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Red Quinoa hydrolysates with antioxidant bioactive properties on oxidative stress-induced *Saccharomyces cerevisiae*

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ABSTRACT

Quinoa (*Chenopodium quinoa Willd*) is a pseudo-cereal of great interest for its nutritional value. Specifically, enzymatic hydrolysis of quinoa proteins has shown several biological activities. The purpose of this study was to investigate the influence of enzymatic hydrolysis on antioxidant by the oxygen radical absorbance capacity (ORAC) method and sensory properties of white, red and black quinoa varieties, and to evaluate the *in vivo* antioxidant capacity of the most promising quinoa hydrolysate using *Saccharomyces cerevisiae* BY4741 as an experimental model. The results showed a hydrolysate from red quinoa seeds with a promising sensory profile and antioxidant activity. Although more studies in experimental models and human trials will be necessary to corroborate the antioxidant effect and the mechanisms of action involved, the results obtained may allow the development of new plant-based foods with antioxidant properties scientifically supported and useful in the prevention and/or the treatment of pathologies related to oxidative stress.

1. Introduction

The redox balance, in which oxidative species are rapidly counteracted by antioxidant defence mechanisms, is an essential equation in maintaining health in the organism. Different physiological processes such as activation of transcription factors, apoptosis, immune status and protein phosphorylation reactions are dependent on adequate levels of ROS (Rajendran et al., 2014). When there is an excess of oxidative molecules that cannot be neutralized by the antioxidant defence, a state of oxidative stress is produced. This imbalance is associated with the appearance and development of different pathological processes such as cardiovascular diseases, inflammatory diseases and neurological disorders (Sharifi-Rad et al., 2020). In this situation, it has been shown that the consumption of plant-based foods with a high content of antioxidant compounds leads to an increase in the body's antioxidant defence systems and plasma antioxidant status, which protects against various cardiovascular risk factors (Garcia & Blesso, 2021). Among exogenous antioxidants, those that can be obtained through food or in the form of nutritional supplements, have gained much attention as a natural source of antioxidant compounds. In this group stand out carotenoids, phenolic compounds, organosulfur compounds, bioactive peptides and vitamins C and E, among others. These antioxidant compounds have different mechanisms of action, such as activation of antioxidant enzymes, chelation of metals, blocking lipid peroxidation or elimination of superoxide radicals (Pisoschi et al., 2021).

Quinoa (*Chenopodium quinoa Willd*) is a pseudo-cereal originating in South America of great interest for its nutritional characteristics, agronomic versatility and contribution to food security (FAO, 2011). Although more than 250 species of quinoa have been described, only few species are used for human nutrition (Pathan & Siddiqui, 2022). Among them, white quinoa is the most researched and marketed, followed by red and black quinoa. (Piñuel et al., 2019). Quinoa stands out as an important source of starch, fiber and protein of high biological value as it contains all the essential amino acids (Morales et al., 2020). Quinoa is also a good source of bioactive compounds with antioxidant properties such as triterpenoid saponins, phytoecdysteroids, phenolic compounds, betalains and bioactive peptides (Lin et al., 2019). Specifically, bioactive peptides are inactive amino acid sequences present in

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the native protein, which are released after a process of enzymatic hydrolysis, fermentation or gastrointestinal digestion (Peighambardoust et al., 2021). Once released, these bioactive peptide sequences have demonstrated biological activities *in vitro*, in silico and in murine animal models, such as anticancer, antioxidant, antimicrobial, antithrombotic, antihypertensive, antidiabetic, opioid, immunomodulatory and hypocholesterolemic (Pérez-Gregorio et al., 2020). Specifically, enzymatic hydrolysis of quinoa proteins has shown several biological activities, being antioxidant activity the most studied (Morales et al., 2020). In addition, techno-functional properties of quinoa have been improved as a consequence of the enzymatic hydrolysis process (Daliri et al., 2021). For all these reasons, quinoa emerges as a healthy and sustainable alternative for obtaining functional foods and an alternative to the consumption of animal-based foods.

Although *in vitro* assays to evaluate the antioxidant potential of quinoa hydrolysates are commonly used for their simplicity, there are very few *in vivo* studies to extrapolate these antioxidant effects to humans. *Saccharomyces cerevisiae* is a model organism that has proven useful as screening tool of oxidative stress, since it shares with mammals many of the antioxidant defence systems such as glutathione peroxidase (GPX), superoxide dismutase (SOD) or cytosolic catalase 1 (CTT1) (Gao et al., 2019). In addition, this model system has a number of advantages: Easy handling and genetic manipulation; short life cycle facilitates the study of the mechanisms involved in the ageing process; enables research with millions of cells in low-volume cultures; contains a high degree of conservation of many of the cellular processes common to yeast and human cells (Pérez de Vega et al., 2020; Yalcin & Lee, 2019). Different antioxidant food compounds such as resveratrol, quercetin or astaxanthin have shown to protect against oxidative stress caused by oxidants in *S.cerevisiae* (Dani et al., 2008; Sudharshan & Dyavaiah, 2021). So far, research on *S.cerevisiae* to assess the antioxidant potential of protein hydrolysates is very limited.

Therefore, the purpose of this study was to investigate the influence of enzymatic hydrolysis on antioxidant and sensory properties of white, red and black quinoa varieties, and to evaluate the *in vivo* antioxidant capacity of the most promising quinoa hydrolysate using *Saccharomyces cerevisiae* BY4741 as an experimental model.

2. Materials and methods

2.1. Preparation of quinoa hydrolysate samples

White, red and black quinoa seeds were purchased from Naturquinoa (Madrid, Spain), and ground in a stone mill (Conasi, Jaén, Spain). All quinoa varieties were diluted in distilled water at a ratio of 1:12.5 (w/v). Enzymatic hydrolysis was carried out for 24 h, according to the conditions declared by manufacturers, with the food-grade enzymes: Alcalase 2.4 LFG (E.C. 3.4.21.61, from *Bacillus Licheniformis;* Novozymes (Copenhagen, Denmark)); BC Pepsin 1:3000 (E.C.3.4.23.1, from *pork stomach*), Promod 11P 100TU (E.C.3.4.22.2), Flavorpro 786MDP (from *Bacillus* sp. and *Carica papaya*), Flavorpro 795MDP (from mixed source), Flavorpro 373MDP (from *Bacillus* spp *inc Bacillus subtilis)*, Promod 950L (from microbial), Promod 279P (from *Aspergillus oryzae)*, Promod 24P (from *B. subtilis)* and Flavorpro 750MDP (from *Aspergillus)* were provided by Biocatalysts (Cardiff, UK). From each enzymatic hydrolysis reaction, six aliquots were collected at different time intervals (0, 1, 2, 4, 6 and 24 h), with inactivation carried out according to the manufacturer's instructions. The hydrolysates were centrifugated at 4500 rpm for 15 min, to remove any seed remnant, and the supernatants stored at –20 °C till further processing. Protein concentration was determined by the Detergent Compatible (DC) protein assay (Bio-Rad Laboratories, United Stated) following manufacturer's instructions, and comparing OD at 750 nm by using a bovine serum albumin (5–50 μg/μL) curve standard.

2.2. Basic evaluation of sensory characteristics of quinoa hydrolysates

The basic organoleptic aspects (appearance, colour, odour, texture and flavour) were evaluated with the aim of discarding those hydrolysates with strange or unpleasant flavour properties, and selecting those ones potentially acceptable to consumers. For this purpose, a basic acceptance test was used to examine the acceptability of the hydrolysate or to determine whether one or more samples were more acceptable than others. The sensory evaluation of the samples was carried out by an internal tasting panel of the Food Science Research Institute (CIAL), and in all cases the analyses were evaluated by a trained panel of between 10 and 12 people with an age between 22 and 60 years.

2.3. Determination of in vitro antioxidant capacity of quinoa hydrolysates

Antioxidant capacity of biological sample or food can be determined *in vitro* by the oxygen radical absorbance capacity (ORAC) method. The ORAC assay was carried out as previously described by Garcés-Rimón et al. (2016). Samples and reagents were dissolved in 75 mM phosphate-buffer saline (PBS) (pH 7.4). Reactions were carried out in a black 96-well plate (Corning, Kennebunk, ME, USA), in 200 μL final volume, containing 20 μL of sample or Trolox solution (0.2–2 nmol/L), 120 μL 1.17 mmol/L fluorescein solution and 60 μL of 1.3 g/100 mL 2, 2′-azo-bis-(2-methylpropionamide) dihydrochloride (AAPH) solution (all reagents from Sigma Aldrich, USA). The fluorescence was evaluated with a fluorimeter (SpectraMax M2; Molecular Devices) at 40 °C every 55 s for 95 min, with excitation and emission wavelengths set at 485 nm and 520 nm, respectively. All samples were tested out in triplicate; in addition, in each plate a full standard curve with Trolox solution (0.2–2 nmol/L) was added. The ORAC activity, antioxidant capacity *in vitro*, was expressed as μmol of Trolox equivalents (TEs) per g of protein.

2.4. Determination of total phenolic compounds content (TPCs) in quinoa hydrolysates

Total phenolic compounds (TPCs) were determined according to the modified Folin-Ciocalteu colorimetric method as previously described (Garcés-Rimón et al., 2016). Briefly, reduction of Folin-Ciocalteu reagent by phenolic compounds is measured as absorbance at 765 nm, against a standard calibration curve. The reaction was carried out in 96-well microplates (Corning 9018, Spain), with up to 24 μL of the sample (quinoa hydrolysate, standard solution or water), 47 μL of 10mL/100 mL Folin-Ciocalteu reagent, and 189 μL of 700 mmol/L Na_2CO_3 in a total volume of 260 μL. After mixing all reaction components, absorbance was recorded at 765 nm using a microplate reader (Biotek PowerWave™ XS, BioTek Instruments, Winooski, VT, USA). TPCs content was determined by absorbance assignment to calibration curve using standard solutions of gallic acid (50–1500 μmol/L), and expressed as of mg gallic acid equivalents (GAE) per 100 g of quinoa.

2.5. In vivo evaluation of antioxidant capacity of selected quinoa hydrolysates using the yeast model Saccharomyces cerevisiae

In vivo antioxidant capacity is measured as recovery of yeast cellular activity after oxidative insult by comparing mean values of growth rate, survivors or relative survivors, against to stressed yeast cells, to values similar to those presented by unstressed yeast cells or shown by yeast cells challenged with known antioxidant compounds (Bonache et al., 2018). In this study, we used the haploid *Saccharomyces cerevisiae* strain BY4741 subjected to oxidative stress following the procedure previously described by Mattenberger et al. (2017). Briefly, a starting culture was set up with 10 μL of glycerol stock culture in 5 mL of YPD (1 g/100 mL yeast extract, 2 g/100 mL bactopeptone, and 2 g/100 mL glucose) at 28 °C for 24 h. From this starting culture, two cultures were set, one (50 μL) reinoculated in 5 mL of YPD (as unstressed control), and the second (50 μL) reinoculated in 5 mL of YPOxD medium (1 g/100 mL yeast extract, 2

g/100 mL bactopeptone, 1 g/100 mL glucose and 3 mM H_2O_2) as oxidative stress induced. Tubes were then incubated at 28 °C, 190 rpm for 24h, along with uninoculated media as negative controls. Oxidative-stress induced-yeasts were then challenged to the selected quinoa hydrolysates at 4 doses (1 μL, 5 μL, 10 μL and 20 μL), with at least 5 replicates (technical replicates), to determine yeast growth parameters and survivors.

Yeast growth parameters (growth rate and carrying capacity) were determined by measuring growth on a Bioscreen C plate reader and incubator (Oy Growth curves Ab Ldt., Turku, Finland), as determined previously (Bonache et al., 2018; Mattenberger et al., 2017). Briefly, yeast cultures subjected to oxidative stress (grown on YPOxD) were diluted to an initial optical density OD_{600} of ~0.1 in 180 μL (per well), and added up to 20 μL of testing samples, solvent (PBS or 50 mL/100 mL methanol), reference antioxidants (2 mg/mL vitamin C, 2 mg/mL resveratrol) or uninoculated media (YPOxD; as control, named as Ctrl (OxD)). Optical density was measured in Honeycomb plates (10 × 10 wells, Oy Growth Curves Ab), including negative controls (uninoculated media YPD or YPOxD), and unstressed yeasts (only grown in YPD) or completely stressed yeast cells (grown in YPOxD without any antioxidant compound), with Bioscreen set at filter 600 nm (brown), 28 °C, OD_{600} measurements every 15 min for 4–5 days ± 1 h, and with high and continuous shaking. Each quinoa hydrolysate or control samples were tested with at least 3 technical replicates. Growth rate (r) and Carrying capacity (k) were determined from corrected OD_{600} values after adjusting observed values to the logistic equation common in ecology and evolution using Growthcurver v.0.3.1 library in RStudio v April 1, 1106 defined by (Sprouffske & Wagner, 2016).

$$N_t = K / [1 + (K - N_0 / N_0)\, e^{-rt}]$$

Where N_t is the number of cells at time t, N_0 is the population size at starting, k or carrying capacity is the maximum possible population size in a particular environment, and r or intrinsic growth rate, is the growth rate that would occur if there were no imposed restrictions on total population size. Growthcurver determines the best values of k, r and N_0 for the growth curve data using the implementation of the non-linear least-squares Levenberg-Marquard algorithm included in the R package (Sprouffske & Wagner, 2016).

Survivors (viable cells after stressing insult) were determined as colony forming units (CFUs) after exposure to the oxidative stress and challenged to the tested quinoa hydrolysates or reference antioxidants (vitamin C or resveratrol), from the Bioscreen assay. For this, challenged cultures from each Honeycomb plate were serially diluted in 96-well plates with 180 μL of sterile 10 mL/100 mL glycerol (by transferring 20 μL from each row to the next). Subsequently, they were spotted on YPDA plates (1 g/100 mL yeast extract, 2 g/100 mL bactopeptone, 2 g/100 mL glucose and 1.5 g/100 mL bactoagar), transferring 1.65 μL of each well on a Petri dish (90 × 15 mm) with an 8x6-pin replicator (Sigma-Aldrich, USA) to conform an 8 × 6 spots array. Each serial dilution was plated in triplicate. Plates were incubated for 48 h at 28 °C. After incubation, CFUs were determined as:

$$\text{Survivors (CFUs } \mu L^{-1}) = [(C_n \text{ x DF}) / 1.65\ \mu L]$$

Where C_n is the number of colonies per spot, DF is the dilution factor, and 1.65 μL is the volume deposited by each pin (of the SIGMA 8 × 6 array replica plater). In addition, we determined the Relative Survivors as:

$$\text{Relative Survivors (a.u.)} = [\text{Sample CFUs } \mu L^{-1} / \text{average Ctrl(OxD) CFUs } \mu L^{-1}]$$

2.6. Statistical analysis

The results of the *in vitro* determination of ORAC or TPC were presented as the mean value ± standard deviation (SD), whereas *in vivo* determination of antioxidant capacity measured as recovery of growth rate and CFUs or relative survivors were expressed as mean value ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to determine the differences between group values, followed by Bonferroni *post hoc* test at a significance level $p < 0.05$. Pearson's correlation was calculated to test the linear relationships between the phenolic content and the antioxidant activity determined with *in vitro* assays. Statistical analyses were performed with GraphPad Prism version 9.0.2 for Mac OS (Graph-Pad Software, United States).

Fig. 1: shows an outline of the methodology used in this study.

3. Results and discussion

3.1. Sensory features of quinoa hydrolysates

The different hydrolysates from quinoa varieties were evaluated for their basic organoleptic aspects (appearance, colour, odour, texture and flavour) by a trained tasting panel. All participants indicated that at t0 (without hydrolysis) all quinoa varieties presented lighter tones with respect to the rest of the hydrolysates and a pleasant flavour, although in the black quinoa both properties were observed to a lesser degree. The hydrolysates obtained with Promod 279P, Flavorpro 373MDP, Flavorpro 750MDP and Alcalase 2.4 LFG showed the most promising results after basic sensory evaluation (Table 1 and Supplementary tables 1A-1C). The rest of the enzymes used triggered unpleasant sensory characteristics related to texture, appearance, flavour and/or odour from the early stages of hydrolysis (results not shown). In general, the selected hydrolysates showed good sensory characteristics that deteriorated as the hydrolysis process progressed (Table 1 and Supplementary tables 1A-1C). After 6 h of enzymatic hydrolysis, most of the hydrolysates exhibited browning and the appearance of bitter tones, which caused a deterioration of the basic sensory characteristics. The red quinoa hydrolysate produced with Alcalase 2.4 LFG with duration of approximately 1–4 h was the most promising, considering the basic sensory aspects analysed. In the case of the black and white quinoa hydrolysates obtained with Alcalase 2.4 LFG in those time periods were identified bitter, acidic and astringent nuances, which negatively affected the sensorial (Table 1).

The removal of bitter tastes is considered one of the major challenges in the industrial application of food protein hydrolysates, and a key factor in the acceptability of that new food or ingredient. In general, bitter taste is strongly correlated with the presence of hydrophobic peptide molecules (Karami & Akbari-Adergani, 2019). Moreover, the intensity of this bitter taste linked to the peptide fractions seems to be dependent on the degree of hydrolysis of the native protein and the molecular weight of the resulting peptides. During the enzymatic hydrolysis process is triggered the release of hydrophobic amino acids related to the bitter taste, being a phenomenon dependent on the enzyme and the duration of hydrolysis (Görgüç et al., 2020). This could explain why hydrolysis of a prolonged duration presents unfavourable organoleptic properties and justifies that after 6 h most of the quinoa hydrolysates obtained had bitter and/or unpleasant flavours. The browning observed during the course of hydrolysis could be the result of the Maillard reaction, due to the non-enzymatic bonding between the carbonyl groups of the reducing sugars and the free amino acids that leads the formation of melanoidins providing that characteristic brownish hue (Starowicz & Zieliński, 2019). Different factors such as temperature, pH, and duration of heat treatment or substrate concentration determine the development of this reaction. It has been shown that the process of enzymatic hydrolysis in plant matrices favours browning, which increases as the temperature and duration of hydrolysis (Kim et al., 2004).This justified that for the next points, we selected hydrolysates of between 1 and 4 h.

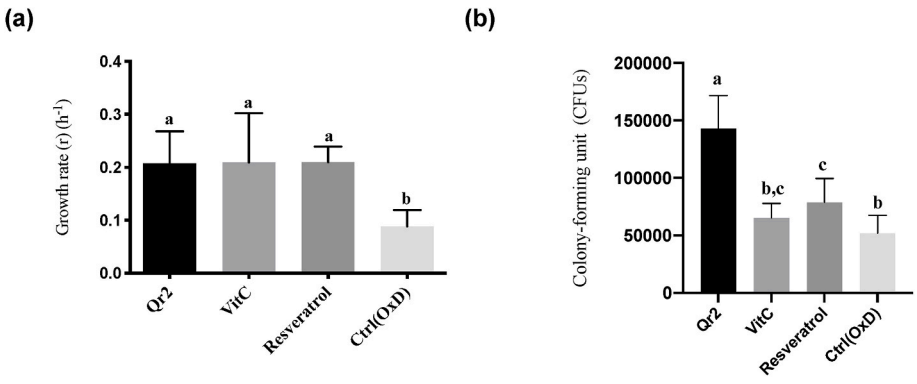

Fig. 1. *In vivo* antioxidant capacity of quinoa hydrolysates determined as recovery of cellular functions after oxidative insult. (**a**) Comparison of the growth rate r (in h^{-1}) (mean ± SEM) of yeast cells subjected to oxidative stress and challenged to red quinoa hydrolysate (Qr2), vitamin C (VitC) or resveratrol against the control group under continuous oxidative treatment (Ctrl (OxD)). Significant differences are indicated with different letters ($p < 0.05$); (**b**) Comparison of the survivors (mean Colony-forming unit ((CFUs) ± SEM) of yeast cells subjected to oxidative stress and challenged to red quinoa hydrolysate (Qr2), vitamin C (VitC) or resveratrol against the control group under continuous oxidative treatment (Ctrl-OxD). Significant differences are indicated with different letters ($p < 0.05$).

Table 1
Basic sensory evaluation of hydrolysates produced with Alcalase 2.4LFG on the different quinoa varieties.

Duration of hydrolysis (h)	White quinoa	Red quinoa	Black quinoa
0	Whitish colour that decants with time. Pleasant taste.	Whitish colour that decants with time, neutral odour. Pleasant taste.	Less whitish in colour. Decants with time. Pleasant smell and neutral flavour with bitter nuances.
1	Sweet smell and taste superior to t0	White colour, neutral odour, decanted with time. Pleasant taste	White colour, pleasant odour and taste with a marked astringency
2	It begins to have bitter fermentation nuances	White colour, neutral odour, decanted with time. Pleasant taste	White colour, pleasant odour and taste with less astringency than t1
4	It begins to have bitter fermentation nuances	White colour, neutral odour, decanted with time. Pleasant taste	White colour, pleasant odour and taste with a bitter aftertaste

3.2. *In vitro antioxidant activity determination of quinoa hydrolysates*

Antioxidant activity was determined by the ORAC method as μmol TE/g for undigested and digested quinoa samples, with the best sensory profiles hydrolysis enzymes (Table 2 for white quinoa, Table 3 for red quinoa, and Table 4 for black quinoa).

All non-hydrolysed quinoa samples showed modest antioxidant activity, although this activity was significantly higher in red quinoa (153.9 μmol ET/g protein) with respect to black quinoa (125.1 μmol ET/g protein) ($p < 0.01$) and slightly higher, but showing no significant difference with respect to white quinoa (145.6 μmol ET/g protein) ($p > 0.05$). White quinoa hydrolysates presented lower antioxidant activity after enzymatic hydrolysis, when compared to red and black quinoa hydrolysates. These data are in line with previous studies, where red quinoa showed the highest antioxidant activity among the different quinoa varieties, showing also variable results depending on geographical origin of red quinoa (Han et al., 2019; Liu et al., 2020).

In this study, the best results of antioxidant activity in the different quinoa varieties and at shorter hydrolysis times (times of less than 6 h, at which time the sensory profile is negatively affected) were obtained with Alcalase 2.4 LFG and Flavorpro 750MDP with maximum ORAC values of 1698.7 and 1815.5 μmol TE/g protein, respectively.

These results show that the antioxidant activity of the quinoa hydrolysates was similar or higher compared to other hydrolysates

Table 2
Antioxidant activity (mean ± SD as μmol TE/g) of white quinoa hydrolysates.

Time (h)	Promod 279P	Flavorpro 373MDP	Flavorpro 750MDP	Alcalase 2.4 LFG
0	145.6 ± 9.0	145.6 ± 9.0	145.6 ± 9.0	145.6 ± 9.0
1	413.3 ± 14.7*	432.1 ± 6.4*	229.5 ± 19.8*	842.6 ± 7.3*
2	399.5 ± 35.5*	473.0 ± 3.8*	584.8 ± 13.9*	884.5 ± 1.8*
4	494.1 ± 19.0*	693.7 ± 10.0*	1179.5 ± 7.8*	608.3 ± 2.0*
6	801.2 ± 34.0*	592.8 ± 14.4*	1009.4 ± 4.5*	696.4 ± 3.4*
24	291.4 ± 9.6*	542.4 ± 7.0*	774.1 ± 14.2*	732.2 ± 1.9*

*means statistical difference $p < 0.05$, One-way ANOVA with Bonferroni *post hoc* test *vs* non-hydrolysed white quinoa.

Table 3
Antioxidant activity (mean ± SD as μmol TE/g) of red quinoa hydrolysates.

Time (h)	Promod 279P	Flavorpro 373MDP	Flavorpro 750MDP	Alcalase 2.4 LFG
0	153.9 ± 7.5	153.9 ± 7.5	153.9 ± 7.5	153.9 ± 7.5
1	534.1 ± 5.3*	1251.9 ± 19.5*	811.8 ± 6.9*	879.4 ± 24.0*
2	597.1 ± 17.1*	892.6 ± 6.3*	689.6 ± 12.9*	1118.4 ± 17.9*
4	790.9 ± 23.0*	461.8 ± 9.7*	1815.5 ± 23.0*	1300.7 ± 31.7*
6	944.9 ± 14.7*	679.2 ± 9.3*	1068.8 ± 18.1*	1133.8 ± 34.6*
24	1200.2 ± 41.5*	1293.6 ± 9.4*	1429.3 ± 17.4*	1164.5 ± 19.8*

*means statistical difference $p < 0.05$, One-way ANOVA with Bonferroni *post hoc* test *vs* non-hydrolysed red quinoa.

Table 4
Antioxidant activity (mean ± SD as μmol TE/g) of black quinoa hydrolysates.

Time (h)	Promod 279P	Flavorpro 373MDP	Flavorpro 750MDP	Alcalase 2.4 LFG
0	125.1 ± 6.2	125.1 ± 6.2	125.1 ± 6.2	125.1 ± 6.2
1	320.2 ± 31.6*	971.8 ± 33.2*	203.6 ± 10.7*	1698.7 ± 6.7*
2	382.2 ± 26.5*	1225.3 ± 8.9*	467.9 ± 17.6*	1205.3 ± 5.2*
4	884.5 ± 45.1*	1445.9 ± 5.4*	799.8 ± 15.3*	1536.7 ± 6.2*
6	729.7 ± 7.4*	2204.3 ± 6.1*	657.7 ± 13.9*	1868.8 ± 5.9*
24	1191.7 ± 44.1*	948.9 ± 19.5*	586.7 ± 17.4*	1539.0 ± 5.5*

*means statistical difference $p < 0.05$, One-way ANOVA with Bonferroni *post hoc* test *vs* non-hydrolysed black quinoa.

obtained in different plant food matrices. A black bean hydrolysate obtained with pepsin and pancreatin presented 1769 μmol ET/g protein, as determined with the same analytical method used in our study (López-Barrios et al., 2016).

Karami and Akbari-Adergani (2019), described that the type of peptidase and the enzymatic hydrolysis conditions determine the degree of hydrolysis and the resulting peptides, and thus also their potential antioxidant activity. In the present study, alcalase was one of the best performing proteases, which is in agreement with the results obtained in other studies with protein hydrolysates from quinoa and other vegetables. Chirinos et al. (2020), found that quinoa protein hydrolysates in the range of 120–240 min with Alcalase 2.4L showed higher antioxidant activity with respect to those obtained with Neutrase 5.0BG or Flavourzyme 500L. In this case the antioxidant activity was performed with the decolorization assay with the cation radical ABTS-+. In another study, Mahdavi-Yekta et al. (2019) evaluated the optimal hydrolysis conditions to obtain a quinoa protein hydrolysate with alcalase and pepsin with higher antioxidant activity. They found that a hydrolysis duration of 150 min caused superior antioxidant activity. Likewise, antioxidant activity was found to be associated with the degree of hydrolysis, suggesting the release of low molecular weight peptide sequences with a higher antioxidant potential as the hydrolysis process proceeded. The specificity of alkaline proteases such as alcalase allows the release of peptides of smaller molecular size during the enzymatic hydrolysis process. Moreover, endopeptidase enzymes including alcalase also cause the cleavage of the bonds at the terminal ends, which induces the release of low molecular weight peptide fractions with higher antioxidant potential (Gong et al., 2020). In general, quinoa protein hydrolysates with a molecular weight lower than 5 kDa have shown higher antioxidant activity relative to those with a higher molecular weight (Guo et al., 2021).

Similarly, numerous works have demonstrated the usefulness of using the enzyme alcalase to produce antioxidant peptides from plant proteins. In this sense, the peanut protein hydrolysate with alcalase showed a higher antioxidant capacity than the hydrolysate obtained with pepsin (Nyo & Nguyen, 2019). Logarušić et al. (2020) evaluated the antioxidant activity of flaxseed hydrolysates obtained with different proteases using the ORAC method. They found that flaxseed hydrolysates with alcalase showed values of 1928 μmol ET/g protein, higher than those obtained with neutrase and protamex. Chirinos et al. (2021) also observed that a *Lupinus mutabilis* protein hydrolysate using Alcalase 2.4 L presented a higher antioxidant activity than with Neutrase 5.0 BG and Flavourzyme 500 L. Similarly, the sweet potato protein hydrolysate obtained with alcalase showed higher antioxidant activity than with the other enzymes used. In this study, the peptide fraction smaller than 3 kDa was responsible for the highest antioxidant activity observed in the protein hydrolysates obtained with alcalase (Zhang et al., 2012).

In addition to alcalase, endopeptidases of bacterial origin such as Flavorpro 373MDP have shown high antioxidant activity in different protein sources compared to other enzymes of different origins (Pan et al., 2020; Piotrowicz et al., 2020). Similarly, aminopeptidases of fungal origin, including Flavorpro 750MDP, have also shown great potential for obtaining bioactive peptides with antioxidant activity (Borrajo et al., 2020; Garcés-Rimón et al., 2015). These enzymes also generated in our study hydrolysates with good *in vitro* antioxidant activity. However, sensory properties have been also considered a key factor and, among all the hydrolysates evaluated, those made with Alcalase 2.4 LFG demonstrated a better sensorial profile together with high antioxidant activity.

In general, the enzymatic hydrolysis process caused an increased antioxidant activity in the different quinoa varieties until reaching a maximum value, after which it began to decrease or underwent oscillations, depending on the enzyme used or digestion time (Tables 2–4). This situation suggests the constant formation and degradation of the different antioxidant compounds as the hydrolysis process progressed. It has been described that the presence of antioxidant compounds such as flavonoids, ecdysteroids or betacyanins in coloured quinoa varieties may be responsible for the higher free radical scavenging capacity described in red quinoa (Choque-Quispe et al., 2021).

3.3. Phenolic compounds content of quinoa hydrolysates

As indicated previously, Alcalase 2.4 LFG quinoa hydrolysates demonstrated a better sensorial profile with high antioxidant activity than the other hydrolysates. Being selected for the next step, by determining the gallic acid equivalents according to the Folin-Ciocalteu colorimetric method. The quinoa varieties studied had a higher content of total phenolic compounds before undergoing the enzymatic hydrolysis process, and suffered a gradual decrease during the hydrolysis process (Table 5). White quinoa suffered a slight reduction in the content of phenolic compounds as the hydrolysis process progressed, with significant differences after 4 h of hydrolysis ($p < 0.05$ *vs* white quinoa without hydrolysis). In the case of red quinoa, the content of phenolic compounds decreased after the first hour of hydrolysis, and remained stable during the following hours. Of the different varieties, black quinoa showed a higher content of phenolic compounds, and specifically the highest value found in the non-hydrolysed black quinoa (Table 5).

The results obtained in the untreated quinoa samples are similar to those described in the literature. Diaz Valencia et al. (2018) reported a higher content of total phenolic compounds in black quinoa with 95.9 mg GAE/100 g of quinoa with respect to the rest of the varieties analysed. Furthermore, in the same study the content of phenolic compounds in red and white quinoa did not change significantly. In this sense, it is important to point out that there are also considerable differences in the content of phenolic compounds according to the origin and growing conditions of quinoa. Farajzadeh et al. (2020), reported a significant variability in the different types of quinoa analysed, since the *Chenopodium quinoa giza* variety presented 38.24 ± 2.45 mg GAE/100 g of quinoa compared to the *Chenopodium quinoa samaja* variety with a content of 17.58 ± 1.12 mg GAE/100 g of quinoa. The content of basal phenolic compounds observed in our study for black quinoa was higher than those described for amaranth (57 mg GAE/100g), another pseudo-cereal belonging to the Amaranthaceae family, and similar to the content of other coloured varieties of different legumes such as red lentils (72 mg GAE/100g) or black beans (90 mg GAE/100g) (Rocchetti et al., 2019).

The enzymatic hydrolysis process generally triggered a reduction in the content of phenolic compounds. It is known that different physicochemical factors such as temperature can cause a reduction in phenolic compounds (Sodeifian & Sajadian, 2021). Thermal processing of foods can lead to changes in the content of phenolic compounds, including their separation from other structures present in the matrix and leaching, as well as degradation, polymerization or formation of Maillard reaction products (Massaretto et al., 2011). In addition, the stability of phenolic compounds is temperature dependent, and it has been reported that temperatures above 125 °C induce significant degradation of some antioxidant compounds such as resveratrol, epicatechin and myricetin (Liazid et al., 2007). This could explain the reduction in the content of

Table 5
Total phenolic compounds (TPCs, mean ± SD), expressed as mg Gallic Acid Equivalents (GAE)/100g of quinoa after different periods of hydrolysis with the enzyme Alcalase 2.4 LFG.

Time (h)	White quinoa	Red quinoa	Black quinoa
0	62.8 ± 0.3	68.9 ± 3.2	81.0 ± 2.3
1	52.8 ± 1.4	44.5 ± 2.4*	78.8 ± 5.9
2	54.1 ± 6.1	37.8 ± 2.3*	75.5 ± 4.9
4	48.7 ± 8.3*	42.2 ± 2.3*	72.5 ± 5.0
6	40.1 ± 8.3*	46.1 ± 6.0*	61.0 ± 4,.5*
24	41.1 ± 7.8*	51.1 ± 7.2*	54.9 ± 2.2*

*means statistical difference $p < 0.05$, One-way ANOVA with Bonferroni *post hoc* test *vs* non-hydrolysed quinoa (white, red or black, respectively).

phenolic compounds observed after the hydrolysis process, and it seems reasonable that the increase in temperature to achieve inactivation of the enzyme Alcalase 2.4 LFG could lead to deterioration in the phenolic compound content of the hydrolysates. This reduction of phenolic compounds was not drastic, because were not reached high inactivation temperatures. Dini et al. (2010), observed that the presence of phenolic compounds was reduced from 77.2 mg GAE/100 g quinoa to 28.7 mg GAE/100 g quinoa after undergoing a thermal process by cooking reaching boiling temperature. In this study, a fraction of the phenolic compounds present in quinoa were released during heat treatment, as 14 mg GAE/100 g of quinoa were identified in the cooking water. These losses may be even higher, as it has been reported that a cooking treatment reduced the initial concentration of phenolic compounds in pearled quinoa from 67.8 mg GAE/100 g to 8.7 mg GAE/100 g (Mhada et al., 2020). It should also be taken into account that the hydrolysis process can cause a disruption of the food matrix and trigger the release of phenolic compounds that were bound to different structural complexes (Nickel et al., 2016). In our case, even if this release had occurred during the hydrolysis process when the temperature was lower, it was not observed a release of phenolic compounds sufficient to counteract the degradation suffered due to the increase in temperature to which the hydrolysates were subjected to inactivate the enzyme.

Finally, the oxygen radical scavenging capacity of quinoa hydrolysates (determined by ORAC method) was not correlated with the total content of phenolic compounds ($p > 0.05$). These findings suggest that the observed increase of antioxidant activity of the quinoa hydrolysates tested here was not due to the increase in the content of phenolic compounds, but rather to the release of other compounds with antioxidant potential, such as bioactive peptides, that deserves further research.

3.4. *In vivo* determination of antioxidant capacity of selected quinoa hydrolysates

As indicated previously, the *in vitro* oxygen radical scavenging capacity of the quinoa hydrolysates was higher for the red quinoa hydrolysate produced with Alcalase 2.4 LFG, whereas this antioxidant capacity was not correlated with the total content of phenolic compounds. To ascertain the effective antioxidant bioactive properties of these quinoa hydrolysates, we used an *in vivo* model, the yeast *Saccharomyces cerevisiae*, to determine the overall recovery of cellular processes in such complex matrix as systems approach to determine biological properties of new bioactive products. To this end, we compared the growth rate (r) and survivors (as CFUs) among yeast cells subjected to continuous oxidative stress and those also challenged to the red quinoa hydrolysate (2h) or treated with known antioxidants vitamin C and resveratrol (Fig. 1). Finding that the red quinoa hydrolysate was able to help yeast cells to recover growth rate significantly, compared to the untreated cells, being of similar weight to the recovery obtained with vitamin C or resveratrol (Fig. 1a). Whereas survivors (or viable cells) after oxidative insult and treatment was significantly higher for the red quinoa hydrolysate, than for the known compounds with antioxidant capacity vitamin C or resveratrol (Fig. 1b).

The results obtained support the *in vitro* antioxidant studies and highlight the antioxidant effect of red quinoa hydrolysate, since it exhibited a significant recovery of the growth rate and survivors (Fig. 1). This is important since the bioavailability, reactivity and stability of a given compound after *in vivo* studies can be modified with respect to the antioxidant capacity observed *in vitro*. Studies performed to evaluate the antioxidant capacity of other food matrices or plant protein hydrolysates using *S. cerevisiae* as an experimental model are limited. A polyphenol-enriched cocoa has been shown to exert antioxidant effects in the same model organism used. This polyphenol-enriched cocoa increased tolerance to oxidative stress when compared to controls grown in YPD. However, in this case the survival rate was lower than the reference antioxidant (vitamin C) (Martorell et al., 2011). Therefore, it could be suggested that our red quinoa hydrolysate presents a higher magnitude of change, given that the rate of recovery of growth parameters exceeded those observed with vitamin C. However, it is important to note that in this study two concentrations of H_2O_2 were tested, which differ from those used with our red quinoa hydrolysate. Therefore, these findings should be verified using the same oxidative stress conditions. Quercetin hyperoside or 3-O-galactoside is another compound that has shown antioxidant potential using the same model organism. This flavonoid increased by 30–60% the survival rate of cells subjected to an oxidizing agent (CCl_4 or H_2O_2) in different isogenic strains of *S. cerevisiae*. The isogenic strain deficient in the gene associated with cytosolic catalase T1 (CCT1) expression exhibited a more modest response than the other strains used (deficient in the superoxide dismutase, glutathione, glutathione transferase 1 or glutathione transferase 2 genes). Therefore, these results suggest that the protective effect of hyperoside against oxidative stress induced by CCl_4 or H_2O_2 could be mediated in part by the action of catalase, involved in the passage of H_2O_2 to generate H_2O or O_2 (Gao et al., 2019). Different peptide fractions isolated from a chia protein hydrolysate have also evidenced an antioxidant effect in a model of *S. cerevisiae*. Specifically, peptides derived from chia hydrolysate produced by sequential addition of Alcalase 2.4 LFG and Flavourzyme 1000L increased the survival rate of cells exposed to an oxidizing agent (H_2O_2) by 27% compared to the control (in the absence of an antioxidant agent). This antioxidant activity of the hydrolysate was reported in the peptide fraction smaller than 3 kDa, since it was superior to that observed in the fractions with a higher molecular weight (3–10 kDa) (Silveira Coelho et al., 2019). According with our study, the antioxidant activity of several synthetic polyphenols has also been evaluated. First, *in vitro* assays of antioxidant activity were carried out using the ORAC assay, which is the same analytical procedure employed in the present study. Those polyphenols that showed the highest *in vitro* antioxidant activity were tested in a model of *S. cerevisiae* BY4741, and achieved a significant recovery of the biological functions of the yeast (Pérez de Vega et al., 2020).

We are aware that there are some limitations to this study. One of them is the use of the yeast model to evaluate the antioxidant capacity. This single-cell model was used as screening tool and as a step prior to the study in experimental models, which allows us to select the most promising hydrolysate and thus refine the animal experimentation protocol and reduce the number of animals that will be used in subsequent *in vivo* studies. However, other cell-based methods or assays based on human cell lines could be used to further investigate the antioxidant mechanisms involved in the effect of these food compounds. It is also important to note that these products will be administered orally, and that only some peptide sequences will be able to survive the digestion process and reach their site of action, and therefore human studies demonstrating their efficacy to prevent and/or improve pathologies related to oxidative stress will have to be carried out.

4. Conclusions

In conclusion, in this study we have obtained a hydrolysate from red quinoa seeds with a promising sensory profile that shows antioxidant activity *in vitro* through ORAC assay and *in vivo* in a model of *S. cerevisiae*. Although we are aware that more studies in experimental models and human trials will be necessary to corroborate the antioxidant effect and the mechanisms of action involved, the results obtained allow the development of new plant-based foods with antioxidant properties scientifically supported and useful in the prevention and/or the treatment of pathologies related to oxidative stress. Moreover, another interesting aspect of this work is the possibility of using the hydrolysates obtained from these plant-based matrices to produce new and more sustainable foods and also, the use of pseudocereals, will allow to produce gluten-free and lactose-free foods.

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Miguel Lopez-Moreno: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Beatriz Sabater-Muñoz:** Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **M. Teresa Lopez-Iglesias:** Methodology, Formal analysis, Writing – original draft. **Marta Miguel-Castro:** Conceptualization, Validation, Resources, Writing – original draft, Writing – review & editing, Funding acquisition, Supervision. **Marta Garcés-Rimón:** Validation, Resources, Writing – review & editing, Supervision.

Declaration of competing interest

None

Data availability

Data will be made available on request.

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