**Kinetics of solid-state fermentation of lupin with *Rhizopus oligosporus* based on
nitrogen compounds balance**

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Abstract

Solid-state fermentation might be a potentially effective method to improve the nutritional value of legumes. The objective of this study was to explore this technology with the *Lupinus mutabilis* species using *Rhizopus oligosporus* mold for solid-state fermentation. Three *Lupinus mutabilis* varieties (INIAP-450, INIAP-451 and Criollo) were evaluated including the impact of the grain status (whole or crushed) and the tegument (presence or absence). Kinetic parameters of the fermentation process were studied to define the specific speed of nitrogen formation and the resulting protein digestibility and nutritional quality. After incubation at 28 °C for 96 h, the titratable acidity increased to 0.60%, the pH decreased to 4.03 and the total nitrogen of the crushed grain without tegument reached a value of 108.27 g·kg⁻¹ in Criollo variety. The whole INIAP-450 grain without tegument had the highest soluble nitrogen content (26.18 g·kg⁻¹), whose released occurred at a specific rate of 0.001 h⁻¹. The apparent digestibility of the protein in this variety increased from 82%

in debittered grain to 96%, the concentration of the first limiting amino acids (methionine + cysteine) increased from 20.84 to 27.87 mg·g⁻¹ protein and the protein digestibility corrected amino acid score from 68.32 to 89.35%. Overall, results confirmed the benefits of fermentation for improving the content and quality of protein in three lupin varieties.

Keywords: protein digestibility, solid-state fermentation, lupin, nutritional quality.

Abbreviations

α	specific rate of total or soluble nitrogen related to protein formation
Ct	critical time of total or soluble nitrogen related to protein formation
Cc	critical concentration of total or soluble nitrogen
CWT	crushed grain with tegument
CIT	crushed grain without tegument
DP	digestible protein
PDA	potato dextrose agar
PDCASS	protein digestibility corrected amino acid score
(dN/dt)	rate of total or soluble nitrogen content
$(dN/dt)_m$	maximum rate of total or soluble nitrogen
t	time of total or soluble nitrogen release
UTM	Universal Transverse Mercator (coordinate system)
WWT	whole grain with tegument
WIT	whole grain without tegument

1. Introduction

The *Lupinus* genus includes approximately 300 species of annual and perennial herbs. For centuries, a number of these species had been part of the human diet and animal feed in the

Mediterranean and Andes regions. The nutrient composition of lupin seeds is exceptional, but varies depending on the species (Sedláková et al., 2016). The *L. mutabilis* species is recognized as one of the richest in nutrients, due to its high protein content (Sujak, Kotlarz, & Strobel, 2006) and amino acid composition, which is influenced by the processing conditions and interactions with other food components (Melini et al., 2017). However, debittering must be applied to reduce the anti-nutrient content prior to their consumption (Erbas, 2010; Villacrés et al., 2020a). In *L. mutabilis*, the debittering process leads to an increase in the protein, crude fiber and lipid contents by 553.3, 146.6 and 220.0 g·kg⁻¹, respectively, the ash and starch contents decrease by 19.5 and 15.2 g·kg⁻¹, respectively (Villacrés et al., 2020b). However, there is increasing interest for further nutritional improvement of these grains, particularly those focused on the quantity (Wang et al., 2016) and quality of protein. In fact, lupin has been used for developing based yogurt alternatives (Hickisch et al., 2016) or lupin enriched breads (Villarino et al., 2015) with the objective of improving the protein quality of those foods, preferentially the biological value of those proteins given by the protein digestibility corrected amino acid score.

Solid-state fermentation is a bioprocess applied to some cereals and legumes to improve their nutritional quality and to significantly decrease the contents of anti-nutritive and flatulence-producing compounds without damaging their sensory properties (Mukherjee, Chakraborty & Dutta, 2016). This process is used in the production of fermented foods such as tempeh to obtain enzymes and secondary metabolites and for the bioconversion of organic waste into useful products (Ruiz-Terán & David Owens, 1996). Solid-state fermentation consists of the growth of microorganisms on textured and porous solid materials in the absence of free water, since the microorganisms take advantage of the

water that is absorbed in the solid matrix (Hong, Lee, & Kim, 2004). Various types of microorganisms grow in solid substrates; however, the low water content in this type of fermentation favors the development of fungi. In particular, *Rhizopus oligosporus* has been used for legume fermentation because it produces proteases and amylases (Egounlety & Aworh 2003). The enzymatic activity of *R. oligosporus* softens the soybean, changing the texture and taste of the grain and increasing its nutritional value due to its actions on proteins, lipids and oligosaccharides (Bavia et al., 2012). Despite those nutritional benefits reported with soybean, limited studies have been carried out with other grains. The substitution of soybeans with *L. angustifolius* seed kernels at levels of 50, 75 and 100% has been investigated in tempeh production to decrease the anti-nutrient content of soybean, resulting in higher proportions of total and soluble nitrogen contents (Fudiyansyah et al., 1995). Nevertheless, effects during fermentation are dependent on substrate availability that varies with the grain condition (whole, crushed, and with or without tegument). Cell wall disruption is essential to facilitate the fast growth of *R. oligosporus*, since in soybeans it has been observed that the mycelium did not penetrate more than two cell layers (Nopharatana, Mitchell, & Howes, 2003). The objective of this research was to study the solid fermentation of lupin with the NRRL2710 *Rhizopus oligosporus* strain, monitoring changes in pH, titratable acidity, total and soluble nitrogen contents, and nutritional quality of bitter, debitter and fermented lupin proteins based on PDCAAS (protein digestibility corrected amino acid score) in three varieties of *L. mutabilis*.

2. Materials and methods

2.1 Raw material and microorganism

Three varieties of lupin were used, INIAP-450, INIAP-451 and Criollo, which were grown in the Santa Catalina Experimental Station with the following geographical location:

altitude 3050 m above sea level, UTM latitude 9959382 m S, longitude 17M0772618 m. Solid-state fermentation was performed with the NRRL2710 *R. oligosporus* strain from the Northern regional research laboratory USDA (USA) collection belonging to the Ambato technical university microbiology laboratory.

2.2 Chemicals reagents

Potato dextrose agar (PDA), Tween 80, porcine pancreatic trypsin (Type IX) with 14,190 BAE units·mg⁻¹ protein, bovine pancreatic α-chymotrypsin (Type II), 60 units·mg⁻¹ powder, porcine intestinal peptidase (Grade III), 40 units·g⁻¹ powder. Sodium hydroxide 99% (NaOH), 95-97% sulfuric acid (H₂SO₄), 37% hydrochloric acid fuming (HCl), boric acid (H₃BO₃) ACS grade, absolute ethanol (C₂H₅OH) for analysis EMSURE® ACS, methanol (CH₃OH) ACS grade, methyl red, bromocresol green, catalyst [potassium sulfate (K₂SO₄), cupric sulfate (CuSO₄), selenium dioxide (SeO₂)], potassium dihydrogen phosphate (NaH₂PO₄), Orto-ftaldehido (OPA), 2-mercaptoetanol (2 ME).

2.3 Grain debittering

The lupin grains were debittered using an aqueous heat processes as previously report (Villacrés et al., 2020a). Briefly, grains were immersed in hot water (80 °C) at a 1:3 ratio (grain:water) for 10 h, then cooked at 91°C for 1 h and finally grains were thoroughly washed at 35 °C for 28 h with continuous changes of water.

2.4 Inoculum preparation of *Rhizopus oligosporus*

A liquid medium of 3.9% (w/v) potato dextrose agar (PDA) was prepared; the solution was sterilized for 15 min and poured into Petri dishes; the agar was cooled and solidified. A concentrated solution of the *Rhizopus oligosporus* inoculum (100 mg·mL⁻¹) was used for preparing diluted solutions with densities varying from 3.5 x 10³ to 3.5 x 10⁶ colony

forming units per gram (CFU·g⁻¹), 100 µL of each inoculation density was spread on the agar and incubated for 4 days at 26°C, when abundant spore formation was observed, the propagative structures were collected and suspended in 9 mL sterile water. This suspension (1 mL) was inoculated on 100 g polished rice (variety INIAP-18 provided by the National Institute of Agricultural Research from Ecuador) that had been previously hydrated in sterile distilled water to reach 24% humidity and then sterilized for 10 min at 121 °C. The inoculated rice was incubated for 5 days at 28 °C. Afterwards, the formation of gray spores was observed; these spores were collected, filtered through # 70 mesh (212 µm) and lyophilized at -40°C and -0.7 bar (Labconco, Kansas, USA). This inoculum was stored in hermetically sealed sterile bottles at 4 °C.

The number of propagative structures was counted from one gram of fermented rice mixed with 10 mL of sterile water that had been stirred for one minute to promote spore shedding. This suspension (100 µL) was removed and the volume was brought to 1 mL with distilled water containing 0.1% tween 80. The count was 3.5 x10⁶ spores·mL⁻¹.

2.5 Lupin fermentation

The fermentation of whole lupin grain with tegument (WWT), whole grain without tegument (WIT), crushed grain with tegument (CWT) and crushed grain without tegument (CIT) was evaluated. Preliminary tests were performed preparing dilutions of sporulated fungi ranging from 10⁻¹ to 10⁻⁵ CFU·mL⁻¹ with different moisture contents (50-63%). Based on the results of these tests, 500 µL of 10⁻² dilution was selected for inoculating 50 g of lupin with 50% humidity. Solid-state fermentation was conducted, for which the whole or crushed debittered lupin grain with a particle size of 0.6 mm was pre-dried at 50 °C for 2 h

in a forced air oven (HS122A, Navarra, Spain) to reduce the humidity up to 50%. In the case of dehulled lupin, the tegument was manually removed.

Samples (50 g) were introduced in perforated plastic bags to facilitate gas exchange, moisture retention and protection from external contamination. The packages were sealed and sterilized for 15 min at 121 °C in the case of grain with tegument and at 121 °C for 10 min in the case of grain without tegument. The samples were cooled at 30 °C and then 500 µL of 10^{-2} CFU spore dilution was added, homogenized and incubated at 28 °C. The evolution of fermentation was monitored and samples collected every 24 h for 4 days.

2.6 Chemical analyses

During fermentation, the pH, acidity, total and soluble nitrogen contents, and *in vitro* protein digestibility were analyzed. The pH was measured using the method of AOAC 943.02 (AOAC, 1990). Titratable acidity was measured using the method of AOAC 947.05, with addition 0.01 M NaOH to obtain 8.2 pH (AOAC, 1990). The result was reported as lactic acid percentage, which predominates in fermentation reactions with *R. oligosporus* (Bartkienė et al., 2014). The total nitrogen content was measured using the method of AOAC 955.04 (AOAC, 1995). Soluble nitrogen content was measured using the method of Periago et al. (1996).

2.7 In vitro protein digestibility

Protein digestibility was measured using the method of Lqari et al. (2002). Samples containing 62.5 mg of protein were suspended in water (10 mL) and the pH was brought to 8 with 0.1 M NaOH. An enzymatic solution containing 1.6 mg trypsin ($18 \text{ U} \cdot \text{mg}^{-1}$), 3.1 mg α -chymotrypsin ($40 \text{ U} \cdot \text{mg}^{-1}$) and 1.3 mg protease ($15 \text{ U} \cdot \text{mg}^{-1}$)·mL⁻¹ was added to the protein suspension 1:10 (v/v) ratio. The pH of the mixture was measured exactly after 10

min and the *in vitro* digestibility was calculated as a percentage of digestible protein (DP)

using the following equation:

$$DP = 210.464 - 18.103 \times pH \quad \text{Eq. 1}$$

2.8 Determination of the fermentation kinetics

The total nitrogen content versus fermentation time was adjusted to third and fourth degree polynomial equations. The first, second and third derivatives of the polynomial equations were obtained. The critical time (Ct) was determined from the second or third derivative, which represents the point at which the velocity reaches its maximum value. The Ct was replaced in the first derivative (dN/dt) of the equations and the maximum rate ($(dN/dt)_m$) was obtained. Ct was replaced in the equations of third or fourth degree and the critical concentration (Cc) was obtained. Using these values, the specific rate of nitrogen content related to protein formation (α) was determined by dividing the maximum rate ($(dN/dt)_m$) by Cc (Mitchell, Von Meien, Krieger, & Dalsenter, 2004).

2.9 Estimation of nutritional quality of bitter, debittered and fermented lupin proteins

Amino acids analysis was performed using the method of AOAC 982.30 (AOAC, 1990) with HPLC system. Protein digestibility corrected amino acid scores (PDCAAS) were calculated using the method of World Health Organization, and United Nations University (2007), the limiting amino acid of bitter, debittered and fermented lupin (Villacrés et al., 2020b) and the following formulas:

$$\text{Limiting amino acid score} = \frac{\text{Limiting essential amino acid content of test protein}}{\text{Amino acid requirement pattern}} \quad \text{Eq. (2)}$$

$$\text{PDCAAS} = (\% \text{ protein digestibility} \times \text{amino acid score}) \quad \text{Eq. (3)}$$

2.10 Statistical analysis

The statistical analysis was performed using the Infostat program (Córdoba, Argentina). The normal distribution of the data was verified using the Kolmogorov-Smirnov goodness of fit test. A multifactor analysis of variance (ANOVA) and the Tukey test with a level of 95% ($P<0.05$) significance were applied to establish significant differences between samples. All analyses were performed in triplicate. The data are presented as means $\pm$ standard deviations.

3. Results and discussion

3.1 Analysis of titratable acidity

The titratable acidity of the grain increased with the fermentation time (Fig. 1), which has been associated to the generation of organic acids (Mukherjee et al., 2016). This variation was significantly ($P<0.05$) dependent on the condition (presence or absence of tegument) and variety of grains. The greatest difference was observed in the fermentation of grains with tegument that showed higher acidity (Fig. 1). In the comparison between varieties, Criollo CWT presented the highest acidity (0.60%) at the end of fermentation. During this process, the titratable acidity increased with a latency period of 24 h in whole grains (WWT and WIT), while in crushed grains (CWT and CIT), this increase occurred from the first day of fermentation, which was attributed to a greater contact area for the growth of microorganisms and its metabolic activity (Priatni, Devi, Kardono, & Jayasena, 2013). In whole grain without tegument (WIT), the surface area and volume were reduced, which result in a lower penetration and microbial activity. Similar variations have been reported for bean fermentation (*Phaseolus vulgaris*) (Chelule, Mokoena, & Gqaleni, 2010).

3.2 pH analysis

In tandem with the increase in titratable acidity, a decrease in the pH of lupin seeds was observed with the fermentation time (Fig. 2). This change was influenced by the variety and condition of the grain ($P<0.05$). The pH varied by 14.24% in WWT of INIAP-451 and 33.60% in CIT of INIAP-450 during fermentation. The decrease in pH has been related to protein metabolism (Ruiz-Terán & David Owens, 1996) and the production of organic acids, particularly L-lactic acid, which production depends on the type of fermentation, the strain of microorganism used and the lupin genotype (Bartkienė et al., 2014). A variation in pH (5.0 to 4.55) was also reported for soybeans fermentation with *R. oryzae* (Hong et al., 2004). The pH change observed during fermentation confirmed the suitability of *L. mutabilis* species for growing *R. oligosporus*. Most molds are acidophiles and grow better at pH between 4.5-3.0 (Priatni et al., 2013), at higher pH values the plasma membrane of the organism becomes very unstable (Bartkienė et al., 2014).

The CWT of Criollo variety presented a pH of 4.03 at 96 h of fermentation (Fig. 2), while in the INIAP-450 and INIAP-451 seeds, this parameter was slightly decreased (4.86 and 4.73, respectively). A decrease in pH is a desirable result in fermentation, since the opposite effect reveals an overproduction of ammonia resulting from the decomposition of nitrogen-containing organic compounds (Bartkienė et al., 2014).

3.3 Nitrogen analysis

3.3.1 Total nitrogen content

Variations in the total nitrogen content as a function of the fermentation time are presented in Fig. 3. Significant differences ($P<0.05$) were observed in different varieties and conditions of the grains (WWT, WIT, CWT and CIT). The total nitrogen content increased along fermentation in the three lupin varieties. This result was attributed to the metabolic action during fermentation and subsequent nitrogen incorporation into the mold protein

biomass (Mukherjee et al., 2016). The total nitrogen content reached $108.27 \text{ g}\cdot\text{kg}^{-1}$ at the end of the solid fermentation of CIT of INIAP-450 and Criollo. Thus, the NRRL2710 strain of *R. oligosporus* develops better on substrates without tegument. In fact, it has been previously reported that molds do not have the ability to penetrate the cell walls (Nopharatana et al., 2003), therefore, those must be removed or the grain crushed to increase the nutrient bioavailability. The increase in the total nitrogen content in *L. mutabilis* was comparable to that of cooked and fermented soybeans (Priatni et al., 2013). Likewise, other authors have reported increases in the crude protein and fat contents in soybeans and soybean meals after fermentation with *Aspergillus oryzae* GB-107 (Hong et al., 2004). In fact, an increase in the protein content (13.1 to 28.3%) was described during millet and soybean (36.81 to 51.99%) fermentation (Wang et al., 2008). This increase might result from the proliferation of microorganisms and synthesis of proteins with catalytic activity (enzymes) or from a rearrangement of the protein composition following the degradation of other constituents (Hong et al., 2004).

3.3.2 Kinetics of total nitrogen balance

The content of total and soluble nitrogen (Fig. 3 and Fig. 4) was assimilated to the growth curve of the microorganisms in the logarithmic phase and responded to polynomial regression equations, from which the expressions that describe the kinetics of the solid fermentation of lupin were obtained (Tables 1 and 2). The correlation between the experimental values and those predicted by the model ($R = 0.987$) confirmed the validity of the model used to describe the kinetics of total protein formation. Along fermentation, the total nitrogen concentration significantly ($P < 0.05$) increased at a variable speed as a function of time, variety and condition of the grain, reaching a maximum value of $109.9 \text{ g}\cdot\text{kg}^{-1}$ in Criollo variety (WWT) at 40.8 h (Ct), while INIAP 450 (WWT) presented the

lowest concentration ($82.4 \text{ g}\cdot\text{kg}^{-1}$) at 100 h. Fig. 3 also shows that the exponential phase extended up to 48 hours, except for INIAP-450 (WWT) and INIAP-451 (CWT), which showed certain delay in the increase of total nitrogen synthesis related to protein synthesis. It is already reported the increase of total nitrogen content during solid-state fermentation due to the ability of *R. oligosporus* to metabolize fibers and oligosaccharides releasing, among other compounds, CO_2 and nitrogen, the latter mainly in the form of proteins that are accumulated during fermentation (Abu-Salem & Abou-Arab, 2011). Lupin contains lower amount of starch, because of that mainly sugars as stachyose and sucrose decreased during grain fermentation (Kaczmarska et al., 2017).

The fermentation kinetic parameters indicated that the grain condition significantly affected the magnitude of changes; the absence of tegument and the smaller particle size contributed to increase the C_c , $(dN/dt)_m$ and α values. This last parameter reached its maximum value (0.006 h^{-1}) by 25 h in INIAP-450 (CIT), which was 2.0 times greater than in INIAP-450 (CWT). This result is consistent with the study by Nopharatana et al. (2003), who showed that the disruption of the cell wall is essential to ensure the rapid growth of *R. oligosporus*.

The critical time (C_t) is that in which the protein concentration reaches its maximum value; its determination is important because in this point, the stationary phase and mold sporulation begin. Molds require less time to induce metabolic transformations when the grain structures are sufficiently exposed, as is the case with the Criollo (CIT), which reached a critical concentration of $98.8 \text{ g}\cdot\text{kg}^{-1}$ by 21.5 h, a result that contrasts with the findings obtained in INIAP-450 (WWT) that needed more time (100 h) to reach lower critical concentration of total nitrogen ($82.4 \text{ g}\cdot\text{kg}^{-1}$). This observation is consistent with previous studies examining fermented rapeseed meal and dehulled lupin grain (*Lupinus*

angustifolius) (Priatni et al., 2013). Authors found that the growth of *R. oligosporus* mycelia was influenced by total surface area of grain particles, confirming that the particle size of grain, besides the crude fiber content in fermenting media, affected the growth of *R. oligosporus* mycelia.

3.3.3 Soluble nitrogen content

The soluble nitrogen content varied significantly ($P<0.05$), depending on the variety and grain condition (Fig. 4). Before starting the fermentation process (time 0), soluble protein fraction ranged from 2.82 to 4.84 g·kg⁻¹ among the different samples, and was attributed to the partial protein solubilization during the cooking of the grain. These contents increased with the fermentation process, as a consequence of the increase in amino acid contents reported by Villacrés et al. (2020b). Likewise, findings are consistent with previous studies on fermented soybean pulp, whose total free amino acid content increased by 2.3- to 2.5-fold after fermentation (Vong, Hua, & Liu, 2018). During this process, a ‘turnover’ of proteins and amino acids likely occurs, as the levels of most amino acids that are soluble in a 70% ethanol solution increased significantly. The quantity of free amino acids increases in tempeh products as fermentation progresses (Vong, Hua, & Liu, 2018). The whole or crushed INIAP-450 grain without tegument had the highest soluble nitrogen content (26.18 and 23.66 g·kg⁻¹ dw) at 96 h of fermentation, possibly due to the lower tannin content in this variety (1.44 g·kg⁻¹) compared to INIAP-451 (1.79 g·kg⁻¹) and Criollo (1.85 g·kg⁻¹) (Villacrés et al., 2020c). Tannins have the ability to form reversible complexes with proteins in a pH range of 3.5-7.0, but dissociate at a pH less than 3.5 (Mukherjee et al., 2016). The INIAP-451 grain (with and without tegument, whole or ground) displayed the lowest soluble nitrogen content at the end of the fermentation.

3.3.4 Kinetics for soluble nitrogen content

The kinetics for soluble nitrogen release during fermentation was adjusted to different polynomial equations (Table 2). The INIAP-450 whole grain with tegument (WWT) reached a critical concentration (C_c) of $26.5 \text{ g}\cdot\text{kg}^{-1}$ in 50 h at a specific speed that was 1.5 times higher than that reached by the whole grain without tegument (WIT). INIAP-450 showed a speed that was 9.25 times higher than INIAP-451, which presented the lowest values for the kinetic parameters evaluated (Table 2). In general, the soluble nitrogen formation process occurred 10 times faster than the formation process of total nitrogen. These changes may enhance the digestibility, nutritional status and possible flavor of lupin (Feng et al., 2007), supporting its potential to be valued as a consumer product or functional food ingredient.

3.3.5 *In vitro* protein digestibility

This parameter varied significantly ($P<0.05$) with the variety and grain condition (Fig. 5). Debittered grains (time 0) showed low protein digestibility (75.25-82.31%), similar to the value reported for the following debittered lupin species: *Lupinus albus* (80.72%), *Lupinus barkeri* (78.58%) and *Lupinus montanus* (81.43%) (Guemes-Vera et al., 2012). As the fermentation process progressed, the *in vitro* digestibility increased, reaching a maximum of 96.07% in INIAP-450 (CIT) (Fig. 5). The results of our study are consistent with observations by Nout & Kiers (2005), who found that total *in vitro* digestibility of soybean and cowpea seeds was significantly improved due to cooking and subsequent fungal fermentation. In this regard, the fermentation of soybean with *A. oryzae* was reported to significantly reduce the trypsin inhibitor content and the size of peptide chains, increasing the protein digestibility (Stodolak, & Starzyńska-Janiszewska, 2008; Feng et al., 2007).

Whole grains of the three varieties with tegument (WWT) had lower digestibility values, possibly due to the lower metabolic activity of *R. oligosporus* in the presence of the tegument fiber, a component that reduces the ability of the mold to penetrate the seed and its activity (Nopharatana et al., 2003). Even in the presence of tegument, crushing of the grain favored an increase in digestibility (CWW), which was particularly significant in INIAP-451 crushed grain with tegument, whose digestibility increased by 16.11% compared to the whole grain. Lower value (88.4%) was reported for *Lupinus albus* fermented with *Rhizopus* (Fudiyansyah et al., 1995). Present results indicated that fermented *L. mutabilis* shows potential as a functional ingredient due to its apparent digestibility values, which can be used in the development of new products with improved digestibility.

3.3.6 Effect of debittering and fermentation on the nutritional quality of lupin proteins

Although lupin seeds contain a moderately high amount of proteins, their use as food and feed is limited owing the low digestibility of its proteins and the presence of several antinutritional components (Mukherjee et al., 2016). Because of that different processing methods, like cooking and fermentation, have been proposed to improve the nutritional quality of lupin (Mukherjee et al., 2016). To assess the biological value of the lupin proteins, which depends on the composition of amino acids and the proportions between them, the PDCAAS was evaluated. The first limiting amino acids in *L. mutabilis* are the methionine and cysteine, followed by tryptophan (Table 3). During debittering, besides the removal of quinolizidine alkaloids till safe levels (Villacrés et al., 2020a), there is a concomitant reduction of other nutrients like essential amino acids, principally (methionine, cysteine and tryptophan) (Villacrés et al., 2020b), which are the limiting amino acids in *L.*

mutabilis (Table 3). The fermentation process induced a significant reduction of the tryptophan content ($P<0.05$) with respect to debittered grain. On the other hand, (methionine + cysteine) content increased. After fermentation, (methionine + cysteine) content reached 27.87 mg·g⁻¹ protein (INIAP-450), 27.08 mg·g⁻¹ (INIAP-451) and 26.49 mg·g⁻¹ (Criollo) in the different lupin varieties. It must be stressed that those values exceeded the requirement of preschool child (25.00 mg·g⁻¹ protein).

In addition, the biological value of lupin protein was evaluated based on the PDCAAS (protein digestibility corrected amino acid score). The (methionine + cysteine) score varied with the processes, being 0.92, 0.85 and 1.08 the average for bitter, debittered and fermented lupins, respectively. These values were lower than those for the tryptophan score, which ranged from 0.93 to 2.11 for fermented (INIAP-450) and bitter (INIAP-451), respectively. Consistent with that, the lowest value for the limiting amino acids (methionine + cysteine) corresponded to the debittered grain in the three varieties, resulting in PDCAAS of 68.32% (INIAP-450), 66.74% (INIAP-451) and 70.15% (Criollo). The values for lupin digestibility were similar to those reported for asparagus (65.94%) and potato protein (70.55%) (Suárez et al., 2006). Regarding processed foods, higher value has been reported for a soy drink containing cereals (86%) (Suárez et al., 2006). Therefore, altogether the digestibility increase after debittering and fermentation might be related to the protein hydrolysis that reduces its compact structure, but also the decrease in the antinutrient content might contribute to enhance digestibility (Khalid, Elhardallou & Gubouri, 2016).

4. Conclusions

Fermentation is one approach to improve the nutritional content of *Lupinus mutabilis* and support its valorization. The fermentation process decreased the pH and increased the

acidity, the formation of total and soluble nitrogen, and the *in vitro* protein digestibility in the three lupin varieties evaluated. The analysis of the kinetics of the fermentation process allowed to determine the specific rate of nitrogen formation and to establish the optimal time of the fermentation process, with which a bioreactor and its operating strategy can be designed. The results obtained here confirm that fermentation is an effective method to improve the nutritional value of lupin, as evidenced by the higher total and soluble nitrogen contents, the higher protein digestibility and PDCAAS, which was particularly notable in the INIAP-450 variety (CIT). Fermented lupin has the potential to be used as a valuable food enricher for infants and people with special nutrient requirements and as raw material for the emerging health food industry. However, amino acid composition represents the

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Table 1. Kinetic equations for total nitrogen balance during the solid fermentation of debittered lupin grain obtained from fitting experimental data to fourth degree polynomial equations. Parameters extracted for defining the kinetics are explained in Materials and Methods section.

Variety	Grain condition	Total nitrogen formation curve	R2	Ct (h)	Cc (g kg ⁻¹)	$(dN/dt)_m$ g kg ⁻¹ h ⁻¹	α (h ⁻¹)
INIAP 450	WWT	$1E-07t^4 - 4E-05t^3 + 0.003t^2 - 0.051t + 87.5$	1.00	100.0	82.4	0.25	0.003
	WIT	$-2E-07t^4 + 6E-05t^3 - 0.0079t^2 + 0.5911t + 86.78$	1.00	75.0	105.6	0.08	0.000
	CWT	$5E-07t^4 - 9E-05t^3 + 0.0061t^2 - 0.0463t + 87.5$	1.00	45.0	95.3	0.35	0.003
	CIT	$1E-06t^4 - 0.000t^3 + 0.009t^2 + 0.281t + 86.78$	1.00	25.0	99.8	0.60	0.006
INIAP 451	WWT	$5E-07t^4 - 8E-05t^3 + 0.002t^2 + 0.192t + 88.38$	1.00	40.0	95.4	0.09	0.001
	WIT	$4E-07t^4 - 5E-05t^3 - 0.0014t^2 + 0.494t + 87.587$	1.00	31.2	100.5	0.31	0.003
	CWT	$4E-07t^4 - 8E-05t^3 + 0.0035t^2 + 0.0632t + 88.387$	1.00	49.0	92.9	0.02	0.000
	CIT	$6E-07t^4 - 1E-04t^3 + 0.0002t^2 + 0.4956t + 87.587$	1.00	41.7	103.2	0.16	0.000
CRIOLLO	WWT	$9E-07t^4 - 0.000t^3 + 0.009t^2 + 0.066t + 89.69$	1.00	40.8	109.9	1.04	0.009
	WIT	$8E-07t^4 - 0.000t^3 + 0.006t^2 + 0.264t + 88.97$	1.00	11.2	92.7	0.28	0.003
	CWT	$2E-07t^4 - 4E-05t^3 + 0.000t^2 + 0.305t + 89.69$	1.00	50.0	101.2	0.10	0.001
	CIT	$9E-07t^4 - 0.000t^3 + 0.005t^2 + 0.339t + 88.97$	1.00	21.5	98.8	0.48	0.005

Abbreviations: critical time (Ct) at which the velocity reaches its maximum value; the maximum rate (dN / dt)_m ; the critical concentration (Cc); the specific rate of nitrogen related to protein formation (α).

515 Table 2. Kinetic equations for soluble nitrogen balance during the solid fermentation of debittered lupin grain obtained from fitting
516 experimental data to third or fourth degree polynomial equations. Parameters extracted for defining the kinetics are explained in
517 Materials and Methods section.

Variety	Grain condition	Soluble nitrogen formation curve	R2	Ct (h)	Cc (g Kg ⁻¹)	(dN/dt) _m (g kg ⁻¹ h ⁻¹)	518
							519
INIAP 450	WWT	$1E-06t^4 - 0.0002t^3 + 0.0112t^2 + 0.2887t + 2.828$	1.00	50.0	26.5	0.15	520
	WIT	$1E-06t^4 - 0.000t^3 + 0.011t^2 + 0.289t + 2.82$	1.00	42.8	38.7	1.54	521
	CWT	$4E-07t^4 - 7E-05t^3 + 0.003t^2 + 0.148t + 3.304$	1.00	42.8	10.9	0.14	522
	CIT	$1E-06t^4 - 0.000t^3 + 0.009t^2 + 0.281t + 86.78$	1.00	25.0	99.8	0.60	523
INIAP 451	WWT	$-9E-08t^4 + 9E-06t^3 + 0.001t^2 - 0.0328t + 4.004$	1.00	25.0	26.5	0.15	524
	WIT	$-8E-06t^3 + 0.0013t^2 + 0.03t + 3.443$	1.00	54.2	7.6	0.10	525
	CWT	$7E-06t^3 - 0.0014t^2 + 0.1488t + 88.337$	1.00	47.6	93.9	0.10	526
	CIT	$-2E-07t^4 + 3E-05t^3 - 0.001t^2 + 0.103t + 3.444$	1.00	37.5	7.1	0.11	527
CRIOLLO	WWT	$-3E-07t^4 + 6E-05t^3 - 0.003t^2 + 0.060t + 4.844$	1.00	50.0	6.0	0.06	528
	WIT	$-.5E-06t^3 + 0.0009t^2 + 0.065t + 4.0348$	1.00	60.0	10.09	0.12	529
	CWT	$-2E-07t^4 + 3E-05t^3 + 0.000t^2 - 0.039t + 4.844$	1.00	37.5	6.0	0.04	530
	CIT	$2E-06t^3 - 0.0005t^2 + 0.1412t + 3.974$	1.00	83.3	13.4	0.09	531

535 Abbreviations: critical time (Ct) at which the velocity reaches its maximum value; the maximum rate (dN / dt)_m ; the critical
536 concentration (Cc); the specific rate of nitrogen related to protein formation (α).

537

538 Table 3. Estimation of protein quality of bitter, debittered and fermented lupin based on Protein digestibility corrected amino acid
539 scores (PDCAAS)*.
540

Variety	Grain condition	Grain content mg/g protein		amino acids score		Protein digestibility (%)	PDCAAS (%)
		methionine + cysteine	tryptophan	methionine + cysteine	tryptophan		
INIAP 450	bitter	22.95 ± 0.39	14.12 ± 0.39	0.92 ± 0.02	2.02 ± 0.06	75.00 ± 0.28	69.00 ± 0.82
	debittered	20.84 ± 0.47	12.5 ± 0.44	0.83 ± 0.02	1.78 ± 0.15	82.31 ± 0.36	68.32 ± 0.73
	fermented	27.87 ± 0.84	6.55 ± 0.23	1.11 ± 0.03	0.93 ± 0.62	96.08 ± 0.36	89.35 ± 0.45
INIAP 451	bitter	22.87 ± 0.84	14.8 ± 0.65	0.91 ± 0.02	2.11 ± 0.11	74.49 ± 0.24	67.78 ± 0.48
	debittered	21.55 ± 0.78	12.10 ± 0.31	0.86 ± 0.03	1.73 ± 0.17	77.61 ± 0.47	66.74 ± 0.68
	fermented	27.08 ± 0.14	6.59 ± 0.26	1.08 ± 0.01	0.94 ± 0.62	93.00 ± 0.36	87.00 ± 0.78
CRIOLLO	bitter	23.15 ± 0.40	11.25 ± 0.41	0.93 ± 0.02	1.61 ± 0.22	74.73 ± 0.30	69.50 ± 0.36
	debittered	21.55 ± 0.03	8.12 ± 0.33	0.86 ± 0.01	1.16 ± 0.50	81.58 ± 0.36	70.15 ± 0.42
	fermented	26.49 ± 0.08	6.49 ± 0.26	1.06 ± 0.01	0.93 ± 0.63	94.07 ± 0.37	87.48 ± 0.30
Standard amino acid values for children > 1-y and adults*		25.00	7.00				

*U.S. National Academy of Sciences for the year 2002 (Institute of Medicine, 2002)

Fig. 1. Variations of the titratable acidity (%) during the solid fermentation of debittered lupin grain. WWT: whole grain with tegument, WIT: whole grain without tegument, CWT: crushed grain with tegument, and CIT: crushed grain without tegument.

Fig.2. Variations of pH during the solid fermentation of debittered lupin grain. WWT: whole grain with tegument, WIT: whole grain without tegument, CWT: crushed grain with tegument, and CIT: crushed grain without tegument.

Fig. 3. Variations of the total nitrogen content ($\text{g}\cdot\text{kg}^{-1}\text{dw}$) during the solid fermentation of debittered lupin grain. WWT: whole grain with tegument, WIT: whole grain without tegument, CWT: crushed grain with tegument, and CIT: crushed grain without tegument.

Fig. 4. Variations of the soluble nitrogen content (g·kg⁻¹ dw) during the solid fermentation of debittered lupin grain. WWT: whole grain with tegument, WIT: whole grain without tegument, CWT: crushed grain with tegument, and CIT: crushed grain without tegument.

Fermentation time (h) and grain condition

Fig. 5. Variations of the *in vitro* protein digestibility (% dw) during the solid fermentation of debittered lupin grain. WWT: whole grain with tegument, WIT: whole grain without tegument, CWT: crushed grain with tegument, and CIT: crushed grain without tegument.

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