

Effects of Novel Aging-Delaying Plant Extracts on the Lipid Composition of the Yeast
Saccharomyces Cerevisiae

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ABSTRACT

Effects of Novel Aging-Delaying Plant Extracts on the Lipid Composition of the Yeast

Saccharomyces Cerevisiae

Mélissa McAuley, M.Sc.

A collaboration between the Titorenko laboratory and Idunn Technologies Inc. has led to a recent discovery of 14 plant extracts that delay the chronological mode of aging in the budding yeast *Saccharomyces cerevisiae*. Age-related changes in the abundance of lipid droplets, a site for the deposition and lipolytic degradation of the "neutral" lipids triacylglycerols and steryl esters, are known to play essential roles in defining yeast longevity. Therefore, one objective of my thesis was to investigate how each of the 14 aging-delaying plant extracts influences the number and size of lipid droplets at different stages of yeast chronological aging. I found that 8 of 14 plant extracts delay yeast chronological aging not because they alter the number of droplets or change their size. Because the number and size of lipid droplets are known to be defined by the relative rates of neutral lipid synthesis in the endoplasmic reticulum, neutral lipid deposition in lipid droplets, neutral lipid lipolytic degradation in lipid droplets, fusion of lipid droplets and fragmentation of lipid droplets, I concluded that the aging-delaying effects of these 8 plant extracts in yeast are unlikely to be caused by changes in the relative rates of the above cellular processes. I also found that 6 of 14 aging-delaying plant extracts cause changes in the number or size of lipid droplets only on one or two (out of four) days of cell collection, each corresponding to different stages of the aging process. These findings suggest that such changes in lipid droplets abundance may (or may not) play essential roles in the ability of some (or all) of these 6 plant extracts to delay yeast chronological aging. To address this, further experiments are required. Because the synthesis,

storage and degradation of neutral lipids are integrated into metabolic pathways for other lipid classes, I then used a combination of liquid chromatography and mass spectrometry to examine how 2 of the aging-delaying plant extracts that increase the size of lipid droplets without affecting their number affect the intracellular concentrations of several classes of lipids in chronologically aging *S. cerevisiae*. I found that these 2 aging-delaying plant extracts exhibit different and statistically significant effects on the concentrations of phosphatidylinositol phospholipids, triacylglycerols and free fatty acids. I therefore concluded that there may be 2 different ways of remodeling lipid metabolism in response to 2 different aging-delaying plant extracts. Future studies will test if such specific remodeling of lipid metabolism plays a causal role in the ability of each of these plant extracts to delay chronological aging of *S. cerevisiae*.

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List of Abbreviations

CL, cardiolipin; CR, caloric restriction; D, diauxic growth phase; DAGs, diacylglycerols; DR, dietary restriction; ER, endoplasmic reticulum; L, logarithmic growth phase; LCA, lithocholic acid; LDs, lipid droplets; non-CR, non-caloric restriction; PD, post-diauxic growth phase; ST, stationary growth phase; TAGs, triacylglycerols; WT, wild-type strain.

Introduction

1.1 Aging and Age-Related Diseases

Aging, known as the progressive decline in fitness of an organism over time, is a process affecting us all. It is estimated that by 2026, one in five Canadians will be a senior citizen (Canadian Institutes of Health Research 2010). It has also been reported that the greatest factor for most life-threatening disorders today is age itself (World Health Organization 2017). This unnerving fact has driven more scientists to this field of research over recent years, especially because the molecular mechanisms through which aging occurs appeared to be linked to such devastating age-related diseases as arthritis, diabetes, sarcopenia, stroke, cancer, cardiovascular diseases and neurodegenerative diseases (including Parkinson's, Alzheimer's and Huntington's diseases) (De Cabo et al. 2014). The following nine age-related pathologies are currently known as hallmarks of aging in humans and other evolutionarily distant eukaryotic organisms: 1) genomic instability, 2) telomere attrition, 3) epigenetic alterations, 4) loss of proteostasis, 5) deregulated nutrient sensing, 6) mitochondrial dysfunction, 7) cellular senescence, 8) stem cell exhaustion, and 9) altered intercellular communication (**Figure 1.1**) (Lopez-Otin et al. 2013). Because cellular processes underlying organismal aging have been conserved in the evolution, our knowledge of these hallmarks can facilitate the development of certain dietary and chemical interventions capable of delaying the age-related onset of some (or all) of these essential traits of aging (Lopez-Otin et al. 2013; De Cabo et al. 2014).

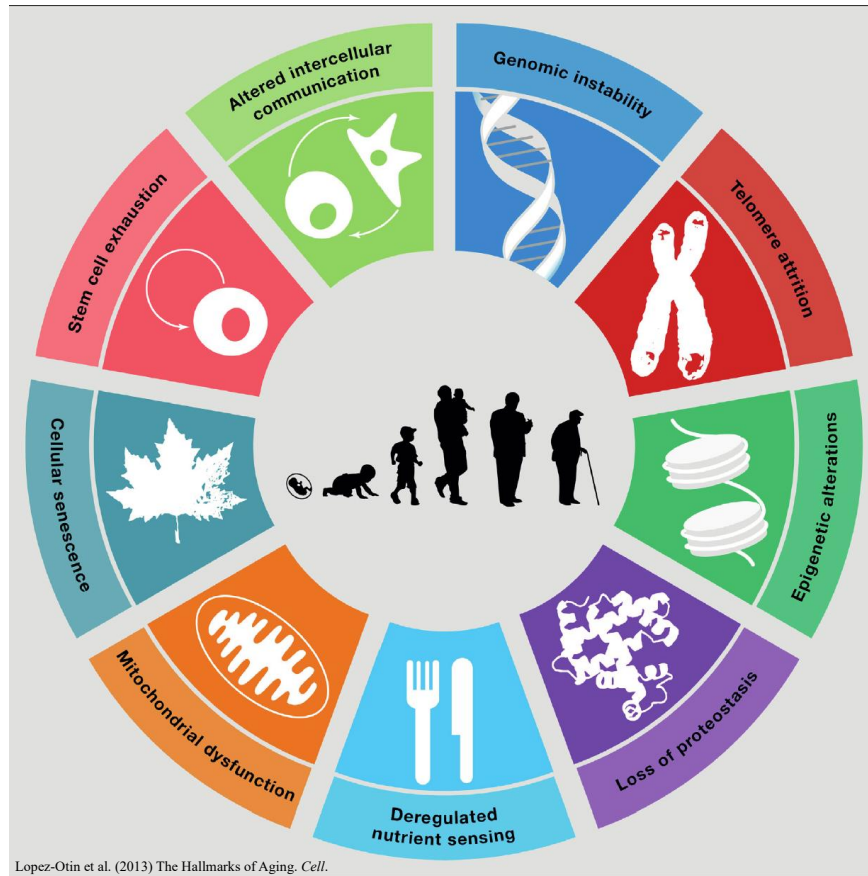


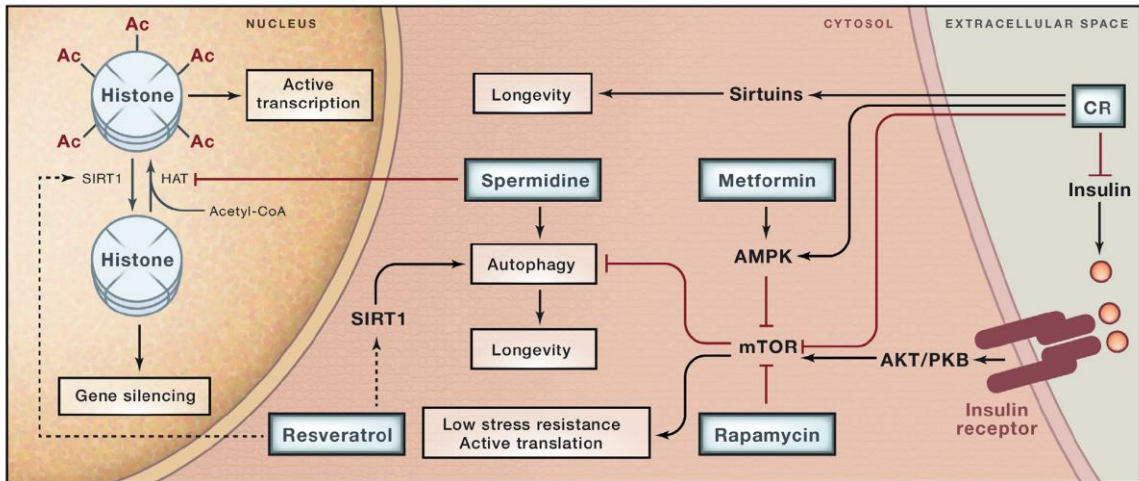
Figure 1.1. The 9 hallmarks of aging: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intracellular communication.

1.2 Some Dietary and Natural Chemical Interventions Can Delay Aging Because They Target Certain Nutrient-Sensing Signaling Pathways

Our fascination with the possibility of living longer has inspired scientists to investigate if the onset and/or progression of the process of biological aging can be delayed by certain dietary interventions or natural chemical compounds produced by microbes, plants or animals. The most effective aging-delaying dietary and chemical interventions that are presently known target certain nutrient-sensing signaling pathways, mitochondrial functionality, autophagic degradation and cellular proteostasis (**Figure 1.2**) (De Cabo et al.

2014). Several dietary and caloric restriction regimens are known as the most potent ways of longevity extension in organisms ranging from the budding yeast *S. cerevisiae* to the long-lived populations of Okinawa, Japan (De Cabo et al. 2014; Brandhorst et al. 2015). All these dietary and caloric restriction regimens change the organismal and intracellular nutrient and energy status, thus regulating a network of nutrient-sensing pathways and protein kinases that converges on the mechanistic target of rapamycin (mTOR) protein complex (Blagosklonny 2009). Likewise, the aging-delaying chemical compound rapamycin has been shown to extend longevity in yeast and other lower eukaryotes by inhibiting the mTOR function, but also to cause some unwanted side-effects (such as diabetes) in mice fed with rapamycin for a prolonged period (Lamming et al. 2012). Functioning through two separate complexes, mTORC1 and mTORC2, mTOR is known to respond to nutrient availability and changes in growth hormone concentrations (Johnson et al. 2013). Because mTOR is a master regulator of cellular growth and metabolism, its inhibition in response to dietary/caloric restriction or rapamycin extends longevity by increasing stress resistance, promoting mitochondrial respiration, reducing insulin/IGF-1-like signaling and activating autophagy (**Figure 1.2**) (Johnson et al. 2013). In addition, the longevity-extending effects of such aging-delaying chemical compounds as metformin and spermidine are due to their abilities to alter the intracellular energy status and activate autophagy (**Figure 1.2**) (De Cabo et al. 2014). Furthermore, it is believed that the aging-delaying compound resveratrol interacts with certain SIRT1 and other deacetylases in the nucleus to activate transcription of genes required for autophagy activation (**Figure 1.2**) (De Cabo et al. 2014). Moreover, the aging-delaying compound lithocholic bile acid has been shown to extend longevity of chronologically aging yeast by changing the lipid

composition of mitochondrial membranes and improving mitochondrial functionality (Medkour et al. 2017).



De Cabo, et al. (2014) The Search for Antiaging Interventions: From Elixirs to Fasting Regimens. *Cell*.

Figure 2. Molecular Targets for Caloric Restriction and Pharmacological Interventions against Premature Aging

Caloric restriction promotes longevity through sirtuin activation as well as through inhibition of the insulin and TOR pathways, which both lead to stress resistance and autophagy activation. Resveratrol, rapamycin, and spermidine exert autophagy-dependent antiaging mechanisms that are exerted both at the cytosolic and nuclear levels. Whether resveratrol directly interacts with the sirtuin family member SIRT1 remains controversial. Metformin promotes AMPK activity and prevents oxidative damage. Ac, acetyl residue; HAT, histone acetyltransferase; AMPK, adenosine monophosphate-activated protein kinase; AKT/PKB, protein kinase B; CR, caloric restriction.

dependent anti-aging mechanisms both at the nuclear and cytosolic levels. Whether resveratrol directly interacts with the sirtuin family member SIRT1 remains controversial. Metformin promotes AMPK activity and prevents oxidative damage. Ac, acetyl residue; HAT, histone acetyltransferase; AMPK, adenosine monophosphate-activated protein kinase; AKT/PKB, protein kinase B.

1.3 The Lipid Composition of a Yeast Cell Affects Its Growth, Functionality, Survival and Death

Lipid synthesis and degradation have previously been linked to certain fat-storage diseases, such as Gaucher disease and obesity; lipid metabolism is considered one of the major factors that influence organismal lifespan and health in evolutionarily distant eukaryotes (National Institute of Neurological Disorders and Stroke 2018) (National Institutes of Health 2014). The ability of lipids to define organismal lifespan and healthspan

is due to the essential roles of lipids in fat synthesis, energy storage and supply, cell signaling, membrane integrity and biogenesis, and cell death (Klug & Daum 2014; Schroeder & Brunet 2015).

The budding yeast *S. cerevisiae* is a useful model organism for studying the metabolism and function of different classes of lipids. Based on the structure and function of lipids, these hydrophobic, water-insoluble molecules can be classified into several major groups as follows: free fatty acids (FFAs), phospholipids, sterols, sphingolipids and nonpolar (i.e. uncharged and therefore also known as neutral) lipids (Klug & Daum 2014).

FFAs are composed of a carboxylic acid with a hydrocarbon tail having varying degrees of (un)saturation (Klug & Daum 2014). *S. cerevisiae* can acquire FFAs through the following three processes: 1) via *de novo* synthesis; 2) via lipolytic degradation of complex lipids, such as phospholipids, sterols, sphingolipids and neutral lipids; and 3) via transport of exogenous FFAs into the cell across the plasma membrane (PM) (Tehlivets et al. 2007). The composition of FFAs in a yeast cell can be changed in response to changes in the nutrient and environmental status of this cell, but under most conditions the major classes of FFAs are palmitoleic acid (C16:1), oleic acid (C18:1), palmitic acid (C16:0) and stearic acid (C18:0), whereas the minor classes of FFAs are myristic acid (C14:0) and cerotic acid (C26:0) (Tehlivets et al. 2007).

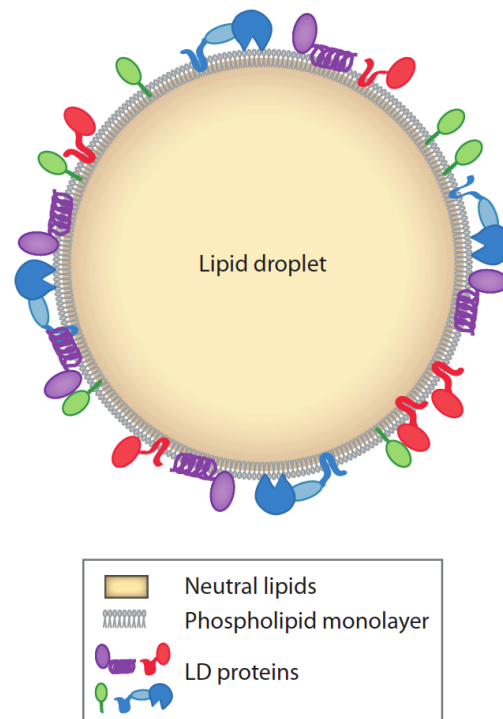
Furthermore, phospholipids, sphingolipids and sterols are important classes of complex lipids that have been found in organellar and cellular membranes; these lipid classes play essential roles in preserving membrane integrity, controlling membrane-based signaling, modulating membrane fusion and regulating cell death (Klug & Daum 2014). Phospholipids are amphipathic lipid molecules having a hydrophobic diacylglycerol (DG)

moiety covalently attached to certain hydrophilic head group (which is different for different classes of phospholipids). The major classes of phospholipids in *S. cerevisiae* are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) (Klug & Daum 2014). Sphingolipids have a ceramide backbone consisting of a sphingoid long-chain base (LCB) which is covalently linked to a FFA. This hydrophobic tail is attached to an inositol-containing molecule which serves as a hydrophilic (polar) head group (Klug & Daum 2014). Phytosphingosine (PHS) and dihydrosphingosine (DHS) are the only types of LCB found in yeast sphingolipids; in most *S. cerevisiae* sphingolipids, they are covalently linked to an FFA called cerotic acid (C26:0) (Klug & Daum 2014). The sterol class of complex lipids in *S. cerevisiae* is represented by ergosterol, which resides mainly in the PM and has been implicated in maintaining the integrity of this cellular membrane (Klug & Daum 2014).

FFAs and ergosterol are known to be toxic if present in concentrations exceeding a threshold (Klug & Daum 2014). A potential lipotoxic effect of FFAs and ergosterol can be weakened by their conversion into triacylglycerols (TAGs) and ergosteryl esters (EEs) (respectively) in the endoplasmic reticulum (ER), and by their subsequent deposition in lipid droplets (Klug & Daum 2014). The lipolytic degradation of TAGs and EEs that have been deposited in lipid droplets provides bulk quantities of FFAs, phospholipids and ergosterol needed for the fast expansion of the cellular surface during rapid growth of a eukaryotic cell (Klug & Daum 2014).

1.4 Lipid Droplets (LDs) Are Dynamic Organelles Implicated in Many Cellular Processes in Yeast and Multicellular Eukaryotes

LDs are cytoplasmic organelles in which a hydrophobic core of neutral lipids (i.e. TAGs and steryl esters or EEs) is surrounded by a phospholipid monolayer in association with several membrane proteins (**Figure 1.3**) (Walther & Farese Jr. 2012). LDs have been found in all presently known eukaryotic organisms; it is believed therefore that the ability to form LDs is one of the earliest evolutionarily traits of eukaryotic cells (Walther & Farese Jr. 2012). The mechanism by which LDs arise in the cytoplasm of a eukaryotic cell is not completely understood, but it is believed that they are formed from the ER through budding (Barbosa et al. 2015).



Walther et al. (2012) Lipid Droplets and Cellular Lipid Metabolism. *Annual Review of Biochemistry*.

Figure 1.3. Schematic overview of the structure of a lipid droplet.

LDs are very abundant in *S. cerevisiae* during stationary phase of culturing (Walther & Farese Jr. 2012). During exponential growth of a yeast culture, the TAG and EE components of LDs undergo intensive lipolysis to provide yeast with FFAs, phospholipids

and ergosterol needed for the rapid expansion of the PM (Walther & Farese Jr. 2012). The major phospholipids present in the LD monolayer are phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) (Walther & Farese Jr. 2012) (Penno et al. 2013).

Although LDs have long been considered only as comparatively static organelles that are used for protecting a eukaryotic cell from the lipotoxic effect of excessive concentrations of FFAs and sterol esters (EEs in yeast), recent studies have implicated LDs in many other vital cellular processes. These cellular processes include the following: 1) the G₁/S phase transition during cell cycle of *S. cerevisiae*, when the accelerated lipolysis of TAGs in LDs provides FFAs and phospholipids for bud formation (Kurat et al. 2009); 2) the establishment of contact sites between LDs and peroxisomes for intensifying peroxisomal β -oxidation of FFAs formed in LDs as products of TAG and EE lipolysis in *S. cerevisiae* (Kohlwein et al. 2013; Barbosa et al. 2015); 3) longevity regulation in the nematode *Caenorhabditis elegans*, by providing neutral lipids as the source of abundant quantities of FFAs needed for maintaining the viability of this organism (Wang et al. 2008); 4) temporal housing of histones prior to their recruitment for nuclear division during embryo segmentation in *Drosophila* (Cermelli et al. 2006); and 5) the regulation of the synthesis and secretion of pro- and anti-aging adipokines or anti-aging lipophilic hormones by white adipose tissue of mice or by cells in the intestine cells of the nematode *C. elegans* (respectively) (Goldberg et al. 2009a).

1.5 Two Different Mechanisms to Delay Yeast Chronological Aging by Differently Altering the Abundance of LDs

One of the recently discovered functions of LDs is their essential role in defining longevity of chronologically aging *S. cerevisiae*. After being synthesized in the ER from FFAs and diacylglycerols (DAGs), the neutral lipids TAGs are delivered to and then stored in LDs (Mitrofanova et al. 2018). The lipolytic degradation of TAGs deposited in LDs yields FFAs and DAGs; these two lipid products of TAG lipolysis can be then utilized for energy production via peroxisomal β -oxidation of FFAs, the formation of phospholipids and sphingolipids for expansion of the PM and organellar membranes, and the synthesis of several signaling lipids involved in some signaling pathways (Mitrofanova et al. 2018). A growing body of evidence indicates that the rates and efficiencies with which TAGs are synthesized from FFAs and DAGs in the ER, delivered to and stored in LDs, and lipolytically degraded to FFAs and DAGs in LDs define yeast chronological lifespan (Goldberg et al. 2009; Mitrofanova et al. 2018; Arlia-Ciommo et al. 2018; Handee et al. 2016; Li et al. 2017). As described below, two different mechanisms for delaying yeast chronological aging by altering these rates and efficiencies (and, thus, by changing the abundance of LDs in yeast cells) have been proposed.

One of these mechanisms operates in yeast grown under caloric restriction (CR) conditions, i.e. in a nutrient-rich medium initially containing 0.2% glucose. Under these conditions, yeast cells do not accumulate ethanol (Goldberg et al. 2009; Arlia-Ciommo et al. 2018). In contrast, yeast cells grown under non-CR conditions (i.e. in a nutrient-rich medium initially containing 2% glucose) accumulate substantial concentrations of ethanol, a product of glucose fermentation (Goldberg et al. 2009). The build-up of ethanol in yeast under non-CR conditions represses the synthesis of the Fox1, Fox2 and Fox3 proteins; each of these proteins is essential for the β -oxidation of FFAs in peroxisomes (**Figure 1.4**)

(Goldberg et al. 2009; Arlia-Ciommo et al. 2018). The resulting inability of peroxisomes to oxidize FFAs causes the accumulation of fatty acyl-CoA esters (which serve as substrate for peroxisomal β -oxidation of FFAs), in peroxisomes (**Figure 1.4**) (Goldberg et al. 2009; Arlia-Ciommo et al. 2018). The build-up of fatty acyl-CoA esters in peroxisomes instigates a negative feedback mechanism that initially slows the lipolysis of TAGs to FFAs and DAGs in LDs, then attenuates the transport of TAGs from the ER to LDs, and ultimately diminishes the synthesis of TAGs from FFAs and DAGs in the ER (**Figure 1.4**) (Goldberg et al. 2009; Arlia-Ciommo et al. 2018). The action of this negative feedback mechanism in yeast grown under non-CR conditions leads to the accumulation of FFAs and DAGs in the ER and LDs. Because both FFAs and DAGs accelerate the onset of an age-related form of regulated cell death (RCD) called liponecrosis (see next section) (Richard et al. 2014; Sheibani et al. 2014), the accumulation of these two lipids shortens yeast chronological lifespan under non-CR conditions. In contrast, because neither FFAs nor DAGs accumulate in yeast under CR conditions, the above mechanism cannot accelerate the chronological mode of aging in *S. cerevisiae* grown under these conditions of low calorie supply (**Figure 1.4**) (Goldberg et al. 2009; Arlia-Ciommo et al. 2018).

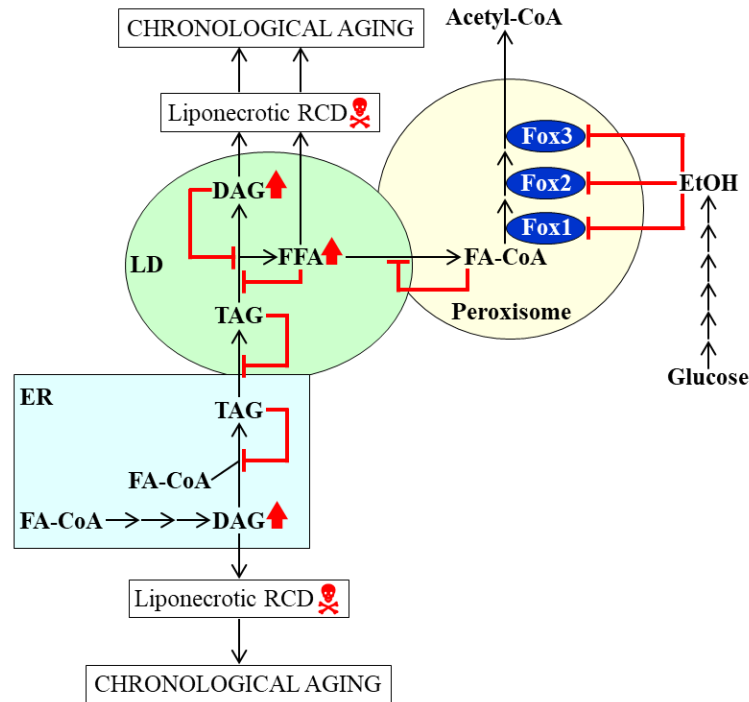


Figure 1.4. A mechanism by which an accumulation of ethanol by yeast cells grown under non-CR conditions on 2% glucose may shorten their chronological lifespan by altering the rates and efficiencies with which TAGs are synthesized from FFAs and DAGs in the ER, delivered to and stored in LDs, and lipolytically degraded to FFAs and DAGs in LDs (Mitrofanova, et al. 2018). Red arrows next to the names of lipid classes denote those of them whose concentrations are increased in non-CR yeast. Inhibition bars displayed in red color signify negative feedback loops. Abbreviations: DAGs, diacylglycerols; ER, the endoplasmic reticulum; FA-CoA, fatty acyl-CoA esters; LDs, lipid droplets; TAGs, triacylglycerols; RCD, regulated cell death.

The other mechanism for delaying yeast chronological aging by altering the rates and efficiencies with which TAGs are synthesized from FFAs and DAGs in the ER, delivered to and stored in LDs, and lipolytically degraded to FFAs and DAGs in LDs has been discovered in yeast grown under non-CR conditions on 2% glucose (Handee et al. 2016; Li et al. 2017). In this so-called "radical sink" mechanism, wild-type (WT) yeast cells that age chronologically under non-CR conditions accumulate reactive oxygen species (ROS) that oxidatively damage the following types of cellular molecules: 1) water-soluble macromolecules (i.e. DNA, RNA and proteins) and small molecules of metabolites;

2) membrane-associated proteins and lipids; and 3) unsaturated FFAs incorporated into TAGs that are deposited in LDs (**Figure 1.5**) (Handee et al. 2016; Li et al. 2017). The build-up of such oxidatively damaged water-soluble and membrane-associated molecules in WT cells accelerates their chronological aging under non-CR conditions (**Figure 1.5**) (Handee et al. 2016; Li et al. 2017). The "radical sink" mechanism of aging delay is activated under CR-conditions in yeast cells carrying the *tgl3Δ*, *tgl4Δ* or *tgl3Δtgl4Δ* mutations (which slow down TAG lipolysis in LDs) and in yeast cells overexpressing the Dgal enzyme of TAG synthesis. All these genetic interventions increase the abundance of LDs because they decelerate LD degradation or accelerate LD formation under non-CR conditions (**Figure 1.5**) (Handee et al. 2016; Li et al. 2017). This increases the abundance of FFAs (including unsaturated FFAs) that are incorporated into TAGs deposited in LDs. Because unsaturated FFAs are very susceptible to oxidative damage, their build-up in LDs decreases their concentrations in the PM and organellar membranes thus allowing LDs act as a radical sink for concentrating oxidatively damaged unsaturated FFAs. By decreasing the concentrations of oxidatively damaged unsaturated FFAs in the PM and cellular membranes, the conversion of LDs into the radical sink lowers the extent of oxidative damage to lipids and proteins associated with these membranes as well as to water-soluble macromolecules and small molecules (**Figure 1.5**) (Handee et al. 2016; Li et al. 2017). Such decline in oxidative damage to various cellular molecules causes a delay of chronological aging of yeast cells that carry the *tgl3Δ*, *tgl4Δ* or *tgl3Δtgl4Δ* mutations or overexpress Dgal and grow under non-CR conditions on 2% glucose (**Figure 1.5**) (Handee et al. 2016; Li et al. 2017).

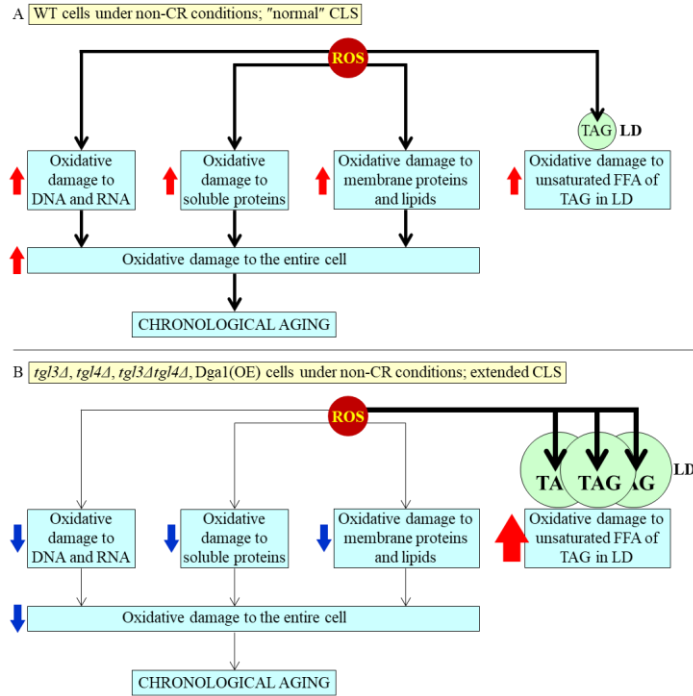


Figure 1.5. A "radical sink" mechanism for the delay of chronological aging in yeast mutants accumulating excessive amounts of LDs under non-CR conditions. The thickness of black arrows is proportional to the extent of oxidative damage to various molecules, degree of oxidative damage to the entire cell or efficiency with which chronological aging is accelerated. Arrows next to the boxes showing names of affected processes denote those of them that are intensified (red arrows) or weakened (blue arrows). (Mitrofanova, et al. 2018). Abbreviations: LD, lipid droplets; TAG, triacylglycerols; ROS, reactive oxygen species.

1.6 Liponecrosis, an age-related form of regulated cell death (RCD)

Liponecrotic RCD can be caused by a brief (for 2 h) exposure of yeast to exogenous palmitoleic acid (POA), a 16-carbon monounsaturated FFA, or DAGs (Goldberg et al. 2010; Richard et al. 2014; Sheibani et al. 2014). Because the number of yeast cells exhibiting hallmarks of liponecrotic RCD increases with chronological age, whereas cell susceptibility to this form of RCD declines with chronological age, liponecrotic RCD is thought to be an age-related form of RCD (Goldberg et al. 2010; Richard et al. 2014; Sheibani et al. 2014; Arlia-Ciommo et al. 2018). The following mechanism of POA-

induced liponecrotic RCD has been established (**Figure 1.6**) (Richard et al. 2014; Sheibani et al. 2014). An exposure of yeast cells to exogenous POA allows this monounsaturated FFA to be incorporated into POA-containing phospholipids that accumulate in the ER membrane, mitochondrial membranes and the PM. The accumulation of POA-containing phospholipids in the PM decreases the concentration of phosphatidylethanolamine (PE) in its outer leaflet; this causes an increase in the PM permeability for small molecules and commits yeast to liponecrotic RCD by allowing an uncontrollable flow of these molecules into the cell (**Figure 1.6**) (Richard et al. 2014; Sheibani et al. 2014). The build-up of excessively high concentrations of POA-containing phospholipids in mitochondrial membranes deteriorates mitochondrial functionality, thus eliciting the excessive generation of ROS in mitochondria (**Figure 1.6**) (Richard et al. 2014; Sheibani et al. 2014). The resulting rise of cellular ROS above a toxic threshold 1) causes an oxidative damage to different cellular organelles, which leads to their excessive autophagic degradation; and 2) elicits an excessive oxidative damage to many cytosolic proteins, which impairs cellular proteostasis. These two processes also contribute to the commitment of yeast to liponecrotic RCD (**Figure 1.6**) (Richard et al. 2014; Sheibani et al. 2014). Yeast cells can use the following four processes to adapt to the cellular stress imposed by POA, thus maintaining viability: 1) peroxisomal oxidation of POA, a process that decreases the incorporation of POA into phospholipids that accumulate in the ER membrane, mitochondrial membranes and the PM; 2) the incorporation of POA into TAGs, a process that also decreases the incorporation of POA into phospholipids; 3) a selective degradation of dysfunctional mitochondria via the mitophagic pathway of cargo-selective autophagy, a process that allows to maintain a healthy population of functional mitochondria required

for the incorporation of POA into TAGs; and 4) a metacaspase-dependent degradation of damaged, dysfunctional and aggregated cytosolic proteins, a process that allows the cell to sustain cellular proteostasis (**Figure 1.6**) (Richard et al. 2014; Sheibani et al. 2014).

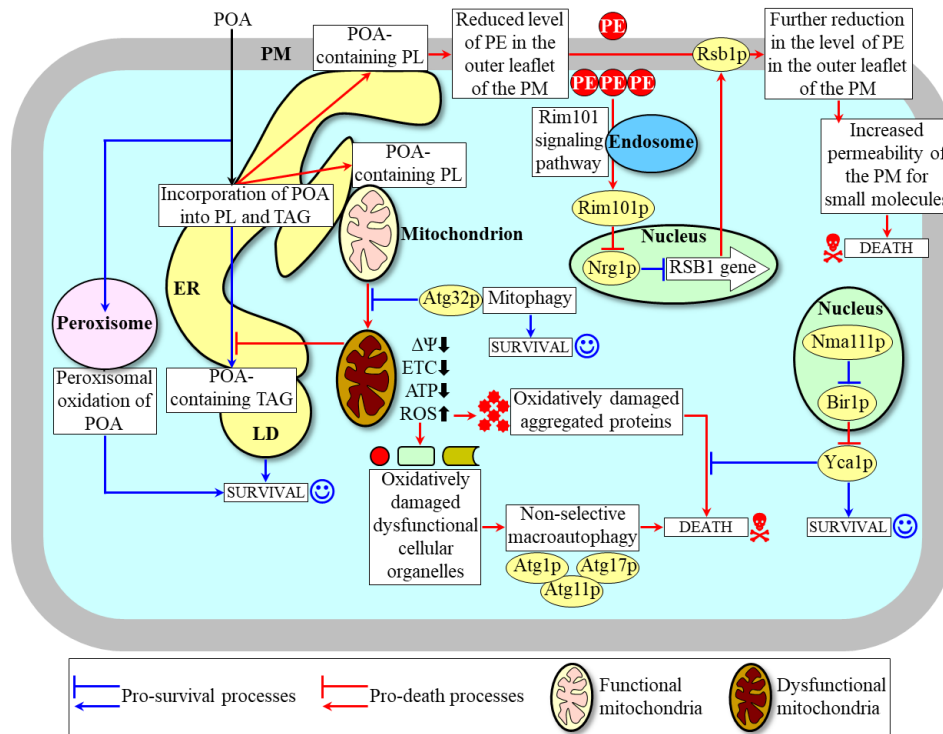


Figure 1.6. A model for a mechanism underlying POA-induced liponecrotic RCD in yeast. Activation arrows and inhibition bars denote pro-death processes (displayed in red color) or pro-survival processes (displayed in blue color) for POA-induced liponecrotic RCD. Abbreviations: ER, the endoplasmic reticulum; PE, phosphatidylethanolamine; PL, phospholipids; PM, the plasma membrane; ROS, reactive oxygen species.

1.7 The Objective of Studies Described in This Thesis

In collaboration with Idunn Technologies Inc., the Titorenko laboratory has recently discovered 14 plant extracts that delay yeast chronological aging. They have been given the following names: PE39, PE42, PE47, PE59, PE64, PE68, PE69, PE72, PE75, PE77, PE78, PE79, PE81 and PE83. Because age-related changes in the abundance of LDs play essential roles in defining the pace of chronological aging in *S. cerevisiae*, one

objective of my thesis was to use live-cell fluorescence microscopy involving BODIPY 493/503 staining for examining how each of the 14 aging-delaying plant extracts affects the number and size of LDs at different consecutive steps of yeast chronological aging. Because the synthesis, storage and degradation of neutral lipids are integrated into metabolic pathways for other lipid classes, my other objective was to use a combination of liquid chromatography and mass spectrometry to examine how 2 of the 14 aging-delaying plant extracts affect the intracellular concentrations of several classes of lipids in chronologically aging *S. cerevisiae*.

2 Effects of Aging-Delaying Plant Extracts on the Number and Size of LDs

2.1 Abstract

Age-related changes in the abundance of lipid droplets, in which the "neutral" lipids triacylglycerols and steryl esters can be stored or lipolytically degraded under different conditions, are known to play essential roles in defining yeast longevity. Therefore, the objective of studies described in this chapter of my thesis was to examine how each of the 14 aging-delaying plant extracts discovered by the Titorenko lab affects the number and size of lipid droplets at different stages of yeast chronological aging (i.e. on different days of cell collection). These extracts have been given the following names: PE26, PE39, PE42, PE47, PE59, PE64, PE68, PE69, PE72, PE75, PE77, PE78, PE79, PE81 and PE83. Each of these plant extracts was used in a concentration showing the highest aging-delaying efficiency. My findings described in this chapter of the thesis indicate that PE69 tends to decrease both the number and size of LDs on all days of cell collection. PE68 decreases the size of LDs only on day 1 and has no effect on the number of LDs on any day of cell collection. PE75 and PE77 increase the size of LDs only on day 2 or day 1 (respectively); none of these two plant extracts alters the number of LDs on any day of cell collection. PE78 and PE81 decrease or increase (respectively) the number of LDs on day 1 or day 4 (respectively); none of them influences the size of LDs on any day of cell collection. I also found that PE39, PE42, PE47, PE59, PE64, PE72, PE79 and PE83 do not change either the number of LDs or their size on any day of cell collection.

2.2 Materials and Methods

Yeast Strains, Growth Conditions and Staining Assays

The *Saccharomyces cerevisiae* wild-type strain BY4742 (*MAT α* Δ *his3* Δ 1 *leu2* Δ 0 *lys2* Δ 0 *ura3* Δ 0) from Thermo Scientific/Open Biosystems was used. Grown in a synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) containing 2% (w/v) glucose and supplemented with 20 mg/l histidine, 30 mg/l leucine, 30 mg/l lysine and 20 mg/l uracil, with and without plant extracts. A 20% stock solution of each plant extract in ethanol was made on the day of cell inoculation into the growth medium at a final concentration of 0.1%, 0.3%, 0.5% and 1.0%. Cells were cultured at 30°C with rotational shaking at 200 rpm in Erlenmeyer flasks at a “flask volume/medium volume” ratio of 5:1. Cells were imaged every 24 h starting at log phase growth and continuing for four consecutive days using BODIPY 493/503.

Cell culture samples were collected at specific time-points and fractions were diluted in dH₂O to determine total number of cells per mL of culture using a hemocytometer. 10 μ L of serial dilutions (1:10 to 1:10³) of cells were applied to the hemocytometer, where each square is calibrated to hold 0.1 μ L. The average of 4 large squares was counted and the concentration of cells in the original flask was calculated as follows: number of cells per large square \times dilution factor \times 10 \times 10,000 = total number of cells per mL of culture. A concentration of 3.5×10^7 cells/mL was used for each culture to have better confluency when imaging.

Chronological Life Span Assay

A sample of cells was taken from a culture at a certain time-point. A fraction of the sample was diluted to determine the total number of cells using a hemocytometer. Another fraction of the cell sample was diluted, and serial dilutions of cells were plated in duplicate onto YP (1% yeast extract, 2% bacto peptone) plates containing 2% glucose as carbon source. After 2 d of incubation at 30°C, the number of colony forming units (CFU) per plate was counted. The number of CFU was defined as the number of viable cells in a sample. For each culture, the percentage of viable cells was calculated as follows: (number of viable cells per ml/total number of cells per ml) × 100. The percentage of viable cells in mid-logarithmic phase was set at 100%.

Live Cell Fluorescence Microscopy and LDs Quantification

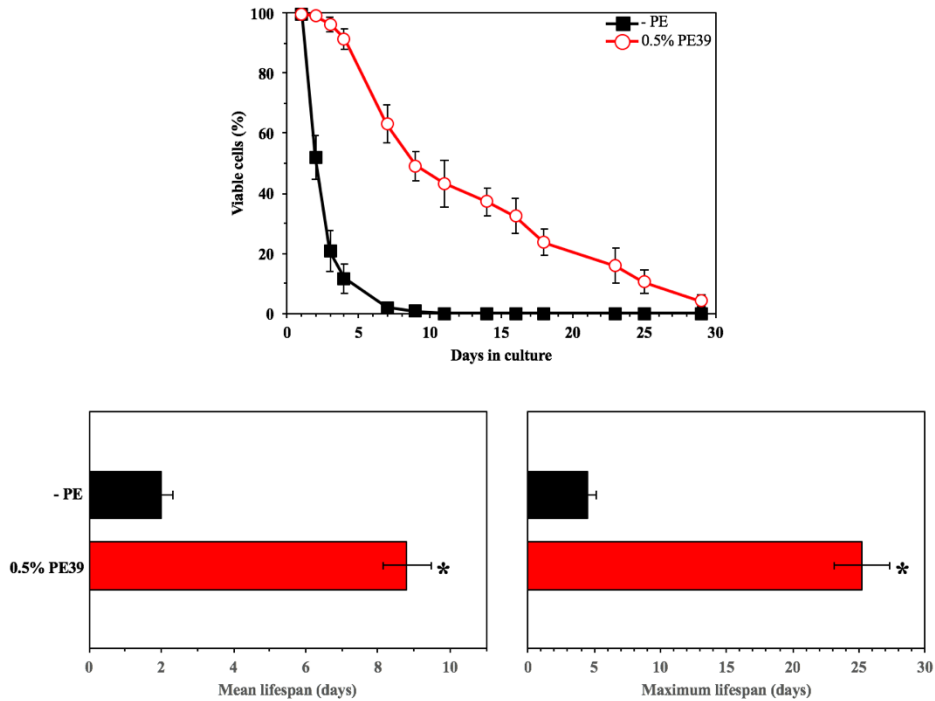
BODIPY 493/503 staining for monitoring neutral lipids deposited in LDs was performed as follows. Formaldehyde-fixed cells were permeabilized by treatment with 0.2% Triton X-100 for 6 min and incubated with 10 μM BODIPY 493/503 (Thermo Fisher Scientific) in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl for 15 min to label LDs. Live imaging was performed on a Leica DM6000B epifluorescence microscope equipped with a high-resolution Hamamatsu Orca ER CCD camera using oil immersion and a 100X objective. Images were acquired with 20-ms exposures using PerkinElmer Volocity software. Image files were exported as TIFFs then opened in ImageJ where counts were performed using the cell counter add-on and cell-to-LD ratios were quantified by a computational macro measuring the total cell fluorescent intensity versus LD intensity for each individual cell.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel's (2017) Analysis ToolPack. All data are presented as mean \pm SD. The p values for comparing the means of two groups (using an unpaired t test) were calculated with the help of GraphPad Prism statistics software.

2.3 Results

The aging-delaying plant extract PE39 extends longevity of chronologically aging yeast. Indeed, I found that 0.5% PE39 statistically significantly increases both the mean and maximum chronological lifespans (CLSs) of wild-type (WT) cells of *S. cerevisiae* grown in YNB medium containing 2% glucose (**Figure 2.3.1**).



I found that 0.5% PE39 does not have a statistically significant effect on the percentage of cells with 1 to 3, 4 to 6, 7 to 9 or > 10 LDs (**Figure 2.3.2**). Thus, 0.5% PE39 does not change the number of LDs in a yeast cell.

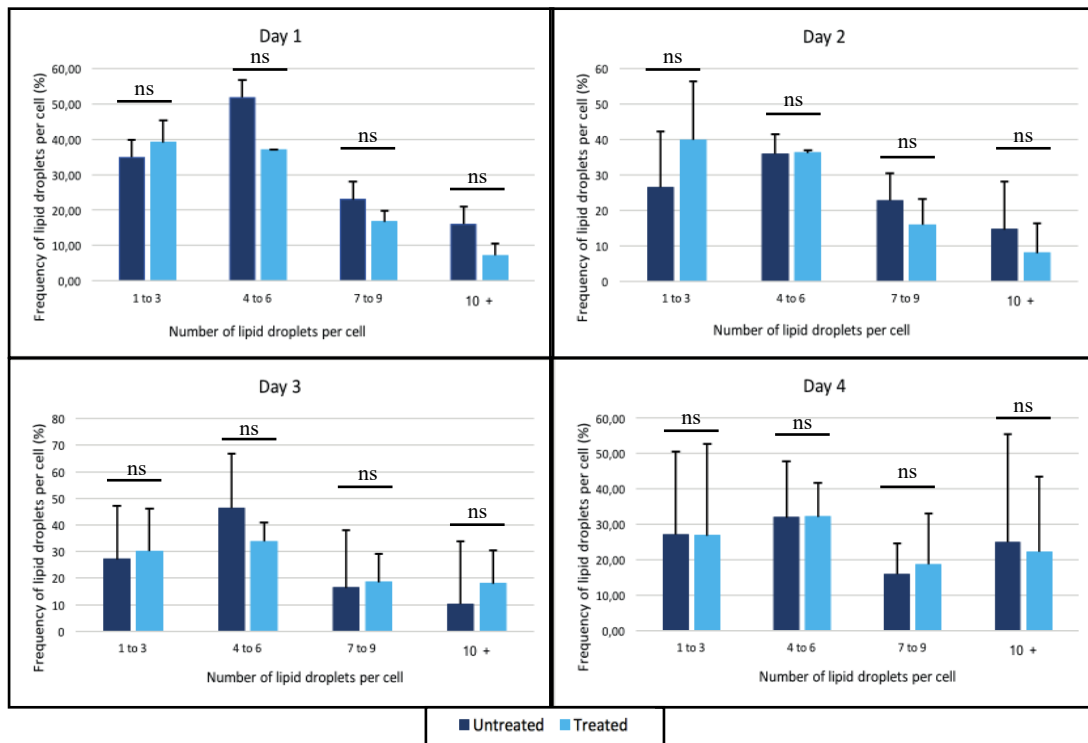


Figure 2.3.2. 0.5% PE39 does not change the number of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE39 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The percentages of cells with certain number of LDs/cell are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

I also found that 0.5% PE39 does not have a statistically significant effect on the ratio "Total volume of LDs per cell/Total volume of this cell" (**Figure 2.3.3**). Thus, 0.5% PE39 does not change the size of LDs in a yeast cell.

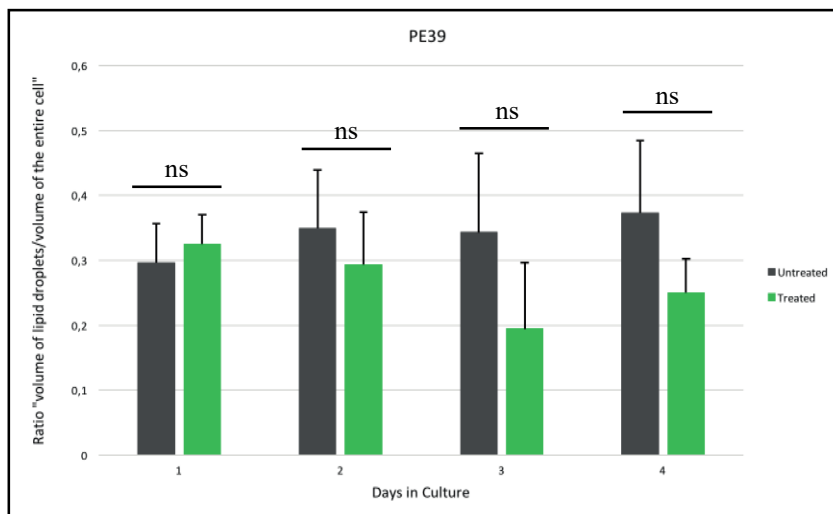


Figure 2.3.3. 0.5% PE39 does not change the size of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE39 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The ratios "Total volume of LDs per cell/Total volume of this cell" are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

The aging-delaying plant extract PE42 extends longevity of chronologically aging yeast. Indeed, I found that 0.5% PE42 statistically significantly increases both the mean and maximum CLSs of WT cells of *S. cerevisiae* grown in YNB medium containing 2% glucose (**Figure 2.3.4**).

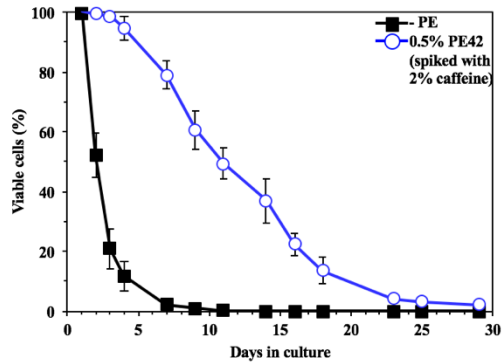
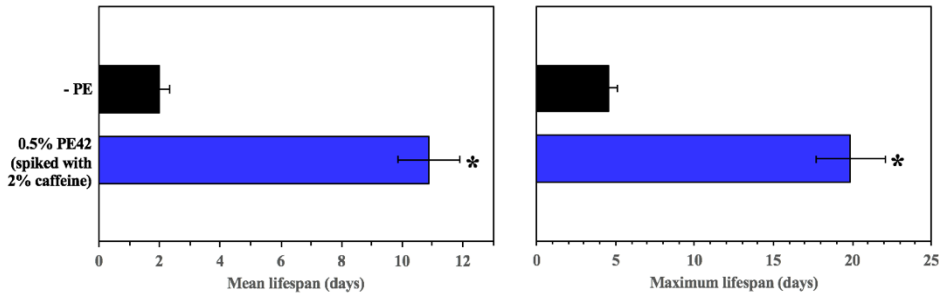


Figure 2.3.4. 0.5% PE42 extends longevity of chronologically aging yeast. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE42 or in its absence. Survival curves, and the mean and maximum CLSs of these cells are shown. Data are presented as means \pm SEM (n = 3; *p < 0.05).



I found that 0.5% PE42 tends to increase the percentage of cells with low number (i.e. 1 to 3) of LDs and to decrease the percentage of cells with high number (i.e. 7 to 9 and > 10 LDs) on most days of cell collection (**Figure 2.3.5**). However, none of these effects of PE42 was statistically significant. I therefore concluded that 0.5% PE42 does not change the number of LDs in a yeast cell.

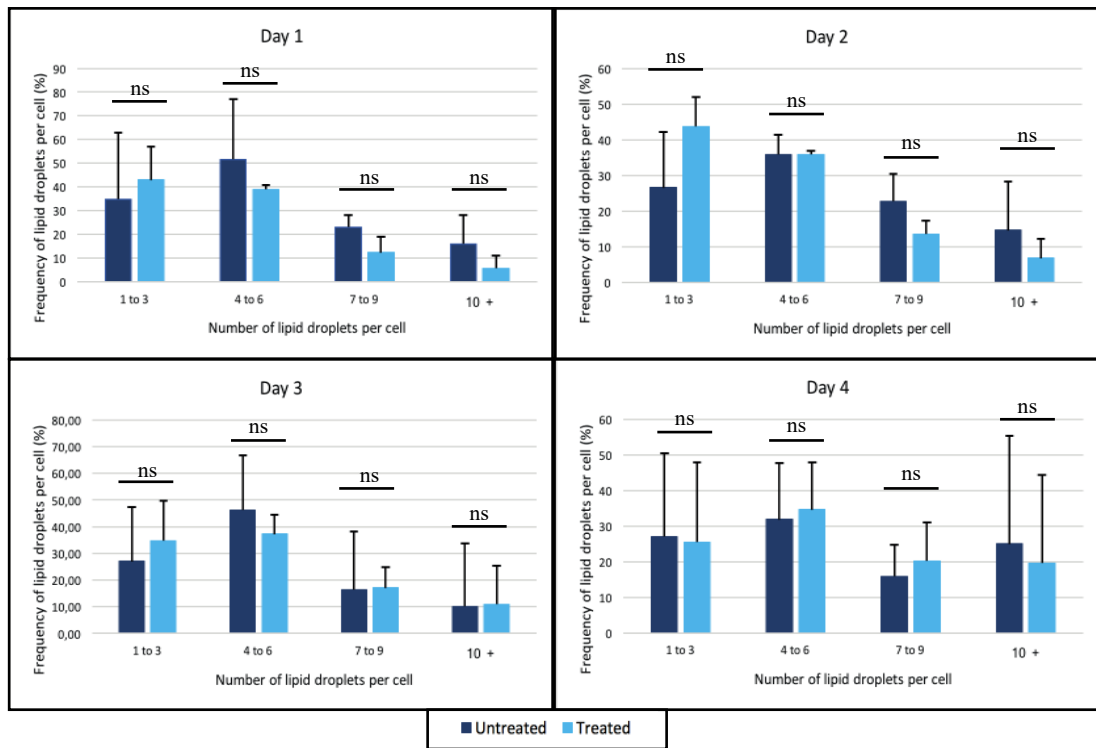


Figure 2.3.5. 0.5% PE42 does not change the number of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE42 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The percentages of cells with certain number of LDs/cell are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

I also found that 0.5% PE42 does not have a statistically significant effect on the ratio "Total volume of LDs per cell/Total volume of this cell" (**Figure 2.3.6**). I therefore concluded that 0.5% PE42 does not change the size of LDs in a yeast cell.

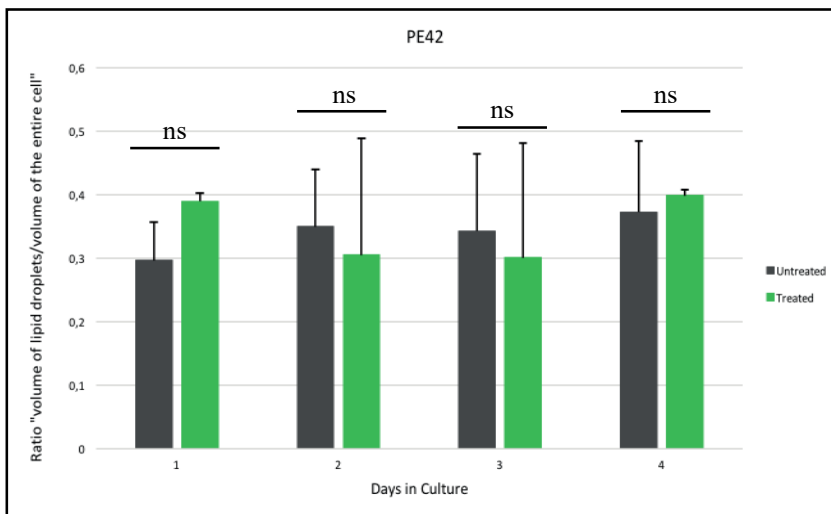


Figure 2.3.6. 0.5% PE42 does not change the size of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE42 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The ratios "Total volume of LDs per cell/Total volume of this cell" are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

The aging-delaying plant extract PE47 extends longevity of chronologically aging yeast. Indeed, I found that 0.3% PE47 statistically significantly increases both the mean and maximum CLSs of WT cells of *S. cerevisiae* grown in YNB medium containing 2% glucose (**Figure 2.3.7**).

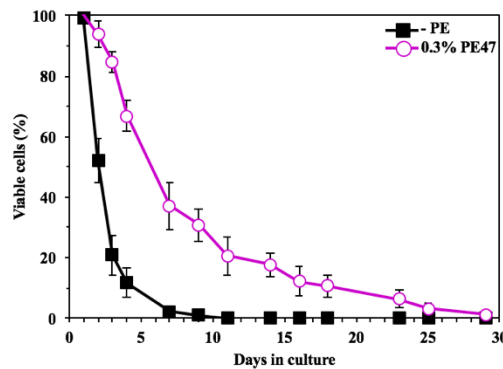
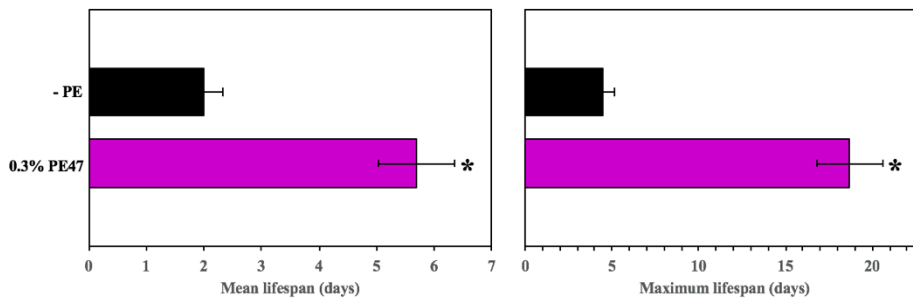


Figure 2.3.7. 0.3% PE47 extends longevity of chronologically aging yeast. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE47 or in its absence. Survival curves, and the mean and maximum CLSs of these cells are shown. Data are presented as means \pm SEM (n = 3; *p < 0.05).



I found that 0.3% PE47 tends to increase the percentage of cells with low number (i.e. 1 to 3 and 4 to 6) of LDs and to decrease the percentage of cells with high number (i.e. 7 to 9 and > 10 LDs) on most days of cell collection (**Figure 2.3.8**). However, none of these effects of PE47 was statistically significant. Therefore, 0.3% PE47 does not change the number of LDs in a yeast cell.

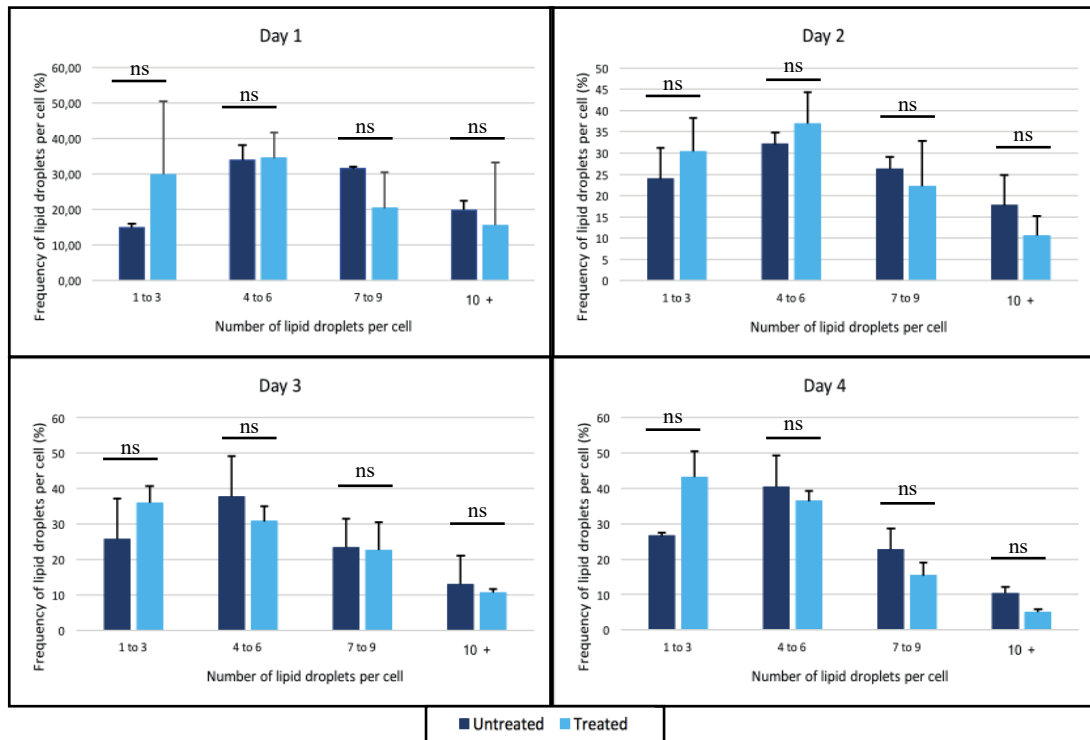


Figure 2.3.8. 0.3% PE47 does not change the number of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE47 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The percentages of cells with certain number of LDs/cell are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

I also found that 0.3% PE47 tends to decrease the ratio "Total volume of LDs per cell/Total volume of this cell" on each day of cell collection (**Figure 2.3.9**). However, none of these effects of PE47 was statistically significant. I therefore concluded that 0.3% PE47 does not change the size of LDs in a yeast cell.

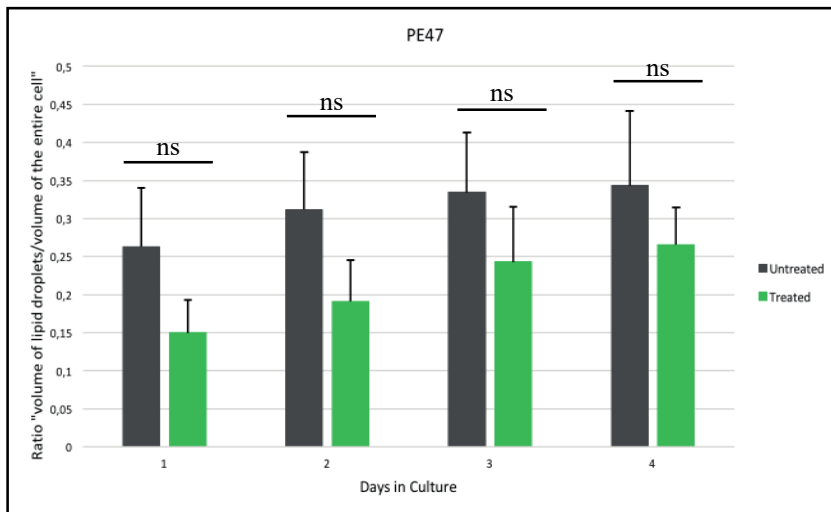


Figure 2.3.9. 0.3% PE47 does not change the size of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE47 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The ratios "Total volume of LDs per cell/Total volume of this

cell" are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

The aging-delaying plant extract PE59 extends longevity of chronologically aging yeast. Indeed, I found that 0.3% PE59 statistically significantly increases both the mean and maximum CLSs of WT cells of *S. cerevisiae* grown in YNB medium containing 2% glucose (**Figure 2.3.10**).

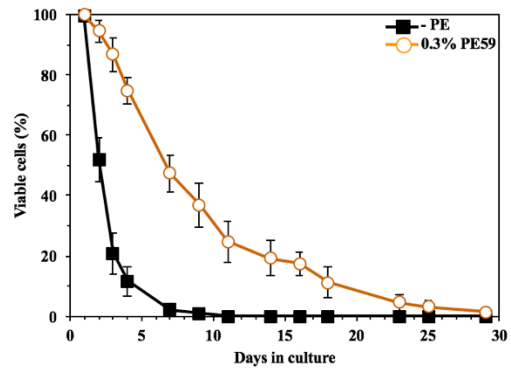
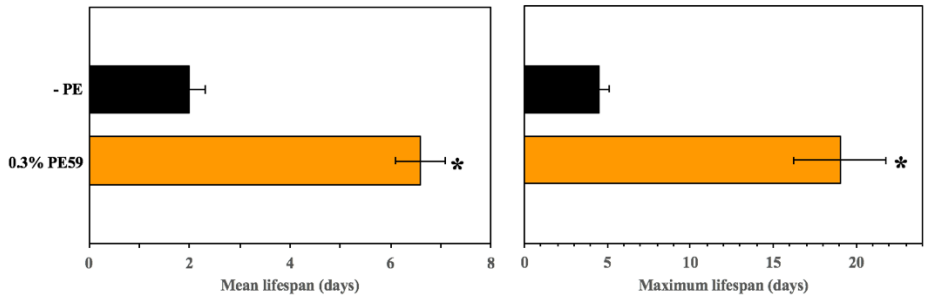


Figure 2.3.10. 0.3% PE59 extends longevity of chronologically aging yeast. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE59 or in its absence. Survival curves, and the mean and maximum CLSs of these cells are shown. Data are presented as means \pm SEM (n = 3; *p < 0.05).



I found that 0.3% PE59 tends to increase the percentage of cells with low number (i.e. 1 to 3) of LDs on most days of cell collection (**Figure 2.3.11**). However, this effect of PE59 wasn't statistically significant. Therefore, 0.3% PE59 does not change the number of LDs in a yeast cell.

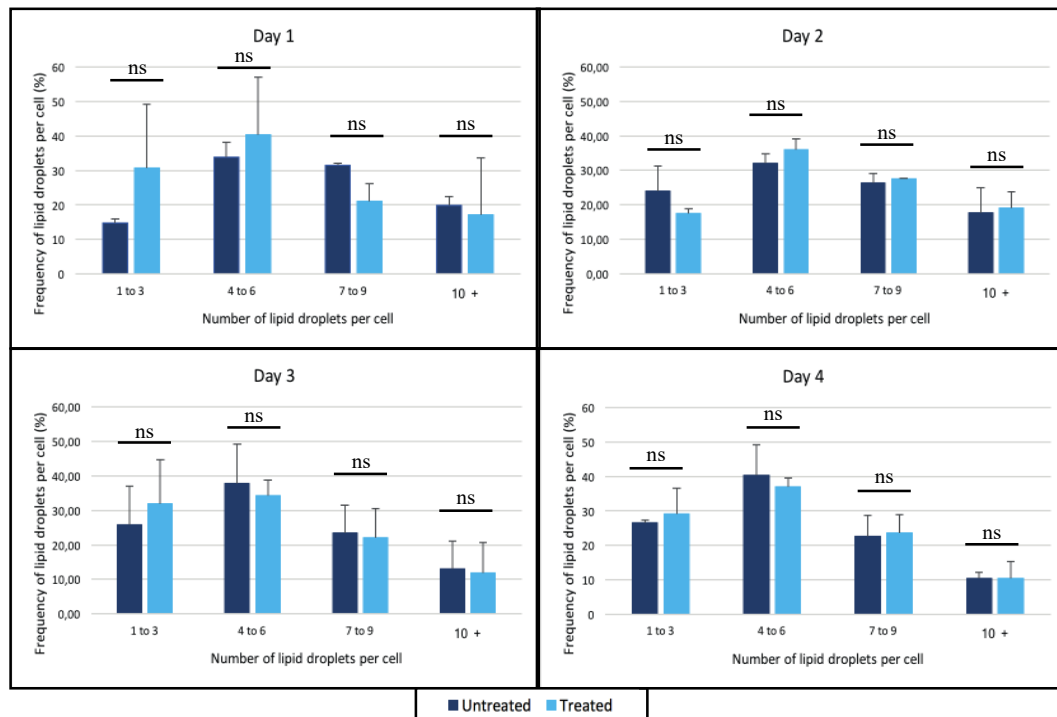


Figure 2.3.11. 0.3% PE59 does not change the number of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE59 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The percentages of cells with certain number of LDs/cell are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

I also found that 0.3% PE59 tends to decrease the ratio "Total volume of LDs per cell/Total volume of this cell" on each day of cell collection (**Figure 2.3.12**). However, none of these effects of PE59 was statistically significant. I therefore concluded that 0.3% PE59 does not change the size of LDs in a yeast cell.

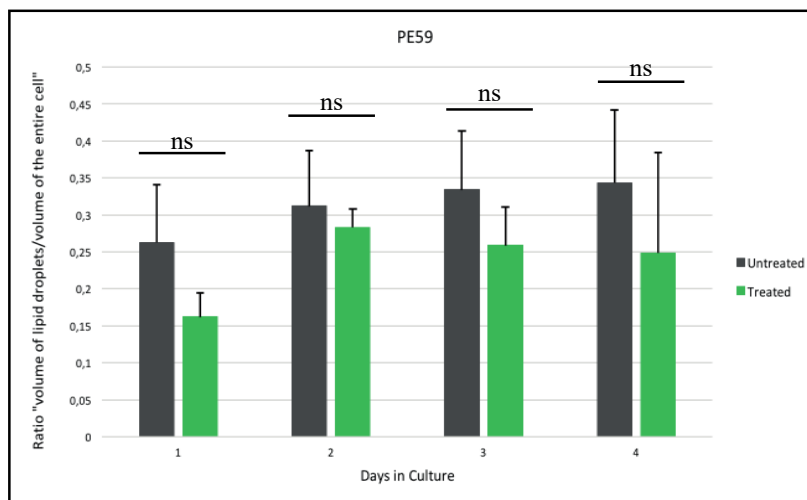


Figure 2.3.12. 0.3% PE59 does not change the size of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE59 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The ratios "Total volume of LDs per cell/Total volume of this cell" are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

The aging-delaying plant extract PE64 extends longevity of chronologically aging yeast. Indeed, I found that 0.1% PE64 statistically significantly increases both the mean and maximum CLSs of WT cells of *S. cerevisiae* grown in YNB medium containing 2% glucose (**Figure 2.3.13**).

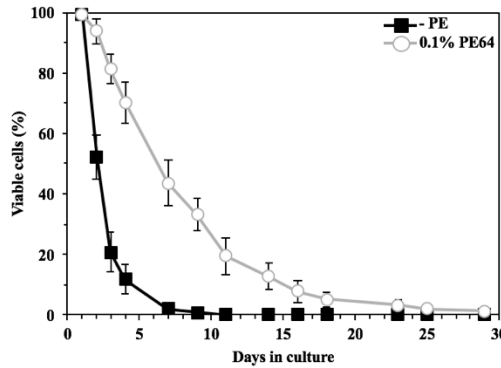
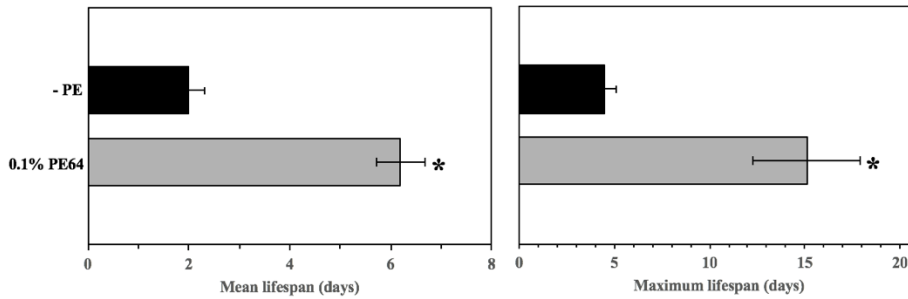


Figure 2.3.13. 0.1% PE64 extends longevity of chronologically aging yeast. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.1% PE64 or in its absence. Survival curves, and the mean and maximum CLSs of these cells are shown. Data are presented as means \pm SEM ($n = 3$; $*p < 0.05$).



I found that 0.1% PE64 tends to increase the percentage of cells with low number (i.e. 1 to 3) and to decrease the percentage of cells with high number (i.e. > 10) of LDs on most days of cell collection (**Figure 2.3.14**). However, none of these effects of PE64 was statistically significant. Therefore, 0.1% PE64 does not change the number of LDs in a yeast cell.

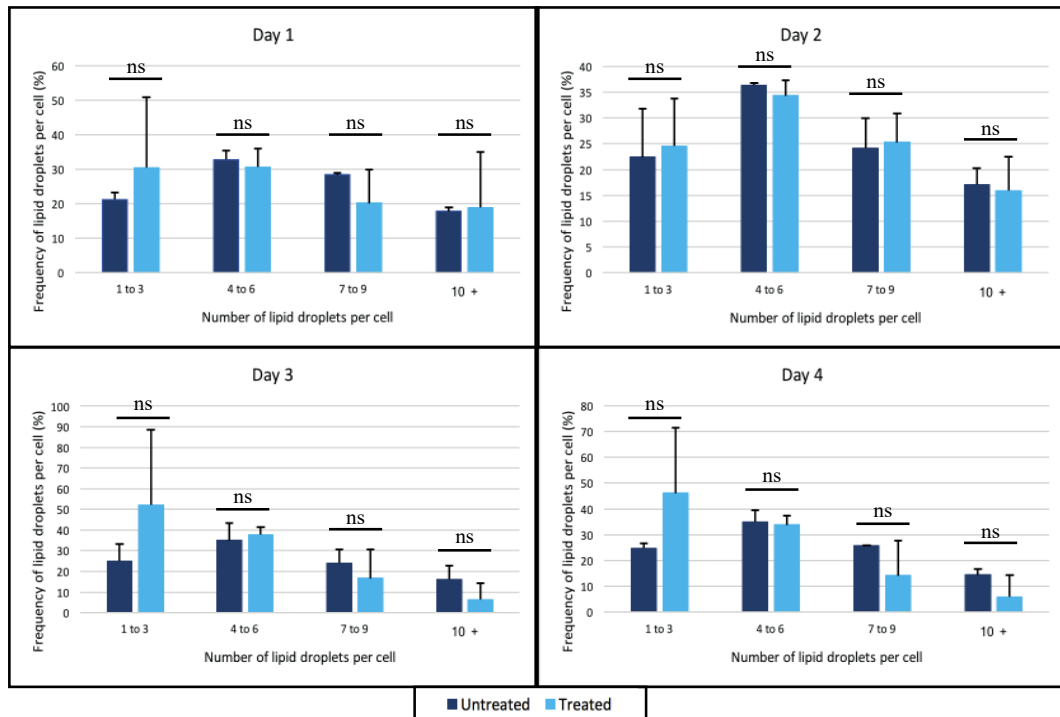


Figure 2.3.14. 0.1% PE64 does not change the number of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.1% PE64 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The percentages of cells with certain number of LDs/cell are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

I also found that 0.1% PE64 tends to decrease the ratio "Total volume of LDs per cell/Total volume of this cell" during initial 3 days of cell collection (**Figure 2.3.15**). However, none of these effects of PE64 was statistically significant. I therefore concluded that 0.1% PE64 does not change the size of LDs in a yeast cell.

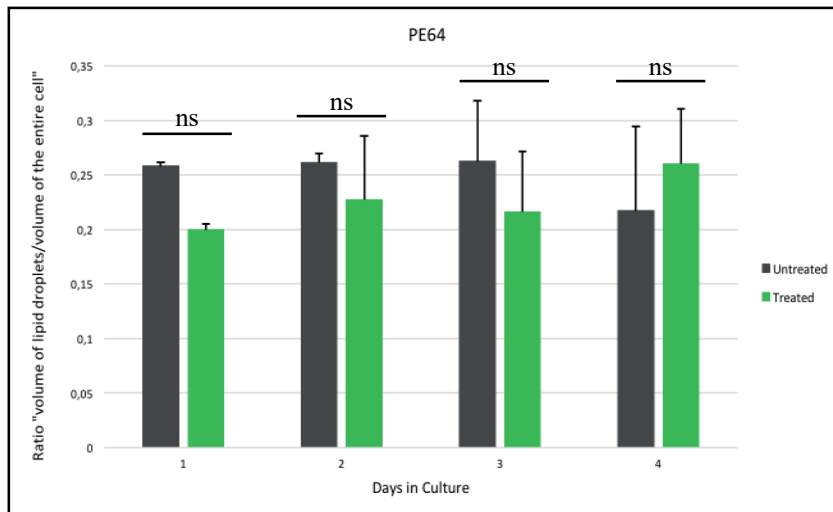


Figure 2.3.15. 0.1% PE64 does not change the size of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.1% PE64 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The ratios "Total volume of LDs per cell/Total volume of this cell" are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

The aging-delaying plant extract PE68 extends longevity of chronologically aging yeast. Indeed, I found that 0.5% PE68 statistically significantly increases both the mean and maximum CLSs of WT cells of *S. cerevisiae* grown in YNB medium containing 2% glucose (**Figure 2.3.16**).

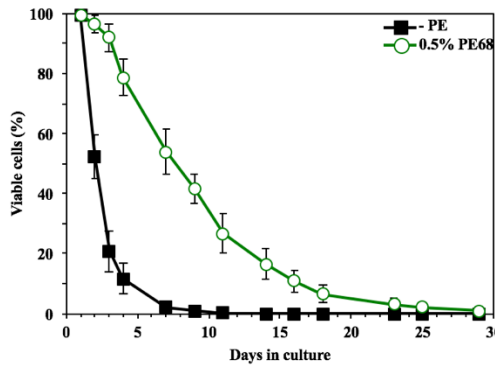
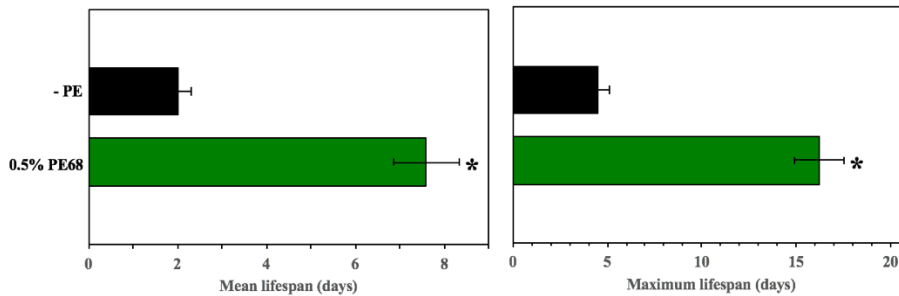


Figure 2.3.16. 0.5% PE68 extends longevity of chronologically aging yeast. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE68 or in its absence. Survival curves, and the mean and maximum CLSs of these cells are shown. Data are presented as means \pm SEM ($n = 3$; $*p < 0.05$).



I found that 0.5% PE68 tends to increase the percentage of cells with low number (i.e. 1 to 3) and to decrease the percentage of cells with high number (i.e. > 10) of LDs on most days of cell collection (**Figure 2.3.17**). However, none of these effects of PE68 was statistically significant. Therefore, 0.5% PE68 does not change the number of LDs in a yeast cell.

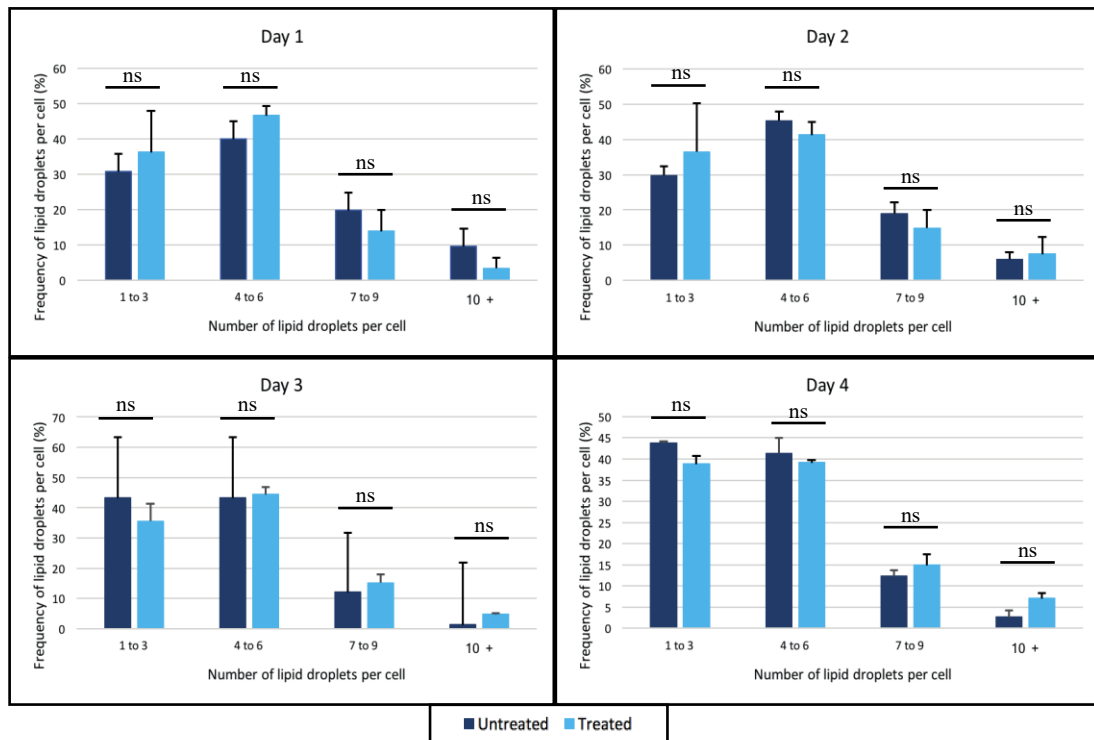


Figure 2.3.17. 0.5% PE68 does not change the number of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE68 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The percentages of cells with certain number of LDs/cell are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

I also found that 0.5% PE68 statistically significantly decreases the ratio "Total volume of LDs per cell/Total volume of this cell" on the first day of cell collection (**Figure 2.3.18**). On three subsequent days of cell collection, 0.5% PE68 tends to decrease the ratio "Total volume of LDs per cell/Total volume of this cell" (**Figure 2.3.18**). However, none of these effects of PE68 was statistically significant. I concluded that 0.5% PE68 significantly decreases the size of LDs in a yeast cell only on day 1 of culturing, but after that does not change the size of LDs in a yeast cell.

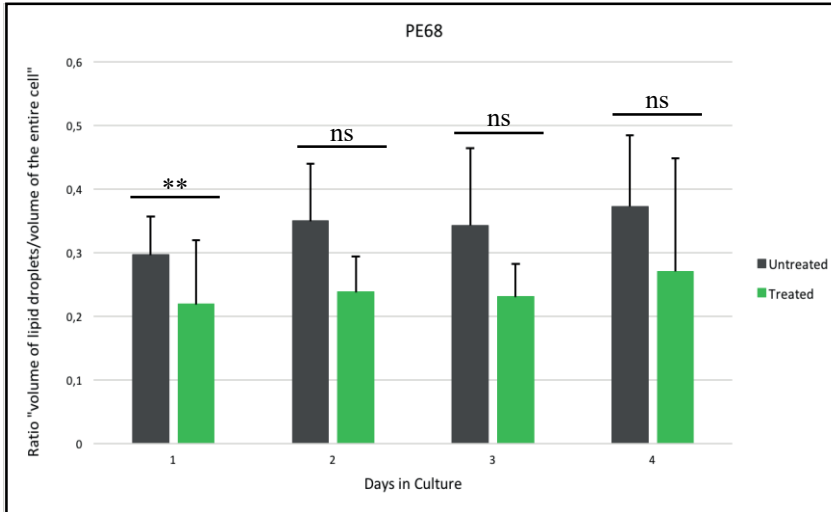


Figure 2.3.18. 0.5% PE68 significantly decreases the size of LDs in a yeast cell only on day 1 of culturing, but after that does not change the size of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE68 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence

microscopy and LDs quantification. The ratios "Total volume of LDs per cell/Total volume of this cell" are shown. Data are presented as means \pm SD (n = 2; *p < 0.01; ns = not significant).

The aging-delaying plant extract PE69 extends longevity of chronologically aging yeast. Indeed, I found that 1% PE69 statistically significantly increases both the mean and maximum CLSs of WT cells of *S. cerevisiae* grown in YNB medium containing 2% glucose (Figure 2.3.19).

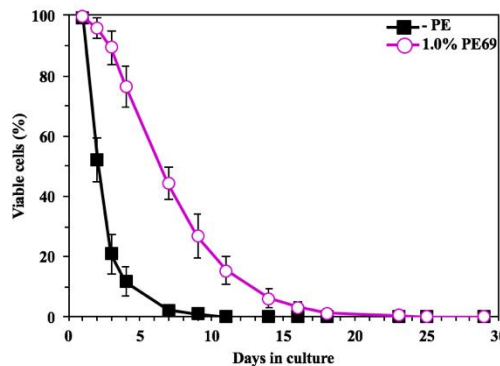
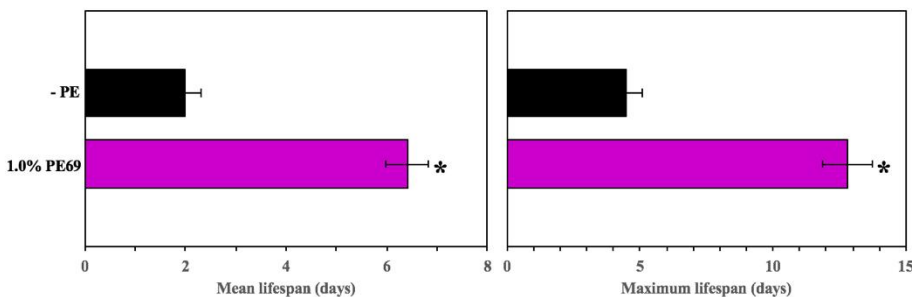


Figure 2.3.19. 1% PE69 extends longevity of chronologically aging yeast. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 1% PE69 or in its absence. Survival curves, and the mean and maximum CLSs of these cells are shown. Data are presented as means \pm SEM (n = 3; *p < 0.05).



In a single experiment conducted so far, I found that 1% PE69 tends to increase the percentage of cells with low number (i.e. 1 to 3) and to decrease the percentage of cells with high number (i.e. 7 to 9 and > 10) of LDs on most days of cell collection (**Figure 2.3.20**).

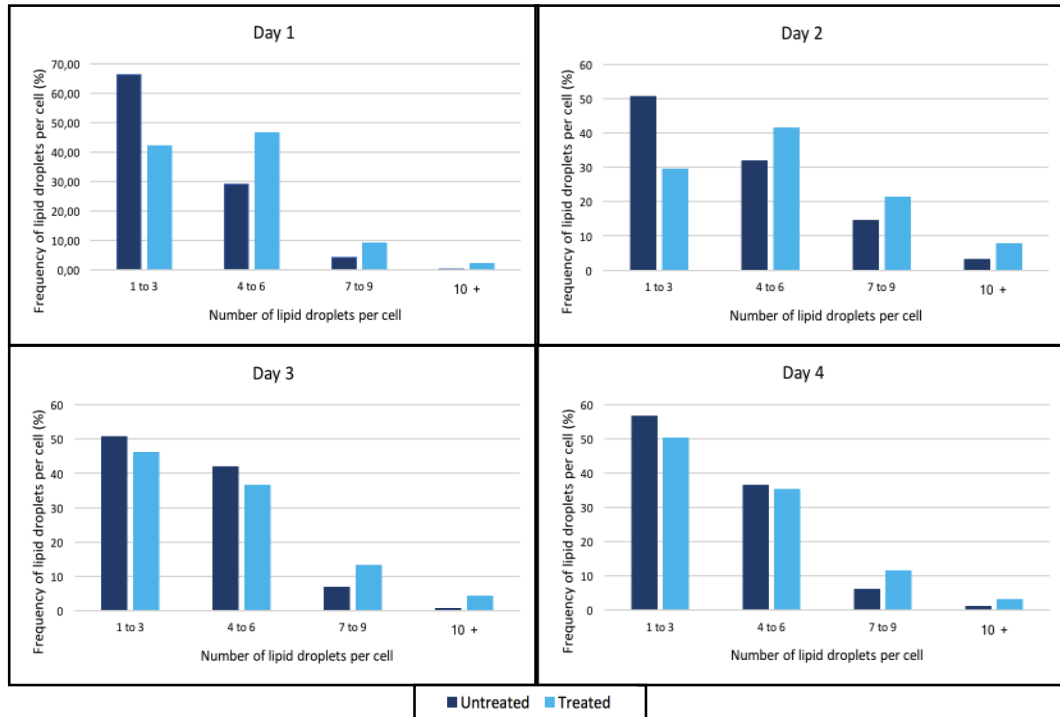


Figure 2.3.20. A single experiment conducted so far indicates that 1% PE69 tends to increase the percentage of cells with low number and to decrease the percentage of cells with high number of LDs on most days of cell collection. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 1% PE69 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The percentages of cells with certain number of LDs/cell are shown. n = 1.

In a single experiment conducted so far, I also found that 1% PE69 decreases the ratio "Total volume of LDs per cell/Total volume of this cell" on days 1, 3 and 4 of cell collection (**Figure 2.3.21**).

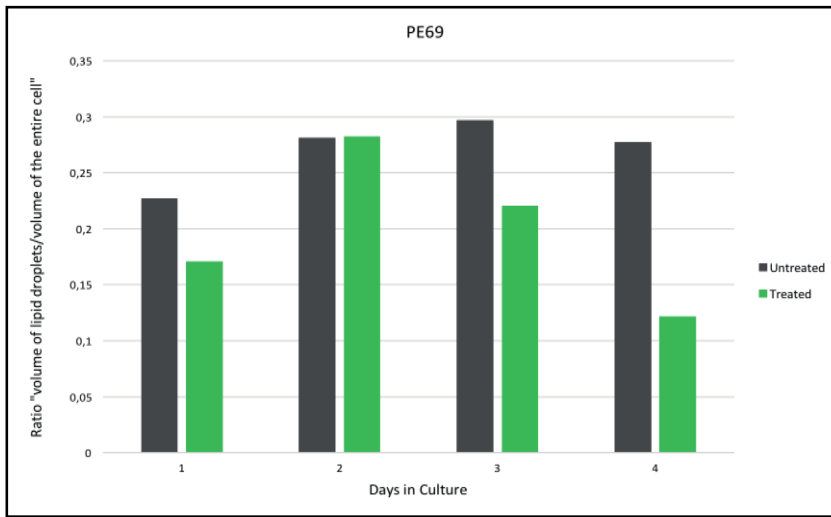


Figure 2.3.21. A single experiment conducted so far indicates that 1% PE69 tends to decrease the size of LDs in a yeast cell on most days of cell collection. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 1% PE69 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence

microscopy and LDs quantification. The ratios "Total volume of LDs per cell/Total volume of this cell" are shown. n = 1.

The aging-delaying plant extract PE72 extends longevity of chronologically aging yeast. Indeed, I found that 0.1% PE72 statistically significantly increases both the mean and maximum CLSs of WT cells of *S. cerevisiae* grown in YNB medium containing 2% glucose (**Figure 2.3.22**).

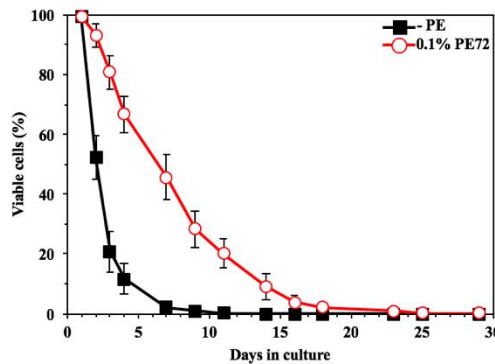
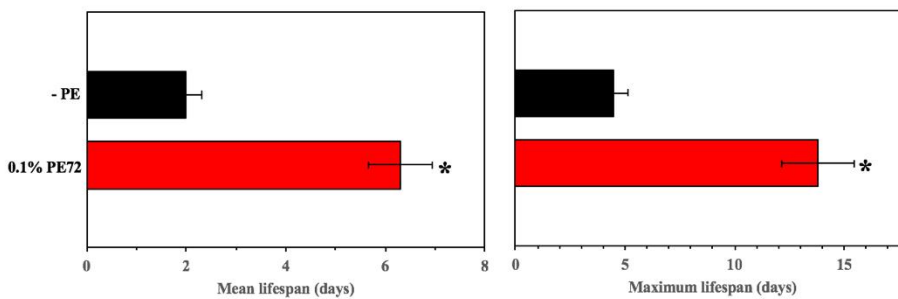


Figure 2.3.22. 0.1% PE72 extends longevity of chronologically aging yeast. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.1% PE72 or in its absence. Survival curves, and the mean and maximum CLSs of these cells are shown. Data are presented as means \pm SEM (n = 3; *p < 0.05).



I found that 0.1% PE72 tends to increase the percentage of cells with low number (i.e. 1 to 3) and to decrease the percentage of cells with high number (i.e. > 10) of LDs on most days of cell collection (**Figure 2.3.23**). However, none of these effects of PE72 was statistically significant. Thus, 0.1% PE72 does not change the number of LDs in a yeast cell.

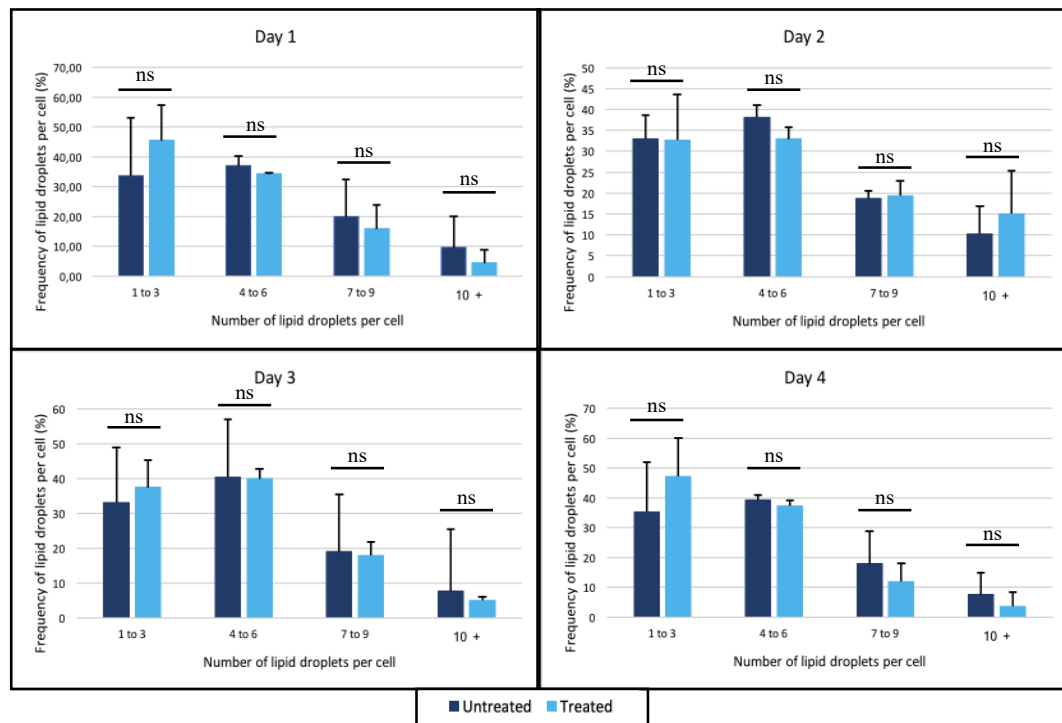


Figure 2.3.23. 0.1% PE72 does not change the number of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.1% PE72 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The percentages of cells with certain number of LDs/cell are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

I also found that 0.1% PE72 tends to increase the ratio "Total volume of LDs per cell/Total volume of this cell" on most days of culturing (**Figure 2.3.24**). However, none of these effects of PE72 was statistically significant. I concluded that 0.1% PE72 does not change the size of LDs in a yeast cell.

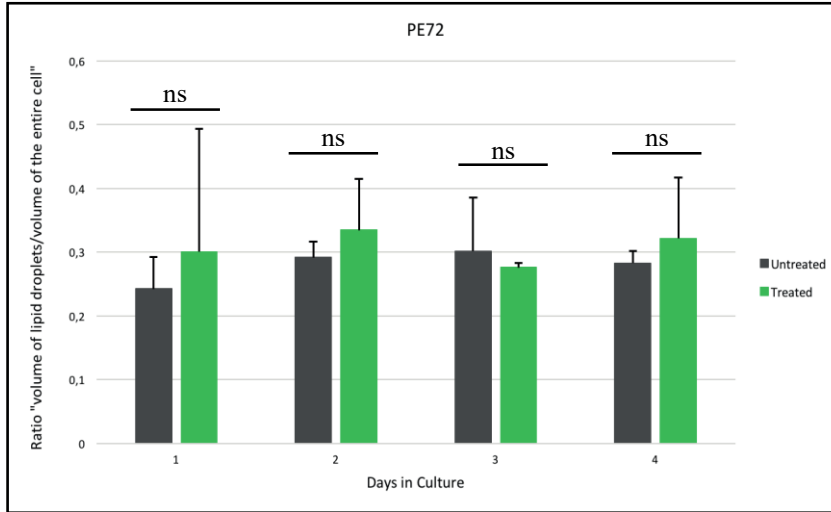


Figure 2.3.24. 0.1% PE72 does not change the size of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.1% PE72 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The ratios "Total volume of LDs per cell/Total volume of this cell" are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

The aging-delaying plant extract PE75 extends longevity of chronologically aging yeast. Indeed, I found that 0.3% PE75 statistically significantly increases both the mean and maximum CLSs of WT cells of *S. cerevisiae* grown in YNB medium containing 2% glucose (Figure 2.3.25).

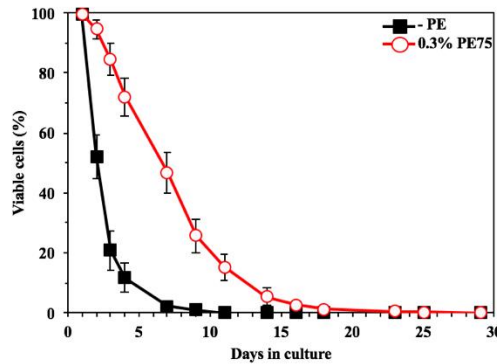
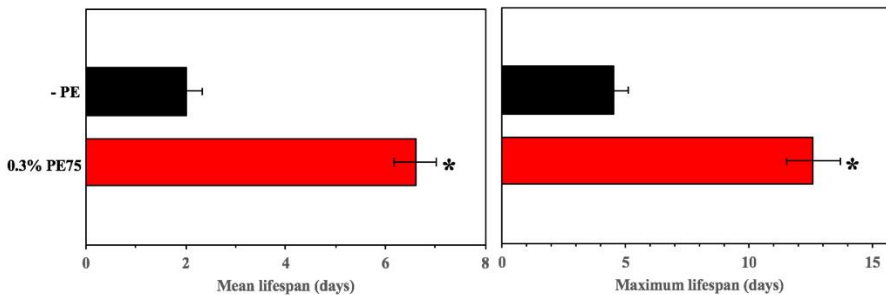


Figure 2.3.25. 0.3% PE75 extends longevity of chronologically aging yeast. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE75 or in its absence. Survival curves, and the mean and maximum CLSs of these cells are shown. Data are presented as means \pm SEM (n = 3; *p < 0.05).



I found that 0.3% PE75 tends to increase the percentage of cells with low number (i.e. 1 to 3 and 4 to 6) and to decrease the percentage of cells with high number (i.e. 7 to 9 and > 10) of LDs on all days of cell collection (**Figure 2.3.26**). However, none of these effects of PE75 was statistically significant. I therefore concluded that 0.3% PE75 does not change the number of LDs in a yeast cell.

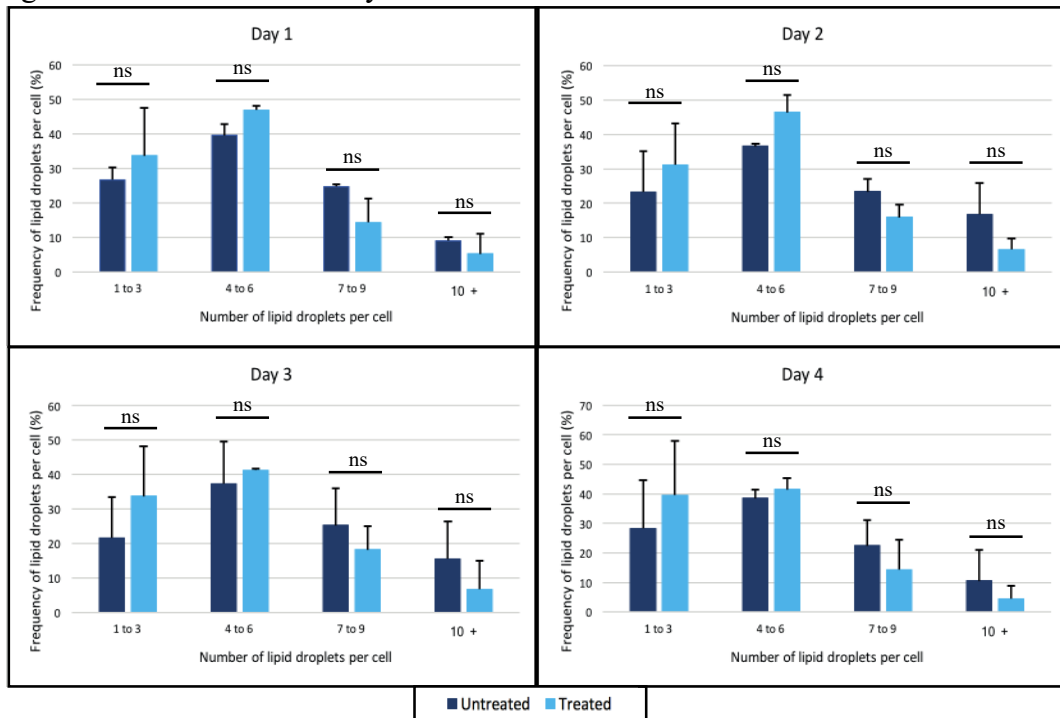


Figure 2.3.26. 0.3% PE75 does not change the number of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE75 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The percentages of cells with certain number of LDs/cell are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

I also found that 0.3% PE75 statistically significantly increases the ratio "Total volume of LDs per cell/Total volume of this cell" only on day 2 of culturing, tends to increase this ratio on day 1 of culturing (but this effect of PE75 is not statistically significant), and has no effect on this ratio on days 3 and 4 of culturing (**Figure 2.3.27**). However, none of these effects of PE72 was statistically significant. I concluded that 0.3%

PE75 increases the size of LDs in a yeast cell on day 2 of culturing but has no effect on the size of LDs in a yeast cell on other days of culturing.

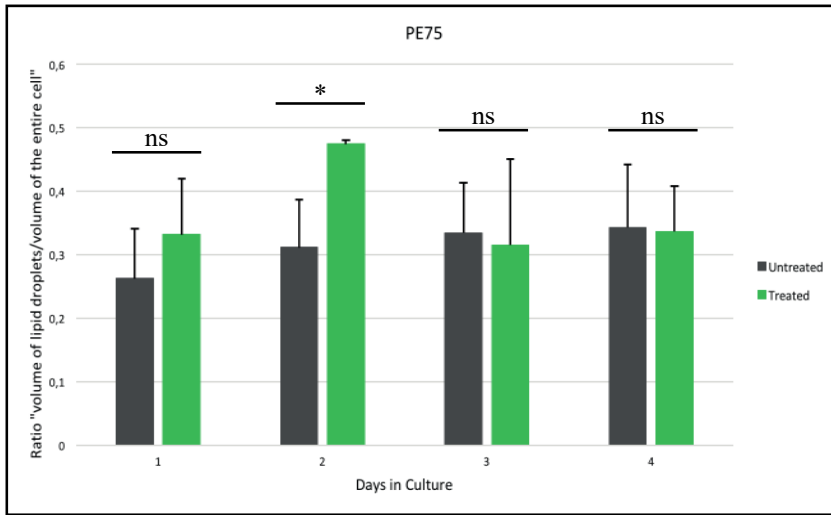


Figure 2.3.27. 0.3% PE75 increases the size of LDs in a yeast cell on day 2 of culturing but has no effect on the size of LDs in a yeast cell on other days of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE75 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503

staining followed by live-cell fluorescence microscopy and LDs quantification. The ratios "Total volume of LDs per cell/Total volume of this cell" are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

The aging-delaying plant extract PE77 extends longevity of chronologically aging yeast. Indeed, I found that 0.5% PE77 statistically significantly increases both the mean and maximum CLSs of WT cells of *S. cerevisiae* grown in YNB medium containing 2% glucose (Figure 2.3.28).

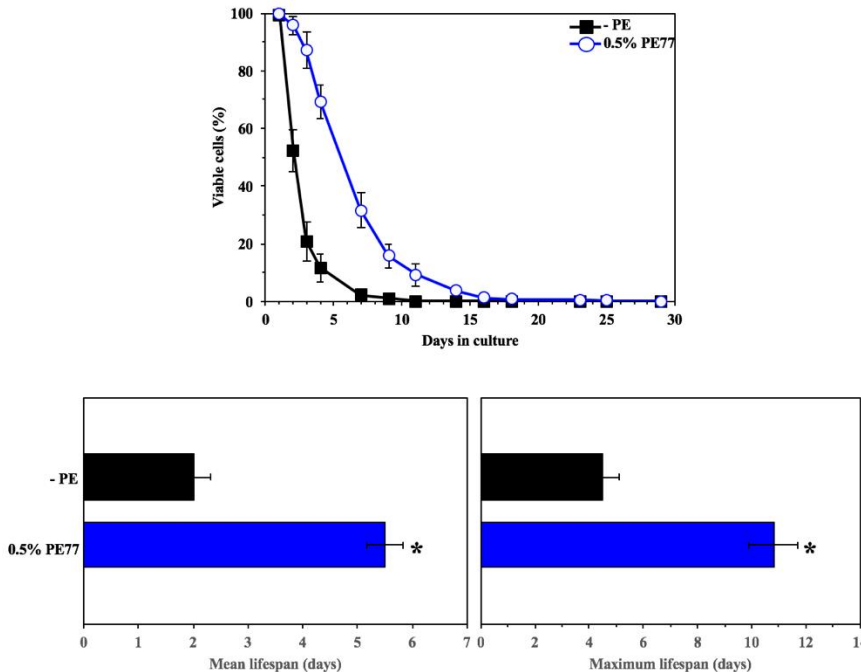


Figure 2.3.28. 0.5% PE77 extends longevity of chronologically aging yeast. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE77 or in its absence. Survival curves, and the mean and maximum CLSs of these cells are shown. Data are presented as means \pm SEM (n = 3; *p < 0.05).

I found that 0.5% PE77 tends to increase the percentage of cells with low number (i.e. 1 to 3 and 4 to 6) and to decrease the percentage of cells with high number (i.e. 7 to 9 and > 10) of LDs on all days of cell collection (**Figure 2.3.29**). However, none of these effects of PE77 was statistically significant. I therefore concluded that 0.5% PE77 does not change the number of LDs in a yeast cell.

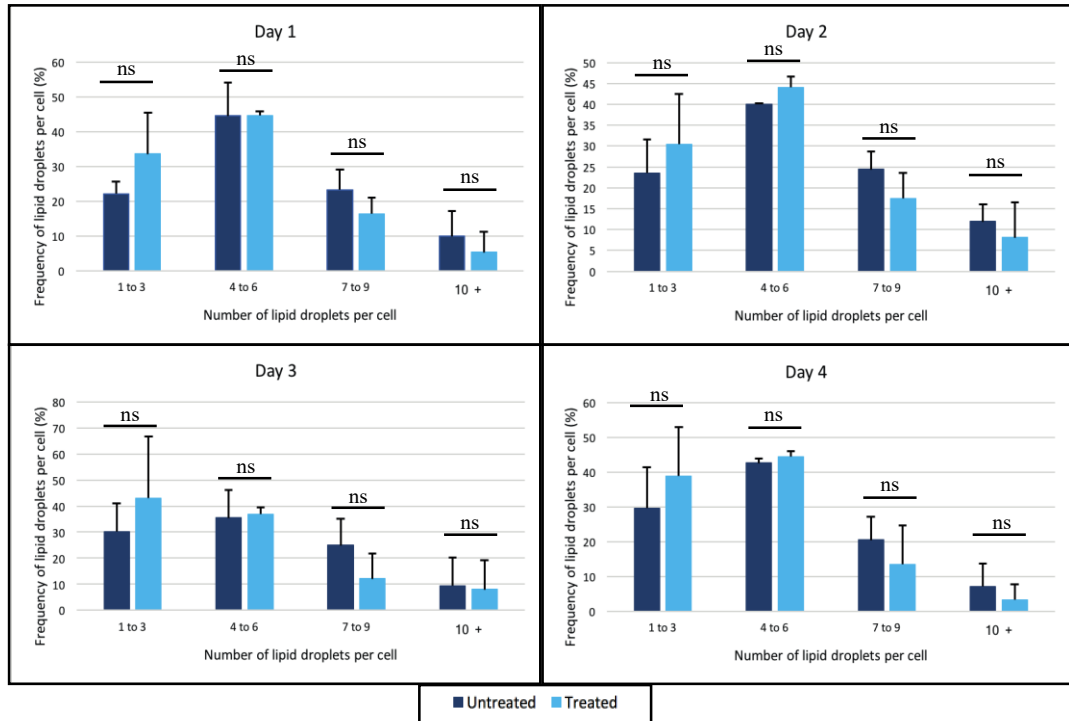


Figure 2.3.29. 0.5% PE77 does not change the number of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE77 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The percentages of cells with certain number of LDs/cell are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

I also found that 0.5% PE77 statistically significantly increases the ratio "Total volume of LDs per cell/Total volume of this cell" only on day 1 of culturing, and tends to increase this ratio on days 2, 3 and 4 of culturing (but this effect of PE77 is not statistically significant) (**Figure 2.3.30**). Thus, 0.5% PE77 increases the size of LDs in a yeast cell on day 1 of culturing but has no effect on the size of LDs in a yeast cell on other days of culturing.

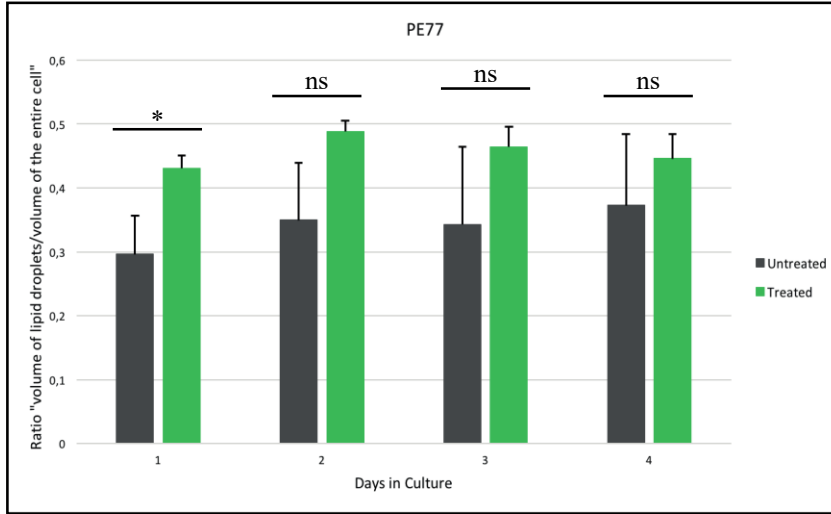


Figure 2.3.30. 0.5% PE77 increases the size of LDs in a yeast cell on day 1 of culturing but has no effect on the size of LDs in a yeast cell on other days of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE77 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-

cell fluorescence microscopy and LDs quantification. The ratios "Total volume of LDs per cell/Total volume of this cell" are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

The aging-delaying plant extract PE78 extends longevity of chronologically aging yeast. Indeed, I found that 0.3% PE78 statistically significantly increases both the mean and maximum CLSs of WT cells of *S. cerevisiae* grown in YNB medium containing 2% glucose (**Figure 2.3.31**).

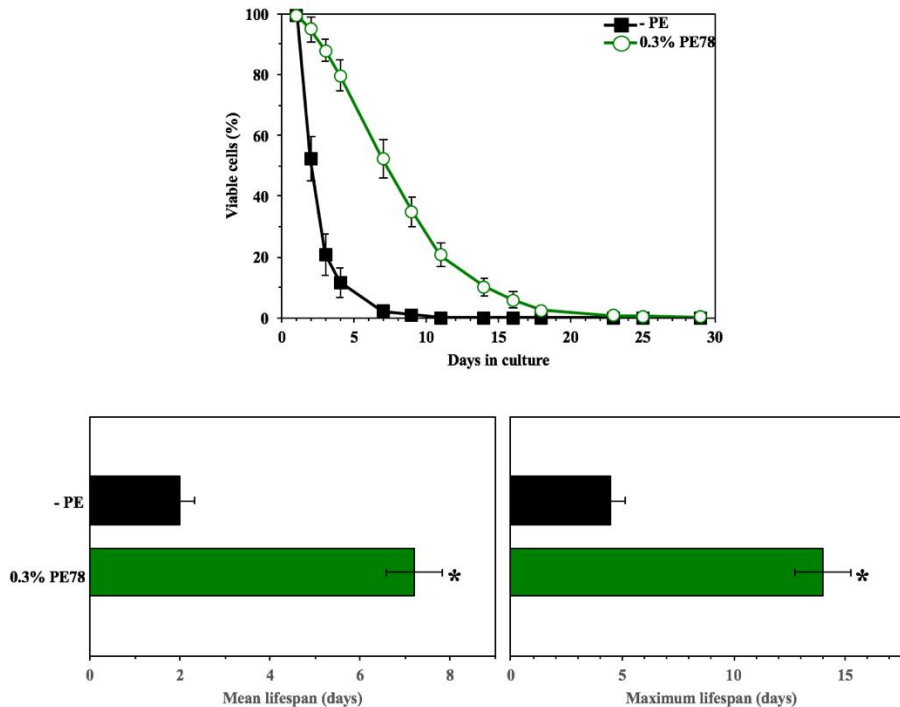


Figure 2.3.31. 0.3% PE78 extends longevity of chronologically aging yeast. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE78 or in its absence. Survival curves, and the mean and maximum CLSs of these cells are shown. Data are presented as means \pm SEM (n = 3; *p < 0.05).

I found that 0.3% PE78 tends to increase the percentage of cells with low number (i.e. 1 to 3 and 4 to 6) and to decrease the percentage of cells with high number (i.e. 7 to 9 and > 10) of LDs on all days of cell collection (**Figure 2.3.32**). However, only a decrease of the percentage of cells with 7 to 9 LDs on day 1 of culturing with 0.3% PE78 was statistically significant. I therefore concluded that 0.3% PE78 decreases the number of LDs in a yeast cell only on day 1 of culturing but does not change the number of LDs in a yeast cell on all other days of culturing.

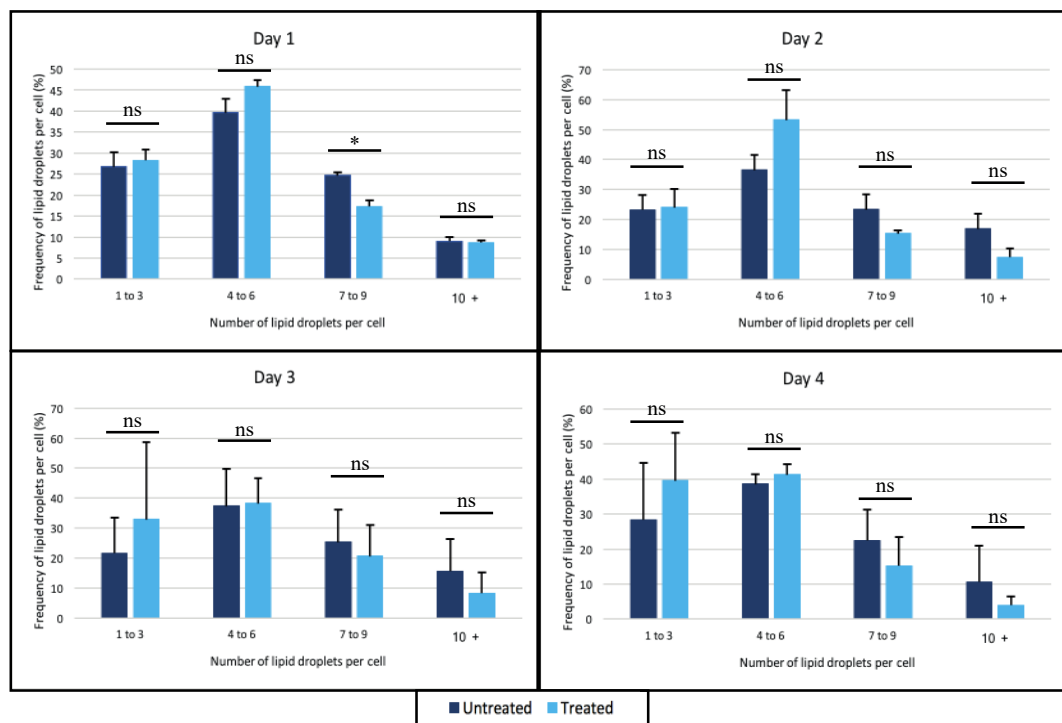


Figure 2.3.32. 0.3% PE78 decreases the number of LDs in a yeast cell only on day 1 of culturing but does not change the number of LDs in a yeast cell on all other days of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE78 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The percentages of cells with certain number of LDs/cell are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

I also found that 0.3% PE78 tends to increase the ratio "Total volume of LDs per cell/Total volume of this cell" on day 1 of culturing, and tends to decrease this ratio on days 2, 3 and 4 of culturing (**Figure 2.3.33**). However, none of these effects of PE78 on

any of the days of culturing was statistically significant. Thus, 0.3% PE78 has no effect on the size of LDs in a yeast cell.

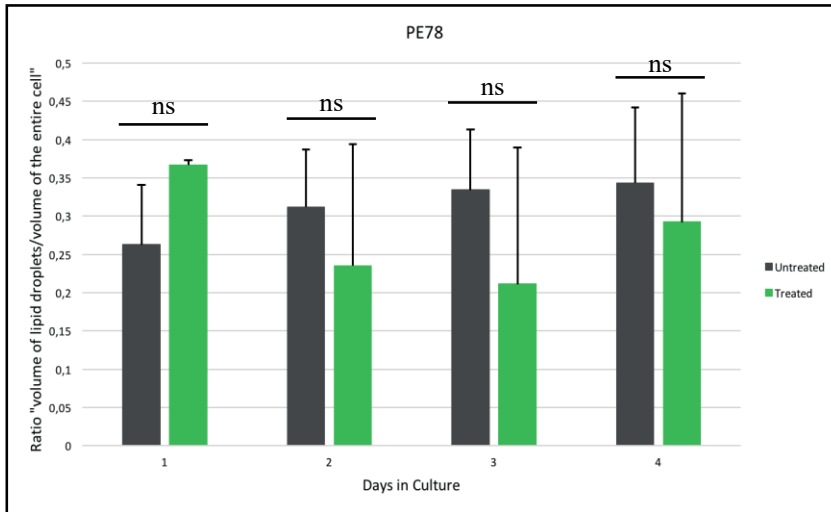


Figure 2.3.33. 0.3% PE78 has no effect on the size of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE78 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The ratios "Total volume of LDs per cell/Total volume of this cell" are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

The aging-delaying plant extract PE79 extends longevity of chronologically aging yeast. Indeed, I found that 0.5% PE79 statistically significantly increases both the mean and maximum CLSs of WT cells of *S. cerevisiae* grown in YNB medium containing 2% glucose (Figure 2.3.34).

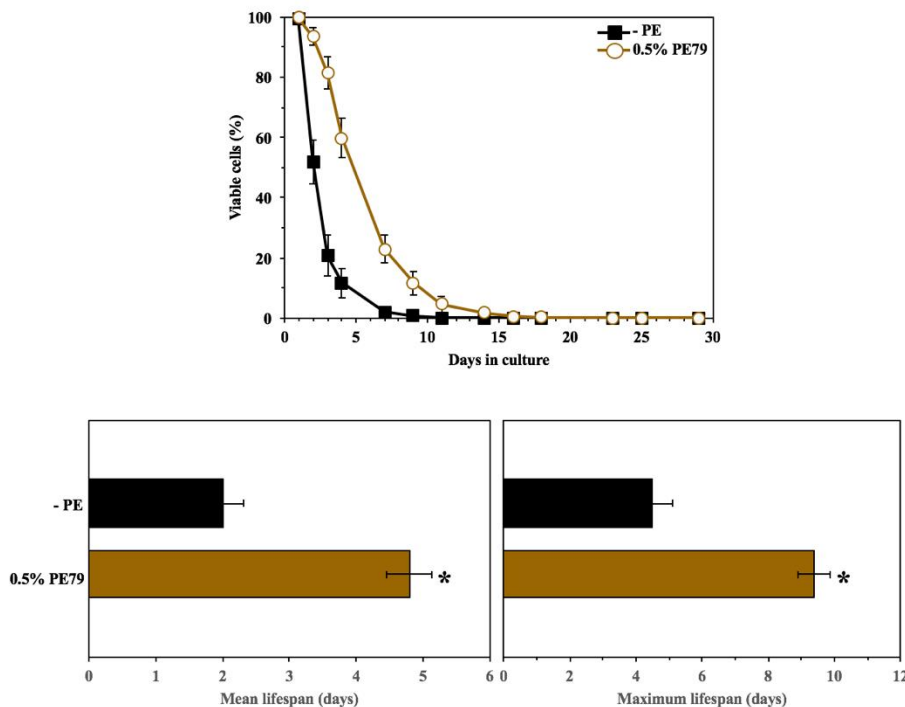


Figure 2.3.34. 0.5% PE79 extends longevity of chronologically aging yeast. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE79 or in its absence. Survival curves, and the mean and maximum CLSs of these cells are shown. Data are presented as means \pm SEM (n = 3; *p < 0.05).

I found that 0.5% PE79 tends to increase the percentage of cells with low number (i.e. 1 to 3 and 4 to 6) and to decrease the percentage of cells with high number (i.e. 7 to 9 and > 10) of LDs on all days of cell collection (**Figure 2.3.35**). However, none of these effects of PE78 was statistically significant. I therefore concluded that 0.5% PE79 does not change the number of LDs in a yeast cell.

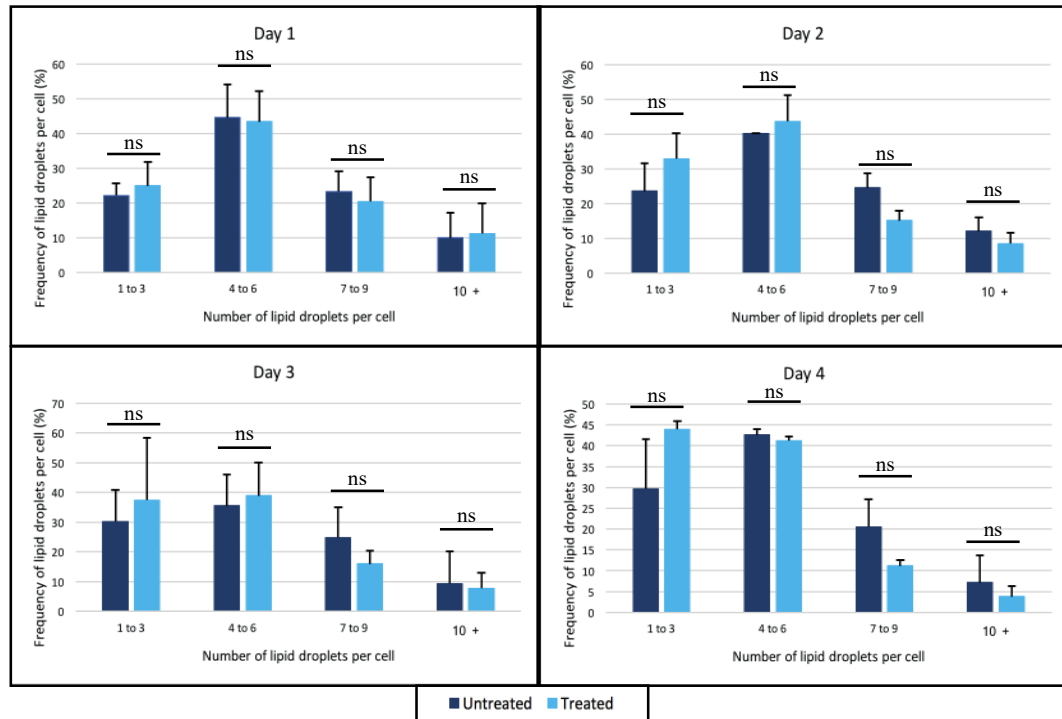


Figure 2.3.35. 0.5% PE79 does not change the number of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE79 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The percentages of cells with certain number of LDs/cell are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

I also found that 0.5% PE79 tends to increase the ratio "Total volume of LDs per cell/Total volume of this cell" on days 1 and 4 of culturing and tends to decrease this ratio on days 2 and 3 of culturing (**Figure 2.3.36**). However, none of these effects of PE79 on any of these days of culturing was statistically significant. I therefore concluded that 0.5% PE79 has no effect on the size of LDs in a yeast cell.

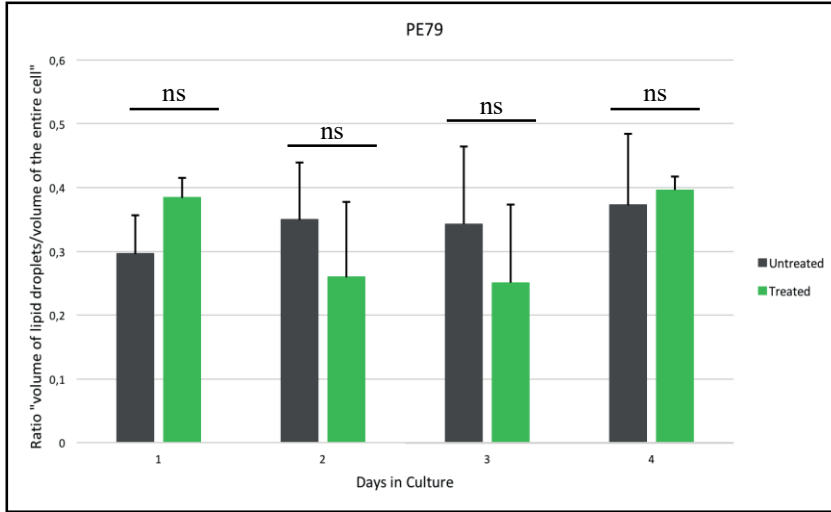


Figure 2.3.36. 0.5% PE79 has no effect on the size of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE79 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The ratios "Total volume of LDs per cell/Total volume of this cell" are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

The aging-delaying plant extract PE81 extends longevity of chronologically aging yeast. Indeed, I found that 0.3% PE81 statistically significantly increases both the mean and maximum CLSs of WT cells of *S. cerevisiae* grown in YNB medium containing 2% glucose (Figure 2.3.37).

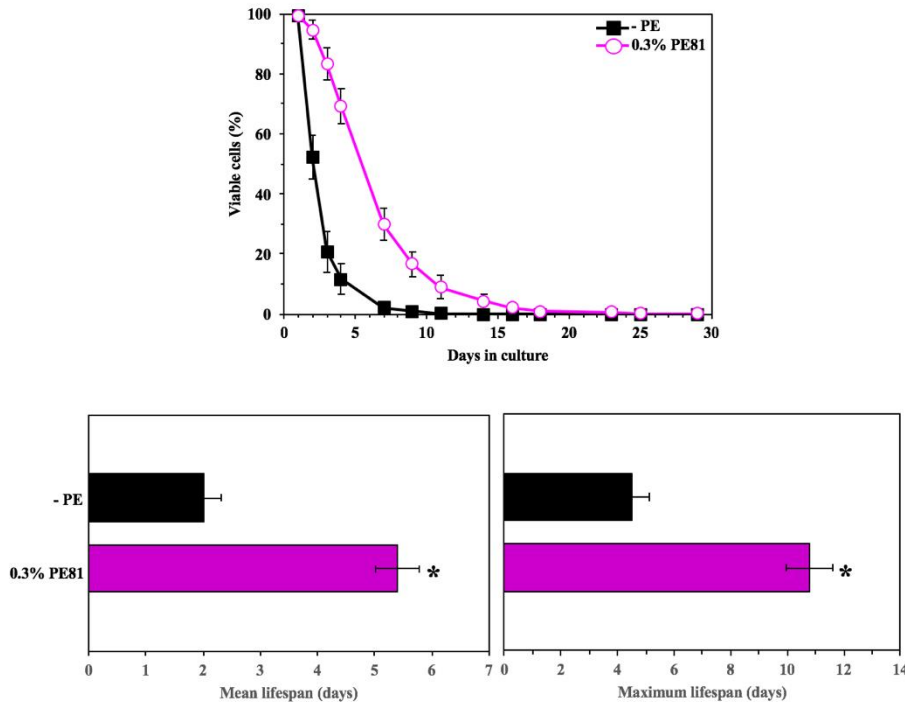


Figure 2.3.37. 0.3% PE81 extends longevity of chronologically aging yeast. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE81 or in its absence. Survival curves, and the mean and maximum CLSs of these cells are shown. Data are presented as means \pm SEM (n = 3; *p < 0.05).

I found that 0.3% PE81 tends to decrease the percentage of cells with low number (i.e. 1 to 3 and 4 to 6) and to increase the percentage of cells with high number (i.e. 7 to 9 and > 10) of LDs on all days of cell collection (**Figure 2.3.38**). However, only a decrease in the percentage of cells with 1 to 3 LDs and an increase in the percentage of cells with 7 to 9 LDs on the day 4 of culturing were statistically significant. Thus, 0.3% PE81 increases the number of LDs in a yeast cell only on day 4 of culturing but does not change the number of LDs in a yeast cell on all other days of culturing.

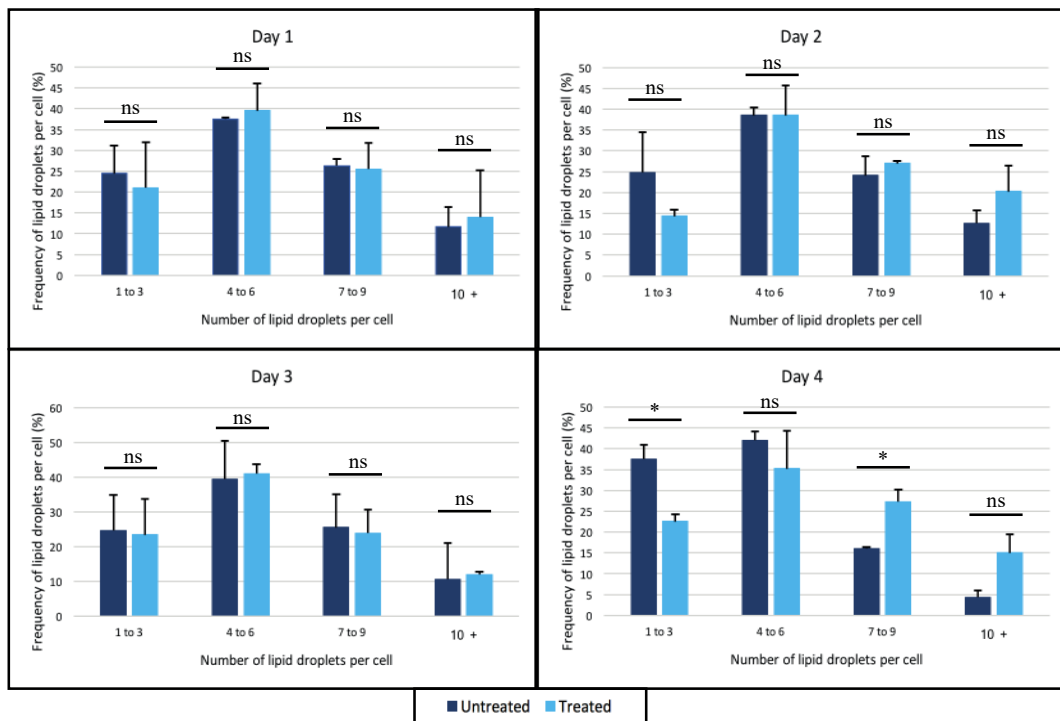


Figure 2.3.38. 0.3% PE81 increases the number of LDs in a yeast cell only on day 4 of culturing but does not change the number of LDs in a yeast cell on all other days of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE81 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The percentages of cells with certain number of LDs/cell are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

I also found that 0.3% PE81 tends to increase the ratio "Total volume of LDs per cell/Total volume of this cell" on all days of culturing (**Figure 2.3.39**). However, none of these effects of PE81 on any of these days of culturing was statistically significant. I therefore concluded that 0.3% PE81 has no effect on the size of LDs in a yeast cell.

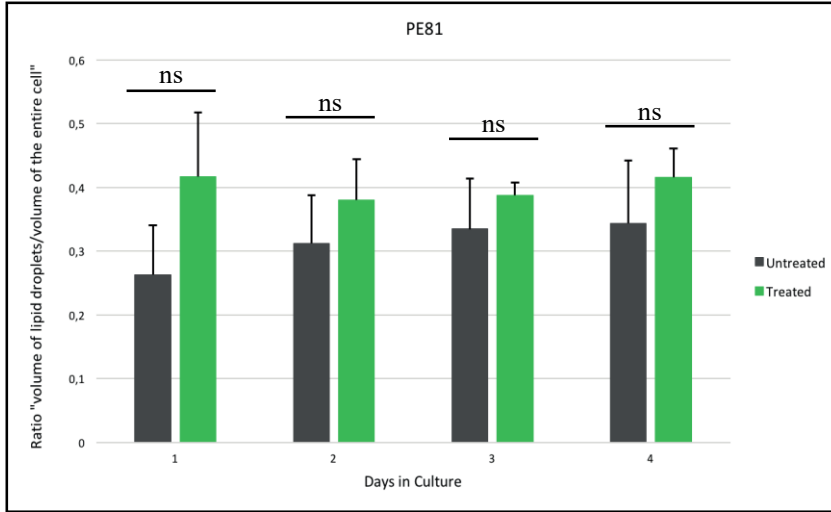


Figure 2.3.39. 0.3% PE81 has no effect on the size of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE81 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The ratios "Total volume of LDs per cell/Total volume of this cell" are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

The aging-delaying plant extract PE83 extends longevity of chronologically aging yeast. Indeed, I found that 0.5% PE83 statistically significantly increases both the mean and maximum CLSs of WT cells of *S. cerevisiae* grown in YNB medium containing 2% glucose (Figure 2.3.40).

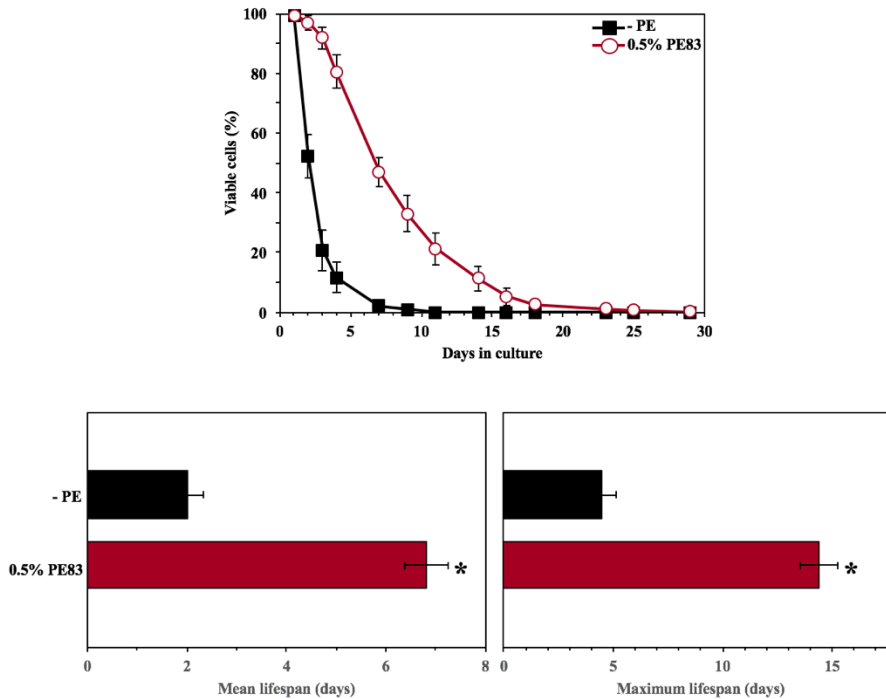


Figure 2.3.40. 0.5% PE83 extends longevity of chronologically aging yeast. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE83 or in its absence. Survival curves, and the mean and maximum CLSs of these cells are shown. Data are presented as means \pm SEM (n = 3; *p < 0.05).

I found that 0.5% PE83 tends to increase the percentage of cells with low number (i.e. 1 to 3) and to decrease the percentage of cells with high number (i.e. 7 to 9) of LDs on all days of cell collection (**Figure 2.3.41**). However, none of these effects of PE83 on any of these days were statistically significant. I therefore concluded that 0.5% PE83 does not change the number of LDs in a yeast cell.

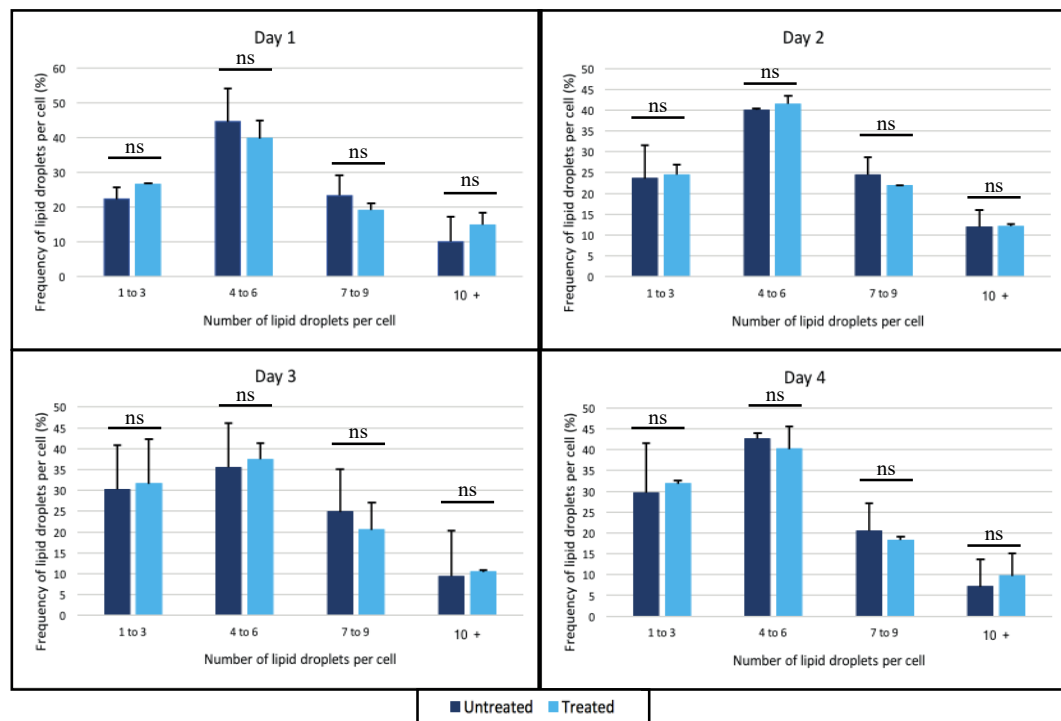


Figure 2.3.41. 0.5% PE83 does not change the number of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE83 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The percentages of cells with certain number of LDs/cell are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

I also found that 0.5% PE83 tends to increase the ratio "Total volume of LDs per cell/Total volume of this cell" on all days of culturing (**Figure 2.3.42**). However, none of these effects of PE83 on any of these days of culturing was statistically significant. I therefore concluded that 0.5% PE83 has no effect on the size of LDs in a yeast cell.

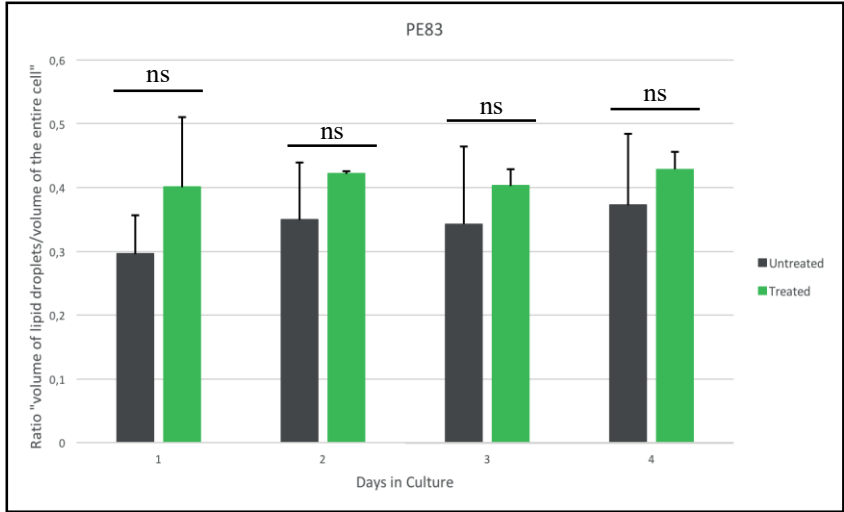


Figure 2.3.42. 0.5% PE83 has no effect on the size of LDs in a yeast cell. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE83 or in its absence. Cells collected on days 1, 2, 3 and 4 of culturing were subjected to BODIPY 493/503 staining followed by live-cell fluorescence microscopy and LDs quantification. The ratios "Total volume of LDs per cell/Total volume of this cell" are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

cell/Total volume of this cell" are shown. Data are presented as means \pm SD (n = 2; ns = not significant).

2.4 Discussion

My live-cell fluorescence microscopy analysis of how each of the 14 aging-delaying plant extracts influences the number and size of LDs revealed the following. PE69 tends to decrease both the number and size of LDs on all days of cell collection; because these effects were observed in a single experiment conducted so far for PE69, the validity of this conclusion will be tested by others in additional experiments with PE69. I found that PE68 decreases the size of LDs only on day 1 and has no effect on the number of LDs on any day of cell collection. Furthermore, PE75 and PE77 increase the size of LDs only on day 2 or day 1 (respectively); none of these two plant extracts alters the number of LDs on any day of cell collection. Moreover, PE78 and PE81 decrease or increase (respectively) the number of LDs on day 1 or day 4 (respectively); none of them influences the size of LDs on any day of cell collection. I also found that PE39, PE42, PE47, PE59, PE64, PE72, PE79 and PE83 do not change either the number of LDs or their size on any day of cell collection.

These findings indicate that PE39, PE42, PE47, PE59, PE64, PE72, PE79 and PE83 delay yeast chronological aging not because they alter the number of LDs or change their size. The number and size of LDs are known to be defined by the relative rates of neutral lipid synthesis in the ER, neutral lipid deposition in LDs, neutral lipid lipolytic degradation in LDs, fusion of LDs and fragmentation of LDs (Kohlwein et al., 2013; Walther and Farese, 2012). Thus, the aging-delaying effects of PE39, PE42, PE47, PE59, PE64, PE72, PE79 and PE83 in yeast are unlikely to be caused by changes in the relative rates of these cellular processes.

My findings that PE68, PE75, PE77, PE78 and PE81 elicits changes in the number or size of LDs only on one or two (out of four) days of cell collection suggest that these changes may (or may not) play essential roles in the ability of some (or all) of these plant extracts to delay yeast chronological aging. To address this, further experiments are required. These future experiments may test how single-gene-deletion mutations eliminating proteins involved in the above processes influence the following: 1) the efficiencies with which PE68, PE75, PE77, PE78 and PE81 extend longevity of chronologically aging yeast; and 2) the size and number of LDs at different stages of the aging process in the presence of a PE (i.e. on different days of culturing yeast in medium supplemented with this PE).

In a single experiment conducted so far, I demonstrated that PE69 tends to decrease both the number and size of LDs on all days of cell collection. Additional experiments with PE69 are required to test the validity of this preliminary conclusion. These experiments will be conducted in the future by other students in the Titorenko laboratory.

3 Effects of Aging-Delaying Plant Extracts on the Cellular Concentrations of Different Classes of Lipids

3.1 Abstract

The synthesis, storage and degradation of neutral lipids are integrated into metabolic pathways for other lipid classes. Therefore, the objective of studies described in this chapter of my thesis was to use a combination of liquid chromatography and mass spectrometry to examine how the aging-delaying plant extracts PE75 and PE77 influence the concentrations of several classes of lipids in yeast cells collected at different stages of chronological aging (i.e. on different days of cell collection). As I found in studies described in the previous chapter, both PE75 and PE77 increase the size of lipid droplets without affecting their number. My findings described in this chapter of the thesis indicate that PE75 and PE77 display different and statistically significant effects on the concentrations of phosphatidylinositol phospholipids, triacylglycerols and free fatty acids. Based on these findings, I infer that there may be two different ways of remodeling lipid metabolism in response to two different aging-delaying plant extracts. It remains to be seen whether any of these two ways of lipid metabolism remodeling may play a causal role in the delay of yeast chronological aging by these plant extracts. It will be also interesting to determine if any of these two ways of lipid metabolism remodeling may influence cellular signaling, vesicular protein trafficking, stress response or other cellular processes.

3.2 Materials and Methods

Yeast Strains, Growth Conditions

The *Saccharomyces cerevisiae* wild-type strain BY4742 (*MAT α* Δ *his3* Δ 1 *leu2* Δ 0 *lys2* Δ 0 *ura3* Δ 0) from Thermo Scientific/Open Biosystems was used. Grown in a synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) containing 2% (w/v) glucose and supplemented with 20 mg/l histidine, 30 mg/l leucine, 30 mg/l lysine and 20 mg/l uracil, with and without plant extracts. A 20% stock solution of each plant extract in ethanol was made on the day of cell inoculation into the growth medium at a final concentration of 0.1%, 0.3%, 0.5% and 1.0%. Cells were cultured at 30°C with rotational shaking at 200 rpm in Erlenmeyer flasks at a “flask volume/medium volume” ratio of 5:1.

Cell Collection for Liquid-Chromatography Mass Spectrometry

A total volume of yeast cell culture was calculated to obtain 5.0×10^7 cells/mL for each sample. These samples were then subjected to centrifugation at $3,000 \times g$ for 5 min at room temperature. The pellet was washed with nanopure H₂O and then washed with 155 mM ammonium bicarbonate (pH 8.0). The final fractions were placed in 1.5-mL microfuge tubes and centrifuged at $20,000 \times g$ for 1 min at room temperature. The pellet was then stored at -80°C until lipid extraction.

Chloroform:Methanol Lipid Extraction

Samples stored at -80°C were thawed on ice and then re-suspended in 400 μL of nanopure water. The samples were transferred to a 15-mL glass centrifuge tube containing approximately 400 μL of 425-600 μM acid-washed glass beads (Sigma Aldrich catalog #G8772), and 2 mL of chloroform:methanol at a 17:1 ratio (Fisher Scientific Catalog # 76-09-3 and 67-56-1) was added. The samples were vortexed for 1 h and centrifuged at $3,000 \times g$ for 6 min. The lower organic phase was transferred to another glass centrifuge tube, 2 mL of a chloroform:methanol at a 2:1 ratio was added to the remaining aqueous phase, which was further vortexed for 1 h and centrifuged at $3,000 \times g$ for 6 min. The lower organic phase was combined with the previous organic phase and the solvent was evaporated through nitrogen gas.

Sample Preparation for LC-MS

500 μL of acetonitrile:2-propanol: H_2O (ratio of 65:35:5) (Fisher Scientific Catalog #s A9551, A4611 and W6212, all LC-MS grade) was added to the lipid film of each sample tube. Each sample was vortexed and sonicated for 15 min and vortexed once more before 100 μL of each sample was transferred to glass vials with inserts used for Agilent1100 well-plate.

Liquid Chromatography

These experiments were performed on an Agilent1100 series LC system (Agilent Technologies) equipped with a binary pump, de-gasser and auto-samples. Lipid species were separated on a reverse-phase column CSH C18 (2.1 mm; 75 mm; pore size 130 \AA ; pH

range of 1-11) (Waters, Milford, MA) coupled to a CSH C18 VanGuard (Waters). The column was maintained at 55°C at a flow-rate of 0.3 mL/min. The mobile phases consisted on (A) 60:40 (v/v) acetonitrile:H₂O and (B) 90:10 (v/v) isopropanol:acetonitrile. For the positive mode electrospray ionization (+) both mobile phases A and B were mixed with 10 mM of ammonium formate (0.631 g/L) (Fisher Scientific Catalog # A115-50). For the negative mode electrospray ionization (-), both mobile phases were mixed with 10 mM ammonium acetate (0.771 g/L) (Fisher Scientific Catalog # 631-61-8). The separation of different lipid species by HPLC was achieved under the following HPLC gradient: 0 to 4 min 90% (A); 4 to 10 min 40% (A); 10 to 21 min 32% (A); 21 to 24 min 3% (A); 24 to 33 min 90% (A). A sample volume of 5 µL and 10 µL was used for the injection in ESI (+) and ESI (-) respectively. Samples were kept in the Agilent1100 well-plate.

Mass Spectrometry

Extracted lipids that were initially separated by HPLC were then analyzed with the help of Thermo Orbitrap Velos Mass Spectrometer equipped with electrospray ionization (ESI) source (Thermo Scientific). Parent ions (MS-1) were detected in positive and negative modes using the FT analyzer at a resolution of 60,000. The MS-1 mass range was from 150-2000 Dalton.

Table 3.1. Thermo Orbitrap Velos mass spectrometer's tune file instrument settings.

Ion Source Type	ESI
Capillary Temperature (°C)	300
Source Heater Temperature (°C)	300
Sheath Gas Flow	10
Aux Gas Flow	5

Table 3.2. Instrument method used for this experiment. HCD: High-energy-induced-collision-dissociation. CID: Collision-induced-dissociation.

Instrument Polarity	Positive	Negative
Activation Type	HCD	CID
Minimum Signal Required	5000	5000
Isolation Width	2.0	2.0
Normalized Collision Energy	55.0	35
Default Charge State	2	2
Activation Time	0.1	10

LC-MS Data Processing

Raw files were analyzed by LipidSearch software (V4.1) from Thermo Fisher. This lipid identification software was used to identify different lipid classes with the help of the molecular fragmentation query language based on the parameters described in **Table 3.3**. In addition to the parameters outlined in **Table 3.3**, an extra manual filter was applied to eliminate any fatty acids containing an odd number of carbons and containing more than one double bond (polyunsaturated fatty acids).

Table 3.3. The parameters for lipid search software (V4.1) used in this experiment. HCD: High-energy-induced-collision-dissociation. CID: Collision-induced-dissociation.

Identification	
Database	Orbitrap
Peak detection	Recall isotope (ON)
Search option	Product search Orbitrap
Search type	Product
Experiment type	LC-MS
Precursor tol	10 ppm
Product tol	HCD (positive mode) 20 ppm
	CID (negative mode) 0.5 Da
Quantitation	
Execute quantitation	ON
Mz tol	-5.0; +5.0
Tol type	ppm
Filter	
Top rank filter	ON

Main node filter	Main isomer peak	
m-score threshold	5.0	
c-score threshold	2.0	
FFA priority	ON	
ID quality filter	A	Lipid class & FA are completely identified
	B	Lipid class & some FA are identified
	C	Lipid class or FA are identified
	D	Lipid identified by other fragment ions (H ₂ O and others)
Lipid Class		
HCD (positive)	PC, SO, TG	
CID (negative)	CER, CL, FA, PE, PG, PI, PS	
Ions		
HCD (positive)	+H, +NH ₄ , +Na	
CID (negative)	-H, -2H, +HCOO	

Statistical Analysis

Statistical analysis was performed using Microsoft Excel's (2017) Analysis ToolPack. All data are presented as mean \pm SD. The *p* values for comparing the means of two groups (using an unpaired *t* test) were calculated with the help of GraphPad Prism statistics software.

3.3 Results

Previous unpublished studies have revealed that PE75 exhibits the highest efficiency of yeast chronological aging if used at the final concentration of 0.3% (w/v). I found that 0.3% PE75 statistically significantly increases the concentration of the phospholipid phosphatidylcholine in yeast cells collected on days 1, 3 and 4 of culturing (**Figure 3.3.1**). I also found that 0.3% PE75 statistically significantly decreases the concentration of phosphatidylcholine in yeast cells collected on day 2 of culturing (**Figure 3.3.1**).

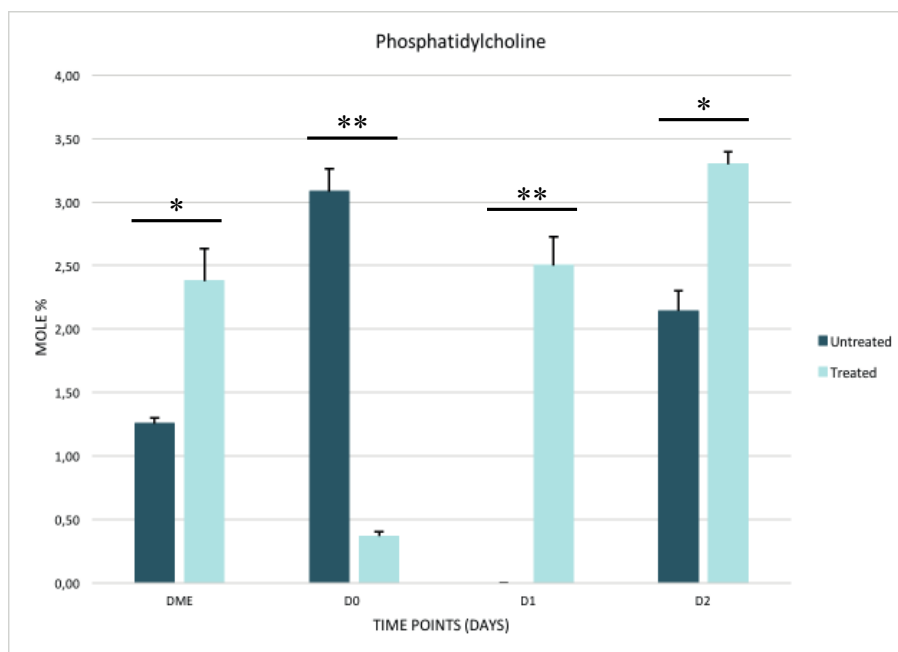


Figure 3.3.1. 0.3% PE75 increases the concentration of phosphatidylcholine in yeast cells collected on days 1, 3 and 4 of culturing, and decreases the concentration of phosphatidylcholine in yeast cells collected on day 2 of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE75 or in its absence. Cell aliquots were

recovered on days 1, 2, 3 and 4 of culturing. Following extraction of lipids from cells recovered on different days of culturing, various lipid classes were identified and quantitated by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of phosphatidylcholine was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD (n = 2; *p < 0.05; **p < 0.01).

I found that 0.3% PE75 statistically significantly increases the concentration of the neutral lipid triacylglycerol in yeast cells collected on day 1 of culturing (**Figure 3.3.2**). In

contrast, 0.3% PE75 has no significant effect on the concentration of triacylglycerol in yeast cells collected on days 2, 3 and 4 of culturing (**Figure 3.3.2**).

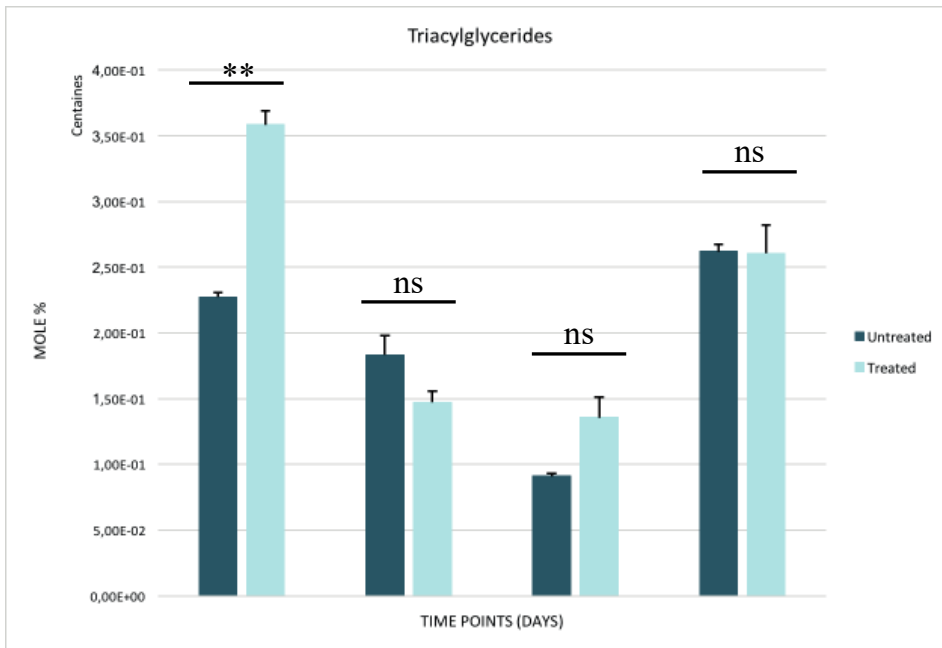


Figure 3.3.2. 0.3% PE75 increases the concentration of the neutral lipid triacylglycerol in yeast cells collected on day 1 of culturing but has no significant effect on the concentration of triacylglycerol in yeast cells collected on days 2, 3 and 4 of culturing. WT cells were

grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE75 or in its absence. Cell aliquots were recovered on days 1, 2, 3 and 4 of culturing. Following extraction of lipids from cells recovered on different days of culturing, various lipid classes were identified and quantitated by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of triacylglycerol was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD (n = 2; **p < 0.01; ns, not significant).

I found that 0.3% PE75 has the following effects on the concentration of cardiolipin, a signature lipid of the mitochondrial inner membrane: 1) it statistically significantly decreases the concentration of cardiolipin in yeast cells collected on days 1 and 2 of culturing; 2) it has no significant effect on the concentration of cardiolipin in yeast cells collected on day 3 of culturing; and 3) it statistically significantly increases the concentration of cardiolipin in yeast cells collected on day 4 of culturing (**Figure 3.3.3**).

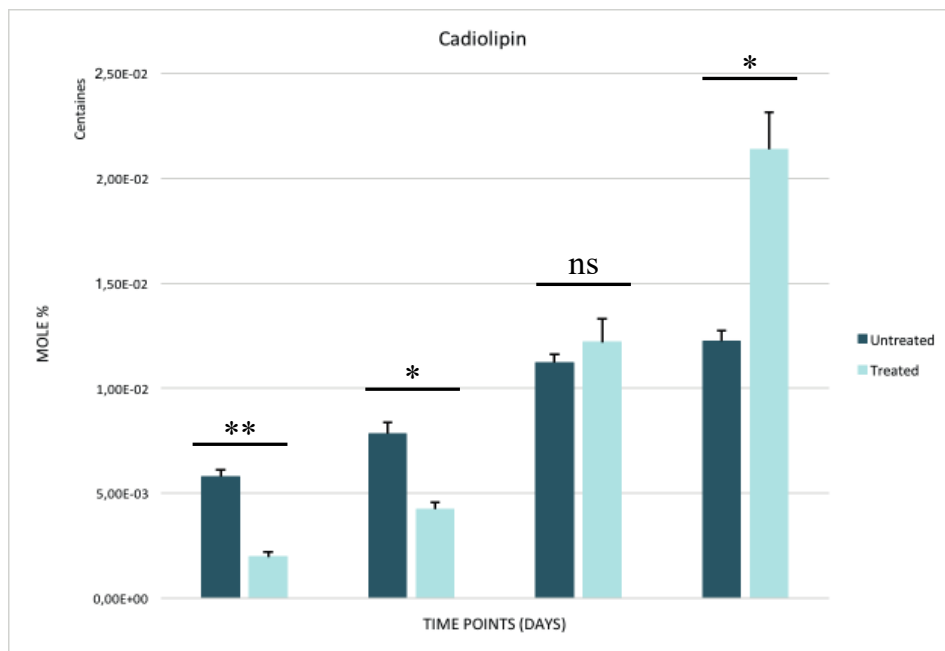


Figure 3.3.3. 0.3% PE75 decreases the concentration of cardiolipin in yeast cells collected on days 1 and 2 of culturing, has no significant effect on the concentration of cardiolipin in yeast cells collected on day 3 of culturing, and increases the concentration of cardiolipin in yeast cells

collected on day 4 of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE75 or in its absence. Cell aliquots were recovered on days 1, 2, 3 and 4 of culturing. Following extraction of lipids from cells recovered on different days of culturing, various lipid classes were identified and quantitated by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of cardiolipin was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD (n = 2; *p < 0.05; **p < 0.01; ns, not significant).

I found that 0.3% PE75 has the following effects on the concentration of the phospholipid phosphatidylethanolamine: 1) it statistically significantly decreases the concentration of phosphatidylethanolamine in yeast cells collected on days 1 and 3 of culturing; 2) it statistically significantly increases the concentration of phosphatidylethanolamine in yeast cells collected on day 3 of culturing; and 3) it has no significant effect on the concentration of phosphatidylethanolamine in yeast cells collected on day 4 of culturing (**Figure 3.3.4**).

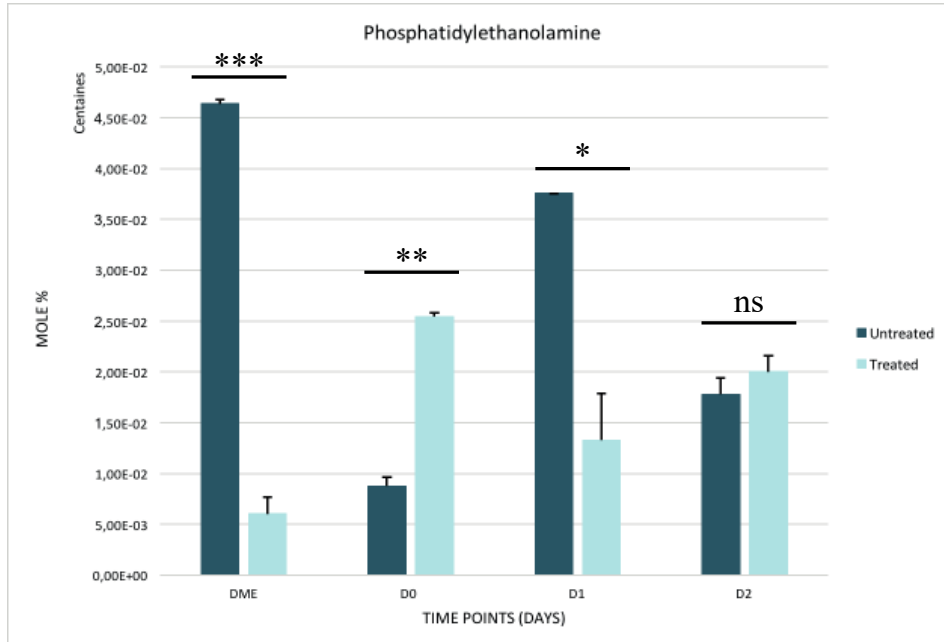


Figure 3.3.4. 0.3% PE75 decreases the concentration of phosphatidylethanolamine in yeast cells collected on days 1 and 3 of culturing, increases the concentration of phosphatidylethanolamine in yeast cells collected on day 3 of culturing and has no significant effect

on the concentration of phosphatidylethanolamine in yeast cells collected on day 4 of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE75 or in its absence. Cell aliquots were recovered on days 1, 2, 3 and 4 of culturing. Following extraction of lipids from cells recovered on different days of culturing, various lipid classes were identified and quantitated by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of phosphatidylethanolamine was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD (n = 2; *p < 0.05; **p < 0.01; ns, not significant).

I found that 0.3% PE75 statistically significantly increases the concentration of the phospholipid phosphatidylinositol in yeast cells collected on each of the 4 days of culturing (Figure 3.3.5).

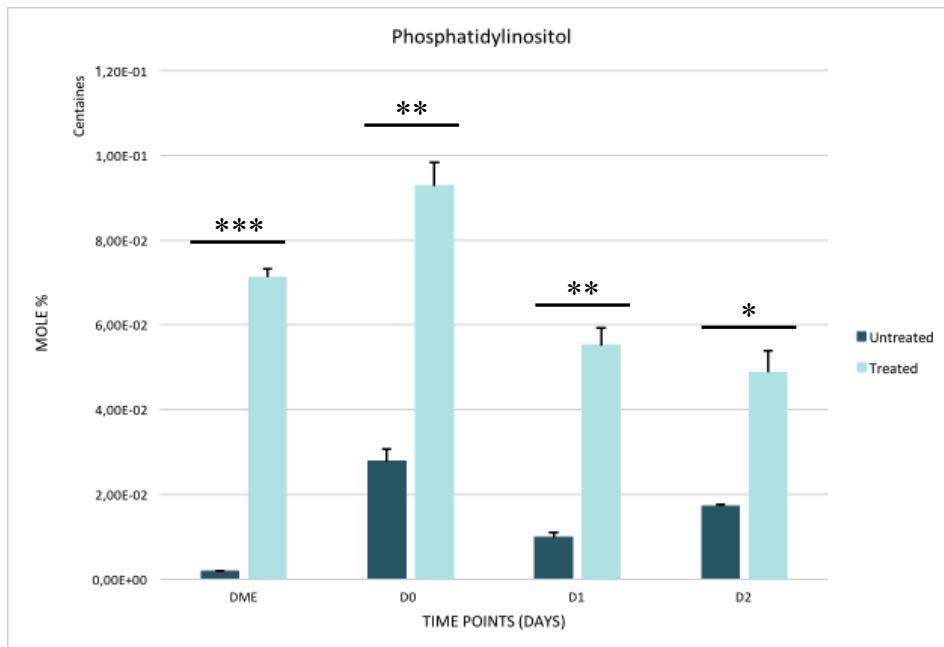


Figure 3.3.5. 0.3% PE75 increases the concentration of the phospholipid phosphatidylinositol in yeast cells collected on each of the 4 days of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE75 or in its absence. Cell aliquots were recovered on

days 1, 2, 3 and 4 of culturing. Following extraction of lipids from cells recovered on different days of culturing, various lipid classes were identified and quantitated by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of phosphatidylinositol was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD (n = 2; *p < 0.05; **p < 0.01; ns, not significant).

I found that 0.3% PE75 has the following effects on the concentration of sphingosine: 1) it statistically significantly increases the concentration of sphingosine in yeast cells collected on day 1 of culturing; 2) it statistically significantly decreases the concentration of sphingosine in yeast cells collected on day 3 of culturing; and 3) it has no significant effect on the concentration of sphingosine in yeast cells collected on days 2 and 4 of culturing (**Figure 3.3.6**).

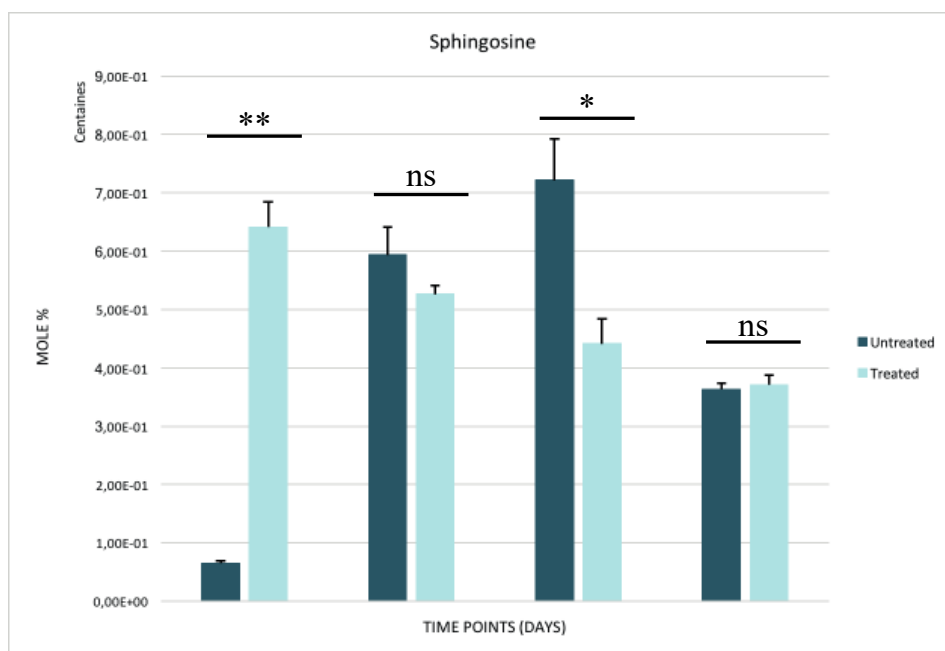


Figure 3.3.6. 0.3% PE75 increases the concentration of sphingosine in yeast cells collected on day 1 of culturing, decreases the concentration of sphingosine in yeast cells collected on day 3 of culturing and has no significant effect on the concentration of sphingosine in yeast cells collected on days

2 and 4 of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE75 or in its absence. Cell aliquots were recovered on days 1, 2, 3 and 4 of culturing. Following extraction of lipids from cells recovered on different days of culturing, various lipid classes were identified and quantitated by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of sphingosine was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD (n = 2; *p < 0.05; **p < 0.01; ns, not significant).

I found that 0.3% PE75 has the following effects on the concentration of ceramide:

- 1) it statistically significantly decreases the concentration of ceramide in yeast cells collected on day 2 of culturing;
- 2) it statistically significantly increases the concentration of ceramide in yeast cells collected on day 4 of culturing;
- and 3) it has no significant effect on the concentration of ceramide in yeast cells collected on days 1 and 3 of culturing (Figure 3.3.7).

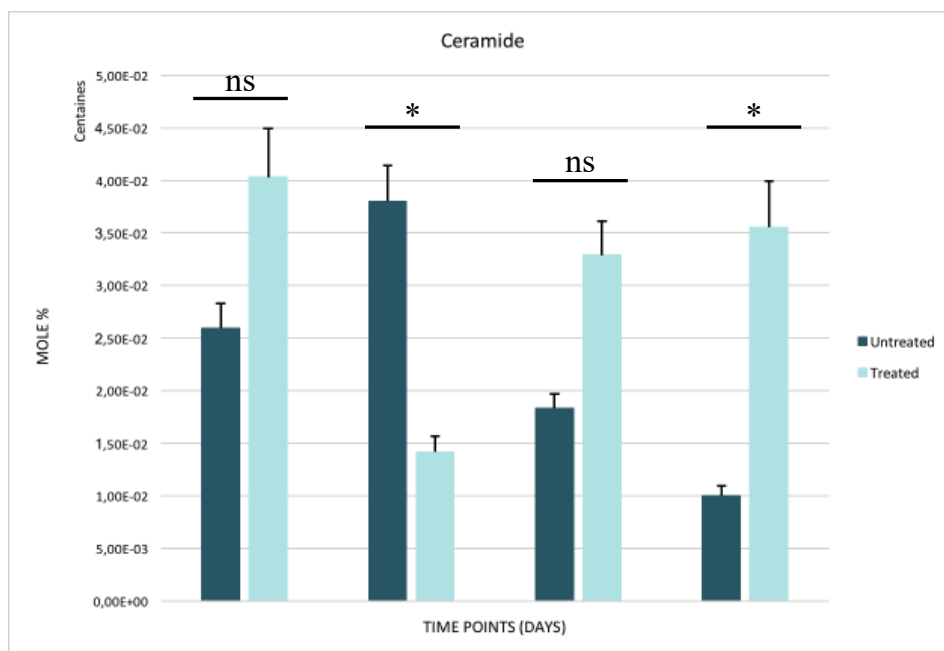


Figure 3.3.7. 0.3% PE75 decreases the concentration of ceramide in yeast cells collected on day 2 of culturing, increases the concentration of ceramide in yeast cells collected on day 4 of culturing and has no significant effect on the concentration of ceramide in yeast cells collected on days 1 and 3 of

culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.3% PE75 or in its absence. Cell aliquots were recovered on days 1, 2, 3 and 4 of culturing. Following extraction of lipids from cells recovered on different days of culturing, various lipid classes were identified and quantitated by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of ceramide was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD (n = 2; *p < 0.05; ns, not significant).

I found that 0.3% PE75 has the following effects on the concentration of free fatty acids: 1) it statistically significantly decreases the concentration of free fatty acids in yeast cells collected on days 1, 3 and 4 of culturing; and 2) it has no significant effect on the concentration of free fatty acids in yeast cells collected on day 2 of culturing (**Figure 3.3.8**).

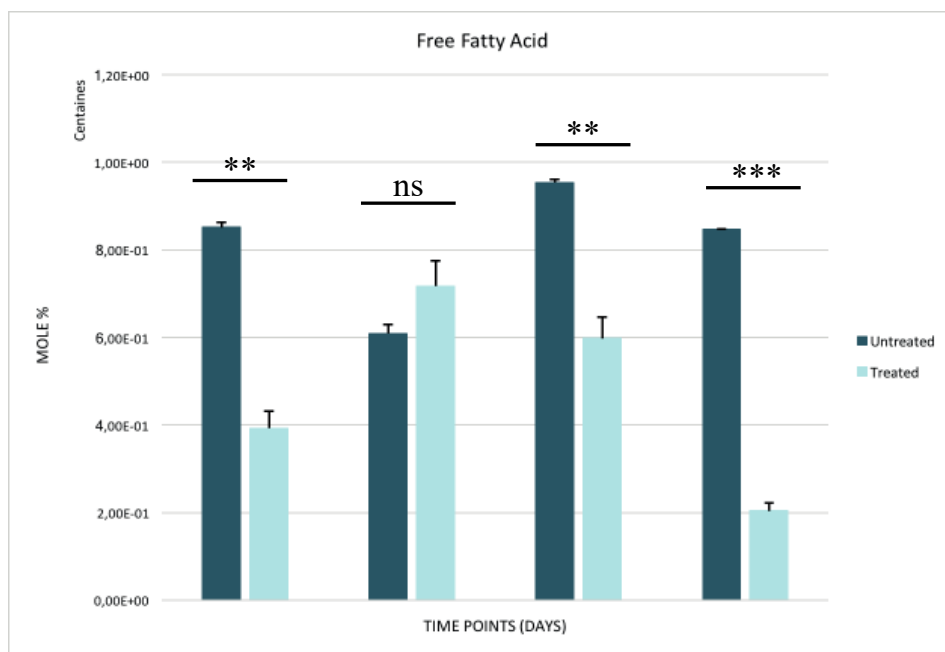


Figure 3.3.8. 0.3% PE75 decreases the concentration of free fatty acids in yeast cells collected on days 1, 3 and 4 of culturing and has no significant effect on the concentration of free fatty acids in yeast cells collected on day 2 of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the

presence of 0.3% PE75 or in its absence. Cell aliquots were recovered on days 1, 2, 3 and 4 of culturing. Following extraction of lipids from cells recovered on different days of culturing, various lipid classes were identified and quantitated by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of free fatty acids was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD (n = 2; *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant).

Previous unpublished studies have revealed that PE77 exhibits the highest efficiency of yeast chronological aging if used at the final concentration of 0.5% (w/v). I found that 0.5% PE77 statistically significantly increases the concentration of the phospholipid phosphatidylcholine in yeast cells collected on day 1 of culturing (**Figure 3.3.9**). I also found that 0.5% PE77 has no significant effect on the concentration of phosphatidylcholine in yeast cells collected on days 2, 3 and 4 of culturing (**Figure 3.3.9**).

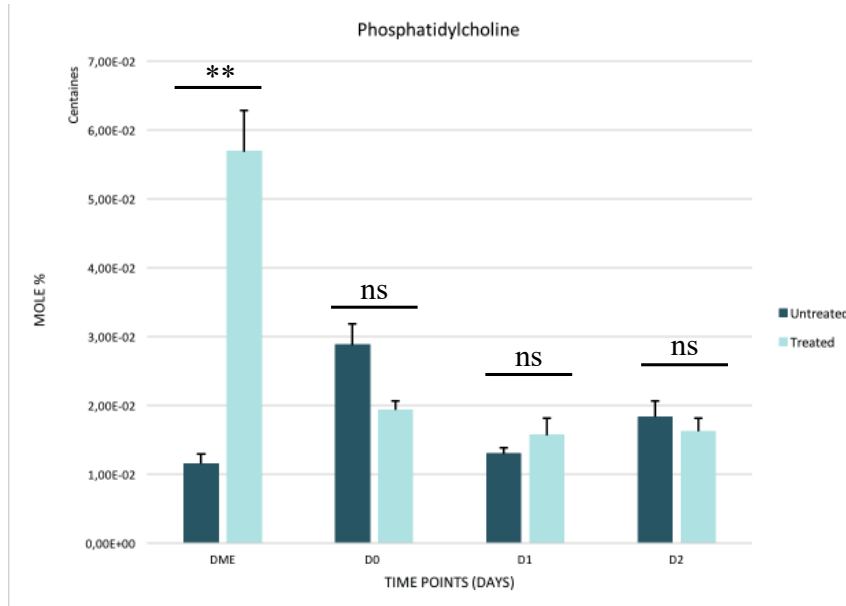


Figure 3.3.9. 0.5% PE77 increases the concentration of the phospholipid phosphatidyl-choline in yeast cells collected on day 1 of culturing and has no significant effect on the concentration of phosphatidyl-choline in yeast cells collected on days 2, 3 and 4 of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE77 or in its absence. Cell aliquots were recovered on days 1, 2, 3 and 4 of culturing.

Following extraction of lipids from cells recovered on different days of culturing, various lipid classes were identified and quantitated by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of phosphatidylcholine was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD ($n = 2$; $**p < 0.01$; ns, not significant).

I found that 0.5% PE77 statistically significantly decreases the concentration of triacylglycerol in yeast cells collected on each of the 4 days of culturing (**Figure 3.3.10**).

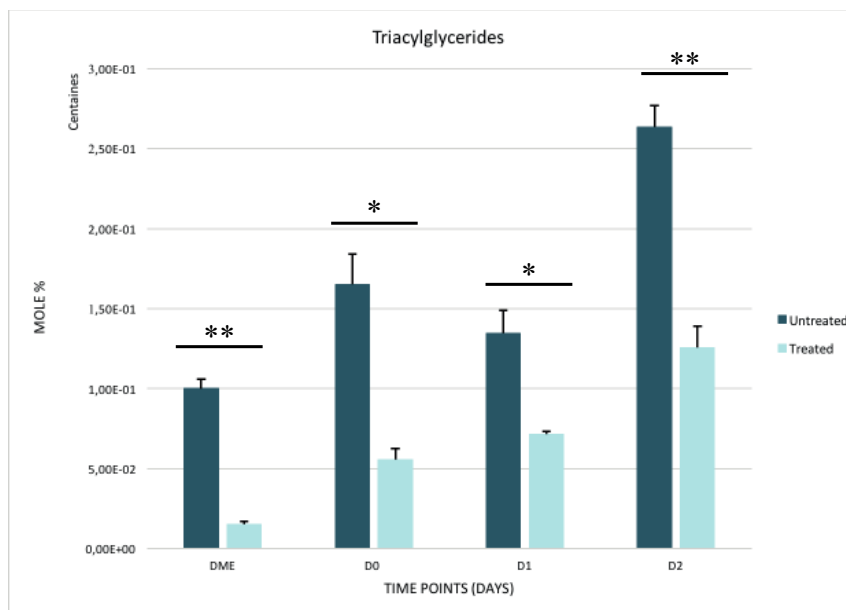


Figure 3.3.10. 0.5% PE77 decreases the concentration of triacylglycerol in yeast cells collected on each of the 4 days of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE77 or in its absence. Cell aliquots were recovered on days 1, 2, 3 and 4 of culturing. Following extraction of lipids from cells recovered on different days of culturing, various lipid classes were identified and quantitated

by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of triacylglycerol was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD ($n = 2$; $**p < 0.01$; ns, not significant).

I found that 0.5% PE77 has the following effects on the concentration of cardiolipin, a signature lipid of the mitochondrial membranes: 1) it statistically significantly decreases the concentration of cardiolipin in yeast cells collected on day 3 of culturing; and 2) it has no significant effect on the concentration of cardiolipin in yeast cells collected on days 1, 2 and 4 of culturing (**Figure 3.3.11**).

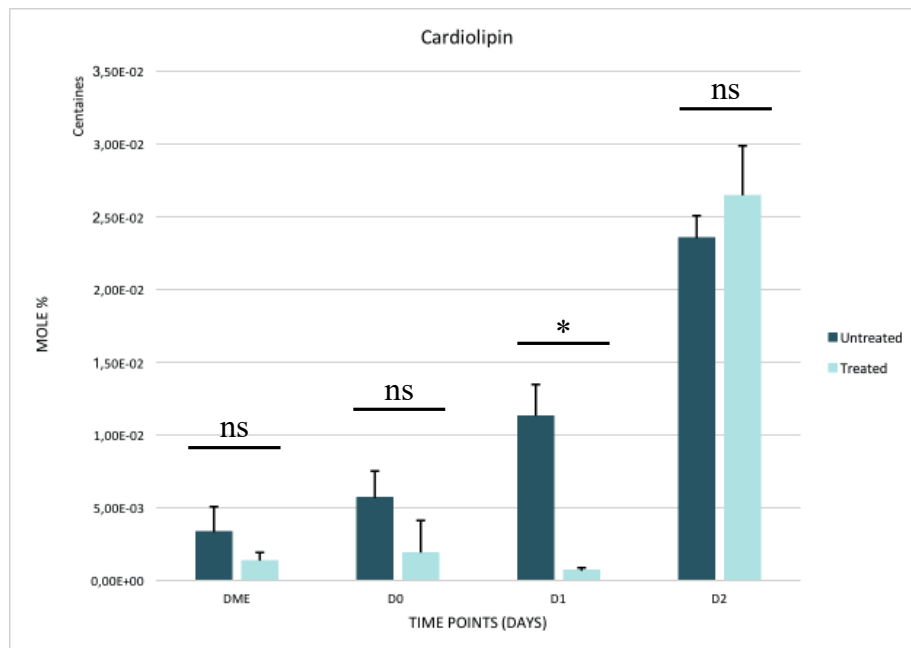


Figure 3.3.11. 0.5% PE77 decreases the concentration of cardiolipin in yeast cells collected on day 3 of culturing and has no significant effect on the concentration of cardiolipin in yeast cells collected on days 1, 2 and 4 of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE77 or in its absence. Cell aliquots were

recovered on days 1, 2, 3 and 4 of culturing. Following extraction of lipids from cells recovered on different days of culturing, various lipid classes were identified and quantitated by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of cardiolipin was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD ($n = 2$; $*p < 0.05$; ns, not significant).

I found that 0.5% PE77 statistically significantly increases the concentration of the phospholipid phosphatidylethanolamine in yeast cells collected on each of the 4 days of culturing (**Figure 3.3.12**).

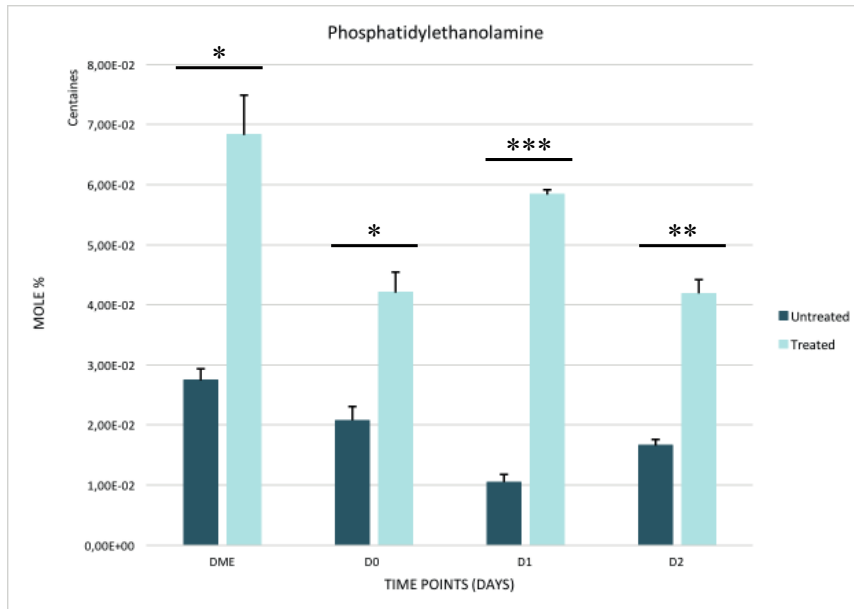


Figure 3.3.12. 0.5% PE77 increases the concentration of the phospholipid phosphatidylethanolamine in yeast cells collected on each of the 4 days of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE77 or in its absence. Cell aliquots were recovered on days 1, 2, 3 and 4 of culturing. Following extraction of lipids from cells recovered on different days of culturing, various

lipid classes were identified and quantitated by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of phosphatidylethanolamine was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD ($n = 2$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

I found that 0.5% PE77 statistically significantly decreases the concentration of the phospholipid phosphatidylinositol in yeast cells collected on each of the 4 days of culturing (Figure 3.3.13).

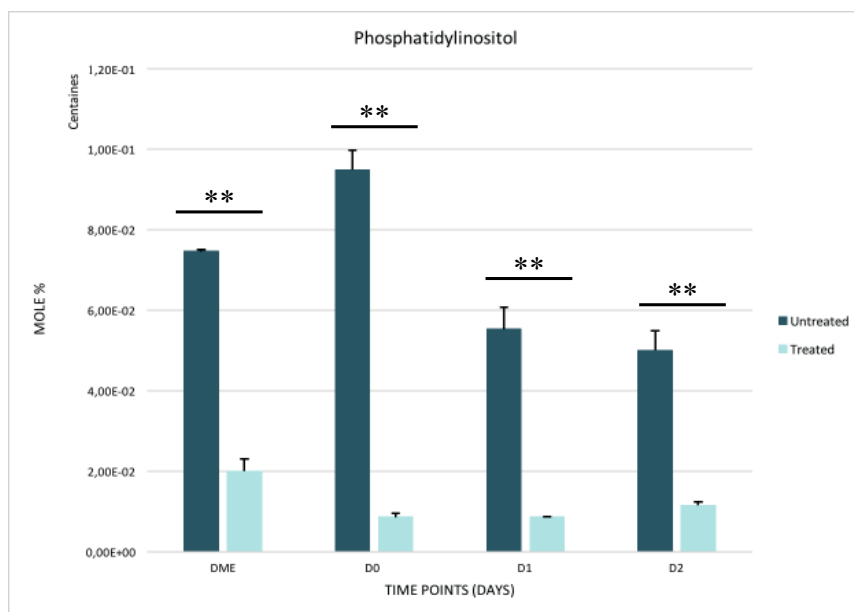


Figure 3.3.13. 0.5% PE77 decreases the concentration of the phospholipid phosphatidylinositol in yeast cells collected on each of the 4 days of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE77 or in its absence. Cell aliquots were recovered on days 1, 2, 3 and 4 of culturing. Following extraction of lipids from cells recovered on different days of culturing, various lipid classes were

identified and quantitated by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of phosphatidylinositol was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD ($n = 2$; ** $p < 0.01$).

I found that 0.5% PE77 has no significant effect on the concentration of sphingosine in yeast cells collected on each of the 4 days of culturing (Figure 3.3.14).

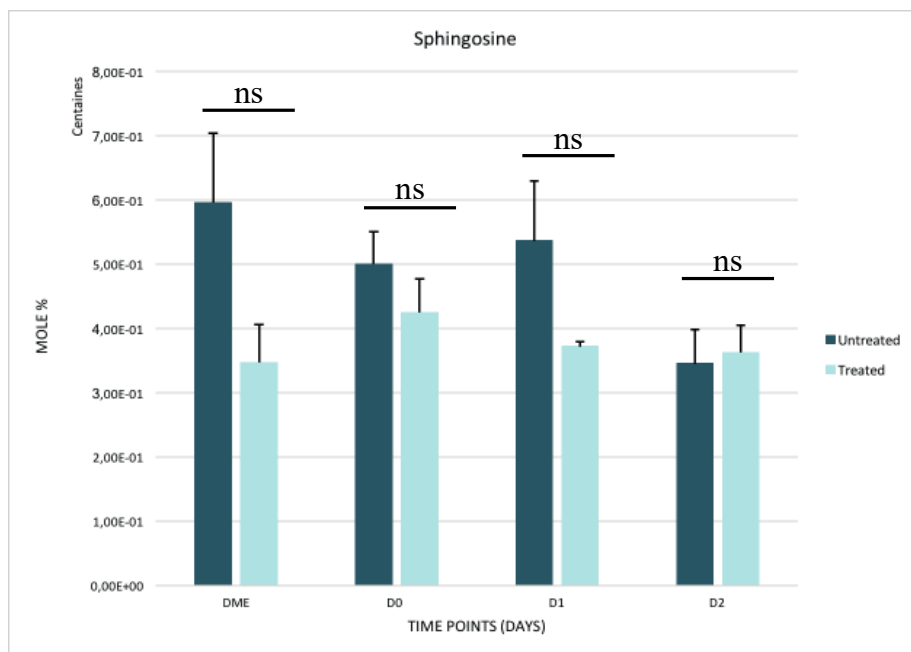


Figure 3.3.14. 0.5% PE77 has no significant effect on the concentration of sphingosine in yeast cells collected on each of the 4 days of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE77 or in its absence. Cell aliquots were recovered on days 1, 2, 3 and 4 of culturing. Following extraction of lipids

from cells recovered on different days of culturing, various lipid classes were identified and quantitated by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of sphingosine was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD (n = 2; ns, not significant).

I found that 0.5% PE77 statistically significantly increases the concentration of ceramide in yeast cells collected on day 1 of culturing and has no significant effect on the concentration of ceramide in yeast cells collected on days 2, 3 and 4 of culturing (Figure 3.3.15).

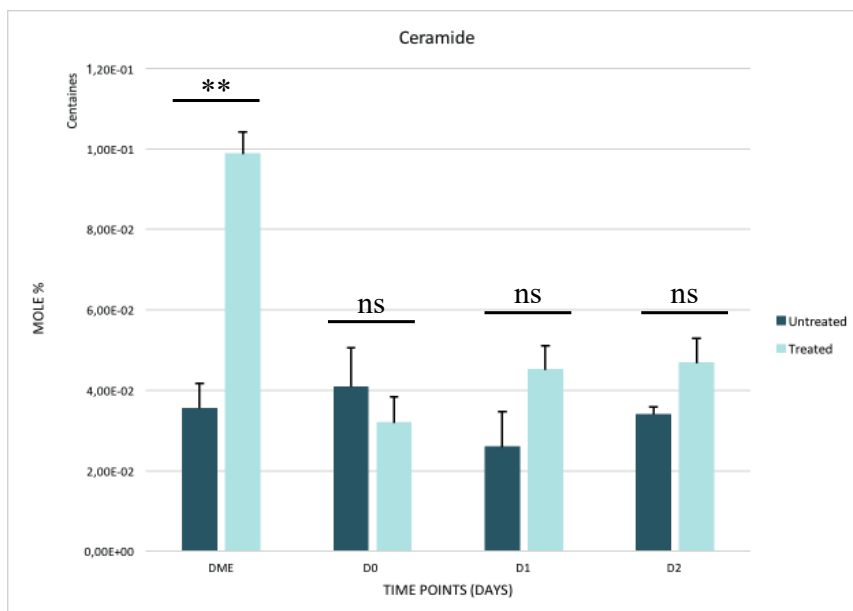


Figure 3.3.15. 0.5% PE77 increases the concentration of ceramide in yeast cells collected on day 1 of culturing and has no significant effect on the concentration of ceramide in yeast cells collected on days 2, 3 and 4 of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE77 or in its absence. Cell aliquots were recovered on days 1, 2, 3 and 4 of culturing. Following extraction of

lipids from cells recovered on different days of culturing, various lipid classes were identified and quantitated by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of ceramide was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD (n = 2; **p < 0.01; ns, not significant).

I found that 0.5% PE77 statistically significantly increases the concentration of free fatty acids in yeast cells collected on each of the 4 days of culturing (**Figure 3.3.16**).

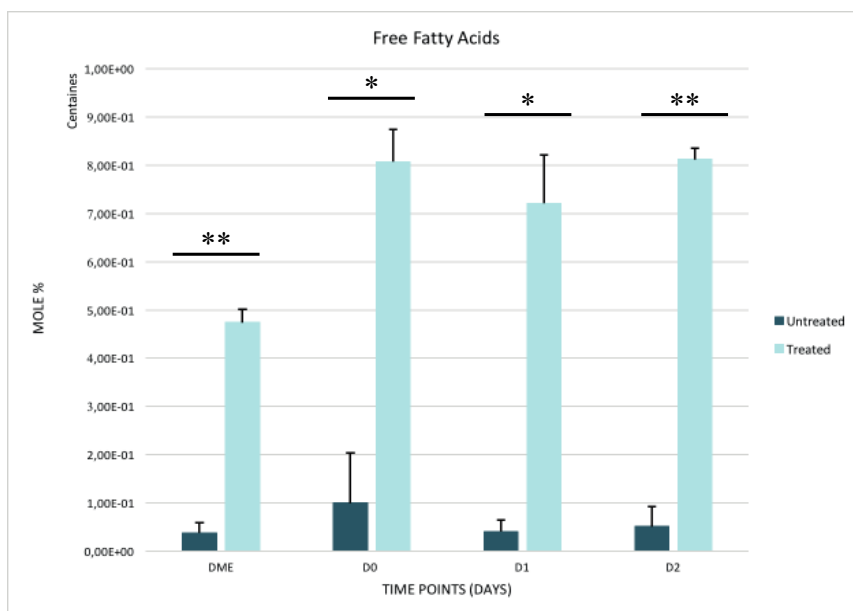


Figure 3.3.16. 0.5% PE77 increases the concentration of free fatty acids in yeast cells collected on each of the 4 days of culturing. WT cells were grown in YNB medium initially containing 2% glucose, in the presence of 0.5% PE77 or in its absence. Cell aliquots were recovered on days 1, 2, 3 and 4 of culturing. Following extraction of lipids from cells recovered on different days of culturing, various lipid classes were identified and quantitated

by a combination of liquid chromatography and tandem mass spectrometry. Based on these data, the relative concentration of free fatty acids was calculated in mol% of all recovered and identified lipid classes. Data are presented as means \pm SD (n = 2; *p < 0.05; **p < 0.01).

3.4 Discussion

My mass spectrometry-based analysis of how the aging-delaying plant extracts PE75 and PE77 influence the concentrations of different classes of lipids in chronologically aging yeast revealed that each of them remodels lipid metabolism in a different way. Because PE77 alters the concentrations of several lipid classes through the entire process of chronological aging (see **Figures 3.3.9 – 3.3.16**), I will first discuss this PE77-dependent way of lipid metabolism remodeling. As schematically depicted in **Figure 3.4.1**, PE77 exhibit the following effects on the concentrations of lipids: 1) it increases the concentrations of phosphatidylethanolamine (PE) and free fatty acids (FFA); and 2) it decreases the concentrations of triacylglycerols (TAG) and phosphatidylinositol (PI). The metabolism of these lipid classes is known to take place in the ER, lipid droplets (LD) and mitochondria, which establish the so-called contact sites for the interorganellar transport of some lipid classes (Kohlwein et al., 2013; Mitrofanova et al., 2018) (**Figures 3.4.1 and 3.4.2**). Based on the observed pattern of changes in lipid concentrations, I suggest the following hypothetical mechanism for the PE77-dependent remodeling of lipid metabolism; 1) the rise in the concentration of PE may be due to an acceleration in PE synthesis within the inner mitochondrial membrane (IMM) and/or an acceleration of PE transport from the IMM to the ER membrane; 2) both the increase in the concentration of FFA and the decrease in the concentration of TAG may be caused by an acceleration of the ER-to-LD transfer of TAG and/or by an acceleration of the lipolytic degradation of TAG into FFA taken place within LD; and 3) the decline in the concentration of PI may be due to a deceleration of the conversion of phosphatidic acid (PA) into PI (**Figure 3.4.1**). The existence of this hypothetical mechanism for the PE77-dependent remodeling of lipid

metabolism will be tested in the future by measuring the rates of metabolic and transport reactions involved in lipid metabolism and transport within the ER, LD and mitochondria (Figure 3.4.2).

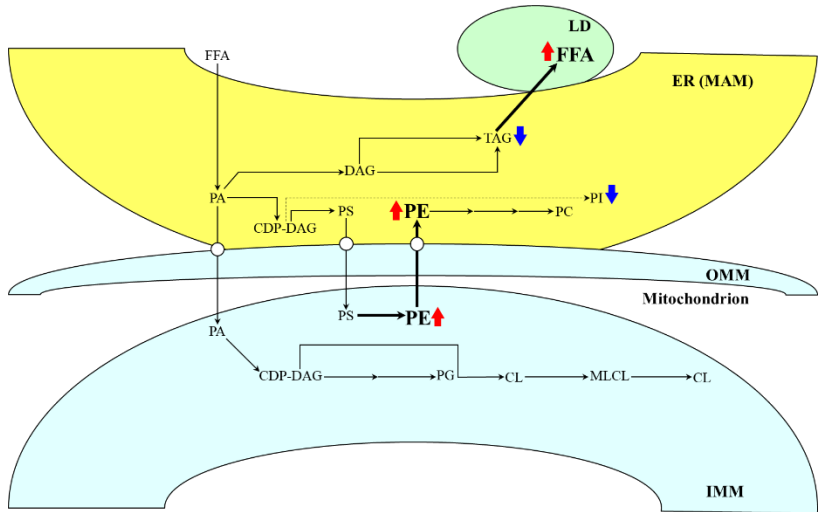


Figure 3.4.1. A hypothetical mechanism for the PE77-dependent remodeling of lipid metabolism in chronologically aging yeast. The thickness of black arrows is proportional to the rates of the corresponding metabolic reactions. Arrows next to the names of lipid classes denote those of them whose concentrations are increased (red arrows) or decreased (blue arrows) in yeast treated with PE77. See text for more details. Abbreviations: ER, the endoplasmic reticulum;

FFA, free fatty acids; IMM, the inner mitochondrial membrane; LD, lipid droplet; OMM, the outer mitochondrial membrane; PE, phosphatidylethanolamine; PI, phosphatidylinositol; TAG, triacylglycerols.

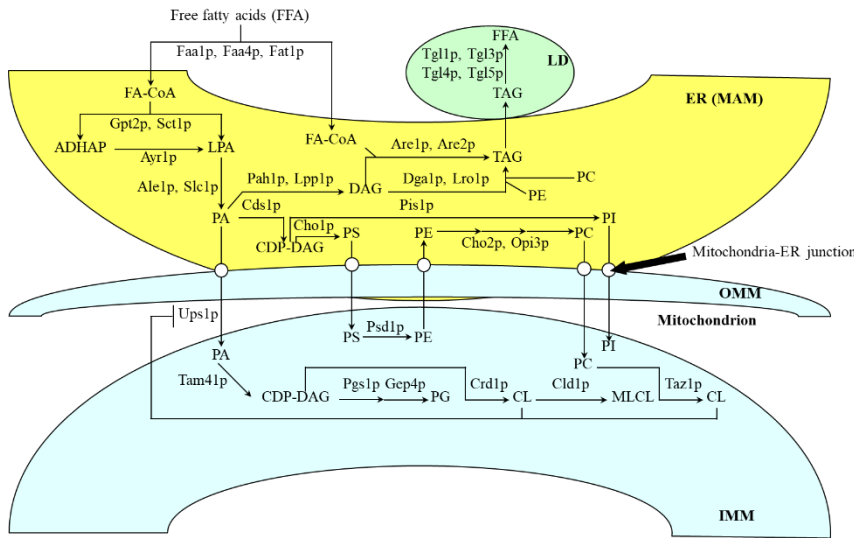


Figure 3.4.2. Lipid metabolism and interorganellar transport in the ER, LD and mitochondria of yeast cells. See text for more details.

It remains to be seen whether this PE77-dependent way of lipid metabolism remodeling may play a causal role in the delay of yeast chronological aging by PE77. This important question will be addressed in the future by examining how single-gene-deletion mutations that eliminate redundant enzymes involved in different reactions of lipid

metabolism and interorganellar transport influence the efficiency with which PE77 delays yeast chronological aging. Although it is known that most of these mutations alter the concentrations of the substrates and products of the reactions affected by the mutations (Kohlwein et al., 2013; Mitrofanova et al., 2018), it will be important to measure the concentrations of different lipid classes in the future experiments addressing a causal relationship between the PE77-dependent lipid metabolism remodeling and the PE77-dependent delay of yeast chronological aging.

All lipid classes whose concentrations are changed in response to exposure of yeast to PE77 play essential roles in such longevity-defining cellular processes as signal transduction, vesicular transport of proteins and stress response (Kohlwein et al., 2013; Mitrofanova et al., 2018). Therefore, as a first step towards addressing the mechanism(s) through which the PE77-dependent remodeling of lipid metabolism may delay yeast chronological aging, it will be important in the future to examine how an exposure of yeast cell to PE77 can influence cellular signaling, vesicular protein trafficking, stress response.

My findings on mass spectrometry-based lipid quantitation indicate that that PE75 remodels metabolism in a different way than PE77. Because PE75 alters the concentrations of several lipid classes differently during different phases of the process of chronological aging (see **Figures 3.3.1 – 3.3.8**), it is a challenge to understand how such diverse alterations may lead to a delay of yeast chronological aging. PE75 exhibit the following effects on the concentrations of lipids: 1) it increases the concentrations of PI during all stages of the process of chronological aging; 2) it decreases the concentration of FFA on 3 out of 4 days of cell collection; 3) it increases the concentration of phosphatidylcholine (PC) on 3 out of 4 days of cell collection (**Figures 3.3.1 – 3.3.8**). The experimental strategy

that has been describe above for PE77 can be also used in the future to test if this PE75-dependent way of lipid metabolism remodeling may play a causal role in the delay of yeast chronological aging by PE75.

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