

Effect of Germination and Fermentation Process on the Antioxidant Compounds of Quinoa Seeds

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Abstract Quinoa (*Chenopodium quinoa*) seed has gained a great interest in the last years, mainly due to its nutritional properties and its content of antioxidant substances with health-promoting properties in humans. In this work, the effect of germination time and fermentation on the levels of antioxidant compounds (ascorbic acid, tocopherol isomers and phenolic compounds) and antioxidant activity of quinoa seeds was evaluated. Fermentation was carried out naturally by the microorganisms present in the seeds or by inoculation with two *Saccharomyces cerevisiae* strains (used for baking and brewing). Ascorbic acid and total tocopherols were significantly increased ($p \leq 0.05$) after 72 h of germination process in comparison with raw quinoa seeds, whilst fermentation caused a decrease in both types of compounds. Phenolic compounds and antioxidant capacity were improved using both bioprocesses, being this effect more noticeable for germination process (101 % of increase after three days of germination). Germination and fermentation proved to be desirable

procedures for producing enriched ingredients with health-promoting antioxidant compounds in a natural way.

Keywords *Chenopodium quinoa* · Germination · Fermentation · Phenolic compounds · Tocopherols · Ascorbic acid · Antioxidant activity

Introduction

Quinoa (*Chenopodium quinoa*) seed is a staple human food in the Andean region. In the last years, it has gained worldwide interest due to its agricultural potential and nutritional properties. In the latter case, quinoa seed has been recognized as an extremely nutritious grain providing relatively high quantity and quality of proteins (compared to traditional cereals), essential fatty acids and dietary fibre [1, 2]. Apart from the macronutrients, quinoa seeds provide a considerable amount of natural antioxidants with health-promoting properties such as phenolic acids, tocopherols, betalains and flavonoids [1, 3]. Also, the fact that quinoa is gluten free is an important feature that explains the baker's and brewer's increasing interest in using quinoa, as it raises the possibility of producing bakery products and beers for celiac people [4, 5].

Quinoa seeds are generally processed before consumption. They are usually extruded, puffed, flaked, or otherwise altered to make desirable commercial products [1, 2]. However, recent literature reviews and Committee reports emphasised the importance of preserving the structure of cereal foods via less drastic hydrothermal and mechanical processes [6]. In this sense, the fermentation and/or germination are viewed as desirable methods for grain processing because of its low cost, low energy requirements, with acceptable and diversified flavours for human consumption [7]. In addition, research has shown that germination and fermentation can not only

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improve nutritional quality and functional properties of several grains but also reduce the anti-nutritional factors [7, 8]. Research on quinoa has mainly focused on composition of the whole seed, protein quality, starch functionality, as well as incorporation into food products made with cereal flours. However, data regarding the effects of different bioprocess on its antioxidant substances is rather scarce.

The objective of the present study was to evaluate the effects of controlled germination and fermentation on the antioxidants compounds and antioxidant activity of quinoa seeds in order to obtain processed quinoa ingredients enhanced in antioxidant substances.

Materials and Methods

Plant Material and Chemicals Quinoa seeds (*Chenopodium quinoa* Willd., var. Real) were obtained from Buenos Aires province, Argentina, during September 2012. Seeds were cleaned and stored in polyethylene containers at room temperature until use. Microorganisms used in this study were two strains of *Saccharomyces cerevisiae* (NBRC 2375 and NBRC 1951, for baker and brewer applications, respectively) obtained from a collection of the Laboratory of Microbiology of the ISA Lille, France. All chemicals and reagents were of analytical and HPLC grade.

Germination Process Quinoa seeds (200 g) were soaked in a 2.5 % sodium hypochlorite solution (5 min) for surface sterilization and then washed with distilled water to neutral pH. Then, seeds were distributed in germination trays on wet laboratory paper and covered with the same wet paper to hydrate the seeds by capillarity. Trays were incubated at 20 °C (90 % relative humidity) in a dark chamber (BF series, Delta Labo, France) during three days. The duration of this germination period was based on the laboratory observation, since in longer periods sprouts overgrow. A sample from 0 to 3 days at 24 h intervals was taken and subsequently dried at 50 °C in a mechanical convection oven to constant weight. Dried sprouts were then milled using a laboratory grinder (Yellow line, A10, IKA-Werke, Staufen, Germany), sieved (0.5 mm), and the obtained flour was stored in plastic bags in a desiccator at 4 °C until further analysis. Germination experiences were performed in triplicate.

Fermentation Process Raw quinoa seeds were milled and sieved (0.5 mm). Suspensions of quinoa flour were prepared in a covered Erlenmeyer flasks with sterile distilled water (100 g /500 mL) and were allowed to ferment spontaneously with the naturally microorganisms present on the seeds (natural fermentation) or were previously autoclaved (121 °C, 15 min) and subsequently inoculated with 10 % (v/v) of two strains of *S. cerevisiae* (for brewing and baking) yeast suspension (10^8 CFU/

mL). Fermentation was carried out in an orbital shaking incubator (Max Q 8000, Thermo Scientific Inc., USA) at 120 rpm for 24 h at 30 °C. After fermentation, an aliquot (50 mL) of fermented quinoa flour suspension was collected and immediately utilized for pH measurement using a Hanna pH-meter (Hi 2211, Hanna instruments, USA). Another sample (100 mL) of suspension was dried (50 °C) to constant weight and the obtained fermented flour was stored in plastic bags in a desiccator at 4 °C until further analysis. Fermentation experiences were performed in triplicate.

Preparation of Extracts Raw, germinated or fermented quinoa flour (2 g) was homogenized with 20 mL of 80 % ethanol. The mixture was kept in agitation for 30 min at 160 rpm in a shaking incubator (Multitron II Infors SARL, Massy, France). Then, the homogenate was centrifuged for 10 min at $10,677\times g$ (Eppendorf Centrifuge 5804, Hamburg, Germany) and the supernatant was removed. The residue was extracted once again at the same conditions. Then, both supernatants were pooled, filtered through 0.45 μm nylon filter (Agilent Technologies, Santa Clara, USA) and stored at -18 °C for further analysis.

Total Phenolic Content (TPC) TPC in extracts was determined using Folin-Ciocalteu reagent, following the method described by Singleton et al. [9]. The results were expressed as milligrams of gallic acid equivalents (GAE) *per* 100 g on dry weight basis (DW).

Antioxidant Activity Antioxidant activity of extracts was evaluated by DPPH radical scavenging activity measured according to Brand-Williams et al. [10]. ABTS⁺ method was carried out according to Kraujalis et al. [11]. Ferric-reducing ability power (FRAP) was measured as previously described by Romero-de Soto et al. [12]. The antioxidant activity in each case was expressed as micromoles (μmol) of Trolox equivalent (TE) 100 g DW.

HPLC Analysis of Phenolic Compounds Reversed phase HPLC method for determination of phenolic acids and flavonoids was carried out with the same HPLC system and the same conditions as has been described in a previous work [13]. The identification of the phenolic compounds was achieved by comparing retention times and UV spectra of the unknown compounds with standards. Phenolic acids and flavonoids were quantified as aglycones in duplicate using standard calibration curves for each identified phenolic compound in the range of 5–500 $\mu\text{g}/\text{mL}$ with correlation coefficients >0.99 .

HPLC Analysis of Ascorbic Acid Quantification of ascorbic acid in fresh samples was carried out according to the procedure described by Kafkas et al. [14]. Separation was carried out in a Lichrocart C-18 column (250 \times 4.6 mm, 5 μm) (Merck Millipore, Darmstadt, Germany). The mobile phase

was aqueous metaphosphoric acid (0.8 %, v/v) pumped at flow rate of 1 mL/min. Detection of L-ascorbic acid was carried out at $\lambda = 245$ nm. Quantification was performed using the external standard method and the amount was expressed as mg per 100 g DW.

HPLC Analysis of Tocopherols Tocopherols concentration in samples was determined in a Hewlett Packard Series 1050 HPLC system (Palo Alto, CA, USA) equipped with an Agilent 1100 fluorescence detector (Santa Clara, CA, USA) operating at $\lambda_{exc} = 292$ nm and $\lambda_{em} = 330$ nm, following the procedure described by Fernández et al. [15]. Separation was carried out in a Lichrosorb Si 60 column (250 × 4.6 mm, 5 μ m) using hexane: isopropanol (99.5:0.5 v/v) as mobile phase, with a flow rate of 1.5 mL/min. The quantification was performed by the external standard method and the results were expressed in mg tocopherol per 100 g DW. Total amount of vitamin E was expressed as α -tocopherol equivalents (α -TE). α -TE is calculated using the formula α -TE = (α -T × 1) + (β -T × 0.5) + (γ -T × 0.1) + (δ -T × 0.03) [16].

Statistical Analysis Experimental results were performed in triplicate and the data are presented as mean ± S.D. Analysis of variance and comparison of treatments means (Tukey's test, $p \leq 0.05$) as well as principal components analysis were performed using InfoStat 2015 software (InfoStat Group, Argentina).

Results and Discussion

Effect of Processing on Ascorbic Acid and Tocopherols Levels The effects of germination and fermentation on the ascorbic acid and tocopherols of quinoa are summarized in

Table 1. The amount of vitamin C detected in unprocessed (raw) seeds was 0.38 mg/100 g and germination showed a progressive increase during different days of process, reaching after 72 h a level of almost 16 times the initial content. This result was in accordance with that published by Lintschinger et al. [17] for 3 days-germinated quinoa seeds. The effect of germination on the vitamin C levels has also been observed in other grains such as wheat, buckwheat, chickpea, lupin and soybean [16–19], indicating that the biosynthesis of vitamin C is a specific metabolic process during germination which seems to be directly implicated in the modulation of initial embryo germination and plant growth, protecting other biological substances from oxidative damage [16]. In contrast to germination, the vitamin C present in raw quinoa disappeared completely after either induced or spontaneous fermentation. This result was in agreement with those reported by other authors in lupin or chickpea seeds [16, 18].

Quinoa lipids have been reported to be generally stable against oxidation, despite their high fat content and degree of unsaturation due to the presence of abundant quantities of tocopherols [4]. Therefore, information on the stability of vitamin E during food processing is essential. Due to antioxidant activity, vitamin E compounds are susceptible to oxidation and degradation, and factors such as light, oxygen, and heat can accelerate vitamin E destruction [20].

The contents of tocopherol isomers in raw quinoa (Table 1) were comparable with those published by other authors [4, 21]. These authors also coincide that γ -tocopherol is the isomer present in larger amounts in quinoa seeds followed by α -tocopherol, whilst β - and δ - are present in smaller amounts. The value observed in this work for α -tocopherol (2.83 mg/100 g DW) was in the range found by others authors in quinoa seeds, which was from 0.59 [22] to 4.64 mg/100 g DW [23]. Interestingly, germination process caused a progressive increase

Table 1 Effect of germination and fermentation on the levels of ascorbic acid and tocopherol isomers of quinoa seeds

Quinoa flour	Ascorbic acid (mg/100 g DW)	Tocopherols (mg/100 g DW)				Vitamin E activity (α -TE/100 g DW)
		α -tocopherol	β -tocopherol	γ -tocopherol	δ -tocopherol	
Raw	0.38 ± 0.17 a	2.83 ± 0.10 d	0.09 ± 0.02 a	4.06 ± 0.01 g	0.31 ± 0.01 b	3.29 ± 0.11 d
Germination						
24 h	1.90 ± 0.11 b	3.60 ± 0.20 e	0.10 ± 0.01 a,b	2.89 ± 0.02 e	0.26 ± 0.01 b	3.95 ± 0.21 e
48 h	4.12 ± 0.31 c	4.64 ± 0.06 f	0.13 ± 0.02 a,b	2.51 ± 0.06 d	0.34 ± 0.11 b	4.96 ± 0.08 f
72 h	6.06 ± 0.33 d	6.63 ± 0.14 g	0.17 ± 0.04 b	3.24 ± 0.32 f	0.43 ± 0.08 b	7.05 ± 0.20 g
Fermentation						
<i>S. cerevisiae</i> (baker's yeast)	n.d.	1.55 ± 0.07 c	n.d.	1.95 ± 0.10 c	n.d.	1.74 ± 0.07 c
<i>S. cerevisiae</i> (brewer's yeast)	n.d.	1.34 ± 0.05 b	n.d.	1.24 ± 0.09 b	n.d.	1.46 ± 0.06 b
Natural	n.d.	0.12 ± 0.01 a	n.d.	0.09 ± 0.01 a	0.03 ± 0.00 a	0.13 ± 0.01 a

Values are expressed as mean ± SD ($n = 3$). Different letters in the same column means significant difference ($p \leq 0.05$) among samples
n.d. not detected

in the content of α -tocopherol, increasing the initial value in 27, 64 and 134 % after 1, 2 and 3 days of process, respectively. At the same time, global germination provides reduction between 20 and 38 % in the content of γ -tocopherol, thus, α -tocopherol was the most abundant isomer in germinated samples. As a consequence of the modification in tocopherol isomers, vitamin E activity increased significantly ($p \leq 0.05$) 114 % compared to initial value at the end of germination process.

No information has been found about the effect of germination on the vitamin E content of quinoa. Even so, different authors have mentioned that α -tocopherol increased its level after the first few days of germination and modifications of the remaining isomers depended on the plant species [16, 18, 24]. Fermentation, however, caused a decrease of all tocopherol isomers, whilst this effect was more noticeable when fermentation was performed naturally in comparison to induced fermentation with both *S. cerevisiae* strains. This fact can be partially explained due to sensitivity of tocopherols in contact with oxygen during agitation in the fermentation process. The formation of carbon dioxide which remains in Erlenmeyer flask during induced fermentation with both *S. cerevisiae* strains might help to reduce the oxidative effect on tocopherols.

Effect of Processing on Total Phenolic Compounds The effect of different applied process on the TPC content of raw and processed quinoa seeds is shown in Fig. 1. Raw quinoa seeds showed a level of 39.3 ± 0.9 mg GAE/100 g DW, which resulted comparable to those reported by other authors [23, 25, 26].

Germination caused a steady increase in TPC content, and rises of 20, 57 and 101 % after 1, 2 and 3 days of germination

were recorded, respectively. This latter result agrees with that reported by Alvarez-Jubete et al. [26] where a 2-fold increase of total phenols in quinoa sprouts after 82 h of germination was found. Phenolic compounds are present in cereals either free (mainly located in the pericarp) or bound to cell wall components (mostly found as an ester on the arabinose side-chains of arabinoxylans and lignin) [8]. The increases observed of TPC during germination can be explained by the action of endogenous esterases synthesized during germination which can lead to the release of cell wall bound phenolic compounds. Also, the biochemical reactions in germinating grain could lead to the synthesis of new phenolic compounds [7]. Fermentation process carried out with different microorganism starters increased similarly TPC levels by about 55 % respect to raw seeds, whilst no significant differences ($p > 0.05$) between the treatments were observed (Fig. 1). Other authors have also demonstrated that fermentation has a positive influence on TPC in some cereals, pseudocereals and legumes [16, 27, 28]. These results can be explained by the fact that levels of bioactive compounds can be modified during fermentation by the metabolic activity of microorganisms. Particularly, several microbial enzymes might induce structural breakdown of cereal cell walls and/or hydrolyse esterified and insoluble-bound phenolics, facilitating their liberation prior to extraction [29, 30].

Effect of Processing on the Phenolic Profile of Quinoa Seeds Table 2 shows that four soluble free phenolic acids (*p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids) and two flavonoids (quercetin and kaempferol) were detected as major

Fig. 1 Total phenolic content (TPC) and antioxidant activity (DPPH[•], ABTS^{•+}, and FRAP) values of raw and processed quinoa seeds. Bars represent standard deviation of three independent replicates

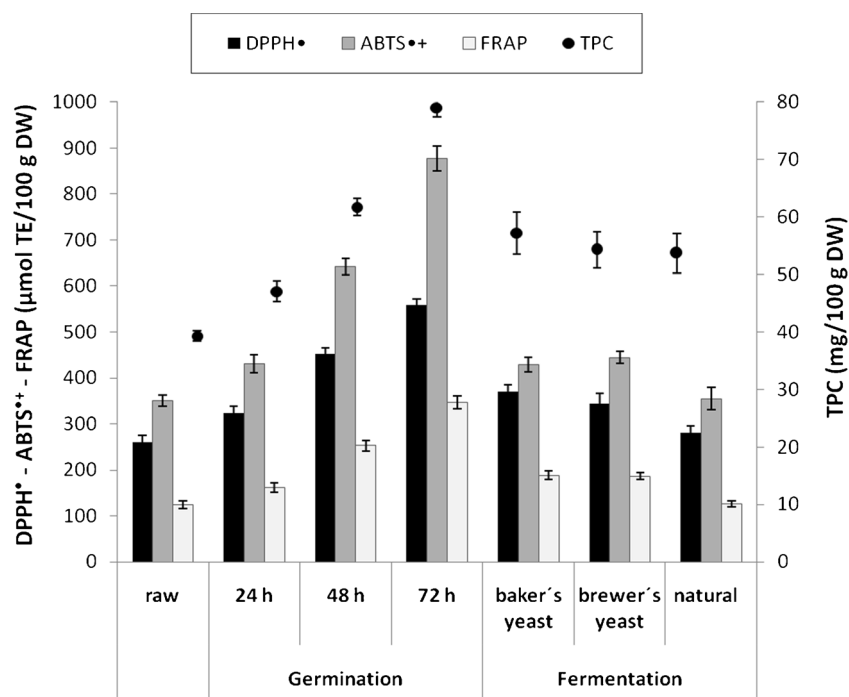


Table 2 Effect of germination and fermentation on levels of major phenolic compounds and pH of quinoa seeds

Quinoa flour	Phenolic compounds (% area relative to raw grain)						pH
	<i>p</i> -OH-benzoic acid	Vanillic acid	<i>p</i> -coumaric acid	Ferulic acid	Quercetin	Kaempferol	
Raw	100	100	100	100	100	100	6.75
Germination							
24 h	148	295	299	215	145	131	ud.
48 h	213	629	965	465	268	117	ud.
72 h	430	975	2177	632	597	185	ud.
Fermentation							
<i>S. cerevisiae</i> (baker's yeast)	831	142	1002	235	n.d.	n.d.	5.50
<i>S. cerevisiae</i> (brewer's yeast)	868	343	813	61	n.d.	n.d.	4.96
Natural	n.d.	n.d.	145	14	n.d.	n.d.	3.92

n.d. not detected, *ud.* not determined

phenolic compounds in raw quinoa seeds. In accordance with Tang et al. [3], vanillic acid was the most abundant of identified phenolic acids in raw quinoa followed by ferulic acid, which levels in this study were 8.8 and 5.7 mg/Kg DW, respectively.

Germination process showed a progressive increase in the levels of all identified phenolic compounds, which was more noticeable for *p*-coumaric and vanillic acids at the end of the process (Table 2). This increase of overall free phenolic contents quantified by HPLC was in accordance with those obtained by Folin-Ciocalteu method, showing, as above mentioned, the hydrolytic effect on complex polyphenols to other simpler ones (thus exhibiting higher levels of assayable phenolic hydroxyl groups) or the *novo* synthesis as sprouting day progressed. However, during fermentation, several changes in the levels of identified phenolic compounds were observed. No flavonoids compounds were detected after either inoculated or natural fermentation. Regarding phenolic acids, treatments carried out with both showed increases in the levels of each identified phenolic acids compared to control, except for ferulic acid, which was only significant ($p \leq 0.05$) in treatments inoculated with baker's yeast. This indicates that both strains of *S. cerevisiae* may produce hydrolytic enzymes capable of releasing soluble conjugated or insoluble bound phenolic acids from quinoa grain, as has been mentioned for other plant matrices [27, 28, 31]. Another possible explanation could be due to considerably higher pH of these fermentation treatments (pH 4.96–5.50, Table 2), which may provide an optimum pH for either endogenous or microbial cell wall degrading enzymes. For example, the increase of phenolic acids in yeast-fermented ray meal has been attributed to pH-mediated activation of endogenous cinnamoyl esterases, which has optimum activity at neutral pH [27].

On the contrary, natural fermentation caused a decreased in the levels of several phenolic acids compared to raw seeds. This might be due to the strong acidity conditions reached in these treatments (pH 3.92, Table 2), which considerably

diminish the activity of the above mentioned enzymes. Also, it has been mentioned that upon certain fermentations, the degradation of phenolic compounds can take place [31, 32]. The degree of influence depends on the microorganism species involved, since it has been reported that during fermentation with some lactobacilli strains certain phenolic acids and flavonoid glucosides were metabolized as a result of the increasing activities of phenolic acid reductase, phenolic acid decarboxylase, and glucosidase [33, 34]. However, more studies of microbes and the activities of their relevant enzymes are needed to establish the precise mechanisms that occur during the natural fermentation of quinoa.

Effect of Processing on the Antioxidant Capacity DPPH[•], ABTS^{•+}, and FRAP methods were chosen to examine the antioxidant activity of processed quinoa samples (Fig. 1), because of their speed, ease and reliability [35]. All evaluated methods showed similar trends in the antioxidant activity values from germinated and fermented samples. Particularly, germination process significantly increased the antioxidant activity, reaching an increase higher than 2-fold at 72 h of process in comparison to raw quinoa, for the three tested methods. Fermentation treatments carried out with both strains of *S. cerevisiae* also showed increases in antioxidant activity compared to raw quinoa, and rises of 43 and 33 % for DPPH[•], 22 and 27 % for ABTS^{•+}, and 51 and 50 % for FRAP using baker's and brewer's yeast were recorded, respectively. Moore et al. [28] also noticed moderate increases in radical scavenging activity of fermented wheat bran extracts using baker's yeast. In contrast, the present study did not show significant changes in antioxidant activity after natural fermentation treatments, thus indicating that the effect depended on the type of fermentation evaluated. The choice of microorganism used in the fermentation process depends on the desired end product, and here only common yeast

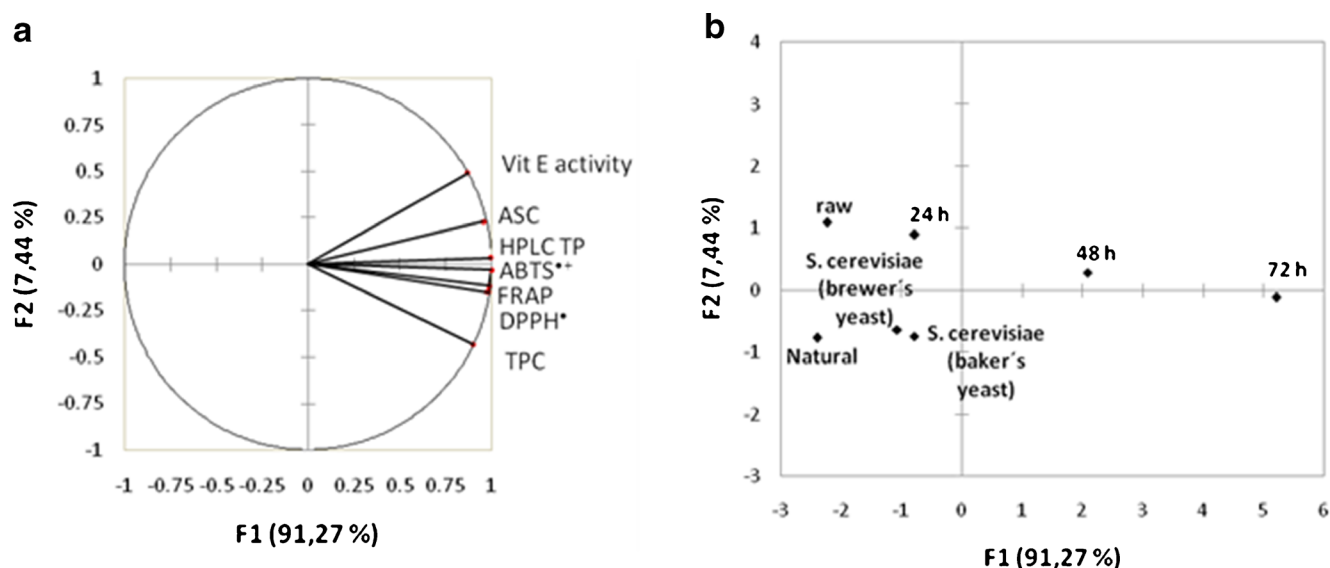


Fig. 2 Plots of principal components analysis showing the evaluated variables (a) and sample locations (b) defined by the first two factors. TPC = total phenolic compounds; ASC = ascorbic acid; HPLC TP = total phenolics quantified by HPLC

strains were evaluated for the current importance of quinoa in baker and brewer products. However, future work evaluating the effect of other microorganisms (bacteria, molds, and yeasts) on antioxidant properties of quinoa is still needed.

On the basis of the results obtained here, the relative contribution of antioxidant compounds (TPC, ascorbic acid, tocopherol isomers, and HPLC identified phenolic contents) to the antioxidant capacity (DPPH method) of raw and processed quinoa seeds was calculated. For better illustration, a plot representing the results of a principal components analysis (PCA) was performed (Fig. 2). The first two principal factors accounted 98.71 % of the total variance, corresponding 91.27 % for Factor 1 (F1) and 7.44 % for Factor 2 (F2). The fact that F1 and F2 are orthogonal assures that information provided by F1 about variability is different than that explained by F2. Figure 2a shows the evaluated variables on quinoa samples (plotted as vectors from the origin) defined by the first two factors. The angles between vectors can be interpreted in terms of correlations between variables, in which an angle close to zero implies that both variables are strongly positively correlated. Thus, the three methods used to evaluate the antioxidant activity were highly correlated between them ($r > 0.979$; $p \leq 0.05$) and with the rest of measured variables. The observed Pearson's coefficients are showed in Table S1. Figure 2b shows the location of raw and bioprocessed samples on the new coordinates. A shift towards positive values on F1 and a decrease on F2 values was observed as germination process progressed, indicating a clear separation between raw samples (with low antioxidant activity, ascorbic acid, and HPLC total phenolic levels) and samples with 72 h of germination (with high antioxidant activity, ascorbic acid, and HPLC total phenolic levels).

Regarding F2, a division between raw samples (with high vitamin E activity) and fermented samples (with high total phenolic content) was observed.

Conclusion

In the present work it was found that the application of controlled germination or fermentation techniques on quinoa seeds significantly increased the antioxidant properties compared with raw seeds. In particular, germination process increased ascorbic acid and total tocopherols levels after 72 h in comparison with raw seeds, whilst fermentations caused a decrease in both types of compounds. Phenolic compounds and antioxidant activity evaluated by three methods were improved by both bioprocesses, being this effect more noticeable for germination process. Moreover, principal component analysis showed strong positive correlation between the evaluated antioxidant compounds, and from them on the antioxidant activity. Thus, germination and fermentation process could therefore offer an excellent strategy to produce ingredients enriched with health-promoting compounds in a natural way that can be used in the formulation of functional foods.

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Compliance with Ethical Standards This article does not contain any studies with human or animal subjects.

Conflict of Interest The authors declare that they have no conflicts of interest.

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