Restrição calórica e extrato de *Spirulina platensis* contra íons ferrosos (Fe\(^{2+}\)) no envelhecimento de células de *Saccharomyces cerevisiae* deletadas do gene SIR2

Caloric restriction and *Spirulina platensis* extract against ferrous ion (Fe\(^{2+}\)) in the aging of *Saccharomyces cerevisiae* cells deleted to the SIR2 gene

Restricción calólica y extracto de *Spirulina platensis* contra el ión ferroso (Fe\(^{2+}\)) en el envejecimiento de células de *Saccharomyces cerevisiae* eliminadas al gen SIR2

Resumo

O processo de envelhecimento é agravado pela presença de uma alta carga de estresse oxidativo associado ao desequilíbrio do corpo em relação a certos metais, com ênfase no ferro. O extrato de *Spirulina platensis* (SP) e a restrição calórica (CR) são intervenções nutricionais capazes de mitigar os efeitos de doenças relacionadas ao envelhecimento. O objetivo deste estudo foi determinar os efeitos de SP e CR contra íons ferrosos no envelhecimento de *Saccharomyces cerevisiae* deletada do gene SIR2. O estudo foi realizado a partir de pesquisa laboratorial quantitativa, utilizando cepas padrão de Saccharomyces cerevisiae (WT) e sir2Δ.
cultivadas em meio YPD de glicose a 2% ou 0,5% (CR), expostas a 0,8 mg / mL de SP e 1mM de Fe²⁺. A viabilidade celular e a lipoperoxidação foram analisadas. Os resultados mostraram sobrevivência celular reduzida e aumento da peroxidação lipídica na deleção do gene SIR2. Resultados estatisticamente significativos foram encontrados após o envelhecimento nos tratamentos WT, SP, CR, SP + Fe²⁺, CR + Fe²⁺. As terapias CR e SP mostraram efeito protetor contra íons ferrosos.

**Palavras-chave:** Antioxidantes; Restrição calórica; Ferro; SIR2; Sirtuinhas; *Spirulina platensis*.

**Abstract**

The aging process is aggravated by the presence of a high load of oxidative stress associated with the body's imbalance concerning certain metals, with emphasis on iron. *Spirulina platensis* extract (SP) and caloric restriction (CR) are nutritional interventions capable to mitigate the effects of aging-related diseases. The objective of this study was to determine the effects of SP and CR against ferrous ion on the aging of *Saccharomyces cerevisiae* deleted of SIR2 gene. The study was carried out based on quantitative laboratory research, using standard strains *Saccharomyces cerevisiae* (WT) and sir2Δ strains, cultured in 2% or 0.5% (CR) glucose YPD media, whether exposed to 0.8 mg/mL SP and 1mM Fe²⁺. Cell viability and lipoperoxidation were analyzed. Results showed reduced cell survival and increased lipid peroxidation in the SIR2 gene deletion. Statistically significant results were found after aging for WT, SP, CR, SP + Fe²⁺, CR + Fe²⁺ treatments. The therapies CR and SP showed a protective effect against ferrous ion.

**Keywords:** Antioxidants; Caloric restriction; Iron; SIR2; Sirtuins; *Spirulina platensis*.

**Resumen**

El proceso de envejecimiento se ve agravado por la presencia de una alta carga de estrés oxidativo asociado con el desequilibrio del cuerpo con respecto a ciertos metales, con énfasis en el hierro. El extracto de *Spirulina platensis* (SP) y la restricción calórica (CR) son intervenciones nutricionales capaces de mitigar los efectos de las enfermedades relacionadas con el envejecimiento. El objetivo de este estudio fue determinar los efectos de SP y CR contra los iones ferrosos sobre el envejecimiento de *Saccharomyces cerevisiae* eliminado del gen SIR2. El estudio se realizó en base a la investigación cuantitativa de laboratorio, utilizando cepas estándar de *Saccharomyces cerevisiae* (WT) y sir2Δ, cultivadas en medio de glucosa YPD al 2% o 0,5% (CR), ya sea expuesto a 0,8 mg / ml SP y Fe²⁺ 1 mM. Se
analizaron la viabilidad celular y la lipoperoxidación. Los resultados mostraron una supervivencia celular reducida y un aumento de la peroxidación lipídica en la deleción del gen SIR2. Se encontraron resultados estadísticamente significativos después del envejecimiento para los tratamientos WT, SP, CR, SP + Fe$^{2+}$, CR + Fe$^{2+}$. Las terapias CR y SP mostraron un efecto protector contra los iones ferrosos.

Palabras clave: Antioxidantes; Restricción calórica; Planchar; SIR2; Sirtuinas; _Spirulina platensis_.

1. Introduction

Aging is a complex process that involves genetic, physiological and stochastic factors. In this process, there is an accumulation of damaged lipids, proteins, and DNA that lead to cell or organ dysfunction (Rusu, et al., 2019; da Costa, et al., 2016). Oxidative stress plays an important role in aging, acting in the regulation and signaling of oxidation reactions. However, oxidative stress at high loads is an aggravating factor in the development of neurodegeneration and the emergence of different disorders (Pascual-Geler, et al., 2019; Sies, 2019; Wojtunik-Kulesza, 2019). Labile iron is one of the compounds that lead to oxidative stress because it catalyzes the formation of highly reactive species (Galaris, et al., 2019). The presence of these metal ions, especially in the brain, can trigger physiological changes that compromise neurons, resulting in neurodegeneration and, consequently, the development of diseases such as Alzheimer, Parkinson and Huntington (Wojtunik-Kulesza, 2019).

The aging process and its disorders over time can be alleviated by nutritional interventions, such as the use of bioactive compounds or caloric restriction, which are associated with the prevention of noncommunicable chronic diseases (NCDs) (Silva, et al., 2019). _Spirulina platensis_ is a bioactive compound with antioxidant, anti-inflammatory, neuroprotective, immunomodulation, antiviral, anticancer, cholesterol-lowering and anti-diabetes properties (Wu, et al., 2016; Galal, et al., 2019). _Spirulina_’s antioxidant system is emphasized in association with phycobiliprotein, C-phycocyanin and allophycocyanin, due to its ability to remove reactive oxygen species (ROS) that cause damage to cells via oxidative stress induction (Wu, et al., 2016; Bertolin, et al., 2017; Galal, et al., 2019).

Calorie restriction, defined as a 10-50% reduction in caloric intake, is another important therapy for healthy and long-lived aging (McCay, 1935; Pifferi & Aujard, 2019). Longevity and carcinogenic reduction promoted by caloric restriction occur due to its action to activate some signaling pathways, such as DNA-PK, and to increase the activity of sirtuin
family proteins. Sirtuins consist of NAD+-dependent deacetylases protein (Nicotinamide Adenine Dinucleotide) that are responsible for the regulation of various cell pathways, including aging progression, primarily by SIRT1 (Libert & Guarente, 2013; Lu, et al., 2019).

SIRT1 is the closest ortholog to *Saccharomyces cerevisiae’s* Sir2, the key modulator of chronological and replicative aging. However, dependence on Sir2 is questioned, as Sir2 activity promotes replicative life but does not promote chronological life (Orlandi, Coppola, et al., 2017; Orlandi, Stamerra, et al. 2017).

Given these perspectives regarding the aging process, we sought to investigate the role of *Spirulina platensis* extract and caloric restriction against ferrous ion (Fe²⁺) in *Saccharomyces cerevisiae* cells deleted to SIR2 gene.

2. Material and Methods

This study is a quantitative laboratory research with methodological support from Pereira et al. (2018).

*Saccharomyces cerevisiae strains*

The *Saccharomyces cerevisiae* strains used were WT strain BY4741 (MATa; his3; leu2; met15; ura3) and SIR2 mutant isogenic to BY4741 except for SIR2 gene, obtained from Euroscarf, Frankfurt, Germany. Strain stocks were maintained in 2% solid YPD medium (1% yeast extract, 2% glucose, 2% peptone and 2% agar) under appropriate conditions to avoid suppressor selection. In the case of mutant strains, the media also contained 0.02% geneticin. For the development of the experiments, cells were grown to half of the exponential phase (0.8 mg dry weight/ml) in 2% YPD or 0.5% liquid medium at 28 °C and 160 rpm, with an average volume ratio 5:1 in the bottle.

*Cellular survival*

According to the method of Manarinno et al. (2008), cell viability was analyzed by triplicate plating on solid YPD medium from appropriate dilution of 400 μg of collected cells. The plates were incubated at 28 °C for 72 h, and then counted. Cell survival was expressed as the percentage of viable cells that survive after exposure to treatments.
Cytotoxicity of Spirulina platensis extract and ferrous ion (Fe²⁺)

The cytotoxicity of Spirulina platensis extract and ferrous ion (Fe²⁺) was tested in Saccharomyces cerevisiae (WT) control cells by cell survival rate, described in item 4.2. Cells were cultivated for 1 h at 28 °C in YPD medium enriched with different concentrations of Spirulina platensis extract (0.08, 0.4 and 0.8 mg/ml) or iron sulfate II (0.5, 1 and 4 mM).

Growing conditions

Yeast cells were cultivated for 1 h at 28 °C under agitation at 160 rpm in the different treatments studied: Standard (YPD - 2% glucose), CR (YPD - 0.5% glucose), standard + Fe²⁺, standard + Spirulina platensis extract, CR + Fe²⁺ and standard + Spirulina platensis extract + Fe²⁺, and then cell survival, as described in item 4.2.

Chronological aging simulation

After cultivation under different growth conditions, the cells were subjected to chronological aging, which consisted of the suspension of 30 mg of centrifuged cells (4000 rpm, 5 min), resuspended in sterile distilled water and centrifuged again (repeated process twice), according to Manarinno et al. (2008). The washed cells were resuspended in 10 mL of sterile water and incubated at 37 °C / 160 rpm for 24h and subsequently determined the cell survival rate, described in section 4.2.

Lipid peroxidation

Lipid peroxidation was determined by the TBARS method (Steels, Learmonth & Watson, 1994). About 50 mg of cells were harvested by centrifugation (600g, 2 min) before and after aging. The cells were washed with ice water and resuspended in 0.5 mL 10% (w/v) trichloroacetic (TCA). The samples were lysed for six cycles of 20 seconds with vortex agitation. The decanted solid was resuspended in TCA and centrifuged. The supernatant was mixed with 0.1 mL of 0.1 M EDTA and 0.6 mL of 1% (w / v) thiobarbituric acid (TBA) at 0.05 M NaOH. The reaction mixture was incubated at 100°C for 15 min and the absorbance of the samples was measured at 532 nm. TBARS concentration was calculated using standard
tetraethoxypropane curve. Tetraethoxypropane is a malonaldehyde precursor (MDA) and results were expressed as nmol MDA·mg⁻¹ cells.

**Statistical analysis**

The results presented in this study are the average of at least three independent experiments and are represented by mean ± standard deviation. Differences between treatments were analyzed by analysis of variance ANOVA and Student’s t (p < 0.05).

**3. Results and Discussion**

**Cytotoxicity of Spirulina platensis extract and ferrous ion (Fe²⁺)**

The different concentrations of *Spirulina platensis* extract tested did not cause cytotoxicity in the control cells. Bertolin, et al. (2017) also observed that the protective effects of phycocyanin on yeast cells are directly proportional to their concentration in the medium. Thus, to evaluate the protective effect of *Spirulina platensis* extract on yeast cells, the highest concentration tested (0.8 mg/mL) was selected.

Regarding ferrous ion, there was a significant decrease in cell survival rate (p < 0.05). This behavior was observed in relation to the increased concentration of ferrous ions in the culture medium. This fact is related to the oxidative stress caused by the accumulation of ferrous ions (Galaris, et al., 2019; Marques, et al., 2019; Nakamura, et al., 2019). Therefore, it was decided to use the concentration of 1 mM Fe²⁺ as a stressor, since at this concentration a moderate effect on cells was observed.

**Caloric restriction dependence on the SIR2 gene**

Figure 1 shows that the sir2Δ strain, compared to the control strain (WT), showed a significant reduction of 20% and 11% in cell survival before aging when submitted to CR (p = 0.027) and CR + Fe²⁺ treatments (p = 0.09), respectively.
Cell survival results, after 24 h aging, are shown in Figure 1. The strain deleted for the SIR2 gene showed a 16% and 11% reduction in viable cell count when compared to WT for the CR (p = 0.01) and CR + Fe^{2+} (p = 0.06) treatments, respectively. Caloric restriction treatment had a positive effect on viable cell count. Standard treatment showed a 36% drop in viable cells for the WT strain and 41% for the sir2Δ strain, whereas in the CR treatment there was an 8% and 3% drop in the WT and sir2Δ strains, respectively, showing significant gain in viable cell count after aging when CR treatment is used.

However, a different behavior was observed when using a medium in oxidative stress. An 8% decrease in cell survival after aging for both strains was observed when using Fe^{2+} treatment, whereas in CR + Fe^{2+} treatment a decrease of 23% and 13% was observed for WT and sir2Δ strains, respectively. Despite this behavior, the final survival percentage for WT cells undergoing CR + Fe^{2+} treatment was significantly higher than Fe^{2+} treatment, showing 44% and 38% cell survival, respectively. For the sir2Δ strain no significant difference was observed. Study by Matsuo et. al. (2017) observed that under oxidative stress, iron supplementation increased the growth of a Saccharomyces cerevisiae culture.

The effect of Sir2 on caloric restriction becomes more evident when we look at the results in Figure 2.
Sir2Δ strain showed higher relative lipid peroxidation compared to the WT strain before and after aging for the CR and CR + Fe²⁺ treatments. When comparing lipid peroxidation data between Standard and CR treatments, we did not observe significant difference, however when using a medium in oxidative stress there is a significant difference between treatments. In Fe²⁺ treatment the relative lipid peroxidation values are 3.35 and 4.06 for WT and sir2Δ strains, respectively. When applying CR treatment, the values are only 2.52 and 2.73 for the WT and sir2Δ strains, respectively. Higher values for lipid peroxidation obtained by Fe²⁺ treatment indicate that CR treatment was able to reduce lipoperoxidation.

**Spirulina platensis extract on cell survival**

Two yeast models (WT and sir2Δ) were used to evaluate the effect of *Spirulina platensis* extract treatment on cell survival under normal and oxidative stress conditions.

Survival rate of sir2Δ and WT strains before aging showed no significant difference (p > 0.05). However, when we analyzed Figure 3, regarding the survival rate after aging, we observed that the WT strains presented higher survival percentage than the mutant strain for the *Spirulina platensis* and *Spirulina + Fe²⁺* treatments, being 27% and 13% higher, respectively. This behavior indicates that the *SIR2* gene plays an important role in the cell survival of *Saccharomyces cerevisiae*.
Figure 3. Cell survival results of *Saccharomyces cerevisiae* control (WT) and sir2Δ mutant strains submitted or not to adaptive treatments with *Spirulina platensis* extract.

![Cell survival results of *Saccharomyces cerevisiae* control (WT) and sir2Δ mutant strains submitted or not to adaptive treatments with *Spirulina platensis* extract.](image)

Source: Author. Cell survival was expressed as the percentage of colony forming units and mean lipid peroxidation before aging and after 24 h of aging. Results represent the mean ± deviation of at least three independent experiments.

When comparing the treatments with *Spirulina* and the control treatments (Standard and Fe²⁺), there was an increase in the survival rate. The survival percentage after aging decreased by 0.73% and 22% from the initial percentage for the WT and sir2Δ strains, respectively, when using treatment with *Spirulina platensis* extract. In the *Spirulina* + Fe²⁺ treatment, the decrease was 14% and 18% for the WT and sir2Δ strains, respectively, being a higher value than the Fe²⁺ treatment. However, the final survival percentage was higher for *Spirulina* + Fe²⁺ treatment, reaching values of 67% for WT strain and 58% for sir2Δ strain, a behavior like that observed with the CR treatment.

The data agree with the lipoperoxidation assay (Figure 4), where a reduction in lipid peroxidation was observed when cells subjected to oxidative stress were treated with *Spirulina platensis* extract. The values were 2.31% and 2.76% for WT and sir2Δ strains, respectively. This fact highlights the ability of *Spirulina platensis* extract to scavenge free radicals.
Figure 4. Lipid peroxidation results of *Saccharomyces cerevisiae* control (WT) and sir2Δ mutant strains submitted or not to adaptive treatments with *Spirulina platensis* extract.

Source: Author. Results represent the mean ± deviation of at least three independent experiments.

**Discussion**

Increased in life expectancy, united with improved quality of life, creates a high demand for innovative therapies against age-related diseases. Thus, the possibility of mitigating human diseases through the understanding of new treatments or food substances with functional properties is very present in the scientific community. In this study, a *Saccharomyces cerevisiae* yeast model was used to explore the potential of *Spirulina platensis* extract and CR treatment against cell aging and to understand the role of the *SIR2* gene. We observed that both treatments, even in a stressful environment, showed an improvement in the resistance of yeast cells to aging, and the WT strains containing the *SIR2* gene showed the best results.

The significant difference between WT and sir2Δ strains is related to the mechanism involved in caloric restriction treatment. The *SIR2* gene mediates the beneficial effects of caloric restriction through mechanisms involving metabolic controls in stress responses to oxidative damage (Burnett, et al., 2011). This mediating effect of Sir2 requires the synthesis of NAD+. Under reduced glucose conditions, *S. cerevisiae* metabolism changes from fermentation to respiration, resulting in a positive NAD+/NADH ratio (Kaeberin, et al., 2005). Sirtuins share a highly conserved catalytic domain consisting of distinct regions that bind NAD+ and protein substrate and are sensitive to cellular NAD+ levels. As NAD+ levels
decrease with aging, NAD+-dependent sirtuins decrease in activity. (Ondracek, et al., 2017). The SIR2 gene promotes replicative shelf life by suppressing homologous recombination between rDNA repeats and the subsequent formation of extrachromosomal rDNA circles (Kaeberin, Mcvey & Guarante, 1999).

A study by Kang et al. (2014) shows that the regulation of oxidative stress by the SIR2 gene is dependent on the cell growth phase, and in the exponential phase the deletion of the SIR2 gene reduces yeast cell resistance to hydrogen peroxide when compared to a control strain (WT) but the resistance increases in the postdiaux and stationary phases. This effect agrees with our findings where cells were cultivated up to mid-exponential phase and in all cases, we observed that control yeast (WT) cells had a higher survival rate. However, even with deletion of the SIR2 gene, yeast cells showed a gain in cell survival and lower lipoperoxidation when compared to control treatments (Standard and Fe²⁺). This fact indicates that part of the protective effect of caloric restriction treatment is due to other factors. Studies show that caloric restriction promotes a change in aerobic metabolism resulting in more efficient electron transport in the mitochondrial respiratory chain, which may lead to a reduction in endogenous ROS production while simultaneously increasing the amount and efficiency of endogenous antioxidant enzyme activity. Furthermore, CR modulates the lipid composition of membranes, decreasing oxidative damage (Pallavi, Giorgio & Pellici, 2012).

Studies by Bertolin et al. (2017) observed that phycocyanin, a pigment present in Spirulina extract, significantly reduces the level of lipid peroxidation in Saccharomyces cerevisiae cultivation. The inhibitory effect on lipid peroxidation may be related to phycocyanin's ability to eliminate hydroxyl radicals, which are the initiators of lipid peroxidation due to their nucleophilic structure (Bertolin, et al., 2017). Other studies have shown that phycocyanin isolated from Spirulina extract is able to bind Fe²⁺ (FeSO₄) and Fe³⁺ (FeCl₃) (Yu, et al., 2002; Bermejo, et al., 2008) and thus avoid the formation of free radicals by iron ions. In addition, Spirulina platensis contains phenolic acids, tocopherols, beta carotene and other vitamins and minerals that are known to exhibit antioxidant properties and eliminate hydroxyl, alkoxy and peroxyl radicals (Miranda, et al., 1998; Abdel-Daim, et al., 2013). Therefore, Spirulina platensis has functional properties that can act to delay disorders related to aging.

For future work, we suggest in vivo studies combining the two therapies used in this work, to maximize the benefits presented.
4. Final Considerations

Observations that treatment using caloric restriction and *Spirulina platensis* extract can improve the cellular survival of a yeast model when subjected to an aging process make these therapies extremely promising in combating aging-related disorders. Therefore, the data presented here open new avenues for the development of preventive therapies against cell damage caused by cell aging and provide important information on the role of sirtuins.

**Conflict of interest**

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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**References**


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