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EFECTO DE LOS POLIFENOLES SOBRE EL CRECIMIENTO Y METABOLISMO DE BACTERIAS LÁCTICAS DEL VINO. POTENCIAL USO COMO ALTERNATIVA AL EMPLEO DE LOS SULFITOS DURANTE LA VINIFICACIÓN.

Almudena García Ruiz

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Memoria presentada por

ALMUDENA GARCÍA RUIZ

Para optar al grado de

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Directoras:

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Que la memoria titulada **"Efecto de los polifenoles sobre el crecimiento y metabolismo de bacterias lácticas del vino. Potencial uso como alternativa al empleo de los sulfitos durante la vinificación",** que presenta Dª. Almudena García Ruiz, para optar al grado de Doctor, se ha realizado bajo su dirección en el Departamento de Biotecnología y Microbiología de Alimentos del Instituto de Investigación en Ciencias de la Alimentación (CIAL), y como directoras de la misma autorizan su presentación.

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Abreviaturas y acrónimos empleados

En este apartado se detallan las abreviaturas y acrónimos utilizados en esta memoria, así como sus correspondientes significados.

AAB: Bacterias acéticas

ANOVA: Análisis de Varianza

AUC: Área Bajo la Curva de caída de fluorescencia

CE: Comunidad Europea

CECT: Colección Española de Cultivos Tipo

CFU/UFC: Unidades Formadoras de Colonias

CSC: Complejo Coloidal de Plata

DAD: Detector Diodo Array

DAO: Enzima Diamino Oxidasa

DGGE: Electroforesis en Gel con Gradiente Desnaturalizante

DMDC: Dicarbonato de Dimetilo

DNA: Ácido Desoxirribonucleico

FA: Fermentación Alcohólica

GC: Cromatografía de Gases

GC-MS: Cromatografía de Gases acoplada a Espectometría de Masas

GOX: Enzima Glucosa Oxidasa

HPLC: Cromatografía de Líquidos de Alta Eficacia

IC_{50}: Concentración que Inhibe al 50% de la población

LAB/BAL: Bacterias Lácticas

LF: Lactoferrina

LSD: Mínima Diferencia Significativa

MAO: Enzima Monoamino Oxidasa

MBC: Concentración Mínima Bactericida

MIC: Concentración Mínima Inhibitoria

MLF/FML: Fermentación Maloláctica

MRS: Medio de cultivo Man, Rogosa y Sharpe para bacterias lácticas

MRSE: Medio líquido de cultivo MRS suplementado con 6% de etanol

MLO: Medio líquido de cultivo *Leuconostoc oeni* para *Oenococcus oeni*

MLOE: Medio líquido de cultivo MLO con 6% de etanol

OAV: Valor de Actividad Odorante

OPA: Ortoftaldialdehido

ORAC: Capacidad de Absorción de Radicales de Oxígeno

PCA: Análisis de Componentes Principales

PCR: Reacción en Cadena de la Polimerasa

PEF: Campo Eléctrico Pulsado

PFGE: Electroforesis en Gel de Campo Pulsado

REA-PFGE: Análisis de Endonucleasas de Restricción por Electroforesis en gel de Campo Pulsado

RNA: Ácido Ribonucleico

RP-HPLC: Cromatografía de Líquidos de Alta Eficacia en Fase Inversa

SPME: Microextracción en Fase Sólida

UA: Unidades de Aroma

UPGMA: Medias Aritméticas por Grupo No Ponderadas

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Resumen

I. RESUMEN

Las bacterias lácticas (BAL) son las responsables de la fermentación maloláctica (FML) en el vino, cuyo principal efecto durante la vinificación es la desacidificación biológica, y la consiguiente mejora de su calidad organoléptica y estabilidad microbiológica. Por otro lado, si esta etapa se lleva a cabo sin control pueden producirse actividades metabólicas bacterianas que alterarían la calidad del vino, tales como la producción de aminas biógenas; cuya presencia afecta a la seguridad sanitaria y exportación de los vinos. Por ello, en bodega se adiciona el anhídrido sulfuroso o dióxido de azufre (SO_2), el cual presenta múltiples propiedades como conservante entre las que se pueden destacar los efectos antioxidante y antimicrobiano, éste último selectivo especialmente frente a BAL. Sin embargo, el empleo de este aditivo está estrictamente regulado debido a que a dosis elevadas puede dar lugar a modificaciones organolépticas y fundamentalmente a riesgos para la salud humana.

Con el objetivo global de reducir el contenido de SO_2 en los vinos, la presente Tesis propone el uso de polifenoles antimicrobianos frente a las BAL del vino como una alternativa total o parcial al empleo de SO_2 en enología.

Inicialmente se evaluó el efecto antimicrobiano de los compuestos fenólicos del vino sobre el crecimiento y viabilidad de BAL, se comprobó que dicha propiedad estaba fuertemente determinada por su estructura química, así como por la concentración y cepa ensayada. El mecanismo implicado en este efecto podría estar basado en interacciones hidrofóbicas entre los compuestos fenólicos y los lípidos de la membrana bacteriana, que producirían daños en la integridad de la membrana y la consiguiente muerte celular. Además, estos polifenoles antimicrobianos destacaron por mostrar un al poder antioxidante, no estando relacionadas estadísticamente ambas propiedades.

Después se determinó la capacidad de degradar aminas biógenas histamina, tiramina y putrescina de un gran número de BAL de origen enológico, evaluándose posteriormente el efecto de los polifenoles y otros componentes de la matriz (etanol y SO2) del vino sobre este metabolismo degradativo. La actividad amino degradativa se halla con baja frecuencia en BAL enológicas y se ve influenciada por los polifenoles y contenido de etanol y SO_2 del vino, lo que hace necesario realizar un mayor número de estudios antes de proceder a su aplicación.

Por último, se procedió a evaluar la eficacia tecnológica de los polifenoles durante la vinificación. En una primera etapa se seleccionaron extractos fenólicos de

origen vegetal con capacidad antimicrobiana frente a bacterias acéticas y lácticas del vino para posteriormente proceder a evaluar su potencial uso como agente antimicrobiano durante la FML. Se observó que la actividad antimicrobiana de los extractos dependía de su composición y contenido fenólico así como de la cepa ensayada y al igual que los polifenoles producían daños en la membrana bacteriana y manifestaban un alto poder antimicrobiano. Por parte, la adición de extractos fenólicos (eucalipto y almendra) durante la FML produjo un consumo más lento del ácido málico y un recuento menor de bacterias, que explicaría este bajo consumo. A su vez, se llevó a cabo la caracterización química y microbiana de los vinos elaborados en presencia/ausencia de extractos fenólicos. El uso de técnicas cromatográficas nos permitió detectar posibles modificaciones en la fracción volátil y fenólica de los vinos tratados o no con extractos fenólicos. Todos los vinos se caracterizaron por un alto descenso en el contenido esteres (>75%) y cambios poco significativos en el perfil fenólico. El empleo de técnicas moleculares nos permitió determinar que *O.oeni* fue la BAL responsable de la FML, observándose una gran diversidad de cepas de esta especie. Las diferencias observadas entre la población microbiana de los diferentes vinos estudio se debieron al tipo de FML (espontáneo o no) y no a la adición de extractos fenólicos durante la elaboración del vino. En conjunto, estos estudios confirman el potencial empleo de los polifenoles como alternativa al SO_2 en enología.

Interés y Objetivos

II. INTERÉS Y OBJETIVOS

Las bacterias lácticas (BAL) son responsables de la fermentación maloláctica (FML) en el vino, cuyo principal efecto y por lo que se busca su desarrollo durante la vinificación es la desacidificación biológica, y la consiguiente mejora de la calidad organoléptica y estabilidad microbiológica de los vinos. Es fundamental que esta etapa se realice de forma controlada, ya que de lo contrario, como resultado de la actividad metabólica bacteriana pueden producirse alteraciones de la calidad organoléptica y seguridad del vino. Entre estas alteraciones cabe destacar la producción de aminas biógenas, cuya presencia en elevadas concentraciones en los alimentos, incluido el vino, supone una preocupación para el sector enólogico y para la Administración, por su potencial efecto tóxico en individuos sensibles.

El anhídrido sulfuroso o dióxido de azufre (SO_2) presenta múltiples propiedades como conservante en la elaboración de los vinos, entre las que destacan los efectos antioxidante y antimicrobiano, especialmente frente a bacterias lácticas. Sin embargo, en los últimos años, existe una tendencia a reducir progresivamente los niveles máximos autorizados en vinificación, debido a que su empleo a dosis elevadas puede generar modificaciones organolépticas indeseables en el producto final y riesgos para la salud humana. Este hecho, junto con la creciente preocupación por parte de los consumidores por el uso de compuestos químicos como conservantes alimentarios, ha promovido un creciente interés en la búsqueda de alternativas. El empleo de productos naturales, entre los que se encuentran los compuestos fenólicos o polifenoles se muestra como una de las posibilidades más prometedoras, debido a que este amplio grupo de compuestos se caracterizan por su doble actividad como antimicrobianos y antioxidantes (García-Ruiz et al., 2008).

En base a lo expuesto, la hipótesis de partida del presente trabajo es que los compuestos fenólicos podrían ser efectivos como aditivos naturales para el control de la fermentación maloláctica, debido a sus propiedades antimicrobianas y antioxidantes, constituyendo una alternativa total o parcial al uso de SO_2 en enología. Además, los polifenoles podrían interferir en la actividad metabólica de las bacterias lácticas del vino, en concreto en la capacidad de degradación de aminas biógenas.

A partir de esta hipótesis, el objetivo de la presente Tesis Doctoral ha sido estudiar el efecto de los polifenoles sobre el crecimiento y metabolismo de bacterias lácticas del

vino con el fin de evaluar su empleo como una alternativa total o parcial al tradicional uso de SO_2 en el control de la fermentación maloláctica del vino.

De una forma más concreta, los objetivos son:

- Evaluar el efecto de los compuestos fenólicos del vino sobre el crecimiento de cepas pertenecientes a las principales especies de bacterias lácticas implicadas en el proceso de fermentación maloláctica y/o causantes de alteraciones de los vinos.

- Realizar un "screening" de cepas de bacterias lácticas aisladas de diferentes nichos enológicos con capacidad para degradar histamina, tiramina y putrescina, las principales aminas biógenas que se pueden encontrar en los vinos, y evaluar el efecto de los polifenoles sobre esta actividad metabólica.

- Seleccionar extractos fenólicos antimicrobianos obtenidos a partir de plantas y diferentes productos vegetales (incluída la vid) con actividad frente a bacterias lácticas de origen enológico. Evaluar la eficacia tecnológica de los extractos fenólicos más activos mediante experimentos de fermentación maloláctica en vinos.

- Caracterizar genéticamente la población de *Oenococcus oeni* representativa de los vinos tratados y no tratados con extractos fenólicos como antimicrobianos, en los experimentos de fermentación maloláctica.

- Establecer los cambios en la composición aromática y polifenólica de vinos tratados y no tratados con extractos fenólicos como antimicrobianos, en los experimentos de fermentación maloláctica.

La realización de esta Tesis Doctoral ha sido posible gracias a la concesión de un contrato de postgrado I3P-CSIC y a una beca predoctoral JAE-CSIC. El trabajo ha sido financiado por el proyecto de investigación AGL2006-04514/ALI ('Efecto de los polifenoles en el crecimiento y metabolismo de bacterias lácticas en vinos. Potencial aplicación como aditivos antimicrobianos en enología') y por el *contrato de investigación OTT20110712 (*'Vinificación más sostenible: empleo de extractos

fenólicos como una alternativa natural a los sulfitos y nuevas vías de valorización de subproductos de vinificación*')*.

Introducción

III. INTRODUCCIÓN

III.1. Vinificación

La vinificación es el conjunto de operaciones puestas en práctica para transformar el jugo o mosto de uva en vino. La fermentación del mosto es un proceso microbiológico complejo que implica interacciones entre levaduras, bacterias y hongos filamentosos (Ribéreau-Gayon y col., 2006) presentes en la uva o procedentes del material de bodega (Fleet y Heard, 1993; Mortimer y Polsinelli, 1999). Como consecuencia de la introducción del mosto en los depósitos de fermentación se reducen las condiciones de aireación; esto favorece el crecimiento de levaduras y bacterias lácticas (BAL) en detrimento de los microorganismos aerobios (bacterias acéticas y hongos). El mosto tiene un alto contenido de azúcares reductores que hace que las levaduras comiencen a transformar estos azúcares en etanol, en la fase conocida como fermentación alcohólica (FA). Durante el transcurso de la FA, las condiciones del medio se modifican (aumento de la concentración de etanol, disminución del pH, etc.), produciéndose una selección natural a favor de aquellos microorganismos mejor adaptados a las nuevas condiciones. Como resultado de este proceso la población de levaduras disminuye, mientras que la población de BAL aumenta, iniciándose entonces la fermentación maloláctica (FML) (Lafon-Lafourcade y col., 1983). Generalmente, la FML se desarrolla tras la FA si las condiciones son favorables, y puede durar entre 5 días y 2 ó 3 semanas, dependiendo de las condiciones físico–químicas del medio y la concentración de ácido málico. Como consecuencia de esta segunda fermentación, aumenta la estabilidad biológica de los vinos así como su calidad y complejidad organoléptica (Versari y col., 1999; Maicas, 2001; Moreno-Arribas y Polo, 2005), especialmente para aquellos que van a ser destinados a envejecimiento en barrica y/o en botella.

III.2. Fermentación maloláctica

La FML es el proceso bioquímico por el cual las BAL presentes en el vino convierten la molécula de ácido L (-) málico en ácido L (+) láctico, liberando una molécula de CO_2 (Figura 1). El ácido málico es uno de los ácidos orgánicos más abundantes del vino; su concentración oscila entre 2 y 10 g/L dependiendo de la región climática de la que proceda la uva, mostrando siempre un mayor contenido en este ácido las uvas que proceden de regiones más frías. En las BAL, la descarboxilación de ácido málico a láctico transcurre mediante una reacción directa catalizada por la enzima maloláctica, que actúa en presencia de los cofactores Mn^{2+} y NAD^{+}. Esta enzima

se ha purificado a partir de diferentes cepas de BAL presentes en la uva y el vino (Lonvaud y Ribereau-Gayon, 1975; Lonvaud-Funel y Strasser de Saad, 1982; Batterman y Radler, 1991). La secuencia del gen bacteriano que codifica la enzima maloláctica se ha caracterizado en *Oenococcoccus oeni* (Labarre y col.., 1996), la principal especie bacteriana responsable de la fermentación maloláctica del vino.

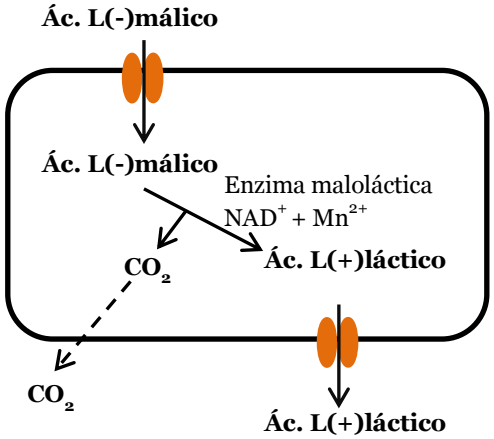


Figura 1. Transformación del ácido L-málico en ácido L-láctico por acción de la enzima maloláctica.

El principal efecto de la FML, y por lo que se busca su desarrollo durante la vinificación, es la desacidificación biológica del vino, que resulta de la transformación de un ácido dicarboxílico (ácido málico) en un ácido monocarboxílico (ácido láctico). Como consecuencia de esta disminución de acidez total, se va a producir un aumento del pH de 0.1-0.2 unidades y un cambio en la calidad organoléptica del vino, al desaparecer el sabor astringente (ácido málico) por otro más suave (ácido láctico). Esta desacidificación es más importante para aquellos vinos que proceden de regiones climáticas frías en los que, como ya se ha mencionado, el contenido de ácido málico en la uva es bastante elevado.

La FML también conlleva otras reacciones enzimáticas y transformaciones metabólicas (Figura 2) que originan compuestos que modifican el aroma y "*flavour*" y la composición y características del producto final. En relación a las implicaciones sobre el perfil aromático del vino, la FML potencia el aroma afrutado y "a mantequilla", y reduce los aromas varietales, desarrollando también otros nuevos aromas de tipo floral, tostado, vainilla, dulce, madera, etc. (Bartowsky y col., 2002; Pozo-Bayón y col.., 2005). Además, este proceso también aumenta el cuerpo, untuosidad y redondez del vino (Jeromel y col., 2008), debido al incremento de polialcoholes y polisacáridos por el metabolismo de las BAL.

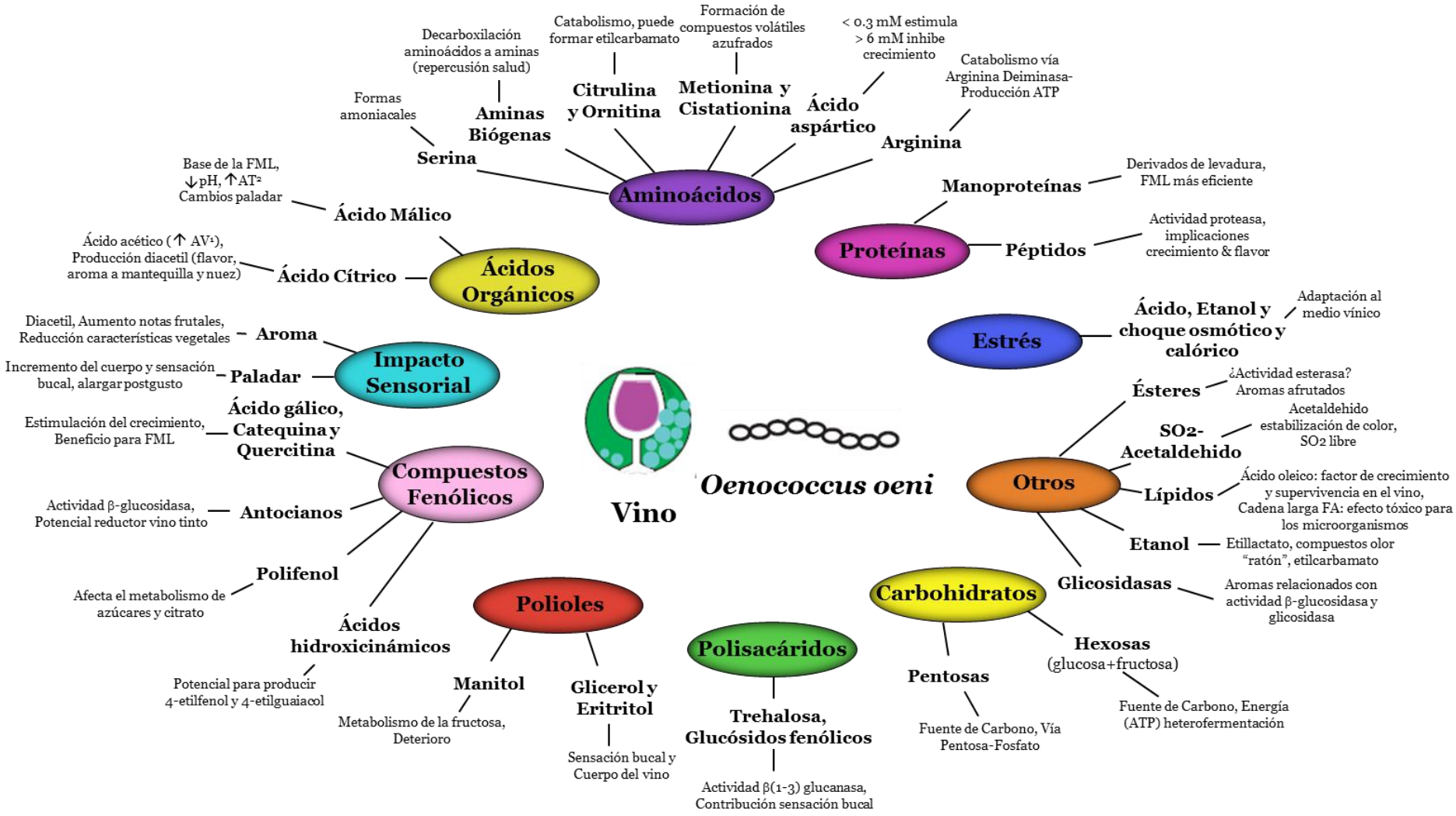


Figura 2. Transformaciones bioquímicas del vino producidas por el metabolismo de *O. oeni* durante la FML (Tomada de Bartowsky y col., 2005)

Por otro lado, también se ha puesto de manifiesto que la FML puede influir en el color del vino, disminuyendo la intensidad del mismo. Esto es debido fundamentalmente a una posible adsorción de antocianos por las paredes celulares bacterianas, a lo que también contribuye la subida de pH y el descenso de los niveles de anhídrido sulfuroso libre (Suárez-Lepe e Iñigo-Leal, 2003). No obstante, los vinos que han llevado a cabo la FML, muestran una mejor estabilización del color, especialmente los vinos tintos (Vivas y col., 2000, Moreno-Arribas y col., 2008).

Por último, es importante añadir que la estabilidad microbiológica del vino se ve favorecida por la FML. Después de este proceso, la concentración de nutrientes es menor y esto impide el crecimiento de otras bacterias y microrganismos alterantes. Además durante la FML, las BAL sintetizan compuestos antimicrobianos como sucede con algunas especies del género *Lactobacillus* que sintetizan polipéptidos con efecto bactericida sobre otras BAL (Rammelsberg y Radler, 1990, Navarro y col., 2002).

III.3. Bacterias lácticas de origen vínico

Bajo el nombre de BAL se engloba un conjunto de microorganismos de una gran diversidad tanto morfológica como fisiológica. El concepto de "bacterias lácticas" como grupo microbiano surgió a principios del siglo XX y responden a la definición general de bacterias Gram-positivas, en forma de cocos, bacilos o coco-bacilos, inmóviles, no esporuladas, anaerobias aerotolerantes, catalasa negativas y desprovistas de citocromos. En consecuencia, presentan un metabolismo estrictamente fermentativo, sintetizando ácido láctico como principal producto de la fermentación de carbohidratos (Axelsson, 2004). Por otro lado, es un grupo, nutricionalmente, complejo, por lo que requieren una gran cantidad de factores nutritivos, tales como aminoácidos, bases nitrogenadas y vitaminas para su crecimiento.

Las BAL son microorganismos muy versátiles y se hallan extensamente distribuidos en la naturaleza. Así, han sido aislados de una gran variedad de productos fermentados, no fermentados e incluso del tracto gastrointestinal de mamíferos. También están implicadas en la fermentación de muchos alimentos y piensos, ya que no existe ninguna indicio de que representen un riesgo para la salud del consumidor, por lo que son consideradas como GRAS (Generally Recognized As Safe) por la *Food and Drug Administration* (FDA) de EEUU. Además, debido a su actividad metabólica sobre azúcares, ácidos orgánicos, proteínas o lípidos estos microorganismos son utilizados en la industria alimentaria, para mejorar el valor nutricional, la preservación

y las características sensoriales de una amplia variedad de productos, tales como leche, bebidas alcohólicas, carnes y vegetales. Así mismo, en los últimos años han logrado gran popularidad debido a la publicación de numerosos trabajos que ponen de manifiesto los beneficios que ejerce la ingesta de determinadas estirpes BAL sobre la salud del consumidor.

Las BAL se pueden clasificar en cocos y bacilos, en función de su morfología. En base a la ruta metabólica de degradación de la glucosa (Tabla 1), las BAL se clasifican como '*homofermentativas*' cuando realizan la glucólisis o '*heterofermentativas*' si llevan a cabo la ruta 6–fosfogluconato/fosfocetolasa. Sin embargo, la glucólisis puede conducir a una fermentación heteroláctica cuando el piruvato es transformado en otros productos como acetato, formiato o etanol (sistema piruvato-formiato liasa), o diacetilo, acetoina y 2,3-butanodiol (ruta diacetilo/acetoina). Por otra parte, algunas BAL consideradas como homofermentativas catabolizan las pentosas mediante la segunda parte de la ruta 6–fosfogluconato/fosfocetolasa, tras su conversión en xilulosa–5–P, formándose cantidades equimolares de ácido acético y láctico. Se considera entonces que son '*heterofermentativas facultativas*'.

Tabla 1. Principales especies de BAL aisladas de mostos y vinos (Pozo-Bayón y col., 2009)

Género	Metabolismo de azúcares	Especie	Etapa de la vinificación
Pediococcus	Homofermentativo	*P. damnosus*	Mosto, FA*, Vino, Vino deteriorado ('viscosidad')
		P. parvulus	Mosto, FA, Vino
		P. pentosaceus	Mosto, FA, Vino
Leuconostoc	Heterofermentativo	*L. mesenteroides*	Uva, Mosto, Vino
Oenococcus	Heterofermentativo	*O. oeni*	Uva, Mosto, FA, FML**, Vino envejecido en barrica
Lactobacillus	Homofermentativa	*L. mali*	Uva, Mosto, Vino
	Heterofermentativa facultativa	*L. plantarum*	Uva, Mosto, Vino, Vino base para producir brandy
		L. casei	Mosto, Vino
	Heterofermentativa	*L. brevis*	Mosto, FA, Vino
		L. hilgardii	Mosto, FA
		L. paracasei	Mosto, Vino
		L. zeae	Vino de crianza biológica
		L. vini	Vino
		L. kunkeei	Uva, FA, FA en vinos deteriorados
		L. lindneri	Uva
		L. kefiri	Uva
		L. vermiforme	Vino
		L. trichodes	Vino deteriorado
		L. fermentum	FA
		L. cellobiosus	FA
		L. nageli	FA en vinos deteriorados

*FA: fermentación alcohólica; **FML: fermentación maloláctica

III.3.1. *Ecología de las bacterias lácticas durante la vinificación*

Las BAL están presentes durante todas las etapas de la elaboración del vino (Figura 3), produciéndose a lo largo de la misma una sucesión en el crecimiento de varias especies (Wibowo y col., 1985; Boulton y cols, 1996; Fugelsang, 1997). Las BAL se pueden aislar de las hojas de la viña, de la uva, del equipamiento de la bodega, de las barricas, etc. (Tabla 1). En el viñedo, la diversidad y densidad poblacional (10^2-10^3 ufc/g uva) de las BAL es muy inferior a la mostrada por las levaduras autóctonas de la uva (Fugelsang, 1997). La población de BAL de esta etapa va a depender del estadío madurativo y sanitario de las uvas, siendo mayoritarias las especies pertenecientes a los géneros *Pediococcus* y *Leuconostoc* (Jackson, 2008).

Durante las primeras etapas de la vinificación (mosto y principio de la FA), la densidad de población de las BAL alcanza una concentración de 10^3–10^4 ufc/mL, siendo las especies mayoritarias durante la misma: *Lactobacillus plantarum*, *L. casei*, *L. hilgardii*, *Leuconostoc mesenteroides* y *Pediococcus damnosus* y en menor proporción *Oenococcus oeni* y *L. brevis* (Wibowo y col., 1985; Lonvaud-Funel y col., 1991; Boulton y col., 1996; Powell y col., 2006). En el tiempo que transcurre entre el final de la FA y el inicio de la FML (densidad BAL= 10^6 ufc/mL) (Wibowo y col., 1985; Lonvaud-Founel, 1999), tiene lugar la fase de multiplicación bacteriana. En esta fase influyen fundamentalmente el pH del medio, el contenido de SO_2, la temperatura y la concentración de etanol (Boulton y col., 1996; Volschenk y col., 2006), siendo las condiciones óptimas para la supervivencia y proliferación de las BAL un pH 3.2-3.4, una temperatura comprendida entre 18 y 22 ºC y una concentración de SO_2 total de 30 mg/L (Lerm y col., 2010). Las condiciones particulares de cada vino, fundamentalmente el contenido en compuestos fenólicos, podrían afectar también al crecimiento de las BAL (Vivas y col., 2000) sin que todavía se conozca suficientemente este proceso. La especie bacteriana que predomina al final de la FA es *O. oeni*. Ésta es la especie mejor adaptada al crecimiento en las difíciles condiciones impuestas por el medio (bajo pH y elevada concentración de etanol) (Davis y col., 1985; Van Vuuren y Dicks, 1993). Aunque se considera que *O.oeni* es la principal especie responsable del desarrollo de la FML en la mayor parte de los vinos, otras especies de los géneros *Lactobacillus* y *Pediococcus,* pueden participar en este proceso, sobre todo en vinos con valores altos del pH.

Una vez que el ácido málico ha sido totalmente consumido por las BAL, es necesario eliminar cualquier población residual de BAL, para evitar alteraciones en etapas más avanzadas de la vinificación. En esta fase, la supervivencia de las BAL

dependerá de las condiciones del medio, especialmente del pH, del contenido en etanol y sobre todo de la concentración de SO_2. En la práctica, la especie *O. oeni* desaparece rápidamente mientras que algunas cepas de los géneros *Pediococcus* y *Lactobacillus* pueden permanecer en bajas concentraciones. Por ello, es una práctica habitual la eliminación de las BAL del vino mediante el sulfitado una vez que todo el ácido málico del vino ha sido degradado. Dado que la efectividad del SO_2 depende del pH, los niveles de esta molécula necesarios para frenar la actividad de las BAL oscilan entre 10-30 mg/L de SO_2 libre en el caso de los vinos con valores de pH comprendidos entre 3.2-3.6 y entre 30-50 mg/L para vinos con valores comprendidos entre 3.5-3.7. Si se trata de vinos con pH superiores, lo que es cada vez más frecuente en el caso de los vinos tintos, la dosis necesaria de SO_2 libre puede llegar incluso a valores cercanos a 100 mg/L (Zamora, 2005).

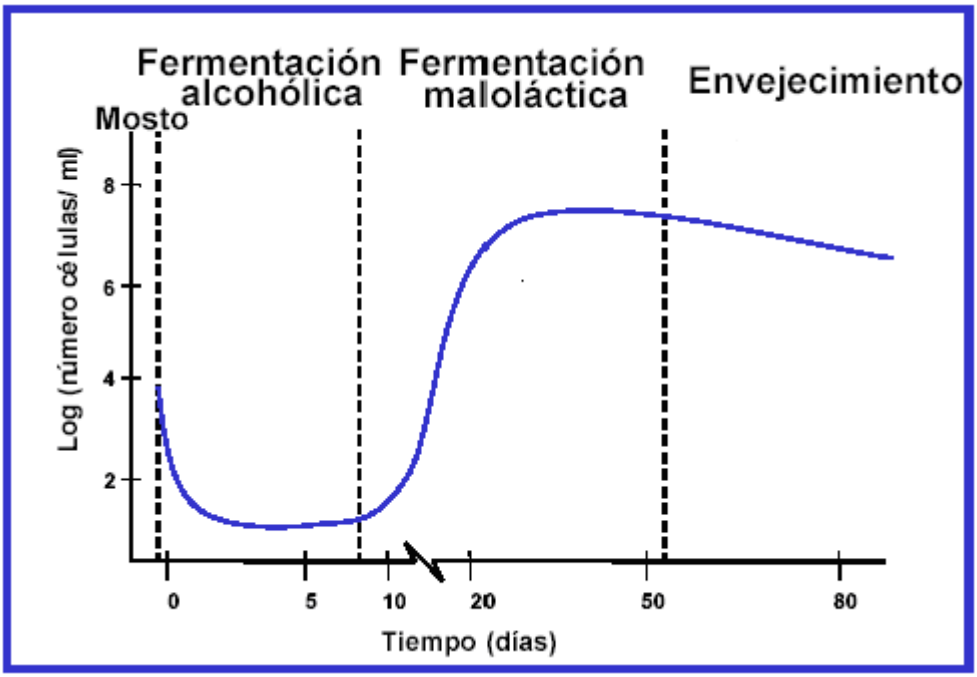


Figura 3. Evolución de la población de bacterias lácticas durante la vinificación de vinos tintos

III.3.2. *Alteraciones del vino debidas a las bacterias lácticas*

En determinadas ocasiones, durante la elaboración industrial del vino, el desarrollo de las BAL y la FML resulta impredecible, ya que puede producirse durante la FA o incluso durante la conservación o envejecimiento del vino. En estos casos, como consecuencia del metabolismo de estas bacterias, se producen cambios en la

composición del vino que se traducen en una alteración de su calidad, convirtiéndolo en algunas ocasiones en un producto no apto para el consumo.

Entre las alteraciones que modifican la calidad organoléptica del vino se encuentran:

- El denominado "picado láctico", que se caracteriza por aumentar considerablemente la acidez volátil del vino (Strasser de Saad y Manca de Nadra, 1992).

- La degradación de glicerol (Garai-Ibabe y col., 2008) y producción de acroleína (Bauer y col., 2010) que al reaccionar con compuestos fenólicos como los taninos puede dar lugar a sabores amargos.

- La producción de polisacáridos extracelulares que van a generar una viscosidad anormal en el vino (Dols-Lafarge y col., 2008; Ciezak y col. 2010).

- La producción de olores desagradables, asociados a la presencia de fenoles volátiles, sintetizados principalmente a partir de los ácidos fenólicos *p*-cumárico y ferúlico (Cavin y col., 1993; Lonvaud-Funel, 1999), y/o bases heterocíclicas asociadas especialmente al metabolismo de ciertos aminoácidos como la ornitina y la lisina (Costello y col., 2001; Swiegers y col., 2005), que otorgan al vino los denominados olores "animal-medicinal" y "orina de ratón", respectivamente.

Como consecuencia del metabolismo de las BAL también se pueden generar compuestos que afecten a la calidad sanitaria del vino, como por ejemplo la formación de precursores del carbamato de etilo, que a dosis elevadas se ha asociado con efectos cancerígenos en animales de experimentación (CalEPA, 1999), o la síntesis de aminas biógenas potencialmente tóxicas (Moreno-Arribas y col., 2000; Landete y col., 2005; Marcobal y col., 2006a; 2006b). El efecto de estas aminas sobre la calidad del vino será descrito con más detalle en el apartado 4 de la presente introducción.

En la mayoría de los casos, se han identificado cepas pertenecientes a los géneros *Lactobacillus* y *Pediococcus* como causantes de estas alteraciones, aunque también se han descrito algunas cepas alterantes de *O. oeni*. Por todo ello, durante la elaboración del vino tiene un especial interés ejercer un buen control sobre la FML.

Existe una gran variedad de *técnicas moleculares* que permiten caracterizar las BAL del vino, así como mejorar el conocimiento de estas bacterias y su papel en el proceso de vinificación (Lonvaud-Funel, 1995). Estas técnicas basadas generalmente en la reacción en cadena de la polimerasa (PCR) nos van a permitir, de forma rápida y sensible, identificar y diferenciar unas especies de BAL de otras e incluso distinguir cepas pertenecientes a una misma especie (Bartowsky y col., 2003b). Entre las técnicas que permiten clasificar las BAL a nivel de especies se encuentran la secuenciación del gen que codifica la subunidad pequeña o 16S del ARN ribosómico (Sato y col., 2001; Narváez-Zapata y col., 2010) o el gen que codifica la subunidad β de la ARN polimerasa (gen *rpoB*) (Renouf y col., 2006) o la electroforesis en gel con gradiente desnaturalizante (DGGE) (López y col., 2003, Renouf y col., 2006; Narváez-Zapata y col., 2010; Ruiz y col., 2010a) (Figura 4). Mientras que los métodos más empleados para caracterizar las BAL hasta el nivel de cepa son la electroforesis en campo pulsado (PFGE) (Gindreau y col., 1997; Zapparoli y col., 2000; Sato y col., 2001;López y col., 2008; Claise y Lonvaud-Funel; 2012), la técnica de RAPD (Random Amplified Polymorphic DNA) (Zavaleta y col., 1997; Zapparoli y col., 2000; Ruiz y col., 2010b; Pérez-Martín y col., 2011) o la secuenciación multilocular o MLST (Multilocus Sequence Typing) (de las Rivas y col., 2004; Bilhère y col., 2009; Bridier y col., 2010). Por otra lado, técnicas como la multiplex PCR permiten de forma simultánea la identificación y tipificación de las BAL (Araque y col., 2009).

Productos PCR en gel de agarosa.

Migración en gel de acrilamida DGGE.

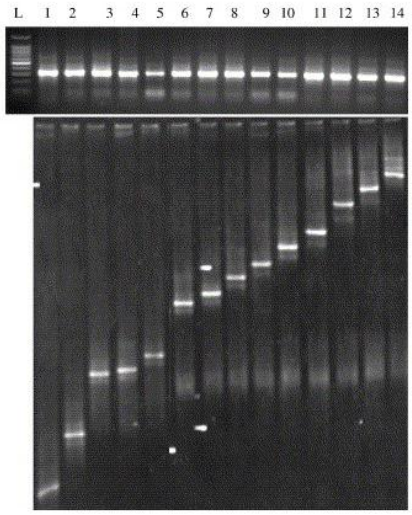


Figura4. Productos *rpoB*-PCR de cocos y especies de *Lactobacillus* aislados de bebidas fermentadas en gel de agarosa y gel DGGE. L: Marcador 100pb; 1: *L. fermentum*; 2: *L. casei*; 3: *L. plantarum*; 4: *Oenococcus oeni*; 5: *L. brevis*; 6: *Pediococcus parvulus*; 7: *L. sakei*; 8: *L. mesenteroides*; 9: *L. hilgardii*; 10: *P. dextrinicus*; 11: *P. pentosaceus*; 12: *P.damnosus*; 13: *L. mali*; 14: *L. buchnerii* (Renouf y col., 2006).

III.4. Aminas biógenas en vinos

Las aminas biógenas son bases nitrogenadas de bajo peso molecular que en los alimentos y bebidas fermentadas se producen generalmente por la descarboxilación de los correspondientes aminoácidos precursores (Silla, 1995). Esta reacción es catalizada por enzimas aminoácido descarboxilasas de origen microbiano. Las aminas biógenas asociadas al vino pueden clasificarse en base a su estructura química en: alifáticas (putrescina, cadaverina, etilamina, metilamina, espermina y espermidina), aromáticas (tiramina, feniletilamina) o heterocíclicas (histamina, triptamina); o en base al número de grupos aminos en: monoaminas (tiramina y feniletilamina), diaminas (putrescina y cadaverina) o poliaminas (espermina y espermidina).

El contenido total de aminas biógenas en el vino varía desde niveles traza hasta concentraciones que pueden llegar a alcanzar los 130 mg/L (Soufleros y col., 1998). Las aminas biógenas mayoritarias y más frecuentemente detectadas en vinos son la histamina, tiramina, putrescina y cadaverina (Figura 5) que se producen a partir de la descarboxilación de los correspondientes aminoácidos precursores, histidina, tirosina, ornitina y lisina, respectivamente. En concentraciones bajas estas aminas resultan esenciales para las funciones metabólicas y fisiológicas de animales, plantas, y microorganismos. Sin embargo, su presencia en elevadas concentraciones es empleada como un marcador de la calidad de los alimentos, incluido el vino. Por otro lado, varios países europeos han impuesto recomendaciones a las concentraciones máximas de histamina en los vinos, como es el caso de Suiza y Austria (10 mg/L), Francia (8 mg/L), Bélgica (5-6 mg/L), Finlandia (5 mg/L), Holanda (3 mg/L) y Alemania (2 mg/L) (Lehtonen, 1996). Este hecho afecta a la importación y exportación de vinos a países de la UE y, a menudo, es causa de trabas comerciales en el mercado internacional.

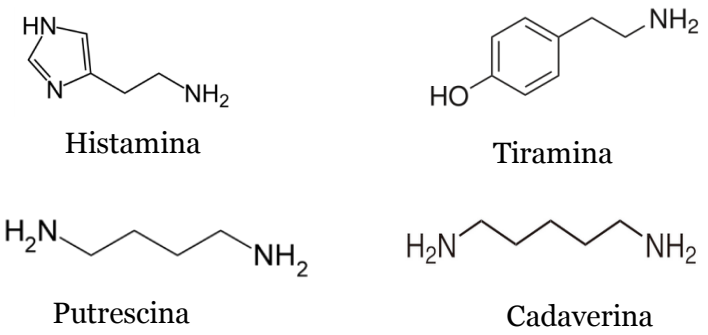


Figura 5. Estructura química de las aminas biógenas más relevantes asociadas al vino.

El problema de la formación de aminas biógenas afecta a numerosos productos alimentarios fermentados como queso, cerveza, algunos embutidos y productos cárnicos fermentados (Fernández-García y col., 1999; Izquierdo-Pulido y col., 2000; Kaniou y col., 2001) que, en general, contienen mayores concentraciones de estos compuestos que los vinos. Sin embargo, en las bebidas alcohólicas, y especialmente en el vino, las aminas biógenas han recibido una especial atención, debido a que el etanol puede aumentar su efecto sobre la salud inhibiendo indirecta o directamente las enzimas encargadas de la detoxificación de estos compuestos (Maynard y Schenker, 1996). El organismo humano tolera fácilmente concentraciones bajas de aminas biógenas, ya que éstas son eficientemente degradadas por las enzimas monoamino oxidasa (MAO) y diamino oxidasa (DAO) en el tracto intestinal (Ten Brink y col., 1990). Estas enzimas transforman las aminas en productos no tóxicos, que son finalmente excretados. Por ejemplo, la histamina puede ser metabolizada por varias rutas enzimáticas (Figura 6). En la primera vía, la estructura del anillo de la histamina es metilada por la histamina N-metiltransferasa (HMT) para formar N-metilhistamina. Este producto puede ser todavía más oxidado por la MAO para formar N-metil imidazol ácido acético. En la segunda vía, la histamina es oxidada por la DAO para formar imidazol ácido acético (Stratton y col., 1991).

Aunque existen diferentes susceptibilidades individuales a la intoxicación por aminas biógenas, se considera que tras la ingestión de cantidades excesivas de las mismas, se pueden iniciar varias reacciones toxicológicas. Las intoxicaciones más notorias son causadas por la histamina, que se ha asociado a dilatación de vasos sanguíneos, capilares y arterias, dando lugar a dolores de cabeza, presión arterial baja, palpitaciones, edemas, vómitos, diarreas, etc. (Taylor, 1986). Otras aminas, como la tiramina y la feniletilamina pueden causar hipertensión y otros síntomas asociados con vasoconstricción causada por la liberación de noradrelanina (especialmente hemorragias en el cerebro y migraña). La putrescina y cadaverina, aunque no tienen efectos tóxicos por sí mismas, puedan aumentar la toxicidad de la histamina, tiramina y feniletilamina, ya que interfieren en las reacciones de detoxificación.

El vino es un sustrato muy susceptible a la producción de aminas biogenas, ya que su elaboración implica no sólo que están disponibles los aminoácidos libres precursores de estas aminas, sino también la posible presencia de microorganismos con actividad enzimática aminoácido descarboxilasa, y algunas condiciones ambientales (ej. pH) favorables para el crecimiento microbiano, así como para la actividad de las enzimas descarboxilasas (Lonvaud-Funel, 1999). Es por ello que, en los últimos años,

hemos asistido a un interés creciente en la bibliografía por el estudio del origen de estos compuestos durante la vinificación y el desarrollo de métodos de detección y cuantificación de aminas biógenas en vinos. Algunas revisiones sobre este tema se pueden encontrar en Ancin-Azpilicueta y col., (2008); Smit y col., (2008) y Pozo-Bayón y col., (2012).

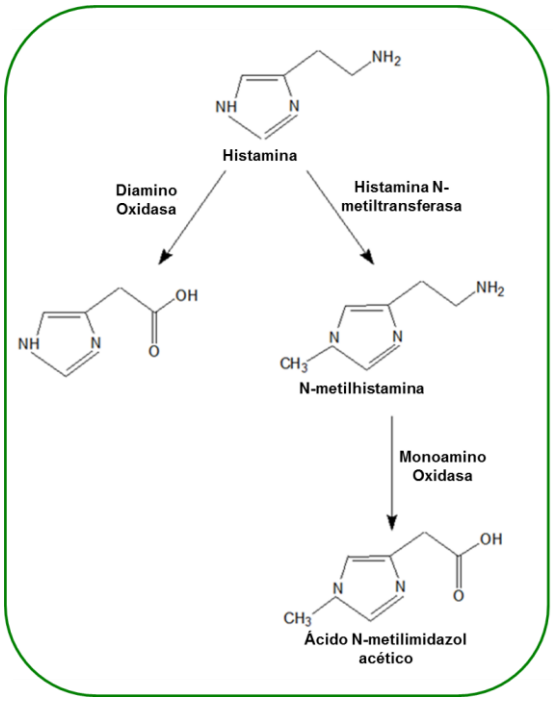


Figura 6. Vías enzimáticas de degradación de la histamina (Tomada de Moreno-Arribas y col., 2010)

Las aminas biógenas pueden estar presentes en la uva, aunque su origen en los vinos está fundamentalmente relacionado con el proceso de vinificación, especialmente como consecuencia de la FML y/o en las etapas posteriores durante el envejecimiento y crianza de los vinos en barrica (Jiménez-Moreno y col., 2003; Marcobal y col., 2006). También las prácticas enológicas empleadas en bodega pueden afectar a la concentración de aminoácidos precursores y/o a la selección de microrganismos con potencial de descarboxilar estos aminoácidos, y por tanto incidir en la evolución del contenido de aminas biógenas en el vino (Martín-Álvarez y col., 2006; Pozo-Bayón y col., 2012). A modo de ejemplo, la tabla 2 resume la información reciente sobre los factores tecnológicos con repercusión en los niveles de aminas biógenas detectados en mostos y vinos.

Tabla 2. Factores tecnológicos relacionados con la formación de aminas biógenas en uvas y vinos

Factores Vitivinícolas	**Bibliografía**
Variedad de uva	Halász *et al.* (1994); Glòria *et al.* (1998); Hajós *et al.* (2000); Cecchini *et al.* (2005); Landete *et al.* (2005b); Bover-Cid *et al.* (2006); Soufleros *et al.* (2007); Del Prete *et al.* (2008)
Fertilización nitrogenada de la viña	Spayd *et al.* (1994); Soufleros *et al.* (2007)
Vintage and region production	Sass-Kiss *et al.* (2000); Herbert *et al.* (2005); Martín-Álvarez *et al.* (2006)
Factores enológicos	
Técnicas de maceración	Bauza *et al.* (1995); Martín-Álvarez *et al.* (2006)
Composición del vino y factores fisico-químicos	Vidal-Carou *et al.* (1990); Lonvaud-Funel and Joyeux (1994); Rollán *et al.* (1995); Moreno-Arribas and Lonvaud-Funel (1999, 2001); Landete *et al.* (2006); Martín-Álvarez *et al.* (2006); Marcobal *et al.* (2006a); Mangani *et al.* (2005); Arena *et al.* (2007)
Condiciones de envejecimiento	Vazquéz-Lasa *et al.* (1998); Moreno and Ancín Azpilicueta (2004); Martín-Álvarez *et al.* (2006); Marcobal *et al.* (2006a); Alcaide-Hidalgo *et al.* (2007); Hernández-Orte *et al.* (2008)

Aunque potencialmente todos los microrganismos asociados con la vinificación pueden intervenir en la acumulación de aminas biógenas en los vinos, se asume que la contribución de las levaduras es mucho menor que la de las BAL, que se consideran los principales microorganismos responsables de la formación de aminas biógenas en vinos (Moreno-Arribas y cols., 2003; Landete y cols., 2005; Marcobal y cols., 2006). Es bien conocido que entre las especies y cepas de BAL del vino, algunas son prácticamente incapaces de producir aminas biogénas, mientras que otras se caracterizan por su elevada capacidad de producción de estos compuestos (Tabla 3). Esta capacidad es frecuente entre los lactobacilos heterofermentativos (*Lactobacillus hilgardii* y *L. brevis*) (Moreno-Arribas y col., 2000), aunque también se han aislado cepas de *Pediococcus* (Landete y col.., 2005a) y de *O. oeni* productoras de histamina (Coton y col., 1998), y *O. oeni* productores de putrescina (Marcobal y col., 2004). En *O. oeni*, la capacidad de producir putrescina está codificada cromosómicamente (Marcobal y col., 2006b), aunque se ha comprobado que tanto la presencia del gen que codifica para la ornitina descarboxilasa como la capacidad para producir putrescina es un característica atípica y poco frecuente en esta especie (Moreno-Arribas y col., 2003). Otros estudios muestran que la presencia de cepas de *O. oeni* productoras de histamina es frecuente durante la FML del vino. En estas bacterias, se ha comprobado que el gen que codifica para la enzima histidina descarboxilasa, implicadas en la producción de histamina, parece que está localizado en un plásmido inestable (Lucas y col., 2008), lo que explica el hecho de que estas cepas pierdan esta capacidad metabólica durante las etapas de cultivo en el laboratorio.

Si bien la información disponible acerca de la capacidad de producción de aminas biógenas por BAL del vino es amplia, se conoce muy poco sobre el potencial de este grupo microbiano en la degradación de estos compuestos. Se ha descrito actividad amino oxidasa en algunas bacterias aisladas de alimentos, como *Micrococcus varians* (Leuschner y col., 1998) y *Staphylococcus xylosus* (Martuscelli y col., 2000; Gardini y col., 2002) aisladas de embutidos, y en BAL empleadas como cultivos iniciadores en el ensilaje de pescado (Enes-Dapkevicius y col., 2000), sin embargo no se ha descrito esta actividad metabólica en ninguna BAL de origen enológico. Tampoco se conoce la influencia de la matriz del vino, y en concreto de componentes mayoritarios, como los polifenoles, en este metabolismo de interés para controlar la concentración final de aminas biógenas del vino.

Tabla 3. Microorganismos asociados a la producción de aminas biógenas durante la vinificación (Moreno-Arribas y col., 2010).

Especie	Función	Amina biógena / Actividad metabólica
Saccharomyces cerevisiae	Levadura fermentadora del vino	Histamina
Brettanomyces bruxellensis	Levadura alterante	Agmatina, feniletilamina, etanolamina
Kloeckera apiculata, Candida stellata, Metschnikowia pulcherrima	Levaduras autóctonas	Agmatina, feniletilamina, etanolamina
Botrytis cinerea	Hongos de los vinos Azsú	Tiramina, putrescina, cadaverina, feniletilamina, espermidina
Lactobacillus spp., *Pediococcus* spp.	Bacterias lácticas fermentadoras y alterantes	Histamina (histidina decarboxilasa)
		Tiramina (tirosina decarboxilasa)
		Putrescina (ornitina decarboxilasa)
		Feniletilamina
Oenococcus oeni	Fermentación maloláctica	Histamina (histidina decarboxilasa)
		Putrescina (ornitina decarboxilasa)

III.5. Anhídrido sulfuroso o dióxido de azufre (SO_2)

El anhídrido sulfuroso o dióxido de azufre (SO_2) es el principal conservante utilizado durante la vinificación para proteger a los vinos de posibles alteraciones. Su uso como conservante enológico se conoce desde la antigüedad, siendo ya utilizado en tiempos de egipcios y romanos para la desinfección y limpieza de bodegas (Frazier y Westhoff, 1978). Pero ha sido en las últimas décadas cuando se han adquirido la mayor parte de los conocimientos científicos sobre su empleo en enología, extendiéndose su uso en operaciones de pre-fermentación durante la vinificación.

En los vinos, este compuesto tiene múltiples propiedades, entre las que se pueden destacar su capacidad antimicrobiana y antioxidante. El SO_2 es un agente antiséptico frente a levaduras y bacterias, presentando un mayor poder antimicrobiano frente a BAL, que frente a levaduras. El SO_2 impide la oxidación no enzimática y enzimática del vino mediante un consumo lento del oxígeno e inhibición de enzimas oxidativas tales como las tirosinasas y lacasas. Además, la unión del SO_2 con el etanol y otros compuestos similares protege los aromas del vino. Por otra parte, también

previene el pardeamiento de los vinos mediante la inactivación de enzimas como la polifenoloxidasa, peroxidasa y proteasas, e inhibe la reacción de Maillard (Ribérau-Gayon y col., 2006).

Generalmente, a las concentraciones en las que están presentes los sulfitos en el vino no existe riesgo para el consumidor. Sin embargo, en los últimos años, existe una tendencia a reducir progresivamente los niveles máximos de SO_2 autorizados en los mostos y vinos, debido al aumento de problemas para la salud humana, preferencias de los consumidores, posibles alteraciones organolépticas en el producto final (olores defectuosos producidos por el propio gas sulfuroso, o por su reducción a sulfhídrico y otros mercaptanos) y a una legislación cada vez más estricta sobre los conservantes (du Toit y Pretorius, 2000; Santos y col., 2012). Aunque en la actualidad, ningún compuesto conocido puede desplazar al SO_2 en todas sus propiedades enológicas, existe un gran interés por la búsqueda de otros conservantes inocuos para la salud que puedan sustituir o al menos complementar la acción del SO_2, permitiendo la reducción de su nivel en los vinos (García-Ruiz y col., 2008; Bartowsky, 2009; Pozo-Bayón y col., 2012; Santos y col., 2012).

III.5.1. *Química y propiedades del SO_2*

Durante la vinificación, las distintas formas químicas del SO_2, libre y combinada, se encuentran en un equilibrio que depende del pH, composición y temperatura del vino. El *SO_2 libre* se define como la fracción presente en forma gaseosa o inorgánica en el vino, mientras que la *fracción combinada* es aquella que se encuentra unida a las diferentes sustancias orgánicas del vino, denominándose *SO_2 total* a la suma de ambas fracciones (Figura 7).

El SO_2 libre al pH del vino está presente en las formas: ácido sulfúrico (H_2SO_3), gas dióxido de azufre (SO_2) y bisulfato de hidrógeno (HSO_3^-). El SO_2 molecular constituye la llamada forma “activa" del SO_2, responsable de la mayor parte de sus propiedades enológicas, las cuales dependen del pH del vino. De la misma manera, podría ser el causante del sabor y olor desagradable en el vino.

La mayor parte del SO_2 adicionado al mosto o al vino está combinado con diversos compuestos orgánicos, tales como: azúcares, polisacáridos, polifenoles, etc. La principal unión del SO_2 con estos compuestos, se produce con el acetaldehído (etanal), generándose un compuesto muy estable y por lo tanto irreversible. Por otro parte, la

unión del anhídrido sulfuroso con azúcares, ácidos, etc., es menor y reversible, denominándose a este dióxido de azufre *SO_2 residual*.

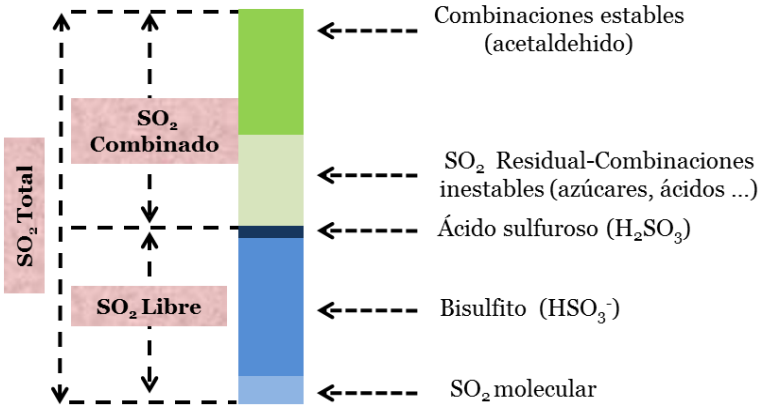


Figura 7. Diferentes formas del SO_2 al pH del vino

El SO_2 combinado es más abundante que el SO_2 libre en el vino. Sin embargo, esta fracción tiene menor relevancia que el SO_2 libre en relación a las propiedades antisépticas y antioxidantes del SO_2 en el vino; a pesar de que su unión con el etanal permite la protección de aromas del vino y hace que el carácter plano del mismo desaparezca.

Los derivados azufrados utilizados habitualmente en enología son el SO_2, y el metabisulfito de sodio y/o de potasio ($Na_2S_2O_5$ y $K_2S_2O_5$), entre otros. Durante la vinificación, estos productos se utilizan fundamentalmente en tres etapas (Figura 8). Una primera, en las uvas o en el mosto durante la etapa prefermentativa, con el objetivo fundamental de prevenir la oxidación del mismo y rebajar la carga microbiana inicial; más adelante, una vez finalizados los procesos de fermentación y previa a las etapas de crianza o conservación de los vinos, para así inhibir el crecimiento de microorganismos alterantes de los vinos; y como último paso, inmediatamente antes del embotellado, con objeto de estabilizar los vinos e impedir cualquier alteración dentro de las botellas. Por otro lado, el SO_2 también se emplea en la limpieza y desinfección de barricas, aunque la reciente revisión de la Directiva Biocidas 98/8 podría conllevar a su prohibición.

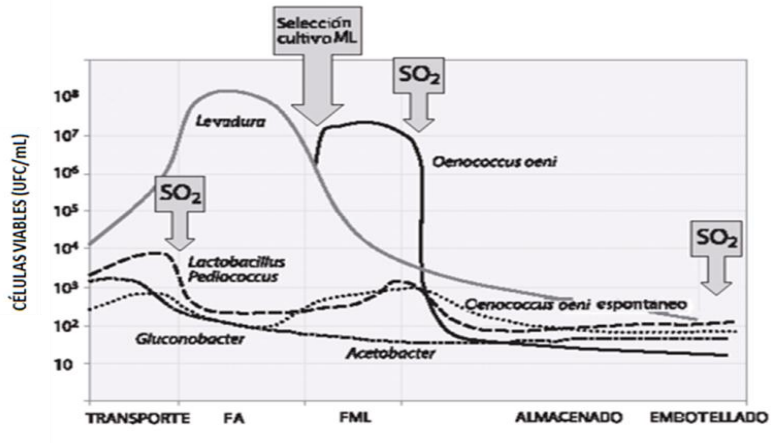


Figura 8. Control del proceso de vinificación mediante la adición de SO_2 (FA: fermentación alcohólica y FML: fermentación maloláctica) (Tomada Krieger, 2008).

III.5.2. *Estudios toxicológicos y aspectos legislativos de la presencia de sulfitos en vino*

Por sus propiedades tecnológicas y bajo coste el SO_2 ha sido ampliamente utilizado en la industria alimentaria (vino, zumo, marisco,...). Sin embargo, algunos estudios han puesto de manifiesto que el empleo de este aditivo puede producir efectos negativos sobre la salud humana, como dolor de cabeza, dificultades respiratorias, diarrea, reacciones alérgicas, fatiga, irritación, hinchazón de cara, labio o garganta... (Taylor y col., 1986; Romano y Suzzi, 1993; Gao y cols., 2002), observándose en los últimos años un aumento en la intolerancia o alta sensibilidad al SO_2, especialmente en personas asmáticas y niños. Este hecho, ha generado una creciente preocupación por parte de los consumidores por el uso de compuestos químicos como conservantes alimentarios y la demanda de la búsqueda de nuevos aditivos naturales. Por otro lado y con el objetivo de aumentar la seguridad de los alimentos, las autoridades europeas han regulado de una forma estricta el uso del SO_2 como conservante alimentario, Directivas 95/2/CE y 2006/52/CE (Tabla 4). En relación al vino, la dosis máxima autorizada por la Organización Internacional de la Viña y el Vino (OIV) es de 150 a 400 mg/L de SO_2 total dependiendo del tipo de vino y de su contenido en materias reductoras. Por otro lado, la Unión Europa (Reglamento Comunidad Europea nº 1493/1999 y 1622/2000) establece que los límites del contenido total de SO_2 en los vinos tintos no podrán exceder de 160 mg/L, y en blancos y rosados de 210 mg/L, mientras en Japón, Estados Unidos, Cánada y Australia el límite de SO_2 total es de 350 mg/L para todos los vinos. Por otra parte, en Estados Unidos, Sudáfrica y la Unión Europea (en concreto desde el 26 de noviembre de 2005, Reglamento nº 1991/2004), la legislación obliga a los

elaboradores, a señalar la presencia de sulfitos en el etiquetado de los vinos, siempre y cuando su nivel exceda de los 10 mg/L. De hecho, en los vinos españoles, es cada vez más frecuente encontrar la indicación *"contiene sulfitos"* en un lugar visible de la etiqueta.

Tabla 4. Concentraciones máximas toleradas de sulfito a nivel europeo en los diferentes alimentos.

Alimento	**Concentración Máxima de SO_2** ($mg\ SO_2/L$ o $mg\ SO_2/Kg$)
Uva de mesa	10
Fruta seca	2000
Coco seco	50
Naranja, pomelo, manzana y piña	50
Jugos concentrados de frutas	250
Patatas deshidratadas	400
Patatas peladas	50
Patatas procesadas	100
Crustáceos cocidos	50
Vino blanco	210
Vino tinto	160
Sidra	200

III.5.3. *Determinación analítica del dióxido de azufre en el vino*

La determinación del SO_2 en el vino es una importante tarea analítica, particularmente en lo que respecta a legislación de seguridad alimentaria, comercio del vino y enología. Para los enólogos y viticultores, la cantidad de SO_2 libre es el valor más importante, ya que proporciona información sobre los procesos de fermentación, mientras que desde un punto de vista legislativo lo es la cantidad total de sulfitos.

Numerosos métodos han sido desarrollados para la determinación de este compuesto en el vino. En general, pueden ser clasificados en dos categorías básicas: a) técnicas que incluyen una destilación inicial para extraer el dióxido de azufre, b) técnicas que utilizan otra reacción química (o procedimiento de separación) para medir el SO_2.

En las bodegas, los métodos más aceptados para la determinación de sulfitos en el vino son el método Ripper (titulación con yodo) (Ripper, 1892; AOAC, 1984) y el método Monier-Williams (destilación + titulación alcalina) (Monier-Williams, 1927; Cunniff, 1995). Ambos procedimientos son lentos y laboriosos y presentan limitaciones, como son una pobre precisión y una baja selectividad (Bruno y col., 1979; Cardwell y col., 1991; Mataix y Lugue de Castro, 1998). Por ello, en los últimos años con el objetivo de minimizar las limitaciones y tiempo de análisis de estos métodos, otros

procedimientos basados en técnicas tales como HPLC, análisis por inyección de flujo (FIA), cromatografía de gases (GC), en combinación con sensores ópticos, métodos electroquímicos, enzimáticos, han sido desarrollados (Tabla 5). Sin embargo, la instrumentación necesaria para la implantación de estas técnicas en bodega es cara y está rara vez presente en los laboratorios de la industria del vino, siendo por ello, y a pesar de sus limitaciones, los métodos Ripper y Monier-Williams, los más utilizados en bodega.

Tabla 5. Metodologías alternativas para la medición de SO_2 libre y total en el vino

SO_2	Separación	Detección	Bibliografía
Libre/Total			
	Cromatografía de gases	Detector fotométrico de llama (FID)	Hamano y col., 1979
	Cromatografía iónica	Electroquímica	Kim y Kim, 1986
	HPLC	Sensor fotométrico	Pizzoferrato y col., 1997
	Electroforesis capilar	UV	Jankovskienė y col., 2003
	Sensor de membrana	Sensor óptico	Silva y col., 2006
	Análisis por inyección secuencial	UV/Vis	Segundo y col., 2001
		Amperométrico	Chinvongamorn y col., 2008
	Análisis por inyección de flujo	Verde malaquita	AOAC, 2005
		Espectofotométrica	Carinhanha y col., 2006
	Sistema de flujo continuo	Sensor piezoeléctrico	Palenzuela y col., 2005
Total			
	Análisis por inyección de flujo	*p*-rosanilina-formaldehido	Linares y col., 1989
		Quimioluminiscencia	Huang y col., 1992
		Potenciométrica	Araújo y col., 1998
		Sensor amperométrico	Corbo y col., 2002
		Conductivímetro	Araújo y col., 2005
	Cromatografía iónica	Conductividad	Cooper y col., 1986
	Membrana bioactiva	Sensor enzimático	Dinçkaya y col., 2007

III.5.4. *Tratamientos complementarios y alternativos al uso del SO_2 en enología*

Las actuales normas legislativas, la preferencia de los consumidores y un aumento de efectos indeseables en la salud humana, justifican una creciente tendencia a reducir los límites máximos permitidos de SO_2 en los mostos y vinos (du Toit y Pretorius, 2000; García-Ruiz y col., 2008; Santos y col., 2012). Aunque en la actualidad, ningún compuesto conocido puede desplazar al SO_2 en todas sus propiedades enológicas, existe un gran interés por la búsqueda de otros conservantes inocuos para la salud que puedan sustituir o al menos complementar la acción del SO_2,

siendo posible así reducir su nivel en el vino (Santos y col., 2012). Este creciente interés científico queda reflejado en los proyectos europeos "Replacement of sulphur dioxide in food Keeling the SAme qualitY and shelf-life of the products" (SOS2SAY) o “WineSul free”, cuyas investigaciones se centran en la búsqueda de alternativas y tecnologías innovadoras que permitan sustituir o reducir al máximo el empleo de SO_2 en diversos alimentos como el vino y zumos.

Las diferentes alternativas propuestas al empleo del SO_2 en el vino, pueden ser clasificadas como físicas, químicas y bioquímicas (Tabla 6), pudiendo ser utilizadas de forma combinada.

III.5.4.1. *Tratamientos físicos*

Tratamientos físicos (Tabla 6), tales como el envasado en atmósfera modificada, almacenamiento bajo control atmosférico, ozono, y otros tratamientos alternativos empleando gases no convencionales han sido aplicados a las uvas de mesa con el fin de prolongar su tiempo de almacenado y vida útil, reduciéndose así las dosis necesarias de SO_2 en la cosecha (Artés-Hernández y col., 2003; 2006).

En los vinos, tecnologías basadas en la radiación ultravioleta (UV) (Valero y col., 2007; Gailunas y col., 2008; Fredericks y col., 2011) y ultrasonido de alta potencia (Jiranek y col., 2008) han sido probados como alternativa al uso de sulfitos debido a que permiten la inactivación de microorganismos presentes en el vino. El empleo de radiación UV ha mostrado propiedades fungicidas (Gailunas y col., 2008) y una reducción significativa de las poblaciones de BAL (Valero y col., 2007); mientras que los mecanismos implicados en la muerte microbiana por ultrasonidos de alta potencia están asociados con un adelgazamiento de las membranas celulares, un calentamiento localizado y por la producción de radicales libres (Fellows, 2000; Butz y Tauscher, 2002).

En los últimos años, el campo eléctrico pulsado (PEF) ha sido empleado durante la vinificación. Esta técnica se basa en la aplicación de cortos (μs) pulsos eléctricos de alto voltaje (>70 kV/cm) (Puértolas y col., 2009) a productos localizados entre dos electrodos. Estudios realizados en mostos y vinos tratados con PEF han mostrado la inactivación de bacterias y levaduras (alteración de la membrana celular y destrucción de algunas enzimas) (Benicho y col., 2002; Heinz y col., 2003; Lustrato y Ranalli, 2009; Lustrato y col., 2010), siendo más sensibles las levaduras. Las características organolépticas de estos vinos no se vieron afectadas (Benicho y col., 2002; Garde-Cerdán y col., 2008; López y col., 2008; Puértolas y col., 2009). Trabajos efectuados a

escala industrial con baja corriente eléctrica, han demostrado la aplicabilidad del PEF para el control de la fermentación del mosto de uva en enología (Lustrato y col., 2003; 2006).

Tabla 6. Tratamientos y compuestos propuestos para controlar el crecimiento de bacterias lácticas en enología.

TRATAMIENTOS FÍSICOS		
Técnicas	**Características Físicas**	**Bibliografía**
Radiación Ultravioleta (UV)*	100 nm – 280 nm	Valero y col., 2007; Fredericks y col., 2011
Ultrasonido de Alta Presión*	20 kHz–10 MHz	Jiranek y cols., 2008
Campo Eléctrico Pulsado*	Pulsos cortos (μs) >70 kV/cm	Garde-Cerdan y col., 2008; Lustrato y col., 2009, 2011
TRATAMIENTOS QUÍMICOS Y BIOQUÍMICOS		
Compuesto	**Características Químicas**	**Bibliografía**
Dicarbonato de dimetilo (DMDC)**	$(CH_3OCO)_2O$	Threfall y Morris, 2002; Divol y col., 2005
Extracto de rábano negro*	Rafanina y Ácido ascórbico	Salaha y col., 2008
Extracto cloroplasto trigo*	Cloroplasto *Triticum aestivum*	Lin y George, 2004
Complejo coloidal de plata (CSC)*	Nanopartículas de plata	Izquierdo-Cañas y col.,2012
Lisozima**	Enzima obtenida de la clara de huevo (129 aminoácidos)	Bartowsky, 2003; Lasanta y col., 2010
Enzimas antimicrobianas*	Enzimas líticas	Blattel y col., 2009
	β-glucanasas	Blatter y col. 2011
Bacteriocina*	Nisina	Bauer y col., 2003, 2005;
	Pediocina PA-1	Rojo-Bezares y col., 2007
Glucosa oxidasa (GOX)*	Síntesis H_2O_2	du Toit y Pretorius, 2000; Malherbe y col., 2003
Péptidos antimicrobianos*	Lactoferrina	Tomita y col., 2002;
	$Lactoferricina_{B17-31}$	Enrique y col., 2007;2009
Lías de levaduras, mosto y vino*	Manoproteínas de levaduras y polisacáridos	Díez y col., 2010
Compuestos fenólicos*	Ácidos hidroxicinámicos	Vivas y col., 1997;
	Ácidos hidroxibenzoicos	García-Ruiz y col., 2008

* métodos/tratamientos en fase de estudio; ** tratamientos autorizados en el vino

5.4.2. *Alternativas químicas y bioquímicas*

En referencia a compuestos químicos con actividad antimicrobiana complementaria al SO_2 (Tabla 6), se ha descrito la utilidad del dicarbonato de dimetilo (DMDC) (E-242) para inhibir la FA y el desarrollo de levaduras no-*Saccharomyces*, permitiendo disminuir la dosis de SO_2 en algunos tipos de vinos como los vinos dulces (Threlfall y Morris, 2002; du Toit y col., 2005). Se ha comprobado que las células de levaduras mueren después de adicionar este compuesto, mientras que con el SO_2 entran

en un estado que se ha denominado 'viable no cultivable' (Divol y col., 2005; Agnolucci y col., 2010), que también han observado algunos autores en el caso de las bacterias acéticas (du Toit y col., 2005) y lácticas (Millet y Lonvaud-Founel, 2000). Sin embargo, se ha demostrado que a las pocas horas de su adición en el vino este compuesto es transformado en metanol, por lo que su efecto es efímero y no se recomienda su uso durante el periodo de almacenaje (Divol y col., 2005). El uso del DMDC está autorizado en Estados Unidos hasta una concentración de 200 ppm, en Australia hasta 200 mg/kg y en Europa, donde ha sido autorizado recientemente hasta un máximo de 200 mg/L (Costa y col., 2008).

Un nuevo producto constituido principalmente por extracto de rábano negro (*Raphanus niger*) y ácido ascórbico ha sido presentado como alternativa enológica al empleo del SO_2 (GEOLIFE, 2004; Salaha y col., 2008). *R. niger* es una variedad de rábano negro cuyo principal compuesto antibacteriano y antifúngico es la rafanina, mientras que el ácido ascórbico y su isómero, el ácido eritórbico, presentan una alta capacidad captadora de moléculas de oxígeno durante la elaboración de mostos y vinos. Ambos compuestos han sido propuestos como posible alternativa al empleo del SO_2 en enología (Fugelsang, 1989).

Otra alternativa para disminuir el contenido de sulfitos en el vino, es el uso de cloroplastos de trigo (*Triticum aestivum*), que reducen los sulfitos a sulfatos inocuos. Se ha demostrado que la preparación de un extracto crudo de estos cloroplastos a una concentración de 5 mg/mL, es capaz de reducir los sulfitos presentes en los vinos blancos comerciales desde 150 ppm a 7.5 ppm, así como disminuir el contenido inicial de sulfitos en vinos tintos hasta un 93% en un tiempo de 45 minutos (Lin y George, 2004). A pesar de que este sencillo proceso biocatalítico parece muy eficaz, barato y valioso para la industria vitivinícola, sería necesario realizar estudios de análisis sensorial para evaluar la calidad de estos vinos.

Una de las últimas alternativas propuestas al empleo de sulfito en enología es el uso de complejos coloidales de plata (CSC) (Izquierdo-Cañas y col., 2012). El efecto antimicrobiano de la plata se conoce desde hace tiempo (Silver y col., 2006), pero ha sido recientemente cuando se ha comenzado a estudiar el efecto antimicrobiano de nanomateriales de plata sobre bacterias Gram-negativas y Gram-positivas, determinándose también su actividad antifúngica y antiviral (Marambio-Jones y Hoek, 2010). En el trabajo realizado por Izquierdo-Cañas y col. (2012) se muestra como los CSC a una concentración de 1 g/kg de uva eran un eficaz antiséptico, capaz de controlar el desarrollo de BAL y acéticas.

Otras alternativas se han centrado en la búsqueda de "*agentes antimicrobianos naturales*" que permitan disminuir el uso de sulfitos en los vinos. Entre estas alternativas hay que destacar la lisozima (1,4-β-N-acetylmuramidasa) (EC 3.2.1.17). La lisozima es una proteína que se obtiene a partir de la clara de huevo, pero que está presente también en varias secreciones mamíferas como pueden ser: la leche, la saliva y las lágrimas. Esta proteína ha sido recientemente introducida en la industria del vino (límite máximo de adición: 500 mg/L (Bartowsky, 2009; Weber y col., 2009)), ofreciendo importantes ventajas para el control de la FML en vinos (Pilatte y col., 2000; Bartowsky, 2003a; Lasanta y col., 2010). La lisozima tiene la capacidad de romper los enlaces β-1,4-glucosídicos presentes en las bacterias Gram-positivas (Proctor y Cunningham, 1988), pero por el contrario posee un efecto limitado o nulo frente a otros microorganismos como bacterias acéticas y levaduras, respectivamente. Estudios realizados con péptidos obtenidos a partir de lisozima modificada por tratamientos térmicos o enzimáticos han permitido aumentar su espectro antibacteriano contra especies de bacterias acéticas, tales como *Gluconobacter oxydans* y *Acetobacter aceti* (Carrillo, 2011). Por otra parte, la actividad antimicrobiana de esta enzima frente a BAL podría verse limitada en el vino por las proantocianidinas de bajo peso molecular (Guzzo y col., 2011), siendo por ello más efectiva en vinos blancos que tintos (Bartowsky y col, 2004; López y col, 2009). Por el contrario, la lisozima no se ve afectada por el contenido de alcohol y es activa al pH en el que transcurre la vinificación, mostrando un efecto neutro sobre la calidad organoléptica de los vinos. Al aumentar el pH del vino aumenta la capacidad antimicrobiana de la lisozima, convirtiéndola en un conservante interesante para prevenir el deterioro de los vinos con un pH alto (Gao y col., 2002; Delfini, 2004). Además, esta proteína no aumenta el pardeamiento de los vinos blancos durante su almacenamiento (Bartowsky y col., 2004), y sus propiedades no se ven modificadas durante las diferentes operaciones tecnológicas (Amati y col., 1996). Por todo ello, la lisozima podría ser empleada para reducir los niveles de SO_2 durante la vinificación (Sonni y col., 2009), autorizándose su uso como aditivo en la elaboración del vino (Resolución OENO 10/97). A pesar de ello, su uso en enología es limitado debido principalmente a los altos costes que conlleva su producción. Otro aspecto a destacar de esta proteína, es que puede provocar en algunos individuos reacciones inmunes mediadas por IgE (Mine y Zhang, 2002; Weber y col., 2009), por lo que su presencia en los alimentos, incluido el vino, es motivo de preocupación. El etiquetado de la lisozima en materia de vinos ha sido regulado por la Comunidad Europea, en el anexo III bis de la Directiva 2007/68/CE que modifica la

Directiva 2000/13/CE, y por países como Australia, Nueva Zelanda, Japón o Estados Unidos.

Estudios recientes, se han centrado en la búsqueda de enzimas antimicrobianas cuya actividad lítica frente a bacterias alterantes del vino sea superior a la observada en la lisozima. Un claro ejemplo de esta búsqueda es el *cocktail* de enzimas exógenas de *Streptomyces* spp.B578 descrito por Blattel y col., (2009), el cual muestra un alto efecto lítico frente a un gran número de bacterias acéticas y lácticas, incluso bajo condiciones de vinificación. Este mismo autor también ha descrito la capacidad de la enzima β-1,3-glucanasa obtenida a partir del hongo *Delftia tsuruhatensis* MV01 para hidrolizar glucanos sintetizados por *Pediococcus parvulus* y otras levaduras presentes en el vino (Blattel y col., 2011). La síntesis de estos glucanos aumenta la viscosidad del vino y por tanto disminuye su calidad. La actividad β-1,3-glucanasa de este hongo es más efectiva frente a levaduras que frente a BAL del vino.

Actualmente, se conoce que otros compuestos de origen peptídico como las bacteriocinas presentan un efecto inhibidor sobre el desarrollo de las BAL. Estos compuestos se caracterizan por ser muy específicos, no aportar color ni olor y no mostrar un efecto tóxico sobre el ser humano (Abee y col., 1995). Además, han sido recibidas con gran interés en la industria láctea, donde son empleadas principalmente como aditivos durante la elaboración de quesos (Martínez-Cuesta y col., 2003). Estudios realizados sobre el posible empleo de la bacteriocina durante la vinificación, han demostrado que son estables a las condiciones en las que transcurre la elaboración del vino (Navarro y col., 2002; Bauer y col., 2003; 2005). Las bacteriocinas pueden ser divididas en tres categorías tal y como describe Cotter y col. 2005, siendo la nisina y la pediocina las más importantes, por su potencial uso en enología. La nisina pertenece a la clase I y es producida por algunas cepas de *Lactococcus lactis*, mientras que la pediocina PA-1, engloba a la clase II y es generada por *Pediococcus acidilactic* PAC1.0. Ambas bacteriocinas manifiestan un efecto inhibidor frente a las BAL presentes en el vino (*Lactobacillus plantarum*, *L. hilgardii*. *L. brevis*, *L. paracasei, L. pentosus, Leuconostoc mesenteroides, Pediococcus pentosaceus* y *O. oeni*) (Bauer y col., 2003; 2005; Rojo-Bezares y col., 2007). Este efecto es causado por la formación de poros en la membrana citoplasmática que permiten la salida de compuestos celulares esenciales. La nisina y pediocina PA-1 se pueden obtener comercialmente como Nisaplin (Danisco, Beaminster, Reino Unido) y ALTA2431 (Quest, Memphis, EE.UU.), respectivamente, estando su uso limitado por patentes de Estados Unidos y Europa. Los avances en genómica contribuirán a la identificación de nuevas bacteriocinas y a una mejor

comprensión de su mecanismo de regulación (Knoll y col., 2008; Navarro y col., 2008; Sáenz y col., 2009). Hoy en día, el uso de bacteriocinas en enología no está autorizado.

Otra posible alternativa para reducir el uso del SO_2 durante la elaboración del vino, sería el empleo de metabolitos con propiedades antimicrobianas, como por ejemplo el peróxido de hidrógeno (H_2O_2) (du Toit y Pretorius, 2000). La glucosa oxidasa (GOX) producida por *Aspergillus niger*, posee status GRAS y es una enzima de gran interés para la industria. La GOX transforma la glucosa en ácido glucónico y H_2O_2, presentando este último un gran efecto/poder antimicrobiano. Se ha demostrado que el H_2O_2 presenta propiedades antimicrobanas frente a bacterias Gram-positivas y Gram-negativas. El gen *gox* se ha expresado en *Saccharomyces cerevisiae*, generándose levaduras transformantes con capacidad de síntesis de la enzima GOX, que inhibe el crecimiento de las bacterias acéticas y lácticas del vino (Malherbe y col., 2003).

En la actualidad, existen cada vez más evidencias de la posible aplicación de algunas proteínas y péptidos antimicrobianos eucarióticos como conservantes alimentarios (Rydlo y col., 2006). Entre otros, los péptidos antimicrobianos derivados de proteínas alimentarias presentan claras ventajas para ser utilizados en la conservación de alimentos (Pellegrini, 2003). La leche es una fuente muy interesante de péptidos antimicrobianos que pueden ser liberados después de la digestión con proteasas. Entre ellas, la lactoferrina (LF), glicoproteína férrica multifuncional, destaca por su amplia gama de propiedades biológicas tales como: actividades antimicrobianas, antivirales, antioxidantes e inmunomoduladoras (Tomita y col., 2002; Orsi, 2004; Wakabayashi y col., 2006; Weinberg, 2007). Además, se ha descrito que presenta capacidad antimicrobiana frente a mohos fitopatógenos (Muñoz y Marcos, 2006). Varias estudios basados en el uso de LF hidrolizadas, como lactoferricina$_{B17-31}$, han demostrado que este péptido presenta propiedades de inhibición del crecimiento y fungicida frente a diversas levaduras vínicas alterantes, como la especie *Zygosaccharomyces bisporu*, pero no frente a cepas comerciales de *Saccharomyces cerevisiae* (Enrique y col., 2007), principal levadura responsable de la FA del vino. Recientemente, también se ha demostrado su actividad antimicrobiana frente a diferentes BAL alterantes del vino (Enrique y col., 2009); siendo necesarios más estudios para conocer su mecanismo de acción y sus efectos sobre la calidad del vino.

Además, se ha analizado el efecto antimicrobiano de manoproteínas de levaduras y polisacáridos obtenidos a partir de lías de levadura, mosto y vino frente a bacterias acéticas y lácticas de origen enológico (Díez y col., 2010). Observándose un mayor efecto antimicrobiano frente a bacterias acéticas que frente a BAL.

Por otra parte, en los últimos años el uso de compuestos fenólicos como conservantes naturales ha adquirido un gran interés científico. Estos compuestos muestran una gran diversidad de efectos biológicos tales como actividad antioxidante, anticancerígena, antiinflamatoria y antimicrobiana (Xia y col., 2010). Extractos fenólicos de uva (Baydar y col., 2004; 2006), piel de almendra (Mandalari y col., 2010), mango (Kaur y col., 2010), cebolla, ajo (Benkeblia y col., 2004), entre otros, han mostrado capacidad antimicrobiana, en medio de cultivo, frente a bacterias patógenas y/o alterantes. Además estudios realizados en ensaladas (Karapinar y Sengun, 2007) y productos cárnicos tales como hamburguesas (Park y Chin, 2010), albóndigas (Fernández-López y col., 2005) y pollo (Kanatt y col., 2010), han demostrado la potencial aplicación de los extractos fenólicos como agentes antimicrobianos y antioxidantes, con el fin de prevenir las enfermedades alimentario y prolongar la vida útil del producto. A continuación se describe el potencial uso de los compuestos fenólicos como alternativa al empleo del SO_2 en el vino.

III.6. Compuestos fenólicos

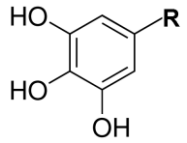
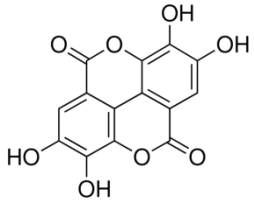
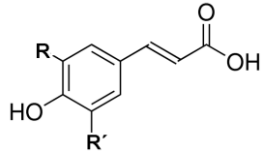
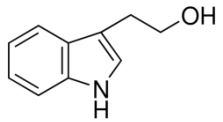
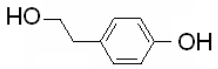
Los polifenoles son constituyentes naturales de la uva, (localizados principalmente en el hollejo y las pepitas) y que pasan al vino durante el proceso de elaboración. Desde un punto de vista químico el término "polifenol" engloba a un grupo muy heterogéneo de compuestos, que se caracterizan por presentar un anillo aromático con al menos un radical hidroxílico y una cadena lateral funcional. Según su estructura química, se subdividen en dos grandes grupos de compuestos: los flavonoides (antocianos, flavonoles, flavanoles, taninos), y los no flavonoides (ácidos benzoicos y cinámicos, alcoholes fenólicos, estilbenos) (Tabla 7). Los compuestos flavonoides se caracterizan por presentar dos anillos fenólicos unidos por un heterociclo.

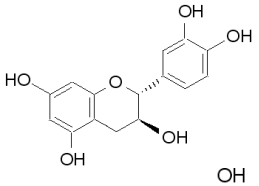
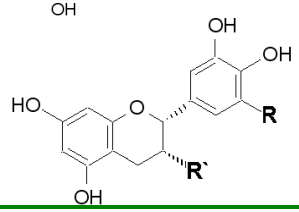
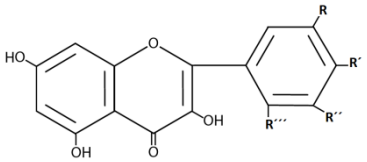
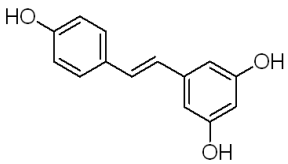
Los polifenoles tienen un gran interés en enología no sólo por ser responsables de muchas de las propiedades organolépticas del vino, fundamentalmente el color y la astringencia (Monagas y col., 2007), sino porque también se les asocian algunos de los efectos fisiológicos beneficiosos derivados del consumo moderado de vino, tales como el poder antioxidante (Balasundram y col., 2006;Xanthopoulou y col., 2010; Xials y col., 2010; Baroni y col., 2012), cardioprotector y vasolidatador, ente otros (Stoclet y col., 1999;2000; Santos-Buelga y Scalbert, 2000; Varache-Lembège y col., 2000; King y col., 2006;). La actividad antioxidante de los compuestos fenólicos se debe a su habilidad para captar radicales libres, donar átomos de hidrógeno o electrones o cationes metálicos (Afanas'ev y col., 1989; Amarowicz y col., 2004). Esta actividad depende de su estructura química y en especial del número y posición de los grupos

hidroxilos, así como de la naturaleza del anillo aromático de sustitución. Al igual que la actividad antioxidante, el resto de propiedades fisiológicas y reactividad química de los fenoles dependen de su estructura química.

La concentración de compuestos fenólicos en el vino está condicionada por diversos factores relacionados con la uva (variedad, calidad de la vendimia, suelo, clima, etc.), y las prácticas enológicas. Durante la vinificación, factores como el tiempo y la temperatura de maceración, la fermentación en contacto con hollejos y pepitas, la adición de enzimas, la concentración de SO_2, el prensado, etc., afectan a la extracción de los compuestos fenólicos de la uva al mosto/vino (Sacchi y col., 2005). La FML también afecta a la composición fenólica del vino, disminuyendo el contenido de antocianos y polifenoles totales (Vrhovsek y col., 2002, Hernández y col., 2006; 2007; Cabrita y col., 2008). Durante el envejecimiento en botella, los antocianos del vino descienden, aunque el contenido de polifenoles totales sufre menos variaciones (Monagas y col., 2005a; 2005b). Todo ello hace que el contenido total de polifenoles se sitúe alrededor de 150-400 mg/L para los vinos blancos y 900-1400 mg/L para los vinos tintos jóvenes, siendo la composición fenólica diferente para ambos tipos de vino. En el caso de los vinos tintos están representados todos los grupos fenólicos mientras que los vinos blancos están constituidos principalmente por ácidos fenólicos y flavonoles (Papadopoulou y col., 2005). En los vinos, la diferencia en el contenido de estos compuestos se atribuye a la diferente composición fenólica de las uvas tintas y blancas, así como a los distintos procesos de vinificación empleado, como por ejemplo la maceración durante la elaboración del vino tinto (Jackson, 2008).

Tabla 7. Estructuras de los principales compuestos fenólicos del vino.

Clase	Estructura Química	Nombre
Ácidos y ésteres hidroxibenzoicos		R= COOH Ácido galico R=$COOCH_3$ Galato de metilo R= $COOCH_2CH_3$ Galato de etilo
		Ácido elágico
Hydroxycinnamic acids		R=R`= H Ácido *p*-cumárico R=R`= OCH_3 Ácido sinápico R= H; R`= $OCH_3$ Ácido ferúlico R= H; R`= OH Ácido cafeico
Alcoholes fenólicos y otros compuestos		Triptofol
		Tirosol

Clase	Estructura Química	Nombre
Flavan-3-oles		[+]-Catequina
		R= H [-]-Epicatequina R= OH [-]-Epigalocatequina R=H; R`= Galato [-]-Galato de epicatequina R=OH; R`=Galato [-]-Galato de epigalocatequina
Flavonoles		R= R´´=R´´= H; R´= OH Kanferol R´´=R´´´´= H; R=R´= OH Quercetina R´´´= H; R= R´=R´´= OH Miricetina R= R´´=H; R´=R´´= OH Morina R´´=R´´= H; R= OCH_3; R´= OH Isorhamnetina
Estilbenos		*trans*-Resveratrol

A modo de resumen, la Tabla 8 recoge el intervalo de variación en la concentración de los principales compuestos fenólicos identificados en vinos tintos jóvenes. Por grupos de compuestos, los ácidos y derivados hidroxibenzoicos representarían el 6.0 % del total; los ácidos y derivados hidroxicinámicos, 1.1 %, los estilbenos, 0.5 %; los alcoholes, 3.8 %; los flavanoles, 15.0 %; los flavonoles, 3.6 %; y las antocianinas, 70.0 %. En proporción muy inferior se encuentran otros derivados antociánicos como los piranoantocianos.

Tabla 8. Principales compuestos fenólicos identificados en vinos tintos jóvenes (García-Ruiz y col.; 2008).

Compuestos Fenólicos	**Concentración** (mg/L)	**Compuestos Fenólicos**	**Concentración** (mg/L)
Ácidos hidroxibenzoicos		*Flavonoles*	
Ácido gálico	10-37	Miricetín-3-glicósidos	1.6-22
Ácido protocatéquico	1.2-4.7	Quercetín-3-glicósidos	1.3-34
Ácido siríngico	4.2-5.8	Kanferol-3-glicósidos	trazas
Ácidos hidroxicinámicos		Isoramnetin-3-glicósidos	trazas
Ácido caftárico	0.7-46	Miricetina	1.7-8
Ácido cutárico	0.7-11	Quercetina	1.9-15
Ácido cafeico	0.3-33	Kanferol	trazas
Acido *p*-cumárico	0.1-8	Isoramnetina	trazas
Estilbenos			
trans-Resveratrol	0.4-2.5	*Antocianinas*	
trans-Resveratrol-3-*O*-glucósido	0.1-3	Delfinidín-3-glucósido	7-11
Alcoholes		Petunidín-3-glucósido	14-25
Tirosol	7-26	Malvidín-3-glucósido	170-260
Triptofol	nd-4.5	Malvidín-3-(6-acetil)-glucósido	23-108
Flavanoles		Malvidín-3-(6-cafeil)-glucósido	3.5-5.6
(+)-Catequina	16-58	Malvidín-3-(6-*p*-cumaril)-	16-28
(-)-Epicatequina	10-38	glucósido	
Procianidinas B1, B2, B3, B4	14-33		

III.6.1. *Interacciones entre compuestos fenólicos y bacterias lácticas del vino*

La interacción entre los polifenoles del vino y las BAL responsables de la FML es bidireccional. Es decir, las BAL pueden metabolizar los compuestos fenólicos presentes en el vino, pero al mismo tiempo el propio metabolismo y crecimiento de las bacterias puede verse afectado por los polifenoles del medio. El balance final de estas interacciones está supeditado a diversos factores como la concentración y estructura química de los compuestos fenólicos (Stead, 1993; Reguant y col., 2000), las

características peculiares de las cepas bacterianas implicadas (Hernández y col., 2007), la presencia de agentes antimicrobianos, etc.

6.1.1. *Metabolismo de los compuestos fenólicos por bacterias lácticas*

Las investigaciones realizadas para determinar el efecto que tienen las BAL sobre los compuestos fenólicos, se han efectuado principalmente en medio de cultivos bacterianos puros y utilizando fenoles de forma individual (Tabla 9). La mayoría de estos estudios, se han centrado en la capacidad metabólica que muestran las BAL para generar compuestos fenólicos volátiles a partir de ácidos hidroxicinámicos, especialmente los ácidos *p*-cumárico y ferúlico (Cavin y col., 1993; Lonvaud-Funel, 1999; Couto y col., 2006). Estas bacterias se caracterizan por presentar actividad cinamato descarboxilasa, por la que los ácidos fenólicos presentes en el vino son transformados en vinil derivados (4-vinilguaiacol y 4-vinilfenol), los cuales a su vez pueden ser posteriormente reducidos enzimáticamente por acción de la vinilfenol reductasa a etil derivados (4-etilguaiacol y 4-etilfenol) (Cavin y col.; 1993; Barthelmebs y col., 2001; Gury y col., 2004; Couto y col., 2006). Los vinil derivados otorgan al vino un olor que recuerda a "fármaco" (Ribéreau-Gayon y col., 2006), mientras que los etil derivados transfieren un olor a "animal" y "medicinal" (Lonvaud-Funel, 1999). Se ha demostrado que cepas bacterianas de los géneros *Pediococcus* y *Lactobacillus* (Moreno-Arribas y Lonvaud-Funel, 1999, Curiel y col., 2010b) y de la especie *O. oeni* (Swiegers y col., 2005) son capaces de sintetizar estos compuestos. Aunque los principales microorganismos responsable de la síntesis de estos fenoles volátiles en el vino no son las BAL sino cepas de las levaduras alterantes *Brettanomyces*/*Dekkera* (Dias y col., 2003).

También se ha estudiado el metabolismo de otros compuestos fenólicos como el ácido gálico y la catequina (Alberto y col., 2004), así como la transformación de los ésteres de ácidos hidroxicinámicos a sus correspondientes ácidos libres como resultado de la actividad cinamil esterasa de las BAL (Hernández y col., 2006; 2007). Por otra parte, se ha demostrado que la actividad polifenol oxidasa de levaduras y BAL modifica el perfil antociánico de uvas y vinos jóvenes (Squadrito y col., 2010), observándose también durante la FML una disminución de ácidos hidroxicinamiltartáricos correlacionado con un aumento de sus formas libres (Cabrita y col., 2008). Landete y col. (2007) han descrito la degradación del ácido protocateico en catecol por cepas de *L. plantarum* aisladas de diferentes fuentes, incluyendo el vino. Este metabolismo parece ser llevado a cabo por enzimas no inducibles, ya que en medios de cultivos en ausencia de fenoles y con extractos celulares también se ha observado (Landete y col., 2007).

Por otro lado, las técnicas moleculares están permitiendo ampliar conocimientos sobre cómo las BAL metabolizan los compuestos fenólicos. Gracias a ellas, en los últimos años se ha podido determinar que las especies *L. plantarum* y *P. pentosaceus* poseen una descarboxilasa inducible que ejerce su actividad sobre el ácido *p*-cumárico, describiéndose además su regulación a nivel molecular (Cavin y col., 1997; Barthelmebs y col., 2000; Licandro-Seraut y col., 2008). Se ha sugerido que esta actividad inducible puede estar implicada en la respuesta a estrés producida por los ácidos fenólicos, convirtiéndolos en compuestos menos tóxicos (Gury y col., 2004). Además, se ha desarrollado un método basado en la amplificación del fragmento del DNA correspondiente al gen *pdc* (ácido fenil descarboxilasa) que permite una identificación preliminar, rápida y sensible, de BAL productoras de fenoles volátiles. Los resultados obtenidos por PCR fueron corroborados mediante análisis de HPLC (de la Rivas y col., 2009). Otros estudios de biología molecular han permitido describir que entre las BAL aisladas de vinos, sólo las bacterias de la especie *L. plantarum* poseen actividad tanasa (Vaquero y col., 2004). La enzima tanasa es una hidrolasa que actúa sobre los taninos y esteres del ácido gálico presentes en el vino, por lo que representa una actividad muy importante en enología por su relación con el color y con fenómenos de enturbiamiento. Esta actividad enzimática también ha sido identificada y cuantificada por análisis de HPLC (Rodríguez y col., 2008a) y caracterizada bioquímicamente mediante ensayos colorimétricos en los que se han utilizado extractos libres de células de *L. plantarum* (Rodríguez y col., 2008b).

Tabla 9. Metabolismo de los compuestos fenólicos por bacterias lácticas del vino.

Bacterias Lácticas	Compuestos Fenolicos	Actividad Metabólica	Bibiliografía
Pediococcus *Lactobacillus* *O. oeni*	Ácido hidroxicinámico (ác. *p*-cumárico y ferúlico)	Ác hidroxicinámica descarboxilasa	Moreno-Arribas y Lonvaud-Funel, 1999; Swiegers y col., 2005
L. hilgardii	Ácido gálico, catequina	Consumo y degradación	Alberto y col., 2004
O. oeni *L. plantarum*	Ésteres ácidos hidroxicinámicos	Cinamil esterasa	Hernández y col., 2006; 2007
Bacterias lácticas	Antocianos	Polifenol Oxidasa	Squadrito y col., 2010
L. plantarum	Ácido protocateico	Producción catecol	Landete y col., 2007
L.plantarum *P. pentosaceus*	Ác. *p*-cumárico	Descarboxilación	Cavin y col., 2007; Licandro-Seraut y col., 2008
L. plantarum	Taninos, ésteres ácido gálico	Tanasa	Vaquero y col., 2004

6.1.2. *Efecto de los compuestos fenólicos en el crecimiento y viabilidad de las bacterias lácticas*

Anteriormente, se ha comentado que los compuestos fenólicos pueden comportarse como activadores o inhibidores del crecimiento bacteriano dependiendo de su estructura química (anillo radical fenólico) y concentración (Reguant y col., 2000; Vivas y col., 1997, Rozès y col., 2003). De este modo, la concentración de ácidos hidroxicinámicos puede tener un efecto crítico sobre las BAL, ya que a concentraciones comprendidas entre 100-250 mg/L la bacteria es capaz de tolerar y a su vez metabolizar dichos compuestos, lo que podría explicar el efecto beneficioso de estos compuestos en el crecimiento, mientras que por el contrario a concentraciones superiores de 500 mg/L tuvieron un efecto tóxico (Stead, 1993).La mayoría de los estudios se han centrado en el análisis del efecto de estos compuestos sobre el metabolismo y crecimiento de *O. oeni*, principal especie responsable de la FML en la mayoría de los vinos, aunque también se ha observado el efecto de los polifenoles sobre diferentes especies del género *Lactobacillus* y en menor medida sobre los géneros *Leuconostoc* y *Pediococcus* (Tabla 10). Así por ejemplo, en *L. hilgardii* se ha demostrado, en sistemas modelo, que el ácido gálico y la catequina a las concentraciones que se encuentran en los vinos, no sólo estimulan su crecimiento sino que además aumentan su población. Este hecho puede relacionarse con la capacidad de *L. hilgardii* para metabolizar estos compuestos durante la fase de crecimiento, los cuales van a proporcionar energía a la célula (Alberto y col., 2001). Además, se ha demostrado que los compuestos fenólicos del vino pueden inhibir la formación de putrescina vía agmitina deiminasa de *L. hilgardii* (Alberto y col., 2007). Por otro lado, se ha descrito que el metabolismo de *O. oeni* se ve afectado por los compuestos fenólicos del vino, favoreciéndose la utilización de azúcares y ácido málico (Vivas y col., 2000; Alberto y col., 2001; Rozès y col., 2003). De este modo, Campos y col. (2009b) han observado que en presencia de los ácidos ferúlico, cafeico y *p*-cumárico una cepa de *O. oeni* es capaz de sintetizar más acetato. Una posible explicación a este hecho, es que la presencia de estos fenoles aumente el consumo de azúcares y mejore el metabolismo del ácido cítrico. Por otro parte, a concentraciones más elevadas, estos compuestos ejercen un efecto negativo sobre el desarrollo bacteriano; observándose una mayor sensibilidad en *O. oeni* que en *L. hilgardii* (Campos y col., 2003; Figueiredo y col., 2008).

Los ácidos hidroxicinámicos libres parecen afectar al crecimiento de *L. plantarum* y otras especies alterantes del género *Lactobacillus*. De este modo, el ácido ferúlico parece ser más efectivo que los ácidos *p*-cumárico y cafeico, aunque algunas

especies son más susceptibles que otras a este efecto. Por el contrario, los ésteres de estos ácidos, al igual que el ácido quínico (no fenólico), no influyeron en el crecimiento de *L. plantarum (*Salih y col., 2000*)*. Por otro lado, Silva y col. (2011) han observado en medios de cultivo que los ácidos cafeico y ferúlico inducen la síntesis de cinamato descarboxilasa en BAL y con ello la producción de fenoles volátiles a partir del ácido *p*-cumárico. Mientras que los taninos inhiben dicha actividad enzimática.

Estudios realizados con *O. oeni* en presencia de epigalocatequina de galato (Theobald y col., 2008) han encontrado un efecto dosis dependiente de este compuesto sobre el crecimiento de *O. oeni*. Un efecto estimulador fue observado a concentraciones entre 400-500 mg/L, por el contrario un efecto inhibidor fue mostrado a concentraciones superiores de 500 mg/L.

Por otro lado, Figueiredo y col., (2008) han descrito el efecto inhibitorio de los distintos aldehídos fenólicos en el crecimiento de *O.oeni,* mostrando que el sinapaldehído es el compuesto más activo, mientras que otros aldehídos como la vainillina y el siringaldehído no tuvieron ningún efecto a las máximas concentraciones ensayadas (500 mg/L).

Más recientemente, se ha demostrado, en medio sintético, que la quercitina posee un efecto pH y dosis dependiente sobre el metabolismo de una cepa de *Lactobacillus plantarum* (Curiel y col., 2010a). Observándose que a pH 5.5 la quercitina acelera el metabolismo de azúcares de esta cepa, así como la producción de ácido láctico a partir de ácido málico; mientras que a pH 6.5 se percibió una fase *lag* de crecimiento más prolongado. Además, se demostró que la quercetina no era catabolizada por *L. plantarum*.

Los mecanismos implicados en la inhibición de las BAL por parte de los compuestos fenólicos no están claros, pudiendo variar en función de la cepa. Se ha descrito que los compuestos fenólicos pueden promover alteraciones tanto a nivel de pared celular como a niveles citoplasmáticos y enzimáticos (Campos y col., 2003, Rodríguez y col., 2009). En una primera base estos compuestos fenólicos pueden alterar la estructura de la membrana plasmática, produciéndose la salida al exterior de componentes esenciales de la célula bacteriana, tales como proteínas, ácidos nucleicos e iones inorgánicos (Johnston y col., 2003), lo cual conduciría a una segunda etapa en la que tendría lugar una muerte celular (Rodríguez y col., 2009). Con el objetivo de demostrar el daño inicial de los compuestos fenólicos del vino sobre cepas de BAL enológicas, Campos y col. (2009b) han observado en suspensiones de *O. oeni* y *L.*

hilgardii, que los ácidos hidroxicinámicos e hidroxibenzoicos mejoran significativamente el flujo hacia el exterior de protones y hacia el interior de potasio y fosfato, mostrando un mayor efecto los ácidos hidroxicinámicos que los ácidos hidroxibenzoicos. Sin embargo, los resultados de inactivación obtenidos en el mismo estudio parecían no correlacionarse completamente con los flujos de iones medidos; lo que puede indicar que el daño ocasionado por los ácidos fenólicos en la membrana de las bacterias es reversible o que en la inactivación de las células por estos fenoles podría estar implicado más de un mecanismo o diana celular (Campos y col., 2009a).

En referencia a los mecanismos de inactivación de las BAL por los taninos, recientemente se ha realizado un estudio que combina técnicas de fisiología y proteómica (Bossi y col., 2007), en el que se observa que en la interacción proteína bacteriana-tanino están implicadas enzimas metabólicas y proteínas funcionales.

Tabla 10. Principales efectos de los compuestos fenólicos sobre las bacterias lácticas del vino.

Compuestos Fenólico	Bacterias Lácticas	Efecto	Bibliografía
Ácidos gálico, catequina, quercetina	*L. hilgardii*	Estimula crecimiento Aumento población	Alberto y col., 2001
Ácidos protocateico, vainillico, cafeico, catequina, rutina	*L. hilgardii*	Inhiben síntesis putrescina vía agmitina deiminasa	Alberto y col., 2007
Ác. hidroxicinámico	*O. oeni*	Aumenta síntesis acetato	Campos y col., 2009b
	O. oeni, Lactobacillus	Inhibe crecimiento	Stead, 1993; Campos y col., 2003; Figueiredo y col.,2008;
	BAL	Induce cinamato descarboxilasa	Silva y col., 2011
Epigalocatequina de galato	*O. oeni*	400-500 mg/L Estimula crecimiento > 500 mg/L Inhibe crecimiento	Theobold y col., 2008
Aldehídos fenólicos	*O. oeni*	Inhiben crecimiento	Figueiredo y col., 2008
Quercetina	*L. plantarum*	pH 5.5 Acelera Metabol. de azúcares y aumenta producción ác. láctico pH 6.5 Prolonga fase lag	Curiel y col., 2010a
Ác. hidroxicinámicos Ác. hidroxibenzoicos	*O. oeni* *L. hilgardii*	Incrementa flujo exterior protones e interior potasio y fosfato	Campos y col., 2009a
Taninos	*L. hilgardii*	Interacción proteína-tanino: alteración metabolismo	Bossi y col., 2007
	BAL	Inhibe cinamato descarboxilas	Silva y col., 2011

Una conclusión general que se obtiene a partir de todos estos estudios, es que el efecto inhibitorio de los polifenoles sobre las BAL del vino es selectivo. Esto lleva a la búsqueda de compuestos fenólicos que puedan inhibir el crecimiento de BAL alterantes del vino, como por ejemplo las especies *L. hilgardii* y *P. pentosaceus*, pero no de aquellas BAL que realizan la FML y aportan efectos positivos a las características del vino, como es el caso de *O. oeni*. Por otro lado, la mayoría de estos trabajos se han realizado en medios sintéticos, siendo necesario llevar a cabo estudios en condiciones de elaboración del vino.

En base a estos antecedentes, la presente Tesis pretende aumentar el conocimiento sobre el efecto que, en base a su estructura química, tienen los compuestos fenólicos sobre el crecimiento y metabolismo de las BAL en el vino, así como evaluar el potencial uso de extractos fenólicos antimicrobianos de origen vegetal como aditivo alternativo total o parcial al SO_2 durante la vinificación.

Resultados

IV. RESULTADOS

En esta sección se exponen los resultados obtenidos durante la presente Tesis Doctoral en base a los objetivos propuestos. Estos resultados se han recogido en 6 publicaciones en revistas incluidas en el Science Citation Index (SCI) y en una patente.

IV.1. Efecto de los compuestos fenólicos del vino en el crecimiento de bacterias lácticas de origen enológico.

Como se describe en la introducción, en la bibliografía científica, se recogen diversos estudios que indican que algunos compuestos fenólicos presentes en el vino, especialmente ácidos hidroxicinámicos y benzoicos, inhiben el crecimiento de determinas especies de BAL de origen vínico (Reguant y col., 2000; Campos y col., 2003, Bloem y col., 2007; Landete y col. 2007; Figueiredo y col., 2008). No obstante, los resultados de estos estudios parecían dispersos en tanto y cuanto se referían sólo a algunos compuestos fenólicos del vino, no empleaban condiciones homogéneas de evaluación (concentración, población microbiana, etc), y expresaban los resultados de modos diversos (% de inhibición, concentración mínima inhibitoria, etc.). Era importante, por tanto, plantear un estudio sistemático para evaluar la capacidad de inhibición de BAL por los compuestos fenólicos del vino, teniendo en cuenta su diversidad estructural (incluyendo, por ejemplo, estilbenos y alcoholes fenólicos, compuestos que no se habían considerado anteriormente) y estableciendo parámetros de inhibición universales que pudieran facilitar la comparativa entre compuestos y cepas procedentes de diversos estudios, laboratorios, etc. También considerábamos interesante incluir, en el diseño experimental, la evaluación de cambios en la morfología celular de las bacterias que nos pudieran arrojar luz sobre los mecanismos implicados en la inhibición del crecimiento de las bacterias lácticas por compuestos fenólicos.

Estas premisas nos llevaron a la selección de 21 compuestos, 18 de ellos representativos de la composición fenólica de los vinos: ácidos y esteres hidroxibenzoicos (ácido gálico, ácido elágico, galato de etilo y galato de metilo), ácidos hidroxicinámicos (ácido ferúlico, ácido p-cumárico, ácido caféico, y ácido sinápico), alcoholes fenólicos y otros compuestos relacionados (tirosol y triptofol), estilbenos

(resveratrol), flavan-3-oles ((+)-catequina, (-)-epicatequina y galato de (-)-epicatequina,), flavonoles (quercetina, miricetnia, kanferol e isoramnetina), y otros 3 compuestos no presentes en el vino, pero relacionados estructuralmente con ellos: morina, (-)-epigalocatequina y galato de (-)-epigalocatequina. De igual forma, seleccionamos dos formas de evaluar la acción inhibidora de los polifenoles sobre las bacterias: *i)* como inactivación de BAL después de la incubación de las bacterias con los fenoles durante un cierto tiempo (parámetros MIC y MBC) **(Publicación I)** y *ii)* como inhibición del crecimiento de la bacteria en presencia de polifenoles (parámetro IC_{50}) **(Publicación II)**. En cuanto a la evaluación de los cambios en la morfología de la bacteria después de su interacción con los polifenoles, se planteó la utilización de la microscopía de epifluorescencia y la microscopía electrónica como técnicas más adecuadas.

Por otro lado, y como se indica en la hipótesis de partida, las propiedades antibacterianas de los polifenoles podrían resultar útiles en el control de proceso de FML del vino, llevada a cabo principalmente por cepas de la especie *Oenococcus oeni*. De igual forma, los polifenoles podrían inhibir el crecimiento de otras especies bacterianas más relacionadas con alteraciones organolépticas en el vino, como *Lactobacillus hilgardii* y *Pediococcus pentosaceus*. Por tanto, en nuestros estudios se han empleado cepas de origen enológico de estas tres especies: *Lactobacillus hilgardii* y *Pediococcus pentosaceus* **(Publicaciones I y II)** y *Oenococcus oeni* **(Publicación II)**. Todas las cepas utilizadas en estos estudios pertenecen a la colección del Instituto de Fermentaciones Industriales (IFI-CA), actualmente incluidas en la colección del Instituto de Investigación en Ciencias de la Alimentación (CIAL).

A continuación se presentan los resultados de este estudio en forma de dos publicaciones:

Publicación I. Inactivación de bacterias lácticas del vino (*Lactobacillus hilgardii* y *Pediococcus pentosaceus*) por compuestos fenólicos del vino.

Publicación II. Estudio comparativo de los efectos de inhibición de los polifenoles del vino sobre el crecimiento de bacterias lácticas de origen enológico.

Publicación I. Inactivación de bacterias lácticas del vino (*Lactobacillus hilgardii* y *Pediococcus pentosaceus*) por compuestos fenólicos del vino.

Almudena García-Ruiz, Begoña Bartolomé, Carolina Cueva, Pedro J. Martín-Álvarez y M. Victoria Moreno-Arribas. Inactivation of oenological lactic acid bacteria (*Lactobacillus hilgardii* and *Pediococcus pentosaceus*) by wine phenolic compound. *Journal of Applied Microbiology,* **2009,** 107: 1042-1053.

Resumen:

El objetivo de este estudio fue investigar la inactivación de dos cepas de *Lactobacillus hilgardii* y *Pediococcus pentosaceus* de origen vínico en presencia de compuestos fenólicos presentes en el vino, así como explorar el mecanismo de acción. Tras un primer "screening" para evaluar el grado de inactivación de las bacterias lácticas por 21 compuestos fenólicos (ácidos hidroxibenzoicos e hidroxicinámicos, alcoholes fenólicos, estilbenos, flavan-3-oles y flavonoles) a ciertas concentraciones, se determinaron los parámetros de supervivencia (MIC y MBC) de los compuestos más activos. En el caso de la cepa *L. hilgardii*, los flavonoles morina y kanferol fueron los compuestos que mostraron mayor inactivación bacteriana (valores de MIC de 1 y 5 mg/L, y de MBC de 7,5 y 50 mg/L, respectivamente). En el caso de la cepa *P. pentosaceus,* los flavonoles también fueron los compuestos con mayor poder de inactivación, con valores de MIC entre 1 y 10 mg/L y valores de MBC entre 7,5 y 300 mg/L. Por medio de microscopía de epifluorescencia y microscopia electrónica de transmisión se observó que los compuestos fenólicos dañaban la membrana celular y promovían la posterior liberación del contenido citoplasmático al medio. A partir de los resultados obtenidos, se concluyó que la actividad antimicrobiana de los compuestos fenólicos del vino frente a *Lactobacillus hilgardii* y *Pediococcus pentosaceus* dependía del compuesto ensayado, y que dicha actividad no sólo producía la inactivación bacteriana sino también la muerte celular. Estos resultados aportan nueva información sobre la capacidad de inactivación de bacterias lácticas del vino por parte de compuestos fenólicos presentes en el mismo, y abren una nueva área de estudio para la selección/obtención de preparaciones fenólicas de origen enológico, con potencial aplicación como alternativa natural al empleo de SO_2 en enología.

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ORIGINAL ARTICLE

Inactivation of oenological lactic acid bacteria (*Lactobacillus hilgardii* and *Pediococcus pentosaceus*) by wine phenolic compounds

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Keywords
antimicrobial activity, antioxidant activity, inactivation mechanism, lactic acid bacteria, phenolic compounds, sulfur dioxide, wine.

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Abstract

Aims: To investigate the inactivation properties of different classes of phenolic compounds present in wine against two wine isolates of *Lactobacillus hilgardii* and *Pediococcus pentosaceus*, and to explore their inactivation mechanism.
Methods and Results: After a first screening of the inactivation potency of 21 phenolic compounds (hydroxybenzoic and hydroxycinnamic acids, phenolic alcohols, stilbenes, flavan-3-ols and flavonols) at specific concentrations, the survival parameters (MIC and MBC) of the most active compounds were determined. For the *L. hilgardii* strain, the flavonols morin and kaempferol showed the strongest inactivation (MIC values of one and 5 mg l^{-1}, and MBC values of 7·5 and 50 mg l^{-1}, respectively). For the *P. pentosaceus* strain, flavonols also showed the strongest inactivation effects, with MIC values between one and 10 mg l^{-1} and MBC values between 7·5 and 300 mg l^{-1}. Observations by epifluorescence and scanning electron microscopy revealed that the phenolics damaged the cell membrane and promoted the subsequent release of the cytoplasm material into the medium.
Conclusions: The antibacterial activity of wine phenolics against *L. hilgardii* and *P. pentosaceus* was dependent on the phenolic compound tested, and led not only to bacteria inactivation, but also to the cell death.
Significance and Impact of the Study: New information about the inactivation properties of wine lactic acid bacteria by phenolic compounds is presented. It opens up a new area of study for selecting/obtaining wine phenolic preparations with potential applications as a natural alternative to SO_2 in winemaking.

Introduction

During winemaking, malolactic fermentation (MLF) reduces the acidity of the wine (by the conversion of L-malic acid into L-lactic acid) and positively contributes to the microbial stability and organoleptic quality of the final product (Moreno-Arribas and Polo 2005). This fermentation is carried out by lactic acid bacteria (LAB) mainly belonging to the genera *Oenococcus*, *Pediococcus*, *Lactobacillus* and *Leuconostoc*. MLF occurs spontaneously during winemaking or can be induced by starter cultures; but in any case, the process has to be kept under control to avoid undesirable bacterial effects. These alterations include the so-called 'lactic disease', the production of off-flavour compounds (Chatonnet *et al.* 1995; Costello and Henschke 2002), and of biogenic amines (Moreno-Arribas *et al.* 2000; Landete *et al.* 2005; Marcobal *et al.* 2006). Winemaking conditions such as temperature, wine pH, SO_2 content, and ethanol concentration are all known to influence the MLF development (Boulton *et al.* 1996). Other wine components, mainly the phenolic compounds, can also affect the growth of LAB (Vivas *et al.* 1997), although this effect is not yet completely understood.

Wine polyphenols comprise different chemical structures including anthocyanins, flavan-3-ols, flavonols,

hydroxybenzoic acids hydroxycinnamic acids, stilbenes, and phenolic alcohols (Fig. 1). Interaction between wine phenolics and LAB can be considered two-way: LAB can degrade wine polyphenols into less-complex structure phenolic metabolites, and, on the other hand, bacteria growth and metabolism can be affected by wine phenolics or even by phenolic metabolites produced by other micro-organisms. The concentration of the phenolic compounds would appear to be critical in this two-way interaction, with the bacteria able to tolerate and even to metabolize the compounds, and/or to be stimulated by low phenolic concentrations, and thus to be inhibited by the presence of the phenolic compounds at relatively high concentrations (Stead 1993).

In relation to the metabolism of wine phenolics by LAB, most of the studies focus on individual compounds being transformed by pure bacterial cultures. Hydroxycinnamic acids (ferulic and *p*-coumaric acids) are well known to be transformed into volatile phenols (4-ethylguaiacol and 4-ethylphenol) by different bacteria species (Cavin *et al.* 1993; Gury *et al.* 2004; Couto *et al.* 2006). Gallic acid and (+)-catechin have also been reported to be degraded to different phenolic metabolites by *L. hilgardii* (Alberto *et al.* 2004). Recently, Landete *et al.* (2007) have reported the degradation of protocatechuic acid to catechol by strains of *L. plantarum* isolated from different sources including wine. This metabolism seemed to be carried out by non-inducible enzymes since a cell-free extract from a culture grown in the absence of the phenolic was also able to metabolize it (Landete *et al.* 2007). Some studies in wine have also shown decreases in the phenolic content after incubation with cells of *L. hilgardii*, which was attributed to the phenolic utilization by bacteria (Alberto *et al.* 2004). Besides this, changes in both the anthocyanin and non-anthocyanin phenolic profiles of wines after MLF have been reported (Hernández *et al.* 2006, 2008; Cabrita *et al.* 2008).

Concerning the inhibition of the growth and metabolism of LAB by wine phenolic compounds, most of the studies refer to *O. oeni*, the predominant bacteria species involved in wine MLF. Reguant *et al.* (2000) have reported that hydroxycinnamic acids inhibited all growth of *O. oeni* at ≥500 mg l^{-1}; *p*-coumaric and ferulic acids being more potent inhibitors than caffeic acid. No inhibitory effects against *O. oeni* were found for gallic acid up to 1 g l^{-1}, and stimulating effects were observed for (+)-catechin (≤100 mg l^{-1}) and quercetin (≤25 mg l^{-1}). Campos *et al.* (2003) found inhibitory effects for both hydroxycinnamic and hydroxybenzoic acids at concentrations of ≥100 mg l^{-1}, the former group being more

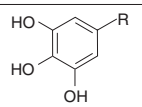
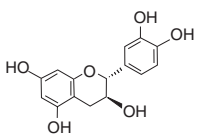
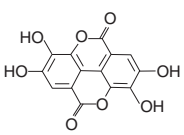
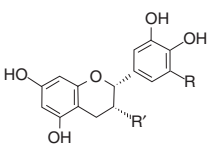
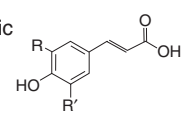
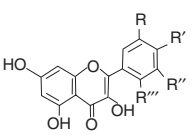
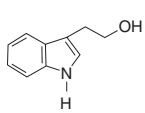
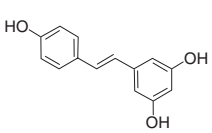
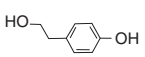
Class	Chemical structure	Name	Class	Chemical structure	Name
Hydroxybenzoic acids and esters		R= COOH Gallic acid R=$COOCH_3$ Methyl gallate R= $COOCH_2CH_3$ Ethyl gallate	Flavan-3-ols		[+]-Catechin R= H; R′= OH [–]-Epicatechin R= R′= OH [–]-Epigallocatechin R=H; R′= gallate [–]-Epicatechin gallate R=OH; R′=gallate [–]-Epigallocatechin gallate
		Ellagic acid			
Hydroxycinnamic acids		R=R′= H *p*-Coumaric acid R=R′= OCH_3 Sinapic acid R= H; R′= OCH_3 Ferulic acid R= H; R′= OH Caffeic acid	Flavonols		R= R″=R‴= H; R′= OH Kaempferol R″=R‴= H; R=R′= OH Quercetin R‴= H; R= R′=R″= OH Myricetin R= R″=H; R′=R‴= OH Morin R″=R‴= H; R= OCH_3; R′= OH Isorhamnetin
Phenolic alcohols and other compounds		Tryptophol	Stilbens		*trans*-Resveratrol
		Tyrosol			

Figure 1 Structure of the phenolic compounds studied.

potent inhibitors than the latter one. Salih *et al.* (2000) also noted that the ester forms of the hydroxycinnamic acids seemed to be less toxic against *O. oeni* than the free forms. Recently, Bloem *et al.* (2007) reported inhibitory effects of different simple phenols and phenolic acids (isoeugenol, eugenol, ferulic acid and vanillic acid) against *O. oeni* at a lower concentration (10 mg l^{-1}). Another phenolic compound found in grape seeds, (−)-epigallocatechin gallate, was found to be toxic for *O.oeni* at ≥500 mg l^{-1} (Theobald *et al.* 2008). More recently, Figueiredo *et al.* (2008) reported the inhibitory effects of different phenolic aldehydes (250 mg l^{-1}) against *O. oeni*, showing sinapaldehyde to have the greatest effect; other aldehydes such as vanillin and syringaldehyde did not affect the growth of the bacteria even at the maximum concentration tested (500 mg l^{-1}). In the same study, quercetin and kaempferol were found to be active inhibitors at concentrations of ≥10 mg l^{-1}, but myricetin (40 mg l^{-1}), (+)-catechin (50 mg l^{-1}) and (−)-epicatechin (50 mg l^{-1}) did not affect the growth of *O. oeni* (Figueiredo *et al.* 2008). On the other hand, the metabolism of *O. oeni* has been seen to be affected by the presence of wine phenolics as they favour the use of sugars and malic acid (Vivas *et al.* 2000; Alberto *et al.* 2001; Rozès *et al.* 2003). Studies with different *Lactobacillus* species have also shown inhibitory effects of hydroxybenzoic acids, hydroxycinnamic acids, flavan-3-ols, flavonols, phenolic aldehydes and other related compounds (Stead 1993; Salih *et al.* 2000; Campos *et al.* 2003; Landete *et al.* 2007; Figueiredo *et al.* 2008). Some of these studies concluded that *O. oeni* seems to be more sensitive to inactivation by phenolic compounds than *L. hilgardii* (Campos *et al.* 2003; Figueiredo *et al.* 2008). Studies about the effects on growth of bacteria species from the genera *Leuconostoc* (Vivas *et al.* 1997) and *Pediococcus* by wine phenolics are quite scarce. But, in any case, all these studies refer to the inhibition effects on the bacterial growth of wine phenolics at certain phenolic concentrations, but no determinations of MIC or MBC have been carried out, with the exception of the study by Landete *et al.* (2007). Both survival parameters MIC and MBC can be useful in comparing the inhibitory potency among phenolic structures, bacteria species, conditions, etc.

The mechanism involved in the inactivation of LAB by wine phenolics is not yet well understood and may vary according to the micro-organism (Figueiredo *et al.* 2008). From works carried out with pathogenic bacteria, some authors propose that these compounds can act on proteins of the bacteria cell membrane causing a series of compounds to leave the cell interior thus producing losses in K^+, glutamic acid, intracellular RNA, etc. as well as an alteration in the composition of fatty acids (Rozès and Perez 1998). Other authors have suggested that phenols adsorb to cell walls, alter the cell casing and even other mechanisms that involve interactions with cellular enzymes (Campos *et al.* 2003). Recently, a contribution towards the elucidation of the mechanisms of tannins on bacteria growth inhibition was made by a combination of physiologic and proteomic approaches (Bossi *et al.* 2007). The effects of tannic acid on cells are deduced by the involvement of metabolic enzymes, and functional proteins on the tannin–protein interaction. On the other hand, phenolic compounds are known to serve oxygen scavenging and reduce the redox potential of wines. This property has been tentatively suggested to be related to the effect of phenolics on the growth and metabolism of LAB (Reguant *et al.* 2000; Theobald *et al.* 2008), but to our knowledge, no relationships between antimicrobial and antioxidant activities of wine phenolics have been found so far.

The aim of this study is to investigate the inactivation properties of different classes of phenolic compounds present in wine (hydroxybenzoic acids and their derivatives, hydroxycinnamic acids, phenolic alcohols and other related compounds, stilbenes flavan-3-ols and flavonols) against two LAB wine isolates of *Lactobacillus hilgardii* and *Pediococcus pentosaceus*. These LAB are considered wine spoilage species due to their potential ability to cause organoleptic and hygienic alterations in wine. After a first screening of the inactivation potency of the phenolics at certain concentrations, the survival parameters (MIC and MBC) of the most active compounds were determined. In order to obtain a greater depth of understanding of the mechanisms involved, changes in cell viability and cell morphology, after incubation with wine phenolics, were observed by epifluorescence and scanning electron microscopy. Additionally, assessment of the oxygen-radical absorbance capacity (ORAC) of the wine phenolics studied was carried out, and the relationship between both antibacterial and antioxidant activities was studied with different statistical techniques.

Materials and methods

Phenolic compounds

Gallic acid, ellagic acid, caffeic acid, (+)-catechin, quercetin, *trans*-resveratrol and myricetin were purchased from Sigma (St Louis, MO, USA); ethylgallate, methylgallate, (−)-epicatechin gallate, (−)-epigallocatechin, (−)-epigallocatechin gallate and isorhamnetin from Extrashyntèse (Genay, France); ferulic acid from Koch-Light Laboratorie Ltd (Colnbrook, Bucks, UK); *p*-coumaric acid, (−)-epicatechin and kaempferol from Fluka (Buchs, Switherland); sinapic acid, tryptophol and tyrosol from Aldrich (Steinheim, Germany), and morin from

Sarshyntex (Merignac, Bordeaux, France). All the phenolic compounds were dissolved in ethanol 60% (v/v). *Cis*-resveratrol was obtained by exposing the *trans*-resveratrol solution to UV light (254 nm) (Bartolomé *et al.* 2000).

Other chemicals

Potassium metabisulfite ($K_2S_2O_5$) was purchased from Panreac Química S.A. (Barcelona, Spain). For the antioxidant activity assay, disodium fluorescein (FL) was purchased from Sigma, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Aldrich) and 2,2′-azobis(2-methyl-propionamidine)-dihydrochloride (APPH; Aldrich), from Aldrich (St Louis, MO, USA).

Lactic acid bacteria and culture media

The two strains used, *Lactobacillus hilgardii* IFI-CA 49 and *Pediococcus pentosaceus* IFI-CA 85, belong to the culture collection of the Institute of Industrial Fermentations (CSIC). Both strains were previously isolated from red wines at the early phase of MLF, and properly identified by 16S rRNA partial gene sequencing as described by Moreno-Arribas and Polo (2008). These strains were kept frozen at −70°C in a sterilized mixture of culture medium and glycerol (50 : 50, v/v). The culture media MRS -based on the formula developed by Caspritz and Radler (1983), and MRS-Agar (containing 1·5% of agar) (pH 6·2) were purchased from Pronadisa (Madrid, Spain). The culture media containing 6% ethanol (MRSE and MRSE-Agar) were prepared by adding ethanol (99·5%, v/v) to the sterilized (121°C, 15 min) medium.

Antibacterial activity assay

The antibacterial assays were performed using the method of López-Expósito *et al.* (2006) adapted to wine model conditions (15% ethanol) and phenolic compounds as inhibitors. Initially, some incubation conditions (i.e. inoculum size, incubation time for bacteria growth and incubation time of the bacteria with the antimicrobial agent) were optimized. Briefly, 100 μl of the de-frozen strain (*Lactobacillus hilgardii* IFI-CA 49 and *Pediococcus pentosaceus* IFI-CA 85) suspension was added to 10 ml of MRS medium, incubated at 30°C for 48 h, and then 100 μl of the suspension was plated on MRSE-Agar. Single bacteria colonies, grown on MRSE-Agar, were inoculated in 10 ml of MRSE and grown at 30°C for 24 h. A total of 300 μl of the bacterial suspension was diluted with 1/50 MRSE. Bacteria were grown at 30°C and organisms at the end of the exponential growth phase were harvested at a density of $1–4 \times 10^8$ colony forming units (CFU) ml^{-1}. The population density was determined by measuring the absorbance at 620 nm. The culture was then centrifuged at 3000 ***g*** for 10 min at 5°C. The pellet of bacteria was washed twice with 10 mmol l^{-1} of sodium acetate-acetic acid buffer (pH 4·6), and the density adjusted to 10^6 CFU ml^{-1}. In a sterile 96-well microplate (Greiner Labortechnik, Frickenhausen, Germany), a total of 50 μl of the suspension was mixed with 50 μl of the antimicrobial agent solution and 100 μl of 10 mmol l^{-1} sodium acetate-acetic acid buffer (pH 4·6) containing 2% MRSE. The ethanol concentration in the mixture was 15%. The mixture was incubated at 30°C for 3 and 6 h, and then plated on MRSE-Agar for colony counting. Assays were conducted in duplicate. The antimicrobial activity was expressed as log No/Nf, where No and Nf were the CFU values corresponding to the bacteria mixtures incubated without (control) and with the antimicrobial agent, respectively. In both cases, the ethanol concentration in the mixtures was the same.

The antibacterial activity of the compounds against *Lactobacillus hilgardii* IFI-CA 49 and *Pediococcus pentosaceus* IFI-CA 85 was initially determined at 0·1 and 1 g l^{-1} for all the phenolics, except for ellagic acid and flavonols, whose concentration was fixed at 0·01 and 0·1 g l^{-1} to ensure complete solubility in the medium.

Determination of MIC and MBC

The MIC was defined as the smallest amount of antimicrobial agent needed to reduce 10–50 times the population of micro-organism of the original inoculum [log (No/Nf) = 1–1·7] after incubation for 3 and 6 h. The MBC was determined as the minimal concentration of the antimicrobial agent that killed over 99·9% of the initial inoculum after incubation for 3 and 6 h. Assays were conducted in duplicate.

Fluorescence microscopy

Cells were observed and photographed with a DM2500 epifluorescence microscope (Leica, Heerbrugg, Switzerland). The LIVE/DEAD *Bac*Light bacterial Viability Kits L7012 (Invitrogen, OR, USA) were used to assess membrane integrity by selective nucleic acid staining. The kit contains two dyes: SYTO 9 (fluorescent green) that penetrates and labels all bacteria, and propidium iodide (fluorescent red) that penetrates only bacteria with damaged membranes, and in these cells suppresses SYTO 9 staining. As a result, live cells stain fluorescent green, and dead cells stain fluorescent red. The bacteria suspension (10^6 CFU ml^{-1}) was mixed with the antimicrobial agent solution and the sodium acetate-acetic acid buffer (10 mmol l^{-1}, pH 4·6) containing 2% MRSE in the proportion indicated above, and was incubated for 3 h at

30°C. After this time, 1 ml of the mixture was mixed with 3 μl of the stain mixture (SYTO 9-propidium iodide, 1 : 1, v/v). After 15 min of incubation in the dark at room temperature, green and red cells were counted under a fluorescence microscope with a long-pass filter (excitation, 420–490 nm; emission, 515 nm). A control without the antimicrobial agent but with the same % ethanol, was carried out in the same way.

Electron microscopy

Bacteria incubated without or with the antimicrobial agent for 6 h were fixed on the culture plate with 4% *p*-formaldehyde (Merck, Darmstadt, Germany) and 2% glutaraldehyde (SERVA, Heidelberg, Germany) in 0·05 mol l^{-1} cacodylate buffer (pH 7·4) for 120 min at room temperature. Cells were then carefully scraped from the plate, centrifuged at 3000 ***g*** for 5 min and the washed pellet post-fixed with 1% OsO_4 and 1% $K_3Fe(CN)_6$ in water for 60 min at 4°C. Cells were dehydrated with ethanol and embedded in Epon (TAAB 812 resin, TAAB Laboratories Equipment Ltd) according to standard procedures. Ultra thin sections were collected on collodion-carbon coated copper grids, stained with uranyl acetate and lead citrate and examined at 80 kV in a JEM 1010 (Jeol, Tokyo, Japan) electron microscope. Electron micrographs were recorded at different orders of magnitude.

Antioxidant activity

The radical scavenging activity of the phenolic compounds was determined by the ORAC method using fluorescein as a fluorescence probe (Dávalos *et al.* 2004). Briefly, the reaction was carried out at 37°C in 75 mmol l^{-1} phosphate buffer (pH 7·4). The final assay mixture (200 μl) contained fluorescein (70 nmol l^{-1}), 2,2′-azobis(2-methyl-propionamidine)-dihydrochloride (12 mmol l^{-1}), and antioxidant [Trolox (1–8 μmol l^{-1}) or phenolic compound (at different concentrations)]. A Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) with 485-P excitation and 520-P emission filters was used. The equipment was controlled by the FLUOSTAR GALAXY software version (4·11-0) for fluorescence measurement. Black 96-well untreated microplates (Nunc, Denmark) were used. The plate was automatically shaken before the first reading and the fluorescence was recorded every minute for 98 min. 2,2′-Azobis (2-methyl-propionamidine)-dihydrochloride and Trolox solutions were prepared daily and fluorescein was diluted from a stock solution (1·17 mmol l^{-1}) in 75 mmol l^{-1} phosphate buffer (pH 7·4). All reaction mixtures were prepared in duplicate and at least three independent runs were performed for each sample. Fluorescence measurements were normalized to the curve of the blank (no antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=98} f_i/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. The net AUC corresponding to a sample was calculated as follows:

$$\text{net AUC} = \mathrm{AUC}_{\text{antioxidant}} - \mathrm{AUC}_{\text{blank}}$$

The net AUC was plotted against the antioxidant concentration and the regression equation of the curve was calculated. The ORAC value was obtained by dividing the slope of the latter curve by the slope of the Trolox curve obtained in the same assay. Final ORAC values were expressed as μmol of Trolox equivalents per mg of compound.

Statistical analysis

To examine the relationships between the activities studied, principal component analysis (from standardized variables) using the STATISTICA program for Windows, ver. 7.1 (StatSoft Inc. 1984–2006, http://www.statsoft.com) was carried out for data processing.

Results

Antibacterial activities of wine phenolic compounds

Most of the phenolic compounds used in this study occur naturally in wine and were chosen because of their different functional group and/or ring substituents (Fig. 1), in an attempt to relate the phenolic chemical structure to their effects on cell viability of LAB. Other phenolic structures not present in wine [morin, (−)-epigallocatechin and (−)-epigallocatechin gallate] were also studied for their structural similarities. At the maximum concentration tested (1 g l^{-1} for all the phenolics, except for ellagic acid and flavonols, whose maximum concentration tested was 0·1 g l^{-1}) most of the phenolic compounds showed inhibition of growth for both *L. hilgardii* and *P. pentosaceus* strains, with the exception of ellagic acid, methyl gallate, sinapic acid, (+)-catechin, (−)-epigallocatechin, (−)-epicatechin gallate, (−)-epigallocatechin gallate and quercetin, that did not exhibit any effect for both strains (Table 1). Only in the case of *L. hilgardii*, there was no indication of inhibitory effects by ferulic acid, tyrosol, (−)-epicatechin, myricetin and isoramnetin. At the

Table 1 Antibacterial activity of the phenolic compounds studied against *Lactobacillus hilgardii* and *Pediococcus pentosaceus* at the concentrations of 10, 100 and 1000 mg l^{-1}

	Antimicrobial activity (expressed as log No/Nf)											
	L. hilgardii IFI-CA 49						*P. pentosaceus* IFI-CA 85					
	1000 mg l^{-1}		100 mg l^{-1}		10 mg l^{-1}		1000 mg l^{-1}		100 mg l^{-1}		10 mg l^{-1}	
	3 h	6 h	3 h	6 h	3 h	6 h	3 h	6 h	3 h	6 h	3 h	6 h
Compounds												
Hydroxybenzoic acids and esters												
Gallic acid	3·63	3·16	n.e.	n.e.			5·56	5·43	0·80	0·78		
Ellagic acid			n.e.	n.e.	n.e.	n.e.			n.e.	n.e.	n.e.	n.e.
Ethyl gallate	3·16	3·26	n.e.	n.e.			5·41	5·48	1·03	0·50		
Methyl gallate	n.e.	n.e.	n.e.	n.e.			n.e.	n.e.	n.e.	n.e.		
Hydroxycinnamic acids												
Ferulic acid	n.e.	n.e.	n.e.	n.e.			6·60	6·31	1·97	1·75		
p-Coumaric acid	6·33	5·81	n.e.	n.e.			6·08	6·04	0·96	0·58		
Caffeic acid	6·14	6·13	n.e.	n.e.			6·20	6·01	n.e.	n.e.		
Sinapic acid	n.e.	n.e.	n.e.	n.e.			n.e.	n.e.	n.e.	n.e.		
Phenolic alcohols												
Tyrosol	n.e.	n.e.	n.e.	n.e.			2·36	2·00	1·13	1·55		
Tryptophol	5·16	2·79	0·71	1·21			5·60	4·18	n.e.	n.e.		
Stilbenes												
trans-Resveratrol	6·46	5·84	n.e.	n.e.			6·07	5·80	1·93	1·55		
Flavan-3-oles												
(+)-Catechin	n.e.	n.e.	n.e.	n.e.			n.e.	n.e.	n.e.	n.e.		
(−)-Epicatechin	n.e.	n.e.	n.e.	n.e.			2·52	2·92	n.e.	n.e.		
(−)-Epigallocatechin	n.e.	n.e.	n.e.	n.e.			n.e.	n.e.	n.e.	n.e.		
(−)-Epicatechin gallate	n.e.	n.e.	n.e.	n.e.			n.e.	n.e.	n.e.	n.e.		
(−)-Epigallocatechin gallate	n.e.	n.e.	n.e.	n.e.			n.e.	n.e.	n.e.	n.e.		
Flavonols												
Quercetin			n.e.	n.e.	n.e.	n.e.			n.e.	n.e.	n.e.	n.e.
Myricetin			n.e.	n.e.	n.e.	n.e.			2·21	2·31	1·02	1·02
Kaempferol			6·03	5·83	1·90	2·20			6·20	6·03	2·04	2·56
Isorhamnetin			n.e.	n.e.	n.e.	n.e.			2·10	4·71	1·63	1·27
Morin			6·79	6·47	6·79	6·47			6·50	6·40	6·40	6·30

n.e., no effect was observed.

minimum concentration tested (0·01 g l^{-1}), morin was the compound that showed the highest inhibition effect (higher Log No/Nf) (Table 1).

In general, the Log No/Nf values were similar or slightly higher for the determinations after 3 h than after 6 h of bacterial exposure to the phenolic compounds, which indicated that inactivation persisted at least for 6 h. It was also found that *P. pentosaceus* IFI-CA 85 was more sensitive to phenolic inactivation than *L. hilgardii* IFI-CA 49; in other words, the *L. hilgardii* strain was more resistant to the action of these compounds.

In an attempt to establish the extent to which phenolic compounds can affect LAB growth during wine-making and to allow a better comparison of the phenolic inhibitory potency among phenolic structures, bacteria species, conditions, etc. the survival parameters (MIC and MBC) were determined for the active compounds reported above (Table 2). For the *L. hilgardii* strain, the flavonols morin and kaempferol showed the strongest inactivation effect; this is to say, the lowest MIC (1 and 5 mg l^{-1}, respectively) and MBC (7·5 and 50 mg l^{-1}, respectively) values. The rest of the compounds studied exhibited values around 100-fold higher for MIC (125–500 mg l^{-1}) and around 50-fold higher for MBC (300–2000 mg l^{-1}). The order among compounds was almost the same for the two survival parameters MIC (morin < kaempferol << resveratrol < gallic acid ≤ caffeic acid < *p*-coumaric acid < tryptophol = ethyl gallate) and MBC (morin < kaempferol << gallic acid < caffeic acid < *p*-coumaric acid < resveratrol < tryptophol < ethyl gallate) (Table 2). For the *P. pentosaceus* strain, flavonols also showed the strongest inactivation effects, with MIC values between 1 and 10 mg l^{-1} and MBC values between 7·5 and 300 mg l^{-1}. The other phenolic compounds

Table 2 MIC and MBC of the phenolic compounds studied against *Lactobacillus hilgardii* and *Pediococcus pentosaceus*

	L. hilgardii IFI-CA 49				*P. pentosaceus* IFI-CA 85			
	MIC (mg l^{-1})		MBC (mg l^{-1})		MIC (mg l^{-1})		MBC (mg l^{-1})	
	3 h	6 h	3 h	6 h	3 h	6 h	3 h	6 h
Compounds								
Gallic acid	300	300	1800	1600	200	200	1000	800
Ethyl gallate	500	500	2000	2000	200	200	1500	1250
Ferulic acid					50	50	900	900
p-Coumaric acid	400	400	1000	1000	200	200	1000	800
Caffeic acid	300	400	900	800	170	170	700	700
Tyrosol					70	70	2000	2000
Tryptophol	500	500	1800	1800	250	100	1400	1250
trans-Resveratrol	125	125	1100	1100	50	50	300	200
(−)-Epicatechin					200	200	2000	2000
Myricetin					10	10	>100	>100
Kaempferol	5	5	50	50	5	5	100	100
Isorhamnetin					10	10	>300	300
Morin	1	1	7·5	7·5	1	1	7·5	7·5

>100, >300 indicate that the MBC must be higher than these values, but it was no possible to test higher concentrations due to lack of solubility

showed values of 50–250 mg l^{-1} for MIC and 300–2000 mg l^{-1} for MBC. The order among compounds was: morin < kaempferol < myricetin = isorhamnetin < resveratrol = ferulic acid< tyrosol < caffeic acid < tryptophol < (−)-epicatechin= gallic acid = *p*-coumaric acid = ethyl gallate for MIC, and morin < kaempferol = myricetin < isorhamnetin < resveratrol < caffeic acid < ferulic acid < gallic acid = *p*-coumaric acid < tryptophol < ethyl gallate < tyrosol = (−)-epicatechin for MBC (Table 2). For this latter strain, it was proven that there were no differences in the survival parameters between the two isomeric forms *trans* and *cis* of resveratrol (data not shown). As seen in the experiment of bacteria inactivation at certain phenolic concentrations (Table 1), the MIC and MBC values were, in general, similar or slightly higher for the determinations after 3 h than after 6 h of bacteria exposure to the phenolic compounds for both *L. hilgardii* and *P. pentosaceus* strains (Table 2). The strain *P. pentosaceus* IFI-CA 85 seemed more sensitive to phenolic inactivation than *L. hilgardii* IFI-CA 49. For instance, ferulic acid, tyrosol, (−)-epicatechin, myricetin and isorhamnetin exhibited inhibitory and bactericide effects against *P. pentosaceus*, but did not affect the growth of the tested strain of *L. hilgardii*. For other compounds, such as morin and *trans*-resveratrol, *P. pentosaceous* showed MIC values from one to two-fold dilution orders lower than those shown by *L. hilgardii* (Table 2).

Additionally, MIC and MBC values of potassium metabisulfite ($K_2S_2O_5$) were determined. For the *L. hilgardii* strain, this chemical showed a MIC value of 25 mg l^{-1} for both 3 and 6 h of bacteria exposure, and a MBC value of 500 and 200 mg l^{-1} for 3 and 6 h, respectively. For the *P. pentosaceus* strain, the MIC value was 75 mg l^{-1} for both 3 and 6 h, and the MBC was 600 and 500 mg l^{-1} for 3 and 6 h, respectively.

Comparatively, $K_2S_2O_5$ showed MIC and MBC values around 5–15-fold higher than those corresponding to kaempferol, but around 2–5-fold lower than resveratrol; this is to say, potassium metabisulfite was less toxic for the bacterial cells than kaempferol, but more toxic than resveratrol.

Microscopy study

Epifluorescence and scanning electron microscopy techniques were applied to observe changes in cell viability and cell morphology after incubation of the LAB with wine phenolics. As examples, Figs 2 and 3 display the micrographs of *P. pentosaceus* cells incubated with two of the most active wine phenolics, kaempferol and *trans*-resveratrol, at their MBCs (100 and 300 mg l^{-1}, respectively). Micrographs corresponding to the controls and the incubations of LAB with potassium metabisulfite (600 mg l^{-1}) were also included. Under epifluorescence microscopy, the cells from the control (Fig. 2a) and from the incubation with potassium metabisulfite (Fig. 2b) shown green fluorescence. However, the number of viable cells seemed lower in the experiment treated with potassium metabisulfite (Fig. 2b). Wine phenolics were showed to damage the bacteria cell membrane, leading to red fluorescence (Fig. 2c,d). In addition, some cell aggregation was observed when the bacteria were incubated with kaempferol (Fig. 2c), a compound that also presented a visible yellow fluorescence by itself (micrograph not shown).

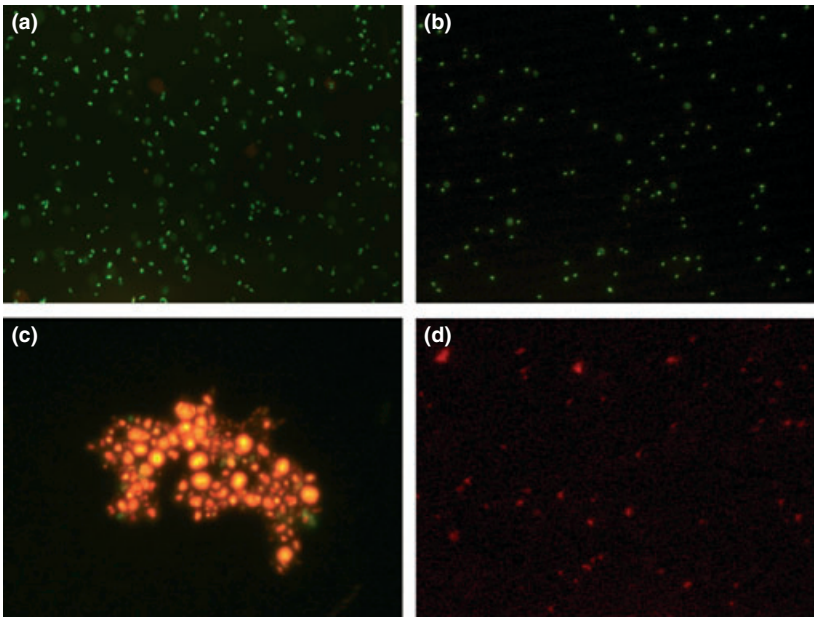


Figure 2 Epifluorescence micrographs (400×) of *Pediococcus pentosaceus* IFI-CA 85 non-incubated and incubated with antimicrobial agents for 3 h: (a) control, (b) incubation with potassium metabisulfite (600 mg l^{-1}), (c) incubation with kaempferol (100 mg l^{-1}) and (d) incubation with *trans*-resveratrol (300 mg l^{-1}).

Confirmation of the harmful effects of wine phenolics in the integrity of the cell membrane was obtained by scanning electron microscopy (Fig. 3c,d). The electron micrograph showed that the treatment with kaempferol at its MBC (100 mg l^{-1}) produced breakdown of the cell membrane and the subsequent release of the cytoplasm material into the medium. The membranes of the cells from the control (Fig. 3a) and from the incubation with

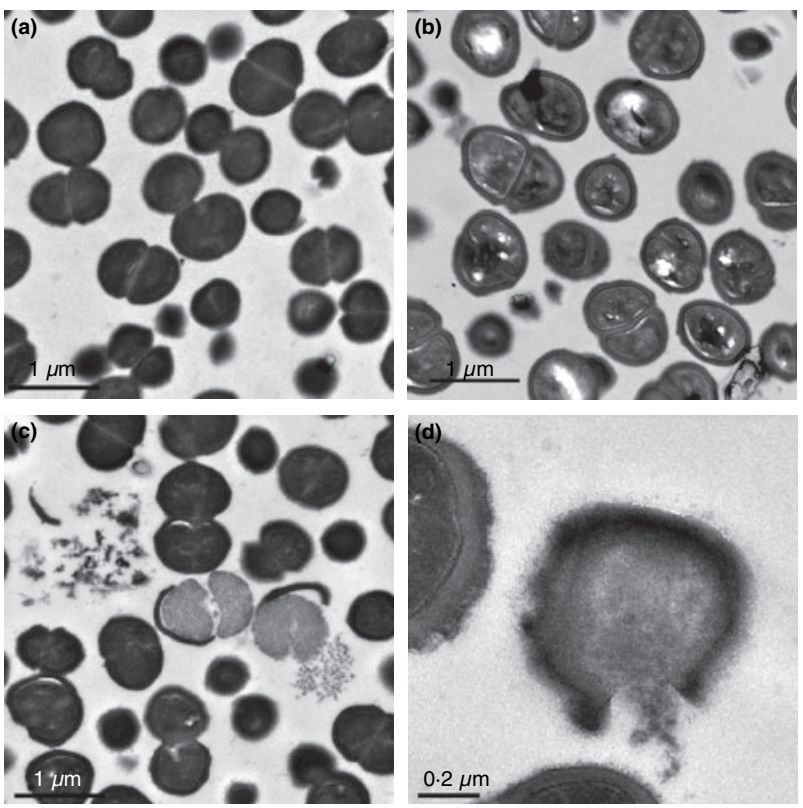


Figure 3 Electron micrographs of ultrathin sections of *Pediococcus pentosaceus* IFI-CA 85 non-incubated and incubated with antimicrobial agents. (a) control, (b) incubation with potassium metabisulfite (600 mg l^{-1}), (c) incubation with kaempferol (100 mg l^{-1}) and (d) incubation with kaempferol (100 mg l^{-1}). Bars = 1 μm (a–c), 0·2 μm (d).

potassium metabisulfite (600 mg l^{-1}) (Fig. 3b) were complete, with the cytoplasm being intact and homogeneously distributed.

Antioxidant activities of phenolic compounds

The ORAC values of the phenolic compounds studied ranged from 10·1 μmol Trolox/mg for gallic acid to 47·6 for *trans*-resveratrol (Table 3). Some features of the phenolic chemical structure seemed to influence the antioxidant activity of the different compounds. For instance, esters (methyl and ethyl gallates) showed higher ORAC values than their corresponding free acid (gallic acid). Methoxylation of the aromatic ring reduced the antioxidant activity of phenolic acid (caffeic acid > ferulic acid).

The relationship between the antibacterial activity (MBC values; Table 2) against *L. hilgardii* and *P. pentosaceus* and the antioxidant activity (ORAC values; Table 3) of the phenolic compounds was investigated by correlation analysis, but a non-significant correlation was obtained for both the *L. hilgardii* ($r = -0{\cdot}3286$, $P = 0{\cdot}427$) and the *P. pentosaceus* ($r = 0{\cdot}2265$, $P = 0{\cdot}457$) strains. Principal component analysis was also applied to study the interrelation between the antibacterial and antioxidant variables (MICs, MBCs and ORAC) considering those phenolic compounds which were most active against both bacteria. Figure 4 displays the distribution of the different phenolic compounds in the plane defined by the first two principal components. The two first principal components explained 93·3% of the total variance of the data. The first principal component, which explains 74·4% of the total variance, was negatively correlated to antimicrobial activity (MIC and MBC values, see loadings in the Fig. 4). The second principal component, which explains 18·9% of the total variance, was mainly correlated to antioxidant activity (−0·901). The two flavanols (morin and kaempferol) were located close together on the right central zone of the plane (high value for PC1 and intermediate for PC2) (Fig. 4). Gallic acid and its ethyl ester were also located together in the left central-upper zone of the plane (low value for PC1 and medium-high value for PC2). The rest of the compounds (tryptophol, *p*-coumaric acid, caffeic acid and *trans*-resveratrol), all exhibiting a C=C bond conjugated with the aromatic ring, were located in the lower part of the plane (low value for PC2). However, they clearly differ in their PC1 value, which can be related to other chemical structure features such as the number of phenolic rings (i.e. two in the case of resveratrol). These results prove similarities and differences among phenolic classes in relation to their antimicrobial and antioxidant properties.

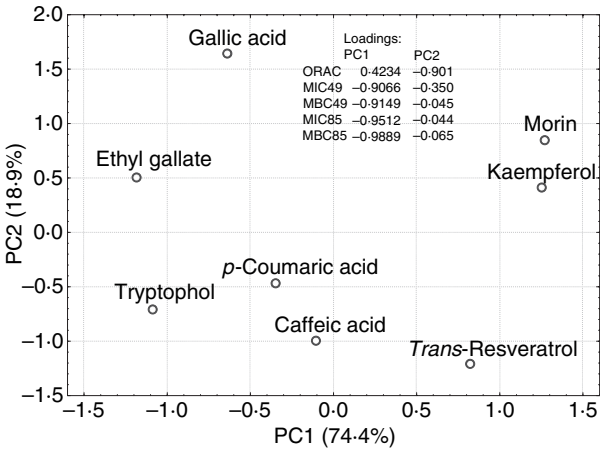


Figure 4 Plot of the phenolic compounds in the plane defined by the two first principal components.

Table 3 Radical scavenging activity (ORAC values) of the phenolic compounds studied

Phenolic compound	ORAC (μmol Trolox mg^{-1})	Phenolic compound	ORAC (μmol Trolox mg^{-1})
Hydroxybenzoic acids and esters		*Flavan-3-ols*	
Gallic acid	10·1	(+)-Catechin	46·8
Ellagic acid	19·8	(−)-Epicatechin	44·0
Ethyl gallate	16·3		
Methyl gallate	14·7		
Hydroxycinnamic acids		*Flavonols*	
Ferulic acid	23·0	Quercetin	33·0
p-Coumaric acid	32·2	Myricetin	15·9
Caffeic acid	39·0	Kaempferol	30·9
Sinapic acid	13·2	Isorhamnetin	32·5
		Morin	25·7
Phenolic alcohols		*Stilbenes*	
Tyrosol	38·4	*trans*-Resveratrol	47·6
Tryptophol	31·8		

Discussion

This study reports new knowledge about the inactivation by the main phenolic compounds present in wine, of less-studied LAB species, *Lactobacillus hilgardii* and *Pediococcus pentosaceus* that may affect wine organoleptic and hygienic properties during winemaking. Cultures were grown in ethanol-containing media in order to simulate the wine environment. Another important contribution of this work is the determination of the survival parameters MIC and MBC for wine phenolic compounds against LAB, which allows a better comparison of the results among different studies as well as a more accurate assessment of the effects of these compounds on the growth of LAB during winemaking.

The results reported here show that the antibacterial activity of wine phenolics against *L. hilgardii* IFI-CA 49 and *P. pentosaceus* IFI-CA 85 was strongly dependent on phenolic structure. The most active compounds belong to the flavonol class, although some of them (e.g. quercetin) did not exhibit any effect at the concentrations tested. In a recent study, Figueiredo *et al.* (2008) have shown that there are no effects of kaempferol, myricetin and quercetin (10 mg l^{-1}) on the growth of *L. hilgardii*, which agrees with the present study. Concerning stilbenes, this study shows that isomerization reactions (from the *trans* to the *cis* form) did not seem to affect the antibacterial activity of resveratrol, the most abundant stilbene found in wine. The alcohols tyrosol and tryptophol are metabolites, which have been respectively formed from tyrosine and tryptophan during yeast fermentation. Both of these have also shown certain inactivation potential against *L. hilgardii* and *P. pentosaceus*. In relation to hydroxycinnamic acids, our results for *L. hilgardii* agreed with those reported by Campos *et al.* (2003), who found that different hydroxycinnamic and hydroxybenzoic acids showed significant inactivation effects at concentrations ≥500 mg l^{-1}, the former group being more potent inhibitors than the latter one. In that study, *p*-coumaric acid caused the greatest decrease in cell viability, and ferulic acid did not show any effect. From our results concerning hydroxycinnamic acids, *p*-coumaric and caffeic were the most potent inhibitors, whereas ferulic and sinapic acids were inactive against *L. hilgardii*. Some features of the hydroxybenzoic acid structure also seemed to influence their antimicrobial properties against *L. hilgardii* and *P. pentosaceus*. Ethylation, and in particular methylation and dimerization of gallic acid, reduced its inactivation potential against these two bacteria. Finally, none of the flavan-3-ol monomers and gallates tested seemed to exert any effects on the growth of *L. hilgardii* and *P. pentosaceus*. Figueiredo *et al.* (2008) also observed no effects of (+)-catechin (≤50 mg l^{-1}) and (−)-epicatechin (≤12·5 mg l^{-1}) on the growth of *L. hilgardii*. Therefore, not only the phenolic class (hydroxybenzoic and hydroxycinnamic acids, phenolic alcohols, stilbenes, flavan-3-ols and flavonols) but also the substituents of the phenolic chemical structure conditioned the antimicrobial properties of wine phenolic compounds against *L. hilgardii* and *P. pentosaceus*.

The results also confirmed differences in bacteria susceptibility to polyphenols among different LAB genera and species. In our case, *P. pentosaceus* IFI-CA 85 was more sensitive to phenolic inactivation than *L. hilgardii* IFI-CA 49. Other authors have proven that *L. hilgardii* is also more resistant to the action of hydroxybenzoic and hydroxycinnamic acids (Campos *et al.* 2003) and phenolic aldehydes, flavonoids and tannins (Figueiredo *et al.* 2008) than *O. oeni*. However, by comparing our data with previous MIC values for *L. plantarum* (Landete *et al.* 2007), it can be seen that this species is even more resistant to the action of phenolic compounds such as *p*-coumaric (MIC = 2000–4000 mg l^{-1}) and caffeic acid (9000–18 000 mg l^{-1}), than the strain of *L. hilgardii* used in this study (MIC = 400 and 300 mg l^{-1} for *p*-coumaric and caffeic acids, respectively).

The mechanisms by which polyphenols inhibit the growth of LAB are not well known. The observations of the cells of *P. pentosaceus* by epifluorescence and scanning electron microscopy, reported in this work, indicate that wine phenolics seem to damage the bacteria cell membrane, which promotes cell death, probably due to alterations in transport and energy-dependent processes, and metabolic pathways that are essential for bacteria viability, as reported for other inhibition agents against other LAB species (Ibrahim *et al.* 1996). It is likely that hydrophobic interactions between membrane lipids and phenolic compounds are involved in this inactivation. The results also show that enological LAB may aggregate in the presence of certain phenolic compounds such as kaempferol. This compound would strongly adhere to the cell membrane, causing its degradation and, therefore, loss of cell viability. The formation of bacterial aggregates has also been reported in studies dealing with other microbial agents such as peptides from dairy proteins and lysozyme with bactericide effect against Gram-negative (i.e. *Escherichia coli*) and Gram-positive (i.e. *Staphylococcus aureus*) species, respectively (Ibrahim *et al.* 1996).

Potassium metabisulfite ($K_2S_2O_5$), the additive usually used in winemaking because its antioxidant and selective antibacterial effect, showed, in our antimicrobial assays, MIC and MBC values for *L. hilgardii* and *P. pentosaceus* in the range of those reported by Rojo-Bezares *et al.* (2007) for other wine LAB. As seen by microscopy, potassium metabisulfite does not lead to cell membrane lysis but affects cell viability. This is in accordance to the results reported by Millet and Lonvaud-Funel (2000), who found that after MLF, and in the following days after sulfiting, *O.oeni* cells entered in a viable but non-culturable (VBNC) state and were no longer culturable on nutrient plates, although they retained some metabolic activity. Evidence of this viable but nonculturable state has also been shown in yeast in botrytis-affected wines (Divol and Lonvaud-Funel 2005) and it also seems to be induced by other sulfite-alternative antimicrobial agents such as dimethyldicarbonate (DMDC) (Divol *et al.* 2005). During wine aging in oak barrels, some micro-organisms were also able to move from the VBNC to the viable state. The results obtained in this work suggested that the phenolic compounds exhibit different antibacterial mechanisms to those reported for potassium metabisulfite,

since they not only inactivate the bacteria but also lead to the cell death, although further research is needed to confirm it.

The antioxidant properties of the phenolic compounds have been tentatively related to their effect on the growth and metabolism of LAB (Reguant *et al.* 2000; Theobald *et al.* 2008). However, the results of this study show that there is no linear correlation between the antibacterial activity (MBC values) and the antioxidant activity (ORAC values) of the different phenolic structures studied. Nevertheless, principal component analysis of both antibacterial and antioxidant variables allowed distribution of the phenolic compounds according to their structural similarities. In seeking for new alternatives to sulfites, both antibacterial and antioxidant properties should be addressed (García-Ruiz *et al.* 2008). The results confirm the potential of phenolic compounds/extracts to be used as an alternative to sulfites in winemaking.

Phenolic compound concentration in wines is conditioned by grape factors (variety, quality of the harvest, soil, climate, etc.) and winemaking conditions (maceration time, temperature, contact with skins and seeds, pressing, etc.). It can be said that the concentrations in wine of most of the phenolic compounds studied in this study are significantly lower than the MIC values against *L. hilgardii* and *P. pentosaceus*. This is the case, for instance, of gallic acid (10–37 mg l^{-1} in young red wines), *p*-coumaric acid (0·1–8 mg l^{-1}), caffeic acid (0·3–33 mg l^{-1}), tyrosol (7–26 mg l^{-1}), tryptophol (<4·5 mg l^{-1}), resveratrol (0·4–2·5 mg l^{-1}) and (−)-epicatechin (10–38 mg l^{-1}) (García-Ruiz *et al.* 2008). However, the flavonols exhibit MIC values closer to their concentration in young red wine: myricetin (1·7–8 mg l^{-1}), kaempferol (<1 mg l^{-1}), isorhamnetin (<1 mg l^{-1}) (García-Ruiz *et al.* 2008). Therefore, it is unlikely that a phenolic compound alone could affect the LAB growth at the concentrations found in wine, but both additive and synergistic effects among all phenolic compounds (150–400 mg l^{-1} for white wines and 900–1400 mg l^{-1} for young red wines; García-Ruiz *et al.* 2008) may promote inactivation of LAB in the wine environment. Further inhibition studies using wine phenolic preparations should be carried out, in order to establish the extent to which these compounds can affect LAB growth and MLF during winemaking.

In summary, this work reports a complete study of the effect of the main classes of wine phenolic compounds on the growth of two strains of *Lactobacillus hilgardii* and *Pediococcus pentosaceus*. Novel and useful information about survival parameters, structure/activity relationship and mechanisms of action of wine phenolic compounds against these two species is provided.

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<u>Publicación II.</u> Estudio comparativo de los efectos de inhibición de los polifenoles del vino sobre el crecimiento de bacterias lácticas de origen enológico.

Almudena García-Ruiz, M. Victoria Moreno-Arribas, Pedro J. Martín-Álvarez, Begoña Bartolomé. Comparative study of the inhibitory effects of wine polyphenols on the growth of enological lactic acid bacteria. *International Journal of Food Microbiology*, **2011,** 145: 426–431.

Resumen:

Este trabajo recoge un estudio comparativo sobre la capacidad inhibitoria de 18 compuestos fenólicos (ácidos y derivados hidroxibenzoicos, ácidos hidroxicinámicos, alcoholes fenólicos y otros compuestos relacionados, estilbenos, flavan-3-oles y flavonoles) frente a diferentes cepas de bacterias lácticas (BAL) de las especies *Oenococcus oeni*, *Lactobacillus hilgardii* y *Pediococcus pentosaceus* aisladas del vino. En general, los flavonoles y estilbenos, mostraron mayor inhibición (valores de IC_{50} más bajos) sobre el crecimiento de las cepas analizadas (0,160 a 0,854 para los flavonoles y 0.307-0.855 g /L para los estilbenos). Los ácidos hidroxicinámicos (IC_{50}<0.470 g/L) y los ácidos y ésteres hidroxibenzoicos (IC_{50}>1 g/L) manifestaron un efecto inhibidor medio, mientras que los alcoholes fenólicos (IC_{50}>2g/L) y flavon-3-oles (efecto no significativo) mostraron el menor efecto sobre el crecimiento de las cepas de BAL estudiadas. En comparación con los aditivos antimicrobianos utilizados durante la elaboración del vino, los valores de IC_{50} de la mayoría de los compuestos fenólicos fueron superiores a los mostrados por el metabisulfito potásico frente a cepas de *O. oeni* (por ejemplo, ~4 veces superior para la quercetina que para el metabisulfito potásico), pero inferiores a los observados frente a las cepas de *L. hilgardii* y *P. pentosaceus* (por ejemplo, ~2 veces inferior para la quercetina). Los valores de IC_{50} de la lisozima frente a *L. hilgardii* y *P. pentosaceus* no fueron significativos, y además, más altos que los correspondientes valores de la mayoría de compuestos fenólicos ensayados frente a las cepas de *O. oeni*, lo que indicaba que la lisozima era menos tóxica para las BAL que los compuestos fenólicos del vino. Por microscopía electrónica de transmisión, se confirmaron daños en la integridad de la membrana celular como consecuencia de la incubación con agentes antimicrobianos. Estos resultados contribuyen al conocimiento sobre la acción inhibidora de los compuestos fenólicos del vino durante el proceso de la fermentación maloláctica, así como sobre el potencial

desarrollo de nuevas alternativas al uso de sulfitos en enología basadas en este tipo de compuestos.

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Comparative study of the inhibitory effects of wine polyphenols on the growth of enological lactic acid bacteria

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ABSTRACT

This paper reports a comparative study of the inhibitory potential of 18 phenolic compounds, including hydroxybenzoic acids and their derivatives, hydroxycinnamic acids, phenolic alcohols and other related compounds, stilbenes, flavan-3-ols and flavonols, on different lactic acid bacteria (LAB) strains of the species *Oenococcus oeni*, *Lactobacillus hilgardii* and *Pediococcus pentosaceus* isolated from wine. In general, flavonols and stilbenes showed the greatest inhibitory effects (lowest IC_{50} values) on the growth of the strains tested (0.160–0.854 for flavonols and 0.307–0.855 g/L for stilbenes). Hydroxycinnamic acids (IC_{50}>0.470 g/L) and hydroxybenzoic acids and esters (IC_{50}>1 g/L) exhibited medium inhibitory effect, and phenolic alcohols (IC_{50}>2 g/L) and flavanol-3-ols (negligible effect) showed the lowest effect on the growth of the LAB strains studied. In comparison to the antimicrobial additives used in winemaking, IC_{50} values of most phenolic compounds were higher than those of potassium metabisulphite for *O. oeni* strains (e.g., around 4-fold higher for quercetin than for potassium metabisulphite), but lower for *L. hilgardii* and *P. pentosaceus* strains (e.g., around 2-fold lower for quercetin). Lysozyme IC_{50} values were negligible for *L. hilgardii* and *P. pentosaceus*, and were higher than those corresponding to most of the phenolic compounds tested for *O. oeni* strains, indicating that lysozyme was less toxic for LAB than the phenolic compounds in wine. Scanning electron microscopy confirmed damage of the cell membrane integrity as a consequence of the incubation with antimicrobial agents. These results contribute to the understanding of the inhibitory action of wine phenolics on the progress of malolactic fermentation, and also to the development of new alternatives to the use of sulphites in enology.

1. Introduction

The three main genera of lactic acid bacteria (LAB) associated with the winemaking process are *Oenococcus*, *Pediococcus* and *Lactobacillus* (Fugelsang, 1997; Wibowo et al., 1985). *Oenococcus oeni* is the species best adapted to growing in the difficult conditions imposed during winemaking (low pH and high ethanol concentration) (Davis et al., 1985; Lonvaud-Funel, 1999; Van Vuuren and Dicks, 1993) and, therefore, the main species of malolactic fermentation (MLF) in wine. Through this process, L-malic acid is decarboxylated into L-lactic acid, which, due to its monocarboxylic nature, imparts a more elegant and round taste to wine (Matthews et al., 2004; Moreno-Arribas and Polo, 2005). The main influence of other LAB species such as *Lactobacillus hilgardii* and *Pediococcus pentosaceus*, on wine quality is to cause alterations to the wine, including the so-called "lactic disease", and the production of off-flavor compounds (Chatonnet et al., 1995; Costello and Henschke, 2002), and biogenic amines (Landete et al., 2005; Marcobal et al., 2006). Sulphur dioxide (SO_2) is the additive most frequently employed to control LAB growth and MLF development during winemaking, because of its antioxidant and selective antimicrobial properties, especially against LAB (Kourakou-Dragona, 1998; Ough and Crowell, 1987). However, nowadays there is a growing tendency to reduce the use of SO_2 in wine processing, since high doses can cause organoleptic alterations in the final product, and especially because of the risks to human health of consuming this substance (Romano and Suzzi, 1993; Taylor et al., 1986). Some alternatives to SO_2 have been introduced based on "natural antimicrobial agents", such as the use of lysozyme, an enzyme obtained from egg white (Bartowsky, 2009; Gerbaux et al., 1997).

Phenolic compounds or polyphenols are natural constituents of grapes and wines. Under the name of wine polyphenols, numerous compounds of different chemical structures are mainly grouped into hydroxybenzoic acids, hydroxycinnamic acids, stilbenes and phenolic alcohols (non-flavonoids), and flavonols, flavan-3-ols, anthocyanins and other flavonoids. Phenolic compounds contribute to the organoleptic characteristics of wine, such as its colour, astringency and bitterness, and have been associated with the cardiovascular protective effects of wine consumption (Pozo-Bayón et al., in press). With regard to MLF, it has been empirically known for years that the phenolic content of grapes and wines can affect the rate and extent of this fermentation (Campos et al., 2009).

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The effects of wine polyphenols on LAB growth and metabolism have been studied for pure compounds against isolated bacteria (Bloem et al., 2007; Campos et al., 2003; Figueiredo et al., 2008; García-Ruiz et al., 2009; Landete et al., 2007; Reguant et al., 2000; Salih et al., 2000; Stead, 1993; Theobald et al., 2008; Vivas et al., 1997), mainly those belonging to the *O. oeni species*. The inhibitory effects of phenolic compounds on LAB have been confirmed and, based on that, polyphenols have been proposed as an alternative to the use of sulphites in controlling the growth and metabolism of LAB during winemaking (Bartowsky, 2009; García-Ruiz et al., 2008).

With regard to the mechanism involved in bacteria inactivation by phenolic compounds, it is thought that in the first stages, polyphenols alter the cell membrane structure producing leakage of bacterial cell constituents such as proteins, nucleic acids and inorganic ions (Johnston et al., 2003; Rodríguez et al., 2009). As an approach to demonstrating the initial damage of wine phenolic compounds on enological LAB strains, Campos et al. (2009) have recently demonstrated that hydroxycinnamic and hydroxybenzoic acids significantly enhanced the proton influx and the potassium and phosphate efflux from *O. oeni* and *L. hilgardii* suspensions, the effect being greater for hydroxycinnamic and hydroxybenzoic acids. However, inactivation results obtained in the same study did not appear to correlate completely with the measured ion effluxes, which may indicate that the membrane damage caused by phenolic acids may be reversible, or that bacterial inactivation by phenolics might involve more than one mechanism or cellular target (Campos et al., 2009).

Another key question that arises from all these studies is about the selectivity of the inhibitory effects of wine polyphenols depending on bacteria species. Moreover, phenolic compounds may inhibit the growth of LAB, leading to desirable species selection by inhibiting, for example, those that can cause wine alterations – such as *L. hilgardii* and *P. pentosaceus* species – but causing minimal alteration to the growth of species that lead to satisfactory MLF, such as *O. oeni*. Some studies have tried to address this question, although comparative studies among different enological LAB species are scarce (Campos et al., 2003; Figueiredo et al., 2008; Salih et al., 2000).

The aim of this study was to compare the inhibitory effects of different classes of phenolic compounds present in wine (hydroxybenzoic acids and their derivatives, hydroxycinnamic acids, phenolic alcohols and other related compounds, stilbenes, flavan-3-ols and flavonols) against different LAB wine isolates of *Oenococcus oeni* (n = 4), *Lactobacillus hilgardii* (n = 1) and *Pediococcus pentosaceus* (n = 1). The inhibitory potency of phenolic compounds has been expressed as IC_{50} in order to allow further comparison between phenolic structures, bacteria species, conditions etc. A principal component analysis (PCA) has been applied to the IC_{50} data to examine the relationship between the inhibitory effects of the antimicrobial compounds and the different enological lactic acid bacteria. Finally, changes in cell morphology, after incubation with wine phenolics, have been observed by scanning electron microscopy in order to obtain a greater depth of understanding of the mechanisms involved.

2. Materials and methods

2.1. Phenolic compounds and other chemicals

Gallic acid, ellagic acid, caffeic acid, (+)-catechin, quercetin, trans-resveratrol and myricetin were purchased from Sigma (St. Louis, MO, USA); isorhamnetin, ethylgallate and methylgallate from Extrasynthèse (Genay, France); ferulic acid from Koch-Light Laboratories Ltd (Colnbrook, Bucks, England); *p*-coumaric acid, (−)-epicatechin and kaempferol from Fluka (Buchs, Switzerland); sinapic acid, tryptophol and tyrosol from Aldrich (Steinheim, Germany), and morin from Sarshyntex (Merignac, Bordeaux, France). Potassium metabisulphite ($K_2S_2O_5$) was purchased from Panreac Química S.A. (Barcelona, Spain). Lysozyme was purchased from Sigma (St. Louis, MO, USA).

Stock solutions of phenolic compounds, lysozyme and potassium metabisulphite (2 g/L, except for ellagic acid and flavonols, 0.2 g/L) were prepared by dissolving antimicrobial compounds in culture media (MRSE and MLOE, see below).

2.2. Lactic acid bacteria and culture media and growth conditions

The six strains used, *Lactobacillus hilgardii* IFI-CA 49, *Pediococcus pentosaceus* IFI-CA 85, *Oenococcus oeni* IFI-CA 17, *O. oeni* IFI-CA 88, *O. oeni* IFI-CA 91, and *O. oeni* IFI-CA 96, belong to the culture collection of the Institute of Industrial Fermentations (CSIC, Madrid). These strains were previously isolated from red wines at the early phase of MLF, and properly identified by 16S rRNA partial gene sequencing as described by Moreno-Arribas and Polo (2008). Among these six strains, *Lactobacillus hilgardii* IFI-CA 49 was found to be a biogenic-amine-producer strain, being able to generate histamine in culture media (results not published). These strains were kept frozen at −70 °C in a sterilized mixture of culture medium and glycerol (50:50, v/v). MRS culture media (pH 6.2) based on the formula developed by Man et al. (1960) were employed for *L. hilgardii* and *P. Pentosaceus*. They were cultivated for 48 h. The culture media MLO (pH 4.8) developed by Caspritz and Radler (1983) were employed for *O. oeni*. This bacterium was cultivated for 72 h. Both media were purchased from Pronadisa (Madrid, Spain). The culture media containing 6% ethanol (MRSE and MLOE) were prepared by adding ethanol (99.5%, v/v) to the sterilized (121 °C, 15 min) media.

2.3. Antibacterial activity assay

The antibacterial assays were performed using the method of Rojo-Bezares et al. (2007), slightly modified. Initially, 200 μL of either the antimicrobial compound solutions (2, 1, 0.5, 0.25, and 0.125 g/L for the phenolic compounds, except for ellagic acid and flavonols that were 0.2, 0.1, 0.05, 0.025, and 0.0125 g/L; and 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.031 g/L for potassium metabisulphite and lysozyme) or the culture medium (MRSE and MLOE) as controls, were placed into the corresponding wells of the microplate. Then, 20 μL of the diluted strain (inoculum of 1×10^6 cfu/mL) were added to all the microplate wells, including the controls. The final assay volume was 220 μL. The microtiter plates were incubated at 30 °C for 48 h (*L. hilgardii* and *P. pentosaceus*) or 72 h (*O. oeni*). Bacterial growth was determined by reading the absorbance at 550 nm in a PolarStar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) which was controlled by the Fluostar Galaxy software (version 4.11-0). Growth-inhibitory activity was expressed as a mean percentage of growth inhibition with respect to a control without antimicrobial compound. Negligible antimicrobial effects were considered when the growth inhibition percentage was <25% at the maximum concentration tested (2 g/L for all phenolic compounds, except for ellagic acid and flavonols, whose maximum concentration tested was 0.2 g/L). For the active compounds, the survival parameter IC_{50} value was defined as the concentration required to obtain 50% inhibition of growth after 48 (*L. hilgardii* and *P. pentosaceus*) or 72 h (*O. oeni*) of incubation and was estimated by sigmoidal dose–response curve with variable slope using the software package Prism 4 for Windows, version 4.3 (GraphPad Software Inc., www.graphpad.com).

2.4. Electron microscopy

Bacteria incubated with or without the antimicrobial agent for 20 h were fixed on the culture plate with 4% *p*-formaldehyde (Merck, Darmstadt, Germany) and 2% glutaraldehyde (SERVA, Heidelberg, Germany) in 0.05 M cacodylate buffer (pH 7.4) for 120 min at room temperature. Cells were then carefully scraped from the plate, centrifuged at 3000 *g* for 5 min, and the washed pellet post-fixed with 1% OsO_4 and 1% $K_3Fe(CN)_6$ in water for 60 min at 4 °C. Cells were dehydrated with ethanol and embedded in Epon (TAAB 812 resin, TAAB

Laboratories Equipment Limited) according to standard procedures. Ultrathin sections were collected on collodion–carbon-coated copper grids, stained with uranyl acetate and lead citrate and examined at 80 kV in a JEM-1010 (JEOL, Tokyo, Japan) electron microscope. Electron micrographs were recorded at different orders of magnitude.

2.5. Statistical analysis

To examine the relationships between the inhibition effects on the different LAB strains studied, principal component analysis (PCA) (from standardized variables) using the STATISTICA program for Windows, version 7.1 (StatSoft. Inc. 1984–2006, www.statsoft.com) was carried out for data processing. In addition, correlation analysis (Pearson's correlation coefficient) was used to investigate the relationship between the IC_{50} and MBC (minimal concentration that killed over 99.9% of the initial inoculum; García-Ruiz et al., 2009) parameters for *L. hilgardii* IFI-CA 49 and *P. pentosaceus* IFI-CA 85.

3. Results

3.1. Inhibitory effects of wine phenolic compounds

With the exception of morin, the compounds used in this study occur naturally in wine at different concentrations and were chosen because of their different functional group and/or ring substituents in an attempt to relate the phenolic chemical structure to their inhibitory effects on the growth of enological LAB. Within the 18 phenolic compounds tested, ellagic acid, tyrosol, (+)-catechin, (−)-epicatechin and isorhamnetin showed negligible inhibitory effects on the growth of the six LAB strains tested (*L. hilgardii* IFI-CA 49, *P. pentosaceus* IFI-CA 85 and *O. oeni* IFI-CA 17, IFI-CA 88, IFI-CA 91 and IFI-CA 96) (Table 1). Moreover, *L. hilgardii* IFI-CA 49 and *P. pentosaceus* IFI-CA 85 were not susceptible to the action of gallic acid, sinapic acid, tryptophol and myricetin; the *O. oeni* IFI-CA 91 and IFI-CA 96 strains were not susceptible to the action of gallic acid and tryptophol either, and none of the *O. oeni* strains tested were susceptible to the action of kaempferol (Table 1). The IC_{50} parameter was determined for the rest of the compounds and strains (Table 1). In general, flavonols and stilbenes showed the greatest inhibitory effect (lowest IC_{50} values) on the growth of the strains tested (0.160–0.854 for flavonols and 0.307–0.855 g/L for stilbenes). Hydroxycinnamic acids (IC_{50}>0.470 g/L) and hydroxybenzoic acids and esters (IC_{50}>1 g/L) exhibited a medium inhibitory effect, and phenolic alcohols (IC_{50}>2 g/L) and flavanol-3-ols (no effect) showed the lowest effect on the growth of the strains studied. In particular, quercetin showed the greatest inhibitory effect on the growth of the *O. oeni* strains IFI-CA 17 (IC_{50} = 0.148 g/L), IFI-CA 88 (0.267 g/L) and IFI-CA 96 (0.165 g/L); *trans*-resveratrol on the growth of *O. oeni* IFI-CA 91 (0.307 g/L); kaempferol on the growth of *L. hilgardii* IFI-CA 49 (0.160 g/L); and morin on the growth of *P. pentosaceus* IFI-CA 85 (0.212 g/L). Based on their IC_{50} values, some compounds such as ferulic acid seemed to exhibit certain selective inhibition against the *O. oeni* and non-*O. oeni* (*L. hilgardii* and *P. pentosaceus*) strains, their IC_{50} values being at least 2-fold lower for the *O. oeni* than for the non-*O. oeni* strains.

Additionally, IC_{50} values of potassium metabisulphite ($K_2S_2O_5$) and lysozyme were determined following the same procedure as for phenolic compounds. Potassium metabisulphite showed lower values of IC_{50} than lysozyme for all the strains tested (Table 1). The IC_{50} values of potassium metabisulphite for *L. hilgardii* and *P. pentosaceus* were significantly higher than those for *O. oeni*; that is to say, potassium metabisulphite was more toxic for the *O. oeni* strains. The same inhibitory selectivity was also observed for lysozyme, which did not exhibit any inhibitory effect against the *L. hilgardii* and *P. pentosaceus* strains tested. Compared to phenolic compounds, the IC_{50} values of potassium metabisulphite were much lower for the *O. oeni* strains (e.g., around 4-fold lower than those corresponding to quercetin), but higher for the *L. hilgardii* and *P. pentosaceus* strains (e.g., around 2-fold higher than those corresponding to quercetin) (Table 1). With regard to lysozyme, its IC_{50} values for the *O. oeni* strains were higher than those corresponding to most of the phenolic compounds tested – especially flavonols and stilbenes – indicating that lysozyme was less toxic for *O. oeni* than phenolic compounds.

Table 1
IC_{50} data of the phenolic compounds studied against strains of *L. hilgardii*, *P. pentosaceus* and *O. oeni*.

Compounds	IC_{50} (g/L)					
	L. hilgardii IFI-CA 49	*P. pentosaceus* IFI-CA 85	*O. oeni* IFI-CA 17	*O. oeni* IFI-CA 88	*O. oeni* IFI-CA 91	*O. oeni* IFI-CA 96
Hydroxybenzoic acids and esters						
Gallic acid	n.e.	n.e.	3.38	3.20	n.e.	n.e.
Ethyl gallate	2.56	2.89	1.16	1.03	1.87	1.36
Methyl gallate	2.50	3.28	1.51	1.79	2.09	2.22
Ellagic acid	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
Hydroxycinnamic acids						
p-Coumaric acid	1.26	0.994	0.807	1.34	0.818	1.44
Ferulic acid	2.11	1.58	0.475	0.685	0.843	0.590
Caffeic acid	2.03	1.72	1.11	1.13	1.22	1.56
Sinapic acid	n.e.	n.e.	1.42	0.918	0.875	1.27
Phenolic alcohols						
Tryptophol	n.e.	n.e.	2.13	2.05	n.e.	n.e.
Tyrosol	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
Stilbenes						
trans-Resveratrol	0.855	0.715	0.381	0.425	0.307	0.698
Flavan-3-ols						
(+)-Catechin	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
(−)-Epicatechin	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
Flavonols						
Myricetin	n.e.	n.e.	0.471	0.307	0.398	0.854
Morin	0.204	0.212	0.580	0.473	0.689	0.297
Quercetin	0.250	0.300	0.148	0.267	0.454	0.165
Kaempferol	0.160	0.300	n.e.	n.e.	n.e.	n.e.
Isorhamnetin	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
Others						
Potassium metabisulphite	0.536	0.578	0.038	0.066	0.135	0.056
Lysozyme	n.e.	n.e.	3.10	2.36	2.64	3.01

n.e.: no effect.

3.2. Statistical analysis of inhibitory activities

PCA was used to examine the relationship between the inhibitory effects of the antimicrobial compounds and the different enological lactic acid bacteria. Two principal components were obtained and explained 96% of the total variation. The first principal component (PC1, 89% of the total variance) was negatively correlated with the IC_{50} values for *L. hilgardii* IFI-CA 49 (−0.91), *P. pentosaceus* IFI-CA 85 (−0.94), and *O. oeni* IFI-CA 17 (−0.97), IFI-CA 88 (−0.93), IFI-CA 91 (−0.96) and IFI-CA 96 (−0.95). The second principal component (PC2, 7% of the total variance) was not correlated with the IC_{50} values for any of the bacteria tested. The scores of the antimicrobial compounds and the loadings of the IC_{50} values for the different bacteria were plotted as a bi-plot in the plane defined by the first two principal components (Fig. 1). A certain grouping was observed with the phenolic compounds according to their chemical structure. The hydroxybenzoic derivatives (methyl and ethyl gallates) were located on the left side of the plot (low values of PC1); these compounds had high IC_{50} values for all the strains tested. Hydroxycinnamic acids (*p*-coumaric, ferulic and caffeic acids) were located in the central part of the plot (medium values of PC1), which corresponded to medium inhibitory effects on the growth of the bacteria tested. The phenolic compounds quercetin, morin and *trans*-resveratrol, together with potassium metabisulphite, were located on the right side of the plot (high values for PC1), indicating that these compounds showed low IC_{50} values for all

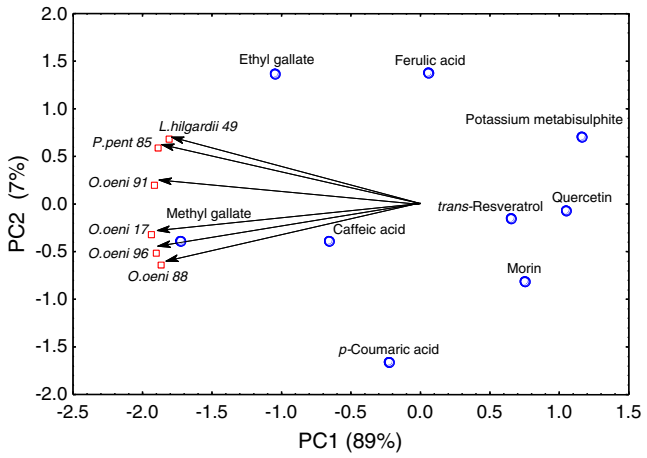

Fig. 1. Plot of the active compounds (ethyl gallate, methyl gallate, *p*-coumaric acid, ferulic acid, caffeic acid, *trans*-resveratrol, morin, quercetin and potassium metabisulphite) and the loadings of the micro-organisms in the plane defined by the first two principal components.

the bacteria tested (Fig. 1). On the other hand, the *L. hilgardii* IFI-CA 49 and *P. pentosaceus* IFI-CA 85 strains showed a similar susceptibility pattern in their response to antimicrobial compounds, as they were closely located in the plot; the *O. oeni* strains were slightly spread towards PC2, and not far from the non-*O. oeni* strains (Fig. 1).

3.3. Comparison between inhibition parameters

In a previous study, the inhibitory effects of wine phenolic compounds on *L. hilgardii* IFI-CA 49 and *P. pentosaceus* IFI-CA 85 were studied by measuring their ability to inactivate the micro-organisms through survival parameters such as MIC (smallest concentration needed to reduce by 10–50 times the population of micro-organisms of the initial inoculum, log (No/Nf) = 1–1.7) and MBC (minimal concentration that killed over 99.9% of the initial inoculum) (García-Ruiz et al., 2009). In order to compare the results obtained in this previous study with those obtained in the present study, a correlation analysis was carried out between the IC_{50} values (Table 1) and the MBC values (García-Ruiz et al., 2009) of the common phenolic compounds active against the two *L. hilgardii* and *P. pentosaceus* strains. Linear and positive correlation was obtained for both *L. hilgardii* IFI-CA 49 ($r = 0.8722$, $P = 0.0105$) and *P. pentosaceus* IFI-CA 85 ($r = 0.9099$, $P = 0.0017$) (Fig. 2), indicating that both evaluation approaches (i.e., inactivation of bacteria through the MBC parameter, and inhibition of bacterial growth through the IC_{50} values) led to similar results in the study of the inhibitory effects of the different wine phenolic compounds on these two enological LAB strains. From our own experience, we concluded that methodologies for evaluating the inhibitory potential of antimicrobial compounds based on absorbance measurements may be quicker and more feasible than those based on colony counting, although attention should be paid to work protocols in order to avoid contamination and to ensure pure bacteria growth.

3.4. Microscopy study

In order to investigate possible changes in cell morphology after incubation of the LAB with antimicrobial agents, the scanning electron microscopy technique was applied. For example, Fig. 3 displays the micrographs of *O. oeni* IFI-CA 96 cells incubated with potassium metabisulphite and some active phenolic compounds of different chemical structures (ethyl gallate, ferulic acid and *trans*-resveratrol) at a concentration of 2 g/L. In all cases, damage to the cell membrane integrity was observed when compared to the control. Incubation with the antimicrobial agents produced a breakdown of the cell membrane and the subsequent release of the cytoplasm material into the medium. Moreover, the proportion of damaged cells seemed to be proportional to the inhibitory potential of the antimicrobial agents: potassium metabisulphite ($IC_{50} = 0.056$ g/L) ≫ ferulic acid (0.590 g/L) ≥ *trans*-resveratrol (0.698 g/L) > ethyl gallate (1.36 g/L) (Table 1).

4. Discussion

Knowledge about the inhibitory action of phenolic compounds on the growth of enological LAB is important in the control of the progress of malolactic fermentation during winemaking, which is known to be affected by the phenolic content and composition of wines, and also in the development of new alternatives to the use of sulphites in enology based on "natural antimicrobial agents" such as plant polyphenols. From the previous data reported in the literature (Bloem et al., 2007; Campos et al., 2003; Figueiredo et al., 2008; García-Ruiz et al., 2009; Landete et al., 2007; Reguant et al., 2000; Salih et al., 2000; Stead, 1993; Theobald et al., 2008; Vivas et al., 1997), this study has expanded the number and type of phenolic compounds tested (a total of 18 compounds corresponding to hydroxybenzoic acids and their derivatives, hydroxycinnamic acids, phenolic alcohols and other related compounds, stilbenes, flavan-3-ols and flavonols) against different enological LAB strains (*O. oeni*, n = 4; *L. hilgardii*, n = 1; and *P. pentosaceus*, n = 1), which has allowed us to better confirm statements about the influence of phenolic chemical structure and bacteria species on the inhibition of LAB growth by wine phenolics. Another contribution of this study is the determination of inhibition parameters (i.e., IC_{50}) for the different compounds tested, allowing a better comparison between chemicals, bacteria species, conditions, etc.,

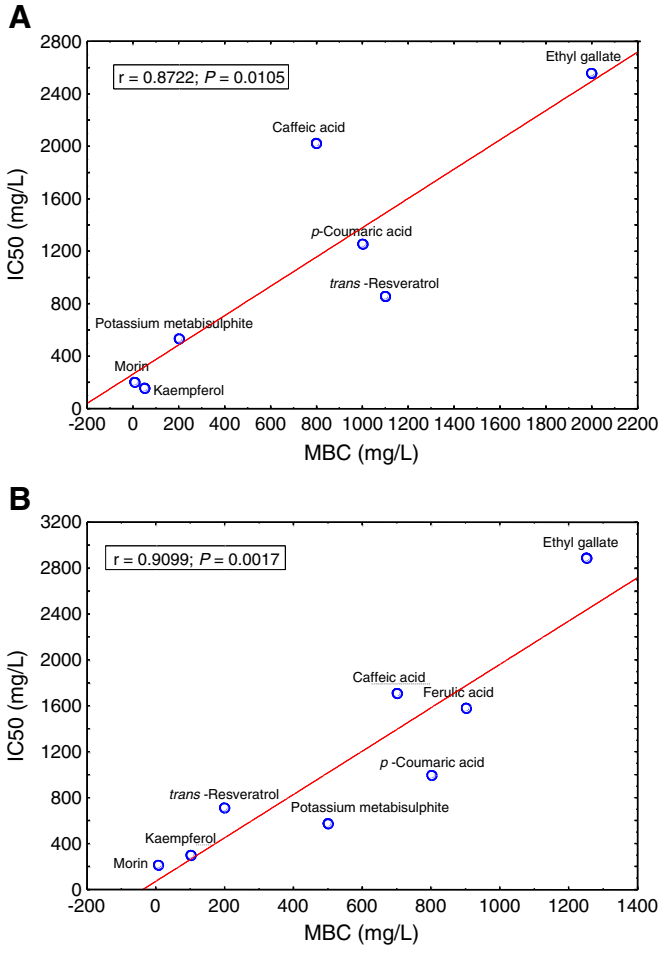

Fig. 2. Linear correlation between IC_{50} and MBC data for *L. hilgardii* IFI-CA 49 (A) and *P. pentosaceus* IFI-CA 85 (B).

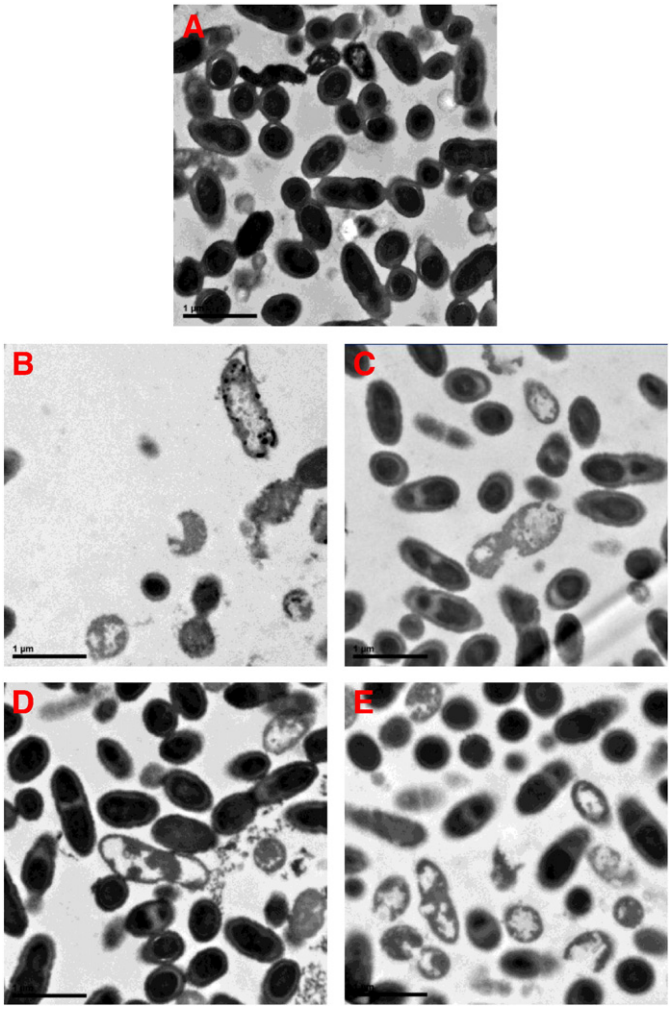

Fig. 3. Electron micrographs of ultrathin sections of *O. oeni* IFI-CA 96 non-incubated and incubated with antimicrobial agents (2 g/L). A: control, B: incubation with potassium metabisulphite, C: incubation with ethyl gallate, D: incubation with ferulic acid, E: incubation with *trans*-resveratrol. Bars = 1 μm.

as well as a more accurate assessment of the effects of these compounds on the growth of LAB during winemaking. With the exception of the studies by Landete et al. (2007) and García-Ruiz et al. (2009), which determined MIC and MBC values, previous studies refer to growth inhibition percentages at certain phenolic concentrations, which makes comparison between them rather difficult.

The results reported in this paper confirm that the antimicrobial activity of wine phenolic compounds against *O. oeni*, *L. hilgardii* and *P. pentosaceus* was strongly dependent on phenolic structure. Differences in the IC_{50} values among the wine phenolic compounds tested were at least of the one-magnitude order for any of the six LAB strains studied (e.g., from 0.160 to 2.56 g/L for *L. hilgardii* IFI-CA 49, Table 1). In general, the inhibitory potential followed the order: flavonols > stilbenes > hydroxycinnamic acids > hydroxybenzoic acids and esters > phenolic alcohols ≫ flavanol-3-ols (no effect), although substituents influenced the inhibitory potential in different ways, depending on the strain. For example, for flavonols, the most active B-ring substitution was 3,4-dihydroxy (quercetin) for the *O. oeni* strains IFI-CA 17, IFI-CA 88 and IFI-CA 96; 3,4,5-trihydroxy (myricetin) for *O. oeni* IFI-CA 91 (0.307 g/L); 4-hydroxy (kaempferol) for *L. hilgardii* IFI-CA 49; and 2,4-dihydroxy (morin) for *P. pentosaceus* IFI-CA 85. With regard to stilbenes, *trans*-resveratrol was one of the phenolic compounds with major antimicrobial activity against *O. oeni*, *P. pentosaceus and L. hilgardii*. With regard to hydroxycinnamic acids, and for the *L. hilgardii* and *P. pentosaceus* strains, the order of activity was: *p*-coumaric acid > ferulic acid ≥ caffeic acid ≫ sinapic acid, which agreed with previous results reported for other LAB species (Landete et al., 2007; Reguant et al., 2000; Stead, 1993). However, there was not a common trend for the *O. oeni* strains, which prevented us from establishing a general structure–activity relationship for hydroxycinnamic acids. On the other hand, the inhibitory potency of hydroxycinnamic acids was greater than that of hydroxybenzoic acid (i.e., gallic acid), as reported by other authors (Campos et al., 2003). Methylation or ethylation of gallic acid (i.e., ethyl and methyl gallates, respectively) slightly increased its inactivation potential against all the species tested, which is in contrast to the results of Landete et al. (2007) for lactobacilli. The flavan-3-ols tested ((+)-catechin and (−)-epicatechin) seemed not to exert any effects on the growth of *O. oeni*, *P. pentosaceus* and *L. hilgardii*, which agreed with the results reported by Reguant et al. (2000) for *O. oeni*, and others for a number of wine LAB species (Figueiredo et al., 2008; Rodríguez et al., 2009; Diez et al., 2010).

Focussing only on hydroxycinnamic and hydroxybenzoic acids, Campos et al. (2003) found that *O. oeni* seemed to be more susceptible to phenolic inactivation than *L. hilgardii*. In the same way, Figueiredo et al. (2008) reported that phenolic aldehydes, flavonoids and tannins were more inhibitory for *O. oeni* than for *L. hilgardii*. For our comparative study of the *O. oeni* (n = 4) and non-*O. oeni* (*L. hilgardii* and *P. pentosaceus*, n = 2) strains and 18 phenolic compounds, we found slight differences in bacteria susceptibility to wine polyphenols, depending on the type of phenolics considered. This was also confirmed by the PCA whose bi-plot showed certain groupings according to their chemical structure (Fig. 1). In contrast, the representation of the loadings of the IC_{50} values for the different bacteria was spread across a small area (Fig. 1), indicating a quite similar susceptibility pattern among the different strains studied in their response to antimicrobial compounds.

The IC_{50} values found in our antimicrobial assay for potassium metabisulphite ($K_2S_2O_5$), the additive most usually used in winemaking because of its antioxidant and selective antibacterial effects, were in the ranges of those reported by Rojo-Bezares et al. (2007) for other wine LAB strains. The susceptibility of the species to potassium metabisulphite was in the order: *O. oeni* ≫ *L. hilgardii* > *P. pentosaceus*, the IC_{50} values corresponding to the *O. oeni* strains around one-magnitude order higher than those corresponding to the non-*O. oeni* studied. This was in agreement with previously reported data (Rojo-Bezares et al., 2007). The other additive tested, lysozyme, was only effective against *O. oeni* but not against *L. hilgardii* and *P. pentosaceus*, which agreed with the results reported by Delfini et al. (2004). In the comparison of the IC_{50} data, *O. oeni* was considerably more susceptible to the action of potassium metabisulphite than to wine phenolic compounds (10-fold higher IC_{50} values), whereas some phenolic compounds can be as effective as this additive in the inhibition of the growth of *L. hilgardii* and *P. pentosaceus*, confirming the potential of phenolic compounds as a good alternative to sulphites in winemaking (Bartowsky, 2009; García-Ruiz et al., 2008).

In a previous study (García-Ruiz et al., 2009), we showed that incubation of *P. pentosaceus* IFI-CA 85 with kaempferol produced a breakdown of the cell membrane and the subsequent release of cytoplasm material into the medium. The same effects were reported in this paper for *O. oeni* IFI-CA 96 in the presence of other wine phenolic compounds, such as ethyl gallate, ferulic acid and *trans*-resveratrol, which confirmed similar mechanisms of membrane disruption. Incubation with potassium metabisulphite also produced a breakdown of the cell membranes of *O. oeni* IFI-CA 96. However, in the previous study with *P. pentosaceus* IFI-CA 85, the membranes of the cells from the incubation with potassium metabisulphite were complete, with the cytoplasm being intact and homogeneously distributed (García-Ruiz et al., 2009). This was explained by the greater susceptibility of *O. oeni* IFI-CA 96 to potassium metabisulphite in comparison to *P. pentosaceus* IFI-CA 85, as was reflected in their IC_{50} values.

In conclusion, these results show that the antimicrobial properties of wine phenolic compounds against *O. oeni*, *P. pentosaceus* and *L. hilgardii*

were conditioned not only by the phenolic type (hydroxybenzoic and hydroxycinnamic acids, phenolic alcohols, stilbenes, flavan-3-ols and flavonols) but also by the substituents of the phenolic chemical structure. Regarding species susceptibility, slight differences were observed between the response of the *O. oeni* and non-*O. oeni* strains to the action of the majority of the wine phenolics tested. This is in contrast to what was observed for potassium metabisulphite, which was more effective for *O. oeni* – the major bacteria species conducting MFL – than for *L. hilgardii* and *P. pentosaceus*, considered to be wine spoilage species. Bearing this in mind, our next goal will be to evaluate the inhibitory effects of plant phenolic extracts, potentially applicable as an alternative to sulphites, on the growth of enological LAB. In comparison to potassium metabisulphite, the application of these extracts may improve strain selection in favour of desirable LAB during winemaking. But in any case, further studies are required in order to assess the impact of this application on the sensory properties of wine.

Acknowledgments

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IV.2. Potencial de bacterias lácticas para degradar aminas biógenas. Influencia de los polifenoles del vino.

Los compuestos fenólicos del vino no sólo inhiben el crecimiento de las BAL – como se ha demostrado en la sección VI.1.-, sino que también pueden modificar el metabolismo bacteriano. Aunque los estudios son escasos, en cepas de *O. oeni*, se había observado, por ejemplo, que el metabolismo de azúcares y ácido málico se favorecía en la presencia de polifenoles del vino, en concentraciones relativamente bajas (Vivas y col. 2000; Alberto y col. 2001; Rozès y col. 2003).

Por otro lado, las aminas biógenas son compuestos potencialmente tóxicos que pueden aparecer en el vino, debido fundamentalmente a la acción de BAL con actividad aminoácido descarboxilasa (Moreno-Arribas y col., 2000; Marcobal y col., 2006). Como estrategias posibles para reducir/eliminar la presencia de aminas biógenas en otros alimentos, se ha descrito el potencial de degradación de estos compuestos por parte de cepas de *Micrococcus varians* (Leuschner y col., 1998) y *Staphylococcus xylosus* (Martuscelli y col., 2000; Gardini y col., 2002) aisladas de embutidos, así como por parte de cultivos de BAL iniciadores en el ensilaje de pescado (*Lactobacillus curvatus* y *Lactobacillus sakei*) (Enes-Dapkevicius y col., 2000), y en productos lácteos (Voigt y Eitenmiller, 1978) y cárnicos (Fadda y col., 2001). No obstante, hasta la fecha de este estudio, no conocíamos ningún trabajo que hubiera investigado la posibilidad de que microorganismos de origen vínico fueran capaces de degradar aminas biógenas.

Por tanto, el objetivo planteado fue doble: por un lado, realizar un "*screening*" de cepas de BAL aisladas de diferentes nichos enológicos con capacidad para degradar aminas biógenas, y por otro lado, evaluar el efecto de los polifenoles en este metabolismo degradativo de aminas por parte de las bacterias lácticas, en comparación con otros antimicrobianos como etanol y SO2, también presentes en el vino.

En el planteamiento experimental, se persiguió llevar a cabo un "*screening*" lo más amplio posible, incluyendo finalmente hasta 85 cepas de BAL aisladas de vinos y otros ecosistemas pertenecientes a las especies *O. oeni*, *Pediococcus parvulus*, *P. pentosaceus*, *Lactobacillus plantarum*, *L. hilgardii*, *L. zeae*, *L. casei*, *L. paracasei*, y *Leuconostoc mesenteroides*, así como cultivos iniciadores comerciales (n=3) y cepas tipo (n=2). Se probó su capacidad degradativa de aminas frente a histamina, tiramina y putrescina, ya que son las aminas encontradas en vinos con más frecuencia (Marcobal y col., 2006).

Una vez que se comprobó que, efectivamente, algunas cepas de BAL de origen enológico eran capaces de degradar aminas biógenas, tanto en medios de cultivo como en el propio medio del vino, se eligió una de las más activas (*L. casei* IFI-CA 52) para estudiar el efecto de los polifenoles y otros antimicrobianos presentes en el vino en esta actividad metabólica. Como material de referencia para este estudio, se eligió el extracto de vino Provinols™ (Seppic, France), previamente testado y caracterizado por nuestro grupo (Sánchez-Patán y col., 2012).

A continuación se presentan los resultados de este estudio en forma de una publicación:

Publicación III. Potencial degradación de aminas biógenas asociada a bacterias lácticas del vino.

<u>Publicación III.</u> Potencial degradación de aminas biógenas asociada a bacterias lácticas del vino.

Almudena García-Ruiz, Eva M. González-Rompinelli, Begoña Bartolomé, M. Victoria Moreno-Arribas. Potential of wine-associated lactic acid bacteria to degrade biogenic amines. *International Journal of Food Microbiology,* **2011**, 148: 115–120.

Resumen:

Se ha demostrado que algunas bacterias lácticas (BAL) aisladas de alimentos fermentados degradan aminas biógenas mediante la producción de enzimas amino-oxidasa. Como consecuencia del poco conocimiento sobre esta propiedad en microorganismos del vino, en el presente trabajo se evaluó la capacidad para degradar histamina, tirosina y putrescina de cepas de BAL (n=85) aisladas del vino y otros nichos ecológicos relacionados, así como la de cultivos iniciadores de la fermentación maloláctica (n=3) y de cepas tipo (n=2). La capacidad de degradar aminas biógenas de estas cepas se determinó por RP-HPLC, tras experimentos en medio de cultivo y fermentaciones malolácticas realizadas a escala de laboratorio. Aunque en diferente grado, el 25% de las cepas aisladas fueron capaces de degradar histamina, el 18% de degradar tiramina y otro 18% de degradar putrescina, mientras que ninguno de los cultivos iniciadores de fermentación maloláctica o cepas tipo fueron capaces de degradar alguna de las aminas ensayadas. Nueve cepas pertenecientes a los géneros *Lactobacillus* y *Pediococcus* mostraron la mayor capacidad amino-degradativa, siendo la mayoría de ellas capaces de degradar de forma simultánea al menos dos de las tres aminas biógenas a estudio. Experimentos realizados con una de las cepas con mayor capacidad amino-degradativa (*L. casei* IFI-CA 52) revelaron que los extractos libres de células mantienen dicha capacidad en comparación con sus suspensiones celulares a pH 4.6, lo que indicaba que las enzimas amino-degradativas fueron extraídas con éxito de las células y su actividad óptima para la degradación de aminas biógenas. Además, se confirmó que componentes del vino como el etanol (12%) y los polifenoles (75 mg / L), y aditivos enológicos como el SO_2 (30mg/L), reducen la capacidad de degradar histamina a pH 4.6 de la cepa *L. casei* IFI-CA 52 en un 80%, 85% y 11% respectivamente, en suspensiones celulares y del 91%, 67% y 50%, respectivamente, en los extractos libres de células. A pesar de esta influencia negativa de la matriz del vino, nuestros resultados demuestran el potencial de las BAL enológicas como una estrategia prometedora para reducir las aminas biógenas en el vino.

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Potential of wine-associated lactic acid bacteria to degrade biogenic amines

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ABSTRACT

Some lactic acid bacteria (LAB) isolated from fermented foods have been proven to degrade biogenic amines through the production of amine oxidase enzymes. Since little is known about this in relation to wine microorganisms, this work examined the ability of LAB strains (n = 85) isolated from wines and other related enological sources, as well as commercial malolactic starter cultures (n = 3) and type strains (n = 2), to degrade histamine, tyramine and putrescine. The biogenic amine-degrading ability of the strains was evaluated by RP-HPLC in culture media and wine malolactic fermentation laboratory experiments. Although at different extent, 25% of the LAB isolates were able to degrade histamine, 18% tyramine and 18% putrescine, whereas none of the commercial malolactic starter cultures or type strains were able to degrade any of the tested amines. The greatest biogenic amine-degrading ability was exhibited by 9 strains belonging to the *Lactobacillus* and *Pediococcus* groups, and most of them were able to simultaneously degrade at least two of the three studied biogenic amines. Further experiments with one of the strains that showed high biogenic amine-degrading ability (*L. casei* IFI-CA 52) revealed that cell-free extracts maintained this ability in comparison to the cell suspensions at pH 4.6, indicating that amine-degrading enzymes were effectively extracted from the cells and their action was optimal in the degradation of biogenic amines. In addition, it was confirmed that wine components such as ethanol (12%) and polyphenols (75 mg/L), and wine additives such as SO_2 (30 mg/L), reduced the histamine-degrading ability of *L. casei* IFI-CA 52 at pH 4.6 by 80%, 85% and 11%, respectively, in cell suspensions, whereas the reduction was 91%, 67% and 50%, respectively, in cell-free extracts. In spite of this adverse influence of the wine matrix, our results proved the potential of wine-associated LAB as a promising strategy to reduce biogenic amines in wine.

1. Introduction

Biogenic amines are a group of biologically active compounds that are widespread in nature. The term 'amine' is used for basic nitrogenous compounds of low molecular weight that are produced within the normal metabolism of humans, animals, plants and microorganisms. In foods and beverages, biogenic amines are formed mainly by the decarboxylation of the corresponding precursor amino acids. This reaction is catalysed by substrate-specific enzymes, decarboxylases, of the microbiota of the food or wine environment.

Some biogenic amines such as histamine, tyramine, putrescine and cadaverine are important for their physiological and toxicological effects on the human body. They may exert either psychoactive or vasoactive effects on sensitive humans. Histamine has been found to cause the most frequent food-borne intoxications associated with biogenic amines; it acts as a mediator and is involved in pathophysiological processes such as allergies and inflammations (Gonzaga et al., 2009). Tyramine can evoke nausea, vomiting, migraine, hypertension and headaches (Shalaby, 1996). Putrescine and cadaverine can increase the negative effect of other amines by interfering with detoxification enzymes that metabolize them (Stratton et al., 1991).

To exhibit these harmful effects the amines need to gain access to the bloodstream. But the existence of a fairly efficient detoxification system in the intestinal tract of mammals prevents biogenic amines from reaching the bloodstream (Taylor, 1985), so they usually do not represent any health hazard to individuals. One of the main detoxification systems is composed of two distinct enzymes, monoamine oxidase (MAO) and diamine oxidase (DAO) (Ten Brink et al., 1990). Mono- and diamine oxidases are present in eukaryotes and have also been described for fungi (i.e. *Aspergillus niger*) (Frébort et al., 2000) and bacteria (Voigt and Eitenmiller, 1978; Murooka et al., 1979; Ishizuka et al., 1993; Yamashita et al., 1993). These enzymes convert amines into non-toxic products, which are further excreted out of the organism.

The main biogenic amines associated with wine are histamine, tyramine and putrescine (Marcobal et al., 2006; Ferreira and Pinho, 2006; Ancín-Azpilicueta et al., 2008; Smit et al., 2008). Their presence in wine is considered as marker molecules of quality loss, and some European countries even have recommendations for the amount of histamine acceptable in wine which impacts on the import and export of wines to these countries. Most fermented foods, such as cheese, fermented sausages and beer, which are consumed more frequently

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than wines, have higher biogenic amine content (Stratton et al., 1991; Izquierdo-Pulido et al., 2000; Fernández et al., 2007). However, the presence of alcohol in wine may enhance the activity of amines because it inhibits monoamine oxidase enzymes (Ten Brink et al., 1990).

The origin of biogenic amines in wines is well documented (Lonvaud-Funel, 2001; Constantini et al., 2009). They are generated either as the result of endogenous decarboxylase-positive micro-organisms in grapes or by the growth of contaminating decarboxylase-positive micro-organisms in the wine (Halász et al., 1994). With regards to wine micro-organisms, a large amount of literature is available on the production of biogenic amines. Several research groups support the view that biogenic amines are formed in winemaking mainly by lactic acid bacteria (LAB) due to the decarboxilation of free amino acids (Coton et al., 1998; Lonvaud-Funel and Joyeux, 1994; Moreno-Arribas et al., 2000; Guerrini et al., 2002; Landete et al., 2005; Constantini et al., 2006; Lucas et al., 2008). It has been reported that during wine storage and ageing, biogenic amine (i.e. histamine and tyramine) concentrations undergo few variations, being observed as a slight decrease of these compounds during the ageing process in oak barrels (Jiménez-Moreno et al., 2003). This might be due to the action of amine oxidase enzymes present in the wines (Ancín-Azpilicueta et al., 2008) although this hypothesis remains to be demonstrated, and to this date no studies have been reported in the literature concerning the degradation of biogenic amines by wine-associated micro-organisms. However, the biogenic amine-degrading ability has been investigated in species such as *Micrococcus varians* (Leuschner et al., 1998) and *Staphylococcus xylosus* (Martuscelli et al., 2000; Gardini et al., 2002) isolated from sausages, in LAB starters from fish silage (*Lactobacillus curvatus* and *Lactobacillus sakei*) (Enes-Dapkevicius et al., 2000), and in dairy (Voigt and Eitenmiller, 1978) and meat (Fadda et al., 2001) products.

The aim of the present paper was to explore the ability of lactic acid bacteria isolated from wines and other related ecosystems to degrade histamine, tyramine and putrescine, which are considered to be the main biogenic amines present in wines. Initially, the ability of a large number of wine-associated LAB strains to degrade biogenic amines was evaluated in culture media and, for the most active strains, their biogenic amine-degrading ability was confirmed in malolactic fermentation experiments. To gain a deeper insight into the biogenic amine-degrading activity exhibit by LAB, and for one of the most active strains (*L. casei* IFI-CA 52), experiments were conducted to show if cell-free extracts were as effective as the whole cells in the degradation of histamine. Finally, the influence of wine components such as ethanol and polyphenols, and wine additives, such as SO_2, on the histamine-degrading activity of *L. casei* IFI-CA 52, was evaluated in both cell-free extracts and cell suspensions.

Table 1
Lactic acid bacteria used in this study[a].

	Species	Source
Isolated strains		
IFI-CA 2, IFI-CA 3, IFI-CA 4, IFI-CA 5, IFI-CA 6, IFI-CA 8, IFI-CA 10, IFI-CA 32, IFI-CA 45	*Oenococcus oeni*	Fermentation lees
IFI-CA 11, IFI-CA 12, IFI-CA 13, IFI-CA 14, IFI-CA 15, IFI-CA 17, IFI-CA 20, IFI-CA 21, IFI-CA 22, IFI-CA 27, IFI-CA 28, IFI-CA 33, IFI-CA 34, IFI-CA 35, IFI-CA 36, IFI-CA 37, IFI-CA 38, IFI-CA 40, IFI-CA 42, IFI-CA 44, IFI-CA 46, IFI-CA 47, IFI-CA 56, IFI-CA 58, IFI-CA 59	*Oenococcus oeni*	Young wine/grape must
IFI-CA 60, IFI-CA 79, IFI-CA 81, IFI-CA 82, IFI-CA 96, IFI-CA 100, IFI-CA 101, IFI-CA 102	*Oenococcus oeni*	Oak barrel-aged wines
IFI-CA 19, IFI-CA 23, IFI-CA 24, IFI-CA 29, IFI-CA 31, IFI-CA 57, IFI-CA 97	*Pediococcus parvulus*	Young wine/grape must
IFI-CA 30, IFI-CA 83, IFI-CA 85	*Pediococcus pentosaceus*	Oak barrel-aged wines
IFI-CA 86	*Pediococcus pentosaceus*	Biologically aged sherry wines
IFI-CA 7, IFI-CA 54, IFI-CA 78, IFI-CA 80, IFI-CA 92	*Lactobacillus plantarum*	Young wine/grape must
IFI-CA 26	*Lactobacillus plantarum*	Fermentation lees
IFI-CA 16, IFI-CA 25, IFI-CA 49, IFI-CA 53, IFI-CA 79, IFI-CA 95,	*Lactobacillus hilgardii*	Young wine/grape must
IFI-CA 41, IFI-CA 108, IFI-CA 111	*Lactobacillus hilgardii*	Biologically aged sherry wines
IFI-CA 50, IFI-CA 131, IFI-CA 140	*Lactobacillus zeae*	Biologically aged sherry wines
IFI-CA 78, IFI-CA 93	*Lactobacillus casei*	Young wine/grape must
IFI-CA 51, IFI-CA 52, IFI-CA 69, FI-CA 115, IFI-CA 124,	*Lactobacillus casei*	Biologically aged sherry wines
IFI-CA 18, IFI-CA 94	*Lactobacillus paracasei*	Young wine/grape must
IFI-CA 125, IFI-CA 136, IFI-CA 137	*Lactobacillus paracasei*	Biologically aged sherry wines
IFI-CA 141, IFI-CA 156	*Leuconostoc mesenteroides*	Biologically aged sherry wines
Commercial malolactic starters		
Uvaferm ALPHA	*Oenococcus oeni*	Lallemand
Viniflora OENOS,	*Oenococcus oeni*	Christian Hansen
Viniferm Oeno 104	*Oenococcus oeni*	Agrovín
Type strains		
30a (ATCC 33222)	*Lactobacillus* sp.	ATCC
CECT 5354 (ATCC 367)	*Lactobacillus brevis*	CECT

[a] ATCC, American Type Culture Collection; CECT, Colección Española de Cultivos Tipo.

2. Materials and methods

2.1. Lactic acid bacteria strains, culture media and growth conditions

Table 1 shows the species and origin of all the strains used in this study. A total of 85 LAB, including *Oenococcus oeni* (42 strains), *Pediococcus parvulus* (7 strains), *P. pentosaceus* (4 strains), *Lactobacillus plantarum* (6 strains), *L. hilgardii* (9 strains), *L. zeae* (3 strains), *L. casei* (7 strains), *L. paracasei* (5 strains) and *Leuconostoc mesenteroides* (2 strains) were used in this study. These strains belong to the bacterial culture collection of the Institute of Industrial Fermentations (IFI), CSIC, Spain. They were previously isolated in our laboratory from musts and wines (young, wood-aged and biologically aged sherry wines) and from winemaking products (fermentation lees) over an 8-year period and properly identified by 16S rRNA partial gene sequencing as described by Moreno-Arribas and Polo (2008). Three *O. oeni* strains isolated from commercial malolactic starter preparations (Uvaferm ALPHA, Viniflora OENOS and Viniferm Oeno 104) that were kindly provided by Lallemand (Ontario, Canada), Christian Hansen (Hørsholm, Denmark) and Agrovín (Alcázar de San Juan, Ciudad Real, Spain) were also used. Additionally, the positive reference biogenic amine producers *Lactobacillus* 30a – a histamine – (Valler et al., 1982) and putrescine-producing (Guirard and Snell, 1980) strain from the American Type Culture Collection in Manassas, Va. (ATCC 33222) – and *L. brevis* CECT 5354 – a tyramine-producing strain (Moreno-Arribas and Lonvaud-Funel, 1999) from the Colección Española de Cultivos Tipo (CECT) – were also included in this study.

These strains were kept frozen at −70 °C in a sterilized mixture of culture medium and glycerol (50:50, v/v). MRS culture media (pH 6.2) based on the formula developed by Man et al. (1960) was employed for *Lactobacillus*, *Pediococcus* and *Leuconostoc*. They were cultivated for 24–48 h. The culture media MLO (pH 4.8) developed by Caspritz and Radler (1983) was employed for *O. oeni*. These bacteria were

cultivated for 3–4 days. Both media were purchased from Pronadisa (Madrid, Spain). All bacteria were incubated at 30 °C.

2.2. Determination of the ability of lactic acid bacteria to degrade biogenic amines

The ability of wine LAB strains to degrade the biogenic amines histamine, tyramine and putrescine was tested in a model system similar to that previously described for other LAB by Enes-Dapkevicius et al. (2000). The broth consisted of MRS or MLO added separately of 0.15 g/L of each amine – histamine dihydrochloride, tyramine or 1,4-diaminobutane dihydrochloride or putrescine – and adjusted to pH 5.5. LAB strains were incubated at 30 °C in this model system in duplicate and on at least two different days. Samples were taken at time 0 and after 48 (LAB non *O. oeni*)–72 (*O. oeni*) hours of incubation.

Additionally, some LAB strains were tested for their potential to degrade histamine, tyramine and putrescine during MLF in a laboratory experiment using a Tempranillo red wine. LAB were cultured and grown on MRS and MLO at 30 °C and 5×10^7 ufc/mL were inoculated into the wine previously enriched with malic acid (2 g/L) and contaminated with histamine (28 mg/L), tyramine (12 mg/L) and putrescine (36 mg/L). The biogenic amines were purchased from (Fluka, Buchs,Switherland). Malolactic fermentation was monitored by the determination of the malic acid concentration of wines using a Malic acid Kit (Megazyme International Ireland Ltd., Bray, Co. Wicklow, Ireland). Biogenic amine degradation was determined by quantitative RP-HPLC analysis, as indicated below.

2.3. Determination of lactic acid bacteria biogenic amine producers

Strains were subcultured at 30 °C in MRS broth for *Lactobacillus* sp., *Pediococcus* and *Leuconostoc*, and MLO broth for *O. oeni*, both of which contained 0.1% of the corresponding amino acid precursor (L-histidine monohydrochlorid, tyrosine di-sodium salt and L-ornithine monohydrochloride), pyridoxal-5′-phosphate (Sigma, St Louis, MO, USA) and growing factors, previously described in Moreno-Arribas et al. (2003). The pH was adjusted to 5.3 and the medium was autoclaved. The precursor amino acids were purchased from Sigma (St. Louis, MO, USA). The ability of bacterial isolates to produce amines (histamine, tyramine and putrescine) was tested by Multiplex PCR, according to Marcobal et al. (2005) and Constantini et al. (2006), and HPLC.

2.4. Influence of wine matrix on the degradation of histamine by L. casei IFI-CA 52 cell- free extracts and whole cells

Two days worth of cultures of the *L. casei* IFI-CA 52 strain, which reached an optical density at 600 nm (Beckman Coulter, DU 800 spectophotometer, Brea, USA) of 2.0, were recovered by centrifugation (3000 *g* for 10 min at 4 °C) using a 3744R Falcon refrigerated centrifuge (Heraeus Sepatech, Biofuge 22R, Hanau, Germany). The cell pellet was washed twice with 0.05 M sodium phosphate buffer (pH 7.0) and suspended in 5 mL of the same buffer. The bacterial suspension was homogenized and the cells were disrupted using an ultrasonic disintegrator (Branson, Digital Sonifier, Danbury, USA) at 150 W, 10×30 s with 30 s of pause, supplied with a thermostatic bath (4 °C). The cell-free extract was separated from the bacterial debris by centrifuging at 14,000 *g* for 15 min at 4 °C.

For the study of the influence of wine components (ethanol and polyphenols) and wine additives (SO_2) on the biogenic amine-degrading ability of *L. casei* IFI-CA 52, the assay mixture contained: cell-free extracts or whole cells, the substrate (histamine dihydrochloride (Fluka, Buchs, Switherland), 50 mg/L) and the buffer to a 2.0 mL final volume. After overnight incubation at 30 °C, the reaction was stopped by the addition of 1 mL hydrochloric acid (HCl) 1 M, and the histamine-degrading activity was determined by HPLC.

For the determination of the optimal pH, 10 mM phosphate buffer pH 7.0 or 10 mM sodium acetate buffer pH 4.6 was used. For the study of the influence of wine components and additives on amine degradation, ethanol (Panreac Química S.A.U., Barcelona, Spain) (12%, final concentration), potassium metabisulphite (Panreac Química S.A., Barcelona, Spain) (30 mg/L) and the commercial wine extract Provinols™ (Seppic, France) (75 and 660 mg/L) were used. The concentrations for the wine extract were selected on the basis of the information provided by the manufacturers (100 mg of Provinols™ corresponds to the polyphenol content of one glass of red wine, 150 mL). Stock solutions of wine extract were prepared beforehand, dissolving the powder in distilled water or in the mixture solution. All the results are the means of three experiments.

2.5. Analysis of biogenic amines

Biogenic amines were analyzed by reversed-phase (RP)-HPLC according to the method described by Marcobal et al. (2005). Briefly, a liquid chromatograph consisting of a Waters 600 controller programmable solvent module (Waters, Milford, MA, USA), a WISP 710B autosampler (Waters, Milford, MA, USA) and an HP 1046-A fluorescence detector (Hewlett Packard) were used. Chromatographic data were collected and analyzed with a Millenium 32 system (Waters, Milford, MA, USA). The separations were performed on a Waters Nova-Pak C18 (150×3.9 mm i.d., 60 Å, 4 μm) column, with a matching guard cartridge of the same type. Samples were submitted to an automatic precolumn derivatization reaction with *o*-phthaldialdehyde (OPA) prior to injection. Derivatized amines were detected using the fluorescence detector (excitation wavelength of 340 nm, and emission wavelength of 425 nm). Samples were previously filtered through Millipore filters (0.45 μm) and then directly injected in duplicate into the HPLC system. All reagents used were HPLC grade.

From the HPLC data, the percentage of biogenic amine degradation was calculated as follows:

$$\%\text{Degradation} = (C_{control} - C_{strain}) / C_{control} * 100$$

where $C_{control}$ is the concentration of the biogenic amine in the control (no strain incubated) and C_{strain} is the concentration in the medium incubated with the strain.

3. Results

3.1. Ability of wine-associated LAB to degrade biogenic amines in culture media

Cell cultures of 85 strains representing 9 species of wine LAB (Table 1) were investigated for their potential to degrade/eliminate histamine, tyramine and putrescine, the major biogenic amines present in wines. None of the LAB strains investigated were able to cause a complete disappearance of histamine, tyramine or putrescine under the experimental conditions used. Among the 85 LAB isolates tested, 25% were able to degrade histamine, 18% tyramine and 18% putrescine, although to different extents. Strains showing a percentage of degradation ≥10% of any of the biogenic amines studied are shown in Table 2. Results concerning the *O. oeni* strains isolated from commercial malolactic starter preparations, as well as those concerning the control positive biogenic amine producers *Lactobacillus* 30a ATCC 33222 and *L. brevis* CECT 5354, were negative, so these strains are not included in Table 2. For this screening of biogenic amine-degrading activity, it would have been worth testing positive control strains of amine oxidase producers, but unfortunately, there are none commercially available.

All of the selected positive strains were able to degrade at least two of the three biogenic amines tested; seven strains were able to degrade histamine, six of them tyramine, and all of them exhibited the

Table 2
Percentage of degradation of the biogenic amines (histamine, tyramine and putrescine) by wine-associated LAB in culture media.

Strains	Degradation (%)[a,b]		
	Histamine	Tyramine	Putrescine
L. casei IFI-CA 52	54	55	65
L. hilgardii IFI-CA 41	n.e.	n.e.	20
L. plantarum IFI-CA 26	33	n.e.	24
L. plantarum IFI-CA 54	23	17	24
O. oeni IFI-CA 32	12	n.e.	16
P. parvulus IFI-CA 31	21	15	53
P. pentosaceus IFI-CA 30	10	12	49
P. pentosaceus IFI-CA 83	19	22	39
P. pentosaceus IFI-CA 86	n.e.	54	69

[a] Activity is expressed as a percentage of control without strain and according to HPLC quantitative biogenic amine results.
[b] Mean values (n = 3); n.e.: no effect was observed.

ability to degrade putrescine (Table 2). The degradation percentages ranged from 10% for histamine degradation by *P. pentosaceus* IFI-CA 30 to 69% for putrescine degradation by *P. pentosaceus* IFI-CA 86. In general, putrescine was degraded to a greater extent than histamine and tyramine by all the selected strains. On the other hand, the highest potential for biogenic amine degradation among LAB seemed to be for the *Lactobacillus* and *Pediococcus* groups, in particular *L. plantarum* and *P. pentosaceus* species. With regards to *O. oeni*, the main LAB species involved in MLF, out of the 42 isolates tested, only *O. oeni* IFI-CA 32 was able to reduce histamine and putrescine, but with low activity (Table 2). Furthermore, the following five strains simultaneously degraded the three biogenic amines: *P. pentosaceus* IFI-CA 30 and IFI-CA 83, *P. parvulus* IFI-CA 31, *L. plantarum* IFI-CA 54 – all of them isolated from red wines – as well as *L. casei* IFI-CA 52, isolated from a sherry wine during its biological aging (Moreno-Arribas and Polo, 2005). This strain exhibited the greatest potential for histamine, tyramine and putrescine degradation (54%, 55% and 65% of degradation, respectively) (Table 2).

3.2. Biogenic amine production by LAB able to degrade histamine, tyramine or putrescine

The nine selected strains exhibiting the highest potential to degrade histamine, tyramine and putrescine in culture media (*L. plantarum* IFI-CA 26, *P. pentosaceus* IFI-CA 30, IFI-CA 83 and IFI-CA 86, *P. parvulus* IFI-CA 31, *O. oeni* IFI-CA 32, *L. hilgardii* IFI-CA 41, *L. casei* IFI-CA 52 and *L. plantarum* IFI-CA 54) were also tested for their ability to produce these compounds (histamine, tyramine and putrescine) in MRS and MLO media spiked with the corresponding amino acid precursors (histidine, tyrosine and ornithine, respectively). None of the lactic acid bacteria tested was able to produce any biogenic amines (results not shown). Furthermore, multiplex PCR assays were performed on these nine strains to test for the presence of decarboxylase genes. None of the strains selected amplified the *hdc*, *tdc* or *odc* genes (results not shown), suggesting that LAB strains able to degrade biogenic amines do not contribute to histamine, tyramine and putrescine formation in wines.

3.3. Ability of selected LAB to degrade biogenic amines in wine malolactic fermentation experiments

The nine selected lactic acid bacteria strains active in culture media were also tested in malolactic fermentation laboratory experiments to evaluate their potential applicability in biogenic amine removal from contaminated wines, which could represent a technological improvement in the resolution of this problem. Table 3 reports the concentrations of amines in wines inoculated with the selected strains in comparison to the control wine (no strain inoculated), after

Table 3
Biogenic amine content (mg/L) in biogenic amine-contaminated wine after MLF fermentation in the presence of amine-degrading LAB[a].

Strains	Histamine	Tyramine	Putrescine
Control	28.02 ± 0.52	12.00 ± 0.15	36.10 ± 0.25
L. casei IFI-CA 52	23.10 ± 0.12	10.16 ± 0.14	33.36 ± 0.47
L. hilgardii IFI-CA 41	28.49 ± 0.60	12.10 ± 0.52	36.69 ± 0.17
L. plantarum IFI-CA 26	27.12 ± 0.12	12.01 ± 0.20	35.85 ± 0.23
L. plantarum IFI-CA 54	28.41 ± 0.27	11.45 ± 0.47	35.65 ± 0.29
O. oeni IFI-CA 32	28.75 ± 0.21	11.58 ± 0.36	36.56 ± 0.25
P. parvulus IFI-CA 31	28.41 ± 0.28	12.41 ± 0.18	36.58 ± 0.41
P. pentosaceus IFI-CA 30	28.14 ± 0.24	12.10 ± 0.15	36.08 ± 0.44
P. pentosaceus IFI-CA 83	27.19 ± 0.15	12.14 ± 0.32	35.14 ± 0.30
P. pentosaceus IFI-CA 86	28.75 ± 0.25	12.57 ± 0.43	34.23 ± 0.21

[a] Mean values ± standard deviations (n = 3).

malolactic fermentation. The concentration of histamine, tyramine and putrescine in the contaminated wine (28 mg/L, 12 mg/L and 36 mg/L, respectively) was not altered after malolactic fermentation either for the control wine or for the wines inoculated with *L. plantarum* IFI-CA 26, *P. pentosaceus* IFI-CA 30, IFI-CA 83 and IFI-CA 86, *P. parvulus* IFI-CA 31, *O. oeni* IFI-CA 32, *L. hilgardii* IFI-CA 41 and *L. plantarum* IFI-CA 54. Only *L. casei* IFI-CA 52 was able to significantly degrade histamine (16% of the initial concentration), tyramine (15%) and putrescine (8%) in the contaminated wine, but at lower percentages than in culture media (Table 2). Therefore, these results indicated that the ability of LAB to reduce biogenic amines was negatively affected by the wine matrix.

3.4. Influence of enological factors on the degradation of histamine by cell suspensions and and cell-free extracts of L. casei IFI-CA 52

To gain a deeper insight into the amine-degrading activity exhibited by LAB, and for one of the most active strain found in previous assays (*L. casei* IFI-CA 52), new experiments were conducted to show whether cell-free extracts were as effective as whole cells in the degradation of biogenic amines. For both cell suspensions and cell-free extracts, the influence of enological conditions (pH, wine components and enological additives) on the biogenic amine-degrading ability of *L. casei* IFI-CA 52 was evaluated. Histamine was used since it is the most controlled biogenic amine in wine trade transactions with certain countries.

The effect of *L. casei* IFI-CA 52 on the degradation of histamine in whole cells and enzymatic crude cell extracts was evaluated in phosphate (pH 7.0) and sodium acetate (pH 4.6) buffer systems. Both pHs (7.0 and 4.6) showed good results for histamine reduction in cell suspensions of *L. casei* IFI-CA 52 (88 and 85% of degradation, respectively) (Table 4). Additionally, at pH 4.6, the histamine-degrading ability of the cell-free extracts (84%) was similar to that of the whole cells, indicating that amine-degrading enzymes were

Table 4
Histamine degradation (%) of cell suspensions and cell-free extracts of *L. casei* IFI-CA 52 in phosphate (pH 7.0) and sodium acetate (pH 4.6). Influence of ethanol, wine polyphenols and SO_2.

	Histamine degradation (%)[a,b]	
	Cell suspensions	Cell-free extracts
Phosphate buffer (pH 7.0)	88	72
Sodium acetate buffer (pH 4.6)	85	84
+ ethanol (12%)	17	7
+ wine polyphenols (75 mg/L)	13	28
+ wine polyphenols (660 mg/L)	n.e.	0.12
+ SO_2 (30 g/L)	75	42

[a] Activity is expressed as a percentage of control and according to HPLC quantitative biogenic amine results;
[b] Mean values (n = 3).

effectively extracted from the cells and their action optimal on the degradation of histamine. However, at pH 7.0 the biogenic amine-degrading ability of *L. casei* IFI-CA 52 was slightly lower (72%) in the cell-free extracts in comparison to the cell suspensions, indicating that either genes encoded amine-degrading enzymes were not totally activated, or induced amine-degrading were not totally extracted from the whole cells or the action of the solubilized enzymes was not optimal at this pH.

Results also showed that the presence of wine components such as ethanol and polyphenols strongly affected the histamine-degrading ability of *L. casei* IFI-CA 52 at pH 4.6, for both cell suspensions and cell-free extracts (Table 4). The addition of 12% ethanol (the average concentration in wine) modified the histamine-degrading ability of *L. casei* IFI-CA 52 down to 17 and 7%, respectively, for cell suspension and cell-free extracts, which meant a reduction of 80% in the ability of the whole cells and of 91% in that of the cell-free extracts. Therefore, amine-degrading enzymes seemed to be more sensitive to the presence of ethanol than the whole cells in terms of their histamine-degrading ability. Wine polyphenols also exhibited an inhibitory effect on the enzyme activity; by adding a concentration of 75 mg/L, only 13 and 28% of the histamine is degraded by whole cells and cell-free extracts, respectively. In the presence of 660 mg/L of Provinols™, only 10% of the histamine was degraded by whole cells and no activity was present in the cell-free extracts. In other words, wine polyphenols (75 and 660 mg/L) seemed to have more effect on the histamine-degrading ability of the whole cells (85 and <100% of reduction, respectively) than on that of the cell-free extracts (67 and 99% of reduction, respectively), indicating that amine-degrading enzymes were less sensitive to the presence of wine polyphenols than the whole cells.

The effect of potassium metabisulphite (SO_2), the additive most employed in winemaking because of its antioxidant and selective antimicrobial properties, was tested at normal concentration (30 mg/L). As observed in Table 4, SO_2 reduced the histamine-degrading ability of *L. casei* IFI-CA 52 down to 75 and 42% respectively for cell suspension and cell-free extracts, which meant a reduction of 11% in the ability of the whole cells and of 50% in that of the cell-free extracts, indicating that amine-degrading enzymes were more sensitive to the presence of SO_2 than the whole cells, as was the case with ethanol.

4. Discussion

Knowledge concerning the origin and factors involved in biogenic amine production in wines is well documented, and recently several reviews on this topic have been published (Ferreira and Pinho, 2006; Ancín-Azpilicueta et al., 2008; Smit et al., 2008; Moreno-Arribas and Polo, 2010). In contrast, there is a lack of studies concerning amine degradation by wine micro-organisms. In this context, this paper reports novel data about the presence of histamine-, tyrosine- and putrescine-degrading enzymatic activities of wine-associated LAB. Of particular interest are the results concerning the degradation of putrescine, since no such degrading ability of any food LAB has previously been reported. The isolates tested belong to the principal species of wine LAB and were selected because they came from wine cellars that often suffer from the problem of biogenic amines in their wines (Marcobal et al., 2004; Marcobal et al., 2006; Martín-Álvarez et al., 2006; Moreno-Arribas and Polo, 2008). Therefore, our results confirmed that, within the natural microbiota of lactic acid bacteria present in wines and other related environments, some species and/or strains possessed the potential to degrade biogenic amines. However, this potential for histamine, tyramine and/or putrescine degradation among wine LAB does not appear to be very frequent, since out of the 85 strains examined, only nine displayed noteworthy amine-degrading activity in culture media. Further studies using other LAB species and/or strains may enable more potent amine-degrading enzyme producers to be identified. However, it was observed that positive strains displayed amine-degrading activity against several biogenic amines simultaneously, in accordance with previous works that also reported the presence of either one or two amines oxidases in other food-fermenting micro-organisms, such as *Micrococcus varians* and *Staphylococcus carnosus* (Leuschner et al., 1998).

The fact that active bacteria which were able to significantly reduce the concentration of biogenic amines in the conditions used in the study came not only from young and wood-aged wines but also from fermentation lees, and especially from biologically aged sherry wines (Table 2), suggests that both fermentation lees and 'flor velum' can be interesting ecological niches for the isolation of potential amine-degrading bacteria.

The potential for amine breakdown proved to be a characteristic related to some species of the genera *Lactobacillus* and *Pediococcus*, which was in agreement with previous works that investigated the distribution of histamine and tyramine oxidase activities among food-fermenting micro-organisms (Leuschner et al., 1998). In this study, the most potent amine-degrading species detected were *L. plantarum*, *P. parvulus* and, in particular, *P. pentosaceus* and *L. casei*, in spite of the fact that strains of these last species have never been reported to degrade histamine, tyramine and/or putrescine. In contrast, the results indicate that, within the natural population of *O. oeni* isolated from wines, the presence of enzymatic activities that degrade histamine, tyramine and/or putrescine was low, suggesting that the potential to reduce amine concentrations in wines is rare in *O. oeni* strains. Regarding commercial malolactic starters, they are regarded as safe with respect to biogenic amine production (Moreno-Arribas et al., 2003; Marcobal et al., 2006). However, to date there has not been any report on the potential role of these starters in the elimination/degradation of biogenic amines in wines, in spite of their wide use in winemaking. In our experiments, none of the commercial malolactic starters tested ($n = 3$) showed any histamine, tyramine or putrescine-degrading ability in culture media, leading to the conclusion that no specific role in the removal of biogenic amines could be attributed to them, although further studies, including a higher number of products, should be carried out.

Once amine-degrading activities of some LAB strains were proven, the next goal was to see if these strains might promote the accumulation of these compounds in wine. Therefore, we tested the production of the most important biogenic amines in wines (histamine, tyramine and putrescine) by the selected positive amine-degrading LAB strains. None of the strains were able to produce these biogenic amines as they did not show the decarboxylase activity necessary for the production of these compounds in wine. Therefore, the biogenic amine-degrading ability of the selected LAB did not appear to be associated with an amine-producing ability.

In order to check their ability to reduce biogenic amines in wine environment strains possessing amine-degrading ability in culture media were also tested in real systems by simulating wine MLF. The *L. casei* IFI-CA 52 strain, displaying high histamine, tyramine and putrescine breakdown in culture media, had a limited effect on these amines during wine MLF, in line with previous works that indicate that the activity *in vitro* of micro-organisms having mono- and diamino-oxidase activities is not quantitatively reproducible *in vivo* (Gardini et al., 2002).

Although no differences in the amine-degrading activity of *L. casei* IFI-CA 52 were found to be affected by pH (4.6 and 7.0), further experiments in the presence of wine components such as ethanol (12%) and polyphenols (75 and 660 mg/L) and wine additives such as SO_2 (30 mg/L) indicated that the wine matrix definitely affected the ability of the strain to degrade histamine, explaining the differences found between the percentage of histamine degradation by *L. casei* IFI-CA 52 in wine (Table 3) and in culture media (Table 2). Although more studies with other LAB species and strains are required to draw final conclusions, these studies suggested that the wine matrix have a

strong effect on the ability of amine-degrading enzymes to reduce undesirable biogenic amines in wine.

The fact that there were no differences in the histamine-degrading ability of the cell suspensions of *L. casei* IFI-CA 52 and their corresponding cell-free extracts indicated that amine-degrading enzymes are intracellular and active at a pH close to wine pH. Therefore, a potential application of amine-degrading strains to prevent the accumulation of biogenic amines in wine could be as starters to be inoculated or as enzymatic preparations to be added to the contaminated wines. Moreover, the wine matrix would influence the efficiency of starters and enzymatic preparations in different ways, as this study also showed that ethanol and SO_2 have more effect on the activity of solubilized amine oxidase enzymes than on whole cells, whereas wine polyphenols showed the opposite (Table 4).

In conclusion, this paper presents, for the first time to our knowledge, a screening of the biogenic amine-degrading ability of wine-associated LAB. Among the many and diverse strains tested, some of them have been found to be active in the degradation of histamine, tyramine and putrescine in culture media and in wine. Although the amine-degrading ability of the active LAB seemed to be good at a pH close to wine pH, wine components such as ethanol and polyphenols and wine additives such as SO_2 might limit this ability, as has been seen in the case of *L. casei* IFI-CA 52. In spite of this adverse influence of the wine matrix, our results prove the potential to prevent/reduce the accumulation of these amines in the final wine. Further investigations are needed in order to evaluate the applicability of this LAB potential in winemaking.

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IV.3. Evaluación de las propiedades antimicrobianas de extractos fenólicos frente a bacterias lácticas en medios de cultivo y en experimentos de FML.

En las secciones anteriores, se ha comprobado que compuestos fenólicos individuales pueden inhibir el crecimiento y metabolismo de BAL del vino. Sin embargo, a nivel práctico, es inviable pensar en la adicción de compuestos individuales (obtenidos por síntesis orgánica) al vino para el control de las BAL, y por tanto de la FML. La posible aplicación tecnológica de las propiedades antimicrobianas de los polifenoles frente a BAL, tendría que pasar necesariamente por el empleo de extractos fenólicos obtenidos por procedimientos técnicos y económicamente viables. Por tanto, en este punto nos planteamos la evaluación de las propiedades antimicrobianas de extractos fenólicos de plantas y otros materiales que pudieran considerarse como procedimientos "naturales" de control de la FML, y, por tanto, como una alternativa total o parcial al empleo de sulfitos.

En la bibliografía, diversos estudios han demostrado la efectividad, frente a patógenos y otras bacterias alterantes, de extractos fenólicos procedentes de diversos orígenes como romero, cacao y aceite de oliva (Bubonja-Sonje y col., 2011), arándano rojo (Côté y col., 2011), frutos rojos (Park y col., 2011), cebollas y ajos (Benkeblia y col., 2004), mango (Kaur y col., 2010), sub-productos (Balasundram y col., 2006), orujo de uva (Özkan y col., 2004), uvas (Baydar y col., 2004; 2006) y piel de almendra (Mandalari y col., 2010), entre otros, aunque la mayoría de los estudios se han realizado en medios de cultivo.

Por tanto, se planteó la selección de un gran número (n=54) de extractos vegetales (calidad alimentaria) procedentes de diferentes orígenes, incluida la uva y los sub-productos vitivinícolas. Lógicamente, algunos de los extractos multicomponentes incluirían en su composición los compuestos fenólicos (p. ej., ácido caféico, quercetina, etc.) cuya actividad antimicrobiana frente a bacterias se habría comprobado previamente, pero otros podrían incluir otras estructuras fenólicas, no consideradas en estudios previos, también con potencial antimicrobiano. En la experimentación, se consideró interesante también realizar una caracterización de los extractos basada en su contenido en polifenoles totales (método de Folin-Ciocalteu) y capacidad antioxidante (método ORAC).

Para la evaluación inicial de las propiedades antimicrobianas de los extractos, se utilizaron las mismas cepas de BAL que se habían empleado en el estudio con compuestos fenólicos individuales (Sección IV.1), más las cepas pertenecientes al

género *Lactobacillus*, *L. casei*-CIAL 52 y *L. plantarum* CIAL 92 Adicionalmente y para ampliar, en parte, el conocimiento sobre el espectro de acción antimicrobiana de estos extractos, en el *screening* también se incluyeron dos especies de bacterias acéticas. De igual forma, además del cálculo de parámetros de inhibición (IC_{50}) que permitieran la comparativa de la capacidad de inhibición entre extractos y cepas, también se utilizó la técnica de microscopia electrónica para evaluar los cambios en la morfología de la bacteria después de su interacción con los extractos fenólicos.

A partir de los resultados de inhibición de las BAL en medio de cultivo, se seleccionó el extracto más activo para una segunda evaluación de su efectividad antimicrobiana durante el proceso de FML del vino. Para ello, se llevó a cabo una experiencia de FML en vinos tintos elaborados a escala industrial, que, una vez en el laboratorio, se inocularon con un cultivo iniciador maloláctico, o bien se mantuvieron en condiciones favorables para el desarrollo de la FML de forma espontánea. En ambos experimentos, se siguió el desarrollo de la FML, determinando el contenido de ácido málico en el vino por una metodología similar a la que se lleva a cabo en bodega.

Finalmente, el extracto seleccionado también se probó en bodega para controlar, desde el punto de vista microbiológico, la etapa de crianza en barrica de vinos blancos, reduciéndose de este modo el empleo de sulfitos durante la vinificación.

A continuación se presentan los resultados de este estudio en forma de una publicación y una patente:

Publicación IV. Extractos fenólicos antimicrobianos capaces de inhibir el crecimiento de bacterias lácticas y la fermentación maloláctica del vino.

Patente I. Procedimiento de elaboración de vino que comprende adicionar un extracto fenólico de origen vegetal con propiedades antimicrobianas frente a bacterias lácticas y/o acéticas.

<u>Publicación IV.</u> Extractos fenólicos antimicrobianos capaces de inhibir el crecimiento de bacterias lácticas y la fermentación maloláctica del vino.

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Resumen:

El propósito de este estudio fue determinar si los extractos fenólicos con propiedades antimicrobianas pueden considerarse una alternativa potencial al uso de dióxido de azufre (SO_2) en el control de la fermentación maloláctica (FML) del vino. Se determinó la inhibición del crecimiento de seis cepas enológicas (*Lactobacillus hilgardii* IFI-CA 49, *Lactobacillus casei* IFI-CA 52, *Lactobacillus plantarum* IFI-CA 92, *Pediococcus pentosaceus* IFI-CA 85, *Oenococcus oeni* IFI-CA 91 y *O. oeni* IFI-CA 96), por extractos fenólicos (n=54) de diferentes orígenes (especias, flores, hojas, frutas, legumbres, semillas, pieles, subproductos agrícolas y otros), calculándose el valor del parámetro de supervivencia IC_{50} para aquellos extractos activos. Un total de 24 extractos mostraron una inhibición significativa del crecimiento de al menos dos de las cepas de BAL estudiadas. Algunos de estos extractos también fueron activos frente a dos bacterias acéticas (*Acetobacter aceti* IFI-CA 106 y *Gluconobacter oxydans* IFI-CA 107). Por microscopía electrónica de transmisión, se observaron daños en la integridad de la membrana celular de las bacterias tras su incubación con el extracto fenólico. Por último, para comprobar la aplicabilidad de los extractos, se adicionó extracto de eucalipto (2g/L) a un vino tinto elaborado a escala industrial, y se monitorizó el proceso de FML en base a la medida de ácido málico residual. La adición del extracto de eucalipto retrasó significativamente el progreso de la FML, tanto la inducida por un iniciador como llevada a cabo espontáneamente, en comparación con el vino control (sin adición de agente microbiano), si bien no de una forma tan efectiva como el $K_2S_2O_5$ (30 mg/L). Estos resultados demuestran la potencial aplicación de extractos fenólicos como agentes antimicrobianos alternativos al uso de sulfitos en la elaboración del vino.

Antimicrobial phenolic extracts able to inhibit lactic acid bacteria growth and wine malolactic fermentation

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Abstract

The purpose of this study was to determine whether phenolic extracts with antimicrobial activity may be considered as an alternative to the use of sulphur dioxide (SO_2) for controlling malolactic fermentation (MLF) in winemaking. Inhibition of the growth of six oenological strains (*Lactobacillus hilgardii* CIAL-49, *Lactobacillus casei* CIAL-52, *Lactobacillus plantarum* CIAL-92, *Pediococcus pentosaceus* CIAL-85, *Oenococcus oeni* CIAL-91 and *O. oeni* CIAL-96) by phenolic extracts (n=54) from different origins (spices, flowers, leaves, fruits, legumes, seeds, skins, agricultural by-products and others) was evaluated, being the survival parameter IC_{50} calculated. . A total of 24 extracts were found to significantly inhibit the growth of at least two of the LAB strains studied. Some of these extracts were also active against two acetic acid bacteria (*Acetobacter aceti* CIAL-106 and *Gluconobacter oxydans* CIAL-107). Scanning electron microscopy of the bacteria cells after incubation with the phenolic extract confirmed damage of the integrity of the cell membrane. Finally, to test the technological applicability of the extracts, the eucalyptus extract was added (2 g/L) to an industrially elaborated red wine, and the progress of the MLF was evaluated by means of residual content of malic acid. Addition of the extract significantly delayed the progress of both inoculated and spontaneous MLF, in comparison to the control wine (no antimicrobial agent added), although not as effective as $K_2S_2O_5$ (30 mg/L). These results demonstrated the potential applicability of phenolic extracts as antimicrobial agents in winemaking.

Keywords: wine, phenolic extracts, lactic acid bacteria, acetic acid bacteria, malolactic fermentation, antimicrobial activity, alternatives to SO_2

Introduction

In wines, lactic acid bacteria (LAB) carry out the process of malolactic fermentation (MLF), which takes place after alcoholic fermentation under favourable conditions. Wine deacidification is the main trigger for MLF, and consists of the conversion of L-malic acid to L-lactic acid resulting in a decrease in titratable acidity and a small increase in pH. MLF also contributes to wine microbial stability and improves the complexity of wine aroma (Versari et al., 1999; Maicas, 2001; Moreno-Arribas and Polo, 2005; Miller et al., 2011).

The bacteria present in the first steps of winemaking (must and the start of fermentation) belong to different species, generally homofermentative ones. The most abundant belong to *Lactobacillus plantarum*, *Lactobacillus hilgardii*, *Leuconostoc mesenteroides* and *Pediococcus sp.*, while to a lesser extent, *Oenococcus oeni* and *Lactobacillus brevis* are also found. Bacterial multiplication takes place in the interval between the end of alcoholic fermentation and the start of MLF. During this stage, the pH of the medium, the SO_2 content, the temperature and the ethanol concentration (Boulton et al., 1996) are the most influential factors. *O. oeni* is the bacteria species predominating at the end of alcoholic fermentation. This is the species best adapted to growing in the difficult conditions imposed by the medium (low pH and high ethanol concentration) (Davis et al., 1985; van Vuuren and Dicks, 1993) and is, therefore, the main species responsible for MLF in most wines. However, some strains of the genera

Pediococcus and *Lactobacillus* can also survive this phase, and most of them are considered to be wine spoilage species. Consequently, if MLF is not well controlled, alterations in wine quality due to bacteria metabolic activity can happen. It is, therefore, common practice to remove LAB by sulphating the wine once malic acid has been mostly degraded.

Sulphurous anhydride or sulphur dioxide (SO_2) has numerous properties as a preservative in winemaking; these include its antioxidant and selective antimicrobial effects, especially against LAB. Nevertheless, and due to increasing health concerns, consumer preference, possible organoleptic alterations in the final product and a tighter legislation regarding preservatives, there is a worldwide trend to reduce SO_2 levels in wine (Du Toit and Pretorius, 2000), with a particular interest within the scientific community in the development of total or partial alternatives to the traditional use of SO_2 in winemaking (García-Ruiz et al., 2008; Bartowsky, 2009;Fredericks et al., 2011; Izquierdo-Cañas et al., 2012).

Over the last two decades, other preservatives from plant, animal and microbial origins have been intensely investigated for practical applications (for a review see Pozo-Bayón et al., 2011). In particular, 'natural' products such as polyphenols have been reported to have a variety of biological effects, including antioxidant, anticarcinogenic, anti-inflammatory and antimicrobial activities (Xia et al., 2010). Phenolic extracts from different vegetal origins, such as rosemary, cocoa, olive oil (Bubonja-Sonje et al., 2011), cranberry (Côté et al., 2011), blueberry (Park et al., 2011), onion, garlic (Benkeblia et al., 2004), mango (Kaur et al., 2010), plant and agricultural by-products (Balasundram et al., 2006), grape pomace (Özkan et al., 2004), grape

(Baydar et al., 2004; 2006) and almond skins (Mandalari et al., 2010), have demonstrated their antimicrobial capacity against numerous spoilage and pathogenic bacteria. Most of these references were in pure culture experiments. Other studies carried out on salad vegetables (Karapinar and Sengun, 2007) and meat products such as fresh pork patties (Park and Chin, 2010), beef meatballs (Fernández-López et al., 2005) and chicken products (Kanatt et al., 2010) have demonstrated the potential application of phenolic extracts as antimicrobial and antioxidant agents in order to prevent food diseases and to prolong the shelf life of final products.

With regard to the potential application of polyphenols as preservatives in wines, most studies have evaluated the effects of pure compounds on isolated bacteria (for a review see García-Ruiz et al., 2008). Recently, the inhibitory effects of the different classes of phenolic compounds present in wine (hydroxybenzoic acids and their derivatives, hydroxycinnamic acids, phenolic alcohols and other related compounds, stilbenes, flavan-3-ols and flavonols) on different LAB wine isolates have been compared (García-Ruiz et al., 2009, 2011), confirming the potential of phenolic compounds as preservatives in winemaking. However, until now, the effectiveness of plant phenolic extracts – which are the products potentially applicable in winemaking – in controlling LAB growth during wine MLF has not been investigated.

With the ultimate goal of developing new alternatives to the use of sulphites in enology, the objective of this work was to evaluate the potential of plant phenolic extracts to inhibit the growth of LAB and the progress of MLF in wines. In the first part of the work, we measured the inhibitory potency of 54 commercial phenolic extracts from different origins on the growth of different enological strains of LAB and acetic

acid bacteria (AAB). Results are expressed as IC_{50} in order to allow further comparison between polyphenol structures and bacteria species and strains. In the second part, the efficacy of one of the most active extracts in pure cultures (the eucalyptus extract) was also tested in wine MLF, occurring either spontaneously or by inoculation with a malolactic starter.

2. Materials and methods

2.1. Phenolic extracts

A total of 54 phenolic extracts were assayed: ***spices (n=5):*** cinnamon, eucalyptus, oregano, rosemary and thyme; ***flowers (n=2):*** camomile and yarrow; ***leaves (n=15):*** green tea (n=3), rock tea, red tea, elder leaves, olive tree leaves, Olixxol® (a commercial formulation from the olive tree), walnut leaves, currant leaves, *Ginkgo biloba,* lady's mantle leaves and vine leaves (n=3); ***fruits (n=8):*** acerola, apple, bitter orange, bilberry, citrus, Citrolive® (a commercial formulation from the citrus tree) and pomegranate (n=2); ***legumes (n=2):*** soy bean and red clover; ***seeds (n=4):*** green coffee and grape seeds (n=3); ***skins (n=6):*** almond skins, Amanda® (a commercial formulation from almond skins) and red grape skins (n=4); ***agricultural by-products (n=3):*** grape pomace (n=2), and Eminol® (a formulation from grape pomace); ***wine (n=1):*** Provinols™ (a formulation from red wine); ***purified tannins (n=7)*****:** grape seed tannins, grape skin tannins, oak tannins, quebracho tannins, Vitaflavan® (a formulation from grape seed tannins) and monomeric and oligomeric fractions from Vitaflavan®; ***others (n=1):*** propolis (Table 1). All phenolic extracts were kindly provided by their producers: Biosearch Life S. A. (Granada, Spain), Agrovin S.L. (Ciudad Real, Spain) and

SilvaTeam (San Michele Mondovì, Italy), except the seed and grape skin tannins which were kindly provided by Dr. Vivas (University of Bordeaux 1, France). In general, the extracts were obtained after maceration of the plant material with aqueous alcoholic mixtures at a temperature between 25-75ºC, following by a drying process to get a final stable solid powder.

2.2. Determination of total phenolic content and antioxidant activity of the extracts

Phenolic extracts (0.05 g) were mixed with 10 mL of methanol/HCl (1000/1, v/v) and sonicated for 5 min followed by a 15 min resting period. The mixture was then centrifuged (3024 *g*, 5 min, 5 ºC) and filtered (0.45 μm) to determine the total phenolic content (total polyphenols, TP). The method of Singleton and Rossi (1965), based on the oxidation of the hydroxyl groups of phenols in basic media by the Folin-Ciocalteu reagent, was used for determining the total phenolic content of the extracts. The results were expressed as mg of gallic acid equivalents per gram of extract. The analysis was performed in triplicate.

The radical scavenging activity of the phenolic extracts was determined by the ORAC (Oxygen-Radical Absorbance Capacity) method using fluorescein as a fluorescence probe (Dávalos et al., 2004). Briefly, the reaction was carried out at 37 ºC in 75 mM phosphate buffer (pH 7.4) and the final assay mixture (200 μl) contained fluorescein (70 nM), 2,2'-azobis(2-methyl-propionamidine)-dihydrochloride (12 mM) and antioxidant (Trolox [1-8 μM] or phenolic extract [at different concentrations]).

ORAC values were expressed as mmol of Trolox equivalents per g of extract. The analysis was performed in triplicate.

Correlation analysis (Pearson's correlation coefficient) was used to investigate the relationship between TP and ORAC parameters, using the STATISTICA program for Windows, version 7.1 (StatSoft. Inc. 1984–2006, www.statsoft.com).

2.3. Culture media and growth conditions

Six strains of LAB, *Lactobacillus hilgardii* CIAL-49, *Lactobacillus casei* CIAL-52, *Lactobacillus plantarum* CIAL-92, *Pediococcus pentosaceus* CIAL-85, *Oenococcus oeni* CIAL-91 and *O. oeni* CIAL-96, and two strains of acetic acid bacteria (AAB) *Acetobacter aceti* CIAL-106 and *Gluconobacter oxydans* CIAL-107, were employed in this study. These strains belong to the bacterial culture collection of CIAL (Instituto de Investigación en Ciencias de la Alimentación, CSIC-UAM). LAB strains were previously isolated from red wines during the early phase of MLF, and properly identified by 16S rRNA partial gene sequencing as described by Moreno-Arribas and Polo (2008). Among these six LAB strains, *Lactobacillus hilgardii* CIAL-49 was found to be a biogenic-amine-producer strain, being able to generate hystamine in culture media (results not published). These strains were kept frozen at -70 ºC in a sterilized mixture of culture medium and glycerol (50:50, v/v). MRS culture media (pH 6.2) based on the formula developed by Man et al. (1960) were employed for *L. hilgardii, L. casei*, *L. plantarum* and *P. pentosaceus.* They were cultivated for 48 h. The culture media MLO (pH 4.8) developed by Caspritz et al. (1983) were employed for *O. oeni*.

These bacteria were cultivated for 72 h. Both media were purchased from Pronadisa (Madrid, Spain). Culture media containing 6% ethanol (MRSE and MLOE) were prepared by adding ethanol (99.5%, v/v) to the sterilized (121 ºC, 15 min) media. AAB were cultivated for 72 h in mannitol culture media (25 g/L n-mannitol [Panreac Química SAU, Barcelona, Spain], 5 g/L yeast extract [Scharlau Chemie S. A., Barcelona, Spain], and 3 g/L peptone [Difco, Becton, Dickinson and Co., Le Pont de Claix, France]).

2.4. Antibacterial activity assay

The antibacterial assays were performed using the method of García-Ruiz et al. (2011). Inhibition of microbial growth by phenolic extracts was determined by the microtiter dilution method, using serial triple dilutions of the antimicrobial agents and initial inocula of 5×10^5 CFU/ml for all the studied micro-organisms. Bacterial growth was determined by reading the absorbance at 550 nm. Growth inhibitory activity was expressed as a mean percentage (%) of growth inhibition with respect to a control without antimicrobial extract. Phenolic extracts were tested at different concentrations from 2 to 0.0625 g/L (final concentration), except for purified tannins whose concentration range was from 1 to 0.0313 g/L, to ensure complete solubility in the medium.

The inhibition percentage was calculated as:

$$\%\text{Inhibition} = 1 - \frac{(TF_{Sample} - T0_{Sample}) - (TF_{Blank} - T0_{Blank})}{(TF_{Growth} - T0_{Growth}) - (TF_{Blank} - T0_{Blank})} \times 100$$

where $T0_{Sample}$ and TF_{Sample} corresponded to the OD_{550} of the strain growth in the presence of the phenolic solution before and after incubation, respectively; $T0_{Blank}$ and TF_{Blank} corresponded to the broth medium with phenolic solution before and after incubation, respectively; and $T0_{Growth}$ and TF_{Growth} corresponded to the strain grown in the absence of the phenolic solution before and after incubation, respectively.

Negligible antimicrobial effects were considered when the growth inhibition percentage was < 25% at the maximum concentration tested (2 g/L). For the active extracts, the survival parameter IC_{50} value was defined as the concentration required to obtain 50% inhibition of growth after 48 (*L. hilgardii, L. casei, L. plantarum* and *P. pentosaceus*) or 72 h (*O. oeni, A. aceti, G. oxydans*) of incubation and was estimated by nonlinear regression using the following sigmoidal dose-response (with variable slope) equation:

$$Y=\text{Bottom} + \frac{(\text{Top-Bottom})}{(1+10\text{^}((\text{LogIC50-X})*\text{Slope}))}$$

where, X represents the logarithm of concentration, Y is the response variable (%Inhibition) which starts at the Bottom and goes to the Top with a sigmoid shape, LogIC50 is the logarithmic of IC_{50}, and Slope represents the slope parameter. The PRISM program for Windows 4.03 (GraphPad Software, Inc., 2005, www.graphpad.com) was used for the estimation of the four parameters. For each data set, the PRISM program also allows comparison of the fit to the previous sigmoidal dose-response model (with 4 parameters) and the fit to the same model with the Bottom and Top parameters constrained to 0 and 100%, respectively.

2.5. Electron microscopy

Bacteria incubated with or without the antimicrobial agent for 20 h were fixed on the culture plate with 4% *p*-formaldehyde (Merck, Darmstadt, Germany) and 2% glutaraldehyde (SERVA, Heidelberg, Germany) in 0.05 M cacodylate buffer (pH 7.4) for 120 min at room temperature. Cells were then carefully scraped from the plate, centrifuged at 3000 *g* for 5 min, and the washed pellet post-fixed with 1% OsO_4 and 1% $K_3Fe(CN)_6$ in water for 60 min at 4 ºC. Cells were dehydrated with ethanol and embedded in Epon (TAAB 812 resin, TAAB Laboratories Equipment Limited) according to standard procedures. Ultrathin sections were collected on collodion-carbon-coated copper grids, stained with uranyl acetate and lead citrate and examined at 80 kV in a JEM-1010 (JEOL, Tokyo, Japan) electron microscope. Electron micrographs were recorded at different orders of magnitude.

2.6. Malolactic fermentation assays in wine

A red wine (var. *Merlot*) (vintage 2009) was elaborated at Bodegas Miguel Torres S.A. (Catalonia, Spain), following their own winemaking procedures. The alcoholic fermentation (AF) was carried out in a controlled form in stainless steel at 25 ± 2 ºC. The end of AF was established by measuring the alcohol degree (13.9 % v/v) and the residual sugar amount (< 3.5 g/L); the wine pH at the end of AF was 3.22. MLF experiments were conducted in laboratory scale, sterile conditions, in 250-mL flasks. Parallel inoculated and spontaneous MLF assays were carried out. The malolactic starter was comprised by a mix of three *O. oeni* strains previously isolated by the winery, and

was inoculated in wine at 3% (v/v). The phenolic extract (eucalyptus extract) was dissolved (2 g/L) in 200 mL of previously inoculated or non-inoculated wine. A control containing no extract was also prepared for both inoculated and spontaneous MLF assays. An extra positive control containing $K_2S_2O_5$ (30 mg/L) as an antimicrobial agent was also prepared for the inoculated MLF assay. Control wines and wines containing phenolic extracts or sulphites, were incubated at 25 ºC in the dark. All the MLF assays were performed in duplicate.

Wine samples were aseptically collected at 14, 19 and 24 days of incubation, and were immediately assayed for L-malic acid content as a marker of the development of MLF. L-malic acid content was determined using an enzymatic kit (Megazyme International Ireland Ltd., Bray, CO. Wicklow, Ireland), and these determinations were carried out in duplicate.

3. Results and Discussion

3.1. Characterization of phenolic extracts

A wide variety of phenolic extracts from different origins were chosen because of their different phenolic composition and content, in an attempt to relate the most appropriate phenolic structures to their inhibitory effects on the growth of enological LAB and AAB. The total phenolic content of the extracts tested (n=54) ranged from 33 mg gallic acid/g for elder leaves to 750 mg gallic acid/g for the monomeric fraction

from Vitaflavan® (Table 1). The purified tannins were the group with the highest total phenolic values (349-750 mg gallic acid/g).

The antioxidant capacity (ORAC value) of the extracts varied from 0.22 mmol Trolox/g (pomegranate #2) to 40.6 mmol Trolox/g (monomeric fraction from Vitaflavan®) (Table 1). The purified tannins were the group with the highest ORAC values whereas the fruits and leaves were the groups with the lowest ORAC values (0.22 – 10.9 mmol Trolox/g and 1.04 – 14.7 mmol Trolox/g, respectively).

To better illustrate the diversity of the extracts, Figure 1 displays the relationship between ORAC values and total phenolic content. A good linear correlation was observed between both variables ($r = 0.9173$, $P < 0.01$), which indicated that polyphenols were largely responsible for the antioxidant properties of the extracts. The purified tannins (shaded points in Figure 1) were widely distributed in the upper-right part of the graph and characterized by high levels of polyphenols and antioxidant capacity.

3.2. Inhibition of LAB growth by phenolic extracts

The antimicrobial effect of the phenolic extracts on the growth of the enological bacteria was measured in terms of IC_{50} (i.e. the concentration required to obtain 50% inhibition of growth after 48 h (*L. hilgardii* CIAL-49, *L. casei* CIAL-52, *L. plantarum* CIAL-92 and *P. pentosaceus* CIAL-85) or 72 h (*O. oeni* CIAL-91 and CIAL-96). In a recent study we concluded that this parameter is quicker and more feasible than methodologies based on colony counting and allows comparison among different

studies as well as a more accurate assessment of the effects of these compounds (García-Ruiz et al., 2011).

To summarize the results, Table 2 reports the IC_{50} values of the phenolic extracts that exhibited antimicrobial activity against two or more LAB strains: a total of 24 from the 54 extracts tested. These active extracts belong to all the different groups of phenolic extracts, with the exception of the flower extract group which showed negligible antimicrobial effects on the growth of the six LAB strains assayed. Only the purified tannins from grape seed and quebracho, as well as the propolis extract, inhibited the growth of the six LAB strains tested, independently of the species, showing the grape seed tannins to have the lowest IC_{50} values (0.41-1.22 g/L) or greatest inhibitory potential. In general, purified tannins exhibited great and wide-ranging antimicrobial effects against the LAB strains studied, which was partly attributed to their higher phenolic content (Table 1).

A certain specificity in the inhibition potential against *O. oeni* (CIAL-91 and CIAL-96) and non-*O. oeni* strains (*L. hilgardii* CIAL-49, *L. casei* CIAL-52, *L. plantarum* CIAL-92 and *P. pentosaceus* CIAL-85) was observed for some phenolic extracts. Non-*O. oeni* strains were specifically inhibited by Eminol®, although the survival parameter IC_{50} was relatively high for all of them (1.60–2.88 g/L) (Table 2). The eucalyptus extract and Amanda® also inhibited the growth of the *Lactobacillus* and *Pediococcus* strains plus the growth of one *O. oeni* strain (CIAL-96 for the eucalyptus extract and CIAL-91 for Amanda®), although the IC_{50} values were relatively high for these latter strains (1.90 g/L for CIAL-96 and 2.63 g/L for CIAL-91). In addition, the eucalyptus extract exhibited the greatest inhibitory effect (lowest IC_{50} values) against

the non-*O. oeni* strains (IC_{50}= 0.16-0.33 g/L for *Lactobacillus* strains and 0.09 g/L for *P. pentosaceus* CIAL-85). The *Ginkgo biloba* extract also inhibited the growth of the four non-*O. oeni* strains (IC_{50}= 1.30-1.86 g/L) and one *O. oeni* strain (CIAL-96), but in this case, the IC_{50} value was lower for the latter (0.82 g/L). Other extracts only active against non-*O. oeni* strains, but not against all of those tested, were: grape seed #2 and almond skin extracts, both active against *Lactobacillus;* grape seed #3 extract, active against *L. hilgardii* CIAL-49 and *L. plantarum* CIAL-92; and soy bean and grape seed #1, active against *P. pentosaceus* CIAL-85 and one *Lactobacillus* strain.

On the other hand, *O. oeni* strains were specifically inhibited by the pomegranate #1 and cinnamon extracts and tannins from grape skins, with the pomegranate #1 extract showing the greatest inhibitory effect against *O. oeni strains* (IC_{50}=0.40 and 0.41 g/L) (Table 2). The grape pomace #2 extract, oak tannins and Vitaflavan® were active against *O. oeni* strains and another non-*O. oeni* strain (*L. plantarum* CIAL-92, *L. casei* CIAL-52 and *L. hilgardii* CIAL-49, respectively). The two purified fractions from Vitaflavan® were also active against the two *O. oeni* strains plus *P. pentosaceus* CIAL-85 and one *Lactobacillus* strain.

The other extracts tested – thyme, red grape skin #4 and grape pomace #1 extracts, and Provinols™– showed no clear specificity in their species antimicrobial pattern (Table 2).

Overall, the results confirmed differences in bacteria susceptibility to phenolic extracts among different LAB genera and species. *L. plantarum* CIAL-92 ($IC_{50\ range}$= 0.16-2.82 g/L) and *O. oeni* CIAL-96 ($IC_{50\ range}$= 0.41-3.00 g/L) were the most sensitive

strains, as they were inhibited by 16 of the 54 extracts tested. In contrast, *P. pentosaceus* CIAL-85 ($IC_{50\ range}$= 0.40-2.35 g/L) was the most resistant species, as its growth was inhibited by only 12 of the total extracts tested.

The antimicrobial activity of the phenolic extracts against LAB seemed not to be directly related to their antioxidant capacity as measured by the ORAC test (Table 1), which was in line with what was found for the pure phenolic compounds present in wine (García-Ruiz et al. 2009).

3.3. Inhibition of the growth of AAB by phenolic extracts

Acetic acid bacteria are always associated with wine spoilage and their presence in wines and consequent negative effects on them have to be strictly controlled (du Toit and Pretorius, 2002; Guillamón and Mas, 2011); however, to our knowledge, the possible impact of polyphenols on AAB growth has not previously been explored. Therefore, as a first exploratory approach, IC_{50} values of some phenolic extracts active against LAB strains (eucalyptus, *Ginkgo biloba* and propolis extracts, Amanda®, and grape seed and quebracho tannins) were determined against two AAB strains (*A. aceti* CIAL-106 and *G. oxydans* CIAL-107) following the same procedure as described for LAB (Table 3).

Tannins from quebracho exhibited the greatest antimicrobial effect (lowest IC_{50} values) against both AAB strains (IC_{50} = 0.11 and 0.15 g/L). Compared to LAB, the IC_{50} values of quebracho tannins were lower for AAB, i.e. these tannins were more

toxic for acetic acid bacteria strains. Amanda® showed similar antimicrobial effects against LAB and AAB strains. In contrast, the eucalyptus extract exhibited a lower inhibitory effect against AAB than against the *Lactobacillus* and *Pediococcus* strains. These results suggest a wide species spectrum for the antimicrobial properties of these phenolic extracts in relation to the winemaking process. In general, several scientific evidences indicate that the antimicrobial activity of phenolic compounds from plant origins is higher against Gram-positive than against Gram-negative micro-organisms (Papadopoulou et al., 2005 Karapinar et al., 2007; Oliveira et al., 2008; Kanatt et al., 2010; Mandalari et al., 2010).

3.4. Microscopy study

To investigate possible changes in cell morphology after incubation of LAB with phenolic extracts, scanning electron microscopy was applied. For example, Figure 2 displays the micrographs of *O.oeni* CIAL-96 cells incubated with tannins from grape seeds (B and C) and with red grape skin #4 extract (D and E). In both cases, damage to the integrity of the cell membrane was observed when compared to the control. Alterations in the integrity of the cell membrane might promote cell death, probably due to alterations in the transport and energy-dependent processes, and metabolic pathways that are essential for bacteria viability (Ibrahim et al., 1996). Similar changes in the morphology of *O. oeni* CIAL-96 were observed after the incubation of the cells with pure phenolic compounds such as ethyl gallate, ferulic acid and *trans*-resveratrol (at a concentration of 2 g/L) (García-Ruiz et al., 2011).

3.5. Effects of addition of phenolic extracts on wine MLF

In order to check whether phenolic extracts have the capacity to affect the growth of lactic acid bacteria and the development of MLF, different assays were carried out on an industrial red wine after alcoholic fermentation. For these experiments, the eucalyptus extract was used because it exhibited low IC50 values (great antimicrobial activity) in culture media, in particular against non-*O. oeni* strains (Table 2). Table 4 shows the results obtained expressed as percentage of malic acid degradation during MLF of control wine and wines treated with the antimicrobial agents (eucalyptus extract or SO_2).

MLF was successfully completed for all wines, although at different rates. For the wine inoculated with the malolatic starter, the content of residual malic acid was negligible after 14 days of incubation in the absence of antimicrobial agents (eucalyptus extract or SO_2). However, when the eucalyptus extract was added to the wine, the consumption of malic acid was delayed, and 10% of the initial malic acid still remained after 14 days of incubation. This effect was lower than that observed in the wine treated with SO_2 (30 mg/L of $K_2S_2O_5$), which retained 89% and 35% of the initial malic acid after 14 and 19 days of incubation, respectively.

As expected, the consumption of malic acid was slower in the non-inoculated wine (spontaneous MLF): 40% of the initial malic acid was retained after 14 days of incubation for the control wine (Table 4). Interestingly and as seen for the inoculated wine, the eucalyptus extract delayed spontaneous MLF and 55% of the initial malic acid remained untransformed after 14 days of incubation. This slower consumption of malic

acid caused by the eucalyptus extract could be due to a longer lag period in the development of the enological LAB (Carreté et al., 2006).

A follow-up of the LAB population was monitored during the MLF experiments (Garcia-Ruiz et al., unpublished results). For both inoculated and non-inoculated wines, the eucalyptus extract led to the lowest CFU/mL values in comparison to the controls and the wines containing the other extracts. In other words, the eucalyptus extract reduced the LAB population, which was associated with the lowest consumption of malic acid. Therefore, in the conditions used in our MLF experiments, both fermentation starters and endogenous wine LAB seemed to be sensitive to the antimicrobial properties of the eucalyptus extract at 2 g/L. Although further experimentation at cellar scale is needed to verify it, to our knowledge, this is the first report of the application of natural extracts in the control of MLF in winemaking.

In summary, this paper reports valuable data on the antioxidant and antimicrobial properties of phenolic extracts from different plant origins. The survival parameter IC_{50} allows comparison of the antimicrobial activity of extracts from other sources or processing procedures, and against other enological bacteria. The results confirm that the antimicrobial activity of vegetable phenolic extracts is strongly dependent on phenolic content and composition as reported by other authors (Shoko et al., 1999; Jayaprakasha et al., 2003; Baydar et al.; 2004, and Özkan et al., 2004) and also on the enological bacteria genera and species assayed. In our case, the eucalyptus extracts and Amanda® (almond skins) showed a positive specificity against non-*O. oeni* strains, and pomegranate#1 and grape pomace#2 extracts demonstrated greater inhibitory effects against *O. oeni* strains. Another contribution of this study is the

application of these antimicrobial phenolic extracts in the control of MLF in an industrially obtained red wine. The results show that the eucalyptus extract delayed the consumption rate of malic acid with respect to the control, both in inoculated and non-inoculated wines. Antimicrobial phenolic extracts, such as the eucalyptus extract tested in this study, could constitute a promising alternative to sulphites in winemaking, although further studies are required in order to assess the impact of this application on the sensory properties of wine.

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1 **Table 1**. Phenolic extracts tested for antimicrobial properties.

Phenolic extract	TP (mg gallic acid/g)	ORAC (mmol Trolox/g)	*Phenolic extract*	TP (mg gallic acid/g)	ORAC (mmol Trolox/g)
***Spices* (n=5)**			***Legumes* (n=2)**		
Cinnamon	112	4.60	Red clover	165	5.98
Eucalyptus	89	1.22	Soy bean	136	7.14
Oregano	137	5.87	***Seeds* (n=4)**		
Rosemary	283	11.5	Grape seed #1	342	10.0
Thyme	147	4.72	Grape seed #2	131	3.95
***Flowers* (n=2)**			Grape seed #3	459	22.7
Camomile	46	1.72	Green coffee	183	6.90
Yarrow	74	1.94	***Skins* (n=6)**		
***Leaves* (n=15)**			AMANDA® (almond skins)	165	9.80
Currant bush leaves	74	1.40	Almond skins	195	9.01
Elder leaves	33	1.26	Red grape skins #1	230	2.91
Ginkgo biloba	168	7.10	Red grape skins #2	161	5.49
Green tea #1	292	6.27	Red grape skins #3	210	6.16
Green tea #2	215	4.78	Red grape skins #4	130	5.02
Green tea #3	537	14.7	***Agricultural by-products* (n=3)**		
Lady's mantle leaves	54	1.04	Grape pomace #1	374	13.3
Olive tree leaves	125	3.82	Grape pomace #2	508	21.4
OLIXXOL® (olive trees)	140	1.41	Eminol® (grape pomace)	34	1.43
Red tea	135	4.01	***Wine* (n=1)**		
Rock tea	87	2.11	Provinols™ (red wine)	474	14.5
Vine #1 leaves	84	2.55	***Purified tannins* (n=7)**		
Vine #2 leaves	60	2.19	Grape seed tannins	434	15.7
Vine #3 leaves	65	2.48	Grape skin tannins	349	16.0
Walnut tree leaves	43	1.41	Oak tannins	355	9.68
***Fruits* (n=8)**			Quebracho tannins	484	17.9
Acerola	177	1.30	Vitaflavan® (grape seed tannins)	629	21.4
Apple	373	7.53	Monomeric fraction from Vitaflavan®	750	40.6
Bilberry	291	10.9	Oligomeric fraction from Vitaflavan®	699	24.8
Bitter orange	37	1.65	***Other* (n=1)**		
CITROLIVE® (Citrus)	n.d.	7.72	Propolis	51	1.81
Citrus	126	9.54			
Pomegranate #1	422	8.42			
Pomegranate #2	68	0.22			

2 T.P. = Total polyphenols, ORAC = Oxygen radical absorbance capacity, n.d.: not determined

3

1 **Table 2**. IC_{50} data of the phenolic extracts active against two or more strains of
2 *lactobacilli*, *pediococci* and *O.oeni*

Phenolic extract	IC50 (g/L)					
	L.hilgardii **CIAL-49**	***L.casei*** **CIAL-52**	***L. plantarum*** **CIAL-92**	***P.pentosaceus*** **CIAL-85**	***O.oeni*** **CIAL-91**	***O.oeni*** **CIAL-96**
Spices						
Cinnamon	n.e.	n.e.	n.e.	n.e.	2.46	2.27
Eucalyptus	0.33	0.24	0.16	0.09	n.e.	1.9
Thyme	n.e.	2.92	n.e.	n.e.	n.e.	2.51
Leaves						
Ginkgo biloba	1.86	1.30	1.49	1.56	n.e.	0.82
Fruits						
Pomegranate #1	n.e.	n.e.	n.e.	n.e.	0.40	0.41
Legumes						
Soy bean	n.e.	1.02	0.78	2.34	n.e.	n.e.
Seeds						
Grape seed #1	0.56	n.e.	1.75	0.40	n.e.	n.e.
Grape seed #2	0.73	1.06	1.68	n.e.	n.e.	n.e.
Grape seed #3	2.69	n.e.	1.00	n.e.	n.e.	n.e.
Skins						
Almond skins	1.59	1.41	0.71	n.e.	n.e.	n.e.
AMANDA® (almond skins)	1.85	1.13	1.15	0.88	2.63	n.e.
Red grape #4	2.45	n.e.	n.e.	n.e.	n.e.	3.00
Agricultural by-products						
Eminol®(grape pomace)	2.79	2.88	1.60	2.07	n.e.	n.e.
Grape pomace #1	1.03	n.e.	0.54	n.e.	3.00	n.e.
Grape pomace #2	n.e.	n.e.	1.16	n.e.	1.64	1.68
Wine						
Provinols™ (red wine)	n.e.	1.56	1.17	1.70	n.e.	0.38
Purified tannins						
Grape seed tannins	1.22	0.55	0.41	1.21	1.05	0.66
Grape skin tannins	n.e.	n.e.	n.e.	n.e.	1.88	1.51
Oak tannins	n.e.	1.9	n.e.	n.e.	0.99	0.75
Quebracho tannins	1.10	1.14	2.82	0.99	0.94	0.89
Vitaflavan® (grape seed tannins)	1.09	n.e.	n.e.	n.e.	2.37	1.25
Monomeric fraction from Vitaflavan®	1.42	n.e.	0.67	0.83	0.97	1.12
Oligomeric fraction	n.e.	1.99	n.e.	2.35	0.95	0.74

from Vitaflavan®						
Other						
Propolis	1.05	1.39	0.94	0.72	2.32	0.91

n.e.: no effect

Table 3. IC_{50} data of selected phenolic extracts against acetic acid bacteria

Phenolic extract	**IC50 (g/L)**	
	***A. aceti* CIAL-106**	***G. oxydans* CIAL-107**
Eucalyptus	0.75	1.20
Ginkgo biloba	0.37	n.e.
Amanda® (Almond skins)	1.85	0.36
Grape seed tannins	1.19	0.52
Quebracho tannins	0.11	0.15
Propolis	2.25	n.e.

n.e.: no effect

Table 4. Percentage of disappearance of residual malic acid during MLF assays in wines.

	RESIDUAL MALIC ACID (%)				
	Inoculated MLF			Spontaneous MLF	
	After 14 days	After 19 days	After 24 days	After 14 days	After 19 days
Control	<0.03	n.d.	n.d.	40	<0.03
+ eucalyptus extract	10	<0.03	n.d.	55	<0.03
+ SO_2	89	35	<0.03	n.d.	n.d.

n.d.: not determined

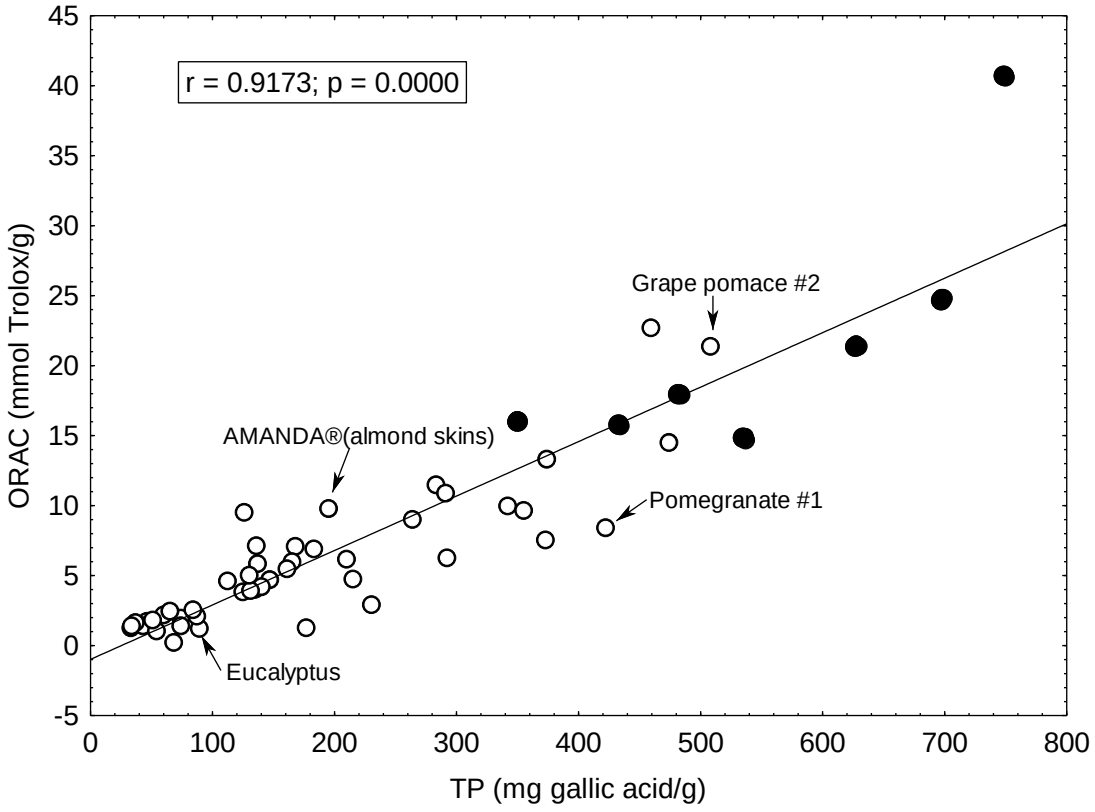
FIGURE LEGENDS

Figure 1. Representation of the antioxidant activity (ORAC value) of phenolic extracts *versus* total phenolic content. Empty circles correspond to plant extract whereas full circles correspond to purified tannins.

Figure 2. Electron micrographs of ultrathin sections of *O. oeni* CIAL 96 non-incubated and incubated with antimicrobial agents. A: control; B, C: incubation with grape seed tannins (1 g/L); D, E: incubation with red grape #4 (2 g/L). Bars = 1 μm (A, B, D), 0.5 μm (E), 0.2 μm (C).

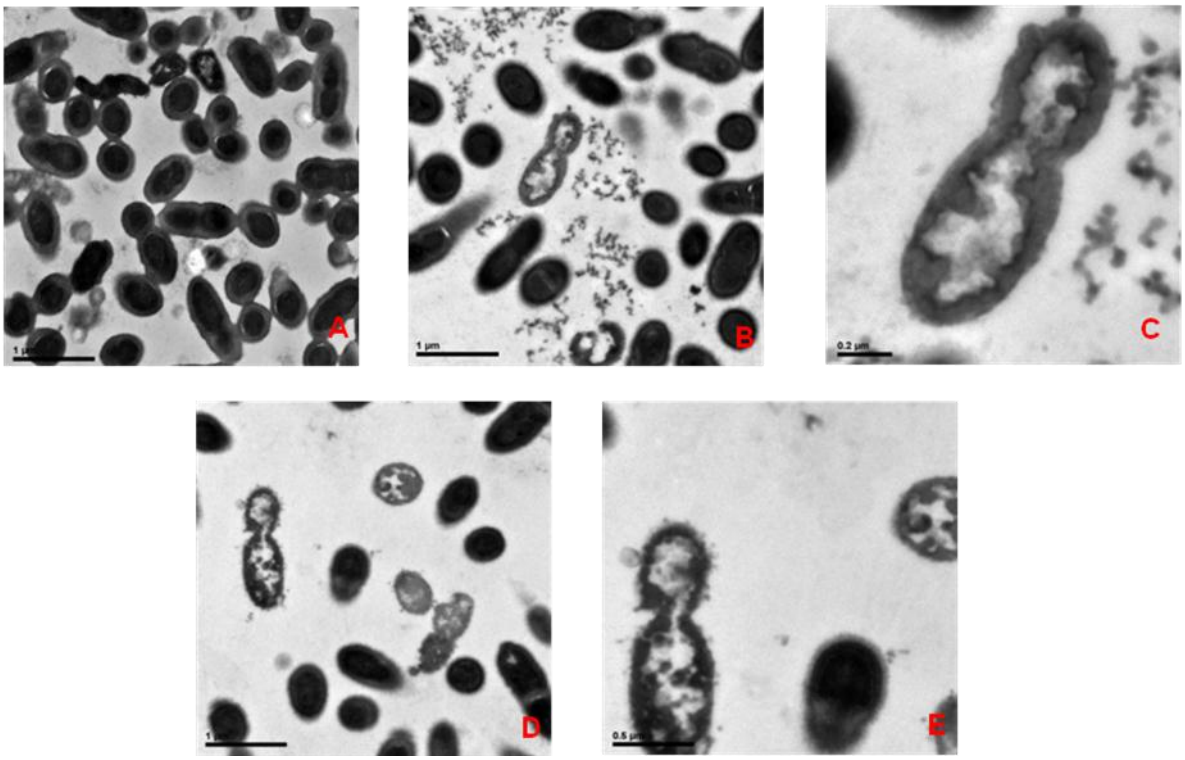
1

2 **Figure 1**


r = 0.9173; p = 0.0000
ORAC (mmol Trolox/g)
TP (mg gallic acid/g)
Grape pomace #2
AMANDA®(almond skins)
Pomegranate #1
Eucalyptus
45
40
35
30
25
20
15
10
5
0
-5
0
100
200
300
400
500
600
700
800

3

Figure 2



<u>Patente</u> Procedimiento de elaboración de vino que comprende adicionar un extracto fenólico de origen vegetal con propiedades antimicrobianas frente a bacterias lácticas y/o acéticas.

Begoña Bartolomé, Almudena García Ruiz, Carolina Cueva Sánchez, Eva González Rompinelli, Juan José Rodríguez Bencomo, Fernando Sánchez Patán, Pedro J. Martín Álvarez, M. Victoria Moreno-Arribas. Oficina Española de Patentes y Marcas. ES P201132134.

Resumen:

Esta invención se refiere al desarrollo de un procedimiento basado en el uso de un extracto fenólico de origen vegetal, durante la elaboración de vino con el fin de controlar el progreso de la fermentación maloláctica (espontánea o inoculada) en vinos tintos, o para controlar desde el punto de vista microbiológico la etapa de crianza en barrica de vinos blancos, evitándose o reduciéndose de este modo el empleo de sulfitos durante la vinificación. Los extractos empleados en la presente invención se caracterizan por mostrar propiedades antimicrobianas (IC_{50} máximo a 3,00 g/L) frente al menos dos especies de bacterias lácticas o acéticas de origen enológico. Así mismo, también muestran un contenido mínimo de polifenoles totales de 50 mg de ácido gálico/g y un valor ORAC mínimo de 1,00 mmol de Trolox/g. Preferiblemente, el procedimiento de elaboración de vino de la invención se caracteriza porque el extracto fenólico vegetal procede de un eucalipto y presenta un valor IC_{50} inferior a 0,5 g/L frente a las especies de bacterias lácticas *Lactobacillus hilgardii*, *L. casei, L. plantarum y Pediococcus pentosaceus.*

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Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica, utilizando la conexión segura de la O.E.P.M. Asimismo, se le ha asignado de forma automática un número de solicitud y una fecha de recepción, conforme al artículo 14.3 del Reglamento para la ejecución de la Ley 11/1986, de 20 de marzo, de Patentes. La fecha de presentación de la solicitud de acuerdo con el art. 22 de la Ley de Patentes, le será comunicada posteriormente.

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IV.4. Cambios en la composición aromática y polifenólica de vinos tratados con extractos fenólicos como antimicrobianos

En sendas experiencias en vinos (Sección IV.3), se encontró que la adición de determinados extractos de plantas ricos en compuestos fenólicos, en concreto, el obtenido de hojas de eucalipto, retrasaba el desarrollo de la FML en vinos tintos, y permitía controlar, desde el punto de vista microbiológico, la etapa de crianza en barrica de vinos blancos, reduciéndose de este modo el empleo de sulfitos durante la vinificación. Antes de pensar en la aplicación real de estos extractos antimicrobianos, era necesario comprobar que la adición de los mismos no produciría modificaciones indeseables en las propiedades organolépticas del vino.

En vista de ello, nuestro siguiente objetivo fue estudiar los posibles cambios organolépticos en los vinos tratados con extractos fenólicos como antimicrobianos. Dentro de los componentes del vino, las fracciones arómatica y polifenólica son, sin duda, las que condicionan las características organolépticas del vino tinto, especialmente el aroma, "*flavour*" y color del mismo (Ribéreau-Gayon et al, 2006). Por tanto, nuestro estudio se centró en los principales compuestos del aroma y compuestos fenólicos presentes en el vino, que incluía esteres, alcoholes, terpenos, C13 nor-isoprenoides, ácidos, fenoles volátiles y lactonas y compuestos furanóicos en el caso de los compuestos de aroma, y antocianos, flavan-3-oles, flavonoles, estilbenos, ácidos y derivados hidroxicinámicos y ácidos benzoicos, en el caso de los compuestos fenólicos.

Los vinos estudiados se refieren a la experimentación descrita en la sección IV.3, en la que se llevó a cabo la FML (inoculada y espontánea) de un vino tinto en presencia del extracto de eucalipto. En este caso también se incluyó una experimentación paralela llevada a cabo con el extracto de piel de almendra en lugar del de eucalipto.

Dado que nuestro propósito era obtener una perspectiva general de los cambios en la composición volátil y fenólica como consecuencia del tratamiento del vino con los extractos antimicrobianos, también se llevó a cabo la aplicación de diferentes tratamientos estadísticos de análisis multivariante a los datos de concentración de los compuestos del aroma y polifenoles individualizados.

A continuación se presentan los resultados de este estudio en forma de una publicación:

Publicación V. Cambios en la composición volátil y fenólica de vinos tratados con extractos vegetales antimicrobianos antes de la fermentación maloláctica.

<u>Publicación V.</u> Cambios en la composición volátil y fenólica de vinos tratados con extractos vegetales antimicrobianos antes de la fermentación maloláctica.

Almudena García Ruiz, Juan José Rodríguez Bencomo, Ignacio Garrido, Pedro J. Martín Álvarez, M. Victoria Moreno Arribas, Begoña Bartolomé. Changes on the volatile and phenolic composition of wine treated with antimicrobial plant extracts before malolactic fermentation (en preparación).

Resumen:

Recientemente se ha propuesto el empleo de extractos vegetales ricos en polifenoles como alternativa a los sulfitos para el control de la fermentación maloláctica (FML). Sin embargo, existe la preocupación de que esta adición de extractos vegetales al vino pueda influir sobre las propiedades organolépticas del vino. En este estudio, se adicionaron dos extractos fenólicos comerciales, hojas de eucalipto y pieles de almendra, a un vino tinto una vez finalizada la fermentación alcohólica. Se evaluaron cambios sobre la composición volátil y fenólica de los vinos después de la FML, ya fuera inducida por inoculación de bacterias o llevada a cabo de forma espontánea y se compararon con los vinos elaborados sin adición (vino control). Respecto al perfil volátil, la mayor diferencia se observó en los vinos tratados con extractos fenólicos e inoculados con *starter* maloláctico. Por otro lado, la composición fenólica no exhibió diferencias significativas entre los vinos estudio, destacando el contenido de algunos compuestos fenólicos como la quercetina, podría relacionarse con la composición fenólica del extracto adicionado, especialmente extracto de eucalipto. Estos resultados proporcionan una base para el conocimiento químico de los vinos elaborados con extracto fenólico como agente antimicrobiano.

Changes on the volatile and phenolic composition of wine treated with antimicrobial plant extracts before malolactic fermentation

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Abstract

Plant extracts rich in polyphenols have recently been proposed as an alternative to sulphites in the control of malolactic fermentation (MLF) in wine. However, a concern that arises about this addition of plant extracts to wine is that it may affect wine organoleptic properties. In this study, two commercial phenolic extracts from eucalyptus leaves and almond skins have been added to a red wine after alcoholic fermentation. Changes on wine volatile and phenolic composition were evaluated after MLF, either induced by inoculated bacteria or carried out spontaneously, and in comparison to the wines not subjected to any addition (control wine). In reference to volatile profile, the greatest variation was observed in the wines treated with phenolic extracts and inoculated with malolactic starter. Furthermore, the content of some esters (ethyl butyrate, ethyl hexanoate, isobutyl acetat and isoamyl acetate) reduced >75% after MLF whereas the concentration of some terpenes and volatile phenol increased in the wines treated with phenolic extracts. On the other hand, the phenolic composition didn't exhibited important significant differences in the wines studied, highlighting the content of some phenolic compounds such as quercetin, could be related with the phenolic composition of the extracts added, especially eucalyptus extract. These results provide a basis in the knowledge of the chemistry composition of wines elaborated with phenolic extract as antimicrobial agent.

1. Introduction

Malolactic fermentation (MLF) in wine is a microbiological process that transforms the L-malic acid in L-lactic acid by the action of lactic acid bacteria (LAB). Normally, MLF is a secondary process which usually follows primary (alcoholic) fermentation of wine but may also occur concurrently. The MLF can occurs spontaneously, however, the use of starter cultures have become of great interest since it could ensure the successful completion of MLF avoiding or reducing the potential risk of the spontaneous MLF that could affect to the quality of the final product (Costantini et al., 2009; Lerm et al., 2010; Swiegers et al., 2005).

In addition of a reduction of the acidity, MLF also positively contributes to the microbial stability and organoleptic wine quality (Costantini et al., 2009; Swiegers et al., 2005). With regard to the sensorial characteristics of wines, in general the studies indicate that the effect of MLF induces a creamier palate, less fruit intensity and more butteriness. On the other hand, other authors have observed that MLF enhanced fruity and buttery notes and reduced the green and grassy aromas (Henick-Kling et al., 1993; Liu, 2002). These effects on the sensorial characteristics could depend on several factors, such as the LAB strain, grape variety and the winemaking practices. The global process of MLF could produce new volatile compounds from the grape compounds such sugars, amino acids etc, transform volatile compounds from grape and generated during alcohol fermentation (AF), and the adsorption phenomena of volatile compounds to wall cells could result in a decreasing of the effective concentration of the volatile compound in the wine headspace (Bartowsky et al., 1995; Lerm et al., 2010; Swiegers et al., 2005). The MLF process should be maintained under control in order to avoid bacterial alterations including the production of undesirable aromas such as volatile phenols (Cavin et al., 1993; Etievant, 1991) and other compounds such as acetic acid

and acetaldehyde by acetic bacteria. In addition, the production of biogenic amines by LAB should be avoided since these compounds could be toxic in sensitive humans (Moreno-Arribas et al., 2009). In order to avoid these problems, the use of sulpites (SO_2) is nowadays widely used in winemaking. Sulphites present interesting preservatives properties such as antioxidant and antimicrobial effects, especially against LAB. However, their use is strictly controlled, since high doses can cause organoleptic alterations in the final product and, especially, owing to the risks to human health of consuming this substance. For that reason, the maximum content of total SO_2 are regulated by European Union (Ruling 1622/2000) limiting to 160 mg/L and 210 mg/L in red and white wines, respectively. In addition, the specification in the label of the presence of sulphites has been recently regulated in the UE (Ruling 1991/2004).

Seeking for alternatives to sulphites is a matter attracting interest from researchers and winemakers (Santos et al., 2012). Dimethyldicarbonate (DMDC), lysozyme and some bateriocins (Nisin and Pediocin) have been considered for several authors as potential antimicrobial alternatives (Bartowsky, 2009; du Toit et al., 2002; Rojo-Bezares et al., 2007; García-Ruiz et al., 2008). On the other hand, phenolic extracts of vegetable origin have been reported to have a variety of biological effects, including antioxidant, anticarcinogenic, anti-inflammatory and antimicrobial activities (Papadopoulou et al., 2005; Baydar et al., 2004; Rodriguez-Vaquero et al., 2007; Garcia Ruiz et al., 2008). Moreover, some studies have evaluated the potential use of the polyphenols wine as alternative antimicrobial agent to sulphites in winemaking (García-Ruiz et al., 2009, 2011). In a previous paper, and after screening a great number of plant extracts for antimicrobial properties against LAB in pure cultures, we tested technological applicability of an extract from eucalyptus leaves during the MLF of a red wine (García-Ruiz et al., submitted). The progress of both inoculated and spontaneous MLF was

found to be delayed by the addition (2 g/L) of the eucalyptus extract, in comparison to the control wine (García-Ruiz et al., submitted). However, a concern that arises about this effective addition of plant extracts to wine is that it may affect wine organoleptic properties. Therefore, the aim of this study was evaluate the changes in the volatile and phenolic composition of wines after being treated with antimicrobial plant extracts during MLF. Apart from the eucalyptus extract previously tested in MLF experiments, a second extract from almond skins – also active against the growth of enological LAB strains (García-Ruiz et al., submitted) - was also selected for the study. MLF experiments induced by inoculated bacteria or spontaneously, were carried out in parallel. To get a global idea of the changes of the addition of the extracts on the volatile and phenolic composition of wines, data of individual compound concentration were also subjected to multivariate analysis.

2. Materials and methods

2.1. Reagents and Solvents

Absolute ethanol p.a. was from Merck (Darmstadt, Germany) and pure water was obtained from a Milli-Q purification system (Millipore). L-(+)-tartaric acid, sodium chloride and sodium hydroxide were from Panreac (Barcelona, Spain). Pure volatile compounds were supplied by Aldrich (Gillingham, UK), Fluka (Buchs, Switzerland), Riedel de Häen (Seelze, Germany) and Firmenich (Geneva, Switzerland). Pure phenolic compounds were purchased from Sigma (St. Louis, MO, USA), Extrasynthèse (Genay, France), Fluka (Buchs, Switzerland) and Aldrich (Steinheim, Germany). The phenolic extracts, eucalyptus leaves and almond skins, were kindly provided by its producer Biosearch Life S. A. (Granada, Spain).

2.2 Microvinification

A red wine (var. *Merlot*) (vintage 2009) was elaborated at Bodegas Miguel Torres S.A. (Catalonia, Spain), following their own winemaking procedures (García-Ruiz et al., submitted). The AF was carried out in a controlled form in stainless steel at 25 ± 2 ºC. The end of AF was established by measuring the alcohol degree (13.9 % v/v) and the residual sugar amount (< 3.5 g/L); the wine pH at the end of AF was 3.22. MLF experiments were conducted in laboratory scale, sterile conditions, in 250-mL flasks. Parallel inoculated and spontaneous MLF assays were carried out. Two selected extracts (eucalyptus leaves and almond skins) were dissolved (2 g/L) in 200 mL of previously inoculated or non-inoculated wine. The malolactic starter was comprised by a mix of three *O. oeni* strains previously isolated by the winery, and was inoculated in wine at 3% (v/v). A control containing no extract was also prepared for both inoculated and spontaneous MLF assays. Wines containing phenolic extracts and control wines were incubated at 25 ºC in the dark. All the MLF assays were performed in duplicate.

Samples to wine volatiles and phenolic analysis were taken after inoculation and at the end MLF. Samples were kept in a freezer (-20ºC) until analysis.

2.3 Volatile composition analysis.

Eight mL of wine sample, forty μL of an internal standards solution (3,4 dimethylphenol, 400 mg/L; 3-octanol, 10 mg/L; and methyl nonanoate, 2.5 mg/L) and 2.3 g of NaCl were added to 20 mL SPME vials and they were sealed with PTFE/Silicon septum (Supelco).

The samples were extracted by SPME fiber of 2 cm length (DVB/CAR/PDMS, Supelco. Bellefonte, PA. USA) and were analyzed by GC-MS. The extraction and chromatography condition were described by Rodríguez-Bencomo et al. (2011). All the analyses were performed in duplicate.

2.4. Phenolic compound analysis

2.4.1. Determination of total phenolic content

The method of Singleton and Rossi (1965) was used for determining the total phenolic content of the extracts. The results were expressed as mg of gallic acid equivalents per gram of extract. The analysis was performed in triplicate.

2.4.2. Analysis of anthocyanic compounds

The analysis of anthocyanic compouds was made according to Monagas et al (Monagas, et al. 2005a) employing a liquid chromatograph Waters (Milford, MA) equipped with a Controller 600-MS, and automatic injector 707 Plus, and a diode array detector (DAD) 996. Determinations were made by duplicate.

2.4.3. Analysis of non-anthocyanic compounds

For the identification and quantification of individual phenolic compounds, a Waters (Milford, MA, USA) liquid chromatography system equipped with a 2695 Alliance separation module, a 2996 DAD, and a 2475 fluorescence detector was used. Separation was performed on a reversed-phase Waters Nova-Pak C18 (250 mm x 4.6 mm, 4μm) column at room temperature. A gradient consisting of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2, v/v/v) was applied as follows: from 0 to 55 min, 100-20 %A, 0-80 %B, 1 mL/min; from 55 to 65 min, 20-0 %A, 80-0 %B, 0-100 % methanol, 1-1.2 mL/min, from 65 to 75 min, 100 % methanol, 1.2 mL/min; and re-equilibration of the column from 75 to 95 min. The DAD detection conditions were 210-360 nm. Quantification was carried out at 280 nm by external standard calibration curves. Due to the lack of commercial standards, hydroxycinnamic derivatives were quantified using the free acid calibration curve, and procyanidins were quantified using the (+)-catechin (Sigma, St. Louis, MO, USA) calibration curve. Analysis was carried out in duplicate.

2.5. Statistical analysis.

The statistical methods used for the data analysis were: Principal Component Analysis (PCA), from standardized variables, to explore the relationship between analyzed variables and between samples; one-way Analysis of Variance (ANOVA) for test the effect of the factor studied (innoculated conditions); Least Significant Difference (LSD) test to compare mean values within each group; and Dunnet test to compare mean values within each group with mean value before MLF. STATISTICA program for Windows version 7.1 was used for data processing (StatSoft, Inc., 2005, www.statsoft.com).

3. Results and discussion

3.1. Volatile compounds

Main wine volatile compounds determined in the wines treated and not treated with antimicrobial plant extracts, corresponded to esters (n=14), alcohols (n=5), terpenes (n=6), C13 nor-isoprenoids (n=3), acids (n=2), voltatile phenols (n=6) and lactone and furanic compounds (n=2) (Table 1). Other compounds such as α- and β-pinene, 5-methylfurfural, and *trans*- and *cis*-whiskey lactone were not detected in any of the wines analysed.

Regarding to the esters, it was first observed that, in general, wines after MLF fermentation (either induced by malolactic starter or carried spontaneously) showed lower content than the wines before this process (Table 1). This was particularly noticeable for ethyl butyrate, ethyl hexanoate, isobutyl acetat and isoamyl acetate whose reduction in its content was >75%. Only ethyl lactate and diethyl succinate experimented great increases after MLF: 6.5 and 7.5 fold higher increase for ethyl lactate, and 10 and 2 fold higher for diethyl succinate, respectively for control wines after inoculated or spontaneous MLF. This increase was coupled to succinic and lactic

acid production during MLF (Ugliano and Moio, 2005) or by hydrolysis in acid medium of the esters. The decrease of concentration after MLF observed for the other esters could be explained by the esterase activity of LABs that has been described by different authors (Davis et al., 1988; Matthews et al., 2007). Some changes were observed in the concentration of ethyl esters and acetates after MLF in the presence of antimicrobial phenolic extracts from eucalyptus leaves and almond skins (Table 1). For both, inoculated and spontaneous MLF, ethyl butyrate, ethyl lactate, butyl acetate and isoamyl acetate showed significantly lower concentration in the wines treated with the eucalyputs extract than in the control wines and the wines treated with the almond extract, with the exception of butyl acetate in the wines treated with the almond extract and subjected to spontaneous MLF. Significant decreases were also observed for ethyl hexanoate and β-phenylethyl acetate in the wines treated with the eucalyptus extract and inoculated with the malolactic starter, but not for the spontaneous MLF. Characteristic of the addition of the almond extract to wines was their significantly higher content of ethyl octanoate, ethyl decanoate, ethyl dodecanoate and ethyl lactate, with the exception of ethyl octanoate and ethyl decanoate in wines carried out spontaneous MLF that showed no significant differences. Addition of both eucalyptus and almond extracts promoted higher concentration of diethyl succinate in wines after MLF in comparison than the control, although differences among the three types of wine were only significant for the spontaneous MLF. Differences in the concentration of esters in wine were explained in terms of the capacity of plant extracts to influence the growth and/or metabolism of LAB, in this way the addition of antimicrobial phenolic extracts could promote an enhancement in the production of succinic acid and hence a higher concentration of diethyl succinate in the treated wines, especially in spontaneous MLF. On the other hand, the lower concentration of ester in the wines treated with eucalyptus

extract could suggest a higher esterase activity in presence of this phenolic extract. Moreover, the inoculated bacteria starter seemed to the more sensitive to the action of the plant extracts since greater changes were observed in the wines subjected to inoculated MLF in comparison to wines carried out spontaneously MLF. This confirms the result obtained in a previous study (Garcia-Ruiz et al., submitted), we showed a different LAB enological susceptibility to the antimicrobial phenolic extract in the medium culture.

As an approach to evaluate the impact on wine aroma of the changes in esters concentration observed after MLF, we calculated the odour activity values (OAV) using the following formula: OAV = Compound concentration/ Compound odour thresholds concentration (bibliography). The OAVs were expressed as unit of aroma (u.a.) (Table 2). Before MLF, the compounds: ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl cinnamate and isoamyl acetate with OAV upper to 10 u.a. were the esters with strong sensorial importance. After MLF, the OAV of all these compounds decreased which contribute to equilibrate the fruity aroma notes of the wines (Etievant, 1991). In the control wines, the total OAV of ester at the end MLF were lower than before MLF, decreasing a 59.2% and 72.1% for inoculated and spontaneous MLF, respectively. The wines treated with eucalyptus extract showed total OAV similar, while the wines added from almond extract exhibited total OAV 2 fold higher in the wine inoculated with malolactic starter (222 units of aroma [u.a.]) than in the wine carried out spontaneous MLF (104 u.a.). Therefore, fruity aromas may be more intense in wines treated with the almond extract, higher total OAV, than in the control wines and wines elaborated with eucalyptus extract.

In relation to the alcohols present in wine, some variations were observed after MLF for either inoculated or spontaneous fermentations (Table 1), although significant

differences were only observed for benzyl alcohol in the spontaneous MLF (a 53% increase for the control wine). After MLF, addition of the eucalyptus extract seemed to reduce the concentration of alcohols in comparison to the control, which is especially noticeable for *cis*-3-hexen-1-ol in the experiment of inoculated MLF (37 % decrease) and for benzyl alcohol in both fermentation experiments (30 and 20% decrease, respectively for inoculated and spontaneous MLF). As seen for some esters, addition of the almond extract led to higher concentrations for benzyl alcohol and β-phenylethyl alcohol in the wine after MLF, although differences were only significant for benzyl alcohol in the case of the spontaneous MLF (17 % increase) and β-phenylethyl alcohol in the case of inoculated MLF (11 % increase). The origin of C6 alcohols (1-hexanol and *cis*- and *trans*-3-hexen-1-ol) may be the enzymatic degradation of unsaturated fatty acids in the grape and the glycosidic aroma precursors too (Etievant, 1991; Baumes, 2009; Ferreira et al., 1995). Concerning benzyl and β-phenylethyl alcohols, their origin should be due to acid or enzymatic hydrolysis of glycosidic aroma precursors or from hydrolysis of esters. The results obtained suggest that the content of these alcohols during MLF depend of the enzymatic on the LAB strains (Ugliano et al., 2003; Izquierdo-Cañas et al., 2008; Hernández-Orte et al., 2009) and other chemical reactions. In any case, the contents of the different alcohols in the wines after MLF were lower than their odour thresholds, except for β-phenylethyl alcohol that showed in all wines aroma values between 3 and 4 a.u. (aroma units) (odour threshold 14 mg/L; Aznar et al., 2003); therefore, this compound could influence the total wine aroma with rose notes (Escudero et al., 2007).

Terpenes and C13 nor-isoprenoids have a varietal origin and they could be present as free volatiles or as glycosidic aroma precursors. These glycosidic aroma precursors could liberate their aglycones during the winemaking or storage of wine by acid or

enzymatic hydrolysis (Etievant, 1991; Baumes, 2009). Several authors have described glycosidase activity of strains of LABs (Grimaldi et al., 2000; 2005a; b; Barbagallo et al., 2004; Hernández-Orte et al., 2009; Gagné et al., 2010) that could produce the liberation of active aromas from their aroma precursors. In our wines, only three of the terpenes analyzed could be quantified: linalool, β-citronellol and nerol, being also detected to level of trace the compounds α-terpineol and limonene (spontaneous MLF). After MLF, significant differences were only observed for linalool in the inoculated MLF (a 21% increase for the control wine). As can be seen, terpinen-4-ol and α-terpienol (inoculated MLF) only were detected in wines treated with eucalyptus extract, being the concentration of terpinen-4-ol 4.5 fold higher in the inoculated wine than in the wine subjected to spontaneous MLF. On the other hand, the lowest concentration of nerol, 2.68 μg/L, was observed in the wine inoculated with malolactic starter and added from eucalyptus extract. In reference to the experiment with almond extract, the concentration of the β-citronellol in the inoculated wine showed significant difference for the control wine before MLF (a 25% increase). In the case C13 nor-isoprenoids, at the final MLF the concentration of α-ionone in both control wines, inoculated and spontaneous MLF, was 2 fold lower than before MLF; while the content of β-damascenone increased slightly (14%) in the wine inoculated with malolactic starter. Furthermore, the wines treated with eucalyptus extract were characterized to show the lowest levels of α-ionone, not being possible to detect this compound in them. In the case of wine elaborated with almond extract, the content of α-ionone was higher (20%) than in the control wines. These results suggest that the glycoside activity of the BAL strains could be more affected by the addition of plant extracts, especially by the eucalyptus which might enhance to the release of bound aroma compounds during

wine-making. The liberation of terpenes and C13 nor-isoprenoids from the aroma precursors by LABs in a model medium during MLF has been previously reported by other authors (Ugliano et al., 2003; Hernández-Orte et al., 2009). In addition of enzymatic processes, other chemical processes could affect to the levels of this varietal compounds such as oxidations in the case of terpenes and chemical reactions in acid medium in the case of C13 nor-isoprenoids (Papadopoulou et al., 2008; Ribereau-Gayon et al., 2006).

All analyzed terpenes presented content lower than their odour thresholds, however among the C13 nor-isoprenoids analyzed (odour thresholds for β-damascenone and α-ionone, 0.05; 2.6 μg/L, respectively; Aznar et al., 2003), β-damascenone and α-ionone were present in contents higher than their odour thresholds in all wines so they contribute to the aroma of these wines.

The main origin of volatile acids in wines is AF and their contents depend on the fermentation conditions, nutrient levels of the musts and the type of yeast used (Ugliano et al., 2009). The formation of volatile acids from lipids during MLF due to lipase activity of LABs has been suggested by Davis et al., 1988. In this study, the content of volatile acids in the control wines didn't change significantly during MLF but some changes were observed in the wines treated with antimicrobial plant extract. With regard to the addition of eucalyptus extract, only the wines subject to inoculated MLF showed significant differences with respect to the control wine, reducing the content of octanoic acid (23%) and increasing the concentration of hexanoic acid (18%). This increment in the content of hexanoic acid was also detected in the wines elaborated with almond extract and inoculated with malolactic starter (a 20% increase for the control wines). In other published works both effects, decrease and increase of concentration of hexanoic and octanoic acids, have been observed (Maicas et al., 1999; Ugliano et al.,

2005; Jeromel et al., 2008; Pozo-Bayón et al., 2005). As the same as esters and alcohols, acids could be involved in many chemical reactions during MLF. Considering the sensorial importance of acids in final wines, the two quantified acids were present in concentrations higher than their odour thresholds (odour thresholds for hexanoic and octanoic acids: 0.42 and 0.5 mg/L, respectively; Escudero et al., 2007). These acids could contribute to the freshness and to equilibrate the fruity aromas of wines (Etievant, 1991).

The volatile phenols analyzed in the control wines, except 4-ethyguaiacol, didn't show significant changes during the MLF. Vinylphenols could be originated during MLF from phenolic acids. In addition, the vinylphenols formed could be enzymatically reduced to the ethylphenols (Lerm et al., 2010). Moreover, these vinylphenols may be originated during MLF from glycosidic aroma precursors (Hernández-Orte et al., 2009) or by yeast of the genera *Brettanomyces* (Etievant, 1991; Suarez et al., 2007). The ability of LABs to produce volatile phenols has been suggested by Nelson, 2008. With respect to the experiment with eucalyptus extract, these wines showed the highest content of volatile phenols (except for 4-vinylphenol), being remarkable the strong increase of the levels of 4-ethylphenol (from 8.34-8.37 to 25.1-30.1 μg/L) and 2,6-dimethoxyphenol (3 and 2 fold higher in the inoculated and spontaneous MLF, respectively, with respect to the control wine) . In contrast, the lowest concentration of 4-vinylphenol (7.18 μg/L) were detected in the wine added with eucalyptus and subjected to inoculated MLF. In addition, 4-ethylguaiacol and 2,6-dimethoxyphenol also presented higher content in the wines added from almond extract than the control wines. These results suggest that the addition of phenolic extracts, especially eucalyptus extract, could affect to the formation and transformation of these compounds during MLF process; in addition in some cases as i.e. 2-methoxy-4-vinylphenol also could

exist a slight cession of this compound from the phenolic extract. Considering the sensorial importance of these volatile phenols, 2-methoxy-4-vinylphenols and eugenol were present at levels higher than their odour thresholds, so could affect to the aroma wine with phenolic, smokey, clove, almond shell and chemical notes (Culleré et al., 2004; Flavornet).

Among the lactones and furanic compounds, only γ-nonalactone was quantified in all samples. During MLF, the content γ-nonalactone in the control wines increased (20%) significantly in the wine inoculated with malolactic starter. This enhancement was also observed in the wines treated with platn extracts. In the case of the wine added from eucalyptus, the concentration γ-nonalactone increased slightly (12%) only in the wine subject to spontaneous MLF while in the wines treated with almond extract this increment was higher to the 50% in both fermentations. This same behavior is reflected on the differences observed among treatments. Lactones could originate during AF, glutamic acid being a possible precursor of γ-lactones, although the formation mechanisms are not clear (Ugliano et al., 2009). Moreover, the origin of this compound also could be the grape aroma precursors present in the wine (Hernández-Orte et al., 2009). In all cases the amounts of γ-nonalactone were below of its odour thresholds (30 μg/L, Escudero et al., 2007).

3.3. Phenolic compounds

Phenolic compounds analysis constitute one of the most important quality parameters of red wines since they contribute to their organoleptic characteristics, particularly colour, astringency, and bitterness (Condelli et al., 2006; Hufnagel et al., 2008 a; b; Monagas et al., 2006). During maturation and aging of wine, phenolics compounds participate in numerous chemical reactions, some of them related to changes in the aroma as the

generation of volatile phenol and interactions π– π that alter the volatility of some compounds due to interaction between the π electron clouds (Terrier et al., 2009). On the other hand, the contents of phenolic compounds can affect the growth of LAB (Vivas et al., 2000), acting as activators or inhibitors of bacterial growth depending on their chemical structure and concentration (Reguant et al., 2000; Vivas et al., 1997; Campos et al., 2003; García-Ruiz et al., 2009; 2011). Table 4 shows results obtained for the determination of TP content and the phenolic compounds (anthocyanin and non-anthocyanin) analysis of red wines tested. As can be seen the TP content of the wines studied didn't change during MLF. Some significant difference was observed in the presence almond extract, showing these wines slight increase TP content (≈10%) than the control wine before MLF.

The anthocyanins are the red pigments of grapes and responsible for the colour of red wines. In our study, the anthocyanins identified, a total of 14 compounds, were: 3-glucosides and 3-acetyl glucosides of delphinidin, cyanidin, peonidin, petunidin and malvidin, as well as the 3-*p*-coumaroyl-glucoside of delphinidin, peonidin, petunidin and malvidin. The table 4 shows the concentration (mg/L) of these compounds before and after MLF. As observed, the simple glucosides and malvidins were the most abundant groups of the anthocyanins in the different wines studied, being malvidin-3-glucoside the major compound (García-Falcón et al., 2007; Monagas et al., 2005a; Moreno-Arribas et al., 2008; Šeruga et al., 2011). As can be noted, at the end of MLF the total anthocyanins content and the concentration of the different anthocyanins decreased, except delphinidin-3-(6-acetyl)-glucoside in the wine inoculated with malolactic starter, perceiving a more declined in the concentration of 3-*p*-coumaroyl-glucoside anthocyanins (>40 and 30% in the inoculated and spontaneous MLF, respectively). This reduction in the content of anthocyanin has also been described by

others authors (Barata et al., 2011; García-Falcón et al., 2007; Monagas et al., 2005a) and could be due mainly to its participation in numerous chemical reactions during the MLF; related especially with the changes in the colour and astringency of wines (Monagas et al., 2005a, 2006). Several differences, especially in the spontaneous MLF, were observed in the wines elaborated with antimicrobial phenolic extract. The experiment realized with eucalyptus extract and subjected to spontaneous MLF was characterized by show changes in the concentration of the compounds: petunidin-3-(6-acetyl)-glucoside, peonidin- and malvidin-3(6-*p*-coumaroyl)-glucoside for the control wine, increasing slightly their concentration (≈10%). In addition, the wine elaborated with almond extract and inoculated with malolactic starter showed a significant increase in the concentration of delphinidin-3-(6-acetyl)-glucoside, 46%, respect to control wine. In contrast, the wines treated with this extract and carried out spontaneous MLF exhibited a lower concentration (≈ 20%) of petunidin-3-(6-acetyl)-glucoside and 3-*p*-coumaroyl-glucoside of delphinidin, peonidin, and malvidin than the control wine.

A total of 17 different non-anthocyanin phenolic compounds were detected in the wines studied (table 4), which according to their chemical structure can be classified into six groups: hydroxybenzoic acids (HBA) and ester (n=3), hydroxycinnamic acids (HCA) (n=4), phenolic alcohol (n=1), stilbenes (n=4), flavan-3-ols (n=3) and flavonols (n=2). As can see in the table 4, the flavan-3-ols was the most abundant group of the non-anthocyanin phenolic compounds in the different wines studied, and the catechin the major compound. At the end of MLF, the wines studied showed a slight reduction in the total content of non-anthocyanin phenolic compounds (<10%).

After MLF the content of some HBC and ester changed in the control wines, increasing the concentration of ethyl gallate in both fermentations and of gallic acid in the wine inoculated with malolactic starter. These compounds are characterized by contribute to

taste of wine, especially astringency. In the experiment with antimicrobial plant extracts, the wines treated with eucalyptus extract exhibited the highest concentration of gallic acid, 36.9 and 35.9mg/L inoculated and spontaneous MLF, respectively. The content of this phenol also increased slightly (10%) in the wines treated with almond extract and subject to MLF but decreased (10%) in the wine inoculated with malolactic starter. The gallic acid originates from the grape itself and by hydrolysis of hydrolyzable and condensed tannins (Terrier et al., 2009). These results suggest that the formation the gallic acid by hydrolysis of hydrolyzable and condensed tannins could be more affected by the eucalyptus extract than by the almond extract. Furthermore, the concentration of ethyl gallate increased slightly in the wine elaborated with eucalyptus and subject to inoculated MLF. The gallic and protocatechuic acids presented contents lower than their sensory thresholds (Table 5).

The color of red wine is also strongly influenced by the presence of HCA, playing an important role in the phenomenon of copigmentation. These compounds, especially ferulic and *p*-coumaric acids, are also well known to be transformed into volatile phenols (4-ethylguaiacol and 4-ethylphenol) by LABs (Cavin et al. 1993; Gury et al. 2004; Couto et al. 2006) and by affect the growth of LABs during winemaking. In our experiment, the content of caffeic acid in the control wines showed a different behavior in function of the type of MLF, increasing significantly (≈50%) in the inoculated wine and decreasing slightly (≈10%) in the wine carried out spontaneous MLF. Moreover, at the end MLF the concentration of *trans*-caftaric and *trans*-coumaric acids were lower in the wine inoculated with malolactic starter and subject to spontaneous MLF, respectively. With regard to addition of plant extracts, the greatest differences were observed in caffeic acid whose concentration decreased in the wine treated with eucalyptus and inoculated with malolactic starter (20%) and in the wine elaborated with

almond extract, especially in the spontaneous MLF (30%). This result suggests that the addition of phenolic extracts could interfere in the reactions of caffeic acid copigmentation. Only the *trans*-caftaric showed content higher than their corresponding astringency thresholds (table 5).

The tyrosol was the only phenolic alcohol distinguished by HPLC-DAD-fluorescence in the wines studied, its concentration in the control wines didn't change during MLF. With regard to the experiments with plant extracts, in the wines treated with eucalyptus extract was observed a slight descent (10%) in the content of tyrosol, whereas in the wines added with almond extract the content of this phenol increased, especially in the wine subject to MLF (34%). The tyrosol is characterized by be an antioxidant potent.

Regarding to resveratrol was detected that the concentration of different forms of the *trans*-isomer reduced in the control wines during MLF, especially in the wines subject to spontaneous MLF whereas the content of the *cis*-isomer increased, particularly in the wines carried out spontaneous MLF. In the wines elaborated with antimicrobial phenolic extract the maximum difference was observed in the concentration of *trans*-resveratrol that were higher in these wines than the control wines, above all in the wines treated with eucalyptus (>35%). This compound has showed antimicrobial properties against LABs (García-Ruiz et al., 2009; 2011) and it has been intensively studied and marked biological activities with regard to the prevention of cardiovascular disease and cancer have been reported (Ito et al. 2003), so a higher content of this compound in wines treated with the extract, especially eucalyptus extract, would be a positive aspect to highlight.

The flavonoids (flavanols and flavonols) are characterized by their beneficial effects on health and by powerful antioxidant. Moreover, the flavonoids contribute to taste of wine, especially astringency and bitterness, and they have exhibited antimicrobial effect

about the development LABs (García-Ruiz et al., 2009; 2011). These compounds are present in red wines in aglycon form, as the glycosides are hydrolyzed during fermentation. In the case of the flavan-3-ols, the concentration of these compounds in the control wines declined after MLF, to exception the Pro C1 in the wine incoulated with malolactic starter. This behavior has also been described by other authors (Gómez-Plaza et al., 2000; Pérez-Magariño et al., 2004; Monagas et al., 2005b, 2006). The wines treated with eucalyptus extract and inoculated with malolactic starter were characterized to show a slight descent (10%) in the content of epicatechin and Pro C1. This reduction was also observed in the wines elaborated with almond skin. Furthermore, the wines added from almond extract exhibited the highest concentration of catechin at the end MLF, 82.9 and 85.6 mg/L inoculated and spontaneous MLF, respectively. In the wine, the catechin interacts with other compounds, i.e. quinone, and it is implicated in polymerization reactions that contribute to the taste of wine. The results suggest that the addition of the almond extract could limit this contribution. Furthermore, the higher concentration of catechin in the wine treated with almond extract could also be due to the phenolic composition of the extract, rich in flavan-3-ols compounds (Garrido et al., 2008). With regard flavonols, these compounds didn't show significant changes in the control wines during MLF. The wines treated with eucalyptus extract highlighted by its high content of quercetin (an increase > 60% for a control wine) and quercetin-3-O-glucoside (an increased 42 and 18% inoculated and spontaneous MLF, respectively). In the case of the wines treated with almond extract the enhancement of quercetin-3-O-glucoside was less (10%). The increment in the concentration of quercetin after the addition of ecualyptus extract could increase the astringency of the wines which could reduce during the aging by polimerization phenomenons. This increment could be provided by the phenolic composition of the

eucalyptus extract. The content of flavanols was lower than sensory thresholds (Table 5) whereas the concentration of flavonols was higher, so these compounds contribute to the astringency of the wine.

3.4. Multivariate Statistical Analysis.

The results discussed above indicate that the addition of these phenolic extracts and the innoculation with a malolactic starter influenced the phenolic and volatile composition of the final wines. Therefore, to complete the information obtained, it is necessary to evaluate the effect of each treatment on all volatile and phenolic compounds studied. This study could be carried out by using different multivariate statistical analyses such as factorial analysis. The first principal component (PC1) obtained of the application of principal component analysis (PCA) explained 49.1% of data variation and presented higher correlation values with isobutyl acetate (-0.957), ethyl butyrate (-0.966), butyl acetate (-0.867), isoamyl acetate (-0.963), ethyl hexanoate (-0.964), hexyl acetate (-0.906), 1-hexanol (0-.856), α-ionone (-0.852), protocatechuic acid (0.802), ethyl gallate (0.906), epicatechin (-0.942) and all analysed anthocyanins, except delphinidin-3-(6-acetyl)-glucoside, showed a negative correlation higher than -0.85. The PC 2 explained 16.4% of data variation and presented higher correlation values with terpinen-4-ol (-0.793), α-terpienol (-0.757), β-phenylethyl acetate (0.722), benzyl alcohol (0.786), 4-ethylphenol (-0.725), 2-methoxy-4-vinylphenol (-0.707), tyrosol (0.701) and cis-resveratrol (0.769). **Figure 1** shows the representation of the samples in the plane defined by the first and second principal components (PC1 and PC2) which explained 65.5% of data variation. As can be seen, PC1 showed negative values for the initial wines (before MLF) while the wines after MLF showed slight higher than zero Therefore, PC1 is mainly showing the separation among wines because of the

differences in the composition before and after of MLF. On the other hand, PC2 showed higher values at the end of MLF for wines of spontaneous fermentation and lower values for inoculated wines, being more differences in the case of wines treated with eucalyptus extract. Therefore, this PC2 is mainly showing a separation among wines due to the differences between inoculated and spontaneous fermentation of some volatile components (benzyl alcohol, terpinen-4-ol, α-terpienol, β-phenylethyl acetate, 4-ethylphenol and 2-methoxy-4-vinylphenol) and some phenolic compounds (tyrosol and cis-resveratrol) that could be related with the differences of enzymatic activities of LABs that carried out the MLF and the modification of these activities due to the addition of the phenolic extracts.

Conclusions

This work reports a complete study about the changes that the addition of antimicrobial plant extracts (eucalyptus leaves and almond skins) produced on the volatile and phenolic composition of red wines during MLF (spontaneous or inoculated with malolactic starter). In reference to the volatile profile, the control wines showed a similar behavior in both MLF, spontaneous or inoculated, whereas in the experiments with phenolic extracts was observed a higher variation in the wines inoculated with malolactic starter than in the wines carried out spontaneous MLF. On the other hand, it should be highlighted the reduction of the content of certain esters (>75%) in all the wines studied as well as the increment in the concentration of some terpenes and volatile phenol in the wines treated with phenolic extracts. Regarding the phenolic compounds, the total phenolic content, in general, did not show statistical differences between the different treatments assayed. However, the content of some phenolic compounds such as quercetin, antimicrobial and antioxidant potent, could be related with the phenolic composition of the extracts added, especially eucalyptus extract. But

in any case, sensory studies are required in order to assess the impact of these treatments on the organoleptic characteristics of wine.

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Table 1. Wine volatile composition before and after malolactic fermentation (MLF) in the absence (control) and presence of plant extracts.

		After MLF					
		Inoculated MFL			Spontaneous MLF		
	Before MLF	Control	+ Eucalyptus extract	+ Almond extract	Control	+ Eucalyptus extract	+ Almond extract
Esters							
Ethyl butyrate	401 ± 78	*71.0 ± 19.9b	*35.1 ± 1.9a	*75.5 ± 20.4b	*57.5 ± 4.2b	*38.7 ± 6.4a	* 46.5 ± 0.9ab
Ethyl 2-methylbutyrate	14.5 ± 2.5	11.9 ± 0.1	13.5 ± 0.3	20.5 ± 7.9	19.2 ± 2.5	14.8 ± 4.2	18.2 ± 0.01
Ethyl hexanoate	641 ± 123	* 131 ± 4b	*67.0 ± 2.0a	*130 ± 8b	*116 ± 0.1	*123 ± 11	*115 ± 8
Ethyl octanoate	917 ± 216	* 538 ± 39a	*424 ± 28a	911 ± 161b	*316 ± 51	*375 ± 17	*360 ± 43
Ethyl decanoate	785 ± 81	* 432 ± 96a	*364 ± 79a	801 ± 77b	*256 ± 18	*279 ± 12	*308 ± 45
Diethyl succinate (mg/L)	0.189 ± 0.032	* 1.99 ±0.16a	* 2.78 ± 0.23a	* 3.43 ± 0.36b	0.435 ± 0.009a	*2.43 ± 0.14c	*1.75 ± 0.20b
Ethyl dodecanoate	182 ± 5	*124 ± 1a	*106 ± 6a	184 ± 12b	*73.9 ± 2.3a	*73.4 ± 2.8a	*85.9 ± 3.3b
Ethyl cinnamate	12.8 ± 0.1	12.9 ± 0.1	12.8 ± 0.1	12.8 ± 0.1	12.8 ± 0.1	*13.1 ± 0.1	12.9 ± 0.1
Ethyl lactate (mg/L)	6.06 ± 0.86	*40.2 ± 9.9b	*25.6 ± 0.4a	*53.6 ± 4.3c	*45.8 ± 1.9b	*32.4 ± 6.3a	*60.6 ± 9.3c
Isobutyl acetate	120 ± 15	*26.2 ± 15.8	*9.39 ± 0.30	*28.0 ± 17.3	*9.87 ± 8.90	*12.2 ± 1.7	*13.8 ± 0.5
Butyl acetate	46.1 ± 12.9	26.1 ± 4.0b	*7.24 ± 0.33a	32.3 ± 1.7b	*20.8 ± 1.4b	*6.27 ± 0.22a	*5.76 ± 0.82a
Isoamyl acetate (mg/L)	2.38 ± 0.42	*0.23 ± 0.30b	*0.1050±0.0002a	*0.232±0.006b	*0.20 ±0.02b	*0.13 ± 0.01a	*0.16 ± 0.02ab
Hexyl acetate	16.8 ± 6.7	5.97 ± 2.05	* 1.40 ± 0.30	5.82 ± 2.79	* 2.94 ± 0.05	*1.75 ± 0.40	* 2.50 ± 0.65
β-Phenylethyl acetate	151 ± 17	142 ± 4b	*96.4 ± 1.5a	139 ± 1b	148 ± 0.2	139 ± 1	149 ± 6
Alcohols							
1-Hexanol	981 ± 148	796 ± 51	728 ± 15	802 ± 11	808 ± 21	766 ± 87	778 ± 44
trans-3-Hexen-1-ol	93.6 ± 11.4	82.0 ± 2.6	78.1 ± 0.7	82.5 ± 2.9	88.4 ± 1.4	84.1 ± 7.8	83.7 ± 5.0
cis-3-Hexen-1-ol	68.8 ± 8.5	51.3 ± 1.4b	* 43.1 ± 2.5a	55.2 ± 1.2b	57.2 ± 0.5	58.8 ± 0.2	48.7 ± 12.7
Benzyl alcohol	273 ± 50	324 ± 7b	228 ± 2a	334 ± 5b	*585 ± 4 b	*469 ± 5a	* 684 ± 25c
β-Phenylethyl alcohol	52.1 ± 3.7	45.3 ± 0.1a	47.6 ± 1.7ab	50.4 ± 1.3b	43.2 ± 1.4	43.4 ± 9.8	44.3 ± 0.6
Terpenes							
Limonene	nd	nd	tr	nd	tr	tr	tr
Linalool	5.80 ± 0.28	*7.03 ± 0.37	*7.39 ± 0.12	*7.21 ± 0.32	6.76 ± 0.17	6.86 ± 1.32	6.75 ± 0.03
Terpinen-4-ol	nd	nd	147 ± 27	nd	nd	32.3 ± 1.1	nd
α-Terpineol	tr	tr	22.0 ± 2.0	tr	tr	tr	tr

β -Citronellol	8.08 ± 0.64	9.25 ± 0.82	9.35 ± 0.92	* 10.1 ± 0.1	7.68 ± 0.33	8.32 ± 1.97	8.45 ± 0.06
Nerol	5.68 ± 1.29	5.12 ± 0.06c	*2.68 ± 0.19a	4.23 ± 0.08 b	6.51 ± 0.33	6.23 ± 0.15	6.42 ± 0.63
C13 nor-isoprenoids							
β -Damascenone	3.86 ± 0.33	4.41 ± 0.27	4.27 ± 0.24	* 4.61 ± 0.01	3.70 ± 0.01	3.05 ± 0.71	3.55 ± 0.15
α -Ionone	5.59 ± 0.72	*2.86 ± 0.08a	*nd	*3.43 ± 0.06b	*2.61 ± 0.11a	*nd	*3.13 ± 0.13b
β -Ionone	tr	tr	tr	tr	tr	tr	tr
Acids							
Hexanoic acid (mg/L)	3.08 ± 0.56	3.55 ± 0.27a	*4.20 ± 0.002b	*4.26 ± 0.01b	2.69 ± 0.10	3.13 ± 0.62	2.89 ± 0.08
Octanoic acid (mg/L)	3.59 ± 0.35	3.70 ± 0.01b	*2.85 ± 0.08a	3.61 ± 0.25b	3.05 ± 0.10	2.89 ± 0.40	3.19 ± 0.02
Volatile phenols							
4-Ethylguaiacol	1.23 ± 0.01	*1.28 ± 0.01a	*1.45 ± 0.01c	*1.29 ± 0.01b	*1.31 ± 0.01a	*1.51 ± 0.01b	*1.34 ± 0.02a
Eugenol	19.1 ± 0.001	19.1 ± 0.01a	*28.6 ± 0.2b	19.3 ± 0.05a	19.0 ± 0.01a	*29.0 ± 0.01b	19.2 ± 0.02a
4-Ethylphenol	8.36 ± 0.07	8.34 ± 0.02a	*30.1 ± 8.20b	8.39 ± 0.02a	8.37 ± 0.01a	*25.1 ± 0.10b	8.43 ± 0.04a
2-Methoxy-4-vinylphenol	433 ± 21	442 ± 10a	*822 ± 87b	528 ± 34a	457 ± 12a	*624 ± 2b	471 ± 1a
2.6-Dimethoxyphenol	94.8 ± 31.9	118 ± 28a	*312 ± 6b	*244 ± 32b	56.7 ± 2.7a	124 ± 12b	117 ± 3b
4-Vinylphenol	14.7 ± 3.5	12.0 ± 2.6b	*7.18 ± 0.14a	10.1 ± 0.3ab	9.02 ± 0.16	12.3 ± 3.3	10.8 ± 0.7
Lactone and furanic compounds							
γ-Nonalactone	11.8 ± 0.8	13.5 ± 0.4a	11.9 ± 0.1a	*21.8 ± 0.9b	* 14.2 ± 0.02a	* 16.9 ± 0.3b	*22.2 ± 1.0c
Furfural	tr	tr	tr	tr	tr	tr	tr

Concentration values in μg/L except indicated.
nd=not detected; tr=traces
* on the left indicates significant differences between before and after MLF ($p<0.05$)
Values with the same letter on the right indicate no statistically significant differences among the three treatments ($p<0.05$)

Table 2. Odour activity values of esters of wines before and after malolactic fermentation (MLF) in the absence (control) and presence of plant extracts.

	Odour Thershold (mg/L)	Before MLF	After MLF					
			Inoculated MLF			Spontaneous MLF		
			Control	+Eucalyptus extract	+Almond extract	Control	+Eucalyptus extract	+Almond extract
Esteres								
Ethyl butyrate	0.02 [a]	20.1	3.55	1.75	3.77	2.88	1.93	2.33
Ethyl 2-methylbutyrate	0.02 [a]	0.81	0.66	0.75	1.14	1.06	0.82	1.01
Ethyl hexanoate	0.01 [a]	45.8	9.38	4.79	9.27	8.30	8.75	8.24
Ethyl octanoate	0.01 [b]	183	108	84.9	182	63.1	74.9	71.9
Ethyl decanoate	0.20 [a]	3.93	2.16	1.82	4.01	1.28	1.39	1.54
Diethyl succinate	200 [a]	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
Ethyl Lactate	154 [a]	<0.10	0.26	0.17	0.35	0.30	0.39	0.39
Ethyl dodecanoate	0.50 [c]	0.36	0.20	0.21	0.40	0.10	0.15	0.20
Ethyl cinnamate	0.001 [d]	12.8	12.9	12.8	12.8	12.8	13.1	12.9
Isobutyl acetate	1.61 [a]	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
Butyl acetate	1.80 [a]	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
Isoamyl acetate	0.03 [a]	79.5	7.61	3.51	7.72	6.70	4.21	5.24
Hexyl acetate	1.50 [b]	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
β-Phenylethyl acetate	0.25 [a]	0.60	0.57	0.39	0.55	0.59	0.55	0.60
Total		347 ± 52	145 ± 8a	111 ± 6 a	222 ± 34b	96.9 ± 11.1	106 ± 2	104 ± 8

Odour Thresholds obtained from referentes: [a] Escudero et al 2007; [b] Aznar et al. 2003; [c] Zea et al. 2001, [d] Escudero et al. 2004.

Values with the same letter on the right indicate no statistically significant differences among the three treatments ($p<0.05$)

Table 3. Wine phenolic composition before and after malolactic fermentation (MLF) in the absence (control) and presence of plant extracts.

		After MLF					
		Inoculated MLF			Spontaneous MLF		
	Before MLF	Control	+ Eucalyptus extract	+ Almond extract	Control	+ Eucalyptus extract	+ Almond extract
Anthocyanins							
Delphinidin-3-glucoside	12.8 ± 0.44	*8.57 ± 1.25	*9.30 ± 0.16	*8.79 ± 0.01	*9.99 ± 0.64	*10.9 ± 0.91	*8.84 ± 0.85
Cyanidin-3-glucoside	1.98 ± 0.07	*1.39 ± 0.14	*1.41 ± 0.01	*1.404±0.001	*1.57 ± 0.10	*1.59 ± 0.04	*1.40 ± 0.10
Peonidin-3-glucoside	14.8 ± 0.45	*9.96 ± 1.12	*9.74 ± 0.06	*10.1 ± 0.03	*11.5 ± 0.60	*11.5 ± 0.54	*9.99 ± 0.86
Petunidin-3-glucoside	15.6 ± 0.51	*10.2 ± 1.37	*10.5 ± 0.14	*10.1 ± 0.11	*11.6 ± 0.81	*12.6 ± 0.82	*10.0 ± 0.87
Malvidin-3-glucoside	80.7 ± 2.13	*55.1 ± 5.69	*54.0 ± 0.16	*55.6 ± 0.38	*62.0 ± 2.99	*62.8 ± 3.22	*55.1 ± 4.78
Delphinidin-3-(6-acetyl)-glucoside	4.84 ± 0.16	*3.16 ± 0.53a	*3.40 ± 0.19a	4.64 ± 0.07b	4.97 ± 0.15	4.36 ± 0.26	5.01 ± 0.49
Cyanidin-3-(6-acetyl)-glucoside	3.55 ± 0.11	*2.32 ± 0.15	*2.47 ± 0.01	*2.27 ± 0.06	*2.42 ± 0.06	*2.35 ± 0.13	*2.20 ± 0.06
Peonidin-3-(6-acetyl)-glucoside	5.13 ± 0.13	*3.60 ± 0.35	*3.42 ± 0.01	*3.57 ± 0.02	*4.14 ± 0.22	*3.97 ± 0.13	*3.50 ± 0.24
Petunidin-3-(6-acetyl)-glucoside	3.96 ± 0.12	*2.79 ± 0.30	*2.87 ± 0.03	*2.67 ± 0.03	*3.10 ± 0.18ab	*3.39 ± 0.18b	*2.60 ± 0.18a
Malvidin-3-(6-acetyl)-glucoside	21.6 ± 0.55	*15.0 ± 1.78	*14.6 ± 0.10	*15.0 ± 0.01	*17.0 ± 0.86b	*17.0 ± 0.75b	*14.4 ± 0.59a
Delphinidin-3-(6-*p*-coumaroyl)-glucoside	2.11 ± 0.18	*1.15 ± 0.01	*1.02 ± 0.08	*1.16 ± 0.02	*1.34 ± 0.10b	*1.53 ± 0.04b	*1.10 ± 0.01a
Peonidin-3-(6-*p*-coumaroyl)-glucoside	4.22 ± 0.24	*2.15 ± 0.29	*2.18 ± 0.03	*2.07 ± 0.01	*2.60 ± 0.17ab	*2.79 ± 0.25b	*2.10 ± 0.15a
Petunidin-3-(6-*p*-coumaroyl)-glucoside	1.92 ± 0.13	*0.96 ± 0.10	*1.07 ± 0.01	*1.11 ± 0.03	*1.30 ± 0.08	*1.17 ± 0.09	*1.17 ± 0.06
Malvidin-3-(6-*p*-coumaroyl)-glucoside	11.9 ± 0.59	*6.12 ± 0.82	*6.19 ± 0.05	*5.91 ± 0.04	*7.32 ± 0.57ab	*7.92 ± 0.75b	*5.88 ± 0.48a
Hydroxybenzoic acids and ester							
Ethyl gallate	18.4 ± 1.09	*20.6 ± 0.15a	*22.2 ± 0.02c	*21.3 ± 0.20b	*21.2 ± 0.15	*22.1 ± 0.44	*21.6 ± 0.04

Gallic acid	27.8 ± 0.59	*31.5 ±0.42b	*36.9 ± 0.11c	*30.3 ± 0.33a	29.1 ± 0.02a	*35.9 ± 0.68c	*31.0 ± 0.06b
Protocatechuic acid	10.4 ± 0.22	10.8 ± 0.18a	*10.9 ± 0.04a	*11.1 ± 0.03a	*10.8 ± 0.01a	*10.8 ± 0.08a	*11.4±0.07b
Hydroxycinnamic acids							
Caffeic acid	4.05 ± 0.06	*6.00 ± 0.15c	*4.89 ± 0.11a	*5.64 ± 0.03b	*3.76 ± 0.04b	3.96 ± 0.06b	*2.68 ± 0.02a
trans-caftaric acid	20.8 ± 0.11	*19.3 ± 0.53a	21.3 ± 0.41b	20.0 ± 0.36ab	21.1± 0.05	21.85 ± 1.69	21.7 ± 0.04
trans p-coumaric acid	4.46 ± 0.55	3.91 ± 0.02b	3.58 ± 0.07a	3.629 ± 0.004a	*3.38 ± 0.10b	3.25 ±0.18ab	*3.02 ± 0.04a
Coutaric acid	3.89 ± 0.21	3.49 ± 0.06a	3.67 ± 0.01b	3.65 ± 0.01b	3.56 ± 0.15a	3.52 ± 0.05a	*4.41 ± 0.00b
Phenolic alcohol							
Tyrosol	28.6 ± 1.23	29.4 ± 0.01b	*26.2 ± 0.18a	*32.0 ± 0.06c	27.0 ± 0.08a	*25.7 ± 0.14a	*35.7 ± 0.15b
Stilbenes							
cis-Resveratrol	0.59 ± 0.02	*0.67 ±0.02b	0.595±0.003a	*0.701±0.001b	*0.75 ± 0.01	*0.68 ± 0.03	*0.74 ± 0.01
trans-Resveratrol	6.73 ± 1.46	5.74 ± 0.14b	7.842±0.003c	5.05 ± 0.07a	4.117 ± 0.002a	8.13 ± 0.02c	5.33 ± 0.07b
cis-Resveratrol-5-O-glucoside	2.41 ± 0.25	2.45 ± 0.01a	2.67 ± 0.02c	2.570 ± 0.004b	2.472±0.003ab	2.37 ± 0.01a	2.533±0.004b
trans-Resveratrol-5-O-glucoside	16.1 ± 3.18	15.0 ± 0.89b	11.1 ± 0.16a	11.8 ± 0.07a	11.4 ± 0.28	10.7 ± 0.22	11.3 ± 0.04
Flavan-3-ols							
Catechin	88.5 ± 3.75	*73.6 ± 0.37a	*75.7 ± 0.69b	82.9 ± 0.30c	80.4 ± 0.02	79.7 ± 7.71	85.6 ± 0.13
Epicatechin	56.0 ± 1.18	*45.3 ± 0.01c	*40.5 ± 0.04a	*42.5 ± 0.01b	*42.7 ± 1.57	*43.2 ± 0.07	*37.0 ± 1.23
Pro C1	36.0 ± 0.96	36.2 ± 0.45b	*32.3 ± 0.58a	*31.5 ± 0.07a	*32.7 ± 1.00b	*32.1 ± 0.20b	*27.9 ± 0.29a
Flavonols							
Quercetin	13.9 ± 1.36	11.7 ± 0.05a	*19.5 ± 0.04c	12.2 ± 0.13b	12.4 ± 0.23a	*20.2 ± 2.65b	14.1 ± 0.33a
Quercetin-3-O-glucoside	18.2 ± 1.15	18.3 ± 0.77a	*26.1 ± 0.03c	20.2 ± 0.06b	20.2 ± 0.19a	*24.0 ± 1.68b	*22.2 ± 0.07b
Total anthocyanins[a]	185 ± 5.43	*122 ± 13.88	*122 ± 1.00	*125 ± 0.25	*141 ± 7.52	*144 ± 7.77	*123 ± 9.73
Total non-anthocyanins[a]	357 ± 1.42	*334 ± 3.44a	*346 ± 2.11b	*337 ± 0.79a	*327 ± 2.40a	348 ± 7.68b	*338 ±2.13ab
Total Phenolics[b]	1578 ± 41.1	1612 ± 41.8	1649 ± 21.4	*1702 ± 40.4	1528 ± 9.09	1657 ± 16.5	*1725 ± 15.5

Concentration values in mg/L

[a] The sum of the anthocyanins profile analysed by HPLC

[b] Total phenolic determinated by Folin-Ciocalteu method

* on the left indicates significant differences in time during MLF ($p<0.05$)

Values with the same letter indicate no statistically significant differences among the treatments ($p<0.05$)

Table 5. Sensory Tresholds values of phenolic compounds in wines.

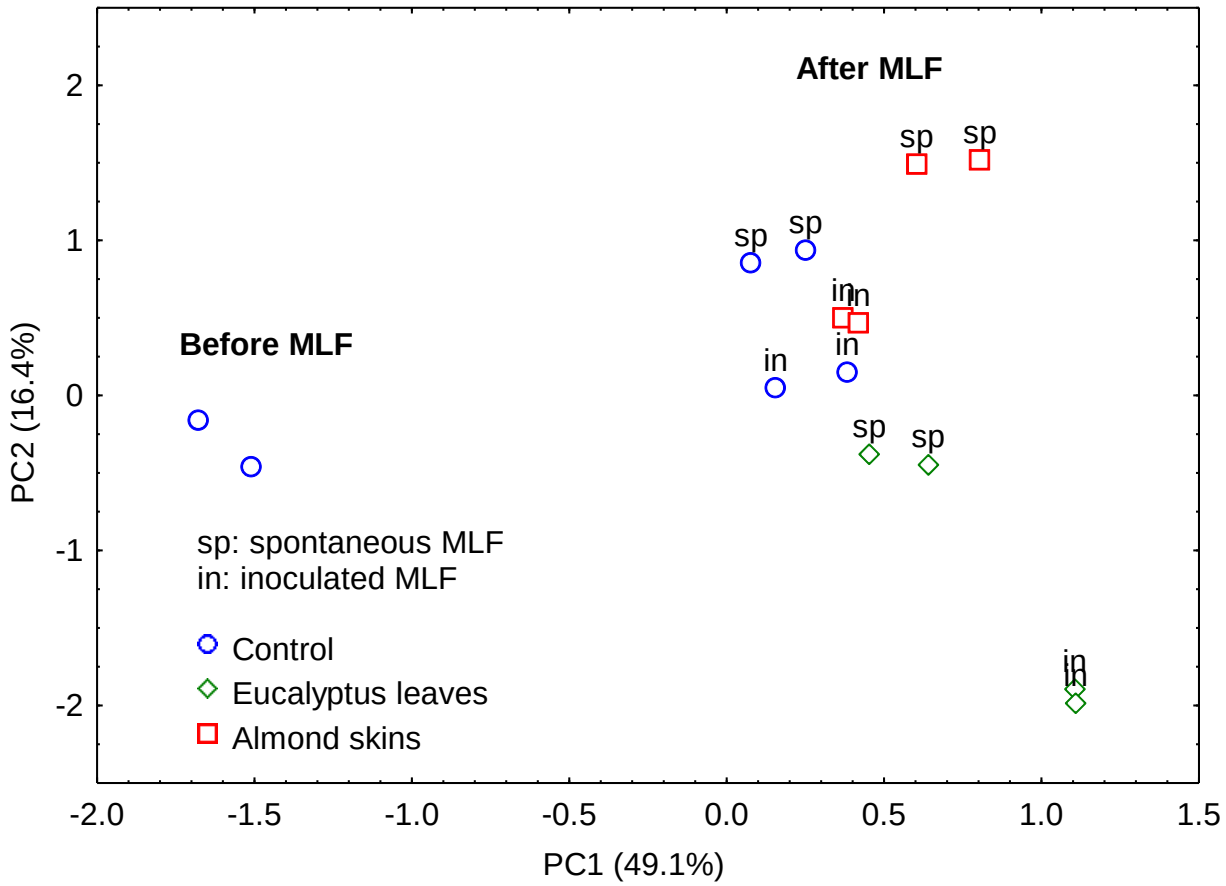
Phenolic Compounds	Sensory Tresholds
Hydroxybenzoic acids and ester	
Gallic acid	50
Protocatechuic acid	32
Hydroxycinnamic acids	
**trans*-caffeic acid	13.0
trans-caftaric acid	5
trans-p-coumaric acid	23
trans-coutaric acid	10
Flavan-3-ols	
Catechin	119
Epicatechin	270
Flavonols	
Quercetin	10.0
Quercetin-3-*O*-glucoside	0.1

Concentration values in mg/L

[a] Sensory Thresholds obtained from referents: Sáenz-Navajas et al., 2010.

Figure Captions

Figure 1: Distribution of wines studied in the plane defined by principal components 1 and 2 obtained from the principal component analysis.


After MLF
Before MLF
sp: spontaneous MLF
in: inoculated MLF
Control
Eucalyptus leaves
Almond skins
PC2 (16.4%)
PC1 (49.1%)
2
1
0
-1
-2
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5

IV.5. Caracterización de la población de *Oenococcus oeni* representativa de los vinos tratados y no tratados con extractos fenólicos como antimicrobianos.

Los avances en las herramientas moleculares, basadas generalmente en las técnicas de PCR, permiten la caracterización rápida y sensible de la mayoría de las BAL del vino. La diversidad intraespecífica de *O.oeni* y la tipificación a nivel de cepa se han realizado mediante el análisis con endonucleasas de restricción, junto con la electroforesis en gel de campo pulsado (REA-PFGE) (Gindreau y col., 1997). Por PCR seguida de electroforesis en gel de gradiente desnaturalizante (DGGE), es posible la visualización de la diversidad de la población microbiana en una comunidad compleja (Pozo-Bayón y col., 2009). Para las bacterias del vino, el gen que codifica para la subunidad beta de la ARN polimerasa (r*poB* gen), que está en copia única en el genoma, se muestra como una de las opciones más fiables para este análisis, ya que proporciona más resolución filogenética que el 16SrRNA (Renouf y col., 2006). El análisis del gen *rpoB* de *O.oeni* proporciona dos bandas cercanas, pero diferentes en los geles de DGGE: la banda L, de menor migración, y la banda H, de mayor migración en el gel. Estas dos secuencias *rpoB* difieren en un sólo nucleótido: una guanidina para L es sustituida por una adenina para H (Renouf y col., 2004). Más recientemente, Renouf y col. (2008) proponen que el estudio de 16 marcadores genéticos en *O.oeni* –entre los que se encuentran marcadores relacionados con la resistencia a estrés ambiental, transporte de metabolitos, y otras funciones esenciales para la célula bacteriana- , posiblemente, podrían estar relacionados con las propiedades enológicas de las cepas de *O. oeni*, como la supervivencia, la multiplicación en el vino y la capacidad de realizar la FML. Esta caracterización genética es importante para entender el mecanismo de selección entre cepas en las primeras etapas de la fermentación.

Teniendo en cuenta que en la bibliografía no se disponía de información a nivel molecular de cómo extractos fenólicos con capacidad antimicrobiana sobre BAL del vino puede afectar a la diversidad de *O. oeni*, y en concreto sobre marcadores genéticos relacionados con los mecanismos moleculares que conducen a la prevalencia de *O.oeni* durante la FML, el objetivo de este trabajo fue describir genéticamente la población de BAL asociadas a los vinos tintos producidos en ausencia/presencia de extractos fenólicos antimicrobianos añadidos antes de la FML, y de caracterizar genéticamente a las cepas de *O. oeni* representativas de estos vinos mediante: *i*) el estudio del gen rpoB, *ii*) la comparación de los patrones de PFGE y *iii*) el análisis de la presencia/ausencia de

marcadores genéticos que parecen estar relacionados con la adaptación de las bacterias lácticas al medio/ambiente del vino.

Los vinos estudiados se refieren a la experimentación descrita en la sección IV.3, en la que se llevó a cabo la FML (inoculada y espontánea) de un vino tinto en presencia del extracto de eucalipto. En este caso, y al igual que en la sección IV.4, también se incluyó una experimentación paralela llevada a cabo con el extracto de piel de almendra en lugar del de eucalipto.

A continuación se presentan los resultados de este estudio en forma de una publicación:

Publicación VI. Caracterización genética de bacterias lácticas aisladas de vinos elaborados con extractos fenólicos como agentes antimicrobianos.

Publicación VI. Caracterización genética de bacterias lácticas aisladas de vinos elaborados con extractos fenólicos como agentes antimicrobianos.

Almudena García Ruiz, Raquel Tabasco, Teresa Requena, Olivier Claisse, Aline Lonvaud-Funel, Carolina Cueva, Begoña Bartolomé, M. Victoria Moreno Arribas. Genetic characterization of lactic acid bacteria from wines treated with phenolic extracts as antimicrobial agents (en preparación)

Resumen:

Técnicas moleculares han sido utilizadas para evaluar la evolución de bacterias lácticas presentes en vinos tintos elaborados en ausencia/presencia de extractos fenólicos antimicrobianos, pieles de almendras y hojas de eucalipto, y caracterizar genéticamente cepas representativas de *Oenococcus oeni*. La monitorización de la población microbiana por *rpoB* PCR-DGGE reveló que *O.oeni* fue la especie responsable de la fermentación maloláctica (FML). Cepas aisladas se identificaron como *O.oeni* mediante las técnicas *rpoB* PCR-DGGE y ARNr 16S. La tipificación de cepas aisladas de *O.oeni* basada en la mutación de la región del gen *rpoB* sugiere una adaptación más favorable de las cepas L (n = 63) que de las cepas H (n = 3) a la FML. La PFGE de cepas aisladas de *O.oeni* mostró 27 perfiles genéticos diferentes, lo que indica una rica biodiversidad de *O.oeni* autóctonas. La caracterización genética de cinco cepas representativas mostró una tendencia a un mayor número de marcadores genéticos relacionados con la adaptación al vino, en el genoma de cepas de vinos tintos fermentados sin adición de extractos fenólicos antimicrobianos que en cepas de vinos elaborados en presencia de extractos fenólicos antimicrobianos. Estos resultados proporcionan una base para una mayor investigación de los mecanismos moleculares y evolutivos que conducen a la prevalencia de *O.oeni* en vinos tratados con polifenoles como inhibidores.

Genetic characterization of lactic acid bacteria from wines treated with phenolic extracts as antimicrobial agents

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Abstract

Molecular techniques have been used to evaluate the evolution of wine-associated lactic acid bacteria from red wines manufactured in the absence/presence of antimicrobial phenolic extracts, almond skins and eucalyptus leaves, and to genetically characterize representative *Oenococcus oeni* strains. Monitoring microbial population by *rpoB* PCR-DGGE revealed that *O.oeni* was the responsible species for malolactic fermentation (MLF). The isolated strains were identified as *O.oeni* species by *rpoB* PCR-DGGE and 16S rRNA techniques. The typing of isolated *O.oeni* strains based in the mutation of *rpoB* gene region suggested a more favorably adaptation of L strains (n=63) than H strains (n=3) to MLF. PFGE analysis of the *O.oeni* isolated showed 27 different genetic profiles, which indicate a rich biodiversity of indigenous *O.oeni* species. The genetic characterization of five representative strains showed a tendency for a higher number of genetic markers related to the adaptation to wine in the genome of strains from red wines fermented without addition of antimicrobial phenolic extracts than strains from wines elaborated in presence antimicrobial phenolic extracts. These results provide a basis for further investigation of the molecular and evolutionary mechanisms leading to the prevalence of *O.oeni* in wines treated with polyphenols as particular inhibitors.

Keywords: phenolic antimicrobial extracts, malolactic fermentation, lactic acid bacteria, genetic characterization

1. Introduction

The malolatic fermentation (MLF) is a biological process that usually occurs once alcoholic fermentation (AF) by yeast is completed (Ruiz *et al.*, 2008). MLF is usually performed by the indigenous lactic acid bacteria (LAB) existing in grapes and wineries,

although sometimes can be induce by starter cultures. These bacterial are the responsible of the degradation of malic acid into lactic acid and carbon dioxide, producing a reduction total acidity (deacidification) of the wine. This deacidification is always accompanied provides additional flavors and stability for wines (Lonvaud-Funel, 1999; Moreno-Arribas and Polo 2005). In the majority of cases, *Oenococcus oeni* is the most tolerant species unfavorable wine conditions (low pH and high ethanol levels), being the main species conducting MLF in wine (Davis *et al.*, 1985; van Vuuren and Dicks, 1993).

Once malic acid is fully transformed, microbial populations are controlled by sulfiting in order to avoid any post-fermentation microbial metabolism that could alter wines organoleptic quality. Most of the bacteria and possible remaining yeasts are sensitive to sulphur dioxide, although effectiveness of SO_2 may be limited by wine pH and other wine components. Thus, in certain conditions *Lactobacillus* and *Pediococcus* may be predominant and induce wine spoilage. Nowadays, it exists a worldwide trend to reduce SO_2 levels in wine; there is a great interest in total or partial natural alternatives to the traditional use of SO_2 in winemaking, such as plant polyphenol (García-Ruiz *et al.*, 2008).

The advances of molecular tools, usually based on polymerase chain reaction (PCR) techniques, have allowed a fast and sensitive characterization of the majority of wine LAB. The intraspecific diversity of *O.oeni* and strain typing also is studied by enzymatic restriction coupled with restriction endonuclease analysis by pulsed-field gel electrophoresis (REA-PFGE) (Gindreau *et al.*, 1997). By PCR followed by Denaturing Gradient Gel Electrophoresis (DGGE) the visualization of the microbial population diversity in a complex community is possible (Pozo-Bayón *et al.*, 2009). Moreover it

includes the detection of the non-cultivable microbiota. DGGE is based on the separation of PCR amplicons of different sequences and same size. For wine bacteria, the gene coding for the *beta* subunit RNA polymerase (*rpoB* gene), which is in unique copy in the genome, is the most reliable target for this analysis. It provides more phylogenetic resolution than the 16S rRNA gene which is repeated, with differences between the copies, leading sometimes to ambiguous profiles (Renouf *et al.*, 2006). Unexpectedly, the *rpoB* analysis showed for *O.oeni* two close but different bands in the DGGE gels: L band as the lower-migrated band, and H band as the higher-migrated band in the gel. These two *rpoB* sequences differed by only one nucleotide: a guanidine for L was substituted by an adenine for H (Renouf *et al.*, 2006). In another study, Renouf *et al.*, (2008) suggest that 16 genetic markers may possibly be linked to oenological properties of *O. oeni* strains, such as survival, multiplication in wine and the ability to perform MLF. This characterization genetic is important to understand the selection mechanism among during the first stages of winemaking.

In a previous study realized by our group (García-Ruiz et al., unpublished) was showed that the addition of the antimicrobial phenolic extract: almond skins and eucalyptus leaves during the elaboration of red wine affected the consumption rate of malic acid during MLF. In presence almond skins the malic acid consumption was faster whereas in presence of eucalyptus leaves extract was slower. The aim of this work was to genetically type wine-associated LAB isolated from red wines manufactured in the absence/presence of antimicrobial phenolic extracts, almond skins and eucalyptus leaves, and to genetically characterize representative *O. oeni* strains by (i) targeting the *rpoB* gene, (ii) comparing the PFGE profiles and (iii) analysing the presence/absence of

oenological genetic markers that seems related to the adaptation of LAB to the wine environment.

2. Materials and Methods

2.1 Malolactic fermentation assays in wine

A red wine (var. *Merlot*) (vintage 2009) was elaborated at Bodegas Miguel Torres S.A. (Catalonia, Spain), following their own winemaking procedures. The AF was carried out in a controlled form in stainless steel at 25 ± 2 ºC. The end of AF was established by measuring the alcohol degree (13.9 % v/v) and the residual sugar amount (< 3.5 g/L); the wine pH at the end of AF was 3.22. MLF experiments were conducted in laboratory scale, sterile conditions, in 250-mL flasks. Parallel inoculated and spontaneous MLF assays were carried out. Two selected extracts (almond skins, eucalyptus leaves) were dissolved (2 g/L) in 200 mL of previously inoculated or non-inoculated wine. The malolactic starter was comprised by a mix of *O. oeni* strains previously isolated by the winery, and was inoculated in wine at 3% (v/v). A control containing no extract was also prepared for both inoculated and spontaneous MLF assays. Wines containing phenolic extracts and control wines were incubated at 25 ºC in the dark. All the MLF assays were performed in duplicate.

Wine samples were aseptically collected at 14, 19 and 24 days of incubation, and were immediately assayed for L-malic acid content as a marker of the development of MLF. L-malic acid content was determined using an enzymatic kit (Megazyme International Ireland Ltd., Bray, CO. Wicklow, Ireland), and these determinations were carried out in duplicate.

Fifty mL of each type of red wine were also aseptically collected (0, 14, 19 and 24 days of incubation) and centrifuged (10 min, 10,000 g, 4 ºC). The pellets were kept in a commercial freezer (-20ºC) until the molecular analysis.

2.2. LAB isolation

Wine samples were diluted in sterile solution and plated on MRS-Agar (Pronadisa, Madrid, Spain), supplemented with 5 g/L fructose (Panreac Química SAU, Barcelona, Spain), 1 g/L D-L malic acid (Panreac Química SAU, Barcelona, Spain), 1 mL Tween 80 (Sigma, St. Louis, USA), and 100 mg/L cycloheximide (Sigma, St. Louis, USA) were also added to the medium to suppress acetic acid and yeast growth. The pH of the medium was adjusted to 4.8 with HCl 37% (Panreac Química SAU, Barcelona, Spain). Plates were incubated anaerobically (Whitehouse Station, New Jersey, USA) at 28 ºC for seven days. At each day's analysis, ten isolated colonies were randomly chosen from plate of convenient dilutions each sample, ensuring that all different colony morphologies were considered. Isolates were sub-cultured onto the same medium until purification. Each pure colony was cultured in liquid medium, with a similar composition that of the plates but without agar and was stored at -80 ºC with 50% (v/v) glycerol (Panreac Química SAU, Barcelona, Spain). LAB strains were identified by sequencing V1 and V2 region of the 16S rRNA gene. The first half of the 16S rRNA gene was sequenced with the forward primer POmod and the reverse primer P3rev and the second half of the gene was sequenced with forward primer 16midfor and the reverse primer PC5 described in the table 1. Sequencing of PCR fragments was carried out at the DNA sequence service of the Centro de Investigaciones Biológicas-CSIC (Madrid, Spain). The resulting sequences were used to search sequences deposited in

database using BLAST algorithm. The identity of the strains was determined on the basis of the highest score.

2.3. *Bacteria strains and culture conditions*

The reference strains *Lactobacillus plantarum* CECT 4645, *Lactobacillus casei* CECT 4045, *Pediococcus parvulus* CECT 4693 and *Oenococcus oeni* CECT 217 from the Spanish Type Culture Collection (CECT) and the LAB isolated from wines were used in this study.

Following CECT recommendations, the *Lactobacillus* and *Pediococcus* species were grown in Man, Rogosa and Sharpe medium (MRS) broth (Pronadisa, Madrid, Spain). *Oenococcus oeni* and LAB isolated from wines were grown in MRS broth (Pronadisa, Madrid, Spain), supplemented with 5 g/L fructose (Panreac Química SAU, Barcelona, Spain) and 1 g/L D-L malic acid (Panreac Química SAU, Barcelona, Spain), pH 4.8 (37% HCl)

2.4. DNA extraction

For PCR-DGGE, the DNA was extracted according to the protocol described by the manufacturer, QIAamp DNA kit (Qiagen, Hilden, Germany). The isolated DNA was stored at -20 ºC until the analyses. DNA concentrations were standardized (100 ng/µL) by measuring optical density at 260 nm with a SmartSpec (+) spectrophotometer (Bio-Rad, Hercules, CA, USA).

For characterization genetic study, strains were cultivated on MRS liquid medium containing: Lactobacilli MRS broth (Difco, Sparks, MD, USA), 10g/L D-L malic acid (Prolabo, Bordeaux, France), pH 4.8 with NaOH 5N. After 3-4 days of incubation,

microbial biomass was collected by centrifugation (5 min, 10,000 *g*, 4 ºC). The supernatant was discarded and the pellet resuspended in 600 µL of 50 mM EDTA, pH 8, with 10 mg/mL of lysozyme (Sigma, St. Louis, MO, USA) and incubated during 1 h at 37ºC. After a second centrifugation (2 min, 10,000 *g*, 4º C), the supernatant was discarded newly and the pellet resuspended with 600 µL of nucleic lysis solution (Promega, Madison, WI, USA) and waved softly with the pipette and incubated during 5 min at 80ºC. Then, 200 µL of protein precipitation solution (Promega, Madison, WI, USA) were added and mixed for 20 s. Cellular fragments were precipitated on ice for 5 min. After another centrifugation (3 min, 10,000 *g*, 4 ºC), the supernatant containing the DNA was transferred to a new microcentrifuge tube containing 600 µL of isopropanol and was gently mixed by inversion. After centrifugation (2 min, 10,000 *g*, 4 ºC), 600 µL of a room temperature 70% ethanol solution was added to the pellet before at final centrifugation (2 min, 10,000 *g*, 4 ºC). Ethanol was carefully removed and the tube dried. Fifty microliters of pour preparation injectable water with 3 µL of RNase (Promega, Madison, WI, USA) was used to rehydrate DNA overnight at 4 ºC. After rehydratation, this DNA stored at -20 ºC. DNA concentrations were standardized (100 ng/µL) by measuring optical density at 260 nm with a SmartSpec (+) spectrophotometer (Bio-Rad, Hercules, CA, USA).

2.5. PCR-DGGE

The PCR-DGGE protocol using *rpoB1, rpoB1o/rpoB2* primers (table 1) and described by Renouf et al. (2006) for bacteria was used with some modifications. The PCR program began with an initial touchdown step in which the annealing temperature was lowered from 59 to 45 ºC in intervals of 1 ºC every cycle. Furthermore, 20 additional

cycles were carried out with an annealing temperature of 45 ºC. Electrophoresis took place in vertically acrylamide (Promega, Madison, WI, USA) gel with denaturing conditions provided by urea (Sigma Chemical Co., St. Louis, MO, USA) and formamide (Sigma Chemical Co., St. Louis, MO, USA). A solution of 100% denaturing consists of 7M urea and 40% (v/v) formamide in milliQwater, being the gradient ranging from 30 to 60%. Ten microliters of PCR amplicons at 50 ng/μL were loaded with high-density marker (GLS). Electrophoresis was run in 1 x TAE buffer at constant temperature (60 ºC) for 10 min at 20 V and subsequently for 16 h at 85 V. After migration, gels were stained with $AgNO_3$ as described by Sanguinetti *et al.* (1994).

2.6. REA-PFGE

Strains were cultivated in 2 mL MRS media supplemented (10g/L D-L malic acid, pH 4.8 with NaOH 5N) for 3-4 days at 28 ºC. The pellet cells were washed twice with 1 x TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) and finally resuspended in 50 μL $T_{100}E$ (10mM Tris - 100mM EDTA, pH 8). The cell suspensions were heated at 50 ºC and mixed an equal volume of a 1% (v/v) agarose (Chromosal Grade Agarosa (Bio-Rad, Hercules, CA, USA)), that was pre-melted and kept at 60ºC. Aliquots were made into moulds to prepare plugs and were kept for 15 min at 4 ºC. The agarose plugs were removed and placed in 1 mL lysis buffer ($T_{100}E$, 10 mg lysozyme (Sigma, St. Louis, MO, USA)) for 3 h at 37 ºC. The lysis buffer was replaced with a 1 mL pronase buffer ($T_{100}E$, 2 mg of Pronase E from *Streptomyces griseus* (Sigma, St. Louis, MO, USA), 1.5% N-lauryl sarcosyl (Sigma, St. Louis, MO, USA)) and incubated for 16 h at 37 ºC. Afterwards the plugs were washed four times in 1 x TE with gentle shaking for 30 min per wash. A one third plug of each strain was digested with *Not*I restriction endonuclease (New England BioLabs, Ipswich, MA, USA) in a volume of 100 μL for

16 h at 25 ºC according to the manufacturer's specifications. The plugs were rinsed with 1 x TE at 4ºC before electrophoresis. The digested DNA fragments were separated by electrophoresis in a 1% agarose gel (Pulse Field Certified Agarose, Bio-Rad (Hercules, CA, USA)) in 0.5 x TBE buffer (0.1M Tris, 0.09M boric acid, 0.01M EDTA, pH 8) with a CHEF-DRIII apparatus (Bio-Rad, Hercules, CA, USA). Electrophoresis was performed at 15 ºC at 6 V/cm: interpolation pulse time of 25 s for 22 h. Gels were stained with ethidium bromide (0.5μg/mL) and photographied under UV light. The low range PFGE Marker (24.0 – 291.0 kb) (New England BioLabs, Ipswich, MA, USA) was used as a size marker and normalization reference. *The DNA fingerprint patterns were analyzed using Bionumerics 5.1 software (Applied Maths, Kortrijk, Belgium). The comparison of profiles obtained was performed with Pearson's product moment correlation coefficient and the Unweighted-Pair Group Method with Arithmetic means (UPGMA).

2.7. Genetic characterization: presence of gene markers.

The presence of 16 genetic markers (Table 1) was determined for *O. oeni* strains isolated during the MLF process. The genetic characterization protocol was performed using the method of Renouf *et al.* (2008). Each 25 μL amplification reaction mixture contained 2 ng DNA template, 12.5 μL custom-made PCR Master Mix (Finnzymes, Espoo, Finland), and 5 pmol of each primer. The reaction mixture was preheated for 5 min at 95 ºC and subjected to 30 cycles, each consisting of denaturing (30 s, 95 ºC), annealing (30 s, 55 ºC), and extension step (30 s, 72 ºC), in an iCycler IQ (Bio-Rad, Hercules, CA, USA). In addition with the convencional negative PCR control run without DNA, a positive control with the DNA of *O. oeni* strains (table 2) were used. These strains belong to the bacterial culture collection of Université Bordeaux Segalen-

ISVV (Bordeaux, France). Amplified products were resolved on MultiNA electrophoresis (Shimadzu Biotech., Kyoto, Japan) using the kit DNA 1000 marker.

3. Results

3.1. Monitoring microbial population

PCR-DGGE has been used to study the evolution of the BAL population from red wines elaborated in absence/presence antimicrobial phenolic antimicrobial extracts, almond skins and eucalyptus leaves. For this analysis, the PCR-*rpoB* amplicons obtained from *Lactobacillus plantarum* CECT 4645, *Lactobacillus casei* CECT 4045, *Pediococcus parvulus* CECT 4693 and *Oenococcus oeni* CECT 217 were used as reference markers. The results revealed a higher number of DGGE profiles in the samples collected at the beginning MLF whereas an only DGGE profile corresponding to the *O.oeni* species was detected, in most cases, in the samples collected at the end MLF. This result confirmed the predominance of *O.oeni* during MLF.

Figure 1 shows the *rpoB* PCR-DGGE gel corresponding to spontaneous MLF red wine in presence/absence antimicrobial phenolic extracts (almond skins and eucalyptus leaves). A maximum of five different bands per samples could be revealed on DGGE gel during MLF, being only possible to identify the lower band corresponding to *O. oeni*. In the control wine these five bands were detected at the start of MLF, being the band corresponding to *O. oeni* the only detected in the following collection days. On the other hand, the wines elaborated in presence antimicrobial phenolic extracts showed five bands in the samples collected at the start and 14 days after the start of MLF (middle of MLF), whereas two bands, upper band and *O.oeni* band, and one band,

O.oeni band, were revealed in the samples collected at the end of MLF of the red wine added from almond skins and from eucalyptus leaves extracts; respectively.

Concerning inoculated MLF red wine in presence/absence antimicrobial phenolic extracts (almond skins and eucalyptus leaves) and SO_2, the PCR-DGGE revealed few bands (results not show) during MLF. As in the spontaneous MLF red wine, a higher number of bands (n=5) was detected in the samples collected at the beginning of the MLF and again it was only possible to identify the lower band, corresponding to the *O.oeni* species. At the end of MLF, a only band corresponding to *O.oeni* was revealed in the wines tested with exception of the sample collected from wine added from eucalyptus leaves extract where again were detected five bands, being the most intensity band the corresponding to *O.oeni*.

3.2. Identification isolated colonies by rpoB PCR-DGGE

A total of 66 colonies isolated from the red wines undergoing spontaneous or inoculated MLF in the presence/absence of antimicrobial phenolic extracts (almond skins and eucalyptus leaves) and SO_2, were subjected to *rpoB* PCR-DGGE assay. A molecular ladder constitute with PCR-*rpoB* amplicons obtained from *O. oeni* CECT 217 was used as reference marker. The *rpoB* PCR-DGGE gel revealed that all isolated colonies belonged to *O. oeni* species. These results were confirming with the obtained in the 16S rRNA gene sequences, where the 100% isolated strains were identificated as *O. oeni*.

As expected, we obtained two different profiles (L and H) corresponding to the two *rpoB* amplicon sequences. In the whole 66 strains collection there were 3 H and 63 L strains. The analysis of the starter also showed strains characterized by L and H bands.

3.3. Genotypic characterization of O.oeni strains

From the PCR-DGGE results, a number of 43 *O.oeni* isolated (Table 3) from spontaneous (n=23) and inoculated (n=16) red wines at different time of MLF performance or from the starter (n=4) were characterized genotypically by REA-PFGE. The number of *O.oeni* selected was higher in the spontaneous MLF red wine than in the inoculated MLF red wine, by assuming a greater microbial biodiversity in the spontaneous MLF red wine.

O. oeni genomic DNA digested with *Not*I yielded 5-11 bands. Cluster analysis and visual inspection of the PFGE profiles of the 43 *O.oeni* isolated revealed 27 genotypes exhibiting specific profiles (Fig. 2), which allowed strain identification. The percentage of similarity between unrelated profiles varied from 20 to 98%. The results showed a clear separation between the *O.oeni* isolated from spontaneous MLF red wines and the *O.oeni* isolated from inoculated MLF red wines (Fig. 2).

The analysis by REA-PFGE *Not*I of the *O. oeni* starters (Fig.3) revealed that the starter 3 (St3) and one colony isolated from spontaneous MLF red wine (CtW.3) presented the same PFGE profile (Fig.2), in other words, were the same *O.oeni* strain. This result showed that this strain is well present in the winery. The rest of starters analyzed (St 2, 5 and 6) were clustered, as expected, together with the colonies isolated from inoculated MLF red wine. However, the percentage of similarity between starters and *O. oeni* isolated from inoculated MLF red wine was low, from 30 to 55%, showing that none of the starters dominated during MLF of the inoculated red wine.

With respect to the *O. oeni* isolated from spontaneous MLF red wines, the analysis by REA-PFGE yielded 5-11 bands; most of the isolated strains showed 7 bands. The 23 *O. oeni* isolated were separated in 14 PFGE profiles different (Fig.2). The strains: Ct.17

and WA.13 exhibited a greater similarity with the colonies isolated from inoculated MLF red wine than with the colonies isolated from spontaneous MLF red wine. This result again demonstrated the domain of the indigenous microflora of the winery on malolactic starters employed in the inoculated MLF red wine. The profiles number 4 and 7 showed the highest number of strains with five and four isolates, respectively. The profile 4 was constituted by strains isolated from red wine elaborated in presence/absence antimicrobial phenolic extract whereas the strains of the profile 7 were isolated from control wine (absence phenolic extract). On the other hand, the profile 3 corresponding to isolated strains from wine elaborated in absence of antimicrobial phenolic extract or with eucalyptus leaves extract was also considered as interesting.

In reference to the *O. oeni* isolated from inoculated MLF red wines, the results by PFGE *Not*I revealed number 7-10 bands; the most of the *O. oeni* isolated showed 8 bands. The 16 *O. oeni* strains were classified in 10 unrelated PFGE profiles (Fig. 2). The profile 13 stood out for being formed by strains isolated from control wine or sulfited wines while the profile 15 was constituted by strains isolated from wine elaborated in presence antimicrobial phenolic extract, Amanda and eucalyptus leaves, or sulfited.

3.4. Genetic characterization: presence of gene markers.

Some strains isolated from both spontaneous and inoculated MLF red wines were characterized genetically for the presence of 16 significant genetic markers (M1 to M16, Table 1): they represented the profiles 3, 4, 7, 13 and 15. As shows the table 4, six out of the 16 markers studied were present in the profiles selected: polysaccharide

biosynthesis export protein (M3), present in the profiles 3 and 7; predicted transcriptional regulators (M7), present in all patterns; hypothetical protein (M8), present in the profiles 7 and 15; alcohol–sugar dehydrogenase (M9), present in all profiles to exception pattern 3; arabinose efflux protein MFS (M11), present only in the pattern 13; and glucosyltransferase involved in cell wall biogenesis (M15), which was present in all profiles to exception of the pattern 13. This result showed a smaller number of markers in the genome of strains from wines elaborated in presence antimicrobial phenolic extracts (profiles 3, 4 and 15) than the strains from wines manufactured without addition of antimicrobial phenolic extracts (profiles 7 and 13).

4. Discussion

In this work, different molecular tools were applied with the aim to analyze the evolution of wine-associated LAB from red wines elaborated in the absence/presence of antimicrobial phenolic compounds (almond skins and eucalyptus leaves) added before MLF and to genetically characterize representative *O.oeni* strains.

Molecular PCR-DGGE was used to study the structure and evolution of LAB community from red wines elaborated in absence/presence antimicrobial phenolic antimicrobial extracts, almond skins and eucalyptus leaves. This technique has been used successfully in monitoring the fermentation of red (Renouf *et al.*, 2006; 2007; Spano *et al.*, 2007) and white (Renouf *et al.*, 2005) wines. The results showed greater microbial diversity at the beginning MLF which it decreased as MLF progressed, with exception the inoculated MLF red wine elaborated with eucalyptus leaves extract. In all the wines analyzed, a total of five bands were detected at the start of MLF but only the

lower band corresponding to *O.oeni* can be identified. At the end of MLF, *O.oeni* was the species predominant in the wines tested. This result was as expected, since many studied had shown before that *O.oeni* is the main responsible species for MLF (Dicks *et al.* 1988; Reguant *et al.* 2003; López *et al*. 2007; Ruiz *et al*. 2010).

The molecular methods *rpoB* PCR-DGGE and 16S rRNA enabled us the identification of 66 strains isolated from spontaneous and inoculated MLF red wines at different time of MLF performance. In both methods, the 100% isolated strains were identified as *O.oeni*. This result again confirmed the dominant of the *O.oeni* species during the MLF of the wines studied. As expected, the *rpoB* analysis showed two different profiles (L and H) corresponding to the two rpoB amplicon sequences. DGGE gels revealed a total of 63 L and 3 H *O.oeni* strains, which suggested a more favorably adaptation of L strains to MLF taking place in this winery. These results agreed with the results of Renouf *et al.* (2009) on the prevalence of L-strains over H-strains during MLF. Out of the four starters two were of H type and two L type.

Identification of the *O.oeni* strains of this study was successfully achieved by PFGE, being *Not*I the restriction enzyme employed for this analysis. This molecular tool is considered as the most powerful method for strain typing (López *et al.*, 2008). The resulting 27 unrelated genotypes out of the total 43 *O.oeni* isolated of this study indicated a rich biodiversity of indigenous *O.oeni* strains in the winery. As observed in the dendogram (Fig. 2), the 27 patterns were separated clearly in two big groups corresponding to the two different types of MLF, spontaneous and inoculated with malolactic starters. Some profiles were more represented than other, as for example the profiles 4, 7, 13, 14 and 18. However, whatever the wine, inoculated or not, there was not a dominant profile that would have shown that some strains would be more or less

tolerant to the antimicrobial phenolic extracts, almond skins and eucalyptus leaves. With respect the starters, one of the starters St.3 was found in the spontaneous fermentation in the control wine (SCtW.03); this showed that this strain was well present in the winery. The profile of St.5 was never found and profiles close, but not identical, to St.2 and St.6 were found in the inoculated wines. The high diversity of strains in the inoculated samples showed the difficulty for the starter to dominate the indigenous microflora.

From PFGE results, some strains were characterized for the presence of 16 oenological markers (M1 to M16). They represented the profiles 3, 4, 7, 13 and 15. Some markers may characterize resistance to environmental stress (M1 and M12), another markers may be important for the metabolites transport (M11, M13 and M14), whereas others may be essential cellular functions (M5, M7 and M15) (Renouf *et al*., 2008). Six out of the 16 markers studied were present in the genome of strains selected (Table 4): M7 in all the strains, M9 in all except pattern 3, M15 in all except profile 13, M8 in patterns 7 and 15, and finally M11 in profile 13. The presence of markers of M7, M9 and M15 in all or almost all characterized strains could indicate that they were essential for the survival of bacteria during MLF. These markers may be responsible of resistance/response to stress by high sugar and ethanol concentrations (M9), cellular functions viz. the cell wall organization (M15) and the transcription (M7). This showed a tendency for a higher number of markers in the genome of strains from wines fermented without addition of antimicrobial phenolic extracts (profiles 7 and 13). These results agreed with Renouf *et al.* (2008), where these 6 markers were present with a higher percentage in the strains selected during industrial winemaking of three wines.

In summary, we concluded that *O.oeni* was the responsible species for MLF in the wines elaborated in absence/presence antimicrobial phenolic extracts, almond skins and eucalyptus leaves. DGGE gels showed a more favorably adaptation of L *O.oeni* strains than H strains to MLF. The high number of profiles revealed in the PFGE analysis indicated a rich biodiversity indigenous *O.oeni* strains in the winery. And finally, the strains from wines manufactured in presence of antimicrobial phenolic extracts (almond skins and eucalyptus leaves) presented differences in their genetic markers in comparison with strains from wines not exposed to antimicrobial phenolic extracts. Furthermore, this study contributes to provide a basis for further investigation of the molecular and evolutionary mechanisms leading to the prevalence of some *O.oeni* strains in wines treated with polyphenols as particular inhibitors.

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Table 1. Primers used in this study.

Genes/Markers	Forward primer (5' → 3')	Reverse primer (5' → 3')	Amplicon length (bp)
16S rRNA			
POmod/P3rev	CAGAGTTTGATCCTGGCTCAG	GGCCGTTACTGACGCTGAG	792-825
16midfor/PC5rev	GGCCGTTACTGACGCTGAG	CTCACTATAGGGATACCTTGT-TACGACTT	767-771
***beta* subunit RNA polymerase**			
*rpo*B1, *rpo*B1o/ *rpo*B2 rev	ATTGACCACTTGGGTAACCGTCG ATCGATCACTTAGGCAATCGTCG	CGCCCGCCGCGCGCGGGGCGG-GGGC ACGATCACGGGTCAAAC-C ACC	250
Marker			
Cadmiun transporting P-type ATPase-M1	GAAGCTCAAGATACCATCC	CGACTTGCACAGATTCC	650
Dps ferritine-M2	TTGGTTAATTCAGCGCCGTTGT	ATTGATCACGATGTCCCAAC	500
Polysaccharide biosyntesis export protein-M3	CTCGTAAGCATGGTTCTCTC	ATTGGTTTGATGAAAAATGG	565
Maltose phosphorylase-M4	ACGCATGATTCCTCATTATTATC	GGTCTTTCAAAATACCATCG	600
Transcriptional regulator-M5	TGGCAAACGTCTCAATCAAC	AGCTTACGGCTGATGCTTT	380
Hypothetical protein-M6	TACTGTTCGTCAGCCGATGT	CTCCCGACAAACTGCTAATG	400
Predicted transcriptional regulators-M7	CAATCAAGCCGGAATAGTT	TGACCAGTTCGAATGAATTC	462
Hypothetical protein-M8	ATGACGCCATTCTATATCCA	ATTTGCCTCGATAGTTTCTG	605
Alcohol-sugar dehydrogenase-M9	GGAAACAATTTACGCTTGC	CGGCCTGTTTGATAAAGAA	471
Copper chaperone-M10	CCTCCTACTTAACCTTGACG	AGTCCCACCTCCTGAATAAA	420
Arabinose efflux protein MFS-M11	TGGCTTAATCCCATCAGAAA	CCAAATTGTCCAGAATACCG	600
Thioredoxin-M12	GTTTCTGAAGACCCGCTTA	TGATGCCCCCTTCGTAAT	300
Glycerol uptake facilitator protein-M13	CTAACGCATTCCTGAAGAAC	CCCAACTATATTCCCAGTGA	602
Arabinose efflux permease-M14	TTTATCTGTCCAAGCAGGT	AATTAGAAGAACGCTGATAGCC	330
Glycosiyltransferases involved in cell wall biogenesis-M15	TGTTAACGATACGAAGCGCG	GAATCACTCCATTCCGTCACC	600
Hypothetical protein lp_3433-M16	AAATAACGCAGGCCAATC	CCATGATTCCTGGTTTACTGAG	569

Table 2. *Oenococcus oeni* strains positive control to genetic characterization

Strains	Marker
O.oeni 7.147	M1-M3, M5-M7
O.oeni 7.135	M4,M8,M9
O.oeni 7.125	M10-M12, M14
O.oeni 10.13	M13, M16
O.oeni 10.10	M15

Table 3. *Oenococcus oeni* strains isolated from spontaneous and inoculated malolactic fermentation red wines elaborated in absence/presence of antimicrobial phenolic extract: Almond skins and eucalyptus leaves; and dioxide sulfure (SO_2).

Red Wine	*Treatment*	*Sampling Time**	*No. O. oeni isolates*	*Representative strains*	*DGGE profiles*	*PFGE profiles*
Spontaneous MLF	Control	0	10	SCtW.00	L	12
				SCtW.03	L	5
				SCtW.06	L	7
				SCtW.09	L	7
		1	10	SCtW.11	L	7
				SCtW.14	L	7
				SCtW.17	L	22
		2	10	SCtW.22	L	1
				SCtW.23	L	4
				SCtW.27	L	4
				SCtW.28	L	3
	Almond skins	1	10	SWA.13	L	21
				SWA.14	L	6
				SWA.15	L	2
				SWA.16	L	8
		2	10	SWA.20	L	9
				SWA.23	L	11
				SWA.25	L	4
				SWA.28	L	11
	Eucalyptus leaves extract	1	10	SWE.10	L	10
				SWE.12	L	3
				SWE.13	L	4
				SWE.14	L	4
Inoculated MLF	Control	0	10	ICtW.01	L	25
				ICtW.08	L	27
		2	10	ICtW.22	L	18
				ICtW.23	L	18
				ICtW.24	H	19
				ICtW.25	H	13
	SO_2	0	10	IS0_2.00	L	13
				IS0_2.01	L	15
		1	10	IS0_2.10	L	17
				IS0_2.13	L	13
		2	10	IS0_2.23	L	23
				IS0_2.24	L	16
	Almond skins	2	10	IWA.24	L	15
				IWA.26	L	18
	Eucalyptus leaves extract	1	10	IWE.12	L	15
				IWE.14	L	14
Starter			10	St.2	H	26
				St.3	L	5
				St.5	H	20
				St.6	L	24

*Sampling time: 0 (start MLF), 1 (middle MLF), 2 (end MLF).

Table 4. Presence (+) or absence (-) of 16 oenological genetic markers

REA-PFGE profiles	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	Total
Control +	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Profile 3	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	3
Profile 4	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	3
Profile 7	-	-	+	-	-	-	+	+	+	-	-	-	-	-	+	-	5
Profile 13	-	-	-	-	-	-	+	-	+	-	+	-	-	-	+	-	4
Profile 15	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	3
Control -	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

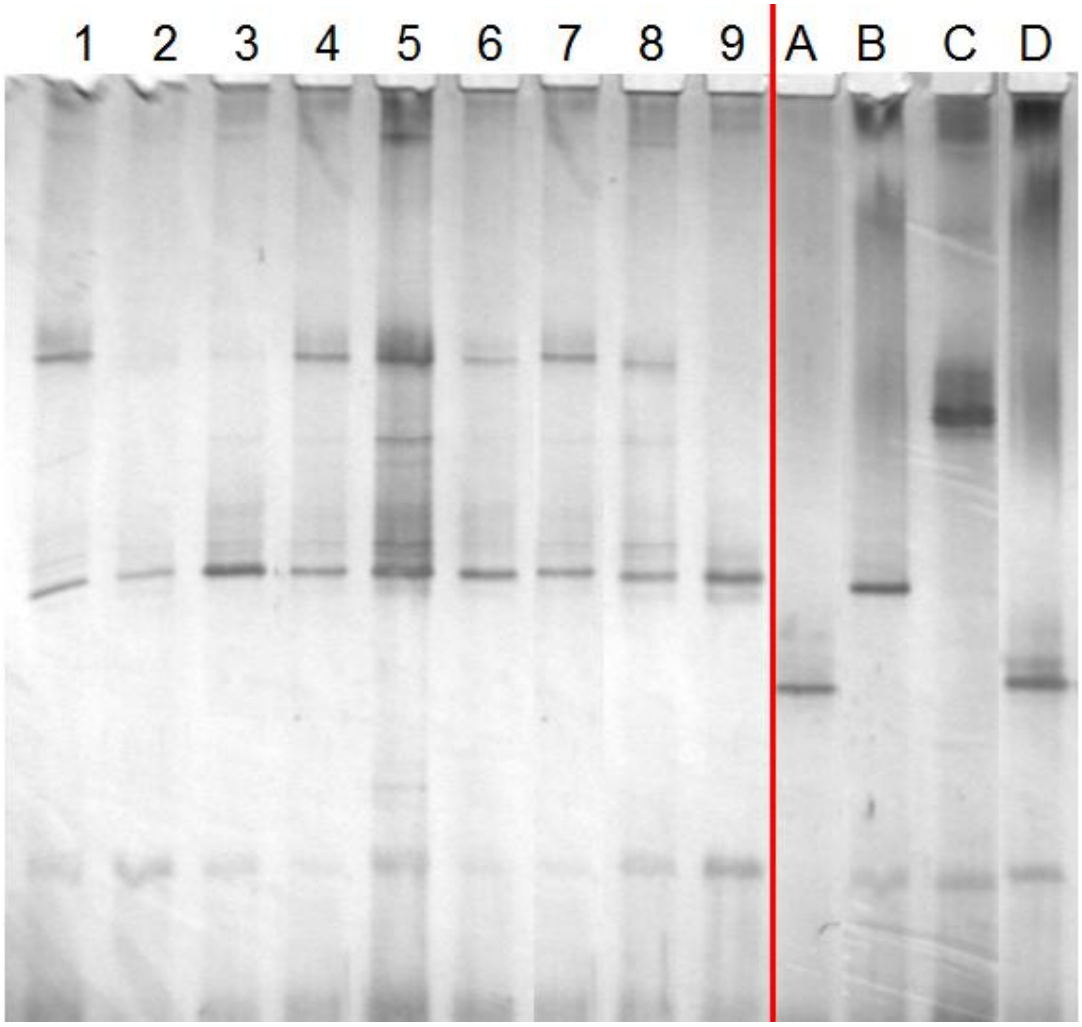

Figure 1. DGGE profiles of wines samples elaborated in presence/absence antimicrobial phenolic extracts during MLF. Lanes 1-3: wine elaborated absence phenolic extract 1: start MLF, 2: middle MLF, 3: end MLF; 4-6: wine added from almond skins 4: start MLF, 5: middle MLF, 6: end MLF; 7-9: wine elaborated with eucalyptus leaves extract 7: start MLF, 8: middle MLF, 9: end MLF. The four last lanes correspond to pure species: lane A, *Lactobacillus casei*, lane B, *Oenococcus oeni*, lane C, *Pediococcus parvulus*, lane D, *Lactobacillus plantarum*.

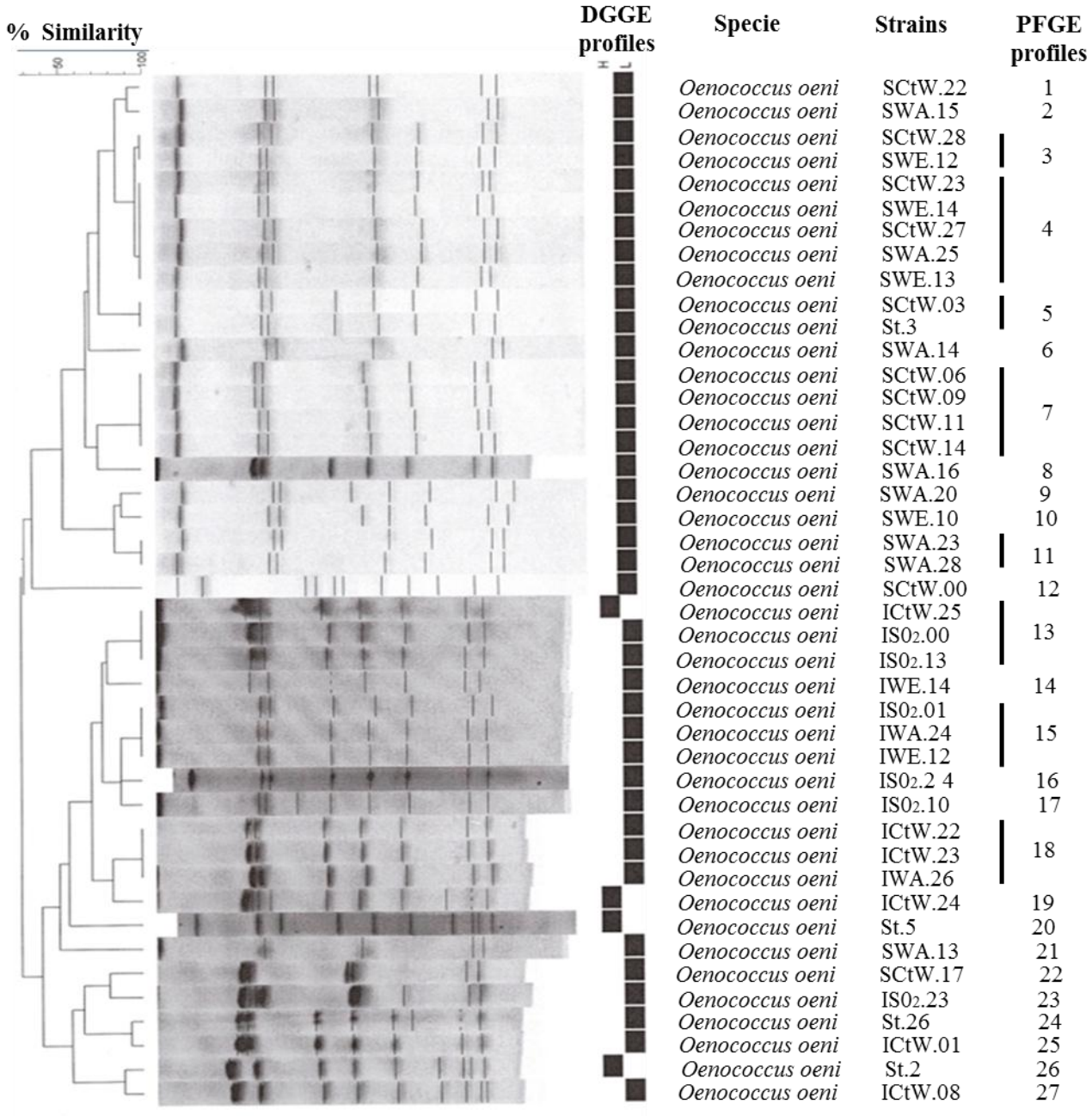

Figure 2. UPGMA dendrogram based on the *Not*I PFGE profiles of the 43 *Oenococcus oeni* strains examined in this studied, which showed 27 patterns unrelated and four *O.oeni* malolactic starters

Discusión General

V. DISCUSIÓN GENERAL

El anhídrido sulfuroso o dióxido de azufre (SO_2) presenta múltiples propiedades como conservante en la elaboración de los vinos, entre las que se pueden destacar los efectos antioxidante y antimicrobiano; especialmente frente a bacterias lácticas (BAL). Es importante que el crecimiento de estas bacterias se realice bajo control, ya que de lo contrario podrían producirse alteraciones de la calidad del vino tales como la generación de aminas biógenas. Por ello, hoy en día el sulfitado constituye un tratamiento indispensable en la tecnología de elaboración y conservación de los vinos. Sin embargo, en los últimos años existe una tendencia a reducir progresivamente los niveles máximos autorizados en los mostos y en los vinos, debido a sus posibles efectos indeseables para la salud. Por ello, existe un gran interés en el desarrollo de alternativas totales o parciales al tradicional uso de SO_2 en la vinificación; siendo el objetivo global de la presente Tesis Doctoral evaluar el potencial empleo de los polifenoles antimicrobianos frente a BAL del vino como una alternativa total o parcial al empleo de SO_2 en enología.

El trabajo de investigación de esta Memoria comprende varias partes. En la primera de ellas, publicaciones I y II, se estudió el efecto antimicrobiano de los polifenoles frente a las BAL en base a su estructura química. Esta parte se completó con estudios de microscopía y determinación de la actividad antioxidante. En la segunda parte, publicación III, se evalúo la capacidad de degradar aminas biógenas de un gran número de BAL así como el efecto que tienen los polifenoles y otros componentes del vino sobre dicho metabolismo degradativo. En la tercera parte, publicación IV, se procedió a analizar la actividad antimicrobiana de diversos extractos fenólicos de origen vegetal así como a evaluar la capacidad tecnológica de estos extractos durante la vinificación. Los resultados de este estudio fueron protegidos bajo patente. Por último, en la cuarta parte (publicaciónes V y VI) se realizó una caracterización química y microbiana de los vinos elaborados con extractos fenólicos.

V.1. Propiedades antimicrobianas de los compuestos fenólicos del vino frente a bacterias lácticas de origen vínico

A los polifenoles se les han atribuido muchas de las propiedades beneficiosas derivadas del consumo moderado del vino, entre las que podemos destacar efectos

cardioprotectores, antioxidantes, antimicrobianos, etc (Xia y col., 2010). Esta última propiedad constituye el eje principal de la presente Tesis, en la que se analizará el efecto de los polifenoles sobre el crecimiento de BAL del vino. Hay que destacar que, bajo nuestro conocimiento, es la primera vez que se realiza un estudio sistemático de la actividad antimicrobiana de todas las familias de polifenoles presentes en los mostos y vinos frente a BAL. Para una fácil comparación de los resultados se determinaron los parámetros de supervivencia, MIC y MBC, e inhibición, IC_{50}.

En la primera etapa de este estudio (Publicación I) nos centramos en la evaluación de la capacidad antimicrobiana de 21 compuestos fenólicos, la mayoría de ellos presentes de forma natural en el mosto y el vino, seleccionados en base a su grupo funcional y/o anillo sustituyente, frente a dos cepas de BAL alterantes: *L. hilgardii* IFI-CA 49 y *P. pentosaceus* IFI-CA 85 (a partir de 2011 el término IFI-CA se cambió por la denominación CIAL). En este trabajo la capacidad antimicrobiana fue determinada por los parámetros de supervivencia MIC y MBC.

Los resultados obtenidos pusieron de manifiesto que el efecto antimicrobiano de los polifenoles frente a las BAL estaba fuertemente determinado por su estructura química (Vivas y col., 1997; Reguant y col., 2000; Rozès y col., 2003), no dependiendo sólo de la familia fenólica sino también del sustituyente químico. A este respecto, se observó que el poder antimicrobiano del ácido gálico fue mayor que el de sus derivados etilados y especialmente metilados y diméricos. Por otro parte, los flavonoles destacaron por ser la familia fenólica más activa frente a las BAL ensayadas, especialmente frente a *P. pentosaceus*. Por el contrario, al igual que en el estudio de Figueiredo y col., (2008), no observamos efecto de los flavan-3-oles sobre el crecimiento de las BAL.

Por otro lado, en la mayoría de los compuestos fenólicos activos no se percibió un efecto inhibidor a concentraciones inferiores a 200 mg/L, lo cual sugería un efecto antimicrobiano dosis dependiente. Este efecto también fue descrito por Campos y col. (2003), en cuyo trabajo no detectaron un importante efecto inhibidor de los ácidos hidroxicinámicos e hidroxibenzoicos sobre *L. hilgardii* a concentraciones inferiores de 500 mg/L.

Finalmente, se observó que la actividad antimicrobiana de los compuestos fenólicos también dependía de la BAL ensayada. En nuestro estudio, *P. pentosaceus* no sólo fue inhibida por un mayor número de compuestos fenólicos sino que también

el valor de sus parámetros de supervivencia, MIC y MBC, fue en casi todos los polifenoles inferior a los observados en *L. hilgardii*, en otras palabras, *P. pentosaceus* mostró una mayor susceptibilidad al efecto inhibidor de los compuestos fenólicos que *L. hilgardii*.

Con el objetivo de comparar el carácter antimicrobiano de los polifenoles y el SO_2, se determinaron los parámetros de supervivencia de *L. hilgardii* y *P. pentosaceus* frente al metabisulfito potásico ($K_2S_2O_5$), principal aditivo empleado en bodega por su efecto antioxidante y antimicrobiano (especialmente frente a BAL). Los valores de MIC y MBC obtenidos se encontraban dentro del rango observado por Rojo-Bezares y col. (2007) para BAL de origen vínico. Estos resultados también reflejaron una menor toxicidad del metabisulfito potásico con respecto a algunos compuestos fenólicos, como por ejemplo el kanferol. En referencia a las cepas ensayadas, los resultados pusieron de manifiesto que *L. hilgardii* fue más susceptible al efecto antimicrobiano del metabisulfito mientras que *P. pentosaceus* lo fue al de los compuestos fenólicos.

Una vez demostrada la capacidad antimicrobiana de los compuestos fenólicos frente a BAL alterantes del vino, el siguiente objetivo era evaluar dicha capacidad frente a la BAL responsable de la FML en la mayoría de los vinos, es decir, frente a *O. oeni* (Publicación II). Debido a que el método empleado para la obtención de los parámetros de supervivencia MIC y MBC era laborioso y prolongado en el tiempo, se optimizó un nuevo método de actividad antimicrobiana basado en la determinación del parámetro de inhibición IC_{50}.

Previamente a la evaluación del parámetro IC_{50} de los polifenoles frente a diferentes cepas de *O. oeni* (IFI-CA 17, IFI-CA 88, IFI-CA 91, IFI-CA 96, se procedió a ensayar nuevamente la actividad antimicrobiana de los compuestos fenólicos frente a las cepas *L. hilgardii* IFI-CA 49 y *P. pentosaceus* IFI-CA 85, con el objetivo de comparar ambos métodos. Obteniéndose que estadísticamente a partir de ambos métodos se obtenían resultados similares. En base a este dato y nuestra experiencia, determinamos que el método a seguir para evaluar la actividad antimicrobiana de los compuestos fenólicos, a partir de este momento, se basaría en la determinación del parámetro de inhibición, IC_{50}, al ser ésta más rápida y factible.

Al igual que en el estudio anterior, se observó que el efecto antimicrobiano de los compuestos fenólicos estaba fuertemente determinado por su estructura química.

En general, el potencial inhibidor de las diferentes familias fenólicas analizadas seguía el siguiente orden: flavonoles > estilbenos > ácidos hidroxicinámicos > ácidos y esteres hidroxibenzoicos > alcoholes fenólicos >> flavan-3-ol (no efecto). El no efecto de los flavanoles analizados, catequina y epicatequina, sobre el crecimiento de *L.hilgarddi*, *P. pentosaceus* y *O. oeni*, también ha sido mostrado por otros autores con respecto a *O.oeni* (Reguant y col., 2000) u otras especies de BAL del vino (Figueiredo y col., 2008; Rodríguez y col., 2009; Diez y col., 2010).

Los resultados obtenidos mostraron, nuevamente, una diferente susceptibilidad de las BAL al efecto antimicrobiano de los compuestos fenólicos. Las cepas de *O. oeni* fueron susceptibles a un mayor número de compuestos fenólicos (n=12-14) que las cepas alterantes *L. hilgardii* y *P. pentosaceus* (n=8). Además, algunos compuestos fenólicos ensayados exhibieron cierta selectividad frente a las BAL. De este modo, el kanferol fue activo sólo frente a especies de BAL no *O. oeni*, mientras que la miricetina sólo lo fue frente a cepas de la especie *O. oeni*. Esta diferente selectividad también fue observada en fenoles activos frente a todas las cepas ensayadas. A modo de ejemplo, se observó que los valores IC_{50} del ácido ferúlico fueron más bajos frente a cepas de *O. oeni* ($IC_{50\ rango}$= 0.475-0.843 g/L) que frente a *L. hilgardii* (IC_{50} = 2.11 g/L) y *P. pentosaceus* (IC_{50} = 1.58 g/L). Este resultado demostraba, de nuevo, un efecto dosis dependiente en la capacidad antimicrobiana de los fenoles.

El análisis de componentes principales mostró cierta agrupación de los compuestos fenólicos en función de su estructura química, en contraste y de acuerdo a sus valores IC_{50} las cepas fueron englobadas en una pequeña área, lo que reflejaba una respuesta similar al efecto antimicrobiano de los polifenoles ensayados. Este resultado confirmaba que la actividad antimicrobiana de los compuestos fenólicos dependía de su estructura química.

La actividad antimicrobiana de los compuestos fenólicos se comparó con la del metabisulfito potásico y la lisozima, ambos aditivos autorizados en enología. La lisozima exhibió escaso o nulo efecto frente a las BAL ensayadas, mientras que el metabisulfito mostró una mayor toxicidad, valores IC_{50} más bajos, frente a *O. oeni* que frente a *L. hilgardii* y *P. pentosaceus*. A partir de estos resultados también se puede concluir que *O. oeni* fue más susceptible al metabisulfito mientras que las bacterias alterantes, *L. hilgardii* y *P. pentosaceus,* fueron más sensibles a los compuestos fenólicos del vino.

Otro de los objetivos fue dilucidar el mecanismo de acción de los compuestos fenólicos sobre la morfología y viabilidad de las BAL. Con tal fin, se realizaron estudios de microscopía de fluorescencia y electrónica de transmisión, los cuales revelaron pérdida de viabilidad bacteriana y daños en la integridad de la membrana; respectivamente. El daño en la membrana bacteriana de las cepas *P.pentosaceus* IFI-CA 85 y *O.oeni* IFI-CA 96 era notable, observándose pérdida de su continuidad así como vertido del contenido citoplasmático al medio extracelular, con la consecuente muerte celular. Rodríguez y col. (2009) también apreciaron daños en la integridad de la membrana tras la exposición de *Lactobacillus plantarum* a compuestos fenólicos. Las microfotografías obtenidas tras la exposición de *P.pentosaceus* IFI-CA 85 y *O.oeni* IFI-CA 96 al metabisulfito potásico, revelaron daños en la integridad de la membrana de *O.oeni* pero no de *P.pentosaceus*. Estos resultados confirmaban una mayor susceptibilidad de *O. oeni* al metabisulfito potásico con respecto a *P. pentosaceus,* tal y como reflejaban sus valores IC_{50}. Los resultados obtenidos a partir de los estudios de microscopía, sugerían que el mecanismo de acción de los compuestos fenólicos se basa fundamentalmente en interacciones hidrofóbicas entre los compuetos fenólicos y los lípidos de la membrana que conducirían a la desintegración de la membrana bacteriana, causando la muerte celular. Esta muerte se debería probablemente a interferencias en procesos biosintéticos indispensables para la viabilidad bacteriana, como son el transporte de nutrientes y/o una respuesta provocada por la estimulación de la liberación de enzimas autolíticas bacterianas. Este hecho ha sido descrito previamente con otros agentes inhibidores y otras especies de BAL (Ibrahim et al., 1996).

Entre las múltiples propiedades por las que se emplean los sulfitos en enología se encuentra su capacidad antioxidante. Es por ello, que a la hora de desarrollar alternativas a su empleo durante la vinificación, resulta importante seleccionar tratamientos con una doble aplicación, antimicrobiana y antioxidante. Aunque las propiedades antioxidantes de los polifenoles del vino ya han sido descritas en la bibliografía, nos resultaba interesante valorar la actividad antioxidante de los compuestos fenólicos seleccionados en este trabajo.

La actividad antioxidante fue medida como capacidad de absorción de radicales de oxígeno (ORAC). Los valores obtenidos comprendían desde 10,1 μmol Trolox/ mg, correspondiente al ácido gálico, hasta los 47,6 correspondiente a *trans-*

resveratrol. Al igual que en el caso de la actividad antimicrobiana, la estructura química de los diferentes compuestos fenólicos influía en su capacidad antioxidante. De este modo, los esteres galato de metilo y de etilo mostraron valores de ORAC superiores a los de su ácido en forma libre. Los valores ORAC de los polifenoles eran superiores a los 4,4 μmol Trolox/mg del ácido ascórbico, aditivo antioxidante utilizado en industria, lo que da indicio de la buena capacidad antioxidante de estos compuestos.

Algunos autores (Reguant y col., 2000; Theobald y col., 2008) han relacionado las propiedades antioxidantes de los compuestos fenólicos con su efecto sobre el crecimiento y metabolismo de las BAL. Sin embargo, los resultados estadísticos de nuestro estudio pusieron de manifiesto una correlación no lineal entre ambas propiedades. En este análisis estadístico se volvió a observar su actividad antioxidante y antimicrobiana de los polifenoles está condicionada por su estructura química.

En resumen, los resultados obtenidos tras la determinación de los parámetros de supervivencia (MIC y MBC) e inhibición (IC_{50}) ponen de manifiesto que el efecto antimicrobiano de los compuestos fenólicos depende de su estructura química y concentración, así como de las características intrínsecas de cada cepa. Este efecto antimicrobiano podría ser debido a la generación de posibles daños en la integridad de la membrana bacteriana. Por último, se ha determinado el carácter antioxidante de estos compuestos, el cual no está relacionado con la actividad antimicrobiana pero si con su estructura química. Todos estos resultados confirmarían el potencial uso de los polifenoles como aditivo alternativo al sulfito en enología.

V.2. Capacidad de bacterias lácticas enológicas para degradar aminas biógenas.

Se ha descrito que algunas BAL aisladas de queso y otros alimentos son capaces de degradar aminas biógenas y reducir su contenido en los alimentos. Esta capacidad no ha sido aún descrita en BAL de origen enológico, siendo por ello el siguiente objetivo de la presente Tesis evaluar la capacidad de distintas cepas de BAL (n=85) aisladas de diferentes nichos enológicos para degradar histamina, tiramina y putrescina, principales aminas biógenas del vino, así como determinar el efecto de los

polifenoles y otros componentes de la matriz del vino (pH, etanol, SO_2) sobre dicho metabolismo (Publicación III).

Con tal fin, se llevaron a cabo experimentos en medios de cultivos enriquecidos con aminas biógenas y FML a escala de laboratorio. En ambos ensayos la capacidad amino-degradativa de las BAL fue analizada y cuantificada por RP-HPLC, al ser la técnica más sensible y utilizada para la detección de aminas biógenas en el vino (Lethonen, 1996; Mafra y cols. 1999; Paleologos y Kontominas, 2004).

Los resultados obtenidos a partir de los experimentos realizados en medio de cultivo mostraron que el 25% de las cepas aisladas eran capaces de degradar histamina, un 18% tiramina y otro 18% putrescina, no observándose esta capacidad en ningún *starters* o cepa tipo analizada. Hay que destacar que es la primera vez que se mostraba la habilidad de degradar putrescina en BAL.

Nueve de las 85 cepas seleccionadas mostraron capacidad amino-degradativa, perteneciendo la mayoría de estas cepas a los géneros *Lactobacillus* (*L. plantarum* IFI-CA 26 e IFI-CA 54, *L. hilgardii* IFI-CA 41, *L. casei* IFI-CA 52) y *Pediococcus* (*P. pentosaceus* IFI-CA 30, IFI-CA 83 e IFI-CA 86, *P. parvulus* IFI-CA 31). Sólo una de las cepas correspondía a la especie *O. oeni* (*O. oeni* IFI-CA 32), poniendo de manifiesto que rara vez la disminución del contenido de aminas biógenas en el vino se debería a esta especie. Este bajo número de bacterias con capacidad amino-degradativa sugería que esta habilidad se halla con baja frecuencia en BAL de origen enológico. *L. casei* IFI-CA 52 destacó por ser la cepa con mayor capacidad amino-degradativa, disminuyendo el contenido inicial de histamina un 54%, el de tiramina un 55% y el de putrescina un 65%. Al igual que *L. casei* IFI-CA 52, todas las cepas con habilidad amino-degradativa disminuyeron el contenido de más de una amina. Este hecho también ha sido descrito en otros microrganismos con esta actividad, como *Micrococcus* varians (Leuschner y col., 1998) y *Staphylococcus xylosus* (Martuscelli y col., 2000; Gardini y col., 2002), donde se determinó la presencia de más de una amino oxidasa. Con respecto al origen de las bacterias que mostraron actividad amino-degradativa, cabe destacar que procedían en un mayor número de lías de fermentación y vinos de crianza biológica, lo que indicaba que ambos nichos enológicos son especialmente atractivos para el crecimiento de BAL con potencial para degradar aminas biógenas.

Por otro lado, se estudió la posible producción de aminas biógenas entre las nueve cepas de BAL con capacidad amino-degradativa. No detectándose ni por RP-HPLC ni por PCR-multiplex cepas productoras de aminas, pudiéndose concluir que la capacidad amino-degradativa de las BAL parecía no estar asociada a la producción de aminas biógenas.

Con el objetivo de evaluar el comportamiento de las cepas seleccionadas durante la elaboración del vino, se realizaron experimentos de FML a escala de laboratorio. Estos experimentos pusieron de manifiesto que ningún vino inoculado, a excepción del inoculado con *L. casei* IFI-CA 52 (disminuyó 16 % histamina, 15% tiramina, 8% putrescina), exhibió diferencias significativas en el contenido de aminas biógenas con respecto al control. Este resultado sugería un efecto negativo de la matriz del vino sobre la capacidad amino-degradativa de las bacterias. Gardini y col. (2002), también han descrito que la actividad mono- y di-amino oxidasa *in vitro* de los microorganismos no era reproducible cuantitativamente en sistema *in vivo*.

Con el propósito de profundizar en el conocimiento de cómo la matriz del vino afecta a la capacidad amino-degradativa de las BAL, se evaluó el efecto de diferentes factores enológicos (pH, etanol, polifenoles y metabisulfito) sobre la capacidad de degradar histamina (amina que determina la transacción del vino) de extractos y células de *L. casei* IFI-CA 52. Los resultados obtenidos en el control no mostraron diferencias significativas entre la habilidad amino-degradativa de los extractos celulares y células enteras de *L. casei* IFI-CA 52, lo que sugería que las enzimas implicadas en esta degradación serían intracelulares. Por otra parte, la capacidad amino-degradativa de los extractos y células de *L. casei* IFI-CA 52 no se vio afectada a los pH ensayados, pH 7 y 4.6 (más próximo al pH del vino), siendo ligeramente superior la degradación de histamina a pH 4.6. Este resultado ponía de manifiesto que las enzimas amino-degradativas serían activas a un pH próximo al vino. Por el contrario, sí se observó un efecto negativo del etanol (12%), de los polifenoles (75 y 660mg/L) y del SO_2 (30mg/L) sobre la degradación de histamina. El efecto negativo del etanol podría ser debido a su efecto inhibidor sobre las enzimas amino-oxidasas, mientras que en el caso de los polifenoles y el SO_2 este efecto podría ser causado por su capacidad antimicrobiana frente a las BAL. En general, estos resultados confirmaban el efecto negativo de la matriz del vino sobre la capacidad amino-degradativa de las BAL, lo cual explicaría los diferentes porcentajes de degradación detectados en medio de cultivo y vino.

En resumen, este estudio pone de manifiesto el posible uso de *starters* de BAL enológicas con capacidad amino-degradativa como procedimiento a seguir para prevenir o reducir la acumulación de aminas biógenas en el vino, siendo necesarios aún más estudios.

V.3. Potencial aplicación tecnológica de extractos fenólicos frente a bacterias lácticas de origen vínico.

Como se ha estudiado y demostrado en la primera parte de la presente Tesis, una potencial alternativa al uso del SO_2 durante la elaboración del vino sería el empleo de compuestos fenólicos con carácter antimicrobiano. Estos compuestos deberán ser adicionados al vino en forma de extractos fenólicos de origen vegetal. Por ello, en la tercera parte de esta Memoria nos centramos en la búsqueda y caracterización de extractos fenólicos antimicrobianos obtenidos a partir de plantas, incluida la vid, así como en la valoración de su eficacia tecnológica durante el proceso de vinificación (Publicaciones IV).

Con el fin de establecer el efecto inhibidor del mayor número de estructuras fenólicas sobre el crecimiento de bacterias vínicas, en la primera parte de este estudio se procedió a ensayar la actividad antimicrobiana de 54 extractos fenólicos vegetales de diverso origen (especias, hojas, frutas, flores, legumbres, semillas, pieles, bioproductos y derivados agrícolas, vino, taninos purificados, otros), composición y contenido fenólico frente a cepas de bacterias lácticas (*L. hilgardii* CIAL 49, *L. casei* CIAL 52, *L. plantarum* CIAL 92, *P. pentosaceus* CIAL 85, *O. oeni* CIAL 91 y CIAL 96) y acéticas (*Acetobacter acetic* CIAL 106 y *Gluconobacter oxydans* CIAL 107). Al igual que en el estudio de los compuestos fenólicos, la capacidad antimicrobiana de estos extractos fue valorada por el parámetro de inhibición IC_{50}.

Previo a este análisis y con el objetivo de una mayor caracterización de los extractos seleccionados, se determinó el contenido fenólico total (método de Singleton y Rossie) y la capacidad antioxidante (método ORAC) de los mismos. La familia de taninos purificados destacó por ser el grupo con mayor contenido fenólico (349-750 mg ácido gálico/g) y mayor capacidad antioxidante (9.68-40.6 mmol Trolox/g). Además, ambas variables fueron relacionadas estadísticamente mediante el coeficiente de Pearson, obteniéndose que la capacidad antioxidante de los extractos fenólicos se debía principalmente a su contenido fenólico.

En referencia a la actividad antimicrobiana, 24 de los 54 extractos inhibieron el crecimiento de al menos dos de las cepas ensayadas, destacando por su alto poder antimicrobiano, valores de IC_{50} más bajo, el grupo de taninos purificados. Por contra, el crecimiento de las BAL no se vio afectado en presencia del grupo flores. Este mayor efecto antimicrobiano de los taninos purificados podría ser atribuido a su alto contenido fenólico. Estos resultados están en consonancia con lo descrito en diversos estudios (Shoko et al., 1999; Jayaprakasha et al., 2003; Baydar et al.; 2004, and Özkan et al., 2004), en los que se ha observado que la actividad antimicrobiana de diferentes extractos dependía de su composición y contenido fenólico.

Con respecto al análisis individual de los extractos seleccionados, sólo los taninos de pepita de uva y quebracho, así como el própolis fueron activos frente a todas las BAL ensayadas. Hay que destacar que algunos extractos fenólicos sólo fueron activos frente a BAL no *O.oeni,* mientras que otros sólo lo fueron frente a cepas de *O.oeni*; es decir, al igual que en el estudio de los compuestos fenólicos, los extractos fenólicos vegetales manifestaban especificidad. De este modo los extracto de Amanda® y eucalipto (valores más de IC_{50}) destacaron por ser activos frente a BAL no *O.oeni* mientras que la granada exhibió los valores más de IC_{50} frente a *O.oeni*.

En referencia a las BAL, se observó una diferente susceptibilidad a los extractos ensayados en función del género y la especie; siendo *L. plantarum* CIAL 92 y *O. oeni* CIAL 96 las cepas sensibles a un mayor número de extractos (16 de los 54). Por el contrario *P. pentosaceus* CIAL 85 destacó por ser la cepa más resistente, al ser inhibida sólo por 12 de los 54 extractos. Este efecto cepa dependiente también fue descrito en el análisis de actividad antimicrobiana de los compuestos fenólicos.

Por otra parte, las bacterias acéticas se caracterizan por ser microrganismos alterantes del vino, siendo éste el primer estudio que analiza la capacidad antimicrobiana de extractos fenólicos frente a estas bacterias. Los resultados revelaron un efecto inhibidor de los extractos fenólicos sobre el crecimiento de estas bacterias, lo cual reflejaba que los extractos fenólicos son activos frente a un amplio espectro de microrganismos.

Con el objetivo de conocer el mecanismo de acción de los extractos fenólicos sobre la morfología y vialidad de las BAL, se realizaron estudios de microscopía electrónica. Estas microfotografías mostraban daños en la integridad de la membrana bacteriana de *O. oeni* CIAL 96 tras un periodo de exposición a extractos fenólicos. Este resultado parecía indicar, que al igual que en el caso de los compuestos fenólicos, el mecanismo de acción antibacteriano de los extractos fenólicos se basaba fundamentalmente en la desintegración de la membrana bacteriana, causando la posterior muerte celular (Ibrahim et al., 1996).

Una posible relación entre la capacidad antioxidante y antimicrobiana de los extractos también se analizó estadísticamente, obteniéndose al igual que en los compuestos fenólicos, que ambas propiedades no estaban relacionadas.

Una vez demostrada que la capacidad antimicrobiana de los extractos fenólicos frente a bacterias acéticas y lácticas dependía de su composición y contenido fenólico así como de la cepa ensayada, se procedió a evaluar su eficacia tecnológica durante la vinificación. Para ello, se desarrolló un procedimiento basado en la adición de extractos fenólicos durante la elaboración de vinos tintos (*var*. Merlot). Esta adición tiene lugar una vez finalizada la fermentación alcohólica. Por su alto efecto antimicrobiano frente a BAL no *O.oeni* el extracto seleccionado para este experimento fue el extracto de eucalipto. La FML de los vinos se realizó de forma paralela bajo condiciones de espontaneidad e inoculación de *starter* maloláctico (*O.oeni*). Todas las FML realizadas concluyeron con éxito, observándose un consumo más lento de ácido málico en los vinos elaborados con extracto de eucalipto, aunque inferior al mostrado en los vinos tratados con SO_2. El recuento de la población microbiana de estos vinos puso de manifiesto un menor número de colonias en los vinos elaborados con extracto de eucalipto, lo cual explicaría el porcentaje más alto de ácido málico en estos vinos. Estos resultados fueron protegidos bajo la solicitud de la patente ESP201132134.

En conjunto, estos resultados junto con los obtenidos en el análisis de compuestos fenólicos demuestran el potencial empleo de los extractos fenólicos como alternativa al uso del SO_2 en enología.

La adición de extractos fenólicos, durante la elaboración del vino puede generar alteraciones en su población microbiana y cambios en su fracción volátil y fenólica. Por ello, en los dos últimos apartados de esta discusión se debatirá sobre

estas posibles modificaciones. En estos apartados también se evalúo el efecto de la adición de almendra durante la vinificación. Este extracto, al igual que el de eucalipto, mostró efecto antimicrobiano sobre el crecimiento de las BAL durante la FML.

V.4. Implicaciones en las propiedades organolépticas (composición aromática y fenólica) y microbiología (caracterización molecular de cepas) de vinos tratados con extractos fenólicos antimicrobianos.

La adición de extractos fenólicos (almendra y eucalipto) durante la elaboración del vino puede producir cambios en su perfil volátil y fenólico que conlleven a alteraciones organolépticas del producto final. Con el objetivo de poder detectar estos cambios se analizó la fracción volátil y fenólica de vinos tintos elaborados en presencia/ausencia de extractos fenólicos al inicio y al final de la FML, utilizándose para ello las técnicas HS-SPME-GC-MS y HPLC-DAD-fluorescencia, respectivamente (Publicación V).

Con respecto al perfil volátil, cabe destacar que su contenido esteárico disminuyó, especialmente en el vino con FML espontánea, llegando en algunos compuestos a ser superior al 75%. Este descenso fue aún mayor en los vinos tratados con eucalipto. Por el contrario, la adición de almendra en vinos inoculados con *starter* no se observó la reducción del contenido de algunos compuestos, como por ejemplo el octanoato de etilo. La disminución del contenido esteárico en el vino puede ser debida a la actividad esterásica de las BAL (Davis y col., 1988; Mathew y col., 2007), la cual como sugerían los resultados obtenidos podría verse afectada por la adición de extractos fenólicos. A su vez, los vinos inoculados con *starter* fueron más sensibles a la adición de extractos fenólicos. Este resultado sugería una diferente susceptibilidad de las BAL al efecto de los extractos fenólicos, tal y como ha sido descrito en el análisis de la actividad antimicrobiana de los extractos fenólicos en medio de cultivo. El análisis de compuestos volátiles también mostró un descenso generalizado de los alcoholes, aunque no tan acentuado como el observado en la familia de los ésteres. Sólo el alcohol bencílico aumentó su concentración durante la fermentación de los vinos tintos con FML espontánea, pudiéndose deber este hecho a la actividad glicosidasa de las BAL (Ugliano y col., 2003; Hernández-Orte y col., 2009). En el caso de los ácidos, se observó que el contenido del ácido hexanoico

aumentó en los vinos tratados con extractos fenólicos, mientras que el contenido del ácido octanoico disminuyó en los vinos tratados con eucalipto. Hay que destacar, que los cambios producidos en estas tres familias, ésteres, alcoholes y ácidos, pueden generar modificaciones en otras propiedades del vino, ya que estos compuestos están implicados en un gran número de reacciones químicas durante la FML. En referencia a los compuestos varietales terpenos y C13 no isopropanoides, se observó un descenso en el contenido de α-ionona, especialmente en los vinos tratados con eucalipto. En los vinos elaborados con extractos fenólicos se detectó un aumento significativo de β-citronellol en los vinos tratados con almendra e inoculados con *starter*, así como el nivel más bajo de nerol en los vinos elaborados con eucalipto e inoculados con *starter*. Otros compuestos como el terpine-4-ol y α-terpineol sólo fueron detectados en vinos elaborados con eucalipto. Estos resultados podrían sugerir que la actividad glicosidasa de las BAL (Ugliano y col., 2003; Hernández-Orte y col., 2009) era modificada en presencia de extractos fenólicos. Además, la adición de extractos fenólicos con carácter antioxidante podría evitar la oxidación de los terpenos durante la vinificación. Respecto a los fenoles volátiles se observó que los vinos adicionados con eucalipto mostraban los niveles más altos de estos compuestos a excepción del 4-etilguayacol y 2,6-dimetoxifenol cuyo contenido más elevado fue observado en los vinos adicionados con almendra. Estos resultados sugerían que los extractos fenólicos podrían aportar compuestos de esta naturaleza, especialmente el eucalipto, así como afectar a la formación y transformación de los fenoles volátiles durante la FML. Por último, la γ-nonalactona fue la única lactona detectada, aumentando su contenido durante la FML de vinos inoculados con *starter*, especialmente los adicionados con almendra.

Desde un punto de vista sensorial, el descenso de ésteres contribuiría a equilibrar el aroma afrutado de los vinos (Etievant, 1991). Los compuestos ácidos, C13 no isoprenoides, 2-methoxy-4vinilophenol, eugenol y γ-nonalactona se encontraban por encima de su umbral de percepción por lo que todos ellos contribuyeron al aroma del vino. Hay que destacar que estos cambios aromáticos se produjeron en todos los vinos, lo cual sugería que este aumento era debido a la evolución de la FML y no a la adición de extractos fenólicos durante la vinificación.

En referencia al perfil fenólico no se observó cambios significativos en el contenido total de estos vinos. Tal y como se describe en la bibliografía (Monagas y col., 2005a; García-Falcón y col., 2007; Barata y col., 2011) el contenido antociánico

descendió a lo largo de la FML. Ello podría ser debido a su participación en un gran número de reacciones químicas durante la FML (Monagas y col., 2005a; 2006). Respecto a los fenoles minoritarios, en los vinos control no se observaron grandes cambios significativos en el contenido de ácidos hidroxibenzoicos e hidroxicinámicos, alcoholes fenólicos, estilbenos y flavonoles, por el contrario la concentración de flavan-3-oles disminuyó. Los vinos elaborados con almendra mostrando un contenido más elevado de tirosol y catequina mientras que los vinos elaborados con eucalipto destacaron por tener un alto contenido de ácido gálico, quercetina y quercetina-3-O-glucoside. El alto contenido de quercetina en los vinos elaborados con eucalipto podría ser aportado por este extracto. En la primera parte de la presente Memoria se describió que la quercetina se caracteriza por tener un alto poder antioxidante (33 μmol Trolox/mg) y un elevado carácter antimicrobiano frente a la BAL (fue el compuesto más activo, valores de IC_{50}= 0.148-0.454), este hecho podría sugerir que la quercetina sería uno de los compuestos responsables del carácter antimicrobiano del extracto de eucalipto. Por otro lado, sólo las concentraciones del ácido *trans*-caftárico y flavonoles fueron superiores a su umbral sensorial. Estas concentraciones más elevadas fueron detectadas desde el principio de la FML, lo cual indica que sería debido a la variedad de uva y no a la adición de extractos fenólicos durante el proceso de vinificación.

Por último, se realizó un estudio conjunto de todas las variables mediante el análisis de componentes principales. Como resultado del mismo, se observó que en la Componente 1 los vinos eran distribuidos en función del efecto de la FML, mientras que en la Componente 2 lo eran en relación al tipo de FML y extracto adicionado.

Estos resultados muestran que la adición de extractos fenólicos tiene un mayor efecto sobre el perfil volátil que sobre la fracción fenólica, pero en ningún caso estas variaciones se reflejan en cambios organolépticos con respecto a los vinos control.

Por otro lado, la adición de extractos fenólicos antimicrobianos en el vino puede producir cambios en su población microbiana. Las técnicas moleculares permiten una rápida y sensible caracterización de los microrganismos. En el presente estudio, técnicas como la PCR, *rpoB*-DGGE y PFGE han sido utilizadas para estudiar la evolución de la población de BAL presentes en vinos tintos elaborados en

presencia/ausencia de extractos fenólicos (eucalipto y almendra) y SO_2 y para caracterizar genéticamente cepas aisladas de *O.oeni*.

La monitorización microbiana mediante *rpoB* PCR-DGGE, mostró una mayor biodiversidad microbiana al principio de la FML; la cual fue paulatinamente disminuyendo a medida que transcurría la fermentación. Al final de la FML, en la mayoría de los vinos sólo se detectó una única banda correspondiente a *O. oeni,* lo cual mostraba la dominancia de esta especie durante la FML (van Vuuren and Dicks, 1993; Claisse y Lonvaud-Funel, 2012). Esta predominancia también quedó reflejada en la identificación (*rpoB* PCR-DGGE y secuenciación del gen 16SrRNA) de cepas aisladas (n=66) de los diferentes vinos, todas fueron identificadas como *O.oeni*. Además, la técnica *rpoB* PCR-DGGE nos permitió diferenciar las cepas de *O.oeni* en dos tipos: L y H (Renouf y col., 2006), detectándose un mayor número de cepas L (63) que H (3). Este dato sugería una adaptación más favorables de las cepas L a los cambios que se producen durante la FML de vinos elaborados en presencia/ausencia de extractos fenólicos. Este resultado coincide con el descrito previamente por Renouf y col. (2009), en cuyo estudio se observó una prevalencia de las cepas L sobre las H durante la FML.

Con el objetivo de alcanzar una identificación hasta el nivel de cepas de las bacterias aisladas se empleó la PFGE, considerada como la técnica más poderosa para la tipificación de microrganismos (López y col., 2008). Un total de 43 colonias identificadas como *O.oeni* fueron seleccionadas para este estudio. Los resultados revelaron 27 perfiles genéticos diferentes y una clara separación de las cepas en función del tipo de FML. El elevado número de perfiles identificados (n=12) en el vino tinto inoculado con *starter*, reflejaba que éste no se había impuesto durante la FML a la microflora indígena de la bodega. Aunque tanto en los vinos con FML espontánea como en los vinos inoculados con *starter* no se observó ningún perfil dominante, si que destacaron algunos de ellos. En el caso de los vinos tintos con FML espontánea los perfiles más numeroso fueron: el perfil 3, correspondiente a cepas presentes en vino en ausencia de extractos y en presencia del extracto de eucalipto, el perfil 4, correspondiente a cepas aisladas de todos los tipos de vinos y el perfil 7, cuyas cepas correspondían a vinos no adicionados con extractos fenólicos. Mientras que en los vinos tintos inoculados con *starter* los perfiles más destacados fueron el perfil 13, correspondiente a cepas aisladas de vinos sin adición de extractos y vinos sulfitados y el perfil 15, cuyas cepas correspondían a vinos adicionados con extractos fenólicos o sulfito. Por último y para una mayor caracterización genética de estos

perfiles se analizaron 16 marcadores genéticos, cuya presencia en el genoma de *O. oeni* está relacionada con una mejor adaptación y supervivencia a las difíciles condiciones en la que transcurre la FML (Renouf y col., 2008). Sólo seis marcados estuvieron presentes en todos (M7 o reguladores de la transcripción, M9 o alcohol-azúcar deshidrogenasa) o en algunos (M3 o proteína exportadora biosíntesis de polisacáridos, presente en los perfiles 3 y 7; presente todos los perfiles; M8 o proteína hipotética, en los perfiles 7 y 15; M11 o proteína MFS de flujo de arabinosa, sólo en el perfil 13, M15 o glucosiltransferasa involucrada en la biogénesis de la pared celular, presente en todos los perfiles a excepción del perfil 13) de los perfiles seleccionados. Estos seis marcadores también fueron detectados con una mayor frecuencia por Renouf y col. (2008), en cepas aisladas durante la vinificación industrial de tres vinos. La presencia de los marcadores M7, M9 y M15 en todos o casi todos los perfiles podría sugerir que son esenciales para la supervivencia/adaptación de las bacterias durante la FML. Además, se observó un mayor número de marcadores en las cepas aisladas de vinos en ausencia de extractos.

En resumen, *O.oeni* fue la especie responsable de la FML, observándose diferencias en la población de BAL en función del tipo de FML (espontánea o no) y no de la presencia/ausencia de extractos fenólicos. A su vez, estos resultados proporcionan una base para una mayor investigación de los mecanismos moleculares y evolutivos que conducen a la prevalencia de algunas cepas de *O. oeni* en vinos tratados con extractos fenólicos como aditivo para el control de la FML y la población de BAL.

Conclusiones

VI. CONCLUSIONES

1. Las metodologías basadas en el cálculo de los parámetros de inactivación (MIC y MBC) e inhibición (IC_{50}) proporcionan resultados similares para la evaluación de la capacidad inhibitoria de los compuestos fenólicos sobre el crecimiento de bacterias lácticas enológicas, y se muestran como métodos rápidos y sencillos que permiten la comparación entre compuestos/extractos y cepas bacterianas.

2. Los compuestos fenólicos del vino, especialmente los flavonoles, presentan capacidad para inhibir el crecimiento de *O. oeni*, la principal especie implicada en la fermentación maloláctica, así como de *L. hilgardii* y *P. pentosaceus,* asociadas a alteraciones del vino. Para *O. oeni*, la mayoría de los compuestos fenólicos mostraron un efecto inhibidor -expresado como IC_{50}- superior al del metabisulfito potásico. El mecanismo de acción antimicrobiana de los polifenoles es diferente al del dióxido de azufre, comprobándose mediante microscopía de electrónica de transmisión que los polifenoles dañan la integridad de la membrana celular bacteriana.

3. Las bacterias lácticas del vino son capaces de degradar las aminas biógenas histamina, tiramina y putrescina. Esta actividad metabólica es más evidente en cepas de los géneros *Lactobacillus* y *Pediococcus,* y está influenciada por los polifenoles y otros componentes de la matriz del vino (etanol y SO_2).

4. Se han seleccionado 12 extractos fenólicos de origen vegetal y distinta composición fenólica con elevada capacidad antimicrobiana (IC_{50} máximo de 3 g/L) frente a bacterias lácticas y acéticas del vino. El extracto de hojas de eucalipto (*Eucalyptus*) mostró la mayor capacidad antimicrobiana (IC_{50} inferior a 0,5 g/L) frente a especies de bacterias lácticas no-*O.oeni* (IC_{50}= 0.16-0.33 g/L para las cepa del género *Lactobacillus*, y 0.09 g/L para la cepa *P. pentosaceus* IFI-CA/CIAL 85).

5. En un experimento a escala de laboratorio sobre vinos tintos elaborados a nivel industrial, se ha conseguido que la adición del extracto de hoja de eucalipto (2 g/L) retrase significativamente la fermentación maloláctica, tanto inducida por un inóculo como llevada a cabo de forma espontánea, aunque el efecto resultó considerablemente inferior al conseguido por el empleo de anhídrido sulfuroso (30 mg/L).

6. En un experimento a escala de bodega sobre vinos blancos sometidos a crianza en madera, se ha encontrado que la adición de un extracto de hoja de eucalipto (0,1 g/L) conjuntamente con una dosis a la mitad de anhídrido sulfuroso (80 mg/L) aseguraba la estabilidad microbiológica de los vinos durante el envejecimiento, lo que confirma la eficacia tecnológica de este tipo de extractos para el control de la fermentación maloláctica y el crecimiento de microorganismos indeseables durante la vinificación.

7. Aunque algunos compuestos del aroma y compuestos fenólicos presentan concentraciones significativamente diferentes entre los vinos tratados y no tratados con extractos fenólicos (hoja de eucalipto y piel de almendra) como agentes microbianos, la adición de estos extractos, en su conjunto, no supondría mayores cambios en la composición volátil y fenólica que los observados en el vino como consecuencia de la fermentación maloláctica, tanto inducida por un inóculo como llevada a cabo de forma espontánea. Por tanto, la adición de extractos fenólicos antimicrobianos durante la elaboración de los vinos tintos, no parece condicionar las propiedades organolépticas asociadas a su composición volátil y fenólica.

8. Aplicando diversas técnicas avanzadas de caracterización molecular, se ha encontrado que las cepas de *O. oeni* aisladas de vinos tintos tratados con extractos fenólicos antimicrobianos (hoja de eucalipto y piel de almendra) presentan un menor número de marcadores genéticos relacionados con la adaptación y supervivencia a las condiciones en las que transcurre la fermentación maloláctica, en comparación con las cepas de la misma especie y aisladas de los vinos no tratados. En nuestro conocimiento, éstos son los primeros indicios de que la acción de los polifenoles sobre las bacterias lácticas representa un mecanismo de selección de especies y cepas, y abren el camino a futuras investigaciones sobre los mecanismos moleculares y evolutivos implicados.

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Anexos

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Review

Potential of phenolic compounds for controlling lactic acid bacteria growth in wine

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Abstract

Lactic acid bacteria are important in enology since they undergo the malolactic fermentation, a process which main effect is the reduction of wine acidity and is almost indispensable in red wine-making. However, if this process is not well controlled during the elaboration of wine, alterations in wine quality due to bacteria metabolic activity can happen. Polyphenols are wine natural components in must and wine that can potentially affect the growth of lactic acid bacteria and the malolactic fermentation. In this paper, after describing the main features of the malolactic fermentation in wine, we review the use of different chemical substances to control growth of lactic acid bacteria in enology. Special attention is given to phenolic compounds, being revised the recent studies about the effect of polyphenols on the growth and metabolism of lactic acid bacteria in wine in order to establish the extent to which these compounds are involved in malolactic fermentation during wine-making. Finally, the potential use of phenolic extracts as new *antimicrobial* agents during wine-making, as a total or partial alternative to traditional treatments mainly using sulphur dioxide (SO_2) is discussed.

Keywords: Wine; Phenolic compounds; Lactic acid bacteria; Antimicrobial activity; Sulphur dioxide

Contents

1. Introduction

In recent studies, carried out in synthetic laboratory media, the effects of some phenolic compounds (mainly phenolic acids and their esters and some flavonols, such as catechin) on some wine lactic acid bacteria species has

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been studied, revealing that, concentrations of these compounds similar to those found in wine, stimulate bacterial growth (Campos, Couto, & Hogg, 2003; Rozès, Arola, & Bordons, 2003). A possible explanation for the stimulating effects of these compounds, is that they serve as substrate for the bacteria. In fact, research carried out by our group (Hernández et al., 2007) and by other groups (Alberto, Farias, & Manca de Nadra, 2001), has shown that some hydroxycinnamic acids and their esters are metabolized during the growth phase of some lactic acid bacteria species. In contrast, at high concentrations, phenolic compounds are toxic for the bacterial cell, which could cause inhibition of their growth (Reguant, Bordons, Arola, & Rozès, 2000; Stead, 1993). Stimulation or inhibition of the growth of lactic acid bacteria by some wine phenolic compounds, lead us to consider whether they are in any way involved in the development of malolactic fermentation in wine and, also, the possibility of evaluating their use as *natural* antimicrobial agents during wine-making. In this paper, after describing the main features of the malolactic fermentation in wine (Section 2), we review the use of different chemical substances to control growth of lactic acid bacteria (Section 3). Phenolic compounds, that naturally occur in grapes and wines (Section 4), have shown to interact with wine lactic acid bacteria (Section 5), which points out their potential use as new antimicrobial agents in enology (Section 6).

2. Lactic acid bacteria in wine and malolactic fermentation

Together with yeasts, lactic acid bacteria are the most important microorganisms in wine-making. Yeasts are responsible for alcoholic fermentation, while lactic acid bacteria carry out the process of malolactic fermentation (MLF), which, under favorable conditions takes place after alcoholic fermentation. The works carried out in recent years, especially since the eighties, have confirmed the essential role of MLF in wine-making, not only because it reduces the wine acidity, which is very important in red wines, but also because it contributes to the microbial stability of the final product and its organoleptic quality (Maicas, 2001; Moreno-Arribas & Polo, 2005; Versari, Parpinello, & Cattaneo, 1999).

Wine lactic acid bacteria have a complex ecology and, as occurred during the production of many other fermented food products, there is a steady growth of lactic acid bacteria during vinification. Lactic acid bacteria may be present during the different steps of wine-making. They can be isolated from vine leaves, grapes, equipment in the wineries, barrels, etc. The bacteria present in the first steps of wine-making (must and the start of fermentation) belong to different species, generally homofermentative ones. The most abundant correspond to *Lactobacillus plantarum*, *Lb. casei*, *Lb. hilgardii*, *Leuconostoc mesenteroides* and *Pediococcus damnosus*. To a lesser extent, *Oenococcus oeni* and *Lb. brevis* are found. Bacterial multiplication takes place in the interval between the end of alcoholic fermentation and the start of malolactic fermentation. During this step, the pH of the medium, the SO_2 contents, the temperature and the ethanol concentration (Boulton, Singleton, Bisson, & Kunkee, 1996) are the most influential factors. However, conditions specific to each wine, mainly the contents of phenolic compounds can also affect the growth of lactic acid bacteria (Vivas, Augustín, & Lonvaud-Funel, 2000), although this effect is not yet completely understood. *O.oeni* is the bacteria species predominating at the end of alcoholic fermentation. This is the species best adapted to growing in difficult conditions imposed by the medium (low pH and high ethanol concentration) (Davis, Wibowo, Eschenbruch, Lee, & Fleet, 1985; Van Vuuren & Dicks, 1993) and is, therefore, the main species responsible for MLF in most wines. However, some strains of the genera *Pediococcus* and *Lactobacillus* can also survive this phase, remaining active during wine production. If proliferation of these lactic acid bacteria species or strains occurs at the wrong time during wine-making, they may diminish the quality and acceptability of the wine. After MLF, bacterial survival depends on the conditions of the medium, especially on the pH, ethanol contents and, also, particularly on the SO_2 concentration. It is, therefore common practice to remove lactic acid bacteria by sulphiting, after all the malic acid in the wine has been degraded. The levels of sulphurous required to slow down the activity of the lactic acid bacteria oscillate between 10 and 30 mg/l of *free* SO_2 in the case of wines with a pH between 3.2 and 3.6 and from 30 to 50 mg/l for wines with pHs from 3.5 to 3.7. For wines with higher pHs, which is increasingly common in wines from warn areas, the dose of *free* SO_2 required can even reach values close to 100 mg/l.

On some occasions, during industrial wine-making, the development of lactic acid bacteria and MLF are unpredictable, since this can occur during alcoholic fermentation or even during storage or ageing. In these cases, as a consequence of the metabolism of these bacteria, changes occur in the wine composition that can alter its quality, in some cases producing a product which is unacceptable for consumption. These alterations include the so-called "lactic disease", the production of undesirable aromas due to the formation of volatile phenols or aromatic heterocyclic substrates (Chatonet, Dubourdieu, & Boidron, 1995; Costello & Henschke, 2002), and the production of biogenic amines (Landete, Ferrer, Polo, & Pardo, 2005; Marcobal, Polo, Martín-Álvarez, Muñoz, & Moreno-Arribas, 2006; Moreno-Arribas, Torlois, Joyeux, Bertrand, & Lonvaud-Funel, 2000). Biogenic amines are important in wines, not only from a toxicological point of view since they can cause undesirable physiological effects in sensitive humans, such as headache, nausea, hypo-or hypertension, cardiac palpitations, and anaphylactic shock, but also because they could cause problems in wine commercial transactions. Generally, strains identified to cause these problems belong to the group of *Lactobacillus* and *Pediococcus*. Therefore, in wine-making, it is especially important to effectively control MLF, to avoid possible

bacterial alterations. On the other hand, although MLF is sometimes difficult to induce in wineries, prevention or inhibition of the growth and development of lactic acid bacteria in wine is also a difficult task.

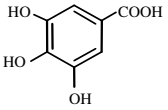
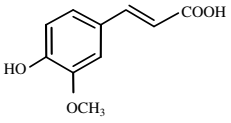
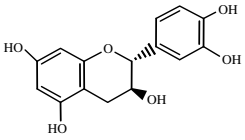
3. The use of SO_2 and complementary substances to control growth of lactic acid bacteria in enology

Sulphur dioxide (SO_2) has numerous properties as a preservative in wines, these include its antioxidant and selective antimicrobial effects, especially against lactic acid bacteria. Today, this is, therefore, considered to be an essential treatment in wine-making. However, the use of this additive is strictly controlled, since high doses can cause organoleptic alterations in the final product (undesirable aromas of the sulphurous gas, or when this is reduced to hydrosuplhate and mercaptanes) and, especially, owing to the risks to human health of consuming this substance. The upper limit permitted by the International Organization of Vine and Wine (OIV) is from 150 to 400 mg/l of total SO_2, depending on the type of wine and its content of reducing matter. However, according to European Union regulations (Ruling n°1622/2000), the total SO_2 content in red wines cannot exceed 160 mg/l, and in white wines it cannot exceed 210 mg/l. On the other hand, in the United States, and also recently in the European Union (specifically from the 26 November 2005, Ruling n° 1991/2004), the legislation requires wine-makers, to specify the presence of sulphites on the wine label, in cases where these exceed 10 mg/l. In fact, in most wines, it is increasingly common to find the specification "*contains sulphites*" on a visible part of the label.

Because of these effects, in recent years there is a growing tendency to reduce the maximum limits permitted in musts and wines. Although as yet, there is no known compound that can replace SO_2 with all its enological properties, there is great interest in the search for other preservatives, harmless to health, that can replace or at least complement the action of SO_2, making it possible to reduce its levels in wines.

With regards products with antimicrobial activity complementary to SO_2 (Table 1), recently dimethyldicarbonate (DMDC) has been described as being able to inhibit alcoholic fermentation and development of yeasts, permitting the dose of SO_2 to be reduced in some types of wines (Divol, Strehaiano, & Lonvaud-Funel, 2005; Threlfall & Morris, 2002). Yeast cells have been shown to die after adding this compound, whereas with SO_2 they enter a "viable state but cannot be cultivated" (Divol et al., 2005), which has also been demonstrated for lactic acid bacteria (Millet & Lonvaud-Funel, 2000). Other alternatives have been introduced based on "natural antimicrobial agents", of which the use of lysozyme is especially important (Bartowsky, 2003; Gerbaux, Villa, Monamy, & Bertrand, 1997), and some antimicrobial peptides or bacteriocins (Du Toit, du Toit, Krieling, & Pretorius, 2002; Navarro, Zarazaga, Sáenz, Ruiz-Larrea, & Torres, 2002) (Table 1).

Table 1
Other compounds proposed to control lactic acid bacteria growth in enology

Compound	Chemical characteristics	References
Dimethyldicarbonate (DMDC)	$(CH_3OCO)_2O$	Threlfall and Morris (2002), Divol et al. (2005)
Lysozyme	Enzyme obtained from egg white (129 amino acids)	Gerbaux et al. (1997), Bartowsky (2003)
Bacteriocins	Nisin (pM < 5000; 34 amino acids)	Radler (1990), Rojo-Bezares et al. (2007)
	Pediocin PD-1 (pM 2866 pI 9,0; optimum pH 5.0 at 25 °C)	Bauer et al. (2003), Bauer et al. (2005)
Polyphenols	Gallic acid 	Vivas et al. (1997), Reguant et al. (2000)
	Ferulic acid 	
	(+)-Catechin 	

In the case of lysozyme, since this was first authorized as an additive in wine-making it has only been used very little due to the high costs of its application. Another aspect to take into account about this protein is that it can cause IgE-mediated (Mine & Zhang, 2002) immune reactions in some individuals so its presence in food products, including wine, can cause some concern. To date, nisin is the only bacteriocin that can be obtained commercially, and although this has been shown to be effective at inhibiting the growth of spoilage bacteria in wines (Radler, 1990; Rojo-Bezares, Sáez, Zarazaga, Torres, & Ruiz-Larrea, 2007), it has not been authorized for use in enology. Other bacteriocins have been described to control the growth of lactic acid bacteria in wine, although the efficacy of these compounds, their mode of action and, especially, their stability during wine-making are still under investigation (Bauer, Hannes, & Dicks, 2003, 2005) (Table 1).

4. Wine phenolic compounds

Phenolic compounds or polyphenols are natural constituents of grapes and wines. Under the name of polyphe-

nols, numerous compounds of different chemical structure are grouped together including: hydroxybenzoic acids, hydroxycinnamic acids, stilbenes, alcohols, flavanols, flavonols, anthocyanins and tannins. These compounds are very important since they are responsible for many of the organoleptic properties of wines, especially, color and astringency. Wine polyphenols are also associated with the beneficial effects associated with moderate wine consumption, especially in relation to cardiovascular diseases. In any case, the structure of a phenolic compound determines its chemical reactivity and its biological properties.

The concentration of phenolic compounds in wine is conditioned by several factors related to the grape (variety, quality of the harvest, soil, climate, etc.) and by enological practices. During wine-making, factors such as maceration time and temperature, fermentation in contact with skins and seeds, the addition of enzymes, the concentration SO_2, the pressing, etc. all affect extraction of phenolic compounds from the grape to the must/wine (Sacchi, Visón, & Adams, 2005). MLF also affects the phenolic composition of wine, reducing the contents of anthocyanins and total polyphenols (Vrhovsek, Vanzo, & Nemanic, 2002). During ageing in the bottle, wine anthocyanin content drops, although the total polyphenol content is less variable (Monagas, Bartolomé, & Gómez-Cordovés, 2005b, 2005a). As a result, the total polyphenol content is around 150–400 mg/l for white wines and 900–1400 mg/l for young red wines.

As a summary, Table 2 shows the whole range of concentrations of the main phenolic compounds identified in young red wines. According to groups of compounds, acids and hydrobenzoic derivatives represent 6% of the total, acids and hydroxycinnamic derivatives 1.1%, stilbenes 0.5%; alcohols 3.8%; flavanols, 15%; flavonols, 3.6%; and anthocyanins, 70%. Other anthocyanin derivatives such as pyranoanthocyanins are present in much lower proportions.

5. Interactions between phenolic compounds and wine lactic acid bacteria

Most studies to date about the interactions between phenolic compounds and lactic acid bacteria in wines refer to the metabolism of hydroxycinnamic acids (ferulic and coumaric acids), by different bacteria species, resulting in the formation of volatile phenols (4-ethylguaiacol and 4-ethylphenol) (Barthelmebs, Diviès, & Cavin, 2001; Cavin, Andioc, Etievant, & Diviès, 1993; Gury, Barthelmebs, Tran, Diviès, & Cavin, 2004). The metabolism of other phenolic compounds such as gallic acid and catechin have also been studied (Alberto, Gómez-Cordovés, & Manca de Nadra, 2004; Vaquero, Marcobal, & Muñoz, 2004). More recently, it has also been reported that *trans*-cafftaric and *trans*-coutaric acids are substrates of wine lactic acid bacteria, that can exhibit cinnamoyl esterase activities during MLF, increasing the concentration of the hydroxycinnamic acids (Hernández et al., 2007, Hernández, Estrella, Carlavilla, Martín-Álvarez, & Moreno-Arribas, 2006).

However, little is known about the effect of wine phenolic compounds on the growth and metabolism of microorganisms, in general, and especially on the lactic acid bacteria that participate in the wine-making process. It has been suggested that phenolic compounds can behave as activators or inhibitors of bacterial growth depending on their chemical structure (substitutions in the phenolic ring) and concentration (Reguant et al., 2000; Vivas, Lonvaud-Funel, & Glories, 1997). For example, it has been demonstrated in *Lb. hilgardii* in culture media that gallic acid and catechin in concentrations found in wines, not only stimulate growth but also increase the bacterial population, owing to their ability to metabolize these compounds during the growth phase, bringing energy to the cell (Alberto et al., 2001). It also seems that they can affect the bacteria metabolism (Rozès et al., 2003; Vivas et al., 2000), since they favor the use of sugars and malic acid (Alberto et al., 2001). On the other hand, at higher concen-

Table 2
Main phenolic compounds identified in young red wines (De Villiers et al., 2005; Monagas et al., 2005a; Monagas et al., 2005b; Soleas et al., 1997)

	Concentration (mg/l)		Concentration (mg/l)
Hydroxibenzoic acids		Flavanols	
Gallic acid	10–37	(+)-Catechin	16–58
Protocatechuic acid	1.2–4.7	(−)-Epicatechin	10–38
Syringic acid	4.2–5.8	Procyanidins B1, B2, B3, B4	14–33
Hydroxycinnamic acids		Flavonols	
Cafftaric acid	0.7–46	Myricetin-3-glycosides	1.6–22
Coutaric acid	0.7–11	Quercetin-3-glycosides	1.3–34
Caffeic acid	0.3–33	Myricetin	1.7–8
p-Coumaric acid	0.1–8	Quercetin	1.9–15
Stilbenes		Anthocyanins	
trans-Resveratrol	0.4–2.5	Delfinidin-3-glucoside	7–11
trans-Resveratrol-3-O-glucoside	0.1–3	Petunidin-3-glucoside	14–25
Alcohols		Malvidin-3-glucoside	170–260
Tyrosol	7–26	Malvidin-3-(6-acetyl)-glucoside	23–108
Tryptophol	nd-4.5	Malvidin-3-(6-caffeoil)-glucoside	3.5–5.6
		Malvidin-3-(6-*p*-coumaroyl)-glucoside	16–28

trations, these compounds have a negative effect on bacterial development. *O.oeni* seems to be more sensitive to inactivation by phenolic compounds than *Lb. hilgardii* (Campos et al., 2003).

Free hydroxycinnamic acids also appear to affect the growth of *Lb. plantarum* and some spoiling species of the group of *Lactobacillus.* Ferulic acid seems to be more effective than *p*-coumaric acid, although some species are more susceptible than others. In contrast, the esters of this acid, as well as the non-phenolic acid, quinnic acid, do not affect growth of *Lb. plantarum* (Salih, Le Quéré, & Drilleau, 2000). Moreover, it has been found that, in a synthetic laboratory environment, the concentration of these compounds can have a critical effect, since the bacteria can tolerate and also metabolize concentrations between 100 and 250 mg/l, which could possibly explain the beneficial effect of these compounds on growth. In contrast, concentrations above 500 mg/l, produce a toxic effect (Stead, 1993). The mechanism of this inhibition is not clear. From these works carried out with pathogenic bacteria, some authors propose that these compounds can act on proteins of the bacteria cell membrane causing a series of compounds to leave the cell interior, producing losses in K^+, glutamic acid, intracellular RNA, etc. as well as an alteration in the composition of fatty acids (Rozès & Perez, 1998). Other authors have suggested that phenols adsorb to the cell walls and alter the cell casing, and even other mechanisms that involve interactions with cellular enzymes (Campos et al., 2003). Recently, a contribution towards the elucidation of the mechanisms of tannins on bacteria growth inhibition was investigated by a combination of physiologic and proteomic approaches (Bossi et al., 2007). The effects of tannic acid on cells are deduced by the involvement of metabolic enzymes, and functional proteins on the tannin–protein interaction.

6. Antimicrobial properties of phenolic compounds

The increased resistance of isolated human and animal pathogens, combined with consumers' growing concern about the use of chemical products as preservatives, has led, over the past few years, to studies being conducted into the application of new efficient antimicrobial products with harmful effects to health. Hence, in recent years, it has gained interest in the study of the antimicrobial properties of phenolic extracts obtained from plants (Ezouberi et al., 2005; Rauha et al., 2000; Zhu, Zhang, & Lo, 2004) and fruits (Puupponen-Pimia, Nohymek, & Hartmann-Schmidin, 2005, 2001). Some studies have been reported in the literature which demonstrate, in growth media, the antimicrobial activity of different phenolic extracts obtained from enological products such as grape seeds (Papadopoulu, Soulti, & Roussis, 2005) and white and red wine (Baydar, Ozkan, & Sagdic, 2004; Rodríguez-Vaquero, Alberto, & MancadeNadra, 2007) against pathogenic bacteria. Phenolic extracts mainly containing phenolic acids, have been described to be more active against bacteria than against yeasts, suggesting that yeasts have a stronger resistance to the action of these compounds. Some attempts have even been made to obtain phenolic fractions, from seeds, with a broad spectrum of activity against bacteria, by "clean" technologies, such as extraction with super-critical fluids, which could constitute a first step for their subsequent development and application in industry (Palma, Taylor, Varela, Cutler, & Cutler, 1999).

As mentioned previously, the efficacy of phenolic compounds as antimicrobial agents against lactic acid bacteria in wine depends on the compound's structure, and is dose-dependent. In general, the antimicrobial effect appears to occur at higher doses than those usually found in wines. Therefore, we must consider that the application of phenolic extracts as antimicrobial agents in wines would be conditioned by possible changes that effective concentrations of these compounds would produce in the physico-chemical (solubility) and organoleptic properties (color, aroma) of the wine. However, it is important to take into account that studies carried out to date (reported above) have been conducted in growth media, in which bacterial growth is favored by the composition and pH of the media. Therefore, the concentration of phenolic compounds required to inhibit growth would be lower in an adverse medium, such as wine (Stead, 1993). On the other hand, antimicrobial activity of phenolic compounds could increase because of synergic effects between them or with other antimicrobial agents, such as SO_2, allowing to reduce the dose of each of them. Finally, when studying the effect of a given phenolic compound, it is important to take into consideration the presence in the wine of other compounds, such as proteins, sugars or oxidants, that can interact with the compound studied, affecting its activity. In any case, studies taking all these factors into consideration are required for establishing the possible applications of phenolics as antimicrobial agents in wine-making.

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Role of Specific Components from Commercial Inactive Dry Yeast Winemaking Preparations on the Growth of Wine Lactic Acid Bacteria

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The role of specific components from inactive dry yeast preparations widely used in winemaking on the growth of three representative wine lactic acid bacteria (*Oenococcus oeni*, *Lactobacillus hilgardii* and *Pediococcus pentosaceus*) has been studied. A pressure liquid extraction technique using solvents of different polarity was employed to obtain extracts with different chemical composition from the inactive dry yeast preparations. Each of the extracts was assayed against the three lactic acid bacteria. Important differences in the effect of the extracts on the growth of the bacteria were observed, which depended on the solvent employed during the extraction, on the type of commercial preparations and on the lactic acid bacteria species. The extracts that exhibited the most different activity were chemically characterized in amino acids, free monosaccharides, monosaccharides from polysaccharides, fatty acids and volatile compounds. In general, specific amino acids and monosaccharides were related to a stimulating effect whereas fatty acid composition and likely some volatile compounds seemed to show an inhibitory effect on the growth of the lactic acid bacteria. These results may provide novel and useful information in trying to obtain better and more specific formulations of winemaking inactive dry yeast preparations

KEYWORDS: Inactive dry yeast preparations; winemaking; lactic acid bacteria; pressure liquid extraction; wine

INTRODUCTION

In recent years, inactive dry yeast (IDY) preparations are gaining interest in the enological industry. These preparations are produced from enological yeasts (*Saccharomyces cerevisiae*) previously inactivated to eliminate their fermentative capacity. Depending on the treatment employed during their manufacturing, yeast extracts, yeast autolysates or cell walls can be obtained (*1*). Among all of them, yeast autolysates are the most commonly commercialized IDY preparations for winemaking applications. They are constituted by a soluble and an insoluble fraction from the cell wall and membranes, obtained after partial autolysis of the yeast (*2*). Depending on their composition IDY can be used for different applications in winemaking. Currently, one of their main applications is to be used for improving alcoholic fermentation and malolactic fermentation (MLF). However, many other IDY preparations are also claimed to enhance the organoleptic characteristics of wines or even to ensure wine safety (*1*, *3*, *4*).

The use of IDY preparations as fermentation enhancers is based on two different action mechanisms. The first one is related to the protective effect of IDY during the rehydration of active dry yeast (ADY) (*5*), and the second one is due to their ability to serve as fermentation nutrients. Regarding the first mechanism, IDY preparations can release insoluble fractions from the yeast cell wall into the rehydration medium, which may form groups of micelle-like sterols that can be incorporated into the ADY membrane, thereby repairing its possible damage (*6*). In addition, IDY preparations may help ADY to adapt their metabolism to the high sugar concentration in musts. Specifically, polyunsaturated fatty acids released from IDY might reduce the osmotic shock of ADY in the musts, thereby acting as protective agents (*7*).

The second mechanism is related to the use of IDY for promoting the growth of wine microorganisms. In this sense, IDY preparations could release yeast's cytoplasm soluble metabolites into the wine (*8*), which, it has been shown, may enhance the alcoholic fermentation rates in nitrogen deficient mediums (*9*). In addition, the insoluble fraction from IDY may also improve the fermentation efficiency in nondeficient nitrogen musts, due to the detoxifying effect of the yeast cell walls (*9*). This effect is based on the adsorption of some toxic metabolites, such as short and medium chain fatty acids, usually associated with stuck or sluggish wine fermentations (*10*, *11*).

Specific IDY preparations are currently being used for enhancing MLF (*1*). This process is important during winemaking for reducing wine's acidity and for improving wine aroma and flavor (*12*). MLF is mainly carried out by *Oenococcus oeni*, although other bacteria belonging to the genera *Lactobacillus* and *Pediococcus* can also be present during winemaking (*13*).

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Table 1. Inactive Dry Yeast (IDY) Preparations Employed in the Present Study

preparation	company	composition[a]
IDY1	1	inactive *S. cerevisiae* rich in polysaccharide + pectinase
IDY2	1	inactive *S. cerevisiae* rich in gluthatione + pectinase + β-glycosidase
IDY3	1	inactive *S. cerevisiae* rich in polysaccharides
IDY4	1	inactive *S. cerevisiae* with antioxidant properties
IDY5	2	inactive *S. cerevisiae* enriched in vitamins and minerals
IDY6	2	*S. cerevisiae* autolysate

[a] In agreement with the data sheet information supplied by the provider.

Although it has been shown that fractions with different molecular weights obtained from noncommercial yeast autolysates and yeast extracts can stimulate the growth of *O. oeni* (*14*−*16*), and besides the increasing number of different types of IDY preparations currently on the market, the literature concerning the effect of commercial winemaking IDY preparations on the MLF, and on their effect on specific wine lactic acid bacteria (LAB), is scarce.

The objective of this work is, therefore, to gain insight on the role of specific components from commercial IDY preparations on the growth of representative species of wine LAB trying to elucidate their action mode.

MATERIALS AND METHODS

Samples. Six commercial IDY preparations, widely used within the enological industry and provided by two different companies, were employed. **Table 1** shows their main characteristics and composition in agreement with the information provided by the manufacturers.

Lactic Acid Bacteria, Culture Media and Growth Conditions. Three bacterial strains corresponding to *Lactobacillus hilgardii* IFI-CA 49, *Pediococcus pentosaceus* IFI-CA 85 and *O. oeni* IFI-CA 96 were essayed. They belonged to the microbial culture collection of the Institute of Industrial Fermentations (CSIC). The bacteria strains were previously isolated from wines, and they were kept frozen at −70 °C in a sterilized mixture of culture medium and glycerol (50% v/v). A MRS culture media (Pronadisa, Madrid, Spain) based on the formula developed by Man et al. (*17*) was used for *L. hilgardii* and *P. pentosaceus*. They were cultivated for 48 h. In addition, a MLO culture media (Pronadisa) developed by Caspritz et al. (*18*) was used for *O. oeni*. This bacterium was cultivated for 3−4 days. In some experiments polyvinyl alcohol at a final concentration of 20 mL L^{-1} (Sigma-Aldrich, Steinheim, Germany) was added to the culture media to improve the solubility of the extracts. All the media were sterilized at 121 °C for 15 min, and in trying to be closer to wine conditions they were supplemented with ethanol to have a final concentration of 60 mL L^{-1}.

Pressure Liquid Extraction (PLE) To Obtain IDY Extracts. The extracts from IDY preparations were obtained by using an accelerated solvent extractor (ASE 200, Dionex Corporation, Sunyvale, CA) equipped with a solvent flow controller. Three solvents of different polarity, ethanol (Scharlau Chemie S.A., Barcelona, Spain), hexane (Panreac Quimica S.A., Barcelona, Spain) and water purified by using a Milli-Q system (Millipore, Inc., Bedford, MA), were employed for each IDY preparation. The extraction conditions were 150 °C, 10342 kPa and 20 min, and they were previously optimized in our laboratory (*19*). All the extractions were performed in 11 mL extraction cells containing 2 g of sample. In the case of water when used as solvent, the extraction cell was filled with three layers in order to prevent the clogging of the cell: first one of sea-sand (4 g) (Panreac Química S.A.), a second layer of the sample (2 g) and a final sand layer on the top of the cell (2 g). Between extractions, a rinse of the complete system was performed in order to overcome any extract carryover. The extracts obtained at all the assayed temperatures were quickly chilled in an ice−water bath to minimize the loss of volatiles and avoiding sample degradation. All the organic solvents were removed by using a Rotavapor R-200 (Büchi Labortechnik AG, Flawil, Switzerland) at 40 °C, while water extracts were dried in a lyophilizer (Labconco, KA, MS).

Determination of the Activity of the IDY Extracts on the Growth of Lactic Acid Bacteria. *Extract Dilution.* The IDY dry extracts that were previously obtained by using ethanol and water were dissolved in the culture media to have a final concentration of 20 mg of dry extract mL^{-1}. The solutions were centrifuged (13000*g*, 10 min) to obtain extracts as clean as possible. From the 20 mg mL^{-1} extract different serial dilutions ranging from 1.25 to 20 mg mL^{-1} were prepared. The IDY extracts obtained with hexane were dissolved in the culture medium supplemented with polyvinyl alcohol to have a final concentration of 5 mg of dry extract mL^{-1} using an Ultraturrax (IKA-Werke GMBH& Co. KG, Staufen, Germany). Serial dilutions ranging from 0.625 to 5 mg mL^{-1} were prepared from the most concentrated one.

Bacterial Inoculum. Briefly, 100 μL of the defrozen strain suspension was added to 10 mL of culture medium, incubated at 30 °C for 48 h for *L. hilgardii* and *P. pentosaceus*, and 72 h for *O. oeni*. Afterward, 100 μL of the suspension was added to 10 mL of medium, and incubated in the same conditions mentioned above. Adequate dilutions to have a final density in the wells of 5×10^5 colony forming units (CFU) mL^{-1} for *L. hilgardii* and *P. pentosaceus*, and 5×10^6 CFU mL^{-1} for *O. oeni* were prepared.

Activity of the IDY Extracts on the Growth of Lactic Acid Bacteria. The activity of the extracts was determined according to the method proposed by Rojo-Bezares et al. (*20*), previously modified in our laboratory (*13*). Prior to the assays, the growth curves of the strains *L. hilgardii* IFI-CA 49, *P. pentosaceus* IFI-CA 85, and *O. oeni* IFI-CA 96 were determined. The activity of the extracts was determined at 24 h for *L. hilgardii* and *P. pentosaceus*, and at 48 h for *O. oeni*, corresponding to a middle point of the exponential growth. For each assay, two 96-well multiplates (Greiner Bio-One, Frickenhausen, Germany) corresponding to the initial and final time were made. Control media wells (containing culture medium), control bacteria wells (containing the culture medium inoculated with bacteria) and sample wells (containing the extracts at different concentrations inoculated with the bacteria) were prepared in triplicate in each plate. The inoculum size was 10% of the total well volume, and the multiwell plates were incubated at 30 °C. Absorbance was measured using a Fluorimeter Fluostar Galaxy at 520 nm (BMG Labtech, Offenburg, Germany); previously the content of the wells was shaken. Finally, the activity of the extracts was determined by comparison of the bacterial growth in the sample wells and in the control bacteria wells, applying eq 1:

$$\%\ \text{activity} = (\Delta OD_{\text{sample}} - \Delta OD_{\text{controlbacteria}})/\Delta OD_{\text{controlbacteria}} \times 100 \quad (1)$$

where ΔOD was the increase in optical density in the final time compared to the initial time.

Chemical Characterization of the IDY Extracts. All the IDY dry extracts were reconstituted in their original solvent (the same employed during the PLE) to have a final concentration of 10 mg of extract mL^{-1}. All the analyses were made in duplicate, and the results were expressed in mg of each chemical component g^{-1} of dry extract.

Amino Acids. Amino acids were analyzed in duplicate by reversed-phase HPLC using a liquid chromatograph, consisting of a Waters 600 controller programmable solvent module (Waters, Milford, MA), a WISP 710B autosampler (Waters), and a HP 104-A fluorescence detector (Hewlett-Packard, Palo Alto, CA). Samples were submitted to automatic precolumn derivatization with *o*-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol (Sigma-Aldrich) following the method described by Moreno-Arribas et al. (*21*). Separation was carried out on a Waters Nova Pack C18 (150 × 3.9 mm i.d., 60 A, 4 μm) column. Detection was performed by fluorescence ($\lambda_{\text{excitation}}$ n = 340 nm; $\lambda_{\text{emission}}$ n = 425 nm), and chromatographic data were collected and analyzed with a Empower-2-2006 system (Waters).

Free Monosaccharides. Monosaccharide analysis was performed according to Núñez et al. (*22*). Briefly, 1 mL of a reconstituted IDY extract in water at 10 mg mL^{-1} was dried in a rotavapor to obtain a dried residue. The dried residue was dissolved in 100 μL of anhydrous pyridine, 100 μL of (trimethylsilyl)imidazole, 100 μL of trimethylchlorosilane, 100 μL of *n*-hexane, and 200 μL of water, which were sequentially added and shaken during each step. Finally, 2 μL of organic phase was injected in split (1/40) into a Hewlett-Packard 6890 gas chromatograph with a flame ionization detector (GC-FID). The injector and detector temperatures were set at 270 °C. For separation, a fused silica Carbowax 20 M column (30 m × 0.25 mm i.d. × 0.5 μm; Quadrex Co., Woodbridge, CT) was used. The oven

temperature was programmed as follows: 175 °C as initial temperature, held for 15 min. In a first ramp, the temperature increased at 15 °C min^{-1} to 200 °C, then held for 13 min. In a second ramp, the temperature increased at 13 °C min^{-1} to 290 °C, held for 20 min. The system was controlled by HP ChemStation software. For quantification, a five point calibration curve of a standard solution including arabinose, xylose, galactose, fructose, glucose and mannose was prepared from 10 to 300 mg L^{-1} and injected in the same conditions as the sample.

Monosaccharides from Polysaccharides. The IDY extracts were hydrolyzed according to Núñez et al. (*22*). For this purpose, 1 mL of a reconstituted extract in water at 10 mg mL^{-1} was hydrolyzed at 110 °C in a stove during 24 h in a closed vial containing 1 mL of 2 M trifluoroacetic acid (Scharlau Quimica S.A.). Afterward, 1 mL of the hydrolyzed sample was dried in a rotavapor and derivatizated and analyzed by GC-FID in the same conditions explained above.

Fatty Acids. For fatty acid determination, the reconstituted extracts in hexane at 10 mg mL^{-1} were previously methylated. To do so, 0.5 mL of extract was dried in a rotavapor. The dried residue was dissolved in a mixture of chloroform:methanol (2:1) at 2 mg mL^{-1}, and then 1 mL of 0.5 N sodium methylate (Supelco, Bellefonte, PA) was added. The reaction took place at 65 °C for 20 min. Then, 0.5 mL of Milli-Q water and 2 mL of hexane were added. The upper layer was separated, and water was removed by anhydrous sodium sulfate. Three microliters of organic phase were injected in split mode (1/20) into an Agilent 6890 gas chromatograph coupled to an Agilent 5973 quadrupole mass spectrometer (GC−MS) (Agilent, Palo Alto, CA). The injector was set at 250 °C. For separation, a Carbowax 20 M (30 m × 0.25 mm i.d. × 0.5 μm; Quadrex Co.) was used. The oven temperature was programmed as follows: 100 °C as initial temperature; first ramp increased at 20 °C min^{-1} to 220 °C, held for 25 min; second ramp, increased at 15 °C min^{-1} to 270 °C and held for 10 min. For the MS system, the temperatures of the manifold and transfer line were 150 and 230 °C, respectively; electron impact mass spectra were recorded at 70 eV ionization volts, and the ionization current was 10 μA. The acquisition was performed in scan mode (from 35 to 450 amu). The TIC signal for each compound was calculated using the data system Agilent MSD ChemStation software (D.01.02 16 version). The identification was carried out by comparison of the retention times and mass spectra of the samples in relation to a commercial standard solution of methyl ester of fatty acids (Supelco 37 Component FAME Mix). An estimation of the percentage of each compound in the sample was obtained by calculating the percentage of TIC area of each compound compared to the sum of TIC area of all the fatty acids identified in the sample.

Volatile Compounds. To determine the volatile compounds in the extracts, 3 μL of the extracts reconstituted at 10 mg mL^{-1} in hexane was directly injected in split mode (1/20) into the GC−MS. The injector was set at 250 °C. For separation, a HP-5 M fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Agilent) was used. The oven temperature was programmed as follows: 40 °C as initial temperature held for 5 min. Then, a first ramp at 4 °C min^{-1} to 200 °C, and a second ramp at 2 °C min^{-1} to 250 °C, held for 5 min. The tentative identification of compounds was carried out by comparison of their mass spectra with those reported in the mass spectrum libraries, NIST98 and Wiley5; moreover, linear retention indexes were experimentally calculated with an *n*-alkane mixture (C5−C30) and compared with those available in the literature. To estimate the proportion of each compound present in the sample, the percentage of TIC area of each volatile compared to the sum of TIC area of all the volatile compounds detected in the sample was calculated.

RESULTS AND DISCUSSION

Pressurized Liquid Extracts from IDY Preparations. In the present work, PLE has been considered a useful technique to obtain extracts of different composition from IDY preparations. Other techniques such as ultrafiltration and dialysis have been also employed in previous works to obtain nitrogen fractions of different molecular weights from yeast autolysates (*14*−*16*, *23*). However, the possibility of using solvents of different polarities during the PLE allows one to obtain extracts with different composition, therefore making easier the study of the effect of compounds from IDY in the growth of lactic acid bacteria. Additional advantages of PLE are its rapidity and the lower amount of solvents required. In addition, the use of fluids at high pressure favors the extraction of analytes trapped into the matrix pores, which are difficult to extract by using other techniques that employ fluids under atmospheric conditions (*24*). In the present work, water, ethanol and hexane were employed as solvents due to the differences in their dielectric constants (78.5, 24.3, and 1.9 respectively), and therefore in their polarity (**Table 2**). As can be seen in **Table 2**, the extraction yields were very different depending on the solvent employed and, to a lesser extent, on the type of IDY preparation. The extraction yields when using water and ethanol (15.5% and 18.2% in average respectively) were much higher than the extraction yields obtained with hexane (2% in average). These results were already suggesting that most of the compounds present on these preparations were more polar than apolar in nature.

Table 2. Yields Obtained (% Dry Weight) in the PLE

		solvents	
type of IDY preparation	hexane (1.9)[a]	ethanol (24.3)	water (78.5)
IDY1	1.4	20.1	23.3
IDY2	0.8	20.1	26.5
IDY3	4.4	16.6	8
IDY4	2.6	15.5	12.2
IDY5	1.3	23.2	14.6
IDY6	1.5	13.7	8.2
average	2	18.2	15.5

[a] Dielectric constant of the solvents.

Effect of IDY Extracts on the Growth of Lactic Acid Bacteria. In general, most of the extracts obtained from the IDY preparations showed an effect on the growth of the three assayed LAB. However, depending on the extracts two opposite effects corresponding to a stimulation or an inhibition on the growth of LAB were found. This already showed that IDY preparations may include specific molecules in their composition that can promote or inhibit the growth of the assayed microorganisms. In addition, it was observed that, independently of the type of extract, the activity (stimulation or inhibition) was directly dependent on the concentration assayed (data not shown). **Table 3** summarizes these results and shows the effect (% activity) of the different extracts at the highest concentration essayed (20 mg mL^{-1} for the IDY extracts obtained with water and ethanol, and 5 mg mL^{-1} for those obtained with hexane) on the growth of the lactic acid bacteria. As can be seen, the differences in activity between different extracts were mainly dependent on the solvent employed during the PLE extraction. In general, the IDY water extracts either stimulated or did not show any effect. The stimulating effect may be due to the presence of some nitrogen compounds, that in the case of yeast autolysates, it has been shown that they may promote the growth of *O. oeni* (*14*−*16*, *25*). Surprisingly, the water extracts obtained from the IDY5 preparation inhibited the growth of all the assayed strains. In addition, the IDY6 water extract also inhibited the growth of *O. oeni*. This fact may be due to the inhibitory activity of some polar compounds, such as specific peptides with molecular weights between 5 and 10 kDa and released from the yeast, which in the presence of ethanol in the medium have been shown may inhibit the growth of *O. oeni* (*23*). On the contrary, the IDY extracts obtained with hexane, and therefore likely richer in nonpolar compounds, inhibited the growth of the three LAB strains. This effect may be related to a high concentration of short- and medium-chain fatty acids from the yeast, which have been shown can inhibit the growth of *O. oeni* (*10*, *26*). The IDY extracts obtained with ethanol showed

Table 3. Effect (% Inhibition or Stimulation) of the IDY Extracts Obtained by PLE Using Water (20 mg/mL), Hexane (5 mg/mL) and Ethanol (20 mg/mL) on the Growth of Lactic Acid Bacteria

		activity (%) of the IDY extract[a]		
type of IDY preparation	solvent[b]	*L. hilgardii*	*P. pentosaceus*	*O. oeni*
IDY1	W	+(186)	+(170)	+(124)
	H	−(59)	−(87)	−(58)
	E	+(149)	+ (24)	−(50)
IDY2	W	+ (12)	+ (29)	−(2)
	E	−(42)	−(36)	−(76)
IDY3	W	+(50)	+(67)	+(152)
	H	−(61)	−(54)	
	E	−(11)	n.a.	−(88)
IDY4	W	+(44)	+(28)	−(6)
	H	−(50)	−(57)	−(7)
	E	−(57)	−(57)	−(49)
IDY5	W	−(28)	−(68)	−(92)
	H	−(91)	−(101)	
	E	−(100)	−(96)	−(112)
IDY6	W	+(98)	n.a.	−(85)
	E	−(56)	−(83)	−(96)

[a] Activity (%) of the IDY extract compared to the control sample (without extract); + denotes a stimulatory effect, whereas − means an inhibitory effect; n.a., no activity was observed. [b] Type of solvent employed during the PLE: W, water; H, hexane; E, ethanol.

an intermediate effect on the growth of LAB between those obtained with water and hexane which could be explained by the intermediate polarity of this solvent and, therefore, by the presence of both types of compounds, those with stimulating and those with inhibitory activity of bacterial growth. Besides of the different effect of the IDY extracts depending on the type of solvent employed during the PLE, the activity of the extracts was also dependent on the type of IDY preparation. In this sense, **Figure 1** shows an example illustrating the effect of water extracts obtained from the six types of commercial IDY preparations on the growth of *O. oeni*. As can be seen, while IDY1 and IDY3 extracts showed a clear stimulation effect, IDY5 and IDY6 showed an inhibition on the growth of *O. oeni*. However, IDY2 and IDY4 did not show any effect. Interestingly, similar behaviors were found among IDY preparations supplied by the same provider and for the same type of application (**Table 1**). For instance, extracts obtained from IDY1 and IDY3 preparations, supplied by provider 1 and recommended for red wines, showed similar effect, while extracts from preparations IDY2 and IDY4 also supplied by provider 1 but for white wines did not show a clear effect on the bacteria growth (**Figure 1**). However, IDY5 and IDY6 extracts, which showed a clear inhibition effect (**Figure 1**), were supplied by a different provider.

Moreover, from **Table 3** it is worth underlining that the three lactic acid bacteria also showed a different susceptibility to the same extract. As an example, the water extract obtained from IDY3 greatly promoted the growth of *O. oeni* (152%), while it moderately stimulated the growth of *L. hilgardii* (50%) and *P. pentosaceus* (67%). These results show important metabolic differences between the three LAB species and/or strains.

To elucidate which compounds from the IDY preparations were the main ones responsible for the observed effects on the LAB growth, a chemical characterization of the extracts from the two IDY preparations which showed the most different activities was performed. Specifically, this study was performed with IDY1 and IDY5 extracts, which in general showed the highest stimulating and inhibition effect on bacterial growth respectively (**Table 3**).

Chemical Characterization of IDY Extracts. As it was explained above, IDY1 and IDY5 extracts were chosen to perform their chemical characterization. For the analysis of amino acids and monosaccharides the water extracts from both IDY preparations were used. In addition, the extracts obtained with hexane were employed to characterize the fatty acid and volatile composition.

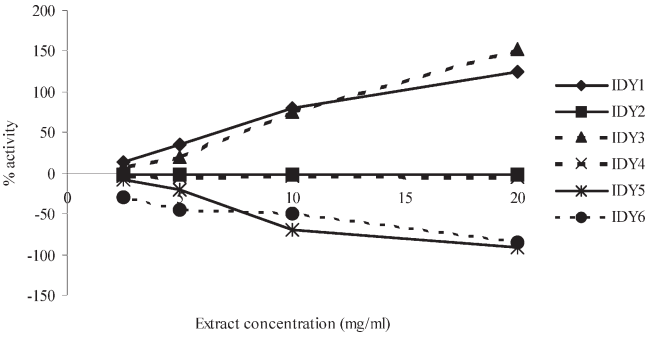

Figure 1. Effect (% activity) of IDY extracts obtained with water on the growth of *O. oeni* IFI-CA 96.

Amino Acids. The amino acid composition of IDY1 and IDY5 extracts is shown in **Figure 2**. As can be seen, the extracts from both preparations showed qualitative and quantitative differences. The total amino acid content was higher in the IDY1 extract (47 mg g^{-1} of dry extract) than in the IDY5 extract (27 mg g^{-1} of dry extract). Taking into consideration that wine LAB are able to use amino acids as a nitrogen source (*16*, *27*, *28*), the extract IDY1 should have provided a higher amount of these compounds for the development of LAB compared to the IDY5 extract. In addition, qualitative differences in the amino acid composition of both IDY extracts were also noticed (**Figure 2**). The major amino acids in the IDY1 extract were α-alanine, γ-aminobutyric, glutamic and aspartic acids, leucine and valine, which is in agreement with previous work performed with yeast autolysates (*14*). Nevertheless, the amino acid composition of the IDY5 extract was different, in which α-alanine was the major amino acid, while aspartic and glutamic acids, glycine, arginine, γ-aminobutyric acid and ornithine were found to a minor extent. The stimulation effect of alanine, valine, leucine, methionine and threonine on the growth of *O. oeni* has been shown in previous work (*28*). All of them were in a higher concentration in the IDY1 extract, which may explain the stimulating effect of this extract on the growth of the three LAB (**Table 3**). Despite the stimulating activity of some amino acids, Vasserot et al. (*29*) have shown that aspartic acid at high concentrations (above 19 mg L^{-1}) could inhibit the growth of *O. oeni*, although they also stated that the inhibition might be reduced in the presence of glutamic acid. In the present work, the aspartic acid concentration of both IDY1 and IDY5 extracts was very similar. However, the IDY1 extract presented higher concentration of glutamic acid compared to the IDY5 extract, and therefore, the former may have reduced the potential inhibitory effect of aspartic acid, which may explain why only the IDY1 extract promoted the growth of *O. oeni* (**Table 3**).

The lower inhibition of the IDY5 extracts in the growth of *L. hilgardii* compared to *P. pentosaceus* and *O. oeni* may be explained by its higher concentration in arginine and ornithine which may specifically promote the growth of *L. hilgardii* (*30*).

Free Monosaccharides and Monosaccharides from Polysaccharides. The results corresponding to the determination of monosaccharides in the IDY water extracts revealed that glucose was the only free monosaccharide detected, whereas mannose and glucose were identified in both extracts after their hydrolysis (**Figure 3**). The concentration corresponding to monosaccharides from polysaccharides was much higher (above 25 mg g^{-1} of dry extract) than that corresponding to free monosaccharides (above 0.5 mg g^{-1} of dry extract), which suggests that probably these preparations were rich in glucoproteins and mannoproteins from the yeast cell

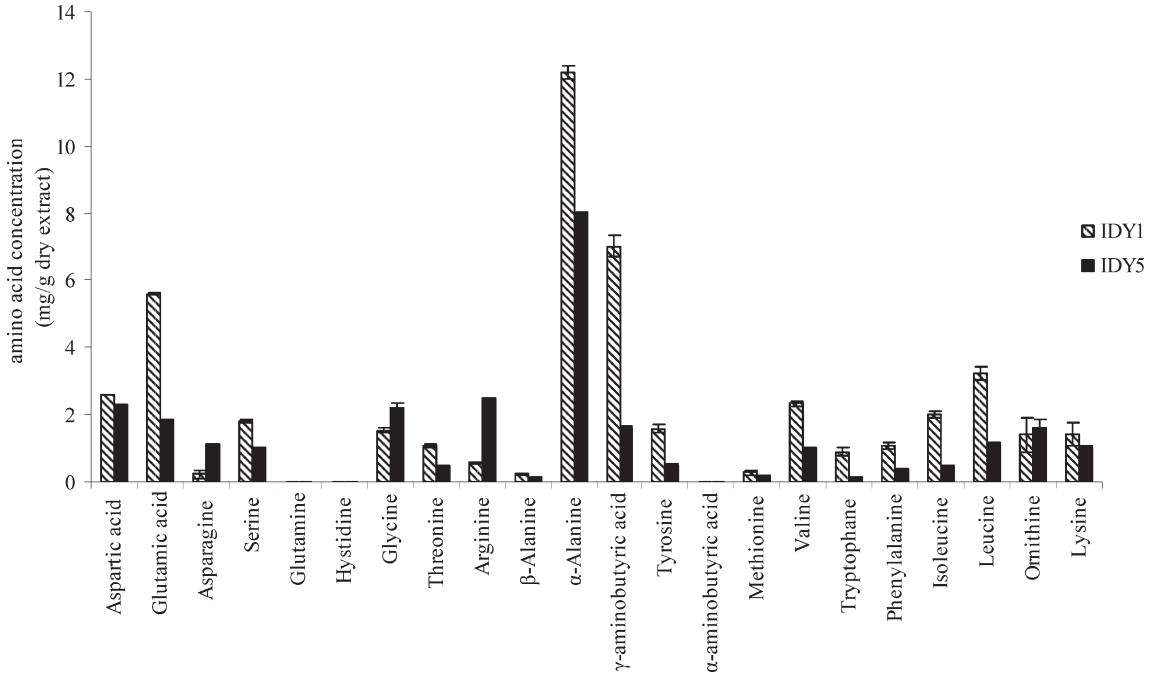

Figure 2. Free amino acid composition of the IDY1 and IDY5 extracts obtained with water.

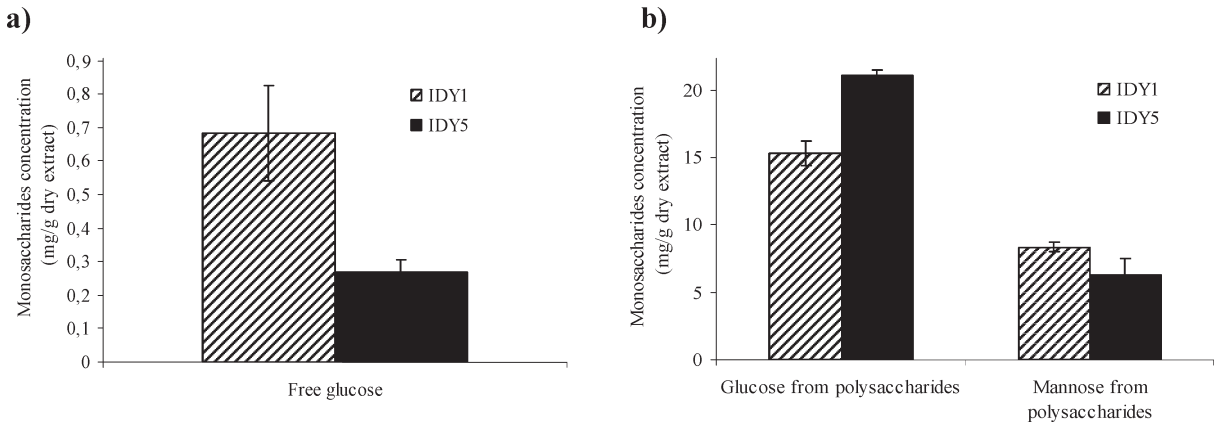

Figure 3. Concentration of free monosaccharides (**a**) and monosaccharides from polysaccharides (**b**) after the hydrolysis of IDY1 and IDY5 extracts obtained with water.

wall (*22*). Differences in monosaccharide concentration in both extracts were not as high as those we found for the amino acid composition. The IDY1 extract showed significantly higher concentration of free glucose, whereas the total content in monosaccharides from polysaccharides was very similar in both extracts, with values of 23.6 and 27.3 mg g^{-1} of dry extract for IDY1 and IDY5 extracts respectively. The ratio glucoproteins/mannoproteins (calculated from the glucose/mannose ratio after the hydrolysis) was 65/35 and 77/23 for IDY1 and IDY5 extracts respectively, showing in both cases a higher concentration of glucoproteins compared to mannoproteins, which is in agreement with the composition of the wall of *Saccharomyces cerevisiae* (*31*). The differences in the ratios between both extracts may be explained by differences during the manufacturing of both preparations, such as the nitrogen content and pH of the culture medium and the temperature and aeration conditions during the growth of the yeast, which, it has been shown, can influence the cell wall composition (*32*).

Free glucose is the most preferred monosaccharide to be consumed by wine LAB (*12*, *33*, *34*). However, the concentration of glucose in IDY1 and IDY5 extracts was very similar, which cannot explain the differences on the LAB growth exhibited by both extracts (**Figure 3**). On the other hand, the effect of polysaccharides from yeast on the growth of some LAB such as *O. oeni* has also been reported (*35*). This effect could be related to the capacity of mannoproteins to adsorb short- and medium-chain fatty acids that can inhibit the growth of some LAB such as *O. oeni* (*36*). In addition, the ability of some LAB with specific enzymatic activities to degrade yeast polysaccharides (e.g. $\beta(1-3)$ glucanase) may improve the nutritional content of the medium, thus promoting bacterial growth (*25*, *37*). Based on these explanations, both extracts IDY1 and IDY5 might have stimulated the growth of the three LAB under study, however, IDY5 not only did not show a promoting effect but rather showed an inhibition effect on the growth of the three LAB, and mainly, on the growth of *O. oeni* (**Table 3**). Therefore IDY5 extracts seemed to contain other components, that may be absent or in lower concentration in the IDY1 preparation.

Fatty Acids. The analysis of fatty acids in the extracts can be of great interest since they can affect the growth of LAB in wines (*36*, *38*). The composition in fatty acids in both extracts (IDY1 and IDY5) is shown in **Table 4**. The percentage of each compound in the sample was calculated as percentage of TIC

Table 4. Fatty Acids Composition of IDY1 and IDY5 Hexane Extracts

			IDY1		IDY5	
peak no.	RT	fatty acids	area ($\times 10^6$)	(%)[a]	area ($\times 10^6$)	(%)
1	3.15	octanoic acid	nd[b]	0	2.47 ± 0.13	0.34 ± 0.02
2	4.25	decanoic acid	nd	0	26.53 ± 0.23	3.65 ± 0.02
3	5.4	dodecanoic acid	nd	nd	2.27 ± 0.02	0.31 ± 0.01
4	6.52	myristic acid (C14:0)	nd	nd	4.97 ± 0.12	0.68 ± 0.01
5	7.92	palmitic acid (C16:0)	16.86 ± 2.61	8.82 ± 0.73	142.43 ± 1.19	19.58 ± 0.12
6	8.2	palmitoleic acid (C:16:1)	71.71 ± 4.81	37.65 ± 0.2	60.44 ± 1.57	8.31 ± 0.34
7	10.01	estearic acid (C18:0)	8.73 ± 1.15	4.57 ± 0.28	40.96 ± 2.72	5.63 ± 0.29
8	10.36	oleic acid (C18:1)	56.08 ± 6.02	29.40 ± 1.03	31.28 ± 4.53	4.30 ± 0.56
9	11.08	linoleic acid (C18:2)	3.63 ± 0.71	1.92 ± 0.51	43.62 ± 3.25	5.99 ± 0.36
10	12.27	α-linolenic acid (C18:3)	nd	nd	5.21 ± 0.59	0.72 ± 0.07
11	20.35	peak 11	33.48 ± 0.11	17.62 ± 1.34	147.43 ± 10.17	20.26 ± 1.10
12	30.8	peak 12	nd	nd	219.71 ± 10.43	30.22 ± 1.88
total			190.49 ± 13.77		727.33 ± 10.67	
∑MCFA[c]			nd	nd	31.28	4.30
∑SFA[d]			25.59	13.40	188.35	25.90
∑UFA[e]			131.42	68.98	140.56	19.32
UFA/SFA			5.14	5.15	0.75	0.75

[a] Normalized TIC signals = (TIC volatile compound/TIC from all volatile compounds) × 100. [b] Not detected. [c] Medium-chain fatty acids. [d] Long-chain saturated fatty acids. [e] Long-chain unsaturated fatty acids.

response compared to the sum of TIC responses from all the fatty acids in the sample. This allowed us to have a relative estimation of the percentage of each compound in the extracts. As can be seen in **Table 4**, the main fatty acids in the IDY extracts included medium-chain fatty acids, such as octanoic, decanoic and dodecanoic acids; long-chain saturated fatty acids such as myristic, palmitic and estearic acids and long-chain unsaturated fatty acids such as palmitoleic, oleic, linoleic and α-linolenic acids. All of them were identified in both extracts, and in general, this composition was in agreement with that corresponding to the plasmatic membrane of active dry yeast (*39*, *40*). Two other compounds that eluted at retention times of 20.35 and 30.80 min (peaks 11 and 12, respectively) were also found. Compared to the total fatty acids content, these compounds were found in larger amount in both extracts. The compound corresponding to peak 11 constituted 20% of the total fatty acid composition of both extracts, and it was tentatively identified as dioctyl adipate. This compound is widely used for the manufacturing of plastic and food packing material (*41*), and it may have migrated from the packaging into the IDY preparations. On the other hand, the compound corresponding to peak 12 was only detected in the IDY5 extract. It was tentatively identified as squalene, an intermediate in the synthesis of ergosterol in yeasts (*42*). Ergosterol can play an important role in the cell, reducing the damage of the plasmatic membrane during the rehydration of the ADY (*6*). Therefore, the ergosterol synthesis may have been promoted during the manufacturing of IDY5 preparation, which may explain the presence of intermediate metabolic products such as squalene. Comparing the fatty acid composition of both extracts, IDY5 showed a higher number of different fatty acids (twelve) compared to IDY1 (six) (**Table 4**). In contrast to what happened with the extract IDY1, the extract IDY5 showed some medium-chain fatty acids, such as α-linolenic acid and squalene. In addition, both extracts showed differences in the composition of saturated and unsaturated fatty acids. The percentage of unsaturated fatty acids (UFA) in IDY1 extract was almost five times higher than the concentration of saturated fatty acids (SFA) (**Table 4**). On the contrary, SFAs were more abundant in the IDY5 extract. These differences might be due to the effect of several factors related to the manufacturing conditions of both preparations, which can affect yeast plasmatic membrane composition such as differences in the nitrogen source (*40*), the aerobic and anaerobic conditions (*43*), the presence of lipids in the culture medium (*43*), the temperature and the species and strain of yeast (*39*) among others. It was previously shown that extracts obtained with hexane from IDY1 and IDY5 preparations inhibited the growth of LAB, although this effect was higher for the IDY5 extract (**Table 3**). This fact may be explained by the greater proportion of fatty acids in the IDY5 extract compared to the IDY1. This is in agreement with the results of Guilloux-Benatier et al. (*26*), who showed the inhibition on the growth of *O. oeni* by a mixture of fatty acids including short-, medium- and long-chain fatty acids. Besides, the proportion of short and medium chain fatty acids was also higher in the IDY5 extracts (**Table 4**). These compounds, and mainly decanoic acid, which represented the 3.6% of the total fatty acid content in IDY5 extract (**Table 4**), can inhibit the growth of some LAB as it has been widely described (*10*, *36*, *44*).

Volatile Compounds. Besides the fatty acid analysis the volatile composition of the hexane extracts from both preparations was also determined. **Table 5** shows the compounds tentatively identified in the samples. The percentage of TIC response of each compound compared to the sum of the TIC from the total volatiles identified in the samples was calculated to have an estimation of the proportion of each volatile compound in the extract. As can be seen, both extracts exhibited larger differences regarding the volatile composition. The IDY5 extract showed the highest number of different volatile compounds, and, in general, the TIC areas were also higher than in the IDY1 extract. In fact, the sum corresponding to the TIC areas of all the volatile compounds identified in the IDY5 extract was almost five times higher than those corresponding to the IDY1 extract. A total of 24 volatile compounds were identified in both samples, 17 of them were identified in the IDY5 extract and 12 in the IDY1. It is worth noticing that the volatile profile of IDY1 was mainly constituted by heterocyclic nitrogen compounds that are products from the reaction between sugars and amino acids and/or peptides present in the IDY preparations, which can take place during the thermal drying, in the last steps of their manufacturing (*19*, *45*). The major volatile compounds tentatively identified in the IDY1 extract were 2-pyrrolidone and 2-ethyl-3,5-dimethylpyrazine. However, IDY5 extract showed a different volatile profile, and besides the heterocyclic volatile nitrogen compounds from Maillard reaction, other compounds such as medium-chain

Table 5. Volatile Compounds Tentatively Identified in the IDY1 and IDY5 Hexane Extracts

			RI			IDY1		IDY5	
peak no.	RT	compounds	exptl[a]	lit.[b]	ID[c]	TIC ($\times 10^6$)	(%)[d]	TIC ($\times 10^6$)	(%)
1	11.25	2,5-dimethylpyrazine	908	913	RI, MS	2.01 ± 0.05	7.36 ± 0.17	nd[e]	nd
2	15.07	2-ethyl-6-methylpyrazine	997	997	RI, MS	0.35 ± 0.01	1.29 ± 0.04	nd	nd
3	15.16	2-ethyl-5-methylpyrazine	999	993	RI, MS	0.53 ± 0.09	1.95 ± 0.05	nd	nd
4	15.23	2,3,5-trimethylpyrazine	1000	1000	RI, MS	3.05 ± 0.1	11.18 ± 0.4	0.75 ± 0.04	0.49 ± 0.00
5	16.03	2-hydroxy-3-methyl-2-cyclopenten-1-one	1020		MS	nd	nd	0.91 ± 0.06	0.60 ± 0.01
6	17.44	2-acetylpyrrole	1055	1060	RI, MS	0.31 ± 0.01	1.15 ± 0.04	1.30 ± 0.26	0.85 ± 0.13
7	17.83	2-pyrrolidone	1064	1076	RI, MS	11.10 ± 0.24	40.69 ± 0.76	6.39 ± 0.61	4.22 ± 0.19
8	18.20	2-ethyl-3,5-dimethylpyrazine	1073	1083	RI, MS	6.37 ± 0.02	23.35 ± 0.01	2.05 ± 0.06	1.36 ± 0.03
9	19.13	isopropylmethoxypyrazine	1096	1097	RI, MS	0.89 ± 0.11	3.25 ± 0.40	nd	nd
10	19.50	3-hydroxy-2-methyl-4*H*-pyran-4-one	1106		MS	nd	nd	21.94 ± 2.62	14.49 ± 1.00
11	20.03	1*H*-pyrrole 5-methyl, 2-carboxaldehyde	1120	1105	RI, MS	nd	nd	1.50 ± 0.14	0.99 ± 0.04
12	21.30	2,3-diethyl-6-methylpyrazine	1153	1158	RI, MS	0.25 ± 0.00	0.91 ± 0.01	nd	nd
13	21.46	3,5-diethyl-2-methylpyrazine	1157	1160	RI, MS	0.82 ± 0.02	2.99 ± 0.1	nd	nd
14	22.11	octanoic acid	1175	1175	RI, MS	nd	nd	8.62 ± 0.12	5.71 ± 0.21
15	23.62	benzothiazole	1215	1221	RI, MS	0.36 ± 0.06	1.32 ± 0.03	1.36 ± 0.13	0.90 ± 0.04
16	24.72	benzeneacetic acid	1246	1254	RI, MS	nd	nd	1.46 ± 0.27	0.97 ± 0.23
17	27.10	2,5-dimethyl-3-isopentylpyrazine	1315	1315	RI, MS	1.24 ± 0.04	4.53 ± 0.16	nd	nd
18	27.65	benzenepropanoic acid	1331	1343	RI, MS	nd	nd	2.39 ± 0.27	1.59 ± 0.26
19	29.00	decanoic acid	1372	1380	RI, MS	nd	nd	81.97 ± 5.12	54.22 ± 0.64
20	29.61	ethyl decanoate	1391	1391	RI, MS	nd	nd	2.15 ± 0.31	1.42 ± 0.13
21	34.80	dodecanoic acid	1560	1567	RI, MS	nd	nd	10.26 ± 1.40	6.82 ± 1.27
22	35.66	dodecanoic acid ethyl ester	1589	1581	RI, MS	nd	nd	3.36 ± 0.47	2.22 ± 0.20
23	40.25	myristic acid	1755	1768	RI, MS	nd	nd	2.51 ± 0.71	1.67 ± 0.55
24	43.81	nonadecane	1893	1900	RI, MS	nd	nd	2.23 ± 0.36	1.47 ± 0.16
		total				27.27 ± 0.1		155.37 ± 0.1	

[a] RIs calculated with an alkane mixture (C5−C30). [b] RIs reported in the literature (NIST web database). [c] Identification method: RI identified by retention index, MS identified by mass spectra (Wiley libraries). [d] Normalized TIC signals = (TIC volatile compound/TIC from all volatile compounds) × 100. [e] Not detected.

fatty acids and their corresponding ethyl esters, such as ethyl decanoate and ethyl dodecanoate, were also identified. In this extract (IDY5), the major compounds corresponded to decanoic acid and the volatile compound tentatively identified such as 3-hydroxy-2-methyl-4*H*-pyran-4-one.

The volatile compounds identified in the two extracts may be responsible for the inhibition on the growth of LAB (**Table 3**). In fact, besides the higher amount of fatty acids detected in the IDY5 extract, the corresponding sterified forms present in greater amount in the IDY5 extract, may also have inhibited the LAB growth (*26*). In addition, the heterocyclic volatile nitrogen compounds present in both preparations could also contribute to the observed inhibitory effect. In fact, it has been previously shown that some of these compounds can have antimicrobial activities (*46*, *47*). However, the effect of these volatiles from IDY on wine LAB deserves further investigation.

In summary, the results from this work have shown that the PLE technique employing solvents of different polarity can be useful to obtain extracts from IDY preparations of different composition which have shown different effect on the growth of LAB. From the chemical characterization of the extracts, amino acids such as alanine, valine, leucine, methionine and threonine and mannose from polysaccharides promoted the growth of LAB while medium-chain fatty acids, such as octanoic, decanoic and dodecanoic acids, and their corresponding esters were more related to an inhibition of the bacterial growth. On the contrary, heterocyclic volatile nitrogen compounds also seemed to show an inhibition effect. Therefore, differences in the proportion of these compounds between the IDY preparations currently available in the market may have different consequences on wine LAB growth. As a whole, in spite of the limited number of LAB strains essayed, the results from this work should be considered as the starting point for deeper research with the objective of looking for more selective formulation of IDY preparations with specific enological applications and without provoking undesirable effects in wines.

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ORIGINAL ARTICLE

Degradation of biogenic amines by vineyard ecosystem fungi. Potential use in winemaking

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Abstract

Aims: To evaluate the ability of grapevine ecosystem fungi to degrade histamine, tyramine and putrescine in synthetic medium and in wines.

Methods and Results: Grapevine and vineyard soil fungi were isolated from four locations of Spain and were subsequently identified by PCR. A total of 44 fungi were evaluated for *in vitro* amine degradation in a microfermentation system. Amine degradation by fungi was assayed by reversed-phase (RP)-HPLC. All fungi were able to degrade at least two different primary amines. Species of *Pencillium citrinum, Alternaria* sp., *Phoma* sp., *Ulocladium chartarum* and *Epicoccum nigrum* were found to exhibit the highest capacity for amine degradation. In a second experiment, cell-free supernatants of *P. citrinum* CIAL-274,760 (CECT 20782) grown in yeast carbon base with histamine, tyramine or putrescine, were tested for their ability to degrade amines in three different wines (red, white and synthetic). The highest levels of biogenic amine degradation were obtained with histamine-induced enzymatic extract.

Conclusion: The study highlighted the ability of grapevine ecosystem fungi to degrade biogenic amines and their potential application for biogenic amines removal in wine.

Significance and Impact of Study: The fungi extracts described in this study may be useful in winemaking to reduce the biogenic amines content of wines, thereby preventing the possible adverse effects on health in sensitive individuals and the trade and export of wine.

Introduction

Biogenic amines are nitrogenous compounds of low molecular weight found in most fermented foods such as cheeses, dairy products, fish, meat, wine and beer (Ten Brink *et al.* 1990; Halàsz *et al.* 1994). These biologically produced amines are essential at low concentrations for normal metabolic and physiological functions in animals, plants and micro-organisms. However, biogenic amines can have adverse effects at high concentrations and pose a health risk for sensitive individuals (Moreno-Arribas *et al.* 2009). A number of countries have implemented upper limits for histamine in food and wine. This development has already started to threaten commercial export transactions and may become more serious and may generate, in a nearby future, a competitive situation between wine industries. The total content of amines in wine varies from trace levels up to 130 mg l^{-1} (Soufleros *et al.* 1998). The most prevalent biogenic amines in wine include histamine, tyramine and putrescine (Bauza *et al.* 1995; Silla Santos 1996; Marcobal *et al.* 2006), which are mainly produced from microbial decarboxylation of the amino acids histidine, tyrosine and ornithine, respectively. Consumption of foods and beverages with high amounts of amines can have toxic effects (Ancín-Azpilicueta *et al.* 2008) that could be more severe in sensitive consumers having a reduced mono- (MAO) and diamino oxidase (DAO) activity (Taylor 1986; Maintz and Novak 2007; Ancín-Az-

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pilicueta *et al.* 2008). Both, MAO, a flavin-containing monoamine oxidase and DAO, a copper-containing amine oxidase or diamine oxidase, are a large group of enzymes catalysing oxidative deamination of amines (Yagodina *et al.* 2002). The activity of these enzymes is maximum under neutral to alkaline conditions, and oxygen is necessary for their action (Beutling 1992). The activity of these enzymes is reduced with the consumption of ethanol, a major compound found in wine, increasing the toxic effect of the biogenic amines (Ten Brink *et al.* 1990).

The high secretory capacity of filamentous fungi has been widely commercially exploited. Recent progress in elucidating primary metabolism pathways in fungi information has been applied to create biotechnologically improved strains (Conesa *et al.* 2001). Enzymatic removal of amines may be a safe and economic way to eliminate these troublesome compounds from wines and other fermented foods. Several kinds of filamentous fungi are known to produce amine oxidase activity when using amines as a sole nitrogen source for growth (Yamada *et al.* 1965, 1966, 1972; Adachi and Yamada 1970; Isobe *et al.* 1982). Two kinds of amine oxidases have been purified and characterized from fungi (Frébort *et al.* 1996, 1997a,b). Additionally, the genome of *Aspergillus niger* contains six genes encoding for amine oxidases. One of those genes has been heterologously expressed in *Saccharomyces cerevisiae* (Kolaříková *et al.* 2009).

Fungi associated with the grapevine ecosystems potentially could be well adapted to utilize biogenic amines in grapes and fermented grape must. To test this hypothesis, we isolated fungi from the soils and living grapevines in four vineyards in central Spain. The fungi were grown in defined medium using a selection of free amines (i.e. histamine, tyramine and putrescine) as the sole nitrogen source using a microfermentation system (Duetz 2007). Amine degradation by fungi was assayed by reversed-phase (RP)-HPLC. Presently, no information exists about the potential of grapevine fungi to degrade biogenic amines. The purposes of this article were as follows: (i) to isolate and identify a set of fungi adapted to the grapevine environment, (ii) to screen these fungi for their ability to degrade histamine, tyramine and putrescine and (iii) to determine whether any of these fungal isolates (with high biogenic amines degradation ability) were able to decrease biogenic amines content in wines.

Materials and methods

Chemicals

Histamine dihydrochloride and 1,4-diaminobutane dihydrochloride (putrescine) were obtained from Fluka (Steinheim, Germany). Tyramine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Fungal isolation

Vineyard soil and plants were sampled at four locations of Spain during the spring of 2008. To isolate endophytic fungi, grapevine stems were cut from grapevine plants, placed in clean paper envelopes and transported to the laboratory at ambient temperature the same day. Samples were stored at 4°C up to 48 h before processing. Bark and leaf bud surfaces were disinfected by sequential 30-s washes in 70% ethanol, 5% sodium hypochlorite, 70% ethanol and sterile water (bark samples), and 70% ethanol and sterile H_2O (leaf bud samples). To obtain xylem samples, grapevine stems were split at the distal end to expose the fresh uncontaminated xylem, and small chips were removed aseptically from the centre of the stem's interior with a sterile scalpel and forceps. After surface decontamination, individual bark fragments, xylem chips and leaf buds were aseptically transferred to each well of 48-well tissue culture plates containing YMC medium [malt extract (Becton Dickinson), 10 g; yeast extract (Becton Dickinson), 2 g; agar (Conda), 20 g; cyclosporin A, 4 mg; streptomycin sulfate, 50 mg; terramycin, 50 mg; distilled H_2O, 1 l]. Eighteen 48-well microplates were prepared per plant (six for bark fragments, six for xylem chips and six for leaf buds). Isolation plates were dried briefly in a laminar flow hood to remove excess liquid from agar surfaces and incubated for 2 weeks at 22°C and 70% relative humidity.

Soil samples were sieved before fungi isolation. Soil aliquots were first washed and separated into particles, and using a particle filtration method to reduce the number of colonies of heavily sporulating fungi (Bills *et al.* 2004). Washed soil particles were plated using a dilution-to-extinction strategy (Collado *et al.* 2007; Sánchez Márquez *et al.* 2011). Approximately 0·5 cm^2 of washed soil particles was resuspended in 30 ml of sterile H_2O. Ten-microlitre aliquots of particle suspensions were pipetted per well into 48-well tissue culture plates containing YMC medium. Nine 48-well microplates were prepared per sample. Isolation plates were dried briefly in a laminar flow hood to remove excess liquid from agar surfaces and incubated for 2 weeks at 22°C and 70% relative humidity.

Generation of fungi inoculums

Emerging fungal colonies from isolation plates were transferred to Yeast Malt Agar [malt extract (Difco), 10 g; yeast extract (Difco), 2 g; bacteriologic agar (Conda), 20 g; distilled H_2O, 1 l] at 22°C for 2 weeks to obtain pure cultures. Three to four mycelial discs were cut from

each 60-mm plate with a sterile Transfer Tube (Spectrum Laboratories, Rancho Dominguez, CA, USA). Mycelia discs were extruded from the Transfer Tube and crushed in the bottom of tubes containing 8 ml of SMYA medium (neopeptone (Difco), 10 g; maltose (Conda), 40 g; yeast extract (Difco), 10 g; bacteriologic agar (Conda), 4 g; distilled H_2O, 1 l) and two cover glasses (22 mm^2). Tubes were agitated on an orbital shaker (200 rev min^{-1}, 5 cm throw), and rotation of the cover glasses continually sheared hyphae and mycelial disc fragments to produce hyphal suspensions consisting of minute hyphal aggregates and fine mycelial pellets. Tubes were agitated 4 days at 22°C in Kühner environmental chambers (ISF-4-V) equipped with inclinable (approximately 75°) tube racks.

Molecular identification

DNA extraction.

Approximately 1 ml of fungi inoculum from each tube was transferred into 96-well plates with a Transfer Tube (Spectrum Laboratories). Total genomic DNA from the different micro-organisms was isolated using a Master Pure ™ Gram Positive DNA Purification kit (Epicentre Biotechnologies) following manufacturer's instruction; slight modifications were made to improve fungi DNA extraction. The modifications carried out were as follows: (i) some centrifugation steps were made twice (the first step of Gram Positive DNA Purification Protocol and the seventh step in the DNA Precipitation), (ii) the volume of isopropanol added for DNA precipitation was 300 μl, followed by a drying step in a Genevac HT-24 vacuum centrifuge at 45°C for 15 min, and (iii) DNA extracts were resuspended in 100 μl of Milli-Q water.

PCR amplification.

DNA extracted was used for PCR amplification. DNAs were subjected to PCR with primers ITS1 and ITS4 (White *et al.* 1990). Reactions were performed in a final volume of 50 μl containing 0·2 mmol l^{-1} of the four dNTPs (Applied Biosystems), 0·05 μmol l^{-1} of each primer, 5 μl of the extracted DNA and 0·5 U *Taq* polymerase (Appligene, Illkirch, France) with its appropriate reaction buffer. Controls without fungi DNA were included for each PCR experiment. Amplifications were performed in a Thermocycler PCR PTC-200 (Bio-Rad), according to the following profile: 40 cycles of 1 min at 95°C, 1 min at 51°C and 2 min at 72°C. Amplifications products were visualized by electrophoresis in 1% agarose gels (Invitrogen E-Gel^R 48 1% (GP) G8008-01) using an Invitrogen E-Base. PCR products were purified using Ilustra GFX 96 PCR Purification Kit (Amersham Biosciences).

DNA sequencing and phylogenetic sequence analysis.

The purified PCR products were used as a template in sequencing reactions with the same primers of PCR amplification. Amplified and cloned DNA fragments were sequenced by using an ABI Prism Dye terminator cycle sequencing kit (Amersham Biosciences). Sequences were assembled and aligned using Genstudio software (Genestudio Inc.). The ITS1-5·8S-ITS2 sequences were aligned with CLUSTAL W (Thompson *et al.* 1994). The phylogenetic analysis was complemented with ITS1-5·8S-ITS2 sequences of fungal species available in GenBank and with similarity searches using BLAST. The data were re-sampled with 1000 bootstrap replicates (Felsenstein 1985) by using the heuristic search option of PAUP (Swofford 1993). The percentage of bootstrap replicates that yielded each grouping was used as a measure of statistical confidence. A grouping found on 95% of the bootstrap replicates was considered statistically significant.

Degradation of biogenic amines by fungi

Forty-four fungi isolates from grapevine plants and soils were screened for their ability to degrade biogenic amines in assay broth consisting of yeast carbon base (Sigma-Aldrich) supplemented with histamine, tyramine or putrescine (0·05 g l^{-1}) as a single nitrogen source to induce amine oxidase activity. Assay broth (pH 4·5) was filter-sterilized (Millipore ExpressTM Plus, 0·22 μm). Before inoculation, a Multidrop Combi (Thermo Fisher Scientific, Inc., Waltham, MA,USA) was used to fill sterilized deepwell 24-well plates with assay broth (4 ml $well^{-1}$). Using transfer tubes, approximately 0·5 cm of each fungi inoculum was transferred to its corresponding well. The fermentation plates were agitated for 10 days at 22°C. Assays were made in duplicate.

Cultured mycelium was separated from the culture broth by filtration (Syringe Filters with Luer tip; Agilent Technologies). As a negative control for degradation of biogenic amines, 1 ml of uninoculated sterile culture broth from a control well was also analysed by reversed-phase high-performance chromatography (RP-HPLC).

Based on primary screening results, five fungi from the grapevine environment were able to degrade biogenic amines, and two generally regarded as safe (GRAS) fungi, *Aspergillus oryzae* CECT 2094 and *Penicillium roqueforti* CECT 2905, obtained from the Spanish Type Culture Collection (CECT) were selected for further experiments. Assays to measure the degradation of biogenic amines were the same as mentioned previously. The culture pH was measured at initial and final incubation time.

Degradation of biogenic amines by fungal enzymes in wine

Three different wines (red, white and synthetic) were selected for the experiment. Red wine (pH 4·06) was selected because of its high natural biogenic amines content (19·33 mg l^{-1} of histamine, 2·07 mg l^{-1} of tyramine and 22·66 mg l^{-1} of putrescine). White wine (pH 3·27) was supplemented with histamine, tyramine and putrescine to have a final concentration of 0·05 g l^{-1} of each amine. Synthetic wine was prepared by mixing 12% ethanol (v/v) (VWR, Leuven, Belgium) and 4 g l^{-1} tartaric acid (Panreac, Barcelona, Spain). After the pH was adjusted to four with NaOH (Panreac), biogenic amines were added at the same concentration as in white wine.

Penicillium citrinum CIAL-274,760 (CECT 20782) was selected for further experiments because of its ability to degrade biogenic amines. To prepare crude extract, approximately 0·5 cm of inoculum was used to inoculate flaks containing 25 ml of assay broth, consisting of Yeast Carbon Base (Sigma) and 0·05 g l^{-1} histamine dihydrochloride (extract A), tyramine hydrochloride (extract B) or putrescine (extract C). All experiments were carried out in duplicate. One flask was prepared plus its corresponding control (amine plus YCB) per amine. The culture was incubated for 1 week on an orbital shaker incubator at 200 (rev min^{-1}), 22°C and 70% relative humidity (RH). Cultured mycelium was separated from the culture broth by filtration (Millipore ExpressTM Plus, 0·22 μm). Filtered supernatant was used as a crude extract. Crude extracts were analysed at least twice by RP-HPLC.

To test whether the crude extracts had the ability to degrade wine biogenic amines, the following steps were carried out: 0·5 ml of crude extract was added to 1 ml of wine. After 18-h incubation at 35°C, the reaction was stopped by the addition of 1·5 ml 1 mol l^{-1} HCl. Samples were filtered and analysed by RP-HPLC. Biogenic amine degradation by the crude extract was expressed as degradation percentage, by comparing the concentration of amines in the sample with respect to its control. Samples that were not used immediately were preserved at −20°C.

Biogenic amines analysis

Biogenic amine degradation was analysed by reversed-phase (RP)-HPLC according to the previously described method (Marcobal *et al.* 2005). Briefly, the liquid chromatography protocol employed a Waters 600 Controller programmable solvent module (Waters, Milford, MA, USA), a WISP 710B autosampler (Waters) and a HP 1046-A fluorescence detector (Hewlett Packard). Chromatographic data were collected and analysed with a Millenium32 system (Waters). The separations were performed on a Waters Nova-Pak C18 (150 × 3·9 mm i.d., 60 Å, 4 μm) column with a matching guard cartridge. Samples were submitted to an automatic precolumn derivatization reaction with *o*-phthaldialdehyde (OPA), prior to injection. Derivatized amines were monitored by fluorescent detection (excitation wavelength of 340 nm, and emission wavelength of 425 nm). Samples were previously filtered through Millipore filters (0·45 μm) and directly injected in duplicate onto the HPLC system. All reagents used were of HPLC grade.

Results

Survey of fungi in the grapevine ecosystem

One of the aims of this study was to isolate a diverse set of fungi representative of the vineyard ecosystem. A total of 224 strains were isolated from the grapevine plants and 66 from the soil (Table 1). The number of isolates per samples, as well as the number of different genera from each, was calculated to compare the richness and diversity of fungi from different sites. The best results regarding number and variety of fungi were obtained from Escuela de la Vid grapevine plants (Table 1).

Table 1 Distribution of fungi isolated from four Spanish vineyard ecosystems

	Number		Number of isolates		Isolates per samples		Number of genera		Unidentified fungus	
Location	Plants	Soils	Plants	Soils	Plants	Soils	Plants	Soils	Plants	Soils
Villamanrique del Tajo (Madrid)	4	–	30	–	7,5	–	12	–	3	–
Escuela de la Vid (Madrid)	5	–	97	–	19,4	–	17	–	17	–
Membrilla (Ciudad Real)	9	2	70	31	7,77	15,5	11	13	14	6
Tortuero (Guadalajara)	6	1	27	35	4,5	35	4	12	11	13
Total	**24**	**3**	**224**	**66**	–	–	**44**	**25**	**45**	**19**

–, no fungi found.

Molecular identification of isolates

Comparisons of nucleotide sequences of different isolates of fungus with sequences in GenBank were able to identify most of the fungi to at least the genus level, with some exceptions. Best GenBank BLAST match identifications and GenBank accession numbers of fungi are provided in Table 3. The majority of the fungi isolated in this study were *Phoma* sp., *Alternaria* sp. and *Fusarium* sp. These genera accounted for 22·8% of all isolates. Unidentifiable fungi were designated as 'unidentified fungus'.

Phylogenetic analysis

To assess the phylogenetic affinities among fungi isolates, ITS sequences were compared against GenBank sequence database using BLAST analysis. A phylogenetic tree was generated by neighbour-joining method, and sequence of reference strains were incorporated into the tree (Fig. 1). Unidentified *Ascomycete* AF502791 and *Microdochium bolleyi* AJ279454 were the most disparate ITS sequences and were not clearly associated with any other grouping of strains. The remaining tree was divided into two main branches (Fig. 1a,b). The first branch with a strong bootstrap (98%) includes reference sequences belonging to orders *Xylariales* and *Sordariales* (class *Sordariomycetes*) (Table 2). Three isolates in this branch could not be associated with any known sequences, suggesting the existence of a new lineage. The other main branch (Fig. 1b) including the majority of the isolates was well supported (81% bootstrap). It was further divided into two sub-branches (Fig. 1c,d) with reasonable support. Branch c included isolates belonging to the orders *Hypocreales*, *Microascales*, *Clalosphaeriales* and *Phyllachoreales* (class *Sordariomycetes*) (Table 2). Branch d seemed to correspond with orders *Capnodiales*, *Botryosphaeriales*, *Dothideales* and *Pleosporales* (class *Dothideomycetes*), *Eurotiales* and *Onygenales* (class *Eurotiomycetes*), *Xylariales* (*Sordariomycetes*) and finally, *Agaricales* (class *Agaromycetes*) (Table 2). Some isolates in these branches could not be associated with any known sequences, especially regarding branch d.

Amine degradation by fungi of the grapevine ecosystem

Forty-four strains isolated from vineyard environment were screened for the ability to degrade histamine, tyramine or putrescine in synthetic medium (Table 3). Out of 44 strains screened, 31 degraded all three amines, 16 strains degraded two amines and three strains degraded only one amine. In this survey, we arbitrarily set the value of 60% degradation as a level insignificant enough to consider that the fungi were able to degrade biogenic amines. *Alternaria* sp. (CIAL-274,707), *E. nigrum* (CIAL-274,672), *P. citrinum* (CIAL-274,760, CECT 20782), *Phoma* sp. (CIAL-274,692) and *U. chartarum* (CIAL-274, 893) were selected for a second experiment because of their high potential to degrade histamine, tyramine and putrescine. Moreover, two GRAS micro-organisms (*A. oryzae* CECT 2094 and *P. roqueforti* CECT 2905) were included in our survey (Table 4). When the assay was repeated with a larger fermentation, all strains maintained their ability to degrade biogenic amines with the exception of *E. nigrum,* for which the histamine degradation percentage decreased from 99·69% (Table 3) to 36·45% (Table 4), and *U. chartarum,* for which putrescine degradation was not detected (Table 4). When the two GRAS fungi were retested, the two strains were able to degrade tyramine and putrescine; however, histamine was only degraded by *P. roqueforti* (Table 4). The pH medium values remained stable for each strain.

Determination of enzymatic degradation of biogenic amines content in wine

Pencillium citrinum (CIAL-274,760, CECT 20782) strain was selected to carry out the enzyme assay because of its high potential to degrade biogenic amines in both experiments (Tables 3 and 4). After growth in a mineral medium supplemented with histamine, tyramine or putrescine (0·05 g l^{-1} final concentration) as a sole source of nitrogen, the supernatant (crude extract) was collected by filtration. The biogenic amines content in crude extracts and their corresponding controls were analysed by RP-HPLC. Biogenic amines (histamine, tyramine or putrescine) only were detected in A, B and C control extracts. 17 Subsequently, free biogenic amines extracts (A, B and C) were used for wine enzyme assays (Fig. 2). When added to wines, the three extracts decreased the biogenic amines content; however, the percentage of degradation varied depending on the type of wine and amine used as the culture's nitrogen source. The highest degradation percentages in biogenic amine content (>80%) were obtained for white wine, regardless of the amine used to induce amine oxidase activity. Culture induction by growth on histamine (extract A) appeared to promote better biogenic amine degradation in white, synthetic and red wines.

Discussion

Biogenic amines are problematic in some wines because of their harmful effects on human health, and they may also alter a wine's organoleptic characteristics, decreasing its quality. In most of the cases, it is the manufacturer's and his winemaking team's responsibility to control the production of biogenic amines, exercising precise controls

Figure 1 Neighbour-joining analysis of vineyard ecosystem fungi isolates from four geographical localizations (Villamanrique del Tajo, Escuela de la Vid, Tortuero and Membrilla) of Spain. Selected reference strains were aligned with vineyard isolates. Statistical support (bootstrap) values were indicated at branches. Horizontal distances are proportional to the distances sequences.

Table 2 Distribution of fungi isolated in this study according to their taxonomical group

Phylum	Class	Order	Family	Species
Ascomycota	*Dothideomycetes*	*Botryosphaeriales*	*Botryosphaeriaceae*	*Botryosphaeria, Dothiorella* and *Microdiplodia* species
		Capnodiales	*Davidiellaceae*	*Cladosporium* and *Davidiella* species
			Not assigned family	*Rachicladosporium* species
		Dothideales	*Dothideaceae*	*Coniozyma* species
			Dothioraceae	*Aureobasidium* species
		Pleosporales	*Didymellaceae*	*Didymella* species
			Leptosphaeriaceae	*Epicoccum* and *Leptosphaeria* species
			Massarinaceae	*Saccharicola* species
			Montagnulaceae	*Paraconiothyrium* species
			Phaeosphaeriaceae	*Phaeosphaeria* and *Stagonospora* species
			Pleosporaceae	*Alternaria, Ulocladium, Embellisia, Pleospora, Lewia, Pyrenochaeta, Didymella* and *Dendryphion* species
			Sporormiaceae	*Sporormia* species
			Not assigned family	*Phoma, Camarosporium* and *Coniothyrum* species
	Eurotiomycetes	*Chaetothyriales*	*Herpotrichiellaceae*	*Exophiala* species
		Eurotiales	*Trichocomaceae*	*Aspergillus* and *Penicillium* species
		Onygenales	Not assigned family	*Geomyces* species
	Sordariomycetes	*Calosphaeriales*	*Calosphaeriaceae*	*Phaeoacremonium* and *Togninia* species
		Hypocreales	*Clavicipitaceae*	Paecilomyces and *Metarrhizium* species
			Hypocreaceae	*Acremonium, Hypocrea* and *Trichoderma* species
			Hypocreomycetidae	*Myrothecium* species
			Nectriaceae	*Fusarium, Nectria* and *Gibberella* species
			Not assigned family	*Acremonium, Acrostalagmus* and *Stachybotrys* species
		Microascales	*Microascaceae*	*Wardomycopsis* and *Scedosporium* species
			Not assigned family	*Microdochium* species
		Phyllachorales	Not assigned family	*Verticillium* species
		Sordariales	*Chaetomiaceae*	*Chaetomium* and Thielavia species
		Xylariales	*Amphisphaeriaceae*	*Discostroma, Pestalotiopsis* and *Truncatella* species
	Not assigned family	Not assigned family	Not assigned family	*Tetracladium, Scolecobasidium* and *Helminthosporium* species
Basidiomycota	*Coelomycetes*			Coelomycete species
	Agaricomycetes	*Agaricales*	*Psathyrellaceae*	*Coprinellus* species

of the factors that would negatively influence their formation. Among these factors are the levels of precursor amino acids and microbial nutrients, wine pH, ethanol levels, sulphite and the phenolic composition of the wine, and especially the activity of decarboxylase-positive endogenous lactic acid bacteria (Marcobal *et al.* 2006; Martín-Álvarez *et al.* 2006; Lucas *et al.* 2008; Marqués *et al.* 2008). However, reducing biogenic amines synthesis in wine is not always possible without affecting the organoleptic characteristics of the commercial product, neither with advanced winemaking technology.

The research on amine degrading enzymes for food industrial applications might have useful applications for wines. Several studies have characterized the amine oxidases involved in amine degradation by filamentous fungi (Yamada *et al.* 1965, 1966, 1972; Adachi and Yamada 1970; Isobe *et al.* 1982; Frébort *et al.* 1996, 1997b); however, nothing is known about the distribution of these enzymes in fungal strains from ecosystems. In this survey, we have demonstrated for the first time the ability of vineyard ecosystem fungi to reduce the biogenic amines content in assay broth as well as in wines. In fungi, most of the amine oxidases have been studied in crude extracts when induced by various amines, mainly *n*-butylamine, methylamine, spermine and agmatine (Isobe *et al.* 1982; Frébort *et al.* 1997a). We selected 44 fungal strains representing the range of genera of fungi from a survey of grapevine ecosystems. The fungal strains were tested for their ability to degrade biogenic amines after being induced by the main biogenic amines found in wines (histamine, tyramine and putrescine). The ability to degrade biogenic amines was noteworthy for many fungi, independent of the amine incorporated into the culture medium (Table 3). These results are consistent with earlier data reported, where 88 fungi species from different origins and,including the genera *Aspergillus* sp., *Fusarium* sp., *Mucor* sp., *Neurospora* sp., and *Monascus* sp., among others, were induced with *n*-butylamine, methylamine or

Table 3 Screening of fungi isolated from grapevine environment that can degrade histamine, tyramine and putrescine (0·05 g l^{-1}) in YCB broth after 10 days of incubation at 22°C

Strain codes	Proposed identification	GenBank accession no.	Histamine degradation (%)	Tyramine degradation (%)	Putrescine degradation (%)
CIAL-274,861	*Acremonium* sp.	JN578630	42·05	96·90	98·94
CIAL-274,707	*Alternaria* sp.	JN545791	99·66	100	100
CIAL-274,722	*Alternaria* sp.	JN578617	99·83	100	100
CIAL-274,736	*Alternaria* sp.	JN578622	99·88	100	100
CIAL-274,737	*Alternaria* sp.	JN545793	99·89	100	100
CIAL-274,767	*Alternaria* sp.	JN578628	100	100	100
CIAL-274,720	*Ascochyta* sp.	JN578616	99·67	100	100
CIAL-274,787	*Cladosporium* sp.	JN578629	80·60	100	99·61
CIAL-274,684	Coelomycete	JN578614	99·42	100	100
CIAL-274,776	Coelomycete (n.s.)		75·94	100	100
CIAL-274,726	*Dendryphion penicillatum*	JN578618	0	99·91	22·80
CIAL-274,659	*Discostroma* sp.	JN578610	88·86	99·98	100
CIAL-274,735	*Discostroma* sp.	JN578621	73·12	100	100
CIAL-274,673	*Embellisia* sp.	JN578612	99·52	100	100
CIAL-274,906	*Embellisia* sp.	JN578641	100	20·08	99·68
CIAL-274,672	*Epicoccum nigrum*	JN578611	99·69	100	100
CIAL-274,667	*Fusarium* sp.	JN545777	2·07	100	100
CIAL-274,763	*Fusarium* sp.	JN578627	19·62	100	100
CIAL-274,683	*Leptosphaeria* sp.	JN545781	35·50	100	100
CIAL-274,696	*Leptosphaeria* sp.	JN545785	99·55	100	100
CIAL-274,897	*Metarhizium anisopliae*	JN545817	0	100	100
CIAL-274,760	*Penicillium citrinum*	JN578626	100	99·91	99·69
CIAL-274,895	*Pestalotiopsis* sp.	JN578635	100	100	99·84
CIAL-274,692	*Phoma* sp.	JN578615	99·64	99·91	99·95
CIAL-274,733	*Phoma* sp.	JN578620	52·14	99·99	100
CIAL-274,741	*Phoma* sp.	JN578623	99·46	100	99·50
CIAL-274,757	*Phoma* sp.	JN578625	100	99·86	99·84
CIAL-274,885	*Phoma* sp.	JN578632	93·79	100	99·82
CIAL-274,896	*Phoma* sp.	JN578636	100	100	100
CIAL-274,903	*Phoma* sp.	JN578639	68·06	100	100
CIAL-274,904	*Scolecobasidium* sp.	JN578640	99·74	64·84	100
CIAL-274,893	*Ulocladium chartarum*	JN578634	99·84	100	100
CIAL-274,899	*Ulocladium chartarum*	JN545819	100	100	100
CIAL-274,670	Unidentified ascomycete	JN545778	79·12	100	100
CIAL-274,674	Unidentified fungus	JN578613	99·65	100	100
CIAL-274,731	Unidentified fungus	JN578619	0	99·98	48·97
CIAL-274,755	Unidentified fungus	JN545794	92·60	0	100
CIAL-274,888	Unidentified fungus	JN578633	100	100	99·77
CIAL-274,901	Unidentified fungus	JN578638	100	100	100
CIAL-274,687	Unidentified fungus (n.s)		5·61	100	88·96
CIAL-274,724	Unidentified fungus (n.s)		37·30	37·93	100
CIAL-274,743	Unidentified Pleosporales	JN578624	99·72	100	99·55
CIAL-274,881	Unidentified Pleosporales	JN578631	51·12	100	99·73
CIAL-274,900	Unidentified Pleosporales	JN578637	100	100	100

n.s., not sequence.

spermine (Frébort *et al.* 1997a). It is thought that amine oxidases allow the fungi to degrade an amine as a source of ammonium for growth; however, the role of these enzymes has not always been well defined (Frébort *et al.* 2000). In a second experiment, we also confirmed that most active fungi retained their ability to degrade biogenic amines (Table 4). It is also important to emphasize that *P. roqueforti* CECT 2905 strain was able to degrade the three studied amines. This finding might be relevant for amine degradation in foods, as the GRAS status makes this fungus attractive for application in products fit for human consumption.

The potential of *P. citrinum* CIAL 274,760 (CECT 20782) extracts for biogenic amines detoxification of

Table 4 Degraded histamine, tyramine and putrescine values (expressed as percentage) in YCB broth with 0·05 g l^{-1} histamine, tyramine or putrescine, starting pH 4·6, 4·5 and 4·5, respectively, after 10 days of incubation at 22°C with fungi grapevine isolates

Strain codes	Origin	Identification	Histamine degradation (%)	Final pH	Tyramine degradation (%)	Final pH	Putrescine degradation (%)	Final pH
CIAL-274,707	Bark grapevine	*Alternaria* sp.	100	4·5	100	4·5	100	4·5
CIAL-274,672	Xylem grapevine	*Epicoccum nigrum*	36·45	4·5	100	4·5	100	4·5
CIAL-274,760	Bark grapevine	*Penicillium citrinum*	100	4	100	4	100	4
CIAL-274,692	Xylem grapevine	*Phoma* sp.	100	4·5	100	4·5	100	4·5
CIAL-274,893	Soil grapevine	*Ulocladium chartarum*	100	5	100	5	ND	5
	CECT 2094	*Aspergillus oryzae*	3·77	4	100	4	100	4
	CECT 2905	*Penicillium roqueforti*	100	4·5	100	4·5	100	4·5

ND, not detected.

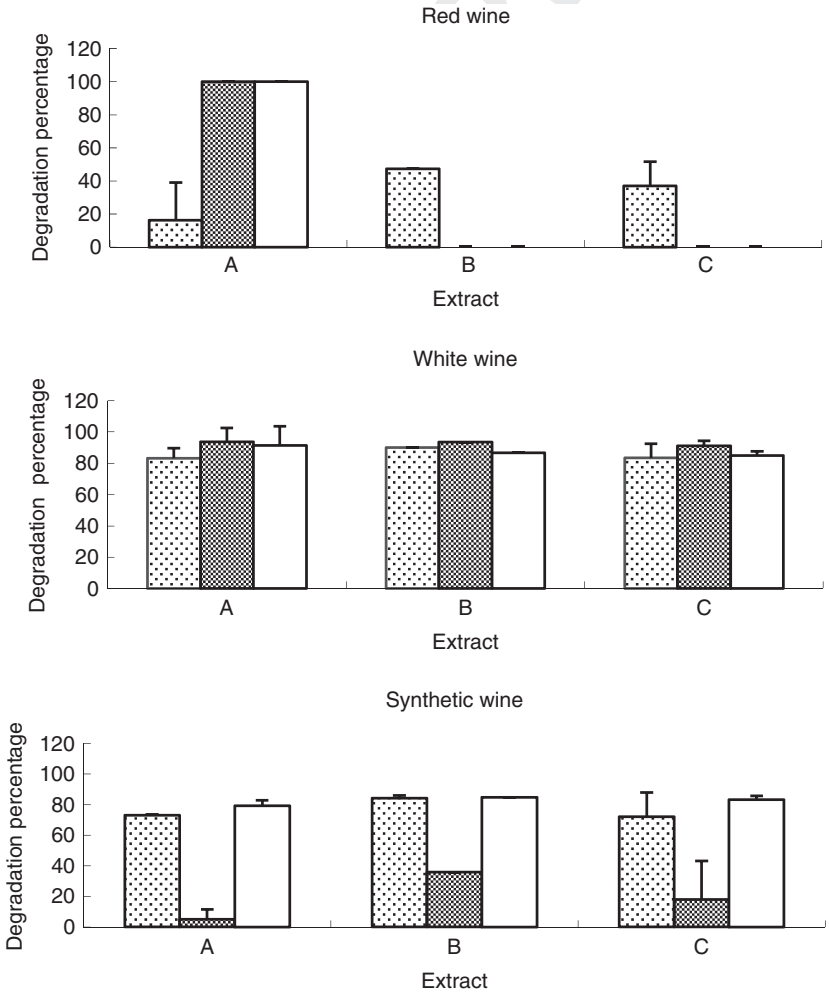

Figure 2 Histamine, tyramine and putrescine degradation measured by RP-HPLC in red, synthetic and white wines with the addition of A, B and C extracts after 18 h of incubation at 35°C. A: Histamine-induced extract; B: Tyramine-induced extract; C: Putrescine-induced extract. (⚃) Histamine; (▩) Tyramine and (□) Putrescine.

wines was further demonstrated in commercial red and white wines and in a synthetic wine, suggesting that the enzymes are active in culture media. Similar results were reported by Frébort *et al.* (2000) with *n*-butylamine-induced amine oxidases of *Aspergillus niger* AKU 3302. In *A. niger*, that amine oxidase was proposed to serve primarily as a detoxifying agent, preventing amines from entering and damaging the fungal cell.

The preparation and industrial applications of the amino oxidase of *A. niger* IMI17454 was described in 1985 (European Patent Application Nº EP0132674A2). Although the authors proposed its use in foods, such as

cheese, beer, must and yeast extracts, specific data were not presented, demonstrating the usefulness under real food production conditions. Based on our results, the amine oxidases from *P. citrinum* CIAL 274,760 (CECT 20782) were active at pH between 4·0 and 5·0; pH values were very similar to those of wines, and clearly lower than the optimal pH reported for *A. niger* IMI17454 amine oxidases (European Patent Application Nº EP0132674A2).

Another important finding was the effectiveness of *P. citrinum* CIAL 274,760 (CECT 20782) in decreasing the biogenic amine content of commercial wines. Red wines, in which the winemaking process normally involves malolactic fermentation, have been clearly shown to have a higher biogenic amine content (especially of histamine, tyramine and putrescine) than *rosé* and white wines, in which malolactic fermentation does not occur or occurs to a lesser degree. The formation of histamine (Herbert *et al.* 2005; Landete *et al.* 2005), tyramine (Vidal-Carou *et al.* 1990; Moreno-Arribas *et al.* 2000) or putrescine (Marcobal *et al.* 2006; Moreno-Arribas and Polo 2008) is commonly associated with lactic acid bacteria and malolactic fermentation or wine storage. Among all biogenic amines, histamine is the most important because many European countries have imposed legal limits for the histamine concentrations, therefore impacting the import and export of wines to EU countries. Therefore, from a commercial point of view, amine oxidase treatments able to decrease histamine and/or to reduce the amine content of red wines would be of great interest. According to our results, histamine was significantly degraded in red wine treated with extracts A, B and C (up to 20, 40 and 38% histamine degradation, respectively); however, we obtained even better results in the white and synthetic wines (Fig. 2). The different phenolic compositions of white and red wines may be associated with these differences. Some phenolic compounds are known to bind proteins (Santos-Buelga and de Freitas 2009), and the differences could be related to their free concentrations rather than to their total concentrations. Therefore, we speculate that anthocyanins present in red wines could affect amine oxidases, modulating the effectiveness of their efficiency in the wine environment.

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