Effect of debittering and solid state fermentation processes on the nutritional content of lupine (*Lupinus mutabilis* Sweet)

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Summary

Currently, there is a growing interest in the search for vegetable protein sources. Lupine, despite its high nutrient content, has been little exploited due to its high content of non-nutritive compounds, especially alkaloids that give it a bitter taste and limit the nutritional potential of the grain. For this reason, the objective of the present study was to evaluate the effect of debittering processes and solid state fermentation on the nutritional content of three lupine varieties (Lupinus mutabilis Sweet). The application of these processes produced significant changes ($P < 0.05$) in the nutritional composition of the three lupine varieties (INIAP-450, INIAP-451 and Criollo). The debittering and fermentation process with *Rhizopus oligosporus* allowed for concentration of protein content and ether extract. Particularly, an increase in the protein content up to 644.55 g kg^{-1} in the Criollo variety. The ether extract increased up to 244.03 g kg^{-1} (INIAP-450), especially in monounsaturated (559.78 g kg^{-1}) and polyunsaturated (293.17 g kg^{-1}) fatty acids. However, a decrease in other components, such as crude and dietary fiber, resistant starch and some macro and micro minerals was determined.

Key Words

Nutritional content, solid fermentation, debittering process, leguminous

1. Introduction

Lupinus is a legume that include numerous species, although only four of them stand out from the nutritional and economic standpoint: *L. angustifulius* (Blue Lupine with narrow leaves), *L. albus* (White Lupine), *L. luteus* (Yellow Lupine) and *L. mutabilis* (Pearl Lupine). The genus *Lupinus mutabilis* Sweet is one of the most common species, because it can grow in poor soils and adapt to extreme conditions, as well as it is able to fix atmospheric nitrogen (Van de Noort, 2016).

Like other legumes, lupine is characterized by having an important nutritional value (Yorgancilar & Bilgiçli, 2014). Specifically, protein content between 41 to 51% has been described, which depends on the species, climatic and crop conditions (Arnoldi, *et al*., 2015; Karnpanit, *et al*., 2016). Likewise, its high content of monounsaturated and polyunsaturated fatty acids, makes it an alternative source of vegetable oil (Villacrés, *et al.*, 2013; Awad-Allah & Elkatry, 2013), and carbohydrates are low in sucrose and starch, with moderate oligosaccharides content that are not digested by human beings but are broken down by the bacterial flora present in the large intestine (Van de Noort, 2016).

Despite its high nutritional value, its use is very limited due to the presence of bitter compounds, specifically alkaloids derived from quinolyzidine (Carvajal-Larenas *et al.*, 2016), which prevent its direct consumption (Repo-Carrasco-V, *et al.*, 2007). To eliminate these components, debittering technologies should be applied. Traditional processes include a hydration phase, followed by cooking that inactivates the germination capacity of the grain and increases the permeability of the cell wall, facilitating the leaching of these compounds through successive washes (Carvajal-Larenas, *et al.*, 2013; Awad-Allah and Elkatry, 2013). Other techniques for the

elimination of alkaloids include hydro-agitation (Carvajal-Larenas et al., 2013) or biological processes such as germination (De Cortes *et al.*, 2005; Khan et al., 2018). Varieties with low alkaloid content have also been developed, but their adaptation to different environments is difficult and they change over time, with a return of the bitter attribute (Awad-Allah and Elkatry, 2013).

Solid state fermentation consists in the growth of microorganisms on substrates with limited water content (Sabu et al., 2002). This technique, originally from Indonesia, was developed from soaked and cooked soy by inoculating *Rhizopus* fungi, to obtain a compact cake formed by a cottony mycelium (Nout & Kiers, 2005). Subsequently, it has been used as a method of food production and preservation, because it improves the nutritional, functional and sensory profile (Blandino, *et al.*, 2003). In the case of legumes, fermentation facilitates the reduction of antinutritional compounds and oligosaccharides and allows increasing protein digestibility (Bartkienė *et al.*, 2014; Pandey, 2003; Karnpanit et al., 2016). This has been confirmed in the case of soybean (Nout & Rombouts, 1990), peas (Nowak & Szebiotko, 1992) and chickpea (Abu-salem & Abou-arab, 2011). Particularly, in the case of lupine, flour from previously fermented lupin with *Pedioccocus acidilactici* has been added to wheat flour to improve the nutritional quality of breads (Bartkiene, *et al.,* 2011). Even a significant increase of vitamin B_{12} content was achieved by fermenting lupin with a starter composed by *R. oryzae* and *P. freudenreichii*, (Rooijackers, Endika, & Smid, 2018). Other researchers fermented *L. mutabilis* with *Rhizopus. oligosporus* to reduce the alkaloid content, reporting the importance of particle size reduction and optimal moisture for fermentation (Ortega-David & Rodriguez-stouvenel, 2014; Carvajal-Larenas *et al.*, 2016). However, this methodology only allowed for the partial detoxification of the lupine.

The aim of this research was to evaluate the combined effect of debittering and solid state fermentation processes, using *Rhizopus oligosporus*, on the nutritional profile of three varieties of *Lupinus mutabilis* of Ecuadorian origin. With that purpose, the fatty acids content, amino acids profiles, dietary fiber, total starch, resistant and mineral content were quantified.

2. Materials and methods

2.1. *Raw material*

Bitter lupine (INIAP-450, INIAP-451 and Criollo) were provided by the National Legumes Program and Andean INIAP Grains. The harvested grain was threshed and classified in a Clirppen MFG.INC.USA equipment, which has a set of sieves with 8, 7 and 6 mm openings. For this study, grains with an average diameter of 7 to 8 mm were used.

In the case of bitter *Lupinus mutabilis*, 2 kg of each variety was used for the analyses. The samples were milled (Retsch KG -5657 Haan, Remscheid, Germany) until a fine powder (250 μm) was obtained. Then, they were packed in polypropylene bags and stored at 12 °C, till further analysis.

2.2. *Debittering the grain*

For the debittering process the thermal-aqueous treatment was applied, which started with the hydration of the grain at an initial temperature of 80 °C for 10 h, a ratio of 1:3 (grain: water) was used. Then, cooking was done in water at 91°C for 1 h, followed by washing with potable water. In this phase a ratio of 1:15 (grain: water) was used. The first washing was done with water at 35 °C for 28 h and the second washing at 18 °C for 45 h. The debittered lupine underwent a drying process in a forced air oven (HS122A) at 60 °C for 8 h. The grain was ground and packed in the same conditions as the bitter lupine.

2.3. *Grain Fermentation*

Rhizopus oligosporus strain NRRL2710 from the NRRL collection (Northern Regional Research Laboratory USDA, USA), belonging to the Microbiology laboratory collection at the Ambato University of Technology, was used. For the fermentation process, the humidity of the debittered grain was reduced to 50% in a forced air oven at 60 °C for two hours. The grain was crushed in a miniprocessor (Oster, Rio de Janeiro, Brazil), and portions of 50 g were packed and sealed in polypropylene bags to be sterilized in autoclave (Webeco, Farjestäden, Germany) at 121 °C for 10 minutes. Then 500 µl of the spore suspension was inoculated to each grain portion and incubated at 28 °C for four days.

Once abundant mycelium formation was observed, the samples were lyophilized in a kit (Labconco Lyph Lock 12, Kansas, USA) at -40 °C and -0.7 bar pressure, for four days. The fermented grain was ground, packed in polypropylene bags and stored at 10 °C, prior to chemical analysis.

2.4. *Chemical analysis*

2.4.1 *Proximate composition*

The determination of protein, fat, crude fiber and ash was carried out using standardized methods (AOAC, 2000). The carbohydrate content was calculated by difference.

2.4.2 *Amino acid profile*

Amino acid profile was carried out by high performance liquid chromatography (AOAC, 2000). The samples were defatted using solvent extraction at room temperature. Then, they were freeze dry (-40^{o}C, -0.7 bar), ground (150 µm) and homogenized. Samples (25 mg) were incubated in an oxygen-free environment in constant-boiling 6 M HCl at 110°C for 22 h. The hydrolyzed samples were concentrated on a rotary evaporator, then 5 ml of citrate buffer pH 2.2 was added before the HPLC

analysis (Shimadzu analyzer). The working conditions were: sample cooler temperature 4^{o}C, oven temperature 60^{o}C, eluent solution flow 0.60 m^{2} /min, injection volume 5 µl, emission length 450 nm, excitation length 350 nm, run time 45 min.

2.4.3 *Fatty acids profile*

The fatty acids were determined by gas chromatography (AOCS, 2005). The oil was extracted from a 50 g sample of ground lupin (150 µm) by refluxing for 6 h with 125 mL of n-hexane in a Soxhlet extractor. The solvent was evaporated under reduced pressure and the oil was recovered, then the esterification was performed on 50 mg of extracted oil, to which 1 mL of 0.5 M KOH in methanol was added. This mixture was placed in sealed test tubes, which were boiled for 30 min in a water bath and then cooled at room temperature. Later, 0.5 mL of the mixture HCl (37 %)/ methanol (1:4) was added and the mixture boiled for 25 min, cooled and mixed with 2 mL of doubly distilled water. The esters were recovered through three successive washes with n-hexane. To this solution was added anhydrous sodium sulfate to eliminate residual water. The supernatant was recovered and the solvent evaporated with nitrogen gas. The extract was diluted with 2 mL of n-hexane and injected into a gas chromatograph (Shimadzu GC-14B). A thermal column TR-FAME, 3 m in length x 0.25 mm in diameter and 0.25 µm pore size was used for separation of fatty acid methyl esters. 100 ºC initial temperature was maintained for 5 minutes and then increased at 4ºC/min rate until reaching 200ºC final temperature, which was maintained for 2 min. A flame ionization detector (air-hydrogen-nitrogen) was used. The split ratio was 1:10, and hydrogen was used as a carrier gas with the flow rate of 0.8 mL/min. The injector and detector temperatures were 250°C and 280°C, respectively. Peak identification of the fatty acids in the analyzed samples was carried out by comparison with retention times of known standards.

158 2.4.4 *Total starch*

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160 The polarimetric determination of starch content is based on the optically activity of
161 starch. Due to the fact that starch cannot be dissolved in water, hydrochloric acid was
162 used. Then sample was clarified, filtered and measured in a polarimeter. The optical
163 rotation of all samples was measured at 20°C by using a sample cell of 200 mm optical
164 path length (AOAC, 2000; Anca *et al*., 2013).

165 2.4.5 *Resistant starch*

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167 Samples were incubated in a shaking water bath with pancreatic α-amylase and
168 amyloglucosidase for 16 h at 37°C. The reaction was terminated by the addition of an
169 equal volume of ethanol and the resistant starch was recovered as a pellet on
170 centrifugation. Resistant starch in the pellet was dissolved in 2 M KOH by vigorously
171 stirring in an ice-water bath over a magnetic stirrer. This solution was neutralised with
172 acetate buffer and the starch was quantitatively hydrolysed to glucose with
173 amyloglucosidase. D-Glucose was measured with glucose oxidase/peroxidase reagent
174 (GOPOD) and this was a measure of the resistant starch content of the sample (AOAC
175 Official Method 2002.02).

176 2.4.6 *Total dietary fiber*

177 Total dietary fiber was determined on samples of dried and defatted following standard
178 methods (AOAC 991.43, AOAC 985.29). Samples were cooked at 100°C with heat
179 stable α-amylase to give gelatinisation, hydrolysis and depolymerisation of starch;
180 incubated at 60°C with protease (to solubilise and depolymerise proteins) and
181 amyloglucosidase (to hydrolyse starch fragments to glucose); and treated with four
182 volumes of ethanol to precipitate soluble fiber and remove depolymerised protein and
183 glucose (from starch). The residue was filtered; washed with 78% ethanol, 95% ethanol,
184 and acetone; dried; and weighed. One duplicate was analysed for protein and the other

was incubated at 525°C to determine ash. The total dietary fiber was the weight of the filtered and dried residue minus the weight of the protein and ash.

2.4.7 *Insoluble and soluble dietary fiber*

The dried lupin sample (1 g) was subjected to enzymatic digestion with thermostable α-amylase, protease and amyloglucosidase, as described in the standard method (AOAC 991.4). The insoluble dietary fiber (IDF) was filtered, and then the residue was washed with hot distilled water. The combined filtrate and water wash solution was precipitated with 4 volumes of 95% ethanol for the determination of soluble dietary fiber (SDF), then the precipitate was filtered and dried. For the final calculation, both residues (IDF and SDS) were corrected by protein, ash and blank.

2.4.8 *Mineral composition*

For the digestion of the samples, the AOAC method 985.35, (2005) was taken as a reference. Samples were burnt in a SNOL 10740 muffle at 525 °C, the ashes dissolved and settled at 25 mL with 0.1 M HNO3 (trace metal grade). Calibration curves were performed by dilution of standards at the specific concentrations for each mineral. The analytical curves were obtained with a linear response for the selected concentration ranges. Mineral analysis was performed by flame spectrophotometry in a Perkin Elmer AAnalyst 400 atomic absorption spectrophotometer, WinLab32AA Software and hollow cathode lamps for each element, except for the phosphorus that was analyzed by colorimetry (AOAC, 2000).

2.5. *Statistic analysis*

The statistical analysis was performed in the Infostat program (Córdoba, Argentina). The normal distribution of the data was verified through the Shapiro-Wilks goodness of fit test. We applied a multifactorial variance design (ANOVA) and the Tukey test with a 95% degree of significance ($P < 0.05$) to establish significant differences between

samples. All analyses were performed in triplicate; the presented data are expressed as the mean ± standard deviation.

3. Results and Discusions

3.1. *Debittering and solis state fermentation effect on the proximal composition of the lupine*

Table 1 indicates the proximal composition of three varieties of lupine grain in a bitter, debittered and fermented state. The statistical analysis confirmed the statistically significant differences between the lupine varieties and the application of the debittering and solid state fermentation processes evaluated, specifically in regard to the protein content, fiber, minerals and carbohydrates.

The bitter lupine protein content, the Criollo variety presented the highest protein content and the lowest in minerals and carbohydrates, while the INIAP-450 variety was characterized by a lower crude fiber content and higher mineral content was quantified in the INIAP-451 variety. The protein contents are within the values recorded by Sujak et al. (2006). However, they are greater than those described for lupine sown in the Andes of Colombia and Peru (Ortega-David, Rodríguez & Zamora-Burbano, 2010). These differences are attributed to the species variation and the climatic conditions (Jacobsen & Mujica, 2008). Regarding the impact of the treatments carried out, the debittering process induced a significant protein increase in the three varieties analyzed, due to the dilution of water-soluble carbohydrates and minerals during the water process (Carvajal-Larenas *et al.*, 2016; Erbas., 2010). This statement corroborated the results of the nitrogen-free extract, which experienced a 70% decrease in relation to bitter lupine. The solid fermentation allowed the debittered grain to reach higher protein content, which might be attributed to *Rhizopus oligosporus* that uses carbohydrates, fiber and some minerals to synthesize protein (Abu-salem & Abou-arab, 2011). It might be

stressed that the increase in protein content caused by fermentation was higher than that reported for other fermented legumes such as: chickpeas (28.85%) (Abu-Salem & Abou-Arab, 2011) and beans (31.6%) (Barampama & Simard, 1995).

The debittering process contributed to the increase of fat content (ether extract), likely due to the loss of aqueous soluble compounds. The fermentation provoked a slight increase in fat. Similarly, to what happen for proteins, the metabolic activity of *R. oligosporus* uses part of the carbohydrates, fiber and minerals to synthesize other compounds such as fat and other bioactive compounds (Khan *et al*., 2015). Concerning the debittering process, crude fiber increased 49.40% (INIAP-450), 15.92% (INIAP-451) and 43.46% (Criollo), with respect to bitter grain, which should be attributed to the insolubility of these compounds in water (García -López *et al.*, 2001). However, fermentation caused the opposite effect, with a decrease of 15.59% (INIAP-450), 0.39% (INIAP-450) and 29.22% (Criollo) with respect to the debittered grain. Moreover, the solubilization of some minerals in the debittering process affected the ash content, registering a reduction of 38.37, 50.25 and 50.73% for INIAP-450, INIAP-451 and Criollo. The fermentation process caused a decrease of the ash (8.63%) in the INIAP-450 variety. In the two remaining varieties there was no significant change in the fermented grain ash with respect to the debittered grain.

3.2. *Debittering and fermentation effect on the lupine amino acid profile*

The evaluation of the amino acid profile confirmed significant differences due to the effect of the variety and the applied process ($P < 0.05$) (Table 2). The arginine, aspartic and glutamic amino acids increased in the three varieties of lupine and lysine acid increased only in the Criollo variety, due to the debittering effect of the grain. The hydrophilic character of the amino acids serine, threonine, cysteine and tyrosine could have contributed to their greater water solubility and reduction in the hydration, cooking

and washing stages of the grains. Fermentation helped to increase the concentration of the following amino acids: valine, methionine, isoleucine, leucine in the three grain varieties while proline increased only in INIAP-451 and Criollo, which could be related to the non-polar character of these amino acids.

Other compounds such as arginine, alanine, lysine and phenylalanine decreased in all three varieties and aspartic acid and serine decreased only in INIAP-451 and Criollo. It is probable that *R. oligosporus* uses part of these amino acids for its metabolic activity (Handoyo & Morita, 2006), which reduces their concentration in the fermented grain compared to the debittered.

3.3. *Debittering and fermentation effect on the lupine fatty acid profile*

The variety and the processes applied significantly affected ($P < 0.05$) the fatty acid composition of the lupine seed (Table 3). In the bitter grain, more than 50% of MUFA, with a predominance of oleic acid. Considering the debittering process, the MUFA of INIAP-451 and Criollo decreased with respect to the bitter grain, but increased in the fermentation process to 57.25% in INIAP-450, 55.80% in INIAP-451 and 54.88% in the Criollo variety. The PUFA, constituted 25% of the lupine oil, with a predominance of linoleic acid, which increased due to the effect of debittering and fermentation. In the case of soy fermentation, an increase in the fat and fatty acids content has been reported by Lee, *et al.,* 1997. The increase in the fatty acids content agree with the metabolic process reported for *Rhizopus oligosporus,* which uses carbohydrates and fiber to synthesize fat and fatty acids (Nout & Kiers, 2005). Regarding the fat quality indexes, it was determined that the PUFA / MUFA ratio exceeds the 0.5 value and the (PUFA + MUFA) / SFA ratio was ≥ 2, which indicates that the debittering or fermented lupine fat complies with the nutritional objectives required (≥ 2) (FAO, 2012). The omega-6 / omega-3 ratio of the debittered grain oil approached the minimum requirement

285 established for good quality oils (5/1) (FAO, 2012). However, in the fermented grain
286 oil, this proportion varied between 25.54 (INIAP-450) to 26.72 (Criollo), due to the
287 increase in linoleic acid and decrease in linolenic acid. FAO (2012) indicates that it is
288 not reasonable to make specific recommendations as long as the intake of the two fatty
289 acids are within the recommended daily values.

290 3.4. *Debittering and fermentation effect on the total starch content, resistant starch and*
291 *dietary fiber of lupine*

292 Concerning starch and dietary fiber, the results showed significant differences ($P < 0.05$)
293 due to the effect of the variety and the processes applied (Table 4). The total starch
294 content and resistant starch of the grain decreased due to the washing during the
295 debittering process, except in the case of the INIAP-451 variety, that kept constant the
296 resistant starch content. The fermentation did not significantly affect the total starch
297 content of the samples, but did produce a significant decrease in the resistant starch,
298 except in the Criollo variety. Nout & Rombouts (1990) pointed out that during the soy
299 fermentation, the enzyme β-glycosidase hydrolyzes the β-glycosides forming aglycones,
300 which are the readily available forms, thus enzyme activities might be responsible of the
301 decrease observed in the resistant starch. Regarding the total dietary fiber, INIAP-451
302 was distinguished by its high content. The debittering process caused an increase in the
303 insoluble fiber in all the varieties under study, at the expense of a decrease in the water
304 soluble compounds of the grain. The fermentation caused a significant decrease in the
305 total dietary fiber, in relation to the debittered grain. Again, this could be attributed to
306 the hydrolytic action of *Rhizopus oligosporus* during fermentation.

307 3.5. *Debittering and fermentation effect on the macro and micro-mineral content of*
308 *lupine*

309 The macro and micronutrients content were also significantly affected by the
310 detoxification processes and variety (Table 5). The most abundant macro-elements of
311 the three bitter grain varieties were potassium and phosphorus. Among the micro-
312 elements iron, manganese and zinc stood out. The debittering favored the increase of the
313 calcium content and a decrease of the other macro and micro elements, with the
314 exception of sodium, which did not experience significant changes in the debittering of
315 the grain. Ertas & Bilcicli (2014) and Van de Noort (2016) observed similar variations
316 in quinoa and amaranth minerals subjected to soaking and cooking processes. Solid
317 fermentation caused a decrease in calcium in INIAP-450 and Criollo, and it reduced
318 phosphorus in INIAP-450. The remaining macro-elements did not show significant
319 variations due to the fermentation effect. According to Omosebi & Otunola (2013), the
320 loss of calcium would be attributed to the metabolic activity of *R. oligosporus*, which
321 requires this mineral for the regulation and/or stimulation of the enzymatic activity
322 responsible for protein metabolism. In the fermented grains, a significant reduction in
323 the manganese, zinc and iron content was observed in the INIAP-451 variety. The
324 decrease in iron might be associated to the formation of nitrogen compounds catalyzed
325 by the nitrogenase enzyme, which requires iron as a cofactor (Viniegra-González,
326 1997). Copper showed an increase with fermentation, possibly due to the dissociation of
327 the complex formed with phytic acid (Ghavidel & Prakash, 2007).

328 **4. Conclusions**

329 The effect of the debittering and solid state fermentation process with *Rhizopus*
330 *oligosporus* affected the nutritional properties in the three evaluated lupine varieties.
331 The debittering increased the protein concentration by 553.30 g kg^{-1} and several amino
332 acids like aspartic acid (76.98 g kg^{-1}), glutamic acid (112.84 g kg^{-1}), arginine (73.59 g
333 kg^{-1}) and leucine (74.93 g kg^{-1}). The ether extract increased by 222.40 g $kg^{-1}$and PUFA

by 292.25 g kg^{-1}. The fermentation helped raise the mentioned contents, specifically proteins (617.85 g kg-1), several essential amino acids as valine (56.75 g kg^{-1}), methionine (42.47 g kg^{-1}), isoleucine (59.27 g kg^{-1}) and leucine (76.11 g kg^{-1}), ether extract (229.80 g kg^{-1}) and PUFA (293.17 g kg^{-1}), but decreased ashes (18.06 g kg^{-1}), nitrogen-free extract (18.38 g kg^{-1}), resistant starch (22.69 g kg^{-1}) and soluble dietary fiber (7.07 g kg^{-1}). Therefore, the processing of lupine by debittering and/or solid-phase fermentation offers an alternative to expand the use of lupine as an ingredient for the fortification of food products.

Conflict of Interest

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

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Table 1. Proximate composition of bitter, debittered and fermented lupine*

	INIAP-450			INIAP-451			Criollo					
	Bitter	**Debittered**	**Fermented**	**Bitter**	**Debittered**	**Fermented**	**Bitter**	**Debittered**	**Fermented**	**Grain condition**	**Variety**	**Interaction**
Protein	470.93 ± 1.40^{g}	546.88± 1.45^{e}	608.15 ± 4.35^{b}	463.87 ± 3.70^{h}	552.42 ± 0.29^{e}	600.85 ± 1.45^{c}	483.83 ± 3.0^{f}	560.59 ± 0.29^{d}	644.55 ± 1.45^{a}	<0.0001	<0.0001	<0.0001
Ether extract	167.13 ± 4.05^{c}	227.50 ± 0.90^{b}	244.03 ± 1.95^{a}	167.73 ± 0.45^{c}	219.97± 3.70^{b}	224.20 ± 7.36^{b}	174.90 ± 0.90^{c}	219.73 ± 4.86^{b}	221.17 ± 2.93^{b}	<0.0001	<0.0001	<0.0001
Crude fiber	92.30 ± 0.50^{f}	137.93 ± 3.85^{b}	116.40 ± 5.14^{cd}	108.03 ± 0.15^{de}	155.23 ± 5.11^{a}	125.70 ± 1.60^{c}	104.00 ± 1.20^{e}	149.23 ± 3.71^{a}	105.60 ± 4.50^{e}	<0.0001	<0.0001	0.0001
Ash	33.25 ± 0.25^{b}	21.50± 0.50^{d}	19.97± 0.50^{e}	37.21 ± 0.25^{a}	18.74 ± 0.75^{ef}	18.24 ± 0.25^{fg}	31.75 ± 0.25^{c}	15.99 ± 0.50^{h}	15.98 ± 1.01^{gh}	<0.0001	<0.0001	<0.0001
Nitrogen Free Extract	236.38 ± 4.30^{a}	66.19 ± 4.92^{c}	11.45 ± 5.1^{e}	223.16 ± 3.52^{a}	53.64 ± 7.37^{c}	31.01 ± 9.72^{d}	205.52 ± 3.00^{b}	54.46 ± 8.49^{c}	12.70 ± 5.04^{e}	0.0001	<0,.0001	0.0001

*Expressed based on g kg $^{-1}$ of dry grain. The values followed by different letters entered in the columns for each variety indicate significant differences (P <0.05). Mean ± standard deviation (n = 3).

Table 2. Amino acid profile of bitter, debittered and fermented lupine*

Aminoacid	INIAP-450			INIAP-451			Criollo			Grain condition	Variety	Interaction
	Bitter	Debittered	Fermented	Bitter	Debittered	Fermented	Bitter	Debittered	Fermented			
Aspartic acid	72.99 ± 0.66^{cd}	77.29 ± 0.19^{a}	72.13 ± 0.55^{cd}	73.36± 0.82^{c}	78.38 ± 0.83^{a}	68.29 ± 0.38^{e}	71.43 ± 0.44^{d}	75.28 ± 0.67^{b}	74.20 ± 0.17^{e}	<0.0001	<0.0001	<0.0001
Serine	60.13 ± 0.74^{a}	59.73 ± 0.44^{a}	56.35 ± 0.74^{b}	60.15± 0.78 ^{a}	58.59± 0.44^{a}	51.98 ± 0.61^{c}	58.95 ± 0.37^{a}	59.28± 0.34^{a}	58.74 ± 0.13^{c}	<0.0001	<0.0001	<0.0001
Glutamic acid	100.45 ± 0.56^{c}	112.95± 0.64^{ab}	114.39± 0.66^{a}	99.45 ± 0.76^{cd}	113.20± 0.42^{ab}	114.17 ± 0.84^{ab}	98.09 ± 0.61^{d}	112.37 ± 0.28^{b}	104.78 ± 0.24^{ab}	0.0082	<0.0001	0.0838
Histidine	53.89 ± 0.67^{a}	50.23 ± 0.71^{cd}	51.52 ± 0.55^{bc}	48.50 ± 0.61^{ef}	48.27± 0.55^{f}	50.82 ± 0.61^{cd}	52.86 ± 0.29^{ab}	49.89 ± 0.11^{de}	50.84 ± 0.11^{cd}	<0.0001	<0.0001	<0.0001
Glycine	55.67 ± 0.82^{a}	55.16± 0.42^{a}	55.36 ± 0.19^{a}	52.25± 0.79^{bc}	51.29 ± 0.62^{c}	52.26 ± 0.87^{bc}	55.44 ± 0.15^{a}	53.87± 0.34^{ab}	53.93 ± 0.12^{bc}	<0.0001	0.0022	0.0023
Arginine	68.35± 0,78^{c}	74.30 ± 0.68^{a}	70.60 ± 0.38^{b}	67.76 ± 0.71^{c}	73.01 ± 0.43^{a}	69.13 ± 0.77^{bc}	68.54 ± 0.36^{c}	73.47± 0.04^{a}	73.17 ± 0.16^{bc}	0.0042	<0.0001	0.2663
Threonine	52.52 ± 0.60^{c}	51.80 ± 0.94^{c}	52.77 ± 0.77^{c}	54.83± 0.48^{ab}	52.44 ± 0.30^{c}	55.53 ± 0.94^{a}	53.39 ± 0.56^{bc}	52.42 ± 0.38^{c}	53.00 ± 0.62^{a}	0.0001	<0.0001	0.0308
Alanine	51.74± 0.75^{bc}	50.65± 0.53^{cd}	49.28 ± 0.67^{e}	53.90 ± 0.63^{a}	51.28 ± 0.52^{c}	49.52± 0.14^{de}	52.96 ± 0.20^{ab}	51.24 ± 0.22^{c}	51.19 ± 0.12^{de}	0.0008	<0.0001	0.0276
Proline	51.10 ± 0.40^{cd}	51.02 ± 0.16^{cd}	51.08 ± 0.81^{cd}	52.03± 0.49^{c}	51.40 ± 0.34^{cd}	54.91 ± 0.13^{a}	53.17± 0.19^{b}	50.91 ± 0.12^{d}	51.19 ± 0.12^{a}	<0.0001	<0.0001	<0.0001
Cystine	45.09 ± 0,40^{a}	38.10 ± 0.42^{d}	40.84 ± 0.74^{c}	44.19 ± 0.37^{ab}	38.32 ± 0.36^{d}	43.48 ± 0.10^{b}	45.01 ± 0.28^{a}	38.77 ± 0.28^{d}	40.54 ± 0.09^{b}	<0.0001	<0.0001	<0.0001
Tyrosine	46.15 ± 0,53^{b}	43.70 ± 0.77^{c}	47.74 ± 0.53^{ab}	47.64± 0.75^{ab}	44.11 ± 0.78^{c}	48.76 ± 0.61^{a}	48.09 ± 0.33^{a}	44.22 ± 0.02^{c}	46.60 ± 0.83^{a}	0.0012	<0.0001	0.331
Valine	54.64 ± 0,54^{cd}	50.71 ± 0.66^{f}	57.04 ± 0.87^{b}	56.05± 0.43^{bc}	53.32 ± 0.77^{de}	58.59 ± 0.14^{a}	54.85± 0.18^{c}	52.92 ± 0.03^{e}	54.62 ± 0.12^{a}	<0.0001	<0.0001	0.0251
Methionine	44.52 ± 0,29^{ab}	38.40± 0.47 ^{f}	42.69 ± 0.84^{cd}	44.05 ± 0.49^{b}	41.51 ± 0.73^{d}	43.85± 0.12^{bc}	45.53 ± 0.40^{a}	39.87 ± 0.02^{e}	40.88 ± 0.09^{bc}	<0.0001	<0.0001	<0.0001
Lysine	60.45 ± 0,30^{b}	61.38± 0.73^{ab}	50.84 ± 0.94^{c}	61.59 ± 0.80^{ab}	62.51 ± 0.29^{a}	50.59 ± 0.52^{c}	60.17 ± 0.26^{b}	62.28 ± 0.73^{a}	60.44± 0.51^{c}	0.037	<0.0001	0.1985
Isoleucine	55.85 ± 0,63^{b}	52.70 ± 0.81^{c}	59.04 ± 0.82^{a}	57.10 ± 0.76^{b}	53.93 ± 0.97^{c}	60.44 ± 0.15^{a}	56.00 ± 0.41^{b}	53.69 ± 0.37^{c}	58.34 ± 0.13^{a}	0.0015	<0.0001	0.4576
Leucine	72.20 ± 0.52^{de}	76.30 ± 0.20^{ab}	77.45 ± 0.45^{a}	73.93 ± 0.82^{cd}	74.58± 0.44^{bc}	76.95 ± 0.74^{a}	71.51 ± 1.05^{e}	73.92 ± 0.41^{cd}	74.55± 0.17^{a}	0.0018	<0.0001	0.0015
Phenylalanine	54.29 ± 0,84^{abc}	55.60 ± 0.47^{a}	51.36 ± 0.49^{d}	53.25± 0.42^{c}	53.88 ± 0.53^{bc}	50.76± 0.38^{d}	54.01 ± 0.60^{bc}	55.32 ± 0.10^{ab}	54.96 ± 0.12^{d}	0.0007	<0.0001	0.2073

*Expressed based on g kg $^{-1}$ of dry grain. The values represent means of ± SD of three repetitions. The mean ± standard deviation followed by a different letter entered in the columns for each variety are significantly different (P <0.05)

Table 3. Fatty acid profile of bitter, debittered and fermented lupine*

	Fatty Acids	INIAP-450			INIAP-451			Criollo			Grain condition	Variety	Interaction
		Bitter	Debittered	Fermented	Bitter	Debittered	Fermented	Bitter	Debittered	Fermented			
SFA	Palmitic acid	104.62± 2.01^{b}	109.00 ± 1.20^{ab}	87.77 ± 1.46^{c}	104.97 ± 1.21^{b}	111.00 ± 1.1^{a}	81.63 ± 1.53^{d}	107.63 ± 2.37^{ab}	108.69 ± 0.31^{ab}	84.17 ± 3.35^{cd}	0.3246	<0.0001	0.0039
SFA	Stearic acid	72.17 ± 1.61^{b}	68.93 ± 2.80^{bc}	48.50 ± 1.04^{f}	76.83 ± 1.93^{a}	67.08± 0.88^{c}	50.40 ± 0,72^{f}	57.00± 0.62^{de}	59.50 ± 0.61^{d}	52.90± 2.27^{ef}	<0.0001	<0.0001	<0.0001
SFA	Arachidic acid	3.20 ± 0.36^{d}	7.83 ± 0.15^{b}	8.03 ± 0.25^{b}	5.45 ± 0.93^{c}	8.80 ± 0.75^{b}	13.87 ± 1.55^{a}	3.96 ± 0.77^{cd}	8.43 ± 1.31^{b}	12.90 ± 0.53^{a}	<0.0001	<0.0001	0.0035
	TOTAL SFA	180.00 ± 3.67^{bc}	185.77 ± 2.06^{ab}	145.30 ± 0.96^{e}	187.25 ± 2.26^{a}	186.88 ± 1.40^{a}	145.90 ± 0,82e	168.60 ± 2.82^{d}	176.63 ± 1.15^{c}	149.97 ± 1.12^{e}	<0.0001	<0.0001	<0.0001
MUFA	Oleic acid	540.83 ± 1.17^{b}	520.30 ± 5.80^{d}	562.07 ± 0.60^{a}	525.07 ± 1.37^{cd}	505.36 ± 2.12^{f}	543.10 ± 1.23^{b}	529.50 ± 1.49^{c}	513.37± 0.76 ^{e}	537.90± 1.11^{b}	<0.0001	<0.0001	<0.0001
MUFA	Oleic Acid Isomer	9.43 ± 0.84^{de}	10.53 ± 0.15^{cd}	10.43± 0.31^{cd}	13.03 ± 0.16^{b}	14.57 ± 0.91^{a}	14.93± 1.81^{b}	8.67 ± 0.35^{e}	9.83 ± 0.51^{cde}	10.90 ± 0.36^{c}	<0.0001	0.0001	0.0027
	TOTAL MUFA	550.27 ± 1.36^{c}	530.83± 5.65^{e}	572.50 ± 0.30^{a}	538.10 ± 1.23^{d}	519.93 ± 1.35^{f}	558.03 ± 0,58^{b}	538.17 ± 1.56^{d}	523.20 ± 0.60^{f}	548.80 ± 1.42^{c}	<0.0001	<0.0001	0.0001
PUFA	Linoleic acid	256.13 ± 3.43^{e}	267.00 ± 2.38^{g}	271.57± 0.64^{de}	261.43± 1.77^{f}	272.16 ± 1.27^{d}	285.31 ± 0.70^{b}	279.50 ± 0..96^{c}	281.13 ± 0.91^{bc}	290.36 ± 1.18^{a}	<0.0001	<0.0001	<0.0001
PUFA	Alpha-linolenic acid	13.60 ± 1.28^{cd}	16.40 ± 1.90^{bc}	10.63 ± 0.31^{d}	13.23 ± 1.36^{cd}	21.03 ± 1.10^{a}	10.77 ± 0.55^{d}	13.73 ± 1.63^{cd}	19.03± 1.05^{ab}	10.87 ± 0.50^{d}	0.0489	<0,0001	0.0164
	TOTAL PUFA	269.73 ± 2.45^{d}	283.40± 3.60^{c}	282.20 ± 0.75^{c}	274.66 ± 3.11^{d}	293.19 ± 0.23^{b}	296.08 ± 0.40^{ab}	293.23 ± 1.91^{b}	300.17 ± 1.07^{a}	301.22 ± 0.72^{a}	<0.0001	<0.0001	0.0001
	PUFA/MUFA	1.50 ± 0.04^{de}	1.53 ± 0.003^{de}	1.94 ± 0.02^{b}	1.47 ± 0.03^{e}	1.57 ± 0.01^{d}	2.03 ± 0.01^{a}	1.74 ± 0.04^{c}	1.70 ± 0.02^{c}	2.01 ± 0.02^{ab}	<0.0001	<0.0001	<0.0001
	(PUFA+MUFA)/SFA	4.56 ± 0.11^{de}	4.38 ± 0.06^{ef}	5.88 ± 0.05^{a}	4.34 ± 0.06^{f}	4.35 ± 0.04^{f}	5.85 ± 0.04^{ab}	4.93 ± 0.10^{c}	4.66 ± 0.04^{d}	5.67 ± 0.05^{b}	<0.0001	<0.0001	<0.0001
	linoleic/linolenic acid ratio	18.83 ± 1.93^{bc}	16.28 ± 1.79 ^{bcd}	25.54 ± 0.73^{a}	19.76 ± 1.96^{b}	12.94 ± 0.75^{d}	26.50 ± 1.42^{a}	20.36 ± 2.39^{b}	14.77± 0.83^{cd}	26.72 ± 1.33^{a}	0.4851	<0,0001	0.1247

*Percentage expressed on g kg $^{-1}$ of dry grain. The values represent means of ± SD of three repetitions. The mean ± standard deviation followed by a different letter entered in the columns for each variety are significantly different (P <0.05). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids

Table 4. Total starch and resistant starch content, dietary fiber of bitter, debittered and fermented lupine*

	INIAP-450			INIAP-451			Criollo					
	Bitter	**Debittered**	**Fermented**	**Bitter**	**Debittered**	**Fermented**	**Bitter**	**Debittered**	**Fermented**	**Grain condition**	**Variety**	**Interaction**
Total Starch	25.83 ± 1.52^{b}	13.06 ± 1.45^{d}	15.71 ± 1.43^{cd}	31.82 ± 1.52^{a}	19.47 ± 1.50^{c}	27.48 ± 1.45^{b}	28.73 ± 1.51^{ab}	13.04± 1.19^{d}	15.79 ± 1.44^{cd}	<0.0001	0.0001	0.0009
Resistant starch	31.91 ± 3.33^{cd}	22.62 ± 0.90^{f}	16.03 ± 1.54^{g}	28.98 ± 1.97^{de}	25.84 ± 1.94^{ef}	14.73 ± 1.04^{g}	61.29 ± 1.63^{a}	39.44 ± 2.33^{b}	37.30 ± 1.37^{bc}	<0.0001	0.0001	<0.0001
Total dietary fiber	324.44 ± 19.61^{f}	501.92 ± 2.70^{b}	449.31 ± 0.13^{e}	491.73 ± 2.54^{bc}	536.14 ± 0.42^{a}	464.98 ± 2.25^{de}	349.31 ± 7.94^{f}	540.29 ± 2.03^{a}	475.44 ± 20.85^{cd}	<0.0001	0.0001	<0.0001
Soluble dietary fiber	14.44 ± 0.15^{d}	5.38 ± 0.52^{g}	2.50 ± 0.25^{h}	20.43 ± 0.33^{a}	17.32 ± 0.16^{b}	11.08 ± 0.10^{e}	19.86 ± 0.07^{a}	16.39 ± 0.14^{c}	7.64 ± 0.04^{f}	<0.0001	0.0001	<0.0001
Insoluble dietary fiber	310.00 ± 19.76^{e}	496.54 ± 3.22^{b}	446.81± 0.37^{d}	471.30 ± 2.21^{c}	518.82 ± 0.59^{a}	452.90 ± 2.35^{cd}	329.44 ± 7.87^{e}	523.90 ± 2.17^{a}	467.80 ± 20.81^{cd}	<0.0001	0.0001	<0.0001

*Expressed based g kg $^{-1}$ dry grain. The values represent means of ± SD of three repetition. The mean ± standard deviation followed by a different letter entered in the columns for each variety are significantly different (P <0.05)

Table 5. Macro and micro minerals of bitter, debittered and fermented lupine content

	Minerals	INIAP-450			INIAP-451			Criollo			Grain condition	Variety	Interaction
		Bitter	Debittered	Fermented	Bitter	Debittered	Fermented	Bitter	Debittered	Fermented			
*Macro elements	Calcium	1.77 ± 0.15^{de}	4.00 ± 0.40^{a}	2.40 ± 0.10^{c}	2.20 ± 0.20^{cd}	3.53 ± 0.06^{ab}	3.50 ± 0.01^{b}	1.67 ± 0.06^{e}	3.07 ± 0.15^{b}	1.17 ± 0.06^{f}	<0.0001	<0.0001	0.0009
	Phosphorus	7.47 ± 0.06^{b}	4.70 ± 0.50^{c}	3.27 ± 0.35^{def}	8.40 ± 0.30^{a}	4.07 ± 0.06^{cd}	4.00 ± 0.60^{cde}	6.67 ± 0.06^{b}	3.13 ± 0.06^{ef}	3.00 ± 0.10^{f}	<0.0001	<0.0001	0.0012
	Magnesium	2.17± 0.06^{b}	0.65 ± 0.05^{cd}	0.56 ± 0.11^{cde}	2.37 ± 0.07^{a}	0.67 ± 0.08^{c}	0.46 ± 0.06^{de}	2.23 ± 0.06^{ab}	0.46 ± 0.06^{de}	0.37 ± 0.06^{e}	0.0008	<0.0001	0.0051
	Potassium	9.80 ± 0.10^{a}	7.43 ± 0.95^{b}	6.80 ± 0.50^{b}	11.23 ± 0.45^{a}	7.40 ± 0.30^{b}	6.53 ± 0.55^{b}	9.87 ± 0.25^{a}	7.80 ± 0.50^{b}	6.90 ± 0.90^{b}	0.385	<0.0001	0.0478
	Sodium	0.14 ± 0.02^{b}	0.12 ± 0.03^{b}	0.11 ± 0.02^{b}	0.15 ± 0.03^{b}	0.14 ± 0.04^{b}	0.13 ± 0.04^{b}	0.17 ± 0.03^{a}	0.16 ± 0.05^{b}	0.28 ± 0.07^{a}	0.0011	0.1743	0.0272
**Micro elements	Copper	6.87 ± 0.31^{a}	1.83 ± 0.22^{fg}	2.97 ± 0.17^{e}	5.85 ± 0.35^{b}	1.28 ± 0.13^{g}	2.18 ± 0.20^{efg}	8.29 ± 0.43^{c}	2.62 ± 0.64^{ef}	4.20 ± 0.12^{d}	<0.0001	<0.0001	0.1136
	Iron	74.33 ± 1.53^{a}	57.70 ± 1.57^{c}	52.67 ± 1.53^{cde}	64.67 ± 2.52^{b}	56.17 ± 0.76^{cd}	46.00 ± 2.00^{f}	56.33 ± 1.53^{cd}	51.33 ± 1.53^{de}	48.00 ± 2.65^{ef}	<0.0001	<0.0001	<0.0001
	Manganese	31.87 ± 0.71^{b}	21.33 ± 2.08^{c}	9.70 ± 0.52^{f}	30.17 ± 0.77^{b}	23.67 ± 0.58^{c}	12.29 ± 0.28^{e}	36.72 ± 0.48^{a}	21.83 ± 0.76^{c}	15.67 ± 0.58^{d}	<0.0001	<0.0001	<0.0001
	Zinc	46.40 ± 1.02^{ef}	69.96 ± 0.14^{c}	29.75 ± 1.52^{h}	49.08 ± 1.02^{de}	97.15 ± 0.79^{a}	39.33 ± 1.53^{g}	51.33 ± 0.58^{d}	83.17 ± 0.76^{b}	44.33 ± 0.58^{f}	<0.0001	<0.0001	<0.0001

*Expressed based g kg $^{-1}$ dry grain. **Expressed based mg kg $^{-1}$ dry grain. The values represent means of ± SD of three repetitions. The mean ± standard deviation followed by a different letter entered in the columns for each variety are significantly different (P <0.05)