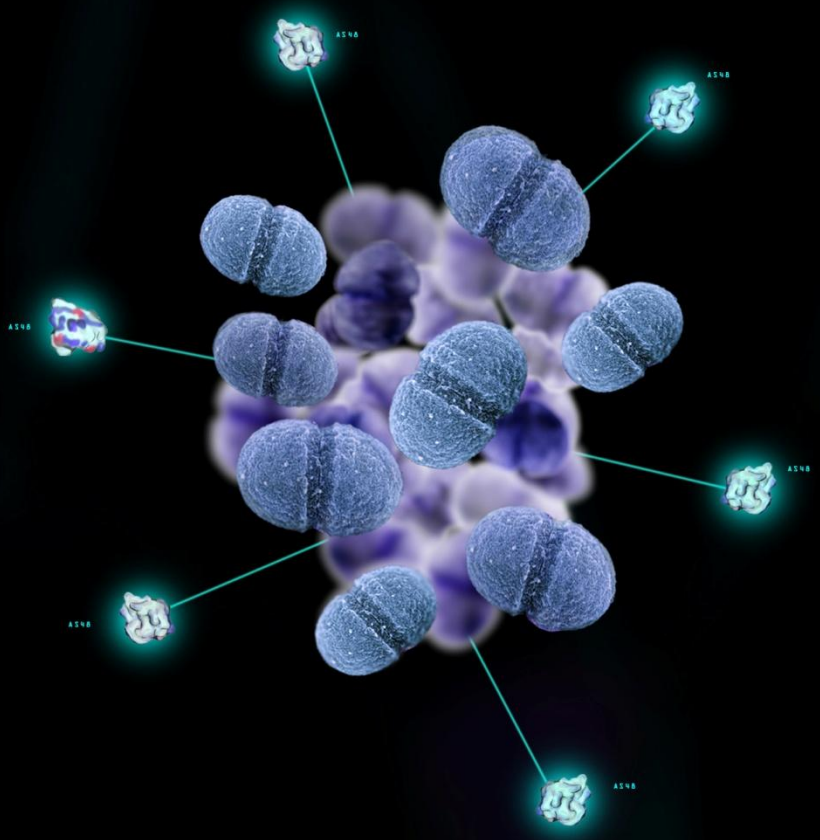
Aplicación de la tecnología de las barreras en el desarrollo de la enterocina AS-48 como bioconservante alimentario. Estudio de probiosis de una cepa productora de AS-48.



Tesis Doctoral

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Editor: Universidad de Granada. Tesis Doctorales
Autor: Alberto Baños Arjona
ISBN: 978-84-9163-218-4
URI: http://hdl.handle.net/10481/46572

Universidad de Granada

FACULTAD DE CIENCIAS. DEPARTAMENTO DE MICROBIOLOGÍA

Programa de Doctorado de Microbiología

APLICACIÓN DE LA TECNOLOGÍA DE LAS BARRERAS EN EL DESARROLLO DE AS-48 COMO BIOCONSERVANTE ALIMENTARIO. ESTUDIO DE PROBIOSIS DE UNA CEPA PRODUCTORA DE AS-48.

Memoria presentada por Alberto Baños Arjona para optar al grado de Doctor en Ciencias por la Universidad de Granada. Esta tesis ha sido dirigida por Eva Valdivia Martínez, Catedrática de la Universidad de Granada y Samir Ananou Jaled, Profesor Titular de la Universidad de Fez.

Granada a 12 de Noviembre de 2015

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Fecha de depósito: 13 de noviembre de 2015

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Traducción de textos: José Manuel García Madero
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Esta tesis ha sido posible gracias a los fondos obtenidos a través del Proyecto de Excelencia de la Junta de Andalucía P07-AGR-02539. Ha sido realizada en el Departamento de Microbiología de la Facultad de Ciencias de la Universidad de Granada y en el departamento de I+D de la empresa DMC Research SLU.

JUNTA DE ANDALUCIA

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RESUMEN

La enterocina AS-48 es un péptido antimicrobiano producido por cepas del género *Enterococcus* (Gálvez et al., 1986). Consta de 70 aminoácidos no modificados y una masa molecular de 7.149,25. Lo singular de esta molécula es su naturaleza circular, establecida a través de un enlace peptídico que une los extremos amino y carboxilo y cierra la estructura (Samyn et al., 1994; Martínez Bueno et al., 1994). En su composición aminoacídica destaca una alta proporción de aminoácidos básicos, lisina y arginina, lo que justifica su carácter fuertemente catiónico. Además, contiene una gran cantidad de residuos hidrofóbicos y de aminoácidos hidrofílicos sin carga neta (Gálvez et al., 1989a). La disposición espacial de ambos tipos de aminoácidos en zonas de la molécula bien diferenciadas confiere a la molécula un carácter anfipático muy importante para su actividad biológica, cuya diana es la membrana citoplásmica bacteriana (Langdon et al., 1998; González et al., 2000; Gálvez et al., 1991). Es su estructura circular, lo que sin duda le confiere una gran estabilidad térmica, a pHs extremos y a altas concentraciones de agentes desnaturalizantes. AS-48 presenta actividad bactericida sobre la mayoría de las bacterias Gram-positivas ensayadas (Gálvez et al., 1989b). Dentro de este amplio espectro de acción, es interesante destacar la sensibilidad de bacterias patógenas transmitidas por los alimentos y/o alterantes de los mismos y la falta de actividad sobre células eucariotas (Gálvez et al., 1989b).

Dadas sus propiedades de espectro de acción y resistencia a agentes físicos y químicos, desde hace algunos años se viene ensayando esta enterocina en alimentos, como alternativa a los conservantes químicos tradicionales, para aumentar la seguridad higiénica y la vida media de diversos productos alimentarios. La aplicación de AS-48 en alimentos se ha realizado mediante producción *in situ* y *ex situ* de la misma, en diversos tipos de alimentos, lácteos, cárnicos y vegetales. Aunque los resultados fueron muy prometedores, las cantidades de AS-48 necesarias para inhibir a patógenos fueron muy superiores a las establecidas previamente en medios de cultivo de laboratorio (Mendoza et al., 1999; Muñoz et al., 2004; 2007; Ananou et al., 2004; Ananou et al., 2005a, 2005b). Las causas de este hecho, que ha sido referido para la mayor parte de las bacteriocinas, son varias: condiciones de procesado del alimento, inestabilidad de la bacteriocina a los cambios de pH del alimento, inactivación por enzimas, interacción con aditivos/ingredientes, adsorción a componentes del alimento, baja solubilidad y distribución irregular en la matriz alimentaria. Estos factores son tanto más importantes en limitar la eficacia de las

bacteriocinas en alimentos cuanto mayor es la complejidad de la matriz alimentaria. En consecuencia, se hace evidente la necesidad de validar la efectividad específica de cada bacteriocina, y sus combinaciones con otros agentes antimicrobianos, en cada alimento y frente a cada organismo diana. Por ello, para rebajar la dosis efectiva de AS-48 se planteó el ensayo de su actividad en alimentos, sola y combinada con otras barreras que puedan potenciar su actividad.

Para este estudio, sobre todo se han seleccionado derivados cárnicos (pescados incluidos), ya que son muy propensos a contaminarse y vehiculizar bacterias patógenas, que se han suplementado con AS-48, sola y combinada con compuestos químicos de grado alimentario así como con barreras físicas implementadas en la conservación de alimentos.

Uno de los modelos alimentarios ha sido el jamón cocido. Según los resultados obtenidos podemos concluir que puede usarse AS-48, sola o combinada con barreras químicas o físicas en la prevención de la contaminación de este alimento por bacterias patógenas como *Listeria monocytogenes* y *Staphylococcus aureus* y en la alteración debida a *Lactobacillus sakei, Brochothrix thermosphacta*, o *Staphylococcus carnosus.* Aunque la enterocina por sí sola es efectiva, la combinación de AS-48 con otras barreras, como tripolifosfatos, pirofosfato sódico, nitritos/nitratos, lactato sódico o acetato sódico (en el caso de los organismos alterantes) puede aumentar su actividad inhibidora, reduciendo la concentración efectiva y proporcionando protección en productos con un nivel de contaminación más elevado. El envasado a vacío ha mostrado ser particularmente efectivo en aumentar la actividad de AS-48 frente a *Lb. sakei* y *B. thermosphacta.*

AS-48 se ha aplicado sola o en combinación con el conservante natural Proallium DMC®, un aromatizante basado en extractos de Aliáceas incorporados en dextrinas, con propiedades antibacterianas bien demostradas. Los resultados obtenidos, aplicando la enterocina AS-48, sola y sobre todo combinada con el extracto aliáceo en diversos tipos de alimentos cárnicos procesados (hamburguesas, chistorras, albóndigas y jamón cocido) frente a *L. monocytogenes, S. aureus* y *Clostrdium perfringens* son muy alentadores, expandiendo el abanico de posibilidades del uso de la enterocina en aquellos productos donde sea compatible el uso de Proallium cuyo afecto antimicrobiano se suma al de AS-48.

También se ha ensayado la eficacia de la enterocina tras ser incorporada en cuatro tipos de recubrimientos comestibles (RC) basados en ésteres de sacarosa y ácidos grasos (sucroésteres), ésteres del ácido acético y mono y digliceridos

(ACETEM), quitosano y gelatina de pescado. Los alimentos estudiados han sido melón, jamón cocido, queso fresco y merluza fresca, aplicando en cada tipo de alimento el RC más adecuado a su naturaleza y estudiando la influencia de la implementación del RC adicionado de AS-48 sobre la contaminación por las bacterias patógenas (*L. monocytogenes* en melón y queso) y alterantes (*Lb. acidipiscis* y *S. carnosus,* en merluza fresca; *Lb. sakei* y *B. thermosphacta* en jamón cocido) seleccionadas. Se ha concluido que la incorporación de AS-48, a cualquiera de los recubrimientos comestibles utilizados, es una estrategia muy válida para la aplicación efectiva de la enterocina en diversos tipos de alimentos, permitiendo el control de bacterias alterantes y patógenas vehiculizadas por los mismos.

En pescado fresco (merluza y salmón) y ahumado (salmón) se ha ensayado la aplicación de tratamientos combinados de AS-48 con el fago lítico de *L. monocytogenes* P-100, así como de ambos agentes por separado para controlar a este patógeno. Los resultados de este estudio indican la capacidad de tanto la enterocina AS-48 como del fago P100 para controlar *L. monocytogenes* en pescado crudo y ahumado. Aunque las dosis no son comparables (hay que tener en cuenta el efecto amplificador del ciclo lítico del fago), AS-48 parece ser más efectiva que el fago. Los mejores resultados se consiguieron por la aplicación simultánea de ambos agentes biológicos, que consiguió eliminar a las listerias en ambos tipos de pescado crudo.

De los estudios de toxicidad subcrónica de la enterocina, llevados a cabo mediante la administración oral continuada de la misma a ratones BALB/c, a una concentración máxima de 200 mg/kg durante 90 días, se puede concluir que AS-48 no produce efectos adversos sobre ninguno de los parámetros evaluados, por lo que es factible su uso como conservante alimentario.

La cepa *E. faecalis* UGRA10, aislada de un queso artesanal de leche de cabra y buena productora de la enterocina AS-48, ha sido estudiada en relación a su potencial uso como probiótico, dada la importancia que se da a la producción de antimicrobianos en ciertos beneficios para el hospedador. Debido al carácter de patógenos oportunistas de algunas cepas de enterococos, se ha hecho especial énfasis en estudiar la bioseguridad de UGRA10.

De los estudios previos realizados *in vitro* cabe destacar la capacidad para hidrolizar caseína y producir enzimas lipasa, cualidades de interés tecnológico. La cepa presenta los genes *gelE, asa1, esp, efa* y *ace* que codifican para putativos factores de virulencia en enterococos aunque más que verdaderos genes de

virulencia, pueden ser considerados como factores de colonización y de permanencia en el tracto gastrointestinal. Las resistencias a antibióticos detectadas en UGRA10 son intrínsecas a la especie y por tanto no transmisibles. En cuanto a su habilidad para sobrevivir a las condiciones del tracto gastrointestinal e implantarse en él, resiste bien el pH ácido, forma biofilmes y tiene una moderada capacidad de adhesión a la línea celular Caco-2, derivada de adenocarcinoma de colon humano. Muy interesante es la interferencia con la adhesión de *L. monocytogenes* a células Caco-2, efectuada por UGRA10, cuando ésta es adicionada antes o conjuntamente con el patógeno.

El estudio de bioseguridad de la cepa UGRA10 en modelo murino administrando la bacteria (10^8 UFC), tanto por vía oral como por vía intravenosa, a animales inmunocompetentes e inmunodeprimidos ha puesto de manifiesto la inocuidad de la cepa sea cual sea la vía. Así, entre los ratones administrados oralmente con UGRA10 y los controles no hubo diferencias en la ingesta, signos de enfermedad y mortalidad, ni en los datos hematológicos ni de bioquímica sanguínea. En cambio, se observó un incremento significativo en la ganancia de peso y en el índice de transformación en los ratones alimentados con el probiótico. No se detectó translocación de la bacteria hacia los órganos linfoides: nódulos linfáticos, bazo e hígado. En los animales administrados vía intravenosa, los enterococos fueron eliminados eficientemente del bazo después de 5 días de la inoculación. No se observó morbilidad ni mortalidad en ninguno de los grupos.

El modelo murino ha servido también para poner de manifiesto la capacidad inmunomoduladora de UGRA10 a través de la estimulación de la proliferación de los esplenocitos activados por mitógenos (LPS y CoA) y del sesgo en la producción de citoquinas, estando incrementada la de IFNγ y disminuida la de TNF-α.

Los ratones alimentados con UGRA10 fueron más resistentes a infecciones por *L. monocytogenes* y *C. perfringens.* El uso paralelo de una cepa mutante derivada de UGRA10, no productora de AS-48, ha proporcionado resultados que apoyan el papel protector de la enterocina en la protección frente a estos patógenos.

El ensayo de UGRA10 AS-48 en peces ha mostrado la utilidad de la cepa UGRA10 y la enterocina AS-48 en el control de patógenos de gran importancia en acuicultura, como es *Lactococcus garviae.* Los estudios previos sobre la toxicidad y bioseguridad de AS-48 y de UGRA10 en pez cebra y en trucha han demostrado la ausencia de efectos adversos. En pez cebra, la administración previa del probiótico incrementó significativamente la tasa de supervivencia respecto al grupo control. En las truchas infectadas con el patógeno, la administración intraperitoneal de AS-48

incrementó la supervivencia de los animales en relación al grupo control. La aplicación de baños periódicos en soluciones con AS-48 fue el tratamiento más efectivo.

Todos estos resultados ponen de manifiesto el potencial tanto de la enterocina AS-48 como de la cepa *E. faecalis* UGRA10 para ser desarrollados como conservante biológico y probiótico, respectivamente.

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INDICE

I. LA CONSERVACIÓN DE LOS ALIMENTOS

1.1. Microorganismos de interés en la conservación y seguridad alimentaria

La conservación de los alimentos pretende, mediante la aplicación de un conjunto de procedimientos y técnicas, impedir o minimizar el crecimiento y actividad de los microorganismos y, así alargar la vida media y proporcionar unos niveles aceptables de seguridad higiénica a los mismos.

A pesar de disponer de una variedad de técnicas de conservación de los alimentos de eficacia demostrada, las enfermedades transmitidas por éstos continúan siendo un problema de importancia en los países industrializados. Así, el informe EFSA "*The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012***"** aporta que durante este período, aparte de los principales patógenos clásicos asociados a los alimentos, como *Salmonella* (82.624 casos), se ha producido un aumento de las infecciones oportunistas ocasionadas por *Campylobacter* (214.779 casos), *Listeria monocytogenes* (1.763 casos) y *Escherichia coli* verotoxigénica (6.043 casos). Esto se atribuye al aumento de la población del grupo denominado YOPIS (Youngs, olders, immunocompromised and pregnants). En la Unión Europea los costos directos e indirectos atribuidos a estas enfermedades se estiman en miles de millones de euros al año (EFSA, 2015).

En España, durante el 2013, se notificaron 792 casos de salmonelosis en humanos, lo que corresponde a una tasa de 0.41 casos por cada 100.000 personas. En comparación a la UE, España se situaría en una posición intermedia (EFSA, 2015). Al igual que en el resto de la UE, en más del 50% de los casos se aisló *S. enteritidis* y *S. typhimurium* y el grupo de edad más afectado fue el de niños de 1 a 4 años que contabilizó aproximadamente una tercera parte de los casos. Además, se declararon en España un total de 7.064 casos confirmados de campilobacteriosis, 140 casos de listeriosis y 28 casos de VTEC (50% del serogrupo O157) (EFSA, 2015).

Para determinar el tipo de tratamiento que se ha de aplicar a un alimento en vistas a aumentar su vida media y su seguridad es preciso conocer cuáles son los microorganismos que pueden colonizarlo más habitualmente. Otra cuestión de gran importancia a este respecto es la procedencia de tales microorganismos. Es, además, necesario hacer una distinción entre los microorganismos que alteran los alimentos y aquellos que los envenenan.

1.1.1. Microorganismos alterantes

Microorganismos alterantes son aquellos que, al crecer sobre los alimentos, degradan sus componentes de forma que cambian sus propiedades organolépticas, de sabor, olor, aroma, textura o color, haciéndolos inaceptables para su consumo. El resultado final es la reducción de la vida media del alimento, con las consiguientes pérdidas económicas (por ejemplo aproximadamente el 20% de las frutas y verduras recolectadas se pierden por deterioro microbiano).

Los diferentes alimentos poseen una microbiota autóctona que, en muchos casos, es la que va a ser responsable de su alteración. En otros, los microorganismos alterantes proceden de fuera del alimento y lo contaminan durante los procesos de manipulación, procesado, transporte y almacenaje. Los agentes microbiológicos causantes de deterioro pueden ser bacterias, hongos y levaduras, siendo bacterias y hongos los más importantes. De todos los microorganismos presentes en un alimento sólo ciertas especies son capaces de multiplicarse de una manera activa sobre el alimento. En el deterioro de alimentos se produce una selección con el tiempo, de forma que la población heterogénea inicial presente en el alimento va quedando reducida a poblaciones más homogéneas y, finalmente, en muchos casos, a un solo tipo de microorganismo que consigue colonizar todo el alimento desplazando a los demás. Por consiguiente, durante el proceso de deterioro se va seleccionando una población o tipo de microorganismos predominantes. Existen una serie de factores como la actividad de agua (aw), el pH o la presencia de ciertos nutrientes que dirigen esta selección y que determinan lo que se denomina resistencia a la colonización de un alimento.

Entre los principales géneros bacterianos responsables de alteración de alimentos encontramos; *Bacillus, Staphylococcus, Micrococcus, Brochothrix, Lactobacillus* y *Leuconostoc*.

1.1.2. Microorganismos patógenos transmitidos por los alimentos

Son aquellos microorganismos que causan enfermedad en los humanos o en los animales y que utilizan a los alimentos, y a menudo también el agua, como vehículos de transmisión. Son responsables de la mayoría de las enfermedades causadas o transmitidas por alimentos, cuyos síntomas se manifiestan mayoritariamente en el tracto intestinal en forma de gastroenteritis, aunque pueden dar lugar a cuadros más extendidos en el organismo que podría llegar incluso a septicemia (en casos no tratados). Las patologías

asociadas al consumo de alimentos contaminados con microorganismos pueden aparecer como casos aislados, cuando el mal procesamiento del alimento se ha producido a nivel particular; pero pueden frecuentemente asociarse a brotes epidémicos más o menos extendidos en el territorio; así, el número de brotes epidémicos asociados a alimentos durante los últimos años en todo el territorio nacional ha oscilado entre 900 y 1.000 brotes anuales (EFSA, 2015).

Las patologías asociadas a transmisión alimentaria pueden ser de dos tipos: infecciones alimentarias producidas por la ingestión de microorganismos viables o intoxicaciones alimentarias producidas como consecuencia de la ingestión de toxinas bacterianas producidas por los microorganismos presentes en los alimentos. En general el crecimiento de los microorganismos patógenos en los alimentos a los niveles de las dosis infectivas medias no suele alterar las propiedades organolépticas de los mismos, por lo que son consumidos sin recelo alguno.

Establecer la relación de un microorganismo particular con una enfermedad y con un alimento requiere de un exhaustivo estudio epidemiológico, que es imprescindible para controlar la enfermedad y establecer el riesgo y los puntos críticos de control en el proceso de producción de los alimentos. Por lo general los patógenos siguen un ciclo de transmisión fecal-oral, siendo los alimentos eslabones de la cadena de transmisión, situados entre el hospedador infectado (enfermo, convaleciente o portador sano) que emite los microorganismos en sus heces, y el individuo sano susceptible de convertirse en un nuevo hospedador.

Entre los principales géneros bacterianos responsables de Enfermedades de Transmisión Alimentaria encontramos; *Salmonella, Shigella, Escherichia, Campylobacter, Yersinia, Vibrio, Listeria, Staphylococcus, Bacillus* y *Clostridium*.

1.2. Métodos de conservación de los alimentos

Sea cual sea el método de conservación que se considere, se trata en realidad de aplicaciones prácticas en el campo de los alimentos de las leyes de Liebig y Shelford, que implican el ajuste de las condiciones ambientales para que se superen los requerimientos de crecimiento y los rangos de tolerancia de los microorganismos, al objeto de impedir el crecimiento de aquellos potencialmente alterantes y/o patógenos. Los procedimientos básicos usados en el control del desarrollo microbiano en los alimentos son:

- Destrucción de todos los microorganismos no deseables mediante tratamientos físicos o químicos e implementación de barreras físicas que impidan la recontaminación.
- Mantenimiento del alimento bajo condiciones ambientales que excluyan o minimicen la actividad microbiana.
- Modificación del alimento mediante procesado o adición de conservantes, que reduzcan su accesibilidad como sustrato para el desarrollo microbiano.

A continuación haremos un breve recorrido por los principales métodos de conservación:

1.2.1. Métodos físicos de conservación de alimentos

Los métodos físicos de conservación de alimentos son aquellos que utilizan tratamientos físicos para inhibir, destruir o retirar microorganismos indeseables y en los que no están implicados aditivos antimicrobianos o productos del metabolismo microbiano (Farkas, 1997).

Procesos físicos de deshidratación. El agua y, más específicamente, la disponibilidad de agua, es uno de los factores más importantes que controlan la alteración de los alimentos. La disponibilidad de agua se mide mediante la actividad de agua (a_w), que se define como la relación, a la misma temperatura, entre la presión de vapor de agua en un alimento, *P*, y la presión de vapor del agua pura, P_0: $a_w = P/P_0$. La conservación de los alimentos mediante deshidratación se basa en que el crecimiento microbiano es inhibido si se elimina el agua disponible ya que la mayoría de las bacterias requieren una a_w mínima de 0.91-0.88.

Conservación en frío. Se trata de mantener los alimentos a temperaturas bajas, situadas por encima del punto de congelación (-2 ºC a 10 ºC). Según el alimento, el periodo de conservación puede ir desde unos pocos días hasta varias semanas. El efecto conservante del frío se basa en que al reducirse la temperatura disminuye la velocidad de las reacciones químicas y el estado de fluidez de los lípidos de la membrana. Las temperaturas usuales de refrigeración permiten sólo el crecimiento de los microorganismos psicrófilos y psicrótrofos, generalmente bacterias.

Congelación. Esta técnica consiste en bajar la temperatura de un alimento hasta -18 ºC y mantenerlo almacenado a ésta o más baja temperatura. Es una técnica muy conveniente pues conserva muy bien sin alterar, sin embargo es energéticamente muy costosa. Aunque la congelación reduce considerablemente el número de células viables en el alimento, no se

puede considerar un proceso de higienización y, en determinadas condiciones, los microorganismos supervivientes pueden proliferar durante la descongelación.

Tratamiento de Altas Temperaturas. Son los métodos más efectivos y más ampliamente usados para destruir microorganismos e inactivar enzimas. Los más importantes son:

- **Pasteurización**: Es uno de los más empleados, consiste en un tratamiento térmico relativamente suave que inactiva enzimas y destruye una amplia población (99-99.9 %) de las células vegetativas. Su principal objetivo es eliminar las bacterias patógenas no esporuladas presentes en los alimentos y, puesto que también destruye una gran proporción de los microorganismos alterantes, aumenta su vida media. Además, los alimentos pasteurizados deben ser almacenados a baja temperatura para evitar la proliferación de los formadores de endosporas. Por todo ello, su vida media depende del tipo de alimento y de las condiciones de pasteurización y almacenamiento.

- **Calentamiento óhmico**: Consiste en aplicar un calentamiento eléctrico directo haciendo pasar una corriente eléctrica a través del alimento con una alta resistencia a la conductividad. Es un proceso de alta temperatura aplicada en un corto tiempo (HTST), que puede pasteurizar y esterilizar el material. Esta tecnología permite una producción continua sin transferencia de calor y además, al ser tan rápido, el daño causado a los alimentos es mínimo y los nutrientes no son destruidos ni alteradas sus cualidades organolépticas. Combinada con un envasado aséptico proporciona un alto efecto conservante (Hugas et al., 2002).

- **Tratamiento dieléctrico o radiofrecuencia**: Tanto este tratamiento como el de microondas se basan en las oscilaciones producidas por las moléculas de agua que producen fricción y, en consecuencia, calor. Además de los efectos letales del calor sobre los microorganismos, los campos electromagnéticos causan cambios iónicos que alteran la permeabilidad y funcionalidad de las membranas y producen la lisis celular.

Irradiación. Sus efectos se ejercen sobre el ADN. Los tipos de radiación empleados son la UV y la ionizante. La radiación UV más destructiva es la de 240-280 nm de longitud de onda (germicida). El principal obstáculo para su empleo en alimentos es su bajo poder de penetración en ellos. Por ello, se emplea sobretodo en alimentos líquidos y loncheados y en la desinfección del aire de cámaras de maduración de quesos y secaderos de embutidos.

Alta Presión Hidrostática (APH). Durante los últimos años varios tratamientos físicos no térmicos, como la alta presión hidrostática han suscitado gran interés, por las posibilidades que ofrecen como herramientas para el procesado y conservación de los alimentos y también para desarrollar productos alimenticios innovadores (Farkas, 1997). Mediante los tratamientos de APH se aplican al alimento presiones hidrostáticas elevadas (300 a 1000 Mpa) que se transfieren a través del mismo de forma instantánea y uniforme, lo que da lugar a cambios en el producto o en sus constituyentes. En general, la APH, a temperatura baja o moderada, causa la destrucción de las células microbianas vegetativas y la inactivación de enzimas, sin alterar las características organolépticas de los productos, dejando, además, las vitaminas intactas. Sin embargo la resistencia de los microorganismos es muy variable dependiendo de la cepa microbiana, de la matriz del alimento, del pH, de la temperatura y del tiempo de exposición.

1.2.2. Conservantes

Hoy en día, los consumidores esperan alimentos libres de patógenos y con una larga vida útil. Aunque algunas mejoras se han conseguido mediante los sistemas de procesamiento y envasado actuales, la utilización de conservantes continúa desempeñando un papel fundamental para garantizar la protección en el suministro de alimentos (Davidson et al., 2002). Además, la globalización de los mercados obliga a buscar métodos de conservación que logren prolongar la vida útil de los productos alimenticios.

Los conservantes químicos son definidos por la Administración de Alimentos y Medicamentos (FDA) de USA como "*cualquier sustancia química que, cuando se añade a los alimentos, impide o retrasa el deterioro de los mismos*", sin incluir la sal común, azúcar, vinagre, especias o aceites esenciales. Los conservantes utilizados para prevenir el deterioro biológico se denominan "antimicrobianos". La función tradicional de los antimicrobianos era la de prolongar la vida útil y preservar la calidad de los alimentos mediante la inhibición de microorganismos alterantes. Sin embargo, cada vez más se han utilizado para la inactivación de microorganismos patógenos (Davidson y Zivanovic, 2003), tal es el caso de la utilización de nitritos en productos cárnicos para el control de *Clostridium botulinum*.

De acuerdo con Davidson (2001), los conservantes alimentarios se pueden encuadrar en dos grupos: conservantes químicos tradicionales, y conservantes naturales.

1.2.2.1. Conservantes químicos tradicionales

Su definición es bastante arbitraria. Un conservante es considerado como tradicional cuando cae dentro de cualquiera de las siguientes categorías: ha sido usado durante muchos años, ha sido aprobado como antimicrobiano alimentario en muchos países, es producido de forma sintética o es inorgánico. Paradójicamente, muchos de los conservantes tradicionales de síntesis se encuentran en la naturaleza, como es el caso del ácido benzoico de los arándanos o el sórbico de las bayas de serbal por lo que también se podrían considerar naturales. Los más importantes incluyen:

Sorbato potásico ($C_6H_7O_2K$). Se trata de la sal de potasio del ácido sórbico (E200), presente de forma natural en algunos vegetales, pero fabricado para su uso como aditivo alimentario por síntesis química. Presenta una mayor actividad a un pH por debajo de 6,5. Inhibe el crecimiento de algunos patógenos como *S. aureus* y *Salmonella* aunque su actividad es fundamentalmente antifúngica. Presenta baja toxicidad y se utiliza en bebidas refrescantes, pastelería, ensaladas, derivados cárnicos y quesos.

Benzoato de sodio (C_6H_5COONa). También conocido como benzoato de sosa o E-211, es una sal del ácido benzoico soluble en agua y ligeramente soluble en alcohol. Se trata de uno de los conservantes de amplio espectro más empleados en el mundo, siendo el primer antimicrobiano aprobado para su uso en los alimentos por parte de la FDA. El ácido benzoico posee actividad antimicrobiana en medio ácido aunque se inactiva en medio alcalino de ahí que su uso esté limitado sólo a aquellos alimentos de naturaleza ácida (Gabel et al., 1921).

Lactato y Diacetato ($C_3H_5O_3Na$, $NaH(C_2H_3O_2)_2$). El ácido láctico es producido abundantemente en el metabolismo de las BAL durante la fermentación. Además de su uso como conservante se utiliza también como agente corrector de pH y como potenciador del sabor. Las sales de lactato (E325 y E326) actúan como agentes bacteriostáticos de amplio espectro incrementando la fase de latencia de los microorganismos. La acción específica de los lactatos se atribuye a mecanismos que interfieren en el metabolismo microbiano tales como la acidificación intracelular y la interferencia del transporte de protones a través de la membrana celular. Además, los lactatos provocan una reducción de los valores de a_w de los alimentos favoreciendo la extensión de su vida útil (Houtsma et al., 1993; Rodríguez, 2005). Diversos autores han descrito la actividad antimicrobiana de los lactatos en productos cárnicos, siendo capaces de inhibir el crecimiento de patógenos como *L. monocytogenes* (Eckert et al., 1997; Geornaras et al., 2013). Por otro lado, el diacetato sódico (E262),

acidificante de origen natural, es también un potente agente antimicrobiano. Diversos estudios han descrito su capacidad para inhibir *L. monocytogenes* en productos cárnicos a concentraciones del 0,25% (Blom et al., 1997). Se ha demostrado que la aplicación combinada de lactato de sodio ($NaC_3H_5O_3$) y diacetato sódico ($C_4H_7NaO_4H_2O$) provoca un efecto sinérgico que incrementa su efecto inhibitorio frente a *L. monocytogenes* (Mbandi y Shelef, 2002).

Nitrito de sodio ($NaNO_2$). Es una sal sódica de la familia de los nitritos. Constituye uno de los aditivos más comunes es en la industria alimentaria (E250), concretamente en el sector cárnico, donde se emplea como conservante y fijador de color en fiambres y embutidos. Suele emplearse en combinación con otras sales como nitratos de sodio y de potasio (E249) formando parte de las denominadas *sales de curado*. Posee un amplio espectro de actividad bactericida resultando muy efectivo en el control de *Clostridium botulinum*. Yarbrough et al. (1980) postularon distintos mecanismos de acción del nitrito de sodio como la interferencia a nivel de la absorción de oxígeno y la fosforilación oxidativa, colapso en el gradiente de protones e inhibición de ciertas enzimas como las aldolasas. Su empleo como aditivo alimentario está muy regulado debido a la capacidad que posee para generar nitrosaminas (agentes cancerígenos).

Sulfitos (E220-E228). Son sales o ésteres del ácido sulfuroso H_2SO_3. Las sales de sulfito contienen el anión SO_3^{2-}, siendo las más importantes el sulfito de sodio y el sulfito de magnesio. Se utilizan para prevenir el crecimiento microbiano, desempeñando un papel fundamental en sectores como el vinícola (Herraiz y Cabezudo 1989). Además poseen actividad antioxidante, inhibiendo el ennegrecimiento enzimático y la reacción de Maillard (Papazian, 1996; Gould, 2000). Los sulfitos poseen el estatus GRAS por la FDA siempre y cuando se usen en cantidades de conformidad con las buenas prácticas de fabricación.

Polifosfatos (E452). Son sales derivadas de fosfatos, como pirofosfato sódico (SAPP), pirofosfato tetrasódico (TSPP), tripolifosfato sódico (STPP), tetrapolifosfato sódico, hexametafosfato sódico (SHMP), y fosfato trisódico (TSP). Estos compuestos tienen numerosas aplicaciones en los alimentos dada su capacidad para formar complejos solubles con iones metálicos. Los polifosfatos se usan ampliamente en productos cárnicos y lácteos donde ejercen un papel estabilizante. Además, poseen cierta actividad antimicrobiana, siendo especialmente efectivos en el control de bacterias Gram-positivas como *C. botulinum* y *S. aureus* (Seward et al., 1982; Shelef y Wang, 1989, Shelef et al., 1990).

1.2.2.2. Conservantes naturales

Como se ha referido más arriba, aún disponiendo de la amplia variedad de métodos de conservación de alimentos de eficacia comprobada, el control de las enfermedades de transmisión alimentaria continua siendo un problema de primera magnitud. A pesar de ello, en los países desarrollados se da la tendencia creciente por parte de los consumidores a demandar alimentos seguros y sin embargo mínimamente procesados. A este hecho debemos añadir la falta de formación por parte del consumidor medio que relaciona cualquier presencia de aditivos en los alimentos (números E de la etiqueta) con productos de producción intensiva o artificiales, lo que ha llevado, en la última década, a la demanda de alimentos más naturales y libres de aditivos químicos. Esta relación, cada vez más frecuente, entre aditivos sintéticos y potenciales riesgos para la salud ha derivado en un intenso control de estas sustancias por parte de las administraciones y autoridades sanitarias, siendo constantemente evaluados tanto en sus propiedades tecnológicas como toxicológicas. De ahí que estén continuamente en revisión y su uso sea cada vez más restrictivo. Debido a estas continuas reevaluaciones que llevará a cabo la EFSA sobre la seguridad de los aditivos, es recomendable buscar alternativas naturales a muchas de estas sustancias que puedan entrar en prohibición en un futuro próximo. Una solución a esta problemática puede conseguirse mediante el empleo de conservantes naturales y bioconservantes.

Conservantes de origen animal. Forman parte del sistema natural de defensa de los organismos. La ovotransferrina, lactoperoxidasa, lactoferrina y lisozima son ejemplos ilustrativos de este tipo de compuestos con actividad antimicrobiana demostrada frente a patógenos alimentarios (de Wir y van Hooydonk 1996; Lönnerdal 2011; Gyawali e Ibrahim, 2014). Otro ejemplo lo constituye la protamina, un péptido antimicrobiano presente de forma natural en células espermáticas de peces, aves y mamíferos (Rodman et al., 1984). La protamina posee un amplio espectro de actividad antimicrobiana frente a bacterias Gram-positivas, Gram-negativas, y hongos (Uyttendaele y Debevere, 1994). Otro conservante de origen animal ampliamente utilizado es el quitosano, un biopolímero policatiónico presente de forma natural en los exoesqueletos de crustáceos y artrópodos (Younes y Rinaudo, 2015). Se trata de un antimicrobiano de amplio espectro, con efectividad en el control de bacterias Gram-positivas y Gram-negativas como *S. aureus, L. monocytogenes, B. cereus, E. coli, y Salmonella typhimurium* (Kong et al., 2010), así como en el control de mohos (Ben-Shalom et al., 2003; Wojdyła et al., 2004). En los últimos años, el quitosano está siendo ampliamente utilizado en la elaboración de recubrimientos comestibles (Valencia-Chamorro et al., 2011; Elsabee y Abdou, 2013).

Conservantes de origen vegetal. Es conocida la riqueza de muchas plantas como fuente de principios bioactivos de interés tecnológico. Por ejemplo, los aceites esenciales se han utilizado durante siglos en medicina tradicional por sus propiedades antisépticas. Los principales grupos funcionales de estas sustancias incluyen terpenos, saponinas, flavonoides y compuestos como el carvacrol, timol o eugenol (Burt, 2004). Esto ha llevado a que los aceites esenciales y otros extractos de plantas sea considerados como potenciales conservantes de alimentos (Richard y Patel 2005; Tajkarimi et al. 2010; Tongnuanchan y Benjakul, 2014). Entre ellos, los extractos de Aliáceas ocupan un lugar destacado por su alta capacidad antimicrobiana de amplio espectro, atribuida principalmente a la presencia de compuestos de tipo tiosulfinato como la alicina (Kyung 2012; Borlinghaus et al., 2014). Por otro lado, las plantas también producen una variedad de péptidos antimicrobianos, muchos de los cuales puede ser usados como bioconservantes de alimentos. Algunos de ellos incluyen tioninas, defensinas y ciclótidos (Padovan et al., 2010).

Bioconservantes. De acuerdo con Stiles (1996), la Bioconservación se puede definir como *la extensión de la vida media y de la seguridad de los alimentos mediante el empleo de su microbiota natural o controlada y/o sus productos antibacterianos.* Los bioconservantes por excelencia son, sin lugar a duda, las bacterias del ácido láctico (BAL) y sus bacteriocinas. La fermentación de alimentos debida a las BAL es, probablemente, después de la desecación, la forma de conservación más antigua. El empleo seguro (no ligado en ningún caso con enfermedad o envenenamiento de los alimentos) de las BAL desde tiempo inmemorial les ha valido, en estos tiempos, el estatus de organismos GRAS (Generally Recognized As Safe: Reconocidos en General como Seguros). Hoy día su uso, ya programado, en las fermentaciones de alimentos, es aceptado por los consumidores como natural y saludable, representando una solución ecológica a la problemática de la conservación de los alimentos, sobretodo de aquellos mínimamente procesados.

Los bacteriófagos también se han señalado como potenciales bioconservantes de alimentos. Se trata de virus que parasitan de manera específica una bacteria huésped, donde se multiplican. Son los microorganismos más abundantes del planeta, encontrándose en grandes cantidades en el medio ambiente (tierra y agua), entre la microbiota intestinal y en los organismos que se utilizan para producir alimentos. Son inocuos para animales y plantas, y poseen una elevada especificidad. Los fagos líticos poseen un mecanismo bactericida, específico y selectivo, lo que ha despertado el interés entre los investigadores, que ven en ellos una herramienta eficaz para el control de bacterias patógenas en diferentes campos, incluido el de la conservación de alimentos. En la actualidad existen preparaciones

comerciales de fagos para el control de *L. monocytogenes* en alimentos como ListShield ™ y Listex ™ P100 (basado en el listeriófago P100). Estos productos han sido aprobados por la FDA y el USDA (Hagens y Loessner, 2014) y se aplican para tratamiento superficial de alimentos e instalaciones. En el mercado también se encuentran disponibles preparaciones de fagos específicos de *Salmonella* (SalmoFresh™) y *E. coli* O157: H7 (EcoShield ™).

1.2.2.3. Nuevos sistemas de aplicación de conservantes

En la actualidad existen novedosos sistemas de aplicación de conservantes que contribuyen a mejorar la seguridad y prolongar la vida útil de los alimentos, facilitando o potenciando la acción de los mismos a la vez que permiten reducir su cantidad y minimizar la presencia de residuos. Entre estos sistemas se incluyen los recubrimientos comestibles, el envasado activo y las tecnologías de higienización vía aérea como la nebulización.

Recubrimientos comestibles (RC). Son capas delgadas de un material biopolímero (proteína o polisacárido como solución hidrocoloide, o como emulsión con lípidos), que son aplicadas por inmersión o pulverización sobre la superficie de un alimento en adición o reemplazo de la corteza natural, y que se comportan principalmente como barreras que reducen la difusión de gases, permitiendo extender la vida útil del alimento (Carrasco et al., 2002; Wu et al., 2002). Además los RC poseen otras funciones como la de mejorar la apariencia y proteger al producto de los daños mecánicos, agentes físico-químicos y de la actividad microbiana (Falguera et al., 2011).

Los RC se pueden elaborar a partir de lípidos, polisacáridos, proteínas o mezclas de estos (Kester y Fennema, 1986). Los polisacáridos como alginatos, almidones, pectinas o quitosano dan lugar a recubrimientos con buenas propiedades de barrera de gases (Gennadios et al., 1997; Krochta, 2002). Dentro de los lípidos empleados para recubrimientos comestibles se encuentran las ceras de abeja, los triglicéridos, monoglicéridos acetilados, ácidos grasos, alcoholes, y esteres de ácidos grasos de sacarosa. Debido a su hidrofobicidad, son empleados como una barrera al vapor de agua (Gennadios et al., 1997). En cuanto a los RC basados en proteínas, estas pueden ser de origen animal y vegetal tales como el colágeno, la gelatina, la caseína y la proteína de soja, entre otras (Krochta, 2002). Las películas y recubrimientos elaborados a partir de proteínas presentan buenas propiedades de barrera frente a los gases pero no frente al agua (Cha y Chinann, 2004).

Además del componente de naturaleza polimérica (matriz), otro componente importante de los RC son los plastificantes. Suelen ser moléculas de bajo peso molecular que se usan para mejorar la flexibilidad de los recubrimientos. Dentro de los agentes plastificantes utilizados más frecuentemente se encuentran: glicerol, polietilénglicol, sorbitol, ácidos grasos y ceras. A los RC también se pueden adicionar agentes de liberación controlada y lubricantes como aceites, emulsificantes y silicona (Baldwin et al., 1995).

La incorporación de aditivos en la matriz puede contribuir a mejorar la funcionalidad de los recubrimientos. Estos aditivos son agregados durante el proceso de elaboración de los recubrimientos y pueden ser antioxidantes, antimicrobianos, aromatizantes, pigmentos o nutrientes (Pascall y Lin, 2013). La adición de agentes antimicrobianos dentro de RC constituye una técnica innovadora en el mantenimiento de la seguridad alimentaria y prolongación de la vida útil de los alimentos mínimamente procesados. El crecimiento de microorganismos en la superficie de productos frescos y cortados es una de las principales causas de su deterioro, pudiendo evitarse mediante la aplicación de RC con propiedades antimicrobianas. Entre los compuestos más utilizados se encuentran los ácidos orgánicos y sus sales (ácido sórbico, propiónico y benzoico), sulfitos, nitritos, bacteriocinas (nisina y pediocina), enzimas (lisozima) y extractos vegetales, entre otros (Brody et al., 2001; Suppakul et al., 2003). Incluso se ha evaluado la combinación de varios antimicrobianos para la detección de sinergias. Así por ejemplo, Pranoto et al., (2005) demostraron la actividad antimicrobiana de un recubrimiento comestible elaborado a base de quitosano, aceite de ajo y nisina, resultando efectivo en el control de *Bacillus cereus*, *E. coli*, *Salmonella typhimurium*, *S. aureus* y *L. monocytogenes.* También se ha estudiado la incorporación de otros compuestos beneficiosos para la salud, tales como microorganismos probióticos. Por ejemplo, Krasaekoopt et al., (2004) describieron la microencapsulación de bacterias prebióticas en perlas de alginato sódico con distintos recubrimientos. Las aplicaciones de los RC han sido objeto de muchas investigaciones para la mejora de la conservación tanto de productos cárnicos, pescado como vegetales y frutas mínimamente procesadas (Dhall, 2013; Sánchez-Ortega et al., 2014; Jasour et al., 2015).

Envasado activo. Se entiende como envase activo al sistema "alimento-envase-entorno" que actúa de forma coordinada para mejorar la calidad del alimento envasado y prolongar su vida útil (Catalá y Gavara, 2001). Con esta definición se amplía el concepto de envase que pasa de ser un mero contenedor pasivo a desempeñar un papel activo en la mejora de la calidad del alimento envasado. Mediante este sistema, el envase corrige las deficiencias del sistema de conservación modificando la composición y/o características del alimento, absorbiendo componentes indeseables o liberando sustancias activas como conservantes.

Como norma general, los principios activos se incorporan en el propio material de envase formando parte del polímero. En los últimos años se han desarrollado múltiples soluciones de envasado activo que permiten minimizar problemas de composición de gases, eliminación de olores extraños y proliferación microbiológica (López-Rubio et al., 2004; Realini y Marcos, 2014; Malhotra et al., 2015).

Los sistema de envasado antimicrobianos incorporan el agente antimicrobiano en el propio polímero con la intención de inhibir la proliferación de microorganismos en el alimento (Han, 2000; Sung et al., 2013). Durante los últimos años, las aplicaciones potenciales de los envases activos antimicrobianos han sido objeto de atención para empresas del sector de envases, encontrándose ya disponibles diferentes sistemas comerciales para la conservación de alimentos (Cha y Chinnan, 2004; Han, 2005).

La acción antimicrobiana en los envases activos puede estar basada en la emisión de sustancias volátiles al espacio de cabeza del envase o en la migración del componente activo del material de envase al alimento, ya que los polímeros permiten una lenta liberación de los aditivos. Un amplio número de sustancias pueden incorporase a materiales poliméricos formando parte como componentes de los mismos. Son muchas las estudiadas: ácidos orgánicos (acético, benzoico, sórbico, cítrico, y propiónico), enzimas (lisozima), bacteriocinas (nisina, pediocina), fungicidas (imazalil), iones metálicos (plata y cobre) y extractos de plantas (romero, tomillo, orégano) (Cha y Chinan, 2004; Han, 2005).

La incorporación de bacteriocinas en filmes de envasado para el control de patógenos ha sido un campo de actuación que ha despertado el interés de los investigadores durante los últimos años. Cooksey et al., (2000) demostraron la efectividad de una película de polietileno (LDPE) que contenía nisina en el control de *S. aureus* y *L. monocytogenes*. Nguyen et al., (2008) demostraron la efectividad de una película de celulosa que incorporaba nisina para el control de *L. monocytogenes* en salchichas envasadas al vacío. Además, la liberación controlada de bacteriocinas desde el envase hacia la superficie de alimentos tiene como ventaja, frente a la inmersión o pulverización directa, la reducción de la inactivación de las bacteriocinas por los componentes de los alimentos (Appendini y Hotchkiss, 2002).

Nebulización de conservantes. Los microorganismos forman parte de los aerosoles biológicos presentes en el aire de muchas industrias alimentarias. Su formación se ve favorecida por altos niveles de humedad y poca ventilación, condiciones que pueden recrearse en determinadas instalaciones como las cámaras de almacenamiento. La

contaminación microbiana durante el proceso de elaboración de los alimentos constituye un serio problema para el sector no solo por los fenómenos de alteración, sino por el riesgo sanitario derivado de la proliferación de patógenos y hongos productores de micotoxinas.

El desarrollo microbiano sobre la superficie de instalaciones y alimentos almacenados puede tener diversos orígenes; en gran parte de los casos, la presencia de microorganismos vehiculados por el aire son los responsables, aunque también pueden proceder de los manipuladores o como consecuencia de deficiencias de higiene en las instalaciones. Por ello, la correcta Limpieza y Desinfección (L+D) de superficies y ambientes resulta de vital importancia para el control de microorganismos alterantes y patógenos. Pero las actuaciones de L+D con detergentes y desinfectantes convencionales no siempre son suficientes para garantizar una buena higiene. Además, la presencia de alimentos hace que no puedan aplicarse ciertos productos cuyos residuos podrían resultar nocivos para el consumidor.

La aplicación adicional de aditivos alimentarios puede complementar a los tratamientos desinfectantes o biocidas comúnmente utilizados en la industria alimentaria. La aplicación aérea de principios activos de origen natural mediante sistemas de microdifusión o nebulización es un concepto novedoso que pone a disposición de las industrias una herramienta para la mejora de la calidad de sus productos, minimizando también los riesgos para la salud de los consumidores. Además, estas metodologías permiten transportar los compuestos activos incluso a las partes más inaccesibles con la ventaja de poder aplicarse en presencia de alimentos sin suponer un riesgo para la salud del consumidor. En la actualidad se encuentran disponibles en el mercado sistemas de microdifusión de ingredientes alimentarios como DECTOCIDE® VA20 (Betelgeux S.L.) basado en la nebulización de pimaricina (E235) o CYCROM PRO (DOMCA S.A.) basado en la nebulización de extractos de cítricos.

II. LAS BACTERIAS DEL ÁCIDO LÁCTICO: CARACTERÍSTICAS GENERALES E INTERÉS TECNOLÓGICO

2.1. Definición y características principales

Las bacterias del ácido láctico (BAL) son un grupo muy heterogéneo de microorganismos que comparten variedad morfológica, fisiológica y metabólica y cuya característica fundamental es la producción de cantidades significativas de ácido láctico como producto principal de su metabolismo fermentativo (Marshall y Law, 1984; Axelsson,

1998). Las BAL son células procariotas, heterótrofos y quimio-organotrofas. Son bacterias Gram-positivas, cocos o bacilos, generalmente inmóviles, no forman esporas, anaerobias y aero-tolerantes/microaerófilas. Las BAL son incapaces de sintetizar el grupo hemo y carecen de muchas actividades enzimáticas, tales como la catalasa, nitrato reductasa y citocromo oxidasa, no producen ni indol ni sulfuro de hidrógeno y son incapaces de hidrolizar la gelatina y sólo algunas especies hidrolizan la caseína. Debido a su baja capacidad biosíntesis, estas bacterias tienen requerimientos nutricionales complejos (aminoácidos, vitaminas, sales, ácidos grasos y carbohidratos fermentables) (Dellaglio et al., 1994).

Hábitat. Las BAL se encuentran ampliamente distribuidas en suelo, aguas, y vegetales dominando en la microbiota fermentadora de ensilados y forrajes. Forman también parte de la microbiota de animales y humanos, donde ocupan muchos nichos ecológicos existentes en la boca y el tracto gastrointestinal y urogenital (Holzapfel et al., 1995; Vandamme et al., 1996; Kelly et al., 1998; Carr et al., 2002; Claesson et al., 2007; Pfeiler y Klaenhammer, 2007). En los diferentes ecosistemas, son capaces de ejercer efectos beneficiosos o, raramente, generar alteraciones biológicas. Así, la presencia de las BAL en el intestino es importante para un correcto funcionamiento del tracto digestivo pero algunas especies están consideradas como patógenas (especies de *Streptococcus* α y/o β-hemolíticos responsables de infecciones intestinales, urogenitales, endocarditis, mastitis y septicemias). Además, otras especies habituales en la cavidad oral (*Lactobacillus*) y el intestino (*Enterococcus* y *Lactobacillus*) pueden tener carácter patógeno oportunista una vez introducidas accidentalmente en el sistema circulatorio (durante una extracción dental, una cirugía o una lesión traumática) (Gasser, 1994).

Un hábitat característico de las BAL son los alimentos fermentados: productos lácteos (yogures, quesos), encurtidos (aceitunas), carnes fermentadas (salami), vino y panes fermentados (Tannock, 2004; Pfeiler y Klaenhammer, 2007; Bautista-Gallego et al., 2012; Bautista-Gallego et al., 2013) siendo responsables de su sabor, aroma y textura. En la actualidad pueden ser adicionadas expresamente a los alimentos, como cultivos iniciadores, en el procesado de éstos, aunque a veces, aquellas que forman parte de la microbiota normal pueden, por el contrario, ocasionar la alteración de las materias primas.

Taxonomía y clasificación. El análisis fenotípico que permite la diferenciación de las BAL se basa en características morfológicas, fisiológicas y de cultivo: observación microscópica, comportamiento/crecimiento en diferentes condiciones de cultivo, ausencia de catalasa, la

producción de enzimas y metabolitos específicos y otras propiedades (Holzapfel et al., 2001; Pot, 2008).

Según el tipo de fermentación que realizan se clasifican en homofermentadoras (homolácticas) estrictas, heterofermentadoras (heterolácticas) estrictas y heterofermentadoras facultativas. Las homolácticas estrictas fermentan los hidratos de carbono dando ácido láctico como producto único, mientras que las heterolácticas producen ácido acético, etanol y CO_2, además de ácido láctico como producto mayoritario. Las BAL homolácticas están representadas por los géneros *Lactococcus, Pediococcus, Enterococcus* y algunas especies de *Lactobacillus*; mientras que el género *Leuconostoc*, y algunas especies de *Lactobacillus* pertenece a las heterolácticas. Sin embargo, dependiendo de la disponibilidad de oxígeno y glucosa, algunas BAL homofermentativas pueden convertirse en heterofermentativas: facultativas (Rhee y Pack, 1980; Murphy et al., 1985; Borch et al., 1991).

Los rasgos fenotípicos que pueden ser de utilidad para la clasificación de las BAL son (Roissart y Luquet, 1994; Holzapfel et al., 2001; Pot, 2008):

- La morfología y las agrupaciones, la movilidad y la producción de cápsulas o pigmento.
- El crecimiento a diferentes pHs, temperaturas y concentraciones de NaCl. Además del crecimiento en presencia de bilis, azul de metileno, etanol u otros inhibidores.
- La producción de CO_2 o de una mezcla de H_2, H_2O_2 o H_2S en medio rico de glucosa, la producción de CO_2 y H_2 a partir de lactato, la producción de NH_3 a partir de arginina y la producción de polisacáridos.
- Perfiles de fermentación de azúcar.
- Hidrólisis de la arginina, esculina, almidón y urea.
- Producción de enzimas específicos: α o β hemólisis, la reducción de cloruro de trifenil tetrazolio, la descarboxilación de tirosina y la actividad lipolítica.

El análisis e identificación de BAL ha experimentado un notable avance en los últimos años, sin duda ligado al desarrollo de las técnicas de biología molecular. Los criterios fenotípicos clasifican las BAL desde el género a la especie, y en menor medida, a la subespecie y a la cepa. Para una diferenciación más precisa, se requiere la utilización de criterios genotípicos (Gevers, 2005). La sencillez y reproducibilidad de estas técnicas, ha

permitido sustituir o completar las técnicas tradicionales por otras que se basan fundamentalmente en el análisis de ADN, ARN o proteínas de los microorganismos o de las propias comunidades microbianas (Holzapfel et al., 2001; Ogier et al., 2002; Giraffa, 2004; Temmerman et al., 2004; Pot, 2008). La principal ventaja de las técnicas de identificación basadas en el estudio del ADN o ARN, es que éstas se han centrado en la detección de secuencias diana específicas de determinados microorganismos en lugar de la expresión fenotípica de los productos codificados por los genes, que puede estar muy influida por el ambiente.

Algunos de los métodos moleculares que se vienen utilizando en la identificación y/o caracterización de BAL son las técnicas de tipo *fingerprinting* (RAPD-PCR, REP-PCR, ERIC-PCR y RISA-PCR), la tipificación de secuencias multilocus (MLST), los métodos basados en la digestión enzimática (electroforesis en campo pulsante, PFGE, y el ribotipado) y las técnicas basadas en la secuenciación y análisis de moléculas consideradas relojes moleculares, siendo el más utilizado el gen ARNr 16S (Holzapfel et al., 2001; Gevers, 2005; Ogier et al., 2002; Ennahar et al., 2003; Temmerman et al., 2004).

Actualmente en el grupo de las BAL se integran los géneros *Aerococcus, Atopobium, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus, Weisella* y *Carnobacterium.* Sin embargo, la denominación BAL a veces se hace extensiva a otros géneros como *Bifidobacterium, Micrococcus, Brevibacterium* y *Propionibacterium* (Stiles y Holzapfel, 1997; Gevers 2002; Patrignani et al., 2006). Se trata de un grupo diverso de géneros de bacterias que presentan en su ADN un porcentaje de G+C menor del 55%, encuadrados en el orden *Lactobacillales* del *Phylum* Firmicutes.

En la Figura 1 se presentan en un dendrograma las relaciones filogenéticas existentes entre los géneros de bacterias lácticas *sensu stricto*.

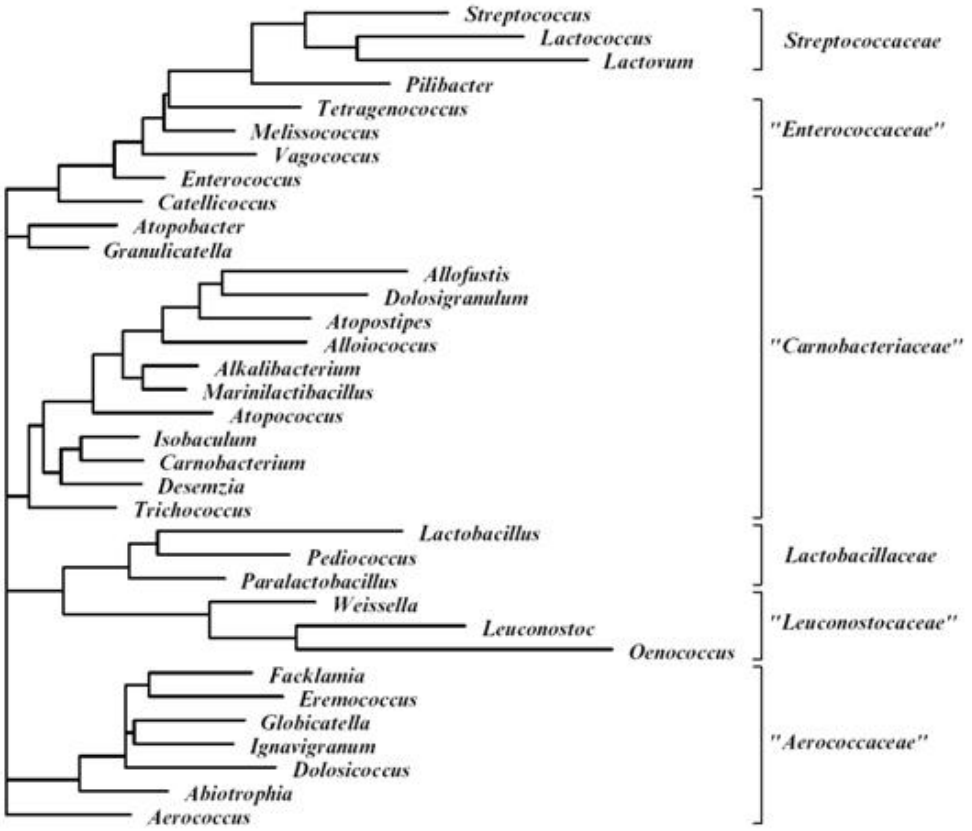

Figura 1. Dendrograma consenso que refleja las relaciones filogenéticas del orden *Lactobacillales* dentro de la clase *Bacilli,* basado en el análisis comparativo de las secuencias del gen ARNr 16S (Ludwig y Schleifer, 2009).

2.2. Importancia industrial de las bacterias lácticas

Entre las propiedades que presentan las BAL, muchas son de interés tecnológico. Tales son la resistencia a los bacteriófagos, las actividades proteolítica y lipolítica (importantes en la maduración del queso), la fermentación de lactosa, galactosa y citrato (responsable del aroma de los productos fermentados frescos), la producción de polisacáridos (implicados en la textura de la crema y de la leche fermentada), su actividad bioconservante -protectora frente a microorganismos alterantes y patógenos de alimentos- y, finalmente, la resistencia a la congelación y a la liofilización (importante en la conservación de fermentos industriales).

La acción de las BAL como bioconservantes de alimentos se atribuye a la bajada controlada del pH que provocan debido a la transformación de los azúcares en ácidos orgánicos, láctico sobre todo, y a la producción de otros metabolitos con actividad antagonista como diacetilo, peróxido de hidrógeno, reuterina y una amplia gama de proteínas antimicrobianas, las bacteriocinas. Las BAL pueden también por sí mismas

contribuir a mejorar nuestra salud dada su actividad como probióticos. Como tales se les ha atribuidos diversas acciones beneficiosas: incrementar la calidad nutritiva de los alimentos al promover la síntesis de vitaminas del complejo B y facilitar la digestión de la lactosa, estimular la respuesta inmune en el epitelio intestinal y controlar las infecciones intestinales, al impedir la implantación de patógenos, entre otras. Por ello, las BAL se consideran importantes componentes de la dieta de humanos y animales.

Algunas especies de *Lactococcus, Lactobacillus, Leuconostoc, Pediococcus* y *Streptococcus* forman parte de algunos cultivos iniciadores industriales contribuyendo a la acidificación y a la proteólisis primaria (Eliskases-Lechner et al., 1999; Hébert et al., 2000; Pfeiler y Klaenhammer, 2007; Gourbeyre et al., 2011).

2.2.1. Cultivos iniciadores

Como se ha referido más arriba, las bacterias lácticas forman parte de la alimentación humana desde la más remota antigüedad al participar de forma natural en la fermentación de numerosos alimentos, particularmente leche y carne. Sin embargo no fue hasta 1873 cuándo Lister aisló los primeros cultivos iniciadores puros y con ello confirmó la implicación bacteriana en la fabricación del queso. El manejo y la manipulación de los cultivos iniciadores no emerge hasta los años 90 del siglo XIX, a través de Conn en Estados Unidos, Storch en Dinamarca y Weigmann en Alemania (Fox, 2002). Más recientemente, el desarrollo extensivo de la industria de transformación de los alimentos, en particular la industria láctea, ha llevado a la producción de fermentos lácteos industriales, capaces de asegurar la calidad del producto (Pfeiler y Klaenhammer, 2007), denominados cultivos iniciadores (CI). No obstante, cabe señalar una importante desventaja de la aplicación de estos CI y es que, dada su biodiversidad limitada, a menudo se deriva en una pérdida de la singularidad del producto original y por tanto de las características que lo han hecho popular (Caplice y Fitzgerald, 1999).

Según Wigley et al., (1999), los cultivos iniciadores pueden ser definidos como preparaciones de una o varias cepas de una o varias especies microbiológicas que inician el proceso de producción de un alimento, generalmente mediante fermentación. Históricamente, un cultivo iniciador era, simplemente una muestra del alimento fermentado que era utilizada en la siguiente elaboración del producto. Este tipo de actuación no siempre daba los resultados deseados por lo que hoy en día un proceso de fermentación tiene que ser predecible y consistente para asegurar la calidad del producto final. De ahí la gran importancia de los cultivos iniciadores comerciales en la actualidad.

Los cultivos iniciadores son utilizados fundamentalmente en la industria láctea, pero cada vez está más extendido su uso en la producción de otros alimentos fermentados tales como la carne, bebidas alcohólicas y productos vegetales. Las bacterias que se usan en la industria alimentaria son seleccionadas en virtud de sus propiedades sobre un tipo concreto de alimento. El metabolismo de este tipo de cultivos altera la composición química, física y biológica del alimento crudo, dando lugar a un producto con unas propiedades organolépticas atractivas para el consumidor.

Para que una cepa pueda ser considerada cultivo iniciador debe carecer de patogenicidad, no producir ningún tipo de sustancia que *per se* o tras su metabolismo sea tóxica, debe ser genéticamente estable y su uso debe estar estandarizado y ser reproducible. Actualmente, el desarrollo industrial de cultivos iniciadores está perfectamente regulado, las cepas utilizadas están estandarizadas e incluso en los últimos años se está trabajando en la selección y mejora de cepas mediante ingeniería genética. El proceso de producción de cultivos iniciadores comerciales incluye el crecimiento de las cepas en cultivos continuos que controlan en todo momento la proporción de cada cepa, la concentración de nutrientes, composición del medio y diversos factores tales como concentración de O_2 o CO_2, pH, etc., para lograr unas condiciones de producción totalmente estandarizadas. Una vez obtenidos los cultivos, son almacenados mediante diversas técnicas en una matriz láctea y posteriormente liofilizados. Los cultivos comerciales liofilizados son los más extendidos, se suelen suministrar en sobres de aluminio conteniendo un número dado de unidades de actividad, según una regla que refiere el número específico de unidades necesarias para la fermentación de 100 kg de alimento (Wigley, 1999).

Las bacterias del ácido láctico, son las responsables de la mayor parte de las fermentaciones conocidas por los humanos y por ello son las que constituyen la mayoría de los CI comerciales. Además, en una serie de alimentos, especialmente los productos lácteos, el uso de CI basados en las BAL, se cree que contribuye a los beneficios que sobre la salud presenta el producto, generalmente a través de la normalización del tracto gastrointestinal tras una enfermedad gástrica (Montville y Wikowski, 1997; Franz et al., 1999). Los iniciadores comerciales más comunes, formados de una sola especie o de varias especies, pertenecen a los géneros *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* et *Bifidobacterium*. (Roissart y Luquet, 1994). El género *Enterococcus* suele aparecer también en aquellos quesos elaborados con leche cruda, aunque actualmente se está incrementando su uso en cultivos iniciadores definidos también en los quesos de elaboración artesanal (Beresford et al., 2001; Cogan, 2002). Otros

iniciadores incluyen los *Streptococcus* y ciertas levaduras. En la medida que las levaduras, al aumentar su volumen, producen alcohol y dióxido de carbono, los productos que las utilizan, tales como el Kéfir, son ligeramente burbujeantes y alcohólicos.

Entre los criterios más importantes que se han utilizado a la hora de seleccionar los CI se encuentran la producción y la sensibilidad al ácido láctico, la actividad enzimática, la capacidad autolítica, la ausencia de patogenicidad y de capacidad de producir ningún tipo de sustancia que sea potencialmente tóxica, la resistencia a fagos, la estabilidad genética, la resistencia a congelación y liofilización y, finalmente, una aplicación estandarizada y reproducible (Carr et al., 2002). Algunas de las propiedades tecnológicas de los CI en ocasiones residen en plásmidos, tales son las relacionadas con el transporte y metabolismo de la lactosa, proteinasas de superficie, transporte de citrato, producción y resistencia de bacteriocinas o resistencia a bacteriófagos. Por ello, las bacterias que se suelen utilizar con frecuencia en la fermentación de alimentos han sido seleccionadas previamente a nivel de cepa, ya que estas características no siempre se encuentran distribuidas entre todos los miembros de una especie, o incluso no se encuentran igualmente adaptados al mismo tipo de sustrato alimentario (Giraffa, 2004).

Función de los cultivos iniciadores. La función principal de las bacterias iniciadoras es la rápida producción de ácido láctico durante el proceso de fermentación de los azúcares reduciendo el pH del alimento. Además de llevar a cabo la acidificación del alimento, que contribuye en el caso de la leche a la coagulación de la caseína, los cultivos iniciadores tienen otra serie de funciones:

- Proporcionar un entorno adecuado en cuanto al potencial redox, el pH y el contenido en humedad, permitiendo el crecimiento de la microbiota secundaria.
- Contribuir a la maduración y a las cualidades organolépticas del producto final mediante sus enzimas, implicadas en la proteólisis y en la conversión de aminoácidos en componentes de sabor característicos de cada queso, producción de polisacáridos que aportan texturas interesantes, producción de componentes de aroma en la fermentación, etc.
- Mejorar la seguridad higiénica de los alimentos y la prevención alteraciones tales como el “hinchamiento tardío” del queso debido a la contaminación por *Clostridium,* caso de incluir bacterias productoras de péptidos antimicrobianos (bacteriocinas).

- Proporcionar ventajas para la salud por las características funcionales y/o potencialmente probióticas de algunas cepas de BAL, producción de nutracéuticos, reducción de factores tóxicos y antinutricionales (p.e. lactosa y galactosa).

Formas de presentación y uso de los cultivos iniciadores. Las industrias lácteas utilizan los CI ya mezclados y preparados por empresas proveedoras (cultivos comerciales), que han sido previamente seleccionados por presentar las propiedades necesarias para conseguir un producto específico con textura, sabor y viscosidad determinados. Las formas más usuales de presentación de los CI comerciales son: cultivos líquidos concentrados, mezcla de cultivos liofilizados y cultivos líquidos concentrados y congelados (Bylud, 2003).

Para su utilización en la producción del queso los CI comerciales (suministrados congelados o más frecuentemente liofilizados, en polvo) pueden ser inoculados directamente en leche en la producción del queso o propagados previamente en una serie sucesiva de etapas a partir del CI comercial. La inoculación directa en leche es la forma de uso más cómoda y que minimiza los riesgos de contaminación por lo que se está generalizando en la actualidad. No obstante hay todavía muchas industrias lácteas que preparan sus propios cultivos iniciadores mediante propagación previa. Para ello el cultivo comercial (cultivo maestro es inoculado en la propia industria quesera en leche desnatada o reconstituida, tratada térmicamente (90-95°C, 30-35 minutos) y re-enfriada, para dar el cultivo madre. Este cultivo madre será el origen de los demás cultivos utilizados en esa industria y se prepara diariamente a partir del que se preparan los cultivos intermedios y los cultivos industriales que son los que, finalmente se emplean en el proceso de producción (coagulación) (Bylud, 2003).

La producción y manejo del cultivo iniciador es uno de los procesos más importantes y difíciles de realizar. Es importante que a lo largo del proceso, se sigan escrupulosamente las indicaciones de uso de la casa proveedora, sobre todo en lo referente al almacenamiento de los CI, las temperaturas y tiempos de cultivo y a la cantidad de CI inoculado en cada caso. También hay que destacar las exigencias de altos niveles de higiene en la propagación de los CI ya que el riesgo de contaminación por levaduras presentes en el ambiente, por mohos y bacteriófagos debe ser lo mínimo posible por lo que deben realizarse en salas especiales provistas de medidas especiales de higiene.

Una alternativa interesante al uso de CI comerciales es el desarrollo de CI a partir de cepas indígenas aisladas de los productos artesanales, que puedan ser utilizados en la

coagulación de la leche, sobre la base de sus propiedades tecnológicas, funcionales e incluso probióticas. Ello permitiría obtener productos fermentados que, sin perder las cualidades sensoriales de interés, sean más homogéneos y mejorados en sus cualidades higiénico-sanitarias-nutricionales.

2.2.2. Cultivos protectores

En ocasiones se hace una distinción entre ambos tipos de cultivos (iniciadores y protectores), aunque en realidad se trata del mismo cultivo utilizado para distintos propósitos y bajo condiciones diferentes. En un cultivo iniciador, la actividad metabólica, por ejemplo la producción de ácidos en el queso, tiene una gran importancia tecnológica, mientras que la actividad antimicrobiana sería un factor secundario. Por el contrario, en un cultivo protector, los objetivos se invierten.

Los cultivos protectores deberían ser considerados siempre como un factor de seguridad adicional y su implantación debe permitir la reducción del riesgo de crecimiento y supervivencia de microorganismos patógenos (Holzapfel et al., 1995). Lo ideal sería, por tanto, desarrollar cultivos en los cuales tanto la actividad metabólica como la actividad antimicrobiana sobre el alimento tuviesen el mismo peso.

III. LAS ACTIVIDADES ANTIMICROBIANAS Y PROBIÓTICAS DE LAS BACTERIAS DEL ÁCIDO LÁCTICO

En la interferencia microbiana común a todos los ecosistemas microbianos, incluidos los alimentos, intervienen dos procesos. El primero es un mecanismo inespecífico que incluye la competición por los nutrientes y por los espacios de colonización (sitios de adhesión) mediante la formación de ambientes inadecuados. El segundo es un mecanismo específico debido a la producción de sustancias antagonistas.

La acción antimicrobiana de las BAL se atribuye a la bajada del pH, por la producción de ácidos láctico y acético sobre todo, y a la producción de otros metabolitos con actividad antagonista como el diacetilo, el peróxido de hidrógeno y las proteínas antimicrobianas denominadas bacteriocinas (Caplice y Fitzgerald, 1999; Cleveland et al., 2001; Ross et al., 2002; Touré et al., 2003). La producción de péptidos antibacterianos sintetizados ribosómicamente es un fenómeno común a todos los organismos vivos. Rasgos comunes a todos ellos son el pequeño tamaño (20-60 residuos), su naturaleza anfifílica/hidrofóbica y,

frecuentemente, su carga positiva a pH fisiológico (Nissen-Meyer y Nes, 1997; Epan y Vogel, 1999).

Actualmente, la producción de bacteriocinas por parte de las bacterias lácticas ha despertado un gran interés biotecnológico y es un factor a tener en cuenta en sistemas alimentarios, con el objetivo de desarrollar cultivos iniciadores o cultivos adicionales que permitan el control y/o eliminación de poblaciones de bacterias indeseables, alterantes o incluso patógenas (Cogan, 2002; Johnson y Lucey, 2006).

Todo lo expuesto convierte a las BAL unas candidatas muy interesantes, no sólo para la industria alimentaria (bioconservantes naturales), sino también para la industria farmacéutica (Montalbán et al., 2011). La bioconservación mediante bacteriocinas de las BAL es un área emergente y prometedora de la Microbiología de los Alimentos. Por ello, y sobretodo por tratarse del objeto de esta memoria, hablaremos mas en detalle de estos antagonistas.

3.1. Las bacteriocinas de las bacterias del ácido láctico

La producción de péptidos antibacterianos sintetizados ribosómicamente es un fenómeno común a todos los organismos vivos. Los de origen bacteriano son referidos generalmente como bacteriocinas. Éstas se definen como *un grupo heterogéneo de sustancias proteicas, de síntesis ribosómica, bien primarias o modificadas, secretadas extracelularmente, y producidas por muchas cepas de bacterias Gram-positivas y Gram-negativas, que, típicamente, tienen un modo de acción bactericida sobre bacterias estrechamente relacionadas* (Tagg, 1976).

Las bacteriocinas producidas por las BAL presentan una serie de características que las convierten en candidatos adecuados para ser usadas como conservantes de alimentos:

- Su naturaleza proteica, por lo que son inactivadas por enzimas proteolíticos del tracto gastrointestinal.
- No han mostrado ser tóxicas ni inmunógenas en animales de experimentación.
- No suelen mostrar actividad frente a eucariotas.
- Muchas de ellas son termorresistentes, conservando su actividad antimicrobiana tras ser sometidas a temperaturas similares a las de pasteurización y esterilización de la leche y otros alimentos (sobre todo las bacteriocinas de pequeño tamaño).

- Varias presentan un amplio espectro antimicrobiano, siendo activas a bajas concentraciones (generalmente inferiores a 10 ppm) frente a la mayoría de las bacterias Gram-positivas patógenas, toxigénicas o alterantes más frecuentes en los alimentos. En determinados casos se ha demostrado que dicha actividad también se extiende a bacterias Gram-negativas dañadas subletalmente por los tratamientos térmicos o por la presencia de agentes potenciadores.
- Dada su naturaleza peptídica y su síntesis ribosómica se facilita la posibilidad de manipulación mediante ingeniería genética lo que incrementa potencialmente la variedad de análogos de péptidos naturales que pueden ser diseñados con las características deseadas. Además en la mayoría de los casos los determinantes genéticos están localizados en plásmidos, lo que también facilita su manipulación genética.

3.1.1. Tipos de bacteriocinas de las bacterias del ácido láctico

Las bacteriocinas de las BAL son muy heterogéneas, variando su tamaño molecular desde unos pocos miles de daltons hasta grandes y complejas estructuras. Atendiendo a sus características estructurales y de actividad biológica, Klaenhammer (1993), estableció cuatro clases de bacteriocinas dentro de las BAL, de las que generalmente se mantienen tres:

- Clase I. Lantibióticos.
- Clase II. Pequeños péptidos no lantibióticos, estables al calor.
- Clase III. Proteínas termolábiles.

La existencia de la cuarta clase de bacteriocinas, moléculas complejas, compuestas de proteína más otros componentes (lípidos, carbohidratos), ha sido descartada con posterioridad, ya que muchas bacteriocinas tienden a formar agregados con componentes celulares así como del medio (Nes et al., 1996). En su lugar, Maqueda et al., (2004) han propuesto la creación de la clase IV de bacteriocinas, de naturaleza cíclica, de las BAL, cuyo prototipo sería la enterocina AS-48, producida por *E. faecalis* S-48, ya que este rasgo estructural se considera de suficiente entidad como para definir una nueva clase.

Desde la primera clasificación propuesta por Klaenhammer (1993), el descubrimiento y la caracterización de numerosas nuevas bacteriocinas de bacterias Gram positivas ha impuesto modificaciones notables en dicha clasificación (Nes et al., 1996; Franz et al., 1999; Van Belkum y Stiles, 2000; Cleveland et al., 2001; Diep y Nes, 2002; Ross et al., 2002; Maqueda et al., 2004; Cotter et al., 2005b; O'Shea et al., 2012). Las bacteriocinas

mejor estudiadas de las BAL son las de las clases I y II, posiblemente debido a que al ser termorresistentes son más adecuadas para ser usadas en la conservación de alimentos.

Actualmente podemos hablar de dos o tres clases distintas de bacteriocinas con varias subclases, siendo sin lugar a dudas la más variada y conflictiva la clase II. Recientemente O'Shea et al., (2012) han propuesto una clasificación en dos grupos distintos, basándose sobre las modificaciones que sufren los péptidos precursores de las bacteriocinas y que confieren las propiedades estructurales características de cada molécula (Cotter et al., 2005b).

Clase I. Se trata de pequeños péptidos hidrófobos (<5 kDa), de síntesis ribosómica, sometidos a une modificación postraduccional y, hasta hace poco, constaba de los lantibióticos exclusivamente. Estos son moléculas singulares estructuralmente por su contenido en aminoácidos modificados, tales como lantionina, metil-lantionina, dideshidroalanina y dideshidrobutirina (que permiten la formación de puentes de sulfuro intra-molecular) (Sahl et al., 1995). El descubrimiento y la caracterización de nuevas bacteriocinas que sufren unas modificaciones postraduccionales, atípicas de los lantibióticos, ha impuesto la subdivisión de la clase I en tres subclases (Rea et al., 2011).

- ***Clase Ia* –Lantibióticos.** Han sido encontrados exclusivamente en bacterias Gram-positivas (McAuliffe et al., 2001). Son altamente termoestables y actúan a nivel de membrana celular mediante una acción doble. De un lado se unen al lípido II (precursor del peptidoglicano), lo que provoca la interrupción de la biosíntesis de la pared celular: Simultáneamente, su inserción en la membrana determina la formación de poros, comprometiendo así la integridad de la membrana citoplasmática de las células diana (Pag y Sahl, 2002). El prototipo de lantibiótico de esta subclase es la nisina, producida por *L. lactis* subsp. *lactis* (Rogers, 1928; Mattick y Hurst, 1944). Es un lantibiótico ampliamente estudiado, muy activo y capaz de producir en las células dianas ambos mecanismos (Wiedemann et al., 2001). Sin embargo, también se han identificado otras dianas celulares, como la fosfatidiletanolamina (una de los principales fosfolípidos de la membrana), que sirve como una molécula de acoplamiento para cinamicina (Machaidze y Seelig, 2003). Existen otros ejemplos de lantibioticos como la lacticina 481 de *L. lactis* (Piard et al., 1993), la citolísina producida por *E. faecalis* con actividad bactericida-hemolítica (Gilmore et al., 1990) y la lacticina 3147 de *L. lactis* (Ryan et al., 1999). Jung en 1991 propuso la creación de dos subgrupos, tipo A (con dos subtipos AI y AII) y tipo B en base a características estructurales y

funcionales. Se han propuesto otras subdivisiones del grupo de lantibióticos sobre la base de la estructura de la molécula, el mecanismo de acción, la vía de maduración y la presencia o ausencia de actividad antimicrobiana (Pag y Sahl, 2002; Cotter et al., 2005a; Willey y Van der Donk, 2007).

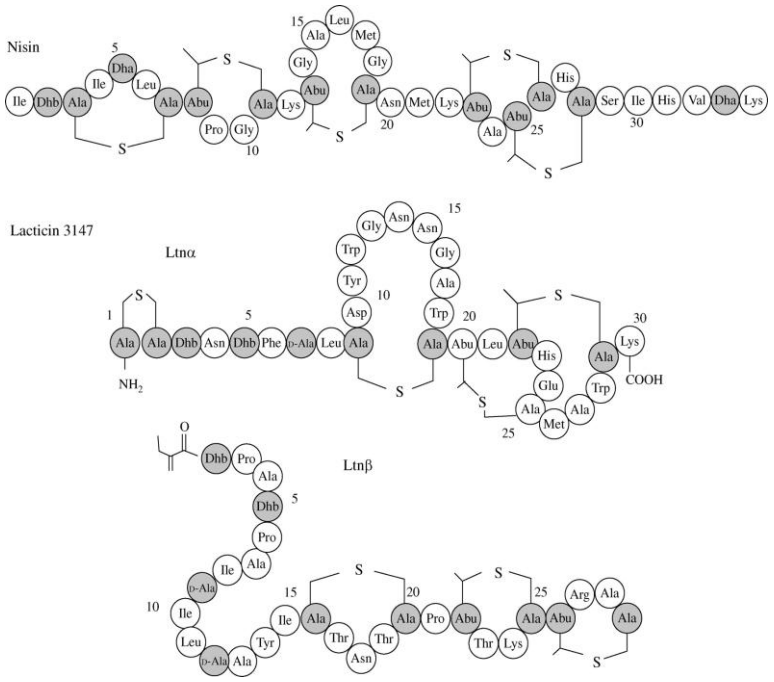

Figura 2. Estructura de los lantibióticos nisina y lacticina 3147. Tomado de Piper et al., (2009)

- ***Clase Ib* –Labirintopeptinas.** Distinguidos por la presencia de labionina (amino acido cíclico), como ejemplo de las labirintopeptinas A1, A2 y A3 producidas por *Actinomadura namibiensis* DSM 6313 (no perteneciente a las bacteriocinas de las LAB) (Meindl et al., 2010).

- ***Clase Ic* -Sactibióticos.** Sus residuos de amino ácidos forman una ligación no frecuente entre la molécula de azufre de cisteína y el carbono α de las fenilalaninas o treoninas (turicina CD producida por *Bacillus thuringiensis* y subtilosina A producida por *B. subtilis*) (Kawulka et al., 2004; Rea et al, 2010b).

Clase II. Pequeños péptidos no modificados. En esta categoría se incluye una serie de péptidos termoestables anfipáticos no modificados, con variadas características genéticas y químicas que generalmente actúan a través de la permeabilización de la membrana celular, provocando la disipación de la fuerza protón-motriz y lisis de las células sensibles (Bruno y Montville, 1993). Se han propuesto varios subgrupos dentro de esta clase de bacteriocinas

dada su naturaleza heterogénea (Nissen-Meyer et al., 2009; Rea et al., 2011; O'Shea et al., 2012).

- ***Clase IIa*** - **Tipo pediocinas.** Se compone de las pediocinas con actividad específica contra *L. monocytogenes* y que poseen el motivo conservado en el extremo N-terminal Y-G-N-G-V. Se caracterizan, además, por presentar al menos un puente disulfuro que une dos residuos de cisteína (Drider et al., 2006). Las pediocinas tienen la ventaja de poseer actividad anti-*Listeria* y ser producidas generalmente por BAL reconocidas como GRAS. Entre ellas se incluyen la pediocina PA-1/AcH producida por cepas del género *Pediococcus* (Henderson et al., 1992; Lozano et al., 1992), carnobacteriocina B2 producida por *Carnobacterium piscicola*, curvacina A de *Lactobacillus curvatus*, leucocina A de *Leuconostoc gelidum,* sakacina A (Holck et al., 1992) y enterocina A de *Enterococcus faecium* (Aymerich et al., 1996). Recientemente, se ha sugerido que el sistema fosfotransferasa para la manosa (PTS-manosa) es una diana de las bacteriocinas de la clase IIa, debido a que se ha observado una correlación entre los niveles de expresión de los componentes del PTS-manosa de las bacterias indicadoras y la sensibilidad a dichas bacteriocinas (Kjos et al., 2009; Opsata et al., 2010).

- ***Clase IIb*** **- Bacteriocinas formadas por dos péptidos.** Bacteriocinas heterodiméricas, que requieren la actividad combinada de dos péptidos complementarios (α y β) para un efecto antimicrobiano óptimo (Oppegard et al., 2007). Estas bacteriocinas presentan un mecanismo de acción que implica la disipación del potencial de membrana, pérdida de iones y/o disminución de las concentraciones intracelulares de ATP (Garneau et al., 2002). Entre ellas encontramos la bactericina ABP-118 producida por *Lactobacillus salivarius,* termofilina 13 por *Streptococcus thermophilus*, plantaricinas S, EF y JK de *Lactobacillus plantarum* (Anderssen et al., 1998), lactococinas G y Q de *Lactococcus lactis* (Nissen-Mayer et al., 1992) y la lactacina F de *L. johnsonii* (Allison et al., 1994).

- ***Clase IIc*** **- Bacteriocinas cíclicas.** Las bacteriocinas pertenecientes a esta clase se agrupan sobre la base de presentar unión covalente de sus extremos C y N, dando lugar a una estructura cíclica, sin principio ni final. Además son en general bastante termostables y resistentes a exoproteasas. Las bacteriocinas circulares descritas hasta la fecha son producidas por bacterias Gram-positivas

pertenecientes al *Phylum* Firmicutes. Son péptidos/proteínas catiónicos y anfifílicos, con acción bactericida por acumulación o inserción en la membrana de las bacterias sensibles causando un incremento en la permeabilidad celular y pérdida de su función de barrera. En todas ellas se encuentra un motivo común, la existencia de 4 o 5 hélices α que estructuralmente se encuentran organizadas encerrando un corazón hidrófobo, muy compacto, con un plegamiento similar al de las saposinas. Tal arquitectura les confiere una estabilidad única y una potente actividad antimicrobiana, en todos los casos ejercida a nivel de membrana. Suelen presentar, además, una distribución asimétrica de las cargas positivas, de manera que los residuos básicos están localizados en algunas hélices y los hidrofóbicos en el resto de la molécula. Hasta la fecha se han descrito 11 bacteriocinas circulares con tamaños que oscilan entre los 58 aa de la gasericinaA/Acidocina A y los 70 de AS-48 (revisado por Maqueda et al., 2008). Este grupo comprende a enterocina AS-48, Gassericina A/reutericina 6/ acidocina B, Butirivibriocina AR10, Uberolisina A, Circularina A, Carnociclina A, Lactociclicina Q y Leucociclicina Q, Garvicina ML y Aureociclicina 4185.

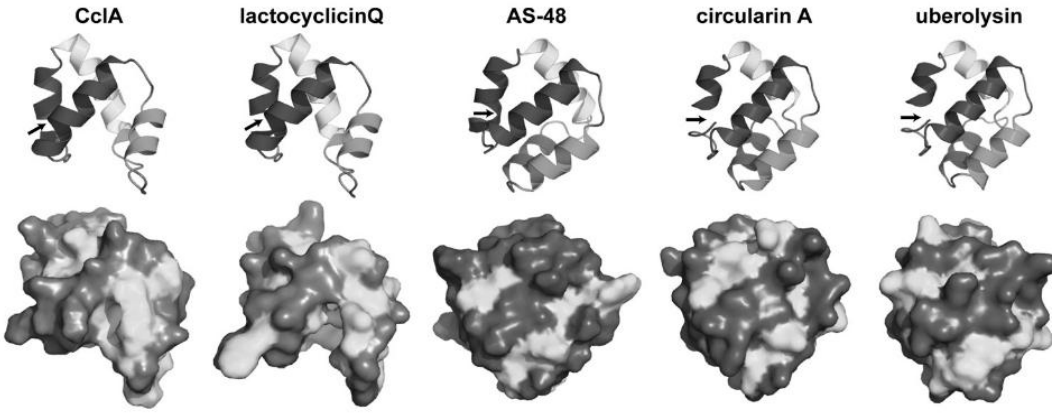

Figura 3. A. Propuesta de dominios estructurales para las bacteriocinas circulares (adaptado de Martin-Visscher et al., 2009).

- ***Clase IId.*** Incluiría el resto de bacteriocinas lineares, sin homología con la pediocina, y con diversos mecanismos y modos de acción. Se trata de bacteriocinas complejas en cuanto a su actividad y/o su estructura proteica. Algunas actúan, como es el caso de las lactococcinas A y B, formando poros, implicando el sistema PTS-manosa en las células sensibles (Diep et al., 2007). Otras mediante una disrupción general de la membrana, como es el caso de la bacteriocina sin péptido líder aurocina A53 (Netz et al., 2002). También, caso de

la lactococcina 972, por la inhibición de la división celular a través de sus interacciones con el lípido II (Martínez et al., 2008), o mediante una acción sobre la inducción de profagos, lo que constituye un nuevo mecanismo de inhibición de las bacteriocinas (Madera et al., 2009). La clase III de la clasificación de Klaenhammer (1993), es mantenida en algunas clasificaciones actuales pero en otras ha desaparecido por ser consideradas como enzimas o bacteriolisinas (Cotter et al., 2005b; O'Shea et al., 2012).

3.1.2. Formas de aplicación de las bacteriocinas en los alimentos

Las estrategias seguidas para la aplicación de las bacteriocinas de las BAL en la conservación de alimentos son diversas:

- Inoculación del alimento con las BAL (como cultivos iniciadores o adjuntos a estos) para que produzcan las bacteriocinas *in situ*.
- Utilización del producto previamente fermentado con la cepa productora de la bacteriocina, como ingrediente en el procesado de alimentos. Dichos preparados tienen la ventaja de que pueden ser reconocidos más fácilmente que otros aditivos, como seguros (GRAS) para su uso directo en alimentos.
- Adición de las bacteriocinas purificadas o semipurificadas. Este método permite aplicar dosis más exactas de la bacteriocina, y por tanto predecir mejor los resultados. Como contrapartida, la bacteriocinas purificadas son consideradas como aditivos, requiriendo autorización expresa para su uso (Fields, 1996).

3.1.3. Producción industrial de bacteriocinas

Para que la producción de las bacteriocinas pueda considerarse viable a nivel industrial, es fundamental que el sustrato de crecimiento de las cepas productoras sea de grado alimentario y de bajo coste y el proceso de recuperación/concentración sea escalable a nivel industrial. Con este objetivo, es cada vez más frecuente la utilización de subproductos de la industria láctea, fundamentalmente lactosueros y lactalbúminas (productos de desecho en las queserías), como medios de crecimiento y de producción alternativos a los medios de cultivo habituales (suministrados por casas comerciales).

El lactosuero es el subproducto líquido generado tras el proceso de cuajado en la fabricación del queso y la lactalbúmina es un subproducto obtenido a partir del lactosuero, que al ser rico en lactosa, constituye un medio de crecimiento idóneo para las BAL. Estos

productos resultan muy económicos y por otro lado, al ser utilizados como medio de crecimiento, se alivia el problema ambiental que supone la eliminación del lactosuero.

Estos subproductos de la industria láctea han sido empleados como medio de crecimiento y producción de la lacticina 3147 (Morgan et al., 2001), la mutacina (Nicolas et al., 2004), la nisina (Liu et al., 2004), la pediocina (Pérez-Guerra et al., 2005), una bacteriocina producida por *B. licheniformis* P40 (Cladera-Olivera et al., 2004) y la bacterocina AS-48 (Ananou et al., 2008). La enterocina AS-48 ha sido producida a partir de un sustrato derivado del lactosuero, Esprion 300, de grado alimentario y de coste económico, 1,53 €/Kg E-300 versus 140 €/Kg BHI. Además, se han establecido las condiciones óptimas de producción (360 UA/ml a 18-20h) en este sustrato: temperatura de incubación de 28 ºC, inoculo de la cepa productora al 8%, E-300 al 5%, glucosa al 1% y pH estabilizado a 6,55; siendo los dos últimos los parámetros clave.

Para obtener preparaciones comercialmente viables de bacteriocinas es necesario aplicar mecanismos de concentración. Actualmente los mecanismos que se perfilan como idóneos para este fin serían:

Filtración tangencial. Es un proceso de filtración en el que el flujo es conducido tangencialmente a la membrana en lugar de perpendicularmente con lo que disminuyen en gran medida los problemas de atascamiento que genera la filtración convencional. Dependiendo del tamaño de poro de la membrana se puede usar para eliminar las células productoras (microfiltración) o para concentrar las moléculas de interés (en este caso las bacteriocinas) en el retenido (ultrafiltración). Esta aplicación es aún muy reciente y se ha empleado en la concentración de proteínas de lactosuero (Atra et al., 2005; Akoum et al., 2005) y en la purificación de la halocina C8 (Li et al., 2003) a partir de medios de cultivo específicos para bacterias halófilas empleando una membrana de 50 kDa.

Secado por electrospray. Es un método utilizado a nivel industrial para la producción de preparaciones de bacteriocinas que permite, tras un proceso de evaporación que aumenta el contenido en sólidos del medio, desecar la muestra, obteniendo un sustrato en polvo con muy poco contenido en células viables y muy activo que puede almacenarse durante varios meses. Mediante este procedimiento se han obtenido preparados activos de lacticina 3147 (Morgan et al., 1999, 2001) patentada en los EE.UU. con el número 6833150, de la mezcla de las bacteriocinas producidas por *Lb. sakei, Lb. salivarius* y *Carnobacterium divergens* (Silva et al., 2002) o de varias cepas de lactococos y lactobacilos a partir de cultivos en leche desnatada o lactosuero (Mauriello et al., 1999). Esta técnica se ha utilizado también

para obtener preparados activos de la enterocina AS-48, libres de células productoras viables, y estable bajo congelación o refrigeración durante meses. Dichas preparaciones han sido activas frente a *L. mococytogenes* y *S. aureus* tanto en medio de cultivo BHI como en leche desnatada (Ananou et al., 2010).

Intercambio iónico. Dado el carácter mayoritariamente catiónico de las bacteriocinas de las BAL el método de elección empleado para purificar un gran número de bacteriocinas ha sido su adsorción sobre matrices de intercambio catiónico y posterior elución a partir de las mismas. Ejemplos de esto son la enterocina AS-48, purificada mediante intercambio iónico enen geles de carboximetil celulosa CM25 (Abriouel et al., 2003), la enterocina P, purificada mediante geles de sefarosa (Gutiérrez et al., 2005), la enterocina F-58, mediante CM25 (Achemchem et al., 2005) o las enterocinas A y B, mediante sefarosa (Ennahar et al., 2001), etc. Este proceso se ha acoplado en algunos casos al de ultrafiltración para lograr un grado de purificación mayor de la bacteriocina, tales son los casos de la halocina C8 (Li et al., 2003) y la enterocina AS-48 (resultados no publicados).

3.1.4. Requisitos generales y aspectos reglamentarios del uso de las bacteriocinas como bioconservantes

A la hora de considerar el uso alimentario de las bacteriocinas es necesario conocer previamente diversos aspectos básicos de las mismas:

- Su espectro inhibidor.
- Las características bioquímicas y genéticas de la cepa productora y de la bacteriocina.
- La sensibilidad de la bacteriocina a los cambios de pH y temperatura.
- Los factores que afectan a su producción.
- El proceso de purificación.
- La concentración efectiva, la forma concreta de la adición de la bacteriocina a los alimentos, y los costes.

3.1.5. Factores que limitan la eficacia de las bacteriocinas en los alimentos

Para que la adición a los alimentos de una bacteriocina sea efectiva, ésta debe conservar sus propiedades bajo los tratamientos que reciba dicho alimento, como cocción, pasteurización, refrigeración, adición de aditivos que varíen su pH y de enzimas que varíen

su composición. Los factores que limitan el uso de las bacteriocinas en los alimentos son, tanto los que afectan a la producción como los que afectan a su eficacia.

Los que limitan la producción son (citando a Daeschel, 1993):

- Baja producción en los alimentos (ambiente inadecuado del alimento).
- Pérdida de la capacidad productora por parte de la cepa.
- Infección por fagos de la cepa productora.
- Antagonismo por otros microorganismos.

Los que limitan la efectividad de las bacteriocinas son (Schillinger et al., 1996; Gálvez et al., 2010):

- Aparición de patógenos y bacterias alterantes resistentes a ellas.
- Adsorción a componentes alimentarios como las partículas de grasa.
- Que sea inactivada o antagonizada por aditivos.
- Baja solubilidad y distribución irregular.
- Inestabilidad ante cambios de pH.

3.1.6. Bacteriocinas empleadas y/o ensayadas en alimentos

Hay un gran número de bacteriocinas que han sido aplicadas en diversos tipos de alimentos para controlar el desarrollo de microorganismos alterantes y patógenos, con diferente grado de eficacia (revisado en Cleveland et al., 2001; Gálvez et al., 2007, 2010; Khan et al, 2010). Hasta la fecha la única bacteriocina cuyo uso alimentario está licenciado es la nisina (E-324). Es un lantibiótico de 34 aminoácidos, producido por *L. lactis*, descrito por primera vez por Rogers en 1928 y ha sido extensamente estudiado por numerosos grupos de investigación. En 1969 un comité de expertos de la Organización Mundial de la Salud dio el visto bueno al uso de este lantibiótico en los alimentos al considerarlo segura. En la actualidad posee el *status* GRAS en más de 45 países y es usada, bajo el nombre comercial de Nisaplin, en productos lácteos, cárnicos y vegetales, lo que permite eliminar o reducir los nitratos en ellos y aplicar tratamientos térmicos moderados y de ahí mejorar en el valor nutricional, aroma, textura y aspecto, además de ahorrar energía (Turtell y Delves-Broughton, 1998; Ross et al., 2002). Respecto a otras bacteriocinas, la nisina posee un espectro de acción, en general más amplio, que afecta a la mayoría de las bacterias Gram-positivas, incluyendo las patógenas transmitidas por alimentos tales como *L. monocytogenes* y *Clostridium.* Aunque las bacterias Gram-negativas no son sensibles a la

nisina, éstas se sensibilizan tras una permeabilización de su membrana externa con compuestos quelantes, o por choque osmótico. Las aplicaciones de la nisina como conservador de alimentos enlatados, productos cárnicos, vegetales fermentados, así como leche y derivados lácteos han sido revisadas en diversas ocasiones (Thomas y Delves-Broghton, 2001; Ross et al., 2002; Gálvez *et al*., 2007; Bilková et al., 2011).

Otra bacteriocina disponible comercialmente es la pediocina, producida por *P. acidilactici,* con diversas aplicaciones en el sector de la conservación de alimentos y de la salud (Mehta et al., 2013). Durante la fermentación de los embutidos fermentados, sola o en combinación con diacetato, la pediocina muestra actividad frente a *L. monocytogenes* y *L. curvatus* (Uhart et al., 2004). También ha dado buenos resultados en el control de *Listeria* en pollo cocido, yogur, ensalada y queso (revisado en Cleveland et al., 2001).

En conservación de alimentos otra bacteriocina ensaya ha sido la lacticina 3147, producida por *L. lactis,* es un lantibiótico que muestra capacidad para reducir hasta 10 veces el nivel de las bacterias lácticas no deseadas responsables de las alteraciones del sabor y la formación de cristales durante la fabricación de los quesos Cheddar (Ross et al., 2002). Igualmente se ha ensayado su eficacia para el control de patógenos alimentarios (Morgan et al., 2001; Suda et al., 2012). Otras bacteriocinas como la MBSa2 y MBSa3 también se han ensayado en el control de *L. monocytogenes* en salami (De Souza Barbosa et al., 2015).

Las enterocinas también han sido ensayadas en la conservación de alimentos, como la enterocina A, para el control de a *L. monocytogenes* en queso (Liu et al., 2008), las enterocinas A y B para el control de patógenos en productos cárnicos (Jofré et al, 2008; 2009) o la enterocina AS-48, ampliamente ensayada frente a diversas bacterias patógenas como *L. monocytogenes, B. cereus, S. aureus, S. choleraesuis*, tanto *in vitro* como en sistemas alimentarios (más detalles en el apartado 4.5. de esta memoria). En la actualidad nuestro grupo de investigación desarrolla un extenso programa para conseguir su aplicación en alimentos.

3.2. El concepto de efecto barrera y la tecnología de las barreras

El concepto de efecto barrera, en el que se basa esta tecnología, fue introducido por primera vez por Leistner en 1978 y hace referencia a que *la estabilidad microbiana y la seguridad, tanto de los alimentos tradicionales como de los nuevos, está basada en una*

combinación de varios factores de conservación, llamados barreras, que los microorganismos allí presentes son incapaces de superar.

En realidad se trata del enunciado conceptual de un hecho, bien conocido desde antiguo, como es el que las complejas interacciones de los diversos parámetros ambientales tales como temperatura, actividad de agua, pH, potencial redox, etc., son de gran importancia para la estabilidad microbiana de los alimentos. A partir del reconocimiento de este efecto se desarrolló la tecnología de las barreras, encaminada a conseguir mejoras en la seguridad y calidad de los alimentos mediante el empleo de una combinación deliberada e inteligente de tales barreras, de forma que actúen sinérgicamente. *Las bases de esta tecnología están en que la aplicación simultánea, a dosis moderadas, de varios factores que actúan a diferentes niveles, es mucho más eficaz que la aplicación de uno sólo, que actúa a un nivel, a dosis muy alta (Leistner, 1999), no solo en lo que se refiere a la estabilidad y seguridad del alimento sino también en lo tocante a sus cualidades sensoriales y a los costes, ya que requiere menos energía durante la producción y el almacenaje.* En la figura 4 se ilustra un ejemplo concreto de aplicación de la misma.

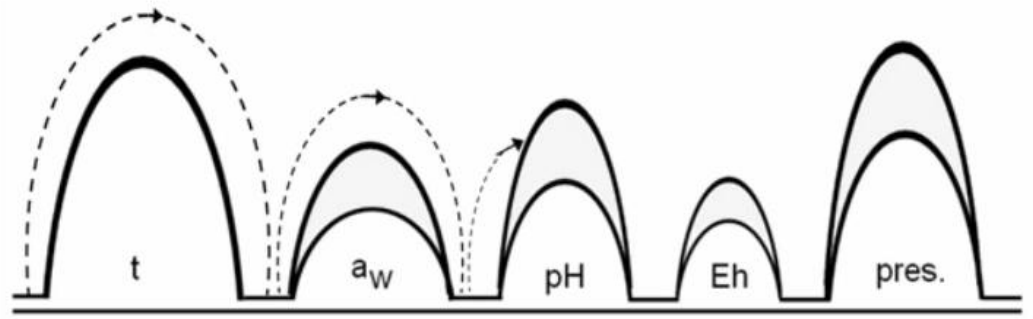

Figura 4. Tecnología de las barreras en la conservación de los alimentos. Se ilustra el ejemplo de un alimento con 5 barreras: alta temperatura durante el procesado (t), actividad de agua limitada (aw), acidez (pH), potencial redox (Eh) y conservantes (pres). Las bacterias del ácido láctico pueden aportar dos de estas barreras, al introducir una importante bajada de pH y al producir diversos compuestos antimicrobianos, p.e. bacteriocinas (Adaptada de Leistner, 1999).

En los países industrializados la tecnología de barreras tiene un gran interés sobre todo en la producción de alimentos mínimamente procesados. De igual forma, es de interés en alimentos refrigerados donde las bajas temperaturas son a menudo la única barrera, ya que la cadena de frío puede romperse. Por ello, en tales alimentos es necesario incorporar controles adicionales, que actúen a modo de guardianes invisibles, tal como son las BAL y sus compuestos antimicrobianos. En los países en vías de desarrollo, donde los alimentos frecuentemente se almacenan sin refrigerar, esta tecnología tendrá sin duda una

importancia capital, ya que permitirá que los alimentos permanezcan estables, seguros y con las características organolépticas adecuadas.

El fundamento último de la tecnología de las barreras es la actuación de los diferentes tratamientos que se aplican simultáneamente sobre dianas múltiples (membrana celular, ADN, enzimas, etc.) ya que en estos casos la reparación de la alteración de la homeostasis celular por las proteínas de choque frente al estrés se hace más difícil (Leistner, 1999). Por ello, para poder combinar los tratamientos de conservación siguiendo este criterio parece conveniente agruparlos de acuerdo con sus dianas celulares. Un ejemplo de proceso multidiana es la aplicación de nisina, que daña la membrana, en combinación con la lisozima y el citrato, que son capaces de alterar la pared celular y penetrar en la célula alterando la homeostasis a diferentes niveles.

3.2.1. Potenciales barreras en la conservación de alimentos

Las principales barreras empleadas en la conservación de alimentos, aplicadas bien como procesos o bien como aditivos son, según Leistner (1999):

- La temperatura (alta o baja).
- La actividad de agua, a_w (alta o baja).
- El pH (alto o bajo).
- El potencial redox, Eh (alto o bajo).
- Atmósfera de envasado (nitrógeno, dióxido de carbono, vacío).
- Sistema envasado (envasado aséptico, envasado activo, recubrimientos, etc.).
- La altas presiones.
- La radiación (microondas, UV).
- Otros procesos físicos (pulsos de alto voltaje, campo magnético oscilante, radiofrecuencia, etc.)
- Antimicrobianos (nitritos, sulfitos, ácidos orgánicos, compuestos naturales de origen vegetal, animal o microbiológico).
- Microbiota competitiva (BAL) y sus compuestos.

3.2.2. Las bacteriocinas en la tecnología de las barreras

Además del uso en solitario de las bacteriocinas, a veces muy discutible por su limitada eficacia en determinados alimentos complejos, en la actualidad existe un gran interés en la búsqueda de combinaciones de ellas que actúen de forma sinérgica frente a

bacterias patógenas en alimentos, y también en la aplicación coordinada de bacteriocinas y tratamientos físicos y químicos moderados, que amplíen, además, su espectro de acción a bacterias Gram-negativas.

Existen numerosos ejemplos de éxito de aplicaciones de bacteriocinas combinadas con otras barreras (revisado en Gálvez et al., 2007, 2010). Citaremos solo algún de ellos:

- La nisina ha sido aplicada en combinación con numerosos tratamientos en el control diversos patógenos: para inhibir *B. cereus* combinada con carvacrol (Periago y Moezelaar, 2001), para inhibir *L. monocytogenes* conjuntamente con el sistema lactoperoxidasa (Boussouel et al., 2000), en combinación con EDTA y otras barreras para inhibir diversos patógenos (Mastromatteo et al., 2010; Wan Norhana et al., 2012; Tavakoli et al., 2015).
- Las altas presiones también han sido utilizadas con diversas cepas productoras de nisina A, bacteriocina TAB 57, enterocina 1 y lacticina 481, para inhibir el crecimiento de *E. coli* en quesos (Rodríguez et al., 2005).
- La enterocina AS-48 ha sido utilizada en combinación con tratamientos químicos para inhibir *S. aureus* y *E. coli* O157:H7 en zumo de manzana (Ananou et al., 2004; 2005c), en combinación con APH para mejora de la calidad microbiológica de chirimoya (Perez-Pulido et al., 2015) y en muchas otras aplicaciones.

3.3. Actividad probiótica de las bacterias lácticas

Las BAL al formar parte de la microbiota habitual del tracto gastrointestinal de humanos y animales desempeñan un papel beneficioso en el mantenimiento de la integridad intestinal y en procesos de inmunomodulación y resistencia a patógenos (Klaenhammer et al., 2005; Gourbeyre et al., 2011). Como probióticos se les ha atribuido diversas acciones beneficiosas como: incrementar la calidad nutritiva de los alimentos, facilitar la digestión de la lactosa y controlar las infecciones intestinales; además de otras supuestas, y no bien demostradas, características beneficiosas, tales como la inhibición del desarrollo de algunos tipos de cánceres y la reducción del colesterol sanguíneo (Gilliland, 1990; Montville y Winkowski, 1997; Franz et al., 1999; Gourbeyre et al., 2011; Sharma y Devi, 2014). Cabe destacar que los efectos probióticos son cepa-dependientes y no se pueden generalizar los efectos positivos de una cepa a toda la especie.

3.3.1. Concepto de probiótico

En los últimos años, se han producido importantes avances en el campo de la Nutrición y Tecnología Alimentaria. La expansión hacia otras áreas científicas como la Inmunología y Biotecnología ha permitido profundizar en la estrecha relación entre dieta y estado de salud, no solo por el valor nutritivo de los alimentos que ingerimos, sino también por sus efectos beneficiosos fruto de las interacciones con la microbiota intestinal. En este contexto, han aparecido los alimentos funcionales, considerados como aquellos que, además de aportar los nutrientes recomendados, ejercen efectos beneficiosos sobre la salud (Diplock et al., 1991). Este efecto beneficioso puede traducirse tanto en el mantenimiento del buen estado de salud como en la reducción del riesgo de padecer una determinada enfermedad.

Los probióticos constituyen uno de los grupos más destacados dentro de los alimentos funcionales ya que incorporan microorganismos viables capaces de modificar e interactuar con la microbiota del hospedador, ejerciendo un efecto beneficioso en la prevención y tratamiento de distintos trastornos gastrointestinales, en la reducción de la intolerancia a la lactosa y en la modulación de la respuesta inmune. Desde muy antiguo, se conoce que el consumo de alimentos fermentados mejora el balance microbiano del intestino en el hospedador. Ya en el año 76 a. C se empleaba la leche fermentada para el tratamiento de diversas afecciones intestinales. Pero no fue hasta el siglo XX cuando se empezó a considerar el efecto beneficioso que determinadas bacterias ejercían sobre la salud de los que las consumían. En 1908, el premio Nóbel Elie Metchnikoff, en su obra *Prolongation of Life*, postuló que el consumo de leches fermentadas tenía un efecto positivo sobre la microbiota del tracto gastrointestinal, con un impacto favorable en la salud humana.

El concepto probiótico, del griego *"pro bios"* (por la vida), probablemente fue empleado por primera vez por Vergio en 1954, cuando analizaba el efecto de los antibióticos sobra la microbiota intestinal. Una década más tarde, Lilly y Stillwell (1965) se referían a los probióticos como microorganismos que promovían el crecimiento de otros microorganismos. En 1989 Fuller redefinió el concepto de probiótico, considerándolo "*aquel suplemento alimenticio integrado por microorganismos vivos que aportan beneficios al hospedador que los consume mediante la mejora de su equilibrio microbiano intestinal*". La FAO y la Organización Mundial de la Salud (OMS) han declarado que existe evidencia científica sobre los beneficios en la salud que producen los probióticos y los ha definido como "*organismos vivos que ingeridos en cantidad adecuada confieren un beneficio para la salud en el hospedador"* (FAO/OMS, 2001).

3.3.2. Efectos beneficiosos de los probióticos en salud humana

Clásicamente se ha atribuido el efecto de los probióticos a su capacidad para modificar la composición de la microbiota intestinal por competición con otras bacterias. Así, los probióticos son microorganismos capaces de prevenir la adherencia, establecimiento y replicación de ciertas bacterias patógenas, debido fundamentalmente a la producción de ácidos orgánicos, peróxidos, péptidos antimicrobianos (bacteriocinas) o por la simple competición por el espacio para unirse al epitelio intestinal. Pero los efectos que han demostrado los probióticos en procesos clínicos sugieren que los mecanismos involucrados van más allá de la simple ocupación del intestino por parte de bacterias beneficiosas en detrimento de las perjudiciales. Se ha demostrado que los probióticos actúan sobre la inmunidad intestinal, ejerciendo un beneficio para el hospedador. El sistema inmunitario intestinal constituye la parte más extensa y compleja del sistema inmunitario. El principal componente de este sistema lo forma el Tejido Linfoide Asociado al Intestino (Gut Associated Lymphoid Tissues, denominado GALT). El GALT está constituido por los folículos linfoides asociados, los ganglios linfáticos mesentéricos, las Placas de Peyer y las poblaciones linfocitarias dispersas que se localizan a lo largo del epitelio intestinal.

Distintos estudios argumentan que ciertos microorganismos probióticos pueden producir una estimulación del GALT (Wan et al., 2015), estimulando la inmunidad intestinal, bien por la producción específica de inmunoglobulinas tipo A (Rinne et al., 2005), activación de células NK y macrófagos o bien por la modulación de la producción de citoquinas (Marin et al., 1998; Arunachalam et al., 2000; Kitazawa et al., 2001; Giorgetti et al., 2015). Pero el efecto de los probióticos sobre la respuesta inmune no se limita a una actuación sobre el tejido intestinal, sino que parece afectar a la inmunidad general. Así, son claros los efectos beneficiosos en distintas afecciones sistémicas como eczemas, alergias y la hipercolesteremia (Gill, 2003).

Los probióticos interactúan no solo directamente con las células epiteliales intestinales a través de sus componentes celulares, tales como ADN, ácidos lipoteicoicos y exopolisacáridos (Lebeer et al., 2010), sino también, indirectamente, a través de la producción de metabolitos activos (Dobson et al., 2012; Giorgetti et al., 2015). Los péptidos y los polisacáridos microbianos pueden activar las vías de señalización y los factores de influencia como la secreción de citoquinas y la permeabilidad intestinal, mejorando así la función de la barrera epitelial. En la actualidad, se investiga para tratar de identificar los posibles mecanismos responsables de estos efectos clínicos.

Existen numerosas revisiones acerca de los efectos beneficiosos de los probióticos a diferentes niveles de actuación (Ouwehand et al., 2002; Gareau et al., 2010 ; Angelakis et al, 2013; Amara y Shibl, 2015; Lunder, 2015; Xie et al., 2015). A continuación presentaremos algunos datos relevantes obtenidos en los estudios sobre los diferentes aspectos beneficiosos atribuidos a los probióticos.

Reducción de la intolerancia a la lactosa. Ciertos probióticos se consideran beneficiosos para mitigar el problema de intolerancia a la lactosa, desorden provocado por una deficiencia congénita del enzima galactosidasa, lo que ocasiona dificultad en la absorción y digestión de este azúcar (Honda et al., 2007). Los individuos que la padecen desarrollan diarrea, flatulencia, dolor abdominal e incluso fiebre tras el consumo de leche, aunque los síntomas varían con el grado de intolerancia. Entre las causas que pueden generar intolerancia a la lactosa se incluyen la alteración en la mucosa intestinal por procesos inflamatorios o por desequilibrios en la composición de la microbiota intestinal.

Estudios en humanos han demostrado que la lactosa en productos lácteos fermentados es asimilada más fácilmente que la misma cantidad presente en la leche (Kolars et al., 1984). Esto podría deberse a que las bacterias del ácido láctico degradan parcialmente la lactosa contenida en estos productos hasta láctico, mejorando la tolerancia a este grupo de alimentos (Gourbeyre et al., 2011; Bautista-Gallego et al., 2013; Sharma y Devi, 2014). Otros estudios relacionan el consumo de *Streptoccocus thermophilus* y *Lb. bulgaricus* con una mejoría en los síntomas de intolerancia a la lactosa (Gorbach et al., 2000).

Tratamiento de enfermedades gastrointestinales. Los probióticos tradicionalmente han sido utilizados en el tratamiento de varias enfermedades gastrointestinales. La diarrea asociada al uso de antibióticos favorece el desarrollo de bacterias oportunistas como *Clostridium difficile*, responsable de la colitis pseudomembranosa en personas tratadas previamente con antibióticos y personas hospitalizadas, en quienes la terapia antimicrobiana es el principal factor de riesgo. Los microorganismos probióticos restauran el balance microbiano y bloquean la proliferación de *C. difficile* disminuyendo significativamente la diarrea (Plummer et al., 2004; Hickson et al., 2007; Bakken et al., 2009; Le Lay et al., 2015). Además, los probióticos pueden ser una alternativa en el manejo de la diarrea asociada a antibióticos en pacientes de edad avanzada (Xie et al., 2015). También, se ha demostrado el efecto beneficioso del consumo de probióticos en la prevención y tratamiento de la diarrea aguda infantil causada por rotavirus (Saavedra et al., 1994; Vanderhoof, 2000).

Saccharomyces boulardii, algunas bifidobacterias y varios lactobacilos (*Lactobacillus* GG; *Lb. rhamnosus, Lb. casei* y *Lb. reuteri*) han demostrado su efecto beneficioso para curar la diarrea, siempre que se administren precozmente al inicio de la enfermedad (Hilton et al., 1977; Isolauri, 2004; Benchimol y Mack, 2004; Auclair et al., 2015).

Aumento de la resistencia a infecciones. La microbiota intestinal representa una importante barrera que cualquier patógeno debe superar para poder iniciar la infección. La prevención de las infecciones intestinales se puede realizar a través de intervenciones dietéticas que reestructuren la microbiota normal mediante la colonización transitoria de microorganismos probióticos. Diversas investigaciones han demostrado la importancia que los probióticos desempeñan en la resistencia a infecciones como las producidas por *E. coli* enterotoxigénica, *L. monocytogenes*, *Salmonella enterica* serovar *Typhimurium*, *Yersinia enterocolitica, Shigella flexneri* and *Legionella pneumophilia* (Gopal et al., 2001; Altenhoefer et al., 2004; Corr et al., 2007; De Montijo-Prieto et al., 2015). Por ejemplo, el consumo de determinados probióticos desempeña un papel fundamental en la erradicación de *H. pylori* en pacientes afectados de úlcera péptica (Johnson-Henry et al., 2005). Los datos disponibles *in vitro* y en animales indican que las bacterias del ácido láctico pueden inhibir el crecimiento de esta bacteria, reduciendo su actividad ureasa necesaria para que el patógeno permanezca en el medio ácido del estómago (Coconnier et al., 1998; Lin et al., 2009; Vitor y Vale, 2011).

Cabe destacar que el potencial probiótico *Enterococcus faecalis* UGRA10, una nueva cepa productora de la enterocina AS-48 (aislada de queso de oveja) recientemente hallada, ha mostrado una alta capacidad de interferir con la adhesión de *Listeria monocytogenes* a células Caco-2 (Cebrián et al., 2012). Pero la resistencia a infecciones por parte de los probióticos no sólo se debe a la capacidad de competencia por la colonización. Se han demostrado mecanismos en los que intervienen, modificando el pH intestinal, estimulando la respuesta inmune o mediante la producción de bacteriocinas (Sherman et al., 2009; Arqués et al., 2015).

Modulación del sistema inmune. Numerosos autores han demostrado claramente que los microorganismos probióticos son capaces de modificar los parámetros inmunitarios (Ashraf y Shah, 2014). La administración continuada de determinados probióticos puede producir un efecto inmunopotenciador, estimulando la proliferación específica de células B y T, lo que se traduce en un incremento de la resistencia a tumores o infecciones y de la eficacia de las inmunizaciones vacunales. Del mismo modo, su efecto inmunomodulador en el huésped

puede deberse a la producción de determinadas citoquinas como el IFN-γ, a la activación de macrófagos, haciéndolos más eficaces en la destrucción de patógenos intracelulares y a la regulación de la producción de diversas interleukinas anti y pro-inflamatorias (Mengheri et al., 2000; Molina et al., 2015).

En el mismo sentido, se ha demostrado que el efecto sobre el sistema inmune difiere para cada cepa. Así, por ejemplo, mientras que *Lb. acidophilus* aumenta la proliferación *ex vivo* de linfocitos de bazo de ratón, otros lactobacilos la inhiben (Kirjavainen et al., 1999). Tambien se ha observado que ciertas cepas de lactobacilos tienen la capacidad de estimular principalmente respuestas hurmonales, mientras que otras promueven la inmunidad celular e inhiben la producción de anticuerpos (Yasui et al., 1999). Perez-Cano et al., (2010) han demostrado que una cepa de *Lb. salivarius* y otra de *Lb. fermentum* aisladas de leche humana son capaces de modular la activación y el perfil de citoquinas de monocitos periféricos sanguíneos y Olivares et al. (2007) encontraron que la ingesta oral de la cepa *Lb. fermentum* CECT5716 potencia la respuesta inmunológica cuando se administra la vacuna contra el virus de la gripe, aumentando el número de células T-cooperadoras tipo 1 las inmunoglobulinas neutralizadoras del virus. Recientemente, Duary et al., (2014) han demostrado que dos cepas probióticas de *Lb. plantarum* ejercen efecto inmunomodulador en células HT-29 estimuladas por lipopolisacárido.

Tratamiento de reacciones de hipersensibilidad. La prevalencia de enfermedades atópicas como el eczema, dermatitis y el asma ha aumentado en los últimos años. Estas afecciones están asociadas a la producción de las interleukinas IL-4, IL-5, y IL-13. El consumo habitual de ciertos probióticos puede ser efectivo en el control de este tipo de respuesta inmune para la prevención de reacciones alérgicas, interviniendo en la secreción reducida de citoquinas proinflamatorias, con un control de la respuesta IgE y una reducción general de la reacción alérgica (Niers et al., 2009; Soh et al., 2009; Vitaliti et al., 2014).

Enfermedades cardiovasculares. Se ha demostrado que la utilización de ciertas cepas de *Lactobacillus* y ciertos subproductos metabólicos de probióticos podría actuar positivamente en la prevención y la terapia de varios síndromes cardiovasculares como son las cardiopatías isquémicas (Oxman et al., 2001) y en la reducción de los niveles de colesterol sérico (Hlivak et al., 2005; Nguyen et al., 2007; Huang et al., 2014; Bosch et al., 2014). A pesar de los avances en el tema, no está bien definido el modo de acción de los probióticos lipo-reductores ni el mecanismo por el cual reducen el colesterol.

Infecciones urogenitales. Diversos estudios han demostrado que ciertos microorganismos presentes en la microbiota vaginal tienen efecto protector por la producción de bacteriocinas y peróxidos con actividad microbicida frente patógenos que alteran este microambiente, como *Prevotella bivia* y *Gardnerella vaginalis* (Atassi et al., 2006; Reid, 2012; Álvarez-Calatayud et al., 2015). La vaginosis bacteriana se produce como consecuencia del crecimiento excesivo de diversas especies de bacterias anaerobias, estando relacionada con la desaparición de los lactobacilos que predominan en la microbiota normal vaginal. La administración por vía vaginal de determinadas cepas de *Lactobacillus* ha demostrado reducir el riesgo de infecciones del tracto urinario y vaginosis bacteriana (McLean y Rosenstein, 2000; Abad y Safdar, 2009).

Enfermedades inflamatorias intestinales. El incremento de algunas enfermedades asociadas a la autoinmunidad y enfermedades inflamatorias intestinales como la enfermedad de Crohn y la colitis ulcerosa, está relacionado con cambios en el microambiente bacteriano del intestino. Esto se debe probablemente a una respuesta autoinmune exagerada frente a antígenos presentes en el lumen intestinal (Podolsky et al., 2002). Estas enfermedades producen una inflamación que puede afectar a cualquier segmento del tracto gastrointestinal, aunque es más frecuente en la región ileocecal y el colon (Gassull y Cabre, 1994). El consumo de probióticos podría ayudar a controlar los cambios generados en la microbiota a través de la modulación de la función de las células T y B, así como de otros mecanismos inmunológicos involucrados, colaborando en la prevención y mejora de estas enfermedades (Ishikawa et al., 2003; Mengheri et al., 2008; Reiff et al., 2009; Saez-Lara et al., 2015).

Algunos autores han demostrado con éxito las ventajas de la aplicación de probióticos en procesos inflamatorios intestinales. Por ejemplo, Ewaschuk et al., (2008) demostraron que cepas de *B. infantis* reducen la permeabilidad y la inflamacion del colon (colitis) en ratones deficientes en IL-10 que desarrollan de manera espontánea colitis en condiciones estándar (Ewaschuk et al., 2008). Otros estudios han demostrado la actividad anti-inflamatoria inducida por el polisacárido PSA, producido por *Bacteriodes fragilis* (bacteria comensal del intestino) así como la protección ejercida en colitis inducida por *Helicobacter hepaticus* (Mazmanian et al., 2008). Estos resultados sugieren que algunos miembros de la microbiota intestinal pueden mejorar la función de barrera y mantener la homeostasis intestinal, desempeñando un importante papel terapéutico de los probióticos en la prevención y tratamiento de patologías intestinales. *Lb. acidophilus* inhibe la colitis desencadenada en ratones por *Citrobacter rodentium* (Chen et al., 2005), mientras que *Lb. fermentum* CECT 5716 protege y revierte la colitis inducida por TNBS disminuyendo IL-6 y

potenciando la vía TLR-MyD88 en un modelo murino (Mañé et al., 2009). De forma similar, Peran et al., (2007) demostraron la actividad anti-inflamatoria de tres cepas de *Bifidobacterium lactis*, *Lb. casei* and *Lb. acidophilus* en un modelo de colitis inducida por TNBS en rata. Estudios recientes realizados por Rodríguez-Nogales et al., (2015) demostraron que la administración, tanto de formas viables como no viables, de la cepa *Lb. fermentum* CECT 5716, atenuaba el proceso inflamatorio (reducción de los niveles IL-1β TNF-α) en ratas con colitis inducida por TNBS.

Cáncer. En la actualidad se desconocen los mecanismos específicos por los que los probióticos pueden ejercer un efecto beneficioso frente a determinados tipos de cáncer colorrectal. Diversos estudios *in vivo* desarrollados en animales de experimentación, han permitido postular algunos de ellos, como la adsorción de carcinógenos (aminas, benzopirenos…), inhibición en la producción de metabolitos potencialmente carcinógenos, alteración de la apoptosis y modulación de la respuesta inflamatoria (Hirayamay y Rafter, 2000; Burns y Rowland, 2000; Akin y Tözün, 2014).

Distintas investigaciones han demostrado que la administración oral de lactobacilos y bifidobacterias reduce las lesiones inducidas por carcinógenos en la mucosa gastrointestinal de animales de experimentación (Pool-Zobel et al., 1996; Goldin et al., 1996). Del mismo modo, se ha demostrado la actividad antitumoral de determinados probióticos (Mego et al., 2005; Thirabunyanon et al., 2009; Fooladi et al., 2015). Por ejemplo, los resultados obtenidos por Fooladi et al., (2015) han mostrado que la administración oral en ratones BALB/c (portadores de cáncer de mama) de *Lb. acidophilus*, como agente inmunoestimulante, podría motivar a la proliferación de las células inmunes e inhibir el crecimiento de células tumorales. Permitiendo, de un lado, aumentar la producción de IFN-γ y, de otro lado, disminuir la producción de IL-4 (conocida como citocinas Th2), en el cultivo de células de bazo. Sus resultados han puesto de manifiesto que el tiempo de supervivencia de los ratones administrados con lactobacilos aumentaba de manera significativa en comparación con la de los ratones control sin tratamiento (Fooladi et al., 2015). También se ha demostrado que la administración de algunas cepas de lactobacilos y bifidobacterias en ratas puede reducir el daño al ADN inducido por carcinógenos y disminuir las lesiones pre-neoplásicas y tumores de colon e hígado (Zsivkovits et al., 2003; Verma y Shukla, 2013). En la tabla 1 se muestra una recapitulacion de algunos efectos beneficiosos demostrados, ejercidos por algunos microorganismos probióticos.

Tabla 1. Efectos beneficiosos para la salud de algunos probibióticos (adaptado de Amara y Shibl, 2015).

Enfermedad	**Cepa**	**Referencia**
Eczema	*Escherichia coli*	Niers et al. (2009), Soh et al., (2009) : Viljanen et al., (2005) ; Wickens et al., (2013) ; Cosenza et at., (2015)
	Bifidobacterium bifidum	
	Bifidobacterium lactis	
	Lactobacillus rhamnosus	
	Lactococcus lactis	
Alergias alimentarias	*Escherichia coli*	Lodinova-Zadnikova et al., (2003)
Inmunidad	*Bacillus circulans* PB7	Bandyopadhyay and Das Mohapatra (2009); Cammarota et al. (2009)
	Lactobacillus plantarum DSMZ 12028	
Efecto de eliminación de antibióticos	*Enterococcus mundtii* ST4SA	Botes et al. (2008), Fukao et al. (2009) Zhou et al. (2005) ; Ling et al., (2015)
	Lactobacillus plantarum 423	
	Lactobacillus brevis KB290	
	Lactobacillus strains	
	Bifidobacterium strains	
Gastroenteritis	*Lactobacillus casei*	Yamada et al. (2009)
Terapia intestinal	*Lactobacillus plantarum* LP299	Kennedy et al. (2000), Strowski and Wiedenmann (2009)
Candidiasis vaginal	*Lactobacillus rhamnosus* GR-1	Martinez et al. (2009) and Chew et al., (2015)
	Lactobacillus reuteri RC-14	
Infecciones urinarias	*Lactobacillus rhamnosus* GR-1	Anukam et al. (2009) and Lee et al., (2013).
	Lactobacillus reuteri RC-14	
Intolerancia a la lactosa	*Lactobacillus acidophilus*	Hawrelak et al. (2003)
Antiinflamatorios no esteroideos	*Escherichia coli* Nissle 1917	Ukena et al. (2005)
Disbiosis intestinal	*Lactobacillus johnsonii* La1	Hawrelak (2003) and Silva et al. (1987)
	Lactobacillus strains	
	Lactobacillus GG	
Síndrome de colon irritable	*Bifidobacterium infantis* 35624	Brenner and Chey (2009), Enck et al. (2009), Whorwell et al. (2006); Tojo González et al., (2015)
	Escherichia coli DSM 17252	
	Bifidobacterium infantis 36524	
Diarrea del viajero	*Lactobacillus GG*	Hawrelak (2003) and Michail and Abernathy (2002)
	Lactobacillus plantarum	
Enteritis por radiación	*Lactobacillus casei* DN-114001	Giralt et al. (2008)
Enfermedad de Croh´n	*Escherichia coli* Nissle 1917	Boudeau et al. (2003)
Prevención cáncer de colon	*Enteroccocus faecium M-74*	Mego et al. (2005)
Colitis ulcerosa	*Lactobacillus acidophilus*	Abdin and Saeid (2008), Adam et al. (2006) and Imaoka et al. (2008)
	Escherichia coli Nissle 1917	
	Bifidobacterium	
Úlcera péptica	*Lactobacillus acidophilus*	Iarovenko et al. (2007); Deguchi et al., (2012)
	Lactobacillus gasseri	
Prevención de atopía	*Lactobacillus rhamnosus GG*	Huure et al. (2008),
Hipercolesterolemia y enfermedades cardiovasculares	*Enteroccocus faecium* M-74	Hlivak et al. (2005), Kiatpapan et al. (2001) and Nyguyen et al. (2007); Bosch et al., (2014)
	Lactobacillus plantarum	
	Propionibacterium freudenreichii	
	Lactobacillus plantarum PH04	

3.3.3. Efectos beneficiosos de los probióticos en producción animal

El crecimiento de los sistemas de producción animal ha favorecido la aparición de enfermedades infecciosas y parasitarias que solían combatirse con el empleo de antibióticos utilizados como promotores del crecimiento o reductores de estrés durante el transporte, mejorando así la calidad de los productos (Courtheyn et al., 2002). Sin embargo, el uso continuo de antibióticos ha provocado el desarrollo de cepas patógenas resistentes, así como el riesgo de la presencia de residuos en los productos de origen animal (Bates et al., 1994; Blake et al., 2003; Marshall and Levy, 2011). Es por ello que la legislación europea, mediante la nueva regulación (EC 1831/2003), prohibió el uso de antibióticos como promotores del crecimiento en Europa a partir del 2006. Desde entonces se ha generado un proceso de investigación y desarrollo de nuevas alternativas seguras e inocuas que no generen resistencias microbianas. Una de las alternativas propuestas es el uso de microorganismos probióticos, los cuales se encuadran en el grupo de suplementos funcionales (Brambilla y De Filippis, 2005; Allen et al., 2013). Muchos autores coinciden en que los probióticos tienen un efecto beneficioso en la fisiología y la salud del hospedador, siendo útiles en la mejora de los indicadores zootécnicos (ganancia de peso y rangos de conversión alimentaria) y en la disminución de procesos infecciosos en animales de interés económico (Schrezenmeir y De Vrese, 2001; Janssens y Van Loo, 2006; Kabir, 2009; Roy et al., 2010; Yeoman y White, 2014). Los mecanismos de acción de los probióticos en animales han sido revisados por varios autores (Simon et al., 2001; Edens, 2003), postulándose dos posibles mecanismos de acción: exclusión competitiva y modulación de la respuesta inmune.

En las explotaciones ganaderas, los animales a menudo son sometidos a diversos elementos de estrés que pueden causar un desequilibrio en la microbiota intestinal, pudiendo constituir un factor de riesgo de ciertas infecciones. Por ejemplo, en la producción porcina, durante el período de lactación y las primeras semanas pos-destete ocurren las mayores pérdidas como consecuencia de factores estresantes, como son la separación de la madre, fin de la inmunidad transmitida por la leche materna o transición a dietas sólidas. Todos estos factores pueden modificar la microbiota gastrointestinal normal y la respuesta inmune del animal, alterando su equilibrio, entre otros efectos (Vanbelle et al., 1990; Matur et al., 2015), lo que conlleva una mayor susceptibilidad frente a trastornos intestinales, infecciones entéricas y presencia de diarrea, así como una disminución de los niveles de producción esperados (Álvarez, 1995; Trevisi et al., 2008). Los problemas entéricos, especialmente en lechones, son una de las principales causas de pérdidas económicas en la industria porcina. Entre las nuevas estrategias se encuentra el uso de probióticos, como

forma preventiva, en la disminución de la carga de patógenos en lechones y en la mejora de cuadros clínicos gastrointestinales propios de este período de la producción porcina. Sin embargo, el uso de probióticos no se reduce sólo a animales jóvenes, sino que también se ha extendido a cerdos de engorde y cerdas reproductoras.

Algunas especies del género *Bacillus* han sido probióticos pioneros en nutrición porcina. Larsen et al., (2014) identificaron diversas cepas bacterianas pertenecientes al género *Bacillus*, aisladas del suelo, de heces y de comida fermentada, e indicaron que constituyen cepas ideales para ser usadas como probióticos en alimentación animal. Por su parte, Aperce et al., (2010) indicaron que *Bacillus licheniformis* y *Bacillus subtilis* tienen cierto efecto inmunomodulador a nivel del epitelio intestinal porcino, lo cual podría explicar la mejora en cuadros diarreicos en período post-destete.

Algunas especies del género *Enterococcus* también han sido objeto de numerosos ensayos a lo largo de los últimos años. Así la suplementación oral con *E. faecium* a lechones, desde el nacimiento hasta el destete, redujo el número de animales que sufrieron diarrea y mejoró su rendimiento (Zeyner y Boldt, 2006). Actualmente, se están llevando a cabo numerosos estudios con cepas de *Lactobacillus*, ya que es bien reconocida su presencia en la microbiota autóctona del cerdo. Estudios, como los de Collado et al., (2007), han mostrado el efecto positivo de especies de *Lactobacillus* como probióticos en estos animales. Recientemente, Sukegawa et al., (2014) han demostrado el efecto de dos cepas probióticas de *E. faecium* en la ganancia de peso en lechones tras el destete. Aunque esta mejora se observó independiente de la administración viva o muerta de las cepas.

Durante las últimas décadas, los antibióticos fueron usados de forma intensiva como aditivos y suplementos del crecimiento en aves de corral con el objetivo de incrementar la producción. Adicionalmente se lograba disminuir el riesgo potencial de desarrollar enfermedades infecciosas por patógenos como *Campylobacter, Salmonella y C. perfringens*, entre otros. Como consecuencia de las restricciones del uso de antibióticos, han resurgido problemas sanitarios en las explotaciones como el incremento de desórdenes intestinales durante la cría (necrosis intestinal por clostridios) o el incremento de patógenos vehiculizados en alimentos como *C. jejuni* y *S. enterica.* Como una posible solución a esta problemática, gran variedad de especies probióticas se vienen usado en producción avícola en los últimos años. La aplicación de probióticos en el sector avícola redunda en animales más sanos, con mayor tasa de crecimiento y en productos más seguros. Esta seguridad se debe a la capacidad de la microbiota bacteriana de competir por los mismos recursos con los microorganismos patógenos. Entre los probióticos aplicados en aves de corral cabe

mencionar las especies pertenecientes a los géneros *Bacillus, Bifidobacterium, Enterococcus, Escherichia, Lactobacillus, Lactococcus, Streptococcus* e incluso a levaduras. Gunther et al., (1995) usó un producto probiótico en pollos de engorde, obteniendo resultados positivos en la ganancia de peso corporal y en la conversión alimenticia con respecto al grupo control. También, Onifade (1997) estudió el efecto de una dieta suplementada con una cepa de *Saccharomyces cerevisiae* en pollos *broilers*. Los grupos tratados mostraron mejor ganancia de peso, mejor conversión alimenticia y mayor rendimiento de la canal. García et al. (2002) observaron una disminución del colesterol total, de las lipoproteínas de baja densidad y de la grasa abdominal en pollos de engorde que consumieron hidrolizado enzimático de crema de levadura *S. cerevisiae*. Recientemente, Zhang et al., (2015) demostraron el efecto beneficioso de la cepa *Lactococcus garvieae* B301 en pollos de engorde, demostrando una menor incidencia de diarrea y mortalidad, así como un incremento del peso corporal en los animales que recibieron la dieta suplementada con el probiótico. Entre los enterococos usados como probióticos probióticos se incluye la cepa SF68 de *E. faecium* y la cepa NCIMB 10415 producida y comercializada por Cerbios-Pharma (Barbengo, Suiza). Dado que los enterococos forman parte de la microbiota mayoritaria en el tracto digestivo de aves de corral, existe un creciente interés por parte de la industria en incorporar estos microorganismos a los piensos destinados a su alimentación (Jin et al., 2000; Kalavathy et al., 2003).

En rumiantes adultos, algunos probióticos también se han aplicado con el objetivo de mejorar la digestión de la fibra por parte de los microorganismos del rumen. Estos probióticos intervendrían en procesos digestivos fundamentales como en la celulolisis ruminal. Con esta finalidad se han usado cepas de *Saccharomyces cerevisiae* en ganado lechero y especies de *Enterococcus* y *Lactobacillus* en ganado de engorde (Nocek and Kautz, 2006). Otros autores han evaluado en ganado ovino diferentes cepas de BAL capaces de reducir la aparición de acidosis ruminal ocasionada por butírico y propiónico (Lettat et al., 2012). Algunas cepas de *Megasphaella elsdenii* y especies de *Propionibacterium* también se han administrado como probióticos para reducir la acumulación de lactato ruminal (Klieve et al., 2003; Stein et al., 2006). En el caso de vacas lecheras se han incluido levaduras vivas en la ración, produciendo ligeros aumentos de la ingestión, de la producción de leche y de la grasa en la misma (Van Vuuren, 2003). Otros autores han demostrado que al alimentar terneros con dietas suplementadas con ciertas especies de *Lactobacillus* se incrementa la ganancia de peso en las primeras semanas de vida (Cruywagen et al., 1996; Timmerman et al., 2005).

En acuicultura, la aplicación de probióticos está destinada principalmente a mejorar el sistema inmunitario y la resistencia a infecciones, siendo por tanto importantes medidas de prevención con las que se pretende reducir el empleo de antibióticos en los animales en cultivo (Merrifield et al., 2010). Su empleo es de especial interés en larvicultura (Bricknell y Dalmo, 2005), aunque también se han demostrado efectos beneficiosos en peces adultos inmunocompetentes (Kesarcodi-Watson et al., 2008; C De et al., 2014).

Los probióticos que se han evaluado en acuicultura pertenecen a un amplio rango de bacterias y levaduras, entre las que destacan especies de *Vibrio*, *Pseudomonas*, *Aeromonas*, *Bacillus*, *Saccharomyces* y de BAL (Merrifield et al., 2010; Dimitroglou et al., 2011; Rombout et al., 2011), siendo numerosos los estudios de eficacia que se han llevado a cabo en el control de patógenos y en la mejora de la respuesta inmune de peces. Por citar algunos ejemplos, Balcázar et al., (2009) demostraron que la administración de *Lactococcus lactis* CLFP 100 y *Leuconostoc mesenteroides* CLFP 196 incrementaba la tasa de supervivencia de truchas desafiadas con *Aeromonas salmonicida*. Kim et al., (2013) informaron de la capacidad de la cepa *Lactococcus lactis* BFE920 para mejorar la respuesta inmune e incrementar la protección frente a infecciones por *Streptococcus iniae* en lenguado. Ramos et al., (2015), recientemente han evaluado los efectos de dos preparaciones comerciales basadas en *Pediococcus acidilactici* y en una mezcla de *Bacillus*, *Pedicoccus*, *Enterococcus*, y *Lactobacillus* en la mejora de la respuesta inmune humoral y del rendimiento zootécnico de truchas arco iris.

3.3.4. Evaluación de microorganismos probióticos

Según las directrices de la FAO, los microorganismos probióticos utilizados en los alimentos deben ser capaces no sólo de sobrevivir al paso por el aparato digestivo, sino también de implantar y proliferar en el intestino. Esto significa que deben ser resistentes a los jugos gástricos y poder crecer en presencia de sales biliares, sustancias que tienen un efecto inhibidor para la mayoría de las bacterias. Además, es conveniente que estos microorganismos presenten antibiosis frente a enteropatógenos por la producción de ácidos, bacteriocinas u otros antimicrobianos y que sean capaces de adherirse a células del epitelio intestinal para poder ejercer sus efectos beneficiosos en el huésped mediante su crecimiento y/o actividad en el cuerpo humano (Tuomola et al., 1998; Jankowska et al., 2008; Kravtsov et al., 2008). Debemos destacar que las propiedades relacionadas con la probiosis no están presentes en todas las cepas de un género o especie y es por ello que, a la hora de definir un probiótico, es necesario hacerlo sobre la cepa concreta y no sobre la especie. Este hecho ha sido reconocido también por la FAO y la OMS. Por tanto no se

pueden extrapolar los beneficios demostrados por una de las cepas a toda la especie, y menos aún al género.

De acuerdo con lo expuesto anteriormente, los probióticos promueven la salud de los que los ingieren, y para que puedan considerarse como tales se establecen ciertos criterios que deben cumplir, entre los que se incluyen:

- Ser bioseguro.
- Permanecer viable en el alimento hasta su consumo.
- Resistir a la acidez gástrica y a las sales biliares.
- Adherirse a células del epitelio intestinal del huésped, siendo capaz de colonizar el tracto gastrointestinal por un determinado periodo de tiempo.
- Presentar antibiosis frente a enteropatógenos.
- Demostrar su efecto inmunomodulador e influir positivamente en el metabolismo del huésped.

La mayoría de los probióticos usados en la actualidad son microorganismos de los géneros *Lactobacillus* y *Bifidobacterium*, aunque también se emplean algunos otros géneros como *Enterococcus* (*E. faecalis, E. faecium*), *Streptococcus* (*S. thermophilus*) y *Propionibacterium* (*P. freudenreichii*). Otras especies pertenecientes a los géneros *Bacillus* (*B. cereus*) y *Escherichia* (*E. coli* Nissle) también se han descrito como probióticos. Aparte de las bacterias, se han descrito levaduras como *Saccharomyces* (*S. boulardii*) y hongos como *Aspergillus* (*A. oryzae*) con propiedades probióticas.

La presencia de las bacterias probióticas en los alimentos no garantiza su funcionalidad y su eficacia, ya que la mayoría de las cepas empleadas podrían ser eliminadas por antagonismo inter-microbiano. Por todo ello, se considera importante la realización de estudios de comprobación del nivel de dichas bacterias beneficiosas en los productos probióticos comerciales y demostrar el efecto antimicrobiano que ejercen estos microorganismos frente a patógenos. En relación a ello, Astashkina et al. (2014) han evaluado algunos productos comerciales que portan la etiqueta “probiótico”. Los resultados ponen de manifiesto que las bacterias beneficiosas alcanzaban los niveles recomendados (10^7 UFC/ml). Los mismos autores evaluaron la actividad antimicrobiana de los productos probióticos (sin tratamiento o con un tratamiento de pasteurización) frente a patógenos como *S. aureus, Salmonella typhimurium* y *Proteus vulgaris*, siendo *Activia*, compuesto principalmente por bifidobacterias, el producto con mayor actividad antimicrobiana

detectada. Sin embargo, dicha actividad inhibitoria desaparecía después de un tratamiento de pasteurización (Astashkina et al., 2014).

3.3.5. Seguridad de los probióticos

Dentro de las BAL, las especies más comúnmente utilizadas como probióticos se incluyen en los géneros *Lactobacillus* y *Bifidobacterium*, aunque algunas formulaciones pueden incluir algunas cepas de *Streptococcus*, *Enterococcus*, *Pediococcus* o *Propionibacterium*. El uso preferencial de lactobacilos y bifidobacterias se debe, por una parte, a que se les considera seguros y, de hecho, muchas especies gozan del estatus GRAS de la FDA. El sistema de cualificación de presunta seguridad (QPS de Qualified Presumption of Safety) ha sido desarrollado por la Autoridad Europea de Seguridad Alimentaria (EFSA) para proporcionar a los comités científicos un sistema interno aplicable a la evaluación del riesgo de los microorganismos introducidos en la cadena alimentaria. Este estatus es equiparable al GRAS. Algunos probióticos como *Lactobacillus y Bifidobacterium* generalmente están reconocidos como seguros porque se llevan utilizando desde hace tiempo en consumo humano, mientras que otros son menos conocidos y su uso podría representar un riesgo para los consumidores. Además, los microorganismos probióticos se emplean en un abanico muy amplio de situaciones, que incluye desde individuos sanos, hasta personas inmunodeprimidas como neonatos, embarazadas, ancianos o enfermos con patologías diversas. En consecuencia, la evaluación de la seguridad de un probiótico debe tener en cuenta, entre otros factores, el tipo de microorganismo en cuestión, la forma de administración y el estado de salud del hospedador.

No obstante, los casos en los que se ha establecido una relación clara entre el consumo de un probiótico y un efecto adverso son extremadamente raros y siempre han involucrado a personas con enfermedades graves subyacentes. Esta incidencia tan baja es especialmente destacable teniendo en cuenta el uso tan extendido de este tipo de productos (Boyle et al., 2006). Las principales propiedades que se evalúan a la hora de definir la seguridad de una cepa se centran en la capacidad para producir sustancias indeseables, en la resistencia a los antibióticos y en su potencial patogenicidad demostrada in vivo (Snydman, 2008; Sanders et al., 2010).

Producción de sustancias indeseables. Dentro de las BAL, algunos géneros como *Lactobacillus* o *Bifidobacterium* carecen de factores de patogenicidad definidos. De hecho los mecanismos que se han propuesto para explicar su implicación en casos de enfermedad son precisamente los que se consideran propiedades deseables como probióticos al facilitar

su implantación y persistencia en el hospedador, como por ejemplo su capacidad de adherencia a las células del hospedador o su resistencia a los mecanismos de defensa de este. Sin embargo estas propiedades podrían facilitar su translocación de la barrera intestinal pudiendo originar sepsis.

En contraste con estos géneros, *Enterococcus* puede presentar numerosos factores de virulencia como la producción de hemolisinas o enzimas hidrolíticas como la ADNasa, gelatinasa o mucinasa (Franz et al., 2001), así como otras enzimas perjudiciales para el organismo, α-quimiotripsina, β-glucoronidasa, β-glucosidasa y N-acetil- β-glucosaminidasa, que se han relacionado con la aparición de determinados cánceres de colon (Parodi 1999, Heavey and Rowland 2004). Aunque la presencia de determinantes de virulencia no tiene porqué desempeñar un papel esencial en el desarrollo de una infección (Franz et al., 2003; Foulquié-Moreno et al., 2006) y a pesar que los enterococos poseen una baja patogenicidad intrínseca (Kayser, 2003), la presencia de estos factores ha impedido su inclusión en el listado QPS, siendo necesaria la evaluación de los aspectos de seguridad para cada cepa en cuestión.

Otro aspecto importante a considerar en relación a la seguridad de los probióticos es la producción de aminas biógenas (Fernández et al., 2015). Estas se producen por descarboxilación de los aminoácidos y aunque ejercen funciones fisiológicas esenciales, su excesiva producción puede inducir trastornos digestivos y respiratorios. Algunos géneros de BAL, como los enterococos, pueden dar lugar a elevadas concentraciones de histamina o tiramina lo que podría ser considerado como un inconveniente para su uso. Por este motivo, la incapacidad de sintetizar aminas biógenas debe formar parte de los criterios de selección de los probióticos (Sanders et al., 2010).

Resistencia a antibióticos y transferencia. La resistencia a antibióticos puede ser natural o adquirida. La resistencia natural no suele ser transmisible y no constituye un motivo de especial preocupación. La resistencia adquirida la presentan determinadas cepas de una especie y es debida, en general, a la adquisición de genes que codifican enzimas que promueven la exportación o inactivación del antibiótico. En este caso los genes responsables suelen estar localizados en elementos transmisibles como plásmidos, y transposones. La principal preocupación con la presencia de genes de resistencia a antibióticos se debe al potencial riesgo de transmisión de estos genes a la microbiota intestinal. Aunque se trata de un riesgo teórico, difícil de evaluar en la práctica, algunos autores han demostrado esta posibilidad de transferencia en modelos *in vivo*. Mater et al., (2008) demostraron la posibilidad de transferencia genética al comprobar que el gen de

resistencia a la vancomicina, vanA, podía ser transferido desde una cepa de *Enterococcus* a un *Lb. acidophilus* en un modelo animal.

Los enterococos han sido referidos como poseedores de un amplio espectro de resistencias tanto naturales como adquiridas a antibióticos (Klare y Werner, 2001). Un ejemplo de resistencia innata es la desarrollada frente a cefalosporinas, aminoglucósidos y polimixinas en *E. faecalis*. Por el contrario, las resistencias a las ampicilinas (especialmente en *E. faecium*), tetraciclinas, macrólidos, cloranfenicol, quinolonas y trimetoprim suele ser adquiridas. La ampicilina, vancomicina y gentamicina son los antibióticos clínicamente más importantes en el tratamiento de infecciones de etiología múltiple. El uso extensivo de ciertos antibióticos como la vancomicina, ha incrementado en los últimos años el porcentaje de cepas de enterococos resistentes a este antibiótico (VRE). De los diferentes fenotipos VRE conocidos hasta la fecha (Leclercq et al., 1992; Klare et al., 2012), los fenotipos vanA y vanB son los de mayor interés clínico, predominando las especies de *E. faecalis* y *E. faecium* entre ellos (Murray, 1998; Cetnikaya et al., 2000). Por ello, resulta esencial definir el patrón de susceptibilidad antibiótica de los organismos candidatos de acuerdo a procedimientos aceptados internacionalmente como es la determinación de la MIC/ID.

Patogenicidad. Se han descrito numerosas metodologías tanto *in vitro* como *in vivo* para evaluar la seguridad de los probióticos. En general, todas son útiles para disponer de la mayor información posible a la hora de identificar las cepas más seguras. Los estudios en animales de experimentación en roedores se han considerado tradicionalmente como una parte esencial en la evaluación de la seguridad de cualquier sustancia que se pretenda administrar a humanos. A pesar de ello, los datos de seguridad obtenidos en estos modelos no siempre son extrapolables a la especie humana (Sanders et al., 2010). La mayoría de los estudios realizados en animales sanos no han mostrado efectos adversos de los probióticos, incluso en ensayos de administración prolongada en el tiempo o de altas ingestas (Ishibashi y Yamazaki, 2001). Por ello, algunos autores han optado por la realización de ensayos con animales inmunodeprimidos o forzando posibles mecanismos de patogenicidad mediante la administración de los probióticos por rutas inusuales como la intravenosa. A pesar de ello, aunque las distintas pruebas *in vitro* y en modelos animales pueden proporcionar información útil durante el proceso de selección de cepas probióticas, los únicos datos que permiten evaluar la seguridad de un microorganismo con certeza se obtienen mediante su evaluación en ensayos clínicos en humanos.

3.3.6. El género *Enterococcus* como probiótico

Los enterococos son cocos Gram-positivos, no esporulados, catalasa y oxidasa negativos, anaerobios facultativos que se presentan individualmente, en pares, o en cadenas y tienen bajo contenido en G+C (<50%). Desde un punto de vista taxonómico, estos cocos Gram-positivos se consideraron *Streptococcus* hasta la década de los 80, cuando se llevó a cabo una reclasificación que introdujo los géneros *Enterococcus* y *Lactococcus* (Schleifer y Kilpper-Bälz, 1987; Devriese et al., 1993; Devriese y Pot, 1995; Hardie y Whiley, 1997). En 1984, Schleifer y Kilpper-Bälz llevaron a cabo estudios de hibridación y secuenciación del ARNr 16S que evidenciaron las diferencias existentes entre las especies *S. faecium* y *S. faecalis* con respecto a otros *Streptococcus*, por lo que propusieron su traslado a un nuevo género, *Enterococcus*. Hasta la fecha se han descrito 37 especies dentro del género *Enterococcus*, que corresponden a 7 grupos diferentes: *E. acquimarinus*, *E. asini*, *E. avium*, *E. caccae*, *E. canintestini*, *E. canis*, *E. camelliae*, *E. casseliflavus*, *E. cecorum*, *E.columbae*, *E. devriesei*, *E. dispar*, *E. durans*, *E. faecalis*, *E. faecium*, *E.gallinarum*, *E. gilvus*, *E. haemoperoxidus*, *E. hermanniensis*, *E. hirae*, *E. italicus*, *E. malodoratus*, *E. moraviensis*, *E. mundtii*, *E. pallens*, *E. phoeniculicola*, *E. pseudoavium*, *E. quebecensis*, *E. raffinosus*, *E. ratti*, *E. saccharolyticus*, *E. silesiacus*, *E.sulfureus*, *E. termitis*, *E. thailandicus*, *E. ureasiticus* y *E. villorum* (Teixeira et al., 2001; Fortina et al., 2004; Carvalho et al., 2006; Franz et al., 2011).

Enterococcus constituye uno de los géneros dentro de las BAL más diverso después de *Lactobacillus* y *Streptococcus*. Se pueden encontrar en diversos hábitats como en el suelo, superficies acuáticas, océanos, aguas residuales, plantas y, sobre todo, en el tracto gastrointestinal de humanos y animales. En este contexto, diversos estudios microbiológicos han demostrado que los enterococos juegan un papel importante en la tecnología de los alimentos como, por ejemplo, en la maduración de algunos quesos tradicionales elaborados con leche cruda o pasteurizada en países mediterráneos (Suzzi et al., 2000; Andrighetto et al., 2001; Giraffa, 2002), o en productos cárnicos fermentados como las salchichas (Giraffa, 2002; Franz et al., 2003; Hugas et al., 2003). Algunas de estas propiedades tecnológicas beneficiosas, tales como el impacto positivo que tienen en la maduración y la producción de aroma de carnes fermentadas y de quesos, así como la segregación de oleuropeínas en las aceitunas, hacen que estas bacterias resulten interesantes para el desarrollo de cultivos iniciadores en la producción de dichos alimentos.

Algunas cepas de especies de *Enterococcus* también se han descrito como probióticos (Fuller, 1989; O´Sullivan et al., 1992; Holzapfel et al., 1998; Franz et al., 2011), siendo *E. faecium* y *E. faecalis* las más utilizadas. Ambas han sido aplicadas tanto como suplementos probióticos en humanos (administración para el tratamiento de diarrea, diarrea

asociada a antibióticos, síndrome del intestino irritable, disminución del colesterol sérico o para la regulación del sistema inmune), como en suplementos para la alimentación animal (Allen et al., 2004; Hlivak et al., 2005; Canani et al., 2007; Enck et al., 2008; Chen et al., 2010; Klingspor et al., 2015). Así, existen preparaciones comerciales farmacéuticas basadas en dos cepas de *Enterococcus* que han sido bien estudiadas para ser usadas como probióticos, se trata de *E. faecium* SF68 (Cylactin® LBC SF86 ME10, F. Hoffmann-Roche S.A. Basilea, Suiza) y *E. faecalis* Symbioflor 1 (SymbioPharm, Herborn, Germany). Pese a todo esto, el uso de los enterococos como probióticos sigue siendo una cuestión polémica, ya que mientras el beneficio probiótico de algunas cepas ha sido bien establecido, el incremento de las enfermedades enterocócicas asociadas con humanos y la resistencia a múltiples antibióticos ha suscitado preocupación en relación a que sus factores de virulencia puedan ser transferidos a otras bacterias del tracto gastrointestinal (Franz et al., 2003).

Recientemente algunos autores se han centrando en la evaluación de cepas probióticas de enterococos productores de bacteriocina. Así por ejemplo, Lauková et al., (2015) han demostrado los efectos beneficiosos de aplicar una cepa bacteriocinogénica de *E. faecium* en la cría de avestruces. En nuestro caso, una buena parte de esta memoria está centrada en la evaluación de las propiedades probióticas de la cepa *E. faecalis* UGRA10, un nuevo productor de enterocina AS-48 aislado de queso de leche de oveja artesanal (Cebrian et al., 2012).

IV. LA ENTEROCINA AS-48

La enterocina AS-48, protagonista de esta memoria, es producida por *E. faecalis* subsp. *liquefaciens* S-48 y otras cepas de enterococos siendo el primer miembro descrito de un nuevo tipo estructural de bacteriocinas de las bacterias del ácido láctico caracterizado por su estructura cíclica y su amplio espectro de acción, que abarca tanto a bacterias Gram-positivas como a algunas Gram-negativas (Gálvez et al., 1985; Gálvez et al., 1986; Samyn et al., 1994).

Los primeros datos sobre la enterocina AS-48 fueron publicados por Gálvez et al. (1985), dentro de un estudio acerca de la producción de sustancias tipo bacteriocina en el género *Enterococcus* (entonces *Streptococcus*). La cepa productora de AS-48, identificada como *Enterococcus* (*Streptococcus*) *faecalis* subsp. *liquefaciens* S-48, produce además una segunda bacteriocina de alto peso molecular (80 kDa), denominada Bc-48 y de espectro de acción bastante limitado (López-Lara et al., 1991). Poco después mediante tratamientos clásicos de curación de plásmidos (bajas concentraciones de anaranjado de acridina y

bromuro de etidio), se obtuvo el mutante A-48-32, que solo produce la enterocina AS-48 (Martínez-Bueno et al., 1990) y ha sido el usado para la producción de AS-48 hasta el hallazgo de la cepa *E. faecalis* UGRA10 (Cebrián et al., 2012).

4.1. Determinantes genéticos de la producción e inmunidad de AS-48

La producción de bacteriocinas es siempre un proceso complejo que requiere la intervención de numerosos genes, además del gen estructural, para su exporte al medio extracelular y la expresión de la inmunidad. En el caso de AS-48, en el que la maduración del pre-péptido exige la formación de un enlace peptídico entre sus extremos amino y carboxilo para originar la molécula cíclica con actividad antibacteriana, la situación es aún más compleja. Los determinantes genéticos relacionados con la maduración, inmunidad y secreción de AS-48–fueron localizados por primera vez en el plásmido conjugativo pMB2 de 68 Kb de *Enterococcus faecalis* S-48 (Martínez Bueno et al., 1990), habiéndose encontrado posteriormente estos genes en otras cepas tanto de *E. faecalis* como *E. faecium,* localizados dentro de plásmidos de características similares (Tomita et al., 1997, Folli et al., 2003, Cebrián et al., 2012; Huang et al., 2013) o incluso en el cromosoma, como es el caso de la variante natural AS48RJ (Abriouel et al., 2005).

En todos los casos, el gen estructural (*as-48A*) codifica para una proteína de 105 aminoácidos de los que los 35 primeros constituyen el péptido señal, que será escindido, teniendo posteriormente lugar la unión covalente entre el Try^{70} terminal y la Met^{1}, para dar lugar a la bacteriocina circular madura (Martínez-Bueno et al., 1994). Tras el gen estructural se dispone un conjunto de 9 genes adicionales, *as-48B, as-48C*, *as-48C1, as-48D, as-48D1, as-48E, as-48F, as-48G* y *as-48H*, (Figura 5) que son responsables de los demás aspectos vinculados con el carácter AS-48 (Martínez-Bueno et al., 1998; Díaz et al., 2003).

Desde el punto de vista transcripcional, la producción/inmunidad de AS-48 requiere la expresión coordinada de los diez genes identificados en el *cluster as-48*, que se realiza a través de 3 ARNm policistrónicos (Fig. 5):

1. $T_{1,}$ de 3,5 Kb, que transcribe el operón *as-48ABC* desde el promotor P_A (Martínez-Bueno et al., 1998). Este ARNm presenta a nivel de la región intergénica entre *as-48A* y *as-48B* unas repeticiones invertidas que conforman una estructura en horquilla, identificada como sitio de procesamiento para las endoribonucleasas. Esto va a permitir que la expresión del gen estructural *as-48A* esté controlada a nivel post-transcripcional pero desacoplada de la traducción de los genes as*-48BC* (Fernández et

al., 2008). De hecho a partir de T_1 se originan dos transcritos diferentes, T_A con una vida media larga, que traduce el gen estructural *as-48A* y T_{BC}, con una vida media muy corta, que traduce las proteínas As-48B y As-48C (Fernández et al., 2008). Este tipo de estructuras secundarias ha sido observado en otras bacteriocinas circulares.

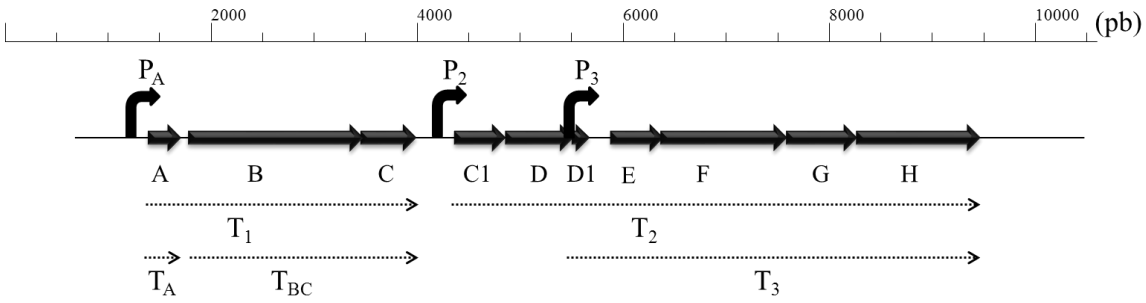

Figura 5. Organización genética del *cluster as-48,* en la que se indica la posición de los tres promotores (P_A , P_2 y P_3), y las ondas de transcripción (líneas discontinuas). En la barra superior se puede ver el tamaño del *cluster* en pares de bases (Cebrián et al., 2014).

2. $T_{2,}$ con 6,4 Kb, que se transcribe desde P_2, permitiendo la expresión del segundo operón, constituído por los genes *as-48C$_1$DD$_1$EFGH* (Díaz et al., 2003; Fernandez et al., 2008).

3. Además de los dos transcritos ya comentados (T_1 y T_2), se ha demostrado recientemente la existencia de un tercero T_3 que se iniciaría a partir de un promotor P_3 localizado aguas arriba del gen *as-48D1,* integrado dentro del gen *as-48D* (Cebrián et al., 2015), que transcribiría de forma independiente del P_2 los cuatro últimos genes *as-48EFGH*, los cuales conforman una segunda bomba de exporte ABC (Díaz et al., 2003).

Desde el punto de vista funcional, el mecanismo de secreción de AS-48 ha sido asignado a las proteínas As-48C$_1$D, las cuales constituyen un sistema de exporte de tipo ABC, de acuerdo con el análisis de homología llevado a cabo con proteínas bien caracterizadas de las bases de datos, en el que AS-48C$_1$ es el dominio integral de membrana y As-48D el dominio de unión al ATP. Mutantes de inserción para estas proteínas conducen a la no producción de AS-48, al igual que ocurre con mutantes de inserción para As-48B, proteína altamente hidrófoba, con hasta 12 posibles regiones transmembrana que se ha mostrado también imprescindible para la producción de AS-48. Parece pues concluyente que las proteínas As-48B junto a As-48C1D, asociadas o no, son las encargadas de la secreción y/o maduración de AS-48 (Martínez-Bueno et al., 1998). As-48C, cuyo gen se encuentra

solapado con *as-48B,* se trata de una proteína presumiblemente organizada en cuatro dominios de inserción en membrana, altamente hidrófoba, con similitud con la familia de proteínas DUF95, para la que se han propuesto funciones protectoras junto a los otros determinantes de inmunidad identificados en el *cluster as-48* (Fernández et al., 2008b), siendo la principal proteína implicada en la inmunidad As-48D_1, péptido de 56 aminoácidos fuertemente hidrófobo (Martínez-Bueno et al., 1998). Es segura, no obstante, la implicación de otros productos génicos de la región genética *as-48,* en el incremento de la resistencia frente a AS-48 cuando es administrado de manera exógena. En este sentido, de acuerdo con resultados experimentales, se ha sugerido que las cuatro últimas proteínas, As-48EFGH, conforman un segundo trasportador ABC implicado en la autoprotección de la cepa productora, siendo As-48E y As-48H dos proteínas hidrofóbicas con dominios de inserción en membrana, As-48G el dominio de unión al ATP y As-48H, de acuerdo con su estructura, una proteína auxiliar implicada en el reciclaje de As-48G (Díaz et al., 2003).

4. 2. Actividad biológica y modo de acción de AS-48

La enterocina AS-48 presenta actividad bactericida sobre la mayoría de las bacterias Gram-positivas ensayadas, siendo especialmente activa frente a cepas de *Enterococcus, Listeria, Bacillus, Planococcus, Mycobacterium, Corynebacterium y Nocardia*. Aunque también afecta a diversas especies de Gram-negativas, estas son mucho menos sensibles debido al efecto protector que les proporciona la membrana externa (Gálvez et al., 1989b). En general, AS-48 no muestra actividad frente a la mayoría de los microorganismos eucariotas ensayados como *Candida albicans* y *Saccharomyces cerevisiae*, las amebas de vida libre, *Naegleria fowleri*, *Acanthamoeba*, eritrocitos y las líneas celulares HeLa y MCDK (Gálvez et al., 1989b).

Dentro de este amplio espectro de acción, es interesante destacar la sensibilidad que muestran bacterias patógenas transmitidas por los alimentos y/o alterantes de los mismos, en condiciones de laboratorio y en sistemas alimentarios, entres las que destaca *L. monocytogenes* (Mendoza et al., 1999), *B. cereus* (Muñoz et al., 2004) y *S. aureus* (Ananou et al., 2004; Ananou et al., 2005b). Muchas otras cepas a priori resistentes, como ciertas bacterias Gram-negativas, se vuelven sensibles cuando se emplean tratamientos combinados, como es el caso de S*. cholerasuis* (Abriouel et al., 1998) y de *E. coli* O157:H7 (Ananou et al., 2005b). Además la presencia de AS-48 a concentraciones inferiores a la mínima bactericida retrasa y disminuye en gran medida (75%) la producción de la enterotoxina A por *S. aureus* (Ananou et al., 2004).

El modo de acción de AS-48 se ha establecido investigando su efecto sobre bacterias intactas, así como sobre protoplastos y vesículas de membrana (Gálvez et al., 1989a, 1989b, 1991). La diana celular primaria de AS-48 es la membrana citoplasmática, y su adición a células sensibles determina el cese inmediato de la captación de precursores y la pérdida de la capacidad para mantener los niveles citoplasmáticos de sodio y potasio. Como consecuencia de la permeabilización de la membrana celular, se produce un rápido colapso del potencial de membrana, de forma similar a como lo hace el inhibidor de ATPasa, diciclo-hexil-carbodiimida, siendo su acción independiente del potencial de membrana. AS-48 actúa también sobre liposomas de fosfatidilcolina, en los que provoca la libre y rápida difusión de pequeñas moléculas (uridina o rubidio), y también la de solutos de mayor tamaño (dextrano) y en los que tratamientos algo más prolongados producen la fusión de las bicapas, dando lugar a agregados multilamelares (Gálvez et al., 1991). Como efecto secundario, en una gran parte de las bacterias ensayadas se observa también un descenso en la densidad óptica de los cultivos, debida a la lisis celular (Gálvez, 1987; Gálvez et al., 1989b). En *E. faecalis*, dicha acción es dependiente de iones Mg^{2+}, siendo atribuible a la actividad de las autolisinas presentes en la pared. Tras los daños letales ocasionados a nivel de membrana se produciría una desregulación de las autolisinas, encargadas de digerir la pared celular (Gálvez et al., 1990). La inducción de bacteriolisis podría tener un gran interés aplicado en productos madurados (por ejemplo quesos), en los que las enzimas intracelulares liberados contribuirían al desarrollo de su sabor.

Mediante la técnica de Langmuir se ha comprobado que es posible la formación de monocapas puras y mixtas de AS-48, lo que ha permitido estudiar mediante esta técnica la interacción de AS-48 con componentes de la membrana citoplasmática de las bacterias. Ello ha permitido determinar que la principal interacción entre AS-48 y el ácido dipalmitoil fosfatídico (mayoritario en la membrana de los enterococos) no es de tipo hidrofóbico sino electrostático, ya que ocurre cuando este lípido está más cargado y el péptido está más desplegado (pH 10.5) (Abriouel et al., 2001) lo que podría explicar el notable incremento de actividad de AS-48 a pH 9 frente a bacterias Gram-negativas (Abriouel et al., 2000).

4. 3. Caracterización bioquímica y estructural

Es una molécula de naturaleza exclusivamente peptídica, por lo que es inactivada por diversas endopeptidadasas (tripsina, proteinasa K o proteasa V-8). No obstante, es resistente a exopeptidasas. Es muy estable a pH ácido (3 a 5), y neutro y a pH alcalino (10-

11) sufre una ligera pérdida de actividad (Gálvez et al., 1986; Gálvez, 1987) y además muy resistente al calor (Abriouel et al., 2001).

En su composición de aminoácidos destaca la ausencia de cisteína así como de aminoácidos modificados como la lantionina, la beta-metil-lantionina u otros residuos deshidratados, lo que la diferencia netamente de los lantibióticos. El análisis de la composición aminoacídica muestra una alta proporción de aminoácidos básicos, que justifica su carácter fuertemente catiónico (pI = 10,5). Además, contiene una gran cantidad de residuos hidrofóbicos (Ala, Pro, Val, Met, Ile, Leu y Phe) y de aminoácidos hidrofílicos sin carga neta (Ser, Gly, Thr, y Tyr) (Gálvez et al., 1989a).

Los intentos realizados para determinar la estructura primaria de AS-48 mediante degradación por el método de Edman fueron infructuosos, lo que sugirió que podía tener el extremo N-terminal bloqueado, hipótesis apoyada por la resistencia a exopeptidasas tales como las carboxipeptidasas A y B. Por ello la estructura primaria completa de AS-48 ha tenido que ser determinada mediante análisis de digeridos obtenidos con endoproteasas Lys-C, Glu-C y quimiotripsina (Samyn et al., 1994).

La masa molecular de AS-48, establecida mediante espectrometría de masas con electrospray es de 7.149,25. Este valor se corresponde exactamente con el calculado sumando la masa de los diferentes residuos de AS-48 y restando la masa de una molécula de agua, que se libera durante la formación de la unión peptídica cabeza-cola, dato que ha sido confirmado por los resultados de secuenciación del gen estructural de AS-48 (Martínez Bueno et al., 1994). La estructura secundaria de esta enterocina (Figura 5), establecida mediante estudios de resonancia magnética nuclear (RMN) está configurada por 5 hélices α-1 (13 aas, Ala9→ Ala21), α–2 (11 aas, Val25→Ala34), α–3 (10 aas, Ser37→Ala45), α–4 (12 aas, Ile51→ Lys62) y α–5 (12 aas, Lys64→Phe5). Esta estructura ha sido confirmada por los estudios tridimensionales (Langdon et al., 1998; González et al., 2000).

Los estudios realizados acerca de la estructura 3D (Figura 6) han permitido conocer la concentración de los aminoácidos básicos y de aquellos otros hidrofóbicos en zonas de la molécula bien diferenciadas, lo que le confiere un carácter anfipático, posiblemente muy importante para su actividad biológica. Tal estructura consiste en un ordenamiento globular de las mencionadas 5 helices-α que encierra un corazón muy hidrofóbico (Langdon et al., 1998). Esta organización da lugar a una fuerte acumulación de cargas positivas en una zona

superficial de la proteína, lo que parece determinante de la actividad formadora de poros en las membranas bacterianas (González et al., 2000).

Así mismo, el estudio termodinámico del desplegamiento de AS-48, mediante calorimetría diferencial de barrido y dicroismo circular, indica una gran estabilidad ya que sólo ocurre un desplegamiento reversible a pH 2,5 y baja fuerza iónica o bien a 25 ºC en presencia de cloruro de guanidina 6,3 M, pero no de urea 8 M. Desde un punto de vista cinético, la renaturalización es detectable en el orden del milisegundos, siendo más rápida incluso de lo que teóricamente se predice, lo que la convierte en una de las proteínas globulares más estables y con mayor velocidad de plegamiento (Cobos et al., 2001, 2002). Hoy es indiscutible que esta gran estabilidad le es conferida por el carácter circular de la molécula. Otro dato estructural de interés es la capacidad de AS-48 para formar agregados de 2, 3, 4, 5, 6 y 7 unidades que conservan las propiedades antigénicas y biológicas del monómero y cuya formación depende del pH y de la concentración de la bacteriocina (Abriouel et al., 2001).

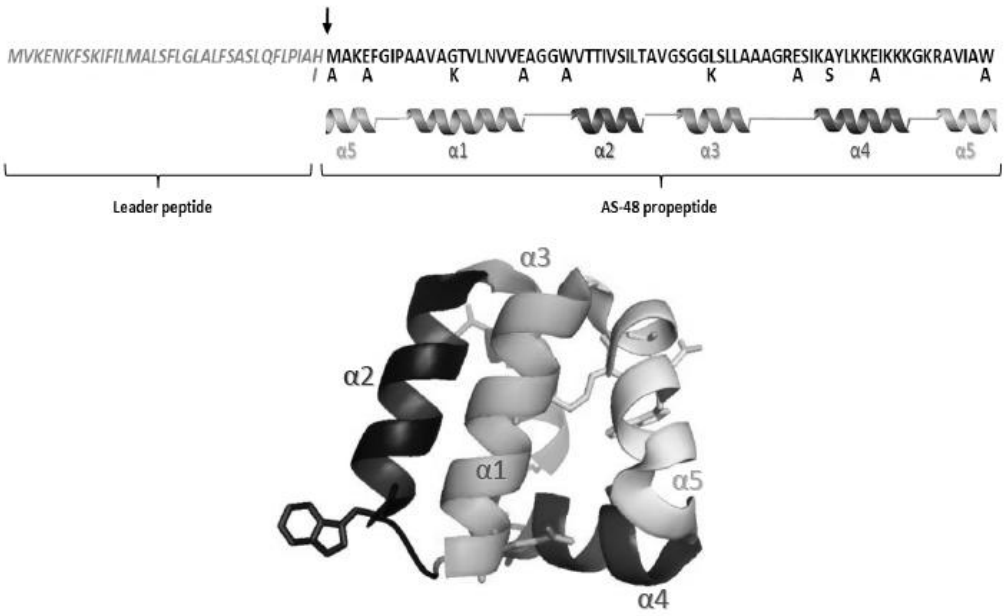

Figura 6. Estructura primaria, secundaria y 3D de AS-48 (Sánchez-Hidalgo et al., 2011)

El análisis cristalográfico llevado a cabo con AS-48 en solución a pH 4,5 y 7,5 ha permitido comprobar, de acuerdo con los estados de agregación encontrados a diferentes pHs, que AS-48 es una molécula mucho más flexible de lo esperado, capaz de adaptar su estructura molecular a las condiciones ambientales. Confirmaron, además, que las moléculas de AS-48 en el cristal adoptan dos formas diméricas, abreviadas como DF-I y DF-II (Figura 6), según las condiciones ambientales en las que se encuentran. Así, las

moléculas en DF-I interaccionan a través de las hélices α1 y α2 lo que supone que el dímero expone una mayor proporción de área cargada accesible al solvente, por lo que es más soluble que el monómero y es la forma en que se encuentra AS-48 en soluciones acuosas. Sin embargo, en ambientes hidrófobos, las moléculas de AS-48 forman los dímeros DF-II, gracias a la interacción entre las hélices hidrófilas α4 y α5. Esta organización permite que la cara hidrófoba se oriente hacia la membrana y la hidrófila al solvente, por lo que se ha propuesto que las moléculas de AS-48 al insertarse en la membrana adoptan tal configuración.

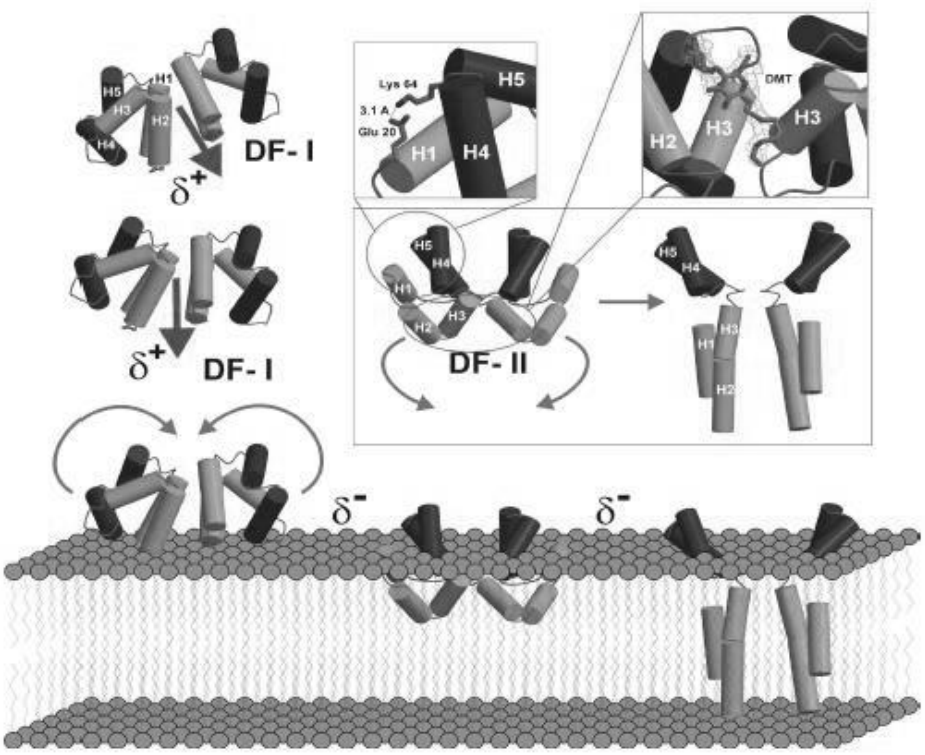

Figura 7. Representación del mecanismo molecular de la inserción de AS-48 en membrana. Se representa la aproximación de la forma DF-I a la membrana y la transición DF-I a DF-II a nivel de la superficie de ésta (Sánchez-Barrena et al., 2003).

Esta hipótesis ha sido revalidada por los estudios de difracción con rayos X realizados con variantes de AS-48 que tienen mutados ciertos residuos de la molécula de manera que la estructura queda estabilizada en la forma DF-I o en la forma DF-II. Los resultados experimentales apoyan que la acción de AS-48 se inicia con la aproximación a la membrana de dímero inactivo soluble en agua a lo que sigue la disociación del mismo y la inserción del péptido en la bicapa lipídica (Cebrián et al., 2015).

4.4. Producción y recuperación de AS-48

AS-48 se produce en medios complejos estándar tanto sólidos como líquidos (caldo de tripticaseína-soja, caldo de infusión de cerebro y corazón) y también en medios complejos diseñados ad hoc e incluso en mínimos definidos aunque en pequeñas cantidades (Gálvez et al., 1986). La cinética de producción de AS-48 sugiere que esta bacteriocina es un metabolito primario puesto que se acumula en el medio de cultivo durante la fase exponencial de crecimiento alcanzándose, en los medios referidos, los máximos títulos en la fase estacionaria temprana (8 h). Aunque sigue produciéndose durante la fase estacionaria, al final de la misma suele tener lugar un declive en los niveles de AS-48 de los cultivos, debido quizás a una adsorción inespecífica a las células productoras (Gálvez et al., 1986) más que a una inactivación enzimática específica.

Más recientemente se ha desarrollado un proceso de producción en un medio de cultivo basado en un subproducto lácteo, la lactalbúmina Esprion 300 (E-300), económico y de grado alimentario que se obtiene a partir del lactosuero generado en la producción de queso. Las condiciones producción establecidas como óptimas en este medio son: 5% de E-300 en agua adicionado de 1% de glucosa e inoculado con 8% de la bacteria productora, incubando a 28 ºC en agitación a pH controlado de 6,55. En estas condiciones el máximo de actividad se alcanza después de 16-18 h y permanece casi constante hasta las 24 h. El factor crítico parece ser la estabilización del pH que, al estar controlado automáticamente por adición de NaOH a demanda, permite que el cultivo siga creciendo hasta alcanzar en torno a 10^{11} UFC/ml (Ananou et al., 2008). Mediante este procedimiento se consigue multiplicar más de 20 veces el rendimiento de AS-48 de los cultivos. En la actualidad se trabaja en el escalado industrial de la producción de AS-48 habiéndose conseguido ya a nivel de planta piloto, en biorreactor de 300 L de capacidad (García-López, 2013).

La recuperación de AS-48 y purificación de la enterocina a partir de los cultivos de la cepa productora se realiza mediante un sencillo proceso de 2 etapas (Gálvez et al., 1989a; Abriouel et al., 2003) la primera del cual es una cromatografía de intercambio catiónico sobre carboximetil sephadex CM-25 del cultivo entero. La enterocina adsorbida es eluida con NaCl 1,5 M y las fracciones activas posteriormente recromatografiadas en HPLC de fase reversa sobre una columna de C18. Otras vías de recuperación de AS-48 han sido ensayadas recientemente, tales como la filtración tangencial a través de membrana cerámica (García-López, 2013). Así mismo se han estudiado formas de estabilizar las preparaciones de AS-48 y de eliminar las células productoras, aprovechando su gran

estabilidad térmica, mediante tratamiento térmico, luz ultravioleta y desecación mediante electrospray (Ananou et al., 2010).

4.5. Aplicación de AS-48 en alimentos y fármacos

Los resultados obtenidos hasta el momento ofrecen perspectivas prometedoras para la aplicación de AS-48 en la industria alimentaria y farmacéutica para el control de patógenos, bien mediante la adición directa de preparaciones de la bacteriocina o mediante el empleo de cepas que la produzcan *in situ*, empleadas éstas como cultivos iniciadores o junto con los cultivos iniciadores habituales.

La eficacia de AS-48 puede ser mejorada mediante el empleo de barreras múltiples, ya que la adición de ciertos compuestos químicos de grado alimenntario, ácidos láctico, acético o cítrico, lactato potásico, ácido nitroso, EDTA, y en especial, de los tripolifosfatos, o de barreras físicas (un choque térmico subletal) potencian la actividad de la enterocina (Ananou et al., 2004, 2005c). Dado el potencial tecnológico de esta bacteriocina como bioconservante, su eficacia ha sido comprobada en diversos sistemas alimentarios tales como leche y queso, productos cárnicos, alimentos de origen vegetal, y en sardinas frescas.

En leche, la eficacia de AS-48 producida *ex situ*, en el control de *S. aureus* es sensiblemente inferior a la mostrada en medios de laboratorio, siendo la misma inversamente proporcional al contenido en grasa. Sin embargo, es notable el control ejercido por la cepa enterocinogénica *E. faecalis* A48-32 sobre los estafilococos en cocultivos realizados en leche desnatada, incluso cuando la cepa productora se inocula a una concentración muy inferior. La eficacia de la bacteriocina producida *in situ* se incrementa de forma importante cuando se aplica un choque térmico subletal (Muñoz et al., 2007).

En productos cárnicos fermentados, modelo salchichón, se ha demostrado la capacidad de las cepas productoras de AS-48 para implantarse y producir eficientemente AS-48. La producción *in situ* de AS-48 (en cocultivo) consiguió eliminar a *Listeria* y tuvo un efecto meramente estabilizador sobre la población de *S. aureus* (Ananou et al. 2005a y b). La adición de AS-48 producida *ex situ* (concentraciones de 20, 30, 40 μg/g) consiguió eliminar ambas bacterias de la masa cárnica si bien a tiempos y concentraciones diferentes, mostrándose *Listeria* mas sensible a la bacteriocina aunque tanto ella como los estafilococos necesitaron de concentraciones efectivas mucho mas altas que las registradas en medios de cultivo de laboratorio. No obstante, en este modelo cárnico, se puede concluir

que el empleo de AS-48, producido *ex situ* o *in situ*, es una buena estrategia para controlar la proliferación de *S. aureus* y de *L. monocytogenes*.

En *fuets* (modelo salchichón de baja acidez), la adición de AS-48 (64 UA/g) causó una drástica inhibición de *L. monocytogens*, y un ligero efecto sobre *S. aureus* y *Salmonella* durante la maduración de los mismos. En los *fuets* adicionados con AS-48 y presurizados (400 MPa), almacenados a temperatura ambiente o bajo refrigeración, los niveles de *L. momocytogenes* fueron inferiores a 5 UFC/g, efecto similar observado con *Salmonella* que sufrió una importante reducción a los 18 d. *S. aureus* fue el más resistente al tratamiento con HHP y la adición de AS-48 tuvo poco efecto (Ananou et al., 2010). Estos resultados indican claramente que la enterocina AS-48 se puede combinar con barreras adicionales tales como HHP para mejorar la seguridad de alimentos de origen cárnico.

En alimentos de origen vegetal, se ha ensayado ensayado AS-48 en verduras y frutas crudas, conservas vegetales, pures y diversos tipos de salsas frente a bacterias patógenas y alterantes (revisado en Abriouel et al., 2010). La enterocina ha mostrado ser muy eficiente en zumos de fruta, tanto en el control de bacterias alterantes como *Alicyclobacillus acidoterrestris* (Grande et al., 2005) y *B. licheniformis* productor de exopolisacárido (Grande et al., 2006b) como en el control de *E. coli* O157:H7 en combinación con los tripolifosfatos o un choque térmico subletal (Ananou et al., 2005c). En conservas de vegetales, AS-48 inactiva eficazmente a *B. coagulans* (Lucas et al., 2006). En alimentos a base de arroz, y en purés de verduras, la adición de AS-48 permite controlar el a *B. cereus* y la producción de su enterotoxina, así como de otras especies de *Bacillus* (Grande et al., 2006 a,b, 2007). La adición de AS-48 a diversos tipos de salsas, permitió también controlar a *S. aureus* (Grande et al., 2007), y el lavado de brotes de semilla con dicha bacteriocina afectó negativamente los niveles de *L. monocytogenes* (Cobo Molinos et al., 2005).

En sardinas frescas (*Sardina pilchardus*), almacenadas en refrigeración (a 5°C) y envasadas en atmósfera normal, atmósfera modificada y en vacío, la adición de AS-48 no tuvo efecto sobre la microbiota mesófila, psicrótrofica y la bacterias Gram-negativas. Sin embargo, AS-48 redujo el nivel de los estafilococos especialmente en las muestras almacenadas bajo vacío. Además, la bacteriocina tuvo efecto negativo sobre el crecimiento de las bacterias productoras de las aminas biógenas tiramina e histamina. Este efecto es, probablemente, el responsable de que los niveles de las aminas biógenas histamina, tiramina, putrescina y cadaverina se mantengan en niveles indetectables, o muy bajos, en las sardinas tratadas con la enterocina. Por todo ello, podemos concluir que la aplicación de

AS-48 prolongó la vida media y la seguridad higiénica de las sardinas frescas refrigeradas en tanto que afecta negativamente a una parte importante de la microbiota normal y reduce las concentraciones de aminas biógenas (Ananou et al., 2014).

En cuanto a la aplicación de AS-48 en el sector farmacéutico, Maqueda et al. (2013) han descrito una composición para el tratamiento y profilaxis de infecciones bacterianas de la piel y mucosas ocasionadas por *Propionibacterium acnes* y *S. aureus* a base de la bacteriocina AS-48 en combinación con la lisozima. Se ha comprobado además que AS-48 sola o en combinación con lisozima carece de actividad hemolítica y de toxicidad sobre la línea celular CCD18 de fibroblastos y sobre la línea epitelial de glándula mamaria humana MCF 10A (resultados no publicados).

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OBJETIVOS

Desde 1985, año en que se refirió por primera vez la existencia de la bacteriocina AS-48, producida por especies de enterococos, se ha ido acumulando un enorme volumen de conocimiento sobre su estructura, actividad biológica, producción y aspectos genéticos. Los estudios estructurales permitieron conocer que se trata de una molécula de estructura (entonces) única circular, por unión peptídica entre sus extremos amino y carboxilo. Esta peculiaridad es probablemente la responsable de varios de sus aspectos de interés tecnológico: resistencia al calor y a diversos agentes químicos desnaturalizantes y estabilidad en un amplio intervalo de pH. Sin embargo, no fue hasta mucho después cuando nos planteamos la posibilidad de su uso biotecnológico como bioconservante alimentario. Desde la primera publicación en este campo, datada de 2005, fue evidente que la eficacia de AS-48 se veía muy disminuida en alimentos, necesitándose concentraciones muy altas (comercialmente poco rentables) de la molécula para lograr resultados satisfactorios en la eliminación o reducción de bacterias patógenas y alterantes. Por ello, se decidió abordar la aplicación de AS-48 dentro del contexto de la teoría de las barreras múltiples, hoy plenamente instaurada en tecnología alimentaria, para rebajar las dosis efectivas de AS-48 y ampliar su espectro de acción. Por otro lado, el auge actual del uso de probióticos y la implicación de sus bacteriocinas en la defensa frente a infecciones intestinales, nos ha llevado a investigar las características probióticas de *Enterococcus faecalis* UGRA10, una nueva cepa productora de AS-48 aislada a partir de un queso artesanal de leche de cabra.

Partiendo de estas premisas se planteó el desarrollo de esta tesis en torno a dos objetivos globales, con varios objetivos específicos dentro de cada uno de ellos:

I. APLICACIÓN DE LA TECNOLOGÍA DE LAS BARRERAS EN EL DESARROLLO DE AS-48 COMO BIOCONSERVANTE ALIMENTARIO.

I.1. Aplicación de la enterocina AS-48 combinada con barreras químicas y físicas para aumentar la seguridad y prolongar la vida útil del jamón cocido.
I.2. Control de bacterias patógenas en productos cárnicos mediante el tratamiento combinado de AS-48 y compuestos organosulfurados derivados de Aliáceas.
I.3. Control de bacterias patógenas y alterantes en alimentos mediante la aplicación superficial de recubrimientos comestibles incorporando la enterocina AS-48.
I.4. Control de *Listeria monocytogenes* en pescado mediante la aplicación combinada de AS-48 y el bacteriófago lítico P-100.
I.5. Estudio de toxicidad oral subcrónica de la enterocina AS-48 en un modelo murino.

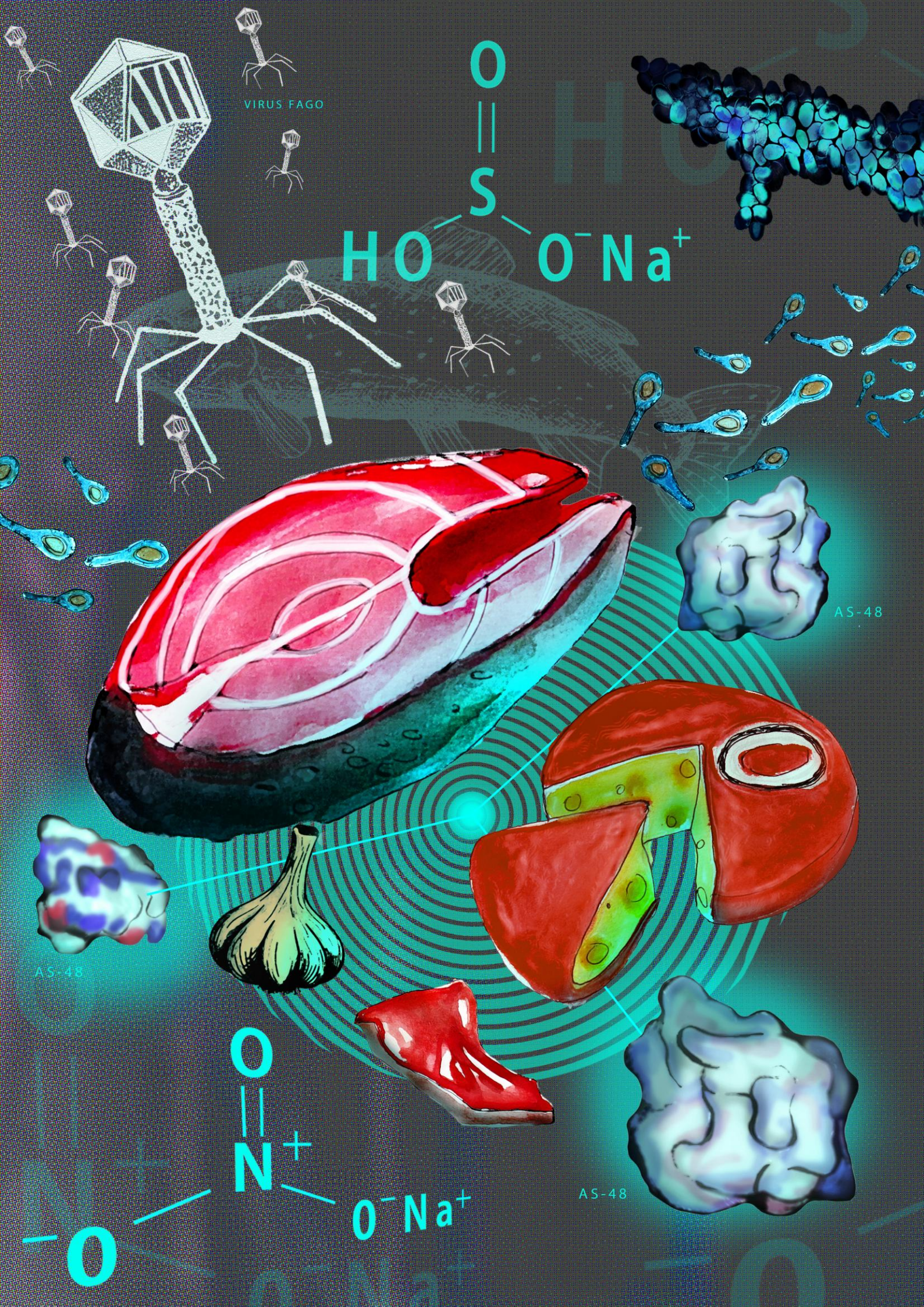
II. ESTUDIO DE PROBIOSIS DE UNA CEPA PRODUCTORA DE AS-48.

II.1. Estudio de las características funcionales, de seguridad y de probiosis de la cepa *E. faecalis* UGRA10.

II.2. Estudio de bioseguridad de la cepa *E. faecalis* UGRA10 en modelo murino.

II.3. Estudio de la capacidad de resistencia a infecciones y de la actividad inmunomoduladora de *E. faecalis* UGRA10 en modelo murino.

II.4. Evaluación de la enterocina AS-48 y la cepa *E. faecalis* UGRA10 en la protección frente a *Lactococcus garvieae* en peces.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BLOQUE I. APLICACIÓN DE LA TECNOLOGÍA DE LAS BARRERAS EN EL DESARROLLO DE AS-48 COMO BIOCONSERVANTE ALIMENTARIO

I.1.a. Effect of combined physico-chemical treatments based on enterocin AS-48 on the control of *Listeria monocytogenes* and *Staphylococcus aureus* in a model cooked ham.

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Abstract

Enterocin AS-48 was tested alone or in combination with chemical preservatives and/or heat against *Listeria monocytogenes* and *Staphylococcus aureus* in a cooked ham model system. AS-48 (20, 40 and 60 μg g^{-1}) alone was active against *L. monocytogenes* at 5 and 15 ºC, but it was not sufficient to avoid regrowth of *Listeria* during the 60 days storage. Combination of AS-48 (40 μg g^{-1}) with nitrite/nitrate, pentasodium tripolyphosphate, sodium benzoate or potassium sorbate improved the anti-listeria effect during storage at 5ºC. The most effective combination was AS-48-nitrite/nitrate (0.007%) that reduced listeria below detection level from the beginning to end of storage. Although much more resistant, *S. aureus* was also inhibited by AS-48 alone at 5ºC, and especially in combinations with nitrite/nitrate, pentasodium tripolyphosphate, sodium lactate and sodium acetate. Best results against both pathogens were obtained when sodium pyrophosphate was applied in combination with 60 μg g^{-1} AS-48. Sublethal heat (60ºC, 2 min) clearly increased AS-48 activity against both *Listeria* and *Staphylococcus.*

Keywords: Biopreservation; hurdle technology; cooked ham; enterocin AS-48; *Listeria monocytogenes*; *Staphylococcus aureus*

Published in Food Control. Volume 21, Issue 4, April 2010, Pages 478-486.

1. Introduction

These days additives are essential part of foods, without nutritional value but intended to improve the hygienic aspect and product shelf-life. This is necessary because although modern technologies and safety concepts have diminished the reported numbers of food-borne illnesses and intoxications, these are far from disappearing. The increasing consumption of precooked foods, ready to eat, prone to temperature abuse, and the importation of raw foods from developing countries are among the main causes of this situation. Meat and meat products may often act as vehicles for bacterial pathogens including *Staphylococcus aureus* and *Listeria monocytogenes,* two representative meat borne pathogens responsible for an important number of food-borne outbreaks in Europe (2369 and 1583 reported cases in 2006, respectively) (EFSA, 2007a). *L. monocytogenes* is of special concern due to its ability to grow during cold storage, and also to its wide spread in the environment (Farber, & Peterkin, 1996). In addition, although listeriosis is rare, its case fatality is notably high (20-30%). According to the Community Summary Reports on Trends and Sources of Zoonoses in the EU in 2004, 2005 and 2006 (EFSA, 2007b) the main foods contaminated with *L. monocytogenes* were meat products with overall ranges of contamination (presence in 25 g) of 0-48%. Collected data also revealed associations with other parameters including: food packaging type and preparation practices (e.g. the use of slicing machines for meat products).

In cooked ham marketing, prime cuts are often vacuum-packaged in order to extend their shelf-life during distribution before preparation of retail cuts. As a result of these practices, ham can become contaminated with pathogens such as *L. monocytogenes,* which can be considered an in house bacterium in processing plants of meat products (Salvat, Toquin, Michel & Colin, 1995).

In spite of these considerations on food safety, many of the chemicals currently licensed for use as food preservatives are increasingly being questioned with regard to their effects on humans, creating pressure on food suppliers to consider the use of natural alternatives to these chemical agents. Among these alternatives, bacteriocins and bacteriocin-producing LAB strains are of great interest since they can more easily earn the status of GRAS (Generally Recognized As Safe) and their products may be regarded as natural biopreservatives (Stiles, 1996), satisfying the consumer demand for foods that are naturally preserved, hygienically safe and also nutrient-rich and minimally processed. To this end, several bacteriocins from LAB like nisin and enterocins A and B have been tested, alone or in combination, with several physico-chemical treatments (modified atmosphere

packaging, high hydrostatic pressure, heat, pH and chemical preservatives) as additional hurdles in meat products with varying degrees of success (Gálvez, Lucas-López, Abriouel, Valdivia & Ben Omar, 2008). However, application of nisin in meat is clearly limited, especially if the pH is above 5.0 (El-Khateib, Yousef & Ockerman, 1993; Fang & Lin, 1994). It is currently applied only on a few meat products, and a high concentration (250 μg g^{-1}) is recommended in these cases (Thomas, Clarkson & Delves-Boughton, 2000).

Enterocin AS-48 is a cationic cyclic bacteriocin produced by *Enterococcus faecalis* S-48, with high stability in a wide range of temperature and pH values, sensitivity to digestive proteases and broad bactericidal activity against most of the Gram-positive bacteria and some Gram-negative bacteria (Gálvez, Maqueda, Valdivia, Quesada & Montoya, 1986; Gálvez, Maqueda, Martínez-Bueno & Valdivia, 1989). These features point to it as a promising alternative to chemical preservatives to be used as a biopreservative in foods. In previous works, we have tested the efficacy of AS-48 to inhibit food-borne bacteria in dairy and vegetable products and also in a meat sausage model system (Ananou, Maqueda, Martínez-Bueno, Gálvez & Valdivia, 2005a, Ananou et al., 2005b; Muñoz et at., 2007; Grande et al., 2006a,b; Grande et al., 2007a,b). However, enterocin amounts required for inhibiting Gram positive bacteria in foods are higher than those required in culture media, especially when the food is more dense and rich in fat and proteins (Cleveland, Montville, Nes & Chikindas, 2001; Ananou et al., 2005a,b). Therefore, in this study we investigated the action of AS-48, alone or combined with moderate heat or several of the chemical hurdles implemented in meat foods on the inhibitory action of AS-48 against *Staphylococcus aureus,* and *Listeria monocytogenes* in a cooked ham meat model.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Enterococcus *faecalis* A-48-32 (Martínez-Bueno, Gálvez, Valdivia & Maqueda, 1990) was used as AS-48 producer. *E. faecalis* S-47 from our collection was used as the standard indicator strain for bacteriocin activity assays. *S. aureus* CECT 976, an enterotoxin A producer strain originally isolated from ham involved in a food poisoning incident, was obtained from the Spanish type culture collection (CECT). *L. monocytogenes* CECT 4032 is a strain associated with a case of meningitis. For meat inoculation, both pathogenic strains were grown overnight on brain heart infusion (BHI) (Scharlau, Barcelona, Spain) at 37 ºC, washed in sterile

saline solution and then inoculated in the meat mixture at the selected concentration. All strain cultures were maintained at 4 ºC on BHI-agar slants.

2.2. Preparation of bacteriocin AS-48

AS-48 was produced by culturing the strain *E. faecalis* A-48-32 in a food-grade whey-derived substrate, Esprion 300 (E-300) (DMV Int., Veghel, Netherland) supplemented with 1% glucose according to Ananou, Muñoz, Gálvez, Martínez-Bueno, Maqueda & Valdivia (2008). The substrate was reconstituted at 5% total solids in sterile distilled water and inoculated (8%) with an 18-h A-48-32 culture in skim milk. Cultivation lasted 18-20 h at 28ºC at a controlled pH of 6.5 by the addition of 5 M NaOH on demand via a 719 S Titrino (Metrohm, Herisau, Suiza) while stirring with a magnetic stirrer to homogeneously distribute the *added* NaOH.

AS-48 was recovered from E-300 cultures by cation exchange chromatography on carboxymethyl Sephadex CM-25 (as described by Abriouel, Valdivia, Martínez-Bueno, Maqueda & Gálvez, 2003). Eluted fractions were tested for bacteriocin activity against the indicator strain S-47 by the agar well diffusion method (Gálvez et al., 1986). The approximate concentration of AS-48 (in µg ml^{-1}) in the preparation was estimated by comparing the diameter of inhibition halo around the well with a titration curve obtained with purified bacteriocin. Before use, the eluted fractions were dialysed at 4 ºC against distilled water through a 2000-Da cut-off membrane to eliminate NaCl and then sterilized by filtration (0.22 µm, Millipore, Belford, MA, USA).

2.3. Manufacture of cooked ham

The cooked ham was prepared with lean pork meat. Meat was coarsely ground through a 12 mm plate and brined. The brine solution contained (in g Kg^{-1}): NaCl, 12; sodium ascorbate, 0.30; sodium phosphate, 2; sodium glutamate, 1 (all from Scharlau); and water, 30. Although cooked ham is usually manufactured implementing nitrate/nitrite as preservative in the brine solution in our experiments we eliminated this preservative with the aims to study the precise effect of the combination of AS-48 with each specific compound, used individually. Ingredients were homogenized and the mix was divided in two batches to one of which was added of AS-48. Then both batches were cooked at 65 ºC during 45 min, cooled to room temperature, inoculated with approx 10^4 cfu g^{-1} of each bacterium (*L. monocytogenes* CECT 4032 and *S. aureus* CECT 976) and subdivided to obtain four batches: a control batch; a batch with added AS-48; a batch containing a different

preservative or a preservative combination -pentasodium tripolyphosphate 0.5%, (STPP, E-451i) sodium nitrate/nitrite 0.015 or 0.007%, (E-251/E-250) sodium pyrophosphate 0.15% (E-450i), sodium acetate 0.2% (E-262), sodium lactate 2% (E-325), potasium benzoate 0.1% (E-211), potasium sorbate 1% (E-202)- and a batch containing AS-48 and preservative or preservative combination. The pH of final product in all batches was 6.0-6.2. All the batches were subdivide in two portions and stored in sterile screw cap bottles at 5 ºC or exceptionally at 15 ºC. Two independent complete experiments were carried out for each preservative. Preservatives were from *Sigma* (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany).

2.4. Sub-lethal heat treatments

In a different experiment series, cooked ham manufactured as above was inoculated with the pathogens. Two batch were separated, one from the meat mixture added of AS-48 (20 µg g^{-1}) and other from this one without AS-48. Afterward both batches were divided into two batches. One batch of each type of meat mixture was heat treated (60 ºC for 2 min each) to produce a sub-lethal cell injury to the inoculated bacteria (killing approx 97% of each population). Finally four independent batches were established: a control batch non heated without AS-48, a second non heated and containing enterocin AS-48 (20 µg g^{-1}), a third one sub-lethal heated without AS-48 and finally another batch heated and containing AS-48 (20 µg g^{-1}). We also investigated the possible synergy between heat, AS-48 and STPP (0.5%) by determining the viability of both inoculated pathogens in two batches heated 60 for 2 min and added of STPP alone or combined with AS-48 (20 µg g^{-1}).

2.5. Cooked ham sampling

Samples from each treatment were extracted in duplicate at selected times (0, 1, 7, 15, 30 and 60 days) to determine viable counts of *S. aureus* and *L. monocytogenes.* For the microbiological determinations, 10 g were aseptically removed and mixed (1:10) with dilution medium (0.1% peptone, 0.85% NaCl). Homogenisation was done in a Masticator blender (IUL, Barcelona, Spain) for 1 min followed by serial 10-fold dilution and plating on the respective selective media: mannitol salt agar (MSA, Scharlau) for *S. aureus* incubated at 37 ºC for 72 h and PALCAM agar (Fluka Chemie GmbH, Buchs, Switzerland) with listeria supplement (Fluka) for *L. monocytogenes* incubated at 37 ºC for 72 h.

2.6. Bacteriocin extraction

Bacteriocin was extracted at selected times from cooked ham according to Garriga, Aymerich, Costa, Monfort & Hugas (2002). Briefly, cooked ham samples were homogenised (1:10) in sodium acetate 50 mM, EDTA 100 mM and Triton X 100 0.2% at pH 5 in a blender for one min, boiled for 10 min, cooled and filtered through a filter paper. The bacteriocin in the liquid phase was precipitated with 300 g l^{-1} ammonium sulphate and the pellet was dissolved in phosphate buffer 50 mM pH 7.2. The sample was heated at 80 ºC for 10 min and then the bacteriocin titre of extracts in arbitrary units (AU) was determined by the by the agar well diffusion method (Gálvez et al., 1986) in order to obtain the approximate concentration of AS-48 (in µg g^{-1}).

2.7. Statistical analyses

The average data from duplicate trials ± standard deviations were determined with the Excel programme (Microsoft Corp., USA). Statistical analyses were performed using the SPSS-PC 14.0 software (SPSS, Chicago, Ill. USA). Data relating to microbiological counts and pH were subjected to ANOVA, using the presence of AS-48, chemical preservative or both as factor. Categories include untreated control ham, ham with enterocin added, ham with preservative or ham with both AS-48 and preservative/s. Mean Square Difference was used as a post-hoc test to determine significant differences between ham manufactured with enterocin AS-48, chemical preservatives or both.

3. Results

3.1. Effect of enterocin AS-48 on the control of Listeria monocytogenes *and* Staphylococcus aureus

AS-48 alone (20, 40 or 60 µg g^{-1}) significantly reduced the viable counts of listeria (P <0.01), specially in the first 7 days at 5ºC in a concentration-dependent way (Fig. 1A). Remarkably, listeria were below detection values (10 cfu g^{-1}) at days 7 and 15 in the batches treated with 40 and 60 µg g^{-1} respectively. Nevertheless, afterwards listeria were able to regrow in all the treated batches, although viable counts for the bacteriocin-treated samples were significantly lower (P <0.01) than the untreated controls for the whole 60 d storage period. Staphylococci were slightly sensitive to AS-48 (Fig. 1B) and the counts were at least 1 log unit lower than in control from day 30 on.

AS-48 (40 µg g^{-1}) was also effective against *Listeria* at 15 ºC (Fig. 1C), although viable counts declined to below the detection level much faster (at day 1) and then recovered partially.

However at 15 ºC AS-48 (40 µg g^{-1}) failed to control *S. aureus* and similar viable count were attained in controls and in treated batches.

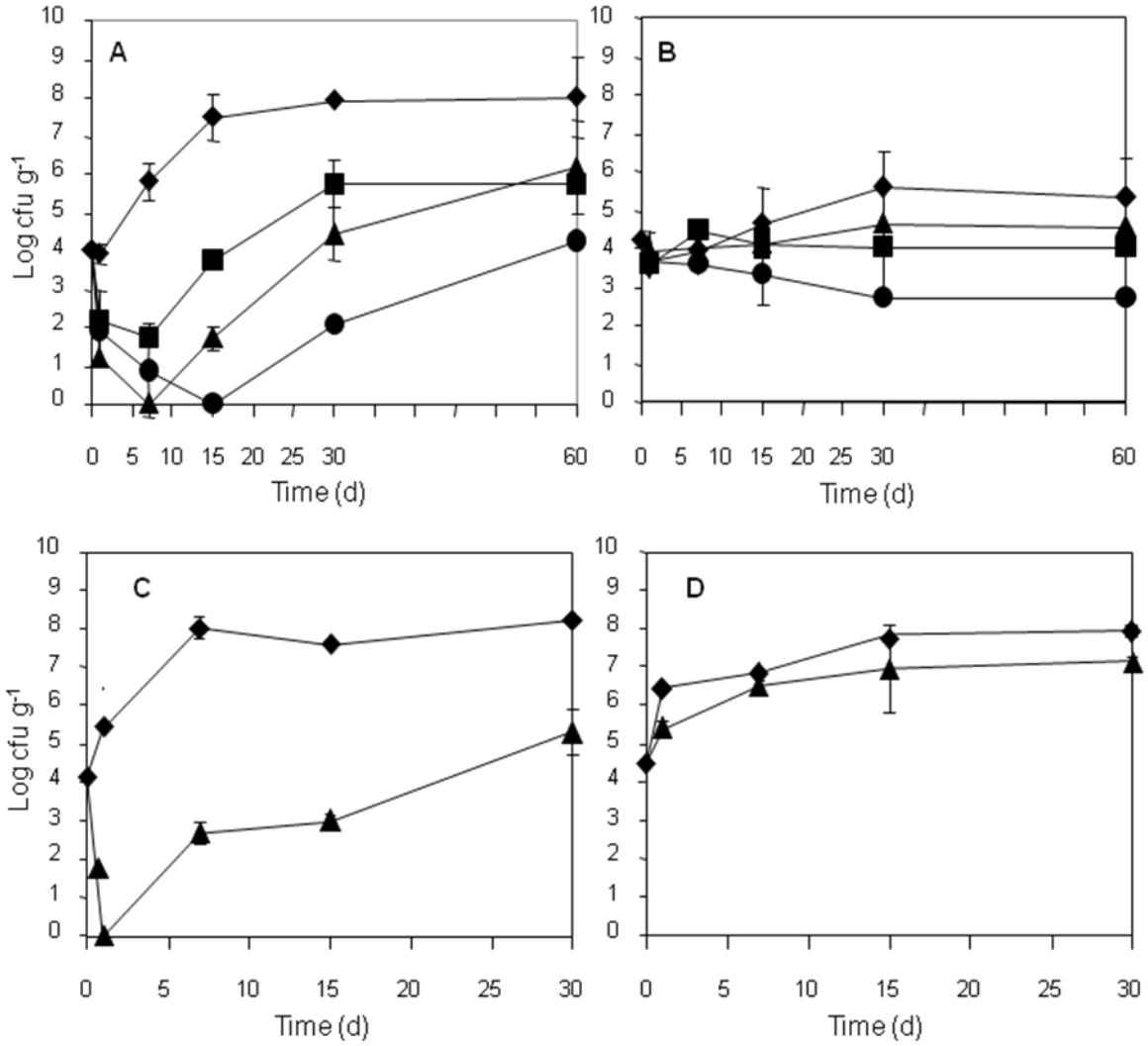

Fig. 1. Effect of different enterocin AS-48 concentrations on the viability of *Listeria monocytogenes* CECT 4032 (A, C) and *Staphylococcus aureus* CECT 976 (B, D) in a model cooked ham stored at 5ºC(A, B) or at 15ºC (C, D) : Control (♦); containing AS-48: 20 µg g^{-1} (■); 40 µg g^{-1} (▲); 60 µg g^{-1} (●). Values are the average ± SD (error bars) of two independent experiments.

3.2. Effect of enterocin AS-48 in combination with chemical preservatives on the control of *Listeria* monocytogenes and *Staphylococcus aureus*

Addition of nitrate/nitrite 0.007% had a very limited effect on growth of *L. monocytogenes* at 5ºC (Fig. 2A). On the contrary a significant inhibitory effect (P <0.01) was observed at this storage temperature against *S. aureus* causing a reduction of 1.94 log units with respect to control from day 30 on (Fig. 2B). Interestingly the combination of nitrate/nitrite

(0.007%) and AS-48 (40 µg g^{-1}) achieved a remarkable increase in the inhibitory effect against both bacteria. This effect was specially significant for *Listeria,* whose counts remained below detection values during storage from day 1 (P <0.001). For *S. aureus* the improved effect was noted from day 15 and reached the maximum at day 30 with count differences between controls and batches treated with both compounds of 3.65 log units. The combined application of AS-48 and a double nitrite/nitrate concentration (0.015%) did not increase the inhibitory effect on *S. aureus* (data not shown). At 15ºC, antilisteria activity of AS-48 was also potentiated by nitrite/nitrate, and regrowth was delayed to day 30, in which viable counts in batches containing both compounds still were much lower (3.05 log units) than in control untreated cultures (8.25 unit log) and in AS-48 treated cultures (5.30 log units) (Fig. 2C). Regarding *S. aureus,* no significant differences were determined between the four different batches stored at 15ºC (Fig. 2D).

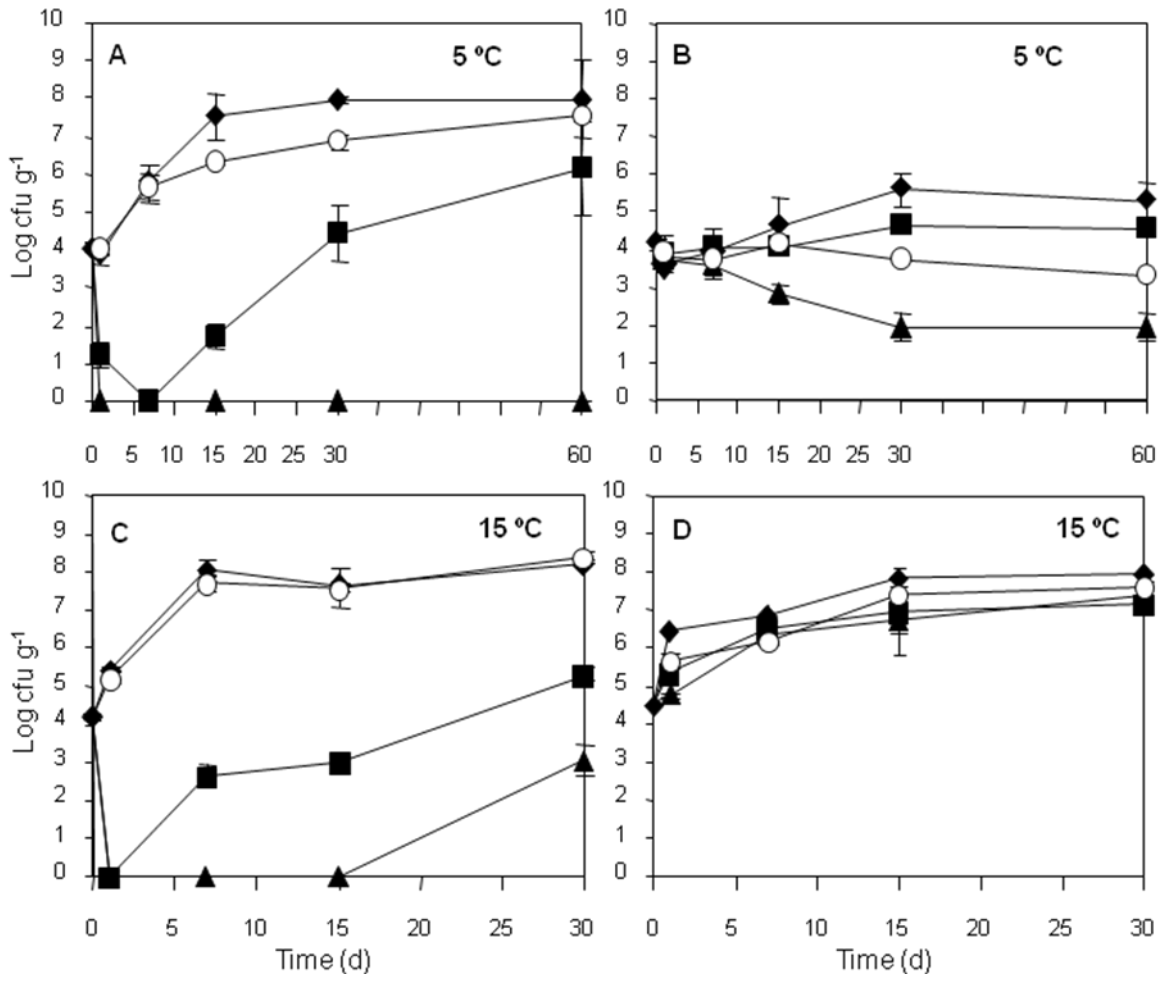

Fig. 2. Effect of enterocin AS-48 (40 µg g^{-1}) combined with nitrate/nitrite (0.007%) on the viability of *Listeria monocytogenes* CECT 4032 (A, C) and *Staphylococcus aureus* CECT 976 (B, D) in a model cooked ham stored at 5 ºC or 15 ºC. Control (♦); containing nitrate/nitrite alone (○); containing AS-48 (■);containing AS-48 and nitrate/nitrite (▲). Values are the average ± SD (error bars) of two independent experiments.

The combination of AS-48 (40 µg g^{-1}) with STPP (0.5%) had an increased bactericidal effect against listeria, causing a delay in regrowth to 15 d and a reduction of approx 4 log units in the maximum counts reached (day 60) compared to samples treated with AS-48 without the chemical (Fig. 3A). By contrast, STPP alone did not exert any inhibitory effect on *Listeria* except at the end of storage. In the case of *S. aureus,* STPP by itself had a remarkable antimicrobial effect. However, the chemical only had an additive effect in combination with AS-48, from day 15 of storage (Fig. 3B).

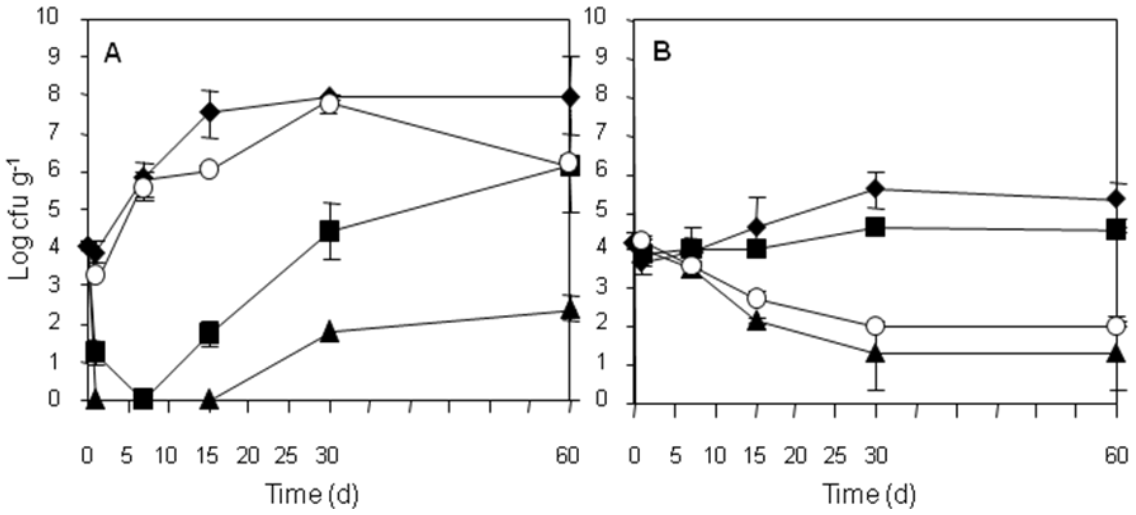

Fig. 3. Effect of enterocin AS-48 (40 µg g^{-1}) combined with sodium tri-polyphosphate (0.5%) on the viability of *Listeria monocytogenes* CECT 4032 (A) and *Staphylococcus aureus* CECT 976 (B) in a model cooked ham stored at 5 ºC. Control (♦);containing sodium tri-polyphosphate alone (○);containing AS-48 (■);containing AS-48 and STPP (▲). Values are the average ± SD (error bars) of two independent experiment.

Treatment with sodium pyrophosphate (0.15%) in combination with AS-48 (40 µg g^{-1}) against *L. monocytogenes* produced no significant differences compared to AS-48 alone (Fig. 4A). Sodium pyrophosphate by itself caused significant inhibition (P <0.01) against *S. aureus* (Fig. 4B), but no significant additional inhibition was observed in combination with AS-48 (40 µg g^{-1}). At 60 d storage, *S. aureus* counts in batches treated with AS-48 and sodium pyrophosphate were the lowest and significantly different (P <0.001) from the untreated batch (2.9 log units versus 5.2 log units in the untreated control batch). Interestingly, when a higher AS-48 concentration of 60 µg g^{-1} was assayed in combination with 0.15% sodium pyrophosphate a significantly higher (P <0.05) antilisteria effect between AS-48 and sodium pyrophosphate was noted from day 1, in which listeria counts fell bellow detection level and remained invariable along the complete storage period (Fig. 4C). This combination also had an

increased inhibitory effect against *S. aureus* from day 15, reducing viable counts below one log unit from day 30 (Fig. 4D).

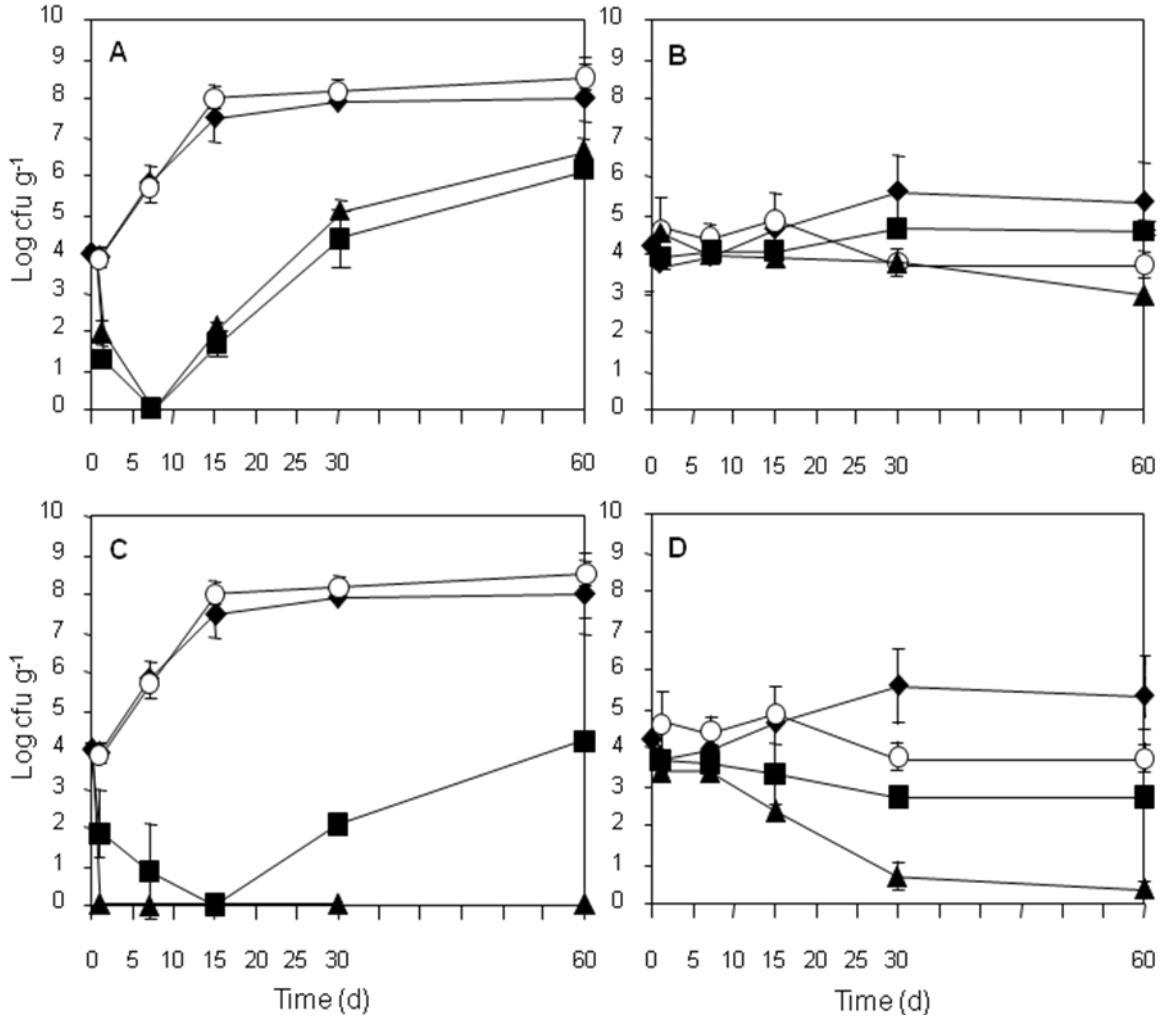

Fig. 4. Effect of enterocin AS-48 (40 µg g^{-1}, A, B) o (60 µg g^{-1}, C, D) combined with sodium pyrophosphate (0.15%) on the viability of *Listeria monocytogenes* CECT 4032 (A, C) and *Staphylococcus aureus* CECT 976 (B, D) in a model cooked ham stored at 5 ºC. Control (♦);containing sodium pyrophosphate alone (○);containing AS-48 (■);containing AS-48 and sodium pyrophosphate (▲). Values are the average ± SD (error bars) of two independent experiments.

The combination of salts of two organic acid extensively used as food preservatives, sodium acetate (0.2%) or sodium lactate (2%), with AS-48 (40 µg g^{-1}) yield an additive rather than a synergistic effect against *S. aureus* (Fig. 5A, B) from day 7 storage. For sodium acetate at day 30 the differences in the counts between the control untreated batches and the batches treated with the acid alone, AS-48 alone or both combined were of 0.80, 1 and 2.40 log units respectively. Similar results were obtained for sodium lactate, although in this case 2% lactate was more efficient than AS-48 in inhibiting *Staphylococcus.* Differences in the counts

determined in batches treated with the acid alone, AS-48 alone or both combined in relation to controls were of 2.20, 1 and 3.20 log units respectively (Fig. 5B). *Listeria* was not affected by any of these preservatives, and their respective combinations with AS-48 neither improved the activity of the enterocin alone (data not shown).

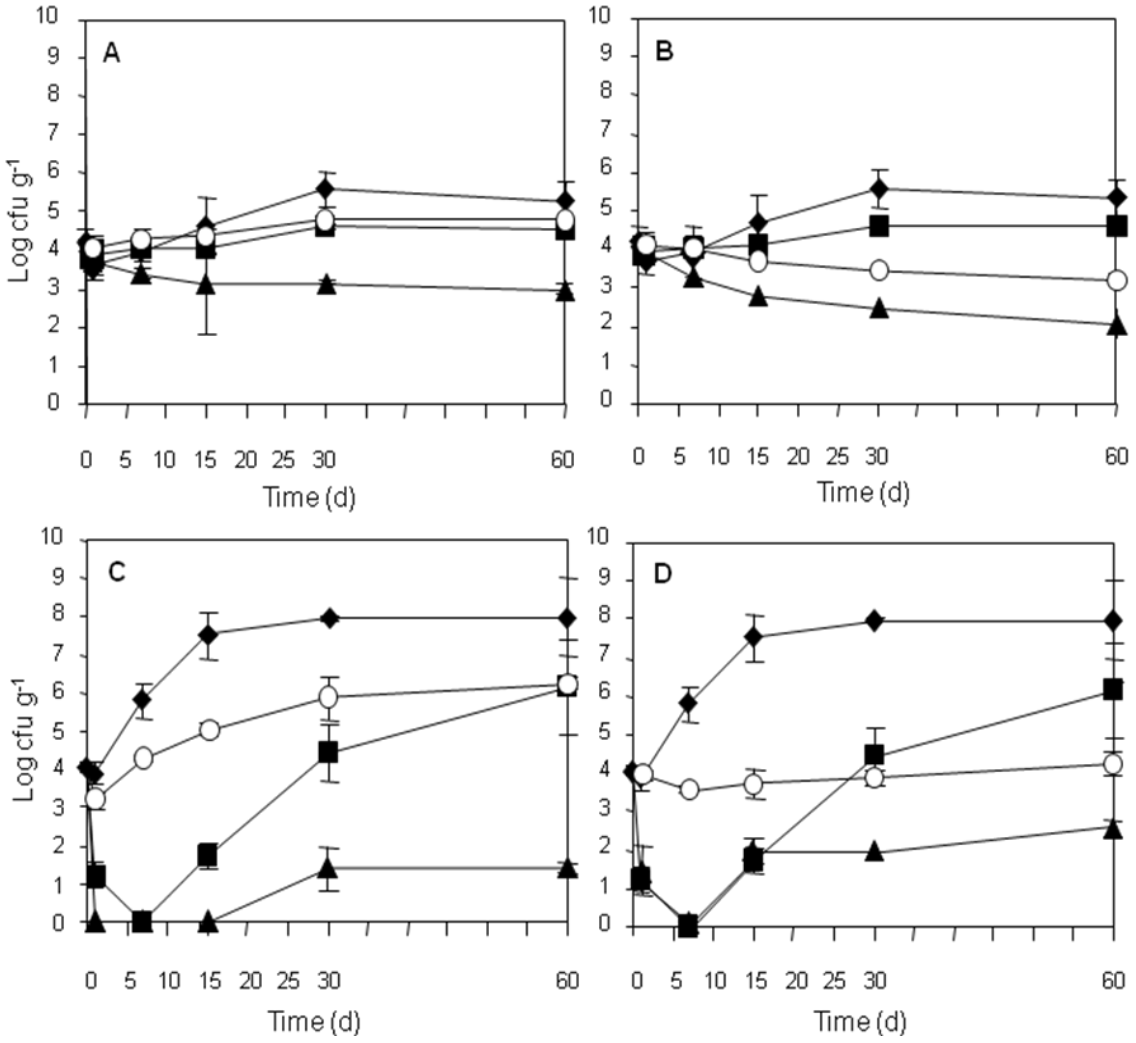

Fig. 5. Effect of enterocin AS-48 (40 µg g^{-1}) combined with sodium acetate (0.2%) (A) or sodium lactate (2%) (B) or sodium benzoate (0.1%) (C) or potasium sorbate (1%) (D) on the viability of *Staphylococcus aureus* CECT 976 (A, B) and *Listeria monocytogenes* CECT 4032 (C, D) in a model cooked ham stored at 5 ºC. Control (♦);containing chemical preservative alone (○);containing AS-48 (■);containing AS-48 and preservative (▲). Values are the average ± SD (error bars) of two independent experiments.

Combined application of sodium benzoate (0.1%) and AS-48 (40 µg g^{-1}) resulted in an increased inhibitory activity on *L. monocytogenes* from day 1 storage, when population decreased below detection values (Fig. 5C). Although listeria were able to regrow after day 15, the counts attained in the batches treated with both antimicrobials (1.4 log units) were significant

lower (P <0.05) than those of the batches treated with only one of the antimicrobials (4.40 and 5.90 log units for batches treated with AS-48 or sodium benzoate respectively). Application of sodium benzoate did not increase the inhibition of *S. aureus* (data not shown). The combination of potassium sorbate and AS-48 (40 µg g^{-1}) was less effective against *L. monocytogenes* compared to benzoate with AS-48. Nevertheless, significant notable differences in viable counts were determined in the batches treated with both compounds (2 and 2.60 log units at 30 and 60 days) with respect to those treated with only sorbate (3.85 and 4.20 log units at 30 and 60 days) or AS-48 (4.40 and 6.15 log units at 30 and 60 days) (Fig. 5D). Therefore, although the simultaneous addition of both antimicrobials did not completely avoid regrowth of listeria, it was able to control population levels at values of approx 2 log units. Regarding *S. aureus,* potassium sorbate did not increase antimicrobial activity of treatments (data not shown).

3.3. Effect of enterocin AS-48 in combination with sub-lethal heat treatment and STPP on the control of Listeria monocytogenes *and* Staphylococcus aureus

Application of sub-lethal heating at 60 ºC for 2 min in combination with AS-48 (20 µg g^{-1}) significantly (P <0.01) improved the antimicrobial effect against the two bacteria (Fig. 6). This effect was especially remarkable for *L. monocytogenes,* whose viable counts decreased below 1 log unit in day 1. Although after day 7-15 of storage *Listeria* regrows slowly, the maximum cell concentrations reached at 60 d was 3.15 log units lower than in batches not heated but treated with the same AS-48 concentration (Fig. 6A). The increase of AS-48 activity against *S. aureus* by heat was especially significant (P <0.01) after 15-30 days of storage (Fig. 6B). When the sub-lethal heat treatment was applied in combination with AS-48 (20 µg g^{-1}) and STPP (0.5%), the results improved remarkably against both bacteria with respect to the separate application of each antimicrobial compound (Fig. 6C, D). The results were particularly relevant for *S. aureus,* given the low susceptibility of this bacterium to the enterocin and the low AS-48 concentration applied.

3.4. Detection of enterocin AS-48 during cooked ham storage

In an attempt to evaluate bacteriocin stability in the cooked ham, we conducted a bacteriocin extraction experiment from meat matrix during the storage experiment. As shown in Fig. 7 at 0 time we recovered most of the 40 µg g^{-1} of AS-48 applied, 35 µg g^{-1}. Afterward the activity recovered declined gradually, and no activity was detected in samples extracted at day 30. In spite of this, in many of the experiments the inhibitory activity was maintained to the end of storage (probably due to damage caused by the bacteriocin at earlier stages).

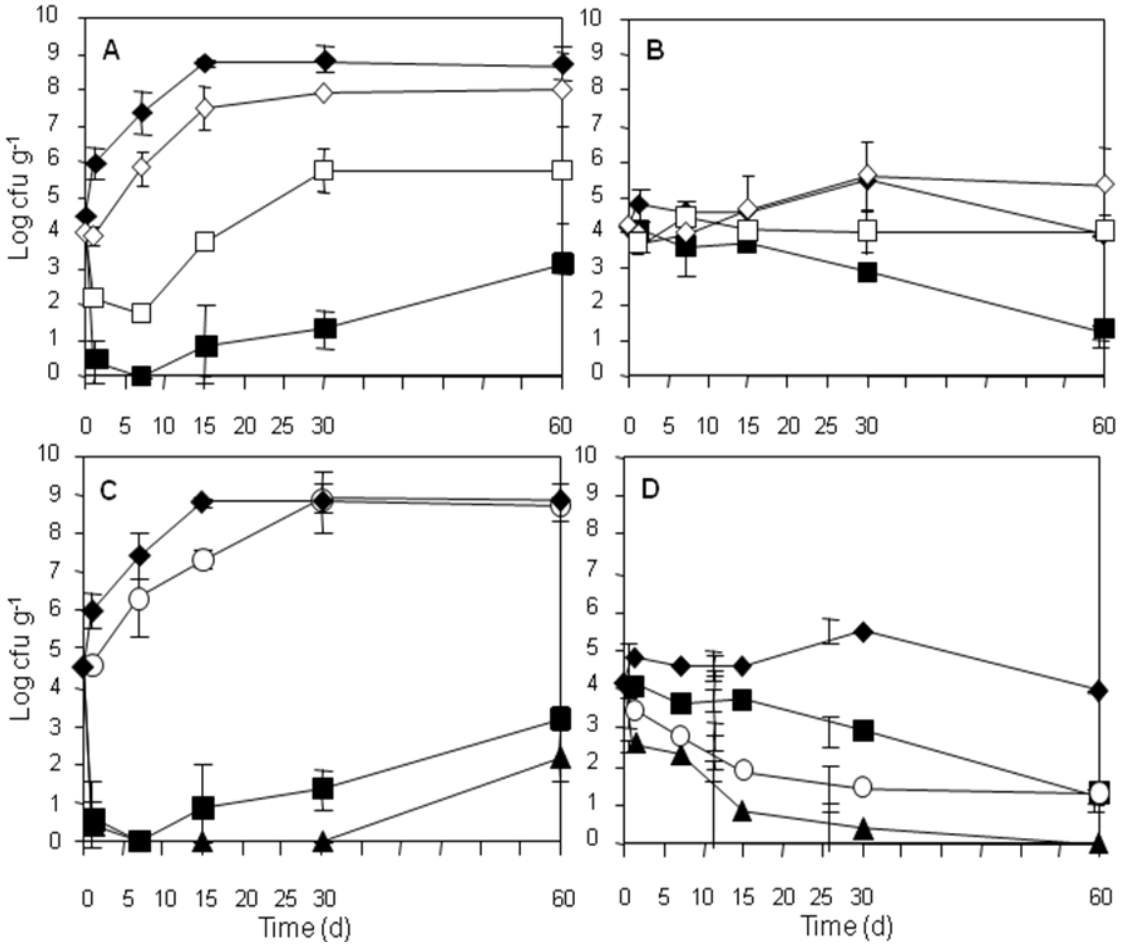

Fig. 6. Effect of enterocin AS-48 (20 µg g^{-1}) combined with heat (60 ºC, 2 min) (A, B) and heat plus STPP (0.5%) (C, D) on the viability of *Listeria monocytogenes* (A, C) and *Staphylococcus aureus* (B, D) in a model cooked ham stored at 5 ºC. A, B) control not heated (◊); control heated (♦); not heated containing AS-48 (□); heated containing AS-48 (■). C, D) control heated (♦); heated containing STPP (○); heated containing AS-48 (■); heated containing AS-48 and STPP (▲). Values are the average ± SD (error bars) of two independent experiments.

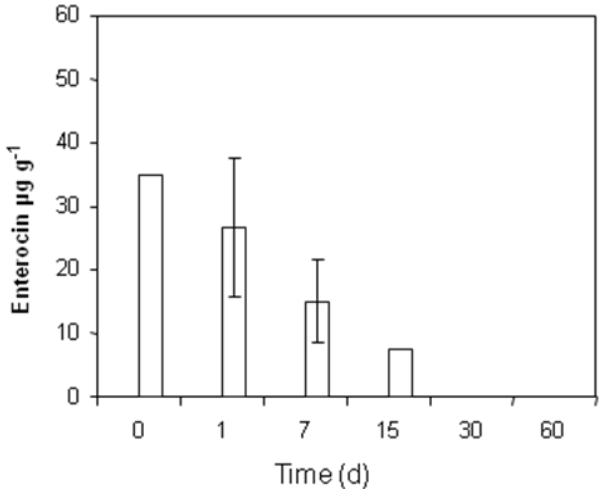

Fig. 7. Extraction of enterocin AS-48 from meat matrix. The concentration of AS-48 added to meat mixture was 40 µg g^{-1}. Values are the average ± SD (error bars) of two independent experiments.

4. *Discussion*

Application of LAB bacteriocins in foods is currently intended as a part of multiple-hurdle technology in order to decrease the efficient concentrations of bacteriocins and chemical preservatives and extend the inhibitory spectrum of bacteriocins to Gram negative bacteria, which are intrinsically resistant. The combination of several antimicrobials is also advantageous for prolonged storages specially for preventing the regrowth of *L. monocytogenes* (Cintas, Casaus, Herranz, Nes & Hernández, 2001). The objective of the present study was to test the efficacy of AS-48 by itself and in combinations with the chemical preservatives currently licensed in meat products and/or heat to control the food-borne pathogens *L. monocytogenes* and *S. aureus* in cooked ham meat model, a ready-to-eat food greatly prone to post-processing contamination due to its high water activity and neutral pH.

Previously we have demonstrated that *L. monocytogenes* was highly susceptible to AS-48 in BHI broth with a minimum bactericidal concentration of 0.1 μg ml^{-1} (Mendoza, Maqueda, Gálvez, Martínez-Bueno & Valdivia, 1999). Nevertheless, since the efficacy of bacteriocins (as the same of the majority of antimicrobial compounds) can be influenced by the chemical composition and the physical conditions of foods (Cleveland et al. (2001) it is necessary to validate the antimicrobial activity in each particular food system to establish the effective concentration and also the most adequate combination of additives or preservative treatments to be applied with the bacteriocin. In the present experiment, AS-48 effectively inhibited *Listeria* in cooked ham in a concentration dependent way at 5 ºC and 15 ºC. Nevertheless even at the higher concentration used (60 μg g^{-1}) it was not possible to avoid the regrowth of listeria after 15-30 storage days at 5 ºC. We can attribute the lower effectiveness of AS-48 in cooked ham compared to BHI broth to a higher retention of the bacteriocin molecules by meat and fat components, to a slower diffusion, and also to the irregular distribution of the bacteriocin molecules and the bacterium in the meat matrix with a higher dry matter content compared to liquid media. Also, results from bacteriocin extraction experiments revealed that bacteriocin levels in the meat decreased markedly after day 7, which could explain regrowth of surviving bacteria. Our previous experiments carried out in a sausage model demonstrated that it was necessary to apply 450 AU g^{-1} (40 μg g^{-1}) to meat mixture to maintain *Listeria* below detection level from 6 d to 9 d incubation at 20 ºC (Ananou et al., 2005b). Nevertheless, the formulation of sausages included, as usual, nitrate/nitrite and sodium pyrophosphate and incubation time was limited to 9 days. Previous experiments carried out by immersion of vegetable foods in solutions containing enterocin AS-48 (12.5-25 μg ml^{-1}) alone or in combination with chemical preservatives showed that

antilisteria effect of enterocin varied according to the type of vegetable and also the storage temperature, with higher efficacy at 15ºC compared to 6ºC (Cobo Molinos et al., 2005). Further experiments carried out in fruits and fruit juices have shown that the antilisteria effect of AS-48 (25 µg ml^{-1}) depended on the fruit type and was much more effective in juices, probably due to the more homogeneous distribution of the enterocin in the liquid food matrix. In this case AS-48 showed also to be in general more effective at low temperatures (6ºC to 20ºC) (Cobo Molino, Abriouel, Ben Omar, Lucas, Valdivia & Gálvez, 2008).

Since it was not possible to avoid regrowth of *Listeria* during storage of cooked ham neither at 5ºC or 15 ºC, we tested combined treatments of AS-48 and different chemical preservatives. The compounds selected are additives licensed by European Parliament and Council Directives on Foods Additives (95/2/EC, 96/85/EC, 98/72/EC) for use in meat products and they have been used at concentrations equal or lesser to those licensed. The most effective combination was AS-48-nitrite/nitrate (0.007%), which reduced listeria below detection level from the first sampling. This is a relevant result since this compound had no effect or almost no effect on viable counts of listeria at this concentration, a half or quarter of the 0.03-0.015% licensed by EC Directives and they are extensively applied as preservatives in most of meat products. To a lesser extent, other combinations of AS-48 (40 µg g^{-1} with other preservatives as STPP, sodium benzoate or potassium sorbate) were also effective in reducing *Listeria* during storage at 5ºC. It is worth noting that although sodium pyrophosphate did not increased the activity of AS-48 at a concentration of 40 µg g^{-1}, the combination of the chemical with a higher AS-48 concentration of 60 µg g^{-1} decreased listeria counts below detection level, thus indicating that it is possible to achieve a complete inactivation of *Listeria* with other more effective combination (e.g. STPP, sodium benzoate or potassium sorbate) by increasing enterocin concentration. The efficacy of chemical preservatives in increasing the antilisteria activity of AS-48 was independent of their measurable anti-listeria activity since, in fact, only sodium benzoate (0.1%) and potassium sorbate (1%) had a clear inhibitory effect on the bacterium. The combined effect of AS-48 and several of the antimicrobial compounds assayed in the present study have been previously tested for *Listeria* inactivation on vegetable foods (Cobo Molino et al., 2005, 2008). The results derived from this study are in general coherent with those presented here with an important exception. So, an improvement in the inactivation of *Listeria* on the surface of vegetable was obtained by combining AS-48 and sodium nitrite, sodium nitrate, chelating agents as trisodium phosphate or trisodium trimetaphosphate, or potassium sorbate. Nevertheless one of the more effective compound in the combined treatments of raw vegetables, sodium lactate, was inefficient when combined with AS-48 in increasing the inhibition of *Listeria* in cooked ham. In fact sodium lactate by itself did not have any adverse

effect on *Listeria.* The antilisteria effect of nisin at 6ºC also has been reported in cooked ham by Jofré, Garriga & Aymerich, (2007). However, after an immediate bactericidal effect in the first day of storage, *L. monocytogenes* rapidly regrew to reach viable counts similar to those of the control. In this case, although the combination with sodium lactate (1.8%) did not eliminate the listeria, it reduced the population levels by at least one log below initial values (approx 4 unit log) along 75 day storage. These results emphasise the needs to test the effectiveness of the enterocin in each food system to establish the precise mode and concentration to be applied.

Another interesting result from this study is the synergy in the antilisteria action between AS-48 and heat. This fact is of technological relevance in cooked products in which AS-48 can represent an additional hurdle to prevent failures in the homogeneous distribution of heat through the whole meat mixture.

In previous experiments we have demonstrated the susceptibility of *S. aureus* to AS-48 in BHI broth, a sausage model system, milk and cheese and in vegetable sauces (Ananou, Valdivia, Martínez-Bueno, Gálvez & Maqueda, 2004; Muñoz et al., 2007; Grande et al., 2007b). As a general rule the AS-48 concentration required to inhibit *S. aureus* CECT 976 in foods is much higher compared with the minimal bactericidal concentration of 15 µg/ml established in BHI broth. E.g. in milk, 50 µg/ml of AS-48 alone was not sufficient to eliminate by complete *S. aureus* CECT 976. We have also proved the effect of combining different antimicrobial treatments with AS-48 on its antistaphylococcal activity. In BHI broth, the combination of AS-48 with sublethal heat achieved a reduction in the minimal bactericidal concentration (Ananou et al., 2004). In vegetable sauces antistaphylococcal activity of AS-48 was significant improved when the enterocin was used in combination with different phenolic compounds and even some of the combinations of enterocin AS-48 and phenolic compounds served to completely inactivate *S. aureus* in sauces. Nevertheless the effect depended largely on the type of food, which in turn had a great influence on the activity of AS-48 as well as the phenolic compounds tested individually (Grande et al., 2007b). The storage temperature was also an important factor in the inhibition of *S. aureus* by AS-48 in sauces being more effective at high (22 ºC) than at low (10 ºC) storage temperatures.

In the present study, in cooked ham, AS-48 applied alone had a slight inhibitory effect on *S. aureus* and yet the higher concentration applied (60 µg g^{-1}) was unable to eliminate the staphylococci. Contrary to the results obtained in vegetable sauces the anti-staphylococci effect of AS-48 was much lesser at 15 ºC. Although neither of the combined treatments of AS-48-chemical preservatives achieved the complete inactivation of *S. aureus* it is

noteworthy that most of them (nitrite/nitrate, STPP, sodium lactate and sodium acetate) improved the inhibitory effect of enterocin. The best results were attained for the combination of AS-48 with nitrate/nitrite or STPP respectively. Interestingly, as the same for *Listeria,* the combination of sodium pyrophosphate with a higher AS-48 concentration of 60 µg g^{-1} decreased the staphylococci counts greatly, especially from the interval 15-30 d when counts decreased to bellow one log unit. A remarkable result is that whereas in untreated controls staphylococci reached viable counts of approx 5.5 log units, counts in the batches treated with both compounds were equal or below 2 log units. Nevertheless, the fact that staphylococcal intoxication results from population levels greater than 10^5 *S. aureus* CFU per gram of contaminated food (Anonymous, 1992) would not make *S. aureus* a major concern if food products are added of AS-48 and properly refrigerated. In their study Jofré et al., (2007) did not achieve a noticeable reduction of *S. aureus* by addition of nisin, lactate or both neither at 6ºC nor 1ºC. Also in this case sublethal heat had an enhancer effect, especially potent in the triple treatment AS-48/heat/STPP. We have described previously the strong anti-staphylococci synergistic effect between AS-48 and a moderate heat treatment (65ºC 5 min) in milk, that lowered to 20 µg ml^{-1} the minimal bactericidal concentration. In the present case the synergistic effect between AS-48 and heat was less remarkable, probably due to the lower temperature applied.

The observed synergy between AS-48 and chemical preservatives and heat can be explained by the fact that bacterial cells sub-lethally injured by different stressing conditions become sensitive to different physical and chemical agents to which healthy cells are resistant (Kalchayagand, Hanlin & Ray, 1992). Anyway, the synergistic effect observed in this complex solid environment was lower compared to previous results obtained in liquid media for AS-48 (Ananou et al., 2004; Muñoz et al., 2007; Grande et al., 2008) reinforcing the suggestion that interaction of AS-48 with food components can interfere with its efficacy.

The general conclusion of this study is that the use of AS-48 alone or combined with other hurdles, such as nitrate/nitrite, STPP and pyrophosphate and/or heat, is an efficient approach to provide an effective protection against *L. monocytogenes* and, to a lesser extent, against *S. aureus,* improving the safety of non-fermented meat foods such as cooked ham. Therefore, according to the present and previous results, the food composition seems to play a key role on the final effect of antimicrobials and AS-48 against both bacteria. Because of this, the adequate combination of hurdles will be dictated by the specific type of food to be preserved.

Acknowledgements

This work was supported by grant (AGL2005-07665-C02-01) from Spanish DGICYT. Samir Ananou received several grants from the Junta de Andalucía (PAI Research Group BIO 160). We would also like to acknowledge the work of Christine Laurin in editing the text.

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I.1.b. Prevention of spoilage by enterocin AS-48 combined with chemical preservatives, under vacuum, or modified atmosphere in a cooked ham model.

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Abstract

The effect of enterocin AS-48 in controlling *Lactobacillus sakei, Brochothrix thermosphacta*, and *Staphylococcus carnosus* in a cooked ham system has been studied. AS-48 alone showed activity against *Lactobacillus*, with 60 µg g^{-1} reducing lactobacilli below detection levels from the beginning to end of storage at 5 ºC. Combinations of 40 µg g^{-1} AS-48 with nitrate/nitrite, pentasodium tripolyphosphate, sodium pyrophosphate, sodium acetate, and sodium lactate reduced *L. sakei* below detection levels from the beginning to end of storage. Even 20 µg g^{-1} of enterocin combined with tripolyphosphate permanently eliminated *L. sakei.* Enterocin AS-48 (40 µg g^{-1}) was also active against *B. thermosphacta* and *S. carnosus*, reducing both bacteria by more than 3 log in the cooked ham. Modified atmosphere (40% CO_2/60% N_2) packaging affected neither bacterial growth nor AS-48 activity against any of the three bacteria. In contrast, storage under vacuum remarkably increased the growth and the inhibitory activity of the enterocin against *B. thermosphacta* and especially against *L. sakei*.

Keywords: *Biopreservation, enterocin AS-48, Lactobacillus sakei, Staphylococcus carnosus, Brochothrix thermosphacta, cooked ham.*

Published in Food Control. Volume 24, Issue 1-2, March–April 2012, Pages 15–22.

1. Introduction

Meats and meat products are good media for the growth of microorganisms. Spoilage in meats by mesophilic or psychrotrophic microorganisms can occur at room temperature or during cold storage of the products. In this respect, cooked ham, due to its characteristics (low salt content of approx. 2 %, pH near 6, and $a_w > 0.945$), is more prone to spoilage than many other meat products. In addition, in cooked ham marketing, ham is sliced and packaged, and then it can become contaminated with spoilage and pathogenic microorganisms and consequently have its shelf-life greatly shortened.

Lactic acid bacteria (LAB) have been found to be major contributors to meat-foods spoilage, with *Lactobacillus* spp. being of special concern (Borch, Kant-Muemansb, & Blixt, 1996; Nychas, Marshall, & Sofos, 2007). Although many strains of *L. sakei* are often used as starters in cured meats, some strains are described as responsible for meat spoilage due to ropy slime production (Aymerich, Garriga, Costa, Monfort & Hugas, 2002; Najjari, Ouzari, Boudabous, & Zagorec, 2008). *B. thermosphacta* is also an important meat and fish spoilage bacterium able to grow both under aerobic and anaerobic conditions (Borch et al., 1996; Holley, 2000). Meats spoiled by *B. thermosphacta* develop an offensive, sour-sweet odor associated mainly with acetoin (Pin, García de Fernando, & Ordóñez, 2002). Staphylococci are microorganisms commonly found on fresh, processed, and even vacuum-packaged meats through contamination from their more common habitat, the skin of animals (Nychas et al., 2007); *S. carnosus* is amongst the most frequent species of coagulase negative staphylococci associated with meat products (Papamanoli, Kotzekidou, Tzanetakis, & Litopoulou-Tzanetaki, 2002). One strategy in food preservation, designed as biopreservation, is based on the use of microorganisms and/or their natural products. Amongst the antimicrobial substances produced by LAB are the antimicrobial peptides known as bacteriocins. The application of LAB bacteriocins in food technology is currently intended through the combination of these antimicrobials with physical and chemical treatments (Gálvez, Abriouel, Lucas-López & Ben Omar, 2007). This approach can overcome major challenges for the present-day food industry by mitigating economic losses due to food spoilage and avoiding the transmission of microbial pathogens through the food chain. At the same time, it has the potential of satisfying growing consumer demand for foods that are ready to eat, fresh-tasting, nutrient and vitamin rich, and minimally-processed.

Enterocin AS-48 is a cationic circular bacteriocin produced by *Enterococcus faecalis* S-48 with broad bactericidal activity against most Gram-positive bacteria, including several pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Mycobacterium* spp.,

Bacillus cereus, and some Gram-negative bacteria (Abriouel, Maqueda, Gálvez, Martínez-Bueno, & Valdivia, 2002; Abriouel, Valdivia, Gálvez, & Maqueda, 1998; Gálvez, Maqueda, Martínez-Bueno, & Valdivia, 1989). The features of AS-48 (a broad spectrum of antimicrobial activity, stability across a wide range of temperatures and pH, and sensitivity to digestive proteases) (Gálvez, Maqueda, Valdivia, Quesada, & Montoya, 1986; Samyn et al., 1994) make it a promising alternative to chemical preservatives for use as a biopreservative in foods. In fact, AS-48 has been shown to be effective in the control of various food-borne pathogens in dairy, meat, and vegetable products (Ananou, Baños, Maqueda, Martínez-Bueno, Gálvez, & Valdivia, 2010; Cobo Molinos, Abriouel, Lucas López, Ben Omar, Valdivia, & Gálvez, 2008; Muñoz et al., 2007) and also against several spoilage bacteria in vegetable and vegetable-derived foods (Grande et al. 2006; Grande et al., 2007). The purpose of the present work was to test the efficacy of AS-48, applied alone or in combination with licensed chemical preservatives or vacuum or modified atmosphere (MA) packaging, in the control of *L. sakei, B. thermosphacta*, and *S. carnosus,* in a cooked ham meat system.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Enterococcus *faecalis* A-48-32 (Martínez-Bueno, Gálvez, Valdivia, & Maqueda, 1990) was used as an AS-48 producer. *E. faecalis* S-47 from our collection was used as the standard indicator strain for bacteriocin activity assays. *L. sakei* CTC 245 was obtained from the IRTA (Institute for Agro-Food Research and Technology, Monells, Girona, Spain) collection. *S. carnosus* CECT 4491 and *B. thermosphacta* CECT 847 were supplied by the Spanish Type Culture Collection (CECT). For meat inoculation, *S. carnosus* and *B. thermosphacta* were grown overnight on brain heart infusion (BHI) and *L. sakei* on Man Rogosa Sharpe broth (MRS, Scharlau, Barcelona, Spain) at 28 ºC, washed in a sterile saline solution, and then inoculated in the meat mixture at the selected concentration. All strains were cultivated routinely on BHI at 28 ºC, and stored at 4 ºC on BHI-agar slants.

2.2. Preparation of bacteriocin AS-48

AS-48 was produced by culturing the strain *E. faecalis* A-48-32 in a food-grade whey-derived substrate, Esprion 300 (E-300) (DMV Int., Veghel, Netherland), supplemented with 1% glucose as described by Ananou, Muñoz, Gálvez, Martínez-Bueno, Maqueda, & Valdivia, 2008.

2.3. Manufacture of cooked ham

The cooked ham was prepared with lean pork meat. The meat was coarsely ground through a 12 mm plate and brined. The brine solution contained (in g Kg^{-1}): NaCl, 12; sodium ascorbate, 0.30; sodium phosphate, 2; sodium glutamate, 1 (all from Scharlau); and water, 30. The ingredients were homogenized by hand during 5 min and the mix was divided into two batches, to one of which was added AS-48. Then both batches were cooked in a bath until the internal temperature reached 65 ºC and then inoculated with approximately 10^4 CFU g^{-1} of the selected bacteria (*L. sakei* CTC 245, *S. carnosus* CECT 4491, and/or *B. thermosphacta* CECT 847) and subdivided into four batches: A) a control batch; B) a batch to which was added AS-48 (20, 40, or 60 µg g^{-1}); C) a batch with added different preservatives (only in the case of *L. sakei* experiments): pentasodium tripolyphosphate 0.5% (STPP, E-451i), sodium nitrate/nitrite 0.015 or 0.007% (E-251/E-250), sodium pyrophosphate 0.15% (E-450i), sodium acetate 0.2% (E-262), sodium lactate 2% (E-325), potasium benzoate 0.1% (E-211), potasium sorbate 1% (E-202); D) a batch to which both AS-48 and preservative were added. Although cooked ham is usually manufactured with nitrate/nitrite as a preservative in the brine solution, in our experiments we eliminated this preservative with the aim of studying the precise effect of the combination of AS-48 with each specific compound, used individually. The pH of the final product in all batches was 6.0-6.2. All the batches were subdivided into two portions and stored in sterile screw cap bottles at 5 ºC or, exceptionally, at 15 ºC. Two complete, independent experiments were carried out. Preservatives were from Sigma (*Sigma*-Aldrich Chemie Gmbh, Steinheim, Germany).

For *L. sakei* sublethal heat treatments, two batches were separated, one from the meat mixture with added AS-48 (20 µg g^{-1}) and the other without AS-48. Afterwards, each batch was divided into a further two batches. One batch of each type of meat mixture was heat-treated (60ºC for 2 min each) to produce a sublethal cell injury to *L. sakei* (killing approx. 99%). Finally, four independent batches were established: an unheated control batch without AS-48, a second unheated batch containing enterocin AS-48, a third batch with sublethal heat without AS-48, and a final batch heated and containing AS-48 (20 µg g^{-1}). Two independent experiments were carried out.

2.4. Packaging conditions

To investigate the influence of packaging conditions on the antimicrobial effect of AS-48 (40 µg g^{-1}) against the spoilage bacteria in cooked ham, samples with and without AS-48 were stored in polyamide-polyethylene plastic bags under different conditions: atmospheric,

vacuum, or MA (40% CO_2/60% N_2) at 5 ºC for 60 d. Vacuum and MA conditions were achieved with a Tecnotrip EVT-10-2-CD-SC (Barcelona, Spain) machine. All samples were stored at 5 ºC for 60 d.

2.5. Microbiological sampling and analysis

Samples from each treatment were withdrawn in duplicate at selected times (0, 1, 7, 15, 30, and 60 days) to determine viable counts of *L. sakei*, *S. carnosus*, and *B. thermosphacta.* For the microbiological determinations, 10 g were aseptically removed and mixed (1:10) with dilution medium (0.1% peptone, 0.85% NaCl). Homogenization was done in a Masticator blender (IUL, Barcelona, Spain) for 1 min followed by serial 10-fold dilution and plating on the respective selective media: Man Rogosa Sharpe agar plus 0.04% sodium azide for *L. sakei*, incubated under anaerobic conditions, mannitol salt agar (MSA, Scharlau) for *S. carnosus* and STAA agar (Oxoid LTD, Basingstoke, Hampshire, England) with added STAA supplement (Oxoid) for *B. thermosphacta,* both incubated under aerobic conditions. All bacteria were incubated at 28 ºC for 48–72 h before counting the colonies.

2.6. Bacteriocin extraction

Bacteriocin was extracted at selected times from cooked ham (as described by Garriga, Aymerich, Costa, Monfort, & Hugas, 2002). Briefly, cooked ham samples were homogenized (1:10) in sodium acetate 50 mM, EDTA 100 mM, and Triton X 100 0.2% at pH 5 in a blender for one min, boiled for 10 min, cooled, and filtered through a filter paper. The bacteriocin in the liquid phase was precipitated with 300 g L^{-1} ammonium sulphate and the pellet was dissolved in phosphate buffer (50 mM, pH 7.2). The sample was heated at 80 ºC for 10 min and then the bacteriocin titre of extracts in arbitrary units (AU) was determined by the agar well diffusion method (Gálvez et al., 1986) to obtain the approximate concentration of AS-48 (in µg g^{-1}).

2.7. Statistical analyses

The average data from duplicate trials ± standard deviations were determined with the Excel program (Microsoft Corp., USA). Statistical analyses were performed using the SPSS-PC 15.0 software (SPSS, Chicago, Ill. USA). Data on microbiological counts were subjected to ANOVA, using the presence of AS-48, the presence of chemical preservatives, or the combination of both as factors. Categories include untreated control ham, ham with added enterocin, ham with preservative(s), or ham with both AS-48 and preservative(s). The

Mean Square Difference was used as a post-hoc test to determine significant differences between ham manufactured with enterocin AS-48, chemical preservatives, or both.

3. Results

3.1. Effect of enterocin AS-48 alone on the control of L. sakei

L. sakei was able to grow in the cooked ham system at 5 ºC and to reach maximum levels of 5 log units (Fig 1A). *Lactobacillus* was very concentration-dependent to AS-48 (20, 40, or 60 µg g^{-1}) when applied in solitary. At 60 µg g^{-1}, the enterocin reduced the counts of lactobacilli below detection levels (<10 CFU g^{-1}) for the entire storage period (1 to 60 d). With the three enterocin concentrations assayed, the antimicrobial effect was significant (P< 0.05) from the first week of storage. The effect of 40 µg g^{-1} of AS-48 was particularly relevant in the first 7–15 days of storage, during which *Lactobacillus* counts remained below detection values, which represented reductions of 4.5 and 5 log units at 7 and 15 d with respect to untreated controls at the same times (Fig. 1A). Although overgrowth of *Lactobacillus* was observed after 15–30 days at this AS-48 concentration, the counts at 60 d of storage were 2.8 log units lower than in untreated batches.

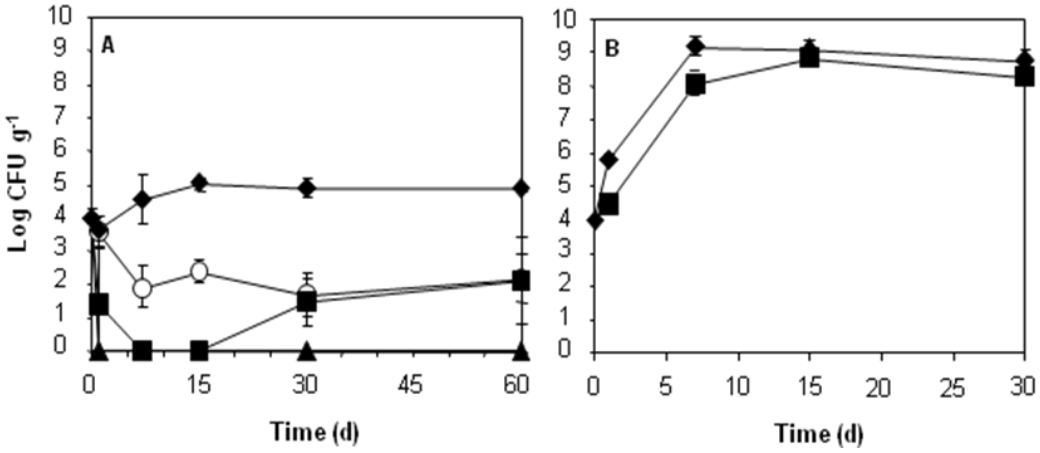

Figure 1. Effect of enterocin AS-48 on the viability of *Lactobacillus sakei* CTC 245 in a cooked ham model stored at 5 ºC (A) or 15 ºC (B). Control (♦); containing AS-48 at a concentration of 20 (○), 40 (■), or 60 (▲)µg g^{-1}. Values are the average ± SD (error bars) of two independent experiments.

We also tested the efficacy of AS-48 (40 µg g^{-1}) to control *L. sakei* at 15 ºC. As expected, *Lactobacillus* growth increased at this higher temperature, reaching 9.2 log units at 7 d versus the 4.5 log units reached at 5 ºC (Fig. 1A, B). Although added AS-48 (40 or 60 µg

g^{-1}) exerted some inhibitory effect on *L. sakei*, mainly in the first 7 d, it was not able to control further growth, reaching cell densities of 8.1 logs at 7 d and 8.3 logs at 30 d.

3.2. Effect of enterocin AS-48 in combination with chemical preservatives and heat on the control of L. sakei

The combinations of AS-48 (40 µg g^{-1}) with sodium lactate (2%), sodium acetate (0.2%), sodium pyrophosphate (0.15 %), STPP (0.5%), and nitrate/nitrite (0.007% and 0.015%) had a clear and significant effect (P<0.05 for the combination AS-48/nitrate/nitrite and P<0.01 for the remaining combined treatments) in controlling *L. sakei* in the cooked ham from the beginning of storage (Fig 2A–F). In batches treated with the combinations of AS-48 and sodium lactate and sodium acetate, lactobacilli counts remained below detection levels from the first day of storage. Combinations of sodium pyrophosphate and STPP attained similar inhibitory results from 1–7 d of storage. Nitrate/nitrite (0.007% and 0.015%) were slightly less effective. Although lactobacilli levels remained close to or below detectable levels from 1–7 days of storage, a slight overgrowth was observed in samples treated with AS-48 and nitrate/nitrite 0.007% from 30 d. All the chemical preservatives had a moderate effect on the growth of *Lactobacillus*, achieving reductions in viable counts ranging from 1 to 2.3 log units from day 7 in most cases (Fig. 2A–F).

Interestingly, when STPP (0.5%) was applied in combination with 20 µg g^{-1}, viable counts of lactobacilli decreased below 1 log unit in days 1–7 and remained undetectable for the remaining storage period (Fig. 3). In contrast, the combined application of sodium benzoate (0.1%) or potassium sorbate (1%) with AS-48 resulted in interference with the *Lactobacillus* inhibition caused by the enterocin since AS-48 was more effective applied in solitary than combined with either of the two above chemicals (results not shown). Sublethal heat treatment of *L. sakei* had no effect on the antimicrobial activity of AS-48 against this bacterium (results not shown).

3.3. Effect of enterocin AS-48 on the control of B. thermosphacta and S. carnosus

B. thermosphacta and *S. carnosus* were also able to grow in the cooked ham system and to reach maximums of 5.45 and 4.35 log units respectively (Fig. 4). Both bacteria were also very sensitive to AS-48 (40 µg g^{-1}). The antimicrobial effect of AS-48 was significant

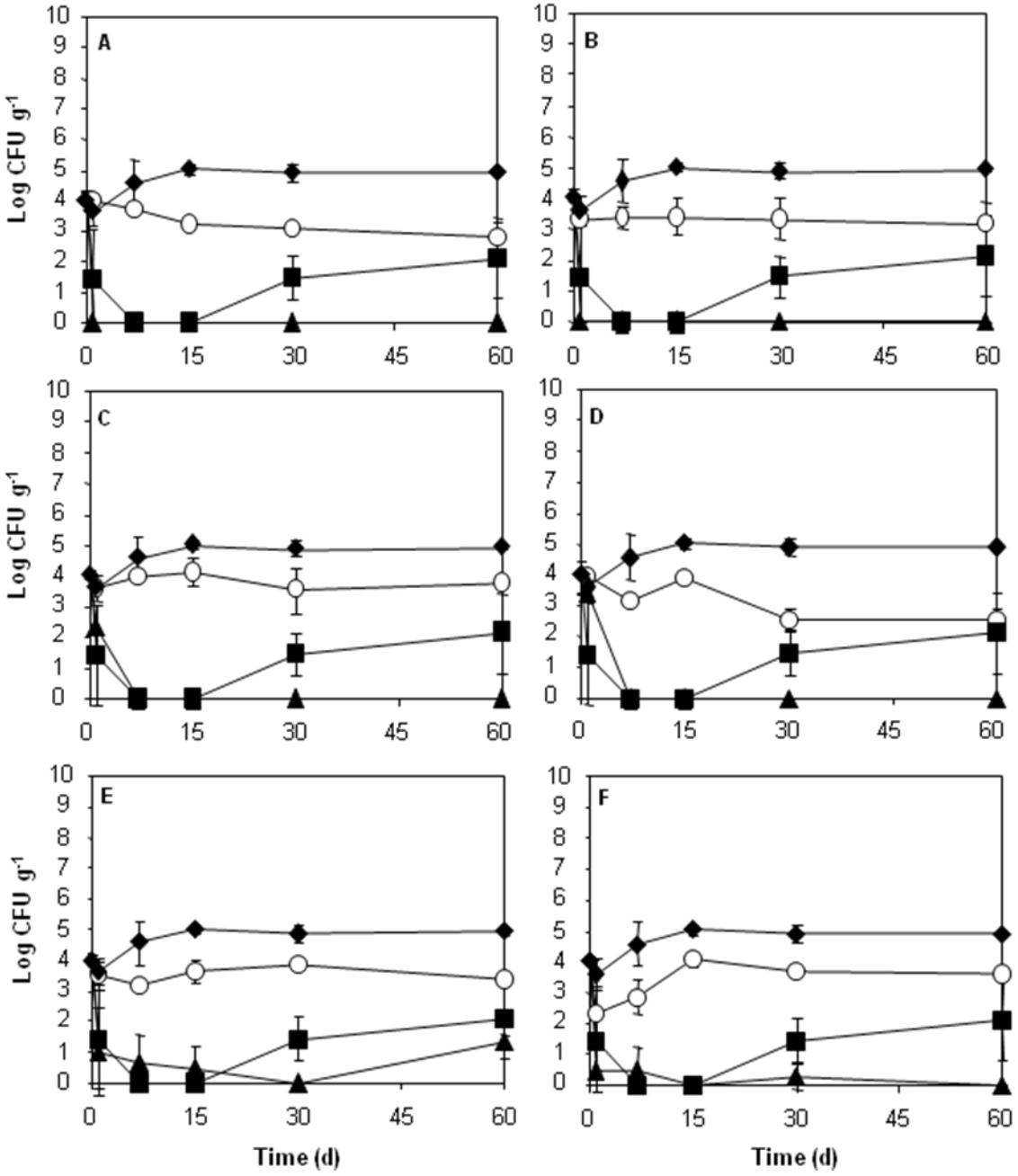

Figure 2. Effect of enterocin AS-48 (40 µg g^{-1}) combined with different chemical preservatives on the viability of *Lactobacillus sakei* CTC 245 in a cooked ham model stored at 5 ºC. Control (♦); containing AS-48 (■); containing preservative alone (○); containing AS-48 and preservative (▲). A) sodium lactate 2%; B) sodium acetate 0.2%; C) sodium pyrophosphate 0.15%; D) STPP 0.5%; E) nitrate/nitrite 0.007%; F) nitrate/nitrite 0.015%. Values are the average ± SD (error bars) of two independent experiments.

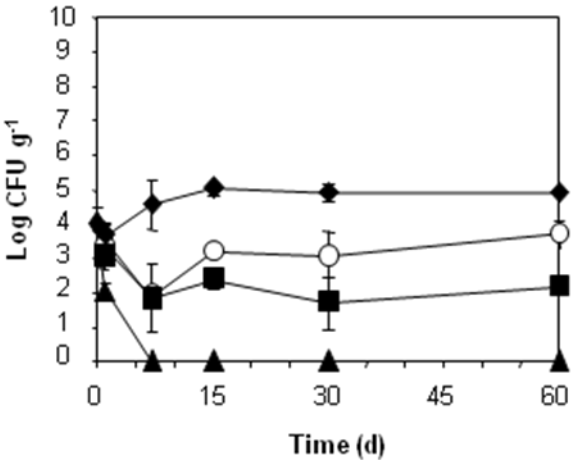

Figure 3. Effect of enterocin AS-48 (20 µg g^{-1}) combined with STPP (0.5%) on the viability of *Lactobacillus sakei* CTC 245 in a cooked ham model stored at 5 ºC. Control (♦); containing AS-48 (■); containing with STPP (○); containing AS-48 and STPP (▲). Values are the average ± SD (error bars) of two independent experiments.

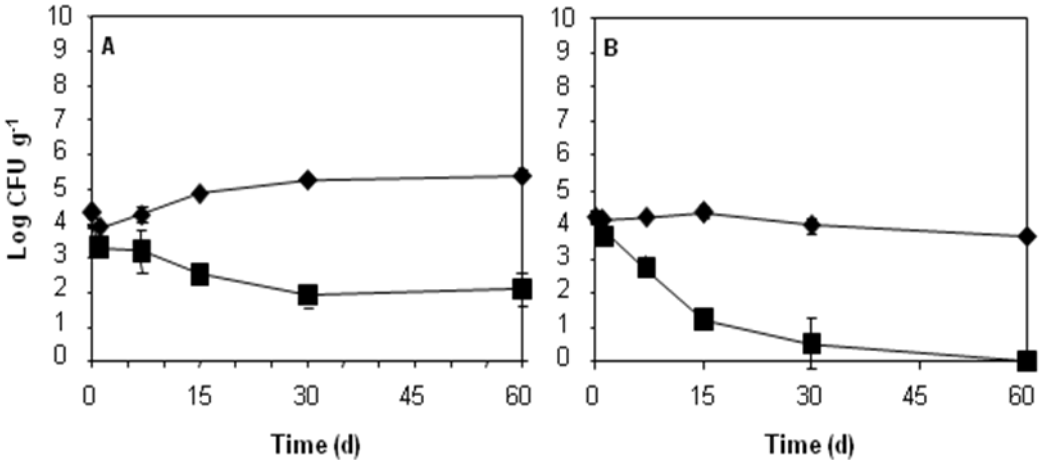

Figure 4. Effect of enterocin AS-48 (40 µg g^{-1}) on the viability of *Brochothrix thermosphacta* CECT 847 (A) and *Staphylococcus carnosus* CECT 4491 (B) in a cooked ham model stored at 5 ºC. Control (♦); containing AS-48 (■). Values are the average ± SD (error bars) of two independent experiments.

(*P*< 0.01) from the first week of storage, reaching reductions of 1 and 1.5 for *B. thermosphacta* and *S. carnosus* at 7 d respectively (Fig. 4A, B). The pattern of AS-48 susceptibility throughout the 60 d storage period at 5 ºC was very similar for *B. thermosphacta* and *S. carnosus,* although *S. carnosus* showed a greater sensitivity to AS-48 than *Brochothrix. B. thermosphacta* counts were 3.2, 2.5, 1.9, and 2.1 log units at 7, 15, 30, and 60 d, respectively (Fig. 4A) in AS-48 treated batches. The counts for untreated ham were 4.3, 4.9, 5.3, and 5.4 log units at the same storage times (significantly different at *P*< 0.05). For staphylococci, counts decreased to 2.7, 1.2, and 0.5 log units at 7, 15, and 30 d

respectively. At 60 d, *S. carnosus* counts were lower than detectable value in AS-48 treated ham. In contrast, in untreated batches staphylococci counts were 4.2, 4.4, 3.9, and 3.6 log units at the same time intervals (significantly different at $P< 0.05$) (Fig. 4B).

3.4. Effect of enterocin AS-48 in combination with vacuum or modified atmosphere (40% CO_2:60% N_2) on the control of L. sakei*,* B. thermosphacta*, and* S. carnosus

No significant differences ($P> 0.05$) were observed in the counts of the three bacteria between the samples treated with AS-48 stored under normal atmosphere and the samples treated with AS-48 and packaged under MA (Fig. 5). The growth of the three bacteria under MA was very similar to growth under normal atmosphere.

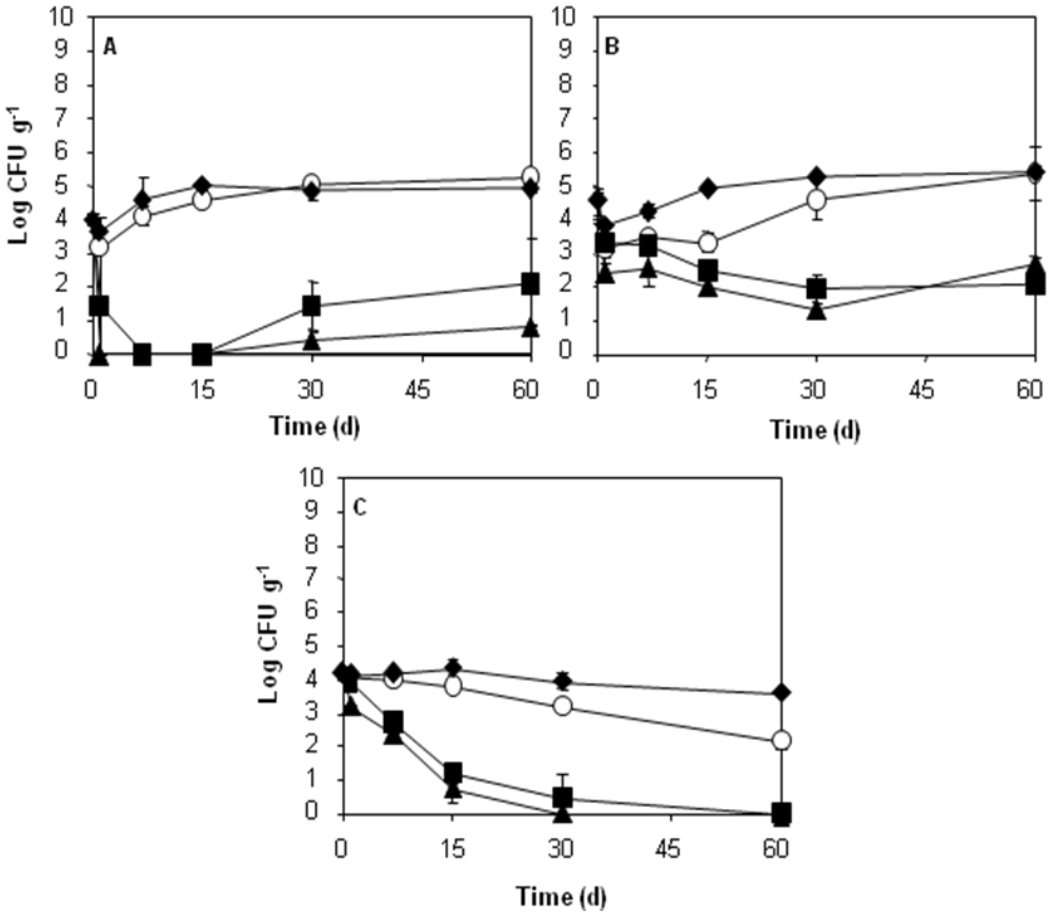

Figure 5. Effect of enterocin AS-48 (40 µg g^{-1}) combined with modified atmosphere (40% CO_2:60% N_2) packaging on the viability of *Lactobacillus sakei* CTC 245 (A), *Brochothrix thermosphacta* CECT 847 (B), and *Staphylococcus carnosus* CECT 4491 (C) in a cooked ham model stored at 5 ºC. Control stored under normal atmosphere (♦); containing AS-48 stored under nomal atmosphere (■); control stored under modified atmosphere (○); containing AS-48 stored under modified atmosphere (▲). Values are the average ± SD (error bars) of two independent experiments.

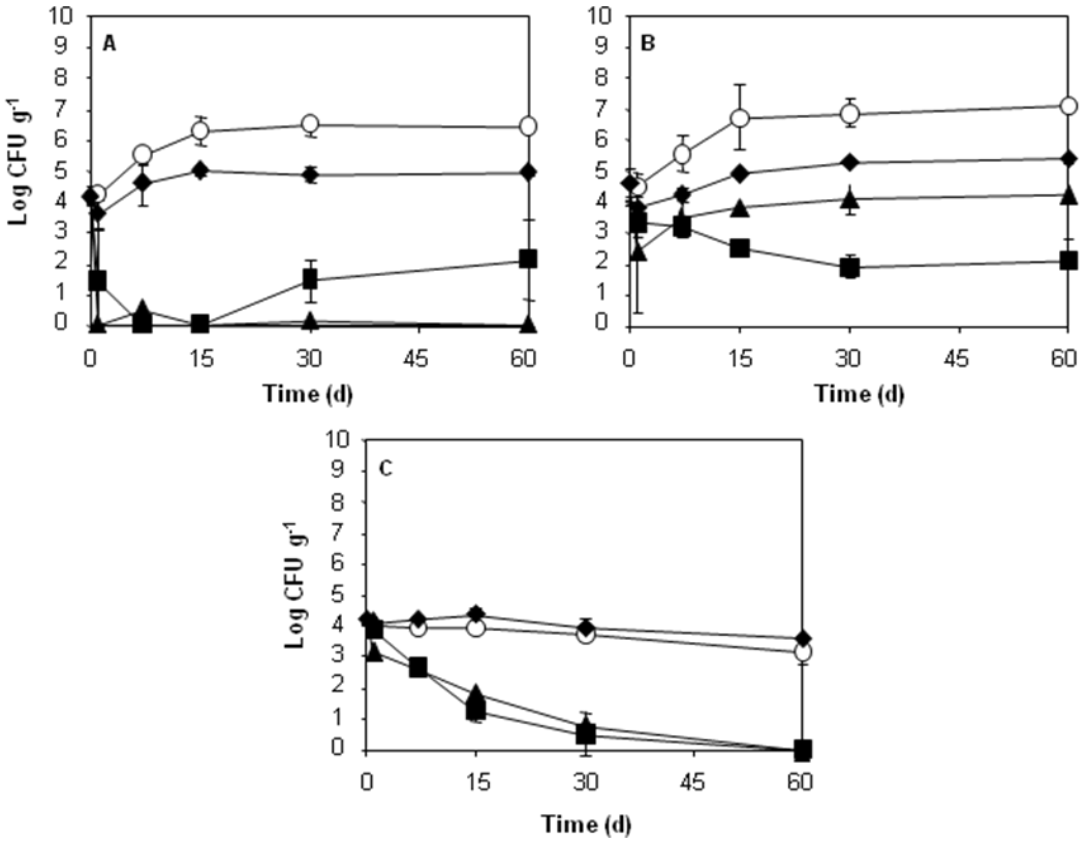

Figure 6. Effect of enterocin AS-48 (40 µg g^{-1}) combined with vacuum packaging on the viability of *Lactobacillus sakei* CTC 245 (A), *Brochothrix thermosphacta* CECT 847 (B), and *Staphylococcus carnosus* CECT 4491 (C) in a cooked ham model stored at 5 ºC. Control stored under normal atmosphere (♦); containing AS-48 stored under normal atmosphere (■); control stored under vacuum (○); containing AS-48 stored under vacuum (▲). Values are the average ± SD (error bars) of two independent experiments

Significant differences were observed in the vacuum-packaged samples with respect to those stored under normal conditions (Fig. 6). Vacuum packaging significantly (P<0.05) improved the growth of *L. sakei* in the control samples, particularly from 15 d storage, achieving counts of 6.3, 6.4, and 6.4 log units at 15, 30, and 60 d compared with the 5, 4.9, and 4.9 log units attained at the same times under normal atmosphere (Fig. 6A). Despite this increased growth, the addition of AS-48 caused a drastic inhibition of *L. sakei,* decreasing its counts to below detection values from day 1 to the end of the experiment. Similar results were obtained for *Brochothrix*, which reached a maximum of 7.1 log units under vacuum versus 5.4 log units under normal atmosphere (significantly different at P<0.05) (Fig. 6B). The efficacy of AS-48 against *Brochothrix* under vacuum was quite similar (in terms of reductions of CFU g^{-1}) to normal atmosphere, especially from day 15, achieving reductions of 2.1, 2.1, 2.9, 2.8, and 2.9 log units at 1, 7, 15, 30, and 60 days respectively versus the

reductions of 0.5, 1, 2.4, 3.3, and 3.3 log units attained under normal atmosphere at the same times. Finally, the *S. carnosus* population showed similar cell densities under both atmospheric conditions and it was also inhibited to the same extent by the enterocin under normal atmosphere and under vacuum (Fig. 6C).

3.5. Detection of enterocin AS-48 during cooked ham storage

Bacteriocin extraction from the meat matrix of several batches was performed at time 0 and during storage. At time 0, recovery was very high, extracting 35 μg g^{-1} out of the 40 μg g^{-1} of AS-48 applied. Afterwards, the activity recovered declined gradually, to 25, 15, and 7 μg g^{-1} at 1, 7, and 15 d, respectively and no activity was detected in samples extracted at days 30 or 60.

3. Discussion

In present work we have tested the efficacy of AS-48, applied alone or in combination with licensed chemical preservatives or vacuum or modified atmosphere (MA) packaging, in the control of *L. sakei, B. thermosphacta*, and *S. carnosus,* in a cooked ham meat system. We have previously demonstrated that enterocin AS-48 is very effective in the control of several spoilage bacteria (mainly *Bacillus* and related genera species but also some lactic acid bacteria) in vegetable foods (Grande et al., 2006; Martínez-Viedma, Abriouel, Ben Omar, Valdivia, Lucas-López, & Gálvez, 2008). Nevertheless, the importance of the chemical composition and the physical conditions of the food in the antimicrobial efficacy of bacteriocins is well recognized (Cleveland, Montville, Nes, & Chikindas, 2001). So, it is necessary to validate the antimicrobial activity of the enterocin for each specific food system against the main microorganisms that challenge its safety and shelf life in order to establish the effective concentration and also the most adequate combination of preservative treatments to be applied with the bacteriocin in this food.

In the present study, AS-48 was assayed against *L. sakei, B. thermosphacta*, and *S. carnosus,* three bacteria reported as very common normal microbiota in meat foods, using a cooked ham system as a food model for the tests. This kind of product is a good target to apply a multiple hurdle system in addition to the few barriers the ham has on its own and which are ineffective against the most common types of post-processing-associated spoilage organisms. In this food system, AS-48 by itself has proven to be very efficient at inhibiting the three challenged bacteria since important reductions of 3-4 log units in CFU g^{-1} were

achieved when 40 µg g^{-1} of the enterocin was applied in solitary. AS-48 activity on *L. sakei* was concentration-dependent, with 60 µg g^{-1} being sufficient to decrease viable counts below detection values during the 60 d of storage at 5 ºC; moreover, 40 µg g^{-1} maintained *Lactobacillus* populations below 2 log CFU g^{-1} throughout the entire storage time. In contrast, in the control lots, the counts reached approximately 5 log units. Despite these good results, we should note that these concentrations of AS-48 are much higher than those necessary to control the spoilage lactic acid bacteria (*Lactobacillus collinoides, Lactobacillus dioliovorans*, and *Pediococcus parvulus*) in apple juice and apple cider (Martínez-Viedma et al., 2008). In apple juice, for instance, an AS-48 concentration of 2.5 µg ml^{-1} reduced LAB viable cell counts below detection levels over the course of 15 d of incubation at 10 and 22 ºC for most strains tested. Although LAB species assayed in cider and apple juice were different to those inoculated in cooked ham, we can attribute the variable efficacy of AS-48 to the food system (solid and with a more complex and heterogeneous matrix) and also probably to the different pH (acidic for juice and cider and close to neutral for cooked ham). These results emphasize the importance of testing the efficacy of bacteriocins in each particular food system against the main spoilage and pathogenic microorganisms. Other factors to be taken into account in explaining the variable efficacy of AS-48 in the two above experiments is the time for which the AS-48 inhibitory effect was evaluated, 2 days for apple juice/cider and 60 days for cooked ham. This is an important factor since very frequently the target microorganism is undetectable at the beginning of treatment but then overgrows during the subsequent storage/incubation time (Ananou et al., 2010). In fact, 40 µg g^{-1} of the enterocin was able to maintain viable counts of lactobacilli below detection levels between 7 and 15 days, but afterwards they had a slow regrowth. The storage temperature also influenced the efficacy of AS-48, being much less effective in the control of *L. sakei* at 15 than at 5 ºC. The influence of temperature in the activity of AS-48 against different bacteria and in different food systems has been studied previously and the results are very heterogenous depending on the target bacterium and the food system (Cobo-Molino et al., 2008; Grande et al., 2006). In the present study, the decreased activity of AS-48 against *L. sakei* at 15 ºC can be attributed to the increased growth of the bacterium at this temperature.

With the aim of decreasing the effective concentrations of AS-48 to be applied in this food system, we tested the combinations of the enterocin and different chemical substances and natural antimicrobials. The compounds selected are additives licensed by the European Parliament and Council Directives on Food Additives (95/2/EC, 96/85/EC, 98/72/EC) for use in meat products, and they have been used at concentrations equal to or lower than those licensed.

We showed previously the increase in AS-48 activity through the combination with chemical preservatives against *L. monocytogenes* and *S. aureus* in the same food system (Ananou et al., 2010). As an example, AS-48 (40 μg g^{-1}) combined with nitrite/nitrate, STPP, sodium benzoate, or potassium sorbate increased its anti-listeria effect and combined with nitrite/nitrate, STPP, sodium lactate, and sodium acetate increased its activity against *S. aureus*.

The combinations of AS-48 with the natural antimicrobials sodium lactate (2%) or sodium acetate (0.2%) increased the activity of AS-48 against *L. sakei* and the viable counts decreased below detection values from day 1 to the end of storage. Organic acids and their salts can potentiate the activity of bacteriocins greatly, whereas acidification enhances the antibacterial activity of both organic acids and bacteriocins (Stiles, 1996; Gálvez et al., 2007). In this case, the enhanced antimicrobial effect of AS-48 cannot be attributed to the acidic pH since organic acid salts were used. We have previously shown that sodium lactate potentiated the activity of AS-48 against *B. cereus* in rice gruel (Grande et al., 2006) and against *L. monocytogenes* in sprouts (Cobo Molinos et al., 2005).

Chelating agents such as sodium pyrophosphate (0.15%) or STPP (0.5%) were also very effective in controlling *L. sakei* in combination with AS-48 in the cooked ham model as the counts remained below detection values from day 7 to the end of storage. The enhancing effect of chelators such as STPP have been reported for other bacteriocins under laboratory conditions and in foods (reviewed in Gálvez et al., 2007). Interestingly, the combination of AS-48 and STPP reduced the effective AS-48 concentration to 20 μg g^{-1}.

The combined application of nitrate/nitrite (0.007% or 0.015%) also improved the activity of AS-48 against lactobacilli. The addition of nitrite also increased the activities of enterocin AS-48 against *B. cereus* (Abriouel et al., 2002) and against *L. monocytogenes* (Ananou et al., 2010). Moreover, the combinations of nisin and nitrite showed increased activity on *Leuconostoc mesenteroides* and *L. monocytogenes* (Gill & Holley, 2000). In contrast, neither sodium benzoate nor potassium sorbate improved the inhibitory activity of AS-48 against *L. sakei.*

Sublethal heat treatment did not increase the activity of AS-48 against *L. sakei*. Nevertheless, in this same food system, we previously showed the synergistic effect between AS-48 and heat against both *L. monocytogenes* and *S. aureus* (Ananou et al., 2010), against *S. aureus* in milk (Muñoz et al., 2004), and against *E. coli* in apple juice (Ananou et al., 2005a).

We wish to draw attention to the different results obtained in this work with respect to the inhibitory activity of AS-48 against *L. sakei* compared with those obtained for *L. monocytogenes* and *S. aureus* in the same food model or with other pathogenic or spoilage bacteria in other foods, when applied alone or combined with different hurdles. This circumstance emphasizes the importance of testing the susceptibility of any bacterium in specific foods to establish the best strategy of bacteriocin application.

Vacuum and MA packaging are the most widely used packaging techniques in the food industry to prolong the shelf life of cooked meat products. Moreover, the results reported by various authors are very controversial. Some reports state that there is no difference in the shelf life of products in the different atmospheres tested (Boerema, Penny, Cummings, & Bell, 1993), whereas others have reported that an MA with 100% N_2 or with CO_2 concentrations lower than 50% (Blickstad & Molin, 1983; Ahvenainen, Skytta, & Kivikataja, 1989; Borch & Nerbrink, 1989) or vacuum (Leisner, Greer, Dilts & Stiles, 1995) gave the best results.

According to our results, MA (40% CO_2:60% N_2) packaging did not affect the growth of *L. sakei, B. thermosphacta*, or *S. carnosus* nor the inhibitory activity of the enterocin against the three bacteria. Nevertheless, vacuum packaging has a notable effect on both growth and AS-48 activity against *L. sakei* since lactobacilli in lots treated with AS-48 stored under vacuum remained below detection values from day 1 of storage. This effect is more remarkable considering that under vacuum, in untreated lots, *L. sakei* reached approximately 2 log units CFU g^{-1} higher than under normal atmosphere, and therefore the differences between the control lots and treated lots were higher than 6 log units from day 15 of storage. These are the first results proving the effect of this factor on AS-48 activity, and they represent an important result since vacuum packaging is a very common practice in the cooked meat food industry. With respect to *B. thermosphacta*, this bacterium is, together with the lactic acid bacteria, the main microbiota present in vacuum-packaged meat products (Borch et al., 1996; Holley, 2000). Although vacuum packaging alone is not enough to prevent *Brochothrix* spoilage, it is a good hurdle for use in combination with others. Various preservatives (lysozime, lactic acid, EDTA, nisin) have been used to increase the inhibitory effect of vacuum. Combinations of nisin, vacuum, and other preservatives have provided satisfactory results (Cutter & Siracusa, 1996; Gill & Holley, 2000). In our experiments, we have proven that *B. thermosphacta* grows better under vacuum than in normal atmosphere (maximum growth of approx. 7 log CFU g^{-1} versus approx. 5 log CFU g^{-1}) and the maximum counts of *Brochothrix* reached in cooked ham treated with AS-48 and stored under vacuum

were higher than those in treated lots stored under normal atmosphere. In any case, the application of AS-48 under vacuum reduced *Brochotrix* populations from approximately 7 log units to 4 log units. This reduction is quantitatively much more relevant that the reduction achieved under normal atmosphere (from 5 log units to 2 log units). Therefore, we can conclude that, in the case of *B. thermosphacta*, the application of vacuum packaging also enhances the inhibitory activity of the enterocin. Finally, in the case of *S. carnosus*, neither bacterial growth nor AS-48 inhibitory activity were enhanced by the storage of cooked ham under vacuum.

5. Conclusions

Based on these results, we conclude that enterocin AS-48 can be used against the spoilage bacteria *L. sakei, B. thermosphacta*, and *S. carnosus* to prolong the shelf life of non-fermented ready-to-eat meat products such as cooked ham. Although a single application of enterocin is very effective, the combination of AS-48 with other hurdles such as STPP, sodium pyrophosphate, nitrate/nitrite, sodium lactate, or sodium acetate enhances the inhibitory action of enterocin against *L. sakei*. This strategy could reduce the effective concentration of the enterocin and also afford protection in products with greater bacterial contamination. Storage under vacuum has proven to be particularly effective in increasing the activity of AS-48 against *L. sakei* and *B. thermosphacta*.

Acknowledgements

This work was supported by Grant (AGL2005-07665-C02-01) from the Spanish DGICYT and Grant (P07-AGR-02539) from the Junta de Andalucía. Samir Ananou received several grants from the Junta de Andalucía (PAI Research Group BIO 160). We would also like to acknowledge the work of Christine Laurin in editing the text.

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I.2. Combined effect of enterocin AS-48 and an organosulfur compound derived from *Allium sp.* to control foodborne pathogens in meat products.

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Abstracts

Non fermented meat products are very prone to microbial contamination and may often act as vehicles for bacterial pathogens. In recent years, the food preservation methods using natural products have become very popular. Several natural extracts from plants and bacteriocins can be considered as effective substitutes to chemical antimicrobial agents traditionally used. The aim of this study was to test the efficacy of enterocin AS-48, applied alone or in combination with an *Allium* extract, to control *Listeria monocytogenes, Staphylocoocus aureus* and *Clostridium perfringens* on burger meat, meatballs, cooked ham and *chistorra*, a type of fast-cure sausage from Spain. The combination of AS-48 (40 µg/g) with different dose of *Allium* extract (500-1000 µg/g) was the most effective treatment for the control of *L. monocytogenes* in both, burger meat and *chistorra* food models, *S. aureus* in meatballs and *C. perfringens* in cooked ham. These results suggest the possibility of dispensing with certain additives such as sulphites or lactate/diacetate for pathogen control in such foods, pointing out the combination of AS-48 and extracts of *Allium* as an effective solution, compatible with a clean labeling in meat industry.

Keywords*:* Biopreservation; hurdle technology; meat; enterocin AS-48; Proallium

Article In-progress.

1. Introduction

Meat products are very prone to microbial contamination and may often act as vehicles for bacterial pathogens responsible for many foodborne illness outbreaks, including *Staphylococcus aureus*, *Clostridium perfringens* and *Listeria monocytogenes*. In order to prevent the growth of these pathogens in meat products, several preservation techniques, such as salting, high hydrostatic pressures, modified atmosphere packaging and chemical preservatives have been used in the meat industry (Latha et al., 2009; Myers et al., 2013; Al-Qadiri et al., 2015).

In recent years, new food preservation methods using natural products have become very popular and numerous efforts have been made to find natural alternatives to control pathogen bacteria in foods. Several natural extracts from plants as well as bacteriocins from lactic acid bacteria have gained the GRAS status (Generally Recognized as Safe) and they can be considered as effective substitutes to antimicrobial chemicals traditionally used.

Garlic (*Allium sativum*) and onion (*Allium cepa*) are members of the *Amarillydaceae* family and have been widely used as food ingredients and medicinal plants. Both of them have been traditionally used for their antimicrobials properties (Corzo-Martínez, et al., 2009; Wilson & Demmig-Adams, 2007), which are attributed to the presence of organosulfur compounds, including alk(en)yl thiosulfinates and thiosulfonates (Block, 2010; Kyung, 2012). Because of the growing interest in the use of natural biologically active ingredients as preservatives, *Allium* extracts containing these organosulfur compounds have been considered as potential natural replacers to synthetic additives (Benkeblia & Lanzotti, 2007) for the increase of shelf-life and safety of foodstuffs. One of these *Allium* extracts is the commercial product Proallium whose antibacterial properties have been previously shown (Ruiz et al., 2010; Llana-Ruiz-Cabello et al., 2015).

The use of bacteriocins or bacteriocin-producing lactic acid bacteria is also receiving increasing interest to be applied as natural preservatives. At this respect, some bacteriocins, including nisin, enterocins A and B, sakacin or pediocin AcH, have been tested, alone or in combination with other preservative treatments, as additional hurdles to control proliferation of pathogens in meat products (Aymerich et al., 2000; Cleveland et al., 2001; Garriga et al., 2002; Katla et al., 2002; Hampikyan et al., 2009).

Enterocin AS-48 is a cationic circular bacteriocin produced by some *Enterococcus faecalis* strains with broad bactericidal activity against most Gram-positive bacteria, including

several pathogens, such as *L. monocytogenes*, *S. aureus*, *Mycobacterium* spp., *Bacillus cereus* as well as some Gram-negative bacteria (Abriouel et al., 2002; Abriouel et al., 1998; Gálvez et al., 1989). Enterocin AS-48 has some desirable characteristics, such as relatively broad antimicrobial activity spectrum, stability in a wide range of pH and temperatures and alack of toxicity (Gálvez et al., 1986; Samyn et al., 1994). These properties make it a promising replacer to synthetic additives in foods. In fact, AS-48 has been shown to be effective in the control of various foodborne pathogens in dairy, meat, and vegetable products (Ananou et al., 2010; Cobo Molinos et al., 2008; Muñoz et al., 2007) and also against several spoilage bacteria in meat products and vegetable-derived foods (Baños et al., 2012; Grande et al., 2006, 2007). Recently, *E. faecalis* UGRA-10, an AS-48 producing strain isolated from a Spanish sheep's cheese, is being investigated due to its probiotic characteristics (Cebrián et al., 2010).

The purpose of the present work was to test the efficacy of AS-48, applied alone or in combination with an *Allium* extract, to control foodborne pathogens on burger meat, meatballs, cooked ham and *chistorra*, a type of fast-cure sausage from Spain.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Enterococcus faecalis UGRA-10 was used as AS-48 producer. *E. faecalis* S-47 from our collection was used as the standard indicator strain for bacteriocin activity assays. For meat inoculation, a total of 30 wild strains of *L. monocytogenes* isolated from different meat industries were used. *S. aureus* CECT 8148, originally isolated from raw meat, was obtained from the Spanish type culture collection (CECT). Five wild strains of *C. perfringens* (CECT 486, CECT 563, CECT 820, CECT 821, CECT 822) isolated from bovine, ovine and meat products were supplied by the CECT. For spores obtainment, *C. perfringens* strains were previously cultured in thioglycolate broth and then transferred into 90 ml Duncan-Strong (DS) sporulating medium (Becton, Dickinson and Company, Le Pont de Claix, France) and anaerobically incubated at 37 °C for 24 h. In order to count the number of heat-resistant spores, cultures were heated at 70 °C for 20 min, serially diluted with phosphate-buffered saline (PBS), plated onto brain heart infusion agar (BHI agar, Scharlau, Barcelona, Spain) and anaerobically incubated under the same conditions. All strains were grown overnight on BHI at 37 ºC, washed in sterile saline solution and then inoculated in the meat mixture at the selected concentration. All strain cultures were maintained at 4 ºC on BHI-agar slants.

2.2. Preparation of Bacteriocin AS-48

AS-48 was produced by culturing the strain *E. faecalis* UGRA-10 in a whey-substrate, Esprion 300 (ES-300) (DMV Int., Veghel, Netherlands), supplemented with 1% glucose (Ananou et al., 2008). AS-48 was recovered from cultures by cation exchange chromatography on carboxymethyl Sephadex CM-25 (as described by Abriouel, et al., 2003). Eluted fractions were tested for bacteriocin activity against the indicator strain S-47 by the agar well diffusion method (Gálvez et al., 1986). The approximate concentration of AS-48 (in µg/ml) in the preparation was estimated by comparing the diameter of inhibition halo around the well with a titration curve obtained from purified bacteriocin. Before use, the eluted fractions were dialysed at 4 ºC against distilled water through a 2000-Da cut-off membrane to eliminate NaCl, and then sterilisd by filtration (0.22 µm, Millipore, Belford, MA, USA).

2.3. *Allium* extract

Proallium® was provided by DOMCA S.A.U (Granada, Spain). Proallium® is a flavouring product based on *Allium* extracts incorporated in a food-grade carrier (corn dextrin).

2.4. Food models

Manufacture of burger meat. The burger patties were prepared by mixing 75% of pork lean, 10% of pork fat, 5% corn starch, 0.30% salt, 0.15% dextrose, 0.05% sodium ascorbate (Scharlau), 0.5% spices (garlic powder, parsley and black pepper) and 9% water. In order to assess the precise effect of AS-48 and Proallium on *L. monocytogenes* in our experiments, the use of sulphites in the formulation was avoided. Only in one of the treatments, considered as control, sodium sulphite was added (Scharlau). The lean and fat pork were obtained from a local butcher. Both were minced separately using a 4 mm hole plate grinder (Mincer N32, Garhe SA, Amorebieta, Vizcaya, Spain). Then, the ingredients were blended for ten minutes using an electric mixer (Kneade Mixer RM-20, MAINCA SL, Granollers, Barcelona, Spain), whereupon meat was inoculated with approximately 10^3 CFU/g of *L. monocytogenes*. The mix was divided into five batches: A) a control batch; B) a batch including sodium sulphite (500 µg/g); C) a batch with AS-48 (40 µg/g); D) a batch with Proallium (800 µg/g); E) a batch with AS-48 (40 µg/g) and Proallium (800 µg/ g). Following to the application of the treatments, 100 grams of every mixture were molded to a size of 10 cm diameter and 1 cm thickness using a hand moulder (R-2186, Bernad SL, Albacete, Spain).

The burgers were placed on a polypropylene tray and stored in polyethylene plastic bags. All batches were stored at 4 ºC for 7 days.

Manufacture of *chistorra*. *Chistorra* sausage was prepared by mixing 70% lean pork and 30% fat pork obtained from a local butcher. Both ingredients were chopped in a 8 mm hole plate grinder, and mixed with paprika (0.25%), salt (0.4%) and garlic (0.6%). Although fresh *chistorra* is usually manufactured adding sodium nitrite as a preservative, we avoided it in our experiments with the aim of studying the precise effect of AS-48 and Proallium on *L. monocytogenes*. In this case we also included an additional batch containing sodium lactate/sodium diacetate (PURASAL OptiForm, PURAC Biochem, Gorinchem, The Netherlands), which is usually used to reduce *Listeria* in this kind of sausages. After being prepared, the mixture was stored in a cold room for 24 h. Then, the meat was inoculated with approximately 10^5 CFU/g of a pool of 30 wild strains of *L. monocytogenes*. The mix was divided into five batches: A) a control batch; B) a batch with sodium lactate/sodium diacetate (20.000 µg/g); C) a batch with AS-48 (40 µg/g); D) a batch with Proallium (1.000 µg/ g); E) a batch with AS-48 (40 µg/g) and Proallium (1.000 µg/g)**.** Afterthe application of the treatments, the meat was stuffed using a hydraulic stuffer (EC-12, MAINCA SL) into natural lamb casings of 22 mm caliber. Finally, the ripening process of the sausages was completed in cold storage at 4 ± 1 ºC for 21 days.

Manufacture of meatballs. The meatballs were prepared by mixing minced pork lean (70 %) with water (15.8 %), breadcrumbs (8 %), egg powder (5 %), salt (1 %) and pepper (0.2 %). As in the previous case, sulphites are usually added as preservative during the meatball preparation but we only added them in one of the batches as a positive control. The meat was inoculated with 10^3 CFU/g of *S. aureus*. Then, the meat batter was divided into five batches; A) a control batch; B) a batch with added sodium sulphite (500 µg/g); C) a batch with AS-48 (40 µg/g); D) a batch with Proallium (800 µg/g); E) a batch with AS-48 (40 µg/g) and Proallium (800 µg/g). After mixing the additives using a homogenizer (TYP UM4, BOSCH, Stuttgart, Germany), meatballs were shaped in 50 g spherical portions. Then, they were placed on a polypropylene tray and stored in polyethylene plastic bags and stored at 4 ± 1 ºC for 10 days.

Manufacture of cooked ham. Cooked ham was prepared by mixing 75% of lean pork meat obtained from a local butcher and 25% of a brine containing (in g/kg): water, 940; NaCl, 48; sodium ascorbate, 6; sodium glutamate, 6 (all from Scharlau). The meat was minced using a 6 mm hole plate grinder and, after being mixed with the brine solution, the ingredients were

homogenized using an electric mixer (RM-20, MAINCA SL) for 25 min. The blend was stored in a cold room for 12 h, whereupon potato starch (7%) was added.

The mixture was inoculated with approximately 10^4 CFU/g of *C. perfringens* spores. Taking into account the well-known sensibility of *C. perfringens* to sodium nitrite, this additive was removed in our experiments with the aim of finding the precise effect of AS-48 on the cooked ham. The blend was divided into four batches: A) a control batch; B) a batch with AS-48 (40 µg/g); C) a batch with Proallium (500 µg/g); D) a batch with AS-48 (40 µg/g) and Proallium (500 µg/g). After the application of the treatments, all batches were stuffed using a hydraulic stuffer (EC-12, MAINCA SL) into synthetic casings of 80 mm caliber (Cryovac, Sealed Air, Elmwood Park, USA) and then cooked in a bath until an internal temperature of 65 ºC. Finally, samples were stored in polyethylene plastic bags under vacuum (Tecnotrip EVT-10-2-CD-SC, Barcelona, Spain) and stored at 4 ± 1 ºC.

2.6. Recovery of bacteria

Samples from all treatments were withdrawn in triplicate at selected times to determine viable counts of *S. aureus*, *C. perfringens* and *L. monocytogenes.* For the microbiological analysis, 25 g samples were aseptically removed and mixed (1:10) with dilution medium (0.1% peptone, 0.85% NaCl). Homogenization was done in a masticator blender (IUL, Barcelona, Spain) for 1 min, followed by serial 10-fold dilution and plating on the respective selective media: *Clostridium perfringens* Agar (Scharlau) for *C. perfringens,* incubated under anaerobic conditions, Mannitol salt agar (MSA, Scharlau) for *S. aureus* and chromogenic selective agar COMPASS Listeria (Biokar Diagnostics, Beauvais, France) for *L. monocytogenes*. All bacteria were incubated at 35 ± 2º C for 48 and 72 h before counting the colonies.

2.7. Statistical analyses

Two independent experiments were carried out for each food model. The average data from duplicate trials ± standard deviations were determined with Excel software (Microsoft Corp., USA). Statistical analyses were performed using the SPSS-PC 15.0 software (SPSS, Chicago, Ill. USA). Data on microbiological counts were subjected to ANOVA, using AS-48, Proallium or the combination of both as factors. Categories included untreated meat samples, meat samples with added enterocin, meat samples with added Proallium and meat samples with both, AS-48 and Proallium.

3. Results and discussion.

In this work we have tested the efficacy of enterocin AS-48, applied alone or in combination with an *Allium* extract, for the control of *L. monocytogenes*, *S. aureus* and *C. perfringens*, in four different meat products: burger meat, meatballs, *chistorra* and cooked ham. These pathogens and their toxins continue to be a threat to consumer safety since they can be transmitted through several raw, cooked or cured meat products, being the origin of several disease outbreaks associated with the consumption of these foods, as reflected in the latest European Union summary report on trends and sources of zoonoses, zoonotic agents and foodborne outbreaks in 2013 (EFSA, 2015).

3.1. Control of L. monocytogenes.

L. monocytogenes has become a major foodborne pathogen responsible for outbreaks of listeriosis that are associated with high morbidity and mortality. The high incidence of Listeria in meat foods can be attributed to its natural resistance, which allows it to overcome the hurdle technologies used in the manufacturing of processed foods e.g. low storage temperatures, mild heat treatments or chemical preservatives). In this context, there are particular concerns about the effects of this microorganism in susceptible individuals (Young, Old, Pregnant women and Immunosuppressed individuals: YOPIS). Therefore, it is necessary to find new methods for the control of this pathogen in foodstuffs. At this respect,the application of enterocin AS-48 in food combined with plant extracts may provide an advantageous approach to reduce the use of traditional synthetic preservatives.

Previously we have shown that *L. monocytogenes* was highly susceptible to enterocin AS-48 in BHI broth with a minimum bactericidal concentration of 0.1 µg/ml (Mendoza et al., 1999). Furthermore, it has also been shown previously that AS-48 is very effective to control of *L. monocytogenes* in meat systems such as fresh sausages, showing a synergistic effect when applied in combination with certain chemical preservatives in cooked ham (Ananou et al., 2005; 2010). Nevertheless, the influence of the chemical composition and physical conditions of the food in the efficacy of bacteriocins is well recognized (Cleveland et al., 2001). Thus, it is necessary to validate the antimicrobial activity of the enterocin and its combinations in each food model.

In this paper, the effectiveness of AS-48 by itself and in combination with an *Allium* extract was tested for the control of *L. monocytogenes* in burger meat and *chistorra* (Fig 1). *Listeria* was able to grow in burger meat models at 4 ºC, reaching maximum levels of 5.2 log

units. In the case of *chistorra* sausage, *Listeria* levels remained more or less stable during the 21 days of maturation. At 40 µg/g, the enterocin reduced the counts of Listeria from the first day after application, with reductions of 1.8 log units compared with the control batch. This difference was increased throughout the shelf life of the meat burger with significant reductions of 2.4 log units in counts compared to the control batch at the 7th day. For the *chistorra* sausage the behaviour was similar. The addition of AS-48 at 40 µg/g significantly decreased the levels of *L. monocytogenes*, with reductions up to 2 log units from the first day of conservation. This difference was maintained during all the storage period to a final level of 1.8 log units in *Listeria* counts in day 21.

Previous experiments carried out in a sausage model demonstrated that it was enough to apply only 40 ppm of AS-48 to maintain Listeria below the detection levels from 6 to 9 days incubated at 20 ºC (Ananou et al., 2005). Nevertheless, the formulation of sausages included, as usual, nitrate/nitrite and sodium pyrophosphate and incubation time was limited to 9 days. We already showed the synergistic effect of nitrate/nitrite and chelating agents as sodium tripolyphosphate on the antilisterial action of AS-48 (Ananou et al., 2010). In our case, the absence of *Listeria* in food models, burger meat and *chistorra* sausage, was not achieved, although no significant regrowth was observed throughout the storage period. It should be mentioned that, although these products are usually manufactured adding sodium nitrite or sulphites as preservatives we avoided these additives in our experiments in order to determine the precise effect of AS-48.

In both applications, AS-48 was more effective for controlling *Listeria* when applied alone than the use of Proallium alone, and it was also more effective than sodium sulphite in burger meat and sodium lactate/sodium diacetate in *chistorra*. The combination of AS-48 with Proallium was the most effective treatment to control *L. monocytogenes* in both burger meat and *chistorra* food models, with far more reductions than any of the treatments applied alone. The viable counts of *Listeria* from day 1 of storage in the batch treated with both antimicrobials were, in burger meat, 2.70, 3.30, 3.53 and 3.40 log units lower than those of the untreated control at 1, 2, 4 and 7 days, respectively. In *chistorra* sausages, the counts were 2.66, 2.95, 2.90 and 2.67 log units lower than those of the untreated control after 1, 7, 14 and 21 days. These results suggest the possibility of dispensing with certain additives such as sulphites or lactate / diacetate for *L. monocytogenes* control in such foods, pointing out than the combination of AS-48 and extracts of *Allium* is an effective solution, compatible with a clean labeling.

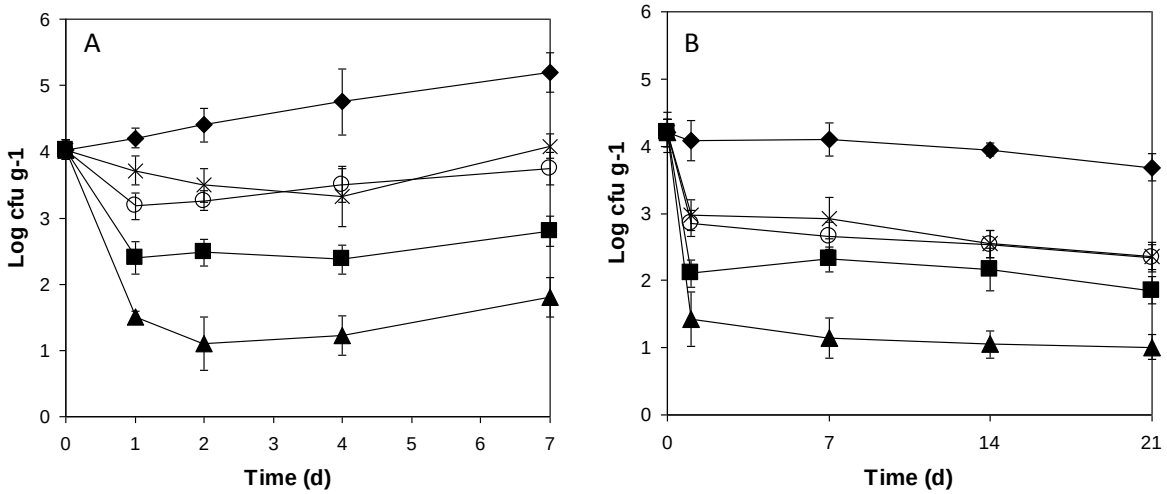

Fig. 1. Effect of enterocin AS-48 (40 µg/g) combined with Allium extract (800 µg/g) on the viability of *Listeria monocytogenes* in a model burguer meat (A) and *chistorra* (B) stored at 4 ºC. Control (♦);containing Allium extract (○); containing sodium sulphite (500 µg/g) (in burger meat) or containing sodium lactate/sodium diacetate (20.000 µg/g) (in *chistorra*) (X); containing AS-48 (■); containing AS-48 and Allium extract (▲). Values are the average ± SD (error bars) of two independent experiments.

3.2. Control of S. aureus

Staphylococcal foodborne disease is one of the most common foodborne diseases worldwide resulting from the contamination of food by preformed staphylococcal enterotoxins (Kadariya et al., 2014). In order to control the development of this pathogen in foods, several LAB bacteriocins, either alone or combined with other hurdles, have been used with varying degrees of success (Ananou et al., 2007; Felicio et al., 2015). In previous experiments the positive effect of combining different antimicrobial treatments with AS-48 on its antistaphylococcal activity in vegetable sauces and cooked ham has been proved (Grande et al., 2007; Ananou et al., 2010).

In this study, the effectiveness of AS-48 alone and in combination with the *Allium* extract was evaluated in the control of *S. aureus* in meatballs (Fig 2). The addition of AS-48 (40 µg/g) slightly reduced the viable counts of *S. aureus* and the counts were at least 0.5 log units lower than in the control batch from day 1. The effect of the enterocin was particularly relevant during the first 5 days of storage, when staphylococci counts remained at least 1 log unit lower than in control. This difference increased slightly during the subsequent conservation days, with 1.3 log units reductions in the levels of *S. aureus* compared to the control batch at the 10th day in meatballs. In comparison with enterocin and sulphites, addition of Proallium has a higher effect with up to 1.46 log units reductions after 10 days of conservation compared with the control batch. The best results in this trial were obtained by

combining AS-48 with Proallium, showing reductions of 1.2, 2.3, 2.6 and 2.5 log units at 1, 5, 7 and 10 days compared to the untreated control. Anyway, the synergistic effect observed in this complex matrix was lower than the previous results obtained in liquid media for AS-48 (Ananou et al., 2004; Muñoz et al., 2007) reinforcing the suggestion that AS-48 interaction with food components can interfere with its efficacy. This apparently lower effectiveness of AS-48 in meatballs compared to BHI broth, in spite of the higher added bacteriocin concentration, could be attributed to a higher retention of the bacteriocin molecules due to the components of meat and fat, which results in a slower diffusion and also inan irregular distribution of the bacteriocin molecules in this matrix. However, based on these results, the combined application of AS-48 and Proallium could be considered as a natural and effective replacer for the sulphites used in the control of *S. aureus* in this type of food.

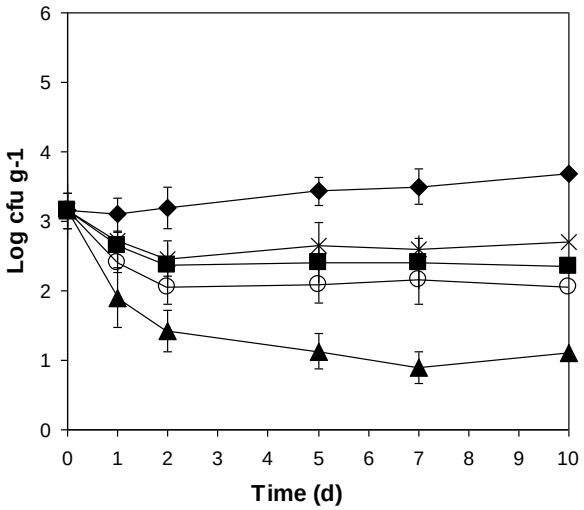

Fig. 2. Effect of enterocin AS-48 (40 µg/g) combined with Allium extract (800 µg/g) on the viability of *Staphylococcus aureus* in a meatball model stored at 4 ºC. Control (♦); containing Allium extract (○); containing sodium sulphite (500 µg/g) (X); containing AS-48 (■); containing AS-48 and Allium extract (▲). Values are the average ± SD (error bars) of two independent experiments.

3.2. Control of C. perfringens

Food poisoning associated with *C. perfringens* is caused by the ingestion of bacteria, which once in the small intestine, release an enterotoxin responsible for the pathological effects. Because of the high heat resistance of the spores of *C. perfringens*, thermal treatments applied to processed meat products are not usually sufficient to inactivate them and the spores usually germinate and the bacteria multiply during subsequent cooling. Therefore, cooked meat products must be cooled rapidly to inhibit spore germination and reduce the subsequent risk by toxin production.

In this paper, we have tested AS-48 against *C. perfringens* in cooked ham, which is a food greatly prone to post-processing contamination due to its high water activity and neutral pH. Firstly, we assessed the growth of *C. perfringens* from spore inoculum in cooked ham that was cooked slowly to 65 °C and then rapidly cooled to 5 ºC. *C. perfringens* was able to grow on the cooked ham system at 4 ºC, reaching a maximum level of 3 log units (Fig 4). In our trial, *C. perfringens* was sensitive to AS-48 (40 μg/g) and the antimicrobial effect of enterocin was significant ($P < 0.05$) from the first week of storage, reaching reductions of 1.4 log units compared to the control batch. These differences remarkably increased during the storage period, with clostridia counts decreasing to 1.3, 1.8, 1.7 and 2.2 log units at 7, 15, 21 and 30 days respectively. Addition of Proallium showed less anti-clostridium effect in comparison with the enterocin, with up to 1.2 log unit reductions after 35 days of storage compared with the control batch. Nevertheless, the best results for the control of this pathogen were obtained by combining AS-48 with *Allium* extract, with reductions of 2, 2.6, 2.7, 2.7 and 3 log units compared to the untreated control at 1, 7, 14, 21 and 35 days.

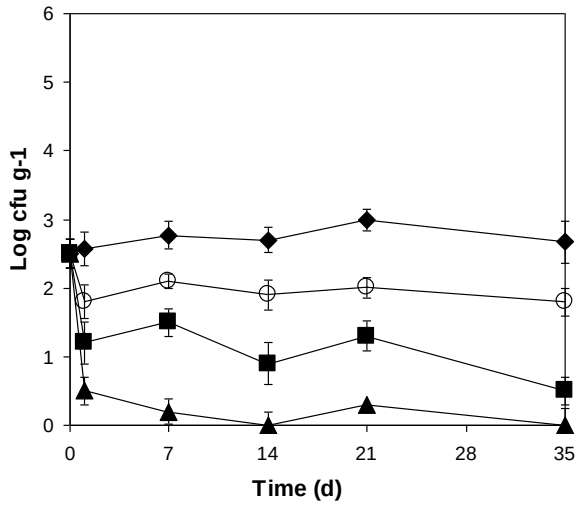

Fig. 3. Effect of enterocin AS-48 (40 μg/g) combined with Allium extract (500 μg/g) on the viability of *Clostridium perfringens* in a cooked ham model stored at 4 ºC. Control (♦); containing Allium extract (○); containing AS-48 (■); containing AS-48 and Allium extract (▲). Values are the average ± SD (error bars) of two independent experiments.

In accordance with our results, recent studies reporting bacteriocins, such as nisin and reuterin, have shown promising results in the inhibition of vegetative cells and spores of *C. perfringens* in vitro assays (Garde et al., 2014), although when they are applied to meat products, their effectiveness is significantly reduced (Udompijitkul et al., 2011). Similarly, Nieto-Lozano et al., (2005) a bacteriostatic effect of the pediocine against *C. perfringens* when applied on raw meat surface. These results suggest that application of enterocin AS-48

may potentially be used to reduce *C. perfringens* in the meat-based products, such as cooked ham, but further studies on food systems are needed to confirm these preliminary findings.

The results presented here show the inhibitory activity of the enterocin AS-48 against *L. monocytogenes* in burger meat and *chistorra*, *S. aureus* in meatballs and *C. perfrigens* in cooked ham. Although AS-48 applied alone was effective for the control of the selected pathogens, its efficacy was greatly enhanced when the enterocin was combined with the natural preservative Allium extract. Previous results have shown the synergistic effect of several chemical food grade preservatives with AS-48 for the control of *S. aureus* and *L. monocytogenes.* The present results expand the range of possibilities for the use of this bacteriocin, alone or in combination with the most appropriate chemical preservative according to the specific case of meat food or bacterial pathogen to be controlled

Acknowledgements

This work was supported by Grant (P07-AGR-02539) from the Junta de Andalucía. We would also like to acknowledge to Piscifactorias Andaluzas S.A. and the work of Jose Manuel García Madero in editing the text.

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I.3. Application of enterocin AS-48 in edible coatings to prevent the spoilage and improve safety of foodstuffs.

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Abstract

The application of edible coating (EC) added with antimicrobial agents is a good strategy to prevent the contamination of food surfaces improving their quality, safety and shelf life. In the present work we assayed the effectiveness of enterocin AS-48, incorporated in four different ECs: sucrose esters of fatty acids (SE), acetic acid esters of monoglycerides (ACETEM), chitosan and fish gelatin, to inhibit pathogen and spoilage bacteria in different food models, during storage at 5 ºC. In cut melons coated with SE-based coatings added of 10 μg/cm^2 AS-48 *Listeria monocytogenes* remained below 1 log CFU/cm^2 during 7 days of storage. In cheese slices covered with ACETEM-based coatings added of 40 μg/cm^2 AS-48, *L. monocytogenes* fell below or close to 1 log CFU/cm^2 within the first 10 d of storage and remained between 1 and 1.7 log CFU/ cm^2 from 10 to 20 days. AS-48 added (25 μg/g) to a fish gelatin-based coating in raw hake fillets achieved significant reductions in *Lactobacillus acidipicis* and *Staphylococcus carnosus* over the entire storage period. In cooked ham, AS-48 (40 μg/g) added to a chitosan-based coating determined that *Lactobacillus sakei* counts decreased below 1 log CFU/cm^2 from 5 d to the end of the storage and *Brochothrix thermosphacta* remained belows 3 log CFU/cm^2 over the entire storage period. In conclusion, safety improvement and extension of shelf life of food products can be achieved by the incorporation of enterocin AS-48 in edible coatings.

Key words: edible coating, enterocin,AS-48, melon, raw hake, cooked ham, fresh cheese

Article in-progress

1. Introduction

Food contamination due to the presence of pathogens and spoilage microorganisms results in large economic losses worldwide all the year round. Outbreaks of foodborne diseases are among the main reasons to find new tools to prevent microbial growth in foodstuffs. Many food-processing technologies have been developed in order to prevent foods from the contamination by pathogens, e.g. thermal processing, irradiation, modified atmosphere packaging and the addition of synthetic antimicrobial compounds. Currently, new technologies are being developed with this aim, such as pulsed electric fields, ultrasounds, high-pressure processes, the use of edible coatings (EC) and new antimicrobial agents (Knorr et al., 2011; Pasha et al., 2014)

Because of the growing consumer demand for chemical preservative-free foods, food manufacturers are now using naturally occurring antimicrobials which have been isolated from natural sources including animals (such as chitosan or lactoferrin), plants (herbs or spices extracts) and microorganisms (Gálvez, 2014). Antimicrobials from microbiological sources, the so-called biopreservatives, are among the most promising tools in food preservation. Some examples are lactic acid bacteria and their bacteriocins (Elsser-Gravesen, 2014)

Bacteriocins are ribosomally synthesised peptidic compounds, lethal to those bacteria closely related to the producing bacteria (Joerger et al., 2003).The use of bacteriocins or bacteriocin-producing lactic acid bacteria is also receiving increasing interest so as to be applied as natural preservatives. With that purpose, some bacteriocins have been tested alone or in combination with other preservative treatments as additional hurdles to control the proliferation of pathogens in foods (Avila et al., 2014; Narayanan et al., 2013; Aymerich et al., 2000).

Enterocin AS-48 is a cationic circular bacteriocin produced by some *Enterococcus faecalis* strains with a broad spectrum of bactericidal activity against most Gram-positive bacteria, including several pathogens, such as *L. monocytogenes*, *S. aureus*, *Mycobacterium* spp., *Bacillus cereus* as well as some Gram-negative bacteria (Abriouel et al., 2002; Gálvez et al., 1989). Enterocin AS-48 has some desirable characteristics, such asa relatively broad antimicrobial activity spectrum, and stability in a wide range of pH and temperatures (Gálvez et al., 1986; Samyn et al., 1994). These properties make it to be a promising alternative to synthetic additives as a biopreservative for foods. In fact, AS-48 has been shown to be effective in the control of various foodborne pathogens in dairy, meat, and vegetable

products (Ananou et al., 2010; Muñoz et al., 2007) and also against several spoilage bacteria in meat products and vegetable-derived foods (Baños et al., 2012; Grandeet al., 2006, 2007).

It is known that microbiological contamination occurs mainly on the surface of foods; therefore, one of the best measures to prevent contamination is the application of antimicrobial agents on the surface. Thus the use of antimicrobial films mixed with bacteriocins can improve the quality, the safety and extend the shelf life of food products (Gálvez et al., 2007). Antimicrobial films may be applied either in the form of packaging (traditional or active) or as a part of the food itself e.g. edible coatings.

Edible coatings (EC) are food grade suspensions which may be delivered by spraying, spreading, or dipping, which upon drying form a thin layer on the food surface. They have been proven to be capable of extending the shelf life and improve the safety of food in different ways. Furthermore, ECs are used to improve the appearance and the mechanical properties of food products, reducing water loss and preventing gas transmission (Falguera et al., 2011; Wu et al., 2002).

Many diverse materials are used in the formulations of ECs. Usually film-forming substances are based on lipids, proteins or polysaccharides, or on a combination of them (Greener-Donhowe & Fennema, 1994). Other materials are also added to improve the structural and mechanical properties of the coating. Lipid-based coatings are based on hydrophobic compounds, such as fatty acids and their esters with glycerol, long-chain monohydric alcohols or other substances. Some of these ingredients are considered as food additives, like esters of fatty acids with sucrose (sucrose esters, E-473) or with other organic acids such as acetic, lactic, tartaric or citric (esters of mono- and diglycerides, E-472).Likewise, protein materials are currently used in ECs. Some examples of substances used in protein-based EC are of plant origin, like corn zein, wheat gluten or soy protein, while others derive from animal sources, like collagen or gelatin. Gelatin is formed from the partial hydrolysis of collagen, forming a flexible and clear film. Polysaccharides can be also a key ingredient in ECs. Most of them come from vegetable sources, like cellulose and starches. Nevertheless, they can also be obtained from algal species (alginate, carageenan) or animal sources (chitin, chitosan). Chitosan, a linear polysaccharide consisting of (1,4)-linked 2-amino-deoxy-b-D-glucan, is a deacetylated derivative of chitin that, after being dissolved in acidic conditions, forms an EC with good antimicrobial and barrier properties (Younes and Rinaudo, 2015; Elsabee and Abdou, 2013).

Currently, edible coatings are commonly used on many commodities, such as candies, fresh fruits and vegetables, and processed meats. The main role of edible coatings is to provide a physical barrier that protects the food against gases and dehydration. But ECs may also act as carriers of functional ingredients, such as antimicrobials to further enhance food stability and safety, leading to the antimicrobial edible coatings (AEC) (Pascall et al., 2013).Considerable research work has been undertaken with antimicrobial agents, such as food preservatives or plant extracts as part of ECs in order to achieve an effective antimicrobial barrier (Jasour et al., 2015; Olaimat et al., 2014; Gniewosz et al., 2013). Bacteriocins are also an interesting option to be included in ECs as they constitute natural preservatives that might avoid or reduce the addition of synthetic compounds to food (Murillo-Martínez et al., 2013).

The purpose of the present study was to evaluate the effectiveness of AS-48 added to four different types of edible coatings, based in sucrose esters of fatty acids (SE, E-473), acetic acid esters of mono and diglycerides (ACETEM, E472a), chitosan and fish gelatin to control pathogens and spoilage bacteria on melon, cooked ham, cheese and hake.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Enterococcus faecalis UGRA10 (Cebrian et al., 2012) was used as AS-48 producer. *E. faecalis* S-47 from our collection was used as the standard indicator strain for bacteriocin activity assays. For in vitro antimicrobial studies, *Listeria monocytogenes* CECT 4032, supplied by the Spanish Type Culture Collection (CECT) was used. For melon inoculation, a total of five wild strains of *L. monocytogenes* from our collection, isolated from different vegetables were used. For cheese inoculation, two serovar 1/2a *Listeria monocytogenes* strains (clones 1 and 2) involved in an outbreak of listeriosis in Austria and Germany (2009-10) due to the consumption of “Quargel” cheese were used (Fretz et al., 2010). *Lactobacillus sakei* CTC 245 were obtained from the Collection of the Institute for Agro-Food Research and Technology (IRTA, Monells, Girona, Spain) and *Brochothrix thermosphacta* CECT 847 was supplied by the CECT. *Lactobacillus acidipiscis* DSM 15836 and *Staphylococcus carnosus* subsp. *utilis* DSM 11677 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), to be used for fish inoculation. Listeria was aerobically incubated at 37 °C for 24 h and the spoilage bacteria were aerobically incubated at 30 °C for 48-72 h. Enterococci, staphylococci, Listeria and *B. thermosphacta* were

maintained at 4 ºC on Brain Heart Infusion-agar (BHA, Scharlau, Barcelona, Spain) slants. Lactobacilli were maintained at 4 ºC on Man Rogose Sharpe-agar (MRS-agar, Scharlau).

2.2. Coatings

Four types of coatings were used in this study, based on different film-forming ingredients: Sucrose esters of fatty acids (SE, E-473), acetic acid esters of mono and diglycerides (ACETEM, E472a), chitosan and fish gelatin.

SE-based coating. FOODCOAT FR DMC (DOMCA S.A.U., Spain) is composed by a blend of fatty acids (mainly lauric acid, palmitic acid and estearic acid) esterified with sucrose in a hydroalcoholic solution. It also contains glycerol as a plasticiser. The production method remains as a trade secret.

Chitosan-based coating. The chitosan coating was prepared by dissolving 20 g of chitosan with a deacetylation degree of 90% (Sigma, U.S.A.) in 1 Kg of a sodium diacetate solution (2% w/w) to obtain the acidic conditions (pH = 4.9) needed to dissolve the biopolymer. It was added by rapid stirring (about 400 rpm) to obtain an opalescent solution. The sample was allowed to stand overnight to achieve the complete solution of chitosan, and then filtered through a sintered glass filter. The sample was finally autoclaved at 121 ºC for 15 min and stored in a refrigerator.

ACETEM-based coating. Recubrimiento comestible R-A2 (Domca SAU, Spain) was used for the coating of cheese. It is composed of an oil-in-water emulsion based on ACETEM and arabic gum (2%) as a thickener. The production method remains as a trade secret.

Fish gelatin-based coating. The fourth coating used in these trials was based on fish gelatin. The gelatin solution was prepared using commercial cold-water soluble fish gelatin (Norland Fish Products, Cranbury, NJ, USA). The film forming solution (FFS) was formulated to contain 5% of gelatin with 1% of sodium alginate (Sigma, USA) and 2% of glycerol as a plasticizer. Firstly, a solution of sodium alginate and glycerol was prepared separately by dissolving them in warm deionised water. After cooling the solution at 4 ºC, the fish gelatin was added while being stirred for 30 min. The product was stored in a refrigerator.

2.3. Diffusion method in solid media

L. monocytogenes CECT 4032 was the organism used to determine in vitro the antimicrobial activity of the EC with and without AS-48. The agar well diffusion method described by Tagg and MacGiven (1979) was used. Stainless steel cylinders with a diameter of 8 mm and a height of 10 mm were placed on a plate containing Mueller Hinton Agar (MHA, Scharlau) (10 ml). Then, 6 ml of soft BHA (BHA plus 0.08% agar) medium inoculated with approx. 10^7 UFC of *L. monocytogenes* CECT were poured onto the plate. After solidification of the overcoating layer, the cylinders were removed, and the holes were filled individually with 100 µl of the sample. Finally, the plates were incubated aerobically at 37 °C for 24-48 h. After incubation, the clear zones were measured using a microcaliper. Each sample was tested in triplicate.

2.4. Broth microdilution antimicrobial assay: determination of Minimum Bactericidal Concentration *(MBC).*

A broth microdilution susceptibility assay was used for the determination of Minimum Bactericidal Concentration (MBC) against *L. monocytogenes* CECT 4032 , as recommended by the National Committee for Clinical Laboratory (NCCLS, 2012). All tests were performed in Mueller-Hinton broth (Scharlau). Enterocin AS-48 and the coatings were dissolved in sterile 1% saline solution. Dilutions (1:1; v/v) were prepared in 96-well microtiter plates to achieve final concentrations ranging from 0.1 to 500 μg/ml. Then, each well received 10 µl of *Listeria* suspension containing 10^7 CFU/ml prepared from an overnight culture, and 100 µl of Mueller-Hinton broth. The wells showing complete lack of growth were selected and 100 µl from each well were deposited on the agar plates and incubated as described previously.The complete absence of growth in the plates at the lowest sample concentration was defined as the MBC. Assays were conducted in duplicate.

2.5. Antilisterial activity in liquid medium

Each edible coating containing AS-48 (100 µg/mL) was diluted (1:9; v/v) into a 0.1% peptone solution containing *L. monocytogenes* (5×10^5 CFU/mL) under continuous mixing in a rotating shaker (VWR International Eurolab S.L. Barcelona, Spain). In addition, coatings activity were tested and a set of coatings without AS-48 was also included as the control. *Listeria* suspensions were incubated at 37 ºC and at 0.25, 0.5 1, 3, 5, and 10 h. 100 µl samples were extracted, serially diluted, and spread onto PCA plates (Scharlau). Plates were incubated aerobically at 37° C for 24 h. The number of colonies was counted and reported as colony-forming units (CFU) per millilitre.

2.6. Food models

Melon. A variety of yellow melon (Indálico) supplied by a local provider was used. Melons were cut in half using a sterile knife. Melons were inoculated in the pulp side surface using a drigalsky handle with a pool of five strains of *L. monocytogenes* to reach a final concentration in food of approx. 5 x 10^2 UFC/cm^2. Previously to be applied, the EC based on sucrose esters (Foodcoat FR DMC), added or not with AS-48, was diluted at 4% in sterile water. Afterwards, different batches were stated by spraying the inoculated melons with the different treatments using an automated spray system (AUTOJET 1550, Spraying System Co.): A) a batch with EC; B) a batch with EC and AS-48 incorporated (10 µg/g final concentration in the food suface coat); C) a batch with and AS-48 incorporated (20 µg/g final concentration in the food coat); D) a batch sprayed with saline solution, the control batch. Each sample received the same amount of saline solution. After the treatments were dried, the melons were covered with transparent film and stored at 4º C. The evolution of *L. monocytogenes* in the food was determined daily for each treatment during 7 days. We have used 3 subsamples (melon halves) for each experimental condition and sampling time. Two independent trials *were* conducted.

Cheese. The assays were performed with a fat free unripened cheese manufactured ad-hoc in the Pilot Plant of DMC Research Center (Granada, Spain). For each trial, 20 L of pasteurized skimmed cow's milk was tempered to 25 ºC. Milk was inoculated with approximately 3 x 10^7 CFU/ml of a commercial mesophilic starter culture (Choozit MA 4001 LYO 5 DCU, DuPont™ Danisco®). Calf rennet (0.0025%, activity 1:10.000, Laboratorios Arroyo, Santander, Spain) was added 2 h after adding the starter. The curds and whey began to separate after 16 h. Then, the curds were cut into cubes of 20 mm in size, deposited into perforated containers, covered with linen cloths and kept overnight at 10 ºC to promote whey drainage. After that, salt was added to curd (1.5%, w/w). The cheese pieces were then packed into sterile containers of 250 ml and refrigerated at 4 ºC. On the day *after*, the cheeses were sliced into 5 mm thick sheets.

Then, they were surface inoculated with a suspension of two strains of *L. monocytogenes* (clones 1 and 2) involved in an outbreak of listeriosis in Austria and Germany due to the consumption of "Quargel" cheese to give a final concentration of Listeria of approx. 10^4 CFU/cm^2. Previously to be applied, the EC based on acetylated monoglicerides, added or not with AS-48, was diluted at 20% in sterile water. Then, the different treatments were sprayed using an automated spray system (AUTOJET 1550) to produce four batches: A) a batch with EC ; B) a batch with EC and AS-48 incorporated (20

µg/g final concentration in the food coat ; C) a batch with EC and AS-48 incorporated (40 µg/g final concentration in the food coat); D) the untreated control. Each sample received the same amount of saline solution. The samples were placed on polystyrene dishes and sealed in a Ziploc bag, and immediately stored at 4 ºC. After 1, 5, 15, and 20 d of storage, samples were taken and the evolution of *L. monocytogenes* in food was determined for each treatment. We have used 3 subsamples (sheets) for each experimental condition and sampling time. Two independent trials were conducted.

Fish. Raw hake and salmon fillets were purchased at a local retail. For each experiment, a series of fish fillets of approximately 100 cm^2 were prepared by slicing with a sterile knife. For the purpose of inoculation, three samples per treatment and sampling time were placed on sterile polystyrene dishes with the flesh side facing up. Then, fish fillet tissue samples were surface inoculated with *Lactobacillus acidipiscis* DSM 15836 and *S. carnosus* subsp *utilis* DSM 11677, two bacteria isolated from fish, to yield a final level of approx. 10^3 CFU/cm^2. Then, the samples were dried for 30 min to allow the attachment of bacterial cells. Before to be applied, the EC based on fish gelatin, added or not with AS-48, was diluted at 10% in sterile water. Afterwards, the different treatments were sprayed using an automated spray system (AUTOJET 1550). Four batches were produced: A) a batch with EC; B) a batch with EC added of AS-48 (25 µg/g final concentration in the food coat); C) untreated control. Each sample received the same amount of saline solution. The different samples were placed on polystyrene dishes and sealed in a Ziploc bag, and immediately stored at 4 ºC. The evolution of bacteria for each treatment was determined for a period of 9 days. We used 3 subsamples (fillets) for each experimental condition and sampling time. Two independent trials *were* conducted.

Cooked ham. Cooked ham was prepared by mixing 75% of lean pork meat from a local butcher and 25% brine solution containing (in g Kg-L): water, 940; sodium chloride, 48; sodium ascorbate, 6; sodium glutamate, 6 (all from Scharlau). The meat was minced using a 6 mm hole grinder plate and mixed and homogenized with the brine solution using an electric mixer (RM-20, MAINCA SL) for 25 min. After, the meat mixture was stored at 5 ºC in a cold room for 12 h, whereupon potato starch (7%) was added. Thereafter, the mixture was stuffed using a hydraulic stuffer (EC-12, MAINCA SL) into synthetic casings of 80 mm caliber (Cryovac, Sealed Air, Elmwood Park, USA). All batches were cooked in a water bath until their final internal temperature reached 65 ºC. Finally, the samples were stored at 5 ºC. The day after, the innards were sliced with an automatic slicer and were surface inoculated with a pool of *L. sakei* CTC 245 and *B. thermosphacta* CECT 847 to a final concentration of approx. 10^3 CFU/cm^2. Before to be applied, the EC based on chitosan, added or not with AS-48, was

diluted at 20% in sterile water Afterwards, the slices were surface exposed to spraying with different mixture coats by means of an automated spray system (AUTOJET 1550) producing four batches: A) a batch with the EC; B) a batch with EC added of AS-48 (20 µg/g final concentration in the food coat); C) a batch with EC added of AS-48 (40 µg/g final concentration in the food coat); D) untreated control. Each sample received the same amount of saline solution. Finally, samples were stored in polyethylene plastic bags under vacuum (Tecnotrip EVT-10-2-CD-SC, Barcelona, Spain) and stored at 5 ºC. After 1, 5, 15, and 20 days of storage, samples were taken and the evolution of the different bacteria in the cooked ham was determined for each treatment. We used 3 subsamples (slices) for each experimental condition and sampling time. Two independent trials were conducted.

2.7. Microbiological analysis

Samples from all treatments were withdrawn in triplicate at selected times to measure viable counts of bacteria. Samples were aseptically removed and mixed (1:10) with dilution medium (0.1% peptone, 0.85% NaCl). Homogenisation was done in a Masticator blender (IUL, Barcelona, Spain) for 1 min followed by serial 10-fold dilution and plating on the respective selective media: Man Rogosa Sharpe agar plus 0.04% sodium azide for *Lb. sakei and Lb. acidipiscis*, manitol saline agar (MSA, Scharlau) for *S. carnosus*, STAA agar (Oxoid LTD, Basingstoke, Hampshire, England) with added STAA supplement (Oxoid) for *B. thermosphacta* and chromogenic selective agar COMPASS Listeria (Biokar Diagnostics, Beauvais, France) for *L. monocytogenes.* Pathogens were incubated aerobically at 37 ºC for 24 h and spoilage bacteria were incubated aerobically at 28 ºC for 48–72 h before counting the *colonies*.

2.8. Statistical Analysis

The average data from duplicate trials ± standard deviations were calculated with Microsoft Excel (Microsoft Corp., USA). Statistical analyses were performed using the SPSS-PC 15.0 software (SPSS, Chicago, Ill. USA). The resulting data of microbiological counts were subjected to ANOVA, using the different edible coatings and their combination with AS-48 as factors. Categories include untreated control food, edible coating treated food, and edible coating supplemented by AS-48 treated food. The Mean Square Difference was used as a post-hoc test to establish significant differences between edible coatings manufactured with or without enterocin AS-48.

3. Results and discussion

Nowadays the use of EC has been extended to almost all sectors of food industry since the application of these products allows us to improve food safety and preservation. The functional characteristics of EC depend directly on the type of film-forming substance used for their manufacturing, but in general, EC provide a physical barrier effect that exert a direct impact on the microbiological food quality, helping to extend the shelf life of foodstuffs. In addition to the barrier effect, some of these film-forming substances, such as SE or chitosan, may exhibit antimicrobial properties, but, beyond this characteristic, one of the great advantages of EC is their potential to act as carriers of antimicrobials. Among these ingredients we find the bacteriocins, e.g. enterocin AS-48, extensively assayed as a *biopreservation* strategy for the control of pathogenic and spoilage bacteria.

In our work, we have evaluated the antimicrobial activity of four EC, based on SE, chitosan, ACETEM and fish gelatin, supplemented or not with enterocin AS-48. Given that some of the coatings could present antimicrobial activity themselves, in vitro assays were undertaken firstly to determine this activity and the improvement of the efficiency as a result of the addition of AS-48 to the coatings. Afterwards, challenge tests were carried out in four food models to test the efficacy of the AEC developed against spoilage bacteria or pathogens such as *L. monocytogenes*. Thus, EC based on SE and ACETEM were tested in the control of *L. monocytogenes* on cut melons and cheese, respectively. Chitosan-coating efficacy was tested in the control of spoilage bacteria in a cooked ham model, and fish gelatin-based coating was tested against spoilage microorganisms in raw hake fillets.

3.1. In vitro antimicrobial activity

L. monocytogenes CECT 4032 was the test organism used to determine in vitro antimicrobial activity. Three methods were used with this purpose: diffusion assay in solid medium, broth microdilution antimicrobial assay to determine the MBC and activity in liquid media. The antilisterial effect on solid medium was carried out using the well agar diffusion assay (Tagg & McGiven, 1971). The clear zones of Listeria growth inhibition produced around the wells are shown in Figure 1 and in Table 1. As expected, in all cases EC containing AS-48 (50 µg/g) showed the highest inhibitory effect against *L. monocytogenes*. In coatings without AS-48, there was no inhibition, except for chitosan-based EC in which a clear *zone* of 15 mm was observed.

The SE coating containing AS-48 had the lowest activity with an inhibition zone of 23 mm, followed by fish gelatin-based EC with 16 mm. The supplemented coatings based on ACETEM and chitosan showed the greatest antimicrobial activity with inhibition zone values of 25 mm each.

MCB of all Ecs, with and without AS-48, was determined by the standard microdilution method (Table 1). As it can be seen, all coatings including AS-48 exhibited a stronger biocidal effect on *L. monocytogenes* compared with the respective ECs not added of AS-48. It is noteworthy that there was not an exact correspondence between the MBC decrease degree due to the incorporation of AS-48 in the four ECs. In other words, the incorporation of AS-48 did not yield a similar increase in the anti-listeria activity of the ECs. Thus, for gelatin-based EC, which, by itself, showed no activity below 1000 µg/mL, the addition of the enterocin conferred it good antilisterial properties, with an estimated MBC of 31.25 µg/mL. In the case of ACETEM-based coating, which MBC was 125 µg/mL, the addition of AS-48 resulted in the same MBC (31.25 µg/mL) than for fish gelatin added of AS-48. Similarly, the chitosan-based EC which, by itself, has a MBC of 4 µg/mLacquired a MBC of 0.4 µg/mL when it was fortified with AS-48. This MBC value is approximate to that of SE-based coating (0.9 µg/mL), but this coating by itself has a MBC as high as 500 µg/mL. Taking into account the extremely low AS-48 MBC (0.1 µg/ml) determined in BHI for a *Listeria* population of 8 log units CFU/mL (Mendoza et al., 1999), it seem appropriate to make some considerations with respect to the anti-listerial activity decrease of the enterocin when it is incorporated in EC. Thus, it is possible that the interactions with the chemical compounds of EC can affect negatively and/or positively to the activity of AS-48. For example, AS-48 can be retained by electrostatic or hydrophobic interactions with the components of the EC thus impeding the action on *Listeria*. On the contrary, some components of EC (e.g. sucrose esters) can act synergistically with the enterocin, increasing its activity.

Antilisterial activity in peptone water was also studied (Figure 2). The population of *L. monocytogenes* in liquid medium without EC proliferated from 5.5 (zero time) to 7.4 log CFU/mL (10 h). The chitosan-based coating was the most effective as it achieved the complete elimination of *Listeria* within the first 30 min of exposure to the product (Figure 2B). The addition of AS-48 to this coating increased the efficacy against the pathogen, shortening the time of elimination to 20 min. SE-based EC also showed a significant ($P < 0.01$) antilisterial activity yielding reductions on the control of 3.8 log CFU/mL within the first hour of exposure and the total elimination after 5 hours of incubation (Figure 2A). In this case, the addition of AS-48 also increased the antibacterial effect, achieving the elimination of *Listeria*

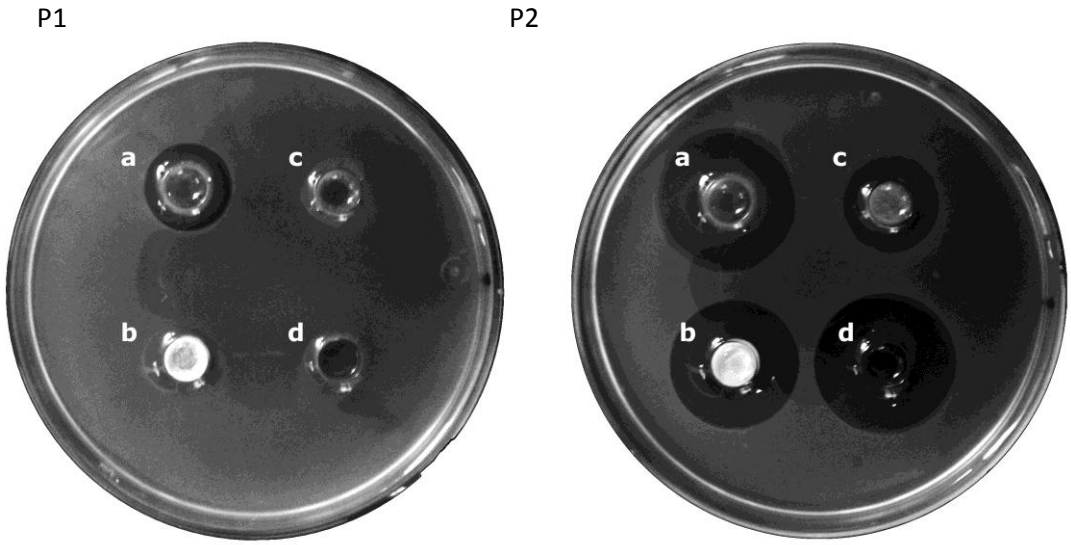

Figure 1. Representative images of inhibition zones against *Listeria monocytogenes* produced by edible coating with and without AS-48 (50 µg/g), assayed by the agar well diffusion test a) Chitosan- based edible coating b) Sucrose esters-based edible coating c) Fish gelatin-based coating d) ACETEM -based coating. P1) Edible coatings alone. P2) Edible coatings with AS-48.

Table 1. Antilisterial activity of several edible coatings with and without enterocin AS-48 (50 µg/g), assayed by the agar well diffusion method and broth microdilution assay (Minimum Bactericidal Concentration, MBC)

	Clear zones (mm) (agar well diffusion method)	**MBC (µg/mL)** (broth microdilution assay)
SE-based edible coating	0	500
SE-based edible coating containing AS-48	23	0.9
Chitosan-based coating	15	4
Chitosan-based coating containing AS-48	25	0.4
ACETEM-based coating	0	125
ACETEM -based coating containing AS-48	25	31.25
Fish gelatin-based coating	0	> 1.000
Fish gelatin-based coating containing AS-48	16	31.25

within the first hour of exposure. The application of the coating based on ACETEM also showed anti-listerial activity in vitro, with reductions in growth that ranged from 1 log in the

first hour of contact, to up to 3.0 log CFU/mL after 10 h. The incorporation of AS-48 to this coating showed a significant ($P < 0.001$) increased effect, achieving a complete elimination of *L. monocytogenes* within the first 15 min of exposure (Figure 2C). Worse results were obtained for fish gelatin-based coating, as it did not exhibit antimicrobial activity in vitro although the addition of AS-48 significantly improved the anti-listerial activity with reductions of up to 3.5 log CFU/mL. Thus, despite an overall improvement in the efficacy, a complete elimination of the pathogen with this AEC was not achieved.

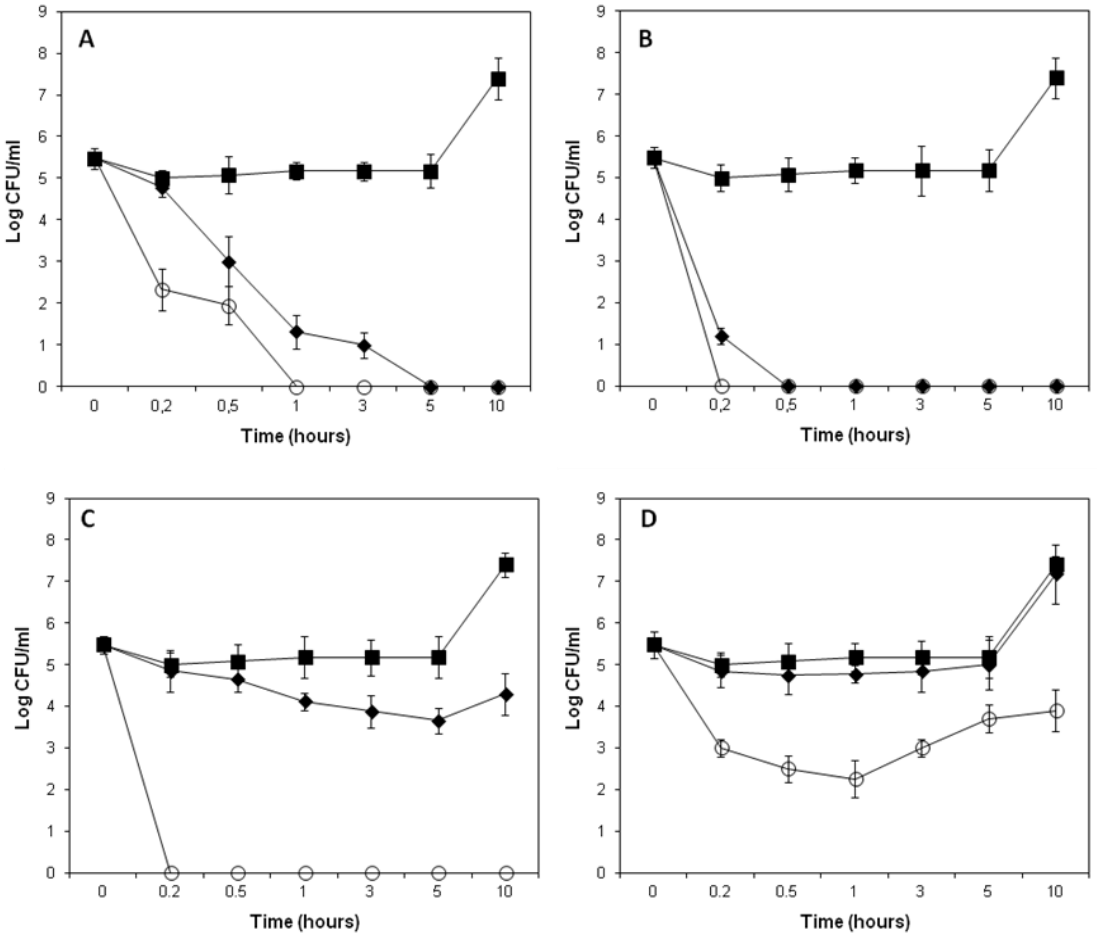

Figure 2. Inhibition effect of enterocin AS-48-incorporated edible coatings against *Listeria monocytogenes* in 0.1% peptone water. A) SE based coating; B) Chitosan based coating; C) ACETEM based coating; D) Fish gelatin based coating. Results expressed as log CFU/mL. Untreated control (■); edible coating alone (♦); edible coating containing AS-48 (10 µg/g) (○).Values are the average ± SD (error bars) of two independent experiments.

3.2. Effectiveness of enterocin AS-48 incorporated into a sucrose esters-based edible coating *in the control of* Listeria monocytogenes *in cut melons.*

The demand for minimally processed and ready-to-eat fruits has considerably increased in the last decades. Preparation steps such as cutting or slicing allow microbial

infiltration in the tissues and therefore, cutters and slicers can be potent sources of contamination. In particular, melons are regarded as potentially hazardous foods because they could favour the growth of pathogenic bacteria due to their low acidity and high water activity. The ability of *L. monocytogenes* to proliferate in this type of array has been shown (Fang et al., 2013). In fact, one of the most recent and best known outbreaks gdue to its magnitude occurred in the USA in 2011 caused by consuming contaminated melons (cantaloupe type), started in an exploitation in Colorado and claimed more than 30 deaths across 28 states (McCollum et al., 2013).

In previous studies, it has been determined the great efficacy of enterocin AS-48 alone and in combination with chemical preservatives against *L. monocytogenes* inoculated in whole and sliced fruits (including melon slices) under different storage conditions (Cobo Molinos et al., 2008). However, this is the first attempt in which the effectiveness against Listeria of AS-48 incorporated into an EC in melon slices is evaluated.

Starting from an initial concentration of 2.6 log units, *L. monocytogenes* proliferated in control batches of melon up to a level of 4.6 log units after 7 days of storage at 4 °C (Figure 3). These results on survival of the Listeria in melons are according with Cobo Molinos et al. (2008) and confirm the risk of *L. monocytogenes* contamination in melons and the need to apply additional measures to prevent survival and proliferation of this pathogen. These authors were able to maintain the Listeria at approx. 2 log CFU/g during 5 days at 6 ºC, but Listeria counts increased above 4 CFU/g after 7 d of storage. The application of FOODCOAT FR slightly reduced the counts of Listeria (around 0.6 log units) compared to the control batch. When this EC was supplemented with AS-48, the concentration of the pathogen was significantly ($P < 0.01$) reduced in a dose-dependent manner (Figure 3). Thus, the coating with 10 µg/g of enterocin AS-48 achieved reductions up to 2.7 log.CFU/cm^2 compared to the control batch after 7 dof storage. The best results were observed at the highest concentration of AS-48 (20 µg/g), achieving reductions of 2.6, 3.6 and 3.9 logCFU/cm^2 after 1, 5 and 7 dof storage, respectively.

One of the major growth segments in the food retail industry is fresh and minimally processed fruits and vegetables. This new market trend has increased the demands to the food industry for seeking new strategies to increase storability and shelf life and to enhance microbial safety of fresh products. Several ECs have been widely used in the IVth range sector with this aim. By regulating the transfer of moisture, oxygen, carbon dioxide and flavouring compounds, ECs have shown the capacity to improve food quality and extend the shelf life of these foodstuffs (Lin et al., 2007). Thus, various authors have shownthe

effectiveness of applying coatings of different nature to improve the quality and extend the shelf life of cut melon (Oms-Oliu et al., 2008; Poverenov et al., 2014).

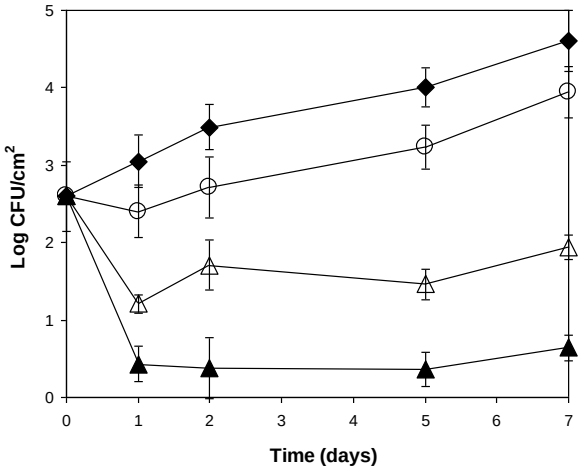

Figure 3. Effect of enterocin AS-48 incorporated in a sucrose esters of fatty acids-based edible coating on the viability of *Listeria monocytogenes* CECT 4032 in cut melons stored at 5 ºC. Control (♦); edible coating alone (○); edible coating containing AS-48 (10 µg/g) (■); edible coating containing AS-48 (20 µg/g) (▲). Values are the average ± SD (error bars) of two independent experiments.

In addition, edible coatings can also carry functional ingredients such as antioxidants, antimicrobials, nutrients, and flavours to further enhance food stability, quality, functionality, and safety. In recent years there has been a growing interest in the application of AECs that work as additional protection against pathogens. Through the addition of antimicrobial agents, such as essential oils or bacteriocins, the antimicrobial activity of these coatings can be extended. For instance, some authors have reported on the effectiveness of incorporating essential oils in alginate-based EC for the control of *Salmonella enteritidis* in melon slices (Raybaudi-Massilia et al., 2008). Ukuku et al. (2002) proved that nisin application as a sanitizer on whole melon surfaces prevented further transfer of the inoculated Listeria to fresh-cut pieces. The same author also evaluated the effects of this bacteriocin in combination with other preservatives such as EDTA, sodium lactate or citric acid for reducing pathogens on whole and fresh-cut melons. Other authors have also proven that nisin reduced *L. monocytogenes* on honeydew melon slices, especially when used in combination with a phage mixture (Leverentz et al., 2003).

In addition, the use of FOODCOAT FR has been reported to delay maturation and reduce weight loss during postharvest storage (Alonso et al., 2004), so it is used in the coating of melon. Its fungistatic properties have also been described (Calvo-Garrido et al., 2013), but it is not the case of its antilisterial effect. In our work, it has been shown that the

incorporation of AS-48 in FOODCOAT FR increases the effectiveness of the SE-based EC against pathogens such as *L. monocytogenes*. The results shown in the present work are in line with those obtained in previous studies as they prove the enhanced bactericidal activity of enterocin AS-48 in combination with sucrose palmitate and sucrose stearate against *L. monocytogenes* in ready-to-eat salads (Cobo Molinos et al., 2009).Therefore, the use of EC combined with AS-48 could be a novel promising approach for extending the shelf life and safety of fresh ready-to-eat fruits.

3.3. Effectiveness of enterocin AS-48 incorporated into an acetylated monoglicerides-based edible coating *in the control of* Listeria monocytogenes *in fresh cheese.*

The large majority of listeriosis cases are caused by foodborne transmission, which accounts for 99% of human cases (Allerberger et al., 2010). Particularly, dairy products are related as one of the main vehicles of *L. monocytogenes* (Gaulin et al., 2012; Jackson et al., 2011), being one of the most important recent outbreaks in Europe, caused by multinational invasive listeriosis linked to Quargel cheese produced in Austria (Fretz et al., 2010). Cheeses are usually produced by methods with a significant risk of cross-contamination with *Listeria*, but a novel approach to reduce this risk could involve the activation of traditionally used ECs in cheese with antimicrobial agents incorporated, such as bacteriocins with anti-listerial activity.

During the ripening period, the specific flavour of naturally ripened cheese is developed. Cheese coating plays an important role in this process. And it is also essential to avoid dehydration and provide an optimal protection during storage and transport. In addition, coatings act as vehicles for the application of antifungal additives such as natamycin or propionates. Among the most popular ECs in cheese are those based on ACETEM or other fatty-acids derivatives. It is well known that ACETEM-based coatings can extend the life of a variety of foods. Liebich et al. (1986) and Prändl et al. (1988) reported the effectiveness of coatings based on acetylated monoglycerides for extending meat freshness and shelf life. Years later, Stuchell et al. (1995) assessed the benefits of applying an ACETEM-based coating to reduce the loss of moisture and to prevent lipid oxidation in salmon. This type of product creates an edible waxy barrier on the surface of the foodstuff that protects it against spoilage and water loss and that is compatible with food additives, such as preservatives, antioxidants or colourings.

In the present work we have evaluated the effectiveness of an EC, based on ACETEM supplemented with AS-48, for the control of *L. monocytogenes* in a fresh cheese

model. *L. monocytogenes* was able to grow in the fresh cheese system at 5 °C, reaching a level of 6.6 log CFU/mL within 20 days (Figure 4). The application of the ACETEM-based EC slightly reduced the counts of Listeria (around 0.5 log) compared to the control batch. The coatings supplemented with AS-48 reduced the concentration of *L. monocytogenes* in a dose-dependent manner. Thus, the coating with 20 µg/g of enterocin AS-48 achieved reductions of approx. 2 log compared to those treated with the coating alone along the entire 20 d period of storage. The best results were observed at the highest concentration of AS-48 (40 µg/g). Even though there was not a complete elimination of *Listeria*, the application of this AEC achieved significant reductions ($P < 0.05$) compared to those batches treated with EC alone: 2.7, 3.8, 3.5 and 4.4 log CFU/mL after 1, 5, 10 and 20 days of storage, respectively. These results prove the efficacy of ACETEM-based ECs including AS-48 on the control of *Listeria* on cheese, being a novel and valid approach to increase the food safety in this food.

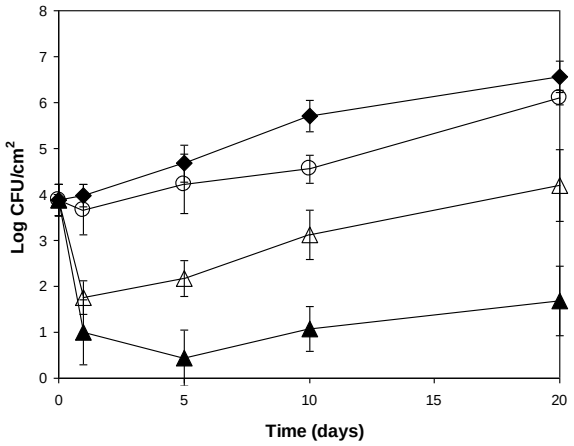

Figure 4. Effect of enterocin AS-48 incorporated in an edible coating based on acetylated monoglicerides on the viability of *Listeria monocytogenes* CECT 4032 (clons 1 and 2) in fresh cheese stored at 5 ºC. Control (♦); edible coating alone (○); edible coating containing AS-48 (20 µg/g) (■); edible coating containing AS-48 (40 µg/g) (▲). Values are the average ± SD (error bars) of two independent experiments.

3.4. Effectiveness of enterocin AS-48 incorporated into an edible coating based on fish gelatin to prevent the spoilage of raw hake fillets

Fishery products are highly perishable. Their shelf life is conditioned upon the loss of sensory properties associated with the growth of spoilage flora responsible for changes in odour, flavour and texture of the fish meat. We can interfere beneficially through the use of ECs on the spoilage microbiota of the fish, by inhibiting its growth and by delaying the emergence of any adverse effects (Heydari et al., 2015). Proteins are considered vital ingredients to form ECs because of their good properties regarding mechanical aspects,

solubility and transparency. Within this type of coatings, those based on gelatin are among the most i*nteresting ones because of its film-forming properties.*

Gelatin is widely used in the food industry because of its nutritional characteristics. It can be obtained from a wide range of sources, but compared to mammalian gelatin, fish gelatin has potential applications. The physical and structural properties of a gelatin-based coating are mainly influenced by the use of plasticisers, such as glycerol, or other additives that improve its gas barrier properties, like alginates or lipid substances. In addition, as for other ECs substrates, the incorporation of natural antimicrobial compounds into fish gelatin extract may add functionality to these ECs by improving the shelf life and safety of food products. The purpose of this experiment was to determine the effect of adding AS-48 into a fish gelatin-based EC in fillets of raw hake stored at 5 ºC against *Lb. acidipiscis* and *S. carnosus,* two bacteria associated to seafood products.

S. carnosus was also able to grow in the raw hake system and to reach a level of 7.1 log CFU/cm^2 in fillets at the end of the storage (Figure 5A). There was no significant effect on the population of staphylococci after the implementation of the fish gelatin-coating, but the addition of AS-48 into the coating conferred it antimicrobial effect against *S. carnosus,* as the counts in the fillets covered with the mixture AS-48/gelatin decreased to 3.1, 2.8, 1.6 and 1.8 log with respect to uncoated control at 1, 4, 6 and 8 days, respectively (Figure 5B). Thus, AS-48 has proven antimicrobial activity when it is added to a protein-based EC against spoilage of raw hake fillets.

Lb. acidipiscis was able to grow in the raw hake system at 5 °C and to reach a level of 6.1 log CFU/cm^2 at the end of the storage (Figure 5B). In fillets coated with the gelatin-based EC alone *Lactobacillus* growth was similar to this observed in uncoated ones. The combination of AS-48 (25 µg/g) with a fish gelatin-based EC had a significant effect in controlling *Lb. acidipiscis* in the raw hake fillets from the beginning of the storage, achieving reductions in the population of lactobacilli of 1.4, 2.4, 2.8 and 2 log CFU/cm^2 after 1, 4 6 and 8 days, respectively with respect to the control batch.

Other authors have shown the ability of coatings based on fish gelatin to extend the shelf life of seafood. For instance, Jiang et al., (2011), showed the effectiveness of gelatin-based coating to delay the microbial growth and extend the shelf life of white shrimps. The effect of adding nisin in protein-based EC for the control of pathogens and spoilage microorganisms has also been shown. Thus, Murillo-Martínez et al. (2013) proved the efficacy of EC based on whey protein isolate (WPI) supplemented with this bacteriocin in the

control of *Listeria innocua* and *B. thermosphacta* Similarly, Lungu and Johnson (2005) showed the anti-listerial activity of an EC based on corn protein supplemented with nisin in turkey frankfurter pieces. In fish gelatin-based EC, analogous trials involving nisin have been conducted on ham and bologna (Gill and Holley, 2000) or Turkey-type sausages (Min et al., 2010). However, up to our knowledge, this is the first attempt to include AS-48 into a fish gelatin-based EC, and in view of the results obtained, it appears to be a promising tool to *control* spoilage bacteria in fishery products.

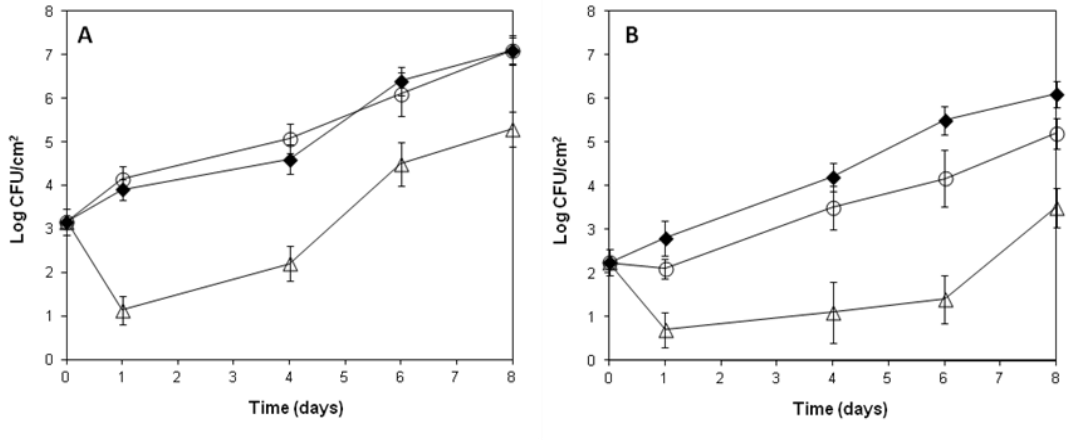

Figure 5. Effect of enterocin AS-48 incorporated in an edible coating based on fish gelatin on the viability of *Staphylococcus carnosus* subsp. *utilis* DSM 11677 (A) and *Lactobacillus acidipiscis* DSM 15836 (B) in raw hake stored at 5 ºC. Control (♦); edible coating alone (○); edible coating containing AS-48 (25 µg/g) (Δ). Values are the average ± SD (error bars) of two independent experiments.

3.5. Effectiveness of enterocin AS-48 incorporated into a chitosan-based edible coating to prevent the spoilage of cooked ham

The shelf life of cooked and sliced meat products is limited mainly because of microbiological spoilage (Vercammen et al., 2011; Kalschne et al., 2015). *B. thermosphacta* and lactic acid bacteria (LAB), suchas *Lactobacillus* and *Leuconostoc* strains contribute actively to the spoilage of these foodstuffs, being identified among the main microbial groups involved in the spoilage of sliced vacuum-packed cooked ham (Vasilopoulos et al., 2008;. Hu et al., 2009; Kreyenschmidt et al., 2010; Vercammen et al., 2011; Kalschne et al., 2015). As a result of bacterial growth, off-flavours, mucosal exudates, gas production and discoloration can be observed (Hu et al., 2009; Audenaert et al., 2010). The extension of the shelf life of meat products by the use of an EC, acting as a barrier and a carrier of antimicrobial substances to inhibit spoilage microorganisms, is one of their most promising applications (Sánchez-Ortega et al., 2014).

In this work, we have tested the efficacy of a chitosan-based EC combined with AS-48 for the control of *Lb. sakei* CTC 245 and *B. thermosphacta* CECT 847 in sliced vacuum packed cooked ham stored at 4 ºC. We have previously shown that enterocin AS-48 incorporated directly as an ingredient to the meat was very effective in the control of these spoilage bacteria in a cooked ham model system (Baños et al., 2012). In this case, we have tested the ability to enhance the antimicrobial properties of a chitosan-based EC applied on the surface of *cooked* ham slices previously inoculated with *Lb. sakei* and *B. thermosphacta*.

Lb. sakei CTC 245 was able to grow in the cooked ham system at 5 ºC up to levels of 6.6 log CFU/cm^2 (Figure 6A). The application of the chitosan-based EC reduced *Lactobacillus* counts in the slices by 1.7 log with respect to the control after 30 d of storage at 5 ºC. The supplementation of EC with AS-48 (20 and 40 µg/g) had a clear dose-dependent antimicrobial effect from the beginning to the end of the storage. Thus the addition of 20 µg/g AS-48 to the EC caused significant ($P < 0.05$) reductions in lactobacilli counts of 1.1, 1.6, 2.1, 2.9 and 4.4 log with respect to control batches at 1, 5, 10, 15 and 30 days respectively. At 40 µg/g, the respective reductions were 1.9, 2.4, 3.2, 4.5 and 5.7 log CFU7cm^2 (significant difference at $P < 0.01$). In comparison with the chitosan-based EC without bacteriocin, the reduction of lactobacilli counts caused by the EC supplemented with 20 µg/g were from 0.85 to 2.7 log units at 1 and 30 days. When the concentration of AS-48 in the EC was 40 µg/g, the counts were reduced from 1.9 logs in the first day up to 5.7 after 30 d of storage at 5 ºC.

B. thermosphacta comprises part of the natural spoilage microbiota of meat. It was also able to grow in the cooked ham system and, on day 20 of storage, bacterial counts reached 7.2 log CFU/cm^2 (Figure 6 B). The application of the EC by itself did not cause significant reductions in *B. thermosphacta* population. Other authors (Soultos et al., 2008), have investigated the effect of chitosan (1% w:w) on *B. thermosphacta's* population in raw sausages reporting a reduction of 1.6 log CFU/cm^2 as compared to untreated samples. The incorporation of 20 µg/g AS-48 conferred antimicrobial properties to the coating as the count reduction with respect to the control reached 2.3 log CFU/cm^2 after 15 days. As in the results obtained previously for the other EC, the effect of the addition of a greater dose of AS-48 (40 µg/g) yield better results, achieving significant reductions ($P < 0.01$) of 1.9, 2.4, 3.3, 4.6 and 5.7 log CFU/cm^2 at 1, 5, 10, 15 and 30 days of storage. These results showed the ability of AS-48 to increase the antimicrobial activity of chitosan-based EC against spoilagebacteria.

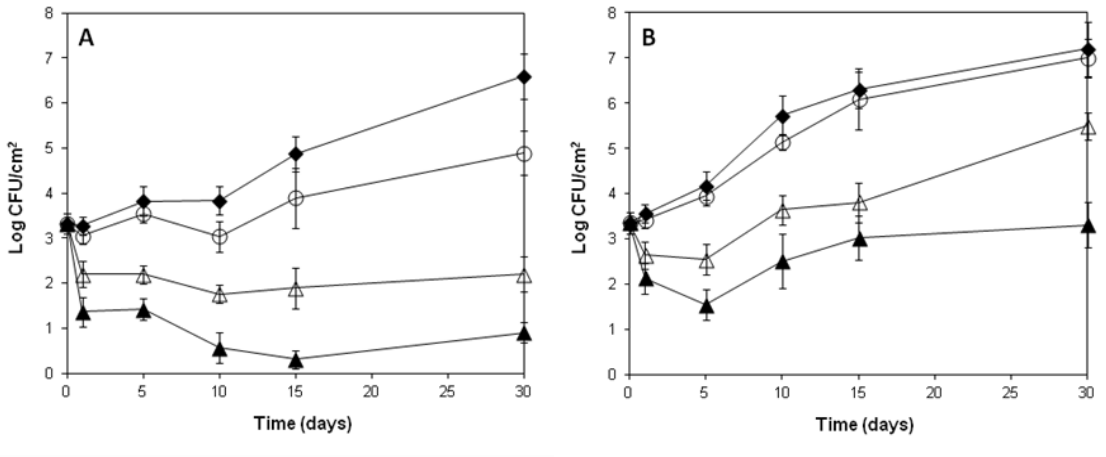

Figure 6. Effect of enterocin AS-48 incorporated to an edible coating based on chitosan on the viability of *Lactobacillus sakei* CTC 245 (A) and *Brochothrix thermosphacta* CECT 847 (B) in sliced vacuum-packaged cooked ham stored at 5 ºC. Control (♦); edible coating alone (○); edible coating containing AS-48 (20 µg/g) (Δ); edible coating containing AS-48 (40 µg/g) (▲). Values are the average ± SD (error bars) of two independent experiments.

In recent years, chitosan−based EC have been used to extend the shelf life of most foodstuffs. For example, applications in meat products have been reported by several authors, as a trial with salami (Moreira et al., 2011) and chicken fillets (Petrou et al., 2012). It has also been used to improve the preservation of seafood. Günlü and Koyun (2012), showed the efficacy of chitosan films to extend the shelf life of sea bass fillets, and more recently, (Wu, 2014) also showed the positive effects of chitosan-based EC on preservation of *white* shrimp.

Combined effects of chitosan with other antimicrobial agents, such as organic acids, essential oils or bacteriocins have also been recently explored. Beverlya et al., (2008) reported the anti-Listeria properties of chitosan-based coatings combined with acetic and lactic acids. Ojagh et al., (2010) reported that the application of a chitosan coating enriched with cinnamon oil reduced counts of *L. monocytogenes* on refrigerated rainbow trout. Among the bacteriocins supplementing ECs, nisin is by far the most studied. Many authors have evaluated nisin effectiveness in chitosan coatings to extend the shelf life and pathogen control products. For instance, chitosan-based coatings combined with nisin or allyl isothiocyanate (AIT) reduced Salmonella contamination in melons (Chen et al., 2012). Guo et al. (2014) reported the effectiveness against *Listeria* of a chitosan-based coating combined with lauric arginate ester (LAE) and nisin on surfaces to RTE meat. However, this is the first evidence of the antimicrobial effect of a chitosan-based

EC combined with AS-48 to inhibit spoilage meat microorganisms, such as *Lb. sakei* and *B. thermosphacta, ensuring* a longer shelf life of sliced vacuum-packaged cooked ham.

The results of the present study indicate that the incorporation of AS-48 into different edible coatings is a good strategy to protect the food against contamination and the proliferation of pathogens, such as spoilage bacteria. The results are influenced by the type of food, the edible coating applied and the target bacteria. Therefore, it will be necessary to establish the optimal conditions regarding EC type and AS-concentration according to the food to be protected *and* the target bacteria.

Acknowledgements

This work *was* supported by Grant (P07-AGR-02539) from the Junta de Andalucía.
We *would* like to acknowledge the work of Jose Manuel García Madero in editing the text.

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I.4. Biocontrol of *Listeria monocytogenes* in fish by enterocin AS-48 and *Listeria* lytic bacteriophage P100.

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Abstract

The purpose of this study was to determine the ability of enterocin AS-48 to control *Listeria monocytogenes* in fish during storage at 4 ºC. AS-48 (0.37 µg/cm^2) was tested singly and in combination with phage P100 (2.3 × 10^7 PFU/cm^2) on a cocktail of 10^3 CFU/cm^2 *L. monocytogenes* strains inoculated in fillet tissues of raw hake and salmon and in smoked salmon. In raw fish, AS-48 alone reduced listeria with respect to the untreated control by 1.68, 2.79, 2.9, and 3.13 log CFU/cm^2 (in hake) and by 1.9, 2.55, 2.8, and 2.8 (in salmon) at 1, 2, 3, and 7 d, respectively. Phage P100 treatment also yields significant reductions (but lower than AS-48) in listeria counts in both raw fishes. A combined treatment of AS-48/P100 eliminated listeria from hake and salmon fillets from 2 and 1 days respectively. In smoked salmon, AS-48 reduced listeria by 2, 3.4, 4.5, 4.25, and 4.25 log CFU/cm^2 with respect to the control at 1, 5, 10, 15, and 30 d, respectively. P100 treatment also reduced listeria counts but in lesser quantities than AS-48. A combined treatment of AS-48/P100 reduced listeria below detection levels from 1 to 15 d; afterwards, a slight listeria reactivation was detected.

Key words: enterocin AS-48; phage P100; *Listeria monocytogenes;* raw fish; smoked salmon

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1. Introduction

Several authors have reported a high prevalence of *Listeria monocytogenes* in raw and processed fish and seafood, leading to a definitive identification of these foods as carriers of *L. monocytogenes* (reviewed in Jami, Ghanbari, Zunabovic, Domig, & Kneifel, 2014). However, since these foods initially carry low concentrations of *L. monocytogenes*, the risk of contamination is frequently caused by storage in conditions favouring the growth of this bacterium. *L. monocytogenes* has been isolated on the surface and intestines of fish and seafood, but the flesh is usually free of the pathogen unless it is contaminated from a different source (Jami et al., 2014). Common routes for contamination of fish with *Listeria* are its spread from the intestinal contents to other tissues, cross-contamination through contact with other food surfaces or with the processing equipment (conveyor belts, trays, skinning and slicing machines, etc.), and storage (Gudmundsdottir, Gudbjornsdottir, Einarsson, Kristinsson, & Kristjansson, 2006). Current intervention strategies for *L. monocytogenes* control in fish and seafood include chemical treatments such as chlorinated products, ozone, and electrolysed oxidizing water (Norhana, Poole, Deeth, & Dykes, 2010; Wang et al., 2010). In addition, physical treatments such as steam application or pulsed light exposure have also been evaluated for *L. monocytogenes* control (Bremer, Monk, Osborne, Hills, & Butler, 2002; Hierro, Ganan, Barroso, & Fernández, 2012).

One strategy in food preservation, designed as biopreservation, is based on the use of microorganisms and/or their natural products. The use of lytic bacteriophages (phages) and antimicrobial peptides produced by LAB, known as bacteriocins, may provide an effective alternative for decontaminating fish and seafood that can contain various bacterial pathogens such as *L. monocytogenes*. Phages are specific obligate parasites that infect bacterial cells. In the absence of a host bacterium, phages exist in a metabolically inert state. Furthermore, their safe nature (due to the inherent specificity for its bacterial host) makes them excellent tools for food safety applications (Mahony, Auliffe, Ross, & Van Sinderen, 2010). In 2007, the U.S. Food and Drug Administration approved phage LISTEX P100 for use for all raw and ready-to-eat foods and LISTEX™ has been accepted as GRAS (Generally Recognised as Safe) by the FDA (GRAS 198) and USDA (GRAS 218) and can be used as a processing aid in all food products susceptible to *L. monocytogenes* contamination. The application of LAB bacteriocins in food technology is currently intended for use in combination with physical and chemical treatments (Gálvez, Abriouel, Lucas-López, & Ben Omar, 2007; Khan, Flint, & Yu, 2010). This approach may resolve major current challenges for the food industry, mitigating economic losses due to food spoilage and avoiding the transmission of microbial pathogens through the food chain. At the same time, it

has the potential of satisfying the growing consumer demand for foods that are ready-to-eat, fresh-tasting, nutrient and vitamin rich, low in fat and salt, and minimally processed.

Enterocin AS-48 is a cationic circular bacteriocin produced by *Enterococcus faecalis* S-48 with broad bactericidal activity against most Gram-positive bacteria, including pathogens such as *L. monocytogenes*, *Staphylococcus aureus*, *Mycobacterium* spp., *Bacillus cereus*, and some Gram-negative bacteria such some *Escherichia coli* and *Salmonella typhimurium* strains (Abriouel, Valdivia, Gálvez, & Maqueda, 1998; Abriouel, Maqueda, Gálvez, Martínez-Bueno, & Valdivia, 2002; Gálvez, Maqueda, Martínez- Bueno, & Valdivia, 1989). The target of AS-48 is the bacterial cell membrane in which the bacteriocin inserts and destabilizes membrane potential, thus leading to pore formation and cell leakage (Sánchez-Barrena et al., 2003). The features of AS-48, a broad spectrum of antimicrobial activity, stability across a wide range of temperatures and pH, and sensitivity to digestive proteases, (Gálvez, Maqueda, Valdivia, Quesada, & Montoya, 1986; Samyn et al., 1994) make it a promising alternative to chemical preservatives for use as a biopreservative in foods. AS-48 has been shown to be effective in the control of various food-borne pathogens in dairy, meat, and vegetable products (Ananou et al., 2010; Cobo Molinos et al., 2008; Muñoz et al., 2007) and also against several spoilage bacteria in meat products and vegetable-derived foods (Baños et al., 2012; Grande et al., 2007). The purpose of the present work was to test the efficacy of AS-48, applied alone or in combination with phage P100 in the control of *L. monocytogenes* in different fish systems. Due to the increasing consumption of these foodstuffs at home and the growing popularity of trendy Japanese-like restaurants, a higher interest in the safety of these products has been paid by consumers and authorities.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. faecalis UGRA10 (Cebrian et al., 2012) was used as AS-48 producer. *E. faecalis* S-47 (from our collection) was used as the standard indicator strain for bacteriocin activity assays. Enterococci were grown in BHI Brain Heart Infusion broth (BHI, Difco™, Becton, Dickinson and Co., Le Pont de Claix, France) at 37° C for 18 h. For fish inoculation, a total of five *L. monocytogenes strains* isolated from different fish industries were used to obtain more detailed information about the anti-listeria activity of AS-48, given the well-known resistance shown by some *L. monocytogenes* strains. *L. monocytogenes* CECT 4032 was used for phage P100 titration. *L. monocytogenes* strains were grown overnight on brain heart infusion

(BHI) (Difco™, Becton, Dickinson and Company, Le Pont de Claix, France) at 37ºC, washed in a sterile saline solution, and then inoculated in fillet tissue samples at the selected concentration. All bacterial cultures were maintained at 4ºC on BHI-agar (BHA) slants.

2.2. Bacteriophage titration

The bacteriophage Listex™ P100, based on listeriophage P100 (Hagens & Loessner, 2014), approved by the U.S. Food and Drug Administration and the U.S. Department of Agriculture, was used in this work. This bacteriophage is strictly lytic and reportedly active against multiple serovars of *L. monocytogenes* (Carlton, Noordman, Biswas, de Meester, & Loessner, 2005). It was obtained from MICREOS BV (Wageningen, The Netherlands). The phage titer was determined using a soft agar overlay assay according to Soni and Nannapaneni (2010) with *L. monocytogenes* CECT 4032 as the host. The phage counts were expressed as plaque-forming units (PFU) per milliliter or square centimetre, as applicable.

2.3. Preparation of bacteriocin AS-48

AS-48 was produced by culturing the strain *E. faecalis* UGRA10 in a food-grade whey-derived substrate (Esprion 300, DMV Int., Veghel, Netherland), supplemented with 1% glucose (as described by Ananou et al., 2008). AS-48 was recovered from cultures by cation exchange chromatography on carboxymethyl Sephadex CM-25 (as in Abriouel, Valdivia, Martínez-Bueno, Maqueda, & Gálvez, 2003). Eluted fractions were tested for bacteriocin activity against the indicator strain S-47 by the agar well diffusion method (Gálvez et al., 1986). The approximate concentration of AS-48 (in µg/ml) in the preparation was estimated by comparing the diameter of inhibition halo around the well with a titration curve obtained with purified bacteriocin. Before use, the eluted fractions were dialysed at 4ºC against distilled water through a 2000-Da cut-off membrane to eliminate NaCl and then sterilized by filtration (0.22 µm, Millipore, Belford, MA, USA).

2.4. Fish fillet tissue preparation and treatment

Challenge test assays were performed according Soni & Nannapaneni (2010). Raw hake fillets, raw salmon fillets and smoked filleted salmon were purchased from a local retail. Fish pieces of approximately 100 cm^2 were cut into slices using a sterile knife. For *Listeria* inoculation, three samples for each treatment and sampling time were placed in sterile polystyrene dishes with the skin face down. Fish samples were then inoculated on the flesh

side with a cocktail suspension of five strains of *L. monocytogenes* (5×10^5 CFU/ml) to yield a final Listeria level of approx. 1×10^3 CFU/cm^2 using a manual spray gun. We have selected this initial level of contamination (10^3 CFU/cm^2) since is a middle value with respect to those found in contaminated foods responsible for *L. monocytogenes* outbreak reports, such as of the Center for Food Safety and Applied Nutrition et al., (2003). The amount of listeria suspension necessary to cover by complete the fillet surface (100 cm^2) was previously determined. Samples were then air dried for 30 min to allow the attachment of pathogen cells. Afterwards, the different treatments were sprayed using an automated spray system (AUTOJET 1550, Spraying System Co.). The first treatment was a physiological saline preparation of phage P100 applied to yield an approximate final concentration of 2.3×10^7 PFU/cm^2. The second treatment was an AS-48 solution (50 µg/mL) applied to yield an approximate final concentration of 0.37 µg/cm^2. Finally, the third treatment consisted of a combination of phage P100 (2.3×10^7 PFU/cm^2) and AS-48 (50 µg/mL). For the untreated controls, each sample received the same amount of saline solution. The different samples were placed in polystyrene dishes (135 x 180 mm, BANDESUR, Alcalá la Real, Spain) with excellent barrier properties against water, oil, fat and oxygen. Then, samples were immediately sealed in Ziplock bags and stored in cool room at 4 ºC. Two independent complete experiments were carried out for each food system for statistical analysis. In each experiment we have used 3 subsamples (fillets) for each experimental condition and sampling time.

2.5. Microbiological sampling and Listeria monocytogenes determination

Samples (100 cm^2) from each treatment were withdrawn in triplicate at selected times to determine viable counts of *L. monocytogenes* and mixed (1/10, w/v) with a dilution medium (0.1% peptone, 0.85% NaCl). Homogenization was performed in a Masticator blender (IUL, Barcelona, Spain) for 1 min followed by serial 10-fold dilution and plating on chromogenic selective agar COMPASS Listeria Agar (Biokar Diagnostics, Pantin, France). All plates were inoculated with 100 µL of the corresponding dilution with the exception of 10^{-1} dilution which was inoculated at 500 µL/plate. Therefore, the detection level was 10 CFU/cm^2. Bacterial cultures were incubated at 37 ± 2 ºC for 24–48 h before counting the colonies.

2.6. Determination of phage P100 stability during storage at 4ºC

To determine the stability of phage P100, 100 cm^2 of raw hake fillet tissue were surface-treated with phage P100 to yield a final concentration of approx. 2.3×10^7 PFU/cm^2

and stored at 4ºC. Phages were counted at 0, 1, 4, and 7 days. Ten grams of fillet sample were homogenized in 90 mL of peptone water, and 1 mL of the homogenate was sterilized by filtration through a 0.22 mm *low protein*-binding *syringe* filter. The filtrate was then tested for PFU counts as described in Subsection 2.2. Two independent complete experiments were carried out.

2.7. Determination of enterocin AS-48 stabilities during storage at 4ºC

Bacteriocin AS-48 was applied to fillet tissue of raw hake and extracted as described by Garriga, Aymerich, Costa, Monfort, & Hugas (2002). Briefly, 100 cm^2 of fillet tissue was sprayed with an AS-48 solution (50 µg/mL, final concentration on a fillet surface of approx. 0.37 µg/cm^2). At selected times, samples were homogenized (1:10) in sodium acetate 50 mM, EDTA 100 mM, and Triton X100 0.2% at pH 5 in a blender for 1 min, boiled for 10 min, cooled, and filtered through a filter paper. The bacteriocin in the liquid phase was precipitated with 300 g/L ammonium sulphate, and the pellet was dissolved in phosphate buffer (50 mM, pH 7.2). The sample was heated at 80 ºC for 10 min and then the bacteriocin titre of extracts was determined as in Subsection 2.3 and expressed as µg/cm^2. Two complete independent experiments were carried out.

2.8. Statistical analyses

The statistics were made from the results of two independent experiments. In each experiment we have used 3 subsamples (fillets) for each experimental condition and sampling time. The average data ± standard deviations were determined with Excel software (Microsoft Corp., USA). Statistical analyses were performed using the SPSS-PC 15.0 software (SPSS, Chicago, Ill. USA). Data on microbiological counts were subjected to ANOVA, using the presence of AS-48, the presence of phage P100, or a combination of both as factors. Categories include untreated control fish fillet tissue, fish fillet tissue with added enterocin, fish fillet tissue with phage P100, and fish fillet tissue with both AS-48 and phage.

3. Results and Discussion

3.1. Stability *of phage P100 in raw fish fillet tissue stored at 4ºC*

Figure 1 shows the stability of phage P100 at 4ºC throughout the 7 d storage of the raw hake fillets. As can be seen, the phage P100 titre was quite stable in hake fillet tissue since, out of the initial approx. 7 log PFU/g applied to food, there was only a slight decrease

in the phage P100 titer (1 log PFU/g) at the end of storage. The stability of phages in foods has been described as being dependent on the food in which is applied. Soni & Nannapaneni (2010), for instance, reported a recovery for P100 in raw salmon similar to the one described by us. Leverentz et al. (2003) found that the titer of a mixture of anti-listeria phages remained between 3.9 and 5.4 log PFU on melon slices, increasing 1 log over a period of 7 days, but declined rapidly to non detectable levels on apple slices within 30 min after application.

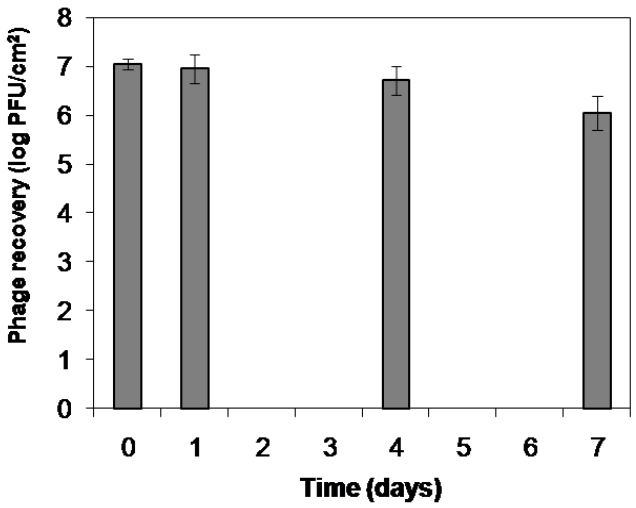

Figure 1. Stability of bacteriophage P100 in raw hake fillets after application at a concentration of approx. 10^7 PFU/cm^2, determined as phage recovered at selected times from the food matrix. Values are the average ± SD (error bars) of two independent experiments.

3.2. Stability of enterocin AS-48 in raw fish fillet tissue stored at 4ºC

Bacteriocin extraction from the raw hake fillet tissue was performed at time 0 and throughout storage. At time 0, we recovered all of the 0.37 µg/cm^2 of AS-48 applied. Afterwards, recovery gradually declined to 0.35, 0.27, and 0.22 µg/cm^2 at 1, 4, and 7 d, respectively (Fig. 2). We also detected an even greater decrease in the recovery of AS-48 during storage in other food model systems such as sausages and cooked ham (Ananou et al., 2005; Ananou et al., 2010). This reduction in the detection of AS-48 may be due to the inactivation of enterocin by components (e.g. proteases) present in the fish tissue and/or to adsorption onto the food matrix. The adsorption issue, as well as its irregular distribution in food, have been reported as two of the main causes negatively influencing antimicrobial efficacy of bacteriocins in foods (Cleveland, Montville, Nes, & Chikindas, 2001). Therefore, the more complex the food matrix is and the greater its content in fat and protein, the higher the adsorption/retention of bacteriocin molecules to them and the less homogenous its distribution in the food. Reinforcing this hypothesis is the fact that we noted greater

effectiveness of AS-48 in fruit juices than in fruits, probably due to the more homogeneous distribution of the enterocin in the liquid food matrix (Cobo Molinos et al., 2008).

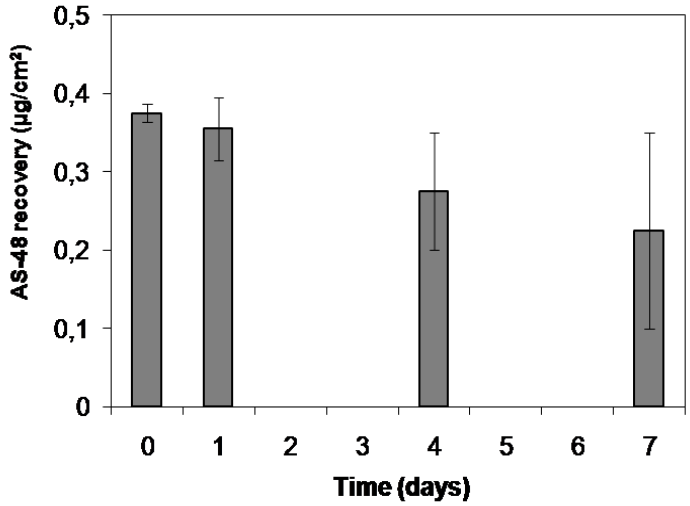

Figure 2. Recovery of enterocin AS-48 from raw hake after spraying at a final concentration of 0.37 µg/cm^2. Values are the average ± SD (error bars) of two independent experiments.

3.3. Effect of enterocin AS-48 alone or combined with phage P100 in the control of Listeria monocytogenes *in fillet tissue of raw hake and salmon and in smoked salmon*

L. monocytogenes was able to grow in the fish fillet of both raw fishes at 4ºC and reached maximum levels of 4.78 log CFU/cm^2 in hake (Fig. 3A) and 4.30 log CFU/cm^2 in salmon at 7 d, the shelf life limit of these types of food (Fig. 3B). AS-48 by itself (0.37 µg/cm^2) significantly reduced ($P < 0.01$) the viable listeria counts from day 1 of storage in both types of raw fillets. In hake, the counts were 1.68, 2.79, 2.9, and 3.13 log CFU/cm^2 lower than the untreated control at 1, 2, 3, and 7 d, respectively. In AS-48-treated raw salmon fillets, listeria counts were 1.9, 2.55, 2.8, and 2.05 CFU/cm^2 log lower than the respective values in untreated samples. Phage P100 also had a relevant effect on *Listeria* growth. Although significant ($P < 0.05$), this anti-listeria effect was less than that achieved by AS-48 when applied in solitary. Thus, in batches of phage-treated hake fillets, the reductions in viable counts were 1.2, 1.85, 2.02, and 1.7 log CFU/cm^2 at 1, 2, 3, and 7 days, respectively (Fig. 3A). In raw salmon fillets, the reductions achieved by treatment with P100 were 0.85, 1.25, 1.0, and 1.06 log CFU/cm^2 at 1, 2, 3, and 7 days (Fig. 3B). The combination of AS-48 with phage P100 had a significant effect ($P < 0.01$) for the control of *L. monocytogenes* in fillets of both types of fish. In these conditions, *Listeria* counts remained below detectable levels (10

CFU/cm^2) from the first and second days of storage in raw hake and salmon respectively (Fig. 3A and Fig. 3B).

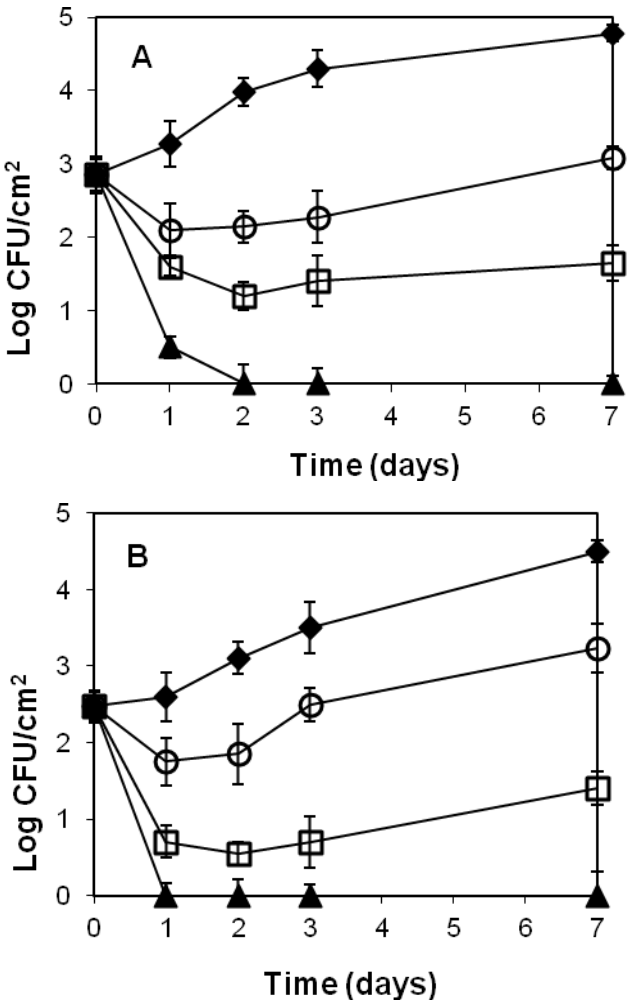

Figure 3. Effect of treatments with enterocin AS-48 (0.37 µg/cm^2) and/or phage P100 (2.3 × 10^7 PFU/cm^2) on the viability of *Listeria monocytogenes* in raw fish models stored at 4ºC. A) Raw hake. B) Raw salmon. control (♦); containing AS-48 (□); containing phage P100 (○); containing AS-48 and phage P100 (▲). Values are the average ± SD (error bars) of two independent experiments.

In fillets of smoked salmon, the listeria counts reached 6.15 log CFU/cm^2 at the end of storage (Fig. 4). Spraying with an AS-48 solution had a significant effect (P < 0.01) as the treated samples showed reductions of 2, 3.4, 4.5, 4.25, and 4.25 log CFU/cm^2 with respect to the untreated control at 1, 5, 10, 15, and 30 d, respectively. This effect was particularly remarkable at 10 d, when *Listeria* counts remained below detection levels (Fig. 4). Although overgrowth of *Listeria* was observed after 10–15 days, the counts at 30 d of storage were significantly lower (4.25 log units, P < 0.01) than in control batches Application of phage P100 alone reduced the viable counts of *Listeria* by 0.85, 2.4, 2.75, 2.34, and 1.58 log CFU/cm^2 relative to the untreated control at 1, 5, 10, 15, and 30 d (significantly different from

control at $P < 0.05$). Combined treatment with AS-48 and phage P100 produced a drastic effect from the first day of storage since the listeria population was kept below detection levels at least between days 1 and 15. A slight regrowth was observed in samples treated with AS-48 and phage P100 from 15 d to 30 d, and the listeria achieved counts of 0.78 log CFU/cm^2 at the end of storage. The regrowth in listeria populations in all samples of smoked salmon and that observed in samples of raw fish treated with AS-48 or phage by separate at the end of storage can be attributed to the inactivation of the bacteriocin, the adsorption to the food matrix, and to the recovery of sublethally damaged listeria (Donnelly, 2002). The implementation of two or more hurdles, such heat, organic acids or nitrate, acting on different cell targets can suppress or diminish the reactivation of sublethally injured bacteria (Leistner, 2000), as it has been found when AS-48 was applied in conjunction with phage P100 (Fig. 3 and 4) in salmon and hake or with nitrate/nitrite, pentasodium tripolyphosphate, and pyrophosphate and/or heat in cooked ham (Ananou et al., 2010).

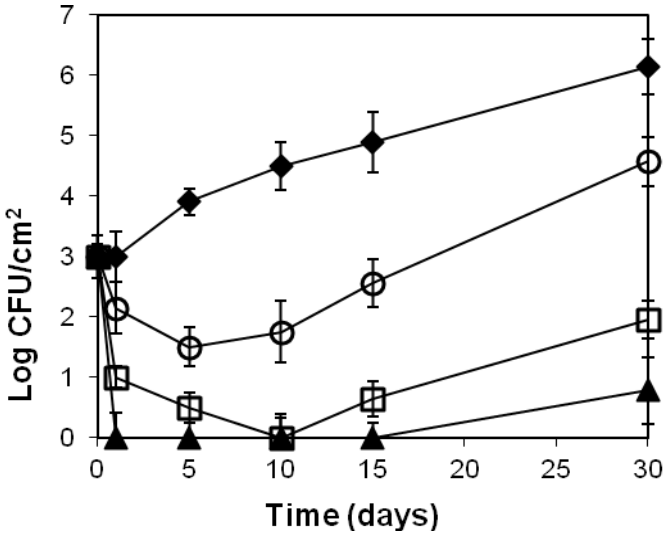

Figure 4. Effect of treatments with enterocin AS-48 (0. 37 µg/cm^2) and or phage P100 (2.3 × 10^7 PFU/cm^2) on the viability of *Listeria monocytogenes* in smoked salmon stored at 4ºC. control (♦); containing AS-48 (□); containing phage P100 (○); containing AS-48 and phage P100 (▲). Values are the average ± SD (error bars) of two independent experiments.

The results presented here indicate the ability of the two biological antimicrobials, enterocin AS-48 and phage P100, to control *L. monocytogenes* in raw and smoked fish products. Separately, AS-48 (0.37 µg/cm^2) and P100 (3.2 × 10^7 PFU/cm^2) produced remarkable reductions in viable listeria counts in all foods tested. Although the dosages cannot be compared due to the marked differences in the nature of the two biological preservatives, AS-48 was much more effective in listeria control. Nevertheless, at the

concentrations used for the two anti-listerials, the complete elimination of the pathogen was not achieved in either case, and it was necessary to apply a combination of enterocin and phage to accomplish this goal. The effective dose of phage P100 has been reported as 7–8 log unit PFU/g, depending on the food tested (Carlton et al., 2005; Soni & Nannapaneni, 2010). Nevertheless, the antilisterial effect of phage P100 is dependent on the environ in which it is applied. Soni & Nannapaneni (2010) claimed that P100 was much more effective in listeria control in a broth model system than in raw salmon fillet tissue. Moreover, Leverentz et al. (2003) found that P100 stability seems to depend on the environ. The causes for this behaviour could be similar to those presented in Subsection 3.2 for bacteriocins. In this respect, we have found that the effect of enterocin AS-48 in food is also greatly affected by physical conditions and chemical compositions (e.g. contents in fat or protein) of foods. In a previous work, for instance, we demonstrated that *L. monocytogenes* CECT 4032 was highly susceptible to AS-48 in BHI broth, with a minimum bactericidal concentration (MBC) of 0.1 µg/mL for a listeria population of 8 log CFU/ml (Mendoza, Maqueda, Gálvez, Martínez-Bueno, & Valdivia, 1999). Further experiments carried out by immersion of vegetable foods in solutions containing AS-48 showed that concentrations of 12.5–25 µg/mL of the enterocin were necessary (alone or even better in combination with chemical preservatives) to achieve effective control of listeria and that the anti-listerial effect of enterocin varied according to the type of vegetable (Cobo Molinos et al., 2005). Even when AS-48 was applied in a cooked ham model at a concentration of 40 µg/g, additional hurdles such as nitrate/nitrite, pentasodium tripolyphosphate, and pyrophosphate and/or heat were needed to provide effective protection against *L. monocytogenes* (Ananou et al., 2010).

We would like to stress the good results achieved, despite the low AS-48 concentration of 0.37 µg/cm^2 used in this work, to protect raw and smoked fish foods against listeria. This fact makes AS-48, alone or combined with phage P100, a promising tool to improve the hygienic quality of raw and smoked fish, particularly with the concern of *L. monocytogenes* contamination.

Acknowledgements

This work was supported by Grant (P07-AGR-02539) from the Junta de Andalucía. We would also like to acknowledge the work of Christine Laurin in editing the text.

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Here we decribe the high suceptibility of *L. monocytogenes* to AS-48.

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This reference is important to learn about the molecular mechanism of action of AS-48.

I.5. Subchronic toxicity study in BALBc mice of enterocin AS-48, an anti-microbial peptide produced by *Enterococcus faecalis* UGRA10.

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Abstract

Few studies report the use of animal models to evaluate the in vivo toxicity of antimicrobial peptides and such investigation is an essential step to ensure its safe use in foods. This study was performed to evaluate any adverse effect of enterocin AS-48, a circular bacteriocin produced by *Enterococcus faecalis* strains, when administered to BALB/c mice at concentrations of 50, 100 and 200 mg/kg for 90 days. Animals dosed with nisin at a dietary concentration of 200 mg/kg served as reference treated group.

There were no deaths and the treatment showed an absence of toxicologically significant effects or clinical signs on body weights, food consumption, urinalysis, haematology, blood biochemistry or gross pathology. Concerning tissue weights, there were no significant differences in the weight of liver, spleen, heart and kidneys between control and mice treated with enterocin AS-48 or nisin. The pathological anatomy study showed the presence of vacuolar degeneration in the hepatocytes of some animals. However, not all animals showed this anomaly and, in any case, the presence was lower than in the group treated with nisin. Thus, no toxicologically significant changes were apparent in mice BALB/c fed a diet containing 0, 50, 100 and 200 mg/kg enterocin AS-48 for 90 days.

Keywords: Bacteriocin, Food additive, enterocin AS-48, Subchronic toxicity, mice.

Article in-progress

1. Introduction

Bacteriocins can be defined as ribosomally synthesized antimicrobial peptides or proteins, which can be posttranslationally modified or not (Jack et al., 1995). Many of these bacteriocins are produced by lactic acid bacteria found in numerous foods. Nisin (E234) is at present the most widely used bacteriocin as a food preservative. Many bacteriocins have been thoroughly characterised so far and there is a broadly knowledge about their structure and mode of action. However, many aspects of these compounds are still unknown. Toxicity data exist for only a few bacteriocins, but the latest research in this field, as well as their long-term use in food suggest that bacteriocins can be safely used. However, most toxicity studies on bacteriocins produced by LAB have been conducted using cell models or based upon acute in vivo tests. Hence the need to focus on the toxicity aspects of these bacteriocins through more detailed studies on chronic and subchronic toxicity.

Enterocin AS-48 is a circular bacteriocin produced by *Enterococcus faecalis* strains from both clinical (Gálvez, et al., 1986; Tomita et al., 1997) and food source, especially from traditional cheeses, including the food-grade strain *E. faecalis* UGRA10 isolated from a farmhouse raw sheep's milk cheese (Cebrian et al., 2012). Enterocin AS-48 was shown to have antibacterial activity exclusively, with no activity against eukaryotic cells, such yeasts and molds, amoeba or red blood cells. Most of the Gram-positive bacteria tested were highly sensitive to enterocin AS-48. Furthermore, early studies showed that many species of Gram-negative bacteria were also inhibited by enterocin AS-48 (Gálvez et al., 1989).

At present, there is a growing interest in using AS-48 as an alternative to conventional preservatives in food. This is due to the great potential of AS-48 to control foodborne pathogens and spoilage bacteria in certain foodstuffs of animal origin, including meats, dairy products and seafood, as well as many different types of vegetable-based foods (Abriouel et al., 2010; Ananou et al., 2010; Baños et al., 2012; Grande et al., 2014; Khan et al., 2010).

Currently, the increasing consumer awareness of the potential health risks associated with chemical additives has led us to investigate the possibility of using bacteriocins produced by lactic acid bacteria as biopreservatives, such as enterocin AS-48. Although numerous studies have dealt with the structure, the mode of action and the effectiveness of this peptide, there are still not enough studies related to the enterocin toxicity. Thus, the aim of the present investigation was to evaluate the subchronic toxicological potential of AS-48 given a diet to BALB/c mice for 90 days.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Enterococcus faecalis UGRA10 (Cebrian et al., 2012) was used as AS-48 producer. *E. faecalis* S-47, from our collection, was used as the standard indicator strain for bacteriocin activity assays. Bacterial cultures were maintained at 4 ºC on BHI-agar (BHA, Schalau, Barcelona, Spain) slants.

2.2. Enterocin AS-48 production

AS-48 was produced by culturing the strain *E. faecalis* UGRA10 in a whey derived substrate, Esprion 300 (ES-300) (DMV Int., Veghel, Netherlands), supplemented with 1% glucose (as described by Ananou et al., 2008). AS-48 was recovered from the cultures by cation exchange chromatography on carboxymethyl Sephadex CM-25 (Abriouel et al., 2003). Eluted fractions were tested for bacteriocin activity against the indicator strain S-47 by the agar well diffusion method (Gálvez et al., 1986). The approximate concentration of AS-48 (in µg/ml) in the preparation was estimated by comparing the diameter of inhibition halo around the well with a titration curve obtained from purified bacteriocin. Before use, the eluted fractions were dialysed at 4 ºC against distilled water through a 2000-Da cut-off membrane to eliminate NaCl, and then sterilised by filtration (0.22 µm, Millipore, Belford, MA, USA). Nisin from *Lactococcus lactis* (N CAS.1414-45-5) used in the present study as reference material was supplied by Siveele B.V. (Breda, The Netherlands) with a concentration of 50%, and a nisin potency of 1.000 IU/mg.

2.3. Diet preparation and analysis

AS-48 and nisin were incorporated at the required levels into an irradiated Global Rodent 2914 powdered diet (provided by the Animal Experimentation Unit of the Universidad de Granada). Diet preparation was developed at the Diet Production Unity of the Animal Experimentation Service of the Universidad de Granada.

Diets with enterocin AS-48 formulated at different concentrations (50, 100 and 200 mg/Kg), nisin (200 mg/Kg) and a control diet with distilled water were prepared. Then, treatment diets were sealed in plastic bags and stored at 4 ºC. In order to maintain the stability of the compounds, diet preparations were developed at 3-week intervals.

2.4. Animals

Ten six-week-old female BALB/c mice were provided by the Animal Experimentation Unit of the Universidad de Granada and were allowed to a 2-week quarantine and acclimation period. After verifying that the mice were in normal health, they were added to the study at the age of 8 weeks. The mice were housed in transparent polypropylene cages on wood chip bedding in an environment-controlled room. Constant conditions of temperature (20–22 ºC) and humidity (50–70%) were maintained, and the room was artificially illuminated to provide a cycle of 12 h of light per day. Diets and city tap water were available ad libitum. The protocol was carried out according to the guidelines of the Helsinki declaration and was approved by the Ethic Committee of Animal Experiment of the Universidad de Granada.

2.5. 90-day feeding study

A similar procedure was followed according to Hagiwara et al. (2010). In order to do so, 50 mice were allocated to five groups (10 mice per group) using a randomised block design, taking into consideration that weight distribution and initial mean body weights between the groups were similar. The animals were fed a diet containing enterocin AS-48 (at doses of 50, 100 and 200 mg/Kg), nisin (200 mg/Kg) and a control diet for 90 days. Possible clinical signs of abnormality, as well as each animal's weights were monitored. Food and water consumption were recorded.

Urinalysis was conducted for ten animals/group at weeks 4, 8 and 12; a semi-quantitative estimation (URIN-10, SPINREACT, Sant Esteve de Bas, Spain) of pH, density, proteins, glucose, ketones, nitrites, bilirubin, leukocytes, occult blood and urobilinogen was included. In addition, urinary sediments were analysed by microscopic examination.

After 90 days, mice were sacrificed by cervical dislocation, and blood was collected by cardiac puncture in EDTA containing tubes in sterile conditions. In order to evaluate the possible changes in physiological functions caused by the oral administration of the bacteriocin, several biochemical and haematological parameters were analysed at the Bioanalysis Unit of the Scientific Instrumentation Centre of the Universidad de Granada. Haematological estimations were carried out using an automatic haematology counter Mythic 22-CT (Orphée, Geneva/Plan-les-Ouates, Switzerland) for the erythrocyte count (RBC), white blood cell count (WBC), haemoglobin (HGB), hematocrit (HCT), platelet count (PLT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular

haemoglobin concentration (MCHC) and percentage of white blood cell differential count. Blood biochemistry determinations were performed with an automatic chemistry analyser BS-200 (Mindray Medical International Ltd., Shenzhen, China). The parameters determined were aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (CRE), glucose (GLU), albumin (ALB), total cholesterol (T-CHO), phospholipid (PL), triglyceride (TG), total protein (TP), sodium (NA), potassium (K) and chlorine (CL).

Furthermore, the following organs from each mouse were removed and weighed: heart, spleen, thymus, kidney and intestine. A full histopathological examination was performed at the laboratories AnaPath (Granada, Spain) on haematoxylin and eosin-stained tissue sections of the liver, kidney, stomach and intestine for all, the control, the nisin and the group of mice fed the highest dose of AS-48.

2.7. Statistical analysis

All results are expressed as the mean ±SD. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and the Student's t−test. Statistical analyses were performed using the SPSS-PC 14.0 software (SPSS, Chicago, Ill. USA) with statistical significance set at $P<0.05$.

3. Results and discussion

In vivo toxicity assessment of each antimicrobial peptide would be an important step forward to consider their use as a food preservative. Few toxicity reports to evaluate in vivo toxicity of LAB bacteriocins have been conducted due to the fact that LAB (and by hence their bacteriocins) are widely found in foods and regarded as safe. Nisin is by far the most studied bacteriocin from a toxicological point of view, with numerous studies on cellular models or experimental animals (Frazer et al., 1962; Hagiwara et al., 2010; Hoover and Steenson, 1993). This work aims to analyse the toxicological effects of oral administration of enterocin AS-48 for 90 days.

No deaths were observed in the control, nisin or AS-48 treated groups during the course of the study. Regarding clinical signs, no signs of abnormality were observed in the control, nisin or AS-48 treated groups during the course of the study. Furthermore, during the experimental protocol, neither noticeable activity nor behavioural changes were observed in mice. During 90-days feeding, diets with nisin and AS-48 had no adverse effects on the food intake of mice, and there were no differences in food intake between the control and the

treated groups (Table 1). No significant differences were observed concerning the body weight gain between the groups (Table 1).

Concerning tissue weights, there were no significant differences in the weight of liver, spleen, thymus, heart and kidney between control and mice treated with enterocin AS-48 or nisin (Table 2). Furthermore, the weight/length ratios of small and large intestine did not statistically differ between the experimental groups. In addition to the weight, the coloration and aspect of the analysed tissues was normal and no significant differences were observed between the groups. Although most of the authors have found no evidences on abnormalities in these aspects, some studies showed low in vivo toxicity for some bacteriocins, such as bovicin HC5, with reduction of weight gain and mild changes in the small intestine of BALB/c mice (Paiva et al., 2013).

Table 1. Body weight and food intake of control and nisin and enterocin AS-48 treated mice.

	Control	AS-48 (mg/Kg)			Nisin
		50	100	200	200
Initial weight (g) time 0 d	21.45 ± 1.04	21.37 ± 1.09	21.34 ± 1.31	21.52 ± 1.42	21.34 ± 1.20
Final weight (g) time 90 d	22.24 ± 1.57	22.69 ± 1.15	22.91 ± 1.25	22.07 ± 1.66	22.25 ± 0.97
Food intake (g/mice/day)	2.95 ± 0.42	2.80 ± 0.62	2.90 ± 0.51	2.75 ± 0.32	2.94 ± 0.24

Values are means ± S.D. for groups of 10 mice.

Table 2. Tissue weights and intestine weight/length ratio of control and mice fed a diet containing nisin and AS-48 for 90 days.

	Control	AS-48 (mg/Kg)			Nisin
		50	100	200	200
Liver (mg)	995.5 ± 55.2	982.2 ± 25.1	988.3 ± 70.2	981.7 ± 94.5	945.1 ± 112.2
Spleen (mg)	92.5 ± 14.5	82.3 ± 11.2	95.1 ± 10.5	91.2 ± 7.5	98.5 ± 15.1
Kidney (mg)	129.2 ± 9.2	140.5 ± 21.4	119.2 ± 19.3	153.2 ± 13.4	175.2 ± 32.3
Small intestine (g/cm)	25.7 ± 5.2	22.7 ± 6.2	28.7 ± 7.1.2	25.7 ± 6.3	27.7 ± 3.3
Large intestine (g/cm)	26.9 ± 6.7	28.5 ± 7.5	30.5 ± 10.5	28.5 ± 8.5	29.5 ± 5.1

Values are means ± S.D. for groups of 10 mice.

In agreement with the absence of clinical symptoms in mice, the biochemical and haematological parameters measured in blood samples did not statistically differ between mice control and mice treated with 50, 100 or 200 mg/Kg enterocin AS-48 (Tables 3 and 4). No significant changes were observed in nisin treated group. Urinalysis also showed no significant differences between control and mice treated with enterocin AS-48 or nisin. A similar subchronic toxicity study showed that nisin A at dietary levels of up to 5% for 90 days caused no adverse effects in rats (Hagiwara et al., 2010). Similarly, Gupta et al., (2008) showed no clinical toxicity signs when nisin was administered to pregnant rats. Dabour et al., (2009) using in vivo experiments showed that repeated doses of pediocin PA-1 (250 mg/day for three consecutive days) did not affect the intestinal microbiota and weight change of the animals.

Table 3. Haematology data of control and mice fed a diet containing nisin and AS-48 for 90 days.

	Control	AS-48 (mg/Kg)			Nisin (mg/Kg)
		50	100	200	200
WBBC (10^3/µL)	0.64 ± 0.21	0.81 ± 0.28	0.80 ± 0.51	1.03 ± 0.23	0.88 ± 0.25
RBC (10^6/µL)	5.27 ± 1.32	5.76 ± 1.32	5.39 ± 1.58	5.86 ± 1.24	6.18 ± 1.29
HGB (g/dL)	7.376 ± 1.10	7.05 ± 1.92	7.52 ± 1.94	8.57 ± 1.08	8.9 ± 1.46
HCT (hematocrit)	20.93 ± 5.23	24.53 ± 12.5	24.32 ± 6.14	24.81 ± 5.35	24.15 ± 6.50
MCV (fL)	42.54 ± 0.51	42.84 ± 1.15	42.44 ± 1.08	42.36 ± 0.52	41.81 ± 1.19
MCH (pg)	16.31 ± 0.32	11.95 ± 0.95	15.06 ± 1.53	17.7 ± 0.93	18.09 ± 0.61
MCHC (g/dL)	38.5 ± 0.58	29.04 ± 1.71	24.6 ± 2.32	37.09 ± 2.24	40.8 ± 1.91
PLT (10^3/µL)	610.32 ± 52.32	643.71 ± 52.51	682.5 ± 76.52	680.5 ± 62.69	625.66 ± 71.5
Leukocyte formula					
LYM%	93.50 ± 3.42	93.50 ± 2.52	92.14 ± 2.70	93.27 ± 1.73	94.20 ± 4.2
MON%	0.35 ± 0.12	0.35 ± 0.05	0.41 ± 0.19	0.36 ± 0.25	0.23 ± 0.5
NEU%	5.08 ± 2.29	4.36 ± 2.24	4.18 ± 2.28	4.55 ± 1.22	3.42 ± 2.2
EOS%	1.71 ± 0.52	2.19 ± 1.63	2.81 ± 1.25	2.16 ± 1.30	2.21 ± 0.4
BAS%	0.36 ± 0.51	0.29 ± 0.53	0.63 ± 0.62	0.04 ± 0.11	0.25 ± 0.61

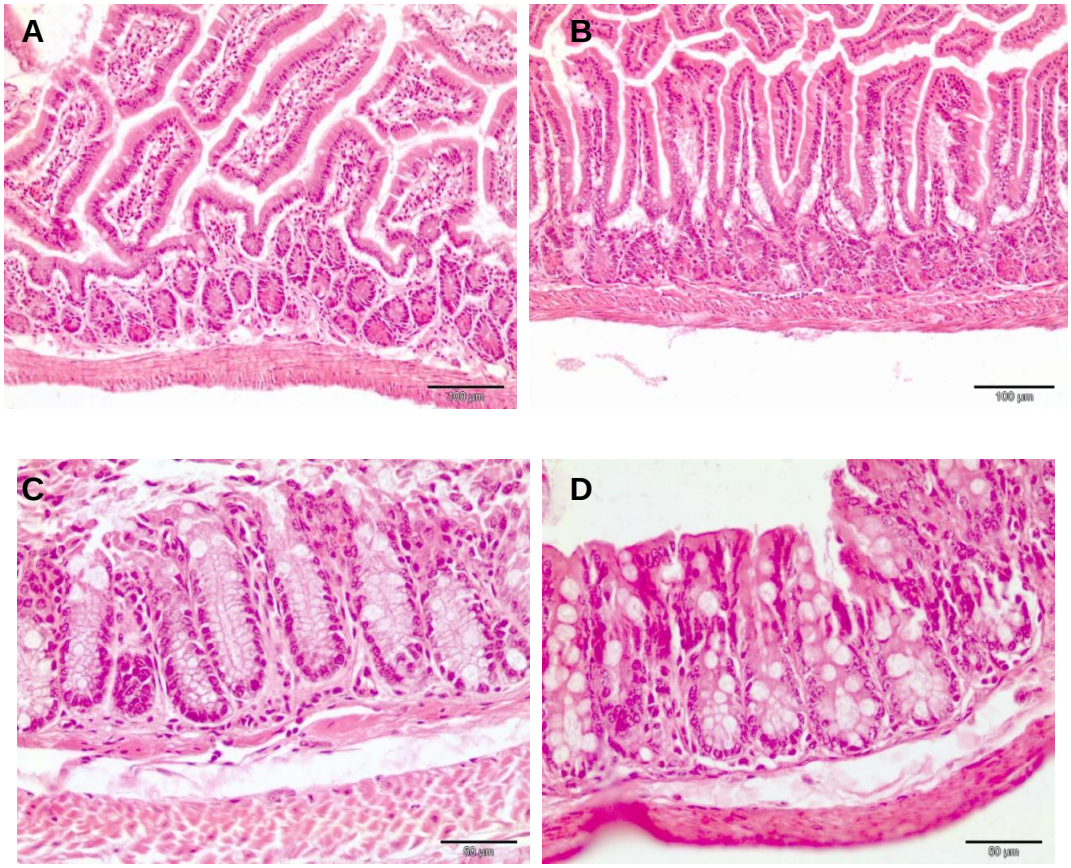
Values are means ± S.D. for groups of 10 mice.

The pathological anatomy study showed no significant abnormalities in the organs tested: heart, spleen, thymus and kidneys, both for control animals and for those treated with AS-48 and nisin. The histopathological study of the small and large intestine showed no significant abnormalities as shown in the picture 1.

Table 4. Blood biochemistry data of control and mice fed a diet containing nisin and AS-48 for 90 days.

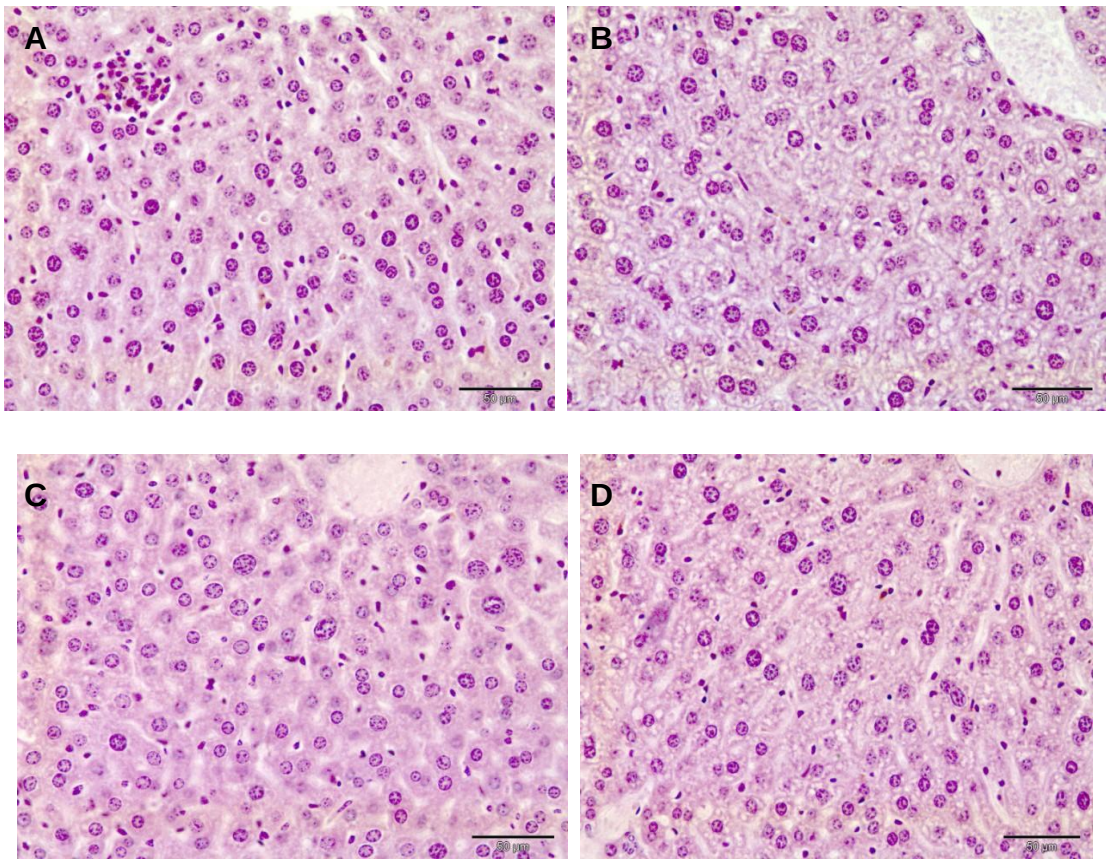
	Control	AS-48 (mg/Kg)			Nisin (mg/Kg)
		50	100	200	200
ALT (U/L)	51.47 ± 5.25	49.41 ± 18.60	57.08 ± 9.55	50.42 ± 6.83	54.89 ± 23.12
AST (U/L)	257.633 ± 75.65	166.82 ± 26.05	252.08 ± 35.89	258.84 ± 25.45	267.31 ± 88.14
ALB (g/dL)	4.66 ± 0.68	4.85 ± 0.58	4.12 ± 0.24	4.85 ± 0.35	4.03 ± 0.79
GLU (mg/dL)	50.35 ± 7.41	47.69 ± 11.04	56.81 ± 12.17	55.34 ± 13.03	58.51 ± 13.85
CRE (mg/dL)	0.26 ± 0.04	0.28 ± 0.05	0.26 ± 0.02	0.3 ± 0.09	0.31 ± 0.03
T-CHO (mg/dL)	84.32 ± 9.53	84.34 ± 6.90	83.29 ± 13.84	85.93 ± 5.43	91.26 ± 7.29
TG (mg/dL)	139.12 ± 24.66	134.84 ± 29.33	133.63 ± 24.24	142.31 ± 2.94	142.31 ± 22.53
TP (g/dL)	6.26 ± 0.51	6.96 ± 0.75	6.76 ± 0.87	6.94 ± 0.74	6.94 ± 0.90
PL (mg/dL)	134.82 ± 12.85	136.25 ± 7.0	130.49 ± 15.22	129.47 ± 6.47	149.67 ± 13.49
NA (mg/dL)	414.32 ± 121.05	514.26 ± 108.02	513.18 ± 94.95	521.62 ± 82.05	526.21 ± 119.61
CL (mg/dL)	310.73 ± 36.68	355.01 ± 27.18	325.09 ± 17.92	323.47 ± 51.74	326.64 ± 42.71

Values are means ± S.D. for groups of 10 mice.



Picture 1. A. Small intestine from control animal. No histopathological lesions. **B.** Small intestine from AS-48 (200 mg/kg). No histopathological lesions. **C.** Large intestine from control animal. No histopathological lesions. **D.** Large intestine from AS-48 (200 mg/Kg). No histopathological lesions.

It has to be mentioned that the most frequent finding was the presence of vacuolar degeneration in the hepatocytes. However, this is generally a reversible change and frequently associated to the intake. Therefore, it is more noticeable in the morning, after the night period, and for this reason it has also been found in the control animals. In this case, the presence was AS-48 dose-dependent. Therefore, the animals treated with higher bacteriocin concentrations showed a higher presence of vacuole. In the case of animals treated with nisin, vacuolar degeneration was found in 5 of 10 animals analysed at a mild to moderated levels, except for one animal in which this finding was insignificant (Picture 2). No animals fed a diet with 50 mg/Kg AS-48 showed vacuolar degeneration but 2 of 10 mice analysed in the group fed the diet containing 100 mg/Kg of AS-48 showed vacuolar degeneration at a moderate or mild level. This abnormality was also found in the group fed the diet containing 200 ppm of AS-48 in 3 of 10 mice analysed at a moderate level.



Picture 2. A. Hepatic tissue from a control animal showing no vacuolar degeneration **B.** Animal from nisin (200 mg/Kg) treated group showing moderate vacuolar degeneration. **C.** Animal from AS-48 (50 mg/Kg) treated group showing no vacuolar degeneration. **D.** Animal from AS-48 (200 mg/Kg) treated group showing moderate vacuolar degeneration.

Vacuolar degeneration is a frequent finding in mice, especially in females. It appears physiologically after food intake. In this case, evidence was greater in the group treated with nisin than in the control group. The mice that received the diet with enterocin AS-48 at concentrations of 100 and 200 mg/Kg showed higher presence compared with the control animals, but showed lower presence than the group treated with nisin. But, since these results are not correlated with biochemical parameters that indicate hepatic function alterations, it seems that these signs are not directly related with the treatments. Nevertheless, complementary studies should be conducted, increasing the doses of bacteriocin or extending the timing to deep this aspect.

In conclusion, the present results indicate that enterocin AS-48 administered at dietary levels of up to 200 mg/Kg for 90 days in BALB/c mice do not cause adverse effects on any parameter examined, and consequently, they may be considered suitable for use as a food preservative.

Acknowledgements

This work was supported by Grant (P07-AGR-02539) from the Junta de Andalucía. We would also like to acknowledge the work of Jose Manuel García Madero in editing the text.

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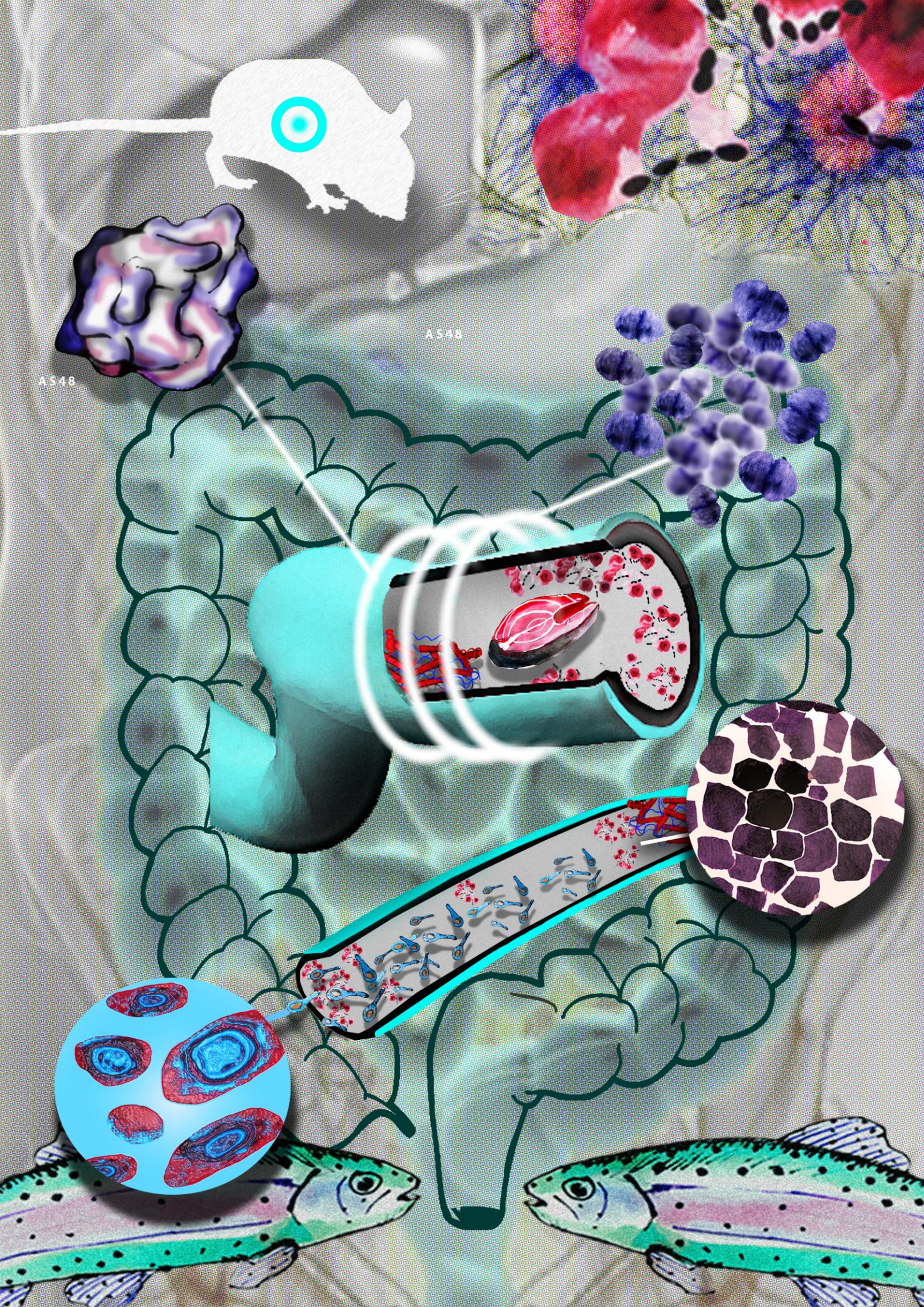
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BLOQUE II. ESTUDIO DE PROBIOSIS DE UNA CEPA PRODUCTORA DE AS-48

II.1. Characterization of functional, safety, and probiotic properties of *Enterococcus faecalis* UGRA10, a new AS-48-producer strain.

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Abstract

Enterococcus faecalis UGRA10, a new AS-48-producer strain, has been isolated from a Spanish sheep's cheese. The inhibitory substance produced by *E. faecalis* UGRA10 was purified and characterized using matrix-assisted laser desorption ionization-time of flight mass spectrometry, confirming its identity with AS-48 enterocin (7.150 Da). Subsequent genetic analysis showed the existence of the *as-48* gene cluster on a plasmid of approximately 70-kb. The UGRA10 strain was examined for safety properties such as enterococci virulence genes, biogenic amine production, and antibiotic resistance. As for most *E. faecalis* strains, PCR amplification revealed the existence of gene encoding for GelE, Asa1, Esp, EfaA, and Ace antigens and for tyrosine decarboxylase. This strain was sensitive to most of the antibiotics tested, being resistant only to aminoglycosides, lincosamide, and pristinamicins. In addition, UGRA10 developed an ability to form biofilms and to adhere to Caco 2 and HeLa 229 cells. More interestingly, this strain shows a high ability to interfere with the adhesion of *Listeria monocytogenes* to Caco 2 cells. Altogether, the results suggest that this broad-spectrum bacteriocin-producing strain has biotechnological potential to be developed as a protective agent in food preservation and as a probiotic.

Keywords*:* enterocin AS-48, *Enterococcus faecalis,* safety properties, probiotic, cell line adhesion*, Listeria monocytogenes* interference.

Published in Food Microbiology. Volume 30, Issue 1, May 2012, Pages 59–67

1. Introduction

The genus *Enterococcus* belongs to a group of microorganisms known as lactic acid bacteria (LAB). This genus consists of non-spore-forming, Gram-positive low G+C content (<50%) catalase negative bacteria that are grouped as individual cocci, in pairs or short chains, and produce lactic acid as the major end product of glucose fermentation. The number of species assigned to this genus has grown continually in recent years, from the 19 species described in 2002 (Giraffa, 2002) to the 34 species currently described in the 2nd edition of Bergey's Manual of Determinative Bacteriology (Svec and Devriese, 2009). They are ubiquitous bacteria, but their primary habitat is the intestine of healthy warm-blooded animals, a complex ecosystem in which a delicate balance exists between the intestinal microbiota and the host; in this system, *E. faecalis* and (to a lesser extent) *E. faecium* are the most dominant species (Kayser, 2003). Moreover, enterococci have been isolated from a wide variety of habitats, including human vaginal secretions (Martín et al., 2008), human milk (Martín et al., 2002), the preen gland secretion of birds (Martin-Platero et al, 2006; Soler et al, 2008), and a broad variety of fermented foods of both animal (milk, cheese, fermented sausages) and vegetable (fermented olives) origins, in which they may have beneficial effects (Foulquié-Moreno et al., 2006).

Enterococci produce a wide array of bacteriocins (enterocins), a family of ribosomally synthesized antimicrobial peptides and proteins with the potential to inhibit the growth of food-borne pathogenic and spoilage bacteria (Franz et al., 2007). Due to their LAB origin, such antimicrobial compounds are attractive to the food industry as they can be more easily licensed for use to improve the hygienic quality and extend the shelf-life of different foods and foodstuffs (Franz et al., 2007; Gálvez et al., 2008). Furthermore, selected *Enterococcus* strains with possible health-promoting capabilities have been used as probiotics in human and animal health promotion to improve the intestinal microbial balance (European Commission, 2011; Foulquié-Moreno et al., 2006; Franz et al., 2011). In fact, some strains are currently in use as therapeutic treatments marketed as Cylactins (Hoffmann-La Roche, Basel, Switzerland), Fargo 688s (Quest International, Naarden, The Netherlands), ECOFLOR (Walthers Health Care, DenHaag, The Netherlands), or Symbioflor 1 (SymbioPharm, Herborn, Germany) (Foulquié-Moreno et al., 2006) to alleviate the symptoms of irritable bowel syndrome and recurrent chronic sinusitis or bronchitis. According to the FAO/WHO regulations (FAO/WHO, 2002), probiotic microorganisms for use in health applications must be non-pathogenic and non-toxic and must survive the transition to the target niche and then persist, serving to protect the host against infection by pathogenic

microorganisms. Antimicrobial activity is thought to be an important means for probiotic bacteria to competitively exclude or inhibit invading bacteria (Carr et al., 2002; Gillior et al., 2008). Nevertheless, enterococci have been associated with a number of opportunistic infections in humans, and they have been implicated as important causes of endocarditis, bacteraemia, and infections of the urinary tract, central nervous system, abdomen, and pelvis (Foulquié-Moreno et al., 2006). Although enterococci do not possess strong virulence factors or toxins, they have several structural and metabolic traits, as well as multiple antibiotic resistances, that some researchers consider to be virulence factors. Nevertheless, none of them have been significantly associated with strains isolated from infections rather than with faecal strains, nor with mortality in patients with bacteraemia (Creti et al., 2004). So, there is growing concern about the dual role of enterococci that has led to an increasing number of studies aimed at discriminating between food-grade and pathogenic strains. Franz et al. (2011), for instance, indicate that the presence of one or more virulence determinants does not necessarily make a strain pathogenic. In fact, in *E. faecalis* Symbioflor 1 (used as a probiotic for more than 20 years without any reported problems), researchers have detected an aggregation substance, collagen-binding protein, capsule formation, and resistance towards reactive oxygen anions (Domann et al., 2007). Nowadays, phylogenomic data and multilocus sequence studies, coupled with virulence research, indicate that a convergence of virulence determinants and antibiotic resistance (often linked to mobile genetic elements) led to the development of specific lineages that cause nosocomial infections (Franz et al., 2011). However, despite intensive ongoing research in this field, there is not yet a global perspective of all the genes and biological processes involved in the pathogenicity of enteroccci. Therefore, keeping in mind the great biotechnological potential of this microbial group, it seems inadvisable to abandon research on their applications as probiotics and in food preservation.

This paper deals with the functional, probiotic, and safety-related properties of the *E. faecalis* UGRA10 strain, a new enterocin AS-48 producer isolated from a handmade sheep's milk cheese. AS-48 is a post-translational modified enterocin with 70 amino acid residues, whose physical-chemical characterization, structure, and genetics have been established elsewhere (reviewed by Maqueda et al., 2004, 2008). AS-48 exhibited several valuable biotechnological features, such as activity against a wide range of Gram-positive and Gram-negative bacteria and stability over a broad range of pH values and temperatures (Gálvez et al., 1986, 1989a, b, c). Furthermore, AS-48 is easy to produce and purify on a large scale using dairy subproducts from whey (Ananou et al., 2008). Therefore, this strain and its bacteriocin are considered potential candidates for improving health and the safety of food

from vegetable, milk, and meat origins (Ananou et al., 2010; Abriouel et al. 2010; Khan et al., 2010).

2. Materials and Methods

2.1. Bacterial strains and culture conditions

The bacterial strains in this study are listed in Table 1. They were used as AS-48 producers, test strains to determine antimicrobial activity, or as positive/negative controls for the specific characteristics investigated. All strains were cultivated routinely on either trypticasein soy broth (TSB, *Escherichia coli* U9, and *Bacillus cereus* LWL1), brain heart infusion (BHI, *Gardnerella vaginalis* HC2), or M-17 (the enterococci) or MRS (the lactobacilli) broths (all from Scharlau, Barcelona, Spain) at 37 ºC, and stored at 4 ºC on the respective agar slants.

Table 1. Bacterial strains used in this work.

Strain	**Characteristics**	**Reference or source**
Enterococcus faecalis T-4-31	AS-48^{+}	Martínez-Bueno et al. (1990)
Enterococcus faecalis S-47	AS-48^{S}	Our collection
Bacillus cereus LWL1	Caseinase^{+} , AS-48^{S}, Gelatinase^{+}	Dufrenne et al. (1995)
Escherichia coli U9	AS-48^{S}, Protease^{-}	Our collection
Enterococcus faecalis FI9190	*asa1*$^{+}$, *gelE*$^{+}$, *hyl*$^{+}$, *cylA*$^{+}$, *esp*$^{+}$	Vankerckhoven et al. (2004)
Enterococcus. faecalis KE2	*efaA*$^{+}$	Creti et al. (2004)
Enterococcus. faecalis KE2	*ace*$^{+}$,	Ben Omar et al. (2004)
Enterococcus faecium G8	*Van A*$^{+}$	Lemcke and Bülte (2000)
Enterococcus faecium G7	*Van B*$^{+}$	Lemcke and Bülte (2000)
Lactobacillus curvatus 09	*tdc*$^{+}$	De las Rivas et al. (2005)
Lactobacillus parabuchneri MC9	*hdc*$^{+}$	De las Rivas et al. (2005)
Lactobacillus plantarum Mc45	Negative control for cells adhesion	Our collection
Listeria monocytogenes	Positive control for Caco-2 cells adhesion	CECT 4032
Gardnerella vaginalis HC2	Positive control for HeLa 229 cell adhesion	Hospital San Cecilio (Granada)

2.2. Isolation of E. faecalis UGRA10

The UGRA10 strain was isolated during a study carried out on the production of antimicrobial substances by LAB from a farmhouse raw sheep's milk cheese (Montefrío, Granada, Spain). For this purpose, 10 g cheese samples were homogenized in 90 ml 2% sodium citrate and then plated in serial dilutions onto MRS agar and M-17 agar. The plates were incubated aerobically at 37 ºC for 48 h and then colonies were selected at random and examined for their inhibitory character by the spot-on-lawn assay. The colonies were replicated onto four sets of M-17 agar plates and incubated for 16 h; afterward, they were screened for bacteriocin production by overlaying with 6 ml of soft-agar inoculated with overnight cultures of different indicator strains. After incubation at 37 ºC, the plates were examined for inhibition zones surrounding individual spots. Strain UGR10 was chosen because it produced a broad-spectrum antimicrobial substance affecting the three indicator *strains* assayed.

2.3. UGRA10 strain identification and characterization

Phenotypic methods. Preliminary identification was based upon phenotypic characteristics, including cell morphology and Gram-staining reaction, catalase activity, ability to grow at 10 and 45 ºC, in the presence of 6.5% (w/v) NaCl or 40% (w/v) bile, and the reaction on bile-aesculin agar. Afterward, the API 50 STREP system (BioMérieux, Marcy l´Etoile, France) was used for species identification. Genotypic methods. Total genomic DNA was extracted from pure culture according to Martín-Platero et al. (2007). Total DNA was used as a template for 16S rDNA amplification using the WO12 and WO1 primers according to Ogier et al. (2002) (Table 2). PCR reactions were performed in a total volume of 50 mL containing 5 mL of 10_ Taq reaction buffer, 1.5 mM of MgCl2, 400 mM of dNTPs, 0.4 mM of the primers WO1 and WO12, 1 U of Taq DNA polymerase (MBL, Córdoba, Spain), and 1 mL of template DNA. The amplification program consisted of an initial denaturing step at 94 ºC for 4 min followed by 30 cycles at 94 ºC for 30 s, 50 ºC for 30 s, and 72 ºC for 60 s, and a final extension of 72 ºC for 2 min. A 700 bp fragment of the 16S rDNA gene containing the V1–V4 variable regions was obtained, purified with a Perfectprep Gel Cleanup kit (Eppendorf, Hamburg, Germany), and sequenced at the Centre for Scientific Instruments of the University of Granada using an ABI PRISM dye-terminator cycle-sequencing ready-reaction automated sequencer (ABI 3100, Applied Biosystems, Madrid, Spain). Homologies were searched for in the BLASTN database (*National* Center for Biotechnology Information) using BLAST (Altschul et al., 1997).

2.4. Identification of the inhibitor produced by the UGRA10 strain

The antagonist substance produced by *E. faecalis* UGRA10 was purified to homogeneity from supernatants of 8 h cultures in complex medium by a two-step procedure based on the adsorption/deadsorption of the bacteriocin on carboxymethyl sephadex CM-25, followed by reverse-phase chromatography on a semi-preparative column (Abriouel et al., 2003). Mass determination of inhibitor was obtained by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using a Voyager-DE PRO spectrometer from Applied Biosystems. Plasmid DNA obtained according to O'Sullivan and Klaenhammer (1993) was used as a template for PCR amplification with specific primers deduced from the *as-48A* structural gene and used in hybridization assays (Martínez-Bueno et al., 1994). The standard procedure for Southern blotting was that described by Sambrook et al. (1989). Pre-hybridization and hybridization with DIG-labelled probes were performed following the recommendations of the Boehringer Mannheim Manual for the DIG DNA labelling and detection kit (Roche Diagnostics GmbH, Mannheim, Germany).

Table 2*. Primer pairs and PCR conditions used in this study.*

Primers	***Specifity***	***PCR conditions***	***Reference***
ORF-X; 5´- AGTGGTTATTCTGAAGG-3´ As48-B5; 5´- GCGGCTCCAATGCT-3	*as48A,B*	*(94ºC 0:30min, 46ºC 0:30min, 72ºC 1:30min) x 30*	Martinez-Bueno et al. (1996)
GEL11; 5´-TATGACAATGCTTTTTGGGAT-3´ GEL12; 5´-AGATGCACCCGAAATAATATA-3´	*gelE*		
HYL n1; 5´-ACAGAAGAGCTGCAGGAAATG-3´ HYL n2; 5´-GACTGACGTCCAAGTTTCCAA-3´	*hyl*		
ASA11; 5´-GCACGCTATTACGAACTATGA-3´ ASA12; 5´-TAAGAAAGAACATCACCACGA-3´	*asa1*	(94ºC 1min, 55ºC 1 min, 72ºC 1 min) x 30	*Vankerckhoven et al. (2004)*
ESP14F; 5´-AGATTTCATCTTTGATTCTTGG-3´ ESP12R; 5´-AATTGATTCTTTAGCATCTGG-3´	*esp*		
CYT I; 5´-ACTCGGGGATTGATAGGC-3´ CYT IIb; 5´-GCTGCTAAAGCTGCGCTT-3´	*cylA*		
EFA-AF; 5´-GCCAATTGGGACAGACCCTC-3´ EFA-AR; 5´-CGCCTTCTGTTCCTTCTTTGGC-3´	*efa*A		*Creti et al. (2004)*
ACE-F; 5´-GAATTGAGCAAAAGTTCAATCG-3´ ACE-R; 5´-GTCTGTCTTTTCACTTGTTTC-3´	*ace*	(94ºC 1min, 55ºC 1 min, 72ºC 1 min) x 30	*Ben Omar et al. (2004)*
VAN-AF; 5'-TCTGCAATAGAGATAGCCGC-3' VAN-AR; 5'-GGAGTAGCTATCCCAGCATT -3'	*vanA*	(94ºC 1min, 55ºC 1 min, 72ºC 1 min) x 30	*Lemcke & Bülte (2000)*
VAN-BF; 5'-GCTCCGCAGCCTGCATGGACA-3' VAN-BR; 5'-ACGATGCCGCCATCCTCCTGC-3'	*vanB*		
JV16HC; 5'-AGATGGTATTGTTTCTTATG-3' JV17HC; 5'-AGACCATACACCATAACCTT-3'	*hdc*		
P2-for; 5'-GAYATNATNGGNATNGGNYTNGAYCARG-3' P1-rev; 5'-CCRTARTCNGGNATAGCRAARTCNGTRTG-3'	*tdc*	*(94ºC 0:30 min, 52ºC 0:30min, 71ºC 2min) x 30*	*De la Rivas et al. (2005)*
ODC- 3; 5'-GTNTTYAAYGCNGAYAARACNTAYTTYGT-3' ODC- 4; 5'-ATNGARTTNAGTTCRCAYTTYTCNGG-3'	*odc*		
WO1; 5'- AGAGTTTGATCMTGGCTC -3' WO12; 5'- TACGCATTTCACCKCTACA -3'	16S rDNA	*(94ºC 0:30min, 50ºC 0:30min, 72ºC 1 min)x30*	*Ogier et al. (2002)*

2.5. Detection of enzymatic activities

Proteolytic activity against casein and gelatin was checked by spotting 5 μl of an overnight culture on milk agar plates (5% milk powder, 1% agar) or BHA supplemented with 0.04% gelatin. DNAse activity was determined by spotting 5 μl of a liquid culture onto DNAse agar plates (Scharlau); after incubation at 37 ºC for 48 h, plates were overlain with HCl 1 N for 5 min. Hydrolysis of gastrointestinal mucine was assayed according to Zhou et al. (2001) in two identical media with added glucose or not. Amylase production was performed according to Giraud et al. (1993). Phytase activity was detected using the procedure described by Bae et al. (1999). Detection of pseudocatalase activity was carried out according to the method described by Knauf et al. (1992). Degradation of raffinose and stacchyose was tested according to Gilliland & Speck (1977). Detection of H_2O_2 production was performed according to Rabe & Hillier. (2003). Haemolysin activity was determined on Columbia Blood Agar (Oxoid, Basingstoke, Hampshire, UK) containing 5% defibrinated horse blood agar. To detect other enzymatic activities, the multiple APIZYM gallery system (Biomerieux, Mercy l'Etoile, France) was used according to the manufacturer's guidelines.

2. 6. Multiplex PCR for detection of virulence determinants

Enterococci virulence genes for gelatinase (*gelE*), hyaluronidase (*hyl*), aggregation substance (*asa1*), enterococcal surface protein (*esp*), cytolysin (*cylA*), adhesion to collagen (*ace*), and cell-wall adhesion (*efaA*) were investigated by multiplex PCR amplifications, using total DNA as described by Martín-Platero et al. (2009). In addition, the presence of resistance genes for vancomycin (*vanA* and *vanB*) and amino acid decarboxylase genes for histidine (*hdc*), ornithine (*odc*), and tyrosine (*tdc*) was determined as described elsewhere (Martín-Platero et al., 2009). The primers used are listed in Table 2.

2.7. Biogenic amine production

The production of biogenic amines in liquid media was tested by inoculating 50 μl of the UGRA10 strain in a microtiter plate containing100 μl/well of the decarboxylase medium (Maijala, 1993), with the addition of 0.2% of each of the precursor amino acids (ornithine, histidine, and tyrosine; Sigma) and also without amino acids (as control). After incubation at 37 ºC for 48 h in aerobic conditions, the production of biogenic amines was qualitatively detected by the colour shift from yellow to purple in the medium. *Escherichia coli* U9 was used as positive control.

2. 8. Determination of antibiotic susceptibilities

Antimicrobial susceptibilities were tested by the semi-automatic Wider system (Francisco Soria Melguizo SA, Madrid, Spain) using the 94B MIC/ID Gram positive panel. The concentration ranges of antibiotics used in the panel are as follows: penicillin (0.12 to 0.5 and 8 mg/ml), ampicillin (0.25 and 2 to 8 mg/ml), amoxicillin/clavulanate (4/2 and 8/4 mg/ml), oxacillin (0.25 and 2 to 4 mg/ml), streptomycin (1,000 mg/ml), gentamicin (2 to 8 and 500 mg/ml), tobramycin (4 and 8 mg/ml), amikacin (8 to 32 mg/ml), vancomycin (1 to 16 mg/ml), teicoplanin (2 to 16 mg/ml), daptomycin (0.5 to 4 mg/ml), levofloxacin (0.5 to 4 mg/ml), erythromycin (0.5 to 4 mg/ml), clindamycin (0.5 and 2 mg/ml), quinupristin/dalfopristin (1 and 2 mg/ml), minociclin (2 to 8 mg/ml), lizenolid (1 to 4 mg/ml), fosfomycin (32 and 64 mg/ml), trimethoprim-sulfamethoxazole (1/19 and 2/38 mg/ml), rifampin (1 and 2 mg/ml), mupirocin (4 and 256 mg/ml), fucidic acid (2 and 16 mg/ml), and nitrofurantoin (32 and 64 mg/ml).

2. 9. Properties related with survival and implantation in in-vitro gastrointestinal conditions

Bile salt hydrolase (BSH) activity. BSH activity was tested by the plate-screening procedure described by Franz et al. (2001b) using sodium taurocholate (Sigma) as bile salt.

Resistance to HCl and oxgall. For testing survival under gastric and intestinal conditions, *E. faecalis* UGRA10 was exposed for 1 h to HCl at pH 3 and consecutively for another hour to oxgall 0.3% (Difco-BBL, Franklin Lakes, NJ, USA) to simulate gastric and intestinal conditions in a two-step procedure based on Rodríguez et al. (2003). Enterococci surviving after each treatment were determined by dilution and plating on TSA medium.

Biofilm formation. The ability of strain UGR10 to form biofilms was quantified essentially as described Martin et al. (2008) by measuring with a microplate reader (Multiskan spectrum; Thermo Fisher Scientific,Vantaa, Finland) the crystal violet retained (OD_{620}) by the biofilm cells formed in microtiter plates at 37 ºC overnight. Each assay was performed in triplicate and repeated twice. *Escherichia coli* U-9 was used as positive control for biofilm formation.

2. 10. Adhesion to cellular lines and inhibition of L. monocytogenes CECT 2032 adhesion

Adhesion assays were conducted as described by Moroni et al. (2006). Enterocyte-like Caco-2 ECACC 86010202 (from colon adenocarcinoma) and Hela 229 ECACC 86090201 (from vaginal cervix carcinoma) (both from the Centre for Scientific Instruments of the University of Granada, Spain) were used in simple adhesion assays. Caco-2 cells were used for listeria interference assays. The cells were aerobically cultured at 37 °C in 25-cm^2

flasks (Falcon, Becton and Dickinson Company, Franklin Lakes, NJ) using RPMI 1640 medium (Biowest, Nuaillé, France) containing 2mM L-glutamine (Sigma), 1mM sodium pyruvate, 50 µM 2-mercaptoethanol (Sigma), 100 UI/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), 5 µg/ml amphotericin B (Sigma), 25 mM HEPES buffer (Biowest), and 10% (vol/vol) fetal bovine serum (Biowest). The cell monolayers were separated from the bottom and suspended in RPMI 1640 with antibiotics and finally seeded in 24-well plates (2 cm^2/well; Falcon) to obtain semiconfluent monolayers in 5 days. Cell cultures were replenished with RPMI 1640 without antibiotics 24 h before the assay was performed.

For individual adhesion assays, cell monolayers in 24-well plates were separately inoculated with 250 µl (10^8 CFU/mll) of each bacterial strain. Plates were then incubated at 37 °C for 30 min, after which free bacteria were eliminated by washing the cell layers twice with PBS. Cells with adherent bacteria were harvested with EDTA-trypsin, centrifuged at 10,000 x *g* for 5 min, suspended in PBS, serially diluted, and finally inoculated on TSA plates. *L. monocytogenes* CECT 4032 and *Bacillus subtilis* CECT 498 were used as positive and negative controls, respectively, in the simple adhesion assays to Caco-2 cells. *Gardnerella vaginalis* HC2 was used as a positive control in simple adhesion assays to HeLa 229 cells.

To test listeria adhesion inhibition by the UGRA10 strain, adhesion of *L. monocytogenes* CECT 4032 and *E. faecalis* UGRA10 to epithelial cells were evaluated separately and in competition assays. Adherent *L. monocytogenes* cells were enumerated on *Listeria* selective medium (PALCAM with added *Listeria* selective supplement, Merck, Darmstadt, Germany) after incubation for 24 to 48 h at 37 °C. Adherent enterococci were enumerated on Kenner faecal agar (Scharlau) after incubation at 37 °C for 24 to 48 h. The individual adhesion capacity was expressed as the number of adherent bacteria divided by the total number of bacteria added, multiplied by 100. For competition assays, cell monolayers in 24-well plates were inoculated with a combination of *Listeria* and *E. faecalis* UGRA10 strain (250 µl) either simultaneously or 1 h before or after one another. The inhibition of *L. monocytogenes* adhesion in the competition assay was expressed as a percentage using the following formula: Inhibition of adhesion = 100(1 - *T*1/*T*2), where *T*1 and *T*2 are the numbers of adherent *Listeria* cells (CFU/well) in the presence and absence of the UGRA10 strain, respectively.

3. Results and Discussion

3.1. Isolation and identification of the UGRA10 producer strain

The UGRA10 strain was selected during a study on bacteriocin production among the LABs isolated from a Spanish sheep's milk cheese. This strain stood out for its broad inhibitory spectrum against the indicator bacteria *E. faecalis* S-47, *Bacillus cereus* LWL1, and *Escherichia coli* U9. Initially, this strain was identified as *E. faecalis* based on phenotypic traits (Gram-positive cocci, catalase negative, growth in 40% bile, 6.5% sodium chloride, at pH 9, and at 45 ºC) (Devriese et al., 1993) and the reactions obtained on the API 20-Strep system. Afterward, a homology analysis of a sequence of 16S rDNA 700-bp fragment obtained by PCR amplification confirmed the assignation of UGRA10 strain to the *E. faecalis* species with a 99% identity (EMBL Nucleotide Sequence Database Accession No: HE613034).

3.2. Identification of the inhibitor produced by the UGRA10 strain

E. faecalis UGRA10 was checked against a wide variety of indicator strains by spot-on-lawn assays, and the results confirmed the broad inhibitory spectrum observed, which was identical to that of *E. faecalis* A-48-32, used as control (result not shown). In addition, PCR amplification using total DNA as template and a pair of specific primers from the structural gene of enterocin AS-48 rendered a PCR product of about 300 bp that, when sequenced, showed a 100% identity with the *as-48A* structural gene. The presence of the complete *as-48* cluster (*as48BCC$_1$DD$_1$EFGH*) encoding for production and immunity to AS-48 was also confirmed by specific PCR (results not shown). Southern hybridization using a probe containing the structural *as-48A* gene revealed a strong label with the higher (>70 kb) of the two plasmids found in the UGRA10 strain (Fig. 1), suggesting that this plasmid harbours the genes involved in the AS-48 character. It is worth noting that the original *E. faecalis* S-48 strain and its derivative *E. faecalis* A-48-32, in which enterocin AS-48 was detected for the first time, possess four plasmids, two big plasmids larger than 23 kb, and two little plasmids smaller than 23 kb (Martínez-Bueno et al., 1990). This fact confirms the non-identity between the S- 48 and UGRA10 strains.

3.2. Technological and biochemical characterization of the UGRA10 strain

The pattern of enzymatic activities for the UGRA10 strain in the APIZYM gallery system and other enzymatic activities, each assayed separately, are shown in Table 3. In the case of casein and gelatin degradation, clear zones around UGRA10 spots were visible on the plates containing each milk or gelatin. Gelatinase is an extracellular metalloprotease, reported as a common trait in strains of *E. faecalis* (Valdivia et al., 1996; Franz et al., 2001a), whose significance in the virulence of enterococci is yet to be clearly understood (Semedo et al., 2003). Casein is a phosphoprotein present in dairy products associated to calcium

phosphate in a soluble complex. Caseine hydrolysis by microorganisms is considered a beneficial trait for LAB implantation in dairy products and in probiotic preparations. Neither phytase, pseudocatalase, DNAse, stacchyose, nor raffinose degradation activities were detected in the UGRA10 strain. Oxygen peroxide was not produced by this strain. Concerning the enzymatic activities detected in the APIZYM strip, it is worth noting the high esterase and esterase lipase C8 activities found. Although enterococci have been frequently referred to as lipolytic and esterolytic, these activities seem to be strain-dependent (revised by Foulquié Moreno et al., 2006). Esterase and esterase lipase activities may help in

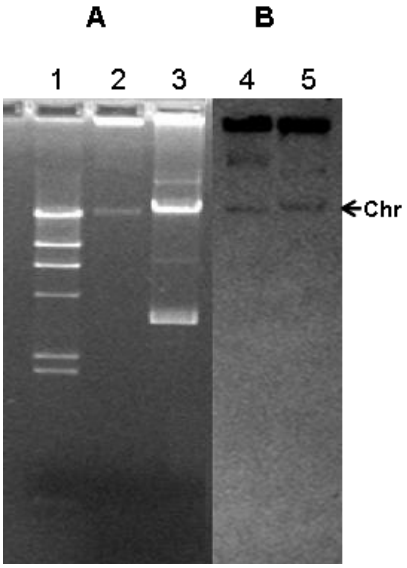

Figure 1. Localization of the gene involved in AS-48 production. (A) Plasmid profile of *E. faecalis* UGRA10. Lane 1: standard molecular weights (λ HindIII); lane 2: *E. faecalis* T-4-31 used as control; lane 3: *E. faecalis* UGRA10. (B) Results of Southern hybridization using a probe containing the structural *as-48A* gene. Lane 4: *E. faecalis* T-4-31; lane 5: *E. faecalis* UGRA10.

Table 3. Results displayed by *Enterococcus faecalis* UGRA-10 in the API ZYM system.

Positive enzymatic activities	**Negative enzymatic activities**
Esterase	Dnase
Lipase esterase C8	Mucinase
Leucine ayilamidase	Amilase
Acid phosphatase	Pseudocatalase
Naphthol-AP-BI-phosphohidrolase	Phytase
β-galactosidase	Stacchyose degradation
β-glucosidase	Raffinose degradation
N-acetil-β-glucosaminidase	Lipase C14Cystine arilamidase
Gelatinase	Valine arilamidase
Caseinase	Tripsine
	α-galactosidase
	α-glucosidase
	α-mannosidase
	α-fucosidase
	α-chymotrypsinase
	β-glucuronidase
	Alkaline phosphatase

transforming fats into fatty acids and glycerol, being particularly useful in dietary preparations for infants and the elderly, and especially indicated for the treatment of metabolic diseases, such as hypercholesterolemia and hyperlipidemia (Huang et al., 2009). In addition, the lipolytic activity of UGRA10 would play an important role in determining aroma when used as a starter or adjunct culture in the making of cheese or fermented milks. Similarly, the □-galactosidase activity observed in UGRA10 may be of interest in subjects with lactose intolerance (De Vrese et al., 2001). Finally, other enzymatic activities detected in this strain were leucine-arylamidase, naphthol-AS-BI-phosphohidrolase, β-glucosidase, and acid phosphatase. Acid phosphatase activity has been reported as significant in improving calcium and phosphorus absorption. N-acetil-β-glucuronidase was weakly detected.

3.3. Safety status of E. faecalis UGRA 10

For detection of the putative virulence determinants referred to in enterococci, we have used a multiplex PCR assay, which is an advantageous method when examining a large number of virulence factors such as those described in enterococci (Dupré et al., 2003; Vankerckhoven et al., 2004), especially when the trait detection requires *in-vivo* assays. The presence of genes encoding aggregation substance (*asa1*), cytolysin activator (*cylA*), surface protein (*esp*), gelatinase (*gelE*), collagen-binding protein (*ace*), and endocarditis antigen (*efaA*) was investigated. The results confirmed that the UGRA10 strain harbours *gelE* (213 pb), *asa1* (375 pb), *esp* (510 pb), *efa* (705 pb), and *ace* genes (1008 pb) (Fig. 2), which encode for proteins conferring ability to adhere to and to colonize eukaryotic cells. These results are not striking considering the high incidence of these putative virulence genes between the *E. faecalis* strains, isolated from different sources, unhealthy or healthy individuals, food or environments (Martín-Platero et al., 2009; Creti et al., 2004). These latter authors analysed the grouping of different virulence genes and showed that most isolates, even commensal ones, harboured four virulence genes. Nevertheless, none of these antigens should be considered as true virulence determinants, but rather as auxiliary factors associated to colonization abilities, promoting the persistence of the bacterium in the gastrointestinal tract (Pillar and Gilmore, 2004; Toledo-Arana *et al*., 2001; Vankerckoven *et al.,* 2004), and hence demonstrating its probiotic properties (Fisher & Phillips, 2009). Nevertheless, the relevant virulence traits attributed to enterococci, encoded by *hyl* (276 pb) or *cyl* (688 pb) genes, were absent in the UGRA10 strain.

Another major concern with respect to the virulence of enterococci is the production of biogenic amines. This is a feature shared by many LABs that produce these compounds in food. Biogenic amines, mainly histamine, have been involved in several intoxications derived

from the eating of cheese (Ten Brick et al., 1990). Although production of these amines has sometimes been attributed to enterococci, its relationship with food poisoning has never been conclusively proven (Giraffa, 2002). Nevertheless, it would seem judicious to evaluate these capabilities in enterococci to be used as starter cultures or probiotics. Thus, the presence of genes for the production of histidine decarboxylase (*hdc,* 367pb), ornithine decarboxylase (*odc,* 1446 pb), and tyrosine decarboxylase (*tdc,* 924 pb) was screened by multiplex PCR using *Lactobacillus curvatus* 09 (*tdc*+) and *Lb. parabuchneri* MC9 (*hdc*+) strains as positive controls. Although no amplification occurred for either *hdc* or *odc*, the *tdc* gene, commonly found in enterococcal isolates, was detected in the UGRA10 strain. According to Psoni et al. (2006), this character does not entail a serious intoxication risk. An assay in a liquid medium confirmed the genetic studies since the production of biogenic amine was detected from tyrosine but not from histidine or ornithine.

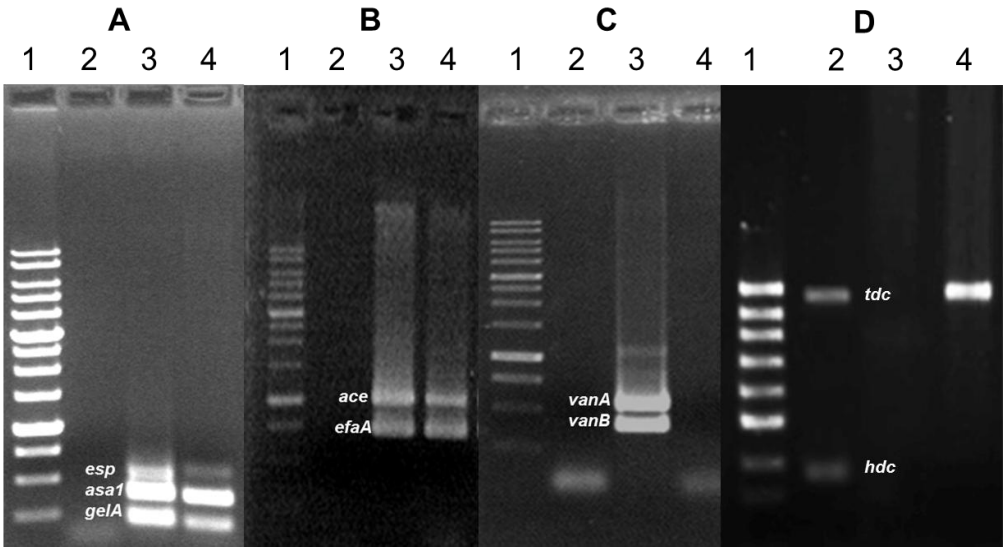

Figure 2. Presence of potential enterococcal virulence factors on *Enterococcus faecalis* UGRA10 as determined by multiplex PCR analysis. A) Lane 1: standard molecular weight (1Kb); lane 2: negative control (asa1, gelE, cylA, esp and hyl genes); lane 3: positive control (*E. faecalis* FI9091). Lane 4*: E. faecalis* UGRA10. B) Lane 1: standard molecular weight (1Kb); lane 2: negative control (ace and efaA genes); lane 3: *E. faecalis* UGRA10; lane 4: positive control (*E. faecalis* KE2). C). Lane 1: standard molecular weight (1Kb); lane 2: negative control (hdc, odc and tdc genes); lane 3: *E. faecalis* UGRA10; lane 4: positive control (*Lb. curvatus* 09 and *Lb. parabuchneri* MC9). D) Lane 1: standard molecular weight (1Kb); lane 2: negative control (vanA and vanB genes); lane 3: *E. faecalis* UGRA10; lane 4: positive control (*E. faecium* G8 and G7).

3. 4. Antibiotic resistance

For safety reasons, a critical criterion in enterococci to be used as co-cultures or starter cultures in foods is the absence of transferable antibiotic resistance (Franz et al., 2001a; De Vuyst et al., 2003). Antibiotic susceptibility of the UGRA10 strain was assayed

using the Wider panel Gram positive 94B MIC/ID, a new computer-assisted image-processing device for bacterial identification and susceptibility testing that has been validated by Canton et al. (2000). The pattern developed by the UGRA10 strain confirms its susceptibility to most of the clinically relevant antibiotics, including vancomycin, although it was resistant to low levels of gentamicin, tobramycin, and amikacin (aminoglycosides), quinupristin/dalfopristin (streptogramin structure), and clindamicin (lincosamide structure).

3. 5. Probiotic properties

To validate the potential of the UGRA10 strain as a probiotic, several properties related with survival in its transit through the gastrointestinal tract and gut colonization have been investigated. These include bile salt hydrolysis, resistance to acid pH, bile tolerance, biofilm formation, and adhesion to different human cellular lines. Although the UGRA 10 strain was able to grow in the presence of bile (40%), it was negative for BSH activity. BSH has frequently been related with bile tolerance in lactic bacteria and also with cholesterol reduction (revised in Begley et al., 2006), and this activity have been reported in *Enterococcus* (Franz et al., 2001b). In contrast, UGRA10 was fairly resistant to acid since the reduction in viable counts after exposure for 1 h at HCl pH 3 was lower than 1 unit log CFU/ml. The transfer of cells challenged by acid to oxgall produced a high reduction in the enterococci population and viable counts fell from 9.15 ± 0.16 log unit CFU/ml (time 0) to 8.55 $\pm$ 0.65 log unit CFU/ml (after 1 h at pH 3) and to 4.53 $\pm$ 0.62 log unit CFU/ml (after a further hour in oxgall). Since this strain was able to grow in bile (40%), the lesser resistance shown against 0.3% bile would be due to its previous exposure to hydrochloric acid, which reinforced the inhibitory action of bile.

The ability to biofilm production is a common property amongst *E. faecalis* isolates from different sources (Creti et al., 2004; Toledo-Arana et al., 2001). Based on our results (OD_{620} = 0.22), we conclude that the UGRA10 strain has the ability to form a well-structured biofilm, a capacity that could be controversial. In effect, though, it may be considered beneficial since, when covering epithelial receptors, the biofilms prevent colonization by other undesirable microorganisms (Chan, 1984; Martin et al., 2008; Moroni et al., 2006). However, this trait has been strongly correlated with the presence of a putative virulence factor, the surface protein Esp, which promotes primary attachment and biofilm formation on abiotic surfaces (Toledo-Arana et al., 2001).

In order to complete the safety study of the UGRA10 strain, *in-vitro* studies on the capability of adhesion to human epithelial cells derived from colon adenocarcinoma (Caco-2

line) and vaginal cervix carcinoma (HeLa 229 line) were also conducted. Figure 3.1 shows the efficiency of UGR10 adhesion to Caco-2 cells. The effectiveness of UGRA10 (13.89%) can be considered as moderate in relation to *L. monocytogenes* (46.23%) and *Lb plantarum* Mc45 (0.65%), used as positive and negative controls, respectively. In any case, this value was greater than that reported for the bifidobacterial species (Moroni et al., 2006). In addition, UGRA10 was also able to adhere to HeLa 229 cells with 21.65% efficiency, which is adequate in relation to that shown by the vaginal pathogen *Gardnerella vaginalis* (35.88%) (Fig. 3.2).

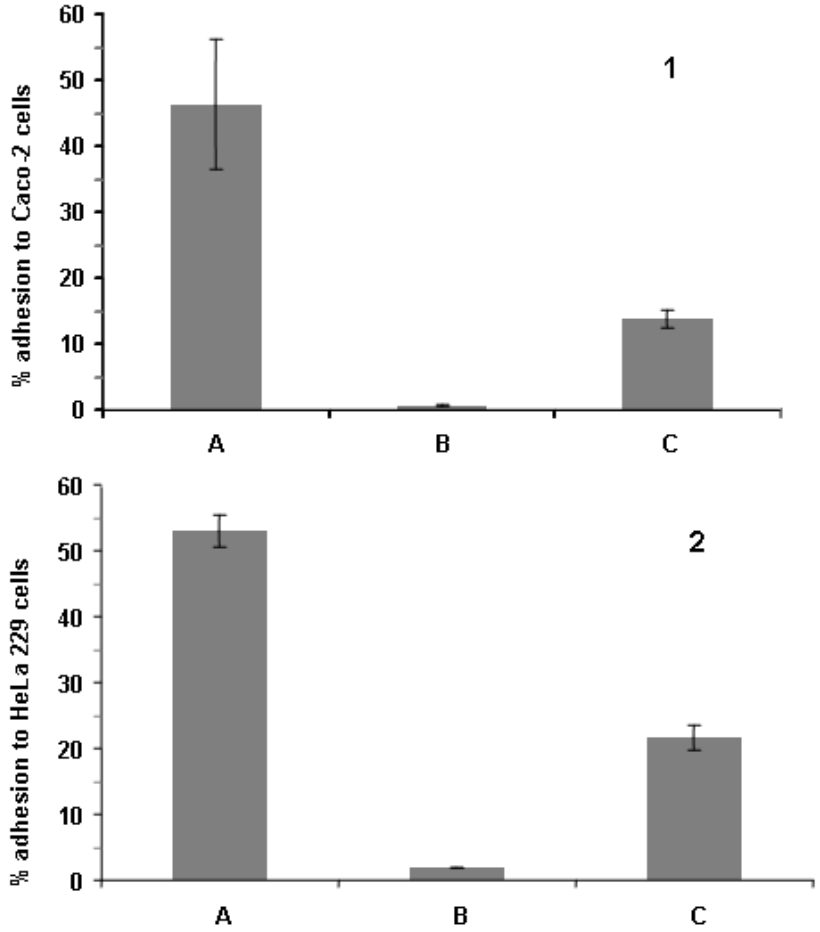

Figure 3. Adhesion of *Enterococcus faecalis* UGRA-10 to Caco-2 and HeLa 229 cell lines. 1): adhesion to Caco-2 cells; A) *L. monocyogenes* CECT 4032, B) *Lb. plantarum* Mc45, C) *E. faecalis* UGRA-10. 2): adhesion to HeLa 229 cells; A) *G. vaginalis* HC2, B) *Lb. plantarum* Mc45, C) *E. faecalis* UGRA-10.

Although the capability of adhesion to epithelial cells is a criterion for defining a new probiotic strain (Salminen et al., 1996; Saarela et al., 2000), currently research also focuses, rather than merely on the simple adhesion of putative probiotics, on its interference with pathogen adhesion. And, in fact, when the UGRA10 strain was added simultaneously to or 1 hour before *Listeria*, a strong inhibition of adhesion could be observed (Fig 4), with

decreases of 99.9% in both cases. In contrast, when UGRA10 was added 1 h after *Listeria*, no significant inhibition of adhesion by this pathogen could be detected. Similarly, inhibition of *Listeria* adhesion by three *Bifidobacterium* strains has also been reported by Moroni et al. (2006), although much poorer results were found by these authors. The adhesion blocking observed here could be attributed to competition for the binding sites on the surface of Caco-2 cells, but also to the inhibitory effect of bacteriocin AS-48 on *Listeria*. In this regard, it is important to take into account that the UGRA10 cells were harvested by centrifugation and washed in PBS prior to being added to the cell monolayer. Thus, in the well, only the AS-48 molecules attached to the UGRA10 cells or those produced during the contact time of the assays would be available.

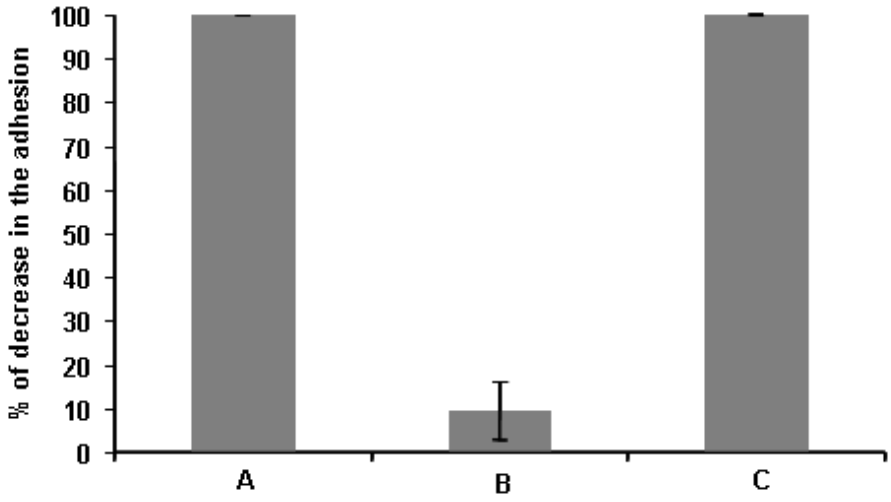

Figure 4. Inhibition of *Listeria monocytogenes* CECT 4032 adhesion by *Enterococcus faecalis* UGRA-10. UGRA 10 strain was inoculated simultaneously with *L. monocytogenes* (A) or 1 hour after (B) or before *L. monocytogenes* (C).

4. Conclusions

For the safety evaluation of bacterial strains, the European Food Safety Authority has proposed a new system to be used in food applications (EFSA, 2004), the “Qualified Presumption of Safety” (QPS), which requires conducting a thorough investigation on biosafety to discard the existence of virulence determinants in such strains, including the presence of hemolysins/cytolysins and the examination of antibiotic resistance. Therefore, we have attempted to ascertain the presence of relevant virulence traits in *E. faecalis* UGRA10, an AS-48 producer strain isolated from a Spanish sheep's milk cheese. We have detected the existence of *gelE, asa1, esp, efaA*, and *ace* genes in the UGR10 strain.

Although considered by several authors as virulence determinants, the presence of the proteins encoded by these genes may be considered as advantageous since they potentially enable the bacterium to colonize the intestine after consumption, to proliferate, and hence to demonstrate its probiotic properties (Fisher & Phillips, 2009). In relation with the other biosafety characteristics, the biogenic amine production and antibiotic resistance, tyramine production, and resistance to aminoglycosides and quinupristin/dalfopristin of this strain may be considered as intrinsic characteristics of the genus *Enterococcus*, particularly of the *E. faecalis* species (Klare et al., 2001) and, therefore, horizontal transmission is unlikely. Furthermore, UGRA10 shows good resistance to pH and moderate resistance to pH/bile, and possesses features that facilitate its implantation in the mucosa, such as the abilities to form biofilms and to adhere to Caco-2 and Hela 229 cells. Nevertheless, the most striking result is the effective blocking that the UGR10 strain exerts in *Listeria* adhesion to Caco-2 cells, which may offer a good way to prevent infection by this pathogen.

In accordance with the above, we conclude that the *E. faecalis* UGRA10 strain possesses desirable characteristics for development as a protective culture in foods and/or as a probiotic, at least for livestock. The interest of using AS-48 producer strains as an adjunct culture in fermented food is heightened by the strong, well-proven activity of this enterocin against spoilage and food-borne pathogens in a wide variety of food (Abriouel et al., 2010; Khan et al., 2010). In addition, this bacteriocin is produced in sufficient amounts in meat and dairy fermented products (Ananou et al., 2005; Muñoz et al., 2004). The effectiveness of AS-48 is reinforced by the fact that the inhibitory action of the enterocin against Gram-positive and Gram-negative bacteria is boosted by several chemical agents or treatments that are commonly implemented in foods or are produced in food fermentations, such as heat, lactic or acetic acids, and nitrite (Khan et al., 2010). In this respect, some authors believe that non-haemolytic antibiotic-sensitive enterocin-producing strains isolated from traditional fermented products can be regarded as safe to be used as starter cultures for fermented products (De Vuyst et al., 2003). It has been proposed that the thin line that separates the dual lifestyle of enterococci (as commensals or pathogens) is the result of an orchestrated interaction between processes involving the sequential action of colonization and potential virulence factors that govern adaptation of bacterial cell physiology during the infection, as well as the host response to the bacterium and its products (Pillar and Gilmore, 2004). In fact, some *E. faecium* and *E. faecalis* strains are used as probiotics and are ingested in high numbers, generally in the form of pharmaceutical preparations, for many years without any reported problems. However, to establish a more accurate balance of risks/benefits in order to determine whether or not the use of this strain to prevent or treat

specific pathogen infections in humans and/or animals is viable, *in-vivo* studies should be designed.

Acknowledgements

This work was supported by the Research Plan of the Junta de Andalucía (research project P07-AGR-02539) and a grant from the GREIB (CEB-005) plan of the University of Granada. R. Cebrián received a fellowship from the University of Granada. We would also like to acknowledge the work of Christine Laurin in editing the text.

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II.2. Safety assessments of *Enterococcus faecalis* UGRA10, a potential probiotic strain AS-48 producer isolated from cheese.

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Abstract

We present here the results of a study on the toxicity and infectivity of *Enterococcus faecalis* UGRA10, a potential probiotic enterocin AS-48 producer strain, in an experimental mouse model. Due to the concern of enterococci as opportunistic pathogens, we have performed the study with immunosuppressed (IS) mice in addition to immunocompetent (IC) ones with the aim of mimicking predisposing disease situations. Furthermore, besides the normal oral via for probiotics administration, we have also used the intravenous inoculation. After oral administration no morbidity or mortality was noted in either of the mice groups. Feed conversion ratio (Kg total food intake/Kg weight gain) was significantly lower in administered IC or IS mice administered UGRA10 than in control animals. No significant differences in hematocrit, WBC, RBC, or HGB neither in blood biochemistry profiles (glucose, urea and GOT) were detected among the four experimental groups. Enterococci were detected in lymph nodes of one of ten mice in both, the control and the UGRA10-administered IC mice. UGRA10 intravenous inoculated was efficiently cleared by spleen of both IC and IS mice after 5 days injection and no morbidity or mortality occurred. Considering these results, we can observe the absence of adverse effects after administering UGRA10 to mice, either orally or intravenously.

Key words: *Enterococcus faecalis* UGRA10; enterocin AS-48; probiotic; in vivo safety assays.

Article in-progress

1. Introduction

Probiotics are defined as “live microorganisms that confer a health benefit to the host when administered in adequate amounts” (FAO/WHO, 2002). Consumption of probiotics beneficially affects lactose intolerance, the incidence of diarrhea and other infectious diseases, mucosal immune responses, blood cholesterol levels, and the induction of cancer. One of the most remarkable benefits of probiotics, documented in several occasions, both in animal models and clinical trials, is the prevention and treatment of a variety of diseases, specially intestinal disorders (reviewed in Gareau et al., 2010)**.** There are some relevant facts about probiotics to consider before staring a therapy based on them: first, not all probiotic strains are appropriate for all ailments and second, probiotics have different underlying mechanisms of action to provide a beneficial effect depending on the strain (Gareau et al., 2010). These mechanisms include: maintenance of appropriate host–microbe interactions and pathogen exclusion, stimulation of mucus secretion, modulation of epithelial barrier function, production of antibacterial factors and activation of the host adaptive immune system (Gareau et al., 2010). Therefore, each potential probiotic strain should be documented and tested independently since extrapolation of data from closely related strains is unreliable. In view of the arguments presented above, it is unlikely that a single probiotic bacterial strain is capable of confering all benefits attributed to probiotics as a whole. It will more likely require the introduction of a combination of strains to accomplish the treatment of all or some of the diseases that are covered by probiotics (Dunne et al., 1999).

Traditionally, lactic acid bacteria (LAB), mainly lactobacilli and bifidobacteria, are the typical probiotic organisms widely used as food components or as non-food preparations in humans or in animal models. Other non LAB organisms, such as *Saccharomyces boulardii*, *Escherichia coli* Nissle 1917 or *Bacillus polyfermenticus* have also been used (Gareau et al., 2010). Enterococci, also belonging to BAL group, are used as probiotics to a lesser extent. The strains of genus *Enterococcus* have been isolated from a wide variety of habitats, including the intestine of healthy warm-blooded animals, human vaginal secretions (Martín et al., 2008), human milk (Martín et al., 2002), the preen gland secretion of birds (Martín-Platero et al., 2006), and a broad variety of fermented foods, in which they may have beneficial effects (Foulquié Moreno et al., 2006). Specifically, strains of *E. faecium, E. faecalis* and *E. durans* predominate in cheese and may play a role in the ripening and aroma production (Foulquié Moreno et al., 2006). In addition, certain *Enterococcus* strains, e.g. *E. faecium* SF68 and *E. faecalis* Symbioflor 1 are successfully used as probiotics to improve human or animal health marketed as Cylactins (Hoffmann-La Roche, Basel, Switzerland) or Symbioflor 1 (SymbioPharm, Herborn, Germany) (Foulquié Moreno et al., 2006; EU, 2011; Franz et al.,

2011) to relieve the symptoms of irritable bowel syndrome and chronic or recurrent sinusitis or bronchitis or to promote growth. Even some strains like *E. faecalis* T-110 have been used in conjunction with other probiotic bacteria LAB to reduce infectious complications after pancreaticoduodenectomy (Nomura et al., 2007). Many others *E. faecium* strains are being used as feed additives for animals (list of them published in the Official Journal of the European Communities) and so far, there have been no reports of diseases caused by them. Nevertheless, the safety of enterococci is nowadays under suspicion due to their implication as opportunistic pathogens in human diseases (Foulquié Moreno et al., 2006; Klare et al., 2001). Therefore, their biotechnological applications, e.g. as starter cultures in fermented foods or as probiotics, should be viewed with caution. Recent studies on the incidence of virulence traits among enterococcal strains isolated from nosocomial infections and foods showed that both types of isolates can harbour certain traits related with virulence (haemolysins, adhesins or invasins and antibiotic resistances) (Ben Omar et al., 2004; Franz et al., 2001). Today, it is generally believed that the virulence of a specific strain is not only the result of the presence of specific virulence determinants alone, but is rather a more intricate process in which other factors are involved, such as the presence of pathogenicity islands (Franz et al., 2011). Based on the above considerations, we cannot guarantee beyond all doubt that enterococci are safe. It is therefore necessary to test the safety for each strain based on the 'body of knowledge' of each strain including virulence determinants, as well as antibiotic resistances (Franz et al., 2011)

At this respect it is noteworthy to emphasize that in the field of probiotic safety, there is a paucity of studies specifically designed to assess safety in contrast to the long history of safe use of many of these microbes in foods. The safety of probiotics is tied to their intended use, which includes consideration of potential vulnerability of the consumer or patient, dose and duration of consumption, and both the manner and frequency of administration (Sanders et al., 2007).

E. faecalis UGRA 10 is a strain isolated from a Spanish sheep's cheese with some desirable characteristics for the development as a protective culture in foods and/or as probiotic. UGRA10 has been examined in vitro for safety properties such as enterococci virulence genes, biogenic amine production, and antibiotic resistance (Cebrian et al., 2012). As for most *E. faecalis* strains, UGRA 10 harbour genes coding for GelE, Asa1, Esp, EfaA, and Ace antigens, related with virulence, as well several authors consider the presence of these genes as advantageous since they potentially enable the bacterium to colonize the intestine and to exert its probiotic abilities (Fisher and Phillips, 2009). This strain produces tyramine and is sensitive to most of the antibiotics tested, being resistant only to

aminoglycosides, lincosamide, and pristinamicins. Since these resistances may be considered as intrinsic characteristics of the genus *Enterococcus,* particularly of the *E. faecalis* species (Klare et al., 2001), horizontal transmission is therefore unlikely. In addition to showing resistance to acidic pH and moderate resistance to bile, UGRA10 possesses other features that facilitate its implantation in the mucosa: ability to form biofilms and to adhere to Caco 2 and HeLa 229 cells. More interestingly, this strain shows a high ability to interfere with the adhesion of *Listeria monocytogenes* to Caco 2 cells which may offer a good way to prevent infection by this pathogen. Moreover, this strain produces a potent antimicrobial substance, the enterocin AS-48, a circular bacteriocin that exhibited several valuable biotechnological features: activity against a wide range of Gram-positive and Gram-negative bacteria, including several food-borne pathogens and food spoiling organisms and stability over a broad range of pH values and temperatures (Gálvez et al., 1986, 1989a, b, c). Therefore, this strain and its bacteriocin are considered potential candidates for improving health and the hygienic quality and for extending the shelf-life of different foods (Baños et al., 2011; Ananou et al., 2010; Abriouel et al., 2010; Khan et al., 2010). However, due to controversial around the enterococci, we deemed it necessary to investigate in vivo, in immunocompetent and immunocompromised animal models, the safety characteristics of UGRA10 strain to establish a more accurate balance of risks/benefits. This would help us to determine whether or not the use of this strain is useful to prevent or treat specific pathogen infections in humans and/or animals. At first, we have evaluated the effect of UGRA10 oral administration to mice on the general health status and on different biochemical and haematological parameters. The potential translocation of this strain from intestine to blood and other organs was also investigated. Then we have also used an intravenous mouse inoculation model to ascertain the invasive potential of UGRA10.

2. Materials and Methods

2.1. Bacterial strain and growth conditions

E. faecalis UGRA10, an AS-48-producer strain, isolated from a Spanish sheep's cheese (Cebrian et al., 2012) was used as a probiotic. A probiotic marketed *E. faecalis* Symbioflor 1 (SymbioPharm, Herborn, Germany), together with *E. faecalis* CECT 5254, a strain isolated from blood of a patient with a bacterial infection, were used as control for the safety bioassay.

Stock cultures were kept at 80 ºC in Brain Hearth Infusion (BHI, Scharlau, Barcelona, Spain) broth added of 50% glycerol. Before use they were inoculated (1%, v/v) in the same

medium and incubated at 37 ºC. In order to grow and count the enterococci from tissues specimens, Slanetz and Bartley agar added of 1% of sterile TTC (2, 3, 5-Triphenyl Tetrazolium Chloride) solution (both from Scharlau) and Blood Columbia agar (Oxoid, Madrid, Spain) were used. All plates were incubated aerobically at 37 ºC for 48 h.

2.2. Animals

Seven week-old female BALB/c mice were purchased from Charles River Laboratories (L'Arbresle, France) and were quarantined and acclimated for 2 weeks. All mice were housed in transparent polypropylene cages on hardwood chip bedding with a 12 h light/dark cycle (8 am – 8 pm) in a controlled atmosphere (temperature 22 ± 2 ºC, humidity 55 ± 2%). The animals were fed *ad libitum* a purified diet (Harlan, Barcelona, Spain) and had free access to water during the experimental protocol. The protocols were carried out according to the guidelines of the Helsinki declaration and in accordance with animal care and use guidelines approved by the Ethic Committee of Animal Experiment of the Universidad de Granada.

2. 3. Immunosuppression treatment

Cyclophosphamide, commercially obtained from Genoxal (Prasfarma S.A., Barcelona, Spain), was used to immunosuppress the animals. It was administered by intraperitoneal injection, according to the protocol described by Sanati et al. [1997]. Each animal received two doses of 150 and 100 mg/Kg of body weight, with an interval of three days in between. This is a common method to induce lymphopaenia (Sharma et al.,1997), which also affects humoral immune response (Ahmed et al., 1984). Both humoral and cell-mediated responses are important components of the immune response controlling infections. This treatment ensures a significant leukopenia from a week after the second injection, as well as a spleen size reduction which results in an important splenomegaly, approx. at day 7.

2. 4. Experimental design with mice

A) UGRA 10 oral administration. After 10 days of acclimation, 40 mice were randomly assigned into four experimental groups (n = 10 per group). Two groups were given cyclophosphamide: immunosuppressed (IS) mice, and the other two groups received no cyclosphosphamide treatment: immunocompetent (IC) mice. The experiment was performed basically as Lara-Villoslada et al., (2009). One group of IS mice and one of the IC received

orally *E. faecalis* UGRA10 strain and the two remaining groups received only the excipient, a phosphate saline solution (PBS, Sigma–Aldrich, France). To prepare UGRA10 suspension, the bacteria were grown on BHI broth at 37 °C for 24 h, harvested by centrifugation, washed twice in sterile PBS solution and resuspended in PBS to a final concentration of 10^9 bacteria /mL. Each animal received orally 100 µL of this solution for 20 days. Oral gavage was performed by intragastric feeding by means of a stainless steel cannula needle purchased from Harvard Apparatus (Edenbridge, UK).

Throughout the experiment, the activity, behaviour, general health (including obvious disease signals and presence of diarrheic faeces) and mortality were observed for each animal on a daily basis. Body weight and food intake were recorded once a week. After 20 days, mice were sacrificed by cervical dislocation. Blood was collected by cardiac puncture in EDTA containing tubes and liver, spleen and mesenteric lymph nodes were removed in sterile conditions and analysed for enterococci presence. For this purpose, tissue samples were homogenised in buffered peptone water (10 g/L) using individually sterilised pestles and 100 µL of homogenate were cultured in BHI broth. The presence of enterococci in liquid BHI cultures positives for growth was confirmed by plating in in Blood Columbia agar and in Slanetz and Bartley agar added of TTC. Positive translocation organ was defined as the organ from which at least one viable bacterial cell was recovered (one colony) (Zhou et al., 2000). The results were expressed as incidence of translocation (number of mice in which CFU were detected/total number of mice).

B) UGRA 10 intravenous administration. After 10 days of acclimation, mice were randomly assigned into two experimental groups (n=27 per group). One group was given cyclophosphamide: IS mice, and the other group, received no cyclosphosphamide treatment: IC mice. Each group was subdivided into three experimental groups (n=9 per group) and received intravenous inoculation with suspensions of different *Enterococcus* strains: *E. faecalis* UGRA10, *E. faecalis* Symbioflor 1 or *E. faecalis* CECT 5254, prepared as described above for UGRA10. Intravenous administration was carried out by inoculation via the retro-orbital plexus of 0.1 mL of viable bacteria (10^9 CFU/mL). Animals were observed daily for morbidity and mortality. Three mice from each group were euthanized by cervical dislocation at different time post-injection (1, 3, and 5 days). Spleen were removed in sterile conditions, weighed and analysed for enterococci presence as follows. Organs were placed in sterile buffered peptone water and homogenised to completion using individually sterilised pestles. During homogenisation, tissues were placed in an ice-water bath inside a biosafety cabinet. Aliquots of each homogenate were serially diluted, plated by duplicate onto Columbia blood

agar and Slanetz Barley agar plus TTC, incubated overnight at 37 ºC, and observed for viable colonies. The results were expressed as number of viable bacteria per gram of spleen.

2.5. Evaluation of biochemical and haematological parameters

In order to evaluate possible changes in the physiological functions, due to the oral administration with the probiotic enterococci, several biochemical and haematological parameters were analysed at the Bioanalysis Unit of the Scientific Instrumentation Centre of the Universidad of Granada. Urea and glucose concentrations and glutamic-oxalacetic transaminase (GOT) activity were measured in plasma samples by means of commercial kits (SpinReact, St. Esteve d'en Bas, Spain) in an automatic chemistry analyser BS-200 (Mindray Medical International Ltd., Shenzhen, China). Blood cell counts (white and red cells), hematocrit and mean corpuscular haemoglobin concentration were also analysed in an automatic haematology counter Mythic 22 CT (Orphée, Geneva/Plan-les-Ouates, Switzerland).

2.6. Statistical Analysis

All results are expressed as the mean±SD. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and *post hoc* least significance tests. The statistical analyses were performed using the SPSS-PC 15.0 software (SPSS, Chicago, Ill. USA).

3. Results and discussion

Due to the increasing association of enterococci with human diseases, the use of *Enterococcus* strains as probiotics remain controversial. In spite of this, several strains belonging to *E. faecalis* and *E. faecium* species have been marketed for use as probiotic in human and, specially, as veterinary feed supplements to prevent or treat several disorders, mainly in the gastrointestinal tract, and to improve the growth (Foulquie-Moreno et al., 2006; Franz et al., 2011). So, although the field of probiotics is characterised by the scarcity of studies on its safety, due to its long history of safe use in food, the especial consideration of genus *Enterococcus* strongly advices on the need of these studies case-by-case in order to well establish the risk/benefit ratio of its consumption. Here we present the results of a study on the in vivo toxicity and infectivity in an experimental mouse model of *E. faecalis* UGRA10, a strain producer of enterocin AS-48 whose functional, safety and probiotic properties have been previously investigated in vitro (Cebrian et al., 2012). Because of the conflictive dual

status of enterococci, as probiotic and opportunistic pathogen, we have considered convenient to use animal models beyond their normal physiological states (immunosuppressed hosts) to allow the detection of any potential pathogenicity of the candidate probiotic strain. Besides that, an unusual via of administration, the intravenous via, has been used in addition to the most common, the oral via, all in order to mimic to some extent the predisposing conditions for certain diseases caused by enterococci.

3.1 Effect of UGRA-10 oral administration in immunocompetent and immunosuppressed mice

Effects on general health status of the animals. During the experimental protocols no noticeable activity or behavioural changes were observed in the mice and no illness or death occurred. There were no differences in the animals' characteristic between treatment and control groups. In addition, oral administration of *E. faecalis* UGRA10 strain had not adverse effects on mice food intake, since there was no difference in food intake between control and treated groups throughout the experiment (Table 1). As shown in Table 1, similar body weight gain (BWG) was observed in IC and IS mice with the exception of the group of IC UGRA10-treated mice. In this group, we determined a greater significant ($P < 0.01$) BWG of 15.32% compared with the values of approx. 8% of the other experimental groups. In addition, significant differences were observed in the feed conversion ratio (FCR, Kg total food intake/Kg weight gain) between control and UGRA10-treated, for both, IC and IS mice. Thus in IC control mice FCR were 2.6 vs. 2.03 in IC UGRA10 administered mice (significant difference at $P < 0.05$). In IS mice, the differences in FCR were higher than in IC mice: 1.31 for UGRA10-treated mice vs. 3.57 for control mice (significance different at $P < 0.01$). The positive effects of probiotics on BWG have been recently reported in the literature for farm animals but it was generally moderate (close or lower than 10%) (Bernardeau and Vernoux, 2013). The hypothetical mode of probiotic action proposed by the different authors has been diverse: greater crude protein retention; greater digestibility of nutrients by production of extracellular degrading enzymes; vitamin production, modulation of the intestinal microbiota or anti-inflammatory properties; competitive exclusion, creation of a hostile environment for pathogen colonization, etc. (Bernardeau and Vernoux, 2013). Recent studies on probiotic effects showed that BWG could also be observed in humans, or not, depending on the species and strains. Nevertheless, the results obtained for farm animal cannot be easily extrapolated to humans, particularly with regard to body weight improvement, because the studies conducted did not use the same microbial strains or the same species and also because animal model studies have been conducted over a reduced period of their short industrial life (Bernardeau and Vernoux, 2013). Our results suggest that UGRA10

administration promotes BWG in mice, especially IS mice. This is a further evidence of the immunomodulatory role of UGRA10 strain, which leads to the reduction of the additional stress induced by the immunosuppression in the gut.

This is supported by the proven immunomodulatory properties of UGRA10 in two types of cells involved in the immune response: HT-29 cells (as a model of epithelial cells) and RAW 264.7 cells (as a model of macrophage cells), in basal and lipopolysaccharide (LPS) induced conditions. Thus, *E. faecalis* UGRA10 in basal conditions, increased the production of IL-1β and TNFα in macrophages but exerts an inhibitory effect on the LPS-induced cytokine (IL-8, TNFα) or nitrite production in these two cell lines (Rodríguez-Nogales et al., 2012). These probiotic properties of UGRA10 can also be attributed to the interference of enterocin AS-48 with pathogenic/competitive bacteria in the gut, as it has been shown for bacteriocin Abp118 produced by *Lactobacillus salivarius* UCC118 (Corr et al., 2007) in an experimental mouse model or even for enterocin AS-48 in Caco 2 cells (Cebrian et al., 2012). Nevertheless, even if the positive effects of UGRA10 on the FCR of IC mice can be attributed to that argued above, the favorable effects of UGRA10 administration on the FCR of IS mice that far exceed the quantity observed in IC mice (1.31 in IS vs. 2.3 in IC mice) is quite inexplicable. So these results would need to be confirmed in future studies.

Table 1. Body weight and food intake of control and probiotic-treated immunocompetent and immunosuppressed mice.

	Immunocompetent mice		Immunosuppressed mice	
	Control	**UGRA 10**	**Control**	**UGRA 10**
Initial weight (g)	18.44 ± 1.75	21.34 ± 1.20	17.2± 1.34	17.88 ± 0.98
Final weight (g)	19.92 ± 1.1	22.25 ± 0.97	18.80 ± 1.2	20.62 ± 0.8
Body weight gain (%)	7.91 ± 0.3	7.92± 0.4	8.67 ± 0.6	15.32 ± 0.4[a]
Food intake (g/mice/day)	3,8 ± 0,35	3,01± 0,40	3,9 ± 0,30	3,6 ± 0,20
Feed Conversion Ratio	2.6	2.03	3.57	1.31

Values are means ± S.D. for groups of 10 mice. [a] Significant different to control at $P < 0.01$

Effects on haematology and blood biochemistry parameters. Although no clinical symptoms revealing alterations in general health status or in body weight were observed in the four experimental groups, we carried out biochemical assays in order to detect adverse sub-clinical effects of UGRA10 administration. These can reveal moderate deficiencies of nutrients or imbalance in nutrient metabolism (Swendseid, 1987). The effect of feeding UGRA10 on haemotological and biochemical blood parameters is shown in Table 2. No significant differences in hematocrit, WBC, RBC, or HGB were detected between the four

experimental groups. In addition, animals UGRA10-treated have similar blood biochemistry profiles (glucose, urea and GOT) to those of the controls. These results suggest that feeding with UGRA10 for a continuous 20-day period has no adverse effect on hematology and blood biochemistry.

Table 2. Hematology and blood biochemistry data from control and probiotic-treated immunocompetent and immunosuppressed mice.

	Immunocompetent mice		Immunosuppressed mice	
	Control	**UGRA 10**	**Control**	**UGRA 10**
Hematocrito (%)	35.32 ± 5.6	34.91 ± 2.2	39.85 ± 2.5	39.56 ± 2.9
WBC (10^3/µL)	3.33 ± 0.9	3.52 ± 1.1	3.82 ± 0.9	3.96 ± 1.2
RBC (10^6/µL)	7.97 ± 1.3	8.02 ± 0.5	8.89 ± 0.9	8.39 ± 0.8
HGB (g/dL)	34.93 ± 0.8	34.84 ± 0.7	34.84 ± 0.2	34.84 ± 1
Glucose (mg/L)	122.82 ± 9.7	148.92 ± 20.8	144.81 ± 21.5	170.97 ± 13.5
Urea (mg/dL)	61.74 ± 15.7	62.78 ± 12.4	66.81 ± 8.9	60.03 ± 4.7
GOT (U/L)	330.21 ± 50.9	252.88 ± 44.6	227.99 ± 31.8	237.29 ± 45.01

WBC, white blood cells ; RBC, red blood cell ; HGB, hemoglobin; GOT, glutamic-oxalacetic transaminase

Values are means ± S.D. for groups of 10 mice.

Bacterial translocation. Bacterial translocation from the gut to mesenteric lymph nodes, spleen and liver was investigated as a measure of the potential of UGRA10 infectivity. The incidence of enterococci in the investigated organs was confirmed by growing the colonies found in BHA in Slanetz and Bartley agar added of TTC. As it is shown in Table 3, although a minimal translocation of bacteria (one animal positive for each group of ten mice) to mesenteric lymph nodes was observed in IC mice, there were no differences in the incidence of translocation between control and treated groups, which suggests that translocation was not related with the treatment. Interestingly, translocation was not detected in either of the two IS mouse groups.

Table 3. Incidence of bacterial translocation to mesenteric lymph nodes, liver and spleen in control and probiotic-treated immunocompetent and immunocompromised mice.

	Immunocompetent mice		Immunosuppressed mice	
	Control	**UGRA 10**	**Control**	**UGRA 10**
Mesenteric lymph	1/10	1/10	0/10	0/10
Liver	0/10	0/10	0/10	0/10
Spleen	0/10	0/10	0/10	0/10

In accordance with the results obtained by analysing the general health status, growth, haematology and blood biochemistry and potential infectivity (bacterial translocation) of IC and IS UGRA10-treated mice, we can conclude that *E. faecalis* UGRA10 strain had not any adverse effect on mice fed 10^9 CFU for 20 days. This suggests a lack of oral toxicity of this potential probiotic strain, even for the IS animals, in spite of the high dose of bacteria administered. In this regard, it is noteworthy the increase in BWG induced by UGRA10 administration in IS mice. This finding, apart from the previous results obtained on the immunomodulatory properties of UGRA10 in cell cultures (Rodriguez-Nogales, 2012), emphasise the importance of this strain for further studies related with inflammatory bowel disease therapies.

3.2 Effect of UGRA-10 intravenous administration in immunocompetent and immunosuppressed mice

As commented above, the concern of enterococci as opportunistic pathogens recommends making special assessments on potential probiotic *Enterococcus* strains. In this sense, we have carried out an experiment in which *E. faecalis* UGRA10 was administered intravenous via retro-orbital plexus, with the aim of finding out the spleen's ability to clear cells of this strain. With comparative purposes, we have administered by the same via *E. faecalis* Symbioflor 1, a probiotic used for the treatment of recurrent diseases of the upper respiratory tract and *E. faecalis* CECT 5254, a strain isolated from a patient's blood with bacterial infection.

After the injection and along the 5 days of the experiment, morbidity and mortality was not observed in any of IC or IS mouse groups. Numbers of viable bacteria in spleen following 1, 3 and 5 days infection with either *E. faecalis* UGRA10, Symbioflor 1 or CECT 5254 were quantified and graphed against infection time (Fig. 1). After 1 day injection, enterococci were detected at levels of 3.66, 3.93 and 4.48 log units/g in the spleens of IC mice infected with UGRA10, Symbioflor1 and CECT 5254 strains respectively (Fig. 1A). At day 3, levels of enterococci in spleen decreased in all mice groups but the analysis of bacterial load in mice revealed that, at this point there was a discernible difference in the number of enterococci recovered from the spleen ($P < 0.05$), with a greater CECT 5254 recovery (2.5 CFU/g spleen) than those of the UGRA10 (0.5 CFU/g spleen). Five days after infection all enterococci were nearly cleared from the spleen, although a residual number (0.3 CFU/g) was detected in mice inoculated with CECT 5254 strain. In IS mice (Fig. 1B), at day 1 of injection, enterococci were recovered at similar levels of 4.23, 4.3 and 4.69 log units/g spleen of UGRA10, Symbioflor1 and CECT 5254 infected mice, respectively. At day 3, the ability of

clearance the enterococci from the spleen of the IS mice was lower than that of the IC animals. Like in IC mice, at this time, levels of enterococci in spleens of mice inoculated with UGRA10 were significant lower than in CECT 5254 administered mice ($P < 0.05$): 2.53 vs.4.09 log CFU/g. At day 5, a low number of enterococci (0.2 log CFU/g) were detected in the spleen of UGRA10-inoculated mice. At this point, the levels of enterococci in the spleen from mice inoculated with strains Symbioflor 1 or CECT 5254 were higher than those of the UGRA10 strain: 1.29 vs. 1.36 CFU/g respectively.

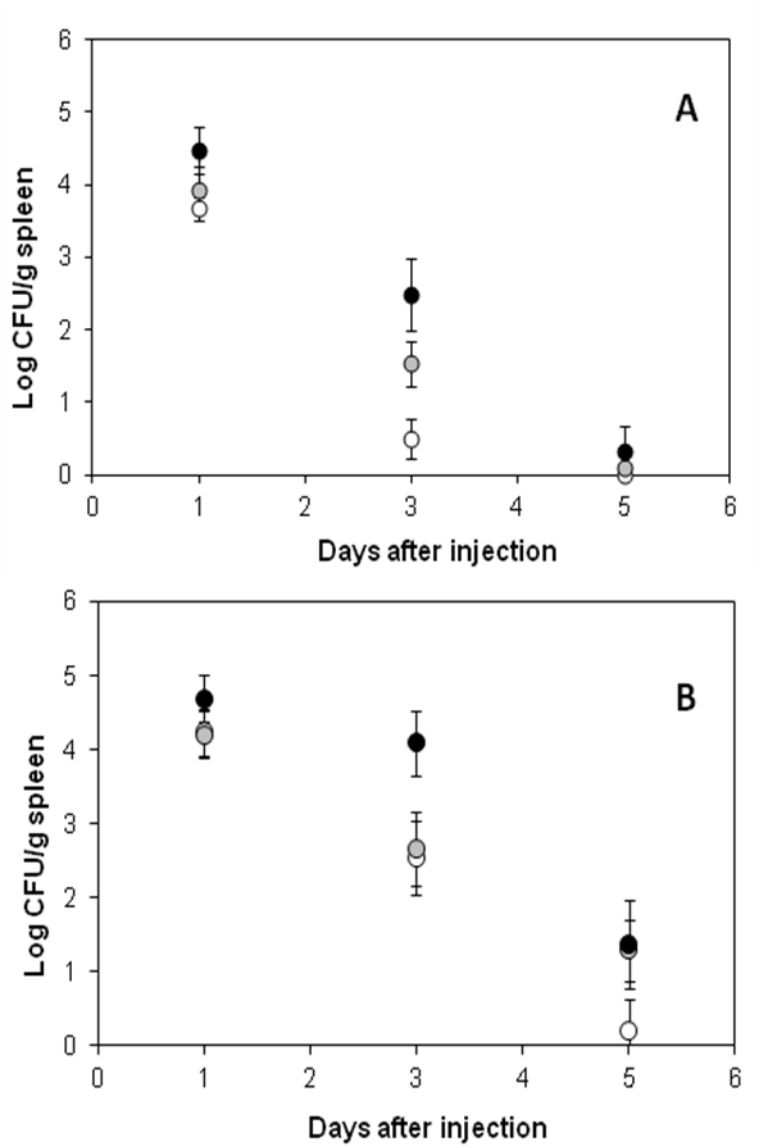

Figure 1. Clearance of bacterial cells from spleen after intravenous injection of 10^8 CFU of three *Enterococcus faecalis* strains in a mouse model. A) Immunocompetent mice; B) Immunosuppressed mice. White circles: *E. faecalis* UGRA10; grey circles*: E. faecalis* Symbioflor 1; black circles: *E. faecalis* CECT 5254. The results are expressed as the means and the standard deviations of results from three mice.

In conclusion, considering the results obtained after administration of *E. faecalis* UGRA10 in immunocompetent and immunosuppressed mice, when combining the normal oral via with the intravenous via, this strain has no adverse effects on the health of the animals, as can be seen from the absence of symptoms of infection or pathological findings regarding to hemological or biochemistry blood parameters. It is also noteworthy to mention the absence of a significant translocation to blood and the rapid clearance of enterococci from the spleen of both IC and IS animals. All these results encourage us to continue the research with future veterinary and human trials to confirm its safety and to evaluate the benefits of the use of the UGRA10 strain, especially with regard to the feed conversion ratio and the protection against pathogens.

Acknowledgements

This work was supported by Grant (P07-AGR-02539) from the Junta de Andalucía. We would also like to acknowledge the work of Jose Manuel García Madero in editing the text.

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II.3. Assessment of probiotic properties of *Enterococcus faecalis UGRA10:* Immunomodulation and protection against pathogens.

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Abstract

Several mechanisms of probiotic action have been proposed, the most common relating to their abilities to modulate the host immune system and to produce antimicrobial substances. However, there are relatively few published reports on the effectiveness of enterococcal strains as probiotics. Thus, the main purpose of this study is to investigate the immunomodulatory potential of the *Enterococcus faecalis* UGRA10 strain and its ability to prevent the invasion by *Listeria monocytogenes* and *Clostridium perfringens* in mice, as well as to determine the importance of AS-48 in the protection against infections. In order to investigate the immunomodulatory properties of UGRA10, we obtained mouse splenocytes from mice orally administered with UGRA10. The cells were treated with Lipopolysaccharide and Concavaline A, and the culture supernatants were collected and levels of TNF-α and IFN-γ were measured. Results indicate that *E. faecalis* UGRA10 is suitable for enhancing immune responses, skewing the cytokine profile to the production of IFNγ and the inhibition of TNF-α. We have also shown here that *E. faecalis* UGRA10 reduces *L. monocytogenes and C. perfringens* infections in mice. A mutant of *E. faecalis* UGRA10, non-producer of enterocin AS-48, was less effective to protect mice against *Listeria*, which suggests that AS-48 is an important mediator in the protection against this pathogen.

Key words: Probiotic, *Enterococcus faecalis* UGRA 10, enterocin AS-48, *Listeria monocytogenes, Clostridium perfringens, immunomodulation*

Article in-progress

1. Introduction

The gastrointestinal microbiota is a complex ecosystem that plays a fundamental role on the induction, training and function of the host immune system, providing essential health benefits to the host. Numerous reports have shown the beneficial effects of microbiota gastrointestinal on human health and well-being (Ouwehand, 2002; Socco et al., 2010; Doré et al., 2010). One of the best proven benefits is the pathogen inhibition by intestinal microbiota that can act as a natural barrier against pathogen exposure (Wallace et al., 2011). Thus, the aim of improving health through consumption of functional foods designed to modulate commensal microbiota or through the introduction of probiotic strains has received much attention in recent years.

According to the general definition given by the Food and Agriculture Organization (FAO), and recently revised by Hill et al., (2014), probiotics are defined as “Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (FAO/WHO, 2001). The scientific and clinical evidence for the therapeutic potential of probiotic bacteria in human and animal health, and especially with regard to the gastrointestinal health, has been increasing in recent years (Collado et al., 2009; Sleator et al., 2015). Some clinical applications of probiotics include the prevention and treatment of gastrointestinal infections (Lebeer et al., 2010; Varankovich et al., 2015) and the reduction of the severity of intestinal inflammation (Sartor et al., 2004; Rook et al., 2005; Reiff et al., 2010; Mazaya et al., 2015).

Several mechanisms of probiotic action have been described. The most common are related to the ability to modulate the host immune system and to produce antimicrobial substances (Collado et al., 2007; Wen et al., 2012; Konieczna et al., 2013; Kanmani et al., 2013). Antimicrobial production is often regarded as an important feature in terms of probiotic efficacy; among these antimicrobial compounds are organic acids, hydrogen peroxide and bacteriocins. Bacteriocins are ribosomally synthesised peptides produced by bacteria that are active against other bacteria and against which the producer strain has a specific immunity mechanism (Cotter et al., 2005). These may enhance their ability to compete against other commensal bacteria which could potentially inhibit colonisation by pathogens (Majeed et al., 2011). Traditionally, bacteriocin production has been considered an important criterion for selecting a probiotic strain, but only few studies have been able to show the precise role of bacteriocin production on the ability of a strain to prevent infections (Corr et al., 2007; Bhardwaj et al., 2010; Dobson et al., 2012).

Regarding to the immunoregulatory properties of probiotics (Macho-Fernandez et al., 2011), mechanisms related to the inhibition of proinflammatory mediator production, such as Tumour Necrosis Factor (TNF-α), induction of anti-inflammatory cytokine production and induction of regulatory T cells (Borchers et al., 2009; Plaza-Diaz et al., 2014), have been described.

The majority of probiotics currently used contain species of lactic acid bacteria (LAB), including *Lactobacillus, Bifidobacterium, Pediococcus* or *Lactococcus*. Furthermore, LAB, includes members of the *Enterococcus* genus. Enterococci are ubiquitous bacteria, whose primary habitat is the intestine of healthy humans and warm-blooded animals. Moreover, they are often found in a wide variety of fermented foods, such as vegetables or cheese (Foulquié Moreno et al., 2006; Martín-Platero et al., 2009). Enterococci produce a wide array of bacteriocins (enterocins) with the potential to inhibit the growth of food-borne pathogenic and spoilage bacteria (Franz et al., 2007). Furthermore, selected *Enterococcus* strains with possible health-promoting capabilities have been used as efficient probiotics for humans and animals (European Commission, 2011; Franz et al., 2011; Foulquié et al., 2006).

In a previous work, we conducted an in vitro study on the functional, probiotic, and safety-related properties of *E. faecalis* UGRA10, an enterocin AS-48 producer strain isolated from a handmade sheep's milk cheese for which we previously showed its ability to inhibit the growth of *Listeria monocytogenes* in vitro, and to interfere with the adhesion of *L. monocytogenes* to cell lines (Cebrian et al., 2012). Enterocin AS-48 is a cationic circular bacteriocin produced by *E. faecalis* S-48 with a broad bactericidal activity against most Gram-positive bacteria, including pathogens, such as *L. monocytogenes*, *Staphylococcus aureus*, *Mycobacterium* spp., *Bacillus cereus*, and some Gram-negative bacteria (Abriouel, et al., 2002; Abriouel et al., 1998; Gálvez et al., 1989). The target of AS-48 is the bacterial cell membrane in which the bacteriocin inserts and destabilises the membrane potential, leading to pore formation and cell leakage (Sánchez-Barrena et al., 2003). The features of AS-48, specially the broad spectrum of antimicrobial activity, the stability across a wide range of temperatures and pH, and the sensitivity to digestive proteases (Gálvez et al., 1986), make it a promising alternative to chemical preservatives for use as a biopreservative in foods. In fact, AS-48 has been shown to be effective in the control of various foodborne pathogens in dairy, meat, vegetable and fish products (Ananou et al., 2014; Ananou et al., 2010; Cobo Molinos et al., 2008; Muñoz et al., 2007) and also against several spoilage bacteria in meat products and vegetable-derived foods (Baños et al., 2012; Grande et al., 2007). Furthermore, other applications of AS-48 focused on the pharmaceutical sector have been described (Maqueda et al., 2013).

In contrast to other probiotic genus, there are relatively few published reports on the effectiveness of enterococcal strains as probiotic and the mechanisms involved, e.g. the role of enterocins in the protection against gastrointestinal infections or gut immunomodulatory activities. Thus, the main purpose of this study was to investigate the immunomodulatory potential of the *E. faecalis* UGRA10 and its ability to prevent gut invasion by *L. monocytogenes* and *C. perfringens* in an experimental mouse model, as well as to highlight the importance of AS-48 in the protection against infections.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Enterococcus faecalis UGRA10, producer of enterocin AS-48, was used as probiotic strain. *E. faecalis* S-47 from our collection was used as the standard indicator strain for bacteriocin activity assays. For mice infection, *L. monocytogenes* CECT 4032 and *C. perfringens* CECT 821 were supplied by the Spanish Type Culture Collection (CECT). Bacterial cultures were maintained at 4º C on BHI-agar (BHA) slants (Scharlau, Barcelona, Spain).

2.2. Obtaining and analysis of AS-48 non producer mutants from E. faecalis UGRA10

In order to investigate the role of enterocin AS-48 in the anti-infective action of UGRA10 strain, we obtained AS-48 negative (AS-48$^-$) mutants from this strain by treatment of cell suspensions with a sublethal concentration of acridine orange for 18 h. The cells surviving the treatment were grown onto BHA plates and the colonies were replicated in the same medium for checking its inhibitory activity against *E. faecalis* S-47. Cells showing no inhibitory activity were selected for further analysis.

The relationship of the mutants AS-48$^-$ with the wild strain was investigated by Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) of the total DNA.. Genomic DNA isolation was based on the method proposed by Martín Platero et al. (2007). ERIC-PCR was carried out according to Martín-Platero et al. (2009).

2.3. Animals

Six-week-old female BALB/c mice were purchased from Charles River Laboratories (L'Arbresle, France) and were quarantined and acclimatised for 2 weeks. The mice were housed in transparent polypropylene cages equipped with HEPA filter cover with a 12 h

light/dark cycle in a controlled atmosphere (temperature 22 ± 2 ºC, humidity 55 ± 2%). The animals were fed *ad libitum* a purified diet (Harlan, Barcelona, Spain) and had free access to water during the experimental protocol. The protocols were carried out according to the guidelines of the Helsinki declaration and in accordance with animal care and use guidelines approved by the Ethic Committee of Animal Experiment of the Universidad de Granada.

2.4. Enterococcal suspensions

E. *faecalis* UGRA10 and AS-48^{-} mutants of UGRA10 strain were grown in BHI broth at 37 ºC for 18 h. After incubation, the cultures were harvested by centrifugation at 6000 x g for 20 min (4 ºC) and washed with sterile phosphate buffered saline (PBS). Bacteria suspensions were prepared at a final concentration of approximately 1 ×10^{9} CFU/mL in sterile PBS.

2.5. *Immunomodulatory properties*

Experimental Design. After 14 days of acclimation, mice were randomly assigned into two different experimental groups (20 per group): a control group receiving orally just the vehicle (PBS) and a probiotic-treated group receiving orally UGRA10 (10^{9} CFU/mice/day) for 30 days. Oral gavage was performed by intragastric stainless steel cannula needle. Throughout the experiment, the animal's activity, behaviour and general health signs were monitored on a daily basis. Mice were sacrificed by cervical dislocation and spleens were removed under sterile conditions and weighed.

Spleen cell proliferation assays. Spleens were removed aseptically from the mice and placed individually into 5 mL of complete RPMI-1640 medium (Sigma-Aldrich, Missouri, USA). Single cell suspensions were prepared by chopping the spleens into small pieces with sterile scissors and then forcing the spleen tissue up and down through a 1.0 mL syringe. After erythrocyte lysis in Red Blood Cell Lysis Buffer (Roche Diagnostics, Mannheim, Germany), splenocytes were washed and suspended in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (0.25 µg/mL). All supplements were from Sigma-Aldrich. Cell suspensions were distributed (5 x 10^{5} cells per well) into 96 well cell culture clusters with flat bottom wells (Corning Costar Co., Tewksbury, USA). *Salmonella typhi* lipopolysaccharide (LPS, Sigma-Aldrich) was used at 2.5 µg/mL as a B-cell mitogen, and concanavalin A (Con A, Sigma-Aldrich) was used at 1 µg/mL as a T-cell mitogen. In each experiment, two cell cultures were

performed: one to assess cell proliferation and the other to determine the cytokine levels in supernatants. After incubation at 37 °C in 5% CO_2 for 3 days, proliferation of spleen cells was measured by colorimetric reading of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction, as described by Mosmann (1983).

Tumour necrosis factor (TNF-α) and gamma interferon (IFN-γ) assays. Spleen cells were cultured with LPS and Con A as described above and after 72 h the supernatants were removed and stored at 20 °C until assayed. TNF-α and IFN-γ were measured using the appropriate ELISA kits (Thermo Scientific, Rockford, USA). The cytokine concentration was interpolated from the appropriate TNF-α and IFN-γ standard curve.

2.6. *Murine model of protection against infections*

Inoculum preparation. *L. monocytogenes* CECT 4032 was grown overnight in BHI broth at 37 ºC. The day after the cells were recovered, washed and finally suspended in PBS solution. The cell concentration was established as routinely by decimal dilutions and plating onto BHA. For *C. perfringens* CECT 821 sporulation, bacteria were cultured previously in thioglycolate broth and then transferred into 90 mL Duncan-Strong (DS) sporulating medium (Becton, Dickinson & Co., Le Pont de Claix, France) and anaerobically incubated at 37 ºC for 4 days to induce sporulation. To count the number of heat-resistant spores, cultures were heated at 70 ºC for 20 min, serially diluted with PBS, plated onto BHA and then anaerobically incubated under the same conditions.

Experimental Design. For *L. monocytogenes* infection, ten mice per group, randomly assigned, were dosed by intragastric feeding by means of a stainless steel cannula needle with 10^9 CFU of *E. faecalis* UGRA10 or the mutant *E. faecalis* UGRA10-AO2 for 20 days. On the day 20, mice were challenged with *L. monocytogenes* CECT 4032 (each mouse was administered by oral pipette with 10^9 CFU of bacteria). Enterococci were administered also during six days after infection. Control mice received a PBS placebo for 20 days before listerial infection. Daily faeces samples were collected. Three days after infection, mice were killed by cervical dislocation, livers and spleens were removed and CFU of *Listeria* per organ was determined as described below.

In addition, a murine model of protection against *C. perfringens* infection was designed. For that, mice were randomly assigned in groups (10 per group) and were dosed by intragastric feeding by means of a stainless steel cannula needle with 10^9 CFU of *E. faecalis* UGRA10 or the mutant *E. faecalis* UGRA10-AO2 for 20 days. On the day 20, mice were challenged with *C. perfringens* CECT 821 (each mouse were administered by oral

pipette with 10^6 CFU of clostridial spores). Enterococci were administered also during ten days after infection. Control mice received a PBS placebo for 20 days before clostridial infection. Daily diarrhoea and disease signs were recorded. The number of deaths was also recorded. Stool samples were periodically collected for C*. perfringens* determination.

Microbiological determinations. For *L. monocytogenes* determination, livers and spleens were aseptically removed, weighed and mixed (1:10, w:v) with dilution vehicle (0.1% peptone, 0.85% NaCl). The homogenisation was prepared by chopping the organs into small pieces with sterile scissors and then forcing the spleen and liver tissues up and down through a 1.0 mL syringe. 10-fold serial dilutions were made and plated on chromogenic selective agar COMPASS Listeria Agar (Biokar Diagnostics, Pantin, France). Bacterial cultures were incubated at 35 ± 2 ºC for 24 to 48 h before colony counting.

To investigate the potential of the *E. faecalis* UGRA10 to prevent the invasion of mice by *C. perfringens,* stool samples were removed, weighed and dissolved in sterile PBS. Samples were serially diluted and plated on Tryptose Sulfite Cycloserine Agar (TSC) plates (Biokar Diagnostics) and cultured anaerobically for 24 to 48 h at 37 ºC before colony counting.

2.7. Statistical analysis

All results are expressed as the mean±SD. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA). The differences between probiotic-treated and control groups were analysed by using Student's *t*-test. Survival curves were estimated by the Kaplan–Meier method and compared by the log-rank test. All statistics were performed using SPSS-PC 15.0 software (SPSS, Chicago, Ill. USA), and the statistical significance level was accepted at $P < 0.05$.

3. Results and discussion

3.1. *Immunomodulatory properties of E. faecalis UGRA10*

The gastrointestinal microbiota is essential to human and animal health, as it contributes to the development and optimal functioning of the intestinal immune system. Numerous reports have suggested that LAB influence the immune system and have the potential to promote beneficial effects on health (Isolauri et al., 2001; Bermudez-Brito et al., 2012). *E. faecalis* UGRA10 is a potential probiotic strain whose ability to interfere the

adhesion of *L. monocytogenes* to Caco-2 cells has been previously shown (Cebrian et al., 2012).

In the present work, we have investigated for the first time, the immunomodulatory effects of the oral administration of UGRA10 in immunocompetent mice. With this aim, we obtained splenocytes from mice orally administered UGRA10 for 30 days and also from untreated mice (controls) and the proliferative response and cytokine production after stimulation with Lipopolysaccharide (LPS) and Concanavalin A (CoA) were measured. As shown in Fig. 1, the proliferative responses of spleen cells taken from mice 30-day-feed with the probiotic when stimulated with LPS and Con A were enhanced by 44% (significant difference at $P<0.01$) and 32% respectively, with regard to the respective cytokine levels of untreated controls. The TNF-α levels of the culture supernatants from splenocytes of UGRA10- treated mice were lower than the LPS-only-mediated control group. On the contrary, CoA-mediated IFN-γ levels in the splenocytes of UGRA10 dosed mice were significantly ($P < 0.01$) increased as compared to the CoA-only-mediated control group (Fig. 2).

Thus *E. faecalis* UGRA10 administration resulted in an increase of the total number of spleen cells, what can lead to a change in the production of molecular mediators of immune response. This is the case of IFN-γ whose levels increased by 46% in splenocytes of UGRA10 feed mice with respect to splenocytes from control. This indicates the usefulness of this probiotic to direct specific responses towards Th1-profiles, which makes this strain an attractive candidate to be used as oral adjuvant (Castro et al., 2008). Recently, Molina et al. (2015) obtained similar results showing a significant increase of IFN-γ secretion in mice, which were orally administered with the *E. faecalis* CECT 712 probiotic strain. Kosaka et al., (2012) also indicated that the intake of *Lactococcus lactis* subsp. *cremoris* FC induces the production of IFN-γ by spleen cells. Hua et al., (2010) showed that IFN-γ levels were higher in mice that consumed Bio-Three, a commercial mixture of *Bacillus mesentericus, Clostridium butyricum and Enterococcus faecalis* probiotic strains. Similarly, Kato et al., (1999) showed the ability of a *Lb. casei* probiotic strain to increase the levels of IFN-γ produced by mouse splenocytes after stimulation with Concanavalin A.

Another immunomodulatory mechanism attributed to probiotics is the anti-inflammatory effect. This effect can be exerted through the modulation of the balance between Th1/Th2 and downregulation of proinflammatory cytokine production (e.g., IL-12, TNF-α) (Lorea-Baroja et al., 2007). In this work, TNF-α levels were reduced 23.5% in splenocytes of UGRA10 feed mice with respect to splenocytes from control. This effect may constitute a mechanism to prevent exacerbated inflammatory responses. In agreement with

our results, recent studies showed the ability of certain *E. faecalis* strains to reduce the expression of TNF-α in vivo (Wang et al., 2014). Han et al., (2015) also showed the anti-inflammatory activity of *L. lactis* NK34 after reducing secretion of TNF-α in RAW 267.7 cells using LPS as an inflammatory mediator. Therefore, *E. faecalis* UGRA10 strain could suppress the inflammatory responses and may help the intestine to maintain the immune balance in response to acute inflammation when exposed to high levels of endotoxin, as occurs in Gram-negative bacterial infections. This immunomodulatory effect on secretion of TNF-α is in accordance with the results obtained by Rodríguez-Nogales et al. (2012) for UGRA10 in cell cultures stimulated or not with LPS. The authors found that, in basal conditions, UGRA10 increased the production of IL-1β and TNF-α in macrophages RAW 264.7 but exerted an inhibitory effect on the LPS-induced cytokine (IL-8, TNF-α) or nitrite production in RAW 264.7 and HT-29 cells.

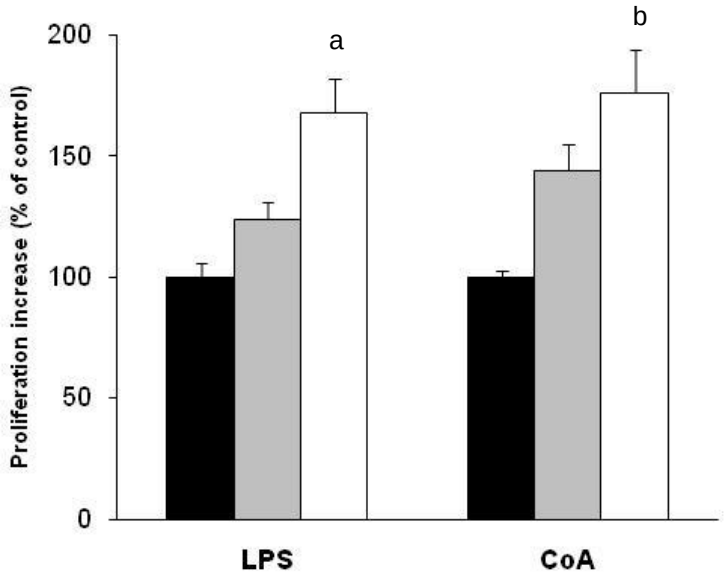

Figure 1. Effect of *E. faecalis* UGRA10 administration on the proliferation of mouse splenocytes. Mice were orally administered UGRA10 (10^9 CFU/mouse/day) for 30 d and after sacrificing them, the splenocytes were extracted, cultured and stimulated with mitogens LPS or Con A. Black bars: proliferation of splenocytes obtained from non-stimulated control mice. Grey bars: proliferation of splenocytes obtained from control mice stimulated with LPS or CoA. White bars: proliferation of splenocytes obtained from UGRA10-treated mice and stimulated with LPS or CoA. The data represents the mean ± SD of two independent experiments. Statistical P values were determined using a two-tailed t-test: (a) $P < 0.01$; (b) $P < 0.05$; compared with controls.

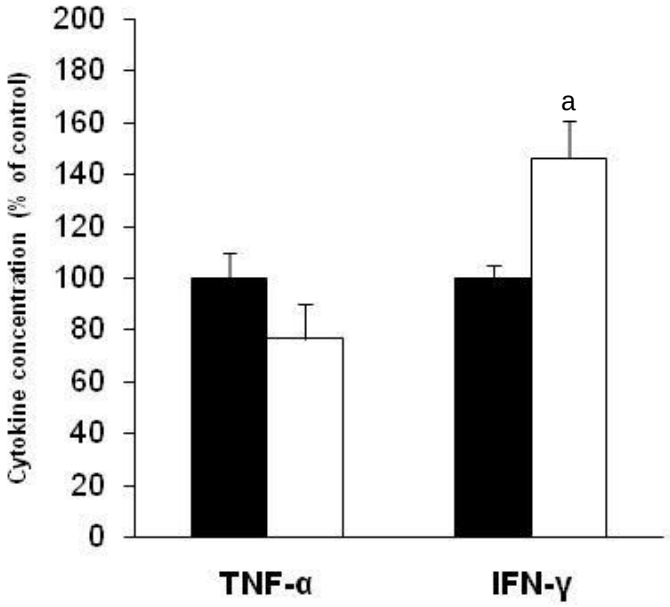

Figure 2. Effect of *E. faecalis* UGRA10 administration on the TNF-α and IFN-γ secretion by mouse splenocytes. Mice were orally administered UGRA10 (10^9 CFU/mouse/day) for 30 d and, after sacrificing them, the splenocytes were extracted, cultured and stimulated with mitogens LPS or Con A. Black bars: cytokine levels from control mice. White bars: cytokine levels from UGRA10 treated mice. The data represents the mean ± SD of two independent experiments. Statistical P values were determined using a two-tailed t-test: (a) $P < 0.01$ compared with controls.

Results shown here indicate that *E. faecalis* UGRA10 strain could be suitable for modulating immune responses and skewing the cytokine profile, the production of IFN-γ and the inhibition of TNF-α and might be investigated as an adjuvant strategy in vaccination or in order to mitigate the inflammatory process by infections and bowel diseases. Hence, we can conclude that *E. faecalis* UGRA10 should be considered a potential probiotic candidate for human and animal applications.

3.2. *Resistance to infections by E. faecalis UGRA 10 administration*

In previous studies Cebrian et al., (2012) showed the ability of UGRA10 strain to inhibit *L. monocytogenes* colonisation in cell line cultures. We showed that when the UGRA10 strain was added simultaneously or 1 h before *Listeria*, a strong inhibition of pathogen-adhesion on Caco-2 cells could be observed. When UGRA10 was added 1 h after *Listeria*, no significant inhibition of adhesion produced by this pathogen was able to be detected. In this work, we have deepened in the UGRA10 ability to inhibit *L. monocytogenes* and *C. perfringens* colonisation in a murine model and we also determined the role of bacteriocin AS-48 as a possible protective mechanism against infection. In order to achieve this goal, we have firstly decided to obtain bacteriocin-negative mutants from UGRA10 strain

to compare the protective effect provided by both, wild and mutant strains. After obtaining the mutants we investigated the relationship of AS-48 negative mutants with the wild strain UGRA10, from which they had been derived by checking the ERIC-PCR pattern of both wild type and mutant strains. As shown in figure 3, there is a high similarity between ERIC-PCR profiles of UGRA10 (line 9) and mutants (lines 1-8).

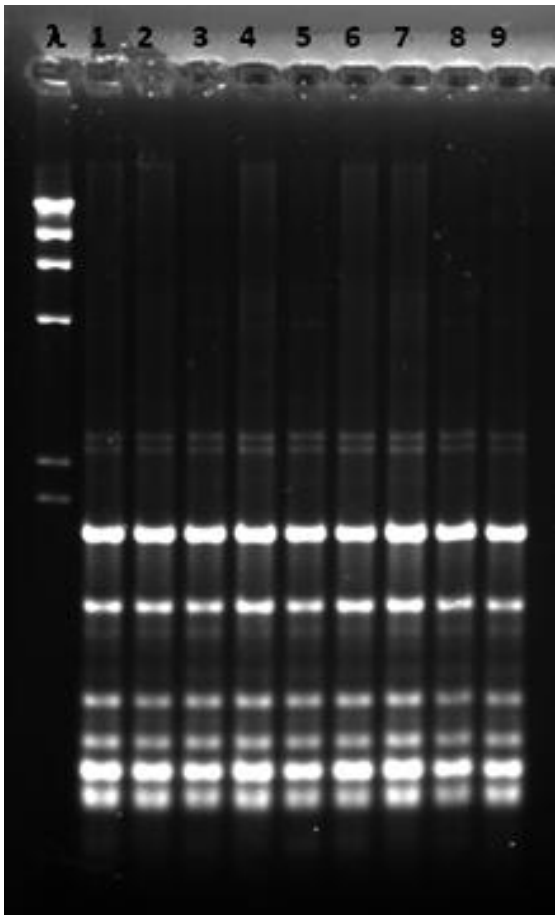

Figure 3. Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) patterns of the total DNA of *E. faecalis* UGRA10 and the AS-48 mutants. Lanes: l, standard molecular weights (l *Hind*III); 1 to 8, mutants AS-48$^-$; 9, wild UGRA10 strain.

3.2.1. Resistance to L. monocytogenes infection by E. faecalis UGRA 10 administration

The determination of *Listeria* number in the murine liver and spleen 3 d after oral inoculation with *L. monocytogenes* is a well established model of infection (Corr et al., 2007). When BALB/c mice were orally infected with *L. monocytogenes* CECT 4032 at 10^9 CFU per mouse, 10^6 *Listeria* reached the liver and the spleen by day 3, which is usual in this infection model. When mice were fed the UGRA10 or UGRA10-AO2 (negative AS-48) strains during 20 days at a dose of 10^9 CFU per mouse and subsequently infected with *L. monocytogenes*,

UGRA10 significantly (*P* <0.05) reduced the numbers of *Listeria* in both, the liver and the spleen (Fig. 4), with reductions of 2.38 and 1.58 log CFU/g compared with the control group. UGRA10-AO2 did not confer protection against *L. monocytogenes,* since mice treated with this mutant showed infection levels similar to those treated with placebo (Fig. 4). The results reveal a potential protective mechanism for the production of AS-48 against the infection by *L. monocytogenes*.

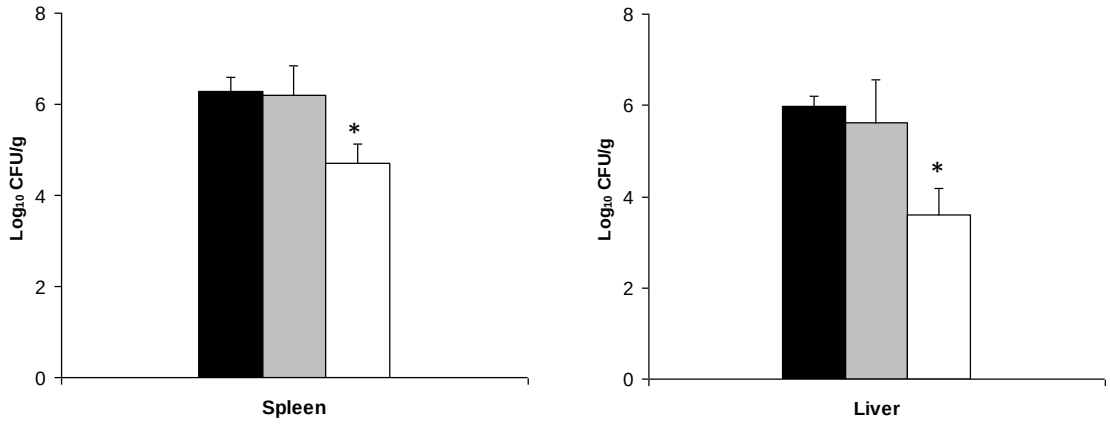

Figure 4. Effect of administration of *E. faecalis* UGRA10 and the AS-48 negative mutant UGRA10-AO2 on *L. monocytogenes* CECT 4032 infection in a murine model. Mice were oral dosed with the bacteria for 20 days before pathogen challenge, and 3 days after infection mice were killed and the number of *Listeria* per gram of spleen and liver was determined. Black bar: Control. Grey bar: UGRA10-AO2 strain; White bar: UGRA10 strain. * *P <0.05*, indicating the statistically significant differences between the numbers of bacteria infecting organs of enterococci administered mice compared with placebo-fed control mice.

3.2.2. Resistance to C. perfringens infection by E. faecalis *UGRA 10 administration*

By following the evolution of *C. perfringens* infection in BALB / c mice a death rate of 50% was recorded in the control group. In the groups fed the UGRA10 strain or its mutant UGRA10-AO2 (negative AS-48) strains, a significant protective effect was observed, with a survival rate of 80% in both groups (Figure 5). The mortality rates in the groups that received UGRA10 or the mutant were significantly lower (*P<0.05*) than in the control group when analysed according to the Kaplan–Meier method.

The microbiological analysis of stool samples allowed us to see the evolution in time of *C. perfringens* counts, expressed as log CFU/g faeces. As it can be seen in Fig. 6, a clear reduction of clostridia in the stools of animals receiving the AS-48 producing strain, *E. faecalis* UGRA10, was observed as the viable counts of *Clostridium* in the group treated with UGRA10 were 0.77, 1.45, 2.32 and 1.70 log lower than those of the untreated control at 3, 6,

8 and 10 post-infection days, respectively. By contrast, in the group of animals that received UGRA10-AO2 (AS-48 negative) *C. perfringens* counts were very similar to those obtained for the control group.

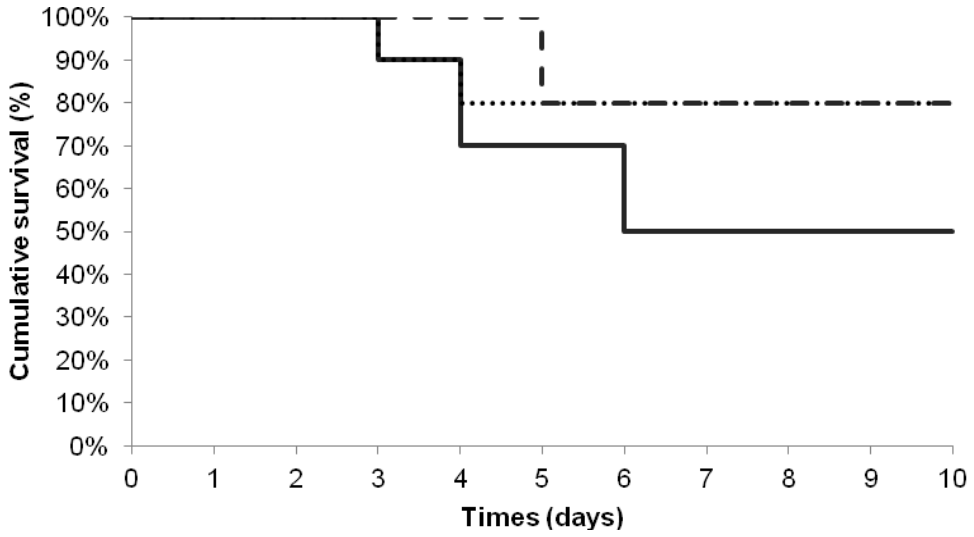

Figure 5. Survival curves of BALB/c mice challenged with *Clostridium perfringens* CECT 821. Control (—); *E. faecalis* UGRA10 fed (·····); Mutant *E. faecalis* UGRA10-AO2 fed (---). Mice were oral dosed with the bacteria for 20 days before pathogen challenge and 10 d after infection.

This decline in *C. perfringens* counts might be explained by the action of AS-48, but considering the survival results, it seems that, apart from enterocin production, other protection mechanisms may be involved in the protection against *C. perfringens,* such as physical interference with pathogen colonisation or immunomodulatory regulation of the host, any or all which would result in an improvement of the natural mechanisms of defence against infections. This could explain the fact that animals receiving the mutant strain UGRA10-AO2 reached a similar protection level than animals treated with UGRA10. To support this hypothesis, we can consider the proven immunomodulatory activity of strain UGRA10 that acts on INF-γ and TNF-α secretions, although we have not evaluated this property in the mutant UGRA10-AO2. The continuous administration of both strains with immunomodulatory properties could reduce the levels of TNF- α and other proinflammatory cytokines produced in excess during infections caused by enteropathogens as it is *C. perfringens*.

There are few studies relating the bacteriocin production with the resistance to infections by pathogens. The most remarkable study addressed to this purpose, is the one of Corr et al., (2007), in which it was shown that Abp18 bacteriocin provided an essential

mechanism for the anti-infective activity of *Lb salivarius* UCC118 against *L. monocytogenes* in an experimental mouse model. In this case, the inhibition of the pathogen was shown to be the direct result of the production of bacteriocin Abp118, as it was shown that a non-bacteriocin-producing isogenic derivative failed to protect mice from infection.

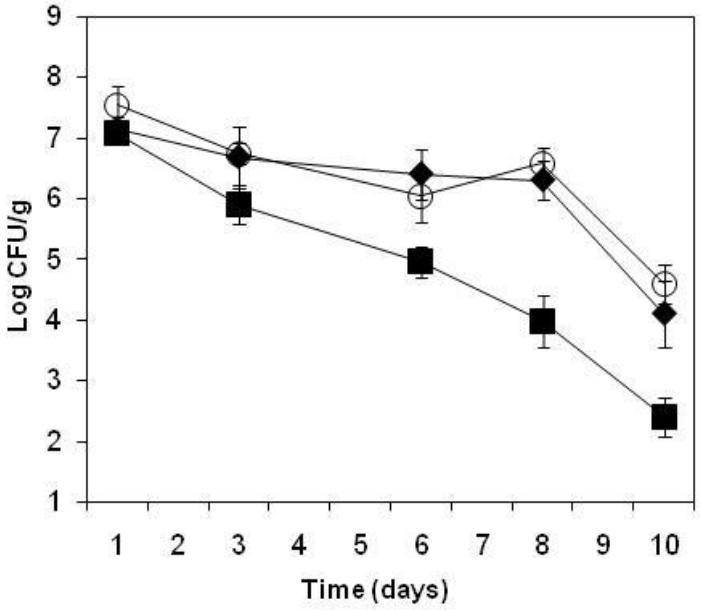

Figure 6. Evolution of viable counts of *Clostridium perfringens* in faeces of BALB/c mice challenged with *Clostridium perfringens* CECT 821. Control (♦); *E. faecalis* UGRA10 (■); Mutant *E. faecalis* UGRA10-AO2 (○). Mice were oral dosed with the bacteria for 20 days before pathogen challenge and 10 days after infection. Periodically, stool samples were recovered for *C. perfringens* determination.

Furthermore, Millette et al. (2008) showed that the pediocin PA-1/AcH producing *P. acidilactici* MM33 strain reduced vancomycin-resistant enterococci (VRE) populations in vivo. In these studies, mice received daily intragastric doses of *P. acidilactici* MM33 or *P. acidilactici* MM33A (a non-pediocin-producing mutant). Levels of VRE in the groups of animals administered the bacteriocin-producing strains were below the detection threshold, whereas VRE levels in mice fed the non-pediocin-producing strain were similar to those of control mice. Recently, Riboulet-Bisson et al., (2012) showed that bacteriocin production by *Lb. salivarius* UCC118 affected the *Bacteroidetes* and *Firmicutes* phyla on mice and pig microbiota. Nevertheless, bacteriocin production may be only partially responsible for the probiotic administration effects, as the authors themselves admitted, and the involvement of other mechanisms must also be considered.

Similar studies to ours have been conducted recently with CRL35 enterocin, a bacteriocin produced by *E. mundtii* CRL35 (Salvucci et al., 2012). These authors evaluated

the effectiveness of this enterocin and its producer strain in a murine model of pregnancy-associated *L. monocytogenes* infection, showing its ability to reduce *Listeria* translocation, with a decrease in pathogen levels both in the spleen and the liver after 3 days of infection in animals treated with the probiotic. Furthermore, no *Listeria* was recovered from the mother's or the foetus' bloodstream of these animals treated with bacteriocin CRL35.

Other authors, however, obtained contradictory results. Bernborn et al. (2006), for example, investigated the ability of the pediocin AcH producer *Lb. plantarum* DDEN11007 to prevent *L. monocytogenes* infection in rats. In this case, the introduction of the probiotic strain prior to *Listeria* inoculation resulted in a subsequent relative increase in pathogens counts in the livers and spleens of animals in comparison with those of control animals. The authors of this study attributed this increase to a lowering of the intestinal pH, suggesting that the production of lactic acid induced virulence gene production in *Listeria*.

4. Conclussions

The results obtained in this study suggest the immunomodulatory effect of UGRA10 strain through the cytokine balance (INF-γ and TNF-α) and also the protective effect of this strain against the infection by *L. monocytogenes* and *C. perfringens* in which the enterocin AS-48 would probably play a significant role. Considerable progress has been achieved with respect to enterocin AS-48 structure, function and regulation. Nevertheless, further research is needed to examine other aspects related to the UGRA10 application as a probiotic, including the strain survival, the dosing regimen or the animal target. In addition, it would be interesting to better understand the factors that control the production of AS-48 in the GI host tract since actively produced in vitro bacteriocins may not necessarily be produced in the GI tract (Kirkup et al., 2006). More detailed studies on aspects related to the ability to produce AS-48 in complex environments or the probiotic properties related to the producer strain regardless of the bacteriocin production are required. Thus this information would lead to a greater knowledge of the bacteriocinogenic probiotic strain *E. faecalis* UGRA10 and the potential applications in human and animal health.

Acknowledgements

This work was supported by Grant (P07-AGR-02539) from the Junta de Andalucía. We would also like to acknowledge the work of Jose Manuel García Madero in editing the text.

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II.4. Protection against *Lactococcus garviae* infection by *Enterococcus faecalis* UGRA10, an enterocin AS-48 producer strain.

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Abstract

Probiotics are emerging as an alternative to antibiotics for the control of fish diseases. The aim of this study was to evaluate in vitro and in vivo the antimicrobial effects of *Enterococcus faecalis* UGRA10, a potential probiotic strain, and its enterocin AS-48, against *Lactococcus garvieae,* causal agent of lactococcosis in the rainbow trout. The Minimum Bactericidal Concentrations of AS-48 against *L. garvieae* CECT 5807, 5806 and 5274 were established in 15.62, 15.62 and 7.81 µg/mL respectively. In broth cultures, the enterocin at 100, 50 and 25 µg/mL was able to eliminate 10^8 CFU/mL of lactococci after 2, 5 and 10 h exposure, respectively. In co-cultures of UGRA10/*L. garvieae* at a 1/10 CFU/mL ratio, lactococci were completely eliminated after 24 h co-cultivation. The studies of UGRA10 biosafety and AS-48 toxicity in zebrafish have shown the lack of adverse effects of both, strain and bacteriocin. Zebrafish challenged with *L. garvieae,* UGRA10 administered in diet 20 d before infection, presented a cumulative survival rate of 70% compared with the 50% recorded for control fish. Trout inoculated with the pathogen and treated with AS-48 by periodically dipping in AS-48 baths, presented a survival rate at the end of the study (20 days) of 60% compared with that of non-treated fish. These results indicate the protective effect of UGRA10 and AS-48 and the potential of these natural products as an alternative to antibiotics for controlling emerging diseases in aquaculture.

Keywords : Probiotic, enterocin AS-48, *Lactococcus garvieae*, bacteriocin, fish pathogen, trout.

Article in-progress

1. Introduction

Aquaculture provides effective means for the intensive production of aquatic organisms under controlled conditions. However, high densities of fish populations in farms produce severe stress conditions which leads to economic and productivity losses due to an increase in infectious diseases (Bondad et al., 2005; Toranzo et al., 2005).

Lactococcus garvieae is a Gram-positive coccus, aerotolerant, non-spore-former, producing haemolysis on blood agar (Eldar et al. , 1996; 1999). *L. garvieae* is an emerging zoonotic pathogen that causes serious infections in humans and animals called lactococcosis (James et al., 2000; Rasmussen et al., 2014; Backes et al., 2015). It has been shown to cause fish diseases with high mortality among wild and cultured fish, including rainbow trout (*Oncorhynchus mykiss*) (Barnes et al., 2002; Vendrell et al., 2006). Lactococcosis causes a variety of clinical signs such as anorexia, melanosis, lethargy, conjunctivitis, haemorrhages and septicemia (Avci et al., 2014). This pathology has great economic and health relevance for the aquaculture sector in the Mediterranean countries, particularly in Spain, the leading producer of trout in the European Union (FAO, 2014; APROMAR, 2014).

In recent years, prevention and control of fish diseases such as lactococcosis have been focused on the use of antibiotics, which generate significant risks to public health by promoting the selection of resistant strains (FAO, 2006), presence of residues in water and animal tissues (Prater, 2005; Matyar, 2007; Vendrell et al., 2012; Di Salvo et al., 2013), as well as alterations in the gastrointestinal microbiota of aquatic species (Nakano, 2007). Furthermore, consumers are increasingly demanding natural products, free of additives such as antibiotics.

Nowadays, vigorous endeavours to prevent these diseases have been focused on good husbandry techniques and vaccination. Although these methods can improve fish health, they appear to be insufficient to prevent disease outbreaks. Furthermore, vaccines are often expensive and highly stressful for the animals (Corripio-Miyar et al. 2007). In aquaculture, there is a growing tendency nowadays to prevent infectious diseases, instead of treating them. Thus, the use of probiotic bacteria is gaining popularity in the aquaculture industry as a sustainable alternative against the use of antibiotics (Pérez-Sánchez et al., 2014; Newaj-Fyzul et al., 2014). The majority of probiotics used in aquaculture belong to lactic acid bacteria (LAB) (Gatesoupe, 2008). *Pediococcus pentosaceus* (Huang et al., 2014), *Lactobacillus plantarum* (Dash et al., 2015), *Lactobacillus rhamnosus* (Nikoskelainen

et al., 2002), *Lactococcus lactis* (Beck et al., 2014) or *Enterococcus faecium* (Chang and Liu 2002; Kim et al., 2012) are examples of LAB that has been considered for use in aquatic organisms.

The use of probiotics in aquaculture offers a wide variety of important health benefits for the host (Tinh et al., 2008). Some of the mechanisms of action of probiotics could be explained by competitive exclusion (Balcázar et al., 2006), enzymatic contribution to digestion (Musa et al., 2009), enhancement of the immune response (Kim et al., 2006; Huang et al., 2014; Akhter et al., 2015) or by the production of inhibitory substances (Kesarcodi-Watson et al., 2008). These substances with antimicrobial effects like lysozyme, hydrogen peroxide or bacteriocins constitute a barrier against the proliferation of opportunistic pathogens (Verschuere et al., 2000).

Bacteriocins are ribosomally synthesized peptidic compounds, lethal to those bacteria closely related to the producing bacteria (Joerger et al., 2003). Bacteriocins such as nisin, pediocin Ac or several enterocins, are efficient weapons to protect intestines from pathogenic invasions, so the production of theses antimicrobial peptides by a strain constitutes a pertinent feature for selecting a probiotic (Riley 1999, Desriac et al., 2010; Dobson et al., 2012). Therefore, bacteriocinogenic strains appear to be excellent candidates since bacteriocins would be used as antibiotic alternatives, whereas bacteria would be used as probiotics (Gillor et al., 2008).

Enterocin AS-48 is a cationic circular bacteriocin produced by *Enterococcus faecalis* S-48 with broad bactericidal activity against most Gram-positive bacteria, including pathogens such as *L. monocytogenes*, *Staphylococcus aureus*, *Mycobacterium* spp., *Bacillus cereus*, and some Gram-negative bacteria (Abriouel et al., 1998; 2002; Gálvez et al., 1989). The target of AS-48 is the bacterial cell membrane in which the bacteriocin inserts and destabilizes the membrane potential, thus leading to pore formation and cell leakage (Sánchez-Barrena et al., 2003). The features of AS-48 (a broad spectrum of antimicrobial activity, stability across a wide range of temperatures and pH, and sensitivity to digestive proteases) (Gálvez et al., 1986) make it a promising alternative to classical chemical preservatives when used as a biopreservative in foods. AS-48 has been shown to be effective for the control of various foodborne pathogens in dairy, meat, vegetable and fish products (Ananou et al., 2014; 2010; Cobo Molinos et al., 2008; Muñoz et al., 2007) and also against several spoilage bacteria in meat products and vegetable-derived foods (Baños et al., 2012; Grande et al., 2007). Moreover, other applications of AS-48 focused on the pharmaceutical sector have been recently reported. (Maqueda et al., 2013).

In a previous work, we isolated from a Spanish sheep's cheese an enterocin AS-48 producing strain, *E. faecalis* UGRA10 (Cebrian et al., 2012), on which an extensive study of its in vitro functional, biosafety and probiotic characteristics was conducted. The present study aims to evaluate the in vitro antimicrobial effects of UGRA10 strain and its enterocin AS-48 against pathogenic strains of *L. garvieae* and the in vivo effectiveness of both, strain and bacteriocin, to protect zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) against this pathogen.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Enterococcus faecalis UGRA10 was used as probiotic and AS-48 producer. *E. faecalis* S-47 from our collection was used as the standard indicator strain for bacteriocin activity assays. *Lactococcus garvieae* CECT 5807 and *Lactococcus garvieae* CECT 5806, supplied by the Spanish Type Culture Collection (CECT), were used as target organisms for in vitro studies. For fish infection, *Lactococcus garvieae* DSMZ 6783, obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), was used. All strain cultures were maintained at 4 ºC on BHI-agar slants (Scharlau, Barcelona, Spain).

2.2. Preparation of UGRA10 and bacteriocin AS-48

AS-48 was produced by culturing the strain *E. faecalis* UGRA10 in a whey-derived substrate, Esprion 300 (ES-300) (DMV Int., Veghel, Netherlands), supplemented with 1% glucose (Ananou et al., 2008). AS-48 was recovered from cultures by cation exchange chromatography on carboxymethyl Sephadex CM-25 (as described by Abriouel et al., 2003). Eluted fractions were tested for bacteriocin activity against the indicator strain S-47 by the agar well diffusion method (Gálvez et al., 1986). The approximate concentration of AS-48 (in µg/mL) in the preparation was estimated by comparing the diameter of the inhibition halo around the well with a titration curve obtained from purified bacteriocin. Before use, the eluted fractions were dialysed at 4 ºC against distilled water through a 2000-Da cut-off membrane to eliminate NaCl, and then sterilised by filtration (0.22 µm, Millipore, Belford, MA, USA).

2.3. Minimum Bactericidal Concentration (MBC)

A broth microdilution susceptibility assay was used for the determination of Minimum Bactericidal Concentration (MBC), as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2012). All tests were performed in Mueller-Hinton broth (Scharlau). Target bacteria were cultured overnight at 25 °C. Enterocin AS-48 dilutions (1:1; v/v) were prepared in 96-well microtiter plates to achieve final concentrations ranging from 0.1 to 500 µg/mL. Afterward, each well received 10 µL of bacterial suspension with 10^7 CFU/mL and 100 µL of Mueller-Hinton broth. Bacteria were aerobically incubated at 25 °C for 24 h. The concentrations showing a complete absence of visual growth were identified, and 100 µL of each culture were transferred onto Columbia Blood agar (Scharlau) plates and incubated at the same conditions of time and temperature as mentioned above. The complete absence of growth on the agar surface at the lowest concentration of AS-48 was defined as the MBC. Assays were conducted in duplicate.

2.4. Time-kill curves

Time-kill curves were performed following the procedure described by Guerillot et al., 1993. Each strain was grown overnight in Mueller-Hinton broth (Scharlau). Then, the initial number of bacteria was adjusted to 10^8 CFU/mL. This suspension was used to perform separate assays in sterile glass flasks. Each assay mixture contained bacterial suspension and different concentrations of AS-48 (100, 50 and 25 µg/mL). A flask without AS-48 was used as a control. The flasks were incubated aerobically at 25 °C with constant agitation. Samples were collected at intervals of 1, 2, 3, 4, 5 and 10 h, then serially diluted, plated in Columbia Blood agar (Scharlau) and incubated at 25 ºC for 24-48 h. Kill curves were represented by expressing the results of log CFU/mL against time.

2.5. Antagonism in co-cultures

Overnight cultures of *E. faecalis* UGRA10 and *L. garvieae* strains were diluted at 10^4 UFC/mL for UGRA10 and at 10^5 UFC/mL for *L. garvieae* into sterile BHI (Scharlau) and incubated at 25 ºC, individually and in co-culture. At selected times of 1, 3, 6, 12 and 24 h, samples were collected and serially diluted into sterile saline solution. Dilutions were plated in triplicate into Slanetz-Bartley agar (Scharlau) and LG-agar, (Chang et al., 2014) for quantification of enterococci and lactococci, respectively, and the average numbers of colonies obtained after 48 h incubation at 25 ºC were used to establish the growth curves of those bacteria cultured alone and co-cultivated.

2.6. Diet preparation

UGRA10 strain was grown in BHI at 30 ºC overnight with agitation in a shaking incubator. After incubation, cells were harvested by centrifugation (2000 × g at 4 °C for 5 min), washed twice with PBS and resuspended in the same buffer. Then, bacterial suspension was adjusted to 10^9 CFU/mL. The commercial feed based on slowly sinking granules with high (50%) content of protein (Discus Gran D-50 Plus, Tadeusz Ogrodnik, Poland) was used as the basal diet for supplementation with UGRA10 strain. Enterococcal suspension was added into the feed, mixing part by part in a mixer (Prospero 900, Kenwood, Barcelona, Spain) reaching a final concentration of 10^8 CFU/g feed. Bacterial concentration in the diet was determined by plate counting onto Slanetz-Bartley agar (Scharlau).

2.7. Animals

Zebrafish. Adult zebrafish (*Danio rerio*) of approx. 4-5 cm in size were obtained from Piscicultura Superior (Barcelona, Spain). The fish were transferred to continuously aerated tanks for two weeks until they were acclimatised. The fish were fed twice daily with commercial fish granules (Discus Gran D-50 Plus). The tanks were maintained with a water temperature of 24–25 ºC, pH 7–7.5 and a photoperiod of 12 h light-dark cycle.

Trout. Rainbow trout (*Oncorhynchus mykiss*) not vaccinated and with average weight of approximately 50 g were obtained from a commercial fish farm (Piscifactorías Andaluzas SA, Granada, Spain). The fish were acclimatised for 2 weeks in 200 L tanks before the start of the trial. All fish were maintained in continuously aerated water at 12 ºC with a photoperiod of 12 h and a water replenishment of 25% every day. The fish were hand-fed with a commercial pelleted high protein feed (Inicio Plus, BioMar Iberia, S.A**.,** Spain) at a rate of 1.5% of biomass per day.

2.8. Biosafety assay in adult zebrafish

Twenty fish were randomly distributed into two tanks of 5 L (10 per group). One tank was inoculated with UGRA10 to a final concentration in water of 10^9 CFU/mL. The non-inoculated group was the control. For a period of 15 days, the health status of the animals was monitored, recording mortality and identifying visible clinical signs such as anorexia, lethargy, exophthalmia or erratic swimming. Then, fish were sacrificed and heart and spleen were removed in sterile conditions. For *E. faecalis* UGRA10 determination, organs were mixed (1:10; w:v) with PBS. The homogenisation was prepared by forcing tissues up and

down through a 1.0 mL syringe in petri plates. Pre-enrichment in BHI (Scharlau) and plated on Slanetz- Bartley agar (Scharlau). Bacterial cultures were incubated at 30 ºC for 48 h before the colony counting. The assay was performed in duplicate.

2.9. Toxicity assay in adult zebrafish

In order to know if the LC50 is greater than the assayed therapeutic dose of AS-48 (2.5 µg/mL administered by dipping bath), a Limit Test was performed using the procedures described in the Guideline for testing of chemicals from the Organization for Economic Cooperation and Development (OECD, 2013). For that purpose, 20 adult zebrafish were randomly transferred into two continuously aerated 5 L tanks for seven days until they were acclimatised. Test conditions included a water temperature of 24-25 °C, pH 7.5 and photoperiod was maintained at 12 h light-dark cycles.

For the Limit Test, a group of 10 fish was exposed to AS-48 (100 µg/mL) for 96 h. During the exposure time fish were not fed. Other tanks without treatment served as control group. The fish were inspected at least after 24, 48, 72 and 96 h. Visible abnormalities and mortalities were recorded. The assay was performed in duplicate.

2.9. Zebrafish model of infection

Experimental infection assays were performed according to the procedure described by Aguado-Urda et al., (2014). Fish were divided into four different tanks (ten animals per tank). During 20 days, animals were fed daily with commercial fish diet, except a group given a UGRA10 supplemented diet (described in section 2.6). Afterwards, zebrafish were anaesthetised with tricaine methanesulfonate (MS-222) and three of the groups were challenged by intraperitoneal injection with 20 µL of *L. garvieae* DSMZ 6783 cells suspended in PBS at a dose of 5 x 10^8 CFU/fish. Bacterial concentration in the inoculum was determined by ten-fold serial dilutions and further plating onto Columbia blood agar (Scharlau). The fourth group of fish was inoculated with 20 µl of sterile PBS as negative control for infection. After inoculation, the group that previously received UGRA10 supplementation continued with the same diet, whereas another group of 10 animals received an UGRA10 supplemented diet from the day of the infection. The control groups (positive and negative for infection) continued on the commercial diet. The zebrafish were observed twice a day for seven days after the infection and mortality was recorded every day for 2 weeks.

2.10. *Trout model of infection*

After the acclimatisation period, eighty trouts were divided into four 200 L tanks, each containing 20 fish. Each group of fish was infected by intraperitoneal injections of 100 µL of *L. garvieae* DSMZ 6783 cells suspended in PBS at a dose of 5 x 10^8 CFU/fish. Afterwards, one group received intraperitoneally an AS-48 solution (a total of 100 µg), a second group received periodically (every 3 days) baths for 30 min in a solution of AS-48 (2.5 µg/mL) and a third group did not receive any treatment (positive control). A fourth group received the same volume of physiological saline solution (negative control). Mortality was monitored for 20 d until no more dead fish were observed. All experimental procedures were conducted in accordance with the European Union legislation on animal experimentation and were carried out according to the guidelines of the Helsinki declaration.

2.11. Statistical Analysis

All results are expressed as the mean±SD. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA). The differences between treated and control groups were analysed by using Student's *t*-test. Survival curves were estimated by the Kaplan–Meier method and compared by the log-rank test. All statistics were performed using SPSS-PC 15.0 software (SPSS, Chicago, Ill. USA), and the statistical significant level was accepted at $P < 0.05$.

3 Results

3.1. In vitro antimicrobial assays

The enterocin AS-48 MBC values obtained, (Table 1) confirm the potential of AS-48 against *L. garvieae* strains. This antimicrobial activity was especially strong against *L. garvieae* CECT 5274, with a MBC of 7.91 µg/mL of AS-48.

The time-kill curves of AS-48 (100, 50 and 25 µg/mL) against *L. garvieae* CECT 5807 and CECT 5806, each assayed separately, are shown in Figure 1. The addition of AS-48 to cultures of any *L. garvieae* strain resulted in a rapid killing of bacteria. A decrease in the bacterial population was observed in cultures added of any bacteriocin concentration and the decrease was, as expected, concentration-dependent. Just after 30 min of exposure to AS-48 (100 µg/mL), the viability of *L. garvieae* CECT 5807 and *L. garvieae* CECT 5806 declined from 7.8 to 6 and 8.1 to 2.5 log CFU/mL, respectively. After 2 h, both strains were

completely inhibited for this concentration. At 50 µg/ml of AS-48, total inhibition of both strains was observed after 5 h of exposure. At 25 µg/ml, the total elimination of CECT 5806 strain was also achieved after 5 h. For *L. garvieae* CECT 5807, the complete inhibition occurred after 5-10 h of exposure.

Table 1. Minimum Bactericidal Concentrations of enterocin AS-48 against *Lactococcus garvieae*, assayed in BHI broth after 24 h at 37 ºC.

	AS-48 (µg/mL)
L. garvieae CECT 5807	15.62
L. garvieae CECT 5806	15.62
L. garvieae CECT 5274	7.81

a Data are expressed as the minimal antimicrobial concentration that caused a complete inhibition of bacteria growth in the three replicates. Range of concentration tested: 500 - 0.1 µg/mL

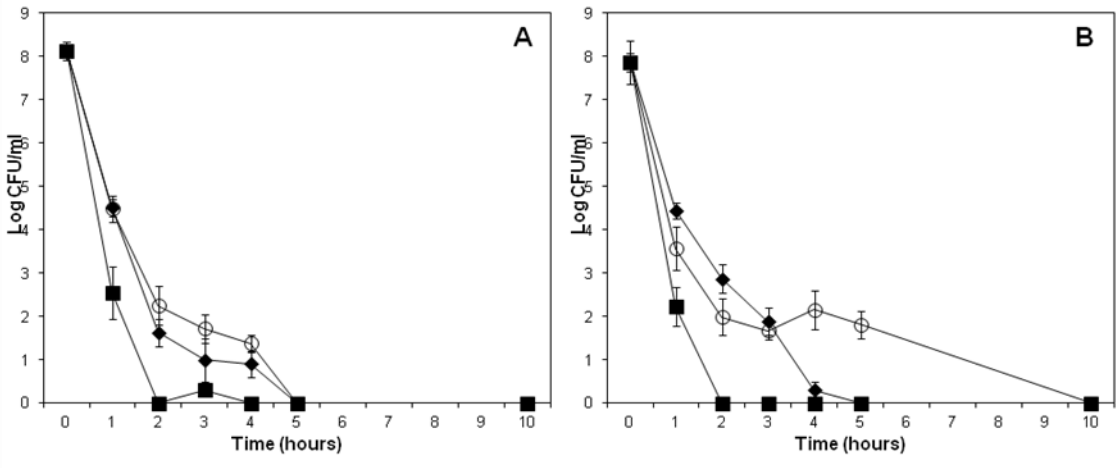

Figure 1. Effect of enterocin AS-48 on the viability of *Lactococcus garvieae CECT* 5806 (A) and L. *garvieae* CECT 5807 (B). The bacteriocin was added to cultures of *L. garvieae* at concentrations of 100 (■), 50 (♦) and 25 (○) µg/mL.

In order to test the antagonism of *E. faecalis* UGRA10 towards *L. garvieae* strains, a model of co-culture of each lactococcus strain (10^5 CFU/mL initial concentration) with the enterococci (10^4 CFU/mL initial concentration) in BHI broth was established. The results reported in Figure 2 show that *L. garvieae* strains in pure culture show a normal pattern of growth, and after a lag phase of 12 h, the culture enter the exponential growth phase to reach approx. 9 log CFU/mL in the stationary phase at 24 h. When *E. faecalis* UGRA10 was

co-cultivated with any of *L. garvieae* strains, an evident antagonistic interaction was observed. Thus, in both cases, the presence of the bacteriocinogenic strain caused reductions in lactococcus viability from 9-12 h of co-culturing, when lactococci assumed the exponential growth in pure cultures. The reductions in lactococcus viability caused by the presence of enterococci were significant (P<0.01) after 12 h of co-cultivation when counts of the pathogen fell dramatically to below the detection level after 24 h, remaining undetectable until the end of the experiment (48 h).

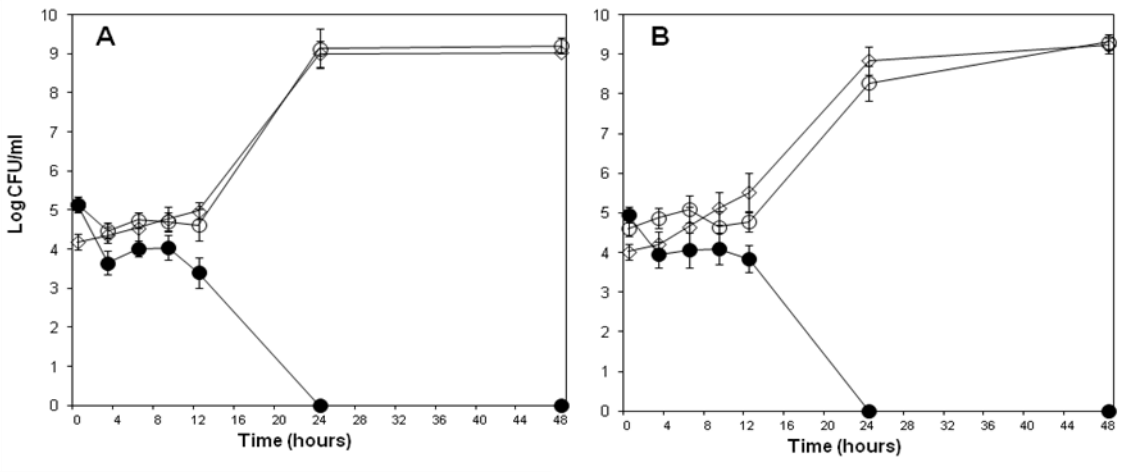

Figure 2. Growth of *Lactococcus garvieae* CECT 5806 (A) and *L. garvieae* CECT 5807 (B) in BHI broth, in pure cultures and in co-cultures with *Enterococcus faecalis* UGRA10. *L. garvieae* in co-culture (•); *L. garvieae* in solitary (o); *E. faecalis* UGRA10 in coculture (◊). Values are the average ± SD (error bars) of two independent experiments.

3.2. Safety assays

According to the EFSA approach (EFSA, 2011), most LAB species are included in the QPS list. However, in the case of *Enterococcus*, a more thorough, strain-specific evaluation is required to assess the risks associated to their intentional use in the food chain. The safety aspects of UGRA10 extensively investigated by both in vitro studies and in vivo experimental models in mice (Cebrian et al., 2012; Baños et al., 2015, unpublished data) have revealed that this strain can be considered as safe. Nevertheless, to gain more certainty on this controversial aspect of enterococci, we have evaluated here the potential capacity of UGRA10 to produce any disease in fish, using a model of adult zebra fish. Once again, the results showed the safety of this strain, as no deaths or signs of disease in fish were recorded. Neither bacteraemia nor translocation of enterococci from the aquatic medium to fish organs, heart and spleen, was observed in any of the experimental groups (Table 2).

Table 2. Incidence of bacterial translocation to heart and spleen in control and probiotic-exposed zebrafish.

	Zebrafish	
	Control	**UGRA10**
Heart	0/10	0/10
Spleen	0/10	0/10

While the application of antibiotics by bath treatment is a common practice in aquaculture, the use of semi-purified bacteriocins for the control of the pathogen by bath treatments is a completely new concept in this area. So, although bacteriocins are generally considered safe and harmless, very little is known about its application in aquaculture, as well as possible adverse effects on the general health of the fish. Therefore, in a first attempt to be aware of the basic principles related to the toxicity of the enterocin AS-48 for application in bath treatments, concentration limit values were established for a zebrafish model according to the OECD Guidelines for testing of chemicals (2013). According to this method, it could be estimated that DL50 for AS-48 is significantly higher than the therapeutic dose chosen, 2.5 µg/mL. Our study revealed that the survival rate of fish exposed to 100 µg/mL of AS-48 for 96 h was 100%. Besides that, fish showed no signs of toxicity. The limit test was performed using 10 fish, and the same number for the control. According to OECD, Binomial theory dictates that when 10 fish are used with zero mortality, there is a 99.9 % confidence that the LC50 is greater than 100 µg/mL. Consequently, the confidence intervals are quite wide.

3.3. Challenge tests

In order to investigate the potential role that *E. faecalis* UGRA10 can play in the control of lactococcosis in fish, challenge tests on zebrafish and rainbow trout were conducted.

Challenge test in zebrafish. In order to investigate the potential role that *E. faecalis* UGRA10 can play in the control of lactococcosis in fish, a challenge test on zebrafish was conducted. The results of zebrafish mortality rates for a 12-d period point to a protective effect of UGRA10 strain against the infection caused by *L. garviae* DSMZ 6783 (Figure 3). Results were obtained by comparing the survival rate of the two groups fed AS-48 with that of the control group that received no treatment (positive control) and with the non-infected

control group (negative control) and presented as cumulative survival rate. Infected fish that have previously received the probiotic (10^8 CFU/g feed) for 20 d before the infection appeared to be significantly more protected (P<0.05) than the controls, recording for these animals a cumulative survival rate of 70%. The animals that started the diet after infection showed a slight delay in the onset of the disease even though the cumulative survival rate at 10 days (50%) was the same as for the control group. The results confirm the usefulness of applying UGRA10 strain for preventive treatment against infections caused by *L. garvieae.* The continued administration of the probiotic would enable better colonization in the animal microbiota, exerting a protective role against pathogenic invasion. The involved mechanisms could be related with the ability of UGRA10 to produce AS-48, and even with the immunomodulatory effects that help to improve the natural defence mechanisms of the animal.

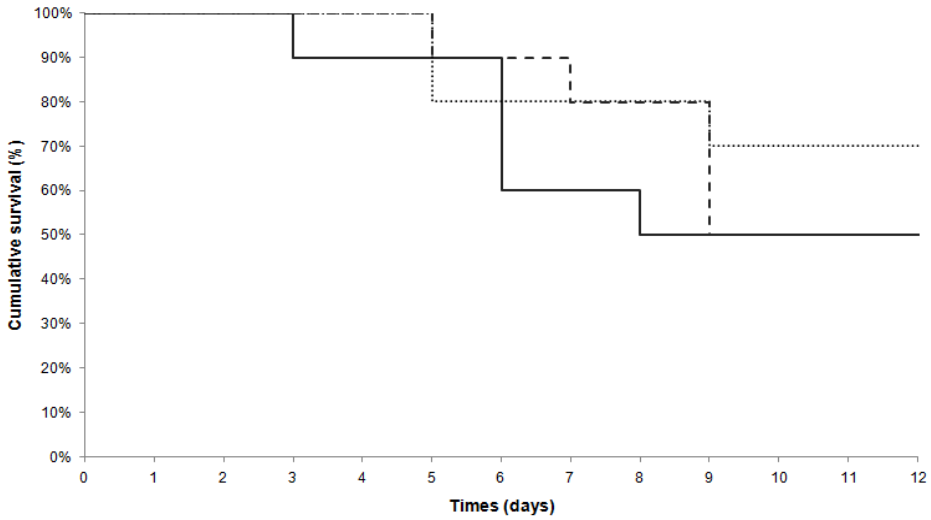

Figure 3. Survival curves of zebrafish challenged with *Lactococcus garvieae* DSMZ 6783 and administered with *Enteroccocus faecalis* UGRA10 (10^8 CFU/g feed) in different conditions. Control not administered with UGRA10 (—); Administered with UGRA10 supplemented diet for 10 d before the challenge (⋯); Administered with UGRA10 supplemented diet just after the challenge (---). The mortality rate was calculated according to the Kaplan–Meier method.

Challenge test in rainbow trout. This test was intended to investigate the potential role of the enterocin AS-48, produced by *E. faecalis* UGRA10, in the control of lactococcosis in fish. For this purpose, AS-48 was administered in two different ways, either by intraperitoneal injection (100 µg) or by periodical bathing with AS-48 solution (2.5 µg/ml). Both treatments were applied just after the infection with *L. garvieae.* The survival rates for both AS-48

treated groups were compared with a control group that received no treatment (positive control) and with the non-infected control group (negative control). For a 20-d period, mortality rates were recorded for each group, and results were presented as cumulative survival rate (Figure 4). Within the control group, inoculated with *L. garvieae* and non-treated with UGRA10 strain, the fish survival rate was 50% after 3 d of infection, compared to 100% survival rate for the group that received intraperitoneally AS-48 and 80% survival rate for the group of fish infected after application of the first AS-48 bath. After one week, the control group showed a 20% survival rate, in comparison with 70% of the group that received periodically AS-48 baths (after 2 baths), and a 100% survival rate for the group treated intraperitoneally with AS-48. After 11 d of infection, all animals of the control group died. The group of animals treated with periodic baths of AS-48 showed a survival rate of 70% (after 3 baths) and the survival rate for the group of fish that received intraperitoneally a single dose of AS-48 was 60%. At the end of the study (day 20), the survival rate of the group treated with AS-48 baths was 60% (after 6 baths), when compared with 30% of the group that received AS-48 intraperitoneally.

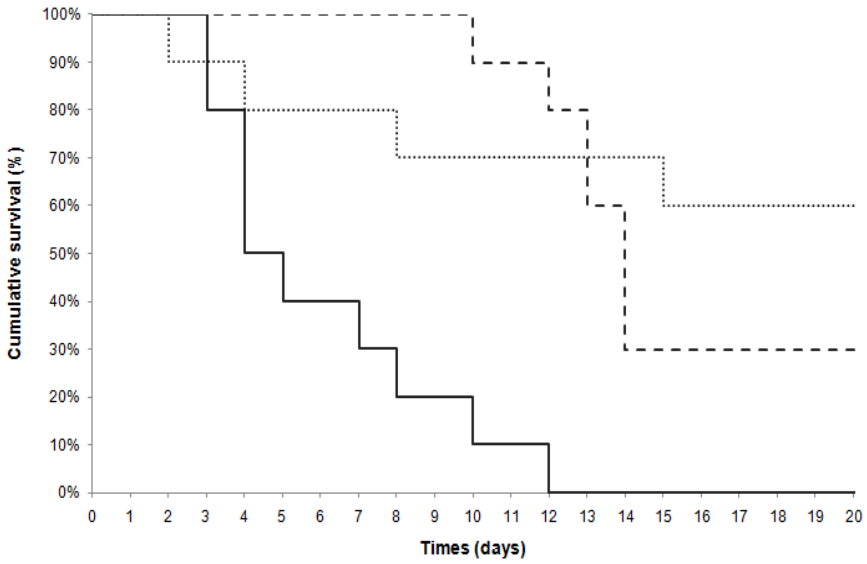

Figure 4. Survival curves of rainbow trout challenged with *L. garvieae* DSMZ 6783 . Control (—); periodical bathing with AS-48 solution (2.5 µg/ml) (⋯⋯); AS-48 intraperitoneal (100 µg) (---). The mortality rate in the groups that received periodically AS-48 baths was significantly lower (P<0.01) than the control group according to the Kaplan–Meier method.

The statistical analysis showed that fish that received the treatment based on immersion baths of AS-48 experienced a significantly lower mortality rate ($P < 0.01$) than that

of the control group. The intraperitoneal application of AS-48 also exerted a protective effect showing a survival rate of 30% at the end of the test. This method of application delayed the onset of infection by approx. one week, but once unleashed, the mortality rates were similar to those of the control group. These results suggest that the application of treatments sustained over time through bath immersions in AS-48 solutions might become an efficient therapy for the control of lactococcosis in rainbow trout.

4. Discussion

Bacteriocin production capacity has been proposed as a key property for the selection of probiotic strains with a potential application in aquaculture, as a natural alternative to the use of therapeutic antibiotics (Desriac et al., 2010). In aquaculture, lactococcosis produced by *L. garviae*, is one of the most serious diseases for both marine and freshwater cultured species (Vendrell et al., 2006). Our study showed the anti-infective activity against *L. garvieae* of *E. faecalis* UGRA10 and the bacteriocin produced by this strain, the enterocin AS-48.

Knowledge about LAB strains with antagonistic activity against pathogens of fish like *L. garviae* is still new and limited today (Sequeiros et al., 2015, Pérez-Sánchez et al., 2011; Lin et al., 2013). Some authors have described the use of bacteriocinogenic LAB strains for the control of fish infections in recent investigations (Muñoz -Atienza et al., 2013).

This study has shown the ability of *E. faecalis* UGRA10 to inhibit *L. garvieae* in co-culture and in experimental infections, reducing mortality in zebrafish previously fed a diet supplemented with probiotics during 20 d before the infection. Other authors have reported the use of other LAB with activity against this pathogen. Thus, Balcázar et al. (2007) showed the activity of *Leuconostoc mesenteroides* CLFP for the control of *L. garvieae.* Vendrell et al. 2008 reported the protective effects of *Lb. plantarum* CLFP 238 against infections by *L. garvieae*. Similar results were obtained by Pérez-Sánchez et al. 2011, showing that supplementing the fish feed with a bacteriocinogenic strain of *Lb. plantarum* mortality rate was reduced in rainbow trout infected with this pathogen. We have also shown the effectiveness of purified enterocin AS-48 against different strains of *L. garvieae* through in vitro and in vivo studies. Although information about bacteriocins with activity against *L. garvieae* is limited, some authors recently reported certain bacteriocins with activity against this pathogen. For example, Lin et al., (2013) showed a similar effect caused by Enterocin P, a bacteriocin produced by *E. faecium* B3-8. Other bacteriocins with potential application in aquaculture due to its activity against *L.garviae* are garvicin A (Maldonado-Barragán et al.,

2013) or nisin Z produced by *L. lactis* TW34 (Sequeiros et al., 2015) and *L. cremoris* WA2-67 (Araújo et al., 2015). Nevertheless, while it has been suggested that LAB bacteriocins may be one of the most powerful weapons to fight against these fish pathogens (Desriac et al 2010), there are still few fish studies investigating the relationship between the bacteriocin production by a strain and its involvement as a defence mechanism against infections. In vivo studies of Araújo et al., (2015) showed the role of Nisin Z production as the main anti-infective mechanism of *Lb. cremoris* WA2-67 against *L. garvieae.* Other authors, such as Schubiger et al. (2015) have shown that the entericidin produced by *Enterobacter* sp. C6-6 is an effective protective mechanism against infections by *Flavobacterium psychrophilum* in rainbow trout.

Moreover, some authors have highlighted the use of bacteriocins as a natural alternative to antibiotics in veterinary medicine and/or aquaculture (Montalbán-López et al., 2011; Nishie et al., 2012; Iyapparaj et al., 2013; Bali et al., 2014**)**. In fact, the application of fully or partially purified bacteriocins may be a useful method for the treatment of some bacterial infections, even with higher percentage of success than that obtained after administration of bacteriocinogenic bacteria. In accordance with that, our study also showed that the single application of a partially purified AS-48 solution administered either intraperitoneally or as bath treatments, exerts a safety and protective effect against lactococcosis in rainbow trout.

Therefore, *E. faecalis* UGRA10, an AS-48 producer strain with proven probiotic properties, represents a promising alternative for application as additive for fish feed. Besides, the application of fully or partially purified AS-48 in baths could be a safe and sustainable tool to prevent and control lactococcosis in fish farms. Overall, our results provide a new testing ground to develop new experiments that confirm the safety and usefulness of UGRA10 and its bacteriocin AS-48 in aquaculture for the control of fish pathogens.

Acknowledgements

This work was supported by Grant (P07-AGR-02539) from the Junta de Andalucía. We would also like to acknowledge to Piscifactorias Andaluzas S.A. and the work of Jose Manuel García Madero in editing the text.

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COMUNICACIONES CIENTÍFICAS

International Conference on Environmental, Industrial and Applied Microbiology
Barcelona. Spain 2015

In vitro and *in vivo* protection against *Lactococcus garviae* infection by the enterocin AS-48 producer strain *Enterococcus faecalis* UGRA10

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The Lactococcosis is a trout disease caused by *L. garvieae*. This pathology has great economic and health relevance for the aquaculture sector in the Mediterranean countries, particularly in Spain, the leading producer of trout in the European Union. The Lactococcosis in trout is associated with an increase in water temperature caused by deficiencies in the quality of the water, and produces a general septicaemia with high mortality rates (50-80% of production). The treatment of this disease requires the administration of expensive antibiotics, which are also difficult to implement and, in many cases, ineffective due to the increasing occurrence of multi-resistant strains of *L. garvieae*.At present, preventive measures against Lactococcosis in fish include the use of vaccines, highly effective but also expensive, and requiring treatments that cause stress to fish. An alternative would be the use of probiotic Lactic Acid Bacteria and their bacteriocins. AS-48 is a broad-spectrum cyclic bacteriocin produced by *Enterococcus* species which is currently being tested against several bacterial pathogens. Recently we have isolated an AS-48 producing strain, *E. faecalis* UGRA10 [1], on which an extensive study of their functional, biosafety and probiotic characteristics is being conducted. The present study aims to evaluate the *in vitro* antimicrobial effects of UGRA10 strain and enterocin AS-48 against pathogenic strains of *L. garvieae* and the *in vivo* effectiveness of both, strain and bacteriocin, to protect zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) against this pathogen.

To evaluate the *in vitro* antimicrobial activity, well diffusion assays were used to provide semi-quantitative measures of antibacterial activity, and Minimum Bactericidal Concentrations (MBC) were determined by micro dilution assay. Furthermore, AS-48 lethal dose curves for each strain of *L. garviae,* at different enterocin concentrations (100, 50 and 25 µg/ml), were performed. In addition cocultures of UGRA10 and the pathogenic lactococci, containing different concentration ratios of both type of bacteria, were prepared in BHI broth at 25ºC, determining the pathogen evolution in time. The results showed the high effectiveness of AS-48 in controlling *L. garviae,* with a MBC average value of 45.3 µg/ml. Likewise, the presence of UGRA10 in cocultures significantly managed the pathogen and differences up to 6 log units in lactococci counts of cocultures, were achieved compared with control cultures of *L. garviae*.

In vivo experiments were conducted with 60 zebrafish divided into three tanks (20 animals per tank) which were experimentally infected with *L. garviae* (by incorporating the pathogen in water at a concentration of 10^7 cfu/ml). Afterward, one group received AS-48 (2 µg/ml) in the water, another group received an inoculum of UGRA10 (to a final concentration in the water of 2.5×10^6 cfu/ml) and a third group had no treatment (control). Regularly, the concentration of *L. garviae* and UGRA10 was quantified in the water and the cumulative mortality was recorded for each group. Another study was carried out with rainbow trout (average weight of 20 g), obtained from a commercial fish farm. In this case, the trouts were infected intraperitoneally with *L. garviae* (10^8 cfu). A first group received *L. garviae* and AS-48 solution (100 µg) intraperitoneally, a second group was infected periodically (every 3 days) and received a bath during 30 minutes with a solution of AS-48 (2 µg/ml) and a third group didn't receive any treatment (control) During the following 10 days mortality was recorded for each group. In both models, the results showed a protective effect of AS-48 when intraperitoneally administered as well as by dipping bath, with reductions in the mortality rates of 50-70%. The results indicate the protective effect of UGRA10 and AS-48 and the potential of these new natural products to replace antibiotics for controlling emerging diseases in aquaculture species.

Keywords: *Lactococcus garviae*; trout; *Enterococcus faecalis*; enterocin AS-48
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Acknowledgement: Piscifactorías Andaluzas S.L., Spain.

VII Reunión de la Red Española de Bacterias Lácticas
Madrid. Spain, 2013

Estudios funcionales, de seguridad y de probiosis de una cepa productora de AS-48

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AS-48 es una bacteriocina circular de amplio espectro de actividad y elevada estabilidad térmica, en un extenso intervalo de pH y en el tiempo. Es producida por especies del género *Enterococcus,* y sus aplicaciones biotecnológicas, en los ámbitos alimentario, clínico y/o veterinario, están siendo abordadas por nuestro grupo de investigación en colaboración con diversas entidades. Recientemente hemos aislado a partir de un queso artesanal una cepa productora de AS-48, UGRA10, que fue caracterizada por su ARNr 16S como *E. faecalis,* sobre la que estamos realizando un extenso estudio de sus características funcionales, de bioseguridad y probióticas, tanto *in vitro* como en cultivos celulares y en animales de experimentación. Sus características funcionales más destacables son sus altas actividades caseinasa, esterasa y esterasa lipasa C8. Mediante PCRs específicas hemos encontrado que UGRA10 presenta los genes *gelE, asa1, esp, efaA* y *ace* y el gen para la tirosin-descarboxilasa, considerados como posibles genes de virulencia enterocócicos. Respecto a su resistencia a antibióticos, (investigada mediante el sistema semi-automático Wider, panel para Gram + 94B MIC/ID) se ha encontrado que es sensible a la mayoría de antibióticos de relevancia clínica, incluida vancomicina, y resistente a bajos niveles de gentamicina, tobramicina, y amikacina (aminoglucósidos), quinupristina/dalfopristina y clindamicina, si bien estas resistencias son consideradas como intrínsecas del género *Enterococcus*, en particular de la especie *E. faecalis*. En cuanto a las propiedades relacionadas con la supervivencia e implantación en el intestino, UGRA10 muestra buena resistencia al pH ácido, moderada resistencia al tratamiento consecutivo pH ácido/sales biliares, forma biofilmes y se adhiere a células Caco-2 y HeLa 229. Pero lo más interesante es su alta capacidad para interferir con la adhesión de *Listeria monocytogenes* a las células Caco-2. Los estudios *in vivo* se han realizado con ratones BALB/c hembras, inmunocompetentes e inmunodeprimidosa. En un primer experimento los ratones fueron administrados durante 21 días, mediante canulación intragástrica, con 10^8 ufc de UGRA10. Durante ese periodo se siguió el estado de salud general de los animales determinando peso, ingesta y presencia de heces diarreicas u otros signos obvios de enfermedad. Al fin del experimento se obtuvieron el hemograma y diversos parámetros bioquímicos en sangre. No se han encontrado diferencias en ninguno de los parámetros entre los animales control, administrados con PBS, y los administrados con UGRA10. Además la ausencia de enterococos en hígado, bazo y ganglios mesentéricos descarta la posibilidad de translocación de la bacteria a través de la mucosa intestinal.

También se ha estudiado la velocidad de aclaramiento de los enterococos por el bazo de los animales determinando la presencia de enterococos en este órgano tras 1, 3 y 5 días después de ser administrados por vía plexo retro orbital con 10^8 ufc de UGR10. En este caso, con propósitos de comparación, se estableció un lote de ratones administrado en idénticas condiciones con la cepa probiótica comercial *E. faecalis* Symbioflor y otro con la cepa de origen clínico *E. faecalis* CECT 5254. UGRA10 fue la cepa aclarada con mayor rapidez, desapareciendo del bazo entre los 3 y 5 días. Le siguieron las cepas Symbioflor y CECT 5254 que fueron detectadas en bazo a los 5 días a niveles de 1,29 y 1,36 log ufc, respectivamente. Un tercer experimento ha sido llevado a cabo para estudiar el efecto protector de UGRA10 frente a la infección por *Listeria monocytogenes.* Para ello los ratones fueron administrados con 10^9 ufc de UGRA 10 durante 20 días y entonces fueron inoculados por igual vía con 10^9 ufc de *L. monocytogenes* CECT 4032 a la vez que se continuó administrando UGRA10 durante los 6 días siguientes a la infección. A los 3 y 6 días de la infección con *Listeria* se determinó la presencia del patógeno en hígado y bazo, encontrándose que los órganos de los ratones alimentados con el probiótico estaban significativamente ($P \leq 0.05$) menos infectados que los administrados sólo con PBS.

Palabras clave: AS-48, UGRA-10, probiótico, características funcionales, bioseguridad.

***Agradecimientos*.** Este trabajo ha sido financiado por el proyecto de excelencia P07-AGR-02539 de la Junta de Andalucía y el proyecto GREIB (CEB-005) de la Universidad de Granada.

Third international symposium on antimicrobial peptides
Lille (Villeneuve d'Ascq). France 2012

New applications of enterocin AS-48 to improve food safety

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Listeria monocytogenes is a foodborne pathogen causing listeriosis. The growth of *L. monocytogenes* has been demonstrated in many foods, with cheeses, meats, and coldsmoked fishes being particularly vulnerable. EC regulations establish a limit of 100 cfu/g in foods for *L. monocytogenes,* whereas in the USA, the FDA regulates zero tolerance. AS-48 is a broad-spectrum cyclic bacteriocin produced by *Enterococcus* species [1] that is now being tested against bacterial foodborne pathogens. In this work we explored the use of AS-48 in the control of *L. monocytogenes* in a variety of foods, fishes, melon, and cheese, alone or combined with chemical, natural, or biological agents. In fresh salmon and hake sprayed with AS-48 (50 ppm) and contaminated with a pool of 5 *L. monocytoges* strains, *Listeria* counts remained below 100 cfu/cm2 from the beginning to end of storage (7 d) at 5ºC. The combination of AS-48 with listeria phages (approx. 107 ufp/cm2) improves AS-48 activity against *Listeria*, reducing it to below detection levels in 1-2 days. In smoked salmon, AS-48 reduced *Listeria* to below 10 ufc/cm2 between 1–15 d storage at 5ºC and below 100 ufc/cm2 for another 15 d of storage. The combination with phages causes a drastic permanent elimination of *L. monocytoges* from day 1 throughout the entire period of storage (30 d). In IV gama melon, AS-48 was applied incorporated in an edible sucrose ester coating at concentrations of 50 and 100 ppm. At 50 ppm of AS-48, listeria counts fell below 100 cfu/cm2 from day 1 to day 7 (end of storage) and at 100 ppm listeria remained below 10 cfu/cm2 for the entire period of storage. In sliced cheese, AS-48 was assayed against two *L. monocytogenes* strains involved in an outbreak of listeriosis in Austria and Germany (2009- 10) due to the consumption of 'Quargel' cheese. AS-48 was applied in a polysaccharidechitosan surface coating. At 100 ppm in the coating, AS-48 maintained *L. monocytogenes* below 100 ufc/cm2 for 15 days of storage at 4ºC.

Keyword: enterocin AS-48, fish, melon, cheese.

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Acknowledgements. This work was supported by a grant (P07-AGR-02539) from the Junta de Andalucía.

I International Congress of food safety
Madrid. Spain. 2012.

Enterocin AS-48, a new natural approach to improve the safety of meat products

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Food applications of bacteriocins from lactic acid bacteria are a welcome alternative to satisfy growing consumer demand for foods that are hygienically safe and also nutrient-rich and minimally processed. AS-48 is a broad-spectrum cyclic bacteriocin produced by *Enterococcus* species that is now being tested against bacterial food-borne pathogens. The purpose of the present study was to determine the effectiveness of AS-48, alone or combined with other natural compounds or vacuum/modified atmosphere, to control *Listeria monocytogenes, Staphylococcus aureus* and *Clostridium perfringens* in three different types of meat products, fresh chistorra, burgers and cooked ham, with the ultimate aim of improving their safety.

In order to test the efficiency of the different treatments we used the Challenge Test. The foods were contaminated with a pool of listeria, staphylococci and/or clostridia strains, from CECT or meat isolated. AS-48 and/or the natural preservatives were added i to a meat mixture and the foods were stored at 5 ± 1ºC for different times, according with their standard half life, and periodically sampled to determine viable counts of the different bacteria.

In chistorra, the best results against *L. monocytogenes* were achieved by the combination of garlic natural extract (150 ppm) and AS-48 (40 ppm) that determined from the beginning a decrease in listeria counts of approx. 3 log units with respect to control. Application of AS-48 alone was also effective but not quite as much.

In burgers, AS-48 (40 ppm) combined with carvacrol (100 ppm), cinnamaldehyde (100 ppm) and garlic extract (50 ppm) was the most effective treatment followed by the application of AS-48 alone, achieving reductions of approx. 3 and 2 log unit compared with control, respectively. The combination of AS-48 and the other natural preservatives was also the most effective treatment in controlling *S. aureus,* which viable counts remained approx. 2 log units below the control.

In cooked ham, AS-48 applied alone was very efficient in the control of *C. perfringens* under normal atmosphere and also under vacuum or modified atmosphere (40%CO_2 : 60% N_2), despite the fact that the growth of clostridia was greatly increased under vacuum.

These results support the viability of the use of AS-48, either alone or combined with other hurdles such as natural preservatives, to improve the safety of meat products.

Keyword: *Listeria monocytogenes, Staphylococcus aureus, Clostridium perfringens*

II International Conference on Antimicrobial Research
Lisbon, Portugal 2012

In vivo protection against *Listeria monocytogenes* infection by the AS-48 producer strain *Enterococcus faecalis* UGRA10

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Recently we have carried out an in vitro study on the functional, probiotic, and safety-related properties of the *E. faecalis* UGRA10, an enterocin AS-48 producer strain isolated from a handmade sheep's milk cheese for which we have demonstrated its ability to interfere with the adhesion of *Listeria monocytogenes* to Caco-2 cells. Enterocin AS-48 is is a cationic cyclic peptide produced by *Enterococcus* strains that present a broad spectrum of bactericidal activity including several food-borne pathogenic bacteria. The production of antimicrobial substances is a desirable property in strains being developed as probiotics since this trait can confer to producer bacterium the ability to kill the pathogens and by hence the prevention of colon epithelium invasion.

The main purpose of this work is to investigate the potential of the *E. faecalis* UGRA10 strain to prevent the invasion of mice by *L. monocytogenes* and also the relevance of AS-48 in the preventive role of UGRA10. To fulfill this purpose, we obtained an enterocin AS-48 negative mutant of UGRA 10 by treatment with a sublethal concentration of acridine orange for 18 hours. The mutant enterocin-negative selected for the experiment, named UGRA 10-AO2, displayed identical plasmid profile and RAPD of genomic DNA than the wild strain and tested negative for the amplification of *as-48* genes.

Seven week-old female Balb/C mice were used for *in vivo* studies. At least ten mice were used per bacterial strain. Mice were dosed by intragastric feeding by means of a stainless steel cannula needle with 10^9 CFU of *E. faecalis* UGRA10 or the mutant *E. faecalis* UGRA10-AO2 for 20 days. On the day 20, mice were challenged with *L. monocytogenes* CECT 4032 (each mice were administered by oral pipette with 10^9 CFU of bacteria). Enterococci were administered also during the six days after infection. Control mice received a PBS placebo for 20 days before listerial infection. Three or six days after infection, mice were killed by cervical dislocation, livers and spleens were excised, and CFU listeria per organ was determined. Results showedes that spleens and livers of probiotic-fed mice were significantly less infected ($P \leq 0.05$) than organs of placebo-fed mice or than organs of enterocin negative mutant-fed mice (gray bars).

Enterococci isolated from probiotic-fed mouse faeces at the end of experiment exhibited an 94% of antilisteria activity versus the percentage of 0% presented by enterococci from placebo-fed mouse faeces and enterococci from AS-48 negative mutant-fed mouse faeces, indicating the ability of survival in gut of UGRA 10 strain.

Keywords: enterocin AS-48; protective effect; *Listeria monocytogenes*; mice.

Acknowledgements. This work was supported by the Research Plan of the Junta de Andalucía (research project P07-AGR-02539) and a grant from the GREIB (CEB-005) plan of the University of Granada.

II International Conference on Antimicrobial Research
Lisbon, Portugal 2012

Innovative air-sanitizing system for the food industry based on vegetable extracts combined with enterocin AS48

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An appropriate sanitization of surfaces and environments in the food industry is of vital importance to control spoilage and pathogenic microorganisms by guaranteeing the production of an excellent quality food, free of microorganisms with a harmful effect on health for the consumers. During manufacture, food can be exposed to microbiological cross-contamination from surfaces and the air, which may give rise to food spoilage and safety issues. The traditional approach to controlling such contamination has been to implement cleaning and disinfection (C/D) regimes. However C and D procedures with conventional detergents and disinfectants are not enough to ensure a good hygiene in the industries, especially in inaccessible areas where biofilms most often develop. Sometimes, the presence of food renders impossible to apply chemical disinfectants. Therefore, serious problems can result due to an excessive proliferation of microorganisms. Air-sanitizing systems appear to be an effective alternative by allowing the disinfection of surfaces and environments through the use of food additives capable of eliminating the microorganisms without leaving chemical residues which may be harmful to health in foods. On this basis, the aim of this study was to evaluate the effectiveness of natural formulate based on two vegetable extracts (from onion and citrus fruits) rich in flavonoids combined with enterocin AS-48 to improve the microbiological quality in environments and facilities where foods are produced or processed.

Minimal bactericidal concentrations (MBCs) were determined for different strains of foodborne pathogens and spoilage bacteria, including *Listeria monocytogenes, Salmonella enteritidis, Campylobacter jejuni, Clostridium perfringens, E. coli* O157:H7*, Staphylococcus aureus, Pseudomonas aeruginosa, Enterobacter sakazaki, Vibrio cholerae, Yersinia enterocolitica, etc.* The results showed a significant effectiveness of the product in low doses. In addition, the effectiveness in biofilms of *Listeria monocytogenes* under laboratory conditions simulating a food processing environment was also evaluated. The results indicated a statistically significant bactericidal action with reductions of bacterial counts by up to 4 log units during the first week.

Finally, tests were performed through the application of the product in 20 rooms of food industries. The product was applied through a cold microdiffusion equipment capable of producing a homogeneous aerosol with a particle size less than 20 µm allowing to reach the most inaccessible areas of the facility. Fogging was carried out for a minimum of 30 min to enable the fog to disperse and the antimicrobial action to occur. After fogging, an additional period of 60 min was required to allow the droplets to settle out of the air and onto the surfaces. After the treatment, the total number of microorganisms suspended in air (cfu/m^3) was quantified observing a reduction in the total microbial load of up to 85%.

In conclusion, our data show that the use of natural extracts of onion and citrus fruits combined with enterocin AS48 is proposed as an innovative and effective alternative to improve sanitation processes in the food industry.

Keywords: enterocin AS-48; vegetable extracts; air-sanitizing; fogging.

Acknowledgements. This work was supported by the Research Plan of the Junta de Andalucía (research project P07-AGR-02539) and a grant from the GREIB (CEB-005) plan of the University of Granada.

Second International Symposium on Antimicrobial Peptides
Saint-Malo, Spain, 2009

Probiotic and biotechnological properties of several AS-48 producer *Enterococcus* strains.

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The production of bacteriocins by bacteria can be an advantageous trait since it can confer the ability to succefully outcompete undesired species, including enteric pathogenic bacteria. So currently the potential use as probiotic of bacteriocin-producing bacteria has received increased attention. Enterocin AS-48 is a cationic cyclic peptide produced by *Enterococcus faecalis* and *E. faecium* strains that presents a broad spectrum of bactericidal activity including several food-borne pathogenic bacteria. The objective of this work was to investigate several properties related with the potential as probiotics of three AS-48 producing *Enterococcus* strains by *in-vitro* studies.

With respect to the production of enzymes degrading biopolymers, none of the strains were able to degrade starch, while the two *E. faecalis* strains (A-48-32 and EFS2) were able to hydrolyse casein. Neither raffinose nor stachyose fermentation was accomplished by any of strains. Phytase activity was detected only in *E. faecium* UJA32-81. Haeme-dependent catalase activity was not found in any of the AS-48 producing strains and neither the production of H_2O_2. Interestingly the three strains were able to form biofilm on the botton of microwell plates.

The presence of some traits related with the virulence was also investigates. We found gelatinase activity in the two *E. faecalis* strains but DNase or mucinase activities were not detected in any of the strains. We also investigated the production of biogenic amines by detecting the presence of decarboxilase genes for the amino-acids arginine, histidine and tyrosine by specific PCRs. All strains gave positive results for tyrosine decarboxilase gene and negative for the other decarboxilase genes. The three strains were able to grow in bile (4%) aesculin agar. All strains were resistant to HCl, pH 3. After HCl treatment cells transferred to oxgall (0.4%) were reduced in aprox. 4 log units. The ability of the strains to adhere to human cells was investigated using Caco-2 intestinal cell line as model. An average adherence of 2 bacterial cells per Caco-2 cell was detected.

Acknowledgements: this research was supported by the Research Plan of the Junta de Andalucía (Research Project P07-AGR-02539).

Second International Symposium on Antimicrobial Peptides
Saint-Malo, Spain, 2009

Application of enterocin as-48 in the control of *Lactobacillus sakei, Staphylococcus carnosus and Brochothrix thermosphacta* in cooked ham

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Food applications of bacteriocins from lactic acid bacteria are a welcome alternative to satisfy growing consumer demand for foods that are hygienically safe and also nutrient rich and minimally processed. Enterocin AS-48 is a cationic cyclic peptide produced by *Enterococcus faecalis* and *E. faecium* strains that presents a broad spectrum of bactericidal activity including several food-borne pathogenic and spoilage bacteria. Besides this feature, the remarkable thermal stability, sensitivity to digestive proteases and its activity in a wide range of pH point to AS-48 to be used as a biopreservative in foods. The purpose of the present study was to determine the effectiveness of AS-48, alone or combined with chemical preservatives, implemented in the manufacture of cooked ham against three main meat spoiling bacteria *L. sakei*, *S. carnosus* and *B. thermosphacta* with the final objective to extend its half life.

For each assay four batches were established with the meat mixture (previously inoculated with *L. sakei*, *S. carnosus* and *B. thermosphacta*): one control batch, one batch added of AS-48 (20 or 40 µg/g), other added with the chemical and other added with the chemical and AS-48. All batches were stored at 5 ºC. At selected times (0, 1, 7, 15, 30 and 60 days) each treatment was sampled, serially diluted and plated in the adequate selective media.

Lb. sakei was very sensitive to AS-48 in solitary but after 15 d it was able to regrow sligthly. The inhibitory action of AS-48 (40 µg/g) was improved by sodium tripolyphosphate (E-452i; 0.5%), sodium pyrophosphate (E-450i; 0.15%), sodium acetate (E-262i, 0.2%) and sodium lactate (E-325, 2%), since *Lactobacillus* population was reduced rapidly (1-7 d) below detection level (<10 cfu/g) and no regrowth occurred afterwards. Not differences were observed between batches treated with AS-48 alone or nitrate-nitrite (0.07%). *St. carnosus* was also very sensitive to AS-48 applied in solitary. Although *Br. thermosphacta* was the less sensitive bacterium assayed, the enterocin achieved a reduction of 2.5-3 log cfu/g with respect to the untreated control. The combination of nitrate/nitrite-TPPS-AS-48 improved the effectiveness of AS-48 against both last bacteria.

These results support the viability of the use of AS-48, alone or in combination with other hurdles such as chemical preservatives, to extend the half life of cooked ham, a meat product specially prone to microbial contamination and spoiling.

Acknowledgements: this research was supported by the Spanish Ministry of Education (Research Project AGL2005-07665-C02-01) and the Research Plan of the Junta de Andalucía (Research Project P07-AGR-02539).

International symposium on meat safety: from abattoir to consumer
Valencia, Spain, 2007

Antilisterial synergism through enterocin AS-48 and chemical preservatices in a meat model system.

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The use of bacteriocins of lactic acid bacteria (LAB) as food biopreservatives is currently intended as part of hurdle technology. This approach allows a lower effective bacteriocin concentration and also a wider range of activity to Gram-negative bacteria. So, presently many investigations on LAB bacteriocins are focused on the possible synergy between these biopreservatives with physical and chemical treatments implemented in foods. This is the case of enterocin AS-48, a cyclic peptide with high tolerance to heat and pH and a broad inhibitory spectrum against Gram-positive and also against some Gram-negative bacteria. In the present study we have assayed the effect of AS-48 (20 and 40 □g/g) in combination with several food-grade chemical preservatives in the control of *Listeria monocytogenes* CECT 4032 in a cooked ham model system during storage (4 ºC). The combination of AS-48 (40 µg/g) and sodium tripolyphosphate (STPP, 0.5 %) reduced viable listeria counts below the minimum detection level (MDL, 1 log cfu/g) from days 1 to15, with a slight recovery of listeria population at days 15-30. The simultaneous application of AS-48 (20 µg/g), STPP (0.5 %) and a sublethal heat shock (60 ºC, 2 min) reduced the listeria population below the MDL for days 1 to 30. Activity of AS-48 (40 µg/g) was also potentiated by sorbate (1 %) and benzoate (0.1%) and specially by nitrate/nitrite (0.015%). No synergy was observed with sodium pyrophosphate. The antilisterial effect of AS-48 alone was higher than each of the chemical preservatives separately.

Acknowledgements: This work was supported by research project AGL2005-07665-C02-01/.

DISCUSION INTEGRADORA

Como se ha referido en la Introducción, la enterocina AS-48 es un péptido antimicrobiano producido por cepas del género *Enterococcus* (Gálvez *et al*., 1985; Gálvez et al., 1986). Consta de 70 aminoácidos no modificados y una masa molecular de 7.149,25, pero lo que hace singular a esta molécula es su naturaleza circular, establecida a través de un enlace peptídico que une los extremos amino y carboxilo y cierra la estructura (Samyn et al., 1994; Martínez Bueno et al., 1994). En su composición aminoacídica destaca una alta proporción de aminoácidos básicos, lisina y arginina, lo que justifica su carácter fuertemente catiónico (pI = 10,5). Además, contiene una gran cantidad de residuos hidrofóbicos y de aminoácidos hidrofílicos sin carga neta (Gálvez et al., 1989a). Estos aminoácidos se estructuran en 5 hélices alfa que conforman una estructura globular bastante compacta. Los estudios realizados acerca de la estructura 3D de AS-48 han permitido conocer la concentración de los aminoácidos básicos y de aquellos otros hidrofóbicos en zonas de la molécula bien diferenciadas, lo que le confiere un carácter anfipático (Langdon et al., 1998; González et al., 2000). Este carácter anfifílico catiónico/hidrofóbico parece ser muy importante para su actividad biológica, que tiene por diana la membrana citoplásmica de la bacterias, en principio los únicos organismos sensibles a ella (Gálvez et al., 1989b; Gálvez et al., 1991). Es su estructura circular, lo que sin duda le confiere una gran estabilidad térmica, a pHs extremos y a altas concentraciones de agentes desnaturalizantes como el cloruro de guanidinio y la urea (Gálvez et al., 1989a; Cobos et al., 2001, 2002).

AS-48 presenta actividad bactericida sobre la mayoría de las bacterias Gram-positivas ensayadas. Dentro de este amplio espectro de acción, es interesante destacar la sensibilidad que muestran bacterias patógenas transmitidas por los alimentos y/o alterantes de los mismos (Gálvez et al., 1989b), entre las que destaca *L. monocytogenes* (Mendoza et al., 1999), *B. cereus* (Muñoz et al., 2007; 2004) y *S. aureus* (Ananou et al., 2004; Ananou et al., 2005b). Muchas otras cepas a priori resistentes, como ciertas bacterias Gram-negativas, se vuelven sensibles cuando se emplean tratamientos combinados, como es el caso de S*. cholerasuis* (Abriouel et al., 1998) y de *E. coli* O157:H7 (Ananou et al., 2005c). Además, la presencia de AS-48 a concentraciones inferiores a la mínima bactericida retrasa y disminuye en gran medida (75%) la producción de la enterotoxina A por *S. aureus* (Ananou et al., 2004).

Por estas propiedades de espectro de acción y resistencia a agentes físicos y químicos, y tras haber acumulado un gran volumen de información básica sobre la estructura, actividad biológica, genética, producción y purificación de AS-48, nos planteamos

rentabilizar estos conocimientos desarrollando posibles aplicaciones para la enterocina y la cepa productora. Esto se vio incentivado por su demostrada falta de actividad sobre células eucariotas lo que presuponía una ausencia de toxicidad. La aplicación más desarrollada de AS-48 es, sin duda, su faceta como bioconservante alimentario. En este campo las bacteriocinas están siendo investigadas como alternativa a los conservantes químicos tradicionales, muchos de ellos cuestionados por sus posibles efectos sobre la salud y, en general, rechazados en mayor o menor media por los consumidores. El uso de las bacteriocinas como conservantes en alimentos se puede realizar mediante dos estrategias básicas: producción *in situ*, inoculando la cepa productora en el alimento (que ha de ser un producto fermentado) y producción *ex situ*, adicionando la bacteriocina previamente producida por fermentación y purificada o semipurificada. Una y otra estrategia presenta ventajas e inconvenientes ya comentados en la Introducción. Al objeto de abaratar sus costes se ha desarrollado un método de obtención de la enterocina cultivando la bacteria productora en un medio a base de un derivado del lactosuero, la lactalbumina Esprion 300, adicionado de glucosa y estabilizando el pH en 6,5 (Ananou et al., 2008).

Los primeros ensayos de aplicación de AS-48 en alimentos se realizaron en quesos inoculados con la cepa productora de AS-48 *E. faecalis* A 48-32 como cultivo adjunto y demostraron en primer lugar la capacidad de ésta para controlar a *B. cereus* (Muñoz et al., 2004) y en un posterior experimento a *S. aureus* (Muñoz et al., 2007). Más tarde, se iniciaron los ensayos en modelos cárnicos, tipo salchichón, que fueron protegidos frente a *L. monocytogenes* y *S. aureus*, bien por la cepa productora A 48-32 o bien mediante la adición de la propia bacteriocina previamente semipurificada por intercambio catiónico (Ananou et al., 2005a, b). Aunque los resultados fueron muy prometedores, las cantidades de AS-48 necesarias para inhibir a ambos patógenos fueron muy superiores a las establecidas previamente en medios de cultivo de laboratorio. Las causas de este hecho, que ha sido referido para la mayor parte de las bacteriocinas (Schillinger et al., 1996; Cleveland, 2001), son varias (Gálvez et al., 2007): condiciones de procesado del alimento, inestabilidad de la bacteriocina a los cambios de pH del alimento, inactivación por enzimas, interacción con aditivos/ingredientes, adsorción a componentes del alimento, baja solubilidad y distribución irregular en la matriz alimentaria. Estos factores son tanto más importantes en limitar la eficacia de las bacteriocinas en alimentos cuanto mayor es la complejidad de la matriz alimentaria. En consecuencia es conveniente validar la efectividad específica de cada bacteriocina y sus combinaciones con otros agentes antimicrobianos en cada alimento y frente a cada microorganismo diana.

Por estas consideraciones y el hecho comprobado de que las bacterias Gram negativas en su gran mayoría presentan una alta resistencia frente a la enterocina, se planteó la necesidad de ensayar su actividad en alimentos combinándola con otras barreras, esto es con agentes químicos o físicos que puedan potenciar su actividad. Citando a Leistner (1999) *"Las bases de esta tecnología están en que la aplicación simultánea, a dosis moderadas, de varios factores que actúan a diferentes niveles, es mucho más eficaz que la aplicación de uno sólo, que actúa a un nivel, a dosis muy alta, no solo en lo que se refiere a la estabilidad y seguridad del alimento sino también en lo tocante a sus cualidades sensoriales y a los costes, ya que requiere menos energía durante la producción y el almacenaje"* Esto es porque la actuación de los diferentes tratamientos que se aplican simultáneamente sobre dianas celulares múltiples (membrana celular, pared, ADN, enzimas, etc.) dificultan o impiden la reparación de la alteración de la homeostasis celular por las proteínas de choque frente al estrés, al agotar las reservas energéticas de la célula. Este hecho es especialmente importante en *L. monocytogenes,* para la cual se ha observado una notable capacidad de recuperación de las células subletalmente dañadas (Donnelly, 2002). Por ello, en la actualidad la aplicación alimentaria de las bacteriocinas se enmarca dentro de la tecnología de las barreras múltiples (Gálvez et al., 2007, 2010).

Las enfermedades infecciosas transmitidas por alimentos siguen representado un importante problema de salud pública en los países desarrollados a pesar de las medidas implementadas para su control. Baste decir que en 2013 se registraron en la Unión Europea (UE) 13.524 casos atribuibles con alta probabilidad a microorganismos o a sus toxinas y otros 28.438 casos con baja probabilidad, agrupados en un total de 5.196 brotes (EFSA, 2015). De ellos un 27,2% fueron atribuibles a carnes y productos cárnicos.

Los productos cárnicos, pues, pueden actuar a menudo como vehículos para bacterias patógenas entre las que se incluyen *S. aureus* y *L. monocytogenes. L. monocytogenes* es responsable de un total de 1.763 casos agrupados en 13 brotes transmitidos por alimentos en la UE en 2013 (EFSA, 2015). Aunque la incidencia de la listeriosis es baja, es considerada un problema de primer orden dada la alta tasa de hospitalización y de letalidad de los afectados, pertenecientes sobre todo al grupo de niños pequeños, ancianos, embarazadas e inmunocomprometidos (YOPIs) (Todd y Noterman, 2011). Los alimentos que se han encontrado que vehiculizan la listeriosis más frecuentemente son las carnes y productos cárnicos y los productos lácteos, aunque los pescados ahumados y crustáceos RTE (ready- to-eat) son también vehículos importantes (Todd y Noterman, 2011). Así mismo, a las toxinas estafilocócicas se les atribuye con gran probabilidad 1.304 casos de intoxicaciones alimentarias y otros 1.899 casos con baja

probabilidad, distribuidos en un total de 386 brotes (EFSA, 2015). Entre los productos cárnicos más consumidos, están los cocidos no fermentados, como es el caso del jamón cocido, el cual debido a su alto contenido en agua (> 0.945), baja acidez (pH próximo a 6) bajo contenido en sal y abundancia de nutrientes, es altamente propenso a contaminarse con microorganismos. Este producto, extensamente consumido en sus diversas variantes comerciales, es embutido y frecuentemente envasado a vacío antes de ser distribuido. Durante su procesado y manipulación puede ser contaminado con bacterias alterantes y patógenas, como es el caso de *Listeria*, considerada bacteria doméstica en las plantas de procesado de productos cárnicos. De hecho, los mayores brotes de listeriosis registrados se han debido a errores de manipulación en las plantas de procesado, que dieron lugar a la contaminación de las máquinas fileteadoras y a la transferencia de *Listeria* al alimento (Todd y Noterman, 2011).

I. Aplicación de la tecnología de las barreras en el desarrollo de AS-48 como bioconservante alimentario.

En el primer capítulo de esta memoria se presentan los resultados de la implementación de AS-48, sola y combinada con barreras químicas y/o físicas en el control de bacterias patógenas, *L. monocytogenes* y *S. aureus* y alterantes (*Lactobacillus sakei, Staphylococcus carnosus* y *Brochothrix thermosfacta*) en un modelo alimentario tipo jamón cocido (Ananou et al., 2010; Baños et al., 2012).

En este sistema AS-48 inhibió efectivamente a *Listeria* en forma dependiente de la dosis, tanto a 5 ºC como a 15 ºC en un periodo de 60 días, siendo mayor la efectividad a temperatura más baja, posiblemente por el crecimiento más lento de la bacteria. Sin embargo, aún a la concentración más alta usada (60 µg/g), no se pudo evitar la recuperación de *Listeria* a partir de los 15-30 días. Teniendo en cuenta que la concentración mínima bactericida determinada para esta misma cepa de *Listeria* en medio BHI fue 0.1 g/mL (Mendoza et al., 1999) se vuelve a confirmar la gran influencia del ambiente en la actividad de AS-48, que ya se había puesto de manifiesto en un modelo alimentario tipo salchichón (Ananou et al., 2005a). En otros experimentos realizados tratando en superficie alimentos vegetales con soluciones de AS-48 o adicionando la enterocina a zumos de frutas también se observó este efecto, si bien la concentración efectiva era algo menor, en torno a 12,5-25 µg/mL. Para evitar la recuperación de *Listeria* se combinó AS-48 con diferentes compuestos químicos licenciados por el reglamento del Parlamento Europeo y del Consejo sobre aditivos alimentarios (95/2/EC, 96/85/EC, 98/72/EC) para uso en productos cárnicos.

La combinación más efectiva fue AS-48 (40 µg/g)-nitrito/nitrato (0.007%) que redujo las listerias por debajo del nivel de detección desde el primer muestreo. Este resultado es relevante ya que los nitritos/nitratos por si mismos no tuvieron efecto sobre *Listeria* a la concentración empleada, que es la cuarta parte o la mitad del 0.03-0.015% autorizado por la directiva de la CE. La combinación de AS-48 con tripolifosfato sódico, benzoato sódico o sorbato potásico también fueron efectivas (aunque menos) en reducir a *Listeria* a 5 ºC. Es interesante que una concentración mayor de AS-48 (60 µg/g) combinada con pirofosfato sódico redujo a *Listeria* por debajo del nivel de detección, lo que indica que sería posible conseguir la eliminación completa del patógeno con combinaciones de concentraciones más altas de la enterocina con los otros compuestos. También es interesante destacar que la mayoría de los conservantes químicos ensayados no presentaron actividad antilisteria significativa a las concentraciones ensayadas. Las combinaciones de AS-48 con lactato sódico y acetato sódico tuvieron un efecto aditivo más que sinergístico, Nuestros resultados coinciden en general con los obtenidos por Cobo Molinos et al., (2005, 2008) en la inactivación de *L. monocytogenes* en superficie de vegetales, con la excepción de que en estos, la mejor combinación fue la de AS-48 con lactato sódico. Jofré et al. (2007) han demostrado el efecto antilisteria de la nisina a 6 ºC en jamón cocido pero en este caso, después de ejercer un efecto bactericida inmediato en el primer día de almacenaje, *Listeria* recuperó rápidamente su crecimiento normal y alcanzó los niveles poblacionales del control. La combinación de nisina con lactato sódico no consiguió eliminar las listerias pero las mantuvo aprox. 1 unidad logarítmica por debajo del control. En este estudio, también se demostró la sinergia entre un choque térmico subletal (60 ºC, 2 min) y AS-48 frente a *Lisiteria.* Esta sinergia ya se había puesto de manifiesto frente *S. aureus* en leche y frente a *E. coli* O157:H7 en zumo de manzana (Muñoz et al., 2007; Ananou et al., 2005c), siendo un resultado de relevancia tecnológica en la contaminación interna de productos alimentarios cocidos en los cuales AS-48 puede representar una barrera adicional que compense los fallos en la distribución homogénea del calor.

Respecto del otro patógeno estudiado en este sistema *S. aureus*, previamente se investigó su susceptibilidad en BHI, en un sistema modelo salchichón, en leche y en alimentos vegetales, aplicado o combinado con diferentes agentes químicos y calor (Ananou et al., 2004; Ananou et al., 2005b; Muñoz et al., 2007; Grande et al., 2007b). Como regla general la concentración requerida para inhibir *S. aureus* CECT976 en alimentos es bastante más alta que la establecida por Ananou et al. (2004) en medio BHI, 15 µg/ml. En el presente estudio, AS-48 por si mismo, incluso a la concentración más alta de 60 µg/g fue incapaz de inhibir a los estafilococos. El efecto inhibidor, contrariamente a lo referido en salsas vegetales, fue mayor a 5 ºC. Ninguna de las combinaciones de AS-48 con

conservantes químicos eliminaron los estafilococos pero es importante destacar que la mayoría de ellos (nitritos/nitratos, tripolifosfatos, lactato sódico, acetato sódico) mejoraron la actividad inhibidora de AS-48, siendo las mejores combinaciones con nitritos/nitratos y tripolifosfatos. Además, al igual que para *Listeria*, la combinación de 60 µg/g de AS-48 con pirofosfato sódico disminuyó notablemente los recuentos de estafilococos (por debajo de 2 log UFC/g) mientras que el control alcanzó aprox. 6 UFC/g. Puesto que las intoxicaciones estafilocócicas se producen cuando las poblaciones de estafilococos son superiores a 5 log UFC/g de alimentos (Anonymous, 1992), las reducciones conseguidas por AS-48 serían suficientes si los alimentos están adecuadamente refrigerados. En el mencionado estudio de Jofré et al. (2007), no se consiguió una reducción importante de *S. aureus* por adición de nisina sola o combinada con lactato sódico. La acción sinérgica del calor sobre la actividad antiestafilocócica de AS-48 fue menos importante que la detectada previamente en leche para esta bacteria posiblemente por la mayor complejidad de la matriz alimentaria y que el tratamiento térmico aplicado fue más bajo.

Los resultados obtenidos en este modelo alimentario corroboran la pérdida de efectividad de las bacteriocinas en general, y de AS-48 en particular, cuando se aplican en alimentos y la necesidad de realizar estudios específicos para cada tipo de ellos. No obstante, se puede concluir que el empleo de AS-48, sólo o especialmente combinado con otras barreras químicas, tales como nitritos/nitratos, tripolifosfatos y pirofosfatos y/o calor es una eficiente estrategia para proporcionar una protección efectiva frente a *L. monocytogenes* y, en menor medida, frente a *S. aureus*, mejorando así la seguridad de los alimentos cárnicos no fermentados como el jamón cocido. La combinación más adecuada de barreras la dictará el tipo específico de alimento en cuestión y los organismos diana.

El estudio realizado sobre la eficacia de AS-48 para prolongar la vida media durante 60 días de este mismo sistema alimentario se ha centrado en la inhibición de tres bacterias asociadas con productos cárnicos e implicadas en su alteración, *Lb. sakei, S. carnosus* y *B. thermosphacta.* AS-48 (40 µg/g) por si misma demostró ser muy eficiente a 5 ºC en inhibir las tres bacterias consiguiéndose reducciones de 3-4 log UFC/g. *L. sakei* fue completamente eliminada desde el primer día y durante todo el almacenaje por aplicación de 60 µg/g de la enterocina y tratada con 40 µg/g permaneció por debajo de 2 log UFC/g durante todo el experimento. No obstante estas concentraciones efectivas son mucho más altas que las encontradas para controlar en zumo y sidra de manzana a las bacterias alterantes *Lactobacillus collinoides, Lactobacillus dioliovorans*, and *Pediococcus parvulus* (Martínez-Viedma et al., 2008). Aunque las especies bacterianas ensayadas fueron diferentes, nosotros atribuimos el diferente comportamiento de AS-48 a la diferente matriz alimentaria,

sólida y más compleja y heterogénea en el caso del jamón cocido y posiblemente también al pH ácido de los zumos. Otro factor a tener en cuenta es el diferente tiempo durante el que se evaluó la actividad de AS-48, 2 días para el zumo/sidra y 60 para el jamón cocido. El crecimiento de células (posiblemente dañadas subletalmente) ya se había observado en este mismo sistema alimentario para listeria y estafilococos. De hecho, 40 µg/g de la enterocina consiguieron mantener bajo los niveles de detección a los lactobacilos entre los días 7 y 15, pero después se inició un lento resurgir de la población. El efecto de la temperatura fue importante siendo AS-48 mucho menos efectiva a 15 que a 5 ºC, posiblemente debido al más rápido crecimiento de la bacteria a mayor temperatura. Se ha estudiado la influencia de la temperatura sobre la actividad de AS-48 frente a diferentes bacterias y en diferentes sistemas alimentarios siendo los resultados muy heterogéneos dependiendo de la bacteria diana y el alimento (Cobo-Molino et al., 2008; Grande et al., 2006). Con el objeto de rebajar la dosis efectiva de AS-48, ésta se combinó con los diferentes conservantes referidos más arriba. La combinación de AS-48 (40 µg/g) con lactato sódico (2%) o acetato sódico (0.2%) aumentó la eficacia de la enterocina ya que *Lb. sakei* fue indetectable desde el día 1 hasta el día 60. Este efecto potenciador no se puede atribuir a la acidificación, al tratarse de sales sódicas, y ha sido referido previamente para el lactato sódico frente a *B. cereus* en derivados de arroz (Grande et al., 2006) y frente a *L. monocytogenes* en brotes (Cobo Molinos et al., 2005). Las combinaciones con los agentes quelantes pirofosfato sódico (0.15%) o tripolifosfato (0.5%) fueron también muy efectivas para controlar a *Lb. sakei* que permaneció indetectable desde el día 7 hasta el fin del almacenaje. Es importante señalar que la aplicación de tripolifosfatos redujo la concentración efectiva de AS-48 a 20 µg/g. El efecto potenciador de los agentes quelantes bajo condiciones de laboratorio y en alimentos sobre la actividad de las bacteriocinas ha sido referida por Gálvez et al. (2007) Los nitritos/nitratos combinados con AS-48 también incrementó su actividad frente a esta bacteria. Otros autores han referido el efecto potenciador de los nitritos sobre la actividad de la nisina frente a *Leuconostoc mesenteroides* y *L. monocytogenes* (Gill & Holley, 2000). Al contrario que para las bacterias antes mencionadas, *L. monocytogenes*, *S. aureus,* o *E. coli* O157:H7, en diferentes alimentos, el tratamiento térmico subletal no incrementó la actividad de la enterocina frente a los lactobacilos.

Hemos ensayado también el efecto que el envasado a vacío o bajo atmósfera modificada (AM) pueda tener sobre la actividad de AS-48. Ambos sistemas de envasado son utilizados extensamente en la industria alimentaria para prolongar la vida media de los productos cárnicos cocidos, aunque existen datos contradictorios al respecto. Según nuestros resultados, la AM (40% CO_2:60% N_2) no afectó el crecimiento de *Lb. sakei, B.*

thermosphacta o *S. carnosus*, ni influyó en la actividad de AS-48 (40 µg/g) frente a las tres bacterias. Sin embargo, el vacío si que tuvo un notable efecto tanto sobre el crecimiento como sobre la actividad de la enterocina sobre *Lb. sakei* ya que los lactobacilos permanecieron indetectables desde el día 1. Este efecto es más destacable si cabe dado que, bajo vacío, esta bacteria alcanzó niveles 2 log UFC/g más altos que bajo atmósfera normal, de manera que entre los lotes control no tratados y los tratados, las diferencias en UFC/g fueron mayores de 6 log a partir de los 15 días. Esta es la primera vez que se demuestra el efecto del vacío sobre la actividad de AS-48 y es un resultado muy interesante ya que el envasado a vacío es una práctica muy habitual para este tipo de alimentos. Con respecto a *B. thermosphacta* se encuentra, junto a las bacterias lácticas, entre la principal microbiota presente en productos cárnicos envasados a vacío (Holley, 2014). Aunque el vacío no es suficiente para prevenir la alteración debida a esta bacteria es una barrera adecuada para ser combinada con otras, tales como lisozima, ácido láctico, EDTA o nisina (Gill y Holley, 2000). Nuestros experimentos pusieron de manifiesto que *B. thermosphacta* crece mejor bajo vacío que en atmósfera normal (aprox 2 log UFC/g más) y que la aplicación de AS-48 redujo de forma importante de 7 log a 4 log UFC/g,) los recuentos de esta bacteria lo que es cuantitativamente mucho más relevante que la reducción causada en atmósfera normal (de 5 log a 2 log UFC/g). Por tanto, podemos concluir que también en el caso de *B. thermosphacta* la aplicación del envasado a vacío aumentó la actividad inhibidora de la enterocina. Finalmente, para *S. carnosus,* no se vieron afectados ni el crecimiento ni la actividad por el envasado a vacío.

Según los resultados obtenidos podemos concluir que puede usarse AS-48, sola o combinada con barreras químicas o físicas en la prevención de la contaminación por bacterias patógenas como *L. monocytogenes* y *S. aureus* y en la alteración del jamón cocido debida a *Lb. sakei, B. thermosphacta*, o *S. carnosus.* Aunque la enterocina por sí sola puede ser muy efectiva, la combinación de AS-48 con otras barreras, como tripolifosfatos, pirofosfato sódico, nitritos/nitratos, lactato sódico o acetato sódico puede aumentar su actividad inhibidora, reduciendo la concentración efectiva y proporcionando protección en productos con un nivel de contaminación más elevado. El envasado a vacío ha mostrado ser particularmente efectivo en aumentar la actividad de AS-48 frente a *Lb. sakei* y *B. thermosphacta.*

Entre los conservantes naturales investigados en la actualidad como alternativas a los aditivos químicos alimentarios en uso, están los extractos de plantas y de ellos destacan los derivados de los miembros de la familia *Amarillydaceae,* ajo (*Allium sativum*) y cebolla (*Allium cepa*), que han sido ampliamente usados por sus propiedades antimicrobianas como

ingredientes en alimentos y en remedios medicinales (Corzo-Martínez, et al., 2009; Wilson & Demmig-Adams, 2007). Tales propiedades antimicrobianas se atribuyen a la presencia de compuestos organosulfurados: alca(que))nil tiosulfinatos y tiosulfonatos (Block, 2010; Kyung, 2012). Uno de estos extractos es el producto comercial Proallium DMC®, un aromatizante basado en extractos de *Allium* incorporados en dextrinas, cuyas propiedades antibacterianas han sido demostradas con anterioridad (Ruiz et al., 2010; Llana-Ruiz-Cabello et al., 2015). En esta memoria hemos ensayado el uso combinado de este extracto con la enterocina AS-48 para inhibir a las bacterias patógenas vehiculizadas por alimentos, *L. monocytogenes*, *S. aureus* y *C. perfringens,* en alimentos cárnicos.

El control de *L. monocytogenes* fue seguido en hamburguesas y en chistorra durante su almacenaje a 4 ºC, durante 7 y 21 días, respectivamente. Ambos alimentos fueron elaborados sin nitritos ni pirofosfato sódico, que forman parte de su formulación habitual, para poder descartar el demostrado efecto sinérgico de ambos compuestos. El tratamiento con AS-48 (40 µg/g) en ambos sistemas alimentarios redujo la población de listerias aprox. 2 log UFC/g desde el primer día, si bien hay que tener en cuenta que en hamburguesas *Listeria* creció hasta un máximo de 5.2 log UFC/g y en chistorra la población permaneció estable. El efecto antilisteria debido a la enterocina fue mayor que el de los otros dos conservantes aplicados por separado, extracto de ajo y sulfito sódico en hamburguesas y extracto de ajo y Purasal en chistorra. La combinación de AS-48 con Proallium tuvo un efecto inhibidor aditivo permaneciendo las listerias por debajo de las 2 log UFC/g en ambos tipos de alimentos desde el primer muestreo (1d). El efecto fue mayor en chistorra donde *Listeria* cayó drásticamente hasta niveles de 1 log UFC/g el primer día, manteniéndose así los restantes 20 días. Aunque no se consiguió eliminar en ninguno de los dos casos a las listerias, no se observó una notoria recuperación de las mismas, sobre todo en chistorra. Estos niveles son, además, aceptables para la mayor parte de los alimentos, según la normativa de la UE. Por tanto, el uso combinado de AS-48 y extracto de *Allium* abre la posibilidad de conseguir un nivel aceptable de seguridad frente a *Listeria* prescindiendo del empleo de conservantes químicos tales como nitritos/nitratos y sulfitos.

El control de *S. aureus* por extracto de *Allium* y AS-48 se realizó en un modelo cárnico tipo albóndigas. La importancia de este patógeno está enfatizada por los brotes de enfermedades ocasionados por toxinas estafilocócicas transmitidos por alimentos (EFSA 2015, Kadariya et al., 2014). El efecto inhibidor de los tres antimicrobianos ensayados por separado, extracto de *Allium*, sulfito sódico y AS-48 (40 µg/g) fue muy significativamente similar y la combinación de AS-48 y extracto aliáceo tuvo un efecto inhibidor aditivo sobre *S. aureus* desde el primer día, manteniéndose la población de estafilococos por debajo de 1 log

UFC/g a partir de los 2-5 días. Otros autores han ensayado el efecto antiestafilocócico de bacteriocinas como la nisina en alimentos, con diverso grado de éxito (Felicio et al., 2015; Jofré et al., 2007).

También en estos casos se produce el efecto obstaculizador que la matriz alimentaria cárnica, más compleja que la de los alimentos líquidos (leche o zumos) o los caldos de cultivo, tiene sobre la efectividad antiestafilocócica y antilisteria de la enterocina, a pesar de lo cual los resultados se pueden considerar como satisfactorios.

Finalmente, se ensayó la combinación de AS-48 con el extracto aliáceo frente a *C. perfringens* en modelo jamón cocido. Las toxinas de los clostridios (*C. perfringens*, *C. botulinum* u otras especies) han sido responsables de 170 brotes asociados a consumo de alimentos contaminados en la UE durante 2013 (EFSA, 2015) lo que representa el 3,3% del total. Normalmente los alimentos se contaminan con sus esporas que resisten los tratamientos térmicos y cuando se enfrían los productos éstas germinan se producen las toxinas. Esta bacteria fue sensible a extracto aliáceo y a AS-48 por separado, si bien ésta segunda fue mucho más efectiva, consiguiendo reducciones de más de 1 log hasta más de 2 log UFC/g a lo largo de los 35 de almacenaje. La combinación de ambos antimicrobianos tuvo un efecto aditivo muy importante ya que los recuentos de los clostridios cayeron por debajo de 1 log el día 1 y se hicieron casi indetectables desde los días 1 a 7 hasta los 35 d. Otros autores han usado a la nisina y al areuterina para inhibir a células vegetativas y esporas de *C. perfringens* obteniendo buenos resultados (Garde et al., 2014), si bien cuando aplicaron el tratamiento a productos cárnicos la efectividad se vio reducida (Udompijitkul et al., 2011). También Nieto-Lozano et al. (2005) han aplicado una pediocina sobre la superficie de carne fresco encontrando que ejercía un efecto bacteriostatico sobre *C. perfringens.*

Los resultados obtenidos aplicando la enterocina AS-48, sola y sobre todo combinada con el extracto aliáceo en diversos tipos de alimentos cárnicos procesados (hamburguesas, chistorras, albóndigas y jamón cocido) frente a *L. monocytogenes*, *S. aureus* y *C. perfringens* son muy alentadores, expandiendo el abanico de posibilidades de uso de la enterocina en aquellos productos donde sea factible el uso de Proallium cuyo afecto antimicrobiano se suma al de AS-48.

Otra estrategia para la aplicación de AS-48 en alimentos abordada en esta memoria ha sido su incorporación en recubrimientos comestibles (RC). Los RC son suspensiones de grado alimentario que pueden ser distribuidas mediante pulverización (espray) u otros

métodos en la superficie de los alimentos, en la cual, tras secarse, forman una fina película. Se ha demostrado que por si mismos, por diversas vías, contribuyen a aumentar la vida media y la seguridad de los alimentos. Además, contribuyen a mejorar las propiedades mecánicas reduciendo la pérdida de agua y la transmisión de gases (Falguera et al., 2011; Wu et al., 2002).Hay diversos tipos de RC según estén basados en lípidos, proteínas, polisacáridos o sus mezclas. Se usan en muchos productos, como dulces, frutas frescas y vegetales y en carnes procesadas. Además de las propiedades mencionadas, los RC pueden funcionar como portadores de ingredientes funcionales como los antimicrobianos, dando lugar a los RC antimicrobianos (Pascall et al., 2013) extensamente investigados sobre todo en lo referente a la incorporación de antimicrobianos de origen natural, como extractos de plantas o bacteriocinas (Gálvez et al., 2007; Jasour et al., 2015; Olaimat et al., 2014; Gniewosz et al., 2013; Murillo-Martínez et al., 2013).

Como parte de uno de los dos principales propósitos de esta memoria, optimizar la aplicación de AS-48 en alimentos, se ha ensayado la eficacia de ésta tras ser incorporada en cuatro tipos diferentes de RC. Los RC empleados han sido los basados en: ésteres de sacarosa y ácidos grasos (sucroésteres, SE, E-473), ésteres del ácido acético y mono y digliceridos (ACETEM, E472a), quitosano y gelatina de pescado. Los alimentos estudiados han sido melón, jamón cocido, queso fresco y merluza fresca. En cada tipo de alimento se ha aplicado el RC más adecuado a la naturaleza del mismo y se ha estudiado la influencia de la implementación del RC sobre la evolución de la contaminación con bacterias alterantes o patógenas seleccionadas.

Previo a su aplicación en el alimento se realizaron experimentos de laboratorio para ver cómo la inclusión de la enterocina afectaba a la actividad anti-listeria de AS-48. Se encontró que los RC que mejor mantenían la actividad inhibidora de la enterocina eran los basados en quitosano y en sucroésteres,

La aplicación de la enterocina AS-48 incluida en un RC basado en SE, a concentraciones de 10 y 20 10 µg/g, sobre la superficie de tajadas de melón redujo significativamente los recuentos de *Listeria,* siendo especialmente importante las reducciones conseguidas con la concentración más alta de la bacteriocina, que mantuvo las listerias por debajo de 1 log UFC/g desde el primer día. Hay que señalar que este alimento ha sido responsable de un brote de listeriosis en 2011 en USA que ocasionó más de 30 muertes en 28 estados (McCollum et al., 2013). En rodajas de melón se había ensayado previamente el efecto de lavados con combinaciones de AS-48 y conservantes químicos sobre *L. monocytogenes,* encontrándose que varios de ellos, especialmente carvacrol y n-

propil p-hidroxibenzoato eran muy eficaces en eliminar al patógeno (Cobo Molinos et al., 2008). El uso de este tipo de RC ha sido estudiado y propuesto por varios autores, ya que se he demostrado que retarda la maduración poscosecha y presenta propiedades fungistáticas (Alonso et al., 2004; Calvo-Garrido et al., 2013).

La actividad antilisteria de AS-48 se ha investigado también, tras ser incorporada a RC basados en ACETEM, aplicada a la superficie de queso fresco. Los productos lácteos están registrados entre los principales vehículos de *L. monocytogenes* (Gaulin et al., 2012; Jackson et al., 2011), siendo uno de los brotes multinacionales más importantes de listeriosis en Europa el ligado a consumo de un queso Quargel producido en Austria (Fretz et al., 2010). La aplicación de estos RC fortalecidos con AS-48 consiguió reducciones significativas en los recuentos de *Listeria,* sobre todo a la concentración más alta usada (40 µg/g). A esta concentración las listerias permanecieron por debajo de 2 log UFC/g mientras que en los controles alcanzaron casi las 7 log UFC/g.

La incorporación de AS-48 en RC ha mostrado ser también eficaz en inhibir bacterias alterantes en pescado crudo (merluza). En este caso, el RC de elección fue el basado en gelatina de pescado y los organismos diana *Lb. acidipiscis* y *S. carnosus,* dos bacterias frecuentemente aisladas de alimentos marinos. Estos alimentos, especialmente los crudos son altamente perecederos y pierden rápidamente sus cualidades sensoriales debido al crecimiento de la microbiota, responsable de cambios en olor, aspecto y textura de la carne del pescado. Para evitar estos efectos indeseables se ha propuesto el uso de RC incorporados con antimicrobianos (Heydari et al., 2015). Entre los EC de elección en este tipo de alimento ocupa un lugar preferente la gelatina, particularmente la obtenida de pescado que ofrece una serie de importantes ventajas (Zhang et al., 2007; Yang et al., 2007). El recubrimiento de los filetes de merluza contaminada con *Lb. acidipiscis* y *S. carnosus* con RC de gelatina de pescado enriquecida con AS-48 (25 µg/g) tuvo un efecto inhibidor sobre ambas bacterias, especialmente sobre los lactobacilos, que estuvieron por debajo de 2 log UFC/g en los primeros 6 días y en torno a 3 log UFC/g a los 8 días. Estos datos significan importantes reducciones respecto a los filetes no recubiertos en los que *Lb. acidipiscis* alcanzó más de 6 log UFC/g. Hay que señalar que los niveles alcanzados por los lactobacilos en los filetes recubiertos con RC adicionada de AS-48 permanecieron durante todo el almacenaje muy por debajo de los niveles máximos de bacterias totales permitidos por la legislación española para pescado fresco y congelado (5×10^5 UFC/g) (MSC, 1991). Otros autores han aplicado RC basados en proteínas suplementados con bacteriocinas como la nisina para proteger productos cárnicos frente a *Listeria* y bacterias alterantes (Lungu y Johnson, 2005; Gill y Holley, 2000; Min et al., 2010).

Para completar el estudio sobre la incorporación de AS-48 en RCs la enterocina se adicionó (20 y 40 µg/g) en RCs basados en quitosano, siguiendo el control que ejercían estas sobre la proliferación de las bacterias alterantes *Lb. sakei* y *B. thermosphacta* en jamón cocido loncheado. De nuevo AS-48 puso de manifiesto su eficacia en controlar ambos microorganismos, siendo los lactobacilos los más afectados por el tratamiento ya que se mantuvieron en torno a 1 log UFC/g de 1 a 7 días y por debajo de 1 log desde los 7 hasta los 30 días. *B. thermosphacta,* también se vio afectada, aunque menos, por el recubrimiento con este RC y los recuentos permanecieron próximos o por debajo de 3 log UFc/g a lo largo de todo el almacenaje (30 d), lo que representa una importante reducción respecto de los niveles poblacionales alcanzados por esta bacteria en las muestras no recubiertas, iguales o superiores a 6 log UFC/g a partir de 10 días. La mayor sensibilidad a AS-48 de *Lb. sakei* con respecto a *B. thermosphacta* en este experimento coincide con la observada en este mismo sistema alimentario cuando AS-48 fue implementada en la masa cárnica (Baños et al., 2012). El uso de RCs basados en quitosanos en productos cárnicos ha sido referido en varias ocasiones (Moreira et al., 2011; Petrou et al., 2012). También se han referido los efectos combinados de los quitosanos con otros agentes antimicrobianos como los ácidos orgánicos o los aceites esenciales (Beverlya et al., 2008; Ojagh et al., 2010). Entre las bacteriocinas usadas como suplementos antimicrobianos de los RCs a base de quitosanos destaca la nisina aplicada para controlar *Salmonella* en melón (Chen et al., 2012) o *Listeria* en carne RTE (Guo et al., 2014).

Concluyendo, la incorporación de AS-48 a recubrimientos comestibles producidos a base de ésteres de sacarosa y ácidos grasos (sucroésteres, SE, E-473), ésteres del ácido acético y mono y digliceridos (ACETEM, E472a), quitosano y gelatina de pescado es una estrategia muy válida para la aplicación efectiva de la enterocina en alimentos de diversos tipos, que permite el control de bacterias alterantes y patógenas vehiculizadas por los mismos.

Dentro de este mismo objetivo de estudio, sobre la potenciación del efecto antimicrobiano de AS-48 por barreras químicas, físicas y biológicas se ha ensayado su acción combinada con un conservante biológico, el fago lítico de *Listeria* P-100, (Carlton et al., 2005) recientemente licenciado para su uso por la FDA. bajo la denominación de LISTEX P100, aceptado como GRAS. Ante la disponibilidad de esta herramienta, nos dispusimos a ensayar la habilidad de AS-48, sola o combinada con el fago P-100, para controlar a *Listeria* en pescado crudos y ahumados, salmón y merluza. Nos motivó a ello el incremento en el consumo incrementado de tales productos que se está produciendo, tanto a nivel doméstico

como en los restaurantes japoneses tan en boga hoy día. Estos productos debido a su bajo nivel de procesado son muy propensos a vehiculizar a *Listeria* (EFSA. 2015; Jami et al., 2014) y están suscitando por ello un creciente interés en las autoridades sanitarias y el público.

Los alimentos fueron pulverizados con preparaciones de AS-40 (50 µg/ml, concentración final en alimento de 0.37 □g/cm^2), fago P100 (concentración final de aprox. 10^7 PFU/cm^2) y sus combinaciones y almacenados a 5 ºC. En ambos pescados crudos, merluza y salmón, los dos conservantes biológicos aplicados por separado tuvieron un significativo efecto anti-listeria, más importante para AS-48. Sin embargo el mejor resultado se consiguió por la aplicación simultánea de los dos agentes que consiguió eliminar a las listerias en ambos tipos de pescado desde los días 1-2 de almacenaje hasta el final del mismo (7 d).

En salmón ahumado la sola aplicación de AS-48 produjo reducciones de hasta 4,25 log UFC/g con respecto al control no tratado. Sin embargo al final del almacenaje se produjo una ligera recuperación de la población de listerias, que alcanzó recuentos de 0,78 log UFC/g a los 30 días de almacenaje. La recuperación de *Listeria* observada en otros modelos alimentarios estudiados en esta memoria, mucho más frecuente cuando se aplica AS-48 en solitario, puede deberse a la inactivación de la bacteriocina, su adsorción a componentes de la matriz alimentaria y/o la recuperación de listerias dañadas subletalmente. La implementación de dos o más barreras, que actúen sobre distintas dianas puede suprimir o disminuir esta reactivación (Leistner, 2000).

Los resultados de este estudio indican la capacidad de tanto la enterocina AS-48 como del fago P100 para controlar *L. monocytogenes* en pescado crudo y ahumado. Aunque las dosis no son comparables (hay que tener en cuenta el efecto amplificador del ciclo lítico del fago), AS-48 parece ser más efectiva que el fago. Los mejores resultados se consiguieron por la aplicación simultánea de los dos agentes que consiguió eliminar a las listerias en ambos tipos de pescado crudo. El empleo de concentraciones más altas de AS-48 en la solución de pulverización que determinen una mayor cantidad de la enterocina retenida en el alimento mejoraría seguramente los resultados.

Finalmente, nos planteamos realizar un estudio sobre los posibles efectos tóxicos que la enterocina AS-48 pudiera tener al ser ingerida con los alimentos, administrándola con la dieta a ratones durante 90 días. Las concentraciones administradas en la dieta fueron de 50, 100 y 200 mg/kg de pienso y además se estableció un grupo control alimentado con

dieta no suplementada y otro grupo alimentado con 200 mg/kg de nisina. Los estudios de toxicidad de las bacteriocinas de bacterias lácticas son escasos, ya que se presupone su inocuidad. La más estudiada a este respecto es, sin duda la nisina, de la que se han hecho numerosos estudios en modelos celulares y animales (Gupta et al., 2008, Frazer et al., 1962; Hagiwara et al., 2010).

El seguimiento de los animales durante el curso del experimento no reveló signos clínicos evidentes ni anormalidades en ninguno de los grupos. Tampoco hubo cambios en el comportamiento, la ingesta ni la ganancia de peso corporal. No se produjeron muertes. Los datos analíticos de la orina recogida periódicamente y los datos hematológicos (recuento de eritrocitos, leucocitos y plaquetas, hemoglobina, hematocrito, volumen corpuscular medio, hemoglobina corpuscular media, concentración de hemoglobina corpuscular media, fórmula leucocitaria) y la bioquímica sanguínea (aspartato aminotransferasa, alanina aminotransferasa, creatinina, glucosa, albúmina, colesterol total, fosfolípidos, triglicéridos, proteínas totales, potasio y cloruros) obtenidos tras el sacrificio de los ratones.

En cuanto al peso de los órganos, no se encontraron diferencias significativas en los pesos de bazo, timo, corazón y riñones entre el grupo control y los tratados ni tampoco en las relaciones peso longitud de los intestinos delgado y grueso. Además la coloración y el aspecto de los tejidos fueron normales. El estudio de anatomía patológica de estómago, riñones e intestino delgado no reveló lesiones histopatológicas en los tejidos de ninguno de ellos. En el hígado de los ratones alimentados con AS-48 apareció con baja frecuencia degeneración vacuolar de los hepatocitos, una alteración generalmente reversible, relacionada con la ingesta, que fue dependiente de la dosis de AS-48. Así, en ninguno de los ratones alimentados con 50 mg/kg de AS-48 apareció esta anomalía y sí estuvo presente en 3 de los 10 ratones analizados en el grupo de animales alimentados con 200 mg/kg. Hay que destacar que en los ratones alimentados con 200 mg/kg de nisina la degeneración vacuolar estuvo presente en 5 de los 10 ratones del grupo.

Podemos concluir pues, que AS-48 administrado continuadamente en la dieta a una concentración máxima de 200 mg/kg durante 90 días no produce efectos adversos sobre ninguno de los parámetros evaluados, lo que en principio no plantea inconvenientes para su uso como conservante alimentario.

II. Estudio de probiosis de una cepa productora de AS-48.

La FAO y la Organización Mundial de la Salud (OMS) han definido los probióticos como "*organismos vivos que ingeridos en cantidad adecuada confieren un beneficio para la salud en el hospedador"* (FAO/OMS, 2001).

Los efectos beneficiosos para la salud humana atribuidos a los probióticos son muy variados, aunque insuficientemente probados en muchas ocasiones, al menos por experimentos *in vivo:* reducción de la intolerancia a la lactosa, protección frente a enfermedades inflamatorias intestinales, aumento de la resistencia a infecciones, modulación del sistema inmune, protección frente a enfermedades cardiovasculares, reducción de reacciones de hipersensibilidad y prevención de ciertos tipos de cáncer.

El uso de los probióticos también se ha propuesto como una alternativa a los antibióticos en producción animal y para paliar el estres al que son sometidos los animales en la cría intensiva. De hecho en nutrición animal se están usando ya diversas cepas perteneciennte a los géneros *Bacillus, Bifidobacterium, Enterococcus, Escherichia, Lactobacillus, Lactococcus, Streptococcus* e incluso a las levaduras. (Ramos et al., 2015; Zhang et al., 2015; Gunther et al., 1995).

En cuanto a las carracterísticas que deben cumplir los microrganismos para ser considerados como probióticos, destacan: ser bioseguro, permanecer viable en el alimento hasta su consumo, ser capace de resistir a la acidez gástrica y a las sales biliares, deben adherirse a células del epitelio intestinal del huésped, siendo capaz de colonizar el tracto gastrointestinal por un determinado periodo de tiempo, presentar antibiosis frente a enteropatógenos y demostrar un efecto inmunomodulador e influir positivamente en el metabolismo del huésped.

Debemos destacar que las propiedades relacionadas con la probiosis no están presentes en todas las cepas de un género o especie y es por ello que, a la hora de definir un probiótico, es necesario hacerlo sobre la cepa concreta y no sobre la especie. Es decir, no se pueden extrapolar los beneficios demostrados por una de las cepas a toda la especie, y menos aún al género.

La mayoría de los probióticos usados en la actualidad son microorganismos de los géneros *Lactobacillus* y *Bifidobacterium*, aunque también se emplean algunos otros géneros como *Enterococcus* (*E. faecalis, E. faecium*), *Streptococcus* (*S. thermophilus*) y

Propionibacterium (*P. freudenreichii*). El uso preferencial de lactobacilos y bifidobacterias se debe, en buena medida a que se les considera seguros y, de hecho, muchas especies gozan del estatus GRAS de la FDA. Otras especies pertenecientes a los géneros *Bacillus* (*B. cereus*) y *Escherichia* (*E. coli* Nissle) también se han descrito como probióticos. Aparte de las bacterias, se han descrito levaduras como *Saccharomyces* (*S. boulardii*) y hongos como *Aspergillus* (*A. oryzae*) con propiedades probióticas.

Mención especial por sus peculiaridades y por ser objeto de esta memoria es el género *Enterococcus.* Se trata de un conjunto de especies incluidas en las BAL que tienen relevancia tecnológica, estando algunas cepas, especialmente de *E. faecium* y *E. faecalis,* propuestas e incluso comercializadas como probióticos (Fuller, 1989; O´Sullivan et al., 1992; Holzapfel et al., 1998; Franz et al., 2011). Pese a todo esto, el uso de estas bacterias como probióticos sigue siendo una cuestión polémica, ya que mientras el beneficio de algunas cepas ha sido bien establecido, el incremento de las enfermedades enterocócicas asociadas con humanos y la resistencia a múltiples antibióticos ha suscitado preocupación en relación a los riesgos de enfermedad y a que sus factores de virulencia puedan ser transferidos a otras bacterias del tracto gastrointestinal (Franz et al., 2003). Es por esto que los aspectos relacionados con la bioseguridad de aquellas cepas de enterococos que sean propuestas para su uso como probióticos deban de ser bien determinados caso por caso.

La segunda parte de esta memoria trata sobre el estudio de las características de probiosis y de bioseguridad de la cepa *E. faecalis* UGRA10, aislada de un queso artesanal de leche de cabra y muy buena productora de la enterocina AS-48. Dada la importancia que se da a la producción de antimicrobianos por parte de una cepa, en relación a su potencial uso como probiótico, nos planteamos realizar un estudio sobre UGRA10 que comenzó por una investigación *in vitro* de sus características tecnológicas, bioquímicas, de bioseguridad y de probiosis. En relación a las características tecnológicas y bioquímicas destacamos la producción de gelatinasa y caseinasa, siendo esta última importante para su implantación en leche, y la actividad esterasa y lipasa-esterasa C8, implicadas en la producción de aroma y en la degradación de las grasas. En cuanto a las características de seguridad, la cepa presenta los genes *gelE, asa1, esp, efa* y *ace* que codifican para la gelatinasa, sustancia de agregación, proteína de superficie, antígeno de endocarditis y proteína de unión al colágeno, respectivamente. Estos genes están ampliamente distribuidos entre las especies del género *Enterococcus* (Martín-Platero et al., 2009), aunque más que verdaderos genes de virulencia, pueden ser considerados como factores auxiliares relacionados con la capacidad de colonización y de permanencia en el tracto gastrointestinal (Pillar y Gillmore, 2004; Toledo-Arana et al., 2001). En cuanto a la producción de aminas biogénicas, se detectó el gen de la

tirosina descarboxilasa, enzima responsable de la producción de tiramina. En lo que se refiere a la resistencia a antibióticos, la cepa resultó sensible a la mayor parte de los antibióticos de relevancia clínica, incluida la vancomicina, aunque fue resistente para aminoglicósidos y quinupristina/dalfopristina. Sin embargo, estas resistencias son consideradas intrínsecas a este género y por tanto, difícilmente transmisibles (Klare et al., 2001).

Respecto a las propiedades probióticas, se han investigado las capacidades relacionadas con la supervivencia en el tracto gastrointestinal e implantación en la mucosa gástrica. UGRA10 fue capaz de crecer en presencia de 40% de bilis aunque no fue capaz de hidrolizar las sales biliares; resultó además bastante resistente a la acidez (pH 3) aunque tras la exposición a pH ácido, se volvió más sensible a las sales biliares. En relación con su capacidad de implantación en el intestino, la bacteria fue capaz de formar biofilmes, lo que puede ser considerado un efecto beneficioso para prevenir la colonización por microorganismos indeseables (Moroni et al., 2006). La capacidad de implantación *in vitro* también se ha investigado mediante el estudio de la adhesión a líneas celulares humanas derivadas de adenocarcinoma de colon (Caco-2) y carcinoma de cérvix vaginal (Hela 229) que fue moderada en ambos casos. Sin embargo, lo realmente importante fue la inhibición de la adhesión de *L. monocytogenes* a células Caco-2 efectuada por UGRA10 cuando ésta se adicionó antes o de forma conjunta con el patógeno.

De acuerdo con estas características determinadas *in vitro*, podemos concluir que *E. faecalis* UGRA10 posee características adecuadas para su desarrollo como cultivo protector en alimentos y/o probiótico. Por ello, nos planteamos continuar los estudios sobre esta cepa en modelos animales, centrados, sobre todo, en los aspectos de bioseguridad y de probiosis (capacidad para modular la respuesta inmune y protección frente a enteropatógenos).

En primer lugar se hizo un estudio de bioseguridad de la cepa UGRA10 en un modelo murino administrando la bacteria (10^8 UFC), tanto por vía oral como por vía intravenosa, a animales inmunocompetentes e inmunodeprimidos. Los ratones administrados oralmente durante 20 días con el probiótico no mostraron diferencias en la ingesta, presencia de signos de enfermedad y mortalidad. Tampoco los datos hematológicos (hematocrito y hemograma) ni de bioquímica sanguínea (glucosa, urea y GOT) difirieron entre los grupos de ratones administrados con UGRA10 y los controles. En cambio se observó un incremento significativo en la ganancia de peso y en el índice de transformación en los ratones alimentados con UGRA10, sobre todo en los inmunodeprimidos. Hay referencias en la literatura acerca del efecto positivo de los probióticos sobre estos

parámetros en animales de granja (Bernardeau and Vernoux, 2013). Diferentes autores han propuesto diversos modos de acción de los probióticos para explicar este efecto: mayor retención de proteína, mayor digestibilidad de los nutrientes, producción de vitaminas, modulación de la microbiota intestinal, propiedades anti-inflamatorias, exclusión competitiva y creación de un ambiente hostil para la colonización por patógenos. (Bernardeau and Vernoux, 2013). Teniendo en cuenta el efecto inmunomodulador demostrado para esta cepa en cultivos celulares (Rodríguez-Nogales et al., 2012) e *in vivo* (esta memoria), pensamos que el incremento en la ganancia de peso debido a la administración de UGRA10, si se confirma en experimentos posteriores, podría deberse a una acción inmunomoduladora de la cepa que llevaría a una reducción del estrés adicional provocado en el intestino por la inmunosupresión. No se detectó translocación de la bacteria hacia los órganos linfoides: nódulos linfáticos, bazo e hígado.

En los animales administrados por vía intravenosa con UGRA10, los enterococos fueron eliminados eficientemente del bazo después de 5 días de la inoculación, tanto en animales inmunocompetentes como en los inmunodeprimidos. Además, no se observó morbilidad ni mortalidad en ninguno de los grupos.

Estos resultados indican la inocuidad de esta cepa y nos incentivaron a profundizar en sus características probióticas relacionadas con su capacidad inmunomoduladora y de protección frente a patógenos.

Con respecto a la capacidad inmunomoduladora de UGRA10, se puso de manifiesto que los esplenocitos procedentes de ratones alimentados con el probiótico y estimulados con los mitógenos LPS y Concanavalina A, mostraban incrementos en la capacidad proliferativa de 44 y 32%, respectivamente, en comparación con los esplenocitos de animales control. Además, se detectó un efecto de sesgo sobre el balance de las citoquinas producidas por estos esplenocitos, estando incrementada la producción de IFNy disminuida la de TNF-α. El efecto estimulador de la producción de IFNγ ha sido demostrado para las potenciales cepas probióticas *E. faecalis* CECT 712 (Molina et al., 2015) y *Lactococcus lactis* subsp. *cremoris* FC (Kosaka et al., 2012). En relación con la capacidad anti-inflamatoria de ciertos probióticos, se ha demostrado la capacidad de algunas cepas de *E. faecalis* para reducir los niveles de TNF-α *in vivo* (Wang et al., 2014).

Los experimentos encaminados a investigar la protección proporcionada por la cepa UGRA10 frente a infecciones por *L. monocytogenes,* mostraron que los recuentos de las listerias en bazo e hígado de los ratones administrados durante 20 días con el probiótico,

eran significativamente inferiores a los de los ratones control. Los animales administrados con el mutante no productor de AS-48 derivado de UGRA10, mostraron un nivel de infección similar al de los controles, lo que apunta al papel que la enterocina AS-48 desempeña en la protección frente al patógeno. El papel protector de la bacteriocina frente a infecciones intestinales ha sido sugerido por varios autores (Gillor et al., 2008) y demostrado de forma concluyente por Corr et al., (2007) para la bacteriocina Abp18 producida por *Lb salivarius* UCC118.

También, UGRA10 fue capaz de ejercer un efecto de protección frente a la infección por *C. perfringens,* ya que la supervivencia en los animales alimentados con el probiótico fue superior a la del grupo control, 80% vs 50%. En este caso, el mutante no productor de AS-48 ofreció el mismo nivel de protección que la cepa salvaje, apuntando a que pueda existir otro mecanismo de protección adicional a la producción de As-48.

Dado los potenciales usos de la cepa como probiótico en animales de cría, nos propusimos ensayarla en el campo de la acuicultura, donde las enfermedades infecciosas representan un grave problema y donde se ha propuesto el uso de probióticos como alternativa a los antibióticos convencionales (Pérez-Sánchez et al., 2014; Newaj-Fyzul et al., 2014). La investigación se centró en una enfermedad emergente de gran incidencia en nuestro país que afecta a la trucha arco iris ocasionada por *Lactococcus garvieae.* En esta investigación se ensayó la eficacia para controlar el patógeno tanto de la enterocina AS-48 como de la cepa productora de la misma, *E. faecalis* UGRA10, estando precedida de estudios de toxicidad y bioseguridad en peces. Los experimentos previos de laboratorio establecieron unas Mínimas Concentraciones Bactericidas para diferentes cepas de *L. garvieae* entre 15,62 y 7,81 µg/mL y demostraron que la cepa UGRA10 era capaz de controlar a los lactococos en cocultivo a una concentración inicial 10 veces inferior. Los estudios de bioseguridad de UGRA10 y de toxicidad de AS-48, realizados sobre pez cebra, pusieron de manifiesto la ausencia de efectos adversos en ambos casos.

Respecto a la protección ejercida por AS-48 y UGRA10 frente a *L. garvieae*, se realizaron dos experimentos utilizando los modelos de infección de pez cebra y trucha arco iris. En el caso del pez cebra, la administración previa del probiótico durante 20 días, incrementó la tasa de supervivencia respecto al grupo control (70% vs 50%). En las truchas infectadas con el patógeno, la administración vía intraperitoneal de AS-48 (100 µg) incrementó la supervivencia de los animales con un 30% de supervivientes, frente al 100% de muertes en el grupo control. El tratamiento a base de baños periódicos de 30 minutos de

duración con AS-48 (2.5 µg/ml) fue el más efectivo, con una supervivencia del 60% al final del experimento.

Este efecto protector frente a infecciones por *L. garvieae* por parte de cepas probióticas bacteriocinogénicas ha sido descrito por algunos autores (Sequeiros et al 2015, Pérez-Sánchez et al. 2011). Investigaciones recientes como las llevadas a cabo por Araujo et al., (2015) incluso han identificado el papel de la producción de bacteriocinas como principal mecanismo de protección frente a la infección por *L. garvieae*, como es el caso de la producción de nisina Z por *Lb. cremoris* WA2-67.

Los resultados obtenidos en los ensayos realizados sobre la cepa *E. faecalis* UGRA10 apoyan firmemente su inocuidad en ratones y peces y su propiedades probióticas, tanto a nivel de inmunomodulación como de protección frente a patógenos, e incentivan posteriores estudios en relación con su uso como probiótico en humanos y en animales.

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Conclusiones

1. En el modelo alimentario jamón cocido, la aplicación combinada de la enterocina AS-48 (40 μg/g) con conservantes químicos nitritos/nitratos, tripolifosfatos y pirofosfatos y/o calor ha mostrado tener efectos sinérgicos y ser una protección muy efectiva frente a la contaminación por *L. monocytogenes*, y en menor medida, frente a *S. aureus*. Para controlar a los alterantes *Lb. sakei* y *B. thermosphacta,* las combinaciones de la enterocina con lactato o acetato sódico también dieron buenos resultados. El envasado a vacío ha mostrado ser particularmente efectivo potenciando la actividad de AS-48 frente a estos microorganismos alterantes.

2. La combinación de AS-48 (40 μg/g) con el aromatizante natural Proallium, extracto derivado de Aliáceas, aplicada a productos cárnicos no fermentados, hamburguesa, chistorra, albóndigas y jamón cocido, mejoró la calidad higiénica de los mismos al producir reducciones significativas de las poblaciones de *L. monocytogenes*, *S. aureus* y *C. perfringens*. Los efectos inhibidores, que fueron aditivos, mantuvieron a *Listeria* por debajo de los niveles tolerados de 2 Log UFC/g y a *S. aureus* y *C. perfringens* por debajo de 1 Log UFC/g, reduciendo el riesgo real de infección/intoxicación.

3. La incorporación de AS-48 en recubrimientos comestibles basados en ésteres de sacarosa y ácidos grasos, ésteres del ácido acético y mono y digliceridos, quitosano y gelatina de pescado, proporciona actividad antimicrobiana a estos recubrimientos y representa una estrategia válida para el control *L. monocytogenes* y bacterias alterantes en alimentos RTE (listos para su consumo).

4. La aplicación de AS-48 a pescados frescos, merluza y salmón y salmón ahumado permite el control eficaz de *L. monocytogenes* que permaneció por debajo de los niveles máximos autorizados (2 Log UFC/g) durante todo el almacenaje. La combinación de la enterocina con el listeriofago P-100 tuvo un efecto aditivo, consiguiéndose la eliminación total de las listerias en ambos tipos de pescado crudo.

5. Se ha demostrado la ausencia de toxicidad de la enterocina AS-48 en un modelo murino tras ser administrada de forma continuada por vía oral durante 90 días. Los parámetros referidos al comportamiento de los animales, ingesta, ganancia de peso, hematología y bioquímica sanguínea no difirieron entre los animales administrados de la enterocina y los del grupo control. Esta carencia de toxicidad se ha confirmado también mediante los estudios en pez cebra.

6. La cepa *E. faecalis* UGRA10, estudiada *in vitro,* presenta interesantes características tecnológicas y de probiosis: hidrólisis de caseína y grasas, resistencia a sales biliares y al pH ácido, formación de biofilmes y presenta moderada capacidad de adhesión a líneas celulares Caco-2 y Hela 229. Pero lo más relevante es su capacidad para inhibir la adhesión de *L. monocytogenes* a Caco-2 cuando es adicionada antes o junto con el patógeno.

7. Se ha detectado en UGRA10 la presencia de los genes *gelE, asa1, esp, efa* y *ace* que codifican para putativos determinantes de virulencia, relacionados con la capacidad de colonización y persistencia en el intestino. Es sensible a la mayor parte de los antibióticos de relevancia clínica, incluida la vancomicina, siendo resistente a aminoglicósidos y quinupristina/dalfopristina. Estas resistencias son consideradas intrínsecas al género *Enterococcus* y por tanto, difícilmente transmisibles.

8. Los ensayos en modelo murino demuestran la bioseguridad de la cepa UGRA10 tanto administrada por vía oral como por vía intravenosa, en animales inmunocompetentes e inmunodeprimidos. Así, la administración oral no produjo cambios en ingesta ni signos de enfermedad. Tampoco los datos hematológicos ni de bioquímica sanguínea se vieron afectados. No se detectó translocación de la bacteria desde el intestino hacia los órganos linfoides. Tras 5 días de ser inoculados intravenosamente, los enterococos fueron eliminados eficientemente por el bazo. La bioseguridad de UGRA10 ha sido confirmada mediante estudios en pez cebra.

9. La capacidad inmunomoduladora de UGRA10 se ha puesto de manifiesto en modelo murino donde se ha demostrado que estimula la proliferación de esplenocitos activados por mitógenos (LPS y CoA) y determina un sesgo en la producción de citoquinas, incrementando los niveles de IFNγ y disminuyendo los de TNF-α.

10. La administración oral de UGRA10 ejerce un efecto protector frente a la infección por los patógenos *L. monocytogenes* y *C. perfringens*, confirmando los resultados de interferencia de adhesión de *Listeria* observados *in vitro.*

11. La administración de UGRA10 o de la enterocina AS-48 a peces cebra y a truchas arcoíris, respectivamente, redujo de forma significativa la tasa de mortalidad ocasionada por la infección por *L. garvieae*. El mejor resultado se obtuvo mediante baños periódicos de los peces en soluciones de AS-48.

12. Podemos concluir que tanto la enterocina AS-48 como la cepa *E. faecalis* UGRA10 poseen un importante potencial biotecnológico para ser usados como conservante alimentario y probiótico en nutrición humana y animal, respectivamente.