Impact of debittering and fermentation processes on the antinutritional and antioxidant compounds in *Lupinus mutabilis* Sweet

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Abstract

Lupin is a nutritive grain, but its use is limited due to its high content of bitter alkaloids and other antinutritional factors, such as phytic acid, tannins, nitrates and trypsin inhibitors (TI), that have undesirable physiological effects. There is increasing interest in finding appropriate methods for reducing the antinutritional compounds in lupin. The objective of this research was to assess the efficacy of a biotechnological process, namely, fungal fermentation, as a debittering process relative to that of conventional aqueous thermal treatment (ATT). We evaluated the effects of these processes on the reduction of antinutritional compounds as well as their potential impacts on enhancing the beneficial antioxidant properties of lupin. Three varieties (INIAP-450, INIAP-451 and Criollo) of the *Lupinus mutabilis* species were studied. The application of ATT and fermentation with *Rhizopus oligosporus* caused decreases in the following antinutrients: nitrates (94.59%), tannins (82.10%), alkaloids (94%), urease activity (93.75%), phytic acid (70.06%) and trypsin inhibitors (76.76%). Ascorbic acid also decreased (79.72%). All values corresponded to

the average in the three varieties evaluated. While the contents of phenols, carotenoids and the antioxidant capacity decreased by 96.83, 49.42 and 96.13%, respectively, due to the debittering process, solid fermentation promoted increases in these compounds and properties in the debittered grain.

Key words

Lupin, Debittering, Cooking, Fermentation, Legume.

1. Introduction

In legumes, the presence of antinutritional compounds, such as protease inhibitors, trypsin, amylase, lectins, antivitamin factors, alkaloids, saponins, tannins, flavones, and isoflavones limits their ability to be consumed (Carvajal-Larenas, Linnemann, Nout, Koziol, & van Boekel, 2016). Specifically, in the case of lupin, consumption is limited by a high content of bitter alkaloids and other antinutritional factors, such as phytic acid and trypsin inhibitors, because they have undesirable physiological effects and can cause acute toxicity (Daverio et al., 2014). Some of these compounds inhibit the activities of specific enzymes (e.g., trypsin and α -amylase) that impair the digestion of protein and starch, reducing the nutritional value of lupin seeds. Other compounds (e.g., tannins) affect mineral utilization (Embaby, 2010; Carvajal-Larenas, et al., 2016).

Conversely, health-related benefits have also been linked to some antinutritional factors in legumes, specifically phytic acid, polyphenols, ascorbic acid and carotenoids (Lampart-Szczapa, Korczak, Nogala-Kalucka & Zawirska-Wojtasiak, 2003). These compounds have been shown to have antioxidant properties as well as beneficial metabolic and physiological effects, such as preventing sclerotic changes in blood vessels and blocking the formation of free radicals (Khan, Karnpanit, Nasar-Abbas, Huma & Jayasena 2015). These effects have been described in some

lupin species, such as *L. albus*, *L. luteus* and *L. angustifolius*, and other wild species (Thambiraj, Reddy, Phillips, & Koyyalamudi, 2019). However, there is little information regarding *Lupinus mutabilis*, despite being one of the most common species due to its ability to grow in poor soils and under extreme climatic conditions.

Although there are different methods to reduce the antinutritional factors (Soetan & Oyewole, 2009), the traditionally called debittering process has been carried out to remove the antinutritional and bitter compounds, making the lupin apt for consumption (Villacrés, Álvarez, & Rosell, 2020a). Lupin debittering treatments facilitate the elimination of antinutritional compounds, such as quinolizidine alkaloids (QAs) and phytic acid (Carvajal-Larenas et al., 2016). Debittering includes lupin hydration, cooking and subsequent washing processes with water. Specifically, cooking reduces the tannin content of lupin by more than 70% (Jiménez-Martínez, Hernández-Sánchez & Dávila-Ortiz, 2001). Treatments employing heat also help reduce trypsin inhibitor and urease activity lowering the nutritional quality of grains. Simultaneously to the removal of toxic compounds, the debittering process results in losses of other nutrients like minerals (Ertaş & Bilgiçli, 2014).

Solid-state grain fermentation with bacterial or fungal species have been applied to reduce antinutritional compounds, such as phytates and tannins, but also to improve the nutritional quality of grains and pulses, given that these compounds affect the bioavailability of minerals, such as calcium, zinc and iron (Ghoshal, Basu, & Shivhare, 2012; Saharan, Sadh, Duhan & Duhan, 2020). Fermentation also increases the polyphenol content and improves grain antioxidant activity because microbial action facilitates the breakdown of cell walls and allows the release or synthesis of antioxidant compounds that act as metal chelators or hydrogen donors to free radicals (Nout & Kiers, 2005). However, this biotechnological strategy has been scarcely

applied to *L. mutabilis* grains. Fernandez-Orozco et al. (2008) studied the impact of fermentation on the antioxidant capacity of *L. angustifolius* and found that there was an increase in total phenolic compounds, peroxy radical-trapping capacity and Trolox equivalent antioxidant capacity under most fermentation conditions.

Therefore, considering the abundance of *L. mutabilis* and the scarce information available on this species, this research was conducted to increase the knowledge about debittering and fermentation processes in grains. The specific objective of this study was to evaluate the impact of debittering and solid fermentation treatments on various antinutritional compounds and antioxidant properties of three *L. mutabilis* varieties (INIAP-450, INIAP-451 and Criollo).

2. Materials and methods

2.1 Raw material

Lupin varieties (INIAP-450, INIAP-451 and Criollo) were provided by the National Legumes Program and INIAP Andean Grains (Ecuador). The harvested grains were threshed and classified in Crippen Mfg. Inc. equipment (Michigan, USA). Grains with an average diameter of 7 to 8 mm were selected for this study and stored at room temperature (16 °C, 65% relative humidity) until analysis. *Rhizopus oligosporus* strain ATCC NRRL2710 was obtained from the Northern Regional Research Laboratory USDA, USA, belonging to the Ambato Technical University Microbiology Laboratory Collection.

2.2 Reagents

The main reagents used in this investigation were the following: BAPA (N-benzoyl-arginine p-nitroanilide), phytic acid kit (Megazyme), potato dextrose agar (Merck), L-ascorbic acid, chlorogenic acid, Folin-Ciocalteu ABTS (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid),

Trolox ($\pm$)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) Sigma Aldrich brand (St. Louis, Missouri, USA).

2.3 Sample preparation methods

For each variety and process, 2 kg of grain was used. The raw *L. mutabilis* seeds were lyophilized (Labconco Lyph lock 12, Kansas, USA) at -40°C and -0.9 bars for four days and then ground (Retsch KG -5657 Haan Remscheid, Germany) to a particle size of 250 μ m. The sample was packed in polypropylene bags and stored at 10°C until analyses. The thermal-aqueous treatment (ATT) was used for the debittering process. The ATT began by soaking the grain at an initial temperature of 80°C for 10 h; a ratio of 1:3 (grain:water) was used. Next, cooking was done in water at 91°C for 1 h, followed by washing with potable water. A ratio of 1:15 (grain:water) was used for washing. Washing was carried out in two stages: first with water at 35°C for 28 h followed by water at 18°C for 45 h (Villacrés et al., 2020a). The debittered lupin was dried in a forced air oven (HS122, Labolan, Navarra, Spain) at 60°C for 8 h. The debittered sample was lyophilized, ground, packed and stored under the same conditions as the bitter grain.

For the fermentation process, the humidity of the debittered grain was reduced to 50% in a forced air oven (HS122, Labolan, Navarra, Spain) at 60°C for 2 h. The grain was crushed in a miniprocessor (Oster, Rio de Janeiro, Brazil), and portions of 50 g were packed and sealed in polypropylene bags for sterilization in an autoclave (Webeco, Farjestäden, Germany) at 121°C for 10 min. Next, 500 μ l of the spore suspension were inoculated to each grain portion and kept in an incubator (Mettmert IN160, Fisher Scientific SI-C/Luis, Madrid, Spain) at 28°C for four days. The grain was then covered with a layer of white fungal mycelia, and the samples were lyophilized in a freeze dryer (Labconco Lyph Lock 12 equipment, Kansas, USA) at -40°C and -0.7 bars for four days. The fermented grain was ground, packed in polypropylene bags and stored at 10°C prior to chemical analyses.

2.4 Analysis of anti-nutritional compounds

Nitrates

Nitrate quantification was performed using the method reported by Cataldo, Maroon, Schrader & Youngs (1975). Samples were previously homogenized and filtered in a K_2SO_4 , 0.34 M solution. The filtrate (0.5 mL) was mixed with 5% salicylic acid and NaOH (4N); Absorbance at 410 nm was measured on a UV-Visible spectrophotometer (Thermo Fisher Scientific 201 Evolution, Madison, WI USA). The nitrate content was expressed in mg per kg (dry weight basis).

Tannins

Tannins were determined with Folin-Denis reagent (AOAC, 1984), using tannic acid as a standard. The absorbance was measured at a wavelength of 680 nm. The tannin content was expressed in mg per 100 g (dry weight basis).

Quinolizidine alkaloids (QAs)

The total alkaloid content was measured following the method described by von Baer, Reimerdes, & Feldheim (1979) with some modifications of the titration process. Specifically, 5 mL of 0.01 N sulfuric acid and two drops of methyl red were added to the concentrated chloroform extract, and the excess acid was titrated with 0.01 N NaOH. For the calculation, 1 mL of 0.01 N H_2SO_4 was equivalent to 2.48 mg of lupanine (Gross et al., 1988).

Residual Urease Activity

Although urease is not related to protein and starch digestion, the urease test has been used as an indirect method for estimating the degree of trypsin inhibition because its inactivation mechanism is nearly identical to that of trypsin inhibition (Yalcin & Basman, 2015). Lupin flour (0.2 g) was dissolved in 10 mL of urea solution (pH 7.0) in a water bath at 30 °C for 30 min. The urea solution was replaced with a phosphate buffer to make the blank. The change in pH caused by the

conversion of urea to ammonia by the urease enzyme in the sample was measured with AACC Method No: 22-90.01 (AACC, 2000).

Phytic acid

Phytic acid determination was conducted by phosphorus colorimetric quantification from a calibration curve with a phosphorus standard at four concentrations (0.5, 2.5, 5.0 and 7.5 ppm) at 655 nm absorbance using Megazyme kit. The results were expressed in g/100 g (dry weight basis).

Trypsin inhibitors (TI)

Trypsin inhibitor activity was measured following the AOCS Official Method (2009). The extraction of TI was performed by mixing 1.00 g of defatted lupin flour with 50.0 mL of 0.01 M NaOH and agitating the resulting suspension for 3 h at room temperature. A final centrifugation step for 10 min at 10000 x g allowed separation of the supernatant (lupin flour extract) for the TI assay.

2.5 Analysis of compounds with antioxidant properties

Ascorbic acid

The ascorbic acid in the samples was extracted with an oxalic acid solution of 0.4% and 20% acetone and quantified using 2,6-dichlorophenol-indophenol (Egoville, Sullivan, Kozempel, & Jones 1988). Absorbance was measured at 520 nm. L-ascorbic acid was used as a standard.

Total carotenoids

The extraction of carotenoids was conducted with cold acetone and petroleum ether according to the methodology described by Rodriguez-Amaya & Kimura (2004). Absorbance of the ether extract was measured at a wavelength of 450 nm. The extinction coefficient of carotenoids in petroleum ether (2500) was considered in the calculation of total carotenoids.

Total phenolic compounds

Phenolic compounds were determined using Folin-Ciocalteu 2N reagent (Waterhouse, 2002) with minor modifications during the extraction. Solvent extraction was carried out using sonication as a pretreatment. Each sample (0.9 g) was suspended in 10 ml of 80% methanol for 2 h, and 5 min of sonication (20 kHz, 100 W) was applied after each 15 min of agitation. Samples were then centrifuged at 4000 rpm and 10°C for 5 min, and the supernatant was collected. This process was repeated twice, and all three supernatants were pooled. Absorbance was measured at 765 nm. Results were expressed in mg chlorogenic acid/100 g sample.

Trolox equivalent antioxidant capacity (TEAC)

This test was based on the reduction of ABTS radical cations (ABTS^{•+}) by antioxidants present in lupin extracts according to the procedure described by Re et al. (1999). Extraction was performed with 80% methanol. A standard Trolox curve (2000 µM) was also prepared; TEAC was expressed as µg Trolox/g (dry weight basis). All samples were analyzed in triplicate.

2.6 Statistical analysis

The data were analyzed by applying two-factorial ANOVA, using the INFOSTAT statistical software package (Universidad de Córdoba, Argentina) to compare the means with respect to variety and the condition of the grain. Tukey's multiple range test was applied to determine significant differences at the 5% level. All analyses were performed in triplicate, and the results are given as the mean ± standard deviation.

3. Results and discussion

3.1 Antinutritional compounds

Nitrates, tannins and quinolizidine alkaloids (QAs)

The results of the quantification of nitrate, tannins and alkaloids, as well as the significant statistical differences ($P<0.05$) between varieties in the condition of the grain (debittered or fermented) and their interaction, are presented in Table 1. The nitrate content in raw seeds varied with grain variety ($P<0.05$) and was the highest in INIAP-451 (40.63 mg/100 g) followed by the Criollo variety and INIAP-450. Values of these compounds were lower than those that have been reported for other vegetables, such as spinach (48.5 mg/100 g) and leaf lettuce (55.5 mg/100 g), where nitrates are concentrated in vacuoles, leaves and transport organs but less abundant in flowers, tubers and seeds (Ranasinghe & Marapana, 2018). The application of ATT caused a reduction in nitrate by 94.84%, 92.95% and 94.43% in INIAP-450, INIAP-451 and Criollo, respectively. These pronounced reductions likely stemmed from the water solubility of nitrates. The reduction in nitrate increased significantly ($P<0.05$) by 3.84% (INIAP-451) and 4.09% (Criollo) in fermented grains relative to debittered grain. The nitrate concentrations in debittered and fermented *L. mutabilis* were much lower than the specified maximum permissible levels that have been reported for lettuce and spinach (1125 mg/100 g) by the regulations of some European countries (Siomos & Dogras, 2000). Other antinutritional compounds are tannins, which affect mineral and protein utilization (Embaby, 2010) and cause growth depression by decreasing the digestibility of protein and carbohydrate (Liener, 1994). In the three varieties of *L. mutabilis* evaluated in this study, the content of tannins depended on variety and grain condition ($P<0.05$). The highest concentration of tannins (975.74 mg/100 g) was recorded in the raw Criollo grain. This concentration is higher than what has been reported in soy (45 mg/100 g) and in raw *Lupinus termis* seeds (753 mg/100 g) but is similar to the concentrations of tannins that have been reported in dehulled seeds of the same variety (816 mg/100 g) (Embaby, 2010). The debittering process resulted in decreases in the contents of tannins in the three varieties by 80.62% relative to the raw grain. Such pronounced decreases are likely explained by the fact that high temperatures

break down the tannin-protein complex, thereby inducing the leaching of tannins in the soaking medium and increasing the digestibility and palatability of the grain (Embaby, 2010). The fermentation of *L. mutabilis* caused an additional decrease in tannins (7.64%) relative to those in the debittered grain, which could be attributed to the production of tannase during fermentation (Khan, Karnpanit, Nasar-Abbas, Huma & Jayasena, 2018). Similar patterns have been observed in *L. campestris* that was debittered in an aqueous system under alkaline conditions where tannin content was reduced by 77% (alkaline aqueous treatment) and 70% (aqueous treatment) (Jiménez-Martínez et al., 2001). The fermentation of *L. angustifolius* L. with *Rhizopus* sp. decreased tannin content by 90.41% (Khan et al., 2018), which is consistent with trends that were observed in *L. mutabilis* in our study.

The content of alkaloids in *L. mutabilis* seeds varied with grain variety and grain condition ($P<0.05$). The raw seeds of the three varieties had values between 3.76–4.47% and were similar to those that have been reported for *L. mutabilis* ecotypes from Perú (3.30–3.10%) (Múzquiz et al., 1989). Alkaloid contents of 3.8%, 2.74% and 1.6% have been reported for *L. albus*, *L. campestris* and *L. angustifolius*, respectively (Jiménez-Martínez et al., 2001; Múzquiz, Burbano, Gorospe & Ródenas, 1989). Variability between species is associated with the amount of nitrogen present in the grain, the intensity of sunlight and the temperature of the growing areas (Carvajal-Larenas et al., 2016). ATT reduced QAs by 91.93% relative to the bitter grain. The water solubility and the low size of QAs likely contributed to their removal from the lupin seeds. Jiménez-Martínez et al. (2001) reported a reduction of 99.96% and 98.95% in QAs of *L. campestris* debittered by aqueous and alkaline treatments. The application of additional techniques such as peeling and autoclaving in *L. campestris* and *L. mutabilis* reduced alkaloids by 55% and 35%, respectively (Jiménez-Martínez, Hernández-Sánchez & Dávila-Ortiz, 2007). Residual QAs of the debittering process were not totally degraded by *R. oligosporus*, and the

following values were recorded in the fermented grain: 0.27% (INIAP-450), 0.29% (INIAP-451) and 0.18% (Criollo), levels that are considered safe for human consumption. The safety limit fixed by the health authorities of the UK, France, Australia and New Zealand for the total amount of alkaloids in lupin flours and derived products is 0.2 g/kg dry matter (Magalhães et al., 2017).

Urease activity, trypsin inhibitors and phytic acid

These compounds are thermolabile proteins that alter the digestion of proteins and inhibit the activity of digestive enzymes that cause the hydrolysis of dietary proteins (Egounlety & Aworh, 2003). Urease inactivation is a reliable indicator of the adequacy of heat processing and hence the degree of trypsin inhibitor activity (Yalcin & Basman, 2015). Multiple comparison tests showed that there was a significant effect of variety and grain condition on the urease activity of lupin samples ($P<0.05$). In the three varieties of *L. mutabilis*, the debittering process caused a substantial reduction (88.42%) in urease activity. In contrast, reductions in urease activity caused by fermentation were comparatively lower: 19.05% (INIAP-450), 31.75% (INIAP-451) and 13.10% (Criollo) (Table 2). The fermented grain of INIAP-450 had the lowest degree of urease activity (0.05 pH difference), indicating that urease was inactivated (Yalcin & Basman, 2015). In soybeans that were cooked, roasted or extruded, urease activity was reduced by 98% (Qin et al., 1996; Yalcin & Basman, 2015). In accordance with the residual urease activity, trypsin inhibitors (TI) were significantly reduced with the application of the debittering and fermentation processes. The bitter grain of three varieties of *L. mutabilis* on average had 1.63 TIU/mg, which was similar to values that have been reported for other lupin species such as *L. exaltatus* (1.37 TIU/mg) and *L. reflexus* (2.05 TIU/mg) (Ruiz-López et al., 2000). A substantial reduction (71.71%) in trypsin inhibitors was observed in debittered grain, but the fermentation generated even more marked reductions: 20.38% (INIAP-450), 5.72% (INIAP-451) and 18.55% (Criollo). Higher values were

reported in cooked soybeans (82.17%) and fermentation of *R. oligosporus* for 48 h (97.42%), which is consistent with the trend that we observed in *L. mutabilis*. Cooking caused a reduction of 86.09% in TIUs in beans; however, fermentation did not change the content of trypsin inhibitors (Egounlety & Aworh, 2003). Phytic acid is often regarded as an antinutrient because of its powerful ability to bind minerals, proteins and starches and thereby decrease their bioavailability. However, in vivo and in vitro studies have demonstrated that phytic acid has preventive as well as therapeutic properties (Mohan, Tresina & Daffodil, 2016). Multiple comparison tests showed that variety and grain condition had significant effects on phytic acid content in *L. mutabilis* ($P<0.05$). On average, the three bitter varieties had 0.312 g phytic acid/100 g, which is similar to values that have been reported for other beans (0.46 g/100 g) but lower compared with other grains and legumes, such as *L. exaltatus* (1.17 g/100 g), *L. albus* (1.42 g/100 g), *L. angustifolius* (1.45 g/100 g) (Múzquiz et al., 1989; Ruiz-López et al., 2000) and soy (1.27 g/100 g) (Egounlety & Aworh, 2003; Carvajal-Larenas et al., 2016).

The initial concentration of 0.312 mg phytic acid/100 g (dry weight basis) was reduced to 0.139 mg phytic acid/100 g (dry weight basis) after the debittering process. These findings are consistent with the results of a previous study (Vijayakumari, Pugalenthil & Vadivel, 2007) that has reported a reduction in phytic acid in soaked and hydrothermally processed *Bauhinia purpurea*. The phytic acid level was reduced by 21.07%–49.76% in the three varieties of *L. mutabilis* after fermentation using *R. oligosporus* (Table 2). The enhanced phytic acid reduction observed in fermented lupin by debittering pretreatment may be correlated with the ability of the fungal phytase to access the substrate. This observation is consistent with the results of Embaby (2010), who found that pretreatments, such as soaking, moistening, pearling, rolling and autoclaving significantly improved the fungal growth of tempeh produced from whole grains and ultimately reduced the contents of antinutritional factors.

3.2 Antioxidant Compounds

In today's world, the scientific community and consumers are not only relying on the nutrient contents of legume crops to make consumption decisions but also aspects of their phytochemical composition, which are often considered equally important. *L. mutabilis* seeds have significant amounts of phytochemicals, including polyphenols, carotenoids and antioxidants, relative to other legume crops. Compounds with antioxidant properties present in the three *L. mutabilis* varieties are shown in Table 3. Multiple comparison tests showed that variety and grain condition caused significant changes in ascorbic acid, antioxidant capacity, total carotenoids and phenols of lupin samples ($P<0.05$).

Ascorbic acid

In the bitter grain, ascorbic acid varied from 5.82 mg/100 g (Criollo) to 13.44 mg/100 g (INIAP-450). These values were similar to those that have been reported for *L. albus* (6.48 mg/100 g) and *L. angustifolius* (5.71 mg/100 g) seeds (Fernandez-Orozco et al., 2008) but higher than values that have been reported for other legumes (3.50 mg/100 g for soybeans and 0.79 mg/100 g for beans) (Moriyama & Oba, 2008).

The debittering process reduced the ascorbic acid content of *L. mutabilis* by 46%, and fermentation with *R. oligosporus* generated a greater reduction (77%) of ascorbic acid relative to the debittered grain. Similar reductions have been reported in soybeans and in cooked beans (Moriyama & Oba, 2008) as well as in fermented lupin with different strains of microorganisms, which possibly use ascorbic acid for its metabolic activities (Fernandez-Orozco et al., 2008). The conditions used for the fermentation of lupin in the present study allowed some ascorbic acid to be retained in the lupin grain.

Total carotenoids

In the bitter grain, total carotenoids varied from 309.87 µg/100 g (Criollo) to 397.46 µg/100 g (INIAP-451) (Table 3). The results were similar to values that have been reported for *L. albus* (470 µg/100 g) but lower than values reported for bitter *L. mutabilis* (1486 µg/100 g) and *L. luteus* (1252 µg/100 g) (El-Difrawi & Hudson, 1979). The debittering process reduced total carotenoids by 49.42%. The temperature used during the debittering treatment appears to be the factor that has the greatest impact on reductions in carotenoids (Kantha & Simpson, 1987). Nevertheless, total carotenoids increased with fermentation of the debittered grain in all varieties: 131.62% (Criollo), 171.62% (INIAP-451) and 192.92% (INIAP-450). These results are consistent with those in soybeans fermented with six strains of *Rhizopus* sp., where carotenoids increased from 9.1 to 11.2 µg/g between 34 and 48 h of fermentation (Denter, Rehm & Bisping, 1998). Presumably, lupin acts as a substrate for *R. oligosporus*, providing carbon, nitrogen, minerals and other growth factors (Villacres, Quelal, Jácome, Cueva & Rosell, 2020b) and producing carotenoids.

Total phenols

An average of 1127 mg chlorogenic acid/100 g was observed in bitter *L. mutabilis*, with the highest value measured from the Criollo variety (1311.13 mg/100 g). These values are within the average reported for raw and bitter *L. mutabilis* seeds (1210 mg/100 g) (Chirinos, Pedreschi, Rogez, Larondelle & Campos, 2013). We observed a significant decrease (96.83%) in total phenolic contents during the debittering process. As these phenols are thermolabile, such reductions likely stem from the soaking and boiling of the grain, which facilitate their thermal and oxidative decomposition and partial leaching. Fermentation caused a substantial increase in total phenols of the three debittered lupin varieties 1320.62% (INIAP-451), 1025.50% (INIAP-450) and 820.58% (Criollo). The glycosidases of *Rhizopus* might hydrolyze the conjugated

polyphenol forms, releasing free polyphenols and, as a consequence, increasing the total phenol content (Lee, Hung & Chon, 2008; Khan et al., 2018).

Similar results have been observed in *Lupinus angustifolius* fermented with *R. oryzae*, where an increase in phenols of 31.45% has been reported (Fernandez-Orozco et al., 2008). The total content of phenols have also been reported to increase by 20.48% and 50.60% relative to unfermented control samples in bean seeds fermented with different strains of *Rhizopus* (Lee et al., 2008).

Trolox equivalent antioxidant capacity (TEAC)

The antioxidant capacity of raw seeds measured by the TEAC assay ranged from 707.73 to 747.57 $\mu\text{mol Trolox/g}$ (dry weight basis) (Table 3). Bitter lupin values from our study were higher than those that have been reported in raw seeds of *L. albus* (202.7 $\mu\text{mol Trolox/g}$) (Chirinos et al., 2013). However, raw seeds of *L. angustifolius* cv. Emir exhibited higher TEAC values (Martínez-Villaluenga et al., 2009). The debittering process caused a decrease of 96.12% in TEAC values; this reduction stemmed from the thermolability and water solubility of phenols. Nonetheless, the fermentation of *L. mutabilis* with *R. oligosporus* increased antioxidant capacity with values that ranged from 340.75 (INIAP-450) to 657.72 $\mu\text{mol trolox/g}$ (dry weight basis) (INIAP-451) relative to debittered grain. This increase might be explained by the ability of some microorganism strains to develop oxidative stress protection mechanisms when they are exposed to reactive oxygen substances (Hur, Lee, Kim, Choi & Kim, 2014). During fermentation, molds produce different enzymes, such as β -glucosidase, which hydrolyze the β -glucosidic bonds of some phenolic compounds, increasing their antioxidant activity. The fermentation of *L. angustifolius* zapatón variety with *Rhizopus* increased antioxidant activity by 10% relative to unfermented grain (Fernandez-Orozco et al., 2008). Increases of 210% and 303% were observed

for free radical-scavenging activity and ferric ion-reducing antioxidant power, respectively, following fermentation of the germinated *Lupinus angustifolius* L. (Khan et al., 2018).

4. Conclusions

The three varieties of *L. mutabilis* in their bitter forms possessed a variety of antinutritional compounds in varying quantities, such as 4.47% of alkaloids (INIAP-451), 975.74 mg tannins/100 g (Criollo) and 0.392 g phytic acid/100 g (Criollo). Nevertheless, bitter lupin varieties also contained functional compounds, such as total phenols (1127.87 mg/100 g), with higher concentrations in the Criollo variety (1311.13 mg/100 g), as well as total carotenoids (3.72 µg/g) and ascorbic acid (9.6 mg/100 g). The impact of debittering and fermentation processes on the aforementioned compounds was analyzed. Debittering caused a substantial reduction in QAs (92.06%), nitrates (94.84%), tannins (83.79%), urease activity (89.51%), trypsin inhibitors (67.44%) and phytic acid (48.46%). However, the contents of total phenols, carotenoids and ascorbic acid were also affected. Fermentation decreased antinutrients and ascorbic acid (63.40%) from debittered grain and increased total carotenoids (165.38%), phenolic compounds (1055.56%) and antioxidant capacity (1515.62%). In this research it was determined that antinutritional compounds with antioxidant properties are concentrated in bitter lupin and that some of these compounds may have pharmacological effects. The outcome of this study may be of great importance to the food industry for the production of novel, fermented food products with improved nutritional value through the fermentation of lupin. The significant concentrations of these phytochemicals in debittered grain suggest that lupin flour could be potentially used in a variety of bakery products.

Conflict of Interest

The authors declare that there is no conflict of interest regarding this publication.

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Table 1

Effect of debittering and fermentation processes on nitrates, tannins and quinolizidine alkaloid content of lupin grain (dry weight basis)

Variety	Grain condition	Nitrates (mg/100 g)	Tannins (mg/100 g)	Alkaloids (%)
INIAP- 450	Bitter	36.61 ± 0.79 ^b	956.20 ± 3.66 ^b	3.76 ± 0.07 ^b
	Debittered	1.89 ± 0.02 ^c	154.97 ± 1.34 ^g	0.30 ± 0.02 ^c
	Fermented	1.47 ± 0.16 ^c	144.48 ± 5.82 ^h	0.27 ± 0.04 ^c
INIAP- 451	Bitter	40.63 ± 0.72 ^a	920.34 ± 4.25 ^c	4.47 ± 0.08 ^a
	Debittered	2.86 ± 0.02 ^c	193.04 ± 2.63 ^e	0.35 ± 0.00 ^c
	Fermented	2.75 ± 0.19 ^c	179.39 ± 1.67 ^f	0.29 ± 0.01 ^c
Criollo	Bitter	37.98 ± 1.29 ^b	975.74 ± 5.24 ^a	3.74 ± 0.14 ^b
	Debittered	2.11 ± 0.04 ^c	204.48 ± 2.81 ^d	0.32 ± 0.01 ^c
	Fermented	2.20 ± 0.02 ^c	185.86 ± 1.51 ^{ef}	0.18 ± 0.04 ^c
<i>P</i> -value	Grain condition	< 0.0001	< 0.0001	< 0.0001
	Variety	< 0.0001	< 0.0001	< 0.0001
	Interaction	< 0.0001	< 0.0001	< 0.0001

Values represent means of three repetitions. The mean ± standard deviation followed by a different letter within columns are significantly different ($P < 0.05$).

Table 2

Effect of debittering and fermentation on urease activity, phytic acid and trypsin inhibitors of lupin grain (dry weight basis)

Variety	Grain condition	Urease activity (pH difference)	Phytic Acid (g/100 g)	Trypsin Inhibitors (TIU/mg sample)
INIAP- 450	Bitter	0.64 ± 0.02^b	0.25 ± 0.01^c	1.50 ± 0.01^c
	Debittered	0.07 ± 0.00^d	0.13 ± 0.02^d	0.43 ± 0.01^f
	Fermented	0.05 ± 0.006^d	0.07 ± 0.01^e	0.32 ± 0.01^h
INIAP- 451	Bitter	0.72 ± 0.01^a	0.30 ± 0.03^b	1.84 ± 0.00^a
	Debittered	0.08 ± 0.01^d	0.16 ± 0.00^d	0.49 ± 0.01^d
	Fermented	0.06 ± 0.00^d	0.13 ± 0.02^d	0.45 ± 0.00^e
Criollo	Bitter	0.58 ± 0.02^c	0.39 ± 0.01^a	1.56 ± 0.00^b
	Debittered	0.07 ± 0.00^d	0.13 ± 0.02^d	$0.47 \pm 0.00^{d,e}$
	Fermented	0.06 ± 0.00^d	0.06 ± 0.01^e	0.37 ± 0.00^g
<i>P</i> -value	Grain condition	< 0.0001	< 0.0001	< 0.0001
	Variety	< 0.0001	< 0.0001	< 0.0001
	Interaction	< 0.0001	< 0.0001	< 0.0001

Values represent means of three repetitions. The mean $\pm$ standard deviation followed by a different letter within columns are significantly different ($P < 0.05$). UTI: Trypsin Inhibitor Units.

Table 3

Effect of debittering and fermentation on the antioxidant content compounds of lupin grain (dry weight basis)

Variety	Grain condition	Ascorbic Acid (mg AA/100 g)	Total carotenoids (µg/g)	Total Phenols (mg CA/100 g)	Trolox equivalent antioxidant capacity (µg Trolox/g)
INIAP -450	Bitter	13.44 ± 0.17 ^a	3.33 ± 5.12 ^d	1034.41 ± 2.04 ^b	747.57 ± 2.90 ^a
	Debittered	7.74 ± 0.17 ^c	1.86 ± 8.87 ^g	30.74 ± 0.88 ^f	18.88 ± 1.53 ^g
	Fermented	3.60 ± 0.37 ^f	5.44 ± 4.46 ^b	345.86 ± 3.63 ^d	340.75 ± 4.24 ^e
INIAP- 451	Bitter	9.54 ± 0.18 ^b	3.97 ± 1.65 ^c	1038.03 ± 2.94 ^b	745.26 ± 8.34 ^a
	Debittered	4.33 ± 0.12 ^e	2.09 ± 5.12 ^f	39.72 ± 0.76 ^f	37.07 ± 2.47 ^f
	Fermented	0.92 ± 0.07 ^g	5.69 ± 5.12 ^a	564.27 ± 7.59 ^c	657.72 ± 4.25 ^c
Criollo	Bitter	5.82 ± 0.12 ^d	3.87 ± 0.00 ^e	1311.13 ± 3.93 ^a	707.73 ± 6.95 ^b
	Debittered	3.41 ± 0.31 ^f	1.30 ± 5.12 ^h	35.72 ± 3.68 ^f	29.27 ± 1.43 ^{f,g}
	Fermented	1.42 ± 0.24 ^g	3.01 ± 0.12 ^e	326.62 ± 2.01 ^e	365.98 ± 4.44 ^d
<i>P</i> -value	Grain condition	<0.0001	<0.0001	<0.0001	<0.0001
	Variety	<0.0001	<0.0001	<0.0001	<0.0001
	Interaction	<0.0001	<0.0001	<0.0001	<0.0001

Values represent means of three repetitions. The mean ± standard deviation followed by a different letter within columns are significantly different ($P < 0.05$). Total phenols: expressed as chlorogenic acid.