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VALIDACIÓN FUNCIONAL DE EXTRACTOS POLIFENÓLICOS DE CACAO MEDIANTE ENSAYOS *IN VIVO* CON ORGANISMOS MODELO

Tesis Doctoral

Presentada por: **Ana Peláez Soto**

Dirigida por: Dra. Patricia Roig Montoya

Dr. José Vicente Gil Ponce

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VNIVERSITAT DE VALÈNCIA Facultat de Farmàcia

La Dra. Patricia Roig Montoya, Profesora Contratada Doctora y el Dr. José Vicente Gil Ponce, Profesor Titular, ambos pertenecientes a la Universitat de València,

CERTIFICAN QUE:

Dña. Ana Peláez Soto, Licenciada en Ciencia y Tecnología de los Alimentos, ha realizado bajo su codirección el trabajo titulado: "Validación funcional de extractos polifenólicos de cacao mediante ensayos *in vivo* con organismos modelo" y autorizan su presentación para optar al título de Doctor por la Universitat de València.

Y para que conste a los efectos oportunos, firman el presente certificado en Valencia, a 19 de octubre de 2016.

Dra. Patricia Roig Montoya

Dr. José Vicente Gil Ponce

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Los polifenoles son los principales antioxidantes de la dieta y a ellos se les atribuyen múltiples propiedades beneficiosas, como la prevención de enfermedades cardiovasculares, tumorales y degenerativas, que pueden aparecer como consecuencia del estrés oxidativo. El cacao representa una fuente interesante de polifenoles en la dieta, dado su amplio consumo y su riqueza en estos compuestos bioactivos. La actividad antioxidante de los polifenoles en general, y en concreto de los polifenoles del cacao, ha sido ampliamente evaluada *in vitro.* Sin embargo, los ensayos *in vitro* no tienen en cuenta aspectos fundamentales en organismos vivos como son la biodisponibilidad y metabolismo, ni proporcionan información sobre los mecanismos *in vivo* de la respuesta celular antioxidante.

Esta tesis doctoral aborda el estudio de la capacidad antioxidante de los polifenoles del cacao *in vivo* mediante el uso de la levadura *Saccharomyces cerevisiae* y del nematodo *Caenorhabditis elegans* como organismos modelo. Para ello, se ha empleado un extracto polifenólico obtenido a partir de un polvo de cacao rico en polifenoles y diversas fracciones cromatográficas obtenidas a partir de dicho extracto, con el fin de identificar los compuestos responsables de los efectos antioxidantes del cacao. Tras la evaluación previa de 26 fracciones correspondientes al extracto completo de cacao, se seleccionaron 7 para su estudio en base a su capacidad protectora antioxidante, las fracciones correspondientes a los compuestos mayoritarios del cacao (teobromina, cafeína, catequina, epicatequina y procianidina B2) y otras dos fracciones que no presentaban ningún compuesto mayoritario.

Para el empleo de *S. cerevisiae*, se ha puesto a punto un método en placa multipocillo que permite estudiar de forma rápida y reproducible la capacidad promotora de respuesta celular antioxidante de ingredientes alimentarios. Se ha demostrado la capacidad protectora antioxidante del extracto de cacao y se han identificado las fracciones más activas, siendo aquellas ricas en catequina, epicatequina y procianidina B2. Por otra parte, se ha utilizado el mutante de deleción *hst3Δ,* confirmándose la implicación de la sirtuina Hst3 en el efecto antioxidante de *S. cerevisiae* mediado por los extractos de cacao y de uva y comprobándose una mayor resistencia del mutante al estrés provocado con H_2O_2 y menadiona. Asimismo, se han llevado a cabo estudios proteómicos y transcriptómicos en la levadura tras haber sido preincubada con

el extracto polifenólico de cacao y posteriormente sometida a estrés oxidativo con peróxido de hidrógeno. Los resultados obtenidos han permitido identificar posibles proteínas y genes implicados en la respuesta a dicho estrés mediada por los polifenoles del cacao. En estas condiciones, se ha observado que el metabolismo de carbohidratos se modifica mediante la represión de la glicolisis y el aumento de la ruta de las pentosas fosfato con el fin de obtener poder reductor para hacer frente al estrés oxidativo. Además, se ha observado la represión de algunos genes implicados en la síntesis de proteínas, mientras que algunos de los enzimas secuestradores de radicales libres fueron rápidamente inducidos por el extracto de cacao, como las catalasas y peroxidasas.

Mediante el uso de *C. elegans*, se ha evaluado la capacidad promotora de respuesta antioxidante tanto del extracto polifenólico completo como de fracciones aisladas, mediante ensayos de estrés oxidativo, estrés térmico y formación de especies reactivas de oxígeno (ROS). Se ha confirmado el efecto protector antioxidante, observado en la levadura, del extracto de cacao y de las fracciones ricas en catequina, epicatequina y procianidina B2 en los ensayos de estrés con peróxido de hidrógeno. Por el contrario, sólo el extracto de cacao ofreció protección antioxidante en el nematodo cuando se llevaron a cabo los ensayos de estrés con Na-arsenito, estrés térmico y formación de ROS. Asimismo, se ha establecido la implicación del factor de trascripción DAF-16 en la protección antioxidante del cacao, efecto que además parece estar mediado por el contenido total de polifenoles del cacao y no como resultado de compuestos aislados.

Polyphenols are the principal dietary antioxidants and they have been associated with several beneficial properties, such as the prevention of cardiovascular, tumoral and degenerative diseases, which can occur as a result of oxidative stress. Cocoa is an important source of dietary polyphenols, due to its wide consumption and its high content of these bioactive compounds. The antioxidant activity of polyphenols in general and of cocoa polyphenols in particular, has been widely evaluated *in vitro*. However, *in vitro* assays do not take into account fundamental aspects of living organisms, such as bioavailability and metabolism, and they do not provide information about the *in vivo* mechanisms of antioxidant cellular response.

This doctoral thesis focuses on the study of the *in vivo* antioxidant capacity of cocoa polyphenols using the yeast *Saccharomyces cerevisiae* and the nematode *Caenorhabditis elegans* as model organisms. To perform this *in vivo* study, a polyphenolic extract obtained from a polyphenol-rich cocoa powder and several chromatographic fractions obtained from the extract were employed to identify the compounds responsible for the antioxidant effects of cocoa. After the preliminary evaluation of 26 fractions corresponding to the full cocoa extract, 7 of them were selected for further studies based on their protective antioxidant capacity, those corresponding to the major compounds of cocoa (theobromine, caffeine, catechin, epicatechin and procyanidin B2) and two fractions with no major compounds.

Using *S. cerevisiae*, a yeast-based method was performed to evaluate the ability of food ingredients to induce an antioxidant cellular response in this model, in a rapid and reproducible way. The antioxidant protective capacity of cocoa extract was proved, identifying fractions rich in catechin, epicatechin and procyanidin B2 as the most active ones. The deletion mutant *hst3Δ* permitted confirmation of the implication of the sirtuin Hst3 in the antioxidant effect of *S. cerevisiae* mediated by the cocoa and grape extracts. It was also found that the mutant had a higher resistance to the oxidative stress induced by H_2O_2 and menadione. Proteomic and transcriptomic studies were performed on the yeast after treatment with the cocoa polyphenolic extract and induction of oxidative stress with hydrogen peroxide. The results permitted the identification of possible proteins and genes involved in the yeast's response to the stress mediated by cocoa polyphenols. In these conditions, it was possible to observe a modification of

carbohydrate metabolism through the repression of glycolysis and the enhancement of the pentose phosphate pathway to obtain redox power to counter oxidative stress. Furthermore, several genes involved in protein synthesis were repressed and some of the radical scavenging enzymes such as peroxidases and catalases were quickly induced by the cocoa extract.

Regarding the use of the *C. elegans* model, the ability of the cocoa extract and the isolated fractions to promote antioxidant response in the worm were evaluated by oxidative stress, thermal stress and reactive oxygen species (ROS) formation assays. It was possible to confirm the antioxidant protective effect of the cocoa extract and the fractions rich in catechin, epicatechin and procyanidin B2, after treatment with hydrogen peroxide; an effect that was previously observed in the yeast model. On the contrary, only the cocoa extract protected the nematode in the oxidative stress assays with Na-arsenite, thermal stress and ROS formation assays. In addition, the involvement of the transcription factor DAF-16 in the antioxidant protection of cocoa was established. This effect seems to be mediated by the total amount of polyphenols in cocoa and not as a result of isolated compounds.

2D-GE	Two-dimensional gel electrophoresis
ABTS	2,2´-azinobis-(3-ethylbenzthiazolin)-6-sulfonic acid
APS	Ammoniumperoxodisulfate
CAT	Catalase
CGC	Caenorhabditis Genetics Center
CIPF	Centro de Investigación Príncipe Felipe
CVD	Cardiovascular disease
DCF	Dichlorodihydrofluorescein
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl-hydrazyl
DTT	Dithiothreitol
EGCG	Epigallocatechin-3-gallate
FMD	Flow-mediated vascular dilatation
FRAP	Ferric reducing-antioxidant power
FUDR	5-fluoro-2´-deoxyuridine
GenRE	Genomic Research Environment
GSH	Glutathione
GST	Glutathione S-transferase
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
HPLC-DAD	High performance liquid chromatography with diode-array detection
HPLC-MS/MS	High performance liquid chromatography-tandem mass spectrometry

ICCO	International Cocoa Organization
LDL	Low density lipoprotein
MAGRAMA	Ministerio de Agricultura, Alimentación y Medio Ambiente
MALDI-MS/MS	Matrix Assisted Laser Desorption/Ionization –mass spectrometry
MAPK	Mitogen-activated protein kinase
NGM	Nematode growth medium
NO	Nitric oxide
ORAC	Oxygen radical absorbance capacity
oxLDL	Oxidized low density lipoprotein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PPP	Pentose phosphate pathway
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SCSIE	Servei Central de Suport a la Investigació
SGD	Saccharomyces Genome Database
SHR	Spontaneously hypertensive rats
SOD	Superoxide dismutase
TCA	Tricarboxylic acid
TFA	Trifluoroacetic acid
TEAC	Trolox equivalent anti-oxidative capacity
YPD	Yeast extract peptone dextrose

INTRODUCTION

1. POLYPHENOLS

Polyphenols are chemical compounds with at least one aromatic ring (C6) with one or more hydroxyl groups. Phenolic compounds are plant secondary metabolites essential for them since they are involved in several processes, such as growth, pigmentation, attraction for pollinators and resistance against pathogens, predators and environmental stresses, thereby enhancing plant survival. Phenolics are the most abundant compounds in plant kingdom, with more than 8000 different structures known today (Crozier, Jaganath & Clifford, 2009; Fraga, Galleano, Verstraeten & Oteiza 2010).

1.1. Classification

Polyphenols can be classified depending on the number and position of their carbon atoms and they are usually conjugated to sugars and organic acids. They can be divided into two major groups: flavonoids and non-flavonoids (Crozier, Jaganath & Clifford, 2009), although other classifications are described as well.

1.1.1. Flavonoids

Flavonoids have 15 carbon atoms with two aromatic rings linked by a three carbon bridge resulting in a C6-C3-C6 structure. The basic skeleton of a flavonoid (Fig. 1) can have several substituents, being sugars very common since most of flavonoids exist naturally as glycosides. Hydroxyl groups and sugars increase the solubility of flavonoids, while methyl groups make them lipophilic. The main sub-classes of dietary flavonoids are flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones and isoflavones. They are the most abundant polyphenols with over 6000 flavonoids identified, widely distributed in fruits, vegetables, seeds, nuts, stems and flowers, as well as derived commodities, such as tea, wine, juice or beer, products which are important constituents of the human diet (Crozier, Jaganath & Clifford, 2009).

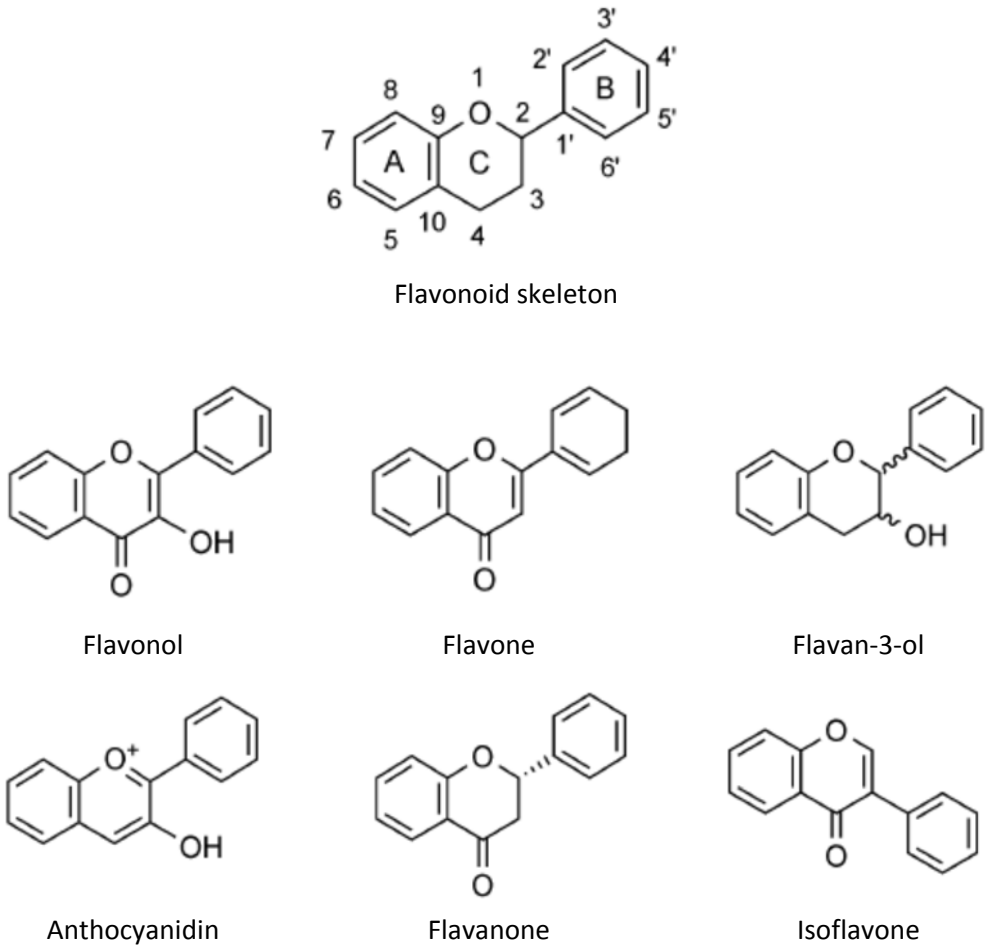


Figure 1. Chemical structure of flavonoids (modified from Crozier, Jaganath & Clifford, 2009).

Flavonols are the widest group of flavonoids, being kampferol and quercetin the most abundant compounds of this group in human diet.

Flavan-3-ols range from simple monomers, such as (+)-catechin and its isomer (-)-epicatechin, to complex structures including oligomeric and polymeric proanthocyanidins also known as condensed tannins. Proanthocyanidins containing only (epi)catechin units are called procyanidins and they are the largest type of proanthocyanidins in plants (Crozier, Jaganath & Clifford, 2009). Unlike other classes of flavonoids, flavanols are not naturally glycosilated, so they can only be found as aglycones in plants and plant derived food products (D'Archivio *et al*., 2007).

Anthocyanidins are the main pigments in nature. They are mainly found in fruits and flowers where they are responsible of red, blue and purple colors, being cyanidin and

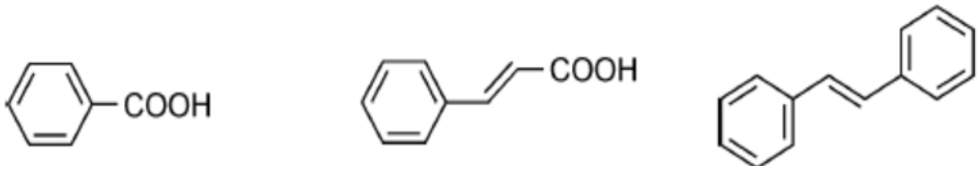
malvidin the most common structures, among others. In plant tissues they are conjugated with sugars and called anthocyanins. Anthocyanidins are less hydrosolubles than its corresponding glycosides (anthocyanins) and they are not found in their free form in nature (Crozier, Jaganath & Clifford, 2009).

With respect to flavanones, the most common aglycones are hesperetin and naringenin, although glycosides are more dominant in nature. The most abundant glycosides of hesperetin are hesperidin and neohesperidin, which are conjugated with rutinose and neohesperidose, respectively. In the case of naringenin, its major glycosides are naringin and narirutin (Khan & Dangles, 2014).

Flavones, such as apigenin and luteolin, are the less common flavonoids. Among the main molecules of isoflavones, the most abundant are genistein and daidzein (Crozier, Jaganath & Clifford, 2009).

1.1.2. Non-flavonoids

The main non-flavonoids compounds (Fig. 2) in human diet are phenolic acids, including hydroxybenzoic (C6-C1) and hydroxycinnamic (C6-C3) acids, such as gallic acid (which is the biosynthetic precursor of hydrolysable tannins) and caffeic acid, respectively, and stilbenes (C6-C2-C6) (Crozier, Jaganath & Clifford, 2009; Khan & Dangles, 2014).



Hydroxybenzoic acid Hydroxycinnamic acid Stilbene

Figure 2. Chemical structure of non-flavonoids (modified from Crozier, Jaganath & Clifford, 2009).

1.2. Main dietary sources of polyphenols

Polyphenols are widely distributed in the plant kingdom and they are the most abundant antioxidants in human diet. In the particular case of Spain (Mediterranean diet), the mean daily intake of these compounds in a whole diet was estimated between 2590 and 3016 mg/person/day (Saura-Calixto, Serrano & Goñi, 2007). Among the richest products in phenolics are fruits and vegetables, tea, coffee, cocoa, wine and beer.

Fruits and vegetables are rich in a wide range of phenolic compounds (Table 1). The species of the genus *Allium* (i.e. onions), *Brassica* (i.e. broccoli), *Lactuca* (i.e. lettuce) and *Lycopersicon* (i.e. tomato) represent a rich source of flavonols, mainly quercetin and kaempferol. In fact, the main contribution of quercetin to the diet is done by commodities such as red onion and garlic, among others. Onions are not only rich in quercetin and kaempferol but also in isorhamnetin and myricetin (Naczk & Shahidi, 2006). Some of them also contain flavones (e.g. celery and parsley). Major flavonoids found in pepper fruits are conjugates of quercetin and luteolin. Tomato contains some flavanones such as naringenin and hesperidin as well as flavonols mainly present in the skin of the fruits, being cherry tomatoes richer than big tomatoes (Naczk & Shahidi, 2006). Carrots are rich in chlorogenic acids. Moreover, the richest legumes in phenolics are the dark ones (e.g. red and black beans). Isoflavones are almost only found in legumes, especially in soy (Crozier, Jaganath & Clifford, 2009; Naczk & Shahidi, 2006).

Flavanols such as catechins can be found in huge amounts in red grapes and apples, but also in stone fruits such as plums and apricots and in a complex mixture in strawberries. Procyanidins type B are present in apples, plums and peaches, among other fruits. Flavanones are characteristic of citric fruits, being naringenin present in big amounts in grapefruit and hesperidin in oranges, lemons and limes (Khan & Dangles, 2014). Although they also contain certain levels of flavones, the most relevant source of these compounds are olives, which are especially rich in luteolin and apigenin. Olives also contain big amounts of phenolic acids (other than hydroxycinnamic and hydroxybenzoic) and other polyphenols such as oleuropein and its aglycone, 3,4-DHPEA-EDA (oleuropein-aglycone di-aldehyde), p-HPEA-EDA (ligstroside-aglycone di-aldehyde), 3,4-DHPEA-EA (oleuropeinaglycone di-aldehyde) and hydroxytyrosol, being olives and olive oil the main contributors of these compounds to the Spanish diet (Tresserra-

Rimbau *et al*., 2013). Anthocyanins are mainly found in red grapes, most of them as malvidin glycosides, although they can also be found in fruits such as blueberry, blackberry, raspberry and strawberry. Quercetin is the main flavonol found in fruits, especially in blueberries and other berries, where there is also kaempferol and myricetin. Blueberries also contain a wide range of phenolic acids such as gallic, caffeic, p-coumaric, ferulic and ellagic acids (Naczk & Shahidi, 2006; phenolexplorer).

Table 1. Main polyphenolic composition of fruits and vegetables (Naczk & Shahidi, 2006).

Phenolic compounds	Dietary sources
Phenolic acids	
Hydroxycinnamic acids	Apricots, blueberries, carrots, cereals, pears, cherries, citrus fruits, oilseeds, peaches, plums, spinach, tomatoes, eggplants
Hydroxybenzoic acids	Blueberries, cereals, cranberries, oilseeds
Flavonoids	
Anthocyanins	Bilberries, black and red currants, blueberries, cherries, chokecherries, grapes, strawberries
Chalcones	Apples
Flavanols	Apples, blueberries, grapes, onions, lettuce
Flavanonols	Grapes
Flavanones	Citrus fruits
Flavonols	Apples, beans, blueberries, buckwheat, cranberries, endive, leeks, lettuce, onions, olive, pepper, tomatoes
Flavones	Citrus fruits, celery, parsley, spinach, rutin
Isoflavones	Soybeans
Tannins	
Condensed	Apples, grapes, peaches, plums, mangosteens, pears
Hydrolysable	Pomegranate, raspberries
Other phenolics	
Stilbenes	Grapes
Lignans	Buckwheat, flaxseed, sesame seed, rye, wheat

Tea has a high content of phenolic compounds of nutritional interest, especially green tea, relevant for the big amount of flavan-3-ols (catechins constitute over 30 % of the dry weight of leaves, being (−)-epigallocatechin-3-gallate (EGCG) the most abundant one). Flavonols (quercetin, kaempferol and its glycosides), flavones (glycosides of apigenin) and phenolic acids (gallic and chlorogenic acids) also occur in tea (Crozier, Jaganath & Clifford, 2009; Lambert & Elias, 2010).

Beer contains a range of polyphenolic compounds, partly from barley (70 %) and partly form hops (30 %). Flavan-3-ols are found in both of them and include monomers such as (+)-catechin and (-)-epicatechin and dimers such as procyanidin B3 and prodelphinidin B3 (Crozier, Jaganath & Clifford, 2009).

Red wine is rich in phenolic compounds due to the high amount of these substances in the skin of grapes. Several studies have related a moderate wine intake with beneficial effects in human health, due to polyphenols, especially flavonoids. Flavan-3-ols (catechin and epicatechin), as well as its polymers (procyanidins), flavonols (quercetin and myricetin) and anthocyanins (glycosides of malvidin, cyanidin, peonidin, delphinidin and petunidin) are the most common polyphenols found in red wine. But other compounds are present in this product as well, such as phenolic acids, for example hydroxybenzoic (gallic acid) and hydroxycinnamic (caffeic acid) acids and stilbenes (resveratrol) which are important non-flavonoid compounds present in grapes and wine (Banc *et al*., 2014).

Cocoa beans are among the richest products in phenolics, being its amount even higher than in black and green tea and red wine, especially for flavan-3-ols catechins (mainly epicatechin) as well as procyanidins and proanthocyanidins. However, the amount of these compounds is highly variable depending on the origin of the cocoa and the manufacturing process of the final product (Andújar, Recio, Giner & Ríos, 2012; Crozier, Jaganath & Clifford, 2009).

Coffee represents the most relevant source of phenolic acids in human diet, specifically hydroxycinnamic acids, given the vast consumption of this product all over the world. In particular, the chlorogenic acid 5-O-caffeoylquinic acid is the most abundant compound among that group (Crozier, Jaganath & Clifford, 2009; Tresserra-Rimbau *et al*., 2013).

1.3. Bioavailability and metabolism of polyphenols

Polyphenol metabolism is not yet fully understood. Polyphenols are usually present in food and beverages in form of glycosides conjugates that can not be absorbed in their original form. Before absorption, they have to be hydrolysed by intestinal enzymes or

by the colonic microbiota. Then, prior to passage into the blood stream, the aglycones undergo major changes, forming sulphate, glucuronide and/or methylated metabolites during absorption. Consequently, molecules arriving to blood and tissues are different from those present in food, thus it is rather complicated to identify all metabolites and evaluate their biological activity (D'Archivio *et al*., 2007). Besides, absorption may be influenced by several factors such as food matrix, heterogeneity of sugars or other functional groups conjugated with the flavan nucleus, molecular size and solubility, gender and genetic differences of consumers, dietary habits, dosage and intestinal microbiota (Banc *et al*., 2014; Rusconi & Conti, 2010).

Although the bioavailability of phenolics is controversial, it has been documented that flavan-3-ol monomers epicatechin and catechin are well absorbed in the intestine (about 22 % and 55 %, respectively), whereas dimers and trimers are weakly absorbed (less than 0.5 %) since their chemical structure and polymerization affect the intestinal absorption (reviewed by Ackar *et al*., 2013). In fact, catechins and procyanidins are not glycosylated in plants and derived food products and thus, their molecular size and solubility might be determining properties for their absorption. Resveratrol has been also proven to be highly absorbed (70 %) and transformed in metabolites that are detected in serum and urine (Banc *et al*., 2014). In general, smaller compounds are better absorbed, being in higher concentration in blood and being more likely to reach the target organ (Ackar *et al*., 2013; Han, Shen & Lou, 2007). It has been described that maximum concentration in plasma rarely exceeds 1 μM after the consumption of 10-100 mg of a single phenolic compound (Scalbert & Williamson, 2000). In the case of unabsorbed compounds, they reach the colon where they are degraded by the colonic microflora into aglycones and metabolized into different aromatic acids (Banc *et al*., 2014).

2. COCOA AND COCOA PRODUCTS

Theobroma cacao L. (family Malvaceae, formerly Sterculiaceae) derived products, such as cocoa powder and chocolates, are extremely consumed all over the world. Botanically, the term "cacao" refers to the tree (native of tropical America) and its fruits.

Cocoa pods contain about 30 to 40 seeds enveloped by a white mucilaginous pulp. Beans (seeds) consist of an outer layer (tegument) enveloping two cotyledons (embryonic) which represent the commercial product and contain mainly lipids (55-60 %), proteins (20 %), as well as starch, polyphenols and alkaloids (Minifie, 2012; Quintero & Díaz, 2004).

Three main cultivar groups of cacao beans are used to make cocoa and chocolate: Criollo (which is highly valued and unusual, less bitter and more aromatic than other beans, from which only 5-10 % of chocolate is prepared), Forastero (which has several sub-varieties, is significantly more resistant than Criollo trees and produces cheaper cocoa beans, representing about 80 % of global chocolate production) and Trinitario (a hybrid of Criollo and Forastero, which is employed in about 10-15 % of chocolate production) (Rusconi & Conti, 2010).

Africa is the largest supplier of cocoa beans in the world (77 % of net world exports), followed by Oceania and Asia (16 %) and America (6 %). With respect to Africa, the most important production and exportation of cocoa beans is carried out in Côte d'Ivoire and Ghana, although Nigeria and Cameroon are also among the five largest producing countries. Regarding Asia and Oceania, Indonesia is by far the responsible for the most important production of cocoa beans. In the case of Latin America, although regional exports are less important, Brazil and Ecuador possess a relevant role in the cocoa production (International Cocoa Organization (ICCO), 2015).

2.1. Manufacturing process and industry applications

Cocoa final products are obtained from the beans by industrial processes. Cocoa sub products are cocoa liquor, cocoa butter, cocoa presscake and cocoa powder. Final products are usually chocolates and other derivatives made with chocolate (e.g toppings, candies and chocolate bars). In addition to traditional uses of chocolate and confectionery products, cocoa butter is also employed in the pharmaceutical industry and in the elaboration of cosmetics products.

The manufacturing process of cocoa products (Fig. 3) starts with the collection of cocoa pods which are then shelled. Beans are consequently fermented, dried, cleaned

and classified. Finally, dried beans are sold to chocolate manufacturers who perform roasted and ground to produce cocoa liquor, cocoa butter and cocoa powder, which will be used to obtain the final product (Fleet & Dircks, 2007).

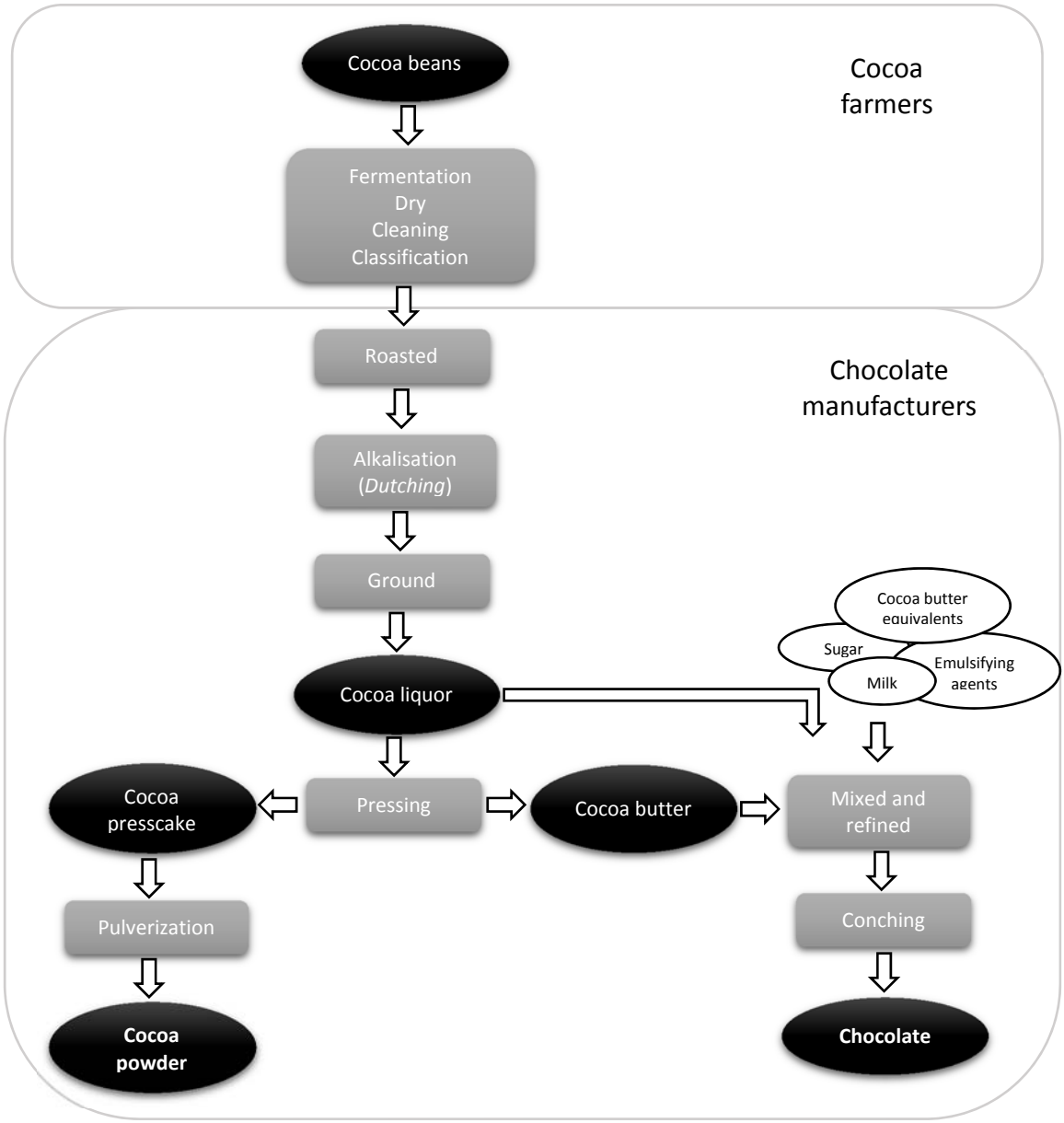


Figure 3. Manufacturing process of cocoa products.

In the particular case of chocolate fabrication (Fig. 3), manufacturers usually proceed as follows. First, beans are roasted to bring out the chocolate flavour and colour. The temperature, time and degree of moisture during roasting depend on the type of beans employed and the sort of chocolate or product required from the process. Shells are

removed from the beans to leave just the cocoa nibs, which undergo an alkalisation treatment (*dutching*), usually with potassium carbonate, to develop flavour and colour. Nibs are then milled to produce cocoa liquor (cocoa particles suspended in cocoa butter). More than one type of bean is commonly used for the products, so different beans have to be mixed together to the required formula. Cocoa butter is extracted from cocoa liquor by pressing, which also lead to the acquisition of a solid mass called cocoa presscake. The amount of butter extracted from liquor is controlled by manufacturers to produce presscake with different proportions of fat. Cocoa butter is used in the manufacture of chocolate while cocoa presscake is broken into small pieces which are then mashed to form cocoa powder. On the other hand, cocoa liquor is used to produce chocolate through the addition of cocoa butter. Other ingredients such as sugar, milk, emulsifying agents and cocoa butter equivalents are also added and mixed in different proportions depending on the type of chocolate being made. The mixture is then refined to obtain a smooth paste that improves the texture of chocolate. Subsequently, a kneading process called conching is carried out to develop flavour and texture. The resulting mixture is tempered, prepared for its different uses (put into moulds or used for enrobing or fillings) and cooled. Finally, chocolate is packaged to be distributed (ICCO, 2015).

Definition, composition and labelling of cocoa products and chocolate are regulated by the Directive 2000/36/EC of the European Parliament and of the Council relating to cocoa and chocolate products intended for human consumption. According to this directive, cocoa powder or cocoa designate the product obtained by converting cleaned, shelled and roasted cocoa beans into powder, which contains not less than 20 % of cocoa butter, calculated according to the weight of the dry matter, and not more than 9 % of water.

According to ICCO, world cocoa production has risen at an average annual growth rate of 3.3 % during the decade 2002-2012. The biggest regional trade of cocoa beans was by far between Africa (the world's largest cocoa producing region) and the European Union (the world's largest cocoa consuming region). Europe remained undoubtedly the largest cocoa processing region during the evaluated interval. The increase of grindings in European countries was estimated at 277,000 tonnes between

2002 and 2012 (corresponding to an average annual growth rate of 2.1 %). Most of the cocoa processing is performed in cocoa importing countries near the major epicenters of cocoa consumption in Europe and North America, with the Netherlands keeping its position as the world's leader in cocoa processing.

2.2. Cocoa consumption

ICCO proposes a way to measure the cocoa consumption at the country or regional level as the amount of cocoa beans used in the manufacture of confectionery, food, beverage or cosmetic products that are actually consumed in that area. This "apparent domestic cocoa consumption" is computed as grindings plus net imports of cocoa and chocolate and chocolate products and expressed as bean equivalents. Cocoa products and the net trade in chocolate and chocolate based products are translated into bean equivalents, based on general assumptions about the cocoa content of chocolate products involved.

According to data provided by ICCO for the decade 2002-2012, primary cocoa consumption (measured as total world grindings of cocoa beans) maintained the upward trend observed in previous periods, growing at an average rate of 2.9 % per annum and representing a total increase of over 915,000 tonnes. The main force behind the strong demand for cocoa powder is the change in global consumption patterns in emerging countries. There are millions of new consumers, including Asia, where demand is focused on powder-based commodities.

Spain is among the leading cocoa consumer countries in the world, after the United States, Germany, France, the United Kingdom, the Russian Federation, Brazil and Japan, according to the data provided by ICCO, for the year 2010/2011, being the world *per capita* consumption of cocoa of 0.61 kg for that year. Figure 4 shows the evolution of cocoa consumption for some of the largest consuming countries from 2002 to 2011. The importance of this product in the European society can be appreciated, since in Germany, for example, cocoa consumption was around 4 kg/head in 2011. In the case of Spain, the consumption level was approximately 2.25 kg/head for the same period.

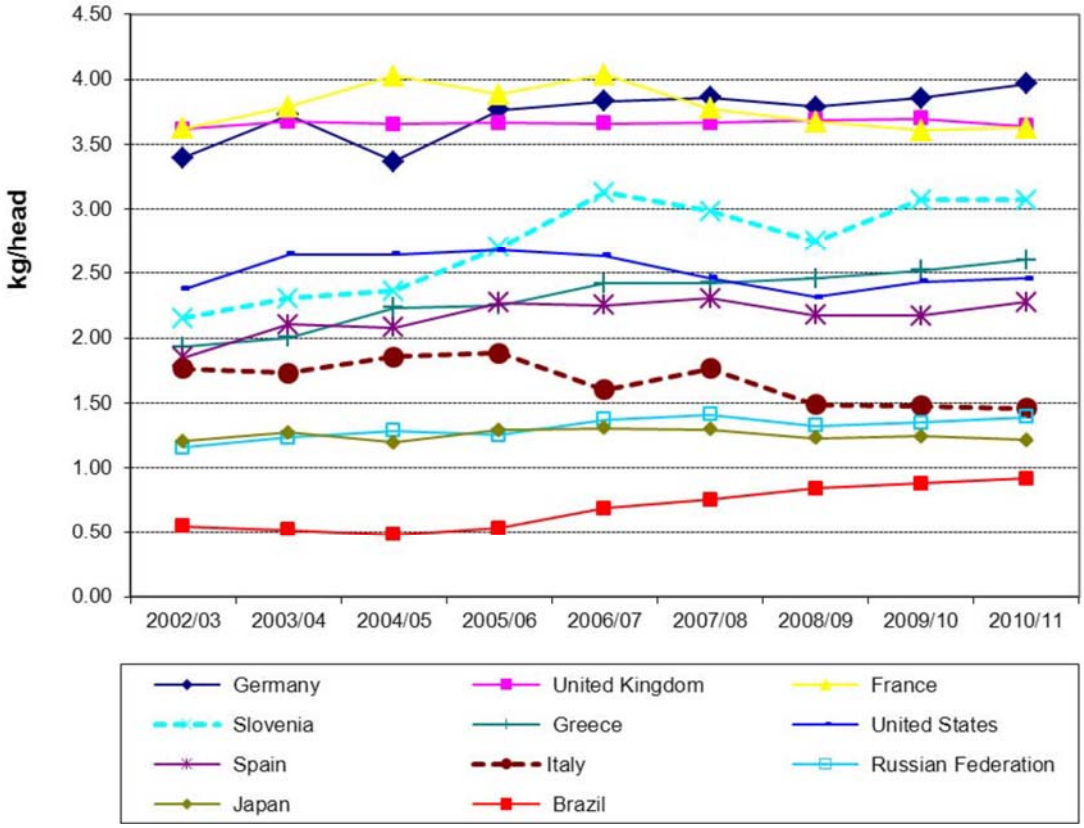


Figure 4. Per caput cocoa consumption (bean equivalents) in the main consuming countries, from 2002 to 2011 (ICCO, 2015).

The Spanish Ministry of Agriculture, Food and Environment (MAGRAMA) collects data on food consumption every month in Spanish households. According to the last published information, the chocolate and cocoa market in Spain reached 1,067 million euros during October 2014, with a consumption of 3.6 kg *per capita* (MAGRAMA, 2015).

2.3. Polyphenol content in cocoa products

Cocoa is a very rich product in phenolic compounds, mainly flavonoids such as polymeric and oligomeric flavanols (procyanidins) and monomeric flavan-3-ols (catechin and epicatechin). The content of polyphenols in unfermented beans is high (12-18 % of dry weight, of which 37 % are monomeric flavan-3-ols and 58 % are polymeric flavanols) (Rusconi & Conti, 2010). Flavonols, flavones and flavanones such as quercetin, apigenin and naringenin, respectively, have been identified in cocoa samples as well (Lamuela-Raventós, Olga, Ibern-Gómez, Pons-Raga & Andrés-Lacueva, 2003; Sánchez-Rabaneda *et al*., 2003). Furthermore, the flavonol glycosides, quercetin-3-*O*-arabinoside,

quercetin-3-*O*-glucoside (isoquercitrin), quercetin-3-*O*-galactoside (hyperoside) and quercetin-3-*O*-glucuronide, and the flavones, apigenin, apigenin-8-*C*-glucoside (vitexin), apigenin-6-*C*-glucoside (isovitexin), luteolin and luteolin-7-*O*-glucoside, have been also found in cocoa (Andrés-Lacueva *et al*., 2000; Sánchez-Rabaneda *et al*., 2003). Two anthocyanins, cyanidin-3-O-arabinoside and cyanidin-3-O-galactoside, and the N-phenylpropernoyl-L-amino acids, N-caffeoyl-L-aspartate, N-coumaroyl-L-aspartate, N-coumaroyl-3-hydroxytyrosine (clovamide) and N-coumaroyltyrosine (deoxyclovamide), were found by Pereira-Caro *et al*. (2012) in cocoa beans. These are some of the compounds present in cocoa but its phenolic composition is much more complex and several studies have been performed in recent years trying to identify as many compounds as possible (Ali, Ranneh, Ismail & Esa, 2013; Cádiz-Gurrea *et al*., 2014; Patras, Milev, Vrancken & Kuhnert, 2014; Pereira-Caro *et al*., 2012).

In addition to these phenolic compounds, methylxanthines are also present in cocoa in significant amounts, being caffeine and theobromine the most important alkaloids (Borchers, Keen, Hannum & Gershwin, 2000; Pereira-Caro *et al*., 2012).

However, although cocoa represents a relevant source of polyphenols, the amount of these compounds considerably fluctuates in processed products such as cocoa powder or chocolate. Great loss of flavonoids occurs during fermentation and processing of cocoa beans (mainly due to roasting and *dutching* steps), up to 90 % in some cases. As a consequence, large variations in flavonoids content are found in commercial cocoa products and many milk chocolate products lose nearly all flavan-3-ols (Crozier, Jaganath & Clifford, 2009). Andrés-Lacueva *et al.* (2008) carried out a study to determine the influence of the manufacturing process on the flavanol and flavonol content of cocoa powder products. They found that alkalization treatment caused a 60 % reduction of cocoa flavonoid content, being epicatechin and quercetin the most affected compounds.

In view of this loss of polyphenols in processed cocoa derivatives, flavonoid-rich cocoa products are being elaborated. To stand up to this problem, Tomás- Barberán *et al*. (2007) performed a new process to develop an unfermented, nonroasted and blanch-treated cocoa powder with higher flavonoid content than a commercial powder. Polyphenol and purine composition of the polyphenol-rich and conventional cocoa

powders are shown in Table 2. Furthermore, they carried out a study in healthy humans to determine the bioavailability of the compounds existing in the enriched cocoa powder and they observed the presence of flavonoid metabolites in plasma, demonstrating the bioavailability of these compounds.

Table 2. Polyphenol and purine composition of cocoa powders (modified from Tomas-Barberán *et al*., 2007). Values are mg/g cocoa powder; t, detected but not quantified; -, not detected.

Compound	Polyphenol-rich cocoa powder	Conventional cocoa powder
Theobromine	17.77	22.14
Caffeoyl aspartate	0.27	-
B1 dimer	3.68	1.12
Trimer	2.33	t
Caffeoyl aspartate (isomer)	3.88	0.37
Tetramer	1.46	-
Tetramer	1.30	-
Catechin	6.46	2.02
Caffeine	4.39	1.95
Trimer	4.36	t
B2 dimer	24.34	2.62
***p*-coumaroyl aspartate**	1.01	t
Tetramer	5.67	-
Epicatechin	25.65	3.30
Trimer	12.71	t
Tetramer	10.85	-
Pentamer	4.82	t
Caffeoyl 3-hydroxy-tyrosine	0.71	t
Dimer	7.19	t
Caffeoyl tyrosine	0.24	t
Quercetin 3-hexoside	0.25	t
Quercetin 3-arabinoside	0.30	-
***p*-coumaroyl tyrosine**	t	-
Quercetin	0.09	t
Oligomers+polymers	8.96	13.93
Total procyanidins	119.78	22.99

As flavonoid-rich products, cocoa derivatives have been shown to possess relevant biological activities translated into human health benefits. These findings have also led to an increased interest in obtaining derived cocoa products with high polyphenol content.

2.4. Beneficial effects of cocoa polyphenols on human health

As a very popular product in European countries, in the past decades cocoa has been extensively studied because of its potential positive effects on human health. Cocoa polyphenols have been associated with some possible beneficial effects since they may act as antioxidant, anticarcinogenic, antiinflammatory, antihepatotoxic, antibacterial, antiviral and antiallergenic compounds (Ackar *et al*., 2013). In this context, indications on some functional cocoa compounds, with beneficial health properties have been published. These statements are related to its high content of monomeric (epicatechin and catechin) and dimeric (procyanidins) flavanols. Consequently, cocoa and chocolate may be considered functional foods but the functional significance of each component has not been correspondingly elucidated.

Actually, polyphenols have most often multitarget actions and depending on the compounds, they could act through nonspecific and/or specific mechanisms (Milenkovic, Jude & Morand, 2013). In fact, the molecular interactions of polyphenols with biological systems are not yet fully understood. Nowadays it is known that polyphenols may offer indirect protection by activating endogenous defense systems (for example, catechin was able to increase catalase (CAT), glutathione S-transferase (GST) and superoxide dismutase (SOD) activities *in vitro* (Du, Guo & Lou, 2007)). And also by modulating cellular signalling processes such as activation of nuclear factor-kappa B (NF-κB), activator protein-1(AP-1) DNA binding, glutathione biosynthesis, phosphoinositide 3 (PI3)-kinase/protein kinase B (Akt) pathway, mitogen-activated protein kinase (MAPK) proteins (extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and P38) activation, and the translocation into the nucleus of nuclear factor erythroid 2 related factor 2 (Nrf2) (Han, Shen & Lou, 2007; Fraga *et al*., 2010). However, more information is needed to understand the mechanisms of action

of polyphenols in general, but also of cocoa phenolics in particular, to support the beneficial effects found in cocoa products. That's why it is worth studying and searching for specific molecular targets of cocoa polyphenols.

As antioxidant and detoxifying agents, polyphenols, specifically flavonoids, exert their protective action by preventing DNA damage caused by ROS or other toxic and carcinogenic agents in several different ways: free radicals scavenging, metals chelation which could participate in the Fenton reaction, antioxidant enzymes modulation and procarcinogenic metabolism alteration by modulating phase I and II enzyme activities (Martin, Goya & Ramos, 2013).

Three main beneficial effects of cocoa polyphenols have been extensively studied: cardiovascular protection, cancer prevention and antioxidant capacity (which is indeed involved in the two previous).

2.4.1. Antioxidant properties

Oxygen is essential for aerobic organisms. Growth under aerobic conditions results in the production of free radicals and other ROS, such as hydrogen peroxide (H_2O_2) or superoxide anions ($O_2^{\cdot-}$), among others (Estruch, 2000). Some ROS are generated endogenously by normal metabolic processes (e.g. respiration, with the main ROS production occurring in the electron transport chain in the mitochondria, and β-oxidation of fatty acids) as well as by exposure to pro-oxidants (e.g. hydrogen peroxide and heavy metals) (Estruch, 2000). Low concentrations of ROS seem to be needed by cells for transduction of cellular signals in processes such as proliferation, apoptosis, inflammation and immune response. However, the excess of these ROS can cause oxidative damage to DNA, lipids and proteins, resulting in an oxidative imbalance of the antioxidant system (Wu *et al*., 2011), which can lead to cell death or to acceleration of cell aging and age-related diseases (Silva *et al*., 2009) Actually, oxidative stress is involved in mutagenesis, carcinogenesis, lipid peroxidation, alterations of the correct membrane function and oxidation and fragmentation of proteins (Lamuela-Raventós, Romero-Pérez, Andrés-Lacueva & Tornero, 2005) and it has been related to several age-related degenerative disorders including cardiovascular and neurodegenerative

diseases, cancer, diabetes and osteoporosis (Scalbert, Manach, Morand, Remesy & Jiménez, 2005).

Since it was shown that polyphenols from food sources had the ability to scavenge free radicals *in vitro*, it was thought that their main role *in vivo* was as antioxidants involved in stress protection. In fact, polyphenols have been reported to be antioxidants because of their capacity to donate electrons and also to form complexes with heavy metal ions (such as Fe^{2+}), thus, avoiding Fenton-like reactions. However, during the last decades, it has been established that the mode of action of these compounds is much more complicated than expected. Indeed, polyphenols could also be involved in the modulation of gene expression and the interaction with cell signalling pathways, since the physiological effects of these compounds may extend beyond the modulation of oxidative stress. In this respect, recent studies have shown that polyphenols could interact with cell signalling cascades regulating the activity of transcription factors and thus affecting the expression of genes.

As antioxidants, one of the main features of polyphenols is to donate hydrogen to free radicals, leading to the formation of stable molecules. Phenolic compounds seem to be incorporated in the membrane lipid bilayers and act as hydrogen donors, leading to the stabilization of free radicals and avoiding the formation of lipid radicals (Dani *et al*., 2008). The antioxidant capacity of cocoa polyphenols has been commonly studied by *in vitro* methods. The most used are the *in vitro* chemical studies to determine the radical-scavenging ability, like the free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging assay (Schinella *et al*., 2010; Tabernero, Serrano & Saura-Calixto, 2006) , the oxygen radical absorbance capacity (ORAC) test (Cádiz-Gurrea *et al*., 2014) and the trolox equivalent anti-oxidative capacity (TEAC) assay (Cádiz-Gurrea *et al*., 2014; Schinella *et al*., 2010; Tabernero, Serrano & Saura-Calixto, 2006) and the determination of the ferric reducing-antioxidant power (FRAP assay) (Cádiz-Gurrea *et al*., 2014; Schinella *et al*., 2010; Tabernero , Serrano & Saura-Calixto, 2006). But these assays present some problems, since they do not evaluate the protective effect on cell survival, the antioxidant effect has been reported to be more marked *in vitro*, using high, nonphysiological concentrations and because several steps are necessary to reach the target organ or tissue before they exert their antioxidant effect (Baroni, Di Paola

Naranjo, García-Ferreyra, Otaiza & Wunderlin, 2012). For that reason, *in vivo* methods would result more useful.

Moreover, several studies endorse the positive effects of cocoa polyphenols on human health, derived from its antioxidant role. It has been shown that a cocoa extract protected human liver cells against oxidative stress-induced apoptosis by reducing ROS production in the presence of a stressor and by modulating the activities of several antioxidants enzymes (Martín *et al*., 2010). Besides, cocoa polyphenols have been described as bioactive compounds because of their metabolic and cardiovascular effects also mainly due to its antioxidant properties, including the inhibition of lipid peroxidation and the protection of LDL-cholesterol against oxidation as well as the decrease of platelet function and inflammation (Andújar, Recio, Giner & Ríos, 2012).

2.4.2. Cardiovascular-protective capacity

Recent meta-analyses of intervention studies have positively correlated cocoa consumption to several cardiovascular risk factors such as blood pressure (Ried, Sullivan, Fakler, Frank & Stocks, 2012), insulin resistance (Hooper *et al*., 2012), lipid profiles (Jia *et al*., 2010) and flow-mediated vascular dilatation (FMD) (Hooper *et al*., 2008). Otherwise, many *in vitro* and *in vivo* experimental data reinforce the idea that polyphenols may mediate these beneficial effects of cocoa (reviewed by Khan *et al*., 2014).

Polyphenols have been proposed as principal mediators in the anti-inflammatory cardio-protective properties of cocoa. It seems that cocoa polyphenols have an effect on the most significant markers of cardiovascular disease (CVD) such as oxidized LDL (oxLDL) (Baba *et al*., 2007; Khan *et al*., 2012), lipid profile (Baba *et al*., 2007; Khan *et al*., 2012), blood pressure (Cienfuegos-Jovellanos *et al*., 2009), nitric oxide (NO) (Quiñones, Sánchez, Muguerza Miguel & Aleixandre, 2011), hemostasis (Murphy *et al*., 2003b) and endothelial dysfunction (Faridi, Njike, Dutta, Ali & Katz, 2008).

With respect to lipid profile, an imbalance in cholesterol levels is associated with the emergence of atherosclerosis and CVD. Besides, oxLDL plays a crucial role in the progression of atherosclerosis. As reviewed by Khan *et al*. (2014), several studies have

established oxLDL as a useful marker for cardiovascular diseases. In this context, cocoa consumption has been related to a decrease in LDL and its oxidation and an increase in HDL (Khan *et al*., 2012; Osakabe *et al*., 2002).

Regarding the vascular systems, cocoa polyphenols have also been studied, being the principal targets the nitric oxide concentration and the endothelial function, together with decreased susceptibility of LDL to oxidation and inhibition of platelet activation and aggregation (Khan *et al*., 2012; Rein *et al*., 2000). As reviewed by Andújar, Recio, Giner & Ríos (2012), preliminary studies carried out in animals and humans suggest a possible regulation of NO bioavailability, influencing endothelial function and, in consequence, blood pressure. In this context, Cienfuegos-Jovellanos *et al*. (2009) demonstrated the antihypertensive effect of a natural flavonoid enriched cocoa powder obtained by an industrial process (CocoanOX) after its administration to spontaneously hypertensive rats (SHR). In brief, this group suggests the possible use of CocoanOX as a functional food ingredient having potential therapeutic effect in the prevention and treatment of hypertension. Further studies carried out by Quiñones, Sánchez, Muguerza, Miguel & Aleixandre (2011) propose that the antihypertensive effect observed after CocoanOX administration to rats could be mediated by an improvement of endothelial release of NO and by a reduction of oxidative stress.

2.4.3. Antitumoral effects

In the past years, a diet rich in fruit and vegetables has been related to a reduced risk of cancer. These facts have led to an intensive research of natural substances present in food with chemopreventive effects, with special interest in polyphenols.

Cocoa products arouse great interest in this field because of its high content in polyphenols and its important consumption by population. In this context, anti-carcinogenic effect of cocoa products has been mainly evaluated *in vitro* (cancer cell lines) as a first approach for further *in vivo* studies, which have been performed in animal models. Epidemiological studies linking cocoa and cancer have been carried out as well.

With respect to cell cultures studies, cocoa polyphenols have been tested to determine its antioxidant and anti-inflammatory properties, as well as for its effects on

apoptosis and proliferation and on angiogenesis and metastasis. Cocoa phenolics could act by modulating the redox status and several key components in signal transduction pathways related to the aforementioned processes.

Studies with experimentation animals (mice and rats) have demonstrated the ability of cocoa and its polyphenols to prevent and/or reduce the initiation or progression of some cancers such as breast, pancreas, lung, thyroid, liver, colon and prostate cancers and leukemia (reviewed by Martin, Goya & Ramos, 2013).

3. *Saccharomyces cerevisiae* AS A MODEL ORGANISM

3.1. General remarks

The unicellular yeast *S. cerevisiae* is commonly used as an eukaryotic model not only for molecular and cellular biology studies but also to identify natural antioxidants. Several characteristics make this organism a versatile model for basic and applied research (Mager & Winderickx, 2005; Outeiro & Giorgini, 2006):

- It is easy to handle in the laboratory and its growth is rapid.
- Several processes such as DNA replication, recombination, protein folding, cell division, metabolism and intracellular transport are conserved in higher eukaryotes organisms, including mammals.
- Its genome is fully sequenced and about 30 % of known human disease-associated genes have yeast orthologues.
- Yeast genes can be easily manipulated.
- There are mutants for each of its 6000 genes, which helps the identification of gene targets of chemicals or stress response pathways and the study of molecular mechanisms associated with cell aging.

3.2. Oxidative stress response in *S. cerevisiae*

The normal aerobic cell metabolism and the exposure to environmental factors such as heavy metal ions and free radical-generating compounds, lead to ROS production

(Estruch, 2000). Although cells have different mechanisms to maintain their redox status, the imbalance between antioxidant defences and ROS generation results in an oxidative stress situation. Oxidative stress causes damage in different cell macromolecules (e.g. lipids, proteins and nucleic acids), which is associated with aging and a range of age-associated human diseases, including neurodegenerative disorders, CVD and cancer. To overcome stress conditions and survive, cells need to obtain a range of stress-adaptation mechanisms among which are the induction of stress proteins, the accumulation of compatible solutes, the modification of membrane composition and the repression of translation, by regulating the corresponding gene expression via stress-triggered signal transduction pathways (Takagi, 2008).

There are many studies about the oxidative stress response mechanisms in *S. cerevisiae* (Drakulic *et al*., 2005; McDonagh, Ogueta, Lasarte, Padilla & Bárcena, 2009; Mulford & Fassler, 2011; Pereira, Herdeiro, Fernandes, Eleutherio & Panek, 2003; Thorpe, Fong, Alic, Higgins & Dawes, 2004). Furthermore, the use of genome-wide screenings of yeast deletion strains has helped to discover novel targets of different chemicals and pharmaceutical compounds (Tucker & Fields, 2004).

Different drugs are used to study the response to oxidative stress in the yeast *S. cerevisiae*. Among them, hydrogen peroxide has been commonly used as an oxidative stress promoter model. This molecule is formed during normal aerobic respiration but also after exposure to several environmental factors. It not only generates oxidative stress damage but also serves as a signalling molecule in the regulation of several biological processes (Veal, Day & Morgan, 2007). The redox-cycling drug menadione is also used to provoke oxidative stress in *S. cerevisiae*. It transfers electrons to molecular oxygen leading to the formation of O_2^- which can act as a precursor of extremely reactive radicals such as hydroxyl radical ($OH^\cdot$).

3.2.1. Genes induced by oxidative stress: metabolic enzymes and ROS scavengers

Aerobic organisms possess several mechanisms to neutralize free radicals generated through metabolism. During oxidative stress response, a transient adaptation to non-toxic doses of H_2O_2 as well as a cross-adaptation to other stresses occur in the cell,

resulting in a protection against toxic ROS. Cells have complex enzymatic and non-enzymatic (chemical scavengers) systems to prevent oxidative damage caused by ROS (Table 3).

Free radical scavenging enzymes (i.e. superoxide dismutases or catalases) represent the first line of cell protection against oxidative damage. The balance between antioxidant enzymes is essential for the elimination of ROS in the cell, avoiding its excessive accumulation. The second line of defense is formed by non-enzymatic scavengers, being glutathione (GSH) the most important one, whose synthesis in the yeast is performed by the enzymes Gsh1 and Gsh2. Various transcription factors are involved in the regulation of genes expression under oxidative stress conditions, among which are Yap1p (yeast AP-1-like) and Skn7p (suppressor of kre null, also known as Pos9p). Both of them are needed for the expression of ROS-detoxifying genes which codify enzymes involved in oxidative stress protection (such as catalases and superoxide dismutases) (Estruch, 2000) with the ultimate goal of restoring the redox homeostasis.

Table 3. Main antioxidant defences in *S. cerevisiae* (modified from Estruch, 2000; Izquierdo, Casas, Muhlenhoff, Lillig & Herrero, 2008; Morano, Grant & Moye-Rowley, 2012; Saccharomyces Genome Database (SGD), 2015).

System	Proteins	Function/localization	Induction by H_2O_2	Transcriptional regulator(s)
Glutathione	Gsh1p	glutathione synthesis (cytoplasm)	Yes	Yap1p
	Gsh2p	glutathione synthesis (cytoplasm)		
Thioredoxins	Trx1p/ Trx2p	thioredoxin (cytoplasm)	Yes	Yap1p/Skn7p
	Trr1p	thioredoxin reductase (cytoplasm)	Yes	Yap1p/Skn7p
	Trx3p	thioredoxin (mitochondria)		
	Trr2p	thioredoxin reductase (mitochondria)		
Glutaredoxin	Grx1p	Glutaredoxin (cytoplasm)	Yes	
	Grx2p	glutaredoxin (cytoplasm, mitochondira)	Yes	
	Grx3p	glutaredoxin (nucleus)		
	Grx4p	glutaredoxin (nucleus)		
	Grx5p	glutaredoxin (mitochondria)		
	Grx6p	glutaredoxin (secretory pathway)		
	Grx7p	glutaredoxin (secretory pathway)		
Superoxide dismutase	Sod1p	Cu/Zn-S0D (cytoplasm)	Yes	Yap1p/Skn7p
	Sod2p	Mn-S0D (mitochondria)	Yes	Yap1p/Skn7p
Catalases	Cta1p	catalase (peroxisome)		
	Ctt1p	catalase (cytoplasm)	Yes	Yap1p/Skn7p
Peroxidases	Gpx1	glutathione peroxidase (cytoplasm)		
	Gpx2	glutathione peroxidase (cytoplasm)		Yap1p/Skn7
	Gpx3	glutathione peroxidase (cytoplasm)		
	Tsa1	peroxiredoxin (cytoplasm)		Yap1p/Skn7
	Tsa2	peroxiredoxin (cytoplasm)	Yes	
	Ahp1	peroxiredoxin (cytoplasm)		Yap1p/Skn7
	Prx1	peroxiredoxin (mitochondira)		
	Dot5	peroxiredoxin (nucleus)		

With respect to the enzymatic defense, two overlapping groups of enzymes can be considered (Fig. 5): one group (including superoxide dismutases, catalases and peroxidases) acts directly detoxifying ROS and the other group (which includes thioredoxin and glutaredoxin systems) acts as redox regulators of protein thiols (Herrero, Ros, Bellí & Cabiscol, 2008).

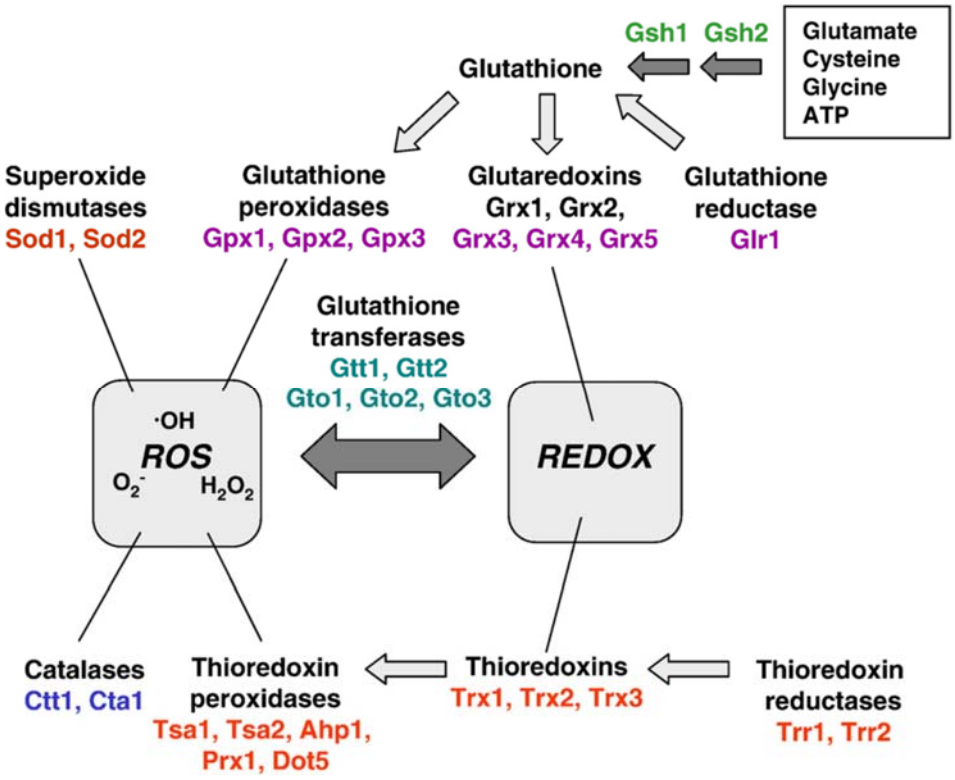


Figure 5. Enzymatic defense against oxidative stress in *S. cerevisiae* (Herrero, Ros, Bellí & Cabiscol, 2008).

Superoxide dismutases (SOD) are the main enzymes involved in the protection against oxidative damage in aerobic organisms. They are a family of metalloproteins having different metallic cofactors. *S. cerevisiae* cells possess two forms: Cu/Zn-SOD (Sod1p) located in the cytoplasm and Mn-SOD (Sod2p) found in the mitochondria. Both of them, *SOD1* and *SOD2*, are induced by hydrogen peroxide in a Yap1p and Skn7p dependent way and could have a role in detoxification of O_2^- produced in the mitochondria respiratory chain through the dismutation reaction of O_2^- into H_2O_2 and O_2 (Estruch, 2000; Herrero, Ros, Bellí & Cabiscol, 2008).

Catalases remove H_2O_2 by catalyzing its reduction to H_2O and O_2 with the help of the redox properties of a heme group complexed to the polypeptide. Two of them exist in yeasts, catalase A and catalase T, located in the peroxisome and the mitochondria, respectively. Catalase A is codified by *CTA1* and its main role seems to be the elimination of H_2O_2 from fatty acid β-oxidation. Catalase T is codified by *CTT1* and its main role in stress response is not yet fully explained although its expression is induced under several stress conditions such as oxidative and osmotic stress, as well as carbon starvation and

freeze-thawing conditions (Estruch, 2000; Herrero, Ros, Bellí & Cabiscol, 2008; Morano, Grant & Moye-Rowley, 2012).

In the case of peroxidases, its role as ROS detoxifiers does not depend on the redox properties of the metal group associated to the enzyme, as in the case of the two aforementioned enzymes. Peroxidases act by the reduction of organic and inorganic peroxides to alcohols with the help of active site cysteine thiols. Thus, electron donors for thiols are essential for peroxidases activity. Depending on this, two types of peroxidases exist: glutathione peroxidases (GPXs), which use GSH, and thioredoxin (TRX) peroxidases (also named peroxiredoxins (PRXs)), which need TRXs. In yeasts, three types of GPXs are found: Gpx1, Gpx2 and Gpx3; as well as five PRXs: cytosolic Tsa1 (cTPxI), Tsa2 (cTPxII) and Ahp1 (cTPxIII), mitochondrial Prx1 (mTPx) and nuclear Dot5 (nTPx) (Herrero, Ros, Bellí & Cabiscol, 2008).

The thioredoxin system is composed by thioredoxin (Trx), thioredoxin reductase (Trr) and NADPH. Two systems are present in the yeast, one of them found in the cytoplasm having two thioredoxins (Trx1p and Trx2p) and a thioredoxin reductase (Trr1p) and the other one in the mitochondria, comprising a thioredoxin (Trx3p) and a thioredoxin reductase (Trr2p). It has been shown that the suppression of *TRX1* and *TRX2* results in a decreased resistance to hydrogen peroxide. Moreover, *TRX2* and *TRR1* are induced by hydrogen peroxide in a Yap1p y Skn7p dependent way (Estruch, 2000).

Glutaredoxins are divided in two subfamilies. The first one protects cells against hydrogen peroxide (Grx2p) and superoxide anions (Grx1p). The second one includes six other members (Grx3-8), present in different subcellular compartments. Grx3/4 are involved in intracellular iron trafficking. Grx5p has a potential role in the protection against oxidative stress during growth in normal conditions as well as after the exposure to oxidant agents such as H_2O_2 and menadione (Estruch, 2000; Morano, Grant & Moye-Rowley, 2012). Grx6/7 are thought to work in the regulation of sulfhydryl in the early secretory pathways under stress conditions. Finally, glutaredoxin Grx8 does not seem to have a function in the oxidative stress response (Morano, Grant & Moye-Rowley, 2012).

Regarding the second line of defense which is formed by non-enzymatic scavengers, the low molecular-weight thiol compound glutathione (ɤ-glutamylcysteinylglycine, GSH)

plays an important role in the prevention of oxidative damage. Gamma glutamylcysteine synthetase (Gsh1) and glutathione synthetase (Gsh2), whose expressions are induced by oxidative stress, are required for the glutathione biosynthetic process. During oxidative stress conditions, glutathione is oxidized to its disulfide form (GSSG). However, glutathione reductase (Glr1) maintains glutathione in its reduced GSH form using NADPH generated in the pentose phosphate pathway. As antioxidant molecule, GSH acts as a cofactor for many protective enzymes such as glutathione transferase (GSTs) and GPXs (Morano, Grant & Moye-Rowley, 2012). Glutathione transferases constitute a bridge between the two defence strategies, since they may share peroxidase and redoxin activities in addition to their GSH-conjugating role (Herrero, Ros, Bellí & Cabiscol, 2008).

3.2.2. Other genes and proteins involved in stress response

Godon *et al*. (1998) carried out a genome-wide characterization of a H_2O_2-inducible stimulus in *S. cerevisiae*. They found 167 differentially expressed proteins in the yeast after exposure to H_2O_2, being the antioxidant defences, heat shock and chaperone proteins, translational apparatus, proteases and carbohydrate metabolism, the cellular functions primarily affected. Metabolic enzymes are also included as H_2O_2-responsive targets. Notable changes happen in carbohydrate metabolism, which seems to be redirected to the regeneration of NADPH (Godon *et al*., 1998). Among the metabolic changes, the repression of the glyceraldehyde-3-phosphate dehydrogenase isozymes *TDH2* and *TDH3* indicates a possible slowdown of glycolysis. Moreover, the hexose phosphate pool would be redirected to the pentose phosphate pathway and the trehalose synthesis through the induction of *PGM2* (phosphoglucomutase), *ZWF1* (glucose-6-phosphate dehydrogenase), *TKL1* and *TKL2* (transketolases), *TAL1* (transaldolase), *UGP1* (UDP-glucose pyrophosphorylase) and *TPS1* (trehalose-6-phosphate synthase) (Estruch, 2000).

Other proteins potentially involved in the oxidative stress response and aging are sirtuins. Sirtuins are a conserved family of nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylases proteins, present in organisms from bacteria to humans. In eukaryotic organisms, sirtuins regulate a wide group of cellular functions

such as cell division cycle and response to DNA damage agents. They are also involved in molecular mechanisms of aging (North & Verdin, 2004). Sirtuins acquired special attention since it was observed that the yeast sirtuin silent information regulator 2 (Sir2) (firstly described as a regulator of transcriptional silencing of mating-type loci, telomeres and ribosomal DNA), prolonged yeast lifespan (Kaeberlein, McVey & Guarente, 1999).

Chromatin silencing leads to a compact structure and needs particular lysine in the extended amino-terminal tail of histones H3 and H4, being histones responsible of regulating processes such as transcription, replication and reparation. These and other residues are acetylated in active chromatin and deacetylated in silent chromatin (Baur, Ungvari, Minor, Le Couteur & de Cabo, 2012; Guarente, 2000). Silencing is used by the yeast to control the expression of genes that codify key regulators factors determining the cellular type, ribosomal DNA levels and correct telomere function (Young & Kirchmaier, 2012). In the particular case of Sir2p, deacetylation of lysine 16 of histone 4 extends the silencing of subtelomeric regions, leading to a more compact structure. Moreover, Sir2p plays a role in longevity, associated to its capacity to increase the genomic stability in silenced regions. It has also been observed that, after a genotoxic stress provoked with H_2O_2, Sir2p undergoes a rearrangement in the genome, leaving the heterochromatic regions to participate in the repair of damaged areas (Oberdoerffer *et al*., 2008), which leads to a transcriptional change similar to that found in aged cells. *S. cerevisiae* genome codifies four more proteins, besides Sir2p, named “sir2 homologues” (Hst1p-Hst4p). Among them, Hst1 presents higher homology with Sir2 although Hst2 is the one expressing in higher amounts in the yeast.

In mammals, seven proteins belong to the sirtuins family (SIRT1-SIRT7), which are located in different cellular compartments and develop different functions (Houtkooper, Pirinen & Auwerx, 2012). Among them, SIRT1 received much more attention than the others because of its possible implication in caloric restriction mediated lifespan extension, the prevention of aging-related disorders and the maintenance of metabolic homeostasis. SIRT1 not only deacetylases histones but also other protein targets such as p53 (which is deacetylated and repressed upon DNA damage or oxidative stress, causing altered apoptosis) and forkhead box O (FOXO) transcription factors (which are crucial regulators of glucose and lipid metabolism and stress response) (Houtkooper,

Pirinen & Auwerx, 2012). SIRT1 also activates the proliferator-activated receptor-γ co-activator 1α (PGC1α) by deacetylation, resulting in the induction of downstream pathways that control the expression of mitochondrial genes. SIRT3 is also becoming important due to its possible involvement in oxidative stress defense by protecting cells from ROS. In fact, it has been shown that during caloric restriction, SIRT3 activates the antioxidant enzyme SOD2 (Qiu, Brown, Hirschey, Verdin & Chen, 2010). Under this conditions, SIRT3 also deacetylates and thus activates isocitrate dehydrogenase 2 (IDH2), which is involved in the tricarboxylic acid cycle (TCA cycle) (Houtkooper, Pirinen & Auwerx, 2012).

Figure 6 shows the interaction of ROS, aging and caloric restriction with SIRT1 and SIRT3, the two closest mammalian homologs sirtuins of yeast Sir2. Activation of these sirtuins leads to an increased oxidative stress resistance, as a result of the deacetylation of the aforementioned proteins, in the cell.

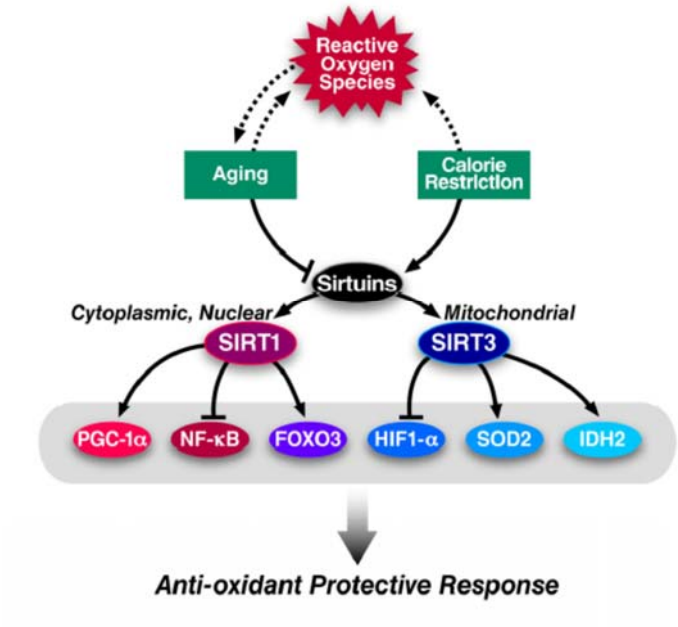


Figure 6. Interaction of ROS, aging and caloric restriction with the mammalian sirtuins SIRT1 and SIRT3. Solid lines indicate strong evidence interaction and dashed lines indicate putative interactions (Merksamer *et al*., 2013).

In view of these findings, it would be interesting to investigate natural compounds with sirtuin modulation properties. In this context, Howitz *et al*. (2003), found small molecules that could modulate the activity of sirtuins, being some of them polyphenols.

Butein, fistein and resveratrol (10 µM each) increased yeast average lifespan of 31 %, 55 % and 70 %, respectively. The capacity of Sir2p to increase lifespan seems to be related to its role in the repetitive DNA stabilization. Ribosomal DNA recombination leads to the formation of extrachromosomal circular DNA which can replicate until toxic levels in old cells (thus decreasing replicative lifespan in yeasts). Results showed that resveratrol decreased the frequency of recombination of ribosomal DNA up to 60 % in a Sir2p dependent way. This effect was also reached by the activation of Sir2p by caloric restriction, resulting in an increased replicative lifespan. Resveratrol not only increased lifespan in yeasts by a Sir2p dependent mechanism, but also in worms and flies (Wood *et al*., 2004).

Martorell *et al*. (2011) found that the sirtuin Hst3p was involved in the yeast protection against oxidative stress conferred by a flavonoid-rich cocoa powder. A transcriptomic study carried out by this group revealed the upregulation of *HST3* after treatment with the cocoa powder. To confirm this results, the knockout mutant in *HST3* gene was used to study the cocoa antioxidant protection against oxidative stress (spots assays). Results showed a different phenotype to that found in the wild-type strain, since the cocoa powder did not protect the *HST3* mutant, indicating the possible involvement of this sirtuin in the effect of cocoa flavonoids.

3.3. Model for the study of bioactive compounds with antioxidant capacity

S. cerevisiae has been used as an *in vivo* model to screen for natural antioxidant compounds, including polyphenols (Baroni, Di Paola Naranjo, García-Ferreyra, Otaiza & Wunderlin, 2012; Belinha *et al*., 2007; Dani *et al*., 2008; Martorell *et al*., 2011; Wu *et al*., 2011; Zhang *et al*., 2011) The yeast model has also been employed to study the polyphenols' molecular mechanisms of action and to identify proteins and targets involved in the yeast oxidative stress response and longevity that could be conserved in mammals (Martorell *et al*., 2011).

Dani *et al*. (2008) studied the capacity of catechin and resveratrol (10 µg/mL) to protect the yeast *S. cerevisiae* against oxidative stress instigated by different oxidative substances (cadmium, carbon tetrachloride and hydrogen peroxide). With the final goal

of trying to find the molecular mechanisms involved in the antioxidant protection conferred by those polyphenols, deletion mutant strains for the genes *SOD1*, *SOD2*, *CTT1* or *GSH1* were used in order to investigate the participation of the antioxidant systems superoxide dismutase, catalase or glutathione. Results showed similar capacity of resveratrol and catechin to protect the yeast from all oxidative conditions, both conferring reduced intracellular oxidation and lipid peroxidation in the yeast. *CTT1* mutant was the only one that did not show an increased tolerance to H_2O_2 after treatment with polyphenols. These results suggest the involvement of *CTT1* in the protective antioxidant effect of polyphenols since its activation by catechin and resveratrol could lead to a reduction of ROS after oxidative stress treatment.

Quercetin was also studied regarding its capacity to increase stress resistance in *S. cerevisiae*. In this context, Belinha *et al.* (2007) observed an increased resistance against hydrogen peroxide in cells pre-incubated with 0.10 mg/mL of quercetin, being this effect correlated with decreased levels of ROS and maintenance of redox homeostasis (measured as glutathione depletion and glutathione oxidation). Protein carbonylation and lipid peroxidation levels were also measured, since oxidative stress is associated with DNA damage, accumulation of oxidized proteins and lipid peroxidation. Quercetin decreased the constitutive quantity of oxidized proteins and lipids. Enzyme activities of superoxide dismutase and catalase were studied as well, but no effect was observed after quercetin treatment, suggesting that this polyphenol improves resistance to oxidative stress by a mechanism which is independent of these endogenous antioxidant defenses. As the antioxidant capacity of polyphenols is also related to the chelation of transition metals, such as iron and copper, quercetin could form complexes with iron. The Aft1p transcription factor increases the expression of genes associated with iron uptake under iron deprivation situations (Rutherford, Jaron & Winge, 2003), for that reason they also analyzed the activation of Aft1p by quercetin, however results showed that this flavonol does not function as iron chelator.

Potential yeast genes modulated by quercetin (300 µM) were studied by Vilaça *et al*. (2012), using DNA microarrays. They found 221 upregulated genes (mainly involved in metabolism, protein fate, cell transport, biogenesis of cellular components and cell defense) and 613 downregulated genes (mainly related to transcription, protein fate,

cell transport, cell cycle and DNA processing, metabolism, biogenesis of cellular components and protein biosynthesis) after treatment with quercetin. Additional studies with deletion mutants in genes encoding for cell wall proteins and trehalose synthesis intermediates were performed to study its implication in the response to oxidative stress mediated by quercetin. The results showed that quercetin induced genes related to carbohydrate metabolism as well as cell integrity pathway, which could lead to a protection against posterior H_2O_2 stress.

With respect to cocoa polyphenols, little is known about its protective mechanism of action in the yeast. In this context, Martorell *et al*. (2011) carried out different experiments to elucidate the metabolic target of a flavonoid-enriched cocoa powder. Oxidative stress assays were performed and the cocoa powder exhibited antioxidant protection in the yeast. Differences in genomic expression profiles of *S*. *cerevisiae* after incubation with the cocoa powder were also studied using DNA arrays. They found, as mentioned above, that cocoa protected the budding yeast against oxidative stress and the sirtuin Hst3p played a role on this effect.

However, nowadays any transcriptomic study has been done in the yeast model to determine the gene expression patterns of the response to oxidative stress mediated by cocoa polyphenols.

4. *Caenorhabditis elegans* AS A MODEL ORGANISM

4.1. General characteristics

C. elegans is a small nematode (adults measure around 1mm), that lives on the soil preferably in warm areas and feeds mainly on bacteria. The body of the worms is fully covered by a thin cuticle and cells are organized into organs. During the adult stage, 959 somatic cells constitute the body, of which 302 are neurons. A digestive system is present, including mouth, pharynx and intestine, as well as sexual organs and a rudimentary nervous system. Since its body is transparent, diverse biological processes can be visualized with microscopic techniques.

Two different sexual forms exist in adult worms: hermaphrodites and males. Reproduction between hermaphrodites and males leads to a bigger genetically variability among the population. *C. elegans* has a very short life cycle (Fig. 7), eggs develop into adults in 2-5 days. Adults live around 2-3 weeks and lay 200-300 eggs.

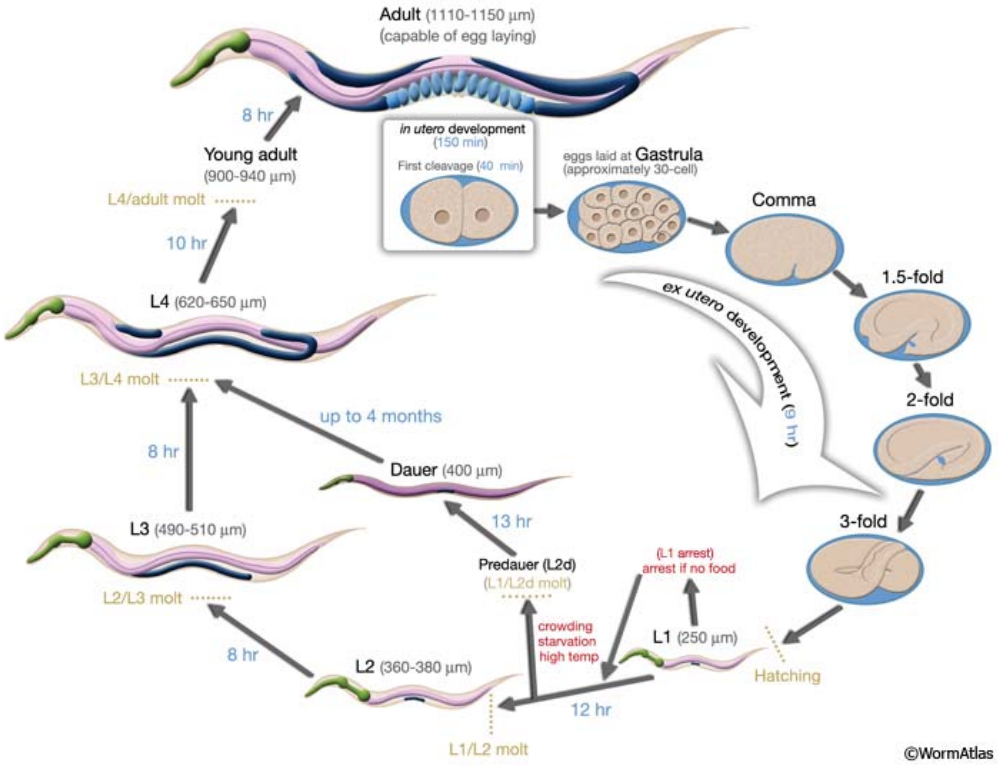


Figure 7. *C. elegans* life cycle at 22 ºC (Wormatlas, 2015).

Although the yeast model is very useful as a first screening of natural compounds with antioxidant properties, it loses effectiveness when results have to be extrapolated to the human being. In that case, the animal model *C. elegans* is of extreme interest, since it presents several advantageous characteristics (González-Manzano *et al*., 2012; Martorell *et al*., 2011):

- It is a multicellular small transparent organism.
- It is easy to handle in the laboratory (where it is maintained on agar plates or liquid cultures and fed with *Escherichia coli*).
- It has a short lifespan and a 3 day life cycle.
- It is not pathogen.

- Its genome is fully sequenced and many mutants are available.
- It exists a strong genes conservation in relation to humans (with 40% of orthologues genes).
- Several molecular mechanisms involved in stress response and aging are evolutionary conserved between worms and mammals.

4.2. Oxidative stress response in *C. elegans*

Similarly to *S. cerevisiae*, the nematode *C. elegans* also possesses several endogenous enzymatic and non-enzymatic defenses to maintain ROS concentration under acceptable levels. SOD, CAT, GPO (glutathione peroxidases) and glutathione act as previously described for the yeast model. Moreover, redox-sensitive signalling pathways are activated under stress conditions as well, leading to the modification of gene expression.

The evolutionary conserved Insulin/Insulin Like Growth Factor (IGF)-I Signalling Pathway (IIS) is involved in longevity and metabolism in different species (including flies and mammals). In the worm, this cascade is needed for the dauer larval formation (an arrested non-aging state during adverse conditions) and the process of aging. Phosphorylation of DAF-2, the *C. elegans* homologue for the Insulin/IGF-1 receptor, activates the IIS cascade resulting in the nuclear exclusion (thus inactivation) of the forkhead FoxO transcription factor DAF-16. In *C. elegans*, an increased in FoxO activity leads to an enhanced longevity, fat storage and stress resistance (Yen, Narasimhan & Tissenbaum, 2011). The transcription factor DAF-16 has received great attention since the targets of this signalling pathway are sod-3 (Honda & Honda, 1999), metallothionein (Barsyte, Lovejoy & Lithgow, 2001), catalase genes ctl-1 and ctl-2 (Murphy *et al*., 2003a), and small heat shock protein (hsp) genes, which are involved in protective and metabolic activities. During stress situations, FoxO proteins translocate into the nucleus. In mammals they are acetylated, mono-ubiquitylated and phosphorylated as a part of the post-translational modifications resulting in the promotion of altered metabolism, stress response or cell death (Chiang *et al*., 2012).

The heat-shock transcription factor HSF-1 and the nuclear factor erythroid-2-related factor 2 (Nrf2, SKN-1 *C. elegans* homologue) are thought to be induced under stress conditions and affected by DAF-2 as well. HSF-1 is induced by heat stress and can regulate the expression of small heat shock proteins. With respect to SKN-1, it regulates the gene expression of phase II detoxifying enzymes and antioxidant proteins such as SOD, GST, GPO or NAD(P)H:quinone oxidoreductase (NQO-1). Oxidative stress results in the translocation of SKN1 into the nucleus (which is present in the cytoplasm under normal conditions) producing molecular changes and the modification of the expression profile (Surh, Kundu & Na, 2008). Specifically, p38 MAPK (mitogen-activated protein kinase) cascade controls SKN1 in the worm and could influence the expression of SOD-3 and other stress response genes. Similar effects were found in the JNK signalling which is also regulated by the ROS level (reviewed by Baumeister, Schaffitzel & Hertweck, 2006) and directly phosphorylates and activates DAF-16 resulting in the induction of target genes such as SOD-3.

In brief, the inhibition of the DAF-2 pathway leads to the nuclear localization and activation of those transcription factors, modifying the expression of several genes involved in a wide range of defense activities including the endogenous stress-response, antimicrobial activity and detoxification of xenobiotic and ROS, as well as processes such as development, metabolism and longevity (reviewed by Baumeister, Schaffitzel & Hertweck, 2006).

As mentioned previously, sirtuins are a family of evolutionary conserved NAD^+ dependent deacetylases proteins, which are involved in several biological processes such as longevity, stress resistance, metabolism and cancer. The worm *C. elegans* possesses four of them (Sir2 paralogs): *sir-2.1*, *sir-2.2*, *sir-2.3* and *sir-2.4*. The most studied is *sir-2.1* because of its homology to human SIRT1 and yeast SIR2, both involved in stress resistance and longevity. Moreover, sirtuins modulate the activity of FoxO (whose deacetylation is thought to promote DNA repair and cell cycle arrest as well as to inhibit apoptosis) and some studies in the worm showed that SIR-2.1 together with 14-3-3 proteins as binding partners increase longevity in a DAF-16 dependent way (Berdichevsky, Viswanathan, Horvitz & Guarente, 2006). However, despite the great interest on SIR-2.1, other less studied sirtuins are becoming more interesting in the

stress protection role, as is the case of SIR-2.4. In this context, Chiang *et al*. (2012) focused on the promotion of DAF-16 relocalization and its potential role in stress resistance modulated by SIR-2.4, concluding that this sirtuin is a novel regulator of DAF-16 transcriptional function during stress conditions in the worm.

In view of current knowledge, recent research on bioactive compounds aims to understand the possible modulation of these antioxidant defenses, sirtuins and redox-sensitive signalling pathways in the worm as a possible explanation of antioxidant effects beyond the simple direct radical scavenging activity.

4.3. Model for the study of bioactive compounds with antioxidant capacity

The nematode *C. elegans* was first introduced as a model organism by Sydney Brenner (1974). Nowadays, this small worm is increasingly used for the study of aging as well as for the involvement of antioxidant compounds in longevity and stress response, due to its short lifespan, its morphological simplicity and its easy maintenance and genetic manipulation. *C. elegans* was one of the first organisms allowing the identification of mutations that increase longevity. Unlike the unicellular yeast, studies in different types of cells and organs can be performed in the worm. In addition, this model is more closely related to mammals (Fontana, Partridge & Longo, 2010).

In recent years, many groups have employed this useful model organism to study the protective beneficial effects of polyphenols (Büchter *et al*., 2013; González-Manzano *et al*., 2012; Havermann, Rohrig, Chovolou, Humpf & Wätjen, 2013; Havermann, Chovolou, Humpf & Wätjen, 2014; Kampkötter *et al*., 2007; Kampkötter *et al*., 2008; Saul, Pietsch, Stürzenbaum, Menzel & Steinberg, 2011; Surco-Laos *et al*., 2011).

Surco-Laos *et al*. (2011) studied the effect of epicatechin and catechin and their methylated metabolites (3'-O-methylepicatechin and 4'-O-methylepicatechin) (200 μM) on the resistance to oxidative and thermal stress of *C. elegans.* They found a protective effect of all tested substances in worms subjected to oxidative stress assessed with juglone (a redox cycler that generates oxidative stress into cells) or to thermal stress. Further studies were carried out by the same group. In this context, González-Manzano *et al*. (2012) analyzed the effect of epicatechin (200 μM) in the worm, with the aim to

identify possible mechanisms involved in the antioxidant effect of this flavonoid. ROS production and glutathione levels were analyzed after treatment with epicatechin and exposure to thermal stress. The results showed a significant reduction of both of them in worms treated with the flavonoid compared to the non-treated control.

The influence of quercetin (100 µM) on *C. elegans* stress resistance was studied by Kampkötter *et al*. (2007b). SOD-3 expression and subcellular DAF-16 localization were also investigated, since these pathways are involved in the oxidative stress response of the worm. Their results showed an increased oxidative stress resistance against juglone in quercetin-treated worms compared to the control. However, they found a repression in SOD-3 levels by quercetin and a nuclear translocation and thus activation of DAF-16. Although activation of DAF-16 has been commonly associated with an induction of SOD-3, different studies have described the requirement of the interaction between 14-3-3 proteins, the histone deacetylases SIR-2.1 and DAF-16 for the up-regulation of SOD-3 and stress resistance in response to stress stimuli (Berdichevsky, Viswanathan, Horvitz & Guarente, 2006) which could explain the contradictory effects found in this study. The authors also suggested that the repression of SOD-3 by quercetin could be due to the strong radical scavenging activity of this flavonol, which, improving the oxidative stress status of cells could lead to a down-regulation of JNK and the p38 MAPK cascades, thus repressing SOD-3.

Büchter *et al*. (2013) studied the antioxidant effects and lifespan-prolonging properties of myricetin (100 µM) in *C. elegans*. Several parameters related to oxidative stress status were tested after treatment with myricetin, such as DAF-16 and SKN-1 activation as well as ROS production and resistance to thermal stress, among others. They found that myricetin activated DAF-16, as it was previously reported for the same flavonoid (Grünz *et al*., 2011) as well as for other flavonoids such as fisetin (Kampkötter *et al*., 2007a), kaempferol (Kampkötter *et al*., 2007a) and quercetin (Kampkötter *et al*., 2007b). On the other hand, they observed that myricetin did not affect the localization of SKN-1, which was, in consequence, not activated by it. However, an activation of the SKN-1 signalling pathway was previously reported for the flavonoids baicalein (Havermann, Rohrig, Chovolou, Humpf & Wätjen, 2013) and epigallocatechin-gallate (Zhang, Jie, Zhang & Zhao, 2009). The effect of myricetin on ROS accumulation was

evaluated as well, finding a strong reduction of ROS formation in worms treated with the flavonoid and incubated at 37 ºC. This parameter was also studied in daf-16 (mu86) mutant nematodes to evaluate whether the transcription factor DAF-16 was necessary for the protective effects of myricetin in the worm. The results showed that the loss of function of DAF-16 almost completely abolished the protective effect of myricetin. Finally, thermal stress (37 ºC) experiments were carried out, but no protective effects of myricetin were observed. The authors concluded that the improvement of lifespan and protection from ROS were not necessarily related to a protection from heat stress despite the thermal-mediated generation of ROS.

Martorell *et al*. (2011), not only carried out experiments in the yeast model as aforementioned, but also in the worm. Particularly, oxidative stress assays were performed in order to investigate the protective effect of cocoa flavonoids (4 mg/mL). The results showed an increase in the worm's oxidative stress resistance after cocoa treatment. Furthermore, they investigated the involvement of the transcription factor DAF-16 and the sirtuin Sir-2.1 in the mechanism of action of cocoa polyphenols during lifespan and oxidative stress response, by using *C*. *elegans* mutant strains. The protective effect against oxidative stress found in the wild-type was not observed in any mutant, indicating that both genes were essential for the resistance to oxidative stress mediated by the cocoa powder. They concluded that the beneficial effects offered by the polyphenol-enriched cocoa powder (regarding lifespan extension and oxidative stress resistance) were produced through a SIR-2.1 and DAF-16 dependent mechanism.

BACKGROUND AND OBJECTIVES

Cocoa and its derived products have attracted great attention since they were shown to have beneficial effects on human health. These positive properties were related mainly to their high amount of polyphenols, which are antioxidants present in plants known for being reported as cardiovascular protector, antitumoral and anti-inflammatory compounds. Polyphenols are not essential in human diet, but they are extensively consumed due to its vast presence in the plant kingdom. The mechanisms of action by which phenolic compounds exert their beneficial effects are not yet fully elucidated. It seems that polyphenols not only act by scavenging ROS or complexing metal ions, but also by modulating signalling pathways involved in a wide range of processes such as aging and stress response. Nevertheless, much more information is required to explain cocoa polyphenols' benefits. Most of these properties have been studied by *in vitro* methods, using, for example, human cell lines, without taking into account the effect in a whole organism. *In vivo* studies have been performed as well, mostly carried out in mice and rats, but the difficulty of these experiments, added to the low bioavailability and complexity of polyphenols, resulted in unclear findings. Nowadays, the use of simple organisms such as the yeast *S*. *cerevisiae* and the worm *C*. *elegans* is becoming more frequent to study protective effects of food compounds with antioxidant and antiaging properties and to identify and understand the molecular mechanisms and signalling pathways responsible for these effects. In the particular case of cocoa, a few works about mechanisms of action or benefits of individual polyphenols in living organisms are currently available, which rise the necessity of additional *in vivo* studies.

Given the interest of the subject, the aim of this work was to study the functional capacity of cocoa polyphenols by *in vivo* assays with the model organisms *S. cerevisiae* and *C. elegans.* To accomplish the main goal, specific objectives were proposed:

1. To characterize analytically and carry out a semi-preparative fractionation of the polyphenolic extract obtained from a polyphenol-rich cocoa powder.
2. To evaluate the capacity of the cocoa extract and its derived fractions to promote oxidative stress response in the yeast *S. cerevisiae*.

3. To identify, by proteomic and transcriptomic studies, yeast proteins and genes probably involved in the oxidative stress response mediated by cocoa polyphenols and to confirm the possible involvement of the selected genes using deletion mutants.

4. To evaluate the ability of the cocoa extract and fractions to promote oxidative stress response in the nematode *C. elegans.*

MATERIALS AND METHODS

1. PLANT SAMPLES AND POLYPHENOL EXTRACTION

The cocoa powder marketed as CocoanOX was supplied by Natraceutical Group (Valencia, Spain). This product has high polyphenol content (12 % w/w) and is produced from unfermented blanch treated, non-roasted cocoa beans (Tomás-Barberán *et al*., 2007). The grape powder was purchased from SECNA, S.A. (Valencia, Spain). To obtain the polyphenol extracts, 20 mL of methanol:water (80:20; v:v) were added to 1 g of cocoa or grape powders. Mixtures were homogenized and sonicated in a bath at 37 ºC for 10 minutes. Then they were centrifuged at 3400 g, 15 min. This procedure was repeated three times and supernatants were mixed and concentrated under reduced pressure in a rotatory evaporator at 35 ºC until a final volume of 6 mL was obtained.

2. FRACTIONATION OF THE COCOA EXTRACT

Cocoa polyphenolic extract was fractionated by semi-preparative HPLC in a Dionex Ultimate 3000 (Sunnyvale, CA, USA) system equipped with an UV-Vis detector coupled to a Foxy Jr. fraction collector. Water-methanol (85:15; v:v) and methanol 100 % were used as solvents A and B, respectively, with a 11 mL/min flow. Elution was carried out as follows: 0 % of B for 33 min and using a gradient to obtain 11.8 % B in 75 min, 29.4 % B in 80 min maintained until 87 min, 88.2 % in 88 min maintained until 93 min, 0 % B in 94 min maintained until 100 min. Separation was carried out in reverse phase with a 250 x 20 mm i.d, 5 µm particle size Ultrabase C18 column. The whole eluted extract was recuperated, separating the main chromatographic peaks in fractions and collecting also the volume eluted between those peaks. Fractions were finally obtained, concentrated under reduced pressure at 35 ºC and conserved at -20 ºC.

3. HPLC-DAD ANALYSIS OF THE COCOA EXTRACT AND FRACTIONS

Polyphenolic extract and all fractions were analyzed by reversed-phase liquid chromatography using a HPLC Dionex Summit system equipped with fluorescence RF 2000 and PDA-100 diode array detectors, a P680 pump and an ASI-100 autosampler. An Ultrabase C18 100 x 4.6 mm i.d and 2.5 µm column was used with a 1 mL/min flow and

0.1 % TFA in water (A) and 0.1 % TFA in methanol (B) as solvents. Elution was carried out using a gradient starting with 10 % B at 10 min, 20 % B at 20 min, 40 % B at 21 min maintained until 23 min, and increasing up to 100 % B at 24 min until 26 min and finally decreasing to 0 % B at 27 min maintained until 35 min. Chromatograms were recorded at 280 nm and at 360 nm by emission of fluorescence after excitation at 278 nm. Standards of procyanidins B1 and B2 (Extrasynthèse, Genay, France), theobromine, caffeine, catechin and epicatechin (Sigma-Aldrich, St. Louis, USA) were used for the identification and quantitative determination of main polyphenols in cocoa samples and fractions.

4. HPLC-MS/MS ANALYSIS OF THE COCOA EXTRACT AND FRACTIONS

The study of fractions was performed using an AB SCIEX TripleTOF™ 5600 LC/MS/MS System, with negative and positive ion detection modes. The LC analysis was carried out using an Agilent 1290 HPLC system with a Waters UPLC C18, 1.7 µm (2.1 x 50 mm) Acquity UPLC BEH column. Gradient elution was carried out with 0.1 % formic acid in H_2O (solvent A) and 0.1 % formic acid in methanol (solvent B) with a constant flow of 0.4 mL/min following the proportions described in Table 4.

Table 4. Multi-step linear gradient used in the LC analysis of cocoa fractions.

Time (min)	% A	% B
0	95	5
2	50	50
13	5	95
15	5	95
15.1	95	5
25	95	5
30	90	10

The conditions established for the MS analysis were the following: Ion source gas 1 (GC1): 50 psi; Ion source gas 2 (GC2): 50 psi; Curtain gas 1: 25 psi; Temperature: 450 ºC; Ion Spray Voltage (ISVF): -4500. The MS was using an IDA acquisition method with two experiments: the survey scan type (TOF-MS) and the dependent scan type (Product Ion) using -30 V of collision energy. Data was evaluated using the qualitatively evaluated

using the XIC manager in the PeakView™ software where it was filtered based on mass error, retention time, isotope ratio % difference and library hit purity score.

5. TOTAL POLYPHENOL DETERMINATION: FOLIN-CIOCALTEU ASSAY

The total polyphenol content of the cocoa extract and fractions was determined by the Folin-Ciocalteu method described by Singleton & Rossi (1965) with some modifications. Briefly, 50 µL of sample and 500 µL of aqueous sodium carbonate solution (2 % sodium carbonate (Panreac, Barcelona, Spain) and 0.4 % sodium hydroxide (Panreac, Barcelona, Spain)) were added in a microcentrifuge tube and vortexed. After 15 min at room temperature, 50 µL of Folin reagent (Folin 2N (Sigma-Aldrich, St. Louis, USA): water; 50:50; v:v) were added and the mixture was stirred and incubated at room temperature for 30 min. The absorbance was finally read at 724 nm in a 96-well plate spectrophotometer reader (BMG Labtech Omega Spectrostar). A calibration curve was prepared with epicatechin concentrations ranging from 10 to 300 mg/L (R^2= 0.9982). The results were expressed as mg of epicatechin equivalents per litre of extract (mg EE/L) and experiments were carried out in triplicate.

6. *IN VITRO* ANTIOXIDANT ASSAYS

6.1. Free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging assay

Total antioxidant activity of samples was determined by the reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). This blue-purple radical becomes decolorized to yellow after the reaction with an antioxidant compound and the reduction of the absorbance can be measured spectrophotometrically. The assay was carried out using a modified version of the method described by Schinella *et al*., 2010. Samples (7.5 µL) were added to 292.5 µL of DPPH (Sigma-Aldrich, St. Louis, USA) 60 µM in methanol 80 %, mixed and incubated for 30 min at room temperature in the dark. The absorbance of the remaining DPPH was then measured at 517 nm against a blank in a 96-well plate spectrophotometer reader (BMG Labtech Omega Spectrostar) and the

radical-scavenging activities of samples, expressed as percentage inhibition of DPPH •, were calculated according to the formula: Inhibition percentage (Ip) = 100 x (A0 - A1)/A0, where A0 is the absorbance value of the blank and A1 is the absorbance value of samples, both checked after 30 min. A calibration curve of epicatechin was prepared with concentrations ranging from 5 to 50 mg/L (R^2= 0.9986) and the results of the fraction experiments were expressed as epicatechin equivalents. Experiments were carried out at least in triplicate.

6.2. Trolox equivalent antioxidant capacity (TEAC) assay

The principle of the TEAC assay is the reductive conversion and decolorization of a stable, blue-green radical solution by an antioxidant. This decolorization, which can be quantified spectrophotometrically, indicates the antioxidant capacity of a compound which is compared to the potency of the synthetic vitamin E derivative Trolox (Calbiochem, Merck, Darmstadt, Germany), used as reference substance. The radical solution was prepared the day before use by mixing equal volumes of an 2,2´-azinobis-(3-ethylbenzthiazolin)-6-sulfonic acid (ABTS) solution (14 mM) and an 4.9 mM ammoniumperoxodisulfate (APS) solution (4.9 mM) and stored in the dark at room temperature. The absorption of this solution (1.4 at a wavelength of 734 nm) was adjusted by dilution with 70 % (v/v) ethanol. Trolox, fractions and pure compounds were measured in a concentration range from 0 to 25 µM by mixing 500 µL of the radical solution with 500 µL of the test solution (in 70 % ethanol). The cocoa extract was standardised to the epicatechin content and measured in a concentration range from 0 to 6 µM epicatechin. The radical scavenging activity was measured spectrophotometrically after two minutes of reaction at 734 nm (Synergy MX, BioTek; Bad Friedrichshall, Germany). Three independent trials were performed.

7. USE OF *S. cerevisiae* AS MODEL ORGANISM

7.1. *S. cerevisiae* strains and maintenance

S. cerevisiae strains used in this work were the wild type strain BY4741 (*MATa; his3*Δ1*; leu2*Δ0*; met15*Δ0*; ura3*Δ0) and 18 haploid deletion mutants strains in the BY4741 background (Table 5) generated by the *Saccharomyces* Genome Deletion Project and obtained from the European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF).

Table 5. List of *S. cerevisiae* BY4741 haploid deletion strains used in this work.

Systematic name	Deleted Gene
YDR099W	*BMH2*
YEL060C	*PRB1*
YER086W	*ILV1*
YER091C	*MET6*
YGL026C	*TRP5*
YGL202W	*ARO8*
YGR192C	*TDH3*
YGR234W	*YHB1*
YJR139C	*HOM6*
YLR309C	*IMH1*
YNL209W	*SSB2*
YNR001C	*CIT1*
YOL057W	*DPP3*
YOR323C	*PRO2*
YOR375C	*DHE4*
YPL138C	*SPP1*
YPR074C	*TKL1*
YOR025W	*HST3*

The strains were grown in YPD medium [2 % (w/v) glucose (Scharlab, Barcelona, Spain), 1 % (w/v) yeast extract (Conda, Madrid, Spain) and 2 % (w/v) peptone (Conda, Madrid, Spain)] with 2 % (w/v) agar (Scharlab, Barcelona, Spain) for plates. Strains were streaked from a frozen glycerol stock to a fresh liquid YPD medium and grown at 28 ºC overnight with shaking. These cultures were diluted in fresh liquid YPD medium and grown again at 28 ºC overnight with shaking. They were then spread over YPD agar plates and incubated at 28 ºC for 72 h to obtain single colonies.

7.2. Development of a yeast-based method to test the ability of food ingredients to promote an antioxidant response in *S. cerevisiae*.

7.2.1. Determination of the oxidant concentrations.

Several experiments were firstly carried out in order to determine the correct oxidant concentration. A single colony of yeast strain was inoculated into 5 mL of fresh liquid YPD medium and incubated for 6 h at 28 ºC with shaking at 40 rpm. An aliquot of 5 µL of the 1/10 dilution of this pre-culture was inoculated into 3 mL of fresh liquid YPD medium for 18 h at 28 ºC with shaking at 40 rpm in a tube rotator. Cells were then harvested by centrifugation at 2700 g, for 10 min, at 20 ºC and resuspended in 3 mL of phosphate-buffered saline (PBS) pH 7.4 (OD_{600} was read to ensure an equal concentration in the next step). An aliquot of this cell suspension was diluted in PBS to a final volume of 3 mL to reach an OD_{600nm} of 0.1 and preadapted incubating 30 min at 28 ºC. To perform the oxidative stress step, a range of H_2O_2 and menadione concentrations were tested. Cells were subsequently incubated for 60 min at 28 ºC with 0.25 to 6 mM H_2O_2 (Merck, Hohenbrunn, Germany) or with 0.1 to 5 mM menadione (Sigma-Aldrich, St. Louis, USA). An untreated control was carried out as well. Oxidants were then removed by centrifugation at 2700 g, for 10 min, at 20 ºC and cells were resuspended in fresh YPD medium for subsequent growth analysis by recording the OD_{600nm}. Cultures were distributed in 96-well microtitre plates with a final volume of 250 µL per well using four replications for each condition combination. Yeast growth was monitored at 30 ºC by reading the $O.D_{600}$ in a 96-well plate spectrophotometer reader

(BMG Labtech Omega Spectrostar) with shaking at 600 rpm for 18 h. Experiments were carried out in triplicate.

Growth ratios curves were calculated for each strain and condition at 5, 9, 12, 16 and 18 h of incubation as the quotient between the growth curve of the culture exposed to oxidative stress and the growth curve of the non-exposed culture.

7.2.2. Performance of the assay to test the ability of food ingredients to promote an antioxidant response in *S. cerevisiae*.

A single colony of yeast strain was inoculated into 5 mL of fresh liquid YPD medium and incubated for 6 h at 28 ºC with shaking at 40 rpm. An aliquot of 5 µL of the 1/10 dilution of this pre-culture was inoculated into 3 mL of fresh liquid YPD medium with the antioxidant ingredients (grape polyphenolic extract, cocoa polyphenolic extract or vitamin C) for 18 h at 28 ºC with shaking at 40 rpm in a tube rotator. Several concentrations of the antioxidant ingredients were assayed (50 mg EE/L to 700 mg EE/L of plant extracts and 0.5 to 25 mM of vitamin C) to induce an intracellular antioxidant response in the yeast. Vitamin C (Sigma-Aldrich, St. Louis, USA) was used as a positive control. Cultures without any antioxidant ingredient were used as negative controls. Cells were harvested by centrifugation at 2700 g, for 10 min, at 20 ºC and resuspended in 3 mL of PBS pH 7.4 (OD_{600} was read to ensure an equal concentration in the next step). An aliquot of this cell suspension was diluted in PBS in a final volume of 3 mL to reach an OD_{600} of 0.1 and preadapted incubating 30 min at 28 ºC. To induce a non-lethal oxidative stress, cells were incubated for 60 min at 28 ºC with H_2O_2 (0.5 mM and 4 mM) or menadione (0.5 mM and 2 mM). An untreated control was carried out for each ingredient. Oxidants were then removed by centrifugation at 2700 g, for 10 min, at 20 ºC and cells were resuspended in fresh YPD medium for subsequent growth analysis by recording the OD_{600nm} as described above. Experiments were carried out in triplicate.

Growth ratios curves were calculated for each condition as previously described. Then, to evaluate the protective antioxidant activity of the ingredients, ‘effect curves’ were constructed by dividing the growth ratio curve of the culture pre-incubated with the ingredient by the growth ratio curve of the culture pre-incubated without it at the

same dose of oxidant. Statistically significant differences ($p<0.05$) were calculated by the Student's t-test.

7.3. Study of the antioxidant activity of cocoa fractions and pure compounds

The developed yeast-based method was then employed to study the antioxidant protection of the fractions and the pure compounds (commercial standards). Four concentrations were used for each fraction, in order to cover a wide range of doses, standardised to the main polyphenol in the fraction or, in the case of fractions without main compounds, standardised to the total polyphenol content (Table 6). The pure compounds were assayed at 10 mg/L. The cocoa polyphenolic extract (350 mg EE/L) was used as a positive control. Experiments were carried out in triplicate. Statistically significant differences ($p<0.05$) were calculated by one-way ANOVA with LSD post-test.

Table 6. Fractions, pure compounds and concentrations studied with the yeast based method in order to establish their antioxidant capacity *in vivo*.

FRACTION (main compound)	F2 (theobromine)	F5	F10 (catechin)	F14 (B2)	F16 (caffeine)	F19 (epicatechin)	F24
CONCENTRATION (mg/L)	5	2	0.5	5	2	5	5
	10	5	1	10	10	10	10
	50	10	2	50	25	50	50
	100	25	5	100	50	100	100
PURE COMPOUND	Theobromine		Catechin	Procyanidin B2	Caffeine	Epicatechin	
CONCENTRATION (mg/L)	10		10	10	10	10	

7.4. Proteomic analysis of the response of *S. cerevisiae* to oxidative stress mediated by cocoa polyphenols

7.4.1. Sample preparation for proteomic analysis

A single colony of yeast strain was inoculated into 5 mL of fresh liquid YPD medium and incubated for 6 h at 28 ºC with shaking at 40 rpm in a vertical tube rotator. An aliquot of 10 µL of this pre-culture was inoculated into flasks with 15 mL of YPD medium, containing or not the cocoa extract (350 mg EE/L), and incubated for 18 h at 28 ºC with shaking at 200 rpm in an orbital shaker. Cells were harvested by centrifugation at 2700 g for 10 min at 20 ºC and resuspended in 15 mL of PBS to avoid carryover of YPD. The cell suspension (10 mL) was diluted 1/20 in PBS (around an optical density at 600 nm of 0.3) and preadapted incubating 30 min at 28 ºC. When cultures (treated or not with the cocoa extract) were subjected to oxidative stress, hydrogen peroxide was added to reach a final concentration of 5 mM and cells were incubated for 90 min at 28 ºC with shaking (200 rpm in an orbital shaker). When no oxidative stress was applied, an equivalent volume of PBS was added and both samples (treated or not with cocoa) were treated exactly the same way to exclude variations due to sample manipulation. Finally, cells were harvested (2700 g for 10 min at 20 ºC) and rinsed with milliQ H_2O. Water was removed by re-centrifugation and the resulting pellet was immediately frozen in liquid nitrogen and stored at -80 ºC. Three biological replicates were carried out per condition.

7.4.2. Protein extraction and two-dimensional gel electrophoresis

Protein extraction of samples was carried out as described by Gómez-Pastor, Pérez-Torrado, Cabiscol & Matallana (2010). Cells were resuspended in 150 µL extraction buffer (8 M urea, 25 mM Tris-HCl pH 8.0), a mixture of protease inhibitors (200 mM phenylmethylsulphonyl fluoride, 20 mM TPcK, 200 mM pepstatin A) and 0.2 g of glass beads. Then, cells were broken in the Fast Prep (MP Bio) at 5.0 ms^{-1} for 45 s on three times. After centrifugation at 16 000 g for 10 min at 4 ºC, the supernatant was sonicated for 10 min and centrifuged again at 16 000 g for 10 min. The protein concentration was determined using a Nanodrop ND-1000 UV/Vis spectrophotometer.

Two-dimensional gel electrophoresis (2D-GE) was carried out at the Proteomic Service of the Centro de Investigación Príncipe Felipe (CIPF) (Valencia, Spain). Immobilized pH gradient (IPG) strips (pI range 3–10, 24 cm, GE Healthcare) were used for protein separation. IGP trips were rehydrated in 450 µL of 2D buffer (7 M urea, 2 M thiourea, 1 % (w/v) NP-40, 4 % dithiothreitol (DTT), 1 % ampholytes (Pharmalite 4–7, GE Healthcare) and 0.002 % bromophenol blue) containing the protein samples (100 µg) overnight at room temperature. Isoelectric focusing was carried out as follows: 500 V for 2h; 500-1000 V for 2 h; 1000-5000 V for 2 h; 5000-8000 V for 4 h and 8000 for 8 h.

For the polyacrylamide gel electrophoresis (PAGE) in the second dimension, the focused IPG strips were equilibrated for 15 min in 50 mM Tris-HCl, pH 8.8, 6 M urea, 30 % (v/v) glycerol, 2 % p/v sodium dodecyl sulfate (SDS), 2 % p/v dithiothreitol (DTT) and then 15 min in the same buffer containing 2.5 % p/v iodoacetamide. Samples were resolved in SDS-PAGE with 12.5 % polyacrylamide gel in a vertical system (Ettan DALT six; GE Healthcare, 26 cm x 20 cm x 1 mm). Electrophoresis was performed at 1 W per gel until the colour front reached the end of the gel.

7.4.3. Protein visualization and images analysis.

Protein visualization was carried out at the Proteomic service of CIPF as follows. Twelve gels (three for each condition) were fixed for 1h in 50 % v/v methanol, 10 % v/v acetic acid following by staining with SYPRO Ruby (BioRad). Gel images were obtained using a high resolution scanner (Typhoon FLA9000, GE Healthcare).

Differences between conditions (cocoa *vs* no cocoa without oxidative stress and cocoa *vs* no cocoa with oxidative stress) were analyzed by image software (PD Quest Advanced, Biorad). Spots that displayed at least 2-fold change in expression and which difference in abundance was statistically significant at the 95 % confidence level (Student's t-test) were considered candidate proteins.

7.4.4. Protein identification by MALDI-MS/MS

Differentially regulated spots were manually extracted. They were then digested with trypsin and identified by mass spectrometry in a MALDI-TOFTOF 5800 (AB Sciex) at the Proteomic Service of the Central Support Service to Experimental Research (SCSIE) of the University of Valencia (Spain). Samples were treated with H_2O/ACN (1:1, v/v) for 10 min twice and then ACN was added until gel dehydration. Liquid was removed and samples were rehydrated with 50 mM NH_4HCO_3 for 5 min, ACN was added then for 15 min, and again for 5 min.

Samples were then digested as follows: proteins in the gel plugs were reduced by 10 mM DTT in 50 mM ammonium bicarbonate and alkylation was carried out with 55 mM iodoacetamide in 50 mM ammonium bicarbonate. The pieces of gel were then rinsed with H_2O and acetonitrile for 2 min and then with acetonitrile for 5 min. 10 µL of trypsin at a final concentration of 5 ng/µL was added to the dry gel and after rehydration 20 µL of 50 mM ammonium bicarbonate were added. Trypsin digestion was carried out at 37 °C overnight. Finally, 3 µL of 10 % trifluoroacetic acid (pH=1) was added to stop the reaction. An aliquot of the above digestion solution (1 µL) was deposited onto a MALDI probe and allowed to dry at room temperature. Then, 0.5 µL of matrix solution (5 mg/mL ACH in 50 % ACN 0.1 % TFA) were added. Spectra were acquired in the positive-ion mode and 5 ions were automatically selected among those of greater intensity, excluding those corresponding to known contaminations. The fragmentation spectrum of each of these ions was obtained. The combined MS and MSMS data were analyzed together with the MASCOT algorithm (Matrixscience). The database used was Expasy_SwissProt.

Genes were grouped into functional categories ($p<0.01$) with the Functional Specification (FunSpec) tool, using the GO Database. GO classification was done with the up and down regulated genes for each condition (with and without oxidative stress).

7.4.5. Study of the ability of the cocoa extract to induce cellular antioxidant response in deletant strains for genes potentially involved in the *S. cerevisiae* response mediated by the cocoa extract.

The yeast-based method was used to study the ability of cocoa extract to affect the phenotype profiling of *S. cerevisiae* mutant strains lacking the genes codifying for the proteins identified as differentially expressed in the proteomic analysis. Growth curves were obtained from the *S. cerevisiae* wild type strain and the selected deletion mutants, after incubation with or without the cocoa polyphenol extract and the presence or absence of oxidative stress induced with two H_2O_2 concentrations (0.5 and 4 mM). Growth ratios and "effect curves" were then calculated as previously reported in paragraph 7.2 of this section and statistical differences ($p<0.05$) were calculated with the Student's t test.

7.5. Transcriptomic analysis of the response of *S. cerevisiae* to oxidative stress mediated by cocoa polyphenols

7.5.1. Sample preparation for transcriptomic analysis

A single colony of yeast strain was inoculated into 5 mL of fresh liquid YPD medium and incubated for 6 h at 28 ºC with shaking at 40 rpm in a vertical tube rotator. An aliquot of 60 µL of this pre-culture was inoculated into a flask with 30 mL of YPD medium, containing or not the cocoa extract (350 mg EE/L), and incubated for 18 h at 28 ºC with shaking at 200 rpm in an orbital shaker. Cells were harvested by centrifugation at 2700 g for 10 min at 20 ºC and resuspended in 30 mL of PBS to avoid carryover of YPD. An aliquot of this cell suspension was diluted 1/20 in 200 mL PBS (to reach an optical density at 600 nm of 0.1) and preadapted incubating 30 min at 28 ºC. When cultures (treated or not with cocoa) were subjected to oxidative stress, hydrogen peroxide was added to reach a final concentration of 1 mM and cells were incubated for 15 and 45 min at 28 ºC with shaking (200 rpm in an orbital shaker). Finally, cells were harvested (2700 g for 10 min at 4 ºC) and rinsed with milliQ H_2O. Water was removed by re-centrifugation and

the resulting pellet was immediately frozen in liquid nitrogen and stored at -80 ºC. Three biological replicates were carried out per condition.

7.5.2. Microarrays analysis

RNA extraction from yeast cultures (with and without cocoa extract, 15 and 45 min with 1 mM H_2O_2) was carried out with the RNeasy® Mini Kit (QIAGEN) according to the manufacturer's instructions. Samples were then treated and analyzed at the Servicio de Genómica y Genética Translacional of the CIPF (Valencia, Spain). RNA was quantified by spectrometry (NanoDrop ND1000, NanoDrop Technologies, Wilminton, Delaware USA) and its quality was confirmed by RNA 6000 Nano Bioanalyzer (Agilent Technologies, Palo Alto, California USA) assay. 150 ng of total RNA were used to produce Cyanine 3-CTP and Cyanine 5-CTP-labeled cRNA using the Low Input Quick Amp Labelling Kit, Two-Color (Agilent p/n 5190-2306) according to the manufacturer's instructions. According to the 'Two-Color Microarray-Based Gene Expression Analysis' protocol Version 6.7 (Agilent p/n G4140-90050), 600 ng of labeled cRNA was hybridized with the Yeast (V2) Gene Expression Microarray, 8x15K (Agilent p/n G4813A-016322) containing 6,256+ *S. cerevisiae* probes. Arrays were scanned in an Agilent Microarray Scanner (Agilent G2565C) according to the manufacturer's protocol and data were extracted using the Agilent Feature Extraction Software 10.10.1.1 following the Agilent protocol GE2_1010_Sep10, grid template 016322_D_F_20120509 and the QC Metric Set GE2_QCMT_Sep10. Data were analyzed with the Babelomics 4.2 Suite. Comparison was carried out using the Limma test with fdr correction ($p<0.0005$).

Genes showing at least two-fold change in expression (in cultures treated with cocoa extract against non-treated cultures) were classified into functional categories ($p<0.05$) with the Genomic Research Environment (GenRE) software. Up and downregulated genes were analyzed independently, for each time point (15 and 45 min of H_2O_2 incubation).

7.5.3. Validation of microarray analysis by RT-qPCR

Validation of microarray analysis was carried out by RT-qPCR. 12 genes were selected: 9 differentially regulated genes in the transcriptomic assays (in cultures treated with cocoa extract against non-treated cultures at 15' or 45' min of stress incubation) and 3 internal standards (Table 7).

Table 7. Selected genes for the validation by RT-qPCR. The incubation time of cultures with hydrogen peroxide at which genes were at least 2-fold up or downregulated is shown.

GENE	Time point	Regulation
GRE2/YOL151W	15'	UP
YIL057C	15'	UP
YJR096W	15'	UP
HSP31/YDR533C	45'	UP
TSA2/YDR453c	45'	UP
CTA1/YDR256C	15', 45'	UP
SRX1/YkL086w	15'	UP
CCP1/YKR066C	45'	UP
RPS17B/YDR447C	45'	DOWN
DOT1/YDR440W	45'	DOWN
OGG1/YML060W	15'	DOWN
REX4 /YOL080C	45'	DOWN
ACT1/YFL039C ***TAF10***/ YDR167W ***UBC6***/ YER100W	Housekeepings	

7.5.3.1. Primers design and verification

Gene sequences were obtained from the SGD. The primers design was then carried out with the program Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) considering the following parameters: a length of 18-23 pb, 70-150 amplicons, 50 % (optimum) G+C with allowable intervals of 30-80 % and a temperature near 62 ºC (optimum) within a range of 60-63 ºC. To avoid dimer formation, primers were checked with the program Gene Runner (Version 3.05) using the parameter dG > -9 kcal/mol. Genes *ACT1*, *TAF10* and *UBC6* were used as housekeepings, since their expression is constitutive. Table 8 shows the corresponding forward/reverse primers for the selected genes, their sequences, their melting temperature and the expected amplified.

Table 8. Primers designed for the validation approach by RT-qPCR. F= forward; R= reverse; Tm= melting temperature.

Gene		Sequence 5'……3'	Tm	Expected amplified
SRX1/YKL086W	F R	ATTCCCACCGCTAGCAAGAC GCCGCCGAAGGCATAATATAG	61.5 62.8	129
CTA1/YDR256C	F R	CCCCAGGTGATGTAGATTTCG AACGCGCTGCTGTATTTGAG	61.6 61.5	137
HSP31/YDR533C	F R	GGCAACCGTTGAAGATGTTG CGCAGAAGCAGGATTCACAC	61.5 62	131
YIL057C	F R	CCTTTGACCGGATTGTTTGG CATTGCGCCATCGTGTTTAC	62.5 62.4	101
GRE2/YOL151W	F R	GAACACATCTTGCGAACTCGTC GCCTTTGCAACATCACGAAC	62.1 61.6	105
TSA2/YDR453C	F R	CCAGAAAAGACGGTGGATTAGG TTCCCTTCGGGTCGATTATG	62 62.1	141
YJR096W	F R	CCTTGCAGCCTACAACTTTGAAC TGTGCATTCCCAATCGGTAG	62.3 61.8	91
CCP1/YKR066C	F R	AACGACGCGAACAACGAAC CCTTGAAGAACTTGTCCTGGTC	62.2 60.1	136
RPS17B/YDR447C	F R	CGTTGACAACCAAACCTCTGAC TGAGCGGAAACGTTGATGAC	61.9 62.2	78
DOT1/YDR440W	F R	TGTTTCATGGACGCATAGTGG GCAGGGCTGAATAAACTTTCG	61.9 61.1	81
OGG1/YML060W	F R	GGCCCAAGGTGTCCTATTCTC CGTAACAATTGCTTCCGTTTCC	62.1 62.8	115
REX4/YOL080C	F R	GACTGCGGATATTCTGGAAGG GGGTGGGATAACATCAATGC	61.1 60	81
ACT1/YFL039C	F R	TTCCCAGGTATTGCCGAAAG GCCAAGATAGAACCACCAATCC	62.2 61.9	125
TAF10/YDR167W	F R	CGTGCAGCAGATTTCACAAC TTGAGCCCGTATTCAGCAAC	60.5 61.2	111
UBC6/YER100W	F R	TGCGGCAAATACAGGTGATG TGTCTCAACGCTTGTTCAGC	62.9 60.2	123

All of them were subsequently verified by PCR in order to confirm the correct DNA amplification. Reactions were performed with PCR buffer (containing Mg_2Cl), 0.5 µM primer F, 0.5 µM primer R, 0.2 mM dNTPs and 0.025 U/µL Taq (Fermentas,Thermo 1.25 U) in a final volume of 25 µL. PCR conditions were 95 ºC for 4 min, followed by 30 cycles of 94/60/72 ºC for 25/25/40 sec, respectively and 72 ºC for 10 min, carried out in a G-Storm thermocycler. The size of the PCR product was verified by electrophoresis in 2 % agarose gel with Gelred.

7.5.3.2. Efficiency curves

For the quantification of gene expression by RT-qPCR, calibration curves with serial dilutions of cDNA (10^{-1} to 10^{-6}) were prepared for each gene. Reactions were performed in 96-well microtiter plates in a LightCycler 480 Real Time PCR (Roche Diagnostics) and data were analyzed with the LightCycler® 480 Software release 1.5.0 version 1.5.0.39. Curves were the mean of three replicates. After the analysis, dilution 10^{-2} of the cDNA samples was chosen for the expression experiments.

7.5.3.3. RT-qPCR reactions

RT-qPCR experiments were carried out as follows: 3 µL of cDNA dil 10^{-2} and 7 µL of Master Mix (1.4 µL nuclease free H_2O, 0.3 µL primer F (10 µM), 0.3 µL primer R (10 µM) and 5 µL of LightCycler® 480 Subr Green I Master 2x (Roche)) were mixed. Three independent cDNAs were analyzed in duplicate. Non template controls were included for each gene and plate. Reaction conditions were 95 ºC for 10 min and 40 cycles of amplification at 95/60/72 ºC for 10/10/10 sec, respectively. Intensity values of fluorescence were collected during the extension steps at 72 ºC. The specificity of the reaction was verified by the analysis of the dissociation curves. Three genes (*ACT1*, *TAF10* and *UBC6*) were used for the normalization of the results (housekeepings). Reactions were performed in 96-well microtiter plates in a LightCycler 480 Real Time PCR (Roche Diagnostics) and data were analyzed in the LightCycler® 480 Software 1.5 with the relative quantification tool using the mean of three housekeepings to calculate the ratio target/reference for each condition (with and without (control) cocoa extract after 15 or 45 min of hydrogen peroxide treatment). Finally, ratios (cocoa ratio/control ratio) of the selected genes were calculated in order to determine the effect of cocoa treatment in gene expression.

8. USE OF *C. elegans* AS MODEL ORGANISM

8.1. *C. elegans* strains and maintenance

C. elegans strains used in this work were the wild type N2 (var. Bristol), CF1038 [*daf-16(mu86)* I.] and TJ356 [*zIs356 IV (pdaf-16::daf-16-gfp; rol-6*)], all of them obtained from the *Caenorhabditis Genetics* Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). *Escherichia coli* OP50 and streptomycin-resistant *E. coli* OP50-1 strains were obtained from the same center. Worms were maintained at 20 ºC on Nematode Growth Medium (NGM) plates [3 g/L NaCl, 2.5 g/L peptone, 17 g/L agar, 1 mL/L cholesterol 5 g/L (in ethanol), 1 mL/L MgSO4 1 M, 25 mL KPO4 1 M pH 6.0 buffer] seeded with the *E. coli* OP50. Age synchronisation of the nematodes was performed by hypochlorite treatment of gravid adults for the determination of the thermal stress resistance and ROS accumulation. Timed egg-laying was used for the arsenite-stress and oxidative stress resistance and the DAF-16-GFP translocation assay.

8.2. Oxidative stress assay with hydrogen peroxide

Gravid worms were placed in standard NGM plates (control) and supplemented NGM plates with cocoa extract (100 mg EE/L) or fraction (10 mg/L of total or main polyphenols depending on the fraction), containing *E. coli* OP50 strain and allowed to lay eggs for 2 h. Adults were then removed and synchronized eggs were grown for 4 days at 20 ºC. Adults were then transferred to basal plates (5.85 g/L NaCl; 17 g/L agar; 50 mL/L KPO4 buffer pH 6.0; 1 mL/L cholesterol 5 g/L in ethanol) with a final concentration of 2 mM hydrogen peroxide and incubated at 20 ºC for 4 h. The viability of worms was then scored by touching them with a platinum wire and considering dead worms those who did not respond to tapping. Worms crawling up the side of the petri dish or suffering from internal hatching were censored. At least three independent trials with 100 adult worms each other were carried out per condition. Statistical significance was determined by one-way ANOVA with LSD post-test. Differences were considered significant at $p<0.05$.

8.3. Na-arsenite stress assay

Synchronized L4 larvae/young adult worms were treated for 3 days with the cocoa extract (100 µM epicatechin or caffeine), 100 µM pure compound (100 mM stock in DMSO), F16 (adjusted to 100 µM caffeine), F19 (adjusted to 100 µM epicatechin) or 0.1 % DMSO and water (control). Incubation was performed at 20 ºC in liquid NGM plates containing 120 µM FUDR (5-fluoro-2´-deoxyuridine) to prevent viable progeny, 1 % (w/v) bovine serum albumin (BSA), 50 µg/mL streptomycin and 10^9 OP50-1/mL as a food source in 35mm petri dishes. 6-days old worms were then transferred into 1.5 mL M9 stress plates containing 4 mM sodium-arsenite, 120 µM FUDR and 10^9 *E. coli* OP50-1/mL and incubated at 20 ºC. Nematodes were scored every day via touch-provoked movement until 100 h of stress-treatment were reached. 30 nematodes per group were analysed. And at least three independent experiments were carried out. Survival curves were represented by the Kaplan-Meier survival analysis with Log Rank test (Mantel-Cox) for each condition. Statistics were performed with GraphPad Prism 6 software (La Jolla, USA). Statistical significance was determined by one-way ANOVA with Dunnett´s post-test. Differences were considered significant at $p<0.05$.

8.3.1. Locomotion measurement

After 72 h of Na-arsenite stress, alive worms treated with the cocoa extract were classified into three groups depending on their locomotion: A) worms moving loosely in the plates; B) motionless worms which started moving lively after touching them and C) motionless worms which barely moved after touching them.

8.4. Measurement of intracellular ROS accumulation (DCF assay)

Non-fluorescent H_2DCF-DA (2',7'- dichlorodihydrofluorescein–diacetate; Sigma) crosses cell membranes and becomes deacetylated to form the non-fluorescent derivative H_2DCF that is trapped inside the cell. Afterwards, H2DCF can be oxidized by intracellular ROS to form fluorescent DCF which is measured in a fluorescence spectrophotometer (excitation wavelength 485 nm; emission wavelength 535 nm). The fluorescence intensity correlates with the intracellular quantity of ROS.

The experiment was carried out as described by Büchter *et al*. (2013) with some modifications. Briefly, L4 larvae/young adult worms incubated with 100 µM pure compound (100 mM stock in DMSO), 100 µM of the main compound in the tested fraction, or the same amount of DMSO and water (control). Incubation was performed at 20 ºC in liquid NGM containing 120 µM FUDR, 1 % BSA, 50 mg/mL streptomycin and 10^9 *E. coli* OP50-1/mL as a food source. After 48 h, animals were washed with 1.5 mL PBST (PBS with 0.1 % Tween 20) for one hour. Subsequently, single worms (eight per group) were transferred individually in 1 µL PBST into each well of a 384-well microtiter plate (Greiner Bio-One, Frickenhausen, Germany) containing 7 µL PBS and finally 2 µL H_2DCF-DA (250 µM in PBS) were added into each well to obtain a final concentration of 50 µM H_2DCF-DA. Wells without nematodes containing 8 µL PBS and the same amount of H_2DCF-DA than samples were used as a background fluorescence. Plates were sealed against evaporation with a black tape (Perkin Elmer, Wellesley, MA, USA). ROS accumulation was induced by thermal stress at 37 ºC and fluorescence was measured at 535 nm (excitation wavelength 485 nm) every 15 min for 12 h in a fluorescence spectrophotometer (Synergy Mx, BioTek; Bad Friedrichshall, Germany). Experiments were carried out at least in triplicate. Statistics were performed with GraphPad Prism 6 software (La Jolla, USA). Statistical significance was determined by one-way ANOVA with Dunnett´s post-test. Differences were considered significant at $p<0.05$.

8.5. Thermal stress measurement (SYTOX assay)

SYTOX® Green Nucleic Acid Stain can cross the damaged cell membranes after thermal stress. The dye then binds to DNA resulting in a bright fluorescence that can be used as a marker for cellular damage and thus for the viability of single nematodes (Gill, Olsen, Sampayo & Lithgow, 2003).

Assays were performed as described by Büchter *et al*. (2013). Worms were treated as described for the DCF assay. To determine the survival of individual nematodes at the lethal temperature of 37 ºC, worms were individually transferred in 1 µL PBST to the wells of a 384-well microtiter plate (Greiner Bio-One, Frickenhausen, Germany) containing 9 µL PBS and finally 10 µL of 2 µM SYTOX® Green Nucleic Acid Stain

(Molecular Probes Inc., Leiden, The Netherlands) in PBS were added (final concentration of 1 µM). The plate was also sealed using black backing tapes (Perkin Elmer, Wellesley, MA, USA) to avoid evaporation. The fluorescence intensity was measured with a fluorescence spectrophotometer (Synergy MX, BioTek; Bad Friedrichshall, Germany) every 15 min for 12 h (excitation wavelength 485 nm; emission wavelength 535 nm). The "virtual times points of death" were calculated for each condition considering a worm dead if its fluorescence intensity was more than 3 times higher than the mean of its initial 3 fluorescence values, as previously established by Gill, Olsen, Sampayo & Lithgow (2003). Eight to sixteen worms were studied per condition and at least three independent experiments were carried out. Resulting survival curves and mean survival times were determined with the Kaplan-Meier survival analysis (Mantel-Cox). Statistics were performed with GraphPad Prism 6 software (La Jolla, USA). Statistical significance was determined by one-way ANOVA with Dunnett´s post-test. Differences were considered significant at $p<0.05$.

8.6. Intracellular localization of DAF-16-GFP

Activation of DAF-16 transcription factor by the cocoa extract, the epicatechin-rich fraction (F19) and epicatechin pure compound was studied using DAF-16::GFP worms (transgenic strain TJ356). L4 larvae/young adults were incubated for 1 h in liquid NGM plates containing 1 % BSA, 50 mg/mL streptomycin and 10^9 *E. coli* OP50-1/mL as a food source, and supplemented with the ingredients of interest: the cocoa extract (adjusted to 100 µM of epicatechin), F19 (adjusted to 100 µM of epicatechin) and epicatechin pure compound 100 µM. A control without ingredient treated in the same conditions was carried out as well. Subsequently, 10 µL medium containing the worms were placed on a microscope slide mixed with 10 µL levamisol 10 mM (anesthetic) and covered with a cover slip. Nuclear translocation of DAF-16-GFP was detected by fluorescence microscopy using a Nikon Eclipse Ni-U and a GFP-filter set (Nikon; Düsseldorf, Germany). 30 worms were used per condition and four experiments were independently carried out. Statistical significance was determined by one-way ANOVA with Dunnett´s post-test. Differences were considered significant at $p<0.05$.

RESULTS

1. COCOA EXTRACT

1.1. Cocoa extract fractionation and characterization

The polyphenolic cocoa extract was characterized by HPLC-MS/MS (Fig. 8). Analyses were performed in positive and negative modes. A total of 28 compounds were identified and confirmed by comparison with the literature and the databases information, having into account their molecular mass and their fragmentation pattern (Table 9). Among the 28 identified compounds, two of them were characterized as methylxantines and the rest as polyphenols (11 phenolic acids, 11 flavanols, 3 flavonols and 1 flavanone).

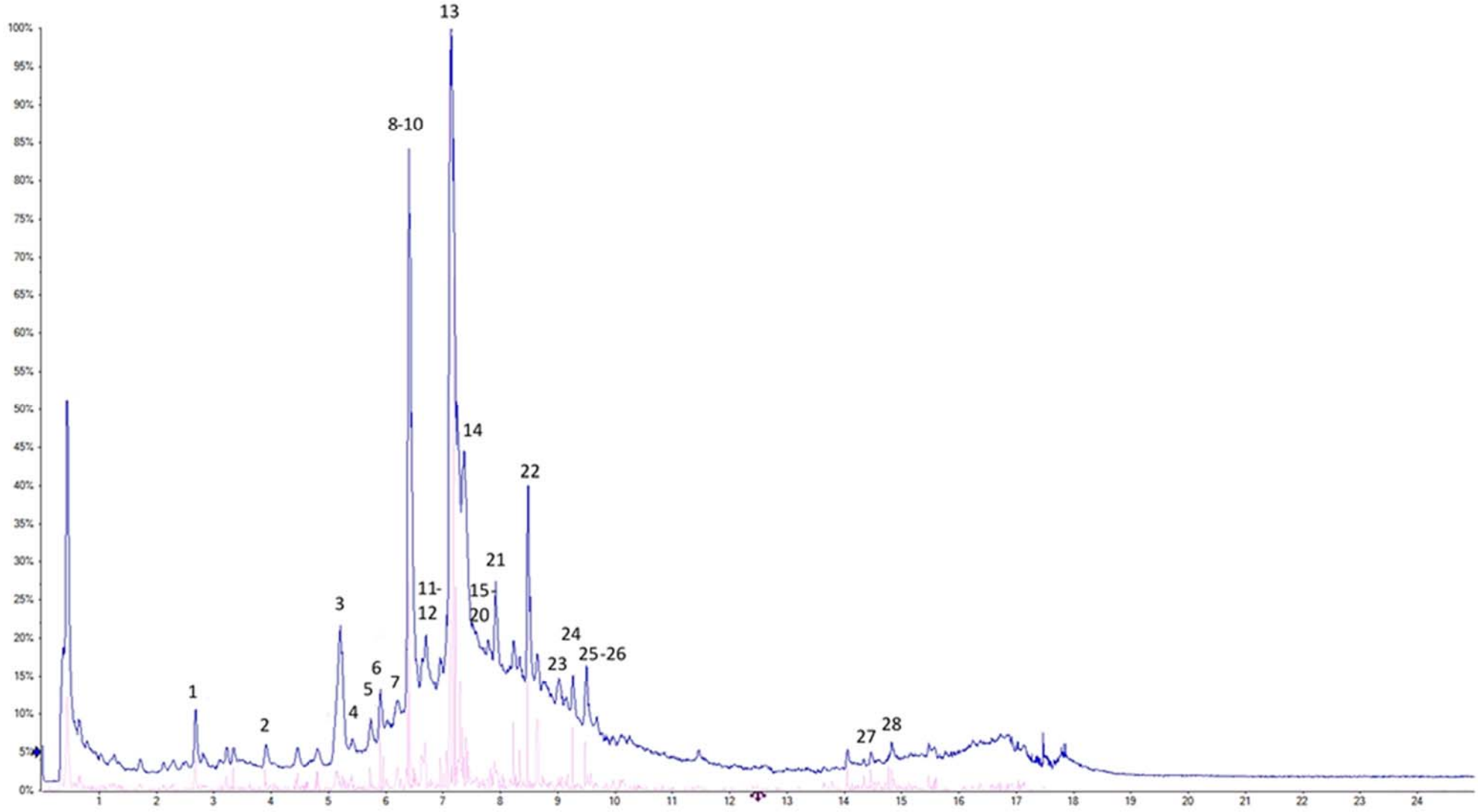


Figure 8. Base peak chromatogram of the cocoa extract. Peak numbers correspond to those collected in Table 9.

Table 9. HPLC MS/MS characterization of cocoa fractions. Identified compounds, molecular mass, ion mode, fragmentation pattern and retention time are shown for each peak number in Figure 8.

Peak number	Compound	M_r	Ion mode	Fragmentation pattern MS^2 (m/z)	R_t (min)	Fraction
1	Theobromine	180	+ -	138, 181, 110, 135, 137, 163 90, 134, 164, 178, 179	2.8	2
2	Procyanidin trimer T2	866	+	247, 289, 291, 409, 427, 559, 577, 579, 715, 867	3.7	2
3	Catechin	290	- +	205, 245, 289, 109, 123, 151, 187, 203, 271 123, 139, 147, 161, 179, 207	5.2	10
4	Caffeoyl aspartic acid (isomer)	295	- +	88, 114, 132, 135, 161, 179 135, 145, 163	5.4 5.6	5
5	Caffeic acid	180	-	91, 107, 134, 135	5.7	5
6	Caffeoyl aspartic acid	295	-	88, 114, 132, 135, 161, 188	5.9	2
7	Procyanidin dimer	578	- +	125, 245, 289, 299, 407, 425, 451, 577 127, 247, 287, 289, 301, 409, 417, 427, 435, 579	6.2	16
8	Caffeine	194	+	110, 123, 138, 195	6.3	16
9	p-coumaroyl aspartic acid	279	-	93, 114, 119, 132, 162, 163, 216, 234, 278	6.4	16
10	Procyanidin B2	578	+	127, 139, 163, 165, 233, 247, 271, 275, 287, 289, 291, 299, 301, 409, 411, 421, 427, 439, 579	6.5	14
11	Procyanidin trimer	886	+	139, 271, 287, 289, 291, 407, 425, 535, 559, 577, 579, 697, 699, 715, 867	6.7	16
12	Procyanidin A pentoside	708	+	271, 287, 299, 407, 425, 431, 437, 451, 539, 557, 559, 709	6.7	16
13	Epicatechin	290	+ -	123, 139, 147, 161, 179, 207 109, 123, 125, 187, 203, 221, 245, 247, 289	7.1	19
14	Chlorogenic acid	354	-	93, 155, 182, 183, 197, 218, 353	7.2	5
15	Caffeoyl 3-hydroxy-tyrosine	359	-	135, 161, 178, 222, 358	7.4	5

Peak number	Compound	M_r	Ion mode	Fragmentation pattern MS^2 (m/z)	R_t (min)	Fraction
16	Procyanidin trimer	866	+	127, 247, 259, 271, 289, 291, 397, 407, 409, 559, 577, 579, 697, 715, 867	7.4	19
17	Coumaric acid	164	+	119, 147, 165	7.5	14
18	Caffeoylquinic acid	354	-	146, 154, 191, 199, 260, 306, 353	7.6	24
19	Procyanidin tetramer T4	1154	+	247, 289, 425, 577, 579, 715, 741, 865, 867, 985	7.7	14
20	Naringenin	272	+	123, 147, 189, 227, 255, 273	7.7	19
21	Kaempferol 3-O-rutinoside	594	-	353, 473, 503, 593	7.8	24
22	Caffeoyl 3-hydroxy-tyrosine (isomer)	359	-	135, 178, 211, 222, 290, 312, 314, 358	8.4	19
23	p-coumaroyl tyrosine	327	- +	99, 119, 134, 145, 206, 267 282, 326, 327, 147	8.9	16
24	Quercetin 3-O-hexoside	464	- +	178, 271, 300, 301, 463 303	9.3	24
25	Procyanidin dimer	578	- +	283, 285, 430, 431, 577 287, 433	9.4	5
26	Quercetin-3-pentoside	434	-	255, 271, 300, 301, 433	9.5	24
27	Procyanidin dimer	578	-	165, 269, 289, 367, 425, 439, 559, 577	14.5	10
28	Caffeoyl tyrosine	343	- +	135, 178, 206, 222, 252, 342 119, 147, 163	15.1	10

26 fractions were obtained from the cocoa extract by semi-preparative HPLC. The fraction collector was programmed to detect and keep the biggest chromatographic peaks in separated fractions and also to keep the whole volume between peaks. Finally, the 26 fractions were concentrated after evaporation under vacuum in a rotatory evaporator and maintained at -20 ºC. Seven of them were selected for further studios (Fig. 9) based on their antioxidant capacity measured in previous experiments with *S. cerevisiae*, those corresponding to theobromine (F2), catechin (F10), procyanidin B2 (F14) and epicatechin (F19) and two fractions without any main compound (F5 and F24).

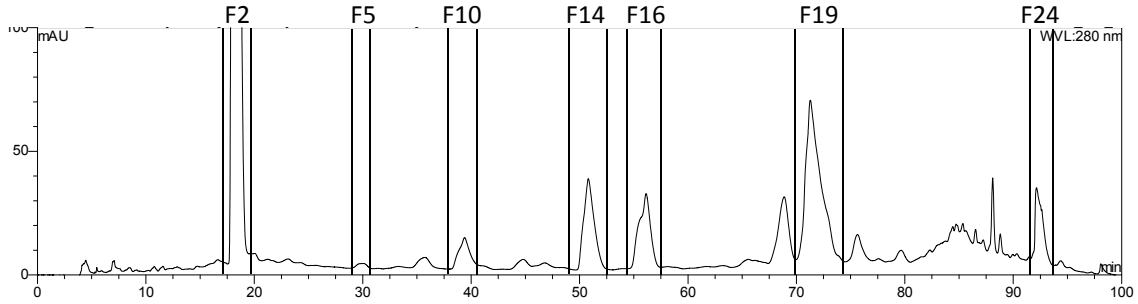


Figure 9. Semi-preparative HPLC chromatogram obtained after the cocoa extract fractionation. The 7 selected fractions to study are shown.

Cocoa fractions were characterized by HPLC-MS/MS as previously described for the cocoa extract. The 28 compounds were assigned to their corresponding fraction (Table 9).

Three different classes of compounds were identified in fraction 2: the methylxantine theobromine ($[M+H]^+$ ion at m/z 181), the phenolic acid caffeoyl aspartic acid ($[M-H]^-$ ion at m/z 294) and the flavonoid procyanidin trimer T2 ($[M+H]^+$ ion at m/z 578), showing a fragmentation pattern (MS^2) similar to the other trimers found in different fractions which are described below (867, 715, 579, 577, 559, 291 and 289).

Phenolic acids were quite abundant in fraction 5, since caffeic acid, an isomer of caffeoyl aspartic acid and chlorogenic acid were found, showing a $[M-H]^-$ spectra at m/z 179, 294 and 353, respectively. Moreover, the caffeoylated amino acid caffeoyl 3-hydroxy-tyrosine ($[M-H]^-$ ion at m/z 358) was identified according to the characterization carried out by previous groups (Locatelli *et al*., 2013; Patras, Milev, Vrancken & Kuhnert,

2014). A procyanidin dimer was detected as well, having the characteristic product ion at m/z 577, in accordance with Tomás-Barberán *et al*. (2007).

Regarding the fraction 10, a procyanidin dimer was identified as well. Catechin, one of the main flavan-3-ols found in cocoa, was present in this fraction, with the main fragment ion at m/z at 289, as described elsewhere (Pereira-Caro *et al*., 2012; Sánchez-Rabaneda *et al*., 2003; Tomás-Barberán *et al*., 2007). Caffeoyl tyrosine ($[M-H]^-$ ion at m/z 342) was also identified.

Two types of procyandins (dimer B2 and tetramer T4) were present in fraction 14. The phenolic acid coumaric acid ($[M+H]^+$ ion at m/z 165) was found as well.

Fraction 16 was rich in caffeine ($[M+H]^+$ m/z 195), as well as in procyanidins (dimer, trimer and procyanidin A pentoside). Moreover, hydroxicinnamic acids such as p-coumaroyl tyrosine ($[M+H]^+$ m/z 326) and p-coumaroyl aspartate ($[M-H]^-$ m/z 278) were present in this fraction.

With respect to fraction 19, epicatechin, another major cocoa falavan-3-ol was detected, having a similar fragmentation pattern as catechin (289, 245, 203, 187, 123 and 109). Procyanidin trimer as well as the flavanone naringenin ($[M+H]^+$ m/z 273), which was found in cocoa samples for the first time by Sánchez-Rabaneda *et al*. (2003), were also present in F19.

Finally, F24, was rich in flavonols, since it was found to contain quercetin derivatives such as quercetin 3-O-hexoside and quercetin 3-O-pentoside, according to $[M-H]^-$ values m/z 463 and 433, respectively, as well as kaempferol 3-O-rutinoside ($[M-H]^-$ ion at m/z 593). The phenolic acid caffeoylquinic acid (m/z 353) was identified in the negative ion mode.

1.1.1. Polyphenol quantification

Total polyphenol content was determined for the cocoa extract and the selected fractions by the Folin-Ciocalteu method (Table 10) and expressed as epicatechin equivalents. In addition, the main polyphenols or methylxanthines corresponding to chromatographic peaks in each fraction were identified and quantified by HPLC-DAD. As

shown in Table 10, fractions 2, 10, 14, 16 and 19 are rich in theobromine, catechin, procyanidin B2, caffeine and epicatechin, respectively. However, cocoa extract is a complex mixture of polyphenols and in fractions 5 and 24 no major compounds were detected by HPLC-DAD.

Table 10. Total polyphenol content of the cocoa extract and fractions and identification of main compounds in the fractions.

Sample	Total polyphenols (g/L epicatechin) [a]	Main compound in the fraction [b]	HPLC quantification (g/L main compound)
Cocoa extract	14.70 ± 1.68	-	-
F2	0.20 ± 0.04	Theobromine	2.14
F5	0.27 ± 0.08	-	-
F10	1.09 ± 0.31	Catechin	0.92
F14	2.39 ± 0.73	Procyanidin B2	1.57
F16	0.39 ± 0.12	Caffeine	0.73
F19	8.99 ± 2.23	Epicatechin	4.21
F24	1.09 ± 0.16	-	-

[a] Results are an average of three different sample preparations ± standard deviations. Duplicate analyses were carried out for each preparation. [b] Cocoa main compounds identified in each fraction against standards by HPLC-DAD. [c] Main compounds quantification against standards calibration curves by HPLC-DAD.

1.2. *In vitro* antioxidant activity of the cocoa extract and fractions

1.2.1. Free radical DPPH scavenging capacity of the cocoa extract and fractions

The *in vitro* radical scavenging properties of the principal flavonoids and methylxanthines in cocoa (pure compounds) as well as the cocoa extract were determined. Ascorbic acid and a commercial red grape powder were also analyzed. A range of concentrations from 5 to 50 mg/L was assayed and the DPPH inhibition percentage after 30 min of reaction was calculated for each sample and concentration. As shown in Figure 10, catechin and epicatechin showed the highest *in vitro* antioxidant capacity. In addition, these two flavanols and the complete cocoa extract were more potent than the well-known antioxidant vitamin C. Procyanidins B1 and B2 presented the same antioxidant capacity, being lower than the activity found in the cocoa extract. Moreover, the red grape extract showed a feeble radical-scavenging activity compared to the aforementioned samples. On the other hand, the methylxanthines caffeine and

theobromine, were the only pure compounds without antiradical properties, since any concentration reduced the free radical DPPH.

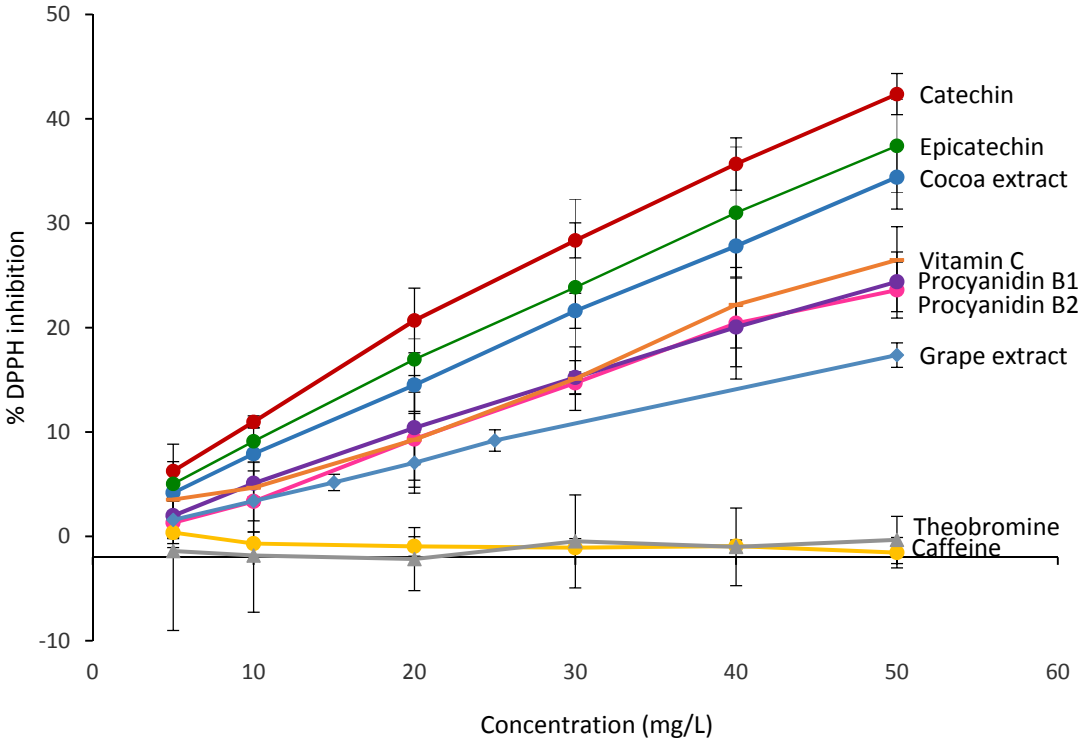


Figure 10. Free radical DPPH scavenging activities of cocoa and red grape extracts, ascorbic acid and main compounds of cocoa (catechin, epicatechin, procyanidins B1 and B2, theobromine and caffeine standards). Results are expressed as percentage of DPPH inhibition. Three independent replicates were carried out. Error bars indicate standard deviation among trials.

The antioxidant capacity of the selected fractions was also analysed by the DPPH methodology. The same volume of each fraction was assayed. The epicatechin-containing fraction (F19) resulted the most significant antioxidant sample (Fig. 11), showing a DPPH inhibition equivalent to 6.54 ± 0.37 g EE/L, followed by F14 (2.51 ± 0.65 g EE/L), rich in procyanidin B2, which was also statistically different from the other fractions. The catechin-rich fraction (F10) showed a very weak antioxidant activity (0.76 ± 0.17 g EE/L), possibly due to the small amount of catechin in that fraction. Similar results were obtained for F24 (with no major compounds), which suggest the presence of some radical-scavengers molecules in its composition. Methylxantines-rich fractions (F2 and F16) as well as fraction F5 showed the smallest antioxidant activity *in vitro* (less than 0.5 g EE/L). On the other hand, when the antioxidant capacity (calculated by the

DPPH methodology) was divided by the total polyphenol content (Table 10), the 7 fractions exhibited very similar values.

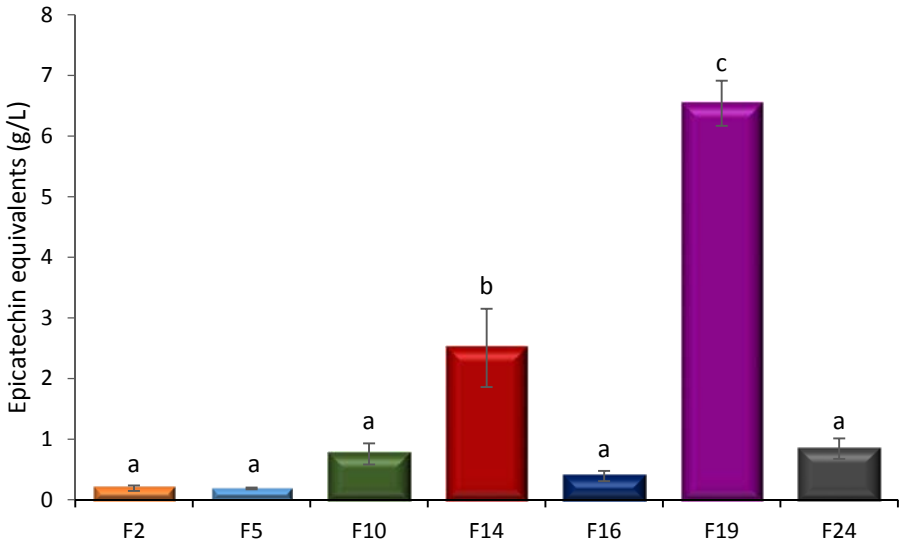


Figure 11. Free radical-scavenging activity of the selected cocoa fractions evaluated by the DPPH assay and expressed as epicatechin equivalents. Assays were carried out with 7.5 µL of each fraction in a total volume of 300 µL. Error bars indicate standard deviation among trials. The letters a, b, c indicate statistically significant differences ($p<0.05$) between fractions determined by one-way ANOVA with LSD test. Three independent experiments were carried out for each fraction.

Finally, fractions composed of a clearly more abundant polyphenol were compared to their corresponding pure compounds at equal concentrations. The comparison showed a higher antioxidant capacity of F16 and F19 (Fig. 12) compared to the same amount of caffeine and epicatechin (standards), respectively. These results suggest the presence of minor compounds with antioxidant capacity in the fractions. Based on these findings both fractions were selected for further studies.

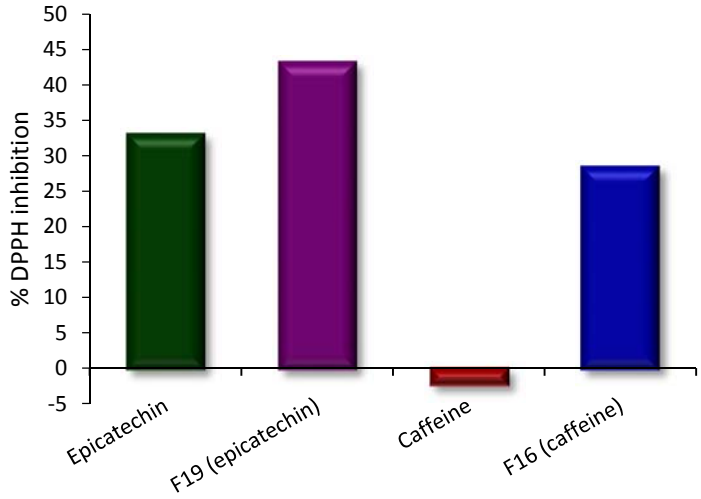


Figure 12. *In vitro* antioxidant activity (DPPH assay) of F16 and F19 cocoa fractions and its corresponding pure compounds caffeine (72.8 mg/L) and epicatechin (42.1 mg/L) respectively, at equal concentrations.

1.2.2. Free radical ABTS scavenging capacity

After a first *in vitro* screening of antioxidant activity using the DPPH method, some fractions showed a bigger antioxidant capacity than the pure compounds, indicating that minor compounds in fractions could contribute to their antioxidant effect. Two fractions were consequently selected for further studies: F16 and F19; as well as the pure compounds mainly found in those fractions: caffeine and epicatechin at the same concentrations, respectively. The ABTS assay was then employed, by measuring the ability of the selected fractions and pure compounds to reduce and decolorize a green radical solution compared to the synthetic vitamin E derivative Trolox (Fig. 13).

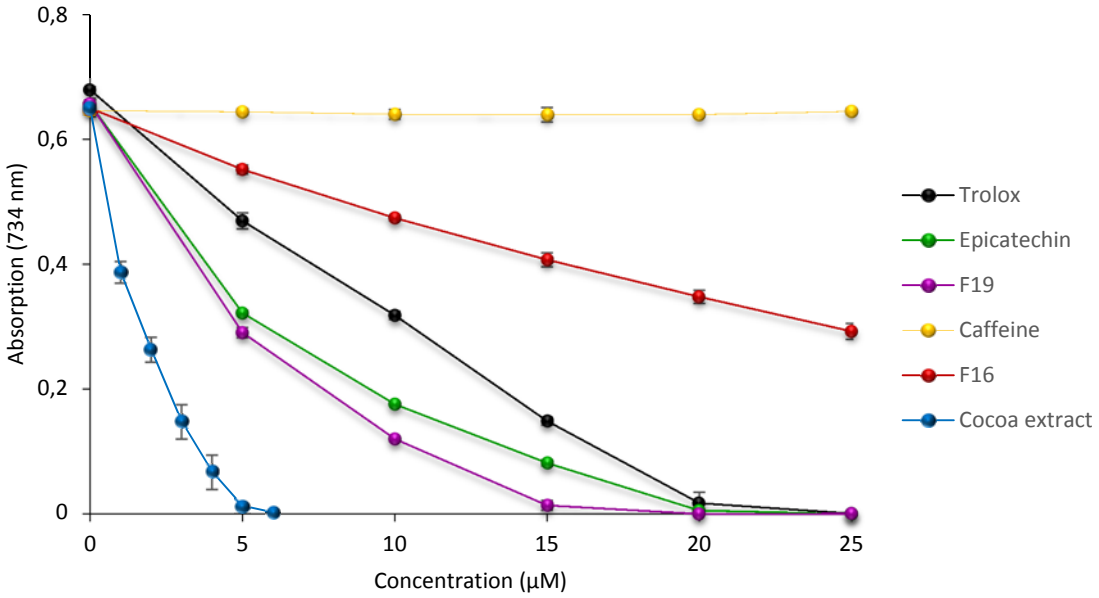


Figure 13. Antioxidant effects of cocoa extract (as epicatechin concentration in the extract), cocoa fractions F16 (0 to 25 µM of caffeine) and F19 (0 to 25 µM of epicatechin) and the corresponding pure compounds caffeine and epicatechin at the same concentrations, respectively, using the *in vitro* ABTS assay. Three independent replicates were carried out. Error bars indicate standard deviation among trials.

Results showed a potent and very similar antioxidant capacity of F19 and epicatechin, being in both cases bigger than the activity of the reference compound Trolox. Otherwise, F16 exhibited a lower radical scavenging capacity compared to Trolox, whilst the pure compound caffeine had no effect at all. In both cases, fractions were more potent than the pure compounds at the same concentration suggesting the presence of minor components with radical scavenging properties which could act in synergy with the main compounds. These findings are in accordance with those found with the DPPH assay.

The polyphenolic cocoa extract was analysed as well (Fig. 13). Its free radical ABTS scavenging effect was far more marked than the other samples. In fact, a concentration equivalent to 5 µM of epicatechin in the global extract, according to the Folin method, were enough to completely reduce the radical solution.

2. USE OF *S. cerevisiae* AS A MODEL TO STUDY THE ABILITY OF COCOA POLYPHENOLS TO INDUCE A CELLULAR ANTIOXIDANT RESPONSE.

2.1. Development of a multiwell assay for screening the ability of food ingredients to induce a cellular antioxidant response in *S. cerevisiae*.

Various methodologies based on the growth of *S. cerevisiae* have been previously adopted for the evaluation of the potential protective effect against oxidative stress of food ingredients. Spots assays are commonly used as qualitative analysis of viability to study different conditions and phenotypes in *S. cerevisiae*. However, this methodology is not always accurate and thus not conclusive. Figure 14 shows the effect of the cocoa extract on cell viability in absence of oxidative stress and after incubation with 0.5 and 4 mM H_2O_2. Although a protective effect conferred by the cocoa extract is visualized, statistical analysis can not be done with these kind of experiments, resulting in the need to find quantitative methods to evaluate bioactive compounds. This could be the case of serial dilutions and count of viable colonies assays, but these experiments are long and tedious, since at least three days are necessary to grow the yeast on agar plates.

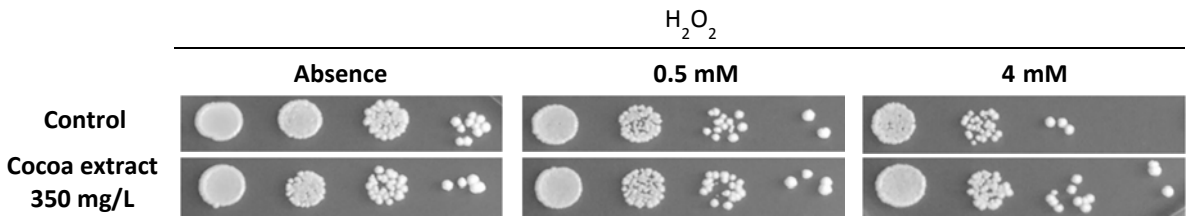


Figure 14. Effect of cocoa extract (350 mg EE/L) on *S. cerevisiae* BY4741 viability compared to a control without ingredient, in absence of oxidative stress and with 0.5 and 4 mM of H_2O_2.

Regarding this problem, a new methodology based on a 96-well plate is proposed in the present work for easy screening of antioxidant response by real time monitoring of the yeast growth. Hydrogen peroxide and menadione have been selected to provoke oxidative stress and the *S. cerevisiae* BY4741 strain has been used as test organism.

2.1.1. Determination of oxidant concentrations for the assays

Growth ratio curves were constructed to establish the most suitable oxidant concentrations for the assays. To do so, we calculated the ratio between the growth curve of the yeast culture obtained after 1 hour of exposure to the oxidant divided by the growth curve obtained in the absence of the oxidant (Fig. 15). In general, the growth ratio curves initially dropped due to the increased phase lag in cultures exposed to the oxidizing agent; subsequently they increased to a greater or lesser extent depending on the dose of the oxidant used. The growth ratio curves of yeast cultures previously stressed with H_2O_2 doses between 0.25 to 6 mM (Fig. 15.A) and with menadione doses between 0.1 to 5 mM (Fig. 15.B) showed that the highest doses of both oxidants led to the complete inhibition of the growth registered up to 18 h. Consequently, the lowest doses resulted in a lower initial decrease in the growth ratio, and almost total recovery at 18 h, reaching a growth ratio near 1. Considering these results, two concentrations of each oxidant were selected to perform the subsequent assays, 0.5 and 4 mM for H_2O_2; and 0.5 and 2 mM for menadione. The lowest concentration selected for both oxidants led to a moderate delay in the growth ratio curve and the highest one produced a severe growth delay so that growth was almost undetectable at 18 h of incubation (Fig. 15). Thus, these two doses of oxidant mimicked weak and severe oxidative stress conditions respectively.

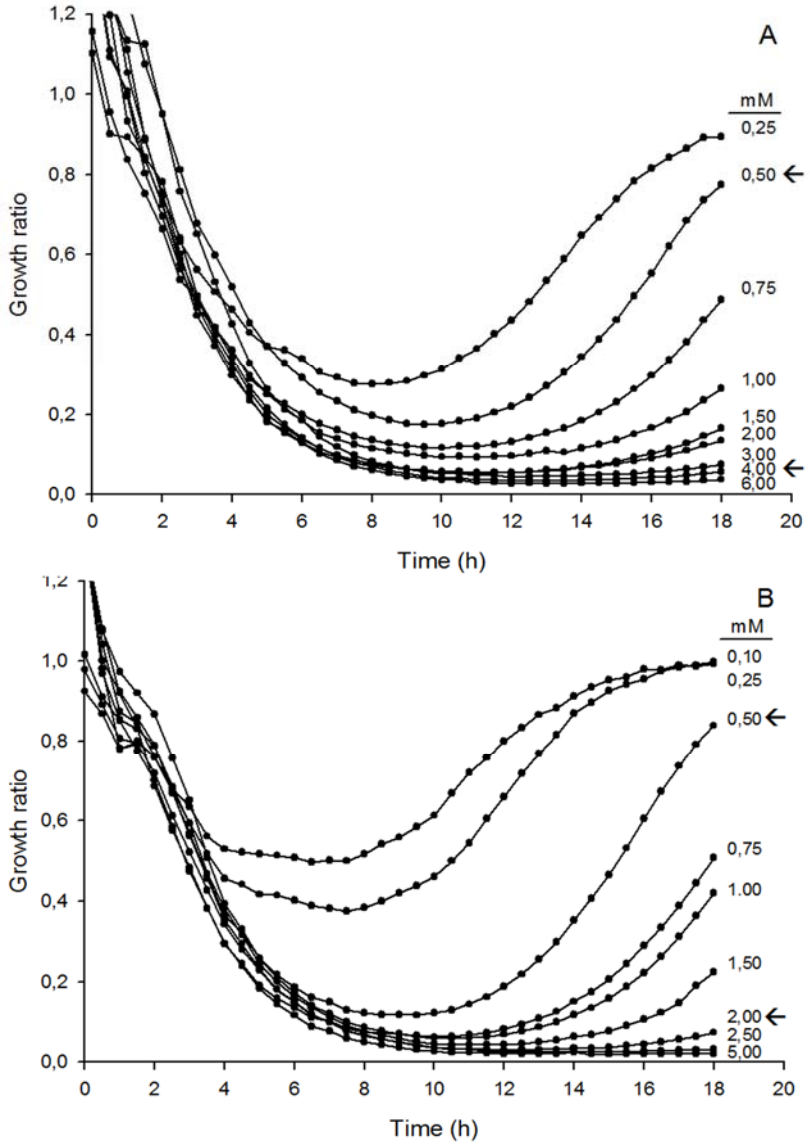


Figure 15. 'Growth ratio curves' of *S. cerevisiae* BY4741 cultures after being stressed for 1 h with ranging concentrations of A) H_2O_2 from 0.25 to 6 mM and B) menadione from 0.1 to 5 mM. Curves were constructed as the ratio between the growth curve of the yeast culture obtained after 1 h of exposure to the oxidant divided by the growth curve obtained in the absence of the oxidant. The arrows indicate the concentrations of each oxidant chosen to carry out the following experiments.

2.1.2. Assay to test the ability of food ingredients to promote an antioxidant response in *S. cerevisiae*

To assay the ability of food ingredients to promote an antioxidant response in *S. cerevisiae*, overnight cultures were pre-incubated in the presence or absence of each ingredient. Subsequently, the cells were washed to minimize extracellular residual ingredients, collected and exposed to the oxidant for 1 h. Then, they were washed,

resuspended in fresh culture media and placed in microtiter plates to monitor growth for 18 h. Polyphenolic extracts from cocoa and red grape and ascorbic acid were tested at six different doses using H_2O_2 and menadione as oxidants at the two concentrations previously chosen (H_2O_2: 0.5 and 4 mM; menadione: 0.5 and 2 mM). The growth curves corresponding to the cultures unexposed to oxidative stress were indistinguishable regardless of the pre-incubation with or without ingredients (data not shown). To evaluate whether the ingredients exerted protective antioxidant activity, we constructed the 'effect curves' by dividing the growth ratio curve of the culture pre-incubated with the ingredient by the growth ratio of the culture pre-incubated without it, both at the same oxidant dose. The effect curve exceeded 1 when the pre-incubation with the ingredient led to cellular antioxidant protection but it was below 1 when it exerted a pro-oxidant effect in the yeast cells.

Figure 16 shows the 'effect curves' for the ingredients assayed at the two H_2O_2 concentrations at six different time values, from 5 to 18 h of incubation. In yeast cultures stressed with 0.5 mM H_2O_2 a significant antioxidant protection was observed after the pre-incubation with ascorbate at 0.5, 2 and 5 mM, markedly at 5 mM. Furthermore, the three highest ascorbate concentrations (10, 15 and 25 mM) exerted pro-oxidant activity (the effect curve was significantly lower than one) (Fig. 16.A). In yeast cultures exposed to 4 mM H_2O_2, only the pre-incubation with 5 mM ascorbate led to a protection against oxidative stress, whereas the three highest doses led to a pro-oxidant effect (Fig. 16.B).

Pre-incubation of yeast cells with the cocoa extract provided a significant protection at the four lowest concentrations tested (50, 100, 200 and 350 mg EE/L) subsequent to oxidative stress with 0.5 and 4 mM H_2O_2 (Fig. 16.C,D).

Treatment of yeast cells with the red grape extract provided protection against stress induced with 0.5 mM H_2O_2 at all concentrations tested (Fig. 16.E). When stress was induced with 4 mM H_2O_2, a statistically significant protection against oxidative stress was only detected in cells pre-incubated with the grape extract at 180 and 260 mg EE/L (Fig. 16.F).

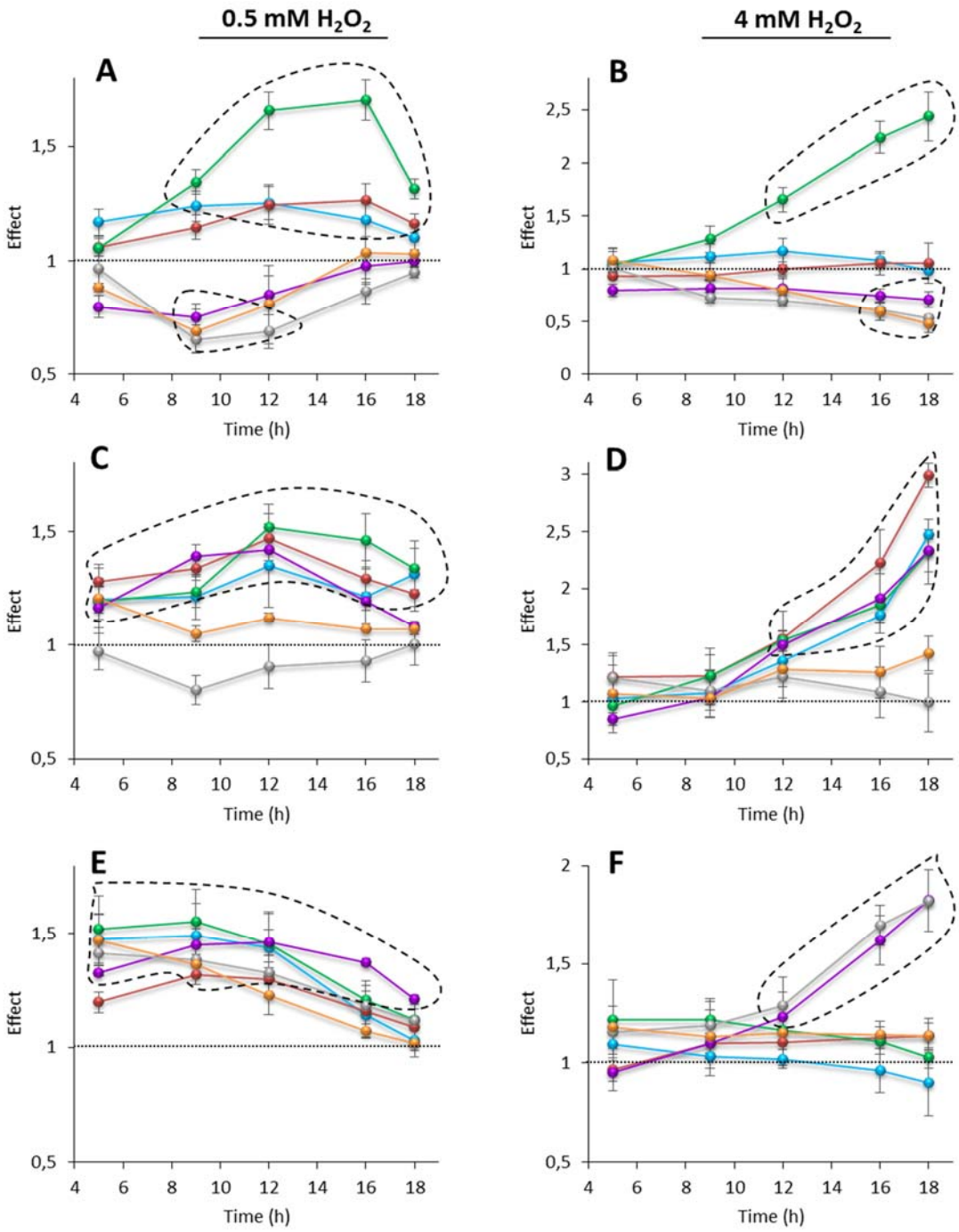


Figure 16. 'Effect curves' of BY4741 yeast strain after oxidative stress assessed with 0.5 mM and 4 mM of H_2O_2. Effect curves were constructed by dividing the growth ratio curve of the culture pre-incubated with the ingredient by the growth ratio curve of the culture pre-incubated without it, both at the same oxidant dose. **A,B)** Ascorbic acid at 0.5 mM (●), 2 mM (●), 5 mM (●), 10 mM (●), 15 mM (●) and 25 mM (●).**C,D)** Cocoa extract at 50 (●), 100 (●), 200 (●), 350 (●), 500 (●) and 700 (●) mg EE/L. **E,F)** Red grape extract at 25 (●), 50 (●), 100 (●), 180 (●), 260 (●) and 370 (●) mg EE/L. Mean values ± standard deviation among trials are represented. Points within the dashed lines indicate statistically significant differences compared to the control without ingredient by the Student's t test at $p<0.05$.

Figure 17 shows the 'effect curves' for the ingredients assayed in the yeast cultures using menadione as oxidant. The treatment with ascorbic acid produced a significant antioxidant response in the yeast at the five highest concentrations (2 to 25 mM) when menadione was applied at 0.5 mM (Fig. 17.A) and at the four highest concentrations (5 to 25 mM) when the oxidative stress was caused with 2 mM menadione (Fig 17.B). The highest antioxidant protection was observed in cells pre-incubated with 10 mM ascorbic acid in both cases.

The pre-incubation of yeast cells with the cocoa extract at 350, 500 and 700 mg EE/L led to a significant antioxidant response at 0.5 mM of menadione (Fig. 17.C) and at 200, 350, 500 and 700 mg EE/L when stress was induced with menadione 2 mM (Fig. 17.D).

When yeast cells were pre-incubated with the grape extract, a significant antioxidant response was observed at the four highest concentrations assayed (100 to 360 mg EE/L) at both 0.5 and 2 mM of menadione (Fig. 17.E,F).

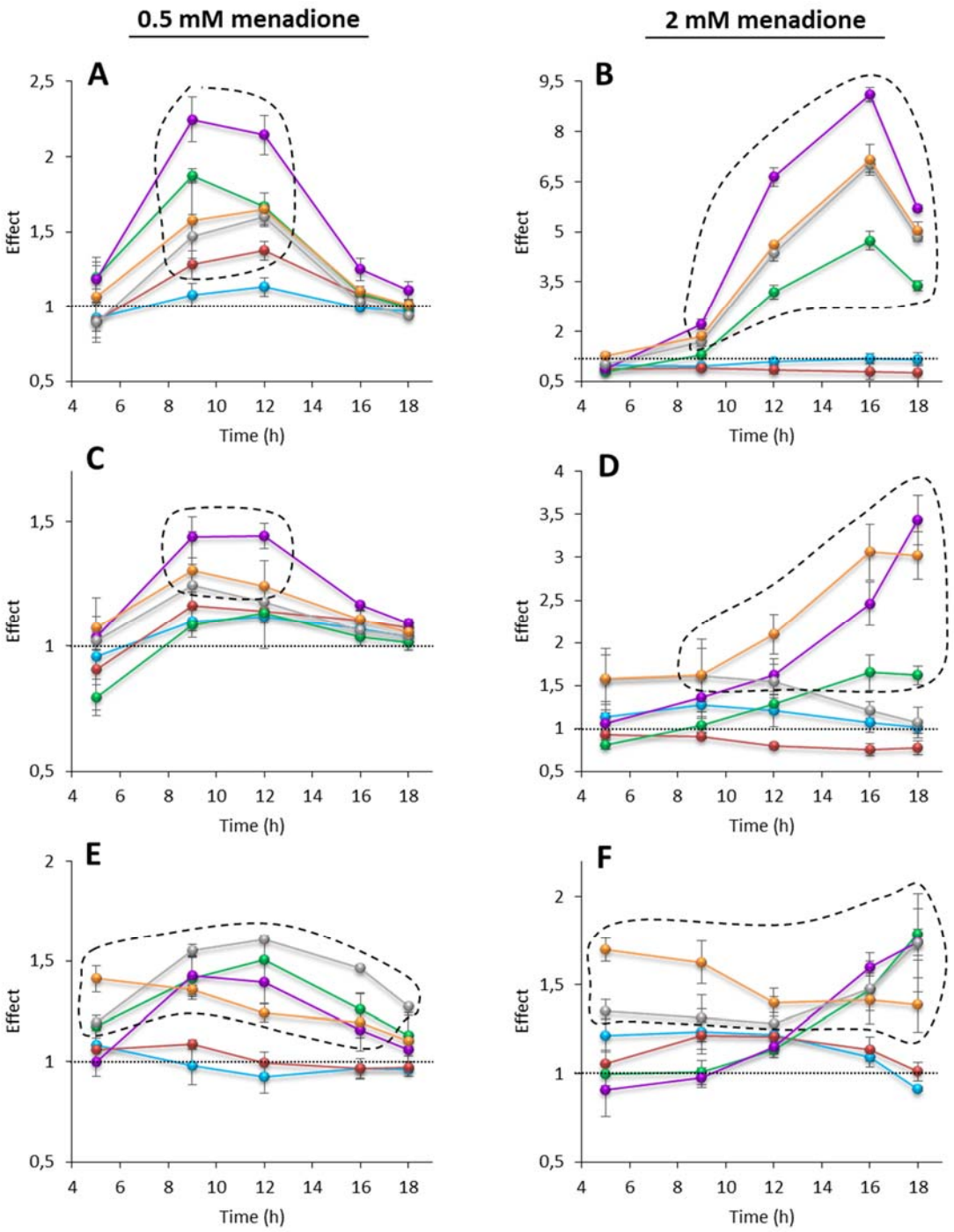


Figure 17. 'Effect curves' of BY4741 yeast strain after oxidative stress assessed with 0.5 mM and 2 mM of menadione. Effect curves were constructed by dividing the growth ratio curve of the culture pre-incubated with the ingredient by the growth ratio curve of the culture pre-incubated without it both at the same oxidant dose. **A,B)** Ascorbic acid at 0.5 mM (●), 2 mM (●), 5 mM (●), 10 mM (●), 15 mM (●) and 25 mM (●).**C,D)** Cocoa extract at 50 (●), 100 (●), 200 (●), 350 (●), 500 (●) and 700 mg EE/L (●) . **E,F)** Red grape extract at 25 (●), 50 (●), 100 (●), 180 (●), 260 (●) and 370 (●) mg EE/L. Mean values ± standard deviation among trials are represented. Points within the dashed lines indicate statistically significant differences compared to control without ingredient by Student's t test at $p<0.05$.

2.1.3. Antioxidant response for the ingredients in the *hst3Δ* mutant strain.

A previous work described that the gene encoding for the Hst3p protein (sirtuin belonging to the Sir2 family of NAD+ dependent deacetylases) was overexpressed 4 times due to the incubation with a cocoa powder in the *S. cerevisiae* BY4741 strain while the *hst3Δ* knockout mutant strain lacked that antioxidant response (Martorell *et al.*, 2011). In the present work, the *hst3Δ* mutant strain was used to conduct assays in the presence and absence of the ingredients, and with both H_2O_2 and menadione as oxidants. One concentration of each ingredient was selected based on the effects shown previously. The growth ratio and effect curves constructed using the *hst3Δ* and the BY4741 strains are shown in Figure 18 for H_2O_2.

The pre-incubation of the *hst3Δ* strain with the cocoa and red grape extracts, and with ascorbate, did not produce the antioxidant response exhibited by the wild-type. The three effect curves for the *hst3Δ* strain were around one, showing no effect due to the pre-incubation with the ingredients at 0.5 and 4 mM H_2O_2 (Fig. 18. A,B). In contrast, a significant antioxidant effect was recorded in the BY4741 wild-type strain for all three ingredients. Interestingly, growth ratio curves for both strains in the absence of the ingredients showed that the *hst3Δ* strain was significantly more resistant to H_2O_2 than the wild-type strain at 0.5 and 4 mM (Fig. 18.C,D).

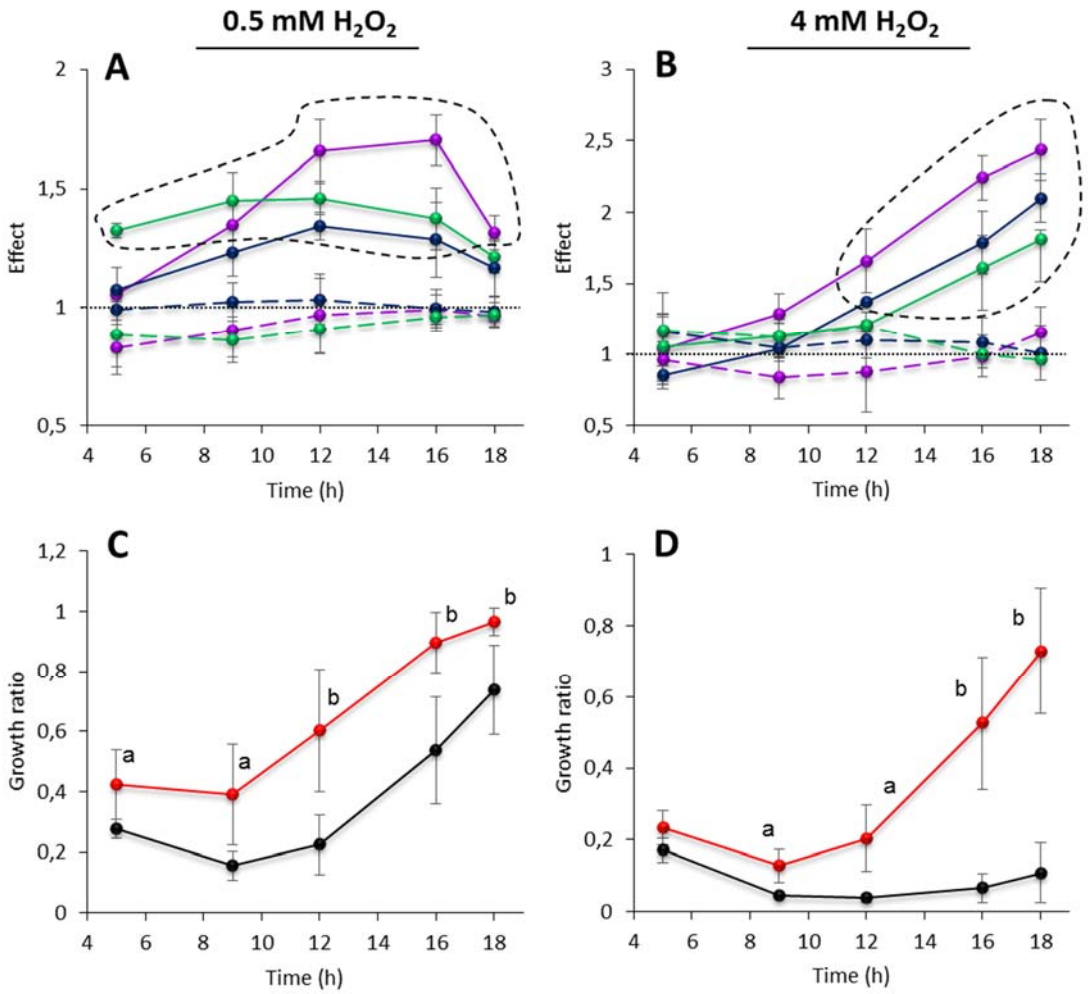


Figure 18. Growth ratios and 'effect curves' of the *hst3Δ* and the BY4741 strains after treatment with 0.5 and 4 mM of H_2O_2. **A,B)** Effect curves of ascorbic acid 5 mM (●), cocoa extract 350 mg EE/l (●) and red grape extract 180 mg EE/L (●) in the wild-type (solid curves) and the *hst3Δ* (dashed curves) strains at 0.5 and 4 mM of H_2O_2. Mean values ± standard deviation among trials are represented. Points within the dashed lines indicate statistically significant differences compared to the control without ingredient by the Student's t test at $p<0.05$. **C,D)** Growth ratios curves of BY4741 (●) and *hst3Δ* (●) strains after treatment with 0.5 and 4 mM of H_2O_2. Mean values ± standard deviation among trials are represented. The letters a and b indicate significant differences between yeast strains by the Student's t test at $p<0.05$ and $p<0.01$, respectively.

A similar response was observed when the oxidant was menadione (Fig. 19). The pre-incubation of the wild-type with both ingredients and the positive control led to a significant antioxidant response whilst no effect was shown in the *hst3Δ* strain pre-incubated with the cocoa and grape extracts. The pre-incubation of the mutant strain with ascorbate showed a significant antioxidant effect both at 0.5 and 2 mM menadione (Fig. 19. A,B). Moreover, the *hst3Δ* strain was significantly more resistant to both concentrations of menadione, as previously described for H_2O_2 (Fig. 19.C,D).

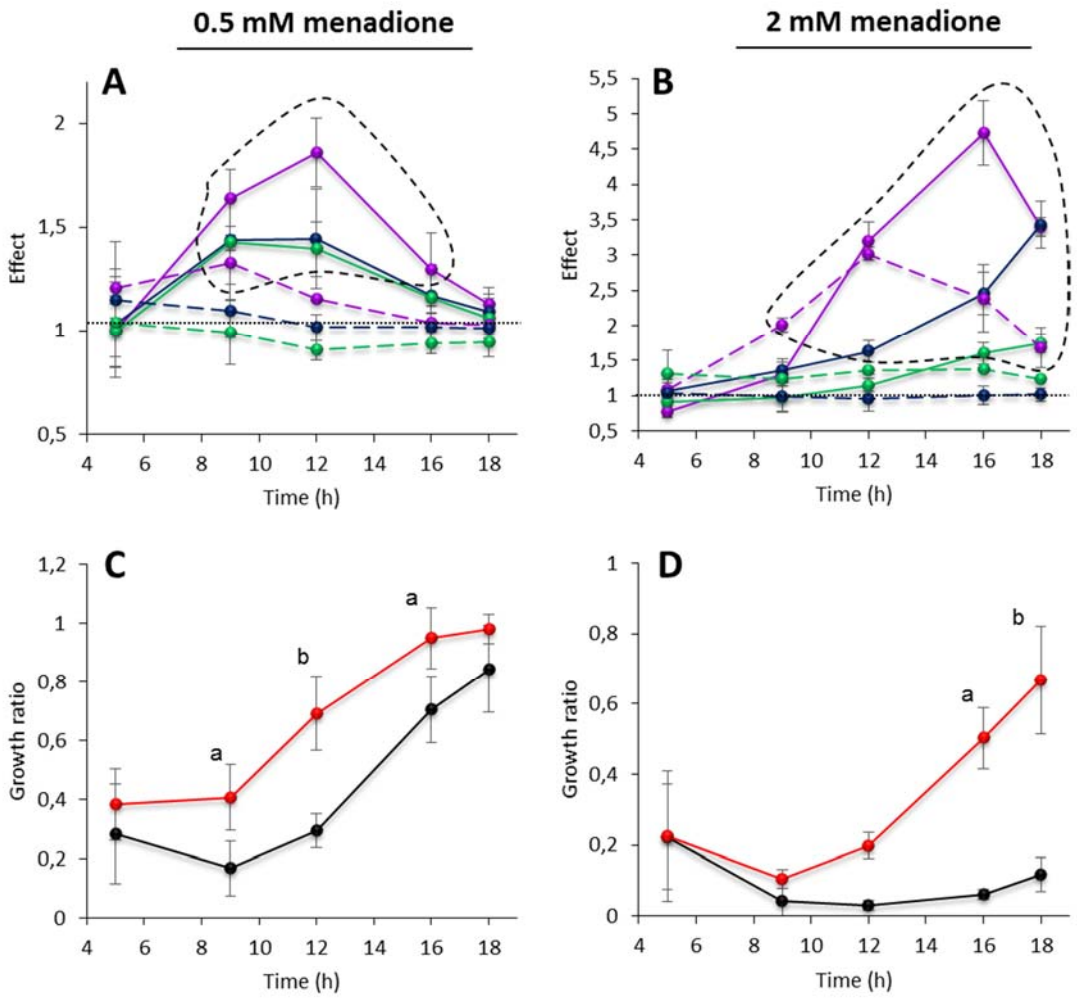


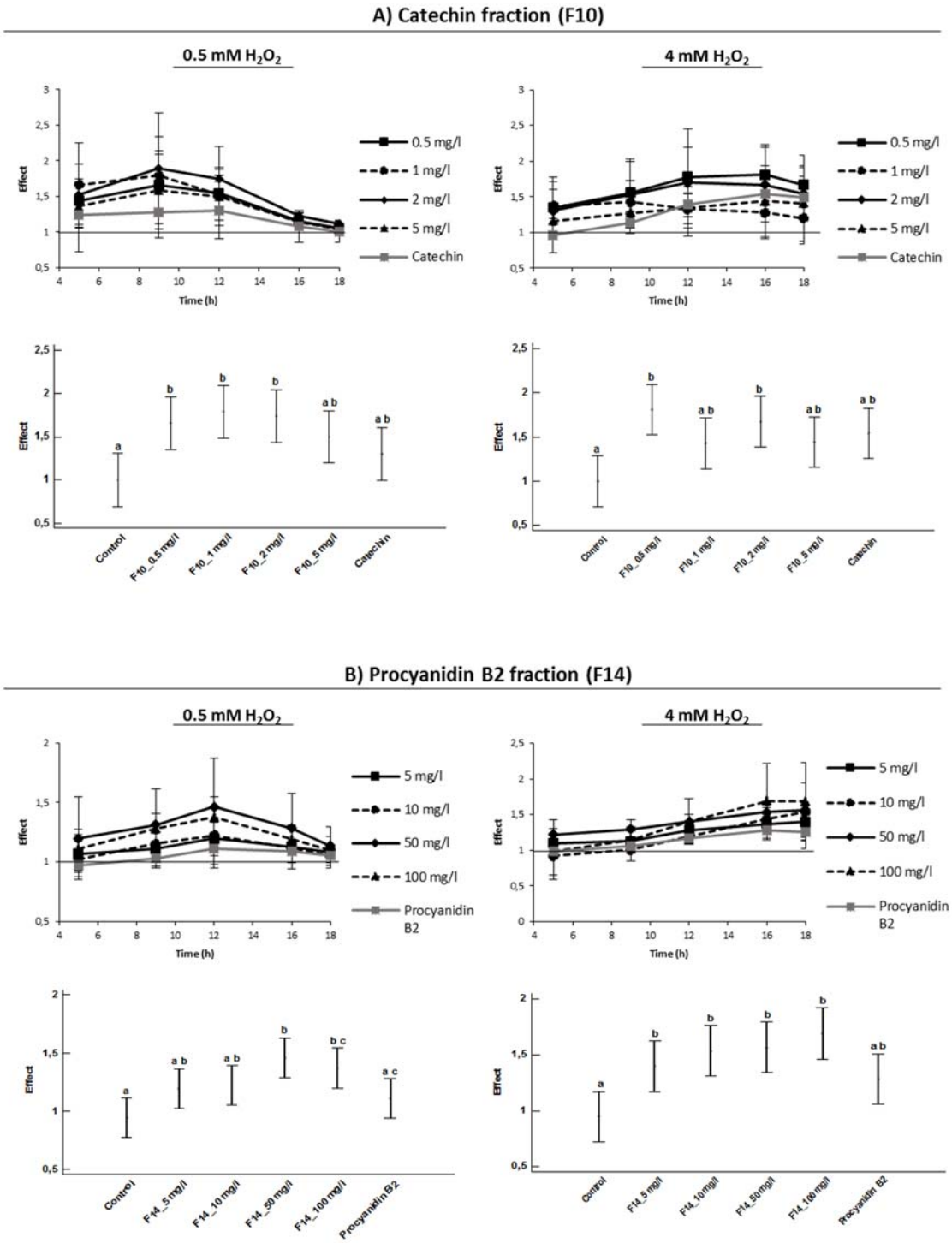
Figure 19. Growth ratios and 'effect curves' of the *hst3Δ* and the BY4741 strains after treatment with 0.5 and 2 mM of menadione. **A,B)** Effect curves of ascorbic acid 5 mM (●), cocoa extract 350 mg EE/L (●) and red grape extract 180 mg EE/L (●) in the wild-type (solid curves) and the *hst3Δ* (dashed curves) strains at 0.5 and 2 mM of menadione. Mean values ± standard deviation among trials is represented. Points within the dashed lines indicate statistically significant differences compared to the control without ingredient by the Student's t test at $p<0.05$. **C,D)** Growth ratios curves of BY4741 (●) and *hst3Δ* (●) strains after treatment with 0.5 and 2 mM of menadione. Mean values ± standard deviation among trials are represented. The letters a and b indicate significant differences between yeast strains by the Student's t test at $p<0.05$ and $p<0.01$, respectively.

2.2. Capacity of cocoa fractions to promote an antioxidant response in *S. cerevisiae*

The *in vivo* antioxidant capacity of cocoa polyphenols in *S. cerevisiae* BY4741 has been studied with a rapid and reproducible yeast-based method previously set up in our group. In this context, the induction of intracellular antioxidant response in *S. cerevisiae* has been tested after incubation with cocoa fractions and subsequently exposition to oxidative stress. After a first screening assay with the 26 fractions (data not shown), 7 of

them were selected for further studies, based on their *in vivo* antioxidant activity: those having in their composition one of the main compounds of cocoa (theobromine, caffeine, catechin, epicatechin or procyanidin B2) and two fractions without any major cocoa compound but showing interesting results in the first yeast screening. All of them were tested at four different concentrations and using hydrogen peroxide at two concentrations as oxidant agent. Pure compounds of theobromine, caffeine, catechin, epicatechin and procyanidin B2 were evaluated as well, in order to compare their effect to that found in the fractions rich in those compounds.

The catechin-containing fraction (F10) as well as the epicatechin-containing fraction (F19) and the procyanidin B2-rich fraction (F14) were the most protective ones (Fig.20). F10 (Fig. 20.A) displayed a statistically significant antioxidant response in the yeast at 0.5 mg/L, 1 mg/L and 2 mg/L of catechin after treatment with 0.5 mM H_2O_2 and at 0.5 mg/L and 2 mg/L of catechin after incubation with 4 mM H_2O_2. This fraction was used in smaller concentrations given its high antioxidant effect previously found (data not shown). Otherwise, the pure compound catechin (10 mg/L) did not protect the cells against oxidative stress. In the case of F14 (Fig. 20.B), when hydrogen peroxide was used at 0.5 mM, the lowest concentrations of procyanidin were not enough to exert an antioxidant effect in *S. cerevisiae*, whereas the highest doses (50 mg/L and 100 mg/L) showed a significant beneficial effect. At 4 mM H_2O_2, all procyanidin B2 concentrations in F14 significantly increased the resistance of the cultures against such stress. However, the pure compound itself (10 mg/L) did not show an antioxidant protection in this experiment. Regarding the epicatechin-rich fraction F19 (Fig. 20.C), all concentrations assayed (5 mg/L, 10 mg/L, 50 mg/L and 100 mg/L of epicatechin) showed an antioxidant protective trend but only the intermediate concentration (10 mg/L) was able to promote a significant antioxidant response in the yeast at both H_2O_2 doses. The pure compound epicatechin (10 mg/L) was protective only at 0.5 mM of H_2O_2, compared to the control without ingredient.


A) Catechin fraction (F10)
0.5 mM H_2O_2
4 mM H_2O_2
Effect
Time (h)
0.5 mg/l
1 mg/l
2 mg/l
5 mg/l
Catechin
a
b
b
b
a b
a b
Control
F10_0.5 mg/l
F10_1 mg/l
F10_2 mg/l
F10_5 mg/l
Catechin
B) Procyanidin B2 fraction (F14)
0.5 mM H_2O_2
4 mM H_2O_2
Effect
Time (h)
5 mg/l
10 mg/l
50 mg/l
100 mg/l
Procyanidin B2
a
a b
a b
b
b c
a c
b
b
b
a b
Control
F14_5 mg/l
F14_10 mg/l
F14_50 mg/l
F14_100 mg/l
Procyanidin B2

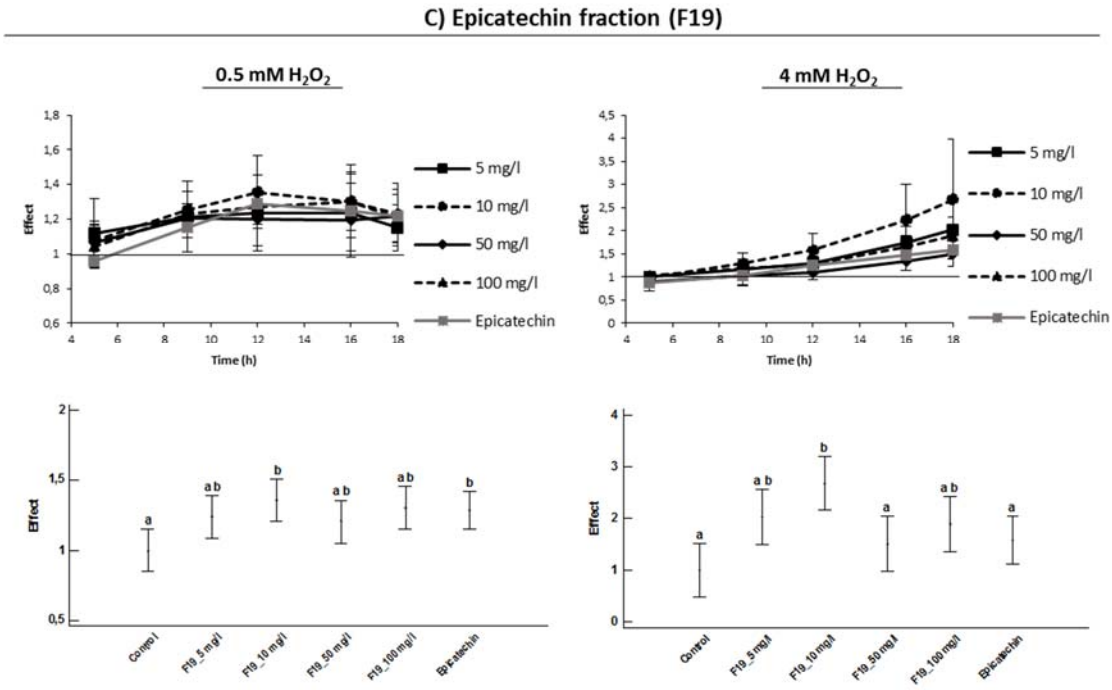


Figure 20. 'Effect curves' of BY4741 yeast strain after incubation with fractions and pure compounds at 0.5 and 4 mM of H_2O_2 and One-way ANOVA with LSD test for the most protective effect time point for each fraction and concentration. **A)** Catechin-containing fraction (F10), at 0.5, 1, 2 and 5 mg catechin/L and the pure compound catechin at 10 mg/L; **B)** Procyanidin B2-containing fraction (F14) at 5, 10, 50, 100 mg procyanidin B2/L and the pure compound procyanidin B2 at 10 mg/L; **C)** Epicatechin-containing fraction (F19) at 5, 10, 50, 100 mg epicatechin/L and the pure compound epicatechin at 10 mg/L. Error bars indicate standard deviation among trials. Different letters indicate statistical significance ($p<0.05$) among conditions. Experiments were carried out at least in triplicate

Methylxantine-rich fractions F2 (mainly containing theobromine) and F16 (rich in caffeine) were not able to protect the yeast against the oxidative stress at any of the assayed concentrations, although the pure compound caffeine itself showed significant antioxidant protection in *S. cerevisiae*, but not the pure compound theobromine. Fractions with no major compounds F5 and F24, did not confer statistically significant protection after treatment of the cultures with a range of concentrations and incubation with hydrogen peroxide.

2.3. Proteomic study for the identification of possible proteins involved in the response of *S. cerevisiae* to oxidative stress mediated by cocoa polyphenols

2.3.1. Proteomic analysis

Once the capacity of the cocoa extract to induce a cellular antioxidant response was confirmed in the yeast model, a proteomic approach was performed to identify proteins potentially involved in this effect. For that, the differential expression of the *S. cerevisiae* proteome was analyzed after treatment with the cocoa extract and in presence and absence of oxidative stress.

2.3.1.1. S. cerevisiae response to cocoa extract

21 statistically significant differential spots ($p<0.05$) were found between both conditions (with and without pre-incubation with the cocoa extract) (Fig. 21), 6 were up-regulated and 15 were down-regulated in presence of cocoa extract. Among these, 15 proteins were identified by MALDI-MS/MS and MASCOT database (Table 11).

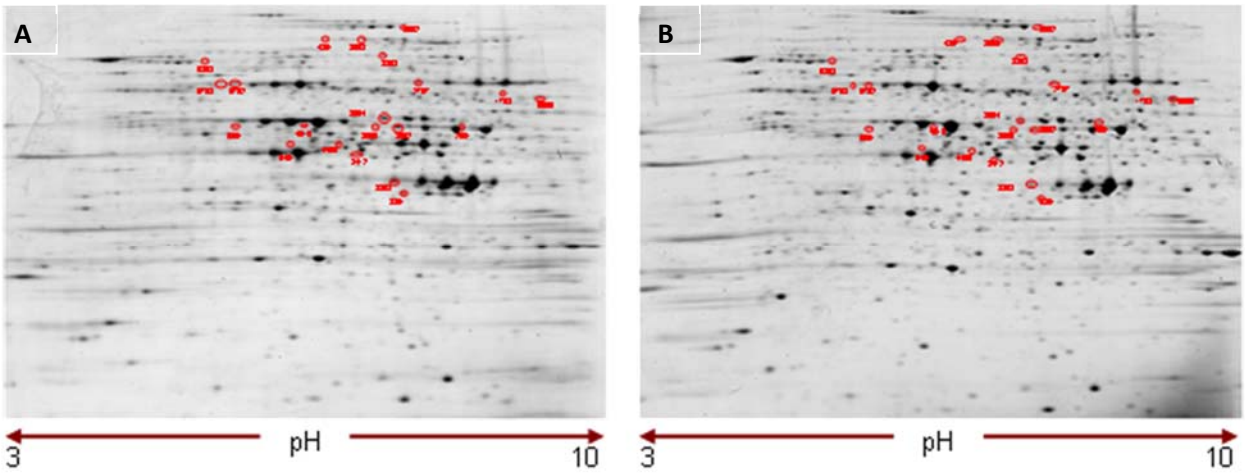


Figure 21. Two dimensional gel electrophoresis protein patterns of *S. cerevisiae* BY4741 cultured with the cocoa extract (A) and without it (B). Proteins were separated in pH 3–10 IPG-strips for the first dimension and in acrylamide 12.5 % gradient gels for the second dimension. Differentially regulated proteins ($p<0.05$) are localized with circles and marked with the spot ID in each gel.

Table 11. Identification of differentially expressed proteins in the *S. cerevisiae* BY4741 culture pre-incubated with the cocoa extract *vs* the yeast culture non pre-incubated with it.

Spot	Protein	Protein name	UniProt ID	Mass (Da)	[a] pI	Mascot score	[b] Matched peptides	[c] %COV	Num MSMS	[d] Fold
1703	Ilv1	Threonine dehydratase, mitochondrial	P00927	64076	8.67	96	18	38	0	+ 2.40
2601	Cit1	Citrate synthase, mitochondrial	P00890	53384	8.23	78	15	31	1	+ 2.56
2707	Cdc19	Pyruvate kinase 1	P00549	54909	7.56	258	32	59	4	- 2.22
3301	Rpl5	60S ribosomal protein L5	P26321	33751	6.36	237	12	48	3	- 2.84
3303	Tdh3	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	35838	6.46	170	14	48	2	- 2.42
3412	Ilv5	Ketol-acid reductoisomerase, mitochondrial	P06168	44512	9.1	129	17	43	2	- 4.90
3502	Pgk1	Phosphoglycerate kinase	P00560	44768	7.11	273	23	53	4	- 2.85
3808	Trp5	Tryptophan synthase	P00931	76977	6.05	82	24	38	1	+ 2.26
3902	Eft1	Elongation factor 2	P32324	93686	5.92	255	37	42	4	+ 2.38
3908	Met6	5-methyltetrahydropteroyl triglutamate--homocysteine methyltransferase	P05694	85978	6.07	115	31	41	1	- 2.03
4405	Yhb1	Flavohemoprotein	P39676	44846	5.86	165	17	57	2	- 2.34
5401	Eft1	Elongation factor 2	P32324	93686	5.92	73	20	20	2	- 2.08
6501	Eno2	Enolase 2	P00925	46942	5.67	131	11	29	2	- 3.89
6702	Frs2	Phenylalanine--tRNA ligase alpha subunit	P15625	57532	5.53	62	8	16	1	- 3.86
6808	Ssb2	Heat shock protein SSB2	P40150	66668	5.37	163	20	33	3	+ 2.09

pI and mass from samples not analysed by MS/MS have been compared to expected results.
[a] pI=Isoelectric point.
[b] Matched peptides=Number of peptides matched from protein in MS/MS query.
[c] Cover=percentage of amino acid sequence of protein covered in MS/MS analysis.
[d]Fold=Relative protein spot intensities of differentially expressed proteins in the cocoa extract condition versus the control condition.

The functional classification of the identified proteins (Table 12) shows the biological process in which a significant number of differentially expressed proteins (up- or down-regulated) are involved. Genes codifying for Rpl5, Eft1, Frs2 and Ssb2 proteins were not located in any functional group. The carbohydrate metabolic process and the cellular amino acid process were the most represented functional categories, with 28.6 % of differentially regulated genes.

Table 12. Functional classification of the differentially expressed proteins in the yeast culture pre-incubated with the cocoa extract compared to the yeast culture not pre-incubated with it. The web-based tool FunSpec was used for the statistical evaluation (p<0.01) using the GO biological process clustering.

GOID	Functional category	p-value	Genes	nº genes	% genes
GO:0006096	glycolysis	1.251e-07	*CDC19, PGK1, TDH3 ENO2*	4	28.6
GO:0006094	gluconeogenesis	3.686e-06	*PGK1, TDH3, ENO2*	3	21.4
GO:0008652	cellular amino acid biosynthetic process	2.062e-05	*ILV1, MET6, TRP5, ILV5*	4	28.6
GO:0009082	branched chain family amino acid biosynthetic process	0.000234	*ILV1, ILV5*	2	14.3
GO:0046356	acetyl-CoA catabolic process	0.001817	*CIT1*	1	7.1
GO:0015671	oxygen transport	0.003632	*YHB1*	1	7.1
GO:0006568	tryptophan metabolic process	0.005443	*TRP5*	1	7.1
GO:0006101	citrate metabolic process	0.005443	*CIT1*	1	7.1
GO:0009097	isoleucine biosynthetic process	0.005443	*ILV1*	1	7.1
GO:0008152	metabolic process	0.005532	*CDC19, ILV1, TRP5, TDH3*	4	28.6
GO:0072593	reactive oxygen species metabolic process	0.007251	*TDH3*	1	7.1
GO:0006567	threonine catabolic process	0.007251	*ILV1*	1	7.1
GO:0009636	response to toxin	0.009057	*YHB1*	1	7.1
GO:0000162	tryptophan biosynthetic process	0.009057	*TRP5*	1	7.1

2.3.1.2. S. cerevisiae response to oxidative stress after pre-incubation with the cocoa extract

When oxidative stress (5 mM H_2O_2) was added to the cultures after the cocoa treatment, the results of the comparative analysis displayed 44 significantly different proteins (p<0.05) from the cultures without cocoa treatment. 6 proteins were up-regulated and 38 were down-regulated in the presence of the cocoa extract after being exposed to 5 mM of H_2O_2. 20 of these 44 differentially expressed proteins were identified by MALDI-MS/MS and MASCOT database (Table 13), 6 were up-regulated and 14 were down-regulated.

Table 13. Identification of differentially expressed proteins in the cocoa extract condition *vs* the control condition without cocoa extract, after being both exposed to 5 mM H_2O_2.

Spot (H_2O_2)	Protein	Protein name	UniProt ID	Mass (Da)	[a] pI	Mascot score	[b] Matched peptides	[c] %COV	Num MSMS	[d] Fold
0905	Cdc48	Cell division control protein 48	P25694	92167	4.82	382	38	44	5	+ 5.38
1108	Tif1	ATP-dependent RNA helicase eIF4A	P10081	44840	5.02	121	12	26	2	- 2.44
1109	Imh1	Golgin IMH1	Q06704	105333	5.52	64	34	36	1	- 3.28
1222	Sec14	SEC14 cytosolic factor	P24280	35107	5.32	52	6	19	1	- 2.29
1311	Aim41	Altered inheritance of mitochondria protein 41, mitochondrial	Q12032	21215	9.6	56	1	39	1	- 2.11
2002	Bmh2	Protein BMH2	P34730	31099	4.82	136	11	36	3	- 4.76
2612	Pro2	Gamma-glutamyl phosphate reductase	P54885	49881	5.37	125	16	46	3	+ 2.91
3107	Hom6	Homoserine dehydrogenase	P31116	38478	6.86	421	19	35	5	- 4.59
3510	Eft1	Elongation factor 2	P32324	93686	5.92	130	24	25	4	- 10.91
3605	Gdh1	NADP-specific glutamate dehydrogenase 1	P07262	49881	5.56	142	17	38	2	- 7.47
4606	Spp1	COMPASS component SPP1	Q03012	42468	6.35	61	15	38	0	+ 2.48
4805	DPP3	Probable dipeptidyl peptidase 3	Q08225	80745	5.86	89	24	35	1	+ 2.87
5411	Aro8	Aromatic/aminoadipate aminotransferase 1	P53090	56371	5.68	181	9	14	2	- 4.77
6205	Prb1	Cerevisin	P09232	69807	5.94	57	8	9	1	- 16.38
6302	Pgk1	Phosphoglycerate kinase	P00560	44768	7.11	89	13	37	2	+ 3.71
6403	Pgk1	Phosphoglycerate kinase	P00560	44768	7.11	231	20	47	4	- 5.98
6801	Met6	5-methyltetrahydropteroyl triglutamate--homocysteine methyltransferase	P05694	85978	6.07	79	23	34	1	- 2.98
7704	Tkl1	Transketolase 1	P23254	73874	6.51	400	27	35	5	+ 9.83
8308	Cdc19	Pyruvate kinase 1	P00549	54909	7.56	352	23	40	5	- 15.63
8508	Tef4	Elongation factor 1-gamma 2	P36008	46605	7.63	99	10	26	1	- 8.43

pI and mass from samples not analysed by MS/MS have been compared to expected results.
[a] pI=Isoelectric point.
[b] Matched peptides=Number of peptides matched from protein in MS/MS query.
[c] Cover=percentage of amino acid sequence of protein covered in MS/MS analysis.
[d]Fold=Relative protein spot intensities of differentially expressed proteins in the cocoa extract condition versus the control condition.

The functional classification of the identified proteins (up- and down- regulated) showed 14 different functional groups (Table 14), being the cellular amino acid biosynthetic process (16.7 %), the metabolic process (27.8 %) and the translation (22.2 %) the categories covering more genes. Genes codifying for Spp1, Prb1 and Imh1 proteins were not significantly grouped in any category.

Table 14. Functional classification of the differentially expressed proteins after hydrogen peroxide treatment in the yeast culture pre-incubated with cocoa extract compared to the yeast culture non pre-incubated with it. The web-based tool FunSpec was used for the statistical evaluation ($p<0.01$) using the GO biological process clustering.

GOID	Functional category	p-value	Genes	nº genes	% genes
GO:0006414	translational elongation	0.00157	*TEF4, EFT1*	2	11.1
GO:0008652	cellular amino acid biosynthetic process	0.002201	*MET6, HOM6, PRO2*	3	16.7
GO:0006096	glycolysis	0.002544	*CDC19, PGK1*	2	11.1
GO:0006520	cellular amino acid metabolic process	0.003115	*HOM6, GDH1*	2	11.1
GO:0009086	methionine biosynthetic process	0.003115	*MET6, HOM6*	2	11.1
GO:0008152	metabolic process	0.004589	*CDC19, HOM6, PRO2 GDH1, TKL1*	5	27.8
GO:0009072	aromatic amino acid family metabolic process	0.005445	*ARO8*	1	5.5
GO:0046470	phosphatidylcholine metabolic process	0.005445	*SEC14*	1	5.5
GO:0009090	homoserine biosynthetic process	0.008157	*HOM6*	1	5.5
GO:0009097	isoleucine biosynthetic process	0.008157	*HOM6*	1	5.5
GO:0051436	negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	0.008157	*BMH2*	1	5.5
GO:0072671	mitochondria-associated protein catabolic process	0.008157	*CDC48*	1	5.5
GO:0006675	mannosyl-inositol phosphorylceramide metabolic process	0.008157	*SEC14*	1	5.5
GO:0006412	translation	0.009444	*TEF4, TIF1, EFT1, AIM41*	4	22.2

2.3.2. Study of the ability of the cocoa extract to induce a cellular antioxidant response in deletant strains for genes potentially involved in the *S. cerevisiae* response mediated by the cocoa extract.

Among the 29 identified proteins, 17 of them were selected based on their significant functional classification or because they are described as involved in the stress defense. Ssb2 (Stress-Seventy Subfamily B), a heat shock protein member of the HPS70 family, was up-regulated after the cocoa treatment but not significantly grouped in any functional category. After the cocoa treatment and oxidative stress induction, Spp1, belonging to a complex which methylates histone H3 on lysine 4 and is required in telomeric transcriptional silencing, was 2.48 times up-regulated. Prb1, a vacuolar proteinase B whose protein abundance increases in response to DNA replication stress, showed a fold change of -16.38 in the same conditions. And Imh1, a protein involved in vesicular transport, was down-regulated as well. None of them were found to belong to

any functional category although they were differentially regulated at the mentioned conditions. All these proteins are described as involved in some stress situations, being interesting for further studies. Otherwise, deletion mutants for genes *CDC19*, *PGK1*, *CDC48*, *ENO2*, *ILV5* and *SEC14* are not viable and therefore they were not available so they were excluded from the confirmation experiments.

Deletant strains of interest were then studied by using the yeast-based multiwell assay and the ability of cocoa polyphenol extract to induce antioxidant response was compared with the BY4741 strain. Growth curves were obtained from the *S. cerevisiae* wild-type strain and the 17 selected deletion mutants, after incubation with or without the cocoa extract and the presence or absence of oxidative stress induced with two H_2O_2 concentrations (0.5 and 4 mM). Growth ratios and "effect curves" were then calculated as previously reported in Material and Methods and statistical differences ($p<0.05$) were calculated with the Student's t test.

PRO2, PRB1, HOM6 and *TDH3* mutant strains of *S. cerevisiae* were significantly more sensitive ($p<0.05$) to one or both hydrogen peroxide concentrations than the wild-type (data not shown). However, the growth ratio curves of *BMH2* mutant strain, displayed a statistically significant resistance ($p<0.05$) against the induced oxidative stress compared to the wild-type (data not shown).

The results of the assays with mutants showed 12 proteins which could be potentially involved in the yeast response to oxidative stress mediated by the cocoa polyphenolic extract. Strains deleted for these 12 genes did not manifest the antioxidant protective effect ($p<0.05$) conferred by the cocoa extract observed in the wild-type, at least at one of the H_2O_2 concentrations tested.

PRO2, *GDH1*, *SSB2*, *YHB1*, *IMH1* and *ARO8* mutant strains showed no statistically significant improved growth at any of the H_2O_2 concentrations tested, after being incubated in the presence of the cocoa extract (Fig. 22). Although *SSB2* and *IMH1* mutant strains showed a very similar behavior compared to the wild-type strain, the antioxidant effect of the cocoa extract was not statistically significant ($p<0.05$), according to the Student's t test, due to the variability found in the experiments.

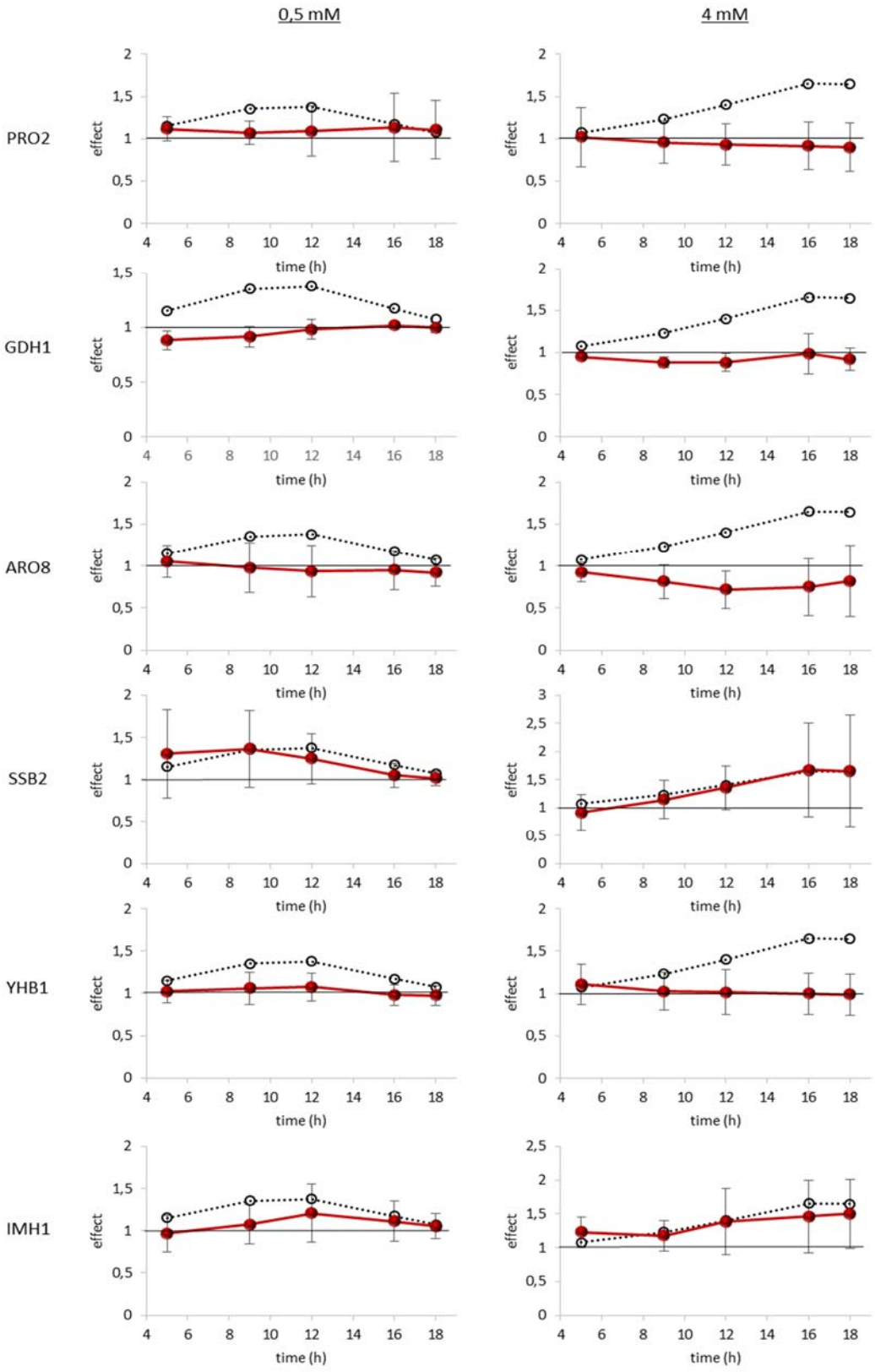


Figure 22. 'Effect curves' of *PRO2, GDH1, SSB2, YHB1, IMH1* and *ARO8* mutant strains after treatment with the cocoa polyphenolic extract and oxidative stress assessed with 0.5 mM and 4 mM H_2O_2. Mean of at least three independent assays is represented. Error bars indicate the standard deviation among trials. Red lines represent the mutant strains effect curves and dashed lines represent the wild-type strain effect curve.

In *PRB1*, *HOM6*, *BMH2* and *ILV1* mutants strains the cocoa extract displayed an antioxidant response only at 0.5 mM H_2O_2, but not at 4 mM H_2O_2 (Fig. 23).

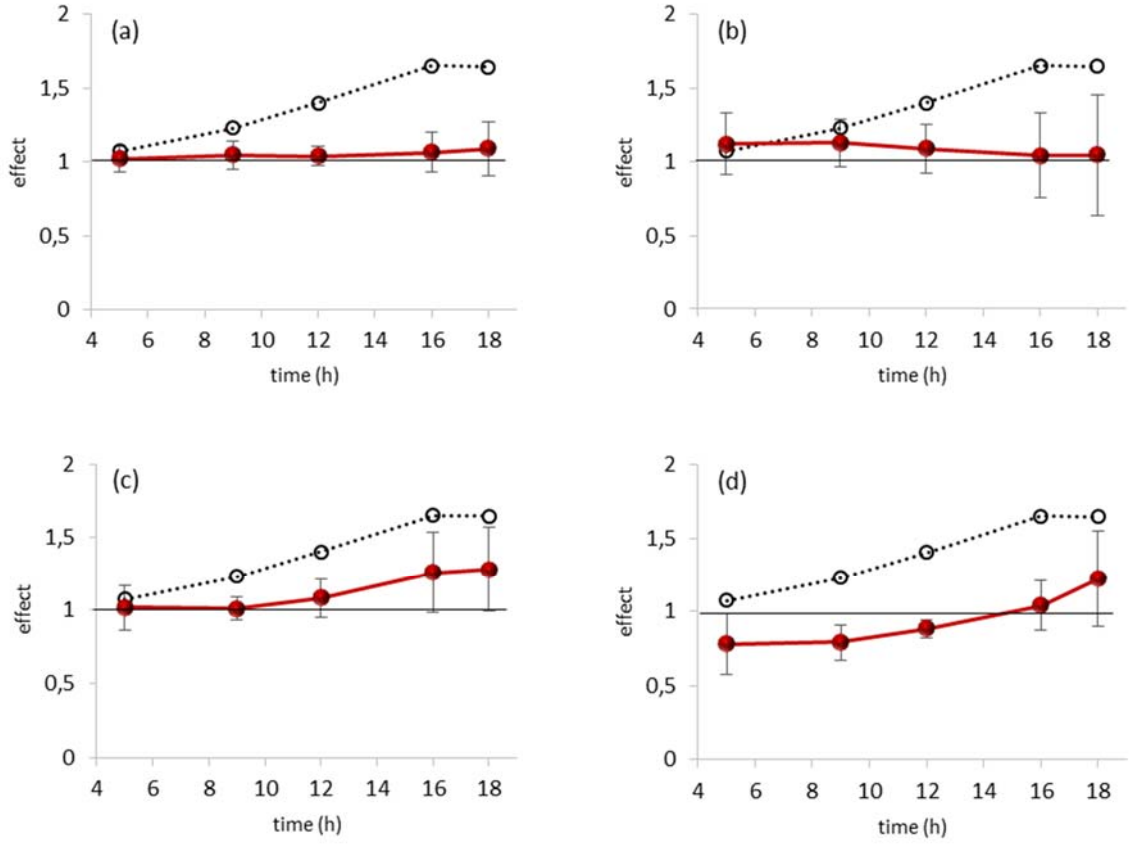


Figure 23. 'Effect curves' of *PRB1* (a), *HOM6* (b), *BMH2* (c) and *ILV1* (d) mutant strains after treatment with the cocoa polyphenolic extract and oxidative stress assessed with 4 mM H_2O_2. Red lines indicate the mutant strains effect curves and dashed lines represent the wild-type strain effect curve at 4 mM H_2O_2. Mean of at least three independent assays is represented. Error bars indicate the standard deviation among trials.

Otherwise, the cocoa extract induced an antioxidant protection against oxidative stress only at 4 mM H_2O_2 in *MET6* and *CIT1* mutants, not being observed at 0.5 mM H_2O_2 (Fig. 24).

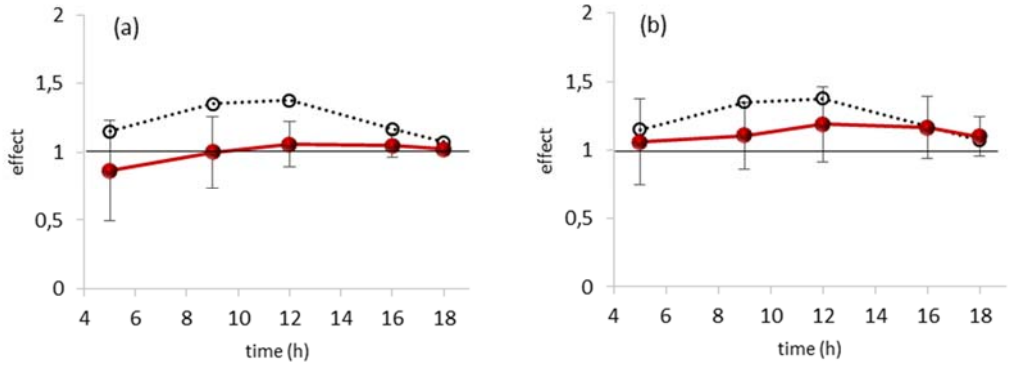


Figure 24. 'Effect curves' of *MET6* (a) and *CIT1* (b) mutant strains after treatment with the cocoa polyphenolic extract and oxidative stress assessed with 0.5 mM H_2O_2. Dashed lines represent the wild-type strain effect curve at 0.5 mM H_2O_2. Mean of at least three independent assays is represented. Error bars indicate the standard deviation among trials.

In five deleted mutant strains (*TDH3*, *TRP5*, *TKL1*, *DPP3* and *SPP1*) the antioxidant protective effect of the cocoa extract ($p<0.05$) was observed at both H_2O_2 concentrations and the phenotype was similar to that found in the wild-type (data not shown). The deletion of these genes does not seem to affect the antioxidant protection phenotype mediated by the cocoa extract. Therefore, more accurate expression studies would be necessary to elucidate their possible involvement in the oxidative stress response of *S. cerevisiae* mediated by cocoa polyphenols.

2.4. Transcriptomic study for the identification of potentially involved genes in the response of *S. cerevisiae* to oxidative stress mediated by cocoa polyphenols

Microarrays experiments were carried out with RNA extracted from yeast cultures incubated in presence and absence of the cocoa extract (350 mg EE/L) and exposed to oxidative stress with hydrogen peroxide 1 mM for 15 and 45 min. Gene expression was then determined in order to get information about the genes modulated by cocoa polyphenols involved in the yeast's oxidative stress resistance. Given the great amount of genes that increased or decreased mRNA expression, only genes showing at least 2-

fold change in expression and an adjusted p-value<0.0005 were taken into account for further analysis. Considering these conditions, a total of 272 differentially regulated genes were found, 121 genes after 15 min of H_2O_2 incubation and 151 after 45 min.

GenRE software was used in order to classify the major biological processes associated to those differentially regulated genes found in the transcriptomic study. Up and down-regulated genes were separately analyzed and classified into functional categories (p<0.05). Complete data are collected in Annex 1.

2.4.1. Genes induced by the cocoa extract

99 genes were up-regulated after 15 min of H_2O_2 incubation and 115 genes after 45 min of treatment. Interestingly, the functional category of cell rescue, defense and virulence was overrepresented at both time points. 16.3 % of the up-regulated genes after 15 min and 17.2 % after 45 min were significantly classified into this group, showing several subcategories such as stress response (oxidative stress, pH stress and heat shock response) as well as detoxification (oxygen and radical detoxification and more specific catalase reaction). Table 15 shows this concrete classification. Almost all the genes included in cell rescue, defense and virulence category were found after 15 and 45 min of stress, except for *PRX1*, *SED1*, *FMP46*, *MSN2*, *SIS1* (found only at 45 min) and *PAI3* (found at 15 min).

In this context, several free radical scavenging enzymes, which represent the first line of cell protection against oxidative damage, were induced by cocoa extract: *CTA1* (codifies the catalase A), *TSA2* (thioredoxin peroxidase), *PRX1* (peroxiredoxin), *CCP1* (cytochrome *c* peroxidase) and *SRX1* (sulfiredoxin). Other stress response genes were up-regulated as well, such as *GRE1* and *GRE2*, *FMP46* (a putative redox protein containing a thioredoxin fold), the heat shock protein *HSP31*, and the stress-responsive transcriptional activator *MSN2*, among others. Moreover, protein degradation genes were also included in the cell defense category, e.g. *MRK1*, which functions not only to activate Msn2p-dependent transcription of stress responsive genes but also in protein degradation and *SIS1* (Type II HSP40 co-chaperone), which is involved in proteasomal degradation of misfolded cytosolic proteins. Finally, it is worth mentioning the presence

of the transcriptional repressor *NRG1* and of *SED1* (which has a possible role in mitochondrial genome maintenance) among the induced genes.

Table 15. Up-regulated genes classified into the overrepresented functional category of cell rescue, defense and virulence, after 15 and/or 45 min of hydrogen peroxide treatment.

Systematic name	Standard name	Time point	Functional category
YBL064C	*PRX1*	45	32.01 stress response 32.01.01 oxidative stress response 32.07 detoxification 32.07.07 oxygen and radical detoxification
YDL079C	*MRK1*	15, 45	32.01 stress response
YDR043C	*NRG1*	15, 45	32.01 stress response 32.01.04 pH stress response
YDR077W	*SED1*	45	32.01 stress response
YDR256C	*CTA1*	15, 45	32.01 stress response 32.01.01 oxidative stress response 32.07 detoxification 32.07.07 oxygen and radical detoxification 32.07.07.01 catalase reaction
YDR453C	*TSA2*	15, 45	32.01 stress response 32.01.01 oxidative stress response 32.07 detoxification 32.07.07 oxygen and radical detoxification
YDR533C	*HSP31*	15, 45	32.01 stress response
YER095W	*RAD51*	15, 45	32.01 stress response
YKL086W	*SRX1*	15, 45	32.01 stress response 32.01.01 oxidative stress response
YKR049C	*FMP46*	45	32.01 stress response 32.01.01 oxidative stress response
YKR066C	*CCP1*	15, 45	32.01 stress response 32.01.01 oxidative stress response 32.07 detoxification 32.07.07 oxygen and radical detoxification
YLR350W	*ORM2*	15, 45	32.01 stress response
YMR037C	*MSN2*	45	32.01 stress response
YMR174C	*PAI3*	15	32.01 stress response
YNL007C	*SIS1*	45	32.01 stress response
YNL042W	*BOP3*	15, 45	
YOL151W	*GRE2*	15, 45	32.01 stress response 32.01.01 oxidative stress response 32.01.04 pH stress response 32.01.05 heat shock response 32.07 detoxification
YOR273C	*TPO4*	15, 45	32.07 detoxification
YPL004C	*LSP1*	15, 45	32.01 stress response 32.01.05 heat shock response
YPL223C	*GRE1*	15, 45	32.01 stress response 32.01.01 oxidative stress response 32.01.04 pH stress response 32.01.05 heat shock response

The energy category was overrepresented at both time points as well, being the functional sub-categories where genes were significantly grouped glycolysis, methylglyoxal bypass, glyoxylate cycle, alcohol fermentation and oxidation of fatty acids.

2.4.2. Genes repressed by the cocoa extract

A smaller number of repressed genes were found after the cocoa treatment and stress induction compared to the induced group. 22 genes were down-regulated after 15 min of H_2O_2 incubation and 36 genes after 45 min of treatment. Some similarities were found among the down-regulated genes, after both stress incubation times. In broad terms, energy and energy-related categories were statistically significant ($p<0.05$) after the GenRe software analysis. However, unlike the induced groups, the sub-categories were electron transport and membrane-associated energy conservation, aerobic respiration and energy generation, among others. Protein synthesis was only found to be significant among the repressed genes after 45 min of H_2O_2 incubation, being the significant categories ribosome biogenesis and the sub-group ribosomal proteins. None of the stress response sub-categories (belonging to cell rescue, defense and virulence) were found among the down-regulated genes. However, homeostasis categories were overrepresented. All data are collected in Annex 1.

2.4.3. Validation of microarray analysis

To carry out the validation of the microarrays results, 7 of the most significantly up- and down-regulated genes were chosen and its expression was determined by RT-qPCR. After the primers design, efficiency curves for the selected genes and the housekeepings (*ACT1*, *TAF10*, *UBC6*) were established (Table 16).

Table 16. Genes used for the microarray validation and efficiency and error values calculated from the calibration curves prepared from cDNA serial dilutions.

GENES	EFFICIENCY	ERROR
GRE2	1.899	0.0125
YJR096W	1.861	0.0082
HSP31	1.905	0.0268
REX4	1.962	0.0191
RPS17B	1.912	0.0246
CCP1	1.838	0.0299
DOT1	1.942	0.0121
ACT1	1.994	0.0201
TAF10	1.944	0.0155
UBC6	2.010	0.0248

RT-qPCR of the 7 selected genes at different time points was subsequently performed (Fig. 25): *GRE2* and YJR096W at 15 min, *HSP31*, *REX4*, *RPS17B* and *DOT1* at 45 min and *CPP1* at both time points. Results showed that both approaches (microarrays and RT-qPCR) were in agreement, despite the differences in quantification due to distinct detection sensitivities of the employed techniques.

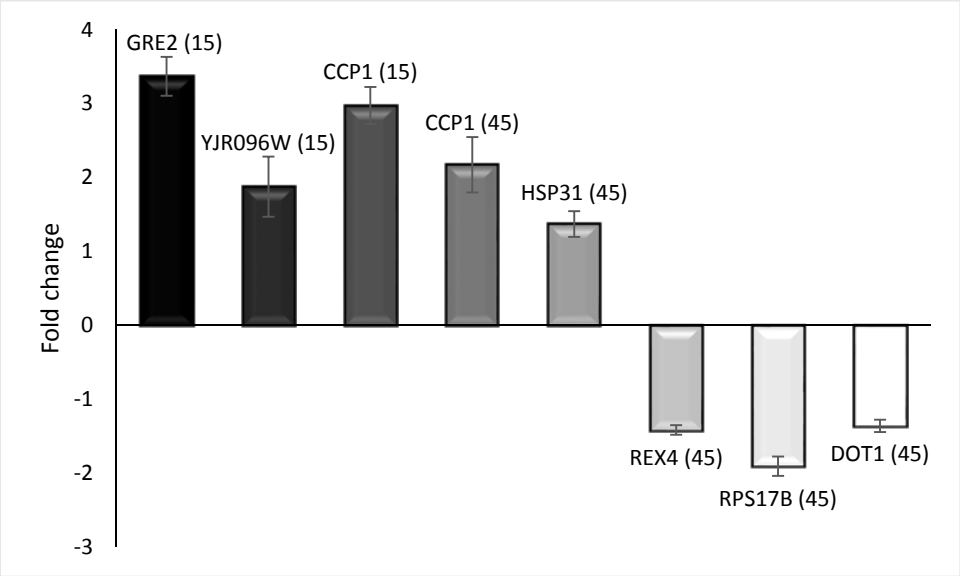


Figure 25. RT-qPCR validation of microarrays results. The fold changes of the 7 selected genes are shown (time point in brackets). Error bars indicate the standard error among replicates. *ACT1*, *TAF10* and *UBC6* were used as internal standards.

3. USE OF *C. elegans* AS A MODEL TO STUDY THE ANTIOXIDANT CAPACITY OF COCOA POLYPHENOLS

3.1. Protective effects of the cocoa extract and fractions against oxidative stress with hydrogen peroxide.

After incubation of age-synchronized worms for 4 days and induction of oxidative stress with 2 mM H_2O_2, the viability of nematodes was measured. The first results (LSD test $p<0.05$) showed that worms fed with the cocoa extract, as well as with fractions 2, 5, 10, 14, 16 and 19 had better resistance to oxidative stress than the control without ingredient (data not shown). F24 was the only fraction that did not show statistically significant antioxidant protection in the worm. Given the few differences found in this experiment, more strict parameters were used in the LSD test. With a p value of 0.001, only fractions rich in theobromine (F2), catechin (F10), procyanidin B2 (F14) and epicatechin (F19) as well as the complete cocoa extract were significantly protective compared to the control (Fig. 26). F10 was the most active fraction, showing a survival rate of 75.7 ± 3.3 % compared to the 50.5 ± 8.9 % survival rate of the control worms, followed by F19 (71.6 ± 3.3 %), F14 (67.7 ± 6.1 %), cocoa extract (66.7 ± 5.5 %) and F2 (65.7 ± 5.0 %). F5, F16 (rich in caffeine) and F24 did not improve the worm's resistance to oxidative stress.

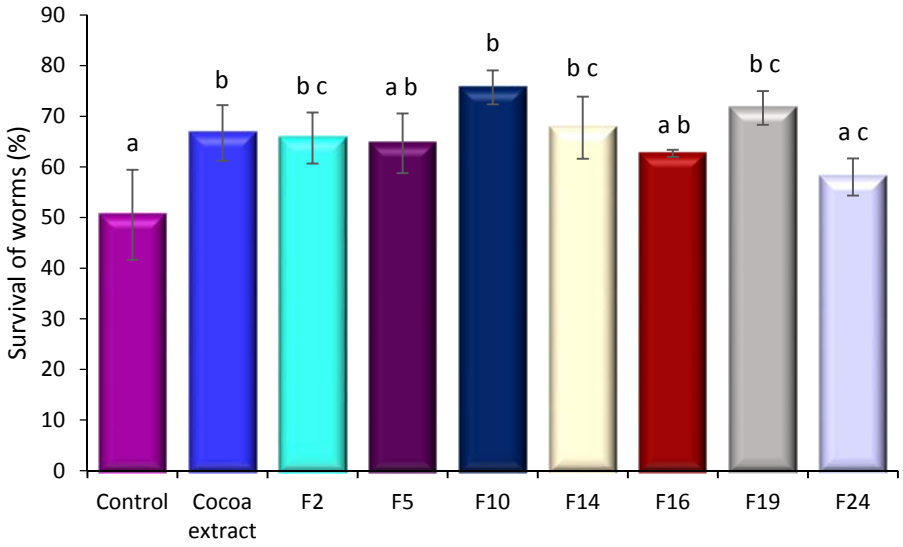


Figure 26. Survival of *C. elegans* N2 (wild-type) treated with 2 mM H_2O_2 on NGM plates, with or without the cocoa extract or fractions supplementation. Error bars indicate standard deviation among three trials (at least 300 worms scored per condition). The letters a, b and c indicate significant differences between conditions by one-way ANOVA with LSD post hoc test ($p<0.001$).

Although statistically significant differences were observed compared to the control, all fractions showed a very similar behavior, being this results not really conclusive. Further studies were, then, performed in order to better understand the antioxidant capacity of fractions in *C. elegans*.

3.2. Effect on ROS accumulation

ROS accumulation was studied by the DCF assay after treatment of worms with the global cocoa extract as well as the selected fractions F16 and F19, which showed higher *in vitro* antioxidant activity compared to the pure compounds, and incubation at 37 ºC.

Regarding the cocoa extract, two standardised concentrations were assayed: 100 µM of caffeine and 100 µM of epicatechin. Both of them conferred a significant reduction of ROS formation in heat stressed worms (Fig. 27) compared to the control. The highest effect was found in the extract adjusted to caffeine, because the standardisation of the cocoa extract to caffeine resulted in a more concentrated extract compared to the standardisation of the cocoa extract to epicatechin, due to the lower amount of caffeine in the complete extract.

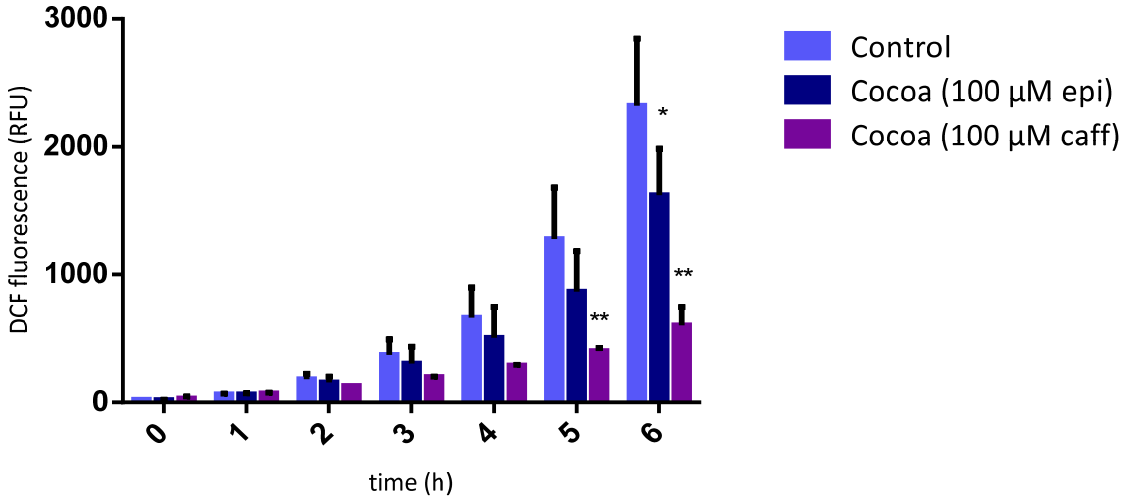


Figure 27. Influence of the cocoa extract on ROS accumulation in heat stressed *C. elegans*. ROS accumulation was measured by DCF fluorescence. Results are the mean of at least three experiments. Error bars indicate standard deviation among trials. Statistical significance was calculated with one-way ANOVA with Dunnett's post hoc test. *p<0.05; **p<0.01 *vs* corresponding control value.

On the other hand, the epicatechin-rich fraction (F19) as well as the pure compound epicatechin caused no significant reduction of the DCF fluorescence, although a slight trend is observed at the latest time points (Fig. 28).

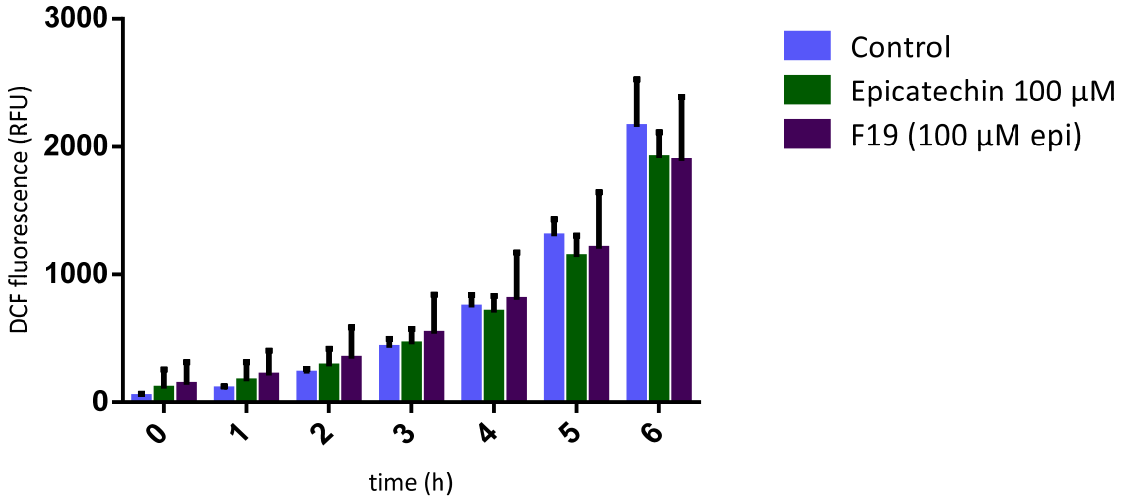


Figure 28. Effect of epicatechin and epicatechin-rich fraction (F19) on ROS accumulation in *C. elegans.* ROS accumulation was measured by an increase in DCF fluorescence. Results are the mean of at least three experiments. Error bars indicate standard deviation among trials. Statistical significance was calculate with one-way ANOVA with Dunnett's post hoc test.

Finally, when the caffeine-rich fraction (F16) and the pure compound caffeine were assayed, no reduction of ROS accumulation was detectable in the worm (Fig. 29).

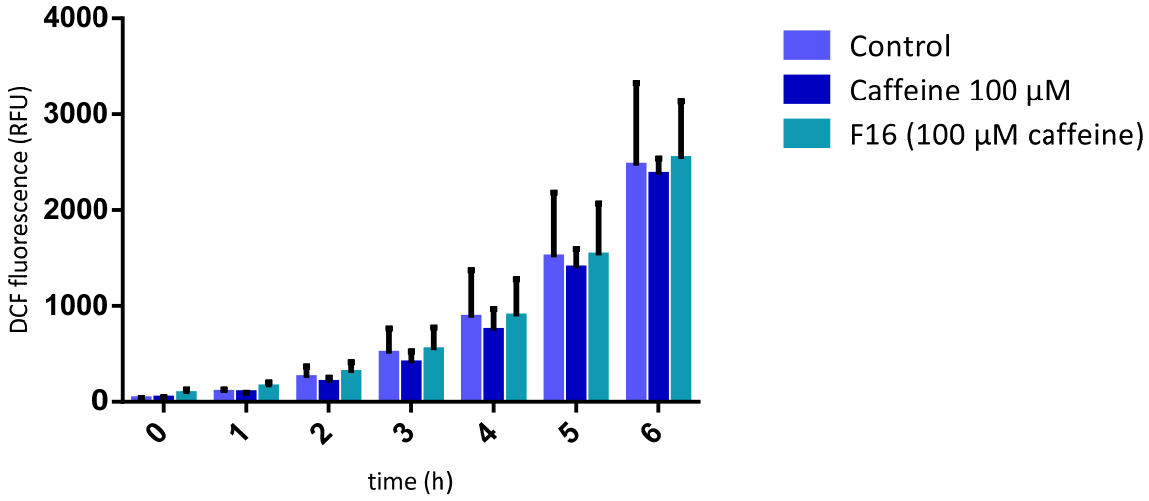


Figure 29. Effect of caffeine and caffeine-rich fraction (F16) on ROS accumulation in *C. elegans.* ROS accumulation was measured by an increase in DCF fluorescence. Results are the mean of at least three experiments. Error bars indicate standard deviation among trials. Statistical significance was calculate with one-way ANOVA with Dunnett's post hoc test.

3.3. Thermal-induced stress protection

Modulation of thermal stress resistance in nematodes was analyzed by the SYTOX® assay. The pre-incubation with the cocoa extract strongly protected *C. elegans* against lethal thermal stress (Fig. 30). The mean life span was 4.479 ± 0.165 h for the control nematodes and 6.052 ± 0.196 h for nematodes treated with the cocoa extract adjusted to 100 µM caffeine, which was more protective than the cocoa extract adjusted to the epicatechin concentration.

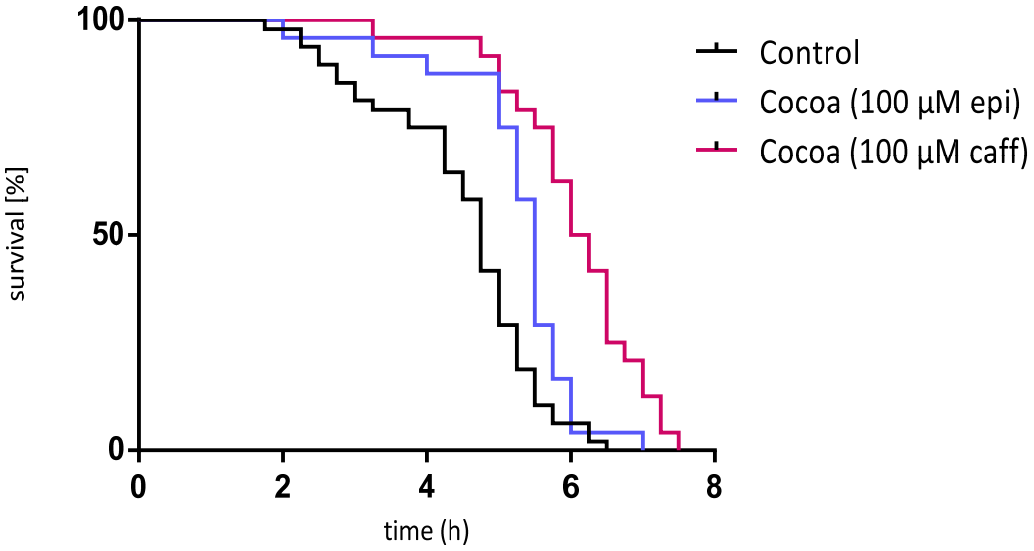


Figure 30.Protective effects of the cocoa extract against thermal-induced stress. The graph shows the percentage of viable nematodes when pre-incubated with the cocoa extract adjusted to 100 µM epicatechin and 100 µM caffeine. Results were evaluated with the Kaplan Meier survival analysis with Log Rank test (Mantel-Cox). The mean of three independent experiments is shown. Corresponding data are summarized in Annex 1.

When the pre-incubation was performed with the epicatechin-rich fraction F19 adjusted to 100 µM epicatechin, a feeble, but non-significant protective effect was found, while in the case of 100 µM epicatechin pure compound, a slight significant protection was detected (Fig. 31).

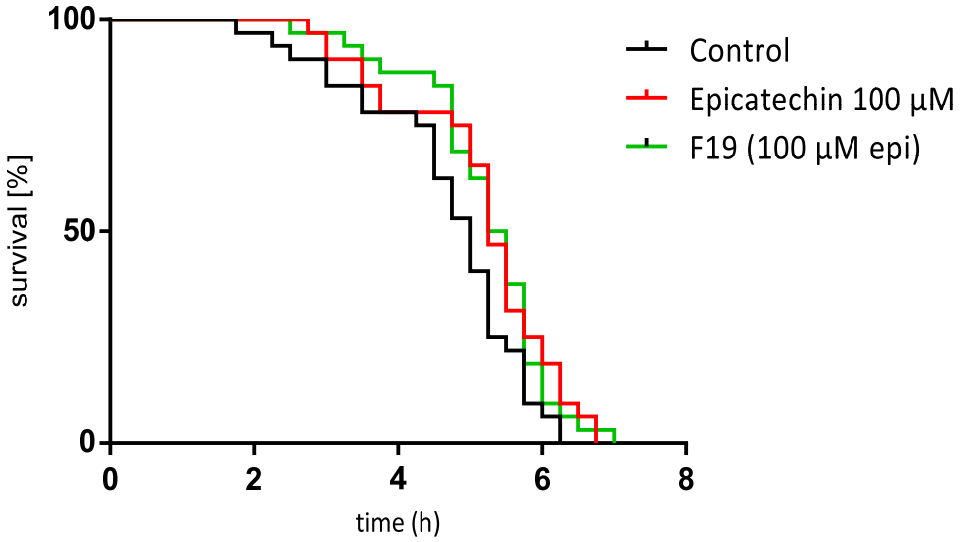


Figure 31. Protection against thermal stress by epicatechin and epicatechin-rich fraction (F19) adjusted to 100 µM epicatechin. The graph shows the percentage of viable nematodes. Results were evaluated with the Kaplan Meier survival analysis with Log Rank test (Mantel-Cox). The mean of at least three independent experiments is represented. Corresponding data are summarized in Annex 1.

In the case of the caffeine-rich fraction F16 adjusted to 100 µM caffeine as well as in the case of 100 µM caffeine (pure compound), no protection was observed (Fig. 32).

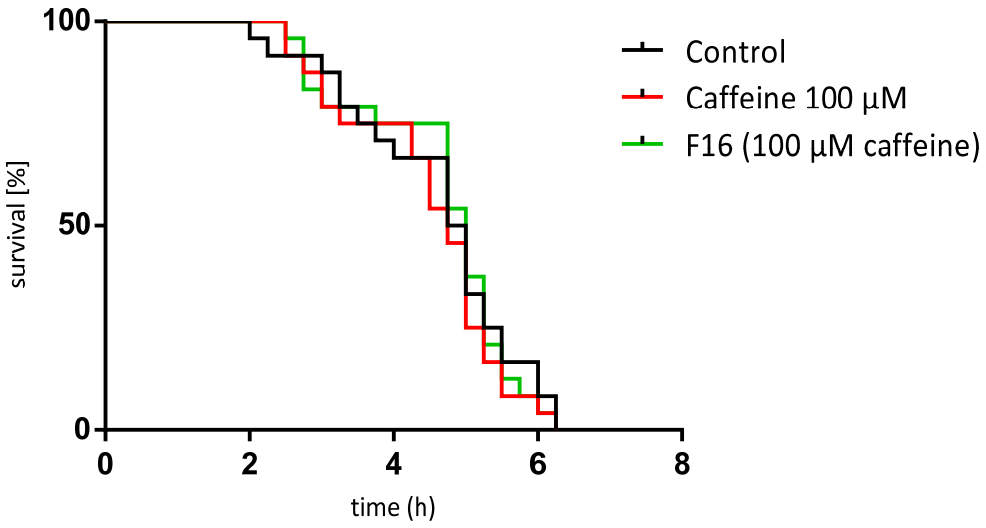


Figure 32. Protection against thermal stress by caffeine and caffeine-rich fraction (F16) adjusted to 100 µM caffeine. The graph shows the percentage of viable nematodes. Results were evaluated with the Kaplan Meier survival analysis with Log Rank test (Mantel-Cox). The mean of three independent experiments is shown. Corresponding data are summarized in Annex 1.

3.4. Protective effects against Na-arsenite induced oxidative stress

Modulation by cocoa polyphenols of *C. elegans* resistance to lethal oxidative stress by sodium-arsenite was analyzed as well. The pre-incubation with the cocoa extract clearly protected the worm against this oxidative stress (Fig. 33). The amount of viable nematodes after 51 h was 61 ± 6.7 % in the control. In contrast, the group of nematodes incubated with the cocoa extract adjusted to 100 µM caffeine showed a viability of 94 ± 6.9 %.

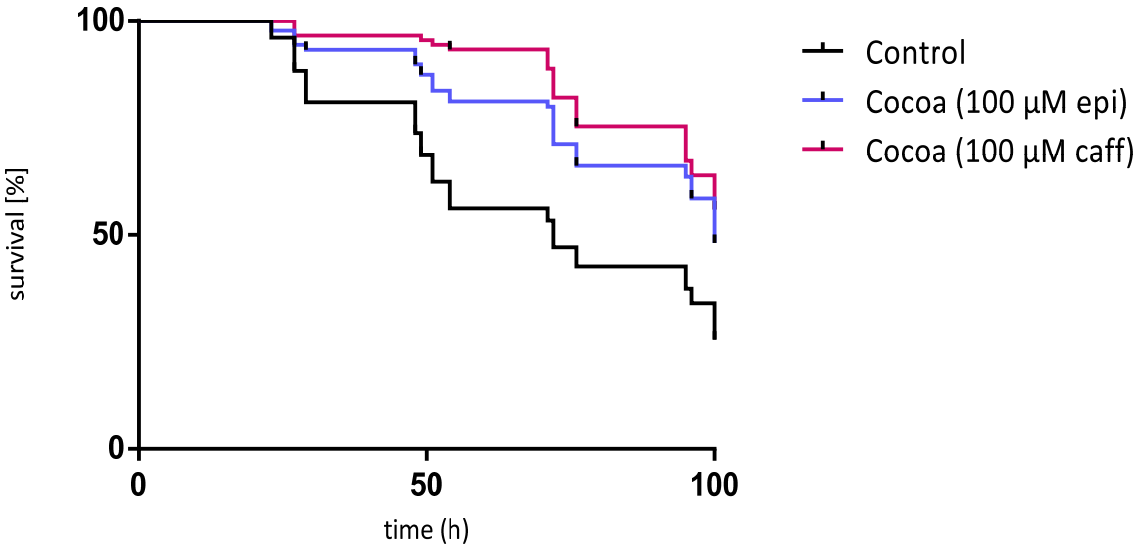


Figure 33. Protective effects of the cocoa extract adjusted to 100 µM epicatechin and 100 µM caffeine against Na-arsenite oxidative stress. The percentage of viable nematodes is shown. Results were evaluated with the Kaplan Meier survival analysis with Log Rank test (Mantel-Cox). The mean of three independent trials is shown.

When the pre-incubation was performed with the epicatechin-rich fraction F19 adjusted to 100 µM epicatechin or the pure compound epicatechin (100 µM), no significant protective effect was found (Fig. 34).

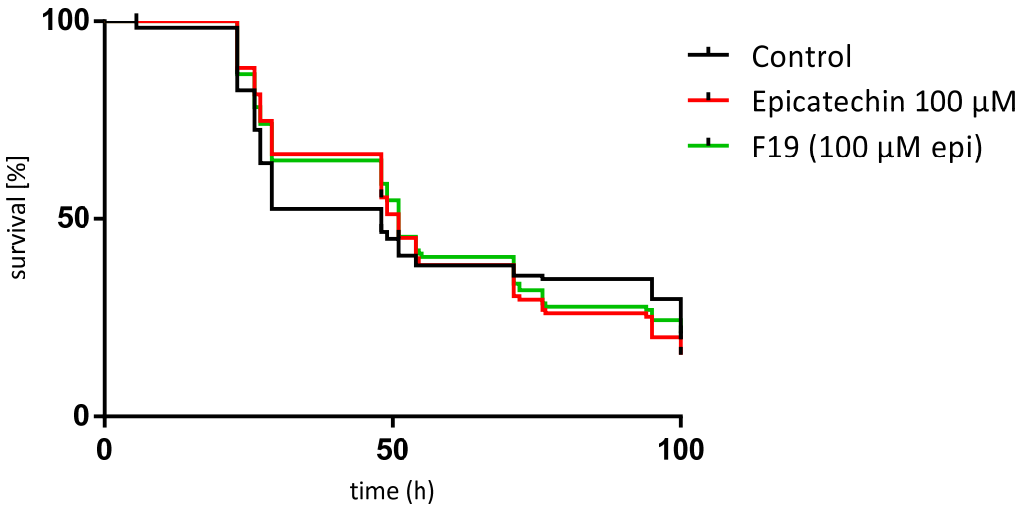


Figure 34. Protective effects of epicatechin (100 µM) and epicatechin-rich fraction (F19) adjusted to 100 µM epicatechin against Na-arsenite oxidative stress. The percentage of viable nematodes is shown. Results were evaluated with the Kaplan Meier survival analysis with Log Rank test (Mantel-Cox). The mean of at least three independent trials is shown.

The caffeine-rich fraction F16 adjusted to 100 µM caffeine did not protect the worm from the Na- arsenite stress. Moreover, 100 µM caffeine (pure compound) significantly decreased the nematodes resistance to such stress, being the median survival rate 54 instead of 77 (Fig. 35), showing a toxic effect.

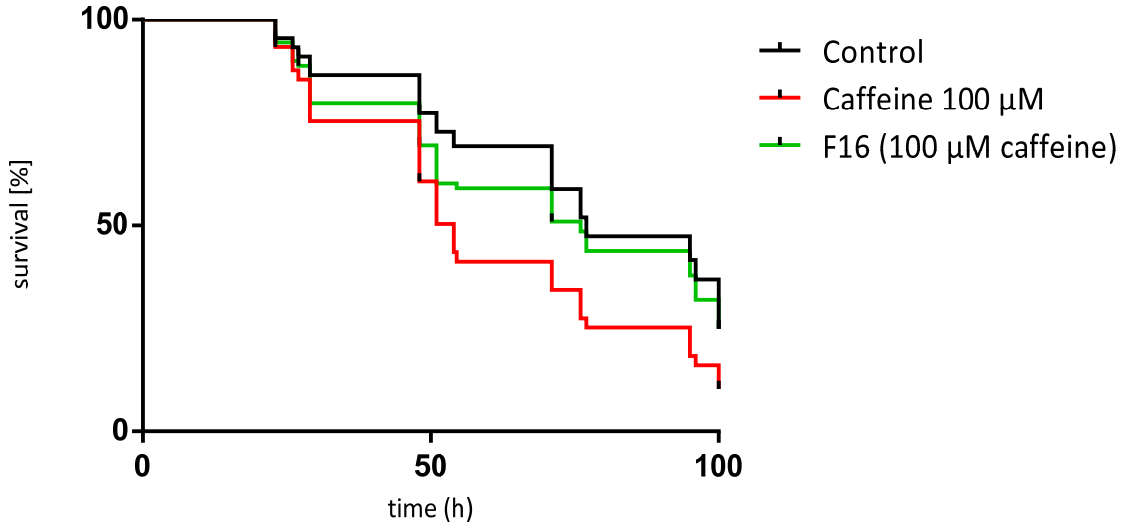


Figure 35. Protective effects of caffeine (100 µM) and caffeine-containing fraction (F16) adjusted to 100 µM caffeine against Na-arsenite oxidative stress. The percentage of viable nematodes is shown. Results were evaluated with the Kaplan Meier survival analysis with Log Rank test (Mantel-Cox). The mean of three independent trials is shown. Corresponding data are summarized in Annexes.

3.4.1. Locomotion

After 72 h of Na-arsenite stress, worms treated with the cocoa extract were classified into three groups depending on their locomotion capacity (Fig. 36): A) worms moving loosely in the plates, B) motionless worms which started moving lively after touching them and C) motionless worms which barely moved after touching them.

Control worms were mainly classified in group C, since most of them barely moved after touching them. Worms treated with the cocoa extract at both concentrations (100 µM of epicatechin and 100 µM of caffeine) showed a very similar behavior, being most of them classified in group A, since they were clearly fit compared to the control worms. In conclusion, the cocoa extract improved the locomotion of Na-arsenite treated worms, after 72 h of incubation.

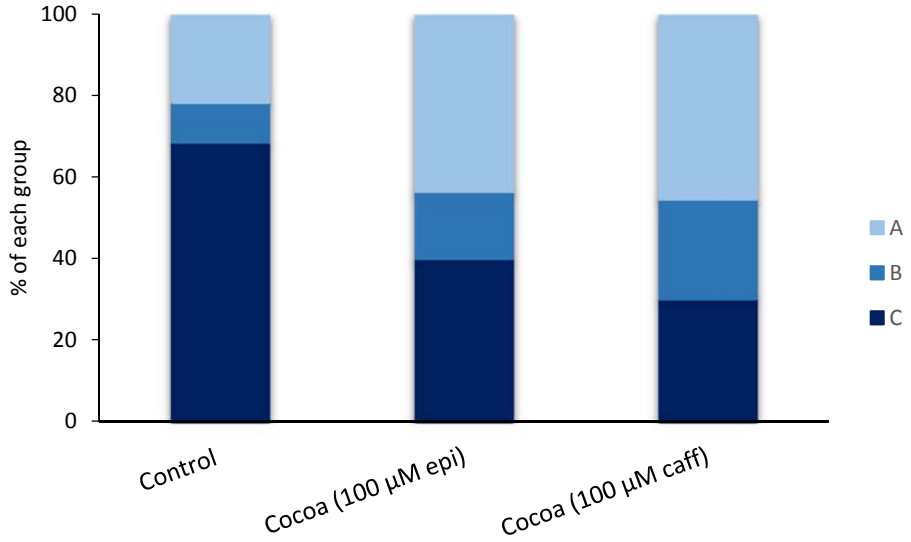


Figure 36. Locomotion capacity of cocoa-treated and Na-arsenite stressed worms. A) nematodes moving loosely in the plates, B) motionless worms which started moving lively after touching them and C) motionless worms which barely moved after touching them.

3.5. Involvement of DAF-16

To evaluate the molecular mechanisms which might be responsible for the increased stress resistance caused by cocoa polyphenols, we analyzed the effect on the FOXO-homologue transcription factor DAF-16. Cocoa extract (adjusted to 100 µM epicatechin), but not F19 adjusted to 100 µM epicatechin or 100 µM epicatechin as a pure compound increased the nuclear localization of DAF-16 (Fig. 37.A). The nuclear localization of DAF-16-GFP is visible by distinct fluorescent dots in the nematode (upper image) in contrast to the diffuse fluorescence of DMSO-treated nematodes (lower image). Using a DAF-16 loss-of-function strain (CF1038; *[daf-16(mu86) I.]*) no reduction of DCF fluorescence was found (Fig. 37.B) and no protection against thermal stress (SYTOX®) was detected (Fig. 37.C) after the treatment of worms with the cocoa extract (100 µM epicatechin), F19 (100 µM epicatechin) and epicatechin pure compound (100 µM).

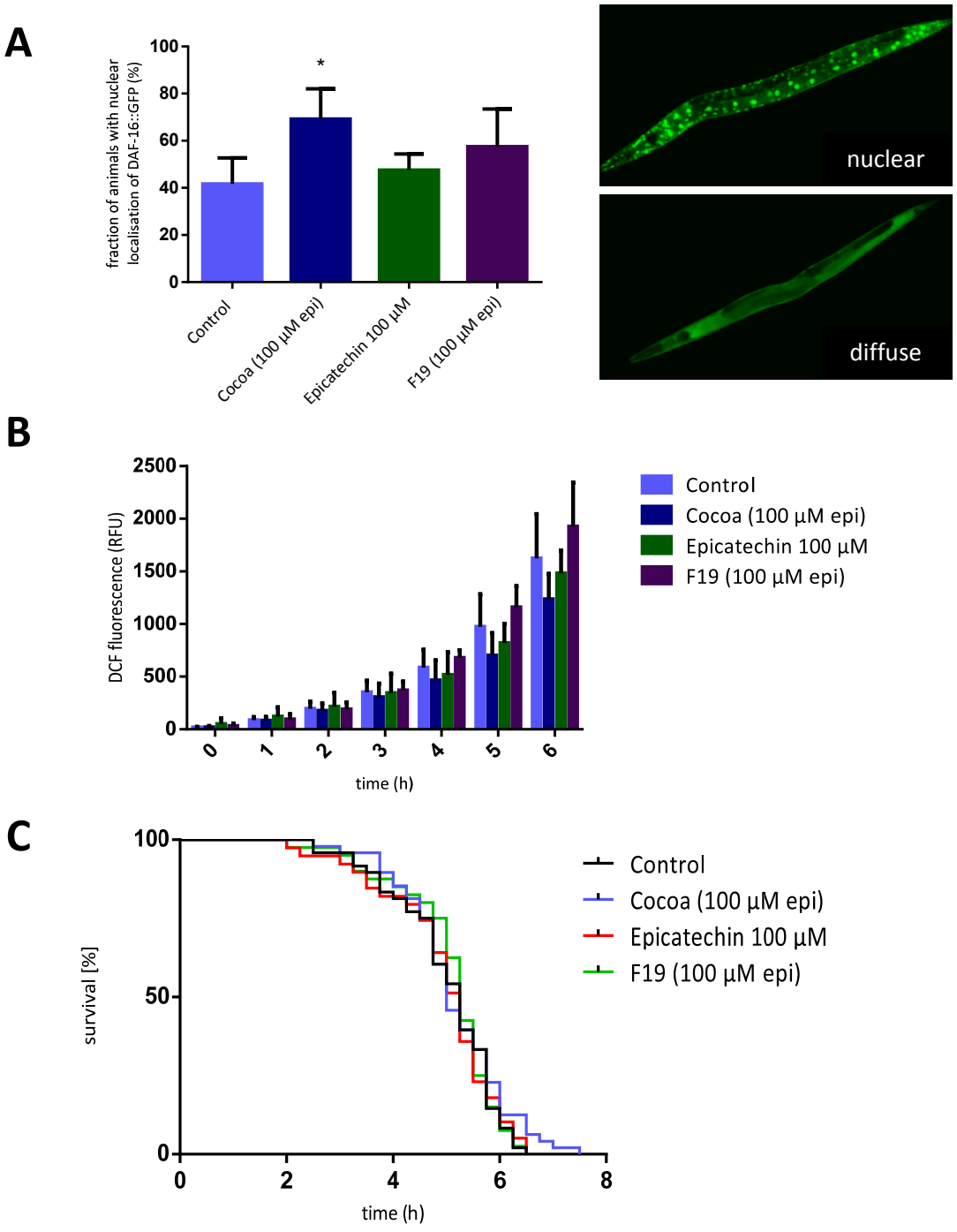


Figure 37. Effect of the cocoa extract adjusted to 100 µM epicatechin, epicatechin 100 µM and epicatechin-containing fraction (F19) adjusted to 100 µM epicatechin on DAF-16. **A)** Localization of the transcription factor DAF-16 on transgenic nematodes (DAF-16-GFP, strain TJ356). The graph shows the fraction of worms having nuclear localization of DAF-16 after treatment with cocoa extract, epicatechin and F19. The mean of 4 experiment and the standard deviation are represented, * $p<0.05$ *vs* corresponding control value; one-way ANOVA with Dunnett´s post hoc test. **B)** ROS accumulation on *daf-16(mu86)* nematodes after treatment with the cocoa extract, epicatechin and F19. Mean and standard deviation of 3 experiments are shown; one way ANOVA with Dunnett´s post hoc test. **C)** Effects of the cocoa extract, epicatechin and epicatechin-containing fraction (F19) on thermal stress in *daf-16(mu86)* nematodes. The graph shows the percentage of viable nematodes. Three experiments and Kaplan Meier survival analysis with Log Rank test (Mantel-Cox) were performed. Corresponding data are summarized in Annex 1.

DISCUSSION

1. COCOA EXTRACT FRACTIONATION AND CHARACTERIZATION

In recent years, cocoa has attracted a great deal of attention due to its potentially beneficial effects on human health, mainly attributed to its high polyphenol content. A growing body of evidence supports these effects on cardiovascular risk through modulation of the endothelial function, inflammation, platelet function, angiotensin-converting enzyme activity and glucose transport. However, the relative impact of these mechanisms remains unclear.

Most of studies are carried out with whole cocoa matrices, which are a complex mixture of polyphenols and other compounds such as methylxantines, leading to a lack of information about the individual compounds responsible for such beneficial properties. In the present work, a cocoa extract was fractionated in order to isolate more purified fractions. A total of 26 fractions were obtained by semi-preparative HPLC and characterized by HPLC-MS/MS. Among them, 7 were selected for further studies on the basis of their antioxidant capacity measured on *S. cerevisiae*. A similar approach was carried out by Cádiz-Gurrea *et al*. (2014), who obtained and characterized a total of 12 cocoa fractions. In analogy to their work, our fractions still turned out to be a mixture of different compounds, procyanidins being widely distributed in most of them.

Different approaches have been commonly employed to determine the antioxidant properties of polyphenols. Regarding the *in vitro* activity, radical-scavenging assays are quite common, such as DPPH or ABTS assays (Oboh *et al*., 2014; Schinella *et al*., 2010). In the present work, the radical-scavenging properties of the cocoa extract and fractions were determined by both tests. The 7 selected fractions were firstly analyzed by the DPPH assay, as well as the cocoa extract and the pure compounds. Afterwards, it was decided to investigate the epicatechin-fraction (F19) and the caffeine-fraction (F16) with the ABTS assay, since both fractions represent important cocoa ingredients which may contribute to the beneficial effects of cocoa. These two fractions were chosen, since the purine alkaloid caffeine possesses no direct antioxidant action compared to the high antioxidant capacity observed in the case of epicatechin. This facilitates a better discrimination between direct antioxidant effects and more specific effects of cocoa ingredients.

We were able to show that the cocoa extract exerts a prominent antioxidant activity in the *in vitro* ABTS and DPPH assays. These results are in line with findings of Oboh *et al*. (2014) who showed the strong radical-scavenging potential of a cocoa extract (DPPH, TEAC-assays).

Baba *et al*. (2000) showed that the antioxidant activity of cocoa powder (protection against oxidation of plasma components in rats) correlated with the plasma levels of (-)-epicatechin (EC) and its metabolites. The epicatechin-containing fraction (F19) turned out to be the most active among the fractions in the DPPH assay, which could be explained by the high amount of epicatechin in the fraction (4.21 g/L). Epicatechin was previously reported as a potent antioxidant *in vitro* (Ruijters, Weseler, Kicken, Haenen & Bast, 2013). In the ABTS test, the antioxidant capacity of F19 (adjusted to epicatechin) was less potent than the cocoa extract but more potent than the reference compound trolox. Since the pure compound epicatechin (100 µM) caused a similar radical-scavenging effect, it has to be suggested that the main radical-scavenging activity of the epicatechin-containing fraction was mediated by this flavonoid.

The procyanidin B2-containing fraction (F14) also showed a strong radical-scavenging effect compared to the other fractions. The antioxidant protective properties of procyanidin B2 were previously demonstrated on Caco2 cells (Rodríguez-Ramiro, Martín, Ramos, Bravo & Goya, 2011). On the contrary, F10, rich in catechin, showed a significantly low antioxidant capacity compared to the aforementioned two flavonol-rich fractions, which could be due to the small concentration of catechin (0.92 g/L) or other antiradical minor compounds present in this sample.

The caffeine-fraction may exert beneficial effects, too. Caffeine and theobromine are the most abundant purine alkaloids (methylxanthines) in cocoa. The physiological effects of methylxanthines are mainly mediated by modulation of adenosine receptors. Chocolate has some psycho-stimulant effects due to the psycho-stimulatory action of caffeine. Epidemiological evidence suggests that theobromine and caffeine improve lung function and produce bronchodilation in asthma patients. However, the low antioxidant capacity of these alkaloids was previously reported by Maleyki & Ismail (2010) and Cádiz-Gurrea *et al*. (2014). Although, caffeine and theobromine as pure compounds caused no radical scavenging effects in our assays, the caffeine-containing

fraction and the theobromine-containing fraction showed a weak antioxidant effect which is mediated by minor phytochemicals in this fraction. According to the HPLC-MS/MS characterization, both fractions contain procyanidins which could confer them the antioxidant capacity found *in vitro*.

With respect to the fractions with no major compound, F5 showed a very feeble radical-scavenging activity. Although this fraction contains caffeic acid and chlorogenic acid, which were reported as antioxidants *in vitro* (Gülçin, 2006; Sato *et al*., 2011) and the well-established procyanidin dimer, its weak activity could be due to a small amount of these compounds in the fraction. F24 had an antioxidant activity equivalent to 0.84 ± 0.17 g/L of epicatechin, being significantly lower than the most potent fractions (F14 and F19) which could be explained by the presence of quercetin derivatives, which have no antiradical properties (Wiczkowski *et al*., 2014).

2. USE OF *S. cerevisiae* AS A MODEL TO STUDY THE ABILITY OF COCOA POLYPHENOLS TO INDUCE AN ANTIOXIDANT RESPONSE.

2.1. Development of a yeast-based method for screening the ability of food ingredients to induce an antioxidant response in *S. cerevisiae*.

Various methodologies based on the growth of *S. cerevisiae* have been used to evaluate the potential protective effect of food ingredients against oxidative stress. These methodologies include serial dilution and spotting on Petri plates (Martorell *et*. *al*, 2011) or yeast growth monitoring in a multiwell plate (Wu *et al*., 2011) in the presence of an oxidant. The present study proposes a new protocol based on the methodology described by Wu *et al*. (2011) for easy screening of the antioxidant response by real-time monitoring of yeast growth after oxidative stress, removing the oxidant and recording cell growth. Unlike other methods, in our approach, the cells grow in the absence of the oxidant, leading to better control and reproducibility and preventing interaction of the oxidants with culture medium components. Hydrogen peroxide and menadione were selected to cause oxidative stress because both are ROS generating compounds but have a different mode of action. H_2O_2 can be reduced by metals via the Fenton reaction producing hydroxyl radicals, whereas menadione can

form superoxide, H_2O_2 and semiquinone radicals. In a study of the sensitivity of knockout *S. cerevisiae* mutant strains to H_2O_2 and menadione, 103 mutant strains were sensitive to both but 254 were specific to H_2O_2 and 37 to menadione (Tucker & Fields, 2004). Moreover an adaptive dose of H_2O_2 confers a cross-adaptive response to a lethal dose of menadione but not vice versa (Drakulic *et al*., 2005). Other ROS and RNS generating compounds could be implemented in the future to provide a wider study of the effects of biologically active compounds.

Growth curves obtained with the spectrophotometric reader were converted into "growth ratio" curves to adjust for plate-to-plate variation. Subsequently, "effect curves" were constructed to achieve an easier visualization of the effect of the ingredients and to perform statistical analysis. The protective antioxidant effect of the cocoa extract and vitamin C, previously described by Martorell *et al*. (2011), was confirmed (using H_2O_2 and menadione as oxidant agents). Furthermore, an antioxidant protective effect was also detected for the red grape extract. This methodology also allowed to study the dose-response relationships of the ingredients. Dose-response curves (Fig. 38) were constructed by considering the statistically significant effect detected in each of the effect curves as the highest effect value for each dose/ingredient treatment. The dose-response curves did not show the classical hermetic pattern (inverted "J" shape) and a pro-oxidant effect of higher concentrations was only found in the case of pre-incubation with vitamin C when stress was induced with H_2O_2 (Fig 38.A). When stress was induced with 0.5 and 2 mM menadione, the effect reached a maximum at the same concentration of vitamin C (10 mM, Fig 38.A), cocoa extract (350 mg EE/L, Fig 38.B) and grape extract (100-260 mg EE/L, Fig 38.C) and the highest concentrations of each ingredient maintained a significant antioxidant effect. However, when H_2O_2 was used to cause stress, the antioxidant effect of vitamin C reached a maximum at 5 mM and the cocoa extract at 100-350 mg EE/L for both H_2O_2 doses (Fig 38.A-B), but the red grape extract reached a maximum between 180-260 mg EE/L when 4 mM of H_2O_2 was applied. The effect was virtually constant for the whole concentration range of red grape extract when stress was induced with 0.5 mM H_2O_2. Similar dose-response curves were reported for the effect of gallic acid and catechin on the mean lifespan of *C. elegans* (Saul, Pietsch, Stürzenbaum, Menzel & Steinberg, 2011).

The dose-dependent functionality and toxicity of plant polyphenols was previously discussed. The beneficial effects of green tea or cocoa polyphenols have been widely reported (Katz, Doughty & Ali, 2011; Lamuela-Raventós, Romero-Pérez, Andrés-Lacueva & Tornero, 2005) but also the harmful effects, mainly related to pro-oxidative activities when they are ingested at high doses (Murakami, Tsubouchi, Fukayama & Yoshino, 2014). Paradoxically the pro-oxidative activities of polyphenols also have been related to anti-cancer effects (Halliwell, 2008; Surh, Kundu & Na, 2008). Thus, dose-response studies are currently required for a better understanding of the health effects of dietary polyphenols and plant extracts.

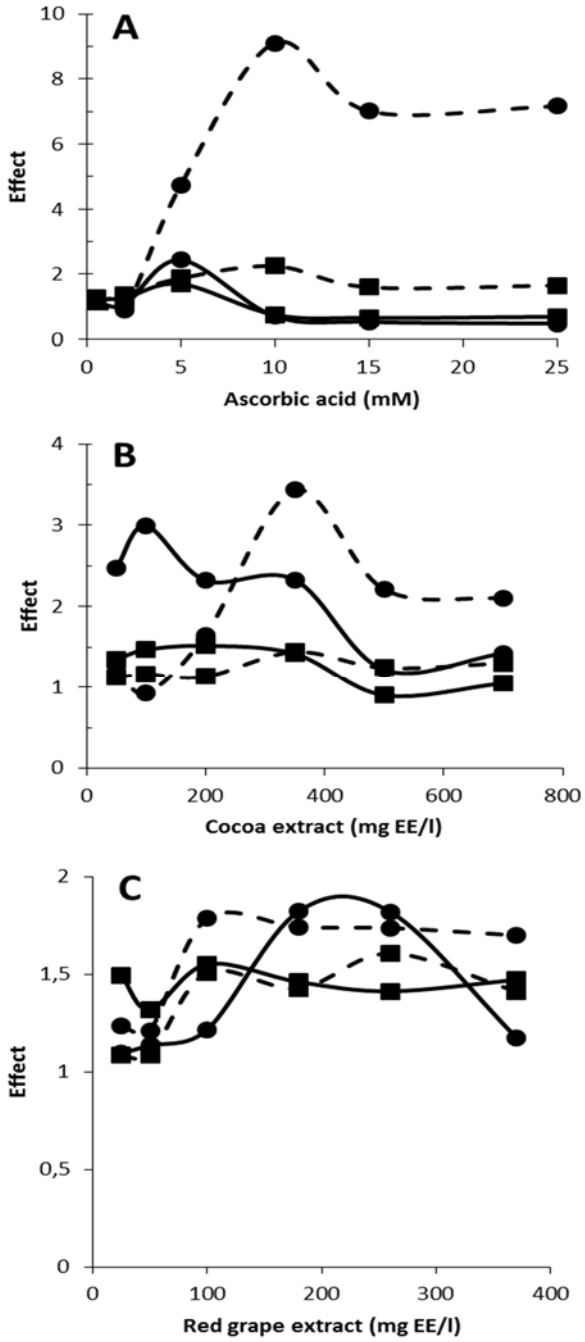


Figure 38. Dose-response curves of vitamin C (A), cocoa extract (B) and red grape extract (C). Curves were constructed considering the greatest statistically significant effect detected in each of the effect curves for BY4741 as the effect value *S. cerevisiae* strain, after treatment with H_2O_2 (solid lines) 0.5 mM (■) or 4 mM (●) and menadione (dashed lines) 0.5 mM (■) or 2 mM (●).

The use of *S. cerevisiae* as a model organism has the advantage that the role of certain genes in both antioxidant and pro-oxidant responses can be studied since all deletion mutant strains are available and there is extensive knowledge about its genome

and proteome. To test the effectiveness of the methodology developed in this work and further investigate the involvement of the Hst3p protein in the cocoa-mediated antioxidant response, as previously described by Martorell *et al*. (2011), the mutant knockout strain *hst3Δ* was studied using H_2O_2 and menadione as oxidant agents. An absence of the protective effect of cocoa and red grape extracts in the mutant strain was clearly stated for both oxidant agents. However the protective effect of vitamin C was only observed in the mutant strain when menadione was added as the oxidative stress agent. Moreover, without ingredient pre-incubation, the comparison of growth ratio curves for *hst3Δ* and wild type strain clearly showed that the deletion of HST3 gen confers a greater resistance to H_2O_2 and menadione. This effect has not been previously reported. This greater resistance of the *hst3Δ* mutant could explain the lack of the protective effect for pre-incubation with cocoa and grape extracts against oxidative stress and the lack of the protective effect of vitamin C when cells are stressed with H_2O_2. According to this hypothesis, the absence of sirtuin Hst3 resulted in an increased resistance to oxidative stress, which is not further increased by subsequent incubation with the extracts. Sirtuins Hst3 and Hst4 play a role in stabilizing the genome during the cell cycle and the lack of both sirtuins leads to increased thermo-sensitivity, genotoxic stress and a higher level of DNA damage by hyperacetylation of histone H3 lysine 56, the target of Hst3 and Hst4 deacetylation (Brachmann *et al*., 1995; Celic *et al*., 2006). However the *hst3Δ* mutation decreases the chronological lifespan (Smith *et al*., 2007), an effect probably not related to the deacetylation of histone H3 and non-histone targets that participate in glucose metabolism were proposed (Wierman & Smith, 2014). The higher resistance to oxidative stress of the *hst3Δ* mutant phenotype revealed in this work is another clue that should be taken into account in efforts made to clarify the roles of the sirtuin Hst3 and its possible participation in the protective effect of cocoa and other polyphenol-rich ingredients against oxidative stress. The methodology developed in this study proved useful to obtain valuable information about the effects of food ingredients on the response of *S. cerevisiae* to oxidative stress and it improved understanding of the genetic targets that support them.

2.2. Capacity of cocoa fractions to promote antioxidant response in the yeast model

The antioxidant protection of fractions was analyzed *in vivo*, using the model organisms *S. cerevisiae* and *C. elegans*. In this context, the protective effect of cocoa (as a whole matrix) was previously established in both models (Martorell *et al*., 2011). We demonstrate that fractions containing catechin, epicatechin or procyanidin B2 were significantly more protective than the others. The results obtained from the yeast experiments showed an increased resistance of cells when incubated with hydrogen peroxide after treatment with F10 (catechin), F14 (procyanidin B2) or F19 (epicatechin). The catechin-rich fraction turned out to be the most potent of them, despite its weak effect *in vitro,* since smaller concentrations (0.5 and 2 mg/L) were able to protect the yeast compared to those used for procyanidin B2 (50 mg/L and 100 mg/L) in F14 and for epicatechin (10 mg/L) in F19. According to our results, the *in vivo* protective effects of green tea catechins are widely studied and demonstrated, as reviewed by Crespy & Williamson, 2004. Regarding epicatechin, previous works correlated the beneficial effects of cocoa powder (antioxidant capacity, cardiovascular protection) with the plasma levels of epicatechin and its metabolites (Baba *et al*., 2000; Schroeter *et al*., 2006). Moreover, Busserolles *et al*. (2006) carried out an *in vivo* approach to study the antioxidant activity of procyanidin-rich plant extracts in rats, showing positive evidence of procyanidins plasma levels. When pure compounds (commercial standards) of catechin, procyanidin B2 and epicatechin were tested at 10 mg/L, none of them protected *S. cerevisiae* against oxidative stress, suggesting that minor compounds are involved in the antioxidant response modulated by fractions. However, other authors have reported the antioxidant capacity of catechin (Dani *et al*., 2008; Surco-Laos *et al*., 2011) and epicatechin (González-Manzano *et al*., 2012) in different *in vivo* approaches, but comparison with our results is difficult due to differences in assays methodologies. Methylxantine rich fractions and fractions with no major compounds were not protective in this model, although the pure compound caffeine displayed a significant antioxidant response in the yeast. Tsoi *et al*. (2015) found similar results both *in vitro* and *in vivo*. Caffeine had relatively low antioxidant effect *in vitro* but it showed a potent antioxidant capacity *in vivo*, since it restored the ORAC level and diminished lipid

peroxidation in the heart tissues of chick embryos damaged by 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH). No effects were found after theobromine treatment.

Overall, these findings are reinforced by several works demonstrating that cocoa flavanols have the ability to act as *in vivo* antioxidants (reviewed by Keen, Holt, Oteiza, Fraga & Schmitz, 2005). It has been shown that pure compounds are not protective in *S. cerevisiae*, so it can be said that enhanced stress resistance is mediated by a mixture of phytochemicals, mainly flavanols, and not by single compounds. Thus, it could be expected that the mixture of phytochemicals present in catechin, epicatechin and procyanidin B2 rich fractions are partly or mainly responsible for the antioxidant effect of the whole cocoa extract. This suggests a possible synergy between cocoa bioactive compounds.

2.3. Proteomic study for the identification of proteins potentially involved in the response of *S. cerevisiae* to oxidative stress mediated by cocoa polyphenols.

It is well known that the adaptation of yeast cells to oxidative stress is ruled by a global gene alteration of expression patterns, including transcription and translation of antioxidant and stress-protective genes. In this work, transcriptome and proteome profiles of *S. cerevisiae* after cocoa extract treatment and/or induction of oxidative stress were established. A similar approach was carried out by Martorell *et al*. (2011). This group evaluated the transcriptional profile of *S. cerevisiae* cultured in the presence of a flavonoid-enriched cocoa powder in order to define the metabolic targets modulated by this ingredient.

In order to investigate the effect of the cocoa extract on *S. cerevisiae* at the proteomic level, 21 statistically significant differential proteins were identified when cultures where incubated in the presence and absence of cocoa polyphenols, which were significantly classified into 14 functional categories. The most overrepresented categories were metabolic process, cellular amino acid biosynthetic process and glycolysis. All of them grouped 28.6 % of the analyzed proteins. Furthermore, all proteins grouped into the glycolysis category (Tdh3, Eno2, Cdc19 and Pgk1) resulted to be

repressed after cocoa treatment, meaning that the cocoa extract causes arrest of glycolysis in *S. cerevisiae*. This metabolic change was previously described as a regulated and conserved oxidative stress response of the yeast. The goal of the glycolysis arrest is to obtain glucose equivalents entering the pentose phosphate pathway (PPP) for the generation of reducing power in the form of NADPH (Ralser *et al*., 2007; Shenton & Grant, 2003) (see below).

Confirmation of proteomic results was carried out using the available deletion mutants in the corresponding genes. In this context, the involvement of Ilv1, Cit1, Met6, Yhb1 and Ssb2 in the yeast response against oxidative stress mediated by cocoa polyphenols was confirmed using the yeast-based method. According to Zhao, *et al*. (1996), flavohemoglobin Yhb1 plays a role in the oxidative stress response. In the present study, this protein was downregulated in the presence of the cocoa extract and the deletion mutant did not show the antioxidant protection conferred by cocoa observed in the wild-type strain. Moreover, this protein was statistically classified ($p<0.01$) into the functional categories of response to toxin and oxygen transport. Although the effect of the cocoa extract was to reduce the amount of this protein in the cell, these results suggest that Yhb1 would be necessary for the cocoa antioxidant protective effect.

Among the up-regulated proteins after treatment with cocoa extract, Ilv1 and Cit1 seem to also be involved in the oxidative stress response of *S. cerevisiae* mediated by cocoa polyphenols. Similarly to Yhb1, deletion mutants in these genes showed a different phenotype from the wild type strain. Cit1 is a citrate synthase which catalyzes citrate formation from acetyl coenzyme A and oxaloacetate in the TCA cycle. This result suggests the enhancement of these pathway in the oxidative stress response, being useful to obtain reduced NADPH, which is involved in replenishing the GSH pools and in keeping catalase in its active form (Kuehne *et al*., 2015). Ssb2 was not classified in any functional group but it was decided to include it in the confirmation assays as it appeared to be up-regulated after cocoa treatment and because of its involvement in the heat stress response, which could provide information about the cross-adaptation occurring in the yeast after H_2O_2 pre-treatment. The results obtained in the deletion

mutant phenotype after cocoa treatment and oxidative stress induction suggested the involvement of this protein in such response.

In order to obtain information about how cocoa polyphenols modulate the *S. cerevisiae* response to oxidative stress, both transcriptomic and proteomic approaches were performed. Hence, when oxidative stress was induced after the cocoa extract treatment in *S. cerevisiae*, 44 differentially regulated proteins were found. Metabolic process and translation were the most representative functional categories during these conditions, with 27.8 % and 22.2 % of the analyzed genes, respectively. Proteins grouped into the translation group (Tef4, Eft1, Tif1 and Aim41) as well as those grouped into the translational elongation group (Tef4 and Eft1), were all downregulated after the cocoa treatment and exposure to oxidative stress. These results are in accordance with the modifications described in the literature. Global inhibition of protein synthesis is a common response to stress conditions to avoid prolonged gene expression during potentially error-prone situations (reviewed by Morano *et al*., 2012). In fact, Shenton *et al*. (2006) showed that *S. cerevisiae* exposure to hydrogen peroxide leads to the inhibition of translation initiation dependent on the Gcn2 protein kinase, which phosphorylates the α-subunit of eukaryotic initiation factor-2 (eIF2 α). Thus, yeast cells respond to such stress with a quick and reversible inhibition of protein synthesis (Godon *et al*., 1998; Shenton *et al*., 2006). However, the involvement of these proteins in the oxidative stress response of the yeast mediated by cocoa polyphenols could not be confirmed with our yeast method due to the lack of viable mutant strains. Furthermore, previous studies carried out by Shenton & Grant (2003) revealed glycolytic and other related enzymes (such as Tdh3 and Eno2) as well as translation factors, as target proteins which are modified in *S. cerevisiae* cells in response to H_2O_2.

Moreover, most of the proteins involved in amino acid metabolic or biosynthetic processes differentially regulated in the proteomic study (Aro8, Hom6, Met6 and Gdh1), proved to be repressed, except for Pro2. The knockout mutants in those downregulated proteins showed a different phenotype from the wild type strain when they were pretreated with the cocoa extract and incubated with H_2O_2, suggesting their involvement in the oxidative stress response of the yeast mediated by cocoa polyphenols. The gamma-glutamyl phosphate reductase Pro2, which catalyzes the

second step in proline biosynthesis, is involved in the yeast's protection against several stresses such as osmolarity and oxidation, according to the bibliography (Liang, Dickman & Becker, 2014; Szabados & Savouré, 2010; Takagi, 2008). In the present work, the *PRO2* knockout mutant was slightly sensitive to H_2O_2 and the validation experiments carried out in 96-well plates, showed no protection of the cocoa extract in this strain after induction of oxidative stress. This result confirms the Pro2 involvement in the oxidative stress response of *S. cerevisiae* modulated by cocoa polyphenols. In conclusion, the cocoa extract could promote amino acid metabolism inhibition by repressing several proteins involved in amino acid metabolic processes, except for Pro2. In the case of Pro2, the cocoa extract leads to an accumulation of proline in the yeast in order to protect it against oxidative stress. Accumulation of this amino acid in engineered yeasts strains show an enhanced stress tolerance, as reviewed by Takagi (2008).

Added metabolic changes take place under oxidative stress. A rapid and regulated response occurs in the yeast, the carbohydrate metabolism being an essential key to maintain the redox status of cells. Specifically, the metabolic flux is switched from glycolysis to the pentose phosphate pathway, in order to obtain redox power in the form of NADPH, which seems to be a conserved response to oxidative stress (Ralser *et al*., 2007). This process was previously described by Shenton & Grant (2003), who found an inhibition of GAPDH, enolase and alcohol dehydrogenase activities after oxidative stress and proposed the inhibition of the glycolytic flux in order to obtain glucose equivalents entering the pentose phosphate pathway for the generation of NADPH. In the present study, a downregulation of glycolytic enzymes (Cdc19 and Pgk1, which were grouped in the same functional category) and the induction of Tkl1 (involved in the pentose phosphate pathway) suggest the inhibition of the glycolysis and the activation of the pentose phosphate pathway which is in agreement with the results aforementioned described in the bibliography (Ralser *et al*., 2007; Shenton & Grant, 2003).

Three other proteins may be involved in *S .cerevisiae* resistance against oxidative stress modulated by cocoa polyphenols. This is the case of Bmh2, which was repressed after cocoa and H_2O_2 treatments and classified into the negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle category. This homologue to mammalian 14-3-3 proteins controls proteome at a post-transcriptional level and it is

involved in Ras/MAPK signalling. With respect to Imh1 (involved in vesicular transport) and Prb1 (vacuolar proteinase B involved in protein degradation), which were not grouped into any functional group, multiwell assays suggested their involvement in the oxidative stress response of the yeast after cocoa treatment.

2.4. Transcriptomic study for the identification of potentially involved genes in the *S. cerevisiae* response to oxidative stress mediated by cocoa polyphenols.

Regarding changes at the transcriptomic level, only experiments involving oxidative stress after cocoa treatment were carried out in the present study, since a previous work (Martorell *et al*., 2011) was already performed to define the single effect of a cocoa powder in *S*. *cerevisiae* transcriptome (see above). Martorell *et al*. (2011) found 20 genes showing a different expression pattern after cocoa treatment (13 down-regulated and 7 up-regulated), most of them of unknown function. They used the deletion mutants of the most over-expressed genes of known function (*YVC1* and *HST3*) and assayed the antioxidant protection of cocoa powder. The results showed that the only phenotype lacking the antioxidant effect was the *HST3* knockout mutant strain.

Given that the transcriptomic response is quite fast, two time points of hydrogen peroxide treatment were analyzed in order to obtain as much information as possible about the modulated genes by cocoa extract in the oxidative stress reaction. Microarrays experiments were carried out with RNA from yeast cultures incubated in the presence and absence of cocoa extract (350 mg EE/L) and exposed to oxidative stress with H_2O_2 1 mM for 15 and 45 min. Gene expression patterns were then determined in order to obtain information about the possible modulation of oxidative stress response genes, by cocoa polyphenols. A total of 272 differentially regulated genes were found ($p<0.0005$), 121 genes after 15 min of H_2O_2 incubation and 151 after 45 min.

Among the up-regulated genes, the functional category of cell rescue, defence and virulence was overrepresented at both time points. Several sub-categories such as stress response (oxidative stress, pH stress and heat shock response) as well as detoxification (oxygen and radical detoxification and more specific catalase reaction) were also of interest. Some of the common free radical scavenging enzymes were induced by cocoa

extract: *CTA1* (codifies catalase A), *TSA2* (thioredoxin peroxidase), *PRX1* (peroxiredoxin), *CCP1* (cytochrome *c* peroxidase) and *SRX1* (sulfiredoxin). These enzymes, which are known to be the first line of cell protection against oxidative damage, where not found in the proteomic assay, suggesting the rapid induction of these genes in response to an oxidative stress situation (proteomic approach was carried out after 90 min of H_2O_2 incubation). Other stress response genes were up-regulated as well, such as *GRE1* and *GRE2*, *FMP46* (a putative redox protein containing a thioredoxin fold), the heat shock protein *HSP31*, and the stress-responsive transcriptional activator *MSN2*, among others. Moreover, protein degradation genes were also included in the cell defense category, e.g. *MRK1*, which functions not only to activate Msn2p-dependent transcription of stress responsive genes but also in protein degradation and *SIS1* (Type II HSP40 co-chaperone), which is involved in proteasomal degradation of misfolded cytosolic proteins. Finally, it is worth mentioning, among the induced genes, the presence of the transcriptional repressor *NRG1* and of *SED1* which has a possible role in mitochondrial genome maintenance. Other categories and sub-categories such as metabolism; energy; cellular transport, transport facilities and transport routes were overrepresented in at least one time point. The major energy sub-categories were the glycolysis methylglyoxal bypass, the glyoxylate cycle, alcohol fermentation and the oxidation of fatty acids. All of them seem to help the yeast to counteract oxidative damage. The glyoxylate cycle permits glucose synthesis from lipids via acetate produced in fatty acid β oxidation. Proteomic results as well as the literature (Ralser *et al*., 2007) showed that PPP is induced during oxidative stress at the expense of glycolysis, so the new glucose would enter the PPP with consequent generation of redox power which can be used by the antioxidant defenses or can enter the methylglyoxal bypass. This pathway was overrepresented among the up regulated genes as well. This branch of the glycolysis pathway converts glucose into methylglyoxal and then into pyruvate, which would enter the TCA cycle, where NADH is generated.

Among the down-regulated genes, the protein synthesis category as well as its sub-categories of ribosome biogenesis and ribosomal proteins were overrepresented only after 45 min of oxidative damage. These results are in accordance with the proteomic insights since a slowdown of protein biosynthetic processes was observed as well.

Similar conclusions were obtained in the works of Godon *et al*. (1998) and Shenton *et al*. (2006), who analyzed the response of *S. cerevisiae* to oxidative stress induced by H_2O_2. Moreover, none of the protein synthesis sub-categories were found after 15 min of stress, suggesting that this cellular mechanism is not immediately executed after the damage. The energy category was also overrepresented among the down-regulated genes. Unlike the energy sub-categories grouping the induced genes, in this case electron transport, respiration and energy generation, were overrepresented, among others, at both time points. Repression of mitochondrial processes involved in respiration seems logical since they represent the major source of intracellular ROS. According to this, it could be hypothesized that during oxidative stress conditions, cells would stop such processes preventing extra ROS formation and they would enhance the fermentation process and alternative pathways to obtain carbon sources and redox power.

In summary, the DNA microarray technique, which covers the entire yeast genome, is extremely helpful to complement the two-dimensional gel studies, which permit visualization of post-transcriptional modifications but only of soluble and abundant proteins (Godon *et al*., 1998). For this reason, differences in the results provided by both techniques are found. The combination of transcriptome and proteome analyses provides a better overview of the molecular mechanisms and metabolic targets involved in the response of *S. cerevisiae* to oxidative stress modulated by the cocoa polyphenolic extract. Nevertheless, complementary expression studies would be needed to further study the involvement of these genes in the *S. cerevisiae* response to oxidative stress mediated by cocoa polyphenols. More incubation time points with different ROS generators would be of interest as well.

3. Use of *C. elegans* AS A MODEL TO STUDY THE ANTIOXIDANT CAPACITY OF COCOA POLYPHENOLS

The model organism *C. elegans* was used to further study of molecular mechanisms of the antioxidant capacity of the cocoa extract and its fractions. It had been previously stated (Martorell *et al*., 2011) that cocoa caused a prolongation of life span in this model organism as well as an increased stress resistance depending on DAF-16. We

investigated the effect of cocoa fractions on this model organism, compared to the complete cocoa extract and the isolated compounds, to identify the active compounds responsible for the beneficial effects.

Martorell *et al*. (2011) also reported that polyphenol-enriched cocoa powder caused a higher degree of protection against oxidative stress in yeast cultures compared to conventional cocoa powder. This suggests a pivotal role of polyphenolic compounds in the stress resistance process. Many polyphenols, e.g. flavonoids, have been shown to be potent antioxidants due to their ability to donate electrons and to stabilize oxidized flavonoid species (semiquione radical). Moreover, flavonoids form complexes with redox-active heavy metal ions, e.g. Fe2+ and Cu2+ that are involved in Fenton-like reactions (reviewed by Wätjen *et al*. 2006).The effects of polyphenols can also be mediated by interference with signal transduction processes. It has been suggested that the beneficial effects of flavonoid-rich cocoa on the endothelial function are mediated by a modulation of nitric oxide synthase (Wang-Polagruto *et al*., 2006). It is also considered that anti-inflammatory effects of polyphenols may be relevant to cardiovascular health (Selmi, Mao, Keen, Schmitz & Gershwin, 2006).

In our first results, all fractions showed very similar behaviour after H_2O_2 treatment of the nematodes, except for F24, which did not show statistically significant ($p<0.05$) antioxidant protection compared to the control. This fraction, which was neither protective *in vitro* nor *in vivo,* was found to contain a mixture of quercetin derivatives as well as kaempferol 3-O-rutinoside, vanillic acid and caffeoylquinic acid. However, fractions containing catechin, epicatechin or procyanidin B2 were among the most potent active fractions ($p<0.001$), supporting the results found in the yeast model. Unexpectedly, the theobromine-containing fraction also proved to be an active sample in the nematode, despite having showed negative results in yeast experiments.

We further analysed the *in vivo* antioxidative potential measured as ROS accumulation (DCF assay). The antioxidant role of cocoa extract was previously shown by Lee *et al*. (2010). They demonstrated that a cocoa phenolic extract (10 µg/mL) dramatically attenuated the H_2O_2-induced accumulation of intracellular ROS in rat liver epithelial cells. In the present study the effect of the cocoa extract on ROS accumulation in *C. elegans* induced by thermal stress was analysed. The cocoa extract (adjusted to an

amount of 100 µM epicatechin or 100 µM caffeine) strongly reduced the ROS formation in the nematode. However, the fractions containing 100 µM epicatechin or caffeine showed no antioxidant effect in this assay. In the case of the epicatechin-containing fraction, the result was not expected, since it has been shown that this flavonoid is able to contribute to the antioxidant capacity in blood plasma (Terao, 1999). Moreover, Gonzalez-Manzano *et al*. (2012) showed that epicatechin reduced the level of thermal-induced intracellular ROS in *C. elegans*, although a concentration of 200 µM was used by this group. Furthermore, the pure compounds epicatechin and caffeine (100 µM) were not able to reduce the ROS accumulation in *C*. *elegans*, which was not unexpected since the cocoa fractions rich in epicatechin and caffeine and adjusted to those concentrations also exerted no significant ROS-scavenging effects. These results suggest the involvement of several compounds in the protective effect of cocoa.

The antioxidative effects detected in the DCF assay are in line with experiments performed to analyse the resistance of *C. elegans* to oxidative stress assessed with Na-arsenite. Only the cocoa extract (adjusted to 100 µM epicatechin or caffeine) but not the fractions or pure compounds revealed a protective effect in this assay. On the contrary, treatment with 100 µM caffeine significantly decreased the resistance against oxidative stress. Since the caffeine-containing cocoa fraction F16 adjusted to 100 µM caffeine attenuates this sensitivity, it is suggested that this fraction contains unknown protective compounds which counteract the adverse effect of caffeine.

The effect of cocoa extract, fractions and pure compounds on stress resistance in *C*. *elegans* was further confirmed by thermal stress experiments. The cocoa extract was able to enhance the resistance of the nematodes. The caffeine-rich cocoa fraction F16 as well as the pure compound caffeine failed to provide any protection. Only 100 µM epicatechin was able to slightly increase stress resistance in the nematode. The epicatechin-containing fraction F19 showed a similar increase in stress resistance, which was not significant ($p=0.057$). We conclude that epicatechin is a secondary plant compound which is responsible for protection against thermal-induced stress in the nematode.

Martorell *et al*. (2011) showed that the protection against H_2O_2-induced stress conferred by the polyphenol-rich cocoa powder was mediated by the transcription

factor DAF-16; therefore, the protective effects of our cocoa extract, epicatechin pure compound and the epicatechin-rich fraction F19 were tested to see if they were able to activate this transcription factor. Using a transgenic *C. elegans* strain expressing GFP-tagged DAF-16, it was observed that only the cocoa extract, but not epicatechin or the epicatechin-containing cocoa fraction, was able to increase the nuclear localization of this transcription factor. In line with this result, the protective effects of the cocoa extract were completely blocked when the experiment was performed in a DAF-16 loss-of-function nematode. The effects of the extract on the accumulation of ROS or on resistance against thermal stress were not detectable in this strain. In addition, epicatechin pure compound did not protect the mutant strain in the thermal-stress assay. With these results it was shown that the DAF-16 transcription is not only directly modulated by the cocoa extract but is also essential for the protective effects of this extract and epicatechin. A role of this evolutionary highly conserved insulin/insulin like growth factor signalling pathway might be relevant in cocoa-induced health effects in other species, too.

The effect of different catechins of cocoa on this pathway has been analyzed by several groups. Abbas & Wink (2009) showed that a treatment with 220 µM epigallocatechingallate (EGCG, 1h) resulted in a rapid nuclear translocation of DAF-16. Analogous results were obtained by Bartholome, Kampkötter, Tanner, Sies & Klotz (2010), demonstrating that treatment with 100 µM EGCG (48 h) increased the amount of nematodes showing nuclear localization of DAF-16 in contrast to untreated *C. elegans*. In this context, it was demonstrated that EGCG significantly up-regulates SOD-3 mRNA expression and enhances SOD-3 expression in transgenic SOD-3::GFP (transgenic strain CF1553) nematodes (Zhang, Jie, Zhang & Zhao, 2009). The flavonoid EGCG (220 µM, 48 h) also suppressed juglone-induced HSP-16.2 expression (Abbas & Wink, 2009). In analogy to the results obtained here with the cocoa extract, Brown *et al*. (2006) showed that 25 µM EGCG does not provoke a significant change in the intracellular ROS level of DAF-16 (loss-of-function) mutant *C. elegans*, while in the wild type strain ROS levels are significantly reduced by the flavonoid.

The inhibitory effects of cocoa extract on the insulin signalling pathway in different cell culture models have been described (Cordero-Herrera, Martín, Bravo, Goya &

Ramos, 2013; Cordero-Herrera, Martín, Goya & Ramos, 2014; Cordero-Herrera *et al*., 2015; Martín *et al*., 2010; Min *et al*., 2013). These effects are in accordance with the results of the present work. An activation of the insulin-receptor (DAF-2) will result in an increased phosphorylation of the transcription factor DAF-16 and therefore a more cytosolic localization. If the kinase cascade is inhibited, DAF-16 phosphorylation is reduced and the transcription factor is able to translocate into the nucleus and regulate the expression of target genes.

In humans, the effects of cocoa/cocoa products on insulin signalling are controversially discussed. Grassi *et al*. (2005) reported that dark chocolate (88 mg flavanols/day, 15 days) decreased blood pressure, serum LDL cholesterol and ameliorated insulin sensitivity in patients with essential hypertension. On the other hand, Stote *et al.* (2012) reported that short-term intake (5 days) of cocoa flavanols did not improve glucose metabolism. No significant changes in glucose or insulin concentrations were detected after consumption of different cocoa beverages (30-900 mg flavanols per day). It also has to be mentioned that cocoa may have advantages for human health but it has to be taken into consideration that consumption of cocoa, especially as sugar-enriched chocolate, is also associated with health problems due to the high caloric intake.

4. GENERAL DISCUSSION

Plant food and beverages have attracted great attention in recent years due to their high levels of polyphenols, which have been described as bioactive compounds. However, most of the studies are carried out with complex food matrices, which are a vast mixture of these phytochemicals, making it difficult to identify the individual compounds responsible for the beneficial properties. Moreover, the mechanisms of action of these compounds are not yet fully elucidated, leading to the need for further studies.

The aim of this thesis was to study the functional capacity of cocoa polyphenols by *in vivo* assays using the model organisms *S. cerevisiae* and *C. elegans*. To achieve this purpose, a polyphenolic cocoa extract was obtained from a 12 % polyphenol content

cocoa powder. This extract was then fractionated and characterized. The capacity of isolated and well defined fractions to induce antioxidant response in both organisms was finally determined.

In order to screen the ability of a wide range of food ingredients to induce an antioxidant response in *S. cerevisiae*, a multiwell assay was developed. The antioxidant protective effect of cocoa polyphenols, previously described by Martorell *et al*. (2011), was confirmed. Moreover, the antioxidant protection of cocoa fractions was also analyzed by this assay. Fractions containing catechin, epicatechin or procyanidin B2 were significantly more protective than the other ones, which did not show positive effects in the yeast model. Previous works have correlated the protective effect of cocoa samples with plasma levels of flavan-3-ols (Baba *et al*., 2000; Schroeter *et al*., 2006). However, when pure compounds were tested, none of them protected *S. cerevisiae* against oxidative stress, suggesting the involvement of minor compounds or the synergy of various compounds in the antioxidant response modulated by cocoa fractions.

Taking into account the protective effect of cocoa extract, proteomic and transcriptomic approaches were employed in order to identify the mechanisms of action and molecular pathways involved in such effect. Genes and proteins potentially involved in the enhanced resistance of *S. cerevisiae* to oxidative stress mediated by cocoa polyphenols were identified. For the first time, proteome and transcriptome profiles of *S. cerevisiae* after cocoa treatment and induction of oxidative stress were established. A similar approach was carried out by Martorell *et al.* (2011) who evaluated the transcriptional profile of the yeast cultured in the presence of a flavonoid-rich cocoa powder (but not exposed to oxidative stress damage assessed with an oxidant agent). Overall, the present work shows that cocoa treatment leads to a glycolysis and protein synthesis slowdown as a protective mechanism of action against oxidative stress. As described in the literature, the repression of glycolysis is a regulated and conserved response of the yeast in order to obtain glucose equivalents entering the PPP for the generation of reducing powder in the form of NADPH (Ralser *et al*., 2007; Shenton & Grant, 2003). Moreover, global inhibition of protein synthesis is also a common response to oxidative stress (Morano *et al*., 2012). In this work, a global reduction of protein synthesis was observed by the repression of translation proteins as well as proteins

involved in amino acid metabolic or biosynthetic processes. Interestingly, the gamma glutamyl phosphate reductase Pro2 was, unlike other proteins, upregulated after cocoa treatment and stress induction and confirmed, using the *PRO2* deletion mutant in the multiwell assay, as a key protein in the yeast protection mediated by cocoa polyphenols. Proline has been previously described as stress protectant. As reviewed by Liang, Dickman & Becker (2014), this amino acid could decrease stress conditions by two general strategies. The first one is the up-regulation of proline biosynthesis and its accumulation in the organism, working as an osmolyte, a chemical chaperone and a direct scavenger of ROS. The second one consists of linking to other metabolic pathways to help maintain cellular energy and $NADP^+$/NADPH balance, activating signalling pathways that promote cell survival, and contributing to other pathways such as the TCA cycle and GSH biosynthesis. Furthermore, the TCA cycle, with the subsequent generation of NADH, seems to also be enhanced by cocoa extract as a protective mechanism against oxidative stress. This is because several related proteins and genes (such as *CIT1*) were up-regulated as well and their mutants showed a different protection phenotype than the wild type. In addition, the common antioxidant defenses (such as catalases and peroxidases) were activated by the cocoa extract as a quick response against oxidative damage.

Sirtuins are a conserved family of nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylases proteins, present in organisms from bacteria to humans. These proteins, which regulate a wide range of cellular functions and which are involved in the molecular mechanism of aging, are receiving special attention due to their possible role in stress protection. Moreover, several natural compounds, including polyphenols, have been described as sirtuins modulators, leading to an increased yeast lifespan (Howitz *et al*., 2003). Martorell *et al*. (2011) found that the sirtuin Hst3p was involved in the protection of *S. cerevisiae* against oxidative stress conferred by a flavonoid-rich cocoa powder. A transcriptomic study carried out by this group revealed the upregulation of *HST3* after treatment with the cocoa powder. To confirm this results, the knockout mutant in *HST3* gene was used to study the cocoa antioxidant protection against oxidative stress (spots assays). Results showed a different phenotype to that found in the wild type strain, since the cocoa powder did not protect the *hst3Δ* mutant,

indicating the possible involvement of this sirtuin in the effect of cocoa flavonoids. In order to test the effectiveness of the yeast-based method developed in this work and obtain additional information about the involvement of Hst3p in the yeast response to oxidative stress modulated by cocoa polyphenols, the *HST3* knockout strain was studied. A lack of antioxidant protection in the mutant strain was observed, as previously described by Martorell *et al*. (2011). However, the *hst3Δ* mutant strain was more resistant to oxidative stress than the wild type, an effect that had not been previously described. This higher resistance could explain the lack of protective effect exhibited after pre-incubation with the cocoa extract and should be taken into account in future works aiming to investigate the role of sirtuin Hst3 in the protective effect of polyphenols against oxidative stress.

The yeast model is very useful as a first screening of antioxidant compounds, but not when results need to be extrapolated to humans. To this end, the use of a model animal is of great interest, such as the nematode *C. elegans,* due to its short lifespan, its morphological simplicity and its easy maintenance and genetic manipulation. Unlike the unicellular yeast, studies on different types of cells and organs can be performed in the worm. Besides, this model is more closely related to mammals (Fontana, Partridge & Longo, 2010).

In analogy to the experiments of Martorell *et al*. (2011), this model organism was used to elucidate the molecular mechanisms of cocoa in detail. This group showed that cocoa caused a prolongation of the life span in this model organism as well as an increased stress resistance depending on DAF-16. We investigated the effect of two cocoa fractions on this model organism compared to the pure cocoa extract and the isolated compounds to identify active compounds responsible for the beneficial effects.

First, oxidative stress experiments were performed in order to confirm the results found in the yeast model. As expected, after treating the worms with the cocoa extract or fractions and inducing oxidative stress with hydrogen peroxide, fractions containing epicatechin, catechin and procyanidin B2 were significantly ($p<0.001$) protective, according to the results found in the yeast model. Further experiments were then performed, using the cocoa extract as well as the epicatechin- and caffeine-rich fractions, showing different results. Only the cocoa extract displayed ROS reduction in

the worm model, but not the two fractions tested nor the corresponding pure compounds. Similar results were obtained after oxidative stress experiments with Na-arsenite. The effect of the extract, fractions and pure compounds on stress resistance in *C. elegans* was further confirmed by thermal stress experiments. Only the cocoa extract was able to clearly enhance the resistance of the nematodes. The pure compound epicatechin was, in this case, slightly protective, suggesting its involvement in the protection against thermal-induced stress in the worm.

Regarding the molecular mechanisms involved in the protective effect of cocoa extract, epicatechin as a pure compound and epicatechin-rich fraction in the worm, the activation of the transcription factor DAF-16 (FOXO homologue) was studied. DAF-16 belongs to the insulin/insulin like growth factor signalling pathway, which is involved in longevity and metabolism in different species. In *C. elegans*, an increase in FoxO activity leads to enhanced longevity, fat storage and stress resistance (Yen, Narasimhan & Tissenbaum, 2011). In this context, Martorell *et al*. (2011) showed that protection against hydrogen peroxide induced stress in worms treated with cocoa powder was mediated by DAF-16. In the present work, only the cocoa extract increased the nuclear localization of DAF-16 in the transgenic worms and the protective effects of cocoa were completely blocked in the DAF-16 knockout mutants. Besides, no significant effect of the extract on the accumulation of ROS nor on resistance against thermal stress were detected in this strain. Taking these results together, the DAF-16 transcription factor is not only directly modulated by the cocoa extract but, according to Martorell *et al.* (2011), also essential for its protective effects, suggesting a role of this evolutionary conserved insulin/insulin like growth factor signalling pathway which might be relevant in cocoa-induced health benefits in other species, too. In addition, the enhanced thermal-stress resistance of the wild-type strain mediated by epicatechin was also blocked in the DAF-16 knockout mutant, which suggest the involvement of DAF-16 in this response, too.

Taking everything into account, it seems necessary to perform several stress studies to further understand the different mechanisms of action involved in the response to each ingredient, since they may have different effects on living organisms.

CONCLUSIONS

1. A multiwell yeast-based methodology has been developed to evaluate the capacity of polyphenolic plant (cocoa and red grape) extracts for inducing the cellular antioxidant response using H_2O_2 or menadione as the causal agents of oxidative stress. The methodology can be extended to analyze extracts prepared from other food ingredients of plant or animal origin, the use of alternative oxidizing agents or the study of different types of stress.

2. Using the yeast-based methodology a possible role for the sirtuin protein Hst3p in the *S. cerevisiae* antioxidant response mediated by the cocoa extract has been inferred from the absence of this response in an *HST3* gene loss-of-function strain. However, it has also been noted that, under the assay conditions used, deletion of the *HST3* gene confers increased resistance to oxidative stress caused by H_2O_2 or menadione which may partly explain the lack of response observed in this mutant strain.

3. Studies carried out with chromatographic fractions of the cocoa extract showed that those fractions rich in catechin, procyanidin B2 and epicatechin promoted significant antioxidant protection in *S. cerevisiae* but that this was not equivalent to that exerted by the pure compounds at the same concentrations. This suggests the participation of other minor compounds in induction of the response.

4. Proteomic and transcriptomic studies of the yeast response to cocoa extract exposure and oxidative stress suggests that a modification of carbohydrate metabolism occurs through repression of glycolysis and up-regulation of the pentose phosphate pathway thus providing greater redox power. Rapid induction of some common antioxidant enzymes such as catalases and peroxidases has also been observed. Experiments with mutant strains deleted for genes identified by the proteomic study indicated that the Pro2, Gdh1, Ssb2, Yhb1, Imh1, Aro8, Prb1, Hom6, Bmh2, Ilv1, Met6 and Cit1 proteins may be involved in the antioxidant response mediated by cocoa extract.

5. As previously observed in the yeast model, cocoa extract and those chromatographic fractions enriched in catechin, procyanidin B2 and epicatechin

also conferred significant protection to *C. elegans* against the oxidative stress induced by H_2O_2. Nevertheless, only the whole cocoa extract elicited a significant response in the nematode to sodium arsenite-induced oxidative stress, thermal stress and ROS formation, suggesting that the greater diversity of compounds present in the whole extract confers the greatest protective capacity.

6. It has been demonstrated that expression of the transcription factor DAF-16 (insulin/insulin-like growth factor) in *C. elegans* is modulated by the cocoa extract but not by epicatechin nor the chromatographic fraction rich in epicatechin. It has been found that DAF-16 is essential for the effect exerted by the cocoa extract on intracellular ROS accumulation and resistance to thermal stress, and it has also been confirmed that this factor is essential for oxidative stress protection in *C. elegans*. Since the insulin signaling pathway is evolutionarily highly conserved it may also play a role in the health effects induced by cocoa in other species.

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ANNEXES

1. Supplementary data

Table I. Differentially regulated genes after cocoa extract treatment and incubation with H_2O_2 (15 and 45 min). Genes having at least 2-fold change in expression and an adjusted p value <0.0005 (Limma test) are showed.

Cocoa vs control 15 min			Cocoa vs control 45 min		
Gene	**statistic**	**adj, p-value**	**Gene**	**statistic**	**adj, p-value**
YIL057C	26,2829	5,24E-05	YIL057C	26,1652	5,27E-05
YBR230C	22,8137	5,24E-05	YBR230C	22,5816	5,31E-05
YOR348C	22,2818	5,24E-05	YDR039C	22,0860	5,31E-05
YDR039C	22,0671	5,24E-05	YDR256C	21,4192	5,31E-05
YDR256C	21,1408	5,69E-05	YOR388C	21,3439	5,31E-05
YOR388C	20,1987	5,69E-05	YOR348C	20,3573	6,05E-05
YDR536W	20,1679	5,69E-05	YNL195C	19,6554	6,37E-05
YNL195C	20,1662	5,69E-05	YOL151W	18,9580	6,45E-05
YBR116C	19,6889	5,77E-05	YKR076W	18,6973	6,45E-05
YOL084W	19,5854	5,77E-05	YOL084W	18,6147	6,45E-05
YHR096C	19,3435	5,77E-05	YIL136W	18,4419	6,45E-05
YIL136W	19,1767	5,77E-05	YDR536W	18,2839	6,45E-05
YOL151W	18,6470	6,31E-05	YDR453C	18,2651	6,45E-05
YCR010C	18,4721	6,42E-05	TC21215	18,1372	6,45E-05
YMR206W	17,7203	7,98E-05	YBR116C	18,0830	6,45E-05
YKR076W	17,1036	9,15E-05	YHR096C	18,0630	6,45E-05
YKL086W	16,9146	9,56E-05	YMR206W	17,8414	6,72E-05
TC21215	16,8289	9,58E-05	YKL086W	17,7175	6,72E-05
YDR043C	16,7270	9,67E-05	YCR010C	17,6910	6,72E-05
YDR453C	16,5181	1,00E-04	YKL217W	16,1368	1,11E-04
YBR298C	16,4258	1,00E-04	YBR298C	16,1173	1,11E-04
YLR142W	16,1941	1,08E-04	YKR066C	15,9061	1,13E-04
YKL217W	16,0364	1,12E-04	YDR018C	15,7219	1,18E-04
YIL160C	15,5653	1,35E-04	YDR043C	15,6914	1,18E-04
YER065C	15,2368	1,52E-04	YLR142W	15,4337	1,21E-04
YHR160C	15,0639	1,61E-04	YIL160C	15,4211	1,21E-04
YDL204W	14,6585	1,90E-04	YOL048C	15,0143	1,43E-04
YOR161C	14,5462	1,96E-04	YHR160C	14,8531	1,50E-04
YDR018C	14,4564	1,97E-04	YPL171C	14,8209	1,50E-04
YPL171C	14,3913	1,97E-04	YOR226C	14,5845	1,62E-04
YOL048C	14,3850	1,97E-04	YER015W	14,5635	1,62E-04
YPL186C	14,3187	1,99E-04	YMR191W	14,4270	1,70E-04
YMR085W	14,2554	2,01E-04	YOR161C	14,2867	1,73E-04
YDR040C	14,2128	2,01E-04	YDR040C	14,2557	1,73E-04
YGL035C	14,1568	2,01E-04	YGL035C	14,2104	1,73E-04
YER015W	14,1218	2,01E-04	YER065C	14,1976	1,73E-04
YLR149C	14,0771	2,02E-04	YLR149C	14,1908	1,73E-04

YEL070W	13,9424	2,12E-04	YDL085W	13,8823	1,95E-04
YLL020C	13,7676	2,27E-04	YDL204W	13,7648	1,98E-04
YPL111W	13,7167	2,28E-04	YLL061W	13,7483	1,98E-04
YMR191W	13,5888	2,36E-04	YPL004C	13,6720	2,00E-04
YLL061W	13,5802	2,36E-04	YMR085W	13,6181	2,02E-04
YPL230W	13,5465	2,36E-04	YLL020C	13,5837	2,02E-04
YBL043W	13,4955	2,38E-04	YDR533C	13,4539	2,10E-04
YKR066C	13,4202	2,39E-04	YPL247C	13,4007	2,12E-04
YMR280C	13,4056	2,39E-04	YMR280C	13,3706	2,12E-04
YDL169C	13,3896	2,39E-04	YPL111W	13,3451	2,12E-04
YER053C-A	13,2135	2,58E-04	YHR002W	13,3038	2,14E-04
YBL086C	13,0870	2,70E-04	YPL186C	13,2636	2,15E-04
YPL004C	13,0661	2,70E-04	YOL126C	13,1794	2,20E-04
YOR292C	12,9924	2,72E-04	YBL043W	13,0570	2,28E-04
YLR252W	12,9733	2,72E-04	YML091C	13,0543	2,28E-04
YNL173C	12,9257	2,72E-04	YNL173C	13,0285	2,28E-04
YPL247C	12,9123	2,72E-04	YER053C-A	12,9816	2,31E-04
YDR533C	12,8580	2,72E-04	YLL023C	12,8093	2,50E-04
YHR002W	12,8534	2,72E-04	YKL093W	12,7455	2,55E-04
YKL093W	12,8359	2,72E-04	YKR009C	12,7245	2,55E-04
YDL214C	12,7899	2,72E-04	YEL070W	12,6368	2,60E-04
YAL034C	12,7842	2,72E-04	YML131W	12,5817	2,60E-04
YML131W	12,7756	2,72E-04	YDL169C	12,5809	2,60E-04
YOR226C	12,6828	2,79E-04	YLR350W	12,5668	2,60E-04
YIL082W-A	12,6780	2,79E-04	YIL082W-A	12,5376	2,60E-04
YMR036C	12,6008	2,84E-04	YDL214C	12,5340	2,60E-04
YDL152W	12,4892	2,95E-04	YBL086C	12,5027	2,60E-04
YOL126C	12,4851	2,95E-04	YPL230W	12,4845	2,60E-04
YDL079C	12,4647	2,95E-04	YAL034C	12,4565	2,60E-04
YOR273C	12,4325	2,96E-04	YER095W	12,4562	2,60E-04
YHR097C	12,3703	3,03E-04	YDR178W	12,4182	2,60E-04
YOR374W	12,3423	3,04E-04	YNR073C	12,4005	2,60E-04
YKL091C	12,2927	3,09E-04	YHR097C	12,3980	2,60E-04
YGR230W	12,2406	3,15E-04	YOR292C	12,3232	2,66E-04
YDL085W	12,1067	3,35E-04	YOR273C	12,3208	2,66E-04
YLR164W	12,0531	3,39E-04	YFL054C	12,2268	2,78E-04
YFL054C	12,0508	3,39E-04	YLR252W	12,2075	2,78E-04
YPL223C	11,9232	3,49E-04	YDR069C	12,1882	2,78E-04
YBR285W	11,9231	3,49E-04	YGL205W	12,1313	2,84E-04
YKR009C	11,9158	3,49E-04	YNR014W	12,0773	2,88E-04
YLR350W	11,8857	3,49E-04	YDL079C	11,9005	3,09E-04
YNR014W	11,8826	3,49E-04	YKL091C	11,8103	3,23E-04
YER095W	11,7210	3,68E-04	YMR090W	11,7894	3,24E-04
YDR178W	11,6835	3,73E-04	YJR019C	11,7560	3,27E-04
YLL023C	11,6197	3,82E-04	YBR203W	11,6730	3,39E-04

YGL205W	11,5421	3,93E-04	YDR077W	11,6659	3,39E-04
YNL042W	11,4515	4,11E-04	YMR036C	11,5610	3,50E-04
YLR328W	11,3587	4,30E-04	YNL007C	11,4829	3,62E-04
YML091C	11,3472	4,30E-04	YLR328W	11,4134	3,71E-04
YNL036W	11,2779	4,44E-04	YER039C	11,3806	3,75E-04
YER054C	11,1984	4,58E-04	YBL064C	11,3426	3,80E-04
YDR114C	11,1700	4,61E-04	YOR374W	11,3249	3,81E-04
YOR052C	11,1557	4,61E-04	YPL135W	11,2987	3,84E-04
YDL110C	11,1283	4,62E-04	YER035W	11,2729	3,87E-04
YNR073C	11,1231	4,62E-04	YCR075C	11,2530	3,89E-04
YLR284C	11,0606	4,72E-04	YLR284C	11,2164	3,92E-04
YMR174C	11,0479	4,72E-04	YNL125C	11,2131	3,92E-04
YER039C	11,0313	4,72E-04	YPL223C	11,1208	4,08E-04
YMR090W	10,9881	4,74E-04	YGR230W	11,0270	4,29E-04
YBR050C	10,9862	4,74E-04	YJL185C	11,0044	4,32E-04
YJR096W	10,9674	4,75E-04	YGR256W	10,9246	4,49E-04
YOR252W	-11,0051	4,74E-04	YLR164W	10,9167	4,49E-04
YER137C	-11,0564	4,72E-04	YKR049C	10,8903	4,50E-04
YDL130W	-11,2103	4,58E-04	YOR049C	10,8901	4,50E-04
YOL080C	-11,6083	3,82E-04	YOL147C	10,8602	4,51E-04
YML060W	-11,7263	3,68E-04	YER054C	10,8545	4,51E-04
YOR314W-A	-11,7580	3,68E-04	YMR037C	10,8518	4,51E-04
YER106W	-11,7853	3,66E-04	YOR052C	10,8018	4,61E-04
YDR526C	-11,9197	3,49E-04	YJL082W	10,7453	4,68E-04
Q0070	-12,6204	2,84E-04	YIL077C	10,7363	4,68E-04
Q0045	-16,4988	1,00E-04	YLR108C	10,7011	4,75E-04
Q0140	-17,2567	8,91E-05	YDR472W	10,6623	4,84E-04
Q0105	-17,4523	8,54E-05	YNL036W	10,6456	4,86E-04
Q0075	-18,3777	6,42E-05	YGR046W	10,6250	4,89E-04
Q0080	-19,0109	5,77E-05	YNL042W	10,6028	4,89E-04
Q0297	-19,1327	5,77E-05	YPL113C	10,5975	4,89E-04
YNL259C	-19,7128	5,77E-05	YHL032C	10,5459	4,97E-04
Q0250	-20,6719	5,69E-05	YOR252W	-10,5814	4,89E-04
Q0055	-21,5432	5,53E-05	YPL090C	-10,5825	4,89E-04
Q0050	-23,2737	5,24E-05	YDR447C	-10,7786	4,62E-04
Q0275	-23,4360	5,24E-05	YML063W	-10,7955	4,61E-04
Q0085	-25,0407	5,24E-05	YOL080C	-11,1315	4,08E-04
Q0130	-30,2608	4,37E-05	YDL130W	-11,4587	3,64E-04
			YDR058C	-11,5539	3,50E-04
			YBL029W	-11,5793	3,50E-04
			YLR062C	-11,5866	3,50E-04
			YDR440W	-11,9480	3,04E-04
			YLR198C	-11,9870	3,00E-04
			YML060W	-12,0724	2,88E-04
			Q0070	-12,5979	2,60E-04

			YAR053W	-13,1681	2,20E-04
			YFL012W-A	-13,5430	2,03E-04
			YLL046C	-13,6707	2,00E-04
			YJL120W	-13,7560	1,98E-04
			YLL030C	-13,9166	1,95E-04
			YBL108W	-15,4772	1,21E-04
			YER137C	-15,5065	1,21E-04
			YOR314W-A	-15,9105	1,13E-04
			Q0045	-16,0170	1,13E-04
			YER106W	-16,9873	8,15E-05
			Q0105	-17,0257	8,15E-05
			YDR526C	-17,5149	6,99E-05
			Q0075	-18,5722	6,45E-05
			YNL259C	-18,9990	6,45E-05
			Q0080	-19,3739	6,45E-05
			Q0140	-19,9555	6,13E-05
			Q0250	-20,1997	6,05E-05
			Q0297	-20,2852	6,05E-05
			Q0055	-21,4737	5,31E-05
			Q0275	-23,5466	5,27E-05
			Q0050	-23,8969	5,27E-05
			Q0085	-24,7528	5,27E-05
			Q0130	-29,4862	5,26E-05

Table II. Functional classification of upregulated genes after treatment with cocoa extract and 15 min of 1mM hydrogen peroxide incubation.

FUNCTIONAL CATEGORY	nº genes	% genes	Genes SET	*p* value
01 METABOLISM	33	35.8	YDL214c YEL070w YKR009c YLR284c YNR073c YDR178w YDR453c YER015w YIL160c YJR096w YOL126c YOR226c YCR010c YER065c YLR142w YML131w YMR280c YOL151w YOR374w YOR388c YGL205w YBR050c YLR328w YDR043c YER054c YGL035c YPL111w YBL086c YDL079c YDR018c YLR164w YMR036c YPL004c	1,05E-02
01.01.03.05 metabolism of arginine	2	2.17	YPL111w YLR142w	3,89E-02
01.01.03.05.02 degradation of arginine	2	2.17	YPL111w YLR142w	6,62E-04
01.04.04 regulation of phosphate metabolism	2	2.17	YPL004c YER054c	2,92E-02
01.05 C-compound and carbohydrate metabolism	16	17.3	YER054c YER065c YLR164w YOR374w YOR388c YGL035c YBL086c YOL126c YJR096w YNR073c YDR043c YBR050c YCR010c YDR178w YEL070w YMR280c	3,12E-03
01.05.25 regulation of C-compound and carbohydrate metabolism	6	6.52	YCR010c YDR043c YMR280c YBR050c YER054c YGL035c	1,17E-02
01.06.05 fatty acid metabolism	4	4.34	YGL205w YKR009c YIL160c YLR284c	4,01E-04
01.20.37 metabolism of peptide derived compounds	1	1.08	YDR453c	4,44E-02
01.20.37.01 metabolism of thioredoxin, glutaredoxin, glutathion	1	1.08	YDR453c	4,44E-02
02 ENERGY	16	17.3	YER054c YMR280c YOL151w YDR178w YOR388c YDL085w YGL205w YKL093w YOR374w YLR164w YLR284c YKR009c YER065c YIL160c YOL126c YPL171c	9,35E-05
02.01.01 glycolysis methylglyoxal bypass	1	1.08	YOL151w	1,50E-02
02.04 glyoxylate cycle	2	2.17	YER065c YOL126c	7,49E-03
02.16 fermentation	3	3.26	YOR388c YDL085w YOR374w	3,30E-02

02.16.01 alcohol fermentation	2	2.17	YOR374w YDL085w	1,56E-02
02.25 oxidation of fatty acids	4	4.34	YKR009c YLR284c YGL205w YIL160c	5,65E-06
20.01 transported compounds (substrates)	17	18.4	YDR178w YKR066c YDR040c YFL054c YBR298c YDR536w YLL061w YHR002w YPL171c YDR039c YOR273c YDL085w YER015w YHR096c YCR010c YKL217w YOR348c	5,59E-03
20.01.03 C-compound and carbohydrate transport	5	5.43	YKL217w YBR298c YCR010c YHR096c YDR536w	2,42E-03
20.01.15 electron transport	4	4.34	YPL171c YDR178w YDL085w YKR066c	3,55E-02
20.03.02.02.01 proton driven symporter	1	1.08	YBR298c	4,44E-02
20.09.03 peroxisomal transport	2	2.17	YER015w YHR160c	3,23E-02
30.05.01.18 transmembrane receptor protein serine/threonine kinase signalling pathways	1	1.08	YDL214c	2,98E-02
32 CELL RESCUE, DEFENSE AND VIRULENCE	15	16.3	YKR066c YOR273c YKL086w YNL042w YPL223c YDR256c YDR533c YLR350w YDL079c YDR043c YDR453c YER095w YMR174c YOL151w YPL004c	1,71E-02
32.01 stress response	13	14.1	YDR453c YER095w YMR174c YKL086w YPL004c YDR043c YDR533c YDR256c YDL079c YLR350w YPL223c YKR066c YOL151w	1,62E-02
32.01.01 oxidative stress response	6	6.52	YDR256c YPL223c YDR453c YKL086w YKR066c YOL151w	1,56E-04
32.01.04 pH stress response	3	3.26	YPL223c YDR043c YOL151w	1,74E-04
32.01.05 heat shock response	3	3.26	YOL151w YPL223c YPL004c	3,10E-03
32.07 detoxification	5	5.43	YDR453c YOL151w YKR066c YOR273c YDR256c	3,07E-02
32.07.07 oxygen and radical detoxification	3	3.26	YDR453c YKR066c YDR256c	6,63E-03
32.07.07.01 catalase reaction	1	1.08	YDR256c	2,98E-02
34.11.03.11 pH response	1	1.08	YDR043c	4,44E-02

Table III. Functional classification of downregulated genes after treatment with cocoa extract and 15 min of 1mM hydrogen peroxide incubation.

FUNCTIONAL CATEGORY	nº genes	% de genes	Genes SET	*p* value
01.03.16 polynucleotide degradation	2	10	YOL080c Q0055	3,14E-02
02 ENERGY	8	40	Q0130 Q0105 Q0050 Q0250 Q0045 Q0275 Q0085 Q0080	1,02E-05
02.11 electron transport and membrane-associated energy conservation	7	35	Q0080 Q0275 Q0130 Q0085 Q0250 Q0105 Q0045	4,75E-10
02.13 respiration	8	40	Q0080 Q0085 Q0130 Q0050 Q0105 Q0275 Q0250 Q0045	5,40E-09
02.13.03 aerobic respiration	4	20	Q0250 Q0045 Q0105 Q0275	9,75E-05
02.45 energy conversion and regeneration	3	15	Q0080 Q0085 Q0130	3,61E-04
02.45.15 energy generation (e.g. ATP synthase)	3	15	Q0085 Q0130 Q0080	3,81E-05
10.01.05 DNA recombination and DNA repair	4	20	Q0070 Q0055 YML060w Q0050	8,03E-03
10.01.05.03 DNA recombination	2	10	Q0070 Q0050	3,48E-02
10.01.05.03.05 DNA integration	2	10	Q0070 Q0050	1,01E-04
10.01.09 DNA restriction or modification	3	15	Q0055 Q0050 Q0070	2,56E-02
10.01.09.03 DNA restriction	3	15	Q0070 Q0050 Q0055	6,41E-06
11.04 RNA processing	4	20	Q0055 Q0070 Q0050 YOL080c	4,92E-02
11.04.03 mRNA processing (splicing, 5'-, 3'-end processing)	3	15	Q0050 Q0055 Q0070	2,14E-02
11.04.03.01 splicing	3	15	Q0050 Q0055 Q0070	1,02E-02

16.07 structural protein binding	2	10	Q0085 Q0130	1,40E-02
20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES	7	35	Q0250 YNL259c Q0085 Q0130 Q0045 Q0275 Q0080	3,99E-02
20.01 transported compounds (substrates)	7	35	Q0045 Q0250 Q0080 Q0275 YNL259c Q0085 Q0130	1,78E-03
20.01.01 ion transport	4	20	Q0130 YNL259c Q0080 Q0085	1,20E-03
20.01.01.01 cation transport (H+, Na+, K+, Ca2+ , NH4+, etc.)	4	20	Q0080 YNL259c Q0130 Q0085	5,65E-04
20.01.01.01.01.01 siderophore-iron transport	1	5	YNL259c	3,85E-02
20.01.15 electron transport	6	30	Q0045 Q0275 Q0085 Q0080 Q0130 Q0250	1,71E-07
20.03 transport facilities	3	15	Q0085 Q0080 Q0130	2,46E-02
20.03.22 transport ATPases	3	15	Q0085 Q0080 Q0130	6,63E-04
20.09.04 mitochondrial transport	3	15	Q0085 Q0080 Q0130	4,38E-03
34.01 homeostasis	4	20	Q0080 Q0085 YNL259c Q0130	2,51E-03
34.01.01 homeostasis of cations	4	20	Q0080 Q0085 YNL259c Q0130	1,64E-03
34.01.01.03 homeostasis of protons	3	15	Q0085 Q0080 Q0130	4,68E-04

Table IV. Functional classification of upregulated genes after treatment with cocoa extract and 45 min of 1mM hydrogen peroxide incubation.

FUNCTIONAL CATEGORY	nº genes	% genes	genes SET	*p* value
01 METABOLISM	37	33.6	YOL126c YOR226c YHL032c YLR328w YMR036c YPL135w YDL214c YKR009c YDR453c YOR388c YGL205w YER054c YGL035c YBL086c YPL004c YNR073c YER035w YIL160c YER065c YML131w YMR280c YOR374w YPL113c YPL111w YLR164w YEL070w YLR284c YDR178w YER015w YGR256w YCR010c YLR142w YOL151w YJR019c YDR043c YDL079c YDR018c	2,11E-02
01.01.03.05 metabolism of arginine	2	1.81	YLR142w YPL111w	5,37E-02
01.01.03.05.02 degradation of arginine	2	1.81	YLR142w YPL111w	9,46E-04
01.04.04 regulation of phosphate metabolism	2	1.81	YPL004c YER054c	4,05E-02
01.05 C-compound and carbohydrate metabolism	17	15.4	YGL035c YOL126c YHL032c YGR256w YBL086c YDR043c YCR010c YDR178w YER054c YER065c YLR164w YOR374w YOR388c YEL070w YPL113c YNR073c YMR280c	8,10E-03
01.06.05 fatty acid metabolism	5	4.54	YJR019c YIL160c YKR009c YLR284c YGL205w	2,83E-04
02 ENERGY	18	16.3	YER054c YOR388c YOL126c YDR178w YDL085w YIL160c YMR280c YOL151w YGL205w YKL093w YLR164w YGR256w YOR374w YLR284c YKR009c YER065c YJR019c YPL171c	1,07E-04
02.01.01 glycolysis methylglyoxal bypass	1	0.90	YOL151w	1,79E-02
02.04 glyoxylate cycle	2	1.81	YER065c YOL126c	1,06E-02
02.16.01 alcohol fermentation	2	1.81	YOR374w YDL085w	2,19E-02
02.25 oxidation of fatty acids	5	4.54	YIL160c YGL205w YKR009c YLR284c YJR019c	1,67E-05
20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES	26	23.6	YDR039c YKR066c YDR536w YLL061w YBR298c YER039c YNL036w YCR010c YHR096c YDR472w YGR046w YHR160c YCR075c YDR069c YHR002w YER015w YNL125c YDR040c YFL054c YDL085w YKL217w YPL171c YOR273c YDR178w YOR049c YOR348c	4,31E-02
20.01 transported compounds (substrates)	20	18.1	YPL171c YER015w YDR178w YDR040c YDR536w YCR075c YDL085w YGR046w YHR096c YOR348c YHR002w YCR010c YOR049c YKR066c YFL054c YBR298c YLL061w YDR039c YOR273c YKL217w	3,37E-03

20.01.03 C-compound and carbohydrate transport	5	4.54	YHR096c YBR298c YCR010c YKL217w YDR536w	5,22E-03
20.01.07 amino acid/amino acid derivatives transport	3	2.72	YOR348c YLL061w YCR075c	4,63E-02
20.01.13 lipid/fatty acid transport	3	2.72	YOR049c YFL054c YER015w	4,38E-02
20.03 transport facilities	9	8.18	YCR075c YDR040c YDR536w YNL125c YFL054c YCR010c YDR039c YBR298c YOR049c	8,33E-03
20.03.02 carrier (electrochemical potential-driven transport)	2	1.81	YCR075c YBR298c	4,05E-02
20.09.03 peroxisomal transport	2	1.81	YER015w YHR160c	4,47E-02
30.05.01.18 transmembrane receptor protein serine/threonine kinase signalling pathways	1	0.90	YDL214c	3,56E-02
32 CELL RESCUE, DEFENSE AND VIRULENCE	19	17.2	YKR066c YDR533c YLR350w YNL007c YKR049c YDR256c YBL064c YKL086w YPL223c YDR043c YDR453c YER095w YDR077w YOR273c YNL042w YDL079c YMR037c YOL151w YPL004c	4,15E-03
32.01 stress response	17	15.4	YDR453c YER095w YBL064c YPL223c YDR077w YPL004c YDL079c YMR037c YKR066c YKL086w YNL007c YDR256c YLR350w YDR043c YDR533c YKR049c YOL151w	2,52E-03
32.01.01 oxidative stress response	8	7.27	YKL086w YKR066c YOL151w YBL064c YDR453c YDR256c YPL223c YKR049c	1,76E-05
32.01.04 pH stress response	3	2.72	YPL223c YOL151w YDR043c	2,95E-04
32.01.05 heat shock response	3	2.72	YPL004c YPL223c YOL151w	5,13E-03
32.07 detoxification	6	5.45	YBL064c YOR273c YOL151w YKR066c YDR256c YDR453c	1,82E-02
32.07.07 oxygen and radical detoxification	4	3.63	YDR453c YDR256c YBL064c YKR066c	1,08E-03
32.07.07.01 catalase reaction	1	0.90	YDR256c	3,56E-02

Table V. Functional classification of downregulated genes after treatment with cocoa extract and 45 min of 1mM hydrogen peroxide incubation.

FUNCTIONAL CATEGORY	nº genes	% genes	genes SET	*p* value
02 ENERGY	8	26.6	Q0050 Q0045 Q0085 Q0130 Q0105 Q0250 Q0275 Q0080	2,79E-04
02.11 electron transport and membrane-associated energy conservation	7	23.3	Q0080 Q0085 Q0250 Q0045 Q0275 Q0130 Q0105	1,16E-08
02.13 respiration	8	26.6	Q0080 Q0250 Q0045 Q0085 Q0130 Q0050 Q0105 Q0275	2,08E-07
02.13.03 aerobic respiration	4	13.3	Q0250 Q0045 Q0105 Q0275	4,92E-04
02.45 energy conversion and regeneration	3	10	Q0080 Q0085 Q0130	1,22E-03
02.45.15 energy generation (e.g. ATP synthase)	3	10	Q0085 Q0130 Q0080	1,33E-04
10.01.05 DNA recombination and DNA repair	4	13.3	Q0070 Q0055 YML060w Q0050	3,30E-02
10.01.05.03.05 DNA integration	2	6.66	Q0070 Q0050	2,29E-04
10.01.09 DNA restriction or modification	4	13.3	Q0050 Q0055 Q0070 YDR440w	1,52E-02
10.01.09.03 DNA restriction	3	10	Q0070 Q0055 Q0050	2,26E-05
11.04.03.01 splicing	3	10	Q0050 Q0055 Q0070	3,07E-02
12 PROTEIN SYNTHESIS	7	23.3	YDL130w YLL046c Q0140 YOL080c YDR447c YML063w YPL090c	7,20E-03
12.01 ribosome biogenesis	7	23.3	YML063w YDR447c YLL046c YDL130w YOL080c Q0140 YPL090c	5,87E-04
12.01.01 ribosomal proteins	5	16.6	YDL130w YDR447c Q0140 YML063w YPL090c	6,25E-03
16.07 structural protein binding	2	6.66	Q0130 Q0085	3,03E-02
20.01 transported compounds (substrates)	7	23.3	Q0250 Q0275 YNL259c Q0085 Q0130 Q0045 Q0080	2,02E-02
20.01.01 ion transport	4	13.3	YNL259c Q0080 Q0085 Q0130	5,62E-03
20.01.01.01 cation transport (H+, Na+, K+, Ca2+ , NH4+, etc.)	4	13.3	Q0080 YNL259c Q0130 Q0085	2,74E-03
20.01.15 electron transport	6	20	Q0045 Q0275 Q0085 Q0080 Q0130 Q0250	2,35E-06

20.03.22 transport ATPases	3	10	Q0130 Q0080 Q0085	2,22E-03
20.09.04 mitochondrial transport	3	10	Q0085 Q0130 Q0080	1,38E-02
34.01 homeostasis	4	13.3	Q0080 YNL259c Q0085 Q0130	1,13E-02
34.01.01 homeostasis of cations	4	13.3	Q0080 YNL259c Q0085 Q0130	7,54E-03
34.01.01.03 homeostasis of protons	3	10	Q0080 Q0085 Q0130	1,58E-03

Table VI. Summary of the data of wild-type (N2) and *daf-16(mu86)* (CF1038) nematodes on thermal stress resistance after treatment with cocoa extract/fractions.

Strain	Exp.	Treatment	Mean lifespan (hours)	SE	*n*	Difference (%)	*p* value
wild-type	3	Control	4.47	0.16	24		
wild-type	3	Cocoa extract adjusted to 100 µM epicatechin	5.25	0.20	24	17.2	0.003
wild-type	3	Cocoa extract adjusted to 100 µM caffeine	6.05	0.19	24	35.1	0.000
wild-type	4	Control	4.66	0.20	32		
wild-type	4	Epicatechin	5.13	0.19	32	10.1	0.034
wild-type	4	Epicatechin-rich fraction	5.20	0.16	32	11.6	0.057
wild-type	3	Control	4.58	0.24	24		
wild-type	3	Caffeine	4.46	0.22	24	-2.5	0.467
wild-type	3	Caffeine-rich fraction	4.63	0.22	24	1.1	0.829
daf-16 (mu86)	3	Control	4.99	0.14	48		
daf-16 (mu86)	3	Cocoa extract adjusted to 100 µM epicatechin	5.14	0.14	48	2.9	0.349
daf-16 (mu86)	3	Epicatechin	4.93	0.17	39	-1.2	0.974
daf-16 (mu86)	3	Epicatechin-rich fraction	5.06	0.15	40	1.5	0.915

2. Resumen en castellano

1. OBJETIVOS

El cacao y sus productos derivados han sido ampliamente estudiados debido a sus efectos beneficiosos para la salud humana. Estas propiedades han sido relacionadas principalmente con su elevado contenido en polifenoles, compuestos antioxidantes presentes en las plantas para los que se han descrito efectos cardioprotectores, antitumorales y antiinflamatorios, entre otros. Aunque los compuestos fenólicos no son esenciales en la dieta humana, son ampliamente consumidos debido a su extensa presencia en el reino vegetal. Los mecanismos de acción por los cuales los polifenoles ejercen sus efectos beneficiosos no están del todo establecidos. Parece que los polifenoles no sólo actúan como secuestradores de especies reactivas de oxígeno o formando complejos con iones metálicos, sino también modulando rutas de señalización implicadas en un amplio rango de procesos como el envejecimiento y la respuesta a estrés. No obstante, se requiere mayor información para explicar las propiedades beneficiosas de los polifenoles. La mayor parte de dichas propiedades han sido estudiadas mediante métodos *in vitro*, empleando, por ejemplo, líneas celulares humanas, las cuales no tienen en cuenta el efecto en un organismo completo. También han sido empleados estudios *in vivo*, la mayoría llevados a cabo en modelo murino, pero la dificultad de estos experimentos sumado a la baja disponibilidad y complejidad de los polifenoles ha dado lugar a resultados poco claros. Actualmente se recurre cada vez más al uso de organismos sencillos, como la levadura *S*. *cerevisiae* y el nematodo *C*. *elegans,* entre otros, para el estudio del efecto protector antioxidante de ingredientes alimentarios, así como para identificar los mecanismos moleculares responsables de dichos efectos. En el caso concreto del cacao, únicamente existen unos pocos estudios sobre el mecanismo de acción o los beneficios de polifenoles individuales en organismos vivos, lo cual pone de manifiesto la necesidad de realizar estudios *in vivo* adicionales.

Dado el interés del tema, el objetivo principal de este trabajo ha sido estudiar la capacidad funcional de los polifenoles del cacao mediante ensayos *in vivo* con los organismos modelo *S. cerevisiae* y *C. elegans.* Para ello, se propusieron los siguientes objetivos específicos:

1. Caracterizar analíticamente y fraccionar un extracto polifenólico obtenido a partir de un polvo de cacao rico en polifenoles.

2. Evaluar la capacidad del extracto de cacao y de las fracciones obtenidas para promover la respuesta antioxidante en la levadura *S. cerevisiae* e identificar los compuestos responsables de dicho efecto.

3. Identificar, mediante ensayos proteómicos y transcriptómicos, posibles proteínas y genes implicados en la respuesta a estrés oxidativo de *S. cerevisiae* mediada por los polifenoles del cacao y confirmar su posible participación utilizando mutantes de deleción.

4. Evaluar la capacidad del extracto de cacao y de las fracciones para promover la respuesta al estrés oxidativo en el nematodo *C. elegans* e identificar los compuestos responsables de dicho efecto.

2. METODOLOGÍA

2.1. Obtención de extractos polifenólicos a partir del polvo de cacao y de uva.

El polvo de cacao, comercializado como CocoanOX fue suministrado por Natraceutical Group (Valencia, España). Este producto contiene un alto contenido en polifenoles (12 % p/p) y se obtiene a partir de granos de cacao no fermentados ni tostados, que han sufrido un tratamiento de blanqueamiento (Tomás-Barberán *et al*., 2007). El polvo de uva fue obtenido de SECNA, S.A. (Valencia, España). Para obtener los extractos polifenólicos, se añadió a 1 g de polvo 20 mL de solución de extracción (metanol:agua; 80:20; v:v). Se homogeneizó y se sonicó en un baño a 37 ºC durante 10 minutos. Después, se centrifugó a 4000 rpm durante 15 minutos y se recogió el sobrenadante. Con el sedimento se repitió 2 veces más el mismo proceso. Los sobrenadantes obtenidos se juntaron y se concentraron en un rotavapor.

2.2. Fraccionamiento del extracto polifenólico de cacao

El extracto de polifenoles de cacao se fraccionó mediante HPLC semi-preparativa con un sistema Dionex Ultimate 3000 (Sunnyvale, CA, USA) equipado con detector UV-Vis acoplado a un colector de fracciones Foxy Jr. Se utilizó agua-metanol (85:15; v:v) (solvente A) y metanol 100 % (solvente B), con un flujo de 11 mL/min. La elución se llevó a cabo de la siguiente manera: 0 % de B durante 33 min y utilizando un gradiente para obtener 11.8 % B a los 75 min, 29.4 % B a los 80 min mantenido hasta los 87 min, 88.2 % a los 88 min mantenido hasta los 93 min, 0 % B a los 94 min mantenido hasta los 100 min. La separación se llevó a cabo en fase reversa con una columna Ultrabase C18, de dimensiones 250 x 20 mm y 5 µm de tamaño de partícula. Se programó el colector de fracciones para recoger todo el extracto eluído, separando en fracciones los principales picos cromatográficos. Las fracciones obtenidas fueron concentradas en un rotavapor a 35 ºC y conservadas a -20 ºC.

2.3. Análisis mediante HPLC-DAD del extracto de cacao y las fracciones

El extracto polifenólico de cacao y las fracciones fueron analizadas mediante cromatografía líquida en fase reversa utilizando un sistema de HPLC Dionex Summit equipado con un detector de fluorescencia RF 2000 y un detector de fotodiodos PDA-100. Se empleó una columna de Ultrabase C18 de 100 x 4.6 mm y 2.5 µm, utilizando 0.1 % TFA en agua (solvente A) y 0.1 % TFA en metanol (solvente B), con un flujo de 1 mL/min. La elución se llevó a cabo utilizando un gradiente empezando con 10 % de B a los 10 min, 20 % B a los 20 min, 40 % B a los 21 min hasta los 23 min, alcanzando el 100 % B a los 24 min hasta los 26 min y finalmente disminuyendo hasta el 0 % B a los 27 min y mantenido hasta los 35 min. El cromatograma se registró a 280 nm y mediante la emisión de fluorescencia a 360 nm tras excitación a 278 nm. Los patrones comerciales de las procianidinas B1 y B2 (Extrasynthèse, Genay, Francia), teobromina, cafeína, catequina y epicatequina (Sigma-Aldrich, St. Louis, USA) fueron utilizados para la identificación y la cuantificación de los principales polifenoles y metilxantinas presentes en el extracto de cacao y las fracciones.

2.4. Análisis mediante HPLC-MS/MS del extracto polifenólico y las fracciones

Se llevó a cabo el estudio de las muestras utilizando un sistema AB SCIEX TripleTOF™ 5600 LC/MS/MS, con detección de iones en modo positivo y negativo. El análisis por cromatografía se realizó utilizando un sistema Agilent 1290 HPLC con una columna Waters UPLC C18, 1.7 µm (2.1 x 50 mm) Acquity UPLC BEH. El gradiente de elución se llevó a cabo con 0.1 % de ácido fórmico en H_2O (solvente A) and 0.1 % de ácido fórmico en metanol (solvente B) con un flujo constante de of 0.4 mL/min siguiendo las siguientes proporciones: 5 % de B al inicio, 50 % de B a los 2 min, 95 % de B a los 13 min hasta los 15 min, disminuyendo a 5 % de B hasta los 25 min y 30 % de B a los 30 min. Las condiciones establecidas para el análisis por MS fueron las siguientes: *Ion source gas* 1 (GC1): 50 psi; *Ion source gas* 2 (GC2): 50 psi; *Curtain gas* 1: 25 psi; temperatura: 450 ºC; *Ion Spray Voltage* (ISVF): -4500. Se utilizó un método de adquisición IDA con dos experimentos: *the survey scan type* (TOF-MS) y *the dependent scan type* (Product Ion) utilizando -30 V de energía de colisión. Los datos se analizaron utilizando el programa XIC manager en el software PeakView™, siendo filtrados según el error de masa, tiempo de retención, % de diferencia de ratio isotópica y *library hit purity score*.

2.5. Determinación de polifenoles totales

El contenido en polifenoles totales de los extractos de cacao y uva y de las fracciones se determinó utilizando el método Folin-Ciocalteau descrito por Singleton & Rossi (1965), con algunas modificaciones. Se añadieron 50 µL de muestra y 500 µL de solución acuosa de carbonato de sodio (Panreac, Barcelona, España) al 2 % e hidróxido de sodio (Panreac, Barcelona, España) al 0.4 % en un tubo eppendorf y la mezcla se agitó. Tras 15 min de incubación en oscuridad a temperatura ambiente, se añadieron 50 µL de reactivo Folin (Folin 2N (Sigma-Aldrich, St. Louis, USA):agua; 50:50; v:v) y la mezcla se agitó e incubó de nuevo en oscuridad durante 30 min, a temperatura ambiente. Posteriormente, se midió la absorbancia a 724 nm en un lector de placas (BMG Labtech Omega Spectrostar). Se preparó una curva de calibrado con 10 a 300 mg/L de

epicatequina (R^2= 0.9982). Los resultados se expresaron como equivalentes de epicatequina (mg EE/L) y los experimentos se realizaron por triplicado.

2.6. Evaluación de la capacidad antioxidante *in vitro* del extracto de cacao y de las fracciones

2.6.1. Ensayo del radical libre DPPH

Se determinó la capacidad antioxidante total de las muestras mediante la reducción del radical libre 2,2-diphenyl-1-picrylhydrazyl (DPPH). El ensayo se llevó a cabo utilizando una versión modificada del método descrito por Schinella *et al*. (2010). Para ello, 7.5 µL de muestra se añadieron a 292.5 µL de DPPH (Sigma-Aldrich, St. Louis, USA) 60 µM en metanol 80 % y la mezcla se incubó 30 min en oscuridad a temperatura ambiente. La absorbancia del DPPH restante se midió a 517 nm frente a un blanco en un lector de placas (BMG Labtech Omega Spectrostar). La actividad secuestradora de radicales libres de las muestras fue calculada de acuerdo a la siguiente fórmula: Porcentaje de inhibición = 100 x (A0 - A1)/A0, donde A0 es el valor de absorbancia del blanco y A1 es el valor de absorbancia de las muestras, ambas medidas tras 30 min de reacción. Una curva de calibrado con epicatequina fue preparada con concentraciones entre 5 y 50 mg/L (R^2= 0.9986) y los resultados fueron expresados como equivalentes de epicatequina. Los experimentos se llevaron a cabo por triplicado.

2.6.2. Ensayo de la capacidad antioxidante equivalente a Trolox (TEAC)

La capacidad antioxidante de las muestras fue también analizada mediante el ensayo TEAC, midiendo la capacidad de un compuesto para reducir una solución radicalaria (ABTS) y comparándola con la actividad del derivado sintético de la vitamina E, Trolox (Calbiochem, Merck, Darmstadt, Alemania). La solución ABTS se preparó el día anterior a su uso mezclando volúmenes iguales de una solución de ácido 2,2´-azinobis-(3-ethylbenzthiazolin)-6-sulfónico (ABTS) (14 mM) y una solución de ammoniumperoxodisulfato (APS) (4.9 mM) y se guardó en oscuridad a temperatura ambiente. La absorbancia de dicha solución fue ajustada mediante su dilución con

etanol 70 % (v/v). Tanto el Trolox como las fracciones fueron ensayadas utilizando concentraciones crecientes de 0 a 25 µM mezclando 500 µL de la solución de radicales con 500 µL de la solución de ensayo (en etanol al 70 %). El extracto de cacao se estandarizó al contenido de epicatequina y se ensayó en un rango de concentraciones de 0 a 6 µM de epicatequin. La actividad secuestradora de radicales libres se midió espectrofotométricamente tras 2 min de reacción a 734 nm (Synergy MX, BioTek; Bad Friedrichshall, Germany). Se llevaron a cabo tres ensayos independientes.

2.7. Utilización de *S. cerevisiae* como organismo modelo

2.7.1. Cepas utilizadas y mantenimiento

Para realizar este trabajo se utilizó la cepa BY4741 de *S. cerevisiae* (*MAT*a; *his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0) y 18 mutantes de deleción para esa misma cepa (Tabla 1), obtenidos del European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF). Las cepas fueron cultivadas en medio YPD [(2 % (p/v) de glucosa (Scharlab, Barcelona, España), 1 % (p/v) de extracto de levadura (Conda, Madrid, España) y 2 % (p/v) de peptona (Conda, Madrid, España)] con 2 % (p/v) de agar (Scharlab, Barcelona, España) para placas. Se sembraron las cepas a partir de un stock de glicerol en medio YPD fresco y se incubaron *overnight* a 28 ºC con agitación. Estos cultivos fueron después diluidos en medio YPD fresco e incubados de nuevo *overnight* a 28 ºC con agitación. Finalmente, se sembraron en placas YPD e incubaron a 28 ºC durante 72 h para la obtención de colonias independientes.

Tabla 1. Lista de mutantes de deleción de la cepa BY4741 utilizados en este trabajo.

Nombre sistemático	Gen delecionado
YDR099W	*BMH2*
YEL060C	*PRB1*
YER086W	*ILV1*
YER091C	*MET6*
YGL026C	*TRP5*
YGL202W	*ARO8*
YGR192C	*TDH3*
YGR234W	*YHB1*
YJR139C	*HOM6*
YLR309C	*IMH1*
YNL209W	*SSB2*
YNR001C	*CIT1*
YOL057W	*DPP3*
YOR323C	*PRO2*
YOR375C	*DHE4*
YPL138C	*SPP1*
YPR074C	*TKL1*
YOR025W	*HST3*

2.7.2. Desarrollo de un método en placa multipocillo para evaluar la capacidad de ingredientes alimentarios para promover una respuesta antioxidante en *S. cerevisiae*.

Una colonia de *S. cerevisiae* fue inoculada en 5 mL de medio YPD fresco e incubada durante 6 h a 28 ºC con agitación a 40 rpm. Una alícuota de 5 µL de la dilución 1/10 de este precultivo se inoculó en 3 mL de medio YPD fresco con los ingredientes antioxidantes a ensayar (extracto de polifenoles de uva, extracto de polifenoles de cacao o vitamina C) durante 18 h a 28 ºC con agitación a 40 rpm. Se ensayaron varias concentraciones de los ingredientes antioxidantes (de 50 mg/L a 700 mg/L de polifenoles totales en los extractos de plantas y de 0.5 a 25 mM de vitamina C) para

inducir una respuesta antioxidante intracelular en la levadura. La vitamina C (Sigma-Aldrich, St. Louis, USA) se utilizó como control positivo y los cultivos sin ingrediente antioxidante como controles negativos. Posteriormente, se centrifugaron los cultivos a 2700 g, durante 10 min, a 20 ºC y se resuspendieron las células en 3 mL de PBS pH 7.4 (se midió la DO_{600} para asegurar la misma concentración en el siguiente paso). Una alícuota de esta suspensión de células se diluyó en un volumen final de 3 mL de PBS para alcanzar una DO_{600} de 0.1 y se preadaptó incubando durante 30 min a 28 ºC. Para inducir estrés oxidativo subletal, una gama de concentraciones de peróxido de hidrógeno (Merck, Hohenbrunn, Alemania) de 0.25 a 6 mM y de menadiona de 0.1 a 5 mM (Sigma-Aldrich, St. Louis, USA) fueron previamente ensayadas con el objetivo de elegir las dos dosis más adecuadas para el ensayo. Se incubaron las células durante 60 min a 28 ºC con H_2O_2 (0.5 mM y 4 mM) o menadiona (0.5 mM y 2 mM). Se llevó a cabo un control sin estrés para cada ingrediente. Los agentes oxidantes fueron posteriormente eliminados por centrifugación a 2700 g, durante 10 min, a 20 ºC y las células se resuspendieron en medio YPD fresco para posteriormente analizar su crecimiento mediante medición de la DO_{600}. Los cultivos se distribuyeron en placas de 96 pocillos con un volumen final de 250 µL por pocillo y cuatro réplicas de cada condición ensayada. Finalmente, se monitorizó el crecimiento de la levadura a 30 ºC mediante la medición de la DO_{600} en un lector espectrofotométrico de placas (BMG Labtech Omega Spectrostar) con agitación a 600 rpm durante 18 h. Los ensayos se llevaron a cabo por triplicado.

Se calcularon las “tasas de crecimiento” para cada condición a 5, 9, 12, 16 y 18 h de incubación, como el cociente entre la curva de crecimiento del cultivo sometido a estrés y la curva de crecimiento del mismo cultivo no expuesto a estrés oxidativo. Posteriormente, para evaluar si los ingredientes mostraban actividad protectora antioxidante, se construyeron las “curvas de efecto” dividiendo el valor de la tasa de crecimiento del cultivo preincubado con el ingrediente entre la tasa de crecimiento del cultivo preincubado sin él para cada una de las dosis de estrés ensayadas. Por último, se determinaron las diferencias estadísticamente significativas ($p<0.05$) mediante el test de la t de Student.

2.7.3. Estudio de la capacidad antioxidante de fracciones de cacao y compuestos puros

Se utilizó el método de crecimiento de *S. cerevisiae* en placa multipocillo previamente puesto a punto para el estudio de la capacidad protectora antioxidante de las fracciones del cacao y los patrones comerciales. Se ensayaron cuatro concentraciones de cada fracción con el objetivo de cubrir un amplio rango de dosis, ajustadas al compuesto mayoritario presente en la fracción o, en el caso de las fracciones que carecían de compuestos mayoritarios, ajustadas al contenido total de polifenoles (Tabla 2). Los patrones comerciales se ensayaron a una concentración de 10 mg/L. El extracto de polifenoles de cacao completo (350 mg EE/L) se utilizó como control positivo. Los experimentos se llevaron a cabo por triplicado. Las diferencias estadísticamente significativas ($p<0.05$) se calcularon mediante el test LSD (one-way ANOVA).

Tabla 2. Fracciones, compuestos puros y concentraciones estudiadas con el método en placa multipocillo.

FRACCIÓN (compuesto principal)	F2 (teobromina)	F5	F10 (catequina)	F14 (B2)	F16 (cafeína)	F19 (epicatequina)	F24
Concentración (mg/L)	5	2	0.5	5	2	5	5
	10	5	1	10	10	10	10
	50	10	2	50	25	50	50
	100	25	5	100	50	100	100
COMPUESTO PURO	Teobromina		Catequina	Procianidina B2	Cafeína	Epicatequina	
Concentración (mg/L)	10		10	10	10	10	

2.8. Análisis proteómico de la respuesta de *S. cerevisiae* al estrés oxidativo mediada por los polifenoles del cacao

2.8.1. Preparación de las muestras para el análisis proteómico

Una colonia de la cepa BY4741 de *S. cerevisiae* fue inoculada en 5 mL de YPD fresco e incubada durante 6 h a 28 ºC con agitación a 40 rpm. Una alícuota de 10 µL de este precultivo se inoculó en matraces con 15 mL de medio YPD, enriquecidos o no con el

extracto de cacao (350 mg EE/L de polifenoles totales), e incubados durante 18 h a 28ºC con agitación a 200 rpm en un agitador orbital. Posteriormente, se recogieron las células por centrifugación a 2700 g durante 10 min a 20 ºC y se resuspendieron en 15 mL de PBS. Se diluyó la suspensión de células (10 mL) 1/20 en PBS (obteniéndose una DO_{600} de 0.3) y preadaptó incubando durante 30 min a 28 ºC. Para someter los cultivos (tratados o no con el extracto de cacao) a estrés oxidativo, se añadió peróxido de hidrógeno hasta alcanzar una concentración final de 5 mM y las células fueron incubadas durante 90 min a 28 ºC con agitación a 200 rpm. Un volumen equivalente de PBS fue añadido a los cultivos no sometidos a estrés oxidativo y ambas muestras (tratadas y no tratadas con extracto de cacao) fueron manipuladas de la misma manera para evitar variaciones en el ensayo. Finalmente, se recogieron las células por centrifugación a 2700 g durante 10 min a 20 ºC y se lavaron con agua milliQ. Se eliminó el agua por centrifugación en las mismas condiciones y el pellet resultante se congeló inmediatamente con nitrógeno líquido y se guardó a -80 ºC. Se llevaron a cabo tres réplicas biológicas por condición.

2.8.2. Extracción de proteínas y separación mediante electroforesis bidimensioanl

La extracción de proteínas de las muestras se llevó a cabo según el protocolo descrito por Gómez-Pastor, Pérez-Torrado, Cabiscol & Matallana (2010). Las células se resuspendieron en 150 µL de tampón de extracción (8 M urea, 25 mM Tris-HCl pH 8.0), una mezcla de inhibidores de proteasas (200 mM phenylmethylsulphonyl fluorido, 20 mM TPcK, 200 mM pepstatina A) y 0.2 g de perlas de vidrio. A continuación, se rompieron las células en un Fast Prep (MP Bio) a 5.0 ms^{-1} durante 45 s en tres veces. Tras centrifugación a 16000 g durante 10 min a 4 ºC, se sonicó el sobrenadante durante 10 min y se volvió a centrifugar en las mismas condiciones. La concentración de proteínas se determinó utilizando un espectrofotómetro Nanodrop ND-1000 UV/Vis.

La electroforesis bidimensional (2D-GE) se llevó a cabo en el Servicio de Proteómica del Centro de Investigación Príncipe Felipe (CIPF) (Valencia, España). Se utilizaron tiras IPG de 24 cm, pH 3–10 (GE Healthcare) para la separación de las proteínas. Dichas tiras fueron rehidratadas *overnight* en 450 µL de tampón 2D (7 M de urea, 2 M de tiourea, 1 % (w/v) de NP-40, 4 % de ditiotreitol (DTT), 1 % de anfolitos (Pharmalite 4–7, GE

Healthcare) y 0.002 % de azul de bromofenol) conteniendo las muestras de proteínas (100 µg) a temperatura ambiente. El enfoque isoeléctrico se llevó a cabo de la siguiente manera: 500 V durante 2h; 500-1000 V durante 2 h; 1000-5000 V durante 2 h; 5000-8000 V durante 4 h y 8000 durante 8 h.

Para la electroforesis de la segunda dimensión en gel de poliacrilamida (PAGE), las tiras IPG enfocadas se equilibraron durante 15 min en 50 mM Tris-HCl, pH 8.8, 6 M urea, 30 % (v/v) de glicerol, 2 % p/v de dodecilsulfato sódico (SDS), 2 % p/v de ditiotreitol (DTT) y después durante 15 min en el mismo tampón conteniendo 2.5 % p/v de iodoacetamida. Las muestras se resolvieron por SDS-PAGE con geles de poliacrilamida al 12.5 % en un sistema vertical (Ettan DALT six; GE Healthcare, 26 cm x 20 cm x 1 mm). La electroforesis se llevó a cabo a 1 W por gel hasta que el color frontal alcanzó el final del gel.

2.8.3. Visualización de proteínas y análisis de imágenes.

La visualización de las proteínas se llevó a cabo en el Servicio de Proteómica del CIPF como se indica a continuación. 12 geles (tres para cada condición) fueron fijados durante 1 h en 50 % v/v de metanol y 10 % v/v de ácido acético y posteriormente se tiñeron con con SYPRO Ruby (BioRad). Las imágenes de los geles se obtuvieron utilizando un escáner de alta resolución (Typhoon FLA9000, GE Healthcare).

Las diferencias entre ambas condiciones (con y sin extracto de cacao, en ausencia de estrés oxidativo, y con y sin extracto de cacao, en presencia de estrés oxidativo) fueron analizadas mediante el software de análisis de imagen PD Quest Advanced (Biorad). Los spots que mostraron al menos una diferencia de expresión de dos veces y cuya diferencia en cantidad fue estadísticamente significativa a un nivel de confianza del 95 % (Student's t-test) fueron consideradas proteínas candidatas.

2.8.4. Identificación de proteínas por MALDI-MS/MS

Los spots que mostraron una expresión diferencial fueron extraídos manualmente y digeridos con tripsina para su identificación por espectrometría de masas en un sistema MALDI-TOFTOF 5800 (AB Sciex) en el Servei Central de Suport a la Investigació (SCSIE) de la Universidad de Valencia (España). Se trataron las muestras con H_20/ACN (1:1, v/v) durante 10 min dos veces y después se añadió ACN hasta la deshidratación del gel. Posteriormente, se rehidrataron las muestras con 50 mM de NH_4HCO_3 durante 5 min y se añadió ACN durante 15 min, y otra vez durante 5 min.

Las muestras fueron posteriormente digeridas de la siguiente manera: las proteínas fueron reducidas con 10 mM de DTT en 50 mM de bicarbonato de amonio y se llevó la alquilación con 55 mM de iodoacetamida en 50 mM de bicarbonato de amonio. Los trozos de gel fueron después lavados con H_2O y acetonitrilo durante 2 min y después acetonitrilo durante 5 min. 10 µL de tripsina a una concentración final de 5 ng/µL fueron añadidos al gel seco y después de su rehidratación, se añadieron 20 µL de 50 mM bicarbonato de amonio. La digestión con tripsina se llevó a cabo a 37 ºC *overnight*. Por último, 3 µL de ácido trifluoroacético al 10 % (pH=1) fueron añadidos para detener la reacción. Una alícuota de la solución de digestión previamente descrita (1 µL) fue depositada en la sonda MALDI y secada a temperatura ambiente. A continuación, se añadieron 0.5 µL de la solución matriz (5 mg/mL de ACH en 50 % de ACN 0.1 % TFA). Se adquirieron los espectros en el modo ión-positivo y cinco iones fueron automáticamente seleccionados entre aquellos que mostraron mayor intensidad, excluyendo los correspondientes a contaminaciones conocidas. Se obtuvo el espectro de fragmentación de cada uno de estos iones. Los datos combinados de MS y MSMS fueron analizados juntos con el algoritmo MASCOT (Matrixscience) y se utilizó la base de datos Expasy_SwissProt.

Las proteínas se clasificaron en categorías funcionales ($p<0.01$) con el programa Functional Specification (FunSpec), a partir de la información obtenida con la base de datos GO. La clasificación se realizó con los genes que codifican para las proteínas que resultaron sobreexpresadas y reprimidas en cada condición (en presencia y ausencia de estrés oxidativo).

2.8.5. Estudio de la capacidad del extracto de cacao para inducir respuesta celular antioxidante en cepas deletantes en genes potencialmente implicados en la respuesta de *S. cerevisiae* mediada por el extracto de cacao.

El método en placa multipocillo se utilizó para estudiar la capacidad del extracto de cacao para afectar el fenotipo de cepas de deleción de *S. cerevisiae* en genes que codifican para las proteínas que resultaron diferencialmente reguladas en el estudio proteómico. Se obtuvieron las curvas de crecimiento de la cepa silvestre de *S. cerevisiae* y de los mutantes de deleción seleccionados, tras su incubación con y sin el extracto polifenólico de cacao y la presencia o ausencia de estrés oxidativo inducido con dos concentraciones de H_2O_2 (0.5 y 4 mM). Posteriormente, se calcularon las "tasas de crecimiento" y las "curvas de efecto" de acuerdo a lo descrito en el apartado 7.2. Las diferencias estadísticamente significativas ($p<0.05$) se calcularon con el test de la t de Student.

2.9. Análisis transcriptómico de la respuesta de *S. cerevisiae* al estrés oxidativo mediada por los polifenoles del cacao.

2.9.1. Preparación de las muestras para el análisis transcriptómico.

Una colonia de *S. cerevisiae* fue inoculada en 5 mL de medio YPD e incubada durante 6 h a 28 ºC con agitación a 40 rpm. Una alícuota de 60 µL de este precultivo fue posteriormente inoculada en un matraz con 30 mL de YPD, conteniendo o no el extracto de polifenoles de cacao (350 mg EE/L) e incubada durante 18 h a 28 ºC con agitación a 200 rpm. Se recogieron las células por centrifugación a 2700 g durante 10 min a 20 ºC y se resuspendieron en 30 mL de PBS. A continuación, una alícuota de esta suspensión de células fue diluida 1/20 en 200 mL de PBS (para alcanzar una DO_{600} de 0.1) y preadaptada incubando durante 30 min a 28 ºC. Para generar estrés oxidativo, se añadió peróxido de hidrógeno a una concentración final de 1 mM a los cultivos y se incubaron durante 15 y 45 min a 28 ºC con agitación a 200 rpm. Finalmente, se recogieron las células por centrifugación a 2700 g durante 10 min a 4 ºC y se lavaron con agua milliQ. Se eliminó el agua por centrifugación y el pellet resultante se congeló inmediatamente con

nitrógeno líquido y se guardó a -80 ºC. Se llevaron a cabo tres réplicas biológicas independientes por condición.

2.9.2. Análisis con microchips

La extracción de ARN de los cultivos (con y sin extracto de cacao a 15 y 45 min de incubación con 1 mM de H_2O_2) se llevó a cabo con el Mini Kit RNeasy® (QIAGEN) de acuerdo con las instrucciones del fabricante. Las muestras fueron posteriormente tratadas y analizadas en el Servicio de Genómica y Genética Translacional del CIPF. El ARN fue cuantificado por espectrofotometría (NanoDrop ND1000, NanoDrop Technologies, Wilminton, Delaware USA) y su calidad fue confirmada mediante un ensayo de ARN 6000 Nano Bioanalyzer (Agilent Technologies, Palo Alto, California USA). 150 ng de ARN total fueron utilizados para producir cRNA marcado con Cyanina 3-CTP y Cyanina 5-CTP utilizando el Kit Low Input Quick Amp Labelling, Two-Color (Agilent p/n 5190-2306) de acuerdo con las instrucciones del fabricante. Siguiendo el protocolo 'Two-Color Microarray-Based Gene Expression Analysis' Versión 6.7 (Agilent p/n G4140-90050), 600 ng de cRNA marcados fueron hibridados con el microchip Yeast (V2) Gene Expression Microarray, 8x15K (Agilent p/n G4813A-016322) conteniendo 6,256+ sondas de *S. cerevisiae*. Los chips fueron escaneados en un escáner Agilent Microarray (Agilent G2565C) siguiendo el protocolo del fabricante y los datos fueron extraídos utilizando el programa Agilent Feature Extraction 10.10.1.1 de acuerdo al protocolo de Agilent GE2_1010_Sep10, grid template 016322_D_F_20120509 y el QC Metric Set GE2_QCMT_Sep10. Se analizaron los datos con el Babelomics 4.2 Suite. La comparación se llevó a cabo utilizando el test Limma con corrección fdr ($p<0.0005$).

Los genes que mostraron una diferencia de expresión de al menos dos veces (en cultivos tratados con cacao frente a los no tratados) fueron clasificados en categorías funcionales ($p<0.05$) con el programa Genomic Research Environment (GenRE). Se analizaron independientemente los genes sobreexpresados y reprimidos, para cada tiempo (15 y 45 min).

2.9.3. Validación de los microchips por RT-qPCR

La validación de los análisis con microchips se llevó a cabo por RT-qPCR. Para ello, se seleccionaron 12 genes: 9 genes regulados diferencialmente (en cultivos tratados con extracto de cacao frente a cultivos no tratados, sometidos a 15 min o 45 min de estrés oxidativo con H_2O_2) y 3 patrones internos (housekeepings) (Tabla 3).

Tabla 3. Genes seleccionados para la validación de los microchips por RT-qPCR. Se muestra el tiempo de incubación de los cultivos con H_2O_2 en el cual los genes mostraron una diferencia de expresión de al menos 2 veces (up=sobreexpresados; down=reprimidos).

GEN	Tiempo	Regulación
GRE2/YOL151W	15’	UP
YIL057C	15’	UP
YJR096W	15’	UP
HSP31/YDR533C	45’	UP
TSA2/YDR453c	45’	UP
CTA1/YDR256C	15’, 45’	UP
SRX1/YKL086W	15’	UP
CCP1/YKR066C	45’	UP
RPS17B/YDR447C	45’	DOWN
DOT1/YDR440W	45’	DOWN
OGG1/YML060W	15’	DOWN
REX4 /YOL080C	45’	DOWN
ACT1/YFL039C		
TAF10/ YDR167W	Housekeepings	
UBC6/ YER100W		

2.9.3.1. Diseño y verificación de los cebadores

La secuencia de los genes se obtuvo de la base de datos SGD. El diseño de los cebadores se llevó a cabo con el programa Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) considerando los siguientes parámetros: una longitud de 18-23 pb, 70-150 amplicones, 50 % (óptimo) G+C

con intervalos admitidos del 30-80 % y una temperatura aproximada de 62 ºC (óptima) con un rango de 60-63 ºC. Para evitar la formación de dímeros, los cebadores fueron verificados con el programa Gene Runner (Versión 3.05) utilizando el parámetro dG > -9 kcal/mol. Los genes *ACT1*, *TAF10* y *UBC6* fueron utilizados como patrones internos, ya que su expresión es constitutiva. La Tabla 4 muestra los correspondientes cebadores forward/reverse para los genes seleccionados, sus secuencias, su temperatura de melting y el amplificado esperado.

Los cebadores fueron posteriormente verificados por PCR con el objetivo de confirmar la correcta amplificación del ADN. Las reacciones se realizaron con tampón de PCR (conteniendo Mg_2Cl), 0.5 µM de cebador F, 0.5 µM de cebador R, 0.2 mM de dNTPs y 0.025 U/µL de Taq (Fermentas,Thermo 1.25 U) en un volumen final de 25 µL. Las condiciones de PCR fueron 95 ºC durante 4 min, seguidos de 30 ciclos de 94/60/72 ºC durante 25/25/40 sec, respectivamente y 72 ºC durante 10 min, llevada a cabo en un termociclador G-Storm. El tamaño del producto de PCR fue verificado por electroforesis en un gel de agarosa al 2 % con Gelred.

Tabla 4. Cebadores diseñados para el ensayo de validación de microchips por RT-qPCR. F= forward; R= reverse; Tm= temperatura de melting.

Gen		Secuencia 5'……3'	Tm	Amplificado esperado
SRX1/YKL086W	F	ATTCCCACCGCTAGCAAGAC	61.5	129
	R	GCCGCCGAAGGCATAATATAG	62.8	
CTA1/YDR256C	F	CCCCAGGTGATGTAGATTTCG	61.6	137
	R	AACGCGCTGCTGTATTTGAG	61.5	
HSP31/YDR533C	F	GGCAACCGTTGAAGATGTTG	61.5	131
	R	CGCAGAAGCAGGATTCACAC	62	
YIL057C	F	CCTTTGACCGGATTGTTTGG	62.5	101
	R	CATTGCGCCATCGTGTTTAC	62.4	
GRE2/YOL151W	F	GAACACATCTTGCGAACTCGTC	62.1	105
	R	GCCTTTGCAACATCACGAAC	61.6	
TSA2/YDR453C	F	CCAGAAAAGACGGTGGATTAGG	62	141
	R	TTCCCTTCGGGTCGATTATG	62.1	
YJR096W	F	CCTTGCAGCCTACAACTTTGAAC	62.3	91
	R	TGTGCATTCCCAATCGGTAG	61.8	
CCP1/YKR066C	F	AACGACGCGAACAACGAAC	62.2	136
	R	CCTTGAAGAACTTGTCCTGGTC	60.1	
RPS17B/YDR447C	F	CGTTGACAACCAAACCTCTGAC	61.9	78
	R	TGAGCGGAAACGTTGATGAC	62.2	
DOT1/YDR440W	F	TGTTTCATGGACGCATAGTGG	61.9	81
	R	GCAGGGCTGAATAAACTTTCG	61.1	
OGG1/YML060W	F	GGCCCAAGGTGTCCTATTCTC	62.1	115
	R	CGTAACAATTGCTTCCGTTTCC	62.8	
REX4/YOL080C	F	GACTGCGGATATTCTGGAAGG	61.1	81
	R	GGGTGGGATAACATCAATGC	60	
ACT1/YFL039C	F	TTCCCAGGTATTGCCGAAAG	62.2	125
	R	GCCAAGATAGAACCACCAATCC	61.9	
TAF10/YDR167W	F	CGTGCAGCAGATTTCACAAC	60.5	111
	R	TTGAGCCCGTATTCAGCAAC	61.2	
UBC6/YER100W	F	TGCGGCAAATACAGGTGATG	62.9	123
	R	TGTCTCAACGCTTGTTCAGC	60.2	

2.9.3.2. Curvas de eficiencia

Para la cuantificación de la expresión génica por RT-qPCR, se prepararon curvas de calibrado con diluciones seriadas de cDNA (10^{-1} a 10^{-6}) para cada gen. Las reacciones se llevaron a cabo en placas de 96 pocillos en un equipo LightCycler 480 Real Time PCR (Roche Diagnostics) y los datos fueron analizados con el Software LightCycler® 480 release 1.5.0 versión 1.5.0.39. Se llevaron a cabo tres réplicas. Tras el análisis, se eligió la dilución 10^{-2} del cDNA para los ensayos de expresión.

2.9.3.3. Reacciones de RT-qPCR

Los experimentos de RT-qPCR se llevaron a cabo como se indica a continuación: 3 µL de la dil 10^{-2} de cDNA y 7 µL de Master Mix (1.4 µL de H_2O libre de nucleasa, 0.3 µL de cebador F (10 µM), 0.3 µL de cebador R (10 µM) y 5 µL de LightCycler® 480 Syber Green I Master 2x (Roche)) fueron mezclados. Tres cDNAs independientes fueron analizados por duplicado. Controles sin cDNA fueron incluidos para cada gen y placa. Las condiciones de reacción fueron 95 ºC durante 10 min y 40 ciclos de amplificación a 95/60/72 ºC durante 10/10/10 sec, respectivamente. Los valores de intensidad de fluorescencia se recogieron durante los pasos de extensión a 72 ºC. La especificidad de la reacción se verificó mediante el análisis de las curvas de disociación. Se utilizaron tres genes (*ACT1*, *TAF10* and *UBC6*) para la normalización de los resultados (housekeepings). Las reacciones se llevaron a cabo en placas de 96 pocillos en un equipo LightCycler 480 Real Time PCR (Roche Diagnostics) y los datos fueron analizados con el Software LightCycler® 480 Software 1.5 con la herramienta de cuantificación relativa utilizando la media de los tres housekeepings para calcular la ratio gen diana/gen de referencia para cada condición (con y sin (control) extracto de cacao tras 15 o 45 min de tratamiento con H_2O_2). Finalmente, las ratios (ratio cacao/ratio control) de los genes seleccionados fueron calculadas para determinar el efecto del tratamiento con extracto de cacao en la expresión génica.

2.10. Utilización de *C. elegans* como organismo modelo

2.10.1. Cepas y mantenimiento

Las cepas de *C. elegans* utilizadas en este trabajo fueron la cepa silvestre N2 (var. Bristol), la cepa CF1038 [*daf-16(mu86)* I.] y la cepa TJ356 [*zIs356 IV (pdaf-16::daf-16-gfp; rol-6*)]. Todas ellas fueron obtenidas del *Caenorhabditis Genetics* Center (CGC). Las cepas de *Escherichia coli* OP50 y OP50-1 (resistente a estreptomicina) se obtuvieron del mismo centro. Los gusanos se mantuvieron a 20º C en placas con Medio de Crecimiento de Nematodo (NGM) [3 g/L de NaCl, 2.5 g/L de peptona, 17 g/L de agar, 1 mL/L de colesterol 5 g/L (en etanol), 1 mL/L de $MgSO_4$ 1 M, 25 mL de tampón KPO_4 1 M pH 6.0] sembradas con *E. coli* OP50. La sincronización de los cultivos se realizó mediante tratamiento con hipoclorito sódico de los adultos grávidos para los ensayos de estrés térmico y acumulación de ROS. En el caso de los ensayos de resistencia a estrés con peróxido de hidrógeno y arsenito sódico y en los ensayos de translocación de DAF-16-GFP, la sincronización se llevó a cabo mediante la puesta de huevos de adultos grávidos durante 2 h.

2.10.2. Ensayos de estrés oxidativo con peróxido de hidrógeno

Se sincronizaron los cultivos en placas de NGM estándar (control) y en placas suplementadas con extracto de cacao (100 mg EE/L) o fracción (10 mg/L de compuesto principal o de polifenoles totales, dependiendo de la fracción), sembradas con *E. coli* OP50 durante 2 h. Los adultos fueron posteriormente eliminados y los huevos sincronizados se incubaron durante 4 días a 20 ºC. Los nuevos adultos se transfirieron a placas de medio basal (5.85 g/L de NaCl; 17 g/L de agar; 50 mL/L de tampón KPO_4 pH 6.0; 1 mL/L colesterol 5 g/L en etanol) con una concentración final de 2 mM H_2O_2 e incubaron a 20 ºC durante 4 h. El recuento de la supervivencia se llevó a cabo mediante un toque suave de los gusanos con un punzón de platino, contando como muertos aquellos que no respondían al estímulo y como censurados a los que subieron por las paredes de las placas o sufrieron eclosión interna de los huevos. Se llevaron a cabo al menos 3 réplicas independientes con 100 adultos cada una para todos los ingredientes

ensayados. Por último, se determinaron las diferencias estadísticamente significativas ($p<0.05$) mediante análisis simple de la varianza (one-way ANOVA) con LSD post-test.

2.10.3. Ensayos de estrés oxidativo con arsenito de sodio

Larvas L4 y adultos jóvenes sincronizados fueron tratados durante 3 días con extracto de cacao (estandarizado a una concentración de 100 µM de epicatequina o cafeína), 100 µM de compuesto puro (stock de epicatequina o cafeína 100 mM en DMSO), F16 (ajustada a 100 µM de cafeína), F19 (ajustada a 100 µM de epicatequina) o 0.1 % DMSO y agua (control). La incubación se llevó a cabo a 20 ºC en placas con medio NGM líquido conteniendo 120 µM de FUDR (5-fluoro-2´-deoxyuridina) para evitar descendencia viable, 1 % (w/v) de seroalbúmina bovina (BSA), 50 µg/mL de esptreptomicina y 10^9 de *E. coli* OP50-1/mL como fuente de alimento, en placas de Petri de 35mm. Los gusanos fueron posteriormente transferidos a placas con 1.5 mL de M9 suplementadas con 4 mM de arsenito de sodio, 120 µM de FUDR y 10^9 de *E. coli* OP50-1/mL e incubadas a 20 ºC. Se midió la viabilidad de los nematodos cada día mediante toques con el punzón hasta las 100 h de tratamiento con el agente estresante. Se representaron las curvas de supervivencia mediante el análisis de supervivencia Kaplan-Meier con el test Log Rank (Mantel-Cox) para cada condición. El análisis estadístico se realizó con el programa GraphPad Prism 6 (La Jolla, USA). Las diferencias estadísticamente significativas ($p<0.05$) se determinaron mediante el análisis de la varianza (one-way ANOVA) con el test Dunnett´s post-test.

2.10.3.1. Ensayos de locomoción

Tras 72 h de estrés con arsenito de sodio, los gusanos vivos fueron clasificados en 3 grupos dependiendo de su capacidad de locomoción: A) gusanos que se mueven activamente en las placas; B) gusanos inmóviles que se mueven activamente tras tocarlos y C) gusanos inmóviles que apenas responden al toque con el punzón.

2.10.4. Ensayo de acumulación de ROS (DCF)

El H_2DCF-DA (2',7'- dichlorodihydrofluoresceina–diacetato; Sigma) atraviesa las membranas celulares transformándose en el derivado no fluorescente H_2DCF el cual se queda atrapado dentro de las células. Posteriormente, este derivado puede ser oxidado por las ROS intracelulares dando lugar al compuesto fluorescente DCF, pudiéndose medir en un espectrofotómetro de fluorescencia (excitación a 485 nm; emisión a 535 nm). La intensidad de la fluorescencia se correlaciona con la cantidad intracelular de ROS.

Los experimentos se llevaron a cabo de acuerdo a lo descrito por Büchter *et al*. (2013) con algunas modificaciones. Se incubaron las larvas L4/adultos jóvenes con 100 µM del compuesto puro (stock de 100 mM en DMSO), 100 µM del compuesto mayoritario en la fracción ensayada o la misma cantidad de DMSO y agua (control). La incubación se realizó a 20 ºC en NGM líquido conteniendo 120 µM de FUDR, 1 % de BSA, 50 mg/mL de estreptomicina y 10^9 de *E. coli* OP50-1/mL. Tras 48 h, los animales fueron lavados con 1.5 mL de PBST (PBS con 0.1 % de Tween 20) durante una hora. A continuación, se transfirieron los gusanos individualmente (8 por grupo) en 1 µL de PBST a cada uno de los pocillos de una placa de 384 pocillos (Greiner Bio-One, Frickenhausen, Germany) conteniendo 7 µL de PBS y, finalmente, se añadieron 2 µL de H_2DCF-DA (250 µM en PBS) a cada uno de los pocillos para obtener una concentración final de 50 µM de H_2DCF-DA. Se utilizaron pocillos sin gusanos con 8 µL de PBS y la misma cantidad de H_2DCF-DA que en las muestras como background de fluorescencia. Se sellaron las placas para evitar la evaporación con una cinta negra adhesiva (Perkin Elmer, Wellesley, MA, USA). La acumulación de ROS se indujo por estrés térmico a 37 ºC y la fluorescencia se midió a 535 nm (excitación a 485 nm) cada 15 min durante 12 h en un espectrofotómetro de fluorescencia (Synergy Mx, BioTek; Bad Friedrichshall, Germany). Los experimentos se llevaron a cabo por triplicado y el análisis estadístico se realizó con el programa GraphPad Prism 6 (La Jolla, USA). Las diferencias estadísticamente significativas ($p<0.05$) se determinaron mediante el análisis simple de la varianza con el test Dunnett´s post-test.

2.10.5. Ensayos de estrés térmico (SYTOX)

El colorante SYTOX® Green puede cruzar las membranas de las células dañadas tras un estrés térmico. Éste se une con el ADN dando lugar a fluorescencia que puede ser utilizada como marcador del daño celular y, por tanto, útil para determinar la viabilidad de los gusanos (Gill, Olsen, Sampayo & Lithgow, 2003).

Los ensayos se realizaron siguiendo el protocolo descrito por Büchter *et al*. (2013). Los gusanos se trataron de la misma manera que para el ensayo del DCF. Para determinar su supervivencia a una temperatura letal de 37 ºC, los gusanos fueron transferidos individualmente en 1 µL de PBST a una placa de 384 pocillos (Greiner Bio-One, Frickenhausen, Germany) conteniendo 9 µL de PBS y, por último, se añadieron 10 µL de SYTOX® Green 2 µM (Molecular Probes Inc., Leiden, The Netherlands) en PBS (concentración final de 1 µM). La placa se selló para evitar evaporación con una cinta negra adhesiva (Perkin Elmer, Wellesley, MA, USA). La intensidad de la fluorescencia se midió con un espectrofotómetro de fluorescencia (Synergy MX, BioTek; Bad Friedrichshall, Germany) cada 15 min durante 12 h (excitación a 485 nm; emisión a 535 nm). El "tiempo virtual de muerte" se calculó para cada condición considerando un gusano muerto si su intensidad de fluorescencia era más de 3 veces superior a la media de sus 3 valores de fluorescencia iniciales, como describieron previamente Gill, Olsen, Sampayo & Lithgow, 2003. Se estudiaron de 8 a 16 gusanos por condición y al menos 3 ensayos independientes se llevaron a cabo. Las curvas de supervivencia y los valores medios de supervivencia se determinaron con el análisis de supervivencia Kaplan-Meier (Mantel-Cox). El análisis estadístico se llevó a cabo con el programa GraphPad Prism 6 (La Jolla, USA). Las diferencias estadísticamente significativas ($p<0.05$) se determinaron mediante el análisis de la varianza (one-way ANOVA) con el test de Dunnett´s post-test.

2.10.6. Localización intracelular de DAF-16-GFP

Se estudió la activación del factor de transcripción DAF-16 mediada por el extracto de cacao y las fracciones seleccionadas (F16 y F19) utilizando los gusanos DAF-16::GFP (cepa transgénica TJ356). Larvas L4/adultos jóvenes fueron incubados durante 1 h en placas de NGM líquido conteniendo 1 % de BSA, 50 mg/mL de estreptomicina y 10^9 de

E. coli OP50-1/mL y suplementadas con los ingredientes de interés: extracto de cacao (ajustado a 100 µM de epicatequina), F19 (ajustada a 100 µM de epicatequina) y epicatequina pura 100 µM. Se preparó un control sin ingredientes tratado en las mismas condiciones. A continuación, 10 µL de medio conteniendo los gusanos se colocaron en un porta mezclados con 10 µL de levamisol 10 mM (anestésico) y se cubrió con un cubre. Se detectó la translocación nuclear de DAF-16-GFP mediante microscopía de fluorescencia utilizando un Nikon Eclipse Ni-U y un set de filtros GFP (Nikon; Düsseldorf, Germany). Se ensayaron 30 gusanos por condición y se llevaron a cabo 4 experimentos independientes. Las diferencias estadísticamente significativas ($p<0.05$) se determinaron mediante el análisis simple de la varianza con el test de Dunnett´s post-test.

3. CONCLUSIONES

1. Se ha puesto a punto un método en placa multipocillo basado en la monitorización del crecimiento de S. cerevisiae demostrándose su utilidad para evaluar la capacidad de extractos polifenólicos de cacao y uva para inducir respuesta celular antioxidante utilizando tanto H_2O_2 como menadiona para causar estrés oxidativo. La aplicación del método es extensible a otras fuentes vegetales o animales y es adaptable al uso de otros agentes oxidantes u otros tipos de estrés.

2. Se ha confirmado la posible implicación de la sirtuina Hst3p en la respuesta de *S. cerevisiae* al estrés oxidativo mediada por el extracto de cacao constatando la ausencia de respuesta en la cepa de pérdida de función para el gen *HST3*. Sin embargo se ha comprobado que la deleción en el gen *HST3* confiere una mayor resistencia al estrés oxidativo provocado con H_2O_2 o menadiona en las condiciones ensayadas, lo que puede explicar en parte la ausencia de respuesta observada en esta cepa.

3. Los ensayos realizados con fracciones cromatográficas del extracto de cacao mostraron que las fracciones ricas en catequina, procianidina B2 y epicatequina promovían respuesta antioxidante en *S. cerevisiae,* la cual no fue equivalente a

la ejercida por los compuestos puros a igual concentración, por lo que se sugiere la participación en la inducción de respuesta de otros componentes minoritarios presentes en dichas fracciones.

4. El estudio transcriptómico y proteómico de la respuesta de levadura a la exposición al extracto de cacao y al estrés oxidativo sugiere que se produce una modificación del metabolismo de carbohidratos mediante la represión de la glicolisis y la activación de la ruta de las pentosas fosfato con el consiguiente incremento de poder reductor. Además, se ha observado una rápida inducción de algunos de los enzimas de actividad antioxidante como catalasas y peroxidasas. Los experimentos con las cepas mutantes por deleción en genes señalados por el estudio proteómico mostraron que las proteínas Pro2, Gdh1, Ssb2, Yhb1, Imh1, Aro8, Prb1, Hom6, Bmh2, Ilv1, Met6 y Cit1 pueden estar implicadas en la respuesta protectora antioxidante mediada por el extracto de cacao.

5. El extracto de cacao y las fracciones cromatográficas ricas en catequina, procianidina B2 y epicatequina protegieron significativamente a *C. elegans* del estrés inducido por H_2O_2, al igual que sucedió en *S. cerevisiae*. Sin embargo, en estudios de estrés inducido con Na-arsenito, estrés térmico y midiendo la formación de ROS intracelular, sólo el extracto completo mostró diferencias significativas, sugiriendo que la mayor riqueza en compuestos del extracto confiere la mayor capacidad protectora.

6. Se ha demostrado que la expresión del factor transcripcional DAF-16 de *C. elegans* (factor de crecimiento tipo insulina) está modulada por el extracto de cacao pero no por la epicatequina ni por la fracción cromatográfica rica en epicatequina. Se ha comprobado que DAF-16 es esencial para que se produzca efecto mediado por el extracto de cacao sobre la acumulación de ROS intracelular y sobre el estrés térmico, confirmándose además que dicho factor es esencial para que se produzca la protección antioxidante en *C. elegans*. La ruta de la señalización de la insulina, dado que se encuentra muy conservada evolutivamente, puede ser también relevante para los efectos saludables inducidos por el cacao en otras especies.