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Use of *Saccharomyces cerevisiae* and *Caenorhabditis elegans* as Model Organisms To Study the Effect of Cocoa Polyphenols in the Resistance to Oxidative Stress

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ABSTRACT: Developing functional foods to improve the quality of life for elderly people has great economic and social impact. Searching for and validating ingredients with *in vivo* antioxidant effects is one of the key steps in developing this kind of food. Here we describe the combined use of simple biological models and transcriptomics to define the functional intracellular molecular targets of a polyphenol-enriched cocoa powder. Cocoa powder supplemented culture medium led to increased resistance to oxidative stress, in both the budding yeast *Saccharomyces cerevisiae* and the nematode *Caenorhabditis elegans*, and, in the latter, lifespan was also increased. These effects are fully dependent on the polyphenols present in the cocoa powder and on the sirtuins Hst3 (yeast) and SIR-2.1 (worm). The transcription factor DAF-16 also plays an important role in the case of the nematode, indicating that the insulin/IGF-1 (insulin-like growth factor) signaling pathway is related with the antioxidative effect of cocoa polyphenols. All in all, these results confirm that this polyphenol-enriched cocoa powder, with antioxidant activity, has great potential use as a functional food ingredient for elderly people. Furthermore, this work reveals the value of using simple biological models to screen for compounds that are of interest for the food and pharmacological industry.

KEYWORDS: *Caenorhabditis elegans*, cocoa polyphenols, functional food, longevity, *Saccharomyces cerevisiae*, sirtuins

INTRODUCTION

Functional foods and nutraceuticals is one of the fastest growing segments of the food industry. The market is driven by an aging population, rising health care costs, advances in food production technology and a growing understanding within the customer population of the link between diet and health.[1] It is estimated that by the year 2050 the elderly population (aged 65 or older) will be double that of children (aged 0−14) for the first time in history. The increase in the elderly population has already taken a toll on health care systems. Moreover, with the rapid growth of aging populations, the prevalence of age-associated diseases has increased, such as neurodegenerative diseases and diabetes. It is well documented that oxygen radicals contribute to aging and play a critical role in age-related disease[2] and that damage to DNA, protein and lipids increases with age in many organisms.[3,4] In this context, although drug development is necessary to solve specific health problems in the population, nutritional intervention using functional foods may be an effective way to improve quality of life. Consequently, studies into the effects of food ingredients with antioxidant properties are of great interest.

There are many studies demonstrating that different compounds or plant extracts with antioxidant properties may extend invertebrate lifespan.[5−8]

Cocoa derivatives are an important source of polyphenols, particularly procyanidins and flavan-3-ols. These polyphenols are of industrial importance as functional ingredients, having been shown to exert beneficial effects on health, particularly with respect to cardiovascular disease.[9−11] Flavonoid-rich cocoa and chocolate products have been proven to possess relevant biological activity.[12−14] Indeed, intake of flavonoid-rich cocoa enhances endothelial function and this property has been linked to the presence of polyphenols.[15,16] Recently, our research group has developed a cocoa powder with high flavonoid content by improving seed-processing methods. This cocoa powder has been obtained from nonfermented cocoa beans without roasting and submitted to a short heat treatment to rapidly inactivate polyphenol oxidase (PPO). Furthermore, satisfactory results have been obtained concerning its bioavailability in human volunteers;[17] therefore, this cocoa powder possesses interesting properties for use as a functional ingredient.

Recently new EU Regulation (EC) 1924/2006 has come into force on nutrition and health claims, regulating the claims concerning nutritional and health properties of foods. This Directive is based on the FUFOSE strategy developed within the European Framework Programs and demands as much scientific information as possible about the functional ingredient or food. To fulfill the directive, experimental evidence demonstrating the metabolic target of a specific ingredient must be presented. Therefore, companies that want to market functional foods should have reliable, rapid and inexpensive models for identification of these metabolic targets.

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Within this context it is important to establish which metabolic target the aforementioned flavonoid-rich cocoa powder acts upon. In order to carry out this evaluation, the yeast *Saccharomyces cerevisiae* and the nematode *Caenorhabditis elegans* have been used as model organisms. The reasons for using *S. cerevisiae* are based on the following: (i) it is easy to handle in the laboratory as millions of cells can be analyzed with just one culture plate; (ii) we have ample knowledge of the biochemistry and physiology of this microorganism; and (iii) its genome, which has been fully sequenced, contains just over 6000 genes and for each there are mutants for both function loss and gain. In fact, molecular knowledge of this yeast species has led to the conclusion that one out of every four of its genes has an orthologous gene in the human genome. For all these reasons, *S. cerevisiae* has become a model organism used to study many biological processes.[18] Furthermore, *C. elegans* has been widely used in aging studies for several reasons: (i) it is a multicellular organism with a fully sequenced genome,[19] (ii) there are available mutant strains for all its described open reading frames, and (iii) it has a short lifespan. This nematode is also revealed to have evolutionarily conserved pathways for aging.[20]

The aim of this work was to evaluate the antioxidative capacity of the aforementioned cocoa powder enriched with flavonoids. To do this, we have developed a protocol using *S. cerevisiae* to study the antioxidant effect of the cocoa powder both quantitatively and qualitatively. Furthermore, we performed several experiments with *C. elegans* to analyze the effect of cocoa antioxidants on lifespan extension. Finally, a genomic approach was performed with mutant strains in both organisms to elucidate the metabolic targets of this food ingredient. Results suggest that treatment with this cocoa powder confers protection against oxidative stress and this protection is mediated by sirtuin proteins through the insulin/IGF-1 signaling pathway.

MATERIALS AND METHODS

Strains and Maintenance Conditions. *S. cerevisiae* strains used in this study were the wild-type strain BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) and its isogenic mutants *hst3*Δ and *yvc1*Δ. In addition the strains BY4742 (MATa; his3D1; leu2D0; lys2D0; ura3D0) and YPH250 (*MAT*a; *ura3-52; lys2-801; ade2-101; trp1-*Δ*1; his3-* Δ*200; leu2-*Δ*1*) were also tested. All strains were obtained from the European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF) and maintained and cultured in YPD medium (2% glucose, 1% yeast extract and 2% peptone) or YNB (Pronadisa).

C. elegans strains N2, Bristol (wild-type); GR1307, *daf-16* (mgDf50); VC199, *sir-2.1* (ok434); and *daf-2* (e1370) were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota and maintained at 20 °C on nematode growth medium (NGM). Strain *Escherichia coli* OP50 used as normal diet for nematodes was requested from the *Caenorhabditis* Genetics Center.

Cocoa Powder Samples. Two different cocoa powders have been used in this study: a natural (conventional) cocoa powder and a flavonoid-enriched cocoa powder. The nutritional profile of both cocoa powders is very similar (data not shown); however the flavonoid-enriched cocoa powder is characterized by having an increased total polyphenol content (12%) compared with conventional cocoa powder (4%). Specifically catechin, epicatechin and total procyanidin levels were found to be significantly higher in this flavonoid-enriched powder (see ref 17 for a more detailed analysis on the polyphenol profile content of both products).

In the experiments with *S. cerevisiae*, polyphenol-enriched cocoa powder was added to the YPD medium at different final concentrations (0.5−8 mg/mL) in order to define the optimal antioxidant dose. Additionally, the resistance to oxidative stress of the polyphenol-enriched cocoa powder was compared with a conventional cocoa powder, not enriched in polyphenols (containing 4% of polyphenols) in order to study the beneficial effect of the polyphenols on the yeast model. Furthermore, the flavonoid-enriched cocoa powder was treated with a commercial protease (15 U/mL, Neutrase, Novozymes) to test the potential increase in the antioxidant effect. After treatment (1 h, 40 °C), the cocoa powder solution was divided into two aliquots, one of which was boiled for enzyme inactivation. Both solutions were used to evaluate yeast survival rates after oxidative stress, using YPD as control medium.

Bioassays using the nematode *C. elegans* were performed in NGM (nematode growth medium; 3 g/LNaCL, 2.5 g/L peptone, 5 g/L cholesterol, 1 M $MgSO_4$, 1 M KPO_4, buffer pH 6.0) agar plates supplemented with the polyphenol-enriched cocoa powder at a final concentration of 4 mg/mL.

***In Vivo* Antioxidant Analysis.** To measure survival after oxidative stress in *S. cerevisiae*, yeast cells were inoculated in fresh liquid YPD medium, with or without the polyphenol-enriched cocoa powder, and incubated for 16 h at 28 °C until reaching 1 unit of OD_{600}. To select the lowest cocoa powder dose that could enhance yeast survival after oxidative stress, cells were exposed to various increasing concentrations of the cocoa powder (0.5−8 mg/mL). Cells were harvested by centrifugation at 3000 rpm for 10 min, rinsed twice in phosphate buffer, and diluted to a final $OD_{600\ nm}$ of 0.3 in phosphate buffer. Both type of cultures, with or without cocoa powder, were subjected to oxidative stress. To do this, the cell suspensions were treated with H_2O_2 (2 and 3 mM) for 1 h at 30 °C with constant shaking (200 rpm). Cells were then collected and washed twice with phosphate buffer and serially diluted before plating them in YPD agar. Viable colonies were counted after 48 h at 30 °C. Furthermore, qualitative analysis of viability was carried out by spot assays. Each cell suspension (5 μL of direct culture and three serial dilutions) was spotted on YPD agar, and after incubation for 48 h at 30 °C, colony density in each drop was observed. All experiments were performed independently in triplicate, and the results were statistically analyzed.

To measure *C. elegans* survival rates after exposure to oxidative stress, we employed synchronized eggs, hatched in NGM (nematode growth medium), on agar plates containing the *E. coli* OP50 strain, in the presence or absence of polyphenol-enriched cocoa powder (4 mg/mL). After 5 days of growth at 20 °C, the worms were transferred to NGM plates containing 2 mM H_2O_2 and left for 5 h. The animals were then washed, and their viability was measured. Worms were considered dead when they no longer responded to prodding. Experiments were carried out in triplicate. The Student *T* test was used to analyze the significance in the viability increase of *C. elegans* between both conditions.

Lifespan Assays. To measure the lifespan of *C. elegans,* synchronized worms of the wild-type strain and the different mutant strains were grown at 20 °C until they reached young adult stage. Worms were then transferred to NGM agar plates or to NGM plates supplemented with polyphenol-enriched cocoa powder (4 mg/mL) and scored every 2 days for viability. The animals were moved periodically to new plates and were scored as dead if they failed to respond to a platinum wire. Three independent assays were carried out with each strain. Survival curves were compared using the log rank survival significance test, provided by GraphPad Prism 4 statistical software package.

***S. cerevisiae* DNA Arrays.** Differences in genomic expression profile of yeasts cultured in the presence of the polyphenol-enriched cocoa powder were studied using DNA arrays containing the full-length open reading frames for 6145 genes of *S. cerevisiae.*

Experiments were independently carried out in triplicate. *S. cerevisiae* BY4741 was cultured overnight at 28 °C and 250 rpm in liquid YPD medium with or without supplementation with polyphenol-enriched cocoa powder (4 mg/mL) until reaching 1 unit of $OD_{600\ nm}$. Cells were

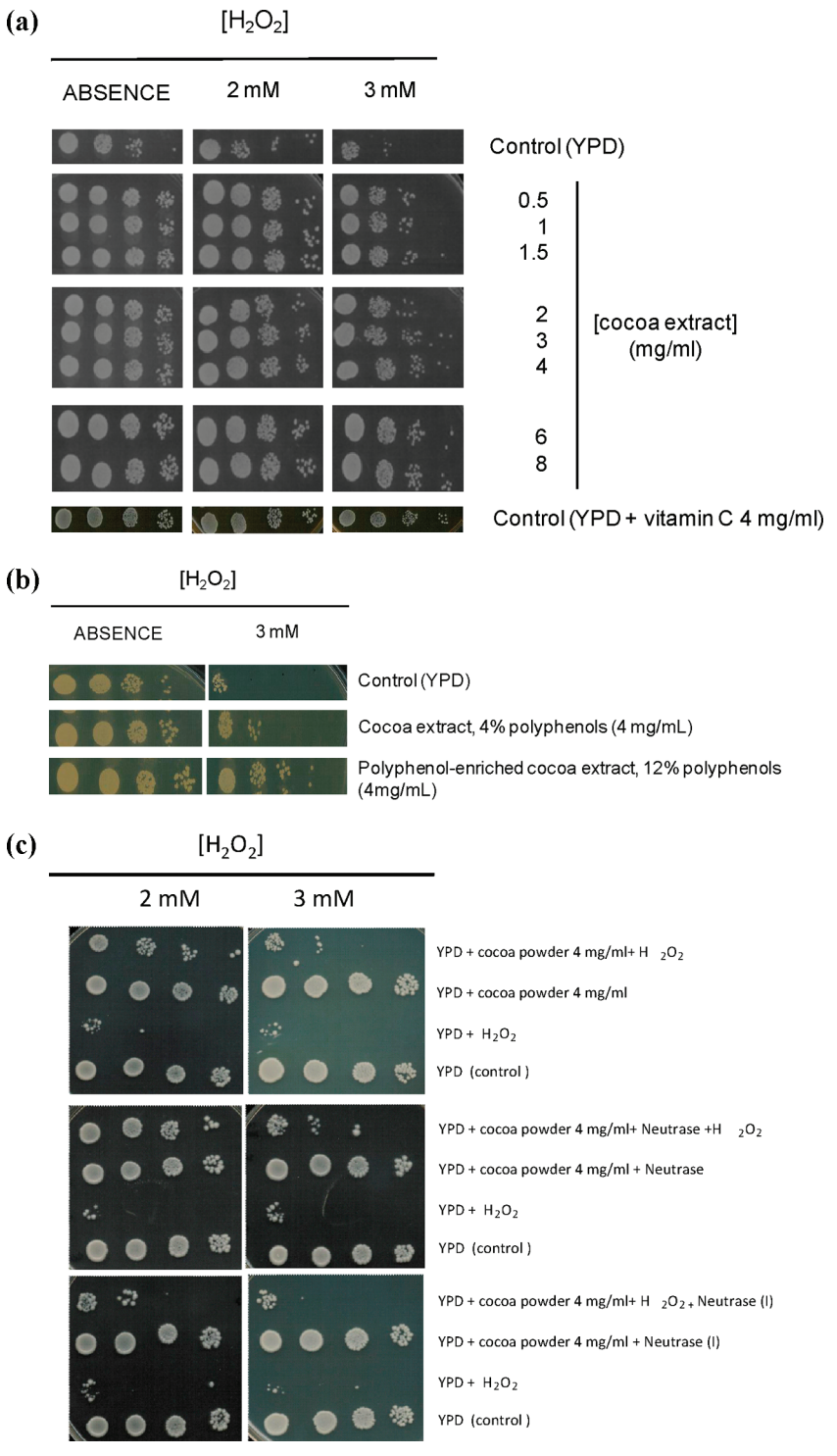

Figure 1. (a) Effects of cocoa powder or vitamin C supplemented culture media on the growth of the *S. cerevisiae* strain BY4741. This strain was cultured in YPD medium with different concentrations of the flavonoid-enriched cocoa powder (from 0.5 to 8 mg/mL). Vitamin C was added at 4 mg/mL. Samples were subjected to strong oxidative stress (2 and 3 mM) in order to observe differences among the different doses of cocoa powder. A control sample was included without cocoa powder supplementation and another without oxidative stress. Cultures of direct yeast suspension and dilutions 10^{-1}, 10^{-2} and 10^{-3} were performed. Serial dilutions are arranged in increasing dilution from left to right. (b) Resistance to oxidative stress of the polyphenol-enriched cocoa powder (12% polyphenols) compared with a conventional cocoa powder (4% polyphenols). (c) Treatment of flavonoid-enriched cocoa powder with a commercial protease (Neutrase) increases the antioxidant properties of the polyphenol-enriched cocoa powder. (I): cocoa powder solution treated with Neutrase and heat inactivated.

collected by centrifugation at 3000 rpm, immediately frozen in liquid nitrogen, and stored at −80 °C for later RNA isolation with phenol−chloroform.[21] cDNA synthesis was carried out using the Fluoroscript cDNA labeling system and oligo(dT). Hybridization and global gene expression analyses were performed by the DNA Chips Section at Valencia University's research support service

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(*Servicio Central de Soporte a la Investigación Experimental, Universitat de València*) using the MicroGridII (BioRobotics) equipment. Pairwise comparisons were done using a sample of BY4741 cultured in YPD medium as the reference. To estimate significantly differentially expressed genes in pairwise comparisons, a *z*-test for independent data was applied, and a *Z*-score was obtained for every gene. The False-Discovery-Rate method was used to monitor the overall false positive error rate.[22] Genes that were overexpressed at least 2-fold in yeast cultured with cocoa powder compared to yeast cultured in control conditions (YPD medium) were considered for further analysis and discussion.

RESULTS

Polyphenol-Enriched Cocoa Powder Has an Antioxidant Effect in *S. cerevisiae*. In the present study, the protective effect of the flavonoid-enriched cocoa powder in the *S. cerevisiae* model was analyzed. Given the disparity among the different methods available to assess antioxidative capacity in *S. cerevisiae*,[23−25] a protocol of acute oxidative stress was optimized in order to test the best conditions to induce oxidative damage in this microorganism. To do so, different parameters were analyzed, such as the pregrowth medium (YNB or YPD), the type of oxidative agent (H_2O_2 or *tert*-butyl) and concentrations thereof (1, 2, or 3 mM), and the cell exposure time to the oxidative agent (15, 30, 45, 60 min and 16 h). In the optimal protocol the wild strain of the yeast is grown for 16 h in YPD medium; cells are recovered by centrifugation at 3000 rpm for 10 min and rinsed twice in phosphate buffer before exposure to the oxidant compound. The highest oxidant effect was observed with H_2O_2, and consequently it was used for all experiments. We determined that concentrations of 2 and 3 mM of H_2O_2 induced high cell death, and no observable differences were determined when a dose of 1 mM was used. These results are in agreement with other studies which use similar doses of H_2O_2 to induce oxidative stress in *S. cerevisiae*.[23] Under these conditions, vitamin C supplementation of BY4741 exhibited high rates of yeast survival after oxidative stress (see below).

After optimization, further experiments were carried out to determine the lowest cocoa powder concentration providing antioxidant protection in yeast cultures. Results showed that an antioxidant effect of the polyphenol-enriched cocoa powder occurred with all doses assayed (Figure 1). Specifically we determined that concentrations between 0.5 and 2 mg/mL of cocoa powder exerted beneficial effects at 2 mM of H_2O_2 (viability of cells was increased at all culture dilutions). In addition, a marked effect in a range of 3 and 8 mg/mL of cocoa powder was observed even at 3 mM of H_2O_2. Therefore, we decided to use 4 mg/mL as an intermediate concentration having a visible effect on yeast growth. This concentration was applied in the oxidative stress assays to evaluate the survival of the wild-type strain BY4741 after treatment with hydrogen peroxide. Using the previously described evaluation protocol, the antioxidative capacity of the flavonoid-enriched cocoa powder was tested *in vivo*. Quantitative assays showed the efficacy of the oxidative stress and extreme reversal thereof achieved by the flavonoid-enriched cocoa powder and the vitamin C in strain BY4741. Thus, yeast survival rate under control conditions (YPD) was 2.63 ± 3.2% after the addition of 2 mM of H_2O_2. Moreover, flavonoid-enriched cocoa (4 mg/mL) was capable of increasing hydrogen peroxide tolerance of yeasts, as the survival rate obtained was 15.39 ± 5.1% (*P*-value ≤0.05), very similar to those observed in cells treated with vitamin C, 19.28 ± 5.1% (*P*-value ≤0.01). Furthermore, using higher doses of H_2O_2 (3 mM) the yeast survival rate determined in YPD (4.02 ± 4.5) was also increased after treatment with the flavonoid-enriched cocoa powder (13.08 ± 5.3%, *P*-value ≤0.05) and the vitamin C (18.81 ± 3.8%, *P*-value ≤0.01). These survival rates are slightly lower than the values obtained with lower concentrations of H_2O_2 in the culture media. Results obtained from qualitative assays also confirmed the antioxidant protection that the cocoa powder and vitamin C conferred to the strain BY4741. The colony density observed in each culture dilution was higher in the cultures with cocoa powder and vitamin C than in YPD cultures, as shown in Figure 1a. To discard the possibility that this was a strain-related effect, the test was repeated with other strains belonging to *S. cerevisiae* species, like YPH250 and BY4742, and similar results were obtained (data not shown).

The resistance to oxidative stress of the polyphenol-enriched cocoa powder was subsequently compared with a conventional cocoa powder not enriched in polyphenols. Lower oxidative stress protection rates were observed after yeast cultures were supplemented with the conventional cocoa powder, demonstrating the beneficial effect of the polyphenols contained in the enriched cocoa powder (Figure 1b).

Finally, the flavonoid-enriched cocoa powder was enzymatically treated with a commercial protease (Neutrase) to investigate its effect on cocoa powder antioxidant properties. We determined that the protease-treated cocoa powder exerted a higher antioxidant effect than untreated cocoa powder (Figure 1c). However, the beneficial effect was lower for the cocoa powder sample with heat inactivated enzyme. These results indicated that proteases could act as breaking the polyphenol−protein complex, and consequently polyphenols or peptides can be released, increasing the resistance to oxidative stress of the sample. In addition, heat inactivation of the cocoa sample could partially inactivate the polyphenols contained, decreasing the resistance to oxidative stress of the product.

Flavonoid-Enriched Cocoa Powder Induces the Expression of the Gene HST3 in *S. cerevisiae*. To discover the metabolic target with which the functional ingredient under study may interact, we evaluated the transcriptional profile of the yeast through the analysis of over/underexpressed genes of strain BY4741, cultured in the presence of the flavonoid-enriched cocoa powder. Total RNA was obtained from strain BY4741 either cultured with or without polyphenol-enriched cocoa powder. Using DNA arrays containing the full-length open reading frames for 6145 genes of *S. cerevisiae* we determined that only twenty genes displayed a different expression pattern between the two test conditions, thirteen of which slightly reduced their expression (Table 1). A list of seven genes was drawn up by analyzing those that increased their expression due to the presence of the ingredient. Only two of these are of known function, one of which is a gene called YVC1, coding for calcium channel, and was found to have a fold chase of 5.8 in the DNA array analysis. This protein would seem to be involved in calcium release from the vacuole when hyperosmotic shock occurs.[26] Another gene that appeared to be more highly expressed in yeasts cultures growing in the presence of the cocoa powder was HST3. This gene encodes Hst3p sirtuin and belongs to the Sir2 family of deacetylases dependent on NAD^+, which is responsible for telomere silencing, cell-cycle progression, resistance to radiation and metabolism of short-chain fatty acids.[27,28] We observed that

Table 1. *S. cerevisiae* ORFs Differentially Expressed after Treatment of the Cells with Cocoa Powder[a]

ORF	gene	molecular function	fold change
YER160C		unknown: DNA polymerase activity, RNA binding, peptidase activity, protein binding, ribonuclease activity?	5.1635
YOR025W	HST3	DNA binding, histone deacetylase (sirtuin)	4.1254
YLR187W	SKG3	unknown: DNA polymerase activity, RNA binding, peptidase activity, protein binding, ribonuclease activity?	3.7530
YJR029W		Unknown: DNA polymerase activity, RNA binding, peptidase activity, protein binding, ribonuclease activity?	3.52
YJR027W		unknown: DNA polymerase activity, RNA binding, peptidase activity, protein binding, ribonuclease activity?	3.31
YOR088W	YVC1	calcium channel activity	5.8681
YER138C		unknown: DNA polymerase activity, RNA binding, peptidase activity, protein binding, ribonuclease activity?	2.47
YLR339C		unknown	0.30
YAL012W	CYS3	cystathionine-gamma-lyase activity	0.29
YMR297W	PRC1	carboxypeptidase C activity	0.25
YOR383C	FIT3	unknown	0.25
YHR182C-A		unknown	0.22
YPR114W		unknown	0.20
YMR303C	ADH2	alcohol dehydrogenase activity	0.20
YFR055W	IRC7	cystathionine beta-lyase activity	0.19
YGL168W		unknown	0.19
YMR316C-A		unknown	0.18
YOL152W	FRE7	ferric-chelate reductase activity	0.14
YDR509W		unknown	0.12
YCR072C		unknown	0.10

[a] The *Saccharomyces* Genome Database (http://yeastgenome.org) was used to retrieve information about specific gene function and biological processes.

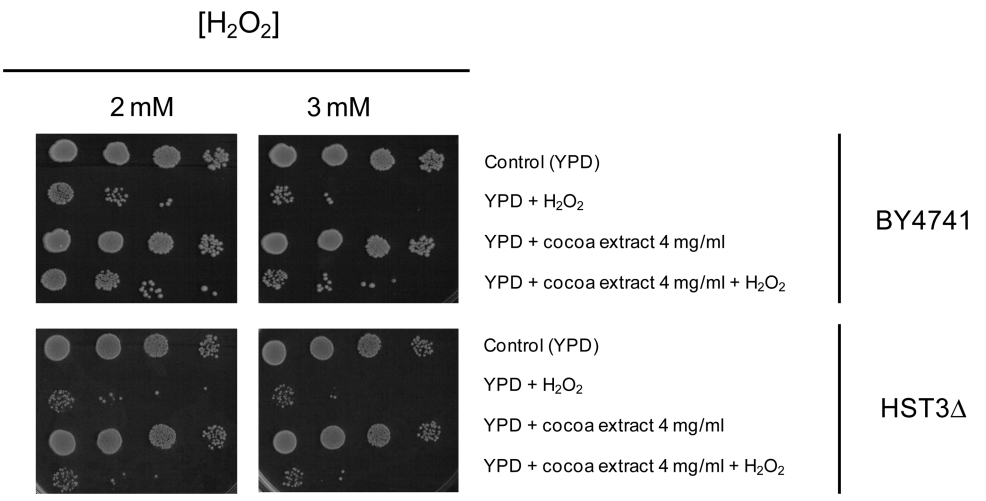

Figure 2. Growth of the *S. cerevisiae* BY4741 strain and the mutant strain *Hst3*Δ cultured in YPD medium and subjected to oxidative stress with 2 or 3 mM H_2O_2 in the presence of the polyphenol-enriched cocoa powder (4 mg/mL).

this gene was overexpressed four times with respect with yeasts cultured in control conditions.

Furthermore, to confirm the results obtained from DNA arrays, mutants in both genes HST3 and YVC1 were obtained from EUROSCARF. They were used to assay the antioxidative effects in the evaluation model developed within the framework of this research study. The only phenotype obtained clearly lacking antioxidative activity was the mutant HST3. In the case of the HST3 knockout mutant strain, results indicated that the assayed cocoa powder did not affect this mutant, thus suggesting that sirtuin induction plays a role in the biological effect of cocoa flavonoids (Figure 2). These results would indicate that the protection upon oxidative stress provided by the flavonoid-enriched cocoa powder is mediated at least by sirtuins in *S. cerevisiae*.

Flavonoid-Enriched Cocoa Powder Provokes Resistance to Oxidative Stress on the Nematode *C. elegans* and Extends Their Lifespan in a SIR-2.1 and DAF-16 Dependent Manner. In order to clarify the function of sirtuin proteins as metabolic targets of cocoa flavonoids, different trials were performed with a more complex model, the nematode *C. elegans*. This nematode has been used by many authors as a model to study the benefits of different compounds and plant extracts on aging-related parameters.[6,29,7] In our trials, we first used wild-type animals (N2 strain) that were grown under control conditions (NGM medium) and NGM medium with 4 mg/

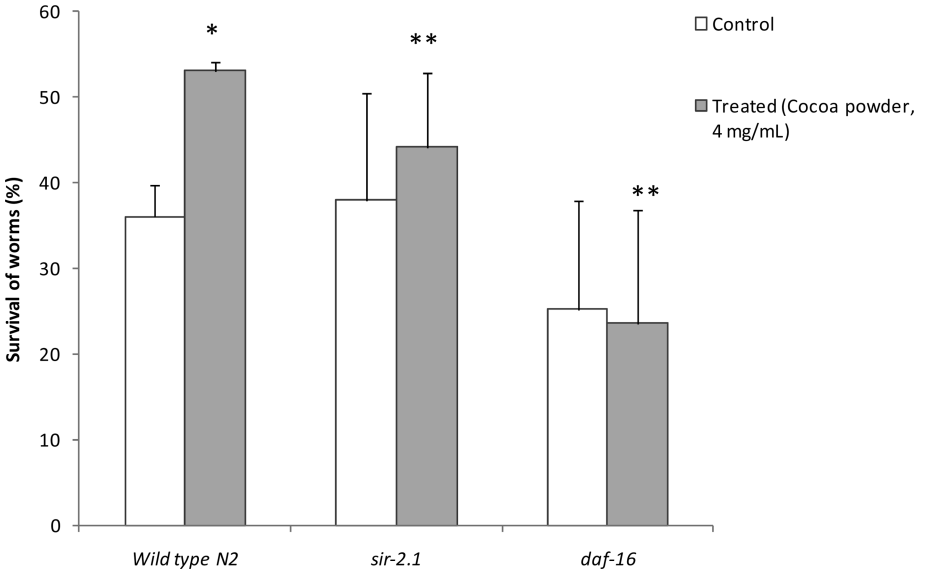

Figure 3. Survival of the *C. elegans N2* (wild-type), *sir-2.1* (ok434) and *daf-16* (mgDf50) strains treated with 2 mM H_2O_2 on NGM plates, with or without polyphenol-enriched cocoa powder supplementation. Error bars indicate standard deviation among three trials (300 worms scored per condition). *Significant at $P \leq 0.05$. **No significant differences with respect to the control (*sir* 2.1, $P = 0.5244$; *daf-16*, $P = 0.8806$).

mL of flavonoid-enriched cocoa powder. Age-synchronized worms were incubated for 5 days and then subjected to oxidative stress with hydrogen peroxide. Worms fed with the cocoa powder were more resistant toward the oxidative stressor hydrogen peroxide. As shown in Figure 3, animals fed with the ingredient prior to receiving oxidative treatment had a survival rate of 53%, compared with the 36% survival rate of control worms. Results were similar when using dead bacteria (data not shown).

Furthermore, as previous results obtained from *S. cerevisiae* assays indicated that the sirtuin Hst3 is a metabolic target of the assayed product, we analyzed the effect of the polyphenol-enriched cocoa powder on a mutant strain for the gene SIR-2.1, the closest *C. elegans* homologue to the yeast Hst3p gene. However, this mutant did not possess resistance to oxidative stress when fed with the ingredient (Figure 3). SIR-2.1 is a NAD^+- dependent protein deacetylase that has been shown to play a key role in lifespan extension in eukaryotes.[30] In order to assess possible effects of the cocoa powder on the worm's lifespan, synchronized populations of the N2 wild-type strain were cultured with or without the ingredient. The presence of cocoa powder increased the lifespan of the wild-type worms by approximately 17% (Figure 4a and Table 2). This effect was fully dependent on the presence of a functional SIR-2.1 gene (Figure 4b and Table 2). No antibacterial activity was observed for the cocoa powder (data not shown), ruling out the possibility of increased lifespan due to the worm feeding on dead bacteria.

Another major determinant of lifespan is the IGF-1 pathway, a transduction signal cascade that ultimately activates the forkhead transcription factor DAF-16, involved in the transcription of longevity-related genes.[31] Also, in some cases the lifespan-extension effects of *sir-2.1* and *daf-16* genes are epistatic.[32,33] To check whether this is so for the cocoa powder, the resistance to oxidative stress and lifespan-extending properties were measured in a *daf-16* mutant. The ingredient failed to exert any effect on *daf-16* mutants (Figures 3 and 4C and Table 2), indicating that the transcription factor is also part of the pathway affected by the cocoa powder. Correlatively, the lack of effect was also found in *daf-2* mutants (lacking the insulin receptor of the insulin/IGF-1 pathway) (Table 2). This is a different scenario from the one observed for other polyphenols like resveratrol, which act directly on SIR-2.1 in a completely DAF-16-independent manner.[8,34]

DISCUSSION

In recent years, the negative role played by reactive oxygen species (ROS) in the aging process has become increasingly accepted.[2] Furthermore, there is evidence that accumulation of damage caused by ROS (which primarily result from mitochondrial metabolism) is related to an etiology of a number of age-related diseases.[35]

To date, many authors have shown an increasing interest in the use of polyphenolic compounds with resistance to oxidative stress as potential therapeutics for aging-related diseases.[23,25] The present study provides evidence that a flavonoid-enriched cocoa powder exerts resistance to oxidative stress in two *in vivo* models, the yeast *S. cerevisiae* and the worm *C. elegans*, after hydrogen peroxide induced oxidative damage. Moreover, the results obtained show that the protection upon oxidative stress provided by the cocoa powder is due to its high polyphenol content (12%), as increased survival was observed in yeast cultures after an oxidative stress when compared with a conventional cocoa powder (4% of polyphenols). Furthermore, the flavonoid-enriched cocoa powder was characterized by significant amounts of catechin and epicatechin.[17] Our results show that there was a marked resistance to oxidative stress in both organisms using 4 mg/mL of the cocoa powder, representing a supplementation of 8 μg/mL of catechin and 12 μg/mL of epicatechin. However, pure flavonoids added directly at the same concentrations as in the cocoa powder to the yeast cultures did not result in antioxidant protection (data not shown). Therefore, we assume that the resistance to oxidative stress was due to the presence of other minor polyphenols or perhaps synergism among the cocoa components. Moreover, proteolysis of the

cocoa powder increased yeast survival after oxidative stress, which might indicate that polyphenols or peptides are released, thereby enhancing the resistance to oxidative stress of the sample.

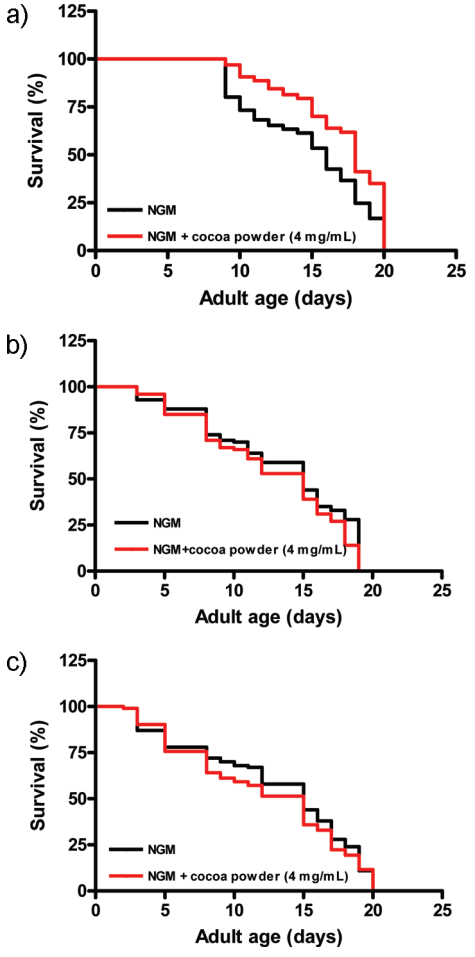

Figure 4. Survival curves of *C. elegans* wild-type strain N2 (a) and mutant strains *sir-2.1* (ok434) (b) and *daf-16* (mgDf50) (c) growing on NGM medium or NGM medium supplemented with 4 mg/mL cocoa powder. Animals were treated with cocoa powder for 3 weeks at 20 °C. The percentage of live animals is plotted against adult age. (a) Increased lifespan in wild-type N2 worms fed with the cocoa powder, log rank X^2 = 13.03, P = 0.0003. Lifespan in *sir-2.1* (b) and *daf-16* (c) mutants was not extended by treatment with 4 mg/mL cocoa powder, log rank X^2 = 2.96, P = 0.0856 and log rank X^2 = 0.47, P = 0.4913 respectively.

Furthermore, a genomic approach was used to determine the biochemical and molecular pathways affected by the flavonoid-enriched cocoa powder. In our studies, we found that polyphenol-enriched cocoa powder activates the sirtuin protein Hst3p in the budding yeast *S. cerevisiae*. Sirtuins encompass a family of proteins that are present in the cell nucleus and exhibit deacetylase activity, promoting longevity in diverse organisms.[36] The nematode *C. elegans* was used to confirm whether the obtained results could be extrapolated to an animal model. Due to its short lifespan, the availability of full genome sequence data and the existence of a wide collection of mutants, this nematode is a perfect model to identify and characterize compounds that delay aging and extend lifespan.[6,29,37] This work shows that the polyphenol-enriched cocoa powder, used as an ingredient that exerts resistance to oxidative stress, produces lifespan extension in *C. elegans*. The beneficial effects of the cocoa powder were analyzed on the genetic level by using *C. elegans* mutant strains, revealing that polyphenol-enriched cocoa powder did not protect *Sir-2.1* and *daf-16* mutant strains upon acute oxidative stress with hydrogen peroxide, and that both genes were essential for resistance to oxidative stress by the cocoa powder. Sirtuin protein Sir-2.1 has been described to promote longevity in eukaryotes[30] and has been reported to increase lifespan in *C. elegans* and *Drosophila melanogaster* through dietary restriction-like mechanisms.[8] The forkhead transcription factor DAF-16 is activated through the IGF-1 pathway promoting transcription of longevity-related genes.[31] Specifically, polyphenol-enriched cocoa powder treatment did not prolong the lifespan of animals lacking SIR-2.1 and DAF-16 genes, suggesting that the effects of cocoa powder are mediated at least through these genes. Furthermore, no effects were observed in *daf-2* mutants, lacking the insulin receptor of the insulin/IGF-1 pathway. Therefore, the beneficial effects exerted by the polyphenol-enriched cocoa powder are produced through a SIR-2.1 and DAF-16 dependent mechanism. In *C. elegans* the transcription factor SKN-1 which is related to vertebrate Nrf proteins promotes expression of detoxification enzymes in response to oxidative stress.[39] Experiments are now in progress in order to study the putative effect of cocoa polyphenols in the expression of this transcriptional factor.

In conclusion, here we report the *in vivo* resistance to oxidative stress of cocoa polyphenols in *S. cerevisiae* and *C. elegans* model organisms. Moreover, lifespan extension of the worms was dependent on SIR-2.1 and DAF-16 proteins. We have also demonstrated that the combined use of transcriptomics and model organisms can facilitate such research, reducing both the time and cost of evaluating compounds with potential interest for industry. Accordingly, we have started high-throughput screen-

Table 2. Effect of the Cocoa Powder on *C. elegans* Lifespan[a]

strain	genotype	treatment	median lifespan (days)	*n*	log rank X^2	*p*-value
N2	wild-type	NGM	15	100		
N2	wild-type	NGM + cocoa powder (4 mg/mL)	18	100	13.03	0.0003*
VC199	*sir 2.1* (ok434)	NGM	15	100		
VC199	*sir 2.1* (ok434)	NGM + cocoa powder (4 mg/mL)	15	100	2.96	0.0856 (NS)
GR1307	*daf-16* (mgDf50)	NGM	15	100		
GR1307	*daf-16* (mgDf50)	NGM + cocoa powder (4 mg/mL)	15	100	0.47	0.4913 (NS)
GR1370	*daf-2* (e1370)	NGM	20	100		
GR1370	*daf-2* (e1370)	NGM + cocoa powder (4 mg/mL)	23	100	1.09	0.2973 (NS)

[a] Lifespan analysis was performed with GraphPad Prism 4 statistical software package. *Significant lifespan extension ($P \leq 0.001$). NS: No significant difference between control and treated conditions.

ing of plant extracts, looking for those with antioxidant activities and impact on longevity. This work indicates the potential use of this polyphenol-enriched cocoa powder as a functional ingredient with resistance to oxidative stress. Further studies should be done in order to identify the exact polyphenol that is responsible for these beneficial effects. Given that some discrepancies exist regarding lifespan studies performed in mammals and simpler organisms, especially where oxidative stress is concerned,[38] confirmation of these results using murine models would be of great interest. Also in these experiments it would be interesting to determine the amount of effective daily dose of polyphenol-enriched cocoa powder.

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