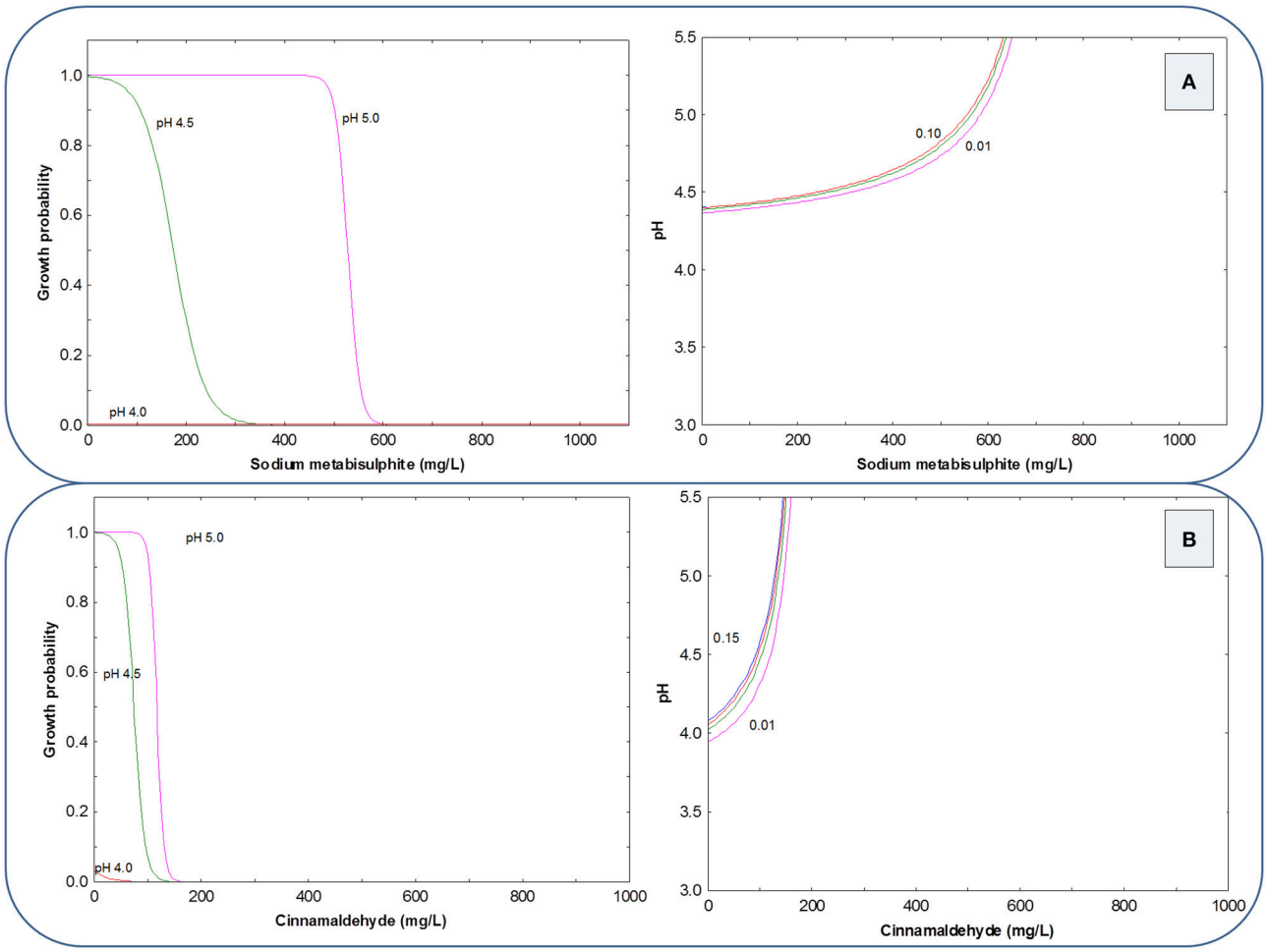

FIGURE 5 | Growth probability and G/NG interfaces of the *Enterobacteriaceae* cocktail as a function of SM (A) and CIN (B) concentrations in a pH range from 3.5 to 5.0, using HCl as acidifying agent.

contrary, the effect of PYR on the inhibitory effect CIN was less appreciable and decreased as the preservative concentration increased leading to complete inhibition above approximately 350 mg/L, regardless of the pH values (**Figure 6B**).

DISCUSSION

The control of spoilage microorganisms is one of the most important aspects of food preservation and also a prominent issue in the stability of table olive packaging. Many of the food preservatives habitually used by industry for this purpose are weak acids, such as sorbic, benzoic, propionic, acetic, and sulphites (Piper, 2011). Weak acids are widely used in low-pH foods, where its inhibitory power increases. Therefore, they could have direct application in table olive packaging, albeit the information on their effects on the main microbial groups found in table olives environment is scarce. This survey tries to elucidate the applicability of SM and CIN as preservatives to control the growth of table olive related microorganisms (bacteria and fungi), as an alternative to classic sorbic, and benzoic acids. To this aim, three different cocktails (LAB, yeast, and *Enterobacteriaceae*) that mimicked the microbiota in real olive packages were assayed. The use of a microbial cocktail rather than individual species is a convenient and faster way of checking the overall effects that inhibitory compounds could have against a specific microbial group. This way, the G/NG boundaries will be obtained for the most resistant strain of the cocktail. This strategy has been successfully used in food microbiology to estimate the overall response of microorganisms as a function of storage conditions or preservatives (Arroyo-López et al., 2012; Leong et al., 2014).

Ratkowsky and Ross (1995) were the first researchers modeling the bacterial G/NG interfaces. Years later, Presser et al. (1998) and Lanciotti et al. (2001) used the visible increase of turbidity to deduce the growth limits of *E. coli, Bacillus cereus, Staphylococcus aureus,* and *Salmonella enteritis.* The application of the logistic/probabilistic models (based on OD) to determine the G/NG boundaries of both bacteria and yeasts is rather habitual in predictive microbiology. Among the environmental variables included in the model, temperature, pH, a_w, organic acids, or preservatives are usual (Boziaris et al., 2006; Valero

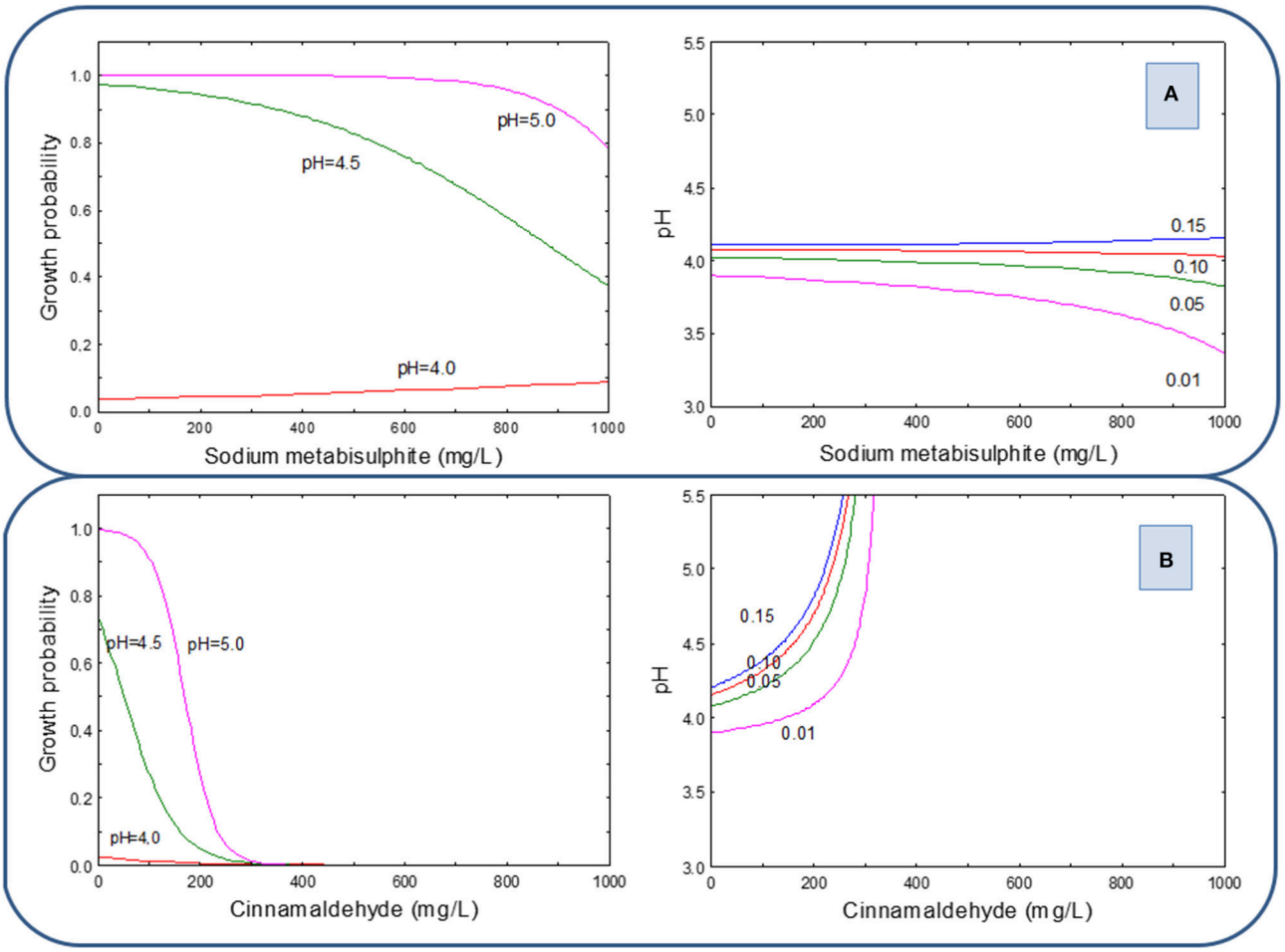

FIGURE 6 | Growth probability and G/NG interfaces of the *Enterobacteriaceae* cocktail as a function of SM (A) and CIN (B) concentrations in a pH range from 3.5 to 5.0, using PYR as an acidifying agent.

et al., 2010; Arroyo-López et al., 2012; Astoreca et al., 2012; Tabanelli et al., 2014). That is, determination of the G/NG interfaces of spoilage and pathogen microorganisms, based on OD, at selected growth probabilities levels has become a standard practice for establishing conditions to avoid economic losses and outbreak food illness, respectively. To our knowledge, this is the first probabilistic model built for SM and CIN using the main microbial groups found in table olive environment as targets.

SO_2 is widely used in both wine and food industries for its antioxidant and antimicrobial properties. Once dissolved in water, SO_2 exists in equilibrium between molecular SO_2, bisulphite, and sulphite forms. This equilibrium is dependent on pH of the medium, with the bisulphite anion being the dominant form under olive packaging conditions (pH between 3.5 and 4.0). Apparently, only molecular SO_2 exerts an antimicrobial action, and its concentration in food depends on of many factors such as pH and temperature (Fugelsang and Edwards, 2007). Thus, its inhibitory power has similar behavior than other weak organic acids such as sorbic and benzoic acids, being pH dependent. In the present study, SM was more useful to control bacteria than yeasts growth. Our results contrast with those obtained by Rojo-Bezares et al. (2007), who found a higher resistance of LAB (12.8 mg/L) than yeasts (1.6 mg/L) against the inhibitory effects of potassium metabisulphite in laboratory media at pH 3.5. On the contrary, the results obtained from this work are in agreement with Chang et al. (1997) who reported that 100 mg/L of sulphite concentration inhibited the growth of *Lactobacillus fermentun* and *Lactobacillus casei,* but it was necessary up to 500 mg/L to inhibit *S. cerevisiae* growth. In line with these results, Taboada-Rodríguez et al. (2013) found that SM did not show any fungicide effect when used as a preservative for dealcoholized red wine, using *Rhodotorula mucillaginosa* and *S. cerevisiae* as target organisms. The only probabilistic model for SO_2 as a function of pH was recently developed by Sturm et al. (2014) for *Dekkera bruxellensis.* These authors found that the effect of SO_2 on yeast G/NG boundaries was considerably affected by the pH of the medium, being necessary lower SO_2 levels as pH decreased. According to the General Standard for Food Additives (CODEX Stand 192-1995, rev 2014) the use of

metabisulphite is permitted for the products included in the Food Category num. 04.2.2.3 (which includes table olives). The recently issued CODEX Standard for Table Olives (CODEX Stan 66-1891 rev 2013) also refers to General Standard in the section related to food additives. However, according to Directive (CE) N° 1129/2011 [European Parliament and Council (EU), 2011], which follows a similar scheme and criterion that the Food Additive Standards issued by the CODEX, the metabisulphite, although allowed for products in the food category num. 04.2.2 (which includes olives), is explicitly excluded for table olives, and yellow peppers in brine. Apparently, the re-introduction of this additive in the Standard issued by the CODEX (rev. 2014) has not implied the subsequent rectification in the European Directive, despite the diverse modifications it has suffered in the last few years. However, metabisulphite was traditionally used in table olives until its temporary removal from the Food Additive Standards issued by the CODEX, which also caused its elimination from the Directive (CE) N° 1129/2011 [European Parliament and Council (EU), 2011] and the Trade Standard for Table Olive (IOOC, 2004). However, after the re-inclusion of the metabisulphite use in CODEX Stan 192-1995, rev 2014) neither of these legislative organisms has updated the metabisulphite status. Nowadays, the discrepancies between the EU legislation and CODEX may lead to disputes and insecurity in the international table olive trade. Thus, studies on the inhibitory effects on table olive related microorganisms are necessary to assist legislators on the homogenization of Standards. Besides, its use in table olives would be convenient due to its antioxidant (browning prevention) and inhibitory effects on the microbial populations (Arroyo-López et al., 2008; Echevarria et al., 2010). Furthermore, SM may also remain as a result of its use as antioxidant during postharvest treatments (Segovia-Bravo et al., 2010) and this carry over effect should also be considered. In a previous study with table olive related microorganisms, this compound had a moderate inhibitory effect in laboratory medium against yeast (MIC value approximately 770 ppm) and especially against LAB cocktails (MIC value 50 ppm; Romero-Gil et al., 2016). However, a concentration of 1500 ppm was not enough to inhibit LAB and yeast populations in real olive fermentations for 2 months, albeit showed a higher inhibitory effect than ascorbic acid (Echevarria et al., 2010). Taking into consideration all these studies, probably the metabisulphite levels necessary to inhibit LAB growth could be compatible with the usual olive packaging procedures. On the contrary, the higher doses necessary to control yeast growth might cause allergic reactions, and headache especially in sensitive persons to this preservative. In the specific case of table olives, its residual current level should be below 100 mg/kg flesh (expressed as sulfur dioxide) as established in the CODEX Stan 192-1995. At this level, any possible health effect would be markedly reduced for most consumers. In wines, the maximum allowable limits for the addition of SO_2 by the OIV is from 150 to 300 mg/L of total SO_2 (OIV, 1998).

The pH of the medium influenced less the inhibitory effect of CIN compared to SM, which is a considerable advantage compared to other weak organic acids, and the own sulphites. Yeasts and *Enterobacteriaceae* were the microbial groups more inhibited by this preservative, and levels of approximately 150 mg/L were enough to prevent their growth. Data obtained in a previous work demonstrated that this organic compound was effective to control table olive microorganisms, but its effect was microbial group dependent, with a higher inhibitory effect against yeast (125 ppm) than on LAB (1060 ppm; Romero-Gil et al., 2016). Its application in olive packaging should be accompanied by sensorial evaluation to determine the influence of the required inhibitory levels on the flavor of the final products due to the characteristic smell of this compound to cinnamon. Recently, CIN was applied to stabilize acidified cucumbers that were adequately preserved free of yeasts (Pérez-Díaz, 2011). Considering the efficient inhibition of yeast in cucumbers, testing CIN against the microorganisms present in table olives may be interesting, especially for the development of new flavored table olives. This compound is obtained from the cinnamon bark. The mechanism of the bactericidal action of CIN against *Listeria monocytogenes*, possible inhibition of glucose uptake, and utilization and effects on membrane permeability, was suggested by Gill and Holley (2004). This compound had both antimicrobial and antioxidant activities when applied to meat, thus, preventing microbial spoilage, and lipid oxidation (Naveena et al., 2013). Carvacrol and CIN were associated with an easier thermal destruction of *E. coli* O157:H7 (Juneja and Friedman, 2008) in raw ground meat: the addition of increasing levels of carvacrol or CIN (range 0.5–1.0%) significantly increased the sensitivity of the microorganism to heat. However, this property would not be of interest for table olives since the application of thermal treatments to the seasoned products would sensibly affect their characteristic flavors. CIN has been reported to show a potential inhibitory effect on methicillin-resistant *S. aureus* biofilm-related to infections (Jia et al., 2011). Recently, CIN has been suggested as a useful compound for the control of *E. coli* at refrigeration temperature (Visvalingam and Holley, 2012). The use of encapsulation is currently observed as an interesting challenge for the application of not only CIN but also other essential (or not) oils (Sagiri et al., 2016).

PYR has a low pK_a value (2.39), a circumstance that allows its use for acidification purposes in table olives. However, a loss of its inhibitory power was observed for SM and CIN preservatives in the presence of this acidifying agent. PYR was useful to control both LAB and yeast growth when applied individually at concentrations around 3200 mg/L (Romero-Gil et al., 2016). This compound was first patented for its preservative properties by Ernst et al. (1979) to stabilize high moisture food products without refrigeration. PYR (and acetaldehyde)-bound sulfur dioxide produced inhibition against wine LAB at a concentration of 5 ppm, albeit the LAB finally degraded such compounds, suggesting that sulfur dioxide-bound PYR could have a bacteriostatic effect rather than a bactericidal action (Wells and Osborne, 2011).

CONCLUSIONS

The *in silico* models obtained have shown that SM and CIN preservatives were very efficient to control the growth of the

main microbial groups found in table olive environment and that HCl is the best acidifying agent. The response of microorganisms as a function of preservative concentration was also different, being the inhibitory effects of SM higher for LAB than for yeast at the same levels of pH, while the opposite behavior was noticed for CIN. The *Enterobacteriaceae* group was markedly affected by the pH of the medium, and they were not able to grow at a pH below 4.0 even in the absence of preservatives. Further verification of these results in synthetic brines as well as validation in real conditions (table olive packaging) are now needed.

AUTHOR CONTRIBUTIONS

VR performed the experimental work. AG and FA designed the work, analyzed the results and written the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01370

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3.3 SECCIÓN III

3.3.1 CAPÍTULO 6:

Survival of foodborne pathogens in natural cracked olive brines.

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Survival of foodborne pathogens in natural cracked olive brines

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ABSTRACT

This work reports the survival (challenge tests) of foodborne pathogen species (*Escherichia coli, Staphylococcus aureus, Listeria monocytogenes,* and *Salmonella enterica*) in *Aloreña de Málaga* table olive brines. The inhibitions were fit using a log-linear model with tail implemented in GInaFIT excel software. The olive brine had a considerable inhibitory effect on the pathogens. The residual (final) populations (*Fp*) after 24 h was below detection limit (<1.30 $\log_{10}$ cfu/mL) for all species assayed. The maximum death rate (k_{max}) was 9.98, 51.37, 38.35 and 53.01 h^{-1}, while the time for 4 $\log_{10}$ reductions ($4D_r$) was 0.96, 0.36, 0.36 and 0.24 h for *E. coli, S. aureus, L. monocytogenes* and *S. enterica,* respectively. Brine dilutions increased *Fp* and *4Dr*, while decreased k_{max}. A cluster analysis showed that *E. coli* had an overall quite different behaviour being the most resistant species, but the others bacteria behaved similarly, especially *S. aureus* and *S. enterica*. Partial Least Squares regression showed that the most influential phenols on microbial survival were EDA (dialdehydic form of decarboxymethyl elenolic acid), HyEDA (EDA linked to hydroxytyrosol), hydroxytyrosol 4-glucoside, tyrosol, and oleoside 11-methyl ester. Results confirm the adverse habitats of table olives for foodborne pathogenic microorganisms.

1. Introduction

Table olives are among the most important fermented vegetable of the food industry with a worldwide production above 2.4 million tons per year (IOC, 2016). They are considered as ready-to-eat products that are consumed without prior cooking. Pasteurization is frequently used to guarantee the stability and safety of fermented olives, but sometimes olives are not subjected to a heat treatment (Garrido-Fernández et al., 1997). This way, microbiota is not always removed thus pathogen contaminations is a potential public health concern. Biogenic amines, mycotoxins, and the presence of pathogens are among the most probable biological hazards found in table olives (Medina-Pradas and Arroyo-Lopez, 2015). Thus, it is necessary to provide scientific data about foodborne pathogen presence/survival on fermented olives, especially in those processed as natural olives, which are commercialized without heat treatment and stabilized only by physicochemical conditions (Garrido-Fernández et al., 1997).

Table olives, as well as other fermented and acidified vegetable foods, have a long history of microbial safety. However, acid-resistant strains of certain pathogenic bacteria have shown that acidic foods may be a source of foodborne diseases (Centers for Disease Control and Prevention, 1996; 1999). Diverse authors have studied the survival and determined the presence of pathogenic bacteria (*Escherichia coli, Listeria monocytogenes, Staphylococcus aureus* and *Salmonella* sp.) in diverse table olive trade preparations (Spyropoulou et al., 2001; Caggia et al., 2004; Pereira et al., 2008; RASFF Portal, 2012a; Argyri et al., 2013; Grounta et al., 2013; Medina et al., 2013; Tataridou and Kotzekidou, 2015). Botulism, associated with *Clostridium botulinum* growth, is certainly the most relevant biohazard in table olives with several reported outbreaks (Debord et al., 1920; Fenicia et al., 1992; Cawthorne et al., 2005; Jalava et al., 2011; Pingeon et al., 2011; RASFF Portal, 2012b). However, the relation between the behaviour of these microorganisms in the olive environment with the presence of phenolic compounds provided by fruits has been scarcely studied. These antimicrobial compounds could limit the growth of microorganisms, mainly in non-alkali-treated olives (Medina et al., 2008; 2009; 2013; Brenes et al., 2011).

Aloreña de Málaga table olives are processed as directly brined green olives under a Spanish Protected Designation of Origin (PDO)

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recognized by the European Union (DOUE, 2012). For cured PDO *Aloreña de Málaga* processing, fruits are harvested, and directly brined in a 60–100 g/L NaCl solution. After fermentation, the olives are cracked, seasoned, and then packaged depending on demand (López-López and Garrido-Fernández, 2006). To preserve their typical green aspect and organoleptic characteristics, fruits are not stabilized by heat treatment. Therefore, safety issues such as the hygienic conditions and studies of foodborne pathogens prevalence in this table olive speciality are of particular interest for risk assessment during storage/shelf life.

The present survey aims to determine the survival of diverse foodborne pathogens (*E. coli, S. aureus, L. monocytogenes,* and *S. enterica*) in the packaging brines of ready-to-eat *Aloreña de Málaga* table olives. Multivariate statistical approaches were used to relate the presence of phenolic compounds to their survivals.

2. Materials and methods

2.1. Preparation of olive brines

PDO *Aloreña de Málaga* fruits were fermented for 3 months in an initial 60 g/L NaCl solution in COPUSAN S.C.A. (Alozaina, Málaga, Spain) during 2013/2014 season. Then, 10 kg of fruits were cracked, washed in water for 24 h to remove excess of salt, and placed in 10 L of 20 g/L of NaCl brine at 4 °C to obtain an equilibrium between fruits and liquid. After 14 days, brines were removed, filter-sterilized (0.2 μm pore size), and analysed to determine its composition (Table 1). Phenolic, oleosidic compounds and reducing sugars in brines were determined by HPLC according to Medina et al. (2013) and Rodríguez-Gómez et al. (2012), respectively. Salt and pH were analysed according to the protocols described by Garrido-Fernández et al. (1997).

The original olive brine (B:10/10) was then diluted with sterile distilled water to reach always a final volume of 10 mL. The following dilutions (olive brine/sterile distilled water) were prepared: i) B:7.5/10 (7.5 mL + 2.5 mL), ii) B:5/10 (5.0 mL + 5.0 mL), iii) B:2.5/10 (2.5 mL + 7.5 mL) and iv) B:1/10 (1.0 mL + 9.0 mL). Also, a medium with only sterile distilled water (C:0/10) was used as a control. Levels of pH, salt, and sugar concentrations in the different dilutions and control were always kept constant by addition of HCl, NaCl, and reducing sugars (glucose, fructose, sucrose, and mannitol), respectively. Therefore, the described above media only differed in the levels of phenolic compounds, which were proportional to the dilution factors.

Table 1
Physicochemical characteristics of the original *Aloreña de Málaga* olive brines used in the present study. Mean absolute deviations obtained from duplicated measurements in parentheses.

Parameter	Compound	Value
Salt (g/l)	NaCl	25.00 (0.00)
pH	[H+]	5.50 (0.01)
Sugars (g/l)	Glucose	6.79 (0.12)
	Fructose	2.12 (0.02)
	Sucrose	0.46 (0.02)
	Mannitol	1.67 (0.01)
	Total	11.04 (0.14)
Phenolic compounds (mM)	Hy	1.04 (0.05)
	Hy 4-Glucoside	0.09 (0.01)
	Tyrosol	0.34 (0.02)
	p-coumaric acid	0.02 (0.01)
	Verbascoside	0.02 (0.01)
	HyEDA	0.14 (0.00)
	Oleuropein	0.16 (0.02)
	Comselogoside	0.00 (0.00)
	EDA	0.13 (0.00)
	Secoxyloganin	0.08 (0.01)
	Secologanoside	0.31 (0.00)
	Oleoside 11-methyl ester	0.28 (0.01)
	Total	2.62 (0.02)

Abbreviations: Hy: hydroxytyrosol. EDA: dialdehydic form of decarboxymethyl elenolic acid. HyEDA: EDA linked to hydroxytyrosol.

The ability of original olive brines to support microbial growth was also confirmed by inoculating the undiluted brine with a strain of *Lactobacillus pentosus* (TOMC-LAB2) and other of *Lodderomyces elongisporus* (TOMC-Y73), isolated previously from diverse table olive processing (Bautista-Gallego et al., 2013; Romero-Gil et al., 2016) and belonging to the Table Olive Microorganisms Collection of Instituto de la Grasa (CSIC-Seville). For this purpose, the microorganisms were cultured at 30 °C in MRS (Oxoid Ltd., Basingstoke, Hampshire, England) and YMPG (Difco, Becton and Dickinson Company, Sparks, MD, USA) broth, washed to remove the culture media and then inoculated in the brines an initial population ca. 5.0 log CFU/mL. The growth was monitored by plating on their respective media.

2.2. Pathogens and inoculum preparation

Four strains of each pathogenic species (*L. monocytogenes, S. aureus, E. coli* and *S. enterica*) were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). Many of them are related with diverse human food poisoning and included in the biosafety level 2. *L. monocytogenes* CECT 4031^T (isolated from rabbit), CECT 4032 (soft cheese), CECT 5366 (human origin) and CECT 7467 (poultry) were cultured at 37 °C in Brain Heart Infusion (BHI, Oxoid) with or without agar (15 g/L). *S. aureus* CECT 86^T (isolated from human pleural fluid), CECT 239 (human lesion), CECT 240 (human lesion) and CECT 976 (ham); *S. enterica* sv. Typhimurium CECT 722^T (natural environment), CECT 443 (human), CECT 4156 (chicken alimentary tract), and *S. enterica* sv. Enteritidis CECT 4300 (unknown); *E. coli* CECT 434 (clinical isolate) and the strains with serotype O157:H7 CECT 4267, CECT 4782 and CECT 5947 (isolated from human faeces) were all cultured at 37 °C in nutrient broth prepared with 5 g/L "Lab-Lemco" powder (Oxoid), 10 g/L of Neutralized bacteriological peptone (Oxoid), 5 g/L NaCl, and 15 g/L of agar in the case of solid medium (pH 7.2).

All these strains were maintained at −80 °C in the adequate culture broths with 20 g/L glycerol. Before the experiments, each strain was cultured in their respective broth media with a previous pre-adaptation phase where NaCl (15 g/L) and pH (5.5) were modified in order to acclimatize the microorganisms to the olive brines. Overnight cultures were centrifuged, washed and re-suspended in sterilized saline solution (9 g/L NaCl). Cocktails of each pathogenic species were prepared by mixing the same quantities of the corresponding strains. The volumes were calculated to obtain ca. 8 log cfu/mL of each strain as initial inoculums. An enumeration of the inoculated cells to confirm the initial population was done in duplicate, using the appropriate medium.

All treatments were dispensed into Eppendorf tubes (1.4 mL), added with 0.1 mL of each bacterial cocktail, and gently mixed. The challenge tests were performed in duplicate at room temperature (20 ± 2 °C), obtaining the average of two individual inoculations. Samples from each tube were removed at different times (0, 0.5, 1, 3, 8 and 24 h), diluted in 1.0 g/L sterile peptone water and plated to count cultivable cells (Spiral Plater Wasp 2, Don Whitley Sci. Ltd., Shirpley, UK) in nutrient agar for *S. aureus, E. coli* and *S. enterica* and in BHI for *L. monocytogenes*.

2.3. Curve fitting of the foodborne pathogen cocktails

The responses of the different pathogen cocktails in the sterile olive brine and their dilutions were fitted using the log-linear

model with a tail (Geeraerd et al., 2000), included in the GInaFIT 1.6 software excel fitting tool (Geeraerd et al., 2005). It has the following expression:

$$\log_{10}(N_t) = \log\left(\left(10\log\hat{}_{10}(Ip) - 10\log\hat{}_{10}(Fp)\right)*\exp(-k_{max}*t) + 10\log\hat{}_{10}(Fp)\right)$$

where the parameters are: N_t, microbial population at time t ($\log_{10}$ cfu/mL); *Ip*, initial microbial population ($\log_{10}$ cfu/mL); *Fp*, final or residual population after 24 h ($\log_{10}$ cfu/mL); and k_{max}, maximum death rate (h^{-1}). The software GInaFIT 1.6 also allows the estimation of *4Dr* (time in hours for a reduction of 4 $\log_{10}$ from *Ip*).

2.4. Statistical analysis

An analysis of variance (ANOVA) was performed by means of the factorial ANOVA module of Statistica 7.1 software package (StatSoft Inc, Tulsa, OK, USA) to determine statistical differences among the response of the several foodborne pathogen cocktails in the original olive brine, their dilutions, and the control. Post-hoc comparisons were achieved by means of the Fisher LSD test.

Cluster analysis is a method for unsupervised learning which tries to find groups containing similar objects. In this work, it was applied based on Pearson correlation coefficient using the pathogen as grouping factor and the fitting parameters as variables. Partial Least Squares (PLS) is a technique that combines features from the Principal Component Analysis and multiple regression, which is particularly useful in case of correlated variables. Its objective is the prediction of a series of dependent variables from a set of independent variables. The prediction is based on the extraction of a series of factors (latent variables) which have the best predictive power. In this study, PLS regression was used to relate the concentration of individual phenolic compounds to the fitted parameters of the foodborne pathogens. Cluster analysis and PLS regression were performed with XLSTAT v.2015.4.01.20116 (Addinsoft, Paris, France). For the analysis, data were centred, standardised and the number of latent variables retained automatically.

3. Results

3.1. Olive brines

The physicochemical characteristics of the non-diluted olive brine are shown in Table 1. The total sugar content was 11.0 g/L, being the glucose (6.8 g/L) and fructose (2.1 g/L) the major components. The total phenolic compound determined by HPLC was 2.62 mM, with hydroxytyrosol (1.04 mM) as the most relevant compound, followed by tyrosol (0.34 mM), secologanoside (0.31 mM), and oleoside 11-methyl ester (0.28 mM). Oleuropein, HyEDA, EDA and Hy-4glucoside were also detected in the brines but at lower concentrations (0.16, 0.14, 0.13 and 0.09 mM, respectively). The pH (5.5) and salt concentration (25 g/L) of the original brine was obtained by placing the fermented fruits in brine for 14 days at 4 °C. Since these levels are not inhibitory for the assayed microorganisms, the possible effects will then be caused exclusively by the phenolic compounds. In all the diluted brines and the control, the pH, salt, and sugars were the same while the phenolic compounds decreased in proportion to the dilution factor.

As a preliminary test to verify the suitability of olive brines to support microbial growth, the brine was inoculated with a strain of yeast and other of lactic acid bacteria, previously isolated from packaged natural green olives and Spanish-style green table olive fermentations, respectively. Both microorganisms grew vigorously (from initial 5.0 to approximately 7.0 $\log_{10}$ cfu/mL in 48 h), as confirmed by plate count. These data supports, then, that the original *Aloreña de Málaga* brine is a good media for the growth of well adapted native microorganisms and inhibition is not due to the absence of fermentable substrates.

3.2. Survival of the foodborne pathogens

The independent inoculation of the four pathogen cocktails in the olive brines obtained from *Aloreña de Málaga* fruits, as well as in their respective dilutions, led to a total of 48 inhibition curves. Their changes in population were fit (Fig. 1) by the log-linear model with a tail (Geeraerd et al., 2000). In most treatments, the fit was good with an R^2 above 0.95 and the Root Mean Sum of Squared Error (RMSE) ranging from 0.000 to 1.730. From the initial population (*Ip*), the model considers a first step of linear inactivation of microorganisms (k_{max}), followed by a second step represented by a tail or residual population (*Fp*). The slope of the linear inactivation phase (Fig. 1) was higher in the undiluted (B:10/10), and first two diluted brines (B:7.5/10 and B:5/10). On the contrary, pathogens kept practically constant their initial population levels (~8 $\log_{10}$ cfu/mL) in the control (C:0/10) through the 24 h study. The behaviour of *E. coli* cocktail in the most diluted brine (B:1/10 and B:2.5/10) was also very similar compared to control. By contrast, for *L. monocytogenes* cocktail, except for the control and B:1/10 media, the response of the pathogen was very similar with small differences among the different treatments.

The inhibition parameters deduced from the fit of the mathematical model to the survival data is shown in Table 2. The *Fp* parameter ranged from 7.92 (*E. coli* in control media) to 1.30 $\log_{10}$ cfu/mL (obtained for many of the species in the undiluted olive brine), the k_{max} parameter ranged from 0.09 (*S. enterica* in the control) to 53.01 h^{-1} (*S. enterica* in the undiluted olive brines), while the *4Dr* parameter ranged from 0.24 (*S. enterica* in the undiluted olive brines) to >24.0 h (for all assayed species in the control). The mean absolute deviations obtained for the fitted parameters from duplicate experiments were low, except for k_{max} in some treatments. In general, as olive brine was diluted, there was an increase in the final remaining population (*Fp*) and time to reduce 4 $\log_{10}$ (*4Dr*) parameters as well as a reduction in the maximum death rate (k_{max}) value (Fig. 2). Thereby, olive brine loss its inhibitory power as dilution increased, fact that was particularly evident for dilutions higher than B:7.5/10 (Fig. 2). The factorial ANOVA analysis of the fitted parameters as a function of categorical variables (pathogen cocktails and type of medium) showed that main effects and interaction of categorical variables were statistically significant ($p < 0.05$) for all parameter assayed (*4Dr*, k_{max}, *Fp*, *Ip*). The RMSE was 1819.18, 0.259, 0.950, and 0.167 for k_{max}, *Fp*, *4Dr*, and *Ip* parameters, respectively, indicating a poorer estimation of effects for k_{max}. The ANOVA shows that *E. coli* was the most resistant microorganism, with the higher statistical significant *Fp* and *4Dr* and the lower k_{max} values for many of the assayed conditions (Fig. 2). Otherwise, *L. monocytogenes* and *S. enterica* were the most sensible microorganisms. Furthermore, even the most diluted media (B:1/10) had a statistically significant higher inhibitory effect on parameters *4Dr* and *Fp* compared to control (Fig. 2).

3.3. Effect of phenolic compounds on the survival of pathogens

The cluster analysis showed that the effect of the polyphenols on *S. enterica* and *S. aureus* in the olive brines were quite similar, although that on *L. monocytogenes* was close but different (Fig. 3). The response of *E. coli* was, on the contrary, rather different (Fig. 3). The segregation is in agreement with its highest *Fp* in brine, the

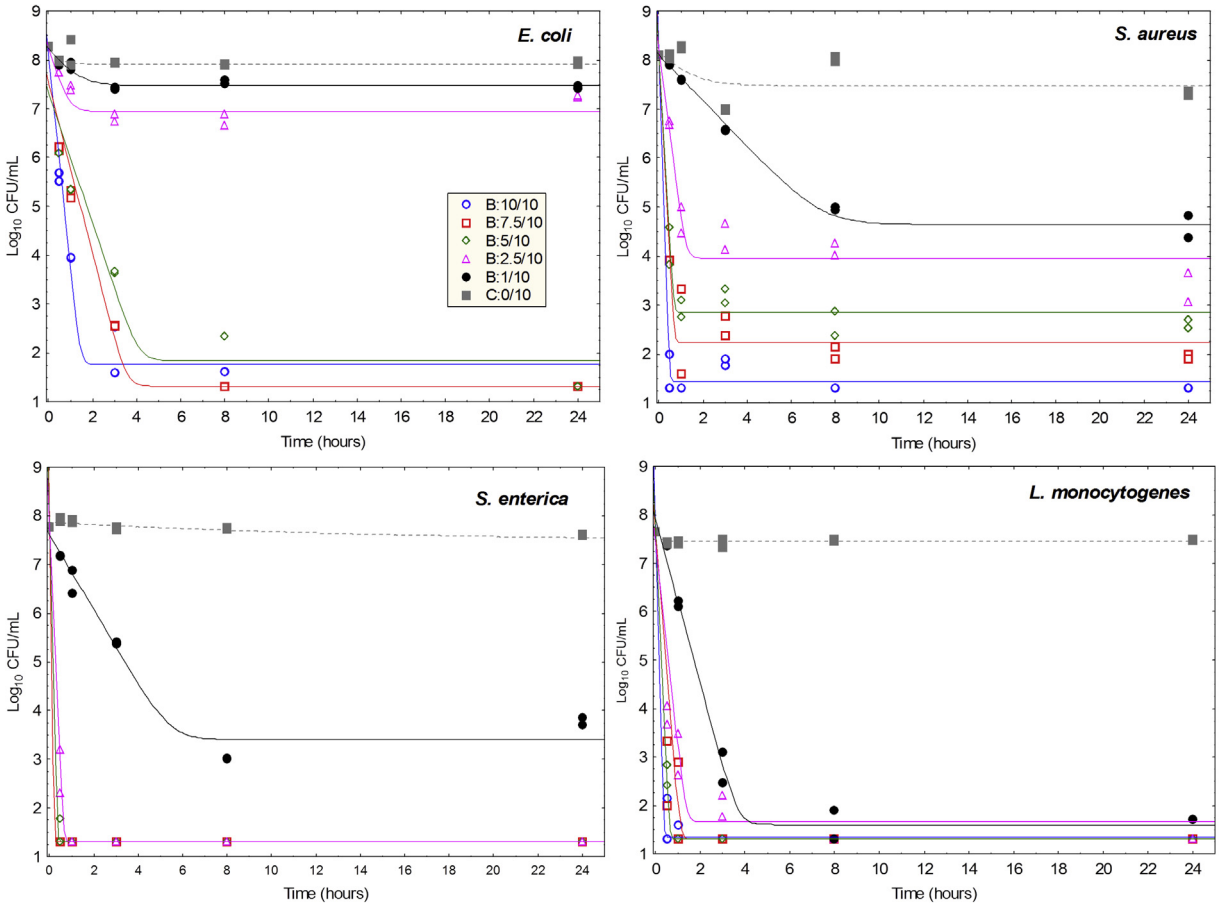

Fig. 1. Fit of the log-linear model with tail to the experimental data of the *E. coli, S. aureus, S. enterica* and *L. monocytogenes* cocktails obtained in the olive brine (B:10/10), their dilutions (B:7.5/10, B:5/10, B:2.5/10, and B:1/10), and the control (C:0/10). For each sampling time, duplicated values are shown. Due to similar responses symbols may overlap. The levels of pH (5.5), salt (25 g/L), and sugars (11 g/L) were kept constant in the undiluted olive brine, diluted samples and control.

longest period of *4Dr* and lowest k_{max} obtained for this species (Table 2).

The PLS regression analysis, using the concentrations of the diverse phenols as independent variables and the parameters obtained from the fitted curves (*Fp, 4Dr,* and k_{max}) as dependent, showed that the parameters *Fp* and *4Dr* for the *S. aureus* and *E. coli* cocktails were highly correlated (>0.80) with all the phenolic compounds while the same parameters obtained for *L. monocytogenes* and *S. enterica* showed a sensibly lower relationship (between 0.60 and 0.70). Similarly, for all the species assayed, the parameter k_{max} was clearly related to all the phenolic compounds with values approximately similar (>0.80). Therefore, the presence of phenolic compounds in the brines led to a stressing environment, which may affected the pathogens' survival selectively.

The number of latent variables retained were 4 and the model explained a high proportion of the variable variances ($R^2X_{cum} = 1.00$; $R^2Y_{cum} = 0.92$) but had a moderate overall fit ($Q^2_{cum} = 0.60$). The contributions of the diverse phenols in the new axes (usually name as *t*) were the following: all the phenols contributed similarly to *t1* (ca. −0.300), the main contributor to *t2* was HyEDA (0.825), with a positive sign. In the case of *t3,* the situation was more complex, and the phenols that most contributed to it were Hy4Glu (0.659) and EDA (−0.612). Finally, Ty (0.492) and Oleos11 (−0.644) were the main contributors to *t4*. The projection onto new axes *t1* and *t2* (Fig. 4) show the overall correlations among the different variables and treatments. All k_{max} were strongly and positively related to the presence of phenols, mainly that of *S. enterica* which was particularly associated to HyEDA and Hy4Glu. This way, higher concentrations of phenolic compounds increased the death rate of foodborne pathogens. On the contrary, the *Fp* and *4Dr* parameters were negatively linked to the presence of phenols. That is, increasing contents of phenols reduce the times to reach *4Dr* and lead to a lower residual population of pathogens. The situation of cases (treatments) moves from left (the highest phenol content is associated to k_{max}) to right, according to the concentrations of phenols (the control, without phenols, is associated with higher survival, *4Dr* and *Fp*). Only the B:7.5/10 treatment is clearly separated from the rest.

4. Discussion

Predictive modelling techniques have been used in the present study to determine the survival of different foodborne pathogens in olive brine. The use of this discipline allows the quantification and comparison of the response of microorganisms in the food matrix by the use of mathematical models and estimation of fitted parameters (McMeekin et al., 1993). In this paper, we have prioritized the selection of strains related with human poisoning rather than a vegetable origin. Albeit strains obtained from vegetable matrixes could be better adapted to olive environment, not all of them can produce food poisoning. This way, we think that information obtained for registered pathogenic strains causing disease in humans will be more useful for further risk assessment.

Table 2
Parameters obtained using the Log linear model with tail to the survival data of the foodborne pathogen cocktails in the original *Aloreña de Málaga* brines and their dilutions. Mean absolute deviations obtained from duplicate experiments in parentheses. *Ec, Sa, Lm* and *Se* stand for the *E. coli, S. aureus, L. monocytones* and *S. enterica* cocktails, respectively.

Brine[a]	Initial population (Ip) $\log_{10}$ CFU/mL				Final population (Fp) $\log_{10}$ CFU/mL				Time for 4 $\log_{10}$ reduction ($4Dr$) (h)				Maximum death rate (k_{max}) (h^{-1})			
	Ec	*Sa*	*Lm*	*Se*	*Ec*	*Sa*	*Lm*	*Se*	*Ec*	*Sa*	*Lm*	*Se*	*Ec*	*Sa*	*Lm*	*Se*
10/10	8.10 (0.03)	8.11 (0.00)	7.64 (0.02)	7.78 (0.00)	1.60 (0.10)	1.42 (0.03)	1.34 (0.04)	1.30 (0.00)	0.96 (0.00)	0.36 (0.12)	0.36 (0.12)	0.36 (0.12)	0.24 (0.00)	9.98 (0.02)	51.37 (22.57)	38.35 (1.68)	53.01 (0.00)
7.5/10	7.54 (0.03)	8.11 (0.00)	7.63 (0.02)	7.78 (0.00)	1.31 (0.00)	2.25 (0.18)	1.30 (0.00)	1.30 (0.00)	2.40 (0.00)	0.48 (0.00)	0.48 (0.00)	1.08 (0.60)	0.24 (0.00)	4.07 (0.01)	19.41 (0.02)	13.23 (6.73)	53.01 (0.00)
5/10	7.35 (0.00)	8.11 (0.00)	7.65 (0.00)	7.78 (0.00)	1.82 (0.00)	2.83 (0.03)	1.30 (0.00)	1.30 (0.00)	3.12 (0.00)	0.60 (0.12)	0.60 (0.12)	0.48 (0.00)	0.36 (0.12)	3.10 (0.02)	18.10 (1.85)	23.27 (1.01)	40.75 (12.25)
2.5/10	8.25 (0.00)	8.21 (0.03)	7.40 (0.24)	7.78 (0.00)	6.94 (0.01)	3.96 (0.02)	1.68 (0.06)	1.30 (0.00)	>24.00 (0.00)	1.32 (0.12)	1.32 (0.12)	0.84 (0.12)	0.48 (0.00)	2.38 (0.11)	7.87 (0.65)	13.97 (4.27)	23.28 (2.16)
1/10	8.25 (0.00)	8.08 (0.05)	7.90 (0.07)	7.61 (0.06)	7.47 (0.01)	4.64 (0.22)	1.65 (0.15)	1.30 (0.00)	>24.00 (0.00)	>24.00 (0.00)	>24.00 (0.00)	2.52 (0.12)	5.88 (0.12)	1.50 (0.27)	1.08 (0.12)	3.92 (0.27)	1.77 (0.02)
Control (0/10)	8.26 (0.00)	8.28 (0.02)	7.67 (0.02)	7.86 (0.03)	7.92 (0.01)	7.48 (0.03)	7.46 (0.03)	7.55 (0.05)	>24.00 (0.00)	>24.00 (0.00)	>24.00 (0.00)	>24.00 (0.00)	>24.00 (0.00)	2.86 (2.43)	1.17 (0.00)	3.13 (3.13)	0.09 (0.03)

[a] Proportion of the original olive brine in a final volume of 10 mL.

Contamination of olives with pathogenic bacteria may be due to poor hygiene and unsanitary procedures by field and processing personnel, inadequate cleaning and sanitizing of processing equipment, and failure to wash the olives prior to brining. Although no severe food poisoning outbreaks have been associated with table olive consumption, recent studies have indicated the survival of certain pathogens in this fermented vegetable. Specifically, the survival of *L. monocytogenes, E. coli, S. enterica* and *S. aureus* has been reported in olive matrix (Spyropoulou et al., 2001; Skandamis and Nychas, 2003; Randazzo et al., 2012; Argyri et al., 2013; Grounta et al., 2013: Medina et al., 2013; Panagou et al., 2013). The results of many of such studies show the rapid decline of the population of these pathogens during processing indicating that the olive environment does not support well pathogen growth. Our results endorse all these previous works, and show that the assayed pathogenic bacteria were usually below the detection limits (<1.3 $\log_{10}$ cfu/mL) in the *Aloreña de Málaga* brines within the first 3 h after inoculation. However, in certain cases, the survival of pathogens can be extended up to 14 days even at low pH (4.2) and high salt concentration (60 g/L) as it was reported for *L. monocytogenes, E. coli* and *Salmonella* in the packaging of lye treated green olives of Halkidiki cv. (Argyri et al., 2013). Grounta et al. (2013) also mentioned a higher survival (>24 h) of pathogenic bacteria in packaged fermented natural black olives Conservolea variety. These data show the importance of specific studies for each table olive trade preparation because of the differences in the survival among foodborne pathogens, as well as the considerable inhibitory effect of *Aloreña de Málaga* brines on pathogen survival.

Recently, Medina et al. (2013) showed that the decline of pathogen populations was variable depending on the composition of the olive brines in phenolic compounds. This way, the time needed to reduce the inoculated pathogen populations of *L. monocytogenes, S. aureus, E. coli* and *S. enterica* by 5 $\log_{10}$ oscillated between less than 5 min and up to 17 days in the least deleterious conditions. The most lethal effect was observed in non-lye treated brines of Hojiblanca and Manzanilla variety, with a higher concentration of HyEDA and EDA phenols than the corresponding lye treated olive brines. Inhibitory compounds in table olives are formed in acid conditions by the action of endogenous enzymes of olive fruits, which hydrolyse the molecule of the oleuropein into HyEDA, and later, EDA. In contrast, these compounds are not detected in any of the brines from olives treated with NaOH, as has already been reported (Medina et al., 2008). EDA and HyEDA, together with oleoside-11-methyl ester, are the substances in olive brines that showed the greatest antimicrobial activity (Brenes et al., 2011; Medina et al., 2009), as it was also confirmed in the present study. The curve fitting approach used in this work give us more detailed information on the pathogen responses in the olive brines (maximum death rate, residual populations, time for 4 $\log_{10}$ reduction, etc.) than previous studies (Medina et al., 2013). Furthermore, the results were also subjected to a multivariate analysis for investigating the possible relationships between individual phenolic compounds and pathogen survival.

In the present study, the effect of HyEDA and EDA was noticed at lower concentrations (~0.1 mM for each compound) compared to values found in other table olive varieties where an inhibitory effect was obtained with values ranging from 0.4 to 0.8 mM (HyEDA) and from 1.1 to 1.2 mM (EDA) (Medina et al., 2013). A lower concentration of antimicrobial compounds requires more time of contact to reduce the initial population. Medina et al. (2009) observed that low concentrations of HyEDA (0.075 mM) are able to reach a 4 $\log_{10}$ reduction of *S. aureus* in 15 min. Data from this work are in line with those previous observed. In fact, the brines tested are less inhibitory when brine dilutions are between 7.5/10 and 2.5/10, which

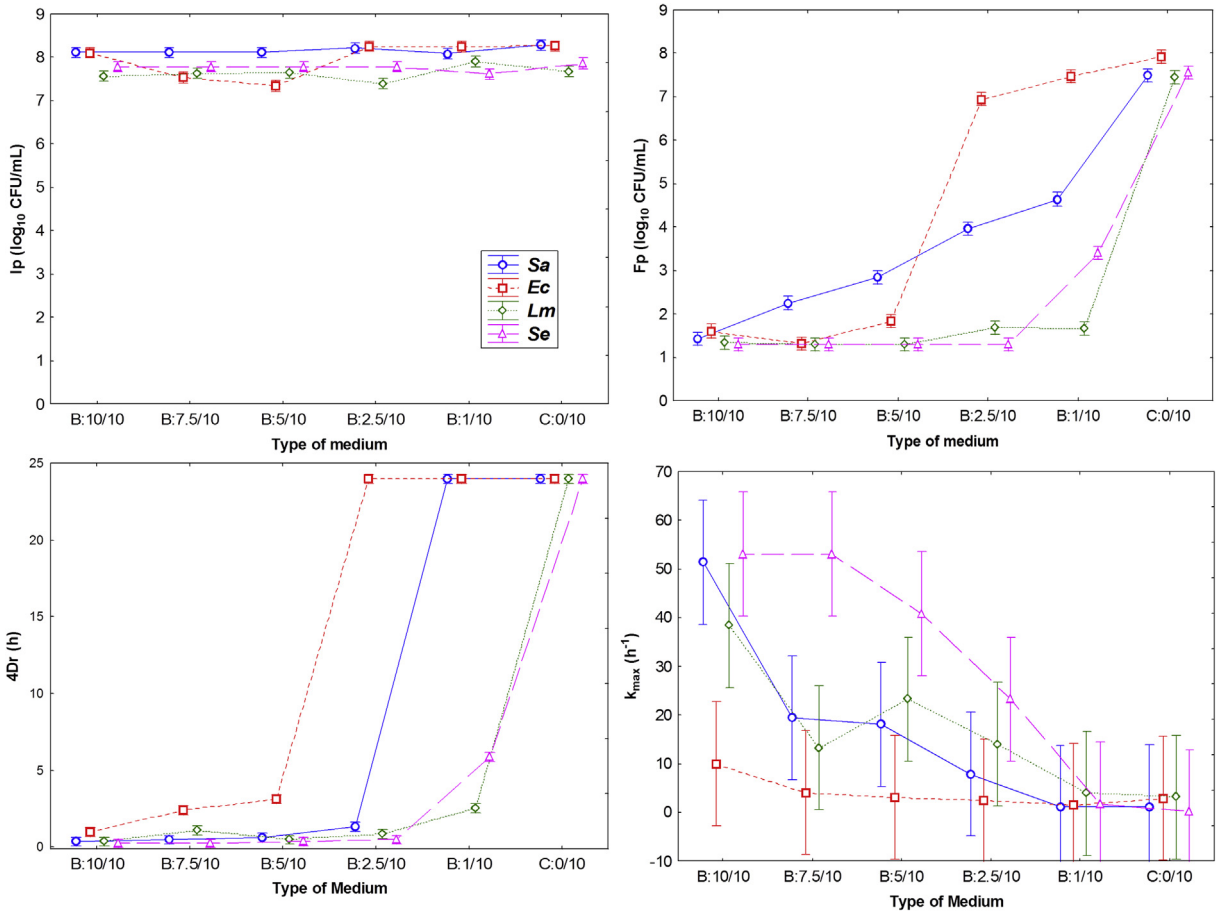

Fig. 2. Graphical representation of the factorial ANOVA analysis carried out for the inhibition parameters *Ip* (initial population), *Fp* (final or residual population), *4Dr* (time for 4 $\log_{10}$ reduction) and k_{max} (maximum death rate) obtained with the log-linear model with tail to the survival data of the foodborne pathogens in the olive brine and their dilutions. *Sa, Ec, Lm* and *Se* stand for the *S. aureus, E. coli, L. monocytogenes* and *S. enterica* cocktails, respectively. B:10/10 stand for the undiluted olive brine, while B:7.5/10, B:5/10, B:2.5/10, B:1/10 stand for their dilutions, respectively. C:0/10 stand for the control. Vertical bars denote 0.95 confidence limits.

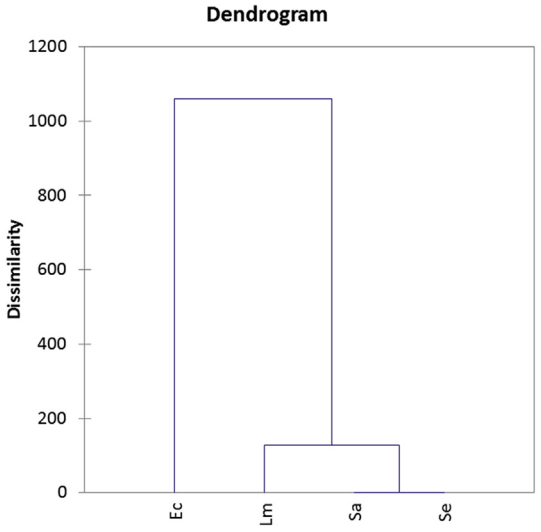

Fig. 3. Cluster analysis grouping pathogens species according to their survival behaviours in olive brines. *Sa, Ec, Lm,* and *Se* stand for the *S. aureus, E. coli, L. monocytogenes,* and *S. enterica* cocktails, respectively.

correspond to a 0.10–0.035 mM concentration rage of HyEDA and the time needed to reach the 4 $\log_{10}$ reduction is longer. Thus, HyEDA concentration found in this *Aloreña de Málaga* olives brine can explain by itself the bactericidal effect exerted against the pathogens tested. However an additive effect was also observed for the three antimicrobial compounds HyEDA, EDA and Ole11Me (Medina et al., 2007). From PLS regression analysis, Hy4Glu has also been correlated with the bactericidal effect exerted by the brines. However, high concentrations of this compound (1.5 mM) did not exert any antimicrobial effect against *L. pentosus* (Medina et al., 2007). Nevertheless, the combined effect of all phenolic substances that may be present in olive brines is ultimately responsible for their antibacterial power (Medina et al., 2007).

In this paper, *E. coli* was the most resistant bacteria, while *S. aureus, L. monocytogenes* and *S. enterica* were the most sensible microorganisms. These data contrast with those obtained by Argyri et al. (2013) and Grounta et al. (2013) who found *L. monocytogenes* as the most resistant pathogen in the olive environment. Medina et al. (2013) studied the survival of different pathogens in various types of olive brines obtained from Hojiblanca, Manzanilla and Gordal varieties. They found that at room temperature *S. aureus* was the most resistant pathogen for all tested brines while *L. monocytogenes* and *S. enterica* were the least resistant to the deleterious effect of the olive brines. These differences in response of pathogenic microorganisms might be mainly due to: i) different origin of strains used, and ii) the different olive matrix composition (phenolic content, pH and salt levels, etc.). Breidt et al. (2007) have preferred the use *E. coli* as the target microorganisms for survival

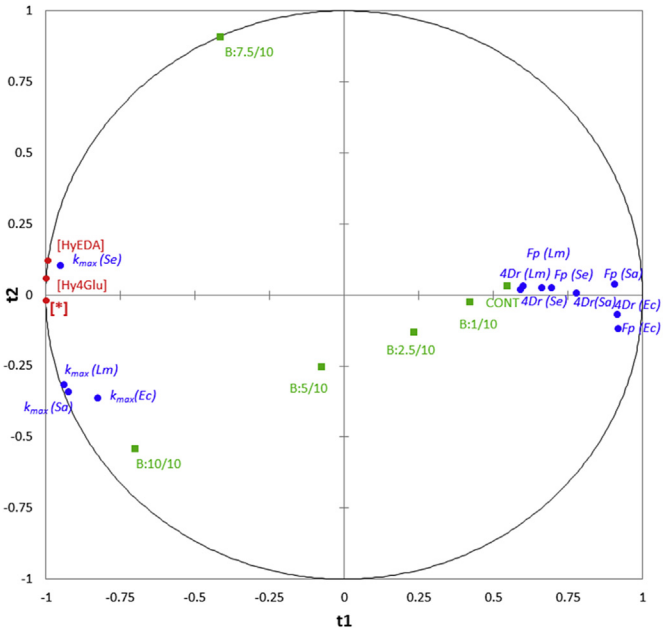

Fig. 4. PLS regression analysis. Projection of variables (independent and dependent) and treatments on the plane of the first two latent variables (*t1* and *t2*). The meanings of the cocktail of pathogens are: *Sa, S. aureus*; *Ec, E. coli*; *Lm, L. monocytogenes* and *Se, S. enterica*. Other abbreviations stands for: *Fp*, final or residual population; k_{max}, maximum death rate; $4D_t$, time to obtain 4 $\log_{10}$ reduction; Hy4Glu, Hydroxytyrosol 4-glucoside; HyEDA, EDA linked to hydroxytyrosol; *, rest of phenolic compounds. Finally, B:10/10 stand for the undiluted olive brine, while B:7.5/10, B:5/10, B:2.5/10, B:1/10 stand for their different dilutions in sterile distilled water. C:0/10 is the reference for the control.

studies in pickles because its higher resistance than other foodborne pathogens, as also confirmed in the present study.

5. Conclusions

Aloreña de Málaga olive brines provide an adverse habitat for the development of foodborne pathogens. *E. coli* was the most resistant microorganisms. The results suggest its use as an appropriate target for further studies concerning olive safety issues. This species showed in less than 1 h a 4 $\log_{10}$ reduction in its initial population, and it was below the limit of detection (<1.30 $\log_{10}$ cfu/mL) after 3 h. The inhibitory power of olive brines was statistically related to the concentration of certain phenolic compounds, and it was disappearing as phenolic compounds were diluted. The pathogenic species tested could have a different survival in real olive packaging, where the presence of preservatives, lower pH levels and higher salt concentrations is usual. This point should be validated in further studies, also taking into account other strains of these pathogens, different physiological history of cells (e.g. acidic or low water activity adaptation) as well as the effect of different initial inoculum levels.

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3.3.2 CAPÍTULO 7:

Foodborne pathogen survival in commercial Aloreña de Málaga table olive packaging.

Foodborne pathogen survival in commercial *Aloreña de Málaga* table olive packaging

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Running title: Survival of pathogens in *Aloreña* packaging

[1] Both authors contributed equally to this work.

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Abstract

This study evaluates the survival of diverse foodborne pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella enterica*) in three commercial *Aloreña de Málaga* table olive presentations (fresh green, traditional, and cured olives). The microbial survival in this green natural table olive speciality was fit using a log-linear regression model implemented in GInaFIT. The contents of sugars, phenolic compounds, additives, salt, pH, and levels of autochthonous microorganisms differed among presentations and affected the survival of microorganisms. The inoculated initial populations of pathogens (7-8 $\log_{10}$ CFU/mL brine) decreased rapidly and, 48 h after inoculation, their counts were always below the detection limit (<1.3 $\log_{10}$ CFU/mL), except for *S. aureus* in the fresh the green presentation (~ 5.0 $\log_{10}$ CFU/mL). The highest maximum death rates (k_{max}) and lowest periods for 4 $\log_{10}$ reductions (*4Dr*) were observed in cured olives but decreased and increased, respectively, from the traditional to the fresh green presentations. *L. monocytogenes* and *S. aureus* were the most resistant species. The multivariate analysis showed that high concentrations of compounds (phenols and sugars) released from the olives were positively associated to *4Dr* and negatively to k_{max}. Conversely, the presence of preservatives reduced *4Dr* and increased k_{max}. This study, executed in real products, pointed out that packaged table olives are adverse habitats for foodborne pathogens but the effects are presentation dependent.

Keywords: Challenge tests; Food safety; Phenolic compounds; Survival models.

1. Introduction

Table olives are one of the most traditional fermented vegetable in the Mediterranean basin, with a worldwide production of around 2.4 million tons per year (IOC, 2016). In addition to chemical treatments, the diverse microbial growth that takes place during fermentation makes them edible and they are responsible for their appreciated organoleptic properties. The control of the microbiota is essential during processing and, particularly, during packaging to guarantee the stability and safety of the final product, especially in those presentations commercialized without heat treatments (Garrido-Fernández et al., 1997).

The predominant microorganisms in table olives are lactic acid bacteria and yeast; however, the presence of pathogenic bacteria such as *Escherichia coli, Listeria monocytogenes, Staphylococcus aureus* and *Salmonella* sp. in table olives from different origins has been reported (Spyropoulou et al., 2001; Caggia et al., 2004; Pereira et al., 2008; RASFF portal 2012a; Argyri et al., 2013; Grounta et al., 2013; Medina et al., 2013; Tataridou and Kotzekidou, 2015). Despite it, the relationship between the physicochemical and microbiological characteristics of olive packaging and the behaviour of foodborne pathogens in the different olive products has been scarcely studied.

The Aloreña de Málaga variety is processed as natural cracked green olives under a Spanish Protected Designation of Origin (PDO) recognized by the European Union (DOUE, 2012). Their peculiar characteristics are related to the production area (climate, edaphology, and geographical location) and presentations, which make their products quite different from other green table olives. *Aloreña de Málaga* usually contains low-to-moderate concentrations of oleuropein (the main bitter compound of

olives), and for this reason, is not subjected to lye debittering. This speciality is frequently seasoned with fennel, thyme, garlic, and pepper, making it rich in aroma. To preserve their typical green aspect and organoleptic characteristics, *Aloreña de Málaga* fruits are not stabilized by heat treatment. Therefore, safety issues related to the hygienic conditions and studies on the prevalence of foodborne pathogens in these table olives (ready to eat) are of particular interest for risk assessment during storage/shelf life.

The PDO regulation includes 3 different presentations of *Aloreña de Málaga* table olives (López-López and Garrido-Fernández, 2006), which are:

i) Fresh green *Aloreña de Málaga* olives. The product is characterized by the immediate cracking after harvesting. Then, the fruits are brined in a 10-11% NaCl solution (in 220 L volume plastic drums), where they remain only for 3 days, after which the partially debittered olives are seasoned and packaged.

ii) Traditional *Aloreña de Málaga* olives. The fruits are also cracked and brined (10-11% NaCl, in 200 L volume plastic drums) after harvesting, but the olives are stored in the NaCl solution for, at least, 20 days. During this period, the fruits undergo a partial fermentation, which intensity depends on the storage time. Then, the olives are seasoned and packaged according to demand, using similar conditions than in the previous (fresh) process.

iii) Cured *Aloreña de Málaga* olives. In this case, the harvested fruits are placed directly in brine (5-6% NaCl, in 10,000 L volume fermentation vessels) where they undergo a full fermentation for a minimum of 90 days. Then, the olives are progressively cracked, seasoned and packaged, according to demand.

In a previous study carried out in our laboratory (Medina et al., 2016), the survival of the foodborne pathogenic species *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. enterica* in a model system composed by sterile brines obtained from fermented *Aloreña de Málaga* fruits was determined. The survival was related to the presence of certain phenolic compounds. The objective of the present study was to investigate the prevalence of these pathogenic species in actual commercial presentations of the *Aloreña de Málaga* speciality, taking into account that the presence of olives may substantially modify the initial packaging media. A multivariate approach was used to associate the pathogens' behaviour with the environmental conditions in the packages (physicochemical parameters, polyphenols, sugars, preservatives and indigenous microbiota), not previously contemplated in the model system.

2. Materials and methods

2.1. Foodborne pathogens

For this survey, 4 strains of each pathogenic species *L. monocytogenes*, *S. aureus*, *E. coli* and *S. enterica* were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). All of them are related to diverse human food poisoning and included in the biosafety level 2. *L. monocytogenes* CECT 4031^T (isolated from rabbit), CECT 4032 (soft cheese), CECT 5366 (human origin) and CECT 7467 (poultry) were cultured at 37ºC in Brain Heart Infusion (BHI, Oxoid LTD, Basingstoke, England) with or without agar (15 g/L). *S. aureus* CECT 86^T (isolated from human pleural fluid), CECT 239 (human lesion), CECT 240 (human lesion) and CECT 976 (ham); *S. enterica* sv. Typhimurium CECT 722^T (natural environment), CECT 443 (human), CECT 4156 (chicken alimentary tract), and *S. enterica* sv. Enteritidis CECT 4300 (unknown); *E. coli* CECT 434 (clinical isolate) and the strains with serotype O157:H7 CECT 4267,

CECT 4782 and CECT 5947 (isolated from human faeces) were all cultured at 37ºC in nutrient broth prepared with 5 g/L "Lab-Lemco" powder (Oxoid), 10 g/L of Neutralized bacteriological peptone (Oxoid), 5 g/L NaCl, and 15 g/L of agar in the case of solid medium (pH 7.2). All pathogen strains were maintained at -80ºC in the adequate culture broths with 20 g/L glycerol until their use.

2.2. Trade presentations packaging

This work was carried out with commercial packages of seasoned cracked *Aloreña de Málaga* olives. The packages were collected in the 2015/2016 season from 4 different industries located in the Guadalhorce Valley (Málaga, Spain) and included the 3 PDO *Aloreña de Málaga* presentations: traditional (Trad), cured (CUR-A and CUR-B), and fresh green (Fresh). Pet packages were filled by industry with 0.7 or 0.5 kg of fruits, seasoning material (a mixture of diced garlic, pepper strips, and small pieces of fennel and thyme) and 0.5 or 0.3 L of cover brine for both cured and fresh or traditional olives, respectively. Twelve different containers (intended for further inoculation) were obtained for each presentation and industry, making a total of 48 table olive packaging. In parallel, 3 packages from each industry were randomly chosen to determine their composition in phenolic, oleosidic compounds, and reducing sugars in brines by HPLC according to Medina et al. (2013) and Rodríguez-Gómez et al. (2012), respectively. Salt, pH, titratable and combined acidity were also determined according to the protocols described by Garrido-Fernández et al. (1997). The additive levels used in each packaging (potassium sorbate, sodium benzoate, citric and ascorbic acid levels) were provided by the industry.

The population levels of the autochthonous microorganisms in the different presentations of *Aloreña de Málaga* table olive packaging were also determined before

inoculation. For this purpose, brine samples were diluted, if necessary, in a sterile saline solution (0.9% NaCl) and plated using a Spiral System model dwScientific (Dow Whitley Scientific Limited, England) on appropriate media. *Enterobacteriaceae* were counted on Crystal-violet Neutral-Red bile glucose (VRBD) agar (Merck, Darmstadt, Germany), lactic acid bacteria (LAB) were spread onto de Man-Rogosa, and Sharpe (MRS) agar (Oxoid) supplemented with 0.02% sodium azide (Sigma, St. Luis, USA), and yeasts were grown on a yeast–malt–peptone–glucose medium (YM) agar (Difco, Becton and Dickinson Company, Sparks, MD, USA) supplemented with oxytetracycline and gentamicin sulphate (0.005%, wt/vol) as selective agents for yeasts. The plates were incubated at 30ºC for 24 (*Enterobacteriaceae)* or 72 h (yeasts and LAB). Then, plates were counted using a Flash&Go (IUL, Barcelona, Spain) image analysis system. Brine counts were expressed as $\log_{10}$ CFU/mL.

2.3. Challenge tests

Before inoculation, each foodborne pathogen strain was cultured in their respective broth media with a previous pre-adaptation phase where NaCl (15 g/L) and pH (5.5) were modified to adapt the microorganisms to the olive brines. Cocktails of each pathogenic species were prepared by mixing equal broth quantities of the corresponding strains. Overnight cultures were centrifuged, and pellets were washed in sterilized saline solution (9 g/L NaCl) and centrifuged again. Ten percent of brine volumes were withdrawn from the olive packaging in sterile conditions and used to re-suspend the cell pellets. After mixing, the strain cocktails were returned as inoculums. The volumes were calculated to obtain ca. 8 log CFU/mL of each strain as initial inoculums in the olive packaging. An enumeration of the inoculated cells to confirm the initial population was done in triplicate, using the appropriate medium.

The challenge tests were performed in triplicate at room temperature (20±2ºC), obtaining the average of 3 individual inoculations. Samples from each olive packaging were removed at different times (0, 1.5, 6, 24, and 48 h), diluted if necessary in 1.0 g/L peptone and plated to count pathogen cultivable cells in appropriate media. Baird-Parker agar (Oxoid) was used for *S. aureus*, MacConkey agar w/sorbitol (Laboratorios Conda, Madrid, Spain) for *E. coli*, Xylose Lysine Desoxycholate Agar (Laboratorios Conda) for *S. enterica* and Palcam *Listeria* agar base supplemented with selective supplement (Laboratorios Conda) for *L. monocytogenes*. The responses of the different pathogen cocktails in the 3 types of *Aloreña de Málaga* table olive packaging were fitted using the log-linear regression model (Bigelow and Esty, 1920), included in the GInaFIT 1.6 software excel fitting tool (Geeraerd et al., 2005). It has the following expression:

$$\mathrm{Log}_{10}(N_t) = \log_{10}(N_0) - k_{max} * t/\ln(10)$$

where the parameters are: N_t, the microbial population at time t ($\log_{10}$ CFU/mL), N_0, initial microbial population ($\log_{10}$ CFU/mL), and k_{max}, maximum death rate (h^{-1}). The software GInaFIT 1.6 also allows the estimation of *4Dr* (time in hours for a reduction of 4 $\log_{10}$ from N_0).

2.4. Statistical analysis

An analysis of variance (ANOVA) was performed using the factorial ANOVA module of Statistica 7.1 software package (StatSoft Inc, Tulsa, OK, USA) to determine statistical differences among the response of the several foodborne pathogen cocktails in the packaging from the 4 industries (only 3 commercial presentations). Post-hoc comparisons were achieved using the Scheffé test.

The data (a matrix consisting of the concentrations of phenols, sugars, organic acids, preservatives, initial LAB and yeast populations, and the parameters deduced from the model fit to the pathogen survival) were also subjected to hierarchical cluster analysis (centred data, Euclidean distance, Ward's method) to disclose the dissimilarities among the diverse treatments, replicates within them, and study the profiles of the presentations. The effect of the diverse variables on the parameters obtained from the fit inhibition models was also studied by Partial Least Square regression (PLS), using centred and standardised data. PLS combines features from the Principal Component Analysis and multiple regression, which application is specifically appropriate in the case of correlated variables. The first step is the selection of latent variables able to explain the maximum proportion of independent variable variances and as much as possible of the dependent variable set. The extracted factors (latent variables) also have the best predictive power of the dependent variable. The great advantage of this statistical technique over classic regression is the available charts that describe the data structure. Thanks to the correlation and loadings, and score plots, the relationship among the variables (the exploratory, dependents, and between both of them) and the proximity of samples and dataset structure are studied (Abdi, 2007). The number of latent variables was retained automatically. The performance of the fit equations obtained from the inhibition response (*4Dr* and k_{max}) as a function of independent variables (phenolic compounds, organic acids, preservatives, and initial microbiota) for each pathogen was checked by cross-validation. The multivariate analyses were performed using XLSTAT v.2015.4.01.20116 software (Addinsoft, Paris, France) and Minitab 15 v. 15.1.20.0 (Minitab Inc., State College, PA).

3. Results

3.1. Characterisation of commercial presentations

The physicochemical characteristics of the 3 industrial olive presentations were analysed (Table 1). The 3 *Aloreña de Málaga* table olive elaborations showed clear differences among them; the highest total sugar content was found in the fresh green (9.6 g/L), followed by traditional (4.63 g/L), and cured (0.42 and 2.03 g/L in samples A and B, respectively), being glucose and fructose the major components (Table 1). The highest total phenolic compound was also observed in the fresh green presentation (8.9 mM) and decreased from traditional (4.9 mM) to cured olives (3.4 and 2.9 mM in samples A and B, respectively). There were also differences in the concentration of specific polyphenols: secoxyloganin, oleuropein, and hydroxytyrosol 4-Glucoside (Hy 4-Glu) were the 3 most abundant compounds in fresh green while their concentrations were lower in the traditional and cured presentations. On the contrary, hydroxytyrosol (Hy) was the phenol with the highest concentrations in traditional and cured, followed by tyrosol (Ty). EDA (the dialdehydic form of decarboxymethyl elenolic acid) was not detected in any sample, while HyEDA (EDA linked to Hy) was only found (at 0.22 mM) in fresh green *Aloreña de Málaga*. The pH, titratable acidity, and salt concentration levels also differed considerably among presentations, ranging from 3.80 (CUR-B) to 4.76 (Fresh), 1.50 (CUR-A) to 5.43 (Trad) g/L (expressed as lactic acid), and 47.30 (CUR-A) to 61.30 (Fresh) g/L NaCl, respectively. The range for combined acidity, 0.02 - 0.05 mEq/L, was narrow. Traditional and cured *Aloreña de Málaga* olives were initially acidified with citric (0.9 – 2.0 g/L) and ascorbic (0.45 – 1.0 g/L) acids and stabilised with different concentrations of potassium sorbate (1.0 – 1.8 g/L) and sodium benzoate (1.0 – 2.20 g/L), while none of them was used in the organic presentation (fresh green).

Regarding the initial presence of autochthonous microorganisms in brine, *Enterobacteriaceae* were always below the detection limit (<1.3 $\log_{10}$ CFU/mL) while LAB and yeasts were found at statistically significant different populations ($p<0.05$). The highest LAB counts were obtained in fresh green presentations (6.7±0.2 $\log_{10}$ CFU/mL), followed by cured olives from industry A (6.4±0.1 $\log_{10}$ CFU/mL), cured olives from industry B (2.4±1.5 $\log_{10}$ CFU/mL), and the traditional packaging (1.33±0.04 $\log_{10}$ CFU/mL). Yeast populations followed a similar trend (4.6±1.2, 4.2±1.2, 2.1±1.1, and 1.8±0.8 $\log_{10}$ CFU/mL, respectively). Therefore, the highest initial microbial population levels were observed in the fresh green, followed by the cured and the traditional presentations.

The great differences in pH, salt, acids, sugar, phenolic compounds, preservatives, and autochthonous microbial populations lead to diverse environmental conditions among the *Aloreña de Málaga* presentations and, presumably, should lead to different survival profiles of pathogens.

3.2. Survival of the foodborne pathogens in olive presentations

The independent inoculation of the 4 pathogen cocktails in the 3 *Aloreña de Málaga* presentations (performed in triplicate) led to a total of 16 different treatments and 48 cases. The individual fit of the changes in populations *vs.* time, using a log-linear regression model (GInaFIT Excel tool), was always good, with an R^2 usually above 0.97. The model assumes a linear inactivation of pathogens (k_{max}) from the initial population level (N_0) to below the detection limit (<1.3 $\log_{10}$ CFU/mL). The responses and fit of the *S. aureus* cocktails in the 3 presentations (including two industries for cured fruits) are reported as an example in Fig. 1; cured olives (CUR-B and CUR-A treatments) showed the highest k_{max} (slope), followed by the traditional and the fresh

green presentations (Fig. 1). The other pathogenic species followed similar trends. Therefore, results consistently indicated that the fresh green presentation had the lowest inhibitory effect on pathogens' survival.

The fitted models estimated N_0 values (Table 2) ranging from 8.5 (*E. coli* in cured olives from industry A) to 7.0 $\log_{10}$ CFU/mL (*L. monocytogenes* in cured olives from industry B). The k_{max} oscillated from 0.14 (*S. aureus* in the fresh green) to 0.67 h^{-1} (*E. coli* in cured olives from industry A), while the *4Dr* parameter ranged from 14 (*E. coli* in cured olives from industry A) to >48 h (*S. aureus* in the fresh green presentation). The standard deviations of the model parameters (obtained from triplicate experiments) were, in general, low. Clearly, the k_{max} and *4Dr* values in the cured olives were higher and lower, respectively, than those found in fresh green and traditional presentations.

The ANOVA showed that the main effects and the interactions of categorical variables (pathogen cocktails and *Aloreña de Málaga* presentation) on the inhibition parameters were statistically significant ($p<0.05$) (except for N_0), indicating significant differences among, at least, two presentations. Particularly, the response of foodborne pathogens was different in traditional and fresh green *Aloreña de Málaga* olives. *S. aureus* was the most statistically resistant microorganism in the fresh green presenttion (highest *4Dr* and lowest k_{max}), while *L. monocytogenes* and *S. aureus* showed great resistance in the traditional product (Fig. 2). Conversely, the survival of the 4 foodborne pathogen species had a very similar behaviour in cured olives (industries A and B), with non-statistically different values for k_{max} and *4Dr* (Fig. 2). According to these results, fresh green and traditional *Aloreña de Málaga* presentations had lower inhibitory effects

and longer survival periods for the two gram-positive than for the two gram-negative pathogenic bacteria assayed.

3.3. Multivariate analysis

The number of variables that may affect the survival of pathogens in packaged olives are numerous and disclosing their specific roles is complex. Furthermore, the concentrations of most compounds in the 3 presentations are proportional and those intentionally added are used at rather similar levels. Hence, the presence of strong correlation among the influential variables is habitual, particularly within and between phenols and sugars (see Table S1 in supplementary material). Also, the presence of salt and preservatives were positively and negatively related to the contents of organic compounds in brine (Table S1 in supplementary material). In addition, the values of *4Dr* and k_{max} (except k_{max} for *E. coli*) were significantly (positive, and negative, respectively) related to most phenols and sugars; that is, they apparently promote the pathogens' survival (high *4Dr* and low k_{max}) (see Table S1). However, as k_{max} for *E. coli* species was hardly related to phenols, it may indicate that the inactivation mechanism of this pathogen could be different.

The correlations among the variables support the convenience of subjecting the data to multivariate analysis. Application of hierarchical clustering led to the segregation of treatments into 3 groups: freshly processed, traditional, and the cured olives (both CUR-A and CUR-B together) (Fig. 3, panel A). The characteristic profiles of clusters (Fig. 3, panel B) indicated marked differences among them in the concentrations of total phenol and sugar contents as well as in the *4Dr* values of the pathogens. After clustering application to the pathogens' survival in the different presentations (Fig. 4, panel A), similarities between *E. coli* and *S. enterica* were evident

as well as between *S. aureus* and *L. monocytogenes* but both groups were, in turn, clearly different. Such similarities can be useful for designing specific strategies to control each group but, in turn, simultaneously efficient against their two pair of pathogens.

The important correlations between independent variables prevent their use for modeling the pathogen survival by just multiple regression. Therefore, the task was achieved by applying PLS regression to the centered values of the variables. The analysis was performed using XLSTAT and Minitab. There were retained 4 latent variables which explained 95% (R^2X=0.974) and 93% (R^2Y=0.931) variances of the independent and dependent variable, respectively and had a global Q^2cum=0.750. Most of the independent variables were strongly related to the *t1* axis (Fig. 4, panel B) while a reduced number (TA, AA, CAc, Yeast, LAB, and pH) were associated to *t2* (Fig. 4, panel B). The graph also shows a similar distribution (correlations with the *t1* axis) of the pathogen survival parameters, *4Dr* always on the right and k_{max} on the left. However, polyphenols (except Hy, Ty) and sugars followed similar trends, presenting positive correlation with the *t1* axis while the preservatives (KS and NaB) were negatively linked to it; that is, the environmental influences of such variables are opposed. In fact, in commercial packages, the presence of high concentrations of most phenols (except Ty and Hy) and sugars are related to high *4Dr* values. In other words, the time to reduce 4 log cycle increased and, subsequently, the k_{max} decreased as the contents in polyphenols and sugars were higher. Apparently, the pathogens managed counteracting the the inhibitory effect of the phenols in brine thanks to simultaneous presence of nutrients. The close situation of phenols and sugars in Fig. 4 was in agreement with the strong correlation found between phenols and sugars (and possible the rest of nutrients released from the olive flesh) due to their similar diffusion rates

and, subsequently, concentrations reached in the brines. This explanation of the pathogen's behaviour is also coherent with the situation of presentations (without practical differences between replicates) on the projections (Fig. 4): the fresh presentations on the right of *t1*, the traditional on an intermediate place (their displacement towards the upper part of the graph may be due to their higher values of CAc, AA, and TA) and the cured olives on the left. The distribution is clearly associated to the expected concentrations of phenols and sugars released in them, according to their processing characteristics. This trend is similar to that observed during the storage of directly brined olives, where the presence of increasing concentrations of nutrients (sugar, mineral, or vitamins) may counteract the inhibitory effect of other compounds such as polyphenols and promote growth of the habitual microflora (Garrido-Fernández et al, 1997).

Ty and Hy show completely opposed linkage with the other phenols. However, this association may be caused by their formation by hydrolysis of other compounds located on the right (which decrease implies an increase in Ty and Hy concentrations) and, therefore, the role of Hy and Ty on k_{max} and *4Dr* should be considered with caution. The position of the pH and initial Yeast and LAB are strongly (negatively) correlated to *t2* and opposed to CAc, AA, and TA (positively linked to *t2*), indicating an opposed effect between both groups of variables. Interestingly, the presence of preservatives is highly (and negatively) correlated to *t1* which, in turn, means high and positive association with k_{max} and, on the contrary, opposed linkage to *4Dr*. Then, the addition of preservatives to the cover brines decreased the pathogen's survival due to their systematic associations with k_{max} (positive) and *4Dr* (negative) in all pathogens. Those presentations with higher contents in KS and NaB (cured) showed higher k_{max} and lower *4Dr* values than presentations with no or low added preservatives (traditional

and fresh green). Therefore, under the packaging conditions of this study, the presence of preservatives was, apparently, more determinant for a rapid decrease in the pathogen populations than the composition of the cover brines (polyphenols, sugars, pH, or total acidity). The results, then, emphasize the essential role that preservatives may have on the pathogen survival in real packaging conditions.

The values of the parameters used for the fit evaluation (Table 3) showed that the models were appropriate, and the *4Dr* and k_{max} could be estimated as a function of the environmental variables, using the equations formed with the coefficients (see Table S2 in supplementary material) obtained from the PLS analysis. The standardized coefficients (which do not depend on the size of the measures) are appropriate parameters for indicating the variables that most contribute to the estimation (Table 4). For example, the positive contributors for *4Dr* of *E. coli* were Coms (1.585), Hy (1.142), pCum (0.863), KS (0.732), while the negative were Hy4Glu (-0.561), HyEDA (-0.659), and LAB (-0.815). In the case of *4Dr* of *S. aureus*, the most outstanding influential variables were pH (0.634), KS (0.753), and NaB (0.527), all of them positive, while the influences of the variables with negative signs were reduced. The contributions to the other *4Dr* or k_{max} parameters survival parameters may be obtained similarly. To emphasize, the peculiar behaviour of *S. enterica* which coefficient profile was rather different from the rest. A graphical image of the good fit and the ability to calculate the survival parameters (*4Dr* and k_{max}) was tested in Minitab, which allows PLS cross-validation. As may be observed (Figs. 5 and 6) there was, in general, a good agreement between the experimentally obtained data and the calculated values.

4. Discussion

Table olives, as well as other fermented and acidified vegetable foods, have a long history of microbial safety. Contamination of olives with pathogenic bacteria may be due accidentally to poor hygiene and unsanitary procedures by field and processing personnel, inadequate cleaning and sanitizing of processing equipment, and failure to wash the olives before brining.

Nevertheless, researchers have recently identified the presence of certain foodborne pathogens such as *L. monocytogenes*, *E. coli*, *S. enterica* and *S. aureus* and they have studied their survival in several olive matrixes (Spyropoulou et al., 2001; Skandamis and Nychas, 2003; Randazzo et al., 2012; Argyri et al., 2013; Grounta et al., 2013: Medina et al., 2013; Panagou et al., 2013; Medina-Pradas and Arroyo-López, 2015). In general, it was concluded that the olive environment not only prevented the pathogen's growth but also promoted a fast decrease of their populations. However, Argyri et al. (2013) reported that *L. monocytogenes*, *E. coli*, and *Salmonella* were inactivated in lye treated green olive Halkidiki *cv.* at low pH (4.2) and high salt concentration (60 g/L) only after 14 days from packaging. Also, a high survival (>24h) of pathogenic bacteria was observed in natural black Conservolea olives packages (Grounta el al., 2013). These data show marked differences in the survivals of foodborne pathogens, which were presentation dependent, and point out the importance of specific studies for each case.

In previous studies executed in our group by Medina et al. (2016), the survival of foodborne pathogenic species in a brine model system obtained from fermented *Aloreña de Málaga* fruits was presented, and the observed inhibition was related to the effects of certain polyphenols. In this work, the investigation was extended to the real commercial presentations of the same olive speciality where the presence of the olives

might modify substantially the environmental conditions in the cover brine due to the progressive release of diverse (nutrients and inhibitory) compounds into the brine. Therefore, in contrast to the studies in just brine, this work also takes into consideration the changes in phenols or sugars due to the presence of olives as well as other variables that can be found in marketed olives like preservatives and autochthonous microorganisms. Considering the numerous variables involved in the experiment and their possible relationships, a multivariate approach for the analysis of results was forced.

Great differences between pathogen responses in brine model system and real products were noticed. The first dissimilarity was related to the type of mathematical model used to fit the survival of pathogens. In brine, the fit was achieved by a log-linear model with a tail (Geeraerd et al., 2000), while in real packaging the data were adjusted to a log-linear regression model without a tail (Bigelow and Esty, 1920). The second great difference was related to the survival of pathogens, which was higher in the commercial products. The total inhibition of pathogen in the brine model system was obtained in less than 24 h for all species, with k_{max} values ranging from 10 (*E. coli*) to 53 h^{-1} (*S. enterica)* and *4Dr* values ranging from 0.36 *(S. aureus* and *L. monocytogenes*) to 0.96 h *(E. coli*). On the contrary, in the commercial packaging, the k_{max} parameter ranged, depending on presentation, from 0.14 (*S. aureus* in green Fresh olives) to 0.67 h^{-1} (*E. coli* in cured olives), while *4Dr* values ranged from 13.92 (*E. coli* in cured olives) to >48 h (*S. aureus* in fresh green fruits). Clearly, the lower k_{max} and higher *4Dr* values obtained in commercial products compared to the brine model system reveal the lower inhibitory power of the former. Apparently, such trend may be associated with the higher presence of nutrients (release from the olives) in the brines of the commercial products. In real packaging, *S. aureus* and *L. monocytogenes* were the more resistant

pathogen, especially in traditional and fresh green *Aloreña de Málaga* presentations, while in brine was *E. coli* (Medina et al., 2016). In the specific case of *S. aureus*, albeit a considerable reduction of the initial inoculum level was noticed in both traditional and fresh green olives, the microorganism was still found in both presentations, after 48h, at 2.0±0.3 $\log_{10}$ CFU/mL in traditional, and 5.0±0.5 $\log_{10}$ CFU/mL, in fresh green olives. These data are in agreement with those obtained by Argyri et al. (2013) and Grounta et al. (2013) who found *L. monocytogenes* as the most resistant pathogen in the olive environment. Medina et al. (2013) also reported a considerable resistant of *S. aureus* in various olive brines obtained from Hojiblanca, Manzanilla, and Gordal varieties.

There were also marked differences among fresh green, traditional, and cured *Aloreña de Málaga* presentations on the presence in their brines of sugars and polyphenols released from the olives. Fresh and traditional presentations are cracked immediately after their arrival to the factory, but they differ on the time elapsed from this operation to packaging: three and longer than 20 days storage in brine, respectively. However, the cured olives are brined directly, subjected to lactic acid fermentation, and, cracked and packaged at demand (usually after 6 months). Therefore, due to the diverse processing conditions, the polyphenols and sugars in the studied presentations were in the following progressive lower order: fresh>traditional>cured (although in this case, olives may also contribute with other compounds from the fermentation). Such circumstances provide diverse inhibitory environments for pathogens.

Packaged *Aloreña de Málaga* olives are stabilized by the addition of sodium benzoate and potassium sorbate for preventing the growth of yeasts which may produce gas, cloudy brines and swollen taps (López-López and Garrido-Fernández, 2006). Although these compounds are not primary intended for preventing the growth of

pathogens, the preservatives have shown a marked influence on the survival of these bacteria. Hence, in the olive presentations studied, microorganisms were exposed not only to the inhibitory effects due to polyphenols (Medina et al., 2007, 2013; 2016) and the growth promotion of diverse concentrations of nutrients but also to a new effect due to the presence of preservatives. As expected, many phenols were not only highly correlated among them but also with the concentrations of glucose and fructose (Table S1 in supplementary material), indicating that, in turn, the levels of both groups of compounds were strongly dependent on the time of storage in the holding solutions (fresh and traditional) or the previous fermentation (cured). Most sugars and polyphenols (except Hy and Ty) were also positively and significantly related to salt (Table S1 in supplementary material), possibly due to the greater leakage caused as the salt levels were higher. Conversely, the presence of potassium sorbate and sodium benzoate was negatively related to most of them (except pH, TA, AA, CCi) (Table S1 in supplementary material). Apparently, their presence in the brine at packaging may have reduced the leakage of sugars and phenols from the olive flesh into the brine.

All these relationships deduced from the multivariate analysis contribute to disclose the strong differences among the pathogen survival *in vitro* and those in commercial presentations. Medina et al. (2016) showed that the microbial survival in the brine model system was linked to EDA, HyEDA, Hy-4Glu, Ty, and oleoside 11-methyl ester. However, in this work, the multivariate study showed that the microbial survival in real products was mainly associated with the presence of preservatives (highly positively and negatively correlated to k_{max} and *4Dr*, respectively); however, the total polyphenols and nutrients are oppositely related (high concentrations imply high *4Dr* and low k_{max} values). Apparently, the presence of olives (with the continuous leakage of compounds from the flesh into the brine) provided an entirely different

environment for pathogen survival. Also, the addition of preservatives introduced new effects that prevailed over those derived from the presence of the olive released compounds.

5. Conclusions

Aloreña de Málaga table olive packaging provides an adverse habitat for the development of foodborne pathogens, although there were clear differences among the diverse commercial presentations. The survival of pathogens in the real product was higher compared to the brine model systems. In this work, *S. aureus* was the most resistant species among pathogens, especially in fresh green olives in which no preservatives were added and despite its high phenolic content. Multivariate analysis showed that, in fact, the possible effect of phenols could be counteracted by the simultaneous higher presence of nutrients (sugars) and that, on the contrary, the presence of preservatives was associated with a reduction of the pathogens' survival. Because of the differences in pathogen survival in the diverse presentations, even of the same olive variety, each commercial product requires specific challenge tests. According to results, *Aloreña de Málaga* products, once processed, should not be distributed before 48h after packaging to ensure pathogen inhibition in case of hypothetical contamination.

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Figure legends

Fig. 1. Fit of the log-linear regression model to the survival data of *S. aureus* cocktail in the 3 industrial presentations. Trad, stands for the Traditional *Aloreña de Málaga* presentation, Fresh for the fresh green style, while CUR-A and CUR-B stand for the cured fruits (two industries).

Fig. 2. Graphical representation of the factorial ANOVA analysis carried out for the inhibition parameters k_{max} (maximum death rate) and *4Dr* (time for 4 $\log_{10}$ reduction) obtained with the log-linear regression model to the survival data of the foodborne pathogens in the different olive presentations. *Sa, Ec, Lm* and *Se* stand for the *S. aureus, E. coli, L. monocytogenes* and *S. enterica* cocktails, respectively. Trad stands for the Traditional *Aloreña de Málaga* presentation, Fresh for the fresh green, while CUR-A and CUR-B stand for the two industries which elaborated cured olives. Vertical bars denote 0.95 confidence limits.

Fig. 3. Cluster analysis of treatments, and their replicates, as a function of the variables studied (polyphenols, sugars, pH, salt, organic acids, preservatives, and fit parameters) (A), and their profiles (B). Trad, stands for the Traditional *Aloreña de Málaga* presentation, Fresh for the fresh green, while CUR-A and CUR-B stand for the cured fruits (two industries). See Table 1 for the rest of abbreviations.

Fig. 4. Cluster analysis of pathogen's behaviour based on the fit parameters (A) and correlation wheel of the variables studied on the plane of the first two PLS components (B). *4Dr*, time in hours for a reduction of 4 $\log_{10}$ from N_0; k_{max}, maximum death rate (h^{-1}); *Ec, Sa, Lm* and *Se* stand for the different microorganisms cocktails assayed, *E. coli, S. aureus, L. monocytogenes* and *S. enterica,* respectively. LAB, lactic acid bacteria. See Table 1 for the meaning of the rest of abbreviations.

Fig. 5. Estimation by PLS of the pathogens' survival fit parameters in four industrial packaging as a function of the environmental variables. Relationships between actual and calculated (by cross-validation) responses for *4Dr* values (h).

Fig. 6. Estimation by PLS of the pathogens' survival fit parameters in four industrial packaging as a function of the environmental variables. Relationship between actual and calculated (by cross-validation) responses for k_{max} values (h^{-1}).

Table 1. Physicochemical characteristics and additives levels of the different *Aloreña de Málaga* commercial packaging brines used in the present study. Standard deviations obtained from triplicated experiments in parentheses.

Parameter	Compound (Abbreviation)	Trad	CUR-A	CUR-B	Fresh
Salt (g/L)	NaCl	56.20 (0.03)^{a}	47.30 (0.04)^{b}	55.10 (0.02)^{a}	61.30 (0.09)^{c}
pH	[H+]	3.95 (0.03)^{a}	4.75 (0.02)^{b}	3.80 (0.01)^{c}	4.76 (0.05)^{b}
Titratable acidity (g/L) (TA)	Expressed as lactic acid	5.43 (0.01)^{a}	1.50 (0.01)^{b}	2.90 (0.01)^{c}	2.37 (0.01)^{d}
Combined acidity (mEq/L) (CA)	-	0.05 (0.00)^{a}	0.04 (0.00)^{b}	0.02 (0.00)^{c}	0.05 (0.00)^{a}
Sugars (g/L)	Glucose (Glu)	3.03 (0.01)^{a}	0.11 (0.01)^{b}	1.17 (0.01)^{c}	6.5 (0.1)^{d}
	Fructose (Fru)	0.68 (0.03)^{a}	0.16 (0.01)^{b}	0.33 (0.02)^{c}	1.93 (0.06)^{d}
	Sucrose (Sac)	0.09 (0.03)^{a}	0.00 (0.00)^{b}	0.03 (0.01)^{b}	0.46 (0.00)^{c}
	Mannitol (Man)	0.82 (0.01)^{a}	0.15 (0.01)^{b}	0.50 (0.01)^{c}	0.64 (0.01)^{d}
	Total sugars (TS)	4.63 (0.08)^{a}	0.42 (0.03)^{b}	2.03 (0.04)^{c}	9.56 (0.21)^{d}
Phenolic compounds (mM)	Hydroxytyrosol (Hy)	2.2 (0.2)^{a}	2.6 (0.1)^{a}	1.4 (0.2)^{b}	1.12 (0.09)^{b}
	Hy 4-Glucoside (Hy4Glu)	0.23 (0.07)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	1.27 (0.17)^{b}
	Tyrosol (Ty)	0.54 (0.08)^{a}	0.81 (0.04)^{b}	0.45 (0.07)^{a}	0.42 (0.09)^{a}
	p-coumaric acid (pCum)	0.02 (0.02)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.03 (0.01)^{a}
	Verbascoside (Verb)	0.2 (0.1)^{a}	0.00 (0.00)^{b}	0.10 (0.01)^{a,b}	0.19 (0.03)^{a}
	HyEDA	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.22 (0.01)^{b}
	Oleuropein (Ole)	0.19 (0.03)^{a}	0.00 (0.00)^{b}	0.12 (0.03)^{a,b}	1.51 (0.10)^{c}
	Comselogoside (Coms)	0.01 (0.00)^{a}	0.00 (0.00)^{a}	0.01 (0.00)^{a}	0.03 (0.00)^{b}
	EDA	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}
	Secoxyloganin (Secox)	0.99 (0.06)^{a}	0.00 (0.00)^{b}	0.44 (0.02)^{c}	2.81 (0.08)^{d}
	Secologanoside (Seclog)	0.20 (0.01)^{a}	0.00 (0.00)^{b}	0.13 (0.01)^{c}	0.65 (0.02)^{d}
	Oleoside 11-methyl ester (Oleo11)	0.38 (0.02)^{a}	0.00 (0.00)^{b}	0.28 (0.02)^{c}	0.60 (0.01)^{d}
	Total phenolic compounds (TPh)	4.9 (0.3)^{a}	3.4 (0.2)^{b}	2.88 (0.09)^{b}	8.9 (0.3)^{c}
Additives (g/L)	Potassium sorbate (KS)	1.0 (0.1)^{a}	1.8 (0.1)^{b}	1.5 (0.2)^{c}	0.0 (0.0)^{d}
	Sodium benzoate (NaB)	1.0 (0.1)^{a}	2.2 (0.1)^{b}	1.5 (0.1)^{c}	0.0 (0.0)^{d}
	Ascorbic acid (AA)	1.0 (0.1)^{a}	0.45 (0.01)^{b}	0.50 (0.01)^{b}	0.0 (0.0)^{c}
	Citric acid (CAc)	2.0 (0.1)^{a}	0.91 (0.01)^{b}	1.50 (0.10)^{c}	0.0 (0.0)^{d}

Notes: EDA: dialdehydic form of decarboxymethyl elenolic acid. HyEDA: EDA linked to hydroxytyrosol. Standard error in parenthesis. Values followed by different superscript letters, within the same row, are significantly different according to the Scheffé posthoc comparison test.

Table 2. Parameters obtained using a Log-linear regression model (Bigelow and Esty, 1920) implemented in GinaFit to the survival data of the foodborne pathogen cocktails in the *Aloreña de Málaga* commercial packaging. Standard deviations obtained from triplicate experiments in parentheses.

Parameter/Pathogen	Trad	CUR-A	CUR-B	Fresh
E. coli				
N_0 ($\log_{10}$ cfu/mL)	8.4 (0.2)^{a}	8.49 (0.05)^{a}	7.12 (0.33)^{b}	7.7 (0.1)^{b}
k_{max} (h^{-1})	0.6 (0.2)^{a}	0.67 (0.03)^{a}	0.59 (0.02)^{a}	0.5 (0.1)^{a}
4Dr (h)	19 (9)^{a}	13.9 (0.6)^{a}	15.92 (0.36)^{a}	21 (7)^{a}
L. monocytogenes				
N_0 ($\log_{10}$ cfu/mL)	8.11 (0.03)^{a}	8.20 (0.05)^{a}	7.04 (0.01)^{b}	7.95 (0.03)^{c}
k_{max} (h^{-1})	0.29 (0.02)^{a}	0.65 (0.01)^{b}	0.54 (0.00)^{c}	0.30 (0.04)^{a}
4Dr (h)	31 (1)^{a}	14.40 (0.00)^{b}	17.1 (0.1)^{b}	31 (4)^{a}
S. aureus				
N_0 ($\log_{10}$ cfu/mL)	8.05 (0.03)^{a}	8.1 (0.2)^{a}	7.51 (0.02)^{b}	7.75 (0.09)^{b}
k_{max} (h^{-1})	0.30 (0.02)^{a,b}	0.5 (0.2)^{b}	0.59 (0.03)^{b,c}	0.14 (0.02)^{a}
4Dr (h)	31 (1)^{a}	19 (7)^{b}	15.8 (0.8)^{b}	>48.00 (0.00)^{c}
S. enterica				
N_0 ($\log_{10}$ cfu/mL)	8.15 (0.04)^{a}	8.1 (0.2)^{a}	7.52 (0.02)^{c}	7.7 (0.2)^{b}
k_{max} (h^{-1})	0.65 (0.00)^{a}	0.64 (0.02)^{a}	0.61 (0.01)^{b}	0.32 (0.01)^{c}
4Dr (h)	14.2 (0.1)^{a}	14.2 (0.5)^{a}	15.12 (0.00)^{a}	29 (1)^{c}

Notes: N_0, initial microbial population, *4Dr*, time for a reduction of 4 $\log_{10}$ from N_0; k_{max}, maximum death rate. Values followed by different superscript letters, within the same row, are significantly different according to the Scheffé posthoc comparison test.

Table 3. Goodness of fit statistics of the models deduced for the estimation of the inhibition curve parameters, according to the pathogen. The number of observations was always 12 and the degree of freedom 1.

Fit parameter	***4Dr***				**k_{max}**			
	Ec	***Sa***	***Lm***	***Se***	***Ec***	***Sa***	***Lm***	***Se***
R^2	0.995	0.995	0.997	<0.999	>0.999	0.995	0.999	>0.999
SD	1.301	3.032	1.538	0.308	0.004	0.049	0.013	0.005
MSE	0.141	0.766	0.197	0.008	<0.001	<0.001	<0.001	<0.001
RMSE	0.376	0.875	0.444	0.089	0.001	0.014	0.004	0.001

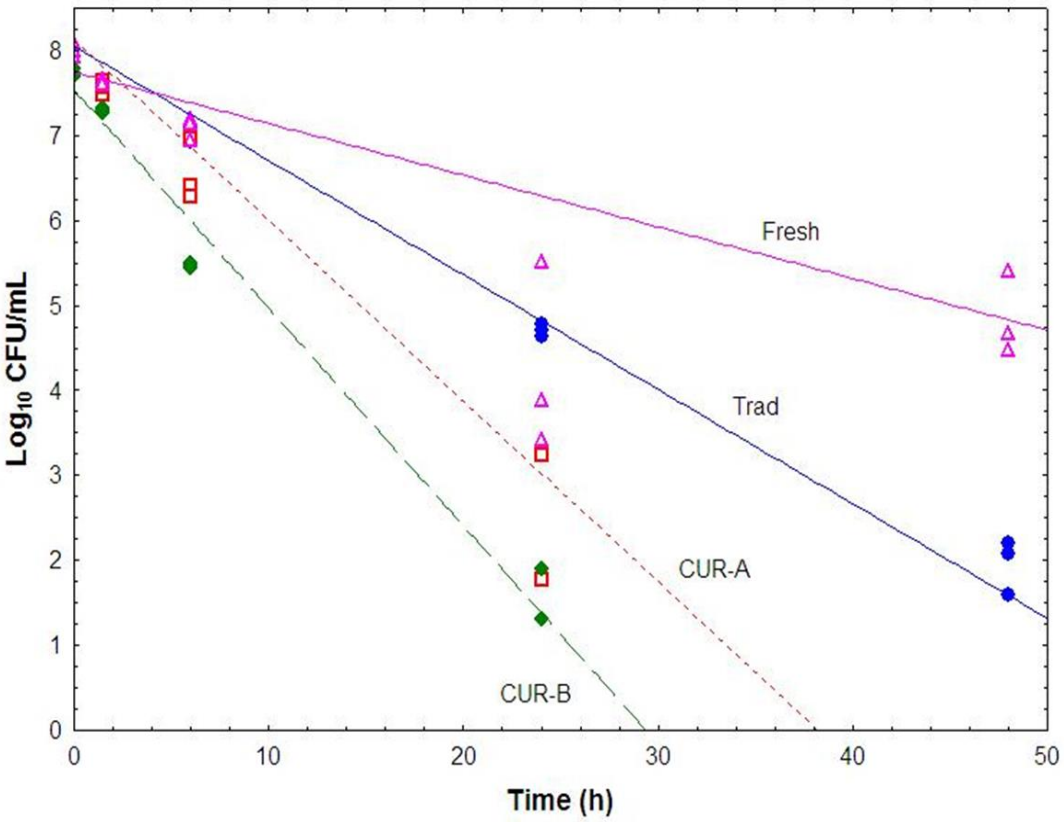
Notes: *4Dr*, time in hours for a reduction of 4 $\log_{10}$ from $N_{0;}$ k_{max}, maximum death rate (h^{-1}); R^2, determination coefficient; SD, standard deviation; MSE, mean square error; RMSE, root mean square error. *Ec*, *Sa*, *Lm* and *Se* stand for the different microorganisms cocktails assayed, *E. coli*, *S. aureus*, *L. monocytogenes* and *S. enterica*, respectively.

Table 4. Standardized coefficients for the different mathematical models.

Variable	*4Dr*				k_{max}			
	Ec	***Sa***	***Lm***	***Se***	***Ec***	***Sa***	***Lm***	***Se***
Hy	**1.142**	0.258	-0.053	-0.180	**-1.028**	-0.302	0.009	0.183
Hy4Glu	**-0.561**	0.377	-0.299	-0.036	0.346	-0.462	0.128	0.020
Ty	**-0.856**	-0.169	0.385	0.065	**0.858**	0.212	-0.196	-0.046
pCum	**0.863**	0.147	-0.163	0.031	**-0.720**	-0.266	0.100	-0.004
Verb	-0.344	-0.105	0.001	0.038	0.131	0.144	-0.003	-0.024
HyEda	**-0.659**	-0.270	0.485	0.243	**0.661**	0.359	-0.254	-0.204
Ole	0.432	0.168	0.036	0.062	-0.297	-0.234	-0.041	-0.047
Coms	**1.585**	0.440	**-0.528**	-0.259	**-1.608**	-0.426	0.243	0.182
Secox	0.286	0.238	0.029	0.036	-0.196	-0.331	-0.060	-0.017
Seclog	0.136	0.244	0.012	0.050	-0.048	-0.354	-0.049	-0.029
Oleo11	0.005	-0.099	0.223	0.061	0.013	0.167	-0.158	-0.064
TPh	0.343	0.298	0.006	0.003	-0.283	-0.387	-0.048	0.012
Glu	-0.056	0.351	-0.092	-0.009	0.037	-0.476	-0.008	0.020
Fru	0.047	0.366	-0.188	-0.016	-0.088	-0.483	0.061	0.021
Sac	-0.009	0.056	0.062	0.059	-0.040	-0.029	-0.047	-0.057
Man	0.018	0.277	0.160	-0.051	0.008	-0.394	-0.194	0.080
TS	-0.030	0.344	-0.088	-0.010	0.007	-0.462	-0.010	0.021
pH	0.495	**0.634**	-0.386	-0.059	-0.381	**-0.897**	0.201	0.080
Salt	-0.099	-0.313	0.512	0.192	0.287	0.372	-0.307	-0.161
TA	-0.136	0.280	0.126	-0.128	0.093	-0.376	-0.178	0.142
CA	-0.225	-0.421	0.412	0.095	0.281	**0.622**	-0.202	-0.118
KS	**0.732**	**0.753**	**-0.543**	-0.194	**-0.622**	**-1.132**	0.257	0.238
NaB	0.373	**0.527**	-0.400	-0.152	-0.351	**-0.778**	0.203	0.181
AA	-0.378	0.294	0.088	-0.104	0.430	-0.484	-0.138	0.137
CAc	0.214	-0.149	0.245	-0.062	-0.175	0.196	-0.171	0.073
LAB	**-0.815**	-0.077	**0.646**	0.214	**0.669**	0.125	-0.367	-0.113
Yeats	0.402	-0.207	-0.356	-0.126	-0.389	0.372	0.208	0.034

Notes: *4Dr*, time in hours for a reduction of 4 $\log_{10}$ from N_0; k_{max}, maximum death rate (h^{-1}); *Ec*, *Sa*, *Lm* and *Se* stand for the different microorganisms cocktails assayed, *E. coli*, *S. aureus*, *L. monocytogenes* and *S. enterica*, respectively. See Table 1 for the meaning of the rest of abbreviations. More relevant contributions are printed in bold.

Figure 1


Log10 CFU/mL
Time (h)
Fresh
Trad
CUR-A
CUR-B
0
1
2
3
4
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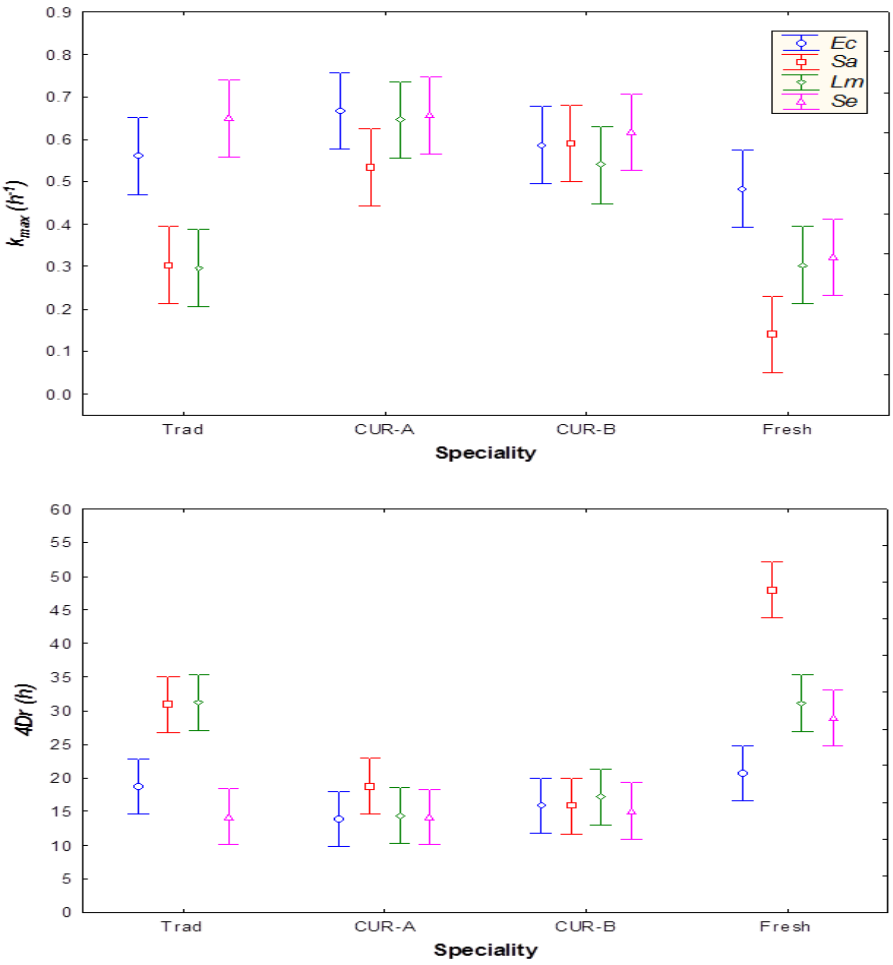
20
30
40
50

Figure 2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k_{max} (h^{-1})
Ec
Sa
Lm
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Trad
CUR-A
CUR-B
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Speciality
60
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5
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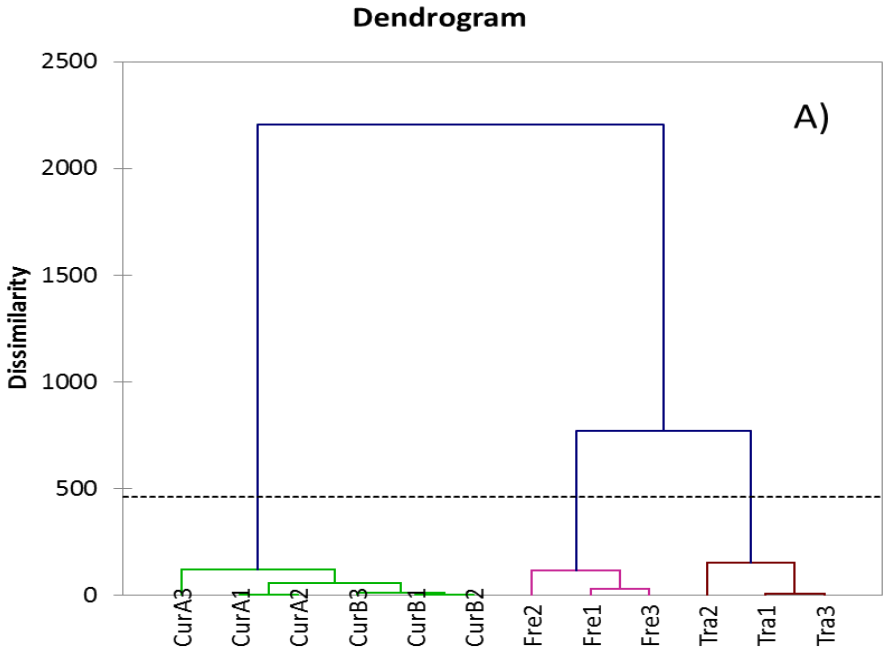
4Dr (h)
Trad
CUR-A
CUR-B
Fresh
Speciality

Figure 3


Dendrogram
A)
Dissimilarity
2500
2000
1500
1000
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0
CurA3
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Fre2
Fre1
Fre3
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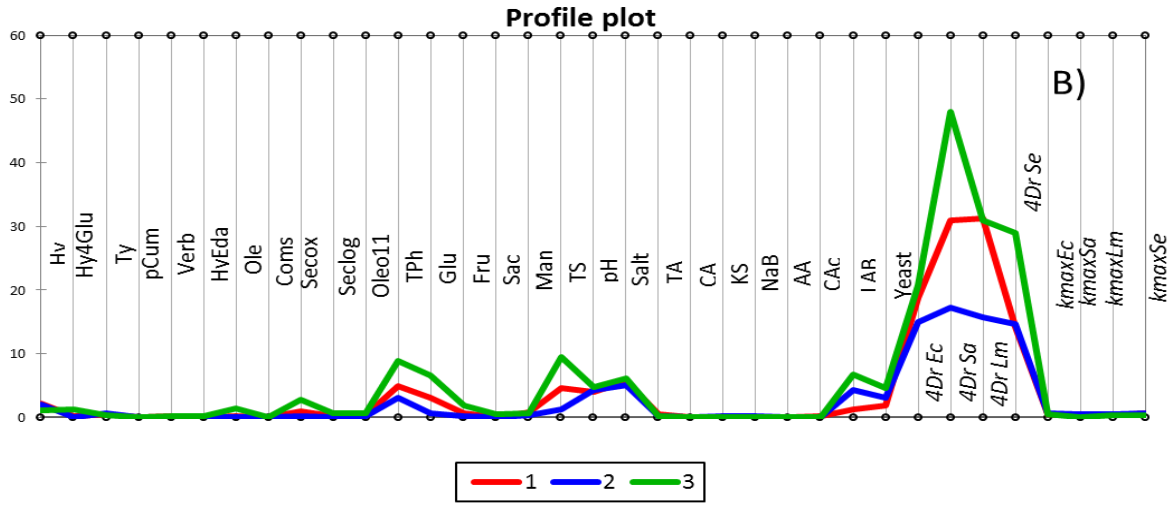
Tra1
Tra3


Profile plot
B)
60
50
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30
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Hy4Glu
Ty
pCum
Verb
HvEda
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Coms
Secox
Seclog
Oleo11
TPh
Glu
Fru
Sac
Man
TS
pH
Salt
TA
CA
KS
NaB
AA
CAc
I AR
Yeast
4Dr Ec
4Dr Sa
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kmaxEc
kmaxSa
kmaxLm
kmaxSe
1
2
3

Figure 4

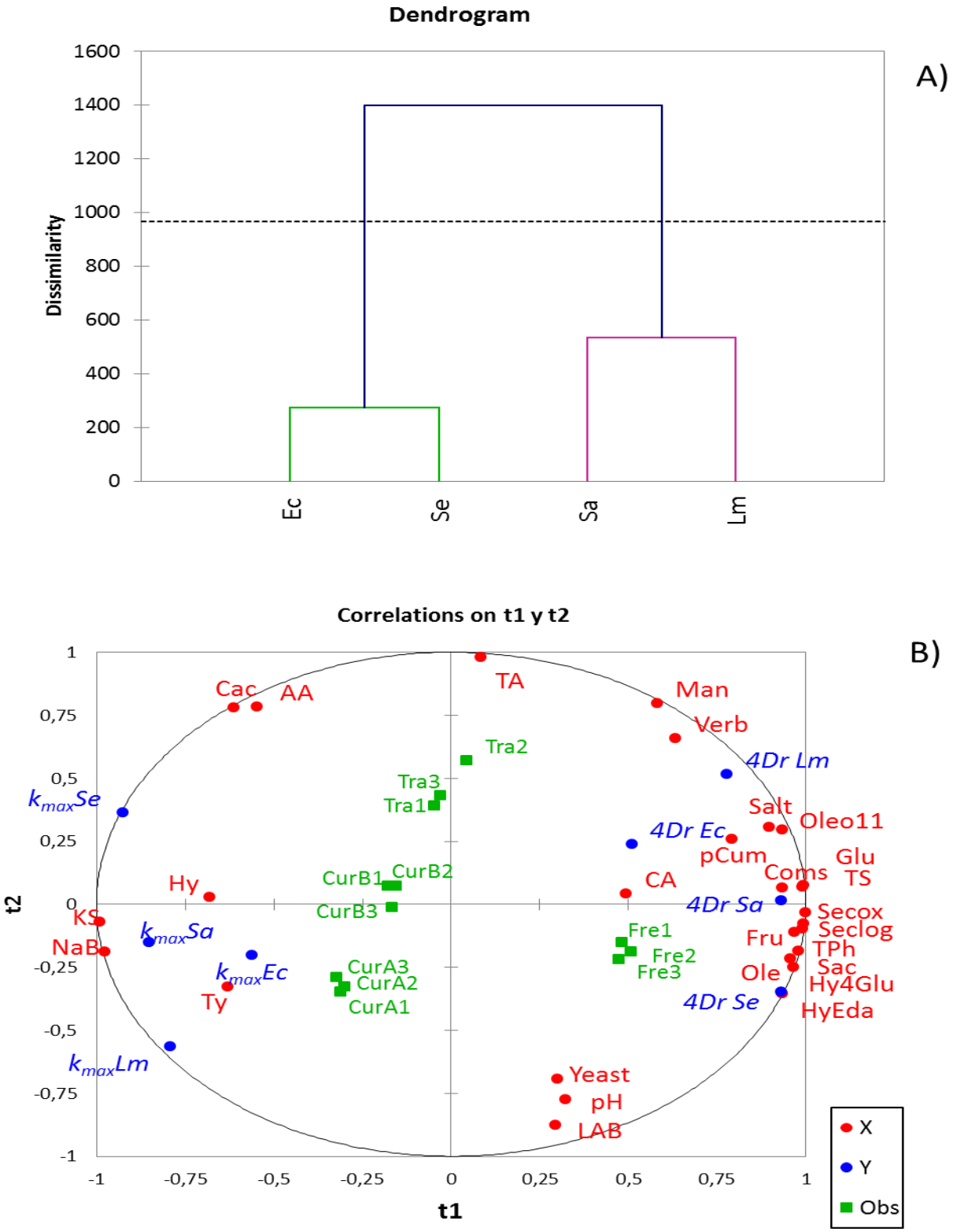

A)
Dendrogram
Dissimilarity
1600
1400
1200
1000
800
600
400
200
0
Ec
Se
Sa
Lm
B)
Correlations on t1 y t2
t2
t1
1
0,75
0,5
0,25
0
-0,25
-0,5
-0,75
-1
-1
-0,75
-0,5
-0,25
0
0,25
0,5
0,75
1
Cac
AA
TA
Man
Verb
Tra2
4Dr Lm
Tra3
Tra1
kmaxSe
Salt
Oleo11
4Dr Ec
pCum
Glu
Coms
TS
Hy
CurB1
CurB2
CA
CurB3
4Dr Sa
Secox
KS
kmaxSa
Fre1
Seclog
Fru
NaB
Fre2
TPh
kmaxEc
Fre3
Sac
CurA3
Ole
Hy4Glu
CurA2
Ty
4Dr Se
HyEda
CurA1
kmaxLm
Yeast
pH
LAB
X
Y
Obs

Figure 5

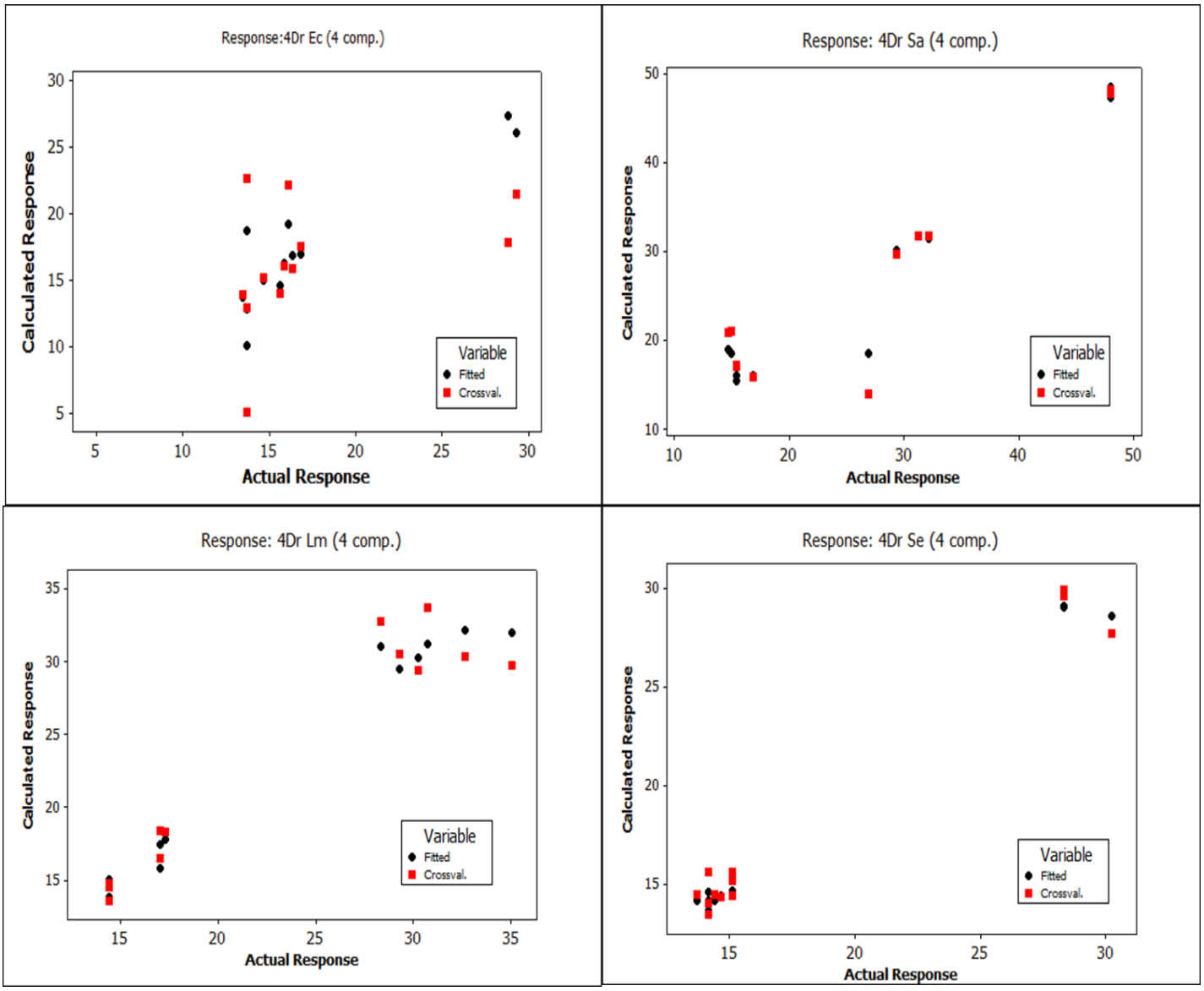
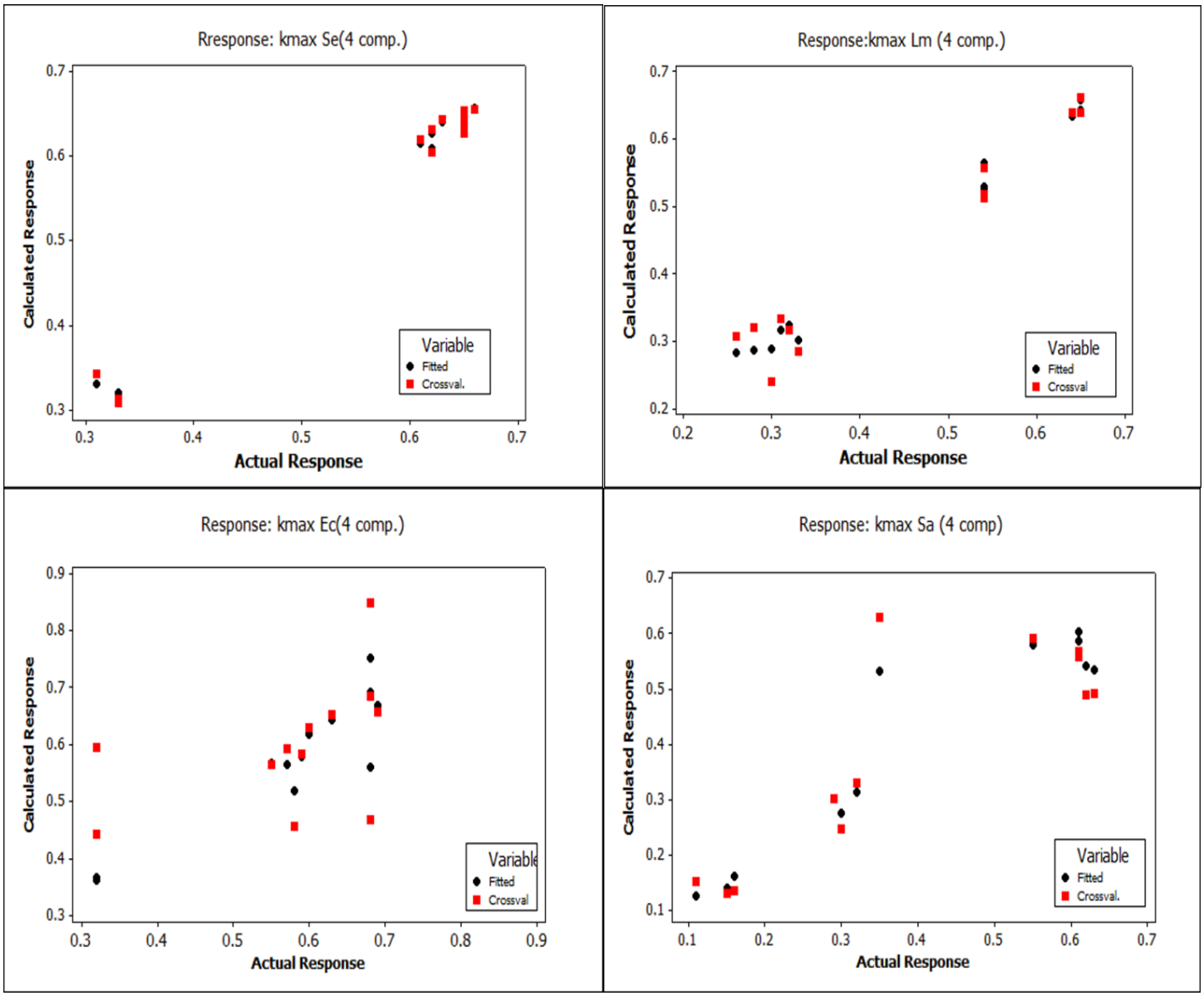

Response:4Dr Ec (4 comp.)
Calculated Response
Actual Response
Variable
Fitted
Crossval.
Response: 4Dr Sa (4 comp.)
Calculated Response
Actual Response
Variable
Fitted
Crossval.
Response: 4Dr Lm (4 comp.)
Calculated Response
Actual Response
Variable
Fitted
Crossval.
Response: 4Dr Se (4 comp.)
Calculated Response
Actual Response
Variable
Fitted
Crossval.

Figure 6


Rresponse: kmax Se(4 comp.)
Calculated Response
Actual Response
Variable
Fitted
Crossval.
Response:kmax Lm (4 comp.)
Calculated Response
Actual Response
Variable
Fitted
Crossval
Response: kmax Ec(4 comp.)
Calculated Response
Actual Response
Variable
Fitted
Crossval
Response: kmax Sa (4 comp)
Calculated Response
Actual Response
Variable
Fitted
Crossval.

Supplementary material

Table S1. Correlation matrix (Pearson) among the diverse variables (independent and dependent) of the data .

Variables	Hy	Hy4Glu	Ty	pCum	Verb	HyEda	Ole	Coms	Secox	Seclog	Oleo11	TPh	Glu	Fru	Sac	Man	TS	pH	Salt	TA	CA	KS	NaB	AA	Cac	LAB	Yeast	4DrEc	4DrSa	4DrLm	4DrSe	kmaxEc	kmaxSa	kmaxLm	kmaxSe
Hy	1	-0,622	0,823	-0,289	-0,34	-0,644	-0,675	-0,775	-0,674	-0,728	-0,765	-0,498	-0,648	-0,649	-0,651	-0,429	-0,652	0,136	-0,822	0,035	0,271	0,666	0,716	0,527	0,414	0,002	-0,022	-0,173	-0,447	-0,346	-0,686	0,287	0,339	0,434	0,708
Hy4Glu	-0,622	1	-0,532	0,646	0,415	0,971	0,972	0,827	0,966	0,966	0,821	0,964	0,953	0,985	0,985	0,395	0,95	0,509	0,767	-0,106	0,53	-0,942	-0,895	-0,662	-0,757	0,476	0,423	0,312	0,923	0,674	0,965	-0,37	-0,83	-0,666	-0,959
Ty	0,823	-0,532	1	-0,313	-0,52	-0,466	-0,524	-0,673	-0,619	-0,632	-0,803	-0,441	-0,638	-0,587	-0,561	-0,699	-0,647	0,389	-0,834	-0,331	0,17	0,635	0,729	0,217	0,074	0,237	0,222	-0,245	-0,455	-0,538	-0,508	0,351	0,41	0,628	0,516
pCum	-0,289	0,646	-0,313	1	0,87	0,641	0,697	0,772	0,765	0,728	0,735	0,799	0,775	0,742	0,723	0,59	0,776	0,223	0,684	0,295	0,629	-0,783	-0,772	-0,223	-0,305	0,125	0,185	0,8	0,782	0,743	0,624	-0,792	-0,763	-0,744	-0,604
Verb	-0,34	0,415	-0,52	0,87	1	0,349	0,441	0,675	0,594	0,554	0,752	0,549	0,645	0,546	0,494	0,846	0,651	-0,269	0,731	0,66	0,373	-0,652	-0,714	0,136	0,121	-0,326	-0,181	0,723	0,58	0,773	0,35	-0,732	-0,611	-0,82	-0,334
HyEda	-0,644	0,971	-0,466	0,641	0,349	1	0,992	0,84	0,944	0,955	0,766	0,943	0,902	0,96	0,981	0,259	0,9	0,581	0,726	-0,269	0,449	-0,9	-0,843	-0,789	-0,852	0,579	0,495	0,365	0,871	0,556	0,997	-0,425	-0,754	-0,547	-0,994
Ole	-0,675	0,972	-0,524	0,697	0,441	0,992	1	0,896	0,973	0,981	0,833	0,96	0,939	0,98	0,992	0,362	0,938	0,5	0,796	-0,166	0,453	-0,941	-0,897	-0,729	-0,788	0,49	0,44	0,44	0,897	0,624	0,988	-0,495	-0,787	-0,625	-0,986
Coms	-0,775	0,827	-0,673	0,772	0,675	0,84	0,896	1	0,919	0,928	0,927	0,847	0,9	0,893	0,893	0,583	0,903	0,143	0,915	0,099	0,276	-0,93	-0,935	-0,532	-0,518	0,15	0,183	0,649	0,8	0,663	0,847	-0,709	-0,704	-0,713	-0,853
Secox	-0,674	0,966	-0,619	0,765	0,594	0,944	0,973	0,919	1	0,996	0,923	0,973	0,992	0,995	0,986	0,56	0,992	0,352	0,884	0,059	0,502	-0,987	-0,968	-0,564	-0,635	0,323	0,318	0,478	0,938	0,773	0,94	-0,527	-0,857	-0,783	-0,933
Seclog	-0,728	0,966	-0,632	0,728	0,554	0,955	0,981	0,928	0,996	1	0,919	0,956	0,981	0,991	0,985	0,526	0,981	0,348	0,89	0,009	0,435	-0,979	-0,961	-0,607	-0,666	0,333	0,328	0,446	0,913	0,729	0,955	-0,503	-0,822	-0,744	-0,952
Oleo11	-0,765	0,821	-0,803	0,735	0,752	0,766	0,833	0,927	0,923	0,919	1	0,829	0,942	0,891	0,857	0,799	0,946	-0,032	0,988	0,355	0,312	-0,946	-0,985	-0,314	-0,33	-0,046	0,025	0,502	0,824	0,84	0,775	-0,561	-0,775	-0,888	-0,773
TPh	-0,498	0,964	-0,441	0,799	0,549	0,943	0,96	0,847	0,973	0,956	0,829	1	0,963	0,98	0,975	0,46	0,961	0,518	0,766	-0,001	0,665	-0,955	-0,906	-0,568	-0,688	0,442	0,417	0,489	0,964	0,758	0,926	-0,516	-0,89	-0,74	-0,913
Glu	-0,648	0,953	-0,638	0,775	0,645	0,902	0,939	0,9	0,992	0,981	0,942	0,963	1	0,986	0,963	0,64	1	0,29	0,898	0,169	0,537	-0,991	-0,981	-0,466	-0,554	0,239	0,257	0,456	0,947	0,835	0,897	-0,501	-0,884	-0,846	-0,888
Fru	-0,649	0,985	-0,587	0,742	0,546	0,96	0,98	0,893	0,995	0,991	0,891	0,98	0,986	1	0,994	0,504	0,985	0,416	0,844	0,002	0,526	-0,978	-0,949	-0,603	-0,683	0,385	0,361	0,436	0,943	0,744	0,955	-0,489	-0,858	-0,747	-0,948
Sac	-0,651	0,985	-0,561	0,723	0,494	0,981	0,992	0,893	0,986	0,985	0,857	0,975	0,963	0,994	1	0,422	0,962	0,471	0,81	-0,095	0,499	-0,961	-0,921	-0,681	-0,744	0,456	0,402	0,447	0,924	0,68	0,977	-0,505	-0,825	-0,68	-0,972
Man	-0,429	0,395	-0,699	0,59	0,846	0,259	0,362	0,583	0,56	0,526	0,799	0,46	0,64	0,504	0,422	1	0,646	-0,475	0,795	0,843	0,262	-0,63	-0,728	0,314	0,28	-0,541	-0,365	0,404	0,54	0,865	0,264	-0,415	-0,599	-0,92	-0,25
TS	-0,652	0,95	-0,647	0,776	0,651	0,9	0,938	0,903	0,992	0,981	0,946	0,961	1	0,985	0,962	0,646	1	0,279	0,903	0,174	0,529	-0,992	-0,983	-0,463	-0,547	0,232	0,249	0,462	0,945	0,836	0,895	-0,508	-0,883	-0,848	-0,886
pH	0,136	0,509	0,389	0,223	-0,269	0,581	0,5	0,143	0,352	0,348	-0,032	0,518	0,29	0,416	0,471	-0,475	0,279	1	-0,111	-0,679	0,589	-0,277	-0,126	-0,67	-0,836	0,916	0,742	0,035	0,443	-0,01	0,544	-0,011	-0,358	0,107	-0,533
Salt	-0,822	0,767	-0,834	0,684	0,731	0,726	0,796	0,915	0,884	0,89	0,988	0,766	0,898	0,844	0,81	0,795	0,903	-0,111	1	0,351	0,196	-0,902	-0,956	-0,3	-0,292	-0,091	0,009	0,477	0,751	0,789	0,741	-0,54	-0,696	-0,849	-0,743
TA	0,035	-0,106	-0,331	0,295	0,66	-0,269	-0,166	0,099	0,059	0,009	0,355	-0,001	0,169	0,002	-0,095	0,843	0,174	-0,679	0,351	1	0,18	-0,158	-0,266	0,77	0,715	-0,803	-0,598	0,208	0,128	0,617	-0,273	-0,166	-0,264	-0,654	0,291
CA	0,271	0,53	0,17	0,629	0,373	0,449	0,453	0,276	0,502	0,435	0,312	0,665	0,537	0,526	0,499	0,262	0,529	0,589	0,196	0,18	1	-0,52	-0,434	-0,046	-0,31	0,35	0,347	0,319	0,685	0,634	0,397	-0,24	-0,709	-0,543	-0,366
KS	0,666	-0,942	0,635	-0,783	-0,652	-0,9	-0,941	-0,93	-0,987	-0,979	-0,946	-0,955	-0,991	-0,978	-0,961	-0,63	-0,992	-0,277	-0,902	-0,158	-0,52	1	0,987	0,482	0,558	-0,217	-0,235	-0,495	-0,924	-0,82	-0,897	0,541	0,851	0,833	0,892
NaB	0,716	-0,895	0,729	-0,772	-0,714	-0,843	-0,897	-0,935	-0,968	-0,961	-0,985	-0,906	-0,981	-0,949	-0,921	-0,728	-0,983	-0,126	-0,956	-0,266	-0,434	0,987	1	0,397	0,446	-0,088	-0,136	-0,502	-0,887	-0,848	-0,846	0,555	0,826	0,877	0,841
AA	0,527	-0,662	0,217	-0,223	0,136	-0,789	-0,729	-0,532	-0,564	-0,607	-0,314	-0,568	-0,466	-0,603	-0,681	0,314	-0,463	-0,67	-0,3	0,77	-0,046	0,482	0,397	1	0,941	-0,789	-0,596	-0,175	-0,439	0,053	-0,799	0,261	0,271	-0,06	0,814
CAc	0,414	-0,757	0,074	-0,305	0,121	-0,852	-0,788	-0,518	-0,635	-0,666	-0,33	-0,688	-0,554	-0,683	-0,744	0,28	-0,547	-0,836	-0,292	0,715	-0,31	0,558	0,446	0,941	1	-0,855	-0,693	-0,117	-0,577	-0,085	-0,844	0,169	0,43	0,048	0,85
LAB	0,002	0,476	0,237	0,125	-0,326	0,579	0,49	0,15	0,323	0,333	-0,046	0,442	0,239	0,385	0,456	-0,541	0,232	0,916	-0,091	-0,803	0,35	-0,217	-0,088	-0,789	-0,855	1	0,836	0,035	0,333	-0,157	0,556	-0,053	-0,206	0,224	-0,556
Yeast	-0,022	0,423	0,222	0,185	-0,181	0,495	0,44	0,183	0,318	0,328	0,025	0,417	0,257	0,361	0,402	-0,365	0,249	0,742	0,009	-0,598	0,347	-0,235	-0,136	-0,596	-0,693	0,836	1	0,132	0,251	-0,107	0,457	-0,139	-0,098	0,137	-0,478
4DrEc	-0,173	0,312	-0,245	0,8	0,723	0,365	0,44	0,649	0,478	0,446	0,502	0,489	0,456	0,436	0,447	0,404	0,462	0,035	0,477	0,208	0,319	-0,495	-0,502	-0,175	-0,117	0,035	0,132	1	0,451	0,39	0,347	-0,984	-0,408	-0,432	-0,347
4DrSa	-0,447	0,923	-0,455	0,782	0,58	0,871	0,897	0,8	0,938	0,913	0,824	0,964	0,947	0,943	0,924	0,54	0,945	0,443	0,751	0,128	0,685	-0,924	-0,887	-0,439	-0,577	0,333	0,251	0,451	1	0,812	0,853	-0,463	-0,976	-0,797	-0,829
4DrLm	-0,346	0,674	-0,538	0,743	0,773	0,556	0,624	0,663	0,773	0,729	0,84	0,758	0,835	0,744	0,68	0,865	0,836	-0,01	0,789	0,617	0,634	-0,82	-0,848	0,053	-0,085	-0,157	-0,107	0,39	0,812	1	0,546	-0,383	-0,851	-0,988	-0,518
4DrSe	-0,686	0,965	-0,508	0,624	0,35	0,997	0,988	0,847	0,94	0,955	0,775	0,926	0,897	0,955	0,977	0,264	0,895	0,544	0,741	-0,273	0,397	-0,897	-0,846	-0,799	-0,844	0,556	0,457	0,347	0,853	0,546	1	-0,415	-0,734	-0,54	-0,998
kmaxEc	0,287	-0,37	0,351	-0,792	-0,732	-0,425	-0,495	-0,709	-0,527	-0,503	-0,561	-0,516	-0,501	-0,489	-0,505	-0,415	-0,508	-0,011	-0,54	-0,166	-0,24	0,541	0,555	0,261	0,169	-0,053	-0,139	-0,984	-0,463	-0,383	-0,415	1	0,402	0,436	0,419
kmaxSa	0,339	-0,83	0,41	-0,763	-0,611	-0,754	-0,787	-0,704	-0,857	-0,822	-0,775	-0,89	-0,884	-0,858	-0,825	-0,599	-0,883	-0,358	-0,696	-0,264	-0,709	0,851	0,826	0,271	0,43	-0,206	-0,098	-0,408	-0,976	-0,851	-0,734	0,402	1	0,829	0,7
kmaxLm	0,434	-0,666	0,628	-0,744	-0,82	-0,547	-0,625	-0,713	-0,783	-0,744	-0,888	-0,74	-0,846	-0,747	-0,68	-0,92	-0,848	0,107	-0,849	-0,654	-0,543	0,833	0,877	-0,06	0,048	0,224	0,137	-0,432	-0,797	-0,988	-0,54	0,436	0,829	1	0,518
kmaxSe	0,708	-0,959	0,516	-0,604	-0,334	-0,994	-0,986	-0,853	-0,933	-0,952	-0,773	-0,913	-0,888	-0,948	-0,972	-0,25	-0,886	-0,533	-0,743	0,291	-0,366	0,892	0,841	0,814	0,85	-0,556	-0,478	-0,347	-0,829	-0,518	-0,998	0,419	0,7	0,518	1

For the meanings of abbreviation see Table 1

Table S2. Model parameters deduced for the prediction of the inhibition curve biological parameters

Variable	4Dr Ec	4Dr Sa	4Dr Lm	4Dr Se	kmax Ec	kmax Sa	kmax Lm	kmax Se
Intercept	-19,75	-66,274	-6,015	14,222	0,966	2,414	0,993	0,526
Hy	9,965	5,447	-0,674	-1,846	-0,205	-0,095	0,002	0,041
Hy4Glu	-5,662	9,21	-4,421	-0,422	0,08	-0,168	0,037	0,005
Ty	-27,75	-13,289	18,319	2,468	0,637	0,247	-0,181	-0,038
pCum	291,364	119,957	-80,786	12,253	-5,565	-3,233	0,959	-0,036
Verb	-18,102	-13,382	0,105	2,324	0,158	0,272	-0,004	-0,032
HyEda	-37,044	-36,713	39,967	16,004	0,851	0,726	-0,406	-0,293
Ole	3,771	3,553	0,461	0,637	-0,059	-0,073	-0,01	-0,01
Coms	675,21	454,39	-330,08	-129,669	-15,692	-6,527	2,944	1,981
Secox	1,433	2,89	0,214	0,21	-0,022	-0,06	-0,009	-0,002
Seclog	2,973	12,949	0,379	1,278	-0,024	-0,279	-0,03	-0,016
Oleo11	0,127	-6,007	8,224	1,792	0,007	0,151	-0,112	-0,041
TPh	0,784	1,651	0,02	0,008	-0,015	-0,032	-0,003	0,001
Glu	-0,124	1,866	-0,296	-0,022	0,002	-0,038	0	0,001
Fru	0,366	6,854	-2,124	-0,147	-0,015	-0,134	0,013	0,004
Sac	-0,257	3,99	2,642	2,042	-0,027	-0,031	-0,039	-0,043
Man	0,385	14,674	5,135	-1,298	0,004	-0,31	-0,12	0,045
TS	-0,046	1,29	-0,199	-0,018	0	-0,026	0	0,001
pH	5,973	18,531	-6,821	-0,832	-0,105	-0,39	0,069	0,025
Salt	-1,055	-8,053	7,976	2,397	0,07	0,142	-0,093	-0,044
TA	-4,991	24,841	6,764	-5,486	0,078	-0,495	-0,185	0,132
CA	-98,733	-447,977	265,315	48,695	2,82	9,812	-2,514	-1,319
KS	57,414	143,119	-62,386	-17,818	-1,117	-3,194	0,574	0,478
NaB	24,887	85,179	-39,089	-11,922	-0,536	-1,866	0,384	0,309
AA	-56,873	107,196	19,422	-18,353	1,479	-2,62	-0,59	0,525
CAc	15,393	-25,857	25,842	-5,264	-0,288	0,507	-0,349	0,134
LAB	-1,737	-0,399	2,019	0,536	0,033	0,01	-0,022	-0,006
Yeast	1,328	-1,655	-1,726	-0,488	-0,029	0,044	0,02	0,003

For the meanings of abbreviation see Table 1

4. CONCLUSIONES

Las conclusiones más relevantes que se obtienen tras el desarrollo de la presente tesis doctoral son las siguientes:

1) Las actuales condiciones de envasado que realizan las empresas del sector utilizando elevadas concentraciones de conservantes (sorbato y benzoato), ha propiciado una considerable reducción de las poblaciones de enterobacterias y levaduras (principales causas de alteración en el pasado), pero, por el contrario, favorece el desarrollo de BAL. Estos microorganismos se han asociado con la aparición de un nuevo tipo de alteración a los 2 meses de envasado caracterizada por la formación de salmueras viscosas y turbias, y por la degradación de la textura de los frutos y aparición de manchas blanquecinas. La alteración, descrita por primera vez en el sector, se relaciona con un genotipo específico de la especie *L. pentosus* O-A-2. Sin embargo, los envasados no representan riesgo microbiológico para los consumidores dada la escasa presencia y supervivencia de enterobacterias. Conclusión obtenida de la siguiente publicación:

Capítulo 1. *Lactobacillus pentosus* is the dominant species in spoilt packaged *Aloreña de Málaga* table olives. LWT – Food Science and Technology 70 (2016) 252-260.

2) Los envasados de aceitunas de mesa *Aloreña de Málaga* siguen presentado problemas de inestabilidad. El estudio de la vida comercial y sensorial de aceitunas *Aloreña de Málaga* envasadas en garrafas PET de 1.6L por un periodo de tiempo superior a los 8 meses, confirma a las poblaciones de BAL como la principal causa de inestabilidad microbiológica, así como una pérdida del valor instrumental de la dureza y tonalidad verde de los frutos a lo largo del envasado. La metodología de análisis sensorial y panel de consumidores utilizado, muestra que la máxima aceptación de los frutos y envases se obtienen entre el periodo comprendido entre los 7 y 40 días de envasado, bajando hasta el 50% en predisposición de compra cuando se sobrepasan los 131 días. Los datos también muestran que los consumidores dan una mayor aceptabilidad inicial para los envases, que disminuye a medida que aumenta el tiempo de almacenamiento, que para los frutos no se modifica prácticamente a lo largo del periodo de estudio. Por lo tanto, se ha de trabajar en el diseño de nuevas presentaciones y materiales de envasado que permitan alargar la vida de mercado de estas aceitunas. Conclusión obtenida del siguiente trabajo:

Capítulo 2. Shelf life of traditional seasoned *Aloreña de Málaga* table olives based on their packages and fruits characteristic.

3) El $ZnCl_2$ es más efectivo que el $ZnSO_4$ para inhibir el crecimiento de levaduras en modelos *in vitro*. El NaCl no mostró ningún tipo de efecto sobre la adición de estas sales, mientras que el Hy mostró un efecto antagónico solo a altas concentraciones. Las interfaces de crecimiento/no crecimiento obtenidas muestran que la inhibición del cóctel de levaduras puede alcanzarse en el rango de 4 – 5 mM de Zn, expresado como $ZnCl_2$. Conclusión obtenida de la siguiente publicación:

Capítulo 3. Effect of zinc formulations, sodium chloride, and hydroxytyrosol on the growth/no-growth boundaries of table olive related yeast. Food Microbiology 57 (2016) 71-80.

4) El MET y el CIN muestran un amplio espectro de inhibición frente a las BAL y levaduras. En el caso del MET, el MIC para las poblaciones de bacterias lácticas fue de 50 mg/L y 772 mg/L para las levaduras. Para el CIN, el valor de MIC es más elevado, 125 mg/L para las levaduras y 1.060 mg/L para las BAL. Su aplicación en envasados de aceitunas *Aloreñas de Málaga* es prometedora ya que, en el caso del MET, también presenta un efecto antioxidante que podría ayudar a retener el color verde de los frutos. Conclusión obtenida de la siguiente publicación:

Capítulo 4. Susceptibility and resistance of lactic acid bacteria and yeasts against preservatives with potential application in table olives. Food Microbiology 54 (2016) 72-79.

5) El pH del medio tiene una gran influencia sobre el efecto inhibidor del MET sobre las poblaciones de BAL, levaduras y enterobacterias (especialmente por debajo de 4,0). Sin embargo, la acción del CIN es independiente del pH. Para un mismo valor de pH, la acidificación con HCl fue más efectiva que con ácido pirúvico. El crecimiento de las poblaciones de enterobacterias se inhibe a pH 4,0 e inferiores, incluso en ausencia de conservantes. Conclusión obtenida de la siguiente publicación:

Capítulo 5. *In silico* logistic model for table olive related microorganisms as a function of sodium metabisulphite, cinnamaldehyde, pH, and type of acidifying agent. Frontiers in Microbiology 7 (2016) 1370.

6) Las salmueras de fermentación de aceitunas *Aloreña de Málaga* muestran un gran poder inhibidor sobre el crecimiento de patógenos, no detectándose la presencia de los mismos a las 24 h tras su inoculación. Estos datos confirman una vez más el hábitat tan adverso que ofrecen las salmueras de aceitunas para los patógenos alimentarios y la gran seguridad microbiológica que presenta este producto. De todos los patógenos estudiados, *E. coli* es el que presenta una mayor supervivencia en las salmueras. La inhibición está relacionada con la presencia en las salmueras de ciertos compuestos antimicrobianos de naturaleza fenólica como el HyEDA, EDA, Hy-4glucosido y Ty. Conclusión obtenida de la siguiente publicación:

Capítulo 6. Survival of foodborne pathogens in natural cracked olivebrines. Food Microbiology 59 (2016) 104-111.

7) En envasados reales, la supervivencia de los patógenos fue diferente según la elaboración de *Aloreña de Málaga* estudiada, pero fue siempre mayor que en las salmueras. La preparación en la que los microorganismos mostraron una mayor supervivencia fue en las verdes frescas ecológicas en las que no se añade ningún tipo de conservante. En los envasados, *S. aureus* resultó ser la especie más resistente. En cualquier caso, los envasados de aceitunas *Aloreñas de Málaga* resultaron ofrecer también un ambiente muy desfavorable para el crecimiento de los patógenos, puesto que no se detectaron tras 48 h de envasado. Por ello, sería aconsejable dejar los envases en el almacén de las industrias durante un periodo de, al menos, 2 días antes de su comercialización como posible medida de seguridad frente a una hipotética contaminación por estos microorganismos. El análisis multivariante realizado confirma que la supervivencia de los microorganismos está una vez más relacionada con la presencia de compuestos fenólicos, la concentración de azúcares y conservantes en las salmueras de envasado cuyos valores dependieron de la forma de elaboración. Conclusión obtenida del siguiente trabajo:

Capítulo 7. Foodborne pathogen survival in comercial *Aloreña de Málaga* table olive packaging.

CURRICULUM VITAE

Licenciada en Biología por la Universidad de Sevilla (2009). Máster en Ciencia y Tecnología de Alimentos y Bebidas Fermentadas por la Universidad Pablo de Olavide (2013). Desde el 2010 trabaja en el Instituto de la Grasa participando en diversos contratos y proyectos de investigación entre los que destaca el proyecto Europeo Probiolives: "*Table olive fermentation with selected strains of probiotic lactic acid bacteria. Towards a new functional food"* y el proyecto motriz de la Junta de Andalucía PrediAlo: "*Control microbiológico de las poblaciones de enterobacterias en envasados de Aceitunas de mesa Aloreña de Málaga*". Antes de la realización de esta Tesis había publicado más de 20 trabajos científicos en revistas internacionales SCI de alto índice de impacto, 3 capítulos de libros y participado en más de 15 congresos nacionales e internacionales, en algunos de ellos siendo parte del comité organizador. También es autora de una patente y un secreto industrial. Su actividad docente queda reflejada al haber participado como profesora en el curso de postgrado y alta especialización "Aislamiento, Caracterización e Identificación Molecular de Microorganismos en Alimentos" (ACIMA), organizado por el CSIC durante los cursos 2014-2015, 2015-2016 y 2016-2017. Socia y fundadora de la EBT Technological Applications for Improvement of the Quality and Safety in Food, S.L.