EFECTO DEL ÁCIDO SUCCÍNICO PRODUCIDO POR LEVADURAS VÍNICAS SOBRE OENOCOCCUS OENI Y LA FERMENTACIÓN MALOLÁCTICA

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Efecto del ácido succínico producido por levaduras vínicas sobre *Oenococcus oeni* y la fermentación maloláctica

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Tesis Doctoral

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FEM CONSTAR que aquest treball, titulat “Efecto del ácido succínico producido por levaduras vínicas sobre *Oenococcus oeni* y la fermentación maloláctica”, que presenta FRANCISCO RAFAEL TORRES GUARDADO per a l’obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia d’aquesta universitat.

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Els directors de la tesi doctoral

Dr. Albert Bordons de Porrata-Doria

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¡En mi corazón, en mi casa y en mi patria!

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Resumen

El ácido succínico es un ácido orgánico que pueden producir las levaduras durante el proceso de vinificación. Este ácido es muy similar en su estructura al ácido málico, y puede impedir que la fermentación maloláctica pueda completarse con éxito si sus niveles llegan a inhibir el desarrollo de *Oenococcus oeni*. En este trabajo se ha evaluado el impacto de este ácido sobre el desarrollo de *O. oeni* y la fermentación maloláctica. Además, también se ha realizado un análisis transcriptómico para determinar la influencia a nivel de expresión génica global en *O. oeni*. Adicionalmente, al ser un ácido que se produce por el metabolismo de las levaduras, se ha evaluado el nivel de producción de ácido succínico por cepas de *Saccharomcyces cerevisiae* y de diversas especies de no-*Saccharomyces*. Como consecuencia de su producción por parte de levaduras y su posible efecto en las bacterias lácticas de vino y la fermentación maloláctica, se cataloga dentro del marco de las interacciones microbianas y su impacto en el producto, con posibles consecuencias en la calidad del vino final.

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1. Introducción

1.1. Bebidas alcohólicas e interacciones microbianas

Las bebidas alcohólicas se obtienen por la fermentación de los jugos o mostos de diferentes frutos o mostos de cereales. Durante este proceso, participan levaduras *Saccharomyces*, no-*Saccharomyces*, bacterias ácido-lácticas (BAL) y algunas bacterias acéticas (Fig. 1). Como consecuencia del metabolismo de estos microrganismos se producen diversos compuestos como alcoholes, ésteres, aldehídos y ácidos orgánicos (Fleet 2003).

El vino y la sidra son bebidas fermentadas que se obtienen a partir de jugos de uva y manzana. Estas bebidas se obtienen por la fermentación alcohólica (FA) llevada a cabo por levaduras y la fermentación maloláctica FML realizada por BAL. Sin embargo, otras bebidas fermentadas como las cervezas se elaboran a partir del mosto de malta, la cual se obtiene por la germinación de granos como la cebada. Durante el proceso de malteado, el almidón contenido en los granos se hidroliza y libera azúcares por la acción enzimas como la amilasa. Posteriormente a la adición de agua y lúpulo, las levaduras comienzan el proceso de fermentación alcohólica (De Roos and De Vuyst 2019; Cordes et al. 2021).

Por otra parte, las bebidas destiladas se obtienen por el proceso de destilación de mostos o jugos fermentados. Su calidad está relacionada al cultivo de donde se obtiene el mosto, los tanques o depósitos de elaboración, el proceso industrial o artesanal y finalmente el añejamiento (Wiśniewska et al. 2016). Sin embargo, independientemente de la bebida alcohólica, todas se obtienen por proceso microbiológico, donde BAL y levaduras son responsables de la calidad y perfil aromático (Azzolini et al. 2010; Izquierdo et al. 2014).

El impacto de la población microbiana y sus interacciones influyen de manera sustancial en la elaboración de las diferentes bebidas alcohólicas. Por lo tanto, hemos descrito la importancia de estas interacciones de forma detallada en el artículo de revisión de Torres-Guardado et al. (2022a), el cual se

incluye en el Anexo 1: Torres-Guardado R, Esteve-Zarzoso B, Reguant C, Bordons A (2022) Microbial interactions in alcoholic beverages (Review). International Microbiology 25, 1-15. https://doi.org/10.1007/s10123-021-00200-1.

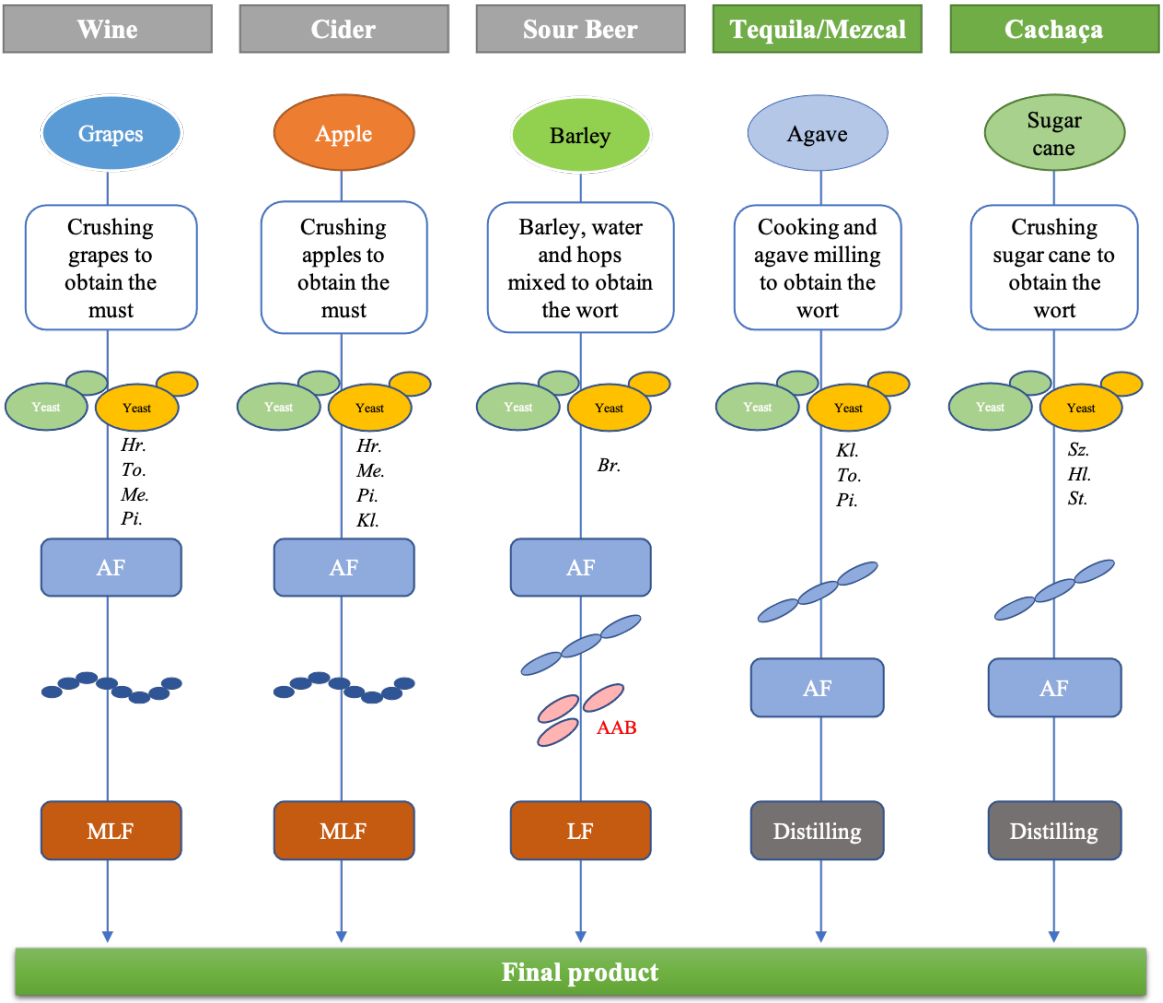

Figura 1. Esquema de los procesos de fermentación de bebidas alcoholicas y destilados (Torres-Guardado et al., 2022). Los organismos principales son: Levaduras *Saccharomyces cerevisiae* (verde) y no-*Saccharomyces* (naranja) como *Hanseniaspora* (*Hr.*), *Torulaspora* (*To.*), *Metschnikowia* (*Me.*), *Pichia* (*Pi.*), *Kluyveromyces* (*Kl.*), *Brettanomyces* (*Br.*), *Schizosaccharomyces* (*Sz.*), *Hansenula* (*Hl.*) y *Candida* (*Ca.*); BAL, bacterias del ácido láctico: *Oenococcus oeni* (azul, forma coco) y *Lactobacillus* (azul, forma bacilo); y BAA, bacterias del ácido acético (rosa, forma bacilo). Fermentación maloláctica (FML) y fermentación láctica (FL).

Este trabajo propone clasificar las interacciones microbianas en los diferentes procesos de fermentación para la obtención de bebidas alcohólicas, no solo con base a la ecología microbiana, sino incluyendo su impacto sobre el producto.

1.2. Interacciones microbianas

Durante los procesos de fermentación se establecen interacciones entre los diferentes microrganismos presentes en el bioproceso, estas interacciones pueden ser positivas o negativas por el efecto que ejerce un microrganismo sobre la población de otro (Tabla 1). No obstante, este efecto está relacionado por la promoción o inhibición del desarrollo y crecimiento, donde actúan diversos mecanismos que determinan cada una de las interacciones (Ivey et al., 2013). Por lo tanto, la naturaleza de los compuestos que los microrganismos liberan al medio en donde se desarrollan, debe ser considerada para clasificar la interacción (Torres-Guardado et al., 2022a).

Desde el punto de vista de la ecología microbiana, las interacciones microbianas se clasifican en dos grupos importantes, negativas y positivas. En el grupo de las interacciones negativas se encuentran el amensalismo y antagonismo. El amensalismo a diferencia del antagonismo indica que un producto de metabolismo primario ejerce un efecto negativo sobre la población de otro microrganismo. En cambio, el antagonismo se define por la acción de un producto dirigido para la inhibición del desarrollo de otra especie o por la competencia de nutrientes.

Por otra parte, en las interacciones positivas figuran el comensalismo y sinergismo. El comensalismo se caracteriza por la interacción de dos especies en la que una de ellas se beneficia sin afectar a la otra. En cambio, cuando dos especies obtienen beneficio mutuo, se establece una interacción de mutualismo o también conocida como sinergismo.

Tabla 1. Principales interacciones en fermentados (adaptación de Ivey et al 2013)

Tipo de interacción	Definición	Referencias
Negativa		
Amensalismo	Interacción donde un producto metabólico de una especie tiene un impacto negativo sobre otra	(Ivey et al. 2013)
Antagonismo	Interacción donde un microrganismo compite por nutrientes o produce compuestos de inhibición	(Little et al. 2008)
Positiva		
Comensalismo	Interacción en la que una especie se beneficia sin dañar a la otra	(Ivey et al. 2013)
Sinergismo	Interacción entre dos especies, en la que las dos obtienen beneficio mutuo	(Little et al. 2008)

1.3. Las levaduras y la fermentación alcohólica

La fermentación alcohólica consiste en la ruptura de azúcares (glucosa y fructosa) para la producción de etanol y CO_2. En principio, las levaduras introducen la glucosa y fructosa por medio de transportadores de membrana hacia el citosol. Posteriormente, los azúcares son transformados por acción enzimática para obtener piruvato, el cual bajo condiciones de anaerobiosis será transformado a etanol durante la fermentación alcohólica (Ribéreau-Gayon et al., 2006).

Las cepas de la especie *Saccharomyces cerevisiae* son las levaduras que principalmente realizan la fermentación alcohólica en la elaboración del vino. Esto es debido a que son resistentes a las diversas condiciones de estrés en el medio, lo que influye en la selección de estas cepas. Por ejemplo, condiciones como anaerobiosis, presencia de sulfitos (García-Ríos et al., 2019), alta concentración de azúcares y presencia de etanol (Cisilotto et al., 2021; Girardi-Piva et al., 2022). Sin embargo, es posible encontrar

otras especies de levaduras y bacterias ácido lácticas que participan en la vinificación puesto que son habitantes naturales del ambiente vitivinícola (Beltran et al., 2002; Franquès et al., 2017). Como consecuencia de esta compleja población microbiana, la fermentación alcohólica (FA) espontánea puede imprimir distintivos locales y estilo al vino ya que están adaptadas a las condiciones de este ambiente enológico (Esteve-Zarzoso et al., 2000). Durante el proceso de vinificación, una amplia variedad de especies de levaduras participa y se establecen dinámicas de población e interacciones, mismas que inciden en la calidad general del vino. Adicionalmente, durante los primeros estadios de la FA es común encontrar especies pertenecientes al grupo de las no-*Saccharomyces*, relacionadas principalmente con una amplia producción de compuestos que enriquecen la complejidad aromática del vino (Bely et al., 2008). No obstante, en general las especies no-*Saccharomyces* no son tolerantes al etanol, lo que implica que a medida que incrementa la concentración de este compuesto en el mosto, las levaduras no-*Saccharomyces* son inhibidas y posteriormente mueren. Subsecuentemente en este procceso de fermentación, las especies *Saccharomyces* sobreviven en el mosto por su resistencia a la concentración presente de etanol, permitiendo que puedan consumir toda la fuente de carbono y terminar con la FA.

De manera interesante, las especies no-*Saccharomyces* fueron consideradas como microrganismos contaminantes, debido a que no eran capaces de terminar la FA y dejar azúcares residuales además de la producción impredecible de compuestos no deseados (Benito, 2018; Medina et al., 2013). Sin embargo, actualmente está descrito que diferentes géneros pertenecientes a este grupo de levaduras son responsables del enriquecimiento del perfil aromático y sensorial en los vinos (Tabla 2).

Tabla 2. Especies de levaduras no-*Saccharomyces* con interés enológico

Especie	Propiedades	Referencia
Hanseniaspora vinae	Alta producción de ésteres Alta producción de β-glucosidasas Alta producción de ésteres	(Del Fresno et al., 2017; Martín-García et al., 2020; Medina-Trujillo et al., 2017; Varela, 2016)
Metschnikowia pulcherrima	Alta producción de ésteres Producción de enzimas hidrolíticas Producción de glicerol	(Hranilovic et al., 2020; Morata et al., 2019; Vicente et al., 2020)
Lachancea thermotolerans	Alta producción de ácido láctico Baja producción de ácido acético Mejora en características de color y estructura	(Benito, 2020; Zhu et al., 2020)
Torulaspora delbrueckii	Reducción acidez volátil Alta producción de glicerol Alta producción de ésteres Producción de manoproteínas	(Belda et al., 2015, 2017; Benito, 2018; Puertas et al., 2017; Renault et al., 2015, 2016)

1.3.1. *Hanseniaspora vinae*

Las especies pertenecientes a este género se han identificado por el aporte de diversas propiedades al vino. En vinos de uva Chardonnay se ha registrado un incremento en los ésteres de acetato y de etilo con el uso de *Hanseniaspora vinae,* los cuales son responsables de los aromas frutales en el vino. No obstante, también se ha registrado una reducción de alcoholes superiores y ácidos grasos de cadena media (Medina et al., 2013). Estos resultados también se han confirmado por Varela et al. (2021), donde registran un aumento en la producción de ésteres como 2-feniletil acetato, acetato de tirosol y diacetato de propanediol. De igual manera, en vino de Albillo Mayor se ha registrado un incremento en la producción de ésteres y alcoholes superiores (del Fresno et al., 2022). Por otra parte, este tipo de levaduras también

pueden producir algunas enzimas hidrolíticas como proteasas, las cuales han demostrado que pueden ser útiles para la estabilización proteica del vino (Martin et al., 2023).

1.3.2. *Metschnikowia pulcherrima*

Las cepas de *Metschnikowia pulcherrima,* se caracterizan principalmente por ser candidatas en la producción de vinos con menor contenido de etanol. No obstante, otras propiedades como el incremento de la concentración de glicerol y la baja producción de acetato son características deseables para al aumento de la complejidad del vino. Además también se caracterizan por la producción de enzimas hidrolíticas como glucosidasas, proteasas y pectinasas, las cuales por su acción favorecen la liberación de nutrientes y producción de aromas varietales (Hranilovic et al., 2020; Morata et al., 2019; Vicente et al., 2020).

1.3.3. *Lachancea thermotolerans*

Este tipo de levaduras son capaces de mejorar la calidad del vino por el incremento de la intensidad colorante, complejidad aromática, la reducción de acidez volátil y la producción de ácido láctico (Benito, 2020). Adicionalmente, en suma a las bondades previamente descritas de este grupo de levaduras, también se ha descrito que estas levaduras son capaces de producir vinos con menor grado alcohólico, debido a su habilidad para redireccionar la ruta de carbono-etanol, lo que representa una ventaja para las regiones en las que llegan a producirse vinos con alto grado etílico como consecuencia del calentamiento global (Benito, 2020; Zhu et al., 2020)

1.3.4. *Torulaspora delbrueckii*

Dentro de las no-*Saccharomyces*, la especie mejor estudiada, comercializada y usada a nivel industrial es *Torulaspora delbrueckii* (Belda et al., 2017). Fenotípicamente se caracteriza por su forma elipsoidal,

ligeramente más pequeña que *S. cerevisiae,* con dimensiones de 2-4 x 3-5 μm. Se reproduce de forma asexual por gemación multilateral (Benito, 2018). Su crecimiento, perfil fermentativo y requerimientos de nitrógeno son muy similares con respecto a *S. cerevisiae,* siendo genéticamente similares (Roca-Mesa et al., 2020). Sin embargo, durante la FA el rendimiento de etanol y los diversos productos son diferentes con respecto a *S. cerevisiae*. Por ejemplo, algunos estudios reportan la disminución del contenido de etanol en co-cultivos de *T. delbrueckii* y en contraste con aquellas fermentaciones llevadas a cabo únicamente por *S. cerevisiae.* Sin embargo, en otros estudios no se reportan diferencias significativas en la producción de etanol, lo cual indica que su producción y rendimiento está sujeto a un efecto cepa-dependiente (Belda et al., 2015; Puertas et al., 2017).

Adicionalmente, otras propiedades alcanzadas por *T. delbrueckii* durante la FA dan soporte al uso e implementación de estas levaduras, ya que se reporta no solo un incremento en la producción de glicerol hasta en 1 g/L en fermentaciones secuenciales (Belda et al., 2017; González-Royo et al., 2015; Puertas et al., 2017), sino que también se incrementa la concentración de mannoproteínas en un 25% más con respecto a *S. cerevisiae,* lo que genera un impacto positivo en la estructura final del vino (Belda et al., 2015). Además, una de las bondades más importantes obtenidas por *T. delbrueckii* es el enriquecimiento en la complejidad aromática, donde se ha reportado el incremento de la producción de ésteres como propanoato de etilo, isobutanoato de etilo, acetato isobutílico, acetato de isoamilo (Belda et al., 2017; Renault et al., 2015) y tioles volátiles, compuestos aromáticos relacionados con notas frutales en vinos blancos (Renault et al., 2016). Más allá del impacto en la textura y perfil aromático que pueden alcanzarse con *T. delbrueckii,* también es posible obtener una disminución en la acidez volátil, principalmente en co-cultivos con *S. cerevisiae.* Algunos estudios reportan valores de 0.13 g/L (Chen et al., 2018), 0.27 g/L (Taillandier and Phong, 2014) y 0.16 g/L (Medina-Trujillo et al., 2017), valores que están dentro del nivel aceptado de 0.8 g/L (Benito, 2018), mismo que representa una alternativa para evitar la producción de acidez volátil que impacta de manera negativa la calidad del vino por su nota característica de vinagre.

1.4. Las bacterias lácticas y la fermentación maloláctica

Las bacterias ácido lácticas tienen un papel importante en la elaboración del vino, básicamente porque pueden llevar a cabo la descarboxilación del ácido L-málico a L-láctico, conocida como fermentación maloláctica (FML), beneficiosa en la mayoría de vinos tintos y algunos blancos y rosados (Morata et al., 2023; Ruiz-de-Villa et al., 2023). Ellas están presentes en la superficie de las uvas y son capaces de desarrollarse en el mosto del vino en un ambiente anaeróbico (Lonvaud-Funel, 1999). Es conocido que durante las diferentes fases del proceso de fermentacion existe una gran variedad de especies (Capozzi et al., 2021). Sin embargo, las especies más importantes pertenecen a los géneros *Lactobacillus*, *Leuconostoc*, *Pediococcus* y *Oenococcus* (Renouf et al., 2005). Las diferentes especies relacionadas con la elaboración del vino están clasificadas en dos grupos importantes: heterofermentativas facultativas y estrictas (Tabla 3). Las heterofermentativas facultativas convierten una molécula de glucosa en dos moléculas de ácido láctico. Sin embargo, en el caso de las pentosas, las convierten en ácido láctico y acético por la ruta pentosa fosfato. Por otra parte, las heterofermentativas estrictas convierten la glucosa en CO_2, ácido láctico, etanol y acético; y ácido láctico o acético en el caso de las pentosas (Ribéreau-Gayon et al., 2006).

Tabla 3. Principales BAL en el vino (Inês and Falco, 2018; Lallemand, 2015; Zheng et al., 2020)

Grupo	Fermentación glucosa	Isómero láctico	Especies
Lactobacilos	Heterofermentativa facultativa grupo 2	L	*Lacticaseibacillus casei*
		DL	*Lactiplantibacillus plantarum*
	Heterofermentativa obligatoria grupo 3	DL	*Levilactobacillus brevis*
		DL	*Lentilactobacillus hilgardi*
		L	*Apilactobacillus kunkeei*
Cocos	Homofermentativo obligatorio	DL	*Pediococcus damnosus*
		DL	*Pediococcus pentosaceus*
	Heterofermentativo obligatorio	D	*Oenococcus oeni*
		D	*Leuconostoc mesenteroides*

Al comienzo de la FA, las especies de BAL están presentes en el mosto en una población aproximada de 10^2 a 10^4 CFU/mL (Lonvaud-Funel, 1999). Sin embargo, durante la FA su población disminuye como consecuencia del incremento en la concentración de etanol y otros inhibidores, haciendo difícil la supervivencia de las especies sensibles (Comitini et al., 2005). Las cepas de *O. oeni* son heterofermentativas, gram positivas, catalasa negativa y microaerofílicas, esto permite que puedan desarrollarse en bajas concentraciones de oxígeno (Dicks et al., 1995). Además, se ha reportado que esta especie se ha aislado de la uva con una mayor proporcion al resto de BAL (Franquès et al., 2017) y que es posible encontrarla durante todo el proceso de elaboración del vino, debido a su habilidad para desarrollarse en las condiciones de estrés (Battistelli et al., 2020), ya que aplican mecanismos para resistir la concentración de etanol, bajo pH, temperatura y empobrecimiento nutritivo (Olguín et al., 2010).

1.4.1. *Oenococcus oeni*

O. oeni pertenece al orden *Lactobacillales* y familia *Lactobacillaceae*, dentro del filo de los Firmicutes (sinónimo del recientemente denominado filo *Bacillota*). Estas bacterias realizan en el vino la

fermentación maloláctica, convirtiendo el L-málico a L-láctico y CO_2. Pero en presencia de carbohidratos fermentables pueden fermentarlos, por su metabolismo heterofermentativo obligatorio. Así, fermentan glucosa o fructosa para obtener D-láctico, CO_2, etanol o acetato (Dicks et al., 1995; Franquès, 2018). Las células de *O. oeni* son esféricas-elipsoidales y de manera típica se presentan en pares o pequeñas cadenas (Capozzi et al., 2021). Genéticamente, *O. oeni* posee un cromosoma circular compacto de 1.8 Mb (Margalef-Català et al., 2017; Mills et al., 2005) y que pudiera reflejar una racionalización genómica durante la adaptación de estas bacterias en el ambiente fermentativo del vino (Borneman et al., 2010).

1.4.2. **Fermentación maloláctica**

La fermentación maloláctica (FML) es una fermentación secundaria en el vino, y la realizan las BAL, como *O. oeni*. Este proceso consiste en la transformación del ácido L-málico en ácido L-láctico, lo cual disminuye la acidez del vino, y a su vez mejora la complejidad aromática y asegura la estabilización microbiana (**Figura 2**). Uno de los compuestos más característicos relacionados con la FML es el diacetilo producido a partir de ácido cítrico. Sin embargo, otros compuestos como ésteres y alcoholes contribuyen en la complejidad aromática después de terminada la FML (Alexandre et al., 2004).

La FML es un proceso enzimático catalizado por la enzima maloláctica. La enzima maloláctica es una proteína dimérica o tetramérica formada por la asociación de un polipéptido de 60 kDa, el cual se fija con los cofactores NAD^+ y Mn^{2+}. La FML constituye una fuente de energía para la célula, debido a que la asimilación del ácido L-málico y la liberación del ácido L-láctico incrementa el pH y genera un flujo de protones, permitiendo así la consevación de energía (Figura 2) (Ribéreau-Gayon et al., 2006).

1. Introducción

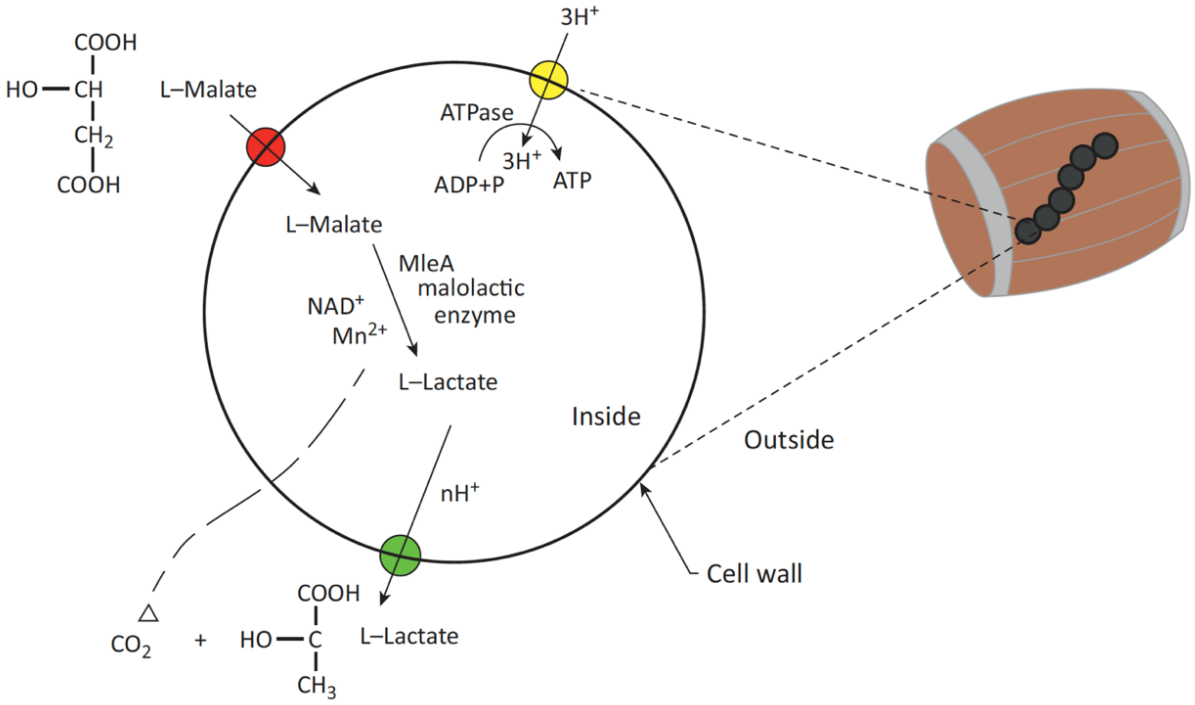

Figura 2. La FML implica el transporte activo del ácido L-málico hacia el interior de la célula por la malato permeasa (MleP, rojo). La descarboxilación del ácido L-málico es realizado por la enzima maloláctica (MleA) y requiere de los cofactores NAD^+ y Mn^{2+}. Posteriormente, el L-láctico es transportado fuera de la célula (verde). El incremento en el pH intracelular y el gradiente de protones confieren una ventaja energética facilitando la producción de ATP (Betteridge et al., 2015).

Además de la descarboxilación del ácido L-málico, otras reacciones se llevan a cabo durante la FML. Por ejemplo, el metabolismo del ácido cítrico que es otra ruta importante en las BAL (**Figura 3)**. El ácido cítrico se encuentra en la uva en concentraciones de 0.5 a 1 g/L (Güler, 2023; Volschenk et al., 2006). Sin embargo, también se produce por el metabolismo de levaduras como consecuencia del ambiente anaerobio. Este compuesto se transporta hacia el interior de las BAL por la citrato permeasa (MaeP) y posteriormente se metaboliza por la enzima citrato liasa (CitE). Como resultado, se libera una molécula de acetato y oxaloacetato, el cual una vez descarboxilado puede producir acetato, butanodiol y diacetilo (Olguín et al., 2009). Por lo tanto, durante este proceso las BAL consumen protones, generan potencial de membrana y gradiente de pH. Otra ventaja del metabolismo del citrato es que las BAL pueden resultar con una mayor producción de biomasa como consecuencia del co-metabolismo del citrato y la glucosa

(Ramos and Santos, 1996; Salou et al., 1994). Además, el diacetilo es uno de los compuestos aromáticos más característicos asociado al desarrollo de la FML.

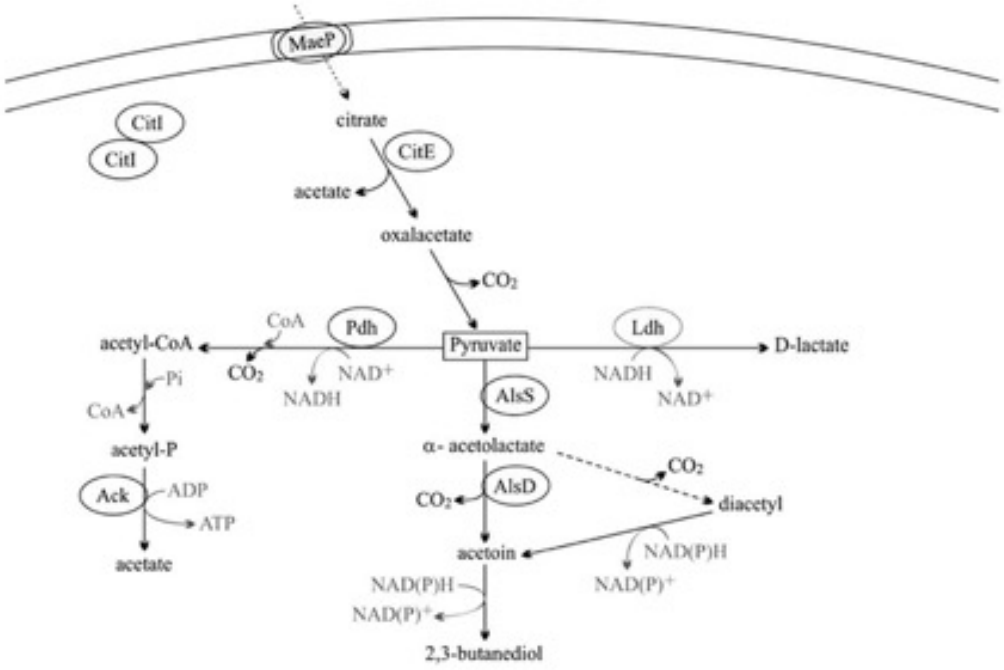

Figura 3. Ruta del metabolismo del citrato en *O. oeni* (Olguín et al., 2009). Enzimas y activadores: CitI – activador trascripcional; MaeP – citrato permeasa; CitE – citrato liasa; Pdh – piruvato deshidrogenasa; AckA – acetate quinasa; Ldh – lactato deshidrogenasa; AlsS – α-acetolactato sintasa; AlsD –α-acetolactato descarboxilasa.

1.4.3. **Estrategias de adaptación de *Oenococcus oeni* al vino**

La superviviencia de *O. oeni* en el vino se asocia a diversas estrategias de adaptación y supervivencia dentro del ambiente enológico. En general, la mayor estrategia de las cepas de *O. oeni* para hacer frente a la presencia de etanol y bajo pH es aplicar alternativas en el transporte de metabolitos y protección de la membrana celular. Bajo esta estrategia, las cepas de *O. oeni* son capaces de adaptarse para superar los obstáculos del ambiente del vino y llevar a cabo la FML (Lonvaud-Funel, 1999; Olguín et al., 2015).

Es conocido que el etanol es un compuesto inhibidor para microrganismos, entre ellos las levaduras y más para las BAL y otras bacterias (Di Martino et al., 2020; Girardi-Piva et al., 2022). Su toxicidad se atribuye al fraccionamiento de la doble capa lipídica de la membrana, causando así la disrupción de la misma (Lonvaud-Funel et al., 1988). Como respuesta a la toxicidad del etanol, se han identificado proteínas de estrés como la proteina Hsp20 (también conocida como Lo18 o Hsp18). Esta proteína ha sido identificada como marcador de estrés en células de *O. oeni* y asociada con la supervivencia en el vino, debido a sus funciones de protección en la membrana (Guzzo et al., 2000).

Otro mecanismo asociaddo a la respuesta al estrés es el metabolismo del citrato, el cual al consumir protones genera potencial de membrana y gradiente de pH, permitiendo así la síntesis de ATP que ayuda a mantener el pH interno. Esta estrategia ha sido confirmada por el incremento en la expresión de genes relacionados con el metabolismo del citrato cuando el etanol está presente, debido a que el etanol modifica la fluidez de membrana y provoca la acidificación intracelular. Por lo tanto, se ha propuesto que el metabolismo del citrato está relacionado con una adaptación a condiciones de acidez en el vino (Olguín et al., 2009). Además, el metabolismo de algunos aminoácidos como glutamina, glutamato y arginina, también juega un papel importante en la homeostásis del pH para la supervivencia en la fase estacionaria de algunas BAL (Gänzle, 2015).

Por otra parte, durante la aclimatación de *O. oeni* a las condiciones adversas del vino se activa la producción de algunos agentes antioxidantes Por ejemplo, algunas BAL implementan la síntesis de glutatión (GSH), un tripéptido no proteico (Glu-Cys-Gly) que actúa como antioxidante y la síntesis de enzimas requeridas para la regulación intracelular del potencial redox (Margalef-Català et al., 2016). Sin embargo, las especies de *O. oeni* no son capaces de producir GSH, pero pueden asimilar este tripéptido del medio donde se desarrollan y también pueden sintetizar enzimas relacionadas con la regulación del potencial redox, incluyendo las que le permitirían utilizar el GSH. Adicionalmente, se han observado cambios en la membrana que estarían también relacionados con la respuesta al estrés, como un

incremento en la producción de la enzima ciclopropano ácido graso sintasa, asociada con el mantenimiento de membrana en respuesta a la acción de radicales libres que inician la peroxidación de lípidos, lo que causa una disminución en la fluidez de membrana (Margalef-Català et al., 2016).

1.5. Interacciones microbianas en vino

Durante la fermentación espontánea es posible encontrar una población compleja de levaduras, hongos miceliales y bacterias. Sin embargo, la ecología microbiana de este ambiente no está estrictamente subordinada al proceso de fermentación, ya que esta población ingresa a la bodega enológica por medio de varios pasos que van desde la vendimia y todos los pasos intermedios hasta llegar al tanque de fermentación (Fleet, 2003; Franquès et al., 2017). Como consecuencia de la diversidad de especies presente en la elaboración de vino, se establecen interacciones microbianas y dinámicas de población, que a su vez generan una amplia producción de compuestos. Por ejemplo, aquellos relacionados con efectos de inhibición como compuestos proteicos (Comitini et al., 2005), etanol, SO_2 (Lemaresquier, 1987), ácidos grasos de cadena media (Lafon-Lafourcade et al., 1984) y ácidos orgánicos (Balmaseda et al., 2018) principalmente producidos por levaduras. No obstante, también se liberan compuestos aromáticos y de estructura que influyen en la calidad general del vino. Por lo tanto, en vista de alcanzar mejores propiedades organolépticas, rendimiento de etanol y reducción en periodos de fermentación, se han implementado diferentes estrategias de inoculación entre levadura-levadura y levadura-bacteria.

1.5.1. Interacciones levadura-levadura

La función principal de las levaduras en la vinificación es la conversión de los azúcares presentes en los mostos de fermentación a etanol. Debido a ello son los organismos de mayor importancia en la elaboración de bebidas alcohólicas. Las especies del género *Saccharomyces* tienen como función principal la producción de etanol y otros compuestos. No obstante, las no-*Saccharomyces* también lo

producen y generan compuestos volátiles que benefician el perfil sensorial en el fermentado (Sadoudi et al., 2012a). En fermentaciones espontáneas en vino, se han encontrado diversas especies de levaduras en el mosto, donde figuran especies pertenecientes a los géneros *Torulaspora*, *Hanseniaspora*, *Metschnikowia* y *Lachancea*, entre otras (Maicas and Mateo, 2023). Este tipo de levaduras están relacionadas con el incremento de la complejidad aromática en el vino, las cuales producen compuestos y enzimas deseables en el proceso de fermentación (Jolly et al., 2014). Sin embargo, de manera general estas levaduras no son buenas fermentadoras por su baja resistencia al etanol, lo cual dificulta el terminar la AF. Por lo tanto, esto implica que sea necesario integrar levaduras *S. cerevisiae* y establecer diferentes estrategias de inoculación para que se pueda finalizar la FA. Por ejemplo, como la fermentación secuencial y co-cultivo entre especies *Saccharomyces* y no-*Saccharomyces*, donde se han realizado diversos estudios de interacción.

Algunos de estos estudios reportan un efecto positivo mediante el uso de especies no-*Saccharomyces* y *S. cerevisiae*. En este caso, se ha registrado un impacto aromático favorable por el incremento de una variedad de compuestos como 2-fenil-etanol (Comitini et al., 2011), ésteres de etilo, glicerol y alcoholes (Izquierdo et al., 2014), acetato de amilo (Sadoudi et al., 2012b), acetato de isoamilo (Comitini et al., 2011; Sadoudi et al., 2012a; Taillandier and Phong, 2014), etil octanoato (Sadoudi et al., 2012a), etil acetato, alcoholes superiores (Clemente-Jimenez et al., 2005; Comitini et al., 2011) y glicerol (Englezos et al., 2019).

1.5.2. **Interacciones levadura-bacteria**

Durante el proceso de vinificación es posible encontrar BAL, las cuales desarrollan la FML después de la FA. La FML disminuye la acidez del vino, impactando de forma positiva en el sabor y haciéndolo menos agresivo al paladar. Además, durante este proceso se generan compuestos involucrados en la composición aromática del vino (Lonvaud-funel, 2014).

La presencia de levaduras y BAL así como la interacción de estos microorganismos en la elaboración del vino, ha tenido un creciente interés, particularmente en la interacción *Saccharomyces cerevisiae/Oenococcus oeni* (Badotti et al., 2014; Sadoudi et al., 2012b). La interacción de estas dos especies ha sido objeto de estudio por la generación y aumento en la concentración de numerosos compuestos volátiles. En condiciones de co-cultivo algunos reportes registran el incremento de compuestos volátiles como etil lactato, ácido propiónico (Rossouw et al., 2012), glicerol (Pan et al., 2011), propanol, 2-feniletanol, etil acetato y etil lactato (Izquierdo et al., 2012). Sin embargo el furfural e hidroximetil furfural disminuyen como resultado de la interacción (Izquierdo et al., 2015).

Por otra parte en fermentación secuencial, también se registra un aumento en la concentración de compuestos volátiles como acetato isobutílico, hexil acetato, 2-feniletil acetato, siringol, hexenol, metanol, isobutanol (Izquierdo et al., 2012) y acetaldehído (Pan et al., 2011). Es importante señalar que además del incremento en la concentración de compuestos volátiles en co-cultivo y fermentación secuencial, los periodos de fermentación se reducen por efecto de la interacción microbiana, lo cual se ha corroborado en diversos estudios con *Saccharomyces cerevisiae/Oenococcus oeni*, donde se reduce el periodo de fermentación maloláctica (Azzolini et al., 2010; Muñoz et al., 2014; Nehme et al., 2008; Rossouw et al., 2012). Por lo tanto, la reducción de períodos de fermentación puede representar una ventaja y alternativa para la elaboración del vino en menor tiempo (Izquierdo et al., 2012).

Los estudios de interacción levadura-BAL realizados hasta hoy muestran las grandes bondades obtenidas como resultado de esta interacción. Sin embargo, la interacción puede influir en la descomposición y generación de compuestos no deseados en la elaboración de bebidas (Mendoza et al., 2010), por lo que el éxito de una fermentación realizada con interacción de microorganismos está sujeto a la elección de las cepas de levaduras y BAL adecuadas (Nehme et al., 2008).

1.5.3. Compuestos promotores de la FML

Durante la fermentación alcohólica, las levaduras liberan al mosto una serie de compuestos como consecuencia de su metabolismo primario y otros por la autolisis celular. Como ejemplo, se ha reportado la producción de ácido pirúvico por levaduras con niveles de entre 98-500 mg/L (Benito et al., 2012; Del Fresno et al., 2017; Mylona et al., 2016), el cual es considerado importante durante la FML por su función como aceptor de electrones, regeneración de NAD^+ (Maicas et al., 2002) y promotor en la producción de diacetilo por su función intermediaria en el metabolismo del ácido cítrico (Mink et al., 2015).

El ácido cítrico se encuentra principalmente en la uva (Güler, 2023; Volschenk et al., 2006), no obstante es otro compuesto que es liberado por levaduras durante la FA. El ácido cítrico puede ser metabolizado por las BAL en la FML. Como consecuencia de su metabolismo, las BAL consumen protones, generan potencial de membrana y gradiente de pH, lo que les permite adaptarse a las condiciones de estrés en el vino (Olguín et al., 2009). Además, durante este proceso se produce diacetilo, el cual ha sido uno de los mayores aromas asociados a la FML. Este compuesto tiene la característica de conferir un nota a mantequilla en bajas concentraciones (1- 4 mg/L), sin embargo puede tener aromas no deseados si la concentracion supera el rango de 5-7 mg/L (Bartowsky and Henschke, 2004).

Por otra parte, como resultado de la lisis celular de las levaduras, diversos compuestos son liberados al vino. Por ejemplo, las manoproteínas las cuales se constituyen por monómeros de manosa y algunos residuos de aminoácidos. Las manoproteínas son benéficas para las BAL, puesto que como estrategia de supervivencia, pueden hidrolizarlas y promover su crecimiento por el aumento del contenido nutritivo del vino (Balmaseda et al., 2021; Diez et al., 2010)

1.5.4. Compuestos inhibidores de la FML

Las levaduras durante la fermentación alcohólica pueden liberar compuestos que generen un impacto negativo sobre la FML y el crecimiento de las BAL. Algunos de estos compuestos pueden ser producidos

por metabolismo primario o seecundario. Además, su origen y efecto sobre el desarrollo de las BAL permite clasificar en términos de ecología microbiana su tipo de interacción.

En los compuestos producidos por metabolismo primario, figura principalmente el etanol. Este compuesto es responsable de la disrupción de la membrana celular (Lonvaud-Funel et al., 1988) y en niveles superiores a 14% (v/v) puede inhibir por completo la FML (Da Silveira et al., 2003; Jiang et al., 2018). Un ejemplo más es el SO_2 , el cual también es un producto del metabolismo de primario de las levaduras (Granuzzo et al., 2023). Este compuesto afecta el desarrollo y crecimiento de *O. oeni* por la inhibición de la actividad de la ATPasa, la cual es responsable de bombear protones del interior de la célula al exterior (Carreté et al., 2002).

Por otra parte, en compuestos producidos por metabolismo secundario, se encuentran los compuestos proteicos (Osborne and Edwards, 2007) y los péptidos antimicrobianos (AMPs) (Branco et al., 2014), asociados con la inhibición del crecimiento y desarrollo de las BAL. Adicionalmente, algunos ácidos grasos de cadena media y ácidos orgánicos como el ácido succínico inhiben el desarrollo de la FML y el crecimiento de las BAL.

1.6. Ácido succínico

El ácido succínico es un ácido orgánico producido por levaduras durante la FA y en conjunto al ácido tartárico y málico confieren estabilidad microbiana a los vinos en relación con la acidez y pH. Adicionalmente, al ácido succínico también se le conoce como ácido 1,4-butanodioico (HOOC-CH_2-CH_2-COOH), con una masa molar de 118g/mol y un pK_{a1} de 4.2 y pK_{a2} de 5.6 (ChemIDplus, 2021). Su estuctura es muy similar a la del ácido málico (HOOC-CHOH-CH_2-COOH), el cual tiene una masa molar de134 g/mol y también puede ser llamado ácido hidroxisuccínico. El umbral organoléptico del ácido succínico en agua es de 35 mg/L. Su nota es amarga-salina y si sus niveles son superiores puede impactar de manera negativa el vino (Coulter and Pretorius, 2007); por lo cual es recomendable que sus niveles

sean reducidos. Por otra parte, cuando el ácido succínico se esterifica puede favorecer la percepción aromática del vino. Por ejemplo, con compuestos como succinato de metilo, succinato de etilo y succinato dietílico, que están asociados con aromas florales y frutales (Gürbüz et al., 2006; Jordán et al., 2002)

1.6.1. **Producción de ácido succínico por levaduras**

El ácido succinico se produce por levaduras principalmente durante los primeros estadios de la FA (Conway and Downey, 1950), pero también puede producirse durante la fase estacionaria (Arikawa et al., 1999; Lamikanra, 1997). Este ácido orgánico se produce principalmente en el ciclo de los ácidos tricarboxílicos o también conocido como ciclo de Krebs. Sin embargo, puede actuar como intermediario en otras rutas metabólicas como en la del ácido γ-aminobutírico (GABA), ácido glioxílico y el ácido metil cítrico (Figura 5). El ácido succínico puede tener un impacto positivo en la calidad general del vino por la formación de ésteres. Su producción depende de diversos factores, como la temperatura, nitrógeno asimilable, oxígeno y amino ácidos y las especies de levaduras (De Klerk 2010).

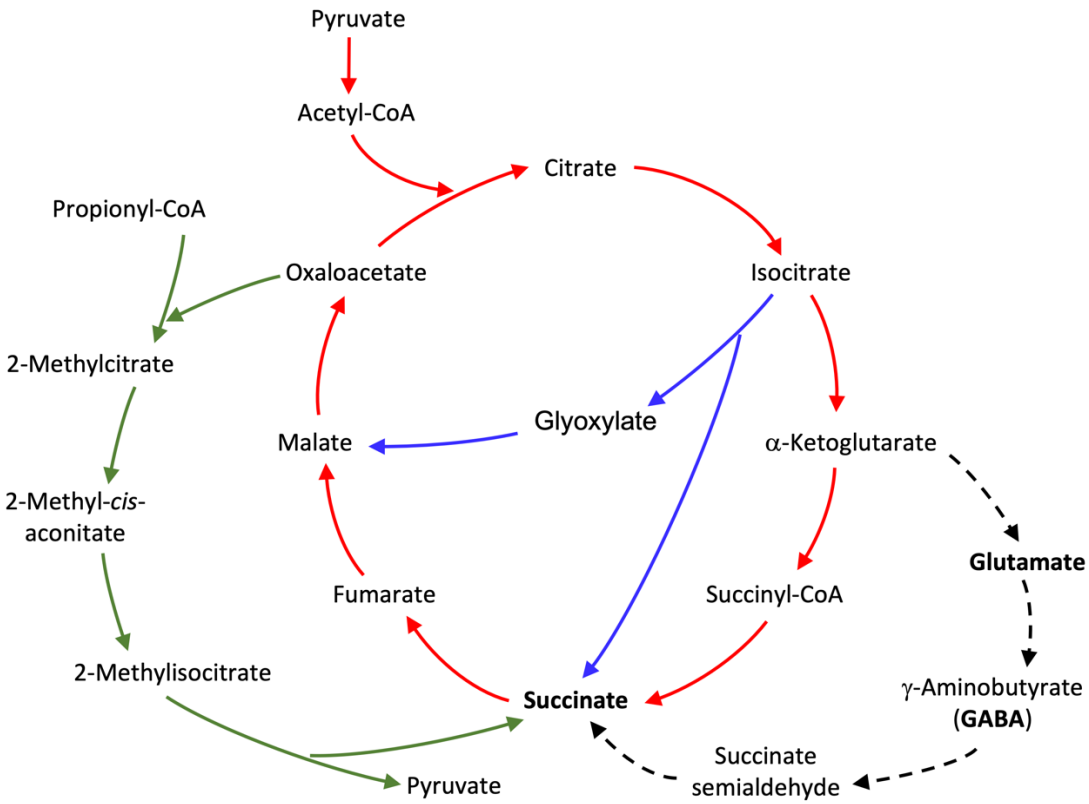

Figura 4. Rutas metabólicas de levaduras y otros organismos donde el succinato actúa como intemediario: ciclo de los ácidos tricarboxílicos (líneas rojas), glioxilato (lineas azules), ácido metil cítrico (líneas verdes) y ruta del ácido γ-aminobutírico (líneas negras). Adaptación de De Klerk (2010) y Freitas e Silva *et al*. (2020).

El ácido succínico puede ser producido por diversas especies de levaduras. La producción de este ácido orgánico según diversos reportes (Tabla 4), puede situarse en un rango de entre 300 hasta 2000 mg /L. Sin embargo, se han reportado valores de hasta 3000 mg/L en vinos tintos australianos, los cuales son superiores en hasta el doble de la concentración de succínico que en vinos blancos (Coulter and Pretorius., 2004).

Las levaduras *S. cerevisiae* pueden producir ácido succínico en niveles de entre 200 mg/L a 1400 mg/L. No obstante, las diferentes especies de levaduras que conforman el grupo de las no-*Saccharomyces*

pueden producirlo en un rango mayor de entre 300 - 3000 mg/L, como se ha comentado. En este grupo podemos encontrar especies como *M. pulcherrima*, *L. thermotolerans, T. delbrueckii* entre otras.

Es importante señalar que la producción de este ácido en cultivos mixtos o secuenciales, la concentración de ácido succínico podría variar según las especies. Por ejemplo, la concentración de ácido succínico obtenida por *S. cerevisiae* es ligeramente superior en cultivo puro con respecto a la interacción de *L. thermotolerans – S. cerevisiae,* con valores de 1470 y 1410 mg/L respectivamente (Vicente et al., 2023). También se ha reportado un incremento en la producción de este compuesto en fermentación secuencial de *T. delbrueckii – S. cerevisiae* con respecto al control con *S. cerevisiae*. No obstante, podría darse el caso en el que no hay diferencia significativa como se muestra con los resultados de *S. cerevisiae* y la interacción simultánea de *T. delbrueckii- S. cerevisiae* (Sgouros et al., 2023).

Por otra parte, además de los factores bióticos y fisicoquímicos, sus niveles de producción pueden ser de hasta el doble en vinos tintos que en vinos blancos (Coulter and Pretorius, 2007). Esta diferencia puede ser explicada por el incremento de GABA en el mosto, debido a la estimulación mecánica y maceración de las uvas, lo cual favorece la producción de ácido succínico por levaduras (De Klerk 2010).

Tabla 4. Producción de ácido succínico por levaduras *Saccharomyces* y no-*Saccharomyces*

Levaduras	Producto	Concentración mg/L	Referencias
S. cerevisiae	Vino	1000	(Coulter and Pretorius, 2007)
S. cerevisiae *S. bayanus*	Vino	3000	(Coulter and Pretorius, 2004)
S. cerevisiae	Mosto sintético	0.752 - 1400	(De Klerk, 2010)
S. cerevisiae	Vino	910	(Muñoz García et al., 2023)
S. cerevisiae	Vino	700	(Sgouros et al., 2023)
S. cerevisiae	Vino	1470	(Vicente et al., 2023)
S. cerevisiae	Mosto sintético	347	(Carbon et al., 2023)
T. delbrueckii	Vino	300	(Ciani and Maccarelli, 1997)
M. pulcherrima *S. malidevorans* *C. stellata*	Vino	900 - 2000	(Contreras et al., 2014)
M. pulcherrima	Mosto sintético	703	(Carbon et al., 2023)
S. cerevisiae QA23	Vino	770	(Zhu et al., 2020)
S.c QA23- *Zygosaccharomyces bailii*		830	
S.c QA23- *T. delbrueckii*		950	
L. thermotolerans- S. cerevisiae *T. delbrueckii - S. cerevisiae*	Vino	700	(Sgouros et al., 2023)
T. delbrueckii - S. cerevisiae		800	
L. thermotolerans- S. cerevisiae	Vino	1410	(Vicente et al., 2023)

1.6.2. **Efectos del ácido succínico sobre bacterias lácticas**

Se ha propuesto que el ácido succínico actúa como inhibidor sobre la FML y *O. oeni* a nivel de la enzima maloláctica (Lonvaud-Funel and Strasser de Saad, 1982). Es de especial interés que el ácido succínico disminuye durante la FML (Yılmaz and Gökmen, 2021) y especialmente en fermentación simultánea FML-FA (Taniasuri et al., 2016). Por consiguiente, la influencia real de este ácido sobre la FML ha sido poco clara. Por lo tanto, debido la falta de información sobre el impacto de este compuesto en condiciones de vinificación, fue necesario evaluar en este trabajo de tesis el efecto de este compuesto sobre el

desarrollo de *O. oeni* y la FML. Además, también es importante estudiar las condiciones que influyen sobre la producción del ácido succínico durante la FA por parte de las diferentes especies de levaduras de interés enológico.

2. Hipótesis y objetivos

El enfoque principal de este trabajo era el estudio de la influencia del ácido succínico sobre la fermentación maloláctica y el desarrollo de *O. oeni* en condiciones de fermentación. Por lo tanto, la hipótesis de este trabajo de tesis fue que **el ácido succínico producido por las levaduras, y especialmente las no-*Sacharomyces*, puede afectar al desarrollo de *Oenococcus oeni* y por lo tanto a la fermentación maloláctica del vino.**

Por lo tanto, el objetivo principal de esta tesis fue estudiar cómo afecta el ácido succínico a *O. oeni* y cómo influye en el desarrollo de la fermentación maloláctica. Otro objetivo importante fue determinar los niveles de ácido succínico producido por las diferentes levaduras y las condiciones que afectan esta producción.

Para cumplir con estos objetivos generales, se plantearon los siguientes objetivos específicos:

1. Evaluar el efecto de diferentes concentraciones de ácido succínico sobre el desarrollo de *O. oeni* y la fermentación maloláctica. (Capítulo 1)
2. Evaluar la inhibición del ácido succínico sobre la fermentación maloláctica realizada por diferentes cepas de *O. oeni*. (Capítulo 2)
3. Evaluar el efecto del ácido succínico sobre la expresión génica global de *O. oeni* mediante análisis transcriptómico. (Capítulo 3)
4. Evaluar la producción de ácido succínico en diferentes especies de levaduras y la influencia de la suplementación de GABA y ácido glutámico sobre la misma. (Capítulo 4)

3. Resultados

CAPÍTULO 1

Influencia del ácido succínico sobre *Oenococcus oeni* y la fermentación maloláctica

En este capítulo se evaluaron diferentes concentraciones de ácido succínico en wine like medium (WLM), con el objetivo de determinar qué concentraciones ejercen un efecto de inhibición sobre el desarrollo de *O. oeni* y la fermentación maloláctica. Los resultados se encuentran en el artículo que se incluye a continuación: Torres-Guardado, R., Rozès, N., Esteve-Zarzoso, B., Reguant, C., and Bordons, A. (2022b). Influence of succinic acid on *Oenococcus oeni* and malolactic fermentation. Oeno One 56, 195–204. https://doi.org/10.20870/oeno-one.2022.56.3.5403

Influence of succinic acid on *Oenococcus oeni* and malolactic fermentation

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Abstract

As a consequence of the alcoholic fermentation carried out by yeasts in wine, several compounds can be delivered to the medium, promoting or inhibiting the malolactic fermentation (MLF) and the lactic acid bacteria, mainly *Oenococcus oeni*. Succinic acid is one of these compounds, and is an example of the interaction between yeasts, including non-*Saccharomyces* species, and *O. oeni*. However, the influence of succinic acid on the MLF has been researched very little as yet. In this work, we study the influence of succinic acid and pH on *O. oeni* CH11 and PSU-1 strains, both during MLF and in resting cell experiments. Moreover, we analysed the relative expression of some significant genes related to stress and malolactic activity to determine how the *O. oeni* strains were affected by the succinic acid. The results showed that the succinic acid can act as a MLF inhibitor at concentrations higher than 1 g/L, but it can be beneficial at 0.5 g/L. This variable effect also depends on the strains and other winemaking conditions, mainly pH, which influences the dissociated and undissociated forms of both acids. The inhibiting effect of succinic seems clearer when it is at a molar concentration higher than that of L-malic acid. Experiments with resting cells have confirmed that *O. oeni* consumes less L-malic acid when succinic acid is higher than 1 g/L. Genetic expression experiments showed that in the presence of succinic acid (2 g/L), gene *hsp18* encoding stress protein was up-regulated in strain CH11, suggesting a good response and adaptation of this strain to stress. On the other hand, genes *mleA* and *mleP,* which are related to malolactic activity, were not affected by succinic acid, except for strain PSU-1 at pH 4.0. Further research is necessary to better understand these effects of succinic acid on *O. oeni* and MLF.

Keywords

Malolactic fermentation, *Oenococcus oeni*, wine, succinic acid, resting cells, *hsp18*, *mleP*

Introduction

Alcoholic fermentation (AF) is the main microbiological process in winemaking, as it converts grape must into wine. This fermentation is carried out by the yeast *Saccharomyces cerevisiae*, although during the early stages non-*Saccharomyces* yeasts are present in the fermenting must (Fleet *et al.*, 1984). There is an increasing interest in these other yeasts (Padilla *et al.*, 2016) due to the production of new aromas (Belda *et al.*, 2017).

The yeast metabolism and the winemaking conditions greatly influence the final composition of the wine. Some compounds produced by yeasts —including non-*Saccharomyces*— during AF have a large impact on the subsequent malolactic fermentation (MLF), carried out mainly by the lactic acid bacterium *Oenococcus oeni* (Lonvaud-Funel, 1999; Bartowsky, 2005; Balmaseda *et al*., 2018; Ferrando *et al.*, 2020; Balmaseda *et al.*, 2021). Some of these compounds are produced by the primary metabolism of yeast, such as ethanol and SO_2 (Arnink and Henick-Kling, 2005), certain organic acids (Lonvaud-Funel and Strasser de Saad, 1982), and medium-chain fatty acids (Guilloux-Benatier, *et al.*, 1998; Lonvaud-Funel *et al.*, 1988). Other compounds are delivered by the secondary metabolism as antimicrobial peptides (Branco *et al.*, 2014) and proteinaceous compounds (Osborne and Edwards, 2007). Among the organic acids derived from the yeast metabolism, succinic acid seems to be the most related to MLF inhibition (Caridi and Corte, 1997; Son *et al*., 2009).

Succinic acid is one of the relevant organic acids in wine, whose main role is to confer microbial stability to wines in relation to acidity and pH. They also preserve the colour and sensory properties of wines. Tartaric and malic acids are generally the most prominent acids in wines, while others such as acetic, succinic, citric, lactic, and pyruvic can exist in minor concentrations (Mendes-Ferreira and Mendes-Faia, 2020). Tartaric and malic acids are already present in grape must, but no succinic or lactic acids are found

in grapes. Instead, succinic acid is usually the predominant non-volatile organic acid formed by yeasts during AF (Thoukis *et al.*, 1965).

Succinic acid is the 1,4-butanedioic acid (HOOC-CH_2-CH_2-COOH), with a m.w. of 118 g/mol, and pK_{a1} of 4.2 and pK_{a2} of 5.6 (ChemIDplus, 2021). Its structure is like that of malic acid (HOOC-CHOH- CH_2-COOH), which has a m.w. of 134 g/mol, and which can also be called hydroxysuccinic acid. The organoleptic threshold of succinic acid in wine is 35 mg/L. It has an unusual bitter-salty taste and excess levels can have a negative impact on the mouthfeel of the wines (Coulter *et al.*, 2004); thus, it may be beneficial to reduce it.

Succinic acid is an intermediate of the tricarboxylic acid cycle (TCA), and it therefore is produced by yeasts during the AF in the early fermentation stages (Conway and Brady, 1950), but also during the stationary phase (Lamikanra, 1997; Arikawa *et al*., 1999). However, it acts as an intermediary in other metabolic pathways such as γ-amino butyric acid (GABA) bypass, glyoxylic acid bypass and the methylcitric acid cycle. Therefore, due to mechanical stimuli and maceration of red grapes, the GABA concentration in must can increase and could encourage yeast to produce succinic acid (De Klerk, 2010). *S. cerevisiae* strains are known to produce succinic acid, with values from 200 mg/L to more than 1 g/L (Heerde and Radler, 1978; Coulter *et al*., 2004; De Klerk, 2010; Zhu *et al*., 2020), thus explaining the increases of this acid in winemaking. This production of succinic acid can influence MLF, which has been shown with a cryotolerant *S. cerevisiae* strain that inhibits MLF (Caridi and Corte, 1997; Son et al., 2009). This organic acid has been described as a possible competitive inhibitor of MLF due to its similarity with L-malic acid (Lonvaud-Funel *et al*., 1988; Caridi and Corte, 1997).

In addition, non-*Saccharomyces* yeasts can also significantly produce succinic acid. For example, Ciani and Maccarelli (1998) found that strains of *Torulaspora delbrueckii* produced it within the range of 0.3 to 0.8 g/L. Moreover, in sequential fermentations of *S. cerevisiae – T. delbrueckii,* succinic acid was produced up to 0.95 g/L (Zhu *et al*., 2020). Moreover, Contreras *et al*. (2014) found productions of 1 to

2 g/L of this acid by strains of *Metschnikowia pulcherrima*, *Schizosaccharomyces malidevorans* and *Candida stellata*.

Besides the above mentioned studies of MLF inhibition by succinic-producing yeasts, the influence of succinic acid *per se* upon MLF and on *O. oeni* has been studied very little (Lonvaud-Funel and Strasser de Saad, 1982). Interestingly, it seems that succinic acid concentration can decrease during MLF (Yilmaz and Gökmen, 2021) and especially in induced simultaneous MLF with AF (Taniasuri *et al.*, 2016). Consequently, its real influence on MLF remains unclear. Due to the lack of information and to clarify the impact of this compound in winemaking conditions, the main aim of this work was to evaluate for the first time the effect of different succinic acid concentrations on *O. oeni* strains in MLF. Having in mind the different dissociated or undissociated forms of succinic acid in function of pH, assays at different pH —3.5 and 4.0— were included in the evaluation of the impact of succinic acid. To better understand the physiological response to succinic acid, the gene expressions related to stress and malolactic activity were also analysed.

Materials and methods

1. Strains and culture conditions

Four strains of *Oenococcus oeni* of diverse origins were initially used: VP41 (Lalvin VP41), from Lallemand Inc. (Montréal, Canada); 1Pw13 (CECT 8893) from our own collection; PSU-1 (ATCC BAA-331), a reference strain with the genome fully annotated; and CH11 (Viniflora CH11), from Chr. Hansen Holding A/S (Hoersholm, Denmark). Before the fermentation assays, cells of these strains were precultured at 28 ºC in an incubator with 10% (v/v) CO_2 in MRS broth supplemented with D, L-malic acid (4 g/L) and fructose (5 g/L) at pH 5.0 for 72 h at least two times prior to experimental use.

2. Influence of succinic acid concentration on the growth of *O. oeni* strains

In order to see the influence on *O. oeni* growth, the above mentioned precultures in supplemented MRS broth were assayed also with three different succinic acid concentrations (0.5, 1 and 2 g/L), and bacterial populations were quantified indirectly by measuring the Abs at 600 nm in a spectrophotometer Polarstar Omega (Biogen, Madrid, Spain).

3. Malolactic fermentation assays with addition of succinic acid

MLF were carried out in wine like media (WLM) (Bordas *et al.*, 2013) containing 2 g/L of L-malic acid, 12% (*v/v*) ethanol, and three different succinic acid concentrations (0.5, 1 and 2 g/L), as well as the control without succinic acid, at pH 3.5 and 4.0, adjusted with NaOH 1N. All fermentations were performed in triplicate in 250 mL bottles at 20 ºC, inoculated with *O. oeni* strains at $2x10^7$ CFU/mL. The samples were taken every 24 h to evaluate the L-malic acid consumption using an enzymatic kit (BioSystems, Barcelona, Spain) and the cells were harvested by centrifugation (6000 x *g*, 10 min). They were then frozen in liquid nitrogen and kept at -80 ºC until the RNA extraction.

4. MLF assays with different proportions of L-malic and succinic acids

To evaluate the influence of the ratio of the two acids on MLF, experiments were carried out in WLM with different concentrations of L-malic acid (0.5, 1, 2 and 3 g/L) and succinic acid (0.5, 1 and 2 g/L). All assays were carried out at pH 3.5 and 4.0, at 20ºC. Control assays without succinic acid were included for each L-malic acid concentration. All experiments were performed in 50 mL Falcon tubes inoculated with $2x10^7$ CFU/mL *O. oeni* and samples were taken every 24 h to evaluate L-malic consumption using an enzymatic kit (BioSystems).

5. Experiments with resting cells

O. oeni cells were grown in 250 mL of MRS broth at 28 ºC to the early stationary phase. They were then harvested by centrifugation at 6000 x *g* for 5 min at room temperature, following Mira de Orduña *et al.* (2000). Cells were resuspended in resting-cell buffer, which contained per litre of deionized water 7.5 g tartaric acid and 1 mL of a mineral solution with 200 g/L $MgSO_4 \cdot 7H_2O$ and 50 g/L $MnSO_2 \cdot 4H_2O$). Then, resting cells were transferred to 50 mL Falcon tubes containing 2 g/L of L-malic acid (control assay) and 12% (*v/v*) of ethanol. The other conditions had the same molar amount of 2 g/L of L- malic acid (14.9 mM) and different proportional molar succinic acid concentrations (3.7 mM, 7.4 mM, 14.9 mM, and 29.8 mM) at pH 3.5 and 4.2. This last pH was chosen because it is the pK_{a1} for succinic acid. The Falcon tubes were placed in a water bath at 25 ºC and stirred gently. Samples were taken periodically and centrifuged at 6000 *g* for 5 min and the supernatants were kept at 4 ºC until the L-malic was analysed with an enzymatic kit (BioSystems).

6. Analysis of gene expression

The *O. oeni* RNA extractions were performed as described by Chomczynski and Sacchi (2006) and then purified with a Roche RNeasy kit according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

cDNA was synthesised from RNA (10 ng/µL) using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) as recommended. To analyse the expression of four genes related to stress and malolactic activity, four pairs of primers (Table 1) were taken from previous works (Beltramo *et al.*, 2006; Desroche *et al.*, 2005; Olguín *et al.*, 2010). They were about 18-22 bp long, contained 50% G/C and had a melting temperature (T_M) above 60 ºC. The *O. oeni gyrA* and *gyrB* genes were used as housekeeping genes (internal control), using the primers described by Desroche *et al.* (2005).

Real-time qPCR was performed following Olguín *et al.* (2010) using a QuantStudio 5 real-time PCR instrument (Thermo Fisher). The results were analysed using the comparative critical threshold (ΔΔCT) method in which the amount of target RNA was adjusted to a reference (internal target RNA) described by Livak and Schmittgen (2001). The relative expression value (RE) was calculated using the Ct values of *gyrA* and *gyrB* and the result is the mean of the two results. The analysis was made from biologically duplicated independent assays and for each sample technical triplicates were analysed by qPCR.

7. Chemical analyses

The organic acid contents (acetic, citric, succinic, L-malic and L-lactic acids) in the final samples of MLF trials were determined by high-performance liquid chromatography (HPLC) following Zhu *et al.* (2020). All samples were filtered previously by injecting them through 0.2 μm Captiva filters (Agilent Technologies). The chromatograms were analysed using the Agilent ChemStation Plus software.

8. Statistical analyses

The data obtained were submitted to one-way ANOVA using the Tukey test, with a confidence interval of 95%, obtaining significative results with a p-value of ≤ 0.05, using the XLSTAT 2021 software (Addinsoft, Paris, France).

Table 1. Gene description and primer sequences used in this work.

Target gene	Description	Forward primer (5′→ 3′)	Reverse primer (5′→ 3′)	Amplicon length (bp)	Reference
gyrA	Gyrase subunit A	CGCCCGACAAACCGCATAAA	CAAGGACTCATAGATTGCCGAA	95	(Desroche *et al*., 2005)
gyrB	Gyrase subunit B	GAGGATGTCCGAGAAGGAATTA	GCCTGCTGGGCATCTGTATTA	107	(Desroche *et al*., 2005)
mleP	Malate permease	GTGCTGACTTATTTGACCCGC	ATGTTCCACGACGACCAACC	141	(Augagneur *et al*., 2007)
mleA	Malolactic enzyme	CCGACAATTGCTGATACAATTGAA	GGCATCAGAAACGACCAGCAG	156	(Beltramo *et al*., 2006)
hsp18	Heat shock protein	CGGTATCAGGAGTTTTGAGTTC	CGTAGTAACTGCGGGAGTAATTC	102	(Beltramo *et al.,* 2006)
atpB	ATPase F_1 F_0 β-subunit	ATACTGATCCGGCTCCGGC	CAGCGGGATAAATACCTTG	93	(Beltramo *et al.*, 2006)

Results and discussion

1. Effect of succinic acid on cells growth

To see the effect of succinic acid concentration on growth of *O. oeni* strains, previous assays were carried out in the same MRS broth used in precultures of fermentation assays. There was a clear lower growth when succinic acid was present for both strains assayed (Supplementary Figure 1), and this effect was progressively significant higher with increasing concentration from 0.5 g/L to 2 g/L of succinic acid. As seen, with 1 g/L of succinic acid the maximum population reached was about 50% (strain PSU-1) or 70% (CH11) of the control, and with 2 g/L it was about 30% for PSU-1 and a mere 20% of the control for strain CH11.

2. Effect of succinic acid on malolactic fermentation

After the first experiments for comparing four different *O. oeni* strains, PSU-1 and CH11 were selected and used exclusively throughout the entire study since they were representative of the two behaviours

observed in response to adding succinic acid. *O. oeni* CH11 showed a stronger inhibition of MLF in the presence of 2 g/L of succinic acid than strain PSU-1. This agrees with the above commented stronger growth inhibition of CH11 at 2 g/L of succinic acid added to MRS medium. The other *O. oeni* strains VP41 and 1Pw13 showed a similar behaviour to CH11, but with longer and slower MLF in all assays (Supplementary Figure 2). Control fermentations in WLM with strain PSU-1, at both pH 3.5 and 4.0, were completed in 120 h, and those with strain CH11 finished in 144 h (Figure 1). The bacterial population in the samples of these WLM fermentations was followed but no increase was detected in any of them, nor in controls (data not shown). It must be taken in account that growth is usually very difficult in the harsh conditions of WLM, similar to wine, with ethanol and low pH, as observed previously (Bordas *et al.,* 2015; Jiang *et al.,* 2018). Nonetheless, the inoculated population is able to survive and finish the MLF.

The results obtained in the presence of succinic acid varied depending on the pH and strains. Succinic acid inhibited the MLF of the two *O. oeni* strains at concentrations of 1 and 2 g/L at pH 3.5, whereas at pH 4 only the MLF with 2 g/L of succinic acid with *O. oeni* CH11 was inhibited in comparison to the control assay (0 g/L succinic acid). At pH 3.5 with 2 g/L of succinic acid, 43% and 34% of the L-malic acid was not consumed by CH11 and PSU-1 respectively, at the time that the control had exhausted all the L-malic acid. This shows that *O. oeni* CH11 was more sensitive to this concentration of succinic acid than PSU-1. The results with 1 and 2 g/L of succinic acid showed that the pH has a relevant influence on the MLF and the inhibitory effect of this organic acid. Meanwhile, at pH 3.5, succinic acid reduced the L-malic acid consumption rate in CH11 and PSU-1; however, at pH 4.0 both strains were able not only to carry out MLF, but also to do so in less time than the control assay. Therefore, pH is an important factor because this physicochemical condition affected the dissociated and undissociated forms of succinic and malic acids, as observed by Augagneur et al. (2007).

Nevertheless, both strains CH11 and PSU-1 showed similar results in the 0.5 g/L succinic acid and in all cases MLF finished about 24 h earlier than in the control assay. This best MLF performance in the presence of 0.5 g/L than without succinic acid was also found for the other two strains 1Pw13 and VP41 (Supplementary Figure 1). Therefore, we have found that this low concentration of succinic acid could be slightly beneficial for *O. oeni* and the MLF, while levels of 1 g/L succinic acid or higher are clearly inhibitory. This probable benefit could be related to the above commented decrease in succinic acid in some MLFs (Taniasuri et al., 2016; Yilmaz and Gökmen, 2021); however, we did not find significant variation in the succinic acid concentration in any case (data not shown).

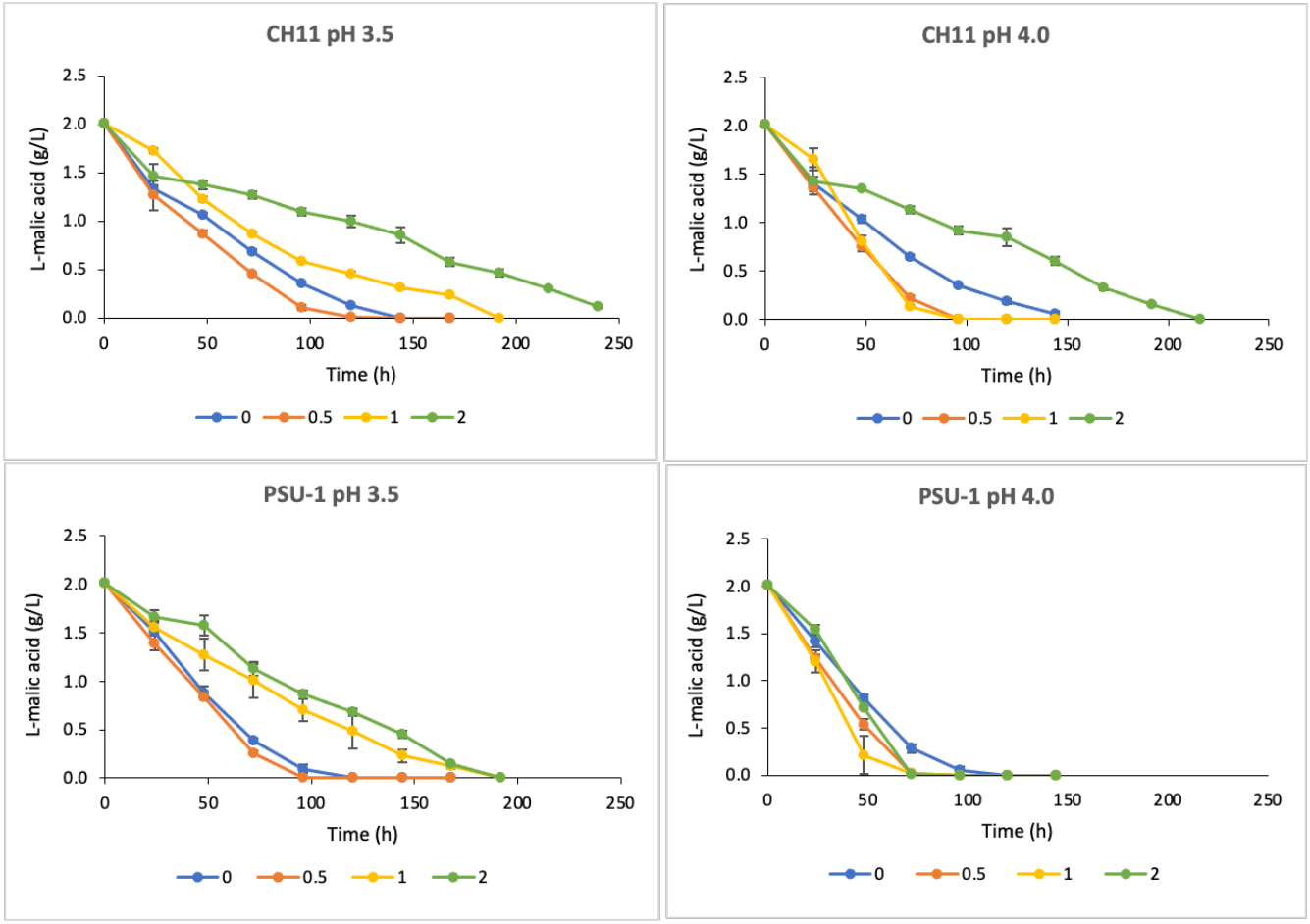

Figure 1. L-malic consumption in WLM with 12% ethanol (v/v) at pH 3.5 and 4.0 by *O. oeni* strains CH11 and PSU-1, in the presence of different concentrations of succinic acid: 0 g/L (control, blue), 0.5 g/L (red), 1 g/L (yellow), and 2 g/L (green). The values are the means of triplicates and the error bars represent the SD values.

Anyway, it is clear that MLF is inhibited by a concentration of 1 g/L or higher of succinic acid for both strains and pHs. This inhibition is probably related to intracellular acidification by this acid, which would lead to a decrease in cells viability, affecting in consequence the MLF. Although, as commented above, we had not detected variation —neither decrease nor decrease— in biomass in these assays with WLM, the inhibition effect on cells growth was clear in the previous assays with MRS medium.

Regarding the important role that pH played in the performance of the MLF with the two strains, the results obtained with 1 and 2 g/L of succinic acid could be related to the dissociated and undissociated forms of succinic and L-malic acids. At pH 3.5, L-malic acid would be present at almost 50% in dissociated monoanionic form (L-malic acid $pK_{a1} = 3.4$) (Tourdot-Maréchal *et al*., 1993), whereas a large fraction of succinic acid (more than 70%) is in its undissociated form (succinic acid $pK_{a1} = 4.2$) (Jansen and van Gulik, 2014). However, at pH 4.0, only around 50% of succinic acid would be undissociated (Jansen and van Gulik, 2014). At pH values above 4.5 the passive diffusion of L-malic acid into *O. oeni* cells would be negligible (Tourdot-Maréchal *et al*. (1993). However, at pH 3.2 the permeability of the cells to the undissociated acid by simple diffusion could represent more than 50% of the total L-malic acid uptake. Considering that succinic acid and L-malic acid have a very similar chemical structure, it would be feasible that undissociated succinic acid could enter the *O. oeni* cell at pH 3.5, producing an intracellular acidification due to the complete deprotonation of succinic acid at the cytosolic pH (*O. oeni* intracellular pH = 5.8-6.1; succinic acid $pk_{a1} = 4.2$ $pk_{a2} = 5.6$). Internal acidification has been associated with the inhibition of MLF by other acidic compounds at pH values close to 3, such as octanoic acid and some phenolic acids (Capucho and Sao Romao 1994, Campos et al. 2003). The cytosolic acidification is known to inhibit the enzymatic activities of *O. oeni* in general and it affects the cells viability, as commented. However, moreover, malolactic activity could be specifically affected due to the changes of the L-malate anionic state inside the cell. According to Acevedo et al. (2020) the optimal substrate for

the malolactic enzyme in *O. oeni* is dianionic L-malate, which is the form present at the standard intracellular *O. oeni* pH (5.8-6.1). The internal acidification produced by succinic acid could convert dianionic L-malate into the monoanionic form, negatively affecting the malolactic enzyme efficiency. Altogether, the internal acidification of *O. oeni*, favoured at a low pH, could explain the stronger inhibition of succinic acid at pH 3.5 than at pH 4 observed in this work.

In regard to the composition of organic acids after MLF, there were no differences in L-lactic acid production (data not shown) because it was in accordance with the L-malic acid consumption. In addition, the amounts of acetic acid in each treatment were in accordance with the citric acid consumption and no statistical differences were found (data not shown). Neither one was above the acetic acid threshold (0.7 g/L) (Drysdale and Fleet 1989).

3. Influence of succinic acid on MLF at different ratios of L-malic and succinic acid

As seen above, the increasing amounts of succinic acid exerted an inhibition effect on the L-malic acid consumption. To understand this effect in more depth, we carried out assays in the same WLM with different ratios of L-malic and succinic acids. In addition, we aimed to determine whether there was an inhibition threshold of succinic acid depending on the initial L-malic acid concentration. At the same time, these assays simulated the real situation of diversity of initial L-malic acid levels (from 0.5 to 3 g/L in these assays) in different climatic zones of winemaking.

To determine this influence, L-malic acid consumption rates were calculated for the different assays, shown in Supplementary Table 1. As seen, the main significant inhibition of succinic acid was when L-malic acid was 0.5 or 1 g/L. To visualize these results better, they are summarized in Figure 2.

There is a significantly bigger inhibition of the consumption rate for most conditions when 2 g/L of succinic acid is present, in agreement with the inhibition of MLF kinetics we found and discussed above. In terms of the pH, a slightly greater L-malic acid consumption can be observed at pH 4.0 than at 3.5, as

expected since the higher pH is less stressful for *O. oeni*. The consumption tendencies are similar for both the strains and the succinic concentrations for the two pHs (Figure 2).

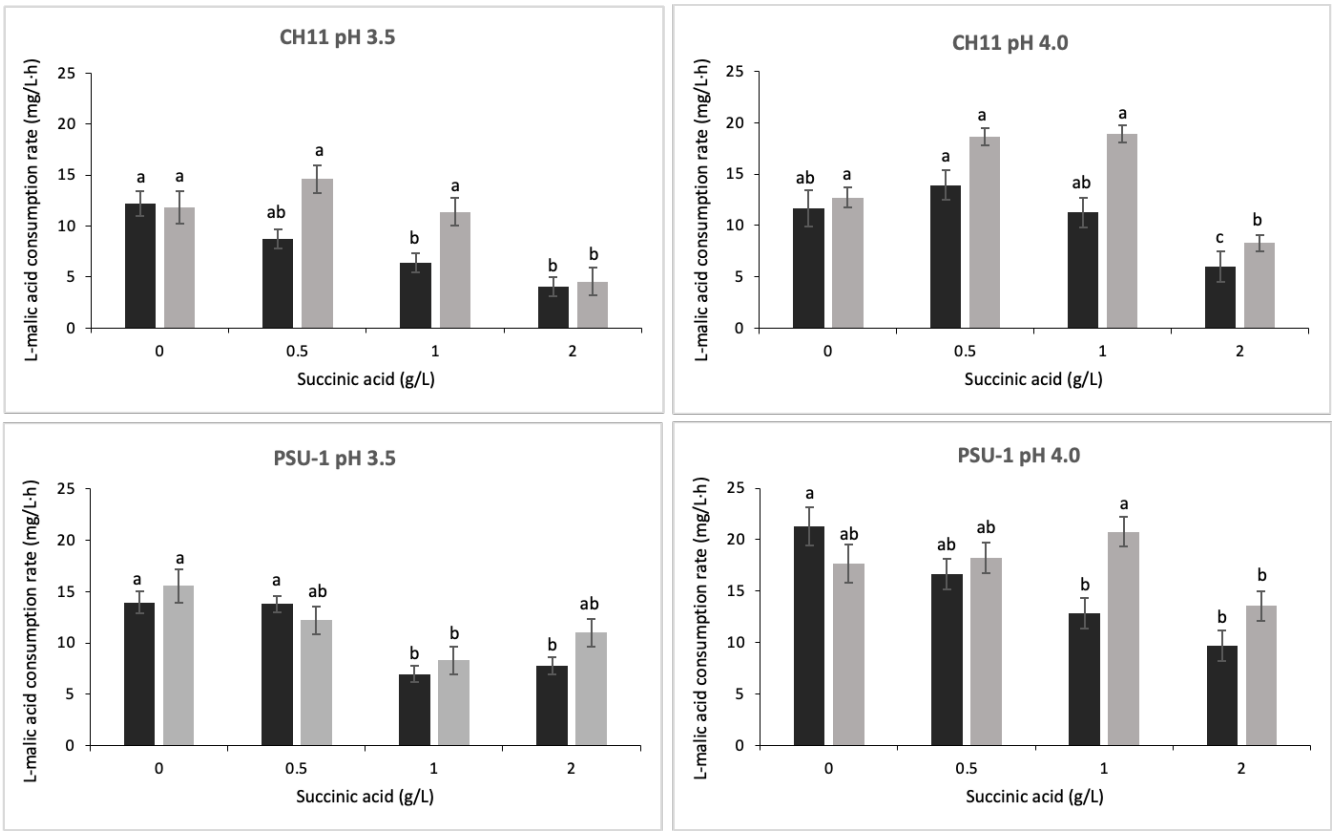

Figure 2. L-malic acid consumption rate (mg/L·h) by *O. oeni* strains CH11 and PSU-1 in WLM with 12% ethanol (v/v) at pH 3.5 and 4.0 with L-malic acid (0.5 g/L [black], and 1 g/L [gray]), at different concentrations of succinic acid. [a-c] Values of columns for each strain and pH are significantly different at $p \leq 0.05$ according to Tukey test.

The most relevant result here is that there is generally more inhibition of the consumption rate at 0.5 g/L of L-malic acid than at 1 g/L, which is clearer for strain CH11 (Figure 2). It seems that succinic acid has an inhibiting effect on the consumption rate of L-malic acid when this is in a lower ratio with respect to succinic acid. That is, succinic acid has a clear inhibition effect on MLF when the concentration of this acid is higher than that of L-malic acid. In addition to the inhibition of MLF due to the already mentioned

intracellular acidification caused by succinic acid, there could be competition between the succinic and L-malic acids for the malolactic enzyme due to their similar chemical structures, as suggested by Lonvaud-Funel and Strasser de Saad (1982).

It is particularly important to take this effect into account nowadays, when it is usual to have a low L-malic acid content in wine, mainly due to climate change that leads to less acidic grapes (Mira de Orduña 2010).

4. The effect of succinic acid on consumption of L-malic acid by resting cells

Unlike the WLM, where *O. oeni* can grow, the resting cell buffer provides a way to monitor the malolactic enzymatic activity without interference from nutrients and metabolic changes related to growth. Therefore, in resting cell assays there is no adaptation phase of growing cells and the effect of succinic is more direct. Cells number was about $5 \cdot 10^9$/mL, since they were harvested from 250 mL at early stationary phase (10^9/mL) and resuspended to a final volume of 50 mL, as said in Methods.

The consumption of 2 g/L (14.9 mM) of L-malic acid by resting cells was very quick (around 80-120 min) for both strains and pH levels and at the different succinic acid concentrations (data not shown). Hence, the high number of resting cells present can consume L-malic rapidly. These short times are similar to those found in similar works (Mira de Orduña *et al.* 2000).

To compare the L-malic acid consumption rate under the different conditions, we considered the amount of L-malic acid for assays with different succinic acid concentrations at the time when control assays had consumed about half of the L-malic acid (Table 2). This middle point of MLF (i.e., about 1 g/L (7.5 mM)) was at about 20 min for the control without succinic acid. Similarly, the data shown are for other sample conditions also taken at about 20 min. The different values of L-malic acid shown for the control at mid-MLF (from 2.08 to 6.82 mM) can be explained because samples were taken every 20 min and the L-malic consumption was very quick. Therefore, these are approximative values for mid-MLF.

We can observe (Table 2), that for most conditions when succinic is present the remaining L-malic acid is significantly higher than the control. Hence, succinic acid slows down the consumption of L-malic acid. Nevertheless, when succinic acid is 3.7 mM (0.5 g/L), the quantity of L-malic acid is significantly lower than the control at mid-MLF, indicating a quicker consumption rate at the lowest concentration of succinic acid. This tendency was not shown for PSU-1 at pH 4.2.

Table 2. L-malic acid amount (mM) for different succinic acid concentrations of the assays with resting cells, when control assay (0 mM succinic acid) has consumed about half of L-malic, *id est*, at middle MLF.

pH	Succinic acid (mM)	CH11	PSU-1
3.5	0	2.08 ± 0.226 ^{bc}	6.82 ± 0.197 ^{d}
	3.7	0.29 ± 0.185 ^{d}	4.59 ± 0.161 ^{e}
	7.4	1.54 ± 0.185 ^{c}	8.05 ± 0.161 ^{c}
	14.9	2.71 ± 0.185 ^{b}	9.00 ± 0.161 ^{b}
	29.8	4.55 ± 0.185 ^{a}	10.74 ± 0.161 ^{a}
4.2	0	4.28 ± 0.583 ^{cd}	3.73 ± 0.248 ^{d}
	3.7	1.98 ± 0.476 ^{d}	5.24 ± 0.203 ^{c}
	7.4	5.54 ± 0.476 ^{bc}	8.67± 0.203 ^{ab}
	14.9	7.01 ± 0.476 ^{ab}	8.97 ± 0.203 ^{a}
	29.8	8.57 ± 0.476 ^{a}	9.30 ± 0.203 ^{b}

$^{a-e}$ Values for different succinic acid concentration for the same pH and strain are significantly different at $p \leq 0.05$, according to Tukey test.

Overall, these results agree with those obtained previously in WLM (Figure 2), where the succinic acid inhibition of MLF was higher for concentrations higher than 1 g/L, that is, more than 7.4 mM of succinic acid.

5. Relative expression of genes related to stress and malolactic activity

In order to evaluate the possible inhibition of succinic acid in relation to the transcription of relevant genes associated with MLF development, we measured the relative expression of the following genes: i) *mleA*, encoding the malolactic enzyme, and *mleP*, encoding the L-malic acid transporter; ii) *atpB*, encoding the β-subunit of the F_1F_0 ATPase, associated with MLF activity and acid stress response (Cox and Henick-Kling 1989, Fortier et al. 2003); and iii) *hsp18*, encoding for one of the most relevant stress proteins of *O. oeni* with a chaperone function, this gene is known to be activated in response to multiple stresses and has been proposed to be a stress adaptation indicator (Coucheney et al. 2005, Beltramo et al. 2006). The analysis was carried out with samples from the assay performed in WLM with the most inhibitory concentration of succinic acid (2 g/L). All samples were taken when the L-malic acid concentration was approximately 1 g/L in each case, at mid-MLF. Samples from cultures without added succinic acid were also analysed as control conditions.

In *O. oeni* CH11, the strain that showed the highest sensitivity to succinic acid, the gene *hsp18* was clearly up-regulated at pH 3.5 and 4.0 (Figure 3). In contrast, the PSU-1 strain did not show overexpression of the *hsp18* gene in any of the assayed conditions. These data suggest that the CH11 strain adopted this strategy to outcome the succinic acid inhibitory effect. However, *O. oeni* PSU-1, the strain least affected by succinic acid, might respond to stress differently, as seen in previous studies (Olguín et al. 2010).

No changes in expression were observed for *atpB* in any of the conditions studied. The transient activation of this gene has been described in response to acid shock (Fortier et al. 2003). In this study, there was no difference in the transcriptional levels of *atpB* at mid-MLF with or without succinic acid.

Surprisingly, *O. oeni* PSU-1 showed a 3.5 and 2.5-fold up-regulation of the genes *mleP* and *mleA* at pH 4 (Figure 3). This could be related to the faster MLF observed under these conditions (2 g/L succinic

acid) with respect to the control condition at the same pH. However, the reason for the enhanced MLF due to the presence of succinic acid remains unclear and needs further research.

The main conclusion of the transcriptional study is that succinic acid does not inhibit the transcription of the *mleA* and *mleP* genes in any of the conditions. Therefore, the inhibition of MLF caused by this compound might be due to the intracellular acidification and possible substrate competition with L-malic acid at the enzymatic level, as previously discussed.

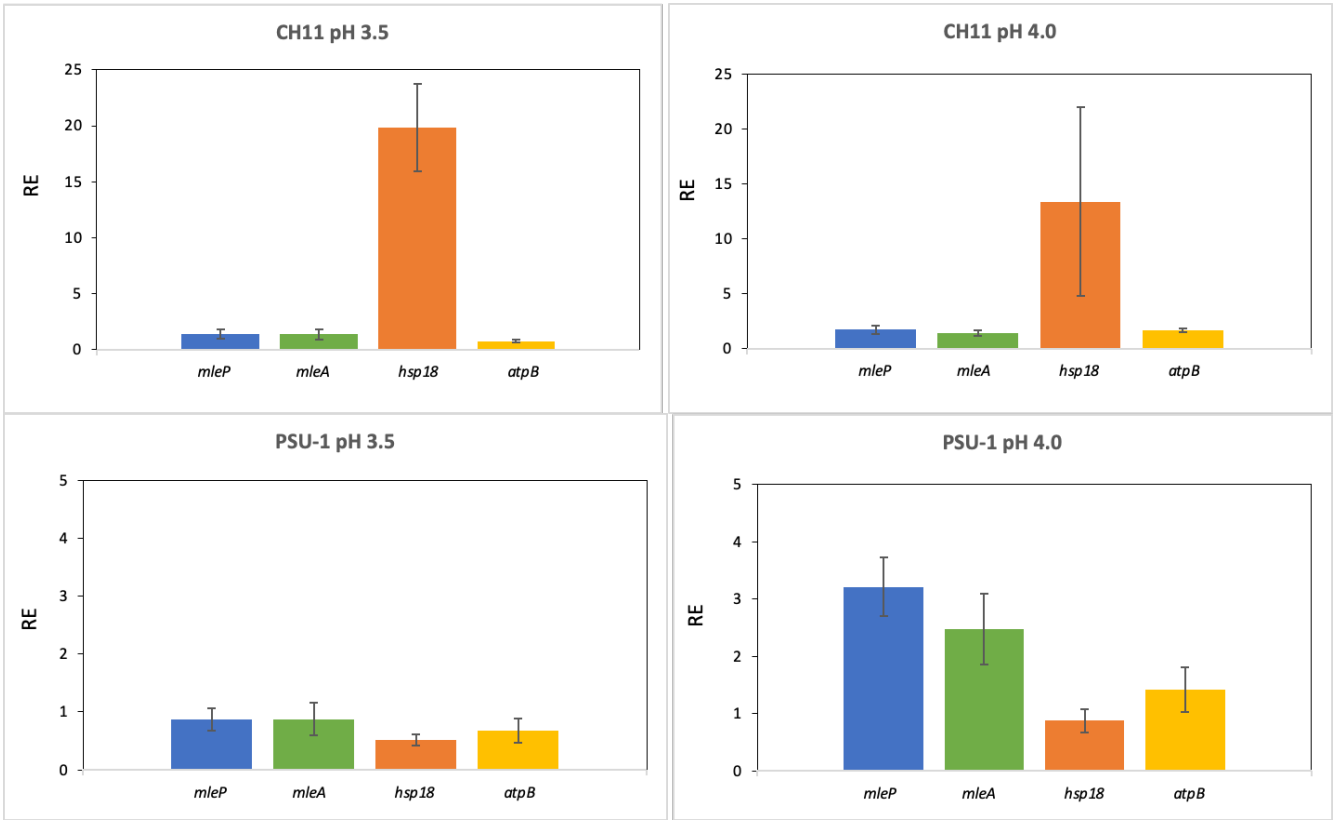

Figure 3. Relative expression (RE) of four genes related to stress (*hsp18* and *atpB*) and malolactic activity (*mleP* and *mleA*) of strains CH11 and PSU-1 of *O. oeni* at middle MLF (approx. 1 g/L L-malic acid) in presence of 2 g/L of succinic acid. The calibrator condition (RE = 1) was the absence of succinic acid. The data shown are mean values between both RE obtained with gene controls *gyrA* and *gyrB* with error bars representing SD values (n=3).

Conclusion

In this work the effect of succinic acid on *O. oeni* and MLF has been evaluated for the first time. It shows that succinic acid can inhibit MLF at concentrations higher than 1 g/L, but it could be beneficial at a lower concentration, near 0.5 g/L. The inhibition is clear when L-malic acid is at a lower ratio with respect to succinic acid. As succinic acid is produced by yeasts, including non-*Saccharomyces* species, this variable effect is an example of an interaction between yeasts and LAB that can be negative or positive depending on the succinic concentration but also on the strains and other winemaking conditions. Therefore, one of the most important factors is the pH because this physicochemical condition influenced the dissociated and undissociated forms of both acids. Consequently, the strain dependent effect can lead to inhibition or promotion of MLF due to the cell response and adaptation. Further research is necessary to understand the molecular mechanisms of the influence of succinic acid on *O. oeni* and possibly the competence of succinic acid against L-malic acid at the enzymatic and transport levels.

Acknowledgements

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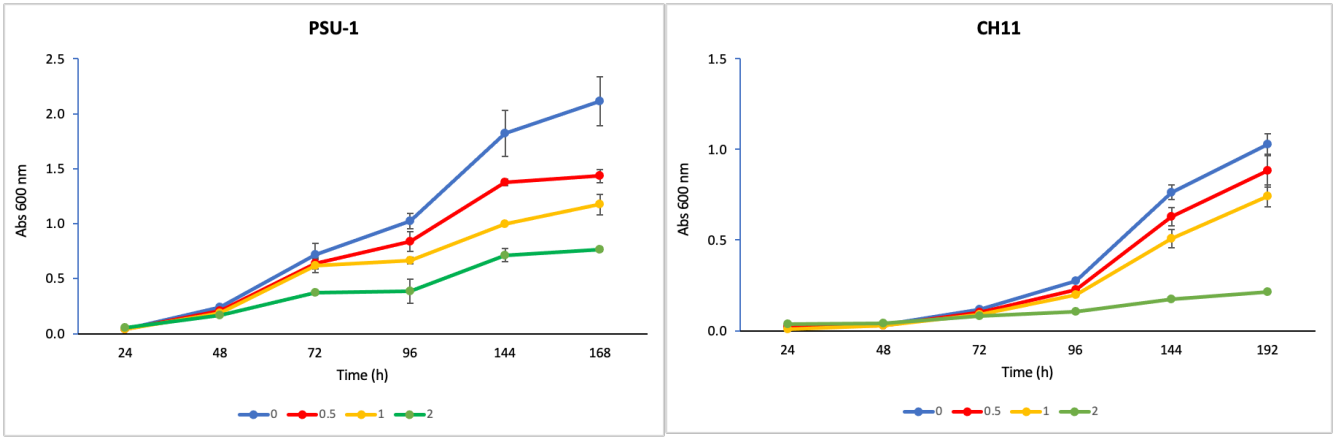
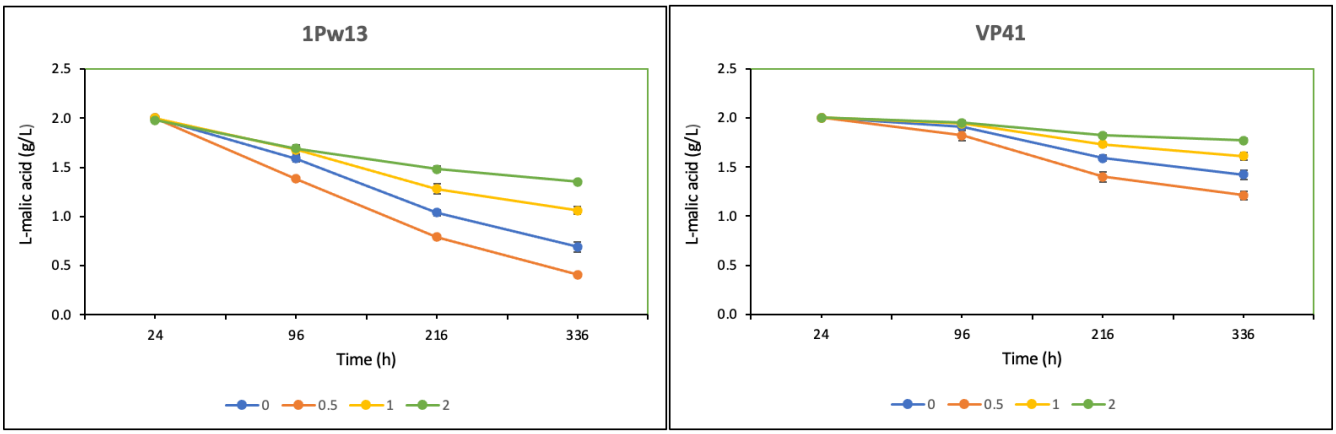
Supplementary Table 1. L-malic acid consumption rate (mg/L·h) by *O. oeni* strains CH11 and PSU-1 in WLM with 12% (v/v) ethanol at pH 3.5 and 4.0 at different proportions of L-malic and succinic acids. The values are the means and SD of triplicates.

		CH11				PSU-1			
		Succinic acid (g/L)							
pH	L-Malic (g/L)	0	0.5	1.0	2.0	0	0.5	1.0	2.0
3.5	0.5	12.19 ± 1.21 ^{a}	8.71 ± 0.94 ^{ab}	6.43 ± 0.94 ^{b}	4.96 ± 0.94 ^{b}	13.94 ± 1.07 ^{a}	13.80 ± 0.80 ^{a}	6.97 ± 0.80 ^{b}	7.77 ± 0.80 ^{b}
	1.0	11.79 ± 1.61 ^{a}	14.61 ± 1.34 ^{a}	11.39 ± 1.34 ^{a}	4.56 ± 0.13 ^{b}	15.54 ± 1.61 ^{a}	12.19 ± 1.34 ^{ab}	8.31 ± 1.34 ^{b}	10.99 ± 1.34 ^{ab}
	2.0	19.56 ± 2.81 ^{a}	26.13 ± 2.28 ^{a}	24.52 ± 2.28 ^{a}	19.7 ± 2.28 ^{a}	22.65 ± 3.48 ^{a}	27.60 ± 2.81 ^{a}	18.89 ± 2.81 ^{a}	21.17 ± 2.81 ^{a}
	3.0	18.63 ± 2.95 ^{a}	25.73 ± 2.41 ^{a}	16.88 ± 2.41 ^{a}	23.05 ± 2.41 ^{a}	18.09 ± 1.07 ^{b}	21.31 ± 0.80 ^{ab}	12.19 ± 0.80 ^{c}	24.52 ± 0.80 ^{a}
4.0	0.5	11.66 ± 1.74 ^{a}	13.94 ± 1.47 ^{a}	11.26 ± 1.47 ^{ab}	6.03 ± 1.47 ^{b}	21.31 ± 1.88 ^{a}	16.62 ± 1.47 ^{ab}	12.86 ± 1.47 ^{b}	9.65 ± 1.47 ^{b}
	1.0	12.73 ± 0.94 ^{b}	18.63 ± 0.80 ^{a}	18.89 ± 0.80 ^{a}	8.31 ± 0.80 ^{c}	17.69 ± 1.88 ^{ab}	18.22 ± 1.47 ^{ab}	20.77 ± 1.47 ^{a}	13.53 ± 1.47 ^{b}
	2.0	15.01 ± 0.94 ^{bc}	21.44 ± 0.80 ^{a}	17.69 ± 0.80 ^{ab}	12.19 ± 0.80 ^{c}	21.44 ± 0.94 ^{b}	23.72 ± 0.80 ^{ab}	24.79 ± 0.80 ^{ab}	26.53 ± 0.80 ^{a}
	3.0	22.51 ± 1.21 ^{a}	26.00 ± 0.94 ^{a}	19.03 ± 0.94 ^{b}	20.37 ± 0.94 ^{b}	25.59 ± 1.61 ^{b}	25.59 ± 1.34 ^{b}	39.26 ± 1.34 ^{a}	37.79 ± 1.34 ^{a}

$^{a-c}$ Values for different succinic acid concentration at each L-malic acid one for the same pH and strain are significantly different at $p \leq 0.05$ according to Tukey test.



Supplementary Figure 1. Influence of succinic acid concentration (0, 0.5, 1 and 2 g/L) on growth of *O. oeni* strains PSU-1 and CH11 in MRS broth at pH 5.0. The values are the means of triplicates and the error bars represent the SD values.



Supplementary Figure 2. L-malic consumption in WLM with 12% ethanol (v/v) at pH 3.5 by *O. oeni* strains 1Pw13 and VP41, in the presence of different concentrations of succinic acid: 0 g/L (control, blue), 0.5 g/L (orange), 1 g/L (yellow), and 2 g/L (green). The values are the means of triplicates and the error bars represent the SD values.

CAPÍTULO 2

Efecto variable del ácido succínico en *Oenococcus oeni* y la fermentación maloláctica según las cepas

En este capítulo se evaluó el efecto que tiene la concentración de 2 g/L de ácido succínico sobre *O. oeni* en wine like medium (WLM). El objetivo fue determinar el efecto que ejerce el ácido succínico sobre diferentes cepas de *O. oeni* en su desarrollo y la fermentación maloláctica. Los resultados se encuentran en el manuscrito en preparación que se incluye a continuación.

Variable effect of succinic acid on *Oenococcus oeni* and malolactic fermentation depending on the strains

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Manuscript in preparation

Abstract

In this work we studied the succinic acid inhibition effect upon the malolactic fermentation (MLF) performance by 15 *Oenococcus oeni* strains, using 2 g/L of succinic acid in wine like medium (WLM). The results showed up that one strain (14P2) was able to overcome the succinic acid inhibition effect during the first five days. However, this succinic acid amount inhibited most of the strains in a mean of 48% at least of 10 experimental days, which confirms our previous results that this succinic acid amount represents an inhibitor level upon the MLF performance. Nonetheless, further research is necessary to understand the mechanisms displayed by some *O. oeni* strains to face the succinic acid inhibition effect.

Keywords: Inhibition, Malolactic fermentation, *Oenococcus oeni*, Succinic acid, Wine.

INTRODUCTION

The malolactic fermentation (MLF) is an important secondary fermentation in wine, carried out by lactic acid bacteria (LAB). The process consist in the removal of L-malic acid to obtain L-lactic acid, which diminishes the wine acidity, improves the aroma complexity and ensures microbiological stability (Alexandre et al., 2004; Lerm et al., 2010). The MLF is a reaction catalyzed by the malolactic enzyme, a dimeric or tetrameric protein formed by the association of 60 kDa polypeptide, that it is fixed with the cofactors NAD^+ and Mn^{2+} (Ribéreau-Gayon et al., 2006). *Oenococcus oeni* strains are the most important LAB in winemaking because they can survive in the harsh physico-chemical conditions of wine, including pH, ethanol content and low nutritional status. However, their growth and MLF can be inhibited for several compounds released during the alcoholic fermentation (AF) (Comitini et al., 2005).

Several compounds delivered by yeast are related with the negative impact on the malolactic fermentation (MLF) performance and *O. oeni* growth, for example, antimicrobial peptides, medium-chain fatty acids and organic acids (Branco et al., 2014; Guilloux-Benatier et al., 1998; Osborne and Edwards, 2007).

The succinic acid is an organic acid produced by yeast, not only during in the early AF stages, but also during the stationary phase (Arikawa et al., 1999; Conway and Downey, 1950; Lamikanra, 1997). Succinic acid is produced mainly by the tricarboxylic acid cycle (TCA) in yeast. Nevertheless, it acts as intermediary in other metabolic pathways such as the γ-amino butyric acid (GABA) bypass, glyoxylic acid bypass and the methylcitric acid (MCA) (De Klerk, 2010). In wine, this acid is characterized by the salty, bitter and sour taste with a threshold of 0.5-2.0 g/L (Coulter and Pretorius, 2007). However, is reported that it can improve the sensory wine properties, due to the increase in aromatic esters as methyl-succinate, ethyl-succinate and diethyl succinate, which are related to fruity aromas (Jordán et al., 2002). Mainly, *Saccharomyces cerevisiae* strains are known to produce succinic acid, with values from 200 mg/L to more than 1 g/L (Coulter and Pretorius, 2007; Zhu et al., 2020). However, non-*Saccharomyces* yeast can also produce succinic acid significantly with values around 0.3-0.8 g/L (Ciani and Maccarelli, 1997), and 0.95 g/L in sequential fermentations with *S. cerevisiae- T. delbrueckii* (Zhu et al., 2020). And above all Contreras et al. (2014) found productions of 1-2 g/L of this acid by strains of *Metschnikowia pulcherrima*, *Schizosaccharomyces malidevorans* and *Candida stellata*. This organic acid has been described as a possible competitive inhibitor of MLF due to its similarity with L-malic acid (Lonvaud-Funel and Strasser de Saad, 1982). Recently, in our research group the succinic acid effect upon the *O. oeni* and malolactic fermentation has been studied. The results showed that the succinic acid could act as inhibitor at concentrations higher than 1 g/L, but it can be beneficial at 0.5 g/L, which depends on strains and other winemaking conditions such as pH, which influences the dissociated and undissociated forms of L-malic and succinic acids. Moreover, the succinic acid inhibition seems to be caused when the molar

concentration was higher than the L-malic acid. Nonetheless, the strain-dependent effect is another factor that is related with the succinic acid inhibition (Torres-Guardado et al., 2022).

Due to the lack of information about how the succinic acid affects MLF performance in different *O. oeni* strains, the aim of this work was to evaluate the inhibition of succinic acid upon the MLF carried out by several *O. oeni* strains, commercial ones and from our group´s collection.

MATERIALS AND METHODS

Strains and cultures

Fifteen strains of *O. oeni* were used in this study (Table 1). Before the fermentation assays, strains were precultured at 28 ºC in MRS broth supplemented with D,L-malic acid (4 g/L) and fructose (5 g/L) at pH 5.0 for 48h at least twice prior to experimental use.

Assays of malolactic fermentation with addition of succinic acid

The MLF were carried out in in wine like media WLM (Bordas et al., 2013), containing 12% (v/v) ethanol, 2 g/L of L-malic acid and 2 g/L of succinic acid. with the respective control with no succinic acid in pH 3.4, adjusted with NaOH 1N. All fermentations were performed by triplicate in 50 mL tubes at 21 ºC were inoculated with the different *O. oeni* strains at $2x10^7$ CFU/mL. The samples were taken every 5 days to evaluate the L-malic acid consumption by enzymatic kit (BioSystems, Barcelona, Spain).

Statistical analysis

Statistically significant differences (ANOVA) with a *p*-value of less than 0.05 were analyzed by XLSTAT 2021 software (Addinsoft, New York, NY, USA).

Table 1. *Oenococcus oeni* strains used in this work.

Strain	Source	Other names [a]	Origin	References
1Pw13	Own collection	CECT 8893	Catalonia, Spain	Franquès *et al*. 2017
10P4	Own collection		Catalonia, Spain	Bordas *et al.* 2013
14P2	Own collection		Catalonia, Spain	Bordas *et al.* 2013
18P7	Own collection		Catalonia, Spain	Bordas *et al.* 2013
1T1	Own collection		Catalonia, Spain	Bordas *et al.* 2013
2T2	Own collection		Catalonia, Spain	Bordas *et al.* 2013
3P2	Own collection		Catalonia, Spain	Bordas *et al.* 2013
8P7	Own collection		Catalonia, Spain	Bordas *et al.* 2013
CH11	Chr. Hansen A/S	Viniflora CH11		
Mf1	Own collection		Catalonia, Spain	Reguant, Bordons 2003
Mf6	Own collection	CECT 7440	Catalonia, Spain	Reguant, Bordons 2003
PSU-1	Reference strain	ATCC BAA-331	Pennsylvania, USA	Mills *et al.* 2005
V-oenos	Chr. Hansen A/S	Viniflora Oenos		
VP41	Lallemand Inc.	Lalvin VP41	Southern Italia	
Z42	Own collection	Fn42	Catalonia, Spain	Masqué, Bordons 1996

[a] CECT: Colección Española de Cultivos Tipo, Valencia, Spain

RESULTS AND DISCUSSION

Effect of succinic acid on MLF done by several *O. oeni* strains

In this experiment, fifteen *O. oeni* strains were tested in WLM with 2 g/L of succinic acid, evaluating the MLF performance by the L-malic consumption in a period of 10 days. As a result of this experiment, inhibition by the succinic acid was found in a mean of 48% at the end of the experimental period (Table 2). This inhibition effect can be explained by the decrease in the L-malic consumption rate because it was observed that in almost all *O. oeni* strains it decreased in around 50% during the first five experimental days (Figure 1). However, the 14P2 strain was the only that showed a minor inhibition in

the L-malic consumption rate with respect to the control trial (16%) and resulting in a final inhibition at 10 days with a value of 6.74 % (Table 2). On the other hand, the Z42 strain presented a high inhibition in the L-malic consumption rate, where it decreased around 88% in presence of succinic acid with respect to the control trial. Interestingly, Z42 strain was the most susceptible to the 2 g/L of succinic acid concentration, due to its consumption of 0.51 g/L of L-malic acid (Figure 2), which represent a final MLF inhibition of 74.5% (Table 2), and consequently leading a residual L-malic amount of 1.49 g/L (Figure 2).

Table 2. L-malic consumption rate (mg/L/h) (0-5 days) in presence of succinic acid (2 g/L) by different *O. oeni* strains, and inhibition (%) at 10 days calculated by residual L-malic acid (g/L).

Strain	L-malic consumption rate	Inhibition at 10 d	Residual L-malic in control	Residual L-malic in media with succinic acid
1Pw13	5.6	59.90	0.03	1.21
10P4	5.8	48.52	0.31	1.13
14P2	10.9	6.74	0.07	0.20
18P7	7.0	39.39	0.02	0.80
1T1	6.9	41.21	0.01	0.83
2T2	7.1	43.43	0.02	0.88
3P2	7.5	40.40	0.02	0.82
8P7	6.9	44.72	0.01	0.90
CH11	5.4	48.15	0.38	1.16
Mf1	7.5	46.00	0	0.92
Mf6	5.5	45.50	0	0.91
PSU-1	5.5	54.84	0.14	1.16
V-oenos	4.7	45.50	0	0.91
VP41	5.1	52.45	0.57	1.32
Z42	2.1	74.50	0	1.49

*The inhibition (%) was calculated in base of the total consumption with respect to the control.

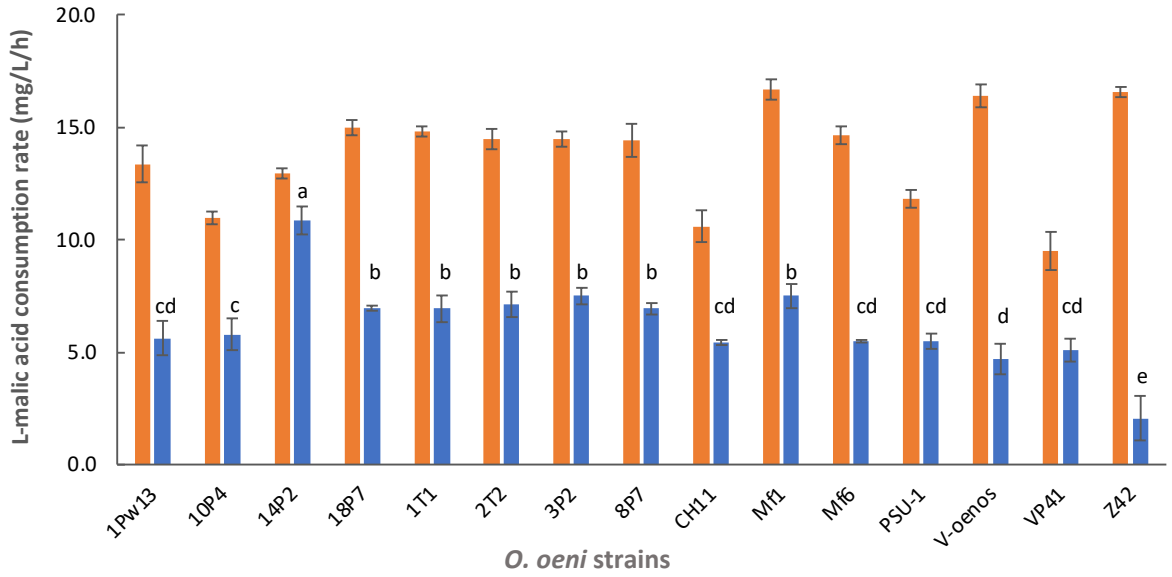

Figure 1. L-malic consumption rate (g/L/h) at 5 days by different *O. oeni* strains in WLM with 2 g/L succinic acid (blue columns) and in controls without it (yellow columns). [a-d] Values are significantly different at $p \leq 0.05$ according to Tukey's post hoc comparison test.

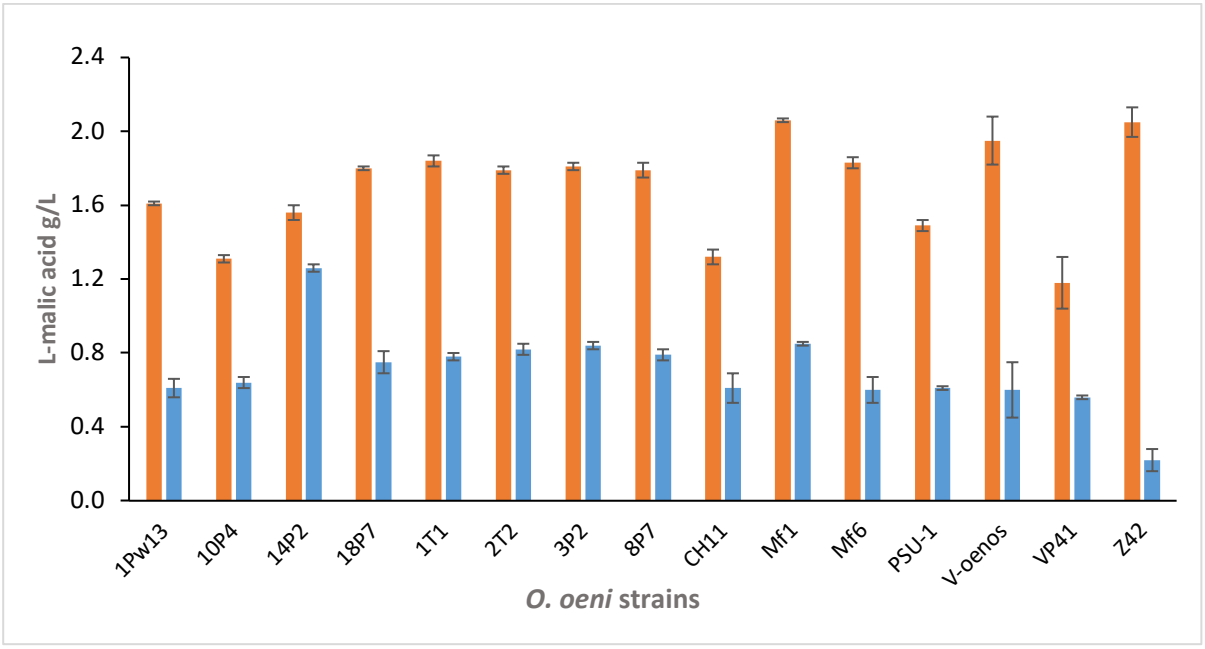

Figure 2. Total L-malic acid consumed at final MLF at 10 days by different *O. oeni* strains in WLM with 2 g/L succinic acid (blue columns) and in controls without it (yellow columns).

The results explained above, showed up that 2 g/L of succinic acid exerted an inhibition effect in all the strains employed in this study, mainly in the first experimental days in which the L-malic acid consumption rate was affected. Nonetheless, the 14P2 strain was able to display cell response strategies to face the succinic acid inhibition effect and consume almost all L-malic acid (Figure 3). In contrast, the Z42 strain presented a different L-malic consumption pattern, in which it led 1.49 g/L of L-malic acid. Therefore, based on the wide range of succinic acid production, not only is important to determine the inhibition effect in MLF, but also the minimal concentration that affects the MLF performance of LAB.

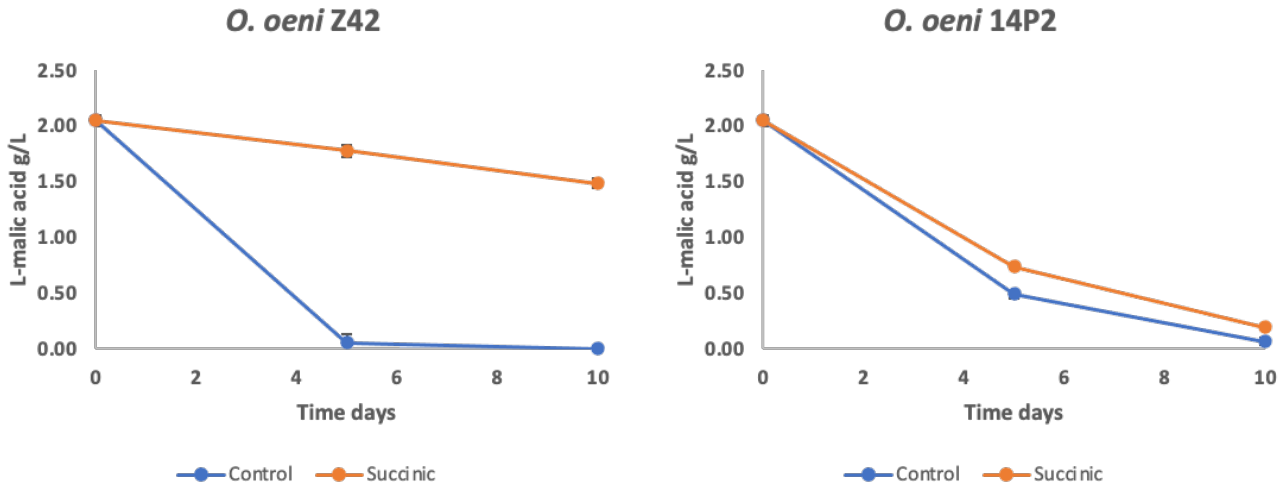

Figure 3. L- malic consumption in WLM by *O. oeni* strains Z42 and 14P2 in presence of succinic acid (2 g/L). The values are the means of triplicates and the error bars represent the SD values.

CONCLUSIONS

This work shows that the succinic acid can inhibit the MLF performance performed by different *O. oeni* strains. The inhibition effect upon the MLF not only is related with the amount of succinic acid contained in the media, but also other important factors such as the pH in the media, the L-malic acid amount, and the strain dependent effect are associated. In this study, only the 14P2 strain was able to overcome the succinic acid inhibition, showing a general inhibition of 6.74% at 10 days. Nonetheless, most of the

strains were affected by succinic acid with 2 g/L at 10 days in a mean of 48%, which confirms our previous results that this succinic acid amount represents an inhibitor level upon the MLF performance. Nonetheless, further research is necessary to understand the mechanisms displayed by some *O. oeni* strains to face the succinic acid inhibition effect.

Acknowledgements

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CAPÍTULO 3

Efecto significativo del ácido succínico sobre la expresión global de genes en *Oenococcus oeni* PSU-1

En este capítulo se evaluó el efecto que ejerce el ácido succínico sobre la expressión del transcriptoma de *O. oeni* PSU-1 durante la fermentación maloláctica en wine like medium (WLM). El objetivo fue determinar qué genes del transcriptoma se ven afectados por la disminución ó sobre expresión de los mismos en presencia del ácido succínico. Los resultados se encuentran en el manuscrito en preparación que se incluye a continuación.

Transcriptomic analysis reveals that succinic acid significantly affects global gene expression of *Oenococcus oeni* PSU-1

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Manuscript in preparation

Abstract

Oenococcus oeni is the predominant lactic acid bacterium performing the malolactic fermentation (MLF) in wine. Succinic acid, produced by yeasts during alcoholic fermentation, can improve the organoleptic properties of wine, but this acid can have a negative impact on *O. oeni* and the MLF, as shown in our previous works. Nowadays levels of succinic acid in wine can be higher because it is produced by the increasingly used non-*Saccharomyces* yeasts. To see the global effect of succinic acid in *O. oeni* PSU-1, in this work transcriptomic analysis by RNA-seq has been performed with triplicate samples of wine-like medium with 2 g/L succinic acid —and control without it—, at the end of MLF. A total of 1638 transcripts of differently expressed genes (DEG) were detected. Near the 25% of these DEG were found to be down-regulated with the half of expression or less, and other 28% of DEG were up-regulated with the double expression or more. These significant DEG were classified into clusters of orthologous groups (COGs). We found that functional categories related to metabolism of nucleotides (F), translation (J), and membrane transport (M) were predominantly down-regulated while those of transport and metabolism of carbohydrates (G), transcription (K), inorganic ions metabolism (P) and defence mechanisms (V) were predominantly up-regulated by succinic acid. Considering the higher upregulation of carbohydrate metabolism genes, we have analysed particularly those belonging to the phosphotransferase system (PTS). This is the first transcriptomic study of the effect of succinic acid on *O oeni*, where we have observed a global cell response with many changes in gene expression, related to the observed growth inhibition and MLF delay by succinic acid.

Keywords

Oenococcus oeni; RNA-seq; Succinic acid; Transcriptomics; Wine

Highlights

- Succinic acid significantly affects global gene expression of *Oenococcus oeni*
- Near 25% genes were noteworthy downregulated by succinic and ca. 28% were upregulated
- Nucleotides and amino acids metabolism was more under expressed by succinic acid
- Carbohydrate transport and metabolism, mainly PTS, was the main upregulated category
- Significant expression changes caused by succinic are related to stress mechanisms

Introduction

Oenococcus oeni is a lactic acid bacterium (LAB) exclusively found in wine environment (Franqués et al. 2017) and it is the predominant species performing the malolactic fermentation (MLF), by decarboxylating L-malic acid into L-lactic acid, and improving wine quality and microbial stability (Davis et al. 1988; Fleet 1998; Lonvaud-Funel 1999; Liu 2002; Bartowsky 2005)

Succinic acid is an organic acid produced by yeasts during the early stages of alcoholic fermentation (De Klerk 2010) and it can improve the sensory wine properties due to the increase in fruity aromatic esters such as methyl-succinate, ethyl-succinate and diethyl succinate (Jordán et al. 2002). *Saccharomyces cerevisiae* strains are known to produce succinic acid at levels of 200 mg/L to 2 g/L (Coulter and Pretorius 2007; De Klerk 2010; Zhu et al. 2020). Non-*Saccharomyces* yeasts can also produce succinic acid, usually in a range between 0.3 and 0.95 g/L (Ciani and Maccarelli 1997; Escribano et al. 2018; Zhu et al. 2020). Moreover, Contreras et al. (2014) found that some strains of *Metschnikowia pulcherrima*, *Schizosaccharomyces malidevorans* and *Candida stellata* produced 1 to 2 g/L of succinic acid.

Succinic acid can have a positive impact on the general quality of wine, as mentioned above, due to ester formation. In contrast, this acid can have a negative impact on LAB development and consequently on MLF performance because it has been reported that it can act as an inhibitor of L-malic decarboxylation

in LAB (Son et al. 2009). Moreover, succinic acid can inhibit MLF performance and *O. oeni* strains (Caridi and Corte 1997; Torres-Guardado et al. 2022).

Therefore, due to the importance of succinic acid production by yeasts, the increasing use of non-*Saccharomyces* strains (Padilla et al. 2016; Belda et al. 2017), and the impact of succinic acid on MLF seen in previous works (Torres-Guardado et al., 2023), the aim of this work was to evaluate the effect of succinic acid on *O. oeni* cells of strain PSU-1 by means of transcriptomics in order to know the global gene expression and analyse which groups of genes are more affected by this acid. A concentration relatively high (2 g/L) of succinic acid was used to see the most significant effects, comparing it to the control assay without this acid.

This work describes the analysis of differential gene expression by massive sequencing of total RNA (RNA-seq) in *O. oeni* samples. RNA quality control, library creation, massive sequencing using Illumina technology and corresponding bioinformatics analysis were performed. RNA-seq represents an aggregated snapshot of the cells' dynamic pool of RNAs, also known as transcriptome (Wang et al. 2009). The use of massive sequencing strategies as RNA-seq for the transcriptome studies is an emerging field that is gaining strength. Unlike microarrays, which are based solely on known transcripts and contained in the array, next-generation sequencing (NGS) technologies provide unprecedented view of the transcriptome of a given tissue or cell type. In addition, they allow the study of other RNA formats, such as long non-coding RNAs or small RNAs (Florea & Salzberg 2013).

Materials and Methods

Strain, culture growth, malolactic fermentation conditions and L-malic quantification

Oenococcus oeni PSU-1 (ATCC BAA-331) is a well-known reference strain of this species with the genome fully annotated. It was cultured at 28ºC in a CO_2 (10%) incubator in MRS broth medium supplemented with D,L-malic acid (4 g/L) and fructose (5 g/L) at pH 5.0. When cultures reached the late

exponential phase (OD_{600nm} = 1.6) it was inoculated at a concentration of $2 \cdot 10^7$ cells/mL in wine like medium (Margalef-Català et al. 2016) with 2 g/L of succinic acid (WLMS) or without this added acid (WLM). The fermentations were carried out at 20 ºC in static conditions, by triplicate.

The population of *O. oeni* was determined by viable counting on solid medium plates at the inoculation time and at the end of MLF using the same medium used for growth (supplemented MRS) with 20 g/L of agar. The L-malic acid consumption was measured enzymatically using the multianalyzer equipment Y15 (Applied Biosystems). MLF were considered as finished when L-malic acid was below 0.2 g/L.

Sample preparation

Samples were obtained by triplicate: control samples (WLM 1, WLM 2, WLM 3) and the treated with succinic acid (WLMS 1, WLMS 2, WLMS 3).

They were taken from WLM at the end of MLF (when [L-malic acid] < 0.1 g/L. Fifty mL of each sample were centrifuged at 4600 ×*g* for 20 min at 4 ºC. The pellet was washed with 10 mM Tris-HCl prepared with diethyl pyrocarbonate-treated water (DEPC), then frozen in liquid nitrogen and kept at -80 ◦C until RNA extraction.

RNA extraction from *O. oeni* cells and RNA quality assessment

It was performed following Margalef-Català et al. (2016). In this way, the cell pellet was defrosted and washed again with 10 mM Tris-HCl DEPC water. A High Pure RNA Isolation Kit (Roche, Mannheim, Germany) was used for the extraction following the manufacturer's instructions, changing the cell lysis for lysozyme dissolved in 10 mM Tris-HCl buffer DEPC at 50 mg/ mL for 30 min at 37 ◦C. Total acid nucleic concentrations were calculated using a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, Bremen, Germany). The extracted RNA was stored at -80 ◦C until RNA analysis.

RNA quality was assessed using the Agilent TapeStation team and the Agilent RNA ScreenTape Assay. RNA quality was assessed using RIN (RNA Integrity Number) and is considered adequate when it is >7 on a scale of 0 to 10.

Transcriptomic analysis

The transcriptomic analysis by RNA-seq was performed by the Centre for Omic Sciences (COS) Joint Unit of the Universitat Rovira i Virgili — Eurecat, according to optimized protocols. The sequencing libraries were created using the Illumina Stranded total RNA Prep, Ligation with Ribo-zero Plus (20040525, Illumina) following the manufacturer's instructions. During this process, the RNA is fragmented, retrotranscribed, and modified to incorporate adaptive sequences and short ones called "indexes" that allow samples to be distinguished in subsequent data analysis. As a result, a sample DNA library is obtained that contains the retrotranscribed and modified RNA fragments so that they can be sequenced.

Each library was quantified by micro-fluid electrolysis in the Agilent TapeStation using the Agilent High Sensitivity Screen Tape kit. From this result, the length and concentration of each sample were determined. Finally, equimolar mixtures, or pools, were created which were taken to a concentration of 750 pM. The pool libraries were sequenced with the NextSeq2000 equipment of the Illumina platform generating up to 50 million 2x76pb reads per sample.

Data analysis included mapping against a reference genome (CP014324.1, *Oenococcus oeni* strain UBOCC-A-315001) using HISAT2 (2.2.1); annotation and quantification of aligned reads with StringTie (2.1.4), and gene expression levels comparison using DESeq2 R package (1.30.0). Samples were normalized by Relative Log Expression (RLE) method and expression levels will be represented in Counts per Million (CPM).

Interpretation of the differentially expressed genes found in transcriptomic analysis

The most significant differentially expressed genes (DEG) in samples with succinic acid related to control without it were selected in base of having a Log2FC < -1 for down-regulated transcripts and up-regulated ones with a Log2FC > 1.

Those most significant DEG were classified into clusters of orthologous groups (COGs) to understand the main biological processes or functional categories affected by succinic acid. In a first approach, FUNAGE-pro web server (De Jong et al. 2022) was used to perform the COG enrichment analysis of DEG.

Considering that FUNAGE-Pro platform analysis allows the classification of only part of the genes, another search of COGs was done manually in the *O. oeni* PSU-1 complete sequence with GenBank database (NCBI 2023) for all selected DEG but limiting their number to those with a higher level of significant different expression, searching for DEG with Log2FC < -3 or Log2FC > 3. The objective was to evaluate those genes and corresponding functions with an expression more affected by the presence of succinic acid.

Analysis of genes related with survival and malolactic performance

To know the detailed effect of succinic acid in *O. oeni* PSU-1, some specific genes related with survival and malolactic performance were searched in the results of DEG obtained in the same transcriptomic analysis. The chosen genes were mostly those related with the different cell mechanisms that are involved in *O. oeni* growth, survival, stress tolerance and malolactic fermentation, such as ATPase, malolactic activity, citrate consumption, membrane modifications, phosphotransferase system (PTS) and others, following previous knowledge (Silveira et al. 2004; Mills et al. 2005; Beltramo et al. 2006; Olguín et al. 2010; Olguín et al. 2010; Jamal et al. 2013; Margalef-Català et al. 2016; Margalef-Català et al. 2017; Balmaseda et al. 2022).

Results and Discussion

Impact of succinic acid on MLF

As expected, the MLF was longer in the presence of succinic acid. In the control condition (WLM) MLF took 6 days to finish, while with succinic acid (WLMS) it took 17 days (Figure 1). A similar negative effect of succinic acid on MLF was observed with *O. oeni* PSU-1 in previous studies (Torres-Guardado et al. 2023). It's suggested that the undissociated succinic acid forms could enter the *O. oeni* cell at wine pH close to 3.5. Internal acidification has been associated with the inhibition of MLF by other acidic compounds at pH values close to 3.0, such as octanoic acid and some phenolic acids (Capucho and San Romão, 1994; Campos *et al.*, 2003). The viable population was determined at the end of MLF and no significant changes were observed between the two conditions. The populations were close to the initial inoculated concentration (data not shown), indicating the survival of the cells, but the lack of growth during MLF as commonly happens in inoculated fermentations.

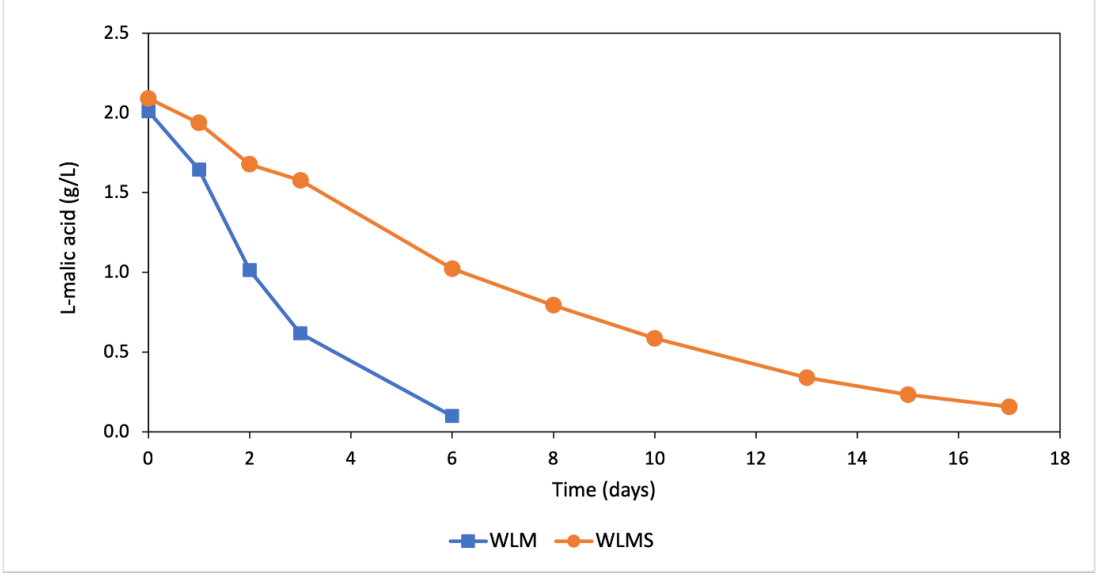


Figure 1. Consumption of L-malic acid by *O. oeni* PSU-1 in the control condition without succinic acid (WLM) and with 2 g/L of succinic acid (WLMS).

RNA quality assessment, library creation and sequencing

The average values of total generated sequencing reads performing RNA-seq on the Illumina platform were 63,819,570 for the three control samples, and 34,646,374 for the three samples with succinic acid. From these, the aligned reads were the 91.35% for the controls and 91.80% for the samples with succinic acid. These more than 30 million generated reads per sample are considered enough for optimal results.

Results of differential expression analysis

Sequencing readings from the alignment were assigned to gene transcripts, and the differential expression analysis was performed from these transcripts.

In Supplementary Table 1 all detected RNAs are displayed, as well as their log of the fold change in base 2 (Log2FC) comparing between two groups of samples. If Log2FC is 1 means duplicate expression, if it is 2 means quadruple, etc. Statistical values (results not shown) for all transcripts were done confirming that the changes were significant. Log2FC change values are marked in red or dark blue depending on whether the transcripts were down or up-regulated, respectively.

Expression results were clearly different for the three samples of *O. oeni* cells grown in WLM with succinic acid in comparison to the three control samples grown without succinic acid, as seen in the PCA graphic (Figure 2), where it is shown that there were no outliers for each group.

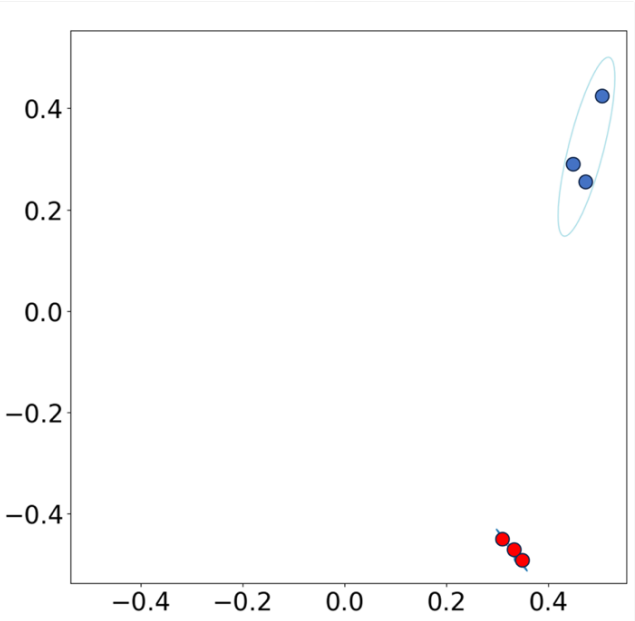


Figure 2. PCA graphics comparing gene expression of *O. oeni* grown with succinic acid (blue dots) regarding control samples (red dots).

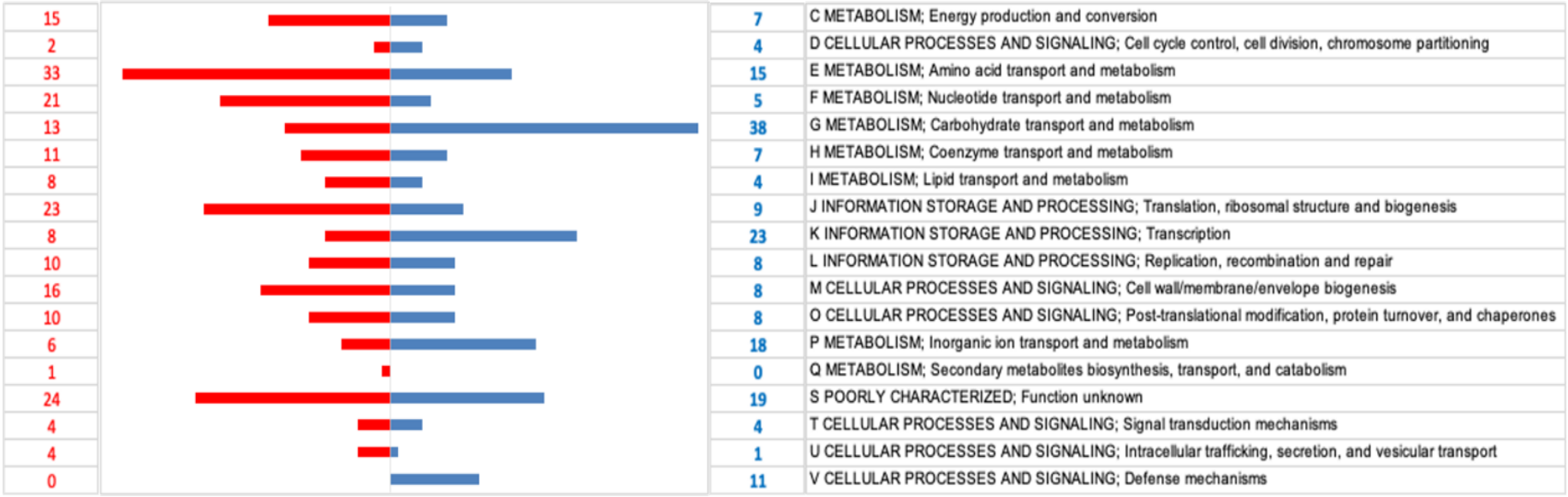
Considering their log of the fold change in base 2 (Log2FC), in this comparison 1638 transcripts out of 1852 (88%) had statistically significant some different expression. This high number (1852) of found transcripts is exceptionally good since *O. oeni* PSU-1 has 1854 genes —of which 1691 are protein coding— with a total DNA of 1.8 Mbp. (NCBI, 2023; Makarova et al., 2006). And this 88% of significant differentially expressed genes (DEG) shows the very important effect of succinic acid on *O. oeni* cells. A total of 405 out of these 1638 DEG were found down-regulated with a Log2FC < -1 and 466 transcripts were found up-regulated with a Log2FC > 1. That means a total of 871 statistically remarkably significant DEG, a 53% of the DEG, of which approximately the half were down- and the other half were up-regulated.

Global analysis of functions affected by succinic acid in *O. oeni* PSU-1

These 871 remarkable DEG with a Log2FC < -1 or > 1 were classified into clusters of orthologous groups (COGs) to understand the main biological processes or functional categories affected by succinic acid. As mentioned, firstly FUNAGE-pro web server was used. Selecting *O. oeni* PSU-1 in this platform, we introduced our DEG list and search for classification data, where COGs and gene ontology (GO) were obtained automatically from many of DEG but not all. We got COGs and GO description for 398 genes: 220 down-regulated and 178 up-regulated (Supplementary Table 2). The other 473 DEG of our list were not classified by FUNAGE-pro platform. Figure 3 shows the number of genes under expressed or overexpressed found with this platform for each representative COG.

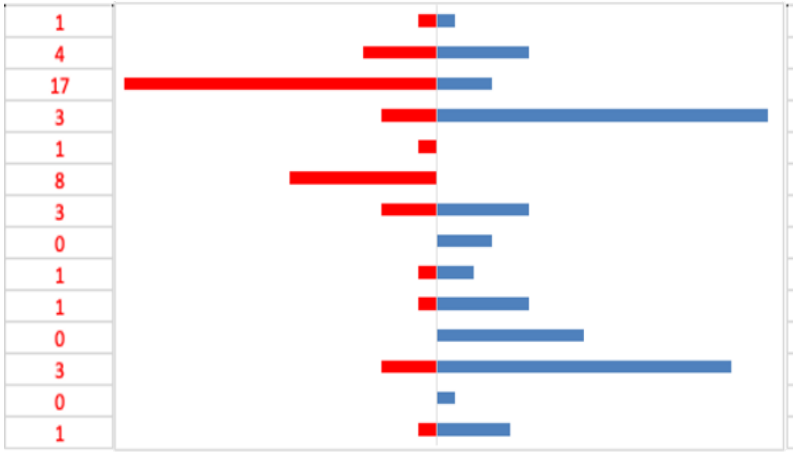
In order to evaluate the most affected genes and considering that FUNAGE-Pro analysis classifies only part of the DEG in COGs (398 out of 871), we proceeded to search for the COGs using GenBank database of the unclassified genes, but limiting the number of DEG to those with a higher level of significant different expression, *id est*, searching for DEG with Log2FC < -3 or Log2FC > 3. In this way, we were able to find 43 highly down-regulated DEGs and 71 highly up-regulated ones. All details of these 114 DEGs, their COG and probable functions or products can be seen in Supplementary Table 3.

As seen in Figure 4, the numbers of DEG of the different found functional categories (COG) are very similar to those found when considering those with Log2FC < -1 or > 1 (Figure 3). This confirms the representativity of the FUNAGE-Pro COG enrichment analysis.



15	7	C METABOLISM; Energy production and conversion
2	4	D CELLULAR PROCESSES AND SIGNALING; Cell cycle control, cell division, chromosome partitioning
33	15	E METABOLISM; Amino acid transport and metabolism
21	5	F METABOLISM; Nucleotide transport and metabolism
13	38	G METABOLISM; Carbohydrate transport and metabolism
11	7	H METABOLISM; Coenzyme transport and metabolism
8	4	I METABOLISM; Lipid transport and metabolism
23	9	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis
8	23	K INFORMATION STORAGE AND PROCESSING; Transcription
10	8	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair
16	8	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis
10	8	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones
6	18	P METABOLISM; Inorganic ion transport and metabolism
1	0	Q METABOLISM; Secondary metabolites biosynthesis, transport, and catabolism
24	19	S POORLY CHARACTERIZED; Function unknown
4	4	T CELLULAR PROCESSES AND SIGNALING; Signal transduction mechanisms
4	1	U CELLULAR PROCESSES AND SIGNALING; Intracellular trafficking, secretion, and vesicular transport
0	11	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms

Figure 3. Number of genes (found with FUNAGE-Pro platform) of *O. oeni* PSU-1 for each representative COG significantly underexpressed (red) or overrexpressed (blue) —with Log2FC < -1 or > 1 respectively— in WLM + 2 g/L succinic acid, according to transcriptomic analysis.



1		1	C METABOLISM; Energy production and conversion
4		5	E METABOLISM; Amino acid transport and metabolism
17		3	F METABOLISM; Nucleotide transport and metabolism
3		18	G METABOLISM; Carbohydrate transport and metabolism
1		0	H METABOLISM; Coenzyme transport and metabolism
8		0	I METABOLISM; Lipid transport and metabolism
3		5	K INFORMATION STORAGE AND PROCESSING; Transcription
0		3	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair
1		2	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis
1		5	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones
0		8	P METABOLISM; Inorganic ion transport and metabolism
3		16	S POORLY CHARACTERIZED; Function unknown
0		1	T CELLULAR PROCESSES AND SIGNALING; Signal transduction mechanisms
1		4	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms

Figure 4. Number of genes (found with FUNAGE-Pro platform and GenBank database NCBI) of *O. oeni* PSU-1 for each representative COG remarkable significantly underexpressed (red) or overrexpressed (blue) —with Log2FC < -3 or > 3 respectively— in WLM + 2 g/L succinic acid, according to transcriptomic analysis.

As seen, there were some functional categories of genes (COG) clearly predominant down-regulated and others clearly up-regulated. Those related to metabolism of nucleotides (F), translation (J) and aminoacid transport and metabolism (E) were predominantly down-regulated while those of transport and metabolism of carbohydrates (G), transcription (K), inorganic ions metabolism (P) and defence mechanisms (V) were predominantly up-regulated.

Some of the previous *O. oeni* transcriptomic studies have evaluated different types of stress associated to wine, including the response to acidic conditions (Olguín et al. 2015; Margalef-Català et al. 2016; Liu et al. 2017). In this study the pH was the same in the control fermentations as in the fermentations with added succinic acid, since the wine like media was adjusted to pH 3.4 in the two conditions in order to evaluate uniquely the inhibitory effect of succinic acid. Therefore, the transcriptomic response cannot be directly associated to a lower external pH. However, as before mentioned, the internal acidification of *O. oeni* cytosol due to succinic acid dissociation could be one of the possible mechanisms of inhibition.

Additionally, it has been described that succinic acid can provoke membrane damage in bacteria such as *Staphylococcus aureus* and *Pseudomonas fluorescens*, being this damage more severe in the Gram + than in the Gram – bacteria (Huang et al. 2022). The alteration of the cell membrane might also be a mechanism of inhibition of succinic acid on *O. oeni* according to the observed results. In this sense, some of the functions associated to DEG are related to transport and cell envelope components as it will be discussed.

Among the significantly inhibited cellular functions, there was nucleotide transport and metabolism category (F). Seven genes organised in an operon encoding for key enzymes in DNA synthesis (from OEOE_RS01235 to OEOE_RS01265) include the genes most downregulated of all the DEG found in this study, showing a reduction in the expression of 8 or more times due to succinic acid presence (Supplementary Table 3). The functions of these genes, such as dihydroorotase and carbamoyl phosphate synthase, are associated to pyrimidine biosynthetic pathway (Kilsrup et al. 2005). The inhibition of

pyrimidine biosynthesis may have a severe impact on the cells. Besides their roles as precursors for RNA and DNA, pyrimidine nucleotides have important roles in the biosynthesis of components present in the cell envelope, including peptidoglycan and exopolysaccharides (EPS). According to the strong downregulation observed in the genes associated to pyrimidine biosynthesis, this would be one of the relevant inhibitory mechanisms affecting *O. oeni* caused by succinic acid. Other essential functions negatively affected by this acid, according to downregulation observed in the associated genes, were translation (J) and aminoacid transport and metabolism (E). Among the genes mostly affected of these functions there are genes involved in the ribosomal assembly and genes encoding peptidases and amino acid transporters (Supplementary Table 3).

Among the functions mostly activated at the transcriptional level there were genes classified in Metabolism and transport of inorganic ions (P) category, being many of them permeases. Two genes associated to the transport of spermidine/putrescine were over-expressed (Supplementary Table 3). The uptake of these two polyamines has been associated with an energy-producing state/membrane potential of the cell in *E. coli* (Kashiwagi et al., 1997), and both putrescine and spermidine protect against oxidative stress (Tkachenko et al., 2001). In another study, the adaptation to conditions of WLM resulted in an over-expression of six out of the eight transporters of these polyamines annotated in PSU-1 genome (Margalef-Català et al. 2018).

Transcription (K) is a functional category clearly activated in response to succinic acid. The most activated genes are transcriptional regulators that would be involved in the complex response of *O. oeni* transcriptome to the inhibition produced by succinic acid, such as genes encoding for GntR (OEOE_RS06440) and TetR (OEOE_RS00850) of transcriptional regulators families (Supplementary Table 3). Very little knowledge is available about transcriptional regulation in *O. oeni*, so further research would be needed to understand the function of each of these regulatory elements. It's worth to notice the

number of up-regulated genes belonging to the mechanisms of cell defence (V) that are mainly encoding for transporter proteins which might be involved in coping with succinic toxicity.

A considerable number of genes associated to carbohydrate metabolism were upregulated. Among these genes, many of them belong to the phosphotransferase system (PTS), associated to sugar transport, that was described by Jamal et al. (2013) in *O. oeni* PSU-1. Table 1 shows the genes described by these authors and the differential expression of these genes they found. The concentration of sugars in the fermentation media was low (0.4 g/L fructose and 0.25 g/L trehalose); however, many of the genes of the PTS system were overexpressed indicating the possible role of sugar metabolism in the response to the stress caused by succinic acid. The up-regulated PTS genes (Table 1) belong mainly to permeases associated to the transport of cellobiose, mannose and trehalose (*celABCD*, *manAB*, *treA*). As before mentioned, trehalose was one of the sugars present in the medium, but WLM it's not expected to have sources of cellobiose or mannose. Jamal et al. (2013) described the difficulty to predict substrate specificity of PTS permeases from sequence comparisons and observed the transcriptional activation of some genes in the presence of sugars not supposed to be the specific one for the studied permease. Such was the case of the *celA* locus, that Jamal et al. (2013) found to be highly overexpressed in the presence of cellobiose but also, to a lesser extent, in the presence of trehalose. Additionally, it has been suggested that PTS may have other substrates different to sugars, since most PTS permeases shown to be able to phosphorylate several substrates in different bacteria (Postma et al. 1993). In this study, the most feasible hypothesis is that PTS system might have been activated in response to succinic acid due to the presence of trehalose and fructose in the media. In other LAB, transcriptomic and proteomic studies have shown the enhanced levels of the glycolytic enzymes under acid, thermal, and osmotic stresses, but without increasing the synthesis of lactic acid (Papadimitrou et al. 2016). LAB such as *Lactiplantibacillus plantarum* and *Lactococcus lactis* modify pyruvate metabolism at the expense of lactic acid increasing the synthesis of basic compounds (e.g. lysine and diacetyl/acetoin), exopolysaccharides (EPS), and/or

glycogen (Bove et al. 2012; Heunis et al. 2014; Zujlan et al. 2014). In this study it was not observed the increase of lactic acid at the end of MLF (data not shown), therefore, some of these mechanisms could be involved in the cell response to succinic acid. In the case of *O. oeni*, the biosynthesis of EPS has been associated to biofilm formation as a mechanism for cell survival under stressful conditions (Dimopolou et al. 2018). The up-regulation of genes encoding glycosyl transferases OEOE_RS07010, OEOE_RS07295 and OEOE_RS07115 was observed in response to succinic acid (Supplementary Table 2). This enzymatic activity has been associated in LAB with biofilm formation and bacterial stress response (Schwab et al. 2007). Further research is needed in order to understand the role of the activation of PTS system under wine stress conditions.

Table 1. Different expression (log2FoldChange, significant negative values in red and positive in green) of *O. oeni* PSU-1 genes related with PTS system, in cells grown in WLMS (succinic acid 2 g/L) compared to control in WLM without succinic acid.

Locus tag	Product (GenBank)	log2FoldCh	Description	Symbol
OEOE_RS03075	HPr his-protein	**-1.15**	phosphocarrier protein HPr	*hpr*
OEOE_RS01045	IIC cellobiose	**1.28**	PTS cellobiose transporter subunit IIC	*celA*
OEOE_RS01050	IIA cellobiose	**1.46**	PTS lactose/cellobiose transporter subunit IIA	*celA*
OEOE_RS01055	IIBcellobiose	**1.72**	PTS sugar transporter subunit IIB	*celA*
OEOE_RS01060	Pβ-glucosidase	0.99	6-phospho-beta-glucosidase	*celA*
OEOE_RS01110	IIA mannose/fructose	0.35	PTS sugar transporter subunit IIA	
OEOE_RS01115	IIA galacticol	0.47	PTS sugar transporter subunit IIA	*galA*
OEOE_RS01120	IIC galacticol	**1.00**	PTS transporter subunit IIC	*galA*
OEOE_RS01125	IIB galacticol	0.68	PTS sugar transporter subunit IIB	*galA*
OEOE_RS01350	IIC cellobiose	**2.18**	PTS sugar transporter subunit IIC	
OEOE_RS01415	IIA glucose	**1.66**	PTS glucose transporter subunit IIA	*celB*
OEOE_RS01420	IIBC β-glucoside	**1.34**	PTS transporter subunit EIIB	*celB*
OEOE_RS01425	IIA β-glucoside / pseudogene	0.49	PTS transporter subunit EIIC	*celB*
OEOE_RS01620	IIB cellobiose	**2.40**	PTS sugar transporter subunit IIB	*celC*
OEOE_RS01625	IIA cellobiose	**4.17**	PTS lactose/cellobiose transporter subunit IIA	*celC*

OEOE_RS01630	6P- β-glucosidase	**4.00**	6-phospho-beta-glucosidase	*celC*
OEOE_RS01635	6P- β-glucosidase	**3.03**	6-phospho-beta-glucosidase	*celC*
OEOE_RS01645	IIC cellobiose	**1.57**	PTS cellobiose transporter subunit IIC	*celC*
OEOE_RS01740	IIBC fructose	**2.75**	PTS sugar transporter subunit IIC	
OEOE_RS01825	IIC mannose	0.84	PTS sugar transporter subunit IIC	*manA*
OEOE_RS01830	IID mannose	**1.15**	PTS system mannose/fructose/sorbose family transporter subunit IID	*manA*
OEOE_RS01835	IIA mannose	**1.31**	PTS sugar transporter subunit IIA	*manA*
OEOE_RS02230	IIAB mannose	**1.51**	PTS sugar transporter subunit IIB	*manB*
OEOE_RS02235	IIC mannose	**1.69**	PTS mannose/fructose/sorbose transporter subunit IIC	*manB*
OEOE_RS02240	IID mannose	**1.93**	PTS system mannose/fructose/sorbose family transporter subunit IID	*manB*
OEOE_RS05815	IIA cellobiose	**1.62**	PTS lactose/cellobiose transporter subunit IIA	*celD*
OEOE_RS05820	IIB cellobiose	**1.86**	PTS sugar transporter subunit IIB	*celD*
OEOE_RS05825	IIC cellobiose / pseudogene	**3.12**	PTS transporter subunit EIIC	*celD*
OEOE_RS05830	6P- β-glucosidase	**2.24**	glycoside hydrolase family 1 protein	*celD*
OEOE_RS06445	P-trehalase	**4.00**	alpha,alpha-phosphotrehalase	*treA*
OEOE_RS06450	IIBC trehalose	**3.48**	PTS transporter subunit EIIC	*treA*
OEOE_RS06455	IIA glucose/trehalose	**2.23**	PTS glucose transporter subunit IIA	*treA*
OEOE_RS07145	IIC ascorbate/ pseudogene	**2.20**	PTS ascorbate transporter subunit IIC	*ascA*
OEOE_RS07150	IIB ascorbate	**1.93**	PTS sugar transporter subunit IIB	*ascA*
OEOE_RS07155	IIA fructose	-0.48	PTS sugar transporter subunit IIA	*ascA*
OEOE_RS01190	mannose-6 phosphate isomerase	0.58	mannose-6-phosphate isomerase, class I	*manA*

Table 2. Different expression (log2FoldChange, significant negative values in red and positive in blue) of some specific genes of *O. oeni* PSU-1 related with main known functions of survival and malolactic performance, in cells grown in WLMS (succinic acid 2 g/L) compared to control in WLM without succinic acid.

Function	Locus tag	Product (GenBank)	log2FC	Description	Symbol
ATPase	OEOE_RS03155	F0F1 ATP synthase subunit A	**-2.37**	F0F1 ATP synthase sub. A	atpB
ATPase	OEOE_RS03160	F0F1 ATP synthase subunit C	**-1.50**		atpE
ATPase	OEOE_RS03165	F0F1 ATP synthase subunit B	**-1.39**		atpF
ATPase	OEOE_RS03170	ATP synthase F1 subunit delta	**-1.34**	ATP synthase subunit delta	atpH
ATPase	OEOE_RS03175	F0F1 ATP synthase subunit alpha	**-1.25**	F0F1 ATP synthase subunit alpha	atpA
ATPase	OEOE_RS03180	ATP synthase F1 subunit gamma	-0.69	ATP synthase subunit gamma	atpG
ATPase	OEOE_RS03185	F0F1 ATP synthase subunit beta	-0.71		atpD
ATPase	OEOE_RS03190	F0F1 ATP synthase subunit epsilon	-0.83	F0F1 ATP synthase subunit epsilon	
Citrate	OEOE_RS02030	citrate (pro-3S)-lyase subunit beta	**1.79**	citrate lyase	citE
Citrate	OEOE_RS02020	[citrate (pro-3S)-lyase] ligase	**1.35**	[citrate [pro-3S]-lyase] ligase	citC
Citrate	OEOE_RS02025	citrate lyase acyl carrier protein	**1.89**	citrate lyase ACP	citD
Citrate	OEOE_RS02035	citrate lyase subunit alpha	**1.73**	citrate lyase subunit alpha	citF
Citrate	OEOE_RS02040	citrate lyase holo-[acyl-carrier protein] synthase	**2.18**		citX
Citrate	OEOE_RS06020	acetate kinase	**-1.95**	acetate kinase	
Citrate	OEOE_RS08225	acetolactate decarboxylase	**-1.67**		budA
Malolactic	OEOE_RS01975	PLP-dependent aminotransferase family protein	**-2.02**		
Malolactic	OEOE_RS01985	D-2-hydroxyacid dehydrogenase, D-Lactate dehydrogenase and D-2-Hydroxyisocaproic acid dehydrogenase	0.25	lactate dehydrogenase	
Malolactic	OEOE_RS02010	malic enzyme-like NAD(P)-binding protein	0.59	malate dehydrogenase	
Malolactic	OEOE_RS02015	AEC family transporter	0.95	malate permease	

Malolactic	OEOE_RS06380	L-lactate dehydrogenase	**-4.33**	
Malolactic	OEOE_RS06985	AEC family transporter	-0.21	malate transporter
Malolactic	OEOE_RS07545	malolactic enzyme	**-1.42**	malolactic enzyme activity
Malolactic	OEOE_RS07550	LysR family transcriptional regulator	0.62	
Stress	OEOE_RS01385	Hsp20/alpha crystallin family protein	**4.44**	heat-shock protein Hsp20
Stress	OEOE_RS02440	CtsR family transcriptional regulator	**1.21**	hypothetical protein
Stress	OEOE_RS02445	ATP-dependent Clp protease ATP-binding subunit	0.89	
Stress	OEOE_RS03060	ATP-dependent Clp protease ATP-binding subunit	**1.38**	
Stress	OEOE_RS08340	ATP-dependent Clp protease ATP-binding subunit ClpX	**-1.31**	ATP-dependent Clp protease ATP-binding subunit

Specific genes related with survival and malolactic performance affected by succinic acid

A selection of genes related with survival and malolactic performance were searched in the results of DEG obtained in the transcriptomic analysis. Table 2 shows their different expression in *O. oeni* cells grown in WLM with succinic acid, including their locus tag, related group of function, products, minimal description and gene symbols.

There was a clear significant different expression in some genes or groups of them. Firstly, all studied eight genes related to ATP synthase were under-expressed, with values of Log2FC from -0.69 to -2.37. The inhibition of *O. oeni* ATPase activity has been described in the presence of different stress factors, such as low pH (Carreté et al. 2002). In this case, the internal acidification provoked by succinic acid might have caused this inhibition. The key gene for malolactic enzyme (locus tag OEOE_RS07545) was also under-expressed, with a value of -1.42. This would explain the slower MLF in the fermentations with added succinic acid.

On the contrary, those related with consumption of citrate were over-expressed, with positive values from 1.35 to 2.18. Citrate metabolism, and the transcriptional activation of some of the citrate lyase operon genes, has been associated to the response to acid stress in *O. oeni* (Olguín et al. 2009). Regarding the stress response associated genes, the very much studied gene with locus tag OEOE_RS01385 for heat-shock protein Hsp20, related to stress tolerance of *O. oeni* in wine (Guzzo et al. 2000), was found also to be over-expressed (Log2FC = 4.44). Also, the gene of the CtsR transcriptional regulator and a ClpP protease encoding gene. These three genes are considered relevant stress biomarkers (Beltramo et al. 2006), which is in accordance with the inhibitory and stressful effect of succinate on *O. oeni* cells observed in this study.

Conclusions

When *O. oeni* PSU-1 was inoculated in wine-like medium with 2 g/L succinic acid, MLF was delayed more than 17 d while the control without this acid was able to finish it in 6 d. This confirmed the previously known negative effect of relatively high levels of succinic acid in wine on *O. oeni* and on MLF. Cell samples at the end of these MLF were used to extract RNA with good quality for transcriptomic analysis by RNA-seq. The RNA libraries were well generated, with more than 30 M reads/sample, which were enough to obtain optimal results.

A total of 1852 transcripts of *O. oeni* PSU-1 —practically the totality of the annotated genes in GenBank— were detected, and the comparison between control and treatment group with succinic gave a total of 1638 transcripts differently expressed (DEG, for genes). This 88% of different gene expression showed the very important effect of succinic acid on *O. oeni* cells, which was clearly confirmed by a PCA graphic. Near the 25% of these DEG were found to be down-regulated with a Log2 Fold Change < -1 —which means the half expression or less—, and other 28% of DEG were up-regulated with a Log2FC > 1 —the double expression or more—. That means a total of 871 DEG with Log2FC < -1 or > 1.

These significant DEG were classified into clusters of orthologous groups (COGs) to understand the main biological processes affected by succinic acid. We found in this global analysis that functional categories equivalent to COG related to metabolism of nucleotides (F), translation (J), and aminoacid transport and metabolism (E) were predominantly down-regulated while those of transport and metabolism of carbohydrates (G), transcription (K), inorganic ions metabolism (P) and defence mechanisms (V) were predominantly up-regulated. Considering the higher upregulation of carbohydrate metabolism genes, we have analysed particularly those belonging to the phosphotransferase system (PTS). We have suggested the reasons for all these changes related to stress caused by succinic acid in base to previous works of observed cell damage in *O. oeni* and other LAB by known inhibiting compounds such as ethanol.

In conclusion, in this first transcriptomic study of the effect of succinic acid on *O oeni*, we have observed a global cell response with many changes in gene expression, but mainly that those related to pyrimidine metabolism were under expressed and those of carbohydrate metabolism, highlighting the role of the PTS system, were the most over expressed.

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CAPÍTULO 4

Producción de ácido succínico por levaduras y la influencia del GABA y el ácido glutámico

En este capítulo fue evaluada la producción de ácido succínico por levaduras *S. cerevisiae* y algunas no-*Saccharomyces*, así como la influencia del GABA y ácido glutámico. El objetivo fue determinar los niveles de producción de levaduras y la influencia de la suplementación de GABA y ácido glutámico. Los resultados pueden encontrarse en el artículo: Torres-Guardado R., Rozès, N., Esteve-Zarzoso, B., Reguant, C., Bordons, A. (2023). Succinic acid production by wine yeasts and the influence of GABA and glutamic acid. International Microbiology. Publicado online 27 Julio, 1-8. https://doi.org/10.1007/s10123-023-00410-9.

Succinic acid production by wine yeasts and the influence of GABA and glutamic acid

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Abstract

As a consequence of alcoholic fermentation (AF) in wine, several compounds are released by yeasts, and some of them are linked to the general quality and mouthfeel perceptions in wine. However, others, such as succinic acid, act as inhibitors, mainly of malolactic fermentation. Succinic acid is produced by non-*Saccharomyces* and *Saccharomyces* yeasts during the initial stages of AF, and the presence of some amino acids such as γ-aminobutyric acid (GABA) and glutamic acid can increase the concentration of succinic acid. However, the influence of these amino acids on succinic acid production has been studied very little to date. In this work, we studied the production of succinic acid by different strains of non-*Saccharomyces* and *Saccharomyces* yeasts during AF in synthetic must, and the influence of the addition of GABA or glutamic acid or a combination of both. The results showed that succinic acid can be produced by non-*Saccharomyces* yeasts with values in the range of 0.2–0.4 g/L. Moreover, the addition of GABA or glutamic acid can increase the concentration of succinic acid produced by some strains to almost 100 mg/L more than the control, while other strains produce less. Consequently, higher succinic acid production by non-*Saccharomyces* yeast in coinoculated fermentations with *S. cerevisiae* strains could represent a risk of inhibiting *Oenococcus oeni* and therefore the MLF.

Keywords

GABA; Glutamic acid; Non-*Saccharomyces*; Succinic acid; Yeast; Wine

Introduction

Winemaking is a complex process that usually involves two important microbiological steps: alcoholic fermentation (AF) carried out by yeasts, which produce ethanol and carbon dioxide by glucose breakdown (Fleet 1998; Ribéreau et al. 2006), and malolactic fermentation (MLF) carried out by lactic acid bacteria (LAB), where L-malic acid is decarboxylated to produce L-lactic acid (Bartowsky 2005; Liu 2002). Besides ethanol and CO_2, yeasts produce other compounds linked to quality and mouthfeel perception, such as polyols, esters and other alcohols (Bartowsky and Pretorius 2009; Dicks et al. 1995; Fleet 2003; Styger et al. 2011). Nonetheless, other compounds such as medium-chain fatty acids (MCFA) and organic acids are also produced (Balmaseda et al. 2018; Guilloux-Benatier et al. 1998).

Succinic acid is an organic acid produced by yeasts during the early stages of alcoholic fermentation (De Klerk 2010) and acts as an intermediary in important metabolic pathways, such as the tricarboxylic acid cycle (TCA), glyoxylic acid shunt, methylcitric acid cycle and GABA shunt (Figure 1). Moreover, this acid can improve the sensory wine properties due to the increase in fruity aromatic esters such as methyl-succinate, ethyl-succinate and diethyl succinate (Jordán et al. 2002).

Saccharomyces cerevisiae strains are known to produce succinic acid at levels of 200 mg/L to 2 g/L (Coulter and Pretorius 2007; De Klerk 2010; Zhu et al. 2020). Non-*Saccharomyces* yeasts can also produce succinic acid, usually in a range between 0.3 and 0.95 g/L (Ciani and Maccarelli 1997; Escribano et al. 2018; Zhu et al. 2020). Moreover, Contreras et al. (2014) found that some strains of *Metschnikowia pulcherrima*, *Schizosaccharomyces malidevorans* and *Candida stellata* produced 1 to 2 g/L of succinic acid.

In this sense, we must consider that there is an increasing oenological interest in these non-*Saccharomyces* yeasts (Padilla et al. 2016) due to the production of new aromas (Belda et al. 2017), and currently, an increasing number of cellars are using them, often inoculating with them prior to *S. cerevisiae*.

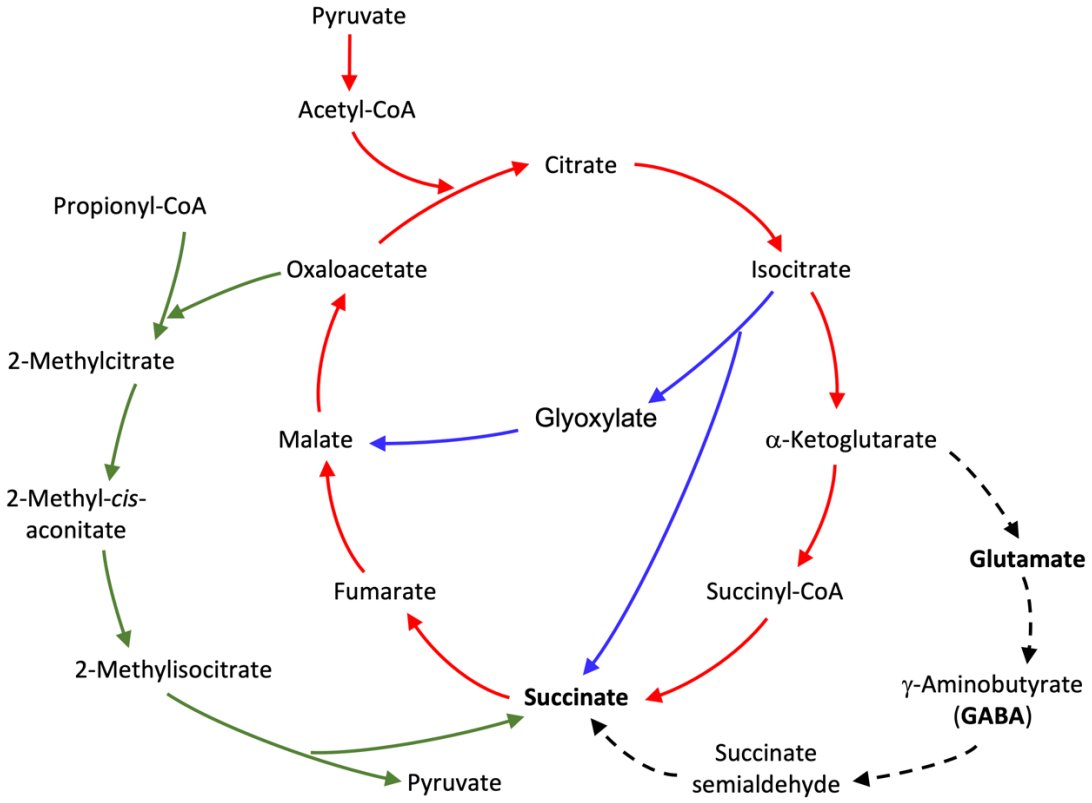

Figure 1. Metabolic pathways of yeasts and many other organisms where succinate acts as an intermediary: tricarboxylic acid cycle (TCA) (red lines), glyoxylate shunt (blue lines), methylcitric acid cycle (green lines) and GABA shunt (black dashed lines). Adapted from De Klerk (2010) and Freitas e Silva et al. (2020).

Succinic acid can have a positive impact on the general quality of wine, as mentioned above, due to ester formation. In contrast, this acid can have a negative impact on LAB development and consequently on MLF performance because it has been reported that it can act as an inhibitor of L-malic decarboxylation in LAB (Lonvaud-Funel and Strasser de Saad 1982; Son et al. 2009). Moreover, succinic acid can inhibit MLF performance and *Oenococcus oeni* strains (Caridi and Corte 1997; Torres-Guardado et al. 2022). There are many factors that influence succinic acid production by yeast, such as temperature, assimilable nitrogen, oxygen and amino acids (De Klerk 2010). Indeed, succinic acid levels in red wines have been reported to be twice that of white wines (Coulter and Pretorius 2007). This difference could be explained

by the increase in GABA in the grape must due to maceration of red grapes, which could favour succinic acid production by yeast (De Klerk 2010).

Therefore, due to the importance of succinic acid production by yeasts, the increasing use of non-*Saccharomyces* strains and the impact of succinic acid on MLF, the aim of this work was to evaluate the influence of GABA and glutamic acid supplementation on succinic acid production by non-*Saccharomyces* yeasts in synthetic must.

Materials and Methods

Yeast strains and culture conditions

Twelve non-*Saccharomyces* strains and two *S. cerevisiae* strains were used in this study (Table 1). Before the fermentation assays, 10^6 cells/mL of each strain were precultured in 12 mL YPD broth (20 g/L of dextrose, 20 g/L of peptone and 10 g/L of yeast extract (Cultimed, Barcelona, Spain)). Yeasts were incubated at 28 °C for 72 h at least twice before experimental use.

Fermentations

Alcoholic fermentations were carried out in synthetic must recently developed by Ruiz-de-Villa et al. (2023) containing 110 g/L glucose, 110 g/L fructose, 5 g/L L-tartaric acid, 2 g/L L-malic acid, 0.5 g/L citric acid, 1.7 g/L yeast nitrogen base w/o amino acids, 50 mg/L NH_4Cl and 1.505 g/L amino acid stock at pH 3.5. This amino acid stock contained, among others, 50 mg/L GABA and 210 mg/L glutamic acid. In addition to this control, three different treatments were performed, where the content of these two amino acids was increased: 100 mg/L GABA, 420 mg/L glutamic acid, and 100 mg/L GABA + 420 mg/L glutamic acid. All fermentations were performed in triplicate in 100 mL of synthetic must in 150 mL bottles at 22 °C. The different yeasts were inoculated at $1x10^6$ cells/mL. Samples were taken at 48 h

to evaluate the succinic acid production and sugar consumption rate, following strategy of Martín-García et al. (2020).

Chemical analyses

The succinic acid content in the final samples of the AF trials was determined by high-performance liquid chromatography (HPLC) following the method of Zhu et al. (2020). All samples were previously filtered by injecting them through 0.2 μm Captiva filters (Agilent Technologies, Santa Clara CA, USA). The chromatograms were analysed using Agilent ChemStation Plus software.

The sugar (glucose + fructose) consumption rate was calculated from the decrease in density (g/cm^3 at 20 °C) from 0 to 48 h and converting it to a sugar concentration, following the recommended conversion of OIV (2022).

Table 1. Yeast strains used in this study.

Strain code	Species	Source	Strain full name
ScK1	*Saccharomyces cerevisiae*	Lallemand Inc.	Lalvin ICV K1 Marquée™
ScQA23	*Saccharomyces cerevisiae*	Lallemand Inc.	Lalvin QA23™
TdBio	*Torulaspora delbrueckii*	Lallemand Inc.	Level 2 Biodiva™
TdNS	*Torulaspora delbrueckii*	Agrovin S.A.	Viniferm NSTD
TdZym	*Torulaspora delbrueckii*	Laffort ®	Zymaflore® Alpha
Td13135	*Torulaspora delbrueckii*	Padilla et al. 2017	CECT 13135
MpFla	*Metschnikowia pulcherrima*	Lallemand Inc.	Flavia™
Mp13131	*Metschnikowia pulcherrima*	Padilla et al. 2017	CECT 13131
Hu10389	*Hanseniaspora uvarum*	CECT	CECT 10389
Hu13130	*Hanseniaspora uvarum*	Padilla et al. 2017	CECT 13130
HvT02	*Hanseniaspora vineae*	Medina et al. 2018	T02/05F
Hv1471	*Hanseniaspora vineae*	CECT	CECT 1471
Sb11109	*Starmerella bacillaris*	CECT	CECT 11109
Sb13129	*Starmerella bacillaris*	Padilla et al. 2017	CECT 13129

* CECT: Spanish Type Culture Collection

Results and Discussion

Succinic acid production by wine yeasts

The production of succinic acid was evaluated in control treatments (the synthetic must contain 50 mg/L GABA and 210 mg/L glutamic acid) at 48 h to determine the total amount produced by every strain. Although the AF were not still finished at 48 h (data not shown), we chose this sampling time because it is known that most succinate is formed from pyruvate by yeasts in the first days of AF (Ribéreau-Gayon et al. 2006), and also because the usual cellar strategy when using non-*Saccharomyces* yeasts is inoculating them first, followed by inoculation with *S. cerevisiae* after 48 h (Martín-García et al. 2020). The results showed that the TdZym and ScK1 strains produced approximately 0.34 g/L (Figure 2), the highest concentration of succinic acid compared with the other strains, in which the HvT02 and ScQA23 strains produced approximately 0.22 g/L. However, for the other strains used in this experiment, there were no significant differences in terms of succinic acid production, ranging between 0.23-0.30 g/L. Other studies reported succinic acid in synthetic must that ranged between 0.3-2.0 g/L (Ciani and Maccarelli 1997; Contreras et al. 2014).

As seen, in some cases there were significant differences in succinic acid production among strains of the same species (Figure 2). This variability is probably related with the wide biodiversity found for other metabolic characteristics in wine yeasts, as has been mentioned by other authors (Romano et al 2008). Variability also depends on whether it is a commercial strain or an isolated one. Obviously, this subject requires further investigation, looking for it in several strains of each species, taking in account their source and isolation place, and looking for possible intraspecific genetic differences.

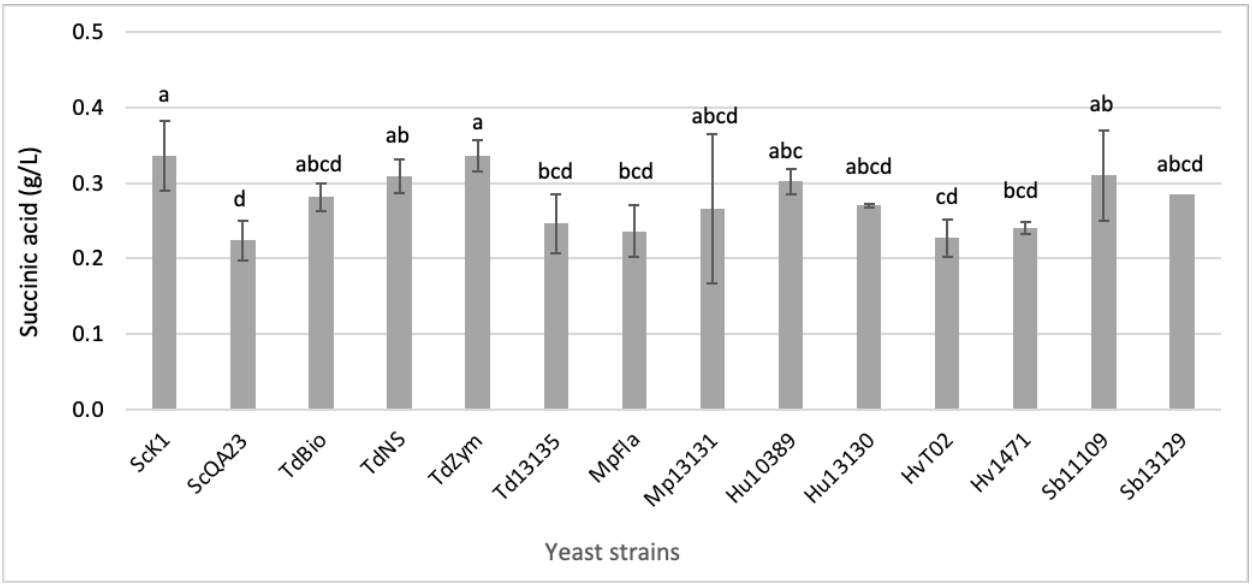

Figure 2. Succinic acid production by different yeast strains (see codes in Table 1) in synthetic must. Values shown are the mean and standard deviation (SD) of triplicates. Different subscript letters ($^{a\text{-}d}$) indicate that yeast treatments are significantly different at $p \leq 0.05$ according to Tukey's post hoc comparison test.

Considering the differences among strains, we also calculated the average values of this acid production for each species (Table 2) in order to see possible significant differences between species. As seen Table 2, two non-*Saccharomyces* species, *H. uvarum* and *S. bacillaris,* produced significantly more succinic acid than the others, with values higher than 0.3 g/L, while *T. delbrueckii* and *M. pulcherrima* produced succinic acid levels like those of *S. cerevisiae*. This is an interestingly positive result since *T. delbrueckii* and *M. pulcherrima* are currently the most used non-*Saccharomyces* species in winemaking (Balmaseda et al. 2021). Escribano et al. (2018) found similar values of succinic acid produced by *T. delbrueckii* in real wines but higher values for *M. pulcherrima* —around 0.62 g/L— and slightly higher values for *S. cerevisiae* —around 0.45 g/L.

Table 2. Succinic acid production by different yeast species in synthetic must. Values shown are the mean values for different strains that were combined for each species. [a-c] Values are significantly different at $p \leq 0.05$ according to a Anova Fisher's least significant difference test.

Species	Succinic acid (g/L)
Saccharomyces cerevisiae	0.252 b
Torulaspora delbrueckii	0.267 b
Metschnikowia pulcherrima	0.269 b
Hanseniaspora uvarum	0.300 a
Hanseniaspora vineae	0.220 c
Starmerella bacillaris	0.315 a

Additionally, it must be considered that in some cases the AF period can be longer than 48 h, resulting in higher amounts of succinic acid produced by non-*Saccharomyces* yeasts. Moreover, the succinic acid levels produced before the sequential inoculation of *S. cerevisiae* could be increased through AF after its inoculation, since sometimes *S. cerevisiae* by itself can produce until 2 g/L of succinic acid (Caridi and Corte 1997; De Klerk 2010).

Consequently, succinic acid production by using non-*Saccharomyces* yeasts in combination with *S. cerevisiae* could represent a risk of stalling MLF because this acid can act as an inhibitor of *O. oeni*. In a previous work, we showed that this acid can exert an inhibitory effect on *O. oeni*, and consequently on MLF development, at concentrations higher than 1 g/L and this inhibition was higher at pH 3.5 than at pH 4.0. This negative effect was more evident when succinic acid was at a molar concentration higher than that of L-malic acid (Torres-Guardado et al. 2022).

Effect of GABA and glutamic acid addition on sugar consumption by yeast strains

In addition to examining succinic acid production, we also evaluated the influence of GABA, glutamic acid, and a combination of the two, when added to the synthetic must, on the development of AF by the different yeast strains. For that purpose, we used the sugar consumption rate obtained during the first 48 h (Table 3). The addition of GABA and glutamic acid exerted slight inhibition on the sugar consumption rate in most strains. However, in some strains, such as TdNS, MpFla, Mp13131, HvT02 and Sb11109, the sugar consumption rate was not affected relative to the control. On the other hand, only two strains (ScK1 and Hv1471) presented increased sugar consumption rate upon amino acid addition to the medium, with a significant increase in the sugar consumption rate of almost 0.1 mg/mL/h (Table 3). Considering the species, we see an inhibition of sugar consumption in most *T. delbrueckii* and *H. uvarum* strains when GABA and/or glutamic acid were added, but there was no variation of sugar consumption in the *M. pulcherrima* strains.

Nitrogen assimilation in yeasts is controlled by nitrogen catabolite repression (NCR) and some species or strains prefer to assimilate N sources in different orders (Gobert et al. 2017). Therefore, we supposed that the decrease in the sugar consumption rate of some strains when concentrations of GABA and glutamic acid were added, can be related as a consequence of regulation of nitrogen metabolism. This could have affected other major pathways, such as sugar and sulphur metabolism, which can led to the production of active intermediates, flavours and end-products (Hirst and Richter, 2016).

Table 3. Sugar consumption rate by different yeast strains (see Table 1 for codes) in synthetic must with increased concentration of GABA, glutamic acid (Glut) or a combination of both. Values shown are the mean and standard deviation (SD) of triplicates.

	Sugar consumption rate (mg/mL/h)			
Strain	Control *	100 mg/L GABA	420 mg/L Glut	100 mg/L GABA and 420 mg/L Glut
ScK1	0.771 ± 0.026 b	0.938 ± 0.022 a	0.938 ± 0.022 a	0.972 ± 0.022 a
ScQA23	0.656 ± 0.022 a	0.486 ± 0.018 b	0.542 ± 0.018 b	0.549 ± 0.018 b
TdBio	0.552 ± 0.022 a	0.340 ± 0.018 b	0.333 ± 0.018 b	0.354 ± 0.018 b
TdNS	0.448 ± 0.022 a	0.417 ± 0.018 a	0.410 ± 0.018 a	0.389 ± 0.018 a
TdZym	0.510 ± 0.013 a	0.424 ± 0.011 b	0.396 ± 0.011 b	0.403 ± 0.011 b
Td13135	0.385 ± 0.013 a	0.326 ± 0.011 b	0.319 ± 0.011 ab	0.333 ± 0.011 b
MpFla	0.260 ± 0.010 a	0.250 ± 0.008 a	0.257 ± 0.008 a	0.271 ± 0.008 a
Mp13131	0.354 ± 0.024 a	0.292 ± 0.020 a	0.278 ± 0.020 a	0.285 ± 0.020 a
Hu10389	0.344 ± 0.020 a	0.250 ± 0.017 b	0.229 ± 0.017 b	0.257 ± 0.017 b
Hu13130	0.344 ± 0.013 a	0.250 ± 0.011 b	0.222 ± 0.011 b	0.264 ± 0.011 b
HvT02	0.542 ± 0.032 a	0.549 ± 0.027 a	0.507 ± 0.027 a	0.535 ± 0.027 a
Hv1471	0.427 ± 0.011 b	0.514 ± 0.009 a	0.521 ± 0.009 a	0.535 ± 0.009 a
Sb11109	0.354 ± 0.017 a	0.368 ± 0.014 a	0.333 ± 0.014 a	0.340 ± 0.014 a
Sb13129	0.417 ± 0.011 a	0.361 ± 0.009 b	0.347 ± 0.009 b	0.361 ± 0.009 b

[a-b] Values for each strain in the same row followed by different letters are significantly different at $p \leq 0.05$ according to a Tukey post-hoc test.

* Control was synthetic must containing 50 mg/L GABA and 210 mg/L glutamic acid.

In contrast, it is remarkable that ScK1 strain showed a significant increase in the C-source consumption rate (Table 3). While other *non-Saccharomyces* yeasts were not affected by increased concentrations of GABA and glutamic acid. Therefore, there is a strain-dependent effect, since variability occur within yeast species, as indicated by Kemsawasd et al. (2015).

Effect of GABA and glutamic acid on succinic acid production

The addition of GABA or glutamic acid or both can differentially affect succinic acid production in different yeast strains (Table 4). For example, in contrast with the control assay (synthetic must

containing 50 mg/L GABA and 210 mg/L glutamic acid), in the experiments where glutamic acid was increased, TdBio, HvT02 and Hv1471 produced almost 50 mg/L more succinic acid. Moreover, this effect was also observed when GABA was added for the same strains HvT02 and Hv1471, which produced approximately 80 mg/L more succinic acid than the control. In contrast, TdBio, Hu10389, Hu13130 and Sb11109 showed a decrease of approximately 50-150 mg/L when both GABA and glutamic acid were added, and this effect was also observed for Hu13130 in the medium where only GABA was increased, which suggests that GABA is responsible for the decrease in succinic acid production.

This effect is likely due to GABA synthesis being an irreversible enzymatic decarboxylation of glutamate (Zhang et al. 2022), catalysed by glutamate decarboxylase (GAD), using pyridoxal phosphate (PLP) as a cofactor (Perpetuini et al. 2020; Yuan et al. 2020) (Figure 1). Thereafter, GABA aminotransferase (Uga1p) transamination produces succinate semialdehyde (SSA), and SSA dehydrogenase (Uga2p) oxidizes SSA to succinate (Pérez et al. 2022). Therefore, although GABA acts as an intermediary in succinic acid production by glutamate, some yeasts produce less succinic acid in media supplemented with GABA than in media supplemented with glutamic acid. These results suggest that some yeast strains decarboxylate glutamate to produce GABA instead of oxidizing it directly. Nonetheless, it is important to note that in some strains the increase in GABA and glutamic acid encouraged succinic acid production.

Table 4. Succinic acid production by different yeast strains in synthetic must with increased concentration of GABA, glutamic acid (Glut) or a combination of both. Values shown are the mean and standard deviation (SD) of triplicates.

	Succinic acid (g/L)			
Strain	Control *	100 mg/L GABA	420 mg/L Glut	100 mg/L GABA and 420 mg/L Glut
ScK1	0.338 ± 0.016 a	0.304 ± 0.013 a	0.301 ± 0.013 a	0.297 ± 0.013 a
ScQA23	0.227 ± 0.029 ab	0.155 ± 0.029 b	0.278 ± 0.029 a	0.192 ± 0.029 ab
TdBio	0.296 ± 0.014 b	0.207 ± 0.012 c	0.344 ± 0.012 a	0.208 ± 0.012 c
TdNS	0.299 ± 0.045 a	0.259 ± 0.037 a	0.327 ± 0.037 a	0.248 ± 0.037 a
TdZym	0.347 ± 0.034 ab	0.347 ± 0.028 ab	0.373 ± 0.028 a	0.253 ± 0.028 b
Td13135	0.235 ± 0.027 a	0.173 ± 0.022 a	0.183 ± 0.022 a	0.185 ± 0.022 a
MpFla	0.237 ± 0.017 a	0.266 ± 0.014 a	0.277 ± 0.014 a	0.258 ± 0.014 a
Mp13131	0.258 ± 0.014 b	0.293 ± 0.011 ab	0.281 ± 0.011 ab	0.313 ± 0.011 a
Hu10389	0.335 ± 0.033 a	0.383 ± 0.027 a	0.157 ± 0.027 b	0.166 ± 0.027 b
Hu13130	0.263 ± 0.023 a	0.177 ± 0.018 b	0.146 ± 0.018 b	0.161 ± 0.018 b
HvT02	0.227 ± 0.017 b	0.307 ± 0.014 a	0.299 ± 0.014 a	0.293 ± 0.014 a
Hv1471	0.245 ± 0.016 b	0.317 ± 0.013 a	0.301 ± 0.013 a	0.306 ± 0.013 a
Sb11109	0.311 ± 0.013 a	0.313 ± 0.010 a	0.307 ± 0.010 a	0.268 ± 0.010 b
Sb13129	0.302 ± 0.021 a	0.359 ± 0.017 a	0.337 ± 0.017 a	0.337 ± 0.017 a

[a-c] Values for each strain in the same row followed by different letters are significantly different at $p \leq 0.05$ according to a Tukey post-hoc test.

* Control was synthetic must containing 50 mg/L GABA and 210 mg/L glutamic acid.

Conclusions

Succinic acid production by non-*Saccharomyces* and *S. cerevisiae* wine yeasts and the influence of GABA and glutamic acid were evaluated. The results show that, considering average values for species, *H. uvarum* and *S. bacillaris* produced significantly more succinic acid than the others, with values higher than 0.3 g/L, while *T. delbrueckii* and *M. pulcherrima* produce amounts of succinic acid like those of *S. cerevisiae*. However, succinic acid production by yeasts is clearly a strain-dependent effect. Nonetheless, a strain-dependent effect was also observed when concentrations of GABA and glutamic acid were

increased in the media, where some strains produced higher amounts of succinic acid in the presence of more quantities of GABA or glutamic acid, while others produced less succinic acid, similar to the mixed GABA-glutamic acid treatment.

Consequently, higher succinic acid production by non-*Saccharomyces* yeast in sequential fermentations with *S. cerevisiae* strains —which are also able to produce succinic acid— could represent a risk of stalling MLF because succinic acid can act as an inhibitor of *O. oeni*.

Further research is necessary to understand the mechanisms of promotion and inhibition of succinic acid production by GABA and glutamic acid in yeast. Besides research in synthetic must like this study, further research is needed also in grape must and in real conditions of winemaking, in spite of its variability. Moreover, it is also necessary to study the best pair of strains in coinoculated fermentations to avoid higher amounts of succinic acid.

Declarations

Author's Contribution

The design of the work was done by all authors. Rafael Torres-Guardado did the experimental work. Rafael Torres-Guardado, Albert Bordons and Cristina Reguant wrote the main manuscript text. Nicolas Rozès and Braulio Esteve-Zarzoso contribute in several parts of the text and some Tables. All authors reviewed the manuscript.

Competing interests

The authors declare that they have no conflict of interest, and that this work does not contain any financial or non-financial competing interests.

Availability of Data and Materials

If someone wants to request the data, the first author Rafael Torres-Guardado can be contacted.

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Compliance with Ethical Standards

This work complies with ethical standards. This article does not contain any studies with human participants or animals performed by any of the authors.

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4. Discusión

La fermentación maloláctica (FML) es un proceso que usualmente se realiza al finalizar la FA y mayoritariamente en vinos tintos y en algunos vinos blancos con elevada acidez. Esta segunda fermentación es realizada por BAL, principalmente por *Oenococcus oeni,* la cual al inicio del proceso de vinificación se encuentra en una población de entre 10^3-10^4 UFC/mL y que posteriormente aumenta para iniciar la FML (Fleet, 2003). Al inicio del proceso de vinificación es posible encontrar una diversidad de microrganismos como levaduras, bacterias y hongos los cuales son habitantes naturales del ambiente vitivinícola y es posible encontrarlas en el viñedo, en la piel de la uva y en la maquinaria de la bodega enológica (Beltran et al., 2002; Franquès et al., 2017). Esta diversidad microbiana puede imprimir distintivos locales y estilo al vino ya que establecen dinámicas e interacciones microbianas que inciden en la calidad general del vino (Esteve-Zarzoso et al., 2000). No obstante, esta diversidad microbiana es capaz de generar compuestos relacionados con efectos de inhibición como compuestos proteicos (Comitini et al., 2005), ethanol, SO_2 (Lemaresquier, 1987), ácidos grasos de cadena media (Lafon-Lafourcade et al., 1984) y ácidos orgánicos (Balmaseda et al., 2018) principalmente producidos por levaduras. Sin embargo, el impacto del ácido succínico no había sido estudiado desde hace 40 años (Lonvaud-Funel and Strasser de Saad, 1982), por lo tanto en esta tesis se ha procedido a estudiar el impacto de este ácido orgánico sobre el desarrollo de *O. oeni* y la FML. También se ha evaluado la producción del ácido succínico con diferentes levaduras pertenecientes al grupo de las no-*Saccharomyces* y algunas cepas *S. cerevisiae,* debido al efecto acumulativo que puede resultar de fermentaciones alcohólicas simultáneas y secuenciales.

Como un primer acercamiento se evaluó la influencia del ácido succínico sobre el desarrollo de *O. oeni* y la FML (**Capitulo 1**). El estudio de la influencia del ácido succínico fue importante por la evaluación del efecto que ejercen las diferentes concentraciones de ácido succínico sobre *O. oeni* y el desarrollo de

la FML, así como la evaluación de la respuesta fisiológica de algunas cepas frente a la presencia del ácido succínico.

De manera seguida y complementaria era necesario estudiar el efecto de inhibición del ácido succínico sobre diferentes cepas de *O. oeni* (**Capítulo 2**). Por lo tanto, se realizó la evaluación de dicho efecto sobre 15 cepas de diferentes orígenes. Además, con base a los resultados obtenidos en el **(Capitulo 1)** donde se observa una sobre expresión de genes relacionados con proteinas de estrés, se realizó el estudio de analisis de transcriptoma global de genes en la cepa PSU-1 **(Capítulo 3)**. También se evaluó la producción de ácido succínico por levaduras (**Capítulo 4**), principalmente por cepas del grupo de las no-*Saccharomyces*. En relación con ello, también era necesario evaluar la influencia de algunos aminoácidos sobre la producción de este ácido orgánico.

El efecto del ácido succínico sobre la FML y el desarrollo de *O. oeni* se evaluó por fermentaciones (**Capítulo 2**), cultivo y ensayos de resting cells (**Capítulo 1**). Los resultados indican que el efecto del ácido succínico sobre el desarrollo de la FML está sujeto a la concentración al pH y también a la respuesta fisiológica de algunas cepas para hacer frente a este ácido orgánico (**Capítulo 1**). Los resultados muestran que el crecimiento de algunas cepas se reduce en presencia de una concentración de 2 g/L de ácido succínico. Asimismo, este efecto de inhibición también ha sido observado sobre la fermentación maloláctica. Sin embargo, no es un efecto generalizado en todas las cepas, puesto que en algunas de ellas se observó que en concentraciones menores a los 0.5 g/L puede representar un efecto benéfico por el incremento en la velocidad de consumo del ácido L-málico. Por otra parte, el análisis de transcripción muestra que algunas cepas son capaces de sobre expresar genes relacionados con la síntesis de proteínas de estrés y actividad maloláctica, adoptando esta estrategia para hacer frente al efecto de inhibición del ácido succínico. Además, los resultados muestran que el ácido succínico no ejerce efecto de inhibición sobre la transcripción de estos genes. Por lo que la inhibición en la FML puede ser causada por una acidificación intracelular y posible competencia con el ácido L-málico a nivel enzimático (**Capítulo 1**).

No obstante, el efecto de inhibición también pudo verse observado en otras cepas, donde este ácido orgánico llega a inhibir la FML en un promedio de 50% al final del periodo de fermentación con una concentración de 2 g/L, lo cual es causado por una disminución en la velocidad de consumo del ácido L-málico. Con base a estos resultados, el ácido succínico puede inhibir el desarrollo de la FML con diferentes cepas en este nivel de concentración de 2 g/L (**Capítulo 2**). No obstante, es fundamental considerar que el efecto de inhibición de este ácido no solo depende de su concentración, sino que también hay factores como el pH y el ratio de proporción entre el ácido L-málico y succínico que influyen sobre su acción (**Capitulo 1**). Es importante señalar que el efecto de inhibición del ácido succínico sobre *O. oeni* y el desarrollo de la FML puede tener su origen en el impacto que causa a nivel de expresión génica. En el análisis de transcriptoma (**Capítulo 3**), se registró un aumento en el tiempo de fermentación en presencia de 2 g/L de ácido succínico, confirmando resultados obtenidos de manera previa en (**Capítulo 1**). Además, fue observado que este ácido afecta de manera global al transcriptoma de la cepa PSU-1, mostrando una disminución en la expresión de genes relacionados con metabolismo de nucleótidos, traducción, transporte y metabolismo de aminoácidos. También fue observada la sobreexpresión de genes relacionados con el transporte y metabolismo de carbohidratos, mecanismos de defensa y metabolismo de compuestos inorgánicos. Estos resultados evidencian el notable impacto que tendría el ácido succínico sobre algunas funciones celulares esenciales en *O. oeni* (**Capítulo 3**).

En vista de que el ácido succínico es un inhibidor de la FML y el desarrollo de *O. oeni* (**Capitulo 1, 2 y 3**), fue sustancial evaluar la producción e influencia de aminoácidos como GABA y ácido glutámico en levaduras no-*Saccharomyces* (**Capítulo 4**).

Los resultados obtenidos muestran que la producción de ácido succínico por parte de levaduras no-*Saccharomyces* están sujetos a un efecto cepa dependiente. No obstante, entre las levaduras del grupo no-*Saccharomyces*, se registró que las levaduras que producen una mayor cantidad de ácido succínico son *H. uvarum* y *S. bacillaris* con valores mayores a 0.3 g/L. En contraste, *T. delbrueckii* y *M.*

pulcherrima producen ácido succínico con valores similares a *S. cerevisiae* de 0.227 g/L. Además, se observó que estos valores pueden incrementarse bajo suplementación de GABA y ácido glutámico, por lo que la interacción de factores de suplementación y selección de cepas de levaduras, podrían incrementar el contenido de ácido succcínico. No obstante, se debe considerar que el aumento de este ácido puede ser aún más elevado en fermentaciones secuenciales, donde la producción podría ser acumulativa por la participación de levaduras no-*Saccharomyces* y *Saccharomyces* **(Capítulo 4)**. Notablemente, el ácido succínico tiene un impacto sobre el desarrollo de la FML y *O. oeni* (**Capitulo 1, 2 y 3**), por lo que su acción debe de clasificarse y catalogarse de acuerdo al marco de interacción propuesto en este trabajo de tesis (**Anexo 1**). En este sentido, la interacción y el efecto que ejerce el ácido succínico sobre el desarrollo de *O. oeni* y la fermentación maloláctica, se clasifica como amensalista. Es importante señalar que las interacciones negativas se dividen en amensalistas y antagonistas, teniendo como diferencia el origen de los productos involucrados en la inhibición. Las interacciones amensalistas se establecen por la inhibición que causa un compuesto de metabolismo primario hacia otro organismo, por lo que en el caso de la inhibición del ácido succínico sobre *O. oeni*, la interacción que se establece es de carácter amensalista.

Finalmente, es necesario realizar más investigaciones relacionadas con el ácido succínico en vinos, ya que como se ha explicado de manera previa su producción es influenciada por parte de algunas especies de levaduras. No obstante, el empleo de estas levaduras a nivel bodega y los índices de producción de ácido succínico deben ser evaluados en condiciones de bodega, puesto que como consecuencia del calentamiento global, la concentración de L-málico ha disminuído. Esto en suma a las concentraciones de ácido succínico podrían causar inhibicion en el desarrollo de *O. oeni* y generar un potencial riesgo de parada de fermentación de la FML.

5. Conclusiones

1. El ácido succínico puede inhibir la fermentación malolática y el desarrollo de *O. oeni* con concentraciones superiores a 1g/L.

2. Las concentraciones de ácido succínico inferiores a 0.5 g/L, pueden ser benefiociosas para el incremento de la velocidad de consumo de ácido L- málico.

3. La proporción de la ratio entre el ácido L-málico y succínico influye sobre el efecto de promoción o inhibición de la FML.

4. El pH como factor fisicoquímico también influye sobre las formas disociadas y no disociadas del ácido succínico, generando así también un efecto de inhibición según las condiciones de pH en el medio.

5. El ácido succínico es inhibidor de la FML en condiciones cercanas a 2 g/L en diferentes cepas de *O. oeni*, lo cual es un valor que debe determinarse antes de comenzar la FML.

6. La producción de ácido succínico por parte de levaduras está asociado a factores como el efecto cepa-dependiente y la presencia de aminoácidos como GABA y ácido glutámico.

7. El ácido succínico producido por levaduras durante la fermentación, puede dar origen a una interacción amensalista con *O. oeni*, originando un menor desarrollo e impactando el desempeño de la fermentación maloláctica.

8. El efecto que el ácido succínico ejerce sobre *O. oeni* puede ser diferente en algunas cepas, puesto que se ha observado un efecto cepa-dependiente.

9. La presencia de ácido succínico (2 g/L) modifica notablemente el transcriptoma de *O. oeni* PSU-1. Cabe destacar la inhibición de la transcripción de genes asociados al metabolismo de nucleótidos, traducción, transporte y metabolismo de aminoácidos y la activación de genes relacionados al metabolismo y transporte de carbohidratos, transcripción y mecanismos de defensa.

10. Algunas especies del grupo de las no-*Saccharomyces* pueden producir una mayor cantidad de ácido succínico que cepas de *S. cerevisiae*, concretamente en el caso de *H. uvarum* y *S. bacillaris*.

6. Referencias

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7. ANEXOS

Anexo 1

Microbial Interactions on Alcoholic Beverages

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REVIEW

Microbial interactions in alcoholic beverages

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Abstract
This review examines the different types of interactions between the microorganisms involved in the fermentation processes of alcoholic beverages produced all over the world from cereals or fruit juices. The alcoholic fermentation converting sugars into ethanol is usually carried out by yeasts, mainly *Saccharomyces cerevisiae*, which can grow directly using fruit sugars, such as those in grapes for wine or apples for cider, or on previously hydrolyzed starch of cereals, such as for beers. Some of these beverages, or the worts obtained from cereals, can be distilled to obtain spirits. Besides *S. cerevisiae*, all alcoholic beverages can contain other microorganisms and especially in spontaneous fermentation when starter cultures are not used. These other microbes are mostly lactic acid bacteria and other yeasts—the non-*Saccharomyces* yeasts. The interactions between all these microorganisms are very diverse and complex, as in any natural occurring ecosystem, including food fermentations. To describe them, we have followed a simplified ecological classification of the interactions. The negative ones are amensalism, by which a metabolic product of one species has a negative effect on others, and antagonism, by which one microbe competes directly with others. The positive interactions are commensalism, by which one species has benefits but no apparent effect on others, and synergism, by which there are benefits for all the microbes and also for the final product. The main interactions in alcoholic beverages are between *S. cerevisiae* and non-*Saccharomyces* and between yeasts and lactic acid bacteria. These interactions can be related to metabolites produced by fermentation such as ethanol, or to secondary metabolites such as proteinaceous toxins, or are feed-related, either by competition for nutrients or by benefit from released compounds during yeast autolysis. The positive or negative effects of these interactions on the organoleptic qualities of the final product are also revised. Focusing mainly on the alcoholic beverages produced by spontaneous fermentations, this paper reviews the interactions between the different yeasts and lactic acid bacteria in wine, cider, beer, and in spirits such as tequila, mezcal and cachaça.

Keywords Amensalism · Antagonism · Commensalism · Spirits · Synergism · Wine

Introduction

Fermentation is one of the oldest methods for preserving food, accessing nutrients, detoxifying products, and enhancing flavors (Ivey et al. 2013). It can benefit food technology in a number of ways. For example, lactic acid bacteria (LAB) are involved in many dairy products as cheese and yoghurt, and their fermentation leads to microbiological stability, nutrient availability, and enhanced mouthfeel (Hickey et al. 2015; Othman et al. 2019). Fermented products can also be obtained from foods other than milk such as meat, fish, fruits, cereals, and plants. For example, fruits and cereals are often used to produce alcoholic beverages and the fermentation process focuses not only on microbial stability and nutrient availability but also on increasing the aromatic profile and mouthfeel perceptions. This review studies how alcoholic beverages such as wine, beer cider, and various worts for elaborating spirits are produced by fermenting microbes, mainly yeasts and LAB. We shall put particular emphasis on how these microorganisms interact with each other and their impact on the final product (Clemente-Jiménez et al. 2005; Del Campo et al. 2008; Dysvik et al. 2019).

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Alcoholic beverages

Alcoholic beverages are obtained by fermenting the juice from different fruits or the worts of cereals (Fig. 1). This figure synthesizes in a simplified way the required types of fermentation and the main involved microorganisms in the different beverages when carried out spontaneously. As can be seen, *Saccharomyces cerevisiae,* yeasts non-*Saccharomyces*, LAB, and some acetic acid bacteria are present.

Wine and cider are fermented products obtained from grape and apple juice, respectively. They are mainly fermented by yeasts—which carry out the alcoholic fermentation (AF)—and also very often by LAB, which carry out the malolactic fermentation (MLF), and during the process alcohol, esters, aldehydes, terpenes, and acids are produced (Fleet 2003). Unlike wine and cider, beer is made not from fruit juice but from liquid wort, which is obtained from malt, the germinated grains of cereals such as barley. In malting the starch fraction is partly broken down into soluble sugars by the action of amylolytic enzymes. After the addition of water and hops, sugars of the obtained wort are then available by the yeasts, which can ferment it (De Roos and De Vuyst 2019; Cordes et al. 2021).

On the other hand, spirits are obtained by distilling fermented must or wort. Their quality is influenced by the crop material, the tanks, the process (industrial or traditional), and ageing (Wiśniewska et al. 2016).

Whatever the type of alcoholic product and elaboration procedure they are made, they are all obtained through a microbiological process, in which yeast and bacteria are responsible for the aromatic profile and quality achieved during fermentation. For this reason, several reports have described the effect of using mixed fermentations for producing beverages and spirits, with particular attention to the aroma, general quality, and microbial dynamics (Azzolini et al. 2010; Izquierdo et al. 2014).

Wine

Several species of microorganisms of indigenous yeasts and LAB are known to be present during spontaneous fermentation of grape must to wine (Fig. 1). In the early steps, the AF is carried out by non-*Saccharomyces* yeasts which contribute to the organoleptic profile and general quality of wine, such as *Hanseniaspora*, *Torulaspora*, *Metschnikowia*, *Pichia, Schizosaccharomyces*, *Starmerella*, and *Lachancea* (Fleet et al. 1984; Beltran et al. 2002; Clemente-Jiménez et al. 2005; Jolly et al. 2014; Benito et al. 2019; Binati et al. 2020; Tufariello et al. 2021). During fermentation, the

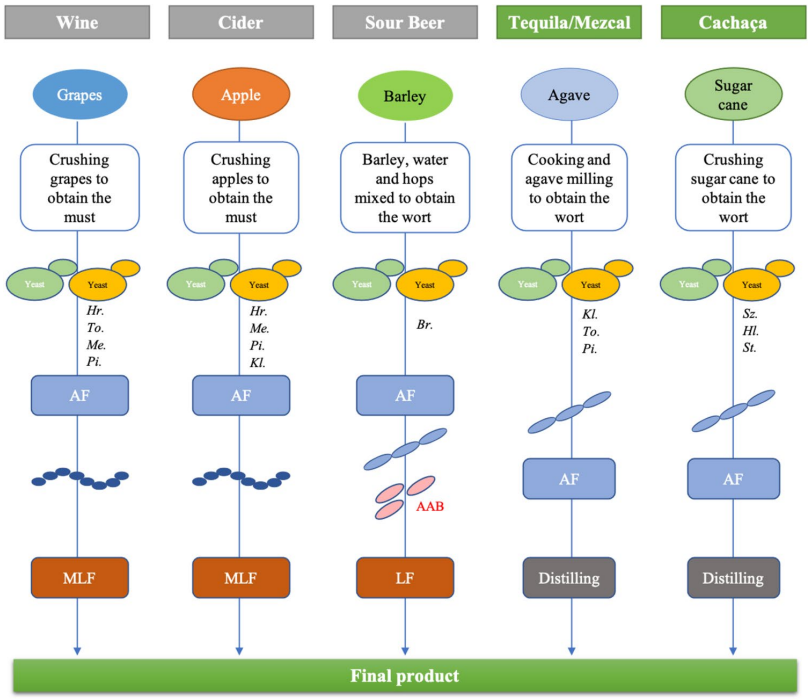

Fig. 1 Scheme of fermentation processes to produce the alcoholic beverages and spirits treated in this work. The main microorganisms (see the text) are shown: Yeasts *Saccharomyces cerevisiae* (green) and non-*Saccharomyces* (orange) such as *Hanseniaspora* (*Hr.*), *Torulaspora* (*To.*), *Metschnikowia* (*Me.*), *Pichia* (*Pi.*), *Kluyveromyces* (*Kl.*), *Brettanomyces* (*Br.*), *Schizosaccharomyces* (*Sz.*), *Hansenula* (*Hl.*), and *Starmerella* (*St.*); LAB, *Oenococcus oeni* (dark blue cocci shape) and *Lactobacillus* (blue bacilli shape); and AAB, acetic acid bacteria (pink bacilli shape). Malolactic fermentation (MLF), alcoholic fermentation (AF) and lactic fermentation (LF)

increasing ethanol level in the must by *S. cerevisiae* usually inhibits most of the non-*Saccharomyces* species due to their lower ethanol resistance, but some non-*Saccharomyces* such as *T. delbrueckii* are able to tolerate relatively high levels of ethanol (Benito 2018). Finally, *S. cerevisiae* increases its population and consumes all the substrate as consequence of its ethanol resistance. Since many years, most big and middle cellars perform AF with *S. cerevisiae* starter cultures and nowadays there is an increasing interest in inoculating additionally some non-*Saccharomyces* such as *T. delbrueckii* or *M. pulcherrima* (Padilla et al. 2016).

In spontaneous uninoculated winemaking, at the end of AF in most red wines with high acidity, the small population of LAB—mainly *O. oeni*—can grow and produce L-lactic acid from the decarboxylation of L-malic acid, the process known as MLF (Wibowo et al. 1985; Lonvaud-Funel 1999; Liu 2002; Alexandre et al. 2004). Many cellars also use *O. oeni* starters to ensure MLF after AF, and it occasionally can be promoted by simultaneous inoculation of LAB with yeasts in the must (Lerm et al. 2010).

Cider

Cider is produced in countries all over the world, and as with wine, the fermentation process used for traditional cider production involves AF and MLF (Cousin et al. 2017). Natural cider production consists of the spontaneous fermentation of apple juice, in which it is fermented by *Saccharomyces* species and non-*Saccharomyces* yeasts such as *Hanseniaspora*, *Pichia*, *Kluyveromyces*, and *Metschnikowia* (Suárez-Vallés et al. 2007; Dierings et al. 2013). Then, it is transformed by LAB, mainly *Lactobacillus*, *Oenococcus*, *Pediococcus*, and *Leuconostoc* (Sánchez et al. 2012; Puertas et al. 2014), which convert L-malic acid into L-lactic acid (Del Campo et al. 2008). Despite the resemblance of cider fermentation with winemaking, there are significant differences in apple juice, such as the high content of fructose—more than 70% of sugars—(Beech 1972), and a higher content of L-malic acid, which affect the microbial diversity in different ways than in winemaking from grapes. Moreover, cider is eventually aged over-lees in order to improve organoleptic properties and foaming (Antón-Díaz et al. 2016).

Beer

Beer is made by AF of the wort, which consists of a mixture of water, barley, and hops (Fig. 1). Yeasts are predominant in brewing, but other microorganisms, mainly bacteria, can have some significant roles (Bokulich and Bamforth 2013). Depending on the brewing process, beers can be classified in four types: lager, ales, red ales, and sour beer or lambic (De Roos and De Vuyst 2019). Traditional sour beers are produced by spontaneous fermentations involving numerous yeast and bacterial species. One of the traits that separates sour beers from ales and lagers is the high concentration of organic acids such as lactic and acetic acids (Dysvik et al 2020). Besides *Saccharomyces* and LAB—mainly *Lactobacillus*—other microorganisms, such as *Brettanomyces* and acetic acid bacteria (AAB), are also important in sour beer fermentations (Steensels et al 2015). Belgian lambic beer is one of the oldest brewed styles, and it is made by spontaneous fermentation in which LAB and AAB are responsible for its sour taste (Spitaels et al. 2015). Unlike cider and wine, the amounts of L-malic acid are so low in beer that the LAB do not carry out the MLF. However, they can survive by converting residual sugars into lactic acid through the homofermentative fermentation (Vriesekoop et al 2012; De Roos and De Vuyst 2019).

Other alcoholic beverages

Besides wine from grapes and cider from apples, many wines are made from fruits juices, sugarcane juice, palm sap (Dharmadhikari 1996; Steinkraus 1997; Harding 2005) and also from flowers such as dandelion wine from *Taraxacum* (Grauso et al. 2019).

There are also many alcoholic beverages other than beer made from cereals. Some are produced by amylolytic starters. This is the case of sake, which is produced in Japan from rice with the starter koji, a combination of strains of the ascomycetes *Aspergillus oryzae* and *A. sojae* (Tamang et al. 2016). Other very popular beverage is chicha, produced in Andean regions from maize, with involvement of several yeasts and some LAB (Mendoza et al. 2017; Rodrigo-Torres et al. 2019) and which traditionally involved human saliva as the source of amylase. Other alcoholic beverages such as mead or the Ethiopian *tej* are produced from honey (Pereira et al. 2009).

Finally, some alcoholic beverages are produced from other plant parts, such as palm juice and beetroot (Tamang et al. 2016), and especially from the juices of *Agave*, such as the Mexican pulque (Lappe-Oliveras et al. 2008), which is produced by the spontaneous fermentation of the agave's sap (*aguamiel*) (Escalante et al. 2016). It is different from the wort used for preparing by distillation the spirits tequila and mezcal, both of which will be discussed below.

Spirits

Being spirits the distilled products from grains or fruits that have already gone through AF, the microorganisms have a role during this initial phase of AF, but logically not during the distillation phase. Nevertheless, during AF the fermenting microorganisms, mainly yeasts, are responsible of other compounds besides ethanol, such as higher alcohols and

esters, that contribute to the aromatic profile of the distillate (Bovo et al 2014).

Some spirits are obtained by direct distillation of wine, such as brandies (mainly Cognac and Armagnac). In this case, the used base wines have similar characteristics to table wines, and they also are prone to MLF due to LAB present after the AF, which can have influence on brandy quality (Du Plessis et al. 2002). Pisco is a sort of brandy produced in winemaking regions of Peru and Chile.

Several spirits named generally as pomace brandies are obtained from grape pomace, *id est*, the solid remains of grapes after winemaking. The most known are marc, grappa, orujo, bagaceira, and many others in several countries. Although the microbiology of these spirits is scarcely known, it seems that besides yeasts, some heterofermentative lactobacilli and AAB would have a role in the grape pomace preparation and silage (Silva et al 2000).

Whisky is obtained by distillation from a fermented grain mash, which can be malted, and because of that, yeasts and LAB acting in malt whisky fermentations will be similar to beer ones. Nevertheless, the hot water extract of the malt—the wort—is not boiled as in a brewery (Van Beek and Priest 2000). Consequently, LAB persist longer and can affect yeast fermentation in various ways and have an influence on aldehyde, ester, and higher alcohol formation (Geddes and Riffkin 1989).

Gin is the distilled product of fermented grain mash, from barley or other grains, in the presence of juniper berries or other aromatic herbs. Vodka is also obtained from cereal grains and from potatoes. Nowadays, gin and vodka are mostly industrial spirits, where the goal is obtaining a neutral alcohol. The fermentation stage is not important and volatile compounds for these products are negligible. Selected yeast strains are used there, and wild yeast and LAB are a contamination problem (Pauley and Maskell 2017).

On the other hand, the spontaneous fermentation process has been reported particularly in spirits obtained from agave and sugarcane must such as tequila or cachaça, respectively (Ferreira et al. 2011; Dierings et al. 2013; Fernández et al. 2014). As we will see below, several reports describe the microbial species associated with the production of these spirits, the microbial interaction, and their influence on the aromatic profile of the final product during the spontaneous fermentation.

Tequila and mezcal

Tequila and mezcal are the ancient spirits produced in Mexico and made from agave (Arrizon et al. 2006; Núñez-Guerrero et al. 2016). The main difference between them is the agave species used. Mezcal can be made from several *Agave* species, although tequila is made only from *Agave tequilana* Weber blue variety. However, tequila and mezcal production involves very similar steps (Narváez-Zapata et al., 2010). Both begin with the harvesting of the mature *Agave* plants (8–12 years old) known as "pines," which are cut and baked to hydrolyze fructans. After baking, the pines are pressed to extract the wort-containing fructose, glucose and sucrose, then this agave wort is allowed to ferment, and finally it is distilled (Narváez-Zapata et al. 2010; González-Robles et al. 2015). Because of the spontaneous fermentation process, the presence of *S. cerevisiae*, non-*Saccharomyces* species such as *Kluyveromyces marxianus, T. delbrueckii, Pichia kluyveri*, and LAB—mainly *Lactobacillus*, *Leuconostoc*, and *Pediococcus*—has been reported in traditional tequila and mezcal distilleries. They all contribute to the complexity of the product's aroma (Lachance 1995; Narváez-Zapata et al. 2010; Verdugo Valdez et al. 2011). Terpenes are one of the most important aromatic compounds that confer distinctive characteristics to the final product, and most of them are released by microbial enzymes—mostly from yeasts—like β-glucosidase and β-xylosidase (Lappe-Oliveras et al. 2008).

Cachaça

Cachaça is a rum-type spirit from Brazil produced by distilling fermented sugarcane must (Badotti et al. 2014). It is made from crushing freshly cut sugarcane to obtain around 20% (w/w) of juice. After the sugarcane has been crushed, a microbial inoculum is prepared by making a mash with undiluted juice, rice, maize flour, salt, and the addition of lemon or orange juice to adjust the pH, a method known as *fermento caipira*. For 5–7 days, fresh sugarcane juice is added to this inoculum, which becomes a complex microbial population after the incorporation of numerous species of microorganisms during harvesting, transport, and crushing, and the addition of water to adjust the sugar concentration. This complex microbial inoculum is constituted by non-*Saccharomyces* species such as *Schizosaccharomyces pombe*, *Hansenula* spp., and *Starmerella* spp. (previously *Candida* spp.) and bacteria as *Bacillus, Lactobacillus*, and *Leuconostoc*. It is added to open vats to carry out the fermentation. Then, AF is often complete in 24 h; thereafter, distillation takes place in a copper still (Freitas et al. 2001). During this spontaneous fermentation process, non-*Saccharomyces* species, *S. cerevisiae* strains, and LAB species such as *Lactobacillus*, *Lactococcus*, and *Weissella* species have been reported to be present (Freitas et al. 2001; Gomes et al. 2010; Ferreira et al. 2013).

Complexity of microorganism communities in alcoholic beverage processes

It is well known that species or strains of yeasts and LAB are largely responsible for improving the complexity of aroma and other sensorial properties in beverages (Fleet 2003).

However, it has also been reported that some microorganisms have a spoiler effect because they increase the volatile acidity of wine (Brizuela et al. 2019), promote oiliness and ropiness in cider (Puertas et al. 2014), generate acidity and ropiness in beer (Geissler et al. 2016), and increase the amount of acetic acid in cachaça (Ferreira et al. 2011).

To increase aroma complexity and prevent the spoilage of beverages and spirits, various inoculating strategies have been studied, two of which are the co-inoculation of yeast and bacteria at the beginning of fermentation (Azzolini et al. 2010) and sequential inoculation non-*Saccharomyces*/S. *cerevisiae* or yeasts/LAB (Bely et al. 2008; Izquierdo et al. 2014). Whatever the inoculation strategy is, yeasts and bacteria constitute a microbial community in the fermentation process. In fact, these communities are complex, because of the biological, chemical, and physical factors that affect the population dynamics and metabolism (Little et al. 2008).

As described above, the fermentation process is not the result of a single microorganism. To have high-quality products, the presence of various species and/or strains is often required, and the predominance of one species or strain over the others is the result of different types of effect. For this reason, the main aim of this study is to review and classify the microbial interactions in beverages and wort for obtaining spirits and their impact on the final product.

Microbial interactions

From a general point of view, we could classify them in terms of whether the effect of the main microorganism involved on the other one is positive or negative. A positive interaction means that there is a benefit. This type of interaction is often found in food technology. For example, one microorganism can induce changes in the media which improves the growth of another. However, the most studied type of interaction in nature is the negative one, in which one of the two microorganisms grows at the expense of the other. This means that the growth of one microorganism prevents the other from growing. This can be because one of the microorganisms produces an antibiotic compound that kills the other or hinders its growth or by nutrient competition among them.

Interactions can also be neutral, that is to say those in which the growth of one microorganism does not affect the other and vice versa. This means that both microorganisms are totally independent of one another.

So, microbial interactions are not easily classified in terms of the benefit they give. For this reason, Ivey et al. (2013) proposed an alternative way of classifying microbial interactions, based on microbial ecology, following Boddy and Wimpenny (1992): amensalism, antagonism, commensalism, and synergism (Table 1). Moreover, in food technology, the metabolites produced during these interactions can either improve or detract the final sensorial properties of foods.

The sections below discuss different interactions and their effects, on wine fermentations in particular, but also on other beverages and spirits, so as to improve our understanding of these interactions. They can take place between species of the same type of microorganism (yeast-yeast, bacteria-bacteria), between species of different types (yeast-bacteria), and between strains of the same species.

Amensalism

Amensalism is a symbiotic relationship in which a metabolite produced by one microorganism affects the growth of another. These compounds are mainly synthetized and excreted to the medium by the primary metabolism. They are not synthetized to protect the microorganism producer; rather, they are the products of the main metabolism of the compounds present in the medium. In addition to the harsh environment created by the alcoholic fermentation, such yeast by-products as SO_2 (Lemaresquier 1987; Carreté et al. 2002; Wells and Osborne 2011) and medium-chain fatty acids (MCFA) (Lonvaud-Funel et al. 1988; Alexandre et al. 2004) can be toxic to LAB. Of course, ethanol is the most known of the inhibitor compounds in alcoholic beverages, being *S. cerevisiae* the species which produces it the most. Ethanol affects negatively every other microorganism, yeast or bacteria, and in wine it is the main inhibitor for *O.*

Table 1 Main microbial interactions in beverages (adapted from Ivey et al. 2013)

Interaction type	Definition	References
Negative		
Amensalism	Interaction where a metabolic product of one species has a negative effect on other species	(Ivey et al. 2013)
Antagonism	Interaction where one microorganism competes for nutrients or produces inhibiting or killer compounds	(Little et al. 2008)
Positive		
Commensalism	Interaction in which one species benefits from the other without doing it any harm	(Ivey et al. 2013)
Synergism	Interaction between two microbial species, where both get benefit from the other	(Little et al. 2008)

oeni (Nehme et al. 2008). The main functional categories of *O. oeni* genes affected by ethanol are metabolite transport and cell wall and membrane biogenesis (Olguín et al. 2015).

Occasionally, yeasts produce some organic acids that can affect also *O. oeni*. So, cryotolerant *S. cerevisiae* strains have been characterized by a high production of succinic acid (Caridi and Corte 1997), and more recent studies agreed with this inhibition effect on MLF (Son et al. 2009).

These negative effects are not exclusive for products from *S. cerevisiae*, since other yeasts can also produce some of these metabolites. For instance, it has been recently shown that *O. oeni* and MLF are inhibited by the high SO_2 and acetic acid produced by several *Hanseniaspora*, especially *H. uvarum* (Ferrando et al. 2020).

However, the inhibitory effect of *S. cerevisiae* on LAB growth and MLF in wine is yeast strain dependent. While some strains have no effect, others inhibit MLF and the growth of LAB (Comitini et al. 2005). Other compounds cause changes in the cell morphology of *O. oeni*, the typical diplococci shape changing to bacilli shape in wines fermented by amensalist strains (Comitini and Ciani 2007).

Nevertheless, it has also been reported that bacteria can inhibit the growth of yeasts. For instance, the presence of *O. oeni* in co-inoculated fermentations not only affects yeast growth but also induces the up-regulated *S. cerevisiae* gene expression coding for lactate dehydrogenase (*DLD3*), lipid biosynthesis (*OAR1*), amino acids (*DIP5*), hexose (*HXT13*), and ammonia (*MEP1*) transporters (Rossouw et al. 2012). The reciprocal inhibition effect has been reported in yeast and LAB in co-fermentations.

The amensalism effect between yeast and LAB is also reported in cachaça (Freitas et al. 2001). In sugarcane fermentation on a laboratory scale, *Limosilactobacillus fermentum* (previously *Lactobacillus fermentum*, Zheng et al. 2020) co-inoculated with *S. cerevisiae* showed a decrease in bacterial population near to 33% (Ferreira et al. 2011). The *S. cerevisiae*/*Lactococcus lactis* interaction in cachaça caused a decrease in *L. lactis* population from 1×10^5 to 1×10^3 cells/mL (Carvalho et al. 2015). In both cases, the main compound responsible for this inhibitory effect was the ethanol released by yeast during AF.

It should be pointed out that the increasing ethanol content does not affect only LAB. Non-*Saccharomyces* yeast can also be inhibited by this compound, which is progressively released by *S. cerevisiae* in spontaneous sugarcane fermentation (Barbosa et al. 2016) or wine fermentations (Wang et al. 2015). Both cases are examples of amensalism interactions between yeasts, in which the main fermenting yeast is *S. cerevisiae*. Some yeast/yeast interactions have been reported in the tequila and mezcal process (Arrizon et al. 2006; Arellano-Plaza et al. 2013). One example is the *Kluyveromyces marxianus*/*S. cerevisiae* interaction in tequila must–type medium, in which the biomass viability was affected not only by the ethanol concentration but also by some unknown compounds released in cell-to-cell contact (Fernández et al. 2014). Another example is the *Hanseniaspora*/*S. cerevisiae* interaction in tequila, in which the population of *Hanseniaspora* gradually decreased to almost 50%, while *S. cerevisiae* remained constant to the end of fermentation, because *S. cerevisiae* is more resistant to ethanol than non-*Saccharomyces* strains (González-Robles et al. 2015).

Antagonism

The main difference between amensalism and antagonism is that in amensalism one metabolite produced by the primary metabolism affects the growth of another. In contrast, in antagonism compounds are associated with the growth inhibition and the killer effect. Secondary metabolism is usually involved in the synthesis of the inhibiting and killer compounds (Little et al. 2008). The killer effect is related with the production and release of several kinds of toxin with low molecular weights and post-translational modifications, which can kill sensitive cells.

Killer yeasts have been known in the wine industry for years. Wine contamination with killer strains can affect negatively AF if it is inoculated with sensitive wine yeasts. Nevertheless, killer starters can be used to control growth and persistence of undesirable yeasts (Van Vuuren and Jacobs 1992). The most well-characterized killer toxins in wine are K1, K2, and K28 of *S. cerevisiae* and others of non-*Saccharomyces*. Most of them are genetically codified by extrachromosomal elements (Mannazzu et al. 2019).

Several wine-related reports have described examples of growth inhibition of *O. oeni* by some strains of *S. cerevisiae*, due to some proteinaceous compounds released by yeasts (Dick et al. 1992; Osborne and Edwards 2007; Branco et al. 2014). This effect was confirmed by Comitini et al. (2005), who showed that the proteinaceous factor concentrated by 7.5 × inhibited the whole *O. oeni* population, which suggests that the effect of proteinaceous compounds is dose dependent. Also, Osborne and Edwards (2007) reported that the compounds released by *S. cerevisiae* had molecular weights between 3 and 10 kDa, and they decreased the *O. oeni* population from 1×10^6 to 1×10^3 cells/mL. Mendoza et al. (2010) also observed a growth inhibition in *O. oeni* of approximately 45% in contrast to the control trial. Similar results were observed by Branco et al. (2014), but in this case, the inhibited microorganisms were also non-*Saccharomyces*, besides *O. oeni*. These authors called these compounds antimicrobial peptides (AMPs). As proposed by Branco et al. (2014) and Albergaria et al. (2010), those AMPs inhibiting other yeasts—and also other fungi—have 4–6 kDa and could be derived from fragments of *S. cerevisiae* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein.

The inhibition mechanisms of AMPs in *O. oeni* are not clearly understood, but some reports suggest that

proteinaceous compounds form membrane channels or pores that destroy the energy potential of sensitive cells (Osborne and Edwards 2007), which also affects MLF by inhibiting the malolactic enzyme (Rizk et al. 2016). In this way, AMPs are not considered as killer factors (Dick et al. 1992) because AMPs are usually small peptides—less than 10 kDa—(Osborne and Edwards 2007), while killer proteins are bigger, from 10 to 40 kDa (Mannazzu et al. 2019).

As mentioned, antagonism includes also the growth inhibition due to competence for nutrients. In spite of the interesting benefits of using non-*Saccharomyces* in wine, growth of these yeasts affects nutrient availability for *S. cerevisiae* during wine fermentation (Medina et al. 2012; Lage et al. 2014). This effect of yeast-yeast competition has been extensively studied at a transcriptional level (Barbosa et al. 2015; Tronchoni et al. 2017; Ruiz et al. 2020).

In wine and cider, this is another negative effect of *S. cerevisiae* and AF on LAB and subsequent MLF, since yeasts exhausts the nutrients, and LAB usually have complex nutrient requirements (Terrade and Mira de Orduña 2009). Therefore, yeast strains with complex nutrient requirements would exhibit an increased antagonistic relationship with LAB (Costello et al. 2003). In this way, it has been recently described that co-inoculation of *S. cerevisiae* with non-*Saccharomyces* yeasts result in a metabolic stimulation of nutrients uptake by yeasts, which could lead to a nutrient deficiency for LAB (Curiel et al. 2017).

The *S. cerevisiae/H. uvarum* interaction has been reported to be antagonistic: *H. uvarum* biomass was reduced as a consequence of co-inoculation with *S. cerevisiae*. The non-*Saccharomyces* increased to 7×10^6 CFU/mL and then decreased to undetectable levels (< 10 CFU/mL) in only 24 h (Li et al. 2020). This effect has also been described elsewhere, and it has been postulated that growth is inhibited by the AMPs, as commented above. During wine fermentation, in the *S. cerevisiae*/*H. uvarum* interaction, the population of *H. uvarum* decreased from 2×10^7 to less than 1×10^6 CFU/mL. This decrease remained constant until the end of the fermentation process, when after 5 days of fermentation, non-viable cells were recorded (Wang et al. 2015). These results are in agreement with those found in the *S. cerevisiae*/*H. guilliermondii* interaction, in which the *H. guilliermondii* yeast population decreased drastically from 2×10^7 to 5×10^2 cells/mL (Albergaria et al. 2010). In this study, the effect was also related to the presence of AMPs < 10 kDa. It was subsequently reported that these AMPs have roles in such subcellular locations as the membrane, cytosol, and nucleus and also in apoptosis (Branco et al. 2014).

Some antagonism interaction due to acetaldehyde production have been described between different strains of *S. cerevisiae* strains and also between *S. cerevisiae* and *S. uvarum* (Cheraiti et al. 2005). Nevertheless, it seems that very low levels of acetaldehyde—less than 100 mg/mL—may reduce the lag phase of ethanol-stressed of some *S. cerevisiae* strains, resulting in short fermentation period (Stanley et al. 1997).

An antagonism interaction has been observed from *M. pulcherrima* against other non-*Saccharomyces* species (Oro et al. 2014). Interestingly, its antimicrobial activity did not have negative influence on *S. cerevisiae*. Instead, *M. pulcherrima* displayed a broad antimicrobial action on undesired wild spoilage yeasts, such as *Brettanomyces*/*Dekkera*, and some *Hanseniaspora* and *Pichia* strains. This antimicrobial activity does not seem due to proteinaceous compounds such as killer phenomenon, but to the pulcherriminic acid, that depletes iron present in the medium, making it not available to the other yeasts (Oro et al. 2014).

Furthermore, other compounds of the secondary metabolism are related to growth inhibition in yeast, such as the aromatic alcohols tyrosol, tryptophol, and 2-phenylethanol, derived from the metabolism of tyrosine, tryptophan, and phenylalanine, respectively. These compounds have been shown to serve as quorum-sensing molecules under low-nitrogen conditions when cultures of *S. cerevisiae* reach a high density in winemaking conditions (Zupan et al. 2013; Avbelj et al. 2016), and they are responsible for reducing yeast cell growth and inducing morphogenesis especially in non-*Saccharomyces* species (González et al. 2018).

An interesting antagonistic interaction in wine fermentations is the inhibition of some non-*Saccharomyces* by *S. cerevisiae* in mixed cultures by means of a cell-to-cell contact-mediated mechanism (Nissen et al. 2003; Nissen and Arneborg 2003). These authors found that the growth arrests of *Lachancea thermotolerans* (previously *Kluyveromyces thermotolerans*) and *T. delbrueckii* seemed to be due neither to nutrient limitation nor to the presence of any growth-inhibitory compound—such as the ethanol—but to a cell-to-cell contact with *S. cerevisiae* cells at high concentrations. Whether or not quorum-sensing molecules are involved in this cell-to-cell communication remains unclear, and further studies within this field are required (Avbelj et al. 2016).

Commensalism

Commensalism is a relationship between species in which one of them benefits from the other without doing it any harm. Alternatively, when both of the microorganisms involved benefit, the relationship can be described as synergism. Usually, synergism is also considered when there are some benefits in the quality of the final product as a result of microbial interaction. One example of commensalism is the co-inoculation of *S. cerevisiae* and *O. oeni* in red wine (Azzolini et al. 2010). It has been reported that in this case malolactic fermentation is completed in fewer days than spontaneous or sequential fermentation. It should be pointed out that, in this co-culture, the carbon source

was not responsible for LAB growth or the enhancement of MLF, because LAB at oenological pH would degrade L-malic acid before sugars (Azzolini et al. 2010). However, fermentations performed by Izquierdo et al. (2014) with *S. cerevisiae*/*O. oeni* in co-culture wine fermentation or simultaneous fermentation of *H. uvarum*/*S. cerevisiae* and *O. oeni* (Du Plessis et al. 2019) have shown that yeasts improve MLF and promote growth. In both cases, the presence of yeasts reduces the time of MLF by about 87% (Izquierdo et al. 2014) or 45% (Du Plessis et al. 2019), in comparison to their respective single-culture LAB controls. Moreover, in cider fermentation, the co-culture of *S. cerevisiae/O. oeni* increased the maximum LAB population to more than 1×10^7 CFU/mL, in contrast to spontaneous fermentation with 1×10^5 CFU/mL (Dierings et al. 2013).

Many other studies have proposed that the improvement in MLF and the effect on LAB growth could be related to the presence of compounds released during simultaneous fermentation or yeast autolysis, mainly nitrogenated compounds such as amino acids, peptides, and proteins (Martínez-Rodríguez et al. 2001; Díez et al. 2010). Other compounds released by yeast during AF are citric, pyruvic, and D-gluconic acids, which are used by LAB to obtain energy: citric acid is metabolized (Balmaseda et al. 2018), pyruvic acid acts as an electron acceptor and growth promotor (Maicas et al. 2002), and D-gluconic acid is an MLF accelerator (Liu et al. 2016). During yeast autolysis, mainly at the end of AF, mannoproteins—heteropolysaccharides constituted mainly by mannans and less than 10% of proteins—are released into the medium (Domizio et al. 2014). In fact some LAB can hydrolyze these compounds, which enhances the nutritional content of the medium and increases the growth rate (Díez et al. 2010). In this way, non-*Saccharomyces* yeasts as *T. delbrueckii* and *M. pulcherrima* have been recently shown to release more mannoproteins in wine, which benefit *O. oeni* and MLF (Ferrando et al. 2020). All these compounds released into the medium as yeast by-products have a positive effect on LAB. In this relationship of commensalism, the bacteria benefit from the products excreted by yeasts during the fermentation process.

Some benefits for *O. oeni* in wine can also appear as consequence of minimizing some inhibiting products from yeasts. This is the case of using non-*Saccharomyces* yeasts, as the above-mentioned *T. delbrueckii* and *M. pulcherrima*, which offer more MLF-friendly conditions than *S. cerevisiae* alone, by lowering SO_2 and medium-chain fatty acid contents (Balmaseda et al. 2021).

Synergism

Synergism can be defined as the relationship between two microbial species in which each one benefits the other (Little et al. 2008). Nevertheless, from a practical point of view, synergism can be also the situation when there are some benefits in the quality of the final product—the alcoholic beverage in our case—as a result of the microbial interaction, in spite of not being a clear benefit for each species. Several authors—for instance, Sadoudi et al. (2012), Wang et al. (2016), and Zhang et al. (2020)—have used this sense of synergism, mainly in winemaking.

There are very few published examples of synergism with benefits for both microorganisms interacting. For instance, Renault et al. (2016) found that the growth of *T. delbrueckii* was improved by *S. cerevisiae* in sequential fermentation, with a population remained in the order of 5×10^6 CFU/mL until the end of fermentation, whereas the population in the pure culture faded out at mid-fermentation.

Synergism interactions have also been reported for LAB during MLF. The *O. oeni* and *Lactiplantibacillus plantarum* (previously *Lactobacillus plantarum*, Zheng et al. 2020) interaction showed better implantation of *O. oeni* in mixed cultures with *L. plantarum* (60–65% implantation) than in single ones (55% of implantation). In this example, there was also an improvement of wine quality, since there was an increase in diethyl succinate—fruity-melon odor—from 5.95 mg/L with *O. oeni* to 7.56 mg/L with mixed cultures. It was positive for the quality of wine, because the increase in ethyl esters improves the sensory attributes in wine (Brizuela et al. 2018).

We discuss below some other cases of synergism in which the benefits are exclusively for the quality of the alcoholic beverage. One example is the case of the *T. delbrueckii*/*S. cerevisiae* interaction in Sauvignon Blanc wine (Sadoudi et al. 2012). When both species were in mixed culture, both of them achieved the same population, about 1×10^8 CFU/mL—the same than in separate cultures—but the benefit was an increasing content of C6 compounds, terpenols and 2-phenylethanol, which suggests that the two species had a cumulative effect of these compounds (Sadoudi et al. 2012).

Another example between these yeast species is the uptake and release of volatile thiols and their precursors when *S. cerevisiae* and *T. delbrueckii* are sequentially inoculated (Sadoudi et al. 2012). Glut-3-sulfanylhexan-1-ol (Glut-3SH) and Cys-3-sulfanylhexan-1-ol (Cys-3SH) are precursors of thiol 3-sulfanylhexan-1-ol (3SH). The fruity aroma in white wines has been attributed to this latter compound (Renault et al. 2016). In pure *T. delbrueckii* cultures, the Glut-3SH degradation produced significant amounts of 3SH and Cys-3SH. However, the cysteinylated precursor is hardly assimilated by *T. delbrueckii* because the transport enzyme Gap1p permease is absent or dysfunctional in this species. But in *S. cerevisiae*, this compound is transported by the membrane protein Gap1p, thus making it able to metabolize the Cys-3SH and release the 3SH in the last stage of AF. The direct consequence of this interaction is that 3SH production

is higher in sequential fermentations than in pure culture fermentations.

In spite of the previously commented antagonistic relationship between *S. cerevisiae* and *H. uvarum*, an improvement of wine quality has been shown sometimes (Hu et al. 2018), with an increase of MCFA and ethyl ester content in mixed fermentation, which lead to wine fruity aroma enhancement.

Results of interaction

The approach proposed by Lemaresquier (1987) was to classify yeast-LAB interactions on the basis of growth promotion or inhibition. Several negative *S. cerevisiae*/*O. oeni* interactions have been described, and the growth inhibition effect is now known to be caused by the presence of proteinaceous compounds in the must (Comitini et al. 2005).

As described above, interactions can be classified from the point of view of microbial ecology. The main interactions described are shown in Table 2, grouped according to the type of interaction reported. Several studies show that the result interaction has on the final product can be different to the positive or negative effects on microorganisms themselves. For example, some amensalism and antagonistic interactions in beverages and spirits positively enhanced the aroma complexity of the product and reduced fermentation time.

The amensalism *S. cerevisiae*/*O. oeni* interaction in synthetic must significantly increased the volatile composition in terms of the amount of ethyl lactate—fruity, buttery flavor—and octanoic acid, soapy, feint fruity flavor, in contrast with the yeast monoculture. Moreover, L-malic acid depleted faster in the mixed fermentation than in the sequential fermentation in synthetic must and was exhausted in 10 days (Rossouw et al. 2012). Another example is the amensalism *S. cerevisiae*/*S. cerevisiae* var. *boulardii* interaction in beer, which decreased the amount of acetic acid in mixed fermentation and increased antioxidant activity. Moreover the survival of *S. cerevisiae* var. *boulardii* conferred added value on the product because it is considered to be a probiotic subspecies (Capece et al. 2018).

Another interaction is the antagonistic *S. cerevisiae*/*H. uvarum,* in which an increase in fruity aroma esters was recorded (Li et al. 2020). And the amensalism yeast/yeast and yeast/LAB interactions in the cachaça fermentation process reported an increase in 2,3-butanedione, ethyl acetate, propionic, and isobutyric and butyric acids in mixed fermentation with *S. cerevisiae*/*L. fermentum* (Ferreira et al. 2011); in isoamyl alcohol, isobutanol and propanol in *S. cerevisiae*/*L. lactis* interaction (Carvalho et al. 2015); and in isoamyl alcohol in the non-*Saccharomyces*/*S. cerevisiae* interaction (Barbosa et al. 2016). The ethanol yield in tequila must type with *K. marxianus*/*S. cerevisiae* also increased in mixed fermentation (Fernández et al. 2014). However, a negative impact on the product has been reported in negative interactions. For instance, the *S. cerevisiae*/*O. oeni* antagonistic interaction in red wine had a negative impact on the product, because the malic acid was not fully depleted (Comitini et al. 2005). This effect was also reported in the amensalism interaction *S. cerevisiae*/*O. oeni,* with a partial L-malic acid degradation, leaving a residual of 40% in white wine (Comitini and Ciani 2007).

Regarding positive interactions, the commensalism *S. cerevisiae*/*O. oeni* interactions show positive effects on the product, because they enhance volatile composition by increasing the propanol, hexyl acetate, ethyl lactate, butyrate, hexanoate, and octanoate concentration (Izquierdo et al. 2014), reducing MLF time in red wine (Azzolini et al. 2010; Du Plessis et al. 2019) and decreasing MLF time and volatile acidity in cider (Dierings et al. 2013). Also, the *O. oeni*/*L. plantarum* synergism interaction showed an interesting increase in diethyl succinate in Malbec wine (Brizuela et al. 2018).

Conclusion

Since Lemaresquier (1987) studied the first yeast/LAB interactions, the negative, positive, and neutral interactions between microorganisms in the winemaking process have been explored. The various microbial interactions in beverages and musts for obtaining distillates have been defined and related to the production of particular compounds, which explain every interaction in the microbial ecology approach. Thus, the study of different compounds and their action gives greater insight into microbial interactions and shows that the pair-strain and inoculation strategies can improve aroma complexity, whether interactions between yeast and yeast and yeast and bacteria are positive or negative.

In this review, we suggest classifying microbial interactions in beverages and spirits as a function, not only of microbial ecology but also of product impact, as a way to further our understanding of how strains behave in fermentation processes, and how they can improve aroma complexity in beverages and spirits. Further studies are necessary to elucidate all the mechanisms involved in microbial interactions, in which genomics, proteomics, and metabolomics play an important role. Omics techniques make it possible to describe up- and down-regulated genes, the action of proteinaceous compounds, and the release of AMPs and quorum-sensing molecules and also to characterize a wide range of aromatic compounds produced during fermentation process.

However, all the studies discussed here mainly focus on species, not on strains. All the strains of the same species

Table 2 Classification of microbial interaction in terms of product impact

Interaction	Product	Microorganisms	Fermentation result	Effect on product	References
Amensalism	White wine	*S. cerevisiae* and *O. oeni*	MLF incomplete	Negative	Comitini and Ciani 2007
	Red wine	*S. cerevisiae* and *O. oeni*	Residual fructose 3.5 g/L	Negative	Muñoz et al. 2014
	Wine	*Hanseniaspora uvarum/H. vinae* and *O. oeni*	Increase in SO_2 and acetic acid	Negative	Ferrando et al. 2020
	Beer	*S. cerevisiae* and *S. cerevisiae var. boulardii*	Increase in antioxidant activity	Positive	Capece et al. 2018
	Tequila	*Kluyveromyces marxianus* and *S. cerevisiae*	Increase in ethanol yield	Positive	Fernández et al. 2014
	Cachaça	Non-*Saccharomyces* and *S. cerevisiae*	Increase in volatile composition	Positive	Barbosa et al. 2016
	Cachaça	*S. cerevisiae* and *Lactococcus lactis*	Increase in volatile composition	Positive	Carvalho et al. 2015
	Cachaça	*Limosilactobacillus fermentum* and *S. cerevisiae*	Increase in volatile composition	Positive	Ferreira et al. 2011
Antagonism	Wine	*S. cerevisiae* killer strains and *S. cerevisiae* sensitive strains	AF incomplete, off-flavors	Negative	Van Vuuren and Jacobs 1992
	Wine	*S. cerevisiae* (antimicrobial peptides) and *O. oeni*	Bacterial inhibition, MLF incomplete	Negative	Dick et al. 1992; Branco et al. 2014
	Wine	*S. cerevisiae* and non-*Saccharomyces*	Cell-to-cell inhibition of non-*Saccharomyces*	Positive	Nissen et al. 2003
	Red wine	*S. cerevisiae* and *H. uvarum*	Increase in fruity esters concentration	Positive	Li et al. 2020
	Red wine	*S. cerevisiae* and *S. uvarum*	Short fermentation period	Positive	Cheraiti et al. 2005
Commensalism	Red wine	*S. cerevisiae* and *O. oeni*	Increase in volatile composition	Positive	Izquierdo et al. 2014
	Red wine	*S. cerevisiae/H. uvarum* and *O. oeni*	Short MLF and increase in fresh and vegetative aroma	Positive	Du Plessis et al. 2019
	Red wine	*S. cerevisiae* and *O. oeni*	Short MLF	Positive	Azzolini et al. 2010
	Wine	*Torulaspora delbrueckii/ Metschnikowia pulcherrima* and *O. oeni*	Short MLF	Positive	Ferrando et al. 2020
	White and red wine	*T. delbrueckii/M. pulcherrima* and *O. oeni*	Short MLF due to lower SO_2 and MCFA	Positive	Balmaseda et al. 2021
	Cider	Non-*Saccharomyces, S. cerevisiae* and *O. oeni*	Short MLF, increase in ethanol yield, and decrease in volatile acidity	Positive	Dierings et al. 2013
Synergism	White wine	*M. pulcherrima, Starmerella bacillaris, T. delbrueckii*, and *S. cerevisiae*	Increase in volatile composition	Positive	Sadoudi et al. 2012
	Red wine	*H. uvarum* and *S. cerevisiae*	Fruity aroma enhancement due to increase in MCFA	Positive	Hu et al. 2018
	Red wine	*O. oeni* and *Lactiplantibacillus plantarum*	Successful MLF and increase in volatile composition	Positive	Brizuela et al. 2018

are known not to exhibit the same behavior. Table 2 shows that the same interaction between yeasts, or between yeasts and LAB, has different results on the final product. This means that not all *S. cerevisiae* strains have the same effect on all the *O. oeni* strains (Arnink and Henick-Kling 2005). Likewise, not all *S. cerevisiae* strains have the same effect on all non-*Saccharomyces* strains, where cultivability loss is not only a species-dependent effect but also a strain-dependent one (Wang et al. 2016). So, future studies should focus on the use of their particular microbial resources as the best

approach to improve the quality of regional alcoholic beverages. In this way, every producer can search for the best combination of microorganisms to get the best final product. Thereby, the market will be less globalized and uniform, and the value of each producer will be increased.

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7. Anexos

ANEXO 2

Tablas suplementarias del Capítulo 3 de Resultados

Supplementary Table 1. Genes (Locus tag) of *Oenococcus oeni* PSU-1 for which transcripts RNAs were detected by RNA-seq, where differential expression (Log2 Fold Change) was found in medium with 2 g/L succinic acid (WLMS) compared to control without it (WLM). Log2FC values are marked in red or dark blue depending on whether the transcripts were down or up-regulated, respectively. Description is the probable locus function.

Locus tag	log2 Fold Change	Description
OEOE_RS00005	-1.78	Chromosomal replication initiator protein dnaA
OEOE_RS00010	-1.25	DNA polymerase III subunit beta
OEOE_RS00015	-0.55	S4-like RNA binding protein
OEOE_RS00020	-0.32	DNA replication and repair protein
OEOE_RS00025	**1.07**	DNA gyrase subunit B
OEOE_RS00030	**2.02**	DNA gyrase subunit A
OEOE_RS00035	-3.34	NH(3)-dependent NAD(+) synthetase
OEOE_RS00040	-0.82	multicopper oxidase
OEOE_RS00045	-0.34	Small ribosomal subunit protein bS6
OEOE_RS00060	-1.03	
OEOE_RS00065	-1.71	Large ribosomal subunit protein bL9
OEOE_RS00070	-1.05	
OEOE_RS00080	-0.35	peptidase
OEOE_RS00085	-0.51	
OEOE_RS00090	0.54	D-aminoacyl-tRNA deacylase
OEOE_RS00095	-0.89	
OEOE_RS00100	-1.75	
OEOE_RS00105	-0.88	
OEOE_RS00110	-1.38	
OEOE_RS00115	-2.28	
OEOE_RS00120	-1.63	2 -hydroxyacid dehydrogenase
OEOE_RS00125	-1.29	mannose-6-phosphate isomerase
OEOE_RS00130	**1.40**	

OEOE_RS00135	**1.39**	
OEOE_RS00140	0.93	
OEOE_RS00145	**1.26**	
OEOE_RS00150	**1.28**	
OEOE_RS00155	**1.33**	
OEOE_RS00160	**1.42**	
OEOE_RS00165	**1.76**	oxidoreductase ion channel protein IolS
OEOE_RS00175	**2.77**	
OEOE_RS00180	**1.85**	
OEOE_RS00185	**1.67**	
OEOE_RS00190	**3.18**	
OEOE_RS00195	**1.03**	
OEOE_RS00200	**2.53**	ketosteroid isomerase
OEOE_RS00205	**3.49**	oxidoreductase
OEOE_RS00210	0.87	
OEOE_RS00215	0.22	XRE family transcriptional regulator
OEOE_RS00220	-0.41	
OEOE_RS00225	0.84	MFS transporter
OEOE_RS00230	-0.52	NAD(P)-dependent oxidoreductase
OEOE_RS00240	-0.93	hypothetical protein
OEOE_RS00245	**2.06**	hypothetical protein
OEOE_RS00250	**2.14**	membrane protein
OEOE_RS00255	**2.03**	membrane protein
OEOE_RS00260	**2.21**	transcriptional regulator
OEOE_RS00270	-1.03	Alkaline shock response membrane anchor protein AmaP
OEOE_RS00275	**1.11**	alpha/beta hydrolase
OEOE_RS00280	**1.15**	hypothetical protein
OEOE_RS00285	**1.17**	transcriptional regulator
OEOE_RS00290	**2.00**	hypothetical protein
OEOE_RS00295	**1.19**	phytoene synthase
OEOE_RS00310	-0.71	hypothetical protein
OEOE_RS00315	-0.48	membrane protein
OEOE_RS00325	0.24	general stress protein
OEOE_RS00330	-0.25	pseudogene
OEOE_RS00335	0.20	hypothetical protein
OEOE_RS00340	0.99	MFS transporter
OEOE_RS00345	-0.31	MFS transporter
OEOE_RS00350	-1.14	diacetyl reductase [(S)-acetoin forming]
OEOE_RS00355	**1.63**	short-chain dehydrogenase
OEOE_RS00360	0.89	transcriptional regulator
OEOE_RS00365	0.82	serine acetyltransferase
OEOE_RS00370	**1.76**	NADPH:quinone reductase

OEOE_RS00375	**2.63**	MarR family transcriptional regulator
OEOE_RS00380	-0.60	hypothetical protein
OEOE_RS00385	-0.46	hypothetical protein
OEOE_RS00390	-0.50	hypothetical protein
OEOE_RS00395	0.69	Hypothetical protein
OEOE_RS00420	0.61	Beta -lactamase class C related penicillin binding protein
OEOE_RS00425	0.63	
OEOE_RS00430	0.76	
OEOE_RS00435	0.82	glutaredoxin
OEOE_RS00440	0.42	
OEOE_RS00445	**4.18**	MFS transporter
OEOE_RS00450	**2.13**	
OEOE_RS00455	0.68	XRE family transcriptional regulator
OEOE_RS00460	**1.33**	
OEOE_RS00475	**1.60**	phosphoesterase
OEOE_RS00485	0.99	response regulator
OEOE_RS00490	**3.37**	histidine kinase
OEOE_RS00495	**2.11**	serine protease
OEOE_RS00500	**2.32**	Ribosomal RNA large subunit methyltransferase H
OEOE_RS00505	-1.19	
OEOE_RS00510	0.86	
OEOE_RS00515	**1.01**	Ribosomal RNA small subunit methyltransferase G
OEOE_RS00520	0.45	
OEOE_RS00525	-0.44	
OEOE_RS00530	0.52	
OEOE_RS00535	0.83	
OEOE_RS00555	0.35	histidine kinase
OEOE_RS00560	0.80	D -alanyl - D -alanine carboxypeptidase
OEOE_RS00565	-2.35	phosphoglyceromutase
OEOE_RS00570	-1.53	
OEOE_RS00575	-1.17	
OEOE_RS00580	**1.79**	
OEOE_RS00590	-0.85	
OEOE_RS00595	-0.47	sugar phosphate isomerase
OEOE_RS00600	**1.38**	gluconate:proton symporter
OEOE_RS00605	**1.42**	
OEOE_RS00610	0.65	
OEOE_RS00620	-0.61	
OEOE_RS00625	-1.18	glucose - 6 -phosphate 1 -dehydrogenase
OEOE_RS00630	0.96	
OEOE_RS00635	-0.54	hemolysin
OEOE_RS00640	-2.20	ribonucleotide reductase

OEOE_RS00645	-3.41	glutaredoxin
OEOE_RS00650	-3.36	ribonucleotide-diphosphate reductase subunit alpha
OEOE_RS00655	-3.67	ribonucleoside-diphosphate reductase
OEOE_RS00660	-0.97	transcriptional regulator
OEOE_RS00665	-0.94	histidine kinase
OEOE_RS00670	0.94	
OEOE_RS00675	0.34	phospholipid phosphatase
OEOE_RS00680	-0.89	
OEOE_RS00690	0.37	MFS transporter
OEOE_RS00700	0.63	Chorismate synthase
OEOE_RS00710	0.63	
OEOE_RS00715	**1.10**	3 -phosphoshikimate 1 -carboxyvinyltransferase
OEOE_RS00720	**1.33**	shikimate kinase
OEOE_RS00725	-0.41	Inosine-uridine nucleoside N-ribohydrolase
OEOE_RS00735	**1.13**	NAD(P)H -hydrate epimerase
OEOE_RS00745	-0.78	Adenine deaminase
OEOE_RS00750	0.24	ribose 5-phosphate isomerase A
OEOE_RS00755	-1.27	ribose -phosphate pyrophosphokinase
OEOE_RS00760	-0.83	
OEOE_RS00765	-0.95	Alanine racemase
OEOE_RS00770	-0.97	ferredoxin--NADP reductase
OEOE_RS00775	-0.53	diguanylate cyclase
OEOE_RS00780	-0.22	
OEOE_RS00785	0.71	
OEOE_RS00790	**1.41**	
OEOE_RS00800	**2.13**	
OEOE_RS00805	0.52	
OEOE_RS00810	**1.44**	
OEOE_RS00815	**1.06**	cobalt ABC transporter permease
OEOE_RS00820	0.31	cobalt ABC transporter
OEOE_RS00830	-0.76	transporter
OEOE_RS00840	**1.95**	
OEOE_RS00845	**2.07**	
OEOE_RS00850	**2.28**	TetR family transcriptional regulator
OEOE_RS00855	**3.77**	
OEOE_RS00875	-0.36	hypothetical protein
OEOE_RS00880	-0.52	
OEOE_RS00890	0.23	Hypoxanthine phosphoribosyltransferase
OEOE_RS00895	-0.33	ATP-dependent zinc metalloprotease
OEOE_RS00900	0.49	disulfide bond formation protein
OEOE_RS00905	0.54	Lysine--tRNA ligase
OEOE_RS00910	-0.98	hypothetical protein

OEOE_RS00915	-0.68	
OEOE_RS00935	**1.56**	transporter
OEOE_RS00940	**1.61**	hemolysin D
OEOE_RS00945	**1.62**	
OEOE_RS00950	**1.80**	
OEOE_RS00955	**2.44**	
OEOE_RS00965	**2.10**	
OEOE_RS00970	**1.40**	
OEOE_RS00975	**1.19**	multidrug ABC transporter ATPase
OEOE_RS00980	0.85	1,4 -beta - N -acetylmuramidase
OEOE_RS00985	-0.29	acetyltransferase
OEOE_RS00990	**1.03**	hydrolase
OEOE_RS00995	0.77	AraC family transcriptional regulator
OEOE_RS01005	**2.41**	hypothetical protein
OEOE_RS01010	**1.28**	
OEOE_RS01015	0.24	D -alanyl - D -alanine dipeptidase
OEOE_RS01020	-1.43	TetR family transcriptional regulator
OEOE_RS01025	-1.12	threonine dehydrogenase
OEOE_RS01030	-0.26	glycosyltransferase
OEOE_RS01035	0.92	membrane protein
OEOE_RS01040	-2.14	hypothetical protein
OEOE_RS01045	**1.28**	PTS sugar transporter subunit IIA
OEOE_RS01050	**1.46**	PTS cellobiose transporter subunit IIA
OEOE_RS01055	**1.72**	PTS sugar transporter
OEOE_RS01060	0.99	6 -phospho -beta -glucosidase
OEOE_RS01065	**1.19**	transcriptional regulator
OEOE_RS01070	-0.74	6 GntR family transcriptional regulator
OEOE_RS01075	**3.11**	ATPase
OEOE_RS01080	**3.17**	araD pseudo
OEOE_RS01085	**1.60**	L-arabinose isomerase
OEOE_RS01095	0.33	pseudogene
OEOE_RS01100	-1.76	aldose 1 -epimerase
OEOE_RS01105	-1.04	transcriptional antiterminator
OEOE_RS01110	0.35	PTS fructose transporter subunit IIA
OEOE_RS01115	0.47	PTS sugar transporter subunit IIA
OEOE_RS01120	**1.00**	PTS galactitol transporter subunit IIC
OEOE_RS01125	0.68	PTS galactitol transporter subunit IIB
OEOE_RS01130	-1.54	
OEOE_RS01135	-0.99	carbonic anhydrase
OEOE_RS01140	-1.68	O-acetylhomoserine aminocarboxypropyltransferase
OEOE_RS01145	**2.48**	
OEOE_RS01150	**2.16**	alpha/β hydrolase

OEOE_RS01155	**1.43**	3-beta-hydroxysteroid dehydrogenase
OEOE_RS01160	-1.06	oxidoreductase ion channel protein IolS
OEOE_RS01165	-1.75	DNA-binding protein
OEOE_RS01170	0.64	TetR family transcriptional regulator
OEOE_RS01175	0.88	manganese transporter
OEOE_RS01185	0.75	
OEOE_RS01190	0.58	mannose - 6 -phosphate isomerase
OEOE_RS01195	0.32	
OEOE_RS01200	0.93	
OEOE_RS01205	**1.46**	
OEOE_RS01210	**1.83**	sugar ABC transporter substrate -binding protein
OEOE_RS01215	**2.03**	sugar ABC transporter permease
OEOE_RS01220	**2.44**	sugar ABC transporter permease
OEOE_RS01225	**2.27**	
OEOE_RS01235	-7.37	aspartate carbamoyltransferase
OEOE_RS01240	-7.15	dihydroorotase
OEOE_RS01245	-6.38	carbamoyl phosphate synthase small subuni
OEOE_RS01250	-6.13	carbamoyl -phosphate synthase large chain
OEOE_RS01255	-5.69	orotidine 5' -phosphate decarboxylase
OEOE_RS01260	-5.53	orotate phosphoribosyltransferase
OEOE_RS01265	-4.62	dihydroorotate dehydrogenase
OEOE_RS01270	-0.89	MFS transporter
OEOE_RS01275	-0.24	
OEOE_RS01280	0.72	peptide ABC transporter substrate-binding protein
OEOE_RS01285	0.25	branched-chain amino acid ABC transporter permease
OEOE_RS01290	0.26	phosphonate ABC transporter ATP-binding protein
OEOE_RS01295	0.57	peptide ABC transporter ATPase
OEOE_RS01300	**1.29**	
OEOE_RS01305	**1.26**	
OEOE_RS01315	0.44	
OEOE_RS01320	0.91	
OEOE_RS01325	0.53	
OEOE_RS01335	-2.54	dihydroxynaphthoic acid synthetase
OEOE_RS01340	-1.91	acyl -CoA synthetase
OEOE_RS01345	-0.82	
OEOE_RS01350	**2.18**	
OEOE_RS01355	**2.10**	
OEOE_RS01360	**1.49**	D-Ala-teichoic acid biosynthesis protein
OEOE_RS01365	**1.30**	D-alanine--D-alanyl carrier protein ligase
OEOE_RS01370	**1.97**	D-alanyl-lipoteichoic acid biosynthesis protein DltB
OEOE_RS01375	**2.71**	D-alanine--poly(phosphoribitol) ligase
OEOE_RS01380	**3.12**	D-alanyl-lipoteichoic acid biosynthesis protein DltD

OEOE_RS01385	**4.44**	heat-shock protein Hsp20
OEOE_RS01390	**1.63**	MFS transporter permease
OEOE_RS01400	**2.16**	
OEOE_RS01405	**1.64**	
OEOE_RS01410	**1.27**	
OEOE_RS01415	**1.66**	
OEOE_RS01420	**1.34**	
OEOE_RS01425	0.49	
OEOE_RS01430	0.23	
OEOE_RS01435	0.43	aldose 1 -epimerase
OEOE_RS01440	0.52	LacI family transcriptional regulator
OEOE_RS01455	-0.44	sodium:solute symporter
OEOE_RS01460	-0.29	
OEOE_RS01465	0.68	
OEOE_RS01475	0.94	ATP -dependent helicase/nuclease subunit A
OEOE_RS01480	0.30	phosphoglycerate mutase
OEOE_RS01485	-1.10	
OEOE_RS01490	-0.71	
OEOE_RS01495	-0.22	
OEOE_RS01500	-0.48	
OEOE_RS01505	**2.02**	
OEOE_RS01510	0.61	deoxyuridine 5' -triphosphate nucleotidohydrolase
OEOE_RS01515	0.94	
OEOE_RS01520	**1.54**	DNA repair protein RadA
OEOE_RS01525	**1.72**	
OEOE_RS01530	**1.08**	nucleoside -triphosphate diphosphatase
OEOE_RS01540	-1.08	
OEOE_RS01545	-0.46	
OEOE_RS01550	-0.18	aldehyde dehydrogenase
OEOE_RS01555	**1.50**	
OEOE_RS01560	0.54	
OEOE_RS01565	**1.98**	lipoate -protein ligase A
OEOE_RS01570	**2.82**	pyruvate dehydrogenase E1 subunit alpha
OEOE_RS01575	**3.45**	2 -oxoisovalerate dehydrogenase subunit β
OEOE_RS01580	**2.77**	dihydrolipoamide acetyltransferase
OEOE_RS01585	**2.37**	dihydrolipoamide dehydrogenase
OEOE_RS01590	0.80	
OEOE_RS01595	-0.42	aspartate aminotransferase
OEOE_RS01615	**2.39**	hypothetical protein
OEOE_RS01620	**2.40**	PTS sugar transporter
OEOE_RS01625	**4.17**	PTS cellobiose transporter subunit IIA
OEOE_RS01630	**4.00**	6 -phospho -beta -glucosidase

OEOE_RS01635	**3.03**	6 -phospho -beta -glucosidase
OEOE_RS01645	**1.57**	PTS fructose transporter subunit IIC
OEOE_RS01650	**1.98**	hypothetical protein
OEOE_RS01655	0.86	acetyltransferase
OEOE_RS01665	0.33	haloacid dehalogenase
OEOE_RS01670	0.97	glycerol uptake permease
OEOE_RS01675	-1.30	thioredoxin
OEOE_RS01680	-1.38	hypothetical protein
OEOE_RS01685	-1.41	hypothetical protein
OEOE_RS01690	-0.99	DNA-binding protein
OEOE_RS01695	-0.31	copper chaperone
OEOE_RS01700	-0.25	Crp/Fnr family transcriptional regulator
OEOE_RS01705	0.74	transposase
OEOE_RS01710	-1.45	metal transporter CorA
OEOE_RS01720	0.54	
OEOE_RS01730	-1.04	
OEOE_RS01740	**2.75**	membrane protein
OEOE_RS01745	**1.51**	
OEOE_RS01750	**1.72**	
OEOE_RS01755	-0.34	
OEOE_RS01760	-1.15	
OEOE_RS01765	-2.10	
OEOE_RS01770	-0.40	
OEOE_RS01775	-0.24	
OEOE_RS01780	-0.16	
OEOE_RS01790	0.21	3-beta-hydroxysteroid dehydrogenase
OEOE_RS01795	-0.51	
OEOE_RS01800	0.67	
OEOE_RS01805	-4.75	uracil transporter
OEOE_RS01810	-1.41	
OEOE_RS01815	-0.44	
OEOE_RS01825	0.84	PTS fructose transporter subunit IIC
OEOE_RS01830	**1.15**	PTS fructose transporter subunit IID
OEOE_RS01835	**1.31**	PTS sugar transporter subunit IIA
OEOE_RS01845	-1.71	MFS transporter permease
OEOE_RS01860	-3.01	4-aminobutyrate aminotransferase
OEOE_RS01865	-2.72	amino acid permease
OEOE_RS01875	0.96	
OEOE_RS01880	0.80	
OEOE_RS01885	0.98	
OEOE_RS01890	**1.20**	
OEOE_RS01895	-0.35	threonine dehydrogenase

OEOE_RS01905	0.47	carbonyl reductase
OEOE_RS01910	**1.36**	
OEOE_RS01920	0.79	
OEOE_RS01940	-0.36	glyceraldehyde - 3 -phosphate dehydrogenase
OEOE_RS01945	0.27	
OEOE_RS01955	0.39	
OEOE_RS01960	-1.89	oxidoreductase
OEOE_RS01965	-0.73	
OEOE_RS01970	0.21	
OEOE_RS01975	-2.02	
OEOE_RS01980	**1.49**	
OEOE_RS01985	0.25	lactate dehydrogenase
OEOE_RS01990	0.46	cysteine ABC transporter ATP -binding protein
OEOE_RS01995	**1.19**	cysteine ABC transporter ATP binding protein
OEOE_RS02000	**1.10**	glucosaminidase
OEOE_RS02005	0.74	citrate lyase
OEOE_RS02010	0.59	malate dehydrogenase
OEOE_RS02015	0.95	malate permease
OEOE_RS02020	**1.35**	[citrate [pro-3S]-lyase] ligase
OEOE_RS02025	**1.89**	citrate lyase ACP
OEOE_RS02030	**1.79**	citrate lyase
OEOE_RS02035	**1.73**	citrate lyase subunit alpha
OEOE_RS02040	**2.18**	
OEOE_RS02045	**2.89**	
OEOE_RS02050	**2.09**	ATP -dependent DNA helicase
OEOE_RS02055	**1.37**	DNA ligase
OEOE_RS02065	**2.04**	ribosome maturation factor RimP
OEOE_RS02070	**1.86**	transcription termination factor NusA
OEOE_RS02075	**1.97**	
OEOE_RS02080	**2.08**	50S ribosomal protein L7ae
OEOE_RS02085	**1.75**	translation initiation factor IF-2
OEOE_RS02090	**1.72**	ribosome-binding factor A
OEOE_RS02095	-2.67	transcriptional regulator
OEOE_RS02100	**1.18**	
OEOE_RS02105	-1.42	
OEOE_RS02110	-1.50	peptide ABC transporter permease
OEOE_RS02115	-1.44	multidrug ABC transporter ATP -binding protein
OEOE_RS02120	-0.73	serine--tRNA ligase
OEOE_RS02125	-0.31	phosphosulfolactate synthase
OEOE_RS02135	-1.18	branched-chain amino acid transporter II carrier protein
OEOE_RS02140	0.80	
OEOE_RS02145	-1.97	

OEOE_RS02150	-0.90	
OEOE_RS02155	-0.67	
OEOE_RS02160	-0.29	
OEOE_RS02165	-1.57	
OEOE_RS02170	-1.95	
OEOE_RS02175	-1.65	
OEOE_RS02180	-0.48	
OEOE_RS02185	-0.53	
OEOE_RS02190	-1.75	
OEOE_RS02195	-2.09	
OEOE_RS02200	-0.75	
OEOE_RS02205	0.54	
OEOE_RS02210	-0.22	flavodoxin
OEOE_RS02215	-0.94	threonyl-tRNA synthase
OEOE_RS02220	-2.20	aminopeptidase C
OEOE_RS02225	-1.85	potassium transporter Kef
OEOE_RS02230	**1.51**	PTS mannose transporter subunit IIAB
OEOE_RS02235	**1.69**	PTS alpha-glucoside transporter subunit IIBC
OEOE_RS02240	**1.93**	PTS mannose transporter subunit IID
OEOE_RS02245	0.90	
OEOE_RS02250	**1.16**	
OEOE_RS02255	**3.44**	
OEOE_RS02260	**5.18**	
OEOE_RS02270	0.77	
OEOE_RS02275	**3.00**	
OEOE_RS02280	**4.14**	
OEOE_RS02285	**3.90**	
OEOE_RS02290	0.57	
OEOE_RS02295	0.64	
OEOE_RS02300	**1.13**	
OEOE_RS02305	-0.29	membrane protein
OEOE_RS02315	0.80	phospholipid phosphatase
OEOE_RS02325	0.90	
OEOE_RS02330	-0.42	PhoB family transcriptional regulator
OEOE_RS02335	0.56	histidine kinase
OEOE_RS02340	**1.45**	FMN-binding protein
OEOE_RS02345	**1.53**	
OEOE_RS02350	0.97	iron reductase
OEOE_RS02355	0.34	
OEOE_RS02365	**1.53**	NADPH:quinone reductase
OEOE_RS02375	**1.06**	ArsR family transcriptional regulator
OEOE_RS02395	**2.28**	hypothetical protein
OEOE_RS02400	0.80	MFS transporter

OEOE_RS02420	0.34	
OEOE_RS02425	0.95	
OEOE_RS02430	**1.38**	
OEOE_RS02435	**1.95**	diguanylate cyclase
OEOE_RS02440	**1.21**	hypothetical protein
OEOE_RS02445	0.89	
OEOE_RS02450	-0.26	
OEOE_RS02455	-0.21	NADH-dependent flavin oxidoreductase
OEOE_RS02460	**2.98**	elongation factor 3
OEOE_RS02465	0.73	2,5-diketo-D-gluconic acid reductase
OEOE_RS02470	0.56	ADP -ribose pyrophosphatase
OEOE_RS02475	**1.39**	
OEOE_RS02480	0.95	
OEOE_RS02485	**1.29**	
OEOE_RS02490	-0.64	
OEOE_RS02500	-1.43	
OEOE_RS02505	-1.04	
OEOE_RS02510	0.41	zinc -dependent alcohol dehydrogenase
OEOE_RS02520	-0.64	
OEOE_RS02525	-1.42	
OEOE_RS02530	-1.04	
OEOE_RS02535	-0.60	
OEOE_RS02545	-0.81	
OEOE_RS02550	-1.17	
OEOE_RS02555	-0.81	xanthine phosphoribosyltransferase
OEOE_RS02560	0.42	
OEOE_RS02565	-0.81	MarR family transcriptional regulator
OEOE_RS02580	**2.87**	hydrolase
OEOE_RS02585	**1.91**	
OEOE_RS02590	**1.45**	
OEOE_RS02595	**1.44**	amidase
OEOE_RS02600	0.37	
OEOE_RS02605	**1.80**	
OEOE_RS02610	**1.16**	
OEOE_RS02615	**1.21**	DNA repair protein RadA
OEOE_RS02620	**1.35**	sulfonate ABC transporter ATP-binding protein
OEOE_RS02630	0.69	
OEOE_RS02635	-0.59	
OEOE_RS02645	0.36	
OEOE_RS02650	0.46	
OEOE_RS02655	0.68	
OEOE_RS02660	0.97	phosphate ABC transporter ATP -binding protein
OEOE_RS02665	**1.05**	

OEOE_RS02670	-1.82	PhoU family transcriptional regulator
OEOE_RS02675	-0.75	
OEOE_RS02690	-0.45	
OEOE_RS02695	0.40	thioredoxin reductase
OEOE_RS02700	0.43	
OEOE_RS02705	0.57	
OEOE_RS02710	**1.84**	sporulation protein
OEOE_RS02720	**3.46**	
OEOE_RS02725	**4.84**	
OEOE_RS02730	0.28	
OEOE_RS02735	-0.51	dipeptidase
OEOE_RS02740	-1.65	
OEOE_RS02745	-1.69	
OEOE_RS02750	-1.41	
OEOE_RS02755	-1.58	
OEOE_RS02760	-2.21	
OEOE_RS02765	-0.95	
OEOE_RS02770	-0.64	elongation factor 4
OEOE_RS02775	-0.48	
OEOE_RS02785	-0.87	NADPH:quinone reductase
OEOE_RS02790	-0.93	
OEOE_RS02795	-0.58	
OEOE_RS02800	-0.57	
OEOE_RS02805	**1.21**	N-acetylmuramoyl-L-alanine amidase
OEOE_RS02810	-0.35	histidyl-tRNA synthetase
OEOE_RS02815	0.34	
OEOE_RS02820	0.24	
OEOE_RS02825	0.50	
OEOE_RS02830	0.98	
OEOE_RS02835	**1.74**	
OEOE_RS02840	-0.67	30S ribosomal protein S10
OEOE_RS02845	-0.68	50S ribosomal protein L3
OEOE_RS02850	-0.61	50S ribosomal protein L4
OEOE_RS02855	-0.50	
OEOE_RS02860	-0.50	50S ribosomal protein L2
OEOE_RS02865	-0.37	30S ribosomal protein S19
OEOE_RS02870	-0.42	50S ribosomal protein L22
OEOE_RS02875	-0.54	30S ribosomal protein S3
OEOE_RS02880	-0.61	50S ribosomal protein L16
OEOE_RS02885	-0.65	50S ribosomal protein L29
OEOE_RS02890	-0.66	30S ribosomal protein S17
OEOE_RS02895	-0.82	50S ribosomal protein L14

OEOE_RS02900	-0.67	50S ribosomal protein L24
OEOE_RS02905	-0.76	50S ribosomal protein L5
OEOE_RS02910	-0.20	30S ribosomal protein S8
OEOE_RS02915	-0.26	50S ribosomal protein L6
OEOE_RS02940	0.28	
OEOE_RS02950	-1.09	
OEOE_RS02955	-0.69	
OEOE_RS02960	-0.46	30S ribosomal protein S11
OEOE_RS02970	0.33	50S ribosomal protein L17
OEOE_RS02975	-0.68	cobalt ABC transporter
OEOE_RS02980	-0.18	cobalt ABC transporter
OEOE_RS02990	0.48	
OEOE_RS02995	-1.59	
OEOE_RS03000	0.36	
OEOE_RS03005	0.34	30S ribosomal protein S9
OEOE_RS03010	-0.27	spermidine/putrescine import ATP-binding protein PotA
OEOE_RS03025	0.51	
OEOE_RS03030	**1.46**	membrane protein
OEOE_RS03035	-1.07	glucosamine--fructose - 6 -phosphate aminotransferase
OEOE_RS03040	-1.21	glucose - 6 -phosphate isomerase
OEOE_RS03045	-0.31	
OEOE_RS03050	-0.66	phosphoglycerate kinase
OEOE_RS03055	**2.06**	membrane protein
OEOE_RS03060	**1.38**	
OEOE_RS03065	-0.39	
OEOE_RS03070	-0.57	
OEOE_RS03075	-1.15	phosphocarrier protein HPr
OEOE_RS03085	0.50	
OEOE_RS03090	0.67	
OEOE_RS03100	-0.56	
OEOE_RS03105	-0.59	
OEOE_RS03110	-0.16	membrane protein
OEOE_RS03115	-1.06	
OEOE_RS03120	-1.64	
OEOE_RS03125	-0.36	
OEOE_RS03130	-0.45	
OEOE_RS03135	-0.17	
OEOE_RS03145	-1.66	
OEOE_RS03150	-1.34	
OEOE_RS03155	-2.37	F0F1 ATP synthase subunit A
OEOE_RS03160	-1.50	
OEOE_RS03165	-1.39	
OEOE_RS03170	-1.34	ATP synthase subunit delta

OEOE_RS03175	-1.25	F0F1 ATP synthase subunit alpha
OEOE_RS03180	-0.69	ATP synthase subunit gamma
OEOE_RS03185	-0.71	
OEOE_RS03190	-0.83	F0F1 ATP synthase subunit epsilon
OEOE_RS03195	-1.69	
OEOE_RS03200	-1.54	rod shape-determining protein
OEOE_RS03205	-1.53	
OEOE_RS03210	-0.71	
OEOE_RS03215	0.48	
OEOE_RS03220	-2.51	
OEOE_RS03225	-0.64	
OEOE_RS03240	-0.43	
OEOE_RS03245	-0.24	
OEOE_RS03250	0.21	
OEOE_RS03260	-0.64	
OEOE_RS03270	-2.45	
OEOE_RS03275	-2.44	
OEOE_RS03280	0.28	
OEOE_RS03285	0.27	
OEOE_RS03290	0.75	
OEOE_RS03295	0.88	
OEOE_RS03300	**1.49**	
OEOE_RS03305	**1.15**	
OEOE_RS03310	0.35	
OEOE_RS03315	-0.29	
OEOE_RS03325	**1.16**	acetoin reductase
OEOE_RS03345	-0.86	
OEOE_RS03350	-0.97	
OEOE_RS03355	-0.56	alpha/ β hydrolase
OEOE_RS03360	-1.56	
OEOE_RS03370	-1.20	
OEOE_RS03380	-0.65	MFS transporter
OEOE_RS03385	**2.64**	chloride channel protein
OEOE_RS03390	0.68	
OEOE_RS03395	-3.85	
OEOE_RS03400	-0.56	
OEOE_RS03405	-0.26	
OEOE_RS03410	-0.30	membrane protein
OEOE_RS03420	-1.58	
OEOE_RS03425	**1.36**	spermidine N1 -acetyltransferase
OEOE_RS03435	0.75	D -alanyl - D -alanine carboxypeptidase
OEOE_RS03440	0.49	acetyl esterase
OEOE_RS03445	**2.57**	multidrug ABC transporter ATPase

OEOE_RS03450	**2.59**	multidrug ABC transporter permease
OEOE_RS03455	**1.51**	transcriptional regulator
OEOE_RS03460	**1.08**	
OEOE_RS03465	**1.93**	
OEOE_RS03470	**2.50**	NADPH-quinone reductase
OEOE_RS03475	**3.30**	MarR family transcriptional regulator
OEOE_RS03480	**2.72**	
OEOE_RS03490	**1.36**	
OEOE_RS03495	**1.51**	
OEOE_RS03500	**3.83**	TetR family transcriptional regulator
OEOE_RS03505	**3.99**	
OEOE_RS03510	**4.08**	
OEOE_RS03515	**3.38**	
OEOE_RS03520	**1.95**	multidrug ABC transporter permease
OEOE_RS03525	**2.59**	
OEOE_RS03530	-0.56	2'-5' RNA ligase
OEOE_RS03535	-0.62	methyltransferase
OEOE_RS03540	0.94	
OEOE_RS03545	**3.39**	hypothetical protein
OEOE_RS03560	**3.20**	threonine dehydrogenase
OEOE_RS03565	0.40	hypothetical protein
OEOE_RS03585	0.74	NADH-flavin reductase
OEOE_RS03590	**1.66**	
OEOE_RS03595	**1.77**	ribulose -phosphate 3 -epimerase
OEOE_RS03600	-0.89	
OEOE_RS03605	-0.51	membrane protein
OEOE_RS03610	-0.65	
OEOE_RS03620	**1.42**	
OEOE_RS03625	-1.88	short-chain dehydrogenase
OEOE_RS03630	-1.66	
OEOE_RS03635	0.44	
OEOE_RS03640	**1.03**	multidrug ABC transporter permease
OEOE_RS03650	**1.03**	
OEOE_RS03655	**1.80**	
OEOE_RS03660	**2.11**	cystathionine β -lyase
OEOE_RS03665	**1.64**	
OEOE_RS03670	0.69	
OEOE_RS03675	-1.92	formate--tetrahydrofolate ligase
OEOE_RS03680	-0.88	50S ribosomal protein L32
OEOE_RS03690	-1.12	
OEOE_RS03695	-1.14	
OEOE_RS03700	-1.38	

OEOE_RS03705	-1.24	4 -hydroxy -tetrahydrodipicolinate synthase
OEOE_RS03710	-0.66	dihydrodipicolinate reductase
OEOE_RS03715	-1.28	
OEOE_RS03720	-1.21	
OEOE_RS03725	-1.55	
OEOE_RS03730	-1.44	5 -formyltetrahydrofolate cyclo -ligase
OEOE_RS03735	-0.85	
OEOE_RS03740	-0.93	
OEOE_RS03745	-2.61	
OEOE_RS03750	-1.52	
OEOE_RS03755	-0.94	metallophosphatase
OEOE_RS03760	-1.46	
OEOE_RS03765	-1.10	Primosomal protein N'
OEOE_RS03770	-1.74	Methionyl-tRNA formyltransferase
OEOE_RS03775	-0.72	
OEOE_RS03780	-0.42	Ser/Thr protein kinase
OEOE_RS03785	-1.25	ribulose -phosphate 3 -epimerase
OEOE_RS03790	-1.40	
OEOE_RS03795	-0.72	elongation factor Tu
OEOE_RS03800	-1.73	XRE family transcriptional regulator
OEOE_RS03805	-0.31	type VI secretion protein ImpB
OEOE_RS03810	0.64	DNA polymerase III subunit alpha
OEOE_RS03820	**1.20**	
OEOE_RS03825	0.93	30S ribosomal protein S16
OEOE_RS03830	0.62	methylated DNA -protein cysteine methyltransferase
OEOE_RS03835	0.78	ribosome maturation factor
OEOE_RS03840	0.61	
OEOE_RS03860	0.68	4-oxalocrotonate tautomerase
OEOE_RS03865	-2.51	
OEOE_RS03870	-1.08	
OEOE_RS03875	-0.76	GTP -binding protein
OEOE_RS03880	0.44	LacI family transcriptional regulator
OEOE_RS03885	**5.54**	
OEOE_RS03890	**4.11**	
OEOE_RS03895	**4.87**	
OEOE_RS03900	**5.79**	
OEOE_RS03905	**5.09**	
OEOE_RS03910	**3.48**	
OEOE_RS03920	-0.66	
OEOE_RS03935	**1.48**	MFS transporter
OEOE_RS03940	0.53	
OEOE_RS03950	-1.42	
OEOE_RS03955	-1.07	

OEOE_RS03960	-0.67	
OEOE_RS03965	**2.28**	
OEOE_RS03970	0.19	2,5 -diketo - D -gluconic acid reductase
OEOE_RS03975	0.67	
OEOE_RS03985	-0.99	energy-coupling factor transporter ATP-binding protein EcfA2
OEOE_RS03990	-1.40	
OEOE_RS03995	-1.64	
OEOE_RS04000	-1.74	hypothetical protein
OEOE_RS04010	-0.39	acetyltransferase
OEOE_RS04015	-0.62	membrane protein
OEOE_RS04020	-2.59	haloacid dehalogenase
OEOE_RS04025	-2.41	hypothetical protein
OEOE_RS04030	-1.66	S -adenosylmethionine synthase
OEOE_RS04035	-1.24	leucine--tRNA ligase
OEOE_RS04040	-1.29	peptide ABC transporter substrate -binding protein
OEOE_RS04045	-2.11	peptide ABC transporter substrate -binding protein
OEOE_RS04050	-1.79	peptide ABC transporter permease
OEOE_RS04055	-1.97	
OEOE_RS04060	-1.89	
OEOE_RS04065	-1.72	
OEOE_RS04070	-0.28	
OEOE_RS04080	-0.97	
OEOE_RS04085	-3.88	deoxyadenosine kinase
OEOE_RS04095	**2.02**	
OEOE_RS04100	**1.51**	
OEOE_RS04105	**1.16**	
OEOE_RS04110	0.46	
OEOE_RS04115	-0.53	
OEOE_RS04120	-0.76	
OEOE_RS04130	-0.84	
OEOE_RS04135	**1.51**	
OEOE_RS04140	**1.48**	
OEOE_RS04150	0.50	
OEOE_RS04155	-0.98	
OEOE_RS04165	-1.45	acetyltransferase
OEOE_RS04170	-0.49	MFS transporter
OEOE_RS04175	0.59	
OEOE_RS04180	-1.85	
OEOE_RS04185	-0.83	
OEOE_RS04190	0.56	FMN reductase
OEOE_RS04195	**3.14**	
OEOE_RS04200	**2.93**	multidrug MFS transporter
OEOE_RS04205	**2.07**	

OEOE_RS04210	**1.49**	
OEOE_RS04215	**1.19**	ribonucleoside -diphosphate reductase
OEOE_RS04220	0.86	ACP phosphodiesterase
OEOE_RS04225	0.67	peptidoglycan interpeptide bridge formation protein
OEOE_RS04230	0.18	
OEOE_RS04235	0.61	sensor histidine kinase
OEOE_RS04240	0.54	
OEOE_RS04245	**1.39**	oxalate:formate antiporter
OEOE_RS04250	**1.12**	
OEOE_RS04255	-0.23	glutathione peroxidase
OEOE_RS04260	-0.81	
OEOE_RS04265	-2.13	
OEOE_RS04270	-2.00	
OEOE_RS04275	0.45	6 -phosphogluconate dehydrogenase
OEOE_RS04280	-0.98	
OEOE_RS04285	-1.15	
OEOE_RS04290	-1.06	aspartate aminotransferase
OEOE_RS04295	-0.32	adenylyltransferase
OEOE_RS04300	-0.89	
OEOE_RS04305	-1.61	
OEOE_RS04310	-0.80	arginine--tRNA ligase
OEOE_RS04315	-0.43	carboxypeptidase
OEOE_RS04320	-2.24	
OEOE_RS04325	-2.08	
OEOE_RS04330	-2.33	
OEOE_RS04335	-2.06	3' -5' exoribonuclease
OEOE_RS04340	-0.95	
OEOE_RS04345	-0.72	
OEOE_RS04350	-0.98	
OEOE_RS04355	-1.05	
OEOE_RS04360	-1.12	
OEOE_RS04365	-1.12	
OEOE_RS04370	-0.26	
OEOE_RS04375	**1.06**	
OEOE_RS04380	**1.30**	
OEOE_RS04385	**1.17**	
OEOE_RS04390	**1.48**	
OEOE_RS04400	0.45	phenylalanine--tRNA ligase subunit alpha
OEOE_RS04405	**1.24**	
OEOE_RS04410	**1.63**	
OEOE_RS04415	-0.22	glucokinase
OEOE_RS04420	-1.40	
OEOE_RS04425	-1.81	

OEOE_RS04435	**1.15**	
OEOE_RS04440	**1.10**	ArsR family transcriptional regulator
OEOE_RS04445	0.57	
OEOE_RS04450	-0.32	
OEOE_RS04455	-0.48	
OEOE_RS04460	-0.48	HIT family hydrolases
OEOE_RS04465	-0.65	membrane protein
OEOE_RS04470	0.63	5'-3'-deoxyribonucleotidase
OEOE_RS04475	0.62	
OEOE_RS04485	0.37	pyruvate oxidase
OEOE_RS04490	**1.27**	
OEOE_RS04500	-3.17	argininosuccinate synthase
OEOE_RS04505	-1.49	
OEOE_RS04510	0.50	
OEOE_RS04520	-0.80	
OEOE_RS04525	-1.18	
OEOE_RS04530	-0.82	hypothetical protein
OEOE_RS04535	-1.08	pyrroline - 5 -carboxylate reductase
OEOE_RS04540	-1.30	
OEOE_RS04545	-1.04	gamma -glutamyl phosphate reductase
OEOE_RS04550	-0.60	
OEOE_RS04555	0.36	
OEOE_RS04560	**1.09**	glutamine synthetase
OEOE_RS04565	0.85	glutamine synthetase
OEOE_RS04570	-1.20	
OEOE_RS04575	-1.02	
OEOE_RS04580	-1.76	
OEOE_RS04585	-0.81	
OEOE_RS04590	-0.50	50S ribosomal protein L21
OEOE_RS04595	-1.03	50S ribosomal protein L27
OEOE_RS04600	-1.22	Xaa-Pro aminopeptidase
OEOE_RS04605	-0.64	
OEOE_RS04610	**2.51**	
OEOE_RS04615	**3.66**	
OEOE_RS04630	0.99	DNA polymerase III, delta subunit
OEOE_RS04635	**1.37**	
OEOE_RS04640	**2.24**	
OEOE_RS04650	-1.34	nucleoside 2 -deoxyribosyltransferase
OEOE_RS04655	-0.37	LexA repressor
OEOE_RS04665	-0.37	
OEOE_RS04670	-0.76	
OEOE_RS04675	0.32	
OEOE_RS04680	-0.42	Small ribosomal subunit protein uS2

OEOE_RS04695	0.49	Ribosome-recycling factor
OEOE_RS04700	0.99	
OEOE_RS04705	**1.19**	
OEOE_RS04710	**1.09**	nucleoside 2 -deoxyribosyltransferase
OEOE_RS04715	0.51	
OEOE_RS04720	0.59	
OEOE_RS04735	0.86	
OEOE_RS04740	**1.67**	endoribonuclease YbeY
OEOE_RS04745	**1.77**	diacylglycerol kinase
OEOE_RS04750	0.36	GTPase Era
OEOE_RS04755	0.34	DNA repair protein RecO
OEOE_RS04760	-3.31	peptidase M20
OEOE_RS04770	-0.42	
OEOE_RS04775	-0.79	glycine--tRNA ligase subunit β
OEOE_RS04780	**2.27**	DNA primase
OEOE_RS04785	**2.22**	RNA polymerase sigma factor RpoD
OEOE_RS04790	**3.63**	SAM-dependent methyltransferase
OEOE_RS04795	0.93	
OEOE_RS04800	-0.56	
OEOE_RS04805	-0.56	DNA polymerase III subunit alpha
OEOE_RS04810	0.35	pyruvate kinase
OEOE_RS04815	-0.96	Tyrosine recombinase xerD
OEOE_RS04820	-0.94	
OEOE_RS04825	-0.43	Segregation and condensation protein B
OEOE_RS04830	-0.18	ribosomal large subunit pseudouridine synthase
OEOE_RS04835	-0.26	
OEOE_RS04840	-0.32	Cytidylate kinase
OEOE_RS04845	0.23	GTPase Der
OEOE_RS04850	-2.18	transcriptional regulator
OEOE_RS04855	-0.46	
OEOE_RS04860	-0.75	
OEOE_RS04865	-0.90	dihydrofolate reductase
OEOE_RS04870	-0.38	
OEOE_RS04875	0.70	lysophospholipase
OEOE_RS04880	0.51	
OEOE_RS04885	0.31	
OEOE_RS04890	-0.22	Ribosome biogenesis GTPase A
OEOE_RS04895	-0.22	
OEOE_RS04900	0.81	DNA-binding protein
OEOE_RS04905	-0.71	DNA topoisomerase I
OEOE_RS04910	-0.56	
OEOE_RS04915	-0.60	tyrosine recombinase XerC

OEOE_RS04920	-1.05	galactose mutarotase
OEOE_RS04930	-0.23	DNA gyrase subunit B
OEOE_RS04935	-1.20	DNA topoisomerase 4 subunit A
OEOE_RS04940	-2.47	
OEOE_RS04945	-1.19	Large ribosomal subunit protein bL33
OEOE_RS04950	-0.59	30S ribosomal protein S14
OEOE_RS04955	-1.57	glutamine amidotransferase
OEOE_RS04960	-1.67	
OEOE_RS04965	-0.52	Uridine kinase
OEOE_RS04970	-0.71	
OEOE_RS04975	0.36	membrane protein
OEOE_RS04980	-2.57	pyridoxal biosynthesis protein
OEOE_RS04985	-2.92	
OEOE_RS04990	-1.78	
OEOE_RS04995	-1.25	
OEOE_RS05005	-1.10	Threonylcarbamoyl-AMP synthase
OEOE_RS05015	0.34	β -galactosidase
OEOE_RS05020	**1.33**	LysR family transcriptional regulator
OEOE_RS05025	-0.20	NADH:flavin oxidoreductase
OEOE_RS05030	-1.05	NADH:flavin oxidoreductase
OEOE_RS05035	-1.28	aspartate racemase
OEOE_RS05040	0.65	tannase
OEOE_RS05050	0.76	
OEOE_RS05055	-2.97	
OEOE_RS05060	**1.41**	
OEOE_RS05065	**1.70**	ABC transporter permease
OEOE_RS05070	**1.91**	methionine import ATP-binding protein MetN 1
OEOE_RS05075	**2.51**	
OEOE_RS05080	-1.42	aminopeptidase N
OEOE_RS05085	**2.05**	
OEOE_RS05090	0.81	
OEOE_RS05095	0.61	
OEOE_RS05105	-0.82	
OEOE_RS05110	-1.78	
OEOE_RS05115	-2.55	
OEOE_RS05120	-2.12	
OEOE_RS05125	-1.80	
OEOE_RS05130	-1.50	
OEOE_RS05135	-1.54	
OEOE_RS05140	-0.22	adenine phosphoribosyltransferase
OEOE_RS05145	-1.00	Single-stranded-DNA-specific exonuclease
OEOE_RS05150	-1.27	Ribonuclease Z

OEOE_RS05155	-1.30	GTPase CgtA
OEOE_RS05165	-0.77	
OEOE_RS05170	-1.41	
OEOE_RS05175	-0.87	
OEOE_RS05180	-0.90	
OEOE_RS05185	-0.63	RNA pseudouridine synthase
OEOE_RS05190	-0.40	pore-forming protein
OEOE_RS05195	-0.99	
OEOE_RS05200	**1.54**	
OEOE_RS05205	**1.98**	
OEOE_RS05210	**2.06**	
OEOE_RS05215	**2.55**	
OEOE_RS05220	**1.65**	
OEOE_RS05225	**1.10**	energy-coupling factor transporter ATP-binding protein EcfA3
OEOE_RS05230	0.78	
OEOE_RS05235	0.18	
OEOE_RS05245	-3.90	oligoendopeptidase F
OEOE_RS05250	-1.13	
OEOE_RS05255	-2.40	Holliday junction DNA helicase RecU
OEOE_RS05260	-2.80	
OEOE_RS05265	-1.55	DNA replication protein DnaD
OEOE_RS05270	-1.09	
OEOE_RS05275	-0.45	
OEOE_RS05280	-0.19	DNA helicase
OEOE_RS05285	-0.96	Mevalonate kinase
OEOE_RS05290	-0.43	diphosphomevalonate decarboxylase
OEOE_RS05295	0.62	
OEOE_RS05310	-0.37	
OEOE_RS05320	-1.62	
OEOE_RS05325	-1.73	
OEOE_RS05330	-2.18	membrane protein
OEOE_RS05335	-0.35	Fe-S cluster formation protein, NifU
OEOE_RS05340	-0.78	Fe -S cluster assembly protein SufB
OEOE_RS05345	-0.84	
OEOE_RS05350	-1.57	
OEOE_RS05355	-2.45	Fe -S cluster assembly ABC -type transport system,. ATPase component
OEOE_RS05360	-2.60	Anaerobic ribonucleoside-triphosphate reductase-activating protein
OEOE_RS05365	-2.61	Ribonucleoside-triphosphate reductase class III catalytic subunit / ribonucleoside-triphosphate reductase
OEOE_RS05370	-0.77	
OEOE_RS05375	-0.96	Arginine deiminase

OEOE_RS05380	0.17	
OEOE_RS05390	-1.01	
OEOE_RS05400	-2.90	GMP synthase
OEOE_RS05405	-3.58	
OEOE_RS05410	-3.70	adenylosuccinate lyase
OEOE_RS05415	-3.74	
OEOE_RS05420	-4.99	inosine - 5 -monophosphate dehydrogenase
OEOE_RS05425	0.60	Phosphoribosylamine--glycine ligase
OEOE_RS05430	-0.59	bifunctional purine biosynthesis protein
OEOE_RS05435	0.52	phosphoribosylglycinamide formyltransferase
OEOE_RS05440	0.41	Phosphoribosylformylglycinamidine cyclo-ligase
OEOE_RS05460	-0.38	Phosphoribosylformylglycinamidine synthase subunit
OEOE_RS05470	0.92	phosphoribosylaminoimidazole carboxylase
OEOE_RS05475	**1.63**	N5-carboxyaminoimidazole ribonucleotide mutase
OEOE_RS05480	-0.91	Isoleucine--tRNA ligase
OEOE_RS05485	-2.00	cell division protein
OEOE_RS05490	-2.69	
OEOE_RS05495	-1.21	Cell division protein
OEOE_RS05500	-0.99	Cell division protein
OEOE_RS05505	-0.39	Cell division protein
OEOE_RS05510	-0.32	
OEOE_RS05515	-0.95	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase
OEOE_RS05520	-1.65	UDP-N-acetylmuramoylalanine--D-glutamate ligase
OEOE_RS05525	-1.20	
OEOE_RS05530	-1.36	
OEOE_RS05535	-2.19	cell division protein
OEOE_RS05540	-1.95	ribosomal RNA small subunit methyltransferase H
OEOE_RS05545	-2.17	transcriptional regulator MraZ
OEOE_RS05550	-0.67	
OEOE_RS05555	**1.09**	
OEOE_RS05560	0.72	
OEOE_RS05565	-0.46	
OEOE_RS05570	-1.94	
OEOE_RS05575	-2.21	pseudouridine synthase
OEOE_RS05580	-3.11	
OEOE_RS05585	-4.20	Oligoendopeptidase F
OEOE_RS05590	0.85	
OEOE_RS05595	0.63	
OEOE_RS05600	0.56	
OEOE_RS05605	-0.22	
OEOE_RS05610	-0.28	Putative pre-16S rRNA nuclease

OEOE_RS05615	-0.38	
OEOE_RS05620	-0.27	Alanine--tRNA ligase
OEOE_RS05625	-0.92	
OEOE_RS05630	-1.25	universal stress protein
OEOE_RS05635	**2.52**	
OEOE_RS05640	**2.73**	
OEOE_RS05645	**2.63**	
OEOE_RS05650	**2.23**	
OEOE_RS05655	-0.42	
OEOE_RS05660	-1.07	cyclopropane -fatty -acyl -phospholipid synthase
OEOE_RS05665	**1.78**	
OEOE_RS05670	0.94	
OEOE_RS05685	-0.79	
OEOE_RS05690	0.51	
OEOE_RS05695	**1.33**	lactate dehydrogenase
OEOE_RS05700	**2.04**	
OEOE_RS05705	**1.25**	
OEOE_RS05710	0.93	
OEOE_RS05715	0.40	
OEOE_RS05725	0.84	metal ABC transporter substrate-binding protein
OEOE_RS05735	-2.51	
OEOE_RS05740	-1.16	glutathione reductase
OEOE_RS05745	-0.40	ATPase AAA
OEOE_RS05750	-0.38	
OEOE_RS05755	0.32	
OEOE_RS05760	-1.34	
OEOE_RS05765	-1.50	haloacid dehalogenase
OEOE_RS05775	-1.18	
OEOE_RS05790	-0.77	
OEOE_RS05795	-0.50	general stress protein
OEOE_RS05810	**1.67**	deoxyribose-phosphate aldolase
OEOE_RS05815	**1.62**	
OEOE_RS05820	**1.86**	
OEOE_RS05825	**3.12**	
OEOE_RS05830	**2.24**	
OEOE_RS05835	-0.39	
OEOE_RS05840	-0.88	
OEOE_RS05845	-1.62	
OEOE_RS05850	-1.32	
OEOE_RS05855	-1.20	
OEOE_RS05860	-0.73	
OEOE_RS05865	-1.21	
OEOE_RS05870	-2.26	

OEOE_RS05875	-2.10	
OEOE_RS05880	-2.23	
OEOE_RS05885	-1.87	
OEOE_RS05890	-2.13	
OEOE_RS05895	-2.10	
OEOE_RS05900	-1.75	
OEOE_RS05905	-1.81	
OEOE_RS05910	-0.75	
OEOE_RS05915	-0.52	RNA -binding protein
OEOE_RS05920	-0.69	cytochrome O ubiquinol oxidase
OEOE_RS05925	-1.04	DNA-directed RNA polymerase subunit omega
OEOE_RS05930	-0.99	Guanylate kinase
OEOE_RS05935	-2.20	
OEOE_RS05940	-0.49	DNA repair protein
OEOE_RS05950	-0.74	
OEOE_RS05955	-0.84	Exodeoxyribonuclease 7 small subunit
OEOE_RS05960	-0.88	Exodeoxyribonuclease 7 large subunit
OEOE_RS05965	-2.05	transcription termination factor NusB
OEOE_RS05970	-2.65	
OEOE_RS05975	-2.66	Elongation factor P
OEOE_RS05980	-0.63	
OEOE_RS05990	-2.48	
OEOE_RS05995	-2.08	
OEOE_RS06000	-1.07	
OEOE_RS06005	-3.08	Transcription elongation factor GreA
OEOE_RS06010	-1.51	
OEOE_RS06015	-0.50	alcohol dehydrogenase
OEOE_RS06020	-1.95	acetate kinase
OEOE_RS06025	-0.89	
OEOE_RS06035	0.49	
OEOE_RS06055	0.31	type II secretion system protein F
OEOE_RS06065	-1.19	peptidase
OEOE_RS06070	-1.08	Phosphopantetheine adenylyltransferase
OEOE_RS06075	0.67	SAM-dependent methyltransferase
OEOE_RS06080	**1.04**	cell division protein
OEOE_RS06085	0.28	
OEOE_RS06090	-0.33	
OEOE_RS06105	**1.18**	
OEOE_RS06110	-1.47	UDP-N-acetylmuramate--L-alanine ligase
OEOE_RS06115	-1.25	
OEOE_RS06120	-2.00	
OEOE_RS06125	-1.58	
OEOE_RS06130	-1.03	

OEOE_RS06135	-0.78	
OEOE_RS06140	-1.18	
OEOE_RS06145	-1.66	
OEOE_RS06150	-1.68	
OEOE_RS06155	-0.49	50S ribosomal protein L20
OEOE_RS06160	-0.24	50S ribosomal protein L35
OEOE_RS06170	**1.10**	
OEOE_RS06175	**1.22**	
OEOE_RS06180	-1.68	30S ribosomal protein S15
OEOE_RS06185	-1.63	30S ribosomal protein S20
OEOE_RS06190	-1.73	DEAD/DEAH box helicase
OEOE_RS06195	-2.25	
OEOE_RS06200	-2.51	metal -dependent hydrolase
OEOE_RS06205	-2.07	Protein translocase subunit yajC
OEOE_RS06210	-0.75	queuine tRNA-ribosyltransferas
OEOE_RS06215	-1.43	NADPH:quinone reductase
OEOE_RS06220	-1.27	
OEOE_RS06225	-0.94	esterase
OEOE_RS06230	-0.18	
OEOE_RS06235	-0.29	
OEOE_RS06240	-2.18	
OEOE_RS06245	-1.46	branched -chain amino acid aminotransferase
OEOE_RS06250	0.42	MarR family transcriptional regulator
OEOE_RS06255	**1.04**	
OEOE_RS06260	0.27	succinate-semialdehyde dehdyrogenase
OEOE_RS06265	-0.51	
OEOE_RS06270	-1.14	
OEOE_RS06275	-1.17	
OEOE_RS06280	-1.11	
OEOE_RS06285	-0.88	
OEOE_RS06290	-1.00	
OEOE_RS06295	-2.22	
OEOE_RS06300	**1.06**	
OEOE_RS06305	**1.04**	molecular chaperone DnaJ
OEOE_RS06315	-0.51	protein GrpE
OEOE_RS06320	-1.78	
OEOE_RS06325	-2.18	Riboflavin biosynthesis protein
OEOE_RS06330	-1.17	tRNA pseudouridine synthase B
OEOE_RS06335	-0.69	elongation factor G (fusA)
OEOE_RS06340	-0.53	
OEOE_RS06350	0.24	
OEOE_RS06360	-1.09	
OEOE_RS06365	-2.35	

OEOE_RS06375	**1.10**	
OEOE_RS06380	-4.33	
OEOE_RS06390	**2.51**	
OEOE_RS06395	-0.65	
OEOE_RS06400	-0.97	alcohol dehydrogenase
OEOE_RS06405	-0.18	
OEOE_RS06410	-1.21	3-deoxy-8-phosphooctulonate synthase
OEOE_RS06415	-1.73	
OEOE_RS06420	-0.68	
OEOE_RS06425	**1.54**	
OEOE_RS06430	**1.59**	1,3 -propanediol dehydrogenase
OEOE_RS06435	**4.76**	
OEOE_RS06440	**4.12**	Transcriptional regulator, GntR family
OEOE_RS06445	**4.00**	Alpha, alpha-phosphotrehalase
OEOE_RS06450	**3.48**	PTS β-glucoside transporter subunit IIABC
OEOE_RS06455	**2.23**	PTS sugar transporter subunit IIA
OEOE_RS06460	0.97	lysophospholipase
OEOE_RS06465	**1.36**	multidrug ABC transporter permease
OEOE_RS06470	**1.59**	ArsR family transcriptional regulator
OEOE_RS06475	0.65	acetyltransferase
OEOE_RS06485	-1.15	
OEOE_RS06495	-1.31	
OEOE_RS06515	0.58	membrane protein
OEOE_RS06520	**2.63**	
OEOE_RS06525	0.45	
OEOE_RS06535	-0.58	NADPH:quinone reductase
OEOE_RS06540	**1.53**	
OEOE_RS06545	-1.86	raiA ribosome -associated inhibitor A
OEOE_RS06550	-0.97	
OEOE_RS06555	-0.73	
OEOE_RS06565	-0.58	acyltransferase
OEOE_RS06570	-0.72	acyl-CoA synthetase
OEOE_RS06575	-0.38	membrane protein
OEOE_RS06580	-0.80	
OEOE_RS06585	-0.60	
OEOE_RS06595	-0.24	
OEOE_RS06600	-0.47	
OEOE_RS06605	-0.36	
OEOE_RS06610	**1.44**	DNA -directed RNA polymerase subunit β'
OEOE_RS06615	**1.20**	DNA -directed RNA polymerase subunit β
OEOE_RS06620	**2.27**	cold -shock protein
OEOE_RS06625	**2.03**	

OEOE_RS06630	**1.44**	
OEOE_RS06635	**1.05**	
OEOE_RS06640	0.90	
OEOE_RS06645	0.34	
OEOE_RS06655	**1.20**	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase
OEOE_RS06660	**1.38**	
OEOE_RS06665	**2.29**	
OEOE_RS06670	**1.65**	
OEOE_RS06675	**1.24**	Cardiolipin synthase
OEOE_RS06685	-1.16	Cell shape-determining protein MreC
OEOE_RS06690	-0.74	
OEOE_RS06695	-0.20	
OEOE_RS06705	0.82	
OEOE_RS06710	0.73	Septation ring formation regulator EzrA
OEOE_RS06715	0.89	30S ribosomal protein S4
OEOE_RS06720	-3.84	Nicotinamide mononucleotide transporter
OEOE_RS06722	0.91	molecular chaperone GroEL
OEOE_RS06730	0.59	Co -chaperonin GroES (HSP10)
OEOE_RS06735	-0.20	transcriptional regulator
OEOE_RS06745	-0.53	
OEOE_RS06750	-1.03	tRNA N6-adenosine threonylcarbamoyltransferase
OEOE_RS06755	-1.24	UDP-glucose 4-epimerase
OEOE_RS06765	-0.56	
OEOE_RS06770	-0.27	
OEOE_RS06775	-1.02	
OEOE_RS06785	0.50	
OEOE_RS06790	0.70	Recombination protein RecR
OEOE_RS06800	**1.54**	
OEOE_RS06805	**1.20**	haloacid dehalogenase
OEOE_RS06810	0.37	deaminase
OEOE_RS06815	0.46	16S RNA G1207 methylase RsmC
OEOE_RS06820	0.42	
OEOE_RS06825	**2.12**	50S ribosomal protein L7/L12
OEOE_RS06830	**2.29**	50S ribosomal protein L10
OEOE_RS06835	**1.08**	50S ribosomal protein L1
OEOE_RS06840	0.87	50S ribosomal protein L11
OEOE_RS06845	**1.57**	
OEOE_RS06850	0.77	gluconokinase
OEOE_RS06855	0.53	Transcription termination/antitermination protein NusG
OEOE_RS06860	-0.38	Protein translocase subunit secE
OEOE_RS06880	-2.09	
OEOE_RS06885	-1.19	

OEOE_RS06890	-0.92	ABC transporter ATP-binding protein
OEOE_RS06895	-0.76	
OEOE_RS06900	-1.24	
OEOE_RS06905	-1.79	peptidoglycan-binding protein
OEOE_RS06915	-0.99	
OEOE_RS06920	**1.10**	
OEOE_RS06925	**1.45**	
OEOE_RS06935	0.41	
OEOE_RS06940	0.72	
OEOE_RS06945	0.83	SsrA-binding protein
OEOE_RS06950	**1.08**	Ribonuclease R
OEOE_RS06955	0.14	ATP-dependent DNA helicase
OEOE_RS06960	-0.76	
OEOE_RS06965	0.45	peptidoglycan interpeptide bridge formation protein
OEOE_RS06970	-0.53	sortase
OEOE_RS06975	-0.65	peptidoglycan interpeptide bridge formation protein
OEOE_RS06980	-0.36	dTDP - 4 -dehydrorhamnose reductase
OEOE_RS06985	-0.21	malate transporter
OEOE_RS06990	**2.20**	dTDP -glucose 4,6 -dehydratase
OEOE_RS06995	**2.10**	dTDP - 4 -dehydrorhamnose 3,5 -epimerase
OEOE_RS07000	**1.98**	glucose - 1 -phosphate thymidylyltransferase
OEOE_RS07005	**3.70**	cell filamentation protein Fic
OEOE_RS07010	**2.04**	glycosyl transferase
OEOE_RS07015	0.89	30S ribosomal protein S4
OEOE_RS07020	-0.92	
OEOE_RS07025	**1.95**	AraC family transcriptional regulator
OEOE_RS07030	-0.51	glycerol-3-phosphate ABC transporter ATP-binding protein
OEOE_RS07035	0.44	glycerol - 3 -phosphate ABC transporter permease
OEOE_RS07040	**1.07**	glycerol - 3 -phosphate ABC transporter permease
OEOE_RS07045	0.64	
OEOE_RS07050	0.38	glycerophosphoryl diester phosphodiesterase
OEOE_RS07055	**3.91**	
OEOE_RS07060	**3.66**	
OEOE_RS07065	**3.50**	
OEOE_RS07070	**4.27**	spermidine/putrescine ABC transporter ATP-binding protein
OEOE_RS07075	**4.43**	spermidine/putrescine ABC transporter ATP-binding protein
OEOE_RS07080	**3.63**	spermidine/putrescine ABC transporter permease
OEOE_RS07085	**3.86**	GntR family transcriptional regulator
OEOE_RS07090	**4.17**	
OEOE_RS07095	**4.36**	allantoin permease
OEOE_RS07100	**4.80**	
OEOE_RS07105	**4.29**	membrane protein

OEOE_RS07110	**2.70**	6 -pyruvoyl -tetrahydropterin synthase
OEOE_RS07115	**2.46**	glycosyl transferase
OEOE_RS07120	**1.54**	Exosortase family protein XrtG
OEOE_RS07125	**1.48**	
OEOE_RS07130	0.48	
OEOE_RS07135	0.31	
OEOE_RS07140	**3.07**	
OEOE_RS07145	**2.20**	
OEOE_RS07150	**1.93**	
OEOE_RS07155	-0.48	
OEOE_RS07160	0.47	
OEOE_RS07165	-0.59	
OEOE_RS07170	0.51	
OEOE_RS07175	-0.26	
OEOE_RS07180	**1.03**	
OEOE_RS07185	**1.26**	
OEOE_RS07190	**1.21**	
OEOE_RS07195	**1.27**	
OEOE_RS07200	-1.16	
OEOE_RS07205	-1.87	
OEOE_RS07215	0.91	
OEOE_RS07220	0.60	
OEOE_RS07230	-1.31	
OEOE_RS07235	-0.98	
OEOE_RS07240	-1.07	
OEOE_RS07245	-0.78	glycosyl transferase family 1
OEOE_RS07250	-1.66	glycosyl transferase
OEOE_RS07255	-0.96	UDP -phosphate galactose phosphotransferase
OEOE_RS07260	-1.18	
OEOE_RS07265	-1.29	
OEOE_RS07270	-1.55	
OEOE_RS07280	**2.29**	
OEOE_RS07285	**2.09**	
OEOE_RS07295	**2.13**	glycosyl transferase
OEOE_RS07300	**2.10**	
OEOE_RS07305	**1.76**	
OEOE_RS07320	-0.99	
OEOE_RS07325	-0.90	
OEOE_RS07330	-0.69	
OEOE_RS07340	-0.67	
OEOE_RS07350	**1.14**	
OEOE_RS07355	-1.33	DNA replication initiation protein
OEOE_RS07360	-1.50	

OEOE_RS07370	**1.02**	
OEOE_RS07375	-0.57	
OEOE_RS07390	-0.19	sodium ABC transporter permease
OEOE_RS07400	-0.96	
OEOE_RS07405	**1.37**	MFS transporter
OEOE_RS07410	-0.43	
OEOE_RS07415	0.35	peptide methionine sulfoxide reductase
OEOE_RS07420	**4.60**	
OEOE_RS07425	**3.24**	cobalt ABC transporter ATPase
OEOE_RS07430	**1.66**	
OEOE_RS07435	0.96	bifunctional protein GlmU
OEOE_RS07440	**1.01**	purine operon repressor
OEOE_RS07445	**1.32**	
OEOE_RS07450	0.59	Threonine synthase
OEOE_RS07455	**1.20**	homoserine dehydrogenase
OEOE_RS07460	**1.01**	Homoserine kinase
OEOE_RS07465	**1.28**	
OEOE_RS07470	0.29	ribosomal RNA small subunit methyltransferase A
OEOE_RS07480	-0.21	
OEOE_RS07485	-0.67	
OEOE_RS07490	-1.47	2,5 -diketo - D -gluconic acid reductase
OEOE_RS07495	-1.21	methionine sulfoxide reductase B
OEOE_RS07500	-0.58	
OEOE_RS07505	-0.23	
OEOE_RS07520	-0.61	cysteine--tRNA ligase
OEOE_RS07525	-1.31	17-β-hydroxysteroid dehydrogenase
OEOE_RS07530	-1.76	D-alanyl-D-alanine carboxypeptidase
OEOE_RS07535	-1.09	S-ribosylhomocysteine lyase
OEOE_RS07540	-1.35	
OEOE_RS07545	-1.42	
OEOE_RS07550	0.62	
OEOE_RS07560	-0.31	membrane protein
OEOE_RS07565	-0.56	capsular polysaccharide biosynthesis protein
OEOE_RS07570	0.56	
OEOE_RS07585	**1.44**	AraC family transcriptional regulator
OEOE_RS07590	**1.12**	
OEOE_RS07595	**1.67**	
OEOE_RS07600	**1.04**	AraC family transcriptional regulator
OEOE_RS07610	-0.22	
OEOE_RS07615	-1.46	uracil phosphoribosyltransferase
OEOE_RS07620	-0.94	
OEOE_RS07625	-1.23	Peptide chain release factor 2

OEOE_RS07630	-2.29	Peptide chain release factor 1
OEOE_RS07645	-3.26	Acetyl-coenzyme A carboxylase carboxyl transferase
OEOE_RS07650	-4.09	3-hydroxyacyl-[acyl-carrier-protein] dehydratase
OEOE_RS07655	-4.56	Acetyl-coenzyme A carboxylase carboxyl transferase
OEOE_RS07660	-4.80	3-oxoacyl-[acyl-carrier-protein] synthase 2
OEOE_RS07665	-4.45	3 -ketoacyl -ACP reductase
OEOE_RS07670	-4.52	ACP S-malonyltransferase
OEOE_RS07675	-4.44	2 -nitropropane dioxygenase
OEOE_RS07680	-4.44	
OEOE_RS07685	-4.61	
OEOE_RS07690	-4.80	
OEOE_RS07695	-4.90	3-hydroxyacyl-[acyl-carrier-protein] dehydratase
OEOE_RS07700	-0.86	
OEOE_RS07705	-1.14	biotin transporter
OEOE_RS07710	-2.29	thymidine kinase
OEOE_RS07715	-2.14	
OEOE_RS07720	-1.93	
OEOE_RS07730	0.75	diacetyl reductase
OEOE_RS07735	-1.13	
OEOE_RS07740	-1.15	
OEOE_RS07745	-4.61	membrane protein
OEOE_RS07750	-2.55	heme ABC transporter ATP-binding protein
OEOE_RS07755	-2.44	ABC transporter permease
OEOE_RS07760	-2.82	sugar ABC transporter permease
OEOE_RS07765	-2.92	
OEOE_RS07770	-1.56	ribose 5 -phosphate isomerase
OEOE_RS07775	-3.84	ribokinase
OEOE_RS07780	-3.32	D -ribose pyranase
OEOE_RS07785	-2.08	Fucose permease
OEOE_RS07790	-1.80	peptide ABC transporter substrate-binding protein
OEOE_RS07795	-1.78	peptide ABC transporter permease
OEOE_RS07800	-2.36	
OEOE_RS07805	-2.66	
OEOE_RS07810	-2.33	
OEOE_RS07815	**1.67**	membrane protein
OEOE_RS07820	**1.43**	glycosyl transferase
OEOE_RS07830	-1.27	
OEOE_RS07835	0.18	thiol -disulfide isomerase
OEOE_RS07845	0.34	
OEOE_RS07855	-2.37	Polyphosphate kinase
OEOE_RS07860	-2.69	peptidase M13
OEOE_RS07870	-0.90	

OEOE_RS07875	-0.99	
OEOE_RS07880	-1.36	
OEOE_RS07885	**1.62**	
OEOE_RS07890	**1.26**	multidrug ABC transporter ATP -binding protein
OEOE_RS07895	-0.53	
OEOE_RS07905	-1.06	peptidylprolyl isomerase
OEOE_RS07910	0.63	
OEOE_RS07915	0.36	alanine racemase
OEOE_RS07920	0.66	holo-ACP synthase
OEOE_RS07925	**1.10**	DEAD/DEAH box helicase
OEOE_RS07930	0.94	UDP-Nacetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase
OEOE_RS07935	**1.96**	
OEOE_RS07945	0.88	
OEOE_RS07950	**1.55**	
OEOE_RS07955	-0.30	saccharopine dehydrogenase
OEOE_RS07960	-0.69	enolase
OEOE_RS07965	-0.25	sugar ABC transporter ATP-binding protein
OEOE_RS07975	0.97	
OEOE_RS07980	**1.49**	
OEOE_RS07985	**1.30**	
OEOE_RS07990	0.53	
OEOE_RS07995	0.65	
OEOE_RS08000	**1.58**	UDP - N -acetylenolpyruvoylglucosamine reductase
OEOE_RS08005	0.46	
OEOE_RS08015	0.27	
OEOE_RS08020	0.68	
OEOE_RS08025	0.34	DNA polymerase III subunit alpha
OEOE_RS08030	0.28	tRNA N6-adenosine threonylcarbamoyltransferase
OEOE_RS08035	0.48	amino acid ABC transporter substrate-binding protein
OEOE_RS08040	0.59	polar amino acid ABC transporter ATPase
OEOE_RS08045	0.57	amino acid ABC transporter permease
OEOE_RS08050	-0.46	
OEOE_RS08055	**1.97**	sugar phosphate isomerase
OEOE_RS08060	**2.31**	
OEOE_RS08075	-0.26	
OEOE_RS08080	**1.05**	RNA methyltransferase
OEOE_RS08085	0.43	lipid kinase
OEOE_RS08100	**2.77**	ammonia permease
OEOE_RS08105	**1.67**	
OEOE_RS08110	**1.57**	
OEOE_RS08115	**2.86**	
OEOE_RS08120	**1.62**	
OEOE_RS08125	0.67	

OEOE_RS08130	0.86	TetR family transcriptional regulator
OEOE_RS08135	**4.85**	
OEOE_RS08140	**5.01**	
OEOE_RS08145	**2.29**	
OEOE_RS08150	**1.01**	macrolide ABC transporter ATP-binding protein
OEOE_RS08155	0.83	threonine dehydrogenase
OEOE_RS08160	-1.25	
OEOE_RS08165	-1.27	
OEOE_RS08170	-1.78	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B
OEOE_RS08175	-2.05	Glutamyl-tRNA(Gln) amidotransferase subunit A
OEOE_RS08180	-2.31	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C
OEOE_RS08185	0.32	
OEOE_RS08190	-1.43	
OEOE_RS08195	-1.72	Regulatory protein
OEOE_RS08200	-1.88	Methionine aminopeptidase
OEOE_RS08205	-0.73	
OEOE_RS08210	-1.58	
OEOE_RS08215	0.89	thioredoxin
OEOE_RS08220	-1.48	Alpha-acetolactate synthase
OEOE_RS08225	-1.67	
OEOE_RS08230	-0.45	glyoxalase
OEOE_RS08240	**1.07**	alcohol dehydrogenase
OEOE_RS08245	**1.18**	fructokinase
OEOE_RS08250	-1.77	
OEOE_RS08255	**1.26**	
OEOE_RS08260	0.96	multidrug ABC transporter ATP -binding protein
OEOE_RS08265	0.52	multidrug ABC transporter ATP -binding protein
OEOE_RS08275	-0.84	glucose transporter
OEOE_RS08280	0.55	
OEOE_RS08285	-0.52	ABC transporter substrate-binding protein
OEOE_RS08290	-0.49	
OEOE_RS08305	0.25	
OEOE_RS08310	**3.27**	
OEOE_RS08315	**3.01**	3-isopropylmalate dehydrogenase
OEOE_RS08320	**2.11**	3-isopropylmalate dehydratase large subunit
OEOE_RS08325	**1.43**	3-isopropylmalate dehydratase small subunit
OEOE_RS08330	0.85	
OEOE_RS08340	-1.31	ATP-dependent Clp protease ATP-binding subunit
OEOE_RS08345	-0.75	Trigger factor
OEOE_RS08360	0.35	RNA polymerase I and III, subunit
OEOE_RS08365	0.60	glucosaminidase
OEOE_RS08375	-1.48	glycosyl transferase

OEOE_RS08380	-2.44	UDP -glucose 6 -dehydrogenase
OEOE_RS08385	-1.11	glycosyl transferase
OEOE_RS08395	-1.93	capsular polysaccharide biosynthesis protein
OEOE_RS08400	-1.70	UDP -galactop yranose mutase
OEOE_RS08405	-0.87	glycosyl transferase
OEOE_RS08410	-0.98	exopolysaccharide biosynthesis protein
OEOE_RS08415	-0.49	multidrug MFS transporter
OEOE_RS08420	0.21	membrane protein
OEOE_RS08425	0.29	methionyl -tRNA synthetase
OEOE_RS08430	**1.20**	amino acid transporter
OEOE_RS08440	**1.59**	ferritin
OEOE_RS08445	**1.48**	hypothetical protein
OEOE_RS08450	-0.64	transposase
OEOE_RS08455	**1.16**	restriction endonuclease
OEOE_RS08465	**1.68**	
OEOE_RS08470	0.27	
OEOE_RS08475	0.21	serine O -acetyltransferase
OEOE_RS08485	0.24	
OEOE_RS08500	**2.10**	
OEOE_RS08505	**2.18**	
OEOE_RS08510	**1.72**	MarR family transcriptional regulator
OEOE_RS08515	0.60	
OEOE_RS08520	-0.61	oxidoreductase
OEOE_RS08525	-0.26	
OEOE_RS08530	**1.31**	
OEOE_RS08540	-0.50	histidine kinase
OEOE_RS08545	-0.33	
OEOE_RS08550	**2.40**	
OEOE_RS08555	**2.47**	
OEOE_RS08560	**1.10**	
OEOE_RS08565	0.73	
OEOE_RS08570	0.95	
OEOE_RS08575	-0.66	
OEOE_RS08580	0.78	alcohol dehydrogenase
OEOE_RS08585	-0.30	alpha -galactosidase
OEOE_RS08590	-0.41	aldo/keto reductase
OEOE_RS08595	-0.90	peptidase C69
OEOE_RS08600	-1.07	50S ribosomal protein L31 type B
OEOE_RS08615	-1.03	DNA -directed RNA polymerase subunit delta
OEOE_RS08620	0.21	
OEOE_RS08635	0.63	
OEOE_RS08640	**1.37**	

OEOE_RS08645	**2.83**	universal stress protein UspA
OEOE_RS08650	**3.32**	
OEOE_RS08655	0.18	
OEOE_RS08660	-1.03	
OEOE_RS08665	-0.58	Phosphoenolpyruvate carboxylase
OEOE_RS08670	-0.44	3-hydroxy-3-methylglutaryl-CoA reductase
OEOE_RS08675	-1.32	tryptophan--tRNA ligase
OEOE_RS08680	**1.01**	
OEOE_RS08685	0.71	
OEOE_RS08690	0.23	
OEOE_RS08700	-0.36	
OEOE_RS08710	0.60	
OEOE_RS08715	-0.36	
OEOE_RS08720	0.99	
OEOE_RS08725	**1.24**	
OEOE_RS08730	-0.45	
OEOE_RS08735	0.65	
OEOE_RS08745	**3.82**	prolyl-tRNA synthetase
OEOE_RS08750	**4.44**	
OEOE_RS08755	0.87	Tyrosine--tRNA ligase
OEOE_RS08765	0.99	
OEOE_RS08770	-1.37	pyruvate oxidase
OEOE_RS08785	0.81	
OEOE_RS08795	-1.24	
OEOE_RS08800	-1.81	
OEOE_RS08810	-0.47	
OEOE_RS08820	**2.02**	
OEOE_RS08830	**1.37**	glycosyl transferase
OEOE_RS08835	**1.62**	
OEOE_RS08840	**3.06**	
OEOE_RS08845	**3.53**	
OEOE_RS08850	**2.76**	
OEOE_RS08855	**2.32**	1,4 -dihydroxy - 2 -naphthoate octaprenyltransferase
OEOE_RS08860	**1.05**	
OEOE_RS08865	**1.77**	cytochrome C oxidase assembly protein
OEOE_RS08870	**1.47**	cytochrome D ubiquinol oxidase subunit
OEOE_RS08875	0.85	pyridine nucleotide-disulfide oxidoreductase
OEOE_RS08880	-2.82	peptidoglycan-binding protein
OEOE_RS08885	-0.48	
OEOE_RS08890	-1.12	CAAX amino protease
OEOE_RS08900	0.50	permease
OEOE_RS08905	0.46	
OEOE_RS08910	0.77	haloacid dehalogenase

OEOE_RS08915	-0.86	
OEOE_RS08920	-0.92	
OEOE_RS08925	0.93	
OEOE_RS08935	**1.81**	O-acetyltransferase
OEOE_RS08940	**1.94**	osmotically inducible protein C
OEOE_RS08945	**1.34**	
OEOE_RS08950	0.67	
OEOE_RS08955	-1.11	
OEOE_RS08965	-0.64	
OEOE_RS08970	-2.44	2,5-diketo-D-gluconic acid reductase
OEOE_RS08975	-0.92	oxidoreductase
OEOE_RS08980	0.31	
OEOE_RS08985	0.22	
OEOE_RS08990	**1.09**	membrane protein
OEOE_RS08995	0.48	ribonuclease P protein component
OEOE_RS09000	0.71	
OEOE_RS09010	**1.88**	
OEOE_RS09015	**1.37**	
OEOE_RS09025	0.28	
OEOE_RS09030	**1.37**	
OEOE_RS09055	-0.68	
OEOE_RS09080	-1.06	
OEOE_RS09095	-0.37	
OEOE_RS09120	-1.13	
OEOE_RS09125	0.59	
OEOE_RS09150	-0.51	
OEOE_RS09155	-0.46	
OEOE_RS09175	0.67	
OEOE_RS09195	**1.12**	
OEOE_RS09210	0.85	
OEOE_RS09225	-0.35	
OEOE_RS09230	-0.68	Ribonuclease P protein component
OEOE_RS09235	-1.50	
OEOE_RS09250	-0.60	
OEOE_RS09255	0.32	
OEOE_RS09265	-1.40	
OEOE_RS09275	-1.03	
OEOE_RS09285	0.67	
OEOE_RS09300	**1.34**	
OEOE_RS09305	-0.71	
OEOE_RS09325	**1.24**	
OEOE_RS09340	-0.69	
OEOE_RS09355	**1.15**	

OEOE_RS09360	**1.12**
OEOE_RS09365	-0.70
OEOE_RS09430	**2.33**
OEOE_RS09445	-0.45
OEOE_RS09450	-1.06
OEOE_RS09460	0.53
OEOE_RS09470	0.29
OEOE_RS09475	**2.76**
OEOE_RS09480	-0.84
OEOE_RS09485	-1.21
OEOE_RS09490	**1.36**
OEOE_RS09500	-0.78
OEOE_RS09505	**2.56**
OEOE_RS09510	**1.57**
OEOE_RS09515	**1.19**
OEOE_RS09525	-0.93
OEOE_RS09530	-1.05
OEOE_RS09535	**2.86**
OEOE_RS09540	-1.54
OEOE_RS09555	0.81
OEOE_RS09560	0.40
OEOE_RS09565	-3.30
OEOE_RS09570	**3.40**
OEOE_RS09575	-0.98
OEOE_RS09580	-0.58
OEOE_RS09585	0.37
OEOE_RS09595	-0.90
OEOE_RS09600	**1.53**
OEOE_RS09605	**2.13**
OEOE_RS09610	**1.87**
OEOE_RS09615	**1.62**
OEOE_RS09620	**2.15**
OEOE_RS09625	**1.28**
OEOE_RS09630	**2.04**
OEOE_RS09635	**1.04**
OEOE_RS09640	0.84
OEOE_RS09650	0.72
OEOE_RS09665	**1.89**
OEOE_RS09670	**1.07**
OEOE_RS09675	0.87
OEOE_RS09680	0.61
OEOE_RS09685	-3.15
OEOE_RS09690	0.70
OEOE_RS09700	0.98

OEOE_RS09705	-1.23
OEOE_RS09710	-0.21
OEOE_RS09715	0.68
OEOE_RS09720	**2.19**
OEOE_RS09725	**2.49**
OEOE_RS09730	-0.28
OEOE_RS09735	0.69
OEOE_RS09740	0.73
OEOE_RS09745	-0.69
OEOE_RS09750	**2.11**
OEOE_RS09755	0.68
OEOE_RS09760	0.87
OEOE_RS09765	**1.10**
OEOE_RS09770	0.35
OEOE_RS09775	0.61
OEOE_RS09780	0.90
OEOE_RS09785	**4.68**
OEOE_RS09790	**5.84**
OEOE_RS09810	-1.80
OEOE_RS09815	**3.87**
OEOE_RS09820	**3.77**
OEOE_RS09825	-0.68
OEOE_RS09830	**1.26**
OEOE_RS09835	-0.76
OEOE_RS09840	-1.68
OEOE_RS09850	0.49
OEOE_RS09870	-1.29
OEOE_RS09875	-0.95
OEOE_RS09880	**1.08**
OEOE_RS09885	0.42
OEOE_RS09890	-0.36
OEOE_RS09895	-0.40
OEOE_RS09900	-1.03
OEOE_RS09905	**1.34**
OEOE_RS09910	**2.46**
OEOE_RS09915	**2.42**
OEOE_RS09920	**2.51**
OEOE_RS09925	**1.58**
OEOE_RS09940	0.79
OEOE_RS09950	-0.26
OEOE_RS09960	**1.35**
OEOE_RS09965	**1.76**
OEOE_RS09970	**1.27**
OEOE_RS09980	-2.70

OEOE_RS09985	-0.32
OEOE_RS09990	-1.22
OEOE_RS10000	0.23

7. Anexos

Supplementary Table 2. Representative functional categories (COG: clusters of orthologous groups) found with FUNAGE-Pro platform for genes (Locus tag) of *Oenococcus oeni* PSU-1 where differential expression (Log2 Fold Change) was found in medium with 2 g/L succinic acid (WLMS) compared to control without it (WLM). Log2FC values are marked in red or dark blue depending on whether the transcripts were down or up-regulated, respectively. Description is the probable locus function. Complete GO description is shown in this file: https://rovira-my.sharepoint.com/:x:/r/personal/39882121-y_epp_urv_cat/_layouts/15/Doc.aspx?sourcedoc=%7B74F5E175-5824-4A57-9B2E-857A184CEED0%7D&file=Supp%20Tab%202b%20COG%20with%20GO%2020230821.xlsx&action=default&mobileredirect=true

Locus tag	Log2 FC	Functional categories (COG)	Description
OEOE_RS00005	-1.78	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	Chromosomal replication initiator protein dnaA
OEOE_RS00010	-1.25	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	DNA polymerase III subunit beta
OEOE_RS00025	1.07	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	DNA gyrase subunit B
OEOE_RS00030	2.02	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	DNA gyrase subunit A
OEOE_RS00035	-3.34	H METABOLISM; Coenzyme transport and metabolism	NH(3)-dependent NAD(+) synthetase
OEOE_RS00065	-1.71	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	Large ribosomal subunit protein bL9
OEOE_RS00070	-1.05	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	
OEOE_RS00115	-2.28	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS00120	-1.63	C METABOLISM; Energy production and conversion	2 -hydroxyacid dehydrogenase
OEOE_RS00135	1.39	C METABOLISM; Energy production and conversion	
OEOE_RS00165	1.76	C METABOLISM; Energy production and conversion	oxidoreductase ion channel protein IolS
OEOE_RS00175	2.77	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS00185	1.67	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS00195	1.03	K INFORMATION STORAGE AND PROCESSING; Transcription	
OEOE_RS00205	3.49	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	oxidoreductase
OEOE_RS00250	2.14	S POORLY CHARACTERIZED; Function unknown	membrane protein
OEOE_RS00295	1.19	I METABOLISM; Lipid transport and metabolism	phytoene synthase
OEOE_RS00350	-1.14	S POORLY CHARACTERIZED; Function unknown	diacetyl reductase [(S)-acetoin forming]
OEOE_RS00355	1.63	S POORLY CHARACTERIZED; Function unknown	short-chain dehydrogenase
OEOE_RS00450	2.13	F METABOLISM; Nucleotide transport and metabolism	
OEOE_RS00490	3.37	T CELLULAR PROCESSES AND SIGNALING; Signal transduction mechanisms	histidine kinase

OEOE_RS00495	2.11	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	serine protease
OEOE_RS00500	2.32	S POORLY CHARACTERIZED; Function unknown	Ribosomal RNA large subunit methyltransferase H
OEOE_RS00515	1.01	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	Ribosomal RNA small subunit methyltransferase G
OEOE_RS00565	-2.35	G METABOLISM; Carbohydrate transport and metabolism	phosphoglyceromutase
OEOE_RS00575	-1.17	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS00605	1.42	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS00645	-3.41	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	glutaredoxin
OEOE_RS00650	-3.36	F METABOLISM; Nucleotide transport and metabolism	ribonucleotide-diphosphate reductase subunit alpha
OEOE_RS00655	-3.67	F METABOLISM; Nucleotide transport and metabolism	ribonucleoside-diphosphate reductase
OEOE_RS00715	1.10	E METABOLISM; Amino acid transport and metabolism	3 -phosphoshikimate 1 -carboxyvinyltransferase
OEOE_RS00720	1.33	E METABOLISM; Amino acid transport and metabolism	shikimate kinase
OEOE_RS00735	1.13	G METABOLISM; Carbohydrate transport and metabolism	NAD(P)H -hydrate epimerase
OEOE_RS00755	-1.27	F METABOLISM; Nucleotide transport and metabolism	ribose -phosphate pyrophosphokinase
OEOE_RS00800	2.13	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS00850	2.28	K INFORMATION STORAGE AND PROCESSING; Transcription	TetR family transcriptional regulator
OEOE_RS00855	3.77	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS00945	1.62	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	
OEOE_RS00950	1.80	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	
OEOE_RS00975	1.19	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	multidrug ABC transporter ATPase
OEOE_RS01025	-1.12	E METABOLISM; Amino acid transport and metabolism	threonine dehydrogenase
OEOE_RS01045	1.28	G METABOLISM; Carbohydrate transport and metabolism	PTS sugar transporter subunit IIA
OEOE_RS01050	1.46	G METABOLISM; Carbohydrate transport and metabolism	PTS cellobiose transporter subunit IIA
OEOE_RS01055	1.72	G METABOLISM; Carbohydrate transport and metabolism	PTS sugar transporter
OEOE_RS01100	-1.76	G METABOLISM; Carbohydrate transport and metabolism	aldose 1 -epimerase
OEOE_RS01150	2.16	E METABOLISM; Amino acid transport and metabolism	alpha/β hydrolase
OEOE_RS01155	1.43	S POORLY CHARACTERIZED; Function unknown	3-beta-hydroxysteroid dehydrogenase
OEOE_RS01160	-1.06	C METABOLISM; Energy production and conversion	oxidoreductase ion channel protein IolS
OEOE_RS01220	2.44	G METABOLISM; Carbohydrate transport and metabolism	sugar ABC transporter permease
OEOE_RS01225	2.27	K INFORMATION STORAGE AND PROCESSING; Transcription	
OEOE_RS01240	-7.15	F METABOLISM; Nucleotide transport and metabolism	dihydroorotase
OEOE_RS01245	-6.38	F METABOLISM; Nucleotide transport and metabolism	carbamoyl phosphate synthase small subuni
OEOE_RS01250	-6.13	F METABOLISM; Nucleotide transport and metabolism	carbamoyl -phosphate synthase large chain

OEOE_RS01255	-5.69	F METABOLISM; Nucleotide transport and metabolism	orotidine 5' -phosphate decarboxylase
OEOE_RS01265	-4.62	F METABOLISM; Nucleotide transport and metabolism	dihydroorotate dehydrogenase
OEOE_RS01335	-2.54	H METABOLISM; Coenzyme transport and metabolism	dihydroxynaphthoic acid synthetase
OEOE_RS01350	2.18	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS01375	2.71	H METABOLISM; Coenzyme transport and metabolism	D-alanine--poly(phosphoribitol) ligase
OEOE_RS01380	3.12	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	D-alanyl-lipoteichoic acid biosynthesis protein DltD
OEOE_RS01385	4.44	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	heat-shock protein Hsp20
OEOE_RS01390	1.63	P METABOLISM; Inorganic ion transport and metabolism	MFS transporter permease
OEOE_RS01415	1.66	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS01420	1.34	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS01520	1.54	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	DNA repair protein RadA
OEOE_RS01525	1.72	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS01530	1.08	F METABOLISM; Nucleotide transport and metabolism	nucleoside -triphosphate diphosphatase
OEOE_RS01565	1.98	H METABOLISM; Coenzyme transport and metabolism	lipoate -protein ligase A
OEOE_RS01570	2.82	C METABOLISM; Energy production and conversion	pyruvate dehydrogenase E1 subunit alpha
OEOE_RS01615	2.39	S POORLY CHARACTERIZED; Function unknown	hypothetical protein
OEOE_RS01620	2.40	G METABOLISM; Carbohydrate transport and metabolism	PTS sugar transporter
OEOE_RS01625	4.17	G METABOLISM; Carbohydrate transport and metabolism	PTS cellobiose transporter subunit IIA
OEOE_RS01630	4.00	G METABOLISM; Carbohydrate transport and metabolism	6 -phospho -beta -glucosidase
OEOE_RS01635	3.03	G METABOLISM; Carbohydrate transport and metabolism	6 -phospho -beta -glucosidase
OEOE_RS01645	1.57	G METABOLISM; Carbohydrate transport and metabolism	PTS fructose transporter subunit IIC
OEOE_RS01675	-1.30	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	thioredoxin
OEOE_RS01730	-1.04	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	
OEOE_RS01830	1.15	G METABOLISM; Carbohydrate transport and metabolism	PTS fructose transporter subunit IID
OEOE_RS01860	-3.01	E METABOLISM; Amino acid transport and metabolism	4-aminobutyrate aminotransferase
OEOE_RS01865	-2.72	E METABOLISM; Amino acid transport and metabolism	amino acid permease
OEOE_RS01960	-1.89	G METABOLISM; Carbohydrate transport and metabolism	oxidoreductase
OEOE_RS01975	-2.02	K INFORMATION STORAGE AND PROCESSING; Transcription	
OEOE_RS01980	1.49	K INFORMATION STORAGE AND PROCESSING; Transcription	
OEOE_RS01995	1.19	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	cysteine ABC transporter ATP binding protein
OEOE_RS02025	1.89	C METABOLISM; Energy production and conversion	citrate lyase ACP

OEOE_RS02040	2.18	H METABOLISM; Coenzyme transport and metabolism	
OEOE_RS02050	2.09	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	ATP -dependent DNA helicase
OEOE_RS02055	1.37	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	DNA ligase
OEOE_RS02065	2.04	S POORLY CHARACTERIZED; Function unknown	ribosome maturation factor RimP
OEOE_RS02070	1.86	K INFORMATION STORAGE AND PROCESSING; Transcription	transcription termination factor NusA
OEOE_RS02075	1.97	K INFORMATION STORAGE AND PROCESSING; Transcription	
OEOE_RS02085	1.75	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	translation initiation factor IF-2
OEOE_RS02090	1.72	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	ribosome-binding factor A
OEOE_RS02095	-2.67	K INFORMATION STORAGE AND PROCESSING; Transcription	transcriptional regulator
OEOE_RS02105	-1.42	F METABOLISM; Nucleotide transport and metabolism	
OEOE_RS02135	-1.18	E METABOLISM; Amino acid transport and metabolism	branched-chain amino acid transporter II carrier protein
OEOE_RS02145	-1.97	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	
OEOE_RS02165	-1.57	I METABOLISM; Lipid transport and metabolism	
OEOE_RS02170	-1.95	I METABOLISM; Lipid transport and metabolism	
OEOE_RS02190	-1.75	U CELLULAR PROCESSES AND SIGNALING; Intracellular trafficking, secretion, and vesicular transport	
OEOE_RS02235	1.69	G METABOLISM; Carbohydrate transport and metabolism	PTS alpha-glucoside transporter subunit IIBC
OEOE_RS02240	1.93	G METABOLISM; Carbohydrate transport and metabolism	PTS mannose transporter subunit IID
OEOE_RS02250	1.16	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	
OEOE_RS02280	4.14	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS02440	1.21	K INFORMATION STORAGE AND PROCESSING; Transcription	hypothetical protein
OEOE_RS02460	2.98	S POORLY CHARACTERIZED; Function unknown	elongation factor 3
OEOE_RS02485	1.29	K INFORMATION STORAGE AND PROCESSING; Transcription	
OEOE_RS02610	1.16	P METABOLISM; Inorganic ion transport and metabolism	
OEOE_RS02615	1.21	P METABOLISM; Inorganic ion transport and metabolism	DNA repair protein RadA
OEOE_RS02665	1.05	P METABOLISM; Inorganic ion transport and metabolism	
OEOE_RS02710	1.84	K INFORMATION STORAGE AND PROCESSING; Transcription	sporulation protein
OEOE_RS02750	-1.41	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	
OEOE_RS02755	-1.58	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	
OEOE_RS02760	-2.21	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	
OEOE_RS02805	1.21	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	N-acetylmuramoyl-L-alanine amidase
OEOE_RS02950	-1.09	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	

OEOE_RS03035	-1.07	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	glucosamine--fructose - 6 -phosphate aminotransferase
OEOE_RS03040	-1.21	G METABOLISM; Carbohydrate transport and metabolism	glucose - 6 -phosphate isomerase
OEOE_RS03060	1.38	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	
OEOE_RS03075	-1.15	G METABOLISM; Carbohydrate transport and metabolism	phosphocarrier protein HPr
OEOE_RS03120	-1.64	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	
OEOE_RS03145	-1.66	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS03150	-1.34	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS03155	-2.37	C METABOLISM; Energy production and conversion	F0F1 ATP synthase subunit A
OEOE_RS03160	-1.50	C METABOLISM; Energy production and conversion	
OEOE_RS03165	-1.39	C METABOLISM; Energy production and conversion	
OEOE_RS03170	-1.34	C METABOLISM; Energy production and conversion	ATP synthase subunit delta
OEOE_RS03175	-1.25	C METABOLISM; Energy production and conversion	F0F1 ATP synthase subunit alpha
OEOE_RS03200	-1.54	D CELLULAR PROCESSES AND SIGNALING; Cell cycle control, cell division, chromosome partitioning	rod shape-determining protein
OEOE_RS03220	-2.51	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	
OEOE_RS03325	1.16	S POORLY CHARACTERIZED; Function unknown	acetoin reductase
OEOE_RS03360	-1.56	C METABOLISM; Energy production and conversion	
OEOE_RS03370	-1.20	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS03425	1.36	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	spermidine N1 -acetyltransferase
OEOE_RS03455	1.51	T CELLULAR PROCESSES AND SIGNALING; Signal transduction mechanisms	transcriptional regulator
OEOE_RS03465	1.93	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS03490	1.36	D CELLULAR PROCESSES AND SIGNALING; Cell cycle control, cell division, chromosome partitioning	
OEOE_RS03495	1.51	D CELLULAR PROCESSES AND SIGNALING; Cell cycle control, cell division, chromosome partitioning	
OEOE_RS03510	4.08	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	
OEOE_RS03525	2.59	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	
OEOE_RS03560	3.20	E METABOLISM; Amino acid transport and metabolism	threonine dehydrogenase
OEOE_RS03590	1.66	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS03595	1.77	E METABOLISM; Amino acid transport and metabolism	ribulose -phosphate 3 -epimerase
OEOE_RS03620	1.42	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS03625	-1.88	S POORLY CHARACTERIZED; Function unknown	short-chain dehydrogenase
OEOE_RS03660	2.11	E METABOLISM; Amino acid transport and metabolism	cystathionine β -lyase
OEOE_RS03665	1.64	E METABOLISM; Amino acid transport and metabolism	

OEOE_RS03675	-1.92	F METABOLISM; Nucleotide transport and metabolism	formate--tetrahydrofolate ligase
OEOE_RS03690	-1.12	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS03695	-1.14	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS03700	-1.38	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS03705	-1.24	E METABOLISM; Amino acid transport and metabolism	4 -hydroxy -tetrahydrodipicolinate synthase
OEOE_RS03715	-1.28	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS03720	-1.21	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS03725	-1.55	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS03730	-1.44	H METABOLISM; Coenzyme transport and metabolism	5 -formyltetrahydrofolate cyclo -ligase
OEOE_RS03745	-2.61	F METABOLISM; Nucleotide transport and metabolism	
OEOE_RS03750	-1.52	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS03760	-1.46	F METABOLISM; Nucleotide transport and metabolism	
OEOE_RS03765	-1.10	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	Primosomal protein N'
OEOE_RS03770	-1.74	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	Methionyl-tRNA formyltransferase
OEOE_RS03785	-1.25	G METABOLISM; Carbohydrate transport and metabolism	ribulose -phosphate 3 -epimerase
OEOE_RS03870	-1.08	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	
OEOE_RS03935	1.48	G METABOLISM; Carbohydrate transport and metabolism	MFS transporter
OEOE_RS03990	-1.40	P METABOLISM; Inorganic ion transport and metabolism	
OEOE_RS04020	-2.59	S POORLY CHARACTERIZED; Function unknown	haloacid dehalogenase
OEOE_RS04030	-1.66	H METABOLISM; Coenzyme transport and metabolism	S -adenosylmethionine synthase
OEOE_RS04035	-1.24	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	leucine--tRNA ligase
OEOE_RS04040	-1.29	E METABOLISM; Amino acid transport and metabolism	peptide ABC transporter substrate -binding protein
OEOE_RS04050	-1.79	P METABOLISM; Inorganic ion transport and metabolism	peptide ABC transporter permease
OEOE_RS04055	-1.97	P METABOLISM; Inorganic ion transport and metabolism	
OEOE_RS04065	-1.72	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS04085	-3.88	F METABOLISM; Nucleotide transport and metabolism	deoxyadenosine kinase
OEOE_RS04215	1.19	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	ribonucleoside -diphosphate reductase
OEOE_RS04245	1.39	G METABOLISM; Carbohydrate transport and metabolism	oxalate:formate antiporter
OEOE_RS04265	-2.13	H METABOLISM; Coenzyme transport and metabolism	
OEOE_RS04285	-1.15	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS04290	-1.06	E METABOLISM; Amino acid transport and metabolism	aspartate aminotransferase

OEOE_RS04320	-2.24	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS04325	-2.08	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	
OEOE_RS04330	-2.33	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS04335	-2.06	S POORLY CHARACTERIZED; Function unknown	3' -5' exoribonuclease
OEOE_RS04355	-1.05	H METABOLISM; Coenzyme transport and metabolism	
OEOE_RS04360	-1.12	H METABOLISM; Coenzyme transport and metabolism	
OEOE_RS04365	-1.12	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS04375	1.06	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS04385	1.17	U CELLULAR PROCESSES AND SIGNALING; Intracellular trafficking, secretion, and vesicular transport	
OEOE_RS04390	1.48	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	
OEOE_RS04405	1.24	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	
OEOE_RS04440	1.10	K INFORMATION STORAGE AND PROCESSING; Transcription	ArsR family transcriptional regulator
OEOE_RS04500	-3.17	E METABOLISM; Amino acid transport and metabolism	argininosuccinate synthase
OEOE_RS04525	-1.18	T CELLULAR PROCESSES AND SIGNALING; Signal transduction mechanisms	
OEOE_RS04535	-1.08	E METABOLISM; Amino acid transport and metabolism	pyrroline - 5 -carboxylate reductase
OEOE_RS04540	-1.30	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS04545	-1.04	E METABOLISM; Amino acid transport and metabolism	gamma -glutamyl phosphate reductase
OEOE_RS04560	1.09	K INFORMATION STORAGE AND PROCESSING; Transcription	glutamine synthetase
OEOE_RS04595	-1.03	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	50S ribosomal protein L27
OEOE_RS04600	-1.22	E METABOLISM; Amino acid transport and metabolism	Xaa-Pro aminopeptidase
OEOE_RS04615	3.66	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	
OEOE_RS04705	1.19	I METABOLISM; Lipid transport and metabolism	
OEOE_RS04740	1.67	F METABOLISM; Nucleotide transport and metabolism	endoribonuclease YbeY
OEOE_RS04760	-3.31	C METABOLISM; Energy production and conversion	peptidase M20
OEOE_RS04780	2.27	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	DNA primase
OEOE_RS04785	2.22	K INFORMATION STORAGE AND PROCESSING; Transcription	RNA polymerase sigma factor RpoD
OEOE_RS04850	-2.18	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	transcriptional regulator
OEOE_RS04945	-1.19	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	Large ribosomal subunit protein bL33
OEOE_RS04955	-1.57	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	glutamine amidotransferase
OEOE_RS04960	-1.67	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS04980	-2.57	H METABOLISM; Coenzyme transport and metabolism	pyridoxal biosynthesis protein

OEOE_RS04985	-2.92	H METABOLISM; Coenzyme transport and metabolism	
OEOE_RS05005	-1.10	H METABOLISM; Coenzyme transport and metabolism	Threonylcarbamoyl-AMP synthase
OEOE_RS05020	1.33	K INFORMATION STORAGE AND PROCESSING; Transcription	LysR family transcriptional regulator
OEOE_RS05035	-1.28	E METABOLISM; Amino acid transport and metabolism	aspartate racemase
OEOE_RS05060	1.41	P METABOLISM; Inorganic ion transport and metabolism	
OEOE_RS05065	1.70	P METABOLISM; Inorganic ion transport and metabolism	ABC transporter permease
OEOE_RS05070	1.91	P METABOLISM; Inorganic ion transport and metabolism	methionine import ATP-binding protein MetN 1
OEOE_RS05075	2.51	T CELLULAR PROCESSES AND SIGNALING; Signal transduction mechanisms	
OEOE_RS05080	-1.42	E METABOLISM; Amino acid transport and metabolism	aminopeptidase N
OEOE_RS05110	-1.78	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS05115	-2.55	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS05120	-2.12	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	
OEOE_RS05125	-1.80	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	
OEOE_RS05150	-1.27	S POORLY CHARACTERIZED; Function unknown	Ribonuclease Z
OEOE_RS05155	-1.30	C METABOLISM; Energy production and conversion	GTPase CgtA
OEOE_RS05215	2.55	H METABOLISM; Coenzyme transport and metabolism	
OEOE_RS05220	1.65	P METABOLISM; Inorganic ion transport and metabolism	
OEOE_RS05225	1.10	P METABOLISM; Inorganic ion transport and metabolism	energy-coupling factor transporter ATP-binding protein EcfA3
OEOE_RS05250	-1.13	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS05255	-2.40	S POORLY CHARACTERIZED; Function unknown	Holliday junction DNA helicase RecU
OEOE_RS05270	-1.09	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	
OEOE_RS05325	-1.73	F METABOLISM; Nucleotide transport and metabolism	
OEOE_RS05350	-1.57	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	
OEOE_RS05360	-2.60	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	Anaerobic ribonucleoside-triphosphate reductase-activating protein
OEOE_RS05365	-2.61	F METABOLISM; Nucleotide transport and metabolism	Ribonucleoside-triphosphate reductase class III catalytic subunit / ribonucleoside-triphosphate reductase
OEOE_RS05400	-2.90	F METABOLISM; Nucleotide transport and metabolism	GMP synthase
OEOE_RS05410	-3.70	F METABOLISM; Nucleotide transport and metabolism	adenylosuccinate lyase
OEOE_RS05415	-3.74	F METABOLISM; Nucleotide transport and metabolism	
OEOE_RS05420	-4.99	F METABOLISM; Nucleotide transport and metabolism	inosine - 5 -monophosphate dehydrogenase
OEOE_RS05475	1.63	F METABOLISM; Nucleotide transport and metabolism	N5-carboxyaminoimidazole ribonucleotide mutase

OEOE_RS05490	-2.69	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS05495	-1.21	S POORLY CHARACTERIZED; Function unknown	Cell division protein
OEOE_RS05520	-1.65	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	UDP-N-acetylmuramoylalanine--D-glutamate ligase
OEOE_RS05525	-1.20	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	
OEOE_RS05540	-1.95	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	ribosomal RNA small subunit methyltransferase H
OEOE_RS05545	-2.17	S POORLY CHARACTERIZED; Function unknown	transcriptional regulator MraZ
OEOE_RS05555	1.09	D CELLULAR PROCESSES AND SIGNALING; Cell cycle control, cell division, chromosome partitioning	
OEOE_RS05570	-1.94	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS05575	-2.21	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	pseudouridine synthase
OEOE_RS05580	-3.11	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS05585	-4.20	E METABOLISM; Amino acid transport and metabolism	Oligoendopeptidase F
OEOE_RS05630	-1.25	T CELLULAR PROCESSES AND SIGNALING; Signal transduction mechanisms	universal stress protein
OEOE_RS05640	2.73	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS05645	2.63	H METABOLISM; Coenzyme transport and metabolism	
OEOE_RS05650	2.23	H METABOLISM; Coenzyme transport and metabolism	
OEOE_RS05695	1.33	C METABOLISM; Energy production and conversion	lactate dehydrogenase
OEOE_RS05700	2.04	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS05705	1.25	K INFORMATION STORAGE AND PROCESSING; Transcription	
OEOE_RS05740	-1.16	C METABOLISM; Energy production and conversion	glutathione reductase
OEOE_RS05815	1.62	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS05820	1.86	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS05830	2.24	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS05965	-2.05	K INFORMATION STORAGE AND PROCESSING; Transcription	transcription termination factor NusB
OEOE_RS05975	-2.66	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	Elongation factor P
OEOE_RS05995	-2.08	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	
OEOE_RS06005	-3.08	K INFORMATION STORAGE AND PROCESSING; Transcription	Transcription elongation factor GreA
OEOE_RS06065	-1.19	T CELLULAR PROCESSES AND SIGNALING; Signal transduction mechanisms	peptidase
OEOE_RS06070	-1.08	H METABOLISM; Coenzyme transport and metabolism	Phosphopantetheine adenylyltransferase
OEOE_RS06080	1.04	D CELLULAR PROCESSES AND SIGNALING; Cell cycle control, cell division, chromosome partitioning	cell division protein
OEOE_RS06105	1.18	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS06110	-1.47	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	UDP-N-acetylmuramate--L-alanine ligase

OEOE_RS06115	-1.25	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	
OEOE_RS06125	-1.58	C METABOLISM; Energy production and conversion	
OEOE_RS06150	-1.68	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	
OEOE_RS06180	-1.68	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	30S ribosomal protein S15
OEOE_RS06190	-1.73	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	DEAD/DEAH box helicase
OEOE_RS06195	-2.25	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	
OEOE_RS06200	-2.51	S POORLY CHARACTERIZED; Function unknown	metal -dependent hydrolase
OEOE_RS06205	-2.07	U CELLULAR PROCESSES AND SIGNALING; Intracellular trafficking, secretion, and vesicular transport	Protein translocase subunit yajC
OEOE_RS06215	-1.43	C METABOLISM; Energy production and conversion	NADPH:quinone reductase
OEOE_RS06245	-1.46	E METABOLISM; Amino acid transport and metabolism	branched -chain amino acid aminotransferase
OEOE_RS06295	-2.22	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS06305	1.04	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	molecular chaperone DnaJ
OEOE_RS06330	-1.17	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	tRNA pseudouridine synthase B
OEOE_RS06365	-2.35	U CELLULAR PROCESSES AND SIGNALING; Intracellular trafficking, secretion, and vesicular transport	
OEOE_RS06410	-1.21	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	3-deoxy-8-phosphooctulonate synthase
OEOE_RS06415	-1.73	U CELLULAR PROCESSES AND SIGNALING; Intracellular trafficking, secretion, and vesicular transport	
OEOE_RS06440	4.12	K INFORMATION STORAGE AND PROCESSING; Transcription	Transcriptional regulator, GntR family
OEOE_RS06445	4.00	G METABOLISM; Carbohydrate transport and metabolism	Alpha, alpha-phosphotrehalase
OEOE_RS06450	3.48	G METABOLISM; Carbohydrate transport and metabolism	PTS β-glucoside transporter subunit IIABC
OEOE_RS06455	2.23	G METABOLISM; Carbohydrate transport and metabolism	PTS sugar transporter subunit IIA
OEOE_RS06465	1.36	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	multidrug ABC transporter permease
OEOE_RS06545	-1.86	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	raiA ribosome -associated inhibitor A
OEOE_RS06610	1.44	K INFORMATION STORAGE AND PROCESSING; Transcription	DNA -directed RNA polymerase subunit β'
OEOE_RS06615	1.20	K INFORMATION STORAGE AND PROCESSING; Transcription	DNA -directed RNA polymerase subunit β
OEOE_RS06620	2.27	K INFORMATION STORAGE AND PROCESSING; Transcription	cold -shock protein
OEOE_RS06635	1.05	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	
OEOE_RS06655	1.20	I METABOLISM; Lipid transport and metabolism	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase
OEOE_RS06675	1.24	I METABOLISM; Lipid transport and metabolism	Cardiolipin synthase
OEOE_RS06750	-1.03	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	tRNA N6-adenosine threonylcarbamoyltransferase

OEOE_RS06755	-1.24	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	UDP-glucose 4-epimerase
OEOE_RS06775	-1.02	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS06800	1.54	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS06825	2.12	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	50S ribosomal protein L7/L12
OEOE_RS06830	2.29	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	50S ribosomal protein L10
OEOE_RS06835	1.08	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	50S ribosomal protein L1
OEOE_RS06880	-2.09	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS06885	-1.19	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS06920	1.10	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS06950	1.08	K INFORMATION STORAGE AND PROCESSING; Transcription	Ribonuclease R
OEOE_RS06995	2.10	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	dTDP - 4 -dehydrorhamnose 3,5 -epimerase
OEOE_RS07000	1.98	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	glucose - 1 -phosphate thymidylyltransferase
OEOE_RS07010	2.04	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	glycosyl transferase
OEOE_RS07040	1.07	G METABOLISM; Carbohydrate transport and metabolism	glycerol - 3 -phosphate ABC transporter permease
OEOE_RS07060	3.66	F METABOLISM; Nucleotide transport and metabolism	
OEOE_RS07070	4.27	E METABOLISM; Amino acid transport and metabolism	spermidine/putrescine ABC transporter ATP-binding protein
OEOE_RS07075	4.43	P METABOLISM; Inorganic ion transport and metabolism	spermidine/putrescine ABC transporter ATP-binding protein
OEOE_RS07080	3.63	P METABOLISM; Inorganic ion transport and metabolism	spermidine/putrescine ABC transporter permease
OEOE_RS07090	4.17	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	
OEOE_RS07100	4.80	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS07115	2.46	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	glycosyl transferase
OEOE_RS07140	3.07	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS07185	1.26	P METABOLISM; Inorganic ion transport and metabolism	
OEOE_RS07195	1.27	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS07240	-1.07	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS07260	-1.18	D CELLULAR PROCESSES AND SIGNALING; Cell cycle control, cell division, chromosome partitioning	
OEOE_RS07270	-1.55	K INFORMATION STORAGE AND PROCESSING; Transcription	
OEOE_RS07295	2.13	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	glycosyl transferase
OEOE_RS07350	1.14	K INFORMATION STORAGE AND PROCESSING; Transcription	
OEOE_RS07425	3.24	P METABOLISM; Inorganic ion transport and metabolism	cobalt ABC transporter ATPase

OEOE_RS07440	1.01	K INFORMATION STORAGE AND PROCESSING; Transcription	purine operon repressor
OEOE_RS07445	1.32	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS07455	1.20	E METABOLISM; Amino acid transport and metabolism	homoserine dehydrogenase
OEOE_RS07465	1.28	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS07495	-1.21	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	methionine sulfoxide reductase B
OEOE_RS07525	-1.31	S POORLY CHARACTERIZED; Function unknown	17-β-hydroxysteroid dehydrogenase
OEOE_RS07535	-1.09	T CELLULAR PROCESSES AND SIGNALING; Signal transduction mechanisms	S-ribosylhomocysteine lyase
OEOE_RS07545	-1.42	C METABOLISM; Energy production and conversion	
OEOE_RS07615	-1.46	F METABOLISM; Nucleotide transport and metabolism	uracil phosphoribosyltransferase
OEOE_RS07625	-1.23	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	Peptide chain release factor 2
OEOE_RS07645	-3.26	I METABOLISM; Lipid transport and metabolism	Acetyl-coenzyme A carboxylase carboxyl transferase
OEOE_RS07650	-4.09	I METABOLISM; Lipid transport and metabolism	3-hydroxyacyl-[acyl-carrier-protein] dehydratase
OEOE_RS07655	-4.56	I METABOLISM; Lipid transport and metabolism	Acetyl-coenzyme A carboxylase carboxyl transferase
OEOE_RS07665	-4.45	S POORLY CHARACTERIZED; Function unknown	3 -ketoacyl -ACP reductase
OEOE_RS07685	-4.61	I METABOLISM; Lipid transport and metabolism	
OEOE_RS07695	-4.90	I METABOLISM; Lipid transport and metabolism	3-hydroxyacyl-[acyl-carrier-protein] dehydratase
OEOE_RS07705	-1.14	S POORLY CHARACTERIZED; Function unknown	biotin transporter
OEOE_RS07710	-2.29	F METABOLISM; Nucleotide transport and metabolism	thymidine kinase
OEOE_RS07715	-2.14	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	
OEOE_RS07720	-1.93	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS07765	-2.92	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS07780	-3.32	G METABOLISM; Carbohydrate transport and metabolism	D -ribose pyranase
OEOE_RS07785	-2.08	G METABOLISM; Carbohydrate transport and metabolism	Fucose permease
OEOE_RS07795	-1.78	P METABOLISM; Inorganic ion transport and metabolism	peptide ABC transporter permease
OEOE_RS07800	-2.36	P METABOLISM; Inorganic ion transport and metabolism	
OEOE_RS07805	-2.66	E METABOLISM; Amino acid transport and metabolism	
OEOE_RS07855	-2.37	P METABOLISM; Inorganic ion transport and metabolism	Polyphosphate kinase
OEOE_RS07860	-2.69	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	peptidase M13
OEOE_RS07885	1.62	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	
OEOE_RS07890	1.26	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	multidrug ABC transporter ATP -binding protein
OEOE_RS07905	-1.06	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	peptidylprolyl isomerase

OEOE_RS07925	1.10	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	DEAD/DEAH box helicase
OEOE_RS07935	1.96	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS07950	1.55	K INFORMATION STORAGE AND PROCESSING; Transcription	
OEOE_RS08080	1.05	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	RNA methyltransferase
OEOE_RS08100	2.77	T CELLULAR PROCESSES AND SIGNALING; Signal transduction mechanisms	ammonia permease
OEOE_RS08110	1.57	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS08115	2.86	G METABOLISM; Carbohydrate transport and metabolism	
OEOE_RS08135	4.85	P METABOLISM; Inorganic ion transport and metabolism	
OEOE_RS08160	-1.25	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	
OEOE_RS08170	-1.78	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B
OEOE_RS08175	-2.05	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	Glutamyl-tRNA(Gln) amidotransferase subunit A
OEOE_RS08180	-2.31	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C
OEOE_RS08195	-1.72	S POORLY CHARACTERIZED; Function unknown	Regulatory protein
OEOE_RS08225	-1.67	Q METABOLISM; Secondary metabolites biosynthesis, transport, and catabolism	
OEOE_RS08245	1.18	G METABOLISM; Carbohydrate transport and metabolism	fructokinase
OEOE_RS08250	-1.77	C METABOLISM; Energy production and conversion	
OEOE_RS08320	2.11	E METABOLISM; Amino acid transport and metabolism	3-isopropylmalate dehydratase large subunit
OEOE_RS08325	1.43	E METABOLISM; Amino acid transport and metabolism	3-isopropylmalate dehydratase small subunit
OEOE_RS08340	-1.31	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	ATP-dependent Clp protease ATP-binding subunit
OEOE_RS08385	-1.11	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	glycosyl transferase
OEOE_RS08400	-1.70	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	UDP -galactop yranose mutase
OEOE_RS08430	1.20	E METABOLISM; Amino acid transport and metabolism	amino acid transporter
OEOE_RS08440	1.59	P METABOLISM; Inorganic ion transport and metabolism	ferritin
OEOE_RS08505	2.18	P METABOLISM; Inorganic ion transport and metabolism	
OEOE_RS08600	-1.07	J INFORMATION STORAGE AND PROCESSING; Translation, ribosomal structure and biogenesis	50S ribosomal protein L31 type B
OEOE_RS08615	-1.03	K INFORMATION STORAGE AND PROCESSING; Transcription	DNA -directed RNA polymerase subunit delta
OEOE_RS08650	3.32	P METABOLISM; Inorganic ion transport and metabolism	
OEOE_RS08660	-1.03	K INFORMATION STORAGE AND PROCESSING; Transcription	
OEOE_RS08745	3.82	S POORLY CHARACTERIZED; Function unknown	prolyl-tRNA synthetase
OEOE_RS08795	-1.24	K INFORMATION STORAGE AND PROCESSING; Transcription	

OEOE_RS08820	2.02	S POORLY CHARACTERIZED; Function unknown	
OEOE_RS08860	1.05	H METABOLISM; Coenzyme transport and metabolism	
OEOE_RS08865	1.77	C METABOLISM; Energy production and conversion	cytochrome C oxidase assembly protein
OEOE_RS08870	1.47	C METABOLISM; Energy production and conversion	cytochrome D ubiquinol oxidase subunit
OEOE_RS08880	-2.82	G METABOLISM; Carbohydrate transport and metabolism	peptidoglycan-binding protein
OEOE_RS08940	1.94	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	osmotically inducible protein C
OEOE_RS08945	1.34	P METABOLISM; Inorganic ion transport and metabolism	
OEOE_RS08955	-1.11	I METABOLISM; Lipid transport and metabolism	
OEOE_RS09010	1.88	K INFORMATION STORAGE AND PROCESSING; Transcription	

Supplementary Table 3. Representative functional categories (COG: clusters of orthologous groups) found with FUNAGE-Pro platform (FU) and with GenBank database (GB) for genes (Locus tag) of *Oenococcus oeni* PSU-1 where remarkable differential expression (Log2 Fold Change < -3 or > 3) was found in medium with 2 g/L succinic acid (WLMS) compared to control without it (WLM). Log2FC values are marked in red or dark blue depending on whether the transcripts were down or up-regulated, respectively. Description with possible functions or products are added, according FU platform or GB database.

Locus tag	**Log2 FC**	**Functional categories (COG)** according to FU or GB		**Description: function, product, other**
OEOE_RS00035	-3.34	H METABOLISM; Coenzyme transport and metabolism	FU	NAD+ synthase (glutamine-hydrolyzing) activity, glutaminase activity
OEOE_RS00190	3.18	G METABOLISM; Carbohydrate transport and metabolism	GB	SLC45 family MFS transporter
OEOE_RS00205	3.49	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	FU	oxidoreductase activity
OEOE_RS00445	4.18	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	GB	MultiDrugR family MFS transporter
OEOE_RS00490	3.37	T CELLULAR PROCESSES AND SIGNALING; Signal transduction mechanisms	FU	phosphorelay sensor kinase activity, osmosensory signaling via phosphorelay pathway
OEOE_RS00645	-3.41	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	FU	protein disulfide oxidoreductase activity, glutaredoxin
OEOE_RS00650	-3.36	F METABOLISM; Nucleotide transport and metabolism	FU	ribonucleoside-diphosphate reductase activity, thioredoxin disulfide as acceptor
OEOE_RS00655	-3.67	F METABOLISM; Nucleotide transport and metabolism	FU	ribonucleoside-diphosphate reductase activity, thioredoxin disulfide as acceptor
OEOE_RS00855	3.77	S POORLY CHARACTERIZED; Function unknown	FU	plasma membrane
OEOE_RS01075	3.11	G METABOLISM; Carbohydrate transport and metabolism	GB	FGGY-family carbohydrate kinase
OEOE_RS01080	3.17	G METABOLISM; Carbohydrate transport and metabolism	GB	L-ribulose-5-phosphate 4-epimerase
OEOE_RS01235	-7.37	F METABOLISM; Nucleotide transport and metabolism	GB	aspartate carbamoyltransferase catalytic subunit
OEOE_RS01240	-7.15	F METABOLISM; Nucleotide transport and metabolism	FU	dihydroorotase activity, 'de novo' UMP biosynthetic process, zinc ion binding
OEOE_RS01245	-6.38	F METABOLISM; Nucleotide transport and metabolism	FU	carbamoyl-phosphate synthase (glutamine-hydrolyzing) activity, arginine biosynthetic process
OEOE_RS01250	-6.13	F METABOLISM; Nucleotide transport and metabolism	FU	carbamoyl-phosphate synthase (glutamine-hydrolyzing) activity, metal ion binding
OEOE_RS01255	-5.69	F METABOLISM; Nucleotide transport and metabolism	FU	orotidine-5'-phosphate decarboxylase activity, 'de novo' pyrimidine nucleobase biosynthetic process
OEOE_RS01260	-5.53	F METABOLISM; Nucleotide transport and metabolism	GB	magnesium ion binding, orotate phosphoribosyltransferase activity, nucleoside metabolic process

OEOE_RS01265	-4.62	F METABOLISM; Nucleotide transport and metabolism	FU	de novo' pyrimidine nucleobase biosynthetic process, dihydroorotate dehydrogenase (fumarate) activity
OEOE_RS01380	3.12	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	FU	D-alanyl-lipoteichoic acid biosynthesis protein DltD
OEOE_RS01385	4.44	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	FU	heat-shock protein Hsp20
OEOE_RS01575	3.45	G METABOLISM; Carbohydrate transport and metabolism	GB	pyruvate dehydrogenase (acetyl-transferring) activity, glycolytic process
OEOE_RS01625	4.17	G METABOLISM; Carbohydrate transport and metabolism	FU	phosphoenolpyruvate-dependent sugar phosphotransferase system
OEOE_RS01630	4.00	G METABOLISM; Carbohydrate transport and metabolism	FU	6-phospho-beta-glucosidase activity, carbohydrate catabolic process
OEOE_RS01635	3.03	G METABOLISM; Carbohydrate transport and metabolism	FU	6-phospho-beta-glucosidase activity, carbohydrate catabolic process
OEOE_RS01805	-4.75	F METABOLISM; Nucleotide transport and metabolism	GB	uracil transmembrane transporter activity
OEOE_RS01860	-3.01	E METABOLISM; Amino acid transport and metabolism	FU	4-aminobutyrate aminotransferase
OEOE_RS02255	3.44	S POORLY CHARACTERIZED; Function unknown	GB	MFS transporter, pseudo
OEOE_RS02260	5.18	S POORLY CHARACTERIZED; Function unknown	GB	aryl-sulfate sulfotransferase, pseudo
OEOE_RS02275	3.00	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	GB	tetratricopeptide repeat protein
OEOE_RS02280	4.14	G METABOLISM; Carbohydrate transport and metabolism	FU	transmembrane transporter activity
OEOE_RS02285	3.90	P METABOLISM; Inorganic ion transport and metabolism	GB	cation transmembrane transporter
OEOE_RS02720	3.46	S POORLY CHARACTERIZED; Function unknown	GB	hypothetical protein
OEOE_RS02725	4.84	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	GB	ATP-dependent Clp protease ATP-binding subunit
OEOE_RS03395	-3.85	K INFORMATION STORAGE AND PROCESSING; Transcription	GB	lys tRNA sintetasa C-terminal
OEOE_RS03475	3.30	K INFORMATION STORAGE AND PROCESSING; Transcription	GB	winged helix DNA-binding protein
OEOE_RS03500	3.83	K INFORMATION STORAGE AND PROCESSING; Transcription	GB	TetR/AcrR family transcriptional regulator
OEOE_RS03505	3.99	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	GB	ABC-type antimicrobial peptide transport system
OEOE_RS03510	4.08	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	FU	plasma membrane, ATP binding
OEOE_RS03515	3.38	K INFORMATION STORAGE AND PROCESSING; Transcription	GB	TetR/AcrR family transcriptional regulator
OEOE_RS03545	3.39	S POORLY CHARACTERIZED; Function unknown	GB	hypothetical protein
OEOE_RS03560	3.20	E METABOLISM; Amino acid transport and metabolism	FU	threonine dehydrogenase
OEOE_RS03885	5.54	S POORLY CHARACTERIZED; Function unknown	GB	extracellular solute-binding protein
OEOE_RS03890	4.11	S POORLY CHARACTERIZED; Function unknown	GB	hypothetical protein
OEOE_RS03895	4.87	S POORLY CHARACTERIZED; Function unknown	GB	hypothetical, Periplasmic_Binding_Protein_Type_2

OEOE_RS03900	5.79	G METABOLISM; Carbohydrate transport and metabolism	GB	sugar ABC transporter permease
OEOE_RS03905	5.09	G METABOLISM; Carbohydrate transport and metabolism	GB	carbohydrate ABC transporter permease
OEOE_RS03910	3.48	G METABOLISM; Carbohydrate transport and metabolism	GB	glycoside hydrolase family 31 protein
OEOE_RS04085	-3.88	F METABOLISM; Nucleotide transport and metabolism	FU	deoxyadenosine kinase
OEOE_RS04195	3.14	S POORLY CHARACTERIZED; Function unknown	GB	DUF4811 domain-containing protein
OEOE_RS04500	-3.17	E METABOLISM; Amino acid transport and metabolism	FU	argininosuccinate synthase activity, arginine biosynthetic process
OEOE_RS04615	3.66	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	FU	plasma membrane, DNA repair, establishment of competence for transformation
OEOE_RS04760	-3.31	C METABOLISM; Energy production and conversion	FU	peptidase M20
OEOE_RS04790	3.63	L INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	GB	class I SAM-dependent methyltransferase
OEOE_RS05245	-3.90	E METABOLISM; Amino acid transport and metabolism	GB	oligoendopeptidase F
OEOE_RS05405	-3.58	F METABOLISM; Nucleotide transport and metabolism	GB	hypoxanthine&guanine phosphoribosyltransferase activity, GMP&IMP salvage
OEOE_RS05410	-3.70	F METABOLISM; Nucleotide transport and metabolism	FU	N6-(1,2-dicarboxyethyl)AMP AMP-lyase (fumarate-forming) activity, 'de novo' IMP&AMP biosynthetic process
OEOE_RS05415	-3.74	F METABOLISM; Nucleotide transport and metabolism	FU	de novo' AMP biosynthetic process, magnesium ion binding
OEOE_RS05420	-4.99	F METABOLISM; Nucleotide transport and metabolism	FU	nucleotide binding, IMP dehydrogenase activity, GMP biosynthetic process
OEOE_RS05580	-3.11	G METABOLISM; Carbohydrate transport and metabolism	FU	NAD+ kinase activity, NADP biosynthetic process, metal ion binding
OEOE_RS05585	-4.20	E METABOLISM; Amino acid transport and metabolism	FU	metalloendopeptidase activity, peptide metabolic process
OEOE_RS05825	3.12	G METABOLISM; Carbohydrate transport and metabolism	GB	PTS transporter subunit EIIC
OEOE_RS06005	-3.08	K INFORMATION STORAGE AND PROCESSING; Transcription	FU	Transcription elongation factor GreA
OEOE_RS06380	-4.33	G METABOLISM; Carbohydrate transport and metabolism	GB	L-lactate dehydrogenase activity, carbohydrate metabolic process
OEOE_RS06435	4.76	S POORLY CHARACTERIZED; Function unknown	GB	hypothetical protein
OEOE_RS06440	4.12	K INFORMATION STORAGE AND PROCESSING; Transcription	FU	Transcriptional regulator, GntR family
OEOE_RS06445	4.00	G METABOLISM; Carbohydrate transport and metabolism	FU	alpha-amylase activity, trehalose catabolic process, alpha,alpha-phosphotrehalase activity
OEOE_RS06450	3.48	G METABOLISM; Carbohydrate transport and metabolism	FU	carbohydrate transmembrane transport, trehalose, phosphoenolpyruvate-dependent sugar phosphotransferase system
OEOE_RS06720	-3.84	F METABOLISM; Nucleotide transport and metabolism	GB	nicotinamide riboside transporter PnuC
OEOE_RS07005	3.70	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	GB	cell filamentation protein Fic
OEOE_RS07055	3.91	F METABOLISM; Nucleotide transport and metabolism	GB	purine nucleobase metabolic process, pyrimidine nucleobase metabolic process
OEOE_RS07060	3.66	F METABOLISM; Nucleotide transport and metabolism	FU	adenine deaminase activity

OEOE_RS07065	3.50	E METABOLISM; Amino acid transport and metabolism	GB	ABC transporter substrate-binding protein
OEOE_RS07070	4.27	E METABOLISM; Amino acid transport and metabolism	FU	spermidine/putrescine ABC transporter ATP-binding protein
OEOE_RS07075	4.43	P METABOLISM; Inorganic ion transport and metabolism	FU	spermidine/putrescine ABC transporter ATP-binding protein
OEOE_RS07080	3.63	P METABOLISM; Inorganic ion transport and metabolism	FU	spermidine/putrescine ABC transporter permease
OEOE_RS07085	3.86	K INFORMATION STORAGE AND PROCESSING; Transcription	GB	GntR family transcriptional regulator
OEOE_RS07090	4.17	O CELLULAR PROCESSES AND SIGNALING; Post-translational modification, protein turnover, and chaperones	FU	hydrolase activity
OEOE_RS07095	4.36	F METABOLISM; Nucleotide transport and metabolism	GB	allantoin permease, cytosine permease
OEOE_RS07100	4.80	G METABOLISM; Carbohydrate transport and metabolism	FU	phosphotransferase activity, alcohol group as acceptor
OEOE_RS07105	4.29	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	GB	Dolichyl-phosphate-mannose-protein
OEOE_RS07140	3.07	G METABOLISM; Carbohydrate transport and metabolism	FU	transketolase activity, pentose-phosphate shunt, metal ion binding
OEOE_RS07420	4.60	C METABOLISM; Energy production and conversion	GB	component interface [polypeptide binding]
OEOE_RS07425	3.24	P METABOLISM; Inorganic ion transport and metabolism	FU	cobalt ABC transporter ATPase
OEOE_RS07645	-3.26	I METABOLISM; Lipid transport and metabolism	FU	Acetyl-coenzyme A carboxylase carboxyl transferase
OEOE_RS07650	-4.09	I METABOLISM; Lipid transport and metabolism	FU	fatty acid biosynthetic process, lipid A, 3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase activity
OEOE_RS07655	-4.56	I METABOLISM; Lipid transport and metabolism	FU	fatty acid biosynthetic process, acetyl-CoA carboxylase activity
OEOE_RS07660	-4.80	I METABOLISM; Lipid transport and metabolism	GB	3-oxoacyl-[acyl-carrier-protein] synthase activity, fatty acid biosynthetic process
OEOE_RS07665	-4.45	S POORLY CHARACTERIZED; Function unknown	FU	3-oxoacyl-[acyl-carrier-protein] reductase (NADPH) activity, fatty acid elongation
OEOE_RS07670	-4.52	I METABOLISM; Lipid transport and metabolism	GB	ACP S-malonyltransferase
OEOE_RS07675	-4.44	V CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	GB	2 -nitropropane dioxygenase
OEOE_RS07680	-4.44	I METABOLISM; Lipid transport and metabolism	GB	acyl carrier activity
OEOE_RS07685	-4.61	I METABOLISM; Lipid transport and metabolism	FU	fatty acid biosynthetic process, 3-oxoacyl-[acyl-carrier-protein] synthase activity
OEOE_RS07690	-4.80	K INFORMATION STORAGE AND PROCESSING; Transcription	GB	MarR family transcriptional regulator
OEOE_RS07695	-4.90	I METABOLISM; Lipid transport and metabolism	FU	3-hydroxyacyl-[acyl-carrier-protein] dehydratase
OEOE_RS07745	-4.61	M CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	GB	Basic membrane lipoprotein Med, periplasmic binding
OEOE_RS07775	-3.84	F METABOLISM; Nucleotide transport and metabolism	GB	ribokinase activity, D-ribose catabolic process
OEOE_RS07780	-3.32	G METABOLISM; Carbohydrate transport and metabolism	FU	D-ribose catabolic process, intramolecular lyase activity
OEOE_RS08135	4.85	P METABOLISM; Inorganic ion transport and metabolism	FU	transmembrane transporter activity

OEOE_RS08140	5.01	S POORLY CHARACTERIZED; Function unknown	GB	DUF4811 domain-containing protein
OEOE_RS08310	3.27	E METABOLISM; Amino acid transport and metabolism	GB	leucine biosynthetic process, 2-isopropylmalate synthase activity
OEOE_RS08315	3.01	E METABOLISM; Amino acid transport and metabolism	GB	leucine biosynthetic process, 2-isopropylmalate synthase activity
OEOE_RS08650	3.32	P METABOLISM; Inorganic ion transport and metabolism	FU	metal ion transmembrane transporter activity
OEOE_RS08745	3.82	S POORLY CHARACTERIZED; Function unknown	FU	translation, aminoacyl-tRNA editing activity, lyase activity
OEOE_RS08750	4.44	S POORLY CHARACTERIZED; Function unknown	GB	hypothetical protein
OEOE_RS08840	3.06	G METABOLISM; Carbohydrate transport and metabolism	GB	beta-galactosidase complex
OEOE_RS08845	3.53	S POORLY CHARACTERIZED; Function unknown	GB	MFS transporter
OEOE_RS09565	-3.30	S POORLY CHARACTERIZED; Function unknown	GB	hypothetical protein
OEOE_RS09570	3.40	S POORLY CHARACTERIZED; Function unknown	GB	MFS transporter
OEOE_RS09685	-3.15	S POORLY CHARACTERIZED; Function unknown	GB	pseudo, DUF2785 domain-containing protein
OEOE_RS09785	4.68	S POORLY CHARACTERIZED; Function unknown	GB	hypothetical protein
OEOE_RS09790	5.84	G METABOLISM; Carbohydrate transport and metabolism	GB	Galactose mutarotase-like, DUF4968 domain-containing protein
OEOE_RS09815	3.87	P METABOLISM; Inorganic ion transport and metabolism	GB	ComEC/Rec2 family competence protein
OEOE_RS09820	3.77	P METABOLISM; Inorganic ion transport and metabolism	GB	ComEC/Rec2 family competence protein

ANEXO 3

CURRICULUM VITAE

de Francisco Rafael Torres Guardado

Posición actual: Profesor en la Universidad Autónoma de Guadalajara, México.

Formación Académica:

- Doctorado

  Doctorado en Enología y Biotecnología, línea de investigación de bacterias lácticas.

  Trabajo de tesis: Efecto del ácido succínico producido por levaduras vínicas sobre *Oenococcus oeni* y la fermentación maloláctica

  Universidad Rovira i Virgili. Tarragona, España. (2018- 2023)

- Maestría

  Maestría en Ciencia y Tecnología, Biotecnología Productiva opción terminal (2017).

  Titulado por el trabajo de tesis: "Estudio de las interacciones bacterias ácido lácticas-levaduras en fermentación para la elaboración de una bebida alcohólica".

  Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco (CIATEJ)

- Licenciatura

  Ingeniero Empresarial Agropecuario (2014).

  Titulado con mención honorífica por el trabajo de tesis "Evaluación del potencial antifúngico de bacterias endófitas del banano (*Musa acuminata*) sobre el desarrollo de cepas de *Fusarium oxysporum cubense,* causante del "Mal de Panamá" y otras cepas pertenecientes al complejo *Fusarium oxysporum".*

  Universidad Autónoma de Guadalajara

Cursos y Diplomados

- Diplomado Sommelier. Worlwide sommelier association- Organización nacional de sommelier de México A.C. Guadalajara Jalisco (2018)
- Curso-taller "Clonación y expresión de genes en los hospederos heterólogos *E. coli* y *Pichia pastoris*" Centro de Investigaciòn y Asistencia en Tecnología y Diseño del Estado de Jalisco. Mayo 2016.
- Curso protección civil: Primeros auxilios, control y combate de fuego, evacuación búsqueda y rescate. Universidad Autónoma de Guadalajara. México. (2022)

- Curso diseño instruccional CANVAS Universidad Autónoma de Guadalajara (2022)

Experiencia Profesional:

- Profesor de ciencias en Universidad Autónoma de Guadalajara (2016-2018, 2021-2023).
- Profesor asociado en Escuela Culinaria Internacional (ECI). Docencia de enología y entrenamiento de sommeliers (2022-2023)
- Profesor asociado en Organización Nacional de Sommeliers (ONSOM). Docencia de enología y entrenamiento de sommeliers. (2023)
- Estudiante de posgrado de tiempo completo en Centro de investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco (2014-2017).
- Asistente en el proyecto de investigación "Endófitos de papa como control biológico y promotores de crecimiento" Universidad Autónoma de Guadalajara- Mc Cain- Green corp. (2013-2015).

Publicaciones en revistas científicas indexadas:

- **Torres-Guardado** R, Esteve-Zarzoso B, Reguant C, Bordons A (2022) Microbial interactions in alcoholic beverages (Review). International Microbiology 25, 1-15 (accepted 28jul2021). FI 3.1 (2021). https://doi.org/10.1007/s10123-021-00200-1
- **Torres-Guardado** R, Rozès N, Esteve-Zarzoso B, Reguant R, Bordons A (2022) Influence of succinic acid on *Oenococcus oeni* and malolactic fermentation. Oeno One 56-3, 195-204 (accepted 1jul2022) FI 3.0 (2021). https://doi.org/10.20870/oeno-one.2022.56.3.5403
- **Torres-Guardado** R., Rozès, N., Esteve-Zarzoso, B., Reguant, C., Bordons, A. (2023). Succinic acid production by wine yeasts and the influence of GABA and glutamic acid. International Microbiology (accepted 21jul2023) FI 3.1 (2022). https://rdcu.be/dhV9d

Publicaciones en libros técnicos:

- Martínez.J, Mora.M, Plascencia.L., Audelo.E., **Guardado.R.**., (2014) Cultivable endophytic bacteria from leaf bases of *Agave tequilana* and their role as plant growth promoters. Brazilian Journal of Microbiology. 45. 4. 1333-1339.
- Castillo.D., Macedo.G., Odriozola.O., García.A., **Torres R.,** Beltrán.M.(2012) innovaciones para el manejo integrado de la sigatoka negra en México. Aportaciones de la investigación básica. Libro Técnico. CICY, INIFAP, U.A. CHAPINGO. UNIVERSIDAD COLIMA. UAG. Páginas: 33-45.

Ponencias y Congresos:

- Sociedad de Química de Guadalajara A.C. Ponencia: "El vino y su rúbrica" Octubre 2021.
- Asociacion Mexicana de Ciencias de los Alimentos. Webinar: Calidad y biotecnología alimentaria. Ponencia: La biotecnología en el proceso de elaboración del vino. Octubre 2020

- III International Symposium on Agave. Guadalajara Jalisco. Noviembre 2016. Co-culture yeast bacteria as candidates for tequila production.
- Latin food 2016 Cancun, Quintana roo (AMECA). Ethanol and osmotic tolerance of lactic acid bacteria involved in agave juice fermentation.
- XVI Congreso Internacional de Biotecnología y Bioingeniería (SMBB) Junio-2015.
- II International Symposium on Agave. Guadalajara Jalisco. Octubre 2014. Centro de investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco.
- 4º congreso Internacional de Biología, Química y Agronomía "innovación para el desarrollo sustentable" Septiembre 2013.Universidad Autónoma de Guadalajara.
- Participación en el 4º congreso Internacional de Biología, Química y Agronomía "innovación para el desarrollo sustentable" septiembre 2013, con el trabajo: Evaluación del potencial antifúngico de bacterias endófitas del banano vs diversas cepas de *Fusarium oxysporum*.
- 16ª Reunión de la Red Española de Bacterias Lácticas, Madrid 11 mayo 2023, póster: Diversidad del efecto del ácido succínico producido por levaduras vinicas sobre Oenococcus oeni. R. Torres-Guardado, N. Rozès, B. Esteve-Zarzoso, C. Reguant, A. Bordons. Publicado en libro de resúmenes (ISBN 978-84-09-51185-3), pág. 60

Idiomas:

- Español: Nativo
- Inglés: TOEFL 480 Puntos.
- Francés: Nivel B1 del Marco Común Europeo de Referencia para las Lenguas.

Distinciones:

- Becario Fundación Carolina (2018-2021), colaboración Gobierno México y Universitat Rovira i Virgili.
- Becario Secretaría de relaciones exteriores México (2018-2021)
- Profesor Distinguido de educación media-superior. Universidad Autónoma de Guadalajara (2018)
- Becario CONACYT (2014-2016)
- Miembro de la Sociedad Mexicana de Biotecnología y Bioingeniería (SMBB)

Intereses:

- Vocación por la investigación y desarrollo de nuevas tecnologías en la industria de la fermentación alcohólica.
- Optimización de procesos fermentativos para el fomento del carácter de origen y terruño.
- Vocación por la docencia en el área de ciencias químicas y biológicas.
- Colaboración local e internacional en el desarrollo de investigación enológica.