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Effect of germination time on protein subunits of *Lupinus angustifolius* L. and its influence on functional properties and protein digestibility

Ciro Baruchs MUÑOZ-LLANDES[1,2], Fabiola Araceli GUZMÁN-ORTIZ[2,3]*, Alma Delia ROMÁN-GUTIÉRREZ[2], Heidi María PALMA-RODRÍGUEZ[1], Javier CASTRO-ROSAS[2], Humberto HERNÁNDEZ-SÁNCHEZ[4], Juan Francisco ZAMORA-NATERA[5], Apolonio VARGAS-TORRES[1]

Abstract

The aim of this research was to evaluate the influence of germination in *Lupinus angustifolius* L. seeds on protein profile, functional properties (FP), protein digestibility, phytic acid concentration and trypsin inhibitory activity, the latter due to its influence on protein digestibility. The germination was development at 26 ºC for 2-7 days. The ungerminated sample showed a protein content of 24.06% that was increased at 3 days of germination. Electrophoretic profile showed that the germination time generates a reduction in the intensity of the bands with molecular weight of 40, 50, 97 and 116 kDa, corresponding to the β and γ-conglutin subunits. At day 7 of germination, the water and oil absorption capacities increased by 146 and 95.23% respectively. The emulsion and foam capacities also increased by 94.04 and 36.24%, respectively. The swelling capacity was reduced by 51.44%. The protein digestibility remained unchanged after germination due to the presence compounds as phytic acid and trypsin inhibitors activity. Three days of germination in *Lupinus angustifolius* are necessary to observe changes in the FP. Germinated *Lupinus angustifolius* flours can have multiple applications in the development of food. Germination time will depend specifically on the application, since the FP change with the germination time.

Keywords: *Lupinus angustifolius*; swelling power; water absorption capacity; conglutin proteins; phytic acid; trypsin inhibitor activity.

Practical Application: *Lupinus angustifolius* germinated flours can be used as an ingredient in functional foods.

1 Introduction

Germination is an effective, low-cost, and simple technique that involves relevant metabolic changes. It is one of the best alternatives that allows modifying the content and composition of nutrients, due to the enzymatic activity that is generated during this process (Guzmán-Ortiz et al., 2019; Elkhalifa & Bernhardt, 2010). The conditions of the germination process modify the content of antinutritional compounds, reducing the concentration of tannins, trypsin inhibitors, phytic acid, lectins, saponins, α-galactosaccharides and others (Modgil & Sood, 2017; Muñoz-Llandes et al., 2019). It has also been reported that with germination the concentration of phenolic compounds such as polyphenols, tocopherols, vitamins, γ-aminobutyric acid increases in legumes as soybeans, chickpea, beans, lentils, lupins and others (Dueñas et al., 2009; Guzmán-Ortiz et al., 2017). Lupins is a seed with high nutritional potential due to its protein content that ranges between 30-40%, is higher compared to conventional legumes (Saastamoinen et al., 2013; Martínez-Villaluenga et al., 2006). Lupinus also contain a high content of essential amino acids, which allows it to have a high nutritional value (Sujak et al., 2006). Wong et al. (2013) reported that the main proteins that constitute of lupins are albumins (13%) and globulins (87%). Most globulins are α and β conglutins, and at lower levels conglutins γ and δ (Duranti et al., 2008). The fraction rich in α and β conglutins shows a high emulsifying capacity (Piornos et al., 2015), while conglutin γ is a protein with greater nutritional value, and a potential hypoglycemic effect (Lovati et al., 2012).

Studies in sprouts of lupins (*Lupinus angustifolius*) are limited. Most of the studies of this seed during germination have been focused on bioactive compounds and antioxidant activity.

It has been shown that during germination microstructural changes of carbohydrates and proteins are generated (de la Rosa-Millán et al., 2019; Gutiérrez-Osnaya et al., 2020). Due to the changes that occur during germination, it is also possible to generate changes in functional properties due to the type of protein that lupins has. Conglutinins are tense active molecules, they have the ability to form emulsion and improve the stability of an oil / water system (Damodaran, 2005). They are amphiphilic proteins, therefore they have the property of adsorbing oil-water at the air-water interface, reducing the surface tension and allowing the formation of foams. The functional properties

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[1] *Instituto de Ciencias Agropecuarias, Universidad Autónoma del Estado de Hidalgo, Rancho Universitario, Tulancingo de Bravo, Hidalgo, México*
[2] *Área Académica de Química, Universidad Autónoma del Estado de Hidalgo, Ciudad del Conocimiento, Mineral de la Reforma, Hidalgo, México*
[3] *CONACYT-Universidad Autónoma del Estado de Hidalgo, Ciudad del Conocimiento, Mineral de la Reforma, Hidalgo, México*
[4] *Departamento de Ingeniería Bioquímica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Unidad Zacatenco, Ciudad de México, México*
[5] *Departamento de Botánica y Zoología, Centro Universitario de Ciencias Biológicas y Agropecuarias, Universidad de Guadalajara, Zapopan, Jalisco, México*
***Corresponding author:** fabiguzman01@yahoo.com.mx*

such as the emulsion capacity and foaming, absorption capacity of water, oil, are desirable in the flours for their incorporation in the development and innovation of food formulations, their functionality depends on the structure of protein, size, as well as interactions with other components as carbohydrates and fats (Ghavidel & Prakash, 2006). It has been reported in *Lupinus angustifolius* that the enzymatic hydrolysis of the protein allows to increase the ability to form foam due to the reduction of the size of the protein (Lqari et al., 2005). Some authors have reported in sorghum that an increase in the germination time allows increasing the oil absorption capacity, foam formation, emulsion capacity and stability (Elbaloula et al., 2014; Ghavidel & Prakash 2006; Setia et al., 2019). In legumes like *Bambara groundnut*, *Phaseolus aureus*, *Vigna catjang*, *Lens culinaris*, *Cicer arietinum* it has also been found increased functional properties as a result of short germination times (1-3 days) (Chinma et al., 2021; Ghavidel & Prakash 2006). Probably the increase in germination time allows increase in functional properties, while germination can modify the structure, concentration and subunits of legumes protein. Changes have been reported in the protein profile of isolates and protein fractions of germinated *Lupinus angustifolius*, showing changes in the intensity of the bands depending on the germination time. Studies that evaluate changes in functional properties depending on the germination time remain limited in this legume seed. Germinated *Lupinus angustifolius* flours may have structural and functional characteristics desirable or improved compared to conventional and ungerminated legumes. In lupins, the effect of germination time on protein subunits and its relationship with functional properties has not been reported. Therefore, the aim of this work was to evaluate the changes in protein subunits, functional properties, protein digestibility, phytic acid concentration and trypsin inhibitory activity, in *Lupinus angustifolius* L. flours subjected to different germination times.

2. Materials and Methods

2.1 Raw material

Lupinus angustifolius L. seeds were obtained from University Center of Biological and Agricultural Sciences of the University of Guadalajara, Mexico.

2.2 Germination

Seeds were washed with distilled water and disinfected with a sodium hypochlorite solution (0.07% w/v). Samples were incubated in a germination chamber at 26 °C and 65% relative humidity for 2 to 7 days (Guzmán-Ortiz et al., 2017). Every 24 h, 300 g of germinated grains was removed from the chamber using a quartet sampling in order to dehydration in a conventional oven at 40 °C until reaching an average 7.5% humidity. The germination index was 95%. Calculated after two days, taking a representative sample to calculate the germination percentage, the number of germinated and non-germinated seeds was recorded. To choose the germinated grains, the rupture of the seed coats and emergence of root were considered (Chiapusio et al., 1997). Grains were then ground and sieved through a 60-mm mesh. In accordance with the method by AACC (American Association of Cereal Chemists, 2000), flours were degreased and stored hermetically for later analysis.

2.3 Protein determination

The protein content was calculated based on nitrogen content, determined according to the Dumas combustion method using a nitrogen analyzer LECO® FP 528 (St. Joseph, MI, USA). A factor of 6.25 was used to calculate the protein content from the nitrogen content (Association of Official Analytical Chemists, 1990).

2.4 Electrophoretic profile

The electrophoretic profile was obtained following the methodology by Laemmli (1970). Polyacrylamide gels were used. A stacking gel of 5% and separating gel of 13% were used. Samples of the germinated and ungerminated *Lupinus angustifolius* L. flours were mixed at a proportion of 1 mg of protein / mL of Trizma base buffer solution (pH 6.8), 0.12 M sodium dodecyl sulfate (SDS), 2 M glycerol, bromophenol blue and 10% (v / v) β-mercaptoethanol. The mix was boiled for 5 min and loaded on the stacking gel lanes (15 µL). The stacking (5%) and separating gels (13%), were subjected to constant and successive 100-V current for 1 h 40 min. The gel staining was carried out with 0.05% (w/v) Coomassie bright blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid with constant shaking for 15 min. The gel was destined by washing and shaking for approximately 12 h in a 1:4:5 (v/v) acetic acid/methanol/water solution. The protein bands for the samples were identified according to the molecular weight marker.

2.5 Functional properties (FP)

Bulk density (BD)

The BD was performed according to the methodology described by Elkhalifa & Bernhardt (2010). Samples (10 g) from different flours (germinated and ungerminated) were placed separately in a 25 mL graduated measuring cylinder. The flours were packed by tapping softly ten times; the final volume of the press flour was determined measured and results were expressed in g/mL.

Water and oil absorption capacity (WAC, OAC)

The WAC and the OAC of the different samples were analyzed according to Elkhalifa & Bernhardt (2010). Three grams of the sample were weighed in a centrifuge tube and suspended in 30 mL of distilled water for WAC and 30 mL of refined sunflower oil for OAC, both at room temperature. The samples were gently shaken for 1 min, then for 10 and 30 min, later were centrifuged at 3000 rpm for 15 min. The supernatant was decanted and tubes were drained for 5 min. The WAC was expressed as the amount of water absorbed per gram of sample and OAC was expressed as the amount of oil absorbed per gram of sample.

Swelling power (SP)

The SP was determined according to Robertson et al. (2000). A sample (0.1 g) of the flour was weighed in a graduated

cylinder filled with distilled water (10 mL). The initial volume was recorded, the sample was gently shaken, and it was left to stand for 16 h. The final volume of the sample was then measured. The SP was calculated by the difference in volumes and dividing by the weight of the sample, reported in mL/g.

Foaming capacity and stability (FC, FS)

To evaluate the FC and FS, was followed the method by Elkhalifa & Bernhardt (2010). Flour sample (2 g) were weighed and mixed with 100 mL of distilled water. The suspension was mixed with vortex at room temperature for 1 min. The contents were placed in a 250 mL graduated measuring cylinder and the foam volume was recorded. The foaming capacity was expressed as the percentage increase in volume.

The FS of the system was determined by measuring the reduction in foam volume depending on time, was measured every 15, 30, 60, 120 min

Emulsifying activity and stability (EA, ES)

The emulsifying properties were measured following the methodology by Elkhalifa & Bernhardt (2010). Flour samples (2 g) were mixed with 20 mL of cold distilled water (4 °C) and 20 mL of refined sunflower oil. The samples were gently shaken for 20 min and centrifuged at 4000 rpm for 10 min. Afterwards, the height of the emulsion layer formed and the EA were calculated using the Equation 1:

$$EA(\%) = \frac{Height\ of\ emulsion\ layer}{Height\ of\ whole\ layer} x100 \quad (1)$$

To assess its stability, the emulsion was heated in a water bath (80 °C) for 30 min and then cooled at room temperature for 20 min. Tubes were centrifuged at 4000 rpm for 10 min and the height of the emulsion layer was measured to calculate the stability of the emulsion created using the Equation 2:

$$ES(\%) = \frac{Height\ of\ emulsion\ layer\ after\ heating}{Height\ of\ whole\ layer} x100 \quad (2)$$

2.6 In vitro protein digestibility (IVPD)

The IVPD was determined according to the report by Tinus et al. (2012). Flour from *Lupinus angustifolius* (62.5 mg protein) was weighed and hydrated in 10 mL milli-Q water at 37 °C for 1 h. Then, pH was adjusted to approximately 8.0 with NaOH 0.1 M/ HCl 0.1 N. A multi-enzymatic solution (10 mL) was prepared with approximately 16 mg trypsin (T0303 Trypsin from porcine pancreas type IX-S, freeze-dried powder, 13000–20000 BAEE units/mg protein), 31 mg chymotrypsin (C4129 α-Chymotrypsin from bovine pancreas Type II, lyophilized powder, P40 units/mg protein), and 13 mg protease (P5147 protease from *streptomyces griseus* Type XIV P3.5 units/mg solid). The multi-enzymatic solution was adjusted to pH 8.0. The sample (10 mL) was added 1 mL of the multienzyme solution and kept under shaking at 37 °C. The pH was automatically recorded every 5 seconds for 15 min. The change in pH 10 min into the digestion (ΔpH 10 min) was used in the following Equation 3 to calculate IVPD:

$$IVPD = 65.66 + 18.10\,\Delta pH\,10min \quad (3)$$

2.7 Phytic acid quantification

Following the method by Vaintraub & Lapteva (1988) and Latta & Eskin (1980), the concentration of phytic acid was determined. First, 0.5 g flour was weighed. Then 10 mL 3.5% HCl was added and continuously stirred for 1 h, followed by centrifugation at 10000 rpm for 10 min. Phytic acid determination was carried using 200 µL extract, 2800 µL distilled water, and 1 mL Wade's reagent (30 mg $FeCl_3 \cdot 6H_2O$ + 300 mg sulfosalicylic acid and 100 mL distilled water). The absorbance was measured at a wavelength of 500 nm. The concentration was calculated based on the calibration curve of sodium phytate from 0 to 160 µg/mL, and the results were expressed in mg of sodium phytate equivalent (SPE) per 100 g of sample dry basis (db).

2.8 Trypsin inhibitor activity

The activity of trypsin inhibitors was determined according to the enzymatic method by Welham & Domoney (2000). N_α-Benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA) diluted with dimethyl sulfoxide (BTC) was used as substrate to assess trypsin control, which was performed by placing 200 µL of a 0.05 M TRIS-HCl buffer solution at pH 7.5, 200 µL of trypsin solution and 500 µL of BTC, incubated at 37 ° C for 10 minutes. The reaction was stopped with the addition of 100 µL of 30% acetic acid. This solution must be 0.4 absorbance units, approximately. For the preparation of the blank, the buffer solution, the trypsin solution, and acetic acid were added to stop the reaction, then the BTC was added. To obtain the sample, 0.025 g were weighed, added 1 mL of 0.05 M HCl, and shaken at 4 °C for 1 h. The sample was centrifuged at 10000 rpm for 10 min, supernatant was collected, and the sample was kept in an ice bath until assessment. The reaction was carried out adding 5 µL of the extract and 195 µL of the regulating solution 0.05 M Tris–HCl pH 7.5 at 37 °C. Afterwards, the mix was added 200 µL of the trypsin solution and 2 min later, 500 µL of the BAPNA solution previously heated at 37 °C. The sample was left standing for 10 min; then 100 µL of 30% acetic acid was added, and the sample was centrifuged at 10000 rpm for 10 min. Absorbance was measured at a wavelength of 410 nm. The same procedure was followed for the assay target, but 200 µL of the trypsin solution were substituted by 200 µL of 1 mM HCl. The concentration of trypsin inhibitors activity was reported trypsin inhibitor units (TIUs) /g of sample db.

2.9 Statistical analysis

Results were expressed as the average of three replications ± standard deviation. Data were analyzed using an analysis of variance (ANOVA). Means were compared using Tukey's test with a confidence level of 95% using SPSS v.16.0 (SPSS, USA).

3 Results and discussion

3.1 Protein content

Table 1 shows the protein content in *Lupinus angustifolius* L. germinated for 2–7 days and the ungerminated sample. The protein concentration is observed to be significantly increased ($p < 0.05$)

Table 1. Protein content of germinated and ungerminated *Lupinus angustifolius* L.

Germination day	Protein* (%)
0	24.0 ± 0.61^{d}
2	30.0 ± 0.81^{bc}
3	31.3 ± 0.65^{bca}
4	29.7 ± 0.45^{b}
5	32.6 ± 1.18^{ca}
6	33.6 ± 0.09^{a}
7	31.6 ± 0.26^{bca}

The results are the average of three determinations ± the standard deviation. a–d letters indicate comparison of means between the samples. Samples with the same letter did not present significant difference using Tukey's test ($p < 0.05$). *Dry basis.

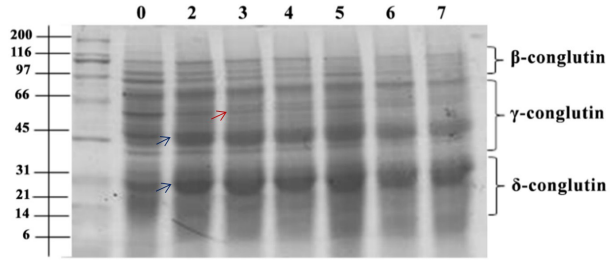

Figure 1. Electrophoretic profile of the flours analyzed, the first lane from left to right indicates the molecular weight marker, line 0 sample ungerminating, line 2-7: samples germinated from 2 to 7 days. The blue arrows indicate the increase intensity between the 21 and 45kDa subunits. The red arrow indicates a slight degradation around 60kDa.

throughout the germination time. The ungerminated sample showed 24.06% content, which was increased to 33.61% by day 6 of germination. Ghumman et al. (2016) reported an increase in lentil (*Lens culinari*) from 24.69 to 27.14% and horse gram (*Macrotyloma uniflorum* L.) from 23.64 to 25.21% after 4 days of germination. Atlaw et al. (2018) have reported an increase in protein in fenugreek (*Trigonellafoenum-graecum Linn*) from day 3 of germination. The time in which the protein presents the highest concentration is dependent on the type of legume and germination conditions. The increase in protein could be the result of the activation of enzymes, such as proteinase, which lead to the release of amino acids and peptides that can be used to create new proteins (Atlaw et al., 2018). During the germination there is a protein synthesis from the duplication of messenger RNA (mRNA) from cell division, and this synthesis is a key component of the plant's structure. However, the behavior of the protein during germination can vary depending on time and temperature of the process (Singh et al., 2017). The germination of *Lupinus angustifolius*, under specific conditions, increases the protein content. The incorporation of germinated flours such as *Lupinus angustifolius* in various food formulations (bakery products, dairy products, sausages, snacks) can allow obtaining foods with higher nutritional value compared to those of non-germinated flours. In addition, the food industry demands the obtaining of modified ingredients through effective and economic techniques with greater nutritional value.

3.2 Protein profile

The most abundant storage proteins in lupine seeds are conglutins, which belong to the globulin fraction and are classified into α-, β-, γ- and δ-conglutins, according to their molecular weight (Burgos-Díaz et al., 2016; Duranti et al., 2008). These protein subunits were evidenced in the electrophoretic profile of the ungerminated sample and samples germinated for different days (Figure 1). The first line of Figure 1 shows the molecular weight marker. Then, samples of the different germination days (2–7 days) are also illustrated. Line 0 represents the ungerminated sample, where all subunits of conglutin (α, β, γ, and δ) were detected. From day 2 of germination (line 2), an increase in the intensity of the bands between 21 and 45 kDa was observed (blue mark). At day 3, was observed a slight degradation around 60 kDa, corresponding to γ-conglutin (red mark). Additionally, smaller subunits were observed around this molecular weight; this is likely because of the protein hydrolysis created by the enzymatic activity. Reports indicate that, during this process, the proteolytic activity is increased between days 2 and 5 after exposure to water (Martinez et al., 2009). On day 5 of germination, was observed a behavior similar to that of day 2; still, the band at 60 kDa was slightly more intense than the rest of the samples (line 5). The sample of day 7 of germination exhibited more visible changes in protein subunits (line 7), the bands observed were less intense, this may be associated with a greater hydrolysis of the protein with the germination. The germination process leads to important changes in protein subunits, considerably reducing the intensity in subunits 40, 50, 97, and 116 kDa from β- and γ-conglutin. Rumiyati et al. (2012) reported similar changes in germination at 25 °C. They found reduced intensity in subunits 40, 50, 60, and 90 kDa from day 5 of germination in *Lupinus angustifolius* L. Furthermore, they reported no changes in the band at 46 kDa, even at 9 days of germination. Gulewicz et al. (2008) also studied *Lupinus angustifolius* germinated at 20 °C for 5 days and found increased intensity in the protein subunit at 20 kDa. The changes in the protein profile during germination are the result of enzyme activation that leads to protein hydrolysis from storage proteins, used as a source of carbon and nitrogen (Duranti et al., 2008). The modification of the protein during germination could be responsible for several changes in the functionality and interaction with other components. In this study similar changes in protein subunits were observed in some days of germination. For that reason, only days 3, 5, and 7 (besides the ungerminated control sample) were selected for the analysis of functional properties.

3.3 Functional properties

Bulk density

Table 2 shows the BD of the different samples analyzed. The ungerminated sample showed a 1.13 g/mL density, which significantly decreased ($p < 0.05$) from day 3 of germination and remained constant until day 7. Elkhalifa & Bernhardt (2010) evaluated the germination time in sorghum and reported a

Table 2. Bulk density, water and oil absorption capacity and swelling power in germinated and ungerminated *Lupinus angustifolius* L.

Germination day	Bulk density (g/mL)	Water absorption capacity (g H_2O/g of sample)	Oil absorption capacity (g of oil/ g of sample)	Swelling power (mL/g)
0	1.1 ± 0.01[a]	1.5 ± 0.26[c]	0.8 ± 0.05[b]	4.1 ± 0.01[b]
3	0.8 ± 0.01[b]	3.2 ± 0.09[b]	1.4 ± 0.11[a]	8.1 ± 0.12[a]
5	0.8 ± 0.02[b]	3.5 ± 0.13[ab]	1.5 ± 0.05[a]	8.2 ± 0.24 [a]
7	0.8 ± 0.051[b]	3.7 ± 0.03[a]	1.6 ± 0.03[a]	2.1 ± 0.09[c]

The results are the average of three determinations ± the standard deviation. a–c letters indicate comparison of means between the samples. Samples with the same letter did not present significant difference using Tukey's test ($p < 0.05$).

decrease of 21% of BD at day 5 of germination. Atlaw et al. (2018) found a 10% reduction in fenugreek (*Trigonellafoenum-graecum Linn*) at day 3 of germination, while Singh et al. (2017) reported decreased density of 8.3% in sorghum germinated for 2 days. These reports seem to indicate that the percentage of decrease in density is closely related to the germination time. Still, in this study, the decrease was constant from day 3 to day 7 of germination (Table 2). This is probably due to the structural changes generated in proteins and carbohydrates as a consequence of enzymatic hydrolysis during germination. By reducing this property, the volume used by the flours decreases, and its storage and industrial transportation become easier. Germination for 3 days at 26 ° C in *Lupinus angustifolius* is enough to reduce density by 28%. Under these germination conditions, it would take *Lupinus angustifolius* L. 3 days to reduce its density by 28%.

Water and oil absorption capacity

Table 2 shows the results of WAC of the different samples. This capacity increased in parallel with the germination time. The ungerminated sample obtained a value of 1.5 g H_2O/g of sample, which was significantly increased from day 3 of germination ($p < 0.05$), reaching 3.7 g H_2O/g by day 7 of germination. Benítez et al. (2013) reported in cowpea (*Vigna unguiculata* L. Walp), dolichos (*Lablab purpureus* L.), jack bean (*Canavalia ensiformis* L.), mucuna (*Stizolobium niveum* L.) increase of this property of 157, 117.6, 142 and 116.6% respectively at 4 days of germination. Ghavidel & Prakash (2006) also found increased WAC in germinated cowpea (*Vigna catjang*) and lentil (*Lens culinaris*) of 115.5 and 148.6% respectively from 2 days of germination. The increase in WAC due to the effect of germination time can be attributed to an increase in the concentration of proteins of lower molecular weight, thus increasing the availability of polar groups capable of interacting with the water in the environment (Ghumman et al., 2016). These data agree with the observations in the electrophoretic profile (Figure 1), since smaller subunits were found with germination time. In addition, the decomposition of polysaccharide molecules generated during germination promotes the interaction with water and, therefore, increases its retention (Elkhalifa & Bernhardt, 2010).

A low WAC in ungerminated flour and during the first days of germination is related to the content of native protein. It is also linked to its capacity to interact with water, mostly due to the structure, conformation, sequence, number, and type of amino acids (Butt & Batool, 2010). Muranyi et al. (2016) reported a lower absorption capacity in protein isolated from *Lupinus angustifolius* L. without germinating (0.85 mL of water / g of protein), this coincides with what was observed in this study. Different WACs by germination allow for alternative uses of these flours. A high WAC is closely related to a soft texture of bakery products. The use of this type of flour in confectionery is desirable since it prevents the solubilization of other proteins without losing WAC. In meat products, germinated lupins flour could be used, it could enhance the texture properties of the final product, improving viscosity, elasticity, adhesion, and consistency (Benítez et al., 2013).

Table 2 shows the OAC of the different samples and it is clear that germination increased OAC. The ungerminated sample showed a value of 0.8 g oil/g of sample, which was significantly from day 3 of germination increased ($p < 0.05$). The increase remained constant until day 7, where was observed a value of 1.64 g oil/g of sample. This may be associated with the degradation of the β and γ subunits of the conglutins observed in the electrophoretic profile throughout the germination time (Figure 1). Lqari et al. (2005) also reported greater oil absorption capacity in conglutin hydrolysates. Singh et al. (2017) reported that OAC increased from 82.26 to 88.12% in sorghum germinated at 30 °C for 48 h. The increase in OAC in germination is likely the result of oil retention by capillarity and protein hydrophobicity. In addition, there is a greater presence of nonpolar amino acid side chains that bind next to oil hydrocarbon chains. This apparent increase in OAC could also be due to the increase in exposure of such chains generated by protein hydrolysis during the germination process. On the other hand, during this process, the native protein is denaturalized, which results in the exposure of a larger lipophilic surface that improves OAC (Elkhalifa & Bernhardt, 2010). These flours can be used in the preparation of foods in which maintaining and protecting the lipid content is necessary, as in infant formulas (Singh & Sharma, 2017). Furthermore, they could be more efficient when compared against conventional legumes like beans and lentils since higher OAC values were obtained in lupins.

Swelling power

The results of the SP of the samples analyzed are shown in Table 2. The ungerminated sample showed a value of 4.16 mL/g. It increased by 97.5% from day 3 of germination and was statistically different from the ungerminated sample ($p < 0.05$). A significant decrease was observed at day 7 of germination ($p < 0.05$). The decrease at day 7 might have been caused by a greater protein denaturalization without molecules able to

interact with the water in the available medium (Waldia et al., 1996). In addition, it must be considered that this property is also affected by the amount of amylopectin present, which is often degraded during germination. In consequence, the swelling capability is affected when the time of the process is longer (Gutiérrez-Osnaya et al., 2020). Obtaining flours with a high swelling capacity allows for the development of foods with elevated humidity content and prevents the syneresis of the final product. In turn, this extends its shelf life, improves the viscosity and texture of the final product (Waldia et al., 1996).

Foaming capacity and stability

The ungerminated *Lupinus angustifolius* L. sample showed 51.66% of foaming capacity (Table 3), significantly different from days 5 and 7 of germination ($p < 0.05$). The increase at day 7 was 36.14% higher when compared against that of the ungerminated sample.

Singh et al. (2017) reported a similar behavior in sorghum germinated for 48 h. They found that the foam formation capacity increased by 16.89% after the process. Setia et al. (2019) also reported an increase of 31.18 and 27.82% in the foam formation capacity of pea (Amarillo variety) and faba bean (Snowdrop variety), respectively at 72h of germination. Germination significantly improves the foam formation capacity, possibly because the activity is promoted by the increased amount of solubilized proteins during germination (Elkhalifa & Bernhardt, 20 10). Hydrolysis of protein from *Lupinus angustifolius* has been reported to increase the ability to foam compared to a native protein due to the reduction in protein size (Lqari et al., 2005). This agrees with what was observed in the electrophoretic profile at 7 days of germination (Figure 1), where degradation of some protein subunits was observed. The increase in foaming capacity can also be related to the decrease in the content of phytates (Table 4) since its union with proteins alters the solubility and its functionality, by decreasing the concentration of phytates they allow the protein to be available. Foam formation was highest on day 7 of germination, however, it was not the most stable due to the smaller size of the protein subunits, possibly the foam was not strong enough to maintain its integrity. Figure 2 shows the foam stability with respect to time in the germinated samples. The ungerminated sample and that germinated for 3 days showed a similar behavior in that stability was constant from 60 min. This behavior is related to the foam formation capacity since these samples showed no significant difference ($p > 0.05$). The sample germinated for 5 days showed a greater stability as time increased. Elkhalifa & Bernhardt (2010), also reported this behavior when germinating sorghum for 48 h. The stability was constant as time was extended since protein denaturalization and increased soluble protein in aqueous interface allow for the creation of hydrophobic interactions. The increased stability is likely due to the denaturalization and reduction in superficial tension of the protein molecules which confer a good FC (Singh et al., 2017).

Results would indicate that the germination of legumes produced a structural change in proteins, increasing their foam formation capacity. This property depends on the amount of

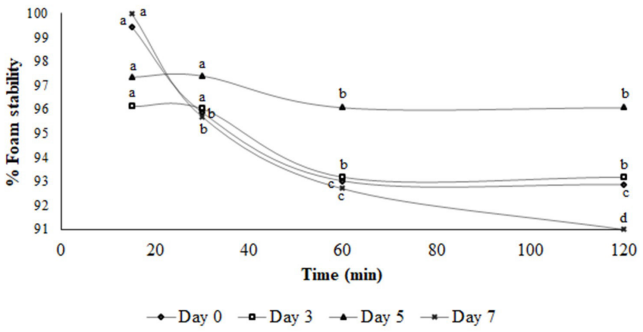

Figure 2. Foam stability of ungerminated *Lupinus angustifolius* L and germinated by 3, 5 and 7 days.

Table 3. Foaming capacity and emulsifying properties of germinated and ungerminated *Lupinus angustifolius* L.

Germination day	Foaming capacity (%)	Emulsifying activity (%)	Emulsifying stability (%)
0	51.6 ± 2.88^{c}	40.3 ± 0.57^{d}	36.2 ± 0.13^{d}
3	55.6 ± 0.57^{cb}	56.7 ± 0.62^{c}	54.7 ± 0.15^{c}
5	59.6 ± 0.57^{b}	64.1 ± 0.14^{b}	62.3 ± 0.51^{b}
7	70.3 ± 1.52^{a}	78.2 ± 0.28^{a}	77.2 ± 0.38^{a}

The results are the average of three determinations ± the standard deviation. a–d letters indicate comparison of means between the samples. Samples with the same letter did not present significant difference using Tukey's test ($p< 0.05$).

Table 4. *In vitro* protein digestibility, phytic acid and trypsin inhibitors activity in germinated and ungerminated *Lupinus angustifolius* L.

Germination day	*In vitro* protein digestibility (%)	Phytic acid (mgSPE*/g of sample)	Trypsin inhibitor activity (TIUs **/g of sample)
0	73.0 ± 4.87^{a}	3.0 ± 0.03^{a}	34.6 ± 0.33^{a}
3	74.3 ± 1.89^{a}	2.3 ± 0.05^{b}	26.3 ± 0.79^{b}
5	71.2 ± 0.70^{a}	2.5 ± 0.09^{b}	17.9 ± 0.45^{c}
7	70.2 ± 2.13^{a}	2.5 ± 0.15^{b}	7.8 ± 0.67^{d}

* Sodium Phytate Equivalents (SPE); ** Trypsin inhibitor units (TIUs). The results are the average of three determinations ± the standard deviation. a–d letters indicate comparison of means between the samples. Samples with the same letter did not present significant difference using Tukey's test ($p< 0.05$)

proteins present, their structure, and their relative ability to denaturalize, precipitate, and reduce the superficial tension in the air-liquid interface of the foam.

Emulsifying activity and stability

Table 3 shows the results obtained in the emulsion properties of the germinated and ungerminated samples. The ungerminated sample showed 40.3% of emulsifying activity, which significantly increase with the germination time progressed ($p < 0.05$), until increasing 94% at day 7 of germination. The germination process affects this property in legumes in different ways. Elkhalifa & Bernhardt (2010) observed that the emulsifying activity increased 33% from the third day of germination. Elbaloula et al. (2014) also found an increase in the emulsifying capacity and stability of approximately 64 and 78% respectively of sorghum *Butanna* on the second day of germination. Germination generates hydrolysis of the protein, leaving subunits of lower molecular weight (Figure 1), which causes the exposure of hydrophobic amino acid residues, producing an increase in EA (Lqari et al., 2005). Also, a high EA could be attributed to elevated levels of solubilized proteins acting as surfactant. In addition, it is likely the result of a change in the equilibrium of Van der Waals forces and their electrostatic repulsive forces (Lawal, 2004). This allows to deduce that germination modifies the structure of the native protein, directly affecting the functional properties of the flours obtained. In ES samples was observed a significant difference ($p < 0.05$). The tendency was the same as in the emulsifying activity: stability was increased along with germination time. These results are similar to those reported by Singh et al. (2017) when sorghum (variety SL 44) was germinated for 2 days. The ES and EA increase as germination time is extended because of a rise in the interactions between protein subunits from native protein hydrolysis when fat is present in the medium. In addition, they are also the result of an increase in the hydrophobic parts of these proteins interacting with the lipids present in the sample (Singh et al., 2017). Soluble proteins are more active on the surface and they are known to promote oil emulsion in water. On the other hand, some types of polysaccharides can help to stabilize the emulsion reaction by increasing the viscosity in the system (Elbaloula et al., 2014). According to these characteristics, germinated flours could be used in bakery products.

3.4 In vitro protein digestibility

Protein has played a key role in the modification of physical properties during germination. However, in *Lupinus angustifolius* under the germination conditions used, its digestibility was not affected. Table 4 shows no significant differences in the samples due to germination ($p > 0.05$). Setia et al. (2019) found a decrease of 1.6 and 3.07% in yellow pea (CDC Amarillo variety) and faba bean (CDC Snowdrop variety), respectively, after 3 days of germination. The changes generated in the protein subunits did not have an impact on the digestibility of the protein through the germination time. Furthermore, the presence of antinutritional compounds as phytic acid and trypsin inhibitors can affect digestibility by forming complexes with the protein and thus preventing hydrolysis and digestion.

3.5 Phytic acid

Table 4 shows the content of phytic acid in germinated and ungerminated *Lupinus angustifolius* L. The ungerminated sample showed a concentration of 3.06 mg sodium phytate equivalents /g of sample and significantly decreased ($p < 0.05$) by 23.33% from day 3 of germination. Reports indicate a reduction of 96% in *Phaseolus vulgaris* germinated for 4 days (Shimelis & Rakshit, 2007). Aguilera et al. (2013) analyzed different non-germinated and germinated for 4 days legume flours to quantify inositol phosphates, verifying that germination caused a reduction of up to 70%. This is likely because the germination process progressively reduces the concentration of those compounds. There are two types of phytases in legumes and they promote a reduction in phytates. One type is constitutive and the other, inductive since it is inducible by germination and/or previous soaking of the grain. Although both types are related to dephosphorylation, constitutive phytase starts hydrolysis during the primary stages of germination, while inducible phytase is synthetized de novo during germination through preexistent mRNA (Greiner & Konietzny, 2010). Guzmán-Ortiz et al. (2019) reported that phytase activity is gradually reduced, probably as a consequence of enzymatic degradation by activated proteases. This could also be related to the fact that phytic acid concentration remains constant after a period of germination. There is no significant difference ($p > 0.05$) when the germination time is extended, possibly because the inductive phytase is not synthesized under the germination conditions used. This is related to protein digestibility since the germination conditions did not allow for phytate degradation. Phytates apparently formed complexes that prevented an increase in protein digestibility (Table 4) because *in vitro* studies have proven that phytates negatively affect proteolytic enzymes in the pancreas.

3.6 Trypsin inhibitor activity

In Table 4 was observed the values of trypsin inhibitor activity from germinated and ungerminated *Lupinus angustifolius* L. The ungerminated sample showed values of 34.64 UTI/g of sample. The germination process proved to have a positive effect from day 3 of germination by generating a significant reduction ($p < 0.05$), which reached 77.45% at day 7, as the germination time progressed. de la Rosa-Millán et al. (2019) reported no modifications in the activity of trypsin inhibitors with germination of black beans (*Phaseolus vulgaris L.*). In contrast, Shimelis & Rakshit (2007) observed a 15.25% decrease in kidney bean (*Phaseolus vulgaris L.*) germinated for 4 days. Kumar et al. (2006) found a decrease of 45.65% in soy germinated at 25 °C for 6 days. The percentage of reduction can vary depending on the germination conditions and the type of legume. Although the activity of trypsin inhibitors is significantly reduced in germination, no impact on protein digestibility was observed. However, it should be considered that the digestibility could also be affected by the presence of phytic acid, which did not decrease significantly. More studies are necessary to clarify the molecular interaction of both compounds with the protein during germination.

4 Conclusions

Germination is an effective method to obtain flours from *Lupinus angustifolius* L. with modified functional properties and enhanced nutritional value compared to the ungerminated lupinus flour. The protein content significantly increased from day 3 of germination, rendering the seed an excellent source of vegetable protein. In addition, there is evidence of protein hydrolysis caused by germination time, which leads to changes in low molecular weight peptide subunits that influence the functional properties of germinated flour. The degradation of the β and γ subunits of the conglutins mainly influenced the OAC, capacity and stability of foaming, more studies are necessary to establish a specific relationship of structural functionality. The WAC and OAC as well as the emulsion and foam capacities increased with germination. This allows for expanding the alternative uses of the flours as modified ingredients in the development of functional foods. The results revealed that germination under these conditions does not affect protein digestibility, even when the activity of trypsin inhibitors decreased. The concentration of phytic acid decreased on the third day of germination, however it remained constant until day 7, this could influence the absence of change in protein digestibility. Three days of germination in *Lupinus angustifolius* L. are necessary to observe changes in the functionality of flours. Extending the time of the process could be suggested depending on the use of the flours obtained. Evaluating effective economic techniques to improve nutritional and technological properties of flours will allow to diversify and enhance their use in the food industry. Finally, according to the modification of their properties with germination, flours could be ideally used in food development.

Disclosure statement

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