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Impact of food supplements on yeast chronological aging

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Dissertação de Mestrado

Mestrado em Ciências da Saúde

Trabalho efetuado sob a orientação da
Doutora Maria de Belém de Sousa Sampaio Marques
E da
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Outubro de 2022

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Agradecimentos

A Deus, por permitir a realização deste trabalho;

À minha família, especialmente minha mãe e irmão, por serem minha base em tudo;

Às minhas orientadoras, pelos ensinamentos e paciência;

Aos colegas e funcionários do laboratório, pelo suporte e apoio.

The work presented in this thesis was performed in the Life and Health Sciences Research Institute (ICVS), University of Minho, Financial support was provided by National funds, through the Foundation for Science and Technology (FCT) - project UIDB/50026/2020 and UIDP/50026/2020.

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Estudo sobre os efeitos de suplementos alimentares no envelhecimento cronológico de leveduras

Resumo

Os adoçantes tornaram-se uma escolha eletiva para substituir o açúcar, particularmente para indivíduos obesos ou para aqueles que não podem tolerar açúcar nas suas dietas, tais como os diabéticos. Os adoçantes apresentam o mesmo sabor doce que o açúcar, no entanto os efeitos por eles promovidos não são bem definidos ou completamente compreendidos. Os adoçantes são classificados como naturais e sintéticos/artificiais e são utilizados para fins terapêuticos, clínicos, e como um substituto palatável do açúcar. Embora muitos adoçantes tenham a sua utilização aprovada por agências internacionais, faltam ainda estudos que os liguem ao envelhecimento, que é um processo biológico complexo e multifatorial determinado pela combinação de fatores genéticos e ambientais. O principal objetivo deste trabalho foi estudar o impacto que a suplementação com adoçantes (sucralose, ciclamato, sacarina, xilitol e stevia) tem no envelhecimento celular. Para atingir os objetivos propostos, o envelhecimento cronológico da levedura *Saccharomyces cerevisiae* foi utilizada como modelo de envelhecimento celular. Os resultados apresentados demonstraram que as células têm uma resposta diferente de acordo com o adoçante usado. Enquanto que o xilitol e a sacarina inibem o crescimento celular, o ciclamato de sódio ou a stevia estimulam o crescimento das células. Adicionalmente, também demonstramos que a suplementação com ciclamato de sódio ou stevia induz uma extensão da longevidade, que foi acompanhada por um aumento da autofagia, sem grandes alterações no perfil do ciclo celular e acumulação de espécies recativas de oxigénio. As análises por HPLC mostraram que as células não são capazes de metabolizar nenhum dos adoçantes, sugerindo que provavelmente estão a sinalizar a ativação de vários mecanismos. Globalmente, estes resultados demonstram a importância destes estudos e abrem novas perspetivas sobre o efeito destes adoçantes no envelhecimento celular.

Palavras-chave: adoçantes; autofagia; envelhecimento; espécies reativas de oxigénio; levedura.

Impact of food supplements on yeast chronological aging

Abstract

Sweeteners have become an elective choice to substitute sugar, particularly for those who may struggle with obesity or cannot tolerate sugar in their diets, such as diabetics. Sweeteners present the same sweet taste as sugar, nevertheless the effects promoted are not well defined or completely understood. Sweeteners are classified into natural and synthetic/artificial and are used for therapeutic and clinical purposes, and as a palatable substitute for sugar. Although many sweeteners have their use approved by international health agencies, there are still lacking studies linking them to aging, which is a complex, multifactorial biological process determined by the combination of genetic and environmental factors. The main objective of this work was to study the impact that supplementation with sweeteners (sucralose, cyclamate, saccharin, xylitol, and stevia) have on chronological aging. To achieve the proposed aims, the chronological lifespan (CLS) of the yeast *Saccharomyces cerevisiae* was used as an aging cellular model. The results presented demonstrated that cells have a different response according to the sweetener. While xylitol and saccharin inhibit cell grow, sodium cyclamate or stevia stimulate it. Furthermore, sodium cyclamate or stevia supplementation are associated with extended CLS, which was accompanied with increased autophagy, without major alterations on cell cycle profile and accumulation of reactive oxygen species. Furthermore, HPLC analyses showed that cells are not able to metabolize none of the sweeteners, suggesting that they are probably signaling the activation of several mechanisms. Overall, these results open new perspectives for the effects of sweeteners on cellular aging.

Keywords: sweeteners; autophagy; aging; reactive oxygen species; yeast.

Index

Abbreviations

AD - Alzheimer's Disease

ADI – Acceptable Daily Intake

AKT - Protein kinase B

ALS - Amyotrophic Lateral Sclerosis

AMPK - Adenosine Monophosphate-Activated Protein Kinase

ATG - Autophagy-related Gene

ATP – Adenosine Triphosphate

BSA - Bovine Serum Albumin

CFU - Colony-Forming Units

CLIPS - Chaperones Linked to Protein Synthesis

CLS - Chronological Life-Span

CR - Calorie Restriction

CTT – Catalase

DDR - DNA Damage Response

DHE - Dihydroethidium

DHR - Dihydrorhodamine

DMSO - Dimethyl Sulfoxide

DNA – Desoxyribonucleic Acid

DR - Dietary Restriction

EFSA - European Food Safety Authority

EDTA - Ethylenediamine Tetraacetic Acid

ETC - Electron Transport Chain

FAO - Food and Agriculture Organization

FDA - Food and Drug Administration

GFP - Green Fluorescent Protein

HPLC - High Performance Liquid Chromatography

HSF - Heat Shock Factor

HSP - Heat Shock or Stress Proteins

IGF – Insulin like Growth Factor

JECFA - Joint Experts Committee on Food Additives

MAPK - Mitogen-Activated Protein Kinase

NADH – Nicotinamide Adenine Dinucleotide

OXPHOS - Oxidative Phosphorylation

PD - Parkinson's Disease

PFK – Phosphofructokinase

PI3K/ PIKKs - Phosphatidylinositol-3-Kinase

PN - Proteostasis Network

Ras/cAMP/PKA - Cyclic Adenosine Monophosphate dependent of Protein Kinase A

RI - Refractive Index

RLS - Replicative Life-Span

RNA – Ribonucleic Acid

ROS - Reactive Oxygen Species

SDS - Sodium Dodecyl Sulfate

SOD - Superoxide Dismutase

S6K - Ribosomal protein S6 Kinase

TGS – Trichlorogalactosucrose

TOR - Target Of Rapamycin

TORC - Target of Rapamycin Complex

UPS - Ubiquitin-Proteasome System

US – United States

UV - Ultraviolet

WHO - World Health Organization

YNB - Yeast Nitrogen Base

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Sugars Substitutes (Sweeteners)

Sugar substitutes, also referred to as sweeteners, are an alternative for those looking to replace glucose in their diet, since these sweeteners provide sweetness without supplying calories or glycemic effects. Due to the increased prevalence of obesity, diabetes *mellitus* and other metabolic-related diseases, the use of substitute sugars became an interesting option in the production of food, drinks, oral hygiene products, as well as pharmaceutical products [1, 2].

Sweeteners can be divided into natural and synthetic or artificial sweeteners. Furthermore, they are classified as nutritive or non-nutritive, both enhancing the flavor and texture of foods. Nutritive sweeteners contain carbohydrates and provide calories (energy). Non-nutritive sweeteners are very low calorie or zero calorie alternatives that provide minimal or no carbohydrates or energy [3, 4]. Their application could relay in a therapeutic strategy, in which they help in weight loss by replacing sugar, protection against dental caries, and help in the regulation of blood glucose levels, or in a non-therapeutic strategy, as a substitute for palatability [3].

The use of substitute sugars must be approved by international agencies such as Codex Alimentarius (Food Code), FDA (Food and Drug Administration) and EFSA (European Food Safety Authority). Despite these rigorous safety reviews, there is still a high level of debate about their use in the food supply, requiring risk assessments to identify potential health problems associated with the consumption of foods containing these sugar substitutes [2, 3]. Currently, the FDA has approved the consumption of eight sweeteners from natural and artificial sources, including steviol glycosides extracted from *Stevia rebaudiana* leaves and extracts obtained from *Siraitia grosvenorii (Swingle)* fruit, also known as Luo Han Guo or monk fruit, as natural sweeteners and saccharin, aspartame, sucralose, neotame, advantame and acesulfame potassium as chemically synthesized sweeteners (artificial sweeteners). European Food Safety Authority has approved 20 sweeteners, among them the same ones approved by the Food and Drug Administration with the addition of artificial cycloclamates and the natural polyol xylitol, among others [5,6]. In this work, five sweeteners or substitute sugars were studied including the natural stevia and xylitol, and the artificial sucralose, saccharin, and sodium cyclamate. This selection was based in the fact that these referred sweeteners are the most common described in the literature associated with diverse applications. In this regard, below we will address what is already known about the sweeteners selected for this study and detail key points of the main target of this work.

1.1.1 Sucralose

Sucralose was discovered in 1976. Structurally, it is a substituted disaccharide, similar to sucrose with 3 chlorine atoms replacing 3 hydroxyl groups. The chemical name of sucralose is 1,6-dichloro-1,6-dideoxy-ß-D-fructofuranosyl-4-chloro-4-deoxy-α-D-galactopyranoside, which has also been described as 4,1′,6'-trichlorogalactosucrose. Sucralose does not have nutritional value, and its average sweetness potency is estimated to be about 600 times higher than sugar [7, 8].

Sucralose is highly water-soluble, stable at high temperatures and has negligible effect on pH or viscosity, making it an ideal sweetener for use in beverages and other foods, including those undergoing heat treatment such as baked goods. In human body, it is not digested into monosaccharides or metabolized for energy; therefore, contributes no calories and does not affect blood glucose levels [9].

A very important step for the approval of a compound and its safe dose are the toxicological tests in which it employs a range of concentrations to characterize potential adverse effects of dose-responses. To do this, the biological fate of the compound in the human body is studied (toxicokinetic), including absorption, distribution, metabolism, and excretion following ingestion, and this step is also important for establishing an Acceptable Daily Intake (ADI) with appropriate safety factors. This ADI is defined as the amount of a food additive expressed on a body weight basis that can be consumed daily over a lifetime without appreciable health risk. In the case of sucralose, it was first approved in 1989 by JECFA [Joint Experts Committee on Food Additives of The Food and Agriculture Organization of the United Nations - World Health Organization - (FAO-WHO)], which established a temporary ADI (t-ADI) of 0 to 3.5 mg/kg/d and in 1991, after further studies, changed to an ADI of 0 to 15 mg/kg/d. Now, sucralose is widely approved by international regulatory agencies as a food additive for sweetening purposes [9] with an ADI of 5mg/Kg body weight [10] The safety of sucralose has been extensively evaluated by regulatory agencies worldwide and in numerous studies, both at the cellular level and in model organisms, which approved globally its use in foods and beverages, as a non-caloric sweetener [8]. Although, sucralose is considered safe for consumption, there are still conflicting findings from the available studies, like the results related to its excretion and metabolization in the body. Some data revealed that part of sucralose absorption occurs in the intestines and the excretion of the remaining portion unchanged in urine and feces [11], while other authors [12] have reported acetylated sucralose metabolites accumulation in the urine and feces of rats. Another study in animals, suggested that sucralose may be recognized as a toxin, because its consumption increased the expression of the

intestinal efflux transporters, particularly P-glycoprotein and cytochrome P450, involved in drug detoxification [13].

1.1.2 Saccharin

Saccharin (1,1-dioxo-1,2-benzothiazol-3-one) is the oldest non-nutritive sweetener approved for food and beverage applications, was discovered by Remson and Fahlberg in 1878 at Johns Hopkins University. It is described that saccharin is not metabolized by the body, is heat stable and has a bitter or metallic taste. In 1977 the FDA tried to embargo the use of saccharin because animal studies showed its implication on cancer development (mainly bladder cancer). However, further studies did not prove a clear causal relationship between saccharin and health risks in humans at normal doses [14, 15, 16]. Guidelines indicate that the saccharin ADI (Acceptable Daily Intake) is about 15 mg/kg of body weight [10].

The saccharin is a water-soluble acid with a pKa of 1.8. Its absorption is increased in animal species with lower stomach pH, such as rabbits and humans, that absorb and eliminate in the urine approximately 85% to 95% of ingested saccharin, with the remainder excreted in the feces. The absorbed saccharin binds reversibly to plasma proteins and it is distributed via the blood to the body organs. The concentrations of saccharin in body tissues are in equilibrium with those in the blood plasma and decrease in parallel with the levels in the general circulation. Besides, saccharin can cross the placenta and appears in fetal tissues of rats, monkeys, and humans [9]. Indeed, studies using the animal model Rhesus monkey showed that saccharin cross the placenta rapidly but to a limited extent and without accumulation in a specific tissue [17].

It is described that exposure at doses of 100 to 400 times the human ADI (Acceptable Daily Intake) does not promote risk of malformations [18]. However, in case–control studies, it was suggested that tabletop use of sweeteners, particularly saccharin, may be associated with bladder cancer, although the differences between subgroups were not statistically significant for saccharin.

1.1.3 Sodium cyclamate

Cyclamate, discovered in 1937, is 30 times sweeter than sucrose, soluble in water, and its solubility can be increased by preparing sodium or calcium salt.

In literature, it is described that cyclamate has low toxicity, however, it is metabolized by intestinal bacteria to cyclohexylamine, which is readily absorbed from the gastrointestinal tract, promoting high toxicity [19]. Indeed, a study performed in rats demonstrated that absorption of

cyclohexylamine induces testicular atrophy in rats [20]. Having into account the possible toxicity of cyclamate, JECFA and the Scientific Committee on Food of the European established a ADI 11 mg/kg/d [10]. About other effects, studies described that prolonged use of sodium cyclamate is associated with bladder cancer development [16, 21]. Furthermore, sodium cyclamate can interact with DNA through the insertion of a helical double strand, which consequently can result in potential cytotoxicity due to loss of nucleic acid sequence of large DNA fragments. Together, the above describe findings showed that although the amount of sodium cyclamate used in the food industry is small, it does not completely rule out its potential harm to human health [22].

1.1.4 Xylitol

Xylitol, a five-carbon sugar alcohol ($C_5H_{12}O_5$) that occurs naturally in fruits and vegetables (plums, strawberries, cauliflower and pumpkin), has a sweetness-time intensity very similar to sucrose, and it is the sweetest of all polyols. Xylitol is best known for its dental benefits, such as reducing the risk of tooth decay [23]. In 1986, FDA declared xylitol as safe for human use. Since then, it has been registered as "generally safe" for utilization in foods, pharmaceuticals, and oral health care products in many countries. Generally, safe amounts of xylitol consumption are 50 g/day for adults and 20 g/day in children, and 50 g in infusion solutions for parenteral nutrition. Substitution of the carbonyl group with an alcohol group hinders its digestion and metabolism, contributing to the low-glycemic and insulinemic properties, in addition to demonstrating a slower gastric emptying, delaying the hunger sensation and food intake [24].

Several clinical trials have indicated that xylitol (gum) products are most effective in reducing tooth decay because they replace cariogenic sucrose, stimulates salivation and inhibits microorganisms' proliferation, particularly *Streptococcus* mutant strains (the main responsible for dental caries). In addition, it is not fermentable and therefore cannot be converted into acids by oral bacteria, helping to restore a proper alkaline/acid balance in the mouth [16, 23]. Xylitol also has other potential health benefits, such as changes in microbiota, which are associated with positive effects on respiratory infections, sinusitis, and acute otitis media. In addition to its prebiotic characteristics, xylitol has also anti-inflammatory effects on immune function [23].

1.1.5 Stevia/ Rebaudioside A

Steviol glycosides-rebaudioside A and stevioside are extracted from the leaves of the *Stevia rebaudiana* plant. In 2008, the FDA allowed gras status for purified rebaudioside A followed by

stevioside. These purified glycosides should not be confused with whole stevia leaves, which contain a number of active components, not all of which are sweet. Stevia is 10-15 times sweeter than sucrose and the human body does not metabolize these sweet glycosides, therefore it is a non-nutritive sweetener [14, 16].

Clinical studies showed that stevia has no effect on blood pressure or blood glucose response, indicating that this sweetener is safe for use by individuals with diabetes. Based on the published research, independent scientific experts in the US and worldwide have concluded that stevia is also safe for people of all ages and an ADI of 4 mg/kg/d (expressed as steviol) has been established. Stevia has a very low acute toxicity, and no allergic reactions are reported [3].

Steviol glycosides are not digested in the upper gastrointestinal tract and are hydrolyzed or degraded only when they come into contact with microbiota in the colon. Microbiota broke the glycosdic bonds, remove the sugar moieties, leaving behind the steviol backbone that is systemically absorbed, glucuronidated in the liver, and excreted via urine in humans and via feces in rats. Mounting evidence demonstrated that steviol glycosides are safe, non-hencariogenic, non-hyperpertensive, and present reduced impact on the gut microbiota. Thus, stevia leaf extract sweeteners are a beneficial and a perfect alternative to apply in sugar and calorie reduction, diabetes, weight management, and healthy lifestyles [25].

1.2 Metabolism of carbohydrates

Carbohydrates are the most abundant macromolecules, in part because of the plant carbohydrates cellulose and starch, both composed of multiple conjugated glucose molecules. Carbohydrates are divided into three major groups based on their structures: (1) simple sugars represented by monosaccharides (glucose, galactose or fructose) and disaccharides (lactose - glucose + galactose, sucrose - glucose + fructose and maltose - glucose + glucose); (2) complex carbohydrates, such as glycogen, starch and cellulose, which are multiple conjugated glucose molecules; and (3) glycoconjugates, which are modified forms of glucose covalently attached to either proteins (glycoproteins) or lipids (glycolipids), which participate in important functions, such as immunity, and as components of cell membranes [26].

The metabolism of carbohydrates uses several metabolic routes that occur according to the needs of the organism: 1) glycolysis - is the metabolic route in which glucose is oxidized to obtain energy for the cells, forming ATP, NADH, and 2 molecules of pyruvate, used in other metabolic routes, to obtain more energy. It is worth noting that glycolysis was the first metabolic

pathway to be elucidated in yeast and muscle extracts in the 1930s, and is an almost universal central pathway of glucose catabolism and the pathway with the highest carbon flux in most cells; 2) gluconeogenesis - activated when the body goes through a long period of fasting, producing glucose from sources other than carbohydrates, such as glycerol, amino acids or lactate; 3) glycogenolysis - a catabolic process in which glycogen stored in the liver and/or muscles is degraded to form glucose being the glycogen from liver the supply to increase blood glucose when needed; and 4) glycogenesis - a pathway for the production of glycogen [27].

The metabolic fate of monosaccharides can be quite diverse. Glucose, galactose, and fructose enter glycolysis by different routes. Glucose becomes glucose 6-phosphate by an ATP-dependent reaction using hexokinases. Galactose enters via the Leloir pathway, in which galactokinase uses ATP to generate galactose 1-phosphate, which is converted to glucose 1-phosphate and subsequently to glucose 6-phosphate by the enzymes galactose-1-P-uridyl transferase and phosphoglucomutase, respectively. In the liver, glucose 6-phosphate can be converted to glucose, while in other tissues it is metabolized through glycolysis. Fructose is mainly metabolized by the liver and to a lesser extent by the small intestine and the kidney. The first step is its phosphorylation to fructose 1-phosphate by fructokinase. Subsequently, it is cut into glyceraldehyde and dihydroxyacetone phosphate by a fructose 1-phosphate aldolase B. The glyceraldehyde is then phosphorylated to glyceraldehyde 3-phosphate, a glycolytic intermediate, by triose kinase. The glycolytic intermediates generated can either proceed through glycolysis and its subsidiary biosynthetic reactions, including fatty acid generation or storage as glycogen. At first glance, it seems that fructose metabolism eventually mirrors glucose metabolism; however, fructose enters glycolysis after the important regulatory step of phosphofructokinase-1 (PFK1) in glycolysis [26].

Yeast *Saccharomyces cerevisiae* cells rapidly convert sugars to ethanol and carbon dioxide at both anaerobic and aerobic conditions. This means that under aerobic conditions, respiration is possible with oxygen as the final electron acceptor, but *S. cerevisiae* exhibits alcoholic fermentation until the sugar reaches a low level, a phenomenon called Crabtree effect [28].

1.3. Aging

Several critical questions have arisen in the field of aging regarding the physiological sources of aging-causing damage, the compensatory responses that try to re-establish homeostasis, the interconnection between the different types of damage and compensatory

responses, and the possibilities to intervene exogenously to delay aging [29]. It is of utmost importance to understand the aging hallmarks and their root causes, as it will allow a better understanding of the key mechanisms that cause aging, thus obtaining a direction for interventions capable of improving and increasing the duration of longevity.

For a better understanding of the complex aging process, at the molecular and cellular levels, López-Otín and colleagues defined nine hallmarks: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, dysregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Fig. 1) [29]. A hallmark is considered a process that occurs during aging progression, which manipulation can result in acceleration or delay of aging process. Nevertheless, the proposed hallmarks do not always meet the described criteria, because it is difficult to fulfil for all criteria, since for example known experimental interventions are not proven to improve aging on all hallmarks.

In the present work, we will focus on markers of genomic instability, loss of proteostasis, and dysregulated nutrient sensing, because much more compelling evidence exists for these hallmarks in the yeast lifespan, and the rest either have limited relevance for natural aging in yeast, or have not been robustly implicated in its aging [30].

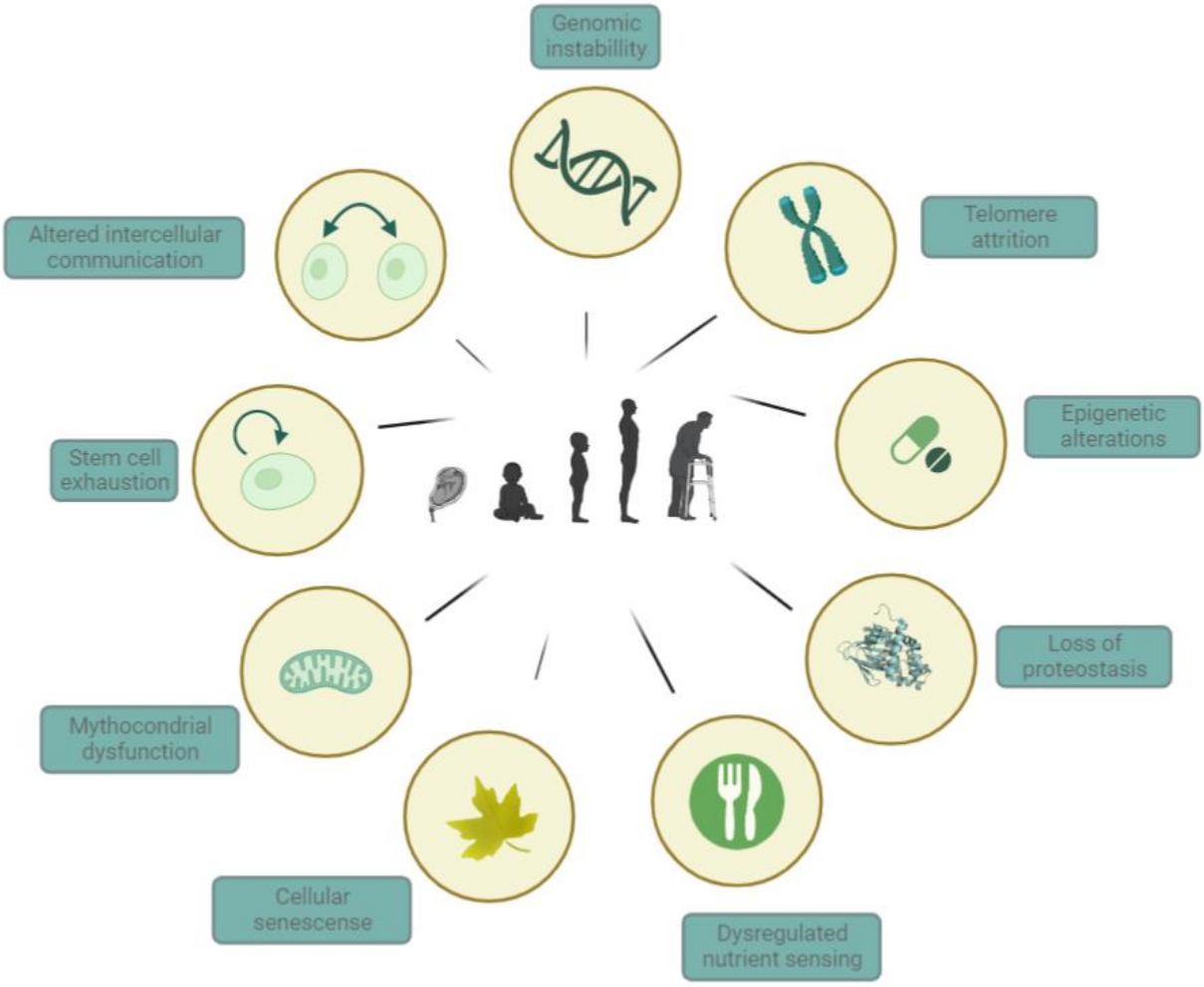

Figure 1: The Hallmarks of aging. Described and adapted from: López-Otín C, Blasco MA, Partridge L, Serrano M. Cell (2013).

1.3.1 Models of aging

As above described, aging is a complex and multifactorial process. Furthermore, to study aging in humans, it takes a lifetime to collect the data. It already known that that many of the aged-associated processes are conserved throughout evolution [31]. Therefore, model organisms such as mice (*Mus musculus*), flies (*Drosophila melanogaster*), worms (*Caenorhabditis elegans*) or yeast (*S. cerevisiae*) can be used as models' organisms to study aging. Their short lifespan is a strong advantage, as well as reduced cost and the simpler ethical concerns in comparison to humans [32].

1.3.1.1 The yeast *S. cerevisiae* as aging model

S. cerevisiae is a model organism in modern cell biology as it is a single-cell eukaryotic microorganism that has a relatively uncomplicated and short life cycle. It is commonly used in food

and beverage industries, besides it is a biological model for the investigation of complex molecular and cellular processes conserved in multicellular organisms, and it has allowed fundamental biological discoveries [33]. It is widely used as an experimental system, due to its easy manipulation and rapid growth compared to animal models. Coupled with the continuous development of new experimental methodologies for manipulating various aspects of its cellular machinery, yeast has served as the primary model organism for molecular and systems biology. Motivated by the availability of its full genome in 1996, as the first eukaryotic organism to be sequenced, an array of functional genomics tools emerged, including a comprehensive collection of yeast deletion mutants, genome-wide over-expression libraries, and green fluorescent protein (GFP)-tagged yeast strains. In addition, it shares with humans a significant fraction of their functional pathways that control key aspects of eukaryotic cell biology, including the cell cycle, metabolism, regulated cell death, protein folding, quality control and degradation, vesicular transport, and many key signaling pathways, such as mitogen-activated protein kinase (MAPK), target of rapamycin (TOR), and insulin/IGF-I signaling pathways [33, 34].

The high simplicity of yeast, when compared to human cells, has been widely explored to accelerate the discovery of new drugs and therapeutic targets in human diseases. In fact, when complemented with more physiologically relevant models where the hits discovered will be validated, yeast can be seen as a powerful first-line screening system for large genetic and chemical libraries [35]. There is no other eukaryotic organism in which so many molecular details have been elucidated by biochemical and genetic analysis in different areas of cellular and molecular biology [36]. Therefore, this work uses this organism as a mechanism to study cellular aging.

Aging studies are becoming increasingly prominent in biomedical research. The reasons for this are obvious. The demographics of the world are rapidly changing, leaving a population with an increasing number of elders and a declining number of working age individuals to support them. Older people tend to have costly chronic diseases that negatively impact their quality of life and functional output. In fact, aging itself is the leading risk factor for an array of diseases that increasingly plague the world population. If researchers can understand aging and modify its rate, the consequences are likely to be a reduced incidence or progression of disease leading to increased health span, allowing older people to keep working and avoid high health care costs [37].

Aging is a complex process associated with accumulation of damage, loss of function and increased vulnerability to disease, leading ultimately to death. Despite the complicated etiology of aging, simple genetic alterations can cause a substantial increase in healthy lifespan in laboratory

model organisms. Many of these longevity-extending mutations decrease the activity of nutrient-signaling pathways, suggesting that they promote a physiological state similar to that experienced during periods of food shortage in nature. Indeed, dietary restriction (DR) or caloric restriction (CR), a reduction in food intake without malnutrition, extends the average and/or maximum life span of various organisms including yeast, flies, worms, fish, rodents and rhesus monkeys [38]. Many damage factors contribute to cellular aging, including chromatin instability, mitochondrial dysfunction, reactive oxygen species, and others. In each single cell aging could be driven by independent damage mechanisms that accumulate with varying rates, resulting in different aged phenotypes in individual cells, or, alternatively, by the deterioration of overall cellular condition, leading to a common aging pathway in all cells. Therefore, single-cell analysis can reveal which scenario actually underlies the aging process [39].

As above described, the budding yeast *S. cerevisiae* is one of the most important model organisms used in aging-related research because of its rapid growth, short division time and low cost in harvest. In comparison with other systems, the relative ease and rapidity with which longevity can be quantified in yeast has allowed rapid progress is defining the molecular mechanisms of aging in this organism and the identification of dozens of factors that modify longevity [31, 40]. To understand the aging related changes that occur in yeast, it is necessary to explore the cellular pathways that vary over time. One study identified and categorized nine cellular and molecular hallmarks of aging most applicable to humans: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, dysregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. These hallmarks together determine the aging phenotype [29].

Two forms of aging - Replicative Lifespan (RLS) and Chronological Lifespan (CLS) - have been described in yeast, both of which are reflected in the aging processes of mammalian cells. The RLS is defined as the number of divisions an individual cell undergoes before dying, while the CLS is the length of time a population remains viable in the post-diauxic and stationary phase, after exhaustion of carbon source [41]. (Fig. 2)

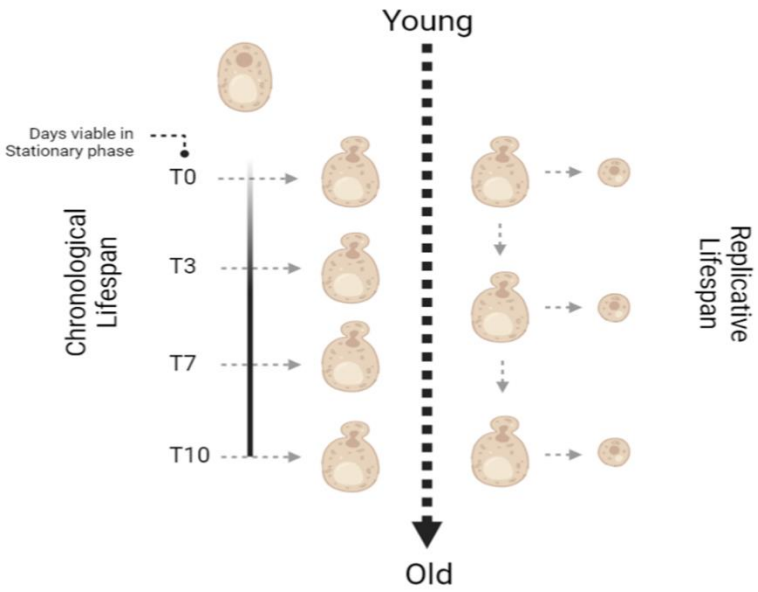

Figure 2 – The two types of yeast cellular aging. Representation of chronological and replicative lifespan of yeast. Adapted from: Kaeberlein M, Burtner CR, and Kennedy BK. PLoS genetics (2007).

1.3.1.1.1. Chronological lifespan

Chronological lifespan (CLS) is the length of time that a non-dividing yeast cell survives, and it is measured by growing cells until the post-diauxic state, following which most cells exit the cell cycle. This post-diauxic phase is the period that begins after depletion of extracellular glucose, in which cells dramatically reduce growth and switch to a mitochondrial respiratory mode of metabolism dependent on the ethanol generated during fermentation. At the end of post-diauxic phase, the stationary phase begins between day 2 and 7, depending on the medium used in the experiment. This phase is characterized by lower metabolic rates and up-regulation of stress-resistance pathways that maintain cells in their quiescent state. Viability, in this case, is defined as the ability to re-enter the cell cycle upon return to nutrient-replete conditions [37, 42].

A growing body of data from studies on model systems indicates that aspects of aging have been conserved throughout evolution, because similar interventions can increase life span among evolutionarily divergent species. One such intervention is CR, which can slow aging in virtually every biological system examined [43]. CLS can be increased by CR, which is accomplished by reducing the glucose concentration from 2% to 0.5% or lower in the initial culture medium, or by transferring yeast cells to water after they have exhausted available nutrients [42].

CLS monitorization of yeast mutants lacking specific genes have allowed to understand how different key molecules affect cellular damage and aging. The main nutrient sensing pathways,

TOR, Sch9 and Ras/PKA, are the master longevity modulators of CLS. Sirtuins have less pronounced anti-aging roles during CLS [44]. Sir2 promote exacerbate autophagy during yeast CLS, which has been linked to the proteotoxic stress induced by proteins associated with age-associated diseases [45, 46]. Reduction of TOR signaling and PKA inhibition result in the activation of many cellular stress responses such as Rim15 or the Msn2/4 or Gis1 transcription factors [37, 47] and the maintenance and arrest of cells in G0/G1 phase of cell cycle promoting lifespan [32]. These key pathways and molecules are described within the hallmarks of cellular aging, as noted below.

1.3.1.2. Aging hallmarks

Aging hallmarks, as above described, are mechanisms that occurs during aging process, which are able to somehow modulate it. Study of these mechanisms will allow to perform future interventions in an attempt to improve longevity. Following, we will address the hallmarks of genomic instability, loss of proteostasis and dysregulated nutrient sensing, particularly focused in the aspects described for the yeast aging.

1.3.1.2.1 DNA damage

DNA can undergo significant changes in its structure, despite its apparent stability. Spontaneous hydrolysis, oxidation and non-enzymatic methylation of DNA nitrogen bases can induce tens of thousands of lesions per day, in addition to environmental agents. These lesions, if left unrepaired, can lead to genomic instability, which is a hallmark of aging [48].

DNA is an important target for time-dependent deterioration, as highlighted by the rapidly expanding family of rare inherited disorders called progeroid syndromes, in which genome maintenance is compromised and many features of aging are accelerated, indicating that DNA is a critical target of aging and that genome maintenance is a major anti-aging mechanism [49]. Thus, eukaryotic cells regulate a set of biological processes collectively titled as DNA Damage Response (DDR) that comprises multiple DNA repair and DNA damage tolerance pathways, as well as cell cycle checkpoints. In yeast, the apical kinases Mec1 and Tel1 (Ataxia Telangiectasia and Rad3-related protein - ATR and Ataxia-Telangiectasia-Mutated - ATM in mammals, respectively) are recruited to sites of injury such as areas with single strand DNA (ssDNA) exposure or double strand break ends respectively, initiating DDR activation. These kinases in turn activate, through phosphorylation, downstream checkpoint kinases Rad53, Chk1 and Dun1 (orthologs of CHK2 and

CHK1 in mammals), via the checkpoint adaptor proteins Rad9 (ortholog of 53BP1 in mammals) and Mrc1. Activation of downstream checkpoint kinases (in yeast, especially Rad53), in addition to phosphorylating targets in distinct pathways, also halt cell cycle progression in G1/S, intra-S or G2/M phases [50]. Hence, for repair to occur, it is necessary to recognize the lesion parallel to the cycle arrest, so that the machinery recruited to the injured site can perform the repair and prevent the transmission of the error, generating mutations.

In this cell cycle process the "continuous events of the growth cycle" (increase in cell mass) and the "discontinuous events of the DNA division cycle" (DNA replication, mitosis, and cell division) need to be tightly coordinated to maintain cell size homeostasis, preventing them from becoming too small or too large [51]. The budding yeast *S. cerevisiae* divides asymmetrically, and regulation of cell cycle progression is achieved predominantly during a narrow interval in the late G1 phase known as START in which it is driven by activation of the transcription factor SBF and Cln3. The execution of START irreversibly commits the cell to a new mitotic cycle and requires the activation of Cdk1 (encoded by the Cdc28 gene), which phosphorylates whi5 (start inhibitor), partially releasing transcription factor SBF and inducing the synthesis of other G1 cyclins (Cln1 and Cln2). The Cln1/2:Cdk1 complexes then accelerate the phosphorylation of whi5 and the activation of SBF, thus promoting the start transition. Nutrient availability also modulates the degree of asymmetry of cell division: poor medium generally produces large mother cells and very small daughter cells, whereas in rich medium the asymmetry between mother and daughter cells is substantially reduced [51, 52, 53].

A correlation exists between shorter CLS and a variety of experimental manipulations that inhibit stationary phase growth arrest in G0/G1, where cells cannot develop replication stress. And it is known that increasing the concentration of glucose in medium also shortens CLS via a mechanism that depends on the AKT homolog Sch9p and results in less frequent stationary phase growth arrest in G0/G1. Besides a strong correlation between longevity in the CLS model and the increased frequency with which stationary phase cells growth arrest in G0/G1, when conserved RAS, TOR and AKT/PKB growth signaling pathways are attenuated by mutations or caloric restriction [32].

In addition, it is already established, that premature aging caused by proteotoxic stress induces a Ras2-dependent growth signaling, a cell cycle re-entry, an activation of DNA damage responses and a degradation of the machinery need to reply to the DNA damage, particularly a

degradation of ribonucleotide reductase 1 (Rnr1), a protein required for the activity of ribonucleotide reductase and dNTP synthesis [54].

1.3.1.2.2. Loss of proteostasis

Proteins are one of the central biological macromolecules that perform and/or mediate almost every biological process in all living organisms, there are about 50 000 different species that are primarily synthesized on ribosome as linear polypeptides, which consist of up to thousands of amino acids. A polypeptide is usually a liner molecule with a high molecular weight, which allows the peptide adopting a multitude of possible conformations, but some of these conformations are thermodynamically unstable under certain conditions, leading to the formation of misfolded structures rather than the native state of a protein [55].

Some conditions as a result of stochastic fluctuations, presence of destabilizing mutations, stress conditions, or metabolic challenges (those that occur during cancer or aging) cause a misfolding of proteins during the life of the cell, potentially generating deleterious "gain-of-function" activities, in part because of their heightened tendency to aggregate. In addition, the cellular capacity to manage the proteome declines during aging and this likely underlies the late onset of neurodegenerative diseases caused by protein misfolding [56]. Thus, the importance in preventing protein aggregation and refolding denatured proteins to their bioactive forms.

Proteostasis is achieved by an integrated network of several hundred proteins, including molecular chaperones and their regulators which assist in *de novo* folding or refolding, and the ubiquitin-proteasome system (UPS) and autophagy system, which mediate the timely removal of irreversibly misfolded and aggregated proteins. Deficiencies in proteostasis have been shown to facilitate the manifestation or progression of numerous diseases, such as neurodegeneration and dementia, type 2 diabetes, peripheral amyloidosis, lysosomal storage disease, cystic fibrosis, cancer, and cardiovascular disease. Aging is also linked to a gradual decline in cellular proteostasis capacity and is a major risk factor for these diseases [57, 58].

The system responsible for maintaining proteins is called the proteostasis network (PN). The PN coordinates protein synthesis, folding, disaggregation or degradation. This definition encompasses the translational machinery, molecular chaperones and cochaperones, the ubiquitin-proteasome system (UPS) and autophagy machinery, which are important for PN. The degradation pathways, the UPS and autophagy, are crucial for PN for cell repair and survival, regulating several processes such as cell cycle, signaling, DNA transcription, repair and translation, among others.

Protein degradation is particularly relevant during aging, where degradation pathways should assist in clearing aging accelerating factors. UPS serves mainly to target individual proteins to the proteasome, while autophagy cleans up larger aggregates or membrane-associated proteins allowing macromolecules and energy recycling [58, 59].

The protein homeostasis that stabilizes and repairs non-native proteins is maintained by a family of proteins called molecular chaperone that, by binding with newly synthesized polypeptides or unfolded proteins, can inhibit undesired intermolecular interactions and aggregations, and then allowing them folding correctly and efficiently into their native forms [55]. In eukaryotic cells, there are two types of chaperones: chaperones linked to protein synthesis (CLIPS) linked to the translation machinery, and the heat shock or stress proteins (HSPs), which protect the proteome from stress [56, 57]. In yeast *S. cerevisiae* proteome is composed of 69 molecular chaperones and their chaperones that commonly assist chaperones in protein folding or unfolding and are classified into different families based on the molecular weight of the monomers (Hsp40, Hsp60, Hsp70, Hsp90, Hsp100 and small Hsp) [58].

The primary route for the disposal of defective proteins in eukaryotic cells is the UPS, it is "challenged" by aging, genetic mutations, and environmental changes that can lead to the accumulation of these defective proteins. For the expression of dozens of proteasomal components to occur, along with other UPS-related genes, eukaryotes have evolved master transcriptional regulators that target all proteasome genes, as well as ubiquitin, ubiquitin ligases, and extrinsic proteasome factors [60]. In this system a cascade of enzymes targets the proteins to be degraded with ubiquitin and the reaction is initiated by the ubiquitin-activating enzyme E1 (Uba1) and activated ubiquitin is transferred to one of the ubiquitin-conjugating enzymes (E2 or Ubc enzymes) for finally, E3 enzymes (ubiquitin ligases) catalyze the formation of isopeptide bonds between lysine residues in substrate proteins and the activated carboxyl group of ubiquitin. In yeast this conjugation machinery is composed by one E1, 11 E2s and 60–100 E3s enzymes [58]. In budding yeast, this master regulation occurs via the transcription factor Rpn4, which has a short half-life of 2 minutes, so will quickly accumulate if the proteasome is impaired [60]. The maintenance of proteasome activity is directly correlated with longevity and in *S. cerevisae*. This preservation is promoted by the overexpression of Ump1, a protein needed for proteasome assembly, increased viability during chronological ageing. Other factors also enhance proteasome activity with the increase of the levels of the transcription factor Rpn4, which regulates the levels of proteasome subunits resulting in increased longevity and resistance to proteotoxic stress [58]. In addition to

the UPS system, another crucial degradation pathway for PN is autophagy, which sequesters misfolded proteins, damaged or aged organelles, and mutated proteins into double membrane vesicles called autophagosomes that fuse to lysosomes/vacuole that in turn degrade the sequestered components [61]. Many autophagy-related genes (ATGs) were first described in *S. cerevisiae*, with forty different Atg proteins reported in yeast and new ones continue to be identified [62, 63]. In this organism, autophagy can be divided into two main types, microautophagy and macroautophagy (hereafter named autophagy), and both of them include nonselective and selective processes. In microautophagy, the cargo is sequestered directly by the vacuole membrane, and the following invagination leads to its delivery into the vacuole lumen. In contrast, during autophagy the cargo is sequestered within cytosolic double-membrane vesicles that subsequently fuse with the vacuole, allowing breakdown of the cargo and recycling of the resulting macromolecules. Nevertheless, the budding yeast *S. cerevisiae* has also another type of selective autophagy—the cytoplasm to vacuole targeting (Cvt) pathway—that operates in nutrient-rich conditions and is responsible for constitutive and selective transport of vacuolar hydrolases such as α-mannosidase (Ams1) and aminopeptidase I (Ape1) to the vacuoles using many of the same molecular components as autophagy [58].

Initiation of autophagy is tightly regulated by Atg1 complex that responds to nutrient starvation by recruiting multiple Atg proteins to the pre-autophagosomal structure proceeding with vesicle nucleation, expansion and completion ending in its fusion with the vacuole. Besides the genetic abrogation and the inhibition by rapamycin of the TOR signaling, other interventions such as caloric restriction (CR) are dependent on autophagy induction that also demonstrated plays a role in the extension of CLS by maintenance and/or improvement of several cellular processes like: turnover and clearance of organelles and aberrant damaged or toxic macromolecules; regulation of reactive oxygen species (ROS) production/accumulation; and regulation of processes that include cell cycle checkpoints and DNA repair [58]. Furthermore, the effects promoted by (CR), a powerful non-genetic manipulation associated with lifespan, are related with a balanced homeostasis between UPS and autophagy activities during aging [64].

Several studies exhibit the various aspects of autophagy that are conserved from yeast to humans, demonstrating the importance of its understanding and manipulation in this model to aid in future treatments of diseases, especially those linked to aging, such as neurodegenerative diseases.

1.3.1.2.3 Dysregulated nutrient sensing

To date, CR is the only non-genetic intervention that has consistently been found to extend both mean and maximum lifespan across a variety of species. Lifespan extension in response to CR is also believed to be helpful in decreasing the incidence of age-related diseases such as cancer, diabetes, atherosclerosis, cardiovascular disease, and neurodegenerative diseases by driving the reduction of cardiac risk factors, improving insulin sensitivity, and enhancing mitochondrial function, as well as reducing oxidative damage of both DNA and RNA [65, 66].

In *S. cerevisiae* the PKA pathway, an essential pathway for growth responds primarily to glucose and other fermentable sugars. While stimulating growth, PKA signaling suppresses stress responses and plays a prominent role in transitions of carbon availability. PKA activation is required for the transcriptional reprogramming occurring upon glucose addition to cells growing on poor carbon sources, and its proper inactivation is also necessary for survival during nutrient-poor conditions. When cultures are subjected to severe carbon starvation during the stationary phase, over activation of the PKA pathway shortens CLS, while mutations that reduce its activity are well known to extend viability [67].

The PKA kinase is a heterotetramer composed of two regulatory (Bcy1) and two catalytic subunits (Tpks) in its inactive form. Activation of the kinase occurs when the second messenger cAMP binds to the regulatory subunits, releasing the catalytic subunits, which are encoded by three partially redundant isoenzymes. Therefore, cAMP levels are key for PKA regulation. Glucose addition to de-repressed cultures induces a transient cAMP increase by the activation of adenylate cyclase (Cyr1) via two branches of the pathway: Ras (the most essential) and the G-protein coupled receptor system [67]. Furthermore, PKA inactivation at diauxic shift is required for proper diauxic transition, post-diauxic growth, and stationary phase survival or CLS. However, very little is known about the mechanisms for PKA inactivation when glucose become depleted at the diauxic shift [67]. This pathway also senses excessive ROS to signal to the Hap2,3,4,5 transcriptional system and down-regulates mitochondrial biogenesis. In other words, increased energy generation and mitochondrial electron transport chain activity is a well-established source of toxic ROS levels, which triggers oxidative damage of macromolecules and metabolic dysfunction [66, 68, 69].

In *S. cerevisiae*, CR is accomplished by reducing glucose in the growth medium, which promotes a down-regulation of the conserved signaling pathways Ras/cAMP/PKA, TOR (target of rapamycin), and TOR's major target the serine/threonine protein kinase Sch9. Deletion of *RAS2*, *TOR1* or *SCH9* enhances cellular protection against thermal and oxidative stresses and extends

yeast CLS. Inhibition of these pathways converges to the activation of stress-resistance transcription factors that positively regulate the expression of protective systems, and these are fundamental changes that mediate part of the effects of CR on CLS, the measurement of cell survival under non-dividing conditions [66, 68, 70].

Under conditions of nutrient scarcity cells need to manage energy generation without undercutting overall cellular physiology, as stress levels increase, protein synthesis is negatively regulated, while protein turnover is increased. Reduced TOR activity by nutrient starvation or genetic manipulation increases stress resistance and extends lifespan in many model organisms, including yeast, worms, and mice [66, 68, 69]. On the other hand, also overnutrition and excessive metabolic activity can threaten cellular function, for example through resulting metabolites or generation of ROS. Deletion of the *TOR1* gene, the main regulator of anabolic pathways that mediate growth under nutrient-rich conditions, extends yeast CLS in part by increasing mitochondrial mass and respiration and promoting adaptive mitochondrial ROS signaling [68, 69, 71]. But under normal, unstressed conditions, protein synthesis is activated by TOR, while protein degradation by autophagy is inhibited. Importantly, TOR is the main negative regulator of autophagy, acting through the phosphorylation of Atg13 and Atg1 or through its downstream target, PP2A (serine/threonine protein phosphatase 2A). TOR is also able to inhibit the activity of the metabolic sensor Snf1, which is a positive regulator of autophagy [66, 69, 71, 72].

It is found then that CR or catalase inactivation extends the CLS of yeast by inducing elevated levels of H_2O_2, which activate superoxide dismutases that inhibit O_{2^-} accumulation [73]. These findings establish a role for hormesis effects of H_2O_2 in promoting longevity induced by CR conditions that are likely to be conserved in complex eukaryotes. In catalase-deficient cells, increased H_2O_2 extends CLS despite parallel increases in oxidative damage. This violates a fundamental tenet of free radical theory that posits oxidative damage as a primary determinant of aging [73, 74, 75].

In sum, data suggest that the reduction in oxidative damage by CR arises from reduced ROS generation and/or enhanced repair of macromolecules, but while these findings are shown to be consistent with the Free Radical/Oxidative Stress Theory of Aging, attention should also be paid to the fact that CR affects numerous other biological pathways, which also could lead to increased life span. Hence, the importance of studying various pathways that lead to aging [76].

CHAPTER 2

AIMS

The main goal of this study was to evaluate the effects promoted by sugar substitutes or sweeteners (particularly stevia extract, saccharin, sucralose, sodium cyclamate and xylitol) on the yeast survival and chronological aging. The project was divided into two specific objectives:

1) To assess the impact of sweeteners supplementation on survival and chronological lifespan;

2) To evaluate if sweeteners can impact on cell-autonomous mechanisms regulating survival and chronological lifespan.

CHAPTER 3

MATERIAL AND METHODS

3.1. Yeast stains and growth conditions

In this work it was used the wild type yeast *S. cerevisiae* strain BY4741 (MAT*a his3 Δ1 leu2Δ0 met15Δ0 ura3Δ0*). Yeast cells were inoculated in synthetic (SD) medium containing 0.67% (w/v) yeast nitrogen base (YNB) without amino acids and 2% (w/v) glucose as carbon source, supplemented with the appropriate amino acids for which the strains were auxotrophic: 100 mg/L uracil; 300 mg/L leucine, 50 mg/L histidine and 50 mg/L methionine. Cultures were grown at 26°C, with shaking at 150 rpm. To evaluate autophagy, the wild type strain was transformed with the plasmid pRS416-GFPAtg8, for the constitutive expression of GFP-Atg8. Yeast cells harboring pRS416-GFP-Atg8 plasmid were cultured in SD without uracil.

3.2. Chronological lifespan assay

To perform the chronological lifespan (CLS) assays, cells were grown until stationary phase, around two days later, and this was considered day 0 of CLS. At this time, the specific sweetener (sucralose, cyclamate, saccharin, xylitol or stevia) was added to a final concentration of 2% in the medium. For determination of cell survival, cellular samples were serially diluted and platted on YPD agar plates, consisting in 0,5% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar and incubated for 2 days at 30°C. Survival was assessed by colony-forming units (CFUs) beginning at day 0 of CLS (when viability was considered to be 100%), and then, every 2-3 days until less than 0.1% of the cells in the culture were viable.

3.3 Spotting assay

Yeasts strains were pre-inoculated on liquid SC media supplemented with 2% glucose and the appropriate amino acids and incubated at 26°C with agitation. After overnight (exponential phase) or 48h (stationary phase) growth, yeasts were inoculated on the same medium, supplemented with 0%, 2%, 5%, 10%, 12% of the sweetener (sucralose, cyclamate, saccharin, xylitol or stevia), at a starting OD_{640} 1 and again incubated at 26°C until during 8h. Equal amounts of each yeast strain at the different conditions were then collected, 5-fold serially diluted using sterile water and 10 µl of each cell suspension was spotted on YPD-agar plates. Plates were incubated at 30°C for 2 days (Fig. 3). Images of plates were acquired with a ChemiDoc XRS System with Quantity One software (BioRad).

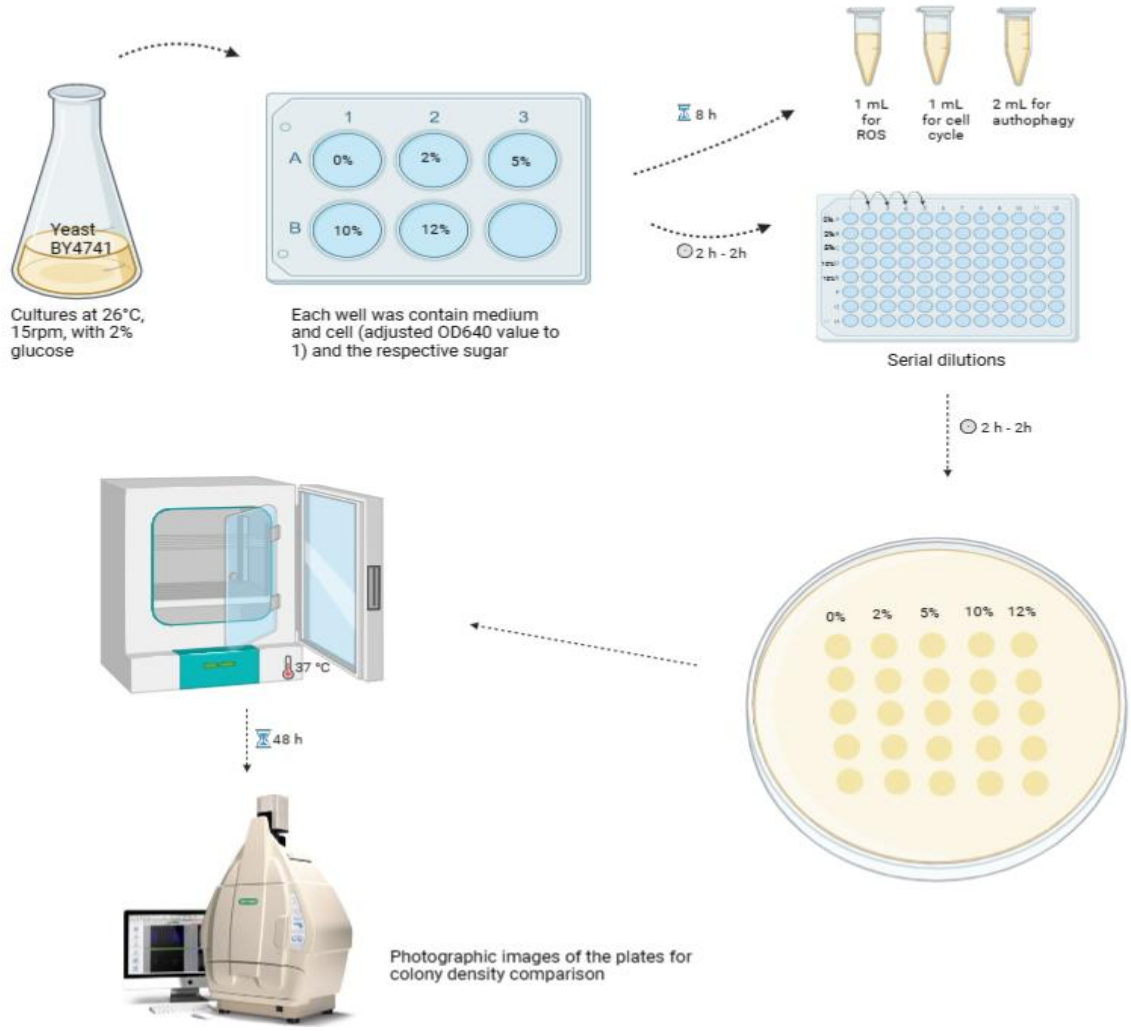

Figure 3: Graphic scheme of the stages of the spotting assay.

3.4 Assessment of intracellular accumulation of reactive oxygen species

Free intracellular reactive oxygen species (ROS), superoxide anion ($O2^{2}$) and hydrogen peroxide (H_2O_2), were measured, respectively, using dihydroethidium (DHE) (Invitrogen, product reference D1168) and dihydrorhodamine 123 (DHR) (Calbiochem, product reference 309825-5MG). Aliquots of cells were collected at indicated time points of CLS and spotting assays. DHE was added to a final concentration of 10µg/mL in dimethyl sulfoxide (DMSO), followed of incubation for 10min at 30°C in the dark. DHR was added at 15µg/ml in absolute ethanol, followed of incubation for 1h at 30°C in the dark. The DHE and DHR signals were detected using BD LSR II (Becton Dickinson, NJ, USA) with a 488-nm excitation laser was used. Signals from 30,000 cells/sample were captured in FITC channel (530 nm±30nm) for DHR or in the PERCP channel (670 nm±30nm), at a flow rate of about 1,000 cells/s. Data collected with the BD LSR II cytometer were processed with FlowJo software (Tree Star Inc., Ashland, OR, USA).

3.5 Cell cycle analysis

For cell cycle analysis, at the desired time points of CLS and spotting assays, cells were collected, fixed with ethanol (80% v/v), washed with sodium citrate buffer 50mM (pH 7,5), followed by centrifugation and resuspension in the same buffer. Next, the samples were sonicated with a 3-pulse 30W internal wave for 1-2 seconds to decrease cell aggregates. And then treated with RNAse 2mg/mL in Tris-EDTA (pH 8,0) at final concentration of 0,25 mg/mL for 1h at 50°C, and subsequent incubation with proteinase K 20mg/mL for 1h at same temperature. DNA was then stained with SYBR Green 10 000X, diluted in Tris-EDTA (pH 8.0), and incubated overnight in dark at 4°C. Before cytometric analysis, was added at the samples Triton 100X diluted in sodium citrate buffer and were sonicated. A BD LSR II (Becton Dickinson, NJ, USA) with a 488-nm excitation laser was used. Signals from 30,000 cells/sample were captured in FITC channel (530 nm ± 30 nm), at a flow rate of about 500 cells/s. Determination of cells in each phase of the cell cycle was performed offline with ModFit LT 3.2 software (Verity Software House Inc., Topsham, ME, USA)

3.6 Autophagy monitoring by the GFP-Atg8 processing assay

To evaluate autophagy, cells were transformed with the plasmid pRS416-GFPAtg8 with fusion gene under the control of the ATG8 endogenous promoter [77]. GFP N-terminally tagged to Atg8 is delivered to the vacuole and given that GFP is more resistant to degradation than Atg8, bulk autophagy leads to accumulation of free GFP in the vacuole. The ratio between free GFP and total GFP is used as a readout for the autophagic flux. GFP-Atg8 and free GFP are detectable by immunoblotting using a GFP-specific antibody (see section Protein quantification and immunoblotting – 3.8).

3.7 Preparation of protein extracts

Cells were washed with 1X phosphate-buffered saline, followed by incubation for 5 minutes at room temperature with 2M lithium acetate. After lithium acetate removal, 0.4 M NaOH was added for 5 min on ice, and then centrifugated, and resuspended in Laemmli loading buffer diluted in β-mercaptoethanol. Followed by boiling at 100°C for 5 minutes and 30 minutes of centrifugation with 13000rpm/min at 4°C. The supernatant was considered the total protein extract.

3.8 Protein quantification and immunoblotting

Total protein quantification was performed with the RC/DC protein assay (Bio-Rad) and processed in accordance with manufacturer's instructions. 20 μg of protein were resolved on a 12% SDS gel and transferred to a nitrocellulose membrane during 12 min at 25V in Trans-Blot Turbo transfer system. Membranes were blocked with tris buffered saline (TBS) with 0.1% Tween 20 (TBST) containing 5% bovine serum albumin (BSA). by incubation with primary antibodies against anti-GFP (1:5000; SICGEN), and anti-PGK (1:5000; Invitrogen) in TBST containing 1% BSA and primary antibody. After washing with TBS, the membranes were incubated with the respective secondary antibody, HRP-conjugated anti-rabbit IgG, anti-mouse IgG or anti-goat IgG at a dilution of 1:5000 in 1% BSA. Membranes were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) or Clarity Western ECL Substrate (Bio-Rad) and images of the immunoblotting were obtained in a ChemiDoc XRS System (Bio-Rad) with Quantity One (Bio-Rad) software.

3.9 Higher Performance Liquid Chromatography (HPLC)

The chromatographic profile of the glucose and sweeteners in each culture medium was analyzed by HPLC. The column used for the separation was the Carbohydrate H+ 8u HyperRez Column, with mobile phase at H_2SO_4 0,0025M in isocratic flux of 0,7mL/min. The analyze occurred at 54°C and was detected by refractive index by detector. The peaks of glucose and sweeteners were previously identified in different concentrations (20%, 10%, 5%, 1%, 0.1%, 0.01%, 0.001%), and the retention time for glucose established was 8,5 minutes, and of the sweeteners according to the Table X. The values of integration of the area of each peak generated for the software were used for create the graphs.

Table 1: Retention time of glucose and sweeteners on HPLC.

Sweeteners	Retention time (minutes)
Glucose	8,5
Saccharin	14
Sucralose	11,5
Xylitol	9,9
Cyclamate	9,8
Stevia	7.54

3.10 Statistical Analysis

The results of this work are presented as mean values and standard error of the mean (SEM) from at least three replicates of independent samples. Statistical analyses were determined with the two-way ANOVA test (GraphPad Prism 7 software, La Jolla, CA, USA). A p value of less than 0.05 was considered significantly different.

CHAPTER 4

RESULTS

Part of the results on this chapter were presented in the following National Congress:

Microbiotec'21 - Congress of microbiology and biotechnology 2021. Nova University Lisbon, Lisbon – Portugal. 2021. The effect of alternative sugars in the yeast *Saccharomyces cerevisiae* fitness. (Poster presentation).

4.1 Effects of sugars substitutes supplementation on yeast survival

In a first approach, we decided to explore the effects on yeast survival of glucose substitution by sweeteners as xylitol, saccharin, sucralose, sodium cyclamate and stevia, commonly used for diverse applications. For such, cells from exponential or stationary phase were replaced for 8 h in SD medium with 2, 5, 10 or 12% of glucose (control) or with 2, 5, 10 or 12% of each sweetener. Exponential cells grew about 12h in SD medium, while stationary cells were cultivated during 48h in the same medium, before to be challenged with the different sweeteners. Afterwards, cells were spotted in 1:10 serial dilutions onto YPD plates.

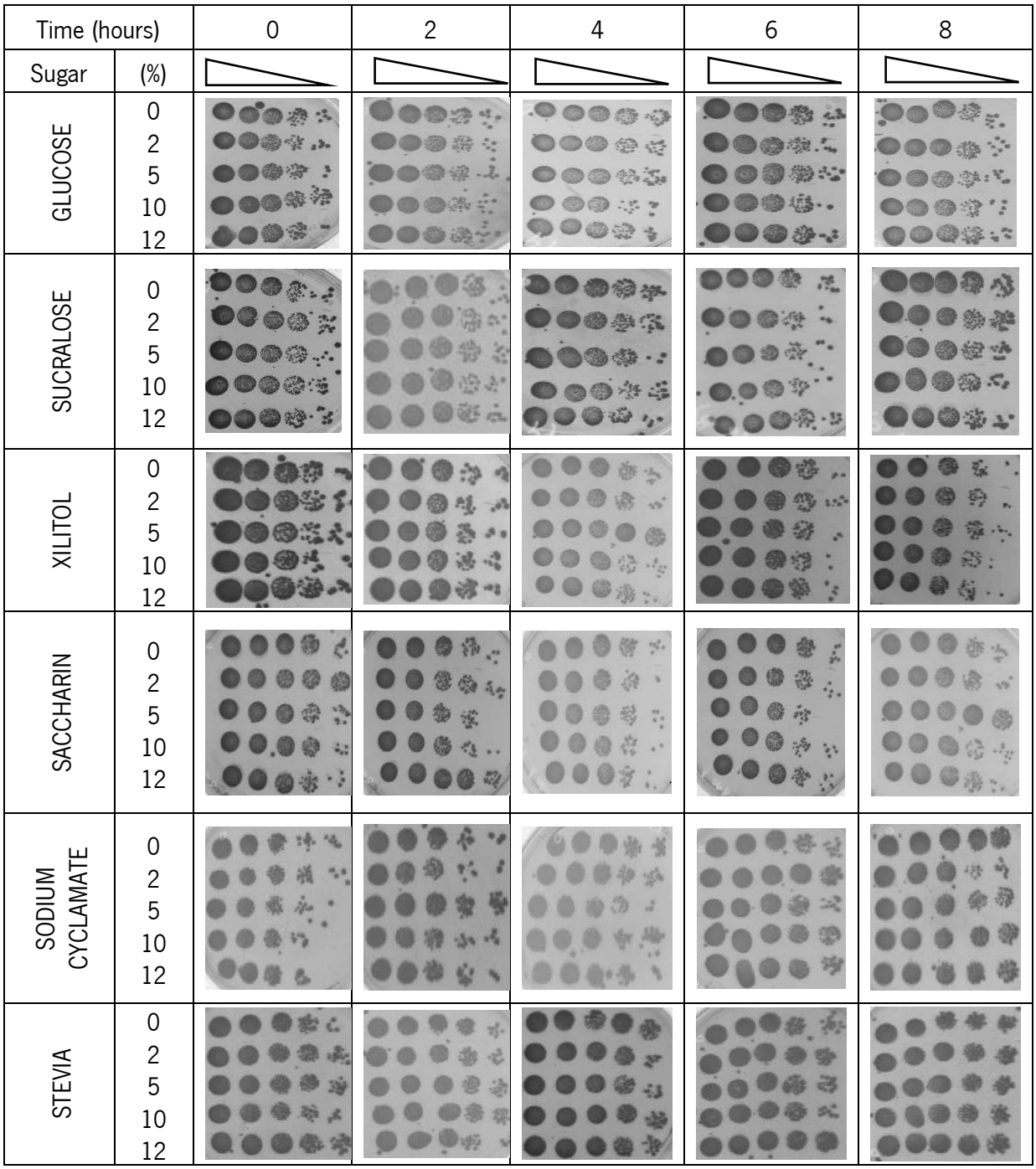

Figure 4. Survival of exponential cells to sweeteners. Survival of exponential By4741 cells to medium supplementation with different concentrations (0%, 2%, 5%, 10%, 12%) of glucose or sweeteners spotted in 5 1:10 serial dilutions. Cell growth was evaluated every 2 hours for a total time of 8 hours of growth counted from the addition of the referred sweeteners.

The results obtained demonstrated that medium supplementation of exponential cells with xylitol and saccharin inhibits cell growth in comparison to medium with glucose. Sucralose supplementation induces a growth behavior similar to glucose, while medium supplementation with sodium cyclamate or stevia stimulates cell growth when compared to glucose conditions (Fig 4).

Concerning the effects promoted by sweeteners supplementation on stationary phase cells, results showed that the tested sweeteners reduce cells' growth, suggesting that stationary phase cells are less resistant to supplementation with substitute sugar sources (Fig 5).

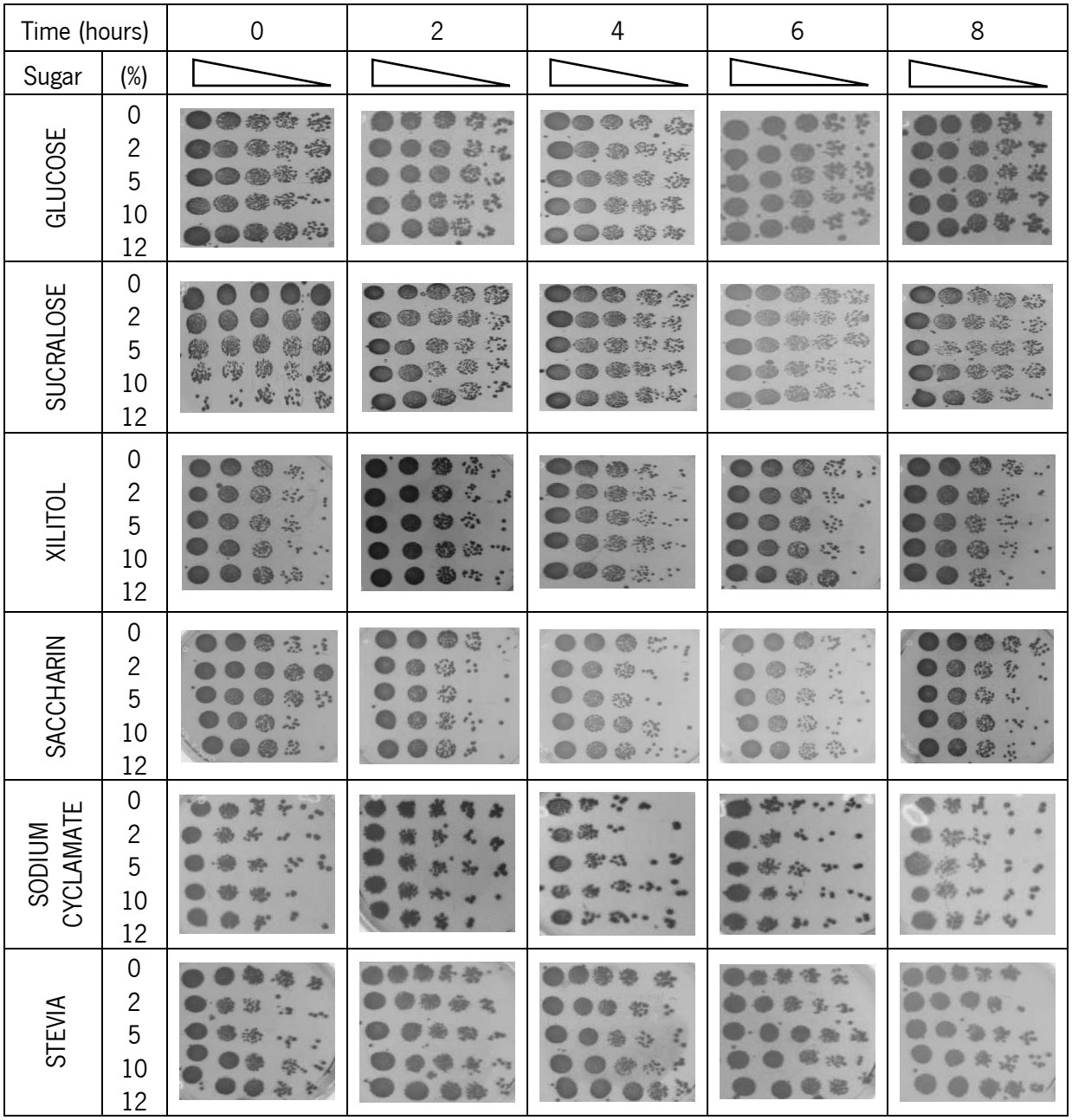

Figure 5. Survival of stationary cells to sweeteners. Survival of stationary By4741 cells to medium supplementation with different concentrations (0%, 2%, 5%, 10%, 12%) of glucose or sweeteners spotted in 5 1:10 serial dilutions. Cell growth were evaluated every 2 hours for a total time of 8 hours of growth counted from the addition of the referred sweeteners.

4.2 The role of autophagy on yeast cells' survival to sweeteners

Autophagy is one of the survival mechanisms of cellular resistance to intrinsic and extrinsic stresses [78]. Autophagy can be induced by limitations in ATP availability or by the lack of essential

nutrients, including glucose and amino acids [79]. Herein, we decided to explore the role of autophagy on cells' survival to sweeteners. For that, after the 8h of supplementation, autophagy flux was evaluated by GFP-Atg8 processing assay. Formation of autophagosomes is dependent of the Atg8 protein that is posteriorly degraded. In yeast cells, GFP is resistant to the vacuolar degradation. Therefore, taking advantage of this fact, we employed the yeast model expressing GFP-Atg8 fusion protein, and the consequent accumulation of free GFP inside the cells, which is directly proportional to the levels of autophagy, was evaluated. Therefore, autophagic flux was evaluated trough the immunoblot analysis of the ratio between free GFP and GFP total (free GFP + GFP-ATG8).

In exponential cells, supplementation with the different glucose concentrations (2, 5, 10 or 12%) did not have a major impact on autophagy flux, although a trend to increase autophagy was observed for 5% glucose (Fig.6A). In contrast, supplementation with xylitol, sodium cyclamate or stevia led to an autophagy flux inhibition, independently of the concentration tested and in comparison, with the levels observed for medium supplemented with glucose (Fig. 6A). Supplementation with saccharin or sucralose led to an increment of autophagy, particularly at concentrations of 5 and 10% (Fig. 6A).

Stationary cells submitted to the different sweetener's supplementation displayed the same pattern as exponential grown cells, however, an even higher autophagy flux was observed with saccharin or sucralose (Fig. 6B).

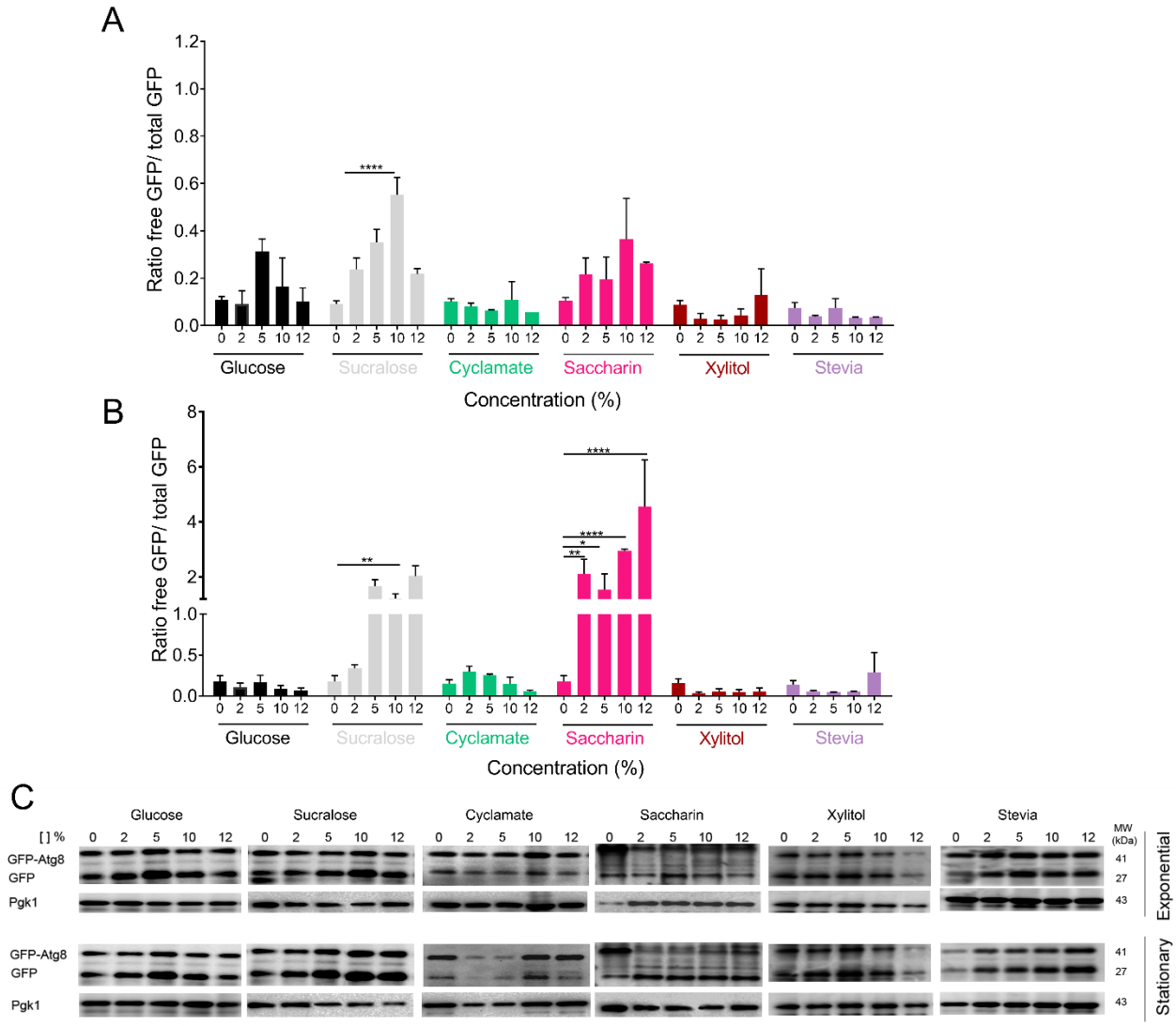

Figure 6: Autophagy flux determined by the GFP-Atg8 processing assay. Densitometric quantification of the ratio of free GFP to total GFP (free GFP + GFP-Atg8) in BY4741 GFP-Atg8 cells in exponential phase (A) and stationary phase (B) supplemented with the control glucose or the sweeteners sucralose, cyclamate, saccharin, xylitol and stevia at concentrations 0%, 2%, 5%, 10% and 12%. (C) Representative blots of the GFP-Atg8 processing assay of exponential or stationary BY4741 cells supplemented with the above refereed sweeteners. Values indicate the mean ± SEM from three independent experiments. Significance of values was determined by two-way ANOVA (*p ≤ 0.05, **p ≤ 0.01; ****p ≤ 0.0001).

4.3 Evaluation of reactive oxygen species (ROS) accumulation induced by sweeteners

Oxidative stress emerges from an enhanced reactive oxygen/nitrogen species (ROS/RNS) generation or from a decay of the antioxidant protective ability, being characterized by the reduced capacity of endogenous systems to fight against the oxidative attack directed towards target biomolecules. Reactive oxygenated/nitrogenated species are represented by superoxide anion radical, hydroxyl, alkoxyl and lipid peroxyl radicals, nitric oxide and peroxynitrite [80]. Herein, we asked how sweeteners supplementation modulates the accumulation of ROS, particularly of

hydrogen peroxide (H_2O_2), and superoxide anion (O_2^{2-}). For that, accumulation of O_2^{2-} was assessed by the fluorescent probe dihydroethidium (DHE) and the accumulation of H_2O_2 levels by the use of the fluorescent probe dihydrorhodamine 123 (DHR), though flow cytometry analysis [73.]. Results revealed that exponential cells supplemented with sodium cyclamate or saccharin displayed a higher percentage of DHE positive cells over the concentration increment, indicating that these cells are accumulating O_2^{2-}. Supplementation with xylitol, sucralose or stevia did not induce accumulation of O_2^{2-}, as seen with glucose supplementation (Fig. 7A).

Concerning the effects promoted by sweeteners supplementation on stationary phase cells, data showed, as expected, that supplementation with higher concentration of glucose led to the increase of DHE positive cells [32]. This phenotype was also observed for stationary cells supplemented with saccharin (Fig. 7B). The remaining supplementations did not induce accumulation of $O2^{2-}$.

Analysis of H_2O_2 accumulation by DHR staining showed that medium supplementation with 5, 10 or 12% of glucose resulted in increased DHR fluorescence intensity for both exponential or stationary phase cells (Fig. 7C and 7D). Regarding the sweeteners, results demonstrated that their supplementation did not result in increased DHR fluorescence, compatible with the absence of H_2O_2 accumulation in exponential phase cells (Fig. 7C). Moreover, supplementation with increasing amount of sodium cyclamate reduced the amount of accumulated H_2O_2. In stationary phase cells, we observed an increase in H_2O_2 levels directly correlated with the increase of sucralose or stevia, nevertheless, the levels were lower than those observed with glucose supplementation (Fig. 7D).

The accumulation of ROS species, particularly O_2^{2-}, appears to be very toxic to the cells [81]. In contrast, moderate levels of H_2O_2, can trigger hormesis, defined as the induction of an adaptive response to stress that protects against the increase of that or other stresses [73].

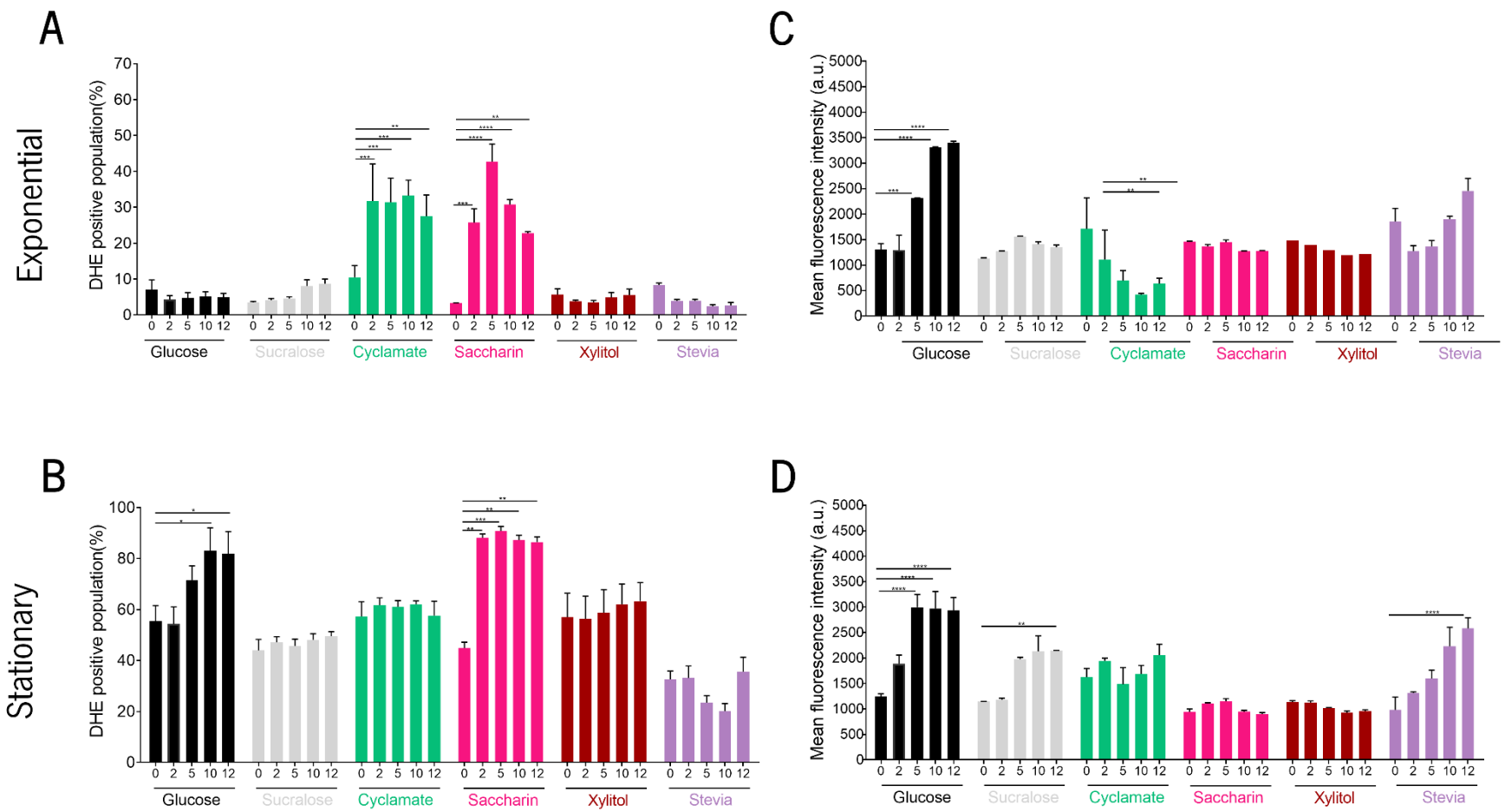

Figure 7: The accumulation of ROS by yeast cells supplemented with different concentrations of glucose or sweeteners. FACS measurements of superoxide anions using the superoxide-specific probe DHE in BY4741 cells in exponential phase (A) and stationary phase (B), and FACS measurements of hydrogen peroxide using the peroxides-specific probe DHR in BY4741 cells in exponential phase (C) and stationary phase (D) supplemented with the control glucose or sucralose, cyclamate, saccharin, xylitol and stevia at concentrations 0%, 2%, 5%, 10% and 12%. Bar graphs indicate the percentage of cells exhibiting elevated levels of intracellular superoxide anion detected by FACS measurements (20.000 cells per sample in three independent experiments) of DHE-positive cells (A and B), and also indicate the DHR mean fluorescence (C and D). Values indicate the mean ± SEM from three independent experiments. Significance of values was determined by two-way ANOVA (***p≤0.001; ****p≤0.0001).

4.4 Cell cycle analysis of cells supplemented with sweeteners

Cell cycle arrest in G0/G1 prevents DNA replication stress (inefficient DNA replication causes DNA damage) that arises when stationary phase cells enter S phase and arrest [82]. Thus, the effects of sweeteners supplementation on cell cycle profile were determined. For that, after the 8h of supplementation in the different conditions, we collected and evaluate the cell cycle, through the evaluation of the DNA content by flow cytometry.

In exponential phase cells, medium supplementation with the different concentrations of glucose was characterized by a regular cell cycle distribution (Fig. 8A). In contrast, supplementation with sodium cyclamate, saccharin, xylitol or stevia induced a pronounced reduction of the number of cells in S phase and increased number in GO/G1 and G2/M phase, compatible with cell cycle arrest in two different checkpoints (Fig. 8C - F).

Relatively to stationary phase cells, as expected, results demonstrated that medium supplementation with increased concentrations of glucose led to an accumulation of cells in S-phase of the cell cycle, suggesting a S-phase arrest promoted by DNA replication stress, as previously reported [54] (Fig.8G). A similar phenotype was also displayed by cells supplemented with increased concentrations of sucralose (Fig.8H). In contrast, medium supplementation with cyclamate, saccharin, xylitol or stevia promoted an efficient accumulation of cells in G0/G1 and G2/M cell cycle phases, similar to the profile observed in exponential growing cells (Fig.8I - L).

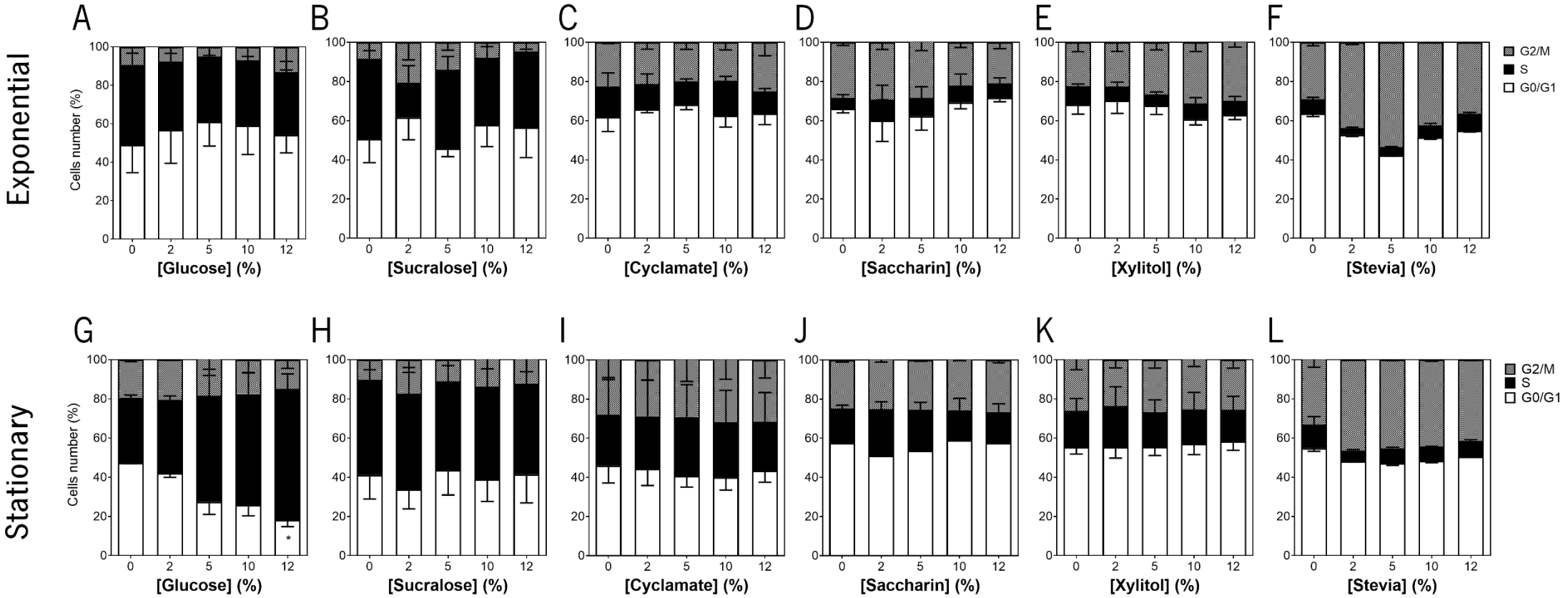

Figure 8: Cell cycle analysis of cells supplemented with sweeteners. Cell cycle analysis evaluated by the measurement of DNA content by flow cytometry of wild-type cells (BY4741) supplemented with sucralose, cyclamate, saccharin, xylitol and stevia, and the control glucose, in different concentrations. The data represents mean ± SEM of three independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA ($p \leq 0.05$), being related to control graph.

4.5 Evaluation of chronological lifespan of yeasts cells supplemented with sweeteners

To uncover whether supplementing the yeast medium with the different sweeteners could impact on the chronological lifespan (CLS), cells were grown in SD medium with 2% glucose, until reaching the stationary phase, about 48h, and then, at day 0 of the CLS, 2% of glucose or 2% of each sweetener (xylitol, saccharin, sucralose, sodium cyclamate or stevia) was added to the medium. The medium supplementation with glucose represents control conditions.

Results obtained showed that medium supplementation with sucralose did not have any impact on CLS in comparison with cells grown in medium supplemented with glucose (Fig. 9A and 9B). In contrast, supplementation with saccharin, sodium cyclamate, xylitol or stevia increased the longevity of the cells (Fig. 9C-F). Importantly, sodium cyclamate has the greatest effect on CLS, with almost 100% of cells still alive after 30 days of CLS (Fig. 9C).

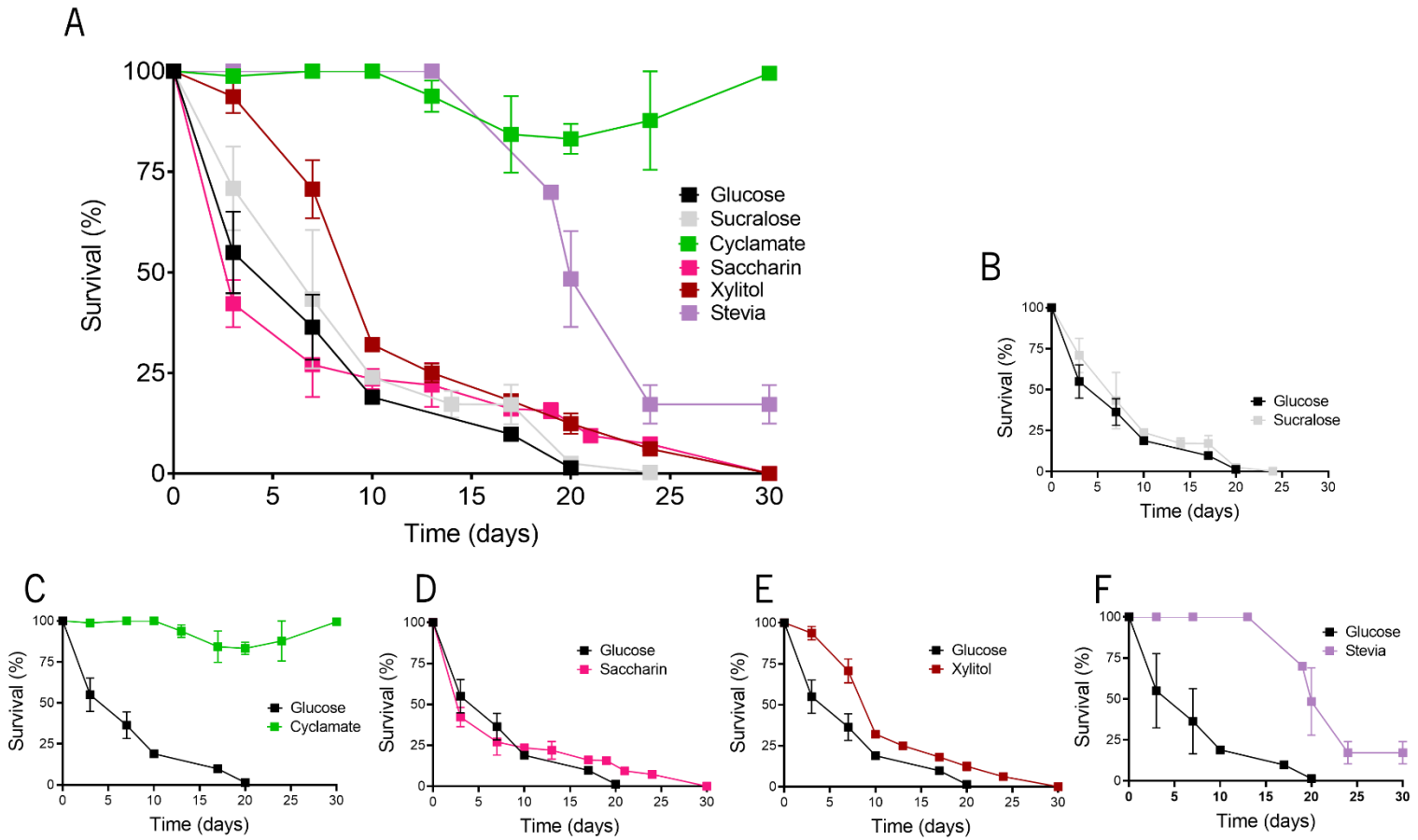

Figure 9: Impact of sweeteners' supplementation on chronological lifespan of yeast. Chronological lifespan of BY4741 cells supplemented with 2% glucose, as control, and 2% of the sweeteners - sucralose (B), cyclamate (C), saccharin (D), xylitol (E) and stevia (F). Cell viability was measured at 2-3 days interval from the day cultures reached stationary phase (day 0) and is expressed as % survival compared to survival at day 0 (100%). The error bars represent the standard error of the mean (SEM). Data significance was determined by t-test ($p \leq 0.05$).

Next, the mean (50% survival) and maximum lifespan (10% survival) were also determined. Mean and maximum chronological lifespan represent 50% and 10% of yeast cell's survival, respectively, and they were determined through the curve fitting of the survival data from pair-matched with the Prism 8 statistical analysis software (GraphPad Software) (Figure 10). The results showed that supplementation with the sweeteners increased the mean and maximum lifespans but only cyclamate, saccharin, xylitol and stevia promoted a statistically significant increase compared to control conditions (glucose) (Figure 10).

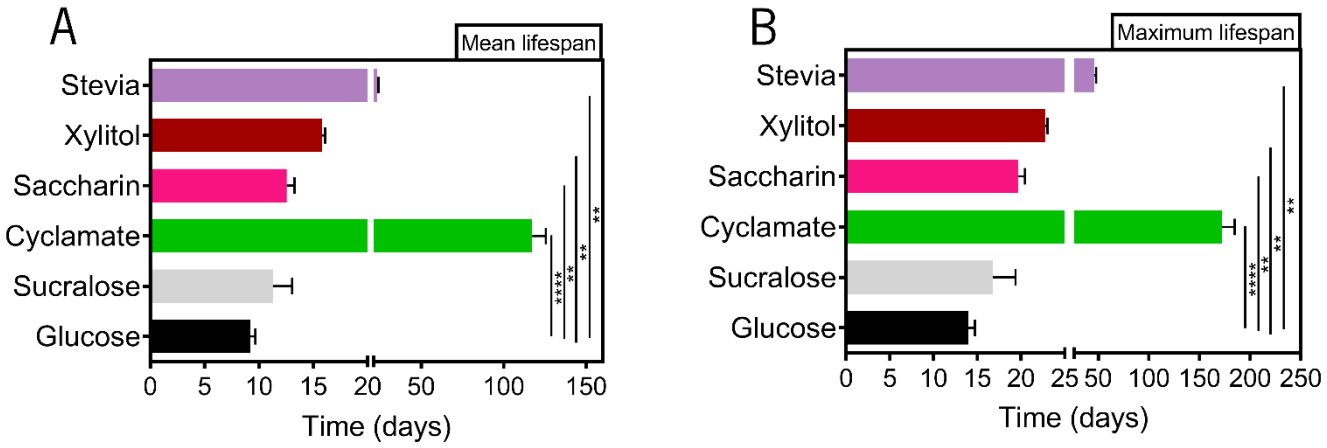

Figure 10: Determination of mean and maximum lifespans of cells. Data significance was determined by *t*-test (**p≤0.01; ***p≤0.001; ****p≤0.0001).

4.6 Evaluation of the sweeteners' metabolization by yeast cells during CLS

Mounting evidence suggested that in humans sweeteners can be metabolized in the large intestine by the commensal bacteria or by gastrointestinal enzymes, such as peptidases, and their remaining products are absorbed in the small intestine and excreted through urine [83]. Therefore, since herein we detected that medium supplementation with different sweeteners has impact of cell longevity, we decide to evaluate if yeast cells are capable of metabolized the tested sweeteners. For such purpose, high-performance liquid chromatography (HPLC), a well-established tool for the analysis of compounds without derivatization [84], was used to determine the amount of each sweetener in the medium, at 0, 3, 7 and 10 days of CLS. The identification of the components of a sample was made by comparing the chromatograms obtained with standards in which the pure sweetener is eluted under the same conditions as the sample to be analyzed, corresponding to a peak in a certain time called retention time, where the component is identified [85]. The standards were obtained by HPLC analysis of samples of sweeteners at different concentrations that allowed

the formation of a calibration curve (graph of the concentration of the component by the area of the peak obtained).

As expected, results demonstrated, as expected, that yeast cells are able to metabolize glucose, since during the days we observed a reduction of glucose concentration in the medium. In contrast, yeasts were not able to metabolize none of the tested sweeteners (Fig.11), suggesting that the effects promoted by these sweeteners must occurs though the modulation of signaling pathways. Nevertheless, this hypothesis needs further investigation.

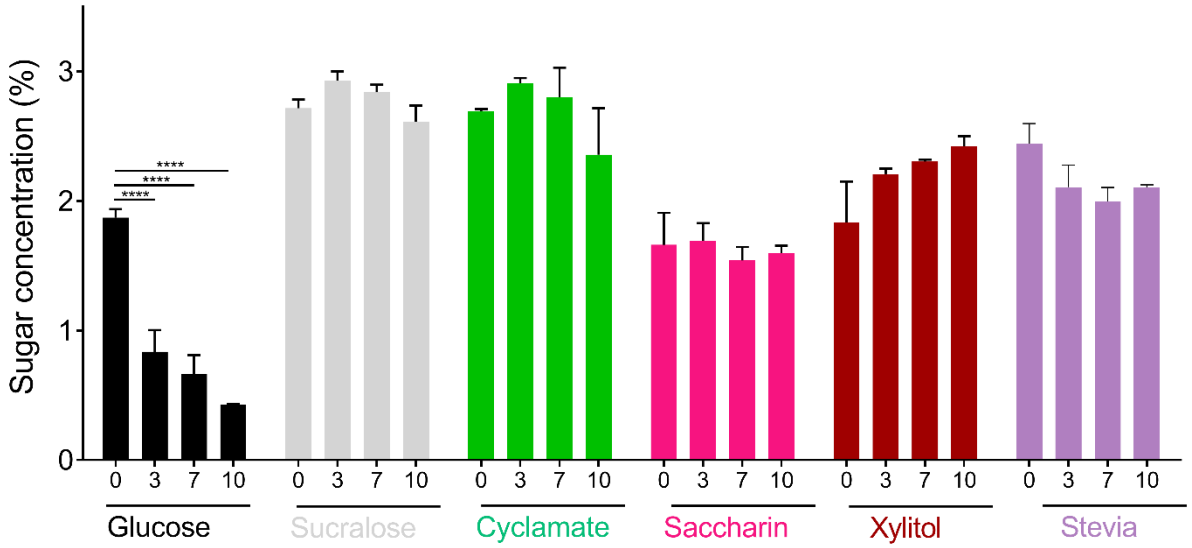

Figure 11: Graphic representation of the glucose and sweeteners concentrations during CLS. Percent concentration of sugar substitutes in yeast growth medium on days 0, 3, 7 and 10 of CLS cells compared to glucose control. The error bars represent the standard error of the mean (SEM). Data significance was determined by t-test ($p \leq 0.05$).

4.7 Evaluating the sweeteners' impact on cell-autonomous mechanisms

Next, we decided to explore how the supplementation with the selected sweeteners impacts in the cell-nonautonomous that are known to govern aging. To do that, at days 0, 3, 7 and 10 days of CLS, we collected cells to evaluate different mechanisms, namely, autophagy, accumulation of ROS and cell cycle.

The effects of sweeteners supplementation on autophagy flux were evaluated by the GFP-Atg8 processing assay, as above described (section 4.2). The results showed that in control conditions (glucose supplementation) there is an increase of autophagy flux along chronological aging (Fig. 12). Concerning the sweeteners supplementation, results showed that supplementation with sucralose, cyclamate or stevia also induced an increase of the autophagy flux during CLS (Fig.

12). In contrast, supplementation with saccharin or xylitol did not promote major alterations in autophagy flux during aging.

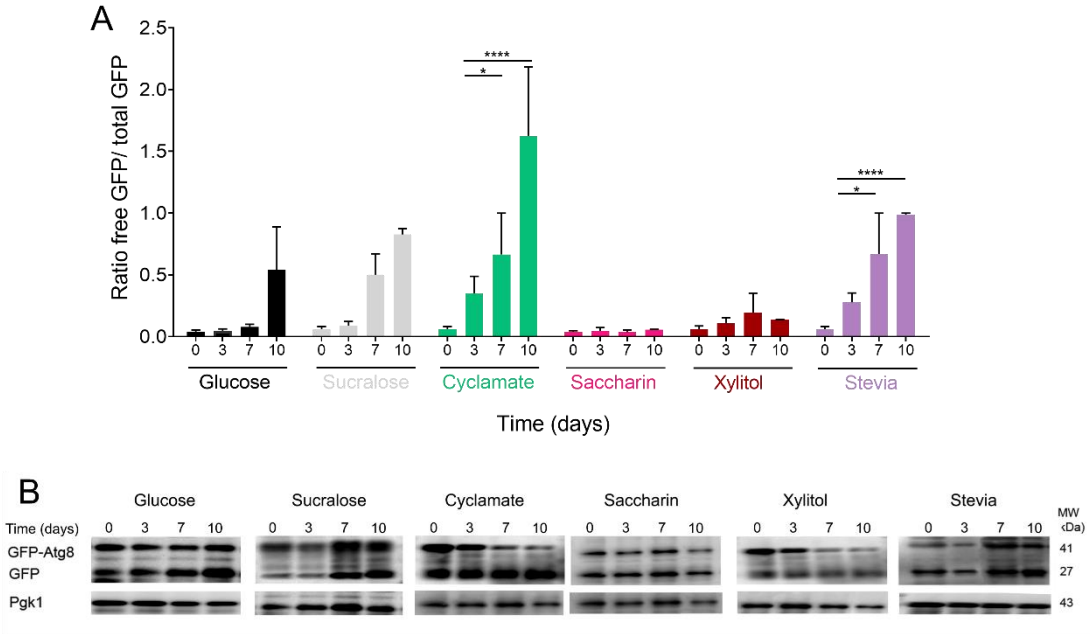

Figure 12: Graphical representation of the GFP-Atg8 processing assay during chronological lifespan (CLS). (A) Densitometric quantification of the ratio of free GFP to total GFP (free GFP + GFP-Atg8) and (B) representative blots of the GFP-Atg8 processing assay of BY4741 cells during the days 0, 3, 7 and 10 of the CLS supplemented with the control glucose or sucralose, cyclamate, saccharin, xylitol and stevia. Values indicate the mean ± SEM from three independent experiments. Significance of values was determined by two-way ANOVA (*$p \leq 0.05$; ****$p \leq 0.0001$).

In the last years, it has been shown that increased ROS may prolong lifespan in yeast and *C. elegans* and that genetic manipulations increasing some ROS, as hydrogen peroxide, do not accelerate aging [73]. Furthermore, it is also described that at certain levels, some ROS species could replenish cellular homeostasis [29, 81]. Therefore, due to the important role of specific ROS species in cellular aging, we decided to evaluate how sweeteners supplementation modulates the accumulation of ROS, particularly of H_2O_2 and O_2^{2-} as described to above in section 4.7. Results revealed that, control cells with glucose displayed higher ROS accumulation, particularly accumulation of O_2^{2-}, during CLS. The same profile, accumulation of ROS during aging, was displayed by cells supplemented with 2% of sucralose, saccharin or cyclamate (Fig.13). Interestingly, the supplementation with natural sweeteners, xylitol and stevia, did not result in a significant ROS accumulation during aging. Stevia supplementation led to the accumulation of increased levels of H_2O_2, early in aging, however we did not detect significant accumulation of the

more toxic ROS species - O_2^{2-}. Regarding stevia, it is important to highlight that this natural sweetener promoted an enhancement of longevity (Fig.13).

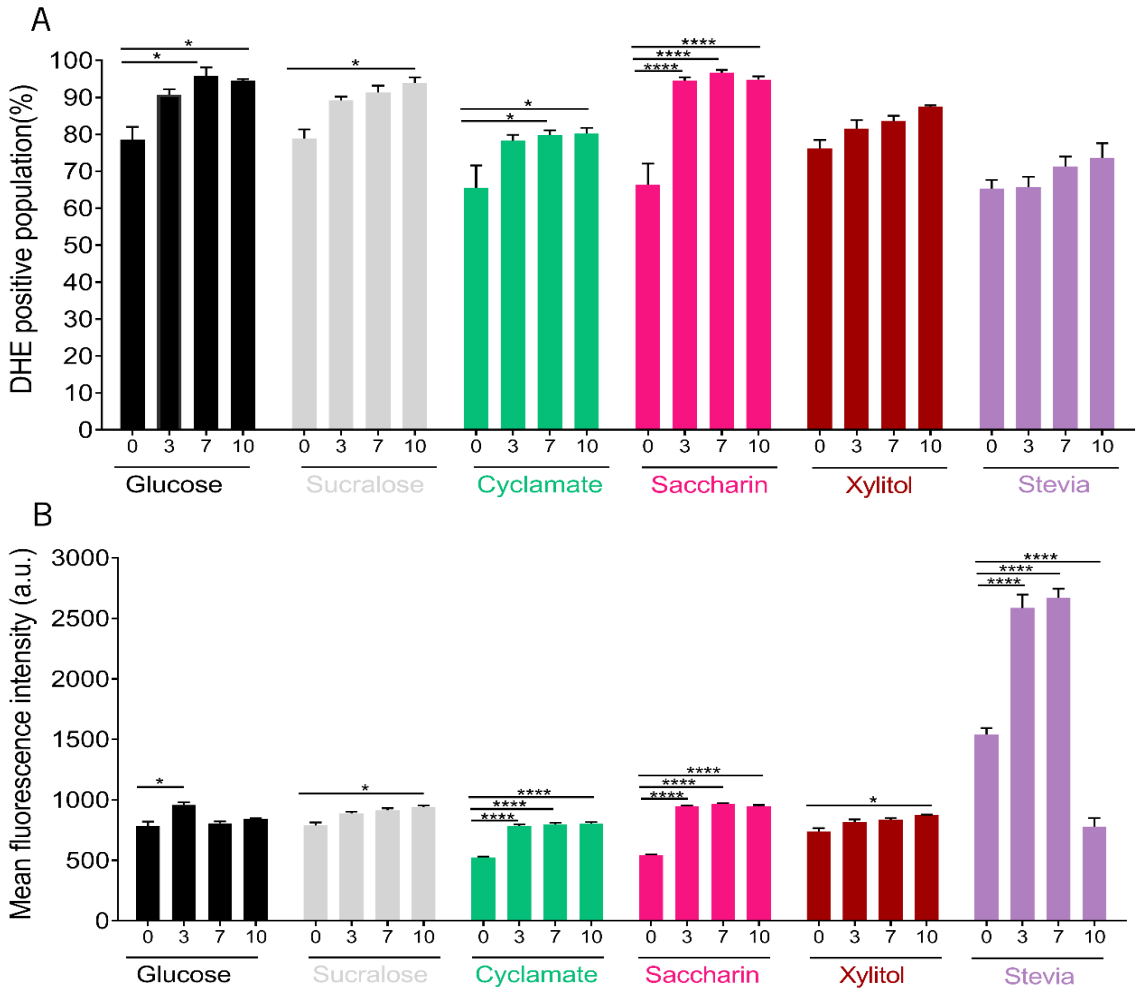

Figure 13: The accumulation of superoxide anion and hydrogen peroxide by yeast cells supplemented with glucose or sweeteners during CLS. FACS measurements of superoxide anions using the superoxide-specific probe DHE (A) and of hydrogen peroxide using the peroxides-specific probe DHR (B) in BY4741 cells supplemented with the control glucose or the sweeteners sucralose, cyclamate, saccharin, xylitol and stevia at concentration 2% from day 0 of CLS. Bar graphs indicate the percentage of cells exhibiting elevated levels of intracellular superoxide anion detected by FACS measurements (20.000 cells per sample in three independent experiments) of DHE-positive cells and indicate the DHR mean fluorescence. Values indicate the mean ± SEM from three independent experiments. Significance of values was determined by two-way ANOVA (*p≤0.05; ***p≤0.001; ****p≤0.0001).

To accomplish the analysis of some of the cell-autonomous mechanisms underlying the aging process, cell cycle profile was also determined. Supplementation with sweeteners did not induce major alterations in cell cycle profile when compared to cells supplemented with glucose (Fig.14). However, it is still possible to observe an increase in G2/M phases as observed in the survival measurements (section 4.4).

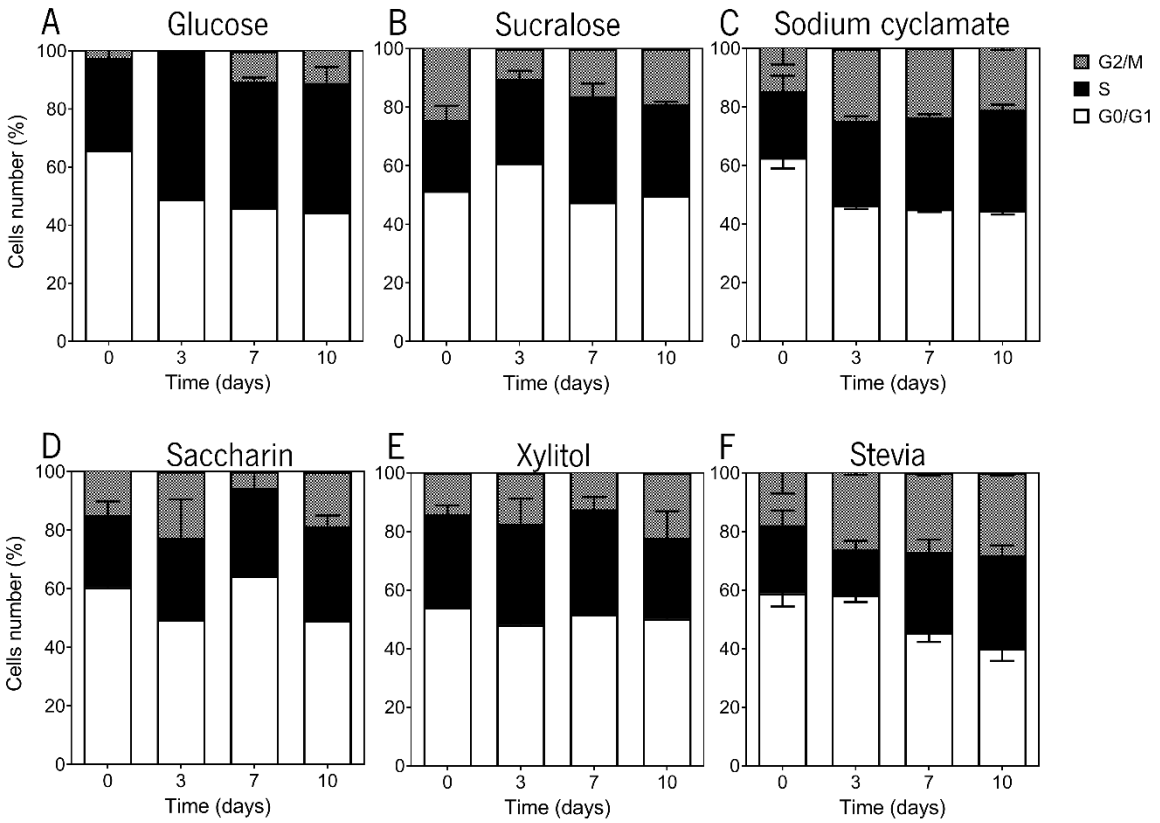

Figure 14: Cell cycle profile during CLS of cells supplemented with sweeteners. Cell cycle analysis evaluated by the measurement of DNA content by flow cytometry of wild-type cells (BY4741) supplemented with the sweeteners sucralose, cyclamate, saccharin, xylitol and stevia, and the control glucose stevia at concentration 2% from day 0 of CLS (days 0, 3, 7 and 10). The data represents mean ± SEM of three independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by two-way ANOVA ($p \leq 0.05$), being related to control graph.

CHAPTER 5

DISCUSSION

The negative impact associated with the consumption of sugar on weight and other metabolic-related diseases has been widely recognized. To counteract these negative effects, people switch from sugar to sweeteners or substitute sugars like sucralose, sodium cyclamate, saccharin, xylitol and stevia. Nevertheless, little is known about the health or toxic effects derived from the consumption of these sweeteners.

Sweeteners are compounds used for the formulation of noncaloric and sugar-free products. In the literature it is described a complex relationship between diseases and sweeteners that needs to be extensively studied for a better understanding. Therefore, in this project, we decided to explore the effects associated with the supplementation of sweeteners, namely, sucralose, sodium cyclamate, saccharin, xylitol and stevia on yeast survival and chronological aging.

Altogether, the data herein obtained is summarized in the table 2. A distinct cellular response to the different used sweetener was observed, in agreement with the literature describing that artificial sweeteners maintain the same palatability but their metabolic routes are different [86].

Table 2: Summary of the results obtained in this project. The summarized data was performed in comparison with results obtained with glucose (- means decrease; + means increase; = means similar)

	Sweetener / Parameters		Sucralose	Sodium Cyclamate	Saccharin	Xylitol	Stevia
	Survival	Exponential	=	+	-	-	+
		Stationary	-	-	-	-	-
	Autophagy	Exponential	+	-	+	-	-
		Stationary	+	-	+	-	-
	ROS accumulation	Exponential	-	+	+	-	-
		Stationary	-	-	+	-	-
	Cell cycle	Exponential	=	Reduction of S-phase arrest	Reduction of S-phase arrest	Reduction of S-phase arrest	Reduction of S-phase arrest
		Stationary	=	Reduction of S-phase arrest	Reduction of S-phase arrest	Reduction of S-phase arrest	Reduction of S-phase arrest
Chronological lifespan	**Longevity**		=	+++	+	+	+
	Autophagy		=/+	+	-	-	+
	ROS accumulation		=	=	=	-	-
	Cell cycle		=	Increased G2/M phases arrest	Increased G2/M phases arrest	Increased G2/M phases arrest	Increased G2/M phases arrest

Concerning the sweeteners effects on survival, globally data herein presented showed that short time supplementation with the selected sweeteners present toxic cellular responses in comparison with glucose, independently of the tested concentrations, since we observed a decreased survival rate, particularly in aged supplemented cells. Furthermore, these toxic effects were promoted by the interaction with distinct pathways, which are sweetener-dependent. In the literature is already possible to find some studies questioning the beneficial effects of the sweeteners (reviewed in [87]).

Concerning sucralose, this is the sweetener that presents a cellular response pattern more similar with glucose. The distinct effect is observed for autophagy activity, which is increased under sucralose supplementation. In the literature, it is already described that in a fly model, a prolonged ingestion of a sucralose-sweetened diet triggers the activation of neuronal AMPK [88], which could consequently induce autophagy. Further studies must be done to confirm the involvement of the Snf1 (yeast homologue of AMPK) in autophagy enhancement. However, the activation of this pathway underlies a toxic response that culminate in decreased survival. In addition, it was also described that in mesenchymal stromal cells, sucralose supplementation is associated with accumulation of ROS and decreased survival [89].

Saccharin supplementation was accompanied by increased autophagy and accumulation of ROS, which reflect decreased survival. Previous studies in a rat model already demonstrated that administration of saccharin increased oxidative status of the liver [90]. Herein, we further showed that apparently autophagy is also involved in the promotion of toxicity. Further studies must be performed to explore the contribution of autophagy to the detected toxicity.

Interestingly, with the exception of sucralose, the supplementation with the sodium cyclamate, saccharin, xylitol or stevia has an impact on cell cycle in comparison with the cell's response to glucose supplementation. With the sweeteners supplementation we detected a reduction in the number of cells arresting in S-phase and increment of cells compatible with cell cycle arrest in two different checkpoints, G0/G1 and G2/M phase. The significance of these effects must be further studies.

Aging is a complex biological process that affects living organisms from the simpler unicellular yeast to higher eukaryotes. It is regulated by highly conserved nutrient and energy signaling networks, orchestrated by master longevity regulators. Furthermore, genetic, pharmacological, or dietary interventions on these conserved pathways enabled for a better understanding of the aging biological phenomenon.

Regarding the studies on aging effects, our experiments are novel and for the first time, we established possible pathways of how sweeteners supplementation may impact on aging progression. Regarding the impact of sweeteners supplementation on the yeast CLS, data herein presented showed that again, the effects on aging are sweetener-dependent. Our data suggested that none of the tested sweetener are metabolized by yeast cell in these experimental conditions. These data were already expected since it is described in the literature (as described in introduction) that they are not metabolized by the human body. Furthermore, considering that the sweeteners were added at time 0 of CLS, it is suggested that the cells used ethanol, a product of fermentation metabolism, as the favored carbon source [84]. In this sense, these data suggests that these sweeteners are able to directly target distinct molecules on the cells, and/or, as already described in the literature, they could affect glucose metabolism or other signaling pathways [91]. Further studies must be accomplished to clarify this aspect.

During CLS, in relation to sucralose, we showed that the effects of its supplementation are similar to those showed by cells supplemented with glucose. Furthermore, supplementation with saccharin or xylitol promoted a slight longevity extension. Interestingly, medium supplementation with sodium cyclamate or stevia increased the cells longevity in comparison to the CLS presented by cells under glucose supplementation. Importantly, the CLS presented by cells supplemented with cyclamate was about 10 times higher. Apparently, this increased longevity was accompanied by enhanced autophagy flux and decreased accumulation of ROS. In addition, cell cycle analysis point to an enhancement of cell proliferation that could also underlay the observed extended longevity. Although, we demonstrated that these sweeteners are not metabolized by cells, they probably are signaling the activation of several mechanisms, including autophagy or ROS detoxification. Indeed, in mammalian cells there is already described a sweet-taste receptor (STR) that has been implicated in a signaling cascade capable to activate metabolism [83]. However, to better understand this connection, particularly between supplementation sodium cyclamate or stevia with longevity, further studies must be performed, since this was the first time that these connections were established.

Sweeteners are used in various food and beverages, and they are very popular in most of the countries. Some research has demonstrated the safety of some sweeteners currently approved. Nevertheless, new research has emerged questioning the safety of these artificial sugars. Thus, further exploration is required with well-designed large-scale studies to better understand the effects of promoted by the use of these sweeteners.

CHAPTER 6

CONCLUSION

From the data generated in this study in which we explored the impact of sweeteners' supplementation on the chronological lifespan of yeast and evaluated the mechanisms triggered by their supplementation, we can conclude that sodium cyclamate, despite being an artificial sweetener was the one that presented the best result in the extension of CLS. However, the effects on longevity were not similar for the other sweeteners, although they also display a trend to increase lifespan, the natural sweeteners did not stand out. But regarding increasing the average and maximum lifespan of the yeast, cyclamate, xylitol or stevia supplementations showed the best results. Up to our knowledge, this is the first time that the effects of sweetener supplementation are studied in an aging cellular model, there is still a long way to go on this path, and further studies must be performed.

In conclusions, we showed that each sweetener has its own cellular response profile, therefore, it is not possible to generalize the conclusions to all the sweeteners. Furthermore, we believe that this work can lead to future investigations that aim to understand how the supplementation of sweeteners can affect the mechanisms related to cellular aging given that these food supplements are so common in human food today and that need to be further investigated.

CHAPTER 7

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