INSTITUTO DE INVESTIGACIONES
QUÍMICO BIOLÓGICAS
UMSNH

UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO
INSTITUTO DE INVESTIGACIONES QUÍMICO-BIOLÓGICAS

# PARTICIPACIÓN DE LA PEROXIDACIÓN DE LÍPIDOS EN LA REGULACIÓN DEL CANAL INESPECÍFICO MITOCONDRIAL (CIML) EN LA MUERTE CELULAR POR ETANOL EN *SACCHAROMYCES CEREVISIAE*

TESIS

que presenta:
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PARA OBTENER EL GRADO DE DOCTORA EN CIENCIAS BIOLÓGICAS

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Morelia, Michoacán. Julio 2023

## DEDICATORIA

A mi familia y amigos, gracias por transitar conmigo esta etapa, gracias por todas sus palabras de aliento que han sido parte integral de esta historia.

# ADRADECIMIENTOS

Al **D.C. Christian Cortés Rojo** por el apoyo en la realización de este proyecto y por su amistad.

A mis sinodales **D.C Rocío del Carmen Montoya Pérez, D.C Xóchitl Pérez Martínez, D.C Mauro Manuel Martínez Pacheco** y **D.C Jesús Campos García** por su apoyo y asesoría.

Al **D.C Alfredo Saavedra Molina** por siempre mantener las puertas abiertas de su laboratorio.

A la **D.C Lorena Martínez Alcántar** por siempre estar en la mejor disposición de enseñar.

Al **M.C Alberto Flores García** por ser el mejor tutor durante mi estancia en el doctorado y por su amistad.

A mis amigos y compañeros de laboratorio de Disfunción mitocondrial, del laboratorio de Fisiología muscular y del laboratorio de Bioquímica gracias por su apoyo.

Al consejo nacional de ciencia y tecnología (**CONACYT**) por la beca otorgada.

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## RESUMEN

La presencia de ácidos grasos poliinsaturados (AGPI) en el entorno puede generar un ambiente desafiante para la levadura *Saccharomyces cerevisiae*. Esto se debe a que los AGPI son propensos a la peroxidación, lo que compromete el correcto funcionamiento de proteínas de membrana esenciales para la levadura, ocasionando la muerte celular. La transición de la permeabilidad por la apertura del canal inespecífico mitocondrial de la levadura (CIML) es un proceso estrictamente controlado que tiene como finalidad el desacoplamiento de la fosforilación oxidativa cuando la producción de energía aumenta para evitar la sobreproducción de especies reactivas de oxígeno (ERO) por la cadena transporte de electrones (CTE). La estabilización de este canal depende de su interacción con la cardiolipina, un fosfolípido mitocondrial susceptible a la peroxidación cuando las levaduras contienen AGPI. Considerando que la acumulación de etanol durante la fermentación es una causa adicional de estrés oxidativo, es posible postular que la presencia de AGPI y etanol en el medio de cultivo de la levadura podría disminuir su sobrevivencia al alterar el funcionamiento del CIML al incrementarse la peroxidación de lípidos y la pérdida de la cardiolipina. Por lo anterior, en este trabajo se investigó el efecto de la combinación de ácido linolénico y etanol en el medio de cultivo de la levadura sobre la sobrevivencia de la levadura, el funcionamiento del CIML y la bioenergética mitocondrial. Se observó un aumento en la muerte celular y en la peroxidación de lípidos. A nivel mitocondrial, se observó una diminución del contenido de cardiolipina, disfunción del CIML y de la actividad de los complejos de la CTE. En contraste, en ausencia de ácido linolénico, se observó una mayor resistencia a la muerte por etanol, mejor preservación del contenido de cardiolipina y del funcionamiento del CIML. Los datos anteriores indican que la ausencia de AGPI en *S. cerevisiae* podría ser un mecanismo para incrementar su resistencia al etanol al disminuir el daño en las membranas y preservar el funcionamiento de la CTE y el CIML. Lo anterior podría tener implicaciones tecnológicas para incrementar la resistencia de la levadura al etanol, en procesos que involucren la acumulación de AGPI en el medio.

**Palabras clave: cardiolipina, CIML, AGPI, etanol, levadura.**

## ABSTRACT

The presence of polyunsaturated fatty acids (PUFA) in the environment can create a challenging environment for the yeast *Saccharomyces cerevisiae*. This is because PUFA are prone to peroxidation, which compromises the proper functioning of essential membrane proteins for yeast, leading to cell death. The permeability transition by the opening of the yeast mitochondrial unspecific channel (YMUC) is a tightly controlled process aimed at uncoupling oxidative phosphorylation when energy production increases to avoid the overproduction of reactive oxygen species (ROS) by the electron transport chain (ETC). Stabilization of this channel depends on its interaction with cardiolipin, a mitochondrial phospholipid highly susceptible to peroxidation when yeast contains PUFA. Considering that ethanol accumulation during fermentation is an additional cause of oxidative stress, it is possible to postulate that the presence of PUFA and ethanol in the yeast culture medium could decrease yeast survival by altering YMUC function through increased lipid peroxidation and loss of cardiolipin. Therefore, in this work, we investigated the effect of the combination of linolenic acid and ethanol in yeast culture medium on yeast survival, YMUC function and mitochondrial bioenergetics. Increased cell death and lipid peroxidation were observed. At the mitochondrial level, decreased cardiolipin content, and dysfunction in both YMUC and ETC complexes were observed. In contrast, in the absence of linolenic acid, increased resistance to ethanol death, better preservation of cardiolipin content and YMUC function were observed. The above data indicate that the absence of PUFA in *S. cerevisiae* could be a mechanism to increase its resistance to ethanol by decreasing membrane damage and preserving the functioning of both ETC and YMUC. This could have technological implications for increasing yeast resistance to ethanol in processes involving PUFA accumulation in the medium.

**Key words: Cardiolipin, YMUC, PUFA, ethanol, yeast.**

# 1. MARCO TEÓRICO

## 1.1 *Saccharomyces cerevisiae*

### 1.1.1 Aspectos generales.

La levadura *Saccharomyces cerevisiae (S. cerevisiae),* comúnmente conocida como levadura de cerveza, es un eucarionte unicelular con morfología ovoide o elipsoidal, rodeado por una pared celular (Figura 1) (Daum 2000).

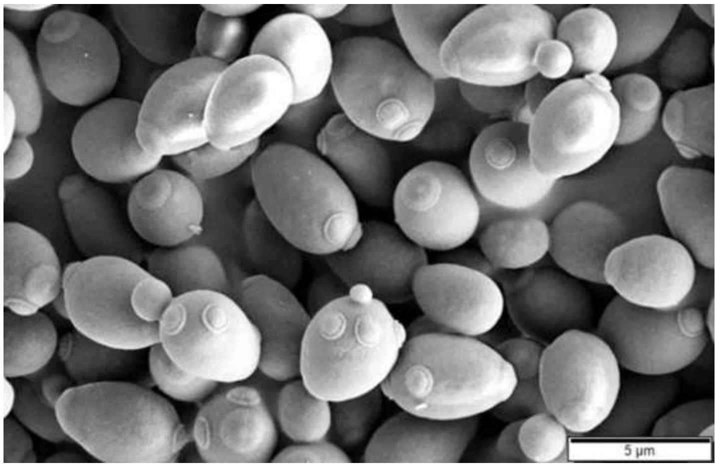

**Figura 1. Microscopia electrónica de la levadura *S. cerevisiae***
(Murtey & Ramasamy, 2016)

En su ciclo de vida alternan dos formas, una haploide y otra diploide. Ambos tipos celulares se reproducen asexualmente a través de la mitosis y son estables. Es una división en ciernes, lo que significa que las células hijas son más pequeñas que las células madre (Duina *et al*., 2014).

### 1.1.2 *S. cerevisiae* y su importancia en la industria.

*S. cerevisiae* es una levadura utilizada en la industria de alimentos, bebidas, biocombustibles productos químicos, enzimas y otros productos industriales, debido a sus propiedades únicas de fácil producción y mantenimiento, así como la capacidad de soportar condiciones estresantes y tener una alta eficiencia de fermentación, rápido crecimiento, uso efectivo de azúcar, la capacidad de producir etanol y tolerar altas concentraciones de etanol (Reis *et al*., 2014).

Además de su participación en la producción de alimentos y combustibles, *S. cerevisiae* también se emplea en la investigación científica como un organismo modelo para estudiar una variedad de procesos biológicos y genéticos. *S. cerevisiae* fue el primer organismo eucarionte cuyo genoma fue secuenciado, debido a esto, puede modificarse fácilmente mediante el uso de ingeniería genética, lo que la convierte en un recurso invaluable para usos biotecnológicos y para la mayoría de las investigaciones sobre especies eucariotas, ya que conserva casi todas las funciones biológicas presentes en eucariotas superiores (Parapouli *et al*., 2020).

### 1.1.3 Metabolismo.

El metabolismo de esta levadura es ampliamente estudiado debido a su importancia en la producción de alimentos y bebidas, así como en la investigación básica de la biología celular. El metabolismo de *S. cerevisiae* es regulado por una compleja red de vías metabólicas y reguladores transcripcionales, lo que permite a la célula su adaptación a diferentes condiciones ambientales (Ostergaard *et al*., 2000). La levadura obtiene energía mediante la respiración o la fermentación (metabolismo aerobio-facultativo), la glucosa es la principal fuente de energía. La levadura utiliza preferentemente la fermentación como vía de obtención de energía (Morales-García *et al*., 2021). El piruvato, el producto final de la glucólisis, ingresa a la mitocondria donde se convierte en $CO_2$ y agua (respiración aeróbica), o puede permanecer en el citosol donde la célula lo transforma primero en acetaldehído y luego en etanol (fermentación). Las células de *S. cerevisiae* modifican su metabolismo fermentativo a uno respiratorio y utilizan el etanol acumulado como sustrato respiratorio para iniciar la fase diaúxica de crecimiento cuando los sustratos fermentativos se agotan y el oxígeno está disponible. En esta transición de metabolismo ocurren las principales reorganizaciones estructurales y funcionales en el metabolismo mitocondrial (Di Bartolomeo *et al*., 2020).

### 1.1.4 Apertura/cierre del canal inespecífico mitocondrial (CIML) como desacoplamiento fisiológico de la respiración.

Tan pronto como la concentración de glucosa externa alcanza una concentración de 0.8 mM, *S. cerevisiae* cambia a un metabolismo respiro-fermentativo que da como resultado la síntesis de etanol. Los niveles altos de glucosa provocan una tasa glucolítica que supera la de la reacción de la piruvato deshidrogenasa, generando así un desbordamiento hacia la piruvato descarboxilasa y la producción de acetaldehído. El acetaldehído se reduce a etanol por la acetaldehído deshidrogenasa 1 (ALDH1) a expensas del NADH producido en la gliceraldehido-3-fosfato deshidrogenasa de la glucolisis, regenerando así el $NAD^+$ necesario para esta reacción. Por otra parte, el etanol es reoxidado por las ALDH1/2 citosólicas y en la mitocondria por la ALDH3 a expensas de $NAD^+$, lo cual genera un incremento en las concentraciones de NADH que, a su vez, es oxidado en la cadena de transporte de electrones (CTE) de la mitocondria (Figura 2). (Otterstedt *et al*., 2004; Bakker *et al.,* 2000).

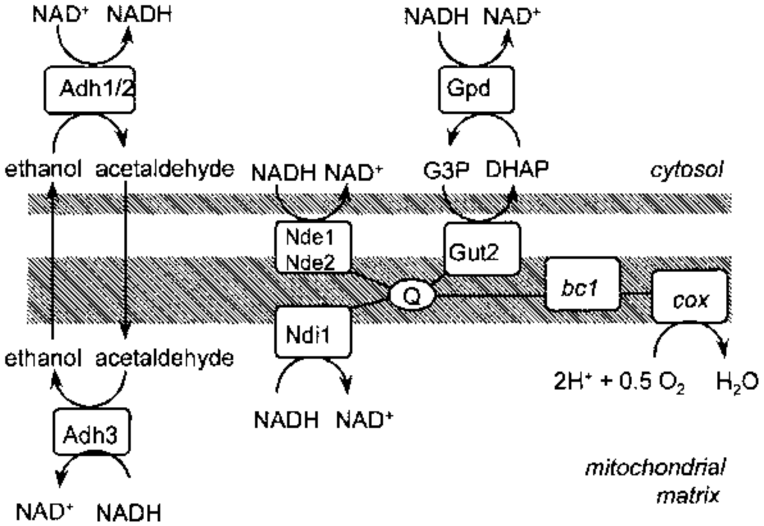

**Figura 2. Metabolismo del acetaldehído y del etanol en *S. cerevisiae*.** Adh 1/2 (acetaldehído deshidrogenasa 1 y 2) Aldh3 (acetaldehído deshidrogenasa 3), Ndi1 (NADH:ubiquinona oxidoreductasa), Nde1 y 2 (NADH deshidrogenasa externa 1 y 2), Gut2 (glicerol-3-fosfato deshidrogenasa), bc1 (complejo citocromo bc1), cox (citocromo oxidasa), G3P (gliceraldehído-3-fosfato), DHAP (dihidroxiacetona fosfato), Gpd (Gliceraldehído-3-fosfato deshidrogenasa) (Bakker *et al.,* 2000).

El exceso de NADH por la oxidación del etanol debe ser reoxidado para funcionar como aceptor de electrones en diversas reacciones catabólicas dependientes de $NAD^+$ (Manon, 1999). Sin embargo, un estado energético con grandes cocientes $NADH/NAD^+$ y ATP/ADP conduce a la rápida reoxidación del NADH por la inhibición enzimática que ejerce el exceso de éste y el alto potencial de membrana mitocondrial generado por el exceso de ATP, lo que limita la velocidad de transporte de electrones y, por lo tanto, la oxidación del NADH en la CTE. Para sortear esta restricción energética, la levadura cuenta con un mecanismo de desacoplamiento fisiológico de la fosforilación oxidativa mitocondrial que lo causa el canal inespecífico mitocondrial de la levadura (CIML) (Guerrero-Castillo *et al.,* 2011). EL CIML es un gran complejo proteico de múltiples subunidades que forma un canal en la membrana mitocondrial interna. En *S. cerevisiae*, el CIML está compuesto por la $F_1F_0$-ATP sintasa, el canal aniónico dependiente de voltaje (CADV) la proteína de la matriz mitocondrial ciclofilina D (CYPD) y el acarreador de Pi (Cabrera-Orefice *et al*., 2015; Gutiérrez-Aguilar *et al*., 2010).

La apertura de la CIML provoca un cambio en la permeabilidad de la mitocondria. En concordancia con la idea de la regulación del CIML en función de los niveles energéticos de la célula, se ha establecido que el ATP es responsable de la apertura de CIML, mientras que Pi y el ADP son responsables de su cierre (Prieto *et al*., 1995). Asimismo, la apertura del CIML depende de la tasa de flujo de electrones a través de la CTE proporcionada por el NADH. La apertura del CIML por el ATP o por sustratos respiratorios incrementa la conductividad de los $H^+$ a través de la membrana interna mitocondrial, una disminución del potencial de membrana mitocondrial ($\Delta\Psi_m$) e incremento en la velocidad de respiración, lo que provoca un aumento en la velocidad de oxidación del NADH. Por el contrario, el cierre del CIML incrementa el $\Delta\Psi_m$ al limitar la conductividad de los $H^+$ a través de la membrana mitocondrial interna (MMI), dando lugar a una disminución del flujo de electrones en la cadena de transporte de electrones (CTE) y de la oxidación de los sustratos respiratorios (Manon S, 1999). Por lo tanto, la apertura del CIML participa en la velocidad de oxidación del NADH citosólico independientemente de su vínculo con la producción de ATP, de esta forma está sujeta su regulación como resultado del potencial de fosforilación (Wallace *et al*., 1994). Asimismo, un incremento en los niveles de $Ca^{2+}$ induce el cierre del CIML (Perez-Vazquez *et al*., 2003; Prieto *et al*., 1992).

Además de regular la velocidad de oxidación del NADH, este mecanismo de desacoplamiento fisiológico regulado en la levadura tiene como finalidad impedir la sobreproducción de especies reactivas de oxígeno (ERO) (Figura 3) producidas en la CTE debido a la diminución de la velocidad de respiración ocasionada por el incremento del $\Delta\psi_m$, lo cual aumenta el tiempo de vida media de los radicales semiquinona producidos en el complejo III de la CTE y la probabilidad de reaccionar con el $O_2$ para formar radical superóxido ($O_2^{\bullet-}$) (Guerrero-Castillo *et al.,* 2011).

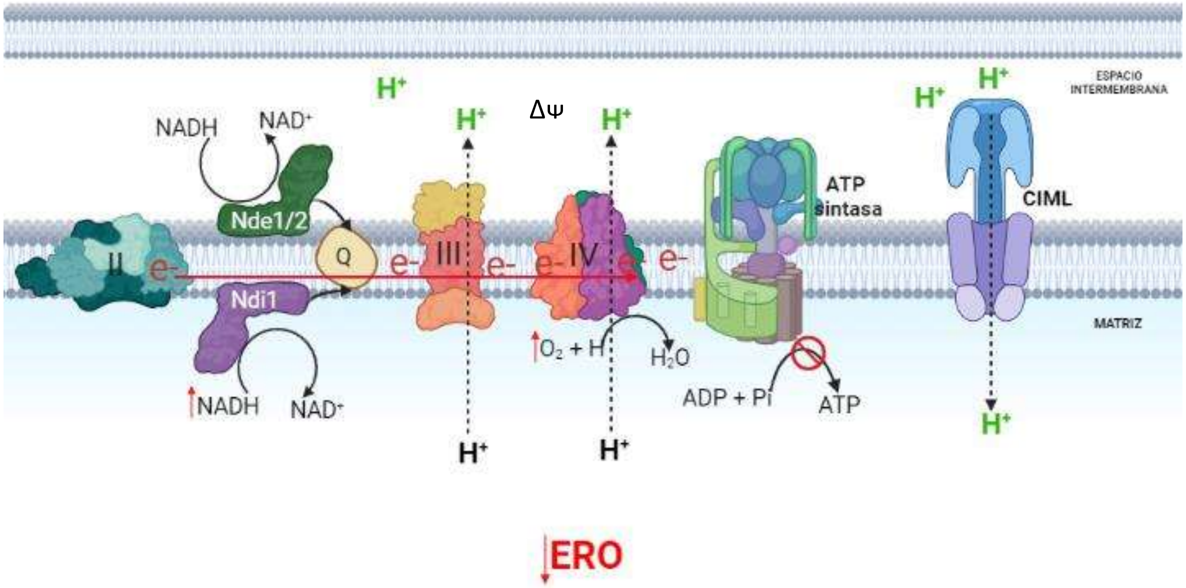

**Figura 3. Desacoplamiento fisiológico de la fosforilación oxidativa por la apertura del CIML.** El desacoplamiento de la respiración evita la sobre producción de ERO producidas por la CTE en la mitocondria. CIML (canal inespecífico mitocondrial de levadura), Nde1 (NADH deshidrogenasa externa 1), Nde2 (NADH deshidrogenasa externa 2), ERO (especies reactivas de oxígeno), ADP (adenosín difosfato), II (complejo II succinato-DCIP oxidorreductasa), III (complejo II succinato-citocromo c oxidorreductasa), IV (citocromo c oxidasa).

### 1.1.5 Participación de la cardiolipina en la estabilización del CIML.

La cardiolipina 1,3-bis(sn-3'-fosfatidil)-sn-glicerol es un lípido, dimérico, compuesto por dos cadenas de fosfatidilglicerol con dos grupos acilo cada una unidas covalentemente a una molécula de glicerol (Figura 4 ) (Gonzalvez & Gottlieb, 2007). En la levadura el ácido oleico (C18:1) y el palmitoleico (C16:1) son los ácidos grasos (AG) presentes en la cardiolipina (Schlame *et al*., 1993). Este fosfolípido tiene una estructura dimérica en la que los dos residuos fosfatidilo son responsables de su afinidad por los iones metálicos divalentes como el calcio y el magnesio (LeCocq & Ballou, 1964). Es un componente fundamental de la MMI, donde desempeña una función en la biogénesis y la fosforilación oxidativa al participar en la estabilización de los super complejos de la CTE (Fry & Grenn, 1981; Joshi *et al*., 2009).

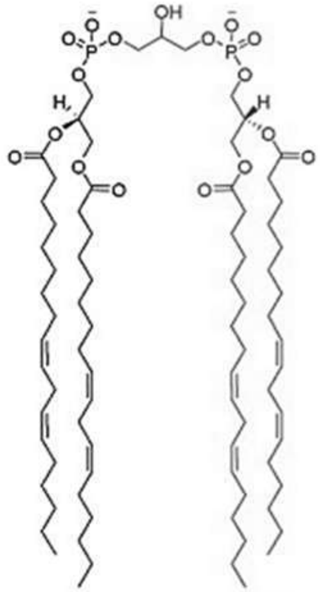

**Figura 4. Estructura de la cardiolipina (1,3 difosfatidil- sn –glicerol)** (Tomada y modificada de Gonzalvez & Gottlieb, 2007).

La estabilización del CIML depende de su interacción con la cardiolipina, (Koshkin & Greenberg, 2002). Se ha demostrado la interacción que tiene la cardiolipina con el CADV, uno de los componentes del poro (Betaneli *et al*., 2012). La cardiolipina interactúa específicamente con el CADV a través de su porción fosfatidilcolina y dos grupos acilo. Esta interacción se produce a través de un sitio de unión ubicado en el CADV que reconoce y se une específicamente a la cardiolipina (Rostovtseva *et al*., 2006). La interacción entre el CADV y la cardiolipina es esencial para mantener la estructura y función de la proteína, y se ha demostrado que la eliminación de la cardiolipina de la membrana mitocondrial externa afecta la función del CADV y la permeabilidad mitocondrial (Koshkin & Greenberg, 2002).

Un incremento en la producción de ERO provoca la peroxidación de la cardiolipina que tiene consecuencias en la síntesis de ATP y en la supervivencia celular. Además, su peroxidación puede interferir en sus interacciones con las proteínas mitocondriales y, por lo tanto, afectar procesos mitocondriales dependientes de la cardiolipina, como el transporte de electrones y la permeabilidad de la membrana mitocondrial interna (Vähäheikkilä *et al*., 2018).

### 1.1.6 Composición de las membranas de la levadura *S. cerevisiae*.

La composición de la membrana celular de la *S. cerevisiae* es compleja: los fosfolípidos, los glucoesfingolípidos, el ergosterol y las proteínas son los constituyentes principales de la membrana biológica de la levadura y cada uno de ellos desempeña una función en la célula (Henderson *et al.,* 2014). Los lípidos particioan en la respuesta al estrés, cuando la levadura se enfrenta a condiciones estresantes, como cambios en la temperatura, pH o disponibilidad de nutrientes, los lípidos de membrana pueden reorganizarse y cambiar su composición para adaptarse a estas condiciones. Esto permite que la levadura mantenga su integridad estructural y función celular en un entorno cambiante (Ferraz *et al*., 2021).

En lo que respecta a la composición lipídica, la fosfatidilcolina, la fosfatidiletanolamina, el fosfatidilinositol, la fosfatidilserina y el ácido fosfatídico constituyen la mayoría de los fosfolípidos de la levadura (Carman & Han 2009). Sin embargo, se observan diferencias en la composición de cada una de las fracciones subcelulares de la levadura, como se muestra en la Tabla 1. En cuanto a los ácidos grasos, los cuatro más frecuentemente esterificados a la fracción de glicerol de los fosfolípidos de levadura son el ácido palmítico (C16:0), el ácido palmitoleico (C16:1), el ácido esteárico (C18:0) y el ácido oleico (C18:1) (Gaspar et al., 2007). Esta composición de AG le confiere a la levadura resistencia al estrés oxidativo, debido a que los AG saturados y monoinsaturados son resistentes a la peroxidación de lípidos (Aguilar-Toral *et al.,* 2014).

| **Fracción subcelular** | **% fosfolípidos totales** | | | | | | |
|---|---|---|---|---|---|---|---|
| | PtdCho | PtdEtn | PtdIns | PtdSer | CL | PA | Otros |
| **Membrana plasmática** | 16.8 | 20.3 | 17.7 | 33.6 | 0.2 | 3.9 | 6.9 |
| **Vesículas secretoras** | 35 | 22.3 | 19.1 | 12.9 | 0.7 | 1.2 | 8.8 |
| **Vacuolas** | 46.5 | 19.4 | 18.3 | 4.4 | 1.6 | 2.1 | 7.7 |
| **Núcleo** | 44.6 | 26.9 | 15.1 | 5.9 | <1.0 | 2.2 | 4.3 |
| **Peroxisomas** | 48.2 | 22.9 | 15.8 | 4.5 | 7 | 1.6 | ND |
| **Microsomas** | 51.3 | 33.4 | 7.5 | 6.6 | 0.4 | 0.2 | 0.5 |
| **Mitocondria** | 40.2 | 26.5 | 14.6 | 3 | 13.3 | 2.4 | ND |
| **Membrana interna** | 38.4 | 24 | 16.2 | 3.8 | 16.1 | 1.5 | ND |
| **Membrana externa** | 45.6 | 32.6 | 10.2 | 1.2 | 5.9 | 4.4 | ND |

**Tabla 1. Composición de fosfolípidos de fracciones subcelulares de *S. cerevisiae*.** PtdCho, fosfatidilcolina; PtdEtn, fosfatidiletanolamina; PtdIns, fosfatidilinositol; PtdSer, fosfatidilserina; CL, cardiolipina; PA, ácido fosfatídico; ND, no detectable (Zinser et al., 1991)

La exposición de la levadura al etanol da como resultado un aumento de la fluidez de la membrana y la consiguiente disminución de su integridad. Esto se debe a que el etanol tiene la capacidad de formar puentes de hidrógeno con los lípidos de membrana, experimentando una reducción en el grosor de la bicapa a medida que el etanol reacciona con el grupo metilo de los ácidos grasos provocando su interdigitalización (Rowe & Cutrera., 1990). Esta alteración ocasiona un aumento en la permeabilidad iónica y cambios en la actividad de proteínas de membrana (Henderson *et al.,* 2014). Existe una relación entre a tolerancia al etanol de la levadura y su composición lipídica, por lo tanto, cambios en la composición de la membrana afectan su capacidad para tolerar el etanol (Mishra & Prasad., 1989).

### 1.1.7 Incorporación de Ácidos grasos polinsaturados (AGPI) por la levadura y su susceptibilidad al estrés oxidativo.

Las cepas de laboratorio de *S. cerevisiae* normalmente crecen en entornos controlados que favorecen un crecimiento rápido; sin embargo, las cepas ambientales de *S. cerevisiae* están expuestas a ambientes mucho más adversos (Parapouli *et al*., 2020), por lo que es de esperar que la composición de sus membranas pueda cambiar. Lo mismo sucede en el contexto de procesos industriales, como es el caso de la utilización de la levadura para la producción de ácidos grasos poliinsaturados (AGPI), en donde *S. cerevisiae* tiene la capacidad de sintetizar estos AGPI a través de la expresión desaturasas y elongasas (Tavares *et al*., 2011). Esto incrementa la sensibilidad de la levadura al estrés, ya que *S. cerevisiae* tiene la capacidad de incorporar AGPI presentes en su entorno a sus membranas. Lo cual resulta en un incremento en la susceptibilidad de las membranas de la levadura al daño oxidativo, ya que, a mayor número de dobles enlaces, mayor es la sensibilidad de un ácido graso a la peroxidación (Wagner et al., 1994). Se ha encontrado que esto es perjudicial para el metabolismo energético mitocondrial de la levadura, ya que la incorporación de AGPI en las membranas mitocondrias incrementa en gran medida la susceptibilidad la sensibilidad de la mitocondria el estrés oxidativo (Cortés-Rojo *et al*., 2009), dando lugar a disfunción de la CTE, incremento en la producción de ERO y una menor viabilidad celular bajo condiciones que no son estresantes para la levadura con su composición de ácidos grasos nativa (Aguilar-Toral *et al.,* 2014).

Un incremento de las ERO por la presencia de AGPI en la levadura está relacionado un aumento en la liberación de hierro por la mitocondria (Cortés-Rojo *et al.,* 2011). El hierro cataliza la producción de radical hidroxilo mediante la reacción de Fenton. A este respecto, se ha demostrado que el etanol incrementa las concentraciones de hierro libre en la mitocondria (Gomez, M., *et al* 2014). La generación de ERO por el etanol está asociada con la liberación de hierro de las enzimas que contienen Fe-S (Pérez-Gallardo *et al*., 2013), lo que ocasiona estrés oxidativo y disfunción mitocondrial.

## 2. JUSTIFICACIÓN

Los ácidos grasos saturados de la levadura contribuyen a su resistencia al estrés oxidativo al ser insensible a la peroxidación lipídica. Sin embargo, la incorporación de AGPI a las membranas de *S. cerevisiae* incrementa su susceptibilidad a la peroxidación lipídica, la cual, junto con el etanol, aumentan la concentración de ERO. La cardiolipina es uno de los blancos más importantes del estrés oxidativo debido a su mayor susceptibilidad a la peroxidación.

La apertura del CIML es un mecanismo de protección contra la producción excesiva de ERO en la mitocondria y su funcionamiento depende de la interacción con la cardiolipina. Por lo tanto, proponemos que la presencia de AGPI incrementa la sensibilidad de *S. cerevisiae* al etanol al promover la peroxidación de la cardiolipina, lo que afecta el funcionamiento del CIML y conduce a un incremento del estrés oxidativo. Esto a su vez provoca un fenotipo de muerte celular exacerbado.

## 3. HIPÓTESIS

La peroxidación de lípidos exacerba la muerte celular por etanol mediante la disfunción del canal inespecífico mitocondrial de *S. cerevisiae.*

## 4. OBJETIVO GENERAL

Demostrar que la peroxidación de lípidos incrementa la muerte celular por etanol en *S. cerevisiae* al promover la disfunción del CIML.

### 4.1 OBJETIVOS ESPECÍFICOS

- Determinar el efecto de la peroxidación de lípidos sobre la inducción de la muerte celular de la levadura.
- Evaluar el efecto de la peroxidación de lípidos sobre la apertura/cierre del CIML.
- Determinar la relación entre la muerte celular de la levadura y los niveles de estrés oxidativo mitocondrial.

# 5. CAPITULO I. EL ÁCIDO LINOLÉNICO Y EL ETANOL EXACERBAN LA MUERTE CELULAR EN *S. CEREVISIAE* AL PROMOVER LA PEROXIDACIÓN LIPÍDICA, LA PÉRDIDA DE CARDIOLIPINA Y LA NECROSIS

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# Linolenic Acid Plus Ethanol Exacerbates Cell Death in *SaccharoMyces cerevisiae* by Promoting Lipid Peroxidation, Cardiolipin Loss, and Necrosis

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**Abstract:** Polyunsaturated fatty acids (PUFA) hypersensitize yeast to oxidative stress. Ethanol accumulation during fermentation is another factor that induces oxidative stress via mitochondrial dysfunction and ROS overproduction. Since this microorganism has raised growing interest as a PUFA factory, we have studied if the combination of PUFA plus ethanol enhances yeast death. Respiration, ROS generation, lipid peroxidation, mitochondrial cardiolipin content, and cell death were assessed in yeast grown in the presence of 10% ethanol (ETOH) or linolenic acid (C18:3), or ethanol plus C18:3 (ETOH+C18:3). Lipid peroxidation and cardiolipin loss were several-fold higher in cells with ETOH+C18:3 than with C18:3. On the contrary, ETOH tended to increase cardiolipin content without inducing changes in lipid peroxidation. This was consistent with a remarkable diminution of cell growth and an exacerbated propidium iodide staining in cells with only ETOH+C18:3. The respiration rate decreased with all the treatments to a similar degree, and this was paralleled with similar increments in ROS between all the treatments. These results indicate that PUFA plus ethanol hypersensitize yeast to necrotic cell death by exacerbating membrane damage and mitochondrial cardiolipin loss, independent of mitochondrial dysfunction and ROS generation. The implications of these observations for some biotechnological applications in yeast and its physiology are discussed.

**Keywords:** PUFA; yeast; alcohol; apoptosis; necrosis; mitochondria; oxidative stress; linolenic acid

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**Citation:** Olmos-Orizaba, B.E.; Arroyo-Peñaloza, J.S.; Martínez-Alcántar, L.; Montoya-Pérez, R.; Flores-García, A.; Rodríguez-Orozco, A.R.; Calderón-Cortés, E.; Saavedra-Molina, A.; Campos-García, J.; Cortés-Rojo, C. Linolenic Acid Plus Ethanol Exacerbates Cell Death in *Saccharomyces cerevisiae* by Promoting Lipid Peroxidation, Cardiolipin Loss, and Necrosis. *Life* **2022**, *12*, 1052. https://doi.org/10.3390/life12071052

Academic Editors: Arnold J. M. Driessen and Rafael A. Casuso

Received: 30 June 2022
Accepted: 12 July 2022

Published: 14 July 2022

## 1. Introduction

*Saccharomyces cerevisiae* is a Crabtree-positive yeast that produces ethanol to overflow pyruvate excess during glucose oxidation. Once glucose decreases to a certain threshold, *S. cerevisiae* utilizes ethanol as a respiratory substrate [1]. Another feature of *S. cerevisiae* is its membrane fatty acid composition, which consists of only saturated and monounsaturated fatty acids (SFA and MUFA, respectively), and has an absence of polyunsaturated fatty acids (PUFA) [2]. This confers yeast membranes a high resistance to lipid peroxidationsince SFA and MUFA are not prone to free radical attack due to the absence of bis-allylic hydrogens [3]. Nevertheless, yeast can incorporate PUFA from its environment into its membranes [4]. PUFA in mitochondrial membranes significantly enhances the sensitivity of yeast mitochondria to lipid peroxidation, leading to the impairment of electron transport chain (ETC) function. In contrast, yeast with native fatty acid composition is highly resistant to the effects of oxidative stress on mitochondria due to an insensitivity to lipid peroxidation [5].

Another challenge for yeast is ethanol accumulation in its surrounding media. Biological membranes are one of the main targets of ethanol. Hence, an adaptive response occurs in the yeast plasma membrane to enhance ethanol tolerance by modifying lipid composition and fluidity. Ethanol increases membrane fluidity, leading to a decrease in membrane integrity [6]. To counteract membrane fluidization, the content of oleic acid (C18:1) increases in the plasma membrane [7], along with the ergosterol/phospholipid ratio [8]. In turn, the increase in ergosterol enhances the activity of proton-pumping ATPase, which establishes a transmembrane proton gradient that drives several vital physiological functions in yeast. It is believed that enhanced ATPase activity participates in ethanol tolerance by counteracting a proton influx elicited by ethanol [9]. Ethanol induces apoptotic cell death by interfering with mitochondrial dynamics [10]. Moreover, ethanol causes oxidative stress and mitochondrial dysfunction by disrupting mitochondrial iron homeostasis and increasing ROS production [11]. On the other hand, cardiolipin is a phospholipid in the inner mitochondrial membrane that is crucial for the function of the ETC, and thus, for cell survival. Cardiolipin is highly susceptible to lipid peroxidation by containing four acyl chains [12]. In yeast, PUFA are readily incorporated into cardiolipin when present in the culture media, and cardiolipin hydroperoxides are produced even in the absence of an oxidative stimulus [13]. This has the potential to impair mitochondrial function since cardiolipin peroxides disrupt the interactions of the ETC with the inner mitochondrial membrane [14].

Yeast is a potential factory for the synthetic production of PUFA via metabolic engineering [15,16]. Increasing the unsaturation of yeast membranes by augmenting the content of oleic acid has been shown to increase ethanol tolerance [17]. However, increased PUFA content in membranes with linoleic (C18:2) or linolenic acid (C18:3) does not enhance ethanol tolerance [18]. In this work, we hypothesize that the presence of PUFA plus ethanol exacerbates cell death in yeast by inducing lipid peroxidation, cardiolipin loss, mitochondrial dysfunction, and necrosis. This is postulated on the basis of ethanol toxicity in yeast via mitochondrial oxidative stress [11], increased susceptibility to oxidative stress in yeast enriched with PUFA [5], and the essentiality of cardiolipin for mitochondrial function [12]. It was found that treatment with C18:3 plus 10% ethanol stimulates necrotic cell death, lipid peroxidation, and cardiolipin loss to a higher degree than treatment with C18:3 alone. On the contrary, ethanol alone tends to increase cardiolipin and does not enhance lipid peroxidation. Moreover, deleterious alterations in respiration and ROS levels were induced to the same extent with any of these treatments. These data suggest that decreased mitochondrial function and ROS overproduction are not factors responsible for the enhanced toxicity of ethanol plus C18:3, but exacerbation in both lipid peroxidation and cardiolipin loss are crucial events that lead to aggravated cell death by necrosis.

## 1. Materials and Methods

### 1.1. Yeast Strains and Growth Conditions

The haploid *S. cerevisiae* strain, BY4741 (*Mat α*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; WT), was obtained from EUROSCARF (Frankfurt, Germany). The cultures ($OD_{600nm}$ = 0.03) were grown in liquid YPD medium (1% yeast extract, 5% peptone, and 2% dextrose) with orbital shaking at 250 rpm and a temperature of 30 °C. Twelve hours later, 10% (*v*/*v*) ethanol was added. Cells were harvested in the early stationary phase (~20 h). For the manipulation of membrane fatty acid content, 1 mM linolenic acid (C18:3) solubilized with 5% (*v*/*v*) Igepal CA-630 was added to YPD medium right before the inoculation with yeast [5].

### 1.2. Determination of Cell Growth

Cell growth was recorded in the prepared cultures, as described above, by measuring the $OD_{600nm}$ every two hours for up to 24 h in a Shimadzu UV2550 spectrophotometer.

### 1.1. Evaluation of Cell Death

Propidium iodide (PI) (Thermo Fisher Scientific, Waltham, MA, USA) was used to assess the percentage of cells that underwent necrosis. The cells were harvested and resuspended in 200 μL of phosphate buffered saline (PBS), and two 1:10 dilutions were made with a binding buffer. The cells were incubated with 1 μL PI for 15 min in the dark at room temperature. This mixture was centrifuged and resuspended at a final volume of one milliliter. Fluorescence was quantified using an Accuri-C6 flow cytometer (BD Biosciences, San Jose, CA, USA). PI fluorescence was assessed with the FL2 channel at 518/617 nm. Forward vs. side scatter (FSC vs. SSC) gating was done to discard cellular debris with low FSC. Positive controls of cell death were carried out by stressing cells with heat shock(50 °C for 90 min). This resulted in an increase in dead cells from 0.2% to 55.2% in control yeast and from 50.8 to 88.3% in C18:3 + ETOH cells (see Supplementary Figure S1).

### 1.2. Lipid Peroxidation Measurement

Lipid peroxidation levels were evaluated in cells by the thiobarbituric acid (TBA) method [19]. Twenty-five mg cells were mixed with 2 mL of an acid mixture containing 0.25 M HCl, 15% (*w*/*v*) trichloroacetic acid, and 0.375% (*w*/*v*) TBA. This mixture was vortexed for 5 s, boiled for 15 min, cooled to room temperature, and centrifuged at 5000 rpm for 5 min. The absorbance of the supernatant was measured at 532 nm on a Shimadzu UV2550 spectrophotometer. The results were calculated using the molar extinction coefficient of malondialdehyde and expressed in nanomoles of TBA-reactive species (TBARS) per milligram cells.

### 1.3. Mitochondria Isolation

Mitochondria were isolated according to the method by Peña et al. [20], with modifications. Cells were harvested and washed twice with deionized water at 5000 rpm for 5 min. The cell pellet was resuspended in a medium containing 0.6 M mannitol, 5 mM MES (10 mM 2-(N-morpholino)ethanesulfonic acid), and 0.1% bovine serum albumin (pH 6.8 with triethanolamine). The cells were resuspended at a ratio of 0.4 g cells/mL medium. This suspension was mixed with 50% (*v*/*v*) glass beads (0.5 mm diameter) andvortexed at high speed for ten cycles of 1 min vortexing–1 min ice. The cell homogenate was centrifuged at 2590 rpm for 5 min. The pellet was discarded and the supernatant was centrifuged at 12,000 rpm for 10 min. Centrifugations were carried out at 4 °C in a Sorvall RC 6+ centrifuge with the SS-34 rotor. The mitochondrial protein concentration was determined by the Biuret method. The intactness of the mitochondrial preparations was assessed by measuring the rate of respiration in phosphorylating state 3 (stimulated with ADP) and state 4 (corresponding to the rate of idling respiration after ADP exhaustion). In our hands, control mitochondria exhibited respiratory control ratios (state 3/state 4) above 3.0 (see Supplementary Figure S2).

### 1.4. Determination of Cardiolipin Levels

Cardiolipin levels were assessed in isolated mitochondria according to the method by Gallet et al. [21]. Ten mg/mL of mitochondrial protein was incubated with 150 μM 10-N-nonyl acridine orange bromide for 15 min at 20 °C in a buffer containing 0.6 M mannitol and 10 mM tris/HCl, pH 7.4. This mixture was centrifuged at 30,000 *g* for 5 min. The unbounded dye in the supernatant was measured by determining its absorbance at 495 nm. The free dye concentration was calculated from a calibration curve obtained with known 10-N-nonyl acridine orange concentrations (0–10 μM). The concentration of cardiolipin was estimated by subtracting the total amount of dye added to the mitochondrial suspension minus the amount of unbounded dye, taking into consideration a stoichiometry of 2 mols of 10-N-nonyl acridine orange/mol cardiolipin. Positive controls for this method were done consisting of a calibration curve with increased concentrations of cardiolipin liposomes vs. the absorbance of acridine orange at 495 nm. This curve shows an increase in acridine orange absorbance that is proportional to the concentration of cardiolipin liposomes, which was saturable at supraphysiological concentrations above 0.2 mg/mL cardiolipin (Supplementary Figure S3).

### 1.1. *Evaluation of Oxygen Consumption*

The in situ mitochondrial respiration was evaluated in whole cells. After 20 h of growth, the cells were starved overnight by transferring them to sterile deionized water in order to exhaust endogenous respiratory substrates. Twelve-and-a-half mg of cells were resuspended in MES-TEA buffer (10 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.0 with triethanolamine) and added to a sealed glass chamber with constant stirring. Oxygen consumption determinations started after the addition of 10 mM of glucose (state 4). Two minutes later, 15 μM of carbonyl cyanide m-chlorophenylhydrazone (CCCP) was addedto stimulate uncoupled respiration (state U). One μg antimycin was added at the end ofthe determinations to abolish ETC activity for discrimination between non-mitochondrial and mitochondrial oxygen consumption. Under our experimental conditions, respiration with antimycin A was negligible (Figure 5A), thereby it was assumed that whole oxygen consumption in yeast was due to mitochondrial ETC activity. The oxygen consumptionrate was determined at room temperature using a Clark-type oxygen electrode coupled toa YSI 5300 oxygen monitor and a computer for data acquisition.

### 1.2. *Determination of ROS Levels*

The cells were washed four times with deionized water and resuspended in 1 mL MES-TEA buffer (pH 6.0). One mg of cells were incubated with constant stirring at 4 °C with 12.5 mM of 2′,7′-dichlorodihydrofluorescein diacetate ($H_2DCFDA$), a cell-permeable probe for ROS. After 20 min, the cells were washed with 50 mM of $KH_2PO_4$ buffer (pH 7.6) to remove the ROS probe remaining outside the cells. The cells were placed in a quartz cuvette at a final volume of 2 mL. Basal fluorescence was followed for 1 min ($\lambda_{ex}$ 485 nm; $\lambda_{em}$ 520 nm). Then, ROS production was stimulated by adding 10 mM of dextrose as a substrate, and the changes in the fluorescence were further followed for 15 min. Fluorescence changes were evaluated in a Shimadzu RF-5301PC. The data were calculated by subtracting the final fluorescence obtained after 15 min of dextrose addition minus the fluorescence detected before dextrose addition, and the result was divided by 15. The results were expressed as the change in arbitrary fluorescence units (AFU) per minute per mg of protein.

### 1.3. *Statistical Analysis*

Data are expressed as the mean ± standard error of $n \geq 5$. Statistical significances: * sign denotes statistically significant differences vs. Control; ¶ sign denotes statistically significant differences vs. C18:3; § sign denotes statistically significant differences vs. ETOH; # sign denotes statistically significant differences vs. C18:3 + ETOH; and n.s. means non-statistical significance ($p < 0.05$, one-way ANOVA, followed by Tukey's post-hoc test).

## 2. Results

### 2.1. *Influence of PUFA on the Effects of Ethanol on Yeast Growth and Cell Death*

Cell growth and death were evaluated to assess whether the presence of PUFA plus ethanol in the culture media exacerbates cell death. Twenty-four-hour growth curves (Figure 1) show that exponential growth in cells with ethanol (ETOH, cyan circles) stops at 12 h of incubation, corresponding to the time at which ethanol was added to the culture. In contrast, the control cells (black circles) keep growing for up to 16 h. There was a slower growth in the cells with linolenic acid (C18:3, blue circles) in comparison to the control group; however, these cells reached the same optical density (OD) as the control cells at 20 h. More deleterious effects were observed in the cells with linolenic acid plus ethanol (C18:3 + ETOH, red circles) since these cells exhibited, as expected, a similar decrease in their growth compared to the C18:3 cells before the addition of ethanol, but ethanol addition at 12 h immediately stopped the growth, thereby yielding the lowest OD among all the treatments. To discard that the deleterious effects of linolenic acid plus ethanol treatment on cell growth were due to the detergent used to solubilize linolenic acid, cells were grown in the presence of the detergent IGEPAL CA-630 and ethanol (IGEPAL + ETOH, yellow circles). IGEPAL + ETOH cells exhibited similar growth as the ETOH cells, indicating that impaired growth by C18:3 + ETOH was not due to the presence of IGEPAL CA-630.

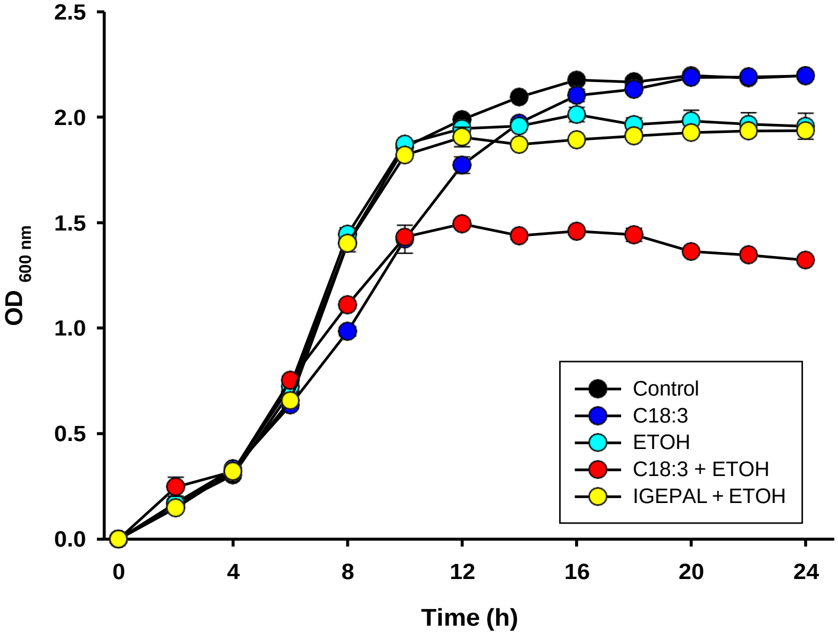

**Figure 1.** Effects of ethanol and C18:3 on yeast growth. Cell growth was assessed by recording the optical density at 600 nm (OD $_{(600nm)}$) for 24 h cultures every two hours. Ethanol was added at the 12th hour of growth, while C18:3 was added at the beginning of the growth.

Regarding cell death, it was observed that cells that were positive for propidium iodide (PI), a marker of necrotic cell death (Figure 2A,B), increased from 0.15 ± 0.02% in control cells to 2.9 ± 0.8% in C18:3 cells. This parameter further increased from 3.0 ± 0.9% in ETOH cells to 38.6 ± 3.6% in the ETOH+C18:3 cells. Overall, these results suggest that the simultaneous presence of PUFA plus ethanol enhances the arrest of yeast growth via a robust induction of necrotic cell death.

### *1.1. Influence of PUFA on Lipid Peroxidation Levels in Yeast Treated with Ethanol*

Lipid peroxidation was assessed to determine if necrotic cell death in ETOH + C18:3 cells was related to enhanced oxidative damage to membrane lipids. Lipid peroxidation levels (Figure 3) were 2.8-fold higher in C18:3 cells than in the control cells. Exacerbated lipid peroxidation was observed in ETOH+C18:3 cells, as lipid peroxidation was 6.8-fold higher in these cells than in control cells. In contrast, no changes in lipid peroxidation were observed in the cells with ETOH. This suggests that the presence of C18:3 exacerbates membrane damage by ethanol, which may be related to the enhanced necrotic cell death phenotype observed in ETOH+C18:3 cells (Figure 2).

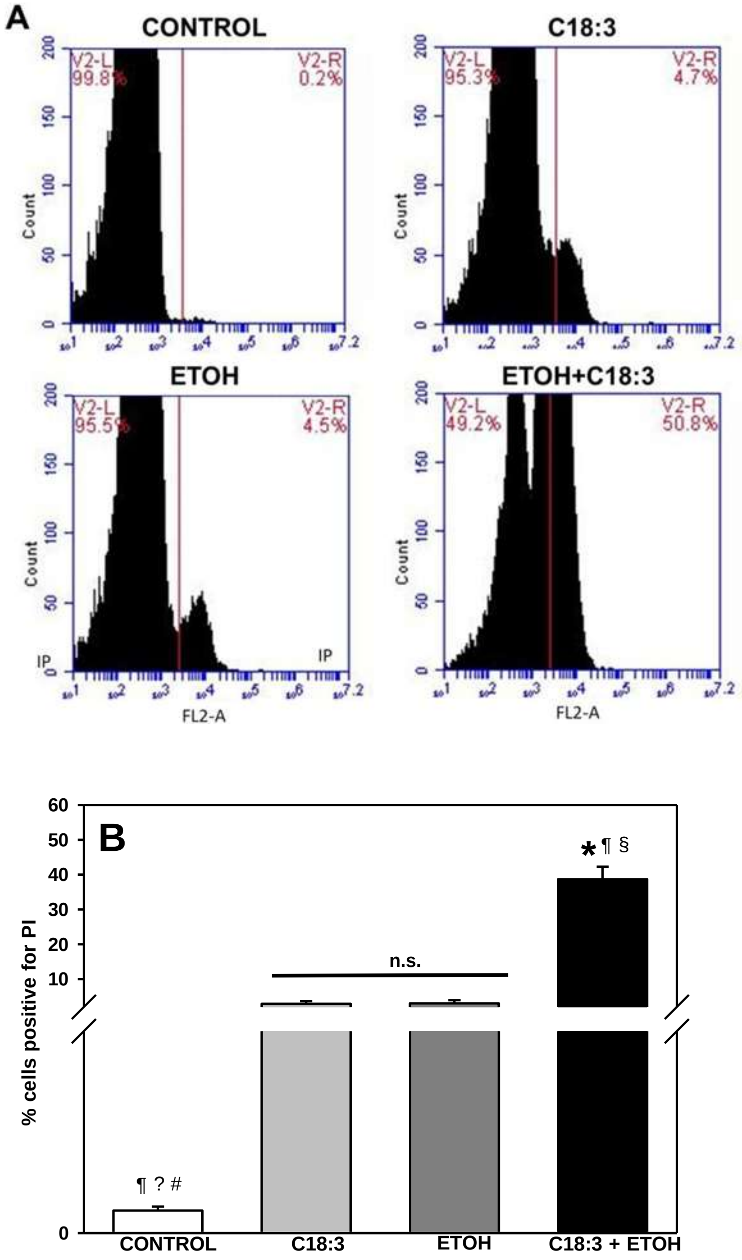

**Figure 2.** Effects of ethanol and C18:3 on yeast cell death. (**A**) Representative flow cytometry univariate histograms analyzing negative (V2-L region) and positive (V2-R region) populations of PI staining. PI staining was analyzed with the FL2-A (518/617) fluorescence channel. The x axis represents the propidium iodide fluorescence in arbitrary units. (**B**) Quantification of the percentage of cells in the V2-L region. The data are presented as the mean ± s.e. of $n \geq 4$. * $p < 0.05$ vs. CTRL; ¶ $p < 0.05$ vs. C18:3; $^{\S}$ $p < 0.05$ vs. ETOH; $^{?,\#}$ $p < 0.05$; n.s. non-statistical significance (one-way ANOVA with Tukey's post-hoc test).

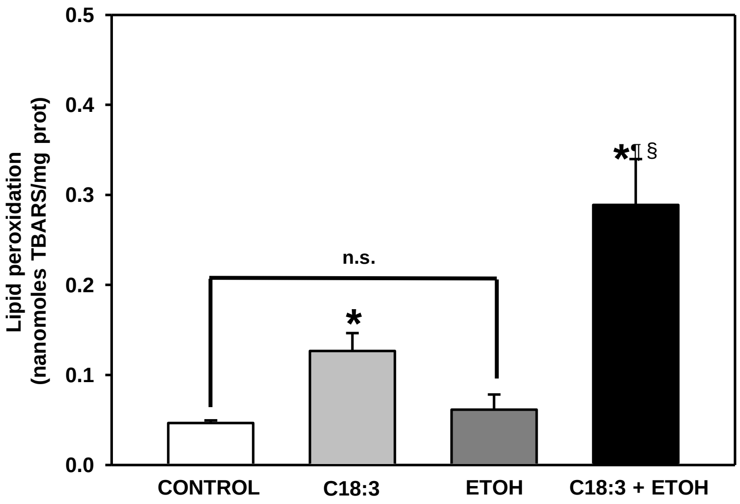

**Figure 3.** Effects of ethanol and C18:3 on lipid peroxidation in yeast cells. The results are expressed as nanomoles of thiobarbituric acid reactive substances (TBARS) per milligram of cells. The data are presented as the mean ± s.e. of $n = 4$. * $p < 0.05$ vs. CTRL; ¶ $p < 0.05$ vs. C18:3; § $p < 0.05$ vs. ETOH; n.s. non-statistical significance (one-way ANOVA with Tukey's post-hoc test).

### 1.1. *Effects of Ethanol and PUFA on Cardiolipin Levels*

Cardiolipin levels were evaluated in isolated mitochondria to determine if exacerbated lipid peroxidation in ETOH+C18:3 cells induces diminution in cardiolipin content. In comparison to the control cells, the presence of PUFA did not modify cardiolipin levels in mitochondria from C18:3 cells (Figure 4) per se. Ethanol tended to increase cardiolipin levels in ETOH cells in a non-statistically significant fashion. On the contrary, ETOH + C18:3 cells exhibited a 73% decrease in cardiolipins levels with respect to control cells. This suggests that enhanced lipid peroxidation in ETOH + C18:3 cells involves damage to mitochondrial membranes and subsequent cardiolipin loss.

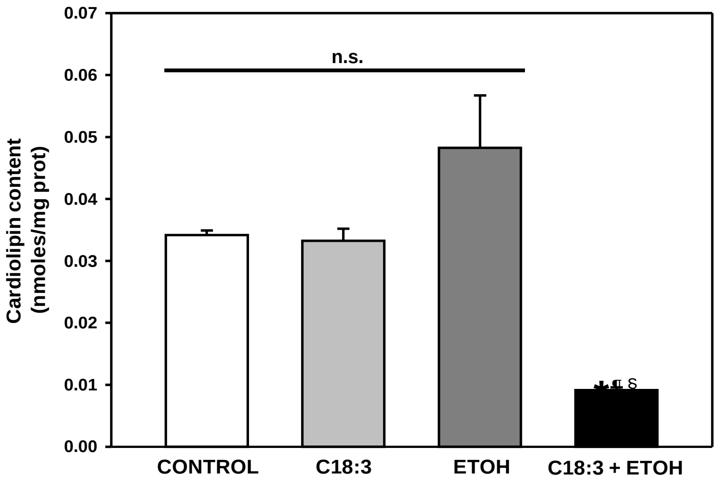

**Figure 4.** Effects of ethanol and C18:3 on cardiolipin levels in yeast mitochondria. The data are presented as the mean ± s.e. of $n = 4$. * $p < 0.05$ vs. CTRL; ¶ $p < 0.05$ vs. C18:3; § $p < 0.05$ vs. ETOH; n.s. non-statistical significance (one-way ANOVA with Tukey's post-hoc test).

### *1.1. Influence of PUFA over the Effects of Ethanol on In Situ Mitochondrial Respiration and ROS Production*

Antimycin A-sensitive respiration in whole yeast cells was assessed to determine if in situ mitochondrial function is impaired to a higher degree with ETOH + C18:3 than with only ETOH or C18:3. As observed in the representative traces of (Figure 5A), idling respiration (i.e., state 4) stimulated with dextrose as a substrate, and uncoupled respiration (i.e., in state U) stimulated with the uncoupler CCCP, decreased to a similar degree with any treatment in comparison to the control. The quantification of respiration data revealed a ~47% decrease in state 4 respiration (Figure 5B) and 52% in state U respiration (Figure 5C). In concordance with this result, representative traces of ROS production revealed an increase in this parameter at the same magnitude with all the treatments in comparison to the control. The quantification of these data revealed an increase in ~66% of all the treatments vs. the control group (Figure 6). Overall, these results suggest that mitochondrial dysfunction and increased ROS generation were common events involved in the deleterious effects of ETOH, C18.3, or ETOH + C18:3. Therefore, exacerbated lipid peroxidation and cardiolipin loss accompanied by mitochondrial dysfunction and ROS production is more important for enhanced necrotic cell death by ETOH + PUFA than only mitochondrial dysfunction or ROS production.

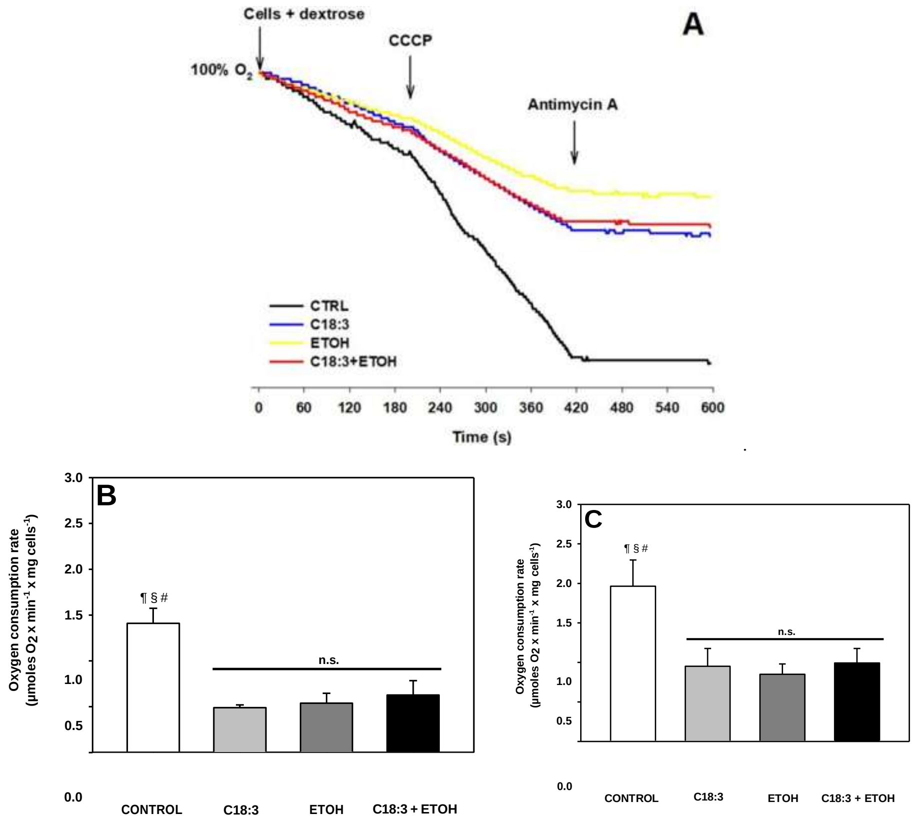

**Figure 5.** Effects of ethanol and C18:3 on in situ mitochondrial respiration in yeast. (**A**) Representative trace of respiration. Dextrose was added as substrate to stimulate state 4 respiration. The uncoupler, CCCP, was added to stimulate uncoupled respiration (state U). Antimycin was added to discriminate between non-mitochondrial and mitochondrial respiration. Quantification of respiration in states 4 (**B**) and U (**C**). The data are presented as the mean ± s.e. of $n \geq 4$. ¶ $p < 0.05$ vs. C18:3; § $p < 0.05$ vs. ETOH; # $p < 0.05$ vs. C18:3 + ETOH; n.s. non-statistical significance (one-way ANOVA with Tukey's post-hoc test).

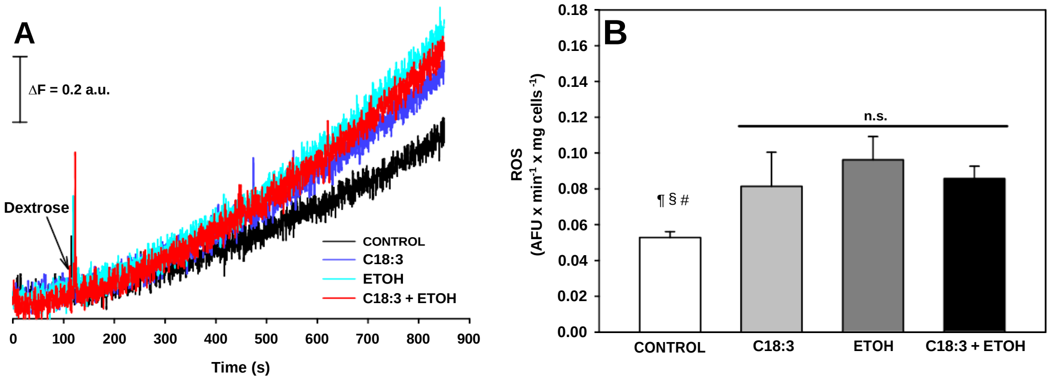

**Figure 6.** Effects of ethanol and C18:3 on reactive oxygen species (ROS) levels in yeast. (**A**) Representative trace of ROS. Cells were incubated with the fluorescent ROS probe $H_2DCFDA$ as indicated in Materials and Methods. Basal fluorescence was recorded and, where indicated, dextrose was added as substrate to stimulate ROS production. (**B**) Quantification of ROS production stimulated with dextrose. The results are expressed as arbitrary fluorescence units (AFU) per minute per milligram of cells. The data are presented as the mean ± s.e. of $n \geq 4$. $^{¶}\,p < 0.05$ vs. C18:3; $^{\S}\,p < 0.05$ vs. ETOH; $^{\#}\,p < 0.05$ vs. C18:3 + ETOH; n.s. non-statistical significance (one-way ANOVA with Tukey's post-hoc test).

## 1. Discussion

The results of the present study show that PUFA plus ethanol severely impaired cell growth (Figure 1) and promotes a necrotic phenotype in the ETOH + C18:3 cells (Figure 2). This was associated with a striking increase in lipid peroxidation (Figure 3) and loss of cardiolipin (Figure 4). In contrast, ethanol in the absence of PUFA (i.e., in the ETOH cells) had no effect on lipid peroxidation levels (Figure 3), had a moderate effect on yeast growth that was similar to that observed in C18:3 cells, tended to increase cardiolipin levels (Figure 4), and slightly induced necrotic cell death like in C18:3 cells (Figure 2). The high levels of lipid peroxidation in the ETOH + C18:3 cells may be related to the ability of ethanol to increase the labile iron pool by disrupting mitochondrial iron homeostasis [11]. This may disrupt overall iron homeostasis in the cell, thereby causing increased levels of free iron in the cytosol [22]. As a consequence, augmented free iron levels could catalyze the generation of the highly oxidant hydroxyl radical ($OH^{\bullet}$) [13], leading to the enhancement of lipid peroxidation as $OH^{\bullet}$ initiates free radical attack in PUFA [23]. In turn, lipid peroxidation increases free iron levels in the mitochondria of yeast grown with PUFA [24]. This may be perpetuating the lipid peroxidation process by establishing a positive feed-forward loop of lipid peroxidation–iron release–lipid peroxidation, thereby explaining the higher levels of lipid peroxidation in ETOH + C18:3 cells (Figure 3). Conversely, as yeast without C18:3 does not contain PUFA in its membranes, there is no site for the attack of $OH^{\bullet}$ radicals in the acyl chains of phospholipids, thereby limiting lipid peroxidation and oxidative stress to other molecules than lipids.

The notable loss of cardiolipin in ETOH + C18:3 cells may be related to the fact that yeast readily incorporates PUFA into cardiolipin acyl chains [13]. Thus, the enrichment of cardiolipin with C18:3 may aggravate lipid peroxidation by ethanol, causing the eventual loss of cardiolipin molecules due to the formation of small aldehydes as final products of lipid peroxidation [3]. There was no decrease in cardiolipin levels in the C18:3 cells (Figure 4) and lipid peroxidation was about a third part of that observed in the ETOH + C18:3 cells. Yeast possesses the cardiolipin phospholipase, Cld1, which remodels peroxidized cardiolipin by substituting peroxidized acyl chains with linoleate [13]. Therefore, the lower levels of lipid peroxidation together with Cld1 activity may be limiting cardiolipin loss in

C18:3 cells. On the contrary, excessive lipid peroxidation in the ETOH + C18:3 cells may be overcoming the ability of Cld1 to remodel cardiolipin, thereby augmenting cardiolipin loss in this group.

Respiration was inhibited equally in C18:3, ETOH, and ETOH+C18:3 cells in both state 4 (Figure 5B) and state U (Figure 5C). A full suppression of respiration in the ETOH + C18:3 cells due to the severe loss of cardiolipin may be expected, as observed in Figure 4. However, as demonstrated previously [25,26], the lack of cardiolipin does not fully impair the rate of respiration in yeast at moderate or high rates of oxidative phosphorylation and under optimal experimental conditions. Thus, the decreased rate of respiration in ETOH + C18:3 cells should be attributed to another factor other than cardiolipin loss, as the respiration rate in these cells was similar to that observed in both C18:3 and ETOH cells—despite the latter two having normal levels of cardiolipin when compared to control cells (Figure 4). The decrease in respiration in ETOH cells agrees with a previous report that 10% ethanol decreases respiration in state 4 and state U, which may be the result of oxidative damage in the mitochondria due to increased levels of free iron [11]. On the other hand, decreased respiration in the C18:3 cells may be associated with a moderate induction of lipid peroxidation (Figure 3), since lipid peroxidation is a key factor impairing the function of the ETC [27]. Thus, these factors may also be contributing to decreased respiration in ETOH+C18:3 cells. In agreement with the inhibition of respiration of a similar magnitude between the ETOH, C18:3, and ETOH + C18.3 cells, there was a parallel increase in ROS production of equal magnitude in all these cells (Figure 6). Therefore, the differences in necrosis, lipid peroxidation, and cardiolipin loss between the ETOH + C18:3 cells and the ETOH and C18:3 cells cannot be attributed to differences in ROS production.

A low percentage of cells underwent cell death, except for the ETOH + C18:3 cells (Figure 2). A high percentage of these cells internalized PI, indicating that ETOH + C18:3 cells die by necrosis. Plasma membrane disruption is a key step in necrotic cell death in both yeast [28] and animals [29]. Lipid peroxidation is a chief factor driving the disruption of both the plasma membrane and organelles in necrosis [29]. Moreover, the oxidation of cardiolipin and diminution in its content in the mitochondria of necrotic cells has been observed [30]. On this basis, it can be hypothesized that exacerbated lipid peroxidation induced by ethanol in ETOH + C18:3 cells (Figure 3) may lead to plasma membrane disruption and necrosis induction, as evidenced by the high percentage of ETOH + C18:3 cells positive to PI (Figure 4).Moreover, the drastic loss of cardiolipin of these cells may be contributing to the necrotic phenotype by decreasing stress resistance, as it has been demonstrated that cardiolipin depletion in yeast decreases mitochondrial fitness in stressful conditions like hyperosmolarity or high temperatures [25,26]. In agreement with this hypothesis, the ETOH cells exhibited negligible rates of necrosis (Figure 2), which may be due to the lipid peroxidation (Figure 3) and cardiolipin levels (Figure 4) remaining unchanged in comparison to the control cells.

*S. cerevisiae* is used in many industrial and biotechnological processes involving large scale fermentations, which implies the accumulation of ethanol [10]. Indeed, *S. cerevisiae* has been engineered to produce PUFA, which has been challenging due to some fatty acids negatively modifying stress tolerance in this yeast [31]. Hence, the study of the effects of PUFA on the tolerance to stress by ethanol is not trivial as it may give valuable information for the engineering of yeast for PUFA production. Taking into consideration the results of this study, the protection of yeast against lipid peroxidation and cardiolipin damage may be a useful strategy to improve yeast fitness during processes involving the accumulation of both ethanol and PUFA. Moreover, our results also highlight that targeting ROS production would not be enough to improve yeast resistance against necrotic cell death by ethanol plus PUFA, since ROS levels were not higher in the ETOH+C18:3 cells than in the C18:3 or ETOH cells (Figure 6). Finally, from a physiological point of view, the absence of PUFA in yeast membranes might be a mechanism for supporting high ethanol concentrations by preventing excessive membrane disruption by lipid peroxidation (Figure 3). This would lead to lower cardiolipin loss (Figure 4), lower necrosis induction

(Figure 2), and a higher survival rate (Figure 1), despite the decreased mitochondrial function (Figure 5) and increased ROS production (Figure 6) observed in the ETOH cells.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/life12071052/s1, Figure S1: Positive controls of necrotic cell death. Cells were grown for 24 h under control conditions or with 10% ethanol plus linolenic acid (ETOH + C18:3). Cells were harvested and incubated for 90 min at 30 °C (control) or 50 °C (heat shock). Cells positive for propidium iodide (PI) were assessed by flow cytometry as indicated in materials and methods. Signals in the V2-L region correspond to live cells; signals in the V2-R region correspond to necrotic, PI—positive cells; Figure S2: Representative time—trace of mitochondrial respiration for assessing quality control of the procedure for mitochondria isolation. 0.5 mg/mL mitochondrial protein was resuspended in 2.5 mL of a buffer with 10 mM MES-TEA (pH 6.0). After exhaustion of 10 mM ADP, respiration back to state 4 (i.e., idling respiration) and 100 s later, 20 mM ADP was added to induce state 3 respiration (i.e., phosphorylating respiration). 5 μg Antimycin A was added before ADP exhaustion to discriminate non-mitochondrial oxygen consumption; Figure S3: Positive control of cardiolipin detection with 10-N-nonyl acridine orange. Cardiolipin liposomes were prepared by sonicating a mixture of 1.7 mg cardiolipin (Sigma-Aldrich, St. Louis, MO, USA)with 1 mL 25 mM $KH_2PO_4$. Six cycles of sonication were applied at 40 W for 2.5 min each under a $N_2$ stream. Liposomes were placed in 1 mL 0.6 M mannitol/10 mM Tris (pH 7.4) at concentrations of 0.03, 0.08, 0.17, 0.27, 0.34 and 0.54 mg/mL. These solutions were mixed with 150 μM\10-N-nonyl acridine orange and incubated for 15 min in ice. Then, these mixtures were centrifuged at 15000 rpm for 5 min. The absorbance of the supernatant was measured at 495 nm.

**Author Contributions:** Conceptualization, C.C.-R.; methodology, J.C.-G. and L.M.-A.; formal analysis, C.C.-R., B.E.O.-O. and A.F.-G.; investigation, B.E.O.-O. and J.S.A.-P.; resources, C.C.-R., A.S.-M., R.M.-P. and J.C.-G.; writing—original draft preparation, B.E.O.-O. and C.C.-R.; writing—review and editing, C.C.-R. and A.R.R.-O.; visualization, C.C.-R. and A.R.R.-O.; supervision, C.C.-R. and E.C.-C.; project administration, C.C.-R. and E.C.-C.; funding acquisition, C.C.-R. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by Programa de Investigación 2021–2022 de la Coordinación de la Investigación Científica—UMSNH, grant number 5872654. The APC was not funded by any agency.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The raw data supporting the conclusions of this article will be made available by the corresponding author, without undue reservation.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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# 6. CAPITULO II. LA DISFUNCIÓN DEL CIML POR EL ÁCIDO LINOLÉNICO Y EL ETANOL PROVOCA LA MUERTE CELULAR DE SACCHAROMYCES CEREVISIAE.

# Yeast mitochondrial unselective channel (YMUC) dysfunction by linolenic acid and ethanol causes cell death in *Saccharomyces cerevisiae*.

## ABSTRACT

Under conditions of energy excess, yeast mitochondria uncouples its respiration via the aperture of a yeast mitochondrial unselective channel (YMUC) to avoid deleterious ROS overproduction. YMUC stabilization depends on cardiolipin, an atypical phospholipid that is highly sensitive to peroxidation due to the presence of four acyl chains. On the other hand, ethanol accumulation during respiro-fermentative metabolism causes oxidative stress. Other source of oxidative stress for yeast is the presence *of* polyunsaturated fatty acids (PUFA) in its environment; as these lipids incorporates into yeast membranes, rendering cells hypersensitive to oxidative stress. Thus, the presence of PUFA and ethanol may overwhelm the ability of yeast to cope with ROS production by impairing the modulation of YMUC due to enhanced lipid peroxidation and further cardiolipin loss. To test this hypothesis, we examined the effects of linolenic acid (C18:3) and ethanol on YMUC modulation, lipid peroxidation, cardiolipin content, respiration and the activity of respiratory complexes. According to the findings of our study, the presence of C18:3 and ethanol together caused yeast necrosis, which was linked to an increase in lipid peroxidation and a decrease in cardiolipin content. These changes contributed to the inability of YMUC and complexes of the electron transport chain to operate properly. Therefore, the death of yeast is caused by CIML malfunction, which is caused by the loss of cardiolipin as a result of lipid peroxidation produced by the presence of C18:3 and ethanol. This loss of cardiolipin ultimately results in the death of yeast.

**Keywords**: ethanol, PUFA, YMUC.

## 1. INTRODUCTION

The lipid composition in *S. cerevisiae* is essential to maintain the structural and functional integrity of the cell membrane and ensure proper cellular functions (Ferraz *et al*., 2021). The presence of polyunsaturated fatty acids (PUFA) in the environment may compromise yeast survival, since PUFA are highly susceptible to peroxidation, thereby increasing the sensitivity of yeast to oxidative stress, mitochondrial dysfunction (Cortés-Rojo *et al*., 2009), aging (Canizal-García *et al*., 2021) and cell death (Farrugia & Balzan, 2012). Recently, it has been shown that linolenic acid (C18:3) plus ethanol exacerbates cell death in yeast by promoting lipid peroxidation, cardiolipin loss, and necrosis (Olmos-Orizaba *et al*., 2022). Ethanol is produced during fermentation and can reach toxic concentrations affecting yeast metabolism. Ethanol is converted into acetaldehyde by alcohol dehydrogenase, increasing the levels of NADH. Acetaldehyde is then oxidized to acetate by acetaldehyde dehydrogenase, producing more NADH. This enhances the NADH/NAD$^+$ ratio, leading to an increase in ROS production in the electron transport chain (ETC) (Zakhari, 2006).

On the other hand, mitochondrial membrane permeability transition (MPT) is a highly regulated process consisting of an increase in the permeability of the mitochondrial membrane to ions and small molecules (Isakova *et al*., 2021). The MPT is involved in cell death induction by deleterious stimuli. However, it has been shown that MPT has also physiological functions, including the regulation of mitochondrial calcium homeostasis (Bernardi and Di Lisa, 2015) (Gutierrez-Aguilar *et al*., 2007), apoptosis induction, and modulation of mitochondrial metabolism in yeast. The latter occurs by uncoupling respiration, preventing the overproduction of ROS that occurs when there is energy excess and a consequent idling in respiration (Cabrera-Orefice *et al*., 2015; Bradshaw and Pfeiffer, 2006). In *S. cerevisiae*, MPT is carried out by the opening of the yeast mitochondrial unspecific channel (YMUC), which is composed of several proteins, including $F_1F_0$-ATP synthase, voltage-dependent anion channel (VDAC), mitochondrial matrix protein cyclophilin D (CYPD), and Pi carrier (Cabrera-Orefice *et al.,* 2015) (Gutiérrez-Aguilar *et al*., 2010).

Cardiolipin, a dimeric phospholipid that stabilizes the structure of several proteins in the mitochondrial inner membrane (MIM), may serve also as a non-protein component of the YMUC, since a yeast mutant in cardiolipin synthesis exhibits a decrease in YMUC opening by ATP (Koshkin & Greenberg, 2002).

Cardiolipin is highly susceptible to lipid peroxidation as it contains four polyunsaturated acyl chains (Paradies *et al.*, 2019). The higher the number of double bonds in an acyl chain, the bigger the susceptibility to lipid peroxidation (Wagner *et al.,* 1994). In this regard, *S. cerevisiae* only contains saturated and monounsaturated fatty acids (SFA and MUFA, respectively) in their membranes, making yeast cells virtually insensitive to lipid peroxidation as SFA and MUFA does not contain bis-allylic methylene hydrogen positions for initiation of lipid peroxidation (Wagner *et al*., 1994; Cortés-Rojo *et al*., 2009). As mentioned before, yeast can become hypersensitive to lipid peroxidation when incorporates PUFA into their membranes, leading to impaired mitochondrial function and necrotic cell death at high ethanol concentrations. Given the role of YMUC on the diminution of ROS overproduction at high concentrations of substrates (i.e. high ethanol), the dependence of YMUC function on cardiolipin, and the high sensitivity of cardiolipin to oxidative stress, we tested if ethanol causes YMUC dysfunction in cells grown in the presence of a PUFA due to lipid peroxidation and cardiolipin loss, leading to impaired mitochondrial function and necrosis induction.

## 2. MATERIALS AND METHODS

### 2.1 Yeast Strains and Growth Conditions

The haploid wild type strain of *S. cerevisiae* BY4741 (*Mat α*; *his3Δ1*; *leu20*; *met15Δ0*; *ura3Δ0*; WT) and the mutant in cardiolipin synthesis *crd1Δ* (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YDL142C::kanMX4*) and in VDAC *por1Δ* (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YNL055c::KanMX4*) were acquired from EUROSCARF (Frankfurt, Germany). The antibiotic G-418 (Merck, USA) was added to cultures inoculated with the mutant strains for selection of mutated cells.

### 2.2 Yeast Culture Conditions and PUFA Addition.

A culture of *S. cerevisiae* with an initial $OD_{600nm}$ of 0.03 was prepared in liquid YPD medium (1% yeast extract, 5% casein peptone and 2% glucose) and incubated for 24 h at 250 rpm and 30°C. Cells were harvested in the late exponential phase (~20 hrs). To increase the unsaturation degree of membrane fatty acids, 1 mM linolenic acid (C18:3) solubilized with 5% (v/v) of Igepal CA-630 was added to the culture medium prior to inoculation with yeast (Cortés-Rojo et al. 2009). $OD_{600nm}$ was registered at the moment of cell harvesting.

### 2.3 Lipid Peroxidation Quantification

The thiobarbituric acid (TBA) method was used to determine lipid peroxidation levels (Buege et al., 1978). Two milliliters of an acid mixture with 0.25 M HCl, 15% (w/v) trichloroacetic acid, and 0.375% (w/v) TBA were mixed with 25 mg of cells. This mixture was stirred for 5 s, incubated in a boiling bath for 15 min, cooled to room temperature, and centrifuged at 5000 rpm for 5 min. The absorbances of the supernatants were determined in a Shimadzu UV2550 spectrophotometer at 532 nm. The results were calculated using the molar extinction coefficient of malondialdehyde and expressed in terms of nanomoles of TBA-reactive species (TBARS) per milligram of cells.

### 2.5. Mitochondria Isolation

Cells were harvested and washed twice at 5000 rpm for 5 minutes with deionized water. Then cells were resuspended in a medium containing 0.6 M mannitol, 5 mM MES (10 mM 2-(N-morpholino) ethanesulfonic acid), and 0.1% bovine serum albumin (pH 6.8 with triethanolamine (TEA)). The cells were resuspended at a concentration of 0.4 g cells per mL of medium. This suspension was homogenized with 50% (v/v) glass beads (0.5 mm diameter) and vortexed for 10 cycles of 1-minute vortexing:1-minute ice at high speed. The cell homogenate was centrifuged for 5 minutes at 2590 rpm. The pellet was discarded, and the supernatant was centrifuged for 10 minutes at 12,000 rpm (Peña et al., 1977). Centrifugations were performed in a Sorvall RC6+ centrifuge at 4°C with the SS-34 rotor. The concentration of mitochondrial protein was measured using the Biuret method.

### 2.6 Evaluation of Cardiolipin Levels

Cardiolipin levels were measured in isolated mitochondria using acridine orange (Gallet et al., 1995). 10 mg/mL mitochondrial protein was treated for 15 minutes at 20 °C with 10 µM 10-N-nonyl acridine orange bromide in a buffer containing mannitol 0.6 M, tris/HCl 10 mM (pH 7.4). This mixture was centrifuged for 10 minutes at 9000 rpm. The absorbance of the supernatant corresponding to the unbound dye was measured at 495 nm. The free dye concentration was determined using a calibration curve made with known concentrations of 10-N-nonyl acridine orange (0-10 µM). The concentration of cardiolipin in mitochondria was estimated by subtracting the total amount of acridine orange added to the mitochondrial solution minus the amount of unbound dye, taking into account a binding stoichiometry of 2 mol 10-N-nonyl acridine orange/mol cardiolipin.

### 2.7 Evaluation of Necrotic Cell Death

Thermo Fisher Scientific's Annexin V-FITC and PI Dead Cell Apoptosis Kit for Flow Cytometry was used for the evaluation of cell death in accordance with the manufacturer's instructions. This kit recognizes dead cells by PI and detects the externalization of phosphatidyl serine in apoptotic cells using recombinant annexin V conjugated with FITC green fluorescent dye. Red fluorescence can be seen in PI-stained necrotic cells. Apoptotic cells exhibit green fluorescence following treatment with both probes, whereas dead cells exhibit red and green fluorescence and living cells exhibit little or no fluorescence.

### 2.8 Assessment of YMUC Opening/closure

YMUC function was evaluated by recording mitochondrial swelling in response to variations in the concentration of free calcium at fixed Pi concentrations. The experiments were carried out in a mixture with 0.6 M mannitol, 5 mM MES (pH 6.8 with TEA), 0.5 mM $Ca^{2+}$, 5 µl/ml ethanol, and 20 mM KCl. Variations in absorbance were detected at 540 nm after the addition of 1.5 mM EGTA. To promote the open state of the YMUC (i.e., instantaneous mitochondrial swelling), 0.6 mM Pi was added to the reaction; to evaluate the ability of pi to keep the YMUC in a closed state at 0.5 mM $Ca^{2+}$ (i.e., very slow mitochondrial swelling), 10 mM Pi was added to the reaction.

### 2.9 Determination of Oxygen Consumption Rate

Whole cells were used to study *in situ* mitochondrial respiration. 12.5 mg of freshly harvested cells were placed in 2.5 mL of 10 mM MES-TEA (2-(N-morpholino)ethanesulfonic acid, pH 6.0) buffer with 10 mM glucose in a sealed chamber with constant stirring. The oxygen consumption rate was assessed at room temperature with a Clark-type oxygen electrode connected to a YSI 5300 oxygen monitor and a computer for data acquisition. To evaluate the maximal rate of respiration, 15 µM of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) was added after two minutes (state U). To evaluate the *in-situ* effects of increased intracellular calcium concentrations on respiration rate, 40 µM of amiodarone, an agent increasing intracellular calcium was added (state A).

## 2.10 Evaluation ETC Complexes Activities

Mitochondria were permeabilized with Triton X-100 (Hallberg et al. 1993). The activities of the complexes were evaluated in a Shimadzu UV2250 spectrophotometer by the method described by Cortés-Rojo et al., 2009. The activity of complex II was evaluated at 600 nm as the succinate:2,6-dichlorophenolindophenol (DCIP) oxidoreductase. The activity of succinate-cytochrome *c* oxidoreductase (complex II-III) was monitored by assessing at 550 nm the antimycin A - sensitive succinate:cytochrome *c* oxidoreductase activity. Complex IV activity was measured by evaluating the oxidation of dithionite-reduced cytochrome *c* at 550 nm.

## 2.11 Statistical Analysis

The data are presented as the mean ± standard error of n>5. Statistically significant differences between groups were assessed with one-way ANOVA and Tukey *post hoc* test with Sigma Plot v11.0. Differences were set at $p < 0.05$. Groups with different letters indicate statistically significant differences between these groups.

# 3. RESULTS

## 3.1 In yeast, the combination of C18:3 and ethanol triggers cell death through necrosis.

Both cell yield and necrotic death were evaluated in yeast treated with C18:3 (+C18:3) and ethanol, as well as in the mutants *por1*Δ and *cdr1*Δ. Cell yield (Figure 1) was four times lower in yeast with +C18:3 plus ethanol in comparison to the control group. In contrast, yeast without C18:3 (-C18:3) was considerably more resistant to ethanol. Regarding the *por1*Δ and *cdr1*Δ mutants, the cell yield was almost negligible in +C18:3 plus ethanol cells compared to the WT -C18:3 plus ethanol cells. It should be stressed that the treatment with C18:3 alone resulted in a lower growth in both the WT strain and the *por1*Δ mutant, up to 1.6 times less. Unexpectedly, in contrast to WT cells, C18:3 did not impair the yield of the *cdr1*Δ mutant in the absence of ethanol.

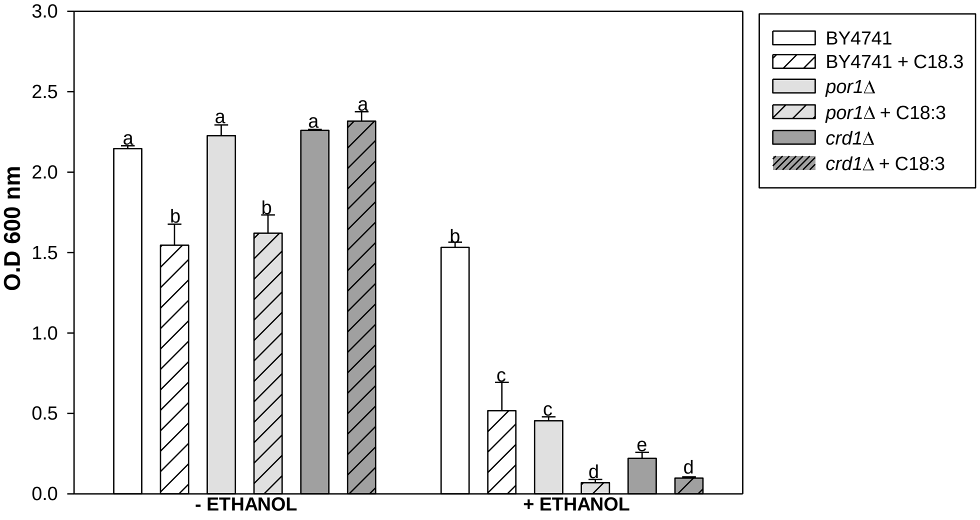

**Figure 1. Effects of ethanol and C18:3 on cell yield.** Cell density was assessed at 600 nm after 24 hours of growth. Ethanol was added at 12 h growth in the culture media. Data are presented as mean ± s.e. (n>5). Different letters indicate statistically significant differences between groups (*p*<0.05, one-way ANOVA with Tukey's post hoc test).

Concerning necrotic cell death, an increase of up to sixty-five times was observed in +C18:3 cells in the absence of ethanol, both in the WT and mutant cells. Moreover, this effect was exacerbated by ethanol, leading to a two hundred and fifty times increase in necrosis in C18:3 plus ethanol cells, irrespectively of the presence of any mutation.

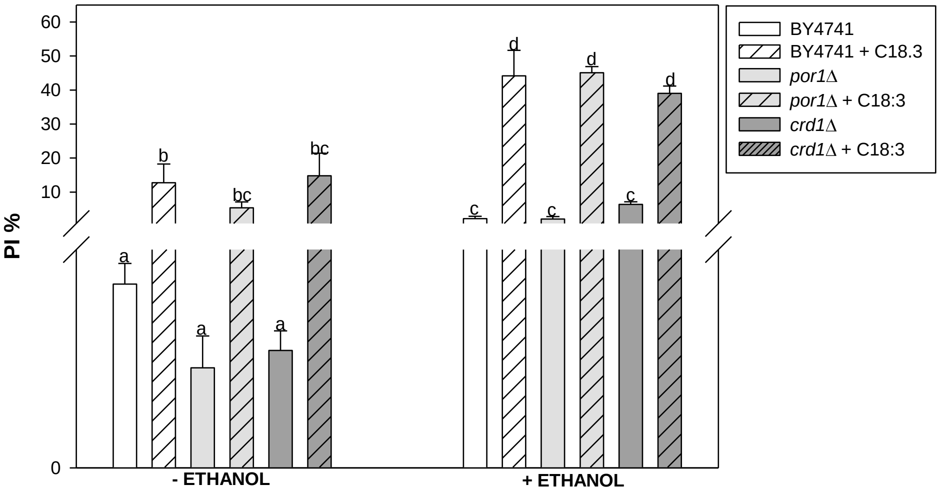

**Figure 2. Effect of C18:3 and ethanol on death by necrosis.** Quantification of the percentage of cells labeled with propidium iodide (PI). Data are presented as mean ± s.e. (n>5). Different letters indicate statistically significant differences between groups ($p$<0.05, one-way ANOVA with Tukey's post hoc test).

### 3.2 The combination of C18:3 plus ethanol increases lipid peroxidation levels and decreases cardiolipin content in yeast.

Lipid peroxidation levels were evaluated in whole cells (Figure 3). A 2.7-fold increase in lipid peroxidation was observed in +C18:3 cells without ethanol in all the strains. Regarding +C18:3 cells plus ethanol, lipid peroxidation increased to a higher degree than in cells without ethanol; nevertheless, this effect was independent of the presence of mutations. In the case of cardiolipin levels (Figure 4), a 3.6-fold decrease was observed in mitochondria of +C18:3 cells plus ethanol from all the groups in comparison to -C18:3 cells without ethanol irrespective of the presence of mutations. In the absence of ethanol, no changes in cardiolipin levels were observed in the +C18:3 cells in comparison to the -C18:3 cells, except by the *por1*Δ mutant, which had lower levels of cardiolipin in the -C18:3 cells compared to the +C18:3 cells. These results suggest that lipid peroxidation and cardiolipin loss in +C18:3 cells is dependent on the presence of ethanol.

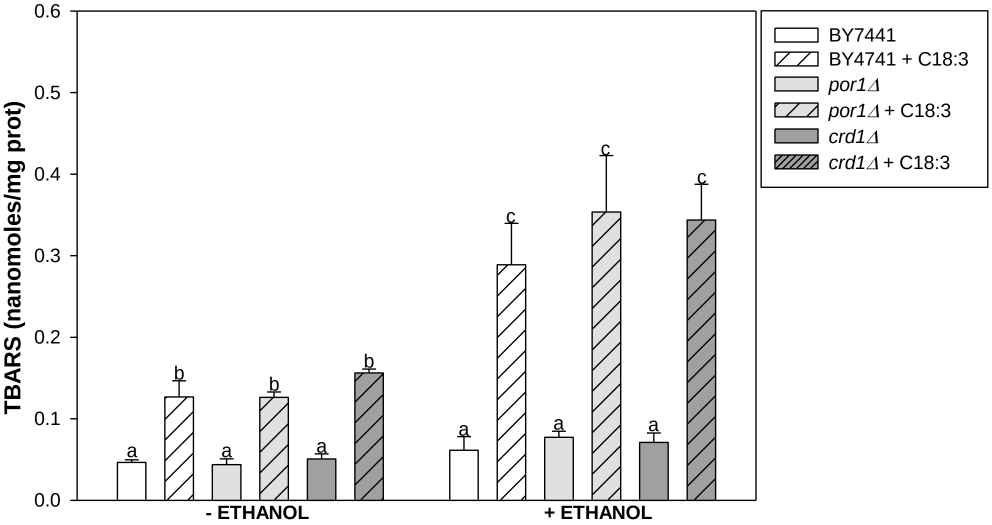

**Figure 3. Effect of C18:3 and ethanol on lipid peroxidation levels.** The results are reported in nanomoles of thiobarbituric acid reactive substances (TBARS) per milligram of cells. Data are presented as mean ± s.e. (n>4). Different letters indicate statistically significant differences between groups ($p$<0.05, one-way ANOVA with Tukey's post hoc test).

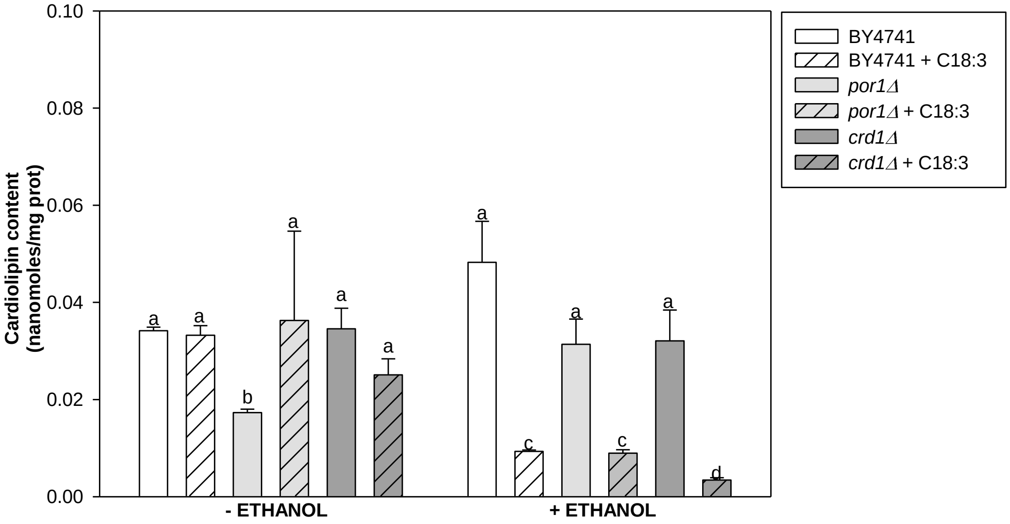

**Figure 4. Effect of C18:3 and ethanol on cardiolipin levels.** Data are presented as mean ± s.e. (n>4). Different letters indicate statistically significant differences between groups ($p$<0.05, one-way ANOVA with Tukey's post hoc test).

### 3.2 Effects of C18:3 and ethanol on mitochondrial swelling and respiration.

YMUC opening was evaluated by determining mitochondrial swelling stimulated by decreasing $Ca^{2+}$ concentrations by EGTA addition and 0.6 mM Pi, which allows YMUC aperture. The magnitude of swelling was quantified (Figure 5). It was observed than in the absence of ethanol, no modifications in YMUC open were observed in all +C18:3 strains in comparison with their counterparts -C18:3. Although there was no statistically significant differences in swelling between the mutants and the WT strain, a tendency for a lower swelling was observed in the *cdr1*Δ mutant. In the presence of ethanol, the swelling was higher in the -C18:3 WT than in the absence of ethanol. The higher induction of YMUC with ethanol was observed in the *cdr1*Δ mutant, leading to an increase of 2.1 times when compared to the condition without ethanol. In contrast, swelling in the *por1*Δ mutant was lower than observed in both the WT and *cdr1*Δ mutants, suggesting a defect in YMUC opening in response to decreased $Ca^{2+}$ concentrations in the *por1*Δ mutant.

In Figure 6 depicts the magnitude of mitochondrial swelling under conditions that keep YMUC closed by a high Pi concentration (10 mM) after Ca2+ quenching by EGTA. In the absence of ethanol, a loss of response to Pi was observed in the +C18:3 WT strain in comparison to the -C18:3 WT strain, and this effect was of a higher magnitude in the presence of ethanol. In contrast, mitochondrial swelling was negligible in the por1Δ mutant, independently of ethanol and C18.3 presence. No significant changes with respect to the -C18:3 WT strain were observed in the -C18:3 cdr1Δ mutant, except that ethanol induced insensitivity to Pi in the +C18:3 cdr1Δ mutant.

On the other hand, when evaluating pore closure through in situ respiration assessment using amiodarone as an inducer of pore closure after increasing $Ca^{2+}$ concentrations, a reduced response to closure was observed in the control strain treated with C18:3 and ethanol, up to 4.2 times, with the pore remaining in the open state. This effect was exacerbated by the mutations.

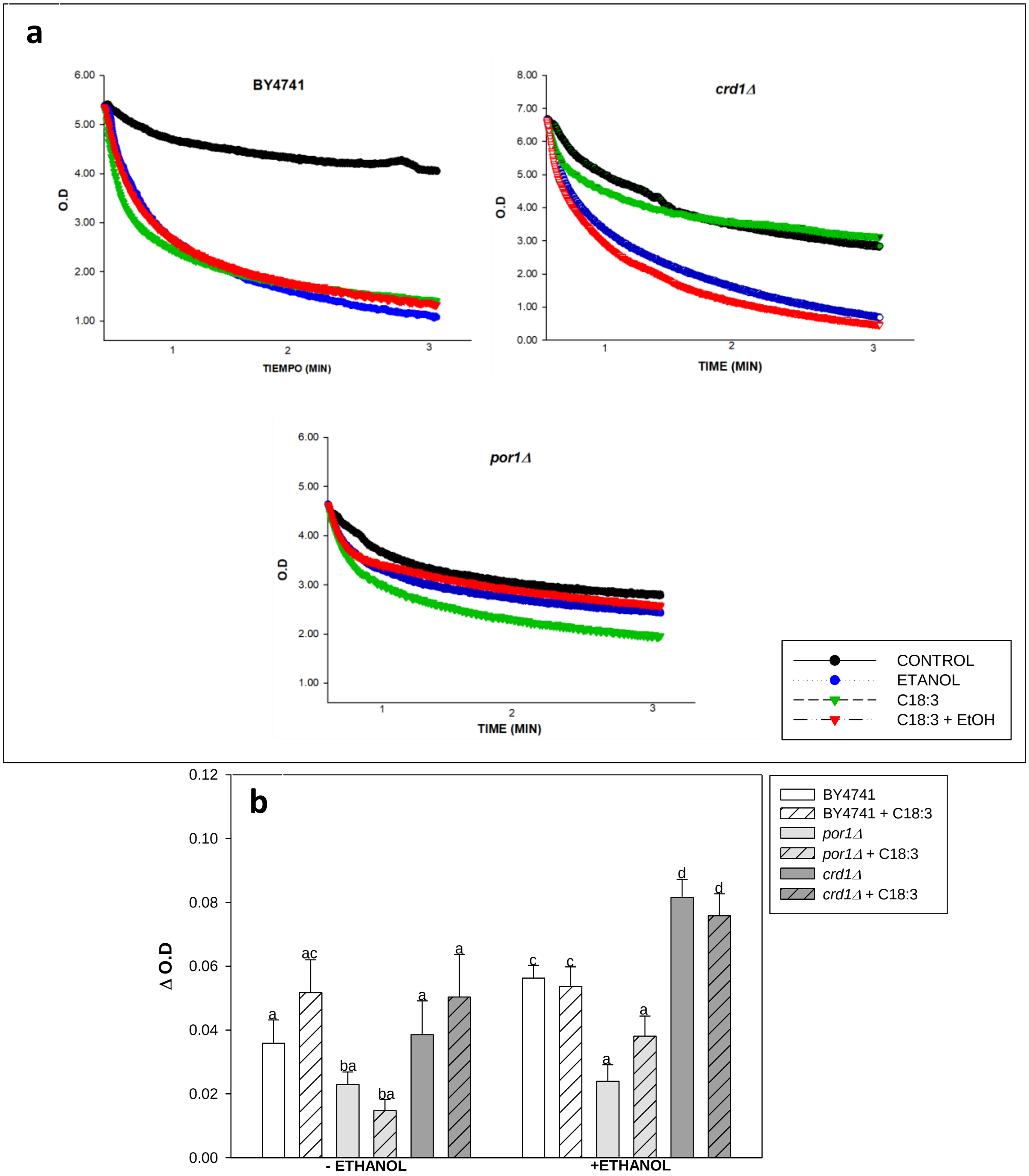

**Figure 5. Effect of C18:3 and ethanol on the induction of YMUC opening. (a)**Changes in mitochondrial swelling at 600 nm were detected and quantified after the addition of 1.5 mM EGTA in the presence of 500 µM Ca$^{2+}$, 5 µl/mL ethanol as respiratory substrate, 20 mM KCl and 0.6 mM Pi to induce YMUC opening. **(b)** Quantification of the changes in the O.D. Data are presented as mean ± s.e. (n>4). Different letters indicate statistically significant differences between groups (*p*<0.05, one-way ANOVA with Tukey's post hoc test)

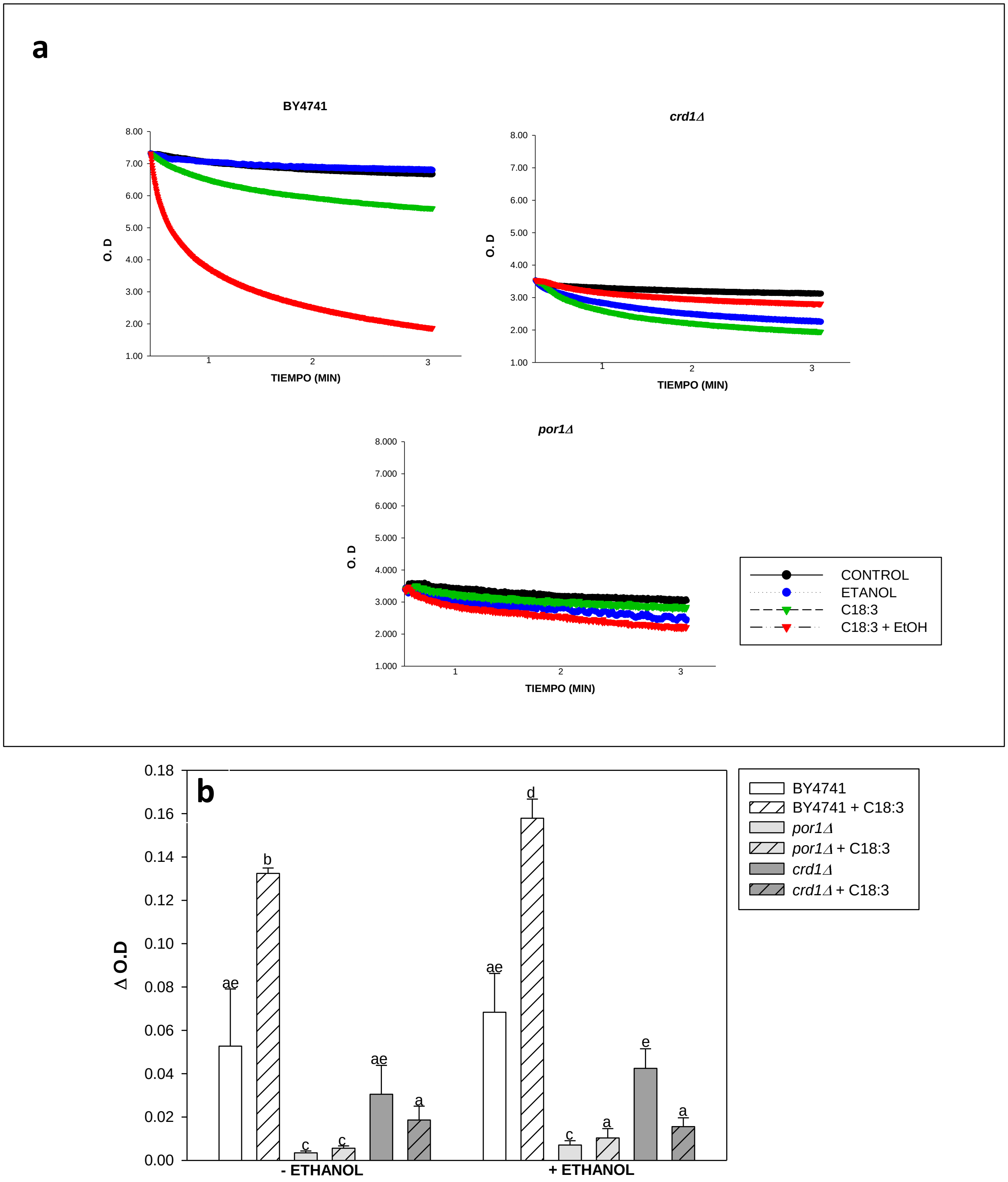

**Figure 6. Effect of C18:3 and ethanol on the induction of YMUC closure. (a)**Changes in mitochondrial swelling at 600 nm were detected and quantified after the addition of 1.5 mM EGTA in the presence of 500 µM $Ca^{2+}$, 5 µl/mL ethanol as respiratory substrate, 20 mM KCl and 10 mM Pi to induce YMUC closure. **(b)** Quantification of the changes in the O.D. Data are presented as mean ± s.e. (n>4). Different letters indicate statistically significant differences between groups (*p*<0.05, one-way ANOVA with Tukey's post hoc test).

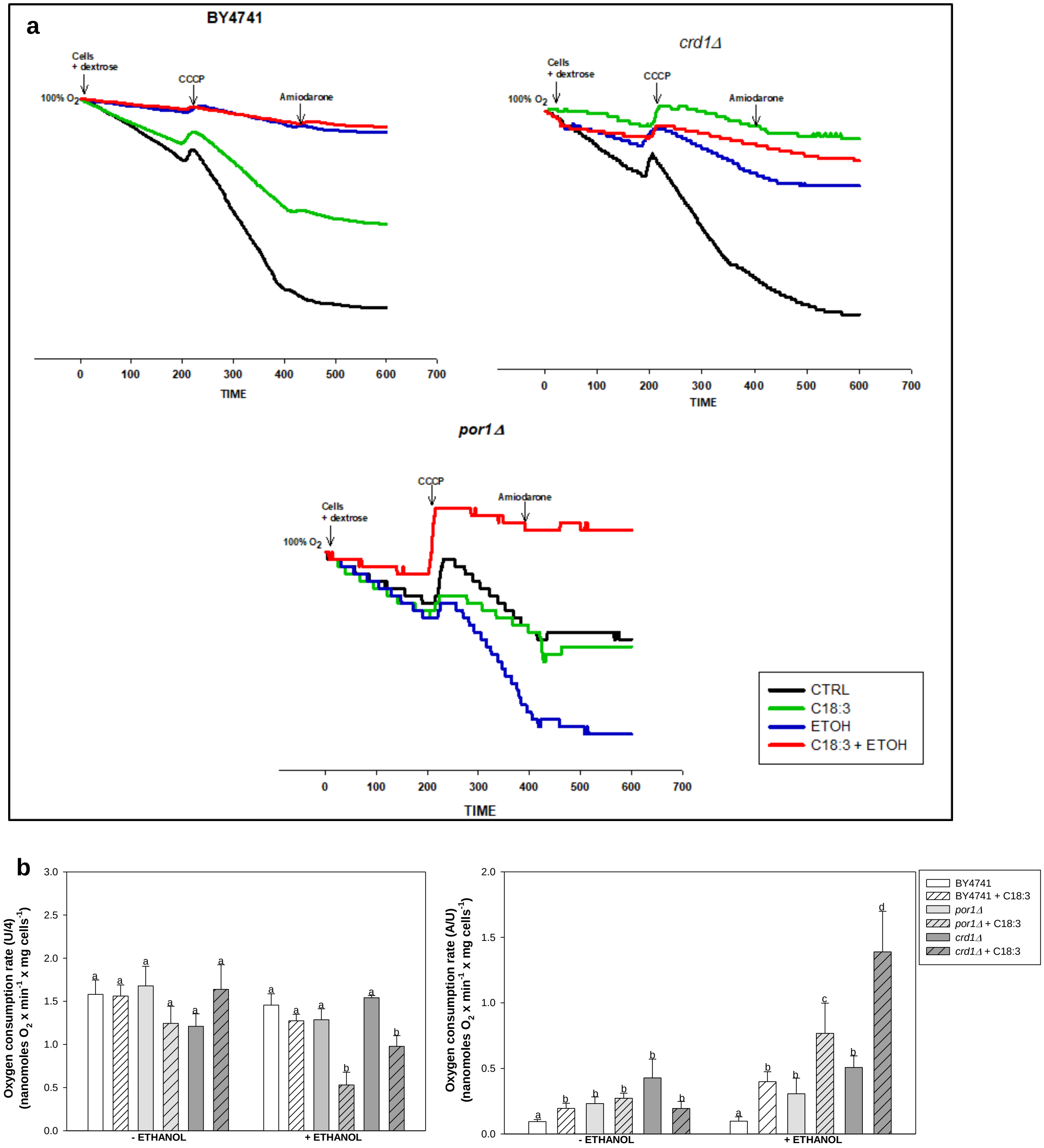

**Figure 7. Effect of C18:3 and ethanol on in situ mitochondrial respiration in yeast. (a)** Representative traces of oxygen consumption and its inhibition by amiodarona, 40 μM of amiodarone were added to induce pore closure**. (b)** Quantification of oxygen consumption**.** Data are presented as mean ± s.e. n>4. Letters indicate statistically significant differences between groups (P<0.05) (1-way ANOVA with Tukey's post hoc test).

### 3.4 Effects of C18:3 and ethanol on electron transport chain complexes.

Figure 8 shows the effects of C18:3 and ethanol on the activities of the ETC complexes. Complex II activity (Figure 8A) decreased in the absence of ethanol and C18:3 4 and 3-fold in the *por1*Δ and *crd1*Δ mutants, respectively, in comparison to the WT. In the +C18:3 WT strain, the activity decreased 50% in comparison to the -C18:3 WT strain; in contrast the presence of C18:3 did not affect complex II activity in both mutants. In the presence of ethanol, the activity in the -C18:3 WT strain decreased more than 50% in comparison to its counterpart without ethanol. In the presence of ethanol, there were no statistically significant differences between the -C18:3 WT strain and the -C18:3 mutants. However, activity decreased significantly in ~~the~~ both the +C18:3 *por1*Δ and *crd1*Δ mutants.

Regarding complex II-III activity in the absence of ethanol, (Figure 8B), the +C18:3 WT displayed a 6.7-fold decrease in comparison to the -C18:3 WT strain. The mutants exhibited a severely impaired activity regardless of the presence of C18:3. In the presence of ethanol, the activity of the -C18:3 WT strain was more than 50-fold lower than the activity of its counterpart without ethanol, while no activity was detected in the +C18:3 WT cells. Regarding the mutant strains, the activity was as low as the activity of the -C18:3 WT strain and no effects were observed in the mutant strains with C18:3. On the other hand, the complex IV activity (Figure 8C) in the WT strain in the absence of ethanol was insensitive to the presence of C18:3. In contrast, this activity was more than three - times lower in both the *por1*Δ and *crd1*Δ mutants regardless the presence of C18:3. The activity in the -C18:3 WT cells was insensitive to ethanol and the activity decreased 50% only in +C18.3 cells plus ethanol. The mutant strains displayed lower values of complex IV activity, except for the -C18:3 *crd1*Δ mutant, which have a higher activity than all the mutants grown with ethanol, although the activity was lower than in the WT strain.

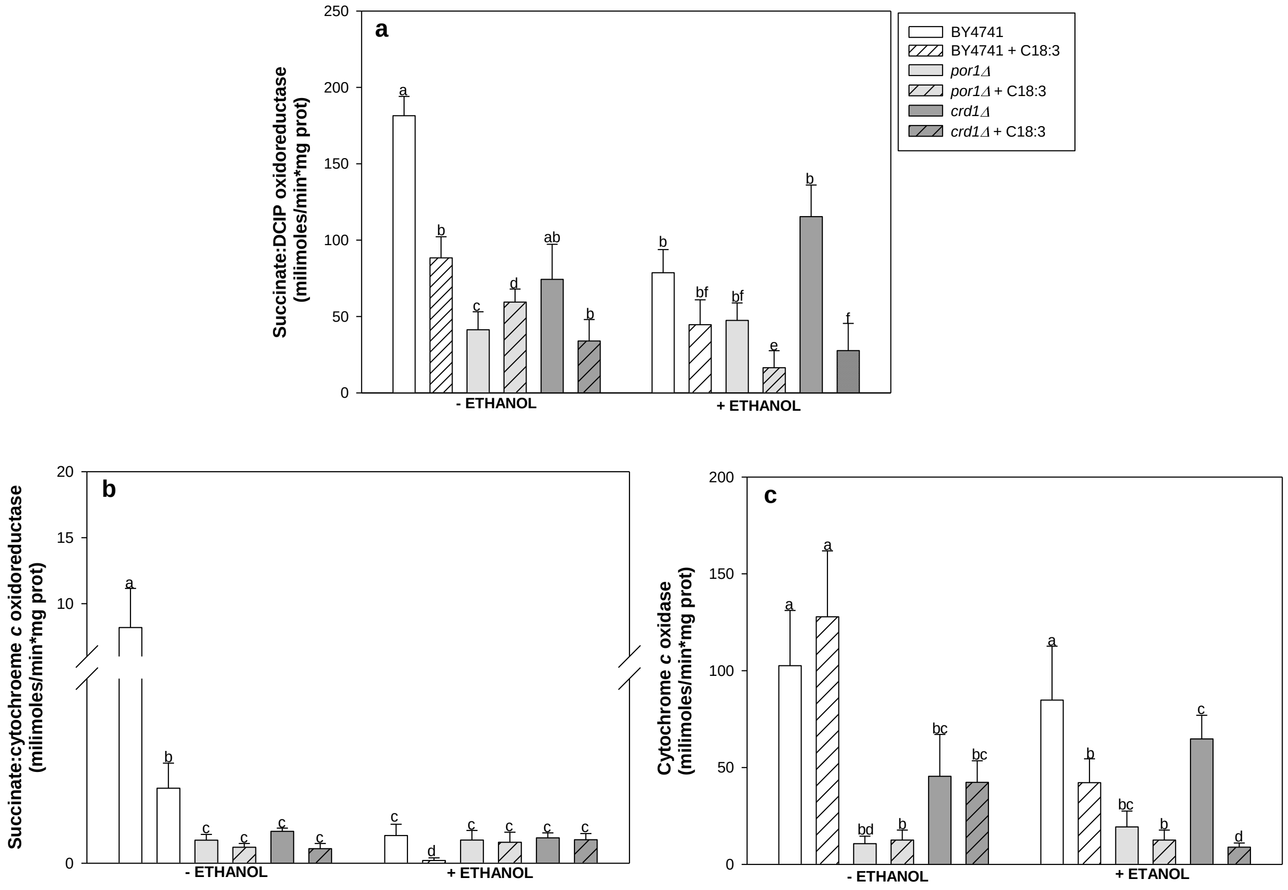

**Figure 8. Effect of C18:3 and ethanol on the activity of electron transport chain complexes.** Data are presented as mean ± s.e. (n>4). Different letters indicate statistically significant differences between groups (*p*<0.05, one-way ANOVA with Tukey's post hoc test).

## 5. DISCUSSION

The yeast *S. cerevisiae* has historically been used in the production of alcoholic beverages and bakery products, and currently has multiple biotechnological uses, such as the production of PUFA and bioethanol. However, due to the effect that ethanol accumulation has on cell viability, its efficiency as a biotechnological tool has been significantly hindered. It is possible that the development of tools to improve microbial tolerance to ethanol and PUFA can be facilitated by an improvement in our understanding of the cellular effect of ethanol toxicity and how the cell reacts to ethanol stress.

The findings in this study show that the combination of ethanol and PUFA decreases the yeast growth (Figure 1) and increases lipid peroxidation (Figure 3). High ethanol concentrations increase ROS production and decrease intracellular antioxidant levels (Pérez-Gallardo *et al*., 2013), which may be the mechanism by which ethanol leads to an increase in lipid peroxidation in yeast containing PUFA. Ethanol plus PUFA causes elevated ROS production (Olmos-Orizaba *et al*., 2022). Cardiolipin is particularly susceptible to ROS-induced oxidation, due to its fatty acid composition and proximity to ROS-generating centers (Paradies *et al*., 2014). The results show that yeast exposed to ethanol plus PUFA had a lower content of cardiolipin (Figure 4), an effect that was exacerbated by the mutation in *por1*Δ and *crd1*Δ. A decrease in cardiolipin content leads to mitochondria dysfunction. Cardiolipin maintains both the stability and activity of ETC complexes, especially complexes III and IV in yeast (Joshi *et al*., 2009). The presence of specific binding sites for cardiolipin suggests that this molecule is essential for the catalytic activity of complexes III and IV, as it acts as an allosteric ligand that stabilizes the proper conformation and activation of these enzymatic complexes (Paradies *et al*., 2019). Our results show that the activity of the ETC complexes was affected by the treatment with ethanol plus PUFA (Figure 8), this effect was exacerbated in the *por1*Δ and *crd1*Δ mutants.

ETC dysfunction increases ROS production and causes oxidative stress in yeast (Zuin *et al*., 2008), inhibiting its growth and causing cell death. In addition, alterations in the ETC lead to alterations in energy metabolism, and thus hinder other metabolic functions. Moreover, it was observed that the increase in lipid peroxidation and the decrease in cardiolipin in yeast with ethanol plus PUFA was related to YMUC impairment (Figures 5 and 6). Evaluating the opening of this pore (Figure 5), it was detected that ethanol plus PUFA leads to greater opening after the addition of the $Ca^{2+}$ chelator EGTA, an effect that was exacerbated by the mutation in *crd1*Δ, while the mutation in *por1*Δ prevented this effect. Since cardiolipin is essential for pore stabilization, structural modifications of cardiolipin due to peroxidation can lead to alterations in protein stabilization (Vähäheikkilä *et al*., 2018), which could explain the prolonged opening effect of the pore caused by the mutation in *crd1*Δ, while the absence of *por1*Δ confirms what has been reported in the literature, where it is proposed that in the absence of VDAC, YMUC becomes desensitized to $Ca^{2+}$ because VDAC contains a $Ca^{2+}$ binding site that promotes YMUC closure. Therefore, even though YMUC still exists in the absence of VDAC, the $Ca^{2+}$-mediated opening control is lost (Gutierrez-Aguilar *et al*., 2007). On the other hand, our results show that there is a partial loss of sensitivity to Pi due to the presence of PUFA (Figure 6), an effect that was further increased by the combination of ethanol and PUFA, while this effect was prevented by the mutations. This is probably due to the fact that the phosphate carrier (Pic) is a protein located in the mitochondrial inner membrane that is known to interact with cardiolipin (Klingenberg 2009). Therefore, this effect would be related to the lower content of cardiolipin observed and the increase in lipid peroxidation in the control strain.

It has been shown that ANT, one of the proteins that is part of the YMUC, is intimately linked to cardiolipin. It has been reported that the presence of cardiolipin is essential for the proper function of ANT and that its absence impairs its function. The crystal arrangement of ANT shows that ANT monomer associates closely with two or three molecules of cardiolipin. It is believed that these strongly bound cardiolipin molecules play an important role in the association of monomers to form the dimer and in stabilizing its dimeric structure, which is the active form of the protein (Nury *et al*., 2006).

Another strategy was used to determine the impact of ethanol and PUFA on YMUC function, which consisted of analyzing *in situ* respiration and response to amiodarone (Figure 7). It is known that this drug increases intracellular $Ca^{2+}$ concentration, which causes pore closure **(**Muend & Rao, 2008) thereby amiodarone was used as an agent inducing YMUC closure. The results obtained revealed that yeasts containing ethanol and PUFA presented a reduced response to amiodarone, and this effect was further intensified in *crd1*Δ and *por1*Δ mutations. These findings confirm what was previously observed in mitochondrial swelling, where it was seen that ethanol and PUFA contribute to YMUC dysfunction, which could be corroborated in whole cells. Among the consequences of YMUC dysfunction, it has been reported that prolonged swelling causes depolarization of the mitochondria, leading to necrotic cell death (Webster *et al*., 2012), which agrees with our results, where we observed greater cell death by necrosis due to the presence of ethanol and PUFA (Figure 2).

## 5. CONCLUSION

The presence of ethanol plus PUFA drive to YMUC malfunction due to lipid peroxidation and cardiolipin loss, causing yeast death by necrosis. Therefore, this confirm the idea that protection of cardiolipin against peroxidative damage may give yeast with higher resistance to the simultaneous presence of PUFA and ethanol**.**

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# 7. DISCUSION GENERAL

Los resultados de este trabajo revelan que la combinación de ácidos grasos poliinsaturados (AGPI) y etanol promovió la muerte celular por necrosis de la levadura (capítulo I, Figura 2), y un aumento en la peroxidación de lípidos (capítulo I, figura 3). La peroxidación de lípidos puede causar daño a nivel mitocondrial debido a la presencia de lípidos insaturados en sus membranas (Schenkel & Bakovic, 2014), lo que las hace susceptibles a la peroxidación. Esto compromete la integridad de la membrana mitocondrial interna (MMI) y el funcionamiento adecuado de las proteínas. El incremento en la peroxidación de lípidos puede afectar significativamente a la cardiolipina y su función en las mitocondrias. Además de su función en la preservación del potencial de membrana y la estructura de la membrana interna de las mitocondrias, la cardiolipina tiene una función estructural y funcional esencial para proteínas involucradas en la respiración mitocondrial (Paradies *et al*., 2014)

La cardiolipina es susceptible a la peroxidación debido a la presencia de ácidos grasos poliinsaturados en su estructura. En la peroxidación de lípidos, las ERO atacan los ácidos grasos poliinsaturados de la cardiolipina, generando productos de peroxidación (Yin & Zhu 2012). Los resultados mostraron un menor contenido de cardiolipina debido a la presencia de AGPI y etanol (capítulo I, figura 4).

La cardiolipina participa en la formación y estabilidad de los complejos proteicos involucrados en la CTE (Paradies *et al*., 2019). El menor contenido de cardiolipina, debido posiblemente a la peroxidación de lípidos, tuvo varias consecuencias negativas en la función mitocondrial, como la inhibición de la actividad de los complejos de la CTE (capítulo II, figura 8). La menor actividad de los complejos observada en el tratamiento con C18:3 y etanol se puede explicar debido a que la peroxidación de lípidos puede alterar la estructura y función de la cardiolipina, lo que afecta la interacción de estas proteínas y disminuye la eficiencia de la CTE. Esto podría relacionarse con la mayor producción de ERO debido a la presencia de C18:3 y etanol (capítulo I, Figura 6B).

Las ERO generadas pueden modificar las proteínas que componen el canal CIML (Kuznetsov *et al*., 2019). Además, la cardiolipina participa en la estabilización de las proteínas que componen lo componen (Koshkin & Greenberg, 2002), por lo tanto, las modificaciones en la cardiolipina debido a la peroxidación junto con el incremento de ERO afectarían la estructura y función CIML, alterando su apertura y cierre regulados normalmente. Nuestros resultados muestran que la presencia de C18:3 y etanol inducen la apertura prolongada del CIML (capítulo II, figuras 6 y 7). La apertura prolongada del CIML puede alterar el equilibrio de protones a través de la MMI, lo que afecta negativamente la generación de ATP. Esto resulta en una disfunción bioenergética y una disminución de la producción de energía. Además, de la liberación de moléculas proapoptóticas, como el citocromo *c*, desde la matriz mitocondrial hacia el citosol. Estas moléculas desencadenan una cascada de eventos que inducen daño oxidativo y estrés celular, comprometiendo la viabilidad celular. Además, la apertura prolongada del CIML afecta el equilibrio iónico dentro de las mitocondrias, lo que provoca una entrada excesiva de iones, como el calcio, y la pérdida de otros metabolitos esenciales. Esto interfiere con las funciones normales de las mitocondrias y puede llevar a la disfunción celular.

Por lo tanto, nuestro resultados sugieren que la peroxidación de lípidos, ocasionada por la presencia de C18:3 y etanol, promovió la pérdida de cardiolipina, lo que causó una alteración en los complejos de la cadena de transporte de electrones (CTE) y promovió una mayor producción de ERO en la mitocondria, contribuyendo al daño oxidativo de los lípidos. Este estrés oxidativo generado por la peroxidación de lípidos también estuvo relacionado con la disfuncionalidad del CIML, que es un componente clave en la regulación de la producción de ERO. Por último, la peroxidación de lípidos alteró la estructura y función de las membranas celulares. La acumulación de productos de peroxidación de lípidos, junto con el hinchamiento prolongado por la apertura prolongada del CIML, promovió la permeabilidad de la membrana, lo que afectaría el equilibrio iónico y provocaría la disfunción de las vías de señalización celular, alteraciones que contribuyen al desarrollo de la necrosis.

## 8. CONCLUSION GENERAL

La muerte de la levadura se produce debido a la disfunción del CIML causada por la pérdida de cardiolipina como resultado de la peroxidación de lípidos debido a la presencia de C18:3 y etanol.

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