VNIVERSITAT DE VALÈNCIA Facultat de Ciències Biològiques

Doctoral Thesis

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December 2017

DEVELOPMENT OF MOLECULAR APPROACHES FOR DIRECT DETECTION AND QUANTIFICATION OF WINE-RELATED MICROORGANISMS

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PhD program in Biomedicine and Biotechnology

ENOLAB
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MCI IViSoCa
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Para la realización de esta tesis, la autora ha sido beneficiaria de una beca predoctoral de investigación del programa Santiago Grisolía concedida por la Generalitat Valenciana según la resolución del 14 de marzo de 2013, por el director general de Universidad Estudios Superiores y Ciencia. Esta tesis ha sido parcialmente financiada con el proyecto AGL2015-71227-R, ERDF, Ministerio de Economía y Competitividad de España.

AGRADECIMIENTOS/AGRADECIMENTOS

Llegando a este momento, me doy cuenta que tengo que agradecerle a muchísima gente que me ayudado a lo largo de esta etapa de mi vida.

Primero me gustaría dar las gracias a mis directores, Sergi Ferrer e Isabel Pardo, por haberme dado la oportunidad de realizar mi tesis doctoral en su laboratorio. Gracias por haberme recibido con los brazos abiertos, por vuestra ayuda, disponibilidad, y enseñanza a nivel científico y personal a lo largo de estos años.

Querría agradecerles también a todos mis compañeros de laboratorio. Muchos de ellos no solo han sido compañero de bancada, si no que GRANDES AMIGOS. Gracias Lucía, Carmen, Olga, Yaiza, Lorena, Isi, Sara, y Liz por todos los momentos que hemos compartido en ese laboratorio. Juntos hacemos un gran equipo y es un placer trabajar con vosotros.

A Toni, por su alegría matinal y por todos los momentos graciosos! Verte por las mañana era sinónimo de día feliz.

A los estudiantes que han estado conmigo, Bea y Pablo. Gracias por toda vuestra ayuda y por todos los momentos divertidos que hemos compartido.

A mis amigas del departamento de Bioquímica. A Inma por haber confiado en mí y en mí trabajo, y por todo el apoyo. A Merche, mi profesora de cuantitativas, por toda la ayuda y por no mirarme mal por ocupar el equipo día tras día!!

También no podría dejar de darle las gracias a Valencia. Una encantadora ciudad en la cual he vivido estupendos momentos y en la cual me he sentido en casa. Sin embargo, yo siempre digo que son las personas las que hacen los lugares. Y yo, afortunadamente, he tenido la suerte de conocer a gente que hizo que mi estancia en Valencia fuese muy feliz y por lo tanto, les quiero dar las gracias.

A la Lucí, por todo tu apoyo, consejos (hasta los de personal shopper), amistad, y buena disposición. He aprendido mucho contigo, mi amiga. A mi hermana mayor, Carmencita! No hay palabras que sean suficientes para agradecerte todo los que has hecho y has representado en mi vida durante los últimos años, y lo sabes. A mi DJ y profesora de español, Olga, no solo por tu ayuda y apoyo, también por toda tu amistad que llega al nivel de compartir ampollas flash. A mi doctor y consejero científico preferido, Javi, por toda tu ayuda, consejos, opiniones, y discursos, con los cuales he aprendido muchísimo. Contigo la monotonía no existe y eso me encanta. A la pareja más guapa de este mundo, Noe y Josep, y a mi sobri Roci. Gracias por todo lo que hemos compartido durante estos años, por todo el apoyo, amistad, y por recibirme en vuestra casa como una reina. A Yaiza, mi nerd informática number one, por todos los momentos graciosos y divertidos que van desde meriendas hasta consejos de sabios. I´m fliping in colors. A mi vecino y fontanero personal, Piotr. Seremos los PoPos para siempre! A mi fallero preferido, Peris, por toda tu disponibilidad, amistad, por los domingos de playa, y por todos los chocolates suizos. A Amparunia, la valenciana disfrazada de alemana. Gracias por todos los momentos divertidos que hemos compartido. A lsa, la reina de la Roda, pero con nivel superior de Valenciano. Gracias por todos los momentos de diversión, y por todo el apoyo y ayuda.

Gracias amigos por todo vuestro apoyo, amistad, y por todos los almuerzos, meriendas, cenas, fiestas, excursiones, viajes, y muchos otros momentos que me han hecho sentir como una autentica española en su nido. Visca Valencia!!!!

A mi familia españolita (Juan, Mamen, Carmencita y Laura) por haberme recibido muchísimas veces, tanto en su casa como en encuentros familiares, y por haberme hecho sentir como una más de la familia. Sois muy especiales.

Espanha e os meus amigos Espanhóis tiveram um papel importante nesta etapa, mas, nem por isso, os meus amigos Portugueses tiveram uma importância menor.

Queria agradecer aos meus amigos de longa data, Vera, Janete, Tete, Trindade, Joana, e Edna. Obrigada pelas vossas visitas e por todos os momentos que fomos privando ao longo dos útimos anos. Sou uma sortuda por vos ter na minha vida.

Em 2013 a minha morada mudou, mas as minhas raízes continuaram bem assentes no mesmo sítio. Porque essas...essas nunca se vão mover por mais longe que eu vá, e é graças a elas e por elas que eu hoje cheguei até aqui. O meu eterno obrigada vai para vocês!

Aos meus avós (Fátima, Zeca, Lurdes e Santos), ao meu mano e cunhada Cláudia, aos meus tios (Mo, Paulo, Manuel, Lourdes, Graças, Zé, Miguel e Nini) e aos meus primos (Miguel, Marco, Ana Mi, Gonçalo, João F. e Tiago). Obrigada a esta fabulosa família que acreditou sempre em mim, que me visitou vezes sem conta, que esteve ligada pelo skype horas a fio, e que me deu toda a força para nunca desistir.

Aos piolhos da família, Tomás, Afonso, Sofia, Mariana, Simão e recentemene o piolhinho Daniel. São vocês a minha fonte de inspiração e foram vocês que me fizeram soltar inúmeras gargalhas mesmo quando parecia que nada tinha piada. Obrigada pequenotes!

Aos meus queridos e fabulosos PAIS, a vocês agradeço-vos por tudo, apesar da palavra”tudo” não suportar todo o vosso esforço e dedicação. É graças a vocês que eu hoje cheguei até aqui. Obrigada!

Ao Homem com quem eu escolhi partilhar a vida. A ti agradeço-te toda a paciência, dedicação, compreensão, apoio, e ajuda. Todos estes elementos foram imprescindíveis para eu conseguir chegar até aqui. Olho para os últimos 4 anos e vejo que esta foi só mais uma prova de fogo a que fomos sujeitos, da qual não só saímos ilesos como mais fortes. Obrigada Hugo!

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ABBREVIATIONS

µm Micrometre

A. *Acetobacter*

AAB Acetic Acid Bacteria

AF Alcoholic Fermentation

B. *Brettanomyces*

bp Base pair

°C Degrees Celsius

CECT Spanish Type Culture Collection

cells/mL Cells per millilitre

CFU/mL Colony-Forming Units per millilitre

CO_2 Carbone dioxide

Ct Cycle threshold

D. *Dekkera*

DNA Deoxyribonucleic Acid

DGGE Denaturing Gradient Gel Electrophoresis

dNTPs Deoxyribonucleotide triphosphate

dsDNA Double-stranded DNA

E Coefficient of Efficiency

EDTA Ethylenediaminetetraacetic Acid

EMA Ethidium Monoazide

FCM Flow cytometry

FRET Fluorescence Resonance Energy Transfer

FISH Fluorescence *In Situ* Hybridization

FITC Fluorescein Isothiocyanate

G. *Gluconobacter*

g/L Gram per litre

Ga *Gluconacetobacter*

H_2O Water

K. *Kozakia*

L Litter

L. *Lactobacillus*

LAB Lactic Acid Bacteria

LAMP Loop-Mediated Isothermal Amplification

Lc. *Leuconostoc*

LFD Lateral Flow Dipstick

LoD Limit of Detection

LoQ Limit of Quantification

mM Millimolar

mg/mL Milligram per millilitre

MLF Malolactic Fermentation

MLO Medium for Leuconostoc Oenos

mol% Mol percent

MRS de Man, Ragosa, and Sharpe Agar

N Normality

NaCl Sodium Chloride

nm Nanometer

O. *Oenococcus*

ABBREVIATIONS

pH -log [H^+]

P. *Pichia*

PCR Polymerase Chain Reaction

Pd. *Pediococcus*

PMA Propidium Monoazide

qLAMP Quantitative LAMP

qPCR Quantitative PCR

rRNA Ribosomic Ribonucleic Acid

RFU Relative Fluorescence Units

RT-PCR Reverse Transcriptase PCR

S. *Saccharomyces*

SDS Sodium Dodecyl Sulfate

SO_2 Sulphur dioxide

TGGE Temperature Gradient Gel Electrophoresis

Tm Melting Temperature

Tt Time threshold

VBNC Viable But Nonculturable

YPD Yeast extract Peptone Dextrose

Z. *Zygosaccharomyces*

RESUMEN

Introducción

El vino es una bebida alcohólica obtenida tras la fermentación de los azúcares del mosto por las levaduras. Aunque esencialmente son las levaduras las que desarrollan el papel principal en la vinificación, algunas bacterias lácticas (LAB)(*Oenococcus oeni, Lactobacillus plantarum*, etc.) realizan un proceso denominado fermentación maloláctica mediante el cual el ácido málico presente en los mostos se transforma en ácido láctico, lo que mejora el gusto del vino. Además de estos microorganismos beneficiosos también se pueden encontrar microorganismos perjudiciales que dan lugar a alteraciones, como son algunas levaduras y LAB, y las bacterias acéticas (AAB).

La necesidad de controlar la calidad de los vinos requiere una monitorización de los microorganismos que intervienen en la vinificación. El uso de métodos microbiológicos clásicos para llevar a cabo el control microbiológico presenta como principales inconvenientes el que requieren mucho tiempo y material muy diverso para dar un resultado de identificación, lo que no permite aplicar medidas correctivas con suficiente antelación para evitar pérdidas de calidad importantes. El sector vinícola, como muchos otros sectores relacionados con la industria alimentaria, requiere métodos más rápidos, específicos, y efectivos para la monitorización de microrganismos del vino, sobre todo de aquellos que producen alteraciones. Las metodologías moleculares que se han desarrollado en los últimos años permiten conseguir resultados en tiempos considerablemente más cortos, son más sensible y más específicos que los métodos clásicos, aunque algunos de ellos, como la PCR cuantitativa (qPCR) requieren de la inversión en equipos costosos y necesitan personal cualificado. Otra desventaja que presentan las técnicas basadas en la PCR es que requieren de la extracción del DNA a partir de las muestras y que muchas veces las reacciones de amplificación se ven inhibidas por componentes de la matriz en la que están presentes las células, como por ejemplo los polifenoles o el etanol, en el caso del vino.

Objetivos

En este trabajo se ha abordado el desarrollo y puesta a punto de métodos moleculares más sencillos, rápidos, sensibles, específicos, y baratos que los existentes en la actualidad para detectar, y cuantificar microrganismos relacionados con el vino (levaduras, bacterias lácticas y bacterias acéticas). Se han desarrollado procedimientos para la detección y cuantificación de levaduras y

bacterias acéticas totales y, en concreto, de las especies más importantes en la vinificación como *Saccharomyces cerevisiae, Brettanomyces bruxellensis, Zygosaccharomyces bailii, L. plantarum,* y *O. oeni.* Las estrategias que se han utilizado para el desarrollo de nuevos métodos se centran fundamentalmente en la supresión del paso de extracción previa de DNA y de los procedimientos de eliminación de inhibidores de la reacción de PCR presentes en las muestras.

Para llevar a cabo este objetivo general, se establecieron los siguientes objetivos específicos:

- Desarrollo de un método de qPCR que permita la detección y la cuantificación de células enteras de microorganismos del vino, independientemente de si éstas están en un estado viable o no.
- Desarrollo de un método de qPCR que permita la detección y cuantificación de células viables de microorganismos del vino, para lo cual se requiere el tratamiento previo con propidio de monoazida (PMA) de las células presentes en una muestra
- Desarrollo de un método basado en la técnica *loop-mediated isothermal amplification* (LAMP) para la detección de células enteras de microorganismos del vino, independientemente de si éstas están en un estado viable o no.
- Desarrollo de un método LAMP cuantitativo para la detección y cuantificación de células enteras de microorganismos del vino, independientemente de si éstas están en un estado viable o no.

Metodología y Resultados

- **Desarrollo de un método qPCR para la detección y cuantificación de células totales (Cells-qPCR)**

Se adaptó la técnica de qPCR para llevar a cabo la detección y cuantificación de levaduras y de bacterias tanto lácticas como acéticas, evitando el paso de extracción de DNA, es decir, utilizando directamente las células enteras. A esta metodología se la denominó Cells-qPCR. Se compararon las cuantificaciones de microorganismos obtenidas por qPCR a partir de células enteras y del DNA extraído a partir de esas células. Los resultados mostraron que el método era menos sensible cuando se usaban células enteras (límite de cuantificación-LoQ=10^5 células/mL) que cuando se

empleaba DNA extraído (LoQ=10^2 células/mL). Sin embargo, los resultados obtenidos con la técnica Cells-qPCR mostraron que existía una proporcionalidad entre los valores de Ct y las concentraciones de células de levaduras y de bacterias lácticas. Las rectas de regresión obtenidas al representar estos datos eran paralelas a las obtenidas cuando se representaban los datos obtenidos a partir de DNA extraído. También se reveló que las curvas de fusión obtenidas en ambos casos (células enteras y DNA extraído) eran especie-específicas. Sin embargo, cuando se trabajó con las bacterias acéticas se obtuvieron resultados inconsistentes y poco reproducibles.

Para mejorar la sensibilidad y efectividad del método de Cells-qPCR, se aumentó el volumen de muestra añadida en 5 veces a cada reacción de amplificación. Los resultados mostraron que el aumento de volumen de la muestra añadida aumentó la sensibilidad del método de Cells-qPCR, ya que el LoQ pasó de 10^5 células/mL a 10^4 células/mL (100 células/tubo de reacción) para *B. bruxellensis* y *L. plantarum*, a 10^3 células/mL (10 células/tubo de reacción) para *S. cerevisiae*, y a 10^2 células/mL (1 célula/tubo de reacción) para *O. oeni*. Además, el aumento de volumen de muestra permitió cuantificar concentraciones celulares de 10^2 células/mL (1 célula/tubo de reacción) de las especies *A. aceti* y *G. oxydans*. El LoQ superior fue 10^8 células/mL para bacterias y 10^7 células/mL para levaduras. Para todas las especies, se observó una linealidad adecuada entre los valores de Ct y la concentración de células (R^2>0,99), confirmando la efectividad del método de Cells-qPCR.

Posteriormente, se evaluó el efecto de matrices complejas, como mostos de uva y vinos blancos y tintos, sobre la cuantificación de microorganismos mediante Cells-qPCR. Se construyeron rectas patrón con concentraciones de células entre 10^2 y 10^8 células/mL suspendidas en estas matrices. Los resultados obtenidos, en mosto y vino blancos, mostraron que el rango de células cuantificables fue similar al obtenido cuando las células estaban suspendidas en medio de cultivo. Los mostos y vinos tintos, se caracterizan por contener una concentración de polifenoles, superior a la de los mostos y vinos blancos. Para comprobar si éstos compuestos inhibían la reacción de amplificación se evaluó el efecto del lavado previo de las células con tampón de lavado TEN suplementado con polivinil pirrolidona (PVP). Para ello, se compararon las rectas patrón obtenidas con concentraciones celulares entre 10^2 y 10^8 células/mL suspendidas en mosto y vino tintos, amplificadas después de un lavado con tampón TEN o amplificadas después de un lavado con tampón TEN suplementado con PVP. Los resultados mostraron que los LoQ obtenidos con células lavadas con tampón TEN suplementado con PVP, era similar al obtenido con células provenientes de medio de cultivo, e igual al obtenido con células suspendidas en mosto y vino blancos. De estos resultados se dedujo que ni

la composición de mosto ni la del vino, fuesen tintos o blancos, afectaba a la reproducibilidad del método, ya que no se observó inhibición de la reacción de amplificación en ningún caso.

De forma resumida, mediante el método desarrollado de Cells-qPCR se logró detectar y cuantificar células enteras de levaduras y de bacterias en medio de cultivo y en mostos y vinos tanto blancos como tintos, en los siguientes rangos de concentraciones: desde 10^4 hasta 10^7 células/mL (10^2-10^5 células por tubo de reacción) para *B. bruxellensis*, desde 10^3 hasta 10^7 células/mL (10-10^5 células por tubo de reacción) para *S. cerevisiae*, desde 10^4 hasta 10^8 células/mL (10^2-10^6 células por tubo de reacción) para *L. plantarum*. Para *O. oeni*, *A. aceti*, y *G. oxydans* el rango de células cuantificables fue desde 10^2 hasta 10^8 células/mL (1-10^6 células por tubo de reacción). Este último LoQ obtenido indicaba que el método permitía discriminar hasta 1 célula por reacción, demostrando la elevada sensibilidad del método desarrollado. Por lo tanto, se demostró que la técnica de Cells-qPCR permitió cuantificar diversos microorganismos del vino en un amplio rango de concentraciones celulares directamente a partir de mostos de uva y vinos, obviando el paso de extracción de DNA.

Tras confirmar la efectividad de método Cells-qPCR, se comprobó la linealidad existente entre los valores de Ct y las concentraciones de células enteras, y se compararon las eficiencias de las rectas patrón obtenidas con células enteras y con DNA extraído, a partir de las propias células, en los diferentes tipos de matrices (medio de cultivo, mostos y vinos blancos y tintos). Los valores de las eficiencias obtenidas fueron, para todos los casos los microorganismos, superiores a 0.84. Además, para las especies de bacterias lácticas y acéticas, no se observaron diferencias significativas (ρ value >0.05) entre las eficiencias obtenidas con DNA y con células enteras.

Mejora de la sensibilidad del método de Cells-qPCR mediante la rotura de la pared de las células

En la industria enológica, un pequeño número de células es suficiente para conducir alteraciones en el producto final. Por lo tanto, la técnica de Cells-qPCR que aquí se propone debería de poseer una alta sensibilidad con el objetivo de detectar muy bajas concentraciones de células. Se evaluó el efecto de la rotura enzimática y de la rotura mecánica de la pared de las células de levaduras, *L. plantarum y O. oeni*, previa a la reacción de Cells-qPCR. Se compararon las rectas patrón construidas a partir de células crecidas en medio de cultivo y con o sin tratamiento de lisis (enzimática o

mecánica). La lisis enzimática de levaduras se llevó a cabo con zimoliasa y la de bacterias con lisozima. El análisis de las rectas patrón reveló que la lisis enzimática no permitía la discriminación de las distintas concentraciones de células, es decir no existía linealidad entre valores de Ct y concentración de células ni de levaduras, ni de *L. plantarum*. Sí existía linealidad cuando las células de *O. oeni* se lisaban con lisozima, sin embargo, los Cts obtenidos, para la misma concentración de células, eran superiores a los obtenidos mediantes células enteras. Estos resultados indicaron que la lisis enzimática era una estrategia inadecuada para aumentar de la sensibilidad del método de Cells-qPCR.

La lisis mecánica de levaduras y bacterias, se llevó a cabo mediante el uso de microesferas de vidrio. Los resultados obtenidos con células de levaduras y de *L plantarum* crecidas en medio de cultivo mostraron que este tipo de lisis aumentó la sensibilidad de la técnica de Cells-qPCR. Cuando se compararon los valores de Ct de las amplificaciones con células lisadas y enteras se observó que, para una misma concentración celular, los valores de Ct eran más bajos en el primer caso y que los LoQ también eran más bajos: 10^2 células/mL (1 célula/tubo de reacción) de *B. bruxellensis*, *S. cerevisiae*, y *L. plantarum*. Además, se observó que todas las rectas obtenidas presentaban unos altos valores de correlación lineal (R^2>0,98). La lisis mecánica de las células previa a la reacción de Cells-qPCR se usó también para la detección y cuantificación específica de *Z. bailii,* una levadura alterante aislada frecuentemente en mostos concentrados. La construcción de rectas patrón demostró que con el procedimiento de lisis mecánica se lograba cuantificar un rango de células desde 10^2 hasta 10^6 células/mL y con coeficientes de correlación (R^2) superiores a 0.99. Sin embargo, la lisis mecánica no aporto ninguna mejora en el caso de las células de *O. oeni*, ya que los Ct obtenidos eran similares a los obtenidos con células enteras.

De manera resumida, los resultados obtenidos demostraron que la lisis mecánica de las células es un proceso rápido, simple y barato, y permite detectar y cuantificar un mayor rango de concentraciones de células de levaduras y de *L. plantarum*, sin tener que recurrir a kits de extracción de DNA.

Tras confirmar el éxito de la lisis mecánica de las células en la cuantificación de células desde medio de cultivo, se evaluó ese mismo efecto en matrices complejas como mostos de uva y vinos blancos y tintos. Para ello, se construyeron rectas patrón mediante el método de Cells-qPCR para la cuantificación de células lisadas mecánicamente de levaduras (*B. bruxellensis*, *S. cerevisiae*, y *Z. bailii*) y de *L. plantarum* en mostos de uva y vinos blancos y tintos. Los resultados obtenidos mostraron que la matriz no influía en la sensibilidad del método, ya que el rango de células

cuantificables fue similar al obtenido desde medio de cultivo. Por lo tanto, el LoQ para todas las especies y matrices fue de 10^2 células/mL. Este resultado demuestra la elevada sensibilidad y versatilidad del método desarrollado ya que es posible detectar hasta una célula por reacción.

Evaluación del efecto matriz en la eficiencia de la técnica de Cells-qPCR empleando células enteras y lisadas

El vino se caracteriza por ser una matriz compleja ya que contienen componentes capaces de inhibir la reacción de PCR, como etanol, taninos, polisacáridos, y polifenoles. Por ello, con la intención de evaluar si la matriz influía en la cuantificación de células mediante Cells-qPCR, se compararon, para cada especie, las rectas patrón y respectivas eficiencias obtenidas en cada matriz. Para levaduras y *L. plantarum*, además de células enteras, el experimento también se realizó mediante células lisadas mecánicamente. Los resultados mostraron que, para todas las especies, no hay diferencias significativas (ρ valor >0.05) en las eficiencias obtenidas entre matrices. Resultados similares fueron obtenidos cuando se llevó a cabo el experimento empleando células lisadas. Por lo tanto, estos resultados indican que la presencia de células en matrices complejas como mostos de uva y vinos, no influye en la eficiencia de la amplificación.

Evaluación de la especificidad de la técnica de Cells-qPCR empleando células enteras y lisadas

Se determinó si la presencia de otros microrganismos influía en la cuantificación de células de *B. bruxellensis, S. cerevisiae*, *Z. bailii*, *L. plantarum*, *O. oeni,* y de bacterias acéticas en una muestra, es decir, si comprometía la especificidad de la técnica. Para ello, se cuantificaron concentraciones celulares de 10^7, 10^5 y 10^3 células/mL de cultivos puros en vino tinto y de esas mismas suspensiones contaminadas con 10^5 células/mL de otras bacterias lácticas o levaduras. Para levaduras y *L. plantarum* se llevaron a cabo experimentos paralelos utilizando células enteras y células lisadas mecánicamente. Los resultados obtenidos mostraron que la presencia de otros microrganismos no influye significativamente (ρ valor >0.05) en la cuantificación del microrganismo diana. Por consiguiente, se puede considerar que el método de Cells-qPCR posee una elevada especificidad, ya que el Ct de diferentes concentraciones de levaduras y bacterias no se vio afectado con la presencia de otros microrganismos.

Aplicación de la técnica Cells-qPCR para el estudio de las poblaciones microbianas en vinificaciones industriales

Tras la optimización de todos los parámetros para para llevar a cabo la técnica de Cells-qPCR, se usó la metodología desarrollada para detectar y cuantificar levaduras y bacterias acéticas totales y las especies individuales *B. bruxellensis, S. cerevisiae, Z. bailii, L. plantarum, O. oeni*, en vinificaciones reales en bodega. Para ello, se realizó el seguimiento de 3 vinificaciones correspondientes a tres variedades de uva tinta distintas: Cabernet Sauvignon, Garnacha y Merlot. Los resultados obtenidos confirmaron la aplicabilidad de la metodología desarrollada en vinificaciones reales, ya que se logró detectar y cuantificar diferentes microrganismos en todos los muestreos realizados. Además, este resultado demostró que la técnica era insensible a la presencia de inhibidores de la reacción de qPCR (polifenoles o etanol), y de otros microrganismos distintos del diana, es decir, era específica.

- **Desarrollo de un método PMA-qPCR para la detección y cuantificación de células viables (PMA-Cells-qPCR)**

Dado que para que se dé una alteración en un vino es necesario que el microorganismo esté vivo, el uso de la qPCR presenta la limitación de que hace una cuantificación de microorganismos totales independientemente de si éstos están vivos o muertos. Es importante por tanto, distinguir entre células vivas y muertas para determinar el número de células activas en ese vino, es decir, el riesgo microbiológico potencial de sufrir alteraciones. La incorporación de agentes intercalantes del DNA como PEMAX, como tratamiento previo a la técnica de qPCR, permite hacerlo. Por ello, se evaluó la utilidad de este agente para diferenciar células vivas y muertas de *B. bruxellensis*, *S. cerevisiae*, *L. plantarum*, y *O. oeni,* mediante la técnica de Cells-qPCR. A esta metodología se la denominó PMA-Cells-qPCR.

Se optimizó la concentración de PEMAX a utilizar en el tratamiento de las células para lograr una buena discriminación entre células vivas y muertas. Para ello, se prepararon, de manera independiente, suspensiones celulares tanto de células vivas como de células muertas por calor (100 °C durante media hora) de cada una de las especies individuales. A continuación se trataron todas las suspensiones celulares con diferentes concentraciones de PEMAX (0, 5, 10, 25, 50, 100 y 200 µM). Los resultados obtenidos mostraron que la concentración óptima de PEMAX para realizar el tratamiento fue de 50 µM para *B. bruxellensis* y *S. cerevisiae*, y de 5 µM para *L. plantarum* y *O. oeni*. A continuación, se evaluó la validez de la técnica de PMA-Cells-qPCR para

detectar y cuantificar células vivas y muertas a partir de medio de cultivo. Para ello, se construyeron rectas patrón aplicando esta técnica a suspensiones celulares de diferentes concentraciones de células, tanto vivas como muertas, y obteniendo el respectivo valor de Ct para las mismas. Los resultados obtenidos confirmaron la posibilidad de discriminar entre células vivas y muertas mediante PMA-Cells-qPCR, y además demostraron que permiten una buena cuantificación en el rango de concentraciones entre 10^3 y 10^6 células/mL para *B. bruxellensis*, entre 10^2 y 10^6 células/mL para *S. cerevisiae*, y entre 10^3 y 10^8 células/mL para *O. oeni*. En el caso de *L. plantarum*, se observó que células muertas amplificaban. Este resultado anómalo indicó que el cebador utilizado posiblemente no era el más adecuado para la metodología empleada.

Considerando el método de PMA-Cells-qPCR adecuado para la diferenciación entre células vivas y muertas de *B. bruxellensis*, *S. cerevisiae*, y *O. oeni* se evaluó su efectividad en muestras de vinos blancos y tintos. Se construyeron, igualmente, rectas patrón a partir de suspensiones con diferentes concentraciones de células, tanto vivas como muertas. En el caso de las levaduras, la sensibilidad del método fue similar a la obtenida en medio de cultivo, no observándose inhibición de la reacción por los componentes de las matrices usadas. Sin embargo, sí se observó disminución del rango de cuantificación en el caso de *O. oeni*, ya que el límite inferior de cuantificación aumentó de 10^3 a 10^5 células/mL.

- **Desarrollo de un método LAMP para la detección y cuantificación de células totales (Cells-LAMP y Cells-qLAMP)**

El LAMP es un método molecular novedoso que permite la amplificación de DNA en condiciones isotérmicas. Este método permite detectar secuencias diana que estén en baja concentración, es capaz de sintetizar grandes cantidades de DNA en un corto espacio de tiempo, es muy específico, dado que reconoce varias secuencias de la región diana y muy rápido puesto que en 1 hora se obtiene el resultado final. Es una técnica que se puede aplicar tanto para la detección como para la cuantificación y en el primer caso de muy fácil interpretación, ya que la existencia del microorganismo diana en una muestra supone un aumento de turbidez en el tubo de reacción, detectable a ojo desnudo o mediante un colorímetro y por tanto, no requiere de equipos de alto coste.

Por ello, se adaptó la técnica de LAMP para conseguir la detección de células enteras de *B. bruxellensis*. A esta metodología se la denominó Cells-LAMP. Se realizaron experimentos de amplificación por LAMP tanto con células enteras como con DNA extraído a partir de las mismas. Los resultados mostraron que tanto en un caso como en otro, se generaba turbidez en los tubos de reacción, pero eso no ocurría en ausencia del microorganismo diana (control negativo). Además, se confirmó que había tenido lugar la reacción de amplificación en las muestras que contenían *B. bruxellensis* por la existencia de bandas de amplificación, que se revelaron mediante electroforesis en gel de agarosa, sin embargo, estas bandas no existían en el control negativo. Estos resultados demostraron que la técnica de LAMP puede ser aplicada directamente para detectar células enteras de *B. bruxellensis*, eliminando la necesidad de realizar una extracción previa de su DNA.

Se diseñaron cebadores específicos para la especie *O. oeni* mediante el uso de programa LAMP Designer. Tras confirmar la especificidad de los cebadores diseñados, se comprobó la aplicabilidad del método Cells-LAMP mediante el uso de células enteras de *O. oeni*. Los resultados confirmaron la detección de células enteras de esta especie, ya que se observó presencia de turbidez en el tubo de reacción y presencia de bandas de amplificación (mediante electroforesis).

A continuación, se confirmó la aplicabilidad del método de amplificación mediante Cells-LAMP para detectar células enteras tanto de *O. oeni* como de *B. bruxellensis* en muestras naturales. Los resultados mostraron que las muestras que contenían células enteras de estas dos especies suspendidas en mostos y vinos blancos y tintos dieron lugar a la aparición de turbidez y de bandas de amplificación, pero no en muestras que no contenían células de las especies diana. Por lo tanto, se pudo demostrar que el método Cells-LAMP permitió detectar estas dos especies, independientemente de la matriz en las cuales se encuentren. Tras comprobar la aplicabilidad del método Cells-LAMP en muestras de vino, se evaluó el rango de concentración de células detectables por la técnica en esa misma matriz. Para ello, se prepararon suspensiones celulares en un rango de concentraciones entre 10^2 y 10^8 células/mL en vino blanco y tinto para cada una de las especies, y se sometieron a reacciones de amplificación mediante LAMP. Los resultados obtenidos, tanto por observación de presencia/ausencia de turbidez como por electroforesis, demostraron que, en el caso de *B. bruxellensis*, la técnica permite detectar un rango de células de entre 10^2 hasta 10^8 células/mL (es decir, desde 1 célula hasta $1x10^6$ células por tubo de reacción) tanto en vino blanco como en vino tinto. Sin embargo, en el caso de *O. oeni*, el rango de células detectables estaba entre $1x10^3$ hasta $1x10^8$ células/mL en vino blanco, y entre $1x10^4$ hasta $1x10^8$ células/mL en vino tinto. La aplicación de una lisis mecánica previa a las células de *O. oeni*, mejoró notablemente el rango de

cuantificación ya que éste se extendía desde 10^2 hasta 10^8 células/mL, tanto en vino blanco como en vino tinto.

Tras optimizar el método de Cells-LAMP para detectar células enteras de *B. bruxellensis* y de *O. oeni*, y con la finalidad de cuantificar estas células, se desarrolló un método de qLAMP basado en el uso de fluoróforos. A esta metodología se la denominó Cells-qLAMP. Se prepararon diferentes concentraciones de células de *B. bruxellensis* y de *O. oeni* (10^3, 10^5 y 10^7 células/mL) y se sometieron a amplificaciones mediante qLAMP. Se compararon los resultados de las amplificaciones obtenidas empleando SYBR Green I y SYTO-9 como fluoróforos.

Los resultados obtenidos mediante Cells-qLAMP con SYBR Green I no fueron adecuados, ya que se observó ausencia de amplificación tanto con células *B. bruxellensis* y de *O. oeni* (no se lograron obtener curvas de fusión específicas con ninguna de las dos especies) lo que demostró falta de especificidad. Sin embargo, los resultados obtenidos mediante Cells-qLAMP con SYTO-9 fueron mucho más alentadores, ya que se logró amplificar células tanto de *B. bruxellensis* y de *O. oeni*, como demostró la aparición de curvas de fusión específicas. Sin embargo, la técnica sólo permitió la cuantificación de las células de *O. oeni*, ya que se observó una buena correlación entre los valores de las Cts y las concentraciones celulares de esta especie en el rango entre 10^2 hasta 10^8, lo cual no ocurrió en el caso de *B. bruxellensis*

Conclusiones

1. Se han desarrollado dos métodos basados en el uso de la técnica de PCR y otros dos basados en la técnica de LAMP que permiten la detección (Cells-LAMP), y la cuantificación tanto de células totales (Cells-qPCR, Cells-qLAMP), como de células viables y muertas (PMA-Cells-qPCR), directamente a partir de mostos y vinos.

2. Se han utilizado sondas específicas, ya descritas en bibliografía o desarrolladas en este trabajo para detectar y cuantificar levaduras y bacterias acéticas totales propias del vino, y, en concreto, las especies más importantes en la vinificación como *S. cerevisiae*, *L. plantarum*, y *O. oeni*, así como las levaduras alterantes *B. bruxellensis* y *Z. bailii*.

3. Los procedimientos desarrollados permiten realizar la detección y cuantificación de las células enteras de esos grupos microbianos o de las especies concretas, sin necesidad de recurrir a la extracción del DNA y directamente a partir de mostos, de vinos, o de medios de cultivo.

4. La aplicación de un tratamiento de lisis mecánica aumenta la sensibilidad de estos cuatro métodos, permitiendo la detección de una única célula presente en el tubo de reacción.

5. La matriz de la cual provengan las células (medios de cultivo, mostos o vinos, tanto blancos como tintos) no inhibe las reacciones de PCR y LAMP. Ello es particularmente importante en el caso de los vinos tintos, donde se encuentran polifenoles y etanol, descritos frecuentemente como inhibidores de las polimerasas. Los límites de detección y cuantificación, y las eficiencias obtenidas en mostos y vinos fueron similares a los obtenidos en medio de cultivo.

6. La presencia de microrganismos, distintos de los que son diana, en las muestras analizadas no influye en los resultados obtenidos con estas técnicas, lo que demuestra que son totalmente específicas.

7. Los métodos desarrollados permiten la detección y cuantificación de células (tanto totales como vivas y muertas) en un plazo de tiempo que varía entre 1 y 5 horas. Estos procedimientos se han aplicado para la detección y cuantificación de levaduras y bacterias en mostos y vinos producidos industrialmente.

8. Las metodologías desarrolladas son económicas, y rápidas y presentan una elevada sensibilidad y especificidad. Sin embargo, se requiere la adquisición de un aparato de PCR cuantitativa, con la excepción de la técnica LAMP utilizada para la detección de microorganismos. Son técnicas transferibles a empresas de servicios al sector enológico y que permiten obtener resultados en plazos lo suficientemente cortos como para que los responsables de producción tomen decisiones a tiempo.

1. INTRODUCTION

1.1 Wine and Winemaking Process

1.2 Microorganisms Present in Wine

1.3 Detection and Quantification of Microorganisms in Wine by Direct Methods

1.4 Influence of Inhibitors on DNA Amplification Dependent Methods

1.1. Wine and Winemaking Process

The conversion of grape juice into wine is a biotechnological tradition dating back over 7000 years (Bartowsky and Pretorius, 2009; Bartowsky, 2009; Mills et al., 2008; Romano and Capece, 2017). Nowadays, the vinification process is not that different from the one used in the time of ancient Egyptians or Greeks. Nevertheless, at the present, there is a much greater control at critical stages from grape harvest to bottling, when some yeasts and bacteria can proliferate (Bartowsky and Pretorius, 2009; Bartowsky, 2009).

Wine is produced through a complex biochemical process involving interactions between fungi, yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) which start in the vineyard and continue throughout the fermentation process until packaging (du Toit et al., 2011; Fleet, 1993, 2003; Fleet et al., 1984). A general scheme of the winemaking steps in the production of white and red wines is presented in **Figure 1**.

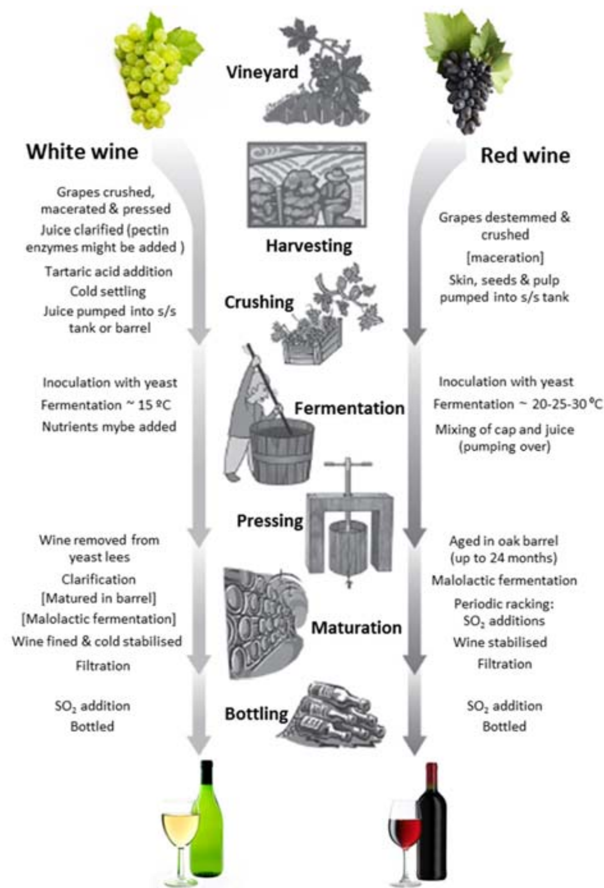

Figure 1-General summary of the main steps in the red and white grape vinification (adapted from Bartowsky and Pretorius (2009)).

The winemaking process includes two main steps carried out by microorganisms: alcoholic fermentation (AF) developed by yeasts, and malolactic fermentation (MLF) developed by LAB (du Toit et al., 2011; Fleet et al., 1984; Vila, 2010).

AF is the anaerobic transformation of sugars, mainly glucose and fructose, into ethanol and CO_2 (Romano and Capece, 2017; Zamora, 2009). At the same time as this reaction occurs, a lot of other biochemical, chemical and physicochemical processes take place, making it possible to turn the grape must into wine. Besides ethanol, several other compounds such as higher alcohols, esters, glycerol, succinic acid, diacetyl, acetoin, and 2,3-butanediol are produced. Without the production of these substances, wine would have little organoleptic interest. Different yeast species participate in spontaneous AF contributing to ethanol and other important flavour compounds production. Nevertheless, in later stages of fermentation, *Saccharomyces cerevisiae* is the predominant yeast given its greater resistance to high ethanol concentration (Zamora, 2009). To ensure a quicker start for fermentation, to out-compete and dominate indigenous yeast strains and provide a wine with distinctive characters, most of the wineries inoculate selected dry strains of *S. cerevisiae* (Krieger-Weber, 2009).

MLF in wine is a secondary fermentation that usually occurs at the end of AF, although sometimes it can occur concurrently. This fermentation is conducted by LAB belonging to the genera *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*, and consists in a biological process of wine decarboxylation in which dicarboxylic L-malic acid is converted to the monocarboxylic L-lactic acid and CO_2. MLF is important in winemaking as it deacidifies the wine, it contributes to microbial stability through the removal of potential carbon sources, and lastly, it contributes to wine aroma through the production of metabolites (Davis et al., 1985; Liu, 2002; Lonvaud-Funel, 1999; Vila, 2010). This deacidification reaction is essential for the production of most red wine and desirable in some white and sparkling base wines. While this secondary fermentation can occur spontaneously, the majority of winemakers, particularly in new world winemaking regions, prefer to minimise the risk of a failed or sluggish MLF, inoculating with a reliable commercially available starter culture. Of all the species of LAB, *Oenococcus oeni* is the most associated with MLF in wine, and it is probably the most adapted species to overcome the harsh environmental wine conditions. Due to its acid tolerance and flavour profile, *O. oeni* represents the majority of commercial MLF starter cultures. Nevertheless, other LAB species as *Lactobacillus plantarum,* have shown interesting results for their ability to induce MLF and overcome wine conditions as low pH and high alcohol levels. Today, *L.*

plantarum is also used as a starter culture to conduct the MLF in wine (Bartowsky et al., 2015; du Toit et al., 2011; Vila, 2010). After MLF, these wines more complex in taste and with an improved mouthfeel, are often subjected to ageing for a long time in barrels and need bottle-ageing to reach their plenitude (Lonvaud-Funel, 1999).

The true challenge for winemakers is to blend an ever-changing "menu" of grapes, soil, and climate with the study of yeast and bacterial metabolism to produce the best possible expression of their chosen wine style (Krieger-Weber, 2009). To produce the best wine with the desired style, flavour formation must be optimized and off-flavours development minimized. Wine aroma and flavour originate from the grape, yeast and bacterial metabolism of grape juice and wood (if used), and such as from chemical reactions during maturation and storage (Bartowsky and Pretorius, 2009).

1.2. Microorganisms Present in Wine

1.2.1. Yeasts

Yeasts are widespread in the natural world. They can be found in flowers, plant leaves, fruits, soil and water (Walker, 1998). Yeasts have a wide-ranging fundamental and industrial importance in science, food, medical, and agricultural disciplines. Traditional industrial attributes of yeasts allow their primary roles in many food fermentations such as beers, ciders, wines, sake, distilled spirits, bakery products, cheeses, sausages, and other fermented foods (Johnson and Echavarri-Erasun, 2011). In the wine industry, the metabolic activities of yeasts can have a profound impact on the composition of the wine, and therefore on its aroma and flavour properties (Bisson and Joshep, 2009).

Taxonomy and physiology

Yeasts constitute a vast, taxonomically heterogeneous and very complex group. Since the first scientific description of *S. cerevisiae*, the development of yeast taxonomy has been undergoing

controversy and with continuous changes in names. Over the years, a number of classifications have been proposed for the ever-increasing number of yeast species described (Deak, 2008b).

Yeasts were traditionally characterized, classified, and identified by morphological and physiological criteria. The first classifications were based on the phenotypic differences between yeasts: cell shape and size, spore formation, cultural characters, fermentation and assimilation of different sugars, assimilation of nitrates, growth-factor needs, and resistance to cycloheximide (Ribéreau-Gayon et al., 2000b). Since the 1970s a large amount of data on the chemical composition of cell walls, capsular polysaccharides, antigenic determinants, and enzyme patterns have provided valuable information for yeast taxonomy. Although biochemical methods have proved to be useful for taxonomic purposes, the exploration of phylogenetic relationships among yeasts became possible only after the introduction of molecular techniques. Various DNA-based methods have reformed the taxonomy and classification of yeasts, including the recognition and identification of species. Nevertheless, sophisticated biochemical and molecular methods, cannot easily be applied in routine identification procedures. Hence, in both classification and identification of yeasts, the morphological and physiological criteria, such as the characteristics of sexual reproduction, are still important (Deak, 2008a).

Yeasts are considered to be unicellular fungi that reproduce by budding (e.g. *Saccharomyces*), direct fission division, (e.g. *Schizosaccharomyces*), or they may also grow as simple irregular filaments (mycelium) (Ribéreau-Gayon et al., 2000b). The shape of the yeast cell and its structural parts are alternatively described as tridimensional objects, such as sphere, globe, egg shaped, cylindrical and olive shaped, or as two dimensional pictures as they appear through the microscope, like circular, elliptical, triangular and bottle shaped (De Becze, 1956).

According to their characteristics of sexual reproduction (teleomorph state), yeasts are divided into three classes of fungi, Ascomycetes, Basidiomycetes, and Deuteromycetes (imperfect fungi). Wine-related yeasts, belonging to Ascomycetes and Basidiomycetes classes, can multiply either asexually by vegetative multiplication or sexually by forming ascospores and basidiospores respectively (Ribéreau-Gayon et al., 2000b).

Of the 100 yeast genera representing more than 700 species, over 20 yeast genera are associated with winemaking: *Aureobasidium*, *Auriculibuller*, *Brettanomyces* (teleomorph *Dekkera*), *Bulleromyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora* (teleomorph *Kloeckera*), *Issatchenka*, *Kluyveromyces*, *Lipomyces*, *Metschnikowia*, *Pichia*, *Rhodosporidium*, *Rhodotorula*,

Saccharomyces, *Saccharomycodes*, *Sporidiobolus*, *Sporobolomyces*, *Schizosaccharomyces*, *Torulaspora*, *Yarrowia*, *Zygoas* and *Zygosaccharomyces* (Bartowsky and Pretorius, 2009; Bisson and Joshep, 2009).

Ecology of yeasts in wine

A large amount of research has focused on the ecology of wine yeasts, concerning the studies in the distribution and succession of species found on the grape and then in grape must and wine.

Yeasts are distributed irregularly on the surface of the grapevine. They are found in small quantities on leaves, in the stem and unripe grapes and they colonize the grape skin during ripening. The number of yeasts on the grape berry, just before the harvest, is between 10^3 and 10^5 cells/cm^2, depending on the geographical situation of the vineyard, the climatic conditions during maturation, the sanitary state of the harvest, the insect pressure (bees, wasps and flies act as vectors of yeast species), and pesticide treatments applied to the vine (Ribéreau-Gayon et al., 2000b).

The change in species on the surface of grapes that occurs during ripening follows a pattern of early dominance by the basidiomycete yeasts, *Aureobasidium*, *Cryptococcus*, *Rhodosporidium*, and *Rhodotorula*, giving way, as the fruit ripens, to the ascomycete yeasts, particularly *Hanseniaspora*, *Metschnikowia*, and *Candida*. The berry damage that occurs later in ripening due to physical or biological factors enriches for these yeasts, as well as fermentative yeasts such as *Saccharomyces* (Bisson and Joshep, 2009; Bisson and Walker, 2015). Occasionally, species in other genera such as *Brettanomyces*, *Saccharomycodes*, *Torulaspora,* and *Zygosaccharomyces* may be present (Fleet, 2003; Fleet, 2008; Fleet and Heard, 1993).

In grape fermentations, one may inoculate with selected yeast or let to ferment by the native microbiota present on the grape and in the winery. In either case, the fermentation is eventually dominated by *Saccharomyces*, mainly *S. cerevisiae,* and the related species *Saccharomyces bayanus*. Despite *S. cerevisiae* is the predominant yeast in winemaking, it is not prevalent on wine grapes. Generally, its contribution to microbe population is less than 10-100 CFU/g (Fleet, 2003; Ribéreau-Gayon et al., 2000b). It is well known that non-*Saccharomyces* species (especially species of *Hanseniaspora*, *Candida*, *Pichia,* and *Metschnikowia*) initiate spontaneous fermentation of the grape must, competing with *Saccharomyces* for nutrients, but are soon overtaken by the growth of *S. cerevisiae* that dominates from mid to final stages of the process. The

predominance of *S. cerevisiae* in these stages of the process is a likely result of its high ethanol tolerance as compared with other yeasts present in the wine environment (Fleet, 2008; Fleet and Heard, 1993). Inoculation with *Saccharomyces* leads to a faster domination of the fermentation and more rapid inhibition of the other yeasts present (Bisson and Joshep, 2009).

During the final part of AF (the yeast decline phase), the population of *S. cerevisiae* progressively decreases while still remaining above 10^6 cells/mL. In favourable winemaking conditions, characterized by a rapid and complete exhaustion of sugars, no other yeast species significantly appears at the end of fermentation (Ribéreau-Gayon et al., 2000b).

Beneficial role of yeasts in wine

Yeasts are the prominent organisms in wine production, determinating wine flavour and other qualities by a range of mechanisms and activities. The AF is the main activity by which yeasts transform an acidic, sweet, low flavored grape must into a distinctive, highly flavored alcoholic beverage (Ugliano and Henschke, 2009). They do this by several mechanisms: (i) utilizing grape must constituents, (ii) producing ethanol and other solvents that help to extract flavour and colour components from grape solids, (iii) producing enzymes that transform neutral grape compounds into flavour active compounds, (iv) producing many hundreds of flavour active, secondary metabolites (e.g. acids, alcohols, esters, polyols, aldehydes, ketones, volatile sulphur compounds), and (v) autolytic degradation of dead yeast cells (Fleet, 2003).

The major volatile products of yeast metabolism, ethanol, glycerol, and carbon dioxide, make a relatively small, but, nonetheless, a fundamental contribution to wine flavour. The main groups of compounds that form the "fermentation bouquet" are the organic acids, higher alcohols, esters and, to a lesser extent, aldehydes (Lambrechts and Pretorius, 2000). Nonetheless, it is important taking into account that excessive concentrations of these metabolites (above the threshold value) have a negative impact on the aroma and flavour of wine (Bartowsky and Pretorius, 2009). Many studies have reported the qualitative and quantitative profiles of these metabolites for various strains of *Saccharomyces* and non-*Saccharomyces* yeasts (Fleet, 2003; Fleet, 2008).

Regarding *Saccharomyces* genus, from the seven described species (*S. bayanus*, *Saccharomyces cariocanus*, *S. cerevisiae*, *Saccharomyces kudriavzevii*, *Saccharomyces mikatae*, *Saccharomyces paradoxus*, and *Saccharomyces pastorianus*) (Blondin et al., 2009), *S. cerevisiae*, *S.*

bayanus, and *S. pastorianus* are the only species that have been found during AF (Bisson and Joshep, 2009). Nevertheless, as mentioned above, *S. cerevisiae* is the main species involved in spontaneous AF, being commonly used as starter culture given its optimal technological properties and sensory outcome (Blondin et al., 2009). *S. cerevisiae* cells are elliptical, or occasionally spherical with typical dimensions of 4-8 µm in diameter for spheres, and 4-7 µm wide and 7-10 µm long for the elliptical cells, with vegetative reproduction by multilateral budding (De Becze, 1956; Querol et al., 2003). These yeasts are particularly well adapted to harsh conditions prevailing in grape musts and wines (strong acidity, high sugar or alcohol concentration, presence of sulfites, etc.) (Blondin et al., 2009). In addition to the production of ethanol, *S. cerevisiae* generates many secondary metabolites that are key determinants of wine quality (Fleet and Heard, 1993; Lambrechts and Pretorius, 2000).

<u>Spoilage of wine by yeasts</u>

Yeasts can spoil wine, both during and after fermentation. Uncontrolled yeast growth at either these two stages can alter the chemical composition of wine, detracting from its sensory properties of appearance, aroma, and flavour. If these faults are severe, the wine is rejected by consumers. Thus, wine spoilage constitutes an important concern to wine producers (Fleet, 2003; Loureiro and Malfeito-Ferreira, 2003).

During AF, unacceptable flavours can be produced by excessive concentrations of hydrogen sulfide and other sulphur volatiles, acetic acid, various esters and volatile phenols (Fleet, 2003). *Pichia anomala*, *Metschnikowia pulcherrima* and *Hanseniaspora uvarum* (teleomorph *Kloeckera apiculata*) are known for producing high levels of ethyl acetate and acetic acid before and during initial fermentation steps, leading to serious wine deterioration. Although these species are common winery contaminants, their activity is especially dangerous when associated with damaged berries, which encourage their growth, leading to high initial populations at the beginning of fermentation (Loureiro and Malfeito-Ferreira, 2003).

During bulk storage in tanks and barrels, the wine that is exposed to air, as in incompletely filled tanks or barrels, and which sulphite level is insufficient to prevent yeast growth, quickly develops a surface microbiota of weakly fermentative and oxidative yeasts. *Pichia membranifaciens*, *P. anomala* and *Candida* spp. are the usual species associated with this phenomenon. These species oxidize ethanol, glycerol, and acids, giving wines with unacceptably high levels of acetaldehyde, esters and acetic acid (Fleet, 2003; Loureiro and Malfeito-Ferreira, 2003).

Bulk wines, as well as bottled wines, can also be spoiled by fermentative yeast species of *Zygosaccharomyces*, *Brettanomyces*, *Saccharomyces,* and *Saccharomycodes*. These species cause excessive carbonation, sediments, and haze, and produce acid off-flavours (Fleet, 2003).

Despite the beneficial role of *S. cerevisiae* in the winemaking process, this yeast can resist to high ethanol concentrations, being able to spoil wine after fermentation. If it is not properly eliminated or controlled and residual sugars remain, or if the bottle is contaminated by yeast cells present in the wine bottling line, or on the cork, it can cause refermentation. This has been found mainly in sweet wines, where the fermentable sugar can support growth, and also in semidry bottled wines (Fleet, 1993; Fleet and Heard, 1993; Loureiro and Malfeito-Ferreira, 2003)

The two yeast species regarded as the most significant threats to wine are *Brettanomyces bruxellensis* and *Zygosaccharomyces bailii* (Loureiro and Malfeito-Ferreira, 2003; Thomas and Davenport, 1985; Wedral et al., 2010; Zuehlke et al., 2013). Despite the hostile environment of wine for most microorganisms given its high concentrations of ethanol, acid pH, the presence of SO_2, cool temperatures during cellar storage, as well as the lack of usable carbon sources, nutrients and oxygen, *B. bruxellensis* and *Z. bailii* are able to tolerate these conditions (Loureiro and Malfeito-Ferreira, 2003).

From the five individual species of *Brettanomyces* described (*Brettanomyces anomalus, B. bruxellensis, Brettanomyces custersianus, Brettanomyces naardensis,* and *Brettanomyces nanus*), the species primarily associated with winemaking is *B. bruxellensis (D. bruxellensis)* (Oelofse et al., 2008; Zuehlke et al., 2013) although, *B. anomalus* (*D. anomala*) and *B. custersianus* isolations from must fermentations have been reported in two instants (Oelofse et al., 2008). *B. bruxellensis* has been known for many time as the major cause of worldwide wine deterioration, with significant economic losses for the wine industry (Fugelsang and Edwards, 2007; Oelofse et al., 2008; Wedral et al., 2010). *B. bruxellensis* has an ovoid, ellipsoidal or cylindrical shape, its typical size varies between 2 to 7 µm in length, and reproduces by multilateral budding. Typical colony morphology has been described as matte to glossy, convex, and light to cream in colour (Zuehlke et al., 2013). Despite the distribution of *B. bruxellensis* includes the vineyard, grape musts, and bottle products, wine spoilage usually occurs during fermentation or, most probably in oak barrels. The yeast grows slowly so it usually imparts flavours only when the wine is aged (Wedral et al., 2010; Zuehlke et al., 2013). The main characteristics of oak barrels (new and old) that are beneficial to *Brettanomyces* growth are the porous microstructure, which allows the influx of small amounts of oxygen, and the

presence of cellobiose that can serve as sugar resource (Oelofse et al., 2008; Zuehlke et al., 2013). Metabolites produced by *B. bruxellensis* result in a unique sensory impact known as the "Brett character". The wide array of descriptive aromas include leather, clove, spice, smoky, animal, horse sweat, stable, and medicinal. These aromas are mainly due to produced volatile phenols, although acetic acid and some fatty acids are produced as well. Volatile phenols produced by *B. bruxellensis* include 4-ethylphenol and 4-ethylguaiacol, which are produced from ρ-coumaric and ferulic acid, respectively (Chatonnet et al., 1995; Oelofse et al., 2008; Wedral et al., 2010; Zuehlke et al., 2013). The impact on wine quality becomes obvious as the volatile phenol concentration exceeds the sensory threshold of 700 mg/L (Zuehlke et al., 2013). Red wines are particularly susceptible to *B. bruxellensis* infection due to their lower acidity, higher polyphenol content and barrel aging. The loss of viability in white wines is largely due to the efficacy of SO_2 at low pH. White wines do not seem to have Brett character due to the absence of precursor compounds (Wedral et al., 2010).

From the six individual species of *Zygosaccharomyces* (*Z. bailii*, *Zygosaccharomyces bisporous*, *Zygosaccharomyces kombuchaenis*, *Zygosaccharomyces lentus*, *Zygosaccharomyces mellis*, and *Zygosaccharomyces rouxii*), *Z. bailii*, *Z. bisporous*, and *Z. rouxii*, have been associated with the spoilage of grape must, grape juice concentrate, and wine. Nevertheless, *Z. bailii* is the most frequently isolated species (Zuehlke et al., 2013). *Zygosaccharomyces* has spherical, cylindrical, or ellipsoidal shape, its size varies between 3-9 µm wide and 3-13 µm long, and reproduces by multilateral budding. Typical colony morphology has been described as smooth, convex, and white to cream in colour (Zuehlke et al., 2013). *Z. bailii* spoilage activity manifest as carbon dioxide production within sealed containers of grape juice concentrate or sweetened wine, and the risks of infections are exacerbated if the room temperature is used for storage concentrate juice rather than cooler. An exceptional tolerance to osmotic stress, low pH, ethanol, and a variety of common preservatives and organic acids characterize the ability of *Z. bailii* to contaminate and spoil the wine. Besides carbon dioxide production, *Z. bailii* can produce a variety of sensorial compounds, such as acetic acid, fruity esters, acetoin, and higher order alcohols (Thomas and Davenport, 1985; Zuehlke et al., 2013).

1.2.2. Lactic Acid Bacteria

In general, LAB are widespread in habitats with a rich nutrition supply. They exist in decomposing plant material, fruits, dairy products, fermented meat and fish, beets, potatoes,

beverages, plants, water, and in human and animal cavities (König and Fröhlich, 2009). In wine industry, LAB are a constant concern to winemakers as they can impact the quality of wine positively, by conducting MLF, or negatively, by acting as spoilage organisms.

Taxonomy and physiology

LAB belong to the order *Lactobacillales*, class *Bacilli*, phylum *Firmicutes* and domain *Bacteria* (De Vos, 2011). From the six families belonging to the order *Lactobacillales,* only two (*Lactobacillaceae* and *Leuconostocaceae*) are typically associated with grape and wine. According to Euzéby (1997-2017), *Lactobacillaceae* and *Leuconostocaceae* families currently include 4 and 5 genera, respectively. *Lactobacillus* and *Pediococcus* from *Lactobacillaceae* family and *Leuconostoc*, *Oenococcus* and *Weissella* from *Leuconostocaceae* family, are the five genera commonly found in grape must and wine (König and Fröhlich, 2009).

Initially, the mode of fermentation in combination with physiological characteristics were used as classification criteria to allocate LAB to genera. More recently, with the application of modern taxonomic tools (especially molecular methods) to LAB identification and classification, some flaws in taxonomy based on physiology have been identified although, these attributes still remain very important for the classification of LAB (Liu et al., 2014).

Some of physiological and biochemical tests used as classification criteria to allocate LAB to genera are, the morphology, mode of sugar fermentation, growth at different temperatures, configuration of the lactic acid isomer from glucose, ability to grow at high salt concentrations, acid or alkaline tolerance and chemotaxonomic markers such as fatty acid composition and constituents of the cell wall (Axelsson, 2004). For grape and wine associated LAB genera, the differential physiological and biochemical characters are shown in **Table 1**.

LAB are Gram positive, non-spore forming**,** aero-tolerant, acid-tolerant, organotrophic, and strictly fermentative rods or cocci producing lactic acid as a major catabolic end product from glucose. Their size varies between 0.4-2 µm wide and 0.7-10 µm long, are usually non-motile, catalase negative, have G+C content below 55 mol%, and have requirements for complex growth factors such as vitamins, carbohydrates, minerals and amino acids (König and Fröhlich, 2009).

After morphological traits, sugar fermentation profile is the major factor considered in LAB classification. LAB metabolize sugars mainly by 3 different pathways: strict homofermentative, strict heterofermentative or facultative heterofermentative. The strictly homofermentative species, produce lactic acid as the sole end product of hexose fermentation by the Embden-Meyerhoff pathway. The facultative heterofermentative species, ferment both hexoses and pentoses. The hexoses are fermented from the same pathway than strictly homofermentative species, and the pentoses are fermented into lactic and acetic acid by heterofermentative pentose phosphate pathway. The strictly heterofermentative bacteria do not possess the fructose 1,6-diphosphate aldolase that is characteristic of the Embden-Meyerhoff pathway. They ferment glucose into CO_2, lactic and acetic acid, and ethanol by the pentose phosphate pathway, and pentose into lactic and acid bacteria in the same manner as facultative fermentative bacteria (Ribéreau-Gayon et al., 2006b).

Table 1- Differential characters of grape/wine-related lactic acid bacteria genera (adapted from Dicks and Endo, 2009; König and Fröhlich, 2009).

Character	*Lactobacillaceae*		*Leuconostocaceae*		
	Lactobacillus	*Pediococcus*	*Leuconostoc*	*Oenococcus*	*Weissella*
Morphology (Arrangement)	rods (single/pairs)	round cocci (pairs/tetrads)	spherical to short rods (pairs/chains)		spherical/rod (pairs/chains)
Optimal Growth temperature (°C)*	30-40	25-35	20-30	20-30	15
Carbohydrate fermentation	homo/hetero/ fac-hetero	homo/ fac-hetero	hetero	hetero	hetero
CO_2 from glucose	+/-	-	+	+	+
Lactic acid isomer from glucose	D/L/DL	DL/L	D	D	D/DL
Hydrolysis of arginine	+/-	+/-	-	+/-	+/-
Dextran from sucrose	+/-	+/-	+/-	-	+/-
G+C content (mol %)*	32-55	35-44	38-44	37-43	37-47

+, positive; -, negative; /, or; homo, strictly homofermentative; hetero, strictly heterofermentative; fac-hetero, facultative heterofermentative

*De Vos (2011)

Lactobacillus genus is the largest amongst all LAB and was first described by Beijerinck in 1901 (Liu et al., 2014). In the 1986 edition of *Bergey's Manual of Systematic Bacteriology*, 44

Lactobacillus species were listed (Dicks and Endo, 2009). Since then, the number of validly published species escalated to 224, with 29 subspecies (Euzéby, 1997-2017). Out of 253 described species/subspecies, at least 18 have been isolated from grapes, grape musts and wines (*Lactobacillus brevis*, *Lactobacillus bobalius*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus collinoides*, *Lactobacillus fermentum*, *Lactobacillus fructivorans*, *Lactobacillus hilgardii*, *Lactobacillus kunkeei*, *Lactobacillus lindneri*, *Lactobacillus mali*, *Lactobacillus nagelii*, *Lactobacillus oeni*, *Lactobacillus paracasei*, *Lactobacillus paraplantarum*, *L. plantarum*, *Lactobacillus uvarum* and *Lactobacillus vini* (du Toit et al., 2011).

Species of *Pediococcus* genus were among the first bacteria to be studied by Louis Pasteur in relation to their role in the spoilage of beer. This genus contains 15 species (Euzéby, 1997-2017). *Pediococcus damnosus, Pediococcus inopinatus, Pediococcus parvulus* and *Pediococcus pentosaceus* are the four species that have been found in grape must and wine (König and Fröhlich, 2009).

The LAB included in the '*Leuconostoc* group' were all classified as *Leuconostoc* species. However, in the early 1990s, molecular phylogenetic analyses led to a subdivision of the group into three distinct lineages: the genus *Leuconostoc sensu stricto*, the *Leuconostoc paramesenteroides* group, and the *Leuconostoc oenos* group. In the past 20 years, some *Leuconostoc* species have been reclassified or moved to other genera (Liu et al., 2014). Nowadays the genus *Leuconostoc* contains 24 species and 7 subspecies (Euzéby, 1997-2017). The species that have been isolated from must and wine are *Leuconostoc mesenteroides*, *Leuconostoc citreum* and *Leuconostoc fructosum* (König and Fröhlich, 2009).

Based on phylogenetic studies, in particular, 16S and 23S rRNA sequencing studies, Dicks et al. (1995) proposed the reclassification of the species *Leuconostoc oenos* as *O. oeni*, separating this species from genus *Leuconostoc* and consequently, creating a new genus, the *Oenococcus* genus. More recently, other two species have been described as *Oenococcus*. Therefore, three species belong to this genus, *O. oeni*, *Oenococcus kitaharae* (isolated from a composting distilled sochu residue) (Endo and Okada, 2006), and *Oenococcus alcoholitolerans* (isolated from cachaça and ethanol fermentation processes) (Badotti et al., 2014), but only *O. oeni* has been isolated during winemaking process (König and Fröhlich, 2009).

Based on 16S rRNA sequence analysis, Collins et al. (1993) proposed the description of a new genus *Weissella* for the *Lc. paramesenteroides* group. The *Lc. paramesenteroides* species was

reclassified as *Weissella paramesenteroides,* and also five heterofermentative lactobacilli were assigned to the genus *Weissella* (König and Fröhlich, 2009). This genus contains 22 validly described species (Euzéby, 1997-2017). *W. paramesenteroides* is the only species of this genus isolated from grape must/wine (König and Fröhlich, 2009)

Ecology of lactic acid bacteria in wine

LAB are present in all grape musts and wines. Their ability to multiply depends on the stage of the winemaking process and environmental conditions (Ribéreau-Gayon et al., 2006c). The evolution of LAB population in the production of wines that require MLF is shown in **Figure 2**.

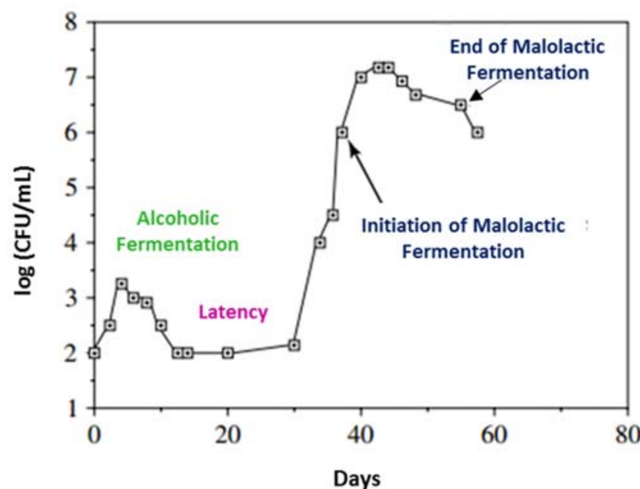

Figure 2-Evolution of lactic acid bacteria population during alcoholic and malolactic fermentation (adapted from Ribéreau-Gayon et al. (2006c)).

On undamaged grape berries, the bacterial population is rarely higher than 10^3 CFU/mL (Lafon-Lafourcade et al., 1983). Nonetheless, the extent of the population depends on maturity and sanity state of the grape. It is mainly correlated with pH; the higher the pH, the higher the total LAB population. The LAB species that can be identified on grapes are *L. plantarum*, *L. casei*, *L. hilgardii*, *L. brevis*, *L. fructivorans*, *Pd. damnosus*, *Pd. pentosaceus, Pd. parvulus*, *Lc. mesenteroides* and *O. oeni* (Lonvaud-Funel, 1999).

In grape must and during the first days of AF, the bacterial population generally increases to a maximum of 10^4 CFU/mL, and the major species of LAB in this stage include *L. plantarum*, *L. casei*, *Lc. mesenteroides*, as well as *O. oeni* to a lesser extent (du Toit et al., 2011). At the end of AF,

total LAB population decreases to around 10^2 CFU/mL. *Lactobacillus* species and *Lc. mesenteroides* progressively disappear or at least are at too low concentration to be detected, while *O. oeni* is the main species identified when AF fermentation is finished (Lonvaud-Funel, 1999). The LAB population decrease, and the natural selection of *O. oeni,* could be attributed to high ethanol concentrations, high SO_2 concentrations, low temperatures, the nutritional status, and competitive interactions with the yeast cultures. Besides these factors, the pH of wine is imperative in determining which species of LAB are present, with values above pH 3.5 favouring the growth of *Lactobacillus* and *Pediococcus* spp., whereas the *O. oeni* population tends to dominate at lower pH values (du Toit et al., 2011).

After the completion of AF, the surviving bacterial population can enter in a latent phase which is followed by the active growth phase. During the active growth phase, the population increases to 10^6 CFU/mL, and the MLF will commence when the total population exceeds 10^6 CFU/mL and sufficient biomass is achieved. During this stage, the temperature is essential in determining the LAB activity. When malic acid is degraded, wines are stabilized by sulfiting, eliminating viable bacteria and blocking microbial growth (Ribéreau- Gayon et al., 2000).

Beneficial role of lactic acid bacteria in wine

LAB have a very complex and diverse metabolism which can induce a range of wine compositional changes that may affect positively the quality of the final product.

The metabolic activities related to the degradation of organic acids, as malic and citric acid, play an important role in the reduction of wine acidity and contribute to its aroma and flavour.

The transformation of malic acid is the most important phenomenon of MLF, producing a biological deacidification of wine. This usually results in a total acidity reduction and an increase in pH. The degradation of malic acid also contributes to organoleptic changes. As the strong and sharp green taste of malic acid is replaced by the less aggressive and milder taste of lactic acid, an improved and softer mouthfeel can be expected (du Toit et al., 2011; Lonvaud-Funel, 1999; Ribéreau-Gayon et al., 2000a). Several wine LAB strains, belonging to the genera *Oenococcus*, *Leuconostoc*, *Lactobacillus,* and *Pediococcus* have the ability to decarboxylate L-malic acid into L-lactic acid and carbon dioxide. Nevertheless, *L. plantarum* and *O. oeni* are the most common species associated with MLF (du Toit et al., 2011).

The citric acid in wine can be degraded by all heterofermentative coco-bacilli (*Leuconostoc* and *Oenococcus*) and facultative lactobacilli (*L. plantarum* and *L. casei*). The most important metabolite of citric acid metabolism is diacetyl, which in wine is perceived as buttery, nutty and/or toasty. At concentrations below 4 mg/L diacetyl is responsible for these pleasant and distinct aromas, and can add complexity to wine (Bartowsky, 2009; du Toit et al., 2011).

Spoilage of wine by lactic acid bacteria

Despite the positive effect in wine, LAB can impact the quality of final product, acting as spoilage organisms. The nature and the extent of wine spoilage by LAB depend on several factors such as the bacterial species, the composition of the wine and vinification practice (du Toit and Pretorius, 2000).

LAB obtain their energy mainly from sugar fermentation, and consequently, the presence of several sugars during winemaking process can be a source of spoilage, particularly due to bacterial growth (Bartowsky and Pretorius, 2009).

When the end of AF is too slow or when it stops, the high sugar concentration, the low pH, and nitrogen deficiency, together with the excretion of toxic yeast metabolites, make the yeast activity very difficult. Under these conditions, bacteria, mainly heterofermentative species, ferment carbohydrates, particularly hexoses, which have not been totally fermented by yeasts, and produce lactic acid and acetic acid, increasing the volatile acidity of the wine. This accident is called “piqûre lactique” or lactic disease (Lonvaud-Funel, 1999).

Fructose metabolism by heterofermentative LAB can result in the formation of mannitol. Mannitol tainted wine (also known as “mannite” disease) is complex and it is usually also accompanied by high acetic acid, D-lactic acid, propanol, butanol, and diacetyl. Such spoiled wine can also be perceived as having a slimy texture with vinegar-estery aroma and slightly sweet taste (Bartowsky, 2009; du Toit and Pretorius, 2000).

Wine spoilage with an increase in viscosity and a thick appearance are called "ropy". Viscosity is attributed to the presence of excess exopolysaccharides such as β-D-glucan, produced from residual sugars by strains of *Pd. damnosus* (Bartowsky and Pretorius, 2009; Bartowsky, 2009; Lonvaud-Funel, 1999). The spoilage may occur when the wine is still in vats. Generally, the wine does not have other defaults except if other microorganisms, yeast or bacteria, are also present.

However, in most cases, the ropiness develops very slowly and becomes evident several weeks or months after bottling (Lonvaud-Funel, 1999).

Wine LAB are able to use glycerol as a carbon source to maintain viability after AF. The degradation of glycerol by certain bacterial strains, mainly *Lactobacillus* sp., harms wine quality producing bitterness in wine. The bitter taste is thought to result from the reaction of red wine phenolics with acrolein. This type of wine spoilage is often referred to as “amertume” (Bartowsky, 2009; du Toit et al., 2011).

Organic acids in wine, primarily citric, tartaric and sorbic acid (when added), can be metabolized by certain LAB to affect the wine quality to a degree that the wine is considered spoiled.

Diacetyl is an intermediate compound of citric acid metabolism and can add pleasant buttery aromas to wine. However, the increase in diacetyl above the threshold value (> 4 mg/L) contributes negatively to the complexity of the wine, giving an undesirable rancid butter-like flavour (Bartowsky, 2009). Also, acetic acid can be produced by citric acid metabolism and contributes to the sensory perception of volatile acidity in wine (du Toit et al., 2011).

Tartaric acid determines the acidity of wine and thus affects characteristics such as odour, colour and flavour. Some wine LAB species are able to metabolize tartaric acid, producing acetic acid, lactic acid, and succinic acid, which might decrease fixed acidity and increase the volatile acidity, giving rise to an unacceptable wine. The degradation can be total or partial, depending on the level of bacterial development, but it always lowers wine quality. This spoilage is named “tourne*”* disease (Ribéreau-Gayon et al., 2000a).

Sorbic acid is widely used as a chemical preservative against yeast in sweetened wine. Certain LAB species, including *O. oeni*, are able to metabolize sorbic acid resulting in a wine defect known as "geranium tone", an off-odour typical of crushed geranium leaves. Acid sorbic is reduced to sorbic alcohol (sorbinol), which then spontaneously reacts with ethanol to form 2-ethoxyhexa-3,5-diene, the compound responsible for the geranium-like odour (du Toit et al., 2011; du Toit and Pretorius, 2000).

Amino acids are important compounds for the growth of LAB, both as a nitrogen, carbon and sulphur source (du Toit et al., 2011).

The metabolism lysine and ornithine amino acids can lead to the formation of several extremely potent and unpleasant nitrogen-heterocycle “mousy” off-flavour compounds. These

compounds are perceived on the back palate as a persistent aftertaste reminiscent of caged mice, and its production appears to be limited to the heterofermentative LAB (*O. oeni*, and some species of *Lactobacillus* and *Pediococcus*) (Bartowsky and Pretorius, 2009; Bartowsky, 2009).

Two groups of substances possibly released in wine, by LAB amino acid metabolism, are undesirable with respect to the healthy and hygienic quality of products: biogenic amines and ethyl carbamate. They are produced during and after fermentation. Some are present in low amounts in grape must (Liu, 2002; Lonvaud-Funel, 1999).

Biogenic amines are produced by the decarboxylation of some amino acid and can cause health problems and sensory defects in wine (König and Fröhlich, 2009). If too high biogenic amine concentrations are ingested, or if the detoxication process is inhibited in humans either by drugs or genetically, possible toxicological effects may occur (Lonvaud-Funel, 1999). In wine, it appears that the lactobacilli and pediococci are the main producers of biogenic amines, although oenococci can also produce them (Liu, 2002).

Ethyl carbamate is known as a possible carcinogen. It is formed through the chemical reaction of ethanol and an ethyl carbamate precursor, such as citrulline and carbamyl phosphate (produced from arginine breakdown by LAB) or urea (produced from arginine breakdown by yeasts) (Mira de Orduña et al., 2000). Heterofermentative LAB have been shown to be active arginine metabolizers, especially *L. hilgardii*. *L. plantarum* has been shown to degrade arginine and this ability has also been identified in *O. oeni* (König and Fröhlich, 2009; Ribéreau-Gayon et al., 2000a).

1.2.3. Acetic Acid Bacteria

AAB are widespread in nature and are commonly found in alcohol and sugar-rich environments as vinegar, wine, beer, cocoa, fruits, flowers and honey (Guillamón and Mas, 2005). They are important microorganisms in food and beverage industries because of their ability to oxidize many types of sugars and alcohols to organic acids as end products during fermentation processes. In wine industry, this capability constitutes spoilage (Fleet, 1993; Sengun and Karabiyikli, 2011).

Taxonomy and physiology

AAB belong to the family *Acetobacteraceae*, order *Rhodospirillales*, class *Alphaproteobacteria,* phylum *Proteobacteria* and domain *Bacteria* (Brenner et al., 2005).

The taxonomy of AAB has undergone many changes the last 30-40 years, and it has not been fully established yet. The reasons for this taxonomic uncertainty are both due to the limited knowledge of the AAB phylogenesis and isolation, identification and preservation difficulties of these bacterial organisms (Sengun and Karabiyikli, 2011). The early classification systems for AAB were based on morphological and biochemical characteristics. Recently, in accordance with the development, and application of new molecular technologies, as DNA-DNA hybridization and analysis of 16S rRNA genes, the AAB are classified as the consensus results of a polyphasic analysis, combining phenotypic, chemotaxonomic and genotypic data (Cleenwerck and De Vos, 2008).

According to Euzéby (1997-2017), this family currently includes 36 genera, of which three, *Acetobacter*, *Gluconobacter,* and *Gluconacetobacter* are the principal genera typically associated with grape and wine. Based mainly on morphological and biochemical characteristics, the genera *Acetobacter* and *Gluconobacter* were described by Beijerink in 1898 and by Asai in 1935 respectively (Cleenwerck and De Vos, 2008), and for many years were considered the only genera belonging to the family *Acetobacteraceae*. Later, on the basis of partial 16S rRNA sequences and chemotaxonomic comparisons of ubiquinone systems, Yamada et al. (1997) proposed the genus *Gluconacetobacter*. As a result, several species of *Acetobacter* genus were allocated in the genus *Gluconacetobacter* (Cleenwerck and De Vos, 2008). Recently, a new genus was isolated from the first time in grape musts, the genus *Kozakia* (Mañes-Lázaro, 2010). This genus was proposed by Lisdiyanti et al. (2002) for four bacterial strains isolated from palm brown sugar and ragi collected in Bali and Yogyakarta, Indonesia.

AAB are Gram-negative, catalase positive, oxidase negative, non-spore forming, ellipsoidal to rod-shaped cells that can occur singles, in pairs or chains. Its size varies between 0.4-1 µm wide and 0.8-4.5 µm long. The optimum pH for the growth of AAB is 5-6.5 while they can grow at lower pH values between 3 and 4. Their metabolism is strictly aerobic (Sievers and Swings, 2005).

Acid production from ethanol and sugars, oxidation of acetate and lactate to CO_2 and H_2O, formation of 2-keto-D-gluconic and 5-keto-D-gluconic acid from D-glucose, ketogenesis from glycerol, growth on carbon sources, formation of water-soluble brown pigments, flagellation, and

motility are some of physiological and biochemical tests used to differentiate the AAB genera (Sievers and Swings, 2005). For grape and wine associated AAB genera, the differential physiological and biochemical characters are shown in **Table 2**.

Table 2- Differential characters of grape/wine-related acetic acid bacteria genera (adapted from Bartowsky and Henschke, 2008; Cleenwerck and De Vos, 2008).

Character	*Acetobacter*	*Gluconacetobacter*	*Gluconobacter*	*Kozakia*
Oxidation of:				
Ethanol to acetic acid	+	+	+	+
Acetic acid to CO_2 and H_2O	+	+	-	w
Lactate to CO_2 and H_2O	+	+/-	-	w
Growth in the presence of:				
0.35 % acetic acid (pH 3.5)	+	+	+	+
30 % glucose	-	+/-	-/w	-
Acid production from:				
D-arabinose	-	-	+	+/–
D-glucose	+/-	+	+	+
D-fructose	-	+	+	-
D-galactose	+/-	+	+	+
D-mannitol	-	+/-	+	-
Raffinose	-	-	-	+
Glycerol	+/-	+	+	+
Ethanol	+	+	+	+
Production of water-soluble brown pigments	-	+/-	+/-	-
Motility and Flagellation	Peritrichous /non-motile	Peritrichous/ non-motile	Polar/ non-motile	Non-motile
Ubiquinone Type	Q9	Q10	Q10	Q10
G+C content (mol %)	52-64	56-67	54-64	56-57

+, positive; -, negative; w, weak positive; /, or

The major distinguishing feature between *Acetobacter* and *Gluconobacter* is the ability to oxidize ethanol to CO_2 via acetic acid. The *Acetobacter* species are able to oxidize ethanol to acetic acid and then to carbon dioxide and water, whereas *Gluconobacter* species do not have a complete citric acid cycle and cannot oxidize ethanol further than acetic acid (Bartowsky and Henschke, 2008).

Acetobacter aceti, *Acetobacter pasteurianus,* and *Gluconobacter oxydans* are the species most frequently found in grape must and wine (Ribéreau-Gayon et al., 2006a). Although, *Gluconacetobacter hansenii* and *Gluconacetobacter liquefaciens* can also be isolated during winemaking (Du Toit and Lambrechts, 2002; González et al., 2004). More recently, other species of *Acetobacter* as *Acetobacter oeni* (Silva et al., 2006), *Acetobacter tropicalis* (Silhavy and Mandl, 2006), *Acetobacter ghanensis*, *Acetobacter syzyygii* (Mañes-Lázaro, 2010), *Acetobacter musti* (Ferrer et al., 2016) and species of *Kozakia* as *Kozakia baliensis* (Mañes-Lázaro, 2010) have been isolated from grape must or wine.

Ecology of acetic acid bacteria in wine

AAB can be present in several stages of the winemaking process, including grape maturation in the vineyard, must preparation, AF, MLF, the ageing phase and packaging (Bartowsky and Henschke, 2008). The factors affecting the development of AAB during the winemaking process are the pH of grape must and wine (5-6.5 are the optimum), the temperature (25-30 °C are the optimum), the SO_2 and ethanol concentration, and the dissolved oxygen in the media (Drysdale and Fleet, 1988; Ribéreau-Gayon et al., 2006a).

On ripe grape, the AAB population varies greatly according to grape health. On healthy grapes, the population is typically small (10^2-10^3 CFU/mL) and it is almost entirely made up of *G. oxydans*. On damaged or infected grapes with fungal diseases as *Botrytis cinerea*, the population can reach upwards of 10^5-10^6 CFU/mL and are mixed, comprising varying proportions of *G. oxydans*, *A. aceti* and *A. pasteurianus* (Guillamón and Mas, 2005).

Grape must and wine are not conducive environments for the growth of bacterial species. Even though, grape must can be a rich, nutritious growth medium. High concentrations of hexoses (glucose and fructose), high acidity, and sulfites provide a highly selective environment for AAB that can limit the growth of *Gluconobacter* species primarily, especially *G. oxydans*. *G. oxydans*, metabolise the small concentrations of ethanol produced by the indigenous yeast during grape must preparation, and during the early stages of AF as a carbon source. During AF, the population of AAB tends to decrease and can fall below 10^2 CFU/mL by the end of AF. This decrease is due to very harsh environment of wine created by high ethanol concentration, low oxygen content and redox potential, low pH and depletion of nutrients, resulting from consumption by yeasts, and restricts

growth to principally AAB genera *Acetobacter* and *Gluconacetobacter* (Bartowsky and Henschke, 2008; Drysdale and Fleet, 1988; Joyeux et al., 1984).

After fermentation, when the wine is transferred from fermentation tanks to other storage vessels, agitation and aeration processes may introduce sufficient oxygen to stimulate the growth of surviving AAB populations from 10^2 to 10^5 CFU/mL predominating *Acetobacter* species (Drysdale and Fleet, 1988; Joyeux et al., 1984; Sengun and Karabiyikli, 2011).

During maturation and storage wine in tank, barrel or bottles, oxygens is either deliberately excluded or carefully controlled, and only when this control fails AAB can proliferate, though, they might remain viable for extended periods in the absence of oxygen (Bartowsky and Henschke, 2008; Joyeux et al., 1984).

Spoilage of wine by acetic acid bacteria

In wine, AAB do not take part in the desirable microbiota, but are rather considered spoilage organisms, being mainly responsible for producing acetic acid and other detrimental metabolites as acetaldehyde and ethyl acetate (Drysdale and Fleet, 1988; Dupuy and Maugenet, 1963; Sponholz and Dittrich, 1984). It has also been reported that excessive growth of AAB on grape berries or in musts can lead incomplete AF, which is caused by an AAB/yeast interaction (Drysdale and Fleet, 1989).

AAB produce acetic acid through the metabolism of ethanol to acetaldehyde by alcohol dehydrogenase and then acetic acid is produced by acetaldehyde dehydrogenase (Bartowsky and Henschke, 2008). Acetic acid is the major volatile acid in wine and is responsible for the generation of pricked wine that is one of the main reasons of wine spoilage. Acetic acid is recognized in wine sensory as contributing with a sour flavour and in a high concentration, a sour flavour with a vinegar-like aroma. Other microorganisms in wine, such as yeasts and LAB can produce acetic acid, nevertheless significant population of AAB is the most known producer of this acid in wine. The intermediate metabolite, acetaldehyde, can also contribute to the sensory spoilage of wine with distinct aroma description; sherry-like, bruised apple or nutty. The ethyl acetate has a pungent, solvent-like aroma, reminiscent of glue or nail polish remover (Guillamón and Mas, 2005).

Many of these aroma characteristics are immediately obvious upon opening a bottle of wine spoiled by ABB, especially the volatile vinegar-like character due to the increase in acetic acid and

ethyl acetate. These wines are often considered to be oxidized with a decrease in fruity aroma (Bartowsky and Henschke, 2008).

Acetaldehyde, and another metabolites as gluconic acid/5-oxofructose and dihydroxyacetone that result from sugars and glycerol oxidation, respectively, have the capacity to bind SO_2. This SO_2 binding reduces the proportion of free SO_2 and therefore its antimicrobial and antioxidant capacity (Guillamón and Mas, 2005).

In addition to the organoleptic changes caused by the production of undesirable metabolites, AAB can also cause physical alterations of the wines by polysaccharides production. The main consequences of the production of these polysaccharides during winemaking are the difficulties it generates in wine filtration (Guillamón and Mas, 2005).

1.3. Detection and Quantification of Microorganisms in Wine by Direct Methods

In order to promote and guide yeast during AF, to verify the growth of the bacteria during MLF, and to obtain better quality wines without economic loss caused by spoilage microorganisms, it is very important to monitor microbial presence during the winemaking process, which implies detecting, identifying and enumerating the responsible microorganisms.

Traditional methods, such as microscopic and plate counting are currently used to monitor the growth of wine microbial populations during fermentation, with phenotypic methods being used for the identification of microbes. Microscopic counting techniques are the most rapid but require at least 10^4 cells/mL (Fugelsang and Edwards, 2007). Plating procedures can quantify lower cell concentrations but are time-consuming as colonies for some wine-related microbes take up to a week or more to appear on a Petri dish. Besides that, cells that are stressed and injured are often not able to grow on synthetic media that contain agents, such as antibiotics, to make them selective towards a specific microorganism, and this can lead to false-negative results. Finally, populations that are numerically less important are not detected by means of platting methods, because they are masked on the plates (Amann et al., 1995; Bokulich, Bamforth, et al., 2012). The classical

phenotypic characteristics of microbes comprise morphological, physiological, and biochemical features (Fugelsang and Edwards, 2007; Vandamme et al., 1996). Nevertheless, these methods show unfortunate limitations, as certain microbes have the capability of altering their phenotypic characteristics due to environmental changes or genetic mutations. Additional weaknesses of phenotypic methods include poor reproducibility, ambiguity of some techniques, extensive logistics for large-scale investigations, and poor discriminatory power (Temmerman et al., 2004).

Although traditional assays have proven to be useful in quality control, they still cannot meet all the demands of the food industry because of their intrinsic limitations (Hanna et al., 2005). For this reason, more sensitive and rapid methods are desired.

The advent of molecular biology brought with it a number of tools to replace traditional methods. Compared to traditional methods, molecular methods are generally faster, more specific, more sensitive and more accurate, allowing a precise study of the microbial populations and their diversity (Ivey and Phister, 2011; Juste et al., 2008). In wine industry, the molecular biology revolution has brought forth significant new advances with application in microbiological analysis during wine production and storage (Mills et al., 2008).

Molecular methods can be used to detect and quantify microorganisms throughout the winemaking process in one of two ways (**Figure 3**).

In a first way, molecular methods are used to identify the organisms that were previously analysed using conventional methods such as plating, which require growth of the microbe. These methods are considered indirect or culture-dependent methods since the analysis of the microbial population is no conducted on the original population.

In a second way, molecular methods are used to analyse the microbial population directly from the sample itself, without the need to isolate and culture the microorganisms present in the sample. These methods are considered direct or culture-independent methods (Ivey and Phister, 2011).

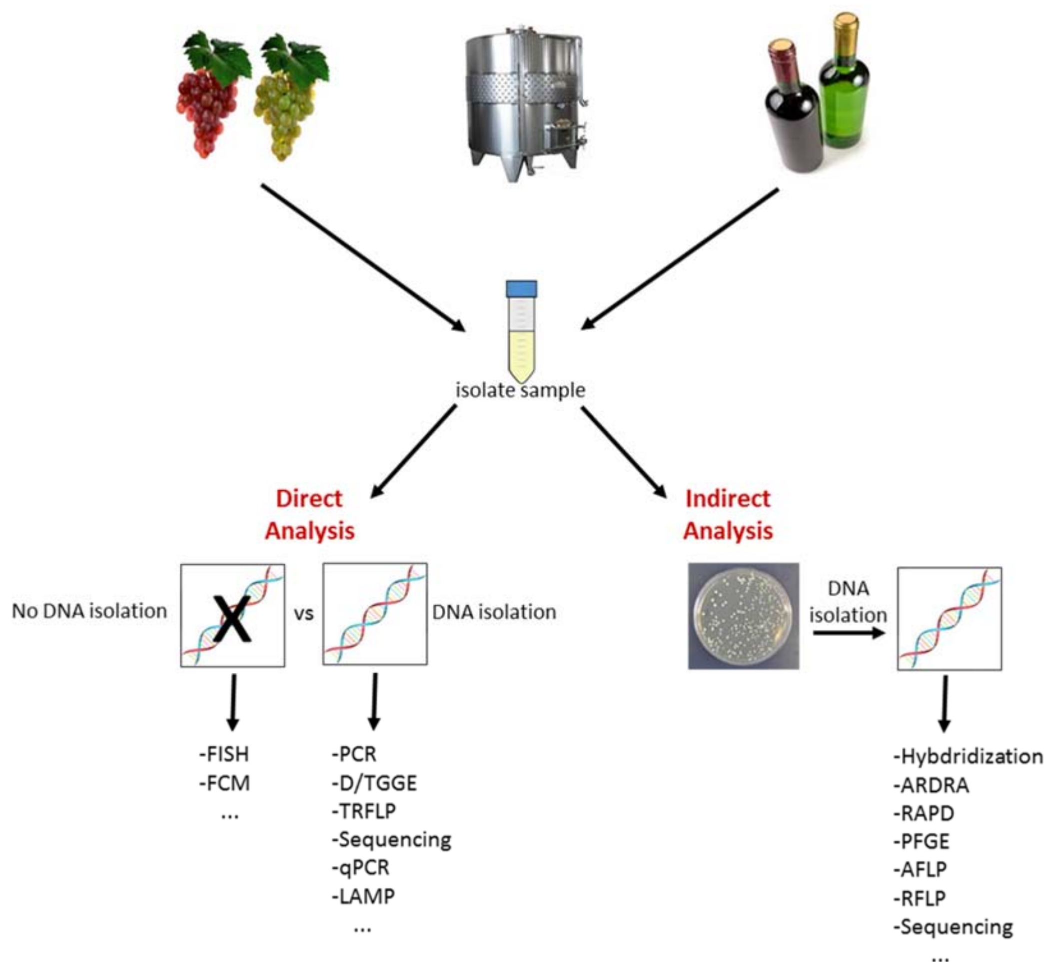

Figure 3- Direct versus indirect methods for microbial population analysis in wine microbiology. **FISH**, Fluorescence *In Situ* Hybridization; **FCM**, Flow Cytometry; **PCR**, Polymerase Chain Reaction; **D/TGGE**, Denaturing/Temperature Gradient Gel Electrophoresis; **TRFLP**, Terminal Restriction Fragment Length Polymorphism **qPCR**, Quantitative PCR; **LAMP**, Loop-Mediated Isothermal Amplification; **ARDRA**, Amplified Ribosomal DNA Restriction Analysis; **RAPD,** Random Amplified Polymorphic DNA; **PFGE**, Pulse-Field Gel Electrophoresis; **AFLP**, Amplified Fragment Length Polymorphism; **RFLP**, Restriction Fragment Length Polymorphism of mitochondrial DNA (adapted from Ivey and Phister (2011)).

In general, direct methods have two major advantages over traditional and indirect methods. First, they can identify nonculturable microbes that are common in wine. These nonculturable microorganisms may be injured, in a viable but nonculturable state (VBNC), or unable to grow in or on the medium chosen for culturing. Nonculturable cells may be injured by the wine-related chemicals such as sorbate, sulfite or ethanol, thus losing the ability to grow on standard culture media. Alternatively, VBNC state is associated with a microbial response to adverse

environmental conditions such as starvations, acid stress, or the presence of antimicrobials. In this case, the cells are metabolically active but are incapable of undergoing the cellular division required for growth in or on a medium. Despite this, in either case, injured or VBNC organisms may still play a role in wine. Regarding the microorganisms unable to grow in or on the medium chosen for culturing, is due to that the real conditions under which most species actually grow in their natural habitat are not always clear. This makes it very difficult to develop universal media for cultivating all species, biasing the obtained results. The second advantage of direct methods is the speed since the microorganisms can be identified in less than 1 day (Ivey and Phister, 2011; Mills et al., 2008).

In the last two decades, direct molecular approaches underwent considerable development in microbial ecology (Postollec et al., 2011). The microorganisms are studied based on DNA, RNA, and proteins, which are the preferred targets for such methods (Cocolin et al., 2013). There are a lot of direct methods and variants, and each one varies in appropriateness, depending upon its speed, cost, sensitivity, and whether they are directed toward community profiling or targeted analysis (Bokulich, Bamforth, et al., 2012). In wine fermentation research and industry, several direct methods including Fluorescence *In Situ* Hybridization (FISH), Flow Cytometry (FCM), Polymerase Chain Reaction (PCR), Denaturing/Temperature Gradient Gel Electrophoresis (D/TGGE), Terminal Restriction Fragment Length Polymorphism (TRFLP), Sequencing, Quantitative PCR (qPCR) and Loop-Mediated Isothermal Amplification (LAMP), have been widely adopted for detect, identify and, in some cases, quantify yeasts and bacteria. FISH and FCM are considered DNA amplification-independent methods. Nevertheless, PCR, D/TGGE, TRFLP, Sequencing, qPCR, and LAMP are considered DNA amplification-dependent methods.

1.3.1. DNA amplification-independent methods

Fluorescence *In Situ* Hybridization

FISH is a targeted analysis technique that employs fluorescently labelled probes typically targeting specific rRNA sequences corresponding to different organisms within a sample. Target cells are immobilized on a microscope glass and then subjected to a permeabilization step in order to allow the probe to penetrate into the cell. These probes are labelled with different fluorophores to allow simultaneous detection of multiple organisms in a mixed population sample. Visualization

is performed with a fluorescence microscope, allowing direct observation of target populations (Bokulich, Bamforth, et al., 2012; Cocolin et al., 2013; Fröhlich et al., 2009).

FISH has been used for rapid identification and quantification of yeasts and LAB species from wine (Blasco et al., 2003; Röder et al., 2007; Xufre et al., 2006).

While accurate and rapid, the use of FISH for the direct detection of microorganisms from wine is not common in the industry. It requires an expensive fluorescent microscope and has the limits of any microscopy-based method. Typically microbes present at levels below 10^3 CFU/mL will not be observed (Ivey and Phister, 2011).

Flow Cytometry

FCM is a quantitative analysis technology which characterizes cell populations at single cell level as cells are illuminated by a laser beam. The intensity of the optical signals generated, which are scattering and/or fluorescence signals (in the case of using fluorescent dyes), is finally correlated to structural and/or functional cell parameters. Scattering signals are related to intrinsic cell parameters (size, shape) since they are obtained without having to stain the sample. The use of fluorochromes in single or multiple analysis supplies additional information about cell structure or functionality. Fluorochromes can also be conjugated to antibodies or oligonucleotides to detect microbial antigens or DNA and RNA sequences directly (Davey, 2002; Díaz et al., 2010).

FISH can be paired with FCM (FISH-FCM) to automatically quantify cell abundances based on sum fluorescence detection as labelled cells pass through the cytometer. The advantage of this technique is that FISH nucleic acid probes are easily designed and less expensive than antibodies or other FCM probes (Bokulich, Bamforth, et al., 2012; Díaz et al., 2010).

FCM has been used in the wine industry to simultaneous detection of *O. oeni* and *S. cerevisiae* (Rodriguez and Thornton, 2008), to detect total yeasts (Bruetschy et al., 1994), to assess the membrane activity of ethanol-stressed *O. oeni* (Silveira et al., 2002), among other applications. Regarding FISH-FCM, it has been in applied to detect and quantify spoilage organisms in wine (Serpaggi et al., 2010), and yeasts in grape must and wine (Lucio et al., 2013).

FCM is a powerful PCR direct method to study microbial suspensions. In biotechnology, the power of this method lies both in the possibility of determining a wide range of cell parameters at

single cell level, and in the ability to obtain information about their distribution within cell populations. Since measurements rates of 1000 to 25000 cells/second can be achieved (depending on the instrument), flow cytometric data sets often represent 10000 to 500000 cells for a given population. This leads to the relatively straight-forward acquisition of statically significant results (Davey, 2002). However, this method has disadvantages, as the high cost of the equipment, the lack of specific knowledge in microbial cell labelling, and the management of the instrumental data obtained, limiting their use in wineries (Bokulich, Bamforth, et al., 2012; Díaz et al., 2010).

1.3.2. DNA amplification-dependent methods

Polymerase Chain Reaction

PCR radically transformed biological science from the time it was discovered by Mullis (1990), who explained that it "lets you pick the piece of DNA you're interested in and have as much of it as you want". PCR uses a thermostable DNA polymerase (Taq polymerase) to amplify genomic sequences of living organisms by *in vitro* DNA synthesis (Mullis, 1990). For that, a pair of oligonucleotide primers complementing to sequences at both ends of the target gene, DNA from the target organism to act as a template, free nucleotides, salts, and DNA polymerase are combined in a reaction mixture, resulting in the replication of the target DNA fragment. PCR has been used to detect target populations. The target gene for the assay can be similar across class or genus or can differ greatly among species, allowing the detection of PCR products at these different levels (Hanna et al., 2005).

Conventional PCR has been developed to identify specific species and genes of wine-related microorganisms (Bartowsky and Henschke, 1999; Zapparoli et al., 1998). Besides conventional PCR methods, other endpoint PCR approaches, as multiplex PCR and two-step PCR (nested-PCR) (González et al., 2006; Ibeas et al., 1996; López et al., 2003; Marcobal et al., 2005), have been developed for several wine yeasts and bacteria. In a multiplex PCR, more than one target sequence can be amplified by using multiple primers in a reaction mixture, saving time and efforts in the laboratory. Nested PCR consists of a double amplification of a DNA template, which uses the amplicon obtained in the first amplification as the second amplification template. In this way, the sensitivity of the analysis is improved.

The two major limitations of using PCR for detection are that it is qualitative, not quantitative (it only provides presence or absence information) and it may not differentiate between viable and nonviable microorganisms (Ivey and Phister, 2011).

Denaturing/Temperature Gradient Gel Electrophoresis

D/TGGE are direct methods for community profiling directly from the environment. These approaches are attractive as they enable detection of individual species, as well as, the overall profiling of community structure changes with time. DGGE is based on separation of DNA fragments of the same length (previously amplified by PCR), but with different base-pair sequences, by polyacrylamide gels containing a linearly increasing gradient of denaturants such as urea and formaldehyde. Double-stranded DNA (dsDNA) will partially separate, arresting electrophoretic mobility once it reaches the point along the chemical gradient at which the least stable section of the DNA becomes denatured. Thus, fragments differing in just a single nucleotide will stop migrating at different concentrations of denaturant. For identification, the separated fragments can be excised for further analysis, often DNA sequencing, or compared to a DNA ladder made of PCR products from known organisms. TGGE method operates under the same principle but uses a temperature gradient increasing with gel depth to separate sequences based on heat denaturation (Bokulich, Bamforth, et al., 2012; Ivey and Phister, 2011).

DGEE and TGEE have been applied to detect and identify of individual species, as well as to monitor the succession of the microbiota during fermentation, both for yeasts and LAB wine-related species (Andorrà et al., 2008; Cocolin et al., 2000; Di Maro et al., 2007; Mills et al., 2002).

These methods became popular for community profiling in wine, however, they are poorly adapted to industrial applications, since many times band extraction or sequencing is necessary, increasing time, labour, and expense. Moreover, some applications of these methods revealed a detection threshold of 10^3 cells/mL and, therefore, cannot detect populations present in low concentrations (Bokulich, Bamforth, et al., 2012).

Terminal Restriction Fragment Length Polymorphism

TRFLP is a high-throughput technique originally developed for assessing the diversity of complex bacterial communities, and for rapidly comparing the community structure and diversity of different ecosystems. (Liu et al., 1997). With this method, a mixed-sample DNA template is amplified by one or more fluorescently labelled primers (5´end), typically universal primers targeting prokaryotic 16S rRNA (Bokulich, Bamforth, et al., 2012), or fungi rRNA internal transcribed spacer (ITS) (Bokulich, Hwang, et al., 2012). The amplified amplicons are then digested by one or more restriction enzymes in separate reactions and separated by capillary electrophoresis, enabling size approximation of fluorescently labelled "terminal" fragments via comparison to a fluorescent molecular marker (usually labelled with a different fluorophore dye to distinguish it from sample peaks). Variation in the presence and location of cutting sites results in different species having terminal fragments of different lengths. Fragment sizes are analysed, true peaks are filtered from noise, and these are compared with a database of target sequences digested by each enzyme (Bokulich, Bamforth, et al., 2012).

TRFLP has been applied to characterize the yeast ecology of sweet, botrytized wine fermentations (Bokulich, Hwang, et al., 2012), and for detection and differentiation of species of LAB and Bacilli in wine and beer (Bokulich and Mills, 2012).

This method can be considered a compromise between speed and specificity of data, making it an attractive option for the analysis of mixed microbial systems with low to moderate diversity. Nevertheless, some microorganisms present in a given sample may not be represented in the database consulted and thus cannot be identified, and some minor organisms may be entirely undetected. Additionally, because electrophoretic mobility is a function of molecular weight, charge, and sequence structure, molecular weight disparity between the different fluorophores used to mark the TRF amplicon size standards and TRF sequences variability can alter the perceived size of TRFs. Finally, it would not be suited for diagnostic testing of pure-culture fermentation, unless spoilage by multiple organisms is expected (Bokulich, Bamforth, et al., 2012).

Sequencing

Illumina and 454 Life Sciences platform sequencing technologies, originally designed for massively parallel genomic sequencing, have been recognized for their utility in profiling complex

microbial systems. The Illumina system, a sequencing-by-synthesis method relying on detection of fluorescent, reversible terminator nucleotides incorporated in growing sequence. The 454 Life Sciences pyrosequencing relies on nuclease base incorporation detection via pyrophosphate release and luciferase activity, facilitate simultaneous sequencing and enumeration of ever amplified transcript in a mixed sample. Thus, generating multimillion sequence libraries from variable regions amplicons enables characterization of the complete microbiome in a given sample. Comparing with DGGE, these two methods provide much more sensitivity and relative quantification (Bokulich, Bamforth, et al., 2012).

Pyrosequencing has been commercially available for longer thus, has been more extensively used for microbial ecology than Illumina sequencing. Nevertheless, this tool was not been used to study beverage fermentation systems. In this field, pyrosequencing is being rapidly overtaken by Illumina given the advantages in cost and sequence coverage (Belda et al., 2017; Bokulich, Bamforth, et al., 2012).

One of the biggest limitation of these methods relays on its inability to address a functional characterization of the microbial communities. In wine, for example, there are many desired microbial functions, mainly related to AF and MLF, and diversity of genes related to those functions may influence winemaking more than just taxonomic diversity. Alternatively, the metagenomics approach, sequencing would also reveal functional genes in addition to rRNA genes, allowing more comprehensive genomic and functional representation through whole-genome sequencing of complete communities. Similarly, the metatranscriptomics approach, by massively parallel sequencing of cDNA transcripts from sum RNA in a sample, revealing a powerful technology for the functional characterization of microbial communities that can reveal both the taxonomic composition and active biochemical functions of the detected microorganisms (Belda et al., 2017; Bokulich, Bamforth, et al., 2012).

Authors like Bokulich, Bamforth, et al. (2012), Bokulich et al. (2016), and David et al. (2014) applied sequencing methods for wine-related microorganisms profiling.

Despite powerful, these methods are still expensive when compared with other community profiling techniques, and for this reason are not suited for routine industrial application (Belda et al., 2017; Bokulich, Bamforth, et al., 2012).

Quantitative PCR

qPCR also called real-time PCR, is a targeted analysis technique for accurately quantifying specific microbial taxa in a given sample based on the number of target sequences (Postollec et al., 2011). This method relies on the basic principles of traditional PCR but, the amount of DNA is measured in real time (after each cycle) via fluorescent dyes that yield increasing fluorescent signal in direct proportion to the number of amplicons generated (Bokulich, Bamforth, et al., 2012; Ivey and Phister, 2011). Computer software records and displays the amount of fluorescence in relative fluorescence units (RFU). Data is collected in the exponential phase of the reaction yield quantitative information on the starting quantity of the amplification target. For this, a baseline threshold, above which sample fluorescence is distinguished from background noise (baseline fluorescence), is set by logarithmically plotting mean RFU against cycle number, and used to determine the cycle threshold (Ct) at which sample crosses the baseline threshold (**Figure 4**). Ct is used to normalize the relative quantities of DNA among samples because Ct reports the number of cycles required to overcome the baseline threshold and is inversely related to original template sequence copy number. Therefore, as the template amount decreases, the cycle number at which significant amplification is seen increases. Sample sequence quantity is calculated by interpolation against a known standard curve, generated from serial dilutions of a known amount of the target DNA (Bokulich, Bamforth, et al., 2012; Hanna et al., 2005; Postollec et al., 2011). The standard curve is also used to assess the performance of qPCR assay by estimating its efficiency and optionally also determining the assay dynamic range, limit of detection (LoD) and limit of quantification (LoQ) (Svec et al., 2015).

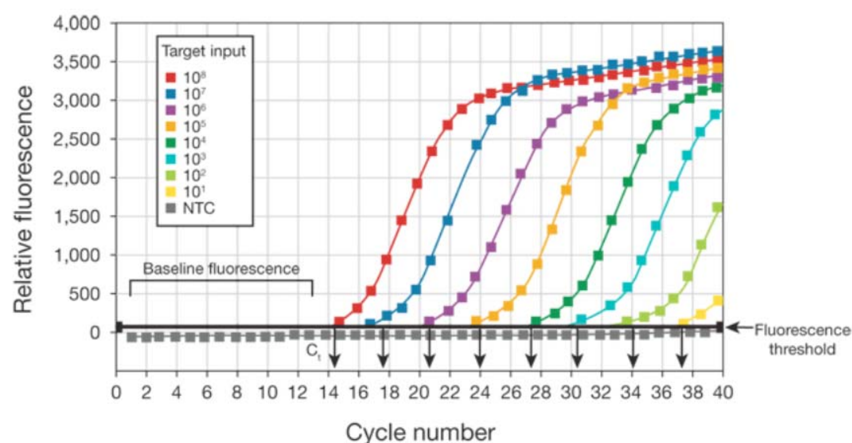

Figure 4- Quantitative PCR amplification plot for ten-fold serial dilutions (ThermoFisher, 2014).

Fluorescence signals can be generated by fluorescent dyes that are specific for dsDNA or by sequence-specific fluorescent oligonucleotide probes.

dsDNA intercalating agents (DNA-binding dyes). DNA-binding dyes (**Figure 5**) bind to dsDNA molecules in the reaction. Excitation of DNA-bound fluorescent dye produces a much stronger fluorescent signal compared to unbound dye. For this reason, an increase in the fluorescence signal occurs during polymerization and this decreases when DNA is denatured. Fluorescent measurements are performed at the end of the elongation step of each PCR cycle and the fluorescent signal is proportional to the amount of PCR product. Moreover, these assays do not require a specific probe to be developed. One of the most frequently used dsDNA dye in qPCR is the SYBR Green I, that has a high binding affinity, and that has an excitation wavelength of about 480_{nm} and an emission wavelength around 520_{nm} (Bokulich, Bamforth, et al., 2012; Hanna et al., 2005; Mills et al., 2008; Wilhelm and Pingoud, 2003). EvaGreen is another dsDNA fluorescent dye used with qPCR. It has been shown to be less inhibitory to PCR than SYBR Green I (Eischeid, 2011; Radvanszky et al., 2015), and stable with high-resolution melt analysis (Mao et al., 2007). Beyond SYBR Green I and EvaGreen, other fluorescent dyes as SYTO (Radvanszky et al., 2015), LCGreen (Wittwer et al., 2003) and Diamond Nucleic Acid (Haines et al., 2016) have been established for use in qPCR.

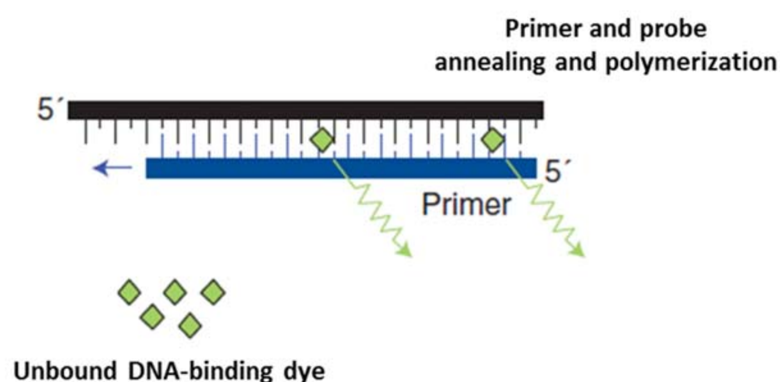

Figure 5-Representation of double-stranded DNA agents/DNA-binding dyes mechanism (adapted from Arya et al. (2005)).

For sequence-specific detection by fluorophore-labelled oligonucleotide probes, the fluorescence signal intensity can be related to the amount of PCR product by a product-dependent decrease of the quench of a reporter fluorophore, or by an increase of the fluorescence resonance

energy transfer (FRET). In FRET, the emissions of a fluorescent dye can be strongly reduced by the presence of another dye, often called the quencher, in close proximity (**Figure 6**) (Wilhelm and Pingoud, 2003).

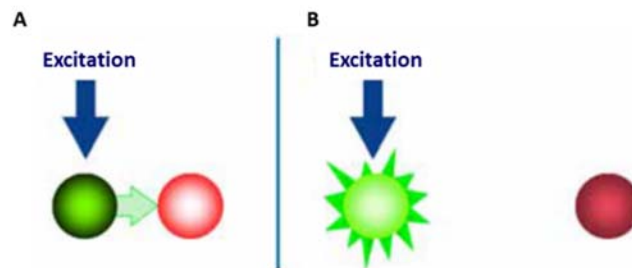

Figure 6- The fluorescence resonance energy transfer (FRET) phenomenon. (**A**) FRET occurs when a green light emitting fluorescent dye is in close proximity to a red light emitting fluorescent dye. (**B**) FRET does not occur when the two fluorescent dyes are not in close proximity (ThermoFisher, 2014).

The FRET and the quench efficiency are strongly dependent on the distance between the fluorophores. Therefore, the PCR-product distance change in the distance between the fluorophores is used to generate the sequence-specific signals. Several formats are used for sequence-specific detection by fluorophore-labelled oligonucleotide probes and in practical terms, most of them are based on a decrease of quench (Wilhelm and Pingoud, 2003).

Hybridization probes. Hybridization probes are used for the only format in which FRET is measured directly (**Figure 7A**). For this, two hybridization probes are used. One of them carries a donor fluorophore at its 3´end, and the other is labelled with an acceptor fluorophore at its 5´end. During the primer-annealing phase, the probes hybridize adjacently on the single-stranded DNA. This brings the two dyes in close proximity allowing FRET. The donor dye in one of the probes transfers energy, allowing the other one to dissipate fluorescence at a different wavelength. The hybridization probe format is used mostly for experiments with LightCycler which have special filters for detection (Arya et al., 2005; Wilhelm and Pingoud, 2003).

TaqMan probes. The TaqMan probe (**Figure7B**) is the most common report mechanism of the sequence-specific detection by fluorophore-labelled oligonucleotide probes. The assay includes a fluorogenic probe that is designed to a region between the two qPCR primers. TaqMan probes are designed with the fluorescent reporter dye on the 5´end and the quenching dye on the 3´end, which quenches all fluorescence excitation of the reporter dye held in proximity. When the probe anneals

to the amplicon, the 5´exonuclease activity of the DNA polymerase cleaves the probe, releasing the reporter dye and produces fluorescence (Hanna et al., 2005) (Wilhelm and Pingoud, 2003).

Molecular beacons. Molecular beacons (**Figure 7C**) are also labelled on both ends. One end is attached to a reporter fluorophore, and the other end is attached to a quencher. However, they are designed to adopt a hairpin-loop structure whilst free in solution to bring the fluorescent dye and the quencher in close proximity for FRET to occur. The loop portion of the molecule is complementary to the amplicon and the stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. During the annealing phase of the PCR, the loop can bind to the PCR. In this conformation, the hairpin is opened and the quenching is relieved, increasing the fluorescence emission (Arya et al., 2005; Hanna et al., 2005; Wilhelm and Pingoud, 2003).

Scorpion primers. The scorpion primers (**Figure 7D**) are structurally and functionally related to molecular beacons, adopting a hairpin-loop configuration with a 5´fluorophhore and 3´quencher but, in this case, the probe element is physically coupled to the primer. The hairpin loop is linked to the 5´-end of a primer via a PCR stopper (non-amplifiable monomer). This chemical modification prevents PCR from copying the hairpin-loop sequence of the scorpion primer that would lead to the detection of non-specific PCR products as primer dimers. After extension of the primer during PCR amplification, the specific probe sequence is able to bind to its complement within the same strand of DNA. This hybridization event opens the hairpin loop so that fluorescence is no longer quenched and an increase in signal is observed. A good discrimination and specificity are achieved using scorpion primers (Arya et al., 2005; Wilhelm and Pingoud, 2003).

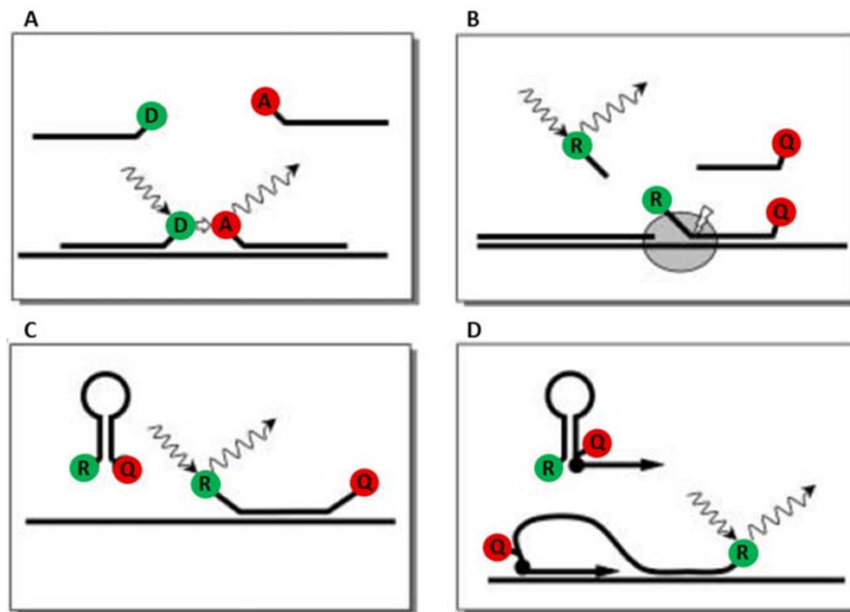

Figure 7- Fluorophore-labelled oligonucleotide probes for sequence-specific detection. (**A**) Hybridization probe, (**B**) TaqMan probe. The grey cycle indicates de Taq polymerase hydrolysing the TaqMan probe, (**C**) Molecular beacon, (**D**) Scorpion primer. A, acceptor; D, donor; Q, quencher; R, reporter (adapted from Wilhelm and Pingoud (2003)).

With sequence-specific fluorescent oligonucleotide probes, nonspecific amplification does not generate signal as specific hybridization between probe and template is necessary. Therefore, this type of amplicon detection benefits from the specificity of DNA-binding dyes, making them less vulnerable to specificity problems. However, DNA-binding dye is a fairly sensitive method that offers greater flexibility and reduced cost as it can be used with any pair of primers for any target. The fact that the DNA-binding dyes are less specific as they bind to any amplified product, target or non-target, can be overcome by melting curve analysis (Arya et al., 2005).

Melting curve analysis is an attractive choice for specificity assessment because it does not add cost to the experiment and can be done right in the PCR reaction tube. Melting curves represent the change in fluorescence observed when individual dsDNA amplicons with incorporate dye dissociate, or "melt" into single-strand DNA as the temperature of the reaction is raised. When the melting point is reached, the fluorescence decrease and a distinction can be made between the desired amplicon and any nonspecific products. The melting point of dsDNA increases with longer length and higher G+C content, and it is recorded subsequent to the amplification of target genes (Hanna et al., 2005; Wilhelm and Pingoud, 2003). This analysis can also be performed for sequence-

specific fluorescent oligonucleotide probes such as hybridization probes and molecular beacons. Nevertheless, TaqMan probes and scorpion primers cannot be used for melting curve analysis because by TaqMan probes the signal generation depends on the hydrolysis of the probe, and by scorpion primer, the probe is integrated into the PCR products (Wilhelm and Pingoud, 2003).

Since its invention in 1996, the number of publications dealing with qPCR has increased nearly exponentially. qPCR offers significant advantages over other molecular methods in terms of the speed by which assays are performed, and the ability to quantify the target microbial population (Wilhelm and Pingoud, 2003). Compared with traditional PCR, qPCR provides several advantages including, increased analytical sensitivity, increased reproducibility, high precision in the obtained results and does not require post-amplification manipulation, hence limiting the risk of contamination (Caraguel et al., 2011; Postollec et al., 2011; Wilhelm and Pingoud, 2003), and reducing the associated costs.

In fermentation research and industry, qPCR presents a rapid quantitative solution for targeted microbial detection. For this reason, many qPCR assays have been developed to detect and enumerate wine microbial groups as AAB (González et al., 2006), LAB (Neeley et al., 2005) and yeasts (Hierro et al., 2006), or wine microbial species as *A. aceti* (Kántor et al., 2014; Torija et al., 2010), *Ga. hansenii*, *G. oxydans*, *A. pasteurianus* (Torija et al., 2010), *L. brevis*, *L. hilgardii* (Kántor et al., 2015), *L. plantarum* (Gyu-Sung et al., 2011; Kántor et al., 2015), *O. oeni* (Pinzani et al., 2004), *Pd. damnosus* (Delaherche et al., 2004), *B. bruxellensis* (Delaherche et al., 2004; Phister and Mills, 2003; Tessonnière et al., 2009; Tofalo et al., 2012; Willenburg and Divol, 2012), *S. cerevisiae* (Martorell et al., 2005) and *Z. bailii* (Rawsthorne and Phister, 2009).

The major qPCR disadvantage is centred on the method´s inability to differentiate viable and non-viable microbes (Elizaquível et al., 2013; Ivey and Phister, 2011; Luo et al., 2010). This drawback is especially relevant when the aim of the analysis is the quantification of the microorganisms in food matrices. It is particularly significant for processed food or foods subjected to long-time storage due to the relative persistence of DNA after death resulting in false positive results (Elizaquível et al., 2013).

In theory, RNA is considered to be a better indicator of cell viability than DNA, as it is thought that RNA degrades more quickly than DNA, and thus would only be found in the metabolically active cell. Therefore, qPCR combined with reverse transcription (RT-PCR), which detects RNA rather than DNA, can overcome this problem (Hanna et al., 2005). Nonetheless, the abundance and stability of

RNA in a cell is heterogeneous and there are some RNA molecules that can also persist in cells for extended time periods after loss of viability, depending on the environmental conditions, the method of cell death, the nature and stability of the RNA, and the region that is amplified. Although not as pronounced as with DNA, this persistence of RNA can lead to false positive results (Nocker and Camper, 2009). Moreover, working with RNA is more demanding as degradation can occur during sampling handling and storage, giving false negative results. These factors make RT-PCR somewhat impractical for a commercial food application (Elizaquível et al., 2013; Hanna et al., 2005).

A solution to differentiate between live and dead cells could be the use of chemicals (Andorrà et al., 2010). The strategy relies on the use of DNA-binding molecules such as propidium monoazide (PMA) or ethidium monoazide (EMA) as sample pre-treatment previous to the qPCR (PMA/EMA-qPCR) (Elizaquível et al., 2013; Nocker and Camper, 2009; Zeng et al., 2016).

PMA and EMA are two DNA-intercalating dyes that penetrate only dead cells with compromised membrane integrity, whereas the intact membranes of viable cells would pose a barrier for the molecules (Bokulich, Bamforth, et al., 2012; Elizaquível et al., 2013; Nocker and Camper, 2006; Nocker and Camper, 2009; Zeng et al., 2016). These biological dyes are positively charged molecules and for this reason, are excluded by intact, negatively charged, bacterial cell-walls, but can enter bacteria with compromised cell-wall/membranes (Nocker and Camper, 2006; Zeng et al., 2016). Once inside the cell, the dye intercalates covalently into the DNA after exposure to strong visible light. The photoreactive azide group on the dye is converted to a high reactive nitrene radical upon photolysis. The nitrene radical reacts with any organic molecule in its proximity including the bound DNA to form a stable covalent nitrogen-carbon bond, thus resulting in cross-linked DNA strands. At the same time, when the cross-linking occurs, the unbound dye, which remains free in solution, is simultaneously inactivated by reacting with water molecules. The covalent crosslinkage of dye to DNA has been shown to result in a strong inhibition of PCR amplification of the modified DNA, and only unmodified DNA from intact cells whose DNA was not crosslinked with dye can be amplified (Elizaquível et al., 2013; Nocker and Camper, 2009; Zeng et al., 2016), as illustrated by **Figure 8**.

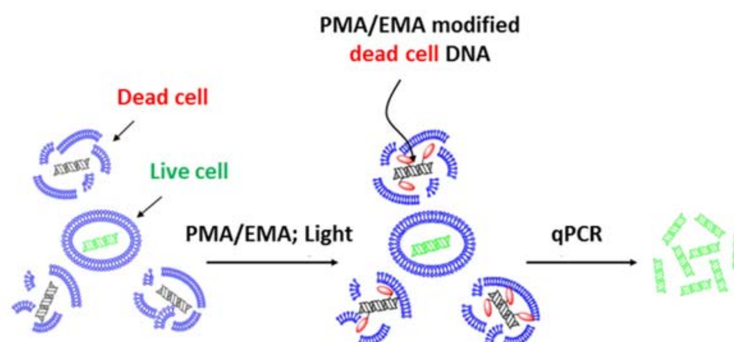

Figure 8- Principle of selective detection of viable cells using propidium monoazide (PMA)/ethidium monoazide (EMA) dyes (adapted from Biotium (2017)).

Although the two dyes behave nearly identically as intercalating stains, they differ in regard to their permeation through cell membrane, concluding that EMA, due to its chemical compositions, is slightly more efficient in signal suppression than PMA, but PMA is more effective than EMA in terms of live-dead discrimination (Elizaquível et al., 2013; Fittipaldi et al., 2012; Zeng et al., 2016). Some studies demonstrated that EMA may penetrate cell with intact membrane (Flekna et al., 2007; Nocker et al., 2006). This problem poses a severe limitation of the use of EMA. A comparative study between both dyes showed that PMA, in contrast to EMA, is efficiently excluded from cells with intact cell membranes, probably due to an increased positive charge (Nocker et al., 2006).

At this moment, new variants of PMA, as PEMAX (Agustí et al., 2017; Randazzo et al., 2016) and PMAxx (Falcó et al., 2017; García-Fontana et al., 2016; Randazzo et al., 2016), are employing to differentiate between live and dead cells. PEMAX is a double dye technology developed by GenIUL. Combining both dyes the DNA from dead cells (with damaged or not damaged membranes) will be neutralized; therefore only DNA from live cells will be detected. PMAxx is a new and improved version of PMA developed by Biotium. This product promises to be much more effective at eliminating PCR amplification of dead cell DNA, and provide the best discrimination between live and dead bacteria.

EMA/PMA treatment combined with qPCR (EMA/PMA-qPCR) has been effectively evaluated to detect and quantify different viable wine-related microorganisms (Andorrà et al., 2010; Lv et al., 2016; Rawsthorne and Phister, 2009; Rizzotti et al., 2015; Shi et al., 2012; Vendrame et al., 2013; Vendrame et al., 2014).

Loop-Mediated Isothermal Amplification

LAMP is a novel technique described by Notomi and coworkers in 2000, which amplifies nucleic acid sequences under isothermal conditions (Notomi et al., 2000). The method requires a set of four special designed primers that recognize six distinct regions of the target and relies on autocycling strand displacing DNA synthesis by the *Bst* polymerase (also called *Gsp* polymerase). The large fragment of *Bst* polymerase, from *Geobacillus stearothermophilus*, can amplify DNA by tearing off double stranded DNA to yield a single strand. The combination of the DNA polymerase and the primer structure enables amplification of the target DNA at a steady temperature (between 60 and 65 °C) (Niessen, 2015; Notomi et al., 2015; Notomi et al., 2000).

The specific four LAMP DNA oligonucleotide primers (two outer and two inner) are designed based on 6 distinct regions of the target gene (**Figure 9**). The outer primers consist of **B3** sequence and of the sequence complementary to F3c that is **F3**. They each recognize one of the six sites and so amplify the entire target region. The inner primers are called the forward inner primer (**FIP**) and the backward inner primer (**BIP**), which are able to recognize two of the six sites. Each one contains two distinct sequences corresponding to the sense and antisense sequence of the target DNA, one for priming in the first stage and the other for self-priming in later stages. The FIP primer contains F1c, a TTTT spacer and the sequence complementary to F2c that is F2. The BIP primer contains the sequence complementary to B1 that is B1c, a TTTT spacer, and B2. The FIP and BIP primers are used in excess, compared to F3 and B3 primers, since they play the major role in the LAMP reaction. The size and sequence of the primers are chosen so that their melting temperature (Tm) fall within certain ranges. The Tm values of F2 and B2 sequences are set between 60 and 65 °C, the optimal temperature of *Bst* polymerase. For F1c and B1c, the Tm values are set slightly higher than those of F2 and B2 in order that a looped out structure is formed immediately after the release of the single-stranded DNA from the template. For outer primers (F3 and B3), the Tm values are set lower than inner primers in order to ensure that synthesis occurs earlier from the inner primers than from the outer primers (Notomi et al., 2000). The sizes of loop between F2 (B2) and F1 (B1) should be from 40 to 60 bp and the length of the amplified DNA region should be less than 300 bp including F2 and B2 (Notomi et al., 2000; Tomita et al., 2008).

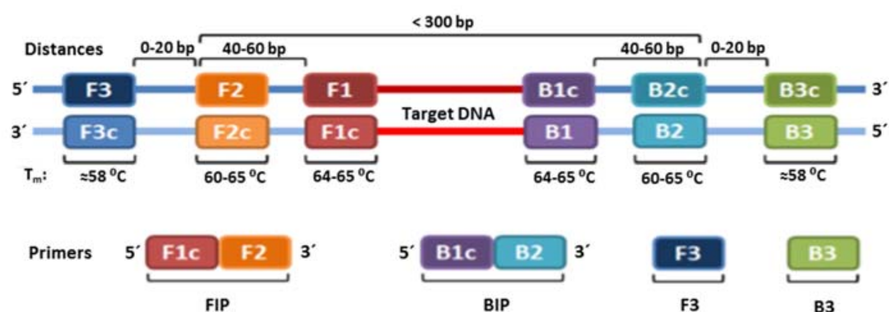

Figure 9- Primers design of the LAMP reaction. B, backward sequence; c, complementary DNA sequence; F, forward sequence (adapted from Santos (2011)).

The mechanism of the LAMP amplification reaction includes three steps: production of starting material, cycling amplification, and elongation and recycling (**Figure 10**) (Notomi et al., 2000; Saharan et al., 2014).

In the initial steps of the LAMP reaction (**Figure 10A**), all four primers are employed, but in the later cycling steps, only the inner primers are used for strand displacement DNA synthesis. The process is initiated by attachment of inner primers to the DNA target. The inner primer FIP hybridizes to F2c in the target DNA (**structure 1**) and initiates complementary strand synthesis (**structure 2**). The outer primer F3 slowly hybridizes to F3c in the target DNA and initiates strand displacement DNA synthesis (**structure 3**), releasing a FIP-linked complementary strand (**structure 4**), which can form a looped out structure at one end. This single-stranded DNA serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis (**structure 5**), leading to the production of a dumbbell form DNA (**structure 6**). This structure then serves as the starting material for the second stage of the LAMP reaction (cycling amplification), which proceeds with the participation of the two inner primers only (Notomi et al., 2000; Parida et al., 2008; Tomita et al., 2008).

To initiate cycling amplification (**Figure 10B**), the starting structure is converted to stem-loop DNA by self-primed DNA synthesis (F1 region), then FIP hybridizes to the loop in the stem-loop DNA (F2c region) and primes strand displacement DNA synthesis (**structure 7**). Subsequent self-primed strand displacement DNA synthesis (**structure 8**) yields one complementary structure of dumbbell structure 7 (**structure 9**) and a structure with a stem elongated twice as long (double copies of the target sequence) and a loop at the opposite end (**structure 11**). Both these products

then serve as a template for a BIP-primed strand displacement reaction in the subsequent steps. After, structure 7 is produced from structure 9. It means that the cycle reaction between structure 7 and 9 is established. Structures 11 and 12 are produced from structures 8 and 10, respectively, and more elongated structures (13-16) are also produced during successive elongation and recycling step (**Figure 10C**) (Notomi et al., 2000; Parida et al., 2008; Tomita et al., 2008).

The final product in LAMP is a mixture of stem-loop DNA with various stem length and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand (Notomi et al., 2000; Parida et al., 2008; Tomita et al., 2008).

For a better understanding of the LAMP technique, video animations can be visualized at Eiken (2005-2017), or at BioLabs (2017).

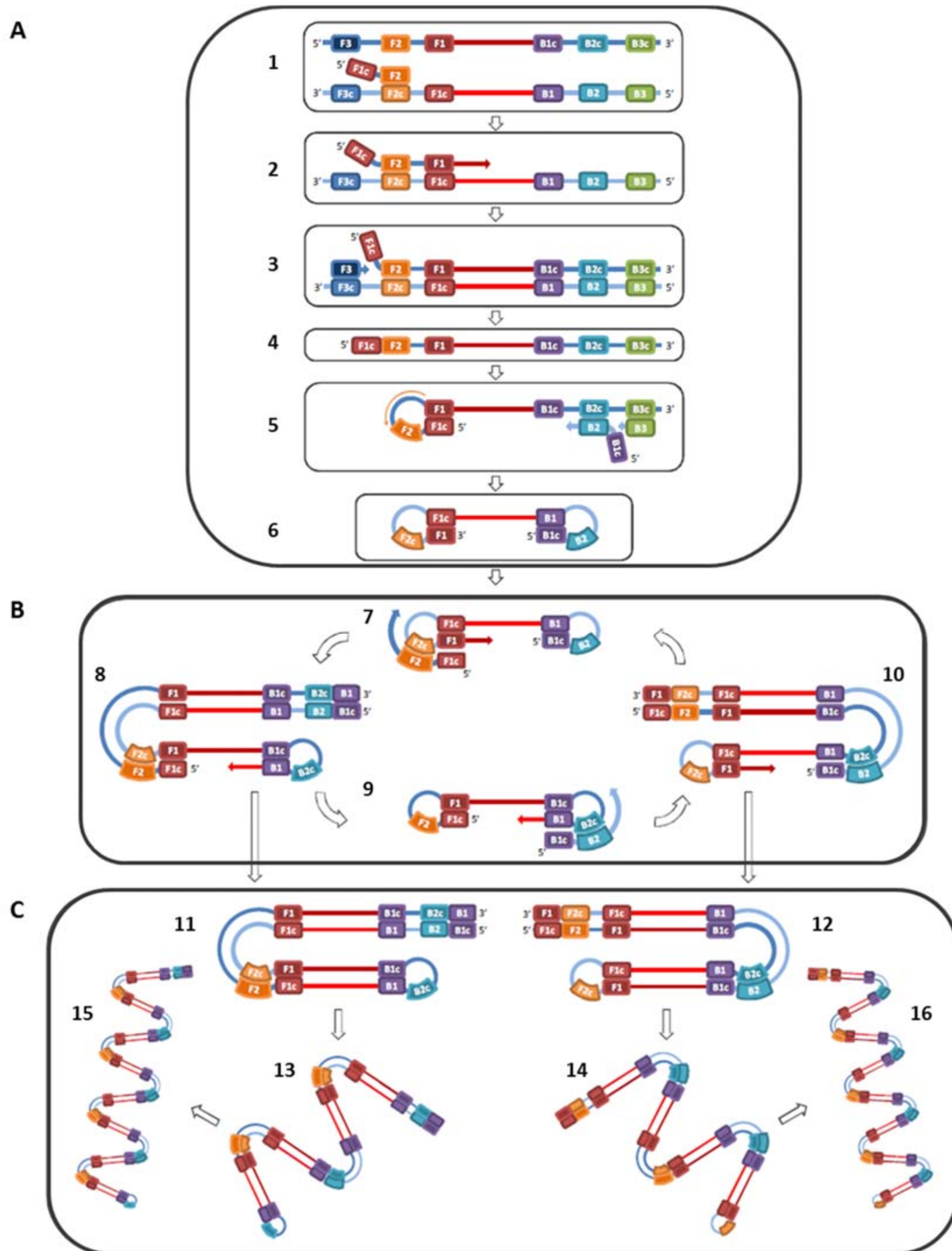

Figure 10- Schematic representation of the mechanism of LAMP amplification reaction. (**A**) starting material producing step [1-6], (**B**) cycling amplification step [7-10], (**C**) elongation and recycling step [11-16]. B, backward sequence; c, complementary DNA sequence; F, forward sequence (adapted from Santos (2011)).

A third pair of primers (loop primers) can optionally be added to the reaction in order to further amplify the amount of DNA produced during LAMP. The loop primers are designed to hybridize with the single-stranded loop structures present in the dumbbell structures as well as in the multimeric DNA formed during autocycling DNA amplification (Nagamine et al., 2002). They prime the production of novel template DNA to which FIB/BIP primers can bind to initiate synthesis of even higher concentration of DNA. Therefore, the addition of one or two loop primers does not increase the speed of amplification but rather increase the rate of the reaction, enabling earlier detection (30 min) of a LAMP signal as compared to a reaction run without loop primers (Nagamine et al., 2002; Niessen, 2015).

A number of methods are available for the detection of LAMP amplification products. Naked eye endpoint monitoring, gel electrophoresis, real-time turbidity, real-time fluorescence, lateral flow dipstick (LFD), and other simpler techniques can be used for monitoring LAMP reaction (Kumar et al., 2017; Niessen, 2015; Zhang et al., 2014).

Naked eye monitoring by observing precipitate. The DNA polymerization reaction mediated by *Bst* polymerase releases pyrophosphate ions from deoxyribonucleotide triphosphate (dNTPs) as a by-product. When a large amount of pyrophosphate ions are produced, they react with magnesium ions in the reaction buffer, yielding a white precipitate. In a successful LAMP reaction, a white precipitation (turbidity) can be observed (**Figure 11A**) (Kumar et al., 2017; Niessen, 2015; Notomi et al., 2015; Tomita et al., 2008; Zhang et al., 2014). Therefore, the presence or absence of turbidity as an endpoint measurement allows easy detection of LAMP amplification results and does not require additional costly specialized equipment. Furthermore, there is no risk of cross-contamination of amplicons because the contents of the reaction tube are not exposed to the atmosphere. This method is sufficient for a preliminary detection determination of the LAMP reaction, especially for rapid testing (Mori et al., 2001; Zhang et al., 2014).

Naked eye monitoring employing DNA-binding dyes. The tube containing the amplified LAMP products can also be visualized in the presence of fluorescent intercalating dye, under natural light or under UV lamp. Under natural light, the formation of the dsDNA complex causes a visible colour change of the dye, and under UV light the fluorescence intensity increases (**Figure 11B**) (Parida et al., 2008; Saharan et al., 2014; Tomita et al., 2008). At present, several fluorescent dyes as SYBR Green, ethidium bromide, Quan-iT PicoGreen (Niessen, 2015; Zhang et al., 2014), SYTO-9, YOPRO1 (Niessen, 2015), and GeneFinder (Zhang et al., 2014), have been applied for qualitative LAMP monitoring. The employment of DNA-binding dyes leads to increased sensitivity when

compared with visual turbidity measurements. Nevertheless, it is associated with higher running costs. Note that this kind of method usually requires two steps, amplification and dye addition. For this reason, opening the reaction tube after amplification must be made carefully to prevent carry-over contamination (Zhang et al., 2014).

Naked eye monitoring employing colorimetric indicators. Another class of naked eye methods utilizes indirect colorimetric indicators. Calcein and hydroxyl naphthol blue are two examples of colorimetric indicators (Niessen, 2015; Zhang et al., 2014). Regarding calcein, it is a metal indicator that yields strong fluorescence by forming complexes with divalent metallic ions, such as calcium and magnesium (Tomita et al., 2008). Before the LAMP amplification reaction, calcein molecules combine with manganous ions, quenching fluorescence. At this stage, the LAMP reaction solution appears orange. As the LAMP reaction proceeds in the presence of target DNA, calcein molecules give up manganous ions to newly generated pyrophosphate ions thereby recovering their green fluorescence. In addition, the green fluorescence signal is further intensified as calcein combines with magnesium ions (**Figure 11C**) (Niessen, 2015; Parida et al., 2008; Tomita et al., 2008; Zhang et al., 2014). Regarding hydroxyl naphthol blue, it develops a purple colour in the presence of Mg^{2+}. During the amplification process, a significant amount of insoluble magnesium pyrophosphate is produced, causing a major decrease of Mg^{2+} concentration in the solution. This reduction in concentration causes the colour change of hydroxyl naphthol blue from purple to blue (Niessen, 2015; Saharan et al., 2014; Zhang et al., 2014). From these features, the presence of fluorescence can indicate the presence of target DNA and visual detection can be achieved without opening the tube, thus preventing carry-over contamination with post-amplification products (Parida et al., 2008; Zhang et al., 2014).

Gel electrophoresis. Gel electrophoresis, followed by ethidium bromide staining and UV transillumination, is a traditional method for monitoring the LAMP amplicons. The positive post-LAMP reaction mixture subjected to gel electrophoresis produces many bands of different sizes in a reproducible ladder-like pattern (**Figure 11D**) (Niessen, 2015; Zhang et al., 2014). The obtained LAMP products can size from a few hundred bp up to several kilobases forming a smear of high molecular weight DNA (Niessen, 2015). Due to a massive production of DNA during LAMP, the risk of cross-contamination of samples by aerosolized product is very high and may compromise analytical results (Niessen, 2015; Zhang et al., 2014). In addition, this method is only suitable for qualitative analysis at the endpoint. Nevertheless, techniques relying on indirect detection methods

as turbidity and colorimetric, may not be able to distinguish between real and false positives under some unexpected cases when non-specific amplification occurs. Using gel electrophoresis the length of amplicons can be recognized directly and hence, the risk of non-specific detection is reduced (Zhang et al., 2014).

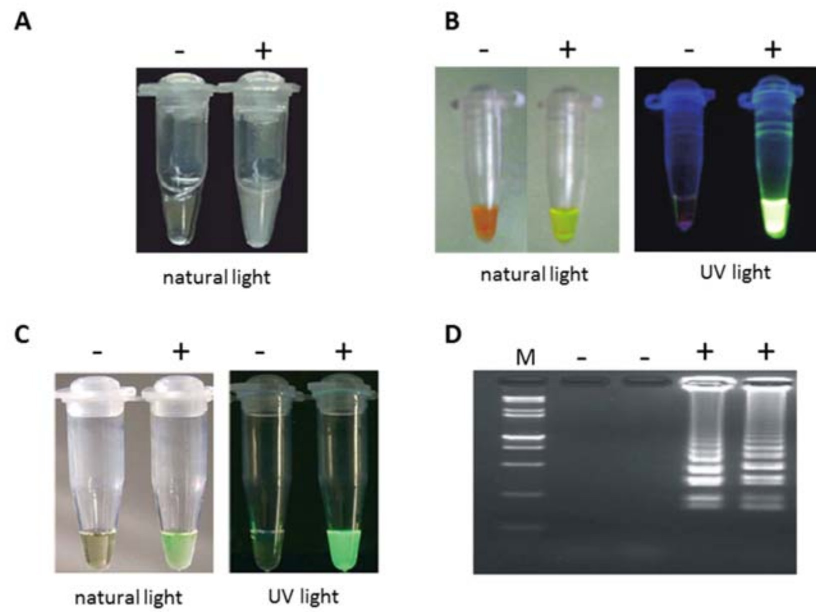

Figure 11-Endpoint monitoring of LAMP amplification. (**A**) naked eye monitoring by observing precipitate (turbidity) (adapted from Mori and Notomi, 2009), (B) naked eye monitoring, employing DNA-binding dyes (SYBR Green I) under natural and UV light (adapted from Parida et al., 2006), (C) naked eye monitoring employing colorimetric indicators (calcein), under natural and UV light (adapted from Tomita et al., 2008), (D) gel electrophoresis (adapted from Zhou et al. (2014)). +, positive; - negative; M, DNA ladder.

Measurement of turbidity, fluorescence, or ion concentration, can be performed in a quantitative real-time format: quantitative LAMP (qLAMP).

Real-time turbidity. The turbidity which occurs upon precipitation of magnesium pyrophosphate was found to be proportional to the concentration of template DNA initially present in a LAMP reaction. As a consequence, the turbidity change during amplification can be monitored in real-time with optical instruments, including turbidimeters and spectrophotometers (Niessen, 2015; Zhang et al., 2014). The qLAMP method by real-time turbidity is capable of quantitative monitoring with high automation, not requiring reagents/indicators. Here is no running cost except

for the turbidimeter or spectrophotometer. Moreover, the risk of amplicon contamination is eliminated (Zhang et al., 2014).

Real-time fluorescence. The dyes employed in the naked eye endpoint determination of the LAMP reaction can also be applied in real-time monitoring. Regarding DNA-binding dyes, their bind to the dsDNA and consequent change in optical signal can be used to monitor the process of the LAMP reaction in real-time. In contrast to qPCR where DNA amplification is a function of the number of Ct, in qLAMP reaction, the biosynthesis of DNA is a continuous process and therefore, DNA amplification is a function of incubation time rather than cycle number. Therefore, the quantitative estimation of target concentrations in a given qLAMP reaction has to be done by comparison of the time needed for the signal to reach a positive signal (Tt value) (Niessen, 2015; Zhang et al., 2014). Several fluorescent dyes such as SYBR Green I, SYTO-82 (Oscorbin et al., 2016; Zhang et al., 2014), SYBR Gold, SYTO-9, SYTO-13, and EvaGreen have already been used for qLAMP (Oscorbin et al., 2016). Generally, the fluorescence-based real-time monitoring of LAMP reaction is considerably faster (>50 %) than that performed by real-time turbidity. Moreover, real-time fluorescence method is more sensitive and the sensitivity is less affected by the presence of opaque substances in the mixture, such as protein. Nevertheless, it is associated with higher running costs (Zhang et al., 2014).

Other methods as LFD can be used for the detection of LAMP products.

Lateral flow dipstick. The LFD is an immunochromatographic technique utilizing antibody capture followed by secondary antibody labelling. On the LFD strip, an antibody specific to biotin is immobilized at the test line (Zhang et al., 2014). This strip detects biotin-labeled amplicons upon hybridization to a fluorescein isothiocyanate (FITC)-labelled DNA probe complexed with a gold-labelled anti-FITC antibody. The gold-labelled anti-FITC antibodies are introduced in order to develop a readable output. In a successful reaction, the gold anti-FITC antibodies are trapped at the test line as a triple complex with dsDNA. Non-hybridized FITC probes are bound by gold-labelled anti-FITC to form a double complex and move through the test line to be trapped at the control line (Jaroenram et al., 2009; Rigano et al., 2014; Zhang et al., 2014). For LAMP amplification combined with LFD, the reaction is performed using a biotinilated FIP primer. After, a specific FITC-labelled probe is added to the reaction mixture. This step produces a dual labelled LAMP product. Finally, for detection, a buffer containing anti-FITC antibodies coupled with colloidal gold is mixed with the reaction mixture, and the LFD strip is inserted into the tube (**Figure 12A**). In a positive reaction,

double labelled LAMP products migrate with the buffer flow and are retained at the test line by a biotin ligand, developing a dark band over the time. In the case of a negative reaction, no products are generated and such process does not take place. The control line retains some of the unbound gold-conjugated antibody and produces a control line that should always be visible (**Figure 12B**) (Kaewphinit et al., 2013; Rigano et al., 2014; Zhang et al., 2014). The implementation of LFD enables a specific, rapid, and simple optical detection of LAMP products, and does not require any specialist instrumentation (Jaroenram et al., 2009; Kaewphinit et al., 2013; Kiatpathomchai et al., 2008; Zhang et al., 2014). Thus, it is a competitive candidate for qualitative tests as an endpoint format. Nevertheless, the preparation of the strips is not only time-consuming but also costly.

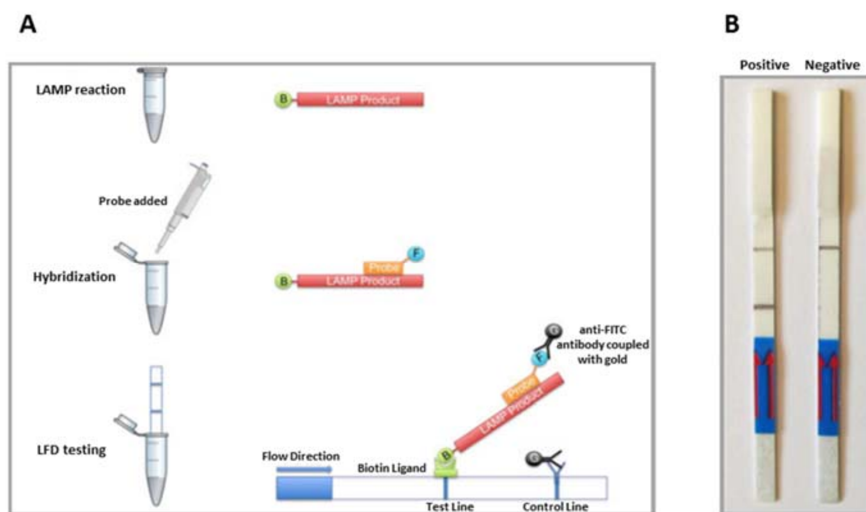

Figure 12- Lateral flow dipstick LAMP evaluation. (**A**) Lateral flow dipstick LAMP procedure; (**B**) Evaluation of results using lateral flow dipstick device (adapted from Rigano et al. (2014)).

Amplification of DNA using isothermal conditions gained increasing attention due to the fact that they can be run with less effort and expense as compared to PCR (Niessen, 2015). Although PCR-based methods are widely used, it requires a thermocycler to carry the DNA amplification through specific temperature phases and, in some cases, the result needs to further analyse by electrophoresis. Moreover, in PCR-based methods, most of the time is passed increasing or decreasing temperatures or incubating at temperatures not suitable for polymerization of DNA (denaturation and hybridization temperatures). LAMP is simple and easy to perform once the appropriate primers are prepared, requiring only four primers, a DNA polymerase, and a regular laboratory water bath or heat block for reaction. Moreover, LAMP reaction combines specificity,

sensitivity, and efficiency. The exceptionally high specificity is due to the fact the set of four primers with six binding sites must hybridize correctly to their target sequence before DNA biosynthesis occurs. LAMP is highly sensitive as small quantities of a gene can be amplified within a short time. Besides that, it seems not to be affected by the presence of non-target DNA in samples. The high amplification efficiency of LAMP is attributed to no time loss of thermal change, and DNA is amplified 10^9-fold in 15-60 min. In addition, both amplification and detection gene can be completed in a single step (Notomi et al., 2015; Notomi et al., 2000; Parida et al., 2008; Saharan et al., 2014; Zhang et al., 2014). Furthermore, the LAMP reaction can be implemented even when the DNA extraction is eliminated, thus greatly reducing the overall assay time and the reaction cost (Dugan et al., 2012; Hill et al., 2008; Poon et al., 2006).

Another feature that contributes to the simplicity and efficiency of the method is the availability of primer design support software as Primer-Explorer (Eiken, 1999-2005), or LAMP Designer (OptiGene, 2017), which facilitate the design of appropriate primers. Proper primer design is crucial for performing LAMP amplification, and with these softwares, candidate primer sets specific to the input target sequence can be automatically produced.

This method can also be used for RNA target, as it is able to amplify, with high efficiency, RNA sequences by reverse transcription LAMP (RT-LAMP) reaction, upon reverse transcriptase (Notomi et al., 2015; Notomi et al., 2000).

Like in qPCR method, the major drawback of LAMP assay is the inability to distinguish viable from dead cells. Nevertheless, the use of EMA and PMA DNA-binding molecules, as sample pre-treatment previous to the LAMP reaction, has also been previously examined (Chen et al., 2011; Lu et al., 2009).

The employment of LAMP assay in wine samples was only described by Hayashi et al. (2007), using this approach to detect and quantify total cells of *B. bruxellensis* by real-time turbidity.

1.4. Influence of Inhibitors on DNA Amplification Dependent Methods

Inhibitors are a major draw-back of DNA amplification. The inhibitors can originate from the sample or may be introduced during sample processing or DNA extraction (Schrader et al., 2012). The inhibitory mode of action of some of these compounds may be linked to both DNA precipitation and denaturation, or to the ability to bind to the polymerase or to denature it (Demeke and Jenkins, 2010; Wilson, 1997). Common inhibitors include various components of body fluids (hemoglobin, immunoglobin G, etc.) food constituents and environmental compounds such as organic and phenolic compounds (bile salts, urea, polysaccharides, phenol, ethanol, etc), glycogen, humic acids, tannic acids, and heavy metals. Other more widespread inhibitors include constituents of bacterial cells, non–target DNA, and contaminants (Schrader et al., 2012; Wilson, 1997). The major consequence of a partial or total inhibition of the amplification is a decreased sensitivity or false-negative results (Schrader et al., 2012).

Wine is considered a complex matrix that contains DNA amplification inhibitors as ethanol, polysaccharides, pigments, and a wide range of polyphenolics (including tannins). These inhibitors can lead to poor DNA isolation and/or amplification efficiency, producing false-negative results or underestimation for quantitative assays (Tessonnière et al., 2009; Wilson, 1997).

Almost all of DNA amplification dependent methods require a previous DNA extraction step. DNA extraction is usually affected by factors such as incomplete cells lysis, DNA sorption to a particular material, coextraction of enzymatic inhibitors, and degradation or damage of DNA (Miller et al., 1999). Despite wine inhibitors can lead a poor DNA extraction, it is important taking into account that inhibitors may be added during DNA extraction step, decreasing the DNA recovery yield. Sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), phenol, chloroform, and ethanol are some examples of substances employed in several DNA extractions that may be necessary for efficient cell lysis, or for the preparation of pure nucleic acids. Nevertheless, they also cause PCR inhibition at certain concentrations. Residual SDS coextracted with DNA can denature DNA polymerase. EDTA at certain concentrations it may inhibit the PCR by complexing $Mg^{2+}$and thus inhibit DNA polymerase activity. Residual phenol, chloroform, and alcohol from the extraction step may contaminate the DNA hence hinders the PCR reaction. Therefore, they can reduce the efficiency and/or reproducibility of DNA amplification, thus contributing to inaccurate results. (Rossen et al.,

1992; Schrader et al., 2012). The choice of DNA extraction method is a trade-off between costs, optimal yield of DNA and removal of substances that could influence the PCR reaction. A procedure that results in an optimal yield of DNA and in the removal of DNA amplification inhibitors is essential (Cankar et al., 2006). In wine matrices, several DNA extraction methodologies have been developed to obtain suitable DNA with no inhibitors interfering (Jara et al., 2008; Longin et al., 2016; Tessonnière et al., 2009). Nevertheless, no standardized DNA extraction method for wine samples is available.

2. OBJECTIVES

In wine industry, rapid, specific, sensitive, and reliable methods to monitor microbial presence during winemaking process are desired. Recent advances in molecular biotechnology have introduced an array of powerful techniques for studying the microbial ecology of beverage fermentations. Nevertheless, most of the newly developed molecular methods are time-consuming, and require expensive reagents and equipment, limiting their use in wineries. Moreover, almost all methods depending on DNA amplification require previous DNA extraction step, which is highlighted as a limitation because it implies a putative error manipulation point, increases reaction costs and times, and can be affected or affect DNA amplification by the presence of inhibitors.

Therefore, the main objective of this work was the development of molecular approaches in order to obtain fast, effective, reliable, and cost-effective methods to detect, and quantify wine-related microorganisms (yeasts, LAB and AAB), directly from grape must and wine samples, and without DNA extraction step requirements.

To carry out this goal the following specific objectives were set out:

- Development of a qPCR method for total cells detection and quantification.

- Development of a PMA-qPCR method for viable cells detection and quantification.

- Development of a LAMP method for total cells detection.

- Development of a qLAMP method for total cells detection and quantification.

3. MATERIAL AND METHODS

3.1 Microorganisms

3.2 Culture Media, Grape Musts and Wines, and Growth Conditions

3.4 Biological Material Preparation

3.5 Microbial Growth Monitoring by Traditional Methods

3.6 Microorganisms Detection and Quantification by Molecular Direct Methods

3.1. Microorganisms

Different species of yeasts, LAB, and AAB from CECT and Enolab culture collections were used in this study (**Table 3**). CECT collection is a Spanish public bank of type cultures, and Enolab collection is a private bank belonging to Enolab research group of Universidad de Valencia.

Table 3- Collection strains used in this study.

	Microorganism	Strain
Yeasts	*B. bruxellensis*	CECT 1451^{T}
	S. cerevisiae	CECT 2056
	Z. bailii	Enolab 5205
LAB	*L. brevis*	CECT 216
	L. plantarum	CECT 748^{T}
	O. oeni	CECT 218
	Pd. damnosus	CECT 4692
	Pd. pentosaceus	CECT 4695
AAB	*A. aceti*	CECT 298^{T}
	A. pasteurianus	CECT 824
	G. oxydans	CECT 4009

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3.2. Culture Media, Grape Musts and Wines, and Growth Conditions

Regarding culture media, yeasts were grown in YPD, AAB and *O. oeni* were grown in MLO, and the remaining LAB species were grown in MRS.

YPD (medium for yeasts growth)

Yeast extract (Pronadisa)	10 g/L
Mycological peptone (Pronadisa)	20 g/L
Glucose (Panreac)	20 g/L
Agar (Pronadisa)	20 g/L (only for solid medium)

MLO (medium for AAB and *O. oeni* growth)

Tryptone (Panreac)	10 g/L
Yeast extract (Pronadisa)	5 g/L
Glucose (Panreac)	10 g/L
Fructose (Scharlau)	5 g/L
$MgS0_4.7H_2O$ (Panreac)	0.2 g/L
$MnSO_4 .H_2O$ (Panreac)	0.05 g/L
Diamonic citrate (Panreac)	3.5 g/L
L-Cysteine (Merck)	0.5 g/L
Tween 80 (Fisher Chemical)	1 mL
Tomato juice extract[a]	300 mL
Agar (Pronadisa)	20 g/L (only for solid medium)[b]

The pH of the medium was adjusted to 4.8 with HCl 10N

[a] Tomato juice extract was prepared macerating overnight a mix of commercial tomato juice (1 L) and distilled water (2 L) at 4 °C. After macerating, the mix was centrifuged for 20 minutes at 14000 rpm (Beckman Coulter Avanti J-E), and the supernatant was filtered through filter paper. This extract was preserved at -20 °C until its use.

[b] For solid medium preparation, the agar was autoclaved separately to prevent its hydrolysis at acid pH. For this, all the ingredients for 1 L of medium were mixed and dissolved only in 500 mL of distilled water. The remaining 500 mL was used to dissolve the agar and autoclave it. Once autoclaved, the agar was mixed with the MLO ingredients pre-autoclaved.

MRS (medium for LAB growth)

MRS Broth (Scharlau)	52 g/L
L-Cysteine (Merck)	0.5 g/L
Agar (Pronadisa)	20 g/L (only for solid medium)

The pH of the medium was adjusted to 6.5 with NaOH 10N

All of the culture media were autoclaved for 30 minutes at 115 °C.

Concerning grape musts and wines, natural samples of white Chardonnay and red Bobal grape varieties were used as matrices for all the experiments (**Table 4**). All samples were previously sterilized by filtration and then frozen at -20 °C until their use.

Table 4- Grape variety and chemical composition of grape must and wine matrices.

Matrix	Grape variety	Chemical composition
White grape must	Chardonnay	Fructose: 85.97 g/L Glucose: 78.47 g/L Citric acid: 1.90 g/L Malic acid: 1.31 g/L Tartaric acid: 10.05 g/L pH: 3.20
White wine		Fructose: 0.00 g/L Glucose: 0.00 g/L Citric acid: 0.09 g/L Malic acid: 0.00 g/L Lactic acid: 2.33 g/L Tartaric acid: 6.9 g/L Ethanol: 10.22 °(v/v) Glycerol: 5.41 g/L pH: 3.20
Red grape must	Bobal	Fructose: 79.11 g/L Glucose: 78.76 g/L Citric acid: 1.48 g/L Malic acid: 1.86 g/L Tartaric acid: 11.78 g/L pH: 3.27
Red wine		Fructose: 6.32 g/L Glucose: 0.51 g/L Citric acid: 1.60 g/L Malic acid. 1.95 g/L Lactic acid: 0.43 g/L Tartaric acid: 9.48 g/L Ethanol: 9.86 °(v/v) Glycerol: 7.51 g/L pH: 3.23

For routine use, the strains were grown in liquid medium at 28 °C for 3-5 days. Each day the number of cells per mL was determined by microscopic counting with Neubauer chamber until the population reached 10^8 cells/mL.

For medium-term conservation (6-8 months), the strains were preserved by cryoprotection method. To carry out this procedure, after growth in liquid medium, the cultures were frozen at -20 °C with 15% (v/v) glycerol.

3.3. Biological Material Preparation

The cultures grown from each species were serially diluted from 10^8 to 10^2 cells/mL in independent experiments using the same culture medium as the diluent. Filtered Natural samples of Chardonnay white grape must and wine, and Bobal red grape must and wine were inoculated with each species from the liquid medium in independent experiments. These cell suspensions were then serially diluted from 10^8 to 10^2 cells/mL, using the same matrix as the diluent to obtain the same matrix composition in all the cell suspensions. After that, three different approaches of biological material preparation, on cells detection and quantification, were evaluated:

1) **Cells wash**, which are named during this study as whole cells;

2) **Cells wash and then, DNA extraction**, which are named during this study as extracted DNA;

3) **Cells wash and then, cells wall disruption** which are named during this study as lysed cells.

3.3.1. Cells wash

Cell suspensions from the culture media, white and red grape musts or white and red wines were centrifuged for 5 minutes at 12000 rpm (LabNet International Inc) being the pellets washed with several solutions:

a) Cell suspensions from culture medium: the pellet was washed twice with milliU water;

b) Cell suspensions from white grape must and white wine: the pellet was washed with milliU water, next with 10% TEN buffer (0.1 M Tris-HCl pH 7.5, 0.05 M EDTA, 0.8 M NaCl) and then twice with milliU water;

c) Cell suspensions from red grape must and red wine: the pellet was washed with milliU water, next with 10% TEN buffer supplemented with Polyvidone 25 (PVP)(2% w/v) (Merck, Darmstadt, Germany), and then twice with milliU water. Only for qPCR experiments, two different buffers were assayed: 10% TEN buffer, or 10% TEN buffer supplemented with PVP (2% w/v).

3.3.2. DNA extraction

The genomic DNA from each pure cell suspension from the culture medium, white and red grape musts and wines was extracted with the commercial extraction kit known as the Ultra Clean® Microbial DNA Isolation Kit (MO BIO) according to the manufacturer´s instructions.

3.3.3. Cell wall disruption

The effect of cell wall disruption, on cells detection and quantification, was evaluated for yeast and LAB species by both enzymatic and mechanical lysis methods.

Enzymatic lysis

Regarding yeast species, the enzymatic lysis was carried out using zymolyase. A final concentration of 6.89 mg/mL of Zymolyase 20T (Seikagaku) in 0.9 M Sorbitol, 0.1 M EDTA pH 7.5 was added to each cell suspension from culture medium. Cells were incubated for 1 hour at 37 °C and washed twice by centrifugation for 5 minutes at 12000 rpm (LabNet International Inc) in milliU water.

Regarding LAB species, the enzymatic lysis was carried out using lysozyme. In this case, as described Blasco (2009), different concentrations of lysozyme were used for each species. A final concentration of 0.1 mg/mL and 1 mg/mL lysozyme (Sigma), in milliU water, was added to each cell suspension of *O. oeni* and *L. plantarum* from culture medium, respectively. Cells were incubated for 25 minutes at 37 °C and washed twice by centrifugation for 5 minutes at 12000 rpm (LabNet International Inc) in milliU water.

Mechanical lysis

Firstly, 425-600 µm-diameter glass beads (Sigma, St. Louis, USA), previously acid washed with hydrochloric acid 37 % (Panreac), were added to each yeast and LAB cell suspension (50% (w/v)) from culture medium, white and red musts and wines. The tubes were shaken in a horizontal microtubes vortex-genie 2 (Scientific Industries) for 10 and 30 minutes, at the maximum rate, to yeast and LAB cell suspensions, respectively.

3.4. Microbial Growth Monitoring by Traditional Methods

3.4.1. Total cells count by microscopy

Total cells count was realized using Neubauer counting chamber (0.0100 mm x 0.0025 mm^2) using the 40X objective from Leica DMLB microscope. The cell population was estimated from the average of cells counted in the 20 squares of the diagonal counting chamber.

3.4.2. Viable cells count by plate

Cell viability was studied by standard plate count technique. The samples were 10-fold serial diluted in sterile saline solution (9 g/L of NaCl, Panreac). A volume of 0.1 mL of the appropriate sample dilutions was spread in duplicate on culture medium plates. These were incubated at 28 °C for 4-7 days until colonies observation. The total number of viable cells on the agar surface was enumerated and expressed in total number of colony forming units per millilitre (CFU/mL).

3.5. Microorganisms Detection and Quantification by Molecular Direct Methods

3.5.1. Total cells detection by PCR

Total cells detection by PCR was carried out with whole cells and extracted DNA, both of them from culture medium. For this purpose, cell suspensions of each species, with a final concentration of 10^6 cells/mL, were obtained, washed, and then DNA was extracted. PCR amplifications with selected primers were performed in independent experiments.

PCR primers

Previously described, general primers for yeasts and AAB, and specific primers for *B. bruxellensis*, *S. cerevisiae*, *Z. bailii*, *O. oeni* and *L. plantarum* species were selected (**Table 5**). For more information about the primers see **Annex 1**.

Table 5- Primers used for PCR and respective target microorganisms.

Microorganism		Primer	Reference
Yeasts	General	YEASTF YEASTR	Hierro et al. (2006)
	B. bruxellensis	DBRUXF DBRUXR	Phister and Mills (2003)
	S. cerevisiae	CESP-F SCER-R	Hierro et al. (2007)
	Z. bailii	ZBF1 ZBR1	Rawsthorne and Phister (2006)
LAB	*L. plantarum*	LacPla1F LacPla1R	Stevenson et al. (2006)
	O. oeni	MalomarF MalomarR	Vendrame et al. (2013)
AAB	General	I2B AABr	Blasco (2009)

PCR reaction conditions

PCR reactions were carried out in a total volume of 50 µL. Each reaction contained 200 µM of dNTPs, 1 µM of each primer, 20 mM of Tris-HCl, pH 8.4, 50 mM of KCl, 2 mM of $MgCl_2$, 2 U of Taq Polymerase (Invitrogen), and 2 µL of whole cells or extracted DNA. All the PCR amplifications were carried out in a Mastercycle Personal 5332 (Eppendorf) under the PCR conditions previously described by Vendrame et al. (2013), and that are shown in **Figure 13**. Negative controls were included at all times. Negative controls were included at all times.

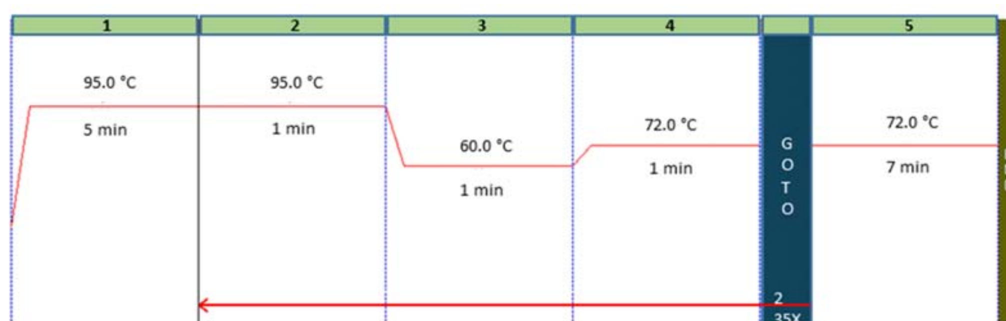


Figure 13- PCR amplification conditions. 1, initial denaturation; 2, denaturation; 3, annealing; 4, extension; 5, final extension.

PCR products were resolved by agarose gel electrophoresis (2% (w/v) in 0.5X Tris-borate-EDTA buffer), stained with 0.5 µg/mL ethidium bromide and visualized by GelPrinter Plus (TDI). A GeneRuler Low-Range Ladder (Thermo Scientific) was used for size standards.

3.5.2. Total cells detection and quantification by quantitative PCR

Total cells detection and quantification by qPCR was carried out using as sample, whole cells, extracted DNA, and lysed cells, from culture medium, white and red grape musts, and white and red wines. For this purpose, cell suspensions of each species, with a final concentration from 10^2 to 10^8 cells/mL were prepared in all matrices. After cell suspensions wash, the DNA was extracted and the cells were lysed, in independent experiments. For cells lysis, both enzymatic and mechanical methods were assayed. qPCR amplifications with selected primers were performed in independent experiments.

Quantitative PCR primers

The primers for all qPCR reactions were the same as the previously used for PCR reactions, being described in section **PCR primers**.

Quantitative PCR reaction conditions

Amplification reactions were carried out in a total volume of 20 µL that contained 1X Hot FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne) and 0.2 µM of each primer. For extracted DNA and lysed cells, volumes of 2 µL and 10 µL were used, respectively. Regarding whole cells, two different volumes were assayed (2 µL and 10 µL). The quantitative PCR amplification conditions for all reactions are shown in **Figure 14**. All the samples were automatically processed for the melting curves analyses of the amplified DNA to determine reaction specificity. As shown in **Figure 14**, the melting curves were obtained by slow heating from 60 °C to 95 °C at 0.5 °C every 5 seconds, with continuous fluorescence collection.

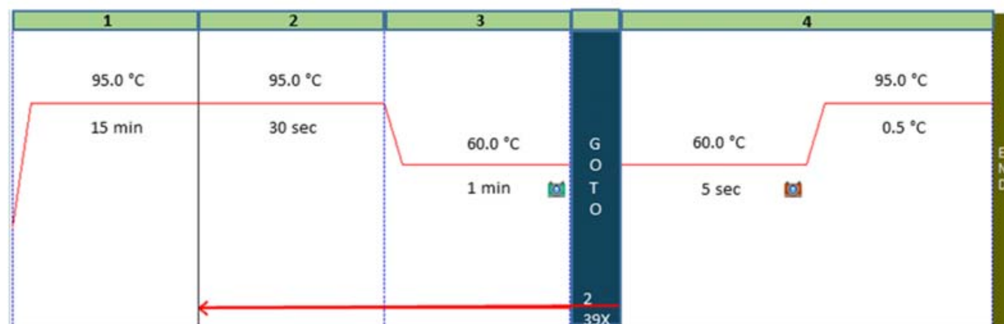


Figure 14- Quantitative PCR amplification conditions. 1, initial denaturation, and DNA polymerase activation; 2, denaturation; 3, annealing/extension; 4, melting curve analysis.

All the analyses were performed in triplicate in a C100^{TM} Thermal Cycler, CFX96^{TM} Real-Time System (BioRad). The Ct was determined automatically by the instrument after setting the baseline at 200 RFU. The data analysis was carried out with the BioRadCFX Manager Software (version 2.1; BioRad). Negative controls were included at all times.

Standard curves construction

Standard curves were created by plotting the Ct values of the qPCR against different concentrations of whole cells, extracted DNA, and lysed cells (10^2 to 10^8 cells/mL). The standard curves were made for each species from the medium, white and red grape must and the white and red wine, using corresponding primer set. The coefficients of efficiency (E) were calculated for each reaction on the basis of the standard curves slopes by the equation $E = 10^{-1/slope} - 1$, as recommended by Bustin et al. (2009).

Industrial wine fermentation monitoring

In order to test the accuracy of the qPCR assay to enumerate yeasts, LAB, and AAB during the winemaking process, samples were taken throughout three spontaneous wine fermentations of Cabernet Sauvignon, Garnacha, and Merlot grape varieties.

Sampling (600 mL) was taken from the three spontaneous wine fermentation at different stages throughout the winemaking process. Wine fermentations were carried out in a winery in the town of Requena (Spain), during the 2015 vintage.

A volume of 500 mL of each sample was sterilised by filtration through 0.22 µm polycarbonate filters, and stored at -20 °C. The remaining volume was used to evaluate the sugars, acids and alcohol content, and to detect and quantify yeasts, LAB, and ABB cells.

The Sugars (fructose, glucose), acids (malic acid, acetic acid, and lactic acid), and alcohol (ethanol) content, in each sampling stage, were determined by HPLC (Frayne, 1986).

The microbial population was determined by qPCR, and also by conventional PCR. Regarding yeasts, viable cells quantification by plate count was also analysed. All the samples were analysed in duplicate. For PCR and qPCR, each sample was washed by centrifugation at 12000 rpm (LabNet International Inc.) with milliU water, next with 10% TEN buffer supplemented with PVP and then, twice with milliU water. *A posteriori*, each sample was subjected to amplification with species-specific primers of *B. bruxellensis*, *S. cerevisiae*, *Z. bailii*, *L. plantarum* and *O. oeni*, and with general yeast and AAB primers. Nevertheless, for yeasts and *L. plantarum* detection and quantification, the samples were previously subjected to mechanical lysis. Except for the first sampling stage, total yeasts and *S. cerevisiae* counts were realized by previous 10 or 100-fold sample dilution.

Conventional PCR reactions were carried out using as sample whole/mechanically lysed cells, following the conditions described in section **PCR reaction conditions**.

The qPCR reactions were carried out using as sample 10 µL of whole/mechanically lysed cells. All qPCR reactions that presented Ct values lower than 33 and fluorescent signal with specific melt curve were considered positive. Increased accuracy was expected by creating a calibration that adapted to wines according to the matrix that they represented. Therefore, the obtained Ct values of each positive reaction were extrapolated to the corresponding standard curve with whole cell or with mechanically lysed cell. The corresponding standard curve is a standard curve that was constructed using as matrix the positive sample previously sterilized by filtration.

For viable cells quantification of yeasts by plate count method, each sample was plated in YPD medium, in duplicate, as described in section **viable cells count by plate**.

A schematic representation of this experimental protocol is shown in **Figure 15**.

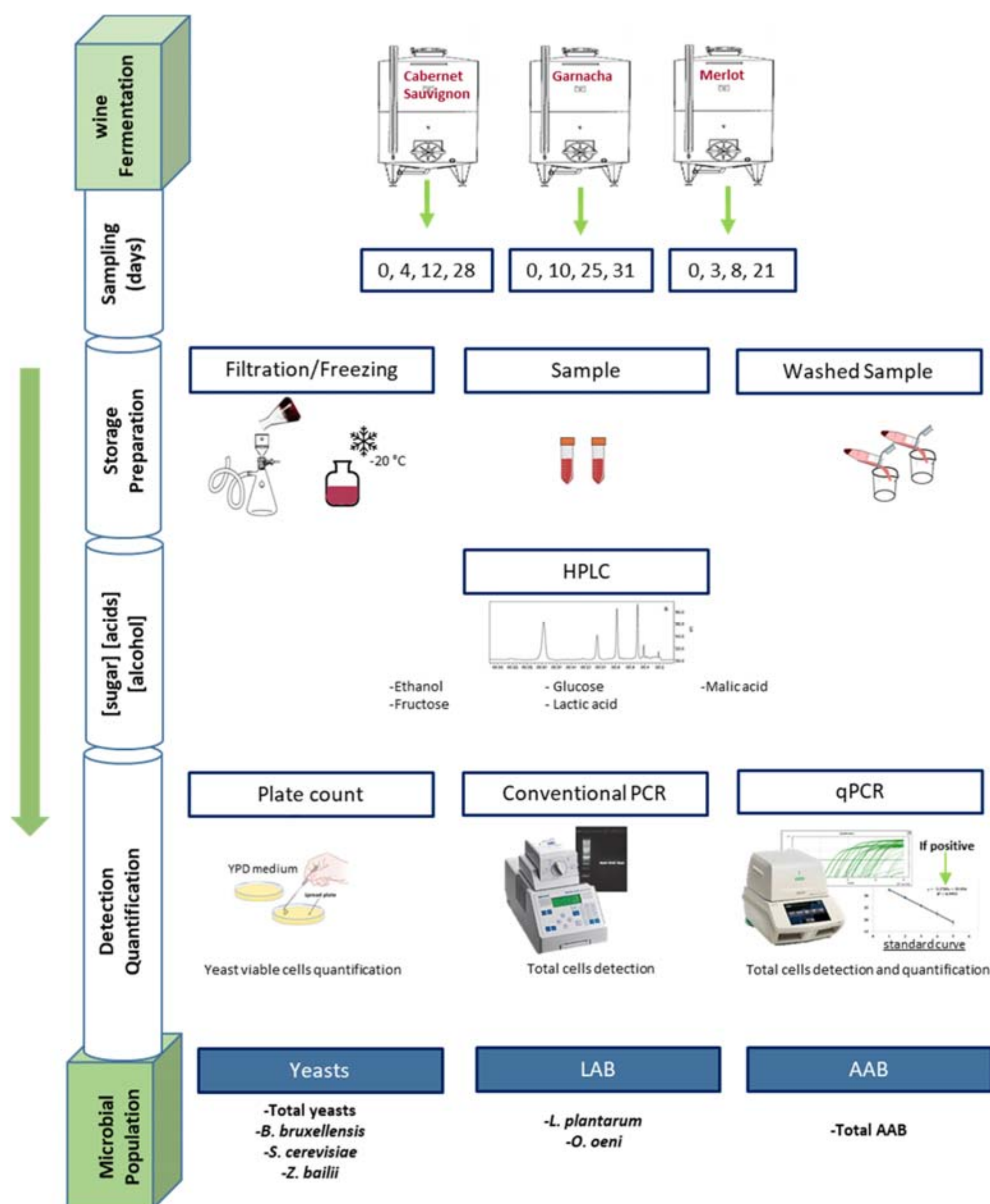


Figure 15-Schematic representation of the experimental protocol used for natural wine fermentation monitoring.

Statistical analysis

All the statistical analyses were performed with version 5.00 of the GraphPad Prism software (California, USA). To determine the significant differences between extracted DNA and whole cells quantification, standard curve efficiencies were analysed by Student´s *t*-test. To determine the effect of matrix and/or wine type on cells quantification ability, standard curve

efficiencies were statistically analysed by one-way ANOVA, followed by Tukey´s multiple comparisons test to determine. To determine the specificity of the qPCR assay, standard curve Cts were statistically analysed by one-way ANOVA, followed by Dunnett´s multiple comparisons test. The one-way ANOVA, followed by Tukey´s multiple comparisons test was also applied to determine the general primers specificity on qPCR standard curves construction. The statistical degree of significance was set at a P value of < 0.05.

3.5.3. Viable cells detection and quantification by propidium monoazide-quantitative PCR

Viable cells detection and quantification by PMA-qPCR was carried out using as sample, mechanically lysed cells, from culture medium and white and red wines. For this purpose, viable and dead cell suspensions of each species were prepared, in independent experiments, in all matrices, and were washed. Dead cells were obtained by heat at 100 °C for 30 minutes in a heat block (Major Science). The lack of cell viability was confirmed by plating. Next, viable and dead cell suspensions were treated with PEMAX, washed twice with milliU water, and subsequently, the cells were mechanically lysed. After that, qPCR amplifications with 10 µL of sample volume, with selected primers were performed, in independent experiments, using both viable and dead cell suspensions.

The qPCR reaction conditions and the selected primers are described in sections **qPCR reaction conditions** and **PCR primers**, respectively.

Optimization of PEMAX concentrations

A 20 mM solution of PEMAX^{TM} (GenIUL) in 20% (v/v) dimethyl sulfoxide (Panreac) was prepared and stored in the dark at -20 °C. To assay the suitable concentration of PEMAX to distinguish between viable and dead cells, both viable and dead cell suspensions of each species were obtained from culture medium. For this, cell suspensions of each species with a final concentration of $3x10^6$ cells/mL were prepared and washed**.** After, each cell suspension was divided into two aliquots; one was heated to obtain heat-killed microorganisms, while the other remained as a viable cell suspension. To each cell aliquot, different amounts of PEMAX were added: 0, 5, 10, 25, 50, 100, 200 µM, and an incubation period of 30 minutes in the dark at room temperature was

performed with constant agitation at the maximum rate (Variomag monoshake, Thermo Electron LED GmbH). Thereafter, the PEMAX-treated cell suspensions were exposed to light using PhAST Blue instrument (GenIUL) for a 15 minutes photo-activation process. Finally, all cell suspensions were washed twice by centrifugation at 1200 rpm (LabNet International Inc) with milliU water, lysed by mechanical lysis, and quantified by qPCR (**Figure 16**). Viable and dead cell suspensions without dye treatment were included at all times as controls.

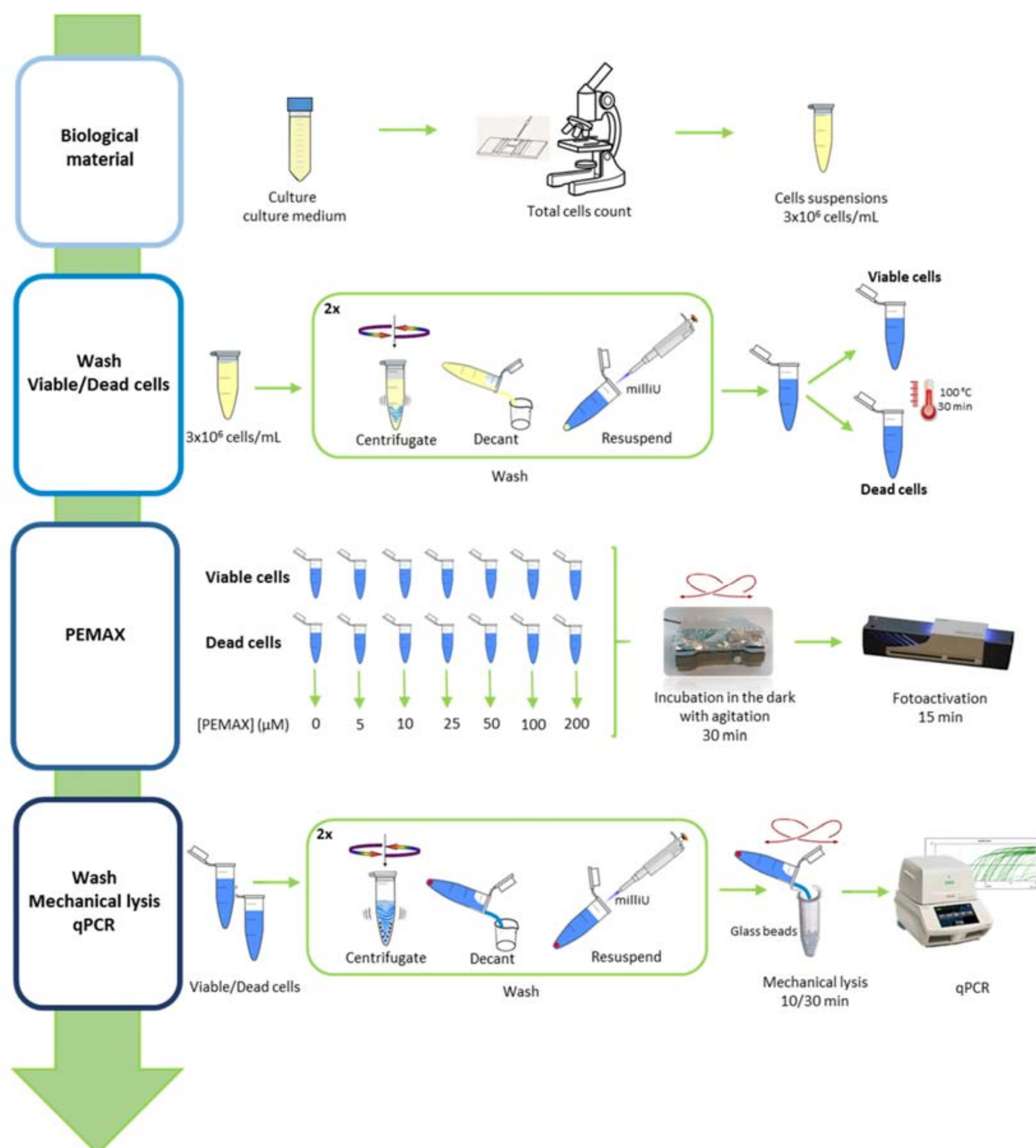


Figure 16- Schematic representation of the experimental protocol used for optimization of PEMAX concentration.

Standard curves construction

Standard curves were created correlating PMA-qPCR and plate counts results. Standard curves were made for each species from the medium, and the white and red wines. For this, cell suspensions comprising only dead cells were previously obtained by heat. After, dead and viable cell suspensions, from 10^2 to 10^8 cells/mL, were prepared in all matrices. Each concentration of viable cell suspensions was enumerated by plate count as described in section **viable cells count by plate** to determine the exact number of viable cells (CFU/mL) in each cell suspension. For dead cell suspensions, only the highest cell concentration was plated. The Ct values produced by the serial dilutions were plotted against the $\log_{10}$ CFU/mL, and the standard curves were constructed. The *E* were calculated for each reaction on the basis of the slope of the standard curves by the equation $E= 10^{-1/slope}-1$, as recommended by Bustin et al. (2009).

Statistical analysis

All the statistical analyses were performed with version 5.00 of the GraphPad Prism software (California, USA). A one-way ANOVA, followed by Tukey´s multiple comparisons test was used to determine the effect of PEMAX concentration on Ct variation of amplification both for viable and dead cells. To determine the significant differences between cells suspension with no PEMAX (0 µM), and cells suspensions with no PEMAX and respective treatment conditions, the Ct values obtained for each condition were analysed by Student´s *t*-test. The statistical degree of significance was set at a *P* value of < 0.05.

3.5.4. Total cells detection by LAMP, and total cells quantification by quantitative LAMP

Total cells detection by LAMP was assayed using as sample extracted DNA, whole or mechanically lysed cells, from culture medium, white and red musts, and white and red wines. For this purpose, cell suspensions of each species, with a final concentration 10^6 cells/mL were prepared in all matrices and were washed. Regarding cell suspensions from culture medium, the DNA was also extracted. Cell suspensions from white and red wines, with a final concentration from 10^2 to 10^8 cells/mL were also assayed. LAMP amplifications with selected/designed primers were performed in independent experiments.

Total cells quantification by qLAMP was carried out using as sample whole and mechanically lysed cells from culture medium. For this purpose, cell suspensions with a final concentration of 10^3, 10^5, and 10^7 cells/mL were prepared and washed. Only for *O. oeni* species, cells suspensions with a final concentration from 10^2 to 10^8 cells/mL were also assayed. qLAMP amplifications with selected/designed primers were performed in independent experiments.

LAMP primers

For specific amplification of *B. bruxellensis*, 5 primers previously described (Hayashi et al., 2009) with target sequence in the ITS region (between the 5.8S and 26S rRNA genes), were selected (**Table 6**). For more information about the primers see **Annex 2**.

Table 6- Sequences of the specific LAMP primers set for *B. bruxellensis.*

Primer	Sequence (5´-3´)
DB1LF1-F3 (	ACATTGCGCCCTCTGG
DB1LF1-B3	TGCTTAAGTTCAGCGGGTC
DB1LF1-FIP	ACCCTCGTGTAATCTCATAACCACTAAGGAGGGCATGCCTGTTTG
DB1LF1-BIP	GATTTAAGGTTTCGGCCGTTCATTATTTTCTCCTACCTGATTTGAGGTCAA
DB1LF1-LoopF	AGTGAGAAGGAAATGACGC

For specific amplification of *O. oeni*, a total of 6 primers were designed based on the 16S rRNA gene. The nucleic acid sequence of 16S rRNA gene of *O. oeni* was searched in GenBank database, and the accession number of the sequence was NR_040810. The sequence was further analysed by LAMP Designer 1.13 software to have the LAMP primers: two outer (F3 and B3), two inner (FIP and BIP), and two loop (loopF and loopB).

The specificity of the designed primers was checked by both conventional PCR and LAMP. PCR reactions were performed with F3/B3 and FIP/BIP primers, in independent experiments, and LAMP reactions were performed with the six primers in the same experiment. Both of them used the bacterial and yeast species from CECT collection listed in **Table 7**. DNA samples from all species were isolated, from cells suspensions of 10^8 cells/mL, as described in section **DNA extraction**. PCR reactions were carried out in a total volume of 50 µL. Each reaction contained 200 µM of dNTPs, 1 µM of each primer, 20 mM of Tris-HCl, pH 8.4, 50 mM of KCl, 2 mM of $MgCl_2$, 2 U of Taq Polymerase

(Invitrogen) and 2 µL of extracted DNA. The reactions were performed in a Mastercycle Personal 5332 (Eppendorf), and the conditions were an initial denaturation at 94 °C for 5 minutes; followed by 35 cycles of denaturing at 94 °C for 30 seconds, annealing at 52 °C for 30 seconds, and extension at 72 °C for 1 minute; with a final extension at 72 °C for 5 minutes. Negative controls were included at all times. The products of the PCR were analysed by gel electrophoresis (2% (w/v) in 0.5X Tris-borate-EDTA buffer), stained with 0.5 µg/mL ethidium bromide and visualized by GelPrinter Plus (TDI). A 1 Kb plus DNA Ladder (Invitrogen) was used for size standards.

Table 7- List of used species for specificity evaluation of *O. oeni* LAMP primers.

	Microorganism	Strain
yeasts	*B. bruxellensis*	CECT 1451^{T}
	S. cerevisiae	CECT 2056
LAB	*L. brevis*	CECT 216
	L. hilgardii	CECT 4786
	L. plantarum	CECT 748^{T}
	Lc. mesenteroides	CECT 394
	O. oeni	CECT 218
	Pd. damnosus	CECT 4692
	Pd. pentosaceus	CECT 4695
AAB	*A. aceti*	CECT 298^{T}
	G. oxydans	CECT 4009

LAMP reaction conditions

The LAMP reactions were performed using *Bst* polymerase 2.0 (New England BioLabs), and following the conditions suggested by the manufacturer. Therefore, the LAMP reactions were carried out in a total volume of 25 µL. Each reaction contained 1.4 mM of dNTPs, 0.2 µM of each outer primer, 1.6 µM of each inner primer, 0.8 µM of each loop primer, 8 mM of $MgCl_2$, (Invitrogen), 1X Isothermal Amplification Buffer, 0.4 U/µL of *Bst* polymerase 2.0, and 2.5 µL of extracted DNA or 12.5 µL of whole/mechanically lysed cells. The LAMP amplifications were carried out in a Mastercycle Personal 5332 (Eppendorf), which was operated at a constant temperature of 62 °C during 1 hour. Negative controls were included at all times. The amplified products were analysed visually by direct detection of turbidity. To confirm the results, the amplified products were also

resolved by agarose gel electrophoresis (2% (w/v) in 0.5X Tris-borate-EDTA buffer), stained with 0.5 µg/mL ethidium bromide and visualized by GelPrinter Plus (TDI). A 1 Kb plus DNA Ladder (Invitrogen) was used for size standards.

Assessment of LAMP assay sensitivity in wine

Cell suspensions of *B. bruxellensis* and *O. oeni*, with a final concentration from 10^2 to 10^8 cells/mL were prepared in white and red wine matrices. After cell suspensions wash, each dilution was used as a template for LAMP assay. Regarding *O. oeni* cell suspensions, mechanically lysed cells were also assayed. The LAMP amplifications with selected primers were performed, in independent experiments, using whole cells or mechanically lysed cells. Sensitivity tests were repeated twice.

Quantitative LAMP reaction conditions

The qLAMP reactions were carried out in a total volume of 25 µL. Two different model systems were used for comparison of SYBR Green I and SYTO-9 fluorescent dyes. Each reaction contained 1.4 mM of dNTPs, 0.2 µM of each outer primer, 1.6 µM of each inner primer, 0.8 µM of each loop primer, 8 mM of $MgCl_2$, (Invitrogen), 1X Isothermal Amplification Buffer, 0.4 U/µL of *Bst* polymerase 2.0 (New England BioLabs), 11.5 µL of whole/mechanically lysed cells, and 0.4 µM of SYTO-9 (Invitrogen) or 1 µL of 10x SYBR Green I (Invitrogen).

The LAMP amplifications were performed in triplicate in a C100^{TM} Thermal Cycler, CFX96^{TM} Real-Time System (BioRad), which was operated at a constant temperature of 62 °C during 1 hour. Fluorescence signals were collected every minute, followed by melting curve analysis obtained by slow heating from 60 °C to 95 °C at 0.5 °C every 5 seconds, with continuous fluorescence collection (**Figure 17**). During the amplification, the fluorescence data were obtained on the 6-carboxyfluorescein (FAM) channel (excitation at 450-495 nm and detection at 510-527 nm). The RFU threshold value was used and threshold time (Tt) was calculated as the time at which the fluorescence equalled the threshold value. The data analysis was carried out with the BioRadCFX Manager Software (version 2.1; BioRad). Negative controls were included at all times.

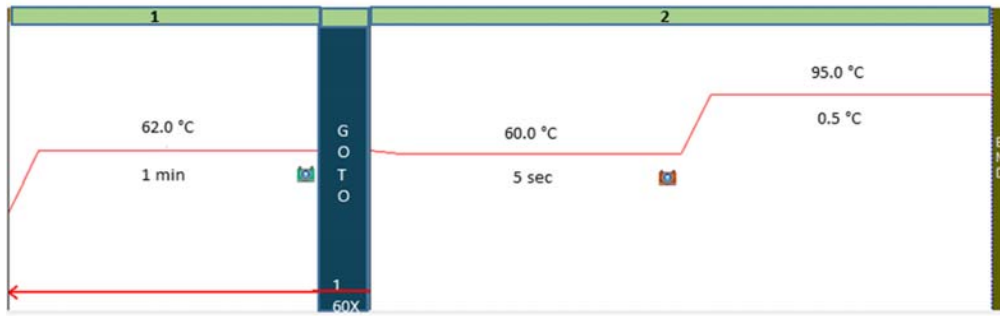


Figure 17- Quantitative LAMP amplification conditions. 1 amplification; 2, melting curve analysis.

Standard curve construction

A standard curve was only made for *O. oeni* from culture medium, and it was created by plotting the Tt values of the qLAMP against different concentrations of cell suspensions (10^2 to 10^8 cells/mL). The *E* was calculated on the basis of the standard curve slope by the equation $E= 10^{-1/slope}-1$, as recommended by Bustin et al. (2009).

4. RESULTS AND DISCUSSION

4.1 Development of a qPCR Method for Direct Total Cells Detection and Quantification (Cells-qPCR)

4.2 Development of a PMA-qPCR Method for Direct Viable Cells Detection and Quantification (PMA-Cells-qPCR)

4.3 Development of a LAMP Method for Direct Total Cells Detection (Cells-LAMP)

4.4 Development of a qLAMP Method for Direct Total Cells Detection and Quantification (Cells-qLAMP)

4.1. Development of a qPCR Method for Direct Total Cells Detection and Quantification (Cells-qPCR)

4.1.1. DNA amplification by conventional PCR directly from whole cells

Firstly, in order to evaluate the applicability of DNA amplification directly from cells, a conventional PCR assay was applied by comparing PCR amplifications from cell suspensions and from the DNA extracted from the same cell suspensions. The assay was applied for all selected primers. *S. cerevisiae* and *A. aceti* were the selected species for total cells detection with general yeast and AAB primers, respectively. The amplifications showed a common fragment with the expected size of both DNA and whole cells from all the tested primers (**Figure 18**), which proved that it is possible to do away with the DNA extraction step for DNA amplification.

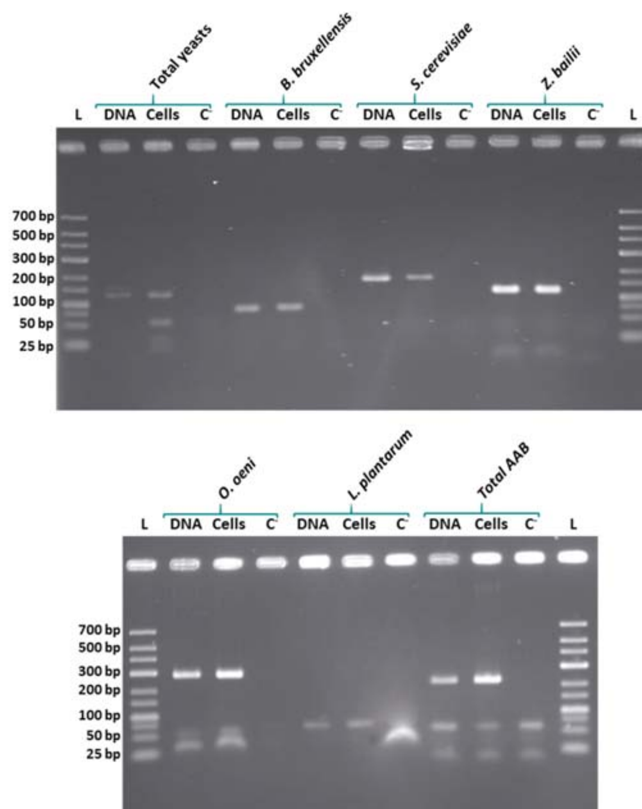


Figure 18- Agarose gel electrophoresis of conventional PCR amplification from DNA and whole cells, of yeasts, *B. bruxellensis*, *S. cerevisiae*, *Z. bailii*, *L. plantarum*, *O. oeni*, and acetic acid bacteria (AAB). C^-, negative control; L, GeneRuler Low-Range Ladder (Thermo Scientific).

4.1.2. Comparing qPCR detection and quantification directly from whole cells and extracted DNA

Considering that is possible amplify DNA directly from cells by conventional PCR, and in order to extend it to qPCR method, qPCR amplifications from whole cells and extracted DNA, were compared. This experiment was carried out with *B. bruxellensis*, *S. cerevisiae*, *L. plantarum*, *O. oeni*, *A. aceti*, and *G. oxydans* pure cultures from culture media. A 2-microliter volume of each DNA and cell concentration were subjected to qPCR with specific primers in independent experiments to obtain amplification curves. The results were reported as Ct *versus* RFU. For the DNA extractions, the assay was linear over seven orders of magnitude for all the tested species, with a correlation coefficient (R^2) higher than 0.99. Regarding whole cells, the assay showed different levels of sensitivity and various LoQs. *B. bruxellensis*, *S. cerevisiae, L. plantarum* and *O. oeni* displayed linearity in the order of four magnitudes and the LoQ was 10^5 cells/mL (=10^3 cells per reaction tube) with R^2 above 0.98 (see **Figure 19** for an example).

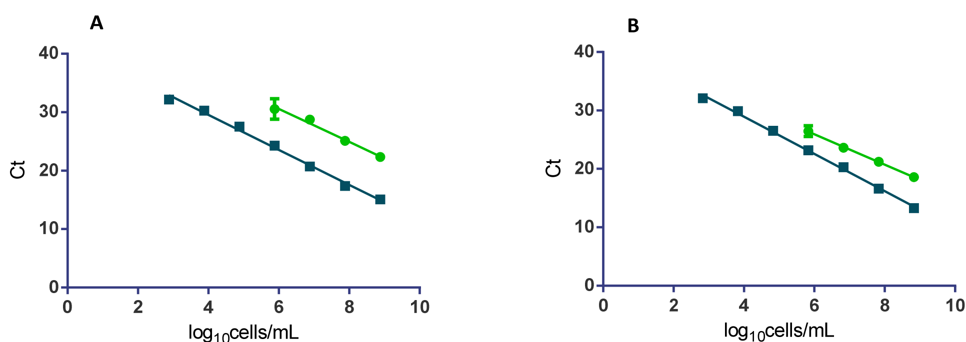


Figure 19- Standard curves obtained by whole cells (●) and extracted DNA (■) with a sample volume of 2 µL qPCR comparison from serial dilutions of (**A**) *B. bruxellensis*, and (**B**) *S. cerevisiae* in culture medium. The Ct values are the averages of three replicates. Errors bars represent standard errors. In all standard curves represented in this work, only linear results were shown.

In spite of the low LoQ observed for whole cells, the Tm obtained for these cell concentrations were species-specific (**Figure 20**). These results ratified and confirmed the effectiveness of the Cells-qPCR procedure to detect and identify these species.

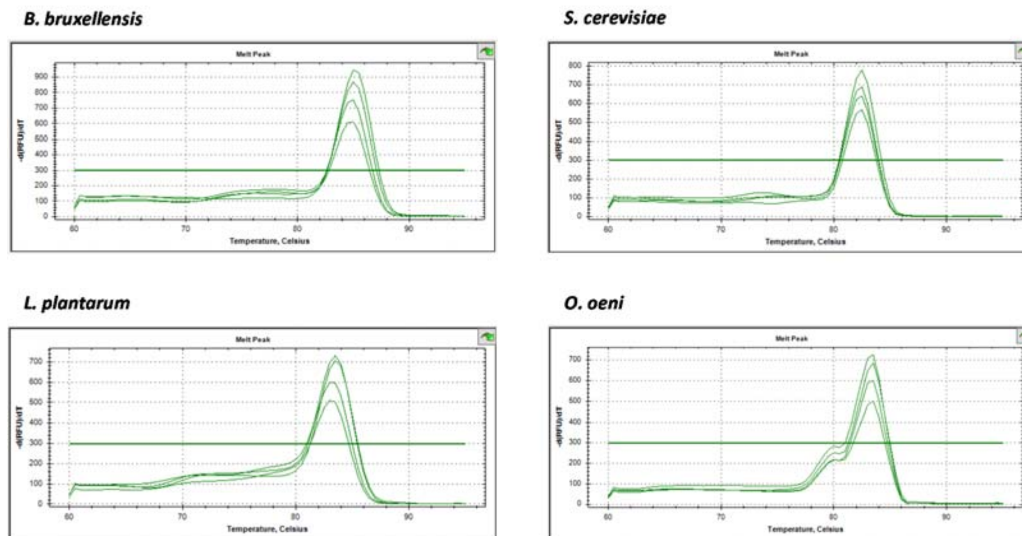


Figure 20- Melting curves analysis of the Cells-qPCR products from *B. bruxellensis*, *S. cerevisiae*, *L. plantarum*, and *O. oeni* species.

The results obtained under these experimental conditions showed lower consistency and reproducibility for both AAB species (*A. aceti* and *G. oxydans*).

In order to decrease the minimum population detectable by Cells-qPCR (10^5 cells/mL) in the former experiment, and to achieve consistent and reproducible AAB cell detection and quantification, higher cell suspension volumes were used in the template. Instead of 2 µL of whole cells, 10 µL were added to obtain 5-fold more cells. For this purpose, the assay was repeated with standard curves with the extracted DNA and whole cells constructed for each species. Therefore, a 2-µL volume of each DNA and a 10-µL volume of each cell suspension were subjected to qPCR amplification in independent experiments with specific primers.

The standard curves obtained for cell quantification showed that higher cell volumes led to an increased sensitivity and higher LoQs, and also to accurate AAB cell detection and quantification (**Figure 21**). For *B. bruxellensis* and *L. plantarum*, the assay recorded a 10-fold lower LoQ (10^4 cells/mL), which corresponded to 100 cells per reaction tube. Nevertheless, the *L. plantarum* standard curve displayed a linearity of over five orders of magnitude, while the *B. bruxellensis* standard curve exhibited one of over four orders of magnitude. For *S. cerevisiae*, the assay recorded a 100-fold lower LoQ (10^3 cells/mL, which corresponded to 10 cells per reaction tube). In short, the quantification ranges obtained from these three species were: *L. plantarum* (10^4-10^8 cells/mL=10^2-

10^6 cells/reaction tube), *B. bruxellensis* (10^4-10^7 cells/mL=10^2-10^5 cells/reaction tube) and *S. cerevisiae* (10^3-10^7 cells/mL=10-10^5 cells/reaction tube). For *O. oeni, A. aceti* and *G. oxydans*, linearity was achieved with values that ranged from 10^2 to 10^8 cells/mL. Therefore, the obtained standard curves had a linearity of over seven orders of magnitude. This LoQ meant that for these species, it was possible to detect as little as 1 cell per reaction tube.

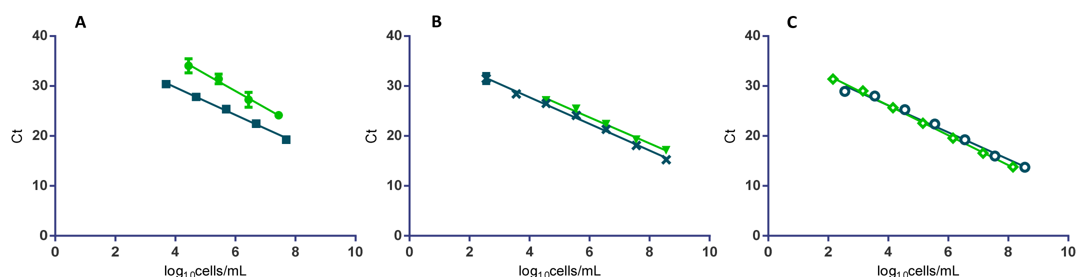


Figure 21- Standard curves obtained by Cells-qPCR with a sample volume of 10 µL from the 10-fold serial dilutions of (**A**) *B. bruxellensis* (●), *S. cerevisiae* (■), (**B**) *L. plantarum* (▼), *O. oeni* (**x**), and (**C**) *A. aceti* (◊), *G. oxydans* (○) in culture media. The Ct values are the averages of three replicates. Error bars represent standard errors.

Regarding yeasts, the *B. bruxellensis* standard Ct values were higher than the *S. cerevisiae* Ct ones. Nevertheless, standard Ct values between LAB species, and also between AAB species were similar. For the AAB species, taking into account that the selected primers were used in this way for the first time, this result means that these primers can be used for qPCR assays, and are also useful for generating standard curves with different AAB species, allowing its detection and quantification with the same sensitivity and specificity. Moreover, the Tm obtained were AAB-specific (**Figure 22**), confirming the specificity of the assay to detect and quantify AAB species. This is very important for winemaking, where lack of any AAB is desired, and a general detection qPCR system run for all the AABs simultaneously is extremely useful.

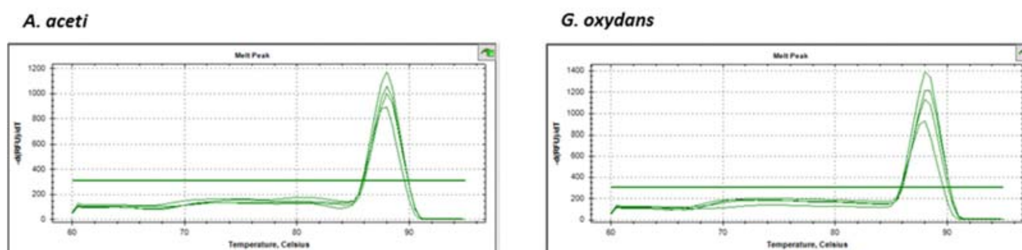


Figure 22- Melting curves analysis of the Cells-qPCR products from *A. aceti*, and *G. oxydans* species.

All the obtained Cells-qPCR standard curves showed a good R^2 (> 0.99), and the minor standard errors among the repetitions revealed that the assays were reproducible and robust. Therefore, these results showed that larger sample volumes improve the assay sensitivity and precision, also minimising errors.

To endorse the Cells-qPCR method, it was tested against the traditional qPCR method using DNA isolated from the same cell suspensions. The results obtained when comparing standard curves showed a good correlation between both the DNA and cell quantification for all the tested species (**Figure 23**).

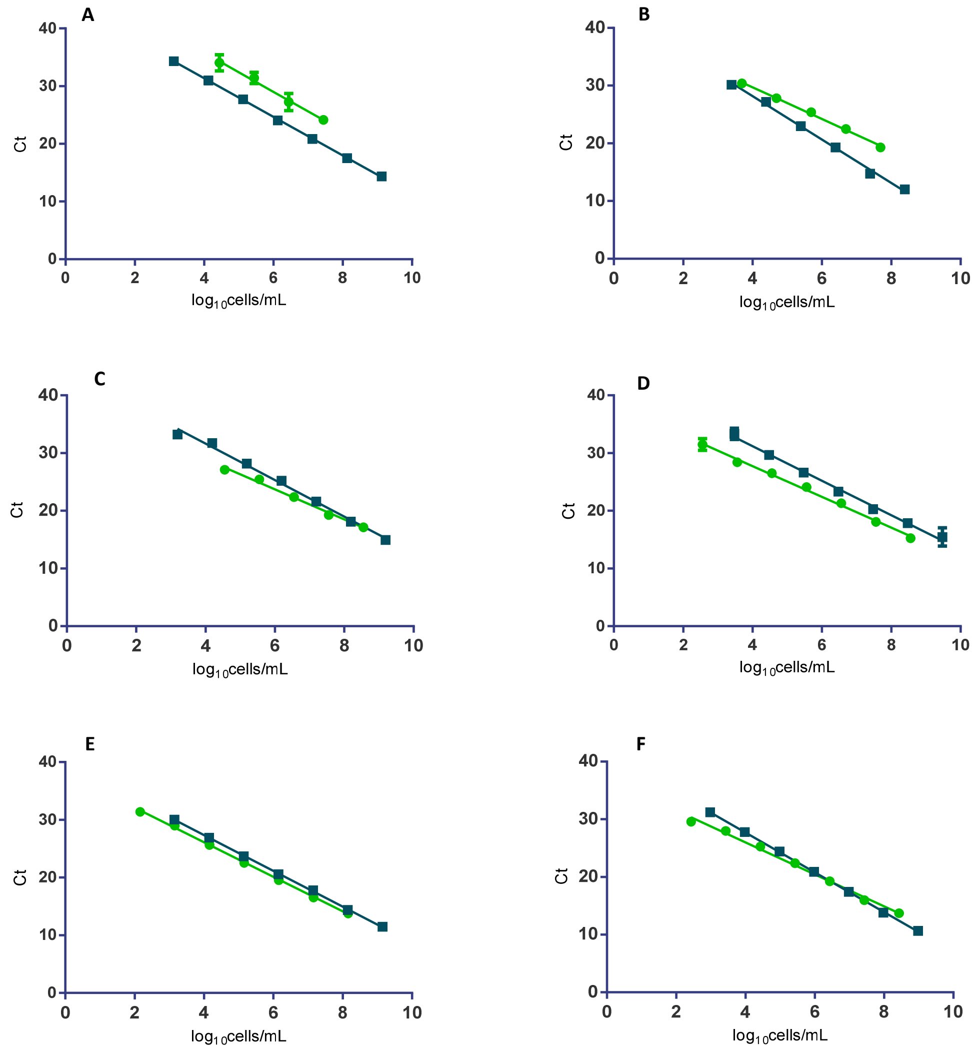


Figure 23- Standard curves obtained by whole cells with a sample volume of 10 µL (●), and extracted DNA with a sample volume of 2 µL (■) qPCR comparison from the 10-fold serial dilutions of (**A**) *B. bruxellensis*, (**B**) *S. cerevisiae*, (**C**) *L plantarum*, (**D**) *O. oeni*, (**E**) *A. aceti*, and (**F**) *G. oxydans* in culture media. The Ct values are the averages of three replicates. Errors bars represent standard errors.

For yeast cells, the Ct value of each dilution was higher than the equivalent sample obtained from DNA. However, a parallelism between both the DNA and cell standard curves was observed. For the LAB and AAB cells, the cell Ct values were similar or lower than the DNA Ct values. These results confirmed the effectiveness of the Cells-qPCR method to quantify different cell concentrations of *B. bruxellensis*, *S. cerevisiae, L. plantarum, O. oeni,* and the AAB species, and to avoid DNA extraction.

4.1.3. White grape must and wine standard curves from whole cells

The ability of the Cells-qPCR assay to detect and quantify yeasts, LAB and AAB in white grape must and wine was evaluated by considering 10 µL to be the best sample volume for the quantification of whole cells. For the analysis, the standard curves for each species were constructed in both white grape must and white wine by Cells-qPCR. For this purpose, the 10-fold serial diluted cells from 10^8 to 10^2 cells/mL were prepared in white grape must/white wine using the same matrix as the diluent to obtain the same matrix composition in all the cell suspensions. This approach allows the direct detection and quantification of microorganisms from grape must and wine samples, which led to more accurate results, independently of the concentration of the microorganism present in the samples. Each cell suspension was then washed with TEN buffer, which was also used by Pinzani et al. (2004) to wash cells before DNA extraction. The data regression analysis (**Figure 24**) showed the effectiveness of the Cells-qPCR methodology to detect and quantify yeasts, LAB and AAB in complex matrices, such as white grape must and white wine, which contain PCR inhibitors, e.g., tannins, polysaccharides and ethanol (Demeke and Jenkins, 2010; Schrader et al., 2012; Wilson, 1997).

The results showed that for both the white grape must and wine standard curves, the LoQs were the same as those obtained for the culture medium: *B. bruxellensis* (10^4-10^7 cells/mL=10^2-10^5 cells/reaction tube), *S. cerevisiae* (10^3-10^7 cells/mL=10-10^5 cells/reaction tube), *L. plantarum* (10^4-10^8 cells/mL=10^2-10^6 cells/reaction tube), *O. oeni*, *A. aceti* and *G. oxydans* (10^2-10^8 cells/mL=1-10^6 cells/reaction tube) (**Figure 24**). Furthermore, the obtained R^2 was higher than 0.98 in all cases. These results were strikingly similar to those obtained for the same cells in a synthetic medium, which suggests that the samples obtained from white grape must and white wine do not affect assay reproducibility for both high and low cell concentrations.

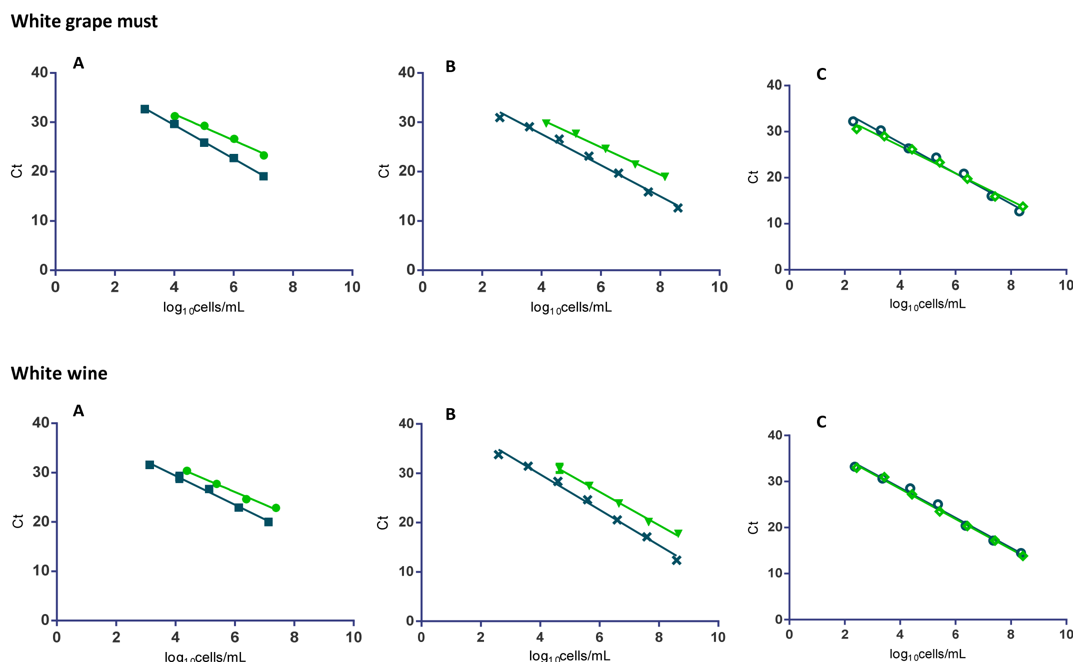


Figure 24- Standard curves obtained by Cells-qPCR from the 10-fold serial dilutions of (**A**) *B. bruxellensis* (●), *S. cerevisiae* (■), (**B**) *L. plantarum* (▼), *O. oeni* (x), and (**C**) *A. aceti* (◊), *G. oxydans* (○) in white grape must and white wine. The Ct values are the averages of three replicates. Error bars represent standard errors.

4.1.4. Red grape must and wine standard curves from whole cells

Tannins, polysaccharides, polyphenols, and ethanol are mainly responsible for PCR inhibition (Demeke and Jenkins, 2010; Schrader et al., 2012; Wilson, 1997). These compounds are present in wine-related matrices, but their inhibitory effect is stronger in the red grape musts and wines with a higher polyphenol concentration than white ones (Martorell et al., 2005; Phister and Mills, 2003; Tofalo et al., 2012). Therefore, in order to construct standard curves from red grape must and wine, 10-fold serial dilutions of cells were prepared as for the construction of the white grape must and wine standard curves. In this case, however, two different buffers were assayed while washing cell suspensions, in independent experiments. The difference between buffers was that one was supplemented with PVP, which is a water-soluble polymer used in winemaking as a fining agent, known for its ability to bind polyphenolic groups (Castillo-Sánchez et al., 2008). PVP has been widely used before DNA extraction steps by other authors (Jara et al., 2008; Tessonnière et al., 2009; Torija et al., 2010) to remove polyphenols from complex matrices such as wine. After

cells wash, with both buffers, the colour comparison between samples showed that cells suspensions washed with TEN buffer supplemented with PVP exhibited less colour (see **Figure 25** for an example).

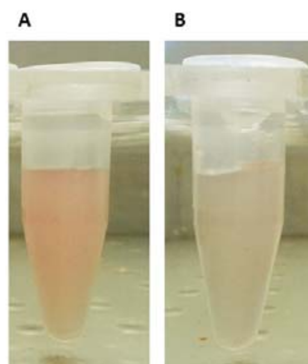


Figure 25- Colour comparison between *B. bruxellensis* cell suspensions from red wine after wash with (**A**) TEN buffer, and (**B**) TEN buffer supplemented with PVP.

Moreover, the results obtained from the cell suspensions, washed using only TEN buffer, showed inhibition (Ct=0) for the cell concentrations above 10^6 cells/mL for all the species and in both matrices.

Nevertheless, when cell suspensions were washed using TEN buffer supplemented with PVP, no inhibition was observed (**Figure 26**).

By analysing these results and taking into account that all the 10-fold serial diluted cells had the same matrix composition, quantification inhibition of high cell concentrations can be explained by potential inhibitors, such as polyphenols, that can be adsorbed by cells; consequently, the higher the cell concentration is, the higher the inhibitors concentration becomes. The use of TEN buffer supplemented with PVP solved this inhibition, which suggests that PVP effectively removes potential inhibitors to allow the quantification of a high cell concentration for all the species by the Cells-qPCR method. The standard curves obtained by cell suspensions, washed with TEN buffer supplemented with PVP, from both matrices showed an R^2 above 0.98. The LoQs for all the species were the same as those obtained from the culture medium, white grape must and white wine (**Figure 26**), which proves the effectiveness of the method with such matrices.

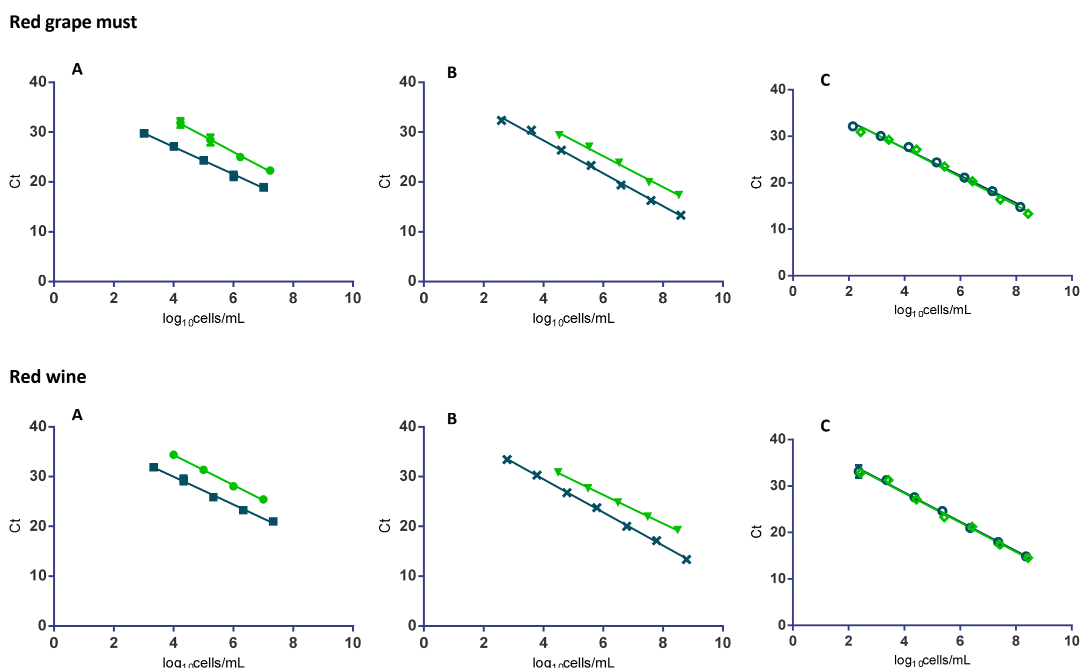


Figure 26- Standard curves obtained by Cells-qPCR from the 10-fold serial dilutions of (**A**) *B. bruxellensis* (●), *S. cerevisiae* (■), (**B**) *L. plantarum* (▼), *O. oeni* (x), and (**C**) *A. aceti* (◊), *G. oxydans* (○) in red grape must and red wine. The Ct values are the averages of three replicates. Error bars represent standard errors.

4.1.5. Effect of whole cells on qPCR efficiency

The robustness and precision of the Cells-qPCR method were tested by correlating DNA and cells' standard curves *E*, obtained from the different matrices of each species. So, three standard curves were constructed from extracted DNA and from whole cells of each matrix and species. Later the results were tested with a *t-test* analysis. The R^2, slopes, and *E* of the obtained standard curves are shown in **Table 8**.

Table 8- Correlation coefficients (R^2), slopes, coefficients of efficiency (*E*), and quantification limits (LoQ) of standard curves obtained from different concentrations of extracted DNA and whole cells from (**A**) yeasts, (**B**) LAB, and (**C**) AAB species in culture medium, and in white and red grape musts and wines[a].

A

Microorganism	Matrix	R^2		Slope		E^b		LoQ (cells/mL)	
		DNA	Cells	DNA	Cells	DNA	Cells	DNA	Cells
	Culture medium	0.996 ± 0.006	0.995 ± 0.004	-3.340 ± 0.009	-3.281 ± 0.077	0.993 ± 0.004	1.019 ± 0.033		
	White must	0.998 ± 0.001	0.993 ± 0.006	-3.222 ± 0.086	-3.025 ± 0.137	1.045 ± 0.039	1.145 ± 0.072		
B. bruxellensis	Red must	0.998 ±0.001	0.985 ± 0.009	-3.133 ± 0.125	-2.995 ± 0.171	1.089 ± 0.061	1.164 ± 0.092	$10^2 – 10^8$	$10^4 – 10^7$
	White wine	0.998 ± 0.001	0.994 ± 0.003	-3.259 ± 0.044	-2.815 ± 0.132	1.027 ± 0.019	1.272 ± 0.091		
	Red wine	0.997 ± 0.002	0.997 ± 0.002	-3.179 ± 0.063	-3.023 ± 0.047	1.064 ± 0.030	1.142 ± 0.025		
	Culture medium	0.996 ± 0.004	0.995 ± 0.004	-3.759 ± 0.022	-2.761 ± 0.027	0.845 ± 0.007	1.303 ± 0.019		
	White must	0.992 ± 0.001	0.994 ± 0.006	-3.132 ± 0.002	-2.925 ± 0.384	1.086 ± 0.001	1.243 ± 0.091		
S. cerevisiae	Red must	0.994 ± 0.002	0.994 ± 0.010	-3.105 ± 0.066	-2.832 ± 0.132	1.100 ± 0.033	1.260 ± 0.083	$10^2 – 10^8$	$10^3 - 10^7$
	White wine	0.996 ± 0.002	0.989 ± 0.006	-3.174 ± 0.045	-2.931 ± 0.105	1.066 ± 0.021	1.197 ± 0.062		
	Red wine	0.995 ± 0.002	0.988 ± 0.005	-2.796± 0.091	-2.791 ± 0.051	1.281 ± 0.060	1.283 ± 0.034		

B

Microorganism	Matrix	R^2		Slope		E^b		LoQ (cells/mL)	
		DNA	Cells	DNA	Cells	DNA	Cells	DNA	Cells
	Culture medium	0.992 ± 0.006	0.996 ± 0.004	-3.165 ± 0.043	-2.862 ± 0.187	1.070 ± 0.021	1.247 ± 0.124		
	White must	0.998 ± 0.001	0.995 ± 0.001	-3.053 ± 0.070	-2.787 ± 0.084	1.127 ± 0.036	1.287 ± 0.058		
L. plantarum	Red must	0.998 ± 0.001	0.992 ± 0.003	-3.155 ± 0.052	-3.117 ± 0.050	1.075 ± 0.025	1.094 ± 0.025	$10^2 – 10^8$	$10^4 – 10^8$
	White wine	0.990 ±0.002	0.993 ± 0.002	-3.038 ± 0.041	-3.388 ± 0.120	1.134 ± 0.022	0.975 ± 0.049		
	Red wine	0.996 ± 0.002	0.997 ± 0.003	-3.177 ± 0.094	-2.780 ± 0.305	1.066 ± 0.043	1.320 ± 0.195		
	Culture medium	0.990 ± 0.011	0.994 ± 0.005	-2.988 ± 0.178	-2.945 ± 0.457	1.169 ± 0.101	1.239 ± 0.250		
	White must	0.999 ± 0.001	0.994 ± 0.005	-3.357 ± 0.049	-3.219 ± 0.125	0.986 ± 0.020	1.048 ± 0.055		
O. oeni	Red must	0.994 ± 0.003	0.996 ± 0.001	-2.974 ± 0.076	-3.298 ± 0.082	1.170 ± 0.042	1.011 ± 0.035	$10^2 – 10^8$	$10^2 – 10^8$
	White wine	0.993 ± 0.002	0.992 ± 0.001	-3.149 ± 0.066	-3.747 ± 0.216	1.078 ± 0.032	0.853 ± 0.063		
	Red wine	0.997 ± 0.002	0.999 ± 0.001	-3.158 ± 0.056	-3.327 ± 0.028	1.074 ± 0.027	0.998 ± 0.012		

C

Microorganism	Matrix	R^2		Slope		E^b		LoQ (cells/mL)	
		DNA	Cells	DNA	Cells	DNA	Cells	DNA	Cells
A. aceti	Culture medium	0.999 ± 0.001	0.998 ± 0.001	-3.096 ± 0.040	-2.974 ± 0.162	1.104 ± 0.020	1.176 ± 0.093		
	White must	0.998 ± 0.001	0.990 ± 0.005	-3.058 ± 0.044	-2.972 ± 0.032	1.124 ± 0.023	1.170 ± 0.018		
	Red must	0.999 ± 0.002	0.985 ± 0.009	-3.373 ± 0.077	-3.048 ± 0.097	0.980 ± 0.032	1.131 ± 0.053	$10^2 – 10^8$	$10^2 – 10^8$
	White wine	0.990 ± 0.001	0.993 ± 0.001	-3.100 ± 0.033	-3.279 ± 0.094	1.102 ± 0.017	1.020 ± 0.041		
	Red wine	0.980 ± 0.009	0.991 ± 0.005	-2.970 ± 0.046	-3.198 ± 0.012	1.172 ± 0.026	1.055 ± 0.012		
G. oxydans	Culture medium	0.999 ± 0.001	0.993 ± 0.001	-3.452 ± 0.063	-3.047 ± 0.164	0.949 ± 0.023	1.136 ± 0.092		
	White must	0.993 ± 0.002	0.986 ± 0.001	-3.161 ± 0.038	-3.313 ± 0.028	1.072 ± 0.018	1.004 ± 0.012		
	Red must	0.996 ± 0.001	0.993 ± 0.004	-3.342 ± 0.129	-2.943 ± 0.105	0.994 ± 0.055	1.190 ± 0.062	$10^2 – 10^8$	$10^2 – 10^8$
	White wine	0.991 ±0.003	0.988 ±0.005	-2.970 ± 0.054	-3.257 ± 0.047	1.172 ± 0.030	1.028 ± 0.021		
	Red wine	0.990 ± 0.005	0.994 ±0.008	-3.112 ± 0.132	-3.151 ± 0.116	1.100 ± 0.069	1.080 ± 0.057		

[a] All values are means and standard errors.

[b] Coefficients of efficiency were estimated by the formula $10^{-1/slope}$-1.

All the obtained *E* were higher than 0.84. After the statistical analysis, no significant differences were observed in the *E* between the extracted DNA and whole cells of the LAB and AAB species, although significant differences were found for the yeast species (**Table 9**).

Table 9- Statistical results of coefficients of efficiency (*E*) between extracted DNA and whole cells of the yeast, LAB and AAB species

Microorganism	DNA´ standard curves *E*	Whole cells´ standard curves *E*	*t*	ρ-value
	Average	Average		
B. bruxellensis	1.043	1.148	2.415	< 0.05
S. cerevisiae	1.076	1.257	2.530	< 0.05
L. plantarum	1.095	1.185	1.349	> 0.05
O. oeni	1.096	1.030	0.926	> 0.05
A. aceti	1.096	1.110	0.313	> 0.05
G. oxydans	1.058	1.087	0.575	> 0.05

This result allows the Cells-qPCR assay authentication with a standard qPCR method that uses DNA as a template and confirms the robustness and precision of this novel methodology.

For the detection and quantification of *O. oeni* and AAB, linearity was obtained with populations between 10^2 and 10^8 cells/mL from both extracted DNA and whole cells in all the matrices (culture medium, white and red grape musts and wines). These results were similar or better than those reported in other studies, where the DNA quantification range was 10^3-10^9 cells/mL for *O. oeni* (Pinzani et al., 2004), and 10^3-10^7 cells/mL (González et al., 2006) for the AAB species. Moreover, these results indicate that it is possible to detect one cell per reaction through Cells-qPCR, which highlights the good sensitivity of the developed method. Regarding the detection and quantification *of L. plantarum*, linearity was obtained with populations that had 10^2 and 10^8 cells/mL from DNA in all the matrices. Similar results have been reported by Gyu-Sung et al. (2011) to quantify *L. plantarum* DNA by qPCR. Nevertheless, whole cells quantification by Cells-qPCR was accurate for the concentrations between 10^4 and 10^8 cells/mL in all the matrices. Thus, using cells instead of DNA narrows the quantification range in this case. For the detection and quantification *of B. bruxellensis* and *S. cerevisiae*, DNA linearity was the same as that obtained for the other species (10^2-10^8 cells/mL) in all the matrices. Regarding whole cell quantification, ranges of 10^4-10^7 cells/mL and 10^3-10^7 cells/mL were obtained for *B. bruxellensis* and *S. cerevisiae,* respectively, in all the matrices. The quantification ranges were similar to those obtained by Delaherche et al. (2004) for

B. bruxellensis and Hierro et al. (2006) for total yeasts, where DNA linearity was obtained between 10^4-10^6 CFU/mL and 10^3-10^6 CFU/mL, respectively. Nevertheless, were lower than those obtained by other authors who detected an LoQ of 10-10^6 CFU/mL (Martorell et al., 2005; Phister and Mills, 2003), 10^2-10^6 cells/mL (Hierro et al., 2007), and 10-10^8 cells/mL (Tofalo et al., 2012). The maximum number of quantifiable cells by Cells-qPCR was 10^7 cells/mL since no linearity was obtained above this level (up to 10^8 cells/mL). Nevertheless, this is not a problem as it is possible to quantify populations larger than 10^7 cells/mL by sample dilution. In addition, sample dilution for musts and wines improved as the inhibitor concentration lowered. As observed for *L. plantarum*, the minimum number of *S. cerevisiae* and *B. bruxellensis* detected and quantified by using whole cells was larger than when DNA was used.

4.1.6. Effect of matrix on Cells-qPCR efficiency by whole cells

Several authors have reported differences when qPCR was performed with DNA extracted from cultures or directly from wine (Hierro et al., 2006; Hierro et al., 2007; Martorell et al., 2005), indicating that complex matrices such as grape must and wine underestimate the population by increasing the Ct values. In the present work, the matrix effect has not altered the quantification range, neither by DNA nor by whole cells, showing that the Cells-qPCR assay is robust and works well on cell samples from grape must and wine matrices. This may be due to qPCR inhibitors, like ethanol, polyphenols, tannins, and polysaccharides, being efficiently removed in white grape must/wine, and in red grape must/wine by prior cell washes with TEN buffer and TEN buffer supplemented with PVP, respectively. Despite this, the effect of the matrix on cell quantification's ability by Cells-qPCR was evaluated, comparing both the standard curves and the efficiencies obtained for each matrix (culture medium, white/red grape must and white/red wine) in each species. Standard curves comparisons showed that the results are very similar between matrices, indicating that the matrix does not affect the Cells-qPCR assay (**Figure 27**). Regarding the efficiencies, that are shown in **Table 8**, they were analysed by a one-way analysis of variance and Tukey´s post-test. No significant differences were observed in the *E* between matrices for all the tested species (ρ value >0.05), which confirms that the matrix has no influence on amplification *E*.

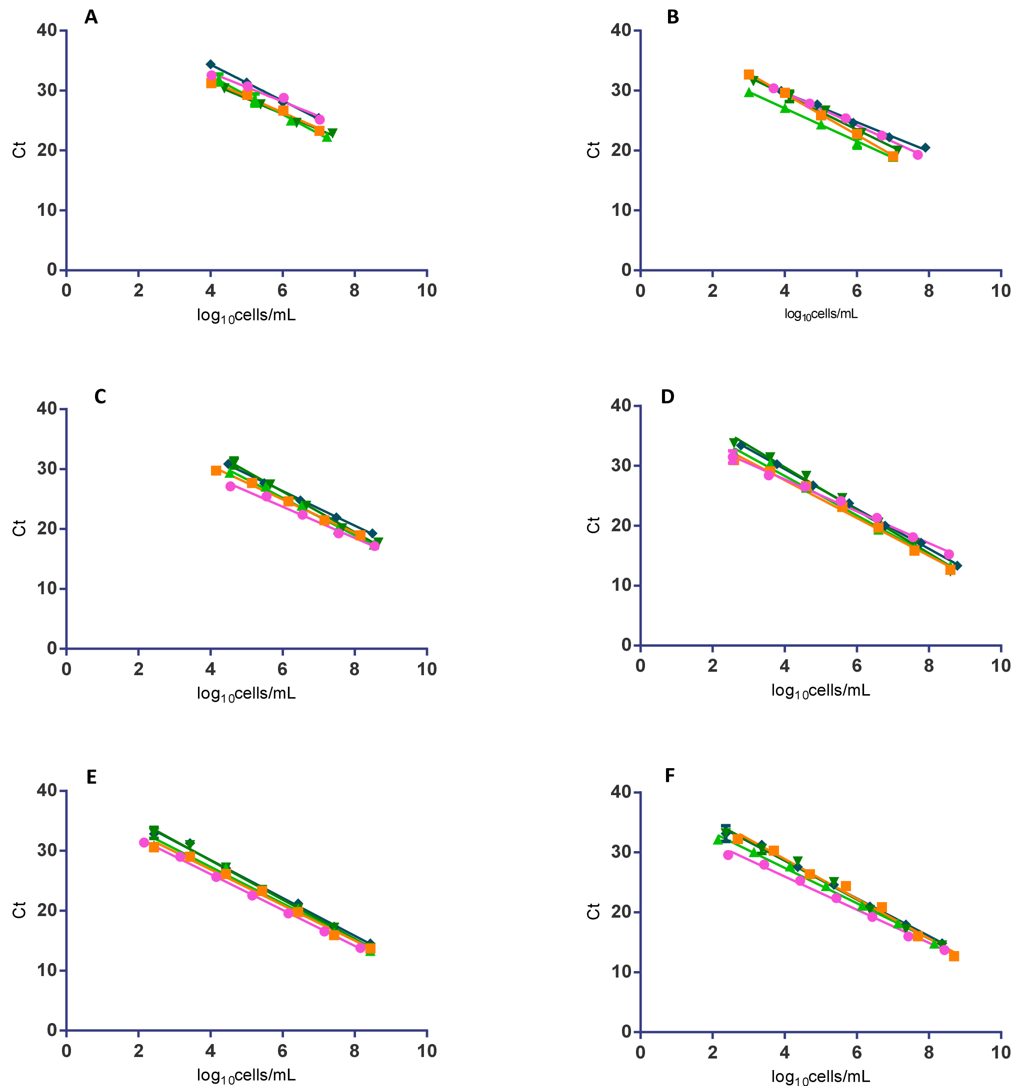


Figure 27- Standard curves comparison in culture medium (●), white grape must (■), red grape must (▲), white wine (▼), and red wine (♦) matrices, obtained by Cells-qPCR from the 10-fold serial dilutions of (**A**) *B. bruxellensis*, (**B**) *S. cerevisiae*, (**C**) *L. plantarum*, (**D**) *O. oeni*, (**E**) *A. aceti*, and (**F**) *G. oxydans*. The Ct values are the averages of three replicates. Error bars represent standard errors.

4.1.7. Cells-qPCR specificity from whole cells

The ability of the Cells-qPCR method to detect and quantify target species in red wine in the presence of other microorganisms was also assayed. For this purpose, three concentrations of each species (10^3, 10^5, 10^7 cells/mL) were combined in red wine as mixed cultures with a fixed cell concentration (10^5 cells/mL) of non-target yeast and LAB, in independent experiments. For *B.*

bruxellensis, *L. plantarum* and the AAB species, the non-target yeast and LAB were *S. cerevisiae* and *O. oeni,* respectively. For *S. cerevisiae*, the non-target yeast and LAB were *B. bruxellensis* and *O. oeni* respectively. For *O. oeni*, the non-target yeast and LAB were *B. bruxellensis* and *L. plantarum,* respectively. A control with no non target microorganisms was always employed. The results were evaluated in terms of the linear regression Ct values and were tested by a one-way analysis of variance and Dunnett´s post-test. No significant differences were found when evaluating Ct values between the pure and mixed cultures (ρ value>0.05), which indicates lack of interference with non-target microorganisms: no significant effects for the presence of the non-target yeasts and LAB were observed on the number of *B. bruxellensis*, *S. cerevisiae*, *L. plantarum*, *O. oeni*, *A. aceti*, and *G. oxydans* (**Figure 28**). These results suggest that the presence of other non-target wine microorganisms in the sample does not significantly affect the specificity of the Cells-qPCR assay at any tested cell concentration, indicating that Cells-qPCR is a useful technique for enumerating yeasts, LAB and ABB species in wine, even in the presence of non-target microorganisms.

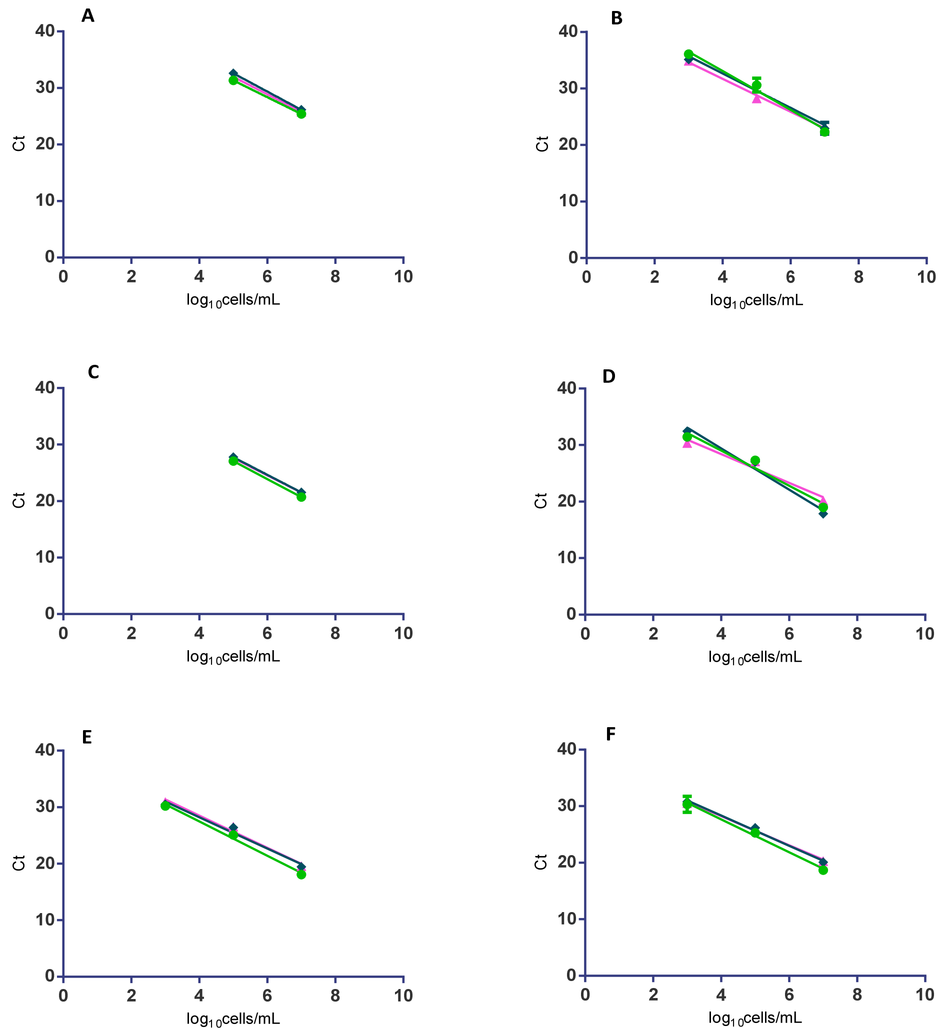


Figure 28- Standard curves obtained from serially diluted pure cultures (●), and mixed cultures with yeasts (▲) or bacteria (♦) Cells-qPCR comparisons in red wine. (**A**) *B. bruxellensis*, (**B**) *S. cerevisiae*, (**C**) *L. plantarum*, (**D**) *O. oeni*, (**E**) *A. aceti*, and (**F**) *G. oxydans*. The Ct values are the averages of three replicates. Error bars represent standard errors.

4.1.8. Cell wall disruption effect in Cells-qPCR sensitivity

In winemaking, a small number of cells may be sufficient to cause spoilage after growth (Deak and Beuchat, 1996). Taking into account this fact, the drawback of using Cells-qPCR method is that for yeasts and *L. plantarum*, the obtained LoQs were high (10^3 cells/mL for *S. cerevisiae,* and 10^4 cells/mL for *B. bruxellensis* and *L. plantarum*).

In order to increase the sensitivity of the assay, the effect of a previous cell wall lysis, by enzymatic and mechanical methods, were evaluated by comparing qPCR amplifications from both cells and lysed cells. In addition to yeasts and *L. plantarum*, the effects of a previous cell wall lysis were also evaluated for *O. oeni*. For this, cultures of each species were serially diluted from 10^7 to 10^2 cells/mL (for yeast species), and from 10^8 to 10^2 cells/mL (for LAB species) in culture medium and then, each dilution was washed and lysed.

Enzymatic lysis

Regarding yeast species, a lytic enzyme, namely Zymolyase 20T, which hydrolyses yeast glucan with the release of soluble carbohydrate (Kitamura et al., 1974), was added to each cell suspension for enzymatic cells lysis. After incubation and washing, cell suspensions were subjected, in independent experiments, to Cells-qPCR quantification using the corresponding species-specific primer set. When comparing the Ct values obtained by the whole cell and enzymatically lysed cell amplifications of each species, as expected, the results showed, that the Cts of the lysed cells were lower than they were for the whole cells. Nevertheless, the differences observed among the Ct values of different cell concentrations for the lysed cells, were scarce (see **Figure 29** for an example), which did not allow good discrimination among the different cell concentrations.

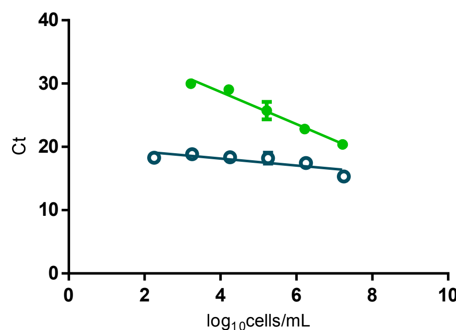


Figure 29- Standard curves obtained by whole cells (●), and enzymatically lysed cells (○) Cells-qPCR comparison from serial dilutions of *S. cerevisiae* in culture medium. The Ct values are the averages of three replicates. Errors bars represent standard errors.

Regarding LAB species, a lytic enzyme namely lysozyme, which degrades the peptidoglycan cell wall of Gram positive bacteria, by cleaving the β (1-4) bond of N-acetylmuramic acid and N-

acetylglucosamine (McKenzie and White, 1991), was added to each one of cell suspensions for enzymatic cells lysis. The bacterial sensitivity to lysozyme seems to vary according to species (Delfini et al., 2004; Dias et al., 2015), and for this reason, different concentrations of lysozyme were used for *L. plantarum* and *O. oeni* species, as described Blasco (2009). After incubation and washing, cell suspensions were subjected, in independent experiments, to Cells-qPCR quantification using the corresponding species-specific primer set. When comparing the Ct values obtained by the whole cell and lysed cell amplifications of *L. plantarum*, the results showed that the Cts obtained by the lysed cells were higher and lower than they were for high and low concentrations of whole cells, respectively, being not possible discriminate different cells concentrations (**Figure 30A**). Regarding *O. oeni* amplifications, the results showed that the Ct values obtained by the lysed cells were, for all cells concentrations, higher than they were for the whole cells (**Figure 30B**), not improving the results. These results could be due to some kind of Cells-qPCR reaction inhibition by lysozyme.

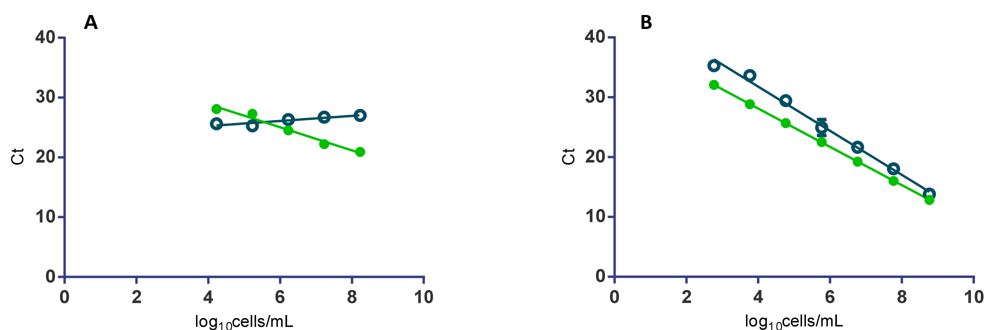


Figure 30- Standard curves obtained by whole cells (●), and enzymatically lysed cells (○) Cells-qPCR comparison from 10-fold serial dilutions of (**A**) *L. plantarum* and (**B**) *O. oeni* in culture media. The Ct values are the averages of three replicates. Errors bars represent standard errors.

As a consequence of the obtained results, the enzymatic lysis strategy to increase the sensitivity of the assay for yeast and LAB species was rejected.

<u>**Mechanical lysis**</u>

For the mechanical lysis, glass beads were added to each cell suspensions and were vortexed during 10 and 30 minutes for yeast and LAB species, respectively. Then, cell suspensions were

subjected, in independent experiments, to Cells-qPCR amplifications using the corresponding species-specific primer set.

For yeast species, the obtained results showed that the cell lysis lowered the Ct values compared with the Cts obtained by whole cells. Furthermore, in this case, a prior mechanical cell lysis can better discriminate different cell concentrations by improving the sensitivity and LoQs of the assay (**Figure 31**). When correlating the whole cell and lysed cell standard curves, the Ct values lowered to at least 6 and 3 Ct for the *B. bruxellensis* and the *S. cerevisiae* lysed cells, respectively, while the LoQs were 100- and 10-fold lower than those obtained by whole cells, respectively.

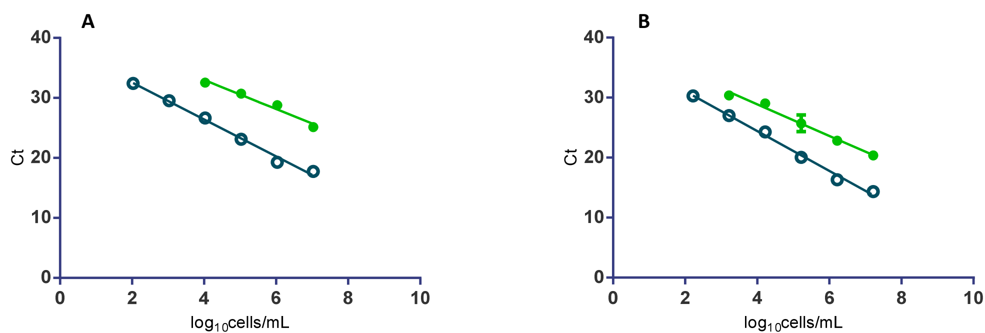


Figure 31- Standard curves obtained by whole cells (●), and mechanically lysed cells (○) Cells-qPCR comparison from 10-fold serial dilutions of (**A**) *B. bruxellensis* and (**B**) *S. cerevisiae* in culture medium. The Ct values are the averages of three replicates.

For lysed cells standard curves, R^2 were higher than 0.99 and linearity was achieved within values that range from 10^2 to 10^7 cells/mL (1-10^5 cells/reaction tube) for both *B. bruxellensis* and *S. cerevisiae*. These LoQs mean that whit these species it is possible to detect as low as 1 cell per reaction tube, which shows the excellent assay sensitivity when performing a mechanical lysis, with no DNA purification step.

By considering that the mechanical lysis was the best option to detect and quantify a wide range of yeast cells with a good efficiency, and in order to endorse this methodology to detect and quantify *Z. bailii* cells, standard curves, comparing qPCR amplifications from both whole and lysed cells, were constructed.

The obtained results showed the effectiveness of the Cells-qPCR method to detect and quantify this species, and the Tm was species-specific, confirming the specificity of the assay (**Figure 32**).

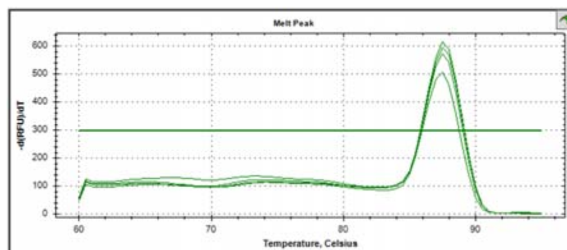


Figure 32- Melting curves analysis of the Cells-qPCR products from *Z. bailii* species.

Moreover, the obtained LoQs were the same for both whole and lysed cells, but the Ct values were lower for lysed cells, which accomplished a better discrimination of different cell concentrations (**Figure 33**). For the lysed cells standard curves, R^2 were higher than 0.99 and linearity was achieved within values that range from 10^2 to 10^6 cells/mL (1-10^4 cells/reaction tube).

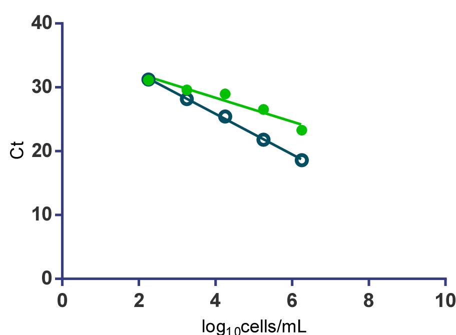


Figure 33- Standard curves obtained by whole cells (●), and mechanically lysed cells (○) Cells-qPCR comparison from 10-fold serial dilutions of *Z. bailii* in culture medium. The Ct values are the averages of three replicates. Errors bars represent standard errors.

Regarding LAB species, the obtained results for *L. plantarum* showed that the cell lysis lowered the Ct values compared with the Cts obtained by whole cells (**Figure 34A**). For lysed cells standard curves, R^2 were higher than 0.99 and linearity was achieved within values that range from 10^2 to 10^8 cells/mL (1-10^6 cells/reaction tube), showing the high assay sensitivity when performing a mechanical lysis.

Nevertheless, for *O. oeni*, no differences were observed between whole and lysed cells LoQs and Ct values (**Figure 34B**), indicating that cell lysis does not improve the assay performance.

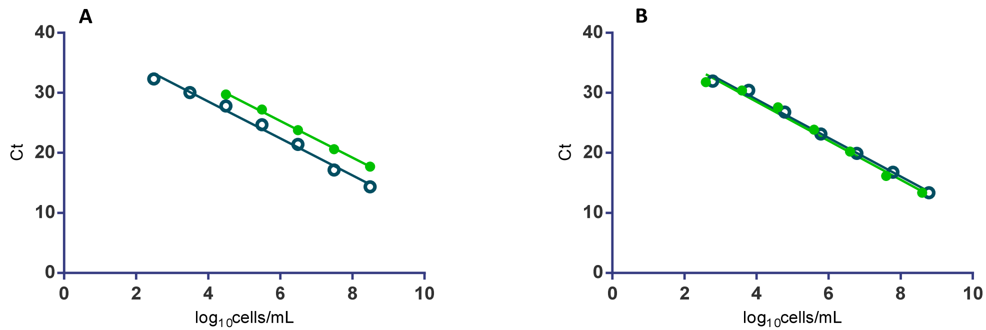


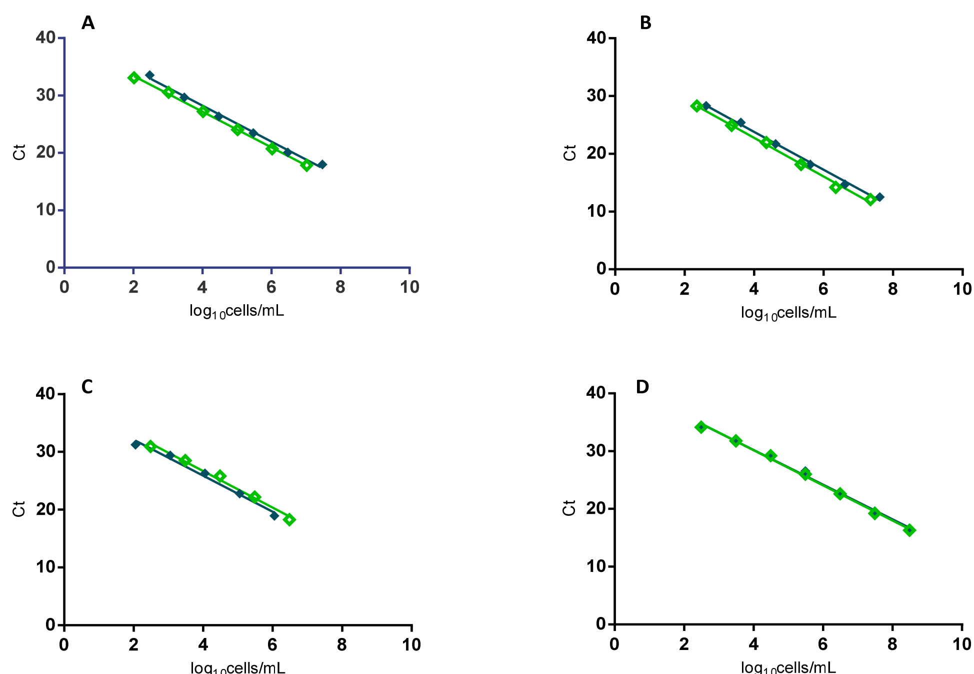
Figure 34- Standard curves obtained by whole cells (●), and mechanically lysed cells (○) Cells-qPCR comparison from 10-fold serial dilutions of (**A**) *L. plantarum* and (**B**) *O. oeni* in culture media. The Ct values are the averages of three replicates.

4.1.9. White/red grape musts and wines standard curves from mechanically lysed cells

By considering that the mechanical lysis was the best option to detect and quantify a high range of yeast and *L. plantarum* cells, standard curves for each species were constructed in grape must and wine matrices in independent experiments. To this end, 10-fold serial diluted cells from 10^8-10^2 cells/mL were prepared in white/red grape musts and wines as for the construction of standard curves from whole cells. All the cell suspensions were then washed, mechanically lysed and quantified by Cells-qPCR.

All the obtained R^2 values were higher than 0.98 and the quantification ranges for all species in all matrices were the same as those obtained for the culture medium (**Figure 35**). These results showed that for lysed cells quantification by Cells-qPCR, the LoQ obtained for all the species in all matrices was 10^2 cells/mL. This result also highlights the method's high sensitivity and sound versatility as it indicates the possibility of detecting one cell per reaction and that the matrix does not interfere with quantification.

White/Red grape must



White/Red wine

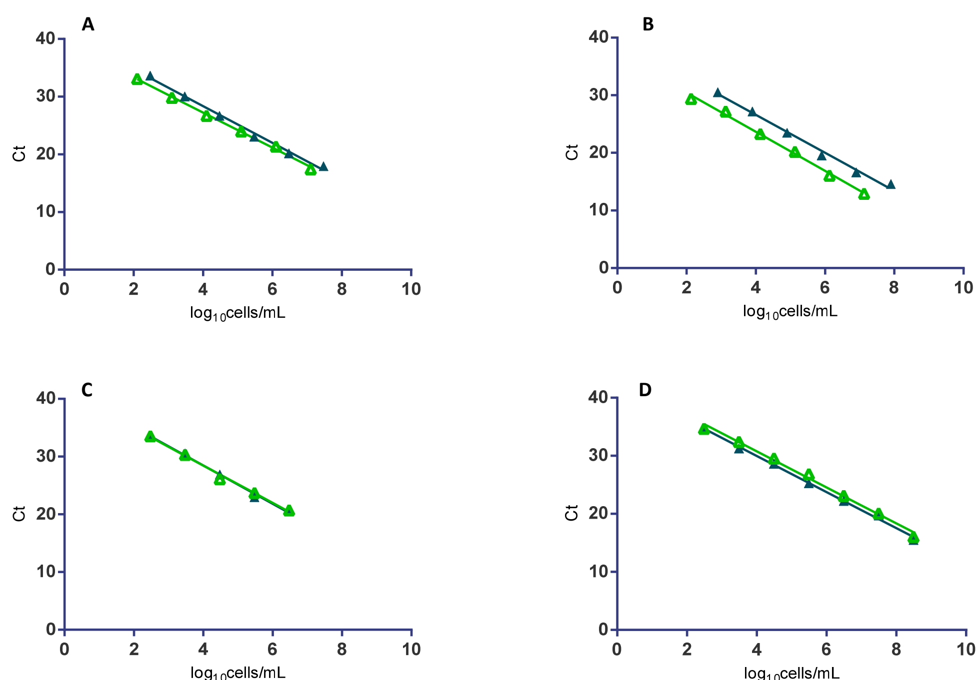


Figure 35- Standard curves obtained by Cells-qPCR from the 10-fold serial dilutions of (**A**) *B. bruxellensis*, (**B**) *S. cerevisiae*, (**C**) *Z. bailii*, and (**D**) *L. plantarum* mechanically lysed cells in white grape must (◊), red grape must (♦), white wine (Δ), and red wine (▲).The Ct values are the averages of three replicates. Error bars represent standard errors.

For the detection and quantification of *B. bruxellensis* and *S. cerevisiae*, linearity was obtained with the populations between 10^2 and 10^7 cells/mL in all the matrices (1 to 10^5 cells per reaction). These results were similar or better than those obtained by other authors, who quantified DNA within ranges of 10^2-10^7 cells/mL (Willenburg and Divol, 2012) and 10^4-10^7 CFU/ml (Delaherche et al., 2004) for *B. bruxellensis*, and within ranges of 10^2-10^6 cells/mL (Hierro et al., 2007) for *S. cerevisiae*. Moreover, the Cells-qPCR methodology was successfully applied to the detection and quantification of *Z. bailii*, and linearity was obtained with populations between 10^2 and 10^6 cells/mL in all the matrices. Nevertheless, Rawsthorne and Phister (2006) have reported DNA linearity within ranges of 10-10^6 CFU/mL using 2 µL of purified DNA as a template.

Regarding *L. plantarum* detection and quantification, linearity was obtained with the population between 10^2 and 10^8 cells/mL in all the matrices (1 to 10^6 cells per reaction). Similar results were reported by Gyu-Sung et al. (2011) to detect and quantify DNA.

4.1.10. Effect of matrix on Cells-qPCR efficiency by mechanically lysed cells

In order to evaluate if the matrix does not interfere on mechanically lysed cell quantification´s ability by Cells-qPCR, three standard curves were constructed from the lysed cell suspensions of each species (*B. bruxellensis*, *S. cerevisiae*, *Z. bailii*, and *L. plantarum*) and matrix in independent experiments. The standard curves R^2, slopes, and *E* are shown in **Table 10**.

Table 10-Correlation coefficients (R^2), slopes, coefficients of efficiency (*E*), and limits of quantification (LoQ) of the standard curves obtained from different concentrations of the mechanically lysed cells from *B. bruxellensis*, *S. cerevisiae*, *Z. bailii,* and *L. plantarum* species in culture media, and in white and red musts and wines[a].

Microorganism	Matrix	R^2	Slope	E^b	LoQ (cells/mL)
B. bruxellensis	Culture medium	0.991 ± 0.001	-3.080 ± 0.070	1.113 ± 0.036	10^2-10^7
	White must	0.997 ± 0.001	-3.110 ± 0.047	1.097 ± 0.023	
	Red must	0.993 ± 0.005	-3.130 ± 0.076	1.088 ± 0.037	
	White wine	0.997 ± 0.001	-3.044 ± 0.047	1.131 ± 0.025	
	Red wine	0.991 ± 0.006	-3.188 ± 0.060	1.060 ± 0.028	
S. cerevisiae	Culture medium	0.992 ± 0.005	-3.316 ± 0.040	1.003 ± 0.040	
	White must	0.997 ± 0.001	-3.345 ± 0.044	0.991 ± 0.018	
	Red must	0.995 ± 0.003	-3.272 ± 0.056	1.022 ± 0.024	
	White wine	0.994 ± 0.001	-3.346 ± 0.034	0.990 ± 0.014	
	Red wine	0.991 ± 0.002	-3.296 ± 0.023	1.011 ± 0.010	
Z. bailii	Culture medium	0.996 ± 0.005	-3.165 ± 0.066	1.071 ± 0.031	10^2-10^6
	White must	0.989 ± 0.005	-3.162 ± 0.056	1.072 ± 0.027	
	Red must	0.997 ± 0.002	-3.252 ± 0.055	1.031 ± 0.025	
	White wine	0.990 ± 0.005	-3.228 ± 0.073	1.042 ± 0.033	
	Red wine	0.994 ± 0.001	-3.313 ± 0.067	1.005 ± 0.028	
L. plantarum	Culture medium	0.988 ± 0.006	-3.074 ± 0.051	1.115 ± 0.026	10^2-10^8
	White must	0.996 ± 0.002	-3.045 ± 0.039	1.131 ± 0.020	
	Red must	0.994 ±0.001	-3.003 ± 0.019	1.153 ± 0.011	
	White wine	0.993 ±0.003	-3.108 ± 0.044	1.098 ± 0.022	
	Red wine	0.996 ±0.001	-3.105 ± 0.025	1.099 ± 0.012	

[a] All values are means and standard errors.
[b] Coefficients of efficiency were estimated by the formula $10^{-1/slope}$-1.

Next, both the standard curves and the *E* obtained for each matrix (culture medium, white/red grape must and white/red wine) in each species were compared. A similarity or parallelism of the linear regression analysis was observed when standard curves were compared in a same graphic (**Figure 36**). Regarding the *E*, they were analysed by a one-way analysis of variance and Tukey´s post-test. No significant differences were observed in the *E* between matrices for all the tested species (ρ value >0.05). This scenario indicates that the presence of the cells in complex matrices, such as grape must and wine, does not influence amplification efficiencies.

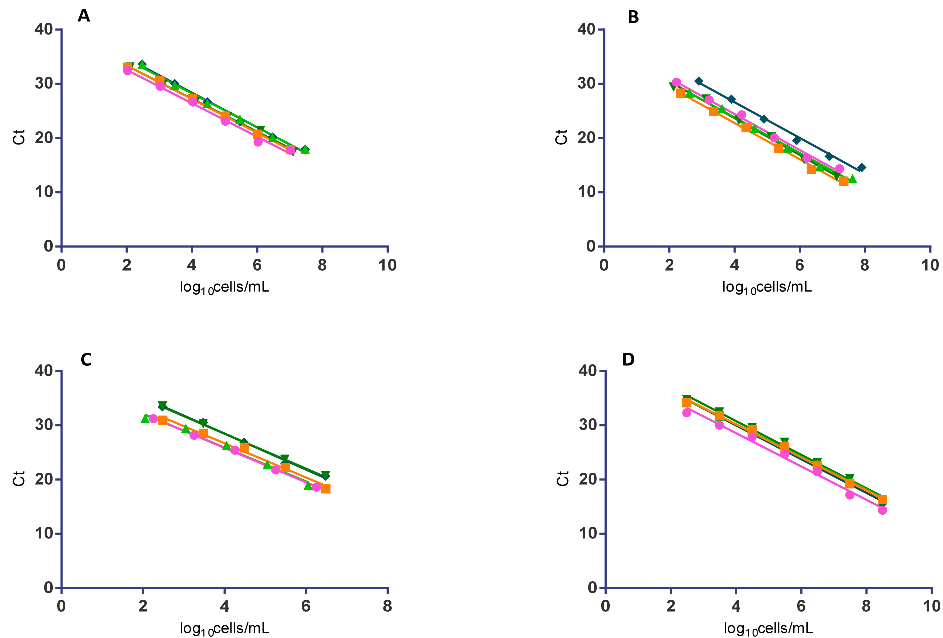


Figure 36- Standard curves comparison in culture medium (●), white grape must (■), red grape must (▲), white wine (▼), and red wine (♦) matrices, obtained by Cells-qPCR from the 10-fold serial dilutions of (**A**) *B. bruxellensis*, (**B**) *S. cerevisiae*, (**C**) *Z. bailii*, and (**D**) *L. plantarum*. The Ct values are the averages of three replicates. Error bars represent standard errors.

4.1.11. Cells-qPCR specificity from lysed cells

The ability of the Cells-qPCR method to detect and quantify the lysed cells of the target yeast species and *L. plantarum* in red wine, and in the presence of other microorganisms, was assayed. To this end, three concentrations of each species (10^3, 10^5, 10^7 cells/mL) were combined in red wine as mixed cultures, with a fixed cell concentration (10^5 cells/mL) of the non-target yeast and LAB, in independent experiments. For *B. bruxellensis*, *Z. bailii,* and *L. plantarum*, the non-target yeast and LAB were *S. cerevisiae* and *O. oeni,* respectively. For *S. cerevisiae*, the non-target yeast and LAB were *B. bruxellensis* and *O. oeni,* respectively. A control with pure target microorganisms was included all the times. Prior to Cells-qPCR quantification, all cells suspensions were washed and subjected to mechanical lysis. The results were evaluated in terms of the linear regression Ct values between the pure and mixed cultures and were tested with a one-way analysis of variance and Dunnett´s post-test. The obtained results showed that the presence of the non-target yeasts and LAB did not significantly impact the specific quantification of *B. bruxellensis*, *S. cerevisiae*, *Z. bailii*, and *L.*

plantarum at any tested concentration (ρ value>0.05) (**Figure 37**), which highlights the assay's high specificity.

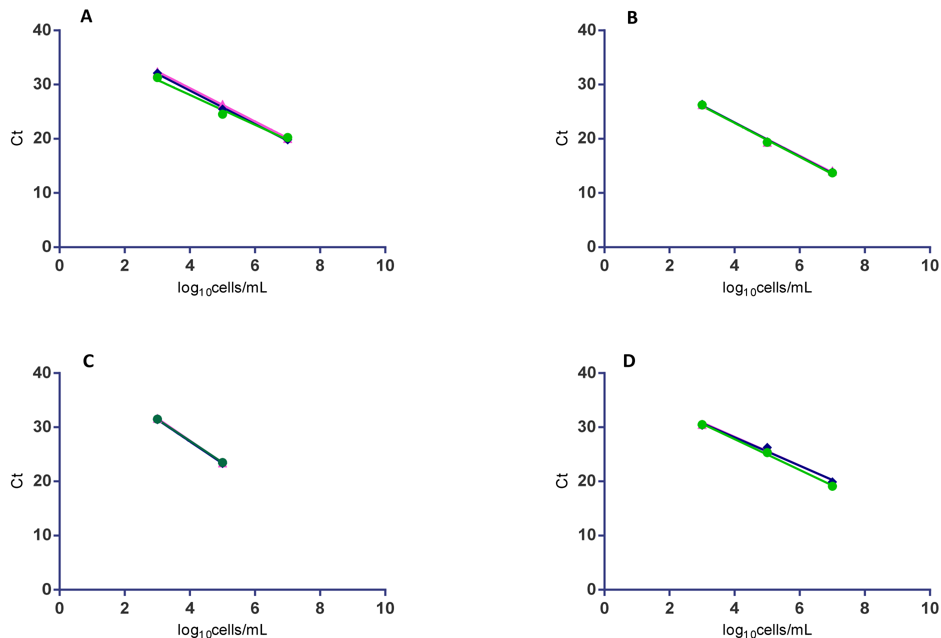


Figure 37- Standard curves obtained from serially diluted pure cultures (●), and mixed cultures with yeasts (▲) or bacteria (♦) Cells-qPCR comparisons in red wine. (**A**) *B. bruxellensis*, (**B**) *S. cerevisiae*, (**C**) *Z. bailii*, and (**D**) *L. plantarum*. The Ct values are the averages of three replicates. Error bars represent standard errors.

4.1.12. Monitoring microbial population in industrial wine fermentations

In order to test the accuracy of the Cells-qPCR assay to enumerate yeasts, LAB, and AAB during the winemaking process, samples were taken during three spontaneous wine fermentations of grape varieties Cabernet Sauvignon, Garnacha, and Merlot.

Each sample was processed in duplicate and examined by correlating the microbial population (total yeasts and total AAB populations, and species of *B. bruxellensis*, *S. cerevisiae*, *Z. bailii, L. plantarum*, and *O. oeni*) determined by Cells-qPCR, and also by PCR. Regarding total yeasts, the cell number determined by Cells-qPCR was also correlated with those obtained by plate counts. Increased accuracy was expected by creating a calibration that adapted to wines according to the matrix that they represented. Therefore, the Ct values obtained from each positive reaction were

extrapolated to the corresponding standard curve with whole or mechanically lysed cells; i.e., a standard curve constructed using as matrix the positive sample previously sterilised by filtration.

Yeasts

For total yeasts and *S. cerevisiae* quantification, with general yeast primer and species-specific primer, respectively, standard curves were constructed for all the sampling days of the three spontaneous wine fermentations. Regarding total yeasts, two standard curves were constructed per sample with *S. cerevisiae* and *Z. bailii* species using the general yeast primer in independent experiments. From these individual standard curves, a general standard curve was constructed for each sample, as suggested by Hierro et al. (2006). The assay was linear over 5 orders of magnitude when recording the population ranges from 10^2 to 10^6 cells/mL for *S. cerevisiae* and *Z. bailii*. For the *Z. bailii* quantification, with a species-specific primer, two standard curves were obtained using Cabernet Sauvignon and Garnacha grape must (t=0) as matrix. For *B. bruxellensis*, only one standard curve was constructed (Merlot, t=21). The R^2, slopes, and efficiencies of the amplifications for the standard curves are shown in **Table 11**.

Table 11- Correlation coefficients (R^2), slopes and coefficients of efficiency (*E*) of the standard curves obtained for total yeast, *B. bruxellensis*, *S. cerevisiae*, and *Z. bailii* detection and quantification during the spontaneous wine fermentations of grape varieties Cabernet Sauvignon, Garnacha and Merlot.

Microorganisms	Grape variety/ Sampling time (days)	R^2	Slope	E^a
Total yeasts	**Cabernet Sauvignon**			
	0	0.984	-3.120	1.092
	4	0.992	-3.131	1.086
	12	0.981	-3.344	0.991
	28	0.983	-3.292	1.013
	Garnacha			
	0	0.991	-3.141	1.081
	10	0.981	-3.228	1.041
	25	0.990	-3.407	0.966
	31	0.985	-3.234	1.038
	Merlot			
	0	0.981	-3.402	0.968
	3	0.990	-3.178	1.064
	8	0.990	-3.139	1.082
	21	0.983	-3.167	1.069
S. cerevisiae	**Cabernet Sauvignon**			
	0	0.995	-3.336	0.994
	4	0.995	-3.059	1.123
	12	0.985	-3.261	1.026
	28	0.994	-3.202	1.053
	Garnacha			
	0	0.988	-3.219	1.045
	10	0.980	-3.565	0.908
	25	0.982	-3.385	0.974
	31	0.992	-3.257	1.028
	Merlot			
	0	0.998	-3.201	1.053
	3	0.993	-3.206	1.051
	8	0.990	-3.220	1.044
	21	0.994	-3.178	1.064
Z. bailii	**Cabernet Sauvignon**			
	0	0.993	-3.475	0.940
	Garnacha			
	0	0.993	-3.351	0.988
B. bruxellensis	**Merlot**			
	21	0.935	-3.367	0.982

[a] Coefficients of efficiency were estimated by the formula $10^{-1/slope}-1$.

The results of plating counts, Cells-qPCR detection/quantification (**Figure 38**), fructose and glucose consumption, and ethanol formation (**Figure 39**) during wine fermentations showed that, as expected, all the processes led to increasing yeast populations, which declined, along with the sugar concentration, in the last fermentation phase and in wine. This decrease is related to the toxic effects of the ethanol that increase along the fermentation process.

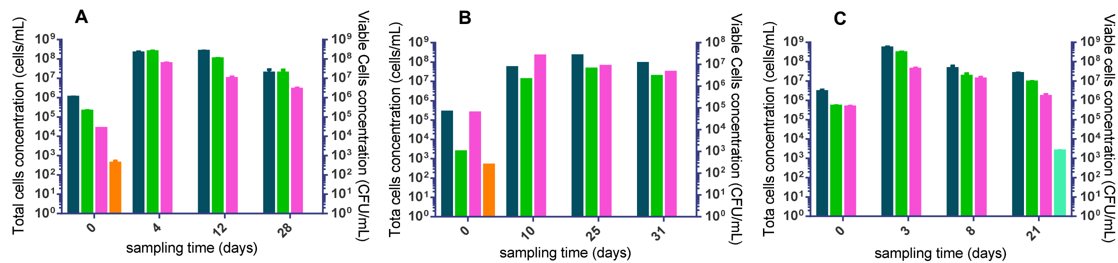


Figure 38- Evolution of total yeasts (■), *B. bruxellensis* (■), *S. cerevisiae* (■), and *Z. bailii* (■) analysed by Cells-qPCR, and viable yeasts (■) analysed by plating, during spontaneous wine fermentations of (**A**) Cabernet Sauvignon, (**B**) Garnacha, and (**C**) Merlot grape varieties. Error bars represent standard errors.

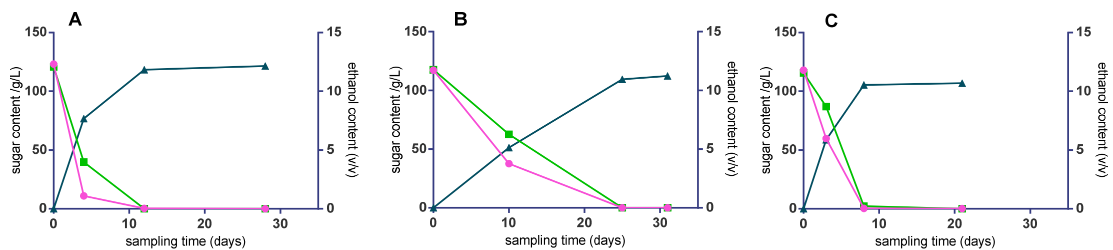


Figure 39-Fructose (■) and glucose (●) consumption, and ethanol (▲) formation during spontaneous wine fermentations of (**A**) Cabernet Sauvignon, (**B**) Garnacha, and (**C**) Merlot grape varieties.

The total yeast counts were about 1 order of magnitude higher with Cells-qPCR than with plating. As fermentations progressed, the viable count determinations made by plating showed that yeast populations died during the winemaking process. Therefore, the lack of correlation between both methods can be explained by the amplification of DNA from dead cells, which could still serve as a template for cells-qPCR quantification. Presence of a viable, but non-culturable population, could be another possible explanation for the lack of correlation between both methods. As suggested by Gyu-Sung et al. (2011), viable, but non-culturable cells, which may have developed during incubation in this hostile environment, may still have been quantified by Cells-qPCR, but not

by plating. Hierro et al. (2006), Hierro et al. (2007), Rawsthorne and Phister (2006), and Tofalo et al. (2012) have also reported that correlations were lacking between the qPCR and plating methods.

The increase in yeast populations was related to an increase in *S. cerevisiae*, which showed the same trend during AF (Cabernet Sauvignon: t=4, Garnacha: t=10, and Cabernet Sauvignon; t=3). As expected, *Z. bailii* and *B. bruxellensis*, which are considered spoilage species, were detected, but only in a few sampling stages and at low concentrations. *Z. bailii* was detected in the grape must (t=0) sampling stage of the Cabernet Sauvignon and Garnacha wine fermentations. After the sampling grape must stage, all the fermentations were sulphited, which could be why *Z. bailii* was not detected in later sampling stages. *B. bruxellensis* was detected only in the last sampling day (t=21) of the Merlot wine fermentation.

The obtained results from Cells-qPCR were compared to that obtained from conventional PCR. A good association between both methods was achieved, however, this association failed for the *B. bruxellensis* and *Z. bailii* detection by conventional PCR, observing absence of PCR single product (see **Figure 40** for an example).

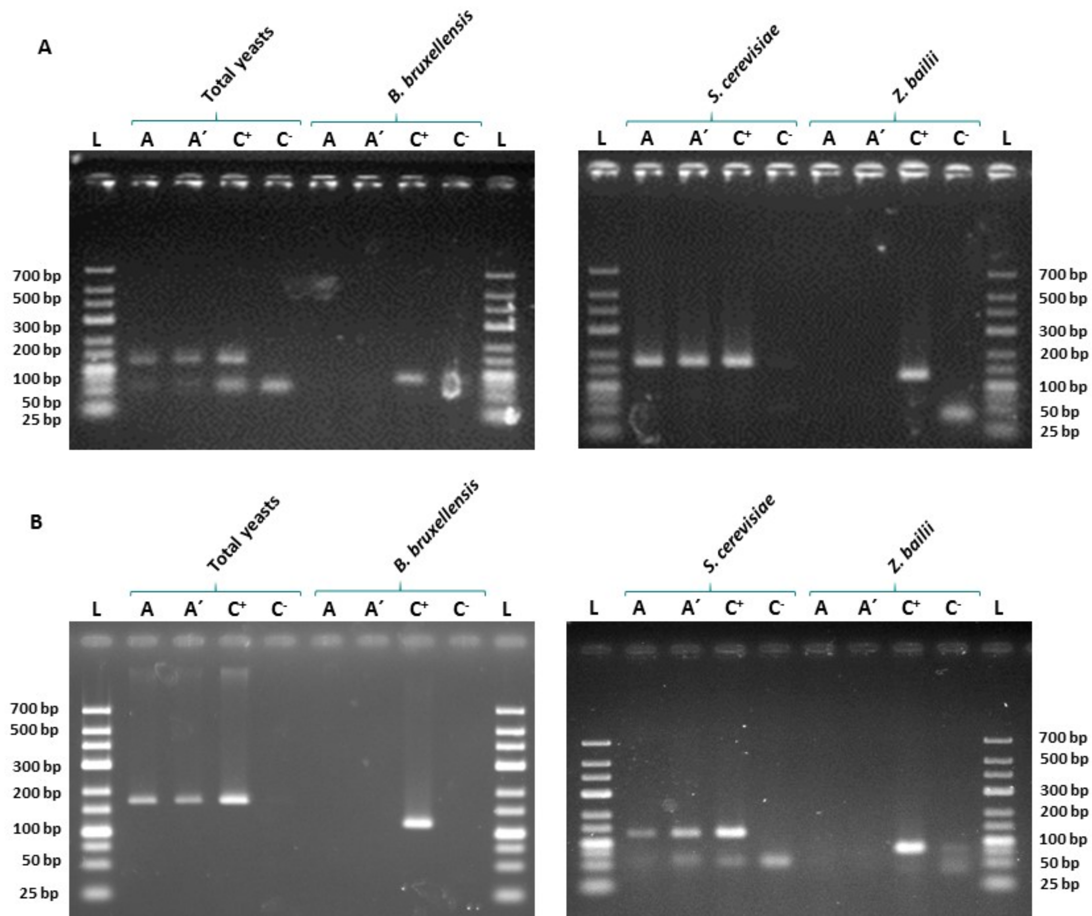


Figure 40- Agarose gel electrophoresis of conventional PCR amplification of total yeasts, *B. bruxellensis, S. cerevisiae*, and *Z. bailii* during spontaneous wine fermentations of (**A**) Cabernet Sauvignon (t=0), and (**B**) Merlot (t=21) grape varieties. L, GeneRuler Low-Range Ladder (Thermo Scientific); A, sample; A´, sample replicate; C^+ positive control; C^-, negative control.

This lack of association probably it was due to the presence of low cells concentration (<10^4 cells/mL) of these species in the samples, and as concluded by other authors (Paiva-Cavalcanti et al., 2009; Sonawane and Tripathi, 2013), qPCR is highly sensitivity method in comparison to conventional PCR.

These results confirm the effectiveness of Cells-qPCR assay to detect and quantify both total yeasts and specific yeast species during natural wine fermentations, overcoming in addition to the presence of inhibitors, the presence of other microorganisms.

LAB

For *L. plantarum and O. oeni* quantification, with species-specific primers, standard curves were constructed, for both species, using as matrix the filtered samples of the last two sampling days of Cabernet Sauvignon (t=12, 28) and Garnacha (t= 25, 31) wine fermentations. Regarding Merlot wine fermentation, standard curves were constructed for both species, just using as matrix the last sampling day sample (t=21). The R^2, slopes, and efficiencies of the amplifications for the standard curves are shown in **Table 12**.

Table 12- Correlation coefficients (R^2), slopes, and coefficients of efficiency (*E*) of the standard curves obtained for *L. plantarum*, and *O. oeni* detection and quantification during the spontaneous wine fermentations of grape varieties Cabernet Sauvignon, Garnacha, and Merlot.

Microorganism	Grape variety/ Sampling time (days)	R^2	Slope	E^a
L. plantarum	**Cabernet Sauvignon**			
	12	0.994	-2.754	1.307
	28	0.992	-2.861	1.236
	Garnacha			
	25	0.996	-3.061	1.122
	31	0.995	-3.035	1.135
	Merlot			
	21	0.994	-2.779	1.290
O. oeni	**Cabernet Sauvignon**			
	12	0,991	-3,042	1,132
	28	0,985	-3,034	1,136
	Garnacha			
	25	0,995	-2,968	1,172
	31	0,995	-2,953	1,181
	Merlot			
	21	0,988	-3,027	1,140

[a] Coefficients of efficiency were estimated by the formula $10^{-1/slope}$-1.

The results of Cells-qPCR detection/quantification (**Figure 41**), malic acid consumption, and lactic acid formation (**Figure 42**) during wine fermentations showed that, as expected, the process led to increasing LAB populations after AF. This result can be explained and confirmed by the observation in the malic acid concentration decrease, and lactic acid concentration increase, which is a direct consequence of the increase of the LAB population during this period.

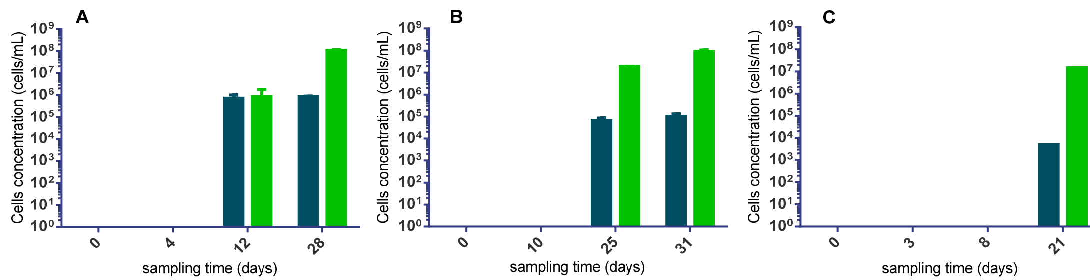


Figure 41- Evolution of *L. plantarum* (■), and *O. oeni* (■) analysed by Cells-qPCR during spontaneous wine fermentations of (**A**) Cabernet Sauvignon, (**B**) Garnacha, and (**C**) Merlot grape varieties. Error bars represent standard errors.

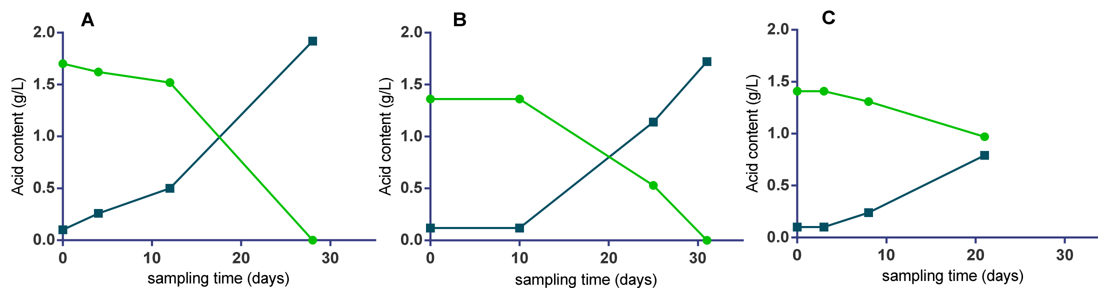


Figure 42- Malic acid (●) consumption, and lactic acid (■) formation during spontaneous wine fermentations of (**A**) Cabernet Sauvignon, (**B**) Garnacha, and (**C**) Merlot grape varieties.

During the first sampling days, neither *O. oeni* nor *L. plantarum* were detected and quantified by Cells-qPCR. This result can be due to that during this period the cell concentrations of these two species were lower than the LoQ of Cells-qPCR method (<10^2 cells/mL).

Regarding *O. oeni,* the total cells count was, in all the cases, higher than the obtained for *L. plantarum*. Moreover, both for Cabernet Sauvignon and Garnacha wine fermentations, the total cells count increased between the last sampling days. These results indicate that in all wine fermentations *O. oeni* was the LAB species responsible for malolactic conversion. This is in line with the expected since that, *O. oeni* is the LAB species that is most often responsible for MLF, and has shown to be able to successfully survive the challenging wine environment (Bauer and Dicks, 2004).

Regarding *L. plantarum*, despite it has shown potential as a starter culture (du Toit et al., 2011), the total cells count was similar in each sampling day of the Cabernet and Garnacha wine fermentations, and in all the cases, lower than the obtained for *O. oeni*, showing that this species was not able to grow during MLF and probably died during this period.

Comparing the results obtained from Cells-qPCR with the ones obtained from conventional PCR showed that the results obtained by both methods were concordant (**Figure 43**), observing the presence of specific band for both species.

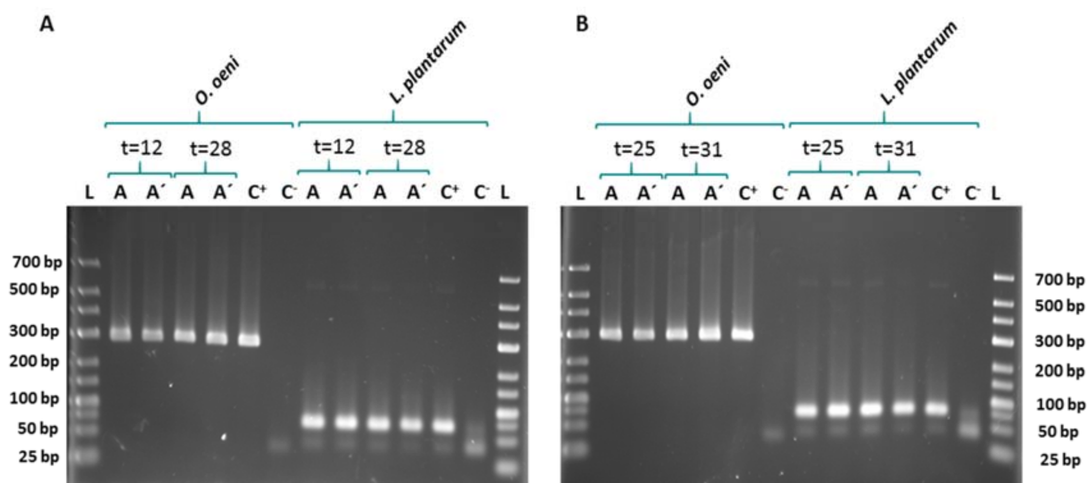


Figure 43- Agarose gel electrophoresis of conventional PCR amplification of *L. plantarum,* and *O. oeni* during spontaneous wine fermentations of (**A**) Cabernet Sauvignon (t=12, t=28), and (**B**) Garnacha (t=25, t=31) grape varieties. A, sample; A´, sample replicate; C^+ positive control; C^-, negative control; L, GeneRuler Low-Range Ladder (Thermo Scientific).

These results confirm the detection observed by Cells-qPCR indicating the effectivity and reliability of the assay. However, it is important to consider that through conventional PCR it is not possible to obtain information regarding the cellular concentration of the microorganisms.

AAB

For total AAB quantification, standard curves were constructed using as matrix the filtered samples of Cabernet Sauvignon and Garnacha grape must (t=0). Regarding Merlot, no standard curves were constructed, as no positive reactions were observed. The R^2, slopes, and efficiencies of the amplifications for the standard curves are shown in **Table 13**.

Table 13- Correlation coefficients (R^2), slopes, and coefficients of efficiency (*E*) of the standard curves obtained for total AAB detection and quantification during the spontaneous wine fermentations of grape varieties Cabernet Sauvignon, Garnacha, and Merlot.

Microorganisms	Grape variety/ Sampling time (day)	R^2	Slope	E^a
Total AAB	**Cabernet Sauvignon**			
	0	0.991	-3.304	1.008
	Garnacha			
	0	0.993	-3.487	0,935

[a] Coefficients of efficiency were estimated by the formula $10^{-1/slope}$-1.

The results of Cells-qPCR detection/quantification (**Figure 44**) during wine fermentations showed that species of AAB were present only in the grape must stage (t=0) of Cabernet Sauvignon and Garnacha wine fermentations. The grape must can be a rich medium for AAB growth due to the high concentration of sugars, high acidity and sulphites, explaining the detection of AAB population at this stage. After that, the lack of detection can be explained by the very harsh condition of wine created during AF (high ethanol concentration, low oxygen content, etc.) which restrict the AAB growth.

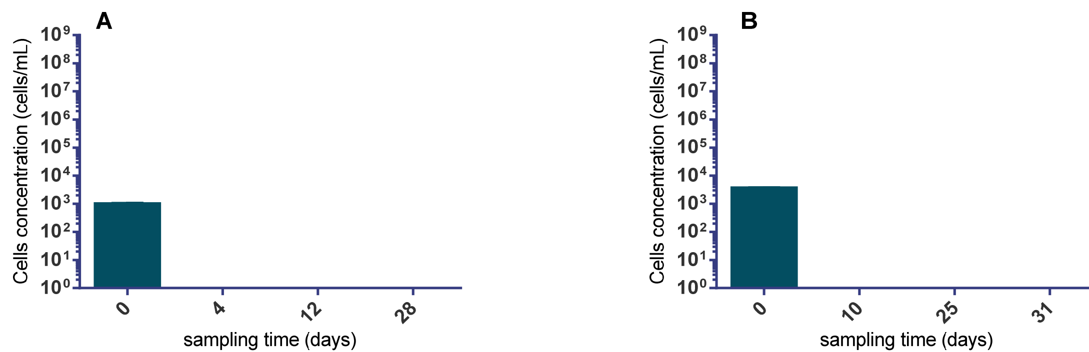


Figure 44- Evolution of total AAB (■) analysed by Cells-qPCR during spontaneous wine fermentations of (**A**) Cabernet Sauvignon, and (**B**) Garnacha. Error bars represent standard errors.

Comparing the results obtained from Cells-qPCR with the ones obtained from conventional PCR, no specific band was detected on the first sampling time of Cabernet Sauvignon and Garnacha wine fermentations, showing the lack of association between both methods. This kind of results also

happened with *B. bruxellensis* and *Z. bailii* detection. Therefore, the reason for this results can be explained by the cells concentration present in the samples, which for AAB was also below 10^4 cells/mL.

4.1.13. Effect of grape variety and wine type on Cells-qPCR efficiency

Considering that both total yeasts as *S. cerevisiae* were detected in all sampling days during the three spontaneous wine fermentations, and in order to check the effect of the different grape varieties, wine type, and matrix interference on the efficiency of the cells quantification by Cells-qPCR were evaluated for all these variables. For this purpose, the standard curves efficiencies obtained for the three natural wine fermentations (**Table 11**) were compared. For both total yeasts and *S. cerevisiae* counts, a one-way analysis of variance and Tukey´s post test were performed by comparing all the efficiencies obtained among the three wine fermentations. No significant differences were observed among wine fermentations (ρ value >0.05), which demonstrates that the assay is reproducible and robust even with different types of wine, which proves that chemical composition and grape variety do not affect both total yeasts as *S. cerevisiae* detection and quantification. These results are confirmed by the comparison of the standard curves in the same graphic (**Figure 45**).

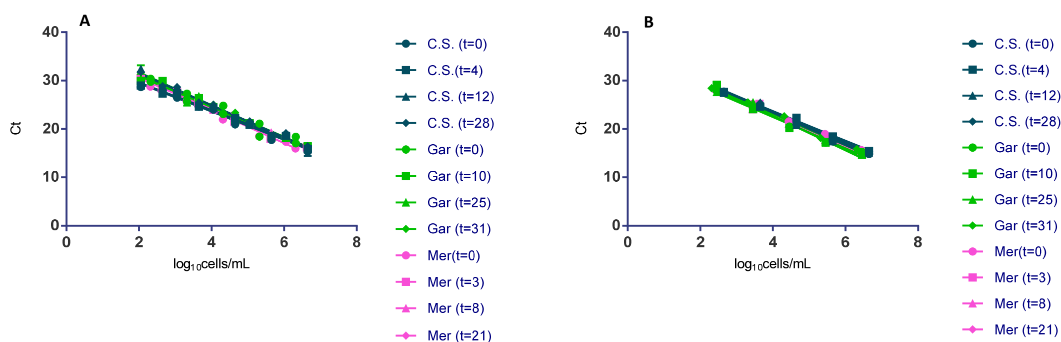


Figure 45- Standard curves comparison in different sampling times of Cabernet Sauvignon, Garnacha, and Merlot spontaneous wine fermentations, obtained by Cells-qPCR from the 10-fold serial dilutions of (**A**) *S. cerevisiae* and *Z. bailii* quantified with total yeast primers, and (**B**) *S. cerevisiae* quantified with species-specific primers. The Ct values are the average of three replicates. Error bars represent standard errors. **C.S**, Cabernet Sauvignon; **Gar**, Garnacha; **Mer**, Merlot; t, sampling time (days).

4.1.14. General primers specificity on Cells-qPCR standard curves construction

The ability of the Cells-qPCR method, with total yeast and AAB primers, to detect and quantify different pure species, and mixed species of yeasts and AAB, respectively, in wine, with the same sensitivity and specificity was evaluated.

For this purpose, regarding yeasts, *B. bruxellensis*, *S. cerevisiae*, and *Z. bailii* cells, with a final concentration of $1x10^6$ cells/mL, were mixed in red wine. The pure cells suspensions of *B. bruxellensis*, *S. cerevisiae* and *Z. bailii,* with a final concentration of $3x10^6$ cells/mL, were also prepared in red wine, in independent experiments. Regarding AAB, the procedure was similar to the one for yeasts. *A. aceti*, *A. pasteurianus,* and *G. oxydans* cells, with a final concentration of $1x10^8$ cells/mL, were mixed in red wine and the pure cells suspensions of *A. aceti*, *A. pasteurianus,* and *G oxydans,* with a final concentration of $3x10^8$ cells/mL, were also prepared in red wine and in independent experiments

All the obtained cell suspensions were 10-fold serially diluted from $3x10^6$/$3x10^8$ to $3x10^2$ cells/mL using the same red wine as a diluent. After washes, all the cell suspensions were subjected to Cells-qPCR detection and quantification using both total yeast and AAB primers, and in independent experiments. For total yeasts, the cell suspensions were previously mechanically lysed.

The results were evaluated in Ct terms and were tested with one-way analysis of variance and Tukey´s test. When comparing the Ct values of the three standard curves obtained by the pure cells suspensions, no significant differences were observed (ρ value >0.05). This suggests that the Cells-qPCR assay with both general primers allow the detection and quantification of different yeasts and AAB species in red wine, and with the same sensitivity and specificity. Moreover, no significant differences were observed when comparing the Ct values of the three standard curves obtained by the pure cells suspensions, and the Ct values obtained by mixed species (ρ value >0.05). The graphic representation of standard curves obtained by pure and mixed cell suspensions corroborate the statistic results (**Figure 46**).

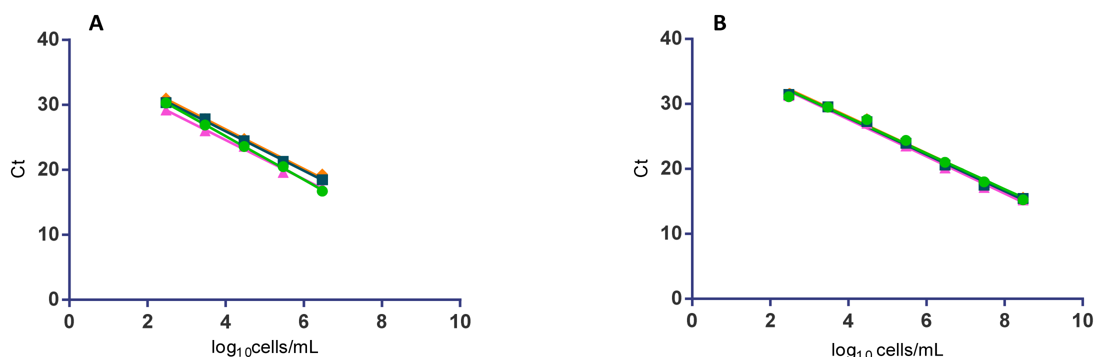


Figure 46- Standard curves obtained from serially diluted pure and mixed cultures in red wine Cells-qPCR comparisons of (**A**) *B. bruxellensis* (■), *S. cerevisiae* (▲), *Z. bailii* (♦), *B. bruxellensis* + *S. cerevisiae* + *Z. bailii*(●)quantified with total yeast primers, and (**B**) *A. aceti* (■), *A. pasteurianus* (▲), *G. oxydans* (♦), *A. aceti* + *A. pasteurianus* + *G. oxydans* (●) quantified with total AAB primers. The Ct values are the averages of three replicates. Error bars represent standard errors.

These results proved the ability of the Cells-qPCR method, with both general primers, to detect and quantify with high sensitivity and specificity, even in the presence of different yeasts/AAB species. Moreover, these results showed that only an individual standard curve with pure or mixed cultures would suffice to quantify both total yeasts and total AAB, reducing time and reaction costs.

4.2. Development of a PMA-qPCR Method for Direct Viable Cells Detection and Quantification (PMA-Cells-qPCR)

In order to selectively detect and quantify live cells of *B. bruxellensis*, *S. cerevisiae*, *L. plantarum*, and *O. oeni*, the DNA-intercalating dye PEMAX was used in conjunction with Cells-qPCR method (PMA-Cells-qPCR).

4.2.1. Determination of the optimal PEMAX concentration

Several methodological parameters may affect PMA efficiency. Among them, the concentration of PMA appears to be one of the key parameters that require optimization for the reliable quantification of viable cells (Elizaquível et al., 2013; Fittipaldi et al., 2012). Moreover, some

authors reported that effective PMA concentration is specific for the studied microorganism, suggesting that PMA concentration should be optimized for different microorganisms to generate the most reliable results (Desneux et al., 2015; Fittipaldi et al., 2012; Lv et al., 2016).

The effect of different PEMAX concentrations (0, 5, 10, 25, 50, 100, and 200 µM) on Cells-qPCR inhibition was studied to determine the optimal PEMAX concentration in order to achieve the highest ΔCt (with PEMAX-without PEMAX). Both viable and dead (heat-killed) cell suspensions of *B. bruxellensis*, *S. cerevisiae*, *L. plantarum*, and *O. oeni* species were obtained from culture media in a final concentration of $3x10^6$ cells/mL to determine the conditions under which the PMA-Cells-qPCR method could be used with the highest efficacy. After washes, PEMAX treatment, and mechanical cell lysis, all cell suspensions were submitted to PMA-Cells-qPCR reaction, in independent experiments.

Regarding *B. bruxellensis*, *S. cerevisiae*, and *L. plantarum* assays, the obtained results showed, as expected, that the ΔCt of dead cells were higher than the ΔCt of viable cells (**Figure 47 A, B, C**). Nevertheless, regarding *O. oeni* assay, the obtained results showed that for almost all tested PEMAX concentrations the ΔCt of dead cells were lower than the ΔCt of viable cells (**Figure 47D**).

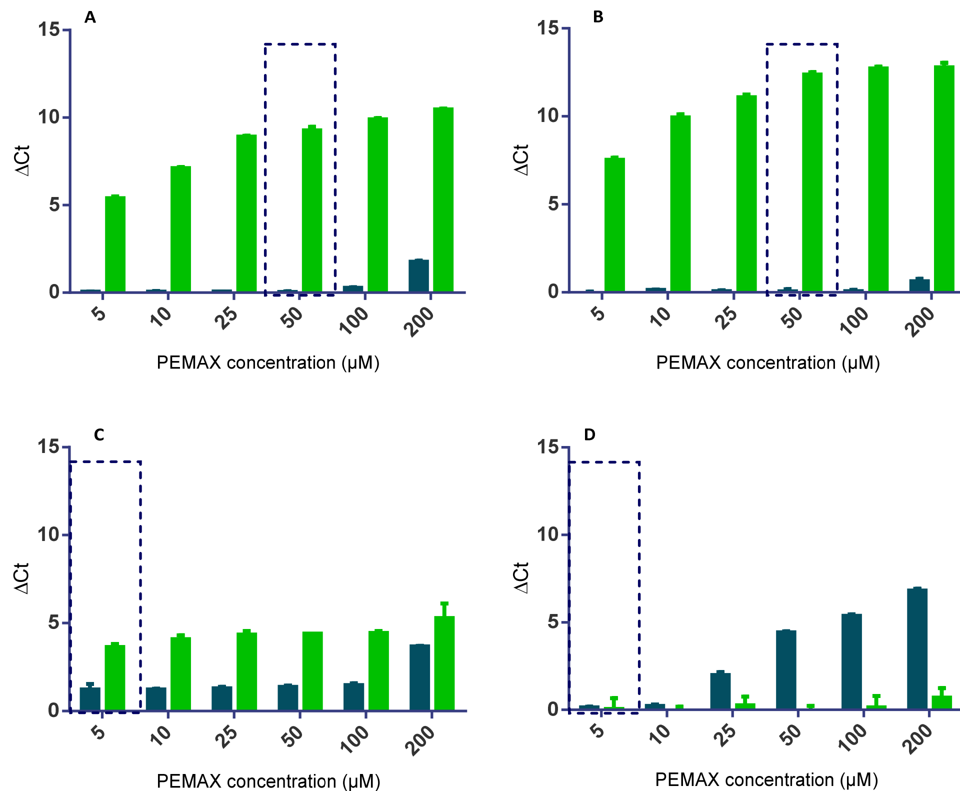


Figure 47- Effect of PEMAX concentration on PMA-Cells-qPCR signals of viable/dead cells of (**A**) *B. bruxellensis*, (**B**) *S. cerevisiae*, (**C**) *L. plantarum*, and (**D**) *O. oeni*. ΔCt was derived by subtracting Ct values of PEMAX treated cells from the values of no PEMAX (0 µM) treated cells. Viable cells (■), dead cells (■), selected PEMAX concentration (dotted rectangles). Error bars represent standard errors.

For *B. bruxellensis* (**Figure 47A**), the average Ct difference between dead cells with PEMAX and with no PEMAX was significantly different (ρ value <0.05) between all PEMAX concentrations, showing that 200 µM was perhaps the best PEMAX concentration to reduce the Cells-qPCR signal of *B. bruxellensis* dead cells. Nevertheless, regarding the average Ct difference between viable cells with PEMAX and with no PEMAX, it was significantly higher (ρ value <0.05) at PEMAX concentrations of 100 and 200 µM, showing that both PEMAX concentrations could inhibit the amplification of DNA from viable cells.

For *S. cerevisiae* (**Figure 47B**), the average Ct difference between dead cells with PEMAX and with no PEMAX was significantly higher (ρ value <0.05) at a PEMAX concentration of 50 µM, and no significant differences (ρ value >0.05) were observed when the PEMAX concentration was further

increased to 100 and 200 µM. Regarding the average Ct difference between viable cells with PEMAX and with no PEMAX, no significant differences (ρ value >0.05) were observed between 5, 10, 25, 50, and 100 µM, showing that only 200 µM could affect amplification of DNA from viable cells.

Regarding *L. plantarum* (**Figure 47C**), the average Ct difference between dead cells with PEMAX and with no PEMAX was significantly higher (ρ value <0.05) at a PEMAX concentration of 200 µM when compared to 5 and 10 µM, and the reduction of Ct values in the PMA-Cells-qPCR did not significantly increase (ρ value >0.05) between PEMAX concentrations of 5, 10, 25, 50, and 100 µM. The average of Ct difference between viable cells with PEMAX and with no PEMAX when the PEMAX concentration was lower than or equal to 100 µM, no significant differences (ρ value >0.05) were found, showing that these PEMAX concentrations do not affect amplification of DNA from viable cells.

Regarding *O. oeni* (**Figure 47D**), the small variation observed of dead cells ΔCt can be explained both by the high Ct values (Ct ≥28) and the lack of specific melt peak (**Figure 48**) obtained for dead cells with no PEMAX (0 µM). These results mean that any PEMAX concentrations can distinguish between viable and dead cells.

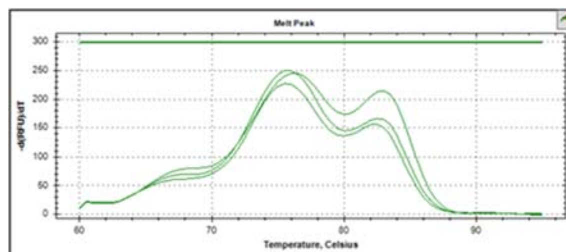


Figure 48- Melting curves analysis of the PMA-Cells-qPCR products from *O. oeni* dead cells with 0 µM of PEMAX

The average Ct difference between *O. oeni* dead cells with PEMAX and with no PEMAX was not significantly different (ρ value >0.05) between all PEMAX concentrations. Nevertheless, the Ct difference between viable cells with PEMAX and with no PEMAX was significantly different (ρ value <0.05) for PEMAX concentration higher or equal of 25 µM, affecting the amplification of DNA from viable cells. When the PEMAX concentration was equal or lower than 10 µM, no significant differences (ρ value >0.05) in Ct values were found between PEMAX treated and those from no PEMAX treated viable cells.

Therefore, the optimization of PEMAX showed that 50 and 5 µM were considered optimal to achieve a compromise between minimal impact on intact cells and maximal signal reduction in the compromised cells of the yeasts and LAB tested species, respectively (**Figure 47**). The selected PEMAX concentrations are lower than the selected by other authors (Udomsil et al., 2016; Vendrame et al., 2013; Vendrame et al., 2014), that used traditional PMA for viable cells enumeration. This result suggests the effectivity of PEMAX for penetrating on cells with membrane damage.

It was speculated that signal reduction of amplification could not be only from the PEMAX dye but also from the PEMAX treatment conditions as washes, light exposure, and incubation. Therefore, beyond the effect of PEMAX on PMA-Cells-qPCR inhibition of viable and dead cells by Ct comparison of cells with PEMAX and with no PEMAX (0 µM), another control with no dye treatment conditions was included in the assays. For this, the Ct obtained by cells suspensions with no PEMAX dye (0 µM) which were subjected to PEMAX treatment conditions (washes, light exposure, and incubation time) were compared with the Ct obtained by cell suspensions with no PEMAX dye and respective treatment conditions. No significant differences (ρ value >0.05) were observed between both controls, both for viable and dead cells of all tested species, showing that PEMAX treatment conditions do not reduce the amplification signal. These results suggest that these treatment conditions do not interfere with the total cells number present in the sample, being adequate for cells suspensions treatment with PEMAX.

4.2.2. Culture media standard curves

Once settled the best conditions for using PEMAX for each species, standard curves were created for each species with viable and dead cells suspensions. To this end, 10-fold serial dilutions from 10^8 to 10^2 cells/mL of both viable and dead cells were prepared in culture media, in independent experiments. The number of viable cells was determined by plate counts for all viable cells suspensions. The two highest cell concentrations (10^8 and 10^7 cells/mL) of dead cells was also platted for each species in order to ensure the absence of viable cells. All the cell suspensions were then washed, treated with PEMAX, mechanically lysed and quantified by PMA-Cells-qPCR.

For all the assays, absence of growth was observed for all cells suspensions comprising only dead microorganisms, demonstrating that the heat treatment allowed to obtain suspensions constituted only by dead cells.

The standard curves were created correlating PMA-Cells-qPCR Ct values and viable plate count results.

Regarding *B. bruxellensis* viable cells quantification, the assay was linear over four orders of magnitude and the LoQs were from 10^3 to 10^6 cells/mL with an R^2 higher than 0.99 and an *E* of 0.87. For low cells concentrations (10^3 cells/mL), the obtained Ct values for viable and dead cells were quite similar. Nevertheless, it was possible to quantify low cells concentrations as specific melt curves were only observed for viable cells. Comparing with Cells-qPCR, these results indicating that PMA-Cells-qPCR leads 1 log sensitivity loss for the detection of *B. bruxellensis* cells. All dead cells Ct values were close to 33 which is similar to the values obtained for negative controls. Nevertheless, when the population of cells was higher, this value decreased slightly (**Figure 49A**).

For *S. cerevisiae* viable cells quantification, the assay was linear over five orders of magnitude and the LoQs were from 10^2 to 10^6 cells/mL with an R^2 higher than 0.99. For all viable cell suspensions specific melt curves were observed, and in no case, the Ct values of viable cells overlap Ct values of dead cells (**Figure 49B**) Moreover, the *E* was 0.99 and the obtained LoQs are similar than the obtained by Cells-qPCR, showing the effectivity and reliability of the assay.

Regarding LAB species viable cells quantification, linearity was obtained over six orders of magnitude with R^2 than 0.99 and *E* of 1.10. In these cases, as observed for *B. bruxellensis*, for the lowest cell concentration (10^3 cells/mL), the Ct values of viable cells almost overlaps the Ct values dead cells. Nevertheless, specific melt curves were also observed only for viable cells suspensions, becoming possible to differentiate between viable and dead cells. Therefore, 10^3-10^8 cells/mL are the LoQs for both LAB species, indicating that PMA-Cells-qPCR leads 1 log sensitivity loss for the detection of *L. plantarum* and *O. oeni* cells when comparing with Cells-qPCR (**Figure 49C, D**).

Comparing with the other species Ct values obtained for *L. plantarum* dead cells suspensions were slightly lower (**Figure 49D**), and the melt curves showed that in almost all cases specific melt curves were observed. These results analysis showed that PEMAX treatment cannot efficiently suppress *L. plantarum* dead cells from PMA-Cells-qPCR. As described by other authors (Contreras et al., 2011; Fittipaldi et al., 2012; Luo et al., 2010; Zeng et al., 2016), signal suppression

from dead cells can show incomplete if the amplicon size of the target gene is too short. Longer amplicons seemed superior to shorter ones in reflecting the extent of cell death induced by the treatment (Fittipaldi et al., 2012). Having this in mind and taking into account that the length of the selected primers is 68 bp, those were considered unsuitable to quantify *L. plantarum* viable cells using PEMAX for suppress dead cells amplification. The selection/design of different primers that codify sequences with longer lengths could be a solution for this limitation.

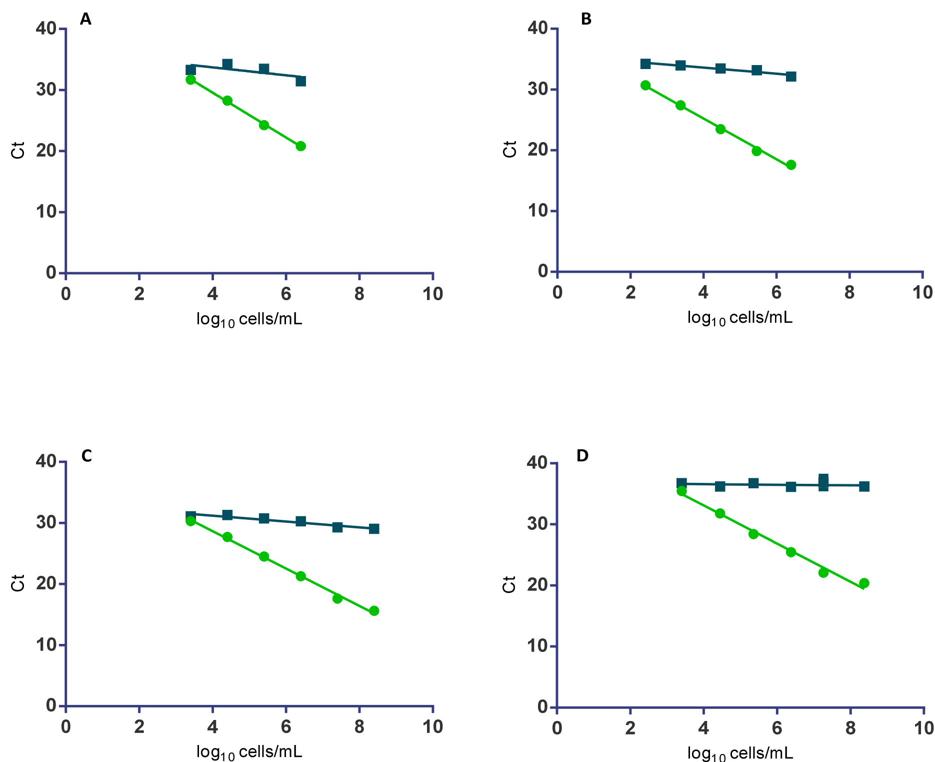


Figure 49- Standard curves obtained by PMA-Cells-qPCR from the 10-fold serial dilutions of (**A**) *B. bruxellensis*, (**B**) *S. cerevisiae*, (**C**) *L. plantarum,* and (**D**) *O. oeni* mechanically lysed viable (●) and dead (■) cells in culture media. The Ct values are the averages of three replicates. Error bars represent standard errors.

4.2.3. White/red wines standard curves

The ability of the PMA-Cells-qPCR methodology to detect and quantify *B. bruxellensis*, *S. cerevisiae*, and *O. oeni* viable cells in white and red wines was also evaluated. For this purpose, standard curves were created for each species applying the same methodology as for culture media standard curves.

As observed for culture media standard curves, absence of growth was observed for all cell suspensions comprising only dead microorganisms, confirming that the heat treatment allowed to obtain suspensions constituted only by dead cells.

The obtained results showed that the PMA-Cells-qPCR methodology can be successfully applied to detect and quantify *B. bruxellensis*, *S. cerevisiae*, and *O. oeni,* both in white and red wines. The regression analysis showed that for viable cells quantification the assays were linear over five orders of magnitude for *S. cerevisiae*, and over four orders of magnitude for *B. bruxellensis* and *O. oeni* (**Figure 50**). The obtained R^2 values were higher than 0.98 for *B. bruxellensis*, and higher than 0.99 for *S. cerevisiae* and *O. oeni*. In white wine standard curves, *E* of 1.04 were obtained for *B bruxellensis* and *S. cerevisiae*, and of 1.02 for *O. oeni*. Regarding red wine standard curves, the *E* were 1.14, 1.045, and 1.13 for *B bruxellensis*, *S. cerevisiae*, and *O. oeni*, respectively.

For standard curves of viable yeast cells, the LoQs were the same as those obtained for the culture medium: *B. bruxellensis* (10^3-10^6 cells/mL=10-10^4 cells/reaction tube), and *S. cerevisiae* (10^2-10^6 cells/mL=1-10^4 cells/reaction tube). Regarding *O. oeni* standard curves, when compared with that obtained from culture medium, the assay recorded 2 log sensitivity loss both for white and red wines. Within this cell concentration ranges, the Ct obtained for dead cells were in all cases over than 31, and no specific melt curves were observed, confirming the exclusive quantification of viable cells.

The obtained LoQs were higher than those obtained by Vendrame et al. (2014), for *B. bruxellensis* and Vendrame et al. (2013) for *O. oeni*, who detected 10 CFU/mL of purified DNA from viable cells in wine matrices. Regarding *S. cerevisiae* regression analysis, the results showed that the use of PEMAX allowed PMA-Cells-qPCR technique to reach LoQ as low as 10^2 cells/mL for viable cells quantification both in white and red wine. This result was better than the obtained by Andorrà et al. (2010), where the LoD was 10^3 cells/mL to quantify DNA of viable cells. Nevertheless, Lv et al. (2016) have reported LoQ of 10^1 CFU/mL using purified DNA of *S. cerevisiae* viable cells as template.

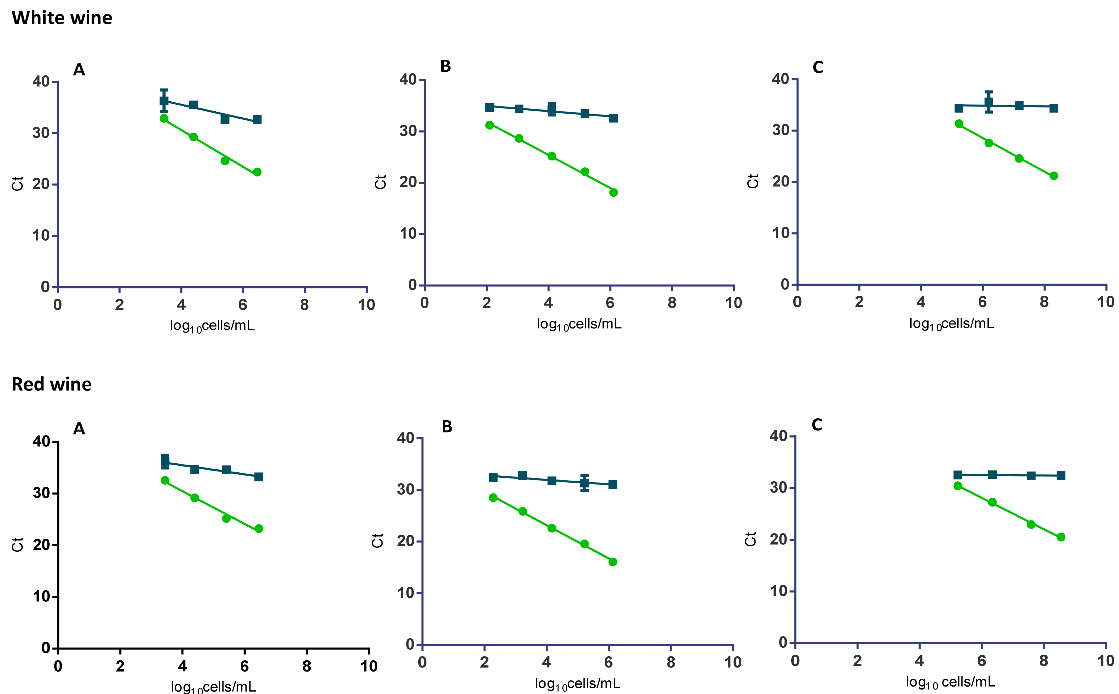


Figure 50- Standard curves obtained by PMA-Cells-qPCR from the 10-fold serial dilutions of (**A**) *B. bruxellensis*, (**B**) *S. cerevisiae*, and (**C**) *O. oeni* mechanically lysed viable (●) and dead (■) cells in white and red wines. The Ct values are the averages of three replicates. Error bars represent standard errors.

4.3. Development of a LAMP Method for Direct Total Cells Detection (Cells-LAMP)

4.3.1. Detection of *B. bruxellensis*

In order to develop a LAMP assay for a direct total cells detection of *B. bruxellensis*, the target products were amplified both from cell suspensions and DNA extracted from the same cell suspensions. This experiment was carried out with *B. bruxellensis* pure cultures from culture medium. A 2.5-microliter volume of extracted DNA and a 12.5-microliter volume of whole cells concentration were subjected to LAMP with specific primers, in independent experiments. For extracted DNA, the sample volume used in each reaction was the suggested by Hayashi et al. (2007). Regarding whole cells, 10-fold more samples volumes were applied in each LAMP reaction as, with higher sample volumes, greater sensitivity was obtained for Cells-qPCR methodology.

The results showed, for both DNA and whole cells, the presence of turbidity by direct observations of the reaction tubes (**Figure 51A**), proving that it is possible to do away with the DNA extraction step for LAMP amplification. Moreover, these results were confirmed resolving the amplified products by agarose gel electrophoresis, showing, as expected, a ladder-like pattern (**Figure 51B**). The lack of both turbidity and ladder-like pattern in negative control sample confirms the specificity of the assay.

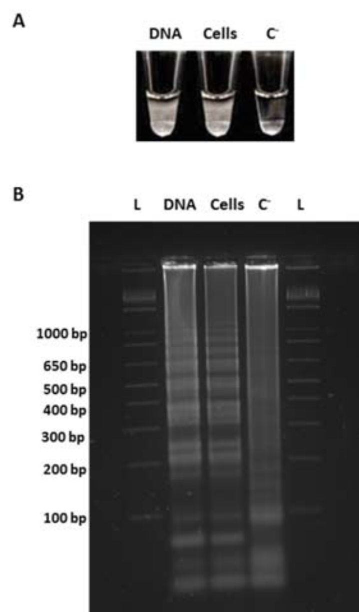


Figure 51- LAMP amplifications from DNA with a sample volume of 2.5 µL, and whole cells with a sample volume of 12.5 µL of *B. bruxellensis* pure cultures. (**A**) Direct observation of turbidity, (**B**) Agarose gel electrophoresis of amplified products. C$^-$, negative control; L, 1 Kb plus DNA Ladder (Invitrogen).

Subsequently, the ability of the Cells-LAMP assay for direct detection of *B. bruxellensis* whole cells in white/red grape musts and wines was evaluated. For this purpose, cells suspensions with a final concentration of 10^6 cells/mL were prepared, in all of the different matrices, in independent experiments. Two samples of *B. bruxellensis* cells suspensions from culture medium were used as positive controls. After wash, all cell suspensions were subjected to LAMP amplification. The increased turbidity along with DNA amplification was observed in all the reaction tubes as well as, ladder-like pattern presence by agarose gel electrophoresis of the respective amplified products (**Figure 52**). Hence, it is possible to confirm the *B. bruxellensis* whole cells

detection by Cells-LAMP reaction, in one hour, and directly from wine-related matrices, overcoming the presence of inhibitors inherent to this kind of matrices.

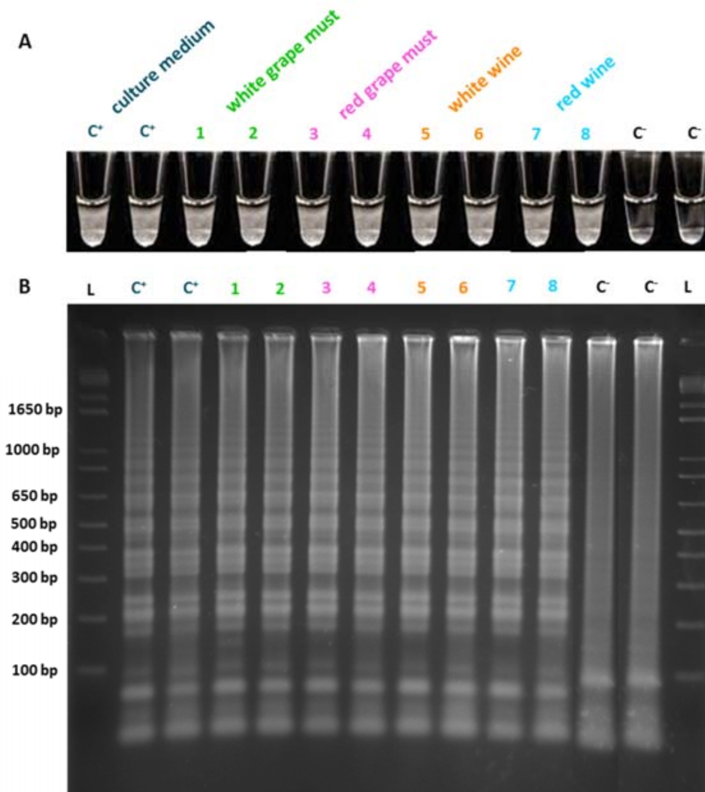


Figure 52- Cells-LAMP amplifications from *B. bruxellensis* cells suspensions from culture medium, white grape must, red grape must, white wine, and red wine. (**A**) Direct observation of turbidity, (**B**) Agarose gel electrophoresis of amplified products. 1, 2, white grape must; 3, 4, red grape must; 5, 6, white wine; 7, 8, red wine; C^+, positive control; C^-, negative control; L, 1 Kb plus DNA Ladder (Invitrogen).

4.3.2. Design and specificity of LAMP primer sets for *O. oeni* detection

Considering that it is possible to amplify DNA directly from *B. bruxellensis* whole cells in culture medium, and in white/red grape musts and wines by Cells-LAMP, and in order to extend it to another species, a set of primers were designed for direct detection of *O. oeni*. Therefore, based on 16S rRNA region and using the LAMP Designer 1.13 software, six LAMP primers (two outer, two inner, and two loop) were carefully designed (**Figure 53**). For more information about the designed primers see **Annex 2**.

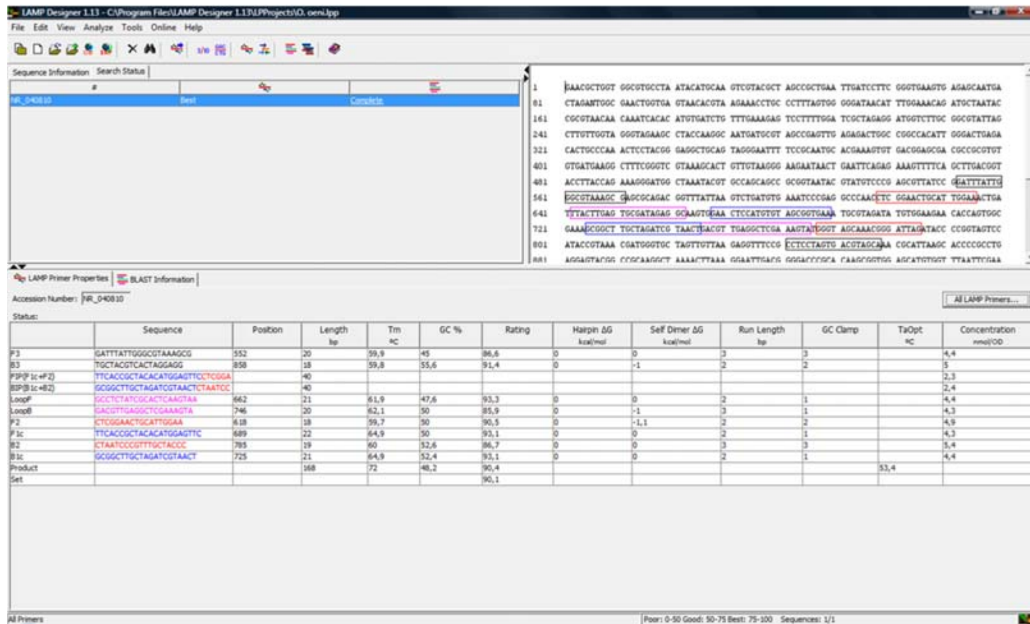


Figure 53- Output obtained by LAMP Designer 1.13 software for *O. oeni* LAMP primers design.

After design, the primers specificity was estimated by both conventional PCR and LAMP reactions with different species of bacteria and yeasts.

Regarding conventional PCR, two independent reactions were performed. With the F3/B3 primers, a fragment with the expected size was observed for *O. oeni*, *Lc. mesenteroides*, *L. brevis*, *G. oxydans*, *S. cerevisiae*, and *B. bruxellensis* (**Figure 54A**). With the FIP/BIP primers, a fragment with the expected size was only observed for *O. oeni* and *Lc. mesenteroides* (**Figure 54B**).

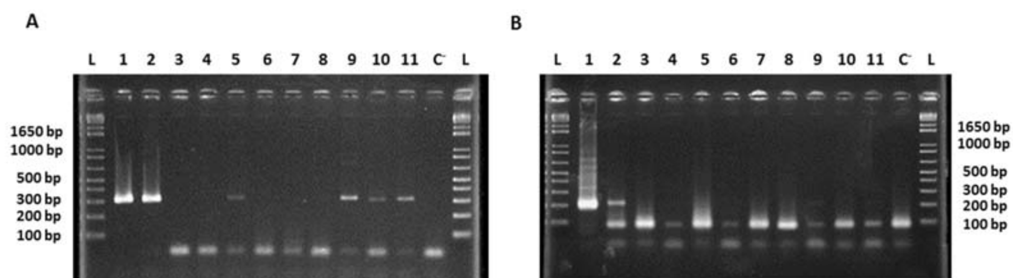


Figure 54- Agarose gel electrophoresis of conventional PCR amplification from extracted DNA of different yeasts and bacteria species. (**A**) Amplification with F3/B3 primers, and (**B**) amplification with FIP/BIP primers. 1, *O. oeni*; 2, *Lc. mesenteroides*; 3, *L. plantarum*; 4, *L. hilgardii*; 5, *L. brevis*; 6, *Pd. damnosus*; 7, *Pd. pentosaceus*; 8, *A. aceti*; 9, *G. oxydans*; 10, *S. cerevisiae*; 11, *B. bruxellensis*; C^-, negative control; L, 1 Kb plus DNA Ladder (Invitrogen).

Nevertheless, in LAMP amplifications with the six primers in the same experiment, increased turbidity and respective ladder-like pattern by agarose gel electrophoresis were only observed when the primer set amplified *O. oeni* species (**Figure 55**). These results showed that despite the obtained results by independent reactions of conventional PCR, LAMP primers designed to target the 16S rRNA gene, were highly specific for *O. oeni* as no amplification with DNA from other of the ten species of bacteria and yeasts was obtained, whereas DNA from *O. oeni* was efficiently amplified.

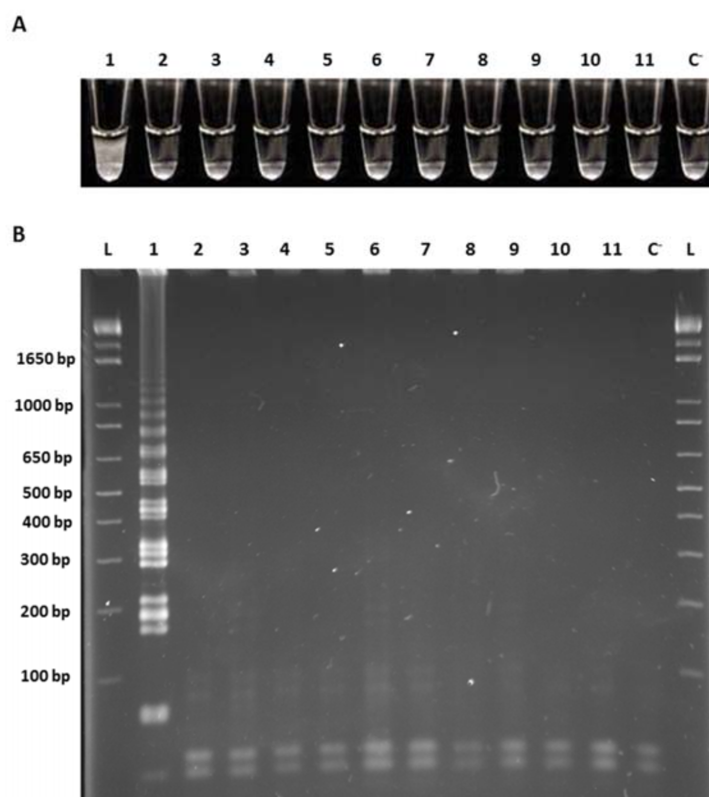


Figure 55- LAMP amplifications from extracted DNA of different yeasts and bacteria species. (**A**) Direct observation of turbidity, (**B**) Agarose gel electrophoresis of amplified products. 1, *O. oeni*; 2, *Lc. mesenteroides*; 3, *L. plantarum*; 4, *L. hilgardii*; 5, *L. brevis*; 6, *Pd. damnosus*; 7, *Pd. pentosaceus*; 8, *A. aceti*; 9, *G. oxydans*; 10, *S. cerevisiae*; 11, *B. bruxellensis*; C^-, negative control; L, 1 Kb plus DNA Ladder (Invitrogen).

4.3.3. Detection of *O. oeni*

Considering that the designed primers are species-specific, the applicability of the Cells-LAMP method for direct *O. oeni* total cells detection, in wine-related matrices, was evaluated.

Therefore, cell suspensions were prepared in culture medium, and in white/ red grapes musts and wines, in independent experiments. After wash, cell suspensions with a final concentration of 10^6 cells/mL were subjected to LAMP amplifications. Positive reactions were observed in all matrices, by turbidity increase (**Figure 56A**). Moreover, ladder-like pattern was also observed after resolving products by agarose gel electrophoresis (**Figure 56B**). These results confirm the method reliability and validity to detect *O. oeni* whole cells directly from wine-related matrices without reaction inhibition by the alcohol, polyphenols, tannins, or other wine inhibitors.

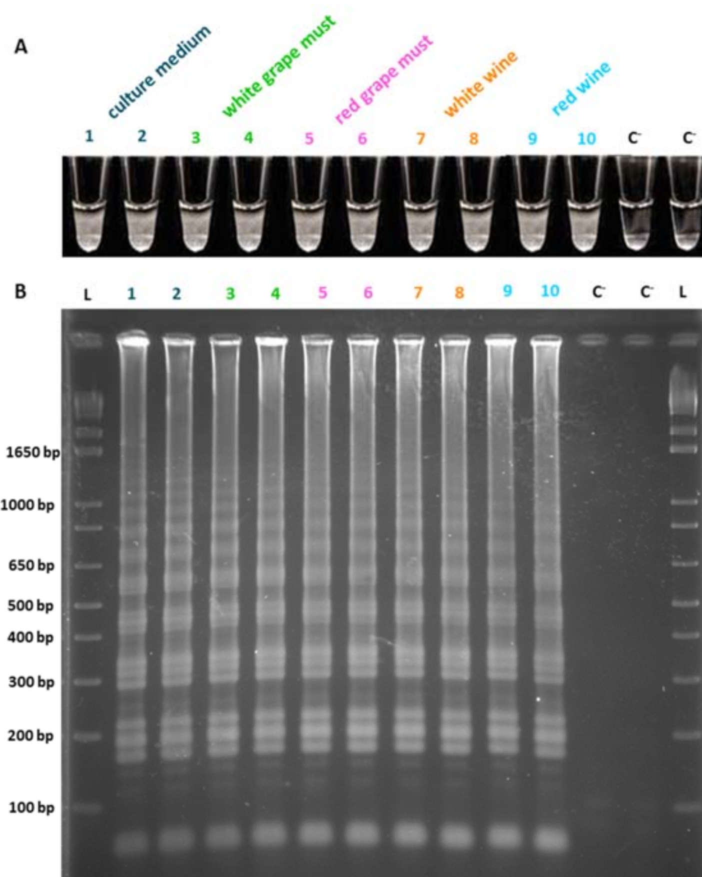


Figure 56- Cells-LAMP amplifications from *O. oeni* cells suspensions from culture medium, white grape must, red grape must, white wine, and red wine. (**A**) Direct observation of turbidity, (**B**) Agarose gel electrophoresis of amplified products. 1, 2, culture medium; 3, 4, white grape must; 5, 6, red grape must; 7, 8, white wine; 9, 10, red wine; C^-, negative control; L, 1 Kb plus DNA Ladder (Invitrogen).

4.3.4. Detection limit of *B. bruxellensis* and *O. oeni* from white and red wines

In order to assess the detection limit of the Cells-LAMP method for the *B. bruxellensis* and *O. oeni* detection in white and red wines, serial dilutions from 10^8 to 10^2 cells/mL were prepared in both matrices and using the same matrix as diluent. After cells wash, all cell suspensions were subjected to LAMP amplification with specific primers, in independent experiments. The results were obtained by direct turbidity observation of the reaction tube followed by products resolving by agarose gel electrophoresis. This experiment was repeated twice

Regarding *B. bruxellensis*, in both experiments, the obtained LoDs were 10^2 cells/mL both in white wine and red wine (see **Figure 57** for an example). This result highlights the method's high sensitivity and versatility as it indicates the possibility of detecting one cell per reaction tube, and that the matrix does not interfere with detection.

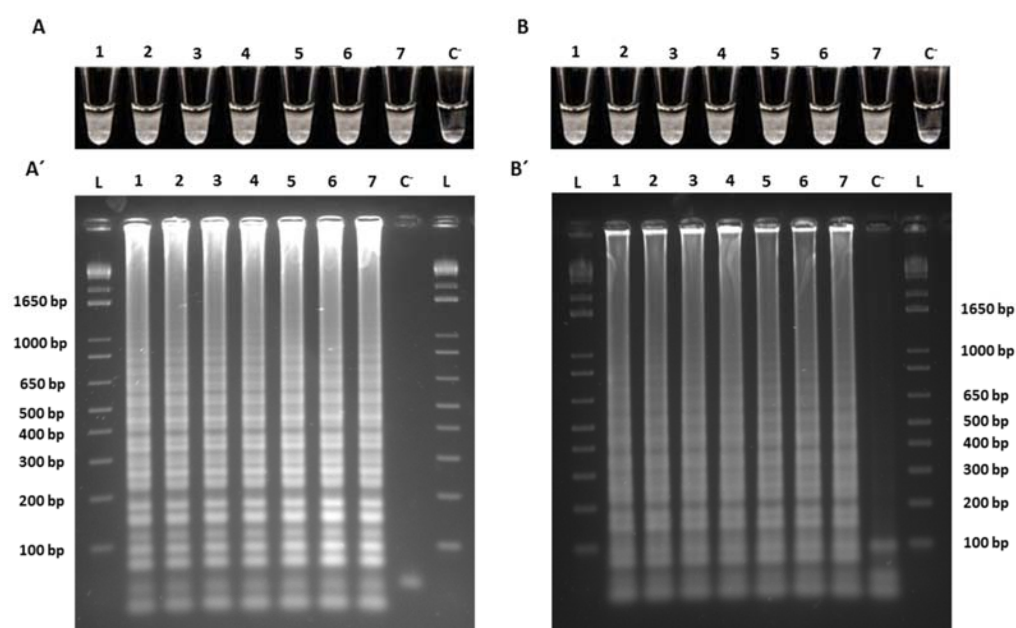


Figure 57- Cells-LAMP amplifications from 10-fold serial dilutions of *B. bruxellensis* whole cells in (**A**) white wine, and (**B**) red wine. A, B, Direct observation of turbidity; A´, B´, Agarose gel electrophoresis of amplified products; 1, 10^8 cells/mL; 2, 10^7 cells/mL; 3, 10^6 cells/ mL; 4, 10^5 cells/ mL; 5, 10^4 cells/ mL; 6, 10^3 cells/mL; 7, 10^2 cells/mL, C^-, negative control; L, 1 Kb plus DNA Ladder (Invitrogen).

The detection limit was higher than the obtained by Hayashi et al. (2007), who detected DNA in wine samples with 10 CFU/mL of *B. bruxellensis*. Nevertheless, it is possible to detect

populations lower than 10^2 cells/mL by sample concentration during the washing step (ten times or more).

Regarding *O. oeni*, in both experiments, the obtained LoDs were 10^3 cells/mL (12 cells/reaction tube) in white wine, and 10^4 cells/mL (120 cells/reaction tube) in red wine (see **Figure 58** as example).

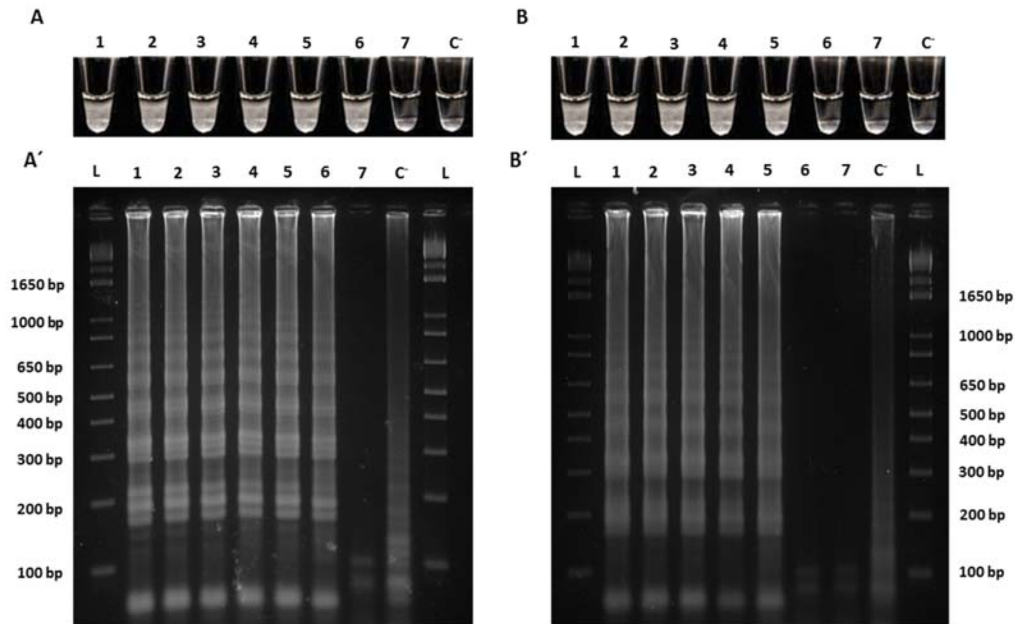


Figure 58- Cells-LAMP amplifications from 10-fold serial dilutions of *O. oeni* whole cells in (**A**) white wine, and (**B**) red wine. A, B, Direct observation of turbidity; A´, B´, Agarose gel electrophoresis of amplified products; 1, 10^8 cells/mL; 2, 10^7 cells/mL; 3, 10^6 cells/ mL; 4, 10^5 cells/ mL; 5, 10^4 cells/ mL; 6, 10^3 cells/mL; 7, 10^2 cells/mL, C^-, negative control; L, 1 Kb plus DNA Ladder (Invitrogen).

In order to decrease the *O. oeni* LoD in both matrices, the effect of a previous cell wall mechanical lysis was evaluated. For this, the assay was repeated but before Cells-LAMP amplification, all cells suspensions were subjected to mechanical lysis. In both experiments, the obtained results showed that the cell lysis lowered the *O. oeni* LoD for 10^2 cell/mL in both matrices. Therefore the LoDs were 10-fold and 100-fold lower in white and red wine, respectively (see **Figure 59** for an example), showing the excellent assay sensitivity when performing a mechanical lysis.

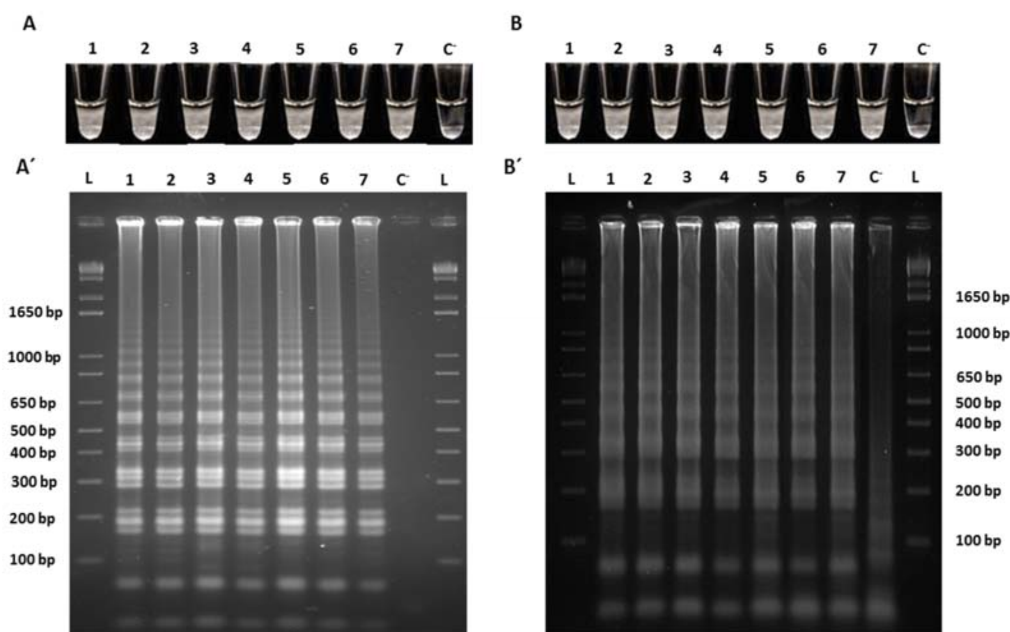


Figure 59- Cells-LAMP amplifications from 10-fold serial dilutions of *O. oeni* lysed cells in (**A**) white wine, and (**B**) red wine. A, B, Direct observation of turbidity; A´, B´, Agarose gel electrophoresis of amplified products; 1, 10^8 cells/mL; 2, 10^7 cells/mL; 3, 10^6 cells/ mL; 4, 10^5 cells/ mL; 5, 10^4 cells/ mL; 6, 10^3 cells/mL; 7, 10^2 cells/mL, C^-, negative control; L, 1 Kb plus DNA Ladder (Invitrogen).

To sum up, the obtained results showed that the developed Cells-LAMP method allows the detection of *B. bruxellensis* and *O. oeni* directly from both grape musts and wines within 60 min from the beginning of the Cells-LAMP reaction. For the detection of both species, the obtained results by observing the turbidity at the endpoint of the reaction were in accordance with the obtained by agarose gel electrophoresis. These results showed that turbidity observation is a reliable method to visualize Cells-LAMP amplifications and results confirmation by agarose gel electrophoresis can be discarded. Moreover, agarose gel electrophoresis requires expensive laboratory infrastructure (electrophoresis equipment, image capture system, etc.), and generally toxic agents (ethidium bromide), making this procedure impracticable for applications in low resource wineries. Nevertheless, it is important to take into account that agarose gel electrophoresis can be useful for specificity evaluation, as it is able to distinguish between real and false positives under some unexpected cases when non-specific amplification occurs (Zhang et al., 2014).

4.4. Development of a qLAMP Method for Direct Total Cells Detection and Quantification (Cells-qLAMP)

4.4.1. SYBR Green I fluorescence-based qLAMP

Considering that is possible detect *B. bruxellensis* and *O. oeni* cells in wine-related matrices by Cells-LAMP, the availability of Cells-qLAMP assay for the direct quantification of both species was also investigated. For this purpose, cell suspensions with a final concentration of 10^3, 10^5, and 10^7 cells/mL were prepared in culture media, were washed, and then were subjected to qLAMP amplification using SYBR Green I as a fluorescent dye, in independent experiments.

Regarding *B. bruxellensis* detection and quantification, amplification curves observation showed that the Cells-qLAMP assay could not efficiently detect and quantify *B. bruxellensis* whole cells (**Figure 60A**). Moreover, the melt peaks indicated lack of specific amplification (**Figure 60B**).

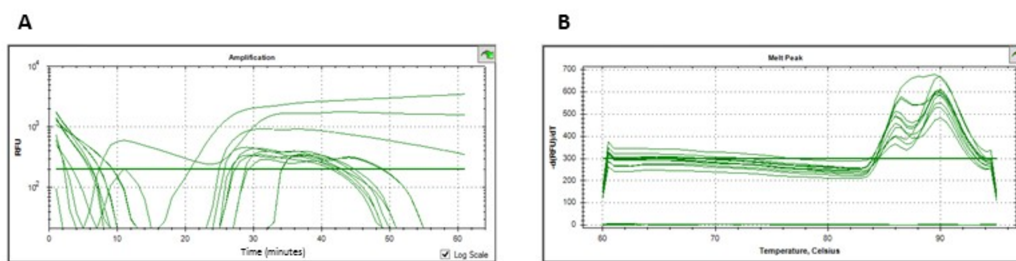


Figure 60- Results of *B. bruxellensis* detection and quantification by SYBR Green I fluorescence-based Cells-qLAMP. (**A**) relative fluorescence units (RFU) vs time, (**B**) melting curves analysis of the amplified products.

Regarding *O. oeni* detection and quantification, all cell suspensions were previously mechanically lysed. The results of Cells-qLAMP amplification were similar to those obtained for *B. bruxellensis*. Under these conditions, the Cells-qLAMP assay with SYBR Green I could not efficiently detect and quantify *O. oeni* whole cells (**Figure 61A**). Moreover, the melt peaks also indicated lack of specific amplification (**Figure 61B**).

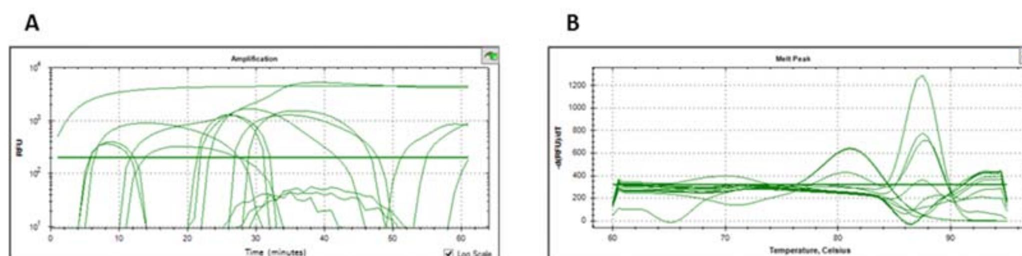


Figure 61- Results of *O. oeni* detection and quantification by SYBR Green I fluorescence-based Cells-qLAMP. (**A**) relative fluorescence units (RFU) vs time, (**B**) melting curves analysis of the amplified products.

These results can be due to SYBR Green I inhibitory effects on DNA amplification that were also reported by other authors (Eischeid, 2011; Oscorbin et al., 2016). Therefore, Cells-qLAMP amplification using SYBR Green I as a fluorescent dye for *B. bruxellensis* and *O. oeni* cells detection and quantification was rejected.

4.4.2. SYTO-9 fluorescence-based qLAMP

Besides SYBR Green I, the SYTO-9 fluorescent dye is also widely used for qLAMP reactions. Moreover, in a comparative study of six fluorescent dyes (SYTO-9, SYTO-13, SYTO-82, SYBR Green I, SYBR Gold, and EvaGreen) for qLAMP, Oscorbin et al. (2016) showed that SYTO-82 and SYTO-9 had the best results. Therefore, the ability of Cells-qLAMP amplifications using SYTO-9 as a fluorescent dye to detect and quantify *B. bruxellensis* and *O. oeni* cells was evaluated.

Cell suspensions with a final concentration of 10^3, 10^5, and 10^7 cells/mL were prepared in culture media, washed, and were then were subjected to qLAMP amplification using SYTO-9 as a fluorescent dye, in independent experiments.

Regarding *B. bruxellensis* detection and quantification, the obtained amplification curves suggested positive results, as typical real-time amplification performance was observed (**Figure 62A**). Moreover, specific melt peaks were also observed (**Figure 62B**).

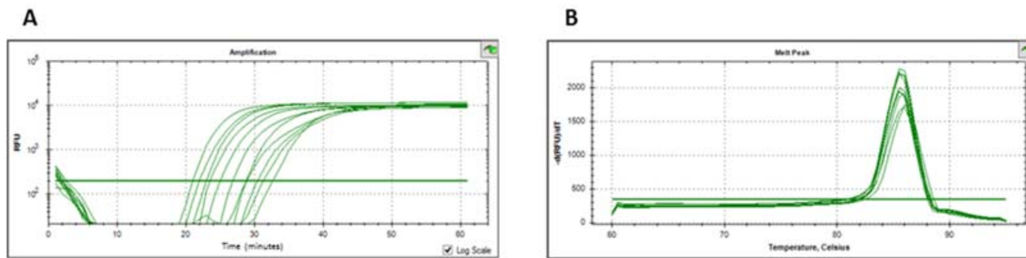


Figure 62- Results of *B. bruxellensis* detection and quantification by SYTO-9 fluorescence-based Cells-qLAMP. (**A**) relative fluorescence units (RFU) vs time, (**B**) melting curves analysis of the amplified products.

Consequently, the quantification ability of the method was tested by correlating the obtained Tt by Cells-qLAMP reaction and the *B. bruxellensis* cells concentration. As shown in **Table 14**, no correlation was observed between both parameters, showing that these reaction conditions do not allow a suitable quantification of *B. bruxellensis* cells. These results can be explained by some kind of inhibition or interference that may occur during amplification, and as a consequence, affects the correct quantification of *B. bruxellensis*.

Table 14- Tt results obtained by SYTO-9 fluorescence-based Cells-qLAMP from *B. bruxellensis* cell suspensions.

Sample	Fluorescent dye	Tt	Cells concentration (log_{10} cells/mL)
B. bruxellensis	SYTO-9	29.56	7.223
		27.61	
		27.42	
		25.82	5.223
		28.94	
		21.64	
		22.73	3.223
		23.66	
		24.31	
Negative Control		31.61	0.000
		32.34	
		31.12	

Regarding *O. oeni* detection and quantification, the obtained amplification curves suggested positive results (**Figure 63A**), and in addition, the observed melt peaks were also specific (**Figure 63B**).

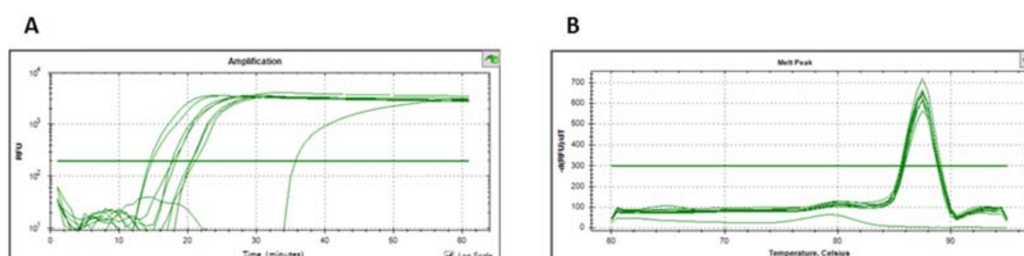


Figure 63- Results of *O. oeni* detection and quantification by SYTO-9 fluorescence-based Cells-qLAMP. (**A**) relative fluorescence units (RFU) vs time, (**B**) melting curves analysis of the amplified products.

Nevertheless, in this case, for negative control the Tt values were high and no specific melt curves were observed. Moreover, a good correlation was observed between Tt of Cells-qLAMP reaction and the *O. oeni* cells concentration (**Table 15**) suggesting that the amplification was reliable and the SYTO-9 fluorescence-based qLAMP assay could be used for *O. oeni* cells detection and quantification.

Table 15- Tt results obtained by SYTO-9 fluorescence-based Cells-qLAMP from *O. oeni* cell suspensions.

Sample	Fluorescent dye	Tt	Cells concentration (log_{10} cells/mL)
O. oeni	SYTO-9	14.03	7.176
		14.76	
		14.49	
		17.84	5.176
		18.23	
		17.60	
		20.51	3.176
		20.45	
		21.09	
Negative Control		35.65	0.000
		34.03	
		34.78	

After that, the sensitivity of the SYTO-9 fluorescence-based Cells-qLAMP to detect and quantify *O. oeni* cells was evaluated. For the analysis, 10-fold serial diluted cells from 10^8 to 10^2 cells/mL were prepared in culture medium, washed, mechanically lysed, and then subjected to qLAMP amplification. The data regression analysis (**Figure 64**) showed that the assay was linear over seven orders of magnitude (10^2 to 10^8 cells/mL) with an R^2 higher than 0.97, and an *E* of 1.23. This result highlights the method's high sensitivity as it indicates the possibility of detecting one cell per reaction.

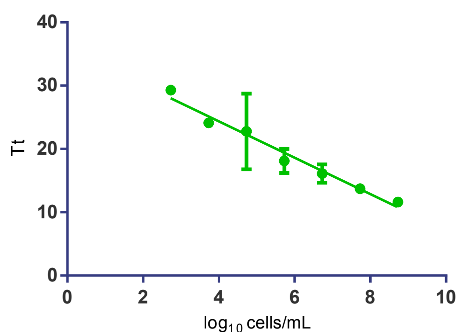


Figure 64- Standard curves obtained by SYTO-9 fluorescence-based Cells-qLAMP from the 10-fold serial dilutions of *O. oeni* in culture medium. The Tt values are the averages of three replicates. Error bars represent standard errors.

The Cells-qLAMP amplifications using SYTO-9 as fluorescence dye showed smaller inhibitory effect to LAMP than SYBR Green I, indicating to be the best option for detection and quantifications.

More experiments with different reaction conditions or fluoresce dye should be performed in order to improve microorganisms quantification directly from wine samples. Nevertheless, great developments were achieved for the quantification of wine-related microorganisms, obtaining good results. Due to its high specificity, high sensitivity, and ease operation, the Cells-qLAMP method has a high potential to be applied in wine industry.

5. CONCLUSIONS

- Two methods based in PCR technique and another two based in LAMP technique have been developed, allowing the detection (Cells-LAMP), and the quantification of both total cells (Cells-qPCR, Cells-qLAMP), and viable and dead cells (PMA-Cells-qPCR), directly from grape musts and wines.

- Specific probes either already described in a bibliography or developed in this work, were used to detect and quantify yeasts and total acetic acid bacteria in wine, and specifically the most important species in winemaking such as *S. cerevisiae*, *L. plantarum,* and *O. oeni*, as well as the spoilage yeasts *B. bruxellensis* and *Z. bailii*.

- The developed procedures allow the detection and quantification of the whole cells of these microbial groups or specific species without DNA extraction or purification requirements and directly from musts, wines or culture media.

- Treatment through mechanical lysis increased the sensitivity of the four methods, allowing the detection of a single cell present in the reaction tube.

- The matrix from which the cells are obtained (culture media, musts or wines, both white and red) does not inhibit PCR and LAMP reactions. This is particularly remarkable for red wines, where polyphenols and ethanol, frequently described as polymerase inhibitors, are found. Moreover, the limits of detection and quantification, and the efficiencies obtained in musts and wines were similar to those obtained in culture medium.

- The presence of other microorganisms, that the ones targeted, in the analysed samples, does not influence the results obtained with these techniques, showing that they are totally specific.

- The developed methods allow the detection and quantification of cells (both total, and viable and dead) in a period of time that varies between 1 to 5 hours. These procedures

were applied for the detection and quantification of yeasts and bacteria in musts and wines produced industrially.

- The developed methodologies are both economic and fast, with high sensitivity and specificity. However, with the exception of the LAMP technique used for the detection of microorganisms, the acquisition of a quantitative PCR apparatus is required. These techniques are transferable to service companies in the wine sector and allow the obtention of results in periods short enough so that those responsible for production can make decisions on time.

6. CONCLUSIONES

- Se han desarrollado dos métodos basados en el uso de la técnica de PCR y otros dos basados en la técnica de LAMP que permiten la detección (Cells-LAMP), y la cuantificación tanto de células totales (Cells-qPCR, Cells-qLAMP), como de células viables y muertas (PMA-Cells-qPCR), directamente a partir de mostos y vinos.

- Se han utilizado sondas específicas, ya descritas en bibliografía o desarrolladas en este trabajo para detectar y cuantificar levaduras y bacterias acéticas totales propias del vino, y, en concreto, las especies más importantes en la vinificación como *S. cerevisiae*, *L. plantarum*, y *O. oeni*, así como las levaduras alterantes *B. bruxellensis* y *Z. bailii*.

- Los procedimientos desarrollados permiten realizar la detección y cuantificación de las células enteras de esos grupos microbianos o de las especies concretas sin necesidad de recurrir a la extracción del DNA y directamente a partir de mostos, de vinos o de medios de cultivo.

- La aplicación de un tratamiento de lisis mecánica aumenta la sensibilidad de estos cuatro métodos, permitiendo la detección de una única célula presente en el tubo de reacción.

- La matriz de la cual provengan las células (medios de cultivo, mostos o vinos, tanto blancos como tintos) no inhibe las reacciones de PCR y LAMP. Ello es particularmente importante en el caso de los vinos tintos, donde se encuentran polifenoles y etanol, descritos frecuentemente como inhibidores de las polimerasas. Los límites de detección y cuantificación, y las eficiencias obtenidas en mostos y vinos fueron similares a los obtenidos en medio de cultivo.

- La presencia de microrganismos, distintos de los que son diana, en las muestras analizadas no influye en los resultados obtenidos con estas técnicas, lo que demuestra que son totalmente específicas.

- Los métodos desarrollados permiten la detección y cuantificación de células (tanto totales como vivas y muertas) en un plazo de tiempo que varía entre 1 y 5 horas. Estos procedimientos se han aplicado para la detección y cuantificación de levaduras y bacterias en mostos y vinos producidos industrialmente.

- Las metodologías desarrolladas son económicas, y rápidas y presentan una elevada sensibilidad y especificidad. Sin embargo, se requiere la adquisición de un aparato de PCR cuantitativa, con la excepción de la técnica LAMP utilizada para la detección de microorganismos. Son técnicas transferibles a empresas de servicios al sector enológico y que permiten obtener resultados en plazos lo suficientemente cortos como para que los responsables de producción tomen decisiones a tiempo.

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8. ANNEXES

8.1. Annex 1

	Microorganism	Primer	Sequence (5´-3´)	Target Gene (locus)	Amplicon Size (bp)	Reference
Yeasts	General	YEASTF YEASTR	GAGTCGAGTTGTTTGGGAATGC TCTCTTTCCAAAGTTCTTTTCATCTTT	Variable D1/D2 domain 26S rRNA	124	Hierro et al. (2006)
Yeasts	*B. bruxellensis*	DBRUXF DBRUXR	GGATGGGTGCACCTGGTTTACAC GAAGGGCCACATTCACGAACCCCG	LSU rRNA (D1/D2)[a]	79	Phister and Mills (2003)
Yeasts	*S. cerevisiae*	CESP-F SCER-R	ATCGAATTTTTGAACGCACATTG CGCAGAGAAACCTCTCTTTGGA	ITS2-5.8S rRNA[b]	175	Hierro et al. (2007)
Yeasts	*Z. bailii*	ZBF1 ZBR1	CATGGTGTTTTGCGCC CGTCCGCCACGAAGTGGTAGA	D1/D2 loop 26S rRNA	122	Rawsthorne and Phister (2006)
LAB	*L. plantarum*	LacPla1F LacPla1R	AGGCGCGGCTGATGTCA CGCGATTGTCTTGGTTTTGTT	*recA*	68	Stevenson et al. (2006)
LAB	*O. oeni*	MalomarF MalomarR	GTTAATCATGCCGAATCG GTCGGAAAGACCCTG	gene coding for the malolactic enzyme	285	Vendrame et al. (2013)
AAB	General	I2B AABr	CCCTACTGCTGCCTCCCGTAGGAGT AGGGATCTATCCACGGGTG	16S rRNA	225	Blasco (2009)

[a]**LSU-rRNA (D1/D2)**, D1-D2 domain of the large subunit domain of the rRNA gene; [b]**ITS2-5.8S rRNA**, region spanning the internal transcribed spacer 2 (ITS2) and the 5.8S rRNA gene

8.2. Annex 2

Microorganism	Primer	Sequence (5´-3´)	Target Gene (locus)	Amplicon Size (bp)	Reference
B. bruxellensis	DB1LF1-F3	ACATTGCGCCCTCTGG	5.8S rRNA	236	Hayashi et al. (2007)
	DB1LF1-B3	TGCTTAAGTTCAGCGGGTC	26S rRNA		
	DB1LF1-FIP	ACCCTCGTGTAATCTCATAACCACTAAGGAGGGCATGCCTGTTTG	5.8S rRNA	222	
	DB1LF1-BIP	GATTTAAGGTTTCGGCCGTTCATTATTTTCTCCTACCTGATTTGAGGTCAA	26S rRNA		
	DB1LF1-LoopF	AGTGAGAAGGAAATGACGC	5.8S rRNA	n.a	
O. oeni	Loo-F3	GATTTATTGGGCGTAAAGCG	16S rRNA	307	Described in this work for the first time
	Loo-B3	TGCTACGTCACTAGGAGG			
	Loo-FIP	TTCACCGCTACACATGGAGTTCCTCGGAACTGCATTGGAA		212	
	Loo-BIP	GCGGCTTGCTAGATCGTAACTCTAATCCCGTTTGCTACCC			
	Loo-LoopF	GCCTCTATCGCACTCAAGTAA		124	
	Loo-LoopB	GACGTTGAGGCTCGAAAGTA			

n.a, not applicable