UNIVERSIDAD DE JAÉN

FACULTAD DE CIENCIAS EXPERIMENTALES
DEPARTAMENTO DE CIENCIAS DE LA SALUD

TESIS DOCTORAL

ANÁLISIS DE LA RESISTENCIA A AGENTES ANTIMICROBIANOS EN BACTERIAS LÁCTICAS AISLADAS DE ACEITUNAS ALOREÑA PARA SU USO COMO PROBIÓTICOS

PRESENTADA POR:
MARÍA DEL CARMEN CASADO MUÑOZ

DIRIGIDA POR:
DRA. D.ª HIKMATE ABRIOUEL HAYANI
DR. D. NABIL BENOMAR EL BAKALI
DR. D. ANTONIO GÁLVEZ DEL POSTIGO RUIZ

JAÉN, JULIO DE 2016

ISBN 978-84-9159-018-7

Análisis de la resistencia a agentes antimicrobianos en bacterias lácticas aisladas de aceitunas Aloreña para su uso como probióticos

Analysis of antimicrobial resistance in Lactic Acid Bacteria isolated from Aloreña olives for use as probiotics

Memoria para optar al grado de Doctor

Jaén, 2016

Fdo: María del Carmen Casado Muñoz

Aspirante al Grado de Doctor

Los Directores del trabajo:

Fdo: Nabil Benomar El Bakali

Fdo: Antonio Gálvez del Postigo Ruiz

Fdo: Hikmate Abriouel Hayani

Área de Microbiología. Dpto. de Ciencias de la Salud
Facultad de Ciencias Experimentales. Universidad de Jaén

Los profesores Dª Hikmate Abriouel Hayani, D Nabil Benomar El Bakali y D. Antonio Gálvez del Postigo Ruiz, pertenecientes al Área de Microbiología del Departamento de Ciencias de la Salud de la Universidad de Jaén

CERTIFICAN: Que el trabajo expuesto en la presente Tesis Doctoral: “Análisis de la resistencia a agentes antimicrobianos en bacterias lácticas aisladas de aceitunas Aloreña para su uso como probióticos” presentado por Dª. María del Carmen Casado Muñoz ha sido realizado bajo nuestra dirección y supervisión, cumpliendo así mismo todas las exigencias para su presentación y defensa para optar al grado de Doctor en la modalidad de Doctorado con Mención Internacional. Parte del trabajo presentado ha sido realizado durante la estancia de la doctoranda en el “Equipe de Chimie Analytique des Molécules Bio-Actives” de la Universidad de Estrasburgo, por un periodo de tres meses.

Jaén, Junio de 2016

Fdo: Nabil Benomar El Bakali

Fdo: Antonio Gálvez del Postigo Ruiz

Fdo: Hikmate Abriouel Hayani

Este trabajo ha sido subvencionado por el MICINN (proyecto AGL2009-08921), el MINECO-FEDER (AGL2013-43571-P), la junta de Andalucía (P08-AGR-4295, ayudas a grupos de investigación “grupo AGR230”), el Plan de Apoyo a la Investigación de la Universidad de Jaén (UJA2014/07/02). La doctoranda ha disfrutado de una beca de formación de personal investigador así como de una ayuda de movilidad en programas de doctorado con mención hacia la excelencia para la realización de estancias en otros centros de I+D (Campus de Excelencia Internacional CeiA3).

Índice

Summary

Lactic acid bacteria (LAB) are widely consumed along with fermented foods and beverages because of their use as starter cultures in fermentation processes for millenia. Furthermore, they are also known for their role as protective cultures or as probiotics. The application of LAB as probiotics has been prompted by their beneficial properties on general health of the consumers and their “QPS” (Qualified Presumption of Safety) status based on a long history of safe use. Recently, several studies revealed that fermented foods have been considered as potential vehicles of antibiotic resistance genes. Gene exchanges may enhance survival of LAB and pathogens and thus represent an important risk within the gastrointestinal tract for spread to other bacteria. The overuse or misuse of antimicrobial agents in human, veterinary, domestic and as disinfectants in food industry has resulted in an important public health risk. For this, the European Scientific Committee on Animal Nutrition (SCAN) and the European Food Safety Authority (EFSA) recommended that LAB strains consumed on a daily basis worldwide should lack acquired or transferable antimicrobial resistance genes prior to considering them safe for human and animal consumption and that any probiotic strain should have QPS status. In order to investigate the susceptibility patterns and possible mechanisms determining resistance to several antibiotics in LAB isolated from Aloreña green table olives, the antimicrobial resistance of *Lactobacillus pentosus* (n=59) and *Leuconostoc pseudomesenteroides* (n=13) was evaluated. The results obtained showed that most *Lb. pentosus* (95%) and all *Lc. pseudomesenteroides* were resistant to at least three antibiotics. Principal component analysis determined that the prevalence of antibiotic resistance in LAB throughout the fermentation process was highly dependent on the fermenter where the fermentation took place. On the other hand, all *Lb. pentosus* and *Lc. pseudomesenteroides* strains were highly sensitive to amoxicillin and ampicillin (MIC ≤ 2 μg/ml), and also to chloramphenicol (MIC ≤ 4 μg/ml), gentamicin and erythromycin (MIC ≤ 16 μg/ml). However, they were phenotypically resistant to streptomycin (83–100%, MIC > 256 μg/ml), vancomycin and teicoplanin (70–100%, MIC > 128 μg/ml), trimethoprim (76% of *Lb. pentosus* and 15% of *Lc. pseudomesenteroides*, MIC > 128 μg/ml), trimethoprim/sulfomethoxazol (71–100%, MIC > 4–64 μg/ml) and cefuroxime (44% of *Lb. pentosus* and 85% of *Lc. pseudomesenteroides*, MIC > 32–128 μg/ml). *Lactobacillus pentosus* was susceptible to tetracycline and clindamycin, while 46% of *Lc. pseudomesenteroides* strains were resistant to these antibiotics. Only *Lb. pentosus* strains were resistant to ciprofloxacin (70%, MIC N 4–64 μg/ml), although no mutations in the quinolone resistance determining regions of the genes encoding GyrA and ParC were found, thus indicating an intrinsic resistance. Similarly, no genes encoding possible transferable resistance determinants for the observed phenotypic resistance were detected by PCR. In some cases, a bimodal distribution of MICs was observed for some antibiotics to which both LAB species exhibited resistance. Nevertheless, such resistances resulted from an intrinsic mechanism, non-transferable or non-acquired resistance determinants which may in part be due to chromosomally encoded efflux pumps (NorA, MepA and MdeA). Results of the present study demonstrate that all *Lb. pentosus* and *Lc. pseudomesenteroides* strains lack transferable resistance-related genes (*cat, bla, blaZ, ermA, ermB, ermC, msrA/B, ereA, ereB, mphA, mefA, tet(M), tet(O), tet(S), tet(W), tet(L), tet(K), aad(E), aac(6′)-Ie-aph(2′)-Ia, aph(2′)-Ib, aph(2′)-Ic, aph(2′)-Id, aph(3′)-IIIa, ant(4′)-Ia, dfrA, dfrD, vanA, vanB, vanC* and *vanE*) and should therefore, according to Qualified Presumption of Safety criteria, be considered safe for future application as starter cultures or as probiotics. On the other hand, biocide susceptibility of LAB was studied and the results obtained revealed that most of them were sensitive to the biocides tested in this study and only 2-15% of the isolates were found to be biocide resistant: *Lc. Pseudomesenteroides* resistant to hexachlorophene and *Lb. pentosus* to cetrimide and hexadecylpiridinium.

With the aim to understand and explain the increased resistance of LAB under changing environmental conditions, their phenotypic and genotypic responses to different physico-chemical stressors, including antimicrobials was investigated. Similar phenotypic responses of LAB were obtained under three stress conditions: antimicrobials, chemicals and UV light. Thus, susceptibility patterns to antibiotics changed: increasing MICs for ampicillin, chloramphenicol, ciprofloxacin, teicoplanin and tetracycline, and decreasing MICs for clindamycin, erythromycin, streptomycin and trimethoprim in most strains. Statistically, cross resistance between different antibiotics was detected in all stress conditions. However, expression profiles of selected genes involved in stress/resistance response (*rpsL, recA, uvrB* and *srtA*) differed depending on the stress parameter, LAB species and

strain, and the target gene. We conclude that, despite the uniform phenotypic response to stresses, the repertoire of induced and repressed genes differs.

In this sense, to understand the resistance mechanisms in a probiotic *Lb. pentosus* under challenging environmental conditions and to discern their utility in fermentative food preparations versus clinical and agricultural risk, proteomic analysis was carried out. *Lactobacillus pentosus* MP-10 exposed to sub-lethal concentrations of antibiotics (amoxicillin, chloramphenicol and tetracycline) and biocides (benzalkonium chloride and triclosan) exhibited differential expression of several genes depending on antimicrobial exposure, such as the up-regulation of protein synthesis, and the down-regulation of carbohydrate metabolism and energy production. The antimicrobials appeared to have altered *Lb. pentosus* MP-physiology to achieve a gain of cellular energy for survival. For example, biocide-adapted *Lb. pentosus* MP-10 exhibited a down-regulated phosphocarrier protein HPr and an unexpressed oxidoreductase. However, protein synthesis was over-expressed in antibiotic- and biocide-adapted cells (ribosomal proteins and glutamyl-tRNA synthetase), possibly to compensate for damaged proteins targeted by antimicrobials. Furthermore, stress proteins, such as NADH peroxidase (Npx) and a small heat shock protein, were only overexpressed in antibiotic-adapted *Lb. pentosus* MP-10. Results showed that adaptation to sub-lethal concentrations of antimicrobials could be a good way to achieve desirable robustness of the probiotic *Lb. pentosus* MP-10 to various environmental and gastrointestinal conditions (e.g., acid and bile stresses).

On the other hand, an advanced strategy to approach safety investigations regarding antibiotic resistances in lactobacilli is to explore genome sequences, i.e. an *in silico* analysis for antibiotic resistance genes. However, the low number of available genome sequences up to date may limit an in-depth understanding of the origin of observed phenotypic resistances, which are either induced or uninduced by the presence of antimicrobials. Some observations about the most important genomic insights that regard antibiotics such as chloramphenicol and glycopeptides in lactobacilli isolated from fermented foods are discussed in this study. *In silico* screening of *vanZ* (glycopeptide resistance) and *cat* (chloramphenicol resistance)-like sequences in *Lactobacillus* species isolated from fermented foods revealed for the first time the occurrence of *vanZ* and *cat* genes in

Lactobacillus species being highly conserved genes in the chromosome of each species, presumably non-transferable. Further studies involving genome sequences of *Lactobacillus* isolated from fermented foods, especially those relying on spontaneous fermentation, is crucial to increase knowledge on the potential presence and spread of antibiotic resistance genes via the food route. For this, we analyzed the genome sequence odf a potential probiotic *Lb. pentosus* MP-10 siolated from Aloreña Green table olives regarding its safety aspects (antibiotic resistance and virulence). The genome sequence of *Lb. pentosus* MP-10 is considered the largest genome among lactobacilli up to date highlighting its ecological flexibility and adaptability. Our data shed new light on the presence of two new genes belonging to Subtype I-E CRISPR system besides other nine genes of two CRISPR clusters (Type I and Type II) considered as immune system against foreign genetic elements which is of great importance in its probiotic and also starter culture application. *In silico* analysis of antibiotic resistance showed the absence of acquired antibiotic resistance genes and that most of the resistance genes were antibiotic efflux genes or resulted from mutation of chromosomal genes. On the other hand, no virulence determinants were found in *Lb. pentosus* MP-10 genome. Thus, we can suggest that *Lb. pentosus* MP-10 could be considered as safe, although additional analysis of its functionality and probiotic-linked mechanisms should be carried out.

Introducción

1. Los probióticos

1.1. Definición e historia de los probióticos

La palabra "probiótico" tiene su origen del latín (*pro* "a favor de") y del griego (*biotikos* "vida") y significa "para la vida" (Gomes *et al.*, 1999), este término fue acuñado por primera vez en 1960 para referirse a suplementos alimenticios promotores del crecimiento en animales (Fuller, 1991). La primera alusión a ellos la realizó el premio Nobel Elie Metchnikoff en 1907 en un libro publicado "Prolongation of Life" (Metchnikoff, 1907). Esta publicación ejerció una gran influencia en la comunidad científica ya que Metchnikoff postulaba que las bacterias que intervenían en la fermentación del yogur contribuían al mantenimiento de la salud mediante la supresión de las "fermentaciones de tipo putrefactivo" de la microbiota intestinal y que ésta era la causa de la longevidad de los campesinos búlgaros, grandes consumidores del yogur. Por entonces, el pediatra francés Henry Tissier observó que los niños con diarrea tenían en sus heces un escaso número de bacterias caracterizadas por una morfología peculiar en forma de Y. Estas bacterias "bífidas" eran, por el contrario, abundantes en los niños sanos (Tissier, 1906). Este último sugirió la posibilidad de administrar estas bacterias a pacientes con diarrea para facilitar el restablecimiento de una microbiota intestinal sana. En 1917, antes del descubrimiento de Alexander Fleming de la penicilina, el profesor alemán Alfred Nissle aisló una cepa no patógena de *Escherichia coli* de las heces de un soldado de la Primera Guerra Mundial que no había desarrollado enterocolitis durante un brote grave de shigellosis. Los trastornos del tracto intestinal frecuentemente eran tratados con bacterias no patógenas viables, para cambiar o reemplazar la microbiota intestinal. La cepa no patógena de *Escherichia coli* de Nissle (1917) es uno de los ejemplos de probióticos no pertenecientes a las bacterias ácido lácticas "BAL" (Nissle, 1916, 1918; Kruis *et al.*, 2004).

En los últimos veinte años, la definición de los probióticos ha recibido varias revisiones para dar lugar a la definición más aceptada por la Organización de Alimentos y Agricultura de las Naciones Unidas (FAO) y la Organización Mundial de la Salud (WHO) en el año 2002 como "suplementos alimenticios microbianos vivos que al ser administrados en cantidades adecuadas benefician al animal huésped" (FAO/WHO, 2002). Ello hace que durante mucho tiempo todos los alimentos fermentados tradicionales se consideraban probióticos. Sin embargo, según la Asociación Científica Internacional de Probióticos y Prebióticos (The International Scientific Association for Probiotics and Prebiotics, ISAPP) dichos alimentos solo se pueden describir como "alimentos que contienen microbiota viva y activa" ya que se desconoce la diversidad microbiana de estos alimentos y los beneficios que ejercen dichos microorganismos sobre la salud del huésped (Hill *et al.*, 2014). En este sentido, la única excepción es el yogur tradicional que contiene una microbiota definida (*Streptococcus salivarius* subsp. *thermophilus* y *Lactobacillus delbrueckii* subsp. *bulgaricus*) (FAO/WHO, 2002) y sus beneficios han sido reconocidos en cuanto a la mejora de la digestibilidad de la lactosa en los individuos intolerantes (EFSA, 2010). Así, el término probiótico solo hace referencia a microbios vivos con efectos beneficiosos sobre la salud (Reid *et al.*, 2003), aunque las preparaciones de células muertas y componentes celulares pueden también ejercer efectos fisiológicos positivos (Adams, 2010; Lau, 2014), así se habla más bien de "Postbióticos" como han sugerido recientemente Tsilingiri y Rescigno (2013). En este sentido, se han producido importantes avances en el campo de la nutrición, dando lugar a los "alimentos funcionales". Un alimento funcional es "Aquel capaz de producir efectos beneficiosos más allá de los efectos nutricionales habituales en un sentido relevante para el estudio del bienestar y salud o reducción del riesgo de enfermedad" según la conferencia consenso celebrada en Madrid en 1998 de la CEE y FUFOSE del *International Life Science Institute Europe* (Martin *et al.*, 2008). Los probióticos constituyen uno de los subgrupos más importantes de los alimentos funcionales (Schrezenenmeir *et al.*, 2001). En un alimento funcional, la acción de los probióticos se complementa con los prebióticos definidos como "ingredientes selectivos que al ser fermentados producen cambios específicos en la composición y/o la actividad de la microbiota gastrointestinal y así confiere beneficios para la salud del huésped" (Blatchford *et al.*, 2014), y dicha mezcla de probióticos y prebióticos se denomina "Simbióticos".

Las principales bacterias probióticas incluyen las BAL representadas por los géneros *Lactobacillus* y *Bifidobacterium*, ambas comensales del tracto gastrointestinal humano y tradicionalmente utilizadas en la fermentación de alimentos (Sanz *et al.*, 2003), pero también las propionibacterias (varias especies del género *Propionibacterium*), bacterias formadoras de esporas (*Bacillus cereus, B. subtilis, B. licheniformis, B. coagulans, B. clausii, B. polyfermenticus, B. megaterium., Paenibacilluspolymyxa*), bacterias Gram-negativas no patógenas (*Escherichia coli*), levaduras (*Saccharomyces cerevisiae, S. boulardii, Debaryomyces hansenii, Phaffia rhodozyma*) y hongos (*Aspergillus*).

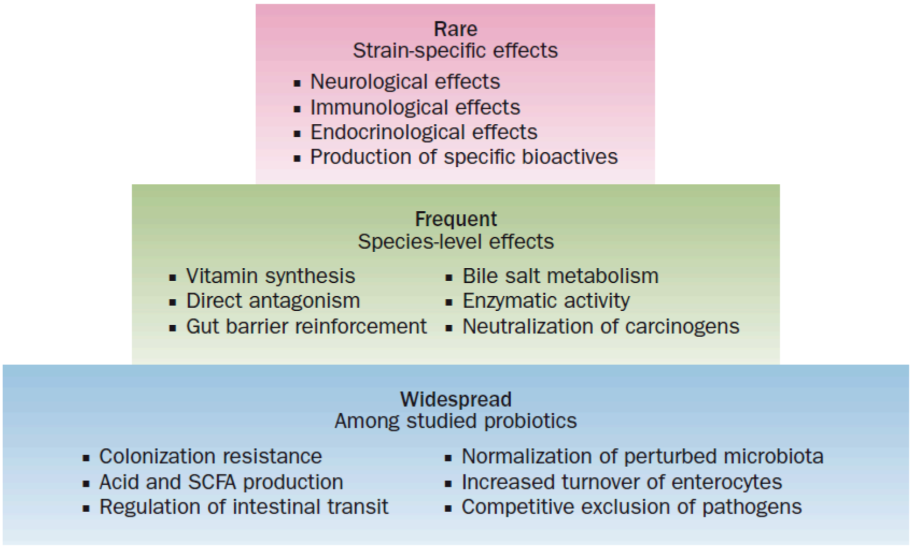


Figura 1. Posible distribución de los efectos ejercidos por las diferentes cepas de probióticos (Hill *et al.*, 2014).

Las bases teóricas que clasifican a una bacteria como probiótico incluyen su seguridad (carencia de genes de virulencia y de resistencia transferibles), su funcionalidad (supervivencia, adherencia a las células intestinales humanas, persistencia en el tracto intestinal humano, colonización del tracto digestivo, producción de antimicrobianos, estimulación del sistema inmune, actividad antigenotóxica y prevención ante patógenos y sus aspectos tecnológicos (propiedades sensoriales, estabilidad ante ácidos y bilis, genes de resistencia a fagos y viabilidad). Sin embargo, algunos de estos mecanismos son ampliamente distribuidos en los géneros probióticos comunes, otros son frecuentemente encontrados en muchas cepas de las especies probióticas y algunos son raros y solamente observados en muy pocas cepas (Figura 1) (Hill *et al.*, 2014). Además, la mayoría de los probióticos son de origen humano, sin embargo en los últimos años se han aislado probióticos de diferentes fuentes (Saarela *et al.*, 2000). Los probióticos tienen numerosos efectos beneficiosos para la salud actuando a nivel del sistema inmune, el sistema endocrino y el sistema nervioso. Además de los efectos beneficiosos conocidos, hoy en día existen hipótesis sobre otras actividades beneficiosas que pudiesen llevar a cabo por estos microorganismos como la reducción de la exposición a carcinógenos y el control del colesterol sanguíneo (ya que al aumentar la actividad de las hidrolasas de las sales biliares que se unen al colesterol, ayudan a su eliminación) (Ferrer *et al.*, 2001), triacilglicéridos e hiperlipidemia (Parvez *et al.*, 2006; Schrezenmeir y Vrese, 2001), longevidad (Matsumoto y Kurihara, 2011; Grompone *et al.*, 2012; Zhao *et al.*, 2013), tratamiento de infecciones vaginales (Homayouni *et al.*, 2014), reducción de la toxicidad por metales pesados (Bisanz *et al.*, 2014), obesidad (Van Baarlen *et al.*, 2011; Drissi *et al.*, 2014), reducción de los daños de la radiación (Ciorba *et al.*, 2012), alivio de los síntomas de la enfermedad celiaca (Sarno *et al.*, 2014) y los daños físico-químicos del estrés (Da Silva *et al.*, 2014). Muchos de los efectos probióticos son mediados a través de la respuesta inmune, particularmente a través del control del balance de citoquinas pro-inflamatorias y anti-inflamatorias.

En poco tiempo el uso de los probióticos se ha incrementado pasando del uso de una sola especie en un principio (*Lb. acidophilus*) al uso de un gran número de especies. Actualmente existen preparados comerciales de probióticos que incluyen especies de *Bacillus*, levaduras (*Saccharomyces cerevisiae*), hongos filamentosos (*Aspergillus oryzae*), *Lb. acidophilus, B. longum, B. infantis*, principalmente (Parvez *et al.*, 2006). Estos productos son recomendados por nutricionistas y médicos. También, se ha investigado la adición de cepas no patógenas de *E. coli* con efectos probióticos para competir con las cepas patógenas (Amores *et al.*, 2004).

1.2. Las bacterias del ácido láctico como probióticos

1.2.1. Generalidades de las bacterias del ácido láctico

Las bacterias del ácido láctico (BAL) son microorganismos procariotas Gram-positivos, heterótrofos y quimioorganotrofos. Son en general inmóviles, no esporuladas (Korhonen, 2010), anaerobias aunque aerotolerantes (microaerófilas) y mesófilas. Carecen de numerosas actividades enzimáticas como catalasa (no obstante, algunas cepas poseen una pseudo-catalasa), nitrato-reductasa y citocromo-oxidasa, no producen indol ni sulfhídrico y solamente algunas especies hidrolizan la caseína. Las BAL pertenecen al filo *Firmicutes* con bajo contenido G+C en su ADN (< 55%), clase *Bacilli*, orden *Lactobacillales* con 6 familias (*Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillaceae, Leuconostoccaceae* y *Streptococcaceae*) y 40 géneros que presentan morfologías diversas (cabe destacar *Aerococcus, Carnobacterium, Enterococcus, Vagococcus, Oenococcus, Lactobacillus, Leuconostoc, Pediococcus Streptococus, Weissella* y *Bifidobacterium* entre otros) (Holzapfel y Wood, 2014). El grupo de las BAL se caracteriza por su gran diversidad fisiológica y genotípica lo cual ha permitido a lo largo de la historia la ocupación de diferentes nichos ecológicos (medio ambiente, alimentos, tracto gastrointestinal, tracto urogenital humano) e incluso ambientes extremos con altas temperaturas (alrededor de 50ºC), bajas temperaturas (0-2ºC), altas concentraciones de sales (hasta el 25% de NaCl), bajos pHs (alrededor de 3.9) o concentraciones fisiológicas de sales biliares (Holzapfel y Wood, 2014).

Estos microorganismos presentan exigencias nutricionales complejas en cuanto a los aminoácidos, péptidos, sales, ácidos grasos y azúcares (Tannock, 2004). Puesto que son acidogénicas por excelencia, su crecimiento mejora considerablemente en presencia de un sistema tampón que neutralice la gran cantidad de ácido que producen (Dellagio *et al.*, 1994). Además toleran altas concentraciones de sales, ácidos y álcalis (Axelsson, 2004).

Las BAL carecen de citocromos y de cadena de transporte electrónico, por tanto no tienen un ciclo de Krebs funcional (Batt, 2000). De hecho, estas bacterias sólo pueden llevar a cabo un metabolismo fermentativo produciendo ácido láctico de forma exclusiva (bacterias homofermentativas estrictas), o bien el ácido láctico y el ácido acético o una mezcla de ambos ácidos, etanol y dióxido de carbono dependiendo de los géneros o incluso de las diferentes especies de un mismo género (bacterias heterofermentativas facultativas o heterofermentativas estrictas). La producción de ácidos orgánicos por las BAL ocasiona siempre una disminución del pH, lo que inhibe el crecimiento de bacterias esporuladas y de otros microorganismos patógenos (Bhatia *et al.*, 1989). Además ciertas bacterias lácticas pueden producir antagonistas específicos como antibióticos y bacteriocinas (Chassy, 1987; Cleveland *et al.*, 2001; Nes *et al.*, 2001). Existen numerosas bacteriocinas producidas por las BAL, capaces de inhibir el crecimiento de microorganismos alterantes de alimentos o de microorganismos patógenos como *Staphylococcus* spp. o *Listeria* spp. (Stiles, 1996).

Las BAL homofermentativas están representadas por los géneros *Lactococcus, Pediococcus, Enterococcus* y muchas especies del género *Lactobacillus*, mientras que *Leuconostoc* y ciertas especies del género *Lactobacillus* pertenecen a las heterofermentativas. No obstante, en función de la disponibilidad del oxígeno y la glucosa, una cepa homofermentativa puede comportarse como heterofermentativa (Rhee y Pack, 1980; Murphy *et al.*, 1985; Borch *et al.*, 1991).

Las BAL poseen propiedades fisiológicas características que el ser humano ha usado en beneficio propio para la fermentación de alimentos. Cabe destacar la resistencia a distintos tipos de bacteriófagos, la actividad proteolítica (importante en la maduración del queso), la utilización del 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), la producción de sustancias inhibidoras de gérmenes nocivos (con acción preventiva de intoxicaciones y toxiinfecciones alimentarias), la adhesión a las mucosas digestivas (que protegen de las agresiones microbianas), y finalmente la alta resistencia a la congelación y a la liofilización (propiedades importantes para la conservación de cepas y fermentos industriales) (Axelson, 1998; Vedamuthu, 2013a; Clark *et al.*, 2014).

El género *Lactobacillus*

El género *Lactobacillus* se caracteriza por su gran diversidad y su compleja filogenia (Schleifer y Ludwig, 1995) lo cual se refleja en el mayor número de especies en relación con todos los géneros que constituyen las BAL (Axelsson *et al.*, 2004). Este género incluye 212 especies descritas en la lista de nombres de procariotas "*List of Prokaryotic names with Standing in Nomenclature* "LPSN"" (www.bacterio.net) que ha sido publicada en enero de 2015 (Abriouel *et al.*, 2015). Suelen poseer una bajo contenido de "G+C" en el genoma, aunque existen algunas excepciones, como en el caso de *Lb. mali* (32%) o *Lb. nasuensis* (59.2%) (Hammes *et al.*, 1992; Cai *et al.*, 2012).

Es posible dividir los lactobacilos en homofermentativos obligados, heterofermentativos facultativos y heterofermentativos obligados (Tannock, 2004; Klaenhammer y de Vos, 2011; Por *et al.*, 1994; Hammes y Vogel, 1995) aunque se ha comprobado que en muchos casos, el producto final depende de las condiciones de cultivo o de la presencia de patógenos. El producto final de su metabolismo, es principalmente el ácido láctico ($C_3H_6O_3$), aunque en el caso de aquellos lactobacilos heterofermentativos irá acompañado de otros compuestos como el acetato, etanol, CO_2, formato o el succinato.

Los lactobacilos son ubicuos en el medio ambiente y en los materiales crudos usados en la producción de alimentos, y de ahí su papel en la producción de alimentos fermentados ha sido establecido durante milenios (revisado por Tamang y Kailasapathy, 2010). Varias especies del género *Lactobacillus* son relevantes en los productos fermentados ya que han sido usadas como cultivos iniciadores y/o protectores de alimentos fermentados vegetales, productos lácteos, salchichas y productos de pescado (Franz *et al.*, 2011; Garrigues, Johansen y Crittenden, 2013; Giraffa *et al.*, 2010; Hansen, 2002; Heller, 2001; Holzapfel, 2002; Leroy y DeVuyst, 1999). El estatus GRAS (Generally Recognized As Safe o generalmente reconocidas como seguras) de los lactobacilos además de sus actividades catabólicas diversas ha hecho que su uso sea ampliamente extendido a una variedad de alimentos fermentados. Además muchas cepas han sido reconocidas como probióticas (Hill *et al.*, 2014), sin embargo no todos los lactobacilos se pueden considerar como probióticos ya que dicha propiedad depende exclusivamente de la cepa en cuestión. Desafortunadamente, a pesar de las multitudes de investigaciones llevadas a cabo para determinar los genes, proteínas u otros componentes celulares responsables de la actividad probiótica, pocos resultados han sido obtenidos en el género *Lactobacillus*. Por lo tanto, la secuenciación del genoma completo de *Lactobacillus* sp. (más de 100 genomas disponibles en la base de datos NCBI: www.ncbi.nlm.nih.gov) y la genómica comparativa permitirá detectar los factores responsables de su actividad probiótica y desvelar aspectos interesantes relacionados con su fisiología, metabolismo y filogenia.

1.2.2. Potencial probiótico de las bacterias del ácido láctico

1.2.2.1. BAL probióticos de origen animal

El principal papel del tracto intestinal es la ingesta y absorción de nutrientes para suplir los requerimientos metabólicos. Un papel importante en este proceso lo lleva a cabo la microbiota intestinal, donde conviven entre 10^{13} y 10^{14} UFC/g de 400 especies diferentes (Hao y Lee, 2004). La comunidad bacteriana en el colon y el intestino delgado (Frank *et al.*, 2007) se caracteriza por la predominancia del filo *Firmicutes*, y en cierto modo de la clase *Bacilli* a la cual pertenece el género *Lactobacillus*. En 1921, Rettger y colaboradores describieron que *Lb. acidophilus* puede sobrevivir en el intestino humano. A partir de entonces se han publicado numerosos estudios con distintos probióticos de origen intestinal.

La microbiota de nuestro tracto gastrointestinal varia mucho de un individuo a otro de la misma especie (Ley *et al.*, 2006), dependiendo del tipo de dieta y del consumo de alimentos funcionales (prebióticos y probióticos). Además, la composición bacteriana intestinal influye en la presencia o no de enfermedades (Carrol *et al.*, 2009) tales como la obesidad (Ley *et al.*, 2005), enfermedades cardiacas (Fava *et al.*, 2006), diabetes (Wen *et al.*, 2008) y enfermedades intestinales (Frank., 2007).

El tubo digestivo de un bebe recién nacido es un medio estéril (Escalante, 2001). El primer contacto con la microbiota se produce en el canal vaginal (cuando se produce un parto natural) y la microbiota fecal materna que se ingiere habitualmente durante el parto (Martin *et al.*, 2008). Gran parte de estos microorganismos mueren al llegar al estómago, debido a su pH ácido, no obstante una pequeña proporción de ellos consiguen alcanzar el intestino, que posee una atmósfera aerobia en el recién nacido, donde se establecen bacterias aerobias como *Escherichia coli* y *Enterococcus* spp. Conforme el recién nacido es alimentado, la proporción de oxígeno presente en el intestino disminuye y organismos anaerobios (o anaerobios facultativos) comienzan a colonizar y a desplazar a la antigua microbiota intestinal del neonato, entre ellas destacan *Lactobacillus* spp. y *Bifidobacterium* spp. (Amores *et al.*, 2004). Una vez finaliza el periodo de lactancia, la microbiota del niño cambia progresivamente, pareciéndose paulatinamente a la composición bacteriana del adulto y apareciendo microorganismos del género

Bacteroides spp. y otros anaerobios estrictos (Amores *et al.*, 2004), principalmente la especificada en la Figura 2. La mayor parte de la concentración de esta microbiota se encuentra en el intestino grueso, concretamente en el colon (Wilson, 2005).

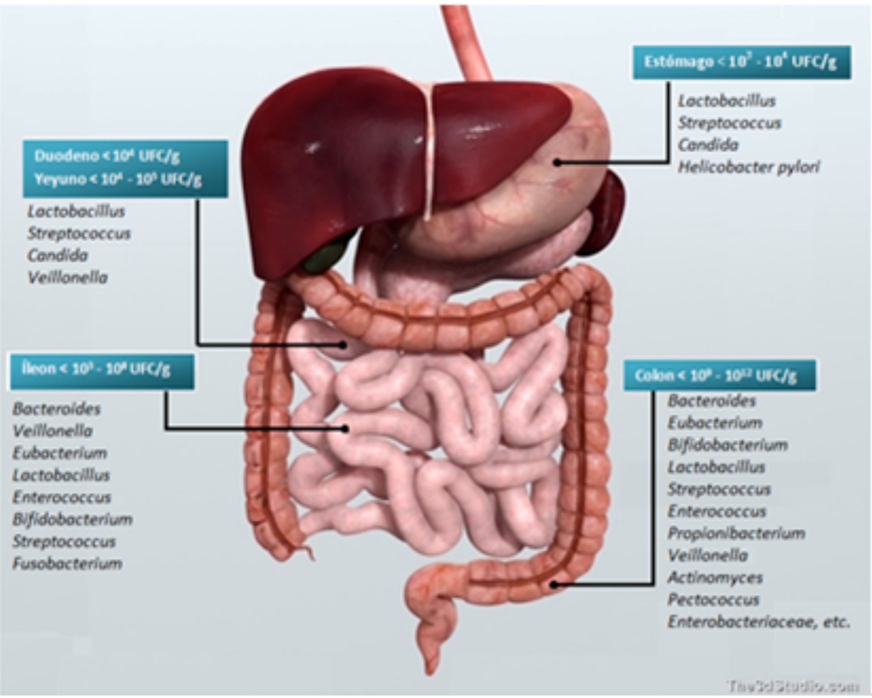


Figura 2. Principales géneros y concentración aproximada (UFC/g) de bacterias encontradas en los diferentes ambientes del tracto digestivo (Delgado, 2013).

No obstante, esta microbiota es diferente incluso entre individuos de la misma especie (genes, alimentación, medio ambiente, tratamiento con antibióticos, estrés, etc). Por lo tanto, al consumir alimentos funcionales se restablece el equilibrio microbiano y se favorece la prevención de enfermedades (Amores *et al.*, 2004). Las bacterias comensales intestinales influyen en la regulación inmunofisiológica del tracto gastrointestinal, que depende de la microbiota endógena que posea el individuo. La demostración de que la microbiota del intestino es uno de los principales constituyentes de la barrera mucosa intestinal, ha provocado el desarrollo de la llamada *"terapia probiótica"*, ésta se basa en la aplicación de microorganismos probióticos potencialmente beneficiosos (Isolauri *et al.*, 2001). La estabilidad de la microbiota intestinal "Eubiosis" es imprescindible para el correcto funcionamiento metabólico del individuo (Amores *et al.*, 2004), teniendo principalmente los siguientes efectos a nivel del sistema inmune:

- Mejora de la resistencia no especifica del hospedador a patógenos intestinales, facilitando la exclusión de los mismos (Perdigón *et al.*, 1995; Hao y Lee, 2004) debido a su adhesión en las áreas del intestino que están disponibles (Brassart y Schiffrin, 1997).
- Ciertas especies de *Lactobacillus, Lactococcus* y *Bifidobacterium* inducen *in vitro* la liberación de las citoquinas proinflamatorias como el TFN-α, y la IL-6 e IL-10, lo que refleja la estimulación de la inmunidad inespecífica (Miettinen *et al.*, 1996).
- *Lactobacillus casei y Lb. bulgaricus* activan la producción de macrófagos (Perdigon *et al.*, 1995).
- *Lactobacillus casei y Lb. acidophilus* activan la fagocitosis de las células inmunitarias en ratones (Perdigon *et al.*, 1995).
- *Lactobacillus rhamnosus GG* incrementa la secreción de anticuerpos del tipo IgA en la fase diarreica de niños infectados con rotavirus (Kaila *et al.*, 1992).
- *Bifidobaterium bifidum* mejora la respuesta inmune frente la ovalbumina y *Bifidobacterium breve* estimula la producción de IgA en ratones infectados de cólera (Moreau *et al.*, 1990).

Existen estudios en los que se ha demostrado que algunos probióticos son capaces de producir metabolitos que modifican directamente la permeabilidad del epitelio intestinal, lo que refuerza la integridad de la barrera epitelial (Madsen *et al.*, 2001).

La fracción de las BAL presentes en el tracto gastrointestinal es baja (< 1% en la mayoría de los individuos), sin embargo las proporciones más altas se encuentran en las cavidades oral y vaginal (Venema y Meijerink, 2015). La microbiota vaginal de una mujer premenstrual está formada principalmente por miembros del filo *Firmicutes*, de ellos entre 10^7-10^8 UFC/g pertenecen al género *Lactobacillus*, esta microbiota protege de infecciones del tracto urogenital (Lepargneur y Rousseau, 2002; Reid y Burton, 2002; Vásquez *et al.*, 2002). En la vagina existen numerosas especies de lactobacilos como *Lb. acidophilus, Lb. fermentum, Lb. casei, Lb. jensenii* (Sobel, 1999), *Lb. brevis, Lb. salivarius* subsp. *salicinius, Lb. gasseri* (Massi *et al.*, 2004), *Lb. crispatus* y *Lb. iners* (Burton *et al.*, 2002). Por otro lado, existen bacterias exógenas, capaces de actuar como probióticos tal es el caso de *Lb. reuteri* MM53 (Ruiz- Palacios *et al.*, 1996), *Lb. acidophilus* NCFM (Reid *et al.*, 1993), *Lb. fermentum* RC-14 (Reid *et al.*, 1987) y *Lb. rhamnosus* GR-1 (Reid *et al.*, 1999) capaces de adherirse a las células urogenitales donde ejercen un efecto beneficioso. Existen numerosos estudios que demuestran que la adherencia de las bacterias probióticas al epitelio vaginal inhibe el crecimiento de otro tipo de microorganismos perniciosos para la salud (Boris *et al.*, 1998; Reid *et al.*, 1990; Zhou *et al.*, 2004) incluyendo bacterias causantes de vaginosis, levaduras causantes de vaginitis (Burton *et al.*, 2002). Estas bacterias también previenen enfermedades de transmisión sexual como el VIH (Burton *et al.*, 2002) y algunas enfermedades causadas por otros microorganismos (McGroarty, 1993; Massi *et al.*, 2003). En este ecosistema, los lactobacilos juegan un papel muy importante acidificando el pH del medio aproximadamente a 4-4.5 (mediante la liberación del ácido láctico y ácidos grasos de cadena corta), liberando peróxido de hidrógeno, bacteriocinas y posiblemente biosurfactantes (Boris y Barbés, 2000). Todo ello inhibe la proliferación de microorganismos patógenos (Reid *et al.*, 2004) como *Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus*, *Streptococcus agalactiae* (Chan *et al.* 1985; Boris *et al.* 1998; Reid, 2000; Osset *et al.* 2001; Mastromarino *et al.* 2002; Zárate y Nader-Macias, 2006), *Candida albicans, E. coli, Gardnerella vaginalis* o *Mobiluncus* spp. por el efecto del bajo pH; *Candida albicans, Gardnerella vaginalis* como consecuencia del H_2O_2 y *Neisseria gonorrhoeae* debido a la acción combinada del pH y el H_2O_2 (Boris y Barbés, 2000), de igual modo desplazan a los microorganismos patógenos mediante la exclusión competitiva por la superficie celular a la que se adhieren o formando biopelículas (Dunne *et al.*, 2001). La formación de biopelículas beneficia a las propias bacterias comensales al favorecer el acceso a nutrientes, escapar de las células inmunitarias del huésped, protegerse de los antimicrobianos e incrementar su supervivencia (Reid, 2001). Desde 1992, el término "probiótico" se usa también para definir las preparaciones intra-vaginales de microorganismos vivos cuyo fin es prevenir las infecciones y restaurar el balance de la microbiota vaginal (McGroarty, 1993).

La microbiota de la cavidad oral es compleja y contiene más de 100 especies bacterianas (Kuramitsu *et al.*, 2007; Keijser *et al.*, 2008). Los lactobacilos (*Lb.*

plantarum, Lb. pentosus, Lb. rhamnosus) presentes en la cavidad oral exhiben efectos beneficiosos para la salud bucal ya que inhiben las bacterias causantes de caries tales como *Streptococcus mutans* (Simark-Mattsson *et al.*, 2007; Kayahan *et al.*, 2014).

1.2.2.2. BAL probióticos de origen alimentario

Los alimentos fermentados son una fuente importante de probióticos, por ejemplo las masas fermentadas, los productos lácteos y los vegetales fermentados. Estos alimentos han sido investigados como fuentes alternativas de nuevas cepas probióticas (Cammarota *et al.*, 2009; Chang *et al.*, 2010). Los lácteos (leches fermentadas, quesos y leche en polvo para bebés) son los productos ampliamente usados como vehículos de probióticos siendo las BAL probióticos frecuentemente encontrados son: *Lb. acidophilus, Lb. gasseri, Lb. helveticus, Lb. johnsonii, Lb. casi, Lb. paracasei, Lb. plantarum, Lb. reuteri, Lb. fermentum y Lb. rhamnosus* (Tamine *et al.*, 2005).

En los últimos años, diferentes estudios se enfocaron sobre la evaluación de la probiosis de las BAL presentes en los materiales crudos de fruta y vegetales (las BAL oscilan entre 2.0 y 5.0 Log_{10} UFC/g) (Spurr, 1994, Di Cagno *et al.*, 2008; Di Cagno *et al.*, 2010a) y también de vegetales fermentados (Swain *et al.*, 2014). Los vegetales y fruta poseen parámetros intrínsecos físicos y químicos que pueden mimetizar las condiciones del tracto gastro-intestinal más que otros ecosistemas alimentarios.

2. Las aceitunas de la variedad "Aloreña" como fuente de probióticos

2.1. Fermentación de la aceituna Aloreña

Durante muchos siglos, la cultura mediterránea se ha centrado en la extracción del aceite de oliva y la elaboración de aceituna de mesa. El cultivo del olivo ha sido la práctica agrícola más importante en varios países mediterráneos tales como España, Italia y Grecia. La producción de aceitunas verdes de mesa a nivel mundial es cerca de 1.700.000 toneladas/año. La mayoría de las aceitunas de mesa pertenecen a los tipos denominados verdes al estilo Español o Sevillano, negras naturales en salmuera (estilo Griego), y las aceitunas verdes al estilo tradicional o casero (IOOC, 2005). En la actualidad numerosas industrias elaboran las aceitunas de mesa según el mismo protocolo (conocido como “estilo español o Sevillano”) transformando la aceituna verde amarga en un producto fermentado estabilizado. Este método incluye una primera etapa de tratamiento con sosa para hidrolizar el glucósido denominado oleuropeína responsable del sabor amargo del fruto. Seguidamente, las aceitunas se sumergen en una salmuera y se dejan fermentar durante un periodo variable de varios meses (1 a 7) (Garrido Fernández *et al.*, 1997), tras el cual las aceitunas se empaquetan en una nueva salmuera acidificada y se ponen en venta.

La aceituna de mesa Manzanilla Aloreña es un producto típico de la región de Guadalhorce en Málaga con Denominación de Origen Protegida (DOP). Esta variedad de aceitunas tiene unas características peculiares que están ligadas al área (clima, pedología y geografía) que las hace diferentes de otras aceitunas verdes de mesa. Además de su color verde-amarillo, su excelente calidad, su buen cociente hueso-pulpa, su sabor y su bajo contenido en oleuropeína (glucósido amargo principal de aceitunas) permiten que se pueda prescindir de la etapa de endulzado por NaOH, siendo eliminada la oleuropeína residual mediante lavados con agua. Las aceitunas de mesa Manzanilla Aloreña se sazonan con frecuencia con hinojo, tomillo, ajo y pimienta, lo que les da más sabor y aroma. A pesar de la importancia de este tipo de aceituna, pocos estudios han sido llevados a cabo para determinar los microorganismos implicados en esta fermentación. Los procesos de elaboración de las aceitunas de mesa Manzanilla Aloreña se llevan a cabo por unas empresas pequeñas y medianas siguiendo unos procedimientos similares y realizando el proceso de fermentación en frío, en patio o en el fermentador. En la actualidad existen varios problemas sin resolver por falta de conocimientos científicos y tecnológicos adecuados sobre la microbiota presente en estas aceitunas. Después de la cosecha, las aceitunas son almacenadas en salmueras en frío o a temperatura ambiente. Las aceitunas almacenadas bajo refrigeración experimentan una fermentación lenta, dando como resultado un alto contenido en azúcares residuales que contribuyen a la formación de gas durante su comercialización con el consiguiente ablandamiento y oscurecimiento del fruto y abombamiento de los envases (Abriouel *et al.*, 2012).

2.2. Aspectos microbiológicos de la aceituna Aloreña

En general, la fermentación de las aceitunas verdes de mesa es un proceso natural y espontáneo que resulta de las actividades competitivas de la microbiota nativa (bacterias lácticas y levaduras), junto con una variedad de microorganismos contaminantes de diferentes fuentes (Garrido-Fernández *et al.*, 1997). Mediante métodos microbiológicos clásicos se ha demostrado que las bacterias del ácido láctico, especialmente *Lb. plantarum* y *Lb. pentosus* son las principales responsables de esta fermentación (Garrido Fernández *et al.*, 1997; Chammem *et al.*, 2005; Abriouel *et al.*, 2012), si bien las levaduras pueden estar también presentes dependiendo de las condiciones ambientales (Ruiz Barba *et al.*, 1994; Leal-Sánchez *et al.*, 2003; Hurtado *et al.*, 2012). Aunque también se detectaron otros géneros, como *Enterococcus, Pediococcus, Leuconostoc* y *Lactococcus* (Borcakli *et al.*, 1993; Ercolini et al, 2006; Floriano et al, 1998). A veces no se produce la cantidad suficiente de ácido láctico durante la fermentación con el consiguiente riesgo de alteración del producto (Fernández Díez, 1983; Garrido Fernández *et al.*, 1995), lo que ha llevado a investigar el desarrollo de inóculos específicos para mejorar la fermentación. Entre las características deseadas de los inóculos está su capacidad para hidrolizar la oleuropeína, su capacidad de implantación y de producir bacteriocinas (Jiménez-Díaz *et al.*, 1993; Ruiz Barba *et al.*, 1994; Leal-Sánchez *et al.*, 2003; Maldonado *et al.*, 2003, 2004), capacidad de crecimiento en las salmueras (Durán *et al.*, 1993) y crecimiento a baja temperatura (Durán Quintana *et al.*, 1999).

En cuanto a las aceitunas Aloreña, estudios previos llevados a cabo sobre la diversidad microbiana y los aspectos microbiológicos de la fermentación natural de dichas aceitunas (Abriouel, 2011a, 2012) han permitido determinar el perfil microbiano presente a lo largo de la fermentación (Abriouel *et al.*, 2011a). Los métodos dependientes de cultivo mediante recuentos en placas permitieron determinar la presencia de mesófilos totales, levaduras y mohos, y las BAL que experimentaron cambios notorios entre las diferentes formas de fermentación (en frío, en patio o en el fermentador) y también entre una empresa y otra. En paralelo, los procedimientos independientes de cultivo usando la técnica PCR-DGGE revelaron la predominancia de las levaduras en las aceitunas fermentadas en frío, mientras que las BAL fueron predominantes en las aceitunas fermentadas en patio o en el fermentador a temperatura ambiente. Los análisis mediante PCR-DGGE determinaron la presencia de *Gordonia* sp./*Pseudomonas* sp. y *Sphingomonas* sp./*Sphingobium* sp./*Sphingopyxis* sp. junto con arqueas halófilas (principalmente haloarchaeon/*Halosarcina pallida* y uncultured archaeon/uncultured haloarchaeon/*Halorubrum orientalis*) y levaduras (*Saccharomyces cerevisiae* y *Candida* cf. *apicola*) en las aceitunas fermentadas en frío. En cuanto a las aceitunas fermentadas en patio, la población microbiana fue más diversa siendo *Gordonia* sp./*Pseudomonas* sp., *Sphingomonas* sp./*Sphingobium* sp./*Sphingopyxis* sp. y *Thalassomonas agarivorans* junto con arqueas halófilas y levaduras (principalmente *S. cerevisiae* y *C.* cf. *apicola*, y también *Pichia* sp., y *Pichia manshurica*/*Pichia galeiformis*) las más destacadas. También, algunas BAL fueron detectadas al final de la fermentación de las aceitunas fermentadas en patio tales como *Lactobacillus pentosus*/*Lb. plantarum* y *Lb. vaccinostercus*/*Lb. suebicus.* En cambio, solamente el fermentador mostró una clara predominancia de las BAL desde el inicio hasta el final de la fermentación (*Lactobacillus* sp., *Lb. paracollinoides* y *Pediococcus* sp.) junto con algunas arqueas halófilas y una población más selectiva de levaduras (*P. manshurica*/*P. galeiformis*). Los estudios llevados a cabo por Abriouel *et al.* (2011a) mostraron la complejidad de las poblaciones microbianas presentes en la fermentación natural de la aceituna Aloreña.

Las BAL responsables de la fermentación de la aceituna Aloreña han sido aisladas y caracterizadas a nivel molecular por Abriouel *et al.* (2012). Una colección de 144 cepas de BAL incluyendo lactobacilos (81.94%), leuconostocs (10.42%) y pediococos (7.64%) fue tipificada e identificada usando la técnica REP-PCR y la secuenciación de los genes *phes* y *rpo* (Figuras 3-5). Los resultados demostraron que todos los lactobacilos fueron identificados como *Lb. pentosus*, los pediococos como *Pediococcus parvulus* y los leuconostocs como *Leuconostoc pseudomesenteroides*. El genotipado de las BAL reveló que las cepas no fueron clonalmente relacionadas y exhibieron un grado considerable de diversidad genómica especialmente los lactobacilos y también los leuconostocs. Algunas cepas exhibieron propiedades tecnológicas de interés tales como la producción de sustancias antimicrobianas activas frente a bacterias patógenas (*Listeria monocytogenes, Bacillus cereus, Staphylococcus aureus, Streptococcus mutans* y *Salmonella entérica*), degradación de azúcares complejos (rafinosa y estaquisa), producción de sales biliares, actividad fitasa y catalasa hemo-dependiente, crecimiento a 10°C y en presencia de 6.5% NaCl, buena capacidad acidificante y resistencia a la congelación. Sin embargo, ninguna de las cepas aisladas mostró actividad proteasa ni amilasa, además dichas cepas no produjeron aminas biógenas. De acuerdo con los datos obtenidos, algunas cepas de las BAL aisladas de la fermentación natural de la aceituna Aloreña pueden tener un gran potencial como probióticos y haciendo de la propia aceituna fermentada como un excelente producto probiótico.

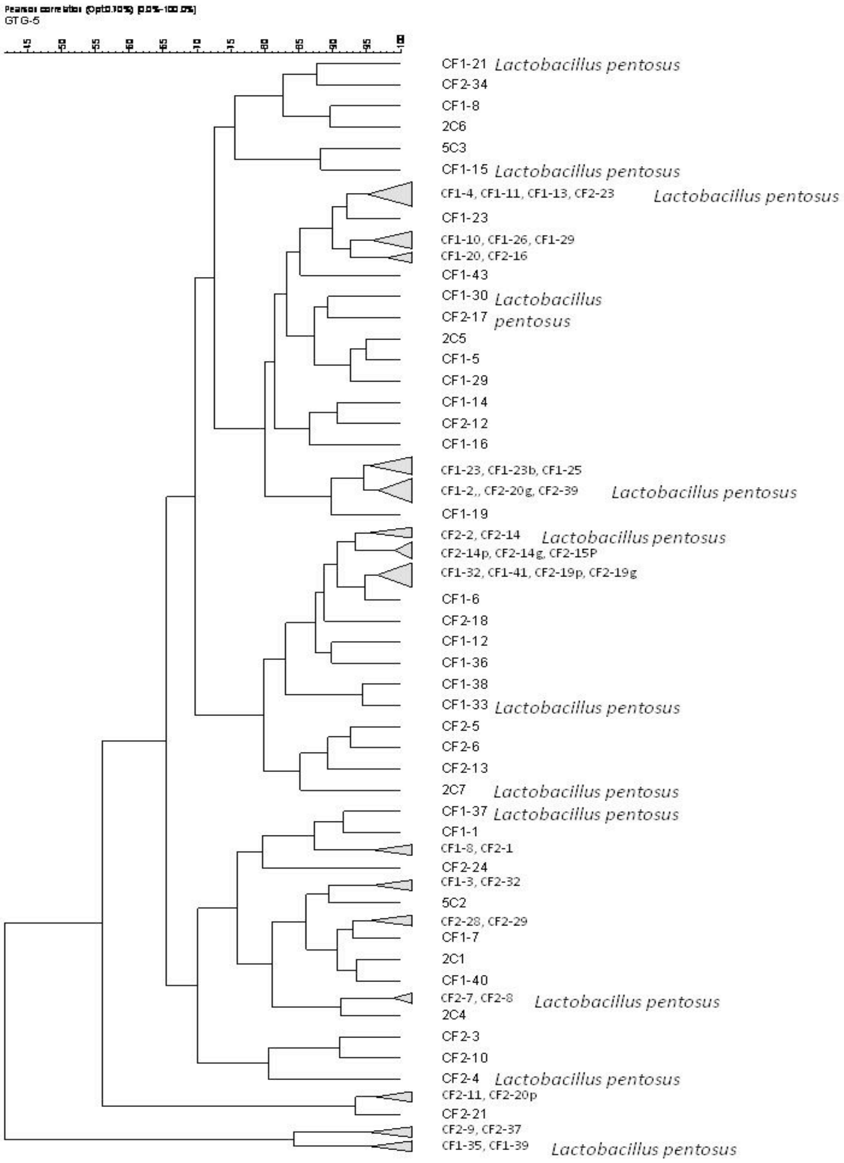


Figura 3. Dendrograma obtenido mediante el tipado de cepas de *Lactobacillus* sp. aisladas de fermentaciones de las aceitunas verdes de mesa Aloreña. Los patrones se agruparon mediante el método UPGMA (Abriouel *et al.*, 2012).

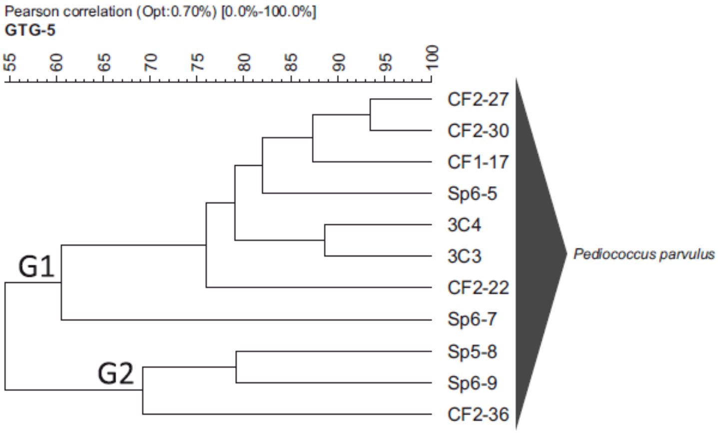


Figura 4. Dendrograma obtenido mediante el tipado de cepas de *Pediococcus parvulus* aisladas de fermentaciones de las aceituna verdes de mesa Aloreña. Los patrones se agruparon mediante el método UPGMA (Abriouel *et al.*, 2012).

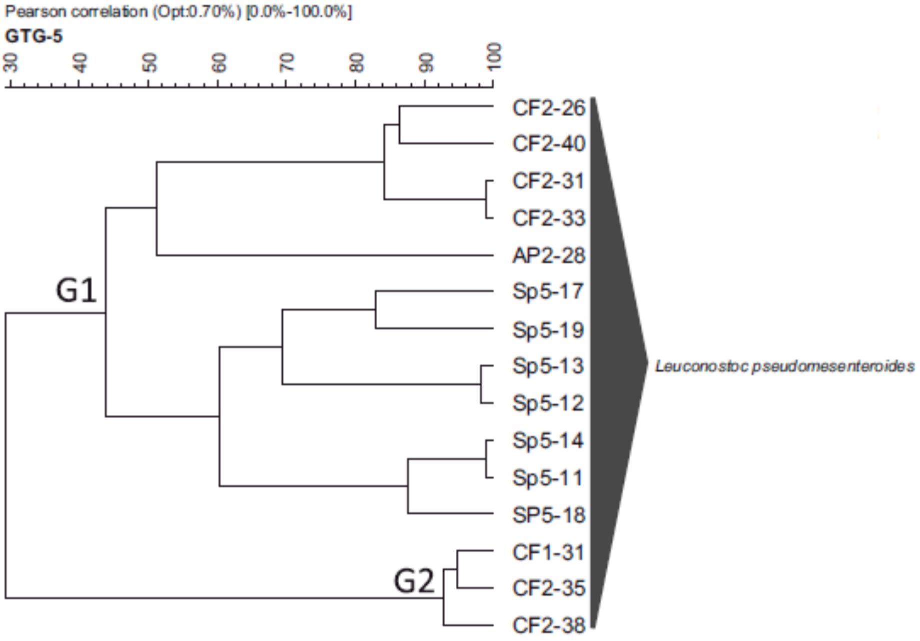


Figura 5. Dendrograma obtenido obtenido mediante el tipado de cepas de *Leuconostoc pseudomesenteroides* aisladas de fermentaciones de las aceituna verdes de mesa Aloreña. Los patrones se agruparon mediante el método UPGMA (Abriouel *et al.*, 2012).

3. Aspectos de seguridad de los probióticos

El análisis de seguridad de las bacterias destinadas para ser usadas como probióticos debe tener en cuenta el tipo de microorganismo usado, el método de administración, el nivel de exposición, el estado de salud de los usuarios y las funciones fisiológicas a desempeñar. En este sentido, pocos efectos adversos han sido descritos a consecuencia de la ingestión de probióticos salvo en el caso de pacientes con condiciones médicas reservadas. Sin embargo, se desconocen cómo se han desarrollado algunas infecciones por cepas de lactobacilos patógenas oportunistas ya que los microorganismos probióticos no son patógenos y se hace difícil identificar aquellas propiedades relacionadas con riesgos para la salud (Sanders *et al.*, 2010). Bernardeau *et al.* (2006) ha calculado el riesgo de las infecciones por *Lactobacillus* en personas sanas, el cual afectó aproximadamente a una persona/10 millones de personas a lo largo de un siglo de consumo de probióticos en Francia. Además, el riesgo de lactobacilemia fue considerado insignificante con menos de un caso por millón de individuos (revisado por Sanders *et al.*, 2010). Estudios retrospectivos han demostrado que el consumo incrementado de un probiótico "*Lactobacillus rhamnosus* GG" en Finlandia no aumento la incidencia de los casos de bacteremia por *Lactobacillus* sp. Igualmente, estudios clínicos llevados a cabo sobre individuos inmunocomprometidos (por ejemplo enfermos del VIH), niños prematuros y pacientes con la enfermedad de Crohn administrados con probióticos no mostraron ningún efecto negativo. Esto demuestra claramente la baja patogenicidad oportunista de los lactobacilos probióticos.

La seguridad de los probióticos ha sido un tema de gran interés especialmente en la Comunidad Europea y esto se refleja en las numerosas publicaciones, opiniones y metodologías sobre cómo determinar dicha seguridad. La Autoridad Europea de Seguridad Alimentaria (European Food Safety Authority, EFSA) fue establecida en 2002 para tratar los aspectos técnicos y científicos de los alimentos y alimentación animal (Regulation No. 178/2002). Sin embargo, los criterios para aprobar un nuevo probiótico quedaron ambiguos. Para ello, el reglamento de la Unión Europea sobre nuevos alimentos (European Union Novel Food regulation, 258/1997 EEC) proporciona un marco regulatorio para los probióticos destinados a su uso en alimentación animal, siendo no regulados los probióticos para humanos. En este sentido, el Comité Científico de la Unión Europea sobre Nutrición Animal (European Union Scientific Committee on Animal Nutrition, 2002) ha propuesto un procedimiento de seguridad denominado "Presunción Cualificada de Seguridad" (Qualified Presumption of Safety, QPS) (EFSA, 2008). La evaluación QPS de una cepa incluye 4 etapas (Sanders *et al.*, 2010): 1) taxonomía de la cepa; 2) proporción de informaciones suficientes sobre la QPS de la cepa; 3) ausencia de patogenicidad y 4) definición de su uso (Figura 6) (EFSA, 2005). Cuando la cepa en cuestión no exhibe ningún riesgo se puede garantizar su seguridad y su estado QPS. En caso contrario, un análisis de seguridad completo es imprescindible para garantizar la seguridad de la cepa. En general, las BAL probióticos gozan de un estatus QPS, aunque hay que tener en cuenta los aspectos de seguridad relacionados con la adquisición de resistencia a antibióticos y determinantes de virulencia. A raíz de un proyecto Europeo *PROSAFE* (Vankerckhoven *et al.*, 2007, 2008) "Evaluación de la Bioseguridad de las Bacterias del Ácido Láctico Probióticas para consumo Humano", diferentes recomendaciones fueron establecidas:

(1) Estudios moleculares para la identificación de la cepa tales como la secuenciación del ADN ribosómico.

(2) Exclusión de los microorganismos que no pertenecen a la distribución de los niveles de resistencia a los antimicrobianos más relevantes que la cepa salvaje.

(3) Ausencia de determinantes de virulencia en las BAL probióticas excepto aquellas propiedades relacionadas con la supervivencia (desconjugación de los ácidos biliares, adhesión a las líneas celulares y proteínas extracelulares).

(4) Además de las Directrices Europeas, estudios sobre la colonización del cuerpo humano por los probióticos deben ser realizadas.

(5) El uso potencial de modelos animales tales como ratones para evaluar la seguridad de las cepas.

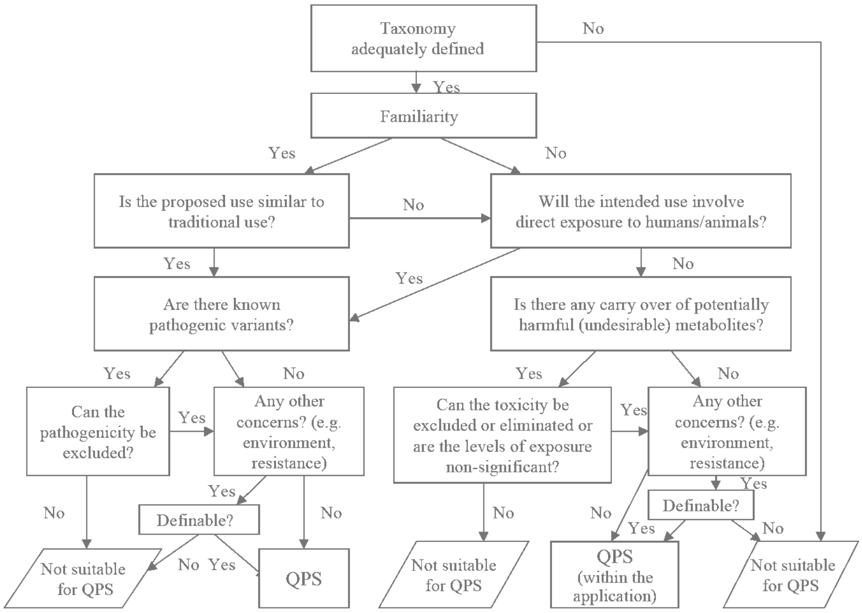


Figura 6. Esquema general para la evaluación de la idoneidad para el estatus QPS de microorganismos (EFSA, 2005).

En 2007, otro proyecto Europeo (ACE-ART) sobre la "Determinación y Evaluación Crítica de la transferibilidad de la resistencia a los antibióticos en la cadena alimentaria" ha determinado que las BAL probióticas pueden actuar como reservorios de genes de resistencia a antibióticos que se pueden transferir a lo largo de la cadena alimentaria.

3.1. Antimicrobianos usados en la industria alimentaria

Un agente antimicrobiano es aquella sustancia producida por microorganismos o sintetizada químicamente que a bajas concentraciones es capaz de inhibir e, incluso, destruir microorganismos sin producir efectos tóxicos en el huésped (Gómez-Lus *et al.*, 2005).

La resistencia de las bacterias a los antimicrobianos es un hecho que se ha incrementado gravemente en los últimos tiempos y resulta un problema potencial para la salud pública. Por lo tanto, es necesario saber que bacterias son consideradas resistentes, para así establecer si esta resistencia pudiese ser transferida a otros microorganismos patógenos y oportunistas (Blázquez *et al.*, 2002).

La cadena alimentaria ha sido reconocida como una de las rutas de transmisión de resistencia a los antibióticos (Teuber *et al.*, 1999; Witte, 2000). Más concretamente, los productos fermentados tradicionales que no han sido sometidos a procesos térmicos antes del consumo y así proporcionan el principal vehículo de resistencias a la microbiota del tracto gastrointestinal humano, donde tiene lugar la transferencia e intercambio de genes de resistencia entre la microbiota intestinal y la microbiota ingerida con los alimentos (Bates *et al.*, 1994; Nikolich *et al.*, 1994). En esta memoria, se describen dos tipos de antimicrobianos de importancia en la industria alimentaria: biocidas y antibióticos.

3.1.1. Biocidas

La legislación europea (Reglamento nº 528/ 2012 del Parlamento europeo y del Consejo de 22 de mayo de 2012, y sucesivas modificaciones) define a los biocidas como:

- Toda sustancia o mezcla, en la forma en que se suministra al usuario, que esté compuesta por, o genere una o más sustancias activas, con la finalidad de destruir, contrarrestar o neutralizar cualquier organismo nocivo, o de impedir su acción o ejercer sobre él un efecto de control de otro tipo, por cualquier medio que no sea una mera acción física o mecánica.
- Toda sustancia o mezcla generada a partir de sustancias o mezclas distintas de las contempladas en el anterior guión, destinadas a ser utilizada con la intención de destruir, contrarrestar o neutralizar cualquier organismo nocivo, o de impedir su acción o ejercer sobre él un efecto de control de otro tipo, por cualquier medio que no sea una acción física o mecánica.

En la industria alimentaria, los biocidas como desinfectantes o como conservantes de alimentos son ampliamente usados para la desinfección de las plantas de producción y de recipientes de alimentos, para la descontaminación de las canales y para el control del crecimiento microbiano en los alimentos y bebidas.

3.1.1.1. Clasificación de los biocidas

Los biocidas requieren aprobación antes de su lanzamiento al mercado, no obstante, al contrario que los antibióticos, cuyo uso se controla minuciosamente, los biocidas se pueden utilizar sin ningún tipo de control. La cantidad total de biocidas producidos y utilizados en la Unión Europea todavía se desconoce, aunque se cree que sea considerablemente mayor que la producción total de antibióticos (Sanidad y Consumidores de la Comisión Europea, 2009).

El número de biocidas conocidos es bastante mayor, pero en general se pueden clasificar de dos formas, según su mecanismo de acción y según su uso. En esta memoria nos vamos a referir únicamente a la primera forma. Según su mecanismo de acción existen dos tipos principales de biocidas: biocidas oxidantes y no oxidantes.

Los **biocidas oxidantes**, tal y como indica su nombre, oxidan químicamente la materia orgánica (material celular, enzimas, proteínas, etc) ya que penetran la pared celular y alteran el metabolismo, teniendo como consecuencia la destrucción de la membrana citoplasmática. Esto se debe a la desnaturalización de las proteínas estructurales como enzimáticas lo que provoca finalmente la muerte de los microorganismos (Diéguez, 2006). En este grupo podemos encontrar el cloro, bromo, yodo, dióxido de cloro, ozono, peróxido de hidrógeno, 6P-oxonia, algunas sales halógenas y de peróxido. En el caso del cloro y sus derivados, normalmente se forman enlaces estables entre el nitrógeno de las proteínas y el cloro, lo cual conduce a la muerte de los microorganismos. Su actividad desinfectante tiene una dependencia del pH y la presencia de grandes cantidades de materia orgánica y de amoníaco en el agua.

Los **biocidas no oxidantes**, no tienen una relación simple entre su estructura química y su mecanismo de acción (Diéguez, 2006), si no que usan diferentes mecanismos en su acción biocida. Generalmente, su efecto se produce mediante el envenenamiento lento de los microorganismos, alterando su metabolismo celular y/o su estructura y provocando así la muerte de los microorganismos. Aunque existen distintos tipos, en general todos los biocidas no oxidantes cumplen los siguientes requisitos: son más estables y persistentes que los biocidas oxidantes y su actividad es independiente del pH siendo el mecanismo de acción propio para cada uno de este tipo de biocidas. Los biocidas no oxidantes se dividen en las siguientes clases:

- Compuestos de amonio cuaternario: son biocidas catiónicos. Sus principios activos contienen como estructura básica al ión amonio NH_4^+, donde cada uno de los hidrógenos está sustituido generalmente por radicales de tipo alquil y aril (Sánchez-Saldaña y Saenz-Anduaga, 2005). Estos compuestos a concentraciones extremadamente bajas son particularmente activos frente a bacterias Gram-positivas. Su efecto sobre las bacterias Gram-negativas es menor requiriendo así concentraciones más elevadas.

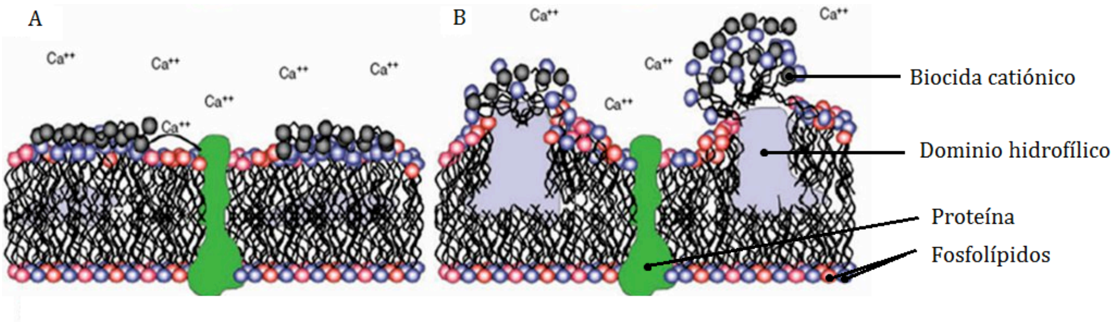


Figura 7. Mecanismo de acción de los biocidas catiónicos. **A.** Primeros estadios de actuación en los que el biocida se adhiere a la pared celular. **B.** Formación de dominios hidrofílicos que disgregan la pared celular (Adaptado de Gilbert y Moore, 2005).

A nivel molecular, su acción antimicrobiana se atribuye a la asociación del amonio (cargado positivamente) con los grupos polares de los fosfolípidos de la membrana, mientras que la cola hidrófoba se integra en el núcleo hidrófobo de la membrana (Figura 7). Estas interacciones conducen a la muerte de la célula debido a la desnaturalización de proteínas y pérdida de la permeabilidad de la membrana celular (López *et al.*, 2010; Sánchez-Saldoña y Saenz-Anduaga, 2005). Los principales compuestos de amonio cuaternario son:

- Cloruro de benzalconio (cloruro de alkilbenzildimetilamonio): con alta efectividad como bacteriostático y bactericida frente a bacterias Gram-positivas. Carece de actividad frente a bacterias Gram-negativas (Nichols, 2003). No obstante, también posee actividad virucida y fungicida (Sánchez-Saldoña y Saenz-Anduaga, 2005).
- Cetrimida (bromuro de hexadeciltrimetilamonio): actúa como agente inhibidor, liberando el nitrógeno y el fósforo de las células. Posee un amplio espectro de

acción actuando frente a bacterias Gram-positivas y Gram-negativas, aunque estas últimas son menos sensibles.

- N-hexadecilpiridinio cloruro (CPC): esta molécula posee actividad bactericida y bacteriostática, contra bacterias Gram-positivas y Gram-negativas. Se piensa que actúa a nivel de la membrana plasmática donde ocurre una atracción entre la carga positiva de la molécula y la carga negativa de los fosfolípidos que constituyen la membrana plasmática bacteriana. Una vez que la molécula se une a la membrana, el extremo no polar del CPC penetra y altera la membrana celular. Esta alteración afecta la regulación osmótica y ocasiona la pérdida del material citoplasmático y posteriormente la muerte celular (López *et al.*, 2010).

- <u>Bis-fenoles:</u> en general, exhiben un amplio espectro de acción, pero tienen poca actividad frente a *Pseudomonas aeruginosa* y mohos (Sánchez-Saldoña y Saenz-Anduaga, 2005). Entre ellos destacan:
  - Triclosan o Irgasan (2,4,4, tricloro-2-hidroxidifenil éter): su mecanismo de acción se basa en la disrupción de la membrana bacteriana a través del bloqueo de la síntesis de lípidos. El triclosán bloquea el sitio activo de la enzima reductasa transportadora de enoil-acil (McMurray *et al.*, 1998; Levy *et al.*, 1999). No obstante, actúa también sobre la síntesis de ácidos nucleicos y proteínas (Sánchez-Saldoña y Saenz-Anduaga, 2005). El triclosán es efectivo frente a algunas bacterias Gram-positivas y la mayoría de las bacterias Gram-negativas, incluyendo las bacterias multirresistentes (Savaje, 1971; Vischer; 1973).
  - Hexaclorofeno (2, 2'-dihidroxi -3,5,6,3',5',6'-hexacloro–difenilmetano): debido a su naturaleza química penetra a través de las membranas celulares de las bacterias, combinándose con las proteínas protoplasmáticas que desnaturaliza y precipita. El hexaclorofeno tiene actividad frente a numerosas bacterias Gram-positivas (Sánchez-Saldoña y Saenz-Anduaga, 2005).

- <u>Biguanidas:</u> Poseen un amplio espectro de actividad antibacteriana, pero su acción como fungicida y virucida es muy limitada. Este biocida interacciona con las cargas negativas de las membranas celulares y altera su permeabilidad

(Quinn *et al.*, 2001; Jeffrey, 1995). La biguanida más representativa es la clorhexidina (1,1′-Hexametilenebis[5-(4-clorofenil)biguanida]). La clorhexidina actúa sobre los fosfolípidos de la membrana citoplasmática, modificando su permeabilidad (Fitzgerald, 1989). Su efecto varía dependiendo de la concentración, a bajas concentraciones altera la permeabilidad osmótica de la membrana e inhibe enzimas del espacio periplasmático. Sin embargo, a concentraciones elevadas origina la precipitación de las proteínas y los ácidos nucleicos (Sánchez-Saldoña y Saenz-Anduaga, 2005). Posee un amplio espectro de acción frente a bacterias Gram-positivas y Gram-negativas (Perapoch *et al.*, 1993).

3.1.1.2. Principales usos de los biocidas

El uso de biocidas, de forma aislada o combinada, es muy diverso. Se utiliza a nivel doméstico, en instalaciones sanitarias y en la industria.

Uso doméstico: se usan para la higiene personal en pastas de dientes, cremas y jabones. También se utilizan para la limpieza y desinfección de superficies (Maillard, 2005).

Instalaciones sanitarias: con el fin de descontaminar superficies, fómites y la piel de los pacientes y personal sanitario. Así como en el tratamiento de infecciones de heridas superficiales.

Industria alimentaria: cuyo principal papel es el de la desinfección de instalaciones y cualquier material que entre en contacto con los alimentos, así como para descontaminar productos cárnicos. También se añaden como conservantes a los productos alimentarios y como desinfectantes al agua potable.

Ganadería: descontaminación de animales, sus derivados y cualquier recinto y material utilizados.

Plantas de tratamiento de agua: se añaden estos compuestos al agua, para evitar la liberación de organismos dañinos al medio ambiente.

Otras aplicaciones industriales cada vez más comunes incluyen la desinfección de torres de refrigeración para evitar la propagación de *Legionella* u otro tipo de bacterias y hongos (Sanidad y consumidores de la Comisión Europea, 2009).

3.1.1.3. Eficacia de los biocidas en la industria alimentaria

En la industria alimentaria, los consumidores demandan en un alimento, que sea saludable, nutritivo y que esté mínimamente procesado con aditivos como conservantes químicos y otros agentes antimicrobianos. No obstante, se ha extendido el uso de biocidas y desinfectantes químicos para garantizar la inocuidad de los alimentos.

Los biocidas juegan un papel esencial en la limitación del desarrollo de enfermedades e infecciones. Estos agentes evitan el crecimiento de microorganismos patógenos y el efecto de sus toxinas a lo largo de la cadena alimentaria en toda una diversidad de soportes, como recipientes, aparatos de agitación, mezcla y elaboración, y también en los envases usados para almacenar los alimentos u incluso en la matriz alimentaria cuando se usan como conservantes. Por todo esto, es de vital importancia el uso de los biocidas para prevenir pérdidas en procesos alimentarios industriales y para proteger la salud del consumidor. En este sentido, el uso de estos agentes se ha incrementado especialmente en el sector de la industria alimentaria, y debido a la emergencia de un gran número de bacterias capaces de tolerar concentraciones importantes de estos antimicrobianos, a la vez que aparecen resistencias cruzadas (detalladas posteriormente), lo cual representa un peligro potencial para la salud pública (Condell *et al.*, 2012).

3.1.2. Antibióticos

En 1928, Alexander Fleming descubrió el primer antibiótico, la penicilina, por el crecimiento en una placa de Petri de un hongo contaminante (*Penicillium notatum*) que inhibía el crecimiento bacteriano (Gómez-Lus *et al.*, 2008). La primera definición de antibiótico fue dada por Waksman y Chevalier en 1944, según ellos los antibióticos eran "sustancias químicas producidas por microorganismos que tienen la propiedad de inhibir o destruir otros microorganismos" (Clardy *et al.*, 2009).

Actualmente se entiende por antibiótico toda sustancia orgánica, sintética o natural, capaz de destruir o inhibir el crecimiento de bacterias generalmente a bajas concentraciones (Gómez-Lus *et al.*, 2008). Su mecanismo de acción se basa en la interferencia en algún punto del metabolismo donde se encuentre su diana.

No obstante, la eficacia de los antibióticos se ha visto alterada por su uso excesivo o incorrecto, que condujo a la aparición y diseminación de bacterias resistentes. Estas bacterias resistentes actúan impidiendo la entrada del antibiótico, modificando o inactivando el antimicrobiano, o bien modificando la diana, o activando los sistemas de exporte. La gran capacidad de las bacterias de mutar y transferir genes resistencia esencialmente presentes en plásmidos y transposones, contribuyen a su diseminación tanto entre bacterias emparentadas y/o patógenas como bacterias no patógenas que representan los reservorios más importantes de dichas resistencias. Las Figuras 8 y 9 muestran cuales son los antibióticos más usados y las dosis empleadas actualmente en los países europeos.

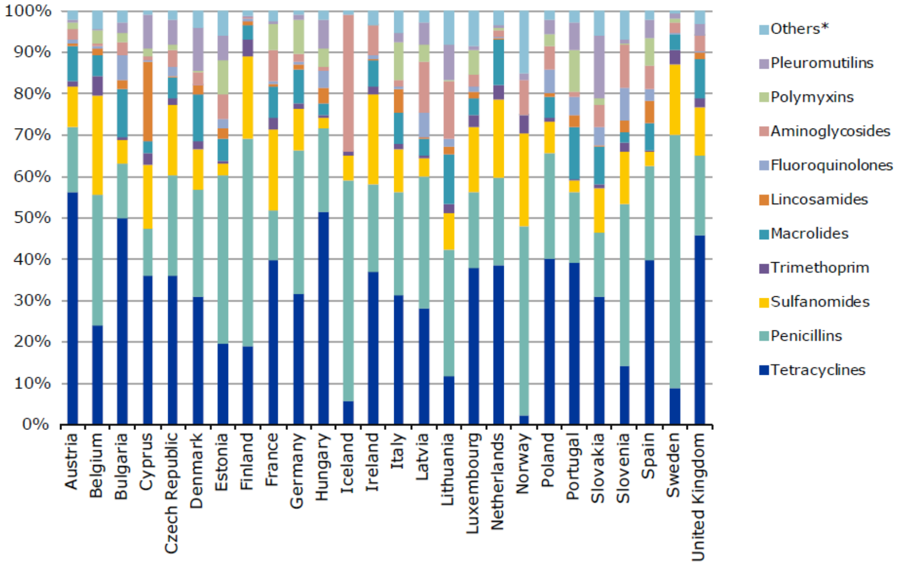


Figura 8. Porcentaje de las ventas totales de diferentes antibióticos para uso veterinario en diferentes países de Europa en mg/PCU (Population Correction Unit) en el año 2013 (European Medicines Agency, 2015).

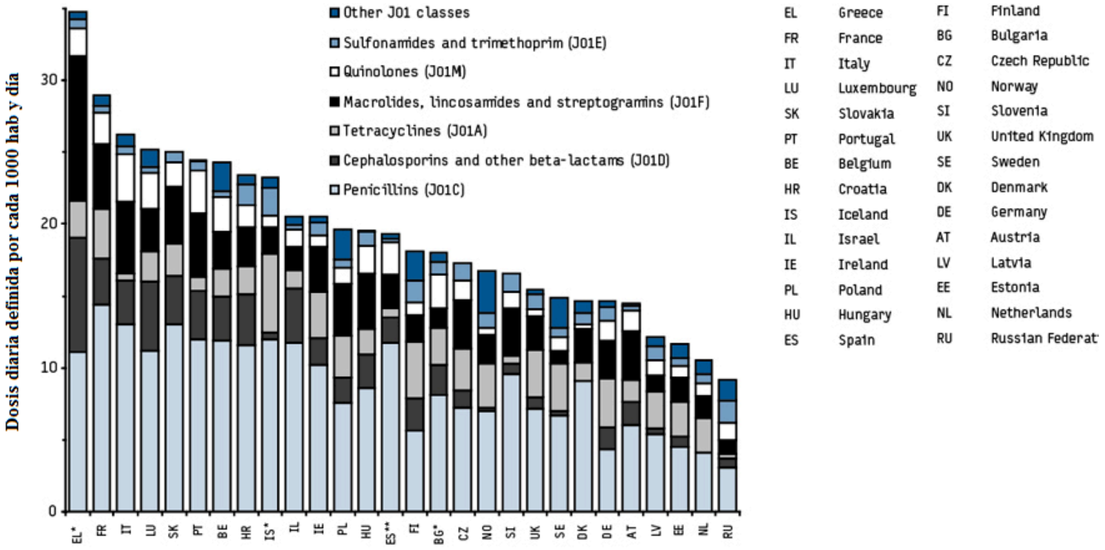


Figura 9. Dosis diaria definida de los principales antibióticos en Europa por cada 1000 habitantes (hab.) y día en 2005 (Modificado de Muller *et al.*, 2007).

3.1.2.1. Clasificación y mecanismos de acción de los antibióticos

Los criterios para la clasificación de los antibióticos son diversos, lo que da lugar a la agrupación según su estructura química (betalactámicos, aminoglucósidos, glicopéptidos, polipéptidos, rifampicinas, tetraciclinas, anfenicoles y macrólidos), según su espectro de acción (antibacterianos, antiviríCos, antifúngicos y antiprotozoarios), según su efecto antimicrobiano (bacteriostáticos, bactericidas y bacteriolíticos) o según su mecanismo de acción (inhibidores de síntesis de la pared celular, inhibidores de permeabilidad, inhibidores de la síntesis proteica, inhibidores de síntesis de ácidos nucleicos e inhibidores de rutas metabólicas) (Gómez-Lus *et al.*, 2008).

El efecto *bacteriostático* se produce si el antimicrobiano inhibe el crecimiento pero las células no mueren. Los agentes bacteriostáticos son inhibidores de la síntesis de proteínas y actúan uniéndose a los ribosomas. Dicha unión no es una unión fuerte y cuando disminuye la concentración del agente, el antimicrobiano se libera de los ribosomas y se reanuda el crecimiento.

Los agentes *bactericidas* matan las células bacterianas pero sin desencadenar lisis o rotura celular. Estos, generalmente se unen fuertemente a sus dianas celulares de acción y no se eliminan por dilución.

Por último, los agentes *bacteriolíticos* provocan la muerte celular por lisis. Dentro de los agentes bacteriolíticos se incluyen los antibióticos que inhiben la síntesis de la pared celular y los compuestos químicos que lesionan la membrana citoplasmática.

Así pues, los antibióticos y otros agentes quimioterapéuticos pueden agruparse según su estructura química o su mecanismo de acción (Figura 10).

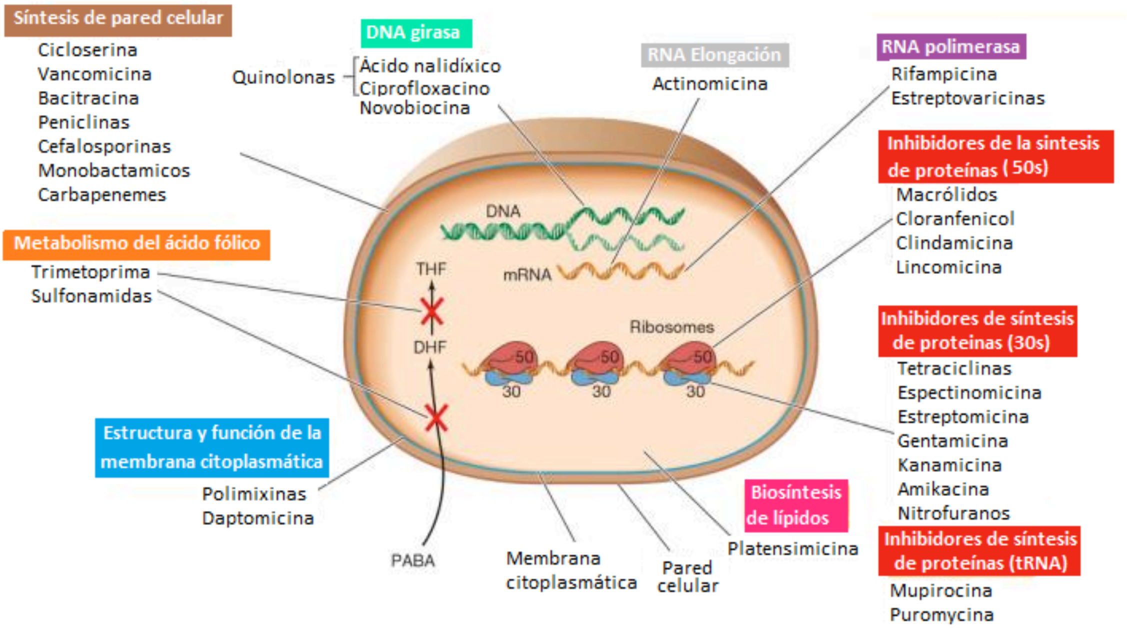


Figura 10. Modo de acción de los principales grupos de antibióticos clasificados dependiendo de las dianas celulares. PABA, ácido para-aminobenzoico; THF, tetrahidrofolato; DHF dihidrofolato; ARNm, ARN mensajero (Adaptado de Madigan *et al.*, 2011).

I. β-lactámicos:

Los β-lactámicos obstaculizan la síntesis de la pared celular en bacterias Gram-positivas, limitando la incorporación del ácido N-acetilmurámico a los mucopéptidos de la pared celular en las últimas etapas de síntesis del peptidoglicano, es por ello que apenas afecta a bacterias Gram-negativas (sus membranas tienen un baja proporción en mucopéptidos) (Romero Cabello, 2007). También inhiben la peptidoglicano transpeptidasa (provocando que no se formen los puentes de unión de la pared celular) y la carboxipeptidasa (responsable de la elongación de la cadena del peptidoglicano) y activan, por otra parte, enzimas autolíticas (Medina Asensio, 2000).

Las penicilinas pertenecen a este grupo, son los primeros antibióticos descubiertos. Se pueden distinguir varios grupos de penicilinas: las penicilinas naturales (penicilinas G y V) de espectro reducido, las penicilinas betalactamasa-resistentes, las penicilinas de amplio espectro, las penicilinas de muy amplio espectro, los penems, los monolactámicos, la fosfomicina y otras penicilinas (cefalosporinas).

II. Aminoglucósidos

Estos antibióticos penetran la célula por transporte activo dependientes de energía (Baeyens *et al.*, 2008) y se unen a los ribosomas bacterianos mediante enlace covalente irreversible a la subunidad 30S (Romero Cabello 2007) interrumpiendo así la síntesis proteica (Gennaro, 2003; Lorenzo *et al.*, 2008). En el caso de las bacterias Gram-negativas, estos antibióticos atraviesan la membrana externa mediante transporte pasivo y posteriormente la membrana interna mediante transporte activo (Baeyens *et al.*, 2008).

La estreptomicina es el antibiótico más antiguo de los aminoglucósidos. Estos antibióticos se usan para el tratamiento de infecciones nosocomiales causadas por bacterias aerobias Gram-negativas (Kotra *et al.*, 2000). Su espectro de acción abarca los bacilos Gram-negativos aerobios (sobretodo algunas especies de la familia *Enterobacteriaceae* y el género *Pseudomonas*) y *S. aureus*. En ocasiones se utilizan en combinación con la penicilina. Aunque no se suelen utilizar ya que son considerados los antibióticos más tóxicos (Lorenzo *et al.*, 2008).

III. Anfenicoles

El principal anfenicol usado en la actualidad es el cloranfenicol, aunque dada su alta toxicidad se usa solo en casos extremos. Su mecanismo de acción se basa en la inhibición de la síntesis proteica al unirse de forma reversible a la subunidad 50S del ribosoma (Mensa *et al.*, 2008) reprimiendo así la síntesis de los aminoácidos (Romero Cabello, 2007).

IV. Polimixinas

Ejercen su efecto a nivel de las membranas bacterianas aumentando su permeabilidad. Destacan principalmente las polimixinas B y E aunque solo tienen actividad frente a bacterias Gram-negativas.

V. Glicopéptidos

Los principales glicopéptidos son la teicoplanina y la vancomicina. Estos antibióticos inhiben la síntesis del peptidoglicano (en una reacción metabólica distinta a los betalactámicos), alteran la permeabilidad de la membrana citoplasmática e inhiben la síntesis del ARN (Mensa *et al.*, 2008).

VI. Macrólidos

Los macrólidos son antibióticos bacteriostáticos y bactericidas (dependiendo del tiempo de exposición), se unen a los ribosomas bacterianos (subunidad 50S) inhibiendo la síntesis proteica (Mensa *et al.*, 2008). Estos, son antibióticos efectivos frente bacterias Gram-positivas, micoplasmas y ciertas especies de *Legionella*. La eritromicina es un macrólido con un amplio margen de seguridad y mínimos efectos adversos, por lo tanto suele ser el más utilizado, muchas veces se emplea como alternativa a la penicilina (Gennaro, 2003).

Dentro de los macrólidos, son importantes las lincosamidas que son un subgrupo integrado por clindamicina y lincomicina, éstas se utilizan frecuentemente en infecciones producidas por bacterias Gram-positivas anaerobias (Romero Cabello, 2007).

VII. Tetraciclinas

Las tetraciclinas son antibióticos bacteriostáticos de amplio espectro (frente a bacterias Gram-positivas y Gram-negativas). Son eficaces frente a cepas de estreptococos, bacilos Gram-negativos, las bacterias del género *Rickettsia* y espiroquetas. Sin embargo, hoy en día, existen muchas cepas resistentes.

Su mecanismo de acción se basa en inhibir reversiblemente la síntesis proteica al unirse a la subunidad ribosómica 30S, esto impide que se una el ARN transferente al ribosoma (Baeyens *et al.*, 2008) imposibilitando así la unión de los aminoácidos a la proteína en formación. Pueden penetrar la célula por difusión pasiva (mediante porinas) o por transporte activo a través de la membrana plasmática (Medina Asensio, 2000).

VIII. Sulfonamidas

Las sulfonamidas son antibióticos bacteriostáticos sintéticos de amplio espectro, que inhiben la síntesis del ácido fólico. Aunque son eficaces contra la mayoría de las bacterias Gram-positivas y muchas bacterias Gram-negativas, hoy en día se utilizan en pocas ocasiones por el gran número de resistencias que ha provocado su uso (Gennaro, 2003; Romero Cabello, 2007).

IX. Quinolonas

Las quinolonas son una clase de antibióticos con efecto bacteriostático, todos ellos de origen sintético. Interaccionan con la ADN-girasa (subunidades α y β) bacteriana impidiendo que el ADN bacteriano esté en su conformación superenrollada, necesaria para el empaquetamiento del ADN en la bacteria (Romero Cabello, 2007).

Se pueden distinguir dos grupos: las quinolonas de primera generación (ácido nalidíxico) que son bacteriostáticas y las de segunda generación (fluorquinolonas) que son bactericidas. El ácido nalidíxico es activo únicamente frente a bacterias Gram-negativas. Las fluorquinolonas son activas frente a la mayoría de los microorganismos, tanto bacterias Gram-negativas como Gram-positivas. Ejemplos de este grupo son el ciprofloxacino, la norfloxacina y la ofloxacina (de Ahumada Vázquez *et al.*, 2002).

X. Otros antimicrobianos

Metronidazol es un antimicrobiano bactericida con actividad exclusiva frente a bacterias anaerobias y protozoos flagelados (Lorenzo *et al.*, 2008). Actúa sobre el ADN modificando su estructura helicoidal y provocando la ruptura de sus hebras (Medina Asensio, 2000).

Nitrofurantoína es un antibiótico cuyo mecanismo de acción es bactericida. Interfiere en los procesos enzimáticos de respiración celular, metabolismo glucídico y síntesis de la pared bacteriana. Es eficaz frente a la mayoría de patógenos del tracto urinario, incluyendo *E. coli*, *Enterobacter, Klebsiella*, ciertas especies del género *Proteus, S. aureus* y *Streptococcus faecalis* (Gennaro, 2003).

Fosfomicina impide la síntesis de la pared celular bacteriana ya que actúa inhibiendo las reacciones de entrecruzamiento de las moléculas del peptidoglicano. Es un antibiótico perteneciente al grupo de los fosfonatos con acción bacteriostática. Tiene amplio espectro aunque su actividad es más pronunciada frente a bacterias Gram-positivas tanto aerobias como anaerobias.

Rifampicina, inhibe los mecanismos de transcripción ya que actúa frente a la ARN-polimerasa, impidiendo que esta enzima se una al ADN, esto trae como consecuencia el bloqueo de la transcripción del ARN (Lorenzo *et al.*, 2008). No obstante, este antibiótico es incapaz de unirse a su homóloga eucariota de tal manera que la síntesis del ARN humano no se ve afectada. Es activa frente a la mayoría de las bacterias Gram-positivas y con actividad variable frente a bacterias Gram-negativas. Es muy utilizado hoy en día para el tratamiento de la tuberculosis (Gennaro, 2003).

3.1.2.2. Eficacia de los antibióticos en la industria alimentaria

Los antibióticos se utilizan en agricultura, ganadería, así como en piscifactorías. En la industria ganadera su uso está muy extendido para el tratamiento y prevención de enfermedades infecciosas (presentándose como premezclas líquidas o sólidas a concentraciones relativamente elevadas), así como para promover el crecimiento del animal con la finalidad de favorecer el control de la microbiota y así se produzca un mayor aprovechamiento de nutrientes y un incremento de peso. En muchas ocasiones el tratamiento empieza de forma empírica, cuando se sospecha que puede haber infección (Pascual Anderson *et al.*, 1999; Cancho Grande *et al.*, 2000).

Como en la industria ganadera, en piscifactorías su uso se reserva al tratamiento de enfermedades infecciosas (Cabello *et al.*, 2006) y su prevención (Kümmerer, 2009). En acuicultura, se usan principalmente la oxitetraciclina, florfenicol, sarafloxacino, eritromicina y sulfonamidas potenciadas con trimetoprima u ormethoprima (Serrano, 2005).

No obstante, después de su administración es preciso un periodo de supresión antes del sacrificio, con el fin de evitar la presencia de residuos (Pascual Anderson *et al.*, 1999) pero pueden liberarse directamente al medio ambiente si pasan al suelo a través del estiércol (Isidori *et al.*, 2005) pudiendo llegar incluso al agua por escorrentía superficial (Park y Choi, 2008). Es por ello que Europa ha prohibido su uso como promotores de crecimiento en animales desde enero de 2006 (European Commission, 2005).

El principal problema del uso de antibióticos en animales es la aparición de resistencias bacterianas (lo que ocurre frecuentemente si se administran de forma continuada a baja concentración) y de alergias en el consumidor (Cancho Grande *et al.*, 2000; Cromwell, 2002). Es por ello que la legislación regula el uso de estas sustancias (Real Decreto 109/1995, Directiva 70/524/CEE, Reglamento CEE nº 2377, Directiva 96/23/CE o el Real Decreto 1749/1998) (Pascual Anderson *et al.*, 1999).

De las 23.000 toneladas de antibióticos producidos en la industria farmacéutica cada año, alrededor del 40% son usados en agricultura (Nawaz *et al.*, 2001). Las infecciones bacterianas en plantas son muy difíciles de controlar y erradicar y a menudo conllevan devastadoras pérdidas económicas para los agricultores. En 1950, se reconoció el potencial de los antibióticos para controlar enfermedades vegetales (McManus, 1999). Para ello, los antibióticos deben ser activos en el interior de la planta, tolerar la oxidación, radiación UV, lluvia y altas temperaturas (Kümmerer, 2009). Los árboles frutales a menudo se tratan con antibióticos con fines profilácticos para el control de infecciones bacterianas (McManus *et al.*, 2002). Su forma de administración es normalmente mediante rociado en forma de pesticidas. Normalmente se utilizan sulfametoxazol, tetraciclina, clortetraciclina (Allen *et al.*, 2010), estreptomicina y oxitetraciclina (Kümmerer, 2009). Todo ello, tiene como consecuencia la aparición de bacterias resistentes a antibióticos en diferentes ambientes: aire, suelo, agua y animales de granja y salvajes por contaminación cruzada (Smith *et al.*, 2005).

3.2. La resistencia a antimicrobianos en las BAL aislados de alimentos fermentados

3.2.1. Evaluación de la resistencia en las BAL

Los antibióticos han sido utilizados a menudo para el tratamiento de infecciones bacterianas en ganadería, lo cual resulta un reservorio muy importante de resistencias bacterianas. Dichas resistencias se pueden transmitir a lo largo de la cadena alimentaria hasta el ser humano, dando como resultado la pérdida de efectividad en el tratamiento de enfermedades tanto en el ser humano como en animales (Abriouel *et al.*, 2015). Esta creciente emergencia de la resistencia a los antimicrobianos ha exigido una evaluación en profundidad de dichos aspectos en las BAL destinadas a ser usadas como probióticos.

Para categorizar si una cepa de las BAL es susceptible o resistente, se llevan a cabo ensayos de susceptibilidad a un rango de antimicrobianos de relevancia en la clínica humana y veterinaria en cada cepa que se pretende usar como probiótico. Para evaluar la susceptibilidad a antibióticos, se utilizan diferentes metodologías cuantitativas y estandarizadas tales como los métodos de dilución en caldo o en agar o también E-tests para la determinación de la concentración mínima inhibitoria (CMI) de acuerdo con las recomendaciones del Instituto de Estándares Clínicos y de Laboratorio (CLSI; www.clsi.org), el Comité Europeo de Evaluación de la Susceptibilidad Antimicrobiana (EUCAST), la Organización Internacional de Normalización (ISO), la Sociedad Británica de Quimioterapia Antimicrobiana (BSAC, UK), la Agencia Francesa de la Seguridad Sanitaria de los Productos para la Salud (AFFSAPS, Francia), el Instituto Alemán de Normalización (DIN, Germany) y la Sociedad Internacional de Quimioterapia/Organización Mundial de la Salud (ISC/WHO) (Abriouel *et al.*, 2015). Sin embargo, los métodos cualitativos o semi-cualitativos para determinar indirectamente la CMI, tales como los métodos de difusión (difusión en disco), son en general inaceptables (EFSA, 2012b). Después de la incubación, la CMI se define como la menor concentración del antimicrobiano que inhibe el crecimiento bacteriano. La Comisión Técnica de Aditivos y Productos o Sustancias utilizados en los Piensos (Comisión FEEDAP) de la Autoridad Europea de Seguridad Alimentaria (EFSA) define los puntos de corte microbiológicos para distinguir entre cepas resistentes a los antimicrobianos de las susceptibles. Por lo tanto, una cepa se clasifica como susceptible (S) cuando se inhibe a una concentración de un antimicrobiano determinado igual o inferior al valor de corte pre-establecido ($S \leq x$ mg/L), y como resistente (R) cuando no es inhibida por una concentración de un antimicrobiano determinado superior al valor de corte pre-establecido ($R > x$ mg/L) de acuerdo con la EFSA (2012b). La actualización periódica de los valores de corte microbiológicos de diferentes antimicrobianos se hace posible gracias a la disponibilidad de los datos de la comunidad científica y otras fuentes pertinentes (por ejemplo, la Agencia Europea de Medicamentos, el Centro Europeo de Prevención y Control de Enfermedades) (EFSA, 2012b). Además de investigar la CMI de un antibiótico para una sola cepa, hay que estudiar la distribución de la CMI en una población de bacterias que pertenecen a una sola unidad taxonómica (especie o género). Una distribución bimodal o multimodal de la CMI usualmente resulta de resistencias adquiridas por adición de genes o mutaciones (Ammor *et al.*, 2008).

3.2.2. Mecanismos de resistencia bacteriana

El comité SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks, 2009), define la resistencia bacteriana como "La capacidad que tienen las bacterias de soportar los efectos nocivos de un agente químico al cual la mayoría de las cepas del mismo microorganismo son susceptibles". Así pues, se puede considerar como una capacidad inherente (intrínseca) o adquirida (extrínseca) de un organismo para resistir los efectos de un agente quimioterapéutico al que es sensible habitualmente.

Hoy en día la resistencia a antimicrobianos es cada vez más evidente. La primera resistencia a biocidas fue descrita entre la década de 1950 y 1960 (Cabrera *et al.*, 2007). En 1946, la mayoría de las cepas de *S. aureus* eran sensibles a la penicilina, hoy en día casi todas las cepas son resistentes a la bencilpenicilina y la mayoría solo se pueden tratar con vancomicina (Cabrera *et al.*, 2007).

En los últimos sesenta años se ha hecho notorio el impacto de la respuesta de estos microorganismos a la presión selectiva que ejercen los antisépticos y desinfectantes, así como los compuestos quimio-terapéuticos más utilizados en los brotes de infecciones en los hospitales a nivel mundial. Incluso en probióticos, han sido documentados doce casos de sepsis bacteriana temporal, nueve casos de bacteriemia, dos casos de endocarditis y un caso de absceso hepático (Boyle *et al.*, 2006).

Desde los primeros estudios realizados en la década en 1940 sobre el mecanismo de acción de la penicilina, fue evidente que esta no era capaz de actuar de la misma manera sobre todos los microorganismos (Hobby *et al.*, 1942; Chain y Duthie 1945; Hobby y Dawson 1946).

La resistencia a antimicrobianos se produce como consecuencia de la interrupción o la alteración de uno o más pasos esenciales en la acción del antimicrobiano, siendo el resultado final, la pérdida total o parcial de la eficacia antimicrobiana (Forbes, 2009). La tolerancia/resistencia fenotípica es la respuesta desarrollada en todas las especies bacterianas a un cambio ambiental incluyendo la presencia de antimicrobianos y que conduce a la supervivencia bacteriana (Novak y Tuomanen, 2001). La tolerancia/resistencia fenotípica se puede deber a la ausencia de la diana del antibiótico o la presencia de la misma con baja afinidad por el substrato (antimicrobiano), la formación de biopelículas, disminución de la permeabilidad (Pages *et al.*, 2008; Fernández, 2012), la formación de un receptor alterado o la alteración de la superficie mediante la modificación del lipopolisacárido, presente en ella. Este último reduce las interacciones moleculares entre la membrana externa y el antimicrobiano, inhibiendo en consecuencia, su penetración y la alteración del número o el tipo de porinas a través de las cuales los antibióticos penetran al interior de las células bacterianas (Corona y Martínez, 2013). También, mediante la expulsión de los antibióticos a través de bombas de exporte que permiten mantener el antibiótico fuera de las envolturas bacterianas y reducir así su actividad tóxica (Corona y Martínez, 2013).

La resistencia genotípica se encuentra codificada en el material genético del microorganismo, bien en el cromosoma, o bien en plásmidos (*plásmidos de resistencia "factores R"*). Una bacteria puede ser intrínsecamente resistente a un determinado antimicrobiano o puede adquirir dicha resistencia mediante mutaciones cromosómicas o transferencia horizontal de genes. La resistencia intrínseca de una determinada especie a un antimicrobiano es la capacidad de dicha bacteria a resistir a la acción de ese antimicrobiano como resultado de las características estructurales o funcionales inherentes (Blair *et al.*, 2015). Los mecanismos más importantes de resistencia intrínseca se muestran en la Figura 11.

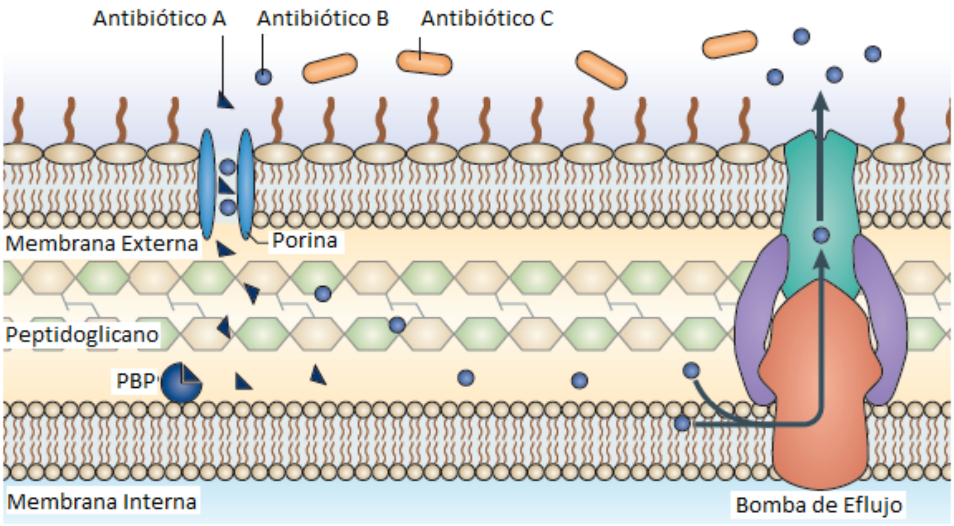


Figura 11. Mecanismos de resistencia intrínseca (Blair *et al.*, 2015).

A nivel fenotípico, las causas de esta resistencia son muy variadas. Pero, fundamentalmente la resistencia a un antimicrobiano se puede producir a dos niveles: inhibición de la entrada y modificación directa del antimicrobiano una vez en el citosol (Blair *et al.*, 2015). La inhibición de la entrada en el microorganismo, se puede producir mediante exporte por bombas de exporte que realizan el transporte activo de antimicrobianos. La sobreexpresión de dichas proteínas, confiere al microorganismo resistencia a alta concentración del antimicrobiano (Blair *et al.*, 2015) ya que la bacteria es capaz de bombearlo hacia fuera (Chopra y Roberts, 2001). Este mecanismo de resistencia no solo se produce en el caso de antibióticos, si no también se produce con un gran número de biocidas. Existe un gran número de familias de estas proteínas halladas en un gran número de microorganismos, entre los que destacan la superfamilia DMT (*Drug/Metabolite Transporter*), la superfamilia MF *(Major Facilitator*), la familia ABC (*ATP-Bindding Cassette*), la familia RND (*Resistance-Nodulation-Division*) y la familia MATE (*Multidrug and Toxic Compound Extrusion*) entre muchas otras (Poole, 2002).

El bloqueo de la entrada de antimicrobiano, también puede utilizar mecanismos pasivos como la permeabilidad reducida a ellos (mucho más común en bacterias Gram-negativas). Este mecanismo de "insensibilidad", se produce para los biocidas, siendo incapaces de alcanzar su diana de acción incluso usados a concentraciones elevadas.

Los microorganismos también inhiben la entrada de los antimicrobianos mediante cambios químicos en la diana del mismo, a nivel de la membrana externa o el espacio citosólico (dependiendo del antimicrobiano). Estos cambios, normalmente se producen por cambios en los aminoácidos que componen las proteínas dando lugar a un cambio conformacional y la pérdida de función (Wright, 2011).

Algunos microorganismos son naturalmente resistentes (es decir intrínsecamente resistentes) a algunos antibióticos. Sin embargo, puede ocurrir que una bacteria sensible a la acción antimicrobiana adquiera mecanismos de resistencia (resistencia extrínseca). La mayor parte de la resistencia a antimicrobianos es debida a transferencia de genes de resistencia, "desrepresión" de ciertos genes cromosomales causada por mutaciones, la inserción de un elemento génico transponible o por adquisición de un elemento génico extracromosomal (plásmido) que porta dicha resistencia (Livermore, 1995). Un microorganismo productor de un antibiótico, desarrolla mecanismos de resistencia al mismo, de forma que neutraliza o destruye su propio antibiótico; los genes que codifican dichos mecanismos de resistencia pueden transferirse ocasionalmente a otros organismos (Blair *et al.*, 2015).

Algunas bacterias poseen genes que codifican para proteínas (normalmente enzimas) que compiten con la diana, actuando como un "bypass" de forma que el antibiótico posee igual o más afinidad por estas enzimas que por la diana, dando como resultado el bloqueo de la acción del mismo (Hawkey, 1998). Esto es lo que ocurre con la llamada proteína de unión a penicilina PBP (Penicillin Binding Protein). Se puede deber a la adquisición un plásmido que codifica para esta enzima o por transposición del mismo por "ADN libre" (Schimtz *et al.*, 2000). Por otro lado, ciertos microorganismos poseen enzimas capaces de modificar químicamente los antibióticos y como consecuencia se produce su inactivación. Existen numerosas enzimas identificadas cuya actividad consiste en degradar y/o modificar los antimicrobianos, entre ellos las betalactamasas (Figura 12) y enzimas que degradan los aminoglucosidos, fenicoles y macrólidos.

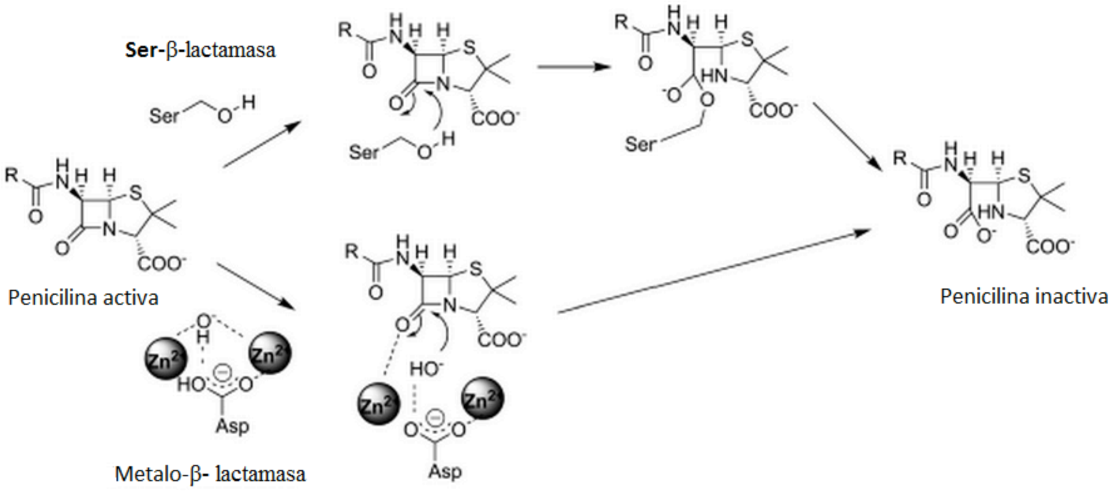


Figura 12. Inactivación química de los antibióticos betalactámicos por la acción de betalactamasas (Wright, 2011).

Existen dos mecanismos de inactivación de antibióticos: la formación de enlaces covalentes de grupos químicos (grupo acil, fosfato, nucleotidil y ribotidil) en determinados "sitios vulnerables" de la molécula, lo que provoca impedimentos estéricos en la unión con las dianas celulares (Wright, 2005) y la activación mediante metales de una molécula de agua nucleofílica (Drawz y Bonomo, 2010). Así, Los principales mecanismos de resistencia a antibióticos quedan recogidos en la Figura 13A.

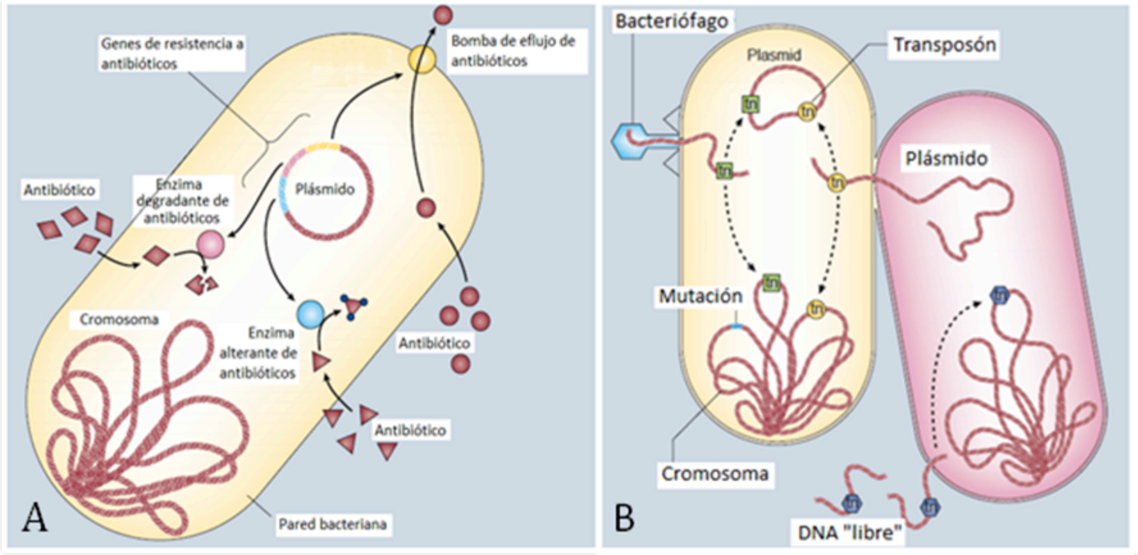


Figura 13. A. Principale**s** mecanismos de resistencias en bacterias (Adaptado de Levy y Marshall, 2004). **B.** Principales mecanismos de adquisición de resistencia a antimicrobianos en bacterias (Adaptado de Levy y Marshall, 2004).

Por otro lado, la adquisición de resistencia (resistencia extrínseca) puede ocurrir mediante la mutación de genes pre-existentes o por transferencia horizontal. Con algunas excepciones, la resistencia intrínseca y la resistencia por mutación es improbable que sea diseminada entre las diferentes especies. En cambio, la transferencia horizontal de genes, y particularmente de los elementos génicos móviles (plásmidos y transposones) se produce con frecuencia (Normark y Normark, 2002).

Tabla 1. Mecanismos de resistencia más representativos según el tipo de antibiótico (Forbes, 2009; Davies y Davies, 2010).

Antibiótico	Mecanismo de Resistencia
Betalactámicos	• Hidrólisis enzimática (Betalactamasas) • Alteración de las dianas (PBPs) • Disminución en la captación y bombas de exporte
Glucopéptidos	• Diana alterada • Exceso de dianas (peptidoglicano)
Aminoglucósidos	• Modificación enzimática (fosforilacion, acetilación, nucleotidilación) • Captación disminuida y bombas de exporte • Diana alterada (mutaciones ribosómicas)
Quinolonas	• Captación disminuida • Diana alterada en las ADN girasas • Acetilación • Exporte • Alteración de la diana
Macrolidos	• Expulsión mediante bombas de exporte o porinas • Diana alterada (ribosomas) • Modificación enzimática (hidrólisis, glicosilación, fosforilación)
Tetraciclinas	• Mono-oxigenación • Exporte • Dianas alteradas
Lincosamidas	• Nucleotidilación • Exporte • Dianas alteradas
Sulfonamidas	• Exporte • Dianas alteradas
Fenicoles	• Modificación enzimática (acetilación) • Exporte • Diana alterada

La resistencia a antibióticos puede ser transferida vertical u horizontalmente de forma natural en las comunidades microbianas, siendo mediada por la difusión clonal de una cepa resistente. En cambio, la diseminación horizontal se basa en la transferencia de genes en bacterias por tres mecanismos identificados: la transformación natural (incorporación de ADN que está libre en el medio extracelular), conjugación (transferencia de ADN entre dos bacterias) y transducción (transferencia por bacteriófagos) (Mathur y Singh, 2005) (Figura 13B). A modo de resumen, la Tabla 1 muestra los mecanismos de resistencia más comunes para cada familia de antibiótico.

3.2.3. Resistencia a antimicrobianos en el género *Lactobacillus*

3.2.3.1. Resistencia a antibióticos

A. Resistencia a antibióticos inhibidores de la síntesis de pared celular

Los antibióticos inhibidores de la síntesis de pared celular a los que el género *Lactobacillus* es susceptible son penicilinas, ampicilinas, oxacilina y piperacilina, inhibidores de betalactamasa y cefalosporinas. En los últimos años, se han encontrado varias cepas de *Lactobacillus* resistentes a penicilina G, pertenecientes a las especies *Lb. rhamnosus*, *Lb. reuteri*, *Lb. plantarun*, *Lb. delbrueckii*, *Lb. casei*, *Lb. helveticus*, *Lb. salivarius*, *Lb. cuvatus* y *Lb. sakei*, aisladas de diferentes alimentos distribuidos por los cinco continentes (Abriouel *et al.*, 2015). Existen estudios que demuestran que determinadas especies de este género son resistentes a ampicilina como son ciertas cepas de *Lb. casei*, *Lb. cuvatus*, *Lb. sakei*, *Lb. helveticus* y *Lb. reuteri*, esto es debido a los genes que codifican para PBP, esta resistencia no es transferible entre bacterias puesto que se encuentra en el cromosoma bacteriano (Rosander *et al.*, 2008).

Especies como son *Lb. rhamnosus, Lb. paracasei, Lb. plantarum* y *Lb. pentosus*, aisladas de alimentos potencialmente funcionales, son resistentes a cefalosporinas y oxacilina (Abriouel *et al.*, 2015). El género *Lactobacillus* es intínsecamente resistente a glicopéptidos y esto es debido a su pared celular que presenta una pequeña modificación en su composición química siendo el peptidoglicano constituido por D-Alanina-D-Lactato en lugar de D-Alanina-D-Alanina. Existe cierta controversia en lo que se refiere al origen de la resistencia a la vancomicina en

Lactobacillus spp., diversas cepas de *Lb. rhamnosus, Lb. paracasei, Lb. salivarus* y *Lb. plantarum* poseen resistencia intrínseca a este antibiótico, mientras que otros estudios demuestran que determinadas cepas de *Lb. delbrueckii, Lb. acidophilus, Lb. johnsonii* y *Lb. crispatus* poseen mecanismos de resistencia adquiridos para el mismo (Abriouel *et al.*, 2015).

B. Resistencia a antibióticos que actúan sobre la síntesis proteica

Las especies pertenecientes al género *Lactobacillus* son susceptibles, generalmente, a los antibióticos que inhiben la síntesis proteica, tales como eritromicina, tetraciclina, clindamicina o cloranfenicol. No obstante se han encontrado cepas resistentes a estos antibióticos de *Lb. plantarum, Lb. pentosus, Lb. bulgaricus, Lb. delbrueckii subsp. lactis, Lb. rhamnosus, Lb. fementus, Lb. helveticus, Lb. brevis, Lb. fermentus, Lb. paracasei, Lb. namurensis* y *Lb. casei* aisladas de diferentes alimentos fermentados. Las principales causas de la resistencia intrínseca a estos antibióticos son la ausencia de un transporte de electrones mediada por el citocromo que permite la captación de los antibióticos (Charteris *et al.*, 2001), cambios en la permeabilidad (Abriouel *et al.*, 2015) y resistencia mediada por degradación enzimática, acetiltransferasas, adenaciltransfersas y fosfotransferasas (Franklin y Snow, 2005). La resistencia a estreptomicina en muchos casos es debida a la presencia de los genes *aph(E)* y *sat* (Ammor *et al.*, 2008). La resistencia adquirida a estos antibióticos ha sido también descrita en diferentes cepas del género *Lactobacillus* encontrándose genes de resistencia tales como *aadE* y *aphA3* en *Lb. curvatus*; *aph(3')-III*, *aadA* y *aadE* en *Lb. casei* y *Lb. pentosus*; *aph(3')-III* y *ant(6)* en *Lb. delbrueckii* subsp. *Bulgaricus*; *aph(3')-III* en *Lb. plantarum* y *Lb. namurensis*, aisladas de alimentos fermentados (Abriouel *et al.*, 2015).

Por otra parte, se han encontrado cepas resistentes a cloranfenicol de *Lb. plantarum, Lb. fermentum, Lb. helveticus, Lb. sakei* y *Lb. namurensis* debido a la presencia del gen *cat* en el cromosoma bacteriano o en plásmidos y que codifica para una acetiltransferasa. En los últimos años, se han aislado de productos fermentados cepas de lactobacilos resistentes a eritromicina debido a la presencia del gen *ermB* tanto en plásmidos como en el cromosoma asociado a elementos móviles. Determinadas cepas aisladas de alimentos fermentados procedentes de la cultura china, alimentos vegetales, productos lácteos y carne fermentada mostraron resistencia a clindamicina y tetraciclina (Abriouel *et al.*, 2015). En muchos casos, esta resistencia se debe a la adquisición del gen *tetM* (presente en el transposon *Tn916* o plásmido), *tetS* y *tetW* que codifican para las proteínas de protección ribosomal implicadas en la resistencia a tetraciclina (Abriouel *et al.*, 2015; Catalouk y Gogebakan, 2004; Danielsen 2002; Gevers *et al.*, 2003; Lin *et al.*, 1996; Simeoni *et al.*, 2008); a *tet*(O) y *tet* (O/W) (Ammor *et al.*, 2008) y *tetL* el cual codifica para una bomba de exporte (Ammor *et al.*, 2008; Thumu & Halami, 2012). La resistencia a lincosamidas (clindamicinas) puede ser también codificada por los genes *erm*, principalmente *ermB, ermC, ermG* y *ermT.*

C. Resistencia a antibióticos inhibidores de la replicación/ transcripción del ADN.

El género *Lactobacillus* es intrínsecamente resistente a quinolonas (ácido nalixídico y ciprofloxacino), pero hoy en día se desconoce el mecanismo de resistencia. La resistencia intrínseca a ciprofloxacino puede deberse a mutaciones de los genes *gyrA* (subunidad A de la ADN girasa) y *parC* (que codifica para una subunidad de la topoisomerasa IV) en la región QRDR (Quinolone Resistance Determining Region) (Hummel *et al.*, 2007), o a otros mecanismos de resistencia pasivos como la estructura de la pared celular, la permeabilidad o incluso a un mecanismo de exporte activo (Abriouel *et al.*, 2015).

D. Resistencia a antibióticos que inhiben la síntesis del ácido fólico.

Los principales antibióticos que afectan la síntesis del ácido fólico son las sulfonamidas y trimetoprima. No existe un patrón típico que indique si este género es o no sensible a este tipo de antibióticos (Abriouel *et al.*, 2015). Así algunas cepas parecen ser sensibles, mientras que otras parecen ser capaces de activar ciertas rutas metabólicas, mediante la síntesis de la enzima dihidrofolato reductasa (Huovinen, 1987).

3.2.3.2. Resistencia a biocidas

La resistencia del género *Lactobacillus* a ciertos biocidas ha sido documentada a nivel fenotípico pero no a nivel genotípico. En 2013, se publicó el primer estudio masivo con 466 cepas de diferentes BAL y cuatro biocidas demostrando que las BAL y particularmente el género *Lactobacillus* poseían baja susceptibilidad a cloruro de benzalconio, clorhexidina, triclosan e hipoclorito sódico, sobre todo las especies de *Lb. plantarum* y *Lb. paracasei*. Este hecho puede ser debido probablemente a ciertas características morfológicas, la alteración de la pared celular, la diferencia de permeabilidad o la sobreexpresión de algunas bombas de exporte (Arioli *et al.*, 2013; Condell *et al.*, 2012; Poole *et al.*, 2002). También, se ha documentado que la supervivencia de un porcentaje alto de lactobacilos tuvo lugar tras la exposición durante un corto periodo de tiempo (3 minutos) a 200 mg/L de cloruro de benzalconio (Sundheim *et al.*, 1993).

Los principales mecanismos de resistencia a biocidas de las bacterias consisten en la alteración de la diana celular y la presencia de bombas de exporte, pero pueden existir otros mecanismos de resistencia como la mutación, la adquisición de genes extracromosómicos (Poole, 2002) e incluso la inactivación genética (Russell, 1997). En contraste con los antibióticos, que tienen dianas celulares muy específicas, los biocidas parecen tener múltiples dianas, por tanto, las mutaciones que afectan la diana son poco frecuentes en las cepas resistentes a biocidas (McDonnell y Russell, 1999).

Las bombas de exporte muestran una amplia especificidad por el substrato que puede ser tanto de tipo antibiótico como biocida (Figura14), las familias más representativas son MF, ABC, RND y la familia MATE entre muchas otras (Poole, 2002). La sobreexpresión de las bombas de exporte puede ser consecuencia, en numerosas ocasiones, de las mutaciones que afectan a represores de genes locales (Webber y Piddock, 2003). El triclosan, es un excelente sustrato de diferentes bombas de exporte, por lo cual es una buena herramienta para seleccionar mutantes reguladores que expresan las bombas de exporte (Schweizer, 2001).

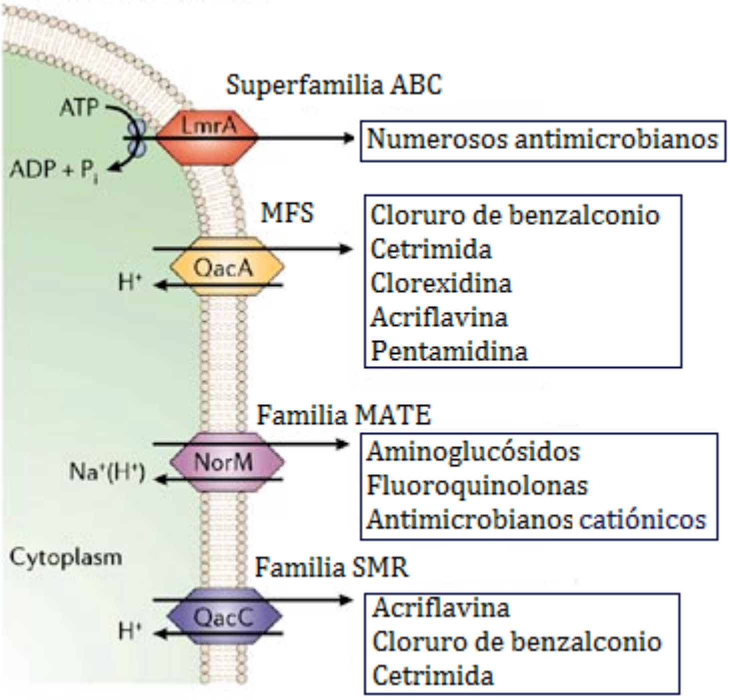


Figura 14. Superfamilias de bombas de exporte más representativas en bacterias Gram-positivas, junto con los antimicrobianos más importantes sobre los que tienen actividad (modificado de Piddock, 2006).

Normalmente el género *Lactobacillus* carece o posee un reducido número de bombas de exporte. En *Lb. brevis*, se observó el gen *horA*, codificante para una bomba de exporte de la superfamilia ABC (Sami *et al.*, 1997; Sakamoto *et al.*, 2001; van de Guchte *et al.*, 2002), homologa a aquella codificada por *lmrA* en *Lc. lactis* (Li y Nikaido, 2009). Por otro lado se ha observado también que varias cepas de *Lactobacillus* spp. tienen proteínas homologas codificadas por el gen *lmrS*, pertenecientes a la familia MF con un 65% de similitud (Floyd *et al.*, 2010). También se ha observado, que la especie *Lb. plantarum* así como *Streptococcus thermophilus* y ciertas especies del género *Bacillus* son capaces de producir la enzima nisinasa que neutraliza la actividad del polipéptido nisina con actividad antimicrobiana (Hurst y Hoover, 1993; Anton *et al.*, 2006).

3.2.4. Perfil de resistencia de las BAL aislados de vegetales fermentados

La gran variedad de alimentos fermentados tradicionales es el resultado de las diferencias en las especies bacterianas predominantes que se encuentran naturalmente en la materia prima de cada región geográfica, las condiciones ambientales locales y los procedimientos de procesamiento tradicionales. Por lo tanto, la amplia gama de productos finales fermentados se caracteriza por una microbiota diversa y una variedad de propiedades organolépticas (Spitaels *et al.*, 2014). Los alimentos tradicionales fermentadas no sólo son atractivos por su valor nutricional (proteínas, minerales, grasas y vitaminas), sabores y consistencias diferentes, pero también presentan oportunidades de subsistencia para los agricultores, procesadores y vendedores. Sin embargo, los alimentos fermentados tradicionales pueden ser considerados como vehículos de bacterias resistentes a los antibióticos y sus genes ya que estas bacterias constituyen un reservorio importante de resistencias que pueden ser transferidos a otras bacterias, incluyendo patógenos y comensales a través de la cadena alimentaria y en el tracto gastrointestinal (Wang *et al.*, 2012; Forslund *et al.*, 2013). En general, la caracterización del perfil de resistencia y virulencia de las bacterias fermentativas en los alimentos tradicionales no se ha realizado de forma rutinaria y en muchos casos, los métodos dependientes de cultivo dejan el 99% de las bacterias sin caracterizar. Varios vegetales fermentados tradicionales se han consumido durante siglos en todo el mundo. Sin embargo, poco se sabe acerca de la incidencia de la resistencia a antibióticos en las BAL, que depende de la zona geográfica, la materia prima, las prácticas de producción y los antibióticos que se utilizan en la agricultura. La evaluación de la resistencia de las BAL exige una mayor atención ya que en 2007 la Autoridad Europea de Seguridad Alimentaria (EFSA) recomendó que las cepas bacterianas que albergan genes de resistencia a antibióticos transferibles deben ser consideradas como peligrosas y no deben ser utilizadas en la alimentación animal, alimentos fermentados y probióticos para los humanos (EFSA, 2007). Así, cuando se comparan con los productos fermentados de origen animal (lácteos y la carne fermentada), la incidencia de las BAL resistentes a los antibióticos es más baja en los productos vegetales fermentados (Pan *et al.*, 2011). Los lactobacilos son los principales protagonistas de la fermentación natural de los vegetales, estas bacterias son intrínsecamente resistentes a muchos antibióticos tales como quinolonas, sulfonamidas y trimetoprima, pero algunas especies son también resistentes a glicopéptidos. Sin embargo, los lactobacilos son susceptibles a todos los inhibidores de la síntesis de proteínas excepto los aminoglucósidos. Varios estudios han sido realizados sobre la evaluación de la resistencia fenotípica a antibióticos en las BAL aislados de los granos de cacao fermentados (Ghana), Koko (Ghana), Dolo y el mosto Pito (Burkina Faso y Ghana). Sin embargo, no hay estudios que revelan dicha resistencia a nivel genotípico.

El género *Lactobacillus* es intrínsecamente resistente a aminoglucósidos (neomicina, kanamicina, estreptomicina y gentamicina), fluoroquinolonas (ciprofloxacino), glicopéptidos (vancomicina y teicoplanina), a ciertos inhibidores de la síntesis de ácido nucleicos (enoxacina, pefloxacina, norfloxacina, ácido nalidíxico y metronidazol) e inhibidores de la síntesis del ácido fólico (sulfamotoxazol, trimetoprima y co-trimexazol) (Charteris *et al.*, 1998; Coppola *et al.*, 2005; Zhou *et al.*, 2005; Patel *et al.*, 2012, Abriouel *et al.*, 2015). Si bien, este género es susceptible a las penicilinas (ampicilina y penicilina G), cloranfenicol, estreptomicina, clindamicina, tetraciclina, eritromicina, ácido linezólico y quinopristina/dalfopristina (Ammor *et al.*, 2007; Katla *et al.*, 2001; Luh *et al.*, 2000; Patel *et al.*, 2012; Abriouel *et al.*, 2015).

En la actualidad, no se ha descrito ningún miembro del género *Lactobacillus* capaz de realizar transformación natural (Ammor *et al.*, 2007). La resistencia extrínseca en el género *Lactobacillus* se puede producir por transposición, adquisición de plásmidos de resistencia. Se han encontrado secuencias de inserción (IS) en el genoma de diferentes especies de *Lactobacillus* (ciertas cepas *Lb. salivarius*, *Lb. helveticus*, *Lb. delbrueckii* subsp. *bulgaricus* y *Lb. amylovorus*), estas secuencias son un indicador de transferencia horizontal de genes lo cual tiene un papel importante en la evolución genómica (Cai *et al.*, 2009; Callanan *et al.*, 2008; Canchaya *et al.*, 2006; Cremonesi *et al.*, 2013; Kaleta *et al.*, 2010; Kant *et al.*, 2011a; Schneider y Lenski 2004). En *Lactobacillus* spp., las secuencias IS suelen flanquear transposones conjugativos del tipo *Tn916* (resistencia a tetraciclina) y *Tn1546* (resistencia a eritromicina) (Devirgiliis *et al.*, 2008; Devirgilis *et al.*, 2009; Fons *et al.*, 1997). En teoría, el género *Lactobacillus* puede adquirir genes del género *Staphylococcus* y *Enterococcus* (Abriouel *et al.*, 2015).

Ciertas especies de lactobacilos pueden adquirir resistencia a antibióticos mediante conjugación por la adquisición de plásmidos con genes de resistencia como el plásmido pLM300 (encontrado en *Lb. fermentum ROT1*) (Ammor *et al.*, 2008) o el plasmido pAMβ1 que puede ser transferido de *Enterococcus faecalis* a

Lb. reuteri (Morelli *et al.*, 1988). Estos plásmidos pueden proporcionar a la bacteria propiedades fenotípicas relacionadas con la resistencia a antimicrobianos, metabolismo de carbohidratos, metabolismo aminoacídico y producción de bacteriocinas (Lavanya *et al.*, 2011). Recientemente, el análisis del perfil de resistencia fenotípico y genotípico de lactobacilos asilados de diferentes vegetales fermentados tradicionales reveló que los genes de resistencia más comunes encontrados en los lactobacilos fueron *erm*(B) y *tet*(M) localizados en el cromosoma y los plásmidos. Por lo tanto, la presencia de estos genes en elementos genéticos móviles puede participar en la diseminación de la resistencia a antibióticos en diferentes ecosistemas.

3.3. Mecanismos de inducción de la resistencia a los antimicrobianos

Una población bacteriana, en principio, es genéticamente homogénea, no obstante, es fisiológicamente heterogénea. Lo que significa que las bacterias de dicha población difieren en su susceptibilidad y que la exposición a antimicrobianos conduce a la selección de la fracción de bacterias fenotípicamente tolerantes. Por lo tanto, se produce una disminución en la mortalidad de la población bacteriana (Wiuff *et al.*, 2004). Existen diferentes mecanismos que inducen la selección de dichas resistencias, entre los cuales destacan los antimicrobianos y diferentes tipos de estrés.

3.3.1. Selección de resistencias mediante antimicrobianos

Un compuesto antimicrobiano puede afectar la estructura de las membranas bacterianas o las rutas biosintéticas esenciales para la multiplicación microbiana. Sin embargo, las bacterias presentan una extraordinaria resistencia a la acción de compuestos tóxicos debido a los mecanismos intrínsecos que protegen las células de la exposición continua a productos químicos nocivos (Fernandes *et al.*, 2003). Esta propiedad puede conducir a la resistencia cruzada en dicho microorganismo. Este tipo de resistencia, se produce cuando un microorganismo es resistente a un antimicrobiano determinado provocando la resistencia frente a otros antimicrobianos a los cuales no hubo una exposición previa.

Normalmente la resistencia cruzada se produce cuando varios antimicrobianos poseen la misma diana o comparten la misma ruta común de sus respectivas dianas (Chapman, 2003), es decir si diferentes antimicrobianos tienen la misma diana celular; si poseen el mismo mecanismo de transporte al interior de la célula; si comparten el mismo mecanismo de resistencia; o si los genes de resistencia son los mismos para ambos antimicrobianos y sean portados por elementos génicos transponibles (Condell *et al.*, 2012).

3.3.2. Selección de resistencias mediante inducción del sistema de reparación de emergencia o respuesta SOS

La activación del sistema de reparación de emergencia (sistema SOS), es un tipo de respuesta que se produce ante un daño celular. Esta respuesta, es capaz de desencadenar una cascada de represiones y desrepresiones génicas que contribuyen a la reparación del genoma bacteriano contribuyendo así a su supervivencia. Este sistema responde a daños producidos en el ADN, a la interrupción del proceso replicativo o a la acumulación de ADN de cadena sencilla (ssADN), lo que desencadena la expresión de diferentes genes que participan en los procesos de reparación, replicación, recombinación y división celular (Jara Ramírez *et al.*, 2005).

La regulación de este sistema se lleva a cabo principalmente por dos proteínas, la proteina LexA que reprime el sistema y la proteína RecA que lo activa (Courcelle *et al.*, 2001; Fernández de Henestrosa *et al.*, 2000). En un estadio normal, es decir en ausencia de daños genómicos, los genes del operón SOS se encuentran unidos con el dímero formado por el represor LexA, reprimiendo la transcripción. Cuando el ADN es dañado o la replicación del mismo se bloquea, se produce un acumulo de ssADN y la proteína RecA se activa, llevando a cabo su actividad coproteasa que promueve la autodegradación del dímero LexA (Figura 15). Todo ello promueve una cascada de reacciones que promueven la desrepresión del sistema y la transcripción de los genes SOS y de otros genes que llevan a cabo la reparación del ADN y la replicación celular, asegurando así la supervivencia bacteriana.

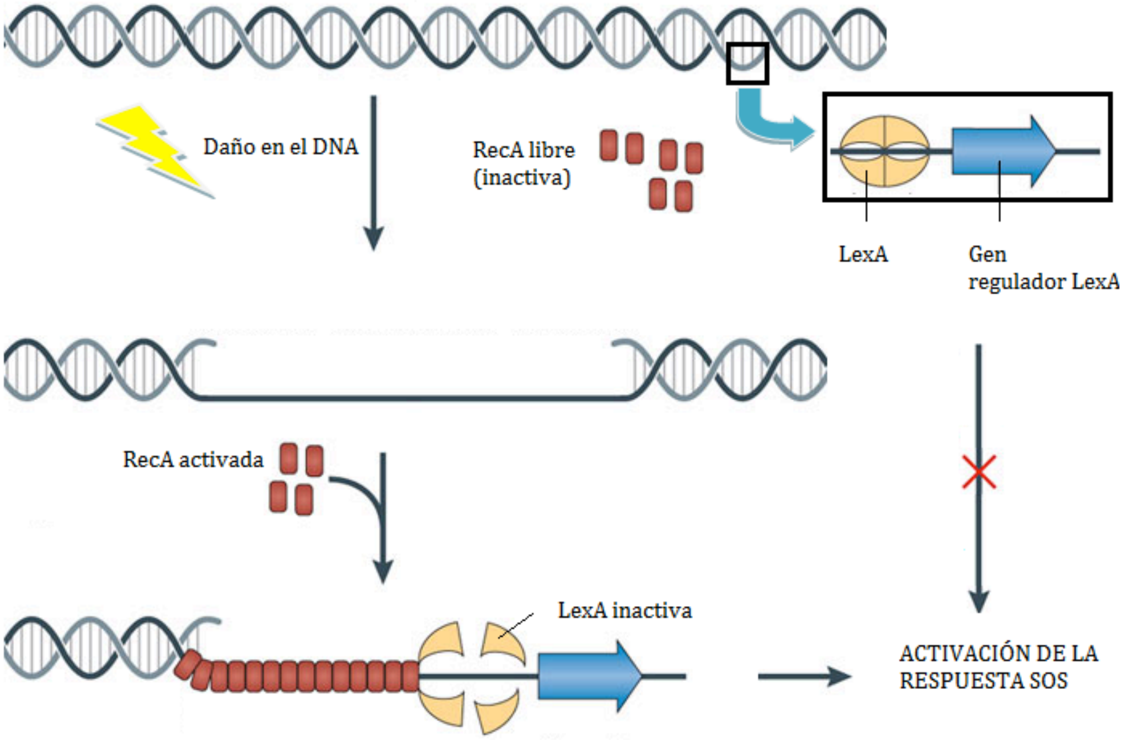


Figura 15. Resumen de la activación del sistema SOS (Modificado de Andersson y Hughes, 2014).

El número de genes expresados para desencadenar la respuesta SOS y los procesos de reparación utilizados es proporcional al grado de inducción del sistema. Para reparar el ADN dañado, entra en juego un tipo de polimerasa diferente de aquella usada normalmente por el microorganismo. Esta polimerasa es menos precisa que las polimerasas replicativas, lo que puede provocar la aparición de mutaciones que pudiesen conllevar a la aparición de resistencias (Cirz *et al.*, 2005). Finalmente, el sistema vuelve a su estado inicial, al repararse la lesión y desaparecer la señal inductora (ssADN), con ello, la proteína RecA vuelve a su estado inactivo, se detiene la hidrolisis de LexA formando un dímero con el ADN y vuelve a producir la represión del regulón SOS volviendo la célula a un estado basal (Jara Ramírez *et al.*, 2005).

Los inductores de esta respuesta son todas aquellas situaciones que causen al microorganismo algún tipo de estrés, entre ellas, un choque térmico, el estrés oxidativo (Foster, 2005), las sales biliares (Bernstein *et al.*, 1999), la exposición a UV, el peróxido de hidrógeno (Goerlich *et al.*, 1989), las altas concentraciones de ssADN (Jara Ramírez *et al.*, 2005) y cierta concentración de antibióticos como quinolonas, betalactámicos, fluoroquinonas, etc (Kohanski *et al.*, 2010). Se ha observado que la inducción con fluoroquinolonas estimula la transferencia horizontal de genes de resistencia (Ubeda *et al.*, 2005).

3.3.3. Selección de resistencias mediante inducción de bombas de exporte

La mayoría de bacterias han demostrado ser capaces de desarrollar un gran número de mecanismos que les han permitido sobrevivir a determinadas concentraciones de antimicrobianos. Uno de estos mecanismos es la inducción de bombas de exporte y que tiene como objetivo final un incremento de la resistencia. La exposición a cualquier antimicrobiano o sustrato de la propia bomba de exporte, puede servir como inductor o regulador de la expresión de las mismas, ya que favorece la sobreexpresión de ellas (Marchetti *et al.*, 2011). Existen muchos tipos de inductores con diferente naturaleza química.

Se ha sugerido que ciertas bombas de exporte a menudo están poco expresadas *in vitro* ya que el inductor de la expresión sólo está presente cuando la bacteria está creciendo en su hábitat natural. Sólo se han identificado unos pocos de estos inductores naturales, son principalmente, las sales biliares y los ácidos grasos y el Triton X-100, este último, induce la expresión del locus *mtrCDE* (codificante para una bomba de exporte de la familia RND) de *Neisseria gonorrhoeae* (Piddock, 2006). Estos mismos inductores naturales, pueden estimular las bombas de exporte de la familia RND (Álvarez-Ortega *et al.*, 2013). Las sales biliares pueden inducir a *B. fragilis, Campylobacter* spp., *E. coli, Salmonella* spp. y *Vibrio cholera* ya que potencian la actividad de las bombas de exporte AcrAB, Bme, CmeABC, VexAb y VexCD. Los ácidos grasos inducen la bomba AcrAB en *E. coli* (Li y Nikaido, 2009).

Los antibióticos pueden inducir o regular la expresión de algunas bombas de exporte a nivel de transcripción génica o traducción a ARNm, por interacción con los sistemas reguladores (van Bambeke *et al.*, 2003). El cloranfenicol, induce las bombas de la familia RND como MexXY o TtgABC en *Burkholderia cenocepacia, P. aeruginosa* y *P. putida* (Li y Nikaido, 2009) y la bomba MexEF-OprN en *P. aeruginosa* (Morita *et al.*, 2013). Las fluoroquinolonas inducen las bombas AcrAB y PatAB en *Salmonella* spp. y *Streptococcus pneumoniae*. Los macrolidos en *P. aeruginosa* inducen MexXY. La bomba de exporte Mef (de la familia MFS) y el transportador Mel (perteneciente a la superfamilia ABC) de *Streptococcus pneumoniae* que confieren resistencia a macrólidos (Piddock, 2006) son inducibles por eritromicina (Ambrose *et al.*, 2005). Las tetracilinas son inductores en *E. coli, P. aeruginosa* y *P. putida* de las bombas de exporte Tet, MexXY y TtgABC. Los aminoglucósidos inducen la bomba de exporte MexXY en *P. aeruginosa* (Li y Nikaido, 2009).

Los biocidas como el cloruro de benzalconio y la clorhexdina inducen en *B. subtilis, E.coli, P. aeruginosa* y *S. aureus*, principalmente las bombas de exporte Blt, Bmr, MexCD-OprJ, MepA, NorA, QacA y QacB (Li y Nikaido, 2009). La clorhexidina es un inductor en *P. aeruginosa* de la bomba MexCD-OprJ (Morita *et al.*, 2013).

La activación de la bomba de exporte NorA (familia MFS) de *S. aureus*, cuyos sustratos son cloranfenicol, fluoroquinolonas y tetraciclina, se puede llevar a cabo mediante IPTG (isopropil- β-D-tiogalactopiranosido) (Yu *et al.*, 2002). También pueden actuar sobre las bombas de exporte de la superfamilia ABC, determinados metabolitos vegetales como la quercetina, un flavonoide, que puede actuar como inhibidor o inductor dependiendo de la concentración utilizada (Rusenova *et al.*, 2009) o las fitoalexinas como la naringenina y la floretina inducen AcrAB en *Erwinia amylovora* (Li y Nikaido, 2009).

Fármacos como el salicilato actúan como inductores de las bombas de exporte AcrAB, CeoAB-OpcM, CmeABC y VceCAB regulando la expresión de los genes *mar* y *mgrA* lo cual regula el gen *sarA* implicado en la resistencia intrínseca. El diazepam en *E. coli* y *K. pneumoniae* promueve la función de las bombas de exporte asociadas con los genes *mar* (Li y Nikaido, 2009).

Las sales como el cloruro sódico actúan como inductores de las bombas de exporte HrdC, AcrAB y otras bombas de la familia RND en *Acinetobacter baumanii* y *Chromohalobacter* spp. Otra sal, el benzoato, es un inductor en *Bacterioides fragilis, E. coli, Klebsiella pneumoniae* de las bombas de exporte asociadas a los gens *mar* (Li y Nikaido, 2009). La bomba AcrAB de *E. coli*, con afinidad a los antibióticos tales como cloranfenicol, eritromicina, ácido fusídico, ácido nalidíxico , rifampicina y tetraciclina, se puede inducir usando 4% de etanol y 0.5 M de NaCl (Ma *et al.*, 1995). Los ácidos fenólicos tales como el ácido salicílico, el ácido t-cinámico y el ácido benzoico son inductores de AcrAB y EmrAB en *Erwinia chrysanthemi*. El indol en *E. coli* y *Salmonella* spp. potencia la actividad de AcrAB, AcrD, AcrEF, CusB, EmrK, MdtA, MdtE y MdtH (Li y Nikaido, 2009). También son capaces de potenciar la actividad de exporte agentes citotóxicos como el bromuro de etidio, la rodamina 6G y el cloruro de tetrafenilfosfonio en *Bacillus subtilits, E. coli, P. aeruginosa* y *S. aureus* (Li y Nikaido, 2009).

4. Las ómicas como herramientas en el análisis de la probiosis de las BAL

Hoy en día, las ciencias ómicas están revolucionando la manera de investigar. La palabra "ómica" es un neologismo de origen latino que significa “conjunto de” y que dependiendo del prefijo utilizado, se referirá al estudio de una materia específica de Biología Molecular (Jiménez-Pranteda *et al.*, 2015). El uso de las nuevas tecnologías basadas en las ómicas moleculares está aumentando rápidamente y probablemente reemplace a los métodos de detección tradicionales (Papadimitriou *et al.*, 2015). Estos estudios se pueden realizar con el fin de ampliar los conocimientos sobre las propiedades moleculares de los probióticos y de las comunicaciones moleculares que se establecen entre ellos y los entornos en los que son capaces de sobrevivir y ejercer sus efectos beneficiosos (Baugher y Klaenhammer, 2011).

Las principales ómicas son la genómica, la transcriptómica y la proteómica sin embargo, muchas de ellas han ido ramificándose en otras a medida que se han obtenido un mayor número de datos (metabolómica, secretómica, fenómica, integrómica, interactómica, metagenómica, metatranscriptómica, etc) (Jiménez-Pranteda *et al.*, 2015).

La genómica es una ciencia basada en datos genéticos globales y detallados de los organismos específicos que viven en un entorno determinado, con el fin de proporcionar un estudio exhaustivo del funcionamiento, el contenido e incluso la evolución y el origen de los genomas. Mediante la genómica es posible detectar y eliminar rápidamente cepas que representen un riesgo potencial para la salud, debido a la presencia de genes de resistencia a antibióticos transferibles o de virulencia (Papadimitriou *et al.*, 2015). Las técnicas utilizadas para el estudio genómico son la secuenciación de ADN y el microarray de ADN (Baugher y Klaenhammer, 2011).

En cuanto a la transcriptómica, ésta proporciona una visión general de los perfiles de expresión génica bajo diferentes condiciones. Como ejemplos, el estudio del perfil de expresión de genes por la microbiota intestinal humana tuvo como finalidad la determinación del impacto de la sobre-expresión génica de ciertos microorganismos en este hábitat (Sonnenburg *et al.*, 2006; Lebeer *et al.*, 2008) o también el estudio de los mecanismos moleculares implicados en las interacciones de los probióticos (Sánchez *et al.*, 2013). Las técnicas más comunes utilizadas en transcriptómica son la secuenciación de ARN, el microarray de ARN, la RT-PCR y la qPCR (Baugher y Klaenhammer, 2011). Sin embargo, tanto en genómica como en transcriptómica la secuenciación de nueva generación (NGS) que hace uso de las tecnologías más avanzadas de secuenciación del material genético y su posterior análisis mediante las herramientas bioinformáticas más adecuadas, está desplazando a las otras técnicas utilizadas ofreciendo datos mucho más precisos y mucho más completos.

La proteómica, por otra parte, implica la identificación y análisis de la expresión, estructura y funciones de las proteínas. Los ensayos realizados con probióticos suelen consistir en el estudio del proteoma en condiciones diferenciales (Baugher y Klaenhammer, 2011), como pueden ser en ensayos de tolerancia bacteriana o resistencia a algún tipo de estrés (Lim *et al.*, 2000; Sánchez *et al.*, 2007; Budin-Verneuil *et al.*, 2007; Lee *et al.*, 2008; Hamon *et al.*, 2011; Wu et al 2011). Existen dos técnicas principales para la identificación proteica: mediante geles de isoeletroenfoque, electroforesis en dos dimensiones e identificación con espectrometría de masas (Gorg *et al.*, 2004) o mediante la identificación de proteínas a través de la cromatografía multidimensional (Washburn *et al.*, 2001).

Las nuevas meta-ómicas (meta-genómica, meta-proteómica y meta-transcriptómica) se centran en el análisis global de las moléculas biológicas de las comunidades microbianas complejas (Sánchez *et al.*, 2013). Las meta-ómicas ofrecen por primera vez las herramientas adecuadas para comprender el comportamiento *in vivo* de los probióticos (Papadimitriou *et al.*, 2015). Estas metodologías son una herramienta clave para entender el ecosistema intestinal microbiano y su modulación dietética, incluyendo a los probióticos (McNulty *et al.*, 2011; Wu *et al.*, 2011; Del Chierico *et al.*, 2012).

Las metodologías meta-genómicas revelan la composición de los ecosistemas complejos y de su Biología (Quin *et al.*, 2010; Upadrasta *et al.*, 2011), además contribuyen al desarrollo de nuevas dianas probióticas, la selección de nuevos criterios probióticos, la identificación de nuevas cepas probióticas, la identificación de diferentes mecanismos de acción e interacción con el hospedador y la determinación de los efectos de los probióticos, entre otros (Gueimonde y Collado, 2012). La aplicación de la meta-proteómica en los probióticos es primordial para entender el comportamiento de los mismos y los efectos de una cepa dada sobre una comunidad bacteriana, como la microbiota del tracto gastrointestinal humano (Benndorf *et al.*, 2009, Chen *et al.*, 2009; Chourey *et al.*, 2010).

En los últimos años, ha surgido el término "probiómica" que hace referencia al conjunto de aplicaciones ómicas capaces de explicar las estructuras probióticas responsables de las interacciones de la microbiota con las células del hospedador. La comprensión del desarrollo del microbioma intestinal humano permitirá una adecuada caracterización de los efectos probióticos en la microbiota comensal de los seres humanos *in vivo* (Baugher y Klaenhammer, 2011). Estos resultados facilitarán, en un futuro, el desarrollo de formulaciones específicas y personalizadas con probióticos dependiendo de las necesidades de cada paciente.

Objetivos

La resistencia a múltiples sustancias antimicrobianas es un problema de salud pública que se viene observando a nivel mundial después de la aparición de los antibióticos. El uso indiscriminado de los antibióticos en terapia clínica y de los biocidas en diversos ámbitos (clínico, industrial, doméstico…etc) junto con la presión selectiva ambiental ejercida por otros agentes (como los empleados en producción agropecuaria o el vertido de residuos industriales tóxicos en aguas y suelos) ha generado una respuesta de supervivencia en los microorganismos, que los capacita para evadir con eficiencia la acción bactericida de muchos agentes (cepas multirresistentes). Entre estas bacterias, encontramos las bacterias lácticas presentes en productos fermentados (por ejemplo las aceitunas fermentadas de mesa) de consumo frecuente, por lo tanto es primordial analizar el perfil de resistencia de dichas bacterias aisladas de la fermentación de aceitunas Aloreña (Guadalhorce, Málaga) frente a diferentes agentes antimicrobianos. En la actualidad se intenta dilucidar si existen mecanismos compartidos de resistencia entre antibióticos y biocidas que permitan a las bacterias lácticas activar genes de respuesta global a las agresiones externas. Además, las prácticas modernas de elaboración y procesado de los alimentos hacen que los microorganismos estén expuestos de forma permanente a diferentes tipos de estrés incluyendo los antimicrobianos, debiendo adoptar diferentes estrategias de supervivencia tales como el desarrollo de mecanismos de resistencia específicos o la alteración de la fisiología. Por lo tanto, nuestro objetivo principal es el estudio de los determinantes genéticos implicados en la resistencia cruzada entre biocidas y antibióticos en bacterias lácticas aisladas de la aceituna Aloreña (DOP, 2008) y además dilucidar la respuesta fenotípica y genotípica de dichas bacterias en un ambiente cambiante (diferentes tipos de estrés). De otra parte, la genómica y la proteómica de una cepa probiótica aislada de la aceituna Aloreña fueron usadas para poner de manifiesto los mecanismos moleculares implicados en la resistencia a antimicrobianos y otros tipos de estrés además de determinar su seguridad con cara a su aplicación como probiótico.

Teniendo en cuenta los argumentos antes mencionados, se plantearon los siguientes objetivos:

1) Determinar la susceptibilidad a biocidas y antibióticos en *Lactobacillus pentosus* y *Leuconostoc pseudomesenteroides* aisladas de la aceituna de mesa Manzanilla Aloreña.

2) Determinar los mecanismos de resistencia a biocidas y antibióticos en *Lactobacillus pentosus* y *Leuconostoc pseudomesenteroides* aisladas de la aceituna de mesa Manzanilla Aloreña.
3) Evaluar la respuesta fenotípica y genotípica de *Lactobacillus pentosus*, aisladas de la aceituna de mesa Manzanilla Aloreña, a diferentes tipos de estrés físico-químico.
4) Conocer los mecanismos de respuesta a nivel proteómico de una cepa probiótica *Lactobacillus pentosus* MP-10 a los antibióticos y biocidas.
5) Analizar *in silico* la resistencia a antibióticos en *Lactobacillus* spp. aisladas de alimentos fermentados.
6) Estudiar la seguridad de *Lactobacillus pentosus* MP-10 a nivel genómico.

Trabajo experimental y resultados

Capítulo I

Análisis de la resistencia a los antimicrobianos de las bacterias del ácido láctico con potencial probiótico

Artículo 1

Antibiotic resistance of *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* isolated from naturally-fermented Aloreña table olives throughout fermentation process

International Journal of Food Microbiology 172 (2014) 110–118

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Antibiotic resistance of *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* isolated from naturally-fermented Aloreña table olives throughout fermentation process

María del Carmen Casado Muñoz, Nabil Benomar, Leyre Lavilla Lerma, Antonio Gálvez, Hikmate Abriouel *

Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaén, 23071 Jaén, Spain

ARTICLE INFO

Article history:
Received 3 April 2013
Received in revised form 5 November 2013
Accepted 24 November 2013
Available online 3 December 2013

Keywords:
Lactobacillus pentosus
Leuconostoc pseudomesenteroides
Antibiotic resistance
Antibiotic resistance genes
Efflux pumps

ABSTRACT

Antimicrobial resistance of *Lactobacillus pentosus* ($n = 59$) and *Leuconostoc pseudomesenteroides* ($n = 13$) isolated from Aloreña green table olives (which are naturally-fermented olives from Málaga, Spain) to 15 antibiotics was evaluated. Most *Lb. pentosus* (95%) and all *Lc. pseudomesenteroides* were resistant to at least three antibiotics. Principal component analysis determined that the prevalence of antibiotic resistance in LAB throughout the fermentation process was highly dependent on the fermenter where the fermentation took place. All *Lb. pentosus* and *Lc. pseudomesenteroides* strains were highly sensitive to amoxicillin and ampicillin (MIC ≤ 2 μg/ml), and also to chloramphenicol (MIC ≤ 4 μg/ml), gentamicin and erythromycin (MIC ≤ 16 μg/ml). However, they were phenotypically resistant to streptomycin (83–100%, MIC > 256 μg/ml), vancomycin and teicoplanin (70–100%, MIC > 128 μg/ml), trimethoprim (76% of *Lb. pentosus* and 15% of *Lc. pseudomesenteroides*, MIC > 128 μg/ml), trimethoprim/sulfomethoxazol (71–100%, MIC $>$ 4–64 μg/ml) and cefuroxime (44% of *Lb. pentosus* and 85% of *Lc. pseudomesenteroides*, MIC $>$ 32–128 μg/ml). *Lb. pentosus* was susceptible to tetracycline and clindamycin, while 46% of *Lc. pseudomesenteroides* strains were resistant to these antibiotics. Only *Lb. pentosus* strains were resistant to ciprofloxacin (70%, MIC $>$ 4–64 μg/ml), although no mutations in the quinolone resistance determining regions of the genes encoding GyrA and ParC were found, thus indicating an intrinsic resistance. Similarly, no genes encoding possible transferable resistance determinants for the observed phenotypic resistance were detected by PCR. In some cases, a bimodal distribution of MICs was observed for some antibiotics to which both LAB species exhibited resistance. Nevertheless, such resistances resulted from an intrinsic mechanism, non-transferable or non-acquired resistance determinants which may in part be due to chromosomally encoded efflux pumps (NorA, MepA and MdeA). Results of the present study demonstrate that all *Lb. pentosus* and *Lc. pseudomesenteroides* strains lack transferable resistance-related genes (*cat*, *bla*, *blaZ*, *ermA*, *ermB*, *ermC*, *msrA/B*, *ereA*, *ereB*, *mphA*, *mefA*, *tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(L), *tet*(K), *aad*(E), *aac(6′)-Ie-aph(2′)-Ia*, *aph(2′)-Ib*, *aph(2′)-Ic*, *aph(2′)-Id*, *aph(3′)-IIIa*, *ant(4′)-Ia*, *dfrA*, *dfrD*, *vanA*, *vanB*, *vanC* and *vanE*) and should therefore, according to Qualified Presumption of Safety criteria, be considered safe for future application as starter cultures or as probiotics.

1. Introduction

Lactic acid bacteria (LAB) are widely consumed along with fermented foods and beverages because of their use as starter cultures in fermentation processes (Caplice and Fitzgerald, 1999; Leroy and De Vuyst, 2004; Wood and Holzapfel, 1995). They are also known for their role as protective cultures as they are involved in producing an arsenal of antimicrobial substances such as lactic acid (and other organic acids), hydrogen peroxide, diacetyl, acetoin, reuterin, reutericyclin, antifungal peptides, and bacteriocins (Holzapfel et al., 1995; Holtzel et al., 2000). In the last decades, LAB have been used as probiotics, with *Bifidobacterium* and *Lactobacillus* being the most commonly used genera (Servin, 2004). The application of LAB as probiotics has been prompted by their beneficial properties on general health of the consumers (Kechagia et al., 2013) and their "QPS" (Qualified Presumption of Safety) status based on a long history of safe use (Anadon et al., 2006; European Commission, SCAN, 2007). In this sense, international regulatory organizations recommended specific prerequisites for approval of a determined strain as feed additive. Accordingly, the European Scientific Committee on Animal Nutrition (European Commission, SCAN, 2005) and the European Food Safety Authority (EFSA, 2012) recommended that LAB strains consumed on a daily basis worldwide should lack acquired or transferable antimicrobial resistance genes prior to considering them safe for human and animal consumption and that any probiotic strain should have QPS status.

---

* Corresponding author at: Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Edif. B3, Universidad de Jaén, Campus Las Lagunillas s/n, 23071 Jaén, Spain. Tel.: +34 953 212003; fax: +34 953 212943.
E-mail address: hikmate@ujaen.es (H. Abriouel).

0168-1605/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.11.025

Table 1
MIC distribution of 15 antibiotics for *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* strains isolated from Aloreña fermented olives.

Antibiotic	Species	No. of isolates with the following MIC range (μg/ml)										ECOFF (μg/ml)
		0.002–≤0.1	>0.1–≤1	>1–≤2	>2–≤4	>4–≤8	>8–≤16	>16–≤32	>32–≤64	>64–≤128	>128	
Amoxicillin	*Lb. pentosus*	30	23	6								$2^{a,b}$
	Lc. pseudomesenteroides		10	3								16^{b}
Ampicillin	*Lb. pentosus*	14	32	13								2^{c}
	Lc. pseudomesenteroides	9	3	1								2^{c}
Cefuroxime	*Lb. pentosus*		11			2	2	18	**21**	**5**		$\geq 32^{e}$
	Lc. pseudomesenteroides		1					1	**11**			$\geq 32^{e}$
Chloramphenicol	*Lb. pentosus*	38	19		2							8^{c}
	Lc. pseudomesenteroides	8	5									4^{c}
Ciprofloxacin	*Lb. pentosus*	7	11			**36**			**5**			$>4^{d}$
	Lc. pseudomesenteroides		5			8						$>32^{a}$
Clindamycin	*Lb. pentosus*	36	22	1								2^{c}
	Lc. pseudomesenteroides	6	1	**6**								1^{c}
Gentamicin	*Lb. pentosus*	38	20				1					16^{c}
	Lc. pseudomesenteroides	6	2				5					16^{c}
Erythromycin	*Lb. pentosus*	45	10			2	2					16^{c}
	Lc. pseudomesenteroides	1	3	9								16^{c}
Kanamycin	*Lb. pentosus*	1	18			4	12	6	18			64^{c}
	Lc. pseudomesenteroides						2	**11**				16^{c}
Streptomycin	*Lb. pentosus*						1		1	8	**49***	$>256^{a}$, NR^{c}
	Lc. pseudomesenteroides										**13**	64^{c}
Teicoplanin	*Lb. pentosus*	3	14								**42**	$\geq 32^{e}$
	Lc. pseudomesenteroides										**13**	$\geq 32^{e}$
Tetracycline	*Lb. pentosus*	13	32	1		6	7					32^{c}
	Lc. pseudomesenteroides		6			1	**6**					8^{c}
Trimethoprim	*Lb. pentosus*	1		8		5	**13**	**8**	**8**	**8**	**8**	8^{c}
	Lc. pseudomesenteroides			11						**1**	**1**	8^{c}
Trimethoprim/ Sulfamethoxazole¥	*Lb. pentosus*			17		**26**		**2**	**14**			$\geq 4^{e}$
	Lc. pseudomesenteroides					**9**		**3**	**1**			$\geq 4^{e}$
Vancomycin	*Lb. pentosus*		11	1							**47**	4^{d}, NR^{c}, $\geq 32^{e}$
	Lc. pseudomesenteroides									**2**	**11**	NR^{c}, $\geq 32^{e}$

The microbiological breakpoint values according to Danielsen and Wind (2003)[a] and Flórez et al. (2005)[b] are given. The EU Commission breakpoint values as suggested by EFSA (2012)[c], European Commission (SCAN) (2005)[d] for *Lb. pentosus* and *Lc. pseudomesenteroides* are described, and in the case of non-described antibiotics we consider the breakpoint values suggested by CLSI (2011)[e] for staphylococci. Resistant strains with a MIC value higher than the breakpoints described in the table are indicated in bold.
ND: not determined, NR: not required.
¥MIC refers to trimethoprim concentrations only. Trimethoprim:sulfamethoxazole was tested in the ratio 1:19.
*The concentration tested of streptomycin was >256 μg/ml.

Recently, several foods have been considered as potential vehicles of antibiotic resistance genes (Bautista-Gallego et al., 2013; Duran and Marshall, 2005; Franz et al., 1999; Zhang et al., 2009) with fermented foods being one of the most important environments where several stresses (low pH, high salt concentration and antimicrobial compounds) and the high number of living bacteria may induce the exchange of such genes. Gene exchanges may enhance survival of LAB and pathogens and thus represent an important risk within the gastrointestinal tract for spread to other bacteria (Salyers et al., 2004; van Reenen and Dicks, 2011). The indiscriminate use of antibiotics in human medicine and animal husbandry during several decades has resulted in an important public health risk. Furthermore, the increasing use of biocides as disinfectants in hospitals and food industries has led to the emergence of cross-resistance phenotypes to clinically important antimicrobial compounds (Fraise, 2002) and to new resistance mechanisms, which impose an additional health risk for consumers or the environment. The development of antimicrobial resistance among bacteria introduced in the food chain is of great concern, thus the EFSA requires that bacteria which are to be introduced into the food chain lack acquired antimicrobial resistance determinants to prevent lateral spread of these (van Reenen and Dicks, 2011). In the present study, the susceptibility patterns and possible mechanisms determining resistance to several antibiotics in *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* strains were investigated. These strains were isolated in a previous study (Abriouel et al., 2012) from Aloreña table olives (which are naturally-fermented olives manufactured by small and medium enterprises from Málaga, Spain), with the aim to select strains that lack acquired antimicrobial resistance genes for possible application as starter cultures or as probiotics. Furthermore, this study allowed us to detect the types and degrees of antibiotic resistance present among the LAB community in the natural olive fermentation environment, and also to determine the prevalence of such phenotypic resistance in LAB throughout the fermentation processes in different producing enterprises.

Table 2
PCR primers used in this study.

Target gene	Primer pair (5′→3′)	Reference
Bla	Bla-forward: CATARTTCCGATAATASMGCC Bla-reverse: CGTSTTTAACTAAGTATSGY	Hummel et al. (2007)
Cat	Catfw1: TTAGGT TATTGGGATAAGTTA Catrev: GCATGRTAACCATCACAWAC	Hummel et al. (2007)
ermA	ErmA1: TCTAAAAAGCATGTAAAAGAA ErmA2: CTTCGATAGTTTATTAATATTAGT	Sutcliffe et al. (1996)
ermB	ErmB1: GAAAAGGTACTCAACCAAATA ErmB2: AGTAACGGTACTTAAATTGTTTAC	Sutcliffe et al. (1996)
ermC	ErmC1: TCAAAACATAATATAGATAAA ErmC2: GCTAATATTGTTTAAATCGTCAAT	Sutcliffe et al. (1996)
msrA/B	MsrA/B1: GCAAATGGTGTAGGTAAGACAACT MsrA/B2: AAGTTATATCATGAATAGATTGTCCTGTTT	Sutcliffe et al. (1996)
ereA	EreA-FW: AACACCCTGAACCCAAGGGACG EreA-RV: CTTCACATCCGGATTCGCTCGA	Sutcliffe et al. (1996)
ereB	EreB-RV: AGAAATGGAGGTTCATACTTACCA EreB-FW: CATATAATCATCACCAATGGCA	Sutcliffe et al. (1996)
mphA	MphA-FW: AACTGTACGCACTTGC Mph-RV: GGTACTCTTCGTTACC	Sutcliffe et al. (1996)
mefA/mefE	MefA/E-FW: AGTATCATTAATCACTAGTGC MefA/E-RV: TTCTTCTGGTACTAAAAGTGG	Sutcliffe et al. (1996)
lsa	Abc2-FW: GGCAATCGCTTGTGTTTTAGCG Abc2-RV: GTGAATCCCATGATGTTGATACC	Singh and Murray (2005)
aac(6′)-Ie-aph(2′)-Ia	FW: CAGGAATTTATCGAAAATGGTAGAAAAG RV: CACAATCGACTAAAGAGTACCAATC	Vakulenko et al. (2003)
aac(6′)-Ie-aph(2′)-Ia	FW: CAGAGCCTTGGGAAGATGAAG RV: CCTCGTGTAATTCATGTTCTGGC	Vakulenko et al. (2003)
aph(2′′)-Ib	FW: CTTGGACGCTGAGATATATGAGCAC RV: GTTTGTAGCAATTCAGAAACACCCTT	Vakulenko et al. (2003)
aph(2″)-Ic-	FW: CCACAATGATAATGACTCAGTTCCC RV: CCACAGCTTCCGATAGCAAGAG	Vakulenko et al. (2003)
aph(2″)-Id	FW: GTGGTTTTTACAGGAATGCCATC RV: CCCTCTTCATACCAATCCATATAACC	Vakulenko et al. (2003)
aph(3′)-IIIa	FW: GGCTAAAATGAGAATATCACCGG RV: CTTTAAAAAATCATACAGCTCGCG	Vakulenko et al. (2003)
ant(4′)-Ia	FW: CAAACTGCTAAATCGGTAGAAGCC RV: GGAAAGTTGACCAGACATTACGAACT	Vakulenko et al. (2003)
tet (L)	tet(L)-2-1: CATTTGGTCTTATTGGATCG tet(L)-2-2: ATTACACTTCCGATTTCGG	Aarestrup et al. (2000)
tet (M)	tet(M)-1: GTTAAATAGTGTTCTTGGAG tet(M)-2: CTAAGATATGGCTCTAACAA	Aarestrup et al. (2000)
tet (O)	Tet(O)-1: GATGGCATACAGGCACAGAC Tet(O)-2: CAATATCACCAGAGCAGGCT	Aarestrup et al. (2000)
tet (S)	Tet(S)-1: TGGAACGCCAGAGAGGTATT Tet(S)-2: ACATAGACAAGCCGTTGACC	Aarestrup et al. (2000)
tet(W)	TetW-FW: GAGAGCCTGCTATATGCCAGC TetW-RV: GGGCGTATCCACAATGTTAAC	Aminov et al. (2001)
vanA	FW: GGGAAAACGACAATTGC RV: GTACAATGCGGCCGTTA	Dutka-Malen et al. (1995)
vanB	FW: ATGGGAAGCCGACAGTC RV: GATTTCGTTCCTCGACC	Dutka-Malen et al. (1995)
vanC1	FW: GCTGAAATATGAAGTAATGACCA RV: CGGCATGGTGTTGATTTCGTT	Miele et al. (1995)
van(E)	VanE1: TGTGGTATCGGAGCTGCAG VanE2: GTCGATTCTCGCTAATCC	Fines et al. (1999)
Bla Z	Bla Z-1: ACTTCAACACCTGCTGCTTTC BlaZ-2: TAGGTTCAGATTGGCCCTTAG	Martineau et al. (2000)
smr	Smr-FW: ATAAGTACTGAAGTTATTGGAAGT Smr-RV: TTCCGAAAATGTTTAACGAAACTA	Bjorland et al. (2001)
Gyr (A)	GyrAfw: CAMCGKCGKATTCTTTACGGAATG GyrArev: TTRTTGATATCRCGBAGCATTTC	Hummel et al. (2007)
Par (C)	ParCfw: TATTCYAAATAYATCATTCARGA ParCrev: GCYTCNGTATAACGCATMGCCG	Hummel et al. (2007)
Dfr(A)	DfrA1: CTTTTCTACGCACTAAATGTAAG DfrA2: CATTATCAATAATTGTCGCTCAC	Liu et al. (2009)
Dfr(D)	DfrD1: GGAAGGGCTTTACCTGACAGAAG DfrD2: CGACATAAGGCAAGAACATAACATA	Liu et al. (2009)
Aad(E)	aadEI: GCAGAACAGGATGAACGTATTCG aadEII: ATCAGTCGGAACTATGTCCC	Klare et al. (2007)
norA	FW: TTTGTTTTCAGTGTCAGAATTTATGTTTG RV: GGCTTGGTGAAATATCAGCTATTAAAC	Patel et al. (2010)
norC	FW: CAGGCAGGATACTTATCAATTAC RV: ATACCAATGACCACAATGAATG	Patel et al. (2010)
norE	norE-F: CTGGCGGCAGCGGTAA norE-R: TGCCATACAGACACCCACCATA	Swick et al. (2011)
mdeA	FW: CTTTCAGGTTACCTTGTTGAATATTTAAAC RV: ATCAATAGGTACTTTAATTGTAGTTCCAAC	Patel et al. (2010)
acrA	acrA-F: CTCTCAGGCAGCTTAGCCCTAA acrA-R: TGCAGAGGTTCAGTTTTGACTGTT	Swick et al. (2011)
acrB	acrB-F: GGTCGATTCCGTTCTCCGTTA acrB-R: CTACCTGGAAGTAAACGTCATTGGT	Swick et al. (2011)
tolC	tolC-F: AAGCCGAAAAACGCAACCT tolC-R: CAGAGTCGGTAAGTGACCATC	Swick et al. (2011)
mdfA	mdfA-F: CATTGGCAGCGATCTCCTTT mdfA-R: TTATAGTCACGACCGACTTCTTTCA	Swick et al. (2011)
mefA	FW: AGTATCATTAATCACTAGTGC RV: TTCTTCTGGTACTAAAAGTGG	Sutcliffe et al. (1996)

2. Material and methods

2.1. Bacterial strains and growth conditions

72 LAB strains (Abriouel et al., 2012) were obtained from Aloreña green table olives naturally-fermented by four small–medium enterprises (SMEs) from Málaga (Spain) and included lactobacilli (59 *Lb. pentosus* strains) and leuconostocs (13 *Lc. pseudomesenteroides* strains). These strains were routinely cultured at 30 ºC in de Man Rogosa and Sharpe (MRS) broth (Fluka, Madrid, Spain) or agar under aerobic conditions for 24–48 h. Strains were kept in 20% glycerol at −80 ºC for long term storage.

2.2. Antibiotic susceptibility testing and MIC determination

MICs of 15 antibiotics encompassing nearly all important classes were determined in LSM broth [90% of IST broth (Oxoid, Madrid, Spain) and 10% MRS broth (Fluka, Madrid, Spain)] (Klare et al., 2005) according to the ISO 10932/IDF 233 standard (ISO, 2010). Pharmacological classes and specific antibiotics employed in this study were: β-lactams (amoxicillin: AMO, ampicillin: AMP and cephalosporin cefuroxime: CFX), quinolone (ciprofloxacin: CIP), lincosamide (clindamycin: CLI), aminoglycosides (gentamicin: GEN, kanamycin: KAN and streptomycin: STR), macrolide (erythromycin: ERY), glycopeptides (teicoplanin: TPL and vancomycin: VAN), sulfonamides (trimethoprim: TMP and trimethoprim/sulfamethoxazole: TMP/SMZ at ratio 1.25/23.75), chloramphenicol: CMP and tetracycline: TET. All antibiotics were from Sigma, with the exception of STR, TET and CIP (Fluka), TMP and SMZ (Almirall), CFX (Norman Laboratories). The microbiological breakpoints of the antibiotics tested (defined also as ECOFF according to the European Food Safety Authority (EFSA, 2012) are shown in Table 1. For the antibiotics not included in the EFSA listing, the microbiological breakpoints were defined according to Danielsen and Wind (2003), Flórez et al. (2005) and the European Commission (European Commission, SCAN, 2005) for *Lactobacillus* and *Leuconostoc*. Furthermore, for the antibiotics for which no breakpoints were defined for *Lactobacillus* and *Leuconostoc* spp. in the literature, such as CFX, TPL and TMP/SMZ, we used the microbiological breakpoints reported by the National Committee for Clinical Laboratory Standards (CLSI, 2011) for staphylococci. We considered MICs higher than the suggested breakpoints as evidence of phenotypic resistance (EFSA, 2012). The accuracy of susceptibility testing was

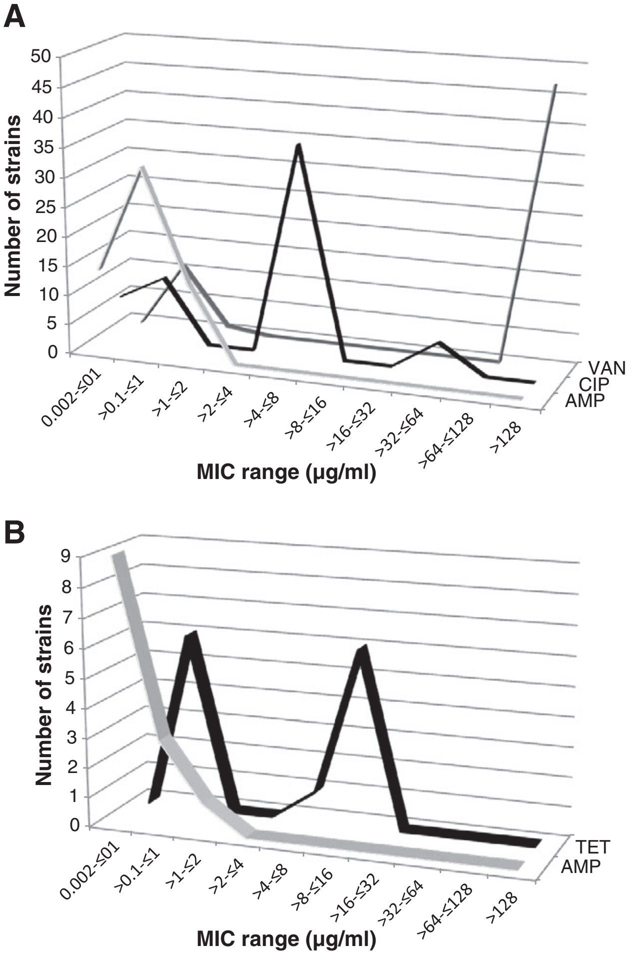


Fig. 1. Distribution of antibiotic MICs for *Lactobacillus pentosus* (A) and *Leuconostoc pseudomesenteroides* (B) strains isolated from Aloreña fermented olives.

monitored by parallel use of the quality control strain *Enterococcus faecalis* ATCC 29212.

2.3. Determination of intrinsic and acquired resistance

According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org), microorganisms without (wild type, WT) and with acquired resistance mechanisms (non-wild type, NWT) to the antibiotic in question are characterized by their MIC values as follows: WT ≤ X µg/ml and NWT > X µg/ml, X being the ECOFF (epidemiological cut-off) value of the corresponding antibiotic for the LAB species studied. The distinction between natural and acquired resistance was determined by analysis of MICs and their distributions according to Stock and Wiedemann (2001). In the case of a population with acquired resistance, plotting the MIC of a particular antibiotic for one species against the number of strains found with the respective MIC usually results in a bimodal distribution (one peak with relatively low MICs which represents the natural population, and one peak with higher MICs which represents the strains with acquired resistance).

2.4. PCR detection of antimicrobial resistance genes

PCR amplifications of well-known structural genes associated with resistance to chloramphenicol (*cat*, the chloramphenicol acetyltransferase gene), β-lactam antibiotics (*bla* and *blaZ*, the β-lactamase genes), macrolides (the *ermA*, *ermB*, *ermC*, *msrA/B*, *ereA*, *ereB*, *mphA*, *mefA*), tetracycline [*tet*(M), *tet*(O), *tet*(S) and *tet*(W), *tet*(L) and *tet*(K)], aminoglucosides [*aad*(E), *aac(6′)-Ie-aph(2′)-Ia*, *aph(2′)-Ib*, *aph(2′)-Ic*, *aph(2′)-Id*, *aph(3′)-IIIa* and *ant(4′)-Ia*], sulfonamides (*dfrA* and *dfrD*) and glycopeptides (*vanA*, *vanB*, *vanC* and *vanE*) were performed using conditions described elsewhere (Aarestrup et al., 2000; Aminov et al., 2001; Dutka-Malen et al., 1995; Fines et al., 1999; Hummel et al., 2007; Klare et al., 2007; Liu et al., 2009; Martineau et al., 2000; Miele et al., 1995; Singh and Murray, 2005; Sutcliffe et al., 1996; Vakulenko et al., 2003) and primers listed in Table 2 (Supplementary Table). Table 2 also includes primers for genes mediating antibiotic resistance through other mechanisms, such as efflux pumps (mdfA, norE, acrA, acrB, tolC, mepA, norA, norC, mefA and mdeA). Template DNA for PCR reactions was prepared as in Jensen et al. (1998).

2.5. DNA sequencing of the QRDR related to ciprofloxacin resistance

To investigate whether observed phenotypic resistance to ciprofloxacin was due to mutations in the quinolone resistance determining regions (QRDR) of the *gyrA* and *parC* genes, the QRDR encoding regions were amplified (Table 2) as described by Hummel et al. (2007). PCR-amplified products purified using Exo Star kit (GE-Healthcare) were sequenced bidirectionally with their corresponding primers and the deduced amino acid sequences were aligned with those retrieved from the GenBank database by using DNAStar CLUSTAL W multiple alignment tool (Lasergene program, version 5.05 (DNASTAR, Inc., Madison, WI, USA).

2.6. Statistical analysis

Statistical analysis of data was accomplished using Excel 2007 and XLSTAT 2012 trial version (2012.4.02 Addinsoft, France) and the correlation between time of fermentation and resistance was determined by principal component analysis (PCA).

3. Results

3.1. Antimicrobial susceptibility testing and MIC distribution profiles

MIC determination of the different antibiotics was performed with 59 *Lb. pentosus* and 13 *Lc. pseudomesenteroides* from Aloreña table olives (including selected LAB with potential probiotic features according to Abriouel et al. (2012). The results obtained (Table 1) indicated that the MICs of β-lactams (amoxicillin and ampicillin), chloramphenicol,

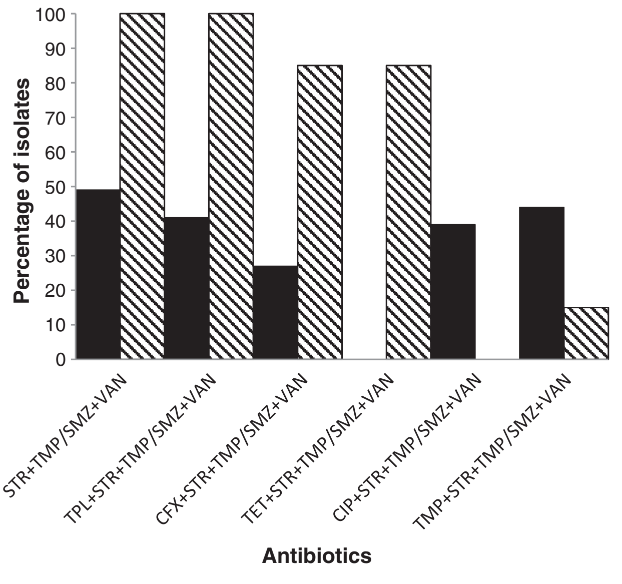


Fig. 2. Multidrug resistance observed in *Lactobacillus pentosus* (■) and *Leuconostoc pseudomesenteroides* (▨) strains isolated from Aloreña fermented olives.

Table 3
Phenotypic and genotypic antibiotic resistance of lactic acid bacteria isolated from Aloreña fermented olives.

Months of fermentation	Species	Phenotypic antibiotic resistance	Resistance gene(s) detected by PCR
Leuconostoc pseudomesenteroides			
3	CF2-26	CFX, KAN, STR, TET, TMP/SMZ, VAN	*acrA*, *mepA*
6	AP2-28	KAN, STR, TMP, TMP/SMZ, VAN	*acrA*, *norA*
	CF1-31	CFX, KAN, STR, TET, TMP/SMZ, VAN	*acrA*
	CF2-31	CFX, KAN, STR, TET, TMP/SMZ, VAN	*acrA*
	CF2-33	STR, TMP, TMP/SMZ, VAN	*mdeA*
	CF2-35	CFX, KAN, STR, TET, TMP/SMZ, VAN	
	CF2-38	CFX, CLI, KAN, STR, TET, TMP/SMZ, VAN	*mepA*
	CF2-40	CFX, KAN, STR, TET, TMP/SMZ, VAN	*mepA*
Other	SP5-11	CFX, CLI, KAN, STR, TET, TMP/SMZ, VAN	*acrA*, *mepA*
	SP5-12	CFX, CLI, KAN, STR, TET, TMP/SMZ, VAN	*acrA*, *mepA*
	SP5-17	CFX, CLI, KAN, STR, TET, TMP/SMZ, VAN	*acrA*, *mepA*
	SP5-18	CFX, CLI, KAN, STR, TET, TMP/SMZ, VAN	*acrA*, *mepA*
	SP5-19	CFX, CLI, KAN, STR, TET, TMP/SMZ, VAN	*acrA*, *mepA*
Lactobacillus pentosus			
1	CF1-1	CFX, STR, VAN	*acrA*, *mepA*
	CF1-3	STR, TMP, TMP/SMZ, VAN	*acrA*, *mepA*
	CF1-4	CFX, CIP, STR, TMP/SMZ, VAN	*acrA*, *mepA*
	CF1-5	CIP, STR, TMP, TMP/SMZ, VAN	*acrA*
	CF1-6	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*acrA*
	CF1-7	STR, VAN	*acrA*
	CF1-8	STR, VAN	*acrA*
	CF1-10	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*acrA*
	CF2-2	CFX, CIP, STR, TMP, TMP/SMZ	*mdeA*
	CF2-3	CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
	CF2-4	CFX, TMP, TMP/SMZ	*mdeA*
	CF2-5	CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
	CF2-6	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
	CF2-7	STR, VAN	*mdeA*
	CF2-9	CFX, CIP, STR, TMP, TMP/SMZ	*mdeA*
	CF2-10	STR, VAN	*mdeA*
2	CF1-12	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*acrA*
	CF1-14	STR, VAN	*acrA*
	CF1-15	CFX, STR, TMP, TMP/SMZ, VAN	*acrA*
	CF1-16	CFX, CIP, STR, TMP, VAN	
	CF1-19	CFX, CIP, STR, TMP, VAN	*acrA*
	CF1-20	STR, TMP, TMP/SMZ, VAN	*acrA*
	CF2-11	CIP, STR, TMP, TMP/SMZ	*acrA*, *norA*, *mdeA*
	CF2-12	CIP, STR, TMP, TMP/SMZ	*acrA*, *norA*, *mdeA*
	CF2-13	CFX, CIP, TMP, TMP/SMZ, VAN	*acrA*, *norA*, *mdeA*
	CF2-15P	CIP, STR, TMP, TMP/SMZ, VAN	*acrA*, *norA*, *mdeA*
	CF2-17	CIP, STR, TMP, TMP/SMZ, VAN	*acrA*, *norA*, *mdeA*
	CF2-18	CFX, CIP, TMP, VAN	*acrA*, *mdeA*
	CF2-19P	CIP, STR, TMP, TMP/SMZ	*acrA*, *norA*, *mdeA*
	LP-1	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
	LP-5	CIP, STR, TMP, TMP/SMZ, VAN	*norA*, *mdeA*
	LP-7	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
	LP-8	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
3	CF1-21	CFX, CIP, STR, TMP, VAN	*acrA*
	CF1-23	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*acrA*
	CF1-25	CIP, STR, TMP, TMP/SMZ, VAN	*acrA*, *norA*, *mdeA*
	CF1-29	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*acrA*, *norA*, *mdeA*
	CF1-30	STR, TMP	*acrA*
	CF2-21	CIP, STR, TMP, TMP/SMZ	*acrA*, *norA*
	CF2-24	CFX, STR, TMP, TMP/SMZ, VAN	
	CF2-28	CIP, STR, TMP/SMZ, VAN	
	AP2-15	CFX, CIP, STR, TMP, VAN	
	AP2-16	CFX, CIP, STR, TMP, TMP/SMZ, VAN	
6	CF1-33	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
	CF1-35	CIP, STR, TMP, TMP/SMZ, VAN	*acrA*, *norA*, *mdeA*
	CF1-36	CFX, STR, TMP, TMP/SMZ, VAN	*acrA*, *mdeA*
	CF1-37	STR, TMP, TMP/SMZ, VAN	*acrA*, *norA*, *mdeA*
	CF1-38	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
	CF1-40	CIP, TMP, TMP/SMZ, VAN	
	CF1-43	CIP, TMP, TMP/SMZ, VAN	*mdeA*
	CF2-34	CIP, STR, TMP, TMP/SMZ, VAN	*norA*
	CF2-39	STR, TMP/SMZ, VAN	
Other	2C1	STR, VAN	
	2C4	CIP, STR, VAN	*acrA*
	2C5	STR	
	2C6	CIP, TMP, TMP/SMZ	
	2C7	CIP	
	5C2	STR	*acrA*
	5C3	CIP, TMP, TMP/SMZ, VAN	*acrA*

Samples CF1 and CF2 corresponded to fermenter 1 and fermenter 2 of SME1, respectively.

gentamicin (aminoglycoside) and erythromycin (macrolide) did not exceed the ECOFF suggested in most cases by the European Food Safety Authority (EFSA, 2012) for both LAB species (*Lb. pentosus* and *Lc. pseudomesenteroides*). However, in the case of amoxicillin, we used the microbiological breakpoint proposed by Flórez et al. (2005) as described in Table 1, since the official ECOFF of amoxicillin has not been designated for *Lb. pentosus* and *Lc. pseudomesenteroides* by the international organizations. Accordingly, relatively narrow unimodal MIC distributions at the low-end concentration range were shown by *Lb. pentosus* and *Lc. pseudomesenteroides* strains for amoxicillin and ampicillin (0.002–2 μg/ml). Concerning the other antibiotics to which both *Lb. pentosus* and *Lc. pseudomesenteroides* were very sensitive, they displayed either uni- or bimodal MIC distributions, depending on the antibiotic used and the LAB concerned (Table 1). Thus, chloramphenicol and erythromycin showed unimodal and bimodal MIC distributions for *Lc. pseudomesenteroides* and *Lb. pentosus*, respectively (Table 1). The bimodal MIC distributions of gentamicin observed could differentiate two sensitive subpopulations (Table 1), the major one at the low-end concentration range (MIC range 0.002–1 μg/ml) and the smallest one with intermediate MICs (>8–16 μg/ml). Regarding ciprofloxacin, only *Lc. pseudomesenteroides* strains were susceptible to this antibiotic (MIC range >0.1–8 μg/ml). However, *Lb. pentosus* showed susceptibility to clindamycin (MIC range 0.002–2 μg/ml), kanamycin (MIC range 0.002–64 μg/ml) and tetracycline (MIC range 0.002–16 μg/ml) (Table 1).

The incidence of phenotypic resistance in *Lb. pentosus* and *Lc. pseudomesenteroides* to some antibiotics varied considerably depending on the LAB genus tested and the antimicrobial used (Table 1). Generally, *Lc. pseudomesenteroides* was more resistant than *Lb. pentosus* to all antibiotics except ciprofloxacin and trimethoprim (Table 1). *Lb. pentosus* strains were phenotypically resistant to streptomycin, vancomycin, trimethoprim, trimethoprim/sulfamethoxazole, teicoplanin and ciprofloxacin in the range of 70–83% (Table 1), and cefuroxime (44%). The phenotypic antibiotic resistances observed for *Lc. pseudomesenteroides* included streptomycin, trimethoprim/sulfamethoxazole, teicoplanin and vancomycin (100%), kanamycin and cefuroxime (85%), clindamycin and tetracycline (46%), and trimethoprim (15%) (Table 1).

For cefuroxime, most strains of *Lb. pentosus* and *Lc. pseudomesenteroides* displayed broader bimodal MIC distributions, which allowed to differentiate two subpopulations: the most sensitive one with low MICs (>0.1–1 μg/ml) and the other one with intermediate-higher MIC values (>4–128 μg/ml), which included both sensitive and resistant strains (Table 1).

Concerning aminoglycosides (kanamycin and streptomycin), resistant *Lc. pseudomesenteroides* strains displayed unimodal MIC distributions (MIC range >16–32 μg/ml for kanamycin and MIC >128 μg/ml for streptomycin). However, *Lb. pentosus* strains showed bimodal MIC distribution for streptomycin, which distinguished between sensitive (MIC range 8–128 μg/ml) and resistant (MIC >256 μg/ml) subpopulations (Table 1).

For sulfonamides (trimethoprim and trimethoprim/sulfamethoxazol) and glycopeptides (teicoplanin and vancomycin), either uni- or bimodal MIC distributions were observed for both *Lb. pentosus* and *Lc. pseudomesenteroides* (Table 1), being resistant to teicoplanin, trimethoprim and vancomycin at a high MIC range (>128 μg/ml for teicoplanin and trimethoprim and >256 μg/ml for vancomycin). Bimodal distributions of teicoplanin and vancomycin for *Lb. pentosus* showed two subpopulations at the low-end (0.002–2 μg/ml) and the high-end (>64–>128 μg/ml) concentration ranges. However the bimodal distribution of trimethoprim MIC values of *Lb. pentosus* strains was broad and positioned at the intermediate-high concentration range (>4–>128 μg/ml) (Table 1).

Regarding tetracycline, only *Lc. pseudomesenteroides* was shown to be resistant to this antibiotic. The bimodal distribution obtained (Table 1, Fig. 1) indicated two subpopulations: one sensitive with low MICs (>0.1–1 μg/ml) and another one resistant with intermediate MICs (>8–16 μg/ml).

The distinction between natural and acquired resistance was determined for those strains of *Lb. pentosus* and *Lc. pseudomesenteroides* which showed phenotypic resistance to some antibiotics and displayed bi- or multimodal distribution of the MICs. Fig. 1 shows an example of the bi-/multimodal distribution of the MICs of ciprofloxacin and vancomycin for *Lb. pentosus* (Fig. 1A), and of tetracycline for *Lc. pseudomesenteroides* (Fig. 1B), in comparison with the normal unimodal MIC distribution for ampicillin to which all *Lb. pentosus* and *Lc. pseudomesenteroides* were susceptible (Fig. 1).

Multi-drug resistance (MDR, defined as resistance to 3 or more different antimicrobials) was observed in 13 *Lc. pseudomesenteroides* strains (100%) and 56 *Lb. pentosus* strains (95%) (Fig. 2, Table 3). All MDR *Lc. pseudomesenteroides* strains were resistant to at least 4 antimicrobials, and 6 were resistant to 7 of them (Fig. 2, Table 3). However, MDR *Lb. pentosus* were resistant to 3 (8 strains), 4 (9 strains), 5 (14 strains), 6 (16 strains) or 7 (9 strains) antibiotics (Fig. 2, Table 3).

3.2. Detection of resistance genes

To identify resistance determinants responsible for the resistance phenotypes observed, all strains were screened by PCR for the presence of resistance genes as described above. Analysis of tetracycline-resistant *Lc. pseudomesenteroides* showed that neither the genes encoding ribosomal protection proteins [*tet*(M), *tet*(O), *tet*(S) or *tet*(W)] nor genes encoding the tetracycline efflux pumps [*tet*(K) or *tet*(L)] were detected

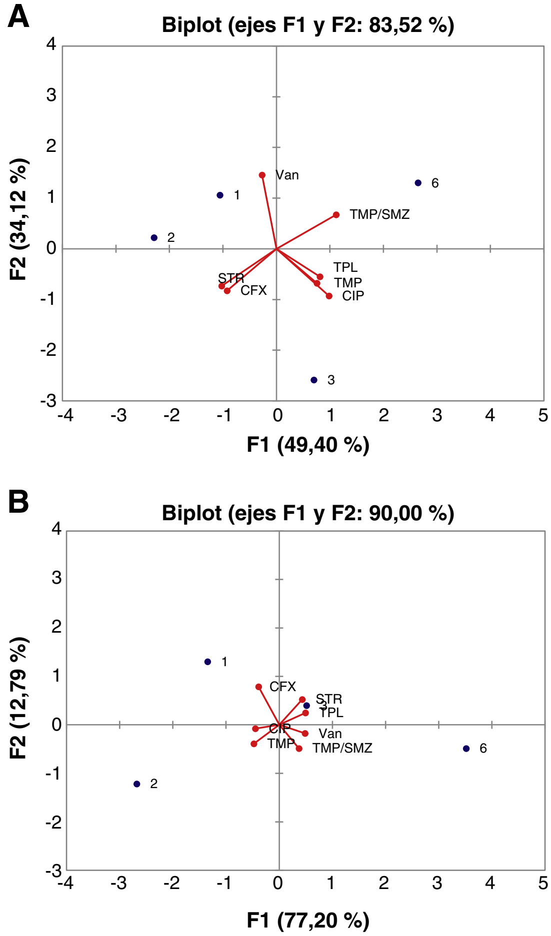


Fig. 3. Biplot of the simultaneous evaluation of the relationship of scores (antimicrobials) and sample variable (months of fermentation in fermenter 1 "A" and fermenter 2 "B" of SME1). Antimicrobials: CFX, cefuroxime; CIP, ciprofloxacin; STR, streptomycin; TPL, teicoplanin; VAN, vancomycin; TMP, trimethoprim and TMP/SMZ, trimethoprim/sulfamethoxazole.

in any strain with an MIC higher than 8 μg/ml. Similar results were obtained for the *lsa* gene responsible for resistance to lincosamides, which was not detected in clindamycin-resistant *Lc. pseudomesenteroides*. Other genes encoding for resistance to aminoglycosides [*aad*(E), *aac(6′)-Ie-aph(2′)-Ia*, *aph(2′)-Ib*, *aph(2′)-Ic*, *aph(2′)-Id*, *aph(3′)-IIIa* and *ant(4′)-Ia*], sulfonamides (*dfrA* and *dfrD*), glycopeptides [*van(A)*, *van(B)*, *van(C)* and *van(E)*], and β-lactam antibiotics (*bla* and *blaZ*) were not detected in any of the LAB strains studied.

Molecular characterization of ciprofloxacin resistance was done by the comparative analysis of the deduced amino acid sequences of the highly conserved quinolone resistance-determining region (QRDR) of *Lb. pentosus* strains with either resistant or sensitive phenotypes. No substitutions could be detected within the QRDR of the GyrA subunit of DNA gyrase (Ser-83 and Asp-87) or the ParC subunit of topoisomerase IV (Ser-80 and Glu-84). Therefore, no insertion sequences, integrons or transposons flanking the genes were involved in the observed resistance to ciprofloxacin.

Concerning efflux pumps implicated in antibiotic resistance, *acrA* was the most commonly detected determinant, i.e. in 69% of *Lc. pseudomesenteroides* and 54% of *Lb. pentosus* (Table 3). Similarly, *mepA* was detected in 62% of *Lc. pseudomesenteroides*, while only 5% of *Lb. pentosus* harbored genes coding for this efflux pump. On the other hand, *mdeA* was detected in 32% of *Lb. pentosus* and only in one *Lc. pseudomesenteroides* strain. Concerning *norA*, 8% of *Lc. pseudomesenteroides* and 22% of *Lb. pentosus* gave positive results in PCR reactions of the corresponding efflux pump (Table 3). With respect to other efflux pumps (*mdfA*, *norE*, *acrB*, *tolC*, *norC* and *mefA*), none of the LAB strains showed positive results in the corresponding PCR reactions.

3.3. Principal component analysis

Principal component analysis (PCA) was used as a mathematical tool to evaluate the relationship among all antibiotic resistances tested in this study as a function of fermentation months (variable). The biplot graph shown in Fig. 3 indicates that during the first, second and six months of fermentation, vancomycin and trimethoprim/sulfamethoxazol were the most relevant antibiotic resistances in fermenter 1. Similarly, vancomycin and trimethoprim/sulfamethoxazol, and also trimethoprim and ciprofloxacin were prevalent resistances in fermenter 2 after two and six months of fermentation (Fig. 3). However, after one month fermentation in fermenter 2, cefuroxime, teicoplanin and streptomycin showed more relevant resistances, being also prevalent after 3 months of fermentation in the same fermenter (Fig. 3). On the other hand, after 3 months fermentation in fermenter 1 several antibiotic resistances were relevant such as ciprofloxacin, teicoplanin, trimethoprim, cefuroxime and streptomycin (Fig. 3).

4. Discussion

The use or rather misuse of antibiotics for decades in bacterial infection treatments, animal husbandry and agriculture (Wegener, 2003) has resulted in the increased emergence of resistant bacteria to modern antibiotics, leading to failure in therapy and causing also evolutionary and ecological problems as were reported by Gillings (2013) about the recruitment of more resistance genes into the resistome and mobilome.

Antibiotic-resistant bacteria represent a great challenge for the food industry, especially LAB which were isolated from several European fermented foods (Maietti et al. 2007; Ouoba et al. 2008; Nawaz et al. 2010; Toomey et al. 2010) such as fermented meat and dairy foods. LAB traditionally used as starter cultures and also as probiotics may act as reservoir of antibiotic-resistance genes which are similar to those found in human pathogens (Flórez et al., 2005) and potentially transferable to other pathogens either in the food matrix or in the gastrointestinal tract (Mathur and Singh, 2005). In the present study, we investigated the antibiotic susceptibility profiles of LAB isolated from a traditional and natural fermentation of Aloreña table olives. Although LAB from this food source were probably not exposed to antibiotics and were therefore not expected to harbor transferable resistances, it could not be assumed that such bacteria would be totally free of antibiotic resistances and transferable antibiotic resistance genes, and their QPS status (EFSA, 2004) would need to be confirmed for further starter culture development as demanded by EFSA (2012) for use of LAB starter strains in foods.

The antibiotic susceptibility profiles of *Lb. pentosus* and *Lc. pseudomesenteroides* strains showed that both are generally quite sensitive to clinically relevant antibiotics such as ampicillin, amoxicillin, erythromycin, chloramphenicol and gentamicin. In general, lactobacilli and leuconostocs are susceptible to antibiotics inhibiting the protein synthesis such as erythromycin, chloramphenicol, clindamycin and tetracycline (Florez et al., 2005; Ammor et al., 2007). However, only *Lb. pentosus* strains were susceptible to tetracycline and clindamycin, while 46% of *Lc. pseudomesenteroides* strains were phenotypically resistant to these antibiotics. In both cases, the bimodal distribution of MICs suggested that some *Lc. pseudomesenteroides* strains possessed acquired resistance for clindamycin and tetracycline. However, the absence of resistance determinants [*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(L) and *tet*(K)], may suggest a new mechanism of resistance which can be due either to acquired genes or to the mutation of indigenous genes (EFSA, 2012). The common resistance mechanism of erythromycin and clindamycin [the so-called macrolide–lincosamide–streptogramin (MLS) phenotype] was not responsible for the observed clindamycin resistance in resistant leuconostocs, since no *erm*, *msrA*/*B*, *mphA* or *mefA* genes were detected. Furthermore, *lsa* gene was not related with clindamycin resistance phenotype in *Lc. pseudomesenteroides* as this gene was absent in resistant bacteria.

The high resistance to teicoplanin and vancomycin (MICs > 128 μg/ml) exhibited by most *Lb. pentosus* strains (71–80%) and all *Lc. pseudomesenteroides* strains (100%) is in agreement with the reported intrinsic resistance of LAB to these antibiotics (Danielsen and Wind, 2003; Ammor et al., 2007; Liu et al., 2009). Glycopeptide resistance of both *Lb. pentosus* and *Lc. pseudomesenteroides* is probably due to the presence of D-Ala-D-lactate in their peptidoglycan, instead of the normal dipeptide D-Ala-D-Ala (Deghorain et al., 2007). As this is a chromosomally mediated and not transferable trait, this would explain why in the present study we did not find any vancomycin resistance gene marker such as the transferable *van* genes (*vanA*, *vanB*, *vanC* and *vanE*).

Intrinsic resistance to aminoglycosides such as streptomycin and kanamycin has been reported to be a general feature of lactobacilli (Danielsen and Wind, 2003), and is thought to result from membrane impermeability (Elkins and Mullis, 2004). The results obtained in the present study showed that *Lb. pentosus* strains (83%) were resistant only to streptomycin (MIC > 256 μg/ml). However, *Lc. pseudomesenteroides* strains (85–100%) were shown to be resistant to both aminoglycosides. The lack of aminoglycoside resistance determinants [*aad*(E), *aac(6′)-Ie-aph(2′)-Ia*, *aph(2′)-Ib*, *aph(2′)-Ic*, *aph(2′)-Id*, *aph(3′)-IIIa* and *ant(4′)-Ia*] and the observed unimodal distribution of MICs at the high-end concentration range suggest an intrinsic resistance to aminoglycosides in *Lc. pseudomesenteroides* strains. Compared to kanamycin and streptomycin, gentamicin showed very effective inhibition at the low-end concentration range (0.002–1 μg/ml), possibly because this aminoglycoside is able to cross the membrane better than other aminoglycosides (Elkins and Mullis, 2004), thus resulting in lower MICs of this antibiotic for both LAB species tested.

All tested *Lc. pseudomesenteroides* and 71% of *Lb. pentosus* exhibiting bimodal or multimodal broad MIC distributions for trimethoprim/sulfamethoxazole, were shown to be resistant. However, trimethoprim displayed bimodal MIC distributions over a broad concentration range, with 76% of *Lb. pentosus* strains but only 15% of *Lc. pseudomesenteroides* strains resistant to this antibiotic. In both cases, none of the strains showed amplification with *dfrA* and *dfrD* primers related with trimethoprim resistance. The European Food Safety Authority (EFSA, 2008) considered that the resistance shown by LAB to trimethoprim, trimethoprim/

sulfamethoxazol and streptomycin is not relevant when tested on LSM medium, because of the interference of its components with the antibacterial activity of these antibiotics. Small portion of LSM broth (10% MRS) contains antagonistic components such as p-aminobenzoic acid (against sulfamethoxazole) and/or thymidine (against trimethoprim) (Klare et al., 2005; Turnidge and Bell, 2005).

The reduced susceptibility to ciprofloxacin reported here for *Lb. pentosus* (70%), exhibiting a multimodal MIC distribution with three different subpopulations, indicated an acquired resistance. However, we found no mutations in the QRDR of the genes encoding GyrA or ParC for ciprofloxacin resistance associated with insertion sequences, integrons, or transposons as reported previously (El Amin et al., 1999; Hummel et al., 2007; Petersen and Jensen, 2004), which indicated an intrinsic mechanism of resistance.

With respect to cefuroxime, LAB are usually more resistant to cephalosporins as reported by Danielsen and Wind (2003) and Coppola et al. (2005). The impermeability of the cell wall is the main mechanism of resistance to inhibitors of cell-wall synthesis, since LAB species lack cytochrome-mediated electron transport (Condon, 1983). However, the cooperation of non-specific mechanisms, such as multidrug transporters (Putman et al., 2001) and defective cell wall autolytic systems (Kim et al., 1982), may be responsible for the differences observed between the strains. In the present study, 44% of *Lb. pentosus* and 85% of *Lc. pseudomesenteroides* were resistant to this antibiotic, but none of the resistant strains harbored acquired resistance determinants (*bla* and *blaZ*).

A multi-resistance profile of LAB isolated from Aloreña table olives was shown throughout the fermentation process. Correlation of different parameters (antibiotics and months of fermentation) showed that the prevalence of antibiotic resistance in LAB was highly dependent on the fermenter where the fermentation took place, since the biotic and abiotic conditions were quite different (Abriouel et al., 2011). Green olives were placed in 6000-liter glass fiber tanks, supplemented with salt (6% wt/vol) and 0.8% (vol/vol) of acetic acid and allowed to ferment for 4 to 7 months at room temperature. Vancomycin and trimethoprim/sulfamethoxazol were the most relevant antibiotic resistances in LAB during the whole fermentation process, regardless of the fermenter sampled. On the other hand, PCA also showed that the most prevalent month was the third month of fermentation when correlated with multiple antibiotic resistance. The intrinsic resistance of LAB to several antibiotics may be partially due to genes that encode multidrug resistance efflux pumps (MDRs), which expel different types of antibiotics and also chemicals (dyes, organic solvents, detergents, biocides and metabolic products). In the present study, *norA* which confers resistance to chloramphenicol and fluoroquinolones (norfloxacin and ciprofloxacin) as reported by several authors (Neyfakh et al., 1993; Truong-Bolduc et al., 2003) was detected in LAB which were both resistant and sensitive to ciprofloxacin, suggesting that *norA* may play another role in some LAB (especially in ciprofloxacin-sensitive strains). Regarding the tripartite complex AcrAB-TolC system which is involved in the efflux of β-lactams, fluoroquinolones, chloramphenicol and tetracycline (Okusu et al., 1996), only AcrA – a fusion protein – was detected in both LAB, while AcrB – a cytoplasmic membrane transporter protein – and TolC – an outer membrane channel protein – were not detected. The absence of AcrB and TolC may hypothetically be due to a point mutation in their corresponding genes, which enabled their detection by PCR, or maybe AcrA does not play a role in antibiotic resistance in this species. NorA and MdeA, both chromosomally encoded MFS (major facilitator superfamily) pumps, and MepA, a MATE-family MDR pump that is also chromosomally encoded (Kaatz et al., 2005), were detected in both LAB species. Multidrug-resistance efflux pumps are encoded in all bacteria and can confer clinically relevant resistance to antibiotics, but it is now understood that these efflux pumps also have a physiological role (Piddock, 2006). In the present study, efflux pumps detected may confer low level resistance to several antibiotics, but they may also play a crucial role in allowing bacteria to survive in their ecological niche (high salt, low pH, antimicrobials such as phenolic compounds from olives, metabolic products).

5. Conclusions

Almost all *Lb. pentosus* (95%) and all *Lc. pseudomesenteroides* strains isolated from naturally-fermented Aloreña green table olives can be regarded as safe because of the absence of acquired resistance determinants. The intrinsic resistance to more than three antibiotics will not become a problem in a medical setting, since they were also highly sensitive to other clinically relevant antibiotics. In this study, the occurrence of intrinsic multi-resistance in both LAB species was due in part to chromosomally encoded efflux pumps such as NorA, MepA and MdeA.

Acknowledgments

This work was supported by grants AGL2009-08921, P08-AGR-4295, Plan propio de la Universidad de Jaén, and Campus de Excelencia Internacional Agroalimentario CeiA3. Leyre Lavilla Lerma was beneficiary of a fellowship from Spanish Ministry of Education and Science.

References

Aarestrup, F.M., Agerso, Y., Gerner-Smidt, P., Madsen, M., Jensen, L.B., 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. Diagn. Microbiol. Infect. Dis. 37, 127–137.

Abriouel, H., Benomar, N., Lucas, R., Gálvez, A., 2011. Culture-independent study of the diversity of microbial populations in brines during fermentation of naturally fermented Aloreña green table olives. Int. J. Food Microbiol. 144, 487–496.

Abriouel, H., Benomar, N., Cobo, A., Caballero, N., Fernández Fuentes, M.A., Pérez-Pulido, R., Gálvez, A., 2012. Characterization of lactic acid bacteria from naturally-fermented Manzanilla Aloreña green table olives. Food Microbiol. 32, 308–316.

Aminov, R.I., Garrigues-Jeanjean, N., Mackie, R.I., 2001. Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. Appl. Environ. Microbiol. 67, 22–32.

Ammor, M.S., Flórez, A.B., Mayo, B., 2007. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiol. 24, 559–570.

Anadon, A., Martinez-Larranaga, M.R., Martinez, M.A., 2006. Probiotics for animal nutrition in the European Union. Regulation and safety assessment. Regul. Toxicol. Pharmacol. 45, 91–95.

Bautista-Gallego, J., Arroyo-López, F.N., Rantsiou, K., Jiménez-Díaz, R., Garrido-Fernández, A., Cocolin, L., 2013. Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. Food Res. Int. 50, 135–142.

Bjorland, J., Sunde, M., Waage, S., 2001. Plasmid-borne smr gene causes resistance to quaternary ammonium compounds in bovine *Staphylococcus aureus*. J. Clin. Microbiol. 39, 3999–4004.

Caplice, E., Fitzgerald, G.F., 1999. Food fermentations: role of microorganisms in food production and preservation. Int. J. Food Microbiol. 50, 131–149.

CLSI: Clinical and Laboratory Standards Institute (CLSI), 2011. Performance standards for antimicrobial susceptibility testing; Twenty-First Informational Supplement. Document M100–S21. no 1, vol. 31. Wayne, PA.

Condon, S., 1983. Aerobic metabolism of lactic acid bacteria. Ir. J. Food Sci. Technol. 7, 15–25.

Coppola, R., Succi, M., Tremonte, P., Reale, A., Salzano, G., Sorrentino, E., 2005. Antibiotic susceptibility of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. Lait 85, 193–204.

Danielsen, M., Wind, A., 2003. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. Int. J. Food Microbiol. 82, 1–11.

Deghorain, M., Goffin, P., Fontaine, L., Mainardi, J.L., Daniel, R., Errington, J., Hallet, B., Hols, P., 2007. Selectivity for D-lactate incorporation into the peptidoglycan precursors of *Lactobacillus plantarum*: role of Aad, a VanX-like D-alanyl-D-alanine dipeptidase. J. Bacteriol. 189, 4332–4337.

Duran, G.M., Marshall, D.L., 2005. Ready-to-eat shrimp as an international vehicle of antibiotic-resistant bacteria. J. Food Prot. 68, 2395–2401.

Dutka-Malen, S., Stefan, E., Courvalin, P., 1995. Detection of glycopeptides resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. 33, 24–27.

El Amin, N.A., Jalal, S., Wretlind, B., 1999. Alterations in GyrA and ParC associated with fluoroquinolone resistance in *Enterococcus faecium*. Antimicrob. Agents Chemother. 43, 947–949.

Elkins, C.A., Mullis, L.B., 2004. Bile-mediated aminoglycoside sensitivity in *Lactobacillus* species likely results from increased membrane permeability attributable to cholic acid. Appl. Environ. Microbiol. 70, 7200–7209.

European Commission (SCAN), 2005. Opinion of the Scientific Committee on Animal Nutrition on the criteria for assessing the safety of microorganisms resistant to antibiotics of human clinical and veterinary importance. European Commission, Brussels.

European Commission (SCAN), 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA J. 587, 1–16.

European Food Safety Authority (EFSA), 2004. EFSA scientific colloquium summary report: QPS—qualified presumption of safety of microorganisms in food and feed. (http://www.efsa.europa.eu/en/colloquiareports/colloquiaqps.htm).

European Food Safety Authority (EFSA), 2008. Scientific opinion of the panel on biological hazards on a request from the European Food Safety Authority on food borne antimicrobial resistance as a biological hazard. EFSA J. 765, 1–87.

European Food Safety Authority (EFSA), 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J. 10, 2740.

Fines, M., Perichon, B., Reynolds, P., Sahm, D.F., Courvalin, P., 1999. VanE, a New Type of Acquired Glycopeptide Resistance in *Enterococcus faecalis* BM4405. Antimicrob. Agents Chemother. 43, 2161–2164.

Flórez, A.B., Delgado, S., Mayo, B., 2005. Antimicrobial susceptibility of lactic acid bacteria isolated from a cheese environment. Can. J. Microbiol. 51, 51–58.

Fraise, A.P., 2002. Susceptibility of antibiotic-resistant cocci to biocides. J. Appl. Microbiol. 92, 158S–162S.

Franz, C.M.A.P., Holzappel, W.H., Stiles, M.E., 1999. Enterococci at the crossroad of food safety. Int. J. Food Microbiol. 47, 1–24.

Gillings, M.R., 2013. Evolutionary consequences of antibiotic use for the resistome, mobilome, and microbial pangenome. Front. Microbiol. 4, 1–9.

Holtzel, A., Ganzle, M.G., Nicholson, G.J., Hammes, W.P., Jung, G., 2000. The first low molecular-weight antibiotic from lactic acid bacteria: reutericyclin, a new tetramic acid. Angew. Chem. Int. Ed. 39, 2766–2768.

Holzapfel, W.H., Geisen, R., Schillinger, U., 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int. J. Food Microbiol. 24, e343–e362.

Hummel, A.S., Hertel, C., Holzapfel, W.H., Franz, C.M.A.P., 2007. Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. Appl. Environ. Microbiol. 73, 730–739.

ISO (International Organization for Standardization), 2010. ISO 10932/IDF 233 standard milk and milk products—determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB).

Jensen, L.B., Ahrens, P., Dons, L., Jones, R.N., Hammerum, A.M., Aarestrup, F.M., 1998. Molecular analysis of Tn1546 in *Enterococcus faecium* isolates from animals and humans. J. Clin. Microbiol. 36, 437–442.

Kaatz, G.W., McAleese, F., Seo, S.M., 2005. Multidrug resistance in *Staphylococcus aureus* due to overexpression of a novel multidrug and toxin extrusion (MATE) transport protein. Antimicrob. Agents Chemother. 49, 1857–1864.

Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., Fakiri, E.M., 2013. Health benefits of probiotics: a review. ISRN Nutrition 2013. (Article ID 481651, 7 pages).

Kim, K.S., Morrison, J.O., Bayer, A.S., 1982. Deficient autolytic enzyme activity in antibiotic-tolerant lactobacilli. Infect. Immun. 36, 582–585.

Klare, I., Konstabel, C., Müller-Bertling, S., et al., 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. Appl. Environ. Microbiol. 71, 8982–8986.

Klare, I., Konstabel, C., Werner, G., Huys, G., Vankerckhoven, V., et al., 2007. Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. J. Antimicrob. Chemother. 59, 900–912.

Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci. Technol. 15, 67–78.

Liu, C., Zhang, Z., Dong, K., Yuan, J., Guop, X., 2009. Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. Biomed. Environ. Sci. 22, 401–412.

Maietti, L., Bonvini, B., Huys, G., Giraffa, G., 2007. Incidence of antibiotic resistance and virulence determinants among *Enterococcus italicus* isolates from dairy products. Syst. Appl. Microbiol. 30, 509–517.

Martineau, F., Picard, F.J., Grenier, L., Roy, P.H., Ouellette, M., Bergeron, M.G., 2000. Multiplex PCR assays for the detection of clinically relevant antibiotic resistance genes in staphylococci isolated from patients infected after cardiac surgery. The ESPIRIT Trial. J. Antimicrob. Chemother. 46, 527–533.

Mathur, S., Singh, R., 2005. Antibiotic resistance in food lactic acid bacteria. Int. J. Food Microbiol. 105, 281–295.

Miele, A., Bandera, M., Goldstein, B.P., 1995. Use of primers selective for vancomycin resistance genes to determine *van* genotype in enterococci and to study gene organization in VanA isolates. Antimicrob. Agents Chemother. 39, 1772–1778.

Nawaz, M., Wang, J., Zhou, A., Ma, C., Wu, X., Moore, J.E., Cherie Millar, B., Xu, J., 2010. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Curr. Microbiol. 62, 1081–1089.

Neyfakh, A.A., Borsch, C.M., Kaatz, G.W., 1993. Fluoroquinolone resistance protein NorA of *Staphylococcus aureus* is a multidrug efflux transporter. Antimicrob. Agents Chemother. 37, 128–129.

Okusu, H., Ma, D., Nikaido, H., 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. 178, 306–308.

Ouoba, L.I., Lei, V., Jensen, L.B., 2008. Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: determination and transferability of the resistance genes to other bacteria. Int. J. Food Microbiol. 121, 217–224.

Patel, D., Kosmidis, C., Seo, S.M., Kaatz, G.W., 2010. Ethidium bromide MIC screening for enhanced efflux pump gene expression or efflux activity in *Staphylococcus aureus*. Antimicrob. Agents Chemother. 54, 5070–5073.

Petersen, A., Jensen, L.B., 2004. Analysis of *gyrA* and *parC* mutations in enterococci from environmental samples with reduced susceptibility to ciprofloxacin. FEMS Microbiol. Lett. 231, 73–76.

Piddock, L.J.V., 2006. Multidrug-resistance efflux pumps—not just for resistance. Nat. Rev. 4, 629–636.

Putman, M., van Veen, H.W., Degener, J.E., Konings, W.N., 2001. The lactococcal secondary multidrug transporter LmrP confers resistance to lincosamides, macrolides, streptogramins and tetracyclines. Microbiology 147, 2873–2880.

Salyers, A.A., Gupta, A., Wang, Y., 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol. 12, 412–416.

Servin, A.L., 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol. Rev. 28, 405–440.

Singh, K.V., Murray, B.E., 2005. Differences in the *Enterococcus faecalis* Isa locus that influence susceptibility to quinupristin-dalfopristin and clindamycin. Antimicrob. Agents Chemother. 49, 32–39.

Stock, I., Wiedemann, B., 2001. Natural antibiotic susceptibilities of *Edwardsiella tarda*, *E. ictaluri*, and *E. hoshinae*. Antimicrob. Agents Chemother. 45, 2245–2255.

Sutcliffe, J., Grebe, T., Tait-Kamradt, A., et al., 1996. Detection of erythromycin-resistant determinants by PCR. Antimicrob. Agents Chemother. 40, 2562–2566.

Swick, M.C., Morgan-Linnell, S.K., Carlson, K.M., Zechiedrich, L., 2011. Expression of multidrug efflux pump genes *acrAB-tolC*, *mdfA*, and *norE* in *Escherichia coli* clinical isolates as a function of fluoroquinolone and multidrug resistance. Antimicrob. Agents Chemother. 55, 921–924.

Toomey, N., Bolton, D., Fanning, S., 2010. Characterisation and transferability of antibiotic resistance genes from lactic acid bacteria isolated from Irish pork and beef abattoirs. Res. Microbiol. 161, 127–135.

Truong-Bolduc, Q.C., Zhang, X., Hooper, D.C., 2003. Characterization of NorR protein, a multifunctional regulator of *norA* expression in *Staphylococcus aureus*. J. Bacteriol. 185, 3127–3138.

Turnidge, J.D., Bell, J.M., 2005. Antimicrobial susceptibility on solid media. In: Lorian, V. (Ed.), Antibiotics in Laboratory Medicine. Williams and Wilkins, Baltimore, pp. 8–60.

Vakulenko, S.B., Donabedian, S.M., Voskresenskiy, A.M., Zervos, M.J., Lerner, S.A., Chow, J.W., 2003. Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. Antimicrob. Agents Chemother. 47, 1423–1426.

van Reenen, C.A., Dicks, L.M., 2011. Horizontal gene transfer amongst probiotic lactic acid bacteria and other intestinal microbiota: what are the possibilities? A review. Arch. Microbiol. M193, 157–168.

Wegener, H.C., 2003. Antibiotics in animal feed and their role in resistance development. Curr. Opin. Microbiol. 6, 439–445.

Wood, B.J.B., Holzapfel, W.H., 1995. The Genera of Lactic Acid Bacteria. Blackie Academic and Professional, London.

Zhang, X.X., Zhang, T., Fang, H.H., 2009. Antibiotic resistance genes in water environment. Appl. Microbiol. Biotechnol. 82, 397–414.

Artículo 2

Biocide tolerance, phenotypic and molecular response of Lactic Acid Bacteria isolated from naturally-fermented Aloreña table to different physico-chemical stresses

Submitted to Food Microbiology

Biocide tolerance, phenotypic and molecular response of Lactic Acid Bacteria isolated from naturally-fermented Aloreña table to different physico-chemical stresses.

María del Carmen Casado Muñoz[1], Nabil Benomar[1], Leyre Lavilla Lerma[1], Charles W. Knapp[2], Antonio Gálvez[1], Hikmate Abriouel[1]*

[1]*Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaén, 23071-Jaén, Spain.*

[2]*Department of Civil and Environmental Engineering, University of Strathclyde, Glasgow, Scotland, United Kingdom.*

[*]*Corresponding author. Present address: Área de Microbiología. Departamento de Ciencias de la Salud. Facultad de Ciencias Experimentales. Edif. B3. Universidad de Jaén. Campus Las Lagunillas s/n. 23071-Jaén, Spain. Tel.: 34-953-212003; fax: 34-953-212943.*

E-mail address: hikmate@ujaen.es

Abstract

Lactic acid bacteria (LAB) isolated throughout the fermentation process of Aloreña table olives were found to be resistant at least to three antibiotics (Casado Muñoz et al., 2014); however, most were sensitive to the biocides tested in this study (with minimum inhibitory concentrations [MIC] below the epidemiological cut-off values). 2-15% of the isolates were found to be biocide resistant: *Leuconostoc Pseudomesenteroides*, which were resistant to hexachlorophene, and *Lactobacillus pentosus* to cetrimide and hexadecylpiridinium.

We analysed the effect of different physico-chemical stresses, including antimicrobials, on the phenotypic and genotypic responses of LAB, providing new insights on how they become resistant in a changing environment. Results indicated that similar phenotypic responses were obtained under three stress conditions: antimicrobials, chemicals and UV light. Susceptibility patterns to antibiotics changed: increasing MICs for ampicillin, chloramphenicol, ciprofloxacin, teicoplanin and tetracycline, and decreasing the MICs for clindamycin, erythromycin, streptomycin and trimethoprim in most strains. Statistically, cross resistance between different antibiotics was detected in all stress conditions. However, expression profiles of selected genes involved in stress/resistance response (*rpsL, recA, uvrB* and *srtA*) differed depending on the stress parameter, LAB species and strain, and the target gene.

We conclude that, despite the uniform phenotypic response to stresses, the repertoire of induced and repressed genes differs. So, a search for a target to improve stress tolerance of LAB, especially those of importance as starter/protective cultures or probiotics, may depend on the individual screening of each strain, even though we could predict the antibiotic phenotypic response to all stresses.

Keywords:

Lactobacillus pentosus, *Leuconostoc pseudomesenteroides*, antibiotics, biocides, stress, quantitative PCR, gene expression

I. INTRODUCTION

Lactic acid fermented foods have been consumed globally for millions of years because of their nutritional value, extensive shelf-life and especially their cultural value. Lactic acid bacteria (LAB), a heterogeneous group of Gram-positive bacteria found widespread in many environments (Schleifer and Ludwig, 1995), are the main microorganisms carrying out the fermentation processes on the vegetables, dairy and meats. They are also used as starter or protective cultures (Caplice and Fitzgerald, 1999; Leroy and De Vuyst, 2004; Wood and Holzapfel, 1995) and as probiotics (Kechagia et al., 2013; Servin, 2004) due to their long history of safe use (Anadon et al., 2006; EFSA, 2007) and several strains having "QPS" (Qualified Presumption of Safety) status (EFSA, 2007). Foodborne LAB are vehicles of antibiotic resistance (AR) genes similar to those found in human pathogens (Korhonen et al., 2010; Mathur and Singh, 2005; Teuber et al., 1999). As such, fermented foods also represent vectors by which AR bacteria can be spread to humans (Franz et al., 2005; Klein et al., 1988; Ross et al., 2002; Reid et al., 2003; Picard et al., 2005). In fact, international organizations have launched criteria addressing the biosafety concerns of starter cultures and probiotic microorganisms; however, nothing could be done with spontaneous fermentations that rely on indigenous microbiota. LAB generally exhibit intrinsic resistance to many antibiotics (e.g., via lowered permeability, enzymatic inactivation and alteration of the target compounds) (Poole, 2002), but also acquired resistance due to chromosomal mutation and mobile genetic elements (e.g., plasmids, transposons, and integrons). The greatest concern is the horizontal gene transfer (Ammor et al., 2007, 2008) due to the higher potential for horizontal dissemination of AR genes (Khachatourians, 1998; European Commission, 2008) to other species.

The emergence of AR has mainly been due to the over-use or the misuse of antimicrobial agents for improper infection control, animal husbandry, and agriculture (Wegener, 2003; Munsch-Alatossava and Alatossava, 2007; Dixon, 2000; Feinman, 1999; SCAN, 1996), including the prophylactic use of antibiotics as animal growth promotion over the last few decades (nowadays banned in several countries), which have contributed to the generation of resistant bacteria in animals and environment. Furthermore, the increased use of biocides as disinfectants (e.g., in clinical setting, industry, and home) at concentrations below the minimum inhibitory concentration (MIC) of targeted bacteria (Holah, 2000) may generate pressures for cross-resistance with antibiotics (Russell, 2000; Meyer and Cookson, 2010; Lavilla Lerma et al., 2015).

Thus, these compounds contribute to AR emergence and decreased treatment efficacy (Chapman, 2003). Sub-inhibitory concentrations of antimicrobials trigger several bacterial responses to ensure their survival: adaptation, mutation, acquisition of mobile resistance genes by horizontal gene transfer (e.g., transformation, conjugation and transduction), over-expression of resistance genes, and efflux pumps (Pearce et al., 1999; Poole, 2002, 2004, 2007; Huet et al., 2008). The genetic basis for the development of AR depends on the genetic fitness of each bacterial strain and available response strategies.

Here, we evaluated biocide tolerance of several antibiotic resistant LAB (Casado Muñoz et al., 2014) isolated from naturally-fermented Aloreña table olives during its fermentation process. Furthermore, we investigated the phenotypic and genotypic responses of LAB to different physico-chemical stressors, including antimicrobials. This is of great relevance in order to understand and explain the increased resistance of these bacteria under changing environmental conditions.

II. MATERIAL AND METHODS

II.1. Bacterial strains and growth conditions. 73 LAB strains, including 13 *Leuconostoc pseudomesenteroides* and 60 *Lactobacillus pentosus* strains isolated from naturally-fermented Aloreña green table olives (Abriouel et al., 2012), were used in this study. These strains were routinely cultured at 30ºC either in Man Rogosa and Sharpe (MRS) broth (Fluka, Madrid, Spain) or on agar under aerobic conditions for 24-48 h. Strains were kept in 20% glycerol at -80ºC for long-term storage.

II.2. Antimicrobial agents. Antimicrobial agents (Tables 1 and 2) included various biocides used in food industry and clinically relevant antibiotics. All antimicrobials were purchased from Sigma Aldrich (Madrid, Spain), except triclosan, which was obtained from Fluka (Madrid, Spain).

II.3. Biocide susceptibility testing, MIC and ECOFF determination. The MICs of the aforementioned biocides were measured in a concentration range from 0.001 µg/mL to 10 µg/mL in LSM broth [90% of IST broth (Oxoid, Madrid, Spain) and 10% MRS broth (Fluka, Madrid, Spain)] (Klare et al., 2005) according to the ISO 10932/IDF 233 standard (ISO, 2010). MICs of all biocides were determined using the NCCLS broth-microdilution method (NCCLS, 2000). After incubation, the MIC was read as the lowest concentration of each antimicrobial agent that inhibited the growth of the strain. All MIC determinations of each antimicrobial against each strain were carried out in triplicate, and reliable results were taken if at least two of the three replicates were in agreement.

ECOFF is defined, from a unimodal distribution of antimicrobial MIC perbacterial species, as the concentration representing ≥ 95% (MIC_{95}) of bacterial populations (Pfaller et al., 2010). These values were determined as reported by Lavilla Lerma, et al. (2015).

II.4. Induction of resistance by different physico-chemical stresses. For induction experiments, seven strains of LAB [*Lc. pseudomesenteroides* AP2-28 and *Lb. pentosus* strains (CF1-16, CF1-25, CF1-35, CF2-11, CF2-15P and CF2-19P)] were selected on the basis of their phenotypic and genotypic resistance profile (Casado Muñoz et al., 2014), and we also included the potentially probiotic strain *Lb. pentosus* MP-10 isolated from naturally-fermented Aloreña green table olives (Abriouel et al., 2011).

Antimicrobials (antibiotics or biocides) were selected on the basis of susceptibility results.

Overnight cultures of different cells were diluted 1:100 in fresh MRS broth and challenged against either triclosan (1 µg/mL), benzalkonium chloride (1 µg/mL), chloramphenicol (5 µg/mL), tetracycline (10 µg/mL) or amoxicillin (0.1 µg/mL). Cells were incubated at 30°C for 48 h and then centrifuged.

Induction of SOS response, a global response to DNA damage involving DNA repair and mutagenesis, was carried out by exposing overnight culture (2 ml) to germicidal UV light (254 nm) for 1, 5 and 10 min, and incubated for 3h at 30°C. After incubation, induced cells were harvested by centrifugation.

To induce the expression of genes coding for multidrug efflux proteins such as NorA and AcrA/B, 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) (Yu et al., 2002) or ethanol (4%) and/or sodium chloride (0.5 M) (Ma et al., 1995) were added, respectively. In all cases, induced cells were cryogenically stored in 20% glycerol at -80ºC until use.

II.5. Antimicrobial susceptibility of induced cells.

MICs of antibiotics tested in this study were determined as reported by Casado Muñoz, et al. (2014). The ECOFFs of antibiotics used in this study were reviewed by Casado Muñoz et al. (2014) as 2 µg/ml for ampicillin in *Lb. pentosus* and *Lc. pseudomesenteroides*; 8 and 4 µg/ml for chloramphenicol in *Lb. pentosus* and *Lc. pseudomesenteroides*, respectively; 4 and 32 µg/ml for ciprofloxacin in *Lb. pentosus* and *Lc. pseudomesenteroides*, respectively; 2 and 1 µg/ml for clindamycin in *Lb. pentosus* and *Lc. pseudomesenteroides*, respectively; 16 µg/ml for erythromycin in *Lb. pentosus* and *Lc. pseudomesenteroides*; >256 and 64 µg/ml for streptomycin in *Lb. pentosus* and *Lc. pseudomesenteroides*, respectively; 32 µg/ml for teicoplanin in *Lb. pentosus* and *Lc. pseudomesenteroides*; 32 and 8 µg/ml for tetracycline in *Lb. pentosus* and *Lc. pseudomesenteroides*, respectively; 8 µg/ml for trimethoprim in *Lb. pentosus* and *Lc. pseudomesenteroides*. Regarding biocides, MICs were determined as described above. There are no data are available about the ECOFF in LAB and the maximum residue level (MRL) recommended by EFSA for each biocide used as disinfectant or pesticide were of 0.01 mg/kg for cetrimide and hexachlorophene, 0.1 mg/kg for benzalkonium chloride and didecyldimethylammonium bromide, 0.2% for triclosan and 5 µg/ml for chlorhexidine (Database of MRLs in the EU; EFSA, 2014).

II.6. Quantitative reverse-transcriptase PCR of stress/resistance following gene expression following induction.

II.6.1. RNA extraction. The design of expression assays of stress/resistance genes (Table 3; *rpsL*, *recA*, *srtA* and *uvrB*) was based on the annotated genome sequence of *Lb. pentosus* MP-10 (Abriouel et al., 2011), and gene transcripts were quantitatively determined after induction of cells under different treatments. Total RNA was isolated in triplicate using the Illustra RNAspin Mini RNA Isolation Kit (GE-Healthcare, Madrid, Spain) according to the manufacturer's instructions. Cells similarly handled without induction were used for comparison. RNA quantification and quality assessment were carried out by UV spectrometry (NanoDrop 2000; Thermo Scientific). RNAs were adjusted to a concentration of 500 ng/ml and frozen at -80ºC until required.

II.6.2. RNA Quantitative reverse transcriptase PCR of stress/resistance genes. For quantitative reverse-transcriptase PCR (qRT-PCR), extracted mRNAs were reverse transcribed to cDNA and quantified via real-time PCR in a single reaction using the SensiFast SYBR & Fluorescein One-Step kit (Bioline, Barcelona, Spain). 16S rRNA gene was used as a housekeeping gene, and a no template control (NTC) was used as negative control. Primers for housekeeping and resistance genes are reported in Table 3. Quantitative PCRs (qPCRs) were performed in triplicate on a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Madrid, Spain). PCR-grade water served as a negative control.

II.7. Statistical analysis. The relationship between physico-chemical stress (antimicrobials, salt and ethanol) and increases in antibiotic MIC was investigated. Pearson correlation coefficients (r) were calculated and determined according to Lavilla Lerma, et al. (2015). Correlations were categorically classified according to Dancey and Reidy (2004), being “strong” correlation when the coefficient is between 0.7-0.9, “moderate” 0.4-0.6, and “weak” 0.1-0.3. In all analyses, a *p*-value <0.05 was considered significant.

III. RESULTS.

III.1. Biocide susceptibility and ECOFF determination. LAB isolated from Aloreña green table olives were generally very sensitive to biocides (75-100% with MIC ≤ 1 µg/ml); however, up to 25% of LAB showed moderate sensitivity to certain biocides (1< MIC ≤ 5 µg/ml) especially *Lb. pentosus* strains to didecyldimethylammonium bromide, triclosan and benzalkonium chloride (Table 1, Fig. 1).

ECOFF values were, in most cases, similar to both LAB species (*Lb. pentosus* versus *Lc. pseudomesenteroides*) except for hexadecylpyridinium chloride and hexachlorophene. The LAB analyzed in this study were, in most cases, sensitive to the biocides. However, 1.66% of the *Lb. pentosus* were resistant to cetrimide or hexadecylpyridinium chloride, and 46% of *Lc. pseudomesenteroides* were resistant to hexadecylpiridinium (Table 1, Fig. 1).

III.2. LAB phenotypic response to physico-chemical stresses. Phenotypic responses to stress induction with different antimicrobials (antibiotics and biocides) were generally similar in all strains analyzed (Table 2). The MICs of ampicillin, chloramphenicol, ciprofloxacin, teicoplanin and tetracycline increased by 340-1000 fold (Table 2). Similarly, after UV light exposure at different time periods (1, 5 and 10 min), the MICs of the mentioned antibiotics increased by 340-750 fold (especially for chloramphenicol and teicoplanin) regardless of the exposure time in most cases (Table 4). Cells induced by IPTG or NaCl+ethanol showed similar results (Table 5); however, when cells were induced by either ethanol or NaCl, MIC increases were observed to the same aforementioned antibiotics and additionally to clindamycin (Table 5).

On the other hand, we observed a decrease in MICs for some antibiotics after induction of different stresses (antimicrobials, UV and chemicals) such as clindamycin, erythromycin, streptomycin and trimethoprim (Tables 2, 4 and 5). Furthermore, the MIC to ampicillin, teicoplanin and ciprofloxacin occasionally declined in some strains (Tables 2, 4 and 5).

III.3. Correlation between MIC of antibiotics and different stresses in LAB. Table 6 shows the strength of the pair-wise relationships of antibiotic MIC increase following stress induction. Pearson's correlation coefficient analysis of values (log-transformed) showed a moderate-strong correlation between some antibiotics especially those exposed to antimicrobials (antibiotics or biocides) (Table 6A). As such, almost all antibiotics significantly correlated with one or two other antibiotics, except for ampicillin, which correlated with three antibiotics (Table 6A). Moderate positive correlations were observed between ampicillin and ciprofloxacin, clindamycin or teicoplanin ($0.372 < r < 0.498$), and also between clindamycin and tetracycline ($r = 0.687$), teicoplanin and trimethoprim ($r = 0.533$), and ciprofloxacin and clindamycin ($r = 0.361$). However, moderate negative correlations were obtained between chloramphenicol and ciprofloxacin ($r = -0.409$) and also between streptomycin and teicoplanin ($r = -0.335$) (Table 6A).

Concerning LAB induced by UV exposure, the MIC increases of some antibiotics positively correlated (Table 6B); moderate correlations were observed between chloramphenicol and teicoplanin ($r = 0.682$), clindamycin and trimethoprim ($r = 0.649$), and ampicillin and tetracycline ($r = 0.528$). However, moderate negative correlation was only obtained between teicoplanin and tetracycline ($r = -0.511$).

Induction with chemicals such as IPTG or ethanol and/or sodium chloride produced strong positive correlation (Table 6C) between chloramphenicol and teicoplanin ($r = 0.739$), and moderate positive correlation between ampicillin and ciprofloxacin ($r = 0.535$). However, a moderate negative correlation was only observed between teicoplanin and tetracycline ($r= -0.483$).

III.4. Genotypic response of LAB to physico-chemical stresses. We selected two strains: *Lb. pentosus* MP-10 and *Lc. pseudomesenteroides* AP2-28 to study the effect of stress on the expression of stress tolerance/resistance genes: *rpsL*, *recA*, *uvrB* and *srtA*. The responses of both bacteria differed depending on the inducer and the gene analyzed (Fig. 2). In respect to *rpsL* gene, up-regulation was observed in *Lc. pseudomesenteroides* AP2-28 after exposure to chloramphenicol, tetracycline, ethanol, NaCl or UV light exposure (5 and 10 min), with transcript abundances increasing 4-5 orders of magnitude (Fig. 2A). Similarly, up-regulation of *rpsL* gene was observed after *Lb. pentosus* MP-10 were exposed to chloramphenicol; however, the same bacteria down-regulated the *rpsL* gene following amoxicillin, benzalkonium and triclosan treatments (Fig. 2A).

RecA expression showed an opposite pattern than the *rpsL* gene (Fig. 2B). The *recA* gene in *Lc. pseudomesenteroides* AP2-28 became down-regulated following amoxicillin, benzalkonium, IPTG, ethanol and NaCl+ethanol induction; and *Lb. pentosus* MP-10 similarly down-regulated *recA* with NaCl (Fig. 2B). However, *Lb.*

pentosus MP-10 up-regulated their *recA* gene when induced by tetracycline and UV exposure (5 and 10 min).

Concerning the *srtA* gene, down-regulation was observed for both *Lc. pseudomesenteroides* AP2-28 and *Lb. pentosus* MP-10 following induction with chloramphenicol, tetracycline and benzalkonium, IPTG and UV exposure (5 and 10 min) (Fig. 2C). Furthermore, amoxicillin, ethanol and NaCl+ethanol down-regulated *srtA* in *Lc. pseudomesenteroides* AP2-28, and NaCl did the same in *Lb. pentosus* MP-10 (Fig. 2C).

Down-regulation of *uvrB* gene expression was observed after stress from amoxicillin, benzalkonium and triclosan, IPTG, ethanol and NaCl+ethanol for *Lc. pseudomesenteroides* AP2-28 (Fig. 2D). However, *uvrB* gene expression was down regulated in *Lb. pentosus* MP-10 after exposure from benzalkonium, IPTG and NaCl (Fig. 2D). On the other hand, we also observed up-regulation of the *uvrB* gene after induction to *Lb. pentosus* MP-10 with chloramphenicol and UV exposure for 5 min (Fig. 2D).

III.5. Correlation between resistance genes in LAB induced by several physico-chemical stresses. Table 7 shows the correlations between expression levels of different resistance genes (*rpsL*, *recA*, *uvrB* and *srtA*) in *Lb. pentosus* MP-10 and *Lc. pseudomesenteroides* AP2-28 following induction with physico-chemical stress. A unique strong positive correlation was detected between *recA* and *uvrB* genes in both *Lc. pseudomesenteroides* AP2-28 (r = 0.930) and *Lb. pentosus* MP-10 (r= 0.770). Concerning the rest of the genes, no correlations were obtained (Table 7).

Discussion

LAB face an array of chemical and environmental stresses in their natural habitats, during food processing and storage, and during passage through gastrointestinal tract. Adaptive responses involve changes in their physiology, behavior and genetic by means of several mechanisms of stress response (Chung et al., 2006; Foster, 2007; Ryall et al., 2012). Phenotypic switching is one strategy adopted by bacteria to ensure their survival and persistence in various environmental conditions (Sousa et al., 2011). In this case, LAB have developed several adaptation strategies to survive in changing and adverse conditions (e.g., pH, salt concentration, temperature, nutrient concentration, etc.) that include the alteration of cellular physiology by controlling stress-response gene expression. The results obtained in this study indicated that stress induction by antimicrobials (biocides or antibiotics), chemicals or UV light changed the pattern of antibiotic susceptibility in LAB isolated from naturally fermented Aloreña table olives. However, at a genotypic level, several stress genes were differentially expressed depending on LAB species and strain, and stressor. Induction patterns were not very clear. Correlating phenotypic and genotypic responses will provide new insights on how bacteria become resistant in a changing environment.

The indiscriminate use of antimicrobials as therapeutic agents and as disinfectants frequently contribute to the development of antimicrobial resistance (Davin-Regli and Pagès, 2012; Dixon, 2000; Hawkey, 2008; McBain et al., 2002). As reported by Casado Muñoz et al. (2014), most *Lb. pentosus* (95%) and all *Lc. pseudomesenteroides* analyzed in the present study were resistant to at least three antibiotics (multidrug resistant). However, here, we showed that they are generally sensitive to biocides (MIC below determined ECOFF), with only 2-15% of bacteria showing any resistance to hexachlorophene (in *Lc. Pseudomesenteroides*) and cetrimide and hexadecylpiridinium in (*Lb. pentosus*). Exposing LAB with sub-lethal concentrations of antibiotics or biocides (sub-MICs) resulted in changes in susceptibility patterns with increased MICs for ampicillin, chloramphenicol, ciprofloxacin, teicoplanin and tetracycline, while decreased MICs were shown for clindamycin, erythromycin, streptomycin and trimethoprim in most strains, which in most cases were originally susceptible to the corresponding antibiotics. Previously, Casado Muñoz et al. (2014) reported that similar LAB used in this study were chloramphenicol-sensitive and resistant to streptomycin and trimethoprim; however, in the present study, susceptibility pattern became inverted after induction with sub-lethal concentrations of antimicrobials.

The emergence of new resistance patterns in LAB induced by sub-lethal concentrations of antimicrobials has ecological and evolutionary consequences. Sub-lethal concentrations of antibiotics could affect mutation rates, horizontal gene transfer and biofilm formation in the natural habitat of LAB and may contribute to the emergence and spread of AR in the environment and food chain (Laureti et al., 2013). Moreover, many authors report that stressors such as heat, salt, acidic and alkaline pH can significantly alter phenotypic AR in food-related pathogens (e.g., Al-Nabulsi et al., 2011; Ganjian et al., 2012; McMahon et al., 2007a, b). In a similar manner, exposure to chemicals or UV light (5 or 10 min) produced the same effects as antimicrobials on antibiotic MICs.

The data suggest that changes in susceptibility patterns following induction in LAB may be due to either the alterations of cell membrane permeability to the corresponding antibiotics or expression profiles of resistance/stress genes. Generally, positive correlations between antibiotics representing different classes and cellular targets suggest either co-resistance (resistance traits carried by the same mobile genetic element such as plasmid, transposon or integron) or cross-resistance mechanisms. In this study, the positive correlations following antimicrobial stress suggest cross-resistance, which rely on unspecific resistance mechanisms with a wide range of activity against antibiotics, such as efflux pumps. Correlations were shared by different physico-chemical stressors (antimicrobials, UV exposure and chemicals) and a repertoire of similar phenotypic and genotypic mechanisms were induced under different conditions. Although phenotypic responses to stress were generally similar under different stress conditions, expression patterns of selected genes involved in tolerance/resistance mechanisms showed differential stress responses.

To determine the mechanisms adopted by LAB to withstand hostile conditions, knowledge about inducible genes under each stress condition is of crucial importance since a balance of different responses is involved in tolerance/resistance. The *rpsL* gene, which encodes the ribosomal protein S12, was over-expressed after induction with all stressors (antibiotics, NaCl, ethanol and UV light) except amoxicillin and biocides. This protein with polyspecific effects may act as RNA chaperone, as reported by Coetzee et al. (1994), with the aim to protect the ribosome structure and function under stress. Increasing *rpsL* gene expression was generally responsible, except for few cases, for increased MICs of ampicillin, chloramphenicol, ciprofloxacin, teicoplanin and tetracycline.

However, *recA* gene was only over-expressed after induction with tetracycline and UV light (5 and 10 min), while an under-expression of this gene was observed in LAB-induced by biocides, amoxicillin, IPTG, NaCl, ethanol and NaCl+ethanol. The gene *recA* encodes the RecA protein, which has a role in SOS regulation system and in many DNA repair pathways such as repairing daughter-strand gaps, double strand breaks and SOS mutagenesis (Cox, 2007). When the SOS response is activated, several genes involved in repair, replication, recombination and cell division are expressed—including the *recA* gene, and genes related to nucleotide excision repair (NER) system (*uvrA*, *uvrB*, *uvrC* and *uvrD* genes). In this study, *uvrB* gene was down regulated under all stress conditions, except after induction with chloramphenicol and UV light during 5 min. It is noteworthy that *recA* and *uvrB* genes had paired expression patterns and being involved in the same repair mechanisms, which were activated only in the case of antibiotics (chloramphenicol or tetracycline) and UV light exposure. Statistical analysis in the present study supports this fact. Thus, the SOS response as an inducible pathway (Van der Veen and Abee, 2011) may partly be involved in the increased MICs of antibiotics after induction with chloramphenicol, tetracycline or UV light; strong positive correlations were obtained for both genes. On the other hand, *srtA* virulence gene, which encodes an enzyme that anchors surface proteins to the cell wall (Mazmanian et al., 199), was under-regulated under all stress conditions. The genes *srtA* and *srtB* are responsible for cross-linking LPXTG motif-containing surface proteins to peptidoglycan, thus the down-regulation of *srtA* gene under stress condition may be responsible for decreased MICs of clindamycin, erythromycin, streptomycin and trimethoprim in the majority of cases by altering the permeability of cellular membranes. Hesketh et al. (2011) obtained similar results, with the repression of the gene encoding sortase under drug stress.

In general, to ensure survival, bacteria maintain the SOS response under tight control and rigorously transcribe select genes to adequately address the stress. So, the analysis of mechanisms adopted by *Lc. pseudomesenteroides* AP2-28 and *Lb. pentosus* MP-10 may help explain how LAB survive each stress condition, and why they change their susceptibility patterns. Induction assays by antibiotics inhibiting protein synthesis (chloramphenicol or tetracycline) caused over-expression of *rpsL* (*Lc. pseudomesenteroides* AP2-28 and *Lb. pentosus* MP-10), *recA* and *uvrB* (*Lb. pentosus*

MP-10) genes; however, down-regulation of the *srtA* gene was observed (*Lb. pentosus* MP-10 and *Lc. pseudomesenteroides* AP2-28). Thus, as mentioned above, to survive under antibiotic stress LAB use repair and protection mechanisms by switching on involved genes such as *rpsL*, *recA* and *uvrB*—depending on the antibiotic used and the LAB strain—and thus increasing the MICs of some antibiotics. In parallel, the down-regulation of *srtB* gene may be responsible for the higher sensitivity of LAB to other antibiotics (clindamycin, erythromycin, streptomycin and trimethoprim). However, induction assays by biocides (benzalkonium or triclosan) caused down-regulation of *rpsL* (*Lb. pentosus* MP-10), *recA* (*Lc. pseudomesenteroides* AP2-28), *srtA* and *uvrB* (*Lb. pentosus* MP-10 and *Lc. pseudomesenteroides* AP2-28) genes. Thus, induction by antibiotics or biocides does not involve the same repertoire of genes (except for the down-regulated *srtA* gene) or mechanisms since both antimicrobials have different cellular targets; furthermore, SOS response was not activated by biocide stress. The similar phenotypic response to antibiotic/biocide stress may suggest that other specific genes are involved in this response, or unspecific mechanisms may be involved such as efflux pumps (Buffet-Bataillon et al., 2012).

Regarding chemicals, induction with either NaCl, ethanol, or both resulted in the same expression profil—over-expressing *rpsL* gene (*Lc. pseudomesenteroides* AP2-28) and down-regulating *recA*, *uvrB* and *srtA* genes (*Lb. pentosus* MP-10 induced by NaCl and *Lc. pseudomesenteroides* AP2-28 induced by ethanol or NaCl+ethanol). Over-expression of *rpsL* gene was attributed to its role in ribosome protection as mentioned previously; however, the down-regulation of genes involved in SOS response suggest that salt and ethanol stresses do not activate this system in LAB. As reported previously for antimicrobial stress, *srtA* gene down-regulation was related with membrane permeability alteration. On the other hand, UV light exposure over-expressed *rpsL*, *recA* and *uvrB* genes suggesting that both repair and protection mechanisms were induced by UV exposure, while *srtA* gene was repressed as in other cases.

Conclusions

We conclude that, in spite of the uniform phenotypic responses to several stresses, the repertoire of induced and repressed genes differed according to the type of stress and LAB strain; although, strong positive correlations were obtained with *recA* and *uvrB* genes under several stress conditions. So, the search for a mechanism to improve stress tolerance of LAB, especially those with importance as starter/protective cultures or probiotics, will depend on individual screening of each strain; although, we can predict the antibiotic phenotypic response to stress with this data. Overall we speculate that *rpsL* gene could be a biomarker of robustness/resistance in LAB, although further studies must be required to elucidate the correlation between induced *rpsL* gene with robustness.

Acknowledgments

This work was supported by grants AGL2013-43571-P (Ministerio de Economía y Competitividad, MINECO, FEDER), AGL2009-08921 (Ministerio de Economía y Competitividad, MINECO) and UJA2014/07/02 (Plan Propio de la Universidad de Jaén). The authors are grateful to technical and human support provided by CICT of University of Jaén (UJA, MINECO, Junta de Andalucía, FEDER).

References

Abriouel, H., Benomar, N., Pérez Pulido, R., Martínez Cañamero, M., Gálvez, A., 2011. Annotated genome sequence of *Lactobacillus pentosus* MP-10 with probiotic potential from naturally-fermented Alorena green table olives. J. Bacteriol. 193, 4559-4560.

Abriouel, H., Benomar, N., Cobo, A., Caballero, N., Fernández Fuentes, M.A., Pérez-Pulido, R., Gálvez, A., 2012. Characterization of lactic acid bacteria from naturally-fermented Manzanilla Aloreña green table olives. Food Microbiol. 32, 308-316.

Al-Nabulsi, A.A., Osaili, T.M., Elabedeen, N.A., Jaradat, Z.W., Shaker, R.R., Kheirallah, K.A., Tarazi, Y.H., Holley, R.A., 2011. Impact of environmental stress desiccation, acidity, alkalinity, heat or cold on antibiotic susceptibility of *Cronobacter sakazakii*. Int. J. Food Microbiol. 146, 137-143.

Ammor, M.S., Florez, A.B., Mayo, B., 2007. Antibiotic resistance in nonenterococcal lactic acid bacteria and bifidobacteria. Food Microbiol. 24, 559-570.

Ammor, M.S., Florez, A.B., vanHoek, A.H., de los Reyes-Gavilan, C.G., Aarts, H.J., Margolles, A., Mayo, B., 2008. Molecular Characterization of Intrinsic and Acquired Antibiotic Resistance in Lactic Acid Bacteria and Bifidobacteria. J. Mol. Microbiol. Biotechnol. 14, 6-15.

Anadon, A., Martinez-Larranaga, M.R., Martinez, M.A., 2006. Probiotics for animal nutrition in the European Union. Regulation and safety assessment. Regul. Toxicol. Pharmacol. 45, 91–95.

Buffet-Bataillon, S., Le Jeune, A., Le Gall-David, S., Bonnaure-Mallet, M., Jolivet-Gougeon, A., 2012. Molecular mechanisms of higher MICs of antibiotics and quaternary ammonium compounds for *Escherichia coli* isolated from bacteraemia. J. Antimicrob. Chemother. 67, 2837-2842.

Caplice, E., Fitzgerald, G.F., 1999. Food fermentations: role of microorganisms in food production and preservation. Int. J. Food Microbiol. 50, 131–149.

Casado Muñoz, M.C., Benomar, N., Lavilla Lerma, L., Gálvez, A., Abriouel, H., 2014. Antibiotic resistance of *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* isolated from naturally-fermented Aloreña table olives throughout fermentation process. Int. J. Food Microbiol. 172, 110-118.

Chapman, J.S., 2003. Biocide resistance mechanisms. Int Biodeter. Biodegrad. 51, 133-138.

Coetzee, T., Herschlag, D., Belfort, M., 1994. *Escherichia coli* proteins, including ribosomal protein S12, facilitate in vitro splicing of phage T4 introns by acting as RNA chaperones. Genes Dev. 8, 1575-1588.

Cho, G.-S., Hanak, A., Huch, M., Holzapfel, W.H., Franz C.M.A.P. 2010. Investigation into the Potential of Bacteriocinogenic *Lactobacillus plantarum* BFE 5092 for Biopreservation of Raw Turkey Meat. Probiotics Antimicro. Proteins 2, 241–249.

Chung, H.J., Bang, W., Drake, M.A., 2006. Stress Response of *Escherichia coli*. Compr. Rev. Food Sci. Food Saf. 5, 52-64.

Cox, M.M., 2007. Regulation of bacterial RecA protein function. Crit. Rev. Biochem. Mol. Biol. 42, 41-63.

Dancey, C., Reidy, J., 2004. Statistics without maths for psychology: using SPSS for windows. Prentice Hall, London.

Davin-Regli, A., Pagès, J.M., 2012. Cross-resistance between biocides and 393 antimicrobials: an emerging question. Rev. Sci. Tech. 31, 89-104.

Dixon B. 2000. Antibiotics as growth promoters: risks and alternatives. ASM News 66, 264–265.

EFSA (European Food Safety Authority), 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA J. 587, 1-16.

European Commission, 2008. Technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. EFSA J. 1-15.

European Food Safety Authority, 2014. Reasoned opinion on the dietary risk assessment for proposed temporary maximum residue levels (MRLs) of didecyldimethylammonium chloride (DDAC) and benzalkonium chloride (BAC). EFSA J. 12, 3675.

Feinman, S.E., 1999. Antibiotics in animal feeds—drug resistance revisited. ASM News 64, 24–29.

Foster, P.L., 2007. Stress-induced mutagenesis in bacteria. Crit. Rev. Biochem. Mol. Biol. 42, 373–397.

Franz, C.M.A.P., Hummel, A., Holzapfel, W.H., 2005. Problems related to the safety assessment of lactic acid bacteria starter cultures and probiotics. Mitt. Lebensm. Hyg. 96, 39–65.

Ganjian, H., Nikokar, I., Tieshayar, A., Mostafaei, A., Amirmozafari, N., Kiani, S., 2012. Effects of salt stress on the antimicrobial drug resistance and protein profile of *Staphylococcus aureus*. Jundishapur. J. Microbiol. 5, 328-331.

Hawkey, P.M., 2008. The growing burden of antimicrobial resistance. J. Antimicrob. Chemother. 62(S1), i1-9.

Hesketh, A., Hill, C., Mokhtar, J., Novotna, G., Tran, N., Bibb, M., Hong, H.-J., 2011. Genome-wide dynamics of a bacterial response to antibiotics that target the cell envelope. BMC Genomics 12, 226.

Holah, J.T., 2000. Cleaning and disinfection. In: Dennis, C., Stringer M.F. (eds.), Chilled Foods: a Comprehensive Guide ed. Woodhead Publishing Cambridge, UK, pp. 397–428.

Huet, A.A., Raygada, J.L., Mendiratta, K., Seo, S.M., Kaatz, G.W., 2008. Multidrug efflux pump overexpression in *Staphylococcus aureus* after single and multiple *in vitro* exposures to biocides and dyes. Microbiol. 154, 3144-53.

ISO (International Organization for Standardization), 2010. ISO 10932/IDF 233 standard milk and milk products—determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB).

Khachatourians, G.G., 1998. Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. Can. Med. Assoc. J. 159, 1129-1136.

Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., Fakiri, E.M., 2013. Health benefits of probiotics: a review. ISRN Nutr. 48165.

Klare, I., Konstabel, C., Müller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., Swings, J., Goossens, H., Witte, W., 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. Appl. Environ. Microbiol. 71, 8982-6.

Klein, G., A. Pack, and G. Reuter. 1998. Antibiotic resistance patterns of enterococci and occurrence of vancomycin-resistant enterococci in raw minced beef and pork in Germany. Appl. Environ. Microbiol. 64, 1825–1830.

Korhonen, J., 2010. Antibiotic Resistance of Lactic Acid Bacteria. Dissertation in Forestru and Natural Sciences. Department Bioscience, University of Eastern Finland Kuopio.

Laureti, L., Matic, I., Gutierrez, A., 2013. Bacterial Responses and Genome Instability Induced by Subinhibitory Concentrations of Antibiotics. Antibiotics 2, 100-114.

Lavilla Lerma, L., Benomar, N., Casado Muñoz, M.C., Gálvez, A., Abriouel, H., 2015. Correlation between antibiotic and biocide resistance in mesophilic and psychrotrophic *Pseudomonas* spp. isolated from slaughterhouse surfaces throughout meat chain production. Food Microbiol. 51, 33-44.

Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci. Technol. 15, 67–78.

Ma, D., Cook, D.N., Alberti, M., Pon, N.G., Nikaido, H. and Hearst, J.E., 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. 16, 45–55.

Mathur, S., Singh, R., 2005. Antibiotic resistance in food lactic acid bacteria-a review. Int. J. Food Microbiol. 105, 281–295.

Mazmanian, S.K., Liu, G., Ton-That, H., Schneewind, O., 1999. *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. Science 285, 760–763.

McBain, A.J., Rickard, A.H., Gilbert, P., 2002. Possible implications of biocide accumulation in the environment on the prevalence of bacterial antibiotic resistance. J. Ind. Microbiol. 29, 326–330.

McMahon, M.A.S., Xu, J., Moore, J.E., Blair, I.S., McDowell, D.A., 2007a. Environmental stress and antibiotic resistance in food-related pathogens. Appl. Environ. Microbiol. 73, 211–217.

McMahon, M.A.S., Blair, I.S., Moore, J.E., McDowell, D.A., 2007b. The rate of horizontal transmission of antibiotic resistance plasmids is increased in food preservation-stressed bacteria. J. Appl. Microbiol. 103, 1883–1888.

Meyer, B., Cookson, B., 2010. Does microbial resistance or adaptation to biocides create a hazard in infection prevention and control?. J. Hosp. Infect. 76, 200-205.

Munsch-Alatossava, P., Alatossava, T., 2007. Antibiotic resistance of raw milk associated psychrotrophic bacteria. Microbiol. Res. 162, 115–123.

NCCLS (National Committee For Clinical Laboratory Standards), 2000. Performance standards for antimicrobial disk susceptibility test-approved standard (document M2-A7). 7th ed. NCCLS, Pennsylvania, USA, ISBN 1-56-238208-X.

Patel, D., Kosmidis, C., Seo, S.M., Kaatz, G.W., 2010. Ethidium bromide MIC screening for enhanced efflux pump gene expression or efflux activity in *Staphylococcus aureus*. Antimicrob. Agents Chemother. 54, 5070-5073.

Pearce, H., Messager, S., Maillard, J-Y, 1999. Effect of biocides commonly used in the hospital environment on the transfer of antibiotic-resistance genes in *Staphylococcus aureus*. J. Hosp. Infect. 43, 101-8.

Pfaller, M.A., Andesb, D., Diekemaa, D.J., Espinel-Ingroffc, A., Sheehand, D., The CLSI Subcommittee for Antifungal Susceptibility Testing, 2010. Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and Candida: Time for harmonization of CLSI and EUCAST broth microdilution methods. Drug Resist. Updat. 13, 180-195.

Picard, C., Fioramonti, J., Francois, A., Robinson, T., Neant, F., Matuchansky, C., 2005. Review article: bifidobacteria as probiotic agents — physiological effects and clinical benefits. Aliment. Pharmacol. Ther. 22, 495-512.

Poole, K., 2002. Mechanisms of bacterial biocide and antibiotic resistance. J. Appl. Microbiol. 92, 55S–64S.

Poole, K., 2004. Acquired resistance. In: Fraise, A.P., Lambert, P.A., Maillard, J.-Y. (Eds.), Principles and practice of disinfection, preservation and sterilization. 4th ed. Oxford, Blackwell Scientific Publication, pp.170-83.

Poole, K., 2007. Efflux pumps as antimicrobial resistance mechanisms. Ann. Med. 39, 162-76.

Reid, G., Jass, J., Sebulsky, M.T., McCormick, J.K., 2003. Potential uses of probiotics in clinical practice. Clin. Microbiol. Rev. 16, 658-672.

Ross, R.P., Fitzgerald, G., Collins, K., Stanton, C., 2002. Cheese delivering biocultures - probiotic cheese. Aust. J. Dairy Technol. 57, 71-78.

Russell, A.D., 2000. Do biocides select for antibiotic resistance?. J. Pharm. Pharmacol. 52, 227-233.

Ben Ryall, G.E., Ferenci, T., 2012. Culture history and population heterogeneity as determinants of bacterial adaptation: the adaptomics of a single environmental transition. Microbiol. Mol. Biol. Rev. 76, 3597-625.

SCAN (Scientific Committee for Animal Nutrition), 1996. Report of the Scientific Committee for Animal Nutrition (SCAN) on the possible risk for humans on the use of avoparcin as feed additive. Office for EC Publications, Luxemburg City, Luxemburg.

Schleifer, K.H., Ludwig, W., 1995. Phylogenetic relationships of lactic acid bacteria. In: Wood, B.J.B., Holzapfel, W.H. (Eds.), The Genera of Lactic Acid Bacteria. Blackie Academic & Professional, London, pp. 7–18.

Servin, A.L., 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol. Rev. 28, 405–440.

Sousa, A.M., Machado, I., Pereira, M.O., 2011. Phenotypic switching: an opportunity to bacteria thrive 2011. In: Méndez-Vilas, A. (Ed.), Science against microbial pathogens: communicating current research and technological advances, pp. 252-262.

Teuber, M., Meile, L., Schwarz, F., 1999. Acquired antibiotic resistance in lactic acid bacteria from food. Anton. Leeuw. 76, 115–137.

Van der Veen, S., Abee, T., 2011. Bacterial SOS response: A food safety perspective. Curr. Opin. Biotech. 22, 136–142.

Wegener, H.C., 2003. Antibiotics in animal feed and their role in resistance development. Curr. Opin. Microbiol. 6, 439–445.

Wood, B.J.B., Holzapfel, W.H., 1995. The Genera of Lactic Acid Bacteria. Blackie Academic and Professional, London.

Yu, J.L., Grinius, L., Hooper, D.C., 2002. NorA functions as a multidrug efflux protein in both cytoplasmic membrane vesicles and reconstituted proteoliposomes. J Bacteriol. 184, 1370–1377.

Figure legends:

Figure 1. Susceptibility of *Lactobacillus pentosus* MP-10 (A) and *Leuconostoc pseudomesenteroides* AP2-28 (B) to biocides and determination of ECOFFs. Arrows indicated ECOFF value in each case.

Figure 2. Effect of physico-chemical stresses on expression of stress tolerance/resistance genes: *rpsL* (A), *recA* (B), *srtA* (C) and *uvrB* (D) in *Lb. pentosus* MP-10 and *Lc. pseudomesenteroides* AP2-28. Antimicrobials: benzalkonium chloride (BZ), triclosan (TC), amoxicillin (AMX), chloramphenicol (Cm) and tetracycline (TET). Et, ethanol.

Table 1. MIC distribution of seven biocides against LAB isolated from naturally-fermented Aloreña green table olives.

Biocide	**Species**	**No. of isolates with the following MIC range (µg/ml)**				ECOFF (µg/ml)	**% of resistant strains** [a]
		0.001<MIC≤0.01	**0.01<MIC≤0.1**	**0.1<MIC≤1**	**1<MIC≤5**		
Benzalkonium chloride	*Lb. pentosus*		17	27	16	5	0
	Lc. pseudomesenteroides		5	7	1	5	0
Cetrimide	*Lb. pentosus*	2	36	21	**1**	1	1.66
	Lc. pseudomesenteroides		8	5		1	0
Hexadecylpyridinium chloride	*Lb. pentosus*	3	26	30	**1**	1	1.66
	Lc. pseudomesenteroides	6	1	**6**		0.1	46
Chlorhexidine	*Lb. pentosus*	5	34	17	4	5	0
	Lc. pseudomesenteroides		9	3	1	5	0
Didecyldimethylammonium chloride	*Lb. pentosus*	1	23	19	17	5	0
	Lc. pseudomesenteroides		11	1	1	5	0
Hexachlorophene	*Lb. pentosus*	35	21	4		1	0
	Lc. pseudomesenteroides	11	2			0.1	0
Triclosan	*Lb. pentosus*		1	43	16	5	0
	Lc. pseudomesenteroides			12	1	5	0

Bold signifies the corresponding strains are resistant showing MIC value above the ECOFF.

[a] % of strains for which MICs are > ECOFF.

Table 2. Effect of antimicrobials on antibiotic MIC in eight LAB isolated from naturally-fermented Aloreña green table olives.

Antimicrobial	Strains	Fold increase (↗) or decrease (↘) in MIC of different antibiotics								
		AMP	**CHL**	**CIP**	**CLI**	**ERY**	**STR**	**TEC**	**TET**	**TMP**
Benzalkonium chloride	*Lc. pseudomesenteroides* AP2-28	↘2	↗2	↗3	S	S	S	↘8	↗2	S
	Lb. pentosus CF1-16	S	↗12.5	↗7	S	S	S	↗340	↗8	S
	Lb. pentosus CF1-25	↗100	↗2	↗14	S	S	S	↘2	↗80	S
	Lb. pentosus CF1-35	↗5	↗12.5	↗7	S	S	S	↘2	↗80	S
	Lb. pentosus CF2-11	↗5	↗500	↗2	S	S	S	↗170	↗8	S
	Lb. pentosus CF2-15p	↗10	↗200	↗2	S	S	S	↗340	↗8	S
	Lb. pentosus CF2-19p	↗10	↗500	↗7	S	S	S	↗340	↗2	↗15
	Lb. pentosus MP-10	↗10	↗125	↗4	S	S	S	↗34	↗2	S
Triclosan	*Lc. pseudomesenteroides* AP2-28	S	↗2	↗7	S	S	S	↘8	↗2	S
	Lb. pentosus CF1-16	↗5	↗2.5	↗7	S	S	S	↗340	↗8	S
	Lb. pentosus CF1-25	↗100	↗1	↗7	S	S	S	↘2	↗20	S
	Lb. pentosus CF1-35	↗10	↗5	↗3	S	S	S	↘2	↗80	S
	Lb. pentosus CF2-11	↗5	↗200	↗1	S	S	S	↗55	↗8	S
	Lb. pentosus CF2-15p	↗10	↗200	↗2	S	S	S	↗340	↗2	S
	Lb. pentosus CF2-19p	↗10	↗200	↗7	S	S	S	↗340	↗8	↗15
	Lb. pentosus MP-10	↗10	↗25	↗4	S	S	S	↗34	↘4	S
Amoxicillin	*Lc. pseudomesenteroides* AP2-28	S	↗1	↗2	S	S	S	↘8	S	S
	Lb. pentosus CF1-16	↗5	↗12.5	↗14	↗5	S	S	↗340	↗68	S
	Lb. pentosus CF1-25	↗100	↗7.5	↗14	↗200	S	S	↘2	↗680	S
	Lb. pentosus CF1-35	↗40	↗12.5	↗2	S	S	S	↘2	↗680	S
	Lb. pentosus CF2-11	↗5	↗500	↗1	S	S	S	↗55	↗68	S
	Lb. pentosus CF2-15p	↗10	↗750	↗2	S	S	S	↗340	↗68	↗15
	Lb. pentosus CF2-19p	↗40	↗500	↗3	S	S	S	↗340	↗68	S
	Lb. pentosus MP-10	S	↗25	↘2	S	S	S	↗34	S	S

Chloramphenicol	*Lc. pseudomesenteroides* AP2-28	S	↗2	↗7	S	S	S	↘4	↗2	S
	Lb. pentosus CF1-16	S	↗2.5	↗7	S	S	S	↗170	↗8	S
	Lb. pentosus CF1-25	↗100	↗1	↗7	S	S	S	↘2	↗20	S
	Lb. pentosus CF1-35	↗5	↗5	↗3	S	S	S	↘4	↗20	S
	Lb. pentosus CF2-11	↗5	↗200	↗1	S	S	S	↗170	↗8	S
	Lb. pentosus CF2-15p	↗10	↗200	↗2	S	S	S	↗340	↗8	↗20
	Lb. pentosus CF2-19p	↗5	↗200	↗7	S	S	S	↗170	↗2	S
	Lb. pentosus MP-10	↗10	↗50	↗4	S	S	S	↗34	1	S
Tetracycline	*Lc. pseudomesenteroides* AP2-28	↗1	↗7.5	↗3	S	S	↘2	↘32	S	S
	Lb. pentosus CF1-16	↗10	↗12.5	↗14	S	S	↘2	↗20	S	S
	Lb. pentosus CF1-25	↗100	↗2	↗14	S	S	↘2	↘32	S	S
	Lb. pentosus CF1-35	↗50	↗19	↗7	S	S	↘2	↘32	S	S
	Lb. pentosus CF2-11	↗10	↗750	↗2	S	S	↘2	↗20	S	S
	Lb. pentosus CF2-15p	↗30	↗1000	↗2	S	S	↘2	↗20	S	S
	Lb. pentosus CF2-19p	↗10	↗500	↗7	S	S	1	S	S	S
	Lb. pentosus MP-10	↗50	↗250	↗9	↗5	S	1	S	S	S

(↗ and ↘): Strains exhibited increase or decrease of MIC, respectively, for the corresponding antibiotic in comparison with controls under standard conditions.

S: Strains susceptible to the corresponding antibiotic.

Table 3. Primers and PCR conditions used in this study.

Target gene	**Primer**	**Sequence (5'-3')**	**Annealing T° (°C)**	**Reference**
rpsL	rpsMP-10-Fw rpsMP-10-Rv	ATTAATTCGTAAAGGCCGT ACTTCCGTAAAGCCGAGTTA	55	This study
recA	recAMP10-Fw recAMP10-Rv	ATTATGCGGATGGGTGAC AACTTTCAGGGCCATAAAT	53	This study
uvrB	uvrBMP10-Fw uvrBMP10-Rv	TAGTTTCAGATTACCAACC TCACTTGTGCAATCACGT	52	This study
srtA	srtMp10-Fw srtMp10-Rv	TAGTGTTAGTGTCGTTGG TTGAAAACTTCAAATCACTC	52	This study
norA	norA-Fw norA-Rv	TTTGTTTTCAGTGTCAGAATTTATGTTTG GGCTTGGTGAAATATCAGCTATTAAAC	55	Patel et al. (2010)
16S rRNA	16S-Fq RT 16S-Rq RT	TCATGATTTACATTTGAGTG GACCATGCGGTCCAAGTTGTT	55	Cho et al. (2010)

Table 4. Effect of UV exposure on antibiotic MIC in eight LAB isolated from naturally-fermented Aloreña green table olives.

Exposure time to UV light	**Strains**	**Fold increase in MIC of different antibiotics**								
		AMP	**CHL**	**CIP**	**CLI**	**ERY**	**STR**	**TEC**	**TET**	**TMP**
1 min	*Lc. pseudomesenteroides* AP2-28	↗3	↗7.5	↗7	S	S	S	1	↗8	S
	Lb. pentosus CF1-16	S	↗5	↗7	S	S	S	↗340	↗8	S
	Lb. pentosus CF1-25	S	↗5	↗7	S	S	S	1	↗160	S
	Lb. pentosus CF1-35	↗10	↗19	↗3	S	S	S	1	↗160	S
	Lb. pentosus CF2-11	S	↗500	↘2	S	S	S	340	↗16	S
	Lb. pentosus CF2-15p	↗10	↗500	1	S	S	S	340	↗16	S
	Lb. pentosus CF2-19p	↗10	↗500	↗14	S	S	S	340	↗64	S
	Lb. pentosus MP-10	↗10	↗125	↗2	10	S	S	34	↗2	S
5 min	*Lc. pseudomesenteroides* AP2-28	1	↗7.5	↗7	S	S	S	1	↗8	S
	Lb. pentosus CF1-16	S	↗12.5	1	S	S	S	↗340	↗8	S
	Lb. pentosus CF1-25	200	↗7.5	↗3	S	S	S	1	↗160	S
	Lb. pentosus CF1-35	5	↗12.5	↗3	S	S	S	1	↗160	S
	Lb. pentosus CF2-11	S	↗500	1	S	S	S	↗340	↗16	S
	Lb. pentosus CF2-15p	↗10	↗500	↗2	S	S	S	↗340	↗16	S
	Lb. pentosus CF2-19p	↗10	↗750	↗14	S	S	S	↗340	↗16	S
	Lb. pentosus MP-10	↗10	↗125	↗2	↗20	S	S	↗34	↗2	S
10 min	*Lc. pseudomesenteroides* AP2-28	1	↗5	↗3	S	S	↗2.5	1	↗8	-S
	Lb. pentosus CF1-16	S	↗5	↗3	S	S	S	↗340	↗8	S
	Lb. pentosus CF1-25	↗100	↗2	↗3	S	S	S	1	↗160	S
	Lb. pentosus CF1-35	↗10	↗5	↗1	S	S	↗5	1	↗80	S
	Lb. pentosus CF2-11	↗10	↗200	↗1	S	S	S	↗340	↗16	S
	Lb. pentosus CF2-15p	↗10	↗200	↗1	S	S	↗2	↗340	↗16	S
	Lb. pentosus CF2-19p	↗10	↗500	↗3	S	S	↗2.5	↗340	↗8	S
	Lb. pentosus MP-10	↗10	↗50	↗2	↗20	S	S	↗34	↗2	↘2.5

(↗ and ↘): Strains exhibited fold increase or decrease, respectively in the corresponding antibiotic MICs comparing with controls under standard conditions.

S: Strains susceptible to the corresponding antibiotic.

Table 5. Effect of resistance gene inducers on MIC in eight LAB isolated from naturally-fermented Aloreña green table olives.

Gene	**Inducer**	**Strains**	**Fold increase in MIC of different antibiotics**								
			AMP	**CHL**	**CIP**	**CLI**	**ERY**	**STR**	**TEC**	**TET**	**TMP**
norA	IPTG	*Lc. pseudomesenteroides* AP2-28	↘2	↗2	↗1.7	S	S	S	↘4	↗16	S
		Lb. pentosus CF1-16	S	↗5	↗1.7	S	S	S	↗340	↗16	S
		Lb. pentosus CF1-25	S	↗2	↗1.7	S	S	S	1	↗80	S
		Lb. pentosus CF1-35	S	S	1	S	S	S	1	↗80	S
		Lb. pentosus CF2-11	S	↗200	↘4	S	S	S	↗340	↗16	S
		Lb. pentosus CF2-15p	↗5	↗200	↘2	S	S	S	↗340	↗8	S
		Lb. pentosus CF2-19p	↗5	↗200	↗3	S	S	S	↗340	↗2	S
		Lb. pentosus MP-10	↗10	↗50	1	S	S	S	↗34	↗2	S
acrA/B	Ethanol	*Lc. pseudomesenteroides* AP2-28	1	↗5	↗14	↗40	S	S	1	↗8	S
		Lb. pentosus CF1-16	S	↗12.5	↗14	↗20	S	S	↗340	↗16	S
		Lb. pentosus CF1-25	S	↗5	↗14	↗400	S	S	1	↗160	S
		Lb. pentosus CF1-35	↗10	↗12.5	↗14	↗20	S	S	1	↗80	S
		Lb. pentosus CF2-11	S	↗500	↗2	↗200	S	S	↗340	↗16	S
		Lb. pentosus CF2-15p	S	↗500	↗2	↗200	S	S	↗340	↗16	S
		Lb. pentosus CF2-19p	S	↗500	↗14	↗2000	S	S	↗340	↗16	S
		Lb. pentosus MP-10	↗10	↗125	↗8.5	↗20	S	S	↗34	↗2	S
	NaCl	*Lc. pseudomesenteroides* AP2-28	S	↗5	↗14	S	S	S	1	↗16	S
		Lb. pentosus CF1-16	↗5	↗12.5	↗3	↗5	S	S	↗340	↗16	S
		Lb. pentosus CF1-25	S	↗2	↗3	↗100	S	S	↘7	↗80	S
		Lb. pentosus CF1-35	↗10	↗12.5	↗14	S	S	S	1	↗160	S
		Lb. pentosus CF2-11	S	↗500	↗2	S	S	S	↗340	↗16	S
		Lb. pentosus CF2-15p	↗10	↗500	↗2	↗50	S	S	↗340	↗16	S
		Lb. pentosus CF2-19p	↗30	↗500	↗14	S	S	S	↗340	↗16	S
		Lb. pentosus MP-10	↗50	↗125	↗8.5	↗10	S	S	↗34	↗2	S

Ethanol + NaCl	*Lc. pseudomesenteroides* AP2-28	1	↗2	↗7	S	S	S	1	↗16	S
	Lb. pentosus CF1-16	S	↗5	↗7	S	S	S	↗85	↗16	S
	Lb. pentosus CF1-25	S	↗2	↗3	S	S	S	↘7	↗80	S
	Lb. pentosus CF1-35	↗5	↗5	↗7	S	S	S	1	↗80	S
	Lb. pentosus CF2-11	↗5	↗500	1	S	S	S	↗340	↗16	S
	Lb. pentosus CF2-15p	↗5	↗200	1	S	S	S	↗340	↗16	S
	Lb. pentosus CF2-19p	↗10	↗200	↗7	S	S	S	↗340	↗16	S
	Lb. pentosus MP-10	↗30	↗50	↗4	S	S	S	↗34	1	S

(↗ and ↘): Strains exhibited fold increase or decrease, respectively in the corresponding antibiotic MICs comparing with controls under standard conditions.

S: Strains susceptible to the corresponding antibiotic.

Table 6. Correlations between MIC values of antibiotics (log-transformed) following different physico-chemical stress in LAB .

A

Antibiotic	AMP	CHL	CIP	CLI	STR	TEC	TET	TMP
AMP	1							
CHL	-0.165	1						
CIP	**0.498****	**-0.409***	1					
CLI	**0.395***	-0.112	**0.361***	1				
STR	0.077	0.303	0.170	-0.076	1			
TEC	-0.301	0.243	-0.099	-0.124	**-0.335***	1		
TET	**0.372***	-0.142	0.166	**0.687****	-0.159	-0.168	1	
TMP	-0.126	0.291	-0.102	-0.057	-0.153	**0.533****	-0.074	1

B

Antibiotic	AMP	CHL	CIP	CLI	STR	TEC	TET	TMP
AMP	1							
CHL	-0.187	1						
CIP	-0.062	0.255	1					
CLI	-0.067	-0.146	-0.178	1				
STR	-0.095	-0.085	-0.221	-0.148	1			
TEC	-0.295	**0.682****	0.051	-0.307	-0.116	1		
TET	**0.528****	-0.341	0.064	-0.265	-0.049	**-0.511***	1	
TMP	-0.039	-0.124	-0.103	**0.649****	-0.085	-0.177	-0.153	1

C

Antibiotic	AMP	CHL	CIP	CLI	TEC	TET
AMP	1					
CHL	0.092	1				
CIP	**0.535****	-0.145	1			
CLI	-0.147	0.339	0.005	1		
TEC	-0.084	**0.739****	-0.292	0.180	1	
TET	-0.195	-0.317	-0.107	0.018	**-0.483****	1

*Significant correlation at $p < 0.05$ level.

**Significant correlation at $p < 0.01$ level.

Antimicrobials are: AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; STR, streptomycin; TEC, teicoplanin; TET, tetracycline; TMP, trimethoprim. A, stress-related induction with antimicrobials (antibiotics or biocides); B, stress-related induction with UV exposure; C, stress-related induction with chemicals (IPTG, NaCl, ethanol).

Table 7. Correlations between resistance gene abundance (log-transformed) in *Lc. pseudomesenteroides* AP2-28 and *Lb. pentosus* MP-10 following induction by physico-chemical stress.

A

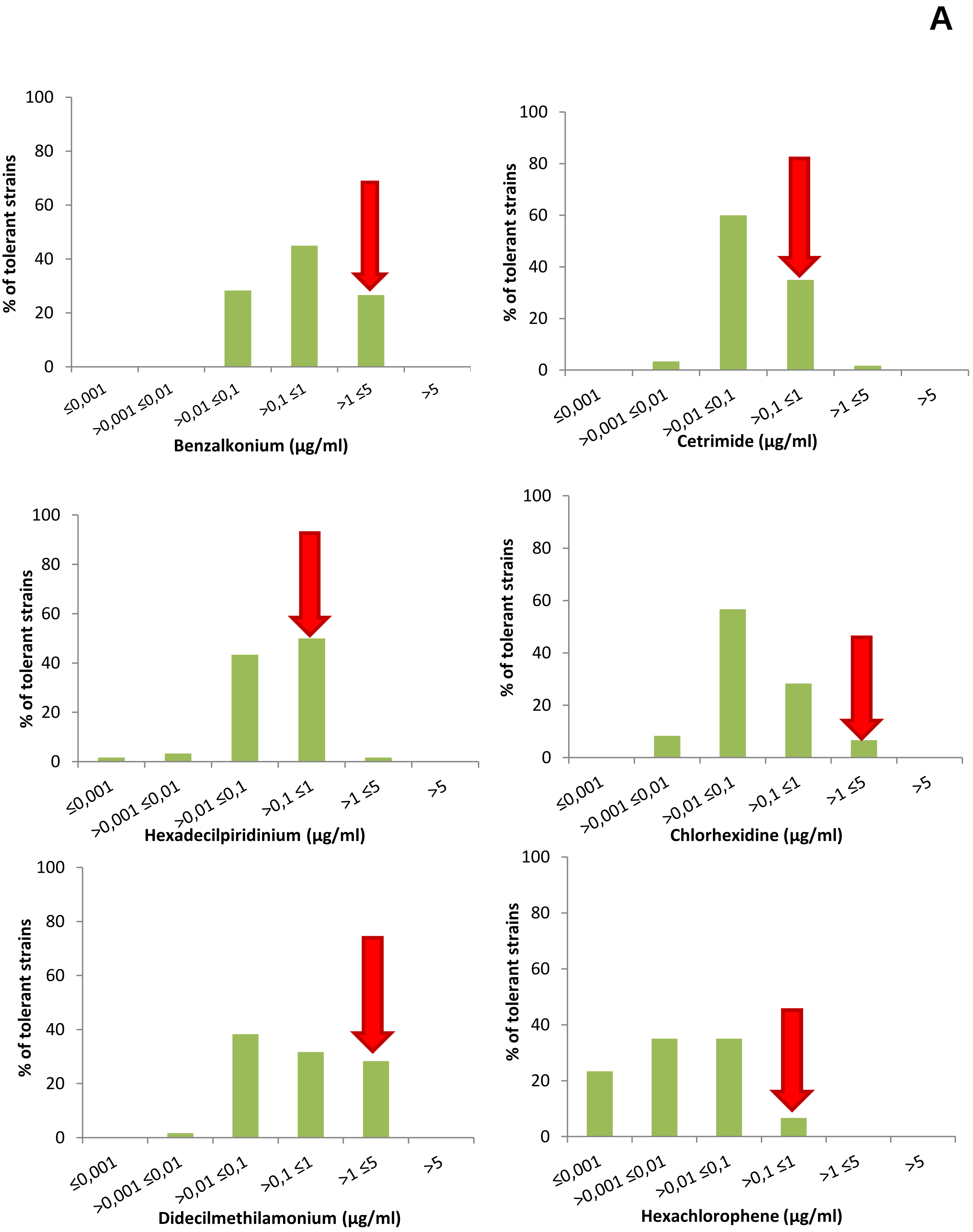
Gene	*rpsL*	*recA*	*strA*	*uvrB*
rpsL	1			
recA	0.121	1		
strA	0.201	0.250	1	
uvrB	0.156	**0.930****	0.318	1

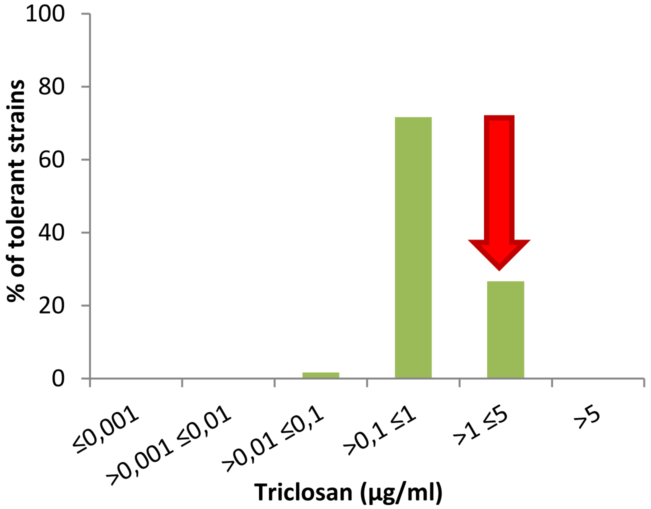
B

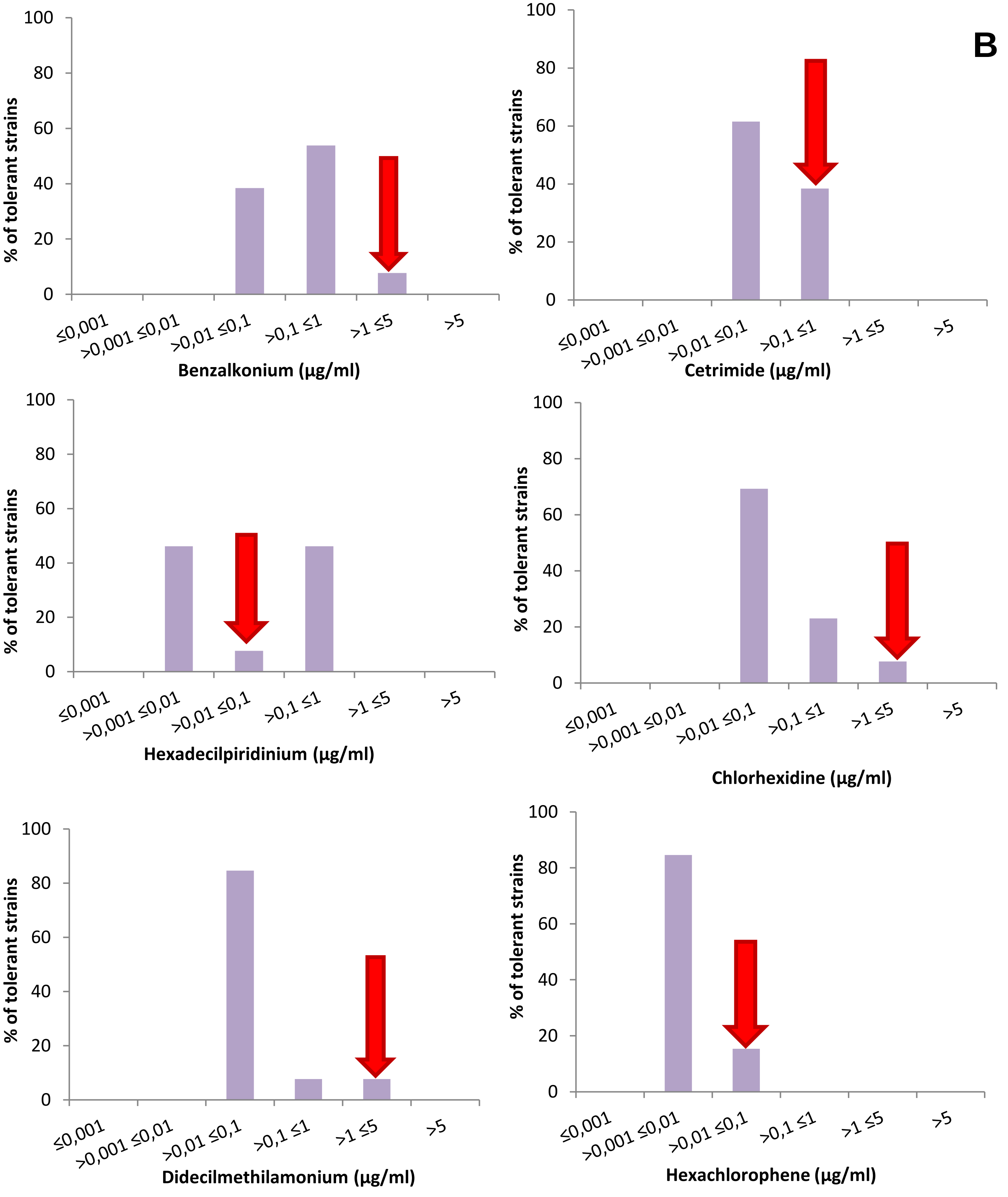
Gene	*rpsL*	*recA*	*strA*	*uvrB*
rpsL	1			
recA	0.037	1		
strA	0.032	-0.535	1	
uvrB	0.592	**0.770****	-0.326	1

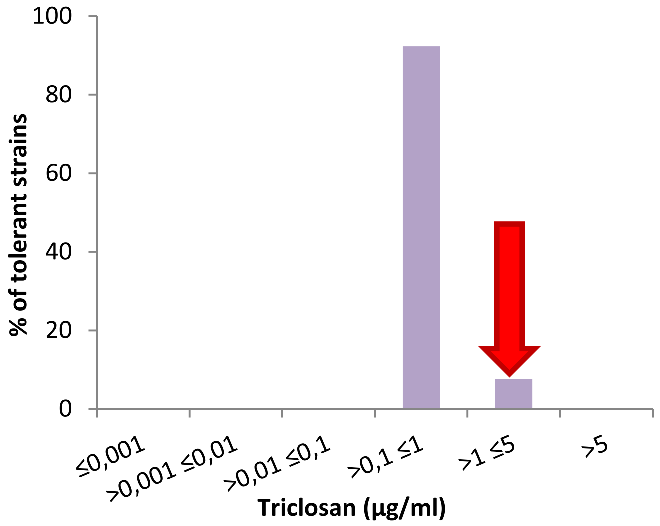
**Significant correlation at $p < 0.01$ level.

rpsL, *recA*, *strA* and *uvrB* resistance genes. A, *Lc. pseudomesenteroides* AP2-28; B, *Lb. pentosus* MP-10.

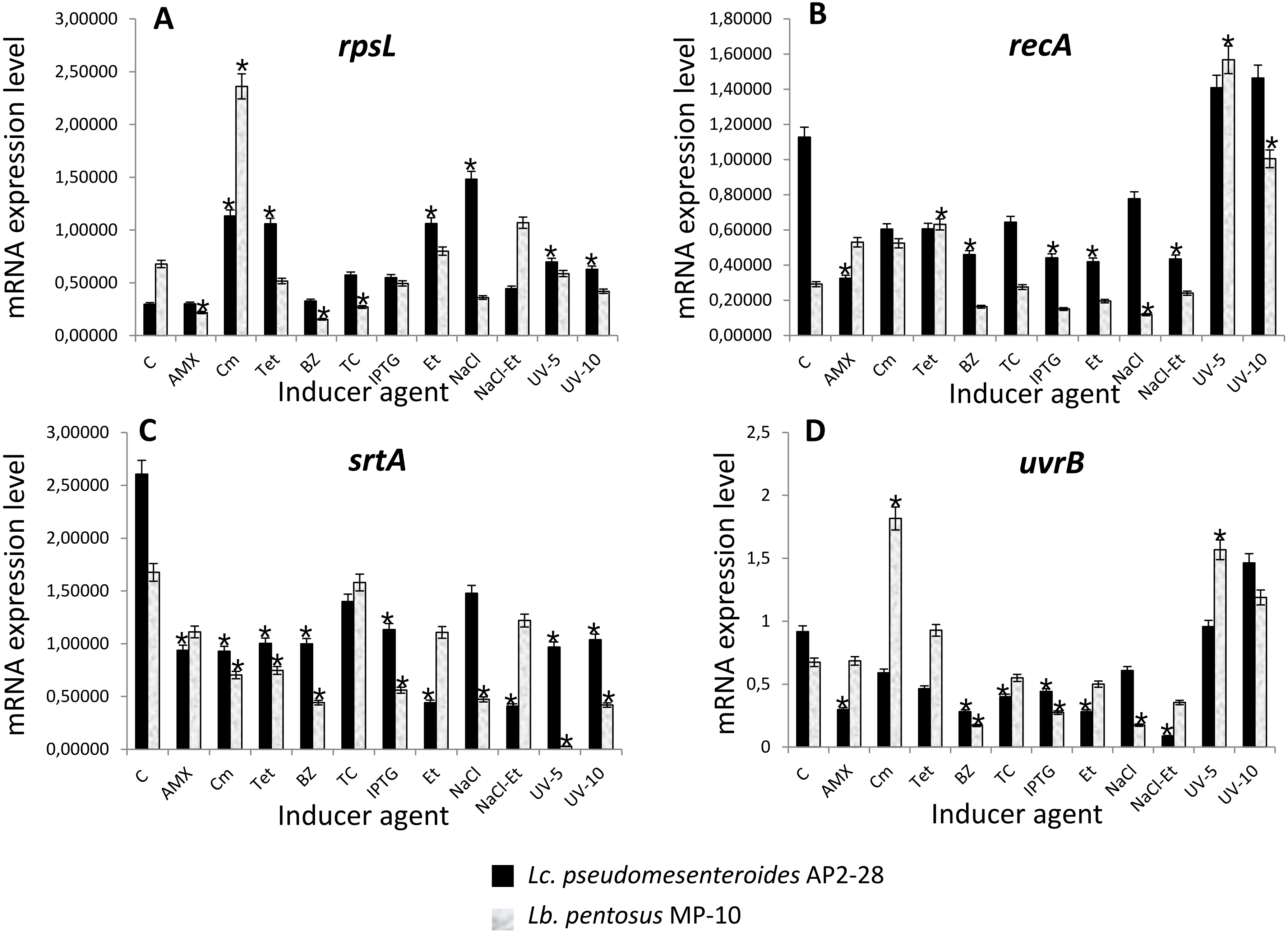

A
% of tolerant strains
100
80
60
40
20
0
≤0,001
>0,001 ≤0,01
>0,01 ≤0,1
>0,1 ≤1
>1 ≤5
>5
Benzalkonium (µg/ml)
Cetrimide (µg/ml)
Hexadecilpiridinium (µg/ml)
Chlorhexidine (µg/ml)
Didecilmethilamonium (µg/ml)
Hexachlorophene (µg/ml)


100
80
60
40
20
0
% of tolerant strains
≤0,001
>0,001 ≤0,01
>0,01 ≤0,1
>0,1 ≤1
>1 ≤5
>5
Triclosan (µg/ml)


B
100
80
60
40
20
0
% of tolerant strains
≤0,001
>0,001 ≤0,01
>0,01 ≤0,1
>0,1 ≤1
>1 ≤5
>5
Benzalkonium (µg/ml)
Cetrimide (µg/ml)
Hexadecilpiridinium (µg/ml)
Chlorhexidine (µg/ml)
Didecilmethilamonium (µg/ml)
Hexachlorophene (µg/ml)



Casado Muñoz et al. **Figure 1**



Casado Muñoz et al.

Figure 2

Artículo 3

Comparative proteomic analysis of a potentially probiotic *Lactobacillus pentosus* MP-10 for the identification of key proteins involved in antibiotic resistance and biocide tolerance

International Journal of Food Microbiology 222 (2016) 8–15

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Comparative proteomic analysis of a potentially probiotic *Lactobacillus pentosus* MP-10 for the identification of key proteins involved in antibiotic resistance and biocide tolerance

María del Carmen Casado Muñoz [a], Nabil Benomar [a], Saïd Ennahar [b], Peter Horvatovich [c], Leyre Lavilla Lerma [a], Charles W. Knapp [d], Antonio Gálvez [a], Hikmate Abriouel [a,]*

[a] *Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaén, 23071 Jaén, Spain*
[b] *Equipe de Chimie Analytique des Molécules Bio-Actives, UMR 7178, IPHC-DSA, Université de Strasbourg, CNRS, 67400 Illkirch-Graffenstaden, France*
[c] *Department of Analytical Biochemistry, Centre for Pharmacy, University of Groningen, Groningen, The Netherlands*
[d] *Department of Civil and Environmental Engineering, University of Strathclyde, Glasgow, Scotland, United Kingdom*

A R T I C L E I N F O

Article history:
Received 27 June 2015
Received in revised form 10 December 2015
Accepted 20 January 2016
Available online 22 January 2016

Keywords:
Comparative proteomics
Lactobacillus pentosus
Probiotics
Antibiotics
Biocides

A B S T R A C T

Probiotic bacterial cultures require resistance mechanisms to avoid stress-related responses under challenging environmental conditions; however, understanding these traits is required to discern their utility in fermentative food preparations, versus clinical and agricultural risk. Here, we compared the proteomic responses of *Lactobacillus pentosus* MP-10, a potentially probiotic lactic acid bacteria isolated from brines of naturally fermented Aloreña green table olives, exposed to sub-lethal concentrations of antibiotics (amoxicillin, chloramphenicol and tetracycline) and biocides (benzalkonium chloride and triclosan). Several genes became differentially expressed depending on antimicrobial exposure, such as the up-regulation of protein synthesis, and the down-regulation of carbohydrate metabolism and energy production. The antimicrobials appeared to have altered *Lb. pentosus* MP-10 physiology to achieve a gain of cellular energy for survival. For example, biocide-adapted *Lb. pentosus* MP-10 exhibited a down-regulated phosphocarrier protein HPr and an unexpressed oxidoreductase. However, protein synthesis was over-expressed in antibiotic- and biocide-adapted cells (ribosomal proteins and glutamyl-tRNA synthetase), possibly to compensate for damaged proteins targeted by antimicrobials. Furthermore, stress proteins, such as NADH peroxidase (Npx) and a small heat shock protein, were only over-expressed in antibiotic-adapted *Lb. pentosus* MP-10. Results showed that adaptation to sub-lethal concentrations of antimicrobials could be a good way to achieve desirable robustness of the probiotic *Lb. pentosus* MP-10 to various environmental and gastrointestinal conditions (e.g., acid and bile stresses).

1. Introduction

Lactobacillus pentosus is the most prevalent species of lactic acid bacteria (LAB) found in naturally-fermented Aloreña table olives (Abriouel et al., 2011, 2012) and Spanish-style green fermented olives (Maldonado-Barragán et al., 2011). Furthermore, these versatile bacteria have been detected in various environmental niches such as plant materials, silage, fermented foods (dairy, vegetable and meat), as well as the oral cavities, gastrointestinal tracts (GIT), and vaginas of humans and animals (Anukam et al., 2013; Okada et al., 1986; Tajabadi et al., 2011; Todorov and Dicks, 2004). Due to their wide distribution and beneficial effects, special and deserved attention was recently given to the application of lactobacilli, especially of vegetable origin, as a starter culture in different fermentations (Rodríguez-Gómez et al., 2014; Ruiz-Barba and Jiménez-Díaz, 2012), as a probiotic in silage (EFSA, 2011), dairy (Anukam and Olise, 2012) and fermented olives (Rodríguez-Gómez et al., 2014), as they provide bio-therapeutic benefits via bacterial pathogen inhibition and an improved immune system. More specifically, *Lb. pentosus* MP-10 isolated from brine of naturally fermented Aloreña olives (Abriouel et al., 2011, 2012) could be used as a probiotic strain due to their ability to inhibit pathogenic bacteria and tolerate low pH (1.5) and bile salts (3%) in the gastrointestinal environment.

Besides the technological and health-promoting effects shown by lactobacilli with probiotic properties, such as production of antimicrobial substances and survival in gastrointestinal tracts, other requirements should be proven to justify their utility. The most important selection criteria for bacterial strains intended for use as probiotics include: 1) intrinsic resistance to antibiotics of human and veterinary importance and 2) lack of transferable resistance genes to avoid the risk of horizontal gene transfer to other bacteria in the food chain and environment

---

* Corresponding author at: Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Edif. B3, Universidad de Jaén, Campus Las Lagunillas s/n, 23071 Jaén, Spain.
E-mail address: hikmate@ujaen.es (H. Abriouel).

http://dx.doi.org/10.1016/j.ijfoodmicro.2016.01.012
0168-1605/

(EFSA, 2008; EFSA Panel on Biological Hazards, 2010). As such, many studies have focused on genotypic methods to highlight the presence or absence of antimicrobial resistance determinants (e.g., Bautista-Gallego et al., 2013; Duran and Marshall, 2005; EFSA, 2012; Franz et al., 1999; Zhang et al., 2009). However, several aspects of bacterial fitness, which develop tolerance or resistance to different antimicrobials used in a clinical setting or disinfection, remain unexplored. Bacterial adaptation to antimicrobials, which was referred by Maisonneuve and Gerdes (2014) as "bacterial persisters," is the intermediary stage that links between sensitive and resistant phenotypes. Thus, more attention should be provided to the potential for bacterial adaptation, such as further induction of cross-resistance to other treatments and modifications in colonization or virulence (Dubois-Brissonnet, 2012). Detecting the mechanisms adopted by different bacteria to resist different drugs in various environmental niches remains important. In this respect, several studies report that physiological modifications occur during adaptation such as differential protein expression, which seems to be concomitant to increased tolerance (Dubois-Brissonnet, 2012) and cross-resistance to other environmental stressors (Karatzas et al., 2007, 2008).

In the last decade, proteomics has been used to study bacterial physiological responses to different stressors; this has progressed significantly with the availability of whole-genome sequences, progress in mass spectrometry and bioinformatics. Proteomics, as a key in the post genomic era, provides useful data to identify new diagnostic markers and therapeutic targets in diseases. Recently, genomic and proteomic analyses of *Lactobacillus* genus have rapidly expanded, especially with *Lb. pentosus* having one of the largest genomes known among LAB (Abriouel et al., 2011; Maldonado-Barragán et al., 2011); however, little is known about the mechanisms adopted by *Lb. pentosus* to tolerate or resist several stressors. This information should be of great concern since the knowledge of these mechanisms could be exploited to improve the functionality of probiotic starter strains and, thus, their health promoting benefits.

The present study aimed to determine the phenotypic and genotypic antimicrobial-resistance profiles of *Lb. pentosus* MP-10 and the selected mechanisms, by which these bacteria adapt under different antimicrobial stress. We compared the proteomic profiles of this strain induced by different antimicrobials (antibiotics or biocides), each with a distinct mechanism of action. The comparative analysis provides valuable knowledge and a broad overview of the key proteins involved in antibiotic and biocide tolerance.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Lb. pentosus MP-10, isolated from naturally-fermented Aloreña green table olives (Abriouel et al., 2011, 2012), was routinely cultured at 30 °C in Man Rogosa and Sharpe (MRS) broth (Fluka, Madrid, Spain) or agar under aerobic conditions for 24–48 h. The strain was stored long-term in 20% glycerol at −80 °C.

2.2. Antimicrobial agents

The antimicrobial agents used in this study were clinically relevant antibiotics: amoxicillin "AMX", ampicillin "AMP", cefuroxime "CFX", chloramphenicol "CMP", ciprofloxacin "CIP", clindamycin "CLI", erythromycin "ERY", gentamicin "GEN", kanamycin "KAN", streptomycin "STR", sulfamethoxazole/trimethoprim "SMZ/TMP", teicoplanin "TC", trimethoprim "TMP", tetracycline "TET" and vancomycin "Van"; and biocides commonly used in the food industry: benzalkonium chloride "BC" and triclosan "TC". All antibiotics and benzalkonium chloride were purchased from Sigma Aldrich (Madrid, Spain); however, triclosan was obtained from Fluka (Madrid, Spain).

2.3. Phenotypic and genotypic antibiotic testing

2.3.1. Antibiotic susceptibility testing and MIC determination

The MICs of the above-mentioned antibiotics were determined for *Lb. pentosus* MP-10 as described by Casado Muñoz et al. (2014) in LSM broth [a mixture of 90% IST broth (Oxoid, Madrid, Spain) and 10% MRS broth (Fluka, Madrid, Spain)] (Klare et al., 2005) according to the ISO 10932/IDF 233 standard (International Organization for Standardization, 2010).

2.3.2. PCR detection of antibiotic resistance genes

PCR amplifications of well-known gene determinants associated with resistance to β-lactam antibiotics (*bla* and *blaZ*, the β-lactamase genes), sulfonamides (*dfrA* and *dfrD*) and glycopeptides (*vanA*, *vanB*, *vanC* and *vanE*) were performed using conditions described elsewhere (Dutka-Malen et al., 1995; Fines et al., 1999; Hummel et al., 2007; Liu et al., 2009; Martineau et al., 2000; Miele et al., 1995). Furthermore, PCR of genes mediating antibiotic resistance through other mechanisms, such as efflux pumps (*mdfA*, *norE*, *acrA*, *acrB*, *tolC*, *mepA*, *norA*, *norC*, *mefA* and *mdeA*), were also performed in the present study. Template DNA for PCR reactions were prepared as reported in Jensen et al. (1998).

2.4. Tolerance induction

Tolerance to antibiotics or biocides was assessed by investigating the ability of *Lb. pentosus* MP-10 to grow in the presence of sub-lethal concentrations of the corresponding antimicrobials, to which the strain was originally sensitive (amoxicillin, chloramphenicol, tetracycline, benzalkonium chloride and triclosan). Tolerant phenotypes were developed by increasing the concentrations of different antimicrobials as described by Casado Muñoz et al. (unpublished data). Briefly, antimicrobial tolerance in *Lb. pentosus* MP-10 was induced by exposure to triclosan (1 μg/ml), benzalkonium chloride (1 μg/ml), cholamphenicol (5 μg/ml), tetracycline (10 μg/ml) or amoxicillin (0.1 μg/ml) at 30 °C for 48 h; cells were then harvested by centrifugation (Casado Muñoz et al., unpublished data). All *Lb. pentosus* isolates were stored in 20% glycerol at −80 °C until use. Isolates were streaked onto MRS-agar; a single colony was selected and subsequently used to inoculate MRS-broth for 24 h at 30 °C. The resulting culture was used to inoculate fresh MRS-broth at a dilution of 1:100. Cultures (both induced and non-induced controls) were harvested at mid-logarithmic growth phase ($OD_{600\ nm} = 0.6$).

2.5. Whole cell protein extraction

The cell pellets obtained, as described above, from isogenic mutants were resuspended in 2 ml of PBS and dispersed into liquid nitrogen with a 200-μl micropipette to obtain cryobeads. Whole-cell protein extraction was done as described by Caballero Gómez et al. (2013). The bacterial beads were ground in liquid nitrogen using a cryogenic grinder (6870 Freezer/Mill, SpexCertiPrep, Stanmore, UK) with three steps of 3 min at a rate of 24 impacts/s. The samples were centrifuged at 5000 × *g* for 5 min (at 4 °C), and the resultant supernatants were filtered through a 0.45-μm pore size filter (Chromafil PET; Macherey-Nagel, Düren, Germany). Proteins were extracted from the filtered supernatants with TRIzol reagent (Euromedex, Souffelweyersheim, France) as previously described (Izquierdo et al., 2009). Protein concentrations were determined using the Bradford protein assay (Bio-Rad) according to the manufacturer's instructions.

2.6. 2-D gel electrophoresis

Protein extracts (150 μg) were loaded onto 17-cm strips with a pH range of 3 to 10 (Bio-Rad), focused for 60,000 V h, and then separated on a 12% SDS-polyacrylamide gel as reported previously (Izquierdo et al., 2009). The gels were stained as described by Candiano et al. (2004) using Bio-Safe Coomassie brilliant blue G-250 (Bio-Rad), which has a reported detection limit of 1 ng for BSA, and scanned on a GS-800 Calibrated Densitometer (Bio-Rad). Three independent experiments were carried out and each assay was performed in triplícate.

2.7. Image analysis

Image analysis of the 2D-GE gels was performed using PD Quest 8.0.1 software (Bio-Rad). Only spots that were present on the three gels were selected for inter-condition comparison. Spot intensities were normalized to the sum of intensities of all valid spots in one gel. For analysis of changes in protein expression during antimicrobial exposure, a protein was considered to be under- or over-produced when changes in normalized spot intensities were at least 1.5-fold at a significance level of $p < 0.05$ (Student's *t* test for paired samples), as previously described (Sánchez et al., 2007). Regarding proteome comparisons between different culture conditions of *Lb. pentosus* MP-10, proteins were considered differentially produced when spot intensities passed the threshold of a twofold difference (one-way ANOVA, p-value < 0.05), as described previously (Izquierdo et al., 2009).

2.8. Protein identification

Spots of interest were subjected to tryptic in-gel digestion as described by Izquierdo et al. (2009) and analyzed by chip–liquid chromatography–quadrupole time-of-flight (chip–LC–QTOF) using an Agilent G6510A QTOF mass spectrometer equipped with an Agilent 1200 Nano LC system and an Agilent HPLC Chip Cube, G4240A (Agilent Technologies, Santa Clara, CA, USA), as described previously (Hamon et al., 2011). Protein identification was performed against the genome of *Lb. pentosus* KCA1 available at the NCBI Website (http://www.ncbi.nlm.nih.gov; accessed 4th November 2014), using the PEAKS DB search engine (Bioinformatics Solutions Inc., Waterloo, Canada). Using the PEAKS inChorus feature, Mascot and PEAKS searches were compared to confirm protein identities and limit the risk of false positives. Scores represent peptide probabilities as calculated using PEAKS DB's Peptide-Spectrum Matching Score (-10 lgP).

2.9. Growth and survival of antimicrobial-induced and non-induced Lb. pentosus MP-10 following exposure to gastric juices

To determine the growth rate of antimicrobial-induced *Lb. pentosus* MP-10 in comparison with control (without induction), overnight cultures were diluted 1/1000 in MRS broth and viable counts were determined by serial dilutions on MRS-agar plates after 4 and 8 h of incubation at 30 °C. Increase in growth rate was determined by the difference between Log_{10} CFU/ml at time × h (4 or 8 h) and Log_{10} CFU/ml at time 0 h. To test if antimicrobial induction of *Lb. pentosus* MP-10 improved its tolerance to acid and bile concentrations, overnight cultures were added (at 2% volume) to simulated gastric juice (pepsin and NaCl) at different conditions: pH 1.5, pH 2.5, 2% bile or 3% bile. The mixtures were incubated at 37 °C for 30 min and viable counts were determined on MRS agar plates as described above. The survival rate was determined according to Bao et al. (2010) by the following equation: survival rate (%) $= (\text{Log}_{10}\ \text{CFU/ml}\ N_1/\text{Log}_{10}\ \text{CFU/ml}\ N_0) \times 100$.

N_1 is the total viable count of *Lb. pentosus* MP-10 after 30 min treatment (at pH 1.5, pH 2.5, 2% bile or 3% bile), and N_0 is the total viable count at time 0 (before treatment).

3. Results

3.1. Antibiotic susceptibility and molecular detection of antibiotic resistance genes in Lb. pentosus MP-10

MIC determinations of the different antibiotics revealed that *Lb. pentosus* MP-10 was sensitive to amoxicillin (MIC = 0.2 μg/ml), ampicillin (MIC = 0.2 μg/ml), chloramphenicol (MIC = 0.04 μg/ml), clindamycin (MIC = 0.2 μg/ml), erythromycin (MIC = 0.1 μg/ml), gentamycin (MIC = 0.8 μg/ml), kanamycin (MIC = 16 μg/ml), streptomycin (MIC = 150 μg/ml) and tetracycline (MIC = 8 μg/ml). However, *Lb. pentosus* MP-10 showed resistance to cefuroxime (MIC = 100 μg/ml), ciprofloxacin (MIC = 8 μg/ml), teicoplanin (MIC > 128 μg/ml), trimethoprim (MIC = 128 μg/ml), trimethoprim/sulfamethoxazole (MIC = 950/50 μg/ml) and vancomycin (MIC > 128 μg/ml). In most cases, resistance or sensitivity was categorized based on the microbiological breakpoints of the antibiotics tested (also defined as ECOFF by the European Food Safety Authority, 2012), which was reviewed by Casado Muñoz et al. (2014).

To identify possible genetic determinants responsible for the resistance phenotypes observed in *Lb. pentosus* MP-10, PCR reactions were performed as described above. However, results revealed an absence of specific resistance determinants, except for the detection of *norA* coding for a multidrug efflux pump.

3.2. Influence of antibiotics on protein expression levels in Lb. pentosus MP-10

Based on antibiotic susceptibility results, amoxicillin, chloramphenicol and tetracycline were selected to carry out tolerance studies. We compared the proteomes of antibiotic-treated and untreated *Lb. pentosus* MP-10 to elucidate the physiological changes resulting from the treatments. 2D-GE analysis of antibiotic-treated cells, collected during mid-exponential growth phase, showed different proteomic profiles depending on the antibiotic used, suggesting various antibiotic stress responses (Fig. 1). Treatment with chloramphenicol, amoxicillin and tetracycline resulted in two, four and six proteins (respectively) that significantly ($p < 0.05$) differed to the pattern from the untreated control (Fig. 1). These proteins were individually excised from duplicate 2D-GE gels, subjected to tryptic digestion, and identified by chip-LC-QTOF and Uniprot database searching (summarized in Table 1). Treatment with amoxicillin or chloramphenicol resulted in an under-expressed CTP synthase (spot 0102), an enzyme involved in nucleotide synthesis that requires ATP for its metabolic function. On the other hand, proteins involved in other metabolic pathways such as carbohydrate metabolism (phosphocarrier protein HPr of the phosphotransferase system “PTS”, spot 4201), homeostasis (NADH peroxidase Npx, spot 6101) and protein synthesis (SSU ribosomal protein S6p, spot 7202) became over-expressed in the presence of amoxicillin. Similarly, three proteins carrying different biological functions were over-expressed in the presence of tetracycline: 6-phosphogluconate dehydrogenase (spot 7605), involved in carbohydrate metabolism; a small heat shock protein (spot 7802) responsible of cell protection; and LSU ribosomal protein L1p (spot 7803), implicated in protein synthesis (Fig. 1, Table 1).

The following three proteins were only expressed in the absence of tetracycline: pyruvate kinase (spot 3102) and NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (spot 4501), which are linked to carbohydrate metabolism, as well as acetaldehyde dehydrogenase (spot 3104), which is involved in alcohol and fat metabolism (Fig. 1, Table 1). In the case of chloramphenicol, the only protein not produced, compared with the untreated control, was 6-phosphofructokinase, which is related to carbohydrate metabolism.

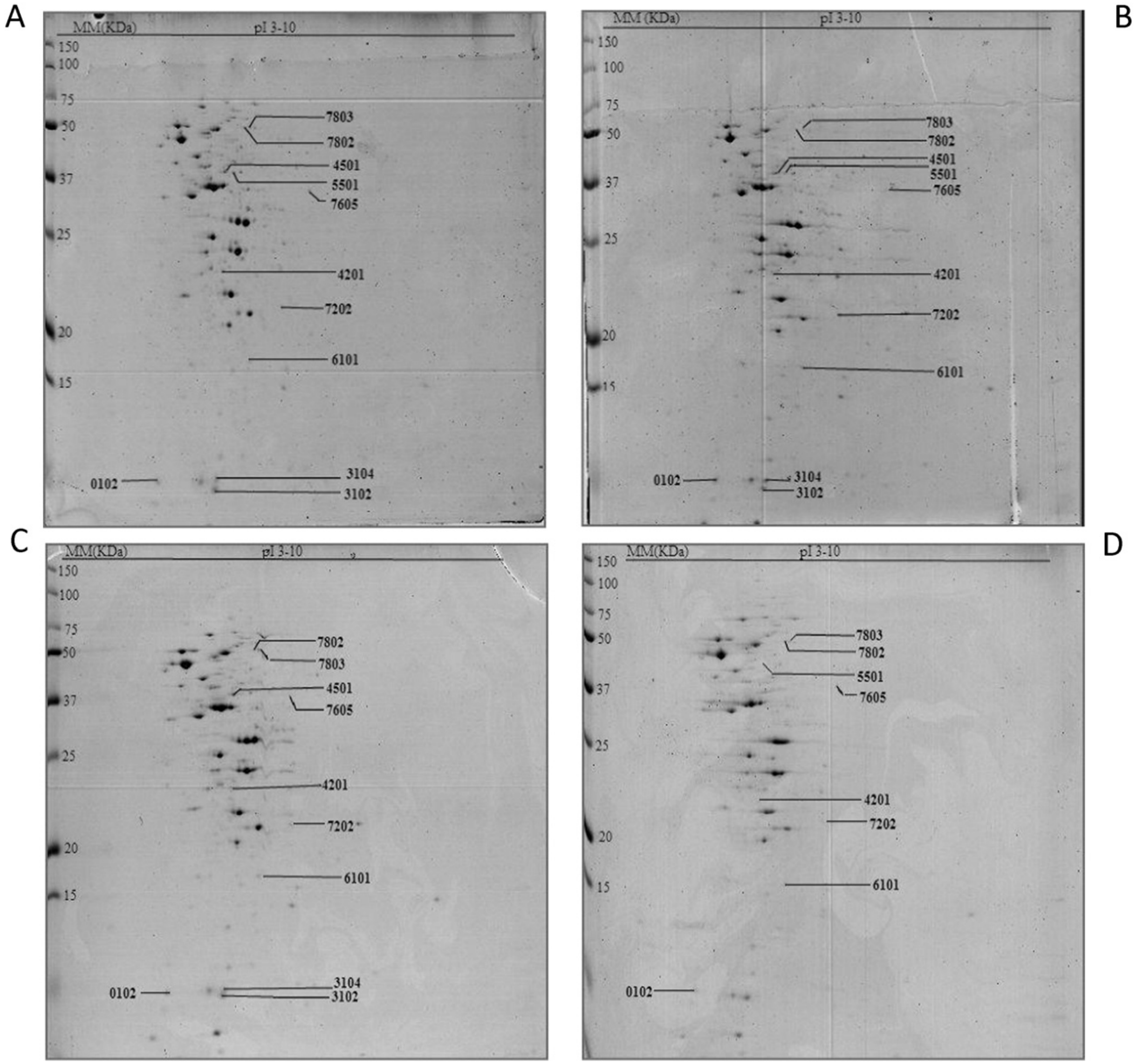


Fig. 1. 2-DE gels of whole cell proteomes from *Lactobacillus pentosus* MP-10 cultured in the absence (A) or presence of amoxicillin (B), chloramphenicol (C) and tetracycline (D). The figure shows representative 2-DE gel pictures (pH range: 4–7) of whole-cell protein lysates from an early stationary phase of *Lb. pentosus* MP-10 spots exhibiting constitutive differential expression between growth of *Lb. pentosus* MP-10 in standard conditions and after induction by antibiotics which were identified by peptide mass fingerprinting and are labeled; the identifications of the spots affected by antibiotics are listed in Table 1.

3.3. Influence of biocides on protein expression levels in Lb. pentosus MP-10

According to biocide susceptibility pattern by *Lb. pentosus* MP-10 (Casado Muñoz et al., unpublished data), we selected benzalkonium chloride and triclosan for further tolerance studies. Following treatment with biocides (benzalkonium chloride or triclosan), the proteomes of *Lb. pentosus* MP-10 were compared with untreated bacteria. The benzalkonium chloride exposure resulted in only one protein significantly ($p < 0.05$) over-expressed in the induced cells: ribosomal subunit interface protein (spot 6603), which is related to protein biosynthesis (Fig. 2, Table 2). However, the proteome of *Lb. pentosus* MP-10 treated with triclosan showed significant ($p < 0.05$) differential expression among three proteins: an over-expressed glutamyl-tRNA synthetase (spot 5801), linked to amino acid starvation; an under-expressed phosphocarrier protein HPr of the PTS (spot 4401), related to carbohydrate metabolism; and no detection of oxidoreductase of the aldo/keto reductase family (spot 5301), involved in energy production and conversion (Fig. 2, Table 2).

3.4. Survival and tolerance responses of antimicrobial-induced Lb. pentosus MP-10

As shown in Table 3, the growth rate was increased in almost all antimicrobial-induced *Lb. pentosus* MP-10 by 0.09–0.32 Log_{10} units after 4 or 8 h incubation at 30 °C except in chloramphenicol-induced cells, which showed the same growth rate as non-induced controls.

Comparison of survival capacity of non-induced and antimicrobial-induced *Lb. pentosus* MP-10 under acid or bile (2 and 3%) stress determined that antimicrobial induction improved the tolerance capacity of *Lb. pentosus* MP-10 at acidic conditions. The bacteria had > 100% survival and they exhibited slightly greater growth than the controls (94% and 100%, at pH 1.5 and 2.5, respectively) (Table 3). Regarding bile tolerance, at both concentrations of 2 and 3% of bile we observed 100% survival, or better, in benzalkonium- and triclosan-induced cells; moreover at 2% bile concentration, chloramphenicol-induced *Lb. pentosus* MP-10 also showed 100% survival (Table 3). However, at 3% bile

Table 1
Proteins differentially expressed in *Lactobacillus pentosus* MP-10 treated with different antibiotics, as compared to control.

Protein identity	Spot no.	Accession number[a]	−10 lgP[b]	Sequence coverage (%)
Proteins over-expressed				
In the presence of amoxicillin:				
Phosphotransferase system, phosphocarrier protein HPr	4201	EIW14262.1	341.26	97
NADH peroxidase Npx (EC 1.11.1.1)	6101	EIW13302.1	372.87	78
SSU ribosomal protein S6p	7202	EIW15333.1	240.77	87
In the presence of tetracycline:				
6-phosphogluconate dehydrogenase, decarboxylating (EC 1.1.1.44)	7605	EIW14063.1	407.61	82
Small heat shock protein	7802	EIW15192.1	315.52	97
LSU ribosomal protein L1p	7803	EIW14839.1	333.50	66
Protein under-expressed				
In the presence of amoxicillin or chloramphenicol:				
CTP synthase (EC 6.3.4.2)	0102	EIW15026.1	384.76	75
Proteins not expressed				
In the presence of chloramphenicol:				
6-Phosphofructokinase (EC 2.7.1.11)	5501	EIW13706.1	358.39	88
In the presence of tetracycline:				
Pyruvate kinase (EC 2.7.1.40)	3102	EIW13705.1	381.86	94
Alcohol dehydrogenase (EC 1.1.1.1); acetaldehyde dehydrogenase (EC 1.2.1.10)	3104	EIW15075.1	317.41	63
NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	4501	EIW14737.1	411.19	91

[a] Accession number in the NCBInr database.
[b] PEAKS DB's Peptide-Spectrum Matching Score.

Table 2
Proteins differentially expressed in *Lactobacillus pentosus* MP-10 treated with different biocides as compared to control.

Protein identity	Spot no.	Accesion number[a]	−10 lgP[b]	Sequence coverage (%)
Proteins over-expressed				
In the presence of benzalkonium chloride:				
Ribosomal subunit interface protein	6603	EIW14771.1	310.89	67
In the presence of triclosan:				
Glutamyl-tRNA synthetase (EC 6.1.1.17)	5801	EIW14887.1	334.84	70
Protein under-expressed				
In the presence of triclosan:				
Phosphotransferase system, phosphocarrier protein HPr	4401	EIW14262.1	181.75	57
Proteins not expressed				
In the presence of triclosan:		EIW12654.1		
Oxidoreductase of aldo/keto reductase family, subgroup 1	5301		322.88	73

[a] Accession number in the NCBInr database.
[b] PEAKS DB's Peptide-Spectrum Matching Score.

concentration, bacteria pre-exposed to amoxicillin, chloramphenicol and tetracycline became less viable (Table 3).

4. Discussion

The importance of probiotic bacteria, which are mainly members of the genera *Lactobacillus* and *Bifidobacterium*, has increasingly become recognized in human and animal nutrition by their contributions to immunological, digestive, and respiratory health. However, according to the Qualified Presumption of Safety (QPS) approach proposed by the European Food Safety Authority (EFSA, 2008), the presence of antibiotic resistance determinants is one of the most important safety selection criteria for bacterial strains intended for use in the food industry, even among bacteria that are generally recognized as "safe". Here, *Lb. pentosus* MP-10 isolated from brines of naturally fermented Aloreña green table olives (Abriouel et al., 2011, 2012) could be regarded as "safe" because of the absence of acquired resistance determinants. Their intrinsic resistance to more than three antibiotics, which relies on chromosomally encoded efflux pumps such as NorA, is unlikely to be an issue from a medical point of view, since *Lb. pentosus* MP-10 remains highly sensitive to other clinically relevant antibiotics.

However, the survival of probiotic bacteria and their beneficial probiotic effects under different environmental conditions, including those encountered in the gastrointestinal tract, may rely on the resistance traits. As such, knowing which proteins are involved in tolerance is important to improve the functionality of probiotic strains under different stress conditions. In the present study, we investigated the proteomic response of probiotic bacteria *Lb. pentosus* MP-10 to antimicrobial stress conditions. Antibiotics and biocides induced adaptations in *Lb. pentosus*

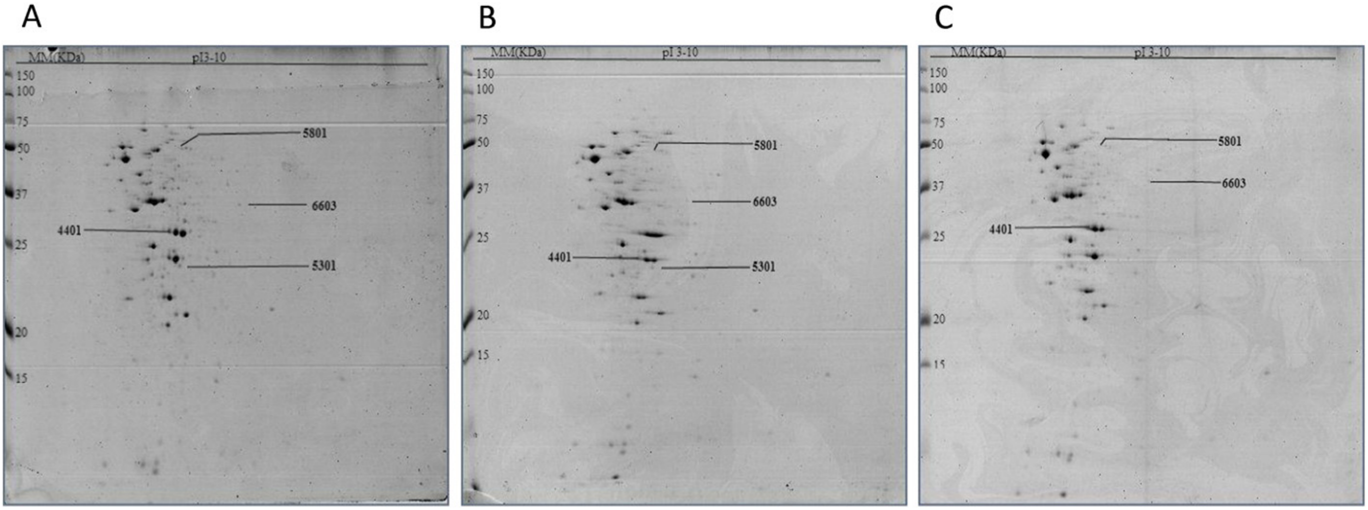


Fig. 2. 2-DE gels of whole cell proteomes from *Lactobacillus pentosus* MP-10 cultured in the absence (A) or presence of benzalkonium chloride (B) and triclosan (C). The figure shows representative 2-DE gel pictures (pH range: 4–7) of whole-cell protein lysates from an early stationary phase of *Lb. pentosus* MP-10 spots exhibiting constitutive differential expression between growth of *Lb. pentosus* MP-10 in standard conditions and after induction by biocides which were identified by peptide mass fingerprinting and are labeled; the identifications of the spots affected by biocides are listed in Table 2.

Table 3
Growth and survival of the antimicrobial-induced *Lactobacillus pentosus* MP-10 after exposition to acidic pH and bile concentrations.

Antimicrobial induced-*Lactobacillus pentosus* MP-10	Increase in growth rate (Log$_{10}$ CFU/ml)[a]		Survival to acidic pH during 30 min (%)		Survival to bile concentration during 30 min (%)	
	4 h	8 h	pH 1.5	pH 2.5	2%	3%
Control (without induction)	0.45 ± 0.17	1.35 ± 0.18	94	100	67	87.5
Benzalkonium chloride	0.76 ± 0.06	1.43 ± 0.07	>100	>100	100	100
Triclosan	0.47 ± 0.24	1.24 ± 0.05	>100	>100	>100	>100
Amoxicillin	0.39 ± 0.06	1.44 ± 0.03	>100	>100	71	29
Chloramphenicol	0.43 ± 0.01	1.34 ± 0.10	>100	>100	>100	40
Tetracycline	0.59 ± 0.05	1.2 ± 0.01	>100	>100	60	64

Each value or percentage in the table represents the mean of triplicate plate count readings from three separate experiments.

[a] Difference between Log$_{10}$ CFU/ml at different times (4 or 8 h) and Log$_{10}$ CFU/ml at time 0.

MP-10 as evidenced by modifications of its proteomic arsenal, with the observed changes being intimately dependent on the antimicrobial used. Overall, antibiotics induced several physiological modifications, possibly due to various mechanisms of action, each targeting a defined cellular structure; in comparison, biocides induced fewer modifications. Adaptation to antibiotics is likely to trigger comparatively more physiological modifications than biocides; several resistance mechanisms to antibiotics have had a longer evolution process to protect bacteria, compared with the more relatively recent exposure to biocides and limited opportunity to develop resistance. Overall, several proteins involved in carbohydrate metabolism like phosphocarrier protein HPr of the PTS, as part of glycolysis-related machinery, and 6-phosphogluconate dehydrogenase of the pentose phosphate pathway were up-regulated after exposure to antibiotics (amoxicillin or tetracycline) targeting different cellular structures. Increasing the level of ATP synthesis (Wilkins et al., 2002) was either required for the increased efflux activity or compensating the low glycolytic capacity (Wouters et al., 2000), and is an important factor for survival under stress conditions. Similar results were obtained with *Bifidobacterium animalis* and *Lactobacillus reuteri* under bile stress (Lee et al., 2008; Sánchez et al., 2007). Furthermore, HPr (histidine-containing protein) protein is not only responsible for carbohydrate uptake; it also plays a regulatory role in sugar metabolism and catabolite repression, depending on protein–protein interactions with many cellular factors (Deutscher et al., 2006). Accordingly, other proteins involved in glycolysis pathways such as 6-phosphofructokinase, and pyruvate kinase and NAD-dependent glyceraldehyde-3-phosphate dehydrogenase were down-regulated in the presence of antibiotics inhibiting protein synthesis — chloramphenicol and tetracycline, respectively. Pyruvate, an end product of glycolysis, is a metabolic key molecule that can be used in a number of different reactions to increase the ATP levels, thus antibiotic stress induced regulation of metabolism by down- or up-regulation of enzymes involved in energy production. These data suggest that, to ensure survival under antibiotic stress, *Lb. pentosus* MP-10 physiology may be altered to achieve a higher cellular energy gain via up- or down-regulation of carbohydrate metabolism (pentose and glycolysis pathways). Under antibiotic stress and subsequent limited energy conditions, PTS transport systems are used rather than ABC transporters (Taranto et al., 1999). These systems are, in fact, more energy efficient as the phosphorylated substrate can directly enter glycolysis or pentose phosphate pathways, conserving ATP. Similarly, Lin et al. (2014) reported that fluctuation of metabolic pathways may represent an antibiotic-resistance mechanism under chlortetracycline stress in *Escherichia coli*.

The interaction of amoxicillin and tetracycline with membrane lipids and proteins induced the over-expression of stress proteins, such as NADH peroxidase Npx and a small heat shock protein, respectively, as a first response of the cell to maintain homeostasis and viability. Furthermore, it has been reported that, besides its role in cell redox homeostasis (degradation of hydrogen peroxide to water and oxygen), Npx of the peroxidase–oxidase–reductase (POR) subgroup of the flavoprotein–disulfide–reductase (FDR) family also contributes to the regeneration of oxidized pyridine nucleotides for glycolysis (Ying, 2006). Small heat shock proteins as "minichaperones" have been associated with enhanced bacterial survival during stress, since they are necessary for normal cellular functions, including growth and stability of DNA and RNA. They also prevent the formation of inclusion bodies (Jakob et al., 1993; Narberhaus, 2002; Veinger et al., 1998), but are not involved in protein re-folding as chaperones.

On the other hand, protein synthesis in *Lb. pentosus* MP-10, exposed to amoxicillin and tetracycline, was up-regulated. However, it has been reported that the proteins involved in cell growth, such as ribosomal proteins, were markedly under-regulated under stress conditions as an energy-saving strategy necessary for protection mechanisms in the cell (Rezzonico et al., 2007). In spite of the fact that ribosomal run-off and transit times are slower upon stressor exposure, stress-regulatory factors are preferentially associated with ribosomes, suggesting increased translation and protein synthesis (Sherman and Qian, 2013). Enhanced protein synthesis may be required to compensate for the proteins damaged as a result of the interaction of antibiotics with the membrane or cytoplasmic proteins, regardless of their cellular target. Some may be involved in metabolism or defense (SOS response and heat shock response). Similarly, Mangalappalli-Illathu and Korber (2006) reported that higher levels of ribosomal proteins associated with increased protein synthesis were important for reduced susceptibility to quaternary ammonium compounds like benzalkonium chloride.

Concerning other metabolic pathways, the enzymes involved in fatty-acid (alcohol dehydrogenase) and pyrimidine biosynthesis (CTP synthase) were down-regulated in the presence of antibiotics. Alterations in fatty-acid biosynthesis may lead to changes in the cell membrane that would favor cell survival in the presence of tetracycline; Rogers et al. (2007) obtained similar results with penicillin-exposed *Streptococcus pneumoniae*. Regarding CTP synthase, this enzyme is required for the biosynthesis of ribo- and deoxyribonucleotides for RNA and DNA replication (Jørgensen et al., 2004). Lowered growth rates obtained just after exposure to antibiotics may have reflected the down-regulation of proteins involved in nucleotide synthesis and fatty acids. However, after antimicrobial exposure, growth rates were either similar or even increased in some antimicrobial-induced cells (e.g., amoxicillin- or benzalkonium-induced cells).

On the other hand, the adaptation of *Lb. pentosus* MP-10 to biocides (benzalkonium chloride or triclosan) induced physiological modifications that are, in part, similar to those caused by antibiotics such as up-regulation of protein synthesis, and down-regulation of carbohydrate metabolism and energy production (Fig. 3). In fact, cross-resistance between antibiotics and biocides was widely reported in literature (e.g., Fraise, 2002; Moken et al., 1997; Randall et al., 2007). Also, in a previous study, *Lb. pentosus* MP-10 pre-adapted to low concentrations of biocides showed increased antibiotic MICs (Casado Muñoz et al., unpublished data), suggesting that the physiological modifications triggered by either a biocide or an antibiotic may provide resistance to the other. Benzalkonium chloride, a disinfectant known to cause membrane damage, specifically induced an over-expression

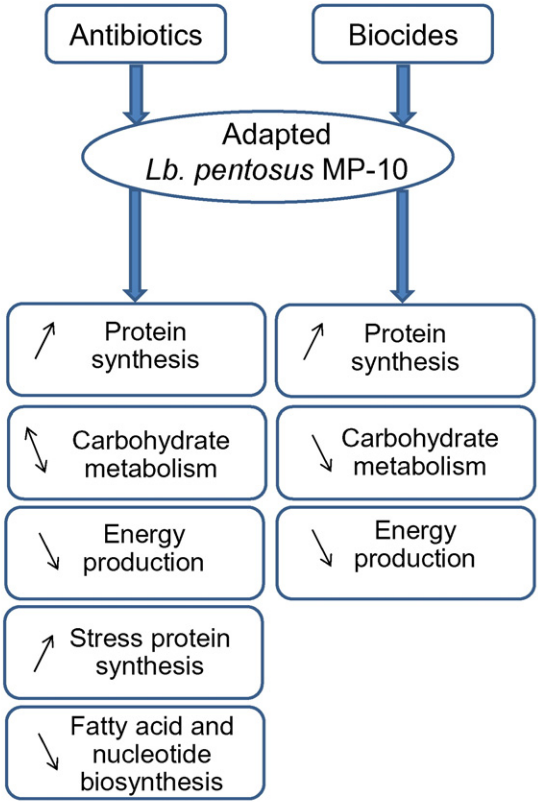


Fig. 3. Schematic representation of the effect of antibiotics and biocides on protein expression in *Lactobacillus pentosus* MP-10.

of ribosomal subunit interface protein related to protein synthesis. However, triclosan caused over-expression of glutamyl-tRNA synthetase, which is considered a key enzyme required for protein biosynthesis. Furthermore, triclosan caused down-regulation of proteins involved in carbohydrate metabolism (phosphocarrier protein HPr) and energy production (oxidoreductase). As stated previously with antibiotics, cells adapted to antimicrobials tended to lower carbohydrate metabolism and energy production, while those involved in protein synthesis were up-regulated to possibly compensate for protein damage as a result of the interaction of biocides with the membrane. Moreover, benzalkonium chloride and triclosan exhibited different adaptation responses, which may be attributed to different mechanisms of action; triclosan acts by inhibiting the enoyl reductase enzyme in fatty acid synthesis (Heath et al., 2002), while benzalkonium chloride has multiple targets in microbial cells (Beumer et al., 2000).

In conclusion, we obtained a better understanding of the proteomic responses of a probiotic bacterium, such as *Lb. pentosus* MP-10 to different antimicrobial stressors. In this sense, we confirmed that antimicrobial stress could enhance bacterial resistance to environmental and gastrointestinal stresses such as acid and bile. Thus, viable counts of some antimicrobial-induced *Lb. pentosus* MP-10 were higher than the non-induced strain. From this information, one could develop strategies to improve the persistence and resistance of this bacterium under different environmental conditions. It has been previously shown that adaptation to different stresses (salt, low pH, bile, high temperature, etc.) could be used as a strategy to enhance the technological performance of probiotic lactobacilli (Corcoran et al., 2006; Desmond et al., 2001; Mills et al., 2011). In our study, pre-stressed *Lb. pentosus* MP-10 exhibited greater viability than those without previous induction (except for a few cases) and had increased tolerance to acidic and high-bile environments than the controls. Here, we describe for the first time that antimicrobial stress adaptation could improve the resistance and robustness of potential probiotic *Lb. pentosus* MP-10 with the aim to withstand conditions where sub-lethal concentrations of antimicrobials and stress conditions (e.g., at low pH or high-bile concentration) may be present, such as the food chain, the environment, or the gastrointestinal tract. On the other hand, this fact has become a great concern since pathogenic bacteria, as they can develop antimicrobial resistance after exposure to antimicrobials, could possibly develop resistance to intestinal conditions. Our results show that *Lb. pentosus* MP-10 responds to the exposure of biocides and antibiotics by adjusting its proteomic arsenal as a survival strategy: up-regulating protein synthesis, including stress proteins, and down-regulating carbohydrate metabolism and energy production (Fig. 3). Further, studies are required to elucidate which proteins are involved in acid and bile tolerance. These aspects should be further emphasized with the aim to achieve desirable robustness of probiotic bacteria in relation to various environmental and gastrointestinal conditions.

Acknowledgments

This work was supported by grants AGL2013-43571-P (Ministerio de Economía y Competitividad, MINECO, FEDER), AGL2009-08921, (Ministerio de Economía y Competitividad, MINECO) and UJA2014/07/02 (Plan Propio de la Universidad de Jaén).

References

Abriouel, H., Benomar, N., Pulido, R.P., Cañamero, M.M., Gálvez, A., 2011. Annotated genome sequence of *Lactobacillus pentosus* MP-10, which has probiotic potential, from naturally fermented Aloreña green table olives. J. Bacteriol. 193, 4559–4560.

Abriouel, H., Benomar, N., Cobo, A., Caballero, N., Fernández Fuentes, M.A., Pérez-Pulido, R., Gálvez, A., 2012. Characterization of lactic acid bacteria from naturally-fermented Manzanilla Aloreña green table olives. Food Microbiol. 32, 308–316.

Anukam, K.C., Olise, N.A., 2012. Development of a novel probiotic yogurt “PENTOYO” with a fully sequenced *Lactobacillus pentosus* KCA1 and its survival during storage at 4 °C. Trop. J. Pharm. Res. 11, 977–981.

Anukam, K.C., Macklaim, J.M., Gloor, G.B., Reid, G., Boekhorst, J., Renckens, B., van Hijum, S., Siezen, R., 2013. Genome sequence of *Lactobacillus pentosus* KCA-1 vaginal isolate from a healthy premenopausal woman. PLoS One 8, e59239.

Bao, Y., Zhang, Y.C., Zhang, Y., Liu, Y., Wang, S.Q., Dong, X.M., Wang, Y.Y., Zhang, H.P., 2010. Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. Food Control 21, 695–701.

Bautista-Gallego, J., Arroyo-López, F.N., Rantsiou, K., Jiménez-Díaz, R., Garrido-Fernández, A., Cocolin, L., 2013. Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. Food Res. Int. 50, 135–142.

Beumer, R., Bloomfield, S.F., Exner, M., Fara, G.M., Nath, K.J., Scott, E., 2000. Microbial resistance and biocides. A Review by the International Scientific Forum on Home Hygiene. International Scientific Forum on Home Hygiene, London, UK (http://www.ifh-homehygiene.org/IntegratedCRD.nsf/111e68ea0824afe1802575070003f039/f309bd6a403617fa80257522005bc5f9?OpenDocument).

Caballero Gómez, N., Abriouel, H., Ennahar, S., Gálvez, A., 2013. Comparative proteomic analysis of *Listeria monocytogenes* exposed to enterocin AS-48 in planktonic and sessile states. Int. J. Food Microbiol. 167, 202–207.

Candiano, G., Bruschi, M., Musante, L., Sabntucci, L., Ghiggeri, G.M., Carnemolla, B., Orecchia, P., Zardi, L., Righetti, P.G., 2004. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25, 1327–1333.

Casado Muñoz, M.C., Benomar, N., Lavilla Lerma, L., Gálvez, A., Abriouel, H., 2014. Antibiotic resistance of *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* isolated from naturally-fermented Aloreña table olives throughout fermentation process. Int. J. Food Microbiol. 172, 110–118.

Corcoran, B.M., Ross, R.P., Fitzgerald, G.F., Dockery, P., Stanton, C., 2006. Enhanced survival of GroESL-overproducing *Lactobacillus paracasei* NFBC 338 under stressful conditions induced by drying. Appl. Environ. Microbiol. 72, 5104–5107.

Desmond, C., Stanton, C., Fitzgerald, G.F., Collins, K., Ross, R.P., 2001. Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying. Int. Dairy J. 11, 801–808.

Deutscher, J., Francke, C., Postma, P.W., 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiol. Mol. Biol. Rev. 70, 939–1031.

Dubois-Brissonnet, F., 2012. Adaptation of *Salmonella* to antimicrobials in food-processing environments. In: Annous, B.A., Gurtler, J.B. (Eds.), Agricultural and Biological Sciences “*Salmonella* — Distribution, Adaptation, Control Measures and Molecular Technologies”. Intech, pp. 123–146.

Duran, G.M., Marshall, D.L., 2005. Ready-to-eat shrimp as an international vehicle of antibiotic-resistant bacteria. J. Food Prot. 68, 2395–2401.

Dutka-Malen, S., Stefan, E., Courvalin, P., 1995. Detection of glycopeptides resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. 33, 24–27.

European Food Safety Authority (EFSA), 2008. Technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. EFSA J. 732, 1–15.

EFSA Panel on Biological Hazards (BIOHAZ), 2010. Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2010 update). EFSA J. 8, 1944.

European Food Safety Authority (EFSA), 2011. Scientific opinion on the safety and efficacy of *Lactobacillus pentosus* (DSM 14025) as a silage additive for all animal species. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). EFSA J. 9, 2449.

European Food Safety Authority (EFSA), 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J. 10, 2740.

Fines, M., Perichon, B., Reynolds, P., Sahm, D.F., Courvalin, P., 1999. VanE, a new type of acquired glycopeptide resistance in *Enterococcus faecalis* BM4405. Antimicrob. Agents Chemother. 43, 2161–2164.

Fraise, A.P., 2002. Susceptibility of antibiotic-resistant cocci to biocides. J. Appl. Microbiol. 92, 158S–162S.

Franz, C.M.A.P., Holzapfel, W.H., Stiles, M.E., 1999. Enterococci at the crossroad of food safety. Int. J. Food Microbiol. 47, 1–24.

Hamon, E., Horvatovich, P., Izquierdo, E., Bringel, F., Marchioni, E., Aoude-Werner, D., Ennahar, S., 2011. Comparative proteomic analysis of *Lactobacillus plantarum* or the identification of key proteins in bile tolerance. BMC Microbiol. 11, 63.

Heath, R.J., White, S.W., Rock, C.O., 2002. Inhibitors of fatty acid synthesis as antimicrobial chemotherapeutics. Appl. Microbiol. Biotechnol. 58, 695–703.

Hummel, A.S., Hertel, C., Holzapfel, W.H., Franz, C.M.A.P., 2007. Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. Appl. Environ. Microbiol. 73, 730–739.

International Organization for Standardization (ISO), 2010. Standard milk and milk products—determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB). ISO 10932/IDF 233. ISO, Geneva, Switzerland.

Izquierdo, E., Horvatovich, P., Marchioni, E., Aoude-Werner, D., Sanz, Y., Ennahar, S., 2009. 2-DE and MS analysis of key proteins in the adhesion of *Lactobacillus plantarum*, a first step toward early selection of probiotics based on bacterial biomarkers. Electrophoresis 30, 949–956.

Jakob, U., Gaestel, M., Engel, K., Buchner, J., 1993. Small heat shock proteins are molecular chaperones. J. Biol. Chem. 268, 1517–1520.

Jensen, L.B., Ahrens, P., Dons, L., Jones, R.N., Hammerum, A.M., Aarestrup, F.M., 1998. Molecular analysis of Tn*1546* in *Enterococcus faecium* isolates from animals and humans. J. Clin. Microbiol. 36, 437–442.

Jørgensen, C.M., Hammer, K., Jensen, P.R., Martinussen, J., 2004. Expression of the *pyrG* gene determines the pool sizes of CTP and dCTP in *Lactococcus lactis*. Eur. J. Biochem. 271, 2438–2445.

Karatzas, K.A.G., Webber, M.A., Jorgensen, F., Woodward, M.J., Piddock, L.J.V., Humphrey, T.J., 2007. Prolonged treatment of *Salmonella enterica* serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. J. Antimicrob. Chemother. 60, 947–955.

Karatzas, K.A.G., Randall, L.P., Webber, M., Piddock, L.J.V., Humphrey, T.J., Woodward, M.J., Coldham, N.G., 2008. Phenotypic and proteomic characterization of multiply antibiotic-resistant variants of *Salmonella enterica* serovar Typhimurium selected following exposure to disinfectants. Appl. Environ. Microbiol. 74, 1508–1516.

Klare, I., Konstabel, C., Müller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., Swings, J., Goossens, H., Witte, W., 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. Appl. Environ. Microbiol. 71, 8982–8986.

Lee, K., Lee, H.G., Choi, Y.J., 2008. Proteomic analysis of the effect of bile salts on the intestinal and probiotic bacterium *Lactobacillus reuteri*. J. Biotechnol. 137, 14–19.

Lin, X., Kang, L., Li, H., Peng, X., 2014. Fluctuation of multiple metabolic pathways is required for *Escherichia coli* in response to chlortetracycline stress. Mol. BioSyst. 10, 901–908.

Liu, C., Zhang, Z., Dong, K., Yuan, J., Guop, X., 2009. Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. Biomed. Environ. Sci. 22, 401–412.

Maisonneuve, E., Gerdes, K., 2014. Molecular mechanisms underlying bacterial persisters. Cell 157, 539–548.

Maldonado-Barragán, A., Caballero-Guerrero, B., Lucena-Padrós, H., Ruiz-Barba, J.L., 2011. Genome sequence of *Lactobacillus pentosus* IG1, a strain isolated from Spanish-style green olive fermentations. j. bacteriol. 193, 5605.

Mangalappalli-Illathu, A.K., Korber, D.R., 2006. Adaptive resistance and differential protein expression of *Salmonella enterica* serovar Enteritidis biofilms exposed to benzalkonium chloride. Antimicrob. Agents Chemother. 50, 3588–3596.

Martineau, F., Picard, F.J., Grenier, L., Roy, P.H., Ouellette, M., Bergeron, M.G., 2000. Multiplex PCR assays for the detection of clinically relevant antibiotic resistance genes in staphylococci isolated from patients infected after cardiac surgery. J. Antimicrob. Chemother. 46, 527–533.

Miele, A., Bandera, M., Goldstein, B.P., 1995. Use of primers selective for vancomycin resistance genes to determine van genotype in enterococci and to study gene organization in VanA isolates. Antimicrob. Agents Chemother. 39, 1772–1778.

Mills, S., Stanton, C., Fitzgerald, G.F., Ross, R.P., 2011. Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. Microb. Cell Fact. 10 (Suppl. 1), S19.

Moken, M.C., McMurry, L.M., Levy, S.B., 1997. Selection of multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the *mar* and *acrAB* loci. Antimicrob. Agents Chemother. 41, 2770–2772.

Narberhaus, F., 2002. Alpha-crystallin-type heat shock proteins: socializing minichaperones in the context of a multichaperone network. Microbiol. Mol. Biol. Rev. 66, 64–93.

Okada, S., Daengsubha, W., Uchimura, T., Ohara, N., Kozaki, M., 1986. Flora of lactic acid bacteria in Miang produced in Northern Thailand. J. Gen. Appl. Microbiol. 32, 57–65.

Randall, L.P., Cooles, S.W., Coldham, N.G., Penuela, E.G., Mott, A.C., Woodward, M.J., Piddock, L.J.V., Webber, M.A., 2007. Commonly used farm disinfectants can select for mutant *Salmonella enterica* serovar Typhimurium with decreased susceptibility to biocides and antibiotics without compromising virulence. J. Antimicrob. Chemother. 60, 1273–1280.

Rezzonico, E., Lariani, S., Barretto, C., Cuanoud, G., Giliberti, G., Delley, M., Arigoni, F., Pessi, G., 2007. Global transcriptome analysis of the heat shock response of *Bifidobacterium longum*. FEMS Microbiol. Lett. 271, 136–145.

Rodríguez-Gómez, F., Romero-Gil, V., García-García, P., Garrido-Fernández, A., Arroyo-López, F.N., 2014. Fortification of table olive packing with the potential probiotic bacteria *Lactobacillus pentosus* TOMC-LAB2. Front. Microbiol. 5, 467.

Rogers, P.D., Liu, T.T., Barker, K.S., Hilliard, G.M., English, B.K., Thornton, J., Swiatlo, E., McDaniel, L.S., 2007. Gene expression profiling of the response of *Streptococcus pneumoniae* to penicillin. J. Antimicrob. Chemother. 59, 616–626.

Ruiz-Barba, J.L., Jiménez-Díaz, R., 2012. A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. Food Microbiol. 30, 253–259.

Sánchez, B., Champomier-Vergès, M.C., Stuer-Lauridsen, B., Ruas-Madiedo, P., Anglade, P., Baraige, F., delos Reyes-Gavilán, C.G., Johansen, E., Zagorec, M., Margolles, A., 2007. Adaptation and response of *Bifidobacterium animalis* subsp. *lactis* to bile: a proteomic and physiological approach. Appl. Environ. Microbiol. 73, 6757–6767.

Sherman, M.Y., Qian, S.B., 2013. Less is more: improving proteostasis by translation slow down. Trends Biochem. Sci. 38, 585–591.

Tajabadi, N., Mardan, M., Abdul Manap, M.Y., Shuhaimi, M., Meimandipour, A., Nateghi, L., 2011. Detection and identification of *Lactobacillus* bacteria found in the honey stomach of the giant honeybee *Apis dorsata*. Apidologie 42, 642–649.

Taranto, M.P., Font de Valdez, G., Perez-Martinez, G., 1999. Evidence of a glucose proton motive force-dependent permease and a fructose phosphoenolpyruvate:phosphotransferase transport system in *Lactobacillus reuteri* CRL 1098. FEMS Microbiol. Lett. 181, 109–112.

Todorov, S.D., Dicks, L.M.T., 2004. Effect of medium components on bacteriocin production by *Lactobacillus pentosus* ST151BR, a strain isolated from beer produced by the fermentation of maize, barley and soy flour. World J. Microbiol. Biotechnol. 20, 643–650.

Veinger, L., Diamant, S., Buchner, J., Goloubinoff, P., 1998. The small heat-shock protein IbpB from *Escherichia coli* stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. J. Biol. Chem. 273, 11032–11037.

Wilkins, J.C., Homer, K.A., Beighton, D., 2002. Analysis of *Streptococcus mutants* proteins modulated by culture under acidic conditions. Appl. Environ. Microbiol. 68, 2382–2390.

Wouters, J.A., Kamphuis, H.H., Hugenholtz, J., Kuipers, O.P., De Vos, W.M., Abee, T., 2000. Changes in glycolytic activity of *Lactococcus lactis* induced by low temperature. Appl. Environ. Microbiol. 66, 3686–3691.

Ying, W., 2006. NAD^+ and NADH in cellular functions and cell death. Front. Biosci. 11, 3129–3148.

Zhang, X.X., Zhang, T., Fang, H.H., 2009. Antibiotic resistance genes in water environment. Appl. Microbiol. Biotechnol. 82, 397–414.

Capítulo II

Visión genómica de los aspectos de seguridad de *Lactobacillus* con potencial probiótico

Artículo 1

New insights in antibiotic resistance of *Lactobacillus* species from fermented foods

Food Research International 78 (2015) 465–481

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

New insights in antibiotic resistance of *Lactobacillus* species from fermented foods

Hikmate Abriouel [a,*], María del Carmen Casado Muñoz [a], Leyre Lavilla Lerma [a], Beatriz Pérez Montoro [a], Wilhelm Bockelmann [b], Rohtraud Pichner [c], Jan Kabisch [b], Gyu-Sung Cho [b], Charles M.A.P. Franz [b], Antonio Gálvez [a], Nabil Benomar [a]

[a] *Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaén, 23071-Jaén, Spain*
[b] *Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Department of Microbiology and Biotechnology, Haid-und-Neu-Str. 9, D-76131 Karlsruhe, Germany*
[c] *Hochschule Fulda, University of Applied Sciences, Department of Nutritional, Food and Consumer Sciences, Leipziger Straße 123, 36037 Fulda, Germany*

ARTICLE INFO

Article history:
Received 28 July 2015
Received in revised form 11 September 2015
Accepted 15 September 2015
Available online 18 September 2015

Keywords:
Lactobacillus spp.
Fermented foods
Phenotypic and genotypic antibiotic resistance
Horizontal gene transfer
In silico analysis

ABSTRACT

Bacteria belonging to the genus *Lactobacillus* are used as starter cultures or that develop naturally as fermenting microbiota in the production of various foods. On the detrimental side, lactobacilli may act as reservoir of antibiotic resistance genes, which can spread to commensal bacteria in humans or animals, or to food-associated pathogens. In the last decade, advances in molecular biology and in genome sequencing have provided more information on antibiotic resistances in foodborne bacteria. The aim of this review was to consider and provide an up-to-date status on phenotypic and genotypic antibiotic resistance profiles in *Lactobacillus* species from fermented foods and also to highlight new information on the distribution of glycopeptide and chloramphenicol resistance genes in *Lactobacillus* genomes. *In silico* screening of *vanZ* (glycopeptide resistance) and *cat* (chloramphenicol resistance)-like sequences in *Lactobacillus* species isolated from fermented foods revealed for the first time the occurrence of *vanZ* and *cat* genes in *Lactobacillus* species being highly conserved genes in the chromosome of each species, presumably non-transferable. Further studies involving genome sequences of *Lactobacillus* isolated from fermented foods, especially those relying on spontaneous fermentation, is crucial to increase knowledge on the potential presence and spread of antibiotic resistance genes via the food route.

Contents

---

[*] Corresponding author at: Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Edif. B3, Universidad de Jaén, Campus Las Lagunillas s/n. 23071-Jaén, Spain.
E-mail address: hikmate@ujaen.es (H. Abriouel).

http://dx.doi.org/10.1016/j.foodres.2015.09.016
0963-9969/

1. Introduction

Members of the genus *Lactobacillus* are Gram-positive, non-spore-forming rods or coccobacilli (Hugenholtz, 1998), catalase-negative (although some strains possess a pseudocatalase), aerotolerant or anaerobic, aciduric or acidophilic and are nutritionally fastidious (Hammes & Vogel, 1995). They generally have a fermentative metabolism, whose primary fermentation end product is lactic acid (Klaenhammer & de Vos, 2011; Tannock, 2004) besides other products such as acetate, ethanol, CO_2, formate and succinate. *Lactobacillus* is the largest genus among lactic acid bacteria (LAB) with over 212 species described to date in *List of Prokaryotic names with Standing in Nomenclature* "LPSN" (January 2015, www.bacterio.net). This genus is characterized by a high level of diversity, which is reflected in its complex phylogeny (Schleifer & Ludwig, 1995). For *Lactobacillus* classification, various schemes have been proposed, based on phenotype, physiology, biochemical characteristics and housekeeping genes such as the 16S rRNA-encoding gene. Nowadays, investigations on the taxonomy of the *Lactobacillus* genus include investigations of a large number of genes or proteins, or indeed the whole genome (phylogenomics). Indeed, genome sequencing and phylogenomics is regarded by some as the way forward from polyphasic taxonomy in bacterial taxonomy (Vandamme & Peeters, 2014). Genome sequencing (more than 100 whole genome sequences available in NCBI database: www.ncbi.nlm.nih.gov) has already paved the way for a better understanding of the genus, which may be subject to future reclassifications (Claesson, van Sinderen, & O'Toole, 2008; Makarova et al., 2006; Zhang, Ye, Yu, & Shi, 2011).

Lactobacilli are ubiquitous in the environment and in raw materials used in food production, thus their role in the production of fermented foods has been reported for millennia (reviewed in Tamang & Kailasapathy, 2010). Numerous species of *Lactobacillus* are relevant in fermented foods as they have been used as starter and/or protective cultures in fermented vegetables, dairy products, sausages and fish (Franz, Cho, & Holzapfel, 2011; Garrigues, Johansen, & Crittenden, 2013; Giraffa, Chanishvili, & Widyastuti, 2010; Hansen, 2002; Heller, 2001; Holzapfel, 2002; Leroy & DeVuyst, 1999). These microorganisms are considered as generally regarded as safe (GRAS) in the U.S.A. due to their long history of use as food processing aids, and some strains of *Lactobacillus* were shown to confer a health benefit on humans and animals (Casas & Dobrogosz, 1997, 2000). In Europe, the suggested Qualified Presumption of Safety "QPS" safety assessment scheme suggested by EFSA is based on the 'body of knowledge' of the species and a safety decision tree which excludes strains that have any history of infection, or which have transferable antibiotic resistance or virulence genes (EFSA, 2012a). Thus, the safety of *Lactobacillus* species should be considered, even though they are common members of the indigenous microbiota (the human mouth, gastrointestinal tract and female genital tract). This is partly due to the fact that some strains may occasionally cause opportunistic infections, especially in elderly and immunocompromised individuals (Cannon, Lee, Bolanos, & Danziger, 2005; Harty, Oakey, Patrikakis, Hume, & Knox, 1994; Schlegel, Lemerle, & Geslin, 1998), and that some strains may carry antibiotic resistance genes that may be transferable. Infections that lactobacilli have been isolated from include dental infections, bacteremia and endocarditis, and in rare cases also other infections (Bennett, Shekhel, Radelet, & Miller, 2014; Franko et al., 2013; Nei et al., 2013; Salminen et al., 1998; Vahabnezhad, Mochon, Wozniak, & Ziring, 2013). The purpose of this review is to summarize the current knowledge on antibiotic resistances and resistance genes in species of the genus *Lactobacillus*, in order to gain an understanding of the potential risk of fermented foods as vehicle for antibiotic-resistant bacteria and for antibiotic resistance genes.

2. Antibiotic resistance in lactobacilli of fermented food origin

Antibiotics are commonly used in animal husbandry to cure or prevent the onset of bacterial infections in agriculture, but antibiotics were also used as growth promoters for decades (forbidden as growth promoters in the EU since January 2006 and South Korea since 2011, however some antibiotics are still allowed in other countries of Asia and in Australia and USA among others), which could have exerted a selective pressure for the emergence and spread of antibiotic-resistant bacteria. The emergence of antibiotic resistances is a complex phenomenon, which involves interactions between humans, animals, bacteria, drugs and the environment (Barbosa & Levy, 2000; Coast & Smith, 2003; O'Brien, 2002). The growing body of evidence which reports on antibiotic resistances in LAB of food origin (Egervärn, Roos, & Lindmark, 2009; Ge, Jiang, & Han, 2007; Marshall, Ochieng, & Levy, 2009; Teuber, Meile, & Schwarz, 1999), albeit at a low incidence, has reflected the global concern of antibiotic resistances in food safety and public health.

Lactobacilli eventually reach the gastro-intestinal tract via ingestion of the high numbers of these bacteria in the fermented foods (typically for fermented foods $>10^8$ CFU/ml; Ali & Mustafa, 2009; Cadena, Villarraga, Luján, & Salcedo, 2006) where they can interact with the resident gut microbiota of the host. Because of the high numbers of microorganisms, the confined conditions and the close cell-to cell contact in this environment, the gastrointestinal tract is an ideal hot spot for gene transfer. The possibility for transfer of bacteria from fermented foods to a gastrointestinal environment underlines the importance of the microbiological quality of fermented foods, especially of traditional fermented foods, which rely on a natural and diverse microbiota present in raw materials. This is less so for industrial fermentations, which rely on a few selected and well-defined starter cultures (Verraes et al., 2013). In this regard, the diversity of traditional Mediterranean, African or Asian fermented food products is a result of differences in bacterial species found naturally in raw materials of each geographical region, local environmental conditions and traditional processing procedures, and the fermented end products are thus characterized by a diverse microbiota as was reported by several authors (Abriouel, Benomar, Lucas, & Gálvez, 2011; Abriouel, Martín-Platero, Maqueda, Valdivia, & Martínez-Bueno, 2008; Abriouel et al., 2006, 2012; Ben Omar et al., 2000; Jokovic et al., 2008, Liu et al., 2011; Nam et al., 2012; Oguntoyinbo et al., 2011; Settani & Moschetti, 2010). Considering the physico-chemical properties of fermented foods, stressors such as salt, low pH and additives could enhance this resistance by switching on specific genes (silent genes), increasing their expression or facilitating their dissemination between strains which coexist in the same place and at the same time (Al-Nabulsi et al., 2011; Ganjian et al., 2012; McMahon, McDowell, & Blair, 2007; Poole, 2012). Clearly, therefore, there is a need for a closer investigation of both the biodiversity of LAB associated with and predominating in the production of traditional fermented foods, as well as their safety regarding acquired and transferable antibiotic resistances.

The role of foodborne lactobacilli as potential reservoirs of antibiotic resistance (AR) has gained more attention, even though these bacteria have historically been considered as "GRAS". Thus, the presence of potentially transferable genes is of great concern and should be considered. Until 1999, there were only a few studies which evaluated acquired antibiotic resistance in lactobacilli (Teuber, 1999). Currently, there are several studies reporting antibiotic resistance in *Lactobacillus* species (Casado Muñoz, Benomar, Lavilla Lerma, Gálvez, & Abriouel, 2014; Hummel, Hertel, Holzapfel, & Franz, 2007; Kastner et al., 2006; Klare et al., 2007; Klein, 2011; Liu, Zhang, Dong, Yuan, & Guo, 2009; Zonenschain, Rebecchi, & Morelli, 2009) and an intrinsic resistance could be shown for many antibiotics (Casado Muñoz, Benomar, Lavilla Lerma, Gálvez, & Abriouel, 2014; Hummel, Hertel, Holzapfel, & Franz, 2007; Klein, 2011). In addition, genes encoding transferable antibiotic resistances were identified and characterized (Ammor et al., 2008; Belletti et al., 2009; Devirgiliis, Coppola, Barile, Colonna, & Perozzi, 2009; Egervärn, Roos, & Lindmark, 2009; Feld, Bielak, Hammer, & Wilcks, 2009).

At present, an additional advantage for assessment of safety and presence of antibiotic resistance genes comes from complete genome sequencing projects as complete genome sequences become more and more available for several *Lactobacillus* species and strains (Lee, Chae, Lee, Lim, & Kim, 2011; Zhang, Ye, Yu, & Shi, 2011). These sequences can be used to monitor for virulence traits as well as transferable antibiotic resistance genes (Bennedsen et al., 2011).

2.1. Methodologies for phenotypic antibiotic resistance determination

As a basic requirement, susceptibility tests to a relevant range of antimicrobials of human and veterinary importance should be carried out for each bacterial strain intended for use as feed additive for humans or animals. Before addressing antibiotic resistances in species within the genus *Lactobacillus*, it is important to describe the methodologies used to determine these and to categorize lactobacilli strains as either susceptible or resistant to specific antimicrobials. To test antibiotic susceptibility in lactobacilli, different standardized, quantitative methods have been developed, such as the broth dilution or agar dilution methods (serial two-fold dilution procedures) or E-tests for the determination of minimum inhibitory concentration (MIC) according to the Clinical and Laboratory Standard Institute (CLSI; www.clsi.org), the European Committee on Antimicrobial Susceptibility Testing (EUCAST), ISO standard, British Society for Antimicrobial Chemotherapy (BSAC, UK), Agence Francaise de Securité Sanitaire des Produits de Santé (AFFSAPS, France) or Deutsches Institut für Normung e.V. (DIN, Germany) and the ISC/WHO (International Society of Chemotherapy/World Health Organization). However, qualitative or semi-qualitative methods to determine MIC indirectly, such as diffusion methods (disk diffusion), are generally not acceptable (EFSA, 2012b). After incubation, the MIC is defined as the lowest concentration of the antimicrobial that inhibits bacterial growth. The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA) defined the microbiological breakpoints to distinguish between antimicrobial resistant strains from the susceptible ones. Hence, a bacterial strain is categorized as susceptible (S) when it is inhibited at a concentration of a specific antimicrobial equal or lower than the established cut-off value ($S \leq x$ mg/L), and as resistant (R), when it is not inhibited at a concentration of a specific antimicrobial higher than the established cut-off value ($R > x$ mg/L) according to EFSA (2012b). A regular update on the microbiological cut-off values of different antimicrobials is reported when data from the scientific community and other relevant sources (e.g., the European Medicines Agency, the European Centre of Diseases Prevention and Control) become available (EFSA, 2012b). Besides investigating the minimal antimicrobial concentration of an antibiotic for a single strain, one may also test for the distribution of a population of bacteria belonging to a single taxonomical unit (species or genus). Bi- or multi-modal MIC distribution usually results from acquired resistances by either added genes or mutation (Ammor et al., 2008).

The MIC of antibiotics expressed in mg/L or μg/ml should be determined in *Lactobacillus* species and strains for each of the following antibiotics: ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol (EFSA, 2012a), taking into consideration different parameters (growth media, inoculum size and incubation conditions such as temperature, incubation time, and composition of the atmosphere) (White et al., 2001) and using relevant quality control strains. In this sense, different media were used for microdilution antibiotic susceptibility testing of lactobacilli taking into consideration the special growth requirements in terms of medium acidity and carbohydrate supplementation. The LAB susceptibility test medium “LSM” broth (Klare et al., 2005) was specifically developed for these bacteria to overcome the disadvantages of standard media for LAB propagation such as MRS broth, since this medium could interfere with some antibiotics (trimethoprim and sulfamethoxazole) as it contains antagonistic components such as *p*-aminobenzoic acid (against sulfamethoxazole) and/or thymidine (against trimethoprim) (Klare et al., 2005; Turnidge & Bell, 2005). Moreover, Muller-Hinton and Iso-Sensitest (IST) media are often not suitable for susceptibility testing of lactobacilli (Klare et al., 2007). Recently, ISO 10932/IDF 233 Standard (ISO, 2010) medium that is based on the LSM formulation was made available for susceptibility testing.

2.2. Phenotypic and genotypic resistance patterns of lactobacilli

Distinction between intrinsic (non-horizontally transmissible) and acquired (which relies on mobile genetic elements such as plasmids, transposons, integrons, or on mutations) resistance in lactobacilli to common antibiotics is necessary. In general, most *Lactobacillus* species are intrinsically resistant to aminoglycosides (neomycin, kanamycin, streptomycin and gentamicin), glycopeptides (vancomycin and teicoplanin), inhibitors of nucleic acid synthesis (ciprofloxacin, enoxacin, pefloxacin, norfloxacin, nalidixic acid, and metronidazole) and inhibitors of folic acid synthesis (sulphamethoxazole, trimethoprim and co-trimoxazole) (Charteris, Kelly, Morelli, & Collins, 1998; Coppola et al., 2005; Patel, Shah, & Prajapati, 2012; Zhou, Pillidge, Gopal, & Gill, 2005). However, they are susceptible to penicillins (ampicillin and penicillin G), chloramphenicol, streptomycin, clindamycin, tetracycline, erythromycin, linezolid and quinupristin/dalfopristin (Ammor, Flórez, & Mayo, 2007; Katla, Kruse, Johnsen, & Herikstad, 2001; Luh et al., 2000; Patel, Shah, & Prajapati, 2012). Susceptibility to bacitracin varies greatly (Coppola et al., 2005; Katla, Kruse, Johnsen, & Herikstad, 2001).

Here, we review the phenotypic and genotypic antibiotic resistance pattern of *Lactobacillus* species isolated from fermented foods (dairy, fermented vegetables and fermented meat), being some of them reviewed by Devirgiliis, Zinno, & Perozzi (2013). In most cases, knowledge of the antibiotic resistance phenotypes may still be important, even in the absence of resistance transfer mechanisms.

2.2.1. Resistance to antibiotics inhibiting cell wall synthesis

In general, *Lactobacillus* species were found to be susceptible to many cell wall synthesis inhibitors, like penicillins (ampicillin, oxacillin and piperacillin) and β-lactamase inhibitors, but relatively more resistant to cephalosporins (cephalotin and cefuroxime, ceftriaxone and cefoxitin). However, a widespread resistance toward penicillins, especially penicillin G, has already been observed in lactobacilli used as probiotics or starter cultures (Charteris, Kelly, Morelli, & Collins, 1998; Danielsen & Wind, 2003), in *Lb. rhamnosus*, *Lb. reuteri* and *Lb. plantarum* isolated from cheese (Belleti et al., 2009; Čanžek Majhenič, Mohar Lorbeg, & Rogelj, 2007; Flórez, Delgado, & Mayo, 2005; Temmerman, Scheirlinck, Huys, & Swings, 2003), in *Lb. delbrueckii* subsp. *bulgaricus* from Chinese yogurts (Zhou et al., 2012), in *Lb. casei* from fermented milk “Dahi” (Soomro & Masud, 2012), in lactobacilli from fermented milk in Burkina Faso (Savadogo, Ouattara, Ilboudo, Karou, & Traore, 2010) or India (Lavanya, Sowmiya, Balaji, & Muthuvelan, 2011), in *Lb. casei* and

Lb. helveticus from fermented milk (Yüksekdağ & Beyatli, 2008), in *Lb. plantarum* from fermented vegetables (Lapsiri, Nitisinprasert, & Wanchaitanawong, 2011; Pérez Pulido et al., 2005), in *Lb. salivarius* from different fermented foods (Nawaz et al., 2011), in *Lactobacillus* sp., *Lb. curvatus* and *Lb. sakei* from fermented dry sausages (Aymerich et al., 2006; Gevers, Danielsen, Huys, & Swings, 2003), in lactobacilli from Nigerian fermented foods and beverages (Olukoya, Ebigwei, Adebawo, & Osiyemi, 1993), in lactobacilli from fermented fish (Sornplang, Leelavatcharamas, Sukon, & Yowarach, 2011) and in lactobacilli from traditional Ethiopian fermented foods (traditional fermented condiments, Awaze and Qotchqotcha and fermented Tef dough) (Dessalegn & Ashenafi, 2010) (Table 1).

Apart from penicillin-resistance, other studies showed that certain lactobacilli (*Lactobacillus* sp. strains not identified at species level, as

Table 1
Overview of antibiotic resistance in the fermented food-associated *Lactobacillus*.

	Fermented foods	*Lactobacillus* species	Antibiotic resistance		References
			Phenotypic resistance*	Genes	
Dairy products	Yogurt	*Lb. acidophilus*	ERY	*ermB*	Nawaz et al. (2011)
		Lb. brevis	TET	*tetM*, *tetS*	Nawaz et al. (2011)
		Lb. delbrueckii subsp. *bulgaricus*	AMP, CM, CTC, KAN, TET, LCM, NEO, GEN, PENG,	*ant*(6), *aph*(3´)*-III*, *tetM*	Zhou et al. (2012)
			ROX, STR	*ermB*	Nawaz et al. (2011)
			ERY	*tetS*	Nawaz et al. (2011)
		Lb. fermentum	TET	*ermB*, *tetM*	Nawaz et al. (2011)
		Lb. kefiri	ERY, TET		
		Lb. plantarum			
	Cheese	*Lactobacillus* sp.	ERY, TET AMP, APR, CEFPO, CIP, COL, GEN, KAN,	*ermB*, *tetM*	van Hoek et al. (2008)
		Lb. casei	NAL, NEO, SPEC, STR, SMX, TMP, VAN	*aadA*, *aadE*, *aph*(3´)*-III*,	Ouoba, Lei, and Jensen (2008)
			ERY, TET		
		Lb. curvatus	DAL, ERY, NOV, TET	*ermB*, *tetM*	van Hoek et al. (2008)
		Lb. fermentum		*ermlF*, *tetM*, *vatE-1*	Gfeller, Roth, Meile, and Teuber (2003)
			ERY, TET		
		Lb. paracasei		*ermB*, *tetM*, *tetW*	van Hoek et al. (2008)
			KAN, STR		Comunian et al. (2010)
		Lb. pentosus	TET	*aadA*, *aadE*, *aph*(3´)*-III*	Han et al. (2013)
		Lb. plantarum		*tetM*	Zago et al. (2011)
			ERY		van Hoek et al. (2008)
		Lb. plantarum		*ermB*	Feld, Bielak, Hammer, and Wilcks (2009)
			TET		
		Lb. sakei		*tetL*, *tetM*	Ammor et al. (2008)
					van Hoek et al. (2008)
	Whey fermented milk	*Lb. salivarius*	TET	*tetM*	Nawaz et al. (2011)
		Lb. vaginalis	ERY	*ermB*	Nawaz et al. (2011)
	Others	*Lb. fermentum*	ERY, TET	*ermB*, *msrC*, *tetK*, *tetL*	Thumu and Halami (2012)
		Lb. plantarum	ERY, TET	*ermB*, *tetL*, *tetW*	Thumu and Halami (2012)
		Lb. plantarum	AMK, BC, CIP, GEN, TMP/SMX, VAN	*vanX*	Liu, Zhang, Dong, Yuan, and Guo (2009)
Fermented vegetables	Pickle	*Lb. plantarum*	TET	*tetM*	van Hoek et al. (2008)
		Lb. salivarius	ERY, TET	*ermB*, *tetM*	Nawaz et al. (2011)
	Fermented vegetables	*Lb. animalis*	ERY, TET	*ermB*, *tetM*	Nawaz et al. (2011)
		Lb. brevis	TET	*tetM*, *tetS*	Nawaz et al. (2011)
		Lb. brevis	CIP, ERY	*ermB*	Pan, Hu, and Wang (2011)
		Lb. fermentum	AMP, CM, CIP, CLI, ERY, KAN, TET	*tetM*	Pan et al. (2011)
		Lb. helveticus	AMP, CM, CLI, KAN	*erm*	Pan et al. (2011)
		Lb. mali	CLI, TET	*tetM*	Pan et al. (2011)
		Lb. namurensis	CM, CIP, CLI, KAN, TET	*aph*(3´)*-III*, *tetM*	Pan et al. (2011)
		Lb. parabuchneri	TET	-	Nawaz et al. (2011)
		Lb. plantarum	TET	*tetM*, *tetS*	Nawaz et al. (2011)
		Lb. plantarum	AMP, CM, CIP, CLI, ERY, KAN, TET	*aph*(3´)*-III*, *erm*, *ermB*, *mefA*,	Pan et al. (2011)
		Lb. salivarius	TET	*tetM*	Nawaz et al. (2011)
		Lb. vaginalis	ERY	*tetM*	Nawaz et al. (2011)
				ermB	
Fermented meat and fish	Fermented meat	*Lb. alimentarius*	TET	*tetM*	Gevers et al. (2003)
		Lb. brevis	ERY, TET	*ermB*, *tetM*	Zonenschain et al. (2009)
		Lb. curvatus	ERY, TET	*ermB*, *tetM*, *tetW*	Zonenschain et al. (2009)
		Lb. curvatus	TET	*tetM*	Gevers, Danielsen, Huys, and Swings (2003)
		Lb. paracasei	ERY, TET	*ermB*, *tetM*	Zonenschain, Rebecchi, and Morelli (2009)
		Lb. paracasei	ERY, TET	*ermB*, *tetM*	Comunian et al. (2010)
		Lb. plantarum	ERY, TET	*ermB*, *ermC*, *tetM*, *tetS*, *tetW*	Zonenschain et al. (2009)
		Lb. plantarum	TET	*ermB*, *tetM*	Gevers et al. (2003)
		Lb. reuteri	ERY, TET	*ermB*, *tetM*	Zonenschain et al. (2009)
		Lb. reuteri	ERY, TET	*ermB*, *tetW*	Thumu and Halami (2012)
		Lb. rhamnosus	ERY, TET	*ermB*, *tetW*	Zonenschain et al. (2009)
		Lb. sakei	ERY, TET	*ermB*, *tetM*, *tetW*	Zonenschain et al. (2009)
		Lb. sakei	TET	*tetM*, *tetK*	Martins et al. (2013)
		Lb. sakei	TET	*tetM*	Gevers et al. (2003)
		Lb. salivarius	ERY, TET	*ermB*, *tetL*, *tetM*, *tetO*, *tetW*	Thumu and Halami (2012)

* AMK, amikacin; AMP, ampicillin; APR, apramycin; BC, bacitracin; CEFPO, cefpodoxime; CIP, ciprofloxacin; CLI, clindamycin; CM, chloramphenicol; COL, colistin; CTC, chlortetracycline; DAL, dalfopristin; ERY, erythromycin; GEN, gentamycin; KAN, kanamycin; LCM, lincomycin; NAL, Nalidixan; NEO, neomycin; NOV, novobiocin; PENG, penicillin G; ROX, roxithromycin; SPEC, Spectinomycin; STR, streptomycin; TET, tetracycline; TMP/SMX, trimethoprim/sulphamethoxazole; VAN, vancomycin.

well as *Lb. plantarum* strains) isolated from dairy products showed resistance to ampicillin (Hleba, Kačániová, Pavelková, & Čuboň, 2012; Rozila et al., 2012; Zhou et al., 2012). Furthermore, ampicillin-resistant lactobacilli were also isolated from Nigerian fermented foods and beverages (Olukoya, Ebigwei, Adebawo, & Osiyemi, 1993), fermented fish (Sornplang, Leelavatcharamas, Sukon, & Yowarach, 2011) as well as fermented milk in India (Lavanya, Sowmiya, Balaji, & Muthuvelan, 2011). Other ampicillin-resistant lactobacilli species were isolated from various products and include *Lb. casei* isolated from fermented milk "Dahi" (Soomro & Masud, 2012), *Lb. curvatus* and *Lb. sakei* (Aymerich et al., 2006) and *Lb. plantarum* from fermented sausages (Pan, Hu, & Wang, 2011), *Lb. fermentum* and *Lb. helveticus* from fermented vegetables (Pan, Hu, & Wang, 2011). Resistance to ampicillin in *Lb. reuteri* was shown to result from point mutations in *pbp* genes which encode the penicillin binding proteins (Pbp1a, Pbp2a and/or Pbp2x), the target of beta-lactam antibiotics. Such resistance is regarded as non-transferable, because the *pbp* genes are located on the chromosome (Rosander, Connolly, & Roos, 2008).

Resistance to cephalosporins and oxacillin was also shown to occur in lactobacilli (especially *Lb. rhamnosus*) isolated from dairy products such as different types of cheeses (Belleti et al., 2009; Coppola et al., 2005; Danielsen & Wind, 2003; Herreros et al., 2005; Hummel, Hertel, Holzapfel, & Franz, 2007). Resistance to these compounds was also shown in *Lb. paracasei* from yogurts (Honi et al., 2013), in *Lb. plantarum* and *Lb. pentosus* from fermented olives (Casado Muñoz, Benomar, Lavilla Lerma, Gálvez, & Abriouel, 2014; Mokhbi, Kaid-Harche, Lamri, Rezki, & Kacem, 2009) and in *Lb. curvatus* and *Lb. fermentum* in fermented sausages (Zdolec et al., 2011).

Condon (1983) showed that cell wall impermeability seems to be the main mechanism of resistance to beta-lactam antibiotics. Furthermore, the cooperation of non-specific mechanisms, such as multi-drug transporters (Putman, van Veen, Degener, & Konings, 2001) and defective cell wall autolytic systems (Kim, Morrison, & Bayer, 1982) may also account for differences between strains within the same species (Ammor, Flórez, & Mayo, 2007).

Concerning resistance to glycopeptides, most *Lactobacillus* species carried intrinsic resistance (Charteris, Kelly, Morelli, & Collins, 1998; Danielsen & Wind, 2003; Fukao et al., 2009; Handwerger, Pucci, Volk, Liu, & Lee, 1994; Klein, Zill, Schindler, & Louwers, 1998; Klein et al., 2000; Mathur & Singh, 2005; Ruoff, Kuritzkes, Wolfson, & Ferraro, 1988; Swenson, Piwnica-Worms, McNamee, & Paul, 1990), which is generally based on the presence of D-Alanine- D-Lactate rather than the D-Ala–D-Ala dipeptide in their peptidoglycan (Ammor et al., 2008; Klein et al., 2000). However, such resistance is different from the enterococcal type which is based on the presence of *vanA*, *vanB* or *vanC* acquired genes (DeLisle & Perl, 2003; Tynkynnen, Singh, & Varmanen, 1998). Klein, Zill, Schindler, & Louwers (1998) and Klein et al. (2000) confirmed that they were unable to show transfer of vancomycin-resistance determinants from *Lb. rhamnosus* to a vancomycin-susceptible enterococcal strain, which provided reassurance on the safety of the *Lactobacillus* strains used as probiotics with regard to their vancomycin resistance. However, an unclear resistance basis of *Lactobacillus* spp. could pose uncertainty about its use as probiotics (EFSA, 2012a). Charteris, Kelly, Morelli, & Collins (2001) and Zhou, Pillidge, Gopal, & Gill (2005) found vancomycin resistance in several probiotic strains of *Lb. rhamnosus.* Similarly, intrinsic resistance to vancomycin was confirmed for *Lb. paracasei*, *Lb. salivarius* and *Lb. plantarum* (Blandino, Milazzo, & Fazio, 2008). However, other studies showed that *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. acidophilus*, *Lb. johnsonii*, and *Lb. crispatus* possessed acquired mechanisms of resistance (Bernardeau, Vernoux, Henri-Dubernet, & Gueguen, 2008; Vescovo, Morelli, & Bottazzi, 1982) to vancomycin. Also, Mater, Langella, Corthier, & Flores (2008) showed that a commercial *Lb. acidophilus* could acquire a *vanA* (encoding high level resistance in enterococci) gene mediated by a Tn*1546* transposon from enterococci *in vitro* and *in vivo* in the gut of mice. Therefore, acquisition and further transfer of resistance genes should be addressed in the safety evaluations of probiotics and starter cultures.

2.2.2. Resistance to antibiotics inhibiting proteins synthesis

Generally, most *Lactobacillus* species are susceptible to antibiotics that are able to inhibit protein synthesis (erythromycin, tetracycline, clindamycin and chloramphenicol) and they also tend to be resistant toward aminoglycosides (streptomycin, kanamycin, neomycin, and gentamicin) (Ammor, Flórez, & Mayo, 2007). Resistance to aminoglycosides was reported in lactobacilli isolated from different fermented foods, such as *Lb. plantarum* from fermented vegetables (Lapsiri, Nitisinprasert, & Wanchaitanawong, 2011), *Lb. pentosus* from naturally fermented olives (Casado Muñoz, Benomar, Lavilla Lerma, Gálvez, & Abriouel, 2014), *Lb. bulgaricus* and *Lb. paracasei* from yogurts (Honi et al., 2013; Zhou et al., 2012), *Lb. helveticus*, *Lb. delbrueckii* subsp. *lactis*, *Lb. rhamnosus* and *Lb. casei* (Belletti et al., 2009), *Lb. casei* (Ouoba, Lei, & Jensen, 2008), *Lb. fermentum* (Gfeller, Roth, Meile, & Teuber, 2003) and *Lb. pentosus* (Han et al., 2013) from cheese, *Lb. plantarum* from dairy products (Liu, Zhang, Dong, Yuan, & Guo, 2009), *Lb. fermentum*, *Lb. helveticus*, *Lb. namurensis* and *Lb. plantarum* from fermented vegetables (Pan, Hu, & Wang, 2011), *Lb. brevis*, *Lb. fermentum* and *Lb. paracasei* subsp. *paracasei* from fermented sausages (Zdolec et al., 2009) and *Lb. plantarum*, *Lb. casei* and *Lb. paracasei* from fermented fish (Liasi et al., 2009). Intrinsic resistance to aminoglycosides may result from various mechanisms and thus can be attributed to the absence of cytochrome-mediated electron transport enabling antibiotic uptake (Charteris, Kelly, Morelli, & Collins, 2001) and to changes in cellular permeability. However, the most important cause of resistance is an enzymatic antibiotic modification by "acetyltransferases (AAC9, adenyltransferases (ANT) and phosphotransferases (APH)", whose encoding genes are usually found on plasmids and transposons (Turnidge & Bell, 2005). The acquired resistance genes *aadE* and *aphA3* have previously been found in *Lb. curvatus* from raw milk cheese (Danielsen, Madsen, Hammer, & Wind, 2005); *aph*(3′)-III, *aadA* and *aadE* genes occurred in *Lb. casei* from dairy products (Ouoba, Lei, & Jensen, 2008) and in *Lb. pentosus* from cheese (Han et al., 2013); *aph*(3′)-III and *ant*(6) genes in *Lb. delbrueckii subsp. bulgaricus* from dairy products (Zhou et al., 2012); *aph*(3′)-III in *Lb. plantarum* and *Lb. namurensis* from Chinese fermented vegetable foods. In the last case, *aph*(3′)-III was found to be located together with other resistance genes on the same plasmid from *E. faecium* SZ109 (Pan, Hu, & Wang, 2011), suggesting a horizontal gene transfer.

As mentioned above, most of *Lactobacillus* species are susceptible to chloramphenicol, erythromycin, clindamycin and tetracycline. However, strains resistant to these agents have also been identified in lactobacilli isolated from several fermented foods. Resistance to chloramphenicol was detected in *Lb. plantarum*, *Lb. fermentum*, *Lb. helveticus* and *Lb. namurensis* isolated from Chinese fermented vegetable foods (Pan, Hu, & Wang, 2011), in *Lb. sakei* from fermented meat products (Martins et al., 2013) and in lactobacilli isolated from Chinese yogurts (Zhou et al., 2012). Resistance to erythromycin was shown to occur in strains of *Lactobacillus* spp. isolated from cheese (Belletti, Monica, Benedetta, Erasmo, Giulia and Fausto, 2009; Comunian et al., 2010; Feld, Bielak, Hammer, & Wilcks, 2009; Gfeller, Roth, Meile, & Teuber, 2003; van Hoek et al., 2008), from dairy products (Hleba, Kačániová, Pavelková, & Čuboň, 2012; Thumu & Halami, 2012), from Chinese fermented vegetable foods (Pan, Hu, & Wang, 2011), from fermented meats (Comunian et al., 2010; Gevers, Danielsen, Huys, & Swings, 2003; Thumu & Halami, 2012; Zonenschain, Rebecchi, & Morelli, 2009) and from a variety of fermented foods (Nawaz et al., 2011) (Table 1).

Resistance to clindamycin was detected in different lactobacilli species isolated from Chinese fermented vegetable foods (Pan, Hu, & Wang, 2011), cheese (Belletti, Monica, Benedetta, Erasmo, Giulia and Fausto, 2009) and fermented vegetables (Lapsiri, Nitisinprasert, &

Wanchaitanawong, 2011) (Table 1). Tetracycline resistance was detected in lactobacilli isolated from various fermented foods such as Chinese fermented vegetable foods (Pan, Hu, & Wang, 2011), cheese (Ammor et al., 2008; Belletti, Monica, Benedetta, Erasmo, Giulia and Fausto, 2009; Comunian et al., 2010; Gfeller, Roth, Meile, & Teuber, 2003; van Hoek et al., 2008; Zago et al., 2011), dairy products (Hleba, Kačániová, Pavelková, & Čuboň, 2012; Thumu & Halami, 2012), from a variety of fermented foods (Nawaz et al., 2011), pickles (van Hoek et al., 2008), fermented vegetables (Pan, Hu, & Wang, 2011), fermented meat (Comunian et al., 2010; Gevers, Danielsen, Huys, & Swings, 2003; Martins et al., 2013; Thumu & Halami, 2012; Zonenschain, Rebecchi, & Morelli, 2009), and African fermented millet porridge (Ouoba, Lei, & Jensen, 2008) (Table 1).

Acquired resistance to tetracycline, erythromycin, clindamycin and chloramphenicol in lactobacilli isolated from fermented foods were found to be mediated by *tet*M and *tet*S genes (encoding ribosomal protection proteins for tetracycline resistance) and *erm*(B) (erythromycin resistance), these being the most commonly observed resistance genes in lactobacilli isolated from a variety of foods, followed by the *cat* (plasmid encoded chloramphenicol acetyltransferase) gene (Cataloluk & Gogebakan, 2004; Danielsen, 2002; Gevers, Danielsen, Huys, & Swings, 2003; Lin, Fung, Wu, & Chung, 1996). The *cat* resistance gene was also detected on the chromosome in different lactobacilli isolated from fermented foods, as we will further discuss in detail.

In the last years, several studies showed that besides *tet*M and *tet*S genes, other tetracycline resistance genes were prevalent in lactobacilli such as the *tet*W gene, encoding a ribosomal protection protein (Comunian et al., 2010; Devirgiliis, Zinno, & Perozzi, 2013; Thumu & Halami, 2012; Zonenschain, Rebecchi, & Morelli, 2009), and the *tet*L efflux gene occurring in lactobacilli from dairy products and fermented meat (Ammor et al., 2008; Thumu & Halami, 2012). Resistance genes *tet*M and *tet*S were identified both on plasmids and the chromosome in different *Lactobacillus* species isolated from different fermented foods (Table 1). Comunian et al. (2010) showed that *tet*M expression requires tight antibiotic-dependent regulation, similarly to tetracycline-resistance genes in *Escherichia coli*. Moreover, this observation supports the results obtained with two strains of *Lb. sakei* isolated from an Italian raw milk cheese (Ammor et al., 2008), where *tet*M was found within transposon-like sequences. Association of *tet*M with widely different MIC values in different strains has been reported by several authors (Flórez et al., 2008; Gevers, Danielsen, Huys, & Swings, 2003). Comunian et al. (2010) showed that increasing transcript levels correlated with increasing MIC values, which suggested that *tet*M transcription is dependent on antibiotic concentration because of an antibiotic concentration-dependent promoter activity. Furthermore, strains harboring more than one tetracycline resistance gene exhibited elevated resistance levels due to synergistic effects of these genes (Ammor et al., 2008). Devirgiliis, Coppola, Barile, Colonna, & Perozzi (2009) and Devirgiliis, Zinno, & Perozzi (2013) reported that *tet*M was present on the Tn*916* transposon in *Lb. paracasei* strains isolated from water buffalo mozzarella, which all display the same MIC value of 32 μg/ml (Devirgiliis, Coppola, Barile, Colonna, & Perozzi, 2009), and also in *Lb. plantarum* M345 (plasmid pLFE1) isolated from raw-milk cheese, and that the transposon is capable of being transferred to different LAB (Feld, Bielak, Hammer, & Wilcks, 2009). However, the plasmid-carried *tet*W gene, more common in human and animal intestinal bacteria, did not exhibit any transferability of the tetracycline resistance gene from *Lb. reuteri* to bacteria of the human gut microbiota (enterococci, bifidobacteria and lactobacilli), as was reported by Egervärn, Lindmark, Olsson, & Roos (2010).

The resistance gene *ermB* located both on plasmids or the chromosome, was detected (Table 1) in several *Lactobacillus* species isolated from a variety of fermented foods (Nawaz et al., 2011), fermented dry sausages (Gevers, Danielsen, Huys, & Swings, 2003; Zonenschain, Rebecchi, & Morelli, 2009), dairy products (Ammor, Flórez, & Mayo, 2007; Feld, Bielak, Hammer, & Wilcks, 2009; van Hoek et al., 2008), traditional Italian fermented foods (Comunian et al., 2010), Indian fermented foods (Thumu & Halami, 2012), Chinese cabbage and fermented sour and hot cabbage (Pan, Hu, & Wang, 2011). The *ermB* gene is often associated with mobile elements, mainly conjugative transposons, and thus these mobile genetic elements may explain their spread (Simeoni et al., 2008). Furthermore, Thumu and Halami (2012) showed that the highly erythromycin-resistant *Lactobacillus* species isolated from fermented dry sausages harbored only the *ermB* gene, and that the cMLS$_B$ (constitutive Macrolide, Lincosamide, and Streptogramin B) phenotype displayed by these lactobacilli was a result of a mutation in the leader peptide of *erm* genes after their continuous expression mainly under exposition of the animals to different antibiotics (Weisblum, 1995). In this sense, the acquired resistance to lincosamide (clindamycin) in lactobacilli, referred to as MLS$_B$ (Macrolide, Lincosamide, Streptogramin B compound) resistance, is frequently associated with the presence of methylase genes, mainly *ermB* gene, but also *ermC* (especially in lactobacilli from fermented meats), *ermG* and *ermT* genes.

Transferable multiresistance in lactobacilli is rare so far, with the exception of *erm-vat-tet* resistance that is plasmid-encoded (pLME300) in a *Lb. fermentum* strain (Gfeller, Roth, Meile, & Teuber, 2003) and *erm-vat-tet-aad-aph-sat* resistance in a *Lb. curvatus* strain (Danielsen, Madsen, Hammer, & Wind, 2005), both isolated from cheeses made from raw milk.

2.2.3. Resistance to antibiotics inhibiting DNA replication/transcription

Lactobacilli seem to be intrinsically resistant to quinolones, *e.g.* nalidixic acid and ciprofloxacin, by a currently unknown resistance mechanism (Hummel, Hertel, Holzapfel, & Franz, 2007). The intrinsic resistance to ciprofloxacin was not found to be associated with mutations in the QRDR of *gyrA* and *parC* as occurred in Gram-positive bacteria (Hummel, Hertel, Holzapfel, & Franz, 2007), thus the intrinsic resistance could have resulted from intrinsic characteristics such as cell wall structure, permeability, or an efflux mechanism.

2.2.4. Resistance to folic acid synthesis inhibitors

Resistance to sulphonamides and trimethoprim has also been reported as an intrinsic feature of the folate auxotrophic lactobacilli (Katla, Kruse, Johnsen, & Herikstad, 2001). In some cases, sulfamethoxazole/trimethoprim phenotypic determination of susceptibility of lactobacilli in some culture media may not be coherent, because certain antagonistic medium components such as p-aminobenzoic acid (PABA) and thymidine may interfere with the antibiotic activity (Turnidge & Bell, 2005). In other cases, the mechanisms of resistance in lactobacilli isolated from fermented foods include cell wall impermeability, alternative metabolic pathways, a dihydrofolate reductase (DHFR), that is insensitive to trimethoprim, overproduction of DHFR and trimethoprim-insensitive transferable DHFRs (Huovinen, 1987).

2.3. Antibiotic resistance and mobile genetic elements in Lactobacillus from fermented foods

Consumption of live *Lactobacillus* spp. included in lactic acid fermented foods as potential vehicles of spread of antibiotic resistance determinants have received increasing attention in recent years. The risks are associated with potential transfer of antibiotic resistance within the gastrointestinal tract from commensal or probiotic bacteria to other bacteria or potential pathogens, thus impairing successful antibiotic treatment of common microbial infections (Klein et al., 2000; Snydman, 2008). The antibiotic resistance genes can be transferred horizontally via mobile elements (plasmids, transposons and integrons) which are responsible for intra- and inter-species transfer of genetic material (van Reenen & Dicks, 2011). Furthermore, the presence of insertion sequences (IS) within bacterial genomes is also an indicator of horizontal gene transfer which is regulated by environmental factors, thus they were detected in some lactobacilli (http://www-is.biotoul.fr/)

such as *Lb. salivarius* UCC118 isolated from the human ileum (Canchaya, Claesson, Fitzgerald, van Sinderen, & O'Toole, 2006), *Lb. helveticus* DPC 4571 isolated from cheese (Callanan et al., 2008), *Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842 isolated from bulgarian yogurt and *Lb. amylovorus* GRL1118 (Kant, Paulin, Alatalo, de Vos, & Palva, 2011a) isolated from intestine among others. The presence of different types of IS elements in lactobacilli suggests their role in genome evolution and thus their role in horizontal gene transfer (Cai, Thompson, Budinich, Broadbent, & Steele, 2009; Cremonesi, Chessa, & Castiglioni, 2013; Kaleta, O'Callaghan, Fitzgerald, Beresford, & Ross, 2010; Schneider & Lenski, 2004). In this context, lactobacilli from fermented foods were investigated regarding the presence of conjugative plasmids or transposons located on the chromosome or on plasmids, which carry single or multiple genes encoding resistance to a diverse array of antibiotics. Furthermore, these strains were also investigated for the presence of IS elements flanking antibiotic resistance genes. The conjugative transposon Tn*916*—an 18-kbp genetic element containing the genetic determinant for tetracycline resistance *tetM*—occurred in *Lb. paracasei* isolated from raw milk and natural whey starter cultures employed in the manufacture of the Italian traditional cheese Mozzarella di Bufala Campana, was capable of horizontal interspecies transfer to the opportunistic pathogen *Enterococcus faecalis* (Devirgiliis, Caravelli, Coppola, Barile, & Perozzi, 2008; Devirgiliis, Coppola, Barile, Colonna, & Perozzi, 2009). A Tn*916*-like transposon was also found in *Lb. sakei* Rits 9 strain isolated from Italian Sola cheese made from raw milk (Ammor et al., 2008), and in other *Lactobacillus* species from different fermented foods (Table 1). The genes *tetL*, *tetK* and *tetW* on the other hand, have been shown to be located on plasmids in different lactobacilli isolated from dairy and fermented meats (Table 1). In the study of Kastner et al. (2006), *Lb. reuteri* SD 2112 isolated from breast milk was shown to harbor the tetracycline resistance gene *tetW* (residing on a plasmid) and the lincosamide resistance gene *lnu*(A). Two plasmids—carrying tetracycline (*tetW*) and lincosamide [*lnu*(A)] resistance genes—were also identified by Rosander, Connolly, & Roos (2008) in a commercial strain of *Lb. reuteri* ATCC 55730 originally isolated from breast milk (Ammor et al., 2008). *Lactobacillus fermentum* isolated from pig feces carried a 5.7 kbp plasmid with an *erm* gene conferring high level erythromycin resistance, which was 98.2% identical to the gene of the enterococcal conjugative transposon Tn*1545* (Fons et al., 1997). In the study of Gfeller, Roth, Meile, & Teuber (2003), a chromosomally located, tetracycline-resistance determinant *tet*M was identified in *Lb. fermentum* ROT1 that was isolated from a raw milk dairy product. A 19,398-bp plasmid (pLME300) found to be present in several erythromycin-resistant strains of *Lb. fermentum* was also isolated and completely sequenced (Ammor et al., 2008).

Shrago and Dobrogosz (1988) showed the conjugal transfer of Group-B streptococcal plasmids to *Lb. plantarum*, while Morelli, Sarra, & Bottazzi (1988) demonstrated that the conjugative plasmid pAMβ1 could be transferred from *Lb. reuteri* to *E. faecalis in vivo*. Thus, lactobacilli like other bacteria, have possibly acquired some antibiotic resistance genes by conjugation from bacteria such as streptococci and enterococci (Ammor, Flórez, & Mayo, 2007; Teuber et al., 1999). On the other hand, transfer of resistance genes by transduction has also been reported (Morelli, Zonenenschain, Del Piano, & Cognein, 2004). Natural transformation has not yet been described in lactobacilli (Ammor, Flórez, & Mayo, 2007), although genes involved in uptake of free DNA have been found in *Lb. reuteri* (Båth, Roos, Wall, & Jonsson, 2005). Most of the *Lactobacillus* species, regardless of their source (plants, meat, silage, sourdough or gastrointestinal tract), contain native plasmids (Wang & Lee, 1997), and frequently appear to harbor multiple different plasmids in a single strain. In lactobacilli, plasmid differences are found in distribution, function and size (Davidson, Kordias, Dobos, & Hillier, 1996; Wang & Lee, 1997). The functions of these plasmids have classically been correlated with phenotypical properties, including drug resistance, carbohydrate metabolism, amino acid metabolism and bacteriocin production (Lavanya, Sowmiya, Balaji, & Muthuvelan, 2011). Auputinan, Tragoolpua, Pruksakorn, & Thongwai (2011) reported that not all lactobacilli from fermented foods harbored plasmids, and those harboring plasmids are characterized by a high diversity not related with the species identity nor the source of isolation. Plasmid curing studies done with lactobacilli isolated from fermented milk indicated the plasmid location of penicillin resistance determinants in all penicillin-resistant *Lactobacillus* strains (Lavanya, Sowmiya, Balaji, & Muthuvelan, 2011).

Acquisition of resistance genes by lactobacilli present in fermented foods could have occurred from staphylococci and enterococci, based on the evidence of homology of mobile genetic elements in those bacteria. Fig. 1 shows the most important resistance gene pool shared in lactobacilli from fermented foods, staphylococci and enterococci. Spread of resistance genes could occur via plasmids or transposons (Fig. 1), such as Tn*916*–Tn*1545* and Tn*1546* type of transposons (Clewell, Flannagan, & Jaworski, 1995; Devirgiliis, Coppola, Barile, Colonna, & Perozzi, 2009; Ammor, Flórez and Mayo, 2007).

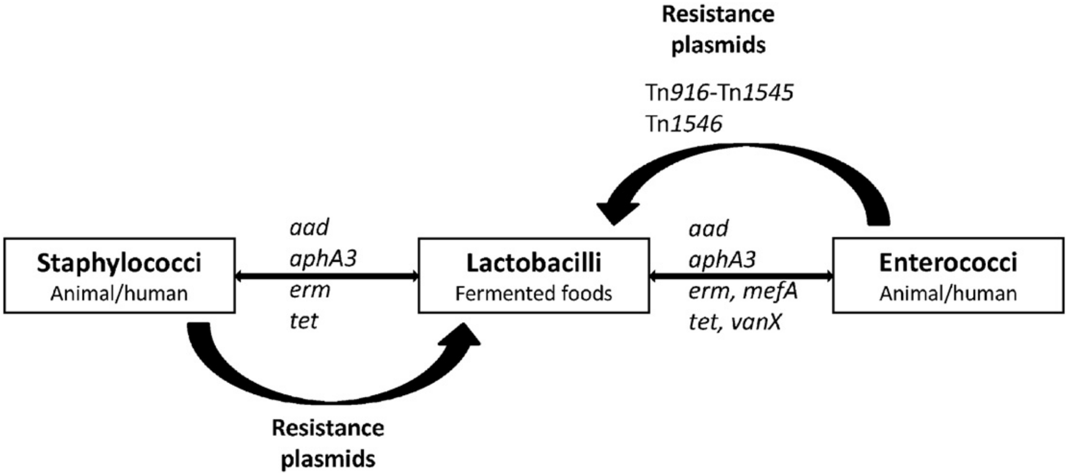


Fig. 1. The most important resistance genes shared by lactobacilli from fermented foods, staphylococci and enterococci, and also the common mobile genetic elements involved in spread of resistance. Genes encoding resistance to: *aad* and *aphA3*, aminoglycosides; *erm*, erythromycin; *mefA*, macrolide; *tet*, tetracycline; *vanX*, vancomycin.

2.4. Novel insights on antibiotic resistances of *Lactobacillus* spp. obtained from whole genome sequence data

An advanced strategy to approach safety investigations regarding antibiotic resistances in lactobacilli is to explore genome sequences, i.e. an in silico analysis for antibiotic resistance genes. However, the low number of available genome sequences up to date may limit an in-depth understanding of the origin of observed phenotypic resistances, which are either induced or uninduced by the presence of antimicrobials. Some observations about the most important genomic insights that regard antibiotics such as chloramphenicol and glycopeptides in lactobacilli isolated from fermented foods are discussed below.

2.4.1. Resistance to chloramphenicol

As explained above, the *cat* gene is frequently found on plasmids associated with various *Lactobacillus* spp. However, a search for this gene in genomic DNA sequences of different lactobacilli available at the National Centre for Biotechnology Information (NCBI) database revealed the presence of *cat* genes on the chromosome of *Lb. brevis* isolated from fermented vegetables, *Lb. plantarum* isolated from dairy products and fermented vegetables, *Lb. fermentum* isolated from dairy products and *Lb. sakei* isolated from fermented sausage and fermented vegetables (Table 2). However, the presence of this gene on the chromosome does not necessarily imply that it is expressed at the phenotypic level, because sometimes the differences detected between phenotypic and genotypic tests are attributed to a mutation in *cat* gene or its regulatory region, as suggested by Hummel, Hertel, Holzapfel, & Franz (2007). Moreover, the analysis of homology of *cat* gene sequences of lactobacilli from fermented foods, using the BLASTn tool for searching against the non-redundant nucleotide database of NCBI, indicated a highly divergent nucleotide sequence region in the *Lactobacillus* genus, being the differences detected between species (Table 2). Fig. 2 shows the evolutionary distances between Cat proteins in different lactobacilli isolated from fermented foods and other environments (especially from silage, intestinal and vaginal microbiota), being all *cat* genes located on the chromosome, except for the *cat* gene of *Lb. reuteri* I5007, that was isolated from healthy weaning piglets and was harbored on the plasmid pLRI04 (Hou et al., 2013). For this, Cat protein sequences were retrieved from the National Centre for Biotechnology Information (NCBI) database and then analyzed to construct the phylogenetic tree (Fig. 2) by using the neighbor-joining method (Bionumerics version 2.5, Applied Maths, Belgium). The resulting phylogenetic tree showed that the chromosomally encoded Cat proteins in lactobacilli isolated from fermented foods and other environments occur in two clusters (G1 and G2). The first cluster G1 subdivided into three subclusters (A, B and C) showed that *Lb. sakei* (subcluster B) and *Lb. buchneri* and *Lb. fermentum* (subcluster C) strains formed different subclusters separated from other Gram-positive bacteria such as *Enterococcus* sp. and *Staphylococcus* sp. (subcluster A). However, the plasmid encoded Cat protein in *Lb. reuteri* I5007 clustered together with *Enterococcus* sp. and *Staphylococcus* sp. in the same subcluster (A). The second cluster (G2) divided into two subclusters—subcluster D and subcluster E—was represented by chromosomally encoded Cat proteins from *Lb. brevis* and *Lb. plantarum* strains, respectively. These data clearly show the high divergence between Cat proteins in lactobacilli species and also a high homology among strains of the same species, regardeless their origin (food or other environments). On the other hand, Cat protein encoded by the *cat* gene from plasmid pLRI04 of *Lb. reuteri* I5007 (belonging to subcluster A) was highly similar to the Cat proteins encoded by *cat* genes located on several plasmids of *S. aureus* (pC194, pKH13 and pKH15), this fact suggesting that the acquisition of the *cat* gene by *Lb. reuteri* I5007 was probably done by horizontal gene transfer from *S. aureus*. Similarly, in the same subcluster A, Cat sequences of *E. faecalis*, *E. faecium* and *S. aureus* (Fig. 2) could also be found to group together, these sharing the same origin as other Cat proteins

Table 2
Percentage of identity of chloramphenicol resistance gene (*cat*) in *Lactobacillus* species isolated from fermented foods.

Lactobacillus strains from fermented foods	Alignment of *cat* gene sequences		References
	Percent identity (%)	Strains (Source of isolation)	
Lb. brevis KB290	100	*Lb. brevis* KB290 (pickle), *Lb. brevis* ATCC 367 (silage)	Fukao, Tomita, Yakabe, Nomura, Ike, and Yajima (2009); Fukao et al. (2013); Makarova et al. (2006)
	95	*Lb. brevis* BSO 464 (brewery)	Bergsveinson, Baecker, Pittet, and Ziola (2015)
Lb. fermentum F-6	100	*Lb. fermentum* F-6 (dairy)	Gao (Unpublished, NCBI Reference Sequence: NC_021235.1)
	99	*Lb. fermentum* 3872 (milk from female), *Lb. fermentum* MTCC 8711 (yogurt)	Jayashree et al. (2013); Karlyshev, Raju, and Abramov (2013)
Lb. plantarum subsp. *plantarum* ST-III and *Lb. plantarum* subsp. *plantarum* P-8	100	*Lb. plantarum* 16 (steep water), *Lb. plantarum* WCFS1 (human saliva), *Lb. plantarum* subsp. *plantarum* P-8 (dairy), *Lb. plantarum* subsp. *plantarum* ST-III (kimchi)	Crowley, Bottacini, Mahony, and van Sinderen (2013); Gao (Unpublished, NCBI Reference Sequence: NC_021224.1); Kleerebezem et al. (2003); Wang, Chen, Ai, Zhou, Zhou, Wang, et al. (2011a)
	99	*Lb. plantarum* JDM1 (human intestinal tract), *Lb. plantarum* ZJ316 (feces), *Lb. plantarum* subsp. *plantarum* NC8 (grass silage), *Lb. plantarum* IPLA88 (sourdough), *Lb. plantarum* 2165 (milk product), *Lb. plantarum* 4_3 (fermented soybean), *Lb. plantarum* Lp90 (wine), *Lb. plantarum* CMPG5300 (vagina of healthy woman), *Lb. plantarum* UCMA 3037 (unpasteurized camembert cheese)	Axelsson et al. (2012); Karlyshev and Abramov (2014); Ladero et al. (2013); Lamontanara et al. (2015); Li et al. (2013); Malik, Siezen, Renckens, Vaneechoutte, Vanderleyden, and Lebeer (2014); Naz et al. (2013); Zhang et al. (2009)
Lb. sakei subsp. *sakei* 23 K	100	*Lb. sakei* subsp. *sakei* 23 K (sausage)	Chaillou et al. (2005)
	99	*Lb. sakei* wikim 22 (kimchi)	Lim et al. (2014)
	98	*Lb. sakei* LTH681 (fermented food), *Lb. sakei* subsp. *sakei* LS25 (fermented sausage)	McLeod, Brede, Rud, and Axelsson (2013); Schmidt, Hertel, and Hammes (1999)

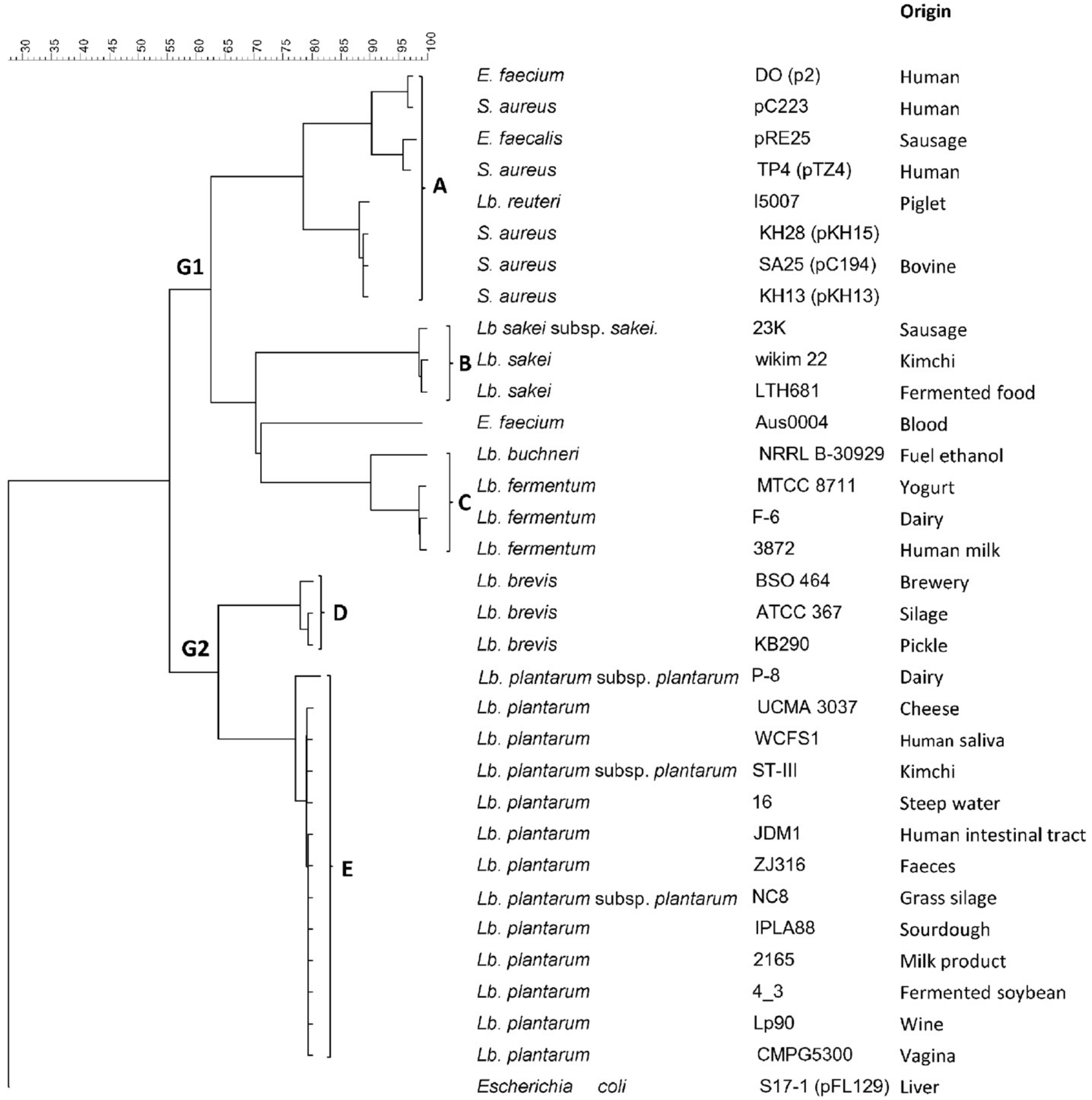


Fig. 2. Phylogenetic tree constructed using the neighbor-joining algorithm to evaluate the distance between Cat proteins from lactobacilli isolated from fermented foods and other bacteria of different sources. Cat protein sequences were retrieved from the National Centre for Biotechnology Information (NCBI) database and then analyzed to construct the phylogenetic tree by using the neighbor-joining method (Bionumerics version 2.5, Applied Maths, Belgium). *Escherichia coli* was used as outgroup bacilli. Abbreviation: *E*, *Enterococcus*; *Lb*, *Lactobacillus*; *S*, *Staphylococcus*.

encoded on plasmids. This fact highlights the importance of *Enterococcus* and *Staphylococcus* genera in contributing to horizontal gene transfer to different other bacterial genera, especially also to certain *Lactobacillus* strains. However, a chromosomally encoded Cat protein in *E. faecium* Aus0004 isolated from blood shared the same origin as *Lb. sakei*, *Lb. buchneri* and *Lb. fermentum* (70% identity, Fig. 2).

In general, lactobacilli from fermented foods did not appear to exhibit a horizontal gene transfer of *cat* genes, since no homology was detected between the chromosomally-encoded *cat* genes from lactobacilli and the plasmid encoded *cat* genes from food and non-food source lactobacilli and other bacteria. However, horizontal gene transfer to food *Lactobacilllus* strains could nevertheless be possible, since *Lb. reuteri* from pigs, whose *cat* gene was plasmid located, was thus probably able to receive the *cat* gene from *Staphylococcus* and *Enterococcus* spp., possibly of an intestinal source. Thus, the mechanism, as well as the origin of the *cat* determinant in lactobacilli from fermented foods, appears to be different from the acquired resistance by plasmids in *Lb. reuteri*, as well as other bacteria occurring in the natural ecosystems such as the intestinal tract of the animals.

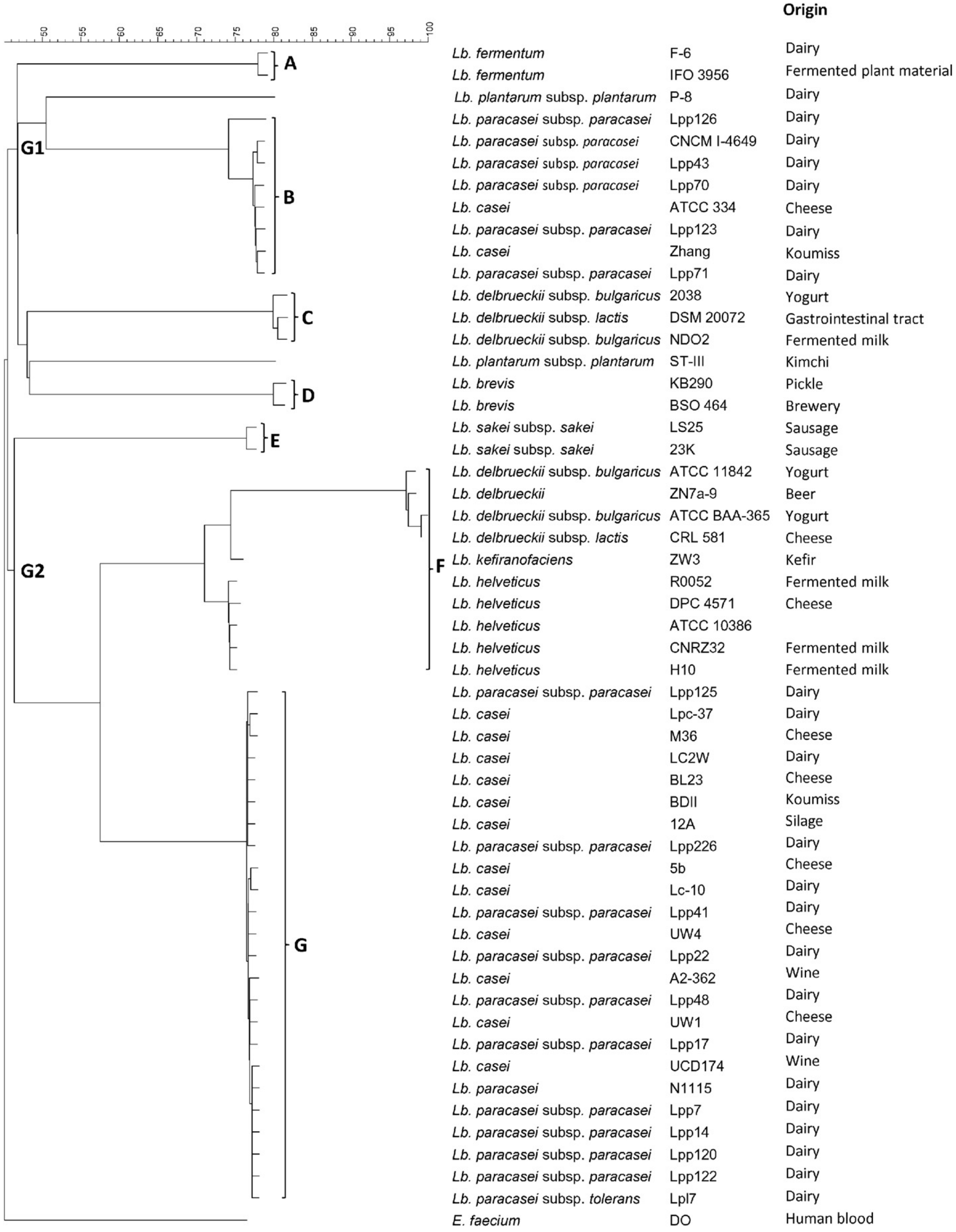
The problem which could be associated with safety assessments of strains regarding antibiotic resistance genes is that no standard

Table 3
Percentage of identity of glycopeptide resistance gene (*vanZ*) in *Lactobacillus* species isolated from fermented foods.

Lactobacillus strains from fermented foods	Alignment of *vanZ* gene sequences		References
	Percent identity (%)	Strains (Source of isolation)	
Lb. brevis KB290	100	*Lb. brevis* KB290 (pickle), *Lb. brevis* ATCC 367 (silage)	Fukao et al. (2013); Makarova et al. (2006)
	99	*Lb. brevis* BSO 464 (brewery)	Bergsveinson et al., Bergsveinson, Baecker, Pittet and Ziola (2015)
Lb. casei BDII, *Lb. casei* BL23 and *Lb. casei* LC2W	100	*Lb. casei* BDII (koumiss), *Lb. casei* BL23 (cheese) and *Lb. casei* LC2W (dairy), *Lb. casei* W56 (probiotic), *Lb. paracasei* subsp. *paracasei* 8700:2 (human gastrointestinal mucosa), *Lb. paracasei* subsp. *paracasei* Lpp226 (artisanal dairy product), *Lb. casei* 12A (corn silage)	Ai et al. (2011); Broadbent et al. (2012); Chen et al. (2011); Hochwind et al. (2012); Mazé, Boel, Zúñiga, Bourand, Loux, Yebra, et al. (2010); Smokvina et al. (2013); Ward et al. (Unpublished, NCBI Reference Sequence: NC_022112.1)
	99	*Lb. casei* LOCK 919 (probiotic from feces), *Lb. casei* str. Zhang (koumiss), *Lb. paracasei* subsp. *paracasei* Lpp125 (artisanal dairy product), *Lb. casei* M36 (cheese), *Lb. casei* Lpc-37 (dairy product), *Lb. casei* Lc-10 (dairy product), *Lb. casei* UW4 (cheese), *Lb. casei* UW1 (cheese), *Lb. casei* 5b (cheese), *Lb. paracasei* subsp. *paracasei* Lpp7 (commercial dairy product), *Lb. paracasei* subsp. *paracasei* Lpp14 (artisanal dairy product), *Lb. paracasei* subsp. *paracasei* Lpp17 (artisanal dairy product), *Lb. paracasei* subsp. *paracasei* Lpp22 (commercial dairy product), *Lb. paracasei* subsp. *paracasei* Lpp41 (commercial dairy product), *Lb. paracasei* subsp. *paracasei* Lpp48 (artisanal dairy product), *Lb. paracasei* subsp. *paracasei* Lpp122 (artisanal dairy product), *Lb. paracasei* subsp. *paracasei* Lpp120 (artisanal dairy product), *Lb. casei* UCD174 (wine), *Lb. paracasei* subsp. *tolerans* Lpl7 (commercial dairy product), *Lb. paracasei* N1115 (traditional homemade dairy product), *Lb. casei* A2-362 (wine)	Broadbent et al. (2012); Ladero et al. (2014); Koryszewska-Bagińska, Bardowski, and Aleksandrzak-Piekarczyk (2014); Smokvina et al. (2013); Wang et al. (2014); Zhang et al. (2010)
	98	*Lb. casei* ATCC 334 (cheese), *Lb. paracasei* subsp. *paracasei* JCM 8130 (milk)	Makarova et al. (2006); Toh et al. (2013)
Lb. casei ATCC 334	100	*Lb. casei* ATCC 334 (cheese), *Lb. casei* UCD174 (wine)	Broadbent et al. (2012); Makarova et al. (2006)
	99	*Lb. casei* BDII (koumiss), *Lb. casei* BL23 (cheese), *Lb. casei* LC2W (dairy), *Lb. casei* W56 (probiotic), *Lb. casei* LOCK 919 (probiotic from feces), *Lb. casei* str. Zhang (koumiss), *Lb. paracasei* N1115 (dairy), *Lb. paracasei* subsp. *paracasei* JCM 8130 (milk), *Lb. paracasei* subsp. *paracasei* 8700:2 (human gastrointestinal mucosa), *Lb. paracasei* subsp. *paracasei* Lpp120 (artisanal dairy product), *Lb. paracasei* subsp. *paracasei* Lpp70 (artisanal dairy product), *Lb. paracasei* subsp. *paracasei* Lpp123 (artisanal dairy product), *Lb. paracasei* subsp. *paracasei* Lpp22 (commercial dairy product), *Lb. casei* UW4 (cheese), *Lb. casei* UW1 (cheese), *Lb. casei* Lc-10 (dairy product), *Lb. casei* 5b (cheese), *Lb. paracasei* subsp. *tolerans* Lpl7 (commercial dairy product), *Lb. casei* M36 (cheese), *Lb. casei* Lpc-37 (dairy product), *Lb. paracasei* subsp. *paracasei* Lpp7 (commercial dairy product), *Lb. paracasei* subsp. *paracasei* Lpp41 (commercial dairy product), *Lb. paracasei* subsp. *paracasei* Lpp71 (artisanal dairy product), *Lb. paracasei* subsp. *paracasei* Lpp126 (artisanal dairy product), *Lb. paracasei* subsp. *paracasei* Lpp22 (commercial dairy product), *Lb. paracasei* subsp. *paracasei* Lpp14 (artisanal dairy product), *Lb. paracasei* subsp. *paracasei* Lpp43 (artisanal dairy product), *Lb. paracasei* subsp. *paracasei* CNCM I-4649 (artisanal dairy product), *Lb. paracasei* subsp. *paracasei* Lpp125 (artisanal dairy product)	Ai et al. (2011); Broadbent et al. (2012); Chen et al. (2011); Hochwind et al. (2012); Koryszewska-Bagińska, Bardowski and Aleksandrzak-Piekarczyk (2014); Ladero et al. (2014); Mazé et al. (2010); Smokvina et al. (2013); Wang et al. (2014); Ward et al. (Unpublished, NCBI Reference Sequence: NC_022112.1); Zhang et al. (2010)
Lb. casei str. Zhang	100	*Lb. casei* str. Zhang (koumiss), *Lb. paracasei* subsp. *paracasei* Lpp17 (artisanal dairy product), *Lb. casei* LOCK 919 (probiotic from feces)	Koryszewska-Bagińska, Bardowski and Aleksandrzak-Piekarczyk (2014); Smokvina et al. (2013); Zhang et al. (2010)
	99	*Lb. casei* BDII (koumiss), *Lb. casei* BL23 (cheese), *Lb. casei* LC2W (dairy), *Lb. casei* W56 (probiotic), *Lb. casei* ATCC 334 (cheese), *Lb. paracasei* N1115 (dairy), *Lb. paracasei* subsp. *paracasei* JCM 8130 (milk), *Lb. casei* A2-362 (wine), *Lb. paracasei* subsp. *paracasei* Lpp123 (artisanal dairy product), *Lb. paracasei* subsp. *paracasei* 8700:2 (human gastrointestinal mucosa)	Ai et al. (2011); Broadbent et al. (2012); Chen et al. (2011); Hochwind et al. (2012); Makarova et al. (2006); Mazé, Boel, Zúñiga, Bourand, Loux, Yebra, et al. (2010); Smokvina et al. (2013); Toh et al. (2013); Ward et al. (Unpublished, NCBI Reference Sequence: NC_022112.1)

Table 3 (*continued*)

Lactobacillus strains from fermented foods	Alignment of *vanZ* gene sequences		References
	Percent identity (%)	Strains (Source of isolation)	
Lb. delbrueckii subsp. *bulgaricus* 2038	100	*Lb. delbrueckii* subsp. *bulgaricus* 2038 (yogurt)	Zheng et al. (2008)
	99	*Lb. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 (yogurt), *Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842 (yogurt)	Makarova et al. (2006); van de Guchte et al. (2006)
	98	*Lb. delbrueckii* subsp. *bulgaricus* ND02 (fermented milk)	Sun et al. (2011)
Lb. delbrueckii subsp. *bulgaricus* ATCC 11842	100	*Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842 (yogurt)	van de Guchte et al. (2006)
	99	*Lb. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 (yogurt), *Lb. delbrueckii* subsp. *bulgaricus* 2038 (yogurt), *Lb. delbrueckii* subsp. *bulgaricus* ND02 (fermented milk), *Lb. delbrueckii* ZN7a-9 (uncooked sour wort of dolo, traditional sorghum malt beer)	Adimpong et al. (2013); Makarova et al. (2006); Sun, Chen, Wang, Zhao, Shao, Guo, et al. (2011); Zheng et al. (2008)
Lb. delbrueckii subsp. *bulgaricus* ATCC BAA-365	100	*Lb. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 (yogurt)	Makarova et al. (2006)
	99	*Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842 (yogurt), *Lb. delbrueckii* subsp. *bulgaricus* 2038 (yogurt), *Lb. delbrueckii* subsp. *bulgaricus* ND02 (fermented milk), *Lb. delbrueckii* ZN7a-9 (uncooked sour wort of dolo, traditional sorghum malt beer), *Lb. delbrueckii* subsp. *lactis* CRL581 (Argentinian hard cheese)	Adimpong et al. (2013); Hebert, Raya, Brown, Font de Valdez, Savoy de Giori, and Taranto (2013); Sun, Chen, Wang, Zhao, Shao, Guo, et al. (2011); van de Guchte et al. (2006) Zheng et al. (2008)
Lb. delbrueckii subsp. *bulgaricus* ND02	100	*Lb. delbrueckii* subsp. *bulgaricus* ND02 (fermented milk)	Sun et al. (2011)
	99	*Lb. delbrueckii* subsp. *lactis* CRL581 (Argentinian hard cheese), *Lb. delbrueckii* subsp. *lactis* DSM 20072 (gastrointestinal tract)	Muzny et al. (Unpublished, GenBank: GL872382.1, Hebert et al. (2013)
	98	*Lb. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 (yogurt), *Lb. delbrueckii* subsp. *bulgaricus* 2038 (yogurt), *Lb. delbrueckii* ZN7a-9 (uncooked sour wort of dolo, traditional sorghum malt beer)	Adimpong et al. (2013); Makarova et al. (2006); Zheng et al. (2008)
	97	*Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842 (yogurt)	van de Guchte et al. (2006)
Lb. fermentum F-6	100	*Lb. fermentum* F-6 (dairy)	Gao (Unpublished, NCBI Reference Sequence: NC_021235.1)
	99	*Lb. fermentum* IFO 3956 (fermented plant material)	Morita et al. (2008)
Lb. fermentum IFO 3956	100	*Lb. fermentum* IFO 3956 (fermented plant material)	Morita et al. (2008)
	99	*Lb. fermentum* F-6 (dairy)	Gao (Unpublished, NCBI Reference Sequence: NC_021235.1)
Lb. helveticus CNRZ32	100	*Lb. helveticus* CNRZ32 (fermented milk), *Lb. helveticus* H9 (dairy), *Lb. helveticus* H10 (fermented milk), *Lb. helveticus* ATCC 10386	Broadbent, Hughes, Welker, Tompkins, and Steele (2013); Zago et al. (Unpublished, NCBI Reference Sequence: NZ_JRQG01000080.1); Zhang et al. (Unpublished, NCBI Reference Sequence: NZ_CP002427.1); Zhao et al. (2011)
	99	*Lb. helveticus* R0052 (fermented milk), *Lb. helveticus* DPC 4571(cheese)	Callanan et al. (2008); Tompkins, Barreau, and Broadbent (2012)
	80	*Lb. amylovorus* GRL1118 (pig ileum), *Lb. amylovorus* GRL 1112 (porcine feces), *Lb. acidophilus* 30SC (swine intestine)	Kant, Paulin, Alatalo, de Vos, and Palva (2011a); Kant, Blom, Pavla, Siezen, and de Vos (2011b); Oh et al. (2011)
Lb. helveticus DPC 4571	100	*Lb. helveticus* DPC 4571 (cheese)	Callanan et al. (2008)
	99	*Lb. helveticus* H9 (dairy), *Lb. helveticus* H10 (fermented milk), *Lb. helveticus* R0052 (fermented milk), *Lb. helveticus* CNRZ32 (fermented milk)	Broadbent, Hughes, Welker, Tompkins and Steele (2013); Tompkins, Barreau, and Broadbent (2012); Zhao et al. (2011)
	80	*Lb. amylovorus* GRL 1118 (pig ileum), *Lb. amylovorus* GRL 1112 (porcine feces), *Lb. acidophilus* 30SC (swine intestine)	Kant, Paulin, Alatalo, de Vos and Palva (2011a); Kant, Blom, Pavla, Siezen and de Vos (2011b); Oh et al. (2011)
Lb. helveticus H10	100	*Lb. helveticus* H9 (dairy), *Lb. helveticus* H10 (fermented milk), *Lb. helveticus* CNRZ32 (fermented milk)	Broadbent et al. (2013); Zhang et al. (Unpublished, NCBI Reference Sequence: NZ_CP002427.1); Zhao et al. (2011)
	99	*Lb. helveticus* R0052 (fermented milk), *Lb. helveticus* DPC 4571 (cheese)	Callanan et al. (2008), Tompkins, Barreau, and Broadbent et al. (2012)
Lb. helveticus R0052	100	*Lb. helveticus* R0052 (fermented milk)	Tompkins, Barreau, and Broadbent et al. (2012)
	99	*Lb. helveticus* H9 (dairy), *Lb. helveticus* H10 (fermented milk), *Lb. helveticus* DPC 4571 (cheese), *Lb. helveticus* CNRZ32 (fermented milk)	Broadbent et al. (2013); Callanan et al. (2008); Zhang et al. (Unpublished, NCBI Reference Sequence: NZ_CP002427.1); Zhao et al. (2011)
Lb. kefiranofaciens ZW3	100	*Lb. kefiranofaciens* ZW3 (kefir)	Wang, Wang, Ahmed, Bai, and Wang et al. (2011a)
Lb. plantarum subsp. *plantarum* ST-III	100	*Lb. plantarum* subsp. *plantarum* ST-III (kimchi)	Wang et al. (2011b)
	98	*Lb. plantarum* JDM1 (human intestinal tract)	Zhang et al. (2009)
Lb. plantarum subsp. *plantarum* P-8	100	*Lb. plantarum* subsp. *plantarum* P-8 (dairy), *Lb. plantarum* subsp. *plantarum* ST-III (kimchi), *Lb. plantarum* WCFS1 (human saliva)	Gao (Unpublished, NCBI Reference Sequence: NC_021224.1); Kleerebezem et al. (2003); Wang et al. (2011b)
	99	*Lb. plantarum* 16, *Lb. plantarum* JDM1 (human intestinal tract), *Lb. plantarum* ZJ316 (feces)	Crowley, Bottacini, Mahony and van Sinderen (2013); Li al. (2013); Zhang et al. (2009)
Lb. sakei subsp. *sakei* 23 K	100	*Lb. sakei* subsp. *sakei* 23 K (sausage)	Chaillou et al. (2005)
	99	*Lb. sakei* subsp. *sakei* LS25 (commercial starter culture for fermented sausage)	McLeod, Brede, Rud and Axelsson (2013)


Origin
50
55
60
65
70
75
80
85
90
95
100
A
B
C
D
E
F
G
G1
G2
Lb. fermentum F-6 Dairy
Lb. fermentum IFO 3956 Fermented plant material
Lb. plantarum subsp. plantarum P-8 Dairy
Lb. paracasei subsp. paracasei Lpp126 Dairy
Lb. paracasei subsp. paracasei CNCM I-4649 Dairy
Lb. paracasei subsp. paracasei Lpp43 Dairy
Lb. paracasei subsp. paracasei Lpp70 Dairy
Lb. casei ATCC 334 Cheese
Lb. paracasei subsp. paracasei Lpp123 Dairy
Lb. casei Zhang Koumiss
Lb. paracasei subsp. paracasei Lpp71 Dairy
Lb. delbrueckii subsp. bulgaricus 2038 Yogurt
Lb. delbrueckii subsp. lactis DSM 20072 Gastrointestinal tract
Lb. delbrueckii subsp. bulgaricus NDO2 Fermented milk
Lb. plantarum subsp. plantarum ST-III Kimchi
Lb. brevis KB290 Pickle
Lb. brevis BSO 464 Brewery
Lb. sakei subsp. sakei LS25 Sausage
Lb. sakei subsp. sakei 23K Sausage
Lb. delbrueckii subsp. bulgaricus ATCC 11842 Yogurt
Lb. delbrueckii ZN7a-9 Beer
Lb. delbrueckii subsp. bulgaricus ATCC BAA-365 Yogurt
Lb. delbrueckii subsp. lactis CRL 581 Cheese
Lb. kefiranofaciens ZW3 Kefir
Lb. helveticus R0052 Fermented milk
Lb. helveticus DPC 4571 Cheese
Lb. helveticus ATCC 10386
Lb. helveticus CNRZ32 Fermented milk
Lb. helveticus H10 Fermented milk
Lb. paracasei subsp. paracasei Lpp125 Dairy
Lb. casei Lpc-37 Dairy
Lb. casei M36 Cheese
Lb. casei LC2W Dairy
Lb. casei BL23 Cheese
Lb. casei BDII Koumiss
Lb. casei 12A Silage
Lb. paracasei subsp. paracasei Lpp226 Dairy
Lb. casei 5b Cheese
Lb. casei Lc-10 Dairy
Lb. paracasei subsp. paracasei Lpp41 Dairy
Lb. casei UW4 Cheese
Lb. paracasei subsp. paracasei Lpp22 Dairy
Lb. casei A2-362 Wine
Lb. paracasei subsp. paracasei Lpp48 Dairy
Lb. casei UW1 Cheese
Lb. paracasei subsp. paracasei Lpp17 Dairy
Lb. casei UCD174 Wine
Lb. paracasei N1115 Dairy
Lb. paracasei subsp. paracasei Lpp7 Dairy
Lb. paracasei subsp. paracasei Lpp14 Dairy
Lb. paracasei subsp. paracasei Lpp120 Dairy
Lb. paracasei subsp. paracasei Lpp122 Dairy
Lb. paracasei subsp. tolerans Lpl7 Dairy
E. faecium DO Human blood

protocols are available to test for transferability of resistance genes (Hummel, Hertel, Holzapfel, & Franz, 2007).

2.4.2. Resistance to glycopeptides

The intrinsic resistance of *Lactobacillus* to glycopeptides is of great concern, since vancomycin and teicoplanin are the antibiotics of choice against majority of Gram-positive bacteria, particularly against multiple drug-resistant enterococci and staphylococci resistant to beta-lactams (Egervärn, 2009; Klare et al., 2007; Reynolds, 1998).

An in silico screening was carried out for the presence of *van* gene-like sequences using the genome sequence informations that are available for *Lactobacillus* species, especially those isolated from fermented foods, in the NCBI genome database. The results showed the presence of *vanZ* genes responsible for glycopeptide resistance in different *Lactobacillus* species such as *Lb. brevis*, *Lb. casei*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. fermentum*, *Lb. helveticus*, *Lb. kefiranofaciens*, *Lb. plantarum* subsp. *plantarum* and *Lb. sakei* subsp. *sakei* isolated especially from dairy products (Table 3).

Screening for chromosomal *vanZ* genes in various *Lactobacillus* species isolated from fermented foods, and also for those from other environments, was done retrieving *vanZ* gene sequences from NCBI's nucleotide database followed by an alignment of each gene using the BLASTn tool for searching against the non-redundant nucleotide database of NCBI. The results obtained (Table 3) indicated that the *vanZ* gene was highly conserved within each species or *Lactobacillus* species group, while no homologs were found in other *Lactobacillus* spp. or other taxa. Consequently, because of this nucleotide sequence divergence between different *Lactobacillus* species, it would be impossible to detect this gene among different species of the genus by PCR. Genomic sequence analysis, therefore, becomes the only way to detect similar genes implicated in glycopeptide resistance.

Phylogenetic analysis of VanZ proteins was used to investigate the evolutionary relationship between all lactobacilli from fermented foods and some lactobacilli isolated from other environments (silage, gastrointestinal tract and vagina) described up to date. For this, VanZ protein sequences were retrieved from the National Centre for Biotechnology Information (NCBI) database and then analyzed to construct the phylogenetic tree (Fig. 3) by using the neighbor-joining method (Bionumerics version 2.5, Applied Maths, Belgium). The resulting neighbor-joining dendrogram from the aligned VanZ protein sequences grouped these sequences into two clusters with seven subclusters (Fig. 3). The results obtained revealed a high divergence in VanZ protein sequences of lactobacilli, which depended in most cases on the species. However, different clusters were obtained in the case of *Lb. casei*/*Lb. paracasei* and *Lb. delbrueckii* belonging to different genomic groups (Fig. 3). Regarding the other species, each one exhibited a single subcluster with a moderate homology of 70–78% identity, except for *Lb. delbrueckii* with 97% identity between strains regardeless their origin. In general, we can conclude that the presence of the *vanZ* gene was not the result of horizontal gene transfer between species and genera due to the high divergence in the encoded VanZ proteins, since in most cases the distribution of the encoding gene is inherently linked to the species or even the phylogenetic group, and the evolutionary changes thus probably occurred as a result of speciation. This fact is of crucial importance, as it leads to the conclusion that this resistance gene would not be subjected to horizontal gene transfer by foodborne lactobacilli in fermented foods to pathogenic bacteria in the gastrointestinal tract. Thus strains carrying the vanZ gene and/or the associated fermented products do not pose a health risk regarding transfer of glycopeptide-resistance genes.

3. Impact of antibiotic resistance in lactobacilli

The emergence of antibiotic resistance in lactobacilli from fermented foods—which have historically been regarded as safe—has a profound impact on safety considerations. Therefore, increased attention should be given to safety assessments regarding antibiotic resistances of LAB, especially those involved in traditional fermented foods. In the absence of a defined starter culture, it is conceivable that the natural fermentation microbiota could include species with transferable resistance genes that may be able to be transferred to pathogenic bacterial species or in the food matrix, or after ingestion if the donor survives, even in the intestinal tract. Thus, the treatment of an infection may be complicated by the subsequent spread of resistance genes. This fact is of great concern, especially in under-developed countries, where the diet relies mainly on fermented foods. Moreover, it is also relevant in developed countries where traditional fermented foods are often considered as delicatessen products, due to their unique taste and aroma. Thus, fermentation that relies on the naturally present microbiota should be standardized in its production methods, including the use of starter cultures, or should at least be investigated for the presence of transferable resistance genes among LAB that occur in such products. Such investigations should focus on mobile elements such as plasmids, transposons or IS, with the aim to evaluate their capability for horizontal transfer of AR genes to pathogens (van Reenen & Dicks, 2011).

The number of infections associated with lactobacilli currently reported is quite small, and there is no evidence of opportunistic infection by lactobacilli from fermented foods. However, the emergence of lactobacilli as reservoirs of antibiotic resistance genes could possibly represent a threat to human health, if antibiotic resistant lactobacilli are ingested in large amounts with fermented foods, and transfer of resistance genes to intestinal bacteria takes place. It also seems likely that antibiotic-resistant lactobacilli would be more difficult to eradicate from polymicrobial infections in immunocompromised individuals, or in individuals suffering any other underlying condition predisposing them to microbial infections. On the other hand, although the risk associated with antibiotic resistant lactobacilli exists, the benefits of consumption of foods with added probiotic microorganisms exceeds the risks, once these microorganisms are internationally considered as generally regarded as safe (GRAS).

4. Conclusions

The body of published work indicates that bacteria naturally present in fermented foods may represent an important reservoir of antibiotic resistance genes. Those bacteria are ingested in large numbers and could possibly transfer antibiotic resistance determinants to intestinal microbiota. Our finding indicates the presence of glycopeptide and chloramphenicol resistance genes in genome sequences of lactobacilli from fermented foods, however no studies were done in this subject which encourage the need of molecular characterization of those genes in lactobacilli found in fermented foods and also probiotics. Furthermore, their ability to transfer antibiotic resistance genes must be considered.

Acknowledgments

We acknowledge research grant AGL2013-43571-P (Ministerio de Economía y Competitividad, MINECO, FEDER).

Fig. 3. Phylogenetic tree constructed using the neighbor-joining algorithm to evaluate the distance between VanZ proteins from lactobacilli isolated from fermented foods. VanZ protein sequences were retrieved from the National Centre for Biotechnology Information (NCBI) database and then analyzed to construct the phylogenetic tree by using the neighbor-joining method (Bionumerics version 2.5, Applied Maths, Belgium). *Enterococcus faecium* DO was used as outgroup LAB. Lactobacilli from other environments (indicated by their origin in the right column) were used in phylogenetic tree construction. Abbreviation: *E*, *Enterococcus*; *Lb*, *Lactobacillus*.

References

Abriouel, H., Ben Omar, N., Lucas López, R., Martínez-Cañaamero, M., Keleke, S., & Gálvez, A. (2006G). Culture-independent analysis of the microbial composition of the African traditional fermented foods poto poto and degue by using three different DNA extraction methods. *International Journal of Food Microbiology*, *111*, 228–233.

Abriouel, H., Martín-Platero, A., Maqueda, M., Valdivia, E., & Martínez-Bueno, M. (2008). Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. *International Journal of Food Microbiology*, *127*, 200–208.

Abriouel, H., Benomar, N., Lucas, R., & Gálvez, A. (2011). Culture-independent study of the diversity of microbial populations in brines during fermentation of naturally fermented Aloreña green table olives. *International Journal of Food Microbiology*, *144*, 487–496.

Abriouel, H., Benomar, N., Cobo, A., Caballero, N., Fernández Fuentes, M.Á., Pérez-Pulido, R., et al. (2012u). Characterization of lactic acid bacteria from naturally-fermented Manzanilla Aloreña green table olives. *Food Microbiology*, *32*, 308–316.

Adimpong, D. B., Nielsen, D. S., Sørensen, K. I., Vogensen, F. K., Sawadogo-Lingani, H., Derkx, P. M. F., et al. (2013,). *Lactobacillus delbrueckii* subsp. *jakobsenii* subsp. nov., isolated from dolo wort; an alcoholic fermented beverage in Burkina Faso. *International Journal of Systematic and Evolutionary Microbiology*, *63*, 3720–3726.

Ai, L., Chen, C., Zhou, F., Wang, L., Zhang, H., Chen, W., et al. (2011). Complete genome sequence of the probiotic strain *Lactobacillus casei* BD-II. *Journal of Bacteriology*, *193*, 3160–3161.

Ali, A. A., & Mustafa, M. M. (2009). Use of starter cultures of lactic acid bacteria and yeasts in the preparation of kisra, a Sudanese fermented food. *Pakistan Journal of Nutrition*, *8*, 1349–1353.

Al-Nabulsi, A. A., Osaili, T. M., Elabedeen, N. A., Jaradat, Z. W., Shaker, R. R., Kheirallah, K. A., et al. (2011t). Impact of environmental stress desiccation, acidity, alkalinity, heat or cold on antibiotic susceptibility of *Cronobacter sakazakii*. *International Journal of Food Microbiology*, *146*, 137–143.

Ammor, M. S., Flórez, A. B., & Mayo, B. (2007). Antibiotic resistance in nonenterococcal lactic acid bacteria and bifidobacteria. *Food Microbiology*, *24*, 559–570.

Ammor, M. S., Florez, A. B., van Hoek, A. H., de Los Reyes-Gavilan, C. G., Aarts, H. J., Margolles, A., et al. (2008s). Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and bifidobacteria. *Journal of Molecular Microbiology and Biotechnology*, *14*, 6–15.

Auputinan, P., Tragoolpua, Y., Pruksakorn, S., & Thongwai, N. (2011). Profiles of plasmids in lactobacilli isolated from fermented foods. *Chiang Mai Journal of Science*, *38*, 648–652.

Axelsson, L., Rud, I., Naterstad, K., Blom, H., Renckens, B., Boekhorst, J., et al. (2012). Genome sequence of the naturally plasmid-free *Lactobacillus plantarum* strain NC8 (CCUG 61730). *Journal of Bacteriology*, *194*, 2391–2392.

Aymerich, T., Martín, B., Garriga, M., Vidal-Carou, M. C., Bover-Cid, S., & Hugas, M. (2006). Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. *Journal of Applied Microbiology*, *100*, 40–49.

Barbosa, T. M., & Levy, S. B. (2000). The impact of antibiotic use on resistance development and persistence. *Drug Resistance Updates*, *3*, 303–311.

Båth, K., Roos, S., Wall, T., & Jonsson, H. (2005). The cell surface of *Lactobacillus reuteri* ATCC 55730 highlighted by identification of 126 extracellular proteins from the genome sequence. *FEMS Microbiology Letters*, *253*, 75–82.

Belletti, N., Monica, G., Benedetta, B., Erasmo, N., Giulia, T., & Fausto, G. (2009). Antibiotic resistance of lactobacilli isolated from two Italian hard cheeses. *Journal of Food Protection*, *72*, 2162–2169.

Ben Omar, N., Ampe, F., Raimbault, M., Guyot, J. -P., & Tailliez, P. (2000). Molecular diversity of lactic acid bacteria from cassava sour starch (Colombia). *Systematic and Applied Microbiology*, *23*, 285–291.

Bennedsen, M., Stuer-Lauridsen, B., Danielsen, M., & Johansen, E. (2011). Screening for antimicrobial resistance genes and virulence factors via genome sequencing. *Applied and Environmental Microbiology*, *77*, 2785–2787.

Bennett, D. M., Shekhel, T., Radelet, M., & Miller, M. D. (2014). Isolated *Lactobacillus* chronic prosthetic knee infection. *Orthopedics*, *37*, 83–86.

Bergsveinson, J., Baecker, N., Pittet, V., & Ziola, B. (2015). Role of plasmids in *Lactobacillus brevis* BSO 464 hop tolerance and beer spoilage. *Applied and Environmental Microbiology*, *81*, 1234–1241.

Bernardeau, M., Vernoux, J. P., Henri-Dubernet, S., & Gueguen, M. (2008). Safety assessment of dairy microorganisms: The *Lactobacillus* genus. *International Journal of Food Microbiology*, *126*, 278–285.

Blandino, G., Milazzo, I., & Fazio, D. (2008). Antibiotic susceptibility of bacterial isolates from probiotic products available in Italy. *Microbial Ecology in Health and Disease*, *20*, 199–203.

Broadbent, J. R., Neeno-Eckwall, E., Stahl, B., Tandee, K., Cai, H., Morovic, W., et al. (2012,). Analysis of the *Lactobacillus casei* supragenome and its influence in species evolution and lifestyle adaptation. *BMC Genomics*, *13*, 533.

Broadbent, J. R., Hughes, J. E., Welker, D. L., Tompkins, T. A., & Steele, J. L. (2013). Complete genome sequence for *Lactobacillus helveticus* CNRZ 32, an industrial cheese starter and cheese flavor adjunct. *Genome Announcements*, *1*, e00590-13. http://dx.doi.org/10.1128/genomeA.00590-13.

Cadena, M. P., Villarraga, E. C., Luján, D. E., & Salcedo, J. G. (2006). Evaluación de la agroindustria del almidón agrio de yuca (*Manihot esculenta* Crantz) en Córdoba y Sucre. *Temas Agrarios*, *11*, 43–53.

Cai, H., Thompson, R., Budinich, M. F., Broadbent, J. R., & Steele, J. L. (2009). Genome sequence and comparative genome analysis of *Lactobacillus casei*: Insights into their niche-associated evolution. *Genome Biology and Evolution*, *1*, 239–257.

Callanan, M., Kaleta, P., O'Callaghan, J., O'Sullivan, O., Jordan, K., McAuliffe, O., et al. (2008e). Genome sequence of *Lactobacillus helveticus*, an organism distinguished by selective gene loss and insertion sequence element expansion. *Journal of Bacteriology*, *190*, 727–735.

Canchaya, C., Claesson, M. J., Fitzgerald, G. F., van Sinderen, D., & O'Toole, P. W. (2006). Diversity of the genus *Lactobacillus* revealed by comparative genomics of five species. *Microbiology*, *152*, 3185–3196.

Cannon, J. P., Lee, T. A., Bolanos, J. T., & Danziger, L. H. (2005). Pathogenic relevance of *Lactobacillus*: A retrospective review of over 200 cases. *European Journal of Clinical Microbiology & Infectious Diseases*, *24*, 31–40.

Čanžek Majhenič, A., Mohar Lorbeg, P., & Rogelj, I. (2007). Characterization of the *Lactobacillus* community in traditional Karst ewe's cheese. *International Journal of Dairy Technology*, *60*, 182–190.

Casado Muñoz, M. C., Benomar, N., Lavilla Lerma, L., Gálvez, A., & Abriouel, H. (2014). Antibiotic resistance of *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* isolated from naturally-fermented Aloreña table olives throughout fermentation process. *International Journal of Food Microbiology*, *172*, 110–118.

Casas, I. A., & Dobrogosz, W. J. (1997). *Lactobacillus reuteri*: An overview of a new probiotic for humans and animals. *Microecology and Therapy*, *25*, 221–231.

Casas, I. A., & Dobrogosz, W. J. (2000). Validation of the probiotic concept: *Lactobacillus reuteri* confers broad-spectrum protection against disease in humans and animals. *Microbial Ecology in Health and Disease*, *12*, 247–285.

Cataloluk, O., & Gogebakan, B. (2004). Presence of drug resistance in intestinal lactobacilli of dairy and human origin in Turkey. *FEMS Microbiology Letters*, *236*, 7–12.

Chaillou, S., Champomier-Vergès, M. C., Cornet, M., Crutz-Le Coq, A. M., Dudez, A. M., Martin, V., et al. (2005i). The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23 K. *Nature Biotechnology*, *23*, 1527–1533.

Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1998). Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *Journal of Applied Microbiology*, *84*, 759–768.

Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (2001). Gradient diffusion antibiotic susceptibility testing of potentially probiotic lactobacilli. *Journal of Food Protection*, *64*, 2007–2014.

Chen, C., Ai, L. Z., Zhou, F. F., Wang, L., Zhang, H., Chen, W., et al. (2011). Complete genome sequence of the probiotic bacterium *Lactobacillus casei* LC2W. *Journal of Bacteriology*, *193*, 3419–3420.

Claesson, M. J., van Sinderen, D., & O'Toole, P. W. (2008). *Lactobacillus* phylogenomics—towards a reclassification of the genus. *International Journal of Systematic and Evolutionary Microbiology*, *58*, 2945–2954.

Clewell, D. B., Flannagan, S. E., & Jaworski, D. D. (1995). Unconstrained bacterial promiscuity: The Tn*916*–Tn*1545* family of conjugative transposons. *Trends in Microbiology*, *3*, 229–236.

Coast, J., & Smith, R. D. (2003). Antimicrobial resistance: Cost and containment. *Expert Review of Anti-Infective Therapy*, *1*, 241–251.

Comunian, R., Daga, E., Dupré, I., Paba, A., Devirgiliis, C., Piccioni, V., et al. (2010). Susceptibility to tetracycline and erythromycin of *Lactobacillus paracasei* strains isolated from traditional Italian fermented foods. *International Journal of Food Microbiology*, *138*, 151–156.

Condon, S. (1983). Aerobic metabolism of lactic acid bacteria. *Irish Journal of Food Science and Technology*, *7*, 15–25.

Coppola, R., Succi, M., Tremonte, P., Reale, A., Salzano, G., & Sorrentino, E. (2005). Antibiotic suscebtibility of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. *Le Lait*, *85*, 193–204.

Cremonesi, P., Chessa, S., & Castiglioni, B. (2013). Genome sequence and analysis of *Lactobacillus helveticus*. *Frontiers in Microbiology*, *3*, 1–12.

Crowley, S., Bottacini, F., Mahony, J., & van Sinderen, D. (2013). Complete genome sequence of *Lactobacillus plantarum* strain 16, a broad-spectrum antifungal-producing lactic acid bacterium. *Genome Announcements*, *1*, e00533-13. http://dx.doi.org/10.1128/genomeA.00533-13.

Danielsen, M. (2002). Characterization of the tetracycline resistance plasmid pMD5057 from *Lactobacillus plantarum* 5057 reveals a composite structure. *Plasmid*, *48*, 98–103.

Danielsen, M., & Wind, A. (2003). Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *International Journal of Food Microbiology*, *82*, 1–11.

Danielsen, M., Madsen, H. L., Hammer, K., & Wind, A. (2005). Investigation of Danish and Italian cheeses made from raw milk and pasteurized milk for the presence of antibiotic resistant lactic acid bacteria and the genetic characterisation of these bacteria. *Poster Lab8 conference: Symposium on lactic acid bacteria: Genetics, metabolism and applications, Egmond aan Zee*.

Davidson, B. E., Kordias, N., Dobos, M., & Hillier, A. J. (1996). Genomic organization of lactic acid bacteria. *Antonie Van Leeuwenhoek*, *70*, 161–183.

DeLisle, S., & Perl, T. M. (2003). Vancomycin-resistant enterococci. *Chest*, *123*(Suppl.), 504S–518S.

Dessalegn, A., & Ashenafi, M. (2010). Evaluation of the probiotic properties and antibiotic resistance of lactic acid bacteria isolated from awaze, qotchqotcha and Tef dough, traditional Ethiopian fermented foods. *Internet Journal of Food Safety*, *12*, 187–191.

Devirgiliis, C., Caravelli, A., Coppola, D., Barile, S., & Perozzi, G. (2008). Antibiotic resistance and microbial composition along the manufacturing process of Mozzarella di Bufala Campana. *International Journal of Food Microbiology*, *128*, 378–384.

Devirgiliis, C., Coppola, D., Barile, S., Colonna, B., & Perozzi, G. (2009). Characterization of the Tn*916* conjugative transposon in a food-borne strain of *Lactobacillus paracasei*. *Applied and Environmental Microbiology*, *75*, 3866–3871.

Devirgiliis, C., Zinno, P., & Perozzi, G. (2013). Update on antibiotic resistance in foodborne *Lactobacillus* and *Lactococcus* species. *Frontiers in Microbiology*, *4*, 301.

Egervärn, M. (2009). Antibiotic Resistance in *Lactobacillus reuteri* and *Lactobacillus plantarum*. *Egervärn, Maria (2009). Diss. (sammanfattning/summary) Uppsala: Sveriges lantbruksuniv., Acta Universitatis agriculturae Sueciae* (pp. 1652–6880) Doctoral thesis.

Egervärn, M., Roos, S., & Lindmark, H. (2009). Identification and characterization of antibiotic resistance genes in *Lactobacillus reuteri* and *Lactobacillus plantarum*. *Journal of Applied Microbiology*, *107*, 1658–1668.

Egervärn, M., Lindmark, H., Olsson, J., & Roos, S. (2010). Transferability of a tetracycline 19 resistance gene from probiotic *Lactobacillus reuteri* to bacteria in the gastrointestinal 20 tract of humans. *Antonie Van Leeuwenhoek*, *97*, 189–200.

European Food Safety Authority (2012a). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in the European Union in 2010. *EFSA Journal*, *10*, 2598.

European Food Safety Authority (2012b). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA Journal*, *10*, 2740.

Feld, L., Bielak, E., Hammer, K., & Wilcks, A. (2009). Characterization of a small erythromycin resistance plasmid pLFE1 from the food-isolate *Lactobacillus plantarum* M345. *Plasmid*, *61*, 159–170.

Flórez, A. B., Delgado, S., & Mayo, B. (2005). Antimicrobial susceptibility of lactic acid bacteria isolated from a cheese environment. *Canadian Journal of Microbiology*, *51*, 51–58.

Flórez, A. B., Ammor, M. S., & Mayo, B. (2008). Identification of *tet*(M) in two *Lactococcus lactis* strains isolated from a Spanish traditional starter-free cheese made of raw milk and conjugative transfer of tetracycline resistance to lactococci and enterococci. *International Journal of Food Microbiology*, *121*, 189–194.

Fons, M., Hégé, T., Ladiré, M., Raibaud, P., Ducluzeau, R., & Maguin, E. (1997). Isolation and characterization of a plasmid from *Lactobacillus fermentum* conferring erythromycin resistance. *Plasmid*, *37*, 199–203.

Franko, B., Vaillant, M., Recule, C., Vautrin, E., Brion, J. P., & Pavese, P. (2013). *Lactobacillus paracasei* endocarditis in a consumer of probiotics. *Médecine et Maladies Infectieuses*, *43*, 171–173.

Franz, C. M. A. P., Cho, G. Y., & Holzapfel, W. H. (2011). Probiotics: Taxonomy and technological features. In N. P. Shah, A. Gomes da Cruz, & J. de Assis Fonseca Faria (Eds.), *Probiotic and Prebiotic Foods: Technology, Stability and Benefits to Human Health*. New York, USA: Nova Science Publishers, Hauppauge.

Fukao, M., Tomita, H., Yakabe, T., Nomura, T., Ike, Y., & Yajima, N. (2009). Assessment of antibiotic resistance in probiotic strain *Lactobacillus brevis* KB290. *Journal of Food Protection*, *72*, 1923–1929.

Fukao, M., Oshima, K., Morita, H., Toh, H., Suda, W., Kim, S. -W., et al. (2013). Genomic analysis by deep sequencing of the probiotic *Lactobacillus brevis* KB290 harboring nine plasmids reveals genomic stability. *PLoS ONE*, *8*, e60521.

Ganjian, H., Nikokar, I., Tieshayar, A., Mostafaei, A., Amirmozafari, N., & Kiani, S. (2012). Effects of salt stress on the antimicrobial drug resistance and protein profile of *Staphylococcus aureus*. *Jundishapur Journal of Microbiology*, *5*, 328–331.

Garrigues, C., Johansen, E., & Crittenden, R. (2013). Pangenomics—an avenue to improved industrial starter cultures and probiotics. *Current Opinion in Biotechnology*, *24*, 187–191.

Ge, B., Jiang, P., & Han, F. (2007). Identification and antimicrobial susceptibility of lactic acid bacteria from retail fermented foods. *Journal of Food Protection*, *70*, 2606–2612.

Gevers, D., Danielsen, M., Huys, G., & Swings, J. (2003). Molecular characterization of *tet*(M) genes in *Lactobacillus* isolates from different types of fermented dry sausage. *Applied and Environmental Microbiology*, *69*, 1270–1275.

Gfeller, K. Y., Roth, M., Meile, L., & Teuber, M. (2003). Sequence and genetic organization of the 19.3-kb erythromycin- and dalfopristin resistance plasmid pLME300 from *Lactobacillus fermentum* ROT1. *Plasmid*, *50*, 190–201.

Giraffa, G., Chanishvili, N., & Widyastuti, Y. (2010). Importance of lactobacilli in food and feed biotechnology. *Research in Microbiology*, *161*, 480–487.

Hammes, W. P., & Vogel, R. F. (1995). The genus *Lactobacillus*. In B. J. B. Wood, & W. H. Holzapfel (Eds.), *The genera of lactic acid bacteria* (pp. 19–54). Glasgow, UK: Blackie Academic and Professional.

Han, J. H., Li, X. F., Gao, W. H., Walczak, P., Zhang, B. L., & Jia, Y. M. (2013). Susceptibility of *Lactobacillus pentosus* strains isolated from fermented products to streptomycin and kanamycin. *International Food Research Journal*, *20*, 1927–1931.

Handwerger, S., Pucci, M. J., Volk, K. J., Liu, J., & Lee, M. S. (1994). Vancomycin-resistant *Leuconostoc mesenteroides* and *Lactobacillus casei* synthesize cytoplasmic peptidoglycan precursors that terminate in lactate. *Journal of Bacteriology*, *176*, 260–264.

Hansen, E. B. (2002). Commercial bacterial starter cultures for fermented foods of the future. *International Journal of Food Microbiology*, *78*, 119–131.

Harty, D. W. S., Oakey, H. J., Patrikakis, M., Hume, E. B., & Knox, K. W. (1994). Pathogenic potential of lactobacilli. *International Journal of Food Microbiology*, *24*, 179–189.

Hebert, E. M., Raya, R. R., Brown, L., Font de Valdez, G., Savoy de Giori, G., & Taranto, M. P. (2013,). Genome sequence of the cheese-starter strain *Lactobacillus delbrueckii* subsp. *lactis* CRL 581. *Genome Announcements*, *1*, e00602–e00613.

Heller, K. J. (2001). Probiotic bacteria in fermented foods: Product characteristics and starter organisms. *The American Journal of Clinical Nutrition*, *73*, 374s–379s.

Herreros, M. A., Sandoval, H., González, L., Castro, J. M, Fresno, J. M., & Tornadijo, M.E. (2005). Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from armada cheese (a Spanish goats' milk cheese). Food Microbiology, 22, 455–459.

Hleba, L., Kačániová, M., Pavelková, A., & Čuboň, J. (2012). Antibiotic resistance of lactobacilli strains isolated from milk and milk products from middle Slovakia. *Journal of Microbiology, Biotechnology and Food Sciences*, *2*, 253–262.

Hochwind, K., Weinmaier, T., Schmid, M., van Hemert, S., Hartmann, A., Rattei, T., et al. (2012a). Draft genome sequence of *Lactobacillus casei* W56. *Journal of Bacteriology*, *194*, 6638.

Holzapfel, W. H. (2002). Appropriate starter culture technologies for small-scale fermentation in developing countries. *International Journal of Food Microbiology*, *75*, 197–212.

Honi, U., Sabrin, F., Islam, T., Islam, E., Billah, M., & Islam, K. D. (2013). Enzymatic activity and antibiotic resistance profile of *Lactobacillus paracasei ssp. paracasei*-1 isolated from regional yogurts of Bangladesh. *Journal of Microbiology Biotechnology and Food Sciences*, *3*, 235–239.

Hou, C., Wang, Q., Zeng, X., Yang, F., Zhang, J., Liu, H., et al. (2013). Complete genome sequence of *Lactobacillus reuteri* I5007, a probiotic strain isolated from healthy piglet. *Journal of Biotechnology*, *179*, 63–64.

Hugenholtz, P. (1998). *The genera of lactic acid bacteria*. London, UK: Blackie Academic and Professional.

Hummel, A. S., Hertel, C., Holzapfel, W. H., & Franz, C. M. A. P. (2007). Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. *Applied and Environmental Microbiology*, *73*, 730–739.

Huovinen, P. (1987). Trimethoprim resistance. *Antimicrobial Agents and Chemotherapy*, *31*, 1451–1456.

International Organization for Standardization (2010). Milk and milk products—determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB). *ISO 10932/IDF 233 standard*.

Jayashree, S., Pooja, S., Pushpanathan, M., Vishnu, U., Sankarasubramanian, J., Rajendhran, J., et al. (2013j). Genome sequence of *Lactobacillus fermentum* strain MTCC 8711, a probiotic bacterium isolated from yogurt. *Genome Announcements*, *1*, e00770-13. http://dx.doi.org/10.1128/genomeA.00770-13.

Jokovic, N., Nikolic, M., Begovic, J., Jovcic, B., Savic, D., & Topisirovic, L. (2008). A survey of the lactic acid bacteria isolated from Serbian artisanal dairy product kajmak. *International Journal of Food Microbiology*, *125*, 305–311.

Kaleta, P., O'Callaghan, J., Fitzgerald, G. F., Beresford, T. P., & Ross, R. P. (2010). Crucial role for insertion sequence elements in *Lactobacillus helveticus* evolution as revealed by interstrain genomic comparison. *Applied and Environmental Microbiology*, *76*, 212–220.

Kant, R., Paulin, L., Alatalo, E., de Vos, W. M., & Palva, A. (2011a). Genome sequence of *Lactobacillus amylovorus* GRL1118, isolated from pig ileum. *Journal of Bacteriology*, *193*, 3147–3148.

Kant, R., Blom, J., Pavla, A., Siezen, R. J., & de Vos, W. M. (2011b). Comparative genomics of *Lactobacillus*. *Microbial Biotechnology*, *4*, 323–332.

Karlyshev, A. V., Raju, K., & Abramov, V. M. (2013). Draft genome sequence of *Lactobacillus fermentum* strain 3872. *Genome Announcements*, *1*, e01006–e01013.

Karlyshev, A. V., & Abramov, V. M. (2014). Draft genome sequence of *Lactobacillus plantarum* 2165. *Genome Announcements*, *2*, e01179-13. http://dx.doi.org/10.1128/genomeA.01179-13.

Kastner, S., Perreten, V., Bleuler, H., Hugenschmidt, G., Lacroix, C., & Meile, L. (2006). Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food. *Systematic and Applied Microbiology*, *29*, 145–155.

Katla, A. K., Kruse, H., Johnsen, G., & Herikstad, H. (2001). Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products. *International Journal of Food Microbiology*, *67*, 147–152.

Kim, K. S., Morrison, J. O., & Bayer, A. S. (1982). Deficient autolytic enzyme activity in antibiotic-tolerant lactobacilli. *Infection and Immunity*, *36*, 582–585.

Klaenhammer, T. R., & de Vos, W. M. (2011). An incredible scientific journey. The evolutionary tale of the lactic acid bacteria. In A. Ledeboer, J. Hugenholtz, J. Kok, W. Konings, & J. Wouters (Eds.), *The 10th LAB symposium. Thirty years of research on lactic acid bacteria* (pp. 1–11). 24 Media Labs.

Klare, I., Konstabel, C., Müller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., et al. (2005t). Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. *Applied and Environmental Microbiology*, *71*, 8982–8986.

Klare, I., Konstabel, C., Werner, G., Huys, G., Vankerckhoven, V., Kahlmeter, G., et al. (2007l). Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. *Journal of Antimicrobial Chemotherapy*, *59*, 900–912.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O. P., Leer, R., et al. (2003r). Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proceedings of the National Academy of Sciences of the United States of America*, *100* (1990–1995).

Klein, G. (2011). Antibiotic resistance and molecular characterization of probiotic and clinical *Lactobacillus* strains in relation to safety aspects of probiotics. *Foodborne Pathogens and Disease*, *8*, 267–281.

Klein, G., Zill, E., Schindler, R., & Louwers, J. (1998). Peritonitis associated with vancomycin-resistant *Lactobacillus rhamnosus* in a continuous ambulatory peritoneal dialysis patient: Organism identification, antibiotic therapy, and case report. *Journal of Clinical Microbiology*, *36*, 1781–1783.

Klein, G., Hallmann, C., Casas, I. A., Abad, J., Louwers, J., & Reuter, G. (2000). Exclusion of *vanA*, *vanB* and *vanC* type glycopeptide resistance in strains of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* used as probiotics by polymerase chain reaction and hybridization methods. *Journal of Applied Microbiology*, *89*, 815–824.

Koryszewska-Bagińska, A., Bardowski, J., & Aleksandrzak-Piekarczyk, T. (2014). Genome sequence of the probiotic strain *Lactobacillus rhamnosus* (formerly *Lactobacillus casei*) LOCK908. *Genome Announcements*, *2*, e00120-14. http://dx.doi.org/10.1128/genomeA.00120-14.

Ladero, V., Alvarez-Sieiro, P., Redruello, B., del Rio, B., Linares, D. M., Cruz Martin, M., et al. (2013i). Draft genome sequence of *Lactobacillus plantarum* strain IPLA 88. *Genome Announcements*, *1*, e00524-13. http://dx.doi.org/10.1128/genomeA.00524-13.

Ladero, V., Herrero-Fresno, A., Martinez, N., del Río, B., Linares, D. M., Fernández, M., et al. (2014,). Genome sequence analysis of the biogenic amine-degrading strain *Lactobacillus casei* 5b. *Genome Announcements*, *2*, e01199-13. http://dx.doi.org/10.1128/genomeA.01199-13.

Lamontanara, A., Caggianiello, G., Orrù, L., Capozzi, V., Michelotti, V., Bayjanov, J. R., et al. (2015,). Draft genome sequence of *Lactobacillus plantarum* Lp90 isolated from wine. *Genome Announcements*, *3*, e00097-15. http://dx.doi.org/10.1128/genomeA.00097-15.

Lapsiri, W., Nitisinprasert, S., & Wanchaitanawong, P. (2011). *Lactobacillus plantarum* strains from fermented vegetables as potential probiotics. *Kasetsart Journal (Natural Science)*, *45*, 1071–1082.

Lavanya, B., Sowmiya, S., Balaji, S., & Muthuvelan, B. (2011). Screening and characterization of lactic acid bacteria from fermented milk. *British Journal of Dairy Sciences*, *2*, 5–10.

Lee, J. H., Chae, J. P., Lee, J. Y., Lim, J. S., & Kim, G. B. (2011). Genome sequence of *Lactobacillus johnsonii* PF01, isolated from piglet feces. *Journal of Bacteriology*, *193*, 5030–5031.

Leroy, F., & De Vuyst, L. (1999). Temperature and pH conditions that prevail during fermentation of sausages are optimal for production of the antilisterial bacteriocin sakacin K. *Applied and Environmental Microbiology*, *65*, 974–981.

Li, X., Gu, Q., Lou, X., Zhang, X., Song, D., Shen, L., et al. (2013). Complete genome sequence of the probiotic *Lactobacillus plantarum* strain ZJ316. *Genome Announcements*, *1*, e00094-13. http://dx.doi.org/10.1128/genomeA.00094-13.

Liasi, S. A., Azmi, T. I., Hassan, M. D., Shuhaimi, M., Rosfarizan, M., & Ariff, A. B. (2009). Antimicrobial activity and antibiotic sensitivity of three isolates of lactic acid bacteria from fermented fish product, Budu. *Malaysian Journal of Microbiology*, *5*, 33–37.

Lim, H. I., Lee, J., Jang, J. Y., Park, H. W., Choi, H. -J., Kim, T. -W., et al. (2014). Draft genome sequence of *Lactobacillus sakei* strain wikim 22, isolated from Kimchi in Chungcheong Province, South Korea. *Genome Announcements*, *2*, e01296-14. http://dx.doi.org/10.1128/genomeA.01296-14.

Lin, C. F., Fung, Z. F., Wu, C. L., & Chung, T. C. (1996). Molecular characterization of a plasmid-borne (pTC82) chloramphenicol resistance determinant (*cat*-TC) from *Lactobacillus reuteri* G4. *Plasmid*, *36*, 116–124.

Liu, C., Zhang, Z. Y., Dong, K., Yuan, J. P., & Guo, X. K. (2009). Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. *Biomedical and Environmental Sciences*, *22*, 401–412.

Liu, S. -B., Qiao, L. -P., He, H. -L., Zhang, Q., Chen, X. -L., Zhou, W. -Z., et al. (2011). Optimization of fermentation conditions and rheological properties of exopolysaccharide produced by deep-sea bacterium *Zunongwangia profunda* SM-A87. *PLoS ONE*, *6*, e26825.

Luh, K. T., Hsueh, P. R., Teng, L. J., Pan, H. J., Chen, Y. C., Lu, J. J., et al. (2000). Quinupristin-dalfopristin resistance among Gram-positive bacteria in Taiwan. *Antimicrobial Agents and Chemotherapy*, *44*, 3374–3380.

Malik, S., Siezen, R. J., Renckens, B., Vaneechoutte, M., Vanderleyden, J., & Lebeer, S. (2014). Draft genome sequence of *Lactobacillus plantarum* CMPG5300, a human vaginal isolate. *Genome Announcements*, *2*, e01149-14. http://dx.doi.org/10.1128/genomeA.01149-14.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., et al. (2006). Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 15,611–15,616.

Marshall, B. M., Ochieng, D. J., & Levy, S. B. (2009). Commensals: Underappreciated reservoir of antibiotic resistance. *Microbe*, *4*, 231–238.

Martins, A., Makita, M., Fernandes, M. H., Fernandes, M. J., Barreto, A. S., & Fraqueza, M. J. (2013). Phenotypic and genotypic characterization of *Lactobacillus sakei* isolated from portuguese fermented meat products for tetracycline resistance. *Poster 59th ICoMST*.

Mater, D. D., Langella, P., Corthier, G., & Flores, M. J. (2008). A probiotic *Lactobacillus* strain can acquire vancomycin resistance during digestive transit in mice. *Journal of Molecular Microbiology and Biotechnology*, *14*, 123–127.

Mathur, S., & Singh, R. (2005). Antibiotic resistance in food lactic acid bacteria-a review. *International Journal of Food Microbiology*, *105*, 281–295.

Mazé, A., Boel, G., Zúñiga, M., Bourand, A., Loux, V., Yebra, M. J., et al. (2010). Complete genome sequence of the probiotic *Lactobacillus casei* strain BL23. *Journal of Bacteriology*, *192*, 2647–2648.

McLeod, A., Brede, D. A., Rud, I., & Axelsson, L. (2013). Genome sequence of *Lactobacillus sakei* subsp. *sakei* LS25, a commercial starter culture strain for fermented sausage. *Genome Announcements*, *1*, e00475-13. http://dx.doi.org/10.1128/genomeA.00475-13.

McMahon, M. A., McDowell, D. A., & Blair, I. S. (2007). The pattern of pleiomorphism in stressed *Salmonella* Virchow populations is nutrient and growth phase dependent. *Letters in Applied Microbiology*, *45*, 276–281.

Mokhbi, A., Kaid-Harche, M., Lamri, K., Rezki, M., & Kacem, M. (2009). Selection of *Lactobacillus plantarum* strains for their use as starter culturesin Algerian olive fermentations. *Grasas y Aceites*, *60*, 82–88.

Morelli, L., Sarra, P. G., & Bottazzi, V. (1988). *In vivo* transfer of pAMßl from *Lactobacillus reuteri* to *Enterococcus faecalis*. *Journal of Applied Bacteriology*, *65*, 371–375.

Morelli, L., Zonenenschain, D., Del Piano, M., & Cognein, P. (2004). Utilization of the intestinal tract as a delivery system for urogenital probiotics. *Journal of Clinical Gastroenterology*, *38*, S107-S100.

Morita, H., Toh, H., Fukuda, S., Horikawa, H., Oshima, K., Suzuki, T., et al. (2008). Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin production. *DNA Research*, *15*, 151–161.

Nam, Y. -D., Lee, S. -Y., & Lim, S. -I. (2012). Microbial community analysis of Korean soybean pastes by next-generation sequencing. *International Journal of Food Microbiology*, *155*, 36–42.

Nawaz, M., Wang, J., Zhou, A., Ma, C., Wu, X., Moore, J. E., et al. (2011). Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. *Current Microbiology*, *62*, 1081–1089.

Naz, S., Tareb, R., Bernardeau, M., Vaisse, M., Lucchetti-Miganeh, C., Rechenmann, M., et al. (2013,). Genome sequence of *Lactobacillus plantarum* strain UCMA 3037. *Genome Announcements*, *1*, e00251-13. http://dx.doi.org/10.1128/genomeA.00251-13.

Nei, T., Inai, S., Mikami, I., Sato, A., Okamoto, J., Yokoshima, K., et al. (2013). Descending necrotizing mediastinitis associated with *Lactobacillus plantarum*. *BMC Infectious Diseases*, *13*, 398.

O'Brien, T. F. (2002). Emergence, spread, and environmental effect of antimicrobial resistance: How use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. *Clinical Infectious Diseases*, *34*, S78–S84.

Oguntoyinbo, F. A., Tourlomousis, P., Gasson, M., & Narbad, A. (2011). Analysis of bacterial communities of traditional fermented West African cereal foods using culture-independent methods. *International Journal of Food Microbiology*, *145*, 205–210.

Oh, S., Roh, H., Ko, H. J., Kim, S., Kim, K. H., Lee, S. E., et al. (2011). Complete genome sequencing of *Lactobacillus acidophilus* 30SC, isolated from swine intestine. *Journal of Bacteriology*, *193*, 2882–2883.

Olukoya, D. K., Ebigwei, S. I., Adebawo, O. O., & Osiyemi, F. O. (1993). Plasmid profiles and antibiotic susceptibility patterns of *Lactobacillus* isolated from fermented foods in Nigeria. *Food Microbiology*, *10*, 279–285.

Ouoba, L. I., Lei, V., & Jensen, L. B. (2008). Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: Determination and transferability of the resistance genes to other bacteria. *International Journal of Food Microbiology*, *121*, 217–224.

Pan, L., Hu, X., & Wang, X. (2011). Assessment of antibiotic resistance of lactic acid bacteria in Chinese fermented foods. *Food Control*, *22*, 1316–1321.

Patel, A. R., Shah, N. P., & Prajapati, J. B. (2012). Antibiotic resistance profile of lactic acid bacteria and their implications in food chain. *World Journal of Dairy & Food Sciences*, *7*, 202–211.

Pérez Pulido, R., Benomar, N., Abriouel, H., Lucas López, R., Martínez Cañamero, M., & Gálvez, A. (2005o). Microbiological study of lactic acid fermentation of caper berries by molecular and culture-dependent methods. *Applied and Environmental Microbiology*, *71*, 7872–7879.

Poole, K. (2012). Efflux mediated antimicrobial resistance. In T. J. Dougherty, & M. J. Pucci (Eds.), *Antibiotic Discoveryand Development* (pp. 349–395). New York, USA: Springer.

Putman, M., van Veen, H. W., Degener, J. E., & Konings, W. N. (2001). The *lactococcal* secondary multidrug transporter LmrP confers resistance to lincosamides, macrolides, streptogramins and tetracyclines. *Microbiology*, *147*, 2873–2880.

Reynolds, P.E. (1998). Control of peptidoglycan synthesis in vancomycin-resistant enterococci: D,D-peptidases and D,D-carboxypeptidases. Cellular and Molecular Life Sciences, 54, 325–331.

Rosander, A., Connolly, E., & Roos, S. (2008). Removal of antibiotic resistance gene-carrying plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain, *L. reuteri* DSM 17938. *Applied and Environmental Microbiology*, *74*, 6032–6040.

Rozila, A., Rajandara, P., Lani, M. N., Siti Hasmah, M., Ezni Suryani, I., Ragupathy, R., et al. (2012,). Antibiotic resistant of *Lactobacillus* isolated from dairy products. *UMT 11th International Annual Symposium on Sustainability Science and Management 09th–11th July 2012*. Terengganu: Malaysia.

Ruoff, K. L., Kuritzkes, D. R., Wolfson, J. S., & Ferraro, M. J. (1988). Vancomycin resistant Gram-positive bacteria isolated from human sources. *Journal of Clinical Microbiology*, *26*, 2064–2068.

Salminen, S., von Wright, A., Morelli, L., Marteau, P., Brassart, D., de Vos, W., et al. (1998l). Demonstration of safety of probiotics—a review. *International Journal of Food Microbiology*, *44*, 93–106.

Savadogo, A., Ouattara, C. A. T., Ilboudo, A. J., Karou, D., & Traore, A. S. (2010). Isolation and susceptibility to antibiotics of bacterial strains from Burkina Faso fermented milk samples. *Advance Journal of Food Science and Technology*, *2*, 91–95.

Schlegel, L., Lemerle, S., & Geslin, P. (1998). *Lactobacillus* species as opportunistic pathogens in immunocompromised patients. *European Journal of Clinical Microbiology & Infectious Diseases*, *17*, 887–888.

Schleifer, K. H., & Ludwig, V. (1995). Phylogenetics relationships of lactic acid bacteria. In B. J. B. Wood, & W. H. Holzapfel (Eds.), *The genera of lactic acid bacteria* (pp. 7–17). Glasgow, UK: Chapman and Hall.

Schmidt, G., Hertel, C., & Hammes, W. P. (1999). Molecular characterization of the dnaK operon of *Lactobacillus sakei* LTH681. *Systematic and Applied Microbiology*, *22*, 321–328.

Schneider, D., & Lenski, R. E. (2004). Dynamics of insertion sequence elements during experimental evolution of bacteria. *Research in Microbiology*, *155*, 319–327.

Settanni, L., & Moschetti, G. (2010). Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. *Food Microbiology*, *27*, 691–697.

Shrago, A. W., & Dobrogosz, W. J. (1988). Conjugal transfer of Group B streptococcal plasmids and co-mobilization of *E. coli–Streptococcus* shuttle plasmids to *L. plantarum*. *Applied and Environmental Microbiology*, *54*, 824–826.

Simeoni, D., Rizzotti, L., Cocconcelli, P., Gazzola, S., Dellaglio, F., & Torriani, S. (2008,). Antibiotic resistance genes and identification of staphylococci collected from the production chain of swine meat commodities. *Food Microbiology*, *25*, 196–201.

Smokvina, T., Wels, M., Polka, J., Chervaux, C., Brisse, S., Boekhorst, J., et al. (2013). *Lactobacillus paracasei* comparative genomics: Towards species pan-genome definition and exploitation of diversity. *PLoS ONE*, *8*, e68731.

Snydman, D. R. (2008). The safety of probiotics. *Clinical Infectious Diseases*, *46*, S104–S111.

Soomro, A. H., & Masud, T. (2012). Probiotic characteristics of *Lactobacillus* spp. isolated from fermented milk product Dahi. *Food Science and Technology Research*, *18*, 91–98.

Sornplang, P., Leelavatcharamas, V., Sukon, P., & Yowarach, S. (2011). Antibiotic resistance of lactic acid bacteria isolated from a fermented fish product, Pla-chom. *Research Journal of Microbiology*, *6*, 898–903.

Sun, Z., Chen, X., Wang, J., Zhao, W., Shao, Y., Guo, Z., et al. (2011). Complete genome sequence of *Lactobacillus delbrueckii* subsp. *bulgaricus* strain ND02. *Journal of Bacteriology*, *193*, 3426–3427.

Swenson, K. I., Piwnica-Worms, H., McNamee, H., & Paul, D. L. (1990). Tyrosine phosphorylation of the gap junction protein connexin43 is required for the pp60v-*src*-induced inhibition of communication. *Cell Regulation*, *1*, 989–1002.

Tamang, J. P., & Kailasapathy, K. (2010). *Fermented foods and beverages of the world (pp. 149–190)*. Boca Raton, USA: CRC Press.

Tannock, G. W. (2004). A special fondness for lactobacilli. *Applied and Environmental Microbiology*, *70*, 3189–3194.

Temmerman, R., Scheirlinck, I., Huys, G., & Swings, J. (2003). Culture-independent analysis of probiotic products by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, *69*, 220–226.

Teuber, M. (1999). Spread of antibiotic resistance with food-borne pathogens. *Cellular and Molecular Life Sciences*, *56*, 755–763.

Teuber, M., Meile, L., & Schwarz, F. (1999). Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie Van Leeuwenhoek*, *76*, 115–137.

Thumu, S. C., & Halami, P. M. (2012). *Presence of erythromycin and tetracycline resistance genes in lactic acid bacteria from fermented foods of Indian origin. 102*. (pp. 541–551). Antonie Van Leeuwenhoek.

Toh, H., Oshima, K., Nakano, A., Takahata, M., Murakami, M., Takaki, T., et al. (2013). Genomic adaptation of the *Lactobacillus casei* group. *PLoS ONE*, *8*, e75073.

Tompkins, T. A., Barreau, G., & Broadbent, J. R. (2012). Complete genome sequence of *Lactobacillus helveticus* R0052, a commercial probiotic strain. *Journal of Bacteriology*, *194*, 6349.

Turnidge, J. D., & Bell, J. M. (2005). Antimicrobial susceptibility on solid media. In A. Lorian (Ed.), *Antibiotics in laboratory medicine* (pp. 8–60). Baltimore, USA: Lippincott Williams and Wilkins.

Tynkynnen, S., Singh, D. V., & Varmanen, P. (1998). Vancomycin resistance factor of *Lactobacillus rhamnosus* GG in relation to enterococcal vancomycin resistance (van) genes. *International Journal of Food Microbiology*, *41*(195), 204.

Vahabnezhad, E., Mochon, A. B., Wozniak, L. J., & Ziring, D. A. (2013). *Lactobacillus* bacteremia associated with probiotic use in a pediatric patient with ulcerative colitis. *Journal of Clinical Gastroenterology*, *47*, 437–439.

van de Guchte, M., Penaud, S., Grimaldi, C., Barbe, V., Bryson, K., Nicolas, P., et al. (2006.). The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 9274–9279.

van Hoek, A. H. A. M., Margolles, A., Domig, K. J., Korhonen, J. M., Życka-Krzesinska, J., Bardowski, J. K., et al. (2008s). Molecular assessment of erythromycin and tetracycline resistance genes in lactic acid bacteria and bifidobacteria and their relation to the phenotypic resistance. *International Journal of Probiotics and Prebiotics*, *3*, 271–280.

van Reenen, C. A., & Dicks, L. M. (2011). Horizontal gene transfer amongst probiotic lactic acid bacteria and other intestinal microbiota: What are the possibilities. A review. *Archives of Microbiology*, *193*, 157–168.

Vandamme, P., & Peeters, C. (2014). Time to revisit polyphasic taxonomy. *Antonie Van Leeuwenhoek*, *106*, 57–65.

Verraes, C., Van Boxstael, S., Van Meervenne, E., Van Coillie, E., Butaye, P., Catry, B., et al. (2013r). Antimicrobial resistance in the food chain: A review. *International Journal of Environmental Research and Public Health*, *10*, 2643–2669.

Vescovo, M., Morelli, L., & Bottazzi, V. (1982). Drug resistance plasmids in *Lactobacillus acidophilus* and *Lactobacillus reuteri*. *Applied and Environmental Microbiology*, *43*, 50–56.

Wang, T. T., & Lee, B. H. (1997). Plasmids in *Lactobacillus*. *Critical Reviews in Biotechnology*, *17*, 227–272.

Wang, Y., Wang, J., Ahmed, Z., Bai, X., & Wang, J. (2011b). Complete genome sequence of *Lactobacillus kefiranofaciens* ZW3. *Journal of Bacteriology*, *193*, 4280–4281.

Wang, Y., Chen, C., Ai, L., Zhou, F., Zhou, Z., Wang, L., et al. (2011a). Complete genome sequence of the probiotic *Lactobacillus plantarum* ST-III. *Journal of Bacteriology*, *193*, 313–314.

Wang, S., Zhu, H., He, F., Luo, Y., Kang, Z., Lu, C., et al. (2014). Whole genome sequence of the probiotic strain *Lactobacillus paracasei* N1115, isolated from traditional Chinese fermented milk. *Genome Announcements*, *2*, e00059-14. http://dx.doi.org/10.1128/genomeA.00059-14.

White, D. G., Acar, J., Anthony, F., Franklin, A., Gupta, R., Nicholls, T., et al. (2001). Antimicrobial resistance: Standardisation and harmonisation of laboratory methodologies for the detection and quantification of antimicrobial resistance. *Revue scientifique et technique (International Office of Epizootics*, *20*, 849–858.

Weisblum, B. (1995). Erythromycin resistance by ribosome modification. Antimicrobial Agents and Chemotherapy, 39, 577–585.

Yüksekdağ, Z. N., & Beyatli, Y. (2008). Antimicrobial activity and antibiotic susceptibility of some lactic acid bacteria isolated different dairy products. *Archiv für Lebensmittelhygiene*, *59*, 216–220.

Zago, M., Fornasari, M. E., Carminati, D., Burns, P., Suarez, V., & Vinderola, G. (2011). Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. *Food Microbiology*, *28*, 1033–1040.

Zdolec, N., Kozačinski, L., Njari, B., Filipović, I., Hadžiosmanović, M., Mioković, B., et al. (2009,). The antimicrobial effect of lactobacilli on some foodborne bacteria. *Archiv für Lebensmittelhygiene*, *60*, 115–119.

Zdolec, N., Filipović, I., Cvrtila Fleck, Ž., Marić, A., Jankuloski, D., Kozačinski, L., et al. (2011i). Antimicrobial susceptibility of lactic acid bacteria isolated from fermented sausages and raw cheese. *Veterinarski arhiv*, *81*, 133–141.

Zhang, Z. -Y., Liu, C., Zhu, Y. -Z., Zhong, Y., Zhu, Y. -Q., Zheng, H. -J., et al. (2009). Complete genome sequence of *Lactobacillus plantarum* JDM1. *Journal of Bacteriology*, *15*, 5020–5021.

Zhang, W., Yu, D., Sun, Z., Wu R., Chen X., Chen W., et al. (2010). Complete genome sequence of *Lactobacillus casei* Zhang, a new probiotic strain isolated from traditional home-made koumiss in Inner Mongolia of China. Journal of Bacteriology, 192, 5268–9.

Zhang, Z. G., Ye, Z. Q., Yu, L., & Shi, P. (2011). Phylogenomic reconstruction of lactic acid bacteria: An update. *BMC Evolutionary Biology*, *11*, 1.

Zhao, W., Chen, Y., Sun, Z., Wang, J., Zhou, Z., Sun, T., et al. (2011). Complete genome sequence of *Lactobacillus helveticus* H10. *Journal of Bacteriology*, *193*, 2666–2667 Back ← Abstract ↓ Descriptors ↓ Top ↑.

Zheng, H. -J., Wang, B. -F., Zhang, X. -L., Han, H., Lu, G., Jin, L., et al. (2008). The complete genome sequence of *Lactobacillus delbrueckii subsp. bulgaricus* 2038. *Trends in Cell & Molecular Biology*, *3*, 15–30.

Zhou, J. S., Pillidge, C. J., Gopal, P. K., & Gill, H. S. (2005). Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. *International Journal of Food Microbiology*, *98*, 211–217.

Zhou, N., Zhang, J. X., Fan, M. T., Wang, J., Guo, G., & Wei, X. Y. (2012). Antibiotic resistance of lactic acid bacteria isolated from Chinese yogurts. *Journal of Dairy Science*, *95*, 4775–4783.

Zonenschain, D., Rebecchi, A., & Morelli, L. (2009). Erythromycin- and tetracycline-resistant lactobacilli in Italian fermented dry sausages. *Journal of Applied Microbiology*, *107*, 1559–1568.

Artículo 2

Complete genome sequence and safety analysis of a potentially probiotic *Lactobacillus pentosus* MP-10 isolated from fermented Aloreña table olives

Complete genome sequence and safety analysis of a potentially probiotic *Lactobacillus pentosus* MP-10 isolated from fermented Aloreña table olives

Hikmate Abriouel[1*], María del Carmen Casado Muñoz[1], Charles Franz[2], Antonio Gálvez[1], Nabil Benomar[1]

[1]Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaén, 23071-Jaén, Spain.

[2]Department of Microbiology and Biotechnology, Max Rubner-Institut, Hermann-Weigmann-Straße 1, 24103 Kiel, Germany.

Abstract

Lactobacillus pentosus MP-10 isolated from naturally fermented Aloreña table olives is a potential probiotic bacteria. In this study, we sequenced the complete genome and we analyzed its safety aspects and defense mechanisms. The whole genome contains 3,698,214 bp with an estimated G+C content of 46.32% and 5 plasmids of (30-50 kb). The annotated genome sequence revealed the presence of 3558 ORFs and 87 structural RNAs. The genome sequence of *Lb. pentosus* MP-10 is considered the largest genome among lactobacilli up to date highlighting its ecological flexibility and adaptability. Our data shed new light on the presence of two new genes belonging to Subtype I-E CRISPR system besides other nine genes of two CRISPR clusters (Type I and Type II) considered as immune system against foreign genetic elements which is of great importance in its probiotic and also starter culture application. *In silico* analysis of antibiotic resistance showed the absence of acquired antibiotic resistance genes and that most of the resistance genes were antibiotic efflux genes or resulted from mutation of chromosomal genes. On the other hand, no virulence determinants were found in *Lb. pentosus* MP-10 genome. Thus, we can suggest that *Lb. pentosus* MP-10 could be considered as safe, although additional analysis of its functionality and probiotic-linked mechanisms should be carried out.

Introduction

Lacobacilli are ubiquitous in the environment and in raw materials used in food production (reviewed in [1], and also they are part of intestinal, vaginal and oral microbiota [2]. As members of the lactic acid bacteria (LAB), they have been used in food fermentation processes for millennia, however in the last decade they were also used as probiotics, and thus when administered as live microorganisms in adequate amounts they confer a health benefit on the host [3]. Lactobacilli and bifidobactria are the main LAB probiotics used being traditionally isolated from human sources (milk and intestinal tract). However, a search for probiotic LAB from non-dairy origin such as fruits and vegetables has increased in the last years because of the increasing lactose intolerance, dyslipidemia, allergy and vegetarianism [4, 5, 6]. Furthermore, those food matrices are characterized by intrinsic physico-chemical features which mimic the conditions found in the gastrointestinal tract since probiotics of vegetable or fruit origin developed mechanisms for adherence to vegetable or fruit surface in a similar way as they could carry out this property on the intestinal surface, besides their tolerance to acids and several stresses. In this way, several researches were focused on the selection of new probiotic candidates [7, 8] being the number of LAB oscillating between 2.0 and 4.0 Log_{10} CFU/g in fruit and vegetable raw materials [9, 10] and of 6-8 Log_{10} CFU/g in fermented foods [11, 12].

Beside the probiotic features of some lactobacilli strains, the safety aspects should be considered as both properties are inherently linked to the strain used and host susceptibility [13]. Although, many *Lactobacillus* spp. are recognized as GRAS (Generally Regarded As Safe; for the USA) or have attained the QPS (Qualified Presumption of Safety; for the European Commission; European Food Safety Authority "EFSA") [14] status, the probiotic and safety of a strain intended to be used as probiotic should be in deep analyzed at genomic scale or "probiogenomics" [15] to reveal the absence of genes involved in virulence or antibiotic resistance transferability besides the presence of other genes involved in their health-promoting traits.

In the present study, we performed complete genome sequencing of a potential probiotic *Lactobacillus pentosus* MP-10 isolated from brines of naturally fermented Aloreña green table olives [16] to provide deep insight into the safety aspects of this strain. In this sense, the presence of antibiotic resistance and virulence determinants was investigated in depth by means of bioinformatic tools.

Materials and Methods

Bacterial strain and growth conditions. *Lactobacillus pentosus* MP-10 isolated from brines of naturally fermented Aloreña green table olives [16] was routinely cultured at 30ºC either in Man Rogosa and Sharpe (MRS) broth (Fluka, Madrid, Spain) or on agar under aerobic conditions for 24-48 h. The strain was kept in 20% glycerol at -80ºC for long-term storage.

Genomic DNA preparation and sequencing. Genomic DNA was isolated from an overnight culture in MRS broth of *Lb. pentosus* MP-10 using the PureGene Core Kit B according to the manufacturers' instructions (QIAGEN, Spain). The quantity and quality of genomic DNA were determined by photometric (NanoDrop) and fluorometric (Picogreen, Promega) methods. Genome sequencing and annotation of *Lb. pentosus* MP-10 were done by Lifesequencing S.L. (Valencia, Spain). The genomic DNA was sheared and size selected to produce ~10-15 Kb insert-size library according to the Procedure and Checklist -20 Kb Template Preparation Using BluePippinTM Size-Selection System protocol (P/N 100-286-000-07), the mean size distribution was 14.718 bp. The sample was sequenced using P6-C4 Polymerase – Chemistry combination, magbead loading, stage start and a data acquisition time of ≥ 4 hrs. The total amount of sequenced material was 1,724Gb of raw data. The resulting reads were assembled de novo using HGAP3.0 approach (SMRT analysis version: 2.3.0, patch #4) with the parameters by default and with the Minimum Seed Read Length set at 6000 bp. Further analysis aimed to circularize the contigs derived from the assemblies was done using the publicly available tool Circlator based on the algorithm reported by Hunt et al. [17]. The assembled genome sequences were annotated using the Prokka annotation pipeline, version 1.11 [18]. This involves predicting tRNA, rRNA, and mRNA genes and signal peptides in the sequences using Aragorn, RNAmmer, Prodigal, and SignalP, respectively [19, 20, 21].

Genomic analysis of safety aspects of *Lactobacillus pentosus* MP-10. The predicted CDSs were annotated by using Blast against the CARD database of antibiotic resistance genes, ResFinder version 2.1(https://cge.cbs.dtu.dk//services/ResFinder/) for acquired antibiotic resistance genes and the MvirDB database of virulence factors (last version downloaded on 10th of January, 2016), and the associated GO terms were obtained by using Swiss-Prot database. Furthermore, we used the Resistance Gene Identifier (RGI) (as part of for CARD tools) software prediction of resistome from protein or nucleotide data based on homology and SNP models.

Nucleotide sequence accession numbers. The complete genome sequence of *Lb. pentosus* MP-10 has been deposited at the EMBL Nucleotide Sequence Database (project no. xxxxx) under accession number xxx.

Results and Discussion

General genomic features of *Lactobacillus pentosus* MP-10. The *Lb. pentosus* MP-10 whole-genome sequence was obtained using a strategy of Pac Bio RSII sequencing technology. The single circular chromosome was of 3,698,214 bp (6 contigs) with an estimated G+C content of 46.32% and 5 plasmids of 30-50 kb (Figure 1). However, the previous draft genome obtained by pyrosequencing technology (GS FLX Titanium system; 454 Life Sciences) revealed the presence of a circular 3,835,873-bp chromosome (108 contigs) and 3 plasmids (pLP-MP10-1 [53 kb], pLP-MP10-2 [35 kb], and pLP-MP10-3 [18 kb]) [16]. The complete and new annotated genome sequence revealed the presence of 3558 ORFs (2971 canonical and 587 Non-Canonical) and 87 structural RNAs (sRNAs) (16 rRNA and 71 tRNA) (Figure 1). The genome sequence of *Lb. pentosus* MP-10 is considered the largest genome among lactobacilli up to date which may reflect the ecological flexibility of this bacterium via metabolic diversity and life-style adaptability as a result of bacterial evolution (gene duplication and horizontal gene transfer “HGT”). We also identified the presence of two CRISPR (clustered regularly interspaced short palindromic repeat) clusters (Type I and Type II) that represent an acquired immune system providing protection against mobile genetic elements (viruses, transposable elements and conjugative plasmids) [22]. The CRISPR associated protein responsible genes (*cas* genes) were detected such as *cas1*, *cas2*, *cas3* and *cas9* genes similar to those of *Streptococcus thermophilus* (Table 1). Furthermore, the coding genes for CRISPR-associated endonucleases Cas1 and Cas2 (*ygbT* and *ygbF* genes), CRISPR system Cascade subunit CasC (*casC* gene) and CRISPR system Cascade subunit CasD (XX999_01592 gene ID of *Lb. pentosus* MP-10) from *Escherichia coli* were detected in *Lb. pentosus* MP-10 chromosome (Table 1). The gene coding for CRISPR-associated endoribonuclease Cse3 in *Thermus thermophilus* was found in *Lb. pentosus* MP-10 chromosome (*cse3* gene). It is noteworthy to highlight that we found two new genes coding for CRISPR-associated protein Cse1 (XX999_01589 gene ID; *cse1_Lpe* gene) and CRISPR-associated protein Cse2 (XX999_0159 gene ID; cse2_Lpe gene) as revealed by the search in the Pfam database and we called them *cse1_Lpe* and *cse2_Lpe* genes belonging to Subtype I-E (Table 1). These data gave new insights into the evolution of bacterial resistance against mobile elements in *Lactobacillus* spp. since *Lb. pentosus* MP-10 possessed a multiple and a variety of CRISPR elements including two new genes *cse1_Lpe* and *cse2_Lpe* as a defense mechanism against foreign genetic elements. This fact is of great relevance for the application of *Lb. pentosus* MP-10 not only as a promising probiotic but also as starter culture at industrial scale.

We also identified 29 transposase related-genes highly represented by 17 Transposase DDE domain proteins, nine transposases (three transposases, two putative transposases, transposase from transposon *Tn916*, two transposase IS200 like proteins and IS2 transposase *TnpB*) and three Transposase Mutator family coding genes. The number of transposase genes present in *Lb. pentosus* MP-10 (9 genes) was lower than other lactobacilli strains such as *Lb. pentosus* KCA1 (25 genes) [23] and *Lb. acidophilus* NCFM (18 genes) [24] which may suggest that insertion element-mediated genome diversification is less frequent in *Lb. pentosus* strains from vegetable environment (Table 2).

***In silico* analysis of safety properties of *Lb. pentosus* MP-10.** To generate genomic insights into the safety aspects of a potential probiotic *Lb. pentosus* MP-10, we surveyed the genes related with antibiotic resistance and virulence factors in the *Lb. pentosus* MP-10 genome.

Antibiotic resistance. Genomic screening for antibiotic resistance genes by using the perfect and strict algorithms in the Resistance Gene Identifier (RGI in the CARD database) revealed that *Lb. pentosus* MP-10 contained resistance genes for several antibiotics such as aminocoumarin, beta-lactam, fluoroquinolone, lincosamide, macrolide, phenicol (including chloramphenicol), rifampin and tetracycline being in most cases antibiotic efflux genes (Figures 2 and 3). In this sense, antibiotic resistance genes for multidrug efflux pumps such as *emeA, lmrD, macB, mepA, arlR* and *fexA* were detected in *Lb. pentosus* MP-10 genome being able to export macrolides (*macB* gene), tetracyclines (*mepA* gene), lincosamides (*lmrD* gene), fluoroquinolones (*arlR* gene and mutation in *gyrB* gene) and phenicols including chloramphenicol (*fexA* gene) (Figure 3). However, previous phenotypic analysis of antibiotic susceptibility of *Lb. pentosus* MP-10 [25] revealed that this strain was sensitive to ampicillin, chloramphenicol, erythromycin and tetracycline. This data can be explained by the fact that the efflux pump genes were not functional under the experimental conditions tested. Furthermore, *PBP1a* gene coding for resistance to beta-lactams and also *mprF* gene mediating antibiotic resistance via aminoacylation and flipping of membrane phospholipids [26] were identified (Figure 3). On the other hand, mutation in *rpoB* and *gyrB* genes conferring resistance to rifampin and fluoroquinolone+aminocoumarin, respectively were found in *Lb. pentosus* MP-10 genome and they were similar to *S.*

aureus rpoB mutants conferring resistance to rifampicin and *Mycobacterium leprae gyrB* conferring resistance to fluoroquinolone (Figure 3).

On the other hand, ResFinder did not detect any acquired antibiotic resistance gene for aminoglycoside, beta-lactam, Colistin, fluoroquinolone, fosfomycin, fusidic acid, MLS-Macrolide, lincosamide and streptogramin B, nitroimidazole, oxazolidinone, phenicol, rifampicin, sulphonamide, trimethoprim, tetracycline and glycopeptide (data not shown).

Antibiotic resistance analysis *in silico* showed the absence of acquired antibiotic resistance genes and that most of the resistance genes are antibiotic efflux genes or resulted from mutation of chromosomal genes.

Virulence. Regarding virulence, alignment of the predicted CDSs against the MvirDB database of virulence factors revealed the presence of Lp2 and Lp3 prophage proteins and alanine racemase from *Lb. plantarum* WCFS1, besides the surface located protein from *Lb. rhamnosus* (>90% identity; Table 3). In this sense, *Lb. pentosus* MP-10 chromosome revealed the presence of Lp3 prophage elements which included DNA packaging genes (encoding small and large terminase, portal protein), head-tail genes (head-to-tail joining) and helicase. Furthermore, several proteins of unknown functions of Lp2 and Lp3 prophages from *Lb. plantarum* WCFS1 were also detected (Table 3). Comparing Lp3 prophage region of *Lb. pentosus* MP-10 and *Lb. plantarum* WCFS1, we observed a strong synteny between prophages from two distinct species of *Lactobacillus* being the comparison only done with proteins with >90% identity (Figure 3). In this case, 9 homologous proteins were shared although both species occupy different ecological niches: human saliva or olives [16, 27]. Those data suggested that different species colonizing different ecosystems may share the same prophages and also their architecture due probably to the interconnection between different habitats via lateral genetic exchange. Similar results were reported by Zhang et al. [28] for other lactobacilli. However, *Lb. pentosus* MP-10 chromosome screening for phage integrases as markers for mobile DNA elements such as prophages determined the presence of two integrases [Putative prophage phiRv2 (XX999_00126) and Phage integrase family protein (XX999_03519)], and also several integrase core domain proteins being all of them located not adjacent to the prophage like-region, thus we can deduce that they are not involved in prophage mobility.

Conclusions. The new annotated genome sequence of *Lb. pentosus* MP-10 is considered the largest genome among lactobacilli up to date which may reflect the ecological flexibility and adaptability of this bacterium. *In silico* analysis of safety properties of *Lb. pentosus* MP-10 revealed the absence of acquired antibiotic resistance genes and that most of the resistance genes are antibiotic efflux genes or resulted from mutation of chromosomal genes. Regarding virulence, we did not find any virulence factor. Thus, we can suggest that *Lb. pentosus* MP-10 could be considered as safe, although additional analysis of its functionality should be carried out.

References

1. Tamang JP, Kailasapathy K: **Fermented foods and beverages of the world.** 2010, Boca Raton, USA: CRC Press, 149–190.
2. Venema K, Meijerink M: **Lactobacilli as probiotics: discovering new functional aspects and target sites.** Probiotics and Prebiotics: Current Research and Future Trends. Edited by: Venema K, do Carmo AP. 2015, Caister Academic Press, UK, 29-42.
3. Food and Agricultural Organization of the United Nations and World Health Organization. Joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food. 2002.
4. Granato D, Branco GF, Nazzaro F, Cruz AG, Faria JAF: **Functional foods and non dairy probiotic food development: trends, concepts, and products.** *Compr Rev Food Sci Food Saf* 2010, 9:292–302.
5. Peres CM, Hernandez-Mendoza A, Peres C, Malcata FX: **Review on fermented plant materials as carriers and sources of potentially probiotic lactic acid bacteria with an emphasis on table olives.** *Trends Food Sci Technol* 2012, 26(1):31-42.
6. Ranadheera RDCS, Baines SK, Adams MC: **Importance of food in probiotic efficacy.** *Food Res Int* 2010, 43, 1–7.
7. Cammarota M, De Rosa M, Stellavato A, Lamberti M, Marzaioli I, Giuliano M: ***In vitro* evaluation of *Lactobacillus plantarum* DSMZ 12028 as a probiotic: emphasis on innate immunity.** Int J Food Microbiol 2009, 135:90–98.
8. Chang J-H, Shim YY, Cha S-K, Chee KM: **Probiotic characteristics of lactic acid bacteria isolated from kimchi.** *J Appl Microbiol* 2010, 109:220–230.
9. Spurr HW: **The microbial ecology of fruit and vegetable surfaces, its relationship to postharvest biocontrol.** Biological Control of Postharvest Diseases: Theory and Practice. Edited by: Wilson C, Wisniewski M. 1994, CRC Press, Boca Raton, FL, 11-23.
10. Di Cagno R, Cardinali G, Minervini G, Antonielli L, Rizzello CG, Ricciuti P, Gobbetti M: **Taxonomic structure of the yeasts and lactic acid bacteria microbiota of pineapple (*Ananas comosus* L. Merr.) and use of autochthonous starters for minimally processing.** *Food Microbiol* 2010, 27:381-389.

11. Bartkiene E, Vidmantiene D, Juodeikiene G, Viskelis P, Urbonaviciene D: **Lactic acid fermentation of tomato: effects on cis/trans lycopene isomer and β-carotene concentration and formation of L(+) and D(-)-lactic acid.** *Food Techn Biotechnol* 2013, 51(4):471-478.
12. Swain MR, Anandharaj M, Ray RC, Rani RP: *Fermented fruits and vegetables of Asia: A potential source of probiotics. Biotechnol Res Int* 2014, 2014:250424.
13. Shanahan F: **A commentary on the safety of probiotics.** *Gastroenterol Clin North Am* 2012, 41(4):869-76.
14. EFSA (European Food Safety Authority): **Scientific Opinion of the Panel on Biological Hazards on the maintenance of the list of QPS microorganisms intentionally added to food or feed.** *The EFSA J* 2008, 923:1-48.
15. Ventura M, O'Flaherty S, Claesson MJ, Turroni F, Klaenhammer TR, van Sinderen D, O'Toole PW: **Genome-scale analyses of health-promoting bacteria: probiogenomics.** *Nat Rev Microbiol* 2009, 7:61–71.
16. Abriouel H, Benomar N, Pérez Pulido R, Martínez Cañamero M, Gálvez A: **Annotated genome sequence of Lactobacillus pentosus MP-10 with probiotic otential from naturally-fermented Alorena green table olives.** *J Bacteriol* 2011, 193:4559-4560.
17. Hunt M, De Silva N, Otto TD, Parkhill J, Keane JA, Harris SR: **Circlator: automated circularization of genome assemblies using long sequencing reads.** *Genome Biol* 2015, 16(1):294.
18. Seemann T: **Prokka: rapid prokaryotic genome annotation.** *Bioinformatics* 2014, 30(14):2068-9.
19. Hyatt D, Chen G-L, LoCascio PF, Land ML, Larimer FW, Hauser LJ: **Prodigal: prokaryotic gene recognition and translation initiation site identification.** *BMC Bioinformatics* 2010, 11:119.
20. Lagesen K, Hallin P, Rødland EA, Stærfeldt H-H, Rognes T, Ussery DW: **RNAmmer: consistent and rapid annotation of ribosomal RNA genes.** *Nucleic Acids Res* 2007, 35:3100-31008.
21. Laslett D, Canback B: **ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences.** *Nucleic Acids Res* 2004, 32:11-16.
22. Horvath P, Barrangou R: **CRISPR/Cas, the immune system of bacteria and archaea.** *Science* 2010, 327:167–170.

23. Anukam KC, Macklaim JM, Gloor GB, Reid G, Boekhorst J, Renckens B, van Hijum SA, Siezen RJ: **Genome Sequence of *Lactobacillus pentosus* KCA1: Vaginal Isolate from a Healthy Premenopausal Woman.** *PLoS ONE* 2013, 8(3):E59239.

24. Altermann E, Russell WM, Azcarate-Peril MA, Barrangou R, Buck BL, McAuliffe O, Souther N, Dobson A, Duong T, Callanan M, Lick S, Hamrick A, Cano R, Klaenhammer TR: **Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM.** *Proc Natl Acad Sci U S A* 2005, 102(11):3906-3912.

25. Casado Muñoz MC, Benomar N, Ennahar S, Horvatovich P, Lavilla Lerma L, Knapp CW, Gálvez A, Abriouel H: **Comparative proteomic analysis of a potentially probiotic *Lactobacillus pentosus* MP-10 for the identification of key proteins involved in antibiotic resistance and biocide tolerance.** *Int J Food Microbiol* 2016, 222:8–15.

26. Ernst CM, Peschel A: **Broad-spectrum antimicrobial peptide resistance by MprF-mediated aminoacylation and flipping of phospholipids.** *Mol Microbiol* 2011, 80:290–299.

27. Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R, Tarchini R, Peters SA, Sandbrink HM, Fiers MW, Stiekema W, Lankhorst RM, Bron PA, Hoffer SM, Groot MN, Kerkhoven R, de Vries M, Ursing B, de Vos WM, Siezen RJ: **Complete genome sequence of *Lactobacillus plantarum* WCFS1.** *Proc Natl Acad Sci U S A* 2003, 100(4):1990-5.

28. Zhang W-Y, Yu D-L, Sun Z-H, Chen W, Hu S-N, Meng H, Zhang H-P: **The comparative analysis of a prophage remnant Lcazh1 in relation to other *Lactobacillus* prophages, particularly Lp3.** *Int J Dairy Technol* 2010, 63:413-417.

Figure legends

Figure 1. Circular representation of the *Lactobacillus pentosus* MP-10 chromosome. The circles from outside to inside are the annotated CDS elements in forward orientation, the annotated CDS elements in the revers orientation, several COG functions, the structural RNA, the GC content and the GC screw.

Figure 2. Analysis of the whole genome of *Lactobacillus pentosus* MP-10 by using the perfect and strict algorithms in the Resistance Gene Identifier (RGI) with overall resistance in the center, resistance classes in the middle, and individual resistance genes on the outer (open reading frames).

Figure 3. Heat-map analysis of antibiotic resistance showing the unique distribution profiles of antibiotic resistance genes in *Lb. pentosus* MP-10 (black: no sequence matching the protein; purple: strict match to known antimicrobial resistance (AMR) gene sequence).

Table 1. Characterization of CRISPR elements detected in *Lactobacillus pentosus* MP-10 genome.

Gene ID	Gene	Gen length (bp)	Protein description	UniProt Reference Clusters (UniRef)	Predicted functions
XX999_00242	*cas9*	4077	CRISPR-associated endonuclease Cas9	UniRef100:G3ECR1	DNA and RNA binding; endonuclease activity; maintenance of CRISPR repeat elements; metal ion binding; defense response to virus
XX999_00243	*cas1*	906	CRISPR-associated endonuclease Cas1	UniRef100:G3ECR2	DNA binding; endonuclease activity; maintenance of CRISPR repeat elements; metal ion binding; defense response to virus
XX999_00244	*cas2*	306	CRISPR-associated endoribonuclease Cas2	UniRef100:G3ECR	Endonuclease activity; maintenance of CRISPR repeat elements; metal ion binding; defense response to virus
XX999_01586	*ygbT*	954	CRISPR-associated endonuclease Cas1	UniRef100:Q46896	DNA binding; cytoplasm; DNA repair; cellular response to DNA damage stimulus; crossover junction endodeoxyribonuclease activity; 5'-flap endonuclease activity; maintenance of CRISPR repeat elements; metal ion binding; defense response to virus
XX999_01587	*ygbF*	900	CRISPR-associated endoribonuclease Cas2	UniRef100:P45956	Endonuclease activity; maintenance of CRISPR repeat elements; defense response to virus
XX999_01588	*cas3*	2748	CRISPR-associated nuclease/helicase Cas3	UniRef100:F2XG53	Nucleic acid binding; helicase activity; deoxyribonuclease activity; ATP binding; DNA metabolic process; metal ion binding
XX999_01589	*cse1_Lpe**	1752	CRISPR-associated protein Cse1 (CRISPR_cse1)	-	-
XX999_01590	*cse2_Lpe**	612	CRISPR-associated protein Cse2 (CRISPR_cse2)	-	-
XX999_01591	*casC*	1080	CRISPR system Cascade subunit CasC	UniRef100:Q46899	RNA binding; protein complex; defense response to virus
XX999_01592	*casD*	726	CRISPR-associated protein (Cas_Cas5)	UniRef100:Q46898	RNA binding; protein complex; defense response to virus
XX999_01593	*cse3*	669	CRISPR-associated endoribonuclease Cse3	UniRef100:Q53WG9	RNA binding; endonuclease activity; defense response to virus

*: New genes founds in this study.

Table 2. Characterization of transposase elements detected in *Lactobacillus pentosus* MP-10 genome.

Gene ID	Gene	Gen length (bp)	Protein description	Protein family
XX999_00061	XX999_00061	252	Transposase	Pfam:PF01527.14
XX999_00069	XX999_00069	582	Transposase, Mutator family	Pfam:PF00872.12
XX999_00112	XX999_00112	504	Transposase DDE domain protein	Pfam:PF01609.15
XX999_00245	XX999_00245	504	Transposase DDE domain protein	Pfam:PF01609.15
XX999_00337	XX999_00337	732	IS2 transposase *TnpB*	CLUSTERS:PRK09409
XX999_00400	XX999_00400	504	Transposase DDE domain protein	Pfam:PF01609.15
XX999_00407	XX999_00407	372	Transposase DDE domain protein	Pfam:PF01609.15
XX999_00611	XX999_00611	504	Transposase DDE domain protein	Pfam:PF01609.15
XX999_00680	*Int-Tn*	1158	Transposase from transposon *Tn916*	UniProtKB:P22886
XX999_01017	XX999_01017	198	Transposase	Pfam:PF01527.14
XX999_01502	XX999_01502	297	Transposase DDE domain protein	Pfam:PF01609.15
XX999_01619	XX999_01619	504	Transposase DDE domain protein	Pfam:PF01609.15
XX999_01924	XX999_01924	1269	Putative transposase	Pfam:PF01385.13
XX999_01925	XX999_01925	441	Transposase IS200 like protein	Pfam:PF01797.10
XX999_02663	XX999_02663	1140	Putative transposase DNA-binding domain protein	Pfam:PF07282.5
XX999_02664	XX999_02664	453	Transposase IS200 like protein	Pfam:PF01797.10
XX999_02834	XX999_02834	297	Transposase DDE domain protein	Pfam:PF01609.15
XX999_02924	XX999_02924	297	Transposase DDE domain protein	Pfam:PF01609.15
XX999_02993	XX999_02993	504	Transposase DDE domain protein	Pfam:PF01609.15
XX999_03221	XX999_03221	372	Transposase DDE domain protein	Pfam:PF01609.15
XX999_03439	XX999_03439	372	Transposase DDE domain protein	Pfam:PF01609.15
XX999_03498	XX999_03498	372	Transposase DDE domain protein	Pfam:PF01609.15
XX999_03585	XX999_03585	504	Transposase DDE domain protein	Pfam:PF01609.15
XX999_03610	XX999_03610	591	Transposase, Mutator family	Pfam:PF00872.12
XX999_03614	XX999_03614	1368	Transposase DDE domain protein	Pfam:PF01609.15
XX999_03618	XX999_03618	504	Transposase DDE domain protein	Pfam:PF01609.15
XX999_03623	XX999_03623	1176	Transposase, Mutator family	Pfam:PF00872.12
XX999_03627	XX999_03627	297	Transposase DDE domain protein	Pfam:PF01609.15
XX999_03642	XX999_03642	252	Transposase	Pfam:PF01527.14

Table 3. Characterization of virulence determinants detected in *Lactobacillus pentosus* MP-10 genome against the MvirDB database of virulence factors.

Gene ID	Identity (%)	Query protein length	Gene product		
			Description	**Protein length**	**Accession**
XX999_00145	92.08	101	Prophage Lp3 protein 21	101	NP_785913
XX999_00131	92.48	266	Prophage Lp3 protein 7	266	NP_785925
XX999_00596	92.53	375	Alanine racemase	375	UniProtKB - O08445
XX999_02401	92.68	127	Prophage Lp2 protein 24	126	NP_785890.1
XX999_00135	93.65	63	Prophage Lp3 protein 10	63	NP_785922.1
XX999_00137	93.80	129	Prophage Lp3 protein 12	129	NP_785920.1
XX999_02409	95.05	101	Prophage Lp2 protein 12	101	NP_785900.1
XX999_02999	95.48	155	Surface located protein	155	UniProtKB - O30701
XX999_01408	95.83	170	Prophage Lp2 protein 16	169	NP_785897.1
XX999_02421	96.00	138	Prophage Lp2 protein 8	127	NP_785904.1
XX999_00141	96.72	368	Prophage Lp3 protein 17 portal protein	366	NP_785917.1
XX999_00138	96.82	157	Prophage Lp3 protein 14 terminase small subunit	157	NP_785919.1
XX999_00132	96.98	464	Prophage Lp3 protein 8 helicase	464	NP_785924.1
XX999_00139	97.53	567	Prophage Lp3 protein 15 terminase large subunit	567	NP_785918.1
XX999_00143	97.70	89	Prophage Lp3 protein 19 head-to-tail joining	89	NP_785915.1
XX999_02397	99.34	152	Prophage Lp2 protein 31	153	NP_785883.1

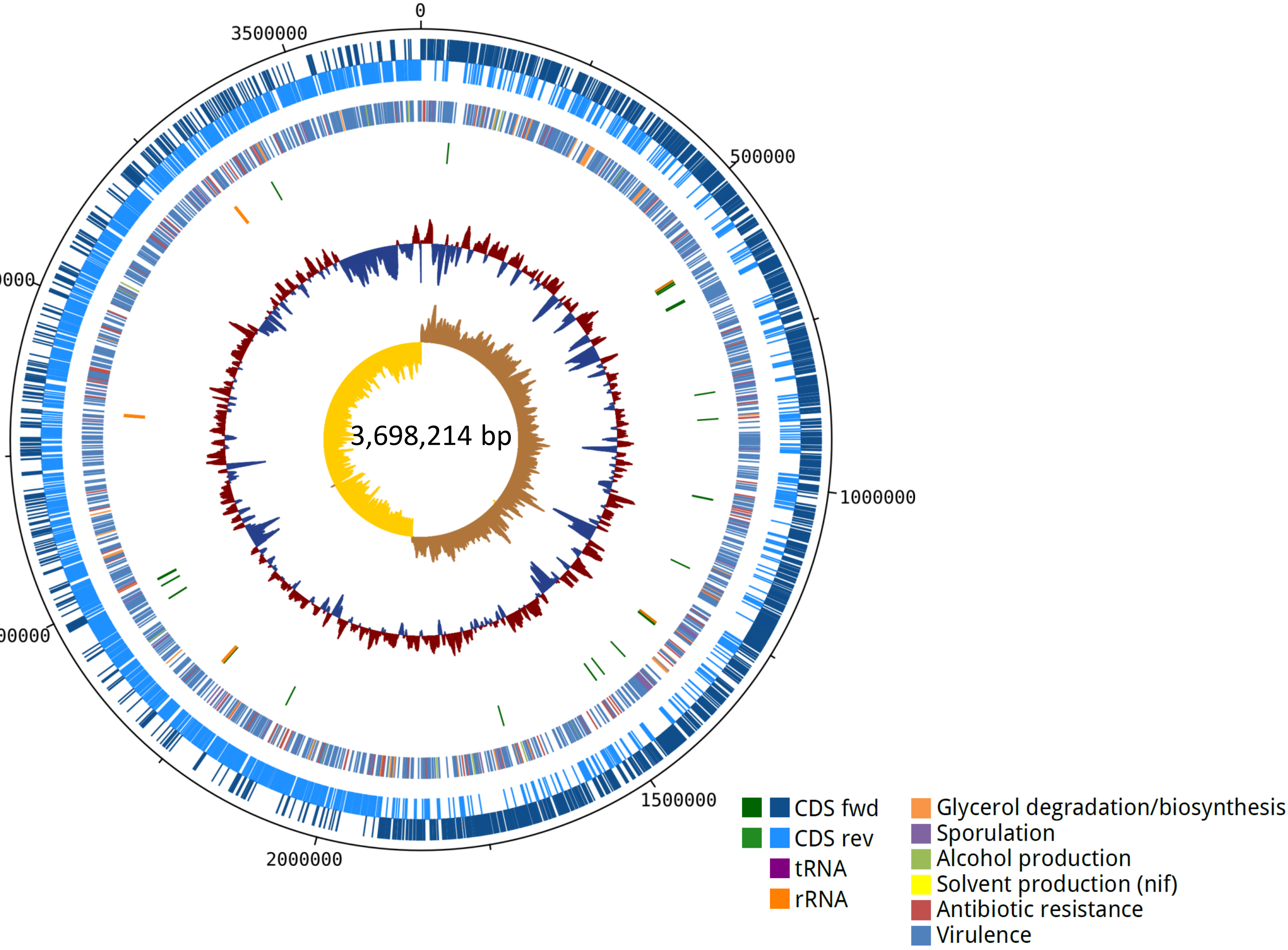

0
500000
1000000
1500000
2000000
2500000
3000000
3500000
3,698,214 bp
CDS fwd
CDS rev
tRNA
rRNA
Glycerol degradation/biosynthesis
Sporulation
Alcohol production
Solvent production (nif)
Antibiotic resistance
Virulence

Figure 1

Abriouel et al. 2016

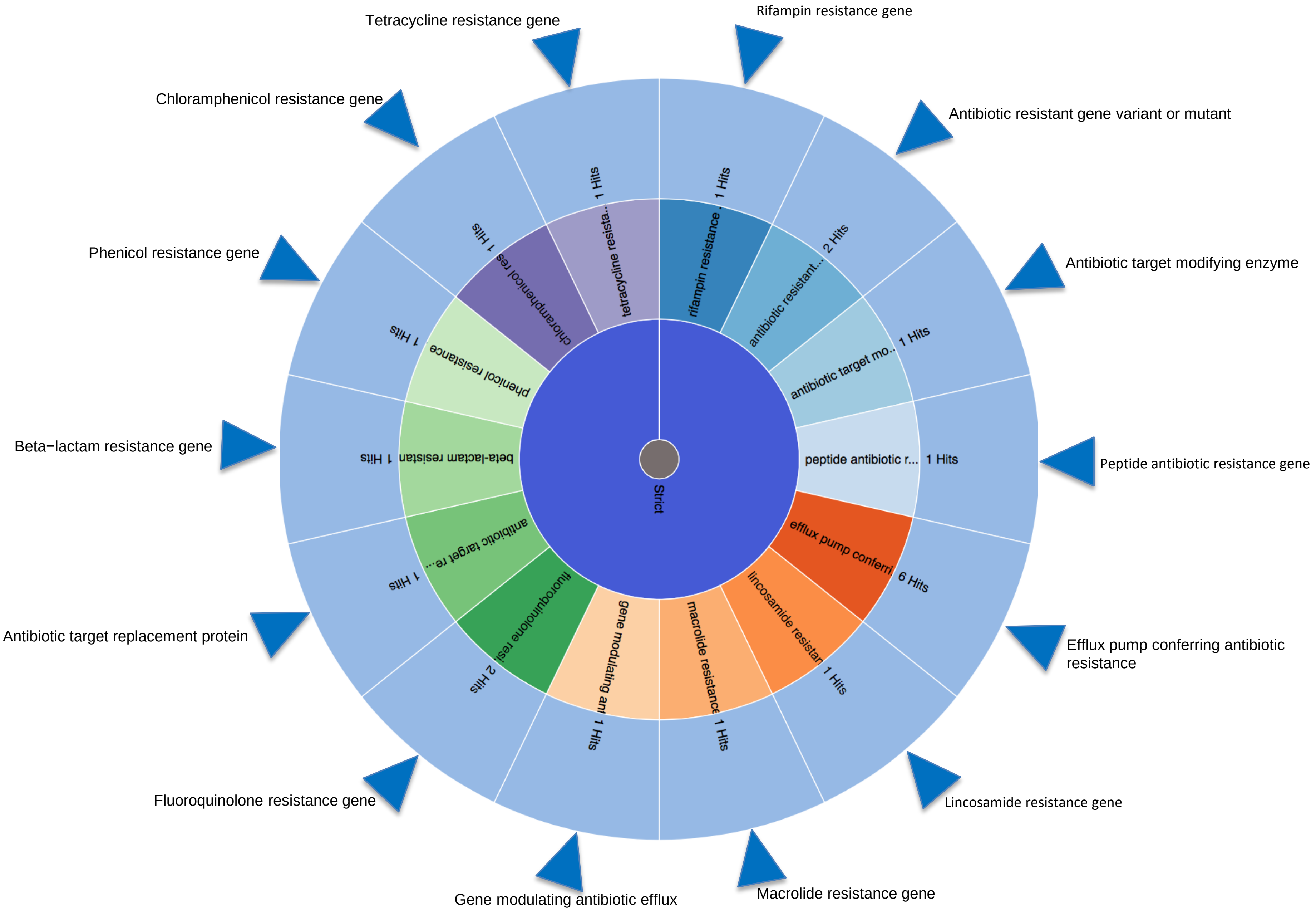

Tetracycline resistance gene
Rifampin resistance gene
Chloramphenicol resistance gene
Antibiotic resistant gene variant or mutant
Phenicol resistance gene
Antibiotic target modifying enzyme
Beta−lactam resistance gene
Peptide antibiotic resistance gene
Antibiotic target replacement protein
Efflux pump conferring antibiotic resistance
Fluoroquinolone resistance gene
Lincosamide resistance gene
Gene modulating antibiotic efflux
Macrolide resistance gene
Strict
tetracycline resista... 1 Hits
rifampin resistance 1 Hits
antibiotic resistant... 2 Hits
antibiotic target mo... 1 Hits
peptide antibiotic r... 1 Hits
efflux pump conferri 6 Hits
lincosamide resistan 1 Hits
macrolide resistance 1 Hits
gene modulating ant 1 Hits
fluoroquinolone resi 2 Hits
antibiotic target re... 1 Hits
beta-lactam resistan 1 Hits
phenicol resistance 1 Hits
chloramphenicol res 1 Hits

Figure 2

Abriouel et al. 2016

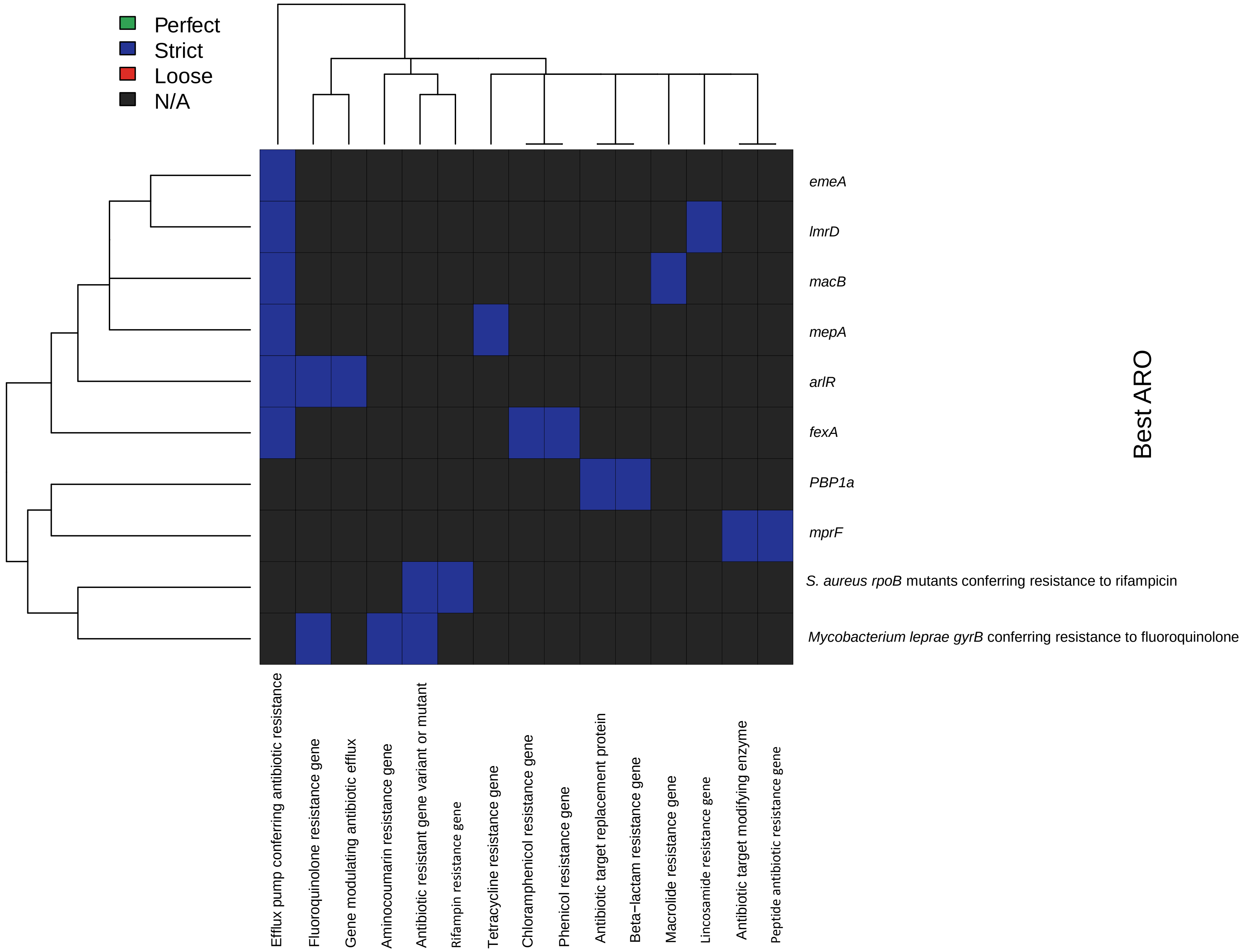

Perfect
Strict
Loose
N/A
emeA
lmrD
macB
mepA
arlR
fexA
PBP1a
mprF
S. aureus rpoB mutants conferring resistance to rifampicin
Mycobacterium leprae gyrB conferring resistance to fluoroquinolone
Best ARO
Efflux pump conferring antibiotic resistance
Fluoroquinolone resistance gene
Gene modulating antibiotic efflux
Aminocoumarin resistance gene
Antibiotic resistant gene variant or mutant
Rifampin resistance gene
Tetracycline resistance gene
Chloramphenicol resistance gene
Phenicol resistance gene
Antibiotic target replacement protein
Beta−lactam resistance gene
Macrolide resistance gene
Lincosamide resistance gene
Antibiotic target modifying enzyme
Peptide antibiotic resistance gene

Figure 3

Abriouel et al. 2016

Discusión general

En las últimas décadas, la seguridad de los probióticos ha despertado un gran interés dentro de la comunidad científica en general y las organizaciones internacionales de la Seguridad Alimentaria/Salud en particular (EFSA, FAO/WHO) que propusieron las pautas necesarias para evaluar la inocuidad y la seguridad de los mismos antes de su aprobación como probióticos. Se ha comprobado que dicha seguridad está altamente ligada a la cepa en cuestión aunque la especie a la que pertenece la cepa tenga un historial de uso seguro, además las últimas evidencias científicas apuntan que muchos rasgos de seguridad son cepa-dependientes. En este contexto, el análisis de la seguridad debe tener en cuenta la naturaleza del microorganismo que se va a utilizar como probiótico (la taxonomía correcta), el método de administración, los niveles de exposición, el estado de salud de los beneficiarios y las funciones fisiológicas a desempeñar por dichos probióticos (Sanders *et al*., 2010). Para ello, el análisis de la seguridad de los microorganismos probióticos está basado en la investigación en profundidad de la presencia de actividades metabólicas perjudiciales, los genes de resistencia a antibióticos transferibles y la patogenicidad de dichas cepas.

La presencia de genes de resistencia transferibles en las cepas probióticas puede presentar un riesgo de transferencia de dichos genes a la microbiota intestinal una vez ingeridos los probióticos, por lo tanto dichos probióticos pueden actuar como reservorios de genes de resistencia a antimicrobianos. La transferencia *in vivo* de genes de resistencia a microorganismos intestinales potencialmente patógenos (comensales o transitorios) es un proceso muy complejo y difícil de demostrar a nivel práctico, sin embargo existen evidencias científicas que han demostrado que por ejemplo el gen *vanA* de resistencia a vancomicina ha sido transferido *in vivo* en un modelo animal de una cepa de *Enterococcus* a una cepa de *Lactobacillus acidophilus*, lo cual confirma que la transferencia de genes puede tener lugar *in vivo* (Mater *et al*., 2008). Esta resistencia adquirida por la adquisición de nuevos genes de resistencia transferibles debe ser diferenciada de la resistencia intrínseca que es inherente al rango taxonómico al cual pertenece la cepa (especie o género) y en el cual el riesgo de transferencia a otros microorganismos es muy bajo. Para ello, en esta memoria hemos analizado diferentes aspectos de seguridad de bacterias lácticas con potencial probiótico aisladas de la fermentación natural de la aceituna Aloreña, en este contexto la resistencia a antibióticos y biocidas ha sido explorada tanto a nivel fenotípico como genotípico. Además, el abordaje de la resistencia desde la perspectiva proteómica y genómica de una cepa probiótica seleccionada se llevó acabo para abarcar más información sobre los mecanismos de resistencia en un ecosistema poco explorado como es el caso de una fermentación espontánea de las aceitunas de mesa.

El uso indiscriminado de antimicrobianos, como agentes terapéuticos y como desinfectantes, puede contribuir al desarrollo de resistencias a estos antimicrobianos (Dixon, 2000; Hawkey, 2008; McBain *et al.,* 2002; Davin-Regli y Pagès, 2012). En las últimas décadas, el uso y sobre todo el abuso de los antibióticos para el tratamiento de las infecciones bacterianas, en ganadería y agricultura (Wegener, 2003), ha tenido como consecuencia un incremento en el número de resistencias bacterianas a los antibióticos modernos y por lo tanto fallos en la terapia y también otras consecuencias a nivel ecológico y evolutivo (Gillings, 2013).

Las bacterias resistentes a antibióticos representan un gran desafío para la industria alimentaria, especialmente las BAL, aisladas de alimentos como carne o productos lácteos fermentados (Maietti *et al.,* 2007; Ouoba *et al.,* 2008; Nawaz *et al.,* 2010; Toomey *et al.,* 2010). Las BAL se utilizan tradicionalmente como cultivos iniciadores de alimentos fermentados y como probióticos, donde pueden actuar como reservorios de genes de resistencia a antibióticos, similares a los encontrados en bacterias patógenas humanas (Flórez *et al.,* 2005), estos genes son potencialmente transferibles a otros microorganismos patógenos de la matriz alimentaria o del tracto gastrointestinal (Mathur y Singh, 2005).

En el presente estudio, hemos evaluado los perfiles de susceptibilidad de las BAL, aisladas de la fermentación natural y tradicional de la aceituna verde de mesa Aloreña, a diferentes antibióticos. Aunque las BAL de este alimento no fueron probablemente expuestas a ningún antibiótico y por lo tanto, *a priori,* no se esperaba que albergasen genes de resistencia transferibles, no se puede suponer que dichas bacterias están totalmente libres de resistencia a antibióticos y de sus genes transferibles. Por lo tanto, su estatus QPS (EFSA, 2004) debe ser confirmado con el fin de usarlas como cultivos iniciadores según las recomendaciones de la Autoridad Europea de Seguridad Alimentaria "EFSA" (EFSA, 2012).

Los perfiles de susceptibilidad a antibióticos de las cepas de *Lactobacillus pentosus* y *Leuconostoc pseudomesenteroides* aisladas de la fermentación de la aceituna Aloreña mostraron que las cepas de ambas especies son en general bastante sensibles a antibióticos clínicamente relevantes tales como ampicilina, amoxicilina, eritromicina, cloranfenicol y gentamicina. En general, los lactobacilos y los leuconostocs son susceptibles a los antibióticos que inhiben la síntesis proteica tales como eritromicina, cloranfenicol, clindamicina y tetraciclina (Flórez *et al.,* 2005; Ammor *et al.,* 2007). Sin embargo, algunas cepas de *Lb. pentosus* fueron susceptibles a tetraciclina y clindamicina, mientras que el 46% de las cepas de *Lc. pseudomesenteroides* fueron fenotípicamente resistentes a estos antibióticos. En ambos casos, la distribución bimodal de las MICs sugiere que algunas cepas de *Lc. pseudomesenteroides* poseían determinantes de resistencia adquirida a clindamicina y tetraciclina. Sin embargo, la ausencia de los determinantes de resistencia [*tet(M), tet(O), tet(S), tet(W), tet(L)* y *tet(K)*] puede sugerir un nuevo mecanismo de resistencia a tetraciclina que ocurre como consecuencia de la presencia de genes de resistencia adquiridos o bien de la mutación de genes intrínsecos (EFSA, 2012). El mecanismo de resistencia común a eritromicina y clindamicina [el fenotipo macrólido-lincosamida-estreptogramina (MLS)] no fue responsable de la resistencia observada a clindamicina en los leuconostocs resistentes, ya que los genes *erm, msrA/B, mphA* o *mefA* no han sido detectados. Además, el gen *lsa* no ha sido relacionado con el fenotipo resistente a clindamicina en *Lc. pseudomesenteroides* puesto que este gen no fue detectado en ninguna cepa resistente a dicho antibiótico.

Los altos niveles de resistencia a teicoplanina y vancomicina (MICs >128 μg/ml) exhibidos por la mayoría de las cepas de *Lb. pentosus* (71-80%) y por todas las cepas de *Lc. pseudomensenteroides* (100%) concuerdan con los datos de resistencia intrínseca descrita en las BAL a estos antibióticos (Danielsen y Wind, 2003; Ammor *et al.,* 2007; Liu *et al.,* 2009). La resistencia a glicopéptidos de *Lb. pentosus* y *Lc. pseudomesenteroides* se debe probablemente a la presencia de *D*-Ala-*D*-lactato en su peptidoglicano en lugar del dipéptido *D*-Ala-*D*-Ala normalmente presente en su pared celular (Deghorain *et al.,* 2007). Dicho rasgo está codificado a nivel cromosómico y no es un determinante transferible, lo cual podría explicar por qué en este estudio no hemos encontrado ningún gen de resistencia a vancomicina tales como son los genes transferibles *van* (*vanA, vanB, vanC* y *vanE*).

La resistencia intrínseca a aminoglucósidos tales como estreptomicina y kanamicina ha sido descrita como una característica inherente de los lactobacilos (Danielsen y Wind, 2003), que resulta como consecuencia de la impermeabilidad de su membrana (Elkins y Mullis, 2004). Los resultados obtenidos en este estudio mostraron que el 83% de las cepas de *Lb. pentosus* eran resistentes a estreptomicina (MIC > 256 µg/ml). Sin embargo, la mayoría de las cepas de *Lc. pseudomesenteroides* (85-100%) fueron resistentes a ambos aminoglucósidos. La ausencia de determinantes de resistencia a aminoglucósidos [*aad(E), aac(6′)-Ie-aph(2′)-Ia, aph(2′)-Ib, aph(2′)-Ic, aph(2′)-Id, aph(3′)-IIIa* y *ant(4′)-Ia*] y la distribución unimodal de las MICs observadas en el rango de concentraciones altas sugieren que estas cepas de *Lc. pseudomesenteroides* son intrínsecamente resistentes a los aminoglucósidos.

Por otra parte, la gentamicina fue muy efectiva inhibiendo el crecimiento de ambas especies en el rango de concentraciones más bajas (0,002-1 µg/ml), posiblemente porque este aminoglucósido ha sido capaz de atravesar la pared bacteriana con mayor facilidad que otros aminoglucósidos tales como la kanamicina y estreptomicina (Elkins y Mullis, 2004), obteniéndose así valores bajos de MICs para este antibiótico en ambas especies de BAL ensayadas.

Todas las cepas de *Lc. pseudomesenteroides* así como el 71% de las cepas de *Lb. pentosus* estudiadas se mostraron resistentes a trimetoprima/sulfametoxazol ya que exhibieron distribuciones bimodales o multimodales de las MICs para dichos antibióticos. Sin embargo, la trimetoprima exhibió una distribución bimodal de la MIC en un amplio rango de concentraciones, con un 76% de las cepas de *Lb. pentosus* y sólo un 15% de las cepas de *Lc. pseudomesenteroides* resistentes a este antibiótico. En ambos casos, ninguna de las cepas mostró amplificación con los cebadores *dfrA* y *dfrD* relacionados con la resistencia a trimetoprima. No obstante, la EFSA (EFSA, 2008) considera que la resistencia mostrada por las BAL a trimetoprima, trimetoprima/sulfametoxazol y estreptomicina no es relevante cuando se ensaya en el medio de cultivo LSM (Klare *et al.,* 2005) ya que los componentes de este medio interfieren en la actividad antibacteriana de estos antibióticos. Una pequeña proporción del caldo LSM (10% de MRS) contiene componentes antagonistas tales como el ácido p-aminobenzóico (contra el sulfametoxazol) y/o la timidina (contra trimetoprima) (Klare *et al.,* 2005; Turnidge y Bell, 2005).

La baja susceptibilidad mostrada por las cepas de *Lb. pentosus* (70% resistentes) a ciprofloxacino, exhibiendo una distribución multimodal de la MIC con tres subpoblaciones diferentes, indica un resistencia adquirida. Sin embargo, no hemos encontrado ninguna mutación de los genes que codifican GyrA o ParC en la región QRDR responsables de la resistencia a ciprofloxacino y que están asociados con secuencias de inserción (IS), integrones o transposones como ha sido descrito previamente (El Amin *et al.,* 1999; Hummel *et al.,* 2007; Petersen y Jensen, 2004), lo cual se trata de un mecanismo de resistencia intrínseco.

En cuanto a cefuroxima, las BAL son generalmente más resistentes a cefalosporinas de acuerdo con Danielsen y Wind (2003) y Coppola *et al.* (2005). La impermeabilidad de la pared celular es el mecanismo principal de resistencia a los inhibidores de síntesis de la pared celular, ya que las especies de las BAL carecen de citocromos en su cadena de transporte electrónico (Condon, 1983). Sin embargo, las diferencias fenotípicas observadas entre las cepas pueden ser el resultado de la cooperación de varios mecanismos de resistencia inespecíficos tales como bombas de exporte (Putman *et al.,* 2001) y los sistemas autolíticos defectuosos de la pared celular (Kim *et al.,* 1982). En este estudio, el 44% de las cepas de *Lb. pentosus* y el 85% de las cepas de *Lc. pseudomesenteroides* fueron resistentes a este antibiótico, pero ninguna de las cepas resistentes albergaron determinantes de resistencia adquirida (*bla* y *blaZ.*).

Las BAL aisladas de las aceitunas verdes de mesa Aloreña a lo largo del proceso de fermentación mostraron un perfil de multirresistencia. La correlación entre los diferentes parámetros (antibióticos y meses de fermentación) indicó que la prevalencia de la resistencia a antibióticos en las BAL dependía principalmente del fermentador donde ocurrió dicho proceso de fermentación, ya que las condiciones bióticas y abióticas fueron bastante diferentes (Abriouel *et al.,* 2011). Las aceitunas verdes se colocaron en tanques de 6000 litros en presencia de sal (6% peso/volumen) y 0.8% de ácido acético y a continuación se dejaron fermentar durante 4 a 7 meses a temperatura ambiente. Las resistencias a vancomicina y trimetoprima/sulfametoxazol fueron las más relevantes en las BAL durante todo el proceso de fermentación, independientemente del fermentador muestreado. Por otra parte, el análisis de componentes principales (PCA) reveló que existe una correlación entre el tercer mes de fermentación y la resistencia múltiple a antibióticos. La resistencia intrínseca de las BAL a varios antibióticos se puede atribuir parcialmente a la presencia de genes que codifican para bombas de exporte de múltiples drogas (MDRs), que expulsan al medio extracelular diferentes tipos de antibióticos así como ciertos compuestos químicos (colorantes, disolventes orgánicos, detergentes, biocidas y productos metabólicos). En este estudio, el gen *norA* que confiere resistencia a cloranfenicol y fluoroquinolonas (norfloxacina y ciprofloxacino) como ha sido descrito por varios autores (Neyfakh *et al.,* 1993; Truong-Bolduc *et al.,* 2003), se detectó en algunas cepas de las BAL tanto resistentes como sensibles a ciprofloxacino, sugieriendo así que *norA* puede jugar un papel diferente en ciertas BAL (especialmente en cepas sensibles a ciprofloxacino).

En cuanto al complejo tripartito AcrAB-TolC, implicado en la expulsión de beta-lactámicos, fluoroquinolonas, cloranfenicol y tetraciclina (Okusu *et al.,* 1996), sólo AcrA -proteína de fusión- fue detectada en ambas especies de BAL, mientras que AcrB -proteína transportadora de la membrana citoplásmica- y TolC -proteína de la membrana externa- no fueron detectados. La ausencia de AcrB y TolC, hipotéticamente, podría deberse a la mutación puntual en los genes correspondientes, lo cual hizo imposible su detección mediante PCR, o tal vez AcrA no desempeña ningún papel en la resistencia a los antibióticos en esta especie. Las bombas de exporte NorA y MdeA pertenecientes a la familia MFS (Major Facilitator Superfamily) y la bomba de exporte MepA (de la familia MATE) (Kaatz et al., 2005) codificadas a nivel cromosómico han sido detectadas en ambas especies de las BAL. Las bombas de exporte de multirresistencia se pueden encontrar en todas las especies bacterianas y pueden conferir una resistencia clínicamente relevante a los antibióticos, pero también se conoce su papel a nivel fisiológico (Piddock, 2006). En este estudio, las bombas de exporte detectadas pueden conferir un nivel bajo de resistencia a varios antibióticos, pero también pueden desempeñar un papel crucial en la supervivencia bacteriana en su nicho ecológico (alta concentración de sal, bajo pH, antimicrobianos tales como los compuestos fenólicos de las aceitunas, productos metabólicos).

Así, la mayoría de las cepas de *Lb. pentosus* (95%) y todas las cepas de *Lc. pseudomesenteroides* aislados de la fermentación natural de la aceituna verde de mesa Aloreña se pueden considerar seguras debido a la ausencia de determinantes de resistencia adquirida. La resistencia intrínseca a más de tres antibióticos no se convertirá en un problema a nivel clínico, ya que estas cepas fueron altamente sensibles a otros antibióticos clínicamente relevantes. En este estudio la ocurrencia de la multirresistencia intrínseca en ambas especies de las BAL fue debida en parte a las bombas de exporte codificadas a nivel cromosómico tales como NorA, MepA y MdeA.

Después de determinar que las BAL aisladas de la fermentación natural de las aceitunas verdes de mesa Aloreña fueron intrínsecamente multirresistentes a diferentes antibióticos, nos pareció interesante determinar la susceptibilidad de estas cepas a diferentes biocidas usados en la industria alimentaria. Para ello, hemos determinado en primer lugar los valores de corte ECOFF (epidemiological cut-off value for resistance) de los biocidas en ambas especies de las BAL (*Lb. pentosus* y *Lc. pseudomesenteroides*) y los resultados demostraron que dichas BAL fueron generalmente sensibles a los biocidas (las MICs están por debajo de los valores ECOFF), detectándose solamente el 2-15% de bacterias resistentes a hexaclorofeno (*Lc. pseudomesenteroides*) y a cetrimida y hexadecilpiridinio (*Lb. pentosus*).

Las BAL se enfrentan a diferentes tipos de estrés químico y ambiental en su hábitat natural, durante el procesamiento y almacenamiento de los alimentos, así como durante su paso a través del tracto gastrointestinal. Las respuestas adaptativas desarrolladas incluyen cambios en su fisiología, comportamiento y genética mediante distintos mecanismos de respuesta al estrés (Chung *et al.,* 2006; Foster, 2007; Ryall *et al.,* 2012). El cambio fenotípico es una de las estrategias adoptadas por las bacterias para garantizar su supervivencia y persistencia bajo diversas condiciones ambientales (Sousa *et al.,* 2011). En este sentido, las BAL desarrollaron varias estrategias de adaptación para sobrevivir bajo condiciones cambiantes y desfavorables (pH, sales, temperatura, concentración de nutrientes…etc), lo cual incluye la alteración de la fisiología celular mediante la expresión de genes de respuesta al estrés. Para entender y explicar el incremento de la resistencia de las BAL aisladas de la fermentación natural de las aceitunas verdes de mesa Aloreña bajo condiciones ambientales cambiantes hemos planteado el siguiente estudio. Los resultados obtenidos indicaron que el estrés inducido por los antimicrobianos (biocidas o antibióticos), compuestos químicos o la luz UV cambió el patrón de susceptibilidad a los antibióticos en las BAL analizadas. Sin embargo, a nivel genotípico, varios genes relacionados con el estrés fueron diferencialmente expresados, dependiendo de la especie y la cepa de las BAL y del tipo de estrés, aunque los patrones de inducción no fueron muy claros. La correlación entre las respuestas fenotípicas y genotípicas nos proporcionó nuevos conocimientos sobre como las bacterias se vuelven resistentes en un ambiente cambiante.

La exposición de las BAL a concentraciones subletales de antibióticos o biocidas (sub-MICs) originó cambios en los patrones de susceptibilidad aumentando las MICs de ampicilina, cloranfenicol, ciprofloxacino, teicoplanina y tetraciclina, mientras que se registró un descenso de las MICs de clindamicina, eritromicina, estreptomicina y trimetoprima en la mayoría de las cepas, las cuales eran originalmente susceptibles en la mayoría de los casos a los antibióticos correspondientes. Como ha sido previamente descrito, las BAL analizadas en este estudio fueron sensibles a cloranfenicol y resistentes a estreptomicina y trimetoprima; sin embargo, dicho patrón de susceptibilidad se invirtió después de la inducción mediante concentraciones sub-letales de antimicrobianos.

La aparición de nuevos patrones de resistencia en las BAL inducidas por concentraciones sub-letales de antimicrobianos tiene consecuencias ecológicas y evolutivas. Las concentraciones sub-letales de antibióticos podrían afectar la tasa de mutación, la transferencia horizontal de genes y la formación de biopelículas en el hábitat natural de las BAL y podrían contribuir a la aparición y diseminación de la resistencia a antibióticos en el medio ambiente y la cadena alimentaria (Laureti *et al.,* 2013). Además, muchos autores describieron que los factores de estrés tales como el calor, la sal, el pH ácido y alcalino pueden alterar de forma significativa el fenotipo de la resistencia a antibióticos en patógenos relacionados con los alimentos (Al-Nabulsi *et al.,* 2011; Ganjian *et al.,* 2012; McMahon *et al.,* 2007a, b). De forma similar, la exposición a compuestos químicos o luz UV (durante 5 o 10 minutos) puede producir los mismos efectos que los antimicrobianos en las MICs de los antibióticos.

Los datos obtenidos en el presente estudio sugieren que los cambios en los patrones de susceptibilidad después de la inducción de las BAL pueden deberse a alteraciones en la permeabilidad de la membrana celular a los antibióticos correspondientes o a diferentes perfiles de expresión de genes de resistencia/estrés. En general, las correlaciones positivas entre los antibióticos que representan diferentes clases y dianas celulares sugieren una co-resistencia (los determinantes de resistencia están portados por el mismo elemento genético móvil tales como plásmido, transposón o integrón) o bien mecanismos de resistencia cruzada entre los diferentes antibióticos. En este estudio, las correlaciones positivas obtenidas tras el estrés con antimicrobianos sugieren la existencia de una resistencia cruzada, la cual se basa en mecanismos de resistencia no específicos con un amplio rango de actividad frente a los antibióticos, tales como las bombas de exporte. Estas correlaciones fueron compartidas por diferentes factores de estrés físico-químicos (agentes antimicrobianos, exposición a los rayos UV y compuestos químicos) siendo inducido un repertorio de mecanismos fenotípicos y genéticos similares bajo diferentes condiciones. Aunque las respuestas fenotípicas fueron generalmente similares bajo diferentes condiciones de estrés, los patrones de expresión de los genes seleccionados implicados en los mecanismos de tolerancia/ resistencia mostraron respuestas diferenciales al estrés.

Para determinar los mecanismos adoptados por las BAL para soportar condiciones hostiles, el conocimiento sobre los genes inducibles bajo cada condición de estrés es de gran importancia, ya que el balance de las diferentes respuestas está implicado en la tolerancia/resistencia. En este sentido, el gen *rpsL* que codifica la proteína ribosomal S12, fue sobre-expresado después de la inducción con todos los factores de estrés (antibióticos, cloruro sódico, etanol y luz UV), exceptuando la amoxicilina y los biocidas (cloruro de benzalconio y triclosán). Esta proteína con efectos múltiples específicos puede actuar como una chaperona de ARN, como ha sido descrito por Coetzee *et al.* (1994), con la finalidad de proteger la estructura y la función del ribosoma bajo condiciones de estrés. El aumento de la expresión del gen *rpsL* fue generalmente responsable, a excepción de pocos casos, del aumento de las MICs de ampicilina, cloranfenicol, ciprofloxacino, teicoplanina y tetraciclina.

Sin embargo, el gen *recA* sólo fue sobre-expresado después de la inducción con tetraciclina y luz UV (durante 5 y 10 minutos), mientras que se observó una disminución de la expresión del mismo en las BAL inducidas por biocidas, amoxicilina, IPTG, cloruro sódico, etanol y la combinación de cloruro sódico y etanol. El gen *recA* codifica la proteína RecA, que juega un papel importante en la regulación del sistema SOS y en muchas rutas de reparación del ADN, tales como la reparación de los gaps de la hebra copia, roturas de la doble cadena o la mutagénesis del sistema SOS (Cox, 2007). Cuando se activa la respuesta SOS, se expresan varios genes implicados en la reparación, replicación, recombinación y la división celular, incluyendo el gen *recA* y los genes (*uvrA, uvrB, uvrC* y *uvrD*) relacionados con el sistema de reparación por escisión de nucleótidos (NER). En este estudio, el gen *uvrB* fue reprimido bajo todas las condiciones de estrés, excepto tras la inducción con cloranfenicol y luz UV durante 5 minutos. Cabe destacar que los genes *recA* y *uvrB* tienen una expresión paralela siendo implicados en los mismos mecanismos de reparación, los cuales han sido solamente activados en el caso de antibióticos (cloranfenicol o tetraciclina) y la exposición a la luz UV. Además, los análisis estadísticos realizados en este estudio apoyan este hecho. Por lo tanto, la respuesta SOS como una ruta inducible (Van der Veen y Abi, 2011) puede parcialmente estar implicada en el aumento de las MICs de antibióticos tras la inducción con cloranfenicol, tetraciclina o la luz UV ya que se obtuvieron fuertes correlaciones positivas para ambos genes.

En cuanto al gen de virulencia *srtA* que codifica una enzima capaz de anclar proteínas de la superficie a la pared celular (Mazmanian *et al.,* 1999), fue reprimido bajo todas las condiciones de estrés. Los genes *srtA* y *srtB* son responsables del entrecruzamiento de proteínas de superficie con motivos LPXTG al peptidoglicano, por lo tanto la represión del gen *srtA* bajo condiciones de estrés podría ser responsable en la mayoría de los casos de la disminución de las MICs de clindamicina, eritromicina, estreptomicina y trimetoprima mediante la alteración de la permeabilidad de las membranas celulares. Hesketh *et al.* (2011) obtuvieron resultados similares con la represión del gen que codifica la enzima sortasa bajo estrés por drogas.

En general, para garantizar la supervivencia, las bacterias mantienen la respuesta SOS bajo un estricto control y transcriben rigurosamente genes selectivos para abordar de manera adecuada la situación del estrés. Entoncés, el análisis de los mecanismos adoptados por *Lc. pseudomesenteroides* AP2-28 y *Lb. pentosus* MP-10 puede ayudar a explicar cómo las BAL sobreviven a diferentes condiciones de estrés y por qué cambian sus patrones de susceptibilidad. Los ensayos de inducción mediante antibióticos que inhiben la síntesis de proteínas (cloranfenicol o tetraciclina) causaron la sobre-expresión de los genes *rpsL* (*Lc. pseudomesenteroides* AP2-28 y *Lb. pentosus* MP-10), *recA* y *uvrB* (*Lb. pentosus* MP-10); sin embargo, se observó una represión del gen *srtA* (*Lb. pentosus* MP-10 y *Lc. pseudomesenteroides* AP2-28). De este modo, como se mencionó anteriormente, las BAL para sobrevivir bajo condiciones de estrés por antibióticos pueden utilizar diferentes mecanismos de reparación y protección activando los genes implicados tales como *rpsL, recA* y *uvrB* -dependiendo del antibiótico utilizado y la cepa de las BAL- y aumentando así las MICs para algunos antibióticos. En paralelo, la represión del gen *srtB* puede ser responsable del incremento de la sensibilidad de las BAL a otros antibióticos (clindamicina, eritromicina, estreptomicina y trimetoprima). Sin embargo, la inducción con biocidas (cloruro de benzalconio o triclosán) causó la represión de los genes *rpsL* (*Lb. pentosus* MP-10), *recA* (*Lc. pseudomesenteroides* AP2-28), *srtA* y *uvrB* (*Lb. pentosus* MP-10 y *Lc. pseudomesenteroides* AP2-28). Por lo tanto, la inducción con antibióticos o biocidas no implica el mismo repertorio de genes (excepto para la represión del gen *srtA*) o mecanismos ya que ambos antimicrobianos tienen diferentes dianas celulares. Además, la respuesta SOS no fue activada por el estrés inducido por biocidas. La respuesta fenotípica similar obtenida tras la inducción con antibiótico/biocida sugiere que hay otros genes específicos implicados en dicha respuesta, o hay mecanismos no específicos tales como las bombas de exporte (Buffet-Bataillon *et al.,* 2012).

En cuanto a los compuestos químicos, la inducción con el cloruro sódico, etanol, o la combinación de ambos, dio lugar a los mismos perfiles de expresión. En este sentido, se obtuvo la sobre-expresión del gen *rpsL* (*Lc. pseudomesenteroides* AP2-28) y la represión de los genes *recA*, *uvrB* y *srtA* (*Lb. pentosus* MP-10 inducida por el cloruro sódico y *Lc. pseudomesenteroides* AP2-28 inducido por etanol o cloruro sódico+etanol). La sobre-expresión del gen *rpsL* se atribuyó a su papel en la protección del ribosoma como ha sido mencionado anteriormente. Sin embargo, la represión de genes implicados en la respuesta SOS sugiere que el estrés producido por el cloruro sódico y el etanol no activa este sistema en las BAL. Como ha sido comentado previamente en el caso del estrés por antimicrobianos, la represión del gen *srtA* fue relacionada con la alteración de la permeabilidad de la membrana. Por otro lado, la exposición a la luz UV produjo la sobre-expresión de los genes *rpsL*, *recA* y *uvrB*, lo cual sugiere que ambos mecanismos de reparación y de protección fueron inducidos en estas condiciones, mientras que el gen *srtA* fue reprimido como ocurrió en los otros casos.

En conclusión, a pesar de la uniformidad de las respuestas fenotípicas a varios tipos de estrés, el repertorio de genes activados y reprimidos fue diferente de acuerdo con el tipo de estrés aplicado y la cepa de las BAL analizada; aunque fuertes correlaciones positivas fueron obtenidas con los genes *recA* y *uvrB* bajo diferentes condiciones de estrés. Por lo tanto, la búsqueda de estrategias para mejorar la tolerancia de las BAL al estrés, especialmente aquellas de importancia como cultivos iniciadores/protectores o como probióticos, va a depender en gran medida del análisis individual de cada cepa. Aunque podemos predecir la respuesta fenotípica al estrés producida por los antibióticos. En general, podemos especular que el gen *rpsL* podría considerarse como un biomarcador de robustez/resistencia en las BAL, aunque estudios posteriores son requeridos para elucidar la correlación entre el gen *rpsL* inducido con la robustez.

Por otro lado, considerando que los lactobacilos probióticos están despertando cada vez más interés en cuanto a su uso en la nutrición humana y animal debido a los beneficios ofrecidos a nivel del sistema inmunológico, el sistema digestivo y el sistema endocrino, planteamos el siguiente estudio sobre una cepa de *Lb. pentosus* con potencial probiótico. En este sentido, teniendo en cuenta los datos expuestos anteriormente para las BAL aisladas de la fermentación natural de la aceituna verde de mesa Aloreña cuyos resultados van a depender de la cepa en cuestión, nos pareció interesante abordar el estudio de la supervivencia de una cepa de *Lb. pentosus* con potencial probiótico bajo diferentes condiciones y desde la perspectiva proteómica. La supervivencia de las bacterias probióticas así como sus efectos beneficiosos bajo diferentes condiciones ambientales (incluyendo las condiciones gastrointestinales) pueden depender de los determinantes de resistencia. Así, es importante conocer las proteínas implicadas en la tolerancia para mejorar la funcionalidad de las cepas probióticas bajo diferentes condiciones de estrés. En este estudio, hemos investigado la respuesta proteómica del probiótico *Lb. pentosus* MP-10 a diferentes condiciones de estrés antimicrobiano. Los antibióticos y biocidas indujeron adaptaciones en *Lb. pentosus* MP-10 mediante la modificación de su perfil proteómico, dichos cambios fueron principalmente dependientes del antimicrobiano usado.

En general, los antibióticos indujeron varias modificaciones fisiológicas, debidas posiblemente a varios mecanismos de acción teniendo cada uno de ellos una diana celular bien definida; en comparación con los biocidas que indujeron pocas modificaciones. La adaptación a los antibióticos parece desencadenar más modificaciones fisiológicas que los biocidas. Este hecho ocurre porque los mecanismos de resistencia a antibióticos bacterianos han experimentado un proceso evolutivo largo para proteger a las bacterias, comparándoles con la relativamente reciente exposición a los biocidas y las pocas oportunidades para desarrollar resistencia a estos últimos. En general, varias proteínas implicadas en el metabolismo de carbohidratos tales como la proteína fosfotransportadora HPr (*Histidine-containing Phosphocarrier protein*) del sistema PTS (sistema fosfotransferasa) –como parte de la maquinaria relacionada con la glicolisis- y 6-fosfogluconato deshidrogenasa perteneciente a la ruta de las pentosas fosfato exhibieron un aumento de su expresión tras la exposición a los antibióticos (amoxicilina y tetraciclina) con diferentes dianas celulares. El incremento de los niveles de síntesis de ATP (Wilkins *et al.,* 2002) fue requerido para la alta actividad de exporte o para compensar la baja capacidad glucolítica (Wouters *et al.,* 2000), lo cual es de gran importancia para la supervivencia bacteriana bajo condiciones de estrés. Resultados similares fueron obtenidos con *Bifidobacterium animalis* y *Lb. reuteri* bajo condiciones de estrés biliar (Lee *et al.,* 2008; Sánchez *et al.,* 2007). Además, la proteína HPr no sólo es responsable del transporte de carbohidratos sino que también desempeña un papel regulador en el metabolismo de azúcares y en la represión catabólica, dependiendo de las interacciones proteína-proteína con diversos factores celulares (Deutscher *et al.,* 2006). En consecuencia, los niveles de expresión de otras proteínas implicadas en las rutas de glucolisis tales como 6-fosfofructoquinasa, y piruvato quinasa y gliceraldehido-3-fosfato deshidrogenasa dependiente de NAD fueron reducidos tras la inducción con antibióticos inhibidores de la síntesis de proteínas tales como cloranfenicol y tetraciclina, respectivamente. El piruvato, como producto final de la glucólisis, es un metabolito clave que puede ser utilizado en diferentes reacciones para aumentar los niveles de ATP, así el estrés con antibióticos indujo la regulación del metabolismo mediante represión o activación de enzimas implicadas en la producción de energía. Estos datos sugieren que para garantizar la supervivencia bajo condiciones de estrés con antibióticos, la fisiología de *Lb. pentosus* MP-10 puede alterarse para lograr un alto rendimiento energético vía la regulación de la activación o represión del metabolismo de carbohidratos (ruta de las pentosas fosfato y la glucólisis). Bajo condiciones de estrés con antibióticos y la subsiguiente limitación energética, los sistemas de transporte PTS fueron usados en lugar de transportadores de la familia ABC (Taranto *et al.,* 1999). De hecho, estos sistemas son más eficientes energéticamente ya que el substrato fosforilado puede entrar directamente en las rutas de glucólisis o de pentosas fosfato conservando ATP. Del mismo modo, Lin *et al.* (2014) describió que la fluctuación de las rutas metabólicas puede representar un mecanismo de resistencia a antibióticos bajo condiciones de estrés con clortetraciclina en *Escherichia coli*.

La interacción de amoxicilina y tetraciclina con los lípidos y proteínas de membrana indujo la sobre-expresión de proteínas de estrés como la NADH peroxidasa Npx y una proteína pequeña de choque térmico, respectivamente. Este hecho ocurre como una primera respuesta celular para mantener la homeostasis y la viabilidad. También, ha sido descrito que además de su papel en la homeostasis celular redox (degradación del peróxido de hidrógeno a agua y oxígeno), la proteína Npx del subgrupo peroxidasa-oxidasa-reductasa (POR) (familia de flavoproteína-disulfuro-reductasa “FDR”) también contribuye a la regeneración de nucleótidos pirimidínicos oxidados para la glucólisis (Ying, 2006). En cuanto a las proteínas pequeñas de choque térmico tales como "minichaperonas", éstas han sido asociadas con la mejora de la supervivencia bacteriana durante el estrés, ya que son necesarias para el funcionamiento normal de las células, incluyendo el crecimiento y la estabilidad del ADN y el ARN. Igualmente, estas proteínas previenen la formación de cuerpos de inclusión (Jakob *et al.,* 1993; Narberhaus, 2002; Veinger *et al.,* 1998), pero no están involucradas en el replegamiento proteico como las chaperonas.

Por otro lado, se incrementaron los niveles de síntesis proteica en *Lb. pentosus* MP-10 tras la exposición con amoxicilina y tetraciclina. Sin embargo, Rezzonico *et al.* (2007) describió que la expresión de las proteínas implicadas en el crecimiento celular (tales como son las proteínas ribosomales) fue marcadamente disminuida bajo condiciones de estrés, como una estrategia de ahorro energético necesario para los mecanismos de protección celular. A pesar de la baja actividad ribosomal tras la exposición al estrés, los factores que regulan dicho estrés están normalmente asociados a los ribosomas, lo cual sugiere un aumento en la síntesis de proteínas (Sherman y Qian, 2013). Este aumento en la síntesis proteica puede ser necesario para compensar el daño que sufren las proteínas de membrana o citoplasmáticas tras su interacción con los antibióticos, independientemente de su diana celular. Algunas de estas proteínas están involucradas en el metabolismo o en la defensa celular (la respuesta SOS y la respuesta al choque térmico). De forma similar, Mangalappalli-Illathu y Korber (2006) describieron que los niveles altos de proteínas ribosomales asociados con un incremento de la síntesis proteica fueron de gran importancia para la reducción de la susceptibilidad a compuestos de amonio cuaternario tales como el cloruro de benzalconio.

Con referencia a otras rutas metabólicas, la expresión de los enzimas implicados en el metabolismo de ácidos grasos (alcohol deshidrogenasa) y en la biosíntesis de nucleótidos pirimidínicos (CTP sintasa) fue reprimido en presencia de antibióticos. Las alteraciones en la biosíntesis de los ácidos grasos pueden provocar cambios en la membrana celular que a su vez favorecerán la supervivencia celular en presencia de tetraciclina, en este sentido Rogers *et al.* (2007) obtuvieron resultados similares con *Streptococcus pneumoniae* expuesta a penicilina. En cuanto a la enzima CTP sintasa, ésta es requerida para la biosíntesis de ribo- y desoxirribonucleótidos necesarias para la replicación del ARN y el ADN (Jørgensen *et al.,* 2004). En este estudio, las bajas tasas de crecimiento obtenidas inmediatamente después de la exposición a los antibióticos pueden reflejar una disminución en la expresión de las proteínas implicadas en la síntesis de nucleótidos y ácidos grasos. Sin embargo, tras la exposición a los antimicrobianos las tasas de crecimiento fueron similares o incluso aumentados en las células inducidas por algunos antimicrobianos (amoxicilina o cloruro de benzalconio).

Por otro lado, la adaptación de *Lb. pentosus* MP-10 a los biocidas (cloruro de benzalconio o triclosán) indujo modificaciones fisiológicas que eran en parte similares a las causadas por los antibióticos tales como el aumento de los niveles de síntesis proteica y la reducción del metabolismo de carbohidratos y de producción de energía (Figura 1). De hecho, la resistencia cruzada entre antibióticos y biocidas ha sido ampliamente descrita en la literatura (Fraise, 2002; Moken *et al.,* 1997; Randall *et al.,* 2007). Además, en el estudio anterior *Lb. pentosus* MP-10 pre-adaptada en presencia de bajas concentraciones de biocidas mostró un aumento de las MICs de antibióticos, lo cual sugiere que las modificaciones fisiológicas desencadenadas por biocida o antibiótico pueden proporcionar resistencia al otro. El cloruro de benzalconio, un desinfectante que daña la membrana celular, indujo específicamente la sobre-expresión de la proteína de interfase de la subunidad ribosomal relacionada con la síntesis de proteínas. Sin embargo, el triclosán causó la sobre-expresión de la glutamil-RNAt sintetasa que está considerada como una enzima clave en la biosíntesis proteica. Además, el triclosán causó un descenso en la expresión de proteínas implicadas en el metabolismo de carbohidratos (la proteína HPr) y en la producción de energía (oxidoresductasa). Al igual que ocurre con la inducción por los antibióticos, las células adaptadas a los antimicrobianos tienden a reducir el metabolismo de carbohidratos y de producción de energía, mientras que las proteínas que participan en la síntesis proteica fueron sobre-expresadas para compensar, posiblemente, el daño proteico resultante de la interacción de los biocidas con la membrana. Además, el cloruro de benzalconio y el triclosán exhibieron diferentes respuestas de adaptación, las cuales pueden ser atribuidas a los diferentes mecanismos de acción de dichos biocidas; el triclosán actúa inhibiendo la enzima enoil reductasa que participa en la síntesis de ácidos grasos (Heath *et al.,* 2002), mientras que el cloruro de benzalconio tiene múltiples dianas celulares (Beumer *et al.,* 2000).

En conclusión, obtuvimos una mejor comprensión de la respuesta proteómica de una bacteria probiótica, tales como es *Lb. pentosus* MP-10 a diferentes antimicrobianos como factores de estrés. En este sentido, hemos confirmado que el estrés antimicrobiano podría potenciar la resistencia bacteriana al estrés ambiental y gastrointestinal tales como los bajos pHs ácidos y las sales biliares. Así, los recuentos de viables de *Lb. pentosus* MP-10 inducida con antimicrobianos fueron mayores que aquellos producidos en la cepa no inducida. A partir de la información, se podrían desarrollar diferentes estrategias para mejorar la persistencia y resistencia de esta bacteria bajo diferentes condiciones ambientales. Como ha sido previamente demostrado, la adaptación a diferentes condiciones de estrés (sal, pH ácido, sales biliares, altas temperaturas…etc) podría ser utilizada como una estrategia para mejorar el rendimiento tecnológico de diferentes cepas de lactobacilos probióticos (Corcoran *et al.,* 2006; Desmond *et al.,* 2001; Mills *et al.,* 2011). En este estudio, *Lb. pentosus* MP-10 inducida exhibió una mayor tasa de viabilidad (excepto para algunos casos) y además mostró una mayor tolerancia a ambientes ácidos y con concentraciones elevadas de sales biliares que los controles sin inducción. En el presente estudio, describimos por primera vez que la adaptación al estrés antimicrobiano podría mejorar la resistencia y la robustez de *Lb. pentosus* MP-10 con potencial probiótico con el fin de hacer frente a condiciones en los cuales concentraciones sub-letales de antimicrobianos y condiciones de estrés (bajo pH y altas concentraciones de bilis) podrían estar presentes, tales como la cadena alimentaria, el medio ambiente o el tracto gastrointestinal. Por otro lado, este hecho es preocupante ya que las bacterias patógenas al desarrollar resistencias antimicrobianas tras la exposición a los antimicrobianos, pueden posiblemente desarrollar resistencias a las condiciones intestinales. Nuestros resultados mostraron que *Lb. pentosus* MP-10 responde a la exposición a biocidas y antibióticos mediante cambios en su proteoma como una estrategia de supervivencia: incrementando la expresión de la síntesis de proteínas incluyendo las proteínas de estrés y disminuyendo la expresión de proteínas implicadas en el metabolismo de carbohidratos y la producción de energía (Figura 1). Por lo tanto, estudios posteriores son requeridos para dilucidar qué proteínas están implicadas en la tolerancia al pH ácido y la bilis. Estos aspectos deben ser enfatizados con el fin de lograr la robustez deseada en las bacterias probióticas en relación con varias condiciones ambientales y gastrointestinales.

Por otro lado, la genómica ofrece nuevas informaciones de interés sobre la resistencia a antimicrobianos y aspectos de patogenicidad ya que mayoritariamente la detección de los determinantes de resistencia o de virulencia por PCR se queda muy limitada a los genes ya conocidos y caracterizados previamente. Sin embargo, el análisis de nuevos genes requiere de la secuenciación del genoma de varias cepas, del análisis bioinformático y de la comparación entre diferentes genomas para dilucidar la presencia de nuevos genes así como los mecanismos de transferencia entre miembros del mismo género o entre géneros distintos. En este sentido, el análisis *in silico* del genoma de los lactobacilos puede ofrecer nuevos conocimientos sobre su seguridad especialmente aquellos aislados de productos fermentados por la importancia de estos alimentos en nuestra dieta y también por los riesgos que supone la transferencia de dichos genes a lo largo de la cadena alimentaria y también a nivel del ecosistema intestinal. Los lactobacilos presentes en alimentos fermentados pueden actuar como potenciales vehículos de determinantes de resistencia a antibióticos no solamente en la cadena alimentaria sino también dentro del tracto gastrointestinal siendo posible la transferencia de genes de las bacterias comensales o probióticas a otras bacterias o incluso a patógenos, por lo tanto dificultando el tratamiento con antibióticos de infecciones comunes (Klein et al., 2000; Snydman, 2008). La transferencia de genes de resistencia a antibióticos se puede realizar horizontalmente mediante elementos móviles (plásmidos, transposones e integrones), lo cual es responsable de la transferencia intra-e inter-individual del material genético (van Reenen y Dicks, 2011). Además la presencia de secuencias de inserción (IS) en los genomas bacterianos indica también una transferencia horizontal de genes que está principalmente regulada por los factores ambientales. Estos elementos han sido detectados en algunos lactobacilos (http://www-is.biotoul.fr/). En este trabajo, hemos analizado *in silico* la seguridad de los lactobacilos aislados de alimentos fermentados en cuanto a la resistencia a antibióticos, y para ello hemos usado las secuencias genómicas disponibles en la base de datos NCBI (National Centre for Biotechnology Information). Hoy en día, disponemos de pocas secuencias genómicas de lactobacilos lo cual puede limitar bastante el análisis en profundidad del origen de la resistencia fenotípica observada en este género, dicha resistencia puede ser inducida o no por los antimicrobianos. En este estudio, hemos llevado a cabo el análisis *in silico* de la distribución de los genes de resistencia a cloranfenicol (*cat*) y glicopéptidos (*vanZ*) en los genomas de lactobacilos.

En cuanto a la resistencia a cloranfenicol, el gen de resistencia *cat* se encuentra frecuentemente en plásmidos de varias especies del género *Lactobacillus*. Sin embargo, la búsqueda de este gen en las secuencias el genómicas de diferentes lactobacilos disponibles en la base de datos NCBI reveló la presencia de *cat* en el cromosoma de *Lb. brevis* aislada de vegetales fermentados, *Lb. plantarum* de productos lácteos y vegetales fermentados, *Lb. fermentum* aislada de productos lácteos y *Lb. sakei* aislada de salchichas y vegetales fermentados. No obstante, la presencia de este gen no siempre implica su expresión a nivel fenotípico, ya que las diferencias observadas entre los ensayos fenotípicos y genotípicos se atribuyen algunas veces a las mutaciones en el gen *cat* o su región reguladora (Hummel *et al.,* 2007). Además, el análisis de la homología de las secuencias del gen *cat* de los lactobacilos aislados de productos fermentados usando la herramienta de búsqueda de nucleótidos no redundantes BLASTn de NCBI, indicó una gran divergencia de las secuencias nucleotídicas de esa región en el género *Lactobacillus* e incluso entre especies. La distancia evolutiva entre las proteínas Cat de diferentes lactobacilos aislados de alimentos fermentados y otros ambientes (especialmente ensilado, microbiota intestinal y vaginal) resultó en 2 grupos G1 y G2 en el árbol filogenético, siendo todos los genes *cat* localizados en el cromosoma excepto el gen *cat* de *Lb. reuteri* I5007 localizado en el plásmido pLRIO4 (Hou *et al.*, 2013). Dicho árbol filogenético mostró una gran divergencia entre las diferentes especies del género *Lactobacillus* y una alta homología entre las cepas de la misma especie independientemente de su origen (alimentos o otros ambientes). Por otro lado, la proteína Cat codificada por el gen *cat* localizado en el plásmido pLRIO4 de *Lb. reuteri* I5007 fue altamente similar a aquellas proteínas codificadas por los genes *cat* localizados en plásmidos de *S. aureus* (pC194, pKH13 y pKH15), lo que sugiere que la adquisición del gen *cat* por *Lb. reuteri* I5007 fue probablemente debida a la transferencia horizontal de genes desde *S. aureus.* De forma similar, *E. faecalis, E. faecium* y *S. aureus* comparten el mismo origen que otras proteínas Cat codificadas en plásmidos. Este hecho subraya la importancia de los géneros *Enterococcus* spp. y *Staphylococcus* spp. en la transferencia horizontal de genes a otros géneros bacterianos, especialmente a algunas cepas de *Lactobacillus*. En general, los lactobacilos aislados de alimentos fermentados no exhiben una transferencia de los genes *cat*, ya que no existe ninguna homología entre los genes *cat* codificados por el cromosoma en los lactobacilos y los genes *cat* codificados por plásmidos en lactobacilos de origen alimentario o otros ambientes. Sin embargo, la transferencia horizontal de genes en cepas de *Lactobacillus* de origen alimentario podría ser posible, ya que el gen *cat* localizado en el plásmido pLRI04 de *Lb. reuteri* I5007 (aislada del intestino de cerdo) fue probablmente capaz de adquirir este gen de *Staphylococcus* spp. y *Enterococcus* spp. posiblemente de origen intestinal. Por lo tanto, el mecanismo y el origen del gen *cat* en los lactobacilos aislados de alimentos fermentados parecen ser diferentes de la resistencia adquirida por los plásmidos en *Lb. reuteri*, así como otras bacterias que se encuentran en sistemas naturales tales como el tracto intestinal de los animales. Sin embargo, el principal problema que podría ser asociado a los ensayos de la seguridad de las cepas en cuanto a los genes de resistencia a antibióticos es la carencia de protocolos estandarizados para el ensayo de la transferibilidad de los genes de resistencia (Hummel *et al*., 2007).

Por otro lado, la resistencia intrínseca de los lactobacilos a los glicopéptidos es preocupante, ya que la vancomicina y la teicoplanina son dos antibióticos de primera elección para el tratamiento de infecciones causadas por la mayoría de las bacterias Gram-positivas, especialmente frente a muchos enterococos y estafilococos multirresistentes a diferentes antimicrobianos y resistentes a beta-lactámicos (Egervärn, 2009; Klare *et al.,* 2007; Reynolds, 1998).

El análisis *in silico* de genes *van* o secuencias relacionadas en las especies de *Lactobacillus* y especialmente aquellas aisladas de alimentos fermentados mostró la presencia de los genes *vanZ* responsables de la resistencia a glicopéptidos en diferentes especies del género *Lactobacillus* tales como *Lb. brevis, Lb. casei, Lb. delbrueckii* subsp*. bulgaricus, Lb. fermentum, Lb. helveticus, Lb. kefiranofaciens, Lb. plantarum* subsp*. plantarum* y *Lb. sakei* subsp. *sakei* aisladas principalmente de productos lácteos. Los análisis del gen *vanZ* localizado en el cromosoma indicaron que dicho gen era muy conservado en cada especie de *Lactobacillus*, pero no se encontraron homólogos en otros lactobacilos u otros taxones. En consecuencia, es casi imposible detectar este gen utilizando la técnica de PCR debido a esta divergencia en la secuencia de los nucleótidos entre las diferentes especies de *Lactobacillus*. Así, el análisis genómico se convierte en la única vía para detectar genes similares implicados en la resistencia a glicopéptidos.

El análisis filogenético de las secuencias proteícas VanZ de los lactobacilos aislados de alimentos fermentados y algunos lactobacilos aislados de otros ambientes (ensilado, tracto gastrointestinal y vagina) reveló una gran divergencia dependiendo en la mayoría de los casos de la especie de *Lactobacillus* estudiada. Sin embargo, diferentes grupos fueron obtenidos en el caso de *Lb. casei*/*Lb. paracasei* y *Lb. delbrueckii* que pertenecen a diferentes grupos genómicos. En cuanto a las demás especies, cada una presentaba un solo subgrupo con una homología moderada del 70% al 78% de identidad, a excepción de *Lb. delbrueckii* que poseía una homología del 97% entre sus cepas independientemente de su origen. De forma general, estos datos sugieren que la presencia del gen *vanZ* no fue el resultado de la transferencia horizontal de genes entre especies o géneros y esto se debe a la gran divergencia en las proteínas VanZ, ya que en la mayoría de los casos la distribución de un gen está vinculada a la especie e incluso al grupo filogenético y los cambios evolutivos producidos probablemente como resultado de la especiación. Este hecho es de gran importancia ya que este gen de resistencia a vancomicina no puede ser transferido horizontalmente por los lactobacilos presentes en los alimentos fermentados a bacterias patógenas en el tracto gastrointestinal. Por lo tanto, las cepas portadoras del gen *vanZ* y/o los productos fermentados asociados a ellas no representan ningún riesgo para la salud en relación a la transferencia de genes de resistencia a glicopéptidos.

Tras encontrar nuevas informaciones sobre la resistencia a antibióticos mediante el análisis *in silico* de genomas de *Lactobacillus* aislados de alimentos fermentados, nos pareció interesante analizar el genoma de *Lb. pentosus* MP-10 con potencial probiótico para dilucidar diferentes aspectos de seguridad. El análisis genómico de las bacterias probióticas puede ofrecer datos de interés sobre su seguridad y su funcionalidad, aunque los lactobacilos se consideran seguros por su larga historia de uso como cultivos iniciadores o protectores en diferentes alimentos tales como los vegetales fermentados, los productos lácteos, productos cárnicos y de pescado (Leroy y DeVuyst, 1999; Heller, 2001; Hansen, 2002; Holzapfel, 2002; Giraffa *et al*., 2010; Franz *et al*., 2011; Garrigues *et al*., 2013). En este sentido, la EFSA considera que el estatus QPS de cualquier cepa destinada a su uso como probiótico debe ser investigada en profundidad para descartar cualquier cepa con algún historial de infección o por posesión de genes transferibles de resistencia a antibióticos o virulencia (EFSA, 2012a). Esto se debe a que algunas cepas del género *Lactobacillus* han sido implicadas en infecciones oportunistas especialmente en personas de avanzada edad y en individuos inmunocomprometidos (Harty *et al.*, 1994; Schlegel *et al.*, 1998; Cannon *et al.*, 2005).

El genoma de *Lb. pentosus* MP-10 fue secuenciado mediante la tecnología de secuenciación de tercera generación. Los resultados obtenidos demostraron que dicho genoma es considerado el más largo dentro del género *Lactobacillus* conteniendo 3,698,214 pb y 5 plásmidos de 30-50 kb. Estos datos reflejan la gran flexibilidad de esta bacteria vía diversidad metabólica y estilos de adaptabilidad como resultado de la evolución bacteriana (duplicación de genes y transferencia horizontal de genes). El análisis *in silico* del genoma de *Lb. pentosus* MP-10 reveló la presencia de regiones implicadas en la defensa a elementos genéticos móviles (virus, elementos transponibles y plásmidos conjugativos) tales como el sistema CRISPR (dos clusters), además de la riqueza y variedad de los genes relacionados con el sistema CRISPR hemos encontrado dos nuevos genes en esta cepa (*cse1_Lpe* y *cse2_Lpe*) lo cual indica que *Lb. pentosus* MP-10 posee varios mecanismos de defensa frente a elementos genéticos exógenos. Este hecho es de gran relevancia para la aplicación de *Lb. pentosus* MP-10 como probiótico y como cultivo iniciador a escala industrial.

En cuanto a la seguridad de *Lb. pentosus* MP-10, el análisis *in silico* del genoma de dicha cepa frente a la base de datos CARD mostró la presencia de genes de resistencia a varios antibióticos relacionados mayoritariamente con bombas de exporte pero algunos también resultaron de la mutación de genes presentes en el cromosoma tal es el caso de los genes *gyrB* y *rpoB* que codifican para la resistencia a fluoroquinolona+aminocoumarin y rifampicina, respectivamente. Por otro lado, no se detectaron genes de resistencia a antibióticos adquiridos lo cual descarta cualquier trasferencia horizontal de genes de resistencia a antimicrobianos.

Además, hemos realizado un análisis *in silico* de la virulencia de *Lb. pentosus* MP-10 usando la base de datos MvirDB y los resultados demostraron la ausencia de genes de virulencia. Sin embargo, hemos detectado la presencia de algunos genes de los profagos Lp2 y Lp3 en el genoma de *Lb. pentosus* MP-10 igualmente encontrados en el genoma de la bacteria probiótica. Aunque ambas cepas (*Lb. pentosus* MP-10 y *Lb. plantarum* WCFS1) provienen de nichos ecológicos diferentes (saliva o aceitunas de mesa), estas dos especies comparten varios genes codificadores de profagos y también su arquitectura lo cual es debido probablemente a la interconexión entre los diferentes ecosistemas.

En conclusión, la cepa *Lb. pentosus* MP-10 se puede considerar segura debido a la ausencia de genes implicados en la virulencia o genes de resistencia adquirida a antibióticos. Aunque para su aplicación como probiótico requiere del análisis *in silico* e *in vivo* de su funcionalidad.

Conclusiones

1.- Casi todas las cepas de *Lactobacillus pentosus* (95%) y *Leuconostoc pseudomesenteroides* aisladas de la fermentación natural de aceitunas verdes de mesa Aloreña pueden ser consideradas como seguras debido a la ausencia de determinantes de resistencia adquiridos.

2.- La resistencia intrínseca a más de tres antibióticos no se convertirá en un problema a nivel clínico, ya que ambas especies de las BAL aisladas de la fermentación natural de aceitunas verdes de mesa Aloreña fueron también muy sensibles a otros antibióticos clínicamente relevantes.

3.- La incidencia de la múltiple resistencia intrínseca en ambas especies de las BAL era debida en parte a bombas de exporte cromosómicamente codificadas tales como NorA, MepA y MdeA.

4.- Las respuestas fenotípicas de las cepas BAL a diferentes tipos de estrés fue uniforme, sin embargo, el repertorio de genes inducidos y reprimidos diferían en función del tipo de estrés y de la cepa BAL; si bien, se obtuvieron correlaciones positivas importantes entre los genes *recA* y *uvrB* bajo varias condiciones de estrés.

5.- La búsqueda de un mecanismo para mejorar la tolerancia al estrés de las BAL, especialmente aquellas con importancia como cultivos iniciadores/protectores o como probióticos, dependerá en gran medida del análisis individual de cada cepa.

6 .- La adaptación al estrés antimicrobiano de *Lactobacillus pentosus* MP-10 podría mejorar su resistencia y su robustez para soportar condiciones ambientales y gastrointestinales.

7.- *Lactobacillus pentosus* MP-10 respondió a la exposición de los biocidas y los antibióticos mediante el ajuste de su arsenal proteómico como una estrategia de supervivencia vía la sobre-expresión de la síntesis de proteínas, incluyendo las proteínas de estrés, y la represión de la producción de energía y el metabolismo de hidratos de carbono.

8.- La presencia de genes altamente divergentes de glicopéptidos (gen *cat*) y cloranfenicol (gen *vanZ*) en los genomas de lactobacilos aislados de alimentos fermentados dependía en la mayoría de los casos de la especie en cuestión.

9.- Las cepas de *Lactobacillus* que llevan los genes de resistencia *cat* o *vanZ* no representan ningún riesgo para la salud en relación con la transferencia de los genes de resistencia a cloranfenicol o glucopéptidos, respectivamente.

10.- La secuenciación genómica reveló que el genoma de *Lactobacillus pentosus* MP-10 es el genoma más grande descrito hasta la fecha en el género *Lactobacillus*.

11.- El análisis *in silico* de las propiedades de seguridad de *Lactobacillus pentosus* MP-10 reveló la ausencia de genes de resistencia a antibióticos adquiridos y que la mayoría de los genes de resistencia eran genes de bombas de exporte o genes cromosómicos con mutaciones. Además, no se detectaron determinantes de virulencia en dicha cepa.

12.- *Lactobacillus pentosus* MP-10 podría ser considerada como segura, aunque un análisis adicional de su funcionalidad debe llevarse a cabo.

Concluding Remarks

1.- Almost all *Lactobacillus pentosus* (95%) and all *Leuconostoc pseudomesenteroides* strains isolated from naturally-fermented Aloreña green table olives can be regarded as safe because of the absence of acquired resistance determinants.

2.- The intrinsic resistance to more than three antibiotics will not become a problem in a medical setting, since both LAB species isolated from naturally-fermented Aloreña green table olives were also highly sensitive to other clinically relevant antibiotics.

3.- The occurrence of intrinsic multi-resistance in both LAB species was due in part to

chromosomally encoded efflux pumps such as NorA, MepA and MdeA.

4.- The phenotypic responses of LAB strains to several stresses was uniform, however the repertoire of induced and repressed genes differed according to the type of stress and LAB strain; although, strong positive correlations were obtained with *recA* and *uvrB* genes under several stress conditions.

5.- The search for a mechanism to improve stress tolerance of LAB, especially those with importance as starter/protective cultures or probiotics, will depend on individual screening of each strain.

6 .- The antimicrobial stress adaptation of *Lactobacillus pentosus* MP-10 could improve its resistance and its robustness to withstand environmental and gastrointestinal conditions.

7.- *Lactobacillus pentosus* MP-10 responded to the exposition of biocides and antibiotics by adjusting its proteomic arsenal as survival strategy by up-regulating protein synthesis, including stress proteins, and down-regulating carbohydrate metabolism and energy production.

8.- The presence of highly divergent glycopeptide (*cat* gene) and chloramphenicol (*vanZ* gene) resistance genes in genome sequences of lactobacilli from fermented foods depended in most cases on the species.

9.- *Lactobacillus* strains carrying *cat* or *vanZ* genes do not pose a health risk regarding transfer of chloramphenicol or glycopeptide-resistance genes, respectively.

10.- Genome sequencing revealed that *Lactobacillus pentosus* MP-10 genome is the most largest genome described up to date in *Lactobacillus* genus.

11.- *In silico* analysis of safety properties of *Lactobacillus pentosus* MP-10 revealed the absence of acquired antibiotic resistance genes and that most of the resistance genes were antibiotic efflux genes or chromosomal genes with mutations. Furthermore, no virulence determinants were detected in this strain.

12.- *Lactobacillus pentosus* MP-10 could be considered as safe, although additional analysis of its functionality should be carried out.

Bibliografía

Aarestrup, F.M., Agerso, Y., Gerner-Smidt, P., Madsen, M., Jensen, L.B. 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. Diagnostic Microbiology and Infectious Disease 37, 127-137.

Abriouel, H., Ben Omar, N., Lucas López, R., Martínez-Cañaamero, M., Keleke, S., Gálvez, A. 2006. Culture-independent analysis of themicrobial composition of the African traditional fermented foods poto poto and degue by using three different DNA extraction methods. International Journal of Food Microbiology 111, 228-233.

Abriouel, H., Martín-Platero, A., Maqueda, M., Valdivia, E., Martínez-Bueno, M. 2008. Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. International Journal of Food Microbiology 127, 200-208.

Abriouel, H., Benomar, N., Lucas, R., Gálvez, A. 2011a. Culture-independent study of the diversity of microbial populations in brines during fermentation of naturally-fermented Aloreña green table olives. International Journal of Food Microbiology 144, 487-496.

Abriouel, H., Benomar, N., Pérez Pulido, R., Cañamero, M.M., Gálvez, A. 2011b. Annotated genome sequence of *Lactobacillus pentosus* MP-10, which has probiotic potential, from naturally fermented Aloreña green table olives. Journal of Bacteriology 193, 4559-4560.

Abriouel, H., Benomar, N., Cobo, A., Caballero, N., Fernández Fuentes, M.A., Pérez-Pulido, R., Gálvez, A. 2012. Characterization of lactic acid bacteria from naturally-fermented Manzanilla Aloreña green table olives. Food Microbiology 32, 308-316.

Abriouel, H., Casado Muñoz, M.C., Lavilla Lerma, L., Pérez Montoro, B., Bockelmann, W., Pichner, R., Kabisch, J., Cho, G.S., Franz, C.M.A.P., Gálvez, A., Benomar, N. 2015. New insights in antibiotic resistance of *Lactobacillus* species from fermented foods. Food Research International 78, 465-481.

Adams, C.A. 2010. The probiotic paradox: live and dead cells are biological response modifiers. Nutrition Research Reviews 23, 37-46.

Adimpong, D.B., Nielsen, D.S., Sørensen, K.I., Vogensen, F.K., Sawadogo-Lingani, H., Derkx, P.M.F., Jespersen, L. 2013. *Lactobacillus delbrueckii* subsp. *jakobsenii* subsp. nov., isolated from dolo wort; an alcoholic fermented beverage in Burkina Faso. International Journal of Systematic and Evolutionary Microbiology 63, 3720-3726.

Ai, L., Chen, C., Zhou, F., Wang, L., Zhang, H., Chen, W., Guo, B. 2011. Complete genome sequence of the probiotic strain *Lactobacillus casei* BD-II. Journal of Bacteriology 193, 3160-3161.

Ali, A.A., Mustafa, M.M. 2009. Use of starter cultures of lactic acid bacteria and yeasts in the preparation of kisra, a Sudanese fermented food. Pakistan Journal of Nutrition 8, 1349-1353.

Allen, H., Donato, K., Wang, J., Cloud-Hansen, H.H., Davies, K.A., Handelsman, J. 2010. Call of the wild: antibiotic resistance genes in natural environments. Nature Reviews Microbiology 8, 251-259.

Al-Nabulsi, A. A., Osaili, T.M., Elabedeen, N.A., Jaradat, Z.W., Shaker, R.R., Kheirallah, K.A., Tarazid, Y.H., Holleye R.A. 2011. Impact of environmental stress desiccation, acidity, alkalinity, heat or cold on antibiotic susceptibility of *Cronobacter sakazakii*. International Journal of Food Microbiology 146, 137-143.

Altermann, E., Russell, W.M., Azcarate-Peril, M.A., Barrangou, R., Buck, B.L., McAuliffe, O., Souther, N., Dobson, A., Duong, T., Callanan, M., Lick, S., Hamrick, A., Cano, R., Klaenhammer, T.R. 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. Proceedings of the National Academy of Sciences U.S.A. 102, 3906-3912.

Alvarez-Ortega, C., Olivares, J., Martínez, J.L. 2013. RND multidrug efflux pumps: what are they good for?. Frontiers in microbiology 4, 1-11.

Ambrose, K.D., Nisbet, R., Stephens, D.S. 2005. Macrolide efflux in *Streptococcus pneumoniae* is mediated by a dual efflux pump (*mel* and *mef*) and is erythromycin inducible. Antimicrobial Agents and Chemotherapy 49, 4203-4209.

Aminov, R.I., Garrigues-Jeanjean, N., Mackie, R.I. 2001. Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. Applied and Environmental Microbiology 67, 22-32.

Ammor M.S., Flórez A.B. Mayo B. 2007. Antibiotic Resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiology 24, 559-570.

Ammor, M.S., Flórez, A.B., Van Hoek, A.H., De Los Reyes-gavilán, C.G., Aarts, H.J., Margolles, A., Mayo, B. 2008. Molecular Characterization of Intrinsic and Acquired Antibiotic Resistance in Lactic Acid Bacteria and Bifidobacteria. Journal of Molecular Microbiology and Biotechnology 14, 6-15.

Amores, R., Calvo, A., Maestre, J.R. Martínez-Hernández, D. 2004. Probióticos. Revisión Española de Quimioterapia 67, 131-139.

Anadon, A., Martinez-Larranaga, M.R., Martinez, M.A. 2006. Probiotics for animal nutrition in the European Union. Regulation and safety assessment. Regulatory Toxicology and Pharmacology 45, 91-95.

Andersson, D.I., Hughes, D. 2014. Microbiological effects of sublethal levels of antibiotics. Nature Reviews Microbiology 12, 465-478.

Anton, R., Barlow, S., Boskou, D., Castle, L., Crebelli, R., Dekant, W., Engel, K., Forsythe, S., Grunow, W., Heinonen, M., Chr. Larsen J., Leclercq, C., Mennes, W., Rosaria Milana, M., Pratt, I., Rietjens, I., Svensson, K., Tobback, P., Toldrá, F. 2006. Opinion of the scientific panel on food Additives, flavourings, processing aids and materials in contact with food on a request from the commission related to the use of nisin (E234) as a food additive. The EFSA Journal 314, 1-16.

Anukam, K.C., Olise, N.A. 2012. Development of a novel probiotic yogurt "PENTOYO" with a fully sequenced *Lactobacillus pentosus* KCA1 and its survival during storage at 4 °C. Tropical Journal of Pharmaceutical Research 11, 977-981.

Anukam, K.C., Macklaim, J.M., Gloor, G.B., Reid, G., Boekhorst, J., Renckens, B., van Hijum, S., Siezen, R. 2013. Genome sequence of *Lactobacillus pentosus* KCA-1 vaginal isolate from a healthy premenopausal woman. PLoS One 8, e59239.

Arioli, S., Elli, M., Ricci, G., Mora, D. 2013. Assessment of the susceptibility of lactic acid bacteria to biocides. International Journal of Food Microbiology 163, 1-5.

Auputinan, P., Tragoolpua, Y., Pruksakorn, S., Thongwai, N. 2011. Profiles of plasmids in lactobacilli isolated from fermented foods. Chiang Mai Journal of Science 38, 648-652.

Axelsson, L. 1998. Lactic acid bacteria: classification and physiology. In: Salminen, S., von Wright, A. (Eds.), Lactic Acid Bacteria Microbiology and Functional Aspects. Marcel Dekker. New York, USA.

Axelsson L. 2004. Lactic acid bacteria: classification and physiology. In: Salminen, S., von Wright, A., Ouwehand, A. (Eds.), Lactic Acid Bacteria Microbiology and Functional Aspects. Marcel Dekker. New York, USA.

Axelsson, L., Rud, I., Naterstad, K., Blom, H., Renckens, B., Boekhorst, J., Kleerebezemd, M., van Hijum, S., Siezen R.J. 2012. Genome sequence of the naturally plasmid-free *Lactobacillus plantarum* strain NC8 (CCUG 61730). Journal of Bacteriology 194, 2391-2392.

Aymerich, T., Martín, B., Garriga, M., Vidal-Carou, M. C., Bover-Cid, S., Hugas, M. 2006. Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. Journal of Applied Microbiology 100, 40-49.

Baeyens, J.M., Del Pozo, E. 2008. Antibióticos aminoglucósidos, tetraciclinas y cloranfenicol. Lorenzo, P., Moreno, A., Lizasoain, I., Leza, J.C., Moro, M.A., Portoles, A. (Eds). In: Velázquez: Farmacología básica y clínica. Editorial Médica Panamericana, Buenos Aires.

Bao, Y., Zhang, Y.C., Zhang, Y., Liu, Y., Wang, S.Q., Dong, X.M., Wang, Y.Y., Zhang, H.P. 2010. Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. Food Control 21, 695-701.

Barbosa, T.M., Levy, S.B. 2000. The impact of antibiotic use on resistance development and persistence. Drug Resistance Updates 3, 303-311.

Bartkiene E., Vidmantiene D., Juodeikiene G., Viskelis P., Urbonaviciene D. 2013. Lactic acid fermentation of tomato: effects on cis/trans lycopene isomer and β-carotene concentration and formation of L(+) and D(-)-lactic acid. Food Technology and Biotechnology 51, 471-478.

Bates, J., Jorderns, J.Z., Griffiths, D.T. 1994. Farm Animals As a Putative Reservoir for Vancomicyn-Resistant Enterococcal Infection in Man. Journal of Antimicrobial Chemotherapy 34, 507-514.

Båth, K., Roos, S., Wall, T., Jonsson, H. 2005. The cell surface of *Lactobacillus reuteri* ATCC 55730 highlighted by identification of 126 extracellular proteins from the genome sequence. FEMS Microbiology Letters, 253, 75-82

Batt, C.A. 2000. *Lactococcus*: Introduction. In: Encyclopedia of Food Microbiology. Academic Press. London, UK.

Baugher, J.L., Klaenhammer, T.R. 2011. Invited review: Application of omics tools to understanding probiotic functionality. Journal of Dairy Science 94, 4753-4765.

Bautista-Gallego, J., Arroyo-López, F.N., Rantsiou, K., Jiménez-Díaz, R., Garrido-Fernández, A., Cocolin, L. 2013. Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. Food Research International 50, 135-142.

Belletti, N., Monica, G., Benedetta, B., Erasmo, N., Giulia, T., Fausto, G. 2009. Antibiotic resistance of lactobacilli isolated from two Italian hard cheeses. Journal of Food Protection 72, 2162-2169.

Ben Omar, N., Ampe, F., Raimbault, M., Guyot, J.P., Tailliez, P. 2000. Molecular diversity of lactic acid bacteria fromcassava sour starch (Colombia). Systematic and Applied Microbiology 23, 285-291.

Benndorf, D., Vogt, C., Jehmlich, N., Schmidt, Y., Thomas, H., Woffendin, G., Shevchenko, A., Richnow, H.H., von Bergen, M. 2009. Improving protein extraction and separationmethods for investigating themetaproteome of anaerobic benzene communities within sediments. Biodegradation 20, 737–750.

Bennedsen, M., Stuer-Lauridsen, B., Danielsen, M., Johansen, E. 2011. Screening for antimicrobial resistance genes and virulence factors via genome sequencing. Applied and Environmental Microbiology 77, 2785-2787.

Bennett, D.M., Shekhel, T., Radelet, M., Miller, M.D. 2014. Isolated *Lactobacillus* chronic prosthetic knee infection. Orthopedics 37, 83-86.

Bergsveinson, J., Baecker, N., Pittet, V., Ziola, B. 2015. Role of plasmids in *Lactobacillus brevis* BSO 464 hop tolerance and beer spoilage. Applied and Environmental Microbiology 81, 1234-1241.

Bernardeau, M., Guguen, M., Vernoux, J.P. 2006. Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. FEMS Microbiology Reviews 30, 487-513.

Bernardeau, M., Vernoux, J.P., Henri-Dubernet, S., Gueguen, M. 2008. Safety assessment of dairy microorganisms: the *Lactobacillus* genus. International Journal of Food Microbiology 126, 278-285.

Bernstein, C., Bernstein, H., Payne, C. M., Beard, S. E., Schneider, J. 1999. Bile salt activation of stress response promoters in *Escherichia coli*. Current Microbiology 39, 68-72.

Beumer, R., Bloomfield, S.F., Exner, M., Fara, G.M., Nath, K.J., Scott, E. 2000. Microbial resistance and biocides. A Review by the International Scientific Forum on Home Hygiene. International Scientific Forum on Home Hygiene, London, UK. Available from: http://www.ifh-homehygiene.org/IntegratedCRD.nsf/111e68ea0824afe1802575070003f039/f309bd6a403617fa80257522005bc5f9?OpenDocument.

Bhatia, S.J., Kochar, N., Abraham, P., Nair, N.G., Mehta, A.P. 1989. *Lactobacillus acidophilus* inhibits growth of *Campylobacter pylori* in vitro. Journal of Clinical Microbiology 27, 2328-2330.

Bisanz, J.E., Enos, M.K., Mwanga, J.R., Changalucha, J., Burton, J.P., Gloor, G.B., Reid, G. 2014. Randomized open-label pilot study of the influence of probiotics and the gut microbiome on toxic metal levels in Tanzanian pregnant women and school children. MBio 5, e01580-14.

Bjorland, J., Sunde, M., Waage, S. 2001. Plasmid-borne smr gene causes resistance to quaternary ammoniumcompounds in bovine *Staphylococcus aureus*. Journal of Clinical Microbiology 39, 3999-4004

Blair, J.M., Webber, M.A., Baylay, A.J., Ogbolu, D.O., Piddock, L.J. 2015. Molecular mechanisms of antibiotic resistance. Nature Reviews Microbiology 13, 42-51.

Blandino, G., Milazzo, I., Fazio, D. 2008. Antibiotic susceptibility of bacterial isolates from probiotic products available in Italy. Microbial Ecology in Health and Disease 20, 199-203.

Blatchford, P., Ansell, J., de Godoy, M.R.C., Fahey, G., Garcia-Mazcorro, J.F., Gibson, G. R., Goh, Y.J., Hotchkiss, A.T., Hutkins, R., LaCroix, C., Rastall, R.A., Reimer, R.A., Schoterman, M., van Sinderen, D., Venema, K., Whelan, K. 2014. Prebiotic mechanisms, functions and applications-A review. International Journal of Probiotics and Prebiotics 8, 109-131.

Blázquez, J., Oliver, A., Gómez-Gómez, J.M. 2002. Mutation and evolution of antibiotic resistance: antibiotics as promoters of antibiotic resistance?. Current Drug Targets 3, 345-349.

Borcakli, M., Ozay, G., Alperden, I. 1993. Fermentation of Turkish olives with traditional and aerated systems. Food Flavours, Ingredients, and Composition, 265-277.

Borch, E., Berg, H., Holst, O. 1991. Heterolactic fermentation by a homofermentative *Lactobacillus* sp. during glucose limitation in anaerobic continuous culture with complete cell recycle. Journal of Applied Bacteriology 71, 265-269.

Boris, S., Suares, J., Vazquez, F., Barbes, C. 1998. Adherence of human lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infection and Immunity 66, 1985-1989.

Boris, S., Barbés, C. 2000. Role played by lactobacilli in controlling the population of vaginal pathogens. Microbes and Infection 2, 543-546.

Boyle, R., Robins-Browne, R.M., Tang, M.L.K. 2006. Probiotic use in clinical practice: what are the risks? American Journal of Clinical Nutrition 83, 1256-1264.

Brassart, D., Schiffrin, E.J. 1997. The use of probiotics to reinforce mucosal defence mechanisms. Trends in Food Science and Technology 8, 321-326.

Broadbent, J.R., Neeno-Eckwall, E., Stahl, B., Tandee, K., Cai, H., Morovic, W., Horvath, P., Heidenreich, J., Perna, N.T., Barrangou, R., Steele, J.L. 2012. Analysis of the *Lactobacillus casei* supragenome and its influence in species evolution and lifestyle adaptation. BMC Genomics 13, 533.

Broadbent, J.R., Hughes, J.E., Welker, D.L., Tompkins, T.A., Steele, J.L. 2013. Complete genome sequence for *Lactobacillus helveticus* CNRZ 32, an industrial cheese starter and cheese flavor adjunct. Genome Announcements 1, e00590-13.

Budin-Verneuil, A., Pichereau, V., Auffray, Y., Ehrlich, D., Maguin, E. 2007. Proteome phenotyping of acid stress-resistant mutants of *Lactococcus lactis* MG1363. Proteomics 7, 2038–2046.

Buffet-Bataillon, S., Le Jeune, A., Le Gall-David, S., Bonnaure-Mallet, M., Jolivet-Gougeon, A. 2012. Molecular mechanisms of higher MICs of antibiotics and quaternary ammonium compounds for *Escherichia coli* isolated from bacteraemia. Journal of Antimicrobial Chemotherapy 67, 2837-2842.

Burton, J.P., Cadieux, P.A., Reid, G. 2002. Improved understanding of the bacterial vaginal microbiota of women before and after probiotic instillation. Applied and Environmental Microbiology 69, 97-101.

Caballero Gómez, N., Abriouel, H., Ennahar, S., Gálvez, A. 2013. Comparative proteomic analysis of *Listeria monocytogenes* exposed to enterocin AS-48 in planktonic and sessile states. International Journal of Food Microbiology 167, 202-207.

Cabello, F.C. 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environmental Microbiology 8, 1137-1144.

Cabrera C.E., Rommel F., Zuñiga A.E. 2007. La resistencia de bacterias a antibioticos, antisépticos y desinfectantes una manifestación de los mecanismos de supervivencia y adaptación. Colombia Médica 38, 149-158.

Cadena, M.P., Villarraga, E.C., Luján, D.E., Salcedo, J.G. 2006. Evaluación de la agroindustria del almidón agrio de yuca (Manihot esculenta Crantz) en Córdoba y Sucre. Temas Agrarios 11, 43-53.

Cai, H., Thompson, R., Budinich, M.F., Broadbent, J.R., Steele, J.L. 2009. Genome sequence and comparative genome analysis of *Lactobacillus casei*: Insights into their niche-associated evolution. Genome Biology and Evolution 1, 239-257.

Cai, Y., Pang, H., Kitahara, M., Ohkuma, M. 2012. *Lactobacillus nasuensis* sp. nov., a lactic acid bacterium isolated from silage, and emended description of the genus *Lactobacillus*. International Journal of Systematic and Evolutionary Microbiology 62, 1140-1144.

Callanan, M., Kaleta, P., O'Callaghan, J., O'Sullivan, O., Jordan, K., McAuliffe, O., Sangrador-Vegas, A., Slattery, L., Fitzgerald, G.F., Beresford, T., Ross, R.P. 2008. Genome sequence of *Lactobacillus helveticus*, an organism distinguished by selective gene loss and insertion sequence element expansion. Journal of Bacteriology 190, 727-735.

Cammarota, M., De Rosa, M., Stellavato, A., Lamberti, M., Marzaioli, I., Giuliano, M. 2009. In vitro evaluation of *Lactobacillus plantarum* DSMZ 12028 as a probiotic: emphasis on innate immunity. International Journal of Food Microbiology 135, 90-98.

Canchaya, C., Claesson, M.J., Fitzgerald, G.F., van Sinderen, D., O'Toole, P.W. 2006. Diversity of the genus *Lactobacillus* revealed by comparative genomics of five species. Microbiology 152, 3185-3196.

Cancho Grande, B., García Falcón, M., Simal Gandára, J. 2000. El uso de los antibióticos en la alimentación animal: perspectiva actual. Ciencia y Tecnología Alimentaria 3, 39-47.

Candiano, G., Bruschi, M., Musante, L., Sabntucci, L., Ghiggeri, G.M., Carnemolla, B., Orecchia, P., Zardi, L., Righetti, P.G. 2004. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25, 1327-1333.

Cannon, J.P., Lee, T.A., Bolanos, J.T., Danziger, L.H. 2005. Pathogenic relevance of *Lactobacillus*: A retrospective review of over 200 cases. European Journal of Clinical Microbiology and Infectious Diseases 24, 31-40.

Čanžek Majhenič, A., Mohar Lorbeg, P., Rogelj, I. 2007. Characterization of the *Lactobacillus* community in traditional Karst ewe's cheese. International Journal of Dairy Technology 60, 182-190.

Caplice, E., Fitzgerald, G.F. 1999. Food fermentations: role of microorganisms in food production and preservation. International Journal of Food Microbiology 50, 131-149.

Carroll, I.M., Threadgill, D.W., Threadgill, D.S. 2009. The gastrointestinal microbiome: A malleable, third genome of mammals. Mammalian Genome 20, 395-403.

Casado Muñoz, M.C., Benomar, N., Lavilla Lerma, L., Gálvez, A., Abriouel, H. 2014. Antibiotic resistance of *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* isolated from naturally-fermented Aloreña table olives throughout fermentation process. International Journal of Food Microbiology 172, 110–118.

Casado Muñoz, M.C., Benomar, N., Ennahar, S., Horvatovich, P., Lavilla Lerma, L., Knapp, C.W., Gálvez, A., Abriouel, H. 2016. Comparative proteomic analysis of a potentially probiotic *Lactobacillus pentosus* MP-10 for the identification of key proteins involved in antibiotic resistance and biocide tolerance. International Journal of Food Microbiology 222, 8-15.

Casas, I.A., Dobrogosz, W.J. 1997. *Lactobacillus reuteri*: An overviewof a newprobiotic for humans and animals. Microecology and Therapy 25, 221-231.

Casas, I.A., Dobrogosz, W.J. 2000. Validation of the probiotic concept: *Lactobacillus reuteri* confers broad-spectrum protection against disease in humans and animals. Microbial Ecology in Health and Disease 12, 247-285.

Cataloluk, O., Gogebakan, B. 2004. Presence of drug resistance in intestinal lactobacilli of dairy and human origin in Turkey. FEMS Microbiology Letters 236, 7-12.

Chaillou, S., Champomier-Vergès, M.C., Cornet, M., Crutz-Le Coq, A.M., Dudez, A.M., Martin, V., Beaufils, S., Darbon-Rongère, E., Bossy, R., Loux, V., Zagorec, M. 2005. The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23 K. Nature Biotechnology 23, 1527-1533.

Chain, E., Duthie, E.S. 1945. Bactericidal and bacteriolytic action of penicillin on the *Staphylococcus*. The Lancet 245, 652-657.

Chammem, N., Kachouri, M., Mejri, M., Peres, C., Boudabous, A., Hamdi, M. 2005. Combined effect of alkali pretreatment and sodium chloride addition on the olive fermentation process. Bioresource Technology 96, 1311-1316.

Chan, R.C.Y., Reid, G., Irvin, R.T., Bruce, A.W., Costerton, J.W. 1985. Competitive exclusion of uropathogens from uroepithelial cells by *Lactobacillus* whole cells and cell wall fragments. Infection and Immunity 47, 84-89.

Chang, J.H., Shim, Y.Y., Cha, S.K., Chee, K.M. 2010. Probiotic characteristics of lactic acid bacteria isolated from kimchi. Journal of Applied Microbiology 109, 220-230.

Chapman, J.S. 2003a. Biocide resistance mechanisms. International Biodeterioration and Biodegradation 51, 133-138.

Chapman, J.S. 2003b. Disinfectant resistance mechanisms, cross-resistance, and co-resistance. International Biodeterioration and Biodegradation 51, 271-276.

Charteris, W.P., Kelly, P.M., Morelli, L., Collins, J.K. 1998. Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. Journal of Applied Microbiology 84, 759-768.

Charteris, W.P., Kelly, P.M., Morelli, L., Collins, J.K. 2001. Gradient diffusion antibiotic susceptibility testing of potentially probiotic lactobacilli. Journal of Food Protection 64, 2007-2014.

Chassy, B.M. 1987. Prospects for the genetic manipulation of lactobacilli. FEMS Microbiology Reviews 46, 297- 312.

Chen, S.N., Rillig, M.C., Wang, W. 2009. Improving soil protein extraction for metaproteome analysis and glomalin-related soil protein detection. Proteomics 9, 4970-4973.

Chen, C., Ai, L.Z., Zhou, F.F., Wang, L., Zhang, H., Chen, W., Guo, B. 2011. Complete genome sequence of the probiotic bacterium *Lactobacillus casei* LC2W. Journal of Bacteriology 193, 3419-3420.

Cho, G.S., Hanak, A., Huch, M., Holzapfel, W.H., Franz C.M.A.P. 2010. Investigation into the Potential of Bacteriocinogenic *Lactobacillus plantarum* BFE 5092 for Biopreservation of Raw Turkey Meat. Probiotics and Antimicrobial Proteins 2, 241-249.

Chopra, I., Roberts, M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiology and Molecular Biology Reviews 65, 232-260.

Chourey, K., Jansson, J., VerBerkmoes, N., Shah, M., Chavarria, K. L., Tom, L.M., Brodie, E.L., Hettich, R.L. 2010. Direct cellular lysis/protein extraction protocol for soil metaproteomics. Journal of Proteome Research 9, 6615–6622.

Chung, H.J., Bang, W., Drake, M.A. 2006. Stress Response of *Escherichia coli*. Comprehensive Reviews in Food Science and Food Safety 5, 52-64.

Ciorba, M.A., Riehl, T.E., Rao, M.S., Moon, C., Ee, X., Nava, G.M., Walker, M.R., Marinshaw, J.M., Stappenbeck, T.S., Stenson, W.F. 2011. *Lactobacillus* probiotic protects intestinal epithelium from radiation injury in a TLR-2/cyclo-oxygenase-2-dependent manner. Gut 61, 829-838.

Cirz, R.T., Chin, J.K., Andes, D.R., de Crécy-Lagard, V., Craig, W.A., Romesberg, F.E. 2005. Inhibition of mutation and combating the evolution of antibiotic resistance. PLoS Biology 3, 1024-1033.

Claesson, M.J., van Sinderen, D., O'Toole, P.W. 2008. *Lactobacillus* phylogenomics-towards a reclassification of the genus. International Journal of Systematic and Evolutionary Microbiology 58, 2945-2954.

Clardy J., Fischbach M.A., Currie C.R. 2009. The natural history of antibiotics. Current Biology 19, R437–R441.

Clark, S., Jung S., Lamsal, B. 2014. Food Processing: Principles and Applications. John Wiley and Sons. Chichester, UK.

Cleveland, J., Montville, T.J., Nes, I.F., Chikindas, M.L. 2001. Bacteriocins: safe, natural antimicrobials for food preservation. International Journal of Food Microbiology 71, 1-20.

Clewell, D.B., Flannagan, S.E., Jaworski, D.D. 1995. Unconstrained bacterial promiscuity: The *Tn916-Tn1545* family of conjugative transposons. Trends in Microbiology 3, 229-236.

CLSI (Clinical and Laboratory Standards Institute). 2011. Performance standards for antimicrobial susceptibility testing; Twenty-First Informational Supplement. Document M100-S21. no 1, vol. 31. Wayne, PA.

Coast, J., Smith, R.D. 2003. Antimicrobial resistance: Cost and containment. Expert Review of Anti-Infective Therapy 1, 241-251.

Coetzee, T., Herschlag, D., Belfort, M. 1994. *Escherichia coli* proteins, including ribosomal protein S12, facilitate in vitro splicing of phage T4 introns by acting as RNA chaperones. Genes and Development 8, 1575-1588.

Comunian, R., Daga, E., Dupré, I., Paba, A., Devirgiliis, C., Piccioni, V., Perozzi, G., Zonenschain, D., Rebecchi, A., Morelli, L., De Lorentiis, A., Giraffa, G. 2010. Susceptibility to tetracycline and erythromycin of *Lactobacillus paracasei* strains isolated from traditional Italian fermented foods. International Journal of Food Microbiology 138, 151-156.

Condell, O., Iversen, C., Cooney, S., Power, K.A., Walsh, C., Burgess, C., Fanning, S. 2012. Efficacy biocides used in the modern food industry to control *Salmonella enterica*, and links between biocide tolerance and resistance to clinically relevant antimicrobial compounds. Applied and Environmental Microbiology 78, 3087-3097.

Condon, S. 1983. Aerobic metabolism of lactic acid bacteria. Irish Journal of Food Science and Technology 7, 15-25.

Coppola, R., Succi, M., Tremonte, P., Reale, A., Salzano, G., Sorrentino, E. 2005. Antibiotic susceptibility of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. Le Lait 85, 193-204.

Corcoran, B.M., Ross, R.P., Fitzgerald, G.F., Dockery, P., Stanton, C. 2006. Enhanced survival of GroESL-overproducing *Lactobacillus paracasei* NFBC 338 under stressful conditions induced by drying. Applied and Environmental Microbiology 72, 5104-5107.

Corona, F., Martinez, J.L. 2013. Phenotypic resistance to antibiotics. Antibiotics 2, 237-255.

Courcelle, J., Khodursky, A., Peter, B., Brown, P.O., Hanawalt, P.C. 2001. Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient *Escherichia coli*. Genetics 158, 41-64.

Cox, M.M. 2007. Regulation of bacterial RecA protein function. Critical Reviews in Biochemistry and Molecular Biology 42, 41-63.

Cremonesi, P., Chessa, S., Castiglioni, B. 2013. Genome sequence and analysis of *Lactobacillus helveticus*. Frontiers in Microbiology 3, 1-12.

Cromwell, G.L. 2002. Why and how antibiotics are used in swine production. Animal Biotechnology 13, 7-27.

Crowley, S., Bottacini, F., Mahony, J., van Sinderen, D. 2013. Complete genome sequence of *Lactobacillus plantarum* strain 16, a broad-spectrum antifungal-producing lactic acid bacterium. Genome Announcements 1, e00533-13.

Da Silva, S., Robbe-Masselot, C., Ait-Belgnaoui, A., Mancuso, A., Mercade-Loubière, M., Salvador-Cartier, C., Gillet M., Ferrier, L., Loubiere, P., Dague, E., Theodorou, V., Mercier-Bonin, M. 2014. Stress disrupts intestinal mucus barrier in rats via mucin O-glycosylation shift: prevention by a probiotic treatment. American Journal of Physiology-Gastrointestinal and Liver Physiology 307, G420-G429.

Dancey, C., Reidy, J. 2004. Statistics without maths for psychology: using SPSS for windows. Prentice Hall, London, UK.

Danielsen, M. 2002. Characterization of the tetracycline resistance plasmid pMD5057 from *Lactobacillus plantarum* 5057 reveals a composite structure. Plasmid 48, 98-103.

Danielsen, M., Wind, A. 2003. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. International Journal of Food Microbiology 82, 1-11.

Danielsen, M., Madsen, H.L., Hammer, K., Wind, A. 2005. Investigation of Danish and Italian cheeses made from raw milk and pasteurized milk for the presence of antibiotic resistant lactic acid bacteria and the genetic characterisation of these bacteria. Poster Lab8 conference: Symposium on lactic acid bacteria: Genetics, metabolism and applications, Egmond aan Zee.

Davidson, B.E., Kordias, N., Dobos, M., Hillier, A.J. 1996. Genomic organization of lactic acid bacteria. Antonie Van Leeuwenhoek 70, 161-183.

Davies, J., Davies, D. 2010. Origins and evolution of antibiotic resistance. Microbiology and Molecular Biology Reviews 74, 417-433.

Davin-Regli, A., Pagès, J.M. 2012. Cross-resistance between biocides and antimicrobials: an emerging question. Scientific and Technical Review 31, 89-104.

de Ahumada Vázquez, J.I., Falcón, M.L., Molina, J.S. 2002. Farmacología práctica: para las diplomaturas en ciencias de la salud. Ediciones Díaz de Santos. Madrid, Spain.

Deghorain, M., Goffin, P., Fontaine, L., Mainardi, J.L., Daniel, R., Errington, J., Hallet, B., Hols, P. 2007. Selectivity for D-lactate incorporation into the peptidoglycan precursors of *Lactobacillus plantarum*: role of Aad, a VanX-like D-alanyl-D-alanine dipeptidase. Journal of Bacteriology 189, 4332-4337.

Del Chierico, F., Masotti, A., Onori, M., Fiscarelli, E., Mancinelli, L., Ricciotti, G., Muraca, M. 2012. MALDI-TOF MS proteomic phenotyping of filamentous and other fungi from clinical origin. Journal of Proteomics 75, 3314-3330.

DeLisle, S., Perl, T.M. 2003. Vancomycin-resistant enterococci. Chest 123 (Suppl.), 504S-518S.

Dellagio, F. 1994. Taxonomie, métabolisme, croissance et génétique des bactéries lactiques. In: de Roissart, H., Luquet, F.M. Bactéries lactiques. Lorica. Uriage, France.

Desmond, C., Stanton, C., Fitzgerald, G.F., Collins, K., Ross, R.P. 2001. Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying. International Dairy Journal 11, 801-808.

Dessalegn, A., Ashenafi, M. 2010. Evaluation of the probiotic properties and antibiotic resistance of lactic acid bacteria isolated from awaze, qotchqotcha and Tef dough, traditional Ethiopian fermented foods. Internet Journal of Food Safety 12, 187-191.

Deutscher, J., Francke, C., Postma, P.W. 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiology and Molecular Biology Reviews 70, 939-1031.

Devirgiliis, C., Caravelli, A., Coppola, D., Barile, S., Perozzi, G. 2008. Antibiotic resistance and microbial composition along the manufacturing process of Mozzarella di Bufala Campana. International Journal of Food Microbiology 128, 378-384.

Devirgiliis, C., Coppola, D., Barile, S., Colonna, B., Perozzi, G. 2009. Characterization of the *Tn916* conjugative transposon in a food-borne strain of *Lactobacillus paracasei*. Applied and Environmental Microbiology 75, 3866-3871.

Devirgiliis, C., Zinno, P., Perozzi, G. 2013. Update on antibiotic resistance in foodborne *Lactobacillus* and *Lactococcus* species. Frontiers in Microbiology 4, 301.

Di Cagno, R., Surico, R.F., Siragusa, S., De Angelis, M., Paradiso, A. Minervini, F., De Gara, L., Gobbetti, M. 2008. Selection and use of autochthonous mixed starter for lactic acid fermentation of carrots, French beans or marrows. International Journal of Food Microbiology 127, 220-228.

Di Cagno, R., Cardinali, G., Minervini, G., Antonielli, L., Rizzello, C.G., Ricciuti, P., Gobbetti, M. 2010a. Taxonomic structure of the yeasts and lactic acid bacteria microbiota of pineapple (*Ananas comosus* L. Merr.) and use of autochthonous starters for minimally processing. Food Microbiology 27, 381-389.

Diéguez, C.C. 2006. Biocidas. Eficacia. Criterios para su evaluación y autorización. Revista de Salud Ambiental 6, 56-60.

Dixon, B. 2000. Antibiotics as growth promoters: risks and alternatives. American Society for Microbiology News 66, 264-265.

Drawz, S.M., Bonomo, R.A. 2010. Three decades of β-lactamase inhibitors. Clinical Microbiology Reviews 23, 160-201.

Drissi, F., Merhej, V., Angelakis, E., El Kaoutari, A., Carriere, F., Henrissat, B., Raoult, D. 2014. Comparative genomics analysis of *Lactobacillus* species associated with weight gain or weight protection. Nutrition and Diabetes 4, e109.

Dubois-Brissonnet, F. 2012. Adaptation of *Salmonella* to antimicrobials in food-processing environments. In: Annous, B.A., Gurtler, J.B. (Eds.). Agricultural and Biological Sciences "*Salmonella*: Distribution, Adaptation, Control Measures and Molecular Technologies". Intech Europe. Rijeka, Croatia.

Dunne, C., O'Mahony, L., Thornton G., Feeny, M., Daly, C., O'Sullivan, G., Collins, J.K. 2001. In vitro selection criteria for probiotic bacteria of human origin: Correlation With in vivo findings. The American Journal of Clinical Nutrition 73, 386-392.

Durán, M.C., García, P., Brenes, M., Garrido, A. 1993. *Lactobacillus plantarum* survival during the first days of ripe olive brining. Systematic and Applied Microbiology 16, 153-158.

Durán, G.M., Marshall, D.L. 2005. Ready-to-eat shrimp as an international vehicle of antibiotic-resistant bacteria. Journal of Food Protection 68, 2395-2401.

Durán Quintana, M., García García, P., Garrido Fernández, A. 1999. Establishment of conditions for green table olive fermentation at low temperature. International Journal of Food Microbiology 51, 133-143.

Dutka-Malen, S., Stefan, E., Courvalin, P. 1995. Detection of glycopeptides resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. Journal of clinical microbiology 33, 24-27.

EFSA (European Food Safety Authority). 2004. EFSA scientific colloquium summary report: QPS-qualified presumption of safety of microorganisms in food and feed. (http://www.efsa.europa.eu/en/colloquiareports/colloquia qps.htm).

EFSA. (European Food Safety Authority). 2005. QPS e Qualified presumption of safety of microorganisms in food and feed. EFSA Scientific Colloquium, 13-14 December 2004. Summary Report, EFSA. Brussels, Belgium.

EFSA (European Food Safety Authority). 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA Journal 587, 1-16.

EFSA (European Food Safety Authority). 2008a. The maintenance of the list of QPS microorganisms intentionally added to food or feed. EFSA Journal 923, 1-48.

EFSA (European Food Safety Authority), 2008b. Scientific opinion of the panel on biological hazards on a request from the European Food Safety Authority on food borne antimicrobial resistance as a biological hazard. EFSA Journal 765, 1-87.

EFSA (European Food Safety Authority), 2008c. Technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. EFSA Journal 732, 1-15.

EFSA (European Food Safety Authority). 2008. Scientific Opinion of the Panel on Biological Hazards on the maintenance of the list of QPS microorganisms intentionally added to food or feed. The EFSA Journal 923, 1-48.

EFSA Panel on Biological Hazards (BIOHAZ), 2010. Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2010 update). EFSA Journal 8, 1944.

EFSA (European Food Safety Authority), 2011. Scientific opinion on the safety and efficacy of Lactobacillus pentosus (DSM 14025) as a silage additive for all animal species. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). EFSA Journal 9, 2449.

EFSA (European Food Safety Authority) (2012a). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in the European Union in 2010. EFSA Journal 10, 2598.

EFSA (European Food Safety Authority) (2012b). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal 10, 2740.

Egervärn, M. 2009. Antibiotic Resistance in *Lactobacillus reuteri* and *Lactobacillus plantarum*. Swedish University of Agricultural Sciences Uppsala. Doctoral Thesis.

Egervärn, M., Roos, S., Lindmark, H. 2009. Identification and characterization of antibiotic resistance genes in *Lactobacillus reuteri* and *Lactobacillus plantarum*. Journal of Applied Microbiology 107, 1658-1668.

Egervärn, M., Lindmark, H., Olsson, J., Roos, S. 2010. Transferability of a tetracycline 19 resistance gene from probiotic *Lactobacillus reuteri* to bacteria in the gastrointestinal tract of humans. Antonie Van Leeuwenhoek 97, 189-200.

El Amin, N.A., Jalal, S.,Wretlind, B. 1999. Alterations in GyrA and ParC associated with fluoroquinolone resistance in *Enterococcus faecium*. Antimicrobial Agents and Chemotherapy 43, 947-949.

Elkins, C.A., Mullis, L.B. 2004. Bile-mediated aminoglycoside sensitivity in *Lactobacillus* species likely results from increased membrane permeability attributable to cholic acid. Applied and Environmental Microbiology 70, 7200-7209.

Ercolini, D., Villania, F., Aponte, M., Mauriello, G. 2006. Fluorescence in situ hybridisation detection of *Lactobacillus plantarum* group on olives to be used in natural fermentations. International Journal of Food Microbiology 112, 291-296.

Ernst, C.M., Peschel, A. 2011. Broad-spectrum antimicrobial peptide resistance by MprF-mediated aminoacylation and flipping of phospholipids. Molecular Microbiology 80, 290-299.

Escalante Lozada, A. 2001. El potencial de la manipulación de la flora intestinal por medios dietéticos sobre la salud humana. Enfermedades Infecciosas y Microbiología 21, 106-114.

EU (Europe Union Commission). 2005. Ban on antibiotics as growth promoters in animal feed enters into effect. Regulation 1831/2003/EC on additives for use in animal nutrition, replacing Directive 70/524/EEC on additives in feed-stuffs, 22 December. Brussels, Belgium.

European Commission (SCAN). 2005. Opinion of the Scientific Committee on Animal Nutrition on the criteria for assessing the safety of microorganisms resistant to antibiotics of human clinical and veterinary importance. European Commission, Brussels.

European Commission (SCAN). 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA Journal 587, 1-16.

European Commission. 2008. Technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. EFSA Journal 1-15.

European Medicines Agency (EMA). 2015. Sales of veterinary antimicrobial agents in 26 EU/EEA countries in 2013. Fifth ESVAC report ESVAC. EMA/387934/2015. London, UK.

FAO/WHO (Food and Agriculture Organization/World Health Organization) Working Group. 2002. Report Drafting guidelines for the evaluation of probiotics in food. London, Canada.

Fava, F., Lovegrove, J.A., Gitau, R., Jackson, K.G., Tuohy, K.M. 2006. The gut microbiota and lipid metabolism: Implications for human health and coronary heart disease. Current Medicinal Chemistry 13, 3005-3021.

Feinman, S.E. 1999. Antibiotics in animal feeds-drug resistance revisited. American Society for Microbiology News 64, 24-29.

Feld, L., Bielak, E., Hammer, K., Wilcks, A. 2009. Characterization of a small erythromycin resistance plasmid pLFE1 from the food-isolate *Lactobacillus plantarum* M345. Plasmid 61, 159-170.

Fernández, A.G., García, P.G., Balbuena, M.B. 1995. Olive fermentations. Biotechnology: Enzymes, Biomass, Food and Feed 9, 593-627.

Fernandes, P., Ferreira, B. S., Cabral, J.M.S. 2003. Solvent tolerance in bacteria: role of efflux pumps and cross-resistance with antibiotics. International Journal of Antimicrobial Agents 22, 211-216.

Fernández, L., Hancock, R.E. 2012. Adaptive and mutational resistance: Role of porins and efflux pumps in drug resistance. Clinical Microbiology Reviews 25, 661-681.

Fernández de Henestrosa, A.R., Ogi, T., Aoyagi, S., Chafin, D., Hayes, J.J., Ohmori, H., Woodgate, R. 2000. Identification of additional genes belonging to the LexA regulon in *Escherichia coli*. Molecular microbiology 35, 1560-1572.

Fernández-Díez, M.J. 1983. Olives. In: Reed, G. (Ed.). Food and Feed Production with Microorganisms. Verlag Chemie. Deerfield Beach, USA.

Ferrer, B., Dalmau, J. 2001. Alimentos Funcionales: Probióticos. Acta Pediatrica Española 59, 150-155.

Fines, M., Perichon, B., Reynolds, P., Sahm, D.F., Courvalin, P. 1999. VanE, a New Type of Acquired Glycopeptide Resistance in *Enterococcus faecalis* BM4405. Antimicrobial Agents and Chemotherapy 43, 2161-2164.

Fitzgerald, K.A., Davis, A., Russell, A.D. 1989. Uptake of 14C-chlorhexidine diacetate to *E. coli* and *P. aeruginosa* and its release by azolectin. FEMS Microbiology Letters 60, 327-332.

Food and Agricultural Organization of the United Nations and World Health Organization. 2002. Joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food. Food and Agricultural Organization of the United Nations. ftp://ftp.fao.org/es/esn/food/wgreport2.pdf.

Flórez, A.B., Delgado, S., Mayo, B. 2005. Antimicrobial susceptibility of lactic acid bacteria isolated from a cheese environment. Canadian Journal of Microbiology 51, 51-58.

Flórez, A.B., Ammor, M.S., Mayo, B. 2008. Identification of *tet(M)* in two *Lactococcus lactis* strains isolated from a Spanish traditional starter-free cheese made of raw milk and conjugative transfer of tetracycline resistance to lactococci and enterococci. International Journal of Food Microbiology 121, 189-194.

Floriano, B., Ruiz-Barba, J.L., Jiménez-Díaz, R. 1998. Purification and genetic characterization of enterocin I from *Enterococcus faecium* 6T1a, a novel antilisterial plasmid-encoded bacteriocin which does not belong to the pediocin family of bacteriocins. Applied and Environmental Microbiology 64, 4883-4890.

Floyd, J.L., Smith, K.P., Kumar, S.H., Floyd, J.T., Varela, M.F. 2010. LmrS is a multidrug efflux pump of the major facilitator superfamily from *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 54, 5406-5412.

Fons, M., Hégé, T., Ladiré, M., Raibaud, P., Ducluzeau, R., Maguin, E. 1997. Isolation and characterization of a plasmid from *Lactobacillus fermentum* conferring erythromycin resistance. Plasmid 37, 199-203.

Forbes, B.A. 2009. Diagnóstico microbiológico. Editorial Médica Panamericana. Buenos Aires, Argentina.

Forslund, K., Sunagawa, S., Kultima, J.R., Mende, D.R., Arumugam, M., Typas, A., Bork, P. 2013. Country-specific antibiotic use practices impact the human gut resistome. Genome Research 23, 1163-1169.

Foster, P.L. 2005. Stress responses and genetic variation in bacteria. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 569, 3-11.

Foster, P.L. 2007. Stress-induced mutagenesis in bacteria. Critical Reviews in Biochemistry and Molecular Biology 42, 373-397.

Fraise, A.P. 2002. Susceptibility of antibiotic-resistant cocci to biocides. Journal of Applied Microbiology 92, 158S-162S.

Frank, D.N., Amand, A.L.S., Feldman, R.A., Boedeker, E.C., Harpaz, N., Pace, N.R. 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proceedings of the National Academy of Sciences 104, 13780-13785.

Franklin, T.J., Snow, G.A. 2005. Biochemistry and molecular biology of antimicrobial drug action. Springer Science and Business Media. New York, USA.

Franko, B., Vaillant, M., Recule, C., Vautrin, E., Brion, J.P., Pavese, P. 2013. *Lactobacillus paracasei* endocarditis in a consumer of probiotics. Médecine et Maladies Infectieuses 43, 171-173.

Franz, C.M.A.P., Holzappel, W.H., Stiles, M.E., 1999. Enterococci at the crossroad of food safety. International Journal of Food Microbiology 47, 1-24.

Franz, C.M.A.P., Hummel, A., Holzapfel, W.H. 2005. Problems related to the safety assessment of lactic acid bacteria starter cultures and probiotics. Mitteilungen aus Lebensmitteluntersuchung und Hygiene 96, 39-65.

Franz, C.M.A.P., Cho, G.Y., Holzapfel, W.H. 2011. Probiotics: Taxonomy and technological features. In: Shah, N. P., Gomes da Cruz, A., de Assis Fonseca Faria J. (Eds.). Probiotic and Prebiotic Foods: Technology, Stability and Benefits to Human Health. Nova Science Publishers. Hauppauge, USA.

Fukao, M., Tomita, H., Yakabe, T., Nomura, T., Ike, Y., Yajima, N. 2009. Assessment of antibiotic resistance in probiotic strain *Lactobacillus brevis* KB290. Journal of Food Protection 72, 1923-1929.

Fukao, M., Oshima, K., Morita, H., Toh, H., Suda, W., Kim, S.W., Suzuki, S., Yakabe, T., Hattori, M., Yajima, N. 2013. Genomic analysis by deep sequencing of the probiotic *Lactobacillus brevis* KB290 harboring nine plasmids reveals genomic stability. PLoS ONE 8, e60521.

Fuller, R. 1991. Probiotics in human medicine. Gut 32, 439-442.

Ganjian, H., Nikokar, I., Tieshayar, A., Mostafaei, A., Amirmozafari, N., Kiani, S. 2012. Effects of salt stress on the antimicrobial drug resistance and protein profile of *Staphylococcus aureus*. Jundishapur Journal of Microbiology 5, 328-331.

Garrido Fernández, A., García García, P., Brenes Balbuena, M. 1995. Olive fermentations. In: Rehm, H.J., Reed, G. (Eds.). Enzymes, biomass, food and feed. VCH. New York, USA.

Garrido-Fernández, A., Fernández Díez, M.J., Adams, M.R. 1997. In: Table Olives: Production and Processing. Chapman and Hall. London, UK.

Garrigues, C., Johansen, E., Crittenden, R. 2013. Pangenomics-an avenue to improved industrial starter cultures and probiotics. Current Opinion in Biotechnology 24, 187-191.

Ge, B., Jiang, P., Han, F. 2007. Identification and antimicrobial susceptibility of lactic acid bacteria from retail fermented foods. Journal of Food Protection 70, 2606-2612.

Gennaro, A.R. 2003. Remington farmacia. Editorial Médica Panamericana. Buenos Aires, Argentina.

Gevers, D., Huys, G., Swings, J. 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiology Letters 205, 31-36.

Gevers, D., Danielsen, M., Huys, G., Swings, J. 2003. Molecular characterization of *tet(M)* genes in *Lactobacillus* isolates from different types of fermented dry sausage. Applied and Environmental Microbiology 69, 1270-1275.

Gfeller, K.Y., Roth, M., Meile, L., Teuber, M. 2003. Sequence and genetic organization of the 19.3-kb erythromycin- and dalfopristin-resistance plasmid pLME300 from *Lactobacillus fermentum* ROT1. Plasmid 50, 190-201.

Gilbert, P., Moore, L.E. 2005. Cationic Antiseptics: diversity of action under a common epithet. Journal of Applied Microbiology 99, 703-715.

Gillings, M.R. 2013. Evolutionary consequences of antibiotic use for the resistome, mobilome, and microbial pangenome. Frontiers in Microbiology 4, 1-9.

Giraffa, G., Chanishvili, N., Widyastuti, Y. 2010. Importance of lactobacilli in food and feed biotechnology. Research in Microbiology 161, 480-487.

Goerlich O., Quillardet A.T., Hofrung, M. 1989. Induction of the SOS response by hydrogen peroxide in various *E. coli* mutants with altered protection against oxidative DNA damage. Journal of Bacteriology 171, 6141-6147.

Gomes M.P. 1999. Agentes probióticos en alimentos: Aspectos fisiológicos y terapéuticos, y aplicaciones tecnológicas. Boletín de Biotecnología 64, 12-22.

Gómez-Lus, M.L., Calvo, A., Bouza, E., Prieto, J. 2005. Quimioterapia antiinfecciosa y antitumoral: Antibióticos y quimioterápicos. Generalidades. In: Velásquez: Farmacología básica y clínica. Editorial Panamericana. Buenos Aires, Argentina.

Gómez-Lus, M.L., Calvo, A., Prieto, J. 2008. Quimioterapia antiinfecciosa y antitumoral: Antibióticos y quimioterápicos. Generalidades. In: Velásquez: Farmacología básica y clínica. Editorial Panamericana. Buenos Aires, Argentina.

Grompone, G., Martorell, P., Llopis, S., González, N., Genovés, S., Mulet, A.P., Fernández-Calero, T., Tiscornia, I., Bollati-Fogolin, M., Chambaud, I., Foligné, B., Monserrat, A., Ramón, D. 2012. Anti-inflammatory *Lactobacillus rhamnosus* CNCM I-3690 strain protects against oxidative stress and increases lifespan in Caenorhabditis elegans. PloS one 7, e52493.

Gorg, A., Weiss, W., Dunn, M.J. 2004. Current two-dimensional electrophoresis technology for proteomics. Proteomics 4, 3665-3685.

Granato, D., Branco, G. F., Nazzaro, F., Cruz, A.G., Faria, J.A.F. 2010. Functional foods and non dairy probiotic food development: trends, concepts, and products. Comprehensive Reviews in Food Science and Food Safety 9, 292-302.

Gueimonde, M., Collado, M.D.C. 2012. Metagenomics and probiotics. Clinical Microbiology and Infection 18, 32-34.

Hammes, W.P., Weiss, N., Holzapfel, W. 1992. The genera *Lactobacillus* and *Carnobacterium*. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., Schleifer, K.H. (Eds.). The Prokaryotes. Springer. New York, USA.

Hammes, W.P., Vogel, R.F. 1995. The genus *Lactobacillus*. In: Wood, B.J.B., Holzapfel, W.H. (Eds.). The genera of lactic acid bacteria. Blackie Academic and Professional. Glasgow, UK.

Hammes, WP., Vogel, R.F. 1995. The genus *Lactobacillus*. In: Wood, B.J.B., Holzapfel W.H. (Eds.). The Genera of Lactic Acid Bacteria, Vol. 2. Blackie Academic and Professional. Glasgow, UK.

Hamon, E., Horvatovich, P., Izquierdo, E., Bringel, F., Marchioni, E., Aoude-Werner, D., Ennahar, S. 2011. Comparative proteomic analysis of *Lactobacillus plantarum* or the identification of key proteins in bile tolerance. BMC Microbiology 11, 63.

Han, J.H., Li, X.F., Gao, W.H., Walczak, P., Zhang, B.L., Jia, Y.M. 2013. Susceptibility of *Lactobacillus pentosus* strains isolated from fermented products to streptomycin and kanamycin. International Food Research Journal 20, 1927-1931.

Handwerger, S., Pucci, M.J., Volk, K.J., Liu, J., Lee, M.S. 1994. Vancomycin-resistant *Leuconostoc mesenteroides* and *Lactobacillus casei* synthesize cytoplasmic peptidoglycan precursors that terminate in lactate. Journal of Bacteriology 176, 260-264.

Hansen, E.B. 2002. Commercial bacterial starter cultures for fermented foods of the future. International Journal of Food Microbiology 78, 119-131.

Hao, W.L., Lee Y.K. 2004. Microflora of the gastrointestinal tract. Public Health Microbiology: Methods and Protocols 268, 491-502.

Harty, D.W.S., Oakey, H.J., Patrikakis, M., Hume, E.B., Knox, K.W. 1994. Pathogenic potential of lactobacilli. International Journal of Food Microbiology 24, 179-189.

Hawkey, P.M. 1998. The origins and molecular basis of antibiotic resistance. British Medical Journal 317, 657.

Heath, R.J., White, S.W., Rock, C.O. 2002. Inhibitors of fatty acid synthesis as antimicrobial chemotherapeutics. Applied Microbiology and Biotechnology 58, 695-703.

Hebert, E.M., Raya, R.R., Brown, L., Font de Valdez, G., Savoy de Giori, G., Taranto, M.P. 2013. Genome sequence of the cheese-starter strain *Lactobacillus delbrueckii* subsp. *lactis* CRL 581. Genome Announcements 1, e00602-13.

Heller, K.J. 2001. Probiotic bacteria in fermented foods: Product characteristics and starter organisms. The American Journal of Clinical Nutrition 73, 374s-379s.

Herreros, M.A., Sandoval, H., González, L., Castro, J.M, Fresno, J.M., Tornadijo, M.E. 2005. Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from armada cheese (a Spanish goats' milk cheese). Food Microbiology 22, 455-459.

Hesketh, A., Hill, C., Mokhtar, J., Novotna, G., Tran, N., Bibb, M., Hong, H.J. 2011. Genome-wide dynamics of a bacterial response to antibiotics that target the cell envelope. BMC Genomics 12, 226.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Sanders, M.E., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C., Sanders, M.E. 2014. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology and Hepatology 11, 506-514.

Hleba, L., Kačániová, M., Pavelková, A., Čuboň, J. 2012. Antibiotic resistance of lactobacilli strains isolated from milk and milk products from middle Slovakia. Journal of Microbiology, Biotechnology and Food Sciences 2, 253-262.

Hobby, G.L., Meyer, K., Chaffee, E. 1942. Observations on the Mechanism of Action of Penicillin. Experimental Biology and Medicine 50, 281-285.

Hobby, G.L., Dawson, M.H. 1946. Effect of Sulfonamides on Action of Penicillin. Journal of Bacteriology 51, 447.

Hochwind, K., Weinmaier, T., Schmid, M., van Hemert, S., Hartmann, A., Rattei, T., Rothballer, M. 2012. Draft genome sequence of *Lactobacillus casei* W56. Journal of Bacteriology 194, 6638.

Holah, J.T. 2000. Cleaning and disinfection. In: Dennis, C., Stringer M.F. (Eds.), Chilled Foods: a Comprehensive Guide. Woodhead Publishing. Cambridge, UK.

Holtzel, A., Ganzle, M.G., Nicholson, G.J., Hammes, W.P., Jung, G. 2000. The first low molecular-weight antibiotic from lactic acid bacteria: reutericyclin, a new tetramic acid. Angewandte Chemie International Edition 39, 2766-2768.

Holzapfel, W.H., Geisen, R., Schillinger, U. 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. International Journal of Food Microbiology 24, 343-362.

Holzapfel, W.H. 2002. Appropriate starter culture technologies for small-scale fermentation in developing countries. International Journal of Food Microbiology 75, 197-212.

Holzapfel, W.H., Wood, B.J. 2014. Lactic acid bacteria: biodiversity and taxonomy. Wiley Blackwell. Hoboken, USA.

Homayouni, A., Bastani, P., Ziyadi, S., Mohammad-Alizadeh-Charandabi, S., Ghalibaf, M., Mortazavian, A.M., Mehrabany, E.V. 2014. Effects of probiotics on the recurrence of bacterial vaginosis: a review. Journal of Lower Genital Tract Disease 18, 79-86.

Honi, U., Sabrin, F., Islam, T., Islam, E., Billah, M., Islam, K.D. 2013. Enzymatic activity and antibiotic resistance profile of *Lactobacillus paracasei ssp. paracasei*-1 isolated from regional yogurts of Bangladesh. Journal of Microbiology Biotechnology and Food Sciences 3, 235-239.

Horvath P., Barrangou R. 2010. CRISPR/Cas, the immune system of bacteria and archaea. Science 327, 167-170.

Hou, C., Wang, Q., Zeng, X., Yang, F., Zhang, J., Liu, H., Ma, X., Qiao, S. 2013. Complete genome sequence of *Lactobacillus reuteri* I5007, a probiotic strain isolated from healthy piglet. Journal of Biotechnology 179, 63-64.

Huet, A.A., Raygada, J.L., Mendiratta, K., Seo, S.M., Kaatz, G.W. 2008. Multidrug efflux pump overexpression in *Staphylococcus aureus* after single and multiple *in vitro* exposures to biocides and dyes. Microbiology 154, 3144-3153.

Hugenholtz, P. 1998. The genera of lactic acid bacteria.Blackie Academic and Professional. London, UK.

Hummel, A.S., Hertel, C., Holzapfel, W.H., Franz, C.M.A.P. 2007. Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. Applied and Environmental Microbiology 73, 730-739.

Hunt, M., De Silva, N., Otto, T.D., Parkhill, J., Keane, J.A., Harris, S.R. 2015. Circlator: automated circularization of genome assemblies using long sequencing reads. Genome Biology 16,1-10.

Huovinen, P. 1987. Trimethoprim resistance. Antimicrobial Agents and Chemotherapy 31, 1451-1456.

Hurst, A., Hoover, D.G. 1993. Nisin. In: Davidson, P.M., Branan, A.L. (Eds.) Antimicrobials in foods. Marcel Dekker Inc. New York, USA.

Hurtado, A., Reguant, C., Bordons, A., Rozès, N. 2012. Lactic acid bacteria from fermented table olives. Food Microbiology 31, 1-8.

Hyatt, D., Chen, G.L., LoCascio, P.F, Land, M.L, Larimer, F.W., Hauser, L.J. 2010. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics 11, 119

IOOC (International Olive Oil Council). 1980. Unified qualitative standard applying to table olives in international trade. Madrid, Spain.

IOOC (International Olive Oil Council). 2005. Table olive. 93rd International Olive Oil Council Session. Madrid, Spain.

Isidori, M., Lavorgna, M., Nardelli, A., Pascarella, L., Parrella, A. 2005. Toxic and genotoxic evaluation of six antibiotics on non-target organisms. Science of the Total Environment 345, 87-98.

ISO (International Organization for Standardization). 2010. Standard milk and milk products-determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB). *ISO 10932/IDF 233 standard*. ISO, Geneva, Switzerland.

Isolauri, E., Sutas, Y., Kankaanpaa, P., Arvilommi, H., Salminen, S. 2001. Probiotics: effects on immunity. The American Journal of Clinical Nutrition 73, 444S-450S.

Izquierdo, E., Horvatovich, P., Marchioni, E., Aoude-Werner, D., Sanz, Y., Ennahar, S. 2009. 2-DE and MS analysis of key proteins in the adhesion of *Lactobacillus plantarum*, a first step toward early selection of probiotics based on bacterial biomarkers. Electrophoresis 30, 949-956.

Jakob, U., Gaestel, M., Engel, K., Buchner, J. 1993. Small heat shock proteins are molecular chaperones. Journal of Biological Chemistry 268, 1517-1520.

Jara Ramírez, M., Campoy Sánchez, S., Barbé García, J. 2005. Análisis de la composición del regulón LexA en el dominio Bacteria. Tesis Doctoral, Universitat Autònoma de Barcelona.

Jayashree, S., Pooja, S., Pushpanathan, M., Vishnu, U., Sankarasubramanian, J., Rajendhran, J., Gunasekaran, P. 2013. Genome sequence of *Lactobacillus fermentum* strain MTCC 8711, a probiotic bacterium isolated from yogurt. Genome Announcements 1, e00770-13.

Jeffrey, D.J. 1995. Chemicals used as disinfectants: Active ingredients and enhancing additives. Revue Scientifique et Technique (International Office of Epizootics) 14, 57-74.

Jensen, L.B., Ahrens, P., Dons, L., Jones, R.N., Hammerum, A.M., Aarestrup, F.M. 1998. Molecular analysis of Tn*1546* in Enterococcus faecium isolates from animals and humans. Journal of Clinical Microbiology 36, 437-442.

Jiménez-Díaz, R., Rios-Sanchez, R.M., Desmazeaud, M., Ruiz-Barba, J.L., Piard, J.C. 1993. Plantaricins S and T, two new bacteriocins produced by *Lactobacillus plantarum* LPCO10 isolated from a green olive fermentation. Applied and Environmental Microbiology 59, 1416-1424.

Jiménez-Pranteda, M.L., Pérez-Davó, A., Monteoliva-Sánchez, M., Ramos-Cormenzana, A., Aguilera, M. 2015. Food omics validation: towards understanding key features for gut microbiota, probiotics and human health. Food Analytical Methods 8, 272-289.

Jokovic, N., Nikolic, M., Begovic, J., Jovcic, B., Savic, D., Topisirovic, L. 2008. A survey of the lactic acid bacteria isolated from Serbian artisanal dairy product kajmak. International Journal of Food Microbiology 125, 305-311.

Jørgensen, C.M., Hammer, K., Jensen, P.R., Martinussen, J. 2004. Expression of the *pyrG* gene determines the pool sizes of CTP and dCTP in *Lactococcus lactis*. Journal of Clinical Microbiology 271, 2438-2445.

Kaatz, G.W., McAleese, F., Seo, S.M. 2005. Multidrug resistance in *Staphylococcus aureus* due to overexpression of a novel multidrug and toxin extrusion (MATE) transport protein. Antimicrobial Agents and Chemotherapy 49, 1857-1864.

Kaila, M., Isolauri, E., Soppi, E., Virtanen, E., Laine, S., Arvilommi, H. 1992. Enhancement of the circulating antibody secreting cell response in human diarrhea by a human lactobacillus strain. Pediatric Research-Nature 32, 141-144.

Kaleta, P., O'Callaghan, J., Fitzgerald, G.F., Beresford, T.P., Ross, R.P. 2010. Crucial role for insertion sequence elements in *Lactobacillus helveticus* evolution as revealed by interstrain genomic comparison. Applied and Environmental Microbiology 76, 212-220.

Kant, R., Paulin, L., Alatalo, E., de Vos, W.M., Palva, A. 2011a. Genome sequence of *Lactobacillus amylovorus* GRL1118, isolated from pig ileum. Journal of Bacteriology 193, 3147-3148.

Kant, R., Blom, J., Pavla, A., Siezen, R.J., de Vos, W.M. 2011b. Comparative genomics of *Lactobacillus*. Microbial Biotechnology 4, 323-332

Karatzas, K.A., Webber, M.A., Jorgensen, F., Woodward, M.J., Piddock, L.J., Humphrey, T.J. 2007. Prolonged treatment of *Salmonella enterica* serovar *Typhimurium* with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. Journal of Antimicrobial Chemotherapy 60, 947-955.

Karatzas, K.A., Randall, L.P., Webber, M., Piddock, L.J.V., Humphrey, T.J., Woodward, M.J., Coldham, N.G. 2008. Phenotypic and proteomic characterization of multiply antibiotic-resistant variants of *Salmonella enterica* serovar *Typhimurium* selected following exposure to disinfectants. Applied and Environmental Microbiology 74, 1508-1516.

Karlyshev, A.V., Raju, K., Abramov, V.M. 2013. Draft genome sequence of *Lactobacillus fermentum* strain 3872. Genome Announcements 1, e01006-13.

Karlyshev, A.V., Abramov, V.M. 2014. Draft genome sequence of *Lactobacillus plantarum* 2165. Genome Announcements 2, e01179-13.

Kastner, S., Perreten, V., Bleuler, H., Hugenschmidt, G., Lacroix, C., Meile, L. 2006. Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food. Systematic and Applied Microbiology 29, 145-155.

Katla, A.K., Kruse, H., Johnsen, G., Herikstad, H. 2001. Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products. International Journal of Food Microbiology 67, 147-152.

Kayahan Kıvanç, A., Tahtacı, S., Kıvanç, M. 2014. Inhibitory activity of lactic acid bacteria against *Streptococcus mutans* and its biofilm. In: Mendez-Vilas, A. (Ed.). Industrial, medical, and environmental applications of microorganisms, current status and trends. Wageningen Academic Publishers. Madrid, Spain.

Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., Fakiri, E.M. 2013. Health benefits of probiotics: a review. ISRN Nutrition 2013. (Article ID 481651, 7 pages).

Keijser, B.J.F., Zaura, E., Huse, S.M., Van Der Vossen, J.M.B.M., Schuren, F.H.J., Montijn, R.C., ten Cate, J.M., Crielaard, W. 2008. Pyrosequencing analysis of the oral microflora of healthy adults. Journal of Dental Research, 87, 1016-1020.

Khachatourians, G.G. 1998. Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. Canadian Medical Association Journal 159, 1129-1136.

Kim, K.S.,Morrison, J.O., Bayer, A.S. 1982. Deficient autolytic enzyme activity in antibiotictolerant lactobacilli. Infection and Immunity 36, 582-585.

Klaenhammer, T.R., de Vos, W.M. 2011. An incredible scientific journey. The evolutionary tale of the lactic acid bacteria. In: Ledeboer, A., Hugenholtz, J., Kok, J., Konings, W., Wouters J. (Eds.). The 10th LAB symposium. Thirty years of research on lactic acid bacteria. Media Labs 24, 1-11.

Klare, I., Konstabel, C., Müller-Bertling, S., S., Reissbrodt, R., Huys, G., Vancanneyt, M., Swings, J., Goossens, H., Witte, W. 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. Applied and Environmental Microbiology 71, 8982-8986.

Klare, I., Konstabel, C., Werner, G., Huys, G., Vankerckhoven, V., Kahlmeter, G., Hildebrandt, B., Müller-Bertling, S., Witte, W., Goossens, H. 2007. Antimicrobial susceptibilities of *Lactobacillus, Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. Journal of Antimicrobial Chemotherapy 59, 900-912.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., Tarchini, R., Peters, S.A., Sandbrink, H.M., Fiers, M.W.E.J., Stiekema, W., Lankhorst, R.M.K., Bron, P.A., Hoffer, S.M., Groot, M.N.N., Kerkhoven, R., de

Vries, M., Ursing, B., de Vos, W.M., Siezen, R.J. 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proceedings of the National Academy of Sciences of the United States of America 100, 1990-1995.

Klein, G. 2011. Antibiotic resistance and molecular characterization of probiotic and clinical *Lactobacillus* strains in relation to safety aspects of probiotics. Foodborne Pathogens and Disease 8, 267-281.

Klein, G., Zill, E., Schindler, R., Louwers, J. 1998. Peritonitis associated with vancomycin-resistant *Lactobacillus rhamnosus* in a continuous ambulatory peritoneal dialysis patient: Organism identification, antibiotic therapy, and case report. Journal of Clinical Microbiology 36, 1781-1783.

Klein, G., Hallmann, C., Casas, I.A., Abad, J., Louwers, J., Reuter, G. 2000. Exclusion of *vanA, vanB and vanC* type glycopeptide resistance in strains of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* used as probiotics by polymerase chain reaction and hybridization methods. Journal of Applied Microbiology 89, 815-824.

Kohanski, M.A., DePristo, M.A., Collins, J.J. 2010. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Molecular Cell 37, 311-320.

Korhonen, J. 2010. Antibiotic Resistance of Lactic Acid Bacteria. Dissertation in Forestru and Natural Sciences. Publications of the University of Eastern Finland, Kuopio, Finland.

Koryszewska-Bagińska, A., Bardowski, J., Aleksandrzak-Piekarczyk, T. 2014. Genome sequence of the probiotic strain *Lactobacillus rhamnosus* (formerly *Lactobacillus casei*) LOCK908. Genome Announcements 2, e00120-14.

Kotra, L.P., Haddad, J., Mobashery, S. 2000. Aminoglycosides: Perspectives on Mechanisms of Action and Resistance and Strategies to Counter Resistance. Antimicrobial Agents and Chemotherapy 44, 3249-3256.

Kruis, W., Frič, P., Pokrotnieks, J., Lukáš, M., Fixa, B., Kaščák, M., Kamm, M.A., Weismueller, J., Beglinger, C., Stolte, M., Schulze, J., Wolff, C. 2004. Maintaining remission of ulcerative colitis with the probiotic *Escherichia coli* Nissle 1917 is as effective as with standard mesalazine. Gut 53, 1617-1623.

Kümmerer, K. 2009. Antibiotics in the aquatic environment-a review-part I. Chemosphere 75, 417-434.

Kuramitsu, H.K., He, X., Lux, R., Anderson, M.H., Shi, W. 2007. Interspecies interactions within oral microbial communities. Microbiology and Molecular Biology Reviews 71, 653-670.

Ladero, V., Álvarez-Sieiro, P., Redruello, B., del Rio, B., Linares, D.M., Cruz Martin, M., Fernández, M., Álvarez, M.A. 2013. Draft genome sequence of *Lactobacillus plantarum* strain IPLA 88. Genome Announcements 1, e00524-13.

Ladero, V., Herrero-Fresno, A., Martínez, N., del Río, B., Linares, D.M., Fernández, M., Martín, M.C., Álvarez, M.A. 2014. Genome sequence analysis of the biogenic amine-degrading strain *Lactobacillus casei* 5b. Genome Announcements 2, e01199-13.

Lagesen, K., Hallin, P., Rødland, E.A., Stærfeldt, H.H., Rognes, T., Ussery, D.W. 2007. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Research 35, 3100-31008.

Lamontanara, A., Caggianiello, G., Orrù, L., Capozzi, V., Michelotti, V., Bayjanov, J.R., Renckens, B., van Hijum, S.A.F.T., Cattivelli, L., Spano, G. 2015. Draft genome sequence of *Lactobacillus plantarum* Lp90 isolated from wine. Genome Announcements 3, e00097-15.

Lapsiri, W., Nitisinprasert, S., Wanchaitanawong, P. 2011. *Lactobacillus plantarum* strains from fermented vegetables as potential probiotics. Kasetsart Journal (Natural Science) 45, 1071-1082.

Laslett, D., Canback, B. 2004. ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences. Nucleic Acids Research 32, 11-16.

Lau, S. 2014. Oral application of bacterial lysate in infancy diminishes the prevalence of atopic dermatitis in children at risk for atopy. Beneficial Microbes 5, 147-149.

Laureti, L., Matic, I., Gutierrez, A. 2013. Bacterial responses and genome instability induced by subinhibitory concentrations of antibiotics. Antibiotics 2, 100-114.

Lavanya, B., Sowmiya, S., Balaji, S., Muthuvelan, B. 2011. Screening and characterization of lactic acid bacteria from fermented milk. British Journal of Dairy Sciences 2, 5-10.

Lavilla Lerma, L., Benomar, N., Casado Muñoz, M.C., Gálvez, A., Abriouel, H. 2015. Correlation between antibiotic and biocide resistance in mesophilic and psychrotrophic *Pseudomonas* spp. isolated from slaughterhouse surfaces throughout meat chain production. Food Microbiology 51, 33-44.

Leal-Sánchez, M.V., Ruiz-Barba, J.L., Sánchez, A.H., Rejano, L., Jiménez-Dıaz, R., Garrido, A. 2003. Fermentation profile and optimization of green olive fermentation using *Lactobacillus plantarum* LPCO10 as a starter culture. Food Microbiology 20, 421-430.

Lebeer, S., Vanderleyden, J., De Keersmaecker, S.C. 2008. Genes and molecules of lactobacilli supporting probiotic action. Microbiology and Molecular Biology Reviews 72, 728–764.

Lee, K., Lee, H.G., Choi, Y.J. 2008. Proteomic analysis of the effect of bile salts on the intestinal and probiotic bacterium *Lactobacillus reuteri*. Journal of Biotechnology 137, 14-19.

Lee, J.H., Chae, J.P., Lee, J.Y., Lim, J.S., Kim, G.B. 2011. Genome sequence of *Lactobacillus johnsonii* PF01, isolated from piglet feces. Journal of Bacteriology 193, 5030-5031.

Lepargneur, J., Rousseau, V. 2002. Role protecteur de la flore de Doderlein. Journal de Gynécologie Obstétrique et Biologie de la Reproduction 31, 485-494.

Leroy, F., De Vuyst, L. 1999. Temperature and pH conditions that prevail during fermentation of sausages are optimal for production of the antilisterial bacteriocin sakacin K. Applied and Environmental Microbiology 65, 974-981.

Leroy, F., De Vuyst, L. 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends in Food Science and Technology 15, 67-78.

Levy, C.W., Roujeinikoval, A., Sedelnikova, S., Baker, P.J., Stuitje, A.R., Slabas, A.R., Rice, D.W., Rafferty, J.B. 1999. Molecular basis of triclosan activity. Nature 398, 383-384.

Levy, S.B., Marshall, B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nature medicine 10, 122-129.

Ley, R.E., Bäckhed, F., Turnbaugh, P., Lozupone, C.A., Knight, R.D., Gordon, J.I. 2005. Obesity alters gut microbial ecology. Proceedings of the National Academy of Sciences of the United States of America 102, 11070-11075.

Ley, R.E., Peterson, D.A., Gordon, J.I. 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124, 837-848.

Li, X.Z., Nikaido, H. 2004. Efflux-mediated drug resistance in bacteria. Drugs 64, 159-204.

Li, X.Z., Nikaido, H. 2009. Efflux-mediated drug resistance in bacteria. Drugs 69, 1555-1623.

Li, X., Gu, Q., Lou, X., Zhang, X., Song, D., Shen, L., Zhao, Y. 2013. Complete genome sequence of the probiotic *Lactobacillus plantarum* strain ZJ316. Genome Announcements 1, e00094-13.

Liasi, S.A., Azmi, T.I., Hassan, M.D., Shuhaimi, M., Rosfarizan, M., Ariff, A.B. 2009. Antimicrobial activity and antibiotic sensitivity of three isolates of lactic acid bacteria from fermented fish product, Budu. Malaysian Journal of Microbiology 5, 33-37.

Lim, E.M., Ehrlich, S.D., Maguin, E. 2000. Identification of stress-inducible proteins in *Lactobacillus delbrueckii* subsp. *bulgaricus*. Electrophoresis 21, 2557–2561.

Lim, H.I., Lee, J., Jang, J.Y., Park, H.W., Choi, H.J., Kim, T.W., Kang, M.R., Lee, J.H. 2014. Draft genome sequence of *Lactobacillus sakei* strain wikim 22, isolated from Kimchi in Chungcheong Province, South Korea. Genome Announcements 2, e01296-14.

Lin, C.F., Fung, Z.F., Wu, C.L., Chung, T.C. 1996. Molecular characterization of a plasmid-borne (pTC82) chloramphenicol resistance determinant (*cat*-TC) from *Lactobacillus reuteri* G4. Plasmid 36, 116-124.

Lin, X., Kang, L., Li, H., Peng, X. 2014. Fluctuation of multiple metabolic pathways is required for *Escherichia coli* in response to chlortetracycline stress. Molecular BioSystems 10, 901-908.

Liu, C., Zhang, Z., Dong, K., Yuan, J., Guop, X. 2009. Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. Biomedical and Environmental Sciences 22, 401-412.

Liu, S.B., Qiao, L.P., He, H.L., Zhang, Q., Chen, X.L., Zhou, W.Z., Zhou, B.C., Zhang, Y.Z. 2011. Optimization of fermentation conditions and rheological properties of exopolysaccharide produced by deep-sea bacterium *Zunongwangia profunda* SM-A87. PLoS ONE 6, e26825.

Livermore, D.M. 1995. beta-Lactamases in laboratory and clinical resistance. Clinical Microbiology Reviews 8, 557-584.

López Aguayo, M.C., Grande Burgos, M.J., Lucas López, R., Gálvez, A. 2010. Resistencia a biocidas de diferentes cepas de *Echerichia coli*. Anales de la Real Academia de Ciencias Veterinarias de Andalucía Oriental 23, 121-136.

Lorenzo, P., Moreno, A., Leza, J.C., Lizasoain, I., Moro, M.A., Portolés, A. 2008. Farmacología Básica y Clínica. Editorial Médica Panamericana. Madrid, Spain.

Luh, K.T., Hsueh, P.R., Teng, L.J., Pan, H.J., Chen, Y.C., Lu, J.J., Wu, J.J., Ho, S.W. 2000. Quinupristindalfopristin resistance among Gram-positive bacteria in Taiwan. Antimicrobial Agents and Chemotherapy 44, 3374-3380.

Ma, D., Cook, D.N., Alberti, M., Pon, N.G., Nikaido, H., Hearst, J.E. 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Molecular Microbiology 16, 45-55.

Madigan, M.T., Martinko M. Parker J. 2011. Brock: Biología de los Microorganismos. Pearson Educación. Madrid, Spain.

Madsen, K., Carnish, A., Soper, P., McKaigney, C., Jijon, H., Yachimec, C., Doyle, J., Jewell, L., De Simone, C. 2001. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology 121, 580-591.

Maietti, L., Bonvini, B., Huys, G., Giraffa, G. 2007. Incidence of antibiotic resistance and virulence determinants among *Enterococcus italicus* isolates from dairy products. Systematic and Applied Microbiology 30, 509-517.

Maillard, J.Y. 2005. Antimicrobial biocides in the health care environment: efficacy, policies, management and perceived problems. Journal of Therapeutics and Clinical Risk Management 1, 307-320.

Maisonneuve, E., Gerdes, K. 2014. Molecular mechanisms underlying bacterial persisters. Cell 157, 539-548.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D.M., Hawkinse, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.H., Díaz-Muñiz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimerd, B., Millsd, D. 2006.

Comparative genomics of the lactic acid bacteria. Proceedings of the National Academy of Sciences of the United States of America 103, 15611-15616.

Maldonado A., Ruiz-Barba J.L., Jiménez-Díaz R. 2003. Purification and genetic characterization of plantaricin NC8, a novel coculture-inducible two-peptide bacteriocin from *Lactobacillus plantarum* NC8. Applied and Environmental Microbiology 69, 383-389

Maldonado, A., Ruiz-Barba, J. L., Jiménez-Díaz, R. 2004. Production of plantaricin NC8 by *Lactobacillus plantarum* NC8 is induced in the presence of different types of gram-positive bacteria. Archives of Microbiology 181, 8-16.

Maldonado-Barragán, A., Caballero-Guerrero, B. Lucena-Padrós, H., Ruiz-Barba, J.L. 2011. Genome sequence of *Lactobacillus pentosus* IG1, a strain isolated from Spanish-style green olive fermentations. Journal of Bacteriology 193, 5605.

Madsen, K.L. 2001. The use of probiotics in gastrointestinal disease. Canadian Journal of Gastroenterology/Journal Canadien de Gastroenterologie 15, 817-822.

Malik, S., Siezen, R.J., Renckens, B., Vaneechoutte, M., Vanderleyden, J., Lebeer, S. 2014. Draft genome sequence of *Lactobacillus plantarum* CMPG5300, a human vaginal isolate. Genome Announcements, 2, e01149-14.

Mangalappalli-Illathu, A.K., Korber, D.R. 2006. Adaptive resistance and differential protein expression of *Salmonella enterica* serovar Enteritidis biofilms exposed to benzalkonium chloride. Antimicrobial Agents and Chemotherapy 50, 3588-3596.

Marchetti, M.L., Errecalde, J., Mestorino, O.N. 2011. Resistencia bacteriana a los antimicrobianos ocasionada por bombas de eflujo. Analecta Veterinaria, 31.

Marshall, B.M., Ochieng, D.J., Levy, S.B. 2009. Commensals: Underappreciated reservoir of antibiotic resistance. Microbe 4, 231-238.

Martin, F.P.J., Wang, Y., Sprenger, N., Yap, I.K., Lundstedt, T., Lek, P., Rezzi, S., Ramadan, Z., van Bladeren, P., Fay, L.B., Kochhar, S., Lindon, J.C., Holmes, E., Nicholson, J.K. 2008. Probiotic modulation of symbiotic gut microbial–host metabolic interactions in a humanized microbiome mouse model. Molecular Systems Biology 4, 157-171.

Martineau, F., Picard, F.J., Grenier, L., Roy, P.H., Ouellette, M., Bergeron, M.G. 2000. Multiplex PCR assays for the detection of clinically relevant antibiotic resistance genes in staphylococci isolated from patients infected after cardiac surgery. Journal of Antimicrobial Chemotherapy 46, 527-533.

Martins, A., Makita, M., Fernandes, M.H., Fernandes, M.J., Barreto, A.S., Fraqueza, M.J. 2013. Phenotypic and genotypic characterization of *Lactobacillus sakei* isolated from portuguese fermented meat products for tetracycline resistance. Poster 59th ICoMST.

Mater, D.D., Langella, P., Corthier, G., Flores, M.J. 2008. A probiotic *Lactobacillus* strain can acquire vancomycin resistance during digestive transit in mice. Journal of Molecular Microbiology and Biotechnology 14, 123-127.

Mathur, S., Singh, R. 2005. Antibiotic resistance in food lactic acid bacteria. International Journal of Food Microbiology 105, 281-295.

Matsumoto, M., Kurihara, S. 2011. Probiotics-induced increase of large intestinal luminal polyamine concentration may promote longevity. Medical Hypotheses 77, 469-472.

Massi, M., Vitali, B., Federici, F., Matteuzzi, D., Brigidi, P. 2003. Identification method based on PCR combined with automated ribotyping for tracking probiotic *Lactobacillus* strains colonizing the human gut and vagina. Journal of Applied Microbiology 96, 777-786.

Mastromarino, P., Brigidi, P., Macchia, S., Maggi, L., Pirovano, F., Trinchieri, V., Conte, U., Matteuzzi, D. 2002. Characterization and selection of vaginal *Lactobacillus* strains for the preparation of vaginal tablets. Journal of Applied Microbiology 93, 884-893.

Mazé, A., Boel, G., Zúñiga, M., Bourand, A., Loux, V., Yebra, M.J., Monedero, V., Correia, K., Jacques, N., Beaufils, S., Poncet, S., Joyet, P., Milohanic, E., Casarégola, S., Auffray, Y., Pérez-Martínez, G., Gibrat, J.F., Zagorec, M., Francke, C., Hartke, A., Deutscher, J. 2010. Complete genome sequence of the probiotic *Lactobacillus casei* strain BL23. Journal of Bacteriology 192, 2647-2648.

Mazmanian, S.K., Liu, G., Ton-That, H., Schneewind, O. 1999. *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. Science 285, 760-763.

McBain, A.J., Rickard, A.H., Gilbert, P. 2002. Possible implications of biocide accumulation in the environment on the prevalence of bacterial antibiotic resistance. Journal of Industrial Microbiology and Biotechnology 29, 326-330.

McDonnell, G., Russell, A.D. 1999. Antiseptics and disinfectants: activity, action, and resistance. Clinical Microbiology Reviews 12, 147-179.

McGroaorty, J.A. 1993. Probiotic use of lactobacilli in the human female urogenital tract. FEMS Immunology and Medical Microbiology 6, 251-264.

McLeod, A., Brede, D.A., Rud, I., Axelsson, L. 2013. Genome sequence of *Lactobacillus sakei* subsp. *sakei* LS25, a commercial starter culture strain for fermented sausage. Genome Announcements, 1, e00475-13.

McMahon, M.A., McDowell, D.A., Blair, I.S. 2007. The pattern of pleiomorphism in stressed *Salmonella* Virchow populations is nutrient and growth phase dependent. Letters in Applied Microbiology 45, 276-281.

McMahon, M.A.S., Xu, J., Moore, J.E., Blair, I.S., McDowell, D.A. 2007a. Environmental stress and antibiotic resistance in food-related pathogens. Applied and Environmental Microbiology 73, 211-217.

McMahon, M.A.S., Blair, I.S., Moore, J.E., McDowell, D.A. 2007b. The rate of horizontal transmission of antibiotic resistance plasmids is increased in food preservation-stressed bacteria. Journal of Applied Microbiology 103, 1883-1888.

McManus, P. 1999. Uso de los antibioticos en el control de enfermedades de las plantas. Enfermedades Infecciosas y Microbiología 19, 192-196.

McManus, P.S., Stockwell, V.O., Sundin, G.W., Jones, A.L. 2002. Antibiotic use in plant agriculture. Annual Review of Phytopathology 40, 443-465.

McMurray, L.M., Oethinger, M., Levy, S.B. 1998. Triclosan targets lipid synthesis. Nature 394, 531-532.

McNulty, N., Yatsunenko, T., Hsiao, A., Faith, J., Muegge, B., Goodman, A. 2011. The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. Science Translational Medicine 3, 106.

Medina Asensio, J. 2000. Guía de antimicrobianos y tratamiento de las infecciones. Ediciones Díaz de Santos. Madrid, Spain.

Mensa J., Gatell, J.M. 2008. Guia de terapeutica antimicrobiana. Masson. Barcelona, Spain.

Metchnikoff, E. 1907. The Prolongation of Life. Putnam's Sons, New York and London.

Meyer, B., Cookson, B. 2010. Does microbial resistance or adaptation to biocides create a hazard in infection prevention and control?. Journal of Hospital Infection 76, 200-205.

Miele, A., Bandera, M., Goldstein, B.P. 1995. Use of primers selective for vancomycin resistance genes to determine *van* genotype in enterococci and to study gene organization in VanA isolates. Antimicrobial Agents and Chemotherapy 39, 1772-1778.

Miettinen, M., Vuopio-Varkila, J., Varkila, K. 1996. Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. Infection and Immunity 64, 5403-5405.

Mills, S., Stanton, C., Fitzgerald, G.F., Ross, R.P. 2011. Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. Microbial Cell Factories 10, S19.

Moken, M.C., McMurry, L.M., Levy, S.B. 1997. Selection of multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the *mar* and *acrAB* loci. Antimicrobial Agents and Chemotherapy 41, 2770-2772.

Mokhbi, A., Kaid-Harche, M., Lamri, K., Rezki, M., Kacem, M. 2009. Selection of *Lactobacillus plantarum* strains for their use as starter culturesin Algerian olive fermentations. Grasas y Aceites 60, 82-88.

Moreau, M.C., Hudault, S., Bridonneau, C. 1990. Systemic antibody response to ovalbumin in gnotobiotic C3H/HeJ mice with *Bifidobacterium bifidum* or *Escherichia coli*. Microecology and Therapy 20,309-12.

Morelli, L., Sarra, P.G., Bottazzi, V. 1988. *In vivo* transfer of pAMßl from *Lactobacillus reuteri* to *Enterococcus faecalis*. Journal of Applied Bacteriology, 65, 371-375.

Morelli, L., Zonenenschain, D., Del Piano, M., Cognein, P. 2004. Utilization of the intestinal tract as a delivery system for urogenital probiotics. Journal of Clinical Gastroenterology 38, S107-S100.

Morita, H., Toh, H., Fukuda, S., Horikawa, H., Oshima, K., Suzuki, T., Murakami, M., Hisamatsu, S., Kato, Y., Takizawa, T., Fukuoka, H., Yoshimura, T., Itoh, K., O'Sullivan, D.J., McKay, L.L., Ohno, H., Kikuchi, J., Masaoka, T., Hattori, M. 2008. Comparative genome analysis of Lactobacillus reuteri and *Lactobacillus* *fermentum* reveal a genomic island for reuterin and cobalamin production. DNA Research 15, 151-161.

Morita, Y., Tomida, J., Kawamura, Y. 2013. Responses of *Pseudomonas aeruginosa* to antimicrobials. Low-dose antibiotics: current status and outlook for the future, 30.

Muller, A., Coenen, S., Monnet, D.L., Goossens, H. 2007. European Surveillance of Antimicrobial Consumption (ESAC): outpatient antibiotic use in Europe, 1998-2005. Eurosurveillance. 12, E071011.

Munsch-Alatossava, P., Alatossava, T. 2007. Antibiotic resistance of raw milk associated psychrotrophic bacteria. Microbiological Research 162, 115-123.

Murphy, M.G., O'Connor, L., Walsh, D., Condon, S. 1985. Oxygen dependent lactate utilization by *Lactobacillus plantarum*. Archives of Microbiology 141, 75-79.

Nam, Y.D., Lee, S.Y., Lim, S.I. 2012. Microbial community analysis of Korean soybean pastes by next-generation sequencing. International Journal of Food Microbiology 155, 36-42.

Narberhaus, F. 2002. Alpha-crystallin-type heat shock proteins: socializing minichaperones in the context of a multichaperone network. Microbiology and Molecular Biology Reviews 66, 64-93.

Nawaz, M.S., Erickson, B.D., Khan, A.A., Khan, S.A., Pothuluri, J.V., Rafii, F., Sutherland, J.B., Wagner, D., Cerniglia, C.E. 2001. Human health impact and regulatory issues involving antimicrobial resistance in the food animal production environment. Regulatory Research Perspectives 1, 1-10 .

Nawaz, M., Wang, J., Zhou, A., Ma, C., Wu, X., Moore, J.E., Cherie Millar, B., Xu, J. 2010. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Current Microbiology 62, 1081-1089.

Nawaz, M., Wang, J., Zhou, A., Ma, C., Wu, X., Moore, J.E., Millar, B.C., Xu J. 2011. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Current Microbiology 62, 1081-1089.

Naz, S., Tareb, R., Bernardeau, M., Vaisse, M., Lucchetti-Miganeh, C., Rechenmann, M., Vernoux, J.P. 2013. Genome sequence of *Lactobacillus plantarum* strain UCMA 3037. Genome Announcements 1, e00251-13.

NCCLS (National Committee For Clinical Laboratory Standards). 2000. Performance standards for antimicrobial disk susceptibility test-approved standard (document M2-A7). 7th ed. NCCLS, Pennsylvania, USA, ISBN 1-56-238208-X.

Nei, T., Inai, S., Mikami, I., Sato, A., Okamoto, J., Yokoshima, K., Nakamizo, M., Haraguchi, S., Sonobe, K., Saito R. 2013. Descending necrotizing mediastinitis associated with *Lactobacillus plantarum*. BMC Infectious Diseases 13, 398.

Nes, I.F., Holo, H., Fimland, G., Hauge, H.H., Nissen-Meyer, J. 2001. Unmodified peptide-bacteriocins (class II) produced by lactic acid bacteria. In: Dutton, C.J., Haxell, M.A., McArthur, H.A.I., Wax, R.G. Peptide antibiotics: discovery, modes of action and application. Marcel Dekker. New York, USA.

Neyfakh, A.A., Borsch, C.M., Kaatz, G.W. 1993. Fluoroquinolone resistance protein NorA of *Staphylococcus aureus* is a multidrug efflux transporter. Antimicrobial Agents and Chemotherapy 37, 128-129.

Nichols, W.K. 2003. Drogas Antiinfecciosas. In: GENNARO, A.R. Remington farmacia. Editorial Médica Panamericana. Buenos Aires, Argentina.

Nikolich, M.P., Hong, G., Shoemaker, N.B., Salyers, A.A. 1994. Evidence for natural horizontal transfer of *tetQ* between bacteria that normally colonize humans and bacteria that normally colonize livestock. Applied and Environmental Microbiology 60, 3255-3260.

Nissle, A. 1916. Ueber die Grundlagen einer neuen ursaechlichen Bekaempfung der pathologischen Darmflora. Deutsche Medizinische Wochenschrift 42, 1181-1184.

Nissle, A. 1918. Die antagonistische behandlung chronischer darmstörungen mit colibakterien. Medizinische Klinik 2, 29-30.

Normark, B.H., Normark, S. 2002. Evolution and spread of Antibiotic Resistance. Journal of Internal Medicine 252, 91-106.

Novak, R., Tuomanen, E.I. 2001. Phenotypic Tolerance of Bacteria. Bacterial Resistance to Antimicrobials, 209.

O'Brien, T.F. 2002. Emergence, spread, and environmental effect of antimicrobial resistance: How use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. Clinical Infectious Diseases 34, S78-S84.

Oguntoyinbo, F.A., Tourlomousis, P., Gasson, M., Narbad, A. 2011. Analysis of bacterial communities of traditional fermented West African cereal foods using cultureindependent methods. International Journal of Food Microbiology 145, 205-210.

Oh, S., Roh, H., Ko, H.J., Kim, S., Kim, K.H., Lee, S.E., Chang, I.S., Kim, S., ChoiI, G. 2011. Complete genome sequencing of *Lactobacillus acidophilus* 30SC, isolated from swine intestine. Journal of Bacteriology 193, 2882-2883.

Okada, S., Daengsubha, W., Uchimura, T., Ohara, N., Kozaki, M. 1986. Flora of lactic acid bacteria in Miang produced in Northern Thailand. The Journal of General and Applied Microbiology 32, 57-65.

Okusu, H., Ma, D., Nikaido, H. 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. Journal of Bacteriology 178, 306-308.

Olukoya, D.K., Ebigwei, S.I., Adebawo, O.O., Osiyemi, F.O. 1993. Plasmid profiles and antibiotic susceptibility patterns of *Lactobacillus* isolated from fermented foods in Nigeria. Food Microbiology 10, 279-285.

Osset, J., Bartolomé, R.M., Garcia, E., Andreu, A. 2001. Assessment of the capacity of *Lactobacillus* to inhibit the growth of uropathogens and block their adhesion to vaginal epithelial cells. Journal of Infectious Diseases 183, 485-491.

Ouoba, L.I., Lei, V., Jensen, L.B. 2008. Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: determination and transferability of the resistance genes to other bacteria. International Journal of Food Microbiology 121, 217-224.

Pan, L., Hu, X., Wang, X. 2011. Assessment of antibiotic resistance of lactic acid bacteria in Chinese fermented foods. Food Control 22, 1316-1321.

Pages, J.M., James, C.E., Winterhalter, M. 2008. The porin and the permeating antibiotic: A selective diffusion barrier in Gram-negative bacteria. Nature Reviews Microbiology 6, 893-903.

Papadimitriou, K., Zoumpopoulou, G., Foligné, B., Alexandraki, V., Kazou, M., Pot, B., Tsakalidou, E. 2015. Discovering probiotic microorganisms: in vitro, in vivo, genetic and omics approaches. Frontiers in microbiology 6, 1-27.

Park, S., Choi, K. 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. Ecotoxicology 17, 526-538.

Parvez, S., Malik, K.A., Ah Kang, S., Kim, H.Y. 2006. Probiotics and their fermented food products are beneficial for health. Journal of Applied Microbiology 100, 1171–1185.

Pascual Anderson, M.R., Calderón y Pascual, V. 1999. Microbiología Alimentaria: Metodología analítica para alimentos y bebidas. Editorial Diaz de Santos. Madrid, Spain.

Patel, D., Kosmidis, C., Seo, S.M., Kaatz, G.W. 2010. Ethidium bromide MIC screening for enhanced efflux pump gene expression or efflux activity in *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 54, 5070-5073.

Patel, A.R., Shah, N.P., Prajapati, J.B. 2012. Antibiotic resistance profile of lactic acid bacteria and their implications in food chain. World Journal of Dairy and Food Sciences 7, 202-211.

Pearce, H., Messager, S., Maillard, J.Y. 1999. Effect of biocides commonly used in the hospital environment on the transfer of antibiotic-resistance genes in *Staphylococcus aureus*. Journal of Hospital Infection 43, 101-108.

Perapoch, J., Salcedo, S., Gallart, A., Peguero, G., Casellas, M., Barroso, C. 1993. Colonización umbilical en recién nacidos normales. Estudio comparativo de cuatro métodos de antisepsia umbilical. Asociación Española de Peditría 39, 195-198.

Perdigón, G., Álvarez, S., Rachid, M., Agüero, G., Gobbato, N. 1995. Imnune system stimulation by probiotics. Symposium: Probiotic Bacteria for Humans: Clinical Systems for Evaluation of Effectiveness. Journal of Dairy Science 78, 1597-1606.

Peres, C.M., Hernandez-Mendoza A., Peres C., Malcata F.X. 2012. Review on fermented plant materials as carriers and sources of potentially probiotic lactic acid bacteria with an emphasis on table olives. Trends in Food Science and Technology 26, 31-42.

Pérez Pulido, R., Benomar, N., Abriouel, H., Lucas López, R., Martínez Cañamero, M., Gálvez, A. 2005. Microbiological study of lactic acid fermentation of caper berries by molecular and culture-dependent methods. Applied and Environmental Microbiology 71, 7872-7879.

Petersen, A., Jensen, L.B. 2004. Analysis of *gyrA* and *parC* mutations in enterococci from environmental samples with reduced susceptibility to ciprofloxacin. FEMS Microbiology Letters 231, 73-76.

Pfaller, M.A., Andesb, D., Diekemaa, D.J., Espinel-Ingroffc, A., Sheehand, D., The CLSI Subcommittee for Antifungal Susceptibility Testing. 2010. Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and Candida: Time for harmonization of CLSI and EUCAST broth microdilution methods. Drug Resistance Updates 13, 180-195.

Picard, C., Fioramonti, J., Francois, A., Robinson, T., Neant, F., Matuchansky, C. 2005. Review article: bifidobacteria as probiotic agents - physiological effects and clinical benefits. Alimentary Pharmacology and Therapeutics 22, 495-512.

Piddock, L.J.V. 2006. Multidrug-resistance efflux pumps - not just for resistance. Nature Reviews Microbiology 4, 629-636.

Poole, K. 2002. Mechanism of bacterial Biocide and Antibiotic Resistance. Journal of Applied Microbiology 92, 55S-64S.

Poole, K. 2004. Acquired resistance. In: Fraise, A.P., Lambert, P.A., Maillard, J.Y. (Eds.), Principles and practice of disinfection, preservation and sterilization. Blackwell Scientific Publication. Oxford, UK.

Poole, K. 2007. Efflux pumps as antimicrobial resistance mechanisms. Annals of Medicine 39, 162-76.

Poole, K. 2012. Efflux mediated antimicrobial resistance. In: Dougherty, T.J., Pucci, M.J. (Eds.), Antibiotic Discoveryand Development. Springer. New York, USA.

Pot, B., Ludwig, W., Kersters, K., Schleifer, K.H. 1994. Taxonomy of lactic acid bacteria. In De Vuyst, L., Vandamme, E.J. (Eds.). Bacteriocins of Lactic Acid Bacteria. Blackie Academic and Professional. London, UK.

Putman, M., van Veen, H.W., Degener, J.E., Konings, W.N. 2001. The lactococcal secondary multidrug transporter LmrP confers resistance to lincosamides, macrolides, streptogramins and tetracyclines. Microbiology 147, 2873-2880.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Wang, J. 2010. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59-65.

Quinn, P.J., Markey, B.K. 2001. Disinfection and disease prevention in veterinary medicine. In: Block S.S. (Ed.). Disinfection, sterilization and preservation. Lippincott, Williams and Wilkins. Philadelphia, USA.

Ranadheera, R.D.C.S., Baines, S.K., Adams, M.C. 2010. Importance of food in probiotic efficacy. Food Research International 43, 1-7.

Randall, L.P., Cooles, S.W., Coldham, N.G., Penuela, E.G., Mott, A.C., Woodward, M.J., Piddock, L.J.V., Webber, M.A. 2007. Commonly used farm disinfectants can select for mutant *Salmonella enterica* serovar Typhimurium with decreased susceptibility to biocides and antibiotics without compromising virulence. Journal of Antimicrobial Chemotherapy 60, 1273-1280.

Regulation (EC) and No. 178/2002. 2002. Regulation of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. Official Journal of the European Union Communities L31, 1-23.

Regulation (EC) No 258/1997. Regulation of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients. Official Journal of the European Union Communities L43, 0001-0007.

Regulation (EU) No. 528/2012. 2012. Regulation (EU) No. 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. Official Journal of the European Union, Legislation 167 27.6. 2012, 1-123.

Reid G. 2000. Probiotic therapy and functional foods for prevention of urinary tract infections: state of the art and science. Current Infectious Disease Reports 2, 518 -522.

Reid, G., Burton, J. 2002. Use of *Lactobacillus* to prevent infection by pathogen bacteria. Microbes and infection 4, 319-324.

Reid, G., Cook, R.L., Bruce, A.W. 1987. Examination of strains of lactobacilli for properties which may influence bacterial interference in the urinary tract. Journal of Urology 138, 330-335.

Reid, G., Mac Groarty, J., Chow, A., Eisen, A., Eisen, A., Costerton, W. 1990. Coaggregation of urogenital bacteria in vitro and in vivo. Current Microbiology 20, 47-52.

Reid, G., Servin, A.L., Bruce, A.W., Busscher, H.J. 1993. Adhesion of three *Lactobacillus* strains to human urinary and intestinal epithelial cells. Microbios 75, 57-65.

Reid, G., Heinemann, C., Velraeds, M., van der Mei, H.C., Busscher, H.J. 1999. Biosurfactants produced by *Lactobacillus*. Methods in Enzymology 310, 426-432.

Reid, G., Habash, M., Vachon, D., Denstedt, J., Riddell, J., Beheshti, M. 2001. Oral fluoroquinolone therapy results in drug adsorption on ureteral stents and prevention of biofilm formation. International Journal of Antimicrobial Agents 17, 317-320.

Reid, G., Jass, J., Sebulsky, M.T., McCormick, J.K. 2003. Potential uses of probiotics in clinical practice. Clinical Microbiology Reviews 16, 658-672.

Reid, G., Burton, J., Devilard, E. 2004. Contemporary Issues in Ob/Gyn and Women's Health: The Rationale for Probiotics in Female Urogenital Healthcare. Medscape General Medicine 6, 49-63.

Rettger, L.F., Cheplin, H.A. 1921. A treatise on the transformation of the intestinal flora with special reference to the implantation of bacillus acidophilus. Yale University Press. London, UK.

Reynolds, P.E. 1998. Control of peptidoglycan synthesis in vancomycin-resistant enterococci: D,D-peptidases and D,D-carboxypeptidases. Cellular and Molecular Life Sciences 54, 325-331.

Rezzonico, E., Lariani, S., Barretto, C., Cuanoud, G., Giliberti, G., Delley, M., Arigoni, F., Pessi, G. 2007. Global transcriptome analysis of the heat shock response of *Bifidobacterium longum*. FEMS Microbiology Letters 271, 136-145.

Rhee, S.K. Pack, M.Y. 1980. Effect of environmental pH on fermentation balance of *Lactobacillus bulgaricus*. Journal of Bacteriology 144 , 217–221.

Rodríguez-Gómez, F., Romero-Gil, V., García-García, P., Garrido-Fernández, A., Arroyo-López, F.N. 2014. Fortification of table olive packing with the potential probiotic bacteria *Lactobacillus pentosus* TOMC-LAB2. Frontiers in Microbiology 5, 467.

Rogers, P.D., Liu, T.T., Barker, K.S., Hilliard, G.M., English, B.K., Thornton, J., Swiatlo, E., McDaniel, L.S. 2007. Gene expression profiling of the response of *Streptococcus pneumoniae* to penicillin. Journal of Antimicrobial Chemotherapy 59, 616-626.

Romero Cabello, R. 2007. Microbiología y Parasitología Humana, Bases etiológicas de las enfermedades infecciosas y parasitarias. Editorial Médica Panamericana. México DF, México.

Rosander, A., Connolly, E., Roos, S. 2008. Removal of antibiotic resistance gene-carrying plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain, *L. reuteri* DSM 17938. Applied and Environmental Microbiology 74, 6032-6040.

Ross, R.P., Fitzgerald, G., Collins, K., Stanton, C. 2002. Cheese delivering biocultures - probiotic cheese. Australian Journal of Dairy Technology 57, 71-78.

Rozila, A., Rajandara, P., Lani, M. N., Siti Hasmah, M., Ezni Suryani, I., Ragupathy, R. 2012. Antibiotic resistant of Lactobacillus isolated from dairy products. UMT 11th International Annual Symposium on Sustainability Science and Management. Terengganu, Malaysia.

Ruiz-Barba, J.L., Jiménez-Díaz, R. 1994. Vitamin and amino acid requirements of *Lactobacillus plantarum* strains isolated from green olive fermentations. Journal of Applied Bacteriology 76, 350-355.

Ruiz-Barba, J.L., Jiménez-Díaz, R. 2012. A novel *Lactobacillus pentosus* - paired starter culture for Spanish-style green olive fermentation. Food Microbiology 30, 253-259.

Ruiz-Palacios, G., Tuz, F., Arteaga, F., Guerrero, M.L., Dohnalek M., Hilty, M. 1996a. Tolerance and fecal colonization with *Lactobacillus reuteri* in children fed a beverage with a mixture of *Lactobacillus* spp. 1090. Pediatric Research - Nature 39, 184.

Ruoff, K.L., Kuritzkes, D.R.,Wolfson, J.S., Ferraro, M.J. 1988. Vancomycin resistant Grampositive bacteria isolated from human sources. Journal of Clinical Microbiology 26, 2064-2068.

Rusenova, N.V., Haritova, A.M., Parvanov, P.R. 2009. Influence of cyclosporine A and quercetine on MIC values of danofloxacin mesylate in *Escherichia coli* strains isolated from poultry. Turkish Journal of Veterinary and Animal Sciences 33, 247-251.

Russell, A.D. 1997. Plasmids and bacterial resistance to biocides. Journal of Applied Microbiology 83, 155-165.

Russell, A.D. 2000. Do biocides select for antibiotic resistance?. Journal of Pharmacy and Pharmacology 52, 227-233.

Ryall, B., Eydallin, G., Ferenci, T. 2012. Culture history and population heterogeneity as determinants of bacterial adaptation: the adaptomics of a single environmental transition. Microbiology and Molecular Biology Reviews 76, 597-625.

Saarela, M., Mogensen, G., Fondén, R., Mättö, J., Mattila-Sandholm, T. 2000. Probiotic bacteria: safety, functional and technological properties. Journal of Biotechnology 84, 197-215.

Sakamoto, K., Margolles, A., van Veen, H.W. Konings, W.N. 2001. Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-binding cassette multidrug transporter HorA. Journal of Bacteriology 183, 5371-5375.

Salminen, S., Bouley, C., Boutron-Ruault, M.C., Cummings, J.H., Franck, A., Gibson, G.R., Isolauri, E., Moreau, M.C., Roberfroid, M., Rowland, I. 1998. Functional food science and gastrointestinal physiology and function. British Journal of Nutrition 80, S147-S171.

Salyers, A.A., Gupta, A., Wang, Y. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends in Microbiology 12, 412-416.

Sami, M., Yamashita, H., Hirono, T., Kadokura, H., Kitamoto, K., Yoda, K., Yamasaki, M. 1997. Hop-resistant *Lactobacillus brevis* contains a novel plasmid harboring a multidrug resistance-like gene. Journal of Fermentation and Bioengineering 84, 1-6.

Sánchez, B., Champomier-Vergès, M.C., Stuer-Lauridsen, B., Ruas-Madiedo, P., Anglade, P., Baraige, F., de los Reyes-Gavilán, C.G., Johansen, E., Zagorec, M., Margolles, A. 2007. Adaptation and response of *Bifidobacterium animalis* subsp. *lactis* to bile: a proteomic and physiological approach. Applied and Environmental Microbiology 73, 6757-6767.

Sánchez, B., Ruiz, L., Gueimonde, M., Margolles, A. 2013. Omics for the study of probiotic microorganisms. Food Research International 54, 1061-1071.

Sánchez-Saldaña L., Sáenz-Anduaga E. 2005. Antisépticos y Desinfectantes. Dermatología Peruana 15, 82-103.

Sanders, M.E., Akkermans, L.M., Haller, D., Hammerman, C., Heimbach, J.T., Hörmannsperger, G., Huys, G. 2010. Safety assessment of probiotics for human use. Gut Microbes 1, 164-185.

Sanidad y Consumidores de la Comisión Europea, European Commission. 2009. Effects of Biocides on antibiotic resistance. Scientific Committees: B-1049. Brussels, Belgium.

Sanz Y., Collado M.C., Dalmau J. 2003. Probióticos: criterios de calidad y orientaciones para el consumo. Acta Pediatrica Española 61, 476-482.

Sarno, M., Lania, G., Cuomo, M., Nigro, F., Passannanti, F., Budelli, A., Fasano, F., Troncone, R., Auricchio, S., Barone, M.V., Nigro, R., Nanayakkara, M. 2014. *Lactobacillus paracasei* CBA L74 interferes with gliadin peptides entrance in Caco-2 cells. International Journal of Food Sciences and Nutrition 65, 953-959.

Savadogo, A., Ouattara, C.A.T., Ilboudo, A.J., Karou, D., Traore, A.S. 2010. Isolation and susceptibility to antibiotics of bacterial strains from Burkina Faso fermented milk samples. Advance Journal of Food Science and Technology 2, 91-95.

Savaje, C.A. 1971. A new bacteriostat for skin care products. Drug and Cosmetic Industry 109, 161-163.

Schlegel, L., Lemerle, S., Geslin, P. 1998. Lactobacillus species as opportunistic pathogens in immunocompromised patients. European Journal of Clinical Microbiology and Infectious Diseases: Official Publication of the European Society of Clinical Microbiology 17, 887-888.

Schleifer, K. H., Ludwig, V. 1995. Phylogenetics relationships of lactic acid bacteria. In: Wood, B.J.B., Holzapfel, W.H. (Eds.). The genera of lactic acid bacteria. Chapman and Hall. Glasgow, UK.

Schleifer, K.H., Ludwig, W. 1995. Phylogenetic relationships of lactic acid bacteria. In: Wood, B.J.B., Holzapfel, W.H. (Eds.). The lactic acid bacteria, vol. 2: The

Genera of Lactic Acid Bacteria. Blackie Academic and Professional. Glasgow, UK.

Schmidt, G., Hertel, C., Hammes, W.P. 1999. Molecular characterization of the dnaK operon of *Lactobacillus sakei* LTH681. Systematic and Applied Microbiology 22, 321-328.

Schmitz, F.J., Sadurski, R., Kray, A., Boos, M., Geisel, R., Kohrer, K., Verhoef, J., Fluit, A.C. 2000. Prevalence of macrolide resistance genes in *Staphylococcus aureus* and *Enterococcus faecium* isolates from 24 European University hospitals. Journal of Antimicrobial Chemotherapy 45, 891-894.

Schneider, D., Lenski, R.E. 2004. Dynamics of insertion sequence elements during experimental evolution of bacteria. Research in Microbiology 155, 319-327.

Schrezenenmeir, J., De Vrese, M. 2001. Probiotics, prebiotics, and symbiotics-approaching a definition. The American Journal of Clinical Nutrition 73, S361-S364.

Schweizer, H.P. 2001. Triclosan: a widely used biocide and its link to antibiotics. FEMS Microbiology Letters 202, 1-7.

SCAN (Scientific Committee for Animal Nutrition). 1996. Report of the Scientific Committee for Animal Nutrition (SCAN) on the possible risk for humans on the use of avoparcin as feed additive. Office for EC Publications, Luxemburg City, Luxemburg.

SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks). 2009. Assessment of the Antibiotic Resistance Effects of Biocides. Brussels, Belgium. European Commission, 1-87.

Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30, 2068-2069.

Serrano, P.H. 2005. Responsible use of antibiotics in aquaculture. FAO Fisheries Technical Paper 469, 1-97.

Servin, A.L. 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiology Reviews 28, 405-440.

Settanni, L., Moschetti, G. 2010. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. Food Microbiology 27, 691-697.

Shanahan, F. 2012. A commentary on the safety of probiotics. Gastroenterology Clinics of North America 41, 869-876.

Sherman, M.Y., Qian, S.B. 2013. Less is more: improving proteostasis by translation slow down. Trends in Biochemical Sciences 38, 585-591.

Shrago, A.W., Dobrogosz, W.J. 1988. Conjugal transfer of Group B streptococcal plasmids and co-mobilization of *E. coli-Streptococcus* shuttle plasmids to *L. plantarum*. Applied and Environmental Microbiology 54, 824-826.

Simark-Mattsson, C., Emilson, C.G., Håkansson, E.G., Jacobsson, C., Roos, K., Holm, S. 2007. *Lactobacillus*-mediated interference of mutans streptococci in caries-free vs. caries-active subjects. European Journal of Oral Sciences 115, 308-314.

Simeoni, D., Rizzotti, L., Cocconcelli, P., Gazzola, S., Dellaglio, F., Torriani, S. 2008. Antibiotic resistance genes and identification of staphylococci collected from the production chain of swine meat commodities. Food Microbiology 25, 196-201.

Singh, K.V., Murray, B.E. 2005. Differences in the *Enterococcus faecalis Isa* locus that influence susceptibility to quinupristin-dalfopristin and clindamycin. Antimicrobial Agents and Chemotherapy 49, 32-39.

Smith, D.L., Dushoff, J., Morris Jr, J.G. 2005. Agricultural antibiotics and human health. PLoS Medicine 2, e232.

Smokvina, T., Wels, M., Polka, J., Chervaux, C., Brisse, S., Boekhorst, J., van Hylckama Vlieg, J.E.T., Siezen, R.J. 2013. *Lactobacillus paracasei* comparative genomics: Towards species pan-genome definition and exploitation of diversity. PLoS ONE 8, e68731.

Snydman, D.R. 2008. The safety of probiotics. Clinical Infectious Diseases 46, S104-S111.

Sobel, J.D. 1999. Biotherapeutic agents as therapy for vaginitis. In: Elmer, G.W., McFarland, L., Surawicz, C. (Eds.). Biotherapeutic agents and infectious diseases. Human Press Inc. Totowa, USA.

Sonnenburg, J.L., Chen, C.T., Gordon, J.I. 2006. Genomic and metabolic studies of the impact of probiotics on amodel gut symbiont and host. PLoS Biology 4, e413.

Sornplang, P., Leelavatcharamas, V., Sukon, P., Yowarach, S. 2011. Antibiotic resistance of lactic acid bacteria isolated from a fermented fish product, Pla-chom. Research Journal of Microbiology 6, 898-903.

Sousa, A.M., Machado, I., Pereira, M.O. 2011. Phenotypic switching: an opportunity to bacteria thrive 2011. In: Méndez-Vilas, A. (Ed.). Science against microbial pathogens: communicating current research and technological advances. Formatexa. Badajoz, Spain.

Spitaels, F., Wieme, A.D., Janssens, M., Aerts, M., Daniel, H.M., Van Landschoot, A., De Vuyst, L., Vandamme, P. 2014. The microbial diversity of traditional spontaneously fermented lambic beer. PloS One 9, e95384.

Spurr, H.W. 1994. The microbial ecology of fruit and vegetable surfaces, its relationship to postharvest biocontrol. In: Wilson, C., Wisniewski, M. (Eds.). Biological Control of Postharvest Diseases: Theory and Practice. CRC Press. Boca Raton, USA.

Stiles, M.E. 1996. Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek 70, 331-345.

Stock, I., Wiedemann, B. 2001. Natural antibiotic susceptibilities of *Edwardsiella tarda, E. ictaluri*, and *E. hoshinae*. Antimicrobial Agents and Chemotherapy 45, 2245-2255.

Sun, Z., Chen, X., Wang, J., Zhao, W., Shao, Y., Guo, Z., Zhang, X., Zhou, Z., Sun, T., Wang, L., Meng, H., Zhang, H., Chen, W. 2011. Complete genome sequence of *Lactobacillus delbrueckii* subsp. *bulgaricus* strain ND02. Journal of Bacteriology 193, 3426-3427.

Sundheim, G., Schulerud, S., Dainty, R.H. 1993. Resistance of lactic acid bacteria isolated from meat products to quaternary ammonium compound. International Biodeterioration and Biodegradation 41, 235-239.

Sutcliffe, J., Grebe, T., Tait-Kamradt, A., Wondrack, L. 1996. Detection of erythromycin-resistant determinants by PCR. Antimicrobial Agents and Chemotherapy 40, 2562-2566.

Swain, M.R., Anandharaj, M., Ray, R.C., Parveen, R. 2014. Fermented fruits and vegetables of Asia: a potential source of probiotics. Biotechnology Research International 2014, 1-11.

Swenson, K.I., Piwnica-Worms, H., McNamee, H., Paul, D.L. 1990. Tyrosine phosphorylation of the gap junction protein connexin43 is required for the pp60v-src-induced inhibition of communication. Cell Regulation 1, 989-1002.

Swick, M.C., Morgan-Linnell, S.K., Carlson, K.M., Zechiedrich, L. 2011. Expression of multidrug efflux pump genes *acrAB-tolC*, *mdfA*, and *norE* in *Escherichia coli* clinical isolates as a function of fluoroquinolone and multidrug resistance. Antimicrobial Agents and Chemotherapy 55, 921-924

Tajabadi, N., Mardan, M., Abdul Manap, M.Y., Shuhaimi, M., Meimandipour, A., Nateghi, L. 2011. Detection and identification of *Lactobacillus* bacteria found in the honey stomach of the giant honeybee *Apis dorsata*. Apidologie 42, 642-649.

Tamang, J.P., Kailasapathy, K. 2010. Fermented foods and beverages of the world. CRC Press. Boca Raton, USA.

Tamine, A.Y., Saarela, M., Korslund, A.S., Mistry, V.V., Shah, N.P. 2005. Production and maintenance of viability of probiotic micro-organisms in dairy products. In: Tamine A.Y. (Ed.). Probiotic Dairy Products. Blackwell Publishing. Oxford, UK.

Tannock, G.W. 2004. A special fondness for lactobacilli. Applied and Environmental Microbiology 70, 3189-3194.

Taranto, M.P., Font de Valdez, G., Perez-Martinez, G. 1999. Evidence of a glucose proton motive force-dependent permease and a fructose phosphoenolpyruvate: phosphotransferase transport system in *Lactobacillus reuteri* CRL 1098. FEMS Microbiology Letters 181, 109-112.

Temmerman, R., Scheirlinck, I., Huys, G., Swings, J. 2003. Culture-independent analysis of probiotic products by denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 69, 220-226.

Teuber, M. 1999. Spread of antibiotic resistancewith food-borne pathogens. Cellular and Molecular Life Sciences 56, 755-763.

Teuber, M., Meile, L., Schwarz, F. 1999. Acquired antibiotic resistance in lactic acid bacteria from food. Antonie Van Leeuwenhoek 76, 115-137.

Thumu, S.C., Halami, P.M. 2012. Presence of erythromycin and tetracycline resistance genes in lactic acid bacteria from fermented foods of Indian origin. Antonie Van Leeuwenhoek 102, 541-551.

Tissier, H. 1906. Traitement des infections intestinales par la methods de transformations de la flore bacterinne de l'intestin. Comptes Rendus de la Société de Biologie 60, 359-362.

Todorov, S.D., Dicks, L.M.T. 2004. Effect of medium components on bacteriocin production by *Lactobacillus pentosus* ST151BR, a strain isolated from beer produced by the fermentation of maize, barley and soy flour. World Journal of Microbiology and Biotechnology 20, 643-650.

Toh, H., Oshima, K., Nakano, A., Takahata, M., Murakami, M., Takaki, T., Nishiyama, H. Igimi, S. Hattori, M. Morita, H. 2013. Genomic adaptation of the *Lactobacillus casei* group. PLoS ONE 8, e75073.

Tompkins, T.A., Barreau, G., Broadbent, J.R. 2012. Complete genome sequence of *Lactobacillus helveticus* R0052, a commercial probiotic strain. Journal of Bacteriology 194, 6349.

Toomey, N., Bolton, D., Fanning, S. 2010. Characterisation and transferability of antibiotic resistance genes from lactic acid bacteria isolated from Irish pork and beef abattoirs. Research in Microbiology 161, 127-135.

Truong-Bolduc, Q.C., Zhang, X., Hooper, D.C. 2003. Characterization of NorR protein, a multifunctional regulator of *norA* expression in *Staphylococcus aureus*. Journal of Bacteriology 185, 3127-3138.

Tsilingiri, K., Rescigno, M. 2013. Postbiotics: what else?. Beneficial Microbes 4, 101-107.

Turnidge, J.D., Bell, J.M. 2005. Antimicrobial susceptibility on solidmedia. In: Lorian, A. (Ed.), Antibiotics in laboratory medicine. Lippincott Williams and Wilkins. Baltimore, USA.

Tynkynnen, S., Singh, D.V., Varmanen, P. 1998. Vancomycin resistance factor of *Lactobacillus rhamnosus* GG in relation to enterococcal vancomycin resistance (*van*) genes. International Journal of Food Microbiology 41, 195-204.

Úbeda, C., Maiques, E., Knecht, E., Lasa, Í., Novick, R.P., Penadés, J.R. 2005. Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci. Molecular Microbiology 56, 836-844

Upadrasta, A., Stanton, C., Hill, C., Fitzgerald, G., Ross, R.P. 2011. Improving the stress tolerance of probiotic cultures: recent trends and future directions. In: Tsakalidou, E., Papadimitriou, K. (Eds.). Stress Responses of Lactic Acid Bacteria. Springer. New York, USA.

Vahabnezhad, E., Mochon, A.B.,Wozniak, L.J., Ziring, D.A. 2013. *Lactobacillus* bacteremia associated with probiotic use in a pediatric patient with ulcerative colitis. Journal of Clinical Gastroenterology 47, 437-439.

Vakulenko, S.B., Donabedian, S.M., Voskresenskiy, A.M., Zervos, M.J., Lerner, S.A., Chow, J.W. 2003. Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. Antimicrobial Agents and Chemotherapy 47, 1423-1426.

van Baarlen, P., Troost, F., van der Meer, C., Hooiveld, G., Boekschoten, M., Brummer, R.J., Kleerebezem, M. 2011. Human mucosal in vivo transcriptome responses to three lactobacilli indicate how probiotics may modulate human cellular pathways. Proceedings of the National Academy of Sciences, 108, 4562-4569.

van Bambeke, F., Glupczynski, Y., Plesiat, P., Pechere, J.C., Tulkens, P.M. 2003. Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy. Journal of Antimicrobial Chemotherapy 51, 1055-1065.

van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S.D., Maguin, E. 2002. Stress responses in lactic acid bacteria. Antonie Van Leeuwenhoek 82, 187-216.

van de Guchte, M., Penaud, S., Grimaldi, C., Barbe, V., Bryson, K., Nicolas, P., Robert, C. Oztas, S., Mangenot, S., Couloux, A., Loux, V., Dervyn, R., Bossy R., Bolotin, A., Batto, J.M., Walunas, T., Gibrat, J.F., Bessières, P., Weissenbach, J., Ehrlich, S.D., Maguin E. 2006. The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. Proceedings of the National Academy of Sciences of the United States of America 103, 9274-9279.

van der Veen, S., Abee, T. 2011. Bacterial SOS response: A food safety perspective. Current Opinion in Biotechnology 22, 136-142.

van Hoek, A.H.A.M., Margolles, A., Domig, K.J., Korhonen, J.M., Życka-Krzesinska, J., Bardowski, J.K., Danielsen, M., UGent, G.H., Morelli L., Aarts H.J.M. 2008. Molecular assessment of erythromycin and tetracycline resistance genes in lactic acid bacteria and bifidobacteria and their relation to the phenotypic resistance. International Journal of Probiotics and Prebiotics 3, 271-280.

van Reenen, C.A., Dicks, L.M. 2011. Horizontal gene transfer amongst probiotic lactic acid bacteria and other intestinal microbiota: what are the possibilities? A review. Archives of Microbiology 193, 157-168.

Vandamme, P., Peeters, C. 2014. Time to revisit polyphasic taxonomy. Antonie Van Leeuwenhoek 106, 57-65.

Vankerckhoven, V., Moreillon, P., Piu, S., Giddey, M., Huys, G., Vancanneyt, M, Goossens, H., Entenza, J.M. 2007. Infectivity of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* isolates in a rat model of experimental endocarditis. Journal of Medical Microbiology 56, 1017-1024.

Vankerckhoven, V., Huys, G., Vancanneyt, M., Vael, C., Klare, I., Romond, M.B., Entenza, J.M., Moreillon, P., Wind, R.D., Knol, J., Wiertz, E., Pot, B., Vaughan, E.E., Kahlmeter, G. 2008. Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. Trends in Food Science and Technology 19, 102-114.

Vásquez, A., Jakobsson, T., Ahrme, S., Forsum, U., Molin, G. 2002. Vaginal *Lactobacillus* flora of healthy Swedish women. International Journal of Food Microbiology 40, 2746-2749.

Vedamuthu, E.R. 2013a. Starter cultures for yogurt and fermented milks. In: Chandan, R.C., Kilara, A. (Eds.). Manufacturing Yogurt and Fermented Milks. Jhon Wiley Publishing. Chichester, UK.

Veinger, L., Diamant, S., Buchner, J., Goloubinoff, P. 1998. The small heat-shock protein IbpB from *Escherichia coli* stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. Journal of Biological Chemistry 273, 11032-11037.

Venema, K., Meijerink, M. 2015. Lactobacilli as Probiotics: Discovering New Functional Aspects and Target Sites. In: Venema, K., Carmo, A.P. (Eds.). Probiotics and prebiotics: Current Research and Future Trends. Caister Academic Press. UK.

Ventura, M., O'Flaherty, S., Claesson, M.J., Turroni, F., Klaenhammer, T.R., van Sinderen, D., O'Toole, P.W. 2009. Genome-scale analyses of health-promoting bacteria: probiogenomics. Nature Reviews Microbiology 7, 61-71.

Verraes, C., van Boxstael, S., van Meervenne, E., van Coillie, E., Butaye, P., Catry, B., de Schaetzen, M.A., Van Huffel, X., Imberechts, H., Dierick, K., Daube, G., Saegerman, C., De Block, J., Dewulf, J., Herman, L. 2013. Antimicrobial resistance in the food chain: A review. International Journal of Environmental Research and Public Health 10, 2643-2669.

Vescovo, M., Morelli, L., Bottazzi, V. 1982. Drug resistance plasmids in *Lactobacillus acidophilus* and *Lactobacillus reuteri*. Applied and Environmental Microbiology 43, 50-56.

Vischer, W.A., Regos, J. 1973. Antimicrobial spectrum of triclosan, a broad-spectrum antimicrobial agent for topical application. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. Erste Abteilung Originale 226, 376-89.

Wang, T.T., Lee, B.H. 1997. Plasmids in *Lactobacillus*. Critical Reviews in Biotechnology 17, 227-272.

Wang, Y., Wang, J., Ahmed, Z., Bai, X., Wang, J. 2011b. Complete genome sequence of *Lactobacillus kefiranofaciens* ZW3. Journal of Bacteriology 193, 4280-4281.

Wang, Y., Chen, C., Ai, L., Zhou, F., Zhou, Z., Wang, L., Zhang, H., Chen, W., Guo, B. 2011a. Complete genome sequence of the probiotic *Lactobacillus plantarum* ST-III. Journal of Bacteriology 193, 313-314.

Wang, H., McEntire, J.C., Zhang, L., Li, X., Doyle, M. 2012. The transfer of antibiotic resistance from food to humans: facts, implications and future directions. Revue scientifique et technique (International Office of Epizootics) 31, 249-260.

Wang, S., Zhu, H., He, F., Luo, Y., Kang, Z., Lu, C., Xue Y., Wang, H. 2014. Whole genome sequence of the probiotic strain *Lactobacillus paracasei* N1115, isolated from traditional Chinese fermented milk. Genome Announcements, 2, e00059-14.

Washburn, M.P., Wolters, D., Yates, J.R. 2001. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nature Biotechnology 19, 242-247.

Webber, M.A., Piddock, L.J.V. 2003. The importance of efflux pumps in bacterial antibiotic resistance. Journal of Antimicrobial Chemotherapy 51, 9-11.

Wegener, H.C. 2003. Antibiotics in animal feed and their role in resistance development. Current Opinion in Microbiology 6, 439-445.

Weisblum, B. 1995. Erythromycin resistance by ribosome modification. Antimicrobial Agents and Chemotherapy 39, 577-585.

Wen, L., Ley, R.E., Volchkov, P.Y., Stranges, P.B., Avanesyan, L., Stonebraker, A. C, Hu, C., Wong, F.S., Szot, G.L., Bluestone, J.A., Gordon, J.I., Chervonsky, A.V. 2008. Innate immunity and intestinal microbiota in the development of type 1 diabetes. Nature 455, 1109-1113.

White, D.G., Acar, J., Anthony, F., Franklin, A., Gupta, R., Nicholls, T., Tamura Y., Thompson S., Threlfall E.J., Vose D., van Vuuren M., Wegener H.C., Costarrica M.L. 2001. Antimicrobial resistance: Standardisation and harmonisation of laboratory methodologies for the detection and quantification of antimicrobial resistance. Revue scientifique et technique 20, 849-858.

Wilkins, J.C., Homer, K.A., Beighton, D. 2002. Analysis of *Streptococcus* mutants proteins modulated by culture under acidic conditions. Journal of Biological Chemistry 68, 2382-2390.

Wilson, M. 2005. The gastrointestinal tract and indigenous microbiota. In: Microbial inhabitants of humans: their ecology and role in health and disease. Cambridge University Press. Cambridge, UK.

Witte, W. 2000. Selective pressure by antibiotic use in livestock. International Journal of Antimicrobial Agents 16, 19–24.

Wiuff, C., Zappala, R.M., Regoes, R.R., Garner, K.N., Baquero, F., Levin, B.R. 2004. Phenotypic tolerance: antibiotic enrichment of noninherited resistance in bacterial populations. Antimicrobial Agents and Chemotherapy 49, 1483-1494.

Wood, B.J.B., Holzapfel, W.H. 1995. The Genera of Lactic Acid Bacteria. Blackie Academic and Professional. London, UK.

Wouters, J.A., Kamphuis, H.H., Hugenholtz, J., Kuipers, O.P., De Vos, W.M., Abee, T. 2000. Changes in glycolytic activity of *Lactococcus lactis* induced by low temperature. Journal of Biological Chemistry 66, 3686-3691.

Wright, G.D. 2005. Bacterial resistance to antibiotics: enzymatic degradation and modification. Advanced Drug Delivery Reviews 57, 1451-1470.

Wright, G.D. 2011. Molecular mechanisms of antibiotic resistance. Chemical Communications 47, 4055-4061.

Wu, G.D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.Y., Keilbaugh, S.A., Bewtra, M., Knights, D., Walters, W.A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H., Bushman, F.D., Lewis, J.D. 2011. Linking long-term dietary patterns with gut microbial enterotypes. Science 334, 105–108.

Ying, W. 2006. NAD$^+$ and NADH in cellular functions and cell death. Frontiers in Bioscience: a journal and virtual library 11, 3129-3148.

Yu, J.L., Grinius, L., Hooper, D.C. 2002. NorA functions as a multidrug efflux protein in both cytoplasmic membrane vesicles and reconstituted proteoliposomes. Journal of Bacteriology 184, 1370-1377.

Yüksekdağ, Z.N., Beyatli, Y. 2008. Antimicrobial activity and antibiotic susceptibility of some lactic acid bacteria isolated different dairy products. Archiv für Lebensmittelhygiene 59, 216-220.

Zago, M., Fornasari, M. E., Carminati, D., Burns, P., Suarez, V., Vinderola, G. 2011. Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. Food Microbiology 28, 1033-1040.

Zarate, G., Nader-Macias, M.E. 2006. Influence of probiotic vaginal lactobacilli on in vitro adhesion of urogenital pathogens to vaginal epithelial cells. Letters in Applied Microbiology 43, 174-180.

Zdolec, N., Kozačinski, L., Njari, B., Filipović, I., Hadžiosmanović, M., Mioković, B., Kuzmanović, Ž., Mitak, M., Samac, D. 2009. The antimicrobial effect of lactobacilli on some foodborne bacteria. Archiv für Lebensmittelhygiene 60, 115-119.

Zdolec, N., Filipović, I., Cvrtila Fleck, Ž., Marić, A., Jankuloski, D., Kozačinski, L., Njari, B. 2011. Antimicrobial susceptibility of lactic acid bacteria isolated from fermented sausages and raw cheese. Veterinarski arhiv 81, 133-141.

Zhang, Z.Y., Liu, C., Zhu, Y.Z., Zhong, Y., Zhu, Y.Q., Zheng, H. J., Zhao, G.P., Wang, S.Y., Guo, X.K. 2009. Complete genome sequence of *Lactobacillus plantarum* JDM1. Journal of Bacteriology 15, 5020-5021.

Zhang, X.X., Zhang, T., Fang, H.H. 2009. Antibiotic resistance genes in water environment. Applied microbiology and biotechnology 82, 397-414.

Zhang, W., Yu, D., Sun, Z., Wu R., Chen X., Chen W., Meng, H., Hu, S., Zhang, H. 2010a. Complete genome sequence of *Lactobacillus casei* Zhang, a new probiotic strain isolated from traditional home-made koumiss in InnerMongolia of China. Journal of Bacteriology 192, 5268-9.

Zhang, W.Y., Yu, D.L., Sun, Z.H., Chen, W., Hu, S.N., Meng, H., Zhang, H.P. 2010b. The comparative analysis of a prophage remnant Lcazh1 in relation to other *Lactobacillus* prophages, particularly Lp3. International Journal of Dairy Technology 63, 413-417.

Zhang, Z.G., Ye, Z.Q., Yu, L., Shi, P. 2011. Phylogenomic reconstruction of lactic acid bacteria: An update. BMC Evolutionary Biology 11, 1.

Zhao, W., Chen, Y., Sun, Z., Wang, J., Zhou, Z., Sun, T., Wang, L., Chen, W., Zhang. H. 2011. Complete genome sequence of *Lactobacillus helveticus* H10. Journal of Bacteriology 193, 2666-2667.

Zhao, H.M., Huang, X.Y., Zuo, Z.Q., Pan, Q.H., Ao, M.Y., Zhou, F., Liu, H.N., Liu, Z.Y., Liu, D.Y. 2013. Probiotics increase T regulatory cells and reduce severity of experimental colitis in mice. World Journal of Gastroenterology 19, 742-749.

Zheng, H.J., Wang, B.F., Zhang, X.L., Han, H., Lu, G., Jin, L., Pu, S.Y., Hu, Q.P., Zhu, G.F., Wang, S.Y., Oda, M., Konno, T., Fu, G., Ji, Z.S., Zhao, G.P. 2008. The complete genome sequence of *Lactobacillus delbrueckii* subsp. *bulgaricus* 2038. Trends in Cell and Molecular Biology 3, 15-30.

Zhou, J.S., Pillidge, C.J., Gopal, P.K., Gill, H.S. 2005. Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. International Journal of Food Microbiology 98, 211-217.

Zhou, N., Zhang, J.X., Fan, M.T., Wang, J., Guo, G., Wei, X.Y. 2012. Antibiotic resistance of lactic acid bacteria isolated from Chinese yogurts. Journal of Dairy Science 95, 4775-4783.

Zonenschain, D., Rebecchi, A., Morelli, L. 2009. Erythromycin- and tetracycline-resistant lactobacilli in Italian fermented dry sausages. Journal of Applied Microbiology 107, 1559-1568.