Tesis Doctoral

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ESTUDIO DEL IMPACTO DEL PROCESO DE DESAMARGADO Y FERMENTACIÓN SOLIDA SOBRE LA COMPOSICIÓN NUTRICIONAL DEL GRANO DE LUPINO Y SU APLICACIÓN EN PANIFICACIÓN

Tesis Doctoral presentada por:

Elena Villacrés Poveda

Dirigida por:

Dra. Cristina Molina Rosell

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Dª Cristina Molina Rosell, Profesor de Investigación del Instituto de Agroquímica y Tecnología de Alimentos del Consejo Superior de Investigaciones Científicas.

Hace constar:

Que la memoria titulada "Estudio del impacto del proceso de desamargado y fermentación solida sobre la composición nutricional del grano de lupino y su aplicación en panificación" presentada por Dña. Elena Villacrés Poveda por la Universidad de Valencia, ha sido realizada bajo su dirección y que reúne las condiciones necesarias para optar al grado de doctor.

Valencia, 30 de Junio de 2020

MOLINA ROSELL M.CRISTINA - DNI 20794643K

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Fecha: 2020.10.09 12:40:54 +02'00'

Dra. Cristina Molina Rosell

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DEDICATORIA

El presente trabajo de investigación lo dedico a los agricultores de mi patria por el esfuerzo de mantener la biodiversidad, incluida la especie *L. mutabilis* para asegurar que haya comida sana y de calidad a nuestro alcance cada día del año, en un planeta en el que, en muchos lugares padecen hambre y desnutrición.

RESUMEN

Entre las leguminosas, el lupino ha sido identificado como promisorio, por su alto contenido y calidad de proteínas y otros nutrientes, apropiados para la producción sostenible y con beneficios potenciales para la salud. A pesar de su alto contenido nutricional, la presencia de alcaloides quinolizidínicos (QAs) en la especie *Lupinus mutabilis* limita la expansión de su uso y consumo. Por lo tanto, el objetivo general de la presente tesis doctoral es mejorar el procesamiento de lupino con respecto a la eficiencia del proceso y la composición nutricional, y validar la aplicabilidad de los productos resultantes en un alimento básico como el pan. Con ese objetivo, se evaluó el efecto de dos tratamientos térmicos, acuoso (ATT) y salino (STT), sobre los QAs y el contenido total de proteínas, así como sobre las características del grano (tamaño, textura y color) en tres variedades de lupino (INIAP-450, INIAP-451 y Criollo). Los dos métodos fueron efectivos para reducir los QAs a niveles seguros de consumo (2,5-3,5 $g \cdot kg^{-1}$ peso seco), pero el STT fue más eficiente en términos del tiempo (58 h frente a 84 h) y el volumen de agua (66 L frente a 127 L) requeridos.

Debido a que el proceso de desamargado causó la pérdida de ciertos nutrientes del grano de lupino, se exploró la fermentación en estado sólido, utilizando *Rhizopus oligosporus*, para mejorar el valor nutricional de tres variedades de *L. mutabilis*, incluyendo el impacto de la condición del grano (entero o triturado) y la presencia de tegumento. Después de la incubación a 28 °C por 96 h, la acidez titulable aumentó a 0,60%, el pH disminuyó a 4,03 y el nitrógeno total del grano triturado sin tegumento alcanzó un valor de 108,27 $g \cdot kg^{-1}$ peso seco, en la variedad Criollo; confirmando el efecto positivo de la fermentación de los granos. El desamargado y la fermentación sólida indujeron cambios significativos en la composición nutricional de las tres variedades de lupino. Concretamente, la aplicación de los dos procesos aumentó el contenido de proteína a 644,55 $g \cdot kg^{-1}$ peso seco (Criollo). Los aminoácidos variaron de forma diferente según el proceso aplicado al grano, así el desamargado causó una disminución de la mayoría de aminoácidos, excepto los ácidos aspártico y glutámico, leucina y fenilalanina. En contraste, al aplicar la fermentación la mayoría de aminoácidos experimentaron un incremento, con relación al grano desamargado, excepto el ácido aspartico, serina, histidina, glicina, arginina, alanina, lisina y fenilalanina. Los ácidos grasos poliinsaturados aumentaron por efecto del desamargado hasta un valor promedio de 292,25 $g \cdot kg^{-1}$; con la fermentación aumentó el contenido de monoinsaturados (559,78 $g \cdot kg^{-1}$ de aceite de lupino) y poliinsaturados (293,17 $g \cdot kg^{-1}$ de

aceite de lupino). Paralelamente, la fermentación causó una disminución del almidón total, almidón resistente, potasio, hierro y zinc, pero el nivel de reducción varió dependiendo de la variedad de lupino. Los procesos de desamargado y fermentación sólida afectaron también a los componentes antinutricionales y las propiedades antioxidantes benéficas de las tres variedades de lupino, causando una disminución de los siguientes antinutrientes: nitratos (94,47%), taninos (82,14%), alcaloides (93,80%), ácido fítico (71,57%) e inhibidores de tripsina (76,83%). La actividad ureasa expresada como diferencia de pH disminuyó hasta 0,05. Los fenoles, los carotenoides y la capacidad antioxidante disminuyeron debido al proceso de desamargado en 96,83; 52,63 y 96,12%, respectivamente, pero mejoraron debido a la fermentación sólida en 1056,87%, 165,42% y 1509,81%, respectivamente.

Cuando las harinas de lupino se probaron en panificación, se obtuvieron panes trigo-lupino con diferentes niveles de substitución (10%, 15%, 20%). El lupino debilitó la masa durante el mezclado, con un tiempo de desarrollo y estabilidad más cortos, especialmente la harina de lupino fermentado (FLF). Las harinas de lupino desamargado (DLF) y fermentado (FLF) redujeron significativamente el volumen de los panes lupino-trigo, particularmente con una sustitución (> 10%). El efecto detrimental observado con mayores niveles de sustitución (20%) fue atenuado usando FLF, sin embargo, los panes elaborados con esta harina, recibieron menores calificaciones sensoriales, debido al sabor ácido detectado por los panelistas. Las dos harinas de lupino permitieron obtener panes lupino-trigo con una composición química similar, aumentando el contenido promedio de proteína, grasa y fibra dietética, en comparación con los panes de trigo.

En general, este estudio dio a conocer las condiciones para realizar un eficiente desamargado del lupino y el impacto de la fermentación en estado sólido para la mejora nutricional del grano, lo que condujo a la obtención de harinas que podrían usarse para reemplazar parcialmente a la harina de trigo en la elaboración de pan, contribuyendo a la sostenibilidad económica de los cultivos de Ecuador.

ABSTRACT

Among legumes, lupin has been identified as particularly promising, characterized by high-quality protein content and other nutrients suitability for sustainable production and potential health benefits. Despite its high nutritional content, the presence of quinolizidine alkaloids (QAs) in the species *Lupinus mutabilis* limits the expansion of its consumption and use. Therefore, the general objective of the present PhD thesis was to improve lupin processing regarding process efficiency and nutritional composition, and to validate the applicability of the resulting products on a staple food like bread. With this goal the effect of two thermal treatments, aqueous (ATT) and saline (STT), on QAs and total protein contents, as well as on grain features (size, texture and color) was evaluated in three lupin varieties (INIAP-450, INIAP-451 and Criollo). Both methods were effective for reducing QAs to safe levels for consumption (2.5-3.5 $g \cdot kg^{-1}$ dry weight), but the STT was more efficient in terms of the time required (58 h vs 84 h) and volume of water (66 L vs 127 L).

Since the debittering process caused the loss of certain lupin nutrients, the solid-state fermentation using *Rhizopus oligosporus* was explored to improve the nutritional value of three *L. mutabilis* varieties, including the impact of the grain status (whole or crushed) and the presence of tegument. 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 $g \cdot kg^{-1}$ dry weight in the Criollo variety; confirming the positive effect of fermentation of the grains. Debittering and solid-state fermentation induced significant changes in the nutritional composition of the three lupin varieties. Regarding macronutrients fermentation increased the protein content of Criollo variety (644.55 $g \cdot kg^{-1}$ dry weight). The amino acids varied differently according to the process applied to the grain, thus debittering caused a decrease in most amino acids, except aspartic and glutamic acids, leucine and phenylalanine. In contrast, with fermentation, most amino acids experienced an increase, in relation to the debittered grain, except aspartic acid, serine, histidine, glycine, arginine, alanine, lysine and phenylalanine. Polyunsaturated fatty acids increased due to debittering to 292.25 $g \cdot kg^{-1}$ of lupine oil (average value); with fermentation, not only the content of monounsaturated fatty acids (559.78 $g \cdot kg^{-1}$ of lupine oil) but also of polyunsaturated (293.16 $g \cdot kg^{-1}$ of lupine oil) was increased. In parallel, fermentation caused a decreased of total starch, resistant starch, potassium, iron and zinc; the extend of the reduction was dependent on the lupin varieties.

Debittering and solid-state fermentation processes affected also the anti-nutritional components and the beneficial antioxidant properties of three lupin varieties, causing a decrease of the following antinutrients: nitrates (94.47%), tannins (82.14%), alkaloids (93.80%), phytic acid (71.57%) and trypsin inhibitors (76.83%). Urease activity expressed as a difference in pH decreased to 0.05. Phenols, carotenoids and antioxidant capacity decrease due to the debittering process by 96.83, 52.63 and 96.12%, respectively, but they were improved due to solid fermentation by 1056.87%, 165.42% and 1509.81%, respectively.

When lupin flours were tested on breadmaking lupin-wheat breads with different levels of substitution (10%, 15%, 20%) were obtained. Lupin weakened the dough during mixing, having shorter development time and stability, especially fermented lupin flour (FLF). Debitter lupin flour (DLF) and FLF significantly reduced the bread volume of the lupin-wheat breads particularly at higher substitution (>10%). Detrimental effects observed at the highest substitutions (20%) were diminished when using FLF, although breads received lower score due to the acidic taste detected by panelists. Both lupin flours provided lupin-wheat breads with rather similar composition, rising the average content of proteins, fat and dietary fiber, compared to wheat breads.

Overall, this study provided the conditions for performing an efficient debittering of lupin and the impact of solid-state fermentation for the nutritional improvement of lupin, leading to flours that could be used to partially replace wheat flour in breadmaking, contributing to the economic sustainability of Ecuador crops.

INDICE GENERAL

INTRODUCCIÓN

Características generales de las leguminosas

El término leguminosa incluye una amplia familia de vegetales que en la última década ha adquirido una gran importancia en alimentación por su alto contenido en proteínas. Esta característica ha propiciado un gran avance en el conocimiento de las leguminosas y su mayor explotación como fuentes de proteínas alternativas.

Leguminosa es una palabra que proviene del latín *legumen* que indica fruto alargado o en espiral (fruto en "legumbre" o "vaina") que contiene varias semillas dispuestas en fila. Las leguminosas, desde el punto de vista botánico, pertenecen a la familia *Fabaceae*, cuyas especies presentan la característica común de producir vainas. Las *Fabaceas* se dividen en tres subfamilias, siendo la *Papilionoideae* la más amplia y prácticamente la única cuyas especies se cultivan para el consumo humano (López-Amorós, 2000). La familia Fabaceae es muy amplia, con 700 géneros y unas 20.000 especies, aunque solo una parte de los géneros es considerada dentro del grupo alimentario de las legumbres.

Las leguminosas tienen importancia desde el punto de vista agrícola, contribuyen a la salud de los suelos y a la mitigación de los efectos del cambio climático mediante sus propiedades de fijación del nitrógeno atmosférico, siendo adecuadas para la recuperación de suelos erosionados y/o degradados. La mayor parte de las leguminosas de grano tienen una gran capacidad de adaptación a las diversas condiciones climáticas de clima y suelo (Valentine *et al*., 2018).

Las leguminosas herbáceas se dividen en función de su utilización agrícola, en leguminosas de grano, hortícola, forrajeras y pascícolas. El término legumbre se reserva para las que se cosechan y se consumen como grano seco, mientras que las de consumo verde como habas, judías verdes o guisantes, se clasifican como hortalizas (Iqbal *et al*., 2006). Otros autores describen como legumbres comestibles la soja, frejol faba, guisante, frejol mungo, frejol rojo pequeño, caupí, frejol, frejol jacinto y palomino (Du *et al*., 2014).

Respecto a la morfología, las legumbres o granos presentan una alta diversidad de tamaños, formas, colores y grosor del tegumento (testa), pero la mayoría de éstas tienen

una estructura similar y cuando madura tienen tres partes estructurales principales: cubierta, cotiledón y embrión. El número de semillas en las vainas es variable oscilando entre 1 a 25 granos en el interior de la vaina (Zornoza-Hernandez *et al*., 2016).

De las legumbres, que pueden ser utilizadas en la alimentación humana y animal, alrededor del 75% se destina al consumo humano, mientras que el uso como pienso representa menos del 15% (Sherasia *et al*., 2017). La utilización de las legumbres como alimentos se incrementa en los países en vías de desarrollo, donde el 90% de su producción se destina a la alimentación humana. Las legumbres presentan una importancia especial para los países de bajos ingresos y con déficit de alimentos, cuyas principales fuentes de proteínas y energía son los productos de origen no animal contribuyendo al 10% de las proteínas diarias recomendadas y a un 5% del aporte energético de la población (Sherasia *et al*., 2017), siendo consideradas una fuente económica de proteínas (Du *et al*., 2014).

Desde el punto de vista nutricional las leguminosas, son importantes como fuente de proteína de origen vegetal, aunque también aportan hidratos de carbono complejos, fibra dietética, minerales, etc. con grandes beneficios para la salud humana. También destaca la presencia de numerosos compuestos con actividad biológica que se utilizan en algunas industrias como la farmaceútica (Van-Wyk and Albrecht, 2008). Entre los factores negativos destaca la presencia de factores antinutritivos que influyen en la digestibilidad proteica y de los carbohidratos, aunque en pequeñas cantidades pueden ser beneficiosos para la salud en la prevención de enfermedades como el cáncer, enfermedades coronarias etc. (Duranti, 2006).

Lupino: cultivo y producción

Los lupinos pertenecen al orden fabales, suborden leguminosae, familia fabaceae, subfamilia faboidea, tribu genisteae, género lupinus (Tapia, 2015). Cerca de 400 especies de lupino han sido encontradas en la naturaleza. Entre ellas, solamente unas pocas especies, como son el lupino blanco (*Lupinus albus*), lupino azúl (*L. angustifolius*) y lupino amarillo (*L. luteus*) han sido extensamente estudiadas por su valor agronómico y características nutricionales. Sin embargo, la especie *L. mutabilis* ha sido escasamente investigada. Esta especie puede crecer en tierras agrícolas marginales

en diversas condiciones ambientales y tienen un alto potencial para incorporarse en varias fuentes de alimentos (Nelson and Hawthorne, 2000).

En la mayor parte del mundo, los lupinos se han usado para alimentación animal, especialmente de vacas, cerdos, aves y acuicultura; lo cual aún persiste (White and Staines, 2007). El interés del lupino como alimento humano ha aumentado significativamente al incrementarse el interés de los consumidores por la alimentación y nutrición. A principios del siglo pasado, se desarrollaron nuevas variedades de lupino llamadas "dulces" bajas en alcaloides (sustancias amargas) y altas en proteínas (Sedláková *et al*., 2016). El desarrollo de variedades con vainas sólidas y con bajo contenido de alcaloides permitió que estos materiales dejaran de ser usados únicamente como abono verde y mejoradores del suelo, para convertirse en leguminosas cultivadas para semillas. Los lupinos también son conocidos por sus hermosas flores. El lupino Russell, un híbrido derivado de *L. polyphyllus*, es ampliamente utilizado como flor ornamental, particularmente en Europa. Algunos lugares de las tierras altas del sur de Nueva Zelanda tienen un color resplandeciente en primavera y principios de verano debido al lupino alpino, una variante del lupino Russell. La flor (bluebonnet) de *L. texensis* es el emblema floral de Texas (Petterson, 2016).

El lupino (*Lupinus* spp.) es una leguminosa que se adapta bien a una variedad de tipos climáticos y de suelo. Existe un amplio consenso sobre que el lupino evolucionó de la tribu primitiva Sophoreae perteneciente a la subfamilia Papilionoideae (plantas de guisantes con flores). Hoy en día, las especies de lupino silvestres cubren casi todas las zonas climáticas: Alaska e Islandia subárcticas, regiones mediterráneas y semidesérticas, las tierras altas de África oriental, México y los Andes, y las tierras bajas subtropicales de las Américas orientales (Petterson, 2016). Desde el punto de vista ecológico, los lupinos se distinguen entre las plantas cultivadas y entre las leguminosas de grano, por su capacidad de crecer en suelos bajos en minerales y en materia orgánica, así como en suelos arenosos y ácidos (Petterson, 2016). La acidez excesiva del suelo es una limitante para los lupinos. Sin embargo, toleran bien un pH 5,0 e incluso inferiores. Estas características, junto con su independencia de la aplicación de fertilizantes nitrogenados, confieren un papel especial a los lupinos en sistemas de producción de bajo costo y en áreas de fertilidad moderada donde otras leguminosas de grano están en desventaja (Lucas *et al*., 2015). El principal valor del cultivo de lupino para los

agricultores es la reposición de nitrógeno al suelo, constituyéndose en una alternativa de los sistemas de rotación de cultivos y la producción de grano comercial.

El mayor productor y exportador mundial de lupino es Australia con el 80-85% de la producción mundial, del cual entre 90-95% se exporta a todo el mundo. Oceanía y Eurasia contribuyen con el 90% de los 1.6 millones de toneladas anuales de la producción mundial, seguidas por los países de África (5-7%) y las Américas (3-5%) (Petterson, 2016). En Australia, 1,3 millones de toneladas de lupino son producidas anualmente. Las dos principales especies cultivadas en Australia son *L. angustifolius* and *L. albus* (Pollard *et al*., 2002).

En Europa dos variedades son cultivadas principalmente: *L. luteus* and *L. albus* (Cowling and Gladstones, 2000). Las regiones de mayor cultivo en Europa son Alemania, Francia, Benelux, España, Polonia, Ucrania y Rusia. En la república Checa el área cultivada es modesta, alcanzando las 7,000 ha y los rendimientos son muy variables. En Europa un ligero aumento del área cultivada y la producción ocurrió durante el periodo 2000-2013, lo que representó el 17,6% de la producción mundial (Lucas *et al.*, 2015). Se estima que cada año son elaborados 500.000 t de productos alimenticios que contienen como ingrediente lupino blanco europeo y/o lupino dulce australiano (Lupin, 2013).

En América del Sur, se cultivan y consumen diferentes especies de lupino. Chile es el único país del mundo donde la producción de lupino se incrementa cada año, con un promedio anual de 70.000 t en el 2013 (FAOStat, 2014). La mayor parte de esta producción es de *L. albus*, que se cultiva en la Región IX y se ha beneficiado de un fuerte esfuerzo de mejoramiento local. Sin embargo, existe un interés creciente en *L. angustifolius y L. luteus*. En Chile un gran porcentaje de lupino es utilizado por la gran industria acuícola chilena del salmón (Ravelo and Planchuelo, 2006). Se estima que el área total cultivada con lupino alcanza las 10,000 ha (Jacobsen and Mujica, 2006).

El llamado lupino andino (*L. mutabilis* Sweet) se cultiva en algunas partes de América del Sur, pero no está presente en Europa a escala comercial (Pollard *et al*., 2002). Aproximadamente 1.895 ha son cultivadas en Bolivia con un rendimiento promedio de 648 $kg \cdot ha^{-1}$, en Ecuador el área cultivada alcanza 5.974 ha con un rendimiento de 700

kg·ha^{-1}, en Perú el rendimiento promedio es de 1,33 kg·ha^{-1} y el área cultivada es de 10.628 ha (Mercado et al., 2018). Los principales centros de producción en Ecuador están en Cotopaxi, con 2.150 ha y 584 t (272 kg·ha^{-1}); Chimborazo es la segunda provincia productora de lupino con 1.013 ha y 230 t (227 kg·ha^{-1}) y Pichincha con 585 ha y 190 t (325 kg·ha^{-1}) como promedio anual. En las provincias de Carchi, Imbabura, Tungurahua y Bolívar se cultivan menores cantidades, con rendimientos de 250 kg·ha^{-1} (Caicedo and Peralta, 2000).

Morfología del grano de lupino

Las semillas de *Lupinus* difieren en tamaño y propiedades físicas, lo que influye sobre el uso tecnológico de las mismas (Grochowicz and Andrejko, 2006). Las semillas de *L. albus* presentan las siguiente propiedades físicas: volumen de las semillas (88,33-90,67 mL), peso de 100 semillas (28,65-38,80 g/100 semillas), diámetro medio (7,04-7,71 mm), tegumento (15,80-16,97% p/p), pH (5,37-5,30), absorción de agua (0,92- 0,98 g·g^{-1}), coeficiente de hidratación (224,09-234,48), coeficiente de hinchamiento (765,83-769,17), capacidad de hinchamiento (0,47-0,36 mL/semilla), índice de hinchamiento (1,51-1,52), tiempo de cocción (7-7,5 h), dureza (198,40-188,51 N·g^{-1}) (Tizazu and Emire, 2010). Estas propiedades desempeñan un rol importante en la selección de tamices y máquinas para el descascarado y en el valor comercial de los granos. Las semillas grandes presentan un mayor valor comercial que las semillas pequeñas para su consumo en fresco, debido a la relación positiva entre peso de semillas y aumento de volumen (Grochowicz and Andrejko, 2006).

Las semillas de *L. mutabilis* poseen un color blanco opaco y una forma ovoide con longitudes variables en las tres dimensiones. El diámetro promedio ortogonal es 7,51 mm, la forma de cada cotiledón es de casquete y el volumen promedio es de 0,3368 mL/semilla. El grano posee un 17% más de diámetro en comparación con la semilla de soya (6,3 mm). El mayor tamaño del lupino frente a otras semillas es un indicador de la mayor capacidad de nutrientes que puede almacenar (Tizazu and Emire, 2010). La semilla está conformada por dos cotiledones y una radícula embrionaria equivalente al 88.97% del peso total. Estos son de color amarillo oscuro debido al contenido de grasa y carotenoides. Su espesor promedio es de 2,40 mm (Ortega *et al*., 2010). Los lupinos

tienen una testa relativamente gruesa en comparación con otras legumbres y en general, se requiere descascarar el grano previo a otros procesos. En estado seco, los cotiledones presentan una estructura rígida con 9,67% de humedad. Al ser hidratados hasta 55% de humedad se tornan elásticos y a una mayor humedad o completamente hidratados (65%) el fenómeno de turgencia hace que se vuelvan quebradizos y sensibles a los esfuerzos mecánicos. El 11,03% de la semilla está compuesta por un tegumento blanco, de textura plástica y resistente. Se estima que su espesor es de 0,20 mm y cubre el borde longitudinal de los cotiledones. El tegumento o testa tiene una baja capacidad de retención de agua y después de hidratado la pierde con facilidad, lo que facilita la absorción de agua por los cotiledones (Tizazu and Emire, 2010). El pH de las semillas de lupino es ácido y varía entre 5,5 y 5,8. La variación está relacionada con el incremento de agua en el grano y el contenido de proteínas que amortiguan los cambios de pH (Grochowicz and Andrejko, 2006).

(a) (b)

Figura 1. Planta (a) y semillas (b) de *Lupinus mutabilis* Sweet.

Composición del grano de lupino

Entre las leguminosas, las semillas de lupino son una de las fuentes más ricas de nutrientes (Kohajdova *et al*., 2011). Las semillas presentan altos contenidos de proteína, lípidos, fibra dietética, minerales y vitaminas, cuya proporción está influenciada por la especie (Martínez-Villaluenga *et al*., 2009). Las semillas de *L. mutabilis* Sweet poseen los niveles más altos de proteína y aceite entre todas las especies domesticas de lupino (Santos *et al*., 1997). La combinación de estas características convierte a *L. mutabilis* en

una alternativa potencialmente superior a las fuentes actuales de proteínas y aceites de origen vegetal en la zona andina y otras regiones.

Proteína

Entre las legumbres, los lupinos han sido identificados como rubros prometedores por su contenido y calidad de proteínas, idoneidad para una producción sostenible y beneficios potenciales para la salud (Lucas *et al*., 2015). Hay algunas variedades de lupinos silvestres y parcialmente domesticadas que contienen en promedio 45% de proteína cruda (Petterson, 2016). El valor más bajo lo presenta *L. angustifolius* y el más alto la especie *L. mutabilis.* Dentro de esta especie, la proteína cruda puede llegar hasta 53 g/100 g peso seco. La variación está asociada con factores genéticos y agronómicos. Al respecto, Haq (1993) menciona que la variabilidad genética de *L. mutabilis* ilustra la adaptación de esta especie a diferentes micro-ambientes y la selección natural. El valor dietético de las proteínas de lupino es mayor que el de los frejoles o guisantes, debido principalmente a las altas concentraciones de aminoácidos esenciales (EAA), lisina (Lys), leucina (Leu) y treonina (Thr) (Starkute *et al.*, 2016). Las principales clases de proteínas que se encuentran en las semillas de leguminosas son las globulinas y las albúminas, seguidas de fracciones menores de prolamina y glutelina (Doxastakis, 2000). En el grupo de las globulinas, el interés en las conglutinas ha aumentado exponencialmente desde que se ha demostrado sus propiedades nutricionales y farmacéuticas beneficiosas en la salud cardiovascular y en el control de la resistencia a la insulina y la diabetes, así como las propiedades antiinflamatorias de γ- y β-conglutina (Fornasini *et al*., 2019). La identificación de las propiedades únicas de las proteínas de *L. mutabilis* abre la puerta a nuevos mercados y eleva el valor nutricional y económico del cultivo. En el caso de la α-conglutina se han observado diferencias dentro de genotipos de *L. mutabilis* (Santos *et al*., 1997), lo que sugiere que estas proteínas pueden tener diferentes funciones entre y dentro de las especies de lupino (Carvajal-Larenas *et al*., 2016). La presencia de ferritina (proteína rica en Fe) en el perfil de proteínas del lupino, aumenta el valor nutricional de este cultivo al ofrecer una forma segura de aumentar la ingesta de hierro en la dieta (Lucas *et al*., 2015). Se ha reportado que la digestibilidad aparente de la proteína de *L. mutabilis* es menor (87,1%) que la de caseína (Petterson, 2016) y puede usarse para mejorar la composición nutricional de diferentes productos y la calidad biológica de las proteínas cuando se usa en

combinación con cereales (Jiménez-Martínez and Dávila-Ortíz, 2006). Por lo expuesto, el lupino se encuentra entre las ocho posibles fuentes de proteína vegetal para su uso en alimentos y que podrían reemplazar a las proteínas de origen animal en las dietas (Dijkstra *et al*., 2003).

Grasas y Ácidos grasos

Las especies de lupino varían ampliamente en su contenido de lípidos. Comparado con la soya, las semillas de lupino cultivadas en Europa tienen un contenido más bajo de grasa, la cual varía de 52,2 a 125,8 $g \cdot kg^{-1}$ peso seco (Sedláková *et al*., 2016). El contenido de lípidos varía de 55 $g \cdot kg^{-1}$ peso seco en *L. luteus* a 246,0 $g \cdot kg^{-1}$ peso seco, en *L. mutabilis*, y entre variedades de esta especie, el contenido de lípidos puede variar de 130,0 a 246,0 $g \cdot kg^{-1}$ peso seco (Berti *et al*., 2013). Esta variación puede ser explicada al menos parcialmente por la influencia de factores genéticos y ambientales (De Carvalho, 2005). El aceite de lupino es una fuente rica de ácidos grasos insaturados. Dentro de los cotiledones, la energía se almacena principalmente en forma de gruesas paredes celulares (alrededor de 25%) y cuerpos oleosos (entre 6 a 14%). La energía metabólica varía ligeramente de 20.320 $kJ \cdot kg^{-1}$ peso seco para *L. angustifolius* a 20.780 $kJ \cdot kg^{-1}$ peso seco para *L. albus*, y 21.640 $kJ \cdot kg^{-1}$ peso seco para *L. luteus*. Estos valores son más bajos que aquellos reportados para *L. mutabilis* (23.070 $kJ \cdot kg^{-1}$ peso seco peso seco) (Carvajal-Larenas *et al*., 2016).

Minerales y Vitaminas

La composición de minerales del lupino crudo presenta amplia variabilidad, el contenido de calcio en el grano cultivado en Europa en peso seco varía de 2,1 a 4,7 $g \cdot kg^{-1}$ peso seco, el fósforo de 4,3 a 7,2 $g \cdot kg^{-1}$ peso seco, el magnesio de 1,2 a 2,2 $g \cdot kg^{-1}$ peso seco, el potasio de 8,6 a 11,1 $g \cdot kg^{-1}$ peso seco, y el sodio de 0,1 a 0,2 $g \cdot kg^{-1}$ peso seco (Kouris-Blazos and Belski, 2016). El contenido de minerales ($g \cdot kg^{-1}$ peso seco) de la harina de lupino producido en Australia (posiblemente de la especie *L. angustifolius*) ha sido reportado como sigue: Fe, 49,0; Ca, 840; Zn, 36; Mg, 1.890; y K, 8.100, valores que se encuentran en el rango de otras legumbres y granos de cereales (Kouris-Blazos and Belski, 2016). Sin embargo, De Carvalho (2005) concluyó que las comparaciones

de minerales resultan un tanto complicadas, debido a la influencia potencial del ambiente de producción y el método analítico reportado en la literatura.

Existen escasos datos disponibles sobre los contenidos de vitaminas en las semillas de lupino. Villarino *et al*., (2014) reportaron el contenido de carotenoides (precursores de la vitamina A) de algunas variedades comerciales de *L. angustifolius* cultivado en la misma estación y localidad de Australia Occidental, con un nivel de 20,1 $\mu g \cdot g^{-1}$ peso seco (luteína 7,6; β-caroteno 5,5; zeaxanthina 4,4, and α-caroteno 2,6 $\mu g \cdot g^{-1}$ peso seco) en la variedad Mandelup. El contenido de vitamina E en el aceite de lupino es similar al de soya pero más bajo que el de girasol y colza (Lampart-Szczapa *et al*., 2003). El contenido de tiamina varió de 0,01 a 6 $\mu g \cdot g^{-1}$ peso seco, la riboflavina de 0,2 a 5 $\mu g \cdot g^{-1}$ peso seco y la niacina de 0,0 a 41 $\mu g \cdot g^{-1}$ peso seco. Valores mayores de niacina fueron reportados por Erbas *et al*. (2005).

Fibra dietética

En comparación con otras legumbres, las semillas de lupino contienen fibra cruda beneficiosa para la dieta. La testa constituye el 30% del peso de la semilla en *L. luteus*, 25% en *L. angustifolius*, 15% en *L. albus* y 12% en *L. mutabilis*, esta última especie tiene el contenido de fibra más bajo entre los lupinos. Una proporción importante de la fibra dietética total de la semilla se encuentra formando parte de las paredes celulares de los cotiledones y está compuesta predominantemente por polisacáridos no lignificados, no celulósicos y sin almidón, con un esqueleto rhamnogalactoronano, con cadenas laterales de galactosa y arabinosa (Evans *et al*., 1993). La testa de las semillas de *L. angustifolius* es muy alta en fibra dietética total (~900 $g \cdot kg^{-1}$ peso seco), principalmente fibra insoluble (Kohajdova *et al*., 2011), un 50% de la cual es celulosa, 13% arabinoxilanos y 30% pectinas (White and Staines, 2007). También están presentes pequeñas cantidades de proteínas, lípidos y cenizas (minerales), y la mayor proporción de fitoquímicos de la semilla se encuentra en la testa (Khan *et al*., 2015). El alto contenido de fibra dietética de la testa y la presencia de niveles significativos de minerales y fitoquímicos indican el potencial de este componente como un ingrediente alimentario con alto contenido de fibra, aunque se sabe poco de su efecto fisiológico en el organismo humano.

Fitoquímicos y capacidad antioxidante

Las semillas de lupino contienen cantidades significativas de fitoquímicos, particularmente polifenoles, fitoesteroles y escualeno (triterpeno) en comparación con otros cultivos de leguminosas. En el lupino, los flavonoides predominan sobre los ácidos fenólicos, lo contrario ocurre en el caso de otras especies. Karamać *et al*. (2018) determinaron que las especies *L. luteus* y *L. angustifolius* presentaron el mayor contenido de fenoles totales. Las variedades comerciales de *L. angustifolius* cultivadas en la misma temporada y localidad de Australia Occidental presentan en el grano un contenido de polifenoles totales entre 1,6 y 1,9 $mg \cdot g^{-1}$ peso seco, expresado como equivalentes de ácido gálico (GAE) y una capacidad antioxidante total entre 2,6 y 5,4 μmol Equivalentes Trolox $(TE) \cdot g^{-1}$ peso seco (Karamać *et al*., 2018). La capacidad antioxidante difiere en especies individuales y variedades de lupino. El cultivar Pootalong de la especie *L. luteus* y el cultivar Kalya de *L. microcarpus* y *L. angustifolius*, mostraron una mayor capacidad antioxidante que otras especies y cultivares (Wang and Clements, 2008). En comparación con las otras fracciones de la semilla, la testa de *L. angustifolius, L. luteus* y *L. albus* mostraron mayor contenido de ácidos fenólicos libres (Lampart-Szczapa *et al*., 2003). Otros autores reportaron un promedio de 0,14 mg de $TE \cdot g^{-1}$ peso seco, para la capacidad antioxidante total de la testa del lupino (Ranilla *et al*., 2009).

Almidón

Los lupinos presentan un bajo contenido de almidón, lo cual contrasta con otras leguminosas como los guisantes y garbanzos que presentan entre 50-70% del peso del cotiledón como almidón, con un bajo contenido de proteínas y aceite, y la soya que contiene entre 15-20% de aceite, algo de almidón y un alto contenido de proteínas. Los bajos niveles de almidón y los altos niveles de carbohidratos fermentables de los lupinos los convierten en un alimento deseable para los rumiantes debido al bajo riesgo de acidosis (Sherasia *et al*., 2017).

Oligosacáridos

Los oligosacáridos de la familia de la rafinosa (RFO) (rafinosa, estaquiosa, verbascosa) forman parte de la fracción de fibra dietética, y se presentan en un alto contenido en las semillas de lupino, en comparación con otras legumbres (Villacrés *et al*., 2015). Los oligosacáridos pueden considerarse factores antinutricionales en grandes cantidades, ya que no pueden ser metabolizados por los animales monogástricos y pasar al colon, donde la digestión bacteriana los convierte en dióxido de carbono, metano e hidrógeno. Por otro lado, también se reporta que los oligosacáridos tienen beneficios para la salud debido a su papel como reguladores osmóticos en el tracto gastrointestinal (Petterson, 2016).

Los RFO son conocidos por sus efectos inductores de flatulencia, aunque no hay evidencia directa de este efecto para los oligosacáridos de lupino. Para semillas cultivadas en Australia Occidental, se reportó un contenido total de RFO que varió significativamente dependiendo del cultivar, con niveles más altos en la variedad Mandelup (168 g·kg^{-1} peso seco) y más bajos en PBA Barlock (76 g·kg^{-1} peso seco) (Karnpanit *et al*., 2016). Sin embargo, Villarino *et al*. (2014) no encontró diferencias significativas en los ROF de las variedades comerciales de *L. angustifolius* cultivados en la misma temporada y ubicación, las que presentaron un valor promedio de 50 g· kg^{-1} peso seco (Johnson *et al.*, 2006). Un estudio realizado por Villacrés *et al*. (2015) en *L. mutabilis*, variedad INIAP-450, mostró que la germinación redujo la rafinosa y la estaquiosa en 98% y 18,33% después de 4 días de germinación a 20 ^{o}C.

Factores antinutricionales

En general, los lupinos son considerados como alimentos con bajo contenido de factores antinutricionales, comparados con otras leguminosas incluida la soya. Los lupinos no contienen lectinas (Petterson, 2016). Las saponinas están presentes en bajas concentraciones (0,057% peso seco) en *L. angustifolius* (Gurfinkel and Rao, 2002), mientras que la harina de soya desengrasada presenta 0,58%, los fréjoles blancos 0,32% y la quinua 0,65%. Hay escasa información disponible sobre el contenido y tipos de taninos en las semillas de lupino. El nivel de taninos condensados en la testa de *L. albus, L. angustifolius y L. luteus* varió entre 13-77 mg de catequina equiv·kg^{-1} peso

seco (Lampart-Szczapa *et al*., 2003). Estos valores contrastan con los niveles reportados en la testa de fréjol, los cuales variaron entre 50-350 g de catequina equiv·kg^{-1} peso seco (Mojica *et al*., 2015). Los inhibidores de proteasas están presentes en niveles muy bajos y son de menor importancia en las especies de lupino (Wink, 2006). La actividad de la tripsina en *L. mutabilis* es considerablemente más baja que la determinada en soja (30,1 TIU·mg^{-1} peso seco) (Haq, 1993) y fréjol común (17-51 TIU·mg^{-1} peso seco) (Guillamon *et al*., 2008). También se reportó ausencia de vicina y convicina para las principales especies de lupino (Múzquiz *et al*., 1989). Los fitatos están presentes en todos los granos incluido el fréjol, que almacena estos compuestos en la testa. La cantidad de ácido fítico reportada para *L. albus*, alcanza un promedio de 14,87 mg·g^{-1} peso seco (Sánchez-Chino *et al*., 2015). Con respecto a los fitoestrogenos, se han reportado niveles más bajos en las semillas de *L. albus* (560 mg·kg^{-1} peso seco) en comparación con el rango reportado para la soya, cuyos contenidos varían entre 1,16-2,74 g·kg^{-1} peso seco (Sirtori *et al*., 2004).

Los alcaloides quinolizidínicos son sintetizados por las especies de lupino y son conocidos por que confieren un sabor amargo y toxicidad anticolinérgica. Se sintetizan a partir de la L-lisina en los tejidos verdes de la planta, luego a través del floema son transportados y almacenados en todos los órganos de la planta, especialmente en las semillas (Gulisano *et al*., 2019). Los lupinos pueden ser clasificados en dulces (contenido de alcaloides <2,88 g·kg^{-1} peso seco) y amargos, aquellos con mayores contenidos de alcaloides. La utilización de las semillas de lupinos amargos ha sido limitada por la presencia de los alcaloides quinolizidinicos, tóxicos y de sabor amargo (Jiménez-Martínez *et al*., 2001). Las industrias alimenticias han establecido un umbral estricto de 2,0 g·kg^{-1} peso seco para el contenido de alcaloides del grano listo para el consumo humano (Frick *et al*., 2017; Cowling and Gladstones, 2000). Los granos de los nuevos cultivares domesticados contienen menos de 2,0 g·kg^{-1} de semillas secas. En contraste con los granos amargos de tipo silvestre que todavía existen en muchos países, los cuales registran valores entre 1 a 40 g·kg^{-1} de semillas secas. Los mayores alcaloides de *L. albus* son la lupanina (0,70 g·kg^{-1} alcaloides totales), albine (0,15 g·kg^{-1} alcaloides totales), 13 α-hydroxylupanina (0,80 g·kg^{-1} alcaloides totales) y esparteína (0,30 g·kg^{-1} alcaloides totales). Además del contenido total, la composición cuantitativa también es importante, ya que los alcaloides en forma individual presentan diferente toxicidad y las

especies de lupino difieren en su composición cuantitativa. El contenido de alcaloides de *L. mutabilis* es más alto que el de otras especies de lupino. Los principales alcaloides reportados en esta especie son lupanina, esparteina, 3-hydroxylupanina, 13-hydroxylupanina, and 4-hydroxylupanina (Carvajal-Larenas *et al*., 2016). Los principales efectos tóxicos de los alcaloides son alteraciones del sistema nervioso central, procesos digestivos, reproductivos y del sistema inmune. No se han reportado muertes de seres humanos asociadas con el consumo de granos amargos de lupino silvestre. En realidad, una persona necesitaría ingerir alrededor de 10 kg de una variedad amarga en poco tiempo para absorber suficientes alcaloides que pongan en riesgo su vida. Los alcaloides de la quinolizidina tienen una vida media corta en humanos y son excretados sin mayores cambios en la orina de las personas (>90%).

Procesamiento

Los procesos de desamargado biólogicos, químicos o acuosos pueden reducir el contenido de alcaloides de las semillas de lupino con diferentes resultados, dependiendo de las condiciones de cada proceso. El procesamiento acuoso es el de mayor aplicación, sin embargo, este es ineficiente, debido a los grandes volúmenes de agua utilizados, largos tiempos de proceso y pérdida de los compuestos hidrosolubles del grano, lo que justifica la necesidad de explorar procesos alternativos que incrementen la sostenibilidad del mismo, acelerando la velocidad de difusión de los alcaloides.

Procesamiento químico

El enfoque químico para extraer los alcaloides incluye las siguientes alternativas: (i) extracción con hexano y soluciones básicas, (ii) extracciones básicas y (iii) extracciones mixtas con alcohol. En las extracciones con hexano y soluciones básicas, las semillas de *L. mutabilis* se trituran y se descascarillan, luego se ponen en contacto con hexano y posteriormente con una solución básica. Con este proceso se extrae entre 80% y 96,9% de alcaloides originales y se requieren entre 3 y 24 horas (Erbas, 2010). Las extracciones básicas de alcaloides en *L. campestris y L. mutabilis* reducen el contenido de alcaloides en un 99,9%, requieren menos de un día para semillas enteras y menos de una hora para harina de lupino (90% atraviesa la malla de 100 mesh). Esto podría deberse a que una reducción en el tamaño de partícula del grano (harina) aumenta el

contacto con el agua, lo que facilita la difusión de alcaloides, especialmente a temperaturas elevadas (Jiménez-Martínez *et al*., 2003; Aguilera *et al*.,1983). El etanol mezclado con hexano o con CO_2 también ha sido propuesto para extraer los alcaloides (Nossack *et al*., 2000). En el primer caso, la semilla es descascarillada y triturada, con lo que se logra una reducción de alcaloides en el orden del 97%, en 20 horas. En el último caso, la semilla es molida a un tamaño de partícula entre 70-100 mesh. El proceso se realiza en 20 min y se logra una reducción de 39,8 $g \cdot kg^{-1}$ de semilla seca. Todos los tratamientos químicos requieren equipos e instalaciones adicionales para lograr operaciones seguras y de eliminación de residuos. Los tratamientos químicos pueden dejar residuos, lo que presenta un riesgo adicional para la salud y puede afectar el sabor del producto. Además, se requieren cantidades considerables de agua (24 a 60 veces el peso de la semilla de lupino, o más) (Carvajal-Larenas *et al*., 2016).

Procesamiento Acuoso

Por siglos los campesinos de los Andes han domesticado, cultivado y utilizado el grano de lupino en su alimentación. La técnica empleada para eliminar los compuestos amargos del grano consiste en remojar las semillas en agua a temperatura ambiente (~ 17 ^oC) durante 14 a 20 h, seguido de una cocción de 0,5 a 2 h y posteriormente se lavan en agua corriente durante 4 o 5 días, con lo cual el 98,4% de alcaloides es eliminado del grano. Sin embargo, este proceso presenta como desventajas: prolongado tiempo de proceso (144 $h \cdot kg^{-1}$), grandes volúmenes de agua (193 $L \cdot kg^{-1}$) y baja calidad sanitaria del grano (Villacrés *et al*., 2000).

En base al proceso tradicional, el tratamiento acuoso es el único que se aplica industrialmente para eliminar los alcaloides y usar el grano como alimento humano. Este proceso reduce el contenido de alcaloides en la semilla entera hasta niveles seguros para el consumo humano (ANZFA, 2001), sin cambiar su sabor natural, lo cual es importante cuando el grano se consume como un snack. Sin embargo, se utilizan cantidades significativas de agua, tiempo prolongados y ocasiona pérdidas de los componentes hidrosolubles (Jiménez-Martínez *et al*., 2003). El tratamiento y la reutilización del agua, así como el aumento de la difusión de alcaloides se contemplan como alternativas para mejorar el procesamiento acuoso de *L. mutabilis*.

Procesamiento biológico

Este proceso se basa en el uso de hongos o bacterias para reducir el contenido de alcaloides y no producen residuos químicos significativos. Sin embargo, requieren operaciones preparatorias del grano, tales como descascarillado, molienda, remojo y cocción. El mínimo tiempo requerido es de dos días, usa cantidades sustanciales de agua y energía y es aplicable a semillas con bajo contenido de alcaloides (inferior al 1%) (Agosin *et al*., 1989). Estudios realizados en *L. albus* aplicando *Lactobacillus acidophilus, L. buchneri, L. cellobiosus y L. fermentum*, para lograr la disminución de alcaloides, resultaron en una reducción del 41,1% del valor inicial (Fritsch *et al*., 2015). Desafortunadamente, en este estudio no se incluyó ningún experimento control para evaluar la pérdida de alcaloides por lixiviación. No obstante, ciertas cepas de bacterias son capaces de degradar los oligosacáridos o el ácido fítico, pero no los alcaloides (Fritsch *et al*., 2015). Con las cepas IST20B e IST40D (no se reporta nombre) sobre una suspensión de harina de *L. albus*, se consiguió una reducción del 50% de alcaloides (Santana and Empis, 2001). Aparentemente, los cultivos ácidos con una alta actividad α-galactosidasa causan la mencionada disminución (Santana and Empis, 2001). La aplicación de *R. oligosporus* sobre *L. mutabilis*, descascarillado, remojado y cocido causó una reducción del 9% de alcaloides (Jiménez-Martínez *et al*., 2007). El efecto de *R. oligosporus* sobre *L. albus* fue dependiente del contenido inicial de alcaloides, no observándose ninguna reducción detectable en el lupino amargo (8,0 $g \cdot kg^{-1}$ peso seco) (Agosin *et al*., 1989). La germinación constituye otro de los procesos que se ha aplicado para reducir el contenido de alcaloides. Villacrés *et al*. (2015) reportaron que la germinación de *L. mutabilis* "INIAP-450" redujo el contenido de alcaloides de 36,0 a 26,30 $g \cdot kg^{-1}$ peso seco, equivalente al 72% del valor inicial después de cuatro días de proceso.

Lupino como ingrediente y aplicaciones en alimentos

Granos, hojuelas, grits y harinas

Después del descascarillado, los granos de lupino se muelen y se tamizan a diferentes tamaños de partícula (<150 a >600 μm). Las hojuelas, los grits, las migas y las harinas tienen tamaños de partícula más grandes en comparación con la harina de lupino,

aunque no se han definido estándares para realizar una clasificación por tamaño. Los estándares estadounidenses para la soya requieren que la harina pase a través de la malla Nro. 100 (0,149 mm), mientras que los granos de diferentes tipos pasan a través de la malla Nro.10 (2,0 mm) a la malla Nro.80 (0,177 mm). Las hojuelas de lupino se fabrican mediante un proceso mecánico propio que difiere de la molienda convencional para obtener partículas con geometría similar a la escama (Villarino *et al*., 2015). El tamaño de partícula del lupino molido es un parámetro de calidad importante en las aplicaciones alimentarias. Villarino *et al*. (2015) reportaron que un aumento en el tamaño de partícula de la harina de lupino añadida al pan de trigo podría condicionar el porcentaje de incorporación. Las semillas de lupino, las hojuelas, los grits o la harina se pueden procesar en varias fracciones, para obtener proteínas aisladas, fibra dietética y subproductos solubles en agua (proteínas y oligosacáridos del "suero"). La harina, proteína o fibra del lupino se pueden agregar a los alimentos para mejorar su calidad nutricional (Villarino *et al*., 2016).

Aislados y concentrados proteicos

Los aislados proteicos de lupino son obtenidos por solubilización de la proteína a pH 9,0, y tras eliminar la porción insoluble (fibra dietética) mediante centrifugación, precipitarla en el punto isoeléctrico (pH 4,5) para obtener una fracción rica en globulinas (α- y β-conglutinas). Esta fracción tiene excelentes propiedades emulsionantes pero poca viscosidad y propiedades formadoras de gel que pueden ser necesarias cuando se usa como ingrediente alimentario. Atendiendo a su funcionalidad, las fracciones de proteína de lupino se han categorizado como fracción E (emulsionante) (α-, β-conglutina) y fracción F (espumante) (rica en γ-conglutina) (Burgos-Díaz *et al*., 2016). Los aislados proteicos de lupino se han utilizado en salchichas como un posible sustituto vegetariano de la clara de huevo (Wong *et al*., 2013).

Fracciones de Fibra dietética

Aunque las fracciones de la fibra dietética del lupino podrían ser utilizadas para enriquecer los alimentos en fibra, su aplicación ha sido muy reducida para tal fin. Sin embargo, se ha reportado el uso de la fibra del grano de lupino como un efectivo

reemplazante de la grasa en salchichas (Archer *et al*., 2004). En la elaboración de pastas, la sustitución de harina de trigo con fibra de lupino (sobre el 10%), incrementó significativamente el nivel de fibra dietética sin disminución de su aceptabilidad global (Clark and Johnson, 2002). El suero ácido-soluble que resulta de la precipitación de las proteínas contiene oligosacáridos, con posible actividad prebiótica (estimula el crecimiento de las bacterias del intestino), aunque actualmente no hay evidencia clínica de esta potencial propiedad benéfica (Johnson *et al*., 2017).

Aceite de lupino

El aceite puede ser extraído de las semillas de lupino por extracción acuosa con pre-tratamientos de extrusión, adición de proteasas (Jung *et al*., 2009) o extracción con solventes. Debido al contenido de aceite en *L. mutabilis* (18-24%) comparado con otras especies de lupino, este nutriente puede enmarcarse en un nicho de productos de bajo costo (Dijkink *et al*., 2008). El aceite de *L. mutabilis* presenta un bajo contenido de ácidos grasos saturados y mayor contenido de insaturados, especialmente ácido oleico (48%), ácido linoleíco (28,17%) y ácido linolénico (2,54%). Con base a estos resultados, los investigadores concluyeron que *L. mutabilis* es una buena fuente de aceite para la alimentación humana, dado que contiene γ-tocoferol y entre los esteroles destacaron el campesterol, estigmasterol, β-sitosterol and D-5 avenasterol (Villacrés *et al*., 2013; Berti *et al*., 2013). Por sus características físicas se ha sugerido su uso en preparaciones frías como ensaladas (Villacrés *et al*., 2010; Villacrés *et al*., 2013).

Aplicación del lupino en alimentos a base de cereales para aumentar el contenido de proteína

La harina de lupino se incorpora en alimentos básicos a base de harina de trigo como el pan, galletas, fideos instantáneos y pastas, con el fin de aumentar el contenido de proteína y fibra dietética (Doxastakis, 2000; Villarino *et al*., 2015). En los fideos instantáneos, una sustitución del 20% aumentó la calidad nutricional sin efecto significativo en la cocción y la calidad sensorial (Jayasena and Nasar-Abbas, 2011).

Las principales barreras para el uso de lupino en productos a base de trigo como el pan, son la baja elasticidad de las proteínas de lupino y la alta capacidad de retención de

agua de la fibra dietética de lupino, lo que produce una matriz de gluten menos interconectada, un bajo volumen de pan, textura dura y poco masticable. Para abordar este problema, algunos investigadores utilizaron un enfoque de modelado estadístico para maximizar la tasa de incorporación de harina de lupino en las piezas de pan y determinaron que un reemplazo del 28% mantuvo la aceptabilidad del pan por parte del consumidor (Villarino *et al*., 2016). Otro desafío en las mezclas trigo-lupino es el regusto indeseable, el cual ha sido descrito como herbáceo, metálico, graso, similar al heno, fréjol, carne o queso. Los consumidores han detectado sabores inusuales en productos horneados (Johnson *et al*., 2006; Hall *et al*., 2005), disminuyendo la aceptabilidad del pan con una incorporación superior al 30% y en galletas con una inclusión mayor al 20% (Jayasena and Nasar-Abbas, 2011). El tostado o el calentamiento previo de las semillas de lupino pueden reducir su sabor a " fréjol" y extender la vida útil de la harina al reducir el desarrollo de rancidez (Bartkiene *et al*., 2013). También se ha investigado el uso de masas madre de lupino en la fabricación del pan para enmascarar sabores indeseables (Bartkiene *et al*., 2011). Otros autores investigaron el efecto de la extrusión en los snacks elaborados con grits de lupino y maíz. Los resultados mostraron un aumento del perfil nutricional, sin afectación de las características físicas de los snacks (Manosalvas *et al*., 2019).

Productos horneados, glaseados y pastas

Diversas investigaciones describen la fortificación del pan con ingredientes de legumbres (Sathe *et al*., 1982; Wang *et al*., 2002; Rosell et *al*., 2009). Las leguminosas contienen relativamente más lisina y menos metionina que los granos de cereal; por lo que la incorporación de 10% de harina de lupino a las harinas de trigo mejora su score de aminoácidos entre 40-70%, en relación con la albúmina de huevo, sin afectar a las características de los productos. La inclusión de niveles superiores puede afectar el volumen del pan, lo cual podría contrarestarse adicionando gluten o empleando harinas procedentes de trigo con mayor fuerza. En Australia, algunos fabricantes de pan utilizan harina de la testa de lupino para aumentar el volumen de los panes ricos en fibra. La harina de *L. albus* se puede agregar a la harina de trigo para la elaboración de los panes tradicionales denominados 'hallulla' y 'marraqueta'.

Hasta el 50% de harina de lupino se puede incorporar para obtener una variedad de pasteles y galletas (Petterson, 2016). Otros autores estudiaron el efecto de la harina de lupino y la harina de trigo sarraceno sobre la calidad nutricional y sensorial de pasteles sin gluten (Levent and Bilgiçli, 2011). La harina de lupino aumentó el contenido de proteínas y minerales, mientras que el trigo sarraceno causó un aumento significativo en los contenidos de potasio y magnesio de los pasteles sin gluten. La sustitución de la harina de trigo con harina de lupino contribuyó a la mejora nutricional de los productos elaborados (Faheid and Hegazi, 1991).

Algunos estudios han demostrado una alta aceptabilidad de la pasta enriquecida con harina de *L. angostifolius*; un 15% de sustitutción de sémolas o harinas de trigo duro por harina de *L. albus* mejoró la calidad de la protetína y la apariencia de los fideos sin afectar las cualidades sensoriales (Jayasena and Nasar-Abbas, 2011; Villarino *et al*., 2016). Otros autores desarrollaron una pasta sin gluten con una nueva formulación basada en harina de arroz y lupino (*L. mutabilis*) y determinaron que el lupino contribuía al contenido de proteínas y minerales (Albuja-Vaca *et al*., 2019).

Imitaciones de la leche

El alto contenido de proteínas y fibras del lupino lo hace adecuado para aplicaciones en bebidas en las que se requieren bajas viscosidades. Hickisch *et al*. (2016) estudiaron la influencia del tratamiento térmico intenso (UHT) sobre una imitación de la leche a base de lupino y determinaron que UHT mejoró las propiedades reológicas (viscosidad aparente, área de la curva de histéresis, flujo, módulo de elasticidad) y propiedades de textura (firmeza, consistencia, cohesión e índice de viscosidad) del yogur. Otros tratamientos térmicos como la pasteurización (80 °C, 10 min) y la ultra alta temperatura (UHT) (140 °C, 10 s) sobre la actividad lipoxigenesa (LOX) y propiedades sensoriales de alternativas lácteas a base de proteínas de lupino, revelaron que los tratamientos térmicos no afectaron significativamente el perfil sensorial, la actividad LOX se redujo aproximadamente un 72% después de la pasteurización y un 90% después del tratamiento UHT (Jacobs *et al*., 2016).

Otras aplicaciones: productos fermentados y mejorador del color

Los lupinos también han sido usados experimentalmente para elaborar miso, de similares características al miso de soya. El "Natto" un producto fermentado japonés y la "Shoyu" la tradicional salsa de soya, pueden ser elaborados con lupino". Algunos japoneses elaboran y consumen los mencionados productos elaborados con lupino. Varios miles de toneladas de lupinos han sido usados comercialmente en Indonesia para la producción de tempeh y pequeños batches de miso a escala comercial han sido vendidos en Japón (Petterson, 2016).

Para potenciar el mercado de *L. mutabilis*, es esencial tener en cuenta las preferencias de los consumidores. Cuando los granos enteros de lupino se comercializan como alimento, el color de la cubierta de la semilla se convierte en un rasgo decisivo para la aceptación de una variedad. La testa de *L. mutabilis* es de color blanco y esta característica es atractiva para los consumidores. Según la variedad, el color puede variar de blanco perla a negro sólido, e incluye los colores beige/amarillo, marrón, marrón oscuro y colores intermedios. La mayoría de las semillas tienen una distribución de color secundario en tonos más oscuros que el color primario (Gulisano *et al*., 2019). Los cotiledones desprovistos de la testa son de color crema, mientras que los grits y harinas son de color amarillo, probablemente debido a la presencia de los pigmentos luteína, zeaxantina y β-caroteno (Wang *et al*., 2008). El color amarillo de la harina tiene un atractivo considerable y es una característica valiosa en muchos productos horneados, pastas y fideos (Petterson, 2016).

Aplicaciones y usos potenciales de *L. mutabilis*

El cultivo de lupino constituye una oportunidad importante como una fuente sustancial de proteínas a través de la agricultura de bajos insumos, tanto en los Andes como en otras partes del mundo. Las semillas de *L. mutabilis* representan una fuente importante y versátil de proteínas. Esta especie emerge como un alimento y/o aditivo alimentario saludable, pero sus aplicaciones potenciales van mucho más allá de los alimentos y apuntan a la utilización de toda la planta.

Las semillas desamargadas se pueden consumir directamente como un snack o como ingrediente de muchos productos y comidas. En la región andina se usan tradicionalmente en sopas, estofados, ensaladas o como materia prima para preparar harina, bebidas tipo leche y margarina (Villacrés *et al*., 2006). Los lupinos también tienen importantes aplicaciones como ingredientes alimentarios de varios productos: harina para ser incluida en varios derivados, concentrados y aislados proteicos con propiedades físicas y funcionales específicas, para usarse como sustitutos de la carne, los huevos, como mejoradores del pan, emulsionantes y para aumentar el contenido de nutrientes de varios productos (Carvajal-Larenas *et al*., 2016). Varias investigaciones han permitido determinar que la ingesta de *L. mutabilis* ayuda a reducir los niveles de glucosa e insulina en sangre, lo que representa una alternativa válida para el tratamiento de enfermedades hiperglucémicas (Fornasini *et al*., 2019). El aceite de *L. mutabilis* representa otro producto atractivo tanto para fines nutracéuticos como cosméticos (Carvajal-Larenas *et al*., 2016). Sin embargo, todas estas aplicaciones se ven limitadas por el procesado previo requerido para el desamargado de los granos.

Potencial nutracéutico del lupino

Las compañías farmacéuticas y nutracéuticas consideran que algunos componentes de lupino son estratégicos, ya que presenta componentes para la prevención y terapia de varios estados patológicos, incluido el síndrome metabólico (un nombre colectivo que hace referencia a la aparición simultánea de obesidad abdominal, aumento del nivel de triglicéridos, aumento de la concentración de colesterol HDL, hipertensión e hiperglucemia). Este síndrome es típico de los países desarrollados y parte de las enfermedades de la civilización (Duranti, 2006). Un número limitado de estudios, reportan el efecto de la ingesta de lupino sobre los factores de riesgo del síndrome metabólico como obesidad, resistencia a la insulina, hipercolesterolemia, diabetes mellitus tipo 2 e hipertensión. Actualmente se prosiguen las investigaciones para corroborar los beneficios metabólicos del consumo de lupino (Fornasini *et al*., 2019). Otros componentes que pueden explotarse en el campo médico son los alcaloides, puesto que estos compuestos tienen actividad farmacológica. Sin embargo, es necesario investigación adicional para validar los resultados preliminares, establecer mecanismos de acción, dosis, protocolos y contraindicaciones (Duranti, 2006).

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OBJETIVOS Y PLAN DE TRABAJO

OBJECTIVES

General Objective

Considering the importance of lupin in Ecuador, the research on this crop will contribute to the following sustainable development goals: Zero hunger (2), Good health and well-being (3), Quality education (4), Industry, innovation and infrastructure (9) and Sustainable cities and communities (11). The general objective of the present doctoral thesis was to improve lupin processing regarding process efficiency and nutritional composition, and to validate the applicability of the resulting products on a staple food like bread.

Specific Objectives

1) Evaluate the effect of two debittering processes on the alkaloid content and quality characteristics of lupin (*Lupinus mutabilis* Sweet)
2) Study the kinetics of solid-state fermentation of lupin with *Rhizopus oligosporus* based on nitrogen compounds balance
3) Determine the effect of debittering and solid-state fermentation processes on the nutritional content of lupin (*L. mutabilis*)
4) Determine the impact of debittering and fermentation processes on the anti-nutritional and antioxidant compounds of lupin (*L. mutabilis)*
5) Evaluate the wheat flour replacement with debittered and fermented lupin in breadmaking and physical and nutritional bread features.

WORKING PLAN

For reaching the previous objectives, different chapters were proposed. Each chapter was focused on a particular objective and the working plan led to results that were compiled in scientific publications, whose references are included.

Specific working packages:

1. Effects of two debittering processes on the alkaloid content and quality characteristics of lupin (*Lupinus mutabilis* Sweet)
   Villacrés Elena, Javier Álvarez, Cristina M. Rosell
   Journal of the Science of Food and Agriculture. (2020), 100: 2166–2175.
   DOI:10.1002/jsfa.10240.
2. Kinetics of solid-state fermentation of lupin with *Rhizopus oligosporus* based on nitrogen compounds balance
   Villacrés Elena, Cristina M. Rosell
   Food Bioscience. 2020. Submitted.
3. Effect of debittering and solid-state fermentation processes on the nutritional content of lupin (*Lupinus mutabilis* Sweet)
   Villacrés Elena, María Belén Quelal, Xiomara Jácome, Gabriela Cueva, Cristina M. Rosell
   International Journal of Food Science and Technology, (2020), 55/6, 2589-2598.
   DOI:10.1111/ijfs.14512.
4. Impact of debittering and fermentation processes on the anti-nutritional and antioxidant compounds of *Lupinus mutabilis* Sweet
   Villacrés Elena, María Belén Quelal, Edgar Fernández, Grace García, Gabriela Cueva, Cristina M. Rosell
   LWT. https://doi.org/10.1016/j.lwt.2020.109745
5. Replacing Wheat Flour with Debittered and Fermented Lupin: Effects on Bread's Physical and Nutritional Features
   Villacrés Elena, Paúl Cueva, Milene Díaz, Cristina M. Rosell
   Plant Food for Human Nutrition, 2020. https://doi.org/10.1007/s11130-020-00844-w

CAPITULO 1

Effects of two debittering processes on the alkaloid content and quality characteristics of lupin (*Lupinus mutabilis* Sweet)

Elena Villacrés[1], Javier Alvarez[1], Cristina Rosell[2]

[1]Nutrition and Quality Department, Instituto Nacional de Investigaciones Agropecuarias, INIAP. Ecuador,

[2]Institute of Agrochemistry and Food Technology, IATA, CSIC, Valencia, Spain,

Credit Authors Statement

Credit Roles:

EV: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Roles/Writing - original draft

JA: Formal statistic analysis

CMR: Conceptualization; Investigation; Supervision; Validation; Writing-review and editing.

Abstract

Background**:** The presence of quinolizidine alkaloids (QAs) in the species *Lupinus mutabilis* Sweet limits the expansion of its consumption and use, despite its high protein content. Therefore, the objective of this research was to determine the effect of two thermal treatments, aqueous (ATT) and saline (STT) on QAs and total protein contents, as well as on the texture (fracturability and hardness), the attributes of the visual perception Hue (*H**), Luminosity (*L**) and chromatism (*C**) and grain size in three lupin varieties (INIAP-450, INIAP-451 and Criollo). In addition, the water consumption of each treatment was measured**.** Results: The debittering process with the ATT helped to concentrate the total nitrogen up to 560 g·kg^{-1} dry weight and decreased the grain hardness to 2,037 gf in the Criollo variety, while the chromatic parameters *H** and *C** increased in the three varieties. The STT was more efficient than the ATT in terms of the time required and the volume of water used to reduce the QAs to safe levels for consumption (2.5-3.5 g·kg^{-1} dry weight). The larger diameter of the grain increased 4 mm (average of the three varieties); the luminosity *L** decreased during cooking to a value of 41.49 in the Criollo variety and then increased to 57.42 during grain washing. Conclusions: the STT was advisable for lupin debittering, although the extent of the effect was dependent on the variety.

Keywords: Quinolizidine alkaloids, protein, lupin, fracturability, chromaticity

1. Introduction

The species *Lupinus mutabilis* Sweet, which is native in the Andes, is currently cultivated in Ecuador, Peru and Bolivia, with a certain level of agronomic and agro-industrial technological development. This specie is known to be resistant to adverse conditions, such as pests, diseases, drought and frost (Jacobsen and Mujica, 2006). Lupin has attracted attention not only as feed supplement but also as grain for human food in the current decade, due to its high protein percentage (340-430 g·kg^{-1} dry weight) and acceptable contents of essential amino acids. In addition, the grain contains vitamins, minerals, unsaturated fat, dietary fiber (Ortega *et al*., 2010; Sujak *et al*., 2006) and phytochemical compounds such as total phenolic compounds and flavonoids (Chirinos *et al*., 2013). However, the use of the species *L. mutabilis* has been limited by the presence of toxic substances, mostly quinolizidine alkaloids (QAs) (Jacobsen and

Mujica, 2008). These compounds confer a very bitter taste (Jiménez-Martínez *et al*., 2003) and a certain degree of toxicity (Alessandro *et al*., 2017). In the seeds of the specie *L. mutabilis*, the alkaloid levels range from 30 to 40 $g \cdot kg^{-1}$ dry weight (Gross *et al*., 1988) and a debittering process must be applied to decrease those levels. The debittered lupin seeds are also referred to as sweet lupin when the alkaloid content has been reduced to less than 2.0 $g \cdot kg^{-1}$ dry weight, which is the maximum concentration currently permitted and adequate for safe consumption (Boschin *et al*., 2008). The FAO recommends that seeds cultivated for human consumption should contain alkaloids less than 1.0 $g \cdot kg^{-1}$ wet weight or 3.3 $g \cdot kg^{-1}$ dry weight. Higher content is sensory perceptible, unpalatable and potentially toxic (Gross *et al*., 1983).

In Andean countries, the grain of *L. mutabilis* is debittered by successive washes with water, which reduces the concentrations of these substances to safe levels for consumption (Chirinos, 2015). The artisanal process uses a large volume of water (193 $m^3 \cdot t^{-1}$) and requires a long process time (5 to 7 days), which turns into a substantial economic cost and significant loss of water soluble nutrients such as some vitamins and mineral, flavonoids, monosaccharides and sucrose (Caicedo and Peralta, 2000). Through this process, including hydration, cooking and washing, the alkaloid level of bitter lupins (0.5-40.0 $g \cdot kg^{-1}$ dry weight) is easily decreased to levels that are safe for human consumption (Erbas, 2010). Several authors determined that the cooking and washing time and number of washing stages significantly influence the final alkaloid content of the lupin grain (Erbas, 2010; Serna-Cock *et al*., 2019; Boschin *et al*., 2008). Recently, a mathematical model was generated to improve the artisanal process (Carvajal-Larenas *et al*., 2013). However, this model has not been validated experimentally. The greatest changes in the physical characteristics and chemical composition of the grain occur during the debittering process. Among the most noticeable are the increase in grain size, difference in color and decrease in its hardness (Erbas, 2010). Texture is an important quality aspect of lupin. It is dictated by the structure of the grain that, in turn, depends on an interaction of chemical components and physical forces (Kamizake *et al*., 2018; Aguilera and Rivera, 1992). When legumes are processed thermally, first turgor is destroyed, leading to a loss of crisp succulence. Blanching, cooking and sterilization affect the tissues of vegetables, resulting in a decrease in hardness that is mainly due to changes in cell-wall pectins during heating that led to the formation of soluble pectins by degradation of methylated pectins. The most common debittering method use water at room temperature for washing the grain

after cooking, resulting in more acceptable sensorial properties than either debittering with 0.5% $NaHCO_3$ at room temperature (~25°C) or debittered with hot water (65°C). However, these processes take between 5 to 6 days (Erbas, 2010). In the case of chickpea, some reduction in the hydration time was reported when was soaked in NaCl (Serna-Cock *et al*., 2019). Nevertheless, when applying it to lupin, cooking with 40% NaCl was needed to decrease up to 51.22% of the alkaloids, but the grain had a salty taste and a subsequent treatment of the process effluents was required to remove the high level of NaCl, making the process costly (Lara, 1999). NaCl increases the porosity of the microstructure of the grain, facilitates the penetration of water and the diffusion of the alkaloids, reduces the interactions between the minerals and the pectin and increases the solubility of the protein (Sievwright and Shipe, 1986). Aqueous and alkaline thermal treatments have been tested for debittering processes. In the case of *L. campestris* and *G. max*, the application of an aqueous thermal treatment resulted in 56 % alkaloids decrease after 3 h, whereas in the case of alkaline treatment a greater decrease (76.5 %) has been observed (Jiménez-Martínez *et al*., 2001). The objective of this study was to improve the debittering process by evaluating the critical factors of the process, including temperature, time and the use of sodium chloride (NaCl), and their effects on QA and total protein contents, as well as on the texture (fracturability and hardness) and grain color. These critical factors were evaluated in three different lupin varieties to check the validity of the conditions.

2. Materials and methods

2.1 Materials

The following varieties of *Lupinus mutabilis* Sweet were used: INIAP-450, INIAP-451 and Criollo, which were grown in the Santa Catalina Experimental Station with the geographic location of altitude 3050 m.a.s.l., latitude UTM 9959382 m S, longitude 17 M0772618 m W. INIAP-450 was obtained by selection from a germplasm population introduced in Peru in 1992, with the identification of ECU-2659 (Peralta *et al*., 2010); INIAP-451 variety was obtained by selection through participatory processes from the ECU-2658-2 line (Caicedo *et al*., 2000) and Criollo variety is a native material from Chimborazo province. The grain harvested was threshed and classified in a *Crippen* Mfg. Inc. (Michigan, USA) using sieves with pore sizes of 10-15 mm. For this study, grains with an average diameter of 10-15 mm were used.

The debittering process was performed in a 46 cm diameter x 17 cm high reactor, which was connected to a digital thermostatic immersion circulator (Cole Parmer Polystat, model 01266-02, USA) to control the temperature and velocity of water flow. The samples were subjected to two different debittering treatments (aqueous and saline thermal). Three batches of each process were performed.

2.2 Aqueous thermal treatment (ATT)

The grains were subjected to an aqueous thermal treatment consisting of three stages: hydration (10 h), cooking (1 h) and washing (73 h), and samples were collected along the treatment (Figure 1). The grain was hydrated with stationary water at an initial temperature of 80 °C for 10 h (T1) at 1:3 ratio (grain: water). Cooking was carried out in water at 91°C for 1 h (T2), a 1:3 ratio (grain: water) was used, and a water change was performed after the first 30 min of cooking. The aqueous washing of the grain was performed with a stirring system (10.6 $L \cdot min^{-1}$), maintaining a ratio of 1:15 (grain: water). The first washing step was made at 35 °C for 28 h, with water changes occurring at the following time intervals: 3 h (T3), 3 h (T4), 16 h (T5), 3 h (T6) and 3 h (T7). In the second stage of washing, the water temperature was maintained at 18 °C for 45 h, and the water was replaced at 18 h (T8), 3 h (T9), 3 h (T10), 18 h (T11) and 3 h (T12). The debittering of the grain through the application of the aqueous thermal treatment took 84 h.

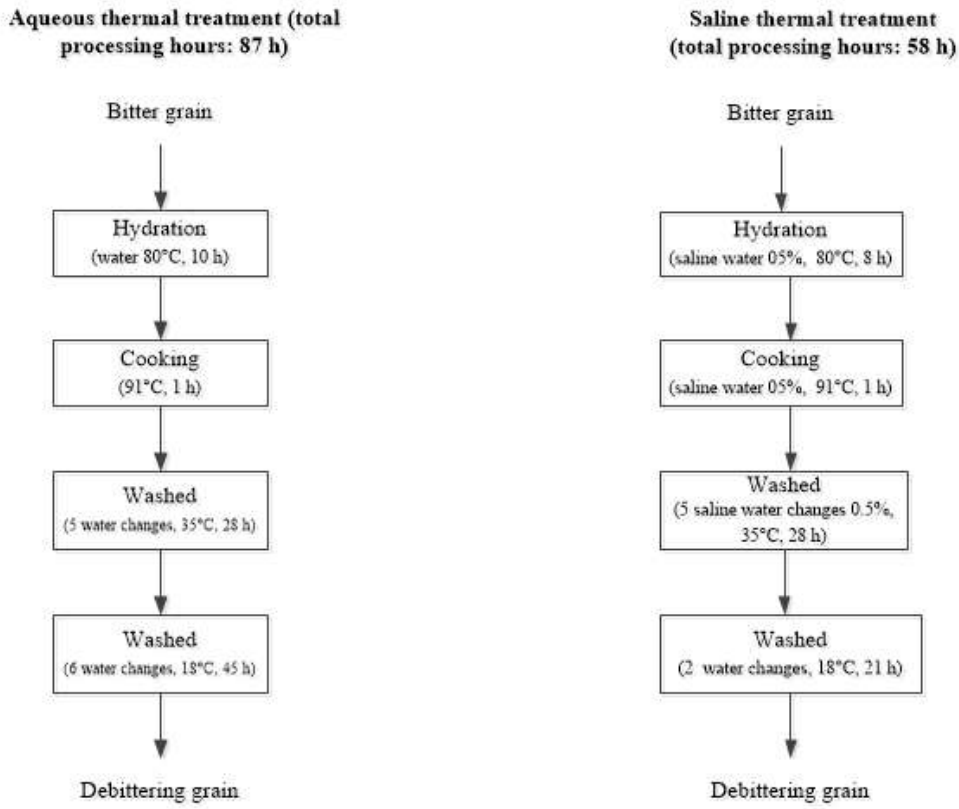

Figure 1. Flowchart of the lupin debittering process with aqueous and saline thermal treatments

2.3 Saline Thermal treatment (STT)

The flowchart of lupin debittering through the application of the saline heat treatments is shown in Figure 1. The saline heat treatment was performed by the addition of 0.5% (w/v) sodium chloride to the water used for hydration, cooking and first wash of the grain at 35 °C (Figure 1). Hydration was performed at an initial temperature of 80 °C for 8 h (T1s), and a 1:3 ratio (grain: saline water) was used in a steady state (without agitation). Then, the grain was cooked at 91 °C for 1 h (T2s) using a 1:3 ratio (grain: saline water); a water change was performed after the first 30 min of cooking. Grain was washed with saline water using a stirring system (10.6 $L \cdot min^{-1}$). In the first 6 h of washing at 35 °C, a ratio of 1:15 (grain: saline water) was used, and for the following washes, the proportions 1:5 and 1:7.5 (grain: saline water) were used. Saline water changes and sampling were performed at the following time intervals: 3 h (T3s), 3 h (T4s), 16 h (T5s), 3 h (T6s) and 3 h (T7s). Afterwards, the saline water was replaced with water at 18 °C to remove the NaCl retained in the grain; then, two water changes

were implemented: 18 h (T8s) and 3 h (T9s). The debittering of the grain through the application of the saline thermal treatment took 58 h.

2.4 Analytical methods

2.4.1 Total nitrogen content

The total nitrogen content was determined according to the standard method 955.04 from AOAC (AOAC, 2005).

2.4.2 Total alkaloid content (QAs)

The method described by Gross *et al*. (1988) was used to determine the total alkaloid contents, with some modification of the titration. Five milliliters of 0.01 N sulfuric acid and two drops of methyl red were added to the concentrated chloroform extract, and the acid excess was titrated with 0.01 N NaOH. For the calculation, 1 mL of 0.01 N H_2SO_4 was equivalent to 2.48 mg of lupanin. The total alkaloid content was reported as the lupanin content (Schoeneberger *et al*., 1983), which is the most abundant alkaloid (55-66% of the total alkaloids) in *L. mutabilis* specie (Frick *et al*., 2017).

2.4.3 Fracturability and hardness

Fracturability and hardness were measured using a previously reported method (Paredes-Pardo, 2012). The grains were compressed in a texture analyzer (texturometer TA-XT2i, Micro Systems, Godalming, UK) with a load cell of 5.00 kg and a stainless-steel cylindrical probe with a diameter of 5 mm (P5) at a speed of 1 $mm \cdot s^{-1}$. The grains were compressed in two cycles up to 50% of their initial height, with an interval of 3 s between each cycle. The strength of the first significant break in the positive area of the first bite defines fracturability. The hardness is the strength of the peak during the first compression cycle (Sahin and Sumnu, 2006).

2.4.4 Color measurement

Color is a quality parameter, particularly when lupin is used as egg substitute. Color measurements were performed using a Portable Spectrophotometer (Lange Spectro-Color d/8° model LZM 268, Chelmsford, United Kingdom) based on the CIE *L*, a*, b** color system. The following attributes of visual sensation were measured: *L** (luminosity), *C** (chromatism) and *H** (hue). The color differences between the raw and

processed lupin seeds were calculated using the following equation: $\Delta E = (\Delta L^{*2} + \Delta C^{*2} + \Delta H^{*2})^{0.5}$, where ΔL^*, ΔC^* and ΔH^* are the differences in the attributes of the visual perception between the raw and processed seeds (Özdemir and Devres, 2000).

2.4.5 Grain size

The larger and smaller diameters of the grains subjected to different treatments were measured with a *Mitutoyo* digital pachymeter (Suzano, Brazil).

2.4.6 Hydration capacity

One hundred grains of lupin with an average diameter of 10-12 mm were weighed and then soaked in water for 8 to 10 h. The surface liquid was drained, and the grains weighed again. The initial weight was subtracted from this value and the amount of embedded water was calculated. Hard seeds do not imbibe water as fast as normal seeds; their weight at the end of soaking does not change significantly and they float in the hydration water. Normal seeds swell and descend to the bottom of the soaking container. Floating grains are counted and the percentage of hydrated grains is established by calculating the difference. This parameter allows know the ability of lupin seeds to rehydrate so that the cooking is more uniform (Williams *et al*., 1988).

2.5 Statistical analysis

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

3. Results and discussion

3.1 Quinolizidine alkaloids

The quinolizidine alkaloids (QAs) contents of the grain gradually decreased as a function of the processing stages and washing time (Figures 2 and 3). In the aqueous

thermal treatment (ATT), after hydration (T1) the content of QAs in the varieties INIAP-450, Criollo and INIAP-451 decreased by 39.8, 35.7 and 29.6 $g \cdot kg^{-1}$ dry weight in relation to raw grain, which was due to the water solubility of the QAs in the seeds, in which the QAs are present in the form of salts that are solubilized in polar solvents such as water (Jiménez-Martínez *et al*., 2001). The initial temperature of the soaking water (80 °C) accelerated its penetration in the grain, requiring 10 h to hydrate 98% of the seeds, compared to 14 h that are typically applied in the artisanal debittering process (Caicedo and Peralta, 2000). In the first stage (T1s) of STT, 98% of the grains were hydrated after 8 h of soaking and the decreases in the QAs contents of the seeds were 34.8, 41.7 and 33.4 $g \cdot kg^{-1}$ dry weight in INIAP-450, INIAP-451 and Criollo, respectively. This decrease is attributed to the NaCl, which increases the porosity of the microstructure of the grain, facilitates the penetration of water and the diffusion of the alkaloids (Sievwright and Shipe, 1986). The osmoactive effect of sodium chloride may have favored the countercurrent mass transfer between grain tissues and the saline solution (Abril and Casp, 2003). Cooking contributed to a greater elimination of the QAs. In the cooking stage (T2) of ATT, the QAs contents of INIAP-450, INIAP-451 and Criollo decreased by 19.3, 20.25 and 11.30%, while in the STT, after cooking (T2s) the QAs contents were reduced to an even greater extent (27.04, 22.79 and 27.02%). During the washing stage, a progressive reduction in the QAs contents was observed in the three varieties evaluated. In ATT, the QAs contents were reduced by 76.66, 75.58 and 72.72% after 28 h of washing the grain at 35 °C (T3-T7). However, with the saline heat treatment (T3s-T7s), at the same washing time a greater reduction in the QAs contents (80.31, 78.88 and 77.05%) was registered in the three varieties (INIAP-450, INIAP-451 and Criollo, respectively).

These results were affected significantly ($P<0.05$) by the processing stage and variety, likely due to the increase in the ionic strength resulting from the dissociation of NaCl, which favors a greater diffusion of the QAs in the extraction medium (Vegas *et al*., 2017).

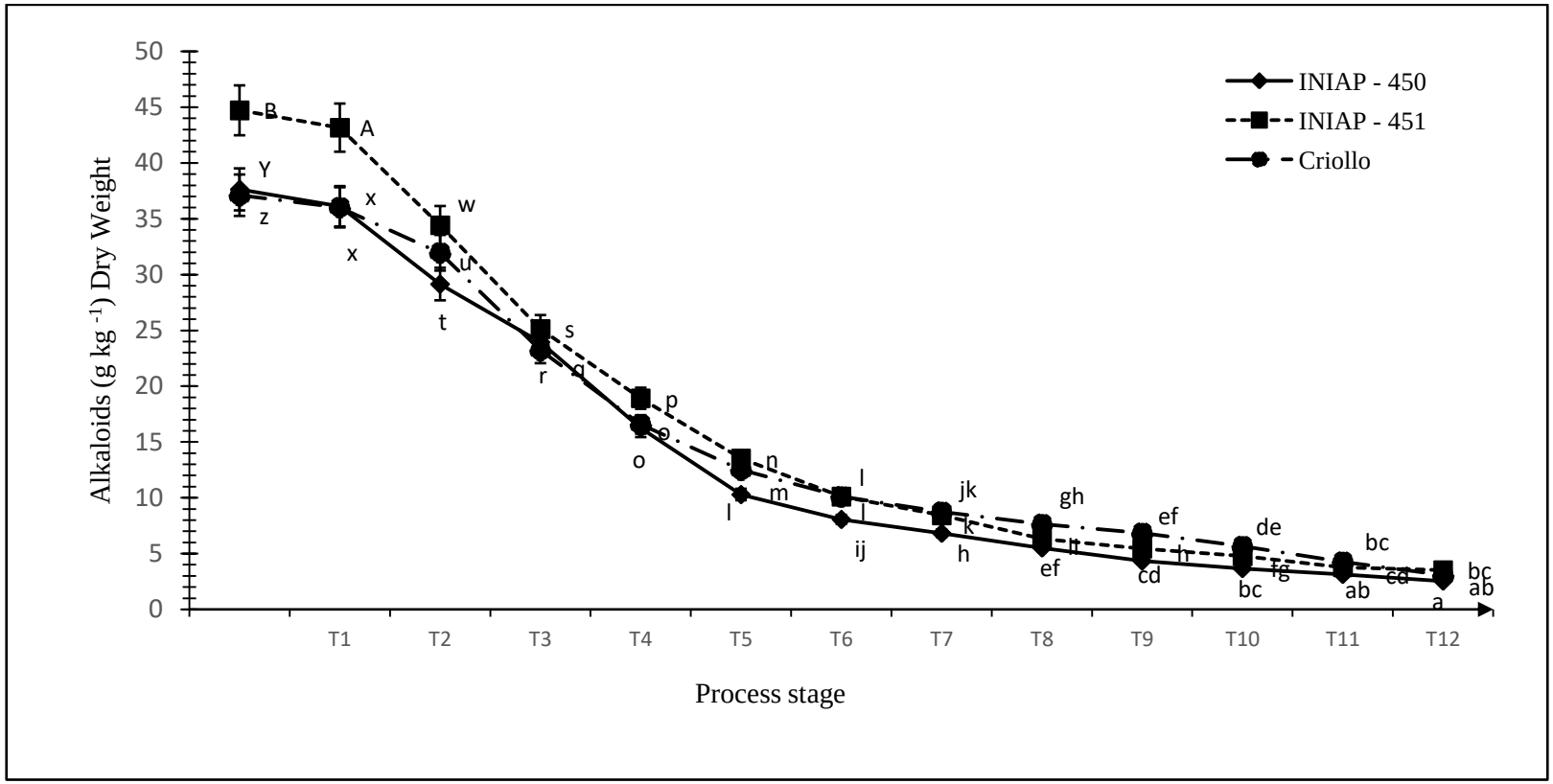

Figure 2. Variation in the total alkaloid content of lupin grain during the aqueous thermal treatment (ATT)

T1 = hydration (80°C, 10 h); T2 = cooking (91°C, 1 h); T3 = washed (35°C, 3 h); T4 = washed (35°C, 3 h); T5 = washed (35°C, 16 h); T6 = washed (35°C, 3 h); T7 = washed (35°C, 3 h); T8 = washed (18°C, 18 h); T9 = washed (18°C, 3 h); T10 = washed (18°C, 3 h); T11 = washed (18°C, 18 h); T12 = washed (18°C, 3 h)

Values followed by different letters within and between columns indicate significant differences ($P < 0.05$). Means ± standard deviations (n = 3).

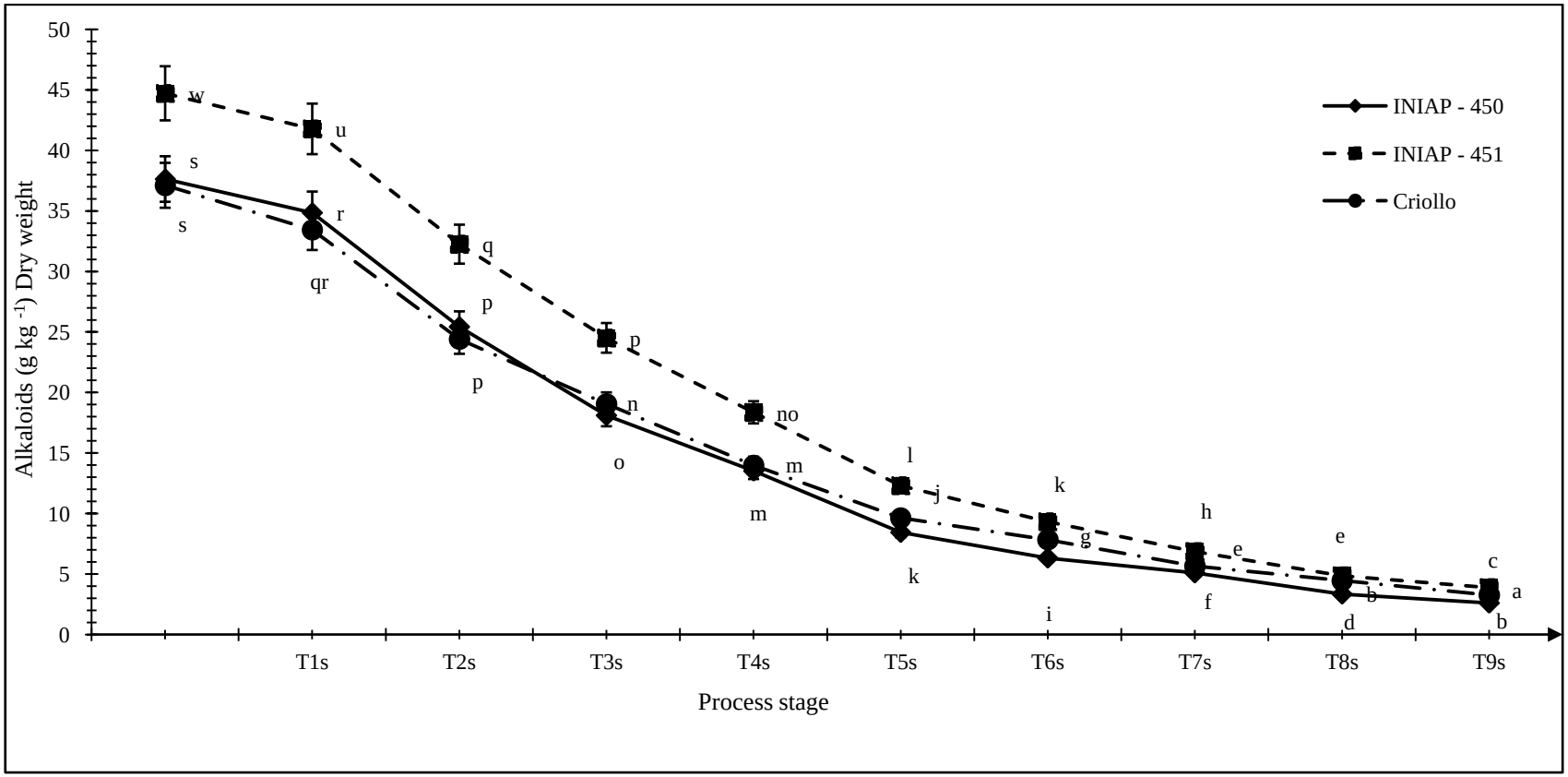

Figure 3. Variations in the total alkaloid content of lupin grain during the saline heat treatment (STT)

T1s = hydration (80°C, 8 h); T2s = cooking (91°C 1 h); T3s = washed (35°C, 3 h); T4s = washed (35°C, 3 h); T6 = washed (35°C, 16 h); T6s = washed (35°C, 3 h); T7s = washed (35°C, 3 h); T8s = washed (18°C, 18 h); T9s = washed (18°C, 3 h)
Values followed by different letters within and between columns indicate significant differences ($P<0.05$). Means ± standard deviations (n = 3)

The alkaloids reduction reached after hydration, cooking and washing stages (T1-T7) of ATT process in INIAP-450, INIAP-451 and Criollo was 81.87, 81.14 and 76.46%, respectively). In the same stages (T1s-T7s) of the STT, the reduction percentages were 86.49, 84.69 and 84.77% in INIAP-450, INIAP-451 and Criollo, respectively. After washing (T7) at 35 °C, a decrease in grain hardness was observed; therefore, the temperature of the washing water was decreased to 18 °C for the last washing stage that lasted 45 h in the aqueous thermal treatment (T8-T12) and 21 h in the saline heat treatment (T8 and T9). In the saline heat treatment, the final wash was performed with water alone to facilitate the removal of the sodium chloride retained in the grain. The alkaloid content of the seeds was affected by the treatment stage and the grain variety ($P<0.05$). In both treatments, after washing at 18 °C, the residual QAs content ranged from 2.5-3.5 $g \cdot kg^{-1}$ dry weight (0.25-0.35% dry weight), a level considered safe for human consumption, as established by FAO (Schoeneberger *et al*., 1982). The debittering process for 1 kg of grain with ATT required 87 h and 96 L of water, with 11 changes, representing a saving of 97 L compared with the artisanal process (Caicedo and Peralta, 2000). The STT was performed in 58 h and 66 L of water was used, with seven changes, which represents a decrease of 26 h in the processing time and a saving of 30 L in the volume of water used compared with the ATT and 127 L with respect to the artisanal process that uses 193 L from rivers or watersheds. In STT, the total volume of water used was similar to that obtained through mathematical modeling (Carvajal-Larenas *et al*., 2015), however, the time required to reduce the QAs content was reduced considerably compared to that study, which mentions 4.4 days as total process time.

3.2 Total nitrogen content

During the debittering process, the chemical composition of the grain in the native state was modified following the application of the two treatments (ATT and STT) (Table 1). The total nitrogen content increased slightly in the three varieties of grain during hydration, which was attributed to the reduction in the content of water-soluble compounds (some vitamins, mineral, flavonoids, monosaccharides and sucrose (Caicedo and Peralta, 2000), including QAs.

Table 1. Variations in the total nitrogen content of the lupin grain* subjected to the aqueous thermal and saline heat treatments

Aqueous thermal treatment stages	INIAP-450	INIAP-451	Criollo	Water used (L)	Saline heat treatment stages	INIAP-450	INIAP-451	Criollo	Water used (L)
Raw grain	75.35 ± 0.22^{wx}	74.22 ± 0.59^{xy}	77.41 ± 0.48^{tu}		Raw grain	75.35 ± 0.22^{q}	74.22 ± 0.59^{r}	77.41 ± 0.48^{no}	
T1	80.13 ± 0.05^{qr}	79.75 ± 0.09^{r}	82.83 ± 0.14^{no}	3	T1s	78.63 ± 0.23^{m}	77.93 ± 0.23^{mn}	80.27 ± 0.23^{kl}	3
T2	74.99 ± 0.14^{xy}	73.59 ± 0.09^{y}	76.58 ± 0.09$^{\mu w}$	3	T2s	72.10 ± 0.23^{s}	72.33 ± 0.23^{s}	77.47 ± 0.23^{no}	3
T3	81.90 ± 0.23^{op}	82.83 ± 0.47^{mn}	87.83 ± 0.14^{fg}	15	T3s	73.64 ± 0.09^{r}	81.20 ± 0.23^{jk}	82.74 ± 0.09^{i}	15
T4	79.57 ± 0.23^{r}	81.67 ± 0.23^{op}	86.10 ± 0.47^{i}	15	T4s	75.37 ± 0.23^{q}	84.47± 0.23^{gh}	85.63 ± 0.47^{def}	15
T5	81.43 ± 0.23^{op}	83.77 ± 0.47^{lm}	87.73 ± 0.23^{fgh}	7.5	T5s	77.93 ± 0.47^{mn}	86.80 ± 0.70^{ab}	87.41 ± 0.09^{a}	5
T6	82.13 ± 0.47^{no}	84.47 ± 0.23^{kl}	88.67 ± 0.23^{cde}	7.5	T6s	76.77 ± 0.23^{op}	84.93 ± 0.23^{fgh}	86.33 ± 0.23^{bcd}	5
T7	81.90 ± 0.70^{op}	78.40 ± 0.23st	88.20 ± 0.23^{efg}	15	T7s	76.30 ± 0.23^{pq}	84.19 ± 0.05 ^{h}	85.87 ± 0.23^{cde}	5
T8	84.70 ± 0.23^{k}	79.10 ± 0.47^{rs}	88.71 ± 0.05^{cde}	7.5	T8s	80.03 ± 0.23^{l}	85.26 ± 0.09^{efg}	86.66 ± 0.09^{abc}	7.5
T9	85.63 ± 0.23^{ij}	80.97 ± 0.47^{pq}	88.95 ± 0.05^{bcd}	7.5	T9s	81.43 ± 0.23^{j}	85.59 ± 0.05^{def}	87.27 ± 0.23^{a}	7.5
T10	86.10 ± 0.23^{i}	84.93 ± 0.23^{jk}	89.18 ± 0.09^{abc}	5					
T11	87.03 ± 0.23^{h}	87.73 ± 0.23^{fgh}	89.41 ± 0.05^{ab}	5					
T12	87.50 ± 0.23^{gh}	88.39 ± 0.05^{def}	89.69 ± 0.05^{a}	5					
P-value	Variety (V)	< 0.0001			P-value	Variety (V)	< 0.0001		
	Treatment stages (TS)	< 0.0001				Treatment stages (TS)	< 0.0001		
	Interaction V*TS	< 0.0001				Interaction V*TS	< 0.0001		

* Percentage based on g·kg^{-1} dry weight. Values followed by different letters within a column indicate significant differences (P< 0.05). Means ± standard deviations (n = 3).

The thermal treatment also improves nutritional value through conversion of native protein into more digestible denatured forms (Prachayawarakorn *et al*., 2004). During cooking, a decrease in the total nitrogen was recorded compared with the hydrated grain. Probably in the cooking process at 91^{o}C globular proteins are not completely denatured, leaving a fraction with the capacity to imbibe water, which is linked to a decrease in the vulnerability to enzymatic attack and to lower digestibility (Belitz *et al*., 1997). Comparing both treatments, the greatest loss was registered with the application of STT in INIAP-450 (8.30%) ($P<0.05$), due to an increase in the solubility of the protein nitrogen induced by a reduction in the number of the electrostatic protein-protein interactions (Belitz *et al*., 1997). When the cooked grain was washed, in both treatments a gradual increase in the total nitrogen concentration was recorded in the three varieties. At the debittering end point, the Criollo variety showed the highest total nitrogen content ($P<0.05$) following the application of ATT (89.69 g·kg^{-1} dry weight) and STT (87.27 g·kg^{-1} dry weight). This increase was attributed to the elimination of some carbohydrates, minerals and QAs during water changes (Jiménez-Martínez *et al*., 2001), while other nutrients, such as protein, remained inside the grain increasing their proportion.

3.3 Fracturability and hardness

Fracturability and hardness are properties that influence the consumer's acceptance of lupin; because of that they were monitored during the debittering process. The greatest effect on the mentioned properties was observed in the cooking stage of ATT and STT treatments (Table 2). Cooking induces the largest alteration in structure and concomitantly in texture. Heating thus allows cells to separate resulting in a softening of the grain. Cell separation has been reported at 76 ^{o}C for soaked lima beans (Aguilera and Rivera, 1992). Heating and soaking also produce changes in the cell inclusions. Protein bodies do not appear to be disrupted, however, deviations from the normal spherical structures were observed, perhaps due to swelling (Jackson and Varriano-Marston, 1981; Varriano-Marston and Jackson, 1981).

The cooking of dried legumes and cereals in a humid environment leads to water migration and simultaneous softening of the grain by degrading the cotyledon tissues in their individual cells, as well as a decrease in the levels of some carbohydrates and an

Table 2. Variations in lupin grain texture parameters following aqueous thermal and saline heat treatments

Aqueous thermal treatment stages	Fracturability (gf)			Hardness (gf)			Saline heat treatment stages	Fracturability (gf)			Hardness (gf)		
	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo		INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo
T1	467±3^{b}	470±4^{b}	462±3^{a}	3,740 ± 160^{i-k}	4,265 ± 359^{k}	4,090 ± 252^{jk}	T1s	470±5^{c}	467± 4^{bc}	462 ±1^{b}	4,367 ± 376 ^{g}	4,137 ± 30 ^{g}	3,421±283^{f}
T2	460±1^{a}	460±2^{a}	459±6^{a}	2,794 ± 320^{b-h}	2,645 ± 301^{a-f}	2,876 ± 329^{b-h}	T2s	462±2^{a}	461 ±2^{a}	459 ± 1^{a}	2,720 ± 456^{a-e}	2,786 ± 313^{a-f}	2,182±236^{a}
T3	461±1^{a}	461±3^{a}	460±1^{a}	3,111 ± 153^{b-h}	2,947 ± 98^{b-h}	3,031 ± 288^{b-i}	T3s	462±2^{a}	462 ±2^{a}	460 ±2^{a}	2,684 ± 514^{a-e}	2,804 ± 288^{a-f}	2,489±238^{a}
T4	459±2^{a}	460±2^{a}	459±3^{a}	3,092 ± 324^{b-i}	2,695 ± 252^{c}	2,501 ± 348^{ab}	T4s	462±2^{a}	462 ±1^{a}	461 ± 1^{a}	2,568 ± 274^{a-e}	2,859 ± 196^{a-f}	2,642 ± 163^{a-e}
T5	459±2^{a}	460±1^{a}	461±1^{a}	3,296 ± 353^{d-i}	2,764 ± 234^{a-g}	2,752 ± 402^{a-h}	T5s	462±2^{a}	462 ±1^{a}	461 ±2^{a}	3,037 ± 309^{c-f}	2,954 ± 258^{b-f}	2,893 ± 332^{b-f}
T6	460±2^{a}	460±1^{a}	460±2^{a}	3,342 ± 573^{e-i}	2,884 ± 258^{bc}	2,788 ± 353^{b-h}	T6s	461±1^{a}	461 ± 3^{a}	461 ±2^{a}	2,431 ± 29^{a-d}	2,646 ± 262^{a-e}	2,346 ± 158^{ab}
T7	460±2^{a}	458±2^{a}	460±1^{a}	3,110 ± 252^{b-i}	2,786 ± 304^{b-h}	2,628 ± 228^{a-e}	T7s	461 ± 1^{a}	461 ±2^{a}	460±2^{a}	2,404 ± 159^{a-c}	2,570 ± 290^{a-e}	2,321 ± 388^{ab}
T8	460±2^{a}	458±2^{a}	461±2^{a}	3,256 ± 321^{c-i}	3,415 ± 241 h^{ij}	2,798 ± 194^{b-h}	T8s	462 ±2^{a}	461 ±3^{a}	461±1^{a}	2,755 ± 351^{a-f}	2,895 ± 128^{b-f}	2,461 ± 456^{a-d}
T9	460±0.2^{a}	460±2^{b}	462±2^{a}	3,360 ± 385^{f-i}	3,433 ± 350 h^{ij}	2,812 ± 133^{b-h}	T9s	462 ±1^{a}	461 ± 2^{a}	463±1^{a}	3,085 ± 216^{def}	3,156 ± 460^{ef}	2,704 ± 422^{a-e}
T10	460± 2^{a}	460±2^{a}	462±1^{a}	3,365 ± 710 g^{-i}	3,433 ± 216 h^{ij}	2,868 ± 271^{b-h}							
T11	461±2^{a}	459±1^{a}	462±1^{a}	3,343 ± 351^{e-i}	2,602 ± 197^{a-d}	2,560 ± 381^{abc}							
T12	460±2^{a}	458±2^{a}	462±1^{a}	2,965 ± 352^{b-h}	2,592 ± 426^{a-d}	2,038 ± 83^{a}							
Variety (V)	0.723			< 0.0001			Variety (V)	0.000			< 0.0001		
Treatment stages (TS)	< 0.0001			< 0.0001			Treatment stages (TS)	< 0.0001			< 0.0001		
Interaction V*TS	< 0.0001			< 0.0001			Interaction V*TS	0.0044			0.0812		

gf = grams of force. Values followed by different letters within a column indicate significant differences ($P < 0.05$). Means ± standard deviations (n = 3).

Increase of the fiber (Prachayawarakorn *et al*., 2004; Linares *et al*., 2005). Following the application of the two evaluated treatments (ATT and STT), the fracturability did not exhibit significant changes during the washing of the grain at 35 °C and 18°C, while the hardness of the three varieties decreased during the washing stage with respect to the hydrated grain, likely due to the increase in water activity (Erbas, 2010). The hardness of the Criollo variety was reduced to 2,038 gf when applying the ATT treatment, resulting in a softer grain at the end of the process. A similar variation was detected in grains subjected to STT, along with a progressive decrease in hardness up to a value of 2,182 gf during grain washing at 35 °C and 18 °C (T8s and T9s). Therefore, the water activity of the seeds increased, whereas the hardness decreased.

3.4 Color of the grain

In addition to the texture, the color of lupin influences the preference of the consumers and their intent to purchase. Tables 3 and 4 show the statistically significant impact ($P< 0.05$) of the variety and treatment stage on grain color. Following the application of ATT, the luminosity (L^*) value ranged from 50.51 to 61.47, while after STT, the variation was higher (41.50-61.47). The darkening of the *Lupinus mutabilis* seeds is attributed to the formation of brown pigments, as a result of Maillard reaction during the cooking, besides to the loss of flavonoid and carotenoids pigments (Erbas, 2010). A decrease in L^* was reported after baking barlotto beans, chickpeas, faba beans, white beans and during processing of soybeans with fluidized superheated-steam (Prachayawarakorn *et al*., 2004). Other authors attribute the reduction in color or darkening of the beans to the coagulation of the proteins (Güzel and Sayar, 2012). The chroma or color level (C^*) was increased during the hydration, cooking and washing of the grain at 35 °C and 18 °C compared with the raw grain. Similar results were obtained with the application of ATT and STT. The attribute that differentiates the color, Hue (H^*) also increased with cooking and washing. Debbitering of the grain with the application of STT caused greater color difference ($\Delta E = 5$ units) in INIAP-451, when the raw and processed seeds were compared. In this case, possibly the browning was partially inhibited by a low concentration of NaCl (Kwak and Lim, 2004).

Table 3. Color variations of lupin grain subjected to aqueous thermal treatment

Aqueous thermal treatment	*L**			*C**			*H**			ΔE		
stages	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo
Raw grain	61.47 ± 3.74^{a}	60.85 ± 1.85^{ab}	58.96 ± 1.86^{abc}	9.18 ± 0.75^{j}	10.51 ± 0.694^{j}	10.88 ± 0.68^{j}	81.47 ± 1.69^{mn}	80.587 ± 1.83^{n}	80.71 ± 0.64^{n}			
T1	56.76 ± 3.81^{a-d}	53.00 ± 2.58^{d-g}	50.51 ± 4.47^{g}	16.79 ± 3.41^{i}	21.221 ± 6.087^{a-h}	17.75 ± 4.49^{hi}	84.26 ± 2.50^{k-n}	81.755 ± 1.77^{mn}	82.81 ± 1.73^{lmn}	10.36±3.28^{h}	13.68 ±6.03^{c-h}	12.27±3.55^{gh}
T2	56.91 ± 2.84^{a-d}	54.93 ± 2.96^{c-g}	53.303 ± 3.28^{d-g}	21.94 ± 1.82^{a-g}	22.515 ± 2.429^{a-d}	22.94± 2.16^{abc}	91.08 ± 1.35^{ab}	90.105 ± 1.64^{abc}	91.31 ± 2.00^{a}	16.91±1.38^{a-c}	16.81 ±1.72^{a-d}	17.45 ±1.79^{a}
T3	54.22 ± 1.71^{c-g}	54.66 ± 2.78^{c-g}	52.961 ± 4.60^{d-g}	19.06 ± 2.27^{d-i}	24.265 ± 2.089^{ab}	21.79± 1.50^{a-g}	89.91 ± 1.53^{abc}	87.935 ± 2.14^{a-j}	89.99 ± 2.48^{abc}	15.15±1.22^{a-g}	17.13 ±1.82^{ab}	16.31±1.43^{a-e}
T4	53.26 ± 3.16^{d-g}	54.39 ± 1.71^{c-g}	54.48 ± 2.12^{c-g}	19.61 ± 1.61^{c-i}	24.688 ± 2.861^{a}	22.44± 1.34^{a-e}	89.52 ± 2.07^{a-d}	87.951 ± 1.55^{a-j}	89.48 ± 2.12^{a-d}	15.96 ±1.19^{a-f}	17.42 ±2.48^{a}	15.47 1.08^{a-g}
T5	56.20 ± 3.03^{a-e}	55.13± 2.60^{c-g}	53.893 ± 3.18^{c-g}	20.07 ± 1.03^{c-i}	21.802 ± 1.268^{a-g}	21.80 ± 2.33^{a-g}	89.30 ± 1.55^{a-f}	86.825 ± 2.26^{c-k}	89.16 ± 2.44^{a-g}	14.78±0.76^{a}-g	14.46 ±1.54^{a-g}	15.28 ±1.52^{a-g}
T6	54.51 ± 4.32^{b-e}	55.56 ± 2.85^{b-g}	53.57 ± 4.06^{d-g}	18.62 ± 1.27^{f-i}	20.173 ± 2.015^{c-i}	21.59 ± 2.33^{a-g}	89.33 ± 1.87a-f	89.047 ± 2.05^{a-g}	87.88 ± 2.50^{b-j}	14.75±1.84^{a-g}	14.38 ±0.95^{a-g}	14.79 ±1.37^{a-g}
T7	56.07 ± 3.81^{c-fg}	52.75 ± 2.05^{d-g}	54.55 ± 3.08^{c-g}	18.59 ± 2.09^{f-i}	20.280 ± 1.862^{c-i}	20.06 ± 1.94^{c-i}	89.73 ± 2.40^{a-d}	86.458 ± 1.62^{d-k}	89.37 ± 1.72^{a-f}	14.38±1.21^{a-g}	14.27 ±1.16^{a-g}	13.86 ±1.22^{b-g}
T8	55.27± 2.76^{c-g}	53.87 ± 1.99^{c-g}	56.48± 2.93^{a-d}	18.19 ± 1.96^{ghi}	22.255 ± 1.877^{a-f}	22.06 ± 1.28^{a-f}	88.61 ± 1.18^{a-i}	85.800 ± 1.77 $^{g-l}$	88.85 ± 1.58^{a-h}	13.44±1.35^{e-h}	14.88 ±1.50^{a-g}	14.40 ±1.37^{a-g}
T9	55.12 ± 3.59^{c-g}	53.54 ± 2.16^{d-g}	50.74 ± 4.01 ^{fg}	19.83 ± 1.97^{c-i}	21.145 ± 2.475^{a-h}	21.55± 1.73^{a-g}	87.98 ± 2.53^{a-j}	85.985 ± 1.80^{f-l}	85.25 ± 2.49^{i-l}	14.63±1.82^{a-g}	14.31 ±1.92^{a-g}	14.79 ±2.59^{a-g}
T10	54.51 ± 3.72^{d-g}	53.25 ± 1.82^{c-g}	54.73 ± 2.72^{c-g}	20.02 ± 2.34^{c-i}	23.143 ± 2.548^{abc}	22.44± 1.49^{a-e}	88.64 ± 1.75^{a-i}	85.538 ± 0.99^{h-l}	87.37 ± 2.40^{c-k}	15.30±1.87^{a}-g	15.80 ±1.49^{a-f}	14.46 ±1.04^{a-g}
T11	56.52 ± 2.63^{a-d}	55.97 ± 2.97^{b-f}	52.29± 1.82^{d-g}	18.67 ± 1.43^{e-i}	20.103 ± 0.734^{c-i}	20.23± 1.40^{c-i}	89.43 ± 1.93^{a-e}	87.717 ± 1.72^{b-j}	86.07 ± 1.95^{e-l}	13.72±1.06^{c-g}	13.31 ±0.76^{e-h}	12.95 ±1.11^{f-h}
T12	54.52 ± 3.88^{c-g}	51.07 ± 2.74^{efg}	54.96 ± 2.56^{c-g}	17.64 ± 1.68^{hi}	20.49 ± 0.96^{b-i}	20.72 ± 1.45^{b-h}	88.40 ± 1.60^{a-i}	84.59 ± 2.60^{j-m}	89.82 ± 2.70^{a-d}	13.55±1.69^{d-h}	14.93 ±1.36^{a-g}	14.47 0.97^{a-g}
Variety (V)	< 0.0001			< 0.0001			< 0.0001			< 0.0001		
Treatment stages (TS)	< 0.0001			< 0.0001			< 0.0001			< 0.0001		
Interaction V*TS	< 0.0001			< 0.0001			< 0.0001			< 0.0001		

Values followed by different letters within a column indicate significant differences (P< 0.05). Means ± standard deviations (n = 10).

Table 4. Color variations of lupin grain subjected to saline thermal treatment

Saline heat treatment stages	*L**			*C**			*H**			ΔE		
	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo
Raw grain	61.47 ± 3.74^{a}	60.85 ± 1.85^{a}	58.97 ± 1.86^{abc}	9.18 ± 0.75^{j}	10.51 ± 0.65^{j}	10.88 ± 0.68^{j}	81.47 ± 1.69^{d}	80.59 ± 1.83^{de}	80.71 ± 0.64^{de}			
T1s	47.24 ± 1.91^{j}	50.72 ± 2.59^{f-j}	41.50 ± 4.77^{k}	16.87 ± 2.30^{i}	15.67 ± 3.58^{i}	20.18 ± 2.20 ^{h}	81.31 ± 0.96^{d}	77.92 ± 1.36^{e}	81.52 ± 2.00^{d}	16.27 ± 2.45^{d-f}	12.05 ± 3.39^{g}	20.15 ± 4.01^{ab}
T2s	51.56 ± 2.64^{e-j}	50.95 ± 2.22^{e-j}	49.52 ± 5.80^{hij}	21.71 ± 1.10^{e-h}	23.45 ± 2.13^{b-g}	25.69 ± 1.65^{ab}	88.95 ± 1.00^{abc}	87.28 ± 0.99^{c}	88.96 ± 3.23^{abc}	17.81 ± 1.50^{a-f}	17.79 ± 1.86^{a-f}	20.42 ± 1.35^{a}
T3s	49.85 ± 2.92 g^{-j}	49.45 ± 3.33^{ij}	50.68 ± 3.89^{f-j}	20.38 ± 0.57^{gh}	24.89 ± 1.81^{a-d}	25.02 ± 1.68^{abc}	89.46 ± 1.23^{abc}	87.28 ± 1.09^{c}	88.63 ± 1.80^{abc}	18.19 ± 1.68^{a-e}	19.82 ± 1.81^{a-c}	18.64 ± 1.73^{a-d}
T4s	55.31 ± 3.48^{b-g}	55.81 ± 1.28^{b-e}	53.00 ± 3.63^{d-i}	22.77 ± 3.24^{b-h}	27.41 ± 3.62^{a}	23.60 ± 1.43^{b-f}	91.12 ± 2.19^{a}	89.50 ± 1.04^{abc}	89.74 ± 2.90^{abc}	18.37 ± 1.80^{a-e}	19.92 ± 2.90^{ab}	17.30 ± 1.03^{b-f}
T5s	59.22 ± 1.08^{ab}	57.42 ± 2.45^{a-d}	55.60 ± 3.21^{b-f}	21.06 ± 1.26^{fgh}	24.72 ± 1.65^{a-e}	25.17 ± 0.91^{ab}	90.40 ± 1.19^{ab}	89.94 ± 2.27^{abc}	88.96 ± 3.08^{abc}	15.14 ± 0.69^{f}	17.68 ± 1.05^{a-f}	17.37 ± 0.72^{b-f}
T6s	55.21 ± 1.67^{b-g}	55.56 ± 3.19^{b-f}	55.73 ± 3.28^{b-e}	21.14 ± 1.81^{fgh}	23.89 ± 2.16^{b-f}	25.25 ± 2.28^{ab}	90.47 ± 1.18^{ab}	89.47 ± 2.56^{abc}	88.79 ± 1.70^{abc}	16.36 ± 1.52^{d-f}	17.40 ± 1.63^{b-f}	17.21 ± 1.89^{b-f}
T7s	56.64 ± 2.07^{a-d}	56.63 ± 3.22^{a-d}	50.47 ± 2.32^{f-j}	21.93 ± 2.50^{c-h}	23.94 ± 1.17^{b-f}	23.60 ± 1.31^{b-f}	89.88 ± 1.71^{abc}	88.49 ± 1.68^{abc}	87.36 ± 1.03^{c}	16.31 ± 1.75^{d-f}	16.53 ± 0.76^{d-f}	16.86 ± 1.06^{c-f}
T8s	54.81 ± 2.15^{b-h}	53.99 ± 3.13^{c-i}	54.53 ± 3.15^{b-h}	21.79 ± 1.81^{d-h}	23.94 ± 1.79^{b-f}	23.20 ± 1.33^{b-h}	90.74 ± 1.20^{a}	88.96 ± 1.98^{abc}	89.84 ± 2.29^{abc}	17.21 ± 1.31^{b-f}	17.65 ± 1.21^{a-f}	16.46 ± 1.11^{d-f}
T9s	57.37 ± 1.60^{a-d}	54.98 ± 3.62^{b-fg}	54.89 ± 1.55^{b-g}	21.24 ± 0.55^{fgh}	24.81 ± 2.08^{a-e}	23.26 ± 1.06^{b-h}	90.24 ± 0.99^{abc}	87.69 ± 1.64^{bc}	89.73 ± 1.31^{abc}	15.55 ± 0.84^{ef}	17.50 ± 1.12^{a-f}	15.97 ± 0.96^{d-f}
Variety (V)	< 0.0001			< 0.0001			< 0.0001			< 0.0001		
Treatment stages (TS)	< 0.0001			< 0.0001			< 0.0001			< 0.0001		
Interaction V*TS	< 0.0001			< 0.0001			< 0.0001			< 0.0001		

Table 5. Variations in lupin grain size following aqueous thermal and saline heat treatments

Aqueous thermal treatment stages	Large diameter (mm)			Small diameter (mm)		
	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo
Raw grain	11.4 ± 0.7^{e}	10.8 ± 0.4^{e}	10.9 ± 0.5^{e}	5.5 ± 0.1^{de}	5.0 ± 0.1^{e}	5.9 ± 0.1^{cd}
T1	14.8 ± 0.8^{a-d}	15.0 ± 0.7^{abc}	15.3 ± 1.0^{a}	6.2 ± 0.1^{a-d}	5.9 ± 0.1^{cd}	6.5 ± 0.1^{abc}
T2	15.2 ± 0.6^{ab}	14.8 ± 0.5^{a-d}	14.5 ± 0.8^{a-d}	6.2 ± 0.1^{a-d}	6.0 ± 0.1^{bcd}	6.5 ± 0.1^{abc}
T3	14.2 ± 0.6^{a-d}	14.6 ± 0.5^{a-d}	14.7 ± 0.7^{a-d}	6.5 ± 0.1^{abc}	6.7 ± 0.1^{abc}	6.8 ± 0.1^{a}
T4	14.0 ± 0.4^{cd}	13.8 ± 0.5^{a-d}	13.9 ± 0.4^{cd}	6.6 ± 0.1^{abc}	6.4 ± 0.1^{abc}	6.7 ± 0.1^{abc}
T5	14.4 ± 0.6^{a-d}	13.8 ± 0.7^{a-d}	14.6 ± 0.7^{a-d}	6.5 ± 0.1^{abc}	5.9 ± 0.1^{cd}	6.7 ± 0.1^{abc}
T6	14.2 ± 0.8^{a-d}	14.0 ± 0.5^{cd}	14.5 ± 0.6^{a-d}	6.7 ± 0.1^{abc}	6.1 ± 0.1^{a-d}	6.8 ± 0.1^{ab}
T7	14.1 ± 0.8^{a-d}	14.0 ± 0.5^{bcd}	13.9± 1.0^{cd}	6.8 ± 0.1^{a}	6.3 ± 0.1^{a-d}	6.8 ± 0.1^{a}
T8	14.9 ± 0.5^{a-d}	14.6 ± 0.6^{a-d}	14.2 ± 0.8^{a-d}	6.6 ± 0.1^{abc}	6.5 ± 0.1^{abc}	6.8 ± 0.1^{ab}
T9	14.5 ± 0.5^{a-d}	14.2 ± 1.0^{a-d}	14.2 ± 1.0^{a-d}	6.7 ± 0.1^{abc}	6.7 ± 0.1^{ab}	6.8 ± 0.1^{a}
T10	14.7 ± 0.7^{a-d}	14.2 ± 0.6^{a-d}	14.8 ± 0.6^{a-d}	6.9 ± 0.1^{a}	6.4 ± 0.1^{abc}	6.7 ± 0.1^{ab}
T11	14.4 ± 0.4^{a-d}	14.4 ± 0.8^{a-d}	14.3± 0.3^{a-d}	6.6 ± 0.1^{abc}	6.4 ± 0.1^{abc}	6.6 ± 0.1^{abc}
T12	14.5 ± 0.5^{a-d}	14.4 ± 0.7^{a-d}	14.2 ± 0.27^{a-d}	6.7 ± 0.1^{abc}	6.4 ± 0.1^{abc}	6.5 ± 0.1^{abc}
Variety (V)	0.07			< 0.0001		
Treatment stages (TS)	< 0.0001			< 0.0001		
Interaction V*TS	0.1024			0.301		

Saline heat treatment stages	Large diameter (mm)			Small diameter (mm)		
	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo
Raw grain	11.4 ± 0.7^{f}	10.8 ± 0.4^{f}	10.9 ± 0.5^{f}	5.5 ± 0.5^{fg}	5.0 ± 0.3 g	5.9 ± 0.4^{ef}
T1s	15.0 ± 1.0^{a-e}	14.6 ± 0.7^{a-e}	14.6 ± 0.8^{a-e}	6.0 ± 0.7^{c-f}	6.1 ± 0.3^{b-f}	6.4 ± 0.3^{a-e}
T2s	14.7 ± 0.7^{a-e}	14.0 ± 1.1^{de}	14.8 ± 1.0^{a-e}	6.3 ± 0.6^{a-e}	6.2 ± 0.6^{b-f}	6.9 ± 0.5^{ab}
T3s	14.0 ± 0.8^{de}	14.2 ± 0.9^{cde}	14.0 ± 0.7^{e}	6.2 ± 0.5^{a-f}	6.1 ± 0.5^{b-f}	6.5 ± 0.6^{a-e}
T4s	15.7 ± 1.02	14.6 ± 0.4^{a-e}	15.3 ± 0.8^{abc}	6.5 ± 0.5^{a-e}	6.3 ± 0.5^{a-e}	6.8 ± 0.5^{abc}
T5s	15.2 ± 0.8^{a-d}	15.1 ± 0.7^{a-e}	15.2 ± 0.5^{a-d}	6.5 ± 0.5^{a-e}	6.0 ± 0.4^{def}	6.8 ± 0.5^{abc}
T6s	15.3 ± 0.9^{abc}	15.1 ± 0.6^{a-e}	15.6 ± 0.8^{ab}	6.6 ± 0.4^{a-e}	6.3 ± 0.6^{a-e}	6.7 ± 0.5^{a-d}
T7s	14.8 ± 0.4^{a-e}	15.2 ± 0.6^{a-e}	15.3 ± 0.7^{abc}	6.4 ± 0.2^{a-e}	6.3 ± 0.3^{a-e}	6.6 ± 0.4^{a-d}
T8s	15.4 ± 0.5^{ab}	15.0 ± 0.6^{a-e}	14.6 ± 0.5^{a-e}	6.9 ± 0.1^{a}	6.4 ± 0.3^{a-e}	6.8 ± 0.4^{ab}
T9s	15.4 ± 0.5^{ab}	14.8 ± 0.6^{a-e}	14.5 ± 0.5^{b-e}	6.9 ± 0.4^{a}	6.6 ± 0.5^{a-e}	6.8 ± 0.2^{ab}
Variety (V)	0.002			< 0.0001		
Treatment stages (TS)	< 0.0001			< 0.0001		
Interaction V*TS	0.0267			0.3003		

Values followed by different letters within a column denote significant differences (P< 0.05). Means ± standard deviations (n = 10).

A variation of 88 to 90.4 for *L** and an increase in the values of *H** and *C**, resulting in a greater intensity of the cream color of the grain, was reported for *Lupinus albus* seeds (Ertaş and Bilgiçli, 2014).

3.5 Grain size

A significant change in the diameters of the three varieties of grain was noted during hydration (T1) and the size increased proportionally to the water absorption capacity of the protein, the components of the fiber, the oligosaccharides and the permeability of the tegument (Table 5). The absorption of water and the increase in grain size continued until the tissues were saturated with water, which was reached in the cooking stage (T2); afterwards, no significant differences were observed in the largest and smallest diameters of grains. In the hydration stage of ATT, the larger diameter of the grain increased 3.4 mm (INIAP-450), 4.2 mm (INIAP-451) and 4.4 mm (Criollo). During cooking, the larger diameter of the grain increased 3.8 mm (INIAP-450), 4.0 mm (INIAP-451), and 3.6 mm (Criollo). In the STT, the larger diameter of the grain increased 0.8 mm (INIAP-450), 1.2 mm (INIAP-451) and 1.0 mm (Criollo). Similar results were reported for lupin debittering, the grain swelled and increased its size, depending on the variety (Schoeneberger et al., 1982). During cooking, the smaller diameter of the grain increased 4.3 mm (INIAP-450), 3.2 mm (INIAP-451) and 3.9 mm (Criollo). The increase in the diameter of the seed depended on their final humidity, thus, when the grain presented 40, 50 and 60% humidity, its size increases by 15, 23 and 24.93%, respectively.

4. Conclusions

Two different debittering processes were applied to three different varieties of *L. mutabilis.* Both processes, ATT and STT, resulted in approximately 80% of the alkaloids removed from the grain during soaking, cooking and the first wash of the grain at 35 °C. In general, the debittering process with ATT and STT exerted positive effects on reducing the QAs and increasing total nitrogen concentrations in the grain of the three varieties. Moreover, the processes improved the grain texture by decreasing hardness and increased grain size. Color was also improved, as evidenced the increase in the tone and chromaticity of the grain, characteristics that affect the consumer's level of acceptance and preference. However, in the saline heat process, the addition of

sodium chloride in the first stages of the process helped to optimize the effect of temperature, agitation and water changes on the reduction of the QAs contents. Considering the economy of the process, the debittering of 1 kg of grain by the SST was achieved in 58 h using 66 L of water, which represents a saving of 26 h in the processing time and 30 L in the volume of water compared with the ATT and 127 L compared with the artisanal process, which uses 193 L and take between 5 to 6 days. Hence, considering the current debittering processes, the saline heat process is advisable for lupin debittering. Nevertheless, further studies will be undertaken pursuing an additional reduction of water and the process time to make it more environmentally friendly.

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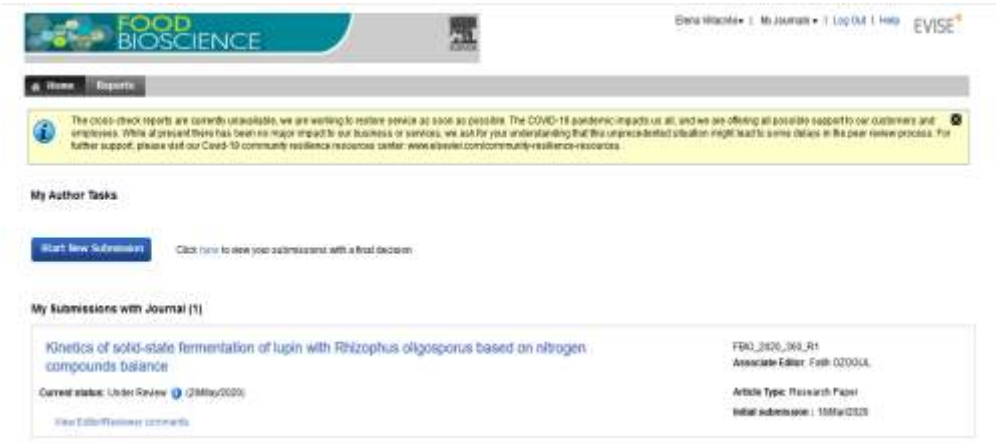
CAPITULO 2

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

Elena Villacrés[1] and Cristina M. Rosell[2]

[1]National Agricultural Research Institute, Mejía, Ecuador
[2]Agrochemistry and Food Technology Institute. IATA, CSIC Valencia, España



Credit Authors Statement

Credit Roles:

EV: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Roles/Writing - original draft

CMR: Conceptualization; Investigation; Supervision; Validation; Writing - review and editing.

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 concentration 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 104.68 $g \cdot kg^{-1}$ dry weigh in the Criollo variety. The crushed INIAP-450 grain without tegument had the highest soluble nitrogen content (99.8 $g \cdot kg^{-1}$ dry weight), whose concentration was reached at a specific rate of 0.006 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 $mg \cdot g^{-1}$ 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 formation
Ct	critical time of total or soluble nitrogen formation
Cc	critical concentration of total or soluble nitrogen formation
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 formation
$(dN/dt)_m$	maximum rate of total or soluble nitrogen formation
t	time of total or soluble nitrogen formation
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 *et al*., 2006) and amino acid composition, which is influenced by the processing conditions and interactions with other food components (Melini *et al*., 2017). However, debittering processes 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 $g \cdot kg^{-1}$ dry weight, respectively, and the ash and starch contents decrease by 19.5 and 15.2 $g \cdot kg^{-1}$ dry weight, 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 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 *et al*., 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 and 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 *et al*., 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 and 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. In fact, only 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 antinutrient 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). In fact, 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 *et al*., 2003). The objective of this research was to study the solid fermentation of lupin with the NRRL2710 *Rhizopus oligosporus* strain, by 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 are 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. 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 BAEE unites per mg protein, bovine pancreatic chymotrypsin (Type II), 60 units per mg poder, porcine intestinal peptidase (Grade III), 40 units per g powder.

Sodium hydroxide 99% (NaOH), sulfuric acid 95-97% (H2SO4), hydrochloric acid fuming 37% (HCl), boric acid ACS grade (H_3BO_3), ethanol absolute for analysis

EMSURE® ACS (C_2H_5OH), methanol ACS grade (CH_3OH), methyl red, bromocresol green, catalyst [potassium sulfate (K_2SO_4), cupric sulfate ($CuSO_4$), selenium dioxide(SeO_2)], Potassium dihydrogen phosphate (NaH_2PO_4), Orto-ftaldehido (OPA), 2-mercaptoethanol (2 ME).

2.3 Grain debittering

The lupin grains were debittered by applying 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 *R. oligosporus* inoculum (100 mg·mL^{-1}) was used for preparing diluted solutions with densities varying from 3.5 x 10^3 to 3.5 x 10^6 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 of sterile water. This suspension (1 mL) was inoculated on 100 g of 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 a # 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 distilled water that had been stirred for one minute to promote spore shedding. This suspension (100 µL) was removed and the volume was increased to 1 mL with distilled water containing 0.1% tween 80. The count obtained was 3.5 x10^6 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 by 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 the 10^{-2} dilution was selected for inoculating 50 g of lupin with 50% humidity. A steady-state fermentation process 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 the 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 according to method AOAC 943.02 (AOAC, 1990). This parameter is the negative decimal logarithm of the activity of hydrogen ions and measures the free protons in a solution. Acidity measures free and bound protons, for its determination it was used the method AOAC 947.05 with the addition of 0.01 M NaOH to obtain a pH of 8.2 (AOAC, 1990). The result was reported as a percentage of lactic acid, which predominates in fermentation reactions with *R. oligosporus* (Bartkienė et al., 2014). The total nitrogen content was determined using the AOAC 955.04 method (AOAC, 1995). Soluble nitrogen content was determined using the method described by Periago *et al*. (1996).

2.7 *In vitro* protein digestibility

Protein digestibility was determined as previously described (Lqari *et al*., 2002). Samples containing 62.5 mg of protein were suspended in 10 mL of water and the pH was adjusted to 8 with 0.1 M NaOH. An enzymatic solution containing 1.6 mg of trypsin (18 $U \cdot mg^{-1}$), 3.1 mg of chymotrypsin (40 $U \cdot mg^{-1}$) and 1.3 mg protease (15 U mg^{-1}) $\cdot mL^{-1}$ was added to the protein suspension at a 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:

$$DP = 210.464 - 18.103x\ pH \qquad \text{Eq. 1}$$

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 third or fourth degree equations and the critical concentration (*Cc*) was obtained. Using these values, the specific rate of nitrogen concentration (α) was determined by dividing the maximum rate $(dN / dt)_m$ by *Cc* (Mitchell et al., 2004).

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

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

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

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

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 ± standard deviations.

3. Results and discussion

3.1 Analysis of titratable acidity

The titratable acidity of the grain increased with the fermentation time (Figure 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 fermentation of grains with tegument showed higher increase of acidity (Figure 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 *et al*., 2013). In whole grain without tegument, 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 *et al*., 2010). The presence of tegument, both in whole and crushed grain, not interfered with fermentation but decreased the evolution of organic acids in WIT. According to Priatni *et al*. (2013) point out that some nutrients and particularly potassium are removed away during the husking grain prior to fermentation and that this deficiency can explain the lower activity of *R. oligosporus* on this substrate.

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 (Figure 2). This change was influenced by the

variety and condition of the grain ($P<0.05$). During fermentation the pH varied from 6.46 to 5.54 in INIAP-451 (WWT) and from 7.32 to 4.90 in INIAP-450 (CIT). The decrease in pH has been related to protein metabolism (Ruiz-Terán and 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, at higher pH values the plasma membrane of the organism becomes very unstable (Priatni *et al*., 2013).

The CWT of Criollo variety presented a pH of 4.03 at 96 h of fermentation (Figure 2), while INIAP-450 and INIAP-451 seeds presented higher values (4.86 and 4.73, respectively).

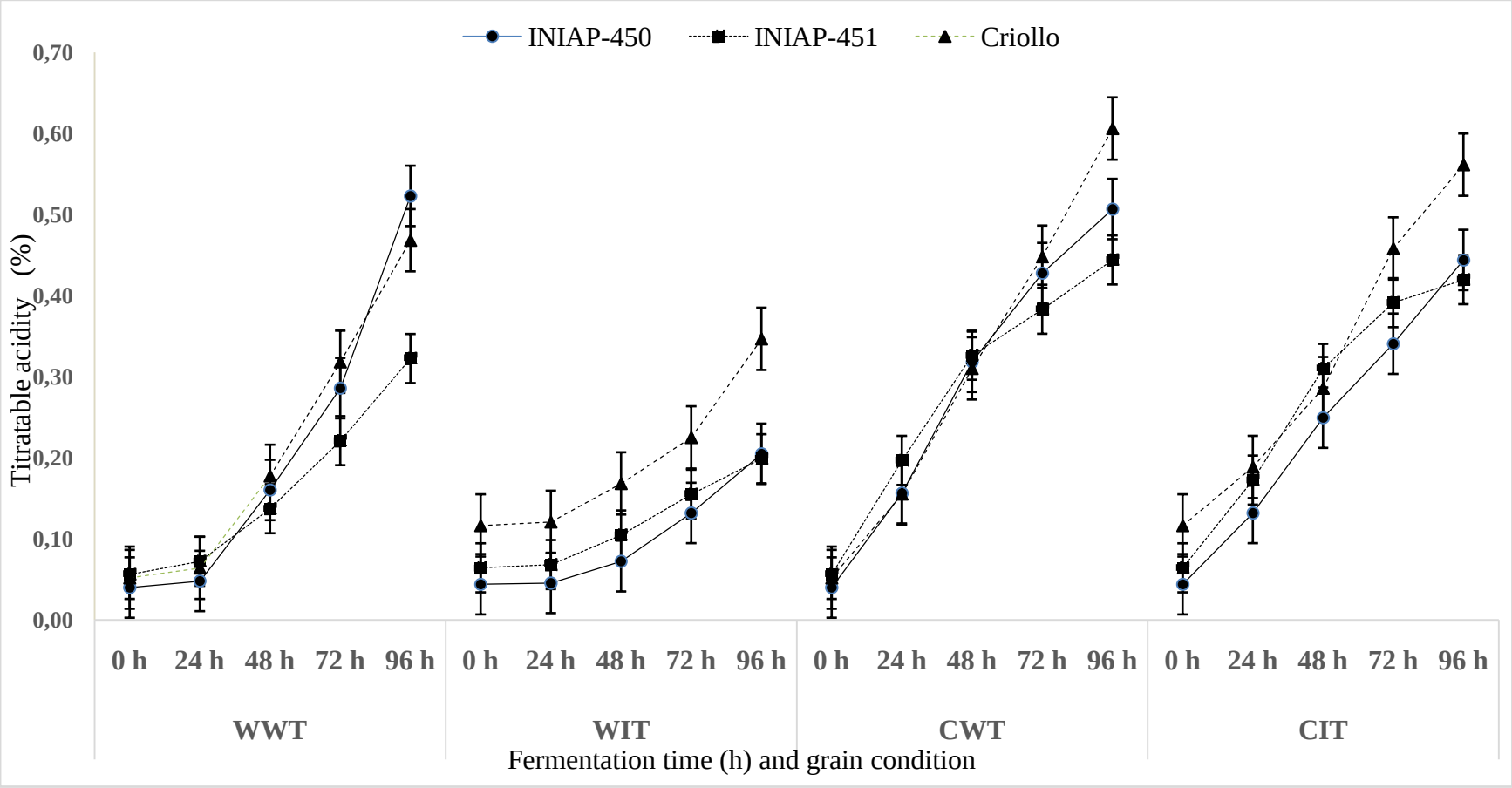


Figure 1. Variations of the titratable acidity (%) during the solid fermentation of debittered lupin grain. WWT: whole grain with tegument, WIT: whole grain without tegument, CWT: crushed grain with tegument, and CIT: crushed grain without tegument.

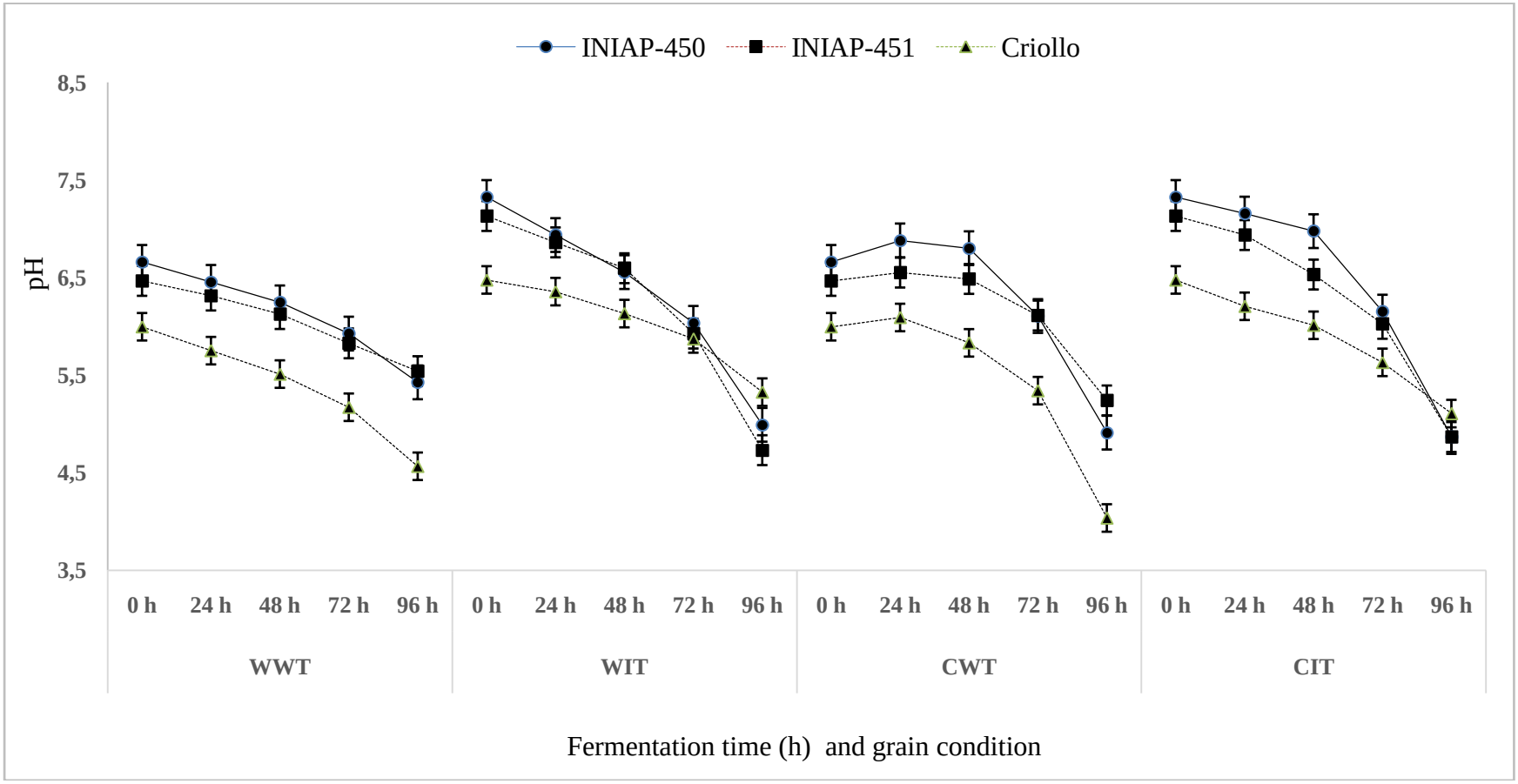


Figure 2. Variations of pH during the solid fermentation of debittered lupin grain. WWT: whole grain with tegument, WIT: whole grain without tegument, CWT: crushed grain with tegument, and CIT: crushed grain without tegument.

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 Figure 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 of nitrogen during fermentation and subsequent incorporation into the mold protein biomass (Mukherjee *et al*., 2016). The total nitrogen content reached 108.27 $g \cdot kg^{-1}$ dry weight 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 kinetic behavior of total nitrogen in the production of fermented lupin

The concentration of total and soluble nitrogen (Figures 3 and 4) followed the growth dynamics of the growth curves of the microorganisms in the exponential phase and responded to polynomial regression equations, from which the expressions that describe the kinetics of the solid-state 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 kinetic behavior of total nitrogen.

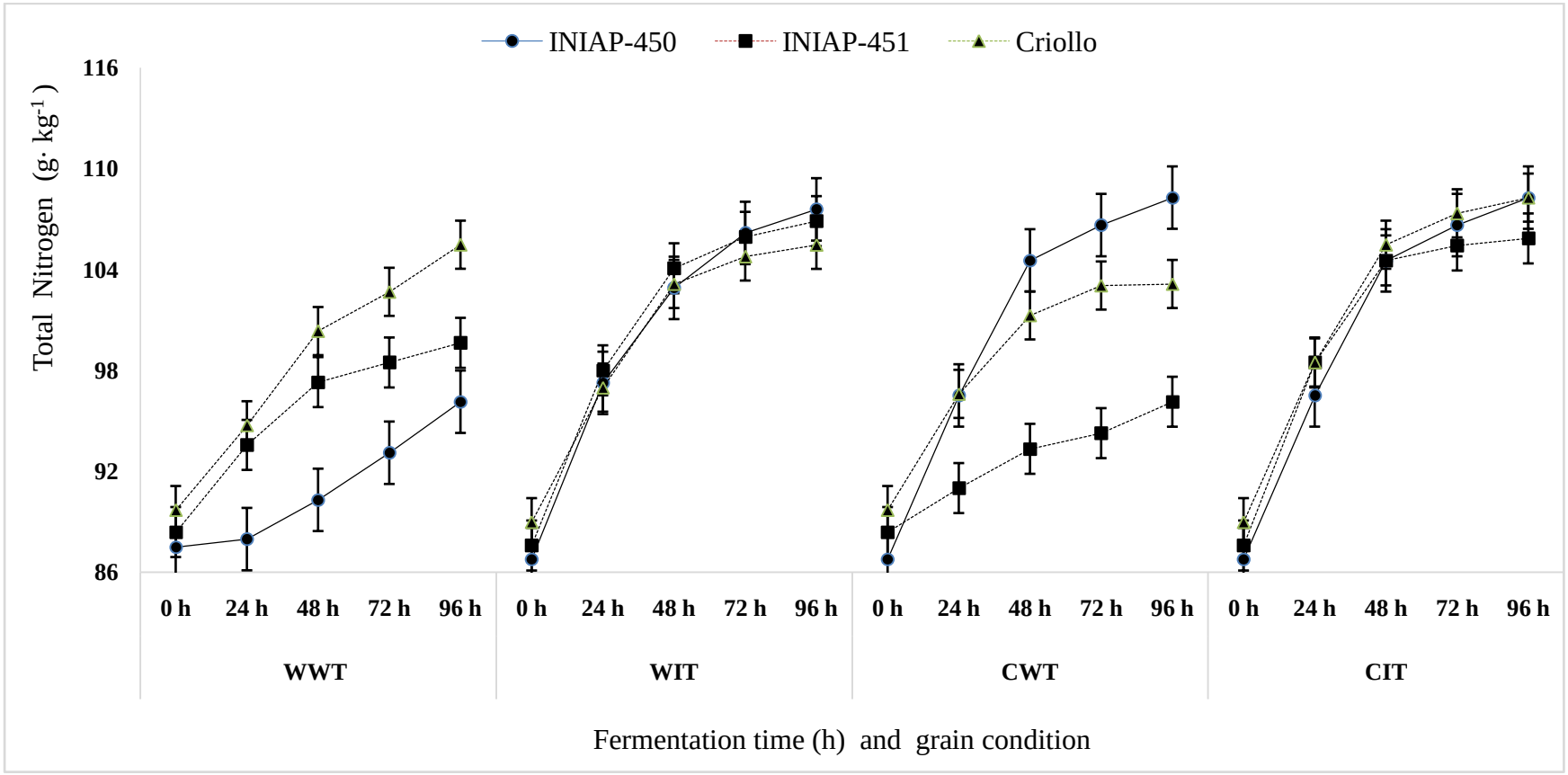


Figure 3. Variations of the total nitrogen content ($g \cdot kg^{-1}$ dry weight) during the solid fermentation of debittered lupin grain. WWT: whole grain with tegument, WIT: whole grain without tegument, CWT: crushed grain with tegument, and CIT: crushed grain without tegument.

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 $g \cdot kg^{-1}$ dry weight, in Criollo variety (WWT) at 40.8 h (*Ct*), while INIAP-450 (WWT) presented the lowest concentration (82.4 $g \cdot kg^{-1}$ dry weight) at 100 h. Figure 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 reaching the concentration of total nitrogen.

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 *Cc*, $(dN / dt)_m$ and α values. This last parameter reached its maximum value (0.009 h^{-1}) by 40.8 h in Ciollo (WWT), which was 3.0 times greater than 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.* Determination of the critical time (*Ct*) 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 total nitrogen (104.7 $g \cdot kg^{-1}$ dry weight) at 30.4 h of fermentation, 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 $g \cdot kg^{-1}$ dry weight). This observation is consistent with previous studies examining fermented rapeseed meal and dehulled lupin grain (*Lupinus angustifolius*) (Priatni *et al*., 2013), who found that the growth of *R. oligosporus* mycelia was influenced by total surface area of grain particles meaning also the particle size of grain and 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 (Figure 4). Before starting the fermentation process (time 0), soluble protein fraction ranged from 2.82 to 4.84 $g \cdot kg^{-1}$ dry weight 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).

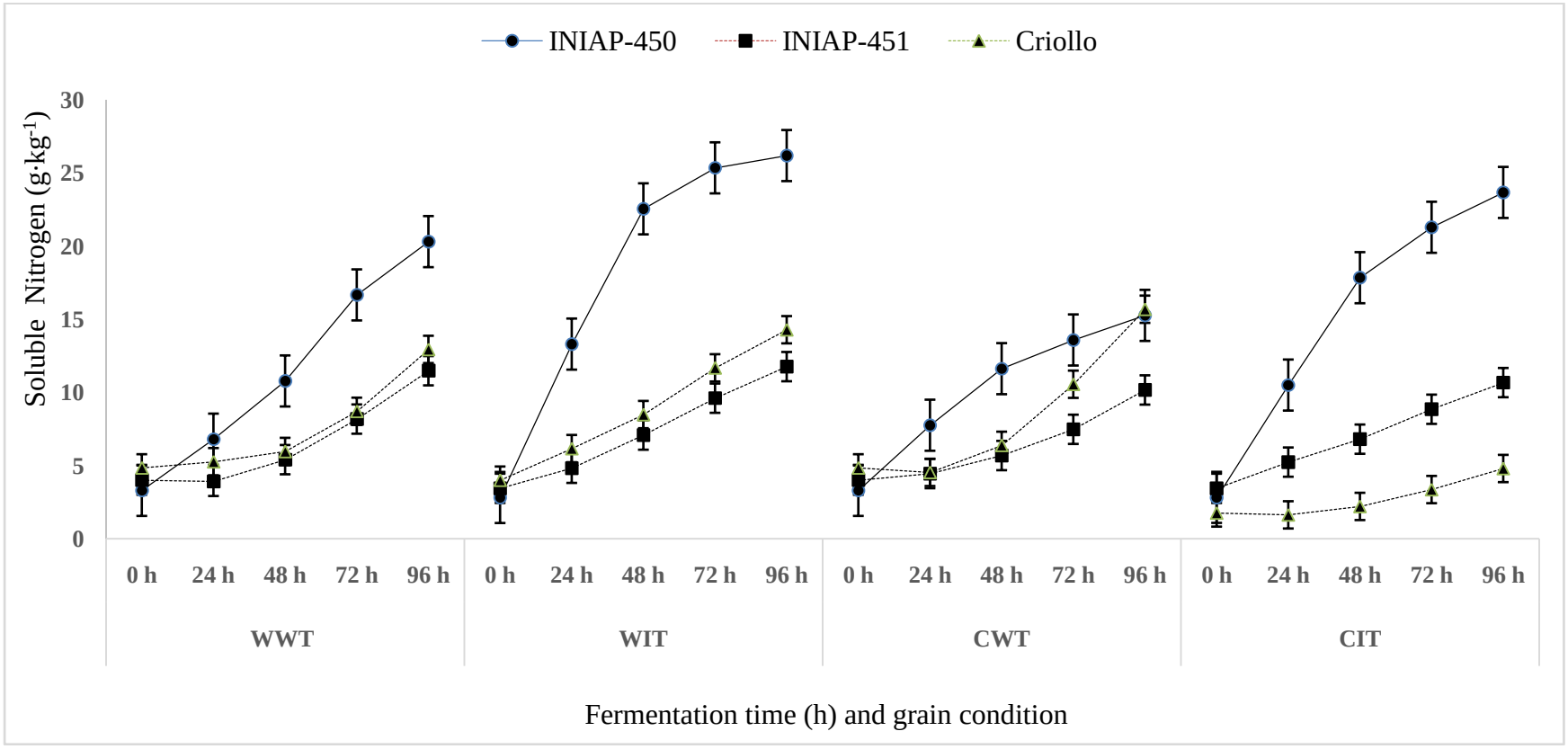


Figure 4. Variations of the soluble nitrogen content ($g \cdot kg^{-1}$ dry weight) during the solid fermentation of debittered lupin grain. WWT: whole grain with tegument, WIT: whole grain without tegument, CWT: crushed grain with tegument, and CIT: crushed grain without tegument.

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 *et al*., 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 *et al*., 2018). The whole or crushed INIAP-450 grain without tegument had the highest soluble nitrogen content (26.18 and 23.66 $g \cdot kg^{-1}$ dry weight) at 96 h of fermentation, possibly due to the lower tannin content in this variety (1.44 $g \cdot kg^{-1}$ dry weight) compared to INIAP-451 (1.79 $g \cdot kg^{-1}$ dry weight) and Criollo (1.85 $g \cdot kg^{-1}$ dry weight) (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 Kinetic behavior of soluble nitrogen in the production of fermented lupin

The progress of soluble nitrogen during fermentation was adjusted to different polynomial equations (Table 2). The INIAP-450 whole grain with tegument (WWT) reached a critical concentration (*Cc*) of 26.5 $g \cdot kg^{-1}$ dry weight, 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). Soluble nitrogen concentration increased 10 times faster than the concentration of total nitrogen in INIAP-450 (WIT) and 451 (WIT, CWT, CIT). These changes may enhance the digestibility, nutritional status and possible flavor of lupin (Feng *et al*., 2007), supporting its potential to be considered a functional food or ingredient.

3.3.5 *In vitro* protein digestibility

This parameter varied significantly ($P<0.05$) with the variety and grain condition (Figure 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 barkery* (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) (Figure 5).

Table 1. Kinetic behavior of total nitrogen 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 concentration	R^2	*Ct* (h)	*Cc* ($g \cdot kg^{-1}$)	$(dN/dt)_m$ $g \cdot kg^{-1} \cdot h^{-1}$	α (h^{-1})
INIAP- 450	WWT	$1E\text{-}07t^4 - 4E\text{-}05t^3 + 0.003t^2 - 0.051t + 87.5$	1.00	100.0	82.4	0.25	0.0030
	WIT	$-2E\text{-}07t^4 + 6E\text{-}05t^3 - 0.0079t^2 + 0.5911t + 86.78$	1.00	75.0	105.6	0.08	0.0007
	CWT	$5E\text{-}07t^4 - 9E\text{-}05t^3 + 0.0061t^2 - 0.0463t + 87.5$	1.00	45.0	95.3	0.35	0.0030
	CIT	$1E\text{-}06t^4 - 0.0001t^3 + 0.009t^2 + 0.281t + 86.78$	1.00	25.0	99.8	0.60	0.0060
INIAP- 451	WWT	$5E\text{-}07t^4 - 8E\text{-}05t^3 + 0.002t^2 + 0.192t + 88.38$	1.00	40.0	95.4	0.09	0.0010
	WIT	$4E\text{-}07t^4 - 5E\text{-}05t^3 - 0.0014t^2 + 0.494t + 87.587$	1.00	31.2	100.5	0.31	0.0031
	CWT	$4E\text{-}07t^4 - 8E\text{-}05t^3 + 0.0035t^2 + 0.0632t + 88.387$	1.00	49.0	92.9	0.02	0.0001
	CIT	$6E\text{-}07t^4 - 1E\text{-}04t^3 + 0.0002t^2 + 0.4956t + 87.587$	1.00	41.7	103.2	0.16	0.0001
CRIOLLO	WWT	$9E\text{-}07t^4 + 0.009t^2 + 0.066t + 89.69$	1.00	40.8	109.9	1.04	0.0092
	WIT	$8E\text{-}07t^4 + 0.006t^{2+} 0.264t + 88.97$	1.00	32.3	103.7	0.70	0.0066
	CWT	$2E\text{-}07t^4 - 4E\text{-}05t^3 + 0.305t + 89.69$	1.00	50.0	101.2	0.10	0.0011
	CIT	$9E\text{-}07t^4 + 0.005t^2 + 0.339t + 88.97$	1.00	30.4	104.7	0.44	0.0042

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 total nitrogen concentration (α).

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

Variety	Grain condition	Soluble nitrogen concentration	R2	*Ct* (h)	*Cc* ($g \cdot Kg^{-1}$)	$(dN/dt)_m$ ($g \cdot kg^{-1} \cdot h^{-1}$)	α (h^{-1})
INIAP 450	WWT	$1E\text{-}06t^4 - 0.0002t^3 + 0.0112t^2 + 0.2887t + 2.828$	1.00	50.0	26.5	0.15	0.0060
	WIT	$1E\text{-}06t^4 - 0.0001t^3 + 0.011t^2 + 0.289t + 2.82$	1.00	42.8	38.7	1.54	0.0040
	CWT	$4E\text{-}07t^4 - 7E\text{-}05t^3 + 0.003t^2 + 0.148t + 3.304$	1.00	42.8	10.9	0.14	0.0010
	CIT	$1E\text{-}06t^4 - 0.0001t^3 + 0.009t^2 + 0.281t + 86.78$	1.00	25.0	99.8	0.60	0.0060
INIAP 451	WWT	$-9E\text{-}08t^4 + 9E\text{-}06t^3 + 0.001t^2 - 0.0328t + 4.004$	1.00	25.0	26.5	0.15	0.0060
	WIT	$-8E\text{-}06t^3 + 0.0013t^2 + 0.03t + 3.443$	1.00	54.2	7.6	0.10	0.0130
	CWT	$7E\text{-}06t^3 - 0.0014t^2 + 0.1488t + 88.337$	1.00	47.6	93.9	0.10	0.0010
	CIT	$-2E\text{-}07t^4 + 3E\text{-}05t^3 - 0.001t^2 + 0.103t + 3.444$	1.00	37.5	7.1	0.11	0.0010
CRIOLLO	WWT	$-3E\text{-}07t^4 + 6E\text{-}05t^3 - 0.003t^2 + 0.060t + 4.844$	1.00	50.0	6.0	0.06	0.0001
	WIT	$-5E\text{-}06t^3 + 0.0009t^2 + 0.065t + 4.0348$	1.00	60.0	10.09	0.12	0.0001
	CWT	$-2E\text{-}07t^4 + 3E\text{-}05t^3 - 0.039t + 4.844$	1.00	37.5	6.0	0.04	0.0070
	CIT	$2E\text{-}06t^3 - 0.0005t^2 + 0.1412t + 3.974$	1.00	83.3	13.4	0.09	0.0071

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 soluble nitrogen concentration (α).

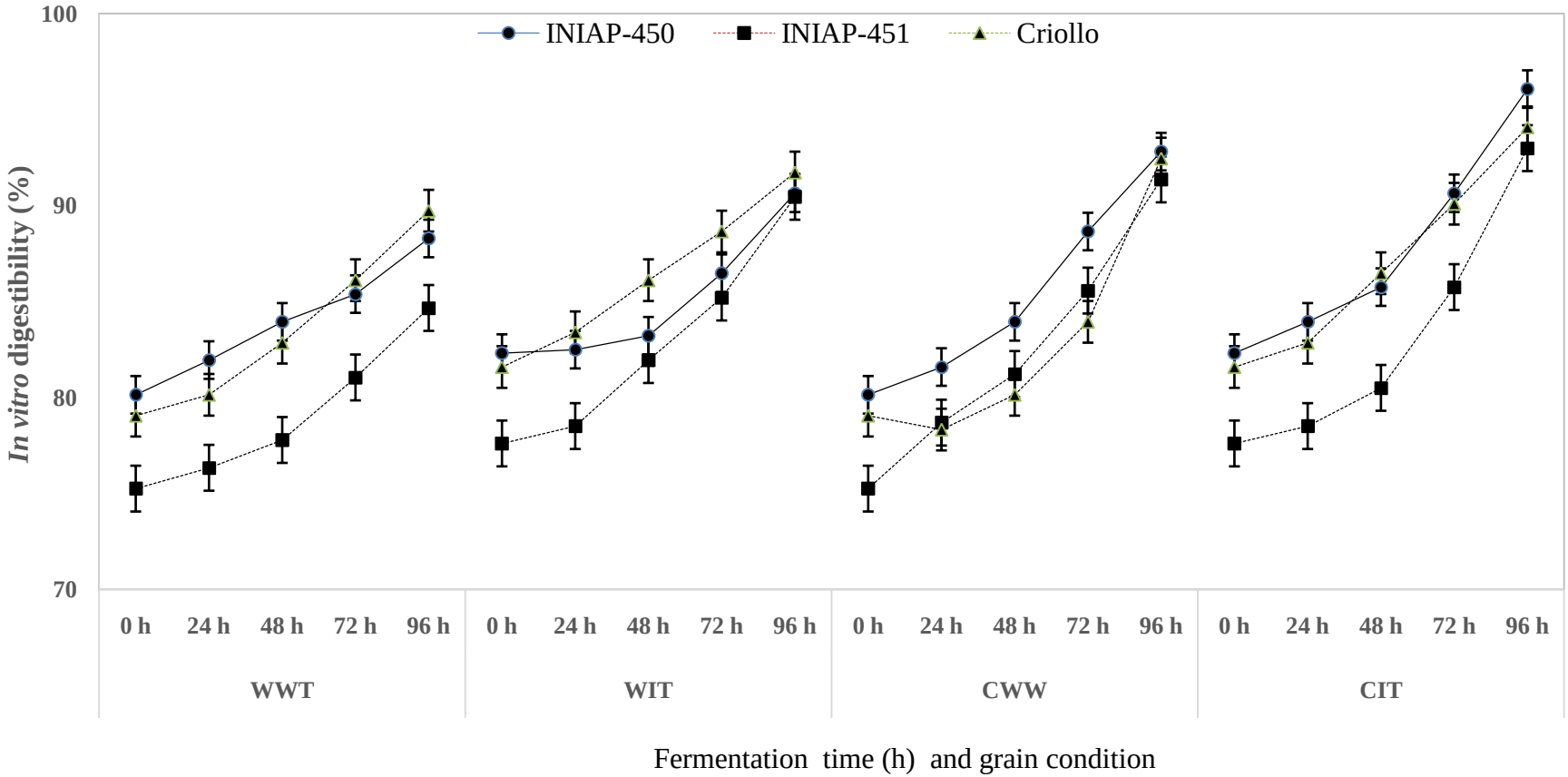


Figure 5. Variations of the *in vitro* protein digestibility (% dry weight) during the solid fermentation of debittered lupin grain. WWT: whole grain with tegument, WIT: whole grain without tegument, CWT: crushed grain with tegument, and CIT: crushed grain without tegument.

The results of our study are consistent with observations by Nout and 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 and Starzyńska-Janiszewska, 2008).

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 are rich in protein, 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 + 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: (methionine + cysteine) and tryptophan, principally (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. In contrast, the

(methionine + cysteine) content increased. After fermentation, the (methionine + cysteine) content reached 27.87 $mg \cdot g^{-1}$ protein (INIAP-450), 27.08 $mg \cdot g^{-1}$ (INIAP-451) and 26.49 $mg \cdot g^{-1}$ protein (Criollo) in the different lupin varieties. It must be stressed that those values exceeded the requirement of preschool child 25.00 $mg \cdot g^{-1}$ 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 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. According to 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% (INIA-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 *et al*., 2016).

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

Variety		mg·g^{-1} protein (dry weight)		amino acids score			
	Grain condition	methionine + cysteine	tryptophan	methionine + cysteine	tryptophan	Protein digestibility (%)	PDCAAS (%)
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, 2005)

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 concentration 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.

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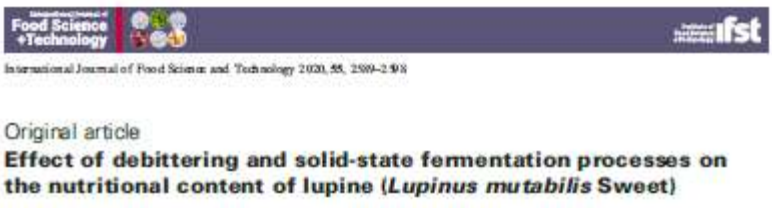
CAPITULO 3

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

Elena Villacrés[1*], María Belén Quelal[1], Xiomara Jácome[2], Gabriela Cueva[2] & Cristina M. Rosell[3]

[1]Departamento de Nutrición y Calidad, Instituto Nacional de Investigaciones Agropecuarias, Mejía, Ecuador
[2]Escuela de Ciencias Químicas, Facultad de Ciencias Exactas y Naturales, Pontificia Universidad Católica del Ecuador, Quito, Ecuador
[3]Institute of Agrochemistry and Food Technology (IATA), CSIC, Valencia, Spain



Credit Authors Statement

Credit Roles:

EV: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Roles/Writing - original draft

MBQ: Formal statistic analysis

XJ: Amino acid analysis

GC: Validation methodology of amino acid analysis by HPLC

CMR: Conceptualization; Investigation; Supervision; Validation; Writing-review and editing.

Abstract

There is a growing interest in vegetable-based sources of proteins. Despite its high nutrient content, lupin has been rarely exploited as a protein source due to the presence of high levels of non-nutritive compounds such as alkaloids, which impart a bitter taste and can be toxic. Here, we evaluated the effect of debittering and solid-state fermentation on the nutritional contents of three lupin varieties (*Lupinus mutabilis* Sweet). These processes induced significant changes ($P<0.05$) in the nutritional composition of the three lupin varieties (INIAP-450, INIAP-451, and Criollo) and increased the protein levels to 644.55 $g \cdot kg^{-1}$ dry weight (Criollo) and the levels of several constituent amino acids such as valine (54.62 $g \cdot kg^{-1}$ dry weight), methionine (40.88 $g \cdot kg^{-1}$ dry weight dry weight), isoleucine (58.34 $g \cdot kg^{-1}$ dry weight) and leucine (74.55 $g \cdot kg^{-1}$ dry weight). The ether extract of INIAP-450 showed increased levels (up to 244.03 $g \cdot kg^{-1}$ dry weight); especially, of monounsaturated fatty acids (572.50 $g \cdot kg^{-1}$) and polyunsaturated fatty acids (282.20 $g \cdot kg^{-1}$) were observed. The ω-6: ω-3 ratio in the debittered grain oils was close to the minimum requirement established for good-quality oils (10:1). However other components decreased, showing levels up to 13.04 $g \cdot kg^{-1}$ (total starch) in the Criollo variety, 22.62 $g \cdot kg^{-1}$ (resistant starch) in INIAP-450, 6.53 $g \cdot kg^{-1}$ (potassium) in INIAP-451, 46 $g \cdot kg^{-1}$ (iron) in INIAP-451, and 29.75 $g \cdot kg^{-1}$ (zinc) in INIAP-450.

Key Words: Nutritional content, solid fermentation, debittering process, leguminous

1. Introduction

Within the legume family, the genus *Lupinus* includes numerous species, and four of these are popular for their nutritional and economic values. These include *Lupinus angustifolius* (blue lupin with narrow leaves), *Lupinus albus* (white lupin), *Lupinus luteus* (yellow lupin), and *Lupinus mutabilis* (pearl lupin). *Lupinus mutabilis* Sweet is one of the most common species because it can grow in poor soils, adapt well to extreme conditions, and fix atmospheric nitrogen (Van de Noort, 2016).

Like other legumes, lupin is characterized with high levels of proteins, fats, dietary fibers, and minerals (Yorgancilar and Bilgiçli, 2014). In particular, its protein content varies between 410 $g \cdot kg^{-1}$ and 510 $g \cdot kg^{-1}$ depending on the species and climatic and

cultivation conditions (Arnoldi *et al*., 2015; Karnpanit *et al*., 2016). The high contents of monounsaturated and polyunsaturated fatty acids (MUFA and PUFA, respectively) make lupin an alternative source of vegetable oil (Villacrés *et al.,* 2013; Awad-Allah and Elkatry, 2013). It has low contents of sucrose and starch and moderate contents of oligosaccharides, which are not digested by humans but are broken down by the bacterial flora present in the large intestine (Van de Noort, 2016).

Despite its high nutritional value, lupin is rarely used owing to the presence of bitter compounds such as alkaloids derived from quinolizidine (Carvajal-Larenas *et al.,* 2016), which prevents its direct consumption (Repo-Carrasco *et al.,* 2007). Elimination of these components may be achieved with debittering technologies. Traditional debittering processes include a hydration phase followed by cooking to inactivate the germination capacity of the grain and increase the permeability of cell wall, thereby facilitating 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 contents have been developed, but their adaptation to different environmental conditions is difficult, and these varieties undergo changes over time that may result in the restoration of the bitter attribute (Awad-Allah and Elkatry, 2013).

Solid-state fermentation is characterized with 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 through the inoculation of *Rhizopus* to obtain a compact cake formed by the cottony mycelium (Nout and Kiers, 2005). This technique has been subsequently used as a method of food production and preservation because it improves nutritional, functional, and sensory profiles (Blandino *et al.,* 2003). Fermentation of legumes reduces the levels of antinutritional compounds and oligosaccharides and enhances protein digestibility (Bartkienė *et al.*, 2014; Pandey, 2003; Karnpanit *et al*., 2016), as confirmed with soybean (Nout and Rombouts, 1990), pea (Nowak and Szebiotko, 1992**)** and chickpea (Abu-salem and Abou-arab, 2011). In particular, the flour from previously fermented lupin with *Pedioccocus acidilactici* was added to wheat flour to improve the nutritional quality of breads (Bartkiene *et al.*, 2011). In addition, a significant increase in the content of vitamin B_{12} was achieved through the fermentation of lupin with a starter comprising *Rhizopus oryzae* and *P. freudenreichii*, (Rooijackers *et al*., 2018). Other researchers fermented *Lupinus*

mutabilis with *Rhizopus oligosporus* to reduce alkaloid contents and reported the importance of particle size reduction and optimum moisture for fermentation (Ortega and Rodriguez-Stouvenel, 2014; Carvajal-Larenas *et al.*, 2016). However, this methodology only allowed for the partial detoxification of lupin. Here, we aimed to evaluate the effect of the combination of debittering and solid-state fermentation processes using *Rhizopus oligosporus* on the nutritional profiles of three varieties of *Lupinus mutabilis* of Ecuadorian origin. We quantified the contents of fatty acids, amino acids, dietary fibers, total starch, and minerals.

2. Materials and methods

2.1 Raw material

Bitter lupin (INIAP-450, INIAP-451, and Criollo) varieties were obtained from the National Legumes Program and Andean INIAP Grains. The harvested grain was threshed and introduced into Crippen MFG.INC.USA equipment, which had a set of sieves with 8-15 mm openings. Grains with an average diameter of 8 to 12 mm were used in this study.

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

2.2 Debittering of grains

We applied the thermal-aqueous treatment for debittering. The process was started with the hydration of the grain at an initial temperature of 80 °C for 10 h at a grain to water ratio of 1:3. This step was followed by cooking in water at 91 °C for 1 h and washing with potable water. In this phase, the grain to water ratio was maintained at 1:15. The first washing step was performed with water at 35 °C for 28 h, while the second washing step was carried out at 18 °C for 45 h. The debittered lupin was subjected to a drying process in a forced air oven (HS122A) at 60 °C for 8 h. The grain was ground and packed under conditions similar to those employed for bitter lupines.

2.3 Solid-state fermentation

Rhizopus oligosporus strain NRRL2710 from the Northern Regional Research Laboratory (NRRL) collection (USDA, USA), belonging to the Microbiology laboratory collection at the Ambato University of Technology, was used in this study. For fermentation, the humidity of the debittered grain was reduced to 50% in a forced air oven at 60 °C for 2 h. The grain was crushed in a miniprocessor (Oster, Rio de Janeiro, Brazil), and portions of 50 g were packed and sealed in polypropylene bags for sterilization in an autoclave (Webeco, Farjestäden, Germany) at 121°C for 10 min. About 500 µL of the spore suspension was inoculated onto each grain portion and incubated at 28 °C for 4 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 4 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 contents of protein, fat, crude fiber, and ash were determined using standardized methods (AOAC, 2000). Carbohydrate content was calculated from the difference.

2.4.2 Amino acid profile

Amino acid profile was evaluated with high-performance liquid chromatography (AOAC, 2000). The samples were defatted using solvent extraction at 20 ^{o}C, ground (150 µm), and homogenized before being weighed (25 mg) into hydrolysis tubes. This process was performed by the incubation of samples in an oxygen-free environment and under constant boiling 6 M hydrochloric acid (HCl) at 110 °C for 22 h. The hydrolyzed samples were concentrated on a rotary evaporator and treated with 5 mL of citrate buffer (pH 2.2); the samples were then applied to the LC-10AS Shimadzu liquid chromatography system, which operated under the following conditions: oven temperature, 60 °C; emission length, 450 nm; sample cooler temperature, 4 °C; eluent solution flow, 0.60 $m^2 \cdot min^{-1}$; excitation length, 350 nm; injection volume, 5 µL; and run time, 45 min.

2.4.3 Fatty acid profile

Fatty acids were determined with 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. In total, 50 mg of the extracted oil was subjected to esterification and treated with 1 mL of 0.5 M potassium hydroxide (KOH) in methanol. This mixture was placed in sealed test tubes, which were boiled for 30 min in a water bath. The tubes were cooled to room temperature and treated with 0.5 mL of the mixture hydrochloric acid (HCl) and methanol (1:4). The mixture was boiled for 25 min, cooled, and mixed with 2 mL of double-distilled water. The esters were recovered through three successive washes with n-hexane (chromatography grade) and treated with anhydrous sodium sulfate to eliminate residual water. The supernatant was recovered, and the solvent was evaporated with nitrogen gas. The extract was diluted with 2 mL n-hexane and injected into a gas GC-14B Shimadzu chromatography system. A thermal TR-FAME column (3 m in length, 0.25 mm in diameter, and 0.25 μm pore size) was used for the separation of fatty acid methyl esters. The initial temperature was maintained at 100 °C for 5 min and then increased at a rate of 4 °C/min to 200 °C 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 at a flow rate of 0.8 $mL \cdot min^{-1}$. The injector and detector temperatures were 250 °C and 280 °C, respectively. Peak identification of fatty acids in the analyzed samples was carried out by comparison with retention times of known standards.

2.4.4 Total starch

Polarimetric determination of starch content is based on the optical activity of starch. As starch cannot be dissolved in water, HCl was used. After dissolution, the samples were clarified, filtrated, and measured in a polarimeter. The optical rotation of all samples was measured at 20 °C using a sample cell with an optical path length of 200 mm (AOAC, 2000; Fărcaş *et al*., 2013).

2.4.5 Resistant starch

Samples were incubated in a shaking water bath with pancreatic α-amylase and amyloglucosidase for 16 h at 37 °C. During this incubation period, non-resistant starch

was solubilized and hydrolyzed to D-glucose by the combined action of the two enzymes. The reaction was terminated with the addition of an equal volume of ethanol, and the resistant starch was recovered as a pellet by centrifugation. The pellet was washed twice in ethanol (50% v/v) and centrifuged. Free liquid was removed by decantation, and the resistant starch in the pellet was dissolved in 2 M KOH by vigorously stirring in an ice-water bath with a magnetic stirrer. This solution was neutralized with acetate buffer, and the starch was hydrolyzed to glucose with amyloglucosidase. D-Glucose level was measured with glucose oxidase/peroxidase reagent (GOPOD) and served as the measure of the resistant starch present in the sample (AOAC Official Method 2002.02).

2.4.6 Total dietary fiber

Total dietary fiber content was determined for dried and defatted samples. Samples were cooked at 100 °C with heat-stable α-amylase to achieve gelatinization, hydrolysis, and depolymerization of starch, incubated at 60 °C with protease (to solubilize and depolymerize proteins) and amyloglucosidase (to hydrolyze starch fragments to glucose), and treated with four volumes of ethanol to precipitate soluble fibers and remove depolymerized protein and glucose (from starch). The residue was filtered, washed with 78% ethanol, 95% ethanol, and acetone and then dried and weighed. One duplicate was analyzed for protein level, while the other was incubated at 525 °C to determine ash content. Total dietary fiber was the weight of the filtered and dried residue minus the weight of the protein and ash (AOAC 991.43, AOAC 985.29).

2.4.7 Insoluble and soluble dietary fibers

Dried lupin samples (1 g) were subjected to enzymatic digestion with thermostable α-amylase, protease, and amyloglucosidase. The insoluble dietary fiber was filtered, and then the residue was washed with hot distilled water. The combined filtrate and water wash solution was precipitated with four volumes of 95% ethanol to determine levels of soluble dietary fiber; the precipitate was filtered and dried. For final calculation, both residues (insoluble dietary fiber and soluble dietary fibers) were corrected for protein and ash levels with an appropriate blank (AOAC 991.4).

2.4.8 Mineral composition

For the digestion of samples, the AOAC method 985.35 (2005) was used; the samples were calcined in a 48000 Thermolyne furnace (Waltham, MA USA) at 525 °C, and the ashes were dissolved in 25 mL of 0.1 M nitric acid (HNO_3; trace metal grade). Calibration curves were prepared by the dilution of standards for each mineral at specific concentrations. Analytical curves were obtained with a linear response for the selected concentration range. Mineral analysis was performed with flame spectrophotometry in an AA-7000 atomic absorption spectrophotometer (Shimadzu, Kyoto, Japan), except for phosphorus that was analyzed with colorimetry (AOAC, 2000).

2.5 Statistical analysis

Statistical analysis was performed with the Infostat program (Córdoba, Argentina). The normal distribution of the data was verified with the Shapiro-Wilks goodness of fit test. We applied a multifactorial variance design (analysis of variance [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 Discussions

3.1 Debittering and solid-state fermentation effect on the proximal composition of lupin

Proximal composition of the three varieties of lupin in bitter, debittered, and fermented states is presented in Table 1. Statistical analysis confirmed the significant differences in protein, fiber, mineral, and carbohydrate contents between these lupin varieties following the application of debittering and solid-state fermentation processes.

Among the bitter lupin varieties, Criollo showed the highest content of protein and the lowest levels of minerals and carbohydrates. INIAP-450 variety showed lower levels of crude fibers and higher levels of minerals than INIAP-451 variety. The protein contents were within the values recorded by Sujak *et al*. (2006) but higher than those reported in lupin sown in the Andes of Colombia and Peru (Ortega *et al*., 2010). These differences may be attributed to the variations in species and climatic conditions (Jacobsen and

Mujica, 2008). Considering the impact of treatments, debittering process induced a significant increase in protein levels in the three varieties analyzed owing to the dilution of water-soluble carbohydrates and minerals (Carvajal-Larenas *et al.*, 2016; Erbas, 2010). This observation corroborated the results of nitrogen-free extract, which showed a 70% decrease in the debittered lupin compared to the case for the bitter lupin. Solid-state fermentation resulted in an increase in the protein content of debittered grains, probably owing to the assimilation of carbohydrates, fibers, and some minerals by *Rhizopus oligosporus* to synthesize proteins (Abu-salem and Abou-arab, 2011). The increase in the protein content caused by fermentation was higher than that reported for other fermented legumes such as chickpeas (288.50 $g \cdot kg^{-1}$ dry weight) (Abu-Salem and Abou-Arab, 2011) and beans (316.00 $g \cdot kg^{-1}$ dry weight) (Barampama and Simard, 1995).

Debittering process increased the content of fats (ether extract), likely due to the loss of aqueous soluble compounds. Fermentation induced a slight increase in fat levels. Consistent with the observation reported for protein levels, the metabolic activity of *Rhizopus oligosporus* resulted in the consumption of a part of carbohydrates, fibers, and minerals to synthesize compounds such as fats and other bioactive compounds (Khan *et al.*, 2015). In addition, the content of crude fiber increased by 49.40% (INIAP-450),

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

	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 valus as g·kg^{-1} dry weight. The values with different superscript letters entered in the columns for each variety indicate significant differences (P< 0.05). Mean ± standard deviation (n = 3)

15.92% (INIAP-451), and 43.46% (Criollo) after the debittering with respect to bitter grains, probably owing to the insolubility of these compounds in water (García-López et al., 2001). However, fermentation exerted the opposite effect and decreased the crude fiber content by 15.61% (INIAP-450), 19.02% (INIAP-451), and 29.24% (Criollo). Moreover, solubilization of some minerals during debittering affected the ash content, as evident from the reduction of 38.37%, 50.25%, and 50.73% in INIAP-450, INIAP-451, and Criollo, respectively. Fermentation also decreased of the level of ash (8.63%) in INIAP-450 variety but had no significant effect on the ash content of the other two varieties.

3.2. Debittering and solid-state fermentation effect on the amino acid profile of lupin

The evaluation of the amino acid profile confirmed the significant differences between different varieties depending on the applied process ($P<0.05$) (Table 2). The levels of arginine, aspartic acid, glutamic acid, lysine, leucine and phenylalanine increased in the three varieties of lupin, in response to debittering. The hydrophilic nature of serine, threonine, cystine, tyrosine and valine could have contributed to its higher solubility in water, resulting in the reduction of this amino acid during hydration, cooking, and washing of grain. Comparing the amino acid profile of the debittered and fermented grain, in this latter an increase of most amino acids was determined, except aspartic acid, serine, arginine, alanine, lysine and phenylalanine. *Rhizopus oligosporus* may use a part of these amino acids for its metabolic activity (Handoyo and Morita, 2006), resulting in the reduction in their concentrations in fermented grains as compared to those in debittered grains.

3.3 Debittering and solid-state fermentation effect on the fatty acid profile of lupin

We observed significant effects of the two processes ($P<0.05$) on the fatty acid composition of different varieties of lupin (Table 3). The bitter grains had more than 50% MUFA, with a predominance of oleic acid. MUFA levels decreased in INIAP-450, INIAP-451 and Criollo but increased during the fermentation process by 57.25% in INIAP-450, 55.80% in INIAP-451, and 54.88% in Criollo. PUFA constituted

Table 2. Amino acid profile of bitter, debitter and fermented lupin*

Amino acid	INIAP-450			INIAP-451			CRIOLLO					
	Bitter	Debittered	Fermented	Bitter	Debittered	Fermented	Bitter	Debittered	Fermented	Grain condition	Variety	Interaction
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
Cysteine	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 values as g·kg^{-1} dry weight. The values represent means ± SD of three repetitions. The means ± standard deviations with different superscript letters entered in the columns for each variety are significantly different (P<0.05)

Table 3. Fatty acid profile of bitter, debitter and fermented lupin*

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/SFA		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

*Expressed values as g·kg^{-1} of lupin oil. The values represent means ± SD of three replicates. The means ± standard deviations with different superscript letters are significantly different (P<0.05). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

25% of lupin oil and predominantly included linoleic acid, the levels of which increased after debittering and fermentation. An increase in the fat and fatty acid contents was reported after soy fermentation by Lee *et al.* (1997). The increase in the fatty acid content was consistent with the metabolic process reported for *Rhizopus oligosporus*, which uses carbohydrates and fibers to synthesize fat and fatty acids (Nout and Kiers, 2005). Evaluation of fat quality indices showed that the PUFA/SFA ratio exceeded the value of 1.0, PUFA/MUFA ratio, exceeded the value of 0.5 (not shown) and the (PUFA + MUFA)/SFA ratio was ≥ 2, indicating that the fat content observed after debittering or fermentation met the nutritional objectives (≥ 2) (FAO, 2012). In the good quality oils the ratio between linoleic acid (ω-6) and alpha-linolenic acid (ω-3) varies from 5:1 to 10:1 (FAO, 2012). The oils from the debittered grains were the ones that came closest to this ratio, as follows: 16:1 (INIAP-450), 13:1 (INIAP-451) and 15:1 (Criollo), while the oils from the fermented grains were far from the recommended ratio, as follows: 26:1 (INIAP-450), 27:1 (INIAP-451) and 27:1 (Criollo), probably due to the increase in the linoleic acid level and decrease in the linolenic acid level. FAO (2012) indicates that it is not reasonable to make specific recommendations as long as the intake of the two fatty acids is within the recommended daily values.

3.4 Debittering and solid-state fermentation effect on the total starch, resistant starch, and dietary fiber contents of lupin

We observed significant differences ($P<0.05$) in the contents of starch and dietary fibers after the application of various processes to different grain varieties (Table 4). The contents of total starch and resistant starch decreased after washing during the debittering process in all varieties. Fermentation had no significant effect on the total starch content of INIAP-450 and Criollo but caused a significant decrease in the level of resistant starch in all varieties except Criollo. Nout and Rombouts (1990) found that the enzyme β-glycosidase hydrolyzed the β-glycoside-forming aglycones, the readily available forms of aglycones, during soy fermentation. Thus, enzyme activities may be responsible for the decrease in the levels of resistant starch. The total dietary fiber content was high in INIAP-451. Debittering caused an increase in insoluble fiber levels in all varieties at the expense of a decrease in the levels of water-soluble compounds. Fermentation induced a significant decrease in the level of total dietary fibers as

compared with debittering, probably owing to the hydrolytic action of *Rhizopus oligosporus*.

Table 4. Total starch, resistant starch, and dietary fiber contents of bitter, debittered, and fermented lupin*

	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 values as g·kg^{-1} dry weight. Values represent means ± SD of three replicates. The means ± standard deviations with different superscript letters are significantly different ($P < 0.05$)

3.5 Debittering and solid-state fermentation effect on the macro and micro-mineral content of lupin

The macro and micronutrient contents were significantly affected by the debittering processes in the three varieties (Table 5). The most abundant macro-elements in the three bitter grain varieties were potassium and phosphorus. Among the micro-elements, iron, copper and manganese levels were affected. Debittering increased the content of calcium and decreased the levels of other macro and micro-elements with an exception of sodium, the levels of which showed no significant changes after debittering. Other authors observed similar variations in quinoa and amaranth minerals subjected to soaking and cooking processes (Ertaş and Bilgiçli, 2014; Van de Noort, 2016). Solid-state fermentation caused a decrease in calcium levels in INIAP-450 and Criollo and reduced the levels of phosphorus in INIAP-450. No significant variations were observed in other macro-elements following fermentation. According to Omosebi and Otunola (2013), the loss in calcium may be attributed to the metabolic activity of *Rhizopus oligosporus*, which requires this mineral for the regulation and/or stimulation of the enzymes involved in protein metabolism. In fermented INIAP-451 grain, the levels of manganese, zinc, and iron significantly decreased. The decrease in iron content may be associated with the formation of nitrogen compounds catalyzed by nitrogenase, which requires iron as a cofactor (Viniegra-González, 1997). Copper levels increased after fermentation, possibly due to the dissociation of the complex formed with phytic acid (Ghavidel and Prakash, 2007).

Table 5. Macro- and micro-minerals of bitter, debittered and fermented lupin

	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 values as g·kg^{-1} dry weight. **Expressed as mg·kg^{-1} dry weight. The values represent means ± SDs of three replicates. The means ± standard deviations with different letters are significantly different (P<0.05)

4. Conclusions

The effects of debittering and solid-state fermentation with *Rizophus oligosporus* on the nutritional properties of three lupin varieties were evaluated. Debittering increased the concentrations of protein and several constituent amino acids such as aspartic acid, glutamic acid, arginine, lysine, isoleucine, leucine and phenylalanine. The ether extract and PUFA also increased, which could relate to the dilution of water-soluble carbohydrates and minerals, with an increase in the concentration of compounds that remain in the grain. Fermentation raised the levels of protein and several essential amino acids such as isoleucine, leucine, methionine, cysteine, threonine and valine, as well as ether extract and PUFA but decreased the levels of ash, nitrogen-free extract, resistant starch and soluble dietary fiber, probably due to the requirement of *Rizophus oligosporus* for some specific element of the mentioned compounds or its hydrolytic action. Therefore, the processing of lupin by debittering or solid-state fermentation may serve as an alternative to expand the use of lupin as an ingredient for the fortification of food products.

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CAPITULO 4

Impact of debittering and fermentation processes on the anti-nutritional and antioxidant compounds of *Lupinus mutabilis* Sweet

Elena Villacrés[1*], María Belén Quelal[1], Edgar Fernández[2], Grace Garcìa[3], Gabriela Cueva[2], Cristina M. Rosell[4]

[1]Departament of Nutrition and Quality, National Institute of Agricultural Research, Mejía, Ecuador
[2]School of Chemical Sciences, Faculty of Exact and Natural Sciences, Pontifical Catholic University of Ecuador, Quito, Ecuador
[3]School of Chemical Sciences, Central University of Ecuador, Quito, Ecuador
[4]Institute of Agrochemistry and Food Technology. (IATA-CSIC). Valencia, Spain

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

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Credit Authors Statement
Credit Roles:
EV: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Roles/Writing - original draft
MBQ: Formal statistic analysis
EF: Formal analysis of antinutrients
GG: Formal analysis- polyphenols and carotenoids
GC: Validation methodology of antinutrient analysis
CMR: Conceptualization; Investigation; Supervision; Validation; Writing - review and editing.

Abstract

Lupin is a nutritive grain, but its use is limited due to its high content of bitter alkaloids and other antinutritional factors, such as phytic acid, tannins, nitrates and trypsin inhibitors (TI), that have undesirable physiological effects. There is increasing interest in finding appropriate methods for reducing the antinutritional compounds in lupin. The objective of this research was to assess the efficacy of a biotechnological process, namely, fungal fermentation, as a debittering process relative to that of conventional aqueous thermal treatment (ATT). We evaluated the effects of these processes on the reduction of antinutritional compounds as well as their potential impacts on enhancing the beneficial antioxidant properties of lupin. Three varieties (INIAP-450, INIAP-451 and Criollo) of the *Lupinus mutabilis* species were studied. The application of ATT and fermentation with *Rhizopus oligosporus* caused decreases in the following antinutrients: nitrates (94.47%), tannins (82.14%), alkaloids (93.80%), phytic acid (71.57%) and trypsin inhibitors (76.83%). Urease activity expressed as pH difference decreased to 0.05 in INIAP-450. Ascorbic acid also decreased (79.72%). All values corresponded to the average in the three varieties evaluated. While the contents of phenols, carotenoids and the antioxidant capacity decreased by 96.83, 52.63 and 96.13%, respectively, due to the debittering process, solid fermentation promoted increases in these compounds and properties in the debittered grain.
Key words: Lupin, debittering, cooking, fermentation, legume.

1. Introduction

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

Conversely, health-related benefits have also been linked to some antinutritional factors in legumes, specifically phytic acid, polyphenols, ascorbic acid and carotenoids (Lampart-Szczapa *et al*., 2003). These compounds have been shown to have antioxidant properties as well as beneficial metabolic and physiological effects, such as preventing sclerotic changes in blood vessels and blocking the formation of free radicals (Khan *et al*., 2015). These effects have been described in some lupin species, such as *L. albus, L. luteus* and *L. angustifulius*, and other wild species (Thambiraj *et al*., 2019). However, there is little information regarding *Lupinus mutabilis*, despite being one of the most common species due to its ability to grow in poor soils and under extreme climatic conditions.

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

Solid-state grain fermentation with bacterial or fungal species have been applied to reduce antinutritional compounds, such as phytates and tannins, but also to improve the nutritional quality of grains and pulses, given that these compounds affect the bioavailability of minerals, such as calcium, zinc and iron (Ghoshal *et al*., 2012; Saharan et al., 2020). Fermentation also increases the polyphenol content and improves grain antioxidant activity because microbial action facilitates the breakdown of cell walls and allows the release or synthesis of antioxidant compounds that act as metal chelators or hydrogen donors to free radicals (Nout and Kiers, 2005). However, this biotechnological strategy has been scarcely applied to *L. mutabilis* grains. Fernandez-Orozco *et al*. (2008) studied the impact of fermentation on the antioxidant capacity of *L. angustifolius* and found that there was an increase in total phenolic compounds, peroxyl radical-trapping capacity and Trolox equivalent antioxidant capacity under most fermentation conditions.

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

2. Materials and methods

2.1 Raw material

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

2.2 Reagents

The main reagents used in this investigation were the following: BAPA (N-benzoyl-arginine p-nitroanilide), phytic acid kit (Megazyme), potato dextrose agar (Merck), L-ascorbic acid, chlorogenic acid, Folin-Ciocalteu ABTS (2.2´-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), Trolox (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) Sigma Aldrich brand (St. Louis, Missouri, USA).

2.3 Sample preparation methods

For each variety and process, 2 kg of grain was used. The raw *L. mutabilis* seeds were lyophilized (Labconco Lyph lock 12, Kansas, USA) at -40 °C and -0.9 bars for four days and then ground (Retsch KG -5657 Haan Remscheid, Germany) to a particle size of 250 µm. The sample was packed in polypropylene bags and stored at 10°C until analyses. The thermal-aqueous treatment (ATT) was used for the debittering process. The ATT began by soaking the

grain at an initial temperature of 80 °C for 10 h; a ratio of 1:3 (grain: water) was used. Next, cooking was done in water at 91 °C for 1 h, followed by washing with potable water. A ratio of 1:15 (grain: water) was used for washing. Washing was carried out in two stages: first with water at 35 °C for 28 h followed by water at 18 °C for 45 h (Villacrés *et al*., 2020a). The debittered lupin was dried in a forced air oven (HS122, Labolan, Navarra, Spain) at 60 °C for 8 h. The debittered sample was lyophilized, ground, packed and stored under the same conditions as the bitter grain.

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

2.4 Analysis of anti-nutritional compounds

2.4.1 Nitrates

Nitrate quantification was performed using the method reported by Cataldo *et al*. (1975). Samples were previously homogenized and filtered in a K_2SO_4, 0.34 M solution. The filtrate (0.5 mL) was mixed with 5% salicylic acid and NaOH (4N); Absorbance at 410 nm was measured on a UV-Visible spectrophotometer (Thermo Fisher Scientific 201 Evolution, Madison, WI USA). The nitrate content was expressed in $mg \cdot kg^{-1}$ (dry weight).

2.4.2 Tannins

Tannins were determined with Folin-Denis reagent (AOAC, 1984), using tannic acid as a standard. The absorbance was measured at a wavelength of 680 nm. The tannin content was expressed in $g \cdot kg^{-1}$ (dry weight).

2.4.3 Quinolizidine alkaloids (QAs)

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

2.4.4 Residual Urease Activity

Although urease is not related to protein and starch digestion, the urease test has been used as an indirect method for estimating the degree of trypsin inhibition because its inactivation mechanism is nearly identical to that of trypsin inhibition (Yalcin and Basman, 2015). Lupin flour (0.2 g) was dissolved in 10 mL of urea solution (pH 7.0) in a water bath at 30 ^{o}C for 30 min. The urea solution was replaced with a phosphate buffer to make the blank. The change in pH caused by the conversion of urea to ammonia by the urease enzyme in the sample was measured with AACC Method No: 22-90.01 (AACC, 2000).

2.4.5 Phytic acid

Phytic acid determination was conducted by phosphorus colorimetric quantification from a calibration curve with a phosphorus standard at four concentrations (0.5, 2.5, 5.0 and 7.5 ppm) at 655 nm absorbance using Megazyme kit. The results were expressed in $g \cdot kg^{-1}$dry weight.

2.4.6 Trypsin inhibitors (TI)

Trypsin inhibitor activity was measured following the AOCS Official Method (2009). The extraction of TI was performed by mixing 1 g of defatted lupin flour with 50.0 mL of 0.01 M NaOH and agitating the resulting suspension for 3 h at room temperature. Then a centrifugation step for 10 min at 10,000 x *g* allowed separation of the supernatant (lupin flour extract) for the TI assay.

2.5 Analysis of compounds with antioxidant properties

2.5.1 Ascorbic acid

The ascorbic acid in the samples was extracted with an oxalic acid solution of 0.4% and 20% acetone and quantified using 2.6-dichlorophenol-indophenol (Egoville *et al.*, 1988). Absorbance was measured at 520 nm. L-ascorbic acid was used as a standard.

2.5.2 Total carotenoids

The extraction of carotenoids was conducted with cold acetone and petroleum ether according to the methodology described by Rodriguez-Amaya and Kimura (2004). Absorbance of the ether extract was measured at a wavelength of 450 nm. The extinction coefficient of carotenoids in petroleum ether ($E_{1cm}^{1\%}$ =2500 (L x mol^{-1} x cm^{-1}) was considered in the calculation of total carotenoids.

2.5.3 Total phenolic compounds

Phenolic compounds were determined using Folin-Ciocalteu 2N reagent (Waterhouse, 2002) with minor modifications during the extraction. Solvent extraction was carried out using sonication as a pretreatment. Each sample (0.9 g) was suspended in 10 ml of 80% methanol for 2 h, and 5 min of sonication (20 kHz, 100 W) was applied after each 15 min of agitation. Samples were then centrifuged at 4000 rpm and 10 °C for 5 min, and the supernatant was collected. This process was repeated twice, and all three supernatants were pooled. Absorbance was measured at 765 nm. Results were expressed as chlorogenic acid ($g \cdot kg^{-1}$ dry weight).

2.5.4 Trolox equivalent antioxidant capacity (TEAC)

This test was based on the reduction of ABTS radical cations ($ABTS^{-+}$) by antioxidants present in lupin extracts according to the procedure described by Re et al. (1999). Extraction was performed with 80% methanol. A standard curve was prepared from a stock solution of Trolox (2000 µM trolox$\cdot L^{-1}$), in a concentration range from 100 to 800 µM trolox Eq$\cdot L^{-1}$. TEAC was expressed as µM Trolox Eq$\cdot g^{-1}$ dry weight. All samples were analyzed in triplicate.

2.6 Statistical analysis

The data were analyzed by applying two-factorial ANOVA, using the INFOSTAT statistical software package (Universidad de Córdoba, Argentina) to compare the means with respect to

variety and the condition of the grain. Tukey's multiple range test was applied to determine significant differences at the 5% level. All analyses were performed in triplicate, and the results are given as the mean ± standard deviation.

3. Results and discussion

3.1 Antinutritional compounds

3.1.1 Nitrates, tannins and quinolizidine alkaloids (QAs)

The results of the quantification of nitrate, tannins and alkaloids, as well as the significant statistical differences ($P<0.05$) between varieties in the condition of the grain (debittered or fermented) and their interaction, are presented in Table 1.

Table 1. Effect of debittering and fermentation processes on nitrates, tannins and quinolizidine alkaloid content of lupin grain (dry weight)

Variety	Grain condition	Nitrates ($mg \cdot kg^{-1}$)	Tannins ($g \cdot kg^{-1}$)	Alkaloids ($g \cdot kg^{-1}$)
INIAP- 450	Bitter	366.10 ± 0.79^{b}	9.56 ± 3.66^{b}	37.60 ± 0.07^{b}
	Debittered	18.91 ± 0.02^{c}	1.55 ± 1.34^{g}	3.00 ± 0.02^{c}
	Fermented	14.70 ± 0.16^{c}	1.44 ± 5.82^{h}	2.71 ± 0.04^{c}
INIAP- 451	Bitter	406.30 ± 0.72^{a}	9.20 ± 4.25^{c}	44.72 ± 0.08^{a}
	Debittered	28.62 ± 0.02^{c}	1.93 ± 2.63^{e}	3.50 ± 0.00^{c}
	Fermented	27.50 ± 0.19^{c}	1.79 ± 1.67^{f}	2.92 ± 0.01^{c}
Criollo	Bitter	379.80 ± 1.29^{b}	9.76 ± 5.24^{a}	37.40 ± 0.14^{b}
	Debittered	21.11 ± 0.04^{c}	2.04 ± 2.81^{d}	3.21 ± 0.01^{c}
	Fermented	22.00 ± 0.02^{c}	1.86 ± 1.51^{ef}	1.82 ± 0.04^{c}
p-value	Grain condition	< 0.0001	< 0.0001	< 0.0001
	Variety	< 0.0001	< 0.0001	< 0.0001
	Interaction	< 0.0001	< 0.0001	< 0.0001

Values represent means of three repetitions. The mean ± standard deviation followed by a different letter between the rows for each variety are significantly different ($P<0.05$).

The nitrate content in raw seeds varied with grain variety ($P<0.05$) and was the highest in INIAP-451 (406.3 $mg \cdot kg^{-1}$) followed by the Criollo variety and INIAP-450. Values of these compounds were lower than those that have been reported for other vegetables, such as spinach (485.00 $mg \cdot kg^{-1}$) and leaf lettuce (555.00 $mg \cdot kg^{-1}$), where nitrates are concentrated in vacuoles, leaves and transport organs but less abundant in flowers, tubers and seeds (Ranasinghe and Marapana, 2018). The application of ATT caused a reduction in nitrate by 94.83%, 92.95% and 94.43% in INIAP-450, INIAP-451 and Criollo, respectively. These pronounced reductions likely stemmed from the water solubility of nitrates. The nitrates were reduced ($P<0.05$) by 22.22% (INIAP-450) and 3.91% (INIAP-451) in fermented grains compared to debittered grain. Conversely, in Criollo variety the nitrate level increased by 4.26% compared to the debittered value. The nitrate concentrations in debittered and fermented *L. mutabilis* were slightly higher than the specified maximum permissible levels that have been reported for lettuce and spinach (11.25 $g \cdot kg^{-1}$ dry weight) by the regulations of some European countries (Siomos and Dogras, 2000). Other antinutritional compounds are tannins, which affect mineral and protein utilization (Embaby, 2010) and cause growth depression by decreasing the digestibility of protein and carbohydrate (Liener, 1994). In the three varieties of *L. mutabilis* evaluated in this study, the content of tannins depended on variety and grain condition ($P<0.05$). The highest concentration of tannins (9.76 $g \cdot kg^{-1}$ dry weight) was recorded in the raw Criollo grain. This concentration is higher than what has been reported in soy (0.45 $g \cdot kg^{-1}$ dry weight) and in raw *Lupinus termis* seeds (7.53 $g \cdot kg^{-1}$ dry weight) but is similar to the concentrations of tannins that have been reported in dehulled seeds of the same variety (8.16 $g \cdot kg^{-1}$) (Embaby, 2010). The debittering process resulted in decreases in the contents of tannins in the three varieties by 80.62% relative to the raw grain. Such pronounced decreases are likely explained by the fact that high temperatures break down the tannin-protein complex, thereby inducing the leaching of tannins in the soaking medium and increasing the digestibility and palatability of the grain (Embaby, 2010). The fermentation of *L. mutabilis* caused an additional decrease in tannins (7.64%) relative to those in the debittered grain, which could be attributed to the production of tannase during fermentation (Khan *et al.,* 2018). Similar patterns have been observed in *L. campestris* that was debittered in an aqueous system under alkaline conditions where tannin content was reduced by 77% (alkaline

aqueous treatment) and 70% (aqueous treatment) (Jiménez-Martínez *et al*., 2001). The fermentation of *L. angustifolius* L. with *Rhizopus* sp. decreased tannin content by 90.41% (Khan *et al*., 2018), which is consistent with trends that were observed in *L. mutabilis* in our study. The content of alkaloids in *L. mutabilis* seeds varied with grain variety and grain condition ($P<0.05$). The raw seeds of the three varieties had values between 37.40–44.72 $g \cdot kg^{-1}$ dry weight and were similar to those that have been reported for *L. mutabilis* ecotypes from Perú (33.00–31.00 $g \cdot kg^{-1}$ dry weight) (Múzquiz *et al*., 1989). Alkaloid contents of 38.00 $g \cdot kg^{-1}$, 27.4 $g \cdot kg^{-1}$ and 16.00 $g \cdot kg^{-1}$ dry weight have been reported for *L. albus, L. campestris* and *L. angustifulius*, respectively (Jiménez-Martínez *et al*., 2001; Múzquiz *et al*., 1989). Variability between species is associated with the amount of nitrogen present in the grain, the intensity of sunlight and the temperature of the growing areas (Carvajal-Larenas *et al*., 2016). ATT reduced QAs by 91.86% relative to the bitter grain. The water solubility and the low size of QAs likely contributed to their removal from the lupin seeds. Jiménez-Martínez *et al*. (2001) reported a reduction of 99.96% and 98.95% in QAs of *L. campestris* debittered by aqueous and alkaline treatments. The application of additional techniques such as peeling and autoclaving in *L. campestris* and *L. mutabilis* reduced alkaloids by 55% and 35%, respectively (Jiménez-Martínez *et al*., 2007). Residual QAs of the debittering process were not totally degraded by *R. oligosporus*, and the following values were recorded in the fermented grain: 2.71 $g \cdot kg^{-1}$ dry weight (INIAP-450), 2.92 $g \cdot kg^{-1}$ dry weight (INIAP-451) and 1.82 $g \cdot kg^{-1}$ dry weight (Criollo), levels that are considered safe for human consumption. The safety limit fixed by the health authorities of the UK, France, Australia and New Zealand for the total amount of alkaloids in lupin flours and derived products is 2.3 $g \cdot kg^{-1}$ dry weight (Magalhães et al., 2017).

3.1.2. Urease activity, trypsin inhibitors and phytic acid

These compounds are thermolabile and can alter the digestion of proteins and inhibit the activity of digestive enzymes that cause the hydrolysis of dietary proteins (Egounlety and Aworh, 2003). Urease inactivation is a reliable indicator of the adequacy of heat processing and hence the degree of trypsin inhibitor activity (Yalcin and Basman, 2015). Multiple comparison tests showed that there was a significant effect of variety and grain condition on the urease activity of lupin samples ($P<0.05$).

The debittering process reduced the urease activity, expressed as pH difference to a value of 0.07 (INIAP-450 and Criollo), with fermentation this value decreased to 0.05 in INIAP-450 (Table 2).

Table 2. Effect of debittering and fermentation on urease activity, phytic acid and trypsin inhibitors of lupin grain (dry weight)

Variety	Grain condition	Urease activity (pH difference)	Phytic Acid ($g \cdot kg^{-1}$)	Trypsin Inhibitors ($TIU.mg^{-1}$ sample)
INIAP- 450	Bitter	0.64 ± 0.02^{b}	2.50 ± 0.01^{c}	1.50 ± 0.01^{c}
	Debittered	0.07 ± 0.00^{d}	1.30 ± 0.02^{d}	0.43 ± 0.01^{f}
	Fermented	0.05 ± 0.006^{d}	0.70 ± 0.01^{e}	0.32 ± 0.01^{h}
INIAP- 451	Bitter	0.72 ± 0.01^{a}	3.10 ± 0.03^{b}	1.84 ± 0.00^{a}
	Debittered	0.08 ± 0.01^{d}	1.62 ± 0.00^{d}	0.49 ± 0.01^{d}
	Fermented	0.06 ± 0.00^{d}	1.30 ± 0.02^{d}	0.45 ± 0.00^{e}
Criollo	Bitter	0.58 ± 0.02^{c}	3.91 ± 0.01^{a}	1.56 ± 0.00^{b}
	Debittered	0.07 ± 0.00^{d}	1.32 ± 0.02^{d}	$0.47 \pm 0.00^{d,e}$
	Fermented	0.06 ± 0.00^{d}	0.60 ± 0.01^{e}	0.37 ± 0.00^{g}
p-value	Grain condition	< 0.0001	< 0.0001	< 0.0001
	Variety	< 0.0001	< 0.0001	< 0.0001
	Interaction	< 0.0001	< 0.0001	< 0.0001

Values represent means of three repetitions. The mean ± standard deviation followed by a different letter between the rows for each variety are significantly different ($P<0.05$). UTI: Trypsin Inhibitor Units.

The fermented grain of INIAP-450 had the lowest degree of urease activity (0.05 pH difference), indicating that urease was inactivated (Yalcin and Basman, 2015). In soybeans that were cooked, roasted or extruded, urease activity was reduced by 98% (Qin *et al*., 1996; Yalcin and Basman, 2015). In accordance with the residual urease activity, trypsin inhibitors (TI) were significantly reduced with the application of the debittering and fermentation processes. The bitter grain of three varieties of *L. mutabilis* on average had 1.63 TIU·mg^{-1} dry weight, which was similar to values that have been reported for other lupin species such as *L. exaltatus* (1.37 TIU·mg^{-1}) and *L. reflexus* (2.05 TIU·mg^{-1}) (Ruiz-López *et al*., 2000). A substantial reduction (71.52%) in trypsin inhibitors was observed in debittered grain, but the fermentation produced a further reduction: 25.58% (INIAP-450), 8.16% (INIAP-451) and 21.27% (Criollo). Higher losses were reported in cooked soybeans (82.17%) and fermented with *R. oligosporus* for 48 h (97.42%), which is consistent with the trend that we observed in *L. mutabilis*. In other beans the cooking caused a reduction of 86.09% in trypsin inhibitors; however, fermentation did not change its content (Egounlety and Aworh, 2003). Phytic acid is often regarded as an antinutrient because of its powerful ability to bind minerals, proteins and starches and thereby decrease their bioavailability. However, *in vivo* and *in vitro* studies have demonstrated that phytic acid has preventive as well as therapeutic properties (Mohan *et al*., 2016). Multiple comparison tests showed that variety and grain condition had significant effects on phytic acid content in *L. mutabilis* ($P<0.05$). On average, the three bitter varieties had 3.12 g·kg^{-1} dry weight, which is similar to values that have been reported for other beans (4.6 g·kg^{-1} dry weight) but lower compared with other grains and legumes, such as *L. exaltatus* (11.7 g·kg^{-1}), *L. albus* (14.2 g·kg^{-1} dry weight), *L. angostifolius* (14.5 g·kg^{-1} dry weight) (Múzquiz *et al*., 1989; Ruiz-López *et al*., 2000) and soy (12.7 g·kg^{-1} dry weight) (Egounlety and Aworh, 2003; Carvajal-Larenas *et al*., 2016).

The initial concentration of phytic acid (3.17 mg·kg^{-1} dry weight) was reduced to 1.41 mg·kg^{-1} dry weight after the debittering process. These findings are consistent with the results of a previous study (Vijayakumari *et al*., 2007) that has reported a reduction in phytic acid in soaked and hydrothermally processed *Bauhinia purpurea*. The reduction of phytic acid in lupine grain after fermentation with *R. oligosporus* varied between 19.75-54.54% (Table 2), which may be correlated to the ability of the fungus phytase to

access the substrate. This observation is consistent with the results of Embaby (2010), who found that pretreatments, such as soaking, moistening, pearling, rolling and autoclaving significantly improved the fungal growth of tempeh produced from whole grains and ultimately reduced the contents of antinutritional factors.

3.2 Antioxidant Compounds

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

3.2.1 Ascorbic acid

In the bitter grain, ascorbic acid varied from 58.20 $mg \cdot kg^{-1}$ dry weight (Criollo) to 134.40 $mg \cdot kg^{-1}$ dry weight (INIAP-450). These values were similar to those that have been reported for *L. albus* (64.80 $mg \cdot kg^{-1}$ dry weight) and *L. angustifulius* seeds (57.11 $mg \cdot kg^{-1}$ dry weight) (Fernandez-Orozco *et al*., 2008) but higher than values that have been reported for other legumes (35.0 $mg \cdot kg^{-1}$ dry weight for soybeans and 7.9 $mg \cdot kg^{-1}$ dry weight for beans) (Moriyama and Oba, 2008).

The debittering process reduced the ascorbic acid content of *L. mutabilis* by 46.14%, and fermentation with *R. oligosporus* generated a greater reduction of ascorbic acid relative to the debittered grain. Similar reductions have been reported in soybeans and in cooked beans (Moriyama and Oba, 2008) as well as in fermented lupin with different strains of microorganisms, which possibly use ascorbic acid for its metabolic activities (Fernandez-Orozco *et al*., 2008). The conditions used for the fermentation of lupin in the present study allowed some ascorbic acid to be retained in the lupin grain.

3.2.2 Total carotenoids

In the bitter grain, total carotenoids varied from 3.33 $mg \cdot kg^{-1}$ dry weight (INIAP-450) to 3.97 $mg \cdot kg^{-1}$ dry weight (INIAP-451) (Table 3). The results were similar to values that have been reported for *L. albus* (4.70 $mg \cdot kg^{-1}$ dry weight) but lower than values reported for bitter *L. mutabilis* (14.86 $mg \cdot kg^{-1}$ dry weight) and *L. luteus* (12.52 $mg \cdot kg^{-1}$ dry weight) (El-Difrawi and Hudson, 1979). The debittering process reduced total carotenoids by 52.63%. The temperature used during the debittering treatment appears to be the factor that has the greatest impact on reductions in carotenoids (Kantha *et al*., 1987). Nevertheless, total carotenoids increased with fermentation in the following percentages, in relation to the debittered grain: 131.54% (Criollo), 172.25% (INIAP-451) and 192.47% (INIAP-450). These results are consistent with those in soybeans fermented with six strains of *Rhizopus* sp., where carotenoids increased from 9.1 to 11.2 $mg \cdot kg^{-1}$ dry weight (123%), between 34 and 48 h of fermentation (Denter *et al*., 1998). Presumably, lupin acts as a substrate for *R. oligosporus*, providing carbon, nitrogen, minerals and other growth factors (Villacrés *et al*., 2020b) and producing carotenoids.

3.2.3 Total phenols

An average of 11.27 $g \cdot kg^{-1}$ dry weight as chlorogenic acid was observed in bitter *L. mutabilis*, with the highest value measured from the Criollo variety (13.11 $g \cdot kg^{-1}$ dry weight). These values are within the average reported for raw and bitter *L mutabilis* seeds (12.10 $g \cdot kg^{-1}$ dry weight) (Chirinos *et al*., 2013). We observed a significant decrease (96.83%) in total phenolic contents during the debittering process. As these phenols are thermolabile, such reductions likely stem from the soaking and boiling of the grain, which facilitate their thermal and oxidative decomposition and partial leaching. Fermentation caused a substantial increase in total phenols of the three debittered lupin varieties 1346.15% (INIAP-451), 1016.13% (INIAP-450) and 808.33% (Criollo). The glycosidases of *Rhizopus* might hydrolyze the conjugated polyphenol forms, releasing free polyphenols and, as a consequence, increasing the total phenol content (Lee *et al*., 2008; Khan *et al*., 2018).

Similar results have been observed in *Lupinus angustifolius* fermented with *R. oryzae*, where an increase in phenols of 31.45% has been reported (Fernandez-Orozco *et al*., 2008). The total content of phenols has also been reported to increase by 20.48% and

50.60% relative to unfermented control samples in bean seeds fermented with different strains of *Rhizopus* (Lee *et al*., 2008).

3.2.4 Trolox equivalent antioxidant capacity (TEAC)

The antioxidant capacity of raw seeds measured by the TEAC assay ranged from 707.73 to 747.57 µM Trolox Eq·g^{-1} dry weight (Table 3). Bitter lupin values from our study were higher than those that have been reported in raw seeds of *L. albus* (202.7 µM Trolox Eq·g^{-1} dry weight) (Chirinos *et al*., 2013). However, raw seeds of *L. angustifolius* cv. Emir exhibited higher TEAC values (Martínez-Villaluenga *et al*., 2009). The debittering process caused a decrease of 96.12% in TEAC values; this reduction stemmed from the thermolability and water solubility of phenols. Nonetheless, the fermentation of *L. mutabilis* with *R. oligosporus* increased antioxidant capacity with values that ranged from 340.75 (INIAP-450) to 657.72 µM TroloxEq·g^{-1} dry weight (INIAP-451) relative to debittered grain. This increase might be explained by the ability of some microorganism strains to develop oxidative stress protection mechanisms when they are exposed to reactive oxygen substances (Hur *et al*., 2014). During fermentation, molds produce different enzymes, such as β-glucosidase, which hydrolyze the β-glucosidic bonds of some phenolic compounds, increasing their antioxidant activity. The fermentation of *L. angustifulius*, zapatón variety with *Rhizopus* increased antioxidant activity by 10% relative to unfermented grain (Fernandez-Orozco *et al*., 2008). Increases of 210% and 303% were observed for free radical-scavenging activity and ferric ion-reducing antioxidant power, respectively, following fermentation of germinated seeds of *L. angustifolius* (Khan *et al*., 2018).

Table 3: Effect of debittering and fermentation on the antioxidant content compounds of lupin grain (dry weight)

Variety	Grain condition	Ascorbic Acid ($mg \cdot kg^{-1}$)	Total carotenoids ($mg \cdot kg^{-1}$)	Total Phenols ($g \cdot kg^{-1}$)	Trolox equivalent antioxidant capacity (μM Trolox $Eq \cdot g^{-1}$)
INIAP -450	Bitter	134.4 ± 0.17^{a}	3.33 ± 5.12^{d}	10.34 ± 2.04^{b}	747.57 ± 2.90^{a}
	Debittered	77.4 ± 0.17^{c}	1.86 ± 8.87^{g}	0.31 ± 0.88^{f}	18.88 ± 1.53^{g}
	Fermented	36.0 ± 0.37^{f}	5.44 ± 4.46^{b}	3.46 ± 3.63^{d}	340.75 ± 4.24^{e}
INIAP- 451	Bitter	95.4 ± 0.18^{b}	3.97 ± 1.65^{c}	10.38 ± 2.94^{b}	745.26 ± 8.34^{a}
	Debittered	43.3 ± 0.12^{e}	2.09 ± 5.12^{f}	0.39 ± 0.76^{f}	37.07 ± 2.47^{f}
	Fermented	9.2 ± 0.07^{g}	5.69 ± 5.12^{a}	5.64 ± 7.59^{c}	657.72 ± 4.25^{c}
Criollo	Bitter	58.2 ± 0.12^{d}	3.87 ± 0.00^{e}	13.11 ± 3.93^{a}	707.73 ± 6.95^{b}
	Debittered	34.1 ± 0.31^{f}	1.30 ± 5.12^{h}	0.36 ± 3.68^{f}	$29.27 \pm 1.43^{f,g}$
	Fermented	14.2 ± 0.24^{g}	3.01 ± 0.12^{e}	3.27 ± 2.01^{e}	365.98 ± 4.44^{d}
p-value	Grain condition	<0.0001	<0.0001	<0.0001	<0.0001
	Variety	<0.0001	<0.0001	<0.0001	<0.0001
	Interaction	<0.0001	<0.0001	<0.0001	<0.0001

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

4. Conclusions

The three varieties of *L. mutabilis* in their bitter forms possessed a variety of antinutritional compounds in varying quantities, such as alkaloids, tannins and phytic acid. Nevertheless, bitter lupin varieties also contained functional compounds, such as total phenols, carotenoids and ascorbic acid. The impact of debittering and fermentation processes on the aforementioned compounds was analyzed. Debittering caused a substantial reduction of alkaloids (91.86%), nitrates, tannins, urease activity, trypsin inhibitors and phytic acid. However, the contents of total phenols, carotenoids and ascorbic acid were also affected. Fermentation decreased antinutrients and ascorbic acid from debittered grain and increased total carotenoids, phenolic compounds and antioxidant capacity. In this research it was determined that antinutritional compounds with antioxidant properties are concentrated in bitter lupin, some of these compounds may have pharmacological effects. The outcome of this study may be of great importance to the food industry for the production of novel, fermented food products with improved nutritional value through the fermentation of lupin. The significant concentrations of these phytochemicals in debittered grain suggest that lupin flour could be potentially used in a variety of bakery products.

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CAPITULO 5

Wheat Flour with Debittered and Fermented Lupin: Effects on Bread's Physical and Nutritional Features

Elena Villacrés[1], Paúl Cueva[1, 2], Milene Díaz[2], Cristina M. Rosell[3]

[1]Instituto Nacional de Investigaciones Agropecuarias, INIAP. Quito, Ecuador.
[2] Universidad Central del Ecuador, Facultad de Ciencias Químicas. Quito, Ecuador
[3] Instituto de Agroquímica y Tecnología de Alimentos. IATA, CSIC. Valencia, España


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Elena Villacrés, Paúl Cueva, Milene Díaz & Cristina M. Rosell

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EV: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Roles/Writing - original draft

PC: Formal statistic analysis

MD: validation methods-nutritional analysis of flour and bread

CMR: Conceptualization; Investigation; Supervision; Validation; Writing - review and editing.

Abstract

In this study the breadmaking potential of lupin flour from *L. mutabilis* after being debittered (DLF) and solid state fermented (FLF) was evaluated in lupin-wheat breads. Different levels of substitution (10%, 15%, 20%) were tested on dough rheology and the technological and nutritional (composition and *in vitro* digestibility indexes) properties of breads, as well as acceptability. Lupin weakened the dough during mixing, having shorter development time and stability, especially FLF. Less relevant were the effect of lupin flours along heating-cooling of the doughs recorded with the Mixolab. DLF and FLF significantly affected technological properties of the lupin-wheat breads at higher substitution (>10%), particularly reducing bread volume, crust luminosity, crumb cohesiveness and resilience. Detrimental effects observed at the highest substitutions (20%) were diminished when using FLF, although breads received lower score due to the acidic taste detected by panelists. Both lupin flours provided lupin-wheat breads with rather similar composition, rising the average content of proteins, fat and dietary fiber, compared to wheat breads. Likewise, lupin-wheat breads had significantly lower hydrolytic and glycaemic indexes. Overall, debittered and fermented lupin could be used for enriching wheat breads, although better technological properties were observed with FLF.
Key words: lupin, debittering, fermentation, bread, nutrition, quality.

1. Introduction

Wheat bread constitutes an important part of the diet and remains as staple food across the civilized world (Betoret and Rosell, 2020). Nevertheless, sustainability issues and current consumers' demands have driven the latest innovations in bakery towards sustainable and healthy foods made of either whole grains, alternative grains or even legumes as substitutes for refined wheat. Likewise, wheat replacement in bread recipes for other grains allows reducing wheat importation in non-wheat producers' countries. Lupin (genus *Lupinus*) is an undervalued legume that some decades back was proposed for increasing the nutritional valued of bread (Ballester *et al.*, 1984). However, only some years ago lupin seeds awakened growing interest due to its high protein content (Villarino *et al*., 2016). Because of that, lupin is being used for enriching wheat breads in proteins by directly adding lupin flour from seeds endosperm, or either their proteins isolate or proteins concentrates, with very little changes in product acceptability up to 6% addition (Mubarak, 2001; Paraskevopoulou *et al*., 2012). Conversely, doughs containing lupin products show a reduction in viscoelastic properties that is proportional to the level of lupin products

(Mubarak, 2001; López, 2014; Villarino *et al*., 2015a). Increasing amounts of lupin flour (up to 20%) decrease the bread volume and the crumb texture quality (Villarino *et al*., 2015a), but allows increasing the content in protein and dietary fiber, apart from the content of bioactive compounds like phenols and carotenoids (Villarino *et al*., 2015b). In addition, an intervention study indicated that replacement of wheat bread consumption by lupin enriched breads reduces blood pressure and cardiovascular risk due to the protein and fibre content (Lee *et al*., 2009).

Most of the studies reported for bread enrichment with lupin have been carried out using *L. angustifolius* (Blue Lupin with narrow leaves) (Villarino *et al*., 2014) or *L. albus* (White lupin) (Mubarak, 2001), although other species like *L. luteus* (Yellow lupin) and *L. mutabilis* (Pearl lupin) are greatly produced. In fact, the genus *Lupinus mutabilis* Sweet is one of the most common species because of its adaptation to poor soils and extreme conditions (Van de Noort, 2016), but very scarce information has been reported about its application in breadmaking (Güémes-Vera *et al*., 2008).

Despite the high nutritional value of lupin seeds, its use is very limited due to the presence of bitter compounds, specifically alkaloids derived from quinolizidine (Frick *et al*., 2017). The aqueous debittering process, consistent in several washings, reduces the alkaloids content to safe levels (Cortes-Avendaño *et al*., 2020), but it is quite costly in water and time consuming. This process can be more efficient using thermal treatments (Jimenez-Martínez *et al*., 2009) and saline solutions 0.5% (w/v) for seeds hydration and cooking (Villacrés *et al*., 2020a). Likewise, further nutritional improvement of lupin seeds could be obtained with the solid-state fermentation by using lactic acid bacteria (Bartkiene *et al*., 2013) or fungi (Villacrés *et al*., 2020b). In fact, wholemeal lupin fermented with *Lactobacillus sakei, Pediococcus pentosaceus* or *P. acidilactici* could be added up to 10% as sourdough to wheat flour, improving the rheological properties of dough and wheat-lupin bread volume (Bartkiene *et al*., 2013). Regarding fungi, fermentation of lupin with *Rhizopus oligosporus* induced a further increase of protein levels (Villacrés *et al*., 2020b), but there is no information about its potential application in breadmaking.

The objective of this research was to evaluate the impact of *Lupinus mutabilis* Sweet for wheat flour replacing in breadmaking, and to what extent the solid-state fermentation of lupin could affect the resulting dough and bread quality. With that purpose, different levels of lupin were tested and dough rheological properties as well as nutritional and technological quality of enriched breads were evaluated.

2. Materials and Methods

2.1 Materials

Lupinus mutabilis Sweet, variety INIAP-450, which was grown in the Santa Catalina Experimental Station with the geographic location of altitude 3050 m.a.s.l., latitude UTM 9959382 m S, longitude 17 M0772618 m W was tested. This variety was obtained by selection and evaluation from a germplasm population introduced from Peru in 1992, with the identification of ECU-2659. Commercial wheat flour (WF) for breadmaking (moisture content 14%, ash 0.73%, protein 14.30%, gluten content 33.11%) “Superior France" from Alsuperior S.A. (Quito, Ecuador) was used.

2.2. Production of debittered lupin flour (DLF) and fermented lupin flour (FLF)

The grains were debittered following the aqueous heat process described by Villacrés et al. (2020a). One part of debittered grain (5 kg) was then dried in an air convection cabinet (Labolan HS122A, Navarra, Spain) at 50^{o}C for 6 h, it was cooled down to room temperature and ground in a mill using a 100 µm sieve (Retsch KG -5657 Haan Remscheid, Germany), obtaining the debittered lupin flour (DLF). Other part of debittered grain (5 Kg) was subjected to solid state fermentation following the procedure described by Villacrés *et al*. (2020b). Fermented grains were then dried and ground as above described, to obtain fermented lupin flour (FLF).

2.3. Physical characteristics and chemical composition of flours

pH and total titratable acidity (TTA) were determined after homogenizing 10 g of flour with 90 ml of distilled water, and expressed as milliliters of 0.1 M NaOH needed to reach pH 8.3. Standard methodologies of the (AOAC, 1995) was used to assess: humidity (925.09), protein (total N x 6.25) (955.39), fat (920.39), dietary fiber (991.43) and ash (942.05). The mineral content was determined by atomic absorption spectrophotometry in AA-7000 atomic absorption spectrophotometer (Shimadzu, Kyoto, Japan), except for phosphorus that was colorimetrically analyzed (AOAC, 2000). For the digestion of samples, the AOAC method 985.35 (2005) was followed.

2.4. Dough characterization

Mixolab (Chopin, Villeneuve-la-Garenne Cedex, France) was used to characterize the rheology of the doughs following the standard method AACC (54-60.01). The impact of the lupin flours (DLF and FLF) was evaluated by replacing wheat flour at three levels 10, 15 and 20%. pH and

total titratable acidity (TTA) of lupin-wheat doughs were assessed as described previously for flours.

2.5. Bread making

Bread recipe, based on 100 g flour, was: 5 g sugar, 2 g salt, 9 g seeds oil, 4 g compressed yeast, 3 g dairy powder and the required water assessed in the Mixolab. Ingredients were mixed together in a mixer (Planetaria VFICB7B, Lombardía, Italy) for 7 min. Dough was proofed in a cabinet at 37 °C and 90% relative moisture for 20 min, then dough was divided (~ 170 g), shaped and placed into previously greased stainless steel trays, which were fermented for 1 h at 37 °C. Baking was carried out at 190^{o}C for 25 min in an electric oven (Maquipan UHC-1, Florida, USA). Loaves were cooled down at room temperature for 30 min.

2.6. Bread characterization

Chemical composition of breads was assessed as described previously for flours. The texture was analyzed on a texturometer TA-XT2i, Stable Micro Systems, Godalming, UK, the crust and crumb color were performed using a Portable Spectrophotometer (Lange Spectro-Color d/8° model LZM 268, Chelmsford, United Kingdom) based on the CIE *L**, *a**, *b** color system. The following attributes of visual sensation were measured: *L** (luminosity), *C** (chromatism) and *H** (hue). The determination of specific volume was made according to the method (AACC International. (s.f.) 10-05.01, n.d.), the hydrolytic and glycemic index by the method described by (Goñi *et al*., 1997).

For sensory acceptability, breads were placed on coded white plastic plates and served randomly. Test was performed with twenty-seven trained panelists (14 females and 13 male, ranging in age between 20 and 40 yr) working at the Santa Catalina Experimental Station, INIAP (Quito, Ecuador). Previous group discussion was carried out to define bread characteristics and scores. A 7-point hedonic scale (1 – disliked extremely, 2 – much disliked, 3 – disliked, 4 – liked and did not like, 5 – liked, 6 –a lot, 7 – liked extremely) (Watts *et al*., 1992).

3. Statistical analysis

All analyses were performed in triplicate, the results are given as the mean ± standard deviation. The data were analyzed by applying multifactorial ANOVA, using INFOSTAT statistical software package (Universidad de Córdoba, Argentina), to compare the means with respect to

flour type and substitution level. The Tukey's multiple range test was applied to determine significant differences at the 5% level.

4. Results and Discussion

4.1. Characteristics of debittered and fermented lupin flours and wheat-lupin doughs

There were significant differences ($P<0.05$) on the proximate and minerals composition of the lupin flours, and in consequence wheat replacement induced an enrichment in those compounds ($P<0.05$) (Table 1). The solid-state fermentation significantly increased the protein and fat, with a concomitant reduction in crude fiber, carbohydrate content and minerals (with exception of copper), likely due to the metabolic activity of *Rhizopus oligosporus* (Villacrés *et al.*, 2020b). It must be stressed that the protein content was much higher than the one reported by Mubarak (2001) for deffated lupin flour from *Lupinus albus*, which could be associated to the lupin variety or the debittering process that can greatly affect the nutritional profile of the flours (Curti *et al*., 2018).

Three levels of wheat replacement with lupin flours (DLF, FLF) were tested in the breadmaking process. Lupin flours significantly affected the pH (5.42-6.66) and TTA of the lupin-wheat doughs (Table 1). FLF significantly decreased the pH (5.42) and increased the TTA (7.75 mL 0.1 M NaOH), and the extent of the effect augmented with the level of substitution. In the case of DLF, the pH reduction was only significant with substitutions higher than 10% and significant effect was observed on dough acidity.

When dough rheological properties were evaluated with the Mixolab, the type of lupin flour significantly affected, the development time, dough stability during mixing and heating (C4) and dough consistency after cooling (C5) (Table 2). Water absorption values were in the range reported by Mubarak (2001). Considering the meaning of the Mixolab parameters (Rosell *et al*., 2007), the level of lupin substitution significantly affected the development time of the doughs and C2 related to protein weakening. It has been reported that lupin-wheat doughs had higher water absorption when defatted lupin flour from *Lupinus albus* was used (Mubarak, 2001). DLF progressively decreased the development time when increasing the substitution level, whereas FLF decreased that parameter independently on the level. Despite the gluten reduction when decreasing the relative amount of WF, dough had similar stability to wheat dough or even increased with DLF. Moreover, C2, related to protein weakening, was higher in lupin-wheat doughs at the highest level tested. Results suggested that lupin proteins might be incorporated into the gluten matrix and remained entrapped, giving some consistency during heating. Islam *et al*.

(2011) also suggested that β-conglutins of lupin could be trapped within gluten matrix even after baking, whereas the higher thermal stability of the α-conglutins might explain their no structural integration.

Table 1. Physical characteristics, proximate and minerals composition of debittered (DLF) and fermented (FLF) lupin flours compared to wheat flour

	DLF	FLF	Wheat
pH	6.66±0.03^{b}	5.42±0.025^{a}	5.96±0.05^{a}
TTA (mL 0.1 M NaOH)	0.30±0.001^{b}	7.75±0.002^{a}	0.27±0.06^{b}
Moisture	104.80±0.14^{b}	106.90±0.13^{b}	124.60±0.14^{a}
Ash	21.50±0.50^{a}	19.97±0.50^{a}	6.80±0.01^{b}
Fat	227.51±0.90^{b}	244.00±1.95^{a}	13.91±0.001^{e}
Crude fiber	137.90±3.85^{a}	116.40±5.14^{b}	12.11±0.19^{e}
Protein	546.88±1.45^{b}	608.15±4.35^{a}	144.03±0.01^{c}
Total carbohydrates	66.22±4.92^{b}	11.45±5.10^{c}	823.22±0.08^{a}
Calcium	4.00±0.40^{a}	2.40±0.10^{b}	0.10±0.01^{c}
Phosphorus	4.70±0.50^{a}	3.27±0.35^{b}	2.03±0.01^{c}
Magnesium	0.65±0.05^{a}	0.56±0.11^{b}	0.42±0.01^{c}
Potassium	0.95±0.12^{b}	0.11±0.02^{c}	11.01±0.01^{a}
Sodium	0.12±0.03^{b}	0.11±0.02^{c}	9.00±0.01^{a}
Iron	57.70±1.57^{a}	52.67±1.53^{b}	33.00±0.01^{c}
Zinc	69.96±0.14^{a}	29.75±1.52^{b}	15.00±0.01^{c}
Copper	1.83±0.22^{c}	2.97±0.17^{a}	2.00±0.01^{b}
Manganese	21.33±2.08^{a}	9.70±0.52^{c}	10.00±0.01^{b}

Values followed by different letters between the columns, denote significant differences (P<0.05). Mean ± standard deviation (n=3). Moisture, protein, ash, lipids, crude fiber, total carbohydrates, calcium, phosphorus, magnesium and potassium data are expressed as the g·kg^{-1} dry weight. Sodium, Iron, zinc, copper and manganese are expressed as mg·kg^{-1} dry weight.

Table 2. Effect on wheat flour substitution by debittered and fermented lupin flour on rheological characteristics of dough (Mixolab profile)

	Wheat	Debittered lupin			Fermented lupin		
	0%	10%	15%	20%	10%	15%	20%
Water absorption (%)	64.25 ± 0.35^{c}	66.82 ± 0.02^{b}	62.67 ± 0.98^{c}	63.43 ± 0.07^{c}	62.78 ± 5.31^{c}	70.90 ± 0.73^{a}	61.61 ± 0.43^{d}
Development time (min)	5.28 ± 0.00^{b}	7.45 ± 1.94^{a}	2.60 ± 0.68^{c}	2.12 ± 0.02^{c}	1.39 ± 0.23^{c}	1.21 ± 0.23^{c}	1.08 ± 0.01^{c}
Stability (min)	10.64 ± 0.05^{c}	11.62 ± 0.19^{a}	12.05 ± 0.04^{a}	12.21 ± 0.01^{a}	11.56 ± 1.45^{b}	9.60 ± 0.88^{c}	9.69 ± 1.32^{c}
C2 (Nm)	0.51 ± 0.01^{b}	0.53 ± 0.05^{b}	0.63 ± 0.01^{ab}	0.66 ± 0.01^{a}	0.58 ± 0.04^{b}	0.61 ± 0.04^{ab}	0.65 ± 0.01^{a}
C3 (Nm)	1.61 ± 0.01^{c}	1.53 ± 0.05^{d}	1.54 ± 0.01^{d}	1.57 ± 0.01^{d}	1.84 ± 0.01^{a}	1.73 ± 0.01^{b}	1.71 ± 0.05^{b}
C4 (Nm)	1.29 ± 0.04^{b}	1.06 ± 0.06^{c}	1.06 ± 0.05^{c}	1.03 ± 0.04^{c}	1.79 ± 0.03^{a}	1.77 ± 0.03^{a}	1.68 ± 0.01^{b}
C5 (Nm)	1.42± 0.08^{c}	1.40 ± 0.04^{c}	1.46 ± 0.16^{c}	2.42 ± 0.04^{a}	2.34 ± 0.01^{a}	2.06 ± 0.03^{b}	1.70 ± 0.12^{b}

Values followed by different letters between the columns, denote significant differences (P<0.05). Mean ± standard deviation (n = 3). C2: Protein weakening, C3: Starch gelatinization, C4: amylase activity, C5: starch retrogradation

Starch gelatinization related to C3 was decreased in the presence of DLF but the opposite effect was observed in FLF, in spite of the lower carbohydrate content of this flour (Table 1), thus the other constituents might affect starch gelatinization. FLF doughs showed higher C4, related to the starch stability during heating (Rosell *et al*., 2007). It has been described that in lupin-wheat blends the dough rheology during heating, associated mainly to starch gelatinization, might be masked by the high content of proteins in lupin, consequently (Rosell *et al*., 2009). Again, FLF increased the dough consistency after cooling (C5) and a tendency to decrease it when increasing the level of the FLF flour was envisaged, but the opposite effect was observed with DLF. An increase in C5 has been described when increasing amounts of debittered lupin flour (up to 25%) were blended with wheat, which was related to the interactions between wheat amylose and lupin lipids (one of the major constituents) (Rosell *et al*., 2007). Divergences obtained in C5 with reported results, might be due to the adapted hydration of the doughs used in this study. Nevertheless, considering the possible role of lipids in dough consistency after cooling, it seems that the different lipid profile of DLF and FLF might be responsible of their diverse performance (Villacrés *et al*., 2020b).

4.2.Breads technological properties and acceptability

Regarding parameters related to the technological quality, the lupin treatment significantly affected the specific volume and all color parameters of the crumb, being the effect more marked for breads containing FLF (Table 3). Nevertheless, no significant effect on specific volume was observed with 10% replacement with either of the lupin flours. Higher replacements induced a significant reduction of the specific volume. These results agree with previous findings, attributing that reduction to the gluten replacement by lupin proteins and the level of fiber (Pollard *et al*., 2002; Villarino *et al*., 2015a).

Wheat substitution with lupin significantly reduced the luminosity of the crust, and that effect was intensified when increasing the levels of lupin, significantly in the case of FLF (Table 3). Similar observations have been reported with other lupin-wheat breads (Pollard et al., 2002). Crumb chroma (C^*) significantly increased with both lupin flours, but a steady increase was observed when augmenting the level of FLF, which could be attributed to its higher carotenoids content (5.45 $mg \cdot kg^{-1}$ dry weight) (Villacrés *et al*., 2020c). Similarly, Dervas *et al*. (1999) reported darker crust and yellowish crumbs at levels of substitution higher than 10% with *L. albus*.

The lupin treatment significantly affected the textural parameters, with exception of springiness, whereas the level of substitution significantly affected hardness, chewiness and resilience (Table 3). Specifically, flours type significantly increased the crumb hardness at levels >10% and reduced resilience. The flour type also affected significantly the crumb cohesiveness. Compared to wheat bread, DLF gave similar crumb hardness in the lupin-wheat breads up to 10% substitution, but higher level of substitution resulted in great crumb hardening. Similar hardness has been reported for lupin-wheat breads at those levels of substitution and it has been explained based on gluten dilution (Rosell *et al*., 2009), which has been also observed with lupin protein isolates (10%) that gave more compact crumbs (Lopez and Goldner, 2015). Conversely, 10% FLF substitution gave softer crumbs, and although higher substitution resulted in crumb hardening, the impact what much less than that of DLF. Villarino *et al*. (2015a) studying different varieties of lupins observed that their lipid and protein profile might be responsible of attaining some textural properties similar to wheat breads. Cohesiveness of the crumbs was reduced with the lupin flours but no trend was observed with the level of substitution. Chewiness was even reduced in lupin-wheat breads, compared to wheat bread and only DLF at 20% replacement resulted in a significant increase. Considering that chewiness is inversely related to the easiness of chewing, FLF allowed obtaining more chewy protein enriched wheat breads. Crumbs resilience was significantly reduced with the lupin flours, compared to wheat bread, and the effect was slightly more noticeable with FLF. This decrease could be related to the low specific volume of the breads, having denser crumbs with lower number of gas cells, in consequence the crumb structure takes longer to recover after compression (Matos *et al*., 2014).

The sensory analysis carried out with those breads to check acceptability indicated that only the lupin treatment significantly affected the acceptability, which was similar to wheat bread when using DLF for substitution, but decreased for FLF (Table 3). Panelists attributed the lower acceptance of the FLF-wheat bread due to their acidic taste and flavor. The aroma of lupin-wheat breads has been associated to oxidative degradation of fatty acids or thermal reactions (Paraskevopoulou *et al*., 2012), thus the different fatty acids profile of DLF and FLF (Villacrés *et al*., 2020b) might explain their sensory differences.

Table 3. Effect on wheat flour substitution by debittered (DLF) and fermented (FLF) lupin flour on technological properties and acceptability of bread

	Wheat	DLF			FLF		
		10%	15%	20%	10%	15%	20%
Specific Volume ($cm^3\ g^{-1}$)	5.26±0.04^{a}	5.29±0.10^{a}	4.95±0.14^{b}	4.66±0.03^{c}	5.28±0.03^{a}	4.61±0.05^{c}	4.59±0.03^{c}
*L** crust	53.78±4.74^{a}	52.00±6.06^{a}	48.81± 6.87^{abc}	50.07±5.75^{ab}	47.35±5.91^{abc}	45.14±6.29^{bc}	44.10±4.84^{c}
*C** crust	62.18±10.25^{a}	53.92±12.91^{a}	55.23±11.00^{a}	60.62±11.95^{a}	56.19±9.18^{a}	61.00±9.09^{a}	62.59±6.25^{a}
*h** crust	74.63±2.02^{a}	74.92±2.73^{a}	73.39±3.35^{a}	74.60±2.22^{a}	74.31±2.49^{a}	74.13±2.61^{a}	73.20±1.86^{a}
*L** crumb	60.01±6.37^{a}	51.63±4.04^{d}	52.75±3.77^{d}	57.54±2.03^{b}	53.97±2.71^{c,d}	50.98±2.73^{d}	56.14±1.88^{bc}
*C** crumb	21.48±2.16^{e}	26.53±1.91^{d}	28.43± .95^{c}	27.96±1.62^{c,d}	26.30±0.98^{d}	31.15±2.35^{b}	33.42±1.78^{a}
*h** crumb	80.47±0.70^{b}	80.88±0.38^{b}	83.34±0.38^{a}	83.53±0.23^{a}	82.92±0.43^{a}	82.96±0.32^{a}	80.68±4.52^{b}
Hardness (N)	7.98±0.08^{d}	7.86±0.53^{d}	9.59±0.22^{c}	28.28±0.44^{a}	6.46±0.04^{e}	9.38±0.08^{c}	12.36±0.077^{b}
Springiness	1.37±0.29^{a}	0.99±0.30^{b}	1.13±0.23^{b}	0.96±0.14^{c}	1.18±0.20^{b}	1.12±0.33^{b}	0.85±0.08^{d}
Cohesiveness	0.49±0.02^{a}	0.30±0.06^{d}	0.43±0.06^{b}	0.42±0.01^{b}	0.37±0.03^{c}	0.38±0.05^{bc}	0.39±0.02^{bc}
Chewiness (N)	5.02±0.09^{b}	2.07±145.29^{d}	4.08±0.12^{c}	11.20±0.09^{a}	2.83±0.07^{d}	4.03±0.10^{c}	4.60±0.08^{d}
Resilience	0.28±0.04^{a}	0.10±0.03^{d}	0.20±0.03^{b}	0.24±0.01^{b}	0.14±0.02^{c}	0.15±0.03^{c}	0.16±0.01^{c}
Acceptability	5.20^{a}	5.74^{a}	5.33^{a}	5.41^{a}	4.74^{b}	4.41^{b}	3.52^{c}

Values followed by different letters within columns denote significant differences ($P<0.05$). Mean ± standard deviation (n = 3).

Changes in aroma and taste have been previously reported at 15% substitution with *L. albus* (Campos and El-Dash, 1978). Even lower levels of defatted lupin (9%) has been reported to decrease the overall quality of lupin-wheat breads due to low scoring in texture, crumb color and flavor (Mubarak, 2001).

4.3. Nutritional characteristics of lupin-wheat bread

Bread composition in macro and micronutrients is displayed in Table 4. The multiple factor analysis of variance indicated significant differences ($P<0.05$) promoted by the type of lupine flour (debittering, fermentation) on moisture, ash, fat, crude fiber, protein and carbohydrates content, whereas the level of wheat substitution by lupin additional resulted in significant variation ($P<0.05$) in most nutritional component, except in copper. Compared to wheat bread, breads containing lupin, whatever treatment, had lower moisture content, higher protein, fat and fiber content, which increased with the level of wheat replacement. Despite the higher fiber content, lupin-wheat breads retained less moisture, likely due to the high fat content. Higher moisture content has been reported for breads made with lupin previously fermented with lactic acid bacteria and used as sourdough (Bartkiene *et al*., 2013). For the same level of wheat substitution (20%), lupin enriched breads had similar composition in protein and ash than the one previously reported by Villarino *et al*. (2015b) when compared breads made with debittered lupin from different varieties of *L. angustifolius*, but higher levels of fat and crude protein are obtained in the present study with *L. mutabilis*. Unexpectedly, although FLF had higher protein content than DLF, that difference was not observed in the lupin-wheat breads. Compared to wheat breads, lupin-wheat breads with FLF increased the protein content by 14.71%, 29.75% and 30.53% when the level of substitution was 10%, 15% and 20%, respectively, versus 20.31%, 30.0% and 32.48% obtained with same substitution of DLF. Possibly, nitrogen compounds in FLF were more accessible to yeast during fermentation reducing the theoretical increase. Therefore, initial differences in the nutrient composition of the debittered and fermented lupin flours were not really noticeable in the resulting breads. Regarding the mineral content (Table 4), the flour type and level of substitution affected significantly the amount of all minerals, with exception of copper.

Table 4. Effect on wheat flour substitution by debittered (DLF) and fermented (FLF) lupin flour on proximate (expressed as percentage) and mineral composition, glycemic and hydrolytic index of bread.

	Wheat	DLF			FLF		
		10%	15%	20%	10%	15%	20%
Moisture	371.00±0.44^{a}	344.61±1.93^{b}	349.70±2.10^{b}	314.82±3.56^{d}	304.61±0.15^{e}	334.60±1.18^{c}	307.22±2.17^{de}
Ash	24.71±0.05^{b}	25.30±0.08^{b}	25.82±0.05^{ab}	27.11±0.05^{a}	24.72±0.02^{b}	26.00±0.06^{ab}	27.04±0.07^{a}
Fat	45.82±0.13^{e}	78.20±1.10^{c}	80.11±0.88^{b}	99.50±2.04^{a}	74.72±1.59^{d}	81.30±1.59^{b}	96.92±1.60^{a}
Crude Fiber	11.70±0.12^{b}	27.12±0.13^{b}	32.04±1.09^{a}	35.80±2.01^{a}	22.73±1.12^{c}	28.82±1.41^{b}	33.52±0.26^{a}
Protein	153.63±0.30^{d}	184.80±0.18^{b}	199.71±0.64^{a}	203.50±0.21^{a}	176.20±0.07^{c}	199.31±0.30^{a}	200.51±0.16^{a}
Carbohydrates	768.61±.00^{a}	684.63±0.68^{c}	662.40±0.95^{d}	634.11±1.57^{e}	701.70±0.59^{b}	664.62±0.91^{d}	642.11±0.85^{e}
Calcium	0.42±0.02^{d}	1.05±0.03^{c}	1.22±0.04^{c}	1.32±0.04^{b}	1.10±0.03^{c}	1.33±0.04^{b}	1.42±0.04^{a}
Phosphorus	2.74±0.02^{d}	2.90±0.02^{c}	3.12±0.02^{b}	3.22± 0.02^{a}	3.04±0.02^{b}	3.33±0.02^{a}	3.40±0.02^{a}
Magnesium	0.80±0.01^{c}	0.71±0.01^{d}	0.83±0.01^{b}	0.80±0.01^{c}	0.93±0.01^{a}	0.90±0.01^{a}	0.91±0.01^{a}
Potassium	12.30±0.02^{b}	12.33±0.02^{b}	12.60±0.02^{a}	12.82±0.02^{a}	12.31±0.02^{b}	12.60±0.02^{a}	12.72±0.02^{a}
Sodium	20.00±0.02^{b}	20.00±0.02^{b}	21.00±0.02^{a}	21.00±0.02^{a}	19.00±0.02^{c}	19.00±0.02^{c}	20.00±0.02^{b}
Copper	2.03±0.02	2.00±0.02	2.00±0.02	2.00±0.02	2.01±0.02	2.01±0.02	2.01±0.02
Iron	56.00±0.02^{f}	97.00±0.02^{e}	101.00±0.02^{d}	101.00±0.02^{d}	107.00±0.02^{c}	110.00±0.02^{b}	121.00±0.02^{a}
Manganese	16.00±0.02^{d}	17.00±0.02^{c}	17.00±0.02^{c}	18.00±0.02^{b}	17.00±0.02^{c}	18.00±0.02^{b}	19.00±0.02^{a}
Zinc	18.00±0.02^{f}	23.00±0.02^{c}	24.00±0.02^{b}	25.00±0.02^{a}	20.00±0.02^{e}	22.00±0.02^{d}	24.00±0.02^{b}
Hydrolytic index	100.00±0.00^{a}	51.96±10.96^{b}	50.12±16.60^{b}	49.90±20.44^{b}	49.45±6.77^{b}	45.14±2.52^{c}	39.76±2.67^{d}
Glycemic index	94.61±0.00^{a}	68.24±5.98^{b}	61.07±9.11^{c}	60.11±11.22^{c}	66.86±3.72^{b}	64.49±1.39^{b}	61.54±1.46^{c}
Dietary fibre*	52.8±0.12^{e}	282.5±0.13^{cd}	466.7±1.09^{b}	581.8±2.01^{a}	215.5±1.12^{d}	475.7±0.26^{b}	350.1±1.41^{c}

Values followed by different letters between columns, denote significant differences (P<0.05). Mean ± standard deviation (n = 3). Moisture, protein, ash, lipids, crude fiber, total carbohydrates, calcium, phosphorus, magnesium and potassium data are expressed as the g·kg^{-1} dry weight. Sodium, Iron, zinc, copper and manganese are expressed as mg·kg^{-1} dry weight. *Data are expressed as g·kg^{-1} dry weight.

Nevertheless, compared with the wheat bread, lupin-wheat breads had significantly higher content of calcium, phosphorus, magnesium (only FLF), iron, manganese and zinc, which agrees with the high mineral content of lupin flour (Ertas, 2015). Again, despite the significant differences observed in the lupin flours composition, no great differences were observed between the resulting lupin-wheat breads.

The hydrolytic and glycemic indexes evaluated by *in vitro* methods were affected by both factors, the flour type and the level of substitution (data not shown), with major impact promoted by FLF. Those indexes were significantly lower than the ones obtained for wheat breads, confirming the reduced digestion of the starchy compounds in lupin-wheat breads (Goñi *et al*., 1997). The hypoglycemic effect of the lupin-wheat breads has already been reported and associated to the type of proteins, particularly the γ-conglutins (Sedláková *et al*., 2016). The effect on hydrolytic index was significantly more accentuated in FLF breads and a progressive reduction was observed increasing lupin levels.

Other highly appreciated aspect in breads is the content in dietary fiber and lupin-wheat breads had significantly higher fiber content than wheat bread, particularly in the case of DLF-wheat breads. The DLF flour had significantly higher content of fiber than FLF flour (Table 1). Fiber is reduced during the solid-state fermentation process because *R. oligosporus* partly used it to synthesize fats and bioactive compounds required for its metabolism (Villacrés *et al.*, 2020b). Fiber, besides proteins, of lupin-wheat breads have been associated to low blood pressure (Lee *et al*., 2009).

5. Concluding remarks

Debittered and fermented lupin flours from *L. mutabilis* showed good breadmaking performance at dough and bread level when blended with wheat flour. Lupin-wheat breads without any significant impact on the technological properties could be obtained with 10% wheat substitution. Nevertheless, to further increase the quantities of protein, dietary fiber and minerals in lupin-wheat breads, substitution could be increased up to 20%, although with some detrimental effect on crust luminosity, specific volume, crumb hardness, cohesiveness and resilience. Despite the different proximate composition of debittered and solid-state fermented lupin flours, barely differences were evidenced in the nutritional composition of the lupin-breads, but FLF could be used up

to 20% with less impact on textural properties, and greater reduction on the hydrolytic index, although the acidic taste detected by panelists should be masked.

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DISCUSIÓN GENERAL

Hace cuatro mil años, dos culturas antiguas, la egipcia y la andina, llegaron a domesticar y utilizar dos especies de Lupinus: *Lupinus luteus* en Egipto y *Lupinus mutabilis* en los Andes. En Europa las especies *L. luteus* y *L. albus* se conocen con el nombre de altramuz, se cultivan para forraje y se consumen como una legumbre en toda la zona mediterránea de España, Italia y Grecia. Un caso especial es el de Australia, en donde en la actualidad se cultivan más de 500.000 ha de lupinus dulce (*Lupinus angustifolius*) sobre todo para uso en la alimentación animal y para exportación (Sherasia *et al*., 2017). Esta especie también es cultivada y utilizada en Japón, Corea y varios países de Asia y Europa. En la región Andina el lupino se ha utilizado por miles de años. Restos de sus semillas se han encontrado en las tumbas de la cultura Nazca (100 a 500 a.C.) en la costa desértica del Perú. En el sur, las pinturas representando el lupino en vasos ceremoniales de la cultura Tiahuanaco (500-1.000 d.C.) son una indicación de su amplia distribución (Tapia, 2015). El interés en el lupino andino radica en que es una especie que se adapta a climas fríos, sirve como abono verde, ya que fija 400 kg de nitrógeno por hectárea, es una especie rústica y adaptable a medios ecológicos secos, ubicados entre 2.800 y 3.600 msnm y es la base del sustento de algunas poblaciones rurales pobres (Jacobsen and Mujica, 2008). En los Andes ecuatorianos las especies silvestres de *Lupinus* se pueden encontrar hasta los 4.500 msnm, aunque su mayor distribución está entre los 2.000 y 3.500 msnm (Caicedo and Peralta, 2000). El lupino andino se seleccionó para la alimentación humana y se consume desde Colombia hasta Bolivia (Tapia, 2015).

Los alcaloides impiden la utilización del lupino en alimentación animal o humana sin untratamiento previo y por ello su uso en la antigüedad fue limitado. La búsqueda de especies de lupino sin alcaloides de forma natural fue abordada en Alemania, donde lograron identificar algunas plantas con bajo contenido de alcaloides y con genes capaces de bloquear la síntesis de alcaloides (Petterson, 2016). Estos genes han permitido generar variedades con niveles inocuos de alcaloides, inferiores al 0,05% peso húmedo del grano, dando lugar a los lupinos “dulces” (Petterson, 2016). En *L. mutabilis* el sabor amargo es una característica genética dominante, además los cambios que ocurren entre las diferentes fases fenológicas, la variación morfológica entre las poblaciones de lupino y sus parientes silvestres como resultado del alto nivel de cruzamiento inter-específico, dificultan la obtención de variedades “dulces” (Sedláková,

et al., 2016), por lo que, el procesamiento tecnológico sigue siendo la alternativa para eliminar los alcaloides del grano y hacerlo comestible. El procesamiento acuoso reduce el contenido de alcaloides en la semilla entera hasta niveles seguros para el consumo humano, sin cambiar su sabor natural, lo cual es importante para el aprovechamiento integral del grano. Sin embargo, la aplicación de esta técnica con la especie L. *mutabilis* (con alto contenido de alcaloides) es ineficiente, costosa e insegura, debido al tiempo que dura el proceso (4 a 5 días), la gran cantidad de agua utilizada y las fuentes de procedencia del líquido vital. Esto impulsó la evaluación de los siguientes tratamientos: térmico acuoso (TTA) y térmico salino (TTS) para mejorar la eficiencia del proceso acuoso de desamargado y la calidad del grano. Los dos tratamientos se basaron en el efecto de la temperatura y la hidrosolubilidad de los alcaloides para la remoción del grano. La inclusión de cloruro de sodio (NaCl) en TTS ayudó a disminuir el tiempo a 58 h y el volumen de agua a 66 L, lo que representa un ahorro de 127 L de agua y 38 h con relación al proceso tradicional. Probablemente el NaCl incrementó la micro-estructura porosa del grano, facilitando la penetración de agua y la difusión de alcaloides. Sin embargo, este tratamiento, redujo el contenido de alcaloides, pero también una gran proporción de carbohidratos solubles y minerales, lo que fue compensado por una mayor concentración de otros nutrientes en el grano, especialmente proteína, grasa y fibra dietética, alcanzándose niveles más elevados que los reportados para soya (Carvajal-Larenas *et al*., 2016). La ausencia de sabor amargo, la textura crujiente (dureza 2.182 gf) y la presencia de un color crema (L^*, a^* y b^*; 61,47; 9,18 y 81,52, respectivamente) fueron considerados importantes criterios de calidad logrados con la aplicación de STT, por lo que este método podría ser usado para producir grano desamargado de lupino, a nivel comercial. Los granos de *L. mutabilis* desamargados con los procesos descritos, pueden utilizarse en la alimentación humana, sin peligro de intoxicación. En la zona andina se los consume con un poco de sal como un snack o como base para la preparación de entradas, sopas, guisos, bebidas tipo leche o yogurt (Villacrés *et al*., 2006).

El organismo humano depende de la ingesta nutricional de proteínas para mantener su salud. El suministro de proteína animal tiene impactos ambientales drásticos en el uso de la tierra, la calidad del aire, el agua y los gases de efecto invernadero (Petterson, 2016). La sustitución parcial de proteínas animales por proteínas vegetales podría ser una estrategia prometedora para recudir el impacto ambiental de la nutrición. En este

contexto la fermentación solida con *Rhizopus oligosporus* ayudó a mejorar el contenido nutricional del grano de lupino desamargado, expresado en la mayor concentración del nitrógeno total, el cual alcanzó un valor de 108,27 $g \cdot kg^{-1}$ peso seco (61,73% proteína) al final de la fermentación del grano triturado sin tegumento en las variedades INIAP-450 y Criollo. La digestibilidad aparente de la proteína alcanzó un máximo de 96,07% y el PDCAAS 89,35% en el grano triturado y sin tegumento de la variedad INIAP-450, lo cual podría estar relacionado con la disminución de la estructura compacta de la proteína por efecto de las enzimas de *R. oligosporus* y la disminución de los antinutrientes (Johnson *et al*., 2017). Durante la fermentación, la concentración de nitrógeno soluble y total, siguió una tendencia similar a la curva de crecimiento de los microorganismos en la fase exponencial y respondió a ecuaciones polinomiales de cuarto orden, a partir de las cuales se obtuvieron los parámetros que describen la cinética de fermentación sólida, así como el tiempo óptimo para detener el proceso. Estos resultados son de utilidad para el diseño de un proceso productivo a nivel comercial y su estrategia de operación. También se determinó que el crecimiento de *R. oligosporus* no prospera en el grano amargo de lupino, el cual requiere actividades de preparación del grano, como el remojo, la cocción y lavado del grano y no puede utilizarse como un tratamiento biológico de desamargado. La aplicación de la fermentación sólida, de amplio uso tradicional en el lejano Oriente (Agosin *et al*., 1989) tiene actualmente un gran interés nutricional e industrial en Occidente, para lograr nuevos productos con elevado valor nutritivo que podrían ser usados por determinados grupos de la población como ancianos, niños o utilizarse para suplementar otros alimentos pobres en proteínas.

En relación al efecto de los procesos de desarmargado y fermentación sobre los componentes nutricionales del grano, se determinó que el desamargado ayudó a incrementar el contenido de proteína desde 463,87 hasta 560,60 $g \cdot kg^{-1}$ peso seco (Criollo), a expensas de la pérdida de los compuestos hidrosolubles como algunos carbohidratos y minerales. Un efecto similar se obtuvo con el proceso de fermentación, que aumentó la proteína cruda hasta 644,55 $g \cdot kg^{-1}$ (Criollo), probablemente debido a la transformación de algunos polímeros como el almidón y la fibra en otros componentes (CO_2 y H_2O), favoreciendo la concentración del nitrógeno para formar proteína. Como ocurre en todas las leguminosas, se determinó que los aminoácidos (metionina + cisteína) son los primeros limitantes en las semillas de *L. mutabilis*, con un mayor

contenido de los ácidos glutámico y aspártico, este balance se mantuvo en los granos desamargados y fermentados. No obstante, los habitantes de la zona andina complementan los aminoácidos, mezclando el lupino con cereales como maíz, trigo o quinua. El contenido de grasa de las semillas crudas varió de 167,13 $g \cdot kg^{-1}$ peso seco (INIAP-450) a 174,90 $g \cdot kg^{-1}$ peso seco (Criollo) y se incrementó por efecto del procesamiento hasta 244,03 $g \cdot kg^{-1}$ peso seco en el grano fermentado de INIAP-450. Se determinó que la mayor fracción de la grasa son ácidos grasos insaturados, con una composición semejante al aceite de soya, aproximadamente la mitad de los ácidos grasos correspondieron al ácido oleico con una baja concentración de ácido linolénico, característica que puede favorecer la conservación del aceite, ya que un mayor contenido de este ácido graso oxidaría rápidamente la grasa y podría originar cambios indeseables en el sabor. Los índices de calidad, PUFA/SFA $\geq$ 1, PUFA/MUFA $\geq$ 0,5 y (PUFA + MUFA) /SFA $\geq$ 2, mostraron que la grasa del lupino desamargado o fermentado cumple los requerimientos de calidad para el consumo humano.

Otro componente de interés en el lupino es la fibra y comparando los resultados de los granos crudos con los desamargados, se observó un aumento de la fibra cruda y la fibra dietética hasta 155,23 (INIAP-451) y 540,29 $g \cdot kg^{-1}$ peso seco (Criollo), respectivamente. Estos cambios pueden ser atribuidos a una redistribución de los componentes solubles e insolubles de los polisacáridos no amiláceos, la formación de almidón resistente y productos de la reacción de Maillard durante la cocción del grano (Ertaş and Bilgiçli, 2014). En contraste, la fermentación disminuyó el contenido de fibra bruta y fibra dietética probablemente debido a una mayor solubilización de las sustancias pécticas de estructura ramificada y con mayor susceptibilidad de sufrir una ruptura. Es probable que durante la fermentación los polisacáridos se movilicen proporcionando a *R. oligosporus* cantidades de carbohidratos solubles que pueden ser utilizados para la formación de otros componentes celulares. La composición de la fibra dietética reveló que la fracción insoluble es la más abundante en el grano desamargado de las tres variedades de lupino estudiadas. Por otro lado, INIAP-451 (amargo) presentó contenidos de fibra soluble mayores que las otras dos variedades (20,43 $g \cdot kg^{-1}$ grano seco). Lo que evidencia que los procesos a los que se somete el grano, provocan variaciones en el nivel y composición de la fibra, cambios que dependen del tipo de proceso empleado y de la variedad. En general, los niveles de fibra determinados en *L.*

mutabilis son bajos comparados con otras especies de lupinos. Así *L. albus,* presenta un 25% más de fibra, *L. angostifolius* y *L. luteus*, contienen el doble de fibra que *L. mutabilis* (Johnson *et al*., 2017). Los carbohidratos totales se calcularon a partir de los resultados obtenidos del resto de componentes químicos, por tanto, sus incrementos o reducciones resultaron del comportamiento de dichos componentes. A diferencia de otras leguminosas, en *L. mutabilis* destacó el bajo contenido de almidón que varió de 25,83 a 31,82 $g \cdot kg^{-1}$ peso seco en las tres variedades de grano, valores que se redujeron hasta 13,04 $g \cdot kg^{-1}$ peso seco (Criollo) con el proceso de desamargado, entre las causas probables se citan la solubilización parcial de la amilopectina, las diferencias del tamaño de la semilla, el aumento de la permeabilidad de la membrana y estructura del almidón, lo que facilita la lexiviación de este compuesto en las aguas de remojo, cocción y lavado del grano (Carvajal-Larenas *et al*., 2016). Otro factor puede ser la conversión de una fracción de almidón total en almidón resistente, propiciado por la retrogradación de la amilosa. Sin embargo, los cambios más importantes en la fracción de almidón resistente se produjeron con la fermentación, la cual redujo el contenido de almidón resistente en las tres variedades de lupino consideradas en este estudio, siendo más notable en INIAP-451 (43%), mientras que en Criollo la reducción fue menor (5.42%). Las enzimas hidrolíticas que se activan durante la germinación (α-amilasa y β-amilasa) degradan las sustancias de reserva de los cotiledones, entre las que destaca el almidón (Boye *et al*., 2010). El contenido de este compuesto en la especie *L. mutabilis* contrasta con otras leguminosas como los guisantes y garbanzos que presentan entre 50-70% del peso del cotiledón como almidón (Zornoza-Hernandez *et al.*, 2016).

Comparando los resultados de los granos crudos con los procesados se observó una reducción en el contenido de cenizas y algunos minerales. Por este efecto, en INIAP-451, la reducción de cenizas alcanzó el 49,64% (desamargado) y 2,66% (fermentado). La mayor pérdida de minerales se registró en los granos desamargados, debido a la lixiviación en el agua de remojo, cocción y lavado, con excepción del calcio y zinc, que aumentaron por efecto del mencionado proceso. Posiblemente estos nutrientes se encuentran formando complejos menos hidrosolubles. Otra explicación podría derivarse de una mayor concentración de calcio y zinc en el agua utilizada para el desamargado del grano (Carvajal-Larenas *et al*., 2016). Con la fermentación, disminuyeron la mayoría de macro y micro minerales del grano. Lo cual podría estar asociado al requerimiento de *R. oligosporus* para el desarrollo de sus actividades metabólicas. En

general los niveles de nutrientes determinados en las tres variedades de lupino (INIAP-450, INIAP-451 y Criollo) desamargadas y fermentadas, son mayores que los reportados en otras especies de lupinos (Johnson *et al*., 2017), lo que demuestra el potencial de *L. mutabilis* como fuente alimenticia.

Como ocurre en otras semillas de leguminosas, en el grano de lupino se encuentran algunas sustancias antinutritivas, que limitan el uso directo del grano crudo en la alimentación humana y animal. Algunas de estas sustancias son conocidas como "metabolitos secundarios", están presentes en pequeñas cantidades, pueden tener efectos metabólicos y fisiológicos de interés y presentan cierta actividad biológica (Sánchez-Chino *et al*., 2015).

Existen más de 400 especies del género *Lupinus,* en su forma original todas las especies contienen alcaloides, principios tóxicos que otorgan un sabor amargo a las partes verdes y al grano. Estos compuestos pertenecen al grupo de la quinolizidina derivada de la lisina. Dentro de este grupo, la lupanina es el mayor constituyente (2,5% de los alcaloides totales) en el grano crudo (Sherasia *et al*., 2017). El segundo en importancia es la esparteína y corresponde al 0,32% del grano crudo. Los alcaloides se concentran en los granos, alcanzando niveles entre 1-40 $g \cdot kg^{-1}$ peso seco. La especie *L. mutabilis* presenta el mayor nivel de alcaloides (compuestos de sabor amargo) que hacen las semillas no palatables y tóxicas. La reacción toxicológica a los alcaloides quinolizidínicos varía entre las diferentes especies animales; los peces muestran una elevada sensibilidad a un exceso de estos compuestos (Frick *et al*., 2017). En los humanos y animales, la toxicidad de estos compuestos ha sido demostrada a dosis altas, un adulto tendría que consumir en una sola vez aproximadamente 7 kg de granos desamargados con 1,0 $g \cdot kg^{-1}$ de alcaloides, para provocar una intoxicación aguda (ANZFA, 2001). A medida que aumenta el grado de oxidación disminuye la toxicidad de los alcaloides de lupino y su sabor amargo, por lo que este atributo sensorial es un buen indicador de la toxicidad de la semilla y de protección para el ser humano. La sensibilidad gustativa reacciona frente a un contenido de alcaloides mucho más bajo que el que podría causar daño. El umbral de sabor de la esparteína es aproximadamente 100 veces inferior a la cafeína (patrón comparativo del sabor amargo). El desamargado disminuyó los alcaloides quinolizidínicos en 91,93% y la fermentación un 7,98% adicional.

Los nitratos están presentes en todas las especies vegetales y son una fuente esencial de nitrógeno para el crecimiento de la planta. Sin embargo, cuando estos compuestos se transforman en nitritos pueden causar varias formas de cáncer (Sánchez-Chino *et al*., 2015). Con el proceso de desamargado los nitratos disminuyeron un 94,83%, 92,95% y 94,44% en INIAP-450, INIAP-451 y Criollo, respectivamente. La fermentación produjo una disminución adicional con respecto al grano desamargado: 22,26% (INIAP-450) y 3,91% (INIAP-451). Sin embargo, el efecto contrario se observó en la variedad Criollo, cuyo contenido de nitratos aumentó 4,22% por efecto de la fermentación. Los taninos podrían ser tóxicos en los niveles que se encuentran en el grano crudo de las tres variedades de lupino (9,51 $g \cdot kg^{-1}$ peso seco), sin embargo, el proceso de desamargado disminuyó el contenido de taninos en 80,63% y la fermentación produjo una reducción adicional (7.64%), atribuible a la acción enzimática de *R. oligosporus*.

Los antinutrientes de naturaleza enzimática en las semillas crudas de *L. mutabilis* son bajos, con una diferencia de pH de 0,64 para la actividad ureasa y 1,63 $TIU \cdot mg^{-1}$ para los inhibidores de tripsina. La ureasa es una enzima del lupino, importante por su influencia sobre la calidad de las proteínas, cuya actividad disminuyó hasta 0.07 (diferencia de pH) por efecto del desamargado y 0,05 (diferencia de pH) en INIAP-450 por acción de la fermentación. La presencia de inhibidores de tripsina requiere una elevada cantidad de aminoácidos azufrados en el grano, por lo tanto, el aprovechamiento de las proteínas del lupino disminuye cuando éstas presentan una cantidad limitada de aminoácidos azufrados (Soetan and Oyewole, 2009). Los inhibidores de tripsina disminuyeron con la aplicación de los procesos de desamargado (71,52%) y fementación (18,33%). El ácido fítico registró en las tres variedades de lupino crudo un valor promedio 3,17 $g \cdot kg^{-1}$ peso seco, el cual disminuyó en un 53,99% con el proceso de desamargado y 40,15 % por fermentación del grano.

Junto con los compuestos indeseables, se produce una disminución de otros compuestos que podrían tener efectos beneficiosos, como el ácido ascórbico, el cual disminuyó desde 134,40 $mg \cdot kg^{-1}$ peso seco (INIAP-450, amargo) hasta 9,20 $mg \cdot kg^{-1}$ (INIAP-451, fermentado) y evidencia la termolabilidad de este compuesto durante la cocción del grano y la demanda de este nutriente por *R. oligosporus* durante la fermentación. Una pérdida total de ácido ascórbico fue reportada en lupino fermentado con diferentes

cepas de microorganismos (Fernandez-Orozco *et al*., 2008). El grano de *L. mutabilis* puede significar una fuente promisoria de carotenoides con aplicación en la producción de huevos, ya que el lupino podría ayudar a mejorar el color de la yema, gracias a la presencia de carotenoides, que se encuentran en mayor concentración en el grano fermentado de INIAP-451 (5,69 $mg \cdot kg^{-1}$ peso seco) respecto al desamargado (2,09 $mg \cdot kg^{-1}$ peso seco) de esta misma variedad. La cocción del grano fue el factor con mayor impacto en la pérdida de los carotenoides durante el desamargado. El procesado provocó diferentes comportamientos en el contenido de fenoles totales en las tres variedades estudiadas. El desamargado disminuyó el contenido de fenoles del grano crudo en 96,83%, posiblemente debido a la unión de los polifenoles con otros compuestos como proteínas, otro factor pueden ser las alteraciones en la estructura química de los polifenoles. En contraste, la fermentación provocó un aumento de los fenoles totales: 1346,15% (INIAP-451), 1016,13% (INIAP-450) y 808,33% (Criollo). Esta gran variación en cuanto al comportamiento de los componentes fenólicos podría ser explicada en base al complejo metabolismo bioquímico de *R. oligosporus* durante el proceso fermentativo. Las enzimas endógenas del hongo se activan durante la fermentación provocando diferencias en la composición química de los granos de *L. mutabilis.* Dichas enzimas, como las hidrolasas y polifenoloxidasas, están directamente relacionadas con los compuestos fenólicos y parece que su actividad aumenta durante la fermentación.

Los compuestos fenólicos, el ácido ascórbico, ácido fítico y los carotenoides por sus características estructurales, tienen la facilidad de captar radicales libres, propiedad que les confiere actividad antioxidante. En general, esta actividad fue proporcional al contenido de los mencionados compuestos presentes en el lupino desamargado y fermentado (r = 0.92). La capacidad antioxidante del grano amargo alcanzó un valor promedio de 733,52 µM Trolox $Eq \cdot g^{-1}$. Sin embargo, el proceso de desamargado causó una reducción del 96.12%. Este efecto podría estar asociado con la termolabilidad y la hidrosolubilidad de los fenoles y el ácido ascórbico, que luego pasaron al agua de cocción; otro factor puede ser la degradación o formación de nuevos compuestos. Sin embargo, la fermentación incrementó de forma significativa la capacidad antioxidante de *L. mutabilis* con valores que fluctuaron entre 1150,36-1704,82%. Lo cual podría explicarse por la habilidad de *R. oligosporus* para desarrollar un mecanismo de

protección al estrés oxidativo cuando el hongo es expuesto a sustancias reactivas de oxígeno. Por tanto, la fermentación sólida sería una técnica efectiva para la obtención de metabolitos secundarios como los carotenoides y polifenoles y para aumentar la capacidad antioxidante.

Los granos de lupino desamargados o fermentados son productos perecederos debido a su alto contenido de humedad (60-65%) y nutrientes (Chirinos, 2015). Una alternativa para su conservación fue el secado hasta reducir el contenido de humedad a niveles entre 10 a 12% (Villacrés *et al*., 2006). De la molienda de los granos deshidratados se obtuvo harina, la cual podría ser una alternativa interesante para la elaboración de pan, un producto que goza de amplia aceptabilidad y puede ser almacenado durante más tiempo. Además, considerando el cultivo limitado de trigo, cereal mayoritario para la fabricación de pan, en la zona Andina, la inclusión del lupino podría reducir las importaciones de trigo y por tanto ayudar a mejorar la economía del país. Por ello, se ensayaron tres niveles de sustitución de harina de trigo (10, 15 y 20%) por harina de lupino desamargado (HLD) o fermentado (HLF). El nivel de sustitución afectó el tiempo de desarrollo de las masas, y debilitó su estructura, lo cual fue mayor en las mezclas trigo-lupino al nivel más alto de sustitución. Con una sustitución del 10% no se produjeron cambios sustanciales en las características reológicas de las masas y las características físicas del pan, con relación al producto de trigo. Porcentajes mayores disminuyeron la luminosidad de la miga, la elasticidad, cohesividad, la resiliencia y la aceptabilidad del pan, con un efecto más pronunciado cuando se utilizó HLF en la sustitución. La formulación del pan con harina de lupino desamargado o fermentado presentó mayor contenido de proteína, grasa, fibra, hierro, manganeso y zinc, además de calcio, fosforo y magnesio, aunque solo en el caso de HLF. Adicional al aporte nutricional, el pan elaborado con inclusión de harina de lupino, presentó menor índice hidrolítico y glucémico, propiedades que podrían ayudar a los consumidores preocupados por el cuidado de su salud, especialmente aquellos que padecen enfermedades metabólicas, como la hiperglicemia. Varias investigaciones muestran que la fibra y una fracción de la proteína (γ-conglutina) inhiben a la enzima α-glucosidasa, lo que retarda la digestión de los carbohidratos, la absorción de la glucosa y produce un descenso del índice glicémico (Fornasini *et al*., 2019; Johnson *et al*., 2017). El pan elaborado con la mezcla trigo-lupino tiene un gran potencial para masificar su consumo como producto de alto valor nutritivo y con beneficios para la salud.

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CONCLUSIONES

The main conclusions from the present research are the following:

- Thermal treatments applied to *L. mutabilis*, aqueous (ATT) and saline (STT), resulted in the reduction of approximately 80% of the alkaloids. In general, the debittering process with ATT and STT exerted positive effects on reducing the QAs and increasing protein concentrations in the grain. Moreover, the processes improved the grain texture by decreasing hardness and increasing grain size. However, in the saline process, the sodium chloride helped to optimize the effect of temperature, agitation, and water changes on the reduction of the QAs content. Hence, considering the current debittering processes, the saline heat process is advisable for lupin debittering.

- The analysis of the fermentation kinetics following total and soluble nitrogen allowed determining the specific rate of nitrogen concentration and to establish the optimal time of the fermentation process, which will help in designing bioreactors and its operating strategy. 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).

- Debittering and solid-state fermentation with *Rhizopus oligosporus* may serve as an alternative to expand the use of lupin as an ingredient for the fortification of food products. Debittering increased the concentrations of protein and several constituent amino acids such as aspartic acid, glutamic acid, arginine, lisina, leucina and fenilalanina. Fermentation raised the levels of protein and several essential amino acids such as isoleucine, leucine, methionine, threonine and valine, as well as MUFA and PUFA (INIAP-451 and Criollo), although decreased the levels of ash, resistant starch and soluble dietary fiber.

- The three varieties of *L. mutabilis* in their bitter forms possessed a variety of antinutritional compounds in varying quantities, such as alkaloids, tannins and phytic acid. Nevertheless, bitter lupin varieties also contained functional compounds, such as total phenols, carotenoids and ascorbic acid. Debittering caused a substantial reduction in antinutritional compounds but simultaneously

affected the content of functional compounds. Nevertheless, when debittering is combined with fermentation it could further reduced the antinutrients and increased total carotenoids, phenolic compounds and antioxidant capacity.

- Debittered and fermented lupin flours from *L. mutabilis* showed good breadmaking performance at dough and bread level when blended with wheat flour. Lupin-wheat breads without any significant impact on the technological properties could be obtained with 10% wheat substitution. Nevertheless, to further increase the quantities of protein, dietary fiber and minerals in lupin-wheat breads, substitution could be increased up to 20%, with the concomitant detrimental effect on crust luminosity, specific volume, crumb texture. Although, particularly FLF could be used up to 20% with less impact on textural properties, and greater reduction on the hydrolytic index, although the acidic taste detected by panelists should be masked.

ANEXOS

Research Article

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Effects of two debittering processes on the alkaloid content and quality characteristics of lupin (*Lupinus mutabilis* Sweet)

Elena Villacrés,[a*] Javier Álvarez[a] and Cristina Rosell[b]

Abstract

BACKGROUND: The presence of quinolizidine alkaloids (QAs) in the species *Lupinus mutabilis* Sweet limits the expansion of its consumption and use, despite its high protein content. The objective of this research was therefore to determine the effect of two thermal treatments, aqueous (ATT) and saline (STT), on the QAs and total protein content, as well as on the texture (fracturability and hardness), visual perception attributes – hue (*H), luminosity (*L**) and chromatism (*C**) – and grain size in three lupin varieties (INIAP-450, INIAP-451, and Criollo). The water consumption required by each treatment was also measured.**

RESULTS: The debittering process with ATT helped to concentrate the total nitrogen by 560 g kg^{-1} and decreased the grain hardness to 2037 gf (grams of force) in the Criollo variety, while the chromatic parameters *H and *C** increased in the three varieties. The STT treatment was more efficient than the ATT treatment in terms of the time required and the volume of water used to reduce the QAs to safe levels for consumption (2.5–3.5 g kg^{-1}). The size of the grain increased to four times its original size; the luminosity *L** decreased during cooking to a value of 41.49 in the Criollo variety and then increased to 57.42 during grain washing.**

CONCLUSIONS: The STT treatment is advisable for lupin debittering, although the extent of the effect was dependent on the variety.

Keywords: quinolizidine alkaloids; protein; lupin; fracturability; chromaticity

INTRODUCTION

The species *Lupinus mutabilis* Sweet, which is native to the Andes, is currently cultivated in Ecuador, Peru, and Bolivia, with a certain level of agronomic and agro-industrial technological development. This species is known to be resistant to adverse conditions, such as pests, diseases, drought, and frost.[1] Lupin has attracted attention not only as a feed supplement but also as grain for human food in the current decade, due to its high protein percentage (340–430 g kg^{-1} of dry matter) and acceptable essential amino acid content. The grain also contains vitamins, minerals, unsaturated fat, dietary fiber,[2,3] and phytochemical compounds such as total phenolic compounds and flavonoids.[4] However, the use of *L. mutabilis* has been limited by the presence of toxic substances, mostly quinolizidine alkaloids (QAs).[5] These compounds confer a very bitter taste[6] and a certain degree of toxicity.[7] In *L. mutabilis*, the alkaloid levels range from 30 to 40 g kg^{-1} and a debittering process must be applied to decrease those levels. The debittered lupin seeds are also referred to as sweet lupin when the alkaloid content has been reduced to less than 0.2 g kg^{-1}, which is the maximum concentration currently permitted and adequate for safe consumption.[8] The Food and Agriculture Organization of the United Nations (FAO) recommends that seeds cultivated for human consumption should contain less than 1.0 g kg^{-1} (wet weight) or 3.3 g kg^{-1} (dry weight) alkaloids. A higher content is perceptible, unpalatable, and potentially toxic.[9]

In Andean countries, the grain of *L. mutabilis* is debittered by successive washes with water, which reduce the concentrations of these substances to safe levels for consumption.[10] The artisanal process uses a large volume of water (193 m^3 t^{-1}) and requires a long processing time (5 to 7 days), which implies a substantial economic cost and significant loss of water-soluble nutrients such as vitamins and minerals, flavonoids, monosaccharides, and sucrose.[11] Through this process, including hydration, cooking, and washing, the alkaloid levels of bitter lupins (0.5–40.0 g kg^{-1}) is easily decreased to levels that are safe for human consumption.[12] Several authors determined that the cooking and washing time and number of washing stages significantly influence the final alkaloid content of the lupin grain.[8,12,13] Recently, a mathematical model was generated to improve the artisanal process.[14] However, this model has not been validated experimentally. The greatest changes in the physical characteristics and chemical composition of the grain

* *Correspondence to: E Villacrés, Nutrition and Quality Department, Instituto Nacional de Investigaciones Agropecuarias, INIAP, Panamericana Sur Km 1, Mejía, Quito, Ecuador. E-mail: elena.villacres@iniap.gob.ec*

a *Nutrition and Quality Department, Instituto Nacional de Investigaciones Agropecuarias, INIAP, Quito, Ecuador*

b *Institute of Agrochemistry and Food Technology, IATA, CSIC, Valencia, Spain*

occur during the debittering process. Among the most noticeable are the increase in grain size, difference in color, and decrease in hardness.[12] Texture is an important quality aspect of lupin. It is dictated by the structure of the grain, which, in turn, depends on an interaction of chemical components and physical forces.[15,16] When legumes are processed thermally, first turgor is destroyed, leading to a loss of crisp succulence. Blanching, cooking, and sterilization affect the tissues of vegetables, resulting in a decrease in hardness that is mainly due to changes in cell-wall pectins during heating, which leads to the formation of soluble pectins by degradation of methylated pectins. The most common debittering methods use water at room temperature for washing the grain after cooking, resulting in more acceptable sensorial properties than either debittering with 0.5% $NaHCO_3$ at room temperature (~25 °C) or debittering with hot water (65 °C). However, these processes take between 5 to 6 days.[12] In the case of chickpea, some reduction in the hydration time was reported when it was soaked in NaCl[16]. Nevertheless, when applying it to lupin, cooking with 40% NaCl was needed to decrease up to 51.22% of the alkaloids, but the grain had a salty taste and a subsequent treatment of the process effluents was required to remove the high level of NaCl, making the process costly.[14] NaCl increases the porous microstructure of the grain, facilitates the penetration of water and the diffusion of the alkaloids, reduces the interactions between the minerals and the pectin, and increases the solubility of the proteins.[17] Aqueous and alkaline thermal treatments have been tested for debittering processes. In the case of *L. campestris* and *G. max*, the application of an aqueous thermal treatment resulted in a 56% alkaloid decrease after 3 h, whereas in the case of alkaline treatment a greater decrease (76.5%) was observed.[18] The objective of this study was to improve the debittering process by evaluating the process's critical factors, including temperature, time, and the use of sodium chloride (NaCl), and their effects on QA and total protein content, as well as on the texture (fracturability and hardness) and grain color. These critical factors were evaluated in three different lupin varieties to check the validity of the conditions.

MATERIALS AND METHODS

Materials

The following varieties of *Lupinus mutabilis* Sweet were used: INIAP-450, INIAP-451, and Criollo. These were grown in the Santa Catalina Experimental Station with the geographic location of altitude 3050 m.a.s.l., latitude UTM 9959382 m S, longitude 17 M0772618 m W. INIAP-450 was obtained by selection from a germplasm population introduced in Peru in 1992, with the identification of ECU-2659[19]; the INIAP-451 variety was obtained by selection through participatory processes from the ECU-2658-2 line[20] and the Criollo variety is a native from Chimborazo province. The grain harvested was threshed and classified in a *Crippen* Mfg. Inc. (Michigan, USA) using sieves with pore sizes of 12, 11, and 10 mm. For this study, grains with an average diameter of 10 to 12 mm were used.

The debittering process was performed in a 46 cm diameter × 17 cm high reactor, which was connected to a digital thermostatic immersion circulator (Cole Parmer Polystat, model 01266–02, East Bunker Ct Vernon Hills, US) to control the temperature and velocity of the water flow. The samples were subjected to two different debittering treatments (aqueous and saline thermal). Three batches of each process were performed.

Aqueous thermal treatment (ATT)

The grains were subjected to an aqueous thermal treatment consisting of three stages: hydration (10 h), cooking (1 h), washing (73 h), and samples were collected along the treatment (Fig. 1). The grain was hydrated with stationary water at an initial temperature of 80 °C for 10 h (T1) at a 1:3 ratio (grain: water). Cooking was carried out in water at 91 °C for 1 h (T2), a 1:3 ratio (grain: water) was used, and a water change was performed after the first 30 min of cooking. The aqueous washing of the grain was performed with a stirring system (10.6 L/min), maintaining a ratio of 1:15 (grain: water). The first washing step was made at 35 °C for 28 h, with water changes occurring at the following time intervals: 3 h (T3), 3 h (T4), 16 h (T5), 3 h (T6), and 3 h (T7). In the second stage of washing, the water temperature was maintained at 18 °C for 45 h, and the water was replaced at 18 h (T8), 3 h (T9), 3 h

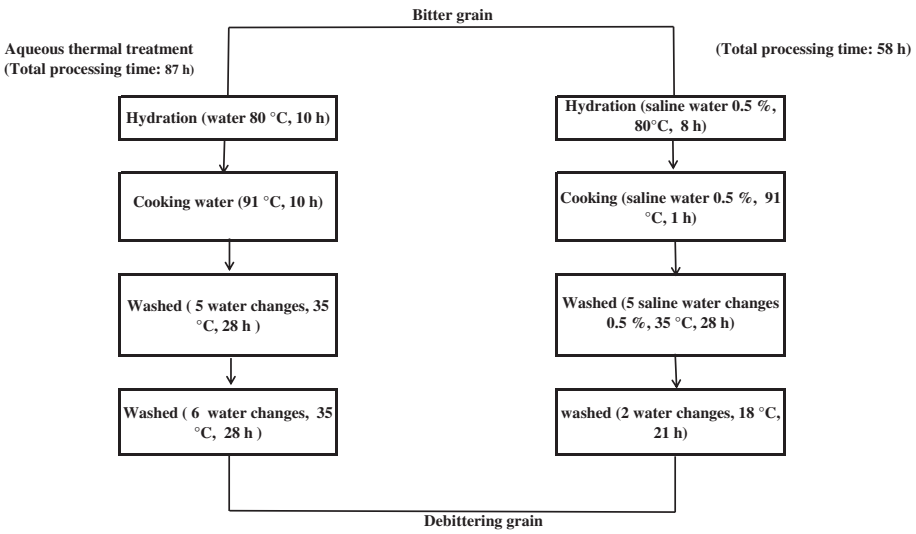

Figure 1. Flowchart of the lupin debittering process with aqueous thermal and saline heat treatments.

(T10), 18 h (T11), and 3 h (T12). The debittering of the grain through the application of aqueous thermal treatment took 84 h.

Saline heat treatment (STT)

A flowchart of lupin debittering through the application of saline heat treatment is shown in Fig. 1. The saline heat treatment was performed by the addition of 0.5% (w/v) sodium chloride to the water used for hydration, cooking, and first wash of the grain at 35 °C (Fig. 1). Hydration was performed at an initial temperature of 80 °C for 8 h (T1 s), and a 1:3 ratio (grain: saline water) was used in a steady state (without agitation). The grain was then cooked at 91 °C for 1 h (T2 s) using a 1:3 ratio (grain: saline water); a water change was performed after the first 30 min of cooking. Grain was washed with saline water using a stirring system (10.6 L/min). In the first 6 h of washing at 35 °C, a ratio of 1:15 (grain: saline water) was used, and for the following washes, the proportions 1:5 and 1:7.5 (grain: saline water) were used. Saline water changes and sampling were performed at the following time intervals: 3 h (T3 s), 3 h (T4 s), 16 h (T5 s), 3 h (T6 s), and 3 h (T7 s). Afterwards, the saline water was replaced with water at 18 °C to remove the NaCl retained in the grain; then, two water changes were implemented: 18 h (T8 s) and 3 h (T9 s).

Analytical methods

Total nitrogen content

The total nitrogen content was determined according to standard method 955.04 from the AOAC.[21]

Total alkaloid content (QAs)

The method described by Gross[22] was used to determine the total alkaloid content, with some modification of the titration. Five milliliters of normal sulfuric acid 0.01 and two drops of methyl red were added to the concentrated chloroform extract, and the acid excess was titrated with 0.01 N NaOH. For the calculation, 1 mL of 0.01 N H_2SO_4 was equivalent to 2.48 mg of lupanin. The total alkaloid content was reported as the lupanin content[23] which is the most abundant alkaloid (55–66%) in the *L. mutabilis* species.[24]

Fracturability and hardness

Fracturability and hardness were measured using a previously reported method.[25] The grains were compressed in a texture analyzer (texturometer TA-XT2i, Micro Systems, Godalming, UK) with a load cell of 5.00 kg and a stainless steel cylindrical probe with a diameter of 5 mm (P5) at a speed of 1 mm·s^{-1}. The grains were compressed in two cycles up to 50% of their initial height, with an interval of 3 s between each cycle. The strength of the first significant break in the positive area of the first bite defines fracturability. The hardness is the strength of the peak during the first compression cycle[26].

Color measurement

Color is a quality parameter, particularly when lupin is used as an egg substitute. Color measurements were performed using a portable spectrophotometer (Lange Spectro-Color d/8° model LZM 268, Chelmsford, UK) based on the CIE L*, a*, b* color system. The following attributes of visual sensation were measured: L* (luminosity), C* (chromatism), and H* (hue). The color differences between the raw and processed lupin seeds were calculated using the following equation: $\Delta E = (\Delta L^*2 + \Delta C^*2 + \Delta H^*2)^{0.5}$, where ΔL*, ΔC* and ΔH* are the differences in the attributes of the visual perception between the raw and processed seeds.[27]

Grain size

The larger and smaller diameters of the grains subjected to different treatments were measured with a Mitutoyo digital pachymeter (Suzano, Brazil).

Hydration capacity

One hundred grains of lupin with an average diameter of 10–12 mm were weighed and then soaked in water for 8 to 10 h. The surface liquid was drained, and the grains were weighed again. The initial weight was subtracted from this value and the amount of embedded water was calculated. Hard seeds do not embed water as fast as normal seeds; their weight at the end of soaking does not change significantly and they float in the hydration water. Normal seeds swell and descend to the bottom of the soaking container. Floating grains are counted and the percentage of hydrated grains is established by calculating

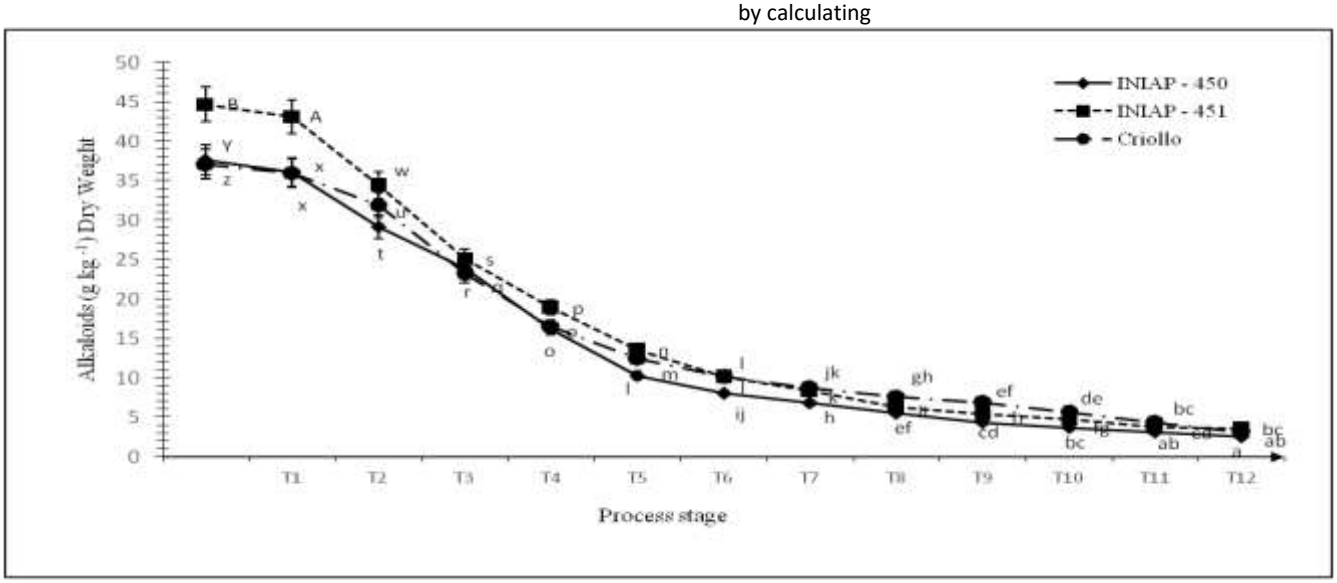

Figure 2. Variation in the total alkaloid content of lupin grain during the aqueous thermal treatment (ATT).

T1 = hydration (80°C, 10 h); T2 = cooking (91°C, 1 h); T3 = washed (35°C, 3 h); T4 = washed (35°C, 3 h); T5 = washed (35°C, 16 h); T6 = washed (35°C, 3 h); T7 = washed (35°C, 3 h); T8 = washed (18°C, 18 h); T9 = washed (18°C, 3 h); T10 = washed (18°C, 3 h); T11 = washed (18°C, 18 h); T12 = washed (18°C, 3 h). Values followed by different letters within and between columns indicate significant differences (P< 0.05). Means ± standard deviations (n = 3).

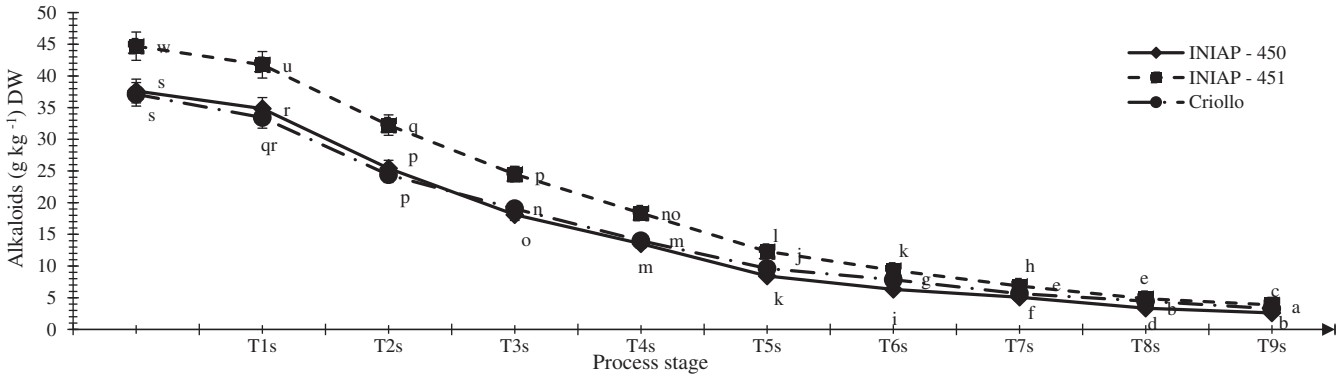

Figure 3. Variations in the total alkaloid content of lupin grain during the saline heat treatment. T1 s = hydration (80 °C, 8 h); T2 s = cooking (91 °C 1 h); T3 s = washed (35 °C, 3 h); T4 s = washed (35 °C, 3 h); T6 = washed (35 °C, 16 h); T6 s = washed (35 °C, 3 h); T7 s = washed (35 °C, 3 h); T8 s = washed (18 °C, 18 h); T9 s = washed (18 °C, 3 h). Values followed by different letters within a column indicate significant differences ($P < 0.05$). Means ± standard deviations (n = 3).

the difference. This parameter provides information about the ability of lupin seeds to rehydrate so that the cooking is more uniform.[28]

Statistical analysis

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

RESULTS AND DISCUSSION

Quinolizidine alkaloids

The quinolizidine alkaloid (QA) content of the grain gradually decreased as a function of the processing stages and washing time (Figs 2 and 3). In the aqueous thermal treatment (ATT), after hydration (T1), the content of QAs in the varieties INIAP-450, INIAP-451 and Criollo decreased by 39.8, 35.7, and 29.6 g kg^{-1} in relation to raw grain, which was due to the water solubility of the QAs in the seeds, in which the QAs are present in the form of salts that are solubilized in polar solvents such as water.[18] The initial temperature of the soaking water (80 °C) accelerated its penetration in the grain, requiring 10 h to hydrate 98% of the seeds, compared to 14 h that are typically applied in the artisanal

Table 1. Variations in the total nitrogen content of the lupin grain* subjected to the aqueous thermal and saline heat treatments

Aqueous thermal treatment stages	INIAP-450	INIAP-451	Criollo	Water used (L)	Saline heat treatment stages	INIAP-450	INIAP-451	Criollo	Water used (L)
Raw grain	75.35 ± 0.22^{wx}	74.22 ± 0.59^{xy}	77.41 ± 0.48^{tu}		Raw grain	75.35 ± 0.22^{q}	74.22 ± 0.59^{r}	77.41 ± 0.48^{no}	
T1	80.13 ± 0.05^{qr}	79.75 ± 0.09^{r}	82.83 ± 0.14^{no}	3	T1 s	78.63 ± 0.23^{m}	77.93 ± 0.23^{mn}	80.27 ± 0.23^{kl}	3
T2	74.99 ± 0.14^{xy}	73.59 ± 0.09^{y}	$76.58 \pm 0.09^{\mu w}$	3	T2 s	72.10 ± 0.23^{s}	72.33 ± 0.23^{s}	77.47 ± 0.23^{no}	3
T3	81.90 ± 0.23^{op}	82.83 ± 0.47^{mn}	87.83 ± 0.14^{fg}	15	T3 s	73.64 ± 0.09^{r}	81.20 ± 0.23^{jk}	82.74 ± 0.09^{i}	15
T4	79.57 ± 0.23^{r}	81.67 ± 0.23^{op}	86.10 ± 0.47^{i}	15	T4 s	75.37 ± 0.23^{q}	84.47 ± 0.23^{gh}	85.63 ± 0.47^{def}	15
T5	81.43 ± 0.23^{op}	83.77 ± 0.47^{lm}	87.73 ± 0.23^{fgh}	7.5	T5 s	77.93 ± 0.47^{mn}	86.80 ± 0.70^{ab}	87.41 ± 0.09^{a}	5
T6	82.13 ± 0.47^{no}	84.47 ± 0.23^{kl}	88.67 ± 0.23^{cde}	7.5	T6 s	76.77 ± 0.23^{op}	84.93 ± 0.23^{fgh}	86.33 ± 0.23^{bcd}	5
T7	81.90 ± 0.70^{op}	78.40 ± 0.23 st	88.20 ± 0.23^{efg}	15	T7 s	76.30 ± 0.23^{pq}	84.19 ± 0.05^{h}	85.87 ± 0.23^{cde}	5
T8	84.70 ± 0.23^{k}	79.10 ± 0.47^{rs}	88.71 ± 0.05^{cde}	7.5	T8 s	80.03 ± 0.23^{l}	85.26 ± 0.09^{efg}	86.66 ± 0.09^{abc}	7.5
T9	85.63 ± 0.23^{ij}	80.97 ± 0.47^{pq}	88.95 ± 0.05^{bcd}	7.5	T9 s	81.43 ± 0.23^{j}	85.59 ± 0.05^{def}	87.27 ± 0.23^{a}	7.5
T10	86.10 ± 0.23^{i}	84.93 ± 0.23^{jk}	89.18 ± 0.09^{abc}	5					
T11	87.03 ± 0.23^{h}	87.73 ± 0.23^{fgh}	89.41 ± 0.05^{ab}	5					
T12	87.50 ± 0.23^{gh}	88.39 ± 0.05^{def}	89.69 ± 0.05^{a}	5					
P-value	Variety (V)	< 0.0001			*P*-value	Variety (V)	< 0.0001		
	Treatment stages (TS)	< 0.0001				Treatment stages (TS)	< 0.0001		
	Interaction V*TS	< 0.0001				Interaction V*TS	< 0.0001		

*Percentage based on g kg^{-1} of dry grain.
Values followed by different letters within a column indicate significant differences ($P < 0.05$). Means ± standard deviations (n = 3).

debittering process.[11] In the first stage (T1 s) of STT, 98% of the grains were hydrated after 8 h of soaking and the decreases in the QAs contents of the seeds were 7.33, 6.71, and 9.97% in INIAP-450, INIAP-451 and Criollo, respectively. This decrease is attributed to the NaCl, which increases the porous microstructure of the grain, and facilitates the penetration of water and the diffusion of the alkaloids.[17] The osmoactive effect of sodium chloride may have favored the countercurrent mass transfer between grain tissues and the saline solution.[29] Cooking contributed to a greater elimination of the QAs. In the cooking stage (T2) of ATT, the QAs contents of INIAP-450, INIAP-451 and Criollo decreased by 19.3, 20.25, and 11.30%, while in the STT, after cooking (T2 s) the QA content was reduced to an even greater extent (27.04, 22.79, and 27.02%). During the washing stage, a progressive reduction in the QA content was observed in the three varieties evaluated. In ATT, the QA content was reduced by 76.66, 75.58, and 72.72% after 28 h of washing the grain at 35 °C (T3-T7). However, with the saline heat treatment (T3 s-T7 s), at the same washing time, a greater reduction in the QA content (80.31, 78.88, and 77.05%) was registered in the three varieties (INIAP-450, INIAP-451 and Criollo, respectively). These results were affected significantly ($P < 0.05$) by the processing stage and variety, probably due to the increase in the ionic strength resulting from the dissociation of NaCl, which favors a greater diffusion of the QAs in the extraction medium.[30] The alkaloid reduction reached after the hydration, cooking, and washing stages (T1-T7) of the ATT process in INIAP-450, INIAP-451, and Criollo was 81.87, 81.14, and 76.46%, respectively). At the same stages (T1 s-T7 s) of the STT, the reduction percentages were 86.49, 84.69, and 84.77% in INIAP-450, INIAP-451, and Criollo, respectively. After washing (T7) at 35 °C, a decrease in grain hardness was observed; the temperature of the washing water was therefore decreased to 18 °C for the last washing stage that lasted 45 h in the aqueous thermal treatment (T8-T12) and 21 h in the saline heat treatment (T8 and T9). In the saline heat treatment, the final wash was performed with water alone to facilitate the removal of the sodium chloride retained in the grain. The alkaloid content of the seeds was affected by the treatment stage and the grain variety ($P < 0.05$).

In both treatments, after washing at 18 °C, the residual QA content ranged from 0.25–0.35% (d.b.), a level considered safe for human consumption, as established by FAO.[23] The debittering process for 1 kg of grain with ATT required 87 h and 96 L of water, with 11 changes, representing a saving of 97 L compared with the artisanal process.[11] The STT was performed in 58 h and 66 L of water was used, with seven changes, which represents a decrease of 26 h in the processing time and a saving of 30 L in the volume of water used compared with the ATT and 127 L with respect to the artisanal process that uses 193 L from rivers or watersheds. In STT, the total volume of water used was similar to that obtained through mathematical modeling[31]; however, the time required to reduce the QAs content was reduced considerably compared to that study, which mentions 4.4 days as the total process time.

Table 2. Variations in lupin grain texture parameters following aqueous thermal and saline heat treatments

Aqueous thermal treatment stages	Fracturability (gf)			Hardness (gf)			Saline heat treatment stages	Fracturability (gf)			Hardness (gf)		
	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo		INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo
T1	467 ± 3^{b}	470 ± 4^{b}	462 ± 3^{a}	3740 ± 160^{i-k}	4265 ± 359^{k}	4090 ± 252^{jk}	T1 s	470 ± 5^{c}	467± 4^{bc}	462 ± 1^{b}	4367 ±376 g	4137 ±30 g	3421 ± 283^{f}
T2	460 ± 1^{a}	460 ± 2^{a}	459 ± 6^{a}	2794 ± 320^{b-h}	2645 ± 301^{a-f}	2876 ± 329^{b-h}	T2 s	462 ± 2^{a}	461 ± 2^{a}	459 ± 1^{a}	2720 ± 456^{a-e}	2786 ± 313^{a-f}	2182 ± 236^{a}
T3	461 ± 1^{a}	461 ± 3^{a}	460 ± 1^{a}	3111 ± 153^{b-h}	2947 ± 98^{b-h}	3031 ± 288^{b-i}	T3 s	462 ± 2^{a}	462 ± 2^{a}	460 ± 2^{a}	2684 ± 514^{a-e}	2804 ± 288^{a-f}	2489 ± 238^{a}
T4	459 ± 2^{a}	460 ± 2^{a}	459 ± 3^{a}	3092 ± 324^{b-i}	2695 ± 252^{c}	2501 ± 348^{ab}	T4 s	462 ± 2^{a}	462 ± 1^{a}	461 ± 1^{a}	2568 ± 274^{a-e}	2859 ± 196^{a-f}	2642 ± 163^{a-e}
T5	459 ± 2^{a}	460 ± 1^{a}	461 ± 1^{a}	3296 ± 353^{d-i}	2764 ± 234^{a-g}	2752 ± 402^{a-h}	T5 s	462 ± 2^{a}	462 ± 1^{a}	461 ± 2^{a}	3037 ± 309^{c-f}	2954 ± 258^{b-f}	2893 ± 332^{b-f}
T6	460 ± 2^{a}	460 ± 1^{a}	460 ± 2^{a}	3342 ± 573^{e-i}	2884 ± 258^{bc}	2788 ± 353^{b-h}	T6 s	461 ± 1^{a}	461 ± 3^{a}	461 ± 2^{a}	2431 ± 29^{a-d}	2646 ± 262^{a-e}	2346 ± 158^{ab}
T7	460 ± 2^{a}	458 ± 2^{a}	460 ± 1^{a}	3110 ± 252^{b-i}	2786 ± 304^{b-h}	2628 ± 228^{a-e}	T7 s	461 ± 1^{a}	461 ± 2^{a}	460 ± 2^{a}	2404 ± 159^{a-c}	2570 ± 290^{a-e}	2321 ± 388^{ab}
T8	460 ± 2^{a}	458 ± 2^{a}	461 ± 2^{a}	3256 ± 321^{c-i}	3415 ± 241 h^{ij}	2798 ± 194^{b-h}	T8 s	462 ± 2^{a}	461 ± 3^{a}	461 ± 1^{a}	2755 ± 351^{a-f}	2895 ± 128^{b-f}	2461 ± 456^{a-d}
T9	460 ± 0.2^{a}	460 ± 2^{b}	462 ± 2^{a}	3360 ± 385^{f-i}	3433 ± 350 h^{ij}	2812 ± 133^{b-h}	T9 s	462 ± 1^{a}	461 ± 2^{a}	463 ± 1^{a}	3085 ± 216^{def}	3156 ± 460^{ef}	2704 ± 422^{a-e}
T10	460 ± 2^{a}	460 ± 2^{a}	462 ± 1^{a}	3365 ± 710 g^{i}	3433 ± 216 h^{ij}	2868 ± 271^{b-h}							
T11	461 ± 2^{a}	459 ± 1^{a}	462 ± 1^{a}	3343 ± 351^{e-i}	2602 ± 197^{a-d}	2560 ± 381^{abc}							
T12	460 ± 2^{a}	458 ± 2^{a}	462 ± 1^{a}	2965 ± 352^{b-h}	2592 ± 426^{a-d}	2038 ± 83^{a}							
Variety (V)		0.723			< 0.0001		Variety (V)		0.000			< 0.0001	
Treatment stages (TS)		< 0.0001			< 0.0001		Treatment stages (TS)		< 0.0001			< 0.0001	
Interaction V*TS		< 0.0001			< 0.0001		Interaction V*TS		0.0044			0.0812	

gf, grams of force. Values followed by different letters within a column indicate significant differences ($P < 0.05$). Means ± standard deviations (n = 3).

Total nitrogen content

During the debittering process, the chemical composition of the grain in the native state was modified following the application of the two treatments (ATT and STT) (Table 1). The total nitrogen content increased slightly in the three varieties of grain during hydration, which was attributed to the reduction in the content of water-soluble compounds (some vitamins, mineral, flavonoids, monosaccharides, and sucrose[11]), including QAs. The thermal

Table 3. Color variations of lupin grain subjected to aqueous thermal treatment

Aqueous thermal treatment stages	L*			C*			H*			ΔE		
	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo
Raw grain	61.47 ± 3.74^{a}	60.85 ± 1.85^{ab}	58.96 ± 1.86^{abc}	9.18 ± 0.75^{j}	10.51 ± 0.694^{j}	10.88 ± 0.68^{j}	81.47 ± 1.69^{mn}	80.587 ± 1.83^{n}	80.71 ± 0.64^{n}			
T1	$56.76 \pm 3.81^{a-d}$	$53.00 \pm 2.58^{d-g}$	50.51 ± 4.47^{g}	16.79 ± 3.41^{i}	$21.221 \pm 6.087^{a-h}$	17.75 ± 4.49^{hi}	$84.26 \pm 2.50^{k-n}$	81.755 ± 1.77^{mn}	82.81 ± 1.73^{lmn}	10.36 ± 3.28^{h}	$13.68 \pm 6.03^{c-h}$	12.27 ± 3.55^{gh}
T2	$56.91 \pm 2.84^{a-d}$	$54.93 \pm 2.96^{c-g}$	$53.303 \pm 3.28^{d-g}$	$21.94 \pm 1.82^{a-g}$	$22.515 \pm 2.429^{a-d}$	22.94 ± 2.16^{abc}	91.08 ± 1.35^{ab}	90.105 ± 1.64^{abc}	91.31 ± 2.00^{a}	$16.91 \pm 1.38^{a-c}$	$16.81 \pm 1.72^{a-d}$	17.45 ± 1.79^{a}
T3	$54.22 \pm 1.71^{c-g}$	$54.66 \pm 2.78^{c-g}$	$52.961 \pm 4.60^{d-g}$	$19.06 \pm 2.27^{d-i}$	24.265 ± 2.089^{ab}	$21.79 \pm 1.50^{a-g}$	89.91 ± 1.53^{abc}	$87.935 \pm 2.14^{a-j}$	89.99 ± 2.48^{abc}	$15.15 \pm 1.22^{a-g}$	17.13 ± 1.82^{ab}	$16.31 \pm 1.43^{a-e}$
T4	$53.26 \pm 3.16^{d-g}$	$54.39 \pm 1.71^{c-g}$	$54.48 \pm 2.12^{c-g}$	$19.61 \pm 1.61^{c-i}$	24.688 ± 2.861^{a}	$22.44 \pm 1.34^{a-e}$	$89.52 \pm 2.07^{a-d}$	$87.951 \pm 1.55^{a-j}$	$89.48 \pm 2.12^{a-d}$	$15.96 \pm 1.19^{a-f}$	17.42 ± 2.48^{a}	$15.47 \pm 1.08^{a-g}$
T5	$56.20 \pm 3.03^{a-e}$	$55.13 \pm 2.60^{c-g}$	$53.893 \pm 3.18^{c-g}$	$20.07 \pm 1.03^{c-i}$	$21.802 \pm 1.268^{a-g}$	$21.80 \pm 2.33^{a-g}$	$89.30 \pm 1.55^{a-f}$	$86.825 \pm 2.26^{c-k}$	$89.16 \pm 2.44^{a-g}$	$14.78 \pm 0.76^{a-g}$	$14.46 \pm 1.54^{a-g}$	$15.28 \pm 1.52^{a-g}$
T6	$54.51 \pm 4.32^{b-e}$	$55.56 \pm 2.85^{b-g}$	$53.57 \pm 4.06^{d-g}$	$18.62 \pm 1.27^{f-i}$	$20.173 \pm 2.015^{c-i}$	$21.59 \pm 2.33^{a-g}$	$89.33 \pm 1.87^{a-f}$	$89.047 \pm 2.05^{a-g}$	$87.88 \pm 2.50^{b-j}$	$14.75 \pm 1.84^{a-g}$	$14.38 \pm 0.95^{a-g}$	$14.79 \pm 1.37^{a-g}$
T7	$56.07 \pm 3.81^{c-fg}$	$52.75 \pm 2.05^{d-g}$	$54.55 \pm 3.08^{c-g}$	$18.59 \pm 2.09^{f-i}$	$20.280 \pm 1.862^{c-i}$	$20.06 \pm 1.94^{c-i}$	$89.73 \pm 2.40^{a-d}$	$86.458 \pm 1.62^{d-k}$	$89.37 \pm 1.72^{a-f}$	$14.38 \pm 1.21^{a-g}$	$14.27 \pm 1.16^{a-g}$	$13.86 \pm 1.22^{b-g}$
T8	$55.27 \pm 2.76^{c-g}$	$53.87 \pm 1.99^{c-g}$	$56.48 \pm 2.93^{a-d}$	18.19 ± 1.96^{ghi}	$22.255 \pm 1.877^{a-f}$	$22.06 \pm 1.28^{a-f}$	$88.61 \pm 1.18^{a-i}$	$85.800 \pm 1.77\ g^{-l}$	$88.85 \pm 1.58^{a-h}$	$13.44 \pm 1.35^{e-h}$	$14.88 \pm 1.50^{a-g}$	$14.40 \pm 1.37^{a-g}$
T9	$55.12 \pm 3.59^{c-g}$	$53.54 \pm 2.16^{d-g}$	50.74 ± 4.01 fg	$19.83 \pm 1.97^{c-i}$	$21.145 \pm 2.475^{a-h}$	$21.55 \pm 1.73^{a-g}$	$87.98 \pm 2.53^{a-j}$	$85.985 \pm 1.80^{f-l}$	$85.25 \pm 2.49^{i-l}$	$14.63 \pm 1.82^{a-g}$	$14.31 \pm 1.92^{a-g}$	$14.79 \pm 2.59^{a-g}$
T10	$54.51 \pm 3.72^{d-g}$	$53.25 \pm 1.82^{c-g}$	$54.73 \pm 2.72^{c-g}$	$20.02 \pm 2.34^{c-i}$	23.143 ± 2.548^{abc}	$22.44 \pm 1.49^{a-e}$	$88.64 \pm 1.75^{a-i}$	$85.538 \pm 0.99^{h-l}$	$87.37 \pm 2.40^{c-k}$	$15.30 \pm 1.87^{a-g}$	$15.80 \pm 1.49^{a-f}$	$14.46 \pm 1.04^{a-g}$
T11	$56.52 \pm 2.63^{a-d}$	$55.97 \pm 2.97^{b-f}$	$52.29 \pm 1.82^{d-g}$	$18.67 \pm 1.43^{e-i}$	$20.103 \pm 0.734^{c-i}$	$20.23 \pm 1.40^{c-i}$	$89.43 \pm 1.93^{a-e}$	$87.717 \pm 1.72^{b-j}$	$86.07 \pm 1.95^{e-l}$	$13.72 \pm 1.06^{c-g}$	$13.31 \pm 0.76^{e-h}$	$12.95 \pm 1.11^{f-h}$
T12	$54.52 \pm 3.88^{c-g}$	51.07 ± 2.74^{efg}	$54.96 \pm 2.56^{c-g}$	17.64 ± 1.68^{hi}	$20.49 \pm 0.96^{b-i}$	$20.72 \pm 1.45^{b-h}$	$88.40 \pm 1.60^{a-i}$	$84.59 \pm 2.60^{j-m}$	$89.82 \pm 2.70^{a-d}$	$13.55 \pm 1.69^{d-h}$	$14.93 \pm 1.36^{a-g}$	$14.47 \pm 0.97^{a-g}$
Variety (V)		< 0.0001			< 0.0001			< 0.0001			< 0.0001	
Treatment stages (TS)		< 0.0001			< 0.0001			< 0.0001			< 0.0001	
Interaction V*TS		< 0.0001			< 0.0001			< 0.0001			< 0.0001	

Values followed by different letters within a column indicate significant differences ($P < 0.05$). Means ± standard deviations (n = 10).

treatment also improved nutritional value through conversion of native protein into more digestible denatured forms.[32]

During cooking, a decrease in the total nitrogen was recorded compared with the hydrated grain. In the cooking process at 91 °C globular proteins are probably not completely denatured, leaving a fraction with the capacity to imbibe water, which is linked to a decrease in the vulnerability to enzymatic attack and to lower digestibility.[33] Comparing both treatments, the greatest loss was registered with the application of STT in INIAP-450 (6.41%) ($P < 0.001$), due to an increase in the solubility of the protein nitrogen induced by a reduction in the number of the electrostatic protein–protein interactions.[33] When the cooked grain was washed, in both treatments, a gradual increase in the total nitrogen concentration was recorded in the three varieties. At the debittering end point, the Criollo variety showed the highest total nitrogen content ($P < 0.05$) following the application of ATT (89.69 g kg^{-1}) and STT (87.27 g kg^{-1}). This increase was attributed to the elimination of some carbohydrates, minerals, and QAs during water changes,[18] while other nutrients, such as protein, remained inside the grain, increasing their proportion.

Fracturability and hardness

Fracturability and hardness are properties that influence the consumer's acceptance of lupin. For this reason, they were monitored during the debittering process. The greatest effect on the properties mentioned was observed in the cooking stage of ATT and STT treatments (Table 2). Cooking induces the largest alteration in structure and concomitantly in texture. Heating thus allows cells to separate, resulting in a softening of the grain. Cell separation has been reported at 76 °C for soaked lima beans.[16] Heating and soaking also produce changes in the cell inclusions. Protein bodies do not appear to be disrupted; however, deviations from the normal spherical structures were observed, perhaps due to swelling.[34,35] The cooking of dried legumes and cereals in a humid environment leads to water migration and simultaneous softening of the grain by degrading the cotyledon tissues in their individual cells, as well as a decrease in the levels of some carbohydrates and an increase in the fiber content.[32,36] Following the application of the two evaluated treatments (ATT and STT), the fracturability did not exhibit significant changes during the washing of the grain at 35 and 18 °C, while the hardness of the three varieties decreased during the washing stage with respect to the hydrated grain, likely due to the increase in water activity.[12] The hardness of the Criollo variety was reduced to 2037 gf (grams of force) when applying the ATT treatment, resulting in a softer grain at the end of the process. A similar variation was detected in grains subjected to STT, along with a progressive decrease in hardness up to a value of 2182 gf during grain washing at 35 and 18 °C (T8 s and T9 s). The water activity of the seeds therefore increased, whereas the hardness decreased.

Color of the grain

In addition to the texture, the color of lupin influences the preference of the consumers and their intent to purchase. Tables 3 and 4 show the statistically significant impact ($P < 0.05$) of the variety and treatment stage on grain color. Following the application of ATT, the luminosity (L^*) value ranged from 61.47 to 50.51, whereas after STT the variation was higher (61.47–41.50). The darkening of the *Lupinus mutabilis* seeds is attributed to the formation of brown pigments, as a result of the Maillard reaction during the cooking, and also the loss of flavonoid and carotenoid pigments.[12] A decrease in L^* was reported after baking barlotto beans, chickpeas, faba beans, white

Table 4. Color variations of lupin grain subjected to saline thermal treatment

Saline heat treatment stages	L^*			C^*			H^*			ΔE		
	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo
Raw grain	61.47 ± 3.74^{a}	60.85 ± 1.85^{a}	58.97 ± 1.86^{abc}	9.18 ± 0.75^{j}	10.51 ± 0.65^{j}	10.88 ± 0.68^{j}	81.47 ± 1.69^{d}	80.59 ± 1.83^{de}	80.71 ± 0.64^{de}			
T1 s	47.24 ± 1.91^{j}	50.72 ± 2.59^{fj}	41.50 ± 4.77^{k}	16.87 ± 2.30^{i}	15.67 ± 3.58^{i}	20.18 ± 2.20^{h}	81.31 ± 0.96^{d}	77.92 ± 1.36^{e}	81.52 ± 2.00^{d}	16.27 ± 2.45^{d-f}	12.05 ± 3.39^{g}	20.15 ± 4.01^{ab}
T2 s	51.56 ± 2.64^{e-j}	50.95 ± 2.22^{e-j}	49.52 ± 5.80^{hij}	21.71 ± 1.10^{e-h}	23.45 ± 2.13^{b-g}	25.69 ± 1.65^{ab}	88.95 ± 1.00^{abc}	87.28 ± 0.99^{c}	88.96 ± 3.23^{abc}	17.81 ± 1.50^{a-f}	17.79 ± 1.86^{a-f}	20.42 ± 1.35^{a}
T3 s	49.85 ± 2.92 g^{j}	49.45 ± 3.33^{ij}	50.68 ± 3.89^{f-j}	20.38 ± 0.57^{gh}	24.89 ± 1.81^{a-d}	25.02 ± 1.68^{abc}	89.46 ± 1.23^{abc}	87.28 ± 1.09^{c}	88.63 ± 1.80^{abc}	18.19 ± 1.68^{a-e}	19.82 ± 1.81^{a-c}	18.64 ± 1.73^{a-d}
T4 s	55.31 ± 3.48^{b-g}	55.81 ± 1.28^{b-e}	53.00 ± 3.63^{d-i}	22.77 ± 3.24^{b-h}	27.41 ± 3.62^{a}	23.60 ± 1.43^{b-f}	91.12 ± 2.19^{a}	89.50 ± 1.04^{abc}	89.74 ± 2.90^{abc}	18.37 ± 1.80^{a-e}	19.92 ± 2.90^{ab}	17.30 ± 1.03^{b-f}
T5 s	59.22 ± 1.08^{ab}	57.42 ± 2.45^{a-d}	55.60 ± 3.21^{b-f}	21.06 ± 1.26^{fgh}	24.72 ± 1.65^{a-e}	25.17 ± 0.91^{ab}	90.40 ± 1.19^{ab}	89.94 ± 2.27^{abc}	88.96 ± 3.08^{abc}	15.14 ± 0.69^{f}	17.68 ± 1.05^{a-f}	17.37 ± 0.72^{b-f}
T6 s	55.21 ± 1.67^{b-g}	55.56 ± 3.19^{b-f}	55.73 ± 3.28^{b-e}	21.14 ± 1.81^{fgh}	23.89 ± 2.16^{b-f}	25.25 ± 2.28^{ab}	90.47 ± 1.18^{ab}	89.47 ± 2.56^{abc}	88.79 ± 1.70^{abc}	16.36 ± 1.52^{d-f}	17.40 ± 1.63^{b-f}	17.21 ± 1.89^{b-f}
T7 s	56.64 ± 2.07^{a-d}	56.63 ± 3.22^{a-d}	50.47 ± 2.32^{f-j}	21.93 ± 2.50^{c-h}	23.94 ± 1.17^{b-f}	23.60 ± 1.31^{b-f}	89.88 ± 1.71^{abc}	88.49 ± 1.68^{abc}	87.36 ± 1.03^{c}	16.31 ± 1.75^{d-f}	16.53 ± 0.76^{d-f}	16.86 ± 1.06^{c-f}
T8 s	54.81 ± 2.15^{b-h}	53.99 ± 3.13^{c-i}	54.53 ± 3.15^{b-h}	21.79 ± 1.81^{d-h}	23.94 ± 1.79^{b-f}	23.20 ± 1.33^{b-h}	90.74 ± 1.20^{a}	88.96 ± 1.98^{abc}	89.84 ± 2.29^{abc}	17.21 ± 1.31^{b-f}	17.65 ± 1.21^{a-f}	16.46 ± 1.11^{d-f}
T9 s	57.37 ± 1.60^{a-d}	54.98 ± 3.62^{b-fg}	54.89 ± 1.55^{b-g}	21.24 ± 0.55^{fgh}	24.81 ± 2.08^{a-e}	23.26 ± 1.06^{b-h}	90.24 ± 0.99^{abc}	87.69 ± 1.64^{bc}	89.73 ± 1.31^{abc}	15.55 ± 0.84^{ef}	17.50 ± 1.12^{a-f}	15.97 ± 0.96^{d-f}
Variety (V)	< 0.0001			< 0.0001			< 0.0001			< 0.0001		
Treatment stages (TS)	< 0.0001			< 0.0001			< 0.0001			< 0.0001		
Interaction V*TS	< 0.0001			< 0.0001			< 0.0001			< 0.0001		

Values followed by different letters within a column indicate significant differences ($P < 0.05$). Means ± standard deviations (n = 10).

Table 5. Variations in lupin grain size following aqueous thermal and saline heat treatments

Aqueous thermal treatment stages	Large diameter (mm)			Small diameter (mm)			Saline heat treatment stages	Large diameter (mm)			Small diameter (mm)		
	INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo		INIAP-450	INIAP-451	Criollo	INIAP-450	INIAP-451	Criollo
Raw grain	11.4 ± 0.7^{e}	10.8 ± 0.4^{e}	10.9 ± 0.5^{e}	5.5 ± 0.1^{de}	5.0 ± 0.1^{e}	5.9 ± 0.1^{cd}	Raw grain	11.4 ± 0.7^{f}	10.8 ± 0.4^{f}	10.9 ± 0.5^{f}	5.5 ± 0.5^{fg}	5.0 ± 0.3 g	5.9 ± 0.4^{ef}
T1	$14.8 \pm 0.8^{a-d}$	15.0 ± 0.7^{abc}	15.3 ± 1.0^{a}	$6.2 \pm 0.1^{a-d}$	5.9 ± 0.1^{cd}	6.5 ± 0.1^{abc}	T1 s	$15.0 \pm 1.0^{a-e}$	$14.6 \pm 0.7^{a-e}$	$14.6 \pm 0.8^{a-e}$	$6.0 \pm 0.7^{c-f}$	$6.1 \pm 0.3^{b-f}$	$6.4 \pm 0.3^{a-e}$
T2	15.2 ± 0.6^{ab}	$14.8 \pm 0.5^{a-d}$	$14.5 \pm 0.8^{a-d}$	$6.2 \pm 0.1^{a-d}$	6.0 ± 0.1^{bcd}	6.5 ± 0.1^{abc}	T2 s	$14.7 \pm 0.7^{a-e}$	14.0 ± 1.1^{de}	$14.8 \pm 1.0^{a-e}$	$6.3 \pm 0.6^{a-e}$	$6.2 \pm 0.6^{b-f}$	6.9 ± 0.5^{ab}
T3	$14.2 \pm 0.6^{a-d}$	$14.6 \pm 0.5^{a-d}$	$14.7 \pm 0.7^{a-d}$	6.5 ± 0.1^{abc}	6.7 ± 0.1^{abc}	6.8 ± 0.1^{a}	T3 s	14.0 ± 0.8^{de}	14.2 ± 0.9^{cde}	14.0 ± 0.7^{e}	$6.2 \pm 0.5^{a-f}$	$6.1 \pm 0.5^{b-f}$	$6.5 \pm 0.6^{a-e}$
T4	14.0 ± 0.4^{cd}	$13.8 \pm 0.5^{a-d}$	13.9 ± 0.4^{cd}	6.6 ± 0.1^{abc}	6.4 ± 0.1^{abc}	6.7 ± 0.1^{abc}	T4 s	15.7 ± 1.02	$14.6 \pm 0.4^{a-e}$	15.3 ± 0.8^{abc}	$6.5 \pm 0.5^{a-e}$	$6.3 \pm 0.5^{a-e}$	6.8 ± 0.5^{abc}
T5	$14.4 \pm 0.6^{a-d}$	$13.8 \pm 0.7^{a-d}$	$14.6 \pm 0.7^{a-d}$	6.5 ± 0.1^{abc}	5.9 ± 0.1^{cd}	6.7 ± 0.1^{abc}	T5 s	$15.2 \pm 0.8^{a-d}$	$15.1 \pm 0.7^{a-e}$	$15.2 \pm 0.5^{a-d}$	$6.5 \pm 0.5^{a-e}$	6.0 ± 0.4^{def}	6.8 ± 0.5^{abc}
T6	$14.2 \pm 0.8^{a-d}$	14.0 ± 0.5^{cd}	$14.5 \pm 0.6^{a-d}$	6.7 ± 0.1^{abc}	$6.1 \pm 0.1^{a-d}$	6.8 ± 0.1^{ab}	T6 s	15.3 ± 0.9^{abc}	$15.1 \pm 0.6^{a-e}$	15.6 ± 0.8^{ab}	$6.6 \pm 0.4^{a-e}$	$6.3 \pm 0.6^{a-e}$	$6.7 \pm 0.5^{a-d}$
T7	$14.1 \pm 0.8^{a-d}$	14.0 ± 0.5^{bcd}	13.9 ± 1.0^{cd}	6.8 ± 0.1^{a}	$6.3 \pm 0.1^{a-d}$	6.8 ± 0.1^{a}	T7 s	$14.8 \pm 0.4^{a-e}$	$15.2 \pm 0.6^{a-e}$	15.3 ± 0.7^{abc}	$6.4 \pm 0.2^{a-e}$	$6.3 \pm 0.3^{a-e}$	$6.6 \pm 0.4^{a-d}$
T8	$14.9 \pm 0.5^{a-d}$	$14.6 \pm 0.6^{a-d}$	$14.2 \pm 0.8^{a-d}$	6.6 ± 0.1^{abc}	6.5 ± 0.1^{abc}	6.8 ± 0.1^{ab}	T8 s	15.4 ± 0.5^{ab}	$15.0 \pm 0.6^{a-e}$	$14.6 \pm 0.5^{a-e}$	6.9 ± 0.1^{a}	$6.4 \pm 0.3^{a-e}$	6.8 ± 0.4^{ab}
T9	$14.5 \pm 0.5^{a-d}$	$14.2 \pm 1.0^{a-d}$	$14.2 \pm 1.0^{a-d}$	6.7 ± 0.1^{abc}	6.7 ± 0.1^{ab}	6.8 ± 0.1^{a}	T9 s	15.4 ± 0.5^{ab}	$14.8 \pm 0.6^{a-e}$	$14.5 \pm 0.5^{b-e}$	6.9 ± 0.4^{a}	$6.6 \pm 0.5^{a-e}$	6.8 ± 0.2^{ab}
T10	$14.7 \pm 0.7^{a-d}$	$14.2 \pm 0.6^{a-d}$	$14.8 \pm 0.6^{a-d}$	6.9 ± 0.1^{a}	6.4 ± 0.1^{abc}	6.7 ± 0.1^{ab}							
T11	$14.4 \pm 0.4^{a-d}$	$14.4 \pm 0.8^{a-d}$	$14.3 \pm 0.3^{a-d}$	6.6 ± 0.1^{abc}	6.4 ± 0.1^{abc}	6.6 ± 0.1^{abc}							
T12	$14.5 \pm 0.5^{a-d}$	$14.4 \pm 0.7^{a-d}$	$14.2 \pm 0.27^{a-d}$	6.7 ± 0.1^{abc}	6.4 ± 0.1^{abc}	6.5 ± 0.1^{abc}							
Variety (V)		0.07			< 0.0001		Variety (V)		0.002			< 0.0001	
Treatment stages (TS)		< 0.0001			< 0.0001		Treatment stages (TS)		< 0.0001			< 0.0001	
Interaction V*TS		0.1024			0.301		Interaction V*TS		0.0267			0.3003	

Values followed by different letters within a column denote significant differences ($P < 0.05$). Means ± standard deviations (n = 10).

beans, and during the processing of soybeans with fluidized superheated steam.[32] Other authors attribute the reduction in color or darkening of the beans to the coagulation of the proteins.[37] The chroma or color level (C^*) was increased during the hydration, cooking, and washing of the grain at 35 and 18 °C compared with the raw grain. Similar results were obtained with the application of ATT and STT. The attribute that differentiates the color, Hue (H^*) also increased with cooking and washing. Debbitering of the grain with the application of STT caused a greater color difference ($\Delta E = 5$ units) in INIAP-451, when the raw and processed seeds were compared. In this case, the browning was possibly partially inhibited by a low concentration of NaCl.[38] A variation of 88 to 90.4 for L^* and an increase in the values of H^* and C^*, resulting in a greater intensity of the cream color of the grain, was reported for *Lupinus albus* seeds.[39]

Grain size

A significant change in the diameters of the three varieties of grain was noted during hydration (T1) and the size increased proportionally to the water absorption capacity of the protein, the components of the fiber, the oligosaccharides, and the permeability of the tegument (Table 5). The absorption of water and the increase in grain size continued until the tissues were saturated with water, which was reached in the cooking stage (T2); afterwards, no significant differences were observed in the largest and smallest diameters of grains. In the hydration stage of ATT, the increase in the larger diameter of the grain was 3.35 times the original size for the INIAP-450 variety, 4.19 for the INIAP-451 variety, and 4.38 for the Criollo variety. During cooking, the increase was 3.74 times the original size for INIAP-450, 4.04 times for INIAP-451 and 3.65 for the Criollo variety. In the STT, the increase in the larger diameter was 3.53 times the original size for INIAP-450 variety, 3.82 for INIAP-451 and 3.70 for Criollo variety. Similar results were reported for lupin debittering, the grain swelled up to three times its original size, depending on the variety.[23] During cooking, the smaller diameter of the grain increased 0.79 times for INIAP-450, 1.14 times for INIAP-451, and 0.98 for the Criollo variety. The increase in the diameter of the seeds depended on their final humidity; thus, when the grain was exposed to 40%, 50%, and 60% humidity, its size increased by 15%, 23% and 24.93%, respectively.

CONCLUSIONS

Two different debittering processes were applied to three different varieties of *L. mutabilis*. Both processes, ATT and STT, resulted in approximately 80% of the alkaloids being removed from the grain during soaking, cooking, and the first wash of the grain at 35 °C. In general, the debittering process with ATT and STT exerted positive effects on reducing the QAs and increasing protein concentrations in the grain in the three varieties. Moreover, the processes improved the grain texture by decreasing hardness and increasing grain size. Color was also improved, as evidenced by the increase in the tone and chromaticity of the grain, characteristics that affect the consumer's level of acceptance and preference. However, in the saline heat process, the addition of sodium chloride in the first stages of the process helped to optimize the effect of temperature, agitation, and water changes on the reduction of the QA content. Considering the economy of the process, the debittering of 1 kg of grain by the SST was achieved in 58 h using 66 L of water, which represents a saving of 26 h in the processing time and 30 L in the volume of water compared with the ATT and 127 L compared with the artisanal process, which uses 193 L and takes between 5 to 6 days. Hence, considering the current debittering processes, the saline heat process is advisable for lupin debittering. Nevertheless, further studies will be undertaken pursuing an additional reduction in water and in the process time, to make it more environmentally friendly.

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Original article

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

Elena Villacrés,[1]* María Belén Quelal,[1] Xiomara Jácome,[2] Gabriela Cueva[2] & Cristina M. Rosell[3]

1 Departamento de Nutrición y Calidad, Instituto Nacional de Investigaciones Agropecuarias, Mejía 1701340, Ecuador
2 Escuela de Ciencias Químicas, Facultad de Ciencias Exactas y Naturales, Pontifica Universidad Católica del Ecuador, Quito 17012184, Ecuador
3 Institute of Agrochemistry and Food Technology (IATA), CSIC, Valencia 746980, Spain

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Summary There is a growing interest in vegetable-based sources of proteins. Despite its high nutrient content, lupine has been rarely exploited as a protein source due to the presence of high levels of non-nutritive compounds such as alkaloids, which impart a bitter taste. Here, we evaluated the effect of debittering and solid-state fermentation on the nutritional contents of three lupine varieties (*Lupinus mutabilis* Sweet). These processes induced significant changes ($P < 0.05$) in the nutritional composition of the three lupine varieties (INIAP-450, INIAP-451 and Criollo) and increased the protein levels to 644.55 g kg^{-1} (Criollo variety) and the levels of several constituent amino acids such as valine (54.62 g kg^{-1}), methionine (42.47 g kg^{-1}), isoleucine (59.27 g kg^{-1}) and leucine (76.32 g kg^{-1}). The ether extract of INIAP-450 showed increased levels (up to 244.03 g kg^{-1}); especially, monounsaturated fatty acids (559.78 g kg^{-1}) and polyunsaturated fatty acids (293.17 g kg^{-1}) were observed. The omega-6/omega-3 ratio in the debittered grain oil reached the minimum requirement established for good-quality oils (5/1). However, the levels of other components decreased, showing levels up to 13.04 g kg^{-1} (total starch) in the Criollo variety, 22.62 g kg^{-1} (resistant starch) in INIAP-450, 6.53 g kg^{-1} (potassium) in INIAP-451, 46 g kg^{-1} (iron) in INIAP-451 and 29.75 g kg^{-1} (zinc) in INIAP-450.

Keywords Debittering process, leguminous, nutritional content, solid fermentation.

Introduction

Within the legume family, the genus *Lupinus* includes numerous species, and four of these are popular for their nutritional and economic values. These include *Lupinus angustifolius* (blue lupine with narrow leaves), *Lupinus albus* (white lupine), *Lupinus luteus* (yellow lupine) and *Lupinus mutabilis* (pearl lupine). *Lupinus mutabilis* Sweet is one of the most common species because it can grow in poor soils, adapt well to extreme conditions and fix atmospheric nitrogen (Van de Noort, 2016).

Like other legumes, lupine is characterised with high levels of proteins, fats, dietary fibres and minerals (Yorgancilar & Bilgiçli, 2014). In particular, its protein content varies between 41% and 51% depending on the species and climatic and cultivation conditions (Arnoldi *et al*., 2015; Karnpanit *et al*., 2016). The high contents of monounsaturated and polyunsaturated fatty acids (MUFA and PUFA, respectively) make lupine an alternative source of vegetable oil (Villacrés *et al*., 2013; Awad-Allah & Elkatry, 2013). It has low contents of sucrose and starch and moderate contents of oligosaccharides, which are not digested by humans but are broken down by the bacterial flora present in the large intestine (Van de Noort, 2016).

Despite its high nutritional value, lupine is rarely used owing to the presence of bitter compounds such as alkaloids derived from quinolyzidine (Carvajal-Larenas *et al*., 2016), which prevents its direct consumption (Repo-Carrasco-V *et al*., 2007). Elimination of these components may be achieved with debittering technologies. Traditional debittering processes include a hydration phase followed by cooking to inactivate the germination capacity of the grain and increase the permeability of cell wall, thereby facilitating leaching of these compounds through successive washes (Carvajal-Larenas *et al*., 2013; Awad-Allah & Elkatry, 2013). Other techniques for the elimination of alkaloids include hydro-agitation (Carvajal-Larenas *et al*., 2013)

*Correspondent: Fax: +593 3007134;
e-mail: elena.villacres@iniap.gob.ec

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or biological processes such as germination (De Cortes *et al.*, 2005; Khan *et al.*, 2018). Varieties with low alkaloid contents have been developed, but their adaptation to different environmental conditions is difficult, and these varieties undergo changes over time that may result in the restoration of the bitter attribute (Awad-Allah & Elkatry, 2013).

Solid-state fermentation is characterised with 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 through the inoculation of *Rhizopus* to obtain a compact cake formed by the cottony mycelium (Nout & Kiers, 2005). This technique has been subsequently used as a method of food production and preservation because it improves nutritional, functional and sensory profiles (Blandino *et al.*, 2003). Fermentation of legumes reduces the levels of antinutritional compounds and oligosaccharides and enhances protein digestibility (Pandey, 2003; Bartkienė *et al.*, 2014; Karnpanit *et al.*, 2016), as confirmed with soybean (Nout & Rombouts, 1990), pea (Nowak & Szebiotko, 1992) and chickpea (Abusalem & Abou-arab, 2011). In particular, the flour from previously fermented lupine with *Pedioccocus acidilactici* was added to wheat flour to improve the nutritional quality of breads (Bartkiene *et al.*, 2011). In addition, a significant increase in the content of vitamin B_{12} was achieved through the fermentation of lupine with a starter comprising *Rhizopus oryzae* and *P. freudenreichii*, (Rooijackers, Endika, & Smid, 2018). Other researchers fermented *L. mutabilis* with *Rhizopus oligosporus* to reduce alkaloid contents and reported the importance of particle size reduction and optimum moisture for fermentation (Ortega-David & Rodriguez-Stouvenel, 2014; Carvajal-Larenas *et al.*, 2016). However, this methodology only allowed for the partial detoxification of lupine.

Here, we aimed to evaluate the effect of the combination of debittering and solid-state fermentation processes using *R. oligosporus* on the nutritional profiles of three varieties of *L. mutabilis* of Ecuadorian origin. We quantified the contents of fatty acids, amino acids, dietary fibres, total starch and minerals.

Materials and methods

Raw material

Bitter lupine (INIAP-450, INIAP-451 and Criollo) varieties were obtained from the National Legumes Program and Andean INIAP Grains. The harvested grain was threshed and introduced into a Crippen MFG. INC. (St. Louis, MI, USA) equipment, which had a set of sieves with 8-, 7- and 6-mm openings. Grains with an average diameter of 7–8 mm were used in this study.

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

Debittering of grains

We applied the thermal-aqueous treatment for debittering. The process was started with the hydration of the grain at an initial temperature of 80 °C for 10 h at a grain-to-water ratio of 1:3. This step was followed by cooking in water at 91 °C for 1 h and washing with potable water. In this phase, the grain-to-water ratio was maintained at 1:15. The first washing step was performed with water at 35 °C for 28 h, while the second washing step was carried out at 18 °C for 45 h. The debittered lupine was subjected to a drying process in a forced air oven (HS122A) at 60 °C for 8 h. The grain was ground and packed under conditions similar to those employed for bitter lupines.

Solid-state fermentation

Rhizopus oligosporus strain NRRL2710 from the Northern Regional Research Laboratory (NRRL) collection (USDA, USA), belonging to the Microbiology laboratory collection at the Ambato University of Technology, was used in this study. For fermentation, the humidity of the debittered grain was reduced to 50% in a forced air oven at 60 °C for 2 h. The grain was crushed in a miniprocessor (Oster, Rio de Janeiro, Brazil), and portions of 50 g were packed and sealed in polypropylene bags for sterilisation in an autoclave (Webeco, Farjestäden, Germany) at 121 °C for 10 min. About 500 μL of the spore suspension was inoculated onto each grain portion and incubated at 28 °C for 4 days.

Once abundant mycelium formation was observed, and the samples were lyophilised in a kit (Labconco Lyph Lock 12, Kansas, USA) at −40 °C and −0.7 bar pressure for 4 days. The fermented grain was ground, packed in polypropylene bags and stored at 10 °C prior to chemical analysis.

Chemical analysis

Proximate composition

The contents of protein, fat, crude fibre and ash were determined using standardised methods (AOAC, 2000). Carbohydrate content was calculated from the difference.

Amino acid profile

Amino acid profile was evaluated with high-performance liquid chromatography (AOAC, 2000). The samples were defatted using solvent extraction at 20 °C, ground (150 μm), and homogenised before being weighed (25 mg) into hydrolysis tubes. This process was

performed by the incubation of samples in an oxygen-free environment and under constant boiling 6 M hydrochloric acid (HCl) at 110 °C for 22 h. The hydrolysed samples were concentrated on a rotary evaporator and treated with 5 mL of citrate buffer (pH 2.2); the samples were then applied to the LC-10AS Shimadzu liquid chromatography system, which operated under the following conditions: oven temperature, 60 °C; emission length, 450 nm; sample cooler temperature, 4 °C; eluent solution flow, 0.60 m^2 min^{-1}; excitation length, 350 nm; injection volume, 5 μL; and run time, 45 min.

Fatty acid profile

Fatty acids were determined with gas chromatography (AOCS, 2005). The oil was extracted from a 50 g sample of ground lupine (150 μm) by refluxing for 6 h with 125 mL n-hexane in a Soxhlet extractor. The solvent was evaporated under reduced pressure, and the oil was recovered. In total, 50 mg of the extracted oil was subjected to esterification and treated with 1 mL of 0.5 M potassium hydroxide (KOH) in methanol. This mixture was placed in sealed test tubes, which were boiled for 30 min in a water bath. The tubes were cooled to room temperature and treated with 0.5 mL of the mixture hydrochloric acid (HCl) and methanol (1:4). The mixture was boiled for 25 min, cooled and mixed with 2 mL of double-distilled water. The esters were recovered through three successive washes with n-hexane (chromatography grade) and treated with anhydrous sodium sulphate to eliminate residual water. The supernatant was recovered, and the solvent was evaporated with nitrogen gas. The extract was diluted with 2 mL n-hexane and injected into a gas GC-14B Shimadzu chromatography system. A thermal TR-FAME column (3 m in length, 0.25 mm in diameter and 0.25 μm pore size) was used for the separation of fatty acid methyl esters. The initial temperature was maintained at 100 °C for 5 min and then increased at a rate of 4 °C min^{-1} to 200 °C final temperature, which was maintained for 2 min. A flame ionisation detector (air–hydrogen–nitrogen) was used. The split ratio was 1:10, and hydrogen was used as a carrier gas at a flow rate of 0.8 mL min^{-1}. The injector and detector temperatures were 250 and 280 °C, respectively. Peak identification of fatty acids in the analysed samples was carried out by comparison with retention times of known standards.

Total starch

Polarimetric determination of starch content is based on the optical activity of starch. As starch cannot be dissolved in water, HCl was used. After dissolution, the samples were clarified, filtrated and measured in a polarimeter. The optical rotation of all samples was measured at 20 °C using a sample cell with an optical path length of 200 mm (AOAC, 2000; Fărcaş *et al*., 2013).

Resistant starch

Samples were incubated in a shaking water bath with pancreatic α-amylase and amyloglucosidase for 16 h at 37 °C. During this incubation period, non-resistant starch was solubilised and hydrolysed to D-glucose by the combined action of the two enzymes. The reaction was terminated with the addition of an equal volume of ethanol, and the resistant starch was recovered as a pellet by centrifugation. The pellet was washed twice in ethanol (50% v/v) and centrifuged. Free liquid was removed by decantation, and the resistant starch in the pellet was dissolved in 2 M KOH by vigorously stirring in an ice-water bath with a magnetic stirrer. This solution was neutralised with acetate buffer, and the starch was hydrolysed to glucose with amyloglucosidase. D-Glucose level was measured with glucose oxidase/peroxidase reagent (GOPOD) and served as the measure of the resistant starch present in the sample (AOAC Official Method 2002.02).

Total dietary fibre

Total dietary fibre content was determined for dried and defatted samples. Samples were cooked at 100 °C with heat-stable α-amylase to achieve gelatinisation, hydrolysis and depolymerisation of starch, incubated at 60 °C with protease (to solubilise and depolymerise proteins) and amyloglucosidase (to hydrolyse starch fragments to glucose) and treated with four volumes of ethanol to precipitate soluble fibres and remove depolymerised protein and glucose (from starch). The residue was filtered, washed with 78% ethanol, 95% ethanol and acetone and then dried and weighed. One duplicate was analysed for protein level, while the other was incubated at 525 °C to determine ash content. Total dietary fibre was the weight of the filtered and dried residue minus the weight of the protein and ash (AOAC 991.43, AOAC 985.29).

Insoluble and soluble dietary fibres

Dried lupine samples (1 g) were subjected to enzymatic digestion with thermostable α-amylase, protease and amyloglucosidase. The insoluble dietary fibre was filtered, and then, the residue was washed with hot distilled water. The combined filtrate and water wash solution was precipitated with four volumes of 95% ethanol to determine levels of soluble dietary fibre; the precipitate was filtered and dried. For final calculation, both residues (insoluble dietary fibre and soluble dietary fibres) were corrected for protein and ash levels with an appropriate blank (AOAC 991.4).

Mineral composition

For the digestion of samples, the AOAC method 985.35 (2005) was used as reference; the samples were calcined in a 48000 Thermolyne furnace (Waltham, MA, USA) at 525 °C, and the ashes were dissolved in

25 mL of 0.1 M nitric acid (HNO_3; trace metal grade). Calibration curves were prepared by the dilution of standards for each mineral at specific concentrations. Analytical curves were obtained with a linear response for the selected concentration range. Mineral analysis was performed with flame spectrophotometry in an AA-7000 atomic absorption spectrophotometer (Shimadzu, Kyoto, Japan), except for phosphorus that was analysed with colorimetry (AOAC, 2000).

Statistical analysis

Statistical analysis was performed with the Infostat program (Córdoba, Argentina). The normal distribution of the data was verified with the Shapiro–Wilk goodness-of-fit test. We applied a multifactorial variance design (analysis of variance [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.

Results and discussions

Debittering and solid-state fermentation effect on the proximal composition of lupine

Table 1 indicates the proximal composition of the three varieties of lupine in bitter, debittered and fermented states. Statistical analysis confirmed the significant differences in protein, fibre, mineral and carbohydrate contents between these lupine varieties following the application of debittering and solid-state fermentation processes.

Among the bitter lupine varieties, Criollo showed the highest content of protein and the lowest levels of minerals and carbohydrates. INIAP-450 variety showed lower levels of crude fibres and higher levels of minerals than INIAP-451 variety. The protein contents were within the values recorded by Sujak *et al.* (2006) but higher than those reported in lupine sown in the Andes of Colombia and Peru (Ortega-David *et al.*, 2010). These differences may be attributed to the variations in species and climatic conditions (Jacobsen & Mujica, 2008). Considering the impact of treatments, debittering process induced a significant increase in protein levels in the three varieties analysed owing to the dilution of water-soluble carbohydrates and minerals (Erbas, 2010; Carvajal-Larenas *et al.*, 2016). This observation corroborated the results of nitrogen-free extract, which showed a 70% decrease in the debittered lupine compared to the case for the bitter lupine. Solid-state fermentation resulted in an increase in the protein content of debittered grains, probably owing to the assimilation of carbohydrates, fibres and some

Table 1 Proximate composition of bitter, debittered and fermented lupine[†]

	INIAP-450			INIAP-451			Criollo			Grain condition	Variety	Interaction
	Bitter	Debittered	Fermented	Bitter	Debittered	Fermented	Bitter	Debittered	Fermented			
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 fibre	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 with different superscript letters entered in the columns for each variety indicate significant differences ($P < 0.05$). Mean ± standard deviation ($n = 3$).

minerals by *R. oligosporus* to synthesise proteins (Abu-salem & Abou-arab, 2011). The increase in the protein content caused by fermentation was higher than that reported for other fermented legumes such as chick-peas (28.85%) (Abu-Salem & Abou-Arab, 2011) and beans (31.6%) (Barampama & Simard, 1995).

Debittering process increased the content of fats (ether extract), likely due to the loss of aqueous soluble compounds. Fermentation induced a slight increase in fat levels. Consistent with the observation reported for protein levels, the metabolic activity of *R. oligosporus* resulted in the consumption of a part of carbohydrates, fibres and minerals to synthesise compounds such as fats and other bioactive compounds (Khan *et al*., 2015). In addition, the content of crude fibre increased by 49.40% (INIAP-450), 15.92% (INIAP-451) and 43.46% (Criollo) after the debittering with respect to bitter grains, probably owing to the insolubility of these compounds in water (García-López *et al*., 2001). However, fermentation exerted the opposite effect and decreased the crude fibre content by 15.59% (INIAP-450), 0.39% (INIAP-450) and 29.22% (Criollo). Moreover, solubilisation of some minerals during debittering affected the ash content, as evident from the reduction of 38.37%, 50.25% and 50.73% in INIAP-450, INIAP-451 and Criollo, respectively. Fermentation also decreased of the level of ash (8.63%) in INIAP-450 variety but had no significant effect on the ash content of the other two varieties.

Debittering and solid-state fermentation effect on the amino acid profile of lupine

The evaluation of the amino acid profile confirmed the significant differences between different varieties depending on the applied process ($P < 0.05$) (Table 2). The levels of arginine, aspartic acid and glutamic acid increased in the three varieties of lupine, while the content of lysine increased only in Criollo in response to debittering. The hydrophilic nature of serine, threonine, cysteine and tyrosine could have contributed to their increased water solubility, resulting in the reduction in their levels during hydration, cooking and washing of grains. Fermentation increased the concentrations of valine, methionine, isoleucine and leucine in the three grain varieties; proline levels increased only in INIAP-451 and Criollo; these phenomena may be related to the non-polar characteristics of these amino acids.

Other amino acids such as arginine, alanine, lysine and phenylalanine decreased in all three varieties, while aspartic acid and serine levels decreased only in INIAP-451 and Criollo. *Rhizopuz oligosporus* may use a part of these amino acids for its metabolic activity (Handoyo & Morita, 2006), resulting in the reduction in their concentrations in fermented grains as compared to those in debittered grains.

Debittering and solid-state fermentation effect on the fatty acid profile of lupine

We observed significant effects of the two processes ($P < 0.05$) on the fatty acid composition of different varieties of lupine (Table 3). The bitter grains had more than 50% MUFA, with a predominance of oleic acid. MUFA levels decreased after the debittering of INIAP-451 and Criollo but increased during the fermentation process by 57.25% in INIAP-450, 55.80% in INIAP-451 and 54.88% in Criollo. PUFA constituted 25% of lupine oil and predominantly included linoleic acid, the levels of which increased after debittering and fermentation. An increase in the fat and fatty acid contents was reported after soy fermentation by Lee *et al*. (1997). The increase in the fatty acid content was consistent with the metabolic process reported for *R. oligosporus*, which uses carbohydrates and fibres to synthesise fat and fatty acids (Nout & Kiers, 2005). Evaluation of fat quality indices showed that the PUFA/MUFA ratio exceeded the value of 0.5, and the (PUFA + MUFA)/SFA ratio was ≥2, indicating that the fat content observed after debittering or fermentation met the nutritional objectives (≥2) (FAO, 2012). The ratio of omega-6/omega-3 in debittered grain oil reached the minimum requirement established for good-quality oils (5/1) (FAO, 2012). However, this proportion varied between 25.54 (INIAP-450) and 26.72 (Criollo) in fermented grain oil owing to the increase in the linoleic acid level and decrease in the linolenic acid level. FAO (2012) indicates that it is not reasonable to make specific recommendations as long as the intake of the two fatty acids is within the recommended daily values.

Debittering and solid-state fermentation effect on the total starch, resistant starch and dietary fibre contents of lupine

We observed significant differences ($P < 0.05$) in the contents of starch and dietary fibres after the application of various processes to different grain varieties (Table 4). The contents of total starch and resistant starch decreased after washing during the debittering process in all varieties except INIAP-451, which showed a constant level of resistant starch. Fermentation had no significant effect on the total starch content of samples but caused a significant decrease in the level of resistant starch in all varieties except Criollo. Nout & Rombouts (1990) found that the enzyme β-glycosidase hydrolysed the β-glycoside-forming aglycones, the readily available forms of aglycones, during soy fermentation. Thus, enzyme activities may be responsible for the decrease in the levels of resistant starch. The total dietary fibre content was high in INIAP-451. Debittering caused an increase in insoluble

Table 2 Amino acid profile of bitter, debittered and fermented lupine[†]

	INIAP-450			INIAP-451			CRIOLLO			Grain		
Amino acid	Bitter	Debittered	Fermented	Bitter	Debittered	Fermented	Bitter	Debittered	Fermented	condition	Variety	Interaction
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 ± SD of three repetitions. The means ± standard deviations with different superscript letters entered in the columns for each variety are significantly different ($P < 0.05$).

fibre levels in all varieties at the expense of a decrease in the levels of water-soluble compounds. Fermentation induced a significant decrease in the level of total dietary fibres as compared with debittering, probably owing to the hydrolytic action of *R. oligosporus*.

Debittering and solid-state fermentation effect on the macro- and micromineral content of lupine

The macro- and micronutrient contents were significantly affected by the debittering processes in the three varieties (Table 5). The most abundant macro-elements in the three bitter grain varieties were potassium and phosphorus. Among the micro-elements, iron, manganese and zinc levels were affected. Debittering increased the content of calcium and decreased the levels of other macro- and micro-elements with an exception of sodium, the levels of which showed no significant changes after debittering. Ertaş & Bilgiçli (2014), Van de Noort (2016) and Chaparro *et al.* (2011) observed similar variations in quinoa and amaranth minerals subjected to soaking and cooking processes. Solid-state fermentation caused a decrease in calcium levels in INIAP-450 and Criollo and reduced the levels of phosphorus in INIAP-450. No significant variations were observed in other macro-elements following fermentation. According to Omosebi & Otunola (2013), the loss in calcium may be attributed to the metabolic activity of *R. oligosporus*, which requires this mineral for the regulation and/or stimulation of the enzymes involved in protein metabolism. In fermented INIAP-451 grain, the levels of manganese, zinc and iron significantly decreased. The decrease in iron content may be associated with the formation of nitrogen compounds catalysed by nitrogenase, which requires iron as a cofactor (Viniegra-González, 1997). Copper levels increased after fermentation, possibly due to the dissociation of the complex formed with phytic acid (Ghavidel & Prakash, 2007).

Conclusions

The effects of debittering and solid-state fermentation with *R. oligosporus* on the nutritional properties of three lupine varieties were evaluated. Debittering increased the concentrations of protein (553.30 g kg^{-1}) and several constituent amino acids such as aspartic acid (76.98 g kg^{-1}), glutamic acid (112.84 g kg^{-1}), arginine (73.59 g kg^{-1}) and leucine (74.93 g kg^{-1}). The ether extract increased by 222.40 g kg^{-1} and PUFA levels increased by 292.25 g kg^{-1}. Fermentation raised the levels of protein (617.85 g kg^{-1}), several essential amino acids such 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}), as well as ether extract (229.80 g kg^{-1}) and PUFA (293.17 g kg^{-1}) but decreased the levels of ash (18.06 g kg^{-1}), nitrogen-free extract (18.38 g kg^{-1}),

Table 3 Fatty acid profile of bitter, debittered and fermented lupine[†]

	INIAP-450			INIAP-451			CRIOLLO			Grain		
Fatty acids	**Bitter**	**Debittered**	**Fermented**	**Bitter**	**Debittered**	**Fermented**	**Bitter**	**Debittered**	**Fermented**	**condition**	**Variety**	**Interaction**
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
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
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
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
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

MUFA, monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA, saturated fatty acids.

[†]Percentage expressed as g kg^{-1} of lupin oil. The values represent means ± SD of three replicates. The means ± standard deviations with different superscript letters are significantly different ($P < 0.05$).

Table 4 Total starch, resistant starch and dietary fibre contents of bitter, debittered and fermented lupine[†]

	INIAP-450			INIAP-451			CRIOLLO			Grain condition	Variety	Interaction
	Bitter	Debittered	Fermented	Bitter	Debittered	Fermented	Bitter	Debittered	Fermented			
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 fibre	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 fibre	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 fibre	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. Values represent means ± SD of three replicates. The means ± standard deviations with different superscript letters are significantly different ($P < 0.05$).

Table 5 Macro- and microminerals of bitter, debittered and fermented lupine

	INIAP-450			INIAP-451			CRIOLLO			Grain condition	Variety	Interaction
Minerals	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 as g kg^{-1} dry grain.

[‡]Expressed as mg kg^{-1} dry grain. The values represent means ± SDs of three replicates. The means ± standard deviations with different letters are significantly different ($P < 0.05$).

resistant starch (22.69 g kg^{-1}) and soluble dietary fibre (7.07 g kg^{-1}). Therefore, the processing of lupine by debittering or solid-state fermentation may serve as an alternative to expand the use of lupine as an ingredient for the fortification of food products.

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Conflict of interest

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

Ethical approval

Ethical approval was not required for this research.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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

Elena Villacrés[a,*], María Belén Quelal[a], Edgar Fernández[b], Grace Garcìa[c], Gabriela Cueva[b], Cristina M. Rosell[d]

[a] Department of Nutrition and Quality, National Institute of Agricultural Research, Mejía, Ecuador
[b] School of Chemical Sciences, Faculty of Exact and Natural Sciences, Pontifical Catholic University of Ecuador, Quito, Ecuador
[c] School of Chemical Sciences, Central University of Ecuador, Quito, Ecuador
[d] Institute of Agrochemistry and Food Technology. (IATA-CSIC), Valencia, Spain

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ABSTRACT

Lupin is a nutritive grain, but its use is limited due to its high content of bitter alkaloids and other antinutritional factors, such as phytic acid, tannins, nitrates and trypsin inhibitors (TI), that have undesirable physiological effects. There is increasing interest in finding appropriate methods for reducing the antinutritional compounds in lupin. The objective of this research was to assess the efficacy of a biotechnological process, namely, fungal fermentation, as a debittering process relative to that of conventional aqueous thermal treatment (ATT). We evaluated the effects of these processes on the reduction of antinutritional compounds as well as their potential impacts on enhancing the beneficial antioxidant properties of lupin. Three varieties (INIAP-450, INIAP-451 and Criollo) of the *Lupinus mutabilis* species were studied. The application of ATT and fermentation with *Rhizopus oligosporus* caused decreases in the following antinutrients: nitrates (94.59%), tannins (82.10%), alkaloids (94%), urease activity (93.75%), phytic acid (70.06%) and trypsin inhibitors (76.76%). Ascorbic acid also decreased (79.72%). All values corresponded to the average in the three varieties evaluated. While the contents of phenols, carotenoids and the antioxidant capacity decreased by 96.83, 49.42 and 96.13%, respectively, due to the debittering process, solid fermentation promoted increases in these compounds and properties in the debittered grain.

1. Introduction

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

Conversely, health-related benefits have also been linked to some antinutritional factors in legumes, specifically phytic acid, polyphenols, ascorbic acid and carotenoids (Lampart-Szczapa, Korczak, Nogala-Kalucka, & Zawirska-Wojtasiak, 2003). These compounds have been shown to have antioxidant properties as well as beneficial metabolic and physiological effects, such as preventing sclerotic changes in blood vessels and blocking the formation of free radicals (Khan, Karnpanit, Nasar-Abbas, Huma, & Jayasena, 2015). These effects have been described in some lupin species, such as *L. albus, L. luteus* and *L. angustifulius*, and other wild species (Thambiraj, Reddy, Phillips, & Koyyalamudi, 2019). However, there is little information regarding *Lupinus mutabilis*, despite being one of the most common species due to its ability to grow in poor soils and under extreme climatic conditions.

Although there are different methods to reduce the antinutritional factors (Soetan & Oyewole, 2009), the traditionally called debittering process has been carried out to remove the antinutritional and bitter compounds, making the lupin apt for consumption (Villacrés, Álvarez, & Rosell, 2020a). Lupin debittering treatments facilitate the elimination of antinutritional compounds, such as quinolizidine alkaloids (QAs) and

* Corresponding author.
E-mail address: elena.villacres@iniap.gob.ec (E. Villacrés).

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phytic acid (Carvajal-Larenas et al., 2016). Debittering includes lupin hydration, cooking and subsequent washing processes with water. Specifically, cooking reduces the tannin content of lupin by more than 70% (Jiménez-Martínez, Hernández-Sánchez & Dávila-Ortiz, 2001). Treatments employing heat also help reduce trypsin inhibitor and urease activity lowering the nutritional quality of grains. Simultaneously to the removal of toxic compounds, the debittering process results in loses of other nutrients like minerals (Ertaş & Bilgiçli, 2014).

Solid-state grain fermentation with bacterial or fungal species have been applied to reduce antinutritional compounds, such as phytates and tannins, but also to improve the nutritional quality of grains and pulses, given that these compounds affect the bioavailability of minerals, such as calcium, zinc and iron (Ghoshal, Basu, & Shivhare, 2012; Saharan, Sadh, Duhan, & Duhan, 2020). Fermentation also increases the polyphenol content and improves grain antioxidant activity because microbial action facilitates the breakdown of cell walls and allows the release or synthesis of antioxidant compounds that act as metal chelators or hydrogen donors to free radicals (Nout & Kiers, 2005). However, this biotechnological strategy has been scarcely applied to *L. mutabilis* grains. Fernandez-Orozco et al. (2008) studied the impact of fermentation on the antioxidant capacity of *L. angustifolius* and found that there was an increase in total phenolic compounds, peroxyl radical-trapping capacity and Trolox equivalent antioxidant capacity under most fermentation conditions.

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

2. Materials and methods

2.1. Raw material

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

2.2. Reagents

The main reagents used in this investigation were the following: BAPA (N-benzoyl-arginine p-nitroanilide), phytic acid kit (Megazyme), potato dextrose agar (Merck), L-ascorbic acid, chlorogenic acid, Folin-Ciocalteu ABTS (2.2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), Trolox (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) Sigma Aldrich brand (St. Louis, Missouri, USA).

2.3. Sample preparation methods

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

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

2.4. Analysis of anti-nutritional compounds

2.4.1. Nitrates

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

2.4.2. Tannins

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

2.4.3. Quinolizidine alkaloids (QAs)

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

2.4.4. Residual urease activity

Although urease is not related to protein and starch digestion, the urease test has been used as an indirect method for estimating the degree of trypsin inhibition because its inactivation mechanism is nearly identical to that of trypsin inhibition (Yalcin & Basman, 2015). Lupin flour (0.2 g) was dissolved in 10 mL of urea solution (pH 7.0) in a water bath at 30 °C for 30 min. The urea solution was replaced with a phosphate buffer to make the blank. The change in pH caused by the conversion of urea to ammonia by the urease enzyme in the sample was measured with AACC Method No: 22–90.01 (AACC, 2000).

2.4.5. Phytic acid

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

2.4.6. Trypsin inhibitors (TI)

Trypsin inhibitor activity was measured following the AOCS Official Method (2009). The extraction of TI was performed by mixing 1.00 g of defatted lupin flour with 50.0 mL of 0.01 M NaOH and agitating the resulting suspension for 3 h at room temperature. A final centrifugation step for 10 min at 10,000 × *g* allowed separation of the supernatant (lupin flour extract) for the TI assay.

2.5. Analysis of compounds with antioxidant properties

2.5.1. Ascorbic acid

The ascorbic acid in the samples was extracted with an oxalic acid solution of 0.4% and 20% acetone and quantified using 2.6-dichlorophenol-indophenol (Egoville, Sullivan, Kozempel, & Jones, 1988). Absorbance was measured at 520 nm. L-ascorbic acid was used as a standard.

2.5.2. Total carotenoids

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

2.5.3. Total phenolic compounds

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

2.5.4. Trolox equivalent antioxidant capacity (TEAC)

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

2.6. Statistical analysis

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

3. Results and discussion

3.1. Antinutritional compounds

3.1.1. Nitrates, tannins and quinolizidine alkaloids (QAs)

The results of the quantification of nitrate, tannins and alkaloids, as well as the significant statistical differences ($P < 0.05$) between varieties in the condition of the grain (debittered or fermented) and their interaction, are presented in Table 1. The nitrate content in raw seeds varied with grain variety ($P < 0.05$) and was the highest in INIAP-451 (40.63 mg/100 g) followed by the Criollo variety and INIAP-450. Values of these compounds were lower than those that have been reported for other vegetables, such as spinach (48.5 mg/100 g) and leaf lettuce (55.5 mg/100 g), where nitrates are concentrated in vacuoles, leaves and transport organs but less abundant in flowers, tubers and seeds (Ranasinghe & Marapana, 2018). The application of ATT caused a reduction in nitrate by 94.84%, 92.95% and 94.43% in INIAP-450, INIAP-451 and Criollo, respectively. These pronounced reductions likely stemmed from the water solubility of nitrates. The reduction in nitrate increased significantly ($P < 0.05$) by 3.84% (INIAP-451) and 4.09% (Criollo) in fermented grains relative to debittered grain. The nitrate concentrations in debittered and fermented *L. mutabilis* were much lower than the specified maximum permissible levels that have been reported for lettuce and spinach (1125 mg/100 g) by the regulations of some European countries (Siomos & Dogras, 2000). Other antinutritional compounds are tannins, which affect mineral and protein utilization (Embaby, 2010) and cause growth depression by decreasing the digestibility of protein and carbohydrate (Liener, 1994). In the three varieties of *L. mutabilis* evaluated in this study, the content of tannins depended on variety and grain condition ($P < 0.05$). The highest concentration of tannins (975.74 mg/100 g) was recorded in the raw Criollo grain. This concentration is higher than what has been reported in soy (45 mg/100 g) and in raw *Lupinus termis* seeds (753 mg/100 g) but is similar to the concentrations of tannins that have been reported in dehulled seeds of the same variety (816 mg/100 g) (Embaby, 2010). The debittering process resulted in decreases in the contents of tannins in the three varieties by 80.62% relative to the raw grain. Such pronounced decreases are likely explained by the fact that high temperatures break down the tannin-protein complex, thereby inducing the leaching of tannins in the soaking medium and increasing the digestibility and palatability of the grain (Embaby, 2010). The fermentation of *L. mutabilis* caused an additional decrease in tannins (7.64%) relative to those in the debittered grain, which could be attributed to the production of tannase during fermentation (Khan, Karnpanit, Nasar-Abbas, Huma, & Jayasena, 2018). Similar patterns have been observed in *L. campestris* that was debittered in an aqueous system under alkaline conditions where tannin content was reduced by 77% (alkaline aqueous treatment) and 70% (aqueous treatment) (Jiménez-Martínez et al., 2001). The fermentation of *L. angustifolius* L. with *Rhizopus* sp. decreased tannin content by 90.41% (Khan et al., 2018), which is consistent with trends that were observed in *L. mutabilis* in our study.

The content of alkaloids in *L. mutabilis* seeds varied with grain variety and grain condition ($P < 0.05$). The raw seeds of the three varieties had values between 3.76 and 4.47% and were similar to those that have been reported for *L. mutabilis* ecotypes from Perú (3.30–3.10%) (Múzquiz, Burbano, Gorospe, & Ródenas, 1989). Alkaloid contents of 3.8%, 2.74% and 1.6% have been reported for *L. albus, L. campestris* and *L. angustifulius*, respectively (Jiménez-Martínez et al., 2001; Múzquiz et al., 1989). Variability between species is associated with the amount of nitrogen present in the grain, the intensity of sunlight and the temperature of the growing areas (Carvajal-Larenas et al., 2016). ATT reduced QAs by 91.93% relative to the bitter grain. The water solubility and the low size of QAs likely contributed to their removal from the lupin seeds. Jiménez-Martínez et al. (2001) reported a reduction of 99.96% and 98.95% in QAs of *L. campestris* debittered by aqueous and alkaline treatments. The application of additional techniques such as peeling and autoclaving in *L. campestris* and *L. mutabilis* reduced alkaloids by 55% and 35%, respectively (Jiménez-Martínez, Hernández-Sánchez, & Dávila-Ortiz, 2007). Residual QAs of the debittering process were not totally degraded by *R. oligosporus*, and the following values were recorded in the fermented grain: 0.27% (INIAP-450), 0.29% (INIAP-451) and 0.18% (Criollo), levels that are considered safe for human consumption. The safety limit fixed by the health authorities of the UK, France, Australia and New Zealand for the total amount of alkaloids in lupin flours and derived products is 0.2 g/kg dry matter (Magalhães et al., 2017).

Table 1
Effect of debittering and fermentation processes on nitrates, tannins and quinolizidine alkaloid content of lupin grain (dry weight basis).

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

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

3.1.2. Urease activity, trypsin inhibitors and phytic acid

These compounds are thermolabile proteins that alter the digestion of proteins and inhibit the activity of digestive enzymes that cause the hydrolysis of dietary proteins (Egounlety & Aworh, 2003). Urease inactivation is a reliable indicator of the adequacy of heat processing and hence the degree of trypsin inhibitor activity (Yalcin & Basman, 2015). Multiple comparison tests showed that there was a significant effect of variety and grain condition on the urease activity of lupin samples ($P < 0.05$). In the three varieties of *L. mutabilis*, the debittering process caused a substantial reduction (88.42%) in urease activity. In contrast, reductions in urease activity caused by fermentation were comparatively lower: 19.05% (INIAP-450), 31.75% (INIAP-451) and 13.10% (Criollo) (Table 2). The fermented grain of INIAP-450 had the lowest degree of urease activity (0.05 pH difference), indicating that urease was inactivated (Yalcin & Basman, 2015). In soybeans that were cooked, roasted or extruded, urease activity was reduced by 98% (Qin et al., 1996; Yalcin & Basman, 2015). In accordance with the residual urease activity, trypsin inhibitors (TI) were significantly reduced with the application of the debittering and fermentation processes. The bitter grain of three varieties of *L. mutabilis* on average had 1.63 TIU/mg, which was similar to values that have been reported for other lupin species such as *L. exaltatus* (1.37 TIU/mg) and *L. reflexus* (2.05 TIU/mg) (Ruiz-López et al., 2000). A substantial reduction (71.71%) in trypsin inhibitors was observed in debittered grain, but the fermentation generated even more marked reductions: 20.38% (INIAP-450), 5.72% (INIAP-451) and 18.55% (Criollo). Higher values were reported in cooked soybeans (82.17%) and fermentation of *R. oligosporus* for 48 h (97.42%), which is consistent with the trend that we observed in *L. mutabilis*. Cooking caused a reduction of 86.09% in TIUs in beans; however, fermentation did not change the content of trypsin inhibitors (Egounlety & Aworh, 2003). Phytic acid is often regarded as an antinutrient because of its powerful ability to bind minerals, proteins and starches and thereby decrease their bioavailability. However, in vivo and in vitro studies have demonstrated that phytic acid has preventive as well as therapeutic properties (Mohan, Tresina, & Daffodil, 2016, pp. 211–220). Multiple comparison tests showed that variety and grain condition had significant effects on phytic acid content in *L. mutabilis* ($P < 0.05$). On average, the three bitter varieties had 0.312 g phytic acid/100 g, which is similar to values that have been reported for other beans (0.46 g/100 g) but lower compared with other grains and legumes, such as *L. exaltatus* (1.17 g/100 g), *L. albus* (1.42 g/100 g), *L. angostifolius* (1.45 g/100 g) (Múzquiz et al., 1989; Ruiz-López et al., 2000) and soy (1.27 g/100 g) (Carvajal-Larenas et al., 2016; Egounlety & Aworh, 2003).

Table 2
Effect of debittering and fermentation on urease activity, phytic acid and trypsin inhibitors of lupin grain (dry weight basis).

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

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

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

3.2. Antioxidant compounds

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

3.2.1. Ascorbic acid

In the bitter grain, ascorbic acid varied from 5.82 mg/100 g

Table 3
Effect of debittering and fermentation on the antioxidant content compounds of lupin grain (dry weight basis).

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

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

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

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

3.2.2. Total carotenoids

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

3.2.3. Total phenols

An average of 1127 mg chlorogenic acid/100 g was observed in bitter *L. mutabilis*, with the highest value measured from the Criollo variety (1311.13 mg/100 g). These values are within the average reported for raw and bitter *L mutabilis* seeds (1210 mg/100 g) (Chirinos, Pedreschi, Rogez, Larondelle, & Campos, 2013). We observed a significant decrease (96.83%) in total phenolic contents during the debittering process. As these phenols are thermolabile, such reductions likely stem from the soaking and boiling of the grain, which facilitate their thermal and oxidative decomposition and partial leaching. Fermentation caused a substantial increase in total phenols of the three debittered lupin varieties 1320.62% (INIAP-451), 1025.50% (INIAP-450) and 820.58% (Criollo). The glycosidases of *Rhizopus* might hydrolyze the conjugated polyphenol forms, releasing free polyphenols and, as a consequence, increasing the total phenol content (Khan et al., 2018; Lee, Hung & Chon, 2008).

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

3.2.4. Trolox equivalent antioxidant capacity (TEAC)

The antioxidant capacity of raw seeds measured by the TEAC assay ranged from 707.73 to 747.57 μmol Trolox/g (dry weight basis) (Table 3). Bitter lupin values from our study were higher than those that have been reported in raw seeds of *L. albus* (202.7 μmol Trolox/g) (Chirinos et al., 2013). However, raw seeds of *L. angustifolius* cv. Emir exhibited higher TEAC values (Martínez-Villaluenga et al., 2009). The debittering process caused a decrease of 96.12% in TEAC values; this reduction stemmed from the thermolability and water solubility of phenols. Nonetheless, the fermentation of *L. mutabilis* with *R. oligosporus* increased antioxidant capacity with values that ranged from 340.75 (INIAP-450) to 657.72 μmol trolox/g (dry weight basis) (INIAP-451) relative to debittered grain. This increase might be explained by the ability of some microorganism strains to develop oxidative stress protection mechanisms when they are exposed to reactive oxygen substances (Hur, Lee, Kim, Choi, & Kim, 2014). During fermentation, molds produce different enzymes, such as β-glucosidase, which hydrolyze the β-glucosidic bonds of some phenolic compounds, increasing their antioxidant activity. The fermentation of *L. angustifulius zapatón* variety with *Rhizopus* increased antioxidant activity by 10% relative to unfermented grain (Fernandez-Orozco et al., 2008). Increases of 210% and 303% were observed for free radical-scavenging activity and ferric ion-reducing antioxidant power, respectively, following fermentation of the germinated *Lupinus angustifolius* L. (Khan et al., 2018).

4. Conclusions

The three varieties of *L. mutabilis* in their bitter forms possessed a variety of antinutritional compounds in varying quantities, such as 4.47% of alkaloids (INIAP-451), 975.74 mg tannins/100 g (Criollo) and 0.392 g phytic acid/100 g (Criollo). Nevertheless, bitter lupin varieties also contained functional compounds, such as total phenols (1127.87 mg/100 g), with higher concentrations in the Criollo variety (1311.13 mg/100 g), as well as total carotenoids (3.72 μg/g) and ascorbic acid (9.6 mg/100 g). The impact of debittering and fermentation processes on the aforementioned compounds was analyzed. Debittering caused a substantial reduction in QAs (92.06%), nitrates (94.84%), tannins

(83.79%), urease activity (89.51%), trypsin inhibitors (67.44%) and phytic acid (48.46%). However, the contents of total phenols, carotenoids and ascorbic acid were also affected. Fermentation decreased antinutrients and ascorbic acid (63.40%) from debittered grain and increased total carotenoids (165.38%), phenolic compounds (1055.56%) and antioxidant capacity (1515.62%). In this research it was determined that antinutritional compounds with antioxidant properties are concentrated in bitter lupin and that some of these compounds may have pharmacological effects. The outcome of this study may be of great importance to the food industry for the production of novel, fermented food products with improved nutritional value through the fermentation of lupin. The significant concentrations of these phytochemicals in debittered grain suggest that lupin flour could be potentially used in a variety of bakery products.

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CRediT authorship contribution statement

Elena Villacrés: Writing - review & editing, Project administration, Supervision, Software. **María Belén Quelal:** Validation, Software, Writing - original draft. **Edgar Fernández:** Investigation, Software. **Grace Garcìa:** Investigation, Software. **Gabriela Cueva:** Conceptualization, Supervision. **Cristina M. Rosell:** Writing - review & editing.

Declaration of competing interest

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

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ORIGINAL PAPER

Replacing Wheat Flour with Debittered and Fermented Lupin: Effects on Bread's Physical and Nutritional Features

Elena Villacrés[1] · Paúl Cueva[1,2] · Milene Díaz[2] · Cristina M. Rosell[3]

Abstract

In this study the breadmaking potential of lupin flour from *L. mutabilis* after being debittered (DLF) and solid state fermented (FLF) was evaluated in lupin-wheat breads. Different levels of substitution (10, 15, 20%) were tested on dough rheology and the technological and nutritional (composition and *in vitro* digestibility indexes) properties of breads, as well as acceptability. Lupin weakened the dough during mixing, having shorter development time and stability, especially FLF. Less relevant was the effect of lupin flours along heating-cooling of the doughs recorded with the Mixolab. DLF and FLF significantly affected technological properties of the lupin-wheat breads at higher substitution (> 10%), particularly reducing bread volume, crust luminosity, crumb cohesiveness and resilience. Detrimental effects observed at the highest substitutions (20%) were diminished when using FLF, although breads received lower score due to the acidic taste detected by panelists. Both lupin flours provided lupin-wheat breads with rather similar composition, rising the average content of proteins, fat and dietary fiber by 0.8, 2.4, 6.5 %, respectively, compared to wheat breads. Likewise, lupin-wheat breads had significantly lower hydrolytic and glycemic indexes. Overall, debittered and fermented lupin could be used for enriching wheat breads, although better technological properties were observed with FLF.

Keywords Lupin · Debittering · Fermentation · Bread · Nutrition · Quality

Introduction

Wheat bread constitutes an important part of the diet and remains as staple food across the civilized world [1]. Nevertheless, sustainability issues and current consumers' demands have driven the latest innovations in bakery towards sustainable and healthy foods made of either whole grains, alternative grains or even legumes as substitutes for refined wheat. Likewise, wheat replacement in bread recipes for other grains allows reducing wheat importation in non-wheat producers' countries. Lupin (genus *Lupinus*) is an undervalued legume that some decades back was proposed for increasing the nutritional value of bread [2]. However, only some years ago lupin seeds awakened growing interest due to its high protein content [3], observing very little changes in product acceptability up to 6% addition [4, 5]. Increasing amounts of lupin flour (up to 20%) decrease the bread volume and the crumb texture quality [6], but allows increasing the content in protein and dietary fiber, apart from the content of bioactive compounds like phenols and carotenoids [7]. Most of the studies reported for bread enrichment with lupin have been carried out using *L. angustifolius* (Blue Lupine with narrow leaves) [8] or *L. albus* (White Lupine) [4]; although *Lupinus mutabilis* Sweet shows better adaptation to poor soils and extreme conditions and limited information exists about its application in breadmaking. Despite the high nutritional value

Highlights
- *L. mutabilis* flour could be used as ingredient for making wheat breads.
- Debittering and solid-state fermentation lead to lupin flours with good breadmaking performance.
- Up to 10% wheat replacement with lupin does not affect bread properties.
- Lupine-wheat breads with fermented lupin flour (20%) presented an improved nutritional profile.

✉ Elena Villacrés
elena.villacres@iniap.gob.ec

[1] Instituto Nacional de Investigaciones Agropecuarias, INIAP, Quito, Ecuador

[2] Facultad de Ciencias Químicas, Universidad Central del Ecuador, Quito, Ecuador

[3] Instituto de Agroquímica y Tecnología de Alimentos. IATA, CSIC, Valencia, España

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of lupin seeds, its use is very limited due to the presence of bitter compounds, specifically alkaloids derived from quinolizidine [9]. The aqueous debittering process, consisting in several washings, reduces the alkaloids content to safe levels [10], but it is quite costly in water and time consuming. This process can be more efficient using thermal treatments [11] and saline solutions (0.5% (w/v) for seeds hydration and cooking [12]. Likewise, further nutritional improvement of lupin seeds could be obtained with the solid-state fermentation by using lactic acid bacteria [13] or fungi [14]. In fact, wholemeal lupin fermented with *Lactobacillus sakei, Pediococcus pentosaceus* or *P. acidilactici* could be added up to 10% as sourdough to wheat flour, improving the rheological properties of dough and wheat-lupine bread volume [13]. Regarding fungi, fermentation of lupin with *Rhizopus oligosporus* induced a further increase of protein levels [14], but there is no information about its potential application in breadmaking.

The objective of this research was to evaluate the impact of *Lupinus mutabilis* Sweet for wheat flour replacing in breadmaking, and to what extent the debittering and solid-state fermentation of lupin could affect the resulting dough and bread quality. With that purpose, different levels of lupin were tested and dough rheological properties as well as nutritional and technological quality of enriched breads were evaluated.

Materials and Methods

Materials

Lupinus mutabilis Sweet, variety INIAP-Andino 450, obtained by selection and evaluation from a germplasm population introduced from Peru in 1992, with the identification of ECU-2659. Commercial wheat flour (WF) for breadmaking (moisture content 14%, ash 0.73%, protein 14.30%, gluten content 33.11%) "Superior France" from Alsuperior S.A. (Quito, Ecuador) was used.

Production of Debittered Lupin Flour (DLF) and Fermented Lupin Flour (FLF)

The grains were debittered following the aqueous heat process [12]. One part of debittered grain (5 kg) was then dried in an air convection cabinet (Labolan HS122A, Navarra, Spain) at 50°C for 6 h, it was cooled down to room temperature and ground in a mill using a 100 μm sieve (Retsch KG -5657 Haan Remscheid, Germany), obtaining the debittered lupin flour (DLF). Other part of debittered grain (5 kg) was subjected to solid state fermentation following the procedure described by Villacrés et al. [14]. Fermented grains were then dried and ground as above described, to obtain fermented lupin flour (FLF).

Physical Characteristics and Chemical Composition of Flours

pH and total titratable acidity (TTA) were determined after homogenizing 10 g of flour with 90 ml of distilled water, and expressed as milliliters of 0.1 m NaOH needed to reach pH 8.3. Standard methodologies of the AOAC was used to assess: humidity (925.09), protein (total N x 6.25) (955.39), fat (920.39), dietary fiber (991.43) and ash (942.05) [15]. The mineral content was determined by atomic absorption spectrophotometry in AA-7000 atomic absorption spectrophotometer (Shimadzu, Kyoto, Japan) following AOAC method 985.35, except for phosphorus that was colorimetrically analyzed [15].

Dough Characterization

Mixolab (Chopin, Villeneuve-la-Garenne Cedex, France) was used to characterize the rheology of the doughs following the standard method AACC (54-60.01) [16]. The impact of the lupin flours (DLF and FLF) was evaluated by replacing wheat flour at three levels 10, 15 and 20%. pH and total titratable acidity (TTA) of lupin-wheat doughs were assessed as described previously for flours.

Bread Making

Bread recipe, based on 100 g flour, was: 5 g sugar, 2 g salt, 9 g sunflower seeds oil, 4 g compressed yeast, 3 g dairy powder and the required water assessed in the Mixolab. Ingredients were mixed together in a mixer (Planetaria VFICB7B, Lombardía, Italy) for 7 min. Dough was proofed in a cabinet at 37 °C and 90% relative moisture for 20 min, then dough was divided (170 g), shaped and placed into previously greased stainless steel trays, which were fermented for 1 h at 37 °C. Baking was carried out at 190°C for 25 min in an electric oven (Maquipan UHC-1, Florida, USA). Loaves were cooled down at room temperature for 30 min.

Bread Characterization

Chemical composition of breads was assessed as described previously for flours. The texture was performed 24 h after the breads were baked, following the AACC method 74 − 09 [18] using a texturometer TA-XT2i, Stable Micro Systems, Godalming, UK. The crust and crumb color were performed using a Portable Spectrophotometer (Lange Spectro-Color d/8° model LZM 268, Chelmsford, United Kingdom) based on the CIE L^*, a^*, b^* color system. The following attributes of visual sensation were measured: L^* (luminosity), C^* (chromatism) and H^* (hue). The determination of specific volume was made according to the method AACC 10-05.01

[16], the hydrolytic and glycemic index by the method described by Goñi et al. [17].

For sensory acceptability, breads were placed on coded white plastic plates and served randomly. Test was performed with twenty seven trained panelists (14 females and 13 male, ranging in age between 20 and 40 year) working at the Santa Catalina Experimental Station, INIAP (Quito, Ecuador). Previous group discussion was carried out to define bread characteristics and scores. A 7-point hedonic scale (1 – disliked extremely, 2 – much disliked, 3 – disliked, 4 – liked and did not like, 5 – liked, 6 –a lot, 7 – liked extremely) [18].

Statistical Analysis

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

Results and Discussion

Characteristics of Debittered and Fermented Lupin Flours and Wheat-lupin Doughs

There were significant differences ($P < 0.05$) on the proximate and minerals composition of the lupin flours (Table 1). The solid-state fermentation significantly increased the protein and fat, with a concomitant reduction in crude fiber, carbohydrate content and minerals (with exception of copper), likely due to the metabolic activity of *Rhizopus oligosporus* [14]. It must be stressed that the protein content was much higher than the one reported by Mubarak [4] for deffated lupin flour from *Lupinus albus*, which could be associated to the lupin variety or the debittering process that can greatly affect the nutritional profile of the flours [7]. Regarding pH and acidity, FLF significantly decreased the pH and increased the TTA (Table 1). Three levels of wheat replacement with lupin flours (DLF, FLF) were tested in the breadmaking process. When dough rheological properties were evaluated with the Mixolab, the type of lupin flour significantly affected, the development time, dough stability during mixing and heating (C4) and dough consistency after cooling (C5) (Table 2). Water absorption values were in the range reported by Mubarak [4]. DLF progressively decreased the development time when increasing the substitution level, whereas FLF decreased that parameter independently on the level. Despite the gluten reduction when decreasing the relative amount of WF, dough had similar stability to wheat dough or even increased with DLF. Moreover, C2 was higher in lupin-wheat doughs at the highest level tested. Results suggested that lupin proteins might be incorporated into the gluten matrix and remained entrapped, giving some consistency during heating. Islam et al. [19] suggested that β-conglutins of lupin could be trapped within gluten matrix even after baking, whereas the higher thermal stability of the α-conglutins might explain their no structural integration. Starch gelatinization related to C3 and its stability during heating (C4) decreased in the presence of DLF but the opposite effect was observed in FLF, in spite of the lower carbohydrate content of this flour (Table 1), thus starch performance might be affected by the other flour constituents like the high content of proteins [20]. Again, FLF increased the dough consistency after cooling (C5) and a tendency to decrease it when increasing the level of the FLF flour was envisaged, but the opposite effect was observed with DLF. An increase in C5 has been described when increasing amounts of debittered lupin flour (up to 25%) were blended with wheat, which was related to the interactions between wheat amylose and lupin lipids (one of the major constituents) [20]. Divergences observed in the present study with DLF might be due to the adapted hydration of the doughs used in this study. Nevertheless, considering the possible role of lipids in dough consistency after cooling, it seems that the different lipid profile of DLF and FLF might be responsible of their diverse performance [14].

Breads Technological Properties and Acceptability

Lupin treatment significantly affected the specific volume and all color parameters of the crumb, being the effect more marked for breads containing FLF (Table 3). Nevertheless, no significant effect on specific volume was observed with 10% replacement with either of the lupin flours. Higher replacements induced a significant reduction of the specific volume. These results agree with previous findings, attributing that reduction to the gluten replacement by lupin proteins and the level of fiber [21, 6].

Wheat substitution with lupin significantly reduced the luminosity of the crust, and that effect was intensified when increasing the levels of lupin, significantly in the case of FLF (Table 3). Similar observations have been reported with other lupin-wheat breads [21]. Crumb chroma (C^*) significantly increased with both lupin flours, but a steady increase was observed when augmenting the level of FLF, which could be attributed to its higher carotenoids content (544.78 μg/100 g) [14]. Similarly, Dervas et al. [22] reported darker crust and yellowish crumbs at levels of substitution higher than 10% with *L. albus*.

The lupin treatment significantly affected the textural parameters, with exception of springiness, whereas the level of substitution significantly affected hardness, chewiness and resilience (Table 3). Specifically, flours type significantly increased the crumb hardness at levels > 10% and reduced

Table 1 Physical characteristics, proximate and minerals composition of debittered (DLF) and fermented (FLF) lupin flours compared to wheat flour

	DLF	FLF	Wheat
pH	6.66 ± 0.03^{b}	5.42 ± 0.025^{a}	5.96 ± 0.05^{a}
TTA (mL 0.1 m NaOH)*	0.30 ± 0.001^{b}	7.75 ± 0.002^{a}	0.27 ± 0.06^{b}
Moisture	104.80 ± 0.14^{b}	106.90 ± 0.13^{b}	124.60 ± 0.14^{a}
Ash	21.50 ± 0.50^{a}	19.97 ± 0.50^{a}	6.80 ± 0.01^{b}
Fat	227.51 ± 0.90^{b}	244.00 ± 1.95^{a}	13.91 ± 0.001^{e}
Crude fiber	137.90 ± 3.85^{a}	116.40 ± 5.14^{b}	12.11 ± 0.19^{e}
Protein	546.88 ± 1.45^{b}	608.15 ± 4.35^{a}	144.03 ± 0.01^{c}
Total carbohydrates	66.22 ± 4.92^{b}	11.45 ± 5.10^{c}	823.22 ± 0.08^{a}
Calcium	4.00 ± 0.40^{a}	2.40 ± 0.10^{b}	0.10 ± 0.01^{c}
Phosphorus	4.70 ± 0.50^{a}	3.27 ± 0.35^{b}	2.03 ± 0.01^{c}
Magnesium	0.65 ± 0.05^{a}	0.56 ± 0.11^{b}	0.42 ± 0.01^{c}
Potassium	0.95 ± 0.12^{b}	0.11 ± 0.02^{c}	11.01 ± 0.01^{a}
Sodium	0.12 ± 0.03^{b}	0.11 ± 0.02^{c}	9.00 ± 0.01^{a}
Iron	57.70 ± 1.57^{a}	52.67 ± 1.53^{b}	33.00 ± 0.01^{c}
Zinc	69.96 ± 0.14^{a}	29.75 ± 1.52^{b}	15.00 ± 0.01^{c}
Copper	1.83 ± 0.22^{c}	2.97 ± 0.17^{a}	2.00 ± 0.01^{b}
Manganese	21.33 ± 2.08^{a}	9.70 ± 0.52^{c}	10.00 ± 0.01^{b}

Values followed by different letters within rows denote significant differences ($P < 0.05$). Mean ± standard deviation ($n = 03$). Moisture, protein, ash, lipids, crude fiber, total carbohydrates, calcium, phosphorus, magnesium and potassium data are expressed as the $g \cdot kg^{-1}$ dry weight of the sample. Sodium, Iron, zinc, copper and manganese are expressed as $mg \cdot kg^{-1}$ (dw)

resilience. The flour type also affected significantly the crumb cohesiveness. Compared to wheat bread, DLF gave similar crumb hardness in the lupin-wheat breads up to 10% substitution, but higher level of substitution resulted in great crumb hardening. Similar hardness has been reported for lupin-wheat breads at those levels of substitution and it has been explained based on gluten dilution [20], which has been also observed with lupin protein isolates (10%) that gave more compact crumbs [23]. Conversely, 10% FLF substitution gave softer crumbs, and although higher substitution resulted in crumb hardening, it was lower than that of DLF. Some authors studying different varieties of lupins observed that their lipid and protein profile might be responsible of attaining some textural properties similar to wheat breads [6]. Cohesiveness of the crumbs was reduced with the lupin flours but no trend was observed with the level of substitution. Chewiness was even reduced in lupin-wheat breads, compared to wheat bread and only DLF at 20% replacement resulted in a significant increase. Considering that chewiness is inversely related to the easiness of chewing, FLF allowed obtaining more chewy protein enriched wheat breads. Crumbs resilience was significantly reduced with the lupin flours, compared to wheat bread, and the effect was slightly more noticeable with FLF. This decrease could be related to the low specific volume of the

Table 2 Effect of wheat flour substitution by debittered (DLF) and fermented lupin (FLF) flour on rheological characteristics of dough (Mixolab profile)

	Wheat	DLF			FLF		
	0%	10%	15%	20%	10%	15%	20%
Water absorption (%)	64.25 ± 0.35^{c}	66.82 ± 0.02^{b}	62.67 ± 0.98^{c}	63.43 ± 0.07^{c}	62.78 ± 5.31^{c}	70.90 ± 0.73^{a}	61.61 ± 0.43^{d}
Development time (min)	5.28 ± 0.00^{b}	7.45 ± 1.94^{a}	2.60 ± 0.68^{c}	2.12 ± 0.02^{c}	1.39 ± 0.23^{c}	1.21 ± 0.23^{c}	1.08 ± 0.01^{c}
Stability (min)	10.64 ± 0.05^{c}	11.62 ± 0.19^{a}	12.05 ± 0.04^{a}	12.21 ± 0.01^{a}	11.56 ± 1.45^{b}	9.60 ± 0.88^{c}	9.69 ± 1.32^{c}
C2 (Nm)	0.51 ± 0.01^{b}	0.53 ± 0.05^{b}	0.63 ± 0.01^{ab}	0.66 ± 0.01^{a}	0.58 ± 0.04^{b}	0.61 ± 0.04^{ab}	0.65 ± 0.01^{a}
C3 (Nm)	1.61 ± 0.01^{c}	1.53 ± 0.05^{d}	1.54 ± 0.01^{d}	1.57 ± 0.01^{d}	1.84 ± 0.01^{a}	1.73 ± 0.01^{b}	1.71 ± 0.05^{b}
C4 (Nm)	1.29 ± 0.04^{b}	1.06 ± 0.06^{c}	1.06 ± 0.05^{c}	1.03 ± 0.04^{c}	1.79 ± 0.03^{a}	1.77 ± 0.03^{a}	1.68 ± 0.01^{b}
C5 (Nm)	1.42 ± 0.08^{c}	1.40 ± 0.04^{c}	1.46 ± 0.16^{c}	2.42 ± 0.04^{a}	2.34 ± 0.01^{a}	2.06 ± 0.03^{b}	1.70 ± 0.12^{b}

Values followed by different letters within rows denote significant differences ($P < 0.05$). Mean ± standard deviation ($n = 03$). C2: Protein weakening, C3: Starch gelatinization, C4: Amylase activity, C5: Starch retrogradation

Table 3 Effect on wheat flour substitution by debittered (DLF) and fermented (FLF) lupin flour on technological properties and acceptability of bread

	Wheat	DLF			FLF		
		10%	15%	20%	10%	15%	20%
Specific volume ($cm^3\ g^{-1}$)	5.26 ± 0.04^{a}	5.29 ± 0.10^{a}	4.95 ± 0.14^{b}	4.66 ± 0.03^{c}	5.28 ± 0.03^{a}	4.61 ± 0.05^{c}	4.59 ± 0.03^{c}
L^* crust	53.78 ± 4.74^{a}	52.00 ± 6.06^{a}	48.81 ± 6.87^{abc}	50.07 ± 5.75^{ab}	47.35 ± 5.91^{abc}	45.14 ± 6.29^{bc}	44.10 ± 4.84^{c}
C^* crust	62.18 ± 10.25^{a}	53.92 ± 12.91^{a}	55.23 ± 11.00^{a}	60.62 ± 11.95^{a}	56.19 ± 9.18^{a}	61.00 ± 9.09^{a}	62.59 ± 6.25^{a}
h^* crust	74.63 ± 2.02^{a}	74.92 ± 2.73^{a}	73.39 ± 3.35^{a}	74.60 ± 2.22^{a}	74.31 ± 2.49^{a}	74.13 ± 2.61^{a}	73.20 ± 1.86^{a}
L^* crumb	60.01 ± 6.37^{a}	51.63 ± 4.04^{d}	52.75 ± 3.77^{d}	57.54 ± 2.03^{b}	$53.97 \pm 2.71^{c,d}$	50.98 ± 2.73^{d}	56.14 ± 1.88^{bc}
C^* crumb	21.48 ± 2.16^{e}	26.53 ± 1.91^{d}	$28.43 \pm .95^{c}$	$27.96 \pm 1.62^{c,d}$	26.30 ± 0.98^{d}	31.15 ± 2.35^{b}	33.42 ± 1.78^{a}
h^* crumb	80.47 ± 0.70^{b}	80.88 ± 0.38^{b}	83.34 ± 0.38^{a}	83.53 ± 0.23^{a}	82.92 ± 0.43^{a}	82.96 ± 0.32^{a}	80.68 ± 4.52^{b}
Hardness (N)	7.98 ± 0.08^{d}	7.86 ± 0.53^{d}	9.59 ± 0.22^{c}	28.28 ± 0.44^{a}	6.46 ± 0.04^{e}	9.38 ± 0.08^{c}	12.36 ± 0.077^{b}
Springiness	1.37 ± 0.29^{a}	0.99 ± 0.30^{b}	1.13 ± 0.23^{b}	0.96 ± 0.14^{c}	1.18 ± 0.20^{b}	1.12 ± 0.33^{b}	0.85 ± 0.08^{d}
Cohesiveness	0.49 ± 0.02^{a}	0.30 ± 0.06^{d}	0.43 ± 0.06^{b}	0.42 ± 0.01^{b}	0.37 ± 0.03^{c}	0.38 ± 0.05^{bc}	0.39 ± 0.02^{bc}
Chewiness (N)	5.02 ± 0.09^{b}	2.07 ± 145.29^{d}	4.08 ± 0.12^{c}	11.20 ± 0.09^{a}	2.83 ± 0.07^{d}	4.03 ± 0.10^{c}	4.60 ± 0.08^{d}
Resilience	0.28 ± 0.04^{a}	0.10 ± 0.03^{d}	0.20 ± 0.03^{b}	0.24 ± 0.01^{b}	0.14 ± 0.02^{c}	0.15 ± 0.03^{c}	0.16 ± 0.01^{c}
Acceptability	5.20^{a}	5.74^{a}	5.33^{a}	5.41^{a}	4.74^{b}	4.41^{b}	3.52^{c}

Values followed by different letters within rows denote significant differences ($P < 0.05$). Mean ± standard deviation ($n = 03$)

breads, having denser crumbs with lower number of gas cells, in consequence the crumb structure takes longer to recover after compression [24]. The sensory analysis carried out with those breads to check acceptability indicated that only the lupin treatment significantly affected the acceptability, which was similar to wheat bread when using DLF for substitution, but decreased for FLF (Table 3). Panelists attributed the lower acceptance of the FLF-wheat bread due to their acidic taste and flavor. The aroma of lupin-wheat breads has been associated to oxidative degradation of fatty acids or thermal reactions [5], thus the different fatty acids profile of DLF and FLF [14] might explain their sensory differences. Changes in aroma and taste have been previously reported at 15% substitution with *L. albus* [25]. Even lower levels of deffated lupin (9%) has been reported to decrease the overall quality of lupin-wheat breads due to low scoring in texture, crumb color and flavor [4].

Nutritional Characteristics of Lupin-wheat Bread

Bread composition in macro and micronutrients is displayed in Table 4. The multiple factor analysis of variance indicated significant differences ($P<0.05$) promoted by flour type (debittering, fermentation) of lupin flour on moisture, ash, fat, crude fiber, protein and carbohydrates content, whereas the level of wheat substitution by lupin additional resulted in significant variation ($P<0.05$) in most nutritional component, except in copper (data not showed). Compared to wheat bread, breads containing lupin, whatever treatment, had lower moisture content, higher protein, fat and fiber content, which increased with the level of wheat replacement. Despite the higher fiber content, lupin-wheat breads retained less moisture, likely due to the high fat content. Higher moisture content has been reported for breads made with lupin previously fermented with lactic acid bacteria and used as sourdough [13]. For the same level of wheat substitution (20%), lupin enriched breads had similar composition in protein and ash than the one previously reported by [7] when compared breads made with debittered lupin from different varieties of *L. angustifolius*, but higher levels of fat and crude protein are obtained in the present study with *L. mutabilis*. Unexpectedly, although FLF had higher protein content than DLF, that difference was not observed in the lupin-wheat breads. Compared to wheat breads, lupin-wheat breads with FLF increased the protein content by 14.71, 29.75 and 30.53% when the level of substitution was 10, 15 and 20%, respectively, versus 20.31, 30.0 and 32.48% obtained with same substitution of DLF. Possibly, nitrogen compounds in FLF were more accessible to yeast during fermentation reducing the theoretical increase. Therefore, initial differences in the nutrient composition of the debittered and fermented lupin flours were not really noticeable in the resulting breads. Regarding the mineral content (Table 4), the flour type and level of substitution affected significantly the amount of all minerals, with exception of copper (results not shown). Nevertheless, compared with the wheat bread, lupin-wheat breads had significantly higher content of calcium, phosphorus, magnesium (only FLF), iron, manganese and zinc, which agrees with the high mineral content of lupin flour [26]. Again, despite the significant differences observed in the lupin flours composition, no great differences were observed between the resulting lupin-wheat breads.

The hydrolytic and glycemic indexes evaluated by *in vitro* methods were affected by both factors, the flour type and the

Table 4 Effect on wheat flour substitution by debittered (DLF) and fermented (FLF) lupin flour on proximate (expressed as percentage), mineral composition, glycemic and hydrolytic index of bread

	Wheat	DLF			FLF		
		10%	15%	20%	10%	15%	20%
Moisture	371.00 ± 0.44^{a}	344.61 ± 1.93^{b}	349.70 ± 2.10^{b}	314.82 ± 3.56^{d}	304.61 ± 0.15^{e}	334.60 ± 1.18^{c}	307.22 ± 2.17^{de}
Ash	24.71 ± 0.05^{b}	25.30 ± 0.08^{b}	25.82 ± 0.05^{ab}	27.11 ± 0.05^{a}	24.72 ± 0.02^{b}	26.00 ± 0.06^{ab}	27.04 ± 0.07^{a}
Fat	45.82 ± 0.13^{e}	78.20 ± 1.10^{c}	80.11 ± 0.88^{b}	99.50 ± 2.04^{a}	74.72 ± 1.59^{d}	81.30 ± 1.59^{b}	96.92 ± 1.60^{a}
Crude fiber	11.70 ± 0.12^{b}	27.12 ± 0.13^{b}	32.04 ± 1.09^{a}	35.80 ± 2.01^{a}	22.73 ± 1.12^{c}	28.82 ± 1.41^{b}	33.52 ± 0.26^{a}
Protein	153.63 ± 0.30^{d}	184.80 ± 0.18^{b}	199.71 ± 0.64^{a}	203.50 ± 0.21^{a}	176.20 ± 0.07^{c}	199.31 ± 0.30^{a}	200.51 ± 0.16^{a}
Carbohydrates	$768.61 \pm .00^{a}$	684.63 ± 0.68^{c}	662.40 ± 0.95^{d}	634.11 ± 1.57^{e}	701.70 ± 0.59^{b}	664.62 ± 0.91^{d}	642.11 ± 0.85^{e}
Calcium	0.42 ± 0.02^{d}	1.05 ± 0.03^{c}	1.22 ± 0.04^{c}	1.32 ± 0.04^{b}	1.10 ± 0.03^{c}	1.33 ± 0.04^{b}	1.42 ± 0.04^{a}
Phosphorus	2.74 ± 0.02^{d}	2.90 ± 0.02^{c}	3.12 ± 0.02^{b}	3.22 ± 0.02^{a}	3.04 ± 0.02^{b}	3.33 ± 0.02^{a}	3.40 ± 0.02^{a}
Magnesium	0.80 ± 0.01^{c}	0.71 ± 0.01^{d}	0.83 ± 0.01^{b}	0.80 ± 0.01^{c}	0.93 ± 0.01^{a}	0.90 ± 0.01^{a}	0.91 ± 0.01^{a}
Potassium	12.30 ± 0.02^{b}	12.33 ± 0.02^{b}	12.60 ± 0.02^{a}	12.82 ± 0.02^{a}	12.31 ± 0.02^{b}	12.60 ± 0.02^{a}	12.72 ± 0.02^{a}
Sodium	20.00 ± 0.02^{b}	20.00 ± 0.02^{b}	21.00 ± 0.02^{a}	21.00 ± 0.02^{a}	19.00 ± 0.02^{c}	19.00 ± 0.02^{c}	20.00 ± 0.02^{b}
Copper	2.03 ± 0.02	2.00 ± 0.02	2.00 ± 0.02	2.00 ± 0.02	2.01 ± 0.02	2.01 ± 0.02	2.01 ± 0.02
Iron	56.00 ± 0.02^{f}	97.00 ± 0.02^{e}	101.00 ± 0.02^{d}	101.00 ± 0.02^{d}	107.00 ± 0.02^{c}	110.00 ± 0.02^{b}	121.00 ± 0.02^{a}
Manganese	16.00 ± 0.02^{d}	17.00 ± 0.02^{c}	17.00 ± 0.02^{c}	18.00 ± 0.02^{b}	17.00 ± 0.02^{c}	18.00 ± 0.02^{b}	19.00 ± 0.02^{a}
Zinc	18.00 ± 0.02^{f}	23.00 ± 0.02^{c}	24.00 ± 0.02^{b}	25.00 ± 0.02^{a}	20.00 ± 0.02^{e}	22.00 ± 0.02^{d}	24.00 ± 0.02^{b}
Hydrolytic index	100.00 ± 0.00^{a}	51.96 ± 10.96^{b}	50.12 ± 16.60^{b}	49.90 ± 20.44^{b}	49.45 ± 6.77^{b}	45.14 ± 2.52^{c}	39.76 ± 2.67^{d}
Glycemic index	94.61 ± 0.00^{a}	68.24 ± 5.98^{b}	61.07 ± 9.11^{c}	60.11 ± 11.22^{c}	66.86 ± 3.72^{b}	64.49 ± 1.39^{b}	61.54 ± 1.46^{c}
Dietary fibre*	52.8 ± 0.12^{e}	282.5 ± 0.13^{cd}	466.7 ± 1.09^{b}	581.8 ± 2.01^{a}	215.5 ± 1.12^{d}	475.7 ± 0.26^{b}	350.1 ± 1.41^{c}

Values followed by different letters within rows denote significant differences ($P < 0.05$). Mean ± standard deviation ($n = 03$). Moisture, protein, ash, lipids, crude fiber, total carbohydrates, calcium, phosphorus, magnesium and potassium data are expressed as the $g \cdot kg^{-1}$ dry weight of the sample. Sodium, Iron, zinc, copper and manganese are expressed as $mg \cdot kg^{-1}$ (dw). * Data are expressed as $g \cdot kg^{-1}$ dry weight of the sample

level of substitution (data not shown), with major impact promoted by FLF. Those indexes were significantly lower than the ones obtained for wheat breads, confirming the reduced digestion of the starchy compounds in lupin-wheat breads [17]. The hypoglycemic effect of the lupin-wheat breads has already been reported and associated to the type of proteins, particularly the γ-conglutins [27]. The effect on hydrolytic index was significantly more accentuated in FLF breads and a progressive reduction was observed increasing lupin levels.

Other highly appreciated aspect in breads is the content in dietary fiber and lupin-wheat breads had significantly higher fiber content than wheat bread, particularly in the case of DLF-wheat breads. The DLF flour had significantly higher content of fiber than FLF flour (Table 1). Fiber is reduced during the solid-state fermentation process because *R. oligosporus* partly used it to synthesize fats and bioactive compounds required for its metabolism [14].

Concluding Remarks

Debittered and fermented lupin flours from *L. mutabilis* showed good breadmaking performance at dough and bread level when blended with wheat flour. Lupin-wheat breads without any significant impact on the technological properties could be obtained with 10% wheat substitution. Nevertheless, to further increase the quantities of protein, dietary fiber and minerals in lupin-wheat breads, substitution could be increased up to 20%, although with some detrimental effect on crust luminosity, specific volume, crumb hardness, cohesiveness and resilience. Despite the different proximate composition of debittered and solid-state fermented lupin flours, barely differences were evidenced in the nutritional composition of the lupin-breads, but FLF could be used up to 20% with less impact on textural properties, and greater reduction on the hydrolytic index, although the acidic taste detected by panelists should be masked.

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Compliance with Ethical Standards

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

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