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INMOVILIZACIÓN DE *OENOCOCCUS OENI* EN GELES HÍBRIDOS DE SÍLICE-ALGINATO Y SU APLICACIÓN PARA LA FERMENTACIÓN MALOLÁCTICA DE VINOS TINTOS

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ABREVIATURAS

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Abreviaturas	Significado
$Si(OR)_4$	Alcóxidos de silicio
AB	Aminas biógenas
BAL	Bacterias ácidolácticas
(G)	Ácido α-L-gulurónico
(M)	Ácido β-D-manurónico
SO_2	Dióxido de azufre
R‘-$Si(OR)_3$	Derivados de los alcóxidos
FA	Fermentación alcohólica
FML	Fermentación maloláctica
Si-OH	Grupo silanol
ROH	Molécula de alcohol
OIV	Organización Internacional de la Viña y el Vino
pH_{ext}	pH externo
pH_{cit}	pH citoplasmático
sHSP	Proteínas de choque térmico
SiO_2	Sílice
TEOS	Tetraetoxisilano
TMOS	Tetrametoxisilano

RESUMEN

RESUMEN

Tradicionalmente, la fermentación maloláctica (FML) ha sido uno de los aspectos más difíciles de controlar en el proceso de vinificación en tinto. Las bacterias ácido lácticas (BAL) protagonistas de la misma son especialmente sensibles a las condiciones fisicoquímicas y ambientales del medio, de ahí que las modificaciones de pH, acidez y grado alcohólico que están sufriendo las uvas y los vinos obtenidos de ellas como consecuencia del cambio climático, estén contribuyendo a dificultar el desarrollo de la FML en vinos en todo el mundo.
Una de las posibles formas de incrementar la tolerancia de las BAL a las condiciones adversas del medio, es su inmovilización en soportes inertes. De esta manera se consigue mejorar su supervivencia y actividad metabólica, reducir la posibilidad de que existan bacterias libres en el vino e incluso facilitar la posibilidad de la reutilización de dichas bacterias inmovilizadas.

Esta tesis doctoral se centra en el desarrollo de una matriz híbrida de sílice-alginato que permite la inmovilización de *Oenococcus oeni* en cápsulas con unas adecuadas propiedades mecánicas y una excelente capacidad de desarrollo de la FML. Los ensayos realizados muestran que la matriz híbrida supone un gran avance con respecto al empleo de alginato solo, a la vez que postulan a este sistema de atrapamiento bacteriano como idóneo para su utilización en vino. Además, las cápsulas bacterianas obtenidas han podido reutilizarse hasta en cinco ocasiones, lo que abarataría su coste y facilitaría su comercialización y uso en bodega.

Los ensayos realizados en vinos con bacterias inmovilizadas frente a bacterias libres, tanto en inoculación secuencial como en coinoculación con levaduras, han puesto de manifiesto una mayor tolerancia de las bacterias inmovilizadas a las condiciones de bajo pH, baja temperatura y elevado grado alcohólico. Las FML llevadas a cabo por dichas bacterias encapsuladas dan lugar a vinos con características sensoriales similares a las producidas por las bacterias libres, con la ventaja de hacerlo en un menor lapso de tiempo y reduciendo, por lo tanto, el intervalo de permanencia del vino en condiciones favorables a la proliferación de microorganismos alterantes.

Para asegurar mejor la posterior estabilidad microbiológica del vino, se han hecho ensayos con vinos tratados con lisozima. En las microvinificaciones realizadas se ha podido constatar de nuevo la ventaja que conlleva la inmovilización en la matriz de sílice-alginato, permitiendo que las bacterias atrapadas en las cápsulas puedan llevar a cabo la FML y respetando la obtención de vinos con características sensoriales similares a las obtenidas con las bacterias libres.

INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. *Oenococcus oeni*

Gracias a Antonie van Leeuwenhoek, quien inventó el microscopio, se observaron por primera vez levaduras en mosto de cerveza. La presencia de esta levadura, denominada como *Saccharomyces* (zuckerpilz, hongo del azúcar) por primera vez en 1838 por Meyen, fue confirmada por estudios de Pasteur en 1866. En esa época Pasteur también describió la presencia de bacterias en el vino, dando así los primeros pasos en el descubrimiento de la fermentación maloláctica (FML), y Balard reconoció que la formación de ácido láctico en el vino se debía a bacterias. Ya al inicio del siglo XX, Koch demostró que la conversión del ácido málico en ácido láctico se debía a la acción de bacterias, las bacterias ácido lácticas (BAL) (Bartowsky, 2017).

Las BAL son un grupo de bacterias muy diverso taxonómicamente hablando, que comparten la característica de producir ácido láctico al fermentar azúcares. Pueden clasificarse en homofermentativas, si producen ácido láctico como principal producto de la fermentación de glucosa; o heterofermentativas si además del ácido láctico, producen otros compuestos como dióxido de carbono, ácido acético y etanol. En general, las BAL pueden caracterizarse como procariotas Gram-positivas, aerobias o anaerobias facultativas, oxidasa, catalasa y bencidina negativas, carecen de citocromos, no reducen los nitratos a nitritos, son gelatinasa negativas y no pueden utilizar el lactato (Carr & Chill, 2002).

Si bien hoy en día se conoce que en el vino puede haber BAL de hasta cuatro géneros distintos (*Lactobacillus, Pediococcus, Leuconostoc* y *Oenococcus*) (Lonvaud-Funel, 1999), inicialmente y de manera general las bacterias del vino fueron denominadas como *Bacterium gracile*. Este término fue sustituyéndose por otros (*Leuconostoc gracile*, *L. citrovorum*, *L. mesenteroides,* entre otros) a medida que se adquiría un mayor conocimiento de las bacterias, hasta que tras un estudio de las diferentes cepas encontradas hasta la fecha, en 1967 Garvie nombraría formalmente a la bacteria como *Leuconostoc oenos*, englobándola así dentro del género *Leuconostoc spp*. (Bartowsky, 2017). Sin embargo, con la llegada de las técnicas moleculares, y debido a las diferencias con el resto de bacterias del género *Leuconostoc spp*. observadas en los estudios filogenéticos (en particular en los estudios de secuenciación de ARNr 16S y 23S), en 1995 se le asignó el género *Oenococcus* con la especie única *Oenococcus oeni*, denominación aceptada y utilizada hoy en día de manera general (Dicks et al., 1995). Posteriormente fue descubierta una nueva especie dentro de este género, *Oenococcus*

kitaharae, obtenida a partir de residuos de la bebida alcohólica japonesa Shochu (Endo & Okada, 2006).

Los análisis de secuencia multilocus han demostrado que la especie *O. oeni* comprende dos linajes principales, denominados grupos A y B, y probablemente al menos un grupo adicional C. El grupo A es el más grande y contiene solo cepas aisladas en vino, mientras que el grupo B incluye cepas tanto de vino como de sidra. También se describieron diversos subgrupos en A y B (Bridier et al., 2010 y Breniaux et al., 2018).

Diversos estudios han informado sobre grupos genéticos de cepas asociadas con regiones geográficas específicas (Marques et al., 2011 y Franquès et al., 2017). De todas las especies de lactobacilos y cocos identificados en el vino, *O. oeni* es la especie que predomina y la máxima responsable del desarrollo de la FML debido a que sus características genómicas únicas han contribuido a una rápida evolución y adaptación al entorno hostil que supone el vino, por lo que posee algunas características inusuales como la tolerancia a la acidez, al etanol y a otros inhibidores naturales del vino (Lonvaud-Funel, 2015; Lorentzen & Lucas, 2019 y Sumby et al., 2019).

El papel principal de *O. oeni* en la vinificación es la desacidificación del vino (Lonvaud-Funel, 1999), proporcionando una mayor estabilidad microbiana al vino al eliminar una fuente de carbono potencialmente disponible para las levaduras y las bacterias no deseables que puedan deteriorarlo. Además, *O. oeni* puede metabolizar una amplia variedad de compuestos procedentes de la uva, las levaduras y la madera de roble de las barricas, liberando de este modo metabolitos secundarios que modificarán el aroma y el sabor del vino (Costello et al., 2013; Betteridge et al., 2015 y Sternes & Borneman, 2016).

1.2. Ecología de las bacterias acidolácticas y evolución de su población

En la naturaleza, las BAL se encuentran en las bayas de uva en concentraciones bajas, menores a las encontradas en las uvas estrujadas de los tanques de fermentación. Las poblaciones varían durante los últimos días de maduración de la uva, oscilando entre 10^2 ufc/ml a 10^4 ufc/ml, dependiendo de las condiciones climáticas, y correlacionándose sobre todo con el pH, de modo que cuanto mayor sea el pH, mayor será la población total de BAL. En esta etapa pueden identificarse hasta 8 o 9 especies de cuatro géneros diferentes: *Lactobacillus, Pediococcus, Leuconostoc* y *Oenococcus*. La población de BAL también varía con el transcurso de la fermentación alcohólica (FA), llegando a un máximo de 10^4 ufc/ml cuando la

concentración de etanol es de 5-6%, y descendiendo hasta 10^2 ufc/ml al final de la FA (Lonvaud-Funel, 1999). A medida que avanza la FA también se ve modificada la diversidad de especies de BAL que pueden encontrarse en el vino, así, la concentración de los géneros *Lactobacillus, Pediococcus* y *Leuconostoc* desciende progresivamente siendo *O. oeni* quien predomina en las etapas finales y una vez concluida la FA (Lonvaud-Funel, 1999; Renouf et al., 2008 y Lonvaud-Funel, 2015).

Durante la FA no sólo se genera etanol, tóxico para las BAL. Las levaduras producen también ácidos grasos como metabolitos secundarios que inhiben el crecimiento de las BAL ya que alteran su membrana. El grado de inhibición varía dependiendo de la concentración del ácido graso, la longitud de su cadena y de la composición del medio. Los ácidos decanoico y dodecanoico, por ejemplo, son fuertes inhibidores del crecimiento de las BAL (Lonvaud-Funel et al., 1988 y Edwards et al., 1990). Además, algunas levaduras generan dióxido de azufre (SO_2) en una cantidad dependiente del tipo de cepa de *Saccharomyces* y de la composición del medio. Las levaduras también pueden consumir los nutrientes del medio generando alguna carencia eventual de estos y dificultando aún más el desarrollo de las BAL (Alexandre et al., 2004).

Tras la FA comienza un rápido crecimiento de *O. oeni* de modo que cuando la población alcanza 10^6 ufc/ml se inicia la FML. Este periodo entre ambas fermentaciones puede ser más o menos largo dependiendo de diversos factores como son la temperatura, el pH y el grado alcohólico (Ribéreau-Gayon et al., 2006).

Otro factor que afecta a la población de las BAL es la presencia de fagos, sin embargo, estos pueden ser inactivados por diversos componentes del vino por lo que su población no es suficiente para exterminar a las BAL. Además, en un vino se encuentran diversas cepas de *O. oeni*, por lo que puesto que los fagos son muy específicos, sólo una parte de la población se vería afectada (Lonvaud-Funel, 1999).

De todas las BAL, probablemente *O. oeni* es la mejor adaptada a todas estas condiciones estresantes. Esta mayor tolerancia de *O. oeni* al etanol y estos otros subproductos de las levaduras permite su selección natural y el predominio de esta especie frente a otras BAL. *O. oeni* posee una hipermutabilidad genética debida a la pérdida de los genes mutS y mutL. Esto podría explicar el elevado nivel de polimorfismo alélico observado entre los aislados de *O. oeni* y la capacidad de adaptación única de este género a ambientes ácidos y alcohólicos en comparación con otras BAL (Marcobal et al., 2008 y Breniaux et al., 2018).

Numerosos estudios moleculares han revelado que existe una gran diversidad de cepas de *O. oeni* que intervienen en la FML del vino. Esta diversidad de cepas se manifiesta tanto a nivel de región geográfica como dentro de las propias fincas vitivinícolas, observándose así

hasta 10 genotipos diferentes simultáneamente en el transcurso de la FML. Además, las cepas son capaces de sobrevivir en bodega durante varias cosechas consecutivas (González-Arenzana et al., 2015 y El Khoury et al., 2017).

Una vez concluidas la FA y la FML se sulfita el vino para obtener una mayor estabilidad microbiológica, limitando así el desarrollo tanto de levaduras y BAL residuales que pudieran permanecer aún en el vino, como la proliferación de otros microorganismos que pudieran alterar las cualidades de este. Sin embargo, puesto que la actividad del SO_2 depende del pH (siendo menos efectivo a pH elevados), en vinos menos ácidos es posible que las bacterias sobrevivan más fácilmente, encontrando poblaciones de hasta 10^5 o 10^6 ufc/ml varios meses después de concluir la vinificación y siendo *Lactobacillus* y *Pediococcus* los géneros más abundantes en este momento. Esto es altamente indeseable ya que se corre el riesgo de que deterioren el vino, por lo que para evitarlo puede realizarse un filtrado para eliminar las bacterias que pudieran haber sobrevivido (Lonvaud-Funel, 1999 y 2015).

1.2.1. Factores ambientales

Existen una serie de factores como son la temperatura, el SO_2, bajo pH, grandes cantidades de taninos, residuos de pesticidas, altos niveles de etanol y ácidos grasos de cadena media, que pueden afectar el crecimiento bacteriano (Wells & Osborne, 2012 y Lasik, 2013). A continuación se detallan los principales:

1.2.1.1. Etanol

Claramente, el etanol es el principal compuesto inhibidor de las BAL producido por *S. cerevisiae*. El grado de tolerancia al etanol varía con el género, la especie y la cepa de BAL, siendo los cocos más sensibles que los lactobacilos (Balmaseda et al., 2018 y Bonomo et al., 2018). De manera general, para un grado alcohólico del 13% vol. más de un 50% de los lactobacilos son capaces de sobrevivir mientras que sólo el 14% de los cocos lo hacen, si bien en el vino las bacterias son capaces de tolerar concentraciones superiores a las mostradas en el laboratorio (Ribéreau-Gayon et al., 2006).

La toxicidad del etanol se ha estudiado ampliamente y está bien documentada. Es debida a que el etanol afecta tanto al estado físico de las membranas como a su capacidad biológica, alterando la interfaz lípido-agua y afectando a la difusión de moléculas a través de la membrana. Se ha observado que la presencia de etanol incrementa la fluidez de la membrana,

lo que a su vez causa acidificación intracelular debido a que la permeabilidad de los protones también es mayor al aumentar la fluidez (Chu-Ky et al., 2005).

A medida que la bacteria se aclimata a un entorno rico en etanol, la modificación de su membrana se pone de manifiesto con un cambio en la composición de sus ácidos grasos. En concreto, se produce un incremento de los ácidos grasos saturados de cadena corta y una disminución de los ácidos grasos de cadena larga. Por tanto, a mayores contenidos en ácidos grasos de cadena corta la bacteria se encuentra más aclimatada al entorno y la degradación del ácido málico es mayor (Bravo-Ferrada et al., 2015).

Múltiples trabajos han demostrado el carácter inhibitorio del etanol. Olguín et al. (2009) observaron que en presencia de etanol, el comportamiento metabólico y transcripcional de *O. oeni* fue diferente al observado cuando el etanol estaba ausente, comprobando que la expresión de los genes de la vía del citrato se vio afectada principalmente por el etanol y en menor medida por el pH. Posteriormente el mismo grupo de investigación observó mediante un análisis transcriptómico que el etanol afectaba principalmente a las funciones de transporte de metabolitos y síntesis de proteínas, ligadas a la biogénesis de la membrana y la pared celular. Se detectó que algunos genes se sobreexpresaron en respuesta al estrés por etanol (por ejemplo, la proteína de choque térmico Hsp20 y una dipeptidasa) y que varias proteínas también se vieron afectadas por la presencia de etanol, comprobándose que en algunos casos el descenso en su concentración podría deberse a la reubicación de las proteínas citosólicas en la membrana, como mecanismo de protección. Estos estudios ponen de manifiesto la importancia de la membrana celular en la respuesta al estrés global (Olguín et al., 2015).

En 2016, gracias al empleo de dos técnicas proteómicas complementarias se confirmó que un gran número de proteínas se ven influenciadas por el estrés por etanol. Se descubrió la relevancia de la regulación de la traducción y la absorción de nitrógeno como vía metabólica clave en la adaptación de *O. oeni* PSU-1 al estrés relacionado con el vino. La biosíntesis de la pared celular y los mecanismos de mantenimiento redox parecen desempeñar también un papel relevante en la protección de *O. oeni* contra el daño celular (Margalef-Català et al., 2016).

Recientemente en un estudio de los flujos metabólicos intracelulares bajo diferentes condiciones de estrés por etanol, se observó que *O. oeni* favorece las reacciones anabólicas relacionadas con las vías de reconstrucción y mantenimiento celular y/o la producción de protectores de estrés; en consecuencia, los requisitos de $NAD(P)^+$ y ATP aumentan con el contenido de etanol, sin relación con el incremento de biomasa. Así, para concentraciones de

etanol del 9 al 12% vol. se requieren 10 y 17 veces más ATP de mantenimiento no asociado al crecimiento respecto a los cultivos sin etanol (Contreras et al., 2018).

1.2.1.2. pH

Tras el etanol, el segundo factor de estrés más importante que afecta a las BAL durante el proceso de vinificación es el pH (Sumby et al., 2019). A diferencia de la mayoría de las bacterias que se desarrollan mejor a un pH externo (pH_{ext}) próximo a la neutralidad, las BAL se consideran acidófilas por lo que se desarrollan activamente en vinos a pH_{ext} alrededor de 3,5, siendo posible el crecimiento a pH_{ext} de 2,9-3,0 aunque este se produce más lentamente. El pH del medio afecta al pH citoplasmático (pH_{cit}) de modo que cuando este desciende hasta determinados valores (normalmente entre 4,7 y 5,5), que dependen de la especie de bacteria, la actividad celular no es posible. La influencia del pH se debe a que el choque ácido (pH_{ext} de entre 3,0 y 4,0) produce un incremento de la rigidez de la membrana plasmática e impide el transporte de sustancias a través de ella, disminuyendo la viabilidad celular. *O. oeni* tiene la capacidad de mantener su pH_{cit} más alto pese a encontrarse con pH_{ext} de 3,5, por lo que se desarrolla bien en este rango de acidez. Para el desarrollo de la FML también hay que considerar el pH óptimo de la enzima maloláctica purificada, que es de 5,9 y por tanto difiere considerablemente del pH óptimo de la bacteria. Desde el punto de vista enológico es preferible valores de pH inferiores a 3,5, puesto que así se evita el consumo de azúcares por parte de las bacterias (evitando así incrementos de acidez volátil). Esto conlleva un retraso en el inicio de la FML, pero esta es de mayor calidad ya que es de carácter homoláctico, produciéndose sólo ácido láctico e implicando una baja subida de la acidez volátil (Nannen & Hutkins, 1991; Chu-Ky et al., 2005; Ribéreau-Gayon et al., 2006 y Hidalgo, 2010). El pH del medio también afecta a la degradación del citrato por parte de las BAL, ya que interviene en la regulación de algunos de los genes involucrados en el metabolismo del citrato y, por lo tanto, influye en las concentraciones de los metabolitos de esta vía. *O. oeni* degrada una mayor cantidad de citratos con pH inferiores a 4,0 (Pretorius et al., 2019).

Estos valores bajos de pH aconsejables en enología son compatibles con el desarrollo de *O. oeni* o *Lactobacillus plantarum,* que pueden incluso desarrollarse a pH de 3,2. Durante la FML, las BAL pueden producir glicosidasas, que escinden los azúcares de los precursores aromáticos y los liberan, y esterasas que producen ésteres aromáticos que mejoran el perfil aromático del vino. Estas actividades enzimáticas junto con su capacidad para desarrollarse a pH bajos hace que se comercialicen habitualmente para inducir la FML en el vino (Cinquanta et al., 2018).

El pH también afecta a la duración de la FML; recientemente se comparó el efecto de una mezcla (50:50) de *O. oeni* y *L. plantarum* con las cepas individuales. La duración de la FML estuvo influenciada por el pH y el mejor rendimiento de *O. oeni* se logró a pH 3,4 en vino blanco. En vinos tintos no se observó diferencia en la duración de la FML entre pH 3,4 y 3,8, aunque a pH 3,8 se observó un aumento en la producción de histamina. Múltiples estudios han confirmado a *O. oeni* como la especie mejor adaptada a vinos de bajo pH, sin embargo, dentro de la propia especie hay cepas mejor adaptadas a un bajo pH por lo que la acidez del vino deberá considerarse como uno de los factores a valorar antes de elegir una cepa comercial para desarrollar la FML (Breniaux et al., 2018 y Cinquanta et al., 2018).

1.2.1.3. Temperatura

Como ocurre con el resto de microorganismos, la temperatura tiene una importancia vital en el desarrollo de las BAL. En laboratorio, las BAL presentan unos óptimos de crecimiento que oscilan entre 20 y 37°C, siendo de 27 a 30°C para *O. oeni*. Sin embargo, estos valores se modifican en un medio como el vino, para el que se considera que el óptimo se encuentra entre 20 y 23°C, condiciones factibles en una vinificación real. Esta temperatura óptima desciende a medida que aumenta el grado alcohólico de los vinos. Por debajo de 14°C el desarrollo de la fermentación se hace muy complicado para la bacteria, mientras que si se superan los 25°C se inhibe la biomasa bacteriana y por ende el desarrollo de la FML, lo que se suma a un mayor riesgo de contaminación microbiana y de una subida de la acidez volátil (Ribéreau-Gayon et al., 2006).

A medida que la temperatura ambiental se aleja de los óptimos para la bacteria, y para proteger a las proteínas de la desnaturalización, particularmente a altas temperaturas, *O. oeni* sintetiza pequeñas proteínas de choque térmico (sHSP), que aumentan la capacidad de tolerancia al calor de un organismo (juega un papel importante en la protección de proteínas y en la estabilización de la membrana celular) y permite la supervivencia en climas adversos (Weidmann, et al., 2017 y Li et al., 2018).

Además la temperatura a la que se desarrolla la FML también afecta al metabolismo de las bacterias y por ende a la composición química de los vinos terminados, afectando así al perfil aromático y en menor medida a la sensación en boca (Sereni et al., 2020). La producción de algunas aminas biógenas (AB) como la tiramina o la triptamina también puede verse afectada por la temperatura (Lorenzo et al., 2017).

La aclimatación de las bacterias puede ayudar a desarrollar mejor su metabolismo; en este sentido se observó como una aclimatación previa a 18 y 21°C condujo a un mayor

consumo de ácido L-málico y una superior supervivencia de la población en vinos Pinot Noir, lo que se atribuyó a una mayor expresión de los genes rmlB y hsp20 (Olguín et al., 2019).

1.2.1.4. Dióxido de azufre

El dióxido de azufre (SO_2) es el conservante más utilizado en la industria enológica. En el vino, el SO_2 actúa como un potente antioxidante, ayudando a reducir los efectos del oxígeno disuelto e inhibiendo las enzimas oxidativas. Además, el SO_2 tiene una alta capacidad antimicrobiana frente a levaduras y BAL y, en menor medida, frente a bacterias acéticas (Rubio-Bretón et al., 2018). Sin embargo, la presencia del SO_2 afecta tanto a estos como a los "microorganismos deseables" del vino. El SO_2 puede existir de manera libre o combinada, variando su actividad antimicrobiana en función de en qué forma se encuentre y de su concentración. El SO_2 molecular es el más activo con respecto a la actividad antimicrobiana, los iones bisulfito muestran una baja actividad antimicrobiana; mientras que el SO_2 combinado sólo ejerce una baja acción antibacteriana, lo que es debido a que solo el SO_2 molecular penetra por simple difusión en lugar de por transporte activo. El pH es un factor importante, pues a mayor pH, menor es la proporción de SO_2 molecular presente con lo que la actividad antimicrobiana se reduce (Ribéreau-Gayon et al., 2006; Quirós et al., 2012 y Lisanti et al., 2019). Las BAL, especialmente *O.oeni*, son más sensibles al SO_2 que otras bacterias no lácticas (Rojo-Bezares et al., 2007 y Maturano et al., 2016). Concentraciones de SO_2 total de 100–150 mg/l, de 50–100 mg/l de SO_2 combinado, o las de 1–10 mg/l de SO_2 libre son suficientes para dificultar el desarrollo de las BAL y pueden producir la detención de la FML. Un sulfitado excesivo puede afectar de manera específica a algunas cepas de *O. oeni* de interés organoléptico ya que la resistencia al SO_2 depende de la cepa. También puede influir la levadura seleccionada en cada caso, por lo que es importante controlar las diferentes cepas a lo largo de la FA y la FML para saber cómo evolucionan y cuál predomina considerando la compatibilidad entre las cepas de levadura y las BAL como criterio principal para seleccionar combinaciones de cultivos iniciadores (Reguant et al., 2005 y Bokulich et al., 2015).

En 2002 se observó que el efecto inhibitorio del SO_2 sobre el crecimiento celular y la duración de la FML está relacionado con la inhibición de la actividad ATPasa (Carreté et al., 2002). Las levaduras, especialmente las del género *Saccharomyces,* son productoras de SO_2 de forma que pueden causar un efecto inhibidor e incluso letal sobre las BAL. Por ello, y para reducir este efecto inhibitorio, un enfoque interesante puede ser el empleo de levaduras no *Saccharomyces* para realizar la FA (Balmaseda et al., 2018). Esta inhibición pone de manifiesto la necesidad de comprender las interacciones microbianas para evaluar correctamente la

compatibilidad levadura-bacteria en el vino, puesto que pueden presentarse efectos de sinergia o efectos antagonistas que afecten al aroma, al equilibrio ácido o a la astringencia del vino (Du Plessis et al., 2017; Bartle et al., 2019; Du Plessis et al., 2019 y Izquierdo-Cañas et al., 2020).

En la actualidad la tendencia es hacia la elaboración de vinos con menores cantidades de sulfitos, lo que supone un desafío para los enólogos por el riesgo de ataque de microorganismos indeseables o de reacciones de oxidación en el vino (Coulon et al., 2019 y Lisanti et al., 2019). Además la Organización Internacional de la Viña y el Vino (OIV) ha ido disminuyendo progresivamente la concentración máxima recomendada en los vinos comercializados (SO_2 total en el momento de la venta al consumidor: 150 mg /l para vinos tintos que contengan un máximo de 4 g/l de sustancias reductoras) (OIV, 2012). En este sentido recientemente se han realizado experimentos para hacer vinificaciones en ausencia de SO_2, empleando microorganismos bioprotectores (como *Lactobacillus plantarum* y *Lachancea thermotolerans*), permitiendo que ambas fermentaciones (FA y FML) tengan lugar al mismo tiempo (Rubio-Bretón et al., 2018).

1.3. Metabolismo de las bacterias acidolácticas

1.3.1. Metabolismo del ácido málico

Al hablar del metabolismo de las BAL debe hacerse especial énfasis en el metabolismo del ácido málico, fenómeno ya observado por Pasteur hace más de un siglo. El término FML fue establecido por considerarse como un paso análogo a la FA con anterioridad al descubrimiento de los mecanismos químicos que tenían lugar en realidad. Sin embargo, desde un punto de vista químico no se trata de una fermentación, sino de la descarboxilación de origen bacteriano del ácido L-málico en ácido L-láctico y CO_2, (Lonvaud-Funel, 1995). Se trata de un proceso complejo que habitualmente tiene lugar tras concluir la FA y que, aunque tradicionalmente se ha considerado como un proceso secundario, desempeña un papel crucial en las características finales de la mayoría de los vinos tintos y de algunos vinos blancos como los elaborados con la variedad Chardonnay y algunos vinos espumosos (Bartowsky, 2008 y Lerm et al., 2010).

Las consecuencias de la FML son principalmente tres: (i) la desacidificación del vino con el consiguiente aumento del pH, (ii) el incremento de la estabilidad microbiana al eliminar del medio un posible sustrato para el desarrollo de microorganismos como es el ácido málico y

(iii) la modificación del perfil aromático del vino en cuestión (Maicas et al., 1999 y Balmaseda et al., 2018).

Existen tres rutas metabólicas posibles para la transformación del ácido málico en láctico. La primera y más utilizada por las BAL vínicas consiste en la conversión directa mediante una reacción catalizada por la malato descarboxilasa o enzima maloláctica y en la que el NAD^+ y el Mn^{2+} actúan como cofactores y no se obtienen subproductos intermedios en la descarboxilación. La tasa de descarboxilación del malato depende de la actividad maloláctica de la bacteria en cuestión. La segunda vía metabólica para la descarboxilación del ácido málico emplea la enzima málica para transformar el ácido L-málico en ácido pirúvico, que es reducido a ácido láctico en una reacción catalizada por la enzima lactato deshidrogenasa. Una tercera vía consiste en la reducción del malato a oxalacetato (mediante acción de la malato deshidrogenasa) seguido de una descarboxilación a piruvato y una reducción a ácido láctico (Lerm et al., 2010). Como ya se ha mencionado en apartados anteriores hay varios tipos de BAL que pueden realizar la FML de las que particularmente *O. oeni* es la mejor adaptada a las duras condiciones del vino (Bartowsky et al., 2015).

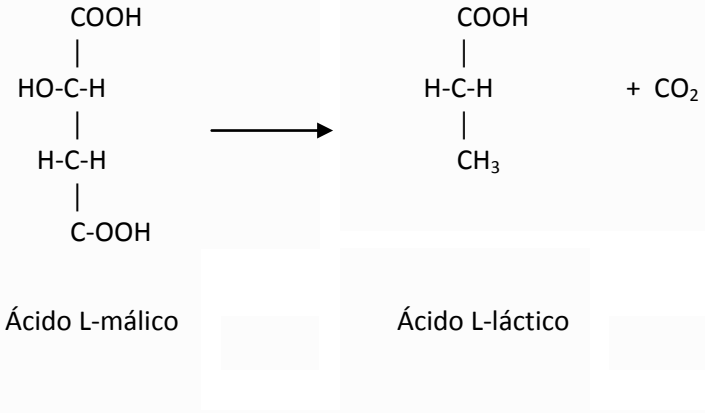


Figura 1. Reacción de transformación del ácido L-málico en ácido L-láctico (extraído de Ribéreau-Gayon et al., 2006).

En 2017 se realizó el primer análisis transcripcional completo de *O. oeni*. Se observó que dentro de la misma especie, en muchos procesos celulares diferentes cepas expresan genes de forma distinta. Se observaron diferencias muy significativas entre diversas cepas, encontrando algunas como la cepa AWRIB419, que conduce la FML a una velocidad sustancialmente más lenta y con una mayor producción de diacetilo. En total, se encontraron trece genes relacionados con el metabolismo del diacetilo que se expresan de forma distinta en función de la cepa de que se trate (Sternes et al., 2017 y Wojdyło et al., 2020).

Los estudios genómicos comparativos de cientos de aislamientos de *O. oeni* han demostrado una variación genómica sustancial entre cepas de *O. oeni*, con una variación de hasta un 10% en los genes codificadores de proteínas observadas entre dos cepas cualesquiera incluidos los que se predice que están involucrados en la utilización y transporte de azúcar, la biosíntesis de exopolisacáridos, la biosíntesis de aminoácidos y la competencia natural (Sternes & Borneman, 2016). Esto pone de manifiesto la importancia de la selección de la cepa adecuada para realizar la FML en cada caso. Con el fin de encontrar la cepa idónea para cada vinificación, puede resultar muy interesante realizar una identificación y selección de cepas autóctonas de las diferentes zonas geográficas que mejor se adapten a las condiciones del vino de esa región (Franquès et al., 2018; Romero et al., 2018 y Manera et al., 2019).

1.3.2. Metabolismo del ácido cítrico

El ácido cítrico se encuentra tanto en el mosto como en el vino, aunque en menor concentración (0,1-1 g/l) que otros ácidos orgánicos como el tartárico o el málico (Muñoz et al., 2011). Algunas BAL como *L. plantarum*, *L. casei*, *O. oeni* y *L. mesenteroides* pueden utilizar citrato durante la FML (serán consideradas citrato positivas). El citrato se transporta a la célula por permeasas de citrato o malato. Una vez en el interior de la célula, el citrato se degrada a acetato y oxaloacetato por la enzima citrato liasa y posteriormente, el oxaloacetato se convierte en piruvato, un intermediario en la producción de compuestos volátiles como el lactato, acetato, diacetilo, acetoína o 2,3-butanodiol (Ramos et al., 1995; Ribéreau-Gayon et al., 2006; Olguín et al., 2009 y Pretorius et al., 2019).

De esos subproductos, quizá el más representativo del metabolismo de las BAL es el diacetilo, responsable del sabor en distintos alimentos como mantequilla, vino, brandy, café tostado y muchos otros alimentos fermentados. El diacetilo se forma como un metabolito intermedio en la descarboxilación reductora del ácido pirúvico a 2,3-butanodiol (Ramos et al., 1995) y su formación y degradación están estrechamente relacionadas con el crecimiento de las BAL. Generalmente, la concentración de diacetilo en el vino aumenta a medida que avanza la FML, exhibiendo niveles máximos cuando se ha consumido aproximadamente el 95% del ácido málico y el 75% del ácido cítrico. Al metabolizarse todo el ácido cítrico, la concentración de diacetilo disminuye hasta valores previos a la FML (Sternes et al., 2017).

1.3.3. Metabolismo de los compuestos aromáticos

La percepción sensorial del vino depende de una elevada cantidad de interacciones entre un gran número de compuestos que interactúan y se combinan mostrando tanto

sinergias como efectos antagonistas (Ryan et al., 2008). Así, el perfil aromático de un vino depende de diversos factores como son (i) la variedad de uva, (ii) el terruño (iii) los microorganismos responsables de las fermentaciones y de otras alteraciones y (iv) las condiciones de envejecimiento y almacenamiento (Styger et al., 2011).

El desarrollo de la FML influye también por tanto en el aroma de los vinos. Las aportaciones más significativas de la FML en el aroma del vino son (i) la mejora de las notas afrutadas debido a la formación de ésteres, como el acetato de etilo, el lactato de etilo, el hexanoato de etilo y el octanoato de etilo; (ii) la aparición de matices mantecosos, en este caso debidos a la producción de compuestos carbonílicos o cetónicos como el diacetilo, la acetoína y el 2,3-butanodiol generados a partir del metabolismo del ácido cítrico por las BAL a través de varias reacciones catalizadas por la citrato liasa; y (iii) una reducción de los matices verdes o herbáceos del vino debido al catabolismo del acetaldehído, lo que resulta en la formación de etanol y acetato. Tras la FML pueden aparecer diferentes matices como notas florales, nueces, levaduras, roble, sudor, picante, tostado, vainilla, ahumado, terroso, amargo, espinoso y miel. Además del aroma, también la FML aumenta el cuerpo y la sensación en la boca del vino y proporciona un sabor más prolongado. Además las BAL contienen enzimas catalíticas (como β-glucosidasas, proteasas, esterasas, citrato liasa y descarboxilasas de ácido fenólico) que producen diversos compuestos aromáticos, por lo que la selección de cepas de BAL que posean esta actividad enzimática puede ser de gran interés para la mejora del aroma durante la vinificación (Liu, 2002; Grimaldi et al., 2005; Styger et al., 2011 y Fia et al., 2018). Estas actividades enzimáticas se ven afectadas principalmente por el pH, la temperatura, el etanol y los azúcares (Olguín et al., 2011 y Martín et al., 2013).

Recientemente se observó que la producción de ésteres hidroxilados ramificados, como el etil 2-hidroxi-3-metilbutanoato y el etil 2-hidroxi-4-metilpentanoato estaba fuertemente influenciada por la cepa bacteriana (Gammacurta et al., 2018). Se comprobó también que determinadas cepas de *O. oeni* producen una mayor cantidad de ésteres que cepas de *Lactobacillus* mostrando una capacidad superior para mejorar el aroma del vino debido a su actividad esterasa, que produce un aumento de compuestos responsables de olores afrutados y cremosos (Brizuela et al., 2018). Esto difiere de lo observado por Wang et al., (2020) quienes advirtieron que los vinos elaborados con diversas cepas de *L. plantarum* eran más aromáticos que los vinos ensayados con distintas cepas de *O. oeni*. Esta variabilidad, y puesto que *O. oeni* muestra una alta diversidad intraespecífica, hace que el factor cepa sea un aspecto fundamental para el correcto devenir de la FML y tendrá una marcada influencia en el aroma de los vinos (Cappello et al., 2017 y Sternes et al., 2017).

Durante el envejecimiento en las barricas de roble las BAL también pueden influir en el sabor y aroma del vino al producir compuestos adicionales derivados del roble como la vainillina, ya que son capaces de transformar un precursor de la vainillina presente en la madera liberando su aroma en el vino. Además las BAL también pueden producir o alterar compuestos que afectan el aroma a través de su propio metabolismo (Bloem et al., 2007).

1.3.4. Metabolismo de los aminoácidos

Los aminoácidos y ocasionalmente los péptidos, proporcionan a las BAL el nitrógeno asimilable que necesitan. Dependiendo de la especie y de las cepas, los aminoácidos requeridos por las BAL del vino varían ampliamente, siendo algunos esenciales y otros activadores del crecimiento y empleándose fundamentalmente para la síntesis de proteínas. Los aminoácidos esenciales para las BAL son los siguientes: alanina, arginina, cisteína, ácido glutámico, histidina, leucina, fenilalanina, serina, triptófano, tirosina y valina. Dependiendo de la cepa, algunos pueden catabolizarse y servir como fuentes de energía (arginina, histidina y tirosina). Aunque su concentración puede ser limitante durante la fase activa de la FA debido a la multiplicación de las levaduras (la arginina puede desaparecer por completo), al final de la FA y debido a la autolisis de las levaduras, se encuentra una amplia variedad y gran cantidad de aminoácidos. Además los aminoácidos que se utilizan para la síntesis de proteínas por la biomasa en crecimiento, también pueden metabolizarse durante la FML, por lo que la concentración de aminoácidos en el vino puede variar debido probablemente a la hidrólisis simultánea de péptidos y proteínas. En lo que a compuestos nitrogenados se refiere, las bases púricas y pirimidínicas juegan un papel importante en el crecimiento. Las necesidades de adenina, guanina, uracilo, timina y timidina también dependen de la cepa, pudiendo no ser esenciales para ella (Ribéreau-Gayon et al., 2006 y Lonvaud-Funel, 2015).

1.3.5. Metabolismo de los compuestos indeseados

Las AB se encuentran comúnmente en muchos alimentos. Se han encontrado altas concentraciones en pescado, chocolate, queso, productos de soja, salchichas, carne procesada y bebidas fermentadas. Durante la vinificación tanto las levaduras como las bacterias pueden participar en la producción de AB. En general las levaduras hacen una contribución menos significativa que las BAL al contenido final de AB en el vino, siendo las más importantes la histamina, la tiramina y la putrescina. Las AB pueden estar presentes en todo tipo de vinos, aunque en los vinos blancos, el contenido es menor que en los vinos tintos (Costantini et al., 2019 y Esposito et al., 2019).

Generalmente, la producción de AB se debe a una descarboxilación de aminoácidos por parte de bacterias. Así, la histamina se forma a partir de histidina mediante la acción de la enzima histidina descarboxilasa, la ornitina descarboxilasa cataliza la descarboxilación de ornitina a putrescina y la tirosina es el precursor de la tiramina producida por la tirosina descarboxilasa (Moreno-Arribas et al., 2003 y Henríquez-Aedo et al., 2016).

La producción de AB puede considerarse un mecanismo de defensa utilizado por las bacterias para sobrevivir mejor en un medio ácido puesto que intervienen en procesos de regulación del pH celular, contribuyendo así a la producción de energía (Spano et al., 2010).

La actividad de estas descarboxilasas depende de varios factores como el pH, la temperatura, y la concentración de azúcares y precursores de aminoácidos. La temperatura es un factor importante, incrementando la actividad enzimática cuando esta es moderada (Marcobal et al., 2006). Se ha demostrado que la biosíntesis de la histidina descarboxilasa y la ornitina descarboxilasa dependen estrechamente de la presencia de altas concentraciones de aminoácidos libres en el medio de crecimiento (Pessione et al., 2005).

En las BAL el pH puede regular las enzimas descarboxilasas mediante activación catalítica y sobreexpresión biosintética, además la máxima síntesis de descarboxilasa ocurre durante la fase estacionaria, cuando el pH es bajo debido a la acumulación de ácido láctico (Pessione et al., 2005 y Pessione et al., 2010) lo que coincidiría con el hecho, anteriormente citado, de que las AB constituyen un mecanismo protector frente a un pH bajo.

La influencia de los azúcares se pone de manifiesto cuando hay una carencia de estos, lo que conlleva una mayor producción de AB debido a que el sistema de transporte de la vía descarboxilativa proporciona energía metabólica (Gardini et al., 2016). En este sentido, recientemente se observó que la presencia de concentraciones crecientes de glucosa y fructosa inhibía la formación de histamina por parte de diversos tipos de bacterias (*Lactobacillus hilgardii, Pediococcus parvulus y O. oeni)* (Gardini et al., 2016 y Costantini et al., 2019). Diferentes estudios han concluido que *O. oeni* contribuye significativamente al contenido general de histamina en los vinos, pero que la capacidad de la especie para producir esta amina varía entre las cepas (Coton et al., 1999 y Guerrini et al., 2002). La tiramina en cambio es producida por bacterias del género *Lactobacillus* (Moreno-Arribas et al., 2000).

Otro compuesto que pueden producir las BAL a partir de la ornitina es la citrulina que, si se libera en el vino, puede ser un precursor del carbamato de etilo. Sin embargo, el carbamato de etilo sintetizado es insignificante en comparación con las cantidades generadas a partir de la urea liberada por las levaduras (Lonvaud-Funel, 2015).

Recientemente Wang et al., (2020) observaron que el empleo de cepas de *L. plantarum* en comparación con *O. oeni*, junto con otras ventajas, condujo a una menor

acumulación de AB en vinos tintos, por lo que sostienen que *L. plantarum* podría servir como un mejor iniciador para realizar la FML del vino tinto.

Respecto al metabolismo de los azúcares por parte de las BAL, este es sumamente indeseable, si bien las BAL suelen fermentar solo pequeñas cantidades de azúcar, por lo que la producción de ácido acético es baja. Si la FA no se controla correctamente y se ralentiza o detiene, las BAL heterofermentativas comienzan a multiplicarse fermentando azúcares en grandes cantidades con la producción de ácido acético, incrementándose de este modo la acidez volátil del vino, alteración conocida como "picado láctico" (Ribéreau-Gayon et al., 2006 y Lonvaud-Funel, 2015).

El ácido tartárico también puede ser metabolizado por las BAL, si bien su metabolismo es distinto al de los ácidos cítrico y málico y causa una enfermedad ya descrita por Pasteur como *tourne* ("rebote" o "vuelta" en castellano) consistente en un descenso de la acidez fija del vino (por la pérdida de ácido tartárico) y un incremento de la acidez volátil, apareciendo un olor a col cocida debido a los compuestos acetoínicos formados (Ribéreau-Gayon et al., 2006).

1.3.6. Interacciones entre bacterias acidolácticas y otros microorganismos

La compatibilidad entre las levaduras y las BAL se ve afectada por parámetros físico-químicos que son específicos de la cepa y del cultivo. Durante la vinificación los microorganismos producen compuestos orgánicos, ácidos grasos y péptidos, entre otros compuestos, que pueden ser estimulantes, inhibidores o no provocar ningún efecto aparente sobre otros microorganismos presentes en la fermentación. Además, esta compatibilidad levadura-BAL está influenciada por diferentes parámetros como la utilización de oxígeno, nitrógeno asimilable y ácido L-málico (Bartle et al., 2019).

Un enfoque distinto al tradicional consiste en realizar fermentaciones simultáneas con levaduras y BAL, con lo que se reduce considerablemente el tiempo de fermentación. En este sentido Sereni et al. (2020) observaron un desarrollo más rápido de la FA y la FML con la inoculación simultánea de levaduras y *O. oeni*, sin que se produjeran impactos adversos en la calidad del vino. Estos resultados van en consonancia con lo observado recientemente en otro estudio en el que se comprobó que el empleo conjunto de *Torulaspora delbrueckii* y *O. oeni* causó un impacto beneficioso tanto para la duración de la FA y la FML como para las propiedades aromáticas y sensoriales del vino y permitió la producción de vinos con un color más intenso (Nardi et al., 2019).

Con el aumento de la popularidad de las fermentaciones autóctonas (no inoculadas), las interacciones de la comunidad microbiana del vino en su conjunto se están volviendo más

importantes y merecen un estudio más a fondo que permita a los enólogos desarrollar prácticas de vinificación más adecuadas (Sumby et al., 2019).

1.4. Problemática actual de la fermentación maloláctica

Como se ha comentado anteriormente, la capacidad de desarrollar la FML por parte de las BAL depende de factores ambientales como el pH, el SO_2, la temperatura, el etanol, la presencia de nutrientes o las interacciones con otros microorganismos. Sin embargo, hoy en día hay que considerar un aspecto adicional. Lamentablemente y pese a los beneficios que conlleva una FML en condiciones adecuadas, el calentamiento global que estamos sufriendo está ejerciendo una profunda influencia sobre la fenología de la vid y la composición de la uva, afectando por tanto a las vinificaciones, la microbiología y la química del vino y a sus aspectos sensoriales (Orduña, 2010 y Vila-Crespo et al., 2010).

Desde principios del siglo XX, la temperatura media mundial ha aumentado aproximadamente 0,78°C, concentrándose dos terceras partes de dicho incremento desde 1960. Aunque las predicciones varían ampliamente, los expertos creen que en el siglo XXI la temperatura global promedio podría aumentar, si no mitigamos las causas antropogénicas del calentamiento global, hasta cifras dramáticas. Si tomamos las medidas necesarias estas cifras podrían rebajarse hasta aproximadamente 1,1°C de incremento de temperatura. Sin embargo, incluso en el punto más bajo, el planeta se enfrenta a resultados que podrían ser catastróficos (Mozell & Thach, 2014).

En lo que a la enología se refiere este incremento de temperatura causaría efectos enormes en las diferentes regiones vinícolas del mundo, llegando a modificar la producción de vino, especialmente en términos de selección de uva, haciendo inviable la producción de determinadas variedades en las zonas actuales y permitiéndola en zonas demasiado frías hoy día para el cultivo de la vid (Hannah et al., 2013). A ello hay que añadir que la viticultura ha cambiado con el paso de los años y en un afán por mejorar la productividad se han incrementado los rendimientos de uva, los abonados inorgánicos del suelo, el regadío y las plantaciones de viña en suelos que quizás no sean los óptimos para su cultivo (Gutiérrez et al., 2012).

El incremento de la temperatura ambiental altera las fechas en que se vendimia, causando que el mosto presente concentraciones demasiado elevadas de azúcar, obteniéndose vinos excesivamente alcohólicos y desequilibrados que presentan una menor acidez y con modificaciones en el color y en las concentraciones de los compuestos aromáticos varietales, pudiendo incluso llegar a inhibirse el metabolismo de la vid. Los mostos con elevadas concentraciones de azúcar ocasionan estrés en las células, obteniéndose una mayor

cantidad de subproductos de fermentación, como el ácido acético. Además estas alteraciones climáticas causan un incremento del pH en la uva, lo que hace que sea necesario realizar una acidificación del mosto para evitar cambios significativos en la ecología microbiana de los mostos y vinos, aumentando así el riesgo de deterioro y degradación organoléptica (Orduña, 2010 y Vila-Crespo et al., 2010).

Otra alteración que afectará al desarrollo de *O. oeni* es el bajo contenido de ácido L-málico existente (el principal substrato utilizable por *O. oeni*), de modo que en algunas regiones cálidas ya se están registrando actualmente valores de ácido L-málico alrededor de 0,5 g/l en los vinos (habitualmente se encuentra en cantidades del orden de 3 g/l) (Palma & Barroso, 2002). Así, esta carencia de nutrientes del vino, frente a los exigentes requisitos nutricionales de *O. oeni* dificulta e incluso compromete la supervivencia de estas bacterias (Reguant et al., 2010).

1.4.1. Estrategias para mejorar la fermentación maloláctica

Para solventar los problemas con que se encuentran los enólogos, en la actualidad existen soluciones enfocadas al viñedo como son proveer de sombra a los cultivos, vendimiar de noche, introducir modificaciones en la poda, mejorar el equilibrio suelo-agua a través de un suministro de riego más efectivo como el riego por goteo, proveer a los suelos de cobertura que evite la erosión, etc. Sin embargo el manejo de los viñedos puede no ser suficiente, por lo que es necesario buscar nuevas estrategias de adaptación que deberán ir acompañadas de una gestión sostenible de los recursos hídricos y la flexibilidad en la legislación (Lereboullet et al., 2013 y Mozell & Thach, 2014).

Debido al calentamiento global se está incrementando el pH del vino, lo que facilita el desarrollo de BAL diferentes a *O. oeni* como pueden ser *Lactobacillus* y *Pediococcus spp.* lo que permite su empleo como cultivos iniciadores (Berbegal et al., 2016 y Wade et al., 2019). Para contrarrestar los efectos del elevado pH del vino se han escogido diferentes estrategias como (i) coinocular *L. plantarum* y *O. oeni* a la vez (pudiendo añadir también levaduras para completar las fermentaciones de manera más rápida y segura (Guzzon et al., 2013)); (ii) emplear *L. plantarum* como acidificante del vino gracias a su capacidad para producir altas concentraciones de ácido láctico; (iii) utilizar *Schizosaccharomyces pombe* para la disminución de la acidez mediante la fermentación maloalcohólica, ya que esta levadura puede consumir el ácido málico presente en el mosto o en el vino con la producción estequiométrica correspondiente de etanol y CO_2, aunque esto puede acarrear la producción de sabores

desagradables asociados a su metabolismo y (iv) técnicas de modificación genética, entre otras (Berbegal et al., 2020).

La modificación genética de las bacterias, puede realizarse siguiendo dos enfoques, métodos recombinantes y no recombinantes. Los primeros suelen consistir en técnicas de alta precisión que añaden o suprimen determinados genes de cuyas funciones e interacciones hay que tener un conocimiento previo. La sobreexpresión de genes nativos o implantados en *O. oeni*, por ejemplo, puede realizarse introduciendo plásmidos como se hace con otros microorganismos, para lo que es necesaria la generación de células competentes o la transferencia forzada de ADN a través de diversas técnicas como la electroporación. En las técnicas no recombinantes no se requiere tal conocimiento previo, si bien se tiene menos control de la situación debido a su aleatoriedad, pudiendo presentarse efectos pleiotrópicos (Betteridge et al., 2015). En esta línea, en 2005, se consiguió expresar por primera vez un gen maloláctico en *S. cerevisiae* de forma que la cepa modificada genéticamente redujo el nivel de L-malato en el mosto de uva a menos de 0,3 g/l en 3 días (Bauer et al., 2005). Aunque se trata de un enfoque prometedor, en la actualidad la OIV prohíbe el uso de organismos modificados genéticamente (OMG).

El momento de la inoculación bacteriana puede variar aunque de manera general se emplea una inoculación secuencial, que consiste en la adición de las BAL seleccionadas una vez ha concluido la FA; o inoculación simultánea (coinoculación), en la que se adicionan las BAL durante la FA de modo que ambos microorganismos conviven durante el proceso (Bartowsky et al., 2015). Se emplean altas densidades celulares (entre 10^7-10^8 ufc/ml) para asegurar el correcto desarrollo del proceso ya que así, factores como el pH y el etanol no son tan críticos y las BAL pueden realizar la FML sin tener que pasar por una fase previa de crecimiento (Maicas, 2001 y Zhang & Lovitt, 2006). A la hora de realizar la coinoculación de diferentes microorganismos deberá tenerse en cuenta la compatibilidad entre ellos, recomendándose el empleo de cultivos iniciadores autóctonos por estar bien adaptados a las condiciones de un área vitivinícola específica (Ruiz et al., 2010 y Bleve et al., 2016). Recientemente se observó que al coinocular *O. oeni* junto con cepas compatibles de *S. cerevisiae* se evita el desarrollo de levaduras no deseadas como *Brettanomyces bruxellensis* (Berbegal et al., 2017).

En mosto blanco de zonas vitivinícolas con clima frío y bajo pH y con alto contenido de alcohol se obtuvieron buenos resultados al coinocular simultáneamente levaduras con dos cepas de *O. oeni*, reduciendo el tiempo total de fermentación y observando concentraciones más altas de ésteres etílicos y de acetatos en comparación con las inoculaciones secuenciales (Knoll et al., 2012).

Una alternativa novedosa para proteger a los microorganismos de las consecuencias del estrés causado por el bajo pH y el etanol es la inmovilización de las BAL. Varios han sido los experimentos realizados en esta línea (Vila-Crespo et al., 2010), obteniéndose bajos niveles de acidez volátil, concentraciones de etanol y glicerol comparables a las obtenidas en una inoculación con BAL libres y una disminución significativa en el tiempo necesario para completar la FA y la FML. Otra forma de protección de las BAL frente a las condiciones de estrés presentes en el vino es mediante el uso de glutatión para la recuperación del equilibrio redox celular (Berbegal et al., 2020).

1.5. Inmovilización celular

Las células inmovilizadas son aquellas que están físicamente confinadas o localizadas en una región determinada del espacio, que permite la retención de su actividad catalítica y, si es posible o incluso necesario, su viabilidad y que pueden usarse repetida y continuamente (Klein & Fritz, 1983). En la naturaleza, muchos microorganismos poseen la capacidad de unirse a diferentes tipos de superficies o introducirse dentro de estructuras naturales, comportamientos relacionados con una inmovilización celular (Lorentzen & Lucas, 2019).

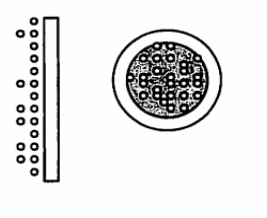
Para que las células inmovilizadas puedan ser aplicables a nivel industrial es necesario que se cumplan una serie de requisitos y propiedades. Por un lado, la matriz de inmovilización debe (i) ser atóxica, (ii) estar fácilmente disponible y ser asequible económicamente, (iii) ser de fácil manejo para una adecuada inmovilización, (iv) permitir una alta carga celular y poseer una resistencia física suficiente. Por otro lado, (v) las células inmovilizadas deben tener una viabilidad prolongada y mantener su actividad metabólica en el soporte elegido (Pilkington et al., 1998).

En los últimos años ha surgido un creciente interés por el empleo de sistemas de inmovilización celular para la elaboración de bebidas fermentadas debido a los beneficios que plantea respecto al uso de células libres. Las principales ventajas del uso de células inmovilizadas son: (i) la protección de las células contra el entorno hostil, (ii) la fácil separación de las células del medio de fermentación, (iii) la posibilidad de reutilizar las células en varios lotes de fermentación, (iv) la posibilidad de un proceso fermentativo en continuo y (v) la alta productividad que se puede obtener debido a la posibilidad de aumentar la concentración celular por unidad de volumen de biorreactor (Kosseva, 2011 y Sekoai et al., 2017).

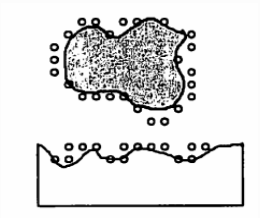
En otras industrias, esta tecnología se ha utilizado para la encapsulación de una amplia gama de productos, como productos farmacéuticos, aceites volátiles, extractos de plantas o enzimas. También se ha empleado en diferentes procesos biotecnológicos como la

encapsulación de probióticos en industrias alimentarias, o en biotransformación y fermentación para producir antibióticos, ácidos orgánicos, enzimas y alcoholes, así como en la descontaminación ambiental y la biorremediación de aguas residuales (Mariangela et al., 2016).

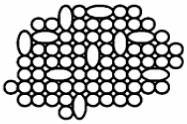
Como se muestra en la Figura 2 y de manera general, los sistemas de inmovilización celular más empleados son los siguientes: (i) inclusión en membranas, (ii) unión a un soporte, (iii) autoagregación celular y (iv) atrapamiento físico dentro de una matriz porosa (Pilkington et al., 1998). A continuación se explica el fundamento de cada una de estas estrategias, profundizando en el atrapamiento físico dentro de la matriz porosa.



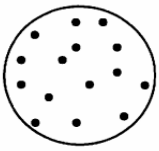
Inclusión celular en membranas



Unión a un soporte



Autoagregación celular



Atrapamiento físico dentro de una matriz porosa

Figura 2. Sistemas de inmovilización celular (extraído de Pilkington et al., 1998).

1.5.1 Inclusión celular en membranas

Como membrana de inclusión celular se pueden utilizar filtros de membrana microporosos, microcápsulas o también puede realizarse la inmovilización celular en una superficie de interacción de dos líquidos inmiscibles, lo que resulta ideal cuando se necesita un subproducto libre de células y una transferencia mínima de compuesto (Kourkoutas et al., 2004).

Esta tecnología se ha estudiado desde hace tiempo para la reutilización celular y en procesos continuos. Así Lebeau et al., (1997) coinmovilizaron *S. cerevisiae* y *Candida shehatae* en una capa de agar plana comprendida entre dos filtros de membrana microporosa. Kargupta

et al., (1998) utilizaron un fermentador de membrana en continuo con eliminación continua de etanol obteniendo un rendimiento superior al obtenido con células libres.

Para el sector enológico, la compañía Millipore desarrolló el sistema "Millispark", un cartucho de filtros de membrana que incluía levaduras seleccionadas para realizar la segunda fermentación de vinos espumosos (Lemonnier, 1996). Sin embargo, se han observado limitaciones a este sistema ya que puede formarse una zona de alta densidad celular en un lado de la membrana que incrementa la resistencia a la difusión molecular, ya sea por acumulación o por desarrollo de microorganismos (Lebeau et al., 1998 y Gryta, 2002).

1.5.2. Unión a un soporte

Este sistema consiste en una unión física entre la superficie de la célula y el soporte en el que pueden intervenir interacciones electrostáticas, enlaces covalentes, fuerzas de Van der Waals y/o por puentes de hidrógeno. Se trata de un mecanismo muy utilizado debido a su simplicidad y bajo coste. Tanto el grosor de la biopelícula de células inmovilizadas como la fuerza de unión entre las células y la superficie pueden ser complicados de determinar. Si la unión entre el soporte y la célula es débil y/o reversible (por ejemplo con el uso de enlaces iónicos, fuerzas de Waals y/o por puentes de hidrógeno) las células pueden liberarse al medio de fermentación donde permanecerán en suspensión. A diferencia del atrapamiento celular, la adsorción es ventajosa porque los nutrientes están en contacto directo con las células inmovilizadas y, por lo tanto, mejora la eficiencia de conversión del sustrato. Ejemplos de soportes sólidos utilizados en este tipo de inmovilización son los materiales celulósicos (dietilaminoetilcelulosa, madera, serrín, etc.) y los materiales inorgánicos (poligorskita, montmorilonita, porcelana porosa, vidrio poroso, etc.). Además pueden utilizarse policationes, quitosano u otros compuestos químicos para aumentar la capacidad de adsorción de los soportes (Norton & Damore, 1994; Silveira Martins et al., 2013 y Sekoai et al., 2018).

1.5.3. Autoagregación celular

La autoagregación celular es la propiedad de algunos microorganismos como mohos, hongos y células vegetales en suspensión de adherirse rápidamente en grupos y sedimentar. Es habitual el empleo de agentes floculantes que mejoran la agregación en cultivos celulares que no floculen por sí mismos. Las levaduras, como *S. cerevisiae,* poseen propiedades de floculación, cuya capacidad varía dependiendo de diferentes factores como la composición de la pared celular, el pH, el O_2 disuelto y la composición del medio (Soares, 2011). En la industria cervecera, la autoagregación de las levaduras es de gran interés ya que mejora la

productividad de la fermentación y la calidad de la cerveza, y facilita la eliminación y recuperación de la levadura (Kourkoutas et al., 2004). Esta propiedad también se emplea como criterio para la selección de cepas de levaduras *S. cerevisiae* para la elaboración de vinos tranquilos y espumosos, con el objetivo de facilitar su eliminación después del proceso fermentativo (Varela et al., 2020).

1.5.4. Atrapamiento físico dentro de una matriz porosa

De todos los sistemas de inmovilización celular, el atrapamiento en una matriz porosa es el más empleado debido a su simplicidad y sus condiciones de trabajo moderadas (Dulieu et al., 1999 y Rother & Nidetzky, 2014).

Hay infinidad de polímeros disponibles para realizar el atrapamiento celular, los ejemplos más característicos son el uso de geles de polisacáridos como alginatos, κ-carragenano, agar, quitosano y ácido poligalacturónico, u otras matrices poliméricas como gelatina, colágeno y alcohol polivinílico. Esta técnica de inmovilización consiste en la inclusión de las células dentro de una matriz porosa que evita que estas difundan en el medio circundante, al tiempo que permite la transferencia masiva de nutrientes y metabolitos. Generalmente, se emplean dos sistemas para el atrapamiento de las células: (i) uno basado en la inclusión de células a través de la matriz porosa hasta que su movilidad se vea obstruida por la presencia de otras células, y (ii) otro donde tiene lugar la formación del material poroso *in situ* en el cultivo celular. Dentro de la matriz porosa se produce un desarrollo celular que dependerá principalmente de la capacidad de difusión de nutrientes y metabolitos a través del material, del impacto de la acumulación de biomasa y metabolitos en la viabilidad celular y del nivel efectivo de penetración del O_2 en la matriz. Todos estos factores pueden provocar que se desarrolle un patrón de crecimiento no homogéneo y que las células cercanas a la superficie puedan comportarse de manera diferente en comparación con las células del interior de la matriz, quienes pueden tener mayores dificultades para desarrollar sus funciones vitales. Otro inconveniente es que las células ubicadas en la superficie externa del soporte pueden multiplicarse y liberarse al medio. Para solventar este inconveniente se han desarrollado sistemas de doble capa con un núcleo interno que contiene las células y una capa externa que impide que las células del núcleo escapen al medio (Kourkoutas et al., 2004; Silveira et al., 2013 y Sekoai et al., 2018).

1.5.4.1. Atrapamiento en geles de alginato

El alginato es el polímero más estudiado para el atrapamiento de células debido a su simplicidad, bajo coste y suaves condiciones de inmovilización que permiten una sencilla manipulación (Lee et al., 2004; Bouallagui & Sayadi, 2006; Remminghorst, 2007; Safarik et al., 2008; Satora et al., 2009; De Vos et al., 2014; Huang et al., 2016 y Kyung-Chul et al., 2016).

El alginato es un polisacárido lineal poliónico e hidrofílico que se extrae de las algas marinas, y cuya estructura está conformada por dos monómeros, el ácido α-L-gulurónico (G) y el ácido β-D-manurónico (M). Estos monómeros se distribuyen en secciones constituyendo homopolímeros de bloques G (-GGG-), M (-MMM-) o heteropolímeros donde los bloques M y G se alternan (-MGMG-). La flexibilidad o rigidez depende del contenido en bloques G de su estructura polimérica. Si posee una mayor cantidad de bloques G generalmente el gel será fuerte pero frágil, mientras que si lo que abundan en mayor proporción son bloques M, el gel formado será suave y elástico. Para su gelificación, el alginato necesita de la presencia de cationes divalentes (excepto el Mg^{2+}), generalmente se emplean Ca^{2+} o Ba^{2+}. La gelificación tiene lugar al producirse una unión a través del catión divalente entre un bloque G de una molécula de alginato con otro bloque G de otra molécula de alginato. Con la unión sucesiva de moléculas de alginato se forma una estructura física denominada modelo de “caja de huevos” (Donati et al., 2005) (ver Figuras 3 y 4). Las sales de alginato más empleadas son las de sodio debido su alta solubilidad en agua fría y característica transición sol-gel de forma instantánea e irreversible ante el Ca^{2+} (Remminghorst, 2007; Funami et al., 2009; Lupo et al., 2012 y Gasperini et al., 2014).

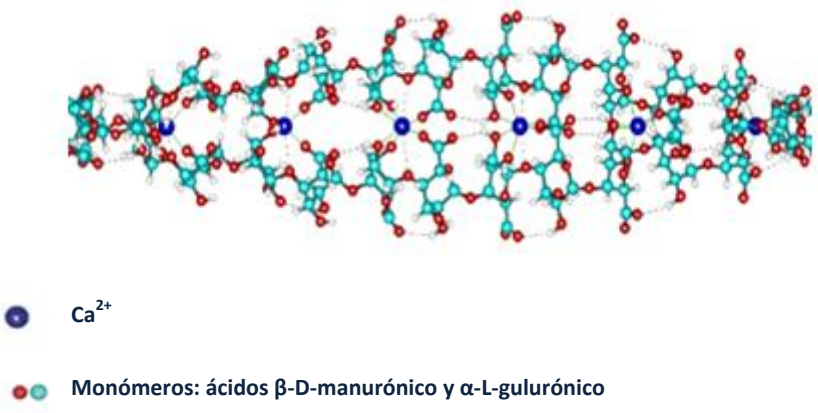


Figura 3. Conformación de la molécula de alginato (extraído de Reddy & Reddy, 2010).

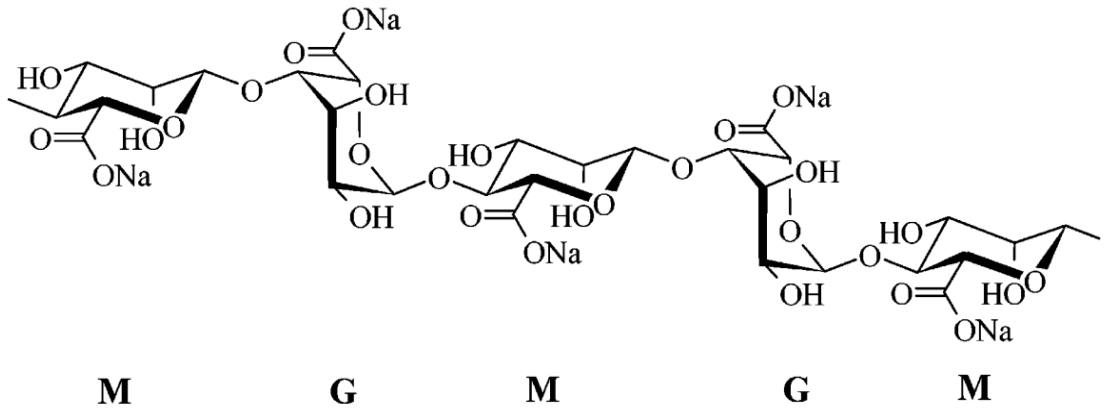


Figura 4. Conformación de los bloques G y M (extraído de Donati et al. 2005).

Pese a sus múltiples virtudes, el gel de alginato posee una baja estabilidad química y una relativa debilidad mecánica, lo que dificulta su implantación a nivel industrial (Coradin et al., 2003). Otra desventaja es que el empleo de alginato está limitado a ambientes con valores de pH medios y baja concentración de fosfatos o citratos, que debido a su afinidad con el Ca^{2+} pueden secuestrar los cationes de la red de alginato desintegrando así la estructura del gel. El Ca^{2+} también puede ser intercambiado por otros cationes por lo que también deberá ser controlada la presencia de cationes que no induzcan la formación del gel, como son los cationes Na^{+} y Mg^{2+} (Mørch & Skjåk-Brik, 1990 y Park & Khang, 1995). Otro de los principales problemas de la inmovilización celular con alginato es la fuga de los microorganismos del interior de los geles hasta el exterior. Además, el crecimiento celular dentro de los geles de alginato puede afectar a sus propiedades mecánicas, ya sea por el crecimiento en sí, o por la formación y acumulación de gases (Martins et al., 1997 y Yu & Zhang, 2007).

Debido a estos inconvenientes el alginato se ha utilizado conjuntamente con otros materiales que permitan aunar las bondades de cada uno de ellos por separado. Esto se ve favorecido por el hecho de ser un polielectrolito, con capacidad de interaccionar con otras moléculas con carga positiva, lo que permite formar sistemas mixtos que muestran mejoras en las propiedades estructurales del gel de alginato dando lugar a los materiales híbridos (Coradin et al., 2003; Perullini, 2009; Ramachandran et al., 2009 y Lupo et al., 2012).

1.5.4.2. Atrapamiento en compuestos inorgánicos. Gel de sílice

Algunos compuestos inorgánicos poseen la capacidad de formar geles. Aunque se han planteado diversas opciones como son los geles de óxido de aluminio (Amoura et al., 2007) y de titanio basados en la química sol-gel (Kessler et al., 2008), es el gel de sílice el material inorgánico más utilizado para la inmovilización de células (Kuncová & Trogl, 2010).

El gel de sílice, además de ser químicamente inerte, muestra algunas propiedades muy ventajosas para la inmovilización de células como son: (i) su biocompatibilidad y resistencia a los microorganismos (Coiffier et al., 2001) (ii) su precio asequible debido a la abundancia de materias primas, y (iii) una relativa transparencia óptica del gel que lo hace apto para la inmovilización de células vegetales (Nguyen-Ngoc & Tran-Minh, 2007). Además, el proceso sol-gel se desarrolla a temperatura ambiente en unas condiciones compatibles con la actividad biológica. El gel de sílice formado mediante el proceso sol-gel tiene una estructura porosa de red tridimensional constituida por SiO_2 (Sakai et al., 2001) que permite la difusión de nutrientes, propiedad esencial para la encapsulación de células. Finalmente, el gel de sílice presenta otras ventajas en comparación con los polímeros orgánicos, entre las que destacan una elevada resistencia mecánica y un bajo grado de hinchamiento (Coiffier et al., 2001).

Tradicionalmente la síntesis del gel de sílice se ha efectuado mediante la ruta de los alcóxidos basada en el empleo de alcóxidos de silicio. Sin embargo para evitar la posible toxicidad celular de sus subproductos se ha desarrollado una ruta acuosa alternativa (Coiffier et al., 2001). A continuación se detallan ambas rutas metabólicas.

1.5.4.2.1. Ruta de los alcóxidos

La ruta de los alcóxidos se basa en el empleo de alcóxidos de silicio ($Si(OR)_4$), como precursores de sílice, donde R pueden ser diferentes grupos orgánicos ($-CH_3$, $-C_2H_5$, etc.). Como precursores también pueden utilizarse derivados de los alcóxidos ($R'-Si(OR)_3$), donde R' puede ser un amplio rango de grupos (por ejemplo $-NH_2$, -Cl, -SH, etc.) que permiten la incorporación de grupos funcionales a la matriz mineral. Estos compuestos sufren una hidrólisis en contacto con el agua liberándose una molécula de alcohol (ROH) y creándose un grupo silanol (Si-OH) (Figura 5), que mediante policondensación puede unirse a otros grupos silanoles rindiendo sílice (SiO_2). Con el tiempo, se producen nanopartículas coloidales, debido a la policondensación, que se enlazan unas con otras para formar una estructura tridimensional que se extiende por el medio líquido, dando lugar al gel (Coradin et al., 2003). La reacción de hidrólisis puede ser catalizada por ácidos minerales, ácido acético, KOH, aminas, KF, HF, alcóxidos de titanio y vanadio (Brinker & Scherer, 1990).

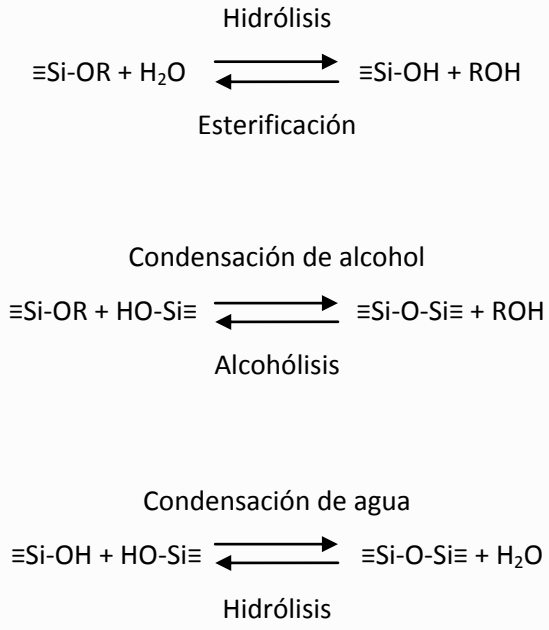


Figura 5. Reacciones de la ruta de los alcóxidos (extraído de Brinker & Scherer, 1990).

Los alcóxidos más empleados en esta ruta son el tetrametoxisilano (TMOS, $Si(OCH_3)_4$) y el tetraetoxisilano (TEOS, $Si(OC_2H_5)_4$) (Brinker & Scherer, 1990). Los alcóxidos son insolubles en agua, por lo que deben usarse solventes orgánicos que pueden alterar la viabilidad celular en los procesos de inmovilización (Brinker & Scherer, 1990 y Shchipunov et al., 2004). Para solventar este inconveniente se han empleado poliolsilicatos cuya solubilidad en agua es mayor. Se ha descrito que el reactivo tetrakis-(2-hidroxietil)-ortosilicato un precursor totalmente soluble en agua, puede formar materiales monolíticos en soluciones acuosas sin la presencia de alcohol y sustituir a los reactivos TMOS y o TEOS (Gill & Ballesteros, 1998 y Meyer et al., 2002). Además, con el objetivo de evitar la presencia de los alcoholes producidos en la fase de hidrólisis, que pueden presentar toxicidad para las células, se ha desarrollado con éxito un método para su eliminación del medio mediante una evaporación controlada a vacío (Nassif et al., 2002 y Ferrer et al., 2003).

1.5.4.2.2. Ruta acuosa

Para solventar los inconvenientes de la ruta de los alcóxidos, se ha desarrollado una ruta acuosa en la que se trabaja a pH neutro y a temperatura ambiente, y no se genera alcohol como subproducto. En esta ruta alternativa se emplea una solución de silicato de sodio como precursor que se gelifica a pH neutro en presencia de las células que se pretenden inmovilizar (Bhatia et al., 2000). Para reducir la concentración de iones Na^+ y/o silicatos y mejorar la

biocompatibilidad de la matriz, puede sustituirse parcialmente el silicato de sodio por nanopartículas de sílice (Coiffier et al., 2001).

Las disoluciones con concentraciones elevadas de silicato de sodio contienen una mezcla de silicatos oligoméricos cargados negativamente. Al diluirse estas soluciones se produce una ruptura de los oligómeros liberándose especies monomoleculares de $[SiO_4H_{4-x}]^{x-}$, donde X aumenta con el pH a partir del punto cero de carga (pH de 3). De este modo dentro del intervalo de pH de 4-9, la especie que predomina es $Si(OH)_4$, predominando la especie $[SiO(OH)_3]^-$ a pH 10. Sin embargo el ácido silícico solo se encuentra en disoluciones muy diluidas ya que en concentraciones elevadas se produce una polimerización mediante reacciones de oxolación (formación de puentes oxígeno) que conducen a la condensación de los grupos silanol (Si-OH) entre las especies cargadas negativamente y las especies neutras del ácido silícico (Figura 6). Estas reacciones son muy dependientes del pH encontrándose el ratio mínimo en las proximidades del punto cero de carga (pH ≈ 3) e incrementando con el pH de forma que a pH 6 es dos veces más rápida que a pH 4 (Coradin & Livage, 2001 y Coradin et al., 2002).

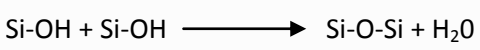


Figura 6. Condensación de grupos silanoles (extraído de Coradin & Livage, 2001).

Tanto en la ruta de los alcóxidos como en la ruta acuosa el resultado es un hidrogel que contiene una fase líquida intersticial y nano- y/o micropartículas con un tamaño aproximado de 2-100 nm formada por silicatos y siloxanos hidrofílicos, y de 0,01–20 nm por siloxanos hidrofóbicos (Gill & Ballesteros, 2000). El secado de los hidrogeles conlleva una reducción de su porosidad, bioactividad y de la capacidad de difusión (Kuncová & Trogl, 2010). Si bien, el tamaño de las partículas de sílice depende del método de síntesis empleado (Hwang & Gu, 2013), el sílice, los silicatos y los hidrogeles de siloxanos hidrófilos presentan volúmenes de poro de 0,9–3,4 ml/g, tamaños de poro de 4–130 nm y áreas superficiales de 700–2100 m^2/g. Durante la fase de envejecimiento del gel, se producen fenómenos de sinéresis y contracción, de forma que los geles envejecidos de sílice muestran volúmenes de poro de 0,6–3,1 ml/g, tamaños de poro de 3–100 nm, áreas superficiales de 600–1700 m^2/g y sinéresis del 5–20% (v/v). Estos hidrogeles envejecidos se pueden someter a un secado controlado, obteniendo xerogeles de sílice y silicatos, con volúmenes de poro de 0,4–2,7 ml/g, tamaños de

poro de 0,5–20 nm, áreas superficiales de 400–1300 m^2/g y sinéresis del 15–85% (Gill & Ballesteros, 2000).

Por tanto, puede decirse que los xerogeles de TMOS son sólidos microporosos con una baja superficie externa mientras que los xerogeles de silicato de sodio y silicato coloidal se consideran mesoporosos (Coiffier et al., 2001).

1.5.4.3. Geles híbridos basados en sílice

Existe un creciente interés en el desarrollo de geles híbridos basados en sílice con compuestos orgánicos, principalmente polisacáridos (celulosa y sus derivados, carragenatos, alginato, etc.) y proteínas (lisozima, colágeno, albúmina, etc.), con el objetivo de combinar las propiedades beneficiosas de ambos tipos de compuestos (Livage & Coradin, 2006). El componente orgánico más estudiado en combinación con las matrices de sílice es el alginato debido a las ventajas que presenta este compuesto para inmovilizar células (Coradin & Livage, 2003). Mediante el proceso sol-gel es posible obtener materiales híbridos de alginato-sílice con propiedades mejoradas respecto del gel de alginato inicial (Pannier et al., 2014). De esta forma, la combinación de ambos materiales permite aunar en una misma matriz la resistencia mecánica y la estabilidad química de los geles de sílice con las propiedades químicas, físicas y biológicas del alginato (Coradin et al., 2003 y Ramachandran et al., 2009).

Por otra parte, se ha demostrado que la presencia del alginato puede actuar como catalizador en el proceso de polimerización del gel de sílice, acelerando por ejemplo la hidrólisis del precursor TMOS o interactuando con los grupos silanol facilitando la nucleación y la formación de una capa de sílice alrededor de la matriz de alginato (Xu et al., 2006). Al hablar de compuestos híbridos de alginato-sílice hay que tener en cuenta que a pH neutro tanto el alginato como el sílice se encuentran cargados negativamente por lo que no se espera una interacción electrostática. Sin embargo, sí pueden formarse otras uniones débiles como puentes de hidrógeno entre la cadena del polisacárido y los grupos silanol (Coradin, 2011). Pese a ello, ambos materiales pueden ser combinados de múltiples formas y a diferentes pH sin que se produzca precipitación y/o separación de las fases, pudiéndose además añadir especies catiónicas que contribuyan a fortalecer esta unión, como la poli-lisina y la poli-arginina (Tam et al., 2011). Un enfoque novedoso e interesante podría ser por tanto la posibilidad de introducir enlaces covalentes entre ambos compuestos que incrementasen la fuerza de la interacción entre el alginato y el sílice (Simó et al., 2017).

En 2006 Carturan et al. diseñaron un método para producir matrices de alginato de calcio usando un eyector de boquilla bajo flujo constante de helio, obteniendo esferas de un

diámetro de 120-140 µm, dependiendo el volumen de la carga de alginato inicial. Posteriormente se añadieron alcóxidos de silicio gaseosos $Si(OEt)_4$ y $MeSi(OEt)_3$ transportados por un flujo de helio. A medida que los alcóxidos se evaporaban, las partículas de sílice se depositaron de manera constante sobre las esferas de alginato generando de esta manera etanol, producido en la formación de gel de sílice. Se calculó que la capa silícea obtenida tenía un grosor que oscilaba entre 0,08 y 0,17 µm (Carturan et al., 2006).

Recientemente varios han sido los experimentos que han estudiado la inmovilización celular empleando geles mixtos de alginato-sílice (Tabla 1). En este sentido Bolyó et al. (2010) con el objetivo de profundizar en el conocimiento sobre las interacciones entre los materiales de fijación y las células y su posible aplicación para el desarrollo de biosensores, inmovilizaron *Saccharomyces carlsbergensis* en gel de sílice (TMOS) o una mezcla de TMOS y alginato. En este campo varios han sido los experimentos realizados, así Ferro et al. (2012) diseñaron un nuevo biosensor para la evaluación de la calidad del medio acuático, inmovilizando para tal fin en hidrogeles de sílice y alginato tres microalgas para su utilización como bioindicadores de toxicidad. Este mismo grupo de investigación ha seguido estudiando el empleo de células de algas inmovilizadas para su uso como biosensores obteniendo resultados satisfactorios (Perullini et al., 2014; y Durrieu et al., 2016). Pannier et al., (2014) inmovilizaron la microalga verde *Chlorella vulgaris* en hidrogeles de alginato reforzados con sílice para demostrar el potencial de la matriz híbrida para el diseño de sistemas de detección basados en células.

Con fines médicos Dandoy et al. (2011) inmovilizaron células humanas dentro de perlas híbridas.

La inmovilización de células en geles de alginato-sílice también se ha utilizado para la eliminación fisicoquímica de contaminantes, Ibrahim et al. (2013) inmovilizaron *Amphibacillus* KSUCr3 con este propósito. Shima et al. (2014) inmovilizaron *Pseudomonas putida YNS* con el objetivo de eliminar fenol y metales pesados del agua contaminada comprobando que las perlas con sílice fueron las más efectivas para la eliminación de Cu y Cd. En este área, Vahidimehr et al. (2020) inmovilizaron paredes celulares de *S. cerevisiae* y *Lactobacillus rhamnosus* en una matriz de sílice posteriormente recubierta de alginato para la eliminación de la aflatoxina M1. Con el mismo objetivo se inmovilizaron paredes celulares de *S. cerevisiae* y *Candida albicans* obteniendo resultados satisfactorios en la eliminación de la toxina y en la reutilización de la matriz (Nejad et al., 2020).

Evstatieva et al. (2014) atraparon micelio y pellets de la cepa *Aspergillus oryzae* PP en matrices híbridas con el fin de aumentar la producción de α-amilasa. Con el objetivo de medir la actividad enzimática y la capacidad de degradación del bisfenol A se inmovilizaron con éxito células enteras del hongo comestible *Agaricus bisporus*, sin necesidad de purificación

enzimática. La adición al alginato de sílice coloidal mejoró la actividad debido a la retención tanto de las células como de la tirosinasa de las células fracturadas, mientras que la actividad observada fue similar a la observada con la tirosinasa aislada inmovilizada en el mismo material (Kampmann et al., 2014; 2015 y 2016).

Otro grupo de investigación propuso un método simple y efectivo para inmovilizar protoplastos en geles de alginato-sílice para su utilización en procesos biológicos celulares como la secreción y el metabolismo (Lei et al., 2015). Para la producción de hidrógeno, Zhu et al. (2018) investigaron el efecto de un recubrimiento de hidrogel de sílice sobre perlas de alginato observando que el recubrimiento redujo la tasa de escape de biomasa. Y Patel et al. (2018) evaluaron cocultivos inmovilizados en alginato al 2% y gel de sílice de los microorganismos metanótrofos *Methylocella tundrae*, *Methyloferula stellata* y *Methylomonas methanica* para mejorar la producción de metanol con su aplicación.

Tabla 1. Publicaciones de inmovilización de células en geles de alginato-sílice (de 2010 a 2020).

CÉLULAS	FINALIDAD	AUTORES
Saccharomyces cerevisiae* y *Lactobacillus rhamnosus	Eliminación de la aflatoxina M1	(Vahidimehr et al., 2020)
Saccharomyces cerevisiae* y *Candida albicans	Eliminación de la aflatoxina M1	(Nejad Khiavi et al.,2020)
Biomasa productora de hidrógeno	Producción de hidrógeno	(Zhu et al., 2018)
Methylocella tundrae*, *Methyloferula stellata* y *Methylomonas	Producción de metanol	(Patel et al., 2018)
Células de algas	Biosensores basados en la medición de la actividad del fotosistema II para la detección de sustancias químicas en aguas pluviales urbanas	(Durrieu et al., 2016)
Agaricus bisporus	Síntesis de ácido 4-dihidroxifenilacético	(Kampmann et al., 2016)
Agaricus bisporus	Medida de la actividad enzimática y degradación de bisfenol A	(Kampmann et al., 2015)
Protoplastos del mesófilo del tabaco	Estudio potencial de procesos biológicos celulares como la secreción y el metabolismo	(Lei et al., 2015)
Chlorella vulgaris, Pseudokirchneriella subcapitata y Chlamydomonas reinhardtii	Protección de los organismos encapsulados de la radiación UV permitiendo la esterilización de dispositivos de encapsulación sin afectar la viabilidad de las células de microalgas atrapadas	(M Perullini et al., 2014)
***Aspergillus oryzae* PP**	Mejora de la actividad alfa-amilasa de *Aspergillus oryzae PP*	(Evstatieva et al., 2014)
Agaricus bisporus	Degradación de bisfenol A	(Kampmann et al., 2014)
Chlorella vulgaris, Pseudokirchneriella subcapitata y Chlamydomonas reinhardtii	Desarrollo de biosensores	(Perullini et al., 2014)
Pseudomonas putida YNS	Eliminación fisicoquímica de fenol y metales pesados	(Shim et al., 2014)
Chlorella vulgaris	Sistemas de detección	(Pannier et al., 2014)
***Amphibacillus* KSUCr3**	Reducción de Cr (VI) mediante células magnéticas inmovilizadas	(Ibrahim et al., 2013)
Chlorella vulgaris, Pseudokirchneriella subcapitata y Chlamydomonas reinhardtii	Desarrollo de biosensores	(Ferro et al., 2012)
Oenococcus oeni	Desarrollo de FML en vino	(Guzzon et al., 2012)
Células humanas modelo HepG2	Trasplante de células	(Dandoy et al., 2011)
Saccharomyces carlsbergensis	Desarrollo de biosensores	(Bolyó et al., 2010)

1.6. Fermentación maloláctica mediante bacterias acidolácticas inmovilizadas

El empleo de BAL seleccionadas inmovilizadas para realizar la FML presenta diversas ventajas respecto al uso de BAL libres, entre las que se pueden destacar: (i) la protección de las BAL contra las condiciones adversas del medio donde tiene lugar la FML, (ii) la posibilidad de reutilizar las bacterias inmovilizadas en sucesivas fermentaciones, (iii) la oportunidad de diseñar procesos fermentativos en continuo, (iv) la capacidad para reducir el tiempo necesario para iniciar la FML y mejorar su productividad al emplearse poblaciones de bacteria elevadas, (v) garantizar que las bacterias seleccionadas para tal efecto conduzcan la FML y (vi) simplificar la separación de las BAL inmovilizadas del vino por un simple proceso de filtración lo que facilita al enólogo decidir la cantidad de ácido málico residual presente en el vino (Maicas, 2001; Kourkoutas et al., 2004 y Vila-Crespo et al., 2010).

Desde que comenzó la investigación en este campo, numerosos han sido los trabajos en los que se han estudiado los efectos del uso de BAL inmovilizadas para conducir la FML, realizándose la inmovilización celular ya sea con polímeros orgánicos naturales como el alginato (Spettoli et al., 1982), el pectato y el quitosano (Kosseva et al., 1998 y Kosseva & Kennedy, 2004), o polímeros orgánicos de síntesis como el gel de polivinil alcohol (Rodríguez-Nogales et al., 2013). También se han empleado materiales orgánicos como la esponja (Maicas et al., 2001), productos celulósicos deslignificados (Agouridis et al., 2005 y Agouridis et al., 2008), así como mazorcas de maíz y pieles y tallos de uva (Genisheva et al., 2013) con resultados satisfactorios.

Diversos grupos de investigación han planteado la posibilidad de inmovilizar simultáneamente levaduras y BAL para realizar simultáneamente la FA y la FML. Así Nedovic et al., (2000) coinmovilizaron *Saccharomyces bayanus* y *Leuconostoc oenos* en matrices de alginato de calcio para la producción de sidra. Se ha inmovilizado *S. cerevisiae* y *Lactobacillus delbrueckii* en esferas de alginato obteniendo vinos con una evaluación sensorial positiva (Aaron et al., 2004). También *S. cerevisiae* se ha coinmovilizado con *O. oeni* en diferentes capas de una matriz de gel de celulosa/almidón para realizar simultáneamente la FA y la FML (Servetas et al., 2013). Genisheva et al., (2014) realizaron FA y FML secuenciales operadas de forma continua mediante *S. cerevisiae* y *O. oeni* inmovilizados en tallos y pieles de uva. Recientemente, Bleve et al. (2016) propusieron la inmovilización simultánea de *S. cerevisiae* y *O. oeni* en esferas de alginato para producir vino Negroamaro.

Menos son los experimentos realizados con inmovilización en matrices híbridas de alginato-sílice. Callone et al. (2008) coinmovilizaron *S. cerevisiae* y *O. oeni* en una matriz

híbrida multicapa de alginato-sílice, confirmando la efectividad del atrapamiento en términos de cantidad de biomasa inmovilizada, mantenimiento de la viabilidad celular y reducción de fugas. Posteriormente el mismo grupo de investigación realizó vinificaciones con células de *O. oeni* atrapadas en microperlas de alginato de calcio, recubiertas con una membrana de sílice obtenidas a partir de TMOS o TEOS en fase gaseosa. Se estudiaron las propiedades de los geles resultantes observando una mejora en sus características físico-químicas y no encontraron diferencias en la composición química de los vinos obtenidos, facilitándose además la FA y la FML simultáneas y la realización de tres FML secuenciales consecutivas empleando la misma biomasa (Guzzon et al., 2012).

En base a estos resultados, la inmovilización de *O. oeni* en matrices híbridas de alginato-sílice podría ser un recurso prometedor para garantizar el crecimiento de las BAL y el adecuado desarrollo de la FML bajo las actuales condiciones enológicas, regidas por el cambio climático y por las recientes prácticas vitivinícolas. Falta todavía mucho conocimiento por adquirir en este campo y sería por tanto muy interesante profundizar en los efectos de la inmovilización en la calidad del vino y en el metabolismo de las BAL.

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OBJETIVOS

2. OBJETIVOS

El principal objetivo de esta tesis fue desarrollar un método eficaz para inmovilizar *Oenococcus oeni* en una matriz híbrida de sílice-alginato con el fin de desarrollar de manera eficiente la fermentación maloláctica (FML) en vinos tintos.

Para lograr el objetivo principal, también se establecieron varios objetivos específicos:

1. Analizar los avances en las técnicas de encapsulación de células con geles de alginato de calcio.

2. Optimizar las condiciones de inmovilización bacteriana en geles mixtos de sílice-alginato y estudiar su estabilidad mecánica, química y operacional.

3. Estudiar el desarrollo de la FML con bacterias inmovilizadas bajo distintas condiciones enológicas.

4. Analizar el efecto del tipo de inóculo y el momento de inoculación en el desarrollo de la FML.

5. Evaluar la composición físico-química y sensorial de los vinos elaborados con bacterias encapsuladas.

ESTRUCTURA

3. ESTRUCTURA

La presente tesis doctoral está dividida en cuatro capítulos en base a las publicaciones científicas obtenidas a partir de la investigación llevada a cabo durante la tesis.

Capítulo 1: Avances en técnicas de encapsulación celular con alginato

- Simó, G.; Fernández-Fernández, E.; Vila-Crespo, J.; Ruipérez V., Rodríguez-Nogales, J.M. (2017). Research progress in coating techniques of alginate gel polymer for cell encapsulation. *Carbohydrate Polymers*, 170, 1-14.

  http://dx.doi.org/10.1016/j.carbpol.2017.04.013

Capítulo 2: Optimización de las condiciones de inmovilización de *Oenococcus oeni*

- Simó, G.; Vila-Crespo, J.; Fernández-Fernández, E.; Ruipérez, V.; Rodríguez-Nogales, J.M. (2017). Highly efficient malolactic fermentation of red wine using encapsulated bacteria in a robust biocomposite of silica-alginate. *Journal of Agricultural and Food Chemistry*, 65, 5188-5197.

  http://dx.doi.org/10.1021/acs.jafc.7b01210

Capítulo 3: Caracterización de la fermentación maloláctica llevada a cabo por *O. oeni* inmovilizada

- Simó, G.; Fernández-Fernández, E.; Vila-Crespo, J.; Ruipérez, V.; Rodríguez-Nogales, J.M. (2017). Silica-alginate encapsulated bacteria to enhance malolactic fermentation performance in stressful environment. *Australian Journal of Grape and Wine Research*, 23, 342-349.

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- Simó, G.; Fernández-Fernández, E.; Vila-Crespo, J.; Ruipérez, V.; Rodríguez-Nogales, J.M. (2019). Effect of stressful malolactic fermentation conditions on the operational and chemical stability of silica-alginate encapsulated *Oenococcus oeni. Food Chemistry*, 276, 643-651.

  https://doi.org/10.1016/j.foodchem.2018.10.025

Capítulo 4: Influencia del sistema de inoculación en el desarrollo de la fermentación maloláctica

- Simó, G.; Fernández-Fernández, E.; Vila-Crespo, J.; Ruipérez, V.; Rodríguez-Nogales, J.M. (2019). Malolactic fermentation induced by silica-alginate encapsulated *Oenococcus oeni* with different inoculation regimes. *Australian Journal of Grape and Wine Research*, 25, 165–172.

  http://dx.doi.org/10.1111/ajgw.12377

- Rodríguez-Nogales, J.M.; Simó, G.; Pérez-Magariño, S.; Cano-Mozo, E.; Fernández-Fernández, E.; Ruipérez, V.; Vila-Crespo, J. (2020). Evaluating the influence of simultaneous inoculation of SiO_2-alginate encapsulated bacteria and yeasts on volatiles, amino acids, biogenic amines and sensory profile of red wine with lysozyme addition. *Food Chemistry*, 327, 1-10.

  https://doi.org/10.1016/j.foodchem.2020.126920

CAPÍTULO 1

4. CAPÍTULO 1: Avances en técnicas de encapsulación celular con alginato

- **4.1. Research progress in coating techniques of alginate gel polymer for cell encapsulation**

Carbohydrate Polymers 170 (2017) 1–14

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Research progress in coating techniques of alginate gel polymer for cell encapsulation

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ABSTRACT

Cell encapsulation is used as a biotechnology tool to solve the technological problems derived from handling and application of cells in a great range of fields. This involves immobilization of the cells within a polymeric gel that permits the preservation of their metabolic activity. Alginate is widely established as the most suitable polymer for cell encapsulation. However, alginate gel capsules suffer several disadvantages because of their lack of mechanical and chemical stability. This review summarizes results of recent advances in coating techniques that include ionic and covalent cross-linking between alginate and coating materials for cell encapsulation as a strategy to solve the disadvantages mentioned before. Throughout this review, physicochemical properties of coated-alginate capsules and the effect of coating process on metabolic activity and viability of immobilized cells have been specially discussed.

Contents

1. Introduction

Cell immobilization is a biotechnology technique used and studied in a wide range of fields such as food production, drug development, environment protection, waste processing and agricultural production. An upsurge of interest in cell immobilization in these fields has arisen recently due to the advantages over the use of free cells. Some of these advantages include protection of cells against harsh environment, easy separation of cells from the reaction medium, possibility of cell reutilization in various batches, feasibility of continuous processing and high productivities due to the possibility of increasing cell concentration per unit of bioreactor volume (Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004). Several techniques have been proposed for cell immobilization such as adsorption of cells on a carrier surface, encapsulation into polymers, self-aggregation by flocculation or

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with cross-linking agents, and finally, cell containment behind barriers (Gallo, Speranza, Corbo, Sinigaglia, & Bevilacqua, 2016). Encapsulation into polymers is the most commonly used cell immobilization technology because of its simplicity and mild working conditions (Dulieu, Poncelet, & Neufeld, 1999).

The most broadly applied and studied polymer for cell encapsulation is alginate (De Vos, Lazarjani, Poncelet, & Faas, 2014). Alginate is an unbranched heteropolysaccharide of 1–4 glycosidically linked β-D-mannuronic (M) and α-L-guluronic (G) acids in varying composition and sequences (Yi, Neufeld, & Poncelet, 2004, Chapter 39) (Fig. 1a). In the presence of divalent cations, such as Ca^{2+} and Ba^{2+}, aqueous solutions of sodium alginate form gels. Gelation takes place through ionic cross-linking of negatively charged carboxyl groups of the alginate chain and divalent metal ions with opposite charges (Gulrez, Al-Assaf, & Phillips, 2003). The binding zone between four G residues of two different chains and a cation is often described as an "egg-box model" (Grant, Morris, Rees, Smith, & Thom, 1973) (Fig. 1b). The distribution and proportions of monomers M and G along the alginate chain determine mechanical, swelling and diffusion properties of the hydrogel (Melvik & Dornish, 2004).

Alginate gels are an appropriate support for immobilized cells since gel formation process is accomplished under mild conditions using biocompatible reagents and the process takes place at room temperature (Yi et al., 2004, Chapter 39). Moreover, entrapment of enzymes and cells into the small pores of alginate gel network is possible, allowing the diffusion of metabolic molecules through the pores.

However, this hydrogel has disadvantages that make its application difficult in some occasions. For example, chelating agents (such as citrate, phosphate, and lactate) or anti-gelling cations (Na^{+} and Mg^{2+}) frequently presented in biological and food applications, can cause a reduction in alginate gel mechanical stability or even the complete liquefaction of the gel (Ching, Bansal, & Bhandari, 2015). More stable alginate gels can be easily formed using either Ba^{2+}, Pb^{2+}, Sr^{2+}, or Cu^{2+}, which have a higher affinity with alginate carboxyl groups. However, these gelling cations are not actually used for cell immobilization because of their potential toxic effect on immobilized cells (Yi et al., 2004, Chapter 39). One of the main problems in cell immobilization applications is the cell leakage from carriers, which results into failure of the immobilization technology. Moreover, the growth of the cells within the alginate gels can affect their mechanical properties, either by the growth itself or by gas formation and accumulation (Martins Dos Santos et al., 1997; Yu, Zhang, & Tan, 2007).

Therefore, coating techniques are under active investigation in order to improve the properties of alginate gel as carrier for cell encapsulation. These techniques have been successfully performed to enhance mechanical and chemical stability of alginate gel and to reduce the swelling behaviour of capsules. In addition, control or/and reduction of cell release and improvement of the viability of encapsulated cells under stress conditions have been studied (Ching et al., 2015; Dulieu et al., 1999; Loh et al., 2012; Melvik & Dornish, 2004; Strand, Skjåk-Bræk, & Gåserød, 2004; Yi et al., 2004, Chapter 39). These coating techniques involve either ionic or covalent interactions between the surface of alginate gel and the coating materials. On one hand, ionic cross-linking coating of the surface of the alginate capsules can be performed because of the alginate ability to form strong electrostatic complexes with polycations (Melvik & Dornish, 2004). These complexes do not dissolve in the presence of Ca^{2+} chelators nor with anti-gelling cations. On the other hand, covalent cross-linking coating includes direct cross-linking of reactive groups of alginate with reagents, photocross-linking (Hertzberg, Kvittingen, Anthonsen, & Skjakbraek, 1992) and silica coating.

Numerous reviews focused on cell encapsulation using alginate gels have been published in the last few years, supporting the relevance and interest of this technology. Table 1 summarizes the most recent reviews and monographs about this topic in chronological order. Some authors have made a general review about cell encapsulation using natural or synthetic matrixes, including alginate (Bhatia, Khattak, & Roberts, 2005; De Vos et al., 2014; Djordjević, Willaert, Gibson, & Nedović, 2016; Gasperini, Mano, & Reis, 2014; Rathore, Desai, Liew, Chan, & Lieng, 2013; Riaz & Masud, 2013; Stolarzewicz et al., 2011; Yi et al., 2004, Chapter 39). More specific reviews have focused on production techniques, physicochemical properties (Ching et al., 2015) and biomedical applications of alginate-based capsules (Lee & Mooney, 2012) (Melvik & Dornish, 2004). However, less attention has been given to the analysis and discussion of strategies to sort out the problems of lack of physicochemical and mechanical stability of alginate capsules and the effect on viability and metabolic activity of the entrapment of the cells. Hence, this review focuses on recent advances of the coating techniques as a strategy to improve physicochemical properties of alginate capsules. An especial emphasis is given to the effect of these techniques on metabolic activity, viability and storage stability of immobilized cells, especially for microbial cells. In the first part of this review, ionic cross-linking coating techniques are reported. In the second part, recent advances in covalent cross-linking coating are described.

2. Ionic cross-linking coating

The formation of an outer layer of polycations on the negatively charged surface of the alginate gel capsule can be realized through ionic interaction between carboxylic groups of alginate and amino terminals of polycations (Ching et al., 2015). This easy procedure can be repeated including a second layer using a polyanion (double-membrane) or consecutively inserting a set of layers of polyanions and polycations (multi-layered membrane). In this way, the opposite charged polyelectrolyte serves as the counter-ion to form the new layer (Schneider et al., 2001). The main advantage of the stepwise coating is that it allows the designing of different layers with separate definition of mechanical and chemical stability, permeability and biocompatibility (Gaumann et al., 2000). Stability of electrostatic complexes can be affected by various factors related to the particular properties (e.g. type and density of reactive groups, molecular weight, chain rigidity, and aromaticity), the concentration of charged polymers and the reaction conditions (e.g. pH, ionic strength and reaction time). Moreover, secondary interactions such as hydrogen bonding, Van der Waals forces, as well as charge transfer and hydrophobic interactions also participate in the robustness of complexes (Prokop, Hunkeler, DiMari, Haralson, & Wang, 1998). The main polycations used to form complexes with alginate are poly-aminoacids (e.g. poly-L-lysine (PLL) and poly-ornithine (PLO)), natural polymers (e.g. chitosan) and synthetic polymers (e.g. polyethyleimine, poly-methylen-*co*-guadine (PMCG), poly(allylamine (PAA), and poly(vinylamine) (PVA))). Table 2 summarizes some examples of immobilized cells into alginate capsules with the ionic cross-linking coating method.

2.1. Poly-aminoacids

The process of coating is relatively simple and the gelation conditions are mild. PLL has been the most commonly used material for coating macro and microcapsules based on alginate gel (Fig. 2) (Melvik & Dornish, 2004; Strand et al., 2004). The alginate-PLL interaction has been extensively studied in the last two decades (Ma et al., 2012; Strand et al., 2004; Thu et al., 1996). On one hand, stability of coating depends on the chain length of PLL. It has been

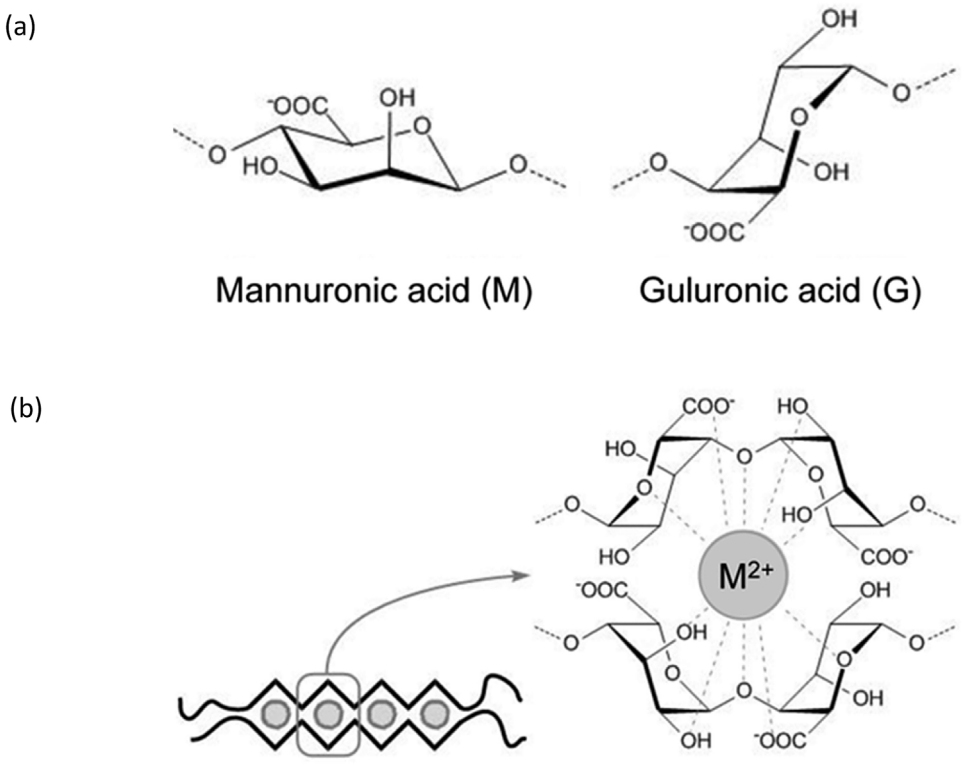

Fig. 1. (a) Chemical structure of alginate monomers: β-D-mannuronic acid (left) and α-L-guluronic acid (right). (b) Interaction between divalent cation and G monomers of alginate in the "egg-box model" (Pistone, Qoragllu, Smistad, & Hiorth, 2015).

Table 1
Recent review papers and monographs related to cell encapsulation into alginate gels.

Title	Year	Reference
Silica–alginate composites for microencapsulation	2003	Coradin et al. (2003)
Alginate as a carrier for cell immobilization	2004	Melvik & Dornish (2004)
Immobilization of cells in polysaccharide gels	2004	Yi et al. (2004), Chapter 39
Polyelectrolytes for cell encapsulation	2005	Bhatia et al. (2005)
Immobilization of yeast on polymeric supports	2011	Stolarzewicz et al. (2011)
Alginate: properties and biomedical applications	2012	Lee & Mooney (2012)
Recent trends and applications of encapsulating materials for probiotic stability	2013	Riaz & Masud (2013)
Microencapsulation of microbial cells	2013	Rathore et al. (2013)
Polymers in cell encapsulation from an enveloped cell perspective	2014	De Vos et al. (2014)
Natural polymers for the microencapsulation of cells	2014	Gasperini et al. (2014)
Alginate gel particles- a review of production techniques and physical properties	2015	Ching et al. (2015)
Immobilized yeast cells and secondary metabolites	2016	Djordjević et al. (2016)

reported that the interaction between alginate and PLL is more effective by reducing the molecular weight of PLL from 55 to 18 kDa. On the other hand, binding is also influenced by the ratio of M and G residues. Alginates with high M content are preferred to increase their affinity with PLL. Binding of PLL can be enhanced by increasing the charge density of the alginate gel surface using inhomogeneous gels. Furthermore, an increase in the strength of PLL-coated alginate capsules is achieved by forming a second layer of alginate to neutralize any excess of PLL on the surface (Krasaekoopt, Bhandari, & Deeth, 2004). An increase of PLL concentration, reaction time and volume ratio of PLL solution in alginate gels resulted in capsules with a thicker membrane, higher mechanical stability and lower swelling degree.

PLO, a polypeptide with a similar structure to PLL, has been explored as an alternative to conventional coating with PLL. PLO-alginate capsules showed enhanced biocompatibility, mechanical stability and hydrophilicity, reduced swelling, less damage under osmotic stress and better diffusion properties than those coated with PLL (Darrabie, Kendall, & Opara, 2005; Loh et al., 2012; Tam et al., 2011).

Other example of coating of alginate capsules with poly-aminoacids is the use of the natural cationic polypeptide protamine. This is an amino acid globular peptide containing a high percentage of cationic arginine (Balhorn, 2007). As protamine exposition (concentration and time) increased, technological properties of capsules decreased significantly (capsule weight, total diameter, and core diameter) due to the shrinkage of alginate matrix during protamine treatment. Coating with protamine also caused an increase of mechanical resistance of capsules and reduction of diffusion of solutes with high molecular weight (Vigo et al., 2004).

Recently, protamine has been employed to design pH-responsive membranes for cell and enzyme immobilization in alginate gels. pH-responsive membrane of capsules was made of a Ca-alginate/protamine composite soft layer and a silica surface rigid layer. Outer silica surface rigid layer was applied to provide a protection to the Ca-alginate/protamine composite soft layer in practical applications. pH-responsive switching function was

Table 2
Cell immobilization into alginate capsules with the ionic cross-linking coating methods.

Coating material	Immobilized cell/molecule	Remarks	References
Alginate/PLL Alginate/PLO	C2C12 myoblast cells	Improved mechanical properties Similar physiological activity of enclosed cells	De Castro, Orive, Hernandez, Gascon, and Pedraz (2005)
Alginate/PLL	*Lactobacillus acidophilus* 547, *Bifidobacterium bifidum* ATCC 1994 and *Lactobacillus casei* 01	Increased thickness of alginate beads Improved protection of cells in the presence of gastric juice	Krasaekoopt et al. (2004)
Alginate/PLO/Alginate	Porcine islet cells	Decreased pore size exclusion limit Improved mechanical properties	Darrabie et al. (2005)
Alginate/PLO/polystyrene sulfonate/ poly(allyamine)	Min-6 cells	Improved mechanical properties No modification of cell metabolic activity	Leung et al. (2009)
Alginate/PLL	*Lactobacillus casei* NCDC 298	No improvement the protection of encapsulated cells from bile salt	Mandal, Hati, Puniya, Khamrui, and Singh (2014)
Alginate/PLL	*Lactobacillus acidophilus* LA5 *Lactobacillus casei* 01	Improved protection of cells in gastric longan juice Improved storage stability	Chaikham, Apichartsrangkoon, George, and Jirarattanarangsri (2013)
Alginate/chitosan	Dairy starter culture	Decreased cell release during fermentation Improved the preservation of strength of the carrier during the storage	Obradovic et al. (2015)
Alginate/chitosan	*Penicillium purpurogenum*	Reduced catalytic activity Improved mechanical strength Improved operation stability and recycling	Cao et al. (2014)
Alginate/chitosan	Acetobacter *sp*. CCTCC M209061	Improved operational stability	Wang et al. (2013)
Alginate/chitosan	Acetobacter *sp*. CCTCC M209061	Improved mechanical strength and swelling-resistance properties Increased resistance for diffusion	Chen et al. (2012)
Alginate/chitosan	*Saccharomyces cerevisiae*	Improved mechanical and chemical stability Reduction cell leakage	Liouni et al. (2008)
Alginate/chitosan	*Saccharomyces cerevisiae*	Improved mechanical and chemical stability No modification of metabolic activity	Li (1996)
Alginate/chitosan	*Streptococcus phocae* PI80	Improved survival during storage	Kanmani et al. (2011)
Alginate/chitosan	*Lactobacillus reuteri* DSM 17938	Improved tolerance of cells towards stress conditions	De Prisco, Maresca, Ongeng, and Mauriello (2015)
Alginate/chitosan	*Lactobacillus plantarum*	Improved cell survival and antibacterial activity	Trabelsi et al. (2014)
Alginate/chitosan	*Lactobacillus bulgaricus*	Improved survival during storage	Koo et al. (2001)
Alginate/PMCG	*Escherichia coli* *Saccharomyces cerevisiae*	No modification of cell growth and metabolic activity	Zhang et al. (2005)
Alginate/protamine	*Lactobacillus casei* CICC 23185	pH responsive membrane for cell protection in stomach	Mei et al. (2014)
Alginate/protamine	Red blood cells	Improved mechanical resistance Reduction of permeability of solute of high molecular weight	Vigo et al. (2004)
Alginate/poly(vinylamine)	IW32 mouse erythroleukemia cells	Improved mechanical resistance Improved cell growth	Wang et al. (1992)

driven by electrostatic interactions between Ca-alginate network and protamine molecules at different pH values (Fig. 3). On one hand, protamine molecules are positively charged when the ambient pH value is higher than a critical pH (close to 4.5, pK_a value of cross-linked alginate hydrogel networks) but lower than pI of protamine (pH values between 10 and 12). So, positive charged protamine molecules are attracted by negatively charged Ca-alginate network in Ca-alginate/protamine composite layer due to electrostatic attraction. Under this pH conditions, diffusion channels of the Ca-alginate network are open, so allowing a high permeability of molecules across the membrane. On the other hand, when the ambient pH value is lower than critical pH of Ca-alginate, the Ca-alginate network does not present any electrical charge. Thus, diffusion channels are closed due to electrostatic repulsion between positively charged protamine molecules and neutral Ca-alginate network. Consequently, there is a reduced cell metabolic activity due to the low permeability of molecules (Mei et al., 2013).

2.2. Chitosan

Chitosan (1,4-linked-2-amino-2-deoxy-β-D-glucan) has been also used as a natural coating agent for alginate capsules. It is a cationic polyelectrolyte soluble in acidic solutions below pH 6.5 due to the presence of amino groups (Liouni, Drichoutis, & Nerantzis, 2008). This material is manufactured by *N*-deacetylation of chitin and leads to the creation of products with different degree of deacetylation and molecular weight. This material is biodegradable, biocompatible and non-toxic, which makes it a suitable product for the coating of alginate capsules.

Binding of chitosan to alginate gel can be described as almost irreversible. Binding between alginate and chitosan is considerably stronger than binding between PLL and alginate (Gåserød, Sannes, & Skjåk-Bræk, 1999). The quantity of chitosan linked to alginate gel is governed by restricted diffusion of chitosan into alginate network. Consequently, factors such as the porosity of alginate gel, the molecular weight of chitosan and the ambient pH may affect the bound amount of chitosan. Gåserød, Smidsrød, & Skjåk-Bræk (1998), Gåserød et al. (1999) studied quantitatively the binding of chitosan to alginate capsules by using radioactive labelled fractions of chitosan. Porosity of the alginate gel increased using homogeneous gels made of high-G alginate and adding high concentrations of calcium ions to pre-formed alginate. Moreover, capsules made by dropping alginate solution into a chitosan solution (one-stage procedure) presented lower amount of chitosan in the capsules than those made by reacting alginate gel in a chitosan solution (two-stage procedure). It was observed that with one-stage procedure, diffusion of chitosan into the capsule core was hindered. It was also reported that high contents of chitosan are obtained with a molecular weight of chitosan of less than 20 kDa and low degree of deacetylation because it shows an increased diffusion coefficient

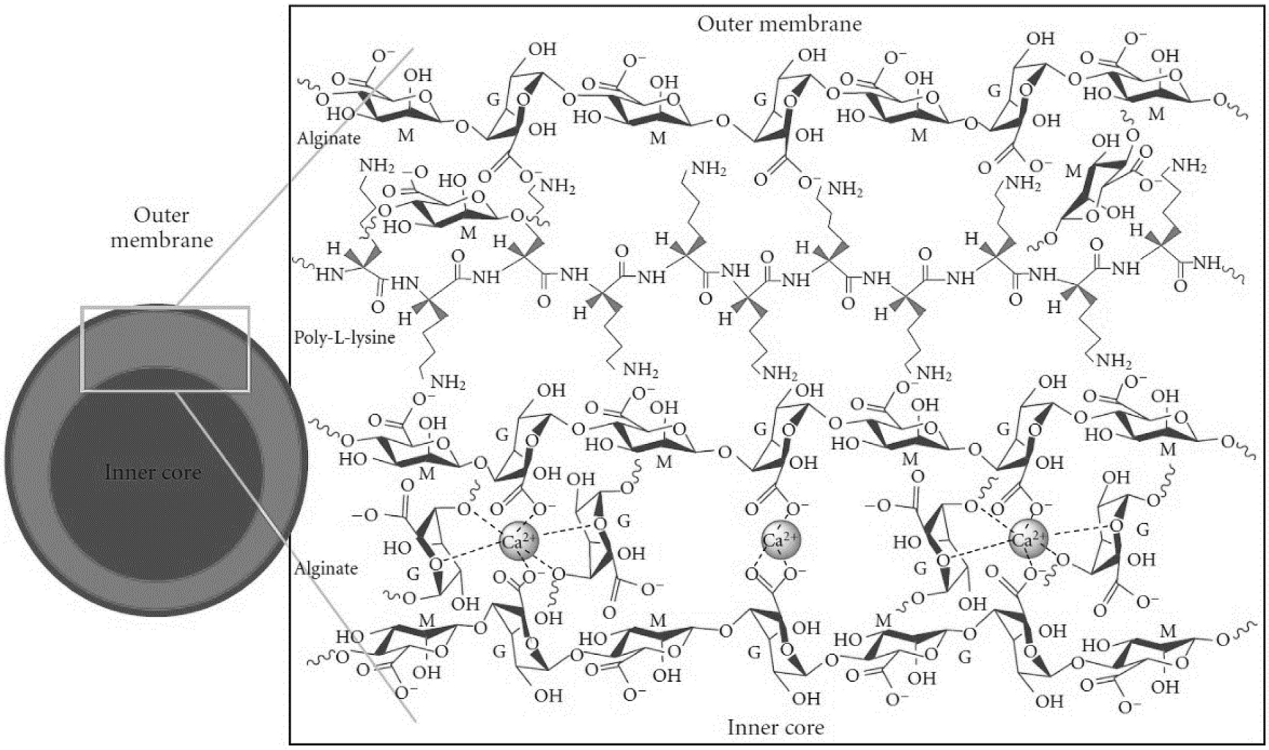

Fig. 2. Scheme of ionic interactions of carboxyl groups of alginate and amino groups of poly-L-lysine (Prakash & Jones, 2005).

through the pores of alginate gel. In addition, reduction of charge density of chitosan (increasing the pH from 4.0 to 6.0) enabled a significant better diffusion of chitosan into the gel network so resulting in an increase of the rate of binding.

Stability of the coated capsules is closely related to the thickness of external membrane of chitosan and therefore, to the amount of chitosan bound to the capsules. It has been reported that factors that increase binding between both polymers (alginate and chitosan) also improve capsule stability (Gåserød et al., 1999). Higher mechanical resistance was found in more homogeneous capsules made by the two-stage method and using chitosan of low molecular weight. Mechanical resistance of the capsules was greater as the chitosan concentration of the coating solution increased. When most of the negatively charged carboxyls on the surface of alginate gel were bound with positively charged amino groups of chitosan, the thickness of the membrane did not grow further, even with higher chitosan concentrations (Cao et al., 2014; Chen et al., 2012; Liouni et al., 2008). The effect of the chitosan solution pH on the mechanical resistance of coated capsules was also studied, showing that an increase of pH from 3.0 to 5.0 notably improved mechanical strength of the capsules (Chen et al., 2012). As indicated above, this behaviour is related to an enhancement of the diffusion of chitosan into alginate capsules due to the decrease of electrostatic interactions between both polymers (Gåserød et al., 1998). Moreover, chitosan as a coating material contributed significantly to preserve the capsules strength with probiotic starter culture during storage (Obradovic et al., 2015).

An increase of stability of alginate capsules coated with chitosan was also observed by a covalent cross-linking of chitosan by bi-functional reagents (e.g. glutaraldehyde) (Chandy, Mooradian, & Rao, 1999) and epoxy compounds (Nishi, Nakajima, & Ikada, 1995). However, these synthetic cross-linkers have the recognized disadvantage of potential cytotoxic effects. A natural alternative has been reported using a naturally derived genipin (iridoid glucoside) from Gardenia fruits (Chen et al., 2007).

A metabolic activity depletion of immobilized cells has been recently observed by Cao et al. (2014). It is probably due to a reduction of permeability of the metabolites in and out of chitosan-coated alginate gel capsules. In this study, hydrolysis of glycyrrhizin with *Penicillium purpurogenum* gradually decreased as chitosan concentration was increased in the coating solution. However, the presence of a chitosan layer on the surface of alginate gel did not modify the metabolic activity of immobilized Acetobacter bacteria used for chiral alcohols biosynthesis (Chen et al., 2012) nor immobilized *S. cerevisiae* employed for ethanol production (Cao et al., 2014; Liouni et al., 2008). An intensification of antibacterial activity of encapsulated *Lactobacillus plantarum* against pathogenic bacteria was reported using chitosan as coating material. Cell protection of microcapsules may induce the production of a highly antibacterial substance (Trabelsi et al., 2014).

Interestingly, addition of chitosan as coating material decreased probiotic bacteria release from alginate capsules during lactic fermentation of a whey beverage (Obradovic et al., 2015; Trabelsi et al., 2014) and yeast release during alcoholic fermentation of glucose (Liouni et al., 2008). However, other authors have observed an instability of the core of chitosan-coated alginate capsules during successive lactic fermentation cycles, reporting an increase in probiotic bacteria release (Voo, Ravindra, Tey, & Chan, 2011).

An intensification of antibacterial activity of encapsulated *Lactobacillus plantarum* against pathogenic bacteria was reported using chitosan as coating material. A cell protection of microcapsules could induce the production of a highly antibacterial substance (Trabelsi et al., 2014).

Storage viability of *Lactobacillus bulgaricus* (Koo, Cho, Huh, Baek, & Park, 2001), *L. plantarum* (Nualkaekul, Cook, Khutoryanskiy, & Charalampopoulos, 2013; Nualkaekul, Lenton, Cook, Khutoryanskiy, & Charalampopoulos, 2012; Trabelsi et al., 2014), and *Bifidobacterium longum* (Nualkaekul et al., 2013) loaded in chitosan-coated alginate was higher than that of free cell cultures. Long-term viability of mammalian cells encapsulated in

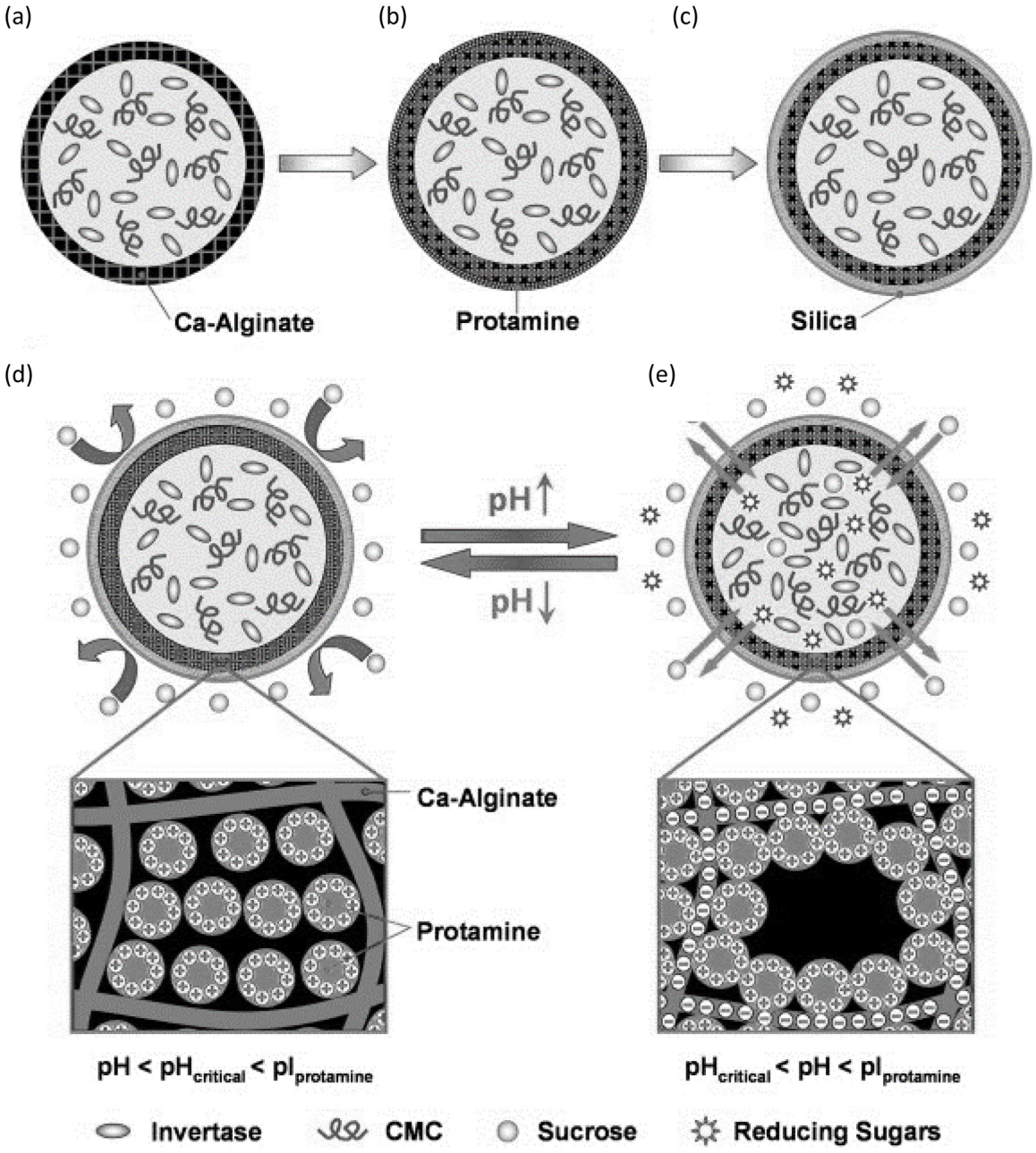

Fig. 3. Schematic representation of the formation process (a–c) and pH-responsive property (d and e) of alginate-protamine-silica capsules. pH-responsive membrane are composed of a soft layer of Ca-alginate-protamine and a rigid layer of silica. (a) Ca-alginate capsule was prepared using a co-extrusion device. (b) Protamine layer was made via electrostatic adsorption. (c) Ca-alginate-protamine-silica capsule was prepared by biosilicification process using sodium silicate. (d) When the capsules are in a solution at a pH lower than critical pH of Ca-alginate networks (close to the pK_a value of alginate network), protamine molecules (positively charged) are not electrostatically attracted by electrically neutral Ca-alginate networks. The diffusion channels of Ca-alginate networks are therefore choked and the permeability of substrates and products across the membrane is very limited. (e) When the capsules are in a solution at a pH lower than pI of protamine and higher than critical pH of Ca-alginate networks, protamine molecules (positively charged) are electrostatically attracted by Ca-alginate networks (negatively charged). The diffusion channels are in open state and the permeability of substrates and products across the membrane is high (Mei et al., 2013).

coated alginate capsules has been also reported (Baruch & Machluf, 2006). Better results were found using the single-stage procedure (the alginate is dropped into the chitosan-calcium solution) than the two-stage procedure (alginate capsules are first formed and coating with chitosan takes place later on). In the single-stage procedure, chitosan is only present at the outer surface of the alginate bead, allowing the existence of a free large alginate hydrogel core, which is probably more favourable to cells. Moreover, these authors found that cell viability was dependent on the molecular weight and the type of chitosan (chitosan chloride and chitosan glutamate). The use of high molecular weight chitosan glutamate and low molecular weight chitosan chloride provided higher cell viability levels.

2.3. Other synthetic polymers

Synthetic polymers offer several advantages over natural polymers, such as the possibility of being synthetized in large quantities, the lack of differences between batches and the fact that they can be easily engineered for the desired properties (De Vos et al., 2014). An exhaustive study of effectivity of different synthetic polymers as coating materials for cell encapsulation was evaluated by Prokop

Table 3
Cell immobilization into alginate capsules with the covalent cross-linking coating methods.

Coating material	Immobilized cell/molecule	Remarks	References
Alginate-glutaraldehyde	*Escherichia coli*	Reduction of cell metabolic activity	Zhang, Prabhu, Lee, and Kim (2010)
Alginate-glutaraldehyde	*Kluyeromyces marxianus*	Improved mechanical resistance No modification of cell metabolic activity	Barranco-Florido, García-Garibay, Gómez-Ruiz, and Azaola (2001)
Alginate-glutaraldehyde	*Pseudomonas* sp. BA2	Reduced metabolic activity	Bódalo Santoyo et al. (1997)
Alginate/polyethylenimine-glutaraldehyde	*Escherichia coli*	Improved mechanical resistance	Trotman et al. (2007)
Alginate/polyethylenimine-glutaraldehyde	*Erwinia sp*. D12	Improved mechanical resistance Modification of cell metabolic activity	Kawaguti, Buzzato, Orsi, Suzuki, and Sato (2006)
Alginate/polyethylenimine-glutaraldehyde	Comamonas terrigea	Improved mechanical resistance Reduced metabolic activity and improved operation stability	Huska et al. (1996)
Alginate/polyethylenimine-glutaraldehyde	*Kluyveromyces marxianus*	Reduced metabolic activity	Tomaska, Gemeiner, Materlin, Sturdik, and Handrikova (1995)
Alginate-functionalized poly(amido amine)-alginate azide	MIN6 cells	Improved mechanical resistance and solute permeability Adequate biocompatibility	Gattas-Asfura, Valdes, Celik, and Stabler (2014)
Alginate-poly(vinyl alcohol)-PLL	CHO SSF3 cells	Improved mechanical resistance No modification of cell growth	Breguet et al. (2005)
Alginate/PLL-poly(ethylene glycol) Alginate/PLL-poly(vinil alcohol)	IW32 cells	Improved mechanical resistance No modification of cell proliferation	Kung et al. (1995)
Alginate/PLL-poly(vinil alcohol)	IW32 cells	Improved mechanical resistance Adequate environment for cell growth	Wang (2000)
Alginate/PLL-poly(methyl vinyl ether-alt-maleic anhydride Alginate/PLL-methacrylic acid-co-2-vinyl-4,4-dimethylazlactone)	Myoblast cells	Improved mechanical stability Modification of solute diffusion Adequate environment for cell growth	Gardner et al. (2012), Gardner et al. (2010), Gardner & Stöver (2011)
Alginate/chitosane-genipin	*Lactobacillus plantarum* 80 HepG2 cells	Improved mechanical characteristics Improved cell viability	Chen et al. (2007)
Alginate-polydopamine	*Saccharomyces cerevisiae*	Improved mechanical stability Improved cell release and cell viability	Kim et al. (2014, 2015)
Alginate-photopolymerized network of acrylate and N-vinylpyrrolidone	C2C12 myoblasts	Improved mechanical resistance Significant cell damage	Shen et al. (2005), Wang et al. (2005)
Alginate/PLL-(N-5-azido-2-nitrobenzoyloxysuccinimide)	Pancreatic islet cells	Improved chemical and mechanical resistance No modification of cell growth	Dusseault et al. (2005)
Alginate/photopolymerized network of activated PLL	Pituitary tumour cells	Improved mechanical strength Permeable to proteins of mid-size molecular weight Adequate environment for cell growth	Lu et al. (2000)
Alginate/photopolymerized network of activated poly(allylamine)	IW32 cells	Improved mechanical strength Adequate environment for cell growth	Lu et al. (2000)
Alginate-(aminopropyltrimethoxysilane)	Pancreatic islet cells	Retained metabolic acidity Adequate permeability to substances of low molecular weight	Sakai et al. (2001)
Alginate-(3-aminopropyltriethosysilane)	*Saccharomyces cerevisiae*	Improved mechanical resistance Reduced cell growth	Ylitervo et al. (2013)
Alginate-(3-aminopropyltriethosysilane)	*Chlorella vulgaris*	Improved stability Adequate environment for cell growth	Pannier et al. (2014)

et al. (1998). Suitability of these materials was analysed focussing on mechanical strength, capsule shape, surface smoothness, stability and swelling or shrinking of coated capsules. Taken together, the results of this study demonstrate that alginate form very stable capsules with poly(allylamine) (PAA) and poly(vinylamine) (PVA).

PAA and PVA, whose chemical structures are very similar to poly-L-lysine, had similar reactivity with alginate. Wang (2000) reported the use of PVA and PAA to replace conventional PLL for coating alginate capsules. PAA formed capsules with similar strength to those prepared from PLL, whereas those prepared from alginate and PVA were the strongest. Release of proteins was lower in PAA-coated alginate capsules than in those uncoated (Sato, Hoshina, & Anzai, 2012). Polyelectrolyte multilayer microcapsules of PLO-alginate with immobilized Min-6 cells were obtained using consecutively polystyrene sulfonate negatively charged and PAA with positive charge. The coating withstood internal pressure generated by continuous cell proliferation and did not affect cell metabolic activity (Leung, Trau, & Nielsen, 2009). PVA coated-alginate capsules were also employed for immobilization of IW32 mouse erythroleukemia cells. Capsules showed a good mechanical strength and presented an adequate environment for cell growth (Wang, Wu, & Wang, 1992).

PMCG has been also tested as a membrane material for coating capsules in cell encapsulation. PMCG is significantly cheaper than PLL and it is liquid at room temperature, thereby facilitating its manipulation (Orive, Hernández, Gascón, Igartua, & Pedraz, 2003). PMCG-coated alginate capsules showed markedly improved mechanical strength compared with the widely used PLL-alginate capsules (Briššová, Lacík, Powers, Anilkumar, & Wang, 1997). Zhang, Yao, & Guan (2003) reported that mechanical strength of PMCG-coated capsules was enhanced as PMCG concentration increased. A higher exposition to PMCG (concentration and time)

caused a reduction of the diameter of PMCG-coated capsules and an increase of their wall thickness. In addition, PMCG-coated capsules showed higher osmotic resistance than those coated with PLL, which meant that polyelectrolyte complexation between alginate and PMCG was able to generate stronger capsules than ionic interaction between alginate and PLL.

Concentration of PMCG was also related to the diffusion characteristics of PMCG-alginate capsules (Zhang et al., 2003). The increasing membrane thickness as long as PMCG concentration rose, resulted in a looser membrane structure and a decrease in mass transfer resistance to solutes with low molecular weight (Orive et al., 2003). Mass transfer characteristics of PMCG-coated capsules were also compared with those capsules coated with PLL and PLO. The results of this study suggest that mass transport of solutes with low molecular weight is faster for PLL and PLO microcapsules than for PMCG formulation, whereas transfer of high molecular mass markers was restricted by all the microcapsule membranes tested (Rosiński et al., 2005).

Furthermore, the effect of PMCG on the growth of different cells has been reported. The presence of free PMCG prevented the growth of *Escherichia coli* and *Saccharomyces cerevisiae*. However, when PMCG was combined with a polyanion to form a complex membrane, the negative effect on the growth of these microorganisms disappeared. Under these conditions, PMCG-coated alginate microcapsules had good biocompatibility with *E. coli* and *S. cerevisiae* (Zhang, Yao, & Guan, 2005). These capsules had also an adequate microenvironment for the growth of mammalian cells, RINm5F, reporting a metabolic activity *in vitro* for up to 60 days for immobilized cells (Orive et al., 2003).

3. Covalent cross-linking coating

A common approach to stabilize alginate gels is covalent cross-linking coating which confers gels improved stability and mechanical strength (Smidsrød & Skjåk-Bræk, 1990). However, covalent cross-linking reagents may be toxic and unreacted chemicals may need to be removed thoroughly from gels (Lee & Mooney, 2012). Table 3 summarizes some examples of immobilized cells into alginate capsules with covalent cross-linking coating methods.

3.1. Cross-linking molecules

Glutaraldehyde (GA) has been widely used as a molecule to obtain cross-linked hydrogel network of alginate (Kawaguti, Buzzato, Orsi, Suzuki, & Sato, 2006; Oh, Kim, Ryu, Rho, & Kim, 2001; Santoyo et al., 1996; Trotman et al., 2007; Zhang, Prabhu, & Lee, 2010). Cross-linking of alginate can be achieved through the reaction of their hydroxyl groups with aldehyde groups of GA (Kim, Yoon, & Ko, 2000) (Fig. 4). Mechanical stability of macrocapsules of alginate gels can be greatly enhanced by their immersion in a bath with this cross-linker under a gently shaker for a few minutes (Barranco-Florido, Garcia-Garibay, Gomez-Ruiz, & Azaola, 2001). Concentration of the hardening agent is a critical factor that can affect cell release and metabolic activity.

Covalent cross-linking of alginate with 0.1% GA reduced the release of immobilized *E. coli* cells, while their metabolic activity decreased as GA concentration increased (Zhang, Prabhu, Lee, 2010). The treatment of alginate capsules (containing L-aminoacylase-producing *Pseudomonas* sp. BA2) with a concentration between 5 and 30 mM of GA caused a significant increase in both substrate conversion and half-life of the biocatalyst, however a higher concentration of the hardening agent decreased cell metabolic activity (Bódalo Santoyo et al., 1997). A GA concentration above 0.2% was associated with enzyme denaturation (Oh et al., 2001).

The use of a combination of GA and polycationic polyethylenimine (PEI) has been employed to increase mechanical stability of the alginate gel (Bódalo Santoyo et al., 1997; Huska et al., 1996; Kawaguti et al., 2006; Tomaska, Gemeiner, Materlin, Sturdik, & Handrikova, 1995; Trotman et al., 2007). Protonated amino groups of PEI form a polyelectrolyte complex with carboxyl groups of alginate gel while free hydroxyl groups of alginate are used for cross-linking with GA (Elnashar, Danial, & Awad, 2009).

Several studies have been done to evaluate metabolic activity of immobilized cells. Metabolic activity assayed on immobilized *Kluyveromyces marxianus* showed that application of PEI and GA caused low values of β-galactosidase activity (Tomaska et al., 1995). Biodegradation of surfactants by immobilized *Comamonas terrigea* in alginate gels was also less at the start of the experiment in capsules treated with GA and PEI, which showed an improved operation stability during two months (Huska et al., 1996). Similar behaviour was observed in cells of *Erwinia* sp. D12 encapsulated in GA-PEI-alginate gels. The isomaltulose production by Erwinia was lower but more stable during the reaction time (Kawaguti et al., 2006). These results have been explained by negative action of GA and PEI on cell metabolic activity and by a reduction of the diffusion of substrates and products of enzymatic reactions (Huska et al., 1996; Kawaguti & Sato, 2008; Tomaska et al., 1995). It has been also observed that optimum concentration of hardening agents depend on the sequence of their application. This effect was observed in the lyase activity of immobilized *E. coli*, where the optimal concentration of GA was increased when PEI was added before GA (Trotman et al., 2007).

Recently, physical properties and cytocompatibility of alginate capsules coated with hyperbranched polymers have been studied. Alginate microbeads were first incubated in 1-methyl-2-(diphenylphosphino) terephthalate (MDT) functionalized poly(amido amine) (PAMAM). Subsequently, a hyperbranched alginate azide (alginate-N_3) was covalently linked to exposed MDT functionalized PAMAM coating. Interlayer covalent bonds were formed between complementary azide and MDT groups via Staudinger ligation (Fig. 5). Coated alginate-N_3 microcapsules showed higher mechanical stability than non-coated capsules. Decreased gel compactness and electrostatic repulsion of alginate-N_3 chains likely contributed to overall decreased swelling of these microcapsules. In comparison to PLL-alginate-coated microcapsules, PAMAM/alginate-N_3 were generally more permselective to dextrans. Finally, *in vivo* biocompatibility of coated alginate capsules was also found comparable to uncoated ones (Gattas-Asfura, Valdes, Celik, & Stabler, 2014).

Covalent grafting with different chemical reagents was also investigated in order to improve mechanical properties of PLL-alginate gels. One strategy was the use of reactive polyanions that possess reactive electrophilic groups capable of forming stable covalent cross-links with the underlying polyamine (PLL), such as poly(methyl vinyl ether-alt-maleic anhydride) (PMM) and poly(methacrylic acid-co-2-vinyl-4,4-dimethylazlactone) (PVV) (Fig. 6). Higher resistance of these gels is due to both electrostatic interaction between negative charge of polyanions and positive charge of PLL and covalent cross-link of reactive polyanions with PLL (Gardner, Potter, & Stöver, 2012).

PLL-alginate gel particles were more stable by increasing concentration of PLL and decreasing molecular weight of PMM, both resulting in a bigger amount of polyelectrolyte bound to the capsule. Permeability studies determined that covalently cross-linked capsules allowed a rapid diffusion of 10–70 kDa dextrans while excluding larger dextrans, indicating a membrane porosity suitable for cell encapsulation (Gardner, Burke, & Stöver, 2010). The reactive polyanions based on PVV could be joined under physiological conditions to PLL-alginate gel beads and spontaneously cross-link with polyamine to form covalent networks of interest for cell encapsula-

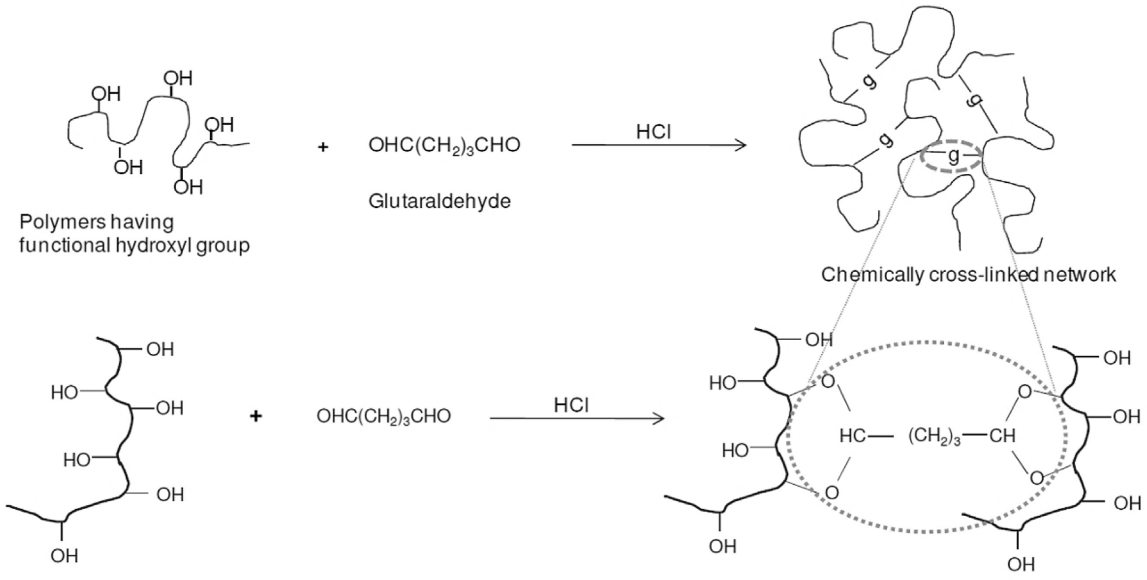

Fig. 4. Reaction scheme of the covalent interaction between the hydroxyl groups of alginate and glutaraldehyde cross-linker (Gulrez et al., 2003).

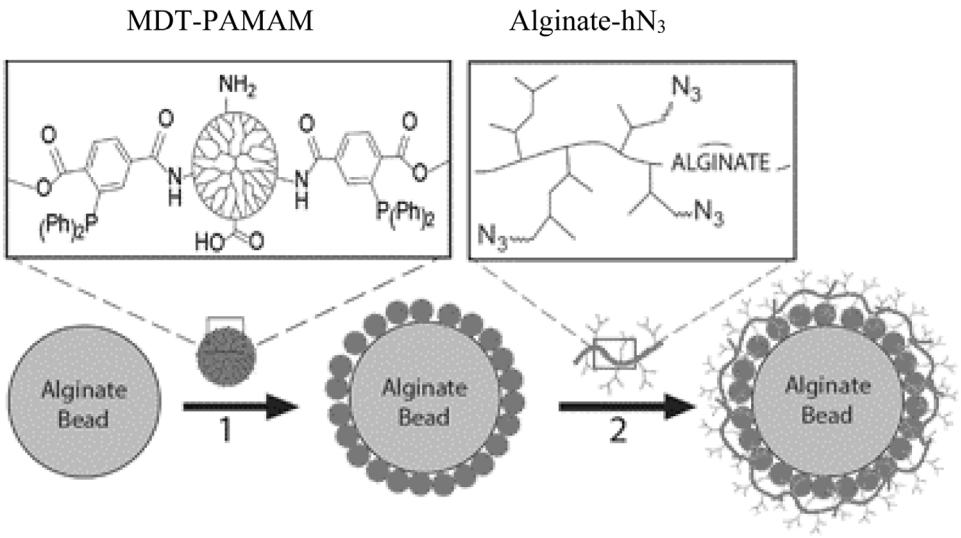

Fig. 5. General scheme of alginate bead coated with 1-methyl-2-(diphenylphosphino) terephthalate (MDT) functionalized poly(amido amine) (PAMAM) and hyperbranched alginate azide (alginate-hN_3). (1) Alginate bead was first coated using MDT functionalized PANAM. (2) Then, a second layer of alginate-hN_3 was added by incubation of PAMAM coated bead with alginate-hN_3 polymer solution (Gattas-Asfura et al., 2014).

tion. These PVV-PLL-alginate capsules showed high stability after a liquefaction treatment with sodium citrate (Gardner & Stöver, 2011). The viability of myoblast cells immobilized in both PVV-PLL-alginate and PMM-PLL-alginate capsules was not affected by the coating process and immobilized cells remained viable for one week *in vitro* (Gardner et al., 2012).

Another strategy has been developed to improve mechanical strength of PLL-alginate capsules based on chemical cross-linking of PLL with tosylated poly(vinylalcohol). A significant improvement of capsular strength was achieved after this type of chemical cross-linking, showing that the frequency of breaking of chemical cross-linked capsules decreased to only 30% as compared to 85% of the conventional microcapsules (Wang, 2000).

Covalent cross-linking of PLL-alginate with tosyl chloride-activated poly(ethylene glycol), cyanuric chloride activated poly(ethylene glycol) and tosyl chloride-activated poly(vinyl alcohol) also improved mechanical stability of the capsules. Likely, the main reaction occurs on the amino groups of PLL, however the covalent reaction could also take place on hydroxyl groups better than carboxyl groups of alginate implicated in chelation with calcium ions. Better results were found using cyanuric poly(ethylene glycol) and tosylated poly(vinyl alcohol) than tosylated poly(ethylene glycol). The three chemically activated polymers did not inhibit the proliferation of IW32 cells (Kung, Wang, Chang, & Wang, 1995).

Transacylation reaction was applied to covalently bound ester groups of poly(vinyl alcohol) to amino groups of PLL and bovine-serum-albumin in alginate gels. Capsules were made by extrusion of a mixture of alginate and bovine-serum-albumin in a hardening bath of poly(ethylene glycol) and calcium ions. Capsules were then suspended in a solution of PLL. Covalent bond formation was initiated by addition of NaOH using a transacylation reaction causing a significant improvement of their mechanical properties. The presence of bovine-serum-albumin was critical to reduce diffusion of NaOH inside the microcapsules and to prevent a reduction of via-

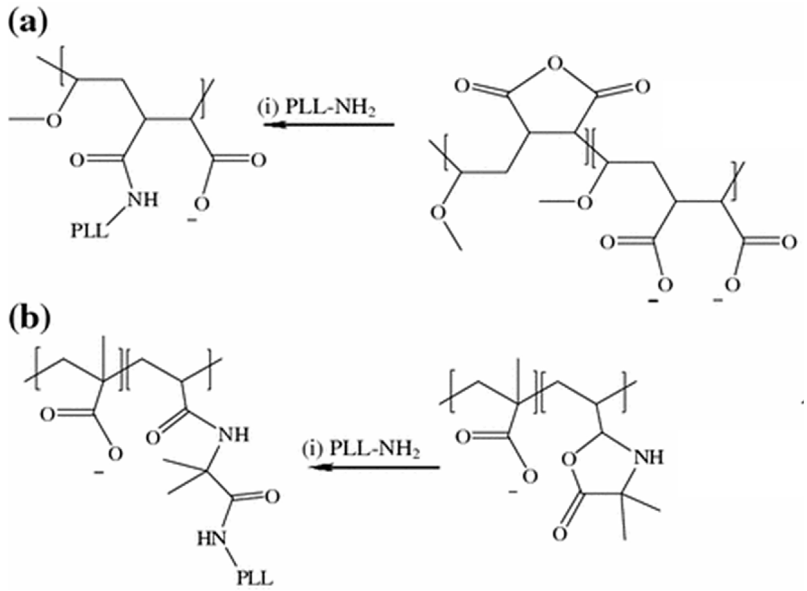

Fig. 6. Scheme of the reaction of (a) poly(methyl vinyl ether-alt-maleic anhydride) (PMM) and (b) poly(methacrylic acid-co-2-vinyl-4,4-dimethylazlactone) (PVV) with amine groups of poly-L-lysine (PLL) (Gardner et al., 2012).

bility of immobilized cells (Breguet, Gugerli, Pernetti, von Stockar, & Marison, 2005).

Recently, alginate capsules have been immersed in a solution of dopamine hydrochloride to form polydopamine-coated alginate beads. This simple polydopamine coating of alginate beads effectively controlled the growth of *S. cerevisiae* inside the capsules and this controlled growth of yeasts prevented undesired cell release from the capsules while maintaining cell viability (Kim et al., 2015). Covalently linked polydopamine shell was mechanically durable and prevented gel swelling. Moreover, it also protected cells from enzymatic attack of alginate lyase and UV irradiation (Kim et al., 2014).

3.2. Photocross-linking

Covalent cross-linking can be also initiated by the use of high energy radiation (Gulrez et al., 2003). Alginate capsules were exposed to sodium acrylate and *N*-vinylpyrrolidone in order to create a covalent network via photopolymerization. These improved capsules showed greater mechanical stability and resistance to osmotic pressure and similar permeability to polyethylene glycol than standard alginate capsules (Wang, Childs, & Chang, 2005). Biological functionality of these photocross-linked alginate capsules were studied then by Shen et al. (2005). The photopolymerization caused significant cell damage, however, cells were able to restore normal growth rates thereafter. Studies of biocompatility confirmed that chemical modification did not adversely affect the properties of these capsules.

PLL modified with a heterobifunctional photoactivatable cross-linker, *N*-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS), was used for coating alginate capsules. Covalent links were created between phenyl azide residue of PLL-ANB-NOS and Ca-alginate capsules upon illumination with UVA (Fig. 7). Coated alginate capsules showed extremely resistance to chemical and mechanical stress and the procedure did not modify viability of immobilized pancreatic islet cells (Dusseault et al., 2005).

An approach to improve mechanical strength of microcapsule employing light-induced photodimerization of polycations has been reported. This method involved partial modification of the amino groups of PLL (Wang 2000) and poly(allylamine) (Lu, Lan, Wang, Chang, & Wang, 2000) with α-phenoxycinnamyldieneacetyl chloride. This strategy has the advantage that there is no release of toxic byproducts during the cross-linking reaction. The photosensitive PLL interacts with alginate capsule via ionic bonding to form water insoluble polymeric membrane and photosensitive α-phenylcinnamylideneacetate moieties pendent to PLL cross-link to form covalent bonds between photosensitive polymers upon light exposure. The irradiation treatment caused a significant improvement of mechanical strength of capsules. Interestingly, pituitary tumour cells grew well within the capsules suggesting that they are suitable for cell entrapment (Wang 2000). Similar results were found using photosensitive poly(allylamine). The capsules prepared from alginate and photosensitive poly(allylamine) upon light exposure exhibited higher mechanical strength compared with those prepared from alginate and poly(allylamine). Moreover, the current method provided an adequate environment for the growth of IW32 cells (Lu, Lan, Wang, Chang, & Wang, 2000).

3.3. Silica coating

The formation of silica gels from aqueous precursors was rendered possible by the development of sol-gel chemistry (Coradin, Nassif, & Livage, 2003). This sol–gel process involves the hydrolysis of alkoxide precursors under acidic or basic conditions with production of alcohol, followed by condensation and polycondensation of hydroxylated units, which leads to the formation of a porous gel. The alcohol can be removed from the sol (prepolymer) under vacuum by rotary evaporation. Commercially available aqueous silica precursors, sodium silicate and colloidal silica (Ludox) can be used in order to avoid the possible toxic effect on cells (Kuncová & Trogl, 2010).

In comparison with organic gels, silica gels present important advantages related to their intrinsic properties (higher mechanical properties, physical and chemical stability towards solvents, pH, light and microbial degradation) and flexibility of the sol-gel process (tailored porosity, and chemical modification using organically modified alkoxides) (Coradin, Allouche, Boissiere, & Livage, 2006). To strengthen alginate gel particles, silica can be used by associating this soft biocompatible organic component (alginate) with the

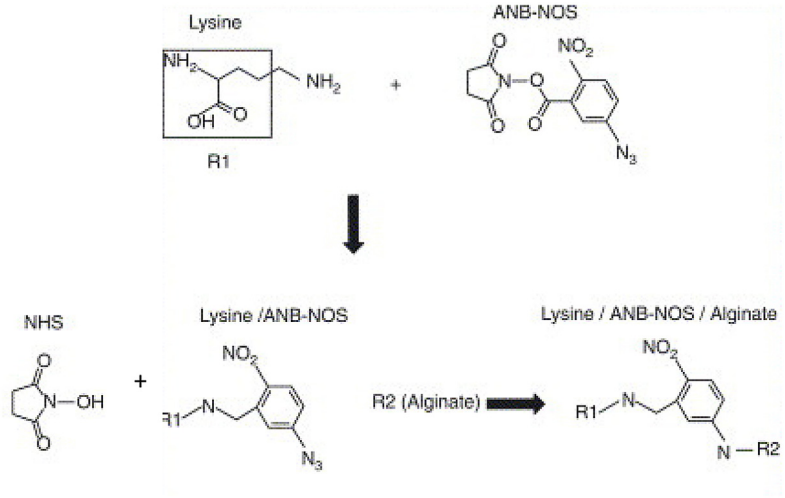

Fig. 7. Reaction scheme of the chemical reaction of *N*-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS) on the primary amine of poly-L-lysine and alginate (Dusseault et al., 2005).

tough, thermostable and non-swelling inorganic silica component (Coradin et al., 2003).

Theoretically, coating of the silicon species with alginate by a layer-by-layer process is not possible, due to the electrostatic repulsion between both negatively charged molecules. One alternative is the use of a polycation previously to coat alginate capsules forming a first layer (positively charged), allowing then the deposition of the silicon species. Coradin, Mercey, Lisnard, and Livage (2001) reported capsules of PLL-alginate coated with sodium silicate and colloidal silica with enhanced mechanical resistance. It was demonstrated that PLL is able to promote the condensation of silica (Coradin & Livage, 2001). Other polycations such as lysozyme and chitosan have been evaluated for the design of silica-coated alginate capsules (Bruyant & Coradin, 2007). Recently, positively charged protamine molecules have been applied for coating the surface of alginate capsules before the treatment with sodium silicate. The layer of inorganic silica was formed due to the electrostatic interaction between positive prolamine and negative sodium silicate (He et al., 2015; Mei et al., 2014).

Sakai, Ono, Ijima, and Kawakami (2001) studied the silica layer deposition using an amino-functionalized silica alkoxide (aminopropyl-trimethoxysilane (APTMS)). APTMS has the potential to interact electrostatically with alginate and to form organic/inorganic hybrid capsules in aqueous media. First, APTMS molecules adsorbed on alginate shells via electrostatic interaction between protonated amino groups of APTMS and carboxyl groups of alginate and then a self-condensation of APTMS occurs to form a polysiloxane network in alginate shell. Pancreatic islet cells encapsulated into APTMS-alginate gels were able to increase their rate of insulin secretion in response to stimulation by a high glucose level. Moreover, the capsules showed good permeability to metabolic substances of low molecular weight such as oxygen, carbon dioxide, and nutrients, while it prevented permeation of substances of high molecular weight as γ.globulin.

Other amino functioned alkoxide precursor, 3-aminopropyltriethoxysilane (APTES), was also studied as coating material of alginate capsules (Fig. 8). The combination of the polyelectrolyte complex and sol-gel synthesis was affected by the pH of the reaction medium and enhanced at acid and basic conditions. Moreover, shell thickness increased as APTES exposition (concentration and time) increased (Kurayama et al., 2010). Alginate-chitosan-alginate capsules with immobilized *S. cerevisiae* were coated with APTES, resulting in capsules with a great chemical and mechanical robustness. The permeability of molecules of low molecular weight from yeast metabolism such as glucose, acetic acid and furfural slightly decreased when increasing APTES concentration (Ylitervo, Franzén, & Taherzadeh, 2013). In addition, immobilized *S. cerevisiae* suitably grew at concentrations below 3.0% of APTES. Recently, APTES was employed to reinforce alginate hydrogels with encapsulated microalgal cells for the design of a cell based sensor array. The immobilization support was flexible enough to allow the proliferation (Pannier, Soltmann, Soltmann, Altenburger, & Schmitt-Jansen, 2014).

4. Conclusions and outlook

Coating techniques have demonstrated great potential as tools to improve disadvantages of cell encapsulation using alginate hydrogels. The effect of ionic cross-linking techniques on mechanical and chemical stability, diffusion properties, porosity and swelling of coated-alginate capsules have been extensively studied over the last decades. Recent advances on covalent cross-linking coating techniques have been successfully developed but in less detail. However, information concerning viability and metabolic activity of cells immobilized into coated-alginate gels is very little. On the whole, ionic cross-linking coating techniques take place under mild and biocompatible conditions, nevertheless, covalent cross-linking reactions can cause cell toxicity, and it is necessary to check the coating protocols in order to select non-toxic reagents or remove the unreacted reagents and by-products. The comparison of different coating methods in order to select the most suitable one for cell encapsulation is very complicated because of the lack of standardized methods to measure physicochemical properties of capsules and cell viability. Moreover, for each application, an individual study is necessary since cell toxic effect depends on both coating protocol and type of cell.

There are numerous challenges that remain in the field of coating techniques of alginate gel for cell encapsulation. Future research studies should focus on the development of new coating protocols of alginate capsules to improve their encapsulation effectiveness in relation to cell viability. The creation of new coating protocols combining ionic and covalent interactions between alginate networks and coating materials may enable future advances in cell encapsulation techniques. Another area of intense study could be the design and application of functionalized alginates as support for cell encapsulation together with coating techniques and the design of stimuli-responsive materials based on coated alginate gel. Moreover, in order to have a deep knowledge of the effect of these

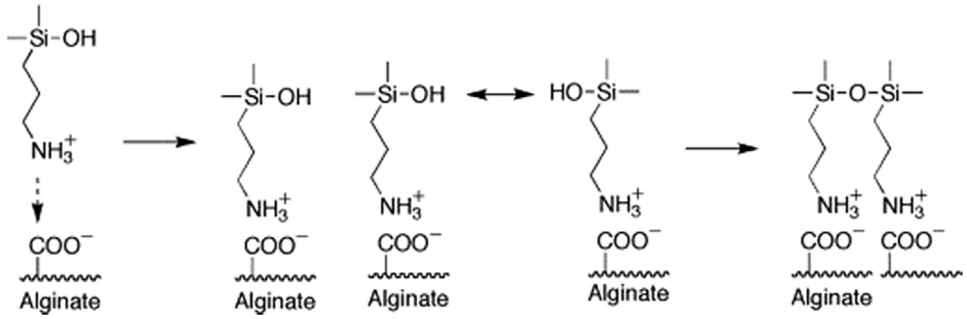

Fig. 8. Electrostatic interaction between carboxyl groups of alginate and amino groups of APTES, and polycondensation of silanol groups to form polysiloxane network (Kurayama et al., 2010).

coating techniques on viability and metabolic activity of encapsulated cells, modern technologies such as metabolome, genome, or transcriptome analysis could be considered.

Despite the development of coating techniques, the mechanical and chemical stability of alginate-based capsules and the viability and metabolic activity of entrapping cells have been improved significantly, there are still limiting factors for the industrial application of this technique. A challenge is the development of a commercially viable production method of coated-capsules under aseptic conditions and on a large scale. Another major challenge is the production of stable capsules for a long period and under easy preservation conditions, without compromising metabolic activity and cell viability. Advances in each of these issues will certainly have a remarkable impact on the development of new industrial applications.

Commercial successes have been obtained mainly for encapsulation of probiotics and some fermenting microorganisms using ionic cross-linking coating techniques, while covalent cross-linking coating methods have not yet been employed commercially. As an industrial application of fermenting microorganism, *Saccharomyces bayanus* immobilized in coated-alginate capsules (Cremanti® from Erbslöh-Cavis, Mainz, Germany) utilising MLR® Technology (Multi Layer Capsule) can be highlighted. This yeast was designed for the elaboration of sparkling wine by traditional methods, avoiding turbidity of wine during its second fermentation in the bottle and saving effort and outlay by elimination of the riddling process. Furthermore, another interesting industrial application of an immobilized yeast has been achieved using a strain of *Schizosaccharomyces pombe* (ProMalic®) created by Proenol (Gaia, Portugal) in collaboration with Lallemand Ind. (Montreal, Canada). This yeast was encapsulated in double-layered alginate beads and performs maloalcoholic fermentation, allowing the conversion of malic acid into ethanol. This is an alternative to traditional malolactic fermentation carried out by lactic acid bacteria in wines. Yeast encapsulation makes possible the addition to grape just at the beginning of alcoholic fermentation and the removal of cells when the desired level of malic acid has been achieved.

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CAPÍTULO 2

5. CAPÍTULO 2: Optimización de las condiciones de inmovilización de *Oenococcus Oeni*

- **5.1. Highly efficient malolactic fermentation of red wine using encapsulated bacteria in a robust biocomposite of silica-alginate**

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Highly Efficient Malolactic Fermentation of Red Wine Using Encapsulated Bacteria in a Robust Biocomposite of Silica-Alginate

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ABSTRACT: Bacteria encapsulation to develop malolactic fermentation emerges as a biotechnological strategy that provides significant advantages over the use of free cells. Two encapsulation methods have been proposed embedding *Oenococcus oeni*, (i) interpenetrated polymer networks of silica and Ca-alginate and (ii) Ca-alginate capsules coated with hydrolyzed 3-aminopropyltriethoxysilane (hAPTES). On the basis of our results, only the first method was suitable for bacteria encapsulation. The optimized silica-alginate capsules exhibited a negligible bacteria release and an increase of 328% and 65% in L-malic acid consumption and mechanical robustness, respectively, compared to untreated alginate capsules. Moreover, studies of capsule stability at different pH and ethanol concentrations in water solutions and in wine indicated a better behavior of silica-alginate capsules than untreated ones. The inclusion of silicates and colloidal silica in alginate capsules containing *O. oeni* improved markedly their capacity to deplete the levels of L-malic acid in red wines and their mechanical robustness and stability.

KEYWORDS: *Oenococcus oeni, encapsulation, bacteria, silicates, alginate*

INTRODUCTION

Malolactic fermentation (MLF) in wines is an essential step to improve the quality of red wines. Lactic acid bacteria (LAB) and, especially *Oenococcus oeni*, are involved in the transformation of L-malic acid with final production of lactic acid and significantly influence the aromatic complexity of wine.[1,2] LAB immobilization has recently received some attention in winemaking because it offers several interesting advantages over the use of free cells. Some of them are the following: (i) immobilization support can improve bacteria protection against adverse conditions of MLF (e.g., extreme pH, high ethanol concentration, presence of inhibitory substances, and low fermentation temperature); (ii) it allows continuous fermentative processes; (iii) it opens the way to bacteria reutilization; (iv) it enhances productivity due to inoculation of higher bacteria concentration; and (v) it allows the conduction of MLF with selected immobilized bacteria.[3−5] Despite the fact that the process of bacterial encapsulation increases the cost of the use of bacteria, the reutilization of the immobilized bacteria in repeated batches and the possibility of design continuous processes have a positive impact on the economic feasibility of MLF of red wine.

Bacteria immobilization involves physical confinement of cells to a region of space, preserving their metabolic activity. Several biotechnological techniques have been developed for cell immobilization, such as (i) immobilization on the surface of a matrix by ionic, covalent, and/or hydrogen bond interactions; (ii) encapsulation into a porous matrix; (iii) cell aggregation with cross-linking reactions or by flocculation; and (iv) cell entrapment into organic or microporous synthetic membranes.[6−8] Among these techniques, cell encapsulation into the matrix has received special attention due to its simplicity, low cost, and mild working conditions.[9,10] A suitable polymeric material for bacteria encapsulation must be able to retain cells, maintain cell viability, and be permeable to gas, nutrients, and metabolites.[8] Natural materials (such as alginate, carrageenan, chitosan, agarose, pectin, gelatin, and chitin) have been widely applied for cell encapsulation because they exhibit these properties and they have low cost and do not generally present cell toxic impurities coming from chemical reactions.[9] Among these materials, alginate is the most common polymer used for cell[10−14] and enzyme[15−17] encapsulation.

Alginate is an ionic polymer composed of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid residues in different sequences.[18] Divalent cations, mainly Ca^{2+} and Ba^{2+}, added to alginate solutions may lead to cross-linking of alginate molecules forming hydrogels compatible with the survival of entrapped cells.[19] However, alginate gels are sensitive to chelating molecules commonly existing in food, such as citrates, phosphates, and lactates and other antigelling cations such as Na^{+} and Mg^{2+}, reducing their chemical stability.[18] Moreover, low mechanical robustness of Ca-alginate gels discourages their implementation for alcohol beverages production.

Diverse strategies have been explored to resolve disadvantages of the application of alginate gel for cell entrapment. Either incorporation of other polymers and/or fillers into alginate structure gel or application of a coating layer to alginate gel have been performed to enhance its mechanical and chemical stability, to increase its biocompatibility and to control

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Table 1. Effect of the Concentration of Sodium Silicate, Colloidal Silica, and pH for the Encapsulation of *O. oeni* into Silica-Alginate Capsules Obtained by the Interpenetrated Polymer Network Method on the L-Malic Acid Consumption by Encapsulated Bacteria, the Capsule Mechanical Strength, and the Population of Released Bacteria from Capsules

	factors			responses[a]		
no.	Na silicate (M)	colloidal silica (M)	pH	L-malic acid consumption (g L^{-1})	mechanical strength (g)	population of released bacteria (cfu mL^{-1} of wine)
1	0.00	0.000	5.15	0.70 ± 0.08	68.9 ± 2.7	1.1×10^1
2	0.08	0.000	5.15	0.64 ± 0.12	76.0 ± 2.7	<10
3	0.00	1.230	5.15	2.92 ± 0.01	72.5 ± 3.7	3.3×10^1
4	0.08	1.230	5.15	2.83 ± 0.03	117.6 ± 8.3	2.0×10^1
5	0.00	0.615	4.00	0.81 ± 0.04	75.8 ± 3.3	<10
6	0.08	0.615	4.00	0.87 ± 0.22	95.1 ± 4.2	<10
7	0.00	0.615	6.30	2.96 ± 0.00	70.4 ± 2.8	6.5×10^1
8	0.08	0.615	6.30	2.25 ± 0.02	90.1 ± 4.3	9.6×10^1
9	0.04	0.000	4.00	0.55 ± 0.14	69.0 ± 2.7	2.1×10^1
10	0.04	1.230	4.00	1.30 ± 0.11	93.9 ± 4.1	4.7×10^1
11	0.04	0.000	6.30	2.97 ± 0.07	69.3 ± 2.3	7.8×10^1
12	0.04	1.230	6.30	2.96 ± 0.07	75.3 ± 5.0	7.0×10^2
13	0.04	0.615	5.15	2.59 ± 0.30	85.8 ± 5.6	6.2×10^1

[a]Each value represents the mean ± standard deviation. MLF conditions: red wine (3.0 g L^{-1} L-malic acid, pH 3.2, 14% (v/v) of alcohol degree), 0.03 g of capsules mL^{-1} of wine, bacterial load of ~9×10^7 cfu mL^{-1} of wine, temperature of 22 °C and 96 h of incubation. Mechanical strength was tested in 20 capsules submerged in sterile 0.2 M Ca_2Cl for 48 h at 22 °C. Population of released bacteria from capsules was tested by immersing 20 capsules (~0.3 g of capsules with a bacterial load of ~3×10^9 cfu g^{-1} of gel) into 10 mL of sterile red wine under stirring (165 rpm) at 22 °C during 24 h of immersion.

cell retention.[20] Recently, improvement of alginate gel stability with silica has been reported by various authors using the sol–gel process.[21−25] The sol–gel process makes it possible to design silica-alginate biocomposites, that have the advantages of the organic polymers (biocompatibility, flexibility, and elasticity) and the inorganic silica component (rigidity, chemical resistance, and thermal stability).[23,26] The sol–gel process led by alkoxide precursors ($Si(OR)_4$) involves formation of silicic acid ($SiOH_4$) upon water hydrolysis with liberation of byproduct alcohol (R–OH). Then, formation of siloxane (Si–O–Si) takes place between silanol groups (Si–OH) of two molecules. Finally, the covalent silica network is obtained by polycondensation of silanols and siloxanes.[27,28] Biocompatibility of alkoxide route may be compromised by the presence of byproduct alcohol. An alternative aqueous route with sodium silicate and colloidal silica has been proposed allowing the formation of silica gel at neutral pH and room temperature, without generation of alcohol as byproduct and therefore eliminating the risk of cell toxicity.[27]

Silica-alginate composites for cell encapsulation have been prepared through the sol–gel process by polycondensation of silica precursors, ionic cross-linking by complexation reaction between carboxyl groups of alginate and divalent cations and intermolecular interaction of silanol/silica with alginate based on the hydrogen link.[29] Lately, 3-aminopropyltriethoxysilane (APTES) has been proposed to form silica-coated alginate composites via electrostatic interaction between carboxyl groups of alginate from the surface of capsules and amino groups of APTES,[30] so reinforcing the alginate structure.[31] Recently, alginate-silica encapsulated *Oenococcus oeni* have been reported to perform MLF in wine.[24,25] Alginate capsules were first coated with an ethanol/water solution of tetraethoxysilane (TEOS) and then with methyltriethoxylsilane (MTES) in the gas phase. MTES-TEOS-alginate capsules loaded with bacteria showed similar metabolic activity and better bacteria leakage reduction and mechanical resistance than untreated ones.[24] These capsules allowed the development of MLF in wines with lysozyme added to eliminate wild LAB and their potential spoilage.[25]

Unlike previous works,[24,25] this study explores two new approaches for *O. oeni* immobilization into silica-alginate biocomposites using aqueous routes based on (i) an interpenetrated polymer networks of silica and Ca-alginate and (ii) a coating for Ca-alginate capsules with APTES. To our knowledge, there has been no work undertaken to apply these strategies for LAB immobilization into silica-alginate capsules and their implementation in MLF. The aim of this work was to optimize the conditions of encapsulation of *O. oeni* into both silica-alginate capsules to perform MLF. Fermentation behavior of encapsulated *O. oeni*, mechanical robustness, and ability to retain bacteria of silica-alginate capsules were evaluated. Finally, the stabilities of silica-alginate capsules at different pH values and ethanol concentrations in water solutions and in red wine were also analyzed.

MATERIALS AND METHODS

Immobilization of *O. oeni* Using the Interpenetrated Polymer Network Method. To immobilize bacteria into interpenetrated polymer networks of silica and alginate, a process based on the mixture of derivatives of silicon with sodium alginate before gelation in the presence of Ca^{2+} was used.[23,32] Sodium silicate (0.00–0.08 M) (Sigma-Aldrich) and colloidal silica (0.00–1.23 M) (Ludox HS40, Sigma-Aldrich) concentrations and immobilization pH (4.0–6.3) were optimized. Thirteen experiments were carried out using a factorial design Box-Behnken for the study of linear, quadratic, and cross-products effects of the three factors each at three levels and three center points (no. 13 was replicated three times) for the estimation of the experimental error (Table 1). Response surface models were fitted to three individual responses (L-malic acid consumption by encapsulated bacteria in red wine (g L^{-1}), mechanical strength of capsules (g), and cell release from capsules in red wine). Statgraphics centurion XVI (v. 16.1.15) software was employed to fit the second-order model to factors (independent variables) using a second order polynomial quadratic equation. The statistical significance of factors was identified using the Pareto diagram ($p < 0.05$). The suitability of constructed models to describe the experimental data was determined

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using the lack of a fit test. Response surface plots were drawn to represent the main and interactive effects of factors on each response. Bacteria encapsulation into biocomposites of silica-alginate was carried out according to the following procedure. The pH of the solution of sodium silicate and colloidal silica in water was adjusted (pH 4.0−6.3) by adding 2 N HCl. Then, 2% of sodium alginate (Panreac, Spain) and freeze-dried bacteria *Oenococcus oeni* (LALVIN VP 41 MBR, Lallemand, France) were added at ∼3 × 10^9 cfu g^{-1} of gel. Before bacteria were added to the alginate solution and according to the manufacturer's instructions, freeze-dried bacteria were rehydrated in 20 times its weight of clean chlorine free water at 20 °C for a maximum of 15 min. Capsules were formed by manually dripping the siliceous material-alginate-cell suspension with a 10 mL syringe (BD Plastipak, Spain) into a sterile 0.2 M $CaCl_2$ (Panreac) under agitation (260 rpm) at 22 °C. The diameter of the nozzle used was 1.78 mm. The height from which the solution was dripped into the gelation bath was maintained at 20 cm. Then, they were maintained in 0.2 M $CaCl_2$ for 2 h and washed with sterile water at 22 °C.

Immobilization of *O. oeni* Using the Coating Method with hAPTES. Briefly, 2% of sodium alginate and bacteria (∼3 × 10^9 cfu g^{-1} of gel) were mixed and dripped into sterile 0.2 M $CaCl_2$ under agitation. Then, the capsules were maintained in 0.2 M $CaCl_2$ for 2 h and washed with sterile water. The resulting capsules were treated with hydrolyzed 3-aminopropyltriethoxysilane (hAPTES) solutions (0.0−2.0%) at different pH (4.0−7.0) and exposure time (15−60 min) at room temperature. APTES (Sigma-Aldrich) was hydrolyzed overnight in water (1 mL of APTES and 9 mL of water) before its use.[30] After coating, capsules were washed again with sterile water before starting MLF. Thirteen experiments were carried out using a factorial design Box-Behnken for the study of linear, quadratic, and cross-products effects of the three factors (hAPTES concentration, time, and pH) each at three levels and three center points (no. 13 was replicated three times for the estimation of the experimental error) (Table 2).

Table 2. Effect of the Concentration of hAPTES, pH, and Reaction Time for the Encapsulation of *O. oeni* into Silica-Alginate Capsules Obtained by the Coating Method on the L-Malic Acid Consumption by Encapsulated Bacteria[a]

	factors			response
no.	hAPTES (%)	pH	time (min)	L-malic acid consumption (g L^{-1})
1	0.50	4.0	37.5	1.57 ± 0.19
2	1.25	7.0	15.0	1.61 ± 0.30
3	1.25	4.0	15.0	1.51 ± 0.29
4	2.00	7.0	37.5	1.55 ± 0.21
5	1.25	4.0	60.0	1.26 ± 0.31
6	0.50	5.5	15.0	1.55 ± 0.28
7	2.00	4.0	37.5	1.57 ± 0.28
8	2.00	5.5	15.0	1.36 ± 0.22
9	2.00	5.5	60.0	1.37 ± 0.34
10	0.50	7.0	37.5	1.42 ± 0.34
11	1.25	7.0	60.0	1.38 ± 0.37
12	0.50	5.5	60.0	1.47 ± 0.29
13	1.25	5.5	37.5	1.72 ± 0.18

[a]Each value represents the mean ± standard deviation. MLF conditions: red wine (3.0 g L^{-1} L-malic acid, pH 3.2, 14% (v/v) of alcohol degree), 0.03 g of capsules mL^{-1} of wine, bacterial load of ∼9 × 10^7 cfu mL^{-1} of wine, temperature of 22 °C, and 96 h of incubation.

The Pareto diagram was employed to identify statistically significant factors ($p < 0.05$). L-Malic acid consumption by encapsulated bacteria in red wine, mechanical strength of capsules, and cell release from capsules in red wine were evaluated.

L-Malic Acid Consumption in Red Wine by Encapsulated Bacteria. Wine was sterilized by filtration through a 0.2 μm pore-size membrane. MLF of red wine of "Tinta de Toro" variety (3.0 g L^{-1} L-malic acid, pH 3.2 and 14% (v/v) of alcohol degree) was developed using encapsulated bacteria. The concentration of 0.03 g of capsules mL^{-1} of wine at 22 °C and a bacterial load of ∼9 × 10^7 cfu mL^{-1} of wine were used. All experiments were carried out in triplicate. A run with free bacteria at a concentration of ∼9 × 10^7 cfu mL^{-1} of wine was also carried. The L-malic acid concentration was determined at 96 h using an enzymatic kit (TDI, Spain), and three independent measurements were realized in each sample. Data were expressed as L-malic acid consumption (g L^{-1}).

Viable Bacteria Release from Capsules. Viable bacteria release from capsules was investigated using a previously published method.[25] A test was performed by immersing 20 capsules (∼0.3 g of capsules with a bacterial load of ∼3 × 10^9 cfu g^{-1} of gel) into 10 mL of sterile red wine under stirring (165 rpm) at 22 °C. The viable bacteria population in wine was tested in Difco Tomato Juice Agar (Difco) (Fluka) at 22 °C after 24 h of immersion using the serial dilution method. Two independent measurements were realized in each dilution sample.

Capsule Mechanical Resistance. Mechanical strength of 20 capsules submerged in sterile 0.2 M Ca_2Cl for 48 h at 22 °C was measured using a previously described method.[33] A double compression test was carried out using a Plus Texture Analyzer-Upgrade (Stable Microsystems, Surrey, U.K.) with a cylindrical probe of 6 mm in diameter. The strength (in grams) required to achieve deformation of 50% capsule size was measured and data were analyzed using Exponent Lite software (v.4.0.13.0, Surrey, U.K.).

Capsule Stability. Capsule stability was carried out in capsules performed with the interpenetrated polymer network method, applying the following conditions of bacteria encapsulation: immobilization pH of 5.6, 0.06 M of sodium silicate, 1.23 M of colloidal silica, and bacterial load of ∼3 × 10^9 cfu g^{-1} of gel. The capsule stability was examined submerging 15 capsules in 10 mL of water solutions with different pH (3.0, 3.3, 3.6, and 3.9) and ethanol concentrations (10, 12, 14 and 16%) and also in red wine (pH 3.2 and 14% of alcohol degree) at 22 °C during 168 h. The capsule weight was determined using a semimicro balance with a readability of 0.1 mg (AS 220/C/2, Radway, Brancka, Poland). The capsule diameter was measured by Software ImageJ 1.47v (National Institute of Health, Bethesda, MD). The alginate release from capsules was analyzed as total polysaccharides using the method proposed by Segarra et al.[34] A calibration curve was built using sodium alginate as a patron (1−25 mg L^{-1}) and three independent measurements were realized in each sample. All experiments were carried out in triplicate.

Statistical Analysis. SPSS v. 17.0 statistical package (SPPS Inc., Chicago, IL) was used for all statistical analyses. Statistical differences were determined by performing a one-way ANOVA followed by a Tukey's posthoc test ($p < 0.05$). In figures, error bars were calculated as standard deviations.

RESULTS AND DISCUSSION

Bacteria Encapsulation Using Interpenetrated Polymer Network Method. Table 1 shows the amount of consumed L-malic acid after 96 h of incubation for the different types of silica-alginate capsules in the red wine. The trial with free bacteria reached a consumption of 2.96 ± 0.01 g L^{-1} of L-malic acid after 96 h of incubation. The Pareto graphic (Figure 1A) shows the statistical significance of immobilizing pH and colloidal silica concentration for L-malic acid consumption ($p < 0.05$), while the inclusion of sodium silicate into alginate gel did not modify the consumption of L-malic acid. Both factors (pH and colloidal silica) presented an estimated positive effect, so that their increase improves the consumption of L-malic acid. Response surface for L-malic acid consumption as a three-dimensional plot of colloidal silica and pH factors is shown in Figure 1B. As colloidal silica concentration increased, consumption of L-malic acid was enhanced, reaching a local maximum (3.0 g L^{-1} of L-malic acid) at pH 6.4 and 1.23 M colloidal silica. Experiments with vacant silica-alginate and

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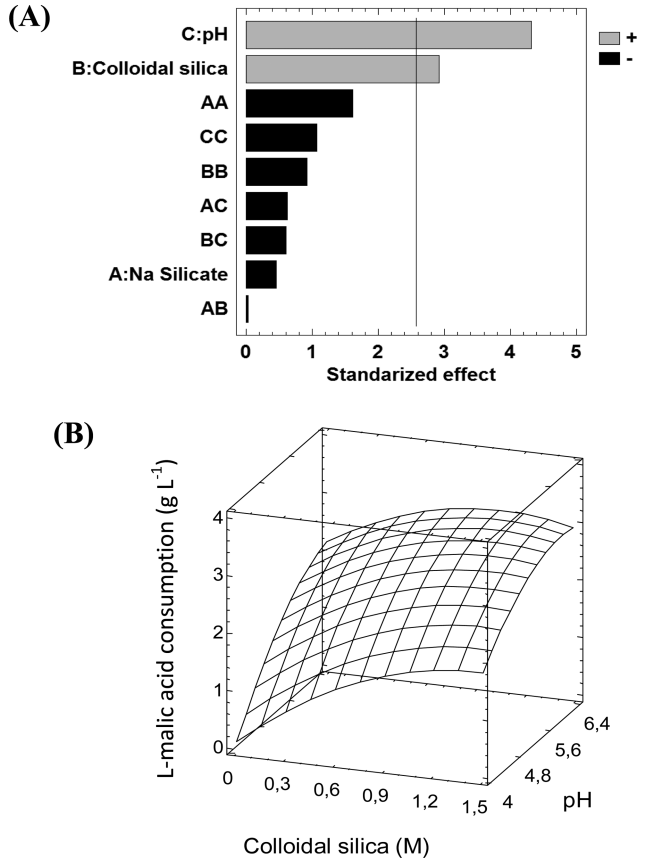

Figure 1. (A) Pareto graphic of the effects calculated for the L-malic acid consumption by encapsulated *O. oeni* using the interpenetrated polymer network method. (B) Response surface plot for the L-malic acid consumption by encapsulated *O. oeni* using the interpenetrated polymer network method as a function of colloid silica concentration (0.00−1.23) and immobilization pH (4.00−6.30) (sodium silicate concentration of 0.04 M). MLF conditions: red wine (3.0 g L^{-1} L-malic acid, pH 3.2, 14% (v/v) of alcohol degree), 0.03 g of capsules mL^{-1} of wine, bacterial load of ~9 × 10^7 cfu mL^{-1} of wine, temperature of 22 °C, and 96 h of incubation.

alginate capsules (without bacteria) have shown that they were unable to reduce the L-malic acid concentration in red wine after 96 h of incubation. pH of bacterial cytoplasm must remain close to neutrality to avoid destruction of macromolecules. To balance the pH gradient between bacteria and wine, bacteria must make an effort to buffer their intracellular pH, thus leading to overexpression of bacterial stress proteins.[35] Furthermore, to preserve cytosolic homeostasis, bacteria have to use their proton pump linked to ATPase.[36] A more alkaline extracellular environment could be supposed in those capsules designed in less acidic pH, which could contribute to the reduction of this stress, so that bacteria would be able to develop their metabolism more comfortably. According to this, other studies have concluded that immobilization pH should be close to the physiological one in order to reduce the potential damage of bacteria during immobilization.[37] Inclusion of siliceous materials as colloidal silicate into alginate gel improved the consumption of L-malic acid, probably due to an increase of the tolerance of bacteria toward harsh conditions of wine (low pH, high ethanol concentration, and low nutrients). It has been described that the partial replacement of sodium silicate by colloidal silicate reduces the environmental stress caused to bacteria, decreasing the cytotoxicity of sodium ions.[37]

Previously, the variability of selected compression test was tested measuring the mechanical strength of 50 capsules of biocomposites 8 and 10. Low variability of data was achieved (coefficients of variation lower than 3.7%), indicating that this compression test was appropriate to quantify this parameter.

An increase in mechanical strength of the different silica-alginate capsules compared to alginate capsules (capsule 1) was observed. This rise was statistically significant for all biocomposites except for 9 and 11 (capsules characterized by low concentration of sodium silicate and without colloidal silica) ($p < 0.05$). The capsule with the highest mechanical strength was 4, followed by 6, 10, and 13. At the tested levels (Figure 2A), only the concentration of sodium silicate and

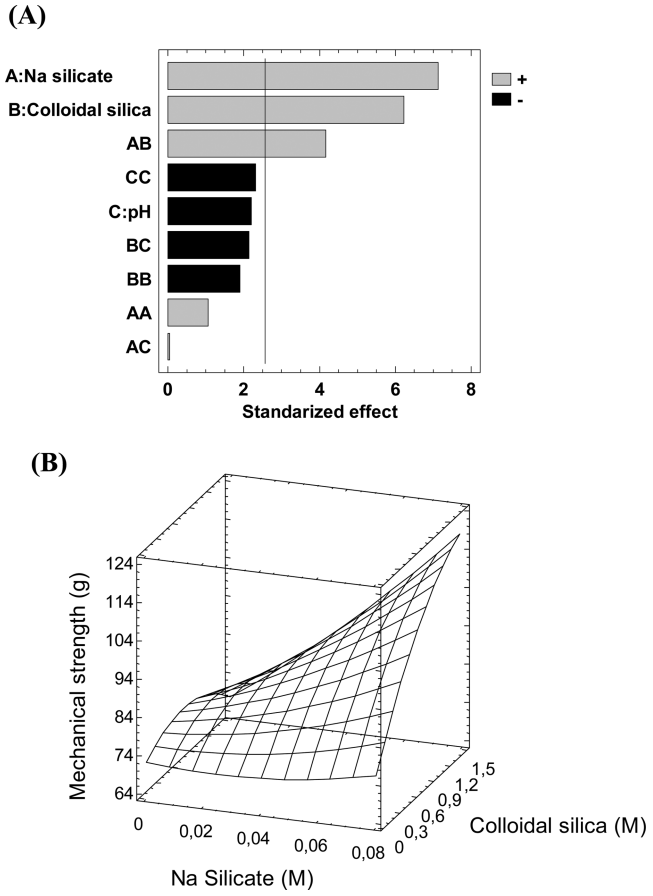

Figure 2. (A) Pareto graphic of the effects calculated for mechanical strength of capsules. (B). Response surface plot for mechanical strength of capsules as a function of sodium silicate (0.00−0.08 M) and colloid silica concentrations (0.00−1.23) (at pH of 5.15). Encapsulation of *O. oeni* using the interpenetrated polymer network method. Experimental conditions: mechanical strength was tested in 20 capsules submerged in sterile 0.2 M Ca_2Cl for 48 h at 22 °C.

colloidal silica and their interaction were statistically significant for this response. Both principal factors had a positive effect, so that an increase in concentration of sodium silicate and colloidal silica improves the gel strength. As shown in Figure 2B, a local optimum was found at the highest concentrations of sodium silicate (0.08 M) and colloidal silica (0.23 M), achieving a mechanical strength of 114.6 g. Moreover, this response was affected by the interaction of both principal factors. At lower levels of colloidal silica, the increase of sodium silicate concentration slightly improved the capsules strength. An

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increase of sodium silicate concentration from 0.00 to 0.08 M enhanced the mechanical strength in 4.1 g in absence of colloidal silica, so mechanical strength reached 44.7 g at 1.23 M colloidal silica. These results confirm that preformed structures of colloidal silica actively participate in the building of SiO_2 networks performed with silicates,[21] enhancing their strength.

Preliminary works have demonstrated that covalent inorganic networks are formed due to the presence of silicate and colloidal silica that strengthen silica-alginate biocomposites.[23] Siloxanes (Si−O−Si) are formed by condensation of silanol groups of silicate (Si−OH), which later are bonded to new silanol groups to produce SiO_2 gels by polycondensation,[27] improving mechanical and chemical stability of silica-alginate gels.[24] Furthermore, the presence of colloidal silica acts as a filler, enhancing matrix stability. Moreover, its rich in silanol groups surface takes part in the reactions of condensation and polycondensation during formation of SiO_2 network with silicates.[21] Finally, it has been described that the homogeneous distribution of silica in an alginate matrix increases significantly its mechanical properties.[38] These organized structures are formed through hydrogen bonds between free hydroxyl groups on the surface of silica particles and carboxyl groups of alginate, enhancing gel rigidity.[39]

Tests to count the released bacteria outside the capsules are shown in Table 1. Neither sodium silicate, colloidal silica, nor pH factors were significant at $p < 0.05$, indicating that the retention capacity of bacteria into capsules are independent of these factors at the levels assayed. In all cases, the percentage of leakage from the initial population was negligible (less than 0.001%). These results indicate the suitable behavior of these silica-alginate composites to bacteria retention.

Bacteria Immobilization Using Coating Method. Coating alginate capsules with hAPTES did not improve their mechanical robustness, with values of mechanical strength between 68.9 and 72.8 g (data not shown). Variance analysis of data showed that strength of hAPTES-treated capsules did not differ from untreated capsules (69.5 ± 4.1 g) and among them. These results do not agree with a recent study that reported that alginate-chitosan-alginate capsules treated with 0.75−3% hAPTES were significantly stronger than control ones.[31] Capsules treated with 3.0% and 1.5% hAPTES showed a 0% and 1% of capsule break, respectively, whereas about 25% of untreated capsules were broken after 6 h of vigorous agitation. Probably, the different tests applied could cause this disagreement. In our study, a compression test was performed, wherein the strength required to reach deformation of 50% capsule size was measured, while a mechanical shear test based on a strong agitation of capsules was applied by Ylitervo et al.[31] As discussed before, the compression test was able to detect the mechanical strength variability of alginate capsules with an interpenetrated polymer network of SiO_2, but it was not able to detect any difference among hAPTES-coated capsules.

L-malic acid consumption of hAPTES-treated capsules is shown in Table 2. hAPTES coating reduced the capsule capacity to metabolize L-malic acid compared to control capsules (with a consumption of 0.86 ± 0.04 g L^{-1}). Pareto graph showed that among three factors only hAPTES exposure time was statistically significant ($p < 0.05$) with a negative effect (Figure 3). Thus, a great reduction in the consumption of L-malic acid was observed increasing hAPTES exposure time. These results are in agreement with those found in alginate-chitosan-alginate capsules treated with hAPTES containing *Saccharomyces cerevisiae*.[31] In this study, untreated capsules showed the highest ethanol production while 0.75% and 1.5% hAPTES-treated capsules had less fermentation capacity. hAPTES not only affects yeast viability but also capsule permeability of low-weight molecules such as glucose and acetic acid. In our study, free bacteria (bacterial load of ∼9 × 10^7 cfu mL^{-1} of wine) exposed at 2.0% and 0.5% hAPTES at pH 5.5 during 60 min accomplished a L-malic acid conversion of about 98%. These results reflect a nontoxic effect of hAPTES on malolactic metabolism of bacteria at the concentrations assayed. On the basis of our results and those found by Ylitervo et al.,[31] the reduction in L-malic acid consumption may be explained by a reduction of L-malic acid transport through the hAPTES-treated capsules. Unfortunately, hAPTES coating of alginate capsules did improve neither their mechanical robustness nor their metabolic activity. Consequently, the method was dropped.

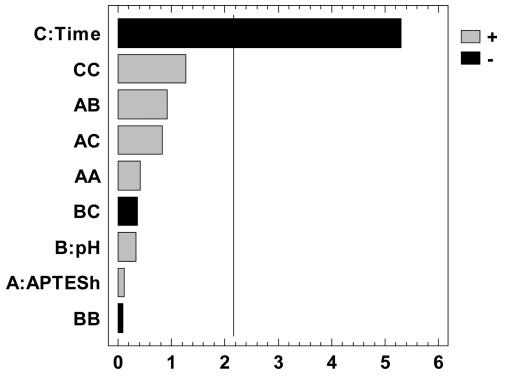

Figure 3. Pareto graphic of the effects calculated for the L-malic acid consumption. Encapsulation of *O. oeni* using the coating method with hAPTES. MLF conditions: red wine (3.0 g L^{-1} L-malic acid, pH 3.2, 14% (v/v) of alcohol degree), 0.03 g of capsules mL^{-1} of wine, bacterial load of ∼9 × 10^7 cfu mL^{-1} of wine, temperature of 22 °C, and 96 h of incubation.

Multiple Response Optimization. In order to search the better experimental conditions for bacteria encapsulation into interpenetrated polymer networks of silica-alginate that maximize consumption of L-malic acid and mechanical strength of capsules, a multiple response optimization was applied. Bacteria release response was omitted from this study because it was not affected by changes in the factors that we had studied. Multiple response optimization was carried out by developing the desirability function,[40] which provides the best value for bacteria encapsulation conditions ensuring compliance with the criteria of maximizing both responses. Several desirability functions can be built using different impact coefficients for both responses. Optimum values for each factor and response using different impact coefficients (1, 2, 3, 4, and 5) for each response are shown in Figure 4. For a better display of the results, the ratio of impact coefficients between L-malic acid consumption and mechanical strength (1/5, 1/4, 1/2, 1/1, 2/1, 3/1, 4/1, and 5/1) was represented in X-axe. Optimum value of colloidal silica concentration was 1.23 M at all ratios assayed. At a ratio of 1/1 (same weight for each response), optimum conditions were obtained at 0.077 M sodium silicate, 1.23 M colloidal silica, and pH 5.4. Under these conditions, theoretical consumption of L-malic acid and mechanical strength were 2.48 g L^{-1} and 111.1 g, respectively. At ratios >1 (higher weight of response consumption of L-malic acid than for mechanical strength), optimum levels of sodium silicate and pH that maximize both responses were lower and higher, respectively.

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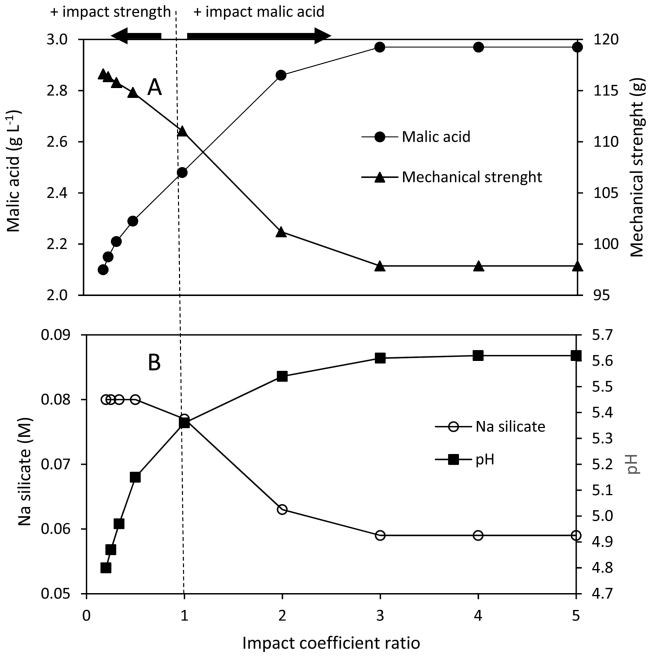

Figure 4. (A) Theoretical optimum values of L-malic acid consumption and mechanical strength obtained from the overall desirability function using different ratios between the impact coefficients for L-malic acid consumption and strength responses. (B) Values of sodium silicate concentration and pH that maximize the responses at the different ratios. The optimum value of colloidal silica concentration was 1.23 M for all ratios assayed.

At a ratio of 3/1 (winemaking conditions where capsules with great metabolic capacity are necessary and their mechanical resistance has less importance), most of the L-malic acid would be transformed (theoretical L-malic acid consumption of 2.97 g L^{-1}) and a strength of 98 g would be observed (corresponding with 0.06 M sodium silicate and 1.23 M colloidal silica at pH 5.6). Sometimes, MLF could take place in configurations where mechanical resistance of capsules is critical (e.g., continuous systems of fermentation in packed-bed column reactor). At a ratio of 1/4, a consumption of 2.15 g L^{-1} of L-malic acid and a strength of 116.4 g could be achieved using 0.08 M sodium silicate, 1.23 M colloidal silica, and a pH 4.9.

Capsule Stability. The effect of siliceous materials on the stability of alginate capsules (made with the interpenetrated polymer network method) was studied in water solutions at different pH and ethanol concentrations and also in red wine. Figure 5 shows an increase in total polysaccharides at the different pH assayed for both alginate and silica-alginate capsules. This rise could be due to the diffusion of unpolymerized alginate from the inside of capsules to wine. The procedure used to form these capsules involves an external polymerization of a drop of sodium alginate as Ca^{2+} diffuses into alginate capsules.[41] Gels with inhomogeneous structure along the diameters of the capsule are built by this gelation mechanism, so the appearing alginate molecules that do not form stable junction zones with Ca^{2+} enable the release out of capsules.[42] In general, this release of alginate was lower in silica-alginate capsules than in untreated ones to 50 h of exposure. Scanning electron micrographs of internal structure of silica-

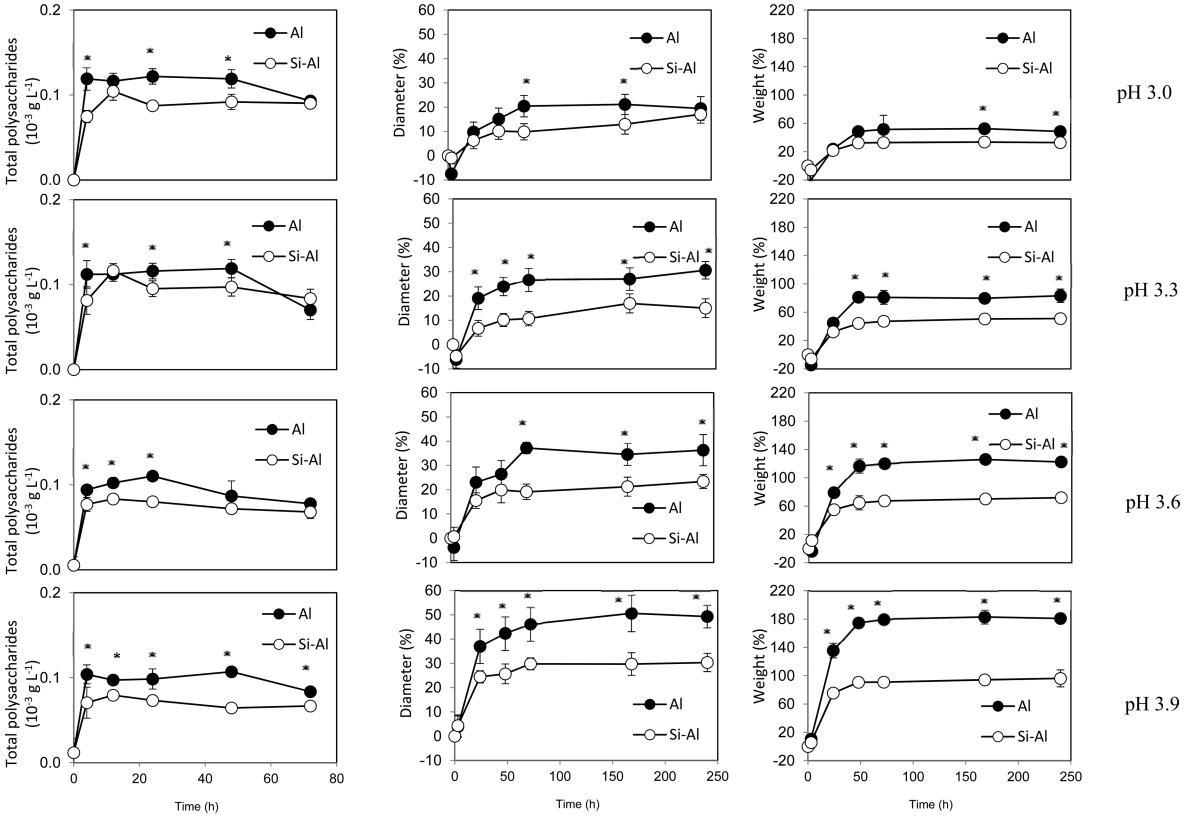

Figure 5. Evolution of release of alginate (expressed as total polysaccharides in 10^{-3} g L^{-1} of sodium alginate) and swelling (expressed as increase of diameter (in %) and weight (in %)) of alginate (Al) and silica-alginate (Si–Al) capsules loaded with bacteria submerged in aqueous solutions at different pH (3.0, 3.3, 3.6, and 3.9). Standard deviations of the assays are represented by error bars. ∗ indicates a statistically significant difference ($p < 0.05$) between both capsules.

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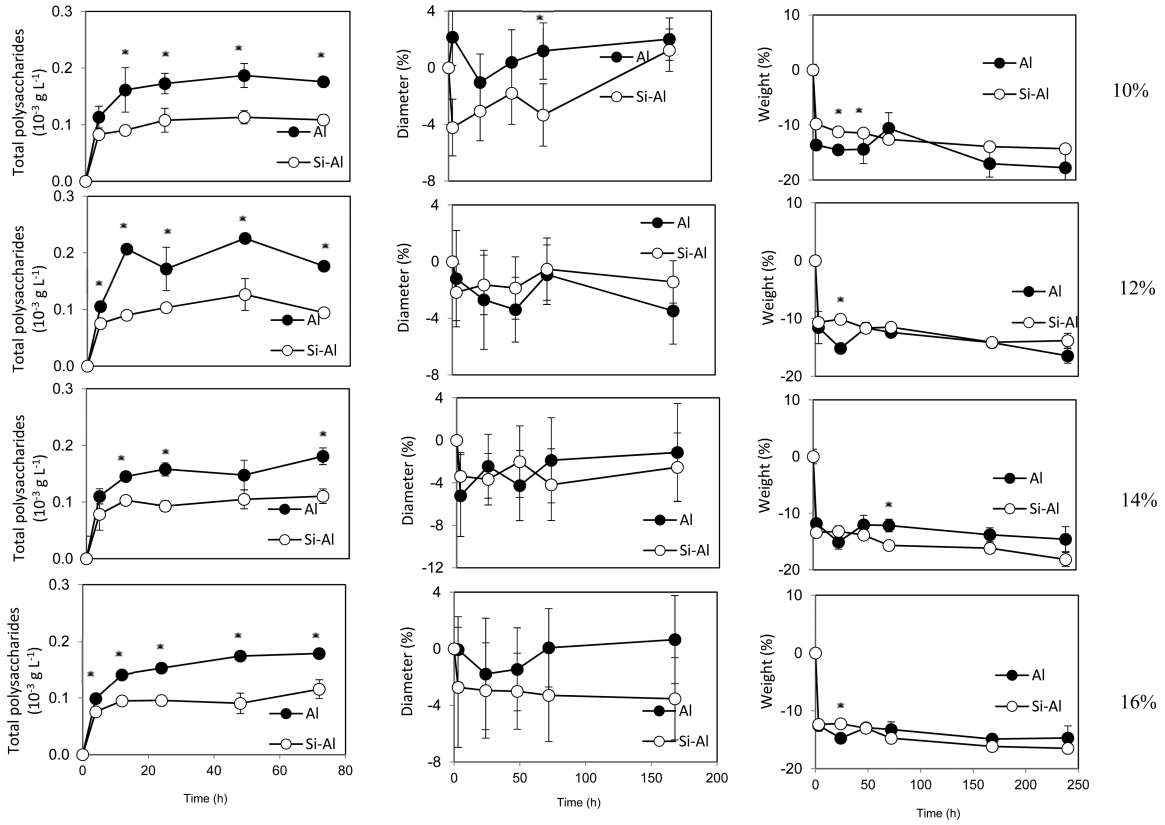

Figure 6. Evolution of release of alginate (expressed as total polysaccharides in 10^{-3} g L^{-1} of sodium alginate) and shrinking (expressed as decrease of diameter (in %) and weight (in %)) of alginate (Al) and silica-alginate (Si−Al) capsules loaded with bacteria submerged in mixtures of ethanol−water (10, 12, 14, and 16%). Standard deviations of the assays are represented by error bars. ∗ indicates a statistically significant difference ($p < 0.05$) between both capsules.

alginate gels have highlighted that the pores of the alginate matrix are filled with the inorganic structures of polymerized silicates, reducing their pore size.[29] Adsorption of silica on the surface of alginate could hinder the diffusion of unpolymerized alginate outside of the capsules. It was noted that the presence of an inorganic network of silica into alginate gels impaired diffusion properties of large biomolecules, such as bovine serum albumin and γ-globulin.[23] After 72 h of exposure, statistically significant differences were not detected between both capsules (apart from pH 3.9).

In order to check diameter uniformity of both alginate and silica-alginate capsules, the diameter of 30 capsules of each type was determined. On one hand, the coefficients of variation for the diameter were small (3.8 and 2.9% for alginate and silica-alginate capsules, respectively), which demonstrated that the diameter variability was slight. These results indicated that the extrusion method employed in this study is suitable to build capsules of diameter uniformity. On the other hand, the presence of an inorganic network of silica involved a slight increase of diameter of silica-alginate capsules (3.54 ± 0.10 mm), compared to alginate capsules (3.17 ± 0.12 mm). In an extrusion process, capsule diameter depends, among other factors, on viscosity of polymer solution,[43] and solution viscosity was higher in biocomposites of silica-alginate than alginate ones. Increases of 50% and 180% of diameter and weight of alginate capsules were observed at pH 3.9 after 72 h of immersion, respectively, while they were only about 30% and 85%, for silica-alginate capsules, respectively. These results support that the inclusion of SiO_2 into the alginate matrix reduces the elasticity and the swelling of the capsules. Interestingly, the pH change had an important effect on capsule diameter and weight for both types of matrix. The highest increases in capsule diameter and weight were monitored at the low acid pH (pH of 3.9) (Figure 5). In studies of alginate gel stability in acid water solutions, Mumper et al.[44] reported that reducing the pH of 4 and 1, a reduction of the capsule diameter was observed. At a pH value lower than the pK_a of mannuronic and guluronic acids (3.38 and 3.36, respectively), the carboxylic acids of these acids are undissociated,[45] and a more compact gel structure is achieved due to the reduction of the electrostatic repulsions between the polymers of alginate.[46] Finally, the differences in diameter and weight between alginate and silica-alginate capsules after 240 h of exposure are higher when increasing the value of pH.

As shown in Figure 6, an increase of total polysaccharide concentration was found for both capsules at all ethanol concentrations assayed. As it was mentioned before, the diffusion of unpolymerized alginate from inside the capsules could be the origin of this rise. Regardless of ethanol concentration, polysaccharide concentration in batches with alginate capsules was higher than that with silica-alginate capsules. In general, the immersion of both alginate and silica-alginate capsules into ethanol−water mixtures reduced their capsule diameter and weight. Torres et al.[47] reported a decrease of 15.2% of the volume of alginate capsules (initial diameter of 3.13 mm) after 4 h of exposition at 20% of ethanol. These authors suggested that a dehydration of capsules took place in water−ethanol mixtures. No statistically significant differences

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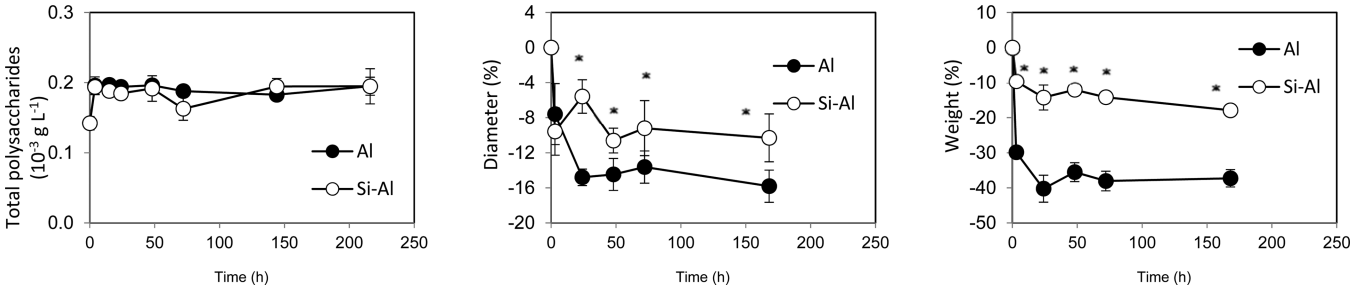

Figure 7. Evolution of release of alginate (expressed as total polysaccharides in 10^{-3} g L^{-1} of sodium alginate) and shrinking (expressed as decrease of diameter (in %) and weight (in %)) of alginate (Al) and silica-alginate (Si−Al) capsules loaded with bacteria submerged in red wines. Standard deviations of the assays are represented by error bars. ∗ indicates a statistically significant difference ($p < 0.05$) between both capsules.

were found in the capsule diameter and weight between alginate and silica-alginate capsules at 240 h of exposure.

A slight increase of total polysaccharides was detected for both capsules at the first 4 h of exposition in wine (Figure 7). After this point (4 h), stabilization in alginate release was observed, not showing significant differences between both capsules at the end of the experiment. The evolution of diameter and weight (expressed as relative percentage) of both alginate and silica-alginate capsules are shown in Figure 7. For both capsules, a reduction of diameter and weight was monitored during their immersion into red wine, being more notable during the first 4 h. These capsules are hydrogels formed mainly by water (between 96 and 99%) and tend to be dehydrated in the presence of alcohols such as ethanol and isopropyl alcohol.[47] Moreover, the decrease in diameter and weight was lower for silica-alginate capsules than for alginate capsules. At 168 h, a decline of diameter of 10.3% and 15.2% was observed for silica-alginate and alginate capsules, respectively. Weight differences between both capsules were more pronounced, showing a decrease of 17.8% and 37.2% for silica-alginate and alginate capsules, respectively. As it was mentioned before, silica-alginate capsules could be less sensible to changes in size due to the presence of the inorganic network that causes more rigidity and resistance to dehydration. On one hand, the incorporation of siliceous products provides chemical and mechanical stability to the capsules of alginate.[23] The addition of sodium silicate leads to the formation of an inorganic network through condensation of silanol groups.[27] The resulted gel presents covalent bond of Si−O, which ensures both inertness and chemical stability.[24] On the other hand, the inclusion of nanoparticles of colloidal silica as filler agent improves the stability of the capsules. Moreover, the silanol surface groups of these nanoparticles can participate in the process of condensation of the SiO_2 matrix.[23]

On the basis of the results of capsule stability in ethanol−water mixtures and at different pH discussed before, it is quite likely that increasing the wine pH, the capsules suffer a lower decrease in diameter and weight, while increasing wine alcohol degree, diameter, and weight are less affected. In order to verify these affirmations, further studies focusing on capsule stability in wines with different alcoholic degree and pH are in progress.

O. oeni was successfully entrapped into capsules based on interpenetrated polymer networks of silicate/silica and alginate. Control of pH and colloidal silica concentration during bacteria encapsulation was crucial to enhance cell fermentation behavior. Inclusion of silicates and colloidal silica markedly improved mechanical robustness of capsules. Silica-alginate capsules also showed effective retention of bacteria. Multiple response optimization was a suitable statistical tool to obtain the optimum conditions of bacteria encapsulation that simultaneously maximized the consumption of L-malic acid in red wines and the mechanical robustness of silica-alginate capsules. Silica-alginate capsule stability was dependent on pH and ethanol concentration in aqueous solutions. An increase of weight and swelling capsules was showed at higher pH (from 3.0 to 3.9), with this behavior more intense for alginate capsules than for silica-alginate ones. Capsule submersion into water−ethanol solutions caused a reduction in their weight and swelling. Silica-alginate capsules were more stable in wines than alginate ones. Regarding the coating method, hAPTES-treated alginate capsules had an inferior fermentation behavior and a similar mechanical robustness than those untreated. Silica-alginate capsules made with interpenetrated polymer networks of silica and alginate demonstrated high mechanical robustness and stability, excellent consumption of L-malic acid, and effective bacteria entrapment. These results confirm the effectiveness of this bacteria encapsulation method and suggest its implementation to develop MLF in batch or continuous systems and at a larger scale of winemaking.

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Notes

The authors declare no competing financial interest.

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CAPÍTULO 3

6. CAPÍTULO 3: Caracterización de la fermentación maloláctica llevada a cabo por *O. oeni* inmovilizada.

- **6.1. Silica-alginate encapsulated bacteria to enhance malolactic fermentation performance in stressful environment**

- **6.2. Effect of stressful malolactic fermentation conditions on the operational and chemical stability of silica-alginate encapsulated *Oenococcus oeni***

Silica–alginate-encapsulated bacteria to enhance malolactic fermentation performance in a stressful environment

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Abstract

Background and Aims: Beginning and completion of malolactic fermentation (MLF) are complicated because of the harsh environment of wine. Encapsulation of lactic acid bacteria could be an attractive strategy to improve their activity and stability. This work evaluates the fermentative capacity of *Oenococcus oeni* encapsulated into silica–alginate (Si–Al) and alginate capsules under adverse winemaking conditions (high alcohol concentration, low pH and low temperature). Mechanical and chemical stability of capsules was also monitored.

Methods and Results: Silica–alginate capsules showed higher malolactic activity and better mechanical and chemical stability than untreated alginate capsules. Their capacity for bacteria retention was similar to that of alginate capsules. Ethanol, pH and fermentation temperature had great influence on fermentative behaviour of free and encapsulated bacteria. Under extreme winemaking conditions, such as high ethanol concentration (13–16%), low pH (3.0–3.3) and low fermentation temperature (13–15°C), the highest consumption of malic acid was achieved using Si–Al capsules. Free and encapsulated bacteria showed similar fermentative profiles in the presence of variable concentration of malic acid and nitrogen in wine.

Conclusions: Encapsulated *O. oeni* in Si–Al gels enhanced MLF in wines with a high concentration of ethanol and low pH and at low fermentation temperature. Moreover, inclusion of an inorganic network of silicates significantly improved the mechanical robustness and chemical stability of alginate capsules.

Significance of the Study: The application of Si–Al-encapsulated bacteria is an interesting strategy to improve MLF in warm climates where wines have higher ethanol concentration and in cool climates with higher acidity and low fermentation temperature.

Keywords*: biocomposites, immobilisation, malolactic fermentation,* Oenococcus oeni*, silica–alginate gel*

Introduction

Malolactic fermentation (MLF) in wines involves transformation of malic acid with final production of lactic acid. Agents developing MLF are lactic acid bacteria (LAB) and especially the species *Oenococcus oeni* (Bauer and Dicks 2004). Malolactic fermentation is difficult to control, and the development of LAB in wine is quite complicated because wine is a rather unfavourable substrate for their growth and metabolism. Besides, climate change is causing an increase in temperature in warm latitudes, which is leading to a rise of sugars in grape must, which in turn results in unbalanced wines with higher alcohol concentration (Vila-Crespo et al. 2010). For this reason, LAB responsible for MLF have even greater difficulty to develop the process (Lerm et al. 2010). Because of these circumstances, the need to improve the conditions for the adaptation of *O. oeni* to this stressful environment arises as an attempt to improve the process of MLF.

Malolactic fermentation usually occurs after alcoholic fermentation. It has been previously reported that at low population, transformation of malic acid by LAB does not occur, requiring a bacterial population in wine of about 10^6 CFU/mL for starting the conversion of malic acid (Vila-Crespo et al. 2010). The population of LAB, which remains low during alcoholic fermentation, increases slowly after this phase, generating a lag period between the end of alcoholic fermentation and the beginning of MLF. For 1 to 3 weeks, until the bacteria population is optimal to start MLF, there is a high risk of undesirable alterations to the wine. Therefore, the use of a technique that allows the inoculation of LAB at high concentration during this period would reduce this lag period.

Several inoculation techniques for LAB can be applied in wine: (i) addition of a given volume of wine in the process of MLF, although it could cause changes in the sensory characters of the wine; (ii) addition of healthy wine lees that have undergone MLF; (iii) addition of an inoculum of autochthonous or selected LAB; (iv) use of yeasts that are able to degrade malic acid; or (v) use of immobilised bacteria (Hidalgo 2003, Zhang and Lovitt 2006, Lerm et al. 2010, Vila-Crespo et al. 2010, Sumby et al. 2014).

Immobilisation of bacteria could present several advantages for the development of MLF, such as (i) increase of LAB stability and activity against the harsh conditions of wine (high ethanol concentration, low pH and low nutrient); (ii) inoculation of encapsulated bacteria at a higher cell density enabling the reduction of the lag phase, enhancing the process productivity and decreasing the risk of microbial contamination; (iii) development of continuous fermentation systems; and (iv) recovery of encapsulated bacteria making possible the reduction of the SO_2 level, a potential allergen (Kourkoutas et al. 2004, Zhang and Lovitt 2006). It has been reported that the performance of immobilised LAB in the degradation of malic acid is high and can exceed even that of free cells (Kosseva et al. 1998). Furthermore, the addition of

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immobilised LAB directly into wine makes them suitable for several consecutive cycles with good yields (Agouridis et al. 2008, Rodríguez-Nogales et al. 2013).

Immobilisation of LAB on a matrix surface and encapsulation into a porous matrix have both been proposed for undertaking MLF in wine (Kourkoutas et al. 2004, Vila-Crespo et al. 2010, Sumby et al. 2014). Mainly, immobilisation of LAB has been tested in organic materials (alginate, carrageenan, calcium pectate gel, chitosan, cellulose sponge, calcium alginate, oak chips and delignified cellulosic material) (Vila-Crespo et al. 2010). Among them, the most popular support for cell immobilisation is calcium alginate gel, which has the advantages of being easy to prepare, is economical and immobilisation conditions are mild (Ching et al. 2017). Moreover, the encapsulation of cells into hydrogels, such as calcium alginate gel, can offer protection to the inside of the cells from stressful environment by an external layer of sacrificial cells (Sun et al. 2007). Interestingly, a recent study reported co-immobilisation of *Saccharomyces cerevisiae* and *O. oeni* in alginate beads as a strategy to carry out simultaneous alcoholic and MLF during winemaking (Bleve et al. 2016). Data showed an improvement in yeast and bacterial activity after immobilisation in comparison with that of free cells.

Although the possibility of immobilising LAB such as *O. oeni* in alginate gel has already been studied, the inconsistent mechanical and chemical properties of this support make attractive the search of new alternatives to improve the stability of the gel and to enhance its commercial applicability (Callone et al. 2008, Guzzon et al. 2012). An alternative to solve this problem is the development of biocomposites of silica–alginate (Si–Al) gels, because the inorganic component promotes an increase in the stability of the gel (Coradin et al. 2006). An ethanol/water solution of tetraethoxysilane (TEOS) or/and methyltriethoxylsilane (MTES) in the gas phase were employed to coat alginate capsules loaded with *O. oeni*. Mechanical stability of the capsules was improved; however, malolactic activity was lower with the TEOS–alginate capsules than that for free cells. Alginate capsules coated with MTES and MTES–TEOS were more efficient in developing MLF (Callone et al. 2008, Guzzon et al. 2012).

Development of silica matrices is possible because of recent advances in sol–gel chemistry allowing the development of gels at room temperature using silica precursors, such as silicon alkoxides. These compounds [$Si(OR)_4$] contain an organic group (e.g. R: CH_3, C_2H_5) that in contact with water undergoes hydrolysis releasing a molecule of alcohol (ROH) and creating a silanol group (SiOH). Then, polycondensation occurs between silanol groups. Over time, polycondensation produces a three-dimensional structure that extends through the liquid medium, resulting in a gel (Coradin et al. 2003). Alkoxides have been previously studied for cell immobilisation; however, their applicability is limited because these precursors are expensive, alcohols generated during hydrolysis of alkoxides can cause cellular stress, and an alternative alcohol extraction involves the use of expensive solvents that can be toxic to cells. An aqueous route has recently been developed on the basis of the use of salts of silicates and colloidal silica, which are cheaper and less aggressive for cells (Kuncová and Trogl 2010). This route has still not been explored for immobilisation of LAB involved in the conduct of MLF.

The aim of this study was to investigate the behaviour of biocomposites of Si–Al as a support for the encapsulation of LAB to develop MLF in red wines. Physicochemical and biological properties of the capsules and performance of MLF under adverse conditions of winemaking (high alcohol concentration and low pH, temperature and nutrient content) have been studied.

Material and methods

Immobilisation of Oenococcus oeni

Alginate and Si–Al gels were employed for the immobilisation of *O. oeni*. To immobilise bacteria into the Si–Al support, an aqueous route based on the mixture of derivatives of silicon (sodium silicate and colloidal silica) with alginate before gelation in the presence of Ca^{2+} was used (Coradin et al. 2003). The method proposed by Trivedi et al. (2008) with modifications was followed under aseptic conditions: the pH of a solution of 0.06 mol/L sodium silicate and 1.23 mol/L colloidal silica (Ludox HS40, Sigma, St Louis, MO, USA) in water was adjusted to 6.29 by adding 2 mol/L HCl. Then, sodium alginate was added to the silica solution until a final concentration of 2% (w/v) was obtained. Finally, bacteria *O. oeni* (LALVIN VP 41 MBR, Lallemand, Montréal, QC, Canada) was added at ~3×10^9 CFU/g of gel. Ten millilitres of this suspension was added dropwise from a sterile syringe into 200 mL of sterile 0.2 mol/L $CaCl_2$ solution under continuous agitation (260 rpm). The capsules were maintained in this solution for 2 h and washed with sterile water (100 mL). The procedure for bacteria entrapment in the alginate gel was similar to that described earlier but omitting the addition of derivatives of silicon.

Malolactic activity of encapsulated cells

Red wine of Tinta de Toro cultivar with a concentration of 2.9 g/L malic acid, pH 3.2 and alcohol concentration of 14% (v/v) was sterilised by filtration through a 0.2μm membrane. Working conditions were the following: gel concentration ~0.03 g/mL of wine at 22°C and bacterial load ~9×10^7 CFU/mL of wine. Consumption of malic acid was evaluated at 96 h of fermentation. The experiment was in triplicate.

Cell release from capsules

The ability of the matrix to retain the immobilised LAB was measured by a modified method proposed by Guzzon et al. (2012): 20 capsules (~0.3 g of capsules with a bacterial load of ~3×10^9 CFU/g of gel) were introduced into 10 mL of sterile red wine with stirring (165 rpm) at 22°C. After 24 h, the population of bacteria in the wine was determined on Difco Tomato Juice Agar (BD/Difco, Sparks, MD, USA) at 22°C. All experiments were in triplicate.

Mechanical resistance of capsules

Mechanical strength of capsules was evaluated by texture profile analysis, a double-compression test in which the strength required to reach deformation of 50% bead size is measured (Serp et al. 2000). For this, a Plus Texture Analyser-Upgrade (Stable Micro Systems, Godalming, England) with a cylindrical probe of 6 mm in diameter accompanied by Exponent Lite software (Stable Micro Systems) to work with the data obtained were used. This parameter was tested in 20 capsules preserved in sterile 0.2 mol/L Ca_2Cl for 48 h at 22°C.

Stability of capsules in red wine

The stability of alginate and Si–Al capsules was determined by submerging 15 capsules with immobilised bacteria in red wine at 22°C (experiments in duplicate). Their mass and diameter were evaluated at 168 h. Capsule diameter was evaluated with software ImageJ 1.47v (National Institutes of Health, Bethesda, MD, USA). Alginate and Si–Al capsules displayed an average

diameter of 3.17 ± 0.12 and 3.54 ± 0.10 mm, respectively. The release of alginate from capsules was determined by measuring the total polysaccharides in wine at 168 h using the method proposed by Segarra et al. (1995). A series of alginate solutions in water with a concentration between 1 and 25 µg/mL was prepared and measured to build the calibration curve. All experiments were in triplicate.

Malolactic fermentation under different winemaking conditions

Malolactic fermentation was monitored in red wine under a range of winemaking conditions, including pH, concentration of ethanol, malic acid and free amino nitrogen (FAN) and fermentation temperature. Red wine from Tinta de Toro grapes was partially dealcoholised and concentrated to 70% of its original volume under reduced pressure in a rotary evaporator. The concentrated wine had the following composition: pH 3.4; malic acid 1.8 g/L; FAN 52 mg/L; alcohol 0.8% (v/v); and free SO_2 0 mg/L. The composition of this wine was modified in order to obtain the required experimental conditions as follows: values of pH 3.0, 3.2, 3.4, 3.6, 3.8 and 4.0 were obtained by adjusting wine pH using acid or basic concentrated solutions; ethanol was added to wine until a final concentration of 11, 12, 13, 14, 15 and 16% (v/v) was achieved; FAN was adjusted to 52, 100, 150, 200 and 250 g/L with diammonium hydrogen phosphate (Panreac, Barcelona, Spain), and the concentration of malic acid was adjusted to 2, 3, 4 and 5 g/L with L-malic acid. Wines were sterilised by filtration through a 0.2µm-cellulose nitrate membrane under sterile conditions. Finally, MLF was run at 13, 16, 19 and 22°C. Alginate and Si–Al capsules were placed in red wine at a concentration of about 0.03 g/mL of wine. Density of free and immobilised bacteria was regulated to about 9×10^7 CFU/mL of wine. The progress of MLF was monitored in triplicate by the evaluation of the concentration of malic acid at 72 h.

Analytical methods

The concentration of FAN was determined with the formol titration method (Shively and Henick-Kling 2001). Alcohol was measured with an ebulliometer (GAP, Barcelona, Spain), SO_2 with a SO_2-Matic 23 (Crison Instruments, Barcelona, Spain), and the concentration of malic acid was determined with an enzyme kit (TDI, Barcelona, Spain). All analyses were in triplicate.

Statistical analysis

Analysis of variance of the experimental data was performed at a significant level of $P = 0.05$ using the SPSS v. 17.0 statistical package (SPPS, Chicago, IL, USA).

Results and discussion

Characterisation of alginate and Si–Al capsules

Biological and physicochemical characterisation of alginate and Si–Al capsules are shown in Figure 1. Malolactic activity of bacteria encapsulated into Si–Al capsules was notably higher than that observed in alginate capsules. Specifically, consumption of 93% of the initial concentration of malic acid was achieved after 96 h of fermentation using Si–Al capsules, in comparison with 23% obtained for alginate capsules (Figure 1a). The presence of an inorganic network of silicates notably improves the consumption of malic acid, probably because of an increase of the tolerance of bacteria towards harsh conditions of wine. The effect of these conditions on malolactic activity of free and encapsulated *O. oeni* will be discussed later. A significant difference for the concentration

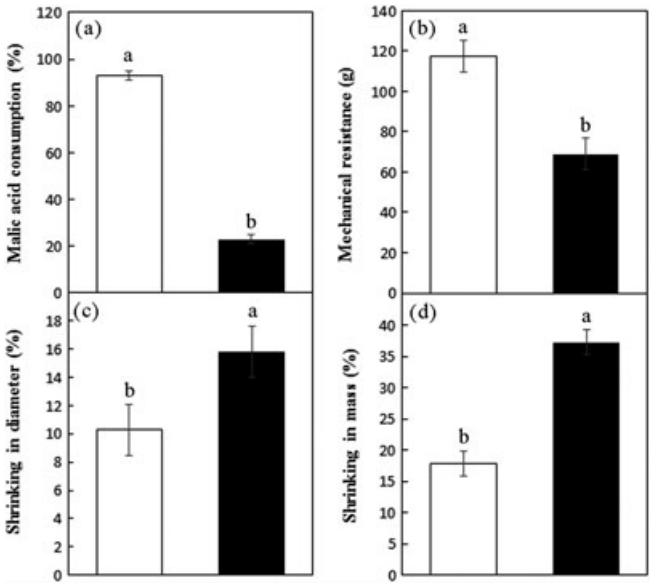

Figure 1. Physicochemical and biological properties of alginate (■) and silica–alginate (□) capsules with encapsulated *Oenococcus oeni*. Different letters in the bars indicate a significant difference between capsules ($P < 0.05$). (a) Malolactic activity expressed as consumption of malic acid (in %) at 96 h of fermentation (initial malic acid concentration 3 g/L) ($n = 3$). (b) Mechanical resistance was calculated on the average of 20 capsules submerged in 0.2 mol/L Ca_2Cl at 22°C for 48 h. (c) Shrinkage in diameter and (d) in mass was calculated from the average of 15 capsules submerged in red wine at 22°C for 168 h (experiments in duplicate). Error bars represent SE.

of malic acid between a wine inoculated with vacant Si–Al capsules (without bacteria) (2.85 ± 0.02 g/L) and an uninoculated wine (2.89 ± 0.03 g/L) was not observed at 96 h, indicating that the concentration of malic acid was not reduced in the absence of bacteria and/or by vacant Si–Al capsules. An identical result was found between a wine inoculated with vacant alginate capsules (2.90 ± 0.03 g/L) and the uninoculated wine.

The mechanical resistance of Si–Al capsules was 117.6 ± 8.3 g, an increase of 70.7% compared with that of alginate capsules (68.9 ± 2.7 g) (Figure 1b). Xu et al. (2006) indicated that the homogeneous distribution of silica particles in an alginate matrix can increase its mechanical properties significantly. The surface of silicate particles contains many free hydroxyl groups (OH) that can form hydrogen bonds with the carboxyl groups (COOH) of alginate forming organised structures that give rigidity to the gel (Liu et al. 2013). Moreover, the presence of cross-linking silicates leads to the formation of an extensive network of covalent inorganic nature (Coradin et al. 2003) that can strengthen the mixed Si–Al gel.

In order to test if a partial disintegration of the capsules had taken place, an aliquot of wine was taken after 168 h of capsule–wine contact and the alginate concentration was determined. A small release of 0.052 µg/mL of alginate was detected for both capsules, corresponding to about a 0.015% of the initial alginate concentration of the capsules. These results show that chemical decomposition of capsules did not take place under these experimental conditions. This negligible release of alginate can be because of the diffusion of unpolymerised alginate from the inside of the capsules to the wine (Strand et al. 2004).

The stability of capsules with entrapped bacteria was tested by determining the modification of their dimension and mass. In both cases, a reduction of diameter and mass was observed after 168 h of wine exposure, being more intense for alginate

capsules than for Si–Al capsules (Figure 1c,d). The diameter of the capsules shrank 15.80 and 10.29% for alginate and Si–Al capsules, respectively, and in addition, the loss of mass was 37.26 and 17.88%, respectively. It has been reported that the exposure of alginate capsules to alcohols (e.g. ethanol and isopropyl alcohol) can cause dehydration because they are hydrogels with a high water concentration of about 96–99% (Torres et al. 2011). It is likely that the inclusion of an inorganic network of silica into the alginate gel reduced the shrinkage of capsules and provoked a strong resistance to dehydration. Condensation of the silanol groups (SiOH) of sodium silicate forms siloxanes (SiOSi), and the polycondensation of silanol and siloxanes generates SiO_2 matrices (Kuncová and Trogl 2010) with strong inertness and chemical stability (Callone et al. 2008). Colloidal silica can also act as a filler reinforcing the stability of capsules, and its surface, rich in silanol groups, can provide reactive sites during the process of polycondensation of the SiO_2 matrix (Coradin et al. 2003). Finally, analysing the retention capacity of the capsules, it was observed that in both alginate and Si–Al matrixes, the proportion of leakage of the initial LAB population (~3×10^9 CFU/g of gel) was minimal, less than 0.0001%.

Performance of encapsulated Oenococcus oeni *under adverse winemaking conditions*

Effect of ethanol stress. Figure 2 shows the consumption of malic acid (expressed as relative proportion) in red wines with an alcohol concentration varying between 11.0 and 16.0% v/v using free bacteria and bacteria encapsulated in alginate and Si–Al gels. For both free and immobilised bacteria, the maximum relative proportion of consumption was achieved at the lowest alcohol concentration. Consumption of malic acid, however, was especially impacted by ethanol, when wine was inoculated with free bacteria, and to a lesser degree with alginate-encapsulated bacteria. The negative and strong influence of the high concentration of ethanol on lactic acid metabolism of LAB is well documented (Lerm et al. 2010, Sumby et al. 2014). Malolactic fermentation and growth of *O. oeni* are clearly inhibited by ethanol causing disruption of the cell membrane structure and alteration of membrane fluidity (Betteridge et al. 2015).

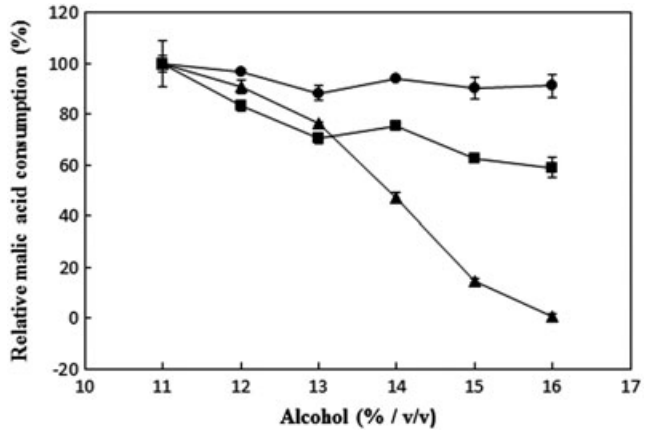

Figure 2. Effect of free *Oenococcus oeni* (▲) and *O. oeni* encapsulated in alginate (■) and in silica–alginate (●) capsules and of alcohol concentration on malic acid consumption (in relative %) in red wines after 72 h of malolactic fermentation. Experimental conditions: pH 3.4, free amino nitrogen 52 mg/L, malic acid 3 g/L and fermentation temperature 22°C. Error bars represent SE ($n = 3$).

For free bacteria, relative consumption of malic acid was less than 50% in the wine with a 14% alcohol, decreasing to only 1% in 16% alcohol. The relative degradation of malic acid, however, was not as strongly affected by ethanol for encapsulated bacteria. At an alcohol concentration of 14%, a relative degradation of 71 and 90% was achieved when wine was inoculated with immobilised bacteria in alginate and Si–Al gels, respectively. The most revealing results were found in wines with the highest alcohol (16% v/v), when malic acid degradation was 55 and 88% with immobilised bacteria in alginate and Si–Al gels, respectively. It is necessary to highlight that the inorganic network of silicates provided a higher ethanol tolerance than that reported for alginate gel.

These results agree with those reported by Rodríguez-Nogales et al. (2013) that showed a higher efficiency in MLF with polyvinyl-alcohol-encapsulated bacteria, as compared with that of free bacteria. The higher stress tolerance to ethanol of immobilised cells has been attributed to various factors. The matrix of immobilisation can provide a protective microenvironment against alcohol stress (Kourkoutas et al. 2004) because of diffusional restrictions that can reduce ethanol concentration inside the matrix. Other studies have reported that alginate-encapsulated cells present a higher level of saturated fatty acids in their cell membranes, with this modification resulting in an improved alcohol tolerance (Junter and Jouenne 2004). In high cell density supports, the intimate cell–cell contact was also reported as a protective mechanism against alcohol stress (Norton et al. 1995). Sun et al. (2007) noted that the non-gelling liquid alginate matrix enhanced the stability of the hydration layer around the cell, increasing cell protection. Recently, transcriptomic and proteomic analysis of *O. oeni* has highlighted that the transport of metabolites and cell wall and membrane biogenesis are the main functional categories affected by ethanol (Olguín et al. 2015). It has also been reported that *O. oeni* has cellular mechanisms that provide a certain resistance to high ethanol concentration, such as the balancing of fatty acid composition of its membrane in response to the fluidising effect of ethanol (Maitre et al. 2014), the synthesis of stress proteins (Maitre et al. 2012) and the relocation of cytosolic proteins in the membrane (Olguín et al. 2015). Such capacity to tolerate high ethanol concentration is dependent on strain and temperature, among other factors (Lerm et al. 2010).

In warm regions, the concentration of grape sugars has been increasing over the past few years as a result of possible climate change, resulting in unbalanced wines with a higher ethanol concentration (Vila-Crespo et al. 2010). Encapsulation of LAB into Si–Al gels provides an attractive biotechnology tool to overcome the greater sugar concentration caused by possible climate change in warm regions.

Effect of low fermentation temperature. Optimal temperature for growth of *O. oeni* is close to 25°C (Lerm et al. 2010), a temperature considerably higher than that usually found in wineries (12–20°C) (Sumby et al. 2014). Low temperature reduces bacteria growth rate, increases the length of the lag phase and decreases the maximum population of LAB (Bauer and Dicks 2004, Betteridge et al. 2015). Below 18°C, a delay in the onset of MLF and an increase in length of MLF are observed. A temperature below 16°C, however, causes an inhibition of growth of *O. oeni* (Lerm et al. 2010), and for that reason, it is recommended to control fermentation temperature at 18–20°C to ensure a fast beginning and termination of MLF (Lerm et al. 2010). These aspects of the effect of temperature

on the development of MLF were studied in wines inoculated with free and immobilised bacteria.

Increase of fermentation temperature from 13 to 22°C stimulated the consumption of malic acid by both free and immobilised bacteria (Figure 3). Free bacteria were quite temperature independent in the range of 16–22°C; however, a significant reduction in their metabolic activity was observed at 13°C. A relative consumption of malic acid of 95% was achieved at 16°C, reducing to 53% at 13°C. For the encapsulated bacteria, a gradual decrease in the consumption of malic acid was noted as the temperature decreased from 22 to 13°C. This behaviour was somewhat more pronounced for bacteria encapsulated into alginate gels.

At the lowest fermentation temperature (13°C), the best results were found for bacteria immobilised into Si–Al gels. An advantage of the immobilised cells for the production of alcoholic beverages is the possibility to operate at low temperature, which leads to a high-quality final product and makes the fermentation safe (Kourkoutas et al. 2004). Moreover, the improved efficiency of Si–Al-encapsulated bacteria at low fermentation temperature could reduce the energy cost of winemaking.

Recently, Servetas et al. (2013) reported the effect of low temperature on simultaneous alcoholic and malolactic wine fermentations using co-immobilisation of two microorganisms (*S. cerevisiae* and *O. oeni*) in a two-layer composite consisting of a tubular delignified cellulosic material covered with starch gel. The data demonstrate the effective performance of both fermentation processes at low temperature (10°C). At this temperature, conversion of malic acid to lactic acid was completed halfway through the alcoholic fermentation. Therefore, both fermentation processes took place at low temperature, pointing at cell entrapment as a suitable strategy for wine quality preservation and reduction of production costs in the wine industry. In addition, some authors have reported that carriers of immobilisation (e.g. pear and potato pieces, delignified brewer's spent grain and kissiris, a volcanic mineral rich in SiO_2) promote alcoholic fermentation of wine (Bakoyianis et al. 1992, Mallios et al. 2004, Kandylis and Koutinas 2008) and beer (Bekatorou et al. 2002, Kopsahelis et al. 2007) at low temperature. Experimental kinetic studies have highlighted that carriers can reduce the activation energy of enzyme reactions. Probably, carriers act as catalysts or promoters of the enzymes of this process (Bardi and Koutinas 1994, Bardi et al. 1996, Kandylis and Koutinas 2008).

Effect of pH stress. The lowest relative proportion of malic acid consumption was noted at the lowest values of wine pH (Figure 4). The growth of *O. oeni* and MLF is affected by the low pH of wine (typically lower than 3.5) (Betteridge et al. 2015). At the beginning of MLF, pH is critical as well as the time taken to complete MLF (Rosi et al. 2003). At pH 3.3 or higher, bacteria growth and survival are less problematic (Lerm et al. 2010), with the greatest MLF activity achieved between pH 3.5 and 4.0 (Bauer and Dicks 2004).

In this study, at pH between 3.6 and 4.0, free and Si–Al-encapsulated bacteria showed the greatest level of relative malic acid degradation (99 and 91% at pH 3.6, respectively), being the lowest for alginate-encapsulated bacteria (67% at pH 3.6). A decrease of wine pH from 3.6 to 3.0 caused a significant reduction of the relative malic acid consumption by free bacteria from 99 to 26%, respectively. Solieri et al. (2010) reported that low pH values (3.0–3.2) are the main stress factor influencing MLF, independently of ethanol concentration (10–13%, v/v). Knoll et al. (2011) indicated that the duration of MLF increased by 34 days when the pH was reduced from 3.8 to 3.2 in wines with 9.8% (v/v) of alcohol, while a partial MLF was observed at pH 3.2 and 11.8% (v/v) ethanol.

Some advances on the physiological alteration of *O. oeni* under pH stress have been secured; however, little attention has been given to immobilised *O. oeni*. It has been reported that *O. oeni* modifies the fatty acid composition of its membrane and induces the expression of specific genes encoding stress proteins in response to low wine pH (Grandvalet et al. 2008) as observed for ethanol stress (Maitre et al. 2012, 2014). Darsonval et al. (2015) demonstrated the involvement of Lo18, a small heat stress protein HSP, in the acid and ethanol tolerance of *O. oeni* using an antisense RNA approach. Moreover, induction of H^+-ATPase activity of *O. oeni* has been related to higher resistance to low pH because of its capacity to regulate cytoplasmic pH against wine pH (Fortier et al. 2003). Recently, Bastard et al. (2016) investigated the tolerance to wine stress of a biofilm culture of *O. oeni*. Biofilm culture represents a natural process of cell immobilisation, where cells

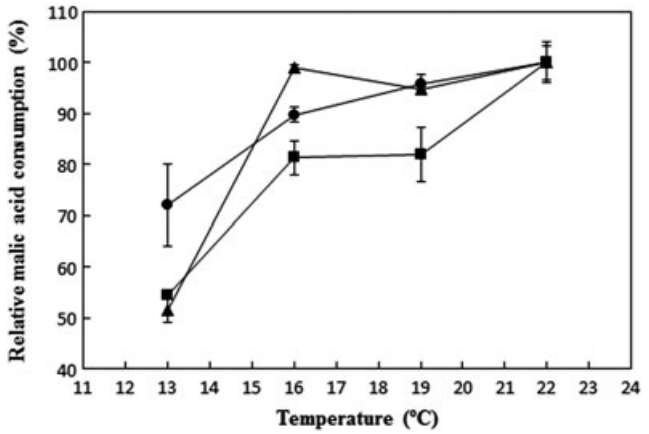

Figure 3. Effect of free *Oenococcus oeni* (▲) and *O. oeni* encapsulated in alginate (■) and silica–alginate (●) capsules and of fermentation temperature on malic acid consumption (in relative %) in red wines after 72 h of malolactic fermentation. Experimental conditions: pH 3.4, alcohol 13% (v/v), free amino nitrogen 52 mg/L and malic acid 3 g/L. Error bars represent SE (n = 3).

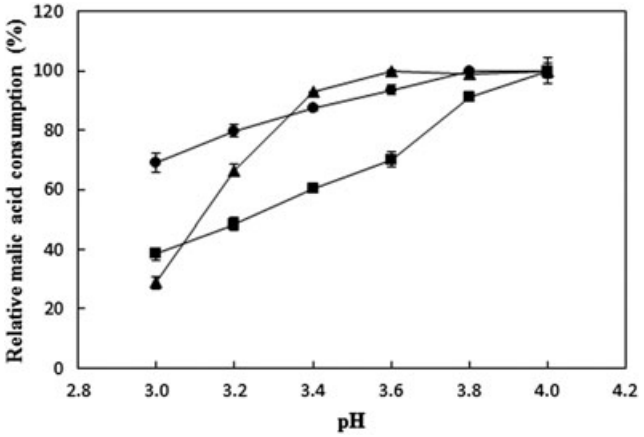

Figure 4. Effect of free *Oenococcus oeni* (▲) and *O. oeni* encapsulated in alginate (■) and silica–alginate (●) capsules and of pH on malic acid consumption (in relative %) in red wines after 72 h of malolactic fermentation. Experimental conditions: alcohol 13% (v/v), free amino nitrogen 52 mg/L, malic acid 3 g/L and fermentation temperature 22°C. Error bars represent SE (n = 3).

are embedded in their own extracellular polymeric substances matrix, providing a protective ambient to microorganisms (Flemming et al. 2007). The biofilm formation of *O. oeni* enhanced stress resistance to wine pH 3.5 and 3.2, showing an increase of the genes involved in the synthesis of extracellular polymeric substances. These results suggest the role of the polysaccharide matrix in increased biofilm resistance.

Interestingly, bacteria immobilisation in Si–Al reduced their sensitivity towards pH between 3.0 and 3.4. At extremely low pH (3.0–3.3), the highest values of malic acid degradation were achieved inoculating Si–Al-encapsulated bacteria. The encapsulation process took place at pH 6.29, so hypothetically, the pH inside the capsules may be higher than wine pH, which could contribute to decrease the pH stress of encapsulated bacteria. In addition, our data suggest that the inclusion of siliceous material can improve the tolerance of encapsulated bacteria at extremely low pH. These results confirm that immobilisation of *O. oeni* into Si–Al hydrogels could reduce problems to develop MLF in cooler climate regions where wine pH varies between 2.8 and 3.2 (Liu 2002).

Effect of wine nitrogen concentration. Efficiency of MLF is affected by the nutrient composition of wine and, especially, its amino acid concentration (Bauer and Dicks 2004) because of the incomplete amino acid biosynthesis ability of LAB. Glutamic acid, valine, arginine, leucine, isoleucine, cysteine and tyrosine have been identified as essential amino acids for LAB (Terrade and de Orduña 2009). In order to determine if the immobilisation process modifies the nutrient requirements for nitrogen, in this work, free and immobilised bacteria were inoculated into wines with variable FAN concentration (from 52 to 250 mg/L). In general, free and encapsulated bacteria showed similar behaviour in the range of nitrogen assayed (Figure 5). Bacteria encapsulated in Si–Al showed a significant increase in the relative malic acid consumption from 90.3 to 100% at 200 and 250 mg/L of FAN, respectively. Likely, the inclusion of the siliceous material into the alginate gel reduced the permeability of Si–Al capsules to amino acids, increasing the diffusion of amino acids through Si–Al capsules at the highest concentration of nitrogen.

Effect of wine malic acid concentration. Malic acid comes from grapes, and its concentration is typically

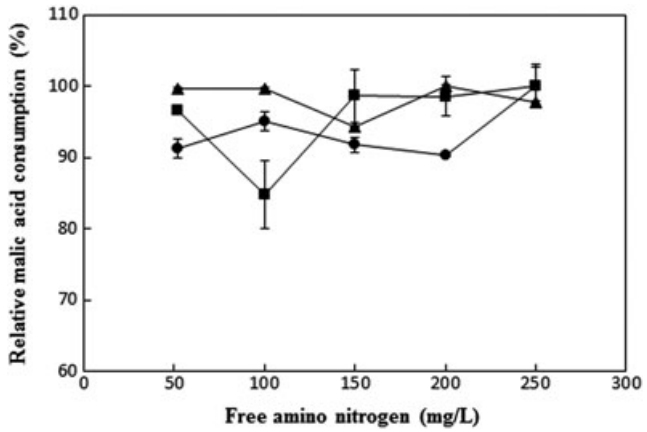

Figure 5. Effect of free *Oenococcus oeni* (▲) and *O. oeni* encapsulated in alginate (■) and silica–alginate (●) capsules and of free amino nitrogen on malic acid consumption (in relative %) in red wines after 72 h of malolactic fermentation. Experimental conditions: pH 3.4, alcohol 13% (v/v), malic acid 3 g/L and fermentation temperature 22°C. Error bars represent SE (n = 3).

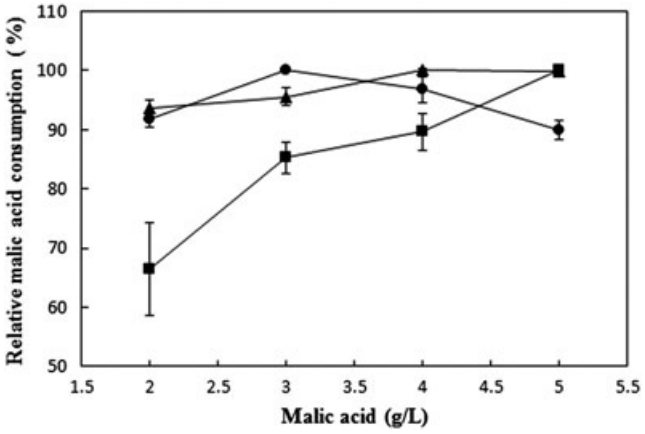

Figure 6. Effect of free *Oenococcus oeni* (▲) and *O. oeni* encapsulated in alginate (■) and silica–alginate (●) capsules and of malic acid concentration on malic acid consumption (in relative %) in red wines after 72 h of malolactic fermentation. Experimental conditions: pH 3.4, alcohol 13% (v/v), free amino nitrogen 52 mg/L and fermentation temperature 22°C. Error bars represent SE (n = 3).

2–5 g/L (Lerm et al. 2010). In this study, the effect of malic acid concentration on MLF was assayed in wines with a malic acid concentration between 2 and 5 g/L (Figure 6). Alginate-encapsulated bacteria were affected by the modification of the initial concentration of malic acid to a greater extent than free and Si–Al-encapsulated bacteria. A lower relative proportion of malic acid consumption by alginate-encapsulated bacteria was observed when decreasing the values of malic acid. At 2 g/L, the relative degradation of malic acid was 66% for alginate-encapsulated bacteria and about 92% for free bacteria and Si–Al-encapsulated bacteria.

Conclusions

The encapsulation of bacteria into an Si–Al gel is an exceptional application for the enhancement of MLF in red wines. Inclusion of an inorganic network of SiO_2 inside an alginate matrix improved bacteria stability and bioactivity and capsule stability. Mechanical resistance and chemical stability of alginate capsules were significantly improved using siliceous materials. The Si–Al capsules showed also a similar cell release to alginate capsules, which makes them suitable for oenological application. Interestingly, malic acid was efficiently metabolised by Si–Al biocomposite-encapsulated bacteria under all the conditions assayed, even under adverse winemaking conditions. Finally, encapsulation within Si–Al support made bacteria more active at high ethanol concentration, low pH and low fermentation temperature. Together, these results indicate that Si–Al bacteria entrapment is a useful and advantageous tool for winemaking, either in warm regions where the ethanol concentration of wines is rising or in cool regions where wines present low pH. Regarding the toxicological aspects of Si–Al biocomposites for winemaking, alginate has been recognised as a 'generally recognised as safe' material and therefore accepted as safe for human consumption (Sosnik 2014). Moreover, silica precursors are being applied in the biomedical fields indicating that they are fully safe for humans (Carturan et al. 2004). These characteristics are suitable for the development of Si–Al biocomposites as a carrier for bacteria encapsulation and for their implementation in the wine industry. Further studies focused on chemical and sensory characteristics of the wines inoculated with encapsulated bacteria into Si–Al biocomposites are necessary prior to their use in wine industry.

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Effect of stressful malolactic fermentation conditions on the operational and chemical stability of silica-alginate encapsulated *Oenococcus oeni*

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ABSTRACT

Oenococcus oeni was encapsulated into inter-penetrated polymer networks of silica-alginate (SiO_2-ALG). Fourier transform infrared spectroscopy analysis proved the presence and the polycondensation of the siliceous material used in SiO_2-ALG capsules. Environmental scanning electron microscopy showed that the structure of SiO_2-ALG biocapsules was rougher than in alginate (ALG) biocapsules. The behaviour of SiO_2-ALG biocapsules was evaluated at pH 3.0–3.6 and alcohol degrees of 12–15%. Repeated-batch malolactic fermentations (MLF) demonstrated that SiO_2-ALG biocapsules can be reused efficiently for five times in either low-pH or high-ethanol wines, while free bacteria only can be used once under the most favourable MLF conditions. The inclusion of siliceous materials into ALG hydrogel improved the stability of the biocapsules, reducing their shrinking and achieving an excellent integrity under winemaking conditions. These results proved the possibility of industrial application of SiO_2-ALG biocapsules in winemaking.

1. Introduction

In the last decade, cell encapsulation has been reported as an alternative to free cells for the enhancement of long-term bio-efficiency in fermentative processes (Kourkoutas, Manojlović, & Nedović, 2010; Maicas, 2001). Different materials have been applied as carriers for cell encapsulation, mainly organic polymers (such as ALG, carrageenan, chitosan, agarose, pectin, gelatine, and chitin). Ionotropic ALG hydrogel has been extensively used for this purpose due to being easy and cost-effective to prepare, the encapsulation conditions are mild for cell, and its porous structure allows the free diffusion of substrates and products. However, the low chemical stability and the poor mechanical strength of ALG hydrogel hinder its industrial implementation (Ching, Bansal, & Bhandari, 2015). Different alternatives have been assessed to improve these disadvantages based on coating techniques (Simó, Fernández-Fernández, Vila-Crespo, Ruipérez, & Rodríguez-Nogales, 2017a) as well as on the incorporation of other polymers and/or fillers into ALG gel (Yi, Neufeld, & Poncelet, 2004).

Previous researches have been done for the improvement of the stability of ALG capsules using covalent polymeric networks of SiO_2 obtained by the sol–gel process (Coradin, Allouche, Boissiere, & Livage, 2006; Kuncová & Trogl, 2010). These inorganic-organic hybrid materials have the advantages of the organic component (ALG) (biocompatibility, elasticity and flexibility) and the inorganic component (SiO_2) (strength, and thermal and chemical stability) (Hwang & Gu, 2013). From a toxicological point of view, SiO_2–ALG capsules are very suitable for food application since (i) ALG is a natural polymer that has been recognized as a “generally recognized as safe” material (Sosnik, 2014), and (ii) siliceous materials are being used for biomedical applications and hence admitted as safe for humans (Carturan, Dal Toso, Boninsegna, & Dal Monte, 2004).

The classic sol-gel process involves the hydrolysis of alkoxide precursors in the presence of water and catalysts (either acids or bases) to form silanol groups (Si-OH) with the releasing of alcohol molecules. Then, condensation of silanol groups takes place to produce siloxanes (Si-O-Si). Finally, polycondensation of silanol and siloxanes occurs, yielding SiO_2 materials (Niederberger & Pinna, 2009). The use of sol-gel chemistry for cell encapsulation presents some risks for cell viability. On one hand, the released alcohol from the hydrolysis of alkoxide precursors may be toxic to cell. On the other hand, the use of cosolvent and catalysts to increase the low water solubility and the reactivity of alkoxide precursors, respectively, may also cause loss of cell viability.

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Some solutions have been reported to solve these drawbacks based on either the removal of alcoholic by-products before cell encapsulation or the use of an aqueous sol-gel process with non-alkoxide precursors (Coradin et al., 2006; Kuncová & Trogl, 2010).

A successful strategy was recently proposed by our research team, in which an easy, economic and efficient cell encapsulation procedure based on inter-penetrated polymer networks of SiO_2 and ALG was developed (Simó, Fernández-Fernández, Vila-Crespo, Ruipérez, & Rodríguez-Nogales, 2017b; Simó, Vila-Crespo, Fernández-Fernández, Ruipérez, & Rodríguez-Nogales, 2017). The hybrid composite was obtained by the sol-gel aqueous route using non-alkoxide silicon precursors (colloidal silica and sodium silicate). The resulting SiO_2-ALG biocapsules with entrapped *O. oeni* bacteria were applied in winemaking to develop MLF. SiO_2-ALG biocapsules presented a remarkable increase in mechanical robustness of 328% and 65% in L-malic acid consumption, observing insignificant bacteria release compared to untreated ALG biocapsules (Simó, Vila-Crespo, et al., 2017). Also, SiO_2-ALG encapsulated *O. oeni* showed an enhanced MLF in wines with high concentration of ethanol (13–16%), low pH (3.0–3.3) and at low fermentation temperatures (13–15 °C) compared to ALG capsules (Simó et al., 2017b).

To the best of our knowledge, only two other works using SiO_2-ALG composites for the encapsulation of *O. oeni* have been reported (Callone, Campostrini, Carturan, Cavazza, & Guzzon, 2008; Guzzon, Carturan, Krieger-Weber, & Cavazza, 2012). Unlike our strategy, both works noted a non-aqueous method based on double-layer coating of ALG capsules embedded with *O. oeni* using tetraethoxysilane and methyltriethoxysilane as alkoxide silicon precursors. The coating procedure did not increase the metabolic activity of bacteria, although there was a notable improvement of the hardness of biocapsules.

One of the potential main advantages claimed for the use of encapsulated cells includes their easy separation and reuse. Industrial implementation of biocapsules depends on the possibility of their recycling for a long period without compromising their metabolic activity and viability. Moreover, biocapsules must be stable along the fermentative period for the success of alcoholic beverage production (Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004). A deep knowledge of the effect of the hostile wine environment on biocapsules stability needs to be addressed to obtain a full exploitation of this potential advantage.

In this context, the encapsulation of *O. oeni* into inter-penetrated polymer networks of SiO_2 and ALG could involve a great enhancement of the operational and chemical stability of the biocapsules to the stressful conditions of MLF. For that, the aim of this study was to evaluate the reusability of Si-ALG biocapsules under unfavourable MLF conditions and to test their long-term chemical stability in harsh wine conditions of low pH and high ethanol concentration. The presence of siliceous materials in the Si-ALG biocomposites and their effect on the external and internal structure of the capsules were also verified by Fourier transform infrared spectra (FTIR) and Environmental scanning electron microscopy (ESEM) analysis.

2. Experimental

2.1. Preparation of SiO_2-ALG biocomposites with encapsulated bacteria

Inter-penetrated polymer networks of silica/silicate and ALG (Simó, Vila-Crespo, et al., 2017) were carried out adjusting the pH of a water solution of 1.23 M colloidal silica (Ludox HS40, Sigma-Aldrich, Spain) and 0.06 M sodium silicate (Sigma-Aldrich) to 6.3 by adding 2 N HCl. Then, sodium ALG (Panreac, Spain) was mixed with the silica/silicate solution until obtaining a final concentration of 2% (w/v). Freeze-dried bacteria (*O. oeni* strain LALVIN VP 41® MBR, Lallemand, France) were rehydrated in water at 20 °C according to the manufacturer's instructions and they were added at a concentration of $\sim 3 \cdot 10^9$ cfu/g of gel. Population of freeze-dried culture was tested in Tomato Juice Agar (Difco, Sparks, MD, USA) at 22 °C. Capsules were made by extrusion of the well-mixed siliceous material-ALG-cell suspension with a 10 mL sterile syringe with a nozzle of 1.78 mm of diameter (BD 166 Plastipak, Spain) into sterile 0.2 M $CaCl_2$ solution under continuous agitation (260 rpm) at 22 °C. Capsules were kept in this solution for 2 h and washed with water at 22 °C. Capsules of ALG were made using the method described for SiO_2-ALG capsules but omitting the addition of siliceous material. All solutions were autoclaved prior to use.

2.2. Environmental scanning electron microscopy (ESEM)

The prepared SiO_2-ALG and ALG biocapsules were dehydrated by freeze-drying at −40 °C. The samples were coated with gold and examined in a Quanta 200FEI ESEM (Hillsboro, Oregon, USA) with a backscattered electron detector at a landing energy of 4.0 keV.

2.3. Fourier transform infrared spectroscopy analysis (FTIR)

Functional groups of freeze-dried SiO_2-ALG and ALG capsules were characterized by FTIR spectrophotometry. The vibrational spectra of the capsules in the 400–4000 cm^{-1} spectral range were measured using a Thermo Scientific Nicolet iS50 FTIR spectrometer (Waltham, MA, USA), equipped with a built-in diamond attenuated total reflection (ATR) system. Spectra of the samples were recorded with a 1 cm^{-1} spectral resolution, and 32 scans.

2.4. Operational stability of biocapsules

Reusability of SiO_2-ALG biocapsules was determined using the same inoculum for five consecutive cycles of MLF under different winemaking conditions. Free bacteria were also used as control. Red wine of "Tinta de Toro" variety (3.0 g/L malic acid, pH 3.4 and alcoholic degree of 14.0%, v/v) was partially dealcoholized to obtain a final alcoholic degree of 11% under reduced pressure in a rotary evaporator. A first batch of wine was made adjusting the alcoholic degree of the concentrated wine to 12.0%, 13.5% and 15.0% with ethanol and at pH 3.4 using acid or basic concentrated solutions. A second lot of wine was set to an alcoholic degree of 13.0% and pH of 3.0, 3.3 and 3.6. MLF was conducted in sterilized red wine (by filtration through a 0.2 µm pore-size) inoculating either free or SiO_2-ALG encapsulated bacteria at a concentration of $\sim 9 \times 10^7$ cfu/mL of wine. Rehydrated culture from freeze-dried *O. oeni* strain (LALVIN VP 41® MBR) was used as free inoculum. Bacterial population of free inoculum was tested in Tomato Juice Agar at 22 °C (Difco). MLF was carried out in triplicate and its progress was monitored by the determination of L-malic acid concentration (in triplicate) using an enzymatic kit (TDI, Barcelona, Spain). After 120 h of incubation at 22 °C, both free and encapsulated bacteria were removed from wine and introduced into a new wine. Free bacteria were recovered by centrifugation (15 min at 2300*g*) while encapsulated ones were separated from wine by filtration using a metallic sieve (nominal sieve opening of ~1.5 mm).

2.5. Long-term chemical stability of biocapsules

For capsule stability evaluation, the same two lots of wine developed for the operation stability study were used. Fifteen SiO_2-ALG biocapsules containing bacteria were immersed in 10 mL of red wine at different pH (3.0, 3.3 and 3.6) and alcoholic degrees (12.0%, 13.5% and 15.0%). ALG capsules were also analysed as control for comparison. Mass and diameter of both SiO_2-ALG and ALG biocapsules were determined along the experimental time at 22 °C. The diameter of biocapsules was measured by software IMAGEJ 1.47v (National Institutes of Health, Bethesda, MD, USA). The mass of biocapsules was determined using a semimicro balance with a readability of 0.1 mg (AS 228 220/C/2, Radway, Brancka, Poland). The release of ALG from both capsules was determined by duplicate after 18 days of immersion in

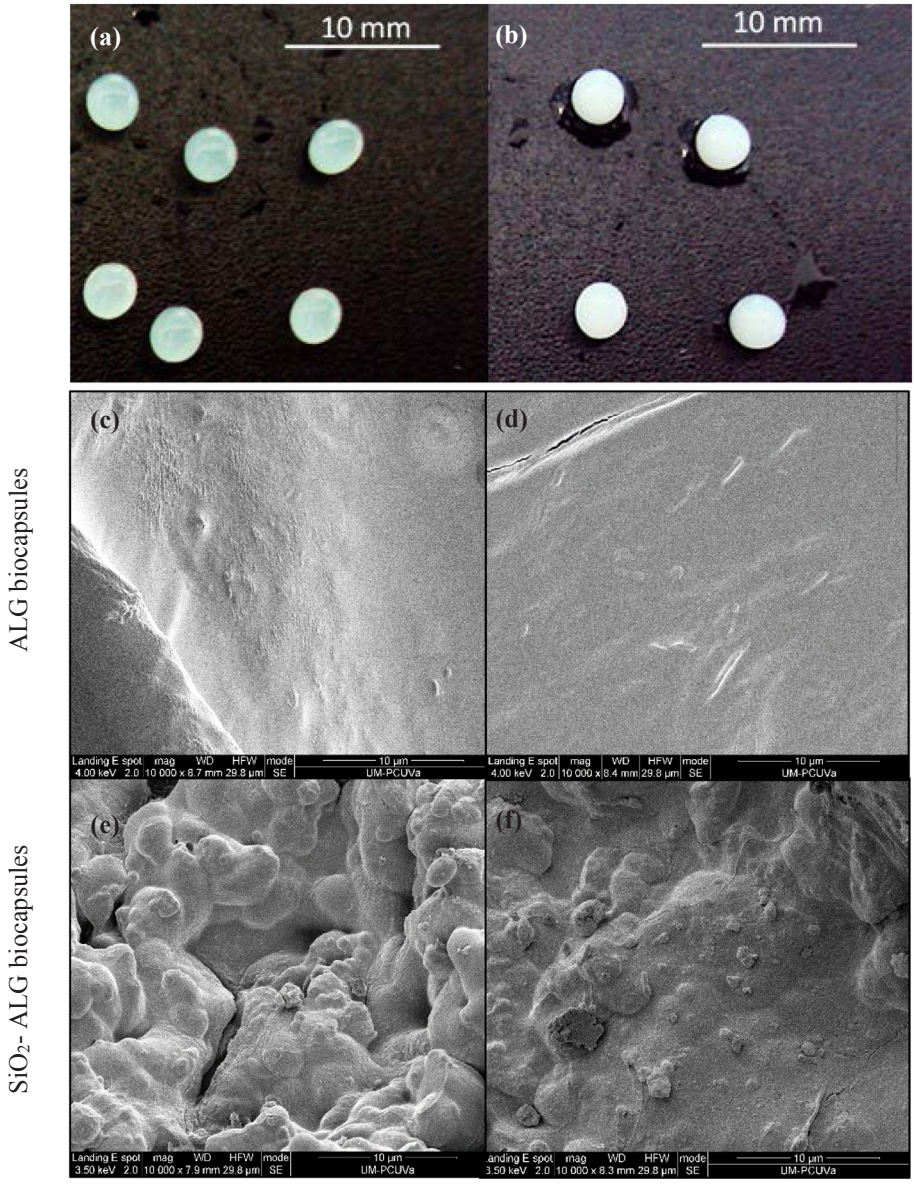

Fig. 1. Optical images of (a) alginate and (b) SiO_2-alginate biocapsules with entrapped *Oenococcus oeni.* (c–f) Environmental scanning electron microscopy (ESEM) of alginate (ALG) and SiO_2-alginate (SiO_2-ALG) biocapsules with entrapped *Oenococcus oeni.* (c, e) External structure of the biocapsules. (d, f) Internal structure of the biocapsules.

wine (fifteen biocapsules in 10 mL of red wine) following the method proposed by Segarra, Lao, Lopez, and De La Torre (1995). All experiments were carried out in triplicate.

2.6. Statistical analysis

SPSS v. 17.0 statistical package (SPPS Inc., Chicago, Ill, USA) was used for all statistical analyses. A variance analysis was carried out to determine statistical differences between samples ($p < 0.05$). In figures, error bars were calculated as standard error.

3. Results and discussion

3.1. Morphological observations

SiO_2-ALG and ALG biocapsules were nearly spherical and homogeneous in size (Fig. 1, images a and b), showing a diameter of 3.24 ± 0.20 and 2.80 ± 0.15 mm, respectively. The sol-gel process using siliceous materials slightly increased the capsule diameter due to the rise of viscosity of ALG solution in presence of colloidal silica/silicate and modified their physical appearance, turning them opaque and white. This behaviour was also observed in alginate-protamine-SiO_2 hybrid capsules where the transparent ALG capsules became opaque and white during the silicification process (Wang et al., 2013).

The surface morphology and the internal structure of ALG and SiO_2-ALG biocapsules containing *O. oeni* were studied by ESEM. Both external and internal structures of ALG biocapsules were relatively smooth (Fig. 1, images c and d, respectively). However, the morphology of both structures changed in the presence of silica/silicate and became rough (Fig. 1, images e and f). A highly porous network was formed by close-packed silica particles. SEM, thermogravimetric analysis and nitrogen adsorption-desorption experiments performed in SiO_2-ALG biocomposites using the sol-gel route with aqueous precursors revealed that ALG polymer filled the macroporosity of hydrogel rather than being strongly entangled with siliceous material (Coradin & Livage, 2003). Compared to ALG biocapsules, the presence of an inorganic network of SiO_2 notably enhanced the bioactivity of SiO_2-ALG encapsulated *O. oeni* (Simó et al., 2017b). This efficiency in L-malic acid

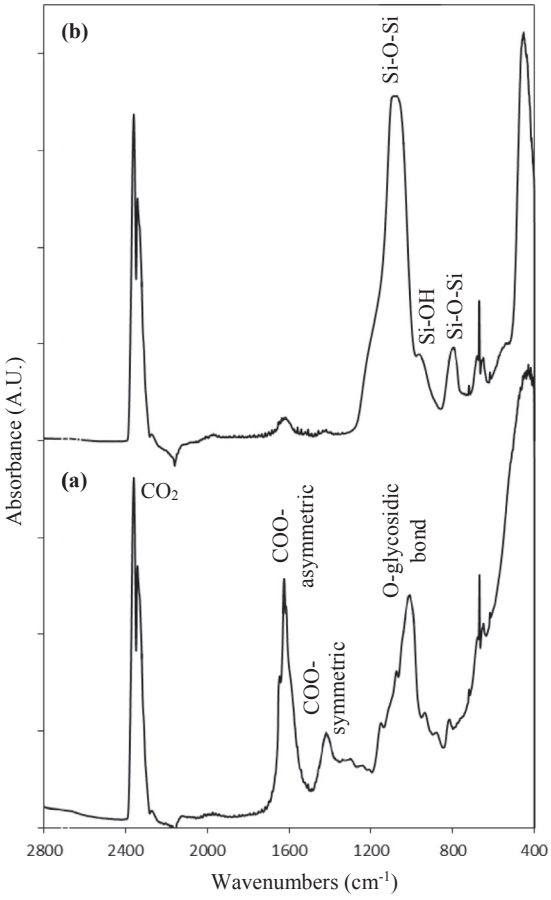

Fig. 2. FTIR-ATR spectra of (a) alginate and (b) SiO_2-alginate capsules.

consumption could be due (at least partly) to the highly porous structure observed in SiO_2-ALG biocapsules that facilitates the substrate accessibility for encapsulated bacteria. This is consistent with the improvement of diffusion characteristics noted in SiO_2-ALG composites (in comparison with ALG hydrogels), evaluating the diffusion coefficients of reduced nicotinamide adenine dinucleotide, an enzymatic cofactor with a molecular weight of about five-times higher than malic acid (Xu, Lu, Li, Jiang, & Wu, 2006).

3.2. Chemical characterization of capsules

Chemical composition of ALG and SiO_2-ALG capsules was characterized by FTIR-ATR spectroscopy. The FTIR spectra of the ALG capsules show characteristic bands displayed for alginic acid (Fig. 2a). According to literature reports (Gómez-Ordóñez & Rupérez, 2011; Pannier, Soltmann, Soltmann, Altenburger, & Schmitt-Jansen, 2014), the bands at 1612 cm^{-1} and 1412 cm^{-1} are assigned to asymmetric and symmetric stretching vibrations of the COO^- of alginic acid, respectively. A broad peak near 1000 cm^{-1} corresponds to vibrational modes of the carbohydrate ring between (1–4)-β-D-mannuronic acid and (1–4)-α-L-guluronic acid of alginic acid (Gómez-Ordóñez & Rupérez, 2011). The FTIR spectra of SiO_2-ALG capsules (Fig. 2b) present the bands associated with symmetric and asymmetric stretching vibrations of the COO^- but in very low intensity, suggesting an interaction of the carboxylate groups of ALG with the siliceous polymer derived from *in situ* gelation of silica/silicate (Xu, Jiang, Lu, Wu, & Yuan, 2006). Under the pH conditions used for bacterial encapsulation (pH 6.3), only hydrogen bonds between hydroxyl groups (–OH) of SiO_2 and carboxyl groups (–COOH) of ALG could take place because the carboxyl groups of ALG are negatively charged and the hydroxyl groups are neutral, whereas SiO_2 presents neutral charge (Coradin & Livage, 2003). A peak at 790 cm^{-1} corresponds to the symmetric stretching of Si–O–Si groups, indicating an extension of SiO_2 polymerization. A peak of high intensity at about 1100 cm^{-1} partially overlapped with the corresponding bands to vibrational modes of the carbohydrate ring of ALG could be assigned to Si–O–Si bending vibration. Finally, the band at 955 cm^{-1}

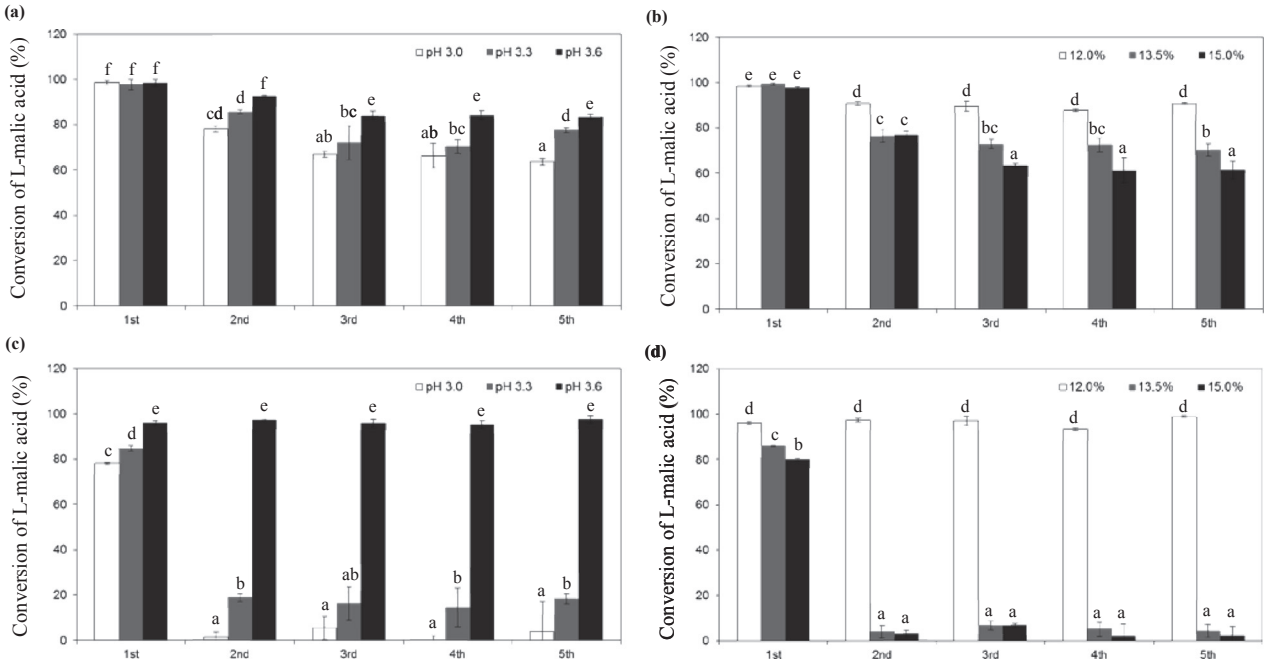

Fig. 3. Conversion of L-malic acid by SiO_2-alginate encapsulated (a, b) and free (c, d) *Oenococcus oeni* in red wines at different pH (3.0, 3.3, and 3.6) and different alcohol degree (12.0, 13.5, and 15.0%) in five subsequent cycles. MLF conditions: red wine (3.0 g/L of L-malic acid), 0.03 g of biocapsules per mL of wine, bacterial load of ~9×10^7 cfu/mL of wine, temperature of 22 °C, and 120 h of incubation. Standard deviations of the assays are represented by error bars (n = 3). Different letters in the bars indicate a significant difference between samples ($p < 0.05$).

corresponds to Si-OH vibration marking also the presence of silica in this capsule (Xu et al., 2006).

3.3. Reusability of SiO_2-ALG biocapsules

Bacterial reuse reduces production cost, thus the study of the operational stability of SiO_2-ALG biocapsules is essential for their industrial implementation. To examine their reusability, repeated-batch MLF were carried out five times using the same inoculum of SiO_2-ALG encapsulated bacteria. Reusability test of free bacteria was also performed as control for comparison. Operational stability of ALG biocapsules was not studied since malolactic activity of ALG-encapsulated bacteria was notably lower than that observed in SiO_2-ALG biocapsules (Simó et al., 2017b).

Fig. 3 shows L-malic acid conversion by both SiO_2-ALG biocapsules and free bacteria along five-repeated cyclic operations in red wine at different pH (3.0, 3.3 and 3.6). Wine pH had a marked effect on malolactic activity of both type of inoculum, achieving the best results at pH 3.6. Malolactic conversion by SiO_2-ALG encapsulated bacteria only decreased by 15.7% after five cycles in wine at pH 3.6 (from 98.4% of conversion in run #1 to 83.0% of conversion in run #5). The decrease of malolactic conversion was calculated as relative percentage as

$$\left(1-\frac{C_{MLF5}}{C_{MLF1}}\right)\times 100$$

where C_{MLF1} and C_{MLF5} are the malolactic conversions of the first and fifth MLF, respectively, observed at each pH, alcoholic degree and type of inoculum. The decreases in L-malic acid conversion were slightly higher at pH 3.3 and 3.0 after five-repeated batches, reaching drops of 20.6% (from 97.6% in run #1 to 77.5% in run #5) and 35.3% (from 98.5% in run #1 to 63.7% in run #5), respectively. Malolactic conversion by free bacteria was stable only at pH 3.6 (13.0% of alcoholic degree) after their reuse for five times. This result was similar to that obtained by four successive reinoculations of cultures of *O. oeni* in Monastrell red wine under favourable MLF conditions (pH 3.5 and 11.1% alcoholic degree) (Maicas, Pardo, & Ferrer, 2000). L-malic acid conversions by free *O. oeni* drastically dropped by 78.4% (from 84.6% in run #1 to 18.3% in run #5) and a 94.9% (from 78.1% in run #1 to 3.97% in run #5) at pH 3.3 and 3.0, respectively.

Free and SiO_2-ALG encapsulated *O. oeni* were also tested in five consecutive MLF processes in wines with different alcoholic degrees (12.0%, 13.5% and 15.0%) to determine if there was deactivation of bacteria after repeated use (Fig. 3). Alcoholic degree notably affected L-malic acid conversion of both free and encapsulated bacteria along the successive MLF, achieving the best results at the lowest alcoholic degree (12.0%). L-malic acid conversion by SiO_2-ALG encapsulated bacteria was maintained at 90.7% after five cycles in favourable winemaking conditions (12.0%), corresponding with a slight reduction of 7.8% (a high conversion of 98.3% was achieved in the first cycle). Drops of 29.5% (from 99.4% in run #1 to 70.1% in run #5) and 36.9% (from 97.6% in run #1 to 61.6% in run #5) in L-malic acid conversion were observed in wines with alcoholic degrees of 13.5% and 15.0%, respectively, after the fifth cycle of use of SiO_2-ALG biocapsules.

L-Malic acid conversion by free bacteria remained almost unchanged (above 98%) in wine with the lowest alcoholic degree (12.0%) after their reuse for five cycles. However, sharp declines of 95.0% (from 85.9% in run #1 to 4.3% in run #5) and 97.0% (from 78.9% in run #1 to 2.3% in run #5) on L-malic conversion were observed at alcoholic degrees of 13.5% and 15.0%, respectively, corroborating the negative impact of ethanol on malolactic activity of *O. oeni* (Bonomo, Di Tomaso, Calabrone, & Salzano, 2018).

Growth and metabolism of *O. oeni* in wine depend on a multitude of parameters (Bauer & Dicks, 2004). It is well documented the adverse effect of low-pH wines as well as high-ethanol wines on the ability of *O. oeni* to survive in this unfavourable environment (Bonomo et al., 2018; Sumby, Grbin, & Jiranek, 2014). Ethanol causes breaking of cell membrane structure and membrane fluidity alterations, affecting mainly transport of metabolites, and cell wall and membrane biogenesis (Olguín et al., 2015). Wine pH also plays a crucial role in the beginning of MLF as well as the time required to complete MLF (Knoll et al., 2011). pH values lower than 3.5 negatively affect the growth of *O. oeni* and reduce their ability to metabolize L-malic acid (Betteridge, Grbin, & Jiranek, 2015; Rosi, Fia, & Canuti, 2003).

Our results have revealed that the encapsulation into SiO_2-ALG hydrogel notably improved the operational stability of bacteria, remaining high malolactic activity after five successive MLF at the harsh environmental conditions of pH 3.0 and 13% of alcoholic degree, as well as pH 3.4 and 15.0% of alcoholic degree. These results agree satisfactorily with a previous study of our group (Simó et al., 2017b). In this study, we found that the inclusion of colloidal silica and silicates into ALG capsules markedly enhanced the malolactic activity of *O. oeni* at low pH, high ethanol, and low fermentation temperatures. Guzzon et al. (2012) reported successful MLF after three cycles in Chardonnay wine (ethanol 12.5%, pH 3.3, malic acid 3.5 g/L) inoculating *O. oeni* entrapped into ALG capsules coated with an organic-silica membrane. Encapsulated *O. oeni* in polyvinyl alcohol gel could be successfully reused through six cycles of MLF in Tempranillo wine (ethanol 14.2%, pH 3.7, malic acid 1.9 g/L), retaining 75% of efficacy after the sixth batch (Rodríguez-Nogales, Vila-Crespo, & Fernández-Fernández, 2013). Conversely, in Monastrell red wine under gentle winemaking conditions (ethanol 11.0%, pH 3.5, malic acid 3.5 g/L), a drop of about 50% of the initial L-malic acid conversion was observed after six consecutive cycles of MLF inoculating *O. oeni* immobilized on positively-charged cellulose sponge (Maicas, Pardo, & Ferrer, 2001). *Lactobacillus casei* cells immobilized on delignified cellulosic material showed a high L-malic acid conversion (80%) in the first cycle of MLF under mild winemaking conditions (ethanol 11.2% and pH 3.5), declining gradually up a conversion of 14.7% in the sixth batch (Agouridis, Bekatorou, Nigam, & Kanellaki, 2005). These results highlight that the success in the reusability of immobilized bacteria strongly depends on both the type of the selected matrix and the immobilization method, as well as MLF conditions and bacterial strain. In our study, the differences in malic acid conversion found in the first cycle of MLF between both type of inoculum (free and Si-ALG encapsulated bacteria) may be lower if the fermentative behaviour of the selected strain was higher at low pH and high ethanol concentration.

The preservation of malolactic activity of SiO_2-ALG encapsulated bacteria along the operation stability assays could be due to the organic-inorganic matrix, which could provide bacterial protection from the unfavourable winemaking conditions (Kourkoutas et al., 2010). An enhancement of the stability of the hydration layer around the cell due to the presence of non-gelling liquid into ALG hydrogel could increase the cell protection (Sun et al., 2007).

3.4. Long-term capsule stability

Together with an adequate operational stability of immobilized bacteria, capsule stability studies also play a decisive role for the industrial implementation of encapsulated bacteria. pH and ethanol concentration in model water solutions markedly affected the stability of ALG-based capsule (Simó, Vila-Crespo, et al., 2017). The weight and swelling of capsules in water increased as pH was higher from 3.0 to 3.9, being more notable this behaviour for ALG capsules than for SiO_2-ALG ones. In contrast, both types of capsules shrank in water-ethanol solutions with more intensity at higher ethanol concentrations (from 10% to 16%) and with less severity in SiO_2-ALG capsules than in ALG ones.

In this study, we evaluate the impact of pH and ethanol concentration on SiO_2-ALG capsule stability using samples of wine at different pH (3.0, 3.3 and 3.6) and alcoholic degrees (12.0%, 13.5% and 15.0%). Simultaneously, ALG capsules were also tested as control

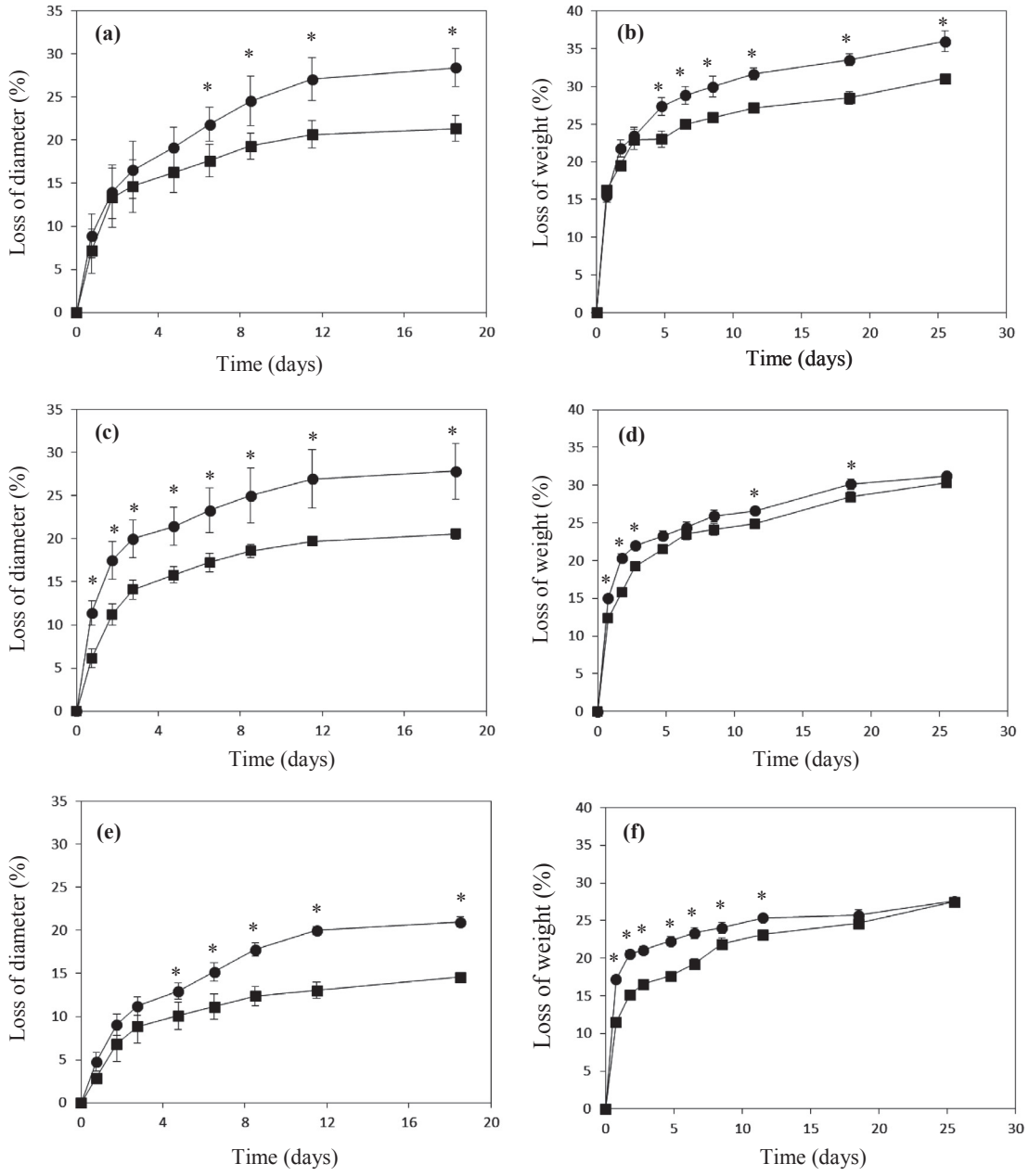

Fig. 4. Evolution of shrinking (expressed as decrease of diameter (in %) and weight (in %)) of alginate (●) and SiO_2-alginate (■) biocapsules loaded with *Oenococcus oeni* submerged in red wines at different pH ((a, b) 3.0, (c, d) 3.3 and (e, f) 3.6). Shrinking was calculated from the average of 20 biocapsules submerged in wine at 22 °C. Standard deviations of the assays are represented by error bars. * indicates a statistically significant difference ($p < 0.05$) between both capsules.

samples to know the effect of silica/silicate on the capsule stability. Pronounced diameter and weight reductions were found when SiO_2-ALG and ALG capsules were exposed to wine, reducing the free volume in the biocapsules (Figs. 4 and 5). These results are consistent with the shrinking process observed in SiO_2-ALG and ALG materials in model water-ethanol solutions (Simó, Vila-Crespo, et al., 2017). On the whole, shrinking was decreased as wine pH increased, being always the loss in diameter lower for SiO_2-ALG capsules than for ALG ones (Fig. 4). After 27.0 days of immersion of capsules in wine, the differences of weight between both capsules were not so clear as those observed for diameter, testing a less drop in weight for SiO_2-ALG capsules than for ALG ones only at pH 3.0. For instance, depletions of 28.4% and 35.9% of diameter and weight of ALG capsules were observed at pH 3.0, respectively, while they were lower for SiO_2-ALG capsules (21.3% and 31.0%, respectively). The differences of shrinking between both capsules can be understood from the reduction of the elasticity and the increase of robustness of capsules due to the inclusion of colloidal silica and silicates into ALG hydrogels (Coradin, Nassif, & Livage, 2003; Simó, Vila-Crespo, et al., 2017). Hydrogels based on ALG tend to shrink when they are exposed to acidic environment (Pasparakis & Bouropoulos, 2006). At a pH value lower than 4.0, the carboxylic groups of mannuronic and guluronic acids of ALG are protonated (pK_a 3.38 and 3.36, respectively) and therefore the electrostatic repulsions among these groups decrease and shrinkage is favoured (Wu, Zhu, Chang, Zhang, & Xiao, 2010).

In parallel with the increase in the alcoholic degree of the wine, the capsules decreased in diameter and weight (Fig. 5). The more the ethanol increased, the more the shrinking increased. To illustrate, reductions of 24.5% and 36.3% were observed at 15.0% of alcoholic degree for ALG capsule diameter and weight, respectively, being the loss of capsule diameter and weight less for SiO_2-ALG (21.4% and 26.8%, respectively). SiO_2-ALG and ALG capsules are hydrogels which composition is mainly water and they are dehydrated in the presence of

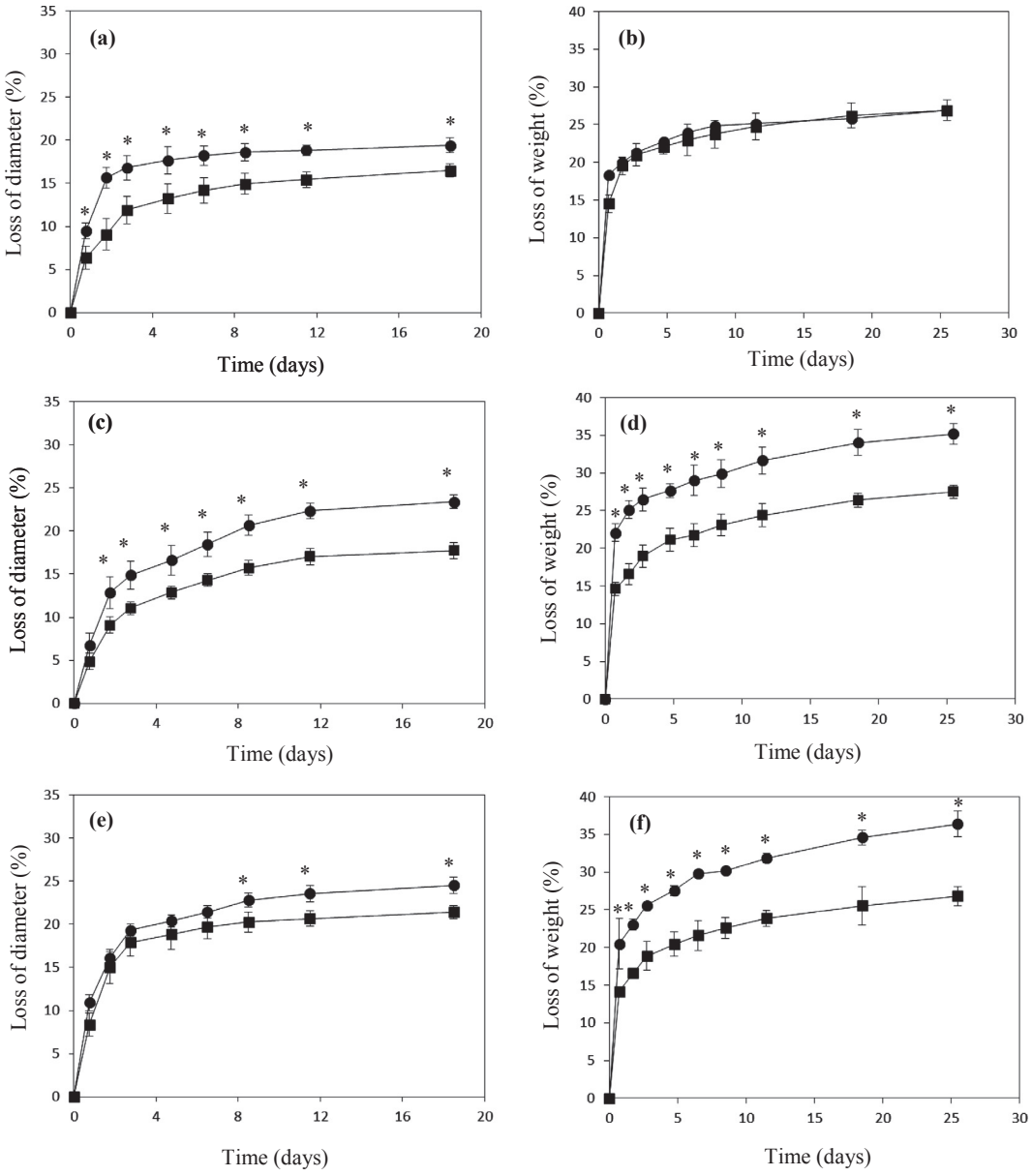

Fig. 5. Evolution of shrinking (expressed as decrease of diameter (in %) and weight (in %)) of alginate (●) and SiO_2-alginate (■) biocapsules loaded with *Oenococcus oeni* submerged in red wines at different alcohol degree ((a, b) 12.0%, (c, d) 13.5% and (e, f) 15.0%). Shrinking was calculated from the average of 20 biocapsules submerged in wine at 22 °C Standard deviations of the assays are represented by error bars. * indicates a statistically significant difference ($p < 0.05$) between both capsules.

alcohols (Torres, Velasquez, & Brito-Arias, 2011). As it was discussed above, the addition of siliceous material into ALG hydrogel causes more rigidity and robustness (Simó, Vila-Crespo, et al., 2017) and hence the degree of shrinking and dehydration of SiO_2-ALG capsules in alcoholic solutions is lower. The higher capacity of SiO_2-ALG capsules to reduce the water loss creates a more hydrophilic microenvironment (Xu et al., 2006), so improving the fermentative performance of bacteria in an ethanol-rich medium as wine.

Finally, to evaluate the capsule stability in wine, the release of ALG polymer from SiO_2-ALG and ALG capsules immersed in wines at different pH and alcoholic degree was evaluated after 18 days (Fig. 6). Very small amounts of released ALG (less than 0.3 mg/L) were found in all wines assayed. The release of ALG was slightly higher in ALG capsules than in SiO_2-ALG ones in wines at pH 3.0 and 3.3. Neither the increase of pH nor the alcoholic degree modified the levels of total polysaccharides. Likely, pH-dependent changes in the electrostatic interactions between SiO_2 and ALG polymer as well as ethanol-subordinate variations in capsule shrinking are not involved in the release of ALG polymer. The diffusion of unpolymerized ALG from the inside of the capsules to the wine could explain this behaviour (Strand, Skjåk-Bræk, & Gåserød, 2004). These results highlight that rupture and chemical decomposition of capsules did not take place under the alcoholic and acid environment of wine indicating the suitability of SiO_2-ALG biocapsules for the development of MLF.

4. Conclusion

Aqueous route using colloidal SiO_2 and sodium silicate as non-alkoxide silicon precursors has been proved as a very suitable strategy to improve the chemical and operational stability of ALG-based biocapsules with entrapped *O. oeni* under hard winemaking conditions. Wine pH and ethanol content play a notable role in the operational stability of both SiO_2-ALG biocapsules and free bacteria being their negative impact most severe for unencapsulated bacteria. SiO_2-ALG

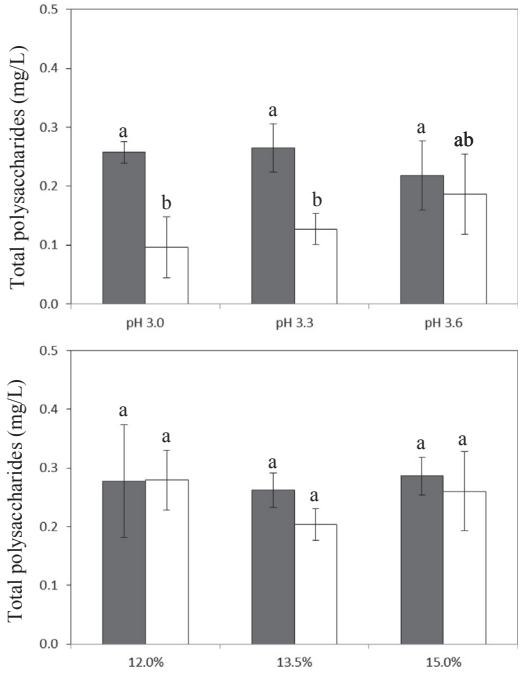

Fig. 6. Release of alginate (expressed as total polysaccharides) from alginate (■) and SiO_2-alginate (□) biocapsules loaded with *Oenococcus oeni* submerged in red wines at different pH (3.0, 3.3 and 3.6) and alcohol degree (12.0%, 13.5% and 15.0%) after 18 days. Standard deviations of the assays are represented by error bars. Different letters in the bars indicate a significant difference between samples ($p < 0.05$).

biocapsules could be reused for at least five cycles in wines at pH 3.0–3.6 as well as with alcoholic degrees of 12.0–15.0%. However, unencapsulated bacteria could be successfully reused neither in wines at pH lower than 3.6 nor in wines with alcoholic degree higher than 12.0%. Combination of ALG and siliceous material enhanced the long-term chemical stability of biocapsules in wines. These results highlight that the implementation of SiO_2-ALG biocapsules with entrapped *O. oeni* may be a promising technique for highly efficient processes in winemaking.

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Declarations of interest

None.

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CAPÍTULO 4

7. CAPÍTULO 4: Influencia del sistema de inoculación en el desarrollo de la fermentación maloláctica.

- **7.1.Malolactic fermentation induced by silica-alginate encapsulated *Oenococcus oeni* with different inoculation regimes**

- **7.2. Evaluating the influence of simultaneous inoculation of SiO2-alginate encapsulated bacteria and yeasts on volatiles, amino acids, biogenic amines and sensory profile of red wine with lysozyme addition**

Malolactic fermentation induced by silica-alginate encapsulated *Oenococcus oeni* with different inoculation regimes

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Abstract

Background and Aims: The encapsulation of *Oenococcus oeni* into silica-alginate (Si-ALG) gels has been previously confirmed as a suitable strategy for a successful malolactic fermentation (MLF). The aim of this study was to evaluate the effect of two inoculation strategies (simultaneous vs sequential) on the performance of MLF with encapsulated *O. oeni* under winemaking conditions.

Methods and Results: Sequential inoculation of Si-ALG biocapsules successfully achieved a complete MLF in high ethanol wines, while free and ALG encapsulated bacteria failed. A simultaneous inoculation with Si-ALG biocapsules provided a significant reduction in time to complete MLF in high sugar and low pH musts. The regime of inoculation did not modify the chemical composition of the wines.

Conclusions: Either sequential or simultaneous inoculation of Si-ALG biocapsules constitute an effective alternative to free bacteria to undertake MLF under stressful conditions.

Significance of the Study: Effective winemaking protocols based on sequential or simultaneous induction of MLF with inoculated Si-ALG encapsulated *O. oeni* have been accomplished. These are of potential interest for winemaking in both warm and cool regions.

Keywords: *co-inoculation, immobilisation, inoculation timing, silicate, wine*

Introduction

Malolactic fermentation (MLF) is a biotechnological process that takes place during the making of most red and some white wines. Malolactic fermentation may occur spontaneously or may be induced by inoculation of selected lactic acid bacteria (LAB), mainly cultures of *Oenococcus oeni*, and involves the conversion of L-malic acid into L-lactic acid and CO_2 (Bauer and Dicks 2004), causing a de-acidification of wine. This process is recommended because an improvement of sensory characteristics (Boido et al. 2009, Antalick et al. 2012) and a microbial stabilisation of wine are achieved (Bartowsky 2005).

Development and metabolism of LAB in wine are complicated because of the stressful conditions of wine [high ethanol concentration, low pH, scarce nutrients, low temperature and the presence of SO_2 (Lerm et al. 2010)]. Currently, MLF is also affected by an increase in temperature because of climate change which is leading to higher concentration of grape sugar and production of high ethanol and unbalanced wines (Mira de Orduña 2010, Vila-Crespo et al. 2010). Because of these circumstances, the development of alternatives to improve the adaptation of LAB to these unfavourable winemaking conditions is necessary.

An explored strategy is the encapsulation of LAB because it can protect bacteria against adverse conditions during MLF (Simó et al. 2017a). Moreover, immobilised cell technology can offer other important advantages for the development of MLF such as: (i) reduction of the lag phase of LAB thus improving productivity of the process and decreasing the risk of microbial contamination; (ii) easy recovery of immobilised bacteria enabling reduction of SO_2 level, a potential allergen; (iii) possibility of reutilisation of encapsulated bacteria in successive lots of wine; and (iv) opportunity of continuous processing (Kourkoutas et al. 2004, 2010, Zhang and Lovitt 2006, Vila-Crespo et al. 2010, Rodríguez-Nogales et al. 2013). Currently, the major challenges for successful application of encapsulated cells are the high cost of the encapsulation process and the difficulty of producing them on a large scale, under aseptic conditions and with high metabolic activity and cell viability. Despite this, some applications for winemaking have been implemented using encapsulated yeasts (Simó et al. 2017a)

Cell encapsulation into alginate (ALG) hydrogel has received special attention because of its simplicity, low cost and mild working conditions (Lee et al. 2004, Bouallagui and Sayadi 2006, Safarik et al. 2008, Satora et al. 2009, Huang et al. 2016, Shin et al. 2016). Low chemical stability and mechanical robustness, however, of ALG hydrogel discourage its implementation for industrial application. Our research group is implementing an innovative process for adaptation of *O. oeni* to the stressful environment of wine based on bacterial encapsulation using robust biocomposites of silica-alginate (Si-ALG) (Simó et al. 2017b). An improvement of ALG gel stability and robustness has been achieved using colloidal silica and silicates. Inclusion of *O. oeni* into these inorganic–organic hydrogels markedly improved its capacity to reduce the concentration of L-malic acid in high ethanol as well as in low pH wines, and at low fermentation temperature (Simó et al. 2017c). These results confirmed

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the greater tolerance of Si-ALG encapsulated LAB to the unfavourable environment of wine.

Together with the encapsulation of *O. oeni*, the time of bacterial inoculation also plays a key role in bacterial adaptation to unfavourable conditions in winemaking. Traditionally, bacterial inoculum is added after alcoholic fermentation (AF) (sequential AF/MLF), and MLF takes place when the bacterial population increases to about 10^6 CFU/mL. Inoculation of high ethanol as well as low pH wines, however, with selected cultures of LAB after completion of AF can cause a large reduction in viable LAB and an increase in the lag phase. Therefore, sluggish or stuck MLF (Zapparoli et al. 2009) and a rise in the risk of spoilage by other wine microorganisms may occur (Di Toro et al. 2015).

Direct inoculation of LAB into grape must at the beginning of AF (simultaneous AF/MLF) is an alternative that enables a gradual adaptation of bacteria to the increasing ethanol concentration in a medium rich in nutrients. This practice is cautiously used by oenologists, fearing that wine quality may be compromised because of the capacity of LAB to consume must sugars and produce acetic acid as a consequence of their heterofermentative metabolism (Antalick et al. 2013). Moreover, an interruption of AF could take place before sugar depletion (Jussier et al. 2006) by a specific yeast strain-bacteria interaction, because yeast growth could be reduced by LAB (Mendoza et al. 2011).

Conversely, recent studies have highlighted the enhanced capacity of *O. oeni* to complete MLF and reduce fermentation time in simultaneous AF/MLF without a notable increase in wine volatile acidity (VA) (Jussier et al. 2006, Zapparoli et al. 2009, Pan et al. 2011, Abrahamse and Bartowsky 2012, Cañas et al. 2012, 2014, Knoll et al. 2012, Antalick et al. 2013, Taniasuri et al. 2016, Tristezza et al. 2016). Furthermore, wines obtained by simultaneous AF/MLF showed higher red fruit and ripe fruit notes (Jussier et al. 2006, Massera et al. 2009, Abrahamse and Bartowsky 2012, Tristezza et al. 2016, Versari et al. 2016). From a technical point of view, wines elaborated by simultaneous AF/MLF would be ready sooner for downstream processes, such as racking, fining and SO_2 addition, improving microbial stability and winemaking efficiency (Jussier et al. 2006).

The aim of this study was to evaluate, for the first time, the performance of free and encapsulated *O. oeni* inoculated at two stages of the winemaking process: (i) at the end of AF (sequential AF/MLF); and (ii) at the beginning of AF (simultaneous AF/MLF) to induce MLF under different winemaking conditions. The trials were carried out with *O. oeni* encapsulated into gels of ALG and Si-ALG.

Materials and methods

Immobilisation of LAB

Oenococcus oeni strain LALVIN VP 41 MBR (Lallemand, Blagnac, France) was encapsulated into both Si-ALG and ALG gels. A procedure based on the mixture of derivatives of silicon with sodium ALG before gelation in the presence of Ca^{2+} was carried out to encapsulate bacteria into Si-ALG biocomposites (Simó et al. 2017b). The pH of a solution of 1.23 mol/L colloidal silica (Ludox HS40, Sigma-Aldrich, Madrid, Spain) and 0.06 mol/L sodium silicate (Sigma-Aldrich) in water was adjusted to 6.29 by adding 2 N HCl. Then, sodium ALG (Panreac, Barcelona, Spain) was mixed with the silica solution until a final concentration of 2% (w/v). Later on, *O. oeni* was added at ~3×10^9 CFU/g of gel. Before adding bacteria to the siliceous material-ALG solution and according to the manufacturer's instructions, freeze-dried bacteria were rehydrated in 20 times their mass of sterilised chlorine-free water at 20°C for a maximum of 15 min. The well-mixed siliceous material-ALG-cell suspension was extruded from a 10 mL sterile syringe (BD 166 Plastipak, Toledo, Spain) into a sterile 0.2 mol/L $CaCl_2$ solution under continuous agitation (260 rpm) at 22°C. The diameter of the nozzle used was 1.78 mm. The height from which the suspension was dripped into the gelation bath was maintained at 20 cm. Capsules were kept in this solution for 2 h and washed with water at 22°C. The encapsulation of bacteria into ALG hydrogels was similar to that reported above but omitting the addition of colloidal silica and sodium silicate. The Si-ALG and ALG encapsulated bacteria were used immediately after preparation.

Fermentation protocols

Wines of this study were prepared from Cabernet Sauvignon grapes from the 2017 harvest in the experimental winery of the School of Agricultural Engineering, University of Valladolid, Palencia, Spain. Grapes were destemmed and crushed, and SO_2 was added (30 mg/L). Wines were made using grape must without skins or seeds. The composition of the grape must [pH 3.9, 22.4°Brix, 3.7 g/L TA (expressed as tartaric acid), 2.2 g/L of malic acid, 15 mg/L of free SO_2] was modified in order to obtain the required experimental conditions. First, sugar concentration was adjusted in two lots of must to: (i) 20.8°Brix by addition of a volume of distilled water; and (ii) 25.3°Brix by adding the same volume of a solution of sucrose. Thus, the composition of the rest of must components remained equal in both musts. Afterwards, a pH value of 3.0 and 3.8 was obtained using concentrated acid or basic solutions. Finally, the concentration of L-malic acid was adjusted to 3.0 g/L with L-malic acid (Sigma-Aldrich). Four lots of grape must with following composition were sterilised by filtration (0.2 μm, cellulose nitrate membrane): must #1, 25.3°Brix and pH 3.0; must #2, 25.3°Brix and pH 3.8; must #3, 20.8°Brix and pH 3.0; and must #4, 20.8°Brix and pH 3.8. Then, musts were inoculated with 0.25 g/L of rehydrated *Saccharomyces cerevisiae* (Lalvin Clos, Lallemand). The completion of AF was estimated by the measurement of the reducing sugar concentration. Free and encapsulated *O. oeni* (into ALG and Si-ALG hydrogels) were inoculated at a concentration of ~9×10^7 CFU/mL of must/wine: (i) 2 days after the yeast inoculation (simultaneous AF/MLF); and (ii) at the end of AF (sequential AF/MLF). A Control wine without inoculating bacteria was also made. The AF and MLF fermentations were carried out in 100 mL Erlenmeyer flasks containing 50 mL of must/wine at 22°C and in duplicate. The flasks were closed with Müller valves, filled with pure sulfuric acid.

Analytical procedures

The AF was monitored by gravimetric determination, evaluating the loss of mass of the flasks because of the production of CO_2. The degradation of L-malic acid concentration during MLF was monitored with an enzymatic kit (TDI, Barcelona, Spain). The methods described by the Organisation Internationale de la Vigne et du Vin (OIV) (2017) were employed for the determination of pH, VA, reducing sugars and alcohol concentration. Colour parameters were determined at 420, 520 and 620 nm. Total polyphenol index (TPI) was quantified at 280 nm (Lan Optics 2000 UV, Labolan, Spain). Analytical determinations were in triplicate.

Statistical analysis

All statistical analyses were undertaken with the SPSS v. 17.0 statistical package (SPPS, Chicago, IL, USA). Statistical differences were determined by a variance analysis followed by a Tukey's post-hoc test ($P < 0.05$).

Results and discussion

Alcoholic fermentation

The AF was completed in about 10 days in all MLF inoculation treatments, independent of the composition of the grape must (Figures 1–2). Similar fermentative kinetics were found in all samples made by either simultaneous or sequential AF/MLF. The kinetics of AF were not affected by the presence of free or encapsulated bacteria during simultaneous AF/MLF (Figure 2). As reported by other authors (Abrahamse and Bartowsky 2012, Knoll et al. 2012, Tristezza et al. 2016), the presence of *O. oeni* during AF in simultaneous AF/MLF assays does not appear to influence the rate of yeast fermentation. The ability of wine LAB, however, to inhibit AF has been reported, having an adverse impact on the production and the quality of the wine (Alexandre et al. 2004). The mechanisms of yeast inhibition by bacteria involve the formation of metabolites, such as acetic acid (Huang et al. 1996) and bacteriocin-like inhibitors (Yurdugül and Bozoglu 2002), and the depletion of either certain nutrients or survival factors required by yeasts (Alexandre et al. 2004). The degradation of yeast cell wall by extracellular bacterial β-1,3-glucanase activity has been also reported as another factor contributing to AF inhibition (Guilloux-Benatier et al. 2000). This disparity in results indicates that the selection of a suitable yeast–bacteria combination avoids the competition for the nutrients and the synthesis of metabolites with potential inhibitory properties (Alexandre et al. 2004, Guzzon et al. 2013), and plays an important role for a successful AF. Our results have demonstrated that the yeast and bacterial strains used in this study were compatible with a simultaneous AF/MLF strategy.

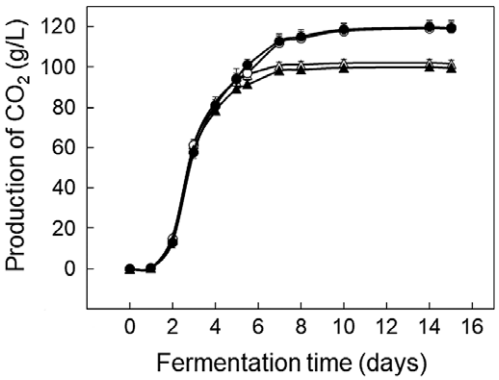

Figure 1. Production of CO_2 during alcoholic fermentation (AF) of Cabernet Sauvignon musts (sequential AF/MLF) at 22°C. Must #1 (25.3°Brix, pH 3.0) (●), must #2 (25.3°Brix, pH 3.8) (○), must #3 (20.8°Brix, pH 3.0) (▲), and must #4 (20.8°Brix, pH 3.8) (△). Standard errors are represented as error bars ($n = 6$).

Malolactic fermentation

The dynamics of MLF were measured along the winemaking processes in both regimes, the sequential AF/MLF (Figure 3) and the simultaneous AF/MLF (Figure 4). The effect of grape must composition (TSS and pH), type of inoculum (encapsulated or free bacteria) and inoculation time (sequential and simultaneous AF/MLF) on the average L-malic acid conversion were analysed 8 days after bacterial inoculation (Figure 5). Significant differences between groups ($P < 0.05$) were found for the four principal variables (TSS, pH, and type of inoculum and inoculation time) and for the following interactions between principal variables: TSS × type of inoculum, TSS × inoculation time and type of inoculum × inoculation time.

The kinetics of MLF were dependent on the composition of the grape must (Figures 3,4). L-Malic acid concentration did not change with respect to the initial value (data not shown) in Control wines (without LAB), demonstrating that MLF development was because of the bacterial inoculum in the remaining samples. The highest average L-malic acid conversion was achieved in wines produced with low sugar grape must (90%) compared with high sugar grape must (66%) (Figure 5). Winemaking with high sugar musts leads to high ethanol wines, which may cause problems with the induction of MLF (Zapparoli et al. 2009). Ethanol is one of the most important factors that affect negatively the metabolism of LAB (Lerm et al. 2010, Sumby et al. 2014). It has been reported that ethanol provokes disruption of cell membrane structure and alteration of membrane fluidity (Betteridge et al. 2015). The average L-malic acid conversion

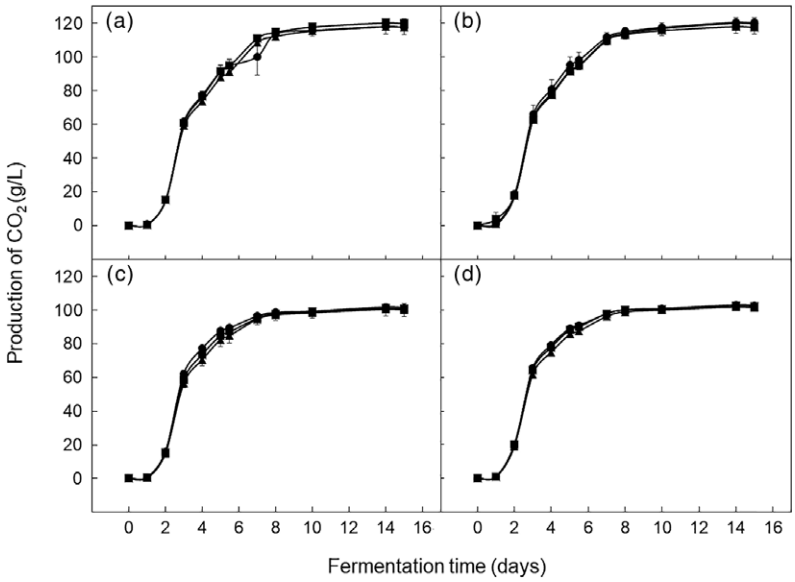

Figure 2. Production of CO_2 during alcoholic fermentation (AF) of Cabernet Sauvignon musts inoculated with free (▲), and alginate (■) and silica-alginate (●) encapsulated *Oenococcus oeni* at the beginning of AF (simultaneous AF/MLF) at 22°C. (a) Must #1 (25.3°Brix, pH 3.0), (b) must #2 (25.3°Brix, pH 3.8), (c) must #3 (20.8°Brix, pH 3.0) and (d) must #4 (20.8°Brix, pH 3.8). Standard errors are represented as error bars ($n = 2$).

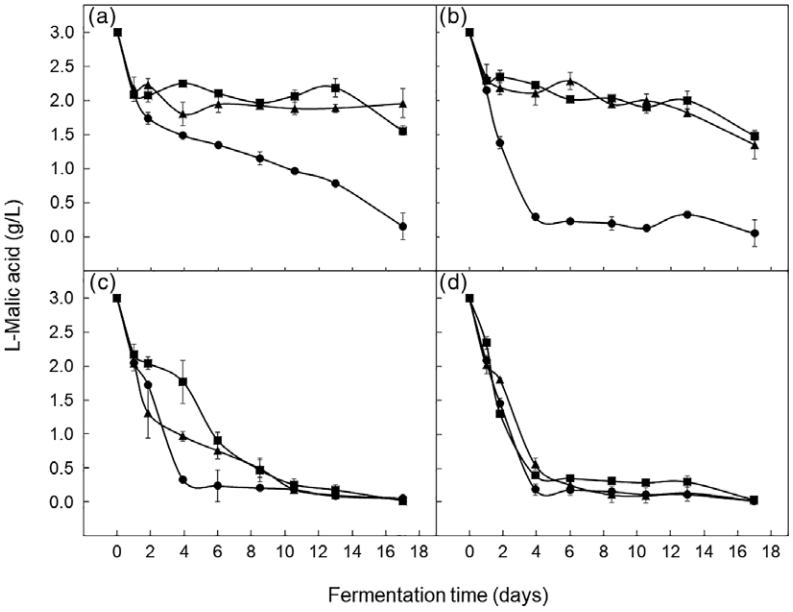

Figure 3. L-Malic acid consumption in Cabernet Sauvignon wines inoculated with free (▲), and alginate (■) and silica-alginate (●) encapsulated *Oenococcus oeni* after alcoholic fermentation (AF) (sequential AF/MLF) at 22°C. (a) Wine from must #1 (25.3°Brix, pH 3.0), (b) wine from must #2 (25.3°Brix, pH 3.8), (c) wine from must #3 (20.8°Brix, pH 3.0) and (d) wine from must #4 (20.8°Brix, pH 3.8). Standard errors are represented as error bars (n = 2).

was reduced from 84 to 71% when pH was reduced from 3.8 to 3.0 (Figure 5). It has been reported that growth of *O. oeni* and development of MLF are negatively influenced by values of pH lower than 3.5 (Rosi et al. 2003, Betteridge et al. 2015), while the highest MLF activity is achieved at pH values of about 3.5–4.0 (Bauer and Dicks 2004).

The combined effect of TSS and pH on malic acid conversion in a sequential AF/MLF regime is shown in Figure 3. A slow reduction of L-malic acid concentration was observed in the wine from grape must #1, characterised by high sugar concentration and low pH. Under these unfavourable conditions, only Si-ALG encapsulated bacteria were able to complete MLF. L-Malic acid, however, was not completely depleted in wines inoculated with ALG encapsulated and free bacteria after 17 days, achieving a low L-malic acid conversion of 48 and 35%, respectively. Conversely, faster L-malic acid depletion was observed during winemaking of grape musts #2, #3 and #4 using Si-ALG encapsulated bacteria (90.7, 89.0 and 94.0% of malic acid conversion, respectively). Moreover, the kinetics of L-malic acid consumption in must #4 were similar with the three types of inoculum, achieving L-malic acid conversion of 96–98% after 8 days. The MLF kinetics of the ALG encapsulated and free bacteria were notably improved in the wines from low sugar musts (#3 and #4) compared with that of wines from high sugar musts (#1 and #2). Solieri et al. (2010) indicated that a low pH value of 3.0–3.2 is the main factor affecting MLF, regardless of ethanol concentration (10–13%). Our results indicated, however, that both low pH and high TSS of must negatively affected malic acid conversion (Figure 3). Similar results were found in Riesling and Chardonnay wines (9.8% of alcohol) when the reduction of pH from 3.8 to 3.2 increased the duration of MLF by up to 34 days, while at pH 3.2 and 11.8% of alcohol a partial MLF was observed (Knoll et al. 2011).

Compared to other inoculation treatments, complete MLF was observed after 4 days (musts #2, #3 and #4) and 17 days (must #1) inoculated with Si-ALG encapsulated bacteria. Slow MLF was found with free bacteria, which completed MLF in wines from musts #3 and #4 after 10 and 6 days, respectively, and they were unable to complete MLF in wines from musts #1 and #2 after 17 days.

Simultaneous AF/MLF had a positive influence on the average of L-malic acid conversion inoculating either

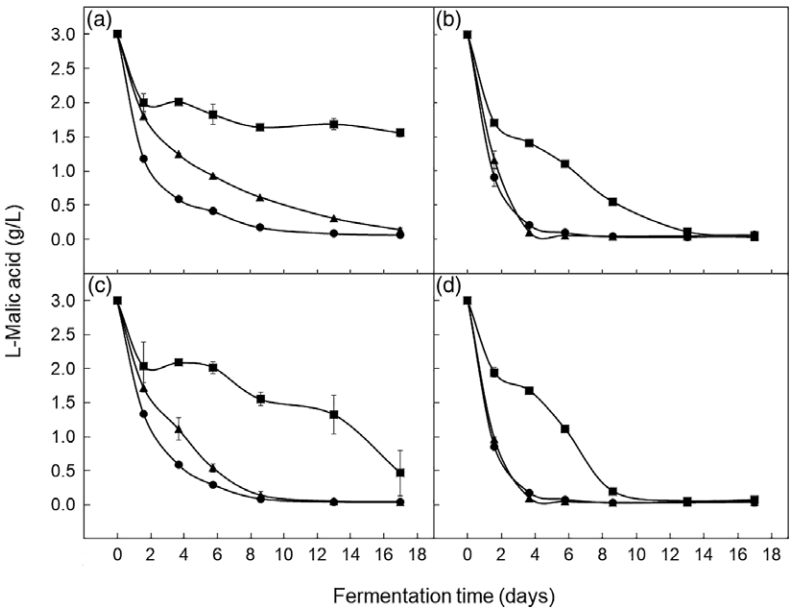

Figure 4. L-Malic acid consumption during winemaking of Cabernet Sauvignon musts inoculated with free (▲), and alginate (■) and silica-alginate (●) encapsulated *Oenococcus oeni* at the beginning of alcoholic fermentation (AF) (simultaneous AF/MLF) at 22°C. (a) Must #1 (25.3°Brix, pH 3.0), (b) must #2 (25.3°Brix, pH 3.8), (c) must #3 (20.8°Brix, pH 3.0) and (d) must #4 (20.8°Brix, pH 3.8). Standard errors are represented as error bars (n = 2).

encapsulated or free bacteria, increasing this parameter from 70 (sequential regime) to 86% (simultaneous regime) (Figure 5). This practice markedly improved MLF behaviour in high sugar grape must (Figure 5c), where an average L-malic conversion of 83% was achieved in comparison with 49% obtained by the sequential regime. Nevertheless, these results were not found in low sugar grape musts where a significant difference for the average of L-malic acid conversion between both inoculation strategies was not observed after 8 days of bacterial inoculation. The combined effect of pH and TSS on malic acid conversion using a simultaneous AF/MLF regimen is shown in Figure 4. In every trial MLF succeeded, regardless of the inoculation treatment; except for musts at pH 3.0 (#1 and #3) inoculated with ALG encapsulated bacteria. In grape must #1, when conditions were more restrictive, simultaneous AF/MLF improved markedly the L-malic acid depletion of grape must inoculated with free and Si-ALG bacteria. L-Malic acid conversion assayed after 8 days increased from 61 and 36% (sequential AF/MLF) to 94 and 79% (simultaneous AF/MLF) with Si-ALG encapsulated and free bacteria, respectively. Simultaneous AF/MLF inoculated with Si-ALG encapsulated and free bacteria resulted in a complete consumption of L-malic acid in 13 and 17 days, respectively. As mentioned before, however, when Si-ALG encapsulated bacteria were inoculated in a sequential inoculation, they required up to 17 days to complete MLF process, while free bacteria were unable to successfully complete the conversion of L-malic acid (Figure 3). In musts #2, #3 and #4, the dynamics of MLF in the simultaneous inoculation were similar using Si-ALG encapsulated and free bacteria, where complete consumption of L-malic acid required 6, 8 and 6 days, respectively.

These results proved that simultaneous AF/MLF was the most efficient inoculation strategy, consistent with other studies (Abrahamse and Bartowsky 2012, Antalick et al. 2013, Cañas et al. 2014, Homich et al. 2016, Tristezza et al. 2016, Versari et al. 2016). Under these winemaking conditions, bacteria were able to induce MLF without a phase of adaptation in grape must. Simultaneous AF/MLF resulted in a valid strategy to allow a gradual bacterial adaptation to the ethanol of the must produced during AF (Zapparoli et al. 2009). During this period, *O. oeni* may be able to activate some cellular mechanisms to adapt to unfavourable conditions, such as low pH and increasing ethanol concentration.

The type of inoculum affected significantly the average of L-malic acid conversion, providing the best results using Si-ALG encapsulated bacteria (90%), followed by free bacteria (79%) and ALG encapsulated bacteria (65%) (Figure 5). In high sugar grape must (Figure 5b), the performance of Si-ALG encapsulated bacteria was superior (87% of conversion) to that obtained inoculating free bacteria (62% of conversion). Both types of inoculum, however, showed similar performance (93–95% of conversion) in low sugar grape must. Differences in performance of Si-ALG encapsulated and free bacteria were observed between sequential and simultaneous AF/MLF (Figure 5f). When bacteria were inoculated after AF (sequential AF/MLF), the highest average of L-malic acid conversion was found with Si-ALG encapsulated bacteria, however, free and Si-ALG encapsulated bacteria did not show a significant difference in simultaneous AF/MLF

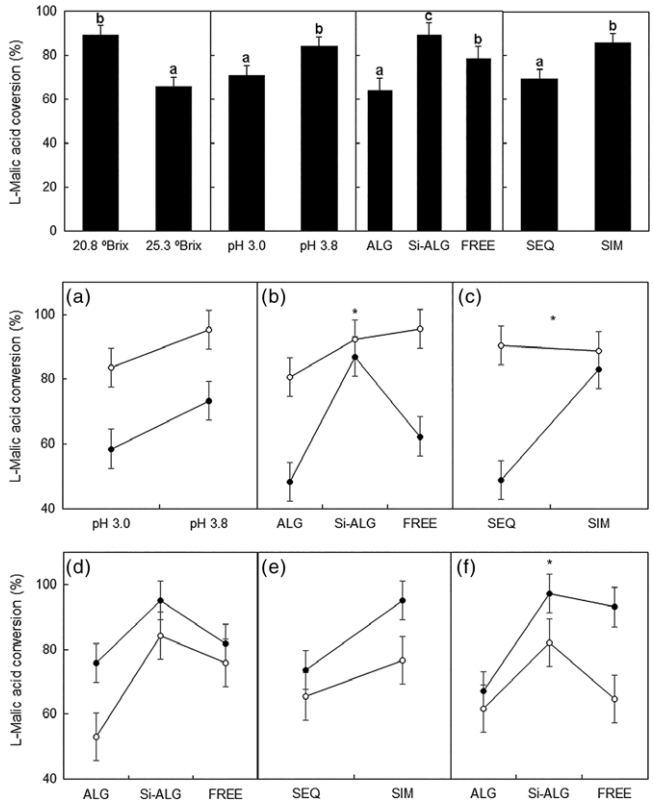

Figure 5. Effect of the grape must composition (TSS and pH), the type of inoculum [alginate (ALG) and silica-alginate (Si-ALG) encapsulated or free bacteria], and the inoculation time [sequential (SEQ) and simultaneous (SIM) AF/MLF] on the average of L-malic acid conversion after 8 days of bacterial inoculation. Standard errors are represented as error bars. Different letters indicate a significant difference at $P < 0.05$. Effect on L-malic consumption depending on (a) pH, (b) type of inoculum and (c) inoculation time of musts with 20.8°Brix (○) and 25.3°Brix (●). Effect on L-malic consumption depending on (d) type of inoculum and (e) inoculation time of musts with pH 3.0 (○) and pH 3.8 (●). Effect on L-malic consumption depending on (f) the type of inoculum performing sequential (○) and simultaneous (●) AF/MLF. *Statistically significant interaction between principal variables ($P < 0.05$).

Table 1. Average of basic chemical composition of Cabernet Sauvignon wines after malolactic fermentation inoculated with free, and alginate and silica-alginate encapsulated *Oenococcus oeni* using simultaneous and sequential alcoholic fermentation/malolactic fermentation.

Factors	Levels	Reducing sugars (g/L)	Alcohol degree (%)	Volatile acid (g/L)	pH	TPI	Colour intensity†	Tonality‡
TSS	20.8	2.83 ± 1.00a	11.8 ± 0.1a	0.21 ± 0.06a	3.41 ± 0.29a	19.2 ± 0.6b	3.26 ± 0.08a	0.97 ± 0.26b
	25.3	4.37 ± 1.06b	14.7 ± 0.1b	0.23 ± 0.05b	3.37 ± 0.33a	18.7 ± 0.3a	3.22 ± 0.05a	0.90 ± 0.23a
pH	3.0	4.17 ± 1.63b	13.3 ± 0.3b	0.24 ± 0.05b	3.12 ± 0.15a	18.9 ± 0.5a	3.65 ± 0.05b	0.49 ± 0.12a
	3.8	3.03 ± 0.74a	13.7 ± 0.3a	0.19 ± 0.06a	3.65 ± 0.15b	19.0 ± 0.9a	2.73 ± 0.05a	1.08 ± 0.18b
Type of inoculum	ALG	3.46 ± 0.42a	13.3 ± 0.4a	0.21 ± 0.04a	3.31 ± 0.20a	19.1 ± 0.3b	3.12 ± 0.03a	0.91 ± 0.22a
	Si-ALG	3.94 ± 1.57a	13.3 ± 0.4a	0.22 ± 0.07a	3.37 ± 0.18ab	18.6 ± 0.5a	3.42 ± 0.06a	0.87 ± 0.17a
	Free	3.38 ± 0.51a	13.3 ± 0.4a	0.22 ± 0.07a	3.47 ± 0.25b	19.2 ± 0.9b	3.31 ± 0.07a	1.02 ± 0.25b
Inoculation time	Sequential AF/MLF	3.72 ± 1.45a	13.3 ± 0.3a	0.23 ± 0.06b	3.34 ± 0.31a	19.2 ± 0.6b	3.32 ± 0.07a	0.96 ± 0.23a
	Simultaneous AF/MLF	3.47 ± 1.32a	13.3 ± 0.3a	0.21 ± 0.06a	3.42 ± 0.30a	18.7 ± 0.8a	3.39 ± 0.07a	0.90 ± 0.19a

Values represent means ± standard error for each level of the factors (n = 12 for TSS, type of inoculum and inoculation time; n = 8 for type of inoculum) and different letters mean a significant difference for each individual factor at $P < 0.05$. AF, alcoholic fermentation; ALG, alginate; MLF, malolactic fermentation; Si-ALG, silica-alginate; TPI, total polyphenol index. †Colour intensity was calculated as $A_{420} + A_{520} + A_{620}$. ‡Tonality was calculated as A_{420}/A_{520}.

inoculation. As previously mentioned, similar L-malic acid evolution by simultaneous AF/MLF was observed inoculating Si-ALG and free bacteria, except for high acid and high sugar grape musts where the depletion of L-malic acid was faster in wines inoculated with Si-ALG bacteria than those inoculated with free bacteria (Figure 4).

The encapsulation of bacteria and yeasts into porous matrices has been applied to improve their catalytic activities in alcohol beverage production (Kourkoutas et al. 2004). A recent study has highlighted that the encapsulation of *O. oeni* into biocomposites of Si-ALG enhanced notably its malolactic activity as compared with that of ALG encapsulated as well as free bacteria (Simó et al. 2017b). The inclusion of siliceous material into ALG capsules increased the tolerance of bacteria towards the stressful environment of wine (low pH, high ethanol and low nutrients) and at low fermentation temperature (Simó et al. 2017c). Rodríguez-Nogales et al. (2013) observed a significant increase in MLF efficiency in high ethanol wines inoculated with encapsulated *O. oeni* into polyvinyl alcohol hydrogel. The success of MLF under winemaking conditions when inoculating Si-ALG encapsulated LAB could be attributed to several factors. The organic–inorganic matrix could offer protection from the harsh environmental conditions of wine, such as alcohol, pH and inhibitors (Kourkoutas et al. 2010, Kosseva 2011). Moreover, an increase in the level of saturated fatty acids of the hydrogel encapsulated cell membrane has been observed in response to ethanol stress, providing a better ethanol tolerance to cells (Junter and Jouenne 2004). Furthermore, it has been reported that cell–cell contact in high cell density matrices enhanced the cell resistance against alcohol stress (Norton et al. 1995). Finally, the non-gelling liquid ALG matrix could improve the stability of the hydration layer around the cell affording protection (Sun et al. 2007).

Wine composition

The composition of the 24 wines after MLF is summarised in Table S1. The average of the final wine composition following MLF for each level of the factors TSS and pH of must, type of inoculum and inoculation time is shown in Table 1. Wines produced with high sugar and low pH grape musts presented a higher value of reducing sugars with an average value of 4.37 and 4.17 g/L, respectively. Wines produced from low pH grape must showed a lower average value of alcohol (13.3%) than those elaborated with high pH grape must (13.7%). Significant differences among type of inoculum and between inoculation regimes were found neither for reducing sugars nor for alcohol concentration, indicating that AF was not affected by the type of inoculum (free or encapsulated bacteria) or by the bacterial inoculation time. All the wines can be considered 'dry' because their reducing sugar level fell below 5 g/L.

The concentration of VA was of neither technological nor legal significance (Table S1), because the sensory threshold and maximum legal concentration for acetic acid range around 1 g/L (Jussier et al. 2006). The type of inoculum, free or encapsulated bacteria, did not affect the average concentration of VA (Table 1). There was a slight increase in VA in wines elaborated with high sugar and high pH grape musts; however, the impact of this difference is limited from a technological and sensory point of view. Sequential inoculation slightly increased the average concentration of VA compared to that of simultaneous inoculation. The results of our study and those of previous studies (Rosi et al. 2003, Knoll et al. 2011, Abrahamse and Bartowsky 2012, Cañas et al. 2012, Homich et al. 2016, Tristezza et al. 2016, Versari et al. 2016) found no evidence for the increase in acetic acid

production because of LAB metabolism in fermenting grape juice using simultaneous AF/MLF. Our results are also consistent with those obtained in a recent study with *S. cerevisiae* and *O. oeni* co-immobilised in ALG gels where inoculation either in simultaneous or in sequential AF/MLF regime did not affect VA (Bleve et al. 2016).

Little difference was found for pH among wines inoculated with free and encapsulated bacteria. This slight difference did not present technological significance. Jussier et al. (2006) found no significant difference with regard to the final value of wine pH between sequential and simultaneous AF/MLF.

The concentration of phenolic substances and colour tonality and intensity values are important factors in the evaluation of wine quality. Overall, there were negligible changes in the TPI and in the colour of wine because of the TSS of grape must and the inoculation time. Wine colour was also affected by must pH, showing the highest colour intensity and the lowest tonality at pH 3.0. The red colour in wine is strongly affected by pH and depends on the proportion of anthocyanin in the flavylium state. As the acidity of wine increases, the proportion of red flavylium anthocyanins also rises (Castañeda-Ovando et al. 2009). Previously, it was shown that similar levels of TPI, colour intensity and tonality were found in wines elaborated by sequential and simultaneous AF/MLF (Abrahamse and Bartowsky 2012, Versari et al. 2016). The use of encapsulated LAB into hydrogels to induce MLF could dilute metabolites and wine colour because ALG and Si-ALG capsules are hydrogels formed by water (about 96–99%) and tend to be dehydrated in the presence of ethanol (Simó et al. 2017b). Moreover, ALG gels have some capacity to adsorb phenolic substances (Massalha et al. 2007). In our study, we did not notice a notable difference in TPI and wine colour characteristics because of the type of inoculum. These results are consistent with the study carried out by Bleve et al. (2016) with *S. cerevisiae* and *O. oeni* co-immobilised in ALG gels. The influence of type of inoculum, however, on colour components should be verified in wines elaborated with skins and under real winemaking conditions.

Conclusions

Inoculation of Si-ALG encapsulated *O. oeni* after AF (sequential AF/MLF) involves a substantial advantage over free bacteria in high sugar as well as in low pH grape must, either enabling to conclude MLF or sensitively reducing the time of this process. A simultaneous AF/MLF strategy may be an alternative to traditional oenological approaches, completing both AF and MLF in a shorter time, and causing no modification in AF kinetics, no increase in VA and no significant variation in basic wine composition. In addition, simultaneous inoculation of yeast and Si-ALG encapsulated bacteria into must of unfavourable composition markedly enhanced the development of MLF compared with free bacteria. These results open the possibility of using both practices (bacterial encapsulation and concomitant yeast–bacteria inoculation), together with a potential recycling of Si-ALG encapsulated *O. oeni* and an implementation of a continuous process. Further studies will examine these factors under commercial winemaking conditions, evaluating the influence of Si-ALG encapsulated bacteria on volatile and sensory characteristics of wines.

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Table S1. Basic composition of the 24 wines after MLF inoculated with free and alginate (ALG) and silica-alginate (Si-ALG) encapsulated *Oenococcus oeni* using simultaneous and sequential AF/MLF of Cabernet Sauvignon.

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Evaluating the influence of simultaneous inoculation of SiO_2-alginate encapsulated bacteria and yeasts on volatiles, amino acids, biogenic amines and sensory profile of red wine with lysozyme addition

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ABSTRACT

The influence of the timing of inoculation (sequential and simultaneous alcoholic fermentation (AF)/malolactic fermentation (MLF)) on the chemical and sensory properties of red wines was studied. The impact of the encapsulation of *Oenococcus oeni* into SiO_2-alginate hydrogel (Si-ALG) and the addition of lysozyme in wines inoculated with encapsulated bacteria were also analysed. There was a significant influence of the timing of inoculation on the volatile composition of the wines just as on the amino acid and biogenic amine content. The wines produced by simultaneous AF/MLF showed the highest contents of some volatile compounds, such as ethyl esters and terpenes, as well as amino acids and tyramine. Bacterial encapsulation affected the volatile and amino acid profile of the wines, while the biogenic amine composition was not modified. The chemical composition of the wines was not altered by the presence of lysozyme. A trained panel did not perceive substantial differences between treatments.

1. Introduction

Red and some white wines can go through a secondary fermentation, known as malolactic fermentation (MLF). Traditionally, this process takes place after alcoholic fermentation (AF). MLF is carried out by lactic acid bacteria (LAB), specifically from the genera *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* (Lerm, Engelbrecht, & du Toit, 2010). Although the LAB play a crucial role in quality of wine, the control of their development during winemaking process is also essential to obtain high-quality wines. In this respect, lysozyme, a hydrolytic enzyme with selective antimicrobial activity for Gram-positive bacteria, is widely used to control LAB with different purposes. Inhibition of wild LAB is often carried out in order to prevent the development of certain species or strains that may cause wine spoilage or stuck AF due to competition between yeast and bacteria. Moreover, prevention of increasing volatile acidity or high levels of biogenic amines can be also achieved. From a technological point of view, lysozyme may reduce the traditional use of sulphur dioxide, required for inhibiting MLF in white wines or microbiological stabilization after MLF (Liburdi, Benucci, & Esti, 2014).

MLF is important because it has a strong influence on wine aroma and taste. LAB are responsible for the production of metabolites and modification of aroma compounds derived from grapes and yeasts (Styger, Prior, & Bauer, 2011). It has been reported that LAB contain catalytic enzymes which are able to hydrolyse grape non-aromatic precursors to generate aromatic compounds (Cappello, Zapparoli, Logrieco, & Bartowsky, 2017). Grape constituents can be also metabolised by LAB causing a modification of the aroma of wine. It has been found that MLF can enhance the formation of esters associated with fruity aroma. A decrease in the concentration of acetaldehyde takes place during MLF reducing the vegetative aroma of wine. The production of carbonyl or ketonic compounds, such as diacetyl, acetoin and 2,3-butanodiol, derived from the metabolism of citric acid has a deep influence on the buttery flavour of wine (Styger et al., 2011). Other volatile compounds modified during MLF, such as volatile fatty acids and higher alcohols, also contribute to the aromatic complexity of wine (Lerm et al., 2010).

Wine amino acid composition can be changed by the metabolism of

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LAB during MLF (Liu, 2002). These compounds play an important role in LAB survival and growth. In particular, LAB need some essential amino acids including glutamic acid, valine, arginine, leucine, isoleucine, as well as cysteine and tyrosine (Lerm et al., 2010). LAB have a limited amino acid biosynthetic capability, however, they are able to release essential amino acids via protein and peptide hydrolysis (Lim, Foo, Loh, Mohamad, & Abdullah, 2019). It has also been reported that the metabolism of amino acids by LAB contributes to the aroma of wine (Moreno-Arribas & Polo, 2009). The metabolism of amino acid is responsible for the synthesis of higher alcohols, aldehydes, ketones and esters during MLF (Styger et al., 2011).

With regard to health issues, the control of biogenic amines in wine is essential because they affect the human health causing undesirable symptoms (Moreno-Arribas & Polo, 2009). Biogenic amines are synthetized by grape metabolism and fermentation processes, mainly MLF, due to undesirable decarboxylase-positive microorganisms (Pardo & Ferrer, 2019). Histamine, tyramine and putrescine are the major biogenic amines in wines and their occurrence is consequence of agronomic factors, winemaking processes, as well as microbiological factors (Martuscelli & Mastrocola, 2019).

The impact of MLF on the composition of volatile compounds, amino acids and biogenic amines in wine can be influenced by both bacterial species and strain employed, the timing of MLF inoculation (Abrahamse & Bartowsky, 2012), the composition of the wine after AF as well as the conditions of fermentation (Lerm et al., 2010).

An extensive knowledge of the response of LAB to the stressful conditions found in wine has been achieved. Nevertheless, in spite of the progress accomplished so far, the risk of a stuck MLF is still high when bacteria grow in unfavourable conditions (high concentration of ethanol, low pH, low levels of nutrients and fermentation temperature, high total concentration of SO_2, or presence of specific inhibitory yeast metabolites) (Sumby, Bartle, Grbin, & Jiranek, 2019).

Bacterial encapsulation has been considered a first-rate strategy to mitigate the stressful conditions of MLF in wine (Vila-Crespo, Rodríguez-Nogales, Fernández-Fernández, & Hernanz-Moral, 2010). It has been reported that the use of immobilized bacteria can improve the development of MLF and modify the chemical and sensory characteristics of wine (Bleve, Tufariello, Vetrano, Mita, & Grieco, 2016; Vila-Crespo et al., 2010). Lately, our research group has developed a biocomposite based on interpenetrated polymer networks of SiO_2 and alginate (ALG) for the adaptation of *Oenococcus oeni* to the stressful environment of wine (Simó, Vila-Crespo, Fernández-Fernández, Ruipérez, & Rodríguez-Nogales, 2017). The capacity of the encapsulated *O. oeni* to reduce the levels of L-malic acid in high ethanol as well as in low pH wines, and at low fermentation temperature was substantially improved compared with free bacteria (Simó, Fernández-Fernández, Vila-Crespo, Ruipérez, & Rodríguez-Nogales, 2017). Both sequential and simultaneous inoculation of encapsulated bacteria turned out to be an effective alternative to free bacteria to reduce the time to complete MLF in high sugar and low pH musts, and the reutilization of encapsulated LAB was successfully carried out to develop successive MLF (Simó, Fernández-Fernández, Vila-Crespo, Ruipérez, & Rodríguez-Nogales, 2019a). Besides, the timing of inoculation did not modify the basic composition of wine (Simó, Fernández-Fernández, Vila-Crespo, Ruipérez, & Rodríguez-Nogales, 2019b).

There is no information on detailed chemical and sensory characterisation of red wines inoculated with SiO_2-ALG encapsulated *O. oeni*. However, several studies focus on these issues in wines inoculated with free bacteria have been reported (Abrahamse & Bartowsky, 2012; Izquierdo-Cañas, Romero, Pérez-Martín, Seseña, & Palop, 2014; Masqué, Romero, Rico, Elórduy, Puig, Capdevila, & Palacios, 2007). Some studies only describe the consumption of malic acid during MLF using SiO_2-ALG immobilized bacteria (Callone, Campostrini, Carturan, Cavazza, & Guzzon, 2008; Guzzon, Carturan, Krieger-Weber, & Cavazza, 2012; Simó et al., 2019b). This study aims to analyse the effect of the timing of MLF (sequential or simultaneous AF/MLF) and the SiO_2-ALG encapsulation of *O. oeni* on the volatile composition, amino acid and biogenic amine content, and sensory properties of red wines. The use of encapsulated bacteria to perform MLF in the presence of lysozyme was also tested.

2. Experimental

2.1. Chemical reagents and standards

Colloidal silica (Ludox HS40), sodium silicate and sodium ALG were from Sigma-Aldrich (Steinheim, Germany). Chromatographic grade reagents were provided by Carlo Erba Reagents (Sabadell, Spain), 2-aminoheptanoic acid (internal standard) and diethylethoxymethylenemalonate (DEEMM) were from Fluka (Sigma-Aldrich, Germany). Milli-Q water was obtained by a Millipore system (Bedford, MA). Helium BIP (99.9997%), air zero (99.998%) and Premier plus hydrogen (99.9998%) for Gas Chromatography (GC) were provided by Carburos Metálicos S.A. (Valladolid, Spain). Major and minor volatile compounds were supplied by Fluka (Buchs, Switzerland), Sigma-Aldrich (Steinheim, Germany) and Alfa Aesar (Lancashire, UK). Amino acid and biogenic amine standards were purchased from Sigma-Aldrich (Steinheim, Germany) and Fluka (Buchs, Switzerland). The remaining reagents were supplied by Panreac (Madrid, Spain).

2.2. Preparation of SiO_2-ALG biocomposites with encapsulated bacteria

Encapsulation of bacteria was performed using an interpenetrated polymer network of SiO_2 and ALG (Simó, Vila-Crespo, et al., 2017). A water solution of 1.23 M colloidal silica (Ludox HS40) and 0.06 M sodium silicate was adjusted to pH 6.3 with 2 N HCl. Then, sodium ALG was added to the silica/silicate solution until obtaining a final concentration of 2% (w/v). Rehydrated freeze-dried bacteria (*O. oeni* strain LALVIN VP 41® MBR, Lallemand, France) were added at a concentration of $\sim 10^{11}$ cfu/g of gel. Count of freeze-dried bacteria was obtained by plate count method with Tomato Juice Agar (Difco, Sparks, MD, USA) at 22 °C. A 10-mL sterile syringe with a nozzle of 1.78 mm of diameter (BD 166 Plastipak, Spain) was employed to create droplets of silica/silicate/ALG/bacteria suspension that were collected in sterile 0.2 M $CaCl_2$ solution under continuous agitation (260 rpm) at ambient temperature. Upon contact with $CaCl_2$ solution, the droplets gelled. Capsules were kept in this solution for 2 h and washed with sterile water before use. All solutions were autoclaved prior to use applying saturated steam under pressure (15 psi), which yields a temperature of 121 °C for 15 min.

2.3. Fermentation protocols

Wines were elaborated with Tempranillo grapes from the 2018 harvest in the experimental winery of the School of Agricultural Engineering (University of Valladolid, Palencia, Spain). After grapes were destemmed and crushed, skins and must were separated and homogenised. Winemaking processes were performed in 20-L stainless steel tanks containing 5.6 L of must and 6.4 kg of skins. The must was sulphited by adding 40 mg/L of SO_2. Chemical composition of the must was determined using the methods described by the Organisation Internationale de la Vigne et du Vin (OIV) (2014) (pH 3.52; 25.8 °Brix; 3.5 g/L of malic acid). Then, musts were inoculated with 0.3 g/L of rehydrated *Saccharomyces cerevisiae* (Lalvin Clos, Lallemand). Grape skins were punched down once daily during AF. AF was monitored daily by gravimetric determination, evaluating the loss of mass of the tanks due to the production of CO_2.

Free and encapsulated *O. oeni* were inoculated at a concentration of $\sim 3.4 \times 10^6$ cfu/mL of must/wine: (i) two days after yeast inoculation (simultaneous AF/MLF) and (ii) at the end of AF (sequential AF/MLF). AF and MLF were carried out at room temperature. After completion of AF the pomaces were pressed with a vertical hydraulic press at 1.5 bar.

Pressed and free-run parts of wine were mixed and transferred to 6.0-L glass bottles to complete MLF. Moreover, musts/wines with 0.3 g/L of lysozyme (Enovin Lyso, Agrovin, Spain), used as antibacterial agent to suppress wild LAB, were also inoculated with encapsulated bacteria by both simultaneous and sequential AF/MLF. Lysozyme was added to the musts/wines simultaneously with the bacterial inoculation. We verified the usefulness of lysozyme by fermentation of a control wine inoculating free bacteria in presence of 0.3 g/L of lysozyme. A total of six treatments were performed: three with sequential AF/MLF (free bacteria, encapsulated bacteria and encapsulated bacteria in presence of lysozyme) and three with simultaneous AF/MLF (free bacteria, encapsulated bacteria and encapsulated bacteria in presence of lysozyme). All treatments (six) were carried out in duplicate. Wines were decanted at the end of MLF and sulphited to reach 30 mg/L of free SO_2. Then, wines were clarified and stabilized (15 days at 6 °C) before bottling without filtration and storing at 6 °C.

2.4. Analyses of volatile compounds

Major volatile compounds were quantified by direct injection of 1 μL of wine. An Agilent 7890A gas chromatograph with a flame ionization detector (FID) was used. The chromatographic and quantification conditions were established by Pérez-Magariño et al. (2019). Samples were injected in split mode (25:1), and volatiles were separated using an Agilent DB-WAX (30 m × 0.25 mm i.d. × 0.25 μm film thickness) capillary column. Minor volatile compounds were extracted by liquid–liquid extraction (Pérez-Magariño et al., 2019). The chromatographic analyses were performed with an HP-6890N gas chromatograph coupled to an HP-5973 inert mass detector. Quantification was carried out using the internal standard quantification method and the quantification ions and internal standard used for each compound. Two internal standards were used, methyl octanoate and 3,4-dimethylphenol. All the analyses were carried out in duplicate.

2.5. Analyses of malic acid, amino acids and biogenic amines

Concentration of L-malic acid was quantified using an enzymatic kit (TDI, Barcelona, Spain). The determination of the amino acids and biogenic amines was carried out following the method described by Ortega-Heras et al. (2014). Aminoenone derivatives were obtained by reaction with DEEMM and after that, they were analysed by liquid chromatography (HPLC) in an Agilent Technologies LC series 1200 with a diode array detection system (DAD) (Agilent, Stuttgart, Germany). Before the injection, the samples were filtered through PVDF filters with a pore size of 0.45 μm. Amino acids and biogenic amines were quantified at 280 nm and the quantification was carried out using the internal standard method and the respective calibration curve of each quantified amino acid or biogenic amine (Ortega-Heras et al., 2014). All the analyses were carried out in duplicate.

2.6. Sensory analysis

A trained panel composed of seven judges (3 men and 4 women; average age: 21 years old) participated in this study. All panellists were trained in 2016, and they had previously participated in wine sensory descriptive analysis studies and had proven wine-judging experience for two years. The descriptive sensory analysis was carried out in the Sensory Science Laboratory of the School of Agricultural Engineering (University of Valladolid, Palencia, Spain) in individual booths. In all the sessions, the samples were served as 25 mL aliquots in standardized wineglasses, which were coded with 3-digit numbers. The serving temperature of the samples was 15 ± 1 °C. Water was provided to rinse mouth between evaluations. The questionnaire was comprised 15 sensory descriptors grouped in three visual descriptors (transparency, tonality, and colour intensity), seven olfactory descriptors (odour intensity, red fruit, black fruit, herbaceous, lactic, acetic, and alcoholic) and five descriptors in the mouth (flavour intensity, bitter, acid, astringency, and persistence). The different descriptors were quantified using 10-cm unstructured intensity scales where 0 corresponded to very low intensity and 10 to high intensity for the respective attribute. Evaluations were carried out in two sessions in which the judges tasted all the wines in quadruplicate, according to a randomized complete block design.

2.7. Statistical analysis

Statistical analysis of the data was carried out by analysis of variance (ANOVA). The Tukey test calculated at a confidence level of 95% was employed to determine significant differences among means. Among pattern recognition tools, Principal Component Analysis (PCA) was carried out with the significant data of volatile compounds and amino acid composition as variables and the six types of wines as samples. Statistical analysis was carried out using IBM SPSS Statistical version 24.0 (IBM Corp. in Armonk, NY).

3. Results and discussion

3.1. Malolactic fermentation

Six combinations of AF/MLF were tested in Tempranillo wines. The inoculum of *O. oeni* (free or encapsulated) was added to the must/wine two days after yeast inoculation (simultaneous AF/MLF) and at the end of AF (sequential AF/MLF). MLF succeeded in all the wines, regardless of the inoculation technique used. The wines produced by simultaneous AF/MLF (24 days of total fermentation) displayed a reduction of the total fermentation time of 10 days compared with those produced by sequential AF/MLF (34 days). These results are in agreement with previous work carried out on a laboratory scale (Simó et al., 2019b) and winery scale (Antalick, Perello, & De Revel, 2013). The wines inoculated with free and encapsulated bacteria needed the same number of days to complete MLF.

Simultaneous and sequential AF/MLF were also carried out with encapsulated bacteria in the presence of lysozyme. A concentration of 0.3 g/L of lysozyme caused a prompt inhibition of malolactic activity of free bacteria, while the same lysozyme concentration had no effect against encapsulated bacteria. These data suggest that the porosity of the immobilization matrix was suitable for the transport of malic acid and other essential nutrients for LAB inside of biocapsules, excluding contact between lysozyme and LAB encapsulated inside the SiO_2-ALG biocomposites. These results provide the opportunity of alternative winemaking processes using combined encapsulated bacteria and lysozyme, which avoid growth and activity of wild or spoilage LAB in wine. Moreover, in the case of inoculation with free bacteria, in order to suppress residual lysozyme activity, the bacteria should be added after bentonite fining and racking, procedures that reduce the organoleptic quality of wine (Weber et al., 2009). The combined use of encapsulated bacteria and lysozyme avoids this disadvantage.

The basic composition of wines inoculated with free, encapsulated bacteria, and encapsulated bacteria in the presence of lysozyme is summarised in Table S1. Regardless of the type of inoculum, similar composition was found after MLF.

After MLF, the concentrations of malic acid in simultaneous AF/MLF wines inoculated with free and encapsulated bacteria, and encapsulated bacteria in the presence of lysozyme were 0.15, 0.20 and 0.20 g/L, respectively. Similar results were found in sequential wines (0.04, 0.20 and 0.20 g/L inoculating free and encapsulated bacteria, and encapsulated bacteria in the presence of lysozyme, respectively).

3.2. Volatile composition

Tables 1 and 2 summarize the concentration of the major and minor volatile compounds, respectively, determined after MLF in wines

Table 1
Concentration of major volatile compounds of wines after MLF inoculated with free and encapsulated (Enc) bacteria, and encapsulated bacteria in presence of lysozyme (Enc+Lys) by sequential (Seq) and simultaneous (Sim) AF/MLF of Tempranillo musts/wines.

Compounds	Type of MLF						Inoculation time		Type of inoculum		
	Sequential AF/MLF			Simultaneous AF/MLF							
	Free	Enc	Enc+Lys	Free	Enc	Enc+Lys	Seq	Sim	Free	Enc	Enc+Lys
Acetaldehyde	1.60 ± 1.89 a	40.9 ± 24.8 b	26.8 ± 0.5 b	33.3 ± 30.5 b	33.2 ± 8.1 b	41.3 ± 0.2 b	23.1 ± 19.9 a	35.9 ± 4.6 a	17.4 ± 22.4 a	37.0 ± 5.5 a	34.1 ± 10.2 a
Ethyl acetate	65.1 ± 1.2 b	55.8 ± 0.7 a	55.6 ± 1.9 a	67.1 ± 1.2 b	55.9 ± 1.6 a	54.2 ± 4.1 a	58.8 ± 5.4 a	59.1 ± 7.0 a	66.1 ± 1.5 b	55.8 ± 0.1 a	54.9 ± 1.3 a
Methanol	139 ± 4 a	141 ± 6 ab	143 ± 6 ab	146 ± 1 ab	148 ± 4.9 ab	150 ± 5 b	141 ± 2 a	148 ± 2 b	142 ± 5 a	144 ± 5 a	147 ± 5 a
Diacetyl	1.55 ± 1.06 a	nd	nd	3.26 ± 3.77 a	nd	nd	0.52 ± 0.90 a	1.09 ± 1.88 a	2.41 ± 1.21	nd	nd
1-Propanol	58.2 ± 0.9 ab	56.7 ± 0.3 a	57.7 ± 0.4 ab	59.6 ± 1.7 b	58.2 ± 0.1 ab	57.7 ± 0.7 ab	57.5 ± 0.7 a	58.5 ± 1.0 b	58.9 ± 1.0 b	57.5 ± 1.0 a	57.7 ± 0.1 a
2-Methyl-1-propanol	43.2 ± 0.6 c	42.2 ± 0.5 bc	40.9 ± 0.3 a	41.7 ± 0.5 ab	41.7 ± 0.6 ab	42.4 ± 0.5 bc	42.1 ± 1.1 a	41.9 ± 0.4 a	42.4 ± 1.0 b	41.9 ± 0.4 ab	41.7 ± 0.9 a
1-Butanol	2.03 ± 0.04 a	2.15 ± 0.13 ab	2.20 ± 0.13 ab	2.30 ± 0.12 b	2.16 ± 0.12 ab	2.13 ± 0.12 ab	2.12 ± 0.09 a	2.19 ± 0.09 a	2.16 ± 0.19 a	2.16 ± 0.00 a	2.16 ± 0.04 a
Isoamylalcohols	334 ± 6 d	331 ± 3 cd	322 ± 2 a	328 ± 3 bc	325 ± 2 ab	324 ± 4 ab	334 ± 6 b	324 ± 2 a	329 ± 4 a	330 ± 4 a	328 ± 1 a

Each value represents the mean ± standard deviation. Different letters mean statistically significant differences at $p < 0.05$. Data expressed in mg/L. nd: not detected.

elaborated by sequential and simultaneous AF/MLF and by the different types of inoculum (free, encapsulated bacteria, and encapsulated bacteria in the presence of lysozyme).

The analysis of variance shows statistically significant differences between sequential and simultaneous AF/MLF for 14 of the 40 volatile compounds evaluated (Table 1 and 2). Higher levels of four ethyl esters (ethyl octanoate, ethyl decanoate, ethyl isovalerate and ethyl lactate), decanoic acid, four terpenes (linalool, α-terpineol, geraniol, β-ionone), methanol and 1-propanol were found in simultaneous AF/FML wines. Increased concentration of esters in Tempranillo and Merlot wines elaborated by simultaneous AF/FML was also observed by Izquierdo-Cañas et al. (2014). In other studies, co-inoculated red wines were higher rated in esters than that obtained by sequential inoculation (Abrahamse & Bartowsky, 2012; Izquierdo-Cañas et al., 2014; Tristezza et al., 2016). Co-inoculated Riesling wines had the highest concentration of alcohol acetates and ethyl esters (Knoll et al., 2012). Increased concentration of terpenes in co-inoculated wines was also observed by Izquierdo-Cañas et al. (2014). As different authors had confirmed in the before mentioned papers, the reasons for the significant differences in the volatile composition between sequential and simultaneous AF/MLF wines depend on both yeast and bacteria metabolism. The better performance of the bacteria, due to low ethanol concentration and higher nutrient availability present in musts, together with the different metabolic behaviour of yeast and bacteria at the different stages in fermentation and, the variation in the interactions yeast-bacteria along the fermentation process are probably the main facts that affect the volatile composition of the wines.

PCA was carried out with the significant data (Fig. 1). Sixteen volatile compounds without statistically significant differences among the six type of AF/MLF were removed from this analysis (ethyl-2-methylbutyrate, isobutyl acetate, hexyl acetate, 2-phenylethyl acetate, isovaleric acid, hexanoic acid, octanoic acid, 1-hexanol, *trans*-3-hexen-1-ol, benzyl alcohol, 2-phenylethanol, β-citronellol, γ-nonalactone, ethyl vanillate, acetovanillone and 4-vinylguaiacol). PC1 (48.05% of total variance) was positively correlated with five of the six significant ethyl esters (ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and ethyl isovalerate), isoamyl acetate, decanoic acid, *cis*-3-hexen-1-ol, β-ionone and five of the eight significant major volatiles (ethyl acetate, diacetyl, 1-propanol, 2-methyl-1-propanol and isoamylalcohols). This component was negatively correlated with ethyl lactate, butyl acetate, 2-ethyl-1-hexanol, three of the four significant terpenes (linalool, α-terpineol and geraniol), γ-butyrolactone, acetaldehyde, methanol and 1-butanol. PC2 (34.23% of total variance) was correlated with positive loading of all volatile compounds except for isoamyl acetate, *cis*-3-hexen-1-ol, 2-methyl-1-propanol and isoamylalcohols. The samples of simultaneous AF/MLF wines were located at positive values of PC2 and characterised by high levels of ethyl esters, decanoic acid and terpenes, compounds which are responsible for the pleasant aroma of wine.

The group of sequential AF/FML wines was located at negative values of PC2, being characterised by high values of isoamyl acetate, *cis*-3-hexen-1-ol, 2-methyl-1-propanol and isoamylalcohols. Isoamyl acetate is an alcohol acetate which shows a fruity character with a characteristic banana odour (Gómez-Míguez, Cacho, Ferreira, Vicario, & Heredia, 2007), while *cis*-3-hexen-1-ol is partly responsible for the green and herbaceous aroma of grapes with negative effect on wine aroma (González-Barreiro, Rial-Otero, Cancho-Grande, & Simal-Gándara, 2015). It has been described that higher alcohols, such as 2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol, seemed to be negatively related to wine quality if they exceed certain values (Sáenz-Navajas et al., 2015). In contrast to these results, Tristezza et al. (2016) observed higher levels of *cis*-3-hexen-1-ol in simultaneous AF/MLF than in sequential AF/MLF wines, while Izquierdo-Cañas et al. (2014) did not found differences between sequential and simultaneous AF/MLF wines. Similar to our results, a significant increase in the concentration of isoamyl acetate was observed in red and

Table 2
Concentration of minority volatile compounds in wines after MLF inoculated with free and encapsulated (Enc) bacteria, and encapsulated bacteria in presence of lysozyme (Enc + Lys) by sequential (Seq) and simultaneous (Sim) AF/MLF of Tempranillo musts/wines.

Compounds	Type of MLF						Inoculation time		Type of inoculum		
	Sequential AF/MLF			Simultaneous AF/MLF							
	Free	Enc	Enc + Lys	Free	Enc	Enc + Lys	Seq	Sim	Free	Enc	Enc + Lys
Ethyl esters											
ethyl butyrate	0.309 ± 0.010 b	0.238 ± 0.018 a	0.237 ± 0.007 a	0.303 ± 0.010 b	0.253 ± 0.018 a	0.231 ± 0.001 a	0.262 ± 0.038 a	0.262 ± 0.034 a	0.306 ± 0.009 b	0.246 ± 0.017 a	0.234 ± 0.005 a
ethyl hexanoate	0.377 ± 0.004 c	0.325 ± 0.013 a	0.332 ± 0.001 a	0.374 ± 0.015 c	0.363 ± 0.019 bc	0.341 ± 0.001 ab	0.345 ± 0.026 a	0.359 ± 0.018 a	0.375 ± 0.009 b	0.344 ± 0.025 a	0.336 ± 0.06 a
ethyl octanoate	0.390 ± 0.004 ab	0.341 ± 0.019 a	0.359 ± 0.001 ab	0.403 ± 0.009 b	0.388 ± 0.024 ab	0.377 ± 0.007 ab	0.363 ± 0.024 a	0.389 ± 0.017 b	0.396 ± 0.011 b	0.364 ± 0.032 a	0.368 ± 0.011 a
ethyl decanoate	0.086 ± 0.004 b	0.056 ± 0.010 a	0.062 ± 0.004 a	0.102 ± 0.000 b	0.093 ± 0.002 b	0.091 ± 0.001 b	0.068 ± 0.015 a	0.095 ± 0.007 b	0.094 ± 0.011 b	0.074 ± 0.022 a	0.076 ± 0.017 a
ethyl 2-methylbutyrate	0.004 ± 0.000 a	0.004 ± 0.000 a	0.004 ± 0.000 a	0.004 ± 0.001 a	0.004 ± 0.001 a	0.004 ± 0.000 a	0.004 ± 0.000 a	0.004 ± 0.000 a	0.004 ± 0.000 a	0.004 ± 0.000 a	0.004 ± 0.000 a
ethyl isovalerate	0.012 ± 0.000 b	0.010 ± 0.001 a	0.010 ± 0.000 a	0.012 ± 0.001 b	0.012 ± 0.001 b	0.011 ± 0.000 ab	0.011 ± 0.001 a	0.012 ± 0.001 b	0.012 ± 0.000 b	0.011 ± 0.001 a	0.011 ± 0.001 a
ethyl lactate	46.0 ± 0.2 a	53.5 ± 2.4 ab	54.9 ± 1.28 ab	49.7 ± 1.6 ab	72.9 ± 4.9 c	61.9 ± 6.7 bc	51.5 ± 4.4 a	61.5 ± 3.1 b	47.8 ± 2.3 a	63.2 ± 11.6 b	58.4 ± 5.7 b
Alcohol acetates											
isobutyl acetate	0.044 ± 0.007 a	0.034 ± 0.005 a	0.034 ± 0.009 a	0.043 ± 0.012 a	0.029 ± 0.005 a	0.026 ± 0.002 a	0.037 ± 0.008 a	0.033 ± 0.010 a	0.044 ± 0.008 a	0.032 ± 0.005 a	0.030 ± 0.007 a
butyl acetate	0.004 ± 0.000 a	0.006 ± 0.000 c	0.005 ± 0.000 bc	0.004 ± 0.000 ab	0.006 ± 0.000 c	0.006 ± 0.000 a	0.005 ± 0.001 a	0.005 ± 0.001 a	0.004 ± 0.000 a	0.006 ± 0.000 b	0.006 ± 0.001 b
isoamyl acetate	0.591 ± 0.019 c	0.449 ± 0.037 ab	0.446 ± 0.027 ab	0.533 ± 0.038 bc	0.428 ± 0.028 ab	0.400 ± 0.040 a	0.495 ± 0.077 b	0.453 ± 0.069 a	0.562 ± 0.042 b	0.438 ± 0.029 a	0.423 ± 0.039 a
hexyl acetate	0.001 ± 0.000 a	0.001 ± 0.000 a	0.001 ± 0.000 a	0.001 ± 0.000 a	0.001 ± 0.000 a	0.001 ± 0.000 a	0.001 ± 0.000 a	0.001 ± 0.000 a	0.001 ± 0.000 a	0.001 ± 0.000 a	0.001 ± 0.000 a
2-phenylethyl acetate	0.011 ± 0.002 a	0.011 ± 0.002 a	0.013 ± 0.002 a	0.010 ± 0.005 a	0.010 ± 0.001 a	0.010 ± 0.002 a	0.012 ± 0.002 a	0.010 ± 0.002 a	0.010 ± 0.003 a	0.010 ± 0.002 a	0.012 ± 0.002 a
Acids											
isovaleric acid	1.08 ± 0.06 a	1.09 ± 0.07 a	1.15 ± 0.00 a	1.1 ± 0.02 a	1.12 ± 0.02 a	1.12 ± 0.07 a	1.11 ± 0.05 a	1.13 ± 0.04 a	1.11 ± 0.05 a	1.11 ± 0.04 a	1.13 ± 0.05 a
hexanoic acid	1.77 ± 0.08 a	1.72 ± 0.06 a	1.77 ± 0.04 a	1.86 ± 0.00 a	1.78 ± 0.06 a	1.72 ± 0.01 a	1.75 ± 0.06 a	1.78 ± 0.07 a	1.81 ± 0.07 a	1.75 ± 0.06 a	1.74 ± 0.04 a
octanoic acid	1.34 ± 0.13 a	1.20 ± 0.09 a	1.27 ± 0.14 a	1.29 ± 0.09 a	1.27 ± 0.03 a	1.19 ± 0.12 a	1.27 ± 0.11 a	1.25 ± 0.08 a	1.31 ± 0.09 a	1.24 ± 0.07 a	1.23 ± 0.12 a
decanoic acid	0.462 ± 0.034 bc	0.308 ± 0.018 a	0.342 ± 0.029 ab	0.559 ± 0.106 c	0.502 ± 0.008 c	0.495 ± 0.034 a	0.371 ± 0.076 a	0.519 ± 0.059 b	0.510 ± 0.085 a	0.405 ± 0.113 a	0.419 ± 0.092 a
Alcohols											
1-hexanol	0.596 ± 0.054 a	0.581 ± 0.055 a	0.569 ± 0.062 a	0.539 ± 0.074 a	0.521 ± 0.027 a	0.514 ± 0.039 a	0.582 ± 0.042 a	0.525 ± 0.041 a	0.568 ± 0.058 a	0.551 ± 0.049 a	0.542 ± 0.053 a
trans-3-hexen-1-ol	0.015 ± 0.001 a	0.015 ± 0.001 a	0.015 ± 0.000 a	0.015 ± 0.002 a	0.015 ± 0.001 a	0.014 ± 0.000 a	0.015 ± 0.001 a	0.015 ± 0.001 a	0.015 ± 0.001 a	0.015 ± 0.001 a	0.014 ± 0.000 a
cis-3-hexen-1-ol	0.075 ± 0.001 b	0.073 ± 0.002 ab	0.071 ± 0.001 ab	0.069 ± 0.004 ab	0.069 ± 0.002 ab	0.067 ± 0.004 a	0.073 ± 0.002 b	0.068 ± 0.003 a	0.072 ± 0.004 a	0.071 ± 0.003 a	0.069 ± 0.003 a
benzyl alcohol	0.311 ± 0.117 a	0.309 ± 0.102 a	0.244 ± 0.012 a	0.210 ± 0.000 a	0.259 ± 0.023 a	0.255 ± 0.018 a	0.288 ± 0.078 a	0.242 ± 0.028 a	0.260 ± 0.089 a	0.284 ± 0.067 a	0.250 ± 0.014 a
2-ethyl-1-hexanol	0.003 ± 0.000 a	0.004 ± 0.001 ab	0.004 ± 0.01 ab	0.003 ± 0.001 a	0.005 ± 0.000 ab	0.006 ± 0.002 b	0.004 ± 0.001 a	0.005 ± 0.002 a	0.003 ± 0.001 a	0.005 ± 0.001 b	0.005 ± 0.002 b
2-phenylethanol	23.9 ± 1.0 a	21.3 ± 2.8 a	22.9 ± 0.5 a	22.8 ± 3.3 a	21.9 ± 0.1 a	20.1 ± 1.2 a	22.7 ± 1.8 a	21.6 ± 2.0 a	23.4 ± 2.1 a	21.6 ± 1.6 a	21.5 ± 1.7 a
Terpenes*											
Linalool	2.18 ± 0.05 a	2.70 ± 0.04 ab	2.86 ± 0.24 ab	2.66 ± 0.37 ab	2.94 ± 0.08 ab	3.29 ± 0.15 b	2.58 ± 0.34 a	2.96 ± 0.34 b	2.42 ± 0.35 a	2.82 ± 0.15 ab	3.07 ± 0.30 b
α-terpineol	0.147 ± 0.091 a	0.405 ± 0.047 ab	0.421 ± 0.126 ab	0.681 ± 0.080 b	0.402 ± 0.236 ab	0.541 ± 0.075 ab	0.325 ± 0.161 a	0.542 ± 0.171 b	0.414 ± 0.316 a	0.40 ± 0.14 a	0.48 ± 0.12 a
β-citronellol	5.05 ± 2.78 a	3.13 ± 0.45 a	3.85 ± 0.52 a	4.22 ± 0.080 a	3.67 ± 0.54 a	3.71 ± 0.27 a	4.01 ± 1.55 a	3.87 ± 0.39 a	4.64 ± 1.67 a	3.40 ± 0.51 a	3.78 ± 0.35 a
geraniol	14.0 ± 1.23 ab	13.4 ± 0.1 a	15.3 ± 0.6 ab	16.1 ± 1.6 b	14.9 ± 0.9 ab	16.0 ± 0.8 b	14.2 ± 1.1 a	15.6 ± 1.1 b	15.1 ± 1.7 a	14.1 ± 1.0 a	15.6 ± 0.7 a
β-ionone	0.557 ± 0.042 ab	0.477 ± 0.081 a	0.550 ± 0.110 ab	0.714 ± 0.158 b	0.614 ± 0.030 ab	0.664 ± 0.050 ab	0.528 ± 0.075 a	0.664 ± 0.087 b	0.636 ± 0.131 a	0.546 ± 0.094 a	0.607 ± 0.096 a
γ-butyrolactone	4.23 ± 0.21 a	5.17 ± 0.00 b	5.26 ± 0.21 b	4.10 ± 0.07 a	5.49 ± 0.39 b	5.61 ± 0.02 b	4.89 ± 0.53 a	5.06 ± 0.78 a	4.16 ± 0.20 a	5.33 ± 0.29 b	5.43 ± 0.23 b
γ-nonalactone*	27.8 ± 0.08 a	27.5 ± 2.0 a	30.5 ± 3.2 a	32.3 ± 2.2 a	31.0 ± 0.4 a	33.7 ± 2.6 a	28.6 ± 2.3 a	32.3 ± 3.7 a	30.0 ± 5.1 a	29.3 ± 2.4 a	32.1 ± 3.0 a
Volatile phenols											
ethyl vanillate	0.211 ± 0.015 a	0.221 ± 0.025 a	0.246 ± 0.055 a	0.318 ± 0.070 a	0.246 ± 0.023 a	0.310 ± 0.012 a	0.226 ± 0.032 a	0.291 ± 0.049 a	0.264 ± 0.074 a	0.233 ± 0.025 a	0.278 ± 0.49 a
acetovanillone*	42.9 ± 1.5 a	43.0 ± 1.5 a	43.5 ± 0.8 a	43.2 ± 2.2 a	43.4 ± 0.7 a	45.0 ± 0.8 a	43.2 ± 1.0 a	43.8 ± 1.4 a	43.1 ± 1.5 a	43.2 ± 1.1 a	44.3 ± 1.1 a
4-vinylguaiacol*	9.83 ± 0.14 a	9.13 ± 0.53 a	8.20 ± 4.60 a	13.73 ± 6.72 a	8.99 ± 2.57 a	12.41 ± 0.32 a	9.05 ± 2.20 a	11.73 ± 3.92 a	11.82 ± 4.51 a	9.71 ± 1.52 a	10.30 ± 2.59 a

Each value represents the mean ± standard deviation. Different letters mean statistically significant differences at $p < 0.05$. Data in mg/L except those marked with an asterisk that are expressed in μg/L.

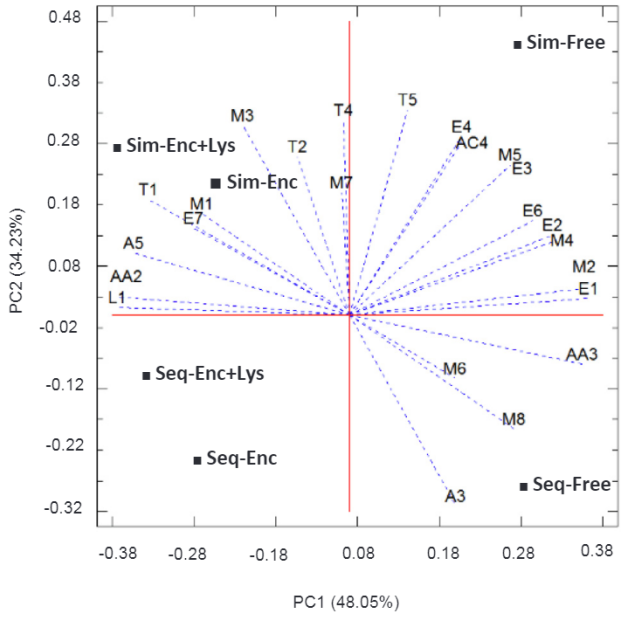

Fig. 1. Loading and score plot of first two principal components for classification of samples of Tempranillo must/wine inoculated with free and encapsulated (Enc) bacteria, and encapsulated bacteria in the presence of lysozyme (Enc + Lys) by sequential (Seq) and simultaneous (Sim) AF/MLF according their volatile composition. Codes: M1: acetaldehyde; M2: ethyl acetate; M3: methanol; M4: diacetyl; M5: 1-propanol; M6: 2-methyl-1-propanol; M7: 1-butanol; M8: isoamylalcohols; E1: ethyl butyrate; E2: ethyl hexanoate; E3: ethyl octanoate; E4: ethyl decanoate; E6: ethyl isovalerate; E7: ethyl lactate; AA2: butyl acetate; AA3: isoamyl acetate; AC4: decanoic acid; A3: *cis*-3-hexen-1-ol; A5: 2-ethyl-1-hexanol; T1: Linalool; T2: α-terpineol; T4: geraniol; T5: β-ionone; L1: γ-butyrolactone.

white wines produced by sequential inoculation (Izquierdo-Cañas et al., 2014; Tristezza et al., 2016). Abrahamse and Bartowsky (2012) have also reported that sequential AF/MLF could lead to an increase in the 2-methyl-1-butanol and 3- methyl-1-butanol.

Cell encapsulation can alter cell growth, physiology and metabolic activity influencing flavour formation during fermentation stage (Genisheva, Teixeira, & Oliveira, 2014). However, little attention has been given to the study of the effect of the bacterial encapsulation on the volatile composition of wine after MLF (Bleve et al., 2016). It has been reported the volatile composition of wines inoculated with immobilized bacteria (Genisheva, Mota, Mussatto, Oliveira, & Teixeira, 2014). However, the volatile composition of these wines elaborated with free bacteria has not been reported, which does not allow a direct comparison between wines inoculated with free and encapsulated bacteria.

Based on our results, the volatile composition of wine also depended on the type of inoculum used (free or encapsulated bacteria). The analysis of variance (Tables 1 and 2) showed that bacterial encapsulation significantly affected the concentration of 15 of the 40 volatile compounds analysed. Statistically significant differences between volatile composition of the wines inoculated with encapsulated bacteria and encapsulated bacteria in the presence of lysozyme were not found. The concentrations of the four major volatiles (ethyl acetate, diacetyl, 1-propanol and 2-methyl-1-propanol) were higher in the wines inoculated with free bacteria than those inoculated with encapsulated bacteria. The former also had the highest content in ethyl esters, except for ethyl lactate. Ethyl butyrate (strawberry and fruity notes), ethyl hexanoate (green apple, fruity, strawberry and anise notes), ethyl octanoate (pineapple, pear and floral notes), ethyl decanoate (fruity, fatty and pleasant notes) and ethyl isovalerate (fruity, pineapple, apple, green and orange notes) make a positive contribution to general quality of wine since they are responsible for fruity and floral sensory properties of wine. Similar results were found in Negroamaro wines inoculated with free bacteria, mainly characterized by high levels of esters, in comparison with wines produced by ALG encapsulated bacteria (Bleve et al., 2016).

The wines inoculated with encapsulated bacteria (in presence or absence of lysozyme) presented statistically significant higher concentrations of five volatile compounds (ethyl lactate, butyl acetate, 2-ethyl-hexanol, linalool and γ-butyrolactone) than those inoculated with free bacteria. Butyl acetate and 2-ethyl-hexanol contribute to fruity and green notes to wine, respectively, while linalool is responsible for floral notes (Styger et al., 2011). Ethyl lactate shows an important contribution to buttery and creamy character of wine (Tristezza et al., 2016), while γ-butyrolactone can confer pleasant aromas of toasted and caramel contributing to the complexity of wine aroma (Sanchez-Palomo, Gomez, Gomez, & Gonzalez, 2012).

On one hand, results of PCA (Fig. 1) showed that the wines simultaneously inoculated with free bacteria are sited on the upper right part of the plane and are characterised by high levels of all fruity ethyl esters, ethyl acetate (fruity note), diacetyl (buttery note) and 1-propanol (alcohol note). On the other hand, the concentrations of ethyl lactate (buttery and creamy notes), butyl acetate (fruity note), 2-ethyl-1-hexanol (floral and citrus notes), linalool (floral and citrus notes), γ-butyrolactone (toasted and caramel notes) and acetaldehyde (fruity and must notes) (Vilanova & Martínez, 2007) were high in the wines simultaneously inoculated with encapsulated bacteria. The wines sequentially inoculated with encapsulated bacteria presented the lowest concentrations of all volatile compounds.

3.3. Amino acid composition

The concentration of amino acids in wines elaborated with different timing of inoculation (sequential and simultaneous AF/MLF) and type of inoculum (free and encapsulated bacteria) is reported in Table 3. As far as we know, the effect of simultaneous AF/MLF, as well as bacterial encapsulation, on the amino acid composition of wine is not yet available in the literature.

The inoculation time and the type of inoculum affected the amino acid composition of the wines. The analysis of variance showed differences among the six wines for all amino acids, except for L-tryptophan, L-glutamine, L-proline and L-leucine. Hydroxyproline, L-valine, L-methionine and L-cysteine were not detected in our wines (Table 3). The wines produced by simultaneous AF/MLF contained higher concentrations of amino acids than those obtained by sequential AF/MLF. This might be due to the yeast-bacteria co-inoculation, that could inhibit the uptake and/or increase the release of amino acids by *S. cerevisiae* and/or *O. oeni*. The final concentration of amino acids in wines is a net balance between utilisation and release of amino acids by both yeasts and bacteria (Chen & Liu, 2016).

Very small differences on the amino acid composition were observed associated with the type of inoculum. The wines inoculated with encapsulated bacteria presented statistically significant higher concentrations of L-glutamic acid, L-serine and L-ornithine hydrochloride and lower concentrations of L-arginine than the wines inoculated with free bacteria. The addition of lysozyme did not modify the concentrations of amino acids, except for L-glutamic acid.

PCA was applied to amino acid composition to elucidate trends in the data and to extract more information on the variability in the wine composition. The amino acids without statistically significant differences among the six type of wines were removed from the analysis (L-tryptophan, L-glutamine, L-proline and L-leucine). PC1 and PC2 displayed 83.65% and 12.37% of variance, respectively (Fig. 2). Fig. 2 shows a clear separation between sequential and simultaneous AF/MLF wines along the PC1 axis. On one hand, the simultaneous AF/MLF wines are sited on the positive values of PC1 that indicates their highest

Table 3
Concentration of aminoacids and biogenic amines in wines after MLF inoculated with free and encapsulated (Enc) bacteria and encapsulated bacteria in presence of lysozyme (Enc + Lys) by sequential (Seq) and simultaneous (Sim) AF/MLF of Tempranillo musts/wines.

Compounds	Type of MLF						Inoculation time		Type of inoculum		
	Sequential AF/MLF			Simultaneous AF/MLF							
	Free	Enc	Enc + Lys	Free	Enc	Enc + Lys	Seq	Sim	Free	Enc	Enc + Lys
Aminoacids											
L-Aspartic acid	7.25 ± 0.48 a	6.96 ± 0.27 a	7.20 ± 0.18 a	9.31 ± 0.87 b	9.93 ± 0.50 bc	10.5 ± 0.44 c	7.14 ± 0.33 a	9.90 ± 0.75 b	8.28 ± 1.28 a	8.44 ± 1.63 a	8.83 ± 1.77 a
L-Glutamic acid	24.0 ± 0.1 a	23.3 ± 0.6 a	31.3 ± 2.2 b	33.2 ± 3.2 bc	36.4 ± 2.6 c	35.3 ± 2.6 c	26.2 ± 3.9 a	35.0 ± 2.9 b	28.6 ± 5.3 a	29.8 ± 7.2 a	33.3 ± 3.1 b
γ-Aminobutyric acid	27.4 ± 4.9 a	25.2 ± 0.7 a	23.8 ± 0.3 a	36.7 ± 8.5 b	39.1 ± 1.0 b	40.8 ± 2.9 b	25.5 ± 3.0 a	38.9 ± 5.0 b	32.1 ± 8.1 a	32.2 ± 7.4 a	32.3 ± 9.3 a
L-Tryptophan	5.28 ± 0.57 a	4.98 ± 0.28 a	4.81 ± 0.08 a	4.96 ± 1.05 a	4.75 ± 0.45 a	4.68 ± 0.43 a	5.02 ± 0.39 a	4.80 ± 0.65 a	5.12 ± 0.80 a	4.87 ± 0.37 a	4.75 ± 0.29 a
L-Asparagine	16.9 ± 2.2 a	15.4 ± 0.2 a	15.5 ± 0.9 a	24.1 ± 1.3 b	22.6 ± 1.9 b	23.5 ± 2.1 b	16.0 ± 1.4 a	23.4 ± 1.7 b	20.5 ± 4.2 a	19.0 ± 4.0 a	19.5 ± 4.5 a
L-Serine	7.51 ± 0.7 a	8.03 ± 0.16 a	7.80 ± 0.74 a	7.92 ± 0.93 a	9.86 ± 0.50 b	10.0 ± 0.92 b	7.78 ± 0.61 a	9.26 ± 1.24 b	7.71 ± 0.83 a	8.95 ± 1.04 b	8.91 ± 1.41 ab
Hydroxyproline	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-Glutamine	19.7 ± 1.5 a	16.8 ± 3.0 a	12.2 ± 8.5 a	18.1 ± 1.9 a	14.5 ± 2.0 a	15.8 ± 1.2 a	16.2 ± 5.7 a	16.1 ± 2.2 a	18.9 ± 1.8 a	15.6 ± 2.7 a	14.0 ± 5.9 a
Glycine	15.0 ± 3.4 a	15.8 ± 0.2ab	13.5 ± 0.5 a	20.4 ± 3.7 bc	21.8 ± 2.3 c	22.3 ± 0.6 c	14.8 ± 2.1 a	21.5 ± 2.5 b	17.7 ± 4.4 a	18.8 ± 3.5 a	17.9 ± 4.8 a
L-Threonine	6.45 ± 0.80 a	6.46 ± 0.15 a	6.45 ± .37 a	8.00 ± 1.00 b	8.59 ± 0.28 b	8.77 ± 0.72 b	6.45 ± 0.47 a	8.45 ± 0.74 b	7.22 ± 1.18 a	7.53 ± 1.16 a	7.61 ± 1.35 a
Alanine	28.7 ± 4.5 a	27.1 ± 0.4 a	26.4 ± 0.6 a	41.3 ± 2.9 b	40.1 ± 1.8 b	42.4 ± 2.9 b	27.4 ± 2.6 a	41.3 ± 2.6 b	35.0 ± 7.6 a	33.6 ± 7.0 a	34.5 ± 8.8 a
L-Proline	1342 ± 100 a	1468 ± 165 a	1241 ± 44 a	918 ± 614 a	1196 ± 53 a	1259 ± 44 a	1351 ± 142 a	1124 ± 358 a	1130 ± 466 a	1332 ± 184 a	1250 ± 42 a
L-Valine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-Methionine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-Cysteine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-Isoleucine	3.40 ± 0.47 a	3.53 ± 0.11 a	4.40 ± 1.25 ab	3.90 ± 0.64 a	5.59 ± 1.69 b	5.49 ± 0.97 b	3.78 ± 0.84 a	4.99 ± 1.34 b	3.65 ± 0.59 a	4.56 ± 1.56 a	4.95 ± 1.19 a
L-Leucine	7.90 ± 1.60 a	7.35 ± 0.24 a	7.19 ± 0.22 a	8.52 ± 5.76 a	9.42 ± 6.28 a	9.08 ± 6.12 a	7.48 ± 0.91 a	9.01 ± 5.49 a	8.21 ± 3.93 a	8.38 ± 4.26 a	8.14 ± 4.13 a
L-Histidine	11.8 ± 3.1 a	11.0 ± 4.0 a	11.3 ± 3.2 a	16.5 ± 0.7 b	17.2 ± 0.5 b	16.9 ± 3.4 b	11.4 ± 3.1 a	16.9 ± 1.8 b	14.1 ± 3.2 a	14.1 ± 4.2 a	14.1 ± 4.3 a
L-Arginine	24.2 ± 2.4 c	14.0 ± 1.4 a	17.9 ± 3.5 ab	35.0 ± 0.8 d	20.0 ± 4.7 b	19.4 ± 1.1 b	18.7 ± 5.0 a	24.8 ± 8.0 b	29.6 ± 6.0 b	17.0 ± 4.5 a	18.6 ± 2.6 a
L-Tyrosine	4.81 ± 0.24 ab	4.49 ± 0.28 a	4.07 ± 0.46 a	5.18 ± 1.63 bc	6.18 ± 0.40 bc	6.62 ± 0.15 c	4.45 ± 0.44 a	5.99 ± 1.08 b	4.99 ± 1.10 a	5.34 ± 0.96 a	5.34 ± 1.40 a
L-Phenylalanine	4.10 ± 0.83 ab	3.80 ± 0.06 a	3.64 ± 0.12 a	5.50 ± 0.31 bc	5.94 ± 0.08 bc	6.12 ± 0.55 c	3.84 ± 0.48 a	5.85 ± 0.43 b	4.80 ± 0.95 a	4.87 ± 1.15 a	4.88 ± 1.38 a
L-Ornithine	10.6 ± 2.8 a	17.9 ± 0.3 b	16.1 ± 0.3 b	18.2 ± 0.6 b	28.3 ± 1.2 c	28.2 ± 1.0 c	14.9 ± 3.6 a	24.9 ± 5.0 b	14.4 ± 4.5 a	23.1 ± 5.6 b	22.1 ± 6.5 b
L-Lysine	11.2 ± 2.1 a	11.0 ± 0.1 a	10.4 ± 0.4 a	16.4 ± 0.5 b	17.9 ± 0.2 b	17.8 ± 1.3 b	10.9 ± 1.2 a	17.4 ± 1.0 b	13.8 ± 3.1 a	14.4 ± 3.7 a	14.1 ± 4.1 a
Biogenic amines											
Histamine	0.609 ± 0.076 b	0.569 ± 0.115 ab	0.549 ± 0.063 ab	0.505 ± 0.150 ab	0.357 ± 0.122 a	0.570 ± 0.050 ab	0.576 ± 0.083 b	0.477 ± 0.140 a	0.557 ± 0.123 a	0.463 ± 0.158 a	0.560 ± 0.054 a
Spermidine	0.688 ± 0.200 a	1.00 ± 0.167 a	0.588 ± 0.061 a	0.922 ± 0.200 a	0.882 ± 0.333 a	0.864 ± 0.234 a	0.759 ± 0.231 a	0.889 ± 0.238 a	0.805 ± 0.224 a	0.941 ± 0.252 a	0.726 ± 0.216 a
Tyramine	0.529 ± 0.166 a	0.439 ± 0.033 a	0.366 ± 0.032 a	1.06 ± 0.175 bc	0.811 ± 0.061 b	1.23 ± 0.137 c	0.445 ± 0.114 a	1.00 ± 0.18 b	0.795 ± 0.326 a	0.725 ± 0.204 a	0.746 ± 0.417 a
Putrescine	5.06 ± 0.20 ab	5.32 ± 0.25 b	4.88 ± 0.08 ab	4.94 ± 0.07 ab	4.78 ± 0.20 a	5.00 ± 0.15 b	5.09 ± 0.26 b	4.91 ± 0.17 a	5.00 ± 0.15 a	5.05 ± 0.36 a	4.94 ± 0.13 a
Cadaverine	0.746 ± 0.041 b	0.755 ± 0.034 b	0.687 ± 0.021 ab	0.719 ± 0.013 ab	0.682 ± 0.021 a	0.714 ± 0.039 ab	0.729 ± 0.043 a	0.705 ± 0.030 a	0.733 ± 0.032 a	0.718 ± 0.047 a	0.700 ± 0.032 a
Phenylethylamine	0.275 ± 0.012 a	0.276 ± 0.022 a	0.283 ± 0.025 a	0.319 ± 0.020 a	0.265 ± 0.011 a	0.284 ± 0.036 a	0.278 ± 0.019 a	0.289 ± 0.032 a	0.297 ± 0.028 a	0.271 ± 0.017 a	0.284 ± 0.029 a

Each value represents the mean ± standard deviation. Different letters mean statistically significant differences at $p < 0.05$. Data expressed in mg/L. nd: not detected.

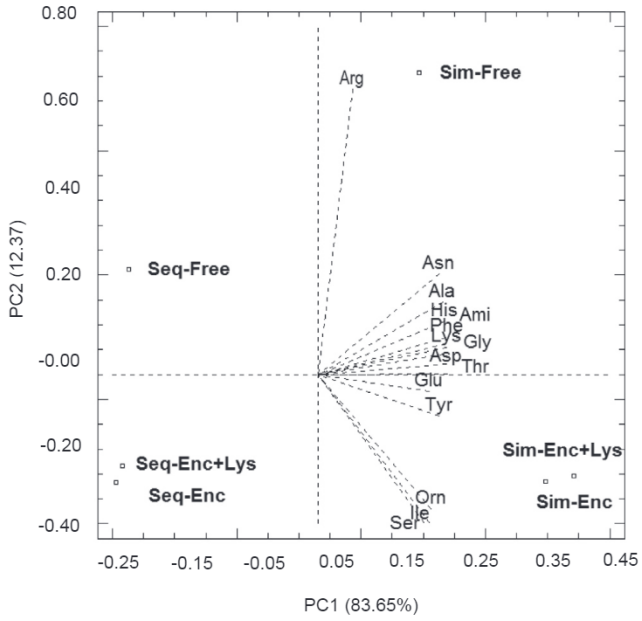

Fig. 2. Loading and score plot of first two principal components for classification of samples of Tempranillo must/wine inoculated with free and encapsulated (Enc) bacteria, and encapsulated bacteria in the presence of lysozyme (Enc + Lys) by sequential (Seq) and simultaneous (Sim) AF/MLF according their amino acid composition. Codes: Codes: Asp: L-aspartic acid; Glu: L-glutamic acid; Ami: γ-Aminobutyric acid; Asn: L-asparagine; Ser: L-serine; Gly: Glycine; Thr: L-Threonine; Ala: Alanine; Ile: L-Isoleucine; His: L-Histidine; Arg: L-Arginine; Tyr: L-Tyrosine; Phe: L-Phenylalanine; Orn: L-Ornithine hydrochloride; Lys: L-Lysine.

concentration of almost all the amino acids, while the sequential AF/MLF wines are sited on the negative zone. On the other hand, PC2 allows the separation of the wines by type of inoculum. The wines produced by encapsulated bacteria both in the presence or absence of lysozyme are localized very close in the plot, denoting their similar composition in amino acids. The wines inoculated with free bacteria were characterized by positive values of PC2, mainly due to the highest content of L-arginine. The wines inoculated with encapsulated bacteria by simultaneous AF/MLF sited on the negative values of PC2 showed the highest concentrations of four amino acids (L-tyrosine, L-ornithine hydrochloride, L-isoleucine and L-serine).

3.4. Biogenic amine composition

In general, the differences found in all biogenic amines among treatments were low, indicating that the consumption of these wines does not involve a hazard to health. Putrescine was the major biogenic amine found in our wines with values between 4.78 and 5.32 mg/L, results in accordance with other authors (Izquierdo-Caña, Pérez-Martín, Romero, Prieto, & Herreros, 2012) (Table 3). As it can be seen, the inoculation time had a different impact on the concentration of biogenic amines. On one hand, the concentrations of histamine and putrescine were higher in the wines elaborated by sequential AF/MLF. Previous studies have provided contradictory evidences on the effect of inoculation time on these biogenic amines. Lower concentrations of histamine and putrescine were found in Zweigelt wines (Jeromel et al., 2018) and Pinotage and Cabernet Sauvignon wines (Smit & du Toit, 2013) produced by co-inoculation than by sequential inoculation, in accordance with our results. On the contrary, Masqué et al. (2007) reported high concentrations of histamine and putrescine in co-inoculated Merlot wines.

On the other hand, the wines obtained by simultaneous AF/MLF showed the greatest concentration of tyramine. This result was consistent with a previous report in Tempranillo wine (Masqué et al., 2007), while other authors underlined a lower concentration for tyramine in wines produced by simultaneous AF/MLF (Izquierdo-Caña et al., 2012; Jeromel et al., 2018). These discrepancies could stem from different must composition as well as yeast and bacteria strains used. Finally, no statistically significant differences were found in the biogenic amine composition between the wines inoculated with free and those with encapsulated bacteria.

3.5. Sensory analysis

Judges only showed statistically significant differences among six wines for one descriptor (lactic in olfactory phase) confirming the sensory similarity of the wines obtained (Table 4). Overall, the different

Table 4
Sensory evaluation of wines after MLF inoculated with free and encapsulated (Enc) bacteria, and encapsulated bacteria in presence of lysozyme (Enc + Lys) using sequential (Seq) and simultaneous (Sim) AF/MLF of Tempranillo musts/wines.

Descriptors	Type of MLF						Inoculation time		Type of inoculum		
	Sequential AF/MLF			Simultaneous AF/MLF							
	Free	Enc	Enc + Lys	Free	Enc	Enc + Lys	Seq	Sim	Free	Enc	Enc + Lys
Visual phase											
Transparency	8.2 ± 1.3 a	8.2 ± 1.1 a	8.1 ± 1.3 a	8.1 ± 1.2 a	8.3 ± 1.1 a	8.0 ± 1.3 a	8.2 ± 1.2 a	8.1 ± 1.2 a	8.1 ± 1.2 a	8.3 ± 1.1 a	8.0 ± 1.3 a
Tonality	7.6 ± 1.2 a	7.1 ± 1.5 a	7.7 ± 1.1 a	7.4 ± 1.4 a	7.6 ± 1.2 a	7.3 ± 1.3 a	7.5 ± 1.3 a	7.4 ± 1.3 a	7.5 ± 1.3 a	7.3 ± 1.4 a	7.5 ± 1.2 a
Colour intensity	7.6 ± 1.3 a	7.8 ± 1.3 a	7.9 ± 1.3 a	8.0 ± 1.3 a	7.8 ± 1.3 a	7.8 ± 1.1 a	7.8 ± 1.3 a	7.9 ± 1.2 a	7.8 ± 1.3 a	7.8 ± 1.3 a	7.8 ± 1.2 a
Olfactory phase											
Odour intensity	7.0 ± 1.1 a	6.8 ± 1.2 a	6.6 ± 1.3 a	7.1 ± 1.0 a	6.6 ± 1.4 a	6.5 ± 1.2 a	6.8 ± 1.2 a	6.7 ± 1.2 a	7.1 ± 1.0 a	6.7 ± 1.3 a	6.5 ± 1.3 a
Red fruit	5.5 ± 1.4 a	5.8 ± 1.2 a	5.7 ± 1.3 a	5.5 ± 1.4 a	5.8 ± 1.3 a	5.7 ± 1.2 a	5.7 ± 1.3 a	5.7 ± 1.3 a	5.7 ± 1.4 a	5.7 ± 1.3 a	5.6 ± 1.3 a
Black fruit	6.0 ± 1.5 a	6.4 ± 1.4 a	6.4 ± 1.1 a	6.3 ± 1.6 a	6.0 ± 1.4 a	6.1 ± 1.2 a	6.3 ± 1.4 a	6.2 ± 1.4 a	6.1 ± 1.5 a	6.2 ± 1.4 a	6.3 ± 1.1 a
Herbaceous	5.4 ± 1.9 a	4.7 ± 1.6 a	5.3 ± 1.5 a	5.3 ± 1.4 a	5.1 ± 1.6 a	5.2 ± 1.4 a	5.1 ± 1.7 a	5.2 ± 1.5 a	5.3 ± 1.7 a	4.9 ± 1.6 a	5.2 ± 1.4 a
Lactic	5.9 ± 1.5 bc	5.7 ± 1.4 abc	5.3 ± 1.4 ab	6.3 ± 1.4 c	5.2 ± 1.5 a	5.4 ± 1.5 abc	5.6 ± 1.4 a	5.6 ± 1.5 a	6.1 ± 1.4 b	5.4 ± 1.5 ab	5.3 ± 1.5 a
Acetic	3.8 ± 1.8 a	3.5 ± 1.8 a	3.7 ± 1.9 a	3.6 ± 2.1 a	3.8 ± 2.4 a	3.9 ± 2.2 a	3.7 ± 1.9 a	3.8 ± 2.2 a	3.7 ± 2.0 a	3.6 ± 2.1 a	3.8 ± 2.0 a
Alcoholic	4.8 ± 2.1 a	4.8 ± 2.1 a	5.0 ± 2.1 a	5.0 ± 1.9 a	4.8 ± 1.9 a	5.0 ± 2.0 a	5.0 ± 2.1 a	4.8 ± 1.9 a	4.9 ± 1.9 a	4.8 ± 2.0 a	5.0 ± 2.0 a
Gustatory phase											
Flavour intensity	7.1 ± 1.0 a	6.8 ± 1.1 a	6.7 ± 1.3 a	6.7 ± 1.0 a	6.7 ± 1.5 a	6.9 ± 1.3 a	6.9 ± 1.1 a	6.8 ± 1.2 a	6.9 ± 1.0 a	6.8 ± 1.3 a	6.8 ± 1.3 a
Bitter	6.0 ± 1.8 a	5.7 ± 1.8 a	5.5 ± 1.9 a	5.4 ± 1.9 a	5.2 ± 2.0 a	5.6 ± 1.9 a	5.7 ± 1.8 a	5.4 ± 1.9 a	5.7 ± 1.9 a	5.5 ± 1.9 a	5.5 ± 1.9 a
Acid	5.7 ± 1.9 a	5.5 ± 1.9 a	5.9 ± 1.7 a	5.8 ± 2.0 a	5.4 ± 2.1 a	5.6 ± 2.2 a	5.7 ± 1.8 a	5.6 ± 2.1 a	5.7 ± 2.0 a	5.5 ± 2.0 a	5.7 ± 2.0 a
Astringency	5.5 ± 1.9 a	4.9 ± 2.0 a	5.1 ± 1.8 a	4.8 ± 1.7 a	4.7 ± 2.1 a	4.9 ± 2.1 a	5.2 ± 1.9 a	4.8 ± 2.0 a	5.1 ± 1.8 a	4.8 ± 2.1 a	5.0 ± 1.9 a
Persistence	6.2 ± 1.5 a	5.9 ± 1.6 a	6.1 ± 1.5 a	6.0 ± 1.6 a	5.5 ± 1.7 a	6.0 ± 1.5 a	6.1 ± 1.6 a	5.8 ± 1.6 a	6.1 ± 1.6 a	5.7 ± 1.7 a	6.0 ± 1.5 a

Each value represents the mean ± standard deviation. Different letters mean statistically significant differences at $p < 0.05$.

chemical composition observed among the wines was not detected by judges. The analysis of variance showed that the inoculation time resulted in no statistically significant differences in the sensory perception of the quality of the wines produced, as described by other authors (Homich, Scheinberg, Elias, & Gardner, 2016; Jussier, Morneau, & De Orduña, 2006). When the wines inoculated with free and encapsulated bacteria were compared, differences in wine sensory attributes were only statistically significant for the lactic descriptor. The wines inoculated with free bacteria had higher score for the lactic descriptor than those inoculated with encapsulated bacteria. This difference may be attributable to the high concentration of diacetyl found in these wines (Table 1).

4. Conclusions

The results obtained in this study confirm previous researches of other authors who have indicated that, regardless of type of inoculum (free or encapsulated bacteria), simultaneous inoculation of wine completed both AF and MLF in a shorter time compared with sequential inoculation fermentations. The inoculation time and the type of inoculum had also effect on the wine volatile composition. A significant increase in the concentration of ethyl esters and terpenes was observed in wines produced by simultaneous AF/MLF. The wines inoculated with free bacteria showed changes in volatile composition compared to those inoculated with encapsulated bacteria, as an enhanced production of ethyl esters and some major volatile compounds. The inoculation time strongly modified the amino acid composition of the wines, while the influence of the type of inoculum was limited. The wines simultaneously inoculated showed the highest concentrations of amino acids and the lowest of biogenic amines, histamine and putrescine. These wines also had the highest content in tyramine. However, the values of the different biogenic amines found in all the wines were low and they do not suppose a negative aspect from a sanitary point of view. Conversely, the type of inoculum had no significant impact on the biogenic amine profile. In addition, the use of lysozyme in the wines inoculated with encapsulated bacteria did not cause a significant variation in wine composition. These results indicate that the combined use of immobilized bacteria and lysozyme allows achieving successfully MLF of red wines, inhibiting the activity of wine wild and spoilage bacteria. Finally, the impact of the chemical differences on the sensory profiles was very limited according to the results of descriptive sensory analysis, which indicates that the quality of the wines was not altered.

CRediT authorship contribution statement

José Manuel Rodríguez-Nogales: Conceptualization, Methodology, Writing - original draft. **Guillermo Simó:** Investigation and Writing - original draft. **Silvia Pérez-Magariño:** Validation, Writing - review & editing. **Estela Cano-Mozo:** Investigation. **Encarnación Fernández-Fernández:** Conceptualization, Methodology, Writing - original draft. **Violeta Ruipérez:** Conceptualization, Methodology, Writing - original draft. **Josefina Vila-Crespo:** Conceptualization, Methodology, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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CONCLUSIONES

8. CONCLUSIONES

La principal conclusión de esta tesis doctoral es la obtención de un método que permite realizar de manera eficiente fermentaciones malolácticas en vinos tintos con bacterias lácticas inmovilizadas en una matriz híbrida de alginato y sílice; consiguiendo una herramienta muy útil y ventajosa para la vinificación. A continuación se exponen las conclusiones más relevantes que se pueden extraer de los diversos trabajos de investigación incluidos en esta tesis.

- Las técnicas de recubrimiento por entrecruzamiento iónico y covalente se muestran como herramientas muy útiles para solventar las desventajas del empleo del alginato de calcio como matriz para la encapsulación celular, observándose mejoras tanto en las propiedades físico-químicas del hidrogel (estabilidad mecánica y química, difusión, porosidad, etc.), como en la actividad metabólica y viabilidad de las células encapsuladas.

- No existen métodos estandarizados que permitan comparar las diferentes técnicas de recubrimiento. Es necesario un estudio individualizado del comportamiento de cada tipo de recubrimiento con las células objeto de estudio en las condiciones concretas de uso.

- Las cápsulas de sílice-alginato con *O. oeni*, utilizando sílice coloidal y silicato de sodio como precursores no alcóxidos, muestran una mayor actividad maloláctica y mejor estabilidad mecánica y química que las elaboradas solo con alginato, siendo su capacidad de retención bacteriana similar a estas últimas.

- La técnica de recubrimiento de las cápsulas de alginato de calcio con 3-aminopropiltrietoxisilano no mejoró ni la robustez mecánica del soporte ni la capacidad de *O. oeni* para degradar el ácido málico.

- Las condiciones vínicas afectan de manera importante a la actividad de *O. oeni*, sin embargo, su encapsulamiento en el entramado de sílice-alginato reduce significativamente el impacto negativo de este ambiente. Los ensayos realizados en condiciones de vinificación extremas de elevadas graduaciones alcohólicas, bajos pH y bajas temperaturas de fermentación revelan un mejor comportamiento de *O. oeni* encapsulada en geles de sílice-alginato.

- Las cápsulas de sílice-alginato con *O. oeni* pueden ser reutilizadas al menos cinco veces en vinos con pH de 3,0-3,6 y grados alcohólicos de 12,0-15,0% vol.

- Tanto en inoculación secuencial como en inoculación simultánea con *S. cerevisiae*, las células de *O. oeni* encapsuladas desarrollan la FML en un periodo de tiempo inferior al de las células libres en mostos de elevada concentración azucarada y bajo pH. Se aprecia más la mejora del empleo de las bacterias encapsuladas en la inoculación simultánea.

- El momento de inoculación bacteriana (secuencial o simultánea) y el tipo de inóculo (libre o encapsulada) afectan a la composición aromática del vino. La inoculación simultánea produce vinos con mayor concentración de ésteres etílicos y terpenos que aquellos con inoculación secuencial, mientras que los vinos inoculados con bacterias libres muestran un mayor contenido en ésteres etílicos y en algunos volátiles mayoritarios respecto al uso de las encapsuladas.

- La encapsulación bacteriana afecta al perfil aminoacídico del vino en menor medida que el momento de inoculación de las bacterias y no modifica su contenido en AB.

- Las diferencias en las características sensoriales de los vinos elaborados con bacterias libres y encapsuladas, tanto en inoculación secuencial como simultánea, no son significativas.

- El empleo de lisozima junto a las bacterias encapsuladas permite un óptimo desarrollo de la FML, sin observarse modificaciones ni en la composición volátil y aminoacídica del vino, ni en su contenido en AB.