UNIVERSIDAD AUTÓNOMA DEL ESTADO DE HIDALGO

Instituto de Ciencias de la Salud

Instituto de Ciencias Básicas e Ingeniería

Instituto de Ciencias Agropecuarias

DOCTORADO EN CIENCIAS DE LOS ALIMENTOS Y SALUD HUMANA

TESIS DOCTORAL

“DESARROLLO DE UNA MATRIZ ALIMENTARIA MEDIANTE LA TECNOLOGÍA DE EXTRUSIÓN CON HARINAS de *Lupinus angustifolius* GERMINADO PARA DETERMINAR SU EFECTO BIOLÓGICO A TRAVÉS DE PRUEBAS *IN VITRO*”

Para obtener el grado de:

Doctor en Ciencias de los Alimentos y Salud Humana

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Asunto: Autorización de impresión

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Por este medio se informa que el comité tutorial asignado al **Mtro. Ciro Baruchs Muñoz Llandes** con número de cuenta **195771**, estudiante del Doctorado en Ciencias de los Alimentos y Salud Humana ha terminado el trabajo de tesis titulado **"Desarrollo de una matriz alimentaria mediante la tecnología de extrusión con harinas de *Lupinus angustifolius* germinado para determinar su efecto biológico a través de pruebas *in vitro*"**, y por lo tanto se autoriza la impresión del documento en extenso propuesto por el/la estudiante después de haber sido revisado, analizado y evaluado de acuerdo a lo estipulado en el Artículo 73, VI del Reglamento General de Estudios de Posgrado. Lo anterior, en función de que, el/la estudiante realizó todas las correcciones, adiciones y/o modificaciones sugeridas por el comité en la revisión previa con fecha 28 de septiembre 2024. Por tal motivo, solicitamos a usted tenga a bien permitir al doctorando dar continuidad al proceso necesario que conlleve a la obtención del grado de Doctor en Ciencias de los Alimentos y Salud Humana.

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Debido a los anterior, el estudiante cumple con los requerimientos de egreso establecidos por el programa de posgrado, al contar con 5 artículos aceptados y publicados. Por lo que solicitamos a usted tenga a bien permitir al doctorando dar continuidad al proceso necesario que conlleve a la obtención del grado de Doctor en Ciencias de los Alimentos y Salud Humana.

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Cada capítulo de esta tesis doctoral ha sido publicado en revistas indexadas Journal Citation Reports (JCR). Evidenciando así la calidad, originalidad, impacto y proyección del trabajo de investigación.

ÍNDICE

Resumen

En este trabajo de investigación se evaluó el efecto del tiempo de germinación de semillas de *Lupinus angustifolius* L. sobre las propiedades tecnofuncionales, calidad nutricional y efecto biológico. Se demostró que a mayor tiempo de germinación las propiedades como capacidad de absorción de agua, absorción de aceite y formación de espuma aumentan de forma significativa. Además, los compuestos antinutricionales como ácido fítico e inhibidores de tripsina disminuyen significativamente con respecto al tiempo de germinación. Por otra parte, se evidenció en el perfil electroforético, que las condiciones de germinación permiten hidrolizar la proteína de almacenamiento, generando fracciones peptídicas de bajo peso molecular, con alto valor biológico y posibles beneficios a la salud humana; demostrando así, que la germinación de esta semilla es una alternativa eficiente parta la obtención de harinas como ingredientes para su utilización en el desarrollo de nuevos alimentos. Posteriormente, se utilizó la harina de Lupinus sin germinar para optimizar el proceso de extrusión y así desarrollar una matriz alimentaria. Se establecieron variables dependientes e independientes y mediante metodología de superficie de respuesta se evaluó el efecto de cada parámetro. Establecidas las condiciones óptimas del proceso de extrusión, se desarrollaron extrudidos con harinas germinadas a diferentes tiempos (3, 5 y 7 días). Estas botanas fueron caracterizadas fisicoquímica y nutricionalmente para determinar el efecto de los procesos de germinación y extrusión sobre cambios y transformaciones morfológicas, estructurales y de digestibilidad *in vitro.* Evidenciando así que la incorporación de harinas, de esta leguminosa germinada en botanas extrudidas, es una alternativa viable que coadyuva a la demanda de este tipo de productos, pero con mayor impacto benéfico sobre la salud del consumidor.

Introducción

La industria de las botanas es una parte de la industria de los alimentos que se encuentra en constante movimiento y expansión; la demanda de este tipo de productos crece de forma exponencial conforme crece la población mundial. Sin embargo, se ha observado un incremento en el consumo de productos con mayor valor nutricional. La cocción por extrusión es una tecnología ampliamente utilizada en la producción de alimentos a base de almidón como cereales para desayuno, galletas, pastas, y principalmente botanas (Cornet et al., 2022). La extrusión es un proceso continuo, versátil, simple y eficiente que se caracteriza por operar con altas temperaturas y tiempos cortos (Ek & Ganjyal, 2020). Durante este proceso, el material que se extrude se somete a múltiples operaciones como la compactación, reducción de tamaño de partícula y degradación molecular, debido al cizallamiento que se genera entre el tornillo y el material utilizado, sin embargo; los parámetros de procesamiento como la humedad de alimentación, velocidad del tornillo y temperatura, también impactan en las propiedades reológicas de la masa fundida, generando cambios fisicoquímicos, transformaciones moleculares, estructurales y nutricionales en el almidón y otros biopolímeros que conforman las materias primas utilizadas (Zhang et al., 2023). Durante el proceso de extrusión se originan diversos fenómenos como la gelatinización del almidón, desnaturalización de proteínas, formación de complejos amilosa-lípidos, oxidación de lípidos, degradación de vitaminas, compuestos antinutricionales y fitoquímicos, así como un incremento en la biodisponibilidad de minerales, aminoácidos y solubilidad de fibra dietética (Gomes et al., 2023).

Diversas materias primas se han incorporado en el desarrollo de snacks por extrusión, para incrementar las propiedades nutricionales del producto terminado, sin embargo, el almidón sigue siendo el componente mayoritario más utilizado, debido a que es un

biopolímero de bajo costo de producción, no tóxico y que se encuentra ampliamente distribuido en fuentes naturales, como los cereales, algunas raíces, tubérculos, tallos, hojas, etc. (Mahmood et al., 2017). Además, este componente, genera múltiples características fisicoquímicas como un alto índice de expansión y baja dureza, parámetros que le confieren al producto final determinada textura (Mohamed, 2023).

La incorporación de nuevas materias primas durante el proceso de extrusión, sigue siendo un reto para la industria de los alimentos, debido a que se generan cambios estructurales que impactan en la reología de las mezclas utilizadas y por lo tanto en la textura del producto final. El uso de harinas de semillas germinadas en el desarrollo de snacks extrudidos, se está convirtiendo en una alternativa prometedora para desarrollar snacks con mayor valor nutricional y múltiples beneficios a la salud humana, debido a que durante la germinación se genera la hidrólisis de nutrientes de reserva (almidón, proteína, fibra y lípidos) por acción enzimática, permitiendo una mayor biodisponibilidad. Además, simultáneamente se activa la síntesis de metabolitos secundarios como compuestos fenólicos, flavonoides y ácido aminobutírico (GABA), compuestos que se les ha relacionado con la prevención de múltiples enfermedades cronicodegenerativas (Ohanenye et al., 2020). Estos cambios que ocurren durante la germinación, pueden impactar en la calidad final de productos extrudidos, generando productos con mayor digestibilidad, concentración de compuestos bioactivos y disponibilidad de aminoácidos, así como un mejoramiento en las propiedades fisicoquímicas como baja densidad aparente y mayor solubilidad en agua (Krapf et al., 2020). Sin embargo, durante el proceso de extrusión los gránulos de almidón sufren cambios fisicoquímicos como la gelatinización y degradación molecular, modificando la estructura y morfología granular, así como el tamaño y distribución de partícula, impactando en parámetros como la digestibilidad, viscosidad, índice de absorción y

solubilidad en agua del producto final (Ye et al., 2018). De igual forma, durante la extrusión los gránulos de almidón pueden forman interacciones con otras biomoléculas como proteínas y fibra, modificando parámetros fisicoquímicos, estructurales y morfológicos en el producto terminado. Es por ello que el objetivo general de la presente investigación fue caracterizar y desarrollar una matriz alimentaria mediante la tecnología de extrusión a base de *Lupinus angustifolius* germinado para determinar su efecto biológico mediante pruebas *in vitro*.

Justificación

Lupinus angustifolius es una leguminosa no convencional de limitado aprovechamiento industrial. Se estima que el 80% de su producción está destinada al consumo animal; sin embargo, su calidad nutricional es de interés científico, debido a su alto contenido de proteína, aporte en fibra, vitaminas y minerales. La germinación es una técnica eficiente que bajo condiciones específicas, permite modificar las propiedades funcionales, tecnológicas y nutricionales de las harinas, convirtiéndolas en un ingrediente modificado para uso alternativo, en el desarrollo de nuevos alimentos con potenciales beneficios a la salud humana. Se ha evidenciado que una dieta con un consumo constante de leguminosas, previene el desarrollo de enfermedades no transmisibles; sin embargo, en México el consumo de germinados es limitado, de ahí la necesidad en desarrollar matrices alimentarias a base de harinas de leguminosas germinadas. La extrusión es una técnica que permite desarrollar matrices alimentarias, principalmente botanas, un alimento de fácil acceso y alto consumo independientemente de la edad, género o estatus social del consumidor. Incorporar harinas de Lupinus germinado es una alternativa viable al uso de esta semilla, ofreciendo al consumidor snacks saludables con mayor aporte proteico, mejor calidad nutricional y posibles beneficios a la salud humana.

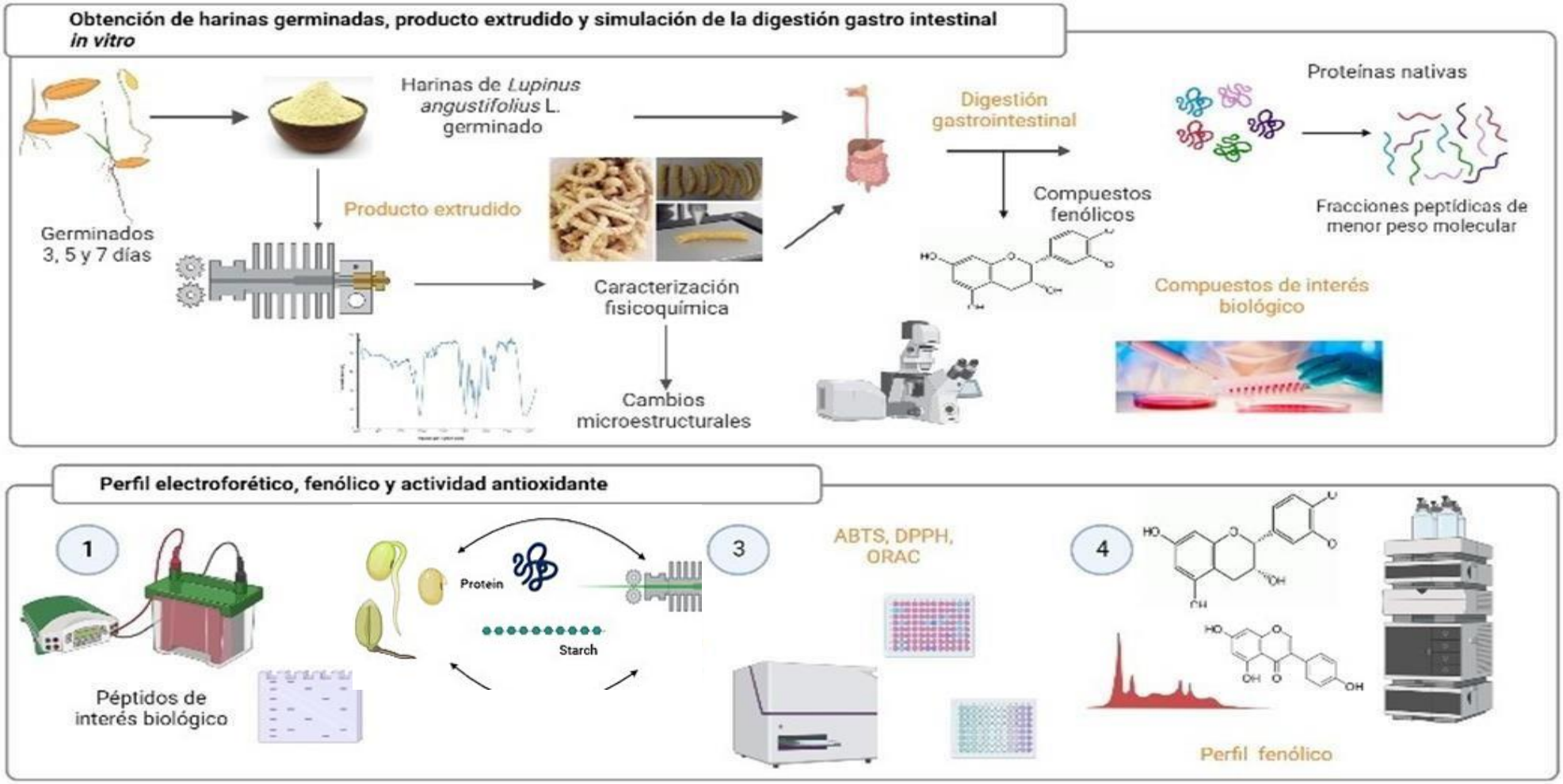
Objetivo General

- “Desarrollar una matriz alimentaria mediante la tecnología de extrusión con *Lupinus angustifolius* germinado para determinar efecto biológico mediante pruebas *in vitro*”

Objetivos específicos

- Obtener harinas de *Lupinus angustifolius* germinado a diferentes tiempos para caracterizar los cambios fisicoquímicos y funcionales por efecto de la germinación.
- Optimizar las condiciones del proceso de extrusión con harina de Lupinus sin germinar y evaluar las propiedades fisicoquímicas del extrudido optimizado.
- Desarrollar botanas mediante el proceso de extrusión con harinas de diferentes días de germinación, bajo las condiciones optimizadas para evaluar su efecto biológico.
- Evaluar el efecto anti-inflamatorio de los extrudidos y harinas de *Lupinus angustifolius* mediante pruebas *in vitro* para evidenciar su efecto biológico.
- Caracterización fisicoquímica y estructural de los extrudidos germinados y sin germinar obtenidos bajo las condiciones óptimas para comprender los cambios en el efecto biológico.

Diagrama metodológico



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Capítulo 1 Effect of Germination on starch

El primer capítulo de esta tesis doctoral es un capítulo de libro titulado Effect of Germination on starch. Este capítulo se publicó en la editorial Springer Nature. El objetivo de este trabajo fue comprender cómo las condiciones de germinación principalmente tiempo y temperatura son factores relevantes que influyen en la activación enzimática y el grado de modificación del almidón, impactando en las propiedades moleculares, fisicoquímicas y morfológicas. Estas modificaciones pueden diversificar su uso y aplicación alimentaria y no alimentaria de las semillas germinadas.

Chapter 19
Effect of Germination on Starch

Ciro Baruchs Muñoz-Llandes, Cristina Martínez-Villaluenga, Heidi María Palma-Rodríguez, Alma Delia Román-Gutiérrez, Javier Castro-Rosas, and Fabiola Araceli Guzmán-Ortiz

19.1 Introduction

Currently, germination has been proposed as a method to modify native starches as an alternative to chemically modified starches, and multiple changes in their properties have been found (Oliveira et al., 2022). Germination is a complex metabolic process in which hydrolytic enzymes deeply modify macromolecules as starch.

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The alterations of the structural, physicochemical, and functional properties of starch after germination can affect the application of starches in industries. It has been reported that germination can reduce the relative crystallinity of starch, elevate gelatinization temperature, and slightly reduce or increase peak viscosity (Xing et al., 2021; Gutiérrez-Osnaya et al., 2020; Li et al., 2017) depending on a number of factors as germination time and temperature, starch composition, crop, and variety (Wang et al., 2020). It is important to identify the most relevant parameters of the germination process that modify starch properties to establish specific conditions that allow for alternative uses and implement a simple and economic method as germination. The functionality of the starch of seed subjected to a germination process has different characteristics from a native starch. During germination enzymes such as amylases, proteases, lipases, phytases, β-glucanases are activated, which are responsible for multiple changes in the structure of the seeds. Starch is one of the main macromolecules affected. When the starch structure is modified due to enzyme hydrolysis, the foaming capacity increases and the size for the molecule is reduced, which are desirable characteristics in the baking industry. The swelling capacity is also decreased and the solubility index increases, which could be caused by a high content of amylose released from the germinated starch, which acts as a swelling inhibitor (AL-Ansi et al., 2021). When the starch is hydrolyzed, water is also allowed to enter the starch granule more easily, which allows it to increase its solubility (Su et al., 2020). Other properties such as the water and oil absorption capacity are also modified because of the changes in starch and protein by germination. Therefore, analyzing the characteristics, structural, morphological, and physicochemical changes of starch granules due to germination is of interest to establish its functionality and diversify and increase its possible application in the food and non-food industry. Therefore, this chapter compiles key information regarding the effect of germination conditions on the composition and physicochemical and structural properties of starch granules from different grains and seeds.

19.2 Germination

The germination process starts when the dry seed containing necessary genetic information absorbs water under optimal time and temperature conditions, which promote the expansion and elongation of the embryo emerging from the seed (Hermann et al., 2007). Germination involves the restoration of the basal metabolism of the seed; key metabolic changes take place before and after the emergence of the radicle.

At the beginning of the germination process, the reserve nutrients are in the form of storage, and it is the key to obtain low molecular-weight molecules such as sugars and essential amino acids, improving their bioavailability. They can still be used during embryo development in germination (Bewley, 2001), a process during which the digestibility of nutrients is improved (Chinma et al., 2021; Muñoz-Llandes et al., 2019).

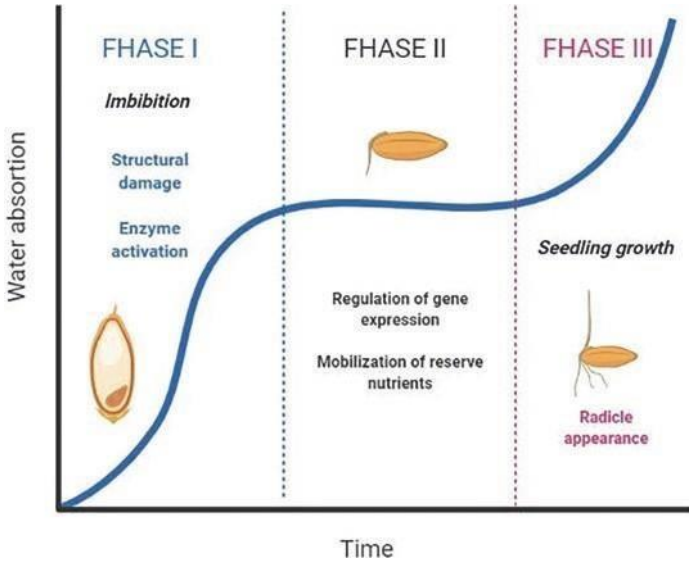

Fig. 19.1 Different phases of germination in the time

The germination process is mainly divided in three stages (Fig. 19.1). Stage I, imbibition, corresponds to water absorption in the seed through the micropyle, a pore on the seed coat (Castillo et al., 2020). The seed cells suffer structural damage due to enzymatic activation, mostly at membrane level, which hydrolyzes structural components of the cell wall. This leads to leaching of low molecular-weight solutes, and then the restoration and restricted leakage of solutes (Bewley et al., 2012). In this stage, the activity of the glycolysis and pentose–phosphate pathways are reestablished, along with the activation of the enzymes involved in the Krebs cycle. Some organelles or structural fractions are repaired while others are hydrolyzed and synthesized, as the mitochondrial membrane of the cells that store lipids. In the embryo, the ribosomal population and mRNA are reduced after rehydration to create polysomes and, later, the proteins necessary for cell structure and metabolic machinery are synthesized (Ballinas et al., 2019). In gramineous plants, there is head and endosperm resistance against the embryo. Then, there is an enzymatic degradation of certain areas of these structures before their emergence. For instance, that of homogalacturonans in the cell wall of the seed, which are esterified by the enzyme pectin methylesterase. This affects the porosity and elasticity of the cell wall, promoting water adsorption (Müller et al., 2013).

Once the seed has absorbed enough water, Stage II starts with the reactivation of different types of metabolism catalyzed by enzymes and gene expression. This triggers the growth and emergence of the radicle, leading to the development of the seedling (Han & Yang, 2015). This step involves a greater energy expenditure, obtained through the mobilization of stored compounds, such as carbohydrates, lipids, and proteins, allowing for the survival of the seed until the seedling can carry

out photosynthesis (Yu et al., 2014). Finally, in Stage III, the radicle emerges through the seed coat (Nonogaki et al., 2010). During the germination of caryopses (cereal grains), the scutellum is involved in the transference of phytohormones gibberellins secreted by the embryo, which indicate thatthe aleurone layer to start or accelerate the synthesis and secrete hydrolytic enzymes. The scutellum then transports hydrolytic products from the endosperm to the embryo (Sreenivasulu et al., 2006). The germination of the seed is regulated by a complex network signaling and regulating gene expression (Han & Yang, 2015). Essentially, this process disturbs the nutritional and physicochemical properties of grains. The time, temperature, type of grain and/or seed, variety, and amylose/amylopectin content are some of the factors that determine the degree of starch modification during germination (Noda et al., 2004; Guzmán-Ortiz et al., 2019). Starch is stored and modified cyclically during seed germination due to the complex enzymatic activity involved, affecting the structural and morphological modification of starch granules.

19.3 Enzymatic Activation

During germination, the major stored carbohydrates are modified by hydrolytic enzymes as α-amylase, β-amylase, and α-glucosidase. Firstly, α-amylase catalyzes the hydrolysis of α-1,4 glycosidic bonds, producing sugars as maltose and glucose, necessary for seedling development. The activity of the enzyme increases considerably during the first days of germination, and it depends on the conditions of the process, mostly time and temperature (Table 19.1). However, it has been reported that, in cereals as wheat, this enzyme also synthesizes *de novo* during the germination process (Krapf et al., 2019; Olaerts et al., 2017; Baranzelli et al., 2018). The enzyme α-amylase plays a key role in starch hydrolysis, generating hydrolysis products that disturb the osmotic balance of the membrane cells; in turn, this leads to a greater water absorption and, eventually, promotes radicle elongation and growth (Bewley, 2001). The stability of this enzyme depends on the acid pH of the endosperm promoting starch mobilization and other physiological processes, such as peptide transport, phytate solubilization, and secretion of hydrolytic enzymes in charge of expanding the cell wall (Macnicol & Jacobsen, 1992).

It is known that α-amylase acts upon starch molecules by breaking the α-1,4 glycosidic bonds between glucose residues of the non-reducing end, creating glucose and maltose from amylose and dextrins from amylopectin (Muslin et al., 2002). Maltose simultaneously turns into glucose after the action of enzyme α-glucosidase. Other enzymes, as dextrinase, hydrolyze the α-1,6 bonds of dextrins to create short chains that will later be hydrolyzed by amylases and create glucose (Stanley et al., 2011). These sugars obtained from starch degradation are absorbed in the scutellum and turn into saccharose. They are carried by the phloem to the embryonic axis where they feed the embryo of the growing seedling through glycolysis and the Krebs cycle (Han & Yang, 2015).

Table 19.1 Effect of germination conditions on α-amylase activity in different seeds

Raw material	α-amylase activity in ungerminated seed	α-amylase activity during germination	Soaking conditions	Germination time	Relative humidity	Temperature of germination	Reference
Waxy brown rice	0.04 U/g	0.35 U/g approx.	N/R	2 days	80%	25 °C	Wang et al. (2020)
		3.19 U/g approx.				30 °C	
		3.19 U/g				35 °C	
Yellow corn	–	6 CU/g	Distilled water (1:3) p/v 14 h, 23 °C	1 day	95–96%	15 °C	Helland et al. (2002)
		7.7 CU/g approx.		2 days			
		8.1 CU/g approx.		3 days			
		8.3 CU/g approx.		4 days			
		12 CU/g approx.		5 days			
		16 CU/g approx.		6 days			
		19 CU/g approx.		7 days			
Rough rice (*Oryza sativa* L.)	1.42 U/g	21.30 U/g	Distilled water 24 h	1 day	90–95%	28–30 °C	Moongngarm (2011)
		45.36 U/g		2 days			
		54.59 U/g		3 days			
		64.46 U/g		4 days			
Barley variety Pokko		290 U/g	Distilled water 5 h	5 days	36%	15 °C	Autio et al. (2001)
		430 U/g	Distilled water 7 h		46%		
		680 U/g	Distilled water 7 h		56%		
Barley variety Kustaa	–	180 U/g	Distilled water 5 h		36%		
		260 U/g	Distilled water 7 h		46%		
		440 U/g	Distilled water 7 h		56%		
Barley variety Arve	–	310 U/g	Distilled water 5 h		36%		
		390 U/g	Distilled water 7 h		46%		
		590 U/g	Distilled water 7 h		56%		

(continued)

Table 19.1 (continued)

Raw material	α-amylase activity in ungerminated seed	α-amylase activity during germination	Soaking conditions	Germination time	Relative humidity	Temperature of germination	Reference
Oats variety Meeri	0.65 U/g	2.75 U/g	Distilled water (1:6) p/v, 4 h, 20 °C	4 days	–	12 °C	Aparicio-García et al. (2020)
		1.57 U/g		2.5 days		14 °C	
		10.81 U/g		6.5 days			
		0.81 U/g		1 day		16 °C	
		4.07 U/g		4 days			
		25.12 U/g		9 days			
		1.47 U/g		2.5 days		18 °C	
		19 U/g		6.5 days			
		5.39 U/g		4 days		20 °C	
Oats variety Barra	0.16 U/g	1.33 U/g	Distilled water (1:6) p/v, 4 h, 20 °C	4 days	–	12 °C	Aparicio-García et al. (2020)
		0.92 U/g		2.5 days		14 °C	
		30.14 U/g		6.5 days			
		0.26 U/g		1 day		16 °C	
		20.97 U/g		4 days			
		1.76 U/g		9 days			
		6.75 U/g		2.5 days		18 °C	
		3.35 U/g		6.5 days			
		14.92 U/g		4 days		20 °C	
Wheat (*Triticum aestivum* L.)	12.62 U/g	30.88 U/g	–	1 day	80%	15–20 ° C	Baranzelli et al. (2018)
		35.93 U/g		2 days			
		39.52 U/g		3 days			
Barley variety *Commander*	2 U/g	4 U/g	Distilled water, 24 h	1 day	–	–	Quek et al. (2019)
		23 U/g		2 days			
		28 U/g		3 days			
		27 U/g		4 days			

Raw material	α-amylase activity in ungerminated seed	α-amylase activity during germination	Soaking conditions	Germination time	Relative humidity	Temperature of germination	Reference
Barley variety Morales	3 U/g	6 U/g	Distilled water, 24 h	1 day	–	–	Quek et al. (2019)
		12 U/g		2 days			
		13 U/g		3 days			
		15 U/g		4 days			
Waxy brown rice	0.054 U/g	0.099 U/g	Distilled water, 12 h, 4 °C	12 h	80%	30 °C	Liu et al. (2022)
		0.483 U/g		1 day			
		0.727 U/g		1.5 days			
		1.180 U/g		2 days			

Unites per gram of sample, UC/g: Ceralpha Units per gram of sample

It has been proven that, in cereal grains, mRNA transcription of α-amylase is activated when glucose is depleted and is suppressed when this sugar is available (Han & Yang, 2015). Reports indicate that the transcription levels of this hydrolase in rice increase in aleurone cells two days after imbibition. Gibberellic acid (GA) is synthesized in the scutellar tissue and makes the transcription factor GAMYB bind to the GA-responsive element as an α-amylase expression promoter (Lee et al., 2014). Then, the enzyme starts its activity and produces hydrolysis in starch.

In seeds of cereals like barley, it has been reported that α-amylase is the most abundant hydrolase produced in aleurone tissue during germination (Jacobsen & Beach, 1985). In wheat, it has been reported that the activity of α-amylase reaches its peak activity around 7–8 days into germination and disappears after 12 days (Zhao & Ma, 2018). In legumes, the behavior of α-amylase has also been evaluated during germination. An increase in α-amylase of up to 200% has been reported in mung bean after 24 h of germination (Rahman et al., 2007). The increase in enzymatic activity leads to a decrease in total starch content and elevated free sugars, although the latter are not always increased. This is because the germ to cover the metabolic requirements for seedling growth can use a fraction. The percentage reduction of the total starch content may vary depending on the germination time. A prolonged germination time favors a greater reduction, after six days of germination and 25 °C in legumes such as green pea, lentil, and mung bean has been reported to reduce 34, 37.06 y 43.38% respectively of total starch (Świeca & Gawlik-Dziki, 2015), while four days after germination the decrease is less than 11.75, 29 and 15% in dolichos, jack bean, and mucuna, respectively (Benítez et al., 2013). However, the type of seed also influences the degree of starch hydrolysis. In lentil (*Lens culinaris* Merr.) and yellow pea (*Pisum sativum* L.); a decrease of 14.77 and 6.55%, respectively, has been reported after 6 days of germination at 25 °C (Xu et al., 2019).

Several factors, such as the composition of the starch granule (amylose/amylopectin), can affect the efficiency of the enzymatic activity. Amylose is quicker and easy to hydrolyze when compared against amylopectin, which has a structure with more complex branching that prevents a fast hydrolyzation (Zheng et al., 2006; Guzmán-Ortiz et al., 2019). A low amylose content (12.5%) in rice germinated for five days shows lower amylase activity versus rice with regular amylose content (20.2%). This proves there is more starch hydrolysis in varieties where the amylose content is superior (Kalita et al., 2017).

Grain humidity is another major factor in enzymatic activation. Some authors have reported that a soaking stage before germination allows triggering hydrolase activation (Table 19.1). When the humidity content is increased the enzymatic activity change (Autio et al., 2001). The soaking process for 24 h, as a step prior to germination, favors the increase in α-amylase activity until 15-fold. If the soaking time is prolonged for a period of 48 h, the enzyme activity can increase up to 43.5 times. This behavior has been studied in rice (Table 19.1) (Moongngarm, 2011). This is likely related to water activity, since a minimum

Table 19.2 Effect of germination on the starch composition of different seeds

Starch source	Native starch			Sprouted starch			Reference
	Total starch (%)	Amylose (%)	Amylopectin (%)	Total starch (%)	Amylose (%)	Amylopectin (%)	
Waxy rice	76	1.16	74.84[a]	N/R	4.88	N/R	Wang et al. (2020)
Quinoa	Mengli variety (MQ) Quinoa gray color						Xing et al. (2021)
	58.18	3.57	54.61[a]	50.50	3.61	46.89[a]	
	Zhongli (ZQ) variety White Quinoa						
	60.40	17.37	43.03[a]	42.07	6.95	35.12[a]	
	Yunnan variety (YQ) Quinoa red color						
	63.12	4.44	58.68[a]	45.51	3.61	41.9[a]	
Chinese rice	Zhengxian variety						Wu et al. (2013)
	78.66	22.01	56.65	42.19	11.32	30.86	
	Nanjing variety						
	77.44	15.10	62.34	41.71	7.32	34.37	
	Yannuo variety						
	76.34	3.97	72.37	43.64	2.24	41	
Rice (*Oryza sativa* L.)	79.89	17.39	62.5[a]	77.11	12.13	64.98	Xu et al. (2012)
Barley	45.7	22.81	22.89[a]	31.67	26.63	5.04	AL-Ansi et al. (2021)
Bambara peanut	43.53	22.61	20.92	42.4	22.48	19.92	Chinma et al. (2021)

[a]Values determined by difference of total starch content with amylose content

of 0.2 is required to activate hydrolases (Larson, 1968; Guzmán-Ortiz et al., 2019). However, when seeds are hydrated in excess water during soaking, they undergo an anaerobiosis process that creates lesions in cell membranes when it is extended for an excessive amount of time, leading to an inability to germinate (Hegarty, 1978).

Temperature also plays an essential role in enzymatic activation. Different germination temperatures (25, 30, and 35 °C) generated different effect on activity levels of α-amylase in waxy brown rice and a significant increase when temperatures are higher (Table 19.1) (Wang et al., 2020). Same effect was found in Meeri and Barra germinated oats (Aparicio-García et al., 2020).

As stated before, enzymes are activated during germination and modify the physicochemical properties of macromolecules as starch. Therefore, it is important to know the alterations in starch granules by germination, so that they can be a relevant ingredient in the food industry.

19.4 Changes in Starch During Germination

19.4.1 Chemical Composition

The amylose/amylopectin ratio can be modified during germination. The proportion of amylose can be significantly reduced versus that of amylopectin since it is more susceptible to hydrolysis by enzymatic action (Kalita et al., 2017). In some rice varieties the total starch, amylose and amylopectin content decreases after germination (Table 19.2) (Wu et al., 2013; Pinkaew et al., 2017).

In addition to the enzymatic activity, the content of amylose and amylopectin can be affected by the amount of phenolic compounds. During germination, phenolic compounds increase significantly (Ferreira et al., 2019), this can cause amylose to interact with small phenolic compound molecules through covalent bonds and create left-handed helical inclusion complexes called V-amylose (Obiro et al., 2012). However, the complex V-amylose, also is possible to form complex with other compounds present in grains and seed such as fatty acids and hydrophobic organic polymers. Amylose-V complexes can affect the physicochemical properties of starch, such as rheology, retrogradation and digestibility, generating resistant starches (Nimz et al., 2004). Still, these interactions depend on the chemical structure of the starch and the type and concentration of the phenolic compound. It could be hypothesized that during germination there is a greater formation of Amylose-V complexes, since germination favors the increase of phenolic compounds (Zhu, 2015). The degree of solubility also plays an important role in the interactions of polyphenols with starch (Han et al., 2020). These interactions and modifications in starch composition can also affect the physicochemical, rheological, and nutritional properties of starch.

On the other hand, the protein content in isolated starch decreases slightly with germination time (Liu et al., 2022). Values of 0.39% have been reported in ungerminated starch and of 0.41, 0.36, 0.37, and 0.35% at 12, 24, 36, and 48 h of germination at 30 °C in waxy brown rice. Variation in temperature does not significantly influence protein content (Wang et al., 2020). Lipid content has been reported as constant throughout the germination time (Al-Ansi et al., 2021). Values of 0.15% in native rice starch and 0.13, 0.16, 0.13, and 0.14% have been found in germination after 12, 24, 36, and 48 h, respectively (Liu et al., 2022). The lipid content in barley starch has also been constant. Additionally, it has been found that the ash content increases along with germination. In germinated rice starch for 12 and 48 h, a high ash content has been reported compared to ungerminated rice (Liu et al., 2022) and Wang et al. (2020).

19.4.2 Swelling Power and Solubility

Starch changes its composition and structure after the germination process, which confers it different properties. Starches can be used as binding agents and thickeners in the development of foods. Still, these parameters depend on the interaction between amylose and amylopectin chains, as well as crystalline and amorphous areas of starch granules, polymerization degree, branching, and molecular weight (Zhang et al., 2005; Ratnayake et al., 2002). The swelling power in native starch is closely related to amylopectin concentration because amylose is a diluent and swelling inhibitor (Singh et al., 2003).

Germination modifies the swelling power of starch granules due to the degradation by enzymatic action, which affects granule hydration. In barley germinated for 24, 48 and 72 h, the swelling power has decreased. This is because of amylose release during germination, due to starch degradation by enzymatic action (AL-Ansi et al., 2021). The amylose released creates a gel structure on the surface of the starch granule, preventing hydration (Yang et al., 2020). A similar behavior was found for germinated mung bean (25 °C for 12–72 h); however, this capacity increases along with the temperature (50–90 °C) (Liu et al., 2020). This occurs since starch granules are partially disintegrated during germination, which exposes the starch chain, increasing hydrophilicity and thus the swelling power (Su et al., 2020). The starch of germinated quinoa (0, 24, and 48 h) has the same behavior as temperature rose, proving that germination deeply affects this property (Xing et al., 2021). However, swelling power is also controlled by intrinsic factors of starch, as the presence of minor compounds as phosphate monoesters and phospholipids (Srichuwong et al., 2005).

Furthermore, solubility is a parameter determined by factors such as particle size, distribution, structure, and granule morphology. These factors are modified by germination, affecting solubility (Xia et al., 2020). In quinoa, the solubility of starch granules increases along with the germination time due to a progressive starch hydrolysis by enzymatic action (AL-Ansi et al., 2021; Xing et al., 2021).

19.4.3 Thermal Properties

Gelatinization, an irreversible transition process, occurs when starch is subjected to high temperatures in excess of water. Starch gelatinization parameters in food processing are of great importance for specific applications. These properties have been evaluated through different techniques as differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR) spectroscopy, and Fourier transform infrared (FTIR) spectrometry. Nevertheless, DSC is most commonly used to analyze the thermal characteristics of starch in foods (Annor et al., 2014).

During germination, branched starch chains are reduced while sugar concentration increases because of the degradation of the double helix bonds of starch (Wu

Table 19.3 Thermal and pasting properties of native and sprouted starches from different seeds

Starch source	Pasting properties				Thermal properties								Germination time and temp.	Reference
	Native starch		Sprouted starch		Native starch				Sprouted starch					
	Peak viscosity	Paste temp. (°C)	Peak viscosity	Paste temp. (°C)	T_0 (°C)	T_C (°C)	ΔH (J/g)	Tp (°C)	T_0 (°C)	T_C (°C)	ΔH (J/g)	Tp (°C)		
Rice	1306.1 (mPa*s)	61.3	1063.7 (mPa*s)	61.9	55.22	77.61	14.44	–	57.16	78.39	12.97	–	2 days, 35 °C	Wang et al. (2020)
			1156.0 (mPa*s)	61.7					57.02	78.07	13.21	–	2 days, 30 °C	
			1194 (mPa*s)	61.4					56.7	78.15	13.81	–	2 days, 25 °C	
Barley	Esmeralda variety												6 days, 25 °C	Gutiérrez-Osnaya et al. (2020)
	2730.50 (cP)	95.33	510.50 (cP)	95.28	–	–	6.46	61.36	–	–	1.56	63.40		
	Perla variety												8 days, 25 °C	
	3101 (cP)	95.43	828.50 (cP)	95.19	–	–	7.93	61.83	–	–	4.96	62.08		
Rice	ZX variety												5 days, 25 °C	Wu et al. (2013)
	1276 (cP)	–	1324 (cP)	–	63.24	74.37	9.68	69.35	62.33	73.81	9.71	68.92		
	NJ variety													
	1451 (cP)	–	1503 (cP)	–	61.66	72.69	10.04	66.78	60.95	72.38	9.97	66.16		
	YN variety													
	1914 (cP)	–	1926 (cP)	–	60.68	71.71	10.15	65.87	60.67	71.57	10.12	65.55		

Starch source	Pasting properties				Thermal properties								Germination time and temp.	Reference
	Native starch		Sprouted starch		Native starch				Sprouted starch					
	Peak viscosity	Paste temp. (°C)	Peak viscosity	Paste temp. (°C)	T_0 (°C)	T_C (°C)	ΔH (J/g)	Tp (°C)	T_0 (°C)	T_C (°C)	ΔH (J/g)	Tp (°C)		
Sorghum (*Sodamchal*)	2466 (cP)	72.7	196 (cP)	72.3	65.3	79.4	17.4	70.3	66.7	79.9	18.4	70.9	2 days, 30 °C	Li et al. (2017)
Millet (*Samdachal*)	2485 (cP)	75.5	972 (cP)	73.5	61.9	82.1	17.5	72.6	62.4	83.4	19.9	71.7	2 days, 30 °C	
Brown rice (*Ilpum*)	990 (cP)	89.9	1076 (cP)	93.2	58.0	72.2	15.8	63.8	58.8	71.7	14.5	64.2	2 days, 30 °C	
Oat (*Choyang*)	914 (cP)	91.5	941 (cP)	94.7	57.4	67.3	13.8	62.2	57.8	68.0	13.9	63.1	2.5 days, 25 °C	
Rice (*Oryza sativa* L.)	3293 (cP)	65.30	2726 (cP)	63.55	59.07	77.98	8.34	65.85	58.74	71.97	6.72	63.97	1 day, 30 °C	Xu et al. (2012)
Mung bean	656 (cP)	78.25	629 (cP)	72.25	61.97	92.71	18.74	69.67	62.01	93.62	22.20	70.18	12 h, 25 °C	Liu et al. (2020)
			588 (cP)	79.05					62.94	96.12	17.53	70.39	1 day, 25 °C	
			653 (cP)	78.65					62.74	95.06	21.64	68.86	1.5 days, 25 °C	
			619 (cP)	79.88					62.75	96.30	15.31	69.55	2 days, 25 °C	
			655 (cP)	79.13					62.87	96.39	15.30	70.72	2.5 days, 25 °C	
			549 (cP)	79.10					63.44	92.55	13.54	70.78	3 days, 25 °C	
Bambara Peanut	1473 (cP)	86.2	1529 (cP)	86.63	67.84	83.05	4.62	72.39	66.9	85.16	2.8	73.48	3 days, 28 °C	Chinma et al. (2021)

(continued)

Table 19.3 (continued)

Starch source	Pasting properties				Thermal properties								Germination time and temp.	Reference
	Native starch		Sprouted starch		Native starch				Sprouted starch					
	Peak viscosity	Paste temp. (°C)	Peak viscosity	Paste temp. (°C)	T_0 (°C)	T_C (°C)	ΔH (J/g)	Tp (°C)	T_0 (°C)	T_C (°C)	ΔH (J/g)	Tp (°C)		
Pea (*Pisum sativum* L.)	Xiwan 1 variety													Gao et al. (2022)
	3205 (cP)	76.75	2453 (cP)	76.58	53.91	74.41	6.40	62.96	59.02	73.95	6.32	64.96	1 day, 25 °C	
			4325 (cP)	74.45					55.44	71.55	7.10	62.49	2 days, 25 °C	
			4735 (cP)	74.25					55.10	71.15	7.00	62.65	3 days, 25 °C	
			2611 (cP)	75.05					61.08	75.52	8.05	67.66	4 days, 25 °C	
	Xiwan 2 variety													
	4608 (cP)	75.88	5261 (cP)	75.00	58.17	72.22	5.94	64.18	58.89	73.55	6.89	63.81	1 day, 25 °C	
			4357 (cP)	73.83					58.29	72.59	8.16	63.56	2 days, 25 °C	
			4235 (cP)	73.48					59.18	74.16	8.23	64.95	3 days, 25 °C	
			3135 (cP)	75.20					61.55	77.19	8.04	67.49	4 days, 25 °C	
Barley	Hot air dried (60 °C, 4 h)													Su et al. (2020)
	2995 (cP)	88.40	3079 (cP)	89.25	55.02	65.23	7.56	58.51	55.67	67.81	9.30	59.65	12 h, 25 °C	
			3243 (cP)	88.38					55.57	65.97	8.66	59.25	1 day, 25 °C	
			4478 (cP)	86.35					55.92	64.94	7.52	59.23	1.5 days, 25 °C	
	Infrared dried (600 W/m2 radiation intensity, 20 °C inlet air, velocity of 0.5 m/s)													
	2995 (cP)	88.40	3140	89.23	55.02	65.23	7.56	58.51	56.40	67.21	8.68	60.19	12 h, 25 °C	
			2490	92.88					56.35	68.26	7.19	61.05	1 day, 25 °C	
			1241	90.83					58.63	70.47	6.24	63.21	1.5 days, 25 °C	

et al., 2013). The alteration of the structure and molecular order of the starch granule led to an increase in gelatinization temperatures and modifications in ΔH values given the differences in the distribution of the size particle, amylose content, and relative crystallinity (Zhang et al., 2020).

When the germination time increases, a degradation of starch double-helical structure is generated, causing a greater demand for energy for the gelatinization the starch, which causes an increase in gelatinization temperature. This modification is due to the changes in granule size and shape, amylose content, and length of amylopectin chain (AL-Ansi et al., 2021). It must also be considered that the presence of some amino acids and peptide fractions can increase the gelatinization temperature (Xu et al., 2017).

Lipids play an important role during gelatinization, since they form a coating on the starch granules, restricting swelling, this causes the gelatinization temperature to increase with germination (Biliaderis & Tonogai, 1991), because the lipids are degraded during the first days of germination. Additionally, the amylose/amylopectin ratio also affects the behavior of gelatinization (Varavinit et al., 2003). In Esmeralda variety barley, the gelatinization temperature has increased after 4 days of germination and Perla variety barley decreases after 2 days of germination (Table 19.2) (Gutiérrez-Osnaya et al., 2020).

The constant increase in gelatinization temperature, as related to germination time, is also linked to sugar accumulation. This behavior occurs in rice, sorghum, and millet at 24 and 48 h of germination (Li et al., 2017). An increase in gelatinization temperature found in adlay (*Coix lacryma-jobi*) seeds germinated for 12, 24, 36, 48, and 60 h (Xu et al., 2017). However, as in several studies shown in Table 19.3, gelatinization enthalpy was significantly reduced due to the germination time. There was a modification in the molecular structure of the amylopectin chains of the starch, representing a loss of double helix order in the crystalline and amorphous regions (Cooke & Gidley, 1992). The type of sugars accumulated because of starch hydrolysis during germination can also affect the behavior of gelatinization enthalpy. Certain sugars, as monosaccharides, reduce gelatinization enthalpy (Baek et al., 2004).

19.4.4 Pasting Characteristics

Pasting properties are closely related to starch functionality. To assess them, starch is heated in an excess of water to obtain a pasting curve and visualize peak viscosity, breakdown, setback, and final viscosity. Peak viscosity indicates the water absorption capacity and how easily starch granules can disintegrate, while breakdown refers to viscosity during a heating period at a constant temperature to have granules swell and break. Setback is the cooling stage and final viscosity indicates the capacity of starch in a solution to create a viscous paste after heating and cooling. The minimum temperature at which viscosity increases is known as pasting temperature (Dhaka & Khatkar, 2015). These properties entirely depend on moisture content and

type of starch; still, it has been proven that germination can modify these properties. In brown rice germinated for 48 h decrease in peak and final viscosities was found (Wang et al., 2020). This effect is likely due to the disruption of the granular structures, amylose leaching, amylopectin degradation, and a disorder of the hierarchical structures of starch (Zhang et al., 2015; Qiao et al., 2019). In addition, a reduction in retrogradation, possibly to the low rearrangement between the molecular chains of starch.

There is a relationship between the reduced viscosity and the morphological changes in starch (Table 19.3). The morphology of the starch granule is modified along the germination time. The structure is lost, and the granule shows small holes with porous texture due to the enzymatic activity that makes starch granules lose water retention capacity while viscosity is reduced in consequence (Simsek et al., 2014; Uthumporn et al., 2010; Xu et al., 2012; Wu et al., 2013).

Several works have demonstrated a decrease in peak viscosity as a result of germination caused by the fractioning of long amylose chains and amylopectin branching in native starch (Oseguera-Toledo et al., 2020; Liu et al., 2022; Wang et al., 2020). The effect of germination on the pasting properties of legumes like lentil, chickpea, and yellow bean has also been studied. Similarly, a progressive reduction in viscosity has been found because of changes in starch granule by the hydrolytic enzymes (Xu et al., 2019; Gutiérrez-Osnaya et al., 2020).

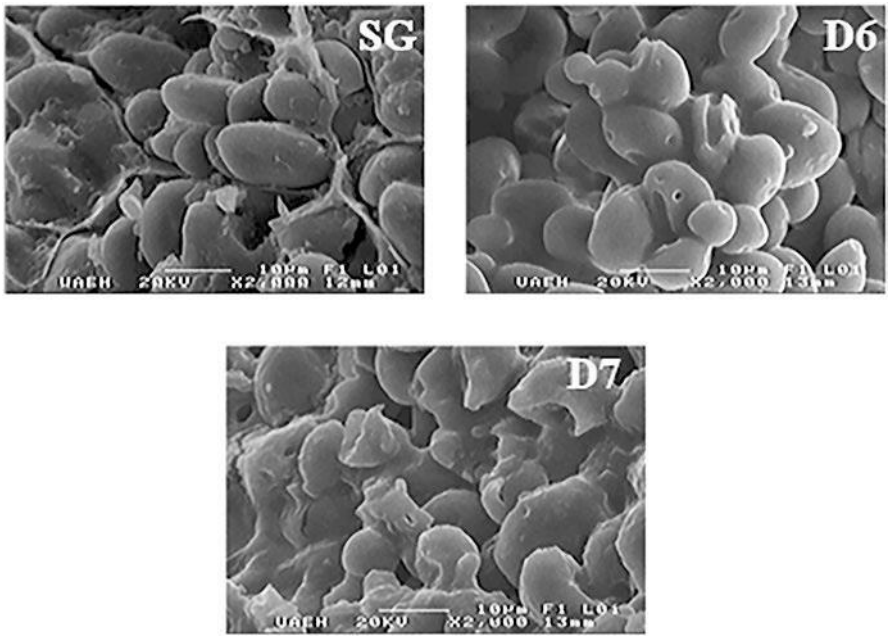

Fig. 19.2 Micrographs of barley variety Perla starch granules: (SG) without germination, (D6) 6 days of germination, (D7) 7 days of germination. (Guitérrez-Osnaya et al., 2020)

19.4.5 Structural and Morphological Changes

The morphology of starch granules is analyzed using different microscopy techniques. The process of starch hydrolysis by enzymatic action produces sugars and modifies and alters granule morphology. Enzymes can penetrate granules and hydrolyze them, creating surface pores and erosion as well as degraded granules (Li et al., 2017). This means starch has been modified by enzymatic action through germination. Some studies have proven that starch granules from cereals are eroded during germination; they show pores and rough surfaces as a result of enzymatic activity (Fig. 19.2) (Gutiérrez-Osnaya et al., 2020; You et al., 2016). In corn and triticale, the enzymatic activity produces large holes on the surface of starch granules (Li et al., 2011, 2012). Correia et al. (2008) evaluated the effect of germination in *Sorghum bicolor* (L.); electron microscopy confirmed the enzymatic hydrolysis of starch granules, which appeared eroded on day 7 of germination at 26 °C. Additionally, the starch granule is released from the existing protein matrix in the seed. The morphology of brown rice starch granules has an irregular polyhedral shape with a relatively smooth surface (Wang et al., 2020). Still, the germination process (35 °C, 48 h) alters granule homogeneity and integrity, and surfaces

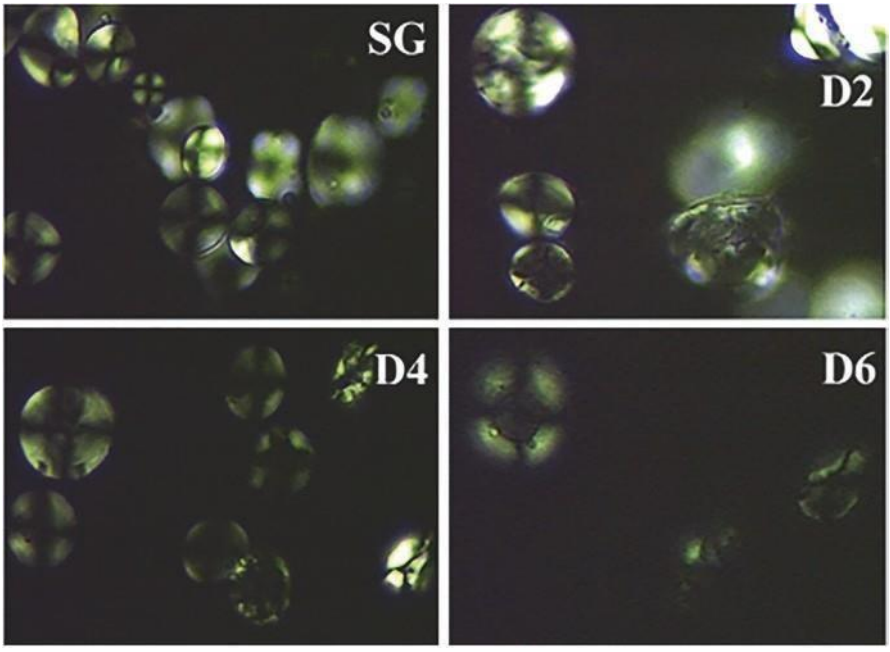

Fig. 19.3 Starch granules of barley variety Esmeralda through polarized light (SG) without germination, (D2) 2 days of germination, (D4) 4 days of germination, (D6) 6 days of germination. (Guitérrez-Osnaya et al., 2020)

show pores and damages as well as a smaller particle size. The compact and homogeneous structure of native starch it is destroyed as the germination time increased, generating a rough and eroded morphology, irregular shapes are generated in starch granules, including dents and holes on the surface. This effect has also been observed in adlay (*Coix lacryma-jobi*), in rice, millet, oat, and sorghum after germination (48 h) (Xu et al., 2017; Li et al., 2017).

On the other hand, the maltese cross or birefringence in starch granules is also modified by the germination process. This morphological characteristic can be visualized under polarized light microscopy and indicates a high order in the structure of amylose and amylopectin layers. The action of α-amylase during germination causes modification in the maltese cross. This morphological change increases as the germination time does (Fig. 19.3), which leads to significant changes in viscosity and gelatinization parameters. From day 4 of germination, birefringence of starch granules is lost, indicating a loss of molecular order in the crystalline region (Jane et al., 2003).

The modification of the starch granule morphology creates a porous surface and reduces viscosity and swelling capacity. This has been observed in barley, mung bean, and quinoa (Gutiérrez-Osnaya et al., 2020; AL-Ansi et al., 2021; Liu et al., 2020; Xing et al., 2021) due to a higher enzymatic activity during the germination process (Wu et al., 2013).

The structural changes in starch by germination can be identified through X-ray diffraction, and starch crystallinity can be calculated from the peaks (Cheetham & Tao, 1998). Several works report a decrease in starch crystallinity in different seeds because of germination conditions (Oseguera-Toledo et al., 2020; Liu et al., 2022; AL-Ansi et al., 2021). The long and organized amylopectin chains in starch represent a relative crystallinity, which can be modified by germination. Relative crystallinity it has been reduced from 24.7% to 23.6% in barley at day 4 of germination when compared against native starch (Gutiérrez-Osnaya et al., 2020). In quinoa, the relative crystallinity also decreases with germination. The MQS variety reduce from 38.62% to 35.24%, while ZQS variety was reduced from 37.41% to 35.46% and YQS, from 38.59% to 37.34% (Xing et al., 2021). Reduced crystallinity has also been reported in other cereals: 35.18% to 32.495% in brown rice, 27.44% to 25.05% in oat, 30.24% to 26.35% in millet, and 35.70% to 32.09% in sorghum (Xing et al., 2021). In legumes like mung bean, relative crystallinity is also decreased from 34.52% to 12.72% after 72 h of germination (Liu et al., 2020). The reduction is a consequence of enzymatic hydrolysis; the presence of shorter amylopectin chains is attributed to the loss of hydrogen bonds in starch helices in the crystalline region (Tarr et al., 2012). Therefore, when the crystalline regions are progressively hydrolyzed, the microcrystalline structure is modified and the interactions between the molecular chains are reduced, this causes a reduction in crystallinity (Liu et al., 2020).

19.4.6 In-Vitro *and* In-Vivo *Digestibility*

Starch digestibility is affected by several factors, such as the concentration and diffusion of the enzyme to the substrate and absorption to the starchy material. Digestion can generate several glucose molecules that are absorbed in the body, it is related with the glycemic index. According to the kinetics of starch digestion, it is classified into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). RDS is a starch fraction that rapidly hydrolyzes into glucose molecules, releasing them after 20 min, according to the method proposed by Englyst et al. (1992), based on the *in vitro* digestion of starch by simulating the stomach and intestine. SDS is the fraction of starch that is slowly hydrolyzed to sugar molecules in the small intestine, in an average time of 20 and 120 min. RD is the portion of starch that remains unhydrolyzed by digestive enzymes and reaches the colon becoming short-chain acids. Native starch digestion can vary depending on the botanical source, granule morphology, presence of pores and channels, amylose/amylopectin content, crystallinity, lamellar density, growth rings, and the presence of other compounds such as proteins, lipids, phenolic compounds. Native starch with a B-type X-ray diffraction pattern shows a high resistance to enzymatic hydrolysis because the long chains form longer and more stable helices, which means that this type of starch has a high content of starch RS. Unlike the native starch of cereals that have a semi-crystalline type A structure, with a high level of short chains and branches, that give them slow starch digestion properties (Zhang et al., 2006).

A thermally, chemically, or enzymatically modified starch undergoes structural changes that will affect its digestibility, depending on the degree of molecular disorder. During germination, the amylopectin chains are reduced (Table 19.2), producing a less crystalline structure due to the loss of hydrogen bonds between the chains, which can favor hydrolysis and increase in digestibility. The enzymatic activation that is generated during the germination process modifies the surface of the starch granules (Fig. 19.2). Through the germination time larger pores appear. The appearance of these perforations in the starch granules can improve the penetration efficiency of the enzymes and favor their digestibility. As germination time progresses, the molecular weight and relative crystallinity of the starch decrease, indicating a partial degradation of the starch, these phenomena may indicate better digestibility (Ma et al., 2020). In maize sprouts at 25 °C, 24 h it has been reported that the RDS contents increase, while the SDS and RS contents decrease with prolonged germination time. In brown rice the SDS increases 10.09%, however the RDS and RS decrease 11.95 and 11.71% respectively at 24 h of germination at 30 °C (Xu et al., 2012).

In other grains the RSD content increased 11.3% in wheat, 12.1% in rice, 20.1% in oats and 24% in corn after 72 h of germination as a result of starch hydrolysis by enzymatic action of amylases and phosphorylases (Kaur & Gill, 2020). The increase in the digestibility of starch during germination is also due to the decrease in interactions of starch with other molecules such as fiber, protein, and lipids.

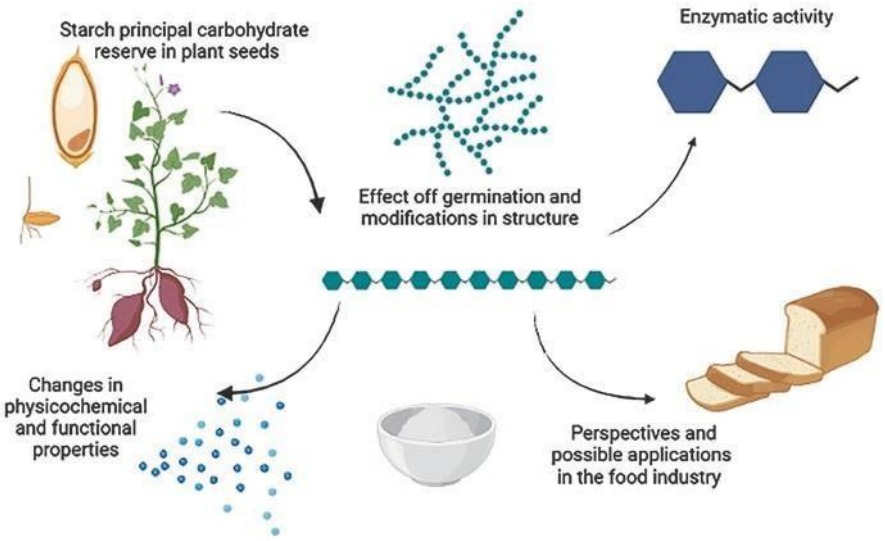

Fig. 19.4 Changes in starch digestibility due to germination

In legumes, in addition to the characteristics of starch, the presence of antinutritional compounds also influence starch digestion (Jeong et al., 2019). In germinated Bambara groundnut flour, a decrease in phytic acid, tannins and trypsin inhibitor activity has been reported with germination time and an increase in starch digestibility from 8.83% to 10.18% at 48 h of germination (Chinma et al., 2021). In mung beans, an increase of 80.1% to 90.6% digestibility at 20 h of germination has also been reported. In chickpea from 79.7% to 88.1% at 60 h and in Cowpea from 80.9% to 91.9% at 24 h (Uppal & Bains, 2012).

The increased digestion of starch with sprouting produces more glucose molecules that can be absorbed into the body. *In vitro* studies reveal a behavioral approach to an *in vivo* system. In the human body, starch digestion through the gastrointestinal tract is mediated by salivary and pancreatic α-amylases hydrolyzing starch to disaccharides and subsequently glucose, which are then hydrolyzed to glucose by the dual action of two border enzymes in brush, maltase-glucoamylase and sucrase-isomaltase (Fig. 19.4) (Quezada-Calvillo et al., 2007). In this sense, starch digestibility has been related to the glycemic index by the amount of glucose released. However, there are different aspects to consider during starch digestibility, the plant cell wall, for example, constitutes a diffusion barrier for amylases as in the case of legumes (Noah et al., 1998). Analyzing the interactions between starch and other macromolecules is of interest, to establish its application in the food industry and its possible benefits to human health.

19.5 Interaction Behavior with Other Polysaccharides and Hydrocolloids

Starch has been widely used as an ingredient in the formulation of different foods to achieve a pleasant texture for the consumer. However, the interaction that it may have with other components modifies the characteristics of the final product. Hydrogen bonding and water competition between starch and other polysaccharides and/or proteins affect the swelling and retrogradation of starch molecules that directly influence the texture of foods. The interactions between soluble polysaccharides and starch in a system help to form a strong gel and increase the viscosity of the gel. The abundant hydroxyl groups of soluble polysaccharides can absorb more water molecules, giving starch granules greater ability to swell (Tu et al., 2021). The use of germinated flours can favor the formation of a strong gel, due to the greater availability of soluble polysaccharides. A higher concentration and accumulation of soluble sugars such as fructose, galactose, melibiose, sucrose, stachyose and verbascose with germination, which allows a greater number of molecules that can interact with other compounds such as hydrocolloids (Goyoaga et al., 2011).

There is a positive correlation between a resistant starch content of native starch and high amylose content (Lin et al., 2021); therefore, starches obtained by germination where the amylose content increases (Table 19.2) can be used to produce resistant starch. Generally, one way to achieve this is through the interactions of starch with polysaccharides or hydrocolloids such as xanthan gums, guar gum, chitosan, arabic, carrageenan, etc. however, by combining starch with these polysaccharides during food processing, texture parameters such as viscosity, gelatinization and digestibility are also modified. The combination of starch with another hydrocolloid such as xanthan gum decreases digestibility. However, another parameter to consider is the viscosity of the mixture, since it increases due to the thickening effect of the hydrocolloid, the leaching of amylose and the swelling power of the granules are reduced (Sasaki, 2020).

By modifying the viscosity, the digestibility is altered due to the limited accessibility that the enzymes have to the starch granules (Brennan, 2005). The digestibility of mung bean starch in combination with hydrocolloids, finding a decrease in the digestibility of resistant starch dependent on the percentage of substitution of the hydrocolloid, reaching a reduction from 44.97% to 10.76% when mixed with xanthan gum at 0.30% and 3.48% when mixed with 0.30% konjac gum (Lin et al., 2021). These results are beneficial and have an impact on human health since these starch/hydrocolloid mixtures can be used in the control of postprandial glucose and insulin response (Qiu et al., 2017; Lim et al., 2003).

On the other hand, carrageenan has a greater effect compared to xanthan gum, pectin, gum arabic and guar gum in restricting the hydrolysis of corn starch during digestibility; In addition, by inhibiting the rapid digestion of starch, the fraction of SDS and the content of RS increased (Tester & Sommerville, 2003). The combination of starch with pectin causes a decrease in the digestibility of starch in the presence of pectin due mainly to two factors: the binding of the enzyme with pectin,

inhibiting its access to the starch granules and the increase in viscosity since the speed is decreased enzyme diffusion (Bai et al., 2017). However, the viscosity parameters depend on the properties of the hydrocolloid used, such as molecular weight and flexibility, concentration, degree of branching, distribution, and charge (Sasaki & Kohyama, 2012). During mung bean germination, starch viscosity parameters tend to decrease depending on process conditions (Liu et al., 2020). The mixture of native mung bean starch with xanthan and konjac gum has been shown to achieve higher values of maximum viscosity and final viscosity compared to native starch, thus modifying its possible application and functionality. In addition, the mixture with hydrocolloids allows to strengthen the formation of gels (Lin et al., 2021). A similar behavior was found in corn starch where xanthan gum interacts with starch granules (Zhang et al., 2018).

Different types and concentrations of hydrocolloids have different effects on starch paste properties. For example, the addition of guar gum decreases the maximum viscosity of wheat starch (Funami et al., 2008). Fenugreek gum increases the peak viscosity time, setback viscosity and final viscosity in corn starch (Ravindran & Matia-Merino, 2009). ß-glucans increased the setback and final viscosity in rice starch (Banchathanakij & Suphantharika, 2009). However, the viscosity and gelatinization properties will depend on the structure and type of hydrocolloid used, due to the possible molecular interactions that they establish with the starch used, as well as the starch, the proportion of the mixture and the method of preparation (BeMiller, 2011).

The functionality of the starch when interacting with other polysaccharides and hydrocolloids can be modified; in addition to modifying the physicochemical properties, its application is diversified. Germinated wheat starch has been used to produce aerogels in combination with polyethylene oxide, due to the increase in water absorption capacity and high degradation temperature, caused by the starch/hydrocolloid interaction, becoming a material of interest for the food industry in the development of absorbent materials and packaging (da Silva et al., 2020).

19.6 Possible Applications in Food Industry

Starch as a food represents the most abundant and relevant digestible polysaccharide. In the food industry, it is mostly used as an ingredient to modify texture, viscosity, adherence, humidity retention, and creation of gels and films. In general, the chemical composition and physicochemical and functional properties, including starch granule morphology, are the major factors to determine a possible application. Germination has proven to be an efficient and cost-effective technique to modify and improve the physicochemical and functional properties and diversify the use and application in foods (Waterschoot et al., 2015; Muñoz-Llandes et al., 2022).

During germination a significant starch degradation is triggered, reducing the viscosity and gelatinization properties. These modified starches are useful in the

development of emulsions as sauces, creams, mayonnaises, and tart fillings (Xing et al., 2021).

Starch hydrolysis during germination involves the accumulation of simple sugars, mostly monosaccharides and oligosaccharides. This concentration is proportional to amylose content; therefore, these starches are desirable in the beer industry. There, these carbohydrates are more available to yeasts during the fermentation process. Additionally, they can be used in foods with a high caloric value, such as candy, infant formulas, and energy drinks for athletes.

The enzymatic activation involved in the process results in the production of depolymerized starches with a greater number of short linear chains that can be used in the creation of stronger thermoreversible gels. These starches can replace the use of fats in formulations that require these gels, significantly reducing the caloric intake. In addition, they produce a texture similar to that of baked goods, noodles, gelatins, emulsion stabilizers as ice-creams, and water-oil emulsions (Liu et al., 2017). The use of these modified starches as fat substitutes is highly relevant to the ice-cream industry due to the presence of discrete domains that imitate the behavior of microstructures in fat particles (Alting et al., 2009). They can also act as an efficient enhancer of creaminess in foods like yoghurt.

On the other hand, the generation of pores on the surface of starch granules, resulting from damage by enzymatic activation during germination, creates a greater capacity for oil absorption. This is a desirable parameter in the development of foods that will undergo a frying process, like nuggets and potatoes, to obtain a crunchy texture that is desirable for consumers (Purcell et al., 2014). Extending the germination time involves a greater capacity to modify starch granules, producing dents on the smooth surface of native starch. This allows for a higher degree of crystallinity, solubility, and swelling power as well as a reduction in viscosity and pasting temperature. This leads for a potential application of modified starches in the innovation of food formulas that require low viscosity, as baked products like cookies, tarts, and pastry (Liu et al., 2020).

19.7 Conclusion

Starch is the most used natural polymer at industrial level; however, the current need and demand for techniques that allow its modification to diversify its use and efficient application is greater. Germination is an environmentally friendly alternative that is simple, efficient, and cost-effective that promotes multiple changes in starch characteristics. It has been proven that the conditions of the germination process are an alternative to modify the structure, morphology, and physicochemical properties of native starch. Then, there is a higher degree of molecular disorder in starch granules when the germination time is increased.

This technique can be used to improve the characteristics of native starches from botanical sources already used and unconventional. The aim is to optimally define their use and application, increasing the quality of the final product in terms of

texture, viscosity, adhesion, springiness, and water retention, among others. Still, the major parameters to consider in germination so that starch granules are modified by enzymatic activity are time and temperature of the process as well as the previous soaking of the seeds used, the botanical source, and starch composition.

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Capítulo 2 Effect of Germination time on protein subunits of *Lupinus angustifolius* L. and its influence on functional properties and protein digestibility

El segundo capítulo de esta tesis doctoral consta de un artículo de investigación titulado *Effect of Germination time on protein subunits of Lupinus angustifolius L. and its influence on functional properties and protein digestibility*. Este trabajo fue publicado en la revista Food Science and Technology (Campinas), con un factor de impacto de 3.1. El objetivo de este trabajo fue determinar los cambios tecnofuncionales perfil electroforético y compuestos antinutricionales en semillas de *Lupinus angustifolius* germinado por diferentes días.

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

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Abstract

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

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

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

1 Introduction

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

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

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

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

2. Materials and Methods

2.1 Raw material

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

2.2 Germination

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

2.3 Protein determination

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

2.4 Electrophoretic profile

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

2.5 Functional properties (FP)

Bulk density (BD)

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

Water and oil absorption capacity (WAC, OAC)

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

Swelling power (SP)

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

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

Foaming capacity and stability (FC, FS)

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

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

Emulsifying activity and stability (EA, ES)

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

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

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

$$ES\ (\%) = \frac{Height\ of\ emulsionlayer\ after\ heating}{Height\ of\ wholelayer} x100 \quad (2)$$

2.6 In vitro protein digestibility (IVPD)

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

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

2.7 Phytic acid quantification

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

2.8 Trypsin inhibitor activity

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

2.9 Statistical analysis

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

3 Results and discussion

3.1 Protein content

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

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

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

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

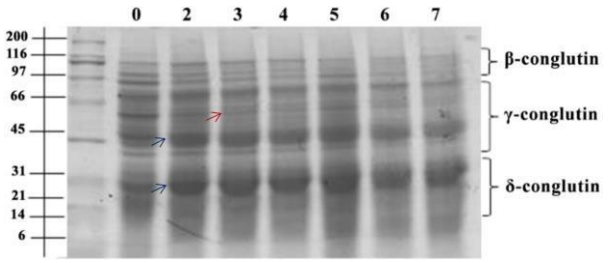

Figure 1. Electrophoretic profile of the flours analyzed, the first lane from left to right indicates the molecular weight marker, line 0 sample ungerminating, line 2-7: samples germinated from 2 to 7 days. The blue arrows indicate the increase intensity between the 21 and 45kDa subunits. The red arrow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

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

3.2 Protein profile

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

3.3 Functional properties

Bulk density

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

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

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

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

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

Water and oil absorption capacity

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

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

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

Swelling power

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

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

Foaming capacity and stability

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

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

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

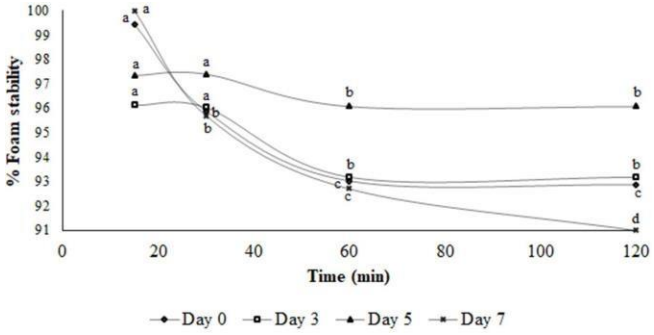

Figure 2. Foam stability of ungerminated *Lupinus angustifolius* L and germinated by 3, 5 and 7 days.

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

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

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

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

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

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

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

Emulsifying activity and stability

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

3.4 In vitro protein digestibility

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

3.5 Phytic acid

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

3.6 Trypsin inhibitor activity

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

4 Conclusions

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

Disclosure statement

All authors possess no conflicts of interest.

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Capítulo 3 Lupinus sprouts a new and potential ingredient in extrusion process: Physicochemical, nutritional and structural evaluation.

El tercer capítulo de esta tesis doctoral consta de un artículo de investigación titulado *Lupinus sprouts a new and potential ingredient in extrusión process: Physicochemical, nutritional and structural evaluation.* Este trabajo fue publicado en la revista Innovative Food Science & Emerging technologies de la editorial Elsevier con un factor de impacto de 6.3. El objetivo de este trabajo fue desarrollar una matriz alimentaria mediante la tecnología de extrusión utilizando como ingrediente las harinas de Lupinus angustifolius germinado a diferentes tiempos.

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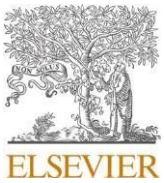

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Lupinus sprouts a new and potential ingredient in extrusion process: Physicochemical, nutritional and structural evaluation

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ABSTRACT

The objective of this study was to determine the optimal conditions of the extrusion cooking process using ungerminated *Lupinus angustifolius* flour, to later develop snacks with germinated flours for 3, 5 and 7 days. The optimal conditions were 16% feed moisture, 152 °C die temperature, and 47/53% lupinus/corn starch proportion. The water absorption and solubility index, bulk density, and ΔE values increased in extrudates with 7 days sprouted flour (EG7) (4.32 g/g, 19.68%, 643.60 kg/m^3, 24.13 respectively). EG7 presented the lowest values in sectional expansion index and hardness (1.69 and 50.98 N respectively). Furthermore, trypsin inhibitors were inactivated during the extrusion process, and a reduction in phytic acid content was observed in the extruded products containing germinated flours. The concentration of phenolic compounds and flavonoids were higher in the extruded products with 47/53% lupinus germinated for 3 days /corn starch proportion. Changes in the components of the secondary structure protein were observed, improving protein digestibility in extrudates with sprouted flours. The combination of processes can be an alternative for the development of a snack with improved physicochemical and nutritional properties.

Industrial relevance: The results show that sprouting and extrusion are emerging techniques in food engineering that, when combined, make it possible to obtain innovative snacks with potential benefits to human health. Lupinus sprouts are a potential ingredient that allows increasing the nutritional, physicochemi cal and functional properties of extruded snacks; offering an alternative of healthy plant-based snacks, given its growing demand by the consumer.

1. Introduction

Germination is a rising technique that improves the nutritional, technological, and functional quality of legume flours, making them a modified ingredient with potential benefits to human health and varied food applications (Huang, Cai, & Xu, 2017). There is a strong industrial interest to incorporate legumes to food development due to its high protein content, low cost of production and lower impact to environmental pollution. Aims of sustainable development can be implanted through innovation in plant-based foods. Grains from *Lupinus angustifolius* are a potential source of vegetable protein. It has been reported that lupin germination increases the content of phenolic compounds, tocopherols, and vitamins C and E, improving the antioxidant capacity (Kamran, Phillips, Harman, & Reddy, 2023). On the other hand, grains germination leads to changes in the composition of isoflavonoids, compounds that take part in the prevention of several chronic degenerative diseases (Aisyah, Vincken, Andini, Mardiah, & Gruppen, 2016). It has been reported that the germination of *Lupinus angustifolius*

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increases the functional properties as water and oil absorption, foaming capacity, and emulsification (Muñoz-Llandes et al., 2022), which favor its use in foods. However, the nutritional and structural properties of raw materials can be modified during processing. Temperature is likely a major factor that alters nutrient bioavailability during cooking. In processes as extrusion cooking, where high temperatures are used, there are reports of modifications in carbohydrates as starch, formation of amylose-lipid complexes, and degradation of compounds as vitamins and pigments (Gulati, Weier, Santra, Subbiah, & Rose, 2016). Proteins are denatured while trypsin inhibitors (non-nutritional compounds) are reduced or inactivated (Orozco-Angelino, Espinosa-Ramírez, & Serna-Saldívar, 2023). These alterations affect protein digestibility after extrusion (Nadeesha Dilrukshi, Torrico, Brennan, & Brennan, 2022). It has been reported that biological actions, such as antioxidant, immunoregulatory, antihypertensive, α-amylase inhibitory, and anti-inflammatory activities from protein of grains like barley, soy, beans, and amaranth increase after extrusion cooking (Montoya-Rodríguez, de Mejía, Dia, Reyes-Moreno, & Milán-Carrillo, 2014; Sharma, Gujral, & Singh, 2012; Sun, 2011; Yao & Ren, 2014). Gao et al. (2020) identified peptides from digested lupinus-based extrudates capable of inhibiting proinflammatory mediators as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1) in RAW 264.7 macrophages induced with lipopolysaccharide (LPS). Paucar-Menacho et al. (2022) reported that the use of germinated wheat flours for 3 days increases the sensory attributes in extrudates, a brown color is generated and its acceptability increases. Similarly, a reduction in the hardness of the final product has been evidenced due to the use of sprouts that have undergone hydrolysis of macromolecules by enzymatic action during germination. The combination of knowledge about the multiple benefits associated with both the germination and the extrusion of food ingredients opens the panorama to a new approach: the use of sprouted grain flours in the preparation of extruded snacks. Although there is abundant information about the advantages that each of these processes can provide individually, the work that has been reported on the synergy of both processes to create value-added products is limited. The combination of germination and extrusion represents an alternative, allowing to maximize the benefits of the grains. The need to carry out additional research becomes evident, with the aim of supporting and demonstrating the advantages of including sprouts in the generation of extrudates. This is based on the molecular transformation that occurs during germination, which causes greater availability and functionality of proteins, as well as the release of simple sugars and the reduction of anti-nutritional compounds. Therefore, the aim of this research was to determine the optimum extrusion cooking conditions using ungerminated *Lupinus angustifolius* flour, to obtain a puffed extrudate, after to replicate the process with germinated flours at different times, and characterize the physicochemical and nutritional properties of the products obtained, evidencing the influence of the use of sprouted flours. In addition, it was sought to establish molecular changes and transformations through Fourier-transform infrared spectroscopy (FT-IR) and scanning electron microscopy (SEM).

2. Materials and methods

2.1. Raw material and sample preparation

Lupinus angustifolius L. grains were obtained from Centro Universitario de Ciencias Biológicas y Agropecuarias at University of Guadalajara, Mexico. The grains were ground in a Raymond mill, 16-in. with a 2-HP engine (Raymond, USA). The flour obtained showed 35 mesh particle size (0.59 mm).

Then, flours were subjected to a defatting process in a Soxhlet extractor using petroleum ether with ratio of 1:4 (sample/solvent) for approximately 6 h, aiming to reduce the fat content to approximately 6%. Subsequently, they were placed in an oven at 40 °C for 1 h to ensure complete solvent evaporation. After, the flours were placed in polyethylene bags, and refrigerated at 4 °C. Commercial corn starch (mi Granero ®) with 12% moisture content and 0% protein was used in order to obtain a puffed extrudate. The ungerminated and germinated flours from lupinus were combined with corn starch to obtain the mixtures to be extruded.

2.2. Extrusion cooking process

Extrusion was carried out in a single-screw extruder (Brabender Instruments Inc., model 19/25DN Duisburg, Germany) equipped with an output die (3 mm diameter) and a compression screw ratio of 3:1. The feed rate was 1.2 kg/h and screw speed of 60 rpm. The extruder barrel has three heating zones. The heating ramp between each zone gradually increased 30 °C until reaching the desired temperature according to a central composite design used. The temperature of the third zone and the temperature of the die were the same according to the experimental design. The Response Surface Methodology (RSM) were used, to analyze independent variables over response ones. The independent variables were feed moisture (X_1), die temperature (X_2), and ungerminated lupinus/corn starch proportion (X_3) (Table 1). The moisture content of the samples was adjusted with water 24 h prior to extrusion. The response variables were water absorption index (WAI), water solubility index (WSI), sectional expansion index (SEI), hardness (H), bulk density (BD), total phenolic content (TPC), total flavonoid content (TFC).

2.3. Physicochemical characterization of extrudates

2.3.1. Water absorption and water solubility indexes (WAI, WSI)

Water absorption index (WAI) and the water solubility index (WSI) were performed according to the Anderson, Conway, and Peplinski (1970) with some modifications. Samples (1 g) were placed in falcon tubes containing 30 mL water at 30 °C, maintained under stirring for 30 min, and centrifuged at 2055 *g* for 10 min. Then, WAI was calculated by weighing the sediment and using the following equation:

$$WAI = \frac{Weight\ of\ sediment\ (g)}{Weight\ of\ sample\ (g)}$$

The supernatant was oven dried at 105 °C for 24 h, and the following equation was used to determine WSI:

$$WSI\ (\%) = \frac{Weight\ of\ dry\ solids\ in\ supernatant\ (g)}{Weight\ of\ dry\ sample\ of\ the\ original\ sample\ (g)} * 100$$

2.3.2. Sectional expansion index (SEI)

The SEI was assessed according to Jin, Hsieh, and Huff (1994) and Alvarez-Martinez, Kondury, and Harper (1988). Ten samples of each run were chosen, and diameters were measured. The values were divided by the die diameter (3 mm), and the following equation was used:

$$SEI = \frac{Sample\ diameter}{Diameter\ of\ extruder\ die}$$

2.3.3. Hardness (H)

The hardness was determined according to the methodology of Pamies, Roudaut, Dacremont, Meste, and Mitchell (2000) with some modifications. A TA-TX2 texture analyzer (Stable Micro Systems, Ltd.,

Table 1
Code leves for the independent varaibles.

	Level				
Variable	−1.682	−1	0	+1	1.682
Feed moisture (%)..... $_1$	16.81	16	18	20	19.19
Die temperature (°C) .....X_2	128.11	120	140	160	151.89
Lupinus proportion (%)*.....X_3	16.22	0	40	80	63.78

* The proportion of lupinus was supplemented with corn starch to complete 100%.

UK) was used to measure the force required to penetrate each extrudate. It was used a load cell of 5.0 kg. 5 cm long extrudates were used. Samples were placed horizontally on the platform and fractured using a 2-mm flat-tip cylinder probe at a speed of 2 mm/s and penetration distance of 3 mm. Thirty-five measurements were obtained per treatment and values were reported in newtons (N).

2.3.4. Bulk density (BD)

The method reported by Wang, Klopfenstein, and Ponte (1993) was used to determine BD. The diameter (d) and length (l) of ten samples from each extrudate were measured. Products were weighed (W) and BD was calculated with the equation below. Results were expressed in kg/m^3.

$$BD = \frac{W}{\pi \left(\frac{d}{2}\right)^2 l}$$

2.3.5. Protein content (P)

The method proposed by Kjeldahl (AOAC, 2006) was used to determine P. The conversion factor used was 6.25.

2.3.6. Total phenolic content (TPC)

The extraction of TPC was carried out according to Dueñas, Hernández, Estrella, and Fernández (2009) with modifications. Ground extrudate (0.5 *g*) was placed in centrifuge tubes and 10 mL 80% methanol was added. The tubes were placed in an orbital shaker away from light for 18 h. Folin–Ciocalteu reagent was used to determine TPC (Singleton, Orthofer, & Lamuela-Raventós, 1999), and absorbances were read at 765 nm. A calibration curve was obtained using gallic acid as standard. Results were expressed as mg of gallic acid equivalents (GAE) per 100 g of the sample, dry base (db).

2.3.7. Total flavonoid content (TFC)

The methods described by Žilić et al. (2011) and Eberhardt, Lee, and Liu (2000) were applied to determine TFC, using the same extract as in TPC. The extract (0.5 mL) was mixed with 0.075 mL 5% $NaNO_2$. After 6 min, 0.15 mL 10% $AlCl_3$ was added and the mixture was left to stand for 5 min. Finally, 0.5 mL 1 M NaOH was added and the volume was adjusted to 2.5 mL with distilled water. Absorbance was read at 510 nm, and a calibration curve was prepared with quercetin as standard. Values were reported as mg of quercetin equivalents (QE) per 100 g of sample db.

2.4. Optimization

Optimization was carried out using the hill climbing technique and Design Expert v7.0 (Stat-Ease Inc., Minneapolis, MN, USA). Multi-response numerical optimization helped to identify the optimum process conditions and apply them to produce extrudates from lupinus flours germinated for 3, 5, and 7 days. The independent variables that showed significant models (Table 2) were used to identify the optimum conditions. The BD and H values were minimized in order to avoid the formation of excessively compact and rigid extrudates. In the same way,

Table 2
Analysis of variance of the different models.

Response	df.	Source	Sum of squares	Mean squares	*F*-valor	*p*-valor
	5	Model	9.35	1.87	7.36	0.0014*
	14	Residual	3.56	0.25		
Water Absorption Index	9	Lack of fit	2.87	0.32	2.34	0.1814
	5	Pure error	0.68	0.14		
	19	Total	12.9			
	9	Model	69.09	7.68	0.94	0.5306
	10	Residual	81.43	8.14		
Water Solubility Index	5	Lack of fit	54.88	10.98	2.07	0.2223
	5	Pure error	26.55	5.31		
	19	Total	150.52			
	5	Model	2.01	0.4	18.21	<0.0001*
	14	Residual	0.31	0.022		
Sectional Expansion Index	9	Lack of fit	0.31	0.034	250.7	<0.0001*
	5	Pure error	6.83E-04	1.37E-04		
	19	Total	2.32			
	6	Model	1.04E+05	17,321.2	17.53	<0.0001*
	13	Residual	12,846	988.15		
Hardness	8	Lack of fit	11,910.1	1488.76	7.95	0.0176*
	5	Pure error	935.9	187.18		
	19	Total	1.17E+05			
	6	Model	2.01E+06	3.35E+05	21.71	<0.0001*
	13	Residual	2.00E+05	15,406.76		
Bulk Density	8	Lack of fit	1.97E+05	24,578.86	33.61	0.0006*
	5	Pure error	3656.96	731.39		
	19	Total	2.21E+06			
	7	Model	252.83	36.12	57.54	<0.0001*
	12	Residual	37.53	0.63		
Protein Content	7	Lack of fit	7.12	1.02	12.31	0.0069*
	5	Pure error	0.41	0.083		
	19	Total	260.36			
	6	Model	13,508.61	2251.44	3.1	0.0414*
	13	Residual	9449.86	726.91		
Total Phenolic Content	8	Lack of fit	7675.95	959.49	2.7	0.144
	5	Pure error	17,732.91	354.78		
	19	Total	22,958.47			
	7	Model	23,192.17	3313.17	5.23	0.0062*
	12	Residual	7597.34	633.11		
Total Flavonoid Content	7	Lack of fit	6806.83	972.4	6.15	0.0313*
	5	Pure error	790.5	158.1		
	19	Total	30,789.51			

* Significant at $p < 0.05$, df: degrees of freedom.

the TPC and TFC values were minimized to prevent possible interactions with proteins that affect their digestibility, while SEI and P were maximized. Values of WAI were established between 2.75 and 5.72. They were determined according to the maximum and minimum values obtained in the runs of the experimental design. In addition to considering that high WAI values can negatively affect the texture of the extrudate, resulting in a soft texture, on the other hand, low WAI values can generate denser products with limited expansion due to low starch gelatinization. Optimum conditions (16% feed moisture, 47/53% lupin/corn starch proportion, and temperature of 152 °C) were used to produce extrudates with 4 different formulations: EUG: extruded with 47% ungerminated lupin flour/53% proportion of corn starch, EG3: extruded with 47% sprouted lupin flour for 3 days/53% proportion of corn starch, EG5: extruded with 47% sprouted lupin flour for 5 days/53% proportion of corn starch and EG7: extruded with 47% sprouted lupin flour for 7 days/53% proportion of corn starch.

2.5. Germination

Grains from *Lupinus angustifolius* were germinated to be included in the extrudate, using the optimum process conditions obtained with ungerminated flour. The germination process was carried out for 3, 5, and 7 days, according to the method described by Guzmán-Ortiz et al. (2017). Lupinus sprouts were dehydrated in a conventional oven (Barnstead Lab-Line, USA) at 40 °C to reach 8% moisture content, approximately. Grains were ground in a 16-in. mill with 91 a 2-HP engine (Raymond, USA). The flour obtained showed 35 mesh particle size (0.59 mm) and degreased as indicated in the section 2.1. The flours were stored in polyethylene bags until use.

2.6. Characterization of extrudates obtained under optimal conditions

2.6.1. Total color difference

Color parameters of the extrudates obtained were determined in a colorimeter (Hunter Associates Laboratory, USA). Color was expressed in CIE-Lab values as L* (whiteness/darkness), a* (redness/greenness), and b* (yellowness/blueness). Three measurements were taken and averaged per sample. The total color difference was calculated using the following equation:

$$\Delta E = \sqrt{(\quad)^2}$$

where subindex 'o' indicates the initial color values of native flour before extrusion.

2.6.2. Proximal chemical composition

The methods established by AOAC (2006) were used to determine ash, protein, crude fiber, and lipids in the extrudates, while carbohydrates were identified by difference (100%). The energy value was determined according to the Regulation (EU) No. 1169/2011of the European Parliament and of the Council of October 25, 2011 (Arribas, Cabellos, Cuadrado, Guillamón, & Pedrosa, 2019) using the following equation:

$$Energy\left(\frac{Kcal}{100\ g\ extruded\ product}\right) = 4(g\ protein + g\ carbohydrates) + 9(g\ fat\ )$$

2.6.3. Phytic acid

The phytic acid content was assessed as described by Vaintraub and Lapteva (1988) and Latta and Eskin (2002). Samples (0.5 g) were added to 10 mL 3.5% HCl (*w*/*v*), kept under constant stirring for 1 h, and centrifuged at 10,000 rpm for 10 min. The determination was carried out by forming a complex between the extract and Wade reagent (30 mg $FeCl_3 \cdot 6H_2O$ +300 mg sulfosalicylic acid and 100 mL distilled water). Absorbance was read at 500 nm, and a calibration curve was prepared with sodium phytate. Results were reported as mg of sodium phytate equivalents (SPE) per gram of sample, db.

2.6.4. Trypsin inhibitor activity

Trypsin inhibitor activity was assessed according to the enzymatic method described by Welham and Domoney (2000) using Nα-Benzoyl-DL-arginine p-nitroanilide hydrochloride (BAPNA) as trypsin substrate. Absorbances were read at 410 nm against a reagent blank. Trypsin inhibitor activity was expressed as trypsin inhibitor units per g of sample (TIU/g).

2.6.5. In vitro protein digestibility

Protein digestibility was identified through the multienzyme technique reported by Tinus, Damour, van Riel, and Sopade (2012). A solution was prepared using the sample and distilled water, maintaining 62.5 mg protein. The solution was incubated at 37 °C for 1 h and pH was adjusted to 8. A multienzyme mix at pH 8 was prepared with the following: 16 mg trypsin from porcine pancreas (type IX-S, T0303), 31 mg chymotrypsin from bovine pancreas (type II, C4129), and 13 mg protease from *streptomyces griseus* (type XIV, P5147). A mixture comprising 1 mL of the multienzyme solution and 10 mL of the sample was maintained in a water bath at 37 °C for 10 min during the digestion process. The pH value at 10 min was used to calculate protein digestibility using the following equation:

$$IVPD = 65.66 + 18.10\Delta pH\ 10\ min$$

2.6.6. Fourier-transform infrared (FT-IR) spectra analysis

The chemical structure of the extrudates was analyzed by infrared spectroscopy (IR) in a Spectrum 2 spectrometer (Perkin-Elmer, USA). The spectra were recorded using at least 12 scans at a resolution of 4 cm^{-1} and a spectra range of 4000–650 cm^{-1} at room temperature (27 °C). Deconvolution of amide I band (1700–1600 cm^{-1}) was done using Origin (v 2021) from the original spectrum where Gaussian peaks were assigned to each component of the protein secondary structure based on the center. The integer of each peak was divided by the sum of all peaks determined to identify the area proportion of every component. For identification, β-sheet around 1638 cm^{-1}, α-helix around 1654 cm^{-1}, β-turn around 1663 cm^{-1} and random coil around 1645 cm^{-1} were considered (Byler & Susi, 1986; Carbonaro & Nucara, 2010; Jackson & Mantsch, 1995; Shevkani, Singh, Kaur, & Rana, 2015; Surewicz, Mantsch, & Chapman, 2002).

2.6.7. Scanning electron microscopy (SEM)

The superficial morphology of all extrudates was determined by scanning electron microscopy (SEM). Longitudinal cuts of each sample were done with a blade (2–3 mm thickness). The sections were mounted on carbon tape and sputter coated with gold (mini sputter, JFC 1600) at 10 mA for 10 s. The coated samples were placed in a scanning electron microscope chamber (JSEM 35CX, Japan Electronic Optical Limited, Japan) and images were recorded (magnification 800×). Measurements were carried out at an accelerating voltage of 15.

2.7. Experimental design and statistical analyses

A rotatable central composite design was used with three independent variables: feed moisture (X_1), extrusion temperature (X_2), and lupinus proportion (X_3) (Table 1). The dependent variables were WAI, WSI, SEI, H, BD, P, TPC, and TFC. Data were analyzed and adjusted to a two-level regression model, and regression coefficients were obtained. The significance of the models was analyzed and RSM and Design Expert 7.1.6 (Stat-Ease, Inc., MN, USA) were used for data analysis. The ANOVA of SEI, H, BD, P and TFC, proved that the models and lack of fit were significant ($p < 0.05$), WAI and TPC the models have a significant effect; however, the lack of fit was not statistically significant ($p > 0.05$) and WSI, the regression model was not significant ($p > 0.05$) (Table 2). The terms, and regression coefficients corresponding to each variable was

determined by analysis of variance (ANOVA) as shown in Tables 3 and 4. All the determinations were carried out in triplicate and were expressed as the mean of the triplicate ± the standard deviation.

3. Results and discussion

3.1. Water absorption index (WAI) and water solubility index (WSI)

WAI is a parameter that reflects the quantity of water taken up by the extruded product, and as such, it is linked to the extent of starch transformation in the product. (Tas & Shah, 2021). The value of R^2 was 0.7244, indicating that 72.44% of variability could be explained by the model obtained. The WAI values ranged from 2.75 to 5.72 g water/g sample. The reduction in lupin content and increase in feed moisture promoted WAI (Fig. 1A). This was possibly because of the intra and intermolecular interactions within the crystalline and amorphous structures of the starch granules with the water available. When starch is gelatinized, its crystalline structure is modified due to the breakage of intramolecular hydrogen bonds, leaving larger numbers of hydroxyl groups exposed to interactions with the available water in the medium, and generates an increase of WAI (Ye, Liu, Luo, Hu, & McClements, 2018). The increase in feed moisture enhanced WAI values thanks to a lubricating and protective effect of water in starch, which reduces viscosity and thus mechanical shear time in the mass. In turn, the total degradation of starch was reduced and allowed for the interaction with the water (Navarro-Cortez et al., 2018). Water acts as plasticizer in polymers, improving chain mobility and providing deformation and fluidity. This reduces viscosity while a shorter time in the die reduces molecular degradation (Lewicki & Jakubczyk, 2004). On the other hand, the increase in lupin content and feed moisture affected WAI values. A similar behavior was reported by Bepary, Wadikar, and Semwal (2022) due to a limited gelatinization of starch content during extrusion. In addition, there was a larger P (18.33%) that affects viscoelasticity properties and reduces drag flow in the barrel. This protects biomolecules from mechanical damage to a large extent and creates more compact products which affect WAI. According to the ANOVA of WSI, the regression model was not significant ($p > 0.05$) (Table 2). The independent variables exerted no significant effect on the WSI.

The decrease in WAI was affected by the increase in the content of lupinus, due to a higher protein content that reduces the starch concentration and the degree of gelatinization is affected, causing low WAI values (Rodríguez-Vidal et al., 2017). A similar behavior was reported by Bepary et al. (2022) in extruded rice bean (*Vigna umbellata*).

WSI indicates the level of degradation of macromolecules, which favor the amount of small molecules solubilized in water (Ye et al., 2018). However, according to the ANOVA of WSI, the regression model was not significant ($p > 0.05$) (Table 2). The independent variables exerted no significant effect on the WSI.

3.2. Sectional expansion index (SEI)

The value corresponding to R^2 was 0.8668, indicating that 86.68% of variability can be explained by the established model. The independent variables with a significant effect are presented in the RSM plot (Fig. 1B), where the values observed are 0.92–2.04. The increase in extrusion temperature (from 120 to 160 °C), the lower lupin content, and increased starch promoted higher SEI values. This is likely due to the enhanced mass viscosity as a result of the high temperatures (153–187 °C) during processing, which promote the formation of larger bubbles and thus an increase in expansion (Navarro-Cortez et al., 2018). Additionally, when temperature is increased, starch gelatinization occurs rapidly while granules start to retain water and expand, contributing to higher SEI. Guzmán-Ortiz et al. (2015) reported a correlation between starch content and the temperature of the extrusion process. On the other hand, a reduced SEI was observed when lupin concentrations were increased and temperature was lower, possibly because of the reduced starch content in the mix that affects the viscoelastic properties.

An increase in the proportion of lupinus in the mix also leads to an increase in the protein content, which exerts an unfavorable influence on the SEI. Because during extrusion, protein aggregation is facilitated due to the breaking of intramolecular disulfide bonds, which favors hydrophobic interactions and promotes the formation of protein aggregates (Zhang et al., 2019). Furthermore, interactions between protein and starch originate during the extrusion process through electrostatic forces, hydrogen bonds, van der Waals forces and hydrophobic interactions, which generate larger complexes and a three-dimensional network (Koch, Emin, & Schuchmann, 2017). On the other hand, starch plays an important role during extrusion by acting as a thickening and binding agent in the protein matrix, generating interactions between proteins and starch that impact the structural, textural and functional properties of the extruded products (Chen, Zhang, Liu, Li, & Wang, 2023). This may lead to limited expansion. On the other hand, low temperature negatively affects SEI values due to inefficient energy transfer in the mix. This inefficiency can decrease the formation of pores and, consequently, restrict the expansion of the extrudate. A similar behavior was reported by Seth, Badwaik, and Ganapathy (2015).

3.3. Hardness (H)

The value of R^2 was 0.89, indicating that 89% of variability could be explained by the model. The values of H were 56.1–299.61 N. The increase in lupin content and feed moisture promoted the increase in H

Table 3
Significance of the P and F values.

Response	Water absorption index		Sectional expansion index		Hardness		Bulk density		Protein content		Total phenolic content		Total flavonoid content	
	P	F	P	F	P	F	P	F	P	F	P	F	P	F
Intercept	0.0014*	7.36	<0.0001*	18.21	<0.0001*	17.53	< 0.0001*	21.71	<0.0001*	57.54	0.0414*	3.1	0.0062*	5.23
X_1	0.0502	4.59	0.8213	0.053	0.0624	4.15	0.4505	0.61	0.1102	2.97	0.0991	3.15	0.3268	1.05
X_2	0.974	1.10E-03	0.0029*	12.99	0.0005*	20.78	< 0.0001*	49.46	0.0054*	11.5	0.8442	0.04	0.0623	4.23
X_3	0.0003*	23.16	0.0006*	19.38	<0.0001*	46.07	0.0010*	17.65	<0.0001*	365.22	0.0284*	6.08	0.0068*	10.63
X_1X_2	0.1201	2.74							0.0987	3.21				
X_1X_3	0.0249*	6.31											0.0449*	5.01
X_2X_3			0.0222*	6.61			0.0151*	7.83	0.0120*	8.73			0.0367*	5.52
X_1^2			<0.0001*	52.05	0.0258*	6.33	< 0.0001*	43.73	0.0161*	7.82	0.1408	2.46	0.0562	4.46
X_2^2					0.0107*	8.86	0.0180*	7.32	0.0611	4.27	0.2573	1.4	0.0243*	6.63
X_3^2					0.0011*	17.36					0.0207*	6.93		

X_1 = Feed moisture (%), X_2 = Die temperature (°C), X_3 = Lupins proprotion (%).
WAI = Water absorbtion index, SEI = Sectional expansion index, H = Hardnees, BD = Bulk density, P = protein content, TPC = Total phenolic content, TFC = Total flavonoid content.

* Significant at $P < 0.05$, df: degrees of freedom.

Table 4
Regression coefficients of the adjusted response surface models for the response variables of the extruded products.

Response	Water absorption index	Sectional expansion index	Hardness	Bulk density	Protein content	Total phenolic content	Total flavonoid content
Intercept	4.28	1.06	152.07	1161	11.5	142.2	88.78
X_1	0.29	—9.25E-03	17.33	26.13	0.37	12.96	6.96
X_2	4.52E-3	0.14	—38.77	—236.22	0.73	—1.46	14
X_3	—0.7	—0.18	57.74	141.12	4.1	17.98	22.2
X_1X_2	0.29				—0.5		
X_1X_3	—0.5						—19.92
X_2X_3		—0.13		122.8	0.83		20.91
X_1^2		0.28	—20.84	—215.14	—0.58	—11.14	13.94
X_2^2			24.65	88.05	—0.43	—8.41	16.98
X_3^2			34.50			—18.69	
R^2	0.7244	0.8668	0.8900	0.9093	0.9711	0.5884	0.7532
Radj²	0.6259	0.8192	0.8392	0.8674	0.9542	0.3984	0.6093

X_1 = Feed moisture (%), X_2 = Die temperature (°C), X_3 = Lupins proprotion (%).
WAI = Water absorbtion index, SEI = Sectional expansion index, H = Hardnees, BD = Bulk density, P = protein content, TPC = Total phenolic compounds, TFC = Total flavonoid content.

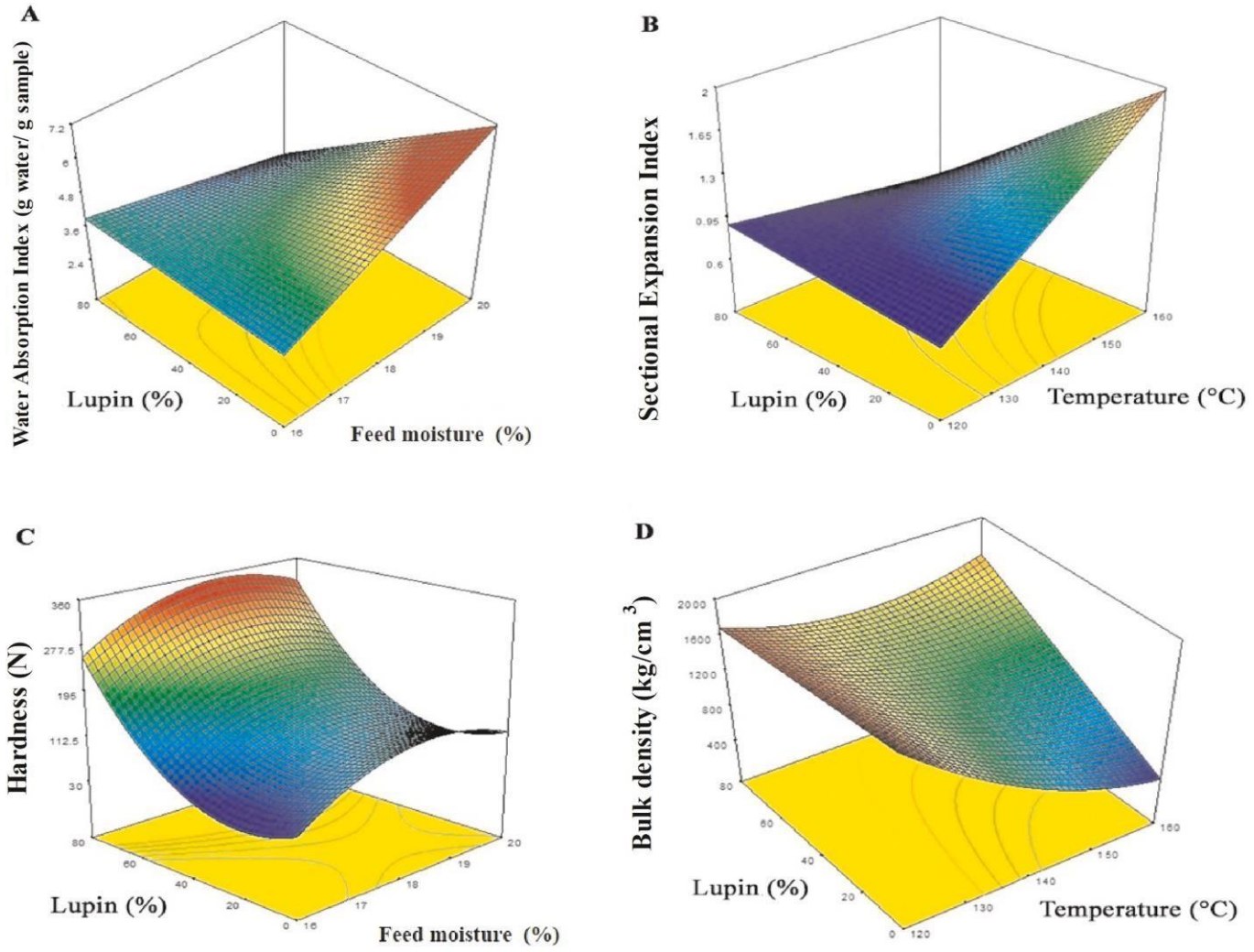

Fig. 1. Response surface graphs for the different models obtained. A) Effect of feed moisture and proportion of lupin on the WAI. B) Effect of temperature and proportion of lupin on BD. C) Effect of temperature and proportion of lupin on the IE. D) Effect of feed moisture and proportion of lupin on H.

(Fig. 1C). A greater lupin content involved larger amounts of protein, favoring the interaction with other molecules (mostly fiber and depolymerized starch fragments) forming complex matrices that limit product expansion and contribute to higher density and hardness during the extrusion process. This generates denser and more compact products that require more force for fracturing (Liu, Hsieh, Heymann, & Huff, 2000). Excessive moisture can hinder pore formation and proper expansion, which can result in a denser structure and therefore higher hardness (Seth et al., 2015), because excess water can reduce energy transfer and mechanical damage.

On the other hand, low lupin contents and moisture reduced H values, likely due to the lower fiber content involved in the formation of

air bubbles, which led to more compact products. A similar behavior was observed by Altan, McCarthy, and Maskan (2008) in extrudates using tomato pomace as fiber source.

3.4. Bulk density (BD)

The value of R^2 was 0.9093, indicating that 90.93% of variability could be explained by the model. The values observed in the treatments ranged from 429.44 to 1482.82 kg/m^3. The increase in lupin content and lower temperature led to a higher BD (Fig. 1D), and when the temperature increase and the lupin content lower, the BD values were reduced. Temperature plays an important role, since it affects the viscosity. At low temperatures, the mixture can have a higher viscosity, generating greater internal resistance to flow through the extruder barrel, which can cause a higher density. On the other hand, the increase in BD when the proportion of lupine increases may be due to a higher protein content in the mixture that modifies the rheological properties during the process, generating a continuous and more uniform protein matrix, without air bubbles and limiting the hydration properties (Filli, Nkama, & Jideani, 2013). A similar behavior was reported by Navarro-Cortez et al. (2018) in extrudates based on blue corn and orange bagasse; they observed a decrease in BD when SEI increased because of the larger volume of the extrudate.

3.5. Protein content (P)

The value of R^2 was 0.9711, indicating that 97.11% of variability might be explained by the model. The values of P ranged from 3.47 to 16.82%. The increase in lupin content and temperature promoted the total P (Fig. 2A). Still, this trend produced more compact products, with higher H and lower SEI. In contrast, lower lupinus levels led to low P, improving SEI and H. A similar behavior was reported by Arribas et al. (2017), who observed that P in extrudates depended on the concentration of the ingredient containing protein.

3.6. Total phenolic and flavonoid contents

The value of R^2 was 0.5884, meaning 58.84% of variability could be explained by the model. The values observed were 8.95–167.69 mg GAE/100 g sample. The highest concentration observed was 18% feed moisture, 140 °C, and 80/20% lupin/corn starch content (Fig. 2B). The increase in moisture and lupin content promoted TPC. Larger concentrations of lupin provided a larger TPC as well. Dueñas et al. (2009) reported phenolic compounds of 8.56 mg GAE/g sample in *Lupinus angustifolius* L. flour. It has been demonstrated that extrusion can denature linkages between phenolic compounds and protein bodies, increasing the concentration of these bioactive compounds after

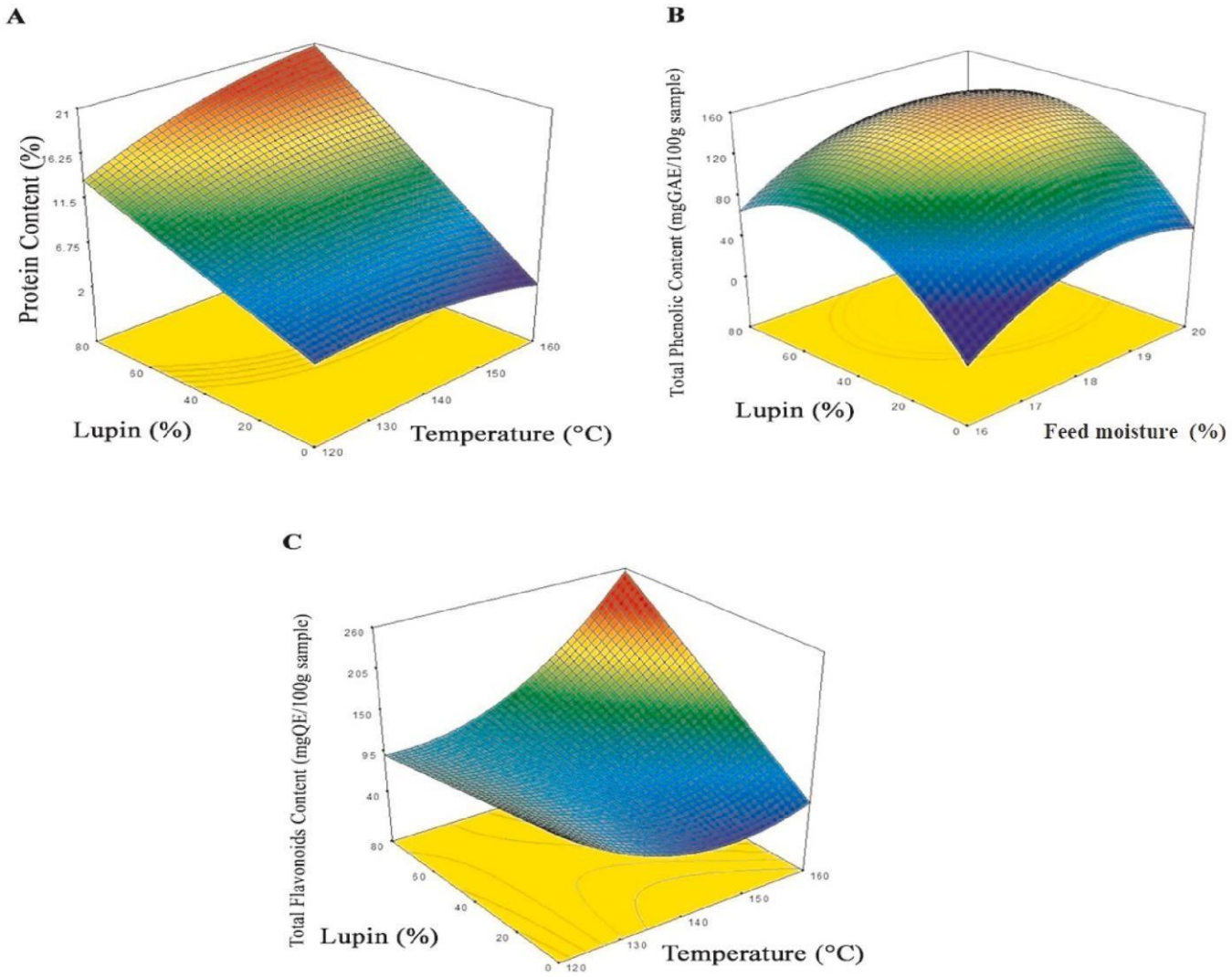

Fig. 2. Response surface plots for the models obtained. A) Effect of temperature and proportion of lupinus on the P. B) Effect of feed moisture and the proportion of lupin on the TPC. C) Effect of temperature and proportion of lupinu on the TFC.

extrusion (Abbas & Ahmad, 2018). Nevertheless, both a lower lupin concentration and higher moisture content reduced TPC.

In TFC, the value of R^2 was 0.7532, indicating that 75.32% of the variability could be explained by the model. The values observed were 72.58–214.09 mg QE/100 g sample. The highest flavonoid concentration was observed at 17% feed moisture, 152 °C, and 64% lupin content (Fig. 2C). The increase in temperature and lupin content promoted TFC. Extrusion allowed to modify the food matrix because of the processing conditions where the covalent interactions between flavonoids and polysaccharides and the cell wall were broken. This released them and thus their concentration in the extrudate increased. Barros et al. (2018) reported a similar behavior in catechin content of lentil extrudates.

3.7. Extrudate optimization

Extrusion was optimized with ungerminated lupin flour (EUG) to use the optimum conditions in the inclusion of germinated lupinus flours. According to multiresponse numerical optimization, the ideal conditions for extrusion when using ungerminated lupin flour were: 16% feed moisture, 152 °C output die temperature, and 47/53% lupin/corn starch proportion. The experimental values of the response variables were similar to those predicted using Design Expert (Table 6). The optimum conditions were employed to develop extrudates including germinated lupin flour (EG3, EG5, and EG7).

3.8. Proximal chemical composition of extrudates with ungerminated and germinated lupinus flour

The chemical compositions of all extrudates are presented in Table 5. The lipids content in EUG was 5.74% with no significant difference regarding extrudate products (EG3, EG5, and EG7) ($p > 0.05$) since degreased flours were used until reaching around 6%. The P value increased significantly ($p < 0.05$) in extrudates from germinated flour. EUG was 11.88% contained and increased to 13.16% in EG3. Those extruded with germinated lupin flour did not present a significant difference ($p > 0.05$). The increase was due to a *de novo* synthesis during germination where mRNA was replicated from cell division to synthesize new proteins that would become part of the germinate structure (Muñoz-Llandes et al., 2022; Singh & Sharma, 2017). This affected the increase in protein in extrudates with germinated flours. The crude fiber content in EUG was 4.17% and increased significantly ($p < 0.05$) in the extrudate with sprouted flour for 7 days (EG7) up to 5.43%. This could be because the pectic substance in the middle lamella (the first layer synthesizing in the cell wall formation of the radicle emerging from the grain) was higher (Banchuen, Thammarutwasik, Ooraikul, Wuttijumnong, & Sirivongpaisal, 2009).

The ash content in EUG was 3.05% and increased significantly ($p < 0.05$) due to the effect of germination up to 4.29% in EG5. This is likely because of the activity of endogenous hydrolytic enzymes during germination which promote mineral release (Atudorei, Stroe, & Codină, 2021). The carbohydrate content in EUG was 75.16%, and a significant decrease ($p < 0.05$) was observed in EG3 and EG5. This decrease is mainly due to the activity of α-amylase and β-amylase enzymes that hydrolyze carbohydrates into simple sugars during germination (Warle, Riar, & Gaikwad, 2015). The energy value in EUG was 399.82 kcal/100 g and it dropped significantly ($p < 0.05$) in extrudates EG5 and EG7. The use of lupinus flours germinated for 5 days allowed to reduce the energy value in germinated extrudates. The nutritional quality of the extrudates improved when the amount of carbohydrates was lower and fiber content and P increased. It has been reported that the extrusion process does not substantially modify the macromolecule content (Ai, Cichy, Harte, Kelly, & Ng, 2016), the variations in the proximal composition of the extrudates obtained are mainly attributed to the germination process that involves various metabolic processes.

3.8.1. Effect of extrusion on physicochemical characteristics

Table 6 shows WAI, WSI, SEI, H, and BD of extrudates from lupin flour ungerminated and germinated at different times obtained under optimum conditions of extrusion. The germination time was observed to affect the physicochemical properties. EUG showed the highest SEI (1.90) and H (72.92 N). Both SEI and H were significantly reduced ($p < 0.05$) along with the germination time. SEI values of 1.80, 1.71, and 1.69 were observed in EG3, EG5, and EG7, respectively. The same behavior was observed in H, with values of 57.92, 54.6, and 50.98 N, respectively. Additionally, BD was higher when germinated flours were used; EUG showed values of 437.45 kg/m³, which statistically increased ($p < 0.05$) (EG7 > EG5, EG3 > EUG) to 643.60 kg/m³ in EG7. Probably at the extrusion temperature (152 °C) the viscosity of the material increases in germinated flours, which limits greater fluidity during extrusion and with it less expansion, greater hardness and density of the extrudate. A similar tendency was observed in WSI (EG7 > EG5 > EG3 > EUG), while EUG showed values of 6.29% that rose to 19.68% in EG7. Sprouted flours during extrusion at 152 °C and 16% moisture content increase the breaking of molecular chains that are easier to break with longer germination time and cause an increase in WSI. The values of WAI the extrudate were also increased when germinated flours were used; however, the highest was observed in EG3. Finally, WSI was significantly greater regarding germination time and reached a maximum of 19.68% in EG7.

The alterations caused by extrusion in germinated flours can be associated to enzymatic activity during germination, involving several metabolic processes. This led to changes in the chemical composition of lupin that affected the physicochemical features of the extrudate. The SEI and BD were possibly affected by the fiber content in germinated flours because the fiber can break the bubbles formed during the extrusion process and can limit the rate of expansion and increasing BD. On the other hand, P increased during germination, enhancing the capacity of polar groups to interact with and retain water in the medium (Ghumman, Kaur, & Singh, 2016), which could lead to a higher WAI. Furthermore, the hydrolysis of macromolecules during germination and extrusion produced structural changes that favored the solubility of the material, as evidenced in a significantly increased WSI ($p < 0.05$) regarding germination time.

The color characteristics of the extrudates were also affected by the

Table 5
Chemical composition of extruded lupinus ungermination and germination for 3, 5 and 7 days.

Sample	Lipids* (%)	Protein* (%)	Fiber* (%)	Ash* (%)	Carbohydrates* (%)	Energy (kcal/100 g of extrudate)
EUG	5.74 ± 0.14^{ab}	11.88 ± 0.14^{b}	4.17 ± 0.35^{b}	3.05 ± 0.03^{c}	75.16 ± 0.41^{a}	399.82 ± 0.73^{a}
EG3	6.31 ± 0.21^{a}	13.16 ± 0.02^{a}	4.64 ± 0.28^{ab}	3.60 ± 0.05^{b}	72.29 ± 0.27^{b}	398.58 ± 0.55^{a}
EG5	5.15 ± 0.28^{b}	12.69 ± 0.06^{a}	5.23 ± 0.11^{ab}	4.29 ± 0.17^{a}	72.63 ± 0.34^{b}	387.65 ± 1.09^{b}
EG7	5.01 ± 0.21^{b}	13.03 ± 0.28^{a}	5.43 ± 0.21^{a}	3.56 ± 0.11^{b}	72.97 ± 0.51^{ab}	389.03 ± 0.76^{b}

Values were expressed as the average of triplicates ± standard deviation. Different superscript letters (a–b) in the same column indicate a significant difference using Tukey's test ($P < 0.05$).

* Results are expressed in dry basis (db). EUG = extruded with lupins flour ungerminating, EG3 = extruded with germinated of lupins by 3 days, EG5 = extruded with germinated of lupins by 5 days, EG7 = extruded with germinated of lupins by 7 days.

Table 6
Physicochemical properties of extruded lupinus ungermination and germination for 3, 5 and 7 days.

Sample	WAI (g/g)	WSI (%)	SEI	H (N)	BD (Kg/m3)	L*	a*	b*	ΔE
Prediction	3.32 c	+ d	1.92 a	71.52 a	434.15 c	a	d	d	d
EUG	3.48 ± 0.09	6.29 ± 0.47	1.90 ± 0.02	72.92 ± 7.07	437.45 ± 38.78	84.55 ± 1.44	2.20 ± 0.20	28.86 ± 0.62	6.26 ± 0.18
EG3	4.84 ± 0.07[a]	10.29 ± 0.58[c]	1.80 ± 0.02[b]	57.92 ± 4.03[b]	567.99 ± 37.44[b]	81.22 ± 0.38[b]	5.70 ± 0.21[c]	36.33 ± 0.40[c]	13.23 ± 0.36[c]
EG5	4.35 ± 0.06[b]	13.32 ± 0.19[b]	1.71 ± 0.01[c]	54.6 ± 1.71[bc]	574.86 ± 49.48[b]	75.48 ± 0.72[c]	9.89 ± 0.50[a]	40.22 ± 0.38[b]	18.39 ± 0.76[b]
EG7	4.32 ± 0.02[b]	19.68 ± 0.20[a]	1.69 ± 0.01[c]	50.98 ± 3.60[c]	643.60 ± 34.99[a]	74.93 ± 0.10[c]	8.68 ± 0.09[b]	42.04 ± 0.18[a]	24.13 ± 1.06[a]

Values were expressed as the average of triplicates ± standard deviation. Different superscript letters (a–d) in the same column indicate a significant difference using Tukey's test ($P < 0.05$). + = the model was not significant. EUG = extruded with lupins flour ungerminating, EG3 = extruded with germinated of lupins by 3 days, EG5 = extruded with germinated of lupins by 5 days, EG7 = extruded with germinated of lupins by 7 days, WAI = Water absorbtion index, SEI = Sectional expansion index, H = Hardnees, BD = Bulk density, P = protein content, TPC = Total phenolic content, TFC = Total flavonoid content, L* = whiteness/darkness), a* = redness/greenness and b* = yellowness/blueness.

use of germinated flours (Fig. S1). It was observed that the parameter L* (luminosity) decreased significantly ($p < 0.05$) in the germinated extrudates (EG7 < EG5 < EG3 < ESG) from 84.55 in EUG to 75.48–74.93 in EG5 and EG7, respectively. Values of a* and b* were significantly higher in extrudates from germinated flour; a* oscillated between 2.21 and 9.89, significantly increasing ($p < 0.05$) along with the germination time to reach a maximum in EG5. The positive b* values indicated a yellowish color and a significant increase ($p < 0.05$) from 28.86 in EUG to 42.04 in EG7. The values of ΔE were significantly increasing in extrudates from germinated flour until reaching maximum in EG7. This occurred likely due to the development of non-enzymatic browning as Maillard reaction and caramelization, which provide more intense brown coloring with germinated flours (Oliveira, Alencar, & Steel, 2018; Ramírez-Rivera et al., 2021). The increase in EG3, EG5, and EG7 could be attributed to the accumulation of simple sugars by enzymatic starch hydrolysis during germination and protein hydrolysis that released free amino acids available for non-enzymatic browning. In addition, in the extrusion process, temperature, shear and pressure generate changes in the structure of the protein and carbohydrates, which makes these compounds more available to form Maillard reactions.

3.9. *Effect of extrusion on non-nutritional compounds of extrudates with ungerminated and germinated lupinus flour*

3.9.1. *Total phenolic content (TPC) and total flavonoid content (TFC)*

Table 7 shows TPC and TFC. As predicted in optimization, EUG presented TPC of 84.11 mg GAE/ 100 g and TFC of 172.62 mg QE/100 g sample. The inclusion of germinated flour in the extrusion increased the concentration of both compounds. A significant rise ($p < 0.05$) was observed in germinated extrudates (EUG < EG3 > EG5 < EG7), peaking in EG3 at TPC of 140.72 mg GAE/100 g sample and TFC of 198.29 mg QE/100 g sample. This might occur because TPC are secondary metabolites expressed by the seedling as a survival and adaptation mechanism, and they were higher during germination. Flavonoids can be synthesized in germination due to the activation of the metabolic phenylpropanoid pathway, where intermediate products can promote the acetyl coenzyme A ester formation that later turn into flavonoids (Ti et al., 2014). Furthermore, extrusion technology releases this type of compounds covalently bound to the cell wall of the used material. Then, they are exposed for quantification after extrusion (Stojceska, Ainsworth, Plunkett, İbanoğlu, & İbanoğlu, 2008). Still, the retention of these compounds in the final extrudate will depend on the extrusion conditions, mostly temperature and moisture (Arribas et al., 2017).

3.9.2. *Trypsin inhibitor activity and phytic acid*

The content of trypsin inhibitor activity and phytic acid is presented in Table 7. Trypsin inhibitors in EUG were 11.95 TIU/g of sample and were significantly reduced ($p < 0.05$) in EG3 to 7.5 TIU/ g. No trypsin inhibitors were detected in EG5 and EG7. It has been reported that the concentration of trypsin inhibitors is reduced along with germination time (Muñoz-Llandes et al., 2022), which would favor their inactivation while using germinated flours in extrusion. Thermal processes involve high temperatures, as extrusion, and lead to deamidation of covalent bonds as peptide bond hydrolysis in aspartic acid residues or the destruction of disulfide bonds, thus deactivating them (Alonso, Orúe, & Marzo, 1998). A similar behavior has been reported in extrudate flours from faba beans, chickpeas, and kidney beans (Abd El-Hady & Habiba, 2003).

Table 7
In vitro digestibility of protein and content of antinutritional compounds of extruded ungerminated and germinated lupinus for 3, 5 and 7 days.

Sample	TPC (mgGAE/ 100 g sample)	TFC (mgQE/ 100 g sample)	Trypsin inhibitor activity (TIUs**/ g of sample)	Phytic acid (mgSPE***/ g of sample)	*In vitro* protein digestibility (%)
Prediction	82.43	169.44			
EUG	84.11 ± 0.02[d]	172.62 ± 0.03[b]	11.95 ± 0.56[a]	0.07 ± 0.01[a]	80.68 ± 0.51[b]
EG3	140.72 ± 0.01	198.29 ± 0.02[a]	7.50[b] ± 0.35	0.05[ab] ± 0.01	72.99 ± 0.12
EG5	101.20 ± 0.03 [c]	145.59 ± 0.01[d]	ND	0.05 ± 0.02 [ab]	74.80 ± 0.38[cd]
EG7	120.71 ± 0.01[b]	165.57 ± 0.02[c]	ND	0.04 ± 0.01[b]	77.69 ± 1.66[bc]
Casein					91.09 ± 1.66[a]

Values were expressed as the average of triplicates ± standard deviation. Different superscript letters (a–d) in the same column indicate a significant difference using Tukey's test ($P < 0.05$).

** Trypsin inhibitor units (TIUs).

*** Sodium Phytate Equivalents (SPE). ND = Not deteced, EUG = extruded with lupins flour ungerminating, EG3 = extruded with germinated of lupins by 3 days, EG5 = extruded with germinated of lupins by 5 days, EG7 = extruded with germinated of lupins by 7 days.

The content of phytic acid in EUG was 0.07 mg SPE/g sample, and it was significantly reduced ($p < 0.05$) to 0.04 mg SPE/g sample in EG7. This behavior might be due to the dephosphorylation of phytic acid into simpler forms during extrusion, as a result of high temperatures that affect viability (Alonso, Aguirre, & Marzo, 2000). The effect of the extrusion process on the decrease in phytic acid can also be confirmed since Muñoz Llandes et al. (2019), reported concentrations of phytic acid in the same variety of ungerminated lupinus of 3.0 mg SPE/g of sample and 2.5 mg SPE/g in 7-day sprouts, which indicates that the extrusion process favored the reduction of phytic acid. Furthermore, germination also contributes to the reduction in this antinutrient because of the activation of endogenous enzymes (phytases and phosphatases). These enzymes hydrolyze phytic acid to release inorganic phosphorus, which needs germination for its growth and development (Muñoz Llandes et al., 2019; Pal et al., 2017).

3.10. In vitro protein digestibility of extrudates

The *in vitro* protein digestibility of the extrudates is presented in Table 7. While EUG showed 80.68% digestibility, that of EG3 was reduced by 9.53% and exhibited no significant difference ($p < 0.05$) when compared to EG5. Digestibility of EG7 reached a maximum of 77.69% and there was no significant difference ($p < 0.05$) regarding EUG. The reduction in protein digestibility in EG3 and EG5 could be linked to the significant increase ($p < 0.05$) in TPC and TFC, because phenols can bind to proteins by interacting with hydrophobic sites on the protein reducing the accessibility of proteases in preference to hydrophobic residues. Phenolic compounds can bind to proteolytic enzymes, reducing protein digestibility by inhibition (Cirkovic Velickovic & Stanic-Vucinic, 2018). The increased digestibility in EG7 could be due to protein hydrolysis by high temperatures, which reorders molecules during thermal denaturation and allows for a better interaction with and access to proteolytic enzymes during digestion (Chávez-Murillo, Veyna-Torres, Cavazos-Tamez, de la Rosa-Millán, & Serna-Saldívar, 2018). In addition, extrusion allowed for the inactivation and/or reduction in non-nutritional compounds, favoring protein digestibility.

3.11. Influence of extrusion on protein secondary structure of ungerminated and germinated lupinus extrudates (FT-IR)

All FT-IR spectra were analyzed to predict the molecular alterations in extrudates from ungerminated and germinated lupinus (EUG, EG3, EG5, and EG7). The spectra are shown in Fig. 3A. A reduction in transmittance intensity was observed in samples from germinated flour (EG3, EG5, and EG7), which presented an alteration in the protein related to germination. This could be due to the decomposition of complex protein molecules in amino acids during germination (Chinma et al., 2022). The intensity of the band at 3283 cm^{-1} was related to the -OH stretch vibrations of water molecules. The band observed at 1648 cm^{-1} was associated to amide I vibrations of proteins, assigned to C−C stretch and N−H bending modes along with C−O stretch vibration of the peptide bond (Kaur & Gill, 2021). The band at 1529 cm^{-1} is associated to amide II vibrations of the protein, and C–N stretch vibrations were assigned with little C–O bending contributions and C–C and N–C stretch vibrations. The band at 1041 cm^{-1} corresponds to C-O-C stretch of glycosidic bonds, probably from carbohydrates (Zhou, Liang, Lü, Kuang, & Xia, 2022).

Amide *I* was found in the range of 1700–1600 cm^{-1} (Sofi et al., 2023) where there was a decrease in absorbance bands corresponding to EG3, EG5, and EG7 as compared to EUG (Fig. 3B). These bands belong to characteristic vibration frequencies that result from the secondary-structure overlapping of different polypeptide components. The specific bands 1667 cm^{-1}, 1658 cm^{-1}, 1630 and 1645 cm^{-1} of β-turn, α-helix, β-sheet and random coil, respectively, were identified in all samples and used for deconvolution and identification of corresponding areas (Carbonaro & Nucara, 2010).

Fig. 4(A – D) shows the curves obtained by deconvolution with 4 Gaussian peaks, where each peak has a specific wave number that corresponds to the structural components of amide I. Table 8 presents the areas of β-sheet, α-helix, and β-turn in the extrudates analyzed. There were changes in the areas of those components that constitute the secondary structure of the protein due to germination and extrusion.

The β-turn content in EUG was 4.05%, and it significantly increased ($p < 0.05$) to 9.18% in EG5. Still, the content was reduced to 1.76% in EG7 when the germination time increased. This reduction is the result of a decrease in carbonyl groups forming hydrogen bonds in β-turn, which is a consequence of the thermal treatment that produces conformational changes (Secundo & Guerrieri, 2005).

The β-sheet content in EUG was 47.90% and was significantly reduced to 43.37% in EG7. The germination process contributed to the modification of the secondary structure in the protein. There are reports on β-sheet reduction due to the breakage of hydrogen bonds in the component and turning into disordered structures (Xu et al., 2020). The proportion of α-helix in EUG was 23.67%, and it was significantly increased ($p < 0.05$) to 33.69% in EG7. This rise was also reported by Beck, Knoerzer, & Arcot, (2017) as a result of thermal treatment during extrusion. Finally, the proportion of random coil in EUG was 24.37% and a significant increase ($p < 0.05$) was observed in EG5, reaching a maximum of 30.84%; however, in EG7 it decreased significantly ($p < 0.05$) to 20.56% due to covalent cross-linking, hydrophobic and hydrogen bond interactions that originate during the extrusion process (Beck et al., 2017). The reduction in β-sheet can be related to the increase in protein digestibility since β-sheets restrict the access to proteolytic enzymes (Zahir, Fogliano, & Capuano, 2021). Sample EG7 presented the lowest β-sheet content and the highest digestibility percentage when compared to EG3 and EG5 (Table 7). The secondary structure of legume proteins has been related to alterations in protein digestibility due to protein transformation by simultaneous unfolding

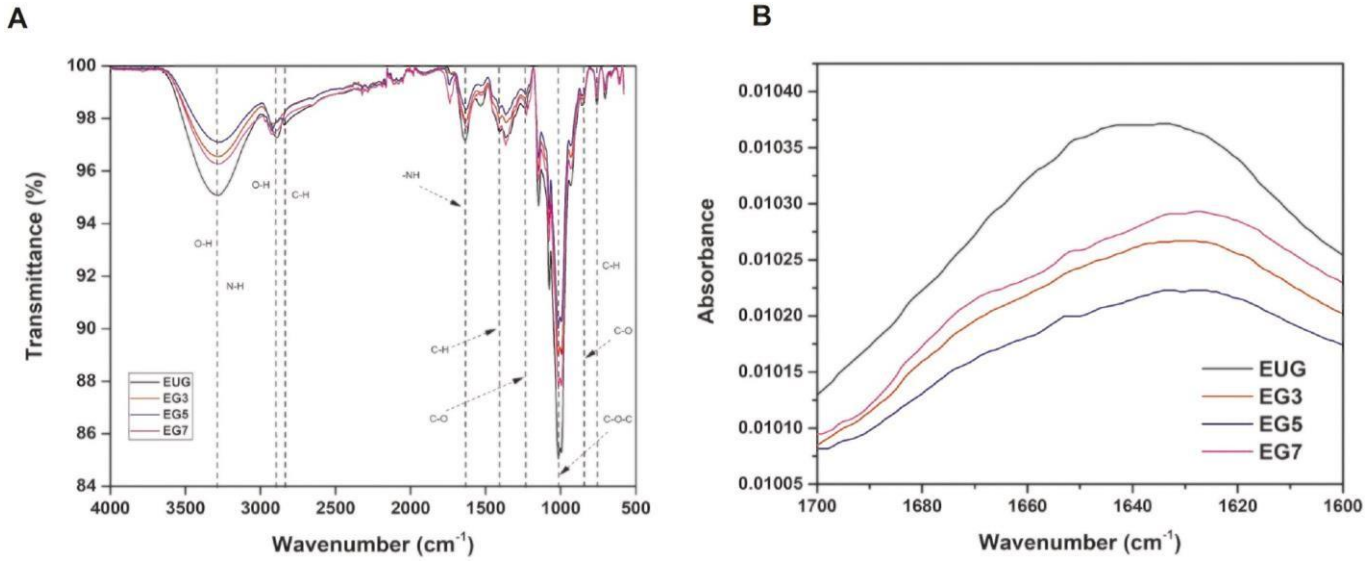

Fig. 3. A) Representative FT-IR spectrum of ungerminated and germinated extrudates. B) Spectrum of ungerminated and germinated extrudates in the amide I region (1700–1600 cm-1).

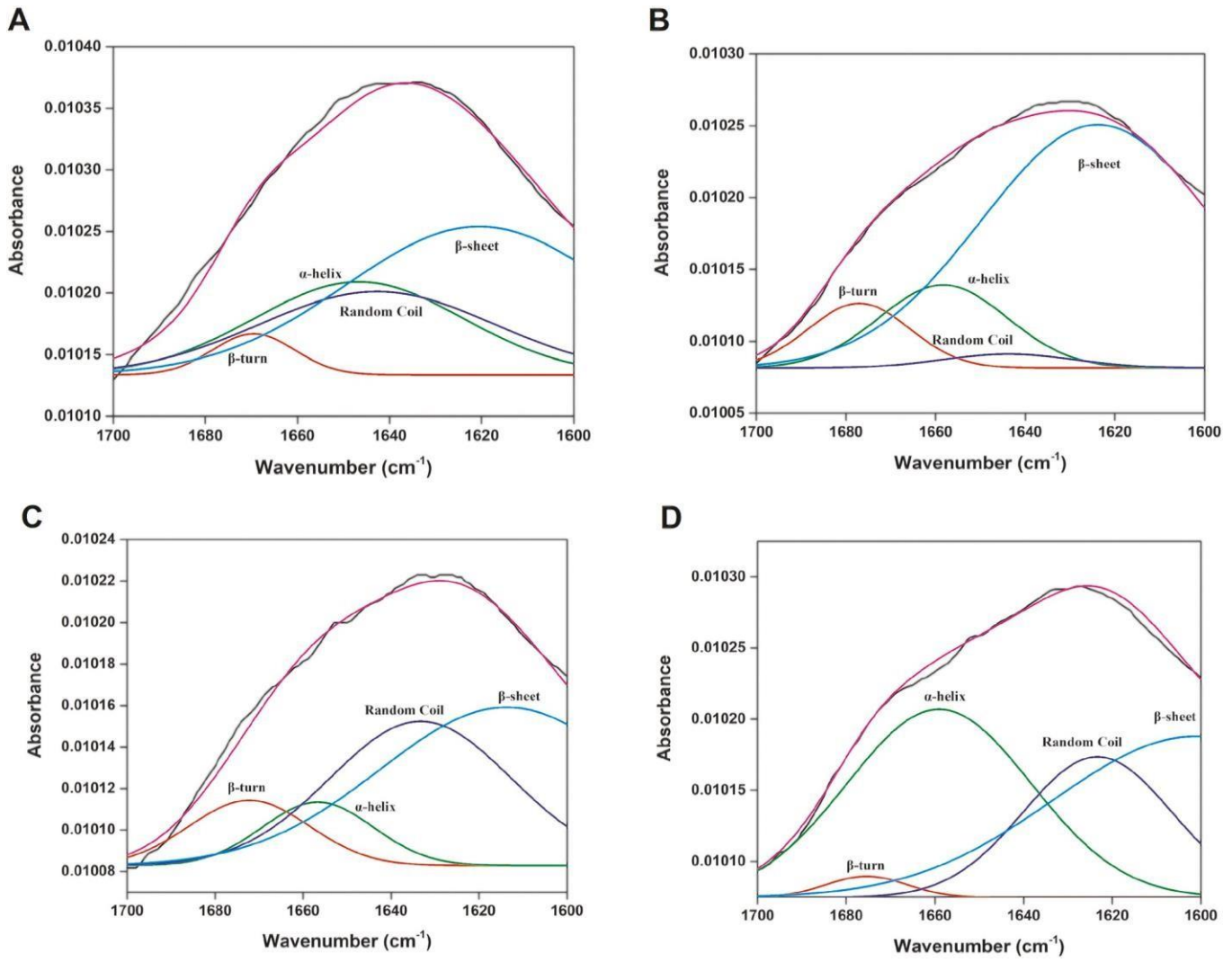

Fig. 4. Spectrum of amide I band deconvoluted of ungerminated and germinated extrudates. A) EUG, B) EG3, C) EG5, D) EG7.

Table 8

Relative proportion of components that constitute the secondary structure of extruded lupinus ungermination and germination for 3, 5 and 7 days.

Sample	β-turn (%)	β-sheet (%)	α-helix (%)	Random Coil (%)
EUG	4.05 ± 0.05^{b}	47.90 ± 0.42^{bc}	23.67 ± 0.09^{b}	24.37 ± 0.58^{b}
EG3	8.72 ± 0.07^{a}	53.04 ± 0.01^{a}	13.84 ± 0.16^{c}	24.14 ± 0.18^{b}
EG5	19.18 ± 0.70^{a}	50.84 ± 1.98^{ab}	9.11 ± 0.60^{d}	30.84 ± 1.93^{a}
EG7	1.76 ± 0.11^{c}	43.37 ± 1.46^{c}	33.69 ± 0.15^{a}	20.56 ± 0.33^{b}

Values were expressed as the average of triplicates ± standard deviation. Different superscript letters (a–c) in the same column indicate a significant difference using Tukey's test ($P < 0.05$). EUG = extruded with lupins flour ungerminating, EG3 = extruded with germinated of lupins by 3 days, EG5 = extruded with germinated of lupins by 5 days, EG7 = extruded with germinated of lupins by 7 days.

and simultaneous crosslinking during thermal treatment. This allows for a better access to digestive enzymes. There are also reports on the linear increase in protein digestibility and reduction in β structures in bean, chickpea, and soybean (Carbonaro, Maselli, & Nucara, 2012).

3.12. SEM

Fig. 5 shows the longitudinal cuts to evaluate the superficial part of the extruded. As observed, EUG showed a rough, uniform, compact, and homogeneous surface as a result of full starch gelatinization. A glue is produced between the proteins and matrix fiber, creating a more rigid superficial structure that prevents air exit and promoting expansion (Wang et al., 2019). Still, the use of germinated flours in extrusion modifies the microstructure. Irregular, heterogenous, and fractured structures were observed in EG3, EG5, and EG7. Sample EG7 presented more open cells in surface products as compared to EG5 and EG3, generating more open spaces on the surface that allow air to escape, limiting its retention in the internal part of the extrudate, which affects its expansion. This is likely due to the use of germinated flours with modified material that underwent hydrolytic alterations in macromolecules, mostly proteins, because of the enzymatic activity during germination. This activity hampers the interaction between proteins and starch during extrusion, and less compact, irregular and fractured surface. This agrees with the physicochemical parameters observed in EG7 (Table 6). The inclusion of germinated.

flour in extrudates improves the texture of the product.

4. Conclusions

The optimum extrusion conditions for the formation of extrudates from *Lupinus angustifolius* flour were: 16% feed moisture, 152 °C die temperature, and 47/53% lupin/corn starch proportion. Under these conditions, extrudates were obtained from flour germinated for 3, 5, and

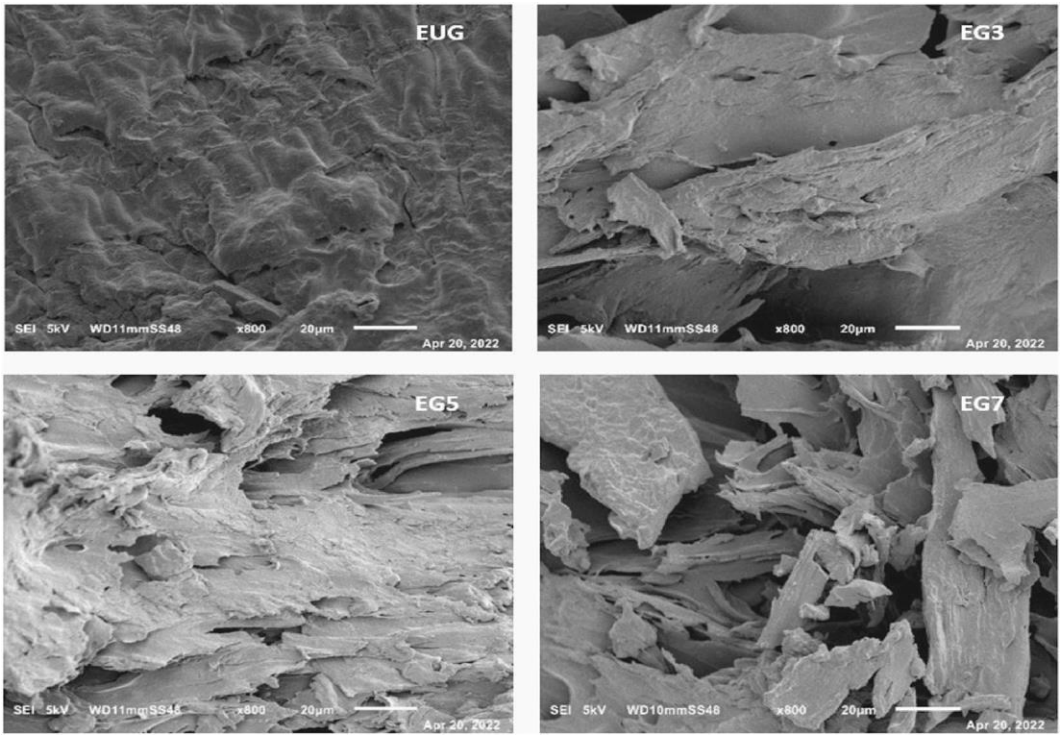

Fig. 5. Micrographs of the surface of ungerminated and germinated extrudates.

7 days and a higher protein content than when ungerminated flour was used. The energy value of extrudates from 5- and 7-day flour was lower. The concentration of total phenol and total flavonoid content was increased when using flour germinated for 3 days, but was reduced at 7 days of germination. The benefits of including germinated flour in extrusion were also observed in the absence of trypsin inhibitors in extrudates with 5- and 7-day germination flour and the reduction in phytates with 7-day flour. The FT-IR analysis evidenced molecular and structural changes, along with differences in the components of the protein secondary structure from germinated extrudates, which affected protein digestibility. The inclusion of germinated flour in extrudates is a potential alternative to obtain functional products with improved physicochemical and nutritional characteristics than the use of ungerminated flour.

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CRediT authorship contribution statement

Ciro B. Muñoz-Llandes: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft. **Heidi M. Palma-Rodríguez:** Methodology, Resources, Investigation. **Luis G. González-Olivares:** Software, Validation, Investigation. **Mirandeli Bautista-Ávila:** Investigation, Visualization. **Alma D. Román-Gutiérrez:** Methodology, Resources, Validation. **Carlos A. Gómez-Aldapa:** Methodology, Resources, Validation. **Fabiola A. Guzmán-Ortiz:** Conceptualization, Investigation, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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Capítulo 4 Incorporation of germinated lupin into corn-based extrudates: Focus on starch digestibility, matrix structure and physicochemical properties.

El cuarto capítulo de esta tesis doctoral consta de un artículo de investigación titulado *Incorporaration of germinated lupin into corn-based extrudates: Focus on starch digestibility, matrix structure and physicochemical properties*. Este trabajo fue publicado en la revista Food Chemistry de la editorial Elsevier con factor de impacto de 8.5. El objetivo de este trabajo de investigación fue determinar los cambios microestructurales en las botanas extrudidas elaboradas con harinas de Lupinus germinado a diferentes tiempos.

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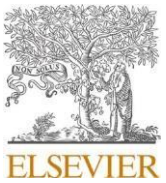

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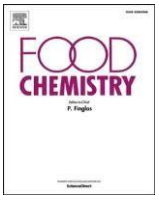

Incorporation of germinated lupin into corn-based extrudates: Focus on starch digestibility, matrix structure and physicochemical properties

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Viscosity and physicochemical properties

ABSTRACT

The research aimed to assess the effects of incorporating germinated *Lupinus angustifolius* flour into corn extrudates for different periods (3, 5, and 7 days), focusing on starch digestibility, morphological structure, thermal, and pasting properties. Extrudate with germinated lupinus flour for 7 days (EG7) significantly increased the content of slowly digestible starch up to 10.56% ($p < 0.05$). Crystallinity increased up to 20% in extrudates with germinated flour compared to extrudates with ungerminated flour (EUG), observing changes at the molecular level by FTIR that impact the thermal and pasting properties. X-ray diffraction revealed angles of 2θ = 11.31, 16.60, 19.91, and 33.04 as a result of the germination and extrusion processes. Microstructural analysis indicated starch-protein interactions influencing changes in calorimetry, viscosity, X-ray diffraction, and digestibility. PCA allowed establishing that the addition of germinated flours significantly affected the properties and microstructural characteristics of extruded products, potentially affecting digestibility and nutritional quality.

1. Introduction

Extruded products play an important role in modern nutrition, offering a unique combination of versatility, convenience, nutritional value, and opportunities for innovation in the food industry. One of the most commonly used polysaccharides in the development of extruded products is starch. During starch processing, non-starch polymers like lipids, fiber, and proteins interact with starch molecules, altering their structure and functionality (Wang et al., 2021). This interaction significantly modifies starch digestibility (Chen et al., 2019). The incorporation of protein sources, such as legumes, in extrusion processes can have a significant influence on the structure and quality of the extrudate. Various studies have developed products using extrusion technology with multiple legumes, such as beans, soy, lentils, chickpeas and peas, among others, as raw materials (Pasqualone et al., 2020; Zhang et al., 2023). However, germinated seeds, which are underutilized and have low consumption rates, are gaining popularity in the development and innovation of healthy products, because germination increases protein content, lowers the content of anti-nutritional compounds, enhances the digestibility of carbohydrates and proteins, and improves the bioavailability of vitamins, minerals, and amino acids (Avezum et al., 2023; Nongmaithem et al., 2024). The addition of germinated seed flours in the development of extruded products has already been reported as a viable alternative, focusing on increasing the phenolic compound content, antioxidant capacity, water solubility, porosity, and sensory attributes of the final product (Fayyaz et al., 2018; Krapf et al., 2019; Paucar-Menacho et al., 2022). Despite this, few studies have explored the use of germinated flours in the development of extruded products, highlighting the structural and physicochemical changes and transformations in the final product due to the effect of germination time. Lupin seeds are an

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important source of protein, which can be modified through germination (Guzmán-Ortiz et al., 2024). Proteins are hydrolyzed during germination by enzymatic action, exposing various residues such as charged groups, phosphate groups, hydrophilic, and hydrophobic groups capable of establishing interactions and modifying their functionality (Ohanenye et al., 2020). However, thermal treatments such as extrusion cooking promote interactions between proteins and starch because proteins denature and lose their structure, exposing new functional groups capable of interacting with starch (Girard & Awika, 2021). The interaction of partially hydrolyzed proteins due to germination effects with starch molecules during the extrusion process, as well as its impact on the microstructural transformation of the final product, have not been widely explored. Despite industrial interest in integrating plant proteins into starch-based foods, such as extruded products, there is a lack of studies on the effects of sprouted lupin flour in such products. In this study, we hypothesize that the incorporation of germinated *Lupinus angustifolius* flour in corn extrudates can significantly improve digestibility and matrix structure in the final product. Additionally, these changes could positively influence the thermal and pasting properties of the starch present in the extrudates, potentially benefiting the nutritional and textural quality of the final product. The use of methodologies such as Fourier-transform infrared (FTIR) spectra, Rapid Visco-Analyzer (RVA), X-ray diffraction (XRD), differential scanning calorimetry (DSC), and microscopy allows for a comprehensive understanding of the physicochemical and structural properties of the extrudate with incorporated germinated lupin flour. The objective was to investigate the effects of incorporating *Lupinus angustifolius* flour into corn extrudates for different periods (3, 5, and 7 days), focusing on starch digestibility, morphological structure, thermal, and pasting properties.

2. Materials and methods

2.1. Sample preparation and germinating

The University Center for Biological and Agricultural Sciences (CUCBA) of the University of Guadalajara, Mexico, provided the seeds of *Lupinus angustifolius* L. utilized in the present research. Cleaning and disinfecting a solution of sodium hypochlorite (0.07% *w/v*) and pure water were used on *Lupinus angustifolius* L. seeds. To encourage germination, the seeds were then immersed in sterile water (1:1 w/v) for a full day. For three, five, and seven days, germination was conducted at 26 °C in a germination chamber (LabTech) with a relative humidity of 65% (Guzmán-Ortiz et al., 2017). The lupin sprouts were dried in a Lab-Line conventional oven at 40 °C until they had about 8% moisture content. Next, the sprouts were pulverized in a rotor mill (TECNAL, model R-TE-651/2). A 35-mesh (0.50 mm) screen was used to sieved the flour. The flours were then kept in plastic bags and chilled after being defatted with petroleum ether to a 6% fat content.

2.2. Extrusion process

The extrusion process was conducted using both ungerminated and germinated lupin flours for 3, 5, and 7 days. The proportion of corn starch mixtures with germinating and ungerminated lupin and the extrusion conditions were determined according to previous studies (Muñoz-Llandes et al., 2023b), where the extrusion process was optimized. Four mixtures of defatted lupin (6% fat) with commercial corn starch (Mi Granero®) were made. The starch contained 12% moisture and 0% protein. The mixtures were made with ungerminated and germinated lupin flour in a proportion of 47/53%: ungerminated lupin flour/corn starch (EUG), germinated lupin flour for three days /corn starch (EG3), germinated lupin flour for five days/corn starch (EG5), and germinated lupin flour for seven days/corn starch (EG7). The mixes were hydrated to a moisture level of 16% twenty-four hours before the extrusion operation. A 1.2 kg/h feed rate was used with a single-screw extruder (type 20DN/8–235-00, Brabender Instruments Inc., CW, Germany). Three heating zones (90, 120, and 152 °C, respectively) were kept at constant temperature within the extruder. Using a die with a 3 mm diameter output and a screw with a 3:1 compression ratio, the screw speed was adjusted to 60 rpm. Following their milling in a rotor mill (model R-TE-651/2, TECNAL), the extrudates were sieved through a 0.42 mm screen. They were then kept in plastic bags until necessary.

2.3. In vitro starch digestibility

The *in vitro* starch digestibility of the extruded products was assessed using a slightly modified version of the Englyst et al. (1992) method. 400 mg of ground extrudate samples were put in centrifuge tubes, and 10 mL of deionized water was added to dissolve them. The tubes were kept for 20 min at 37 °C in a water bath with continuous agitation at 200 rpm. The mixture was then incubated for 30 min at 37 °C after 8 mL of porcine pancreatic α-amylase solution (28 units/mg, Sigma A3176) (5.21 mg/mL) was added. Following the addition of 8 mL of 0.5 M sodium acetate buffer (pH 5.2), the mixture was vortexed for one minute. Lastly, 4 mL of an enzyme solution containing 3260 units/mL of amyloglucosidase (Megazyme) was added. After 20, 120, and 240 min of reaction, 1 mL aliquots were obtained and mixed right away with 2 mL of 80% ethanol. The glucose oxidase-peroxidase reagent (Megazyme) was used to measure the amount of glucose present. The hydrolysis of starch was categorized as follows: rapidly digestible starch (RDS) is digested at 20 min, slowly digestible starch (SDS) at 120 min, and total digestible starch (TDS) at 240 min. These amounts are calculated employing the following formulas:

$$RDS = G_{20}{}^{*}0.9$$

$$SDS = (G_{120} - G_{20})^{*}0.9$$

$$TDS = (G_{240} - G_{20})^{*}0.9$$

$$RS = TS - TDS$$

where G_{20} is the amount of glucose released from hydrolysis at 20 min, G_{120} is the amount of glucose released from hydrolysis at 120 min, and G_{240} is the amount of glucose released from hydrolysis at 240 min. Resistant starch (RS) is represented by the difference between total starch (TS) which represents the total starch content in the samples and TDS. 0.9 is the conversion factor from glucose to starch.

2.4. Fourier-transform infrared (FTIR) spectra

Employing a Spectrum 2 spectrometer (Perkin-Elmer, USA), the molecular spectral information for each extruded product were obtained. At room temperature (27 °C), spectra were captured using a minimum of 12 scans at a 4 cm^{-1} resolution, covering the spectral range of 1200–800 cm^{-1}. The starch's crystalline and amorphous forms have been investigated in the infrared regions that correspond to 995, 1022, and 1047 cm^{-1} (Wang et al., 2015). Spectral deconvolution of the bands was performed from the original spectrum using curve fitting algorithms (Wang et al., 2014). The Origin software was used (Version 7.0, Microcal Inc., Northampton, MA, USA). The values for the ratio of absorbance R1047/1022 and R 1022/995 was calculated by spectral deconvolution by fitting a Gaussian holocrystalline peak in the region of 800–1200 cm^{-1}, which is divided into an amorphous and a crystalline region.

2.5. Pasting properties

The method described by Hagenimana and Ding (2005) was employed to determine the pasting characteristics of the extrudates using both germinated and ungerminated flour. Regarding the analysis, a Rapid Visco-Analyzer (RVA) 4500 (Perten Instrument, Sweden) was used. After dissolving the sample (3 g) in 25 mL of pure water, the suspension was put within an aluminum cylinder in the RVA. The

sample was heated for one minute at 50 °C and five minutes at 95 °C, at a rate of 6 °C/min between the two temperatures. The cooling phase was then carried out, with a 2-min hold period at 50 °C after the temperature was dropped from 95 °C to 50 °C at a rate of 6 °C/min. Centipoises (cP) were used to report the viscosity parameters. Pasting temperature, peak time, peak viscosity, breakdown viscosity, setback viscosity, holding viscosity, and final viscosity values were collected.

2.6. X-ray diffraction (XRD)

An X-ray diffractometer (EquinoX 2000, Inel) operating at 30 kV and 20 mA has been employed to obtain the X-ray diffraction patterns of the extrudates. Diffractograms were acquired at a scanning speed of 2.0°/min over a range of 4–110° (2θ). With Origin software version 10.5.1, relative crystallinity was determined using the method by Colussi et al. (2017).

2.7. Thermal properties

The differential scanning calorimeter (DSC Q2000, TA Instruments Inc., New Castle, DE, USA) method described by Biliaderis (1982) was utilized to ascertain the thermal properties of extrudates. Aluminum capsules (Perkin Elmer, B02190062, USA) containing 2 mg of the sample were sealed and hydrated with 7 μL of distilled water. After being hydrated, the extrudates were warmed at a rate of 10 °C per minute for 30 min at room temperature. The values of the gelatinization temperature range were measured. Onset temperature (To), peak temperature (Tp), conclusion temperature (Tc) °C, the gelatinization temperatures range (Tc - To) (ΔT), and enthalpy of gelatinization (ΔH).

2.8. Scanning electron microscopy (SEM)

According to Jafari et al. (2017), the extrudates were utilized to assess morphology employing scanning electron microscopy. The samples had a small layer of gold applied to them after they were placed on an aluminum plate. The samples coated with gold were then put inside the chamber of a scanning electron microscope (JSEM 35CX; Japan Electronic Optical Limited, Japan), and 30ø magnification photographs were taken. The measurements were carried out at a 15 kV acceleration voltage.

2.9. Confocal laser scanning microscopy (CLSM)

Following the methodology outlined by Ma et al. (2021), CLMS assessed the distribution of components (starch and proteins) in the extrudates, utilizing fluorochromes to visualize the distribution of macromolecules (Moisio et al., 2015). Protein staining was done with fluorescein-5-isothiocyanate (FITC), and carbohydrate labeling was done with rhodamine B. A multiphoton confocal microscope (LSM 710 NLO, Carl Zeiss, Oberkochen, Germany) with a 20ø objective was used to capture the images.

2.10. Statical analysis

Every analytical test was carried out three times. With IBM's SPSS 16.0 statistical software (USA), one-way ANOVA, means, and standard deviations (SD) (Tukey's honest significant difference test (HSD) were computed at a 95% confidence level ($p < 0.05$). The results were subjected to a Principal Components Analysis (PCA) using origin software version 10.5.1.

3. Results and discussion

3.1. In vitro starch digestibility

Table 1 shows the different fractions of *in vitro* starch digestion of the extruded products supplemented with ungerminated and germinated lupin flour at different times (EUG, EG3, EG5, and EG7). The total starch content (TS) in the extrudates ranged from 54.13% to 62.01%. The fraction corresponding to rapidly digestible starch (RDS) in EUG was 52.47% and raised significantly ($p < 0.05$) in EG3 to 61.43%; however, it decreased in EG5 and EG7. Due to the high temperatures that encourage gelatinization and the disintegration of starch granules brought on by the sheer force produced by the screw speed, the extrusion method makes starch more digestible (Kantrong et al., 2018). Nevertheless, this depends on the makeup of the raw materials. According to Cornejo-Ramírez et al. (2015), the activation of hydrolytic enzymes during germination may have caused structural modifications that changed the relationship between storage macromolecules like protein, fiber, and starch, favoring starch digestibility and resulting in the rise in RDS starch in EG3 (61.43%). Certain cereals and legumes exhibit a fluctuation in α-amylase activity during germination, which can be attributed to a range of factors, including variety, germination circumstances, seed genetics, and others. For instance, peak activity in peas has been documented around 4 days post-germination (Yang et al., 2021). This could explain the increase in RDS in the EG3 extrudate; presumably, the activity of enzymes such as α-amylase in lupin is higher at day 3 of germination than at 5 and 7 days. The fraction of slowly digestible starch (SDS) in EUG was 1.31%, and raised significantly ($p < 0.05$) was observed with the incorporation of germinated flours in the extruded products, reaching a maximum value of 10.56% in EG7. Muñoz-Llandes et al. (2022) have shown that *Lupinus angustifolius* seeds exhibit an increase in protein content during germination. This behavior could be the cause of the decline in the digestibility of starch, as during extrusion, higher protein and fiber content tends to trap starch granules within a viscous network, generating protein-starch-fiber interactions that favor the formation of slowly digestible starch. On the other hand, the total digestible starch (TDS) in EUG was 55.84%; however, it decreased significantly to 51.44% in EG5 ($p < 0.05$). According to Téllez-Morales, Herman-Lara, Gómez-Aldapa and Rodríguez-Miranda (2020), this decrease might be the result of crosslinking between starch granules and partially hydrolyzed macromolecules during germination, primarily proteins. This crosslinking creates aggregates, which limit the swelling and gelatinization of starch granules and, in turn, limit their interaction with enzymes during digestion. Additionally, it is important to note that phenolic compounds and flavonoids present in lupin flour could have a significant impact on TDS. A study by Muñoz-Llandes et al. (2023b) has shown that extrudates enriched with lupin sprouts exhibit a higher content of these compounds compared to extrudates made with ungerminated lupin flour. Phenolic compounds have the ability to form non-covalent interactions with starch, which might impact its

Table 1
In vitro starch digestibility and total starch of extruded snacks with ungerminated and germinated lupine flour.

Sample	TS (%)	RDS (%)	SDS (%)	TDS (%)	RS (%)
EUG	58.17 ± 1.06^{ab}	52.47 ± 0.07^{b}	1.31 ± 0.06^{d}	55.84 ± 0.84^{ab}	2.43 ± 0.08^{b}
EG3	62.01 ± 0.49^{a}	61.43 ± 0.04 ^{a}	9.22 ± 0.21^{b}	58.99 ± 0.14^{a}	2.88 ± 0.01^{a}
EG5	54.13 ± 1.15^{b}	46.96 ± 1.77^{c}	4.21 ± 0.07^{c}	51.44 ± 1.06^{c}	2.69 ± 0.08^{ab}
EG7	57.96 ± 1.17^{ab}	50.49 ± 1.23^{bc}	10.56 ± 0. 45^{a}	55.29 ± 1.09^{b}	2.66 ± 0.07^{ab}

Data are the average of three replicates ± standard deviation. Different superscript letters (a-d) in the same column indicate significant differences using Tukey's test ($p < 0.05$). Extruded added with ungerminated lupin flour (EUG); extruded added with germinated lupin flour for 3 days (EG3); extruded added with germinated lupin flour for 5 days (EG5); extruded added with germinated lupin flour for 7 days (EG7). Total starch (TS); Rapidly digestible starch (RDS); Slowly digestible starch (SDS); Total digestible starch (TDS) and Resistant Starch (RS).

digestibility. Hydrogen bonding, hydrophobic interactions, electrostatic interactions, and ionic interactions are some of these interactions (Zhu, 2015); this could explain the observed changes in starch digestibility of the extrudates supplemented with germinated lupin flours. The content of resistant starch (RS) in EUG was 2.43%, and it significantly increased with the use of flour germinated for 3 days, reaching a maximum of 2.88% in EG3. It has been demonstrated that the compact granular structure of starch is destroyed during the extrusion process, and its structure is changed into a porous one that can speed up starch digestion and reduce resistant starch (Gulzar et al., 2021). The addition of germinated flours resulted in an increase in resistant starch, which may be due to crosslinking and aggregate formation between starch and protein. This behavior was observed by Serrano-Sandoval et al. (2022) in corn extrudates added with germinated chickpea flour. Furthermore, moisture promotes starch retrogradation during extrusion, which raises the amount of resistant starch. Water functions as a plasticizer, lowering the starch's glass transition temperature and increasing the flexibility of the starch molecules to reorganize into double helices, which promotes the formation of resistant starch (Sun et al., 2019).

3.2. FTIR analysis

Fig. 1A displays the IR spectrum of extrudates with ungerminated and germinated flour. The region of 1200–800 cm^{-1} observed in Fig. 1A corresponds to fingerprint vibrations associated with C_C, C_O, and C—H stretching and is linked to specific wavelengths of starch structure. Spectral deconvolution was used in this investigation to determine the value absorbance ratios R 1047/1022 and R 1022/995 (Wang et al., 2014). The crystalline and amorphous regions of starch was investigated using the IR spectrum in order to determine whether adding germinated lupin flour to maize extrudates would change these aspects. In accordance with Wang et al. (2015), this range can be described by three main modes with maximum absorbance at 1047, 1022, and 995 cm^{-1}. The band at 1047 cm^{-1} is related to the ordered regions in starch. The 1022 cm^{-1} absorbance band is a result of absorption by stretching modes in amorphous starch, and finally, the band at 995 cm^{-1} results from bonding in hydrated carbohydrate helices (Monroy et al., 2018). These bands were identified in all the analyzed samples. Additionally, spectral bands deconvolution (Fig. 1B) revealed that the areas of individual bands underwent modifications in their intensity when germinated lupin flours were used, indicating changes at the molecular level. The absorbance ratio 1022/995 cm^{-1} is related to the amorphous region, while the intensity ratio 1047/1022 cm^{-1} is associated with the crystalline region (Lu et al., 2021). These ratios were calculated in all samples and shown in Table 2.

Table 2
The ratio of 1047/1022 cm^{-1} and 1022/995 cm^{-1} bands in the FTIR spectra and relative crystallinity by XRD.

Sample	IR ratio 1047/1022 (cm^{-1})	IR ratio 1022/995 (cm^{-1})	Relative crystallinity (%)
EUG	0.84 ± 0.049^{b}	1.54 ± 0.095^{a}	7.24 ± 0.01^{b}
EG3	1.80 ± 0.009^{a}	1.06 ± 0.006^{b}	9.20 ± 0.21^{a}
EG5	1.84 ± 0.001^{a}	1.01 ± 0.075^{b}	8.89 ± 0.38^{a}
EG7	1.87 ± 0.008^{a}	0.98 ± 0.005^{b}	8.35 ± 0.11^{a}

Data are the average of three replicates ± standard deviation. Different superscript (a-b) letters in the same column indicate significant differences using Tukey's test ($P < 0.05$). Extruded added with ungerminated lupin flour (EUG); extruded added with germinated lupin flour for 3 days (EG3); extruded added with germinated lupin flour for 5 days (EG5); extruded added with germinated lupin flour for 7 days (EG7).

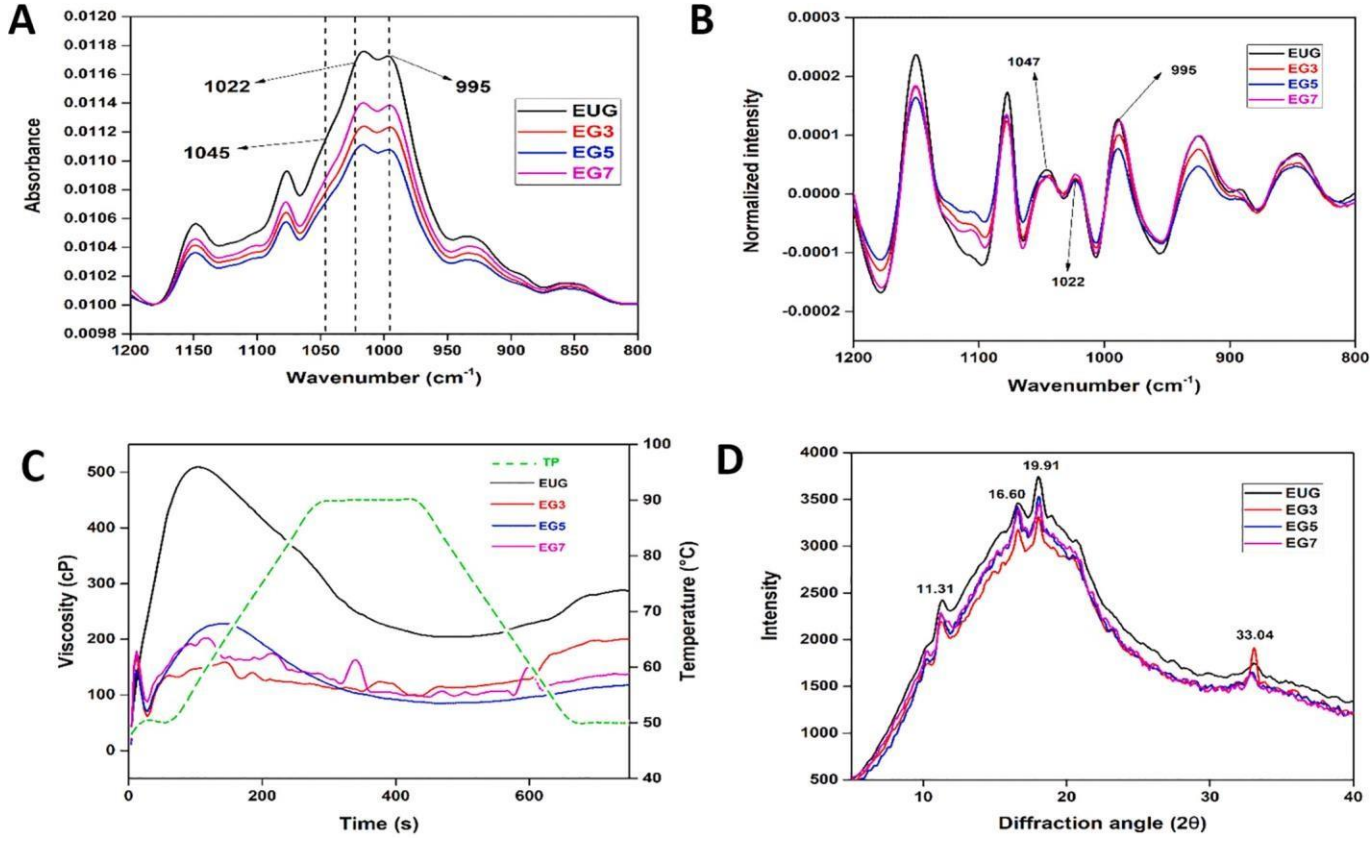

Fig. 1. (A) IR spectrum, (B) IR spectrum deconvoluted, (C) Pasting properties of extruded snacks and (D) XRD patterns of extruded add ed with ungerminated lupin flour (EUG); extruded added with germinated lupin flour for 3 days (EG3); extruded added with g erminated lupin flour for 5 days (EG5); extruded added with germinated lupin flour for 7 days (EG7); TP: temperature profile.

The addition of germinated flours caused significant effects on the crystallinity of the extrudates. EUG had values for the ratio of absorbance 1047/1022 cm^{-1} of 0.84, which significantly increased in extrudates with germinated flour, reaching a maximum of 1.87 in EG7 ($p < 0.05$). This trend indicates an increase in the crystallinity of the extruded product. The increase in crystallinity observed in the extrudates was also evident in the X-ray studies, as a result of the addition of germinated lupinus.

The values absorbance ratio 1022/995 cm^{-1} in EUG was 1.54 and significantly decreased due to the addition of germinated flours, reaching a minimum of 0.98 in EG7 ($p < 0.05$). This behavior may indicate a decrease in the proportion of the amorphous region of starch. These results demonstrate that the inclusion of germinated lupin flour in the extruded product changed the crystalline and amorphous regions of starch. Similar trends were reported by de la Rosa-Millán et al. (2019) in extruded sprouted black bean flours. These differences are attributed to the partial depolymerization of starch and proteins as a consequence of the mechanical damage during extrusion or due to germination process. The alterations in protein during germination most likely promote contact with starch through non-covalent bonds that better retain the double helices; this behavior is comparable to Yu et al. (2022), who reported in rice extrudates that contained glutelin. According to earlier research, the extrusion method modifies the secondary structure of germinating lupin flour protein, increasing the amounts of random coil and α-helix (Muñoz-Llandes et al., 2023b). It is possible that throughout the extrusion process, these proportions are interacting with corn starch more consistently, which could be contributing to the increase in the fraction of slowly digestible starch, as observed in Table 1.

3.3. Pasting properties

The RVA is related to alterations and structural transformations brought about by heat processing and enables the determination of starch viscosity properties. Fig. 1C shows the pasting curves of extrudates added with germinated and ungerminated lupin flour. Table 3 displays the viscosity values of the analyzed extruded products. When the leaching and swelling of starch granules of polymers like amylose are balanced, a rise in viscosity known as peak viscosity is produced (Muñoz-Llandes et al., 2023a). The EUG extrudate exhibited the highest peak viscosity values of 505.5 cP, significantly different from extrudates with germinated lupin flour ($p < 0.05$). EG3 showed the lowest value of 160 cP among the extrudates with germinated flour. Multiple variables, including the distribution and length of the amylopectin branching chain and the existence of other constituents such as protein hydrolysates, lipids, and fiber, limit the union of starch to water molecules, affecting viscosity (Huong et al., 2021; Yang et al., 2023). The decrease in peak viscosity in EG3, EG5, and EG7 may be as a result of having more protein in the lupin flours as a result of germination, resulting in increased interaction with starch granules during extrusion, which limits the water absorption capacity and swelling of starch granules. The stability of the sample under shear and applied temperature is shown by breakdown viscosity (Palabiyik et al., 2016). EUG obtained a value of 302.5 cP in breakdown viscosity, and a significant decrease was observed down to 65 cP in EG3 ($p < 0.05$), indicating greater stability in EUG. When starch granules break, indicating a reduction in viscosity, holding viscosity is the lowest viscosity reached after continued agitation and heating (Wani & Kumar, 2016). EUG obtained a value of 205 cP; however, a significant decrease was observed with the addition of germinated lupin flours, reaching a minimum of 88 cP in EG5, and subsequently increasing significantly to 95.5 cP. This might be the result of interactions between proteins and carbohydrates and amylose chain alignment, which would suggest the presence of these complexes (Iuga & Mironeasa, 2020). On the contrary, setback viscosity reveals that the viscosity of starch suspension rebounds during cooling after heating (Kaushal et al., 2012). A decrease in setback viscosity values demonstrates a low rate of syneresis and starch retrogradation in extruded products (Boahemaa et al., 2024). EUG and EG3 showed the highest setback viscosity values of 86.5 cP and 106.5 cP, respectively. However, in EG5 and EG7, a significant lower ($p < 0.05$) was observed to 31 cP and 45.5 cP, respectively, indicating that the incorporation of 5- and 7-day germinated flours allows for snacks less prone to retrogradation. Final viscosity, is the ability of the extruded product to form a viscous paste after the heating and cooling process (Mendes et al., 2023). It was observed that EUG had a final viscosity of 291.50 cP, and a significant lower ($p < 0.05$) was observed upon adding germinated flours to the extruded product. EG5 showed a minimum of 112.5 cP. This might be the result of partial hydrolysis of macromolecules during germination, mechanical and heat degradation during extrusion, or a decreased polymerization degree during extrusion, which would reduce viscosity (Jacques-Fajardo et al., 2017). Pasting temperature is the lowest temperature needed for starch to gelatinize and to enhance viscosity when heated (Dhaka & Khatkar, 2015). EUG showed the highest pasting temperature of 86.55 °C and significantly decreased ($p < 0.05$) with respect to germination time, reaching a minimum of 61.63 °C in EG7. This indicates that a lower temperature is required to achieve starch gelatinization in extruded products with germinated flours due to the modification by the germination effect on the concentration of the different macromolecules constituting the analyzed sample (de la Rosa-Millán et al., 2019). Finally, significant differences ($p < 0.05$) were observed in the peak time of the different extruded products. EUG reached maximum viscosity at 1.75 min. In EG3 and EG7, a significant increase ($p < 0.05$) in the time required to reach maximum viscosity was observed, as it was 2.37 min, which can be attributed to a lower rate of absorption and swelling of starch granules. The disruption of granular structures, leaching of amylose, and breakdown of starch hierarchical structures (amylose and amylopectin) during the germination process could be the cause of the variation in viscosity qualities (Qiao et al., 2019). However, as the mixture moves through the extruder, the structural alterations brought about by germination in each of the flours may influence the mixture's flow resistance. According to Yang et al. (2023), viscosities decrease with protein hydrolysates. Furthermore, the content of phenolic compounds increases during germination (Li et al., 2023). These compounds can compete with starch for water, which can affect the starch's water absorption and degree of gelatinization during extrusion, reducing the viscosity parameters (Shi et al., 2022; Zhu, 2015). Moreover, the hydroxyl groups of polyphenols can also interact with starch, modifying its hydration properties (Zhu, 2015). Additionally, during extrusion, there is a loss in the crystalline region of starch

Table 3
Pasting properties of extruded snacks with ungerminated and germinated lupin flour.

Sample	Peak viscosity (cP)	Breakdown viscosity (cP)	Holding viscosity (cP)	Setback viscosity (cP)	Final viscosity (cP)	Pasting temperature (°C)	Peak time (min)
EUG	505.5 ± 4.95^{a}	302.5 ± 3.54^{a}	205 ± 1.41^{a}	86.5 ± 4.95^{a}	291.50 ± 3.54^{a}	86.55 ± 2.19^{a}	1.75 ± 0.03^{c}
EG3	160 ± 1.41^{d}	65 ± 2.83^{d}	100.5 ± 3.54^{b}	106.5 ± 9.19^{a}	201 ± 4.24^{b}	70.94 ± 0.85^{b}	2.37 ± 0.05^{a}
EG5	229 ± 2.83^{b}	149.5 ± 4.95^{b}	88 ± 4.24^{c}	31 ± 2.83^{b}	112.5 ± 7.78^{c}	66.66 ± 0.93^{bc}	2.37 ± 0.04^{a}
EG7	214.5 ± 0.70^{c}	126 ± 7.07^{c}	95.5 ± 2.12^{bc}	45.5 ± 2.12^{b}	135 ± 8.49^{c}	61.63 ± 1.59^{c}	2.04 ± 0.04^{b}

Data are the average of three replicates ± standard deviation. Different superscript letters (a-c) in the same column indicate significant differences using Tukey's test ($p < 0.05$). Extruded added with ungerminated lupin flour (EUG); extruded added with germinated lupin flour for 3 days (EG3); extruded added with germinated lupin flour for 5 days (EG5); extruded added with germinated lupin flour for 7 days (EG7).

contributing to the decrease in viscosities (Bresciani et al., 2021). Many factors, including the amount of starch, the molecular structure and order of the degree of crystallinity, starch granules, and the length of the amylose chain, are primarily responsible for the variations in the pasting qualities of the extruded products under analysis (Kharat et al., 2019). However, Li et al. (2020) reported a decrease in viscosities due to differences in protein composition; this could explain the observed changes in the analyzed products since the germination of lupins modifies the structure and composition of the protein (Guzmán-Ortiz et al., 2024; Muñoz-Llandes et al., 2022).

3.4. XRD Analysis

The chains of amylose and amylopectin are organized in a semi-crystalline structure to produce a matrix of starch granules. This structure can be shown by diffracting X-rays.

Starch granules, depending on their botanical source, can be classified as a series of continuous crystalline structures of types A, B, and C (Xiao et al., 2020). According to Wang et al. (2021), corn starch corresponds to type A, and in its X-ray diffraction pattern, it exhibits characteristic peaks at 15°, 17°, 18°, and 23°; however, as shown in Fig. 1D, the extruded products added with non-germinated and germinated lupine flour evidenced peaks at 2θ 11.31, 16.60, 19.91, and 33.04, as a result of modifications by germination and the extrusion process. The relative crystallinity percentage was determined from each diffractogram for each sample (Table 3), observing a value of 7.24 in EUG, which significantly increased with the addition of germinated flours ($p < 0.05$). This is consistent with the results obtained in IR by increasing the ratio of 1047/1022 cm^{-1} in EG3, EG5, and EG7. The extrusion process destroys the double helices of amylopectin, while some of the available lipids can form helical inclusion complexes with amylose molecules, thus modifying the X-ray diffraction pattern. However, there are other factors that impact the structural modification of starch granules. Jafari et al. (2017) reported that a decrease in moisture content and a raised in the exit die temperature in the extruder have a negative effect on starch crystallinity. Limited moisture increases the intensity of shear force, leading to the mechanical breakdown of starch crystallinity through the rupture of molecular bonds. Additionally, the high temperatures used in the extrusion process further promote this behavior. However, contrary to these findings, the results obtained in this study showed an increase in crystallinity due to the use of germinated flours, which was also reflected in starch digestibility, as crystalline regions are less accessible to digestive enzymes. These results are also supported by those obtained from FTIR, which may be attributed to the partial hydrolysis of macromolecules during germination and subsequent polymerization during the extrusion process. Rolandelli et al. (2020) reported an increase in crystallinity in sorghum and quinoa extrudates, attributed to a higher fiber content. However, this behavior will depend on the amylose/amylopectin ratio as well as extrusion conditions, primarily temperature, feed moisture, and compression ratio (Ali et al., 2020).

3.5. Thermal properties

The size and microstructure of the granules, the ratio and concentration of amylose to amylopectin, the crystalline structure, and the starch content of the examined samples all influence the thermal characteristics of starch (Cai et al., 2014). Table 4 shows the gelatinization temperatures for the different extruded products, which allow for characterization of the thermal stability of the starch. The addition of germinated lupin evidences a significant increase ($p < 0.05$) in the gelatinization temperatures (To, Tp, and Tc) as a result of a higher degree of molecular degradation originating during extrusion due to mechanical damage and the high temperatures used. EUG exhibited the highest peak temperature (Tp), with a value of 102.90 °C, without significant differences observed with the EG3 and EG5 samples ($p < 0.05$). However, in the EG7, a significant decrease ($p < 0.05$) was observed as a result of the structural changes in starch, which is consistent with the changes in bands shown in the FTIR studies. The microstructural changes generated by germination in lupin flour impact its composition, affecting the rheology during extrusion and consequently leading to differences in the gelatinization temperatures of the extruded products. According to Gutiérrez-Osnaya et al. (2020), the buildup of simple sugars from starch degradation by enzymatic action during germination caused the temperature of germinated barley to increase. Additionally, amino acids from protein hydrolysis also contributed to this increase in temperature (Xu et al., 2020). This behavior has also been reported in germinated rice, oats, sorghum, and millet for 48 h (Li et al., 2017). On the other hand, EUG exhibited ΔH values of 0.25 (J/g) without significant difference from EG3 ($p > 0.05$); however, a significant increase was observed in EG5 of up to 0.47 (J/g) without showing significant differences with EG7 ($p > 0.05$). The double helices generated by the neighboring outer branches of the amylopectin chains, which unravel and melt during gelatinization, appear to be significantly connected based on the enthalpy of gelatinization of starchy products (Anantha-narayan et al., 2018). This suggests that the addition of germinated lupin flours for a longer period allows for greater interaction between molecules, generating changes in matrix structure of the final product, as evidenced in FTIR and XRD.

Table 4
Thermal properties of extruded snacks with ungerminated and germinated lupin flour.

Sample	*To* (°C)	*Tp* (°C)	*Tc* (°C)	*ΔT* (°C)	*ΔH* (J/g)
EUG	97.99 ± 0.37^{c}	102.90 ± 0.02^{a}	107.61 ± 0.69^{a}	9.61 ± 0.31^{a}	0.25 ± 0.01^{b}
EG3	100.22 ± 0.19^{b}	102.91 ± 0.04^{a}	105.49 ± 0.19^{b}	5.27 ± 0.07^{b}	0.24 ± 0.01^{b}
EG5	101.13 ± 0.09^{a}	102.86 ± 0.07^{ab}	107.04 ± 0.34^{ab}	5.91 ± 0.43^{b}	0.47 ± 0.02^{a}
EG7	100.3 ± 0.01^{ab}	102.70 ± 0.02^{b}	105.74 ± 0.1^{b}	5.42 ± 0.11^{b}	0.45 ± 0.01^{a}

Data are the average of three replicates ± standard deviation. Different superscript (a-c) letters in the same column indicate significant differences using Tukey's test ($P < 0.05$). Extruded added with ungerminated lupin flour (EUG); extruded added with germinated lupinus flour for 3 days (EG3); extruded added with germinated lupin flour for 5 days (EG5); extruded added with germinated lupin flour for 7 days (EG7). Onset temperature of gelatinization (*To)*; peak temperature of gelatinization (*Tp*); conclusion temperature of gelatinization (*Tc*); the gelatinization temperatures range (Tc - To) (*ΔT)*; and the enthalpy changes of gelatinization (*ΔH*).

3.6. Microstructural analysis

3.6.1. SEM

Scanning electron microscopy allowed the analysis of microstructure (Fig. 2) and the observation of possible changes and interactions between corn starch granules and other components, such as protein, due to the addition of germinated lupin flours during the extrusion cooking. It was observed that in the micrographs of EUG and germinated extruded products (EG3, EG5, and EG7), there is no evidence of intact starch granules, meaning that the corn starch used in the blends is completely melted and plasticized, generating amorphous structures due to gelatinization and dextrinization caused by the high temperature and pressure involved in extrusion (Tovar-Jiménez et al., 2015). The cooking of the material used as a result of extrusion led to the formation of disaggregated particles due to the leaching of amylose from the starch granules. This amylose leaching acts as an adhesive material to favor the formation of particles and larger aggregates resulting from the association between proteins and starch (Dura et al., 2014; Jafari et al., 2017). The use of germinated flours during the extrusion process favored the formation of aggregates with thick block-like structures that were heterogeneous and irregular, resulting from the mixture of protein, fiber, and gelatinized starch. A comparable behavior was mentioned by Raza

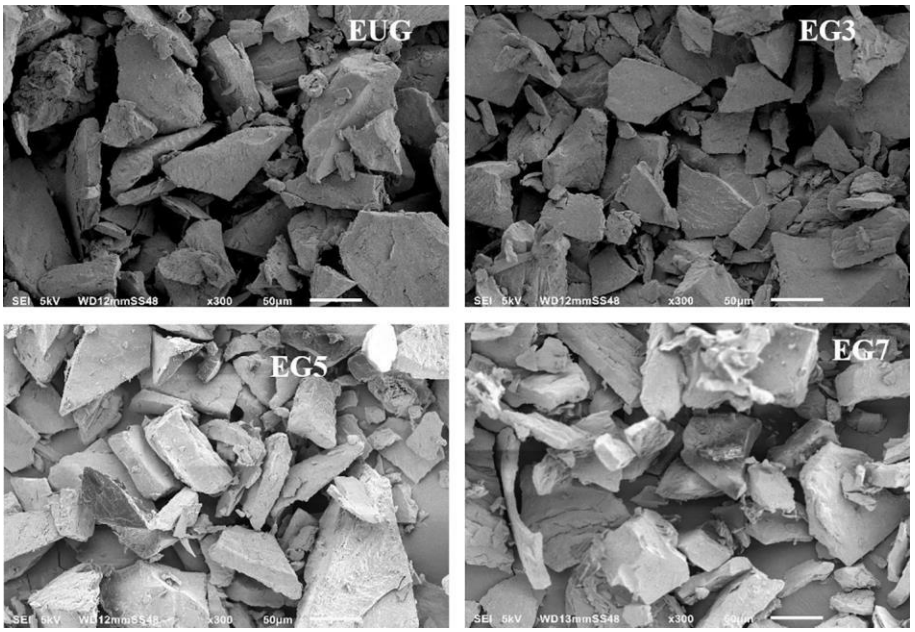

Fig. 2. Scanning electron microscope images of extruded snacks. Extruded added with ungerminated lupin flour (EUG); extruded added with germinated lupin flour for 3 days (EG3); extruded added with germinated lupin flour for 5 days (EG5); extruded added with germinated lupin flour for 7 days (EG7).

et al. (2019) in pea extrudates, where this type of structural morphology originates from a high degree of cross-linking between proteins and starches. The presence of protein from lupin caused the formation of clusters, and the addition of moisture in extrusion enhanced its binding to starch; moreover, the adhesion between starch granules also contributed to the formation of clusters (Yu et al., 2023). The binding of proteins to starch after extrusion could be visualized more clearly with CLSM.

3.6.2. CLSM

The confocal microscopy images of the samples are shown in Fig. 3. Carbohydrates, mainly starch, are observed in red, while proteins are shown in green. Carbohydrates were predominant in all samples due to the proportion of starch used (53% corn starch, 47% lupin). As seen in the SEM micrographs (Fig. 2), complete fusion of starch granules is observed in all samples as a result of cooking due to the extrusion conditions, with no intact granules present, as observed in the DSC results. A comparable behavior was reported by Rolandelli et al. (2021) in corn and sorghum extrudates. In the EUG extrudates, protein aggregates

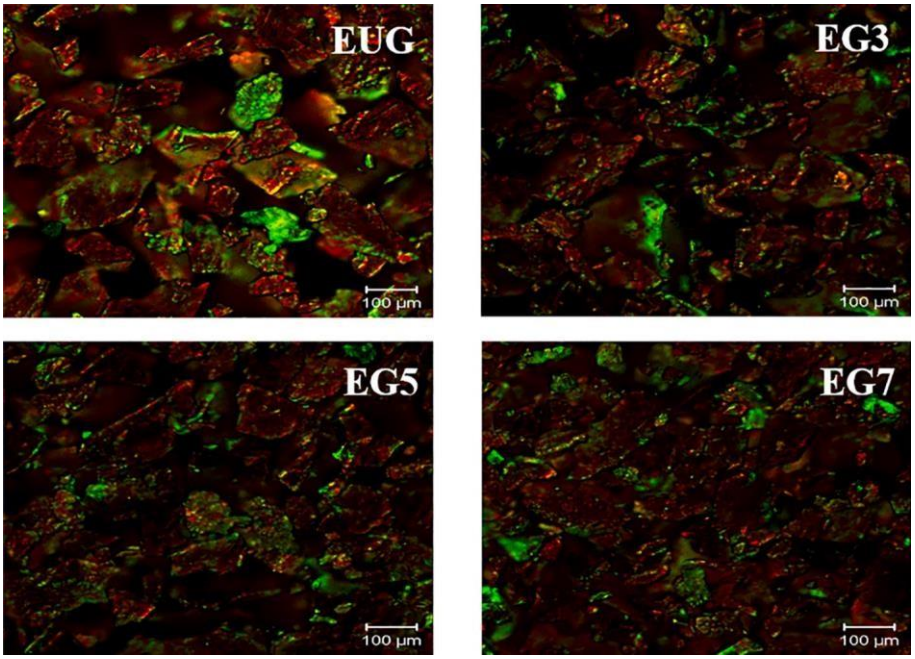

Fig. 3. CLSM micrographs of extruded snacks. Protein staining with fluorescein-5-isothiocyanate (green), and carbohydrate with rhodamine B (red). Extruded added with ungerminated lupin flour (EUG); extruded added with germinated lupin flour for 3 days (EG3); extruded added with germinated lupin flour for 5 days (EG5); extruded added with germinated lupin flour for 7 days (EG7). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were identified, which were more exposed on the surface compared to extrudates made with germinated flours. These protein aggregates are generated as a result of disruption, denaturation, and cross-linking of disulfide bonds between proteins due to the high pressures, temperatures, and mechanical forces employed during the process. This result is in agreement with previous results observed by Ramos-Diaz et al. (2019) in amaranth and quinoa extrudates, where protein conglomerates were also detected, contributing to a final product texture that was brittle and crunchy. In the extrudates with germinated lupin flour (EG3, EG5, and EG7), it was observed that proteins were embedded in the gelatinized starch (Fig. 3). The distribution of proteins increased uniformly and homogeneously along the starch network, thus promoting the formation of a continuous matrix after the extrusion process. This behavior can be attributed to the germination process of lupin seeds, during which reserve proteins are hydrolyzed by enzymatic action, which modifies their structure and affects their subsequent distribution after the extrusion process (Muñoz-Llandes et al., 2023b). A similar phenomenon of homogeneous and uniform distribution of proteins after the extrusion process was observed by Rolandelli et al. (2021) in corn extrudates enriched with red sorghum. This starch and protein distribution observed with CLSM within the extrudate can explain the changes in starch digestibility in the extrudates, as protein embedding in the starch may hinder contact between amylase and particles, thereby reducing the contact site of digestive enzymes (Cao et al., 2023). Furthermore, reports have stated that protein aggregates can occupy spaces in the matrix more efficiently, limiting starch digestion (Huang et al., 2022; Yang et al., 2021), which could also explain the decrease in digestibility observed in extrudates with germinated lupin flour.

3.7. Principal component analysis

To compare the main characteristics of the extrudates developed, a PCA was carried out considering the microstructural data obtained as well as thermal and pasting properties (Fig. 4). The first principal component (PC1) explained 72.42% of the data variation, while the second principal component (PC2) explained 19.20%, achieving a total variation of 91.62% between PC1 and PC2. The extruded products added with germinated lupin flours (EG3, EG5, and EG7) are located in a different quadrant of the plane compared to the extruded with ungerminated flour (EUG), indicating that the addition of germinated flours significantly affected the properties and microstructural characteristics of extruded products. For example, EG7, EG5, and EG3 are highly characterized by higher crystallinity, while EUG is characterized by a higher pasting temperature and peak viscosity. Thus, the PCA allowed us to demonstrate the changes generated by the addition of germinated lupin flours at different times.

4. Conclusion

This study demonstrated how adding germinated lupinus flour affected the extruded samples. The extruded products made with flour from germinated *Lupinus angustifolius* L. presented higher SDS and RS content compared to that extruded with ungerminated flour; changes were observed at the molecular level generating a significant increase in relative crystallinity, these results were evidenced by FTIR and XRD. Through CLSM it is evident that using germinated flours during the extrusion process, generates a greater distribution of lupinus proteins through the starch network, limiting the formation of larger protein aggregates. These changes impacted pasting and thermal properties, showing an increase in enthalpy and a decrease in viscosities. Principal component analysis has revealed that the days of germination of lupinus flours in the production of extrudates result in significant changes in the rheological, thermal and structural properties of the product, which in turn impact the digestion rate. This could have important implications for the food industry, as it could lead to the development of products with greater health benefits for consumers.

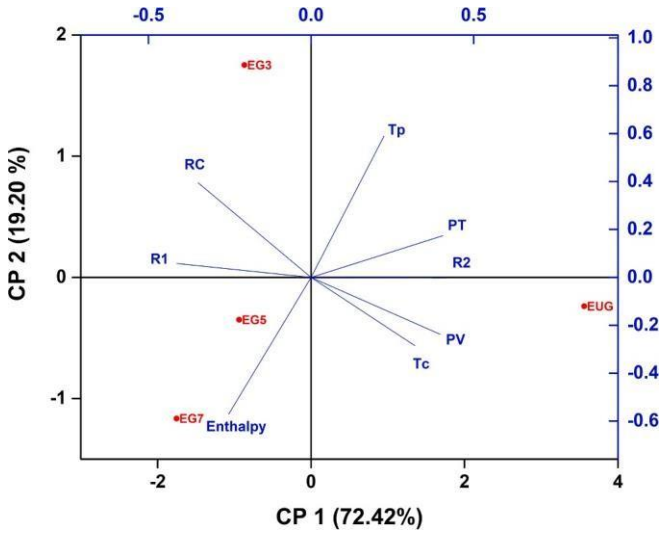

Fig. 4. Principal component plot of descriptive analysis of the extruded samples. Extruded added with ungerminated lupine flour (EUG); extruded added with germinated lupine flour for 3 days (EG3); extruded added with germinated lupine flour for 5 days (EG5); extruded added with germinated lupine flour for 7 days (EG7). relative crystallinity (RC); peak temperature (Tp); conclusion temperature (Tc); pasting temperature (PT); peak viscosity (PV); ratio 1047/1022 cm^{-1} (R1); ratio 1022/995 cm^{-1} (R2).

CRediT authorship contribution statement

Ciro Baruchs Muñoz-Llandes: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Heidi María Palma-Rodríguez:** Resources, Methodology, Formal analysis, Conceptualization. **María de Jesús Perea-Flores:** Resources, Methodology, Formal analysis. **Cristina Martínez-Villaluenga:** Writing – review & editing, Visualization, Methodology, Investigation. **Javier Castro-Rosas:** Methodology, Investigation. **Rene Salgado-Delgado:** Resources, Methodology. **Fabiola Araceli Guzmán-Ortiz:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Capítulo 5 Time matters: Exploring the dynamics of bioactive compounds content, bioaccessibility and antioxidant activity during *Lupinus angustifolius* germination.

El quinto capítulo de esta tesis doctoral consta de un artículo de investigación titulado *Time matters: Exploring the dynamics of bioactive compounds content, bioaccessibility and antioxidant activity during Lupinus angustifolius germination*. Este trabajo fue publicado en la revista Food Research International de la editorial Elsevier con factor de impacto de 7.0. El objetivo de este trabajo de investigación fue determinar el cambio en el perfil fenólico con respecto al tiempo de germinación y evidenciar la bioaccesibilidad de estos compuestos después de la simulación de la digestión gastrointestinal *in vitro*.

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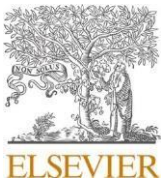

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Time maters: Exploring the dynamics of bioactive compounds content, bioaccessibility and antioxidant activity during *Lupinus angustifolius* germination

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ABSTRACT

Germination is a process that enhances the content of health-promoting secondary metabolites. However, the bioaccessibility of these compounds depends on their stability and solubility throughout the gastrointestinal tract. The study aimed to explore how germination time influences the content and bioaccessibility of γ-aminobutyric acid and polyphenols and antioxidant capacity of lupin (*Lupinus angustifolius* L.) sprouts during simulated gastrointestinal digestion. Gamma-aminobutyric acid showed a decrease following gastrointestinal digestion (GID) whereas phenolic acids and flavonoids exhibited bioaccessibilities of up to 82.56 and 114.20%, respectively. Although the digestion process affected the profile of phenolic acids and flavonoids, certain isoflavonoids identified in 7-day sprouts (G7) showed resistance to GID. Germination not only favored antioxidant activity but also resulted in germinated samples exhibiting greater antioxidant properties than ungerminated counter parts after GID. Intestinal digests from G7 did not show cytotoxicity in RAW 264.7 macrophages, and notably, they showed an outstanding ability to inhibit the production of reactive oxygen species. This suggests potential benefit in mitigating oxidative stress. These findings contribute to understand the dynamic interplay between bioprocessing and digestion in modulating the bioaccessibility of bioactive compounds in lupin, thereby impacting health.

1. Introduction

Currently, there is a global interest in enhancing and maintaining a healthy lifestyle through the consumption of foods rich in bioactive compounds, aiming to prevent various chronic-degenerative diseases associated with oxidative stress (Naliyadhara et al., 2023). Seed sprouts are gaining attention due to their high content of flavonoids, phenolic acids, anthocyanins, and vitamins, which provide various health benefits (Waliat et al., 2023).

Germination involves biochemical changes and transformations that lead to the synthesis of secondary metabolites, serving as a defense mechanism against biotic and abiotic stressors (Quatrin et al., 2020; Rasera et al., 2020; Xia et al., 2017). Throughout seed germination, phenolic acids, flavonoids, vitamins, and free amino acids, particularly γ-aminobutyric acid (GABA), emerge as the primary bioactive compounds synthesized and identified (Chu et al., 2020; Ge et al., 2021; Pilco-Quesada et al., 2020). Germination triggers the activation of several enzymes involved in the synthesis and release of bioactive compounds. The presence of GABA can significantly influence seedling growth regulation during germination by stimulating changes in gene expression and increasing the synthesis of certain metabolites. As germination progresses, the plant undergoes dynamic biochemical changes, potentially enhancing the concentration of phenolic compounds, flavonoids, and other secondary metabolites. Insoluble phenolic compounds are stored in the cell walls through ester and glycosidic bonds. These compounds are synthesized in the intracellular endoplasmic reticulum from glucose as the initial precursor, via various molecular signaling pathways such as the oxidative pentose phosphate pathway, glycolysis, the shikimate pathway, the acetate/malonate pathway, and the phenylpropanoid pathway, through which secondary

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metabolites are synthesized (Agati et al., 2012). Increasing germination time leads to the formation of new cell walls where soluble phenols can be produced and new bound phenols can form, resulting in an increase in these compounds (Gan et al., 2017). Understanding how germination timing affects the concentration and bioaccessibility of phenolic compounds is crucial for several reasons. Firstly, it provides insights into the potential bioaccessibility of these compounds in the human body, shedding light on their effectiveness in conferring health benefits. Secondly, the research aligns with broader interest in functional foods and their role in preventive nutrition.

In the case of *Lupinus angustifolius*, it has been reported that germination at 20 °C for nine days alters the phenolic profile of the seeds, increasing the concentration of flavonols, isoflavones, and flavones, thereby significantly enhancing antioxidant capacity (Dueñas et al., 2009). This alteration is attributed to the synthesis of phytochemicals such as phenolic compounds, which are generated from glucose and aromatic amino acids through oxidative pathways including the pentose phosphate, glycolytic, and shikimate pathways, or compounds like isoflavones, which are synthesized via the malonate and phenylpropanoid pathways (Xu et al., 2020). Similarly, under the same germination conditions, Fernandez-Orozco et al. (2006) observed a significant increase in antioxidant activity concerning germination time due to biochemical changes enabling the production of secondary metabolites during germination. This includes the release of aglycones from conjugated glucosides through enzymatic activation catalyzed by glucosidase (Ribeiro et al., 2006). However, limited research has been conducted to determine the stability of phenolic compounds after the gastrointestinal digestion process, which were initially obtained through germination. Martín-Diana et al. (2021b) report that wheat germination increases the concentration of hydroxycinnamic acids, enhancing their bioaccessibility during digestion as well as their ability to inhibit free radicals. Similarly, it has been reported that germination of black mustard at 25 °C for 48 h increases the bioaccessibility of phenolic compounds with high antioxidant properties such as p-coumaric and sinapic acids after the gastrointestinal digestion process (Rasera et al., 2023). Bioactive compounds exert their biological effect and impact human health only if they remain available for absorption after all phases of gastrointestinal digestion. The metabolism and absorption of phenolic compounds are complex. It is estimated that 48 % of them are digested in the small intestine, 42 % in the large intestine, and only 10 % of the total remain undigested and intact within the food matrix (Tarko et al., 2013). Among all polyphenols, it has been reported that only aglycones can traverse biological membranes because they are highly lipophilic (Domínguez-Avila et al., 2017). However, multiple factors influence the bioavailability of phenolic compounds, including their high capacity to interact with other molecules such as proteins and carbohydrates. Factors such as intestinal environment, pH, and the presence of bile salts also impact their bioavailability (Shahidi & Peng, 2018). Additionally, these compounds can be degraded by bacterial esterases present in the large intestine, limiting their absorption after digestion (Manach et al., 2004). In the case of flavonoids, during gastrointestinal digestion, they undergo multiple reactions such as deglycosylation, glucuronidation, methylation, sulfonation, and hydroxylation, altering their properties for scavenging free radicals (Spencer, 2003). The bioaccessibility of these compounds can be assessed *in vitro*, simulating gastrointestinal digestion quickly and effectively. The INFOGEST method is a standardized protocol that allows the *in vitro* simulation of gastrointestinal digestion, estimating the bioaccessibility of phenolic compounds (Brodkorb et al., 2019). During gastrointestinal digestion, enzymatic action and pH conditions lead to structural changes in macromolecules, resulting in the release of phenolic compounds due to the cleavage of covalent bonds, transitioning from insoluble to soluble molecules and modifying their bioaccessibility (Shahidi & Yeo, 2016). In this context, it is crucial to understand and analyze the fractions of phenolic compounds that are released from the food matrix after *in vitro* gastrointestinal digestion simulation using the INFOGEST method. Therefore, the objective of the present study to explore how germination time influences the content and bioaccessibility of γ-aminobutyric acid and polyphenols and antioxidant capacity of lupin (*Lupinus angustifolius* L.) sprouts during simulated gastrointestinal digestion, aiming to propose efficient strategies that promote the supply of bioactive compounds impacting human health. Lupin seeds, with their nutrient-rich profile, have the potential to contribute to dietary strategies aimed at improving overall health and well-being.

2. Materials and methods

2.1. Materials

Lupin seeds (*Lupinus angustifolius* L.) were provided by the University of Guadalajara (Mexico). Pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, porcine bile extract, fast blue BB, 2,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, vicenin, vitexin, genistein, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), fluorescein, 2,2′-diazobis(2-aminopropane) dihydrochloride (AAPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS•+), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), *tert*-butyl hydroperoxide solution (t-BOOH), o-phthalaldehyde (OPA), and 9-fluorenylmethyl chloroformate (FMOC) were obtained from Sigma-Aldrich, Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin (10,000 U/mL) were purchased from Cultek (Madrid, Spain). Murine RAW 264.7 macrophages were obtained from the American Type Culture Collection (Manassas, VA, USA). The Cell Titer 96 Aqueous One Solution Cell Proliferation Assay kit was acquired from Promega (Madison, WI, USA).

2.2. Germination

Lupin seeds were disinfected using a 0.07 % (w/v) aqueous solution of sodium hypochlorite. Germination process spanned 1 to 7 days and was conducted in a germination chamber (Daihan Labtech CO., LTD, Korea) at 26 °C and 65 % relative humidity (Guzmán-Ortiz et al., 2017). Samples were collected on days 3 (G3), 5 (G5), and 7 (G7) representing different stages of seed development. The germinated grains underwent dehydratation at 40 °C until reaching a moisture content of 7.5 %. Subsequently, the germinated seeds were ground, defatted, and stored in polyethylene bags for further use.

2.3. In vitro simulated gastrointestinal digestion

Flours were digested using the INFOGEST 2.0 protocol for static *in vitro* gastrointestinal digestion (Brodkorb et al., 2019). Initially, 1.25x stocks of simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the detailed INFOGEST 2.0 method.

Raw and germinated flours (3 g) were dispersed in 3 ml of SSF containing 75 U/ml of human salivary amylase (E.C.3.2.1.1, Sigma-Aldrich, St. Louis, MO, USA) and incubated for 2 min at 37 °C and pH 7. The oral digestion was promptly followed by incubation with SGF (50:50, v:v) containing 2,000 U/ml of porcine pepsin from gastric mucosa (E.C. 3.4.23.1, Sigma-Aldrich, St. Louis, MO, USA) at pH 3 and 37 °C for 2 h on an orbital shaker. Gastric digestion was halted by adjusting the pH to 7 with 1 M NaOH.

The intestinal phase was initiated by the addition of SIF (50:50, v:v) containing 100 U/ml of pancreatin from porcine pancreas (Sigma-Aldrich, St. Louis, MO, USA) and porcine bile extract, for a 2-h incubation at 37 °C and pH 7. The intestinal phase was concluded by subjecting the samples to thermal treatment at 80 °C for 10 min. After digestion, samples were lyophilized at —20 °C.

2.4. γ-aminobutyric acid (GABA)

In this study, we assessed the concentration of GABA at various critical stages of lupin (*Lupinus angustifolius* L.) germination, including pre-germination stage and subsequent germination periods 3, 5, and 7 days (referred to as UG, G3, G5, and G7, respectively). Additionally, we monitored GABA levels following gastric (GD) and gastrointestinal digestion (GID) extending our assessment to the evaluation of GABA bioaccessibility. Samples were centrifuged (Eppendorf 5424R, Thermo Fisher Scientific, Madrid, Spain) at 2,500 x g for 15 min. The resulting supernatant was collected for further analysis by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with diode array detection (DAD) on an Agilent 1200 chromatographic system (Agilent Technologies, Inc., Wilmington, DE, EE). USA) as reported by Henderson and Brooks (2010). The Agilent Zorbax Eclipse Plus C18 column (4.6 × 250 mm, with a particle size of 5 μm) was used and maintained at 40 °C. The mobile phases consisted of A: consisted of 10 mM Na_2HPO_4: 10 mM $Na_2B_4O_7$, pH 8.2: 5 mM NaN_3 and B: acetonitrile: methanol: water (45:45:10, v:v:v). Injection of 20 μL of the sample sample was performed with a flow of 1.5 mL/min. Automated derivatization took place in the autosampler using: borate buffer (0.4 M in water, pH 10.2), OPA (10 mg/mL in 0.4 M borate buffer and 3-mercaptopropionic acid), and FMOC (2.5 mg/mL in acetonitrile). The DAD was configured to collect two channels, with signal A at 338 nm and 262 nm. Calibration curves with standard concentrations from 10 to 1000 nmol/mL were used for quantification. The results were expressed as g/100 g of sample on dry weight (dw).

2.5. Extraction and quantification of total free and bound polyphenols

2.5.1. Free polyphenols

The extraction performed following the methodology of Pico et al. (2020), underwent some modifications. Two distinct extractions, employing water and 80 % methanol in 0.1 % formic acid, were conducted. The methanolic extraction aimed to determine the total concentration in the undigested samples and quantify the total amount of bound polyphenols. The aqueous extraction was carried out to determine the available concentration of phenolic compounds post-digestion. Extracted samples were heated to 37 °C in a ThermoMixer Compact (Eppendorf AG, Hamburg, Germany) for 5 min, with intermittent vortex-mixing. Subsequently, the samples underwent sonication (P-Selecta Ultrasons, Barcelona, Spain) for 3 min, followed by centrifugation at 16,000 rpm for 30 min at 20 °C.

For methanolic extraction, 1 mL of 80 % methanol in 0.1 % formic acid was utilized. Samples were vortex-mixed and incubated at 30 °C for 15 min at 2000 rpm in a ThermoMixer Compact (Eppendorf AG, Hamburg, Germany). After centrifugation at 10,000 rpm for 10 min at 5 °C using a Centrifuge 5424 R (Eppendorf AG, Hamburg, Germany), the supernatant was collected. A second extraction was performed on the sediment by adding 1 ml of 70 % acetone in 0.1 % formic acid. The supernatants from both extractions were combined and adjusted to a final volume of 2 mL with deionized water.

To determine free polyphenols, 1 mL of each extract was mixed with 100 μL of 0.1 % Fast Blue BB in distilled water for 1 min. Subsequently, 100 μL of 5 % NaOH was added, and the mixture was incubated in darkness for 120 min to measure absorbance at a wavelength of 420 nm using a Synergy HT microplate reader (BioTek Instruments, Winooski, VT, USA). A calibration curve using gallic acid within the range from 0 to 225 μg/mL was prepared. The results were expressed as milligrams of gallic acid equivalents (GAE) per 100 g on dw.

2.5.2. Bound polyphenols

To determine the bound polyphenols, the methodology described by Martín-Diana et al. (2021a) with slight modifications. The sediment obtained from the extraction of free polyphenols was used. To this sediment, 12 mL of 3 M NaOH was added. The tubes were vortexed until a homogeneous solution was observed, followed by 16 h incubation at room temperature in the dark. Subsequently, the pH of the samples was adjusted to 2 with 6 N HCl, and 7 mL of ethyl acetate was added. After agitation and centrifugation at 17,000 x g, 4 °C for 10 min (RC6 Thermo Fisher Scientific, Madrid, Spain), the supernatant was collected, and the sediment was used for two additional extractions. All supernatants were combined. Acid hydrolysis of the sediment was performed using 2.5 mL of 6 N HCl. The samples underwent a 30-min incubation in a water bath at 85 °C, followed by cooling in an ice bath. Three extractions with ethyl acetate were carried out and all extracts were combined. The solvent was evaporated to dryness using a rotary evaporator, and the dried residues were resuspended in 5 mL of absolute methanol. The subsequent reaction mirrored the procedure for free phenols. Absorbance was measured using a Synergy HT microplate reader (BioTek Instruments, Winooski, VT, United States). A calibration curve with gallic acid ranging from 0 to 225 μg/mL was used. The results were expressed as milligrams equivalent of gallic acid (GAE) per 100 g on dw.

2.5.3. Total flavonoids

The quantification of total flavonoids followed the methodology of (Žilić et al., 2011), with certain modifications. Extraction was conducted using both water and 40 % ethanol. In brief, 500 μl of the extracts were mixed with 75 μL of 5 % $NaNO_2$, then 150 μL of 10 % $AlCl_3$. The moisture was allowed to settle for 5 min. After this period, 500 μL of 1 M NaOH were added, and the volume was adjusted to 2.5 mL with distilled water. The samples were measured at an absorbance of 510 nm. A calibration curve was made using quercetin as a standard within the rage of 0–0.7 mg/mL. The results were expressed as mg of quercetin equivalents (QE) in 100 g on dw.

2.5.4. Bioaccessibility

The percentage of polyphenols released after gastric and intestinal digestion was calculated through the following formula:

$$Bioaccessibility(\%) = (A/B)*100$$

A: Total polyphenols content after *in vitro* digestion

B: Total polyphenols content before *in vitro* digestion

2.5.5. Identification of compounds

Phenolic compounds identification was conducted on water extracts obtained from both undigested 7-days old lupin sprouts and intestinal digestate (UG and G7, respectively). A liquid chromatograph (LC) from the Agilent 1200 series, coupled with a G1315B DAD and an Agilent G6530A precision mass detector was used. Quadrupole Time-of-Flight Mass Spectrometer with Electrospray Ionization Source and JetStream Technology (HPLC-ESI-QTOF-MS), was employed for this analysis. An Agilent ZORBAX Eclipse XDB-C18 column (4.6 mm x 150 mm x 5 μm) (Agilent Technologies, Santa Clara, CA, United States) and DAD chromatograms were used. The extracts were filtered with 0.22 μm syringe filters (Teknokroma OlimPeack). The mobile phases and the execution of MS and MS/MS were performed following the protocol outlined by Martín-Diana et al. (2021b). MassHunter Data Acquisition (version B.05.00) and Qualitative Analysis (version B.07.00) Workstation (Agilent Technologies, Waldbroon, Germany) were used for data processing. Compounds were identified by comparing mass spectra and retention time with the corresponding standard (2,4,-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, vicenin, vitexin, and genistein). In the case of compounds for which standards were not available, identification was based on a prediction of chemical formula from accurate ion mass measurement and confirmed by comparing MSMS with data provided by relevant literature references (Czubinski et al., 2019; Farag et al., 2019; Stobiecki et al., 2010; Wojakowska et al., 2013).

2.6. $ABTS^{\bullet+}$ assay

$ABTS^{\bullet+}$ assay, adapted from Miller & Rice-Evans (1997), was conducted in a 96-well microplate. A solution of $ABTS^{\bullet+}$ was used at a concentration of 2.45 mM, previously adjusted to an absorbance of 0.70 ± 0.02, was prepared. In the microplate, 20 μL of sample extract (both digested and undigested, germinated and ungerminated) was combined with 200 μL of the $ABTS^{\bullet+}$ solution. After 60 min, the absorbance was measured at 734 nm with a microplate reader (Biotek). A calibration curve ranging from 0–400 μM of Trolox was prepared. The results were expressed as μmol Trolox equivalents (TE)/100 g of sample on dw.

2.7. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was carried out according to the method reported by Ou et al. (2001) with minor modifications. The samples and Trolox standard solutions were diluted using 10 mM phosphate buffer at pH 7.4. In a black 96-well microplate, a volume of 25 μL of Trolox, sample and phosphate buffer (blank) were added, along with 125 μL of fluorescein solution. The mixture was incubated at 37 °C for 3 min before adding 25 μL of AAPH solution to initiate the oxidation reaction. Fluorescence was monitored for 120 min using a microplate reader (BioTek Instruments, Winooski, VT, USA) with 485 nm excitation and 520 nm emission filters. A calibration curve ranging from 0 to 160 μM of Trolox was prepared. The results were expressed as μmol TE/100 g of sample on dw.

2.8. DPPH assay

The assay was performed in accordance with the procedure outlined by Thaipong et al. (2006). A 1 mM solution of the $DPPH^{\bullet}$ radical in methanol was prepared, subsequently diluted with methanol and shielded from light. The absorbance of the solution was read at 520 nm and adjusted to 0.8 units. In a black 96-well microplate, 10 μL of each extract and 190 μL of DPPH solution were combined. Absorbance was measured at 515 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA) to determine the percentage of antiradical activity. A calibration curve ranging from 0 to 400 mM Trolox was used. Results were expressed as μmol TE/100 g of sample on dw.

2.9. Cell culture and determination of cell viability

RAW264.7 murine macrophages viability was evaluated through the MTS assay using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay. The macrophages were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % inactivated fetal bovine serum (FBS) and 1 % penicillin/streptomycin within a humidified incubator at 37 °C and 5 % CO_2 for 24 h. Subsequently, cells were seeded in 96-well plates at a density of 2.5×10^4 cells/well. Following overnight incubation at 37 °C and 5 % CO_2, cells were treated with lupin gastric and intestinal digestates at concentrations of 0.5, 1, and 3 mg/mL, diluted with complete DMEM. The cells were then incubated at 37 °C and 5 % CO_2 for an additional 24 h. Afterward, the medium was aspirated, and the cells were washed with PBS. PBS was then replaced with 100 μL of DMEM and 20 μL of CellTiter 96® reagent. The plates were incubated at 37 °C for 45 min and the absorbance at 490 nm was recorded to assess cell viability.

2.10. Reactive oxygen species

Intracellular reactive oxygen species were measured after 24 h treatment of RAW 264.7 macrophages with G7 intestinal digestates at concentrations of 0.5, 1 and 3 mg/mL in complete DMEM. After this incubation period at 37 °C and 5 % CO_2, the medium was collected, and the cells were washed with PBS at 37 °C. Following aspiration of PBS, 200 μL of 10 μM DCFH-DA fluorescent probe solution was added, and treatments were performed in darkness. The cells were then incubated for 30 min at 37 °C and 5 % CO_2. Subsequently, excess DCFH-DA fluorescent probe was removed by aspirating the cell medium, and 200 μL of PBS 37 °C was added. After aspirating the PBS, 200 μL of 2.5 mM t-BOOH was introduced. The incubation continued for 3 h at 37 °C and 5 % CO_2, and fluorescence readings were taken from 0 to 30 min of incubation using fluorescence reader at wavelengths of 485_{ex} and 530_{em} nm.

2.11. Statistical analysis

For statistical analysis, the results, expressed as the average of three replicates ± standard deviation, underwent a one-way Analysis of Variance (ANOVA). The comparison of means was accomplished through Tukey's test with a 95 % confidence level. The statistical software used for analysis was Minitab version 17.1.0.2014. (State College, Pennsylvania, USA).

3. Results and discussion

3.1. Effect of germination time on γ-aminobutyric acid (GABA) content and bioaccessibility in lupin seeds

The GABA content in the analyzed samples is seen in Table 1. It was observed that germination time positively influenced the GABA content. The ungerminated sample (UG) had values of 0.071 g/100 g dw, which increased significantly with progressing germination time (G7 > G5 > G3 > UG). Notably, the GABA content peaked at 0.17 g/100 g dw in G7, representing a 2.5-fold increase compared to UG. This behavior aligns with findings in rice, wheat and soybean sprouts (Baranzelli et al., 2018; Huang et al., 2017; Thomas et al., 2023; Wu et al., 2022; Xu & Hu, 2014). L-glutamic acid serves as precursor for GABA production, achieved through the decarboxylation of L-glutamic acid catalyzed by the enzyme glutamate decarboxylase. This enzymatic process is activated during germination (Peñas et al., 2015). The observed increase in GABA after 7 days of lupin germination maybe attributed to this mechanism. The higher GABA content of lupin sprouts renders them more suitable for human consumption, given that GABA acts as a neurotransmitter with therapeutic implications for central nervous system disorders. Numerous studies have reported the effectiveness of GABA in preventing neurological diseases, improving immunity, reducing blood pressure and alleviating anxiety (Abdou et al., 2006; Inoue et al., 2003; Lątka et al., 2020).

The bioaccessibility of GABA in the body depends on its release from the food matrix, which is influenced by enzymatic degradation and interactions of the molecules during gastrointestinal transit. Therefore, in the present study we assessed the changes in GABA concentration upon lupin seeds and sprouts during the process of digestion. Following gastric digestion (GD), the release of GABA in the ungerminated sample (UG) was 0.055 g/100 g, showing a significant increase with germination time, reaching maximum values of 0.173 g/100 g in G7. However, no significant differences in GABA concentration were observed between undigested lupin sprouts and those subjected to GD ($p > 0.05$). After GD, bioaccessibility was higher in UG (77.58 %) compared to germinated samples (90.89–97.31 %) ($p < 0.05$). After GID, the concentration of GABA in UG remained similar to that of GD, suggesting no additional release of GABA. Conversely, intestinal bioaccessibility of all germinated samples decreased significantly compared to GD ($p < 0.05$). Possibly, this occurs because GABA may form an interaction with other compounds that limit its identification, although Garzón et al. (2020) reports that GABA is probably degraded due to the action of the gastrointestinal environment and the fermentation of the microbiota, more studies are necessary to elucidate the effect. Previous studies by Tuntipopipat et al. (2015) in germinated brown rice (60 %) and Garzón et al. (2020) in fermented sorghum (30 %), reported similar or lower bioaccessibility percentages. These findings underscore the importance

Table 1
Changes in GABA content and bioaccessibility during germination and gastric (GD) and intestinal (GID) digestion.

Sample	γ-aminobutyric acid (g/100 g dw) Undigested	GD	Bioaccessibility (%)	GID	Bioaccessibility (%)
UG	0.071 ± 0.01^{Ca}	0.055 ± 0.002 ^{Da}	77.58 ± 7.67^{Aa}	0.048 ± 0.01^{Ba}	67.55 ± 4.72^{Aa}
G3	0.136 ± 0^{Ba}	0.127 ± 0.006^{Ca}	93.42 ± 5.15^{Aa}	0.081 ± 0.001^{Ab}	59.33 ± 0.72^{Ab}
G5	0.164 ± 0.007^{Aa}	0.149 ± 0.002^{Ba}	90.89 ± 1.68^{Aa}	0.098 ± 0.009^{Ab}	59.59 ± 6.27^{Ab}
G7	0.178 ± 0.006^{Aa}	0.173 ± 0.004^{Aa}	97.31 ± 6.24^{Aa}	0.102 ± 0.005^{Ab}	57.12 ± 1.13^{Ab}

The values are expressed as the mean ± standard deviation.
Different capital letters in each column indicate significant differences between days of germination ($p < 0.05$, Tukey test).
Different lowercase letters in each column indicate significant differences between digestion endpoints ($p < 0.05$, Tukey test).
Abbreviations: UG, ungerminated lupin seeds; G3, lupin germinated for 3 days; G5, lupin germinated for 5 days; G7, lupin germinated for 7 days

of understanding factors affecting the bioaccessibility of GABA.

3.2. Effect of germination time on polyphenols content and bioaccessibility in lupin seeds

The analysis of phenolic content in lupin seeds (UG) and sprouts germinated at different times (G3, G5 and G7) was carried out before and after gastric (GD) and intestinal digestion (GID). The objective was to assess the bioaccessibility of phenolic compounds that could potentially be absorbed by the epithelial layer of the gastrointestinal tract, shedding light on the impact of germination time on their bioaccessibility. Table 2 presents the concentrations of free, conjugated and total phenolic compounds in both the ungerminated and germinated lupin samples. Fig. 1A and B represent the total phenolic content of samples in their undigested and digested states. The UG sample exhibited a total phenolic concentration of 1335.5 mg gallic acid equivalents (GAE)/100 g dw. The main contribution to this concentration came from free phenolic compounds, which represented 76 % of the total phenolic content. The concentration of total phenolic compounds increased significantly ($p < 0.05$) with germination time, reaching 1.4 times higher in G7. In this case, the contribution of free phenolic compounds mirrored that of UG, comprising 78 % of the total phenolic content.

The concentration of conjugated polyphenols demonstrated similarity between G5 and G7, with no significant differences ($p > 0.05$) observed. An increase in enzymatic activity with germination time could explain the higher free phenolic concentration in G7 compared to G5 ($p < 0.05$). Previous studies in *Phaseolus vulgaris* and *Pisum sativum*, adlay seed, Tartary buckwheat (*Fagopyrum tataricum*) and amaranth, reported a similar trend in the concentration of total phenolic compounds with germination time (G.-L. Chen et al., 2016; Y. Chen, Zhu, & Qin, 2022; Popoola, 2022; Martínez, Concha, Velázquez, Martínez, & Ruiz, 2021). The observed increase in total polyphenols during the germination process could also be attributed to the release of bound phenols observed with germination time and the synthesis of polyphenols through the shikimic acid, pentose phosphate and phenylpropanoid pathways (Lin et al., 2016). These processes are integral components of the seed metabolic activities and its defense mechanism against damage caused by pathogens and environmental factors.

Table 2
Content of free, bound and total polyphenols in ungerminated and germinated lupin at different time intervals.

Samples	Free polyphenols (mg GAE/100 g)	Bound polyphenols	Total polyphenols
UG	1144 ± 0 ^{D}	191.78 ± 8.74^{C}	1335.5 ± 8.74^{C}
G3	1349 ± 0^{B}	314.9 ± 17.7^{B}	1663.9 ± 17.7^{B}
G5	1331.05 ± 7.58^{C}	400.05 ± 3.01 ^{A}	1731.10 ± 4.57^{B}
G7	1500.28 ± 3.53 ^{A}	407.7 ± 32.8 ^{A}	1908.0 ± 29.3 ^{A}

The values are expressed as the mean ± standard deviation.
Different capital letters in each column indicate significant differences between days of germination ($p < 0.05$, Tukey test).
Abbreviations: UG, ungerminated lupin seeds; G3, lupin germinated for 3 days; G5, lupin germinated for 5 days; G7, lupin germinated for 7 days

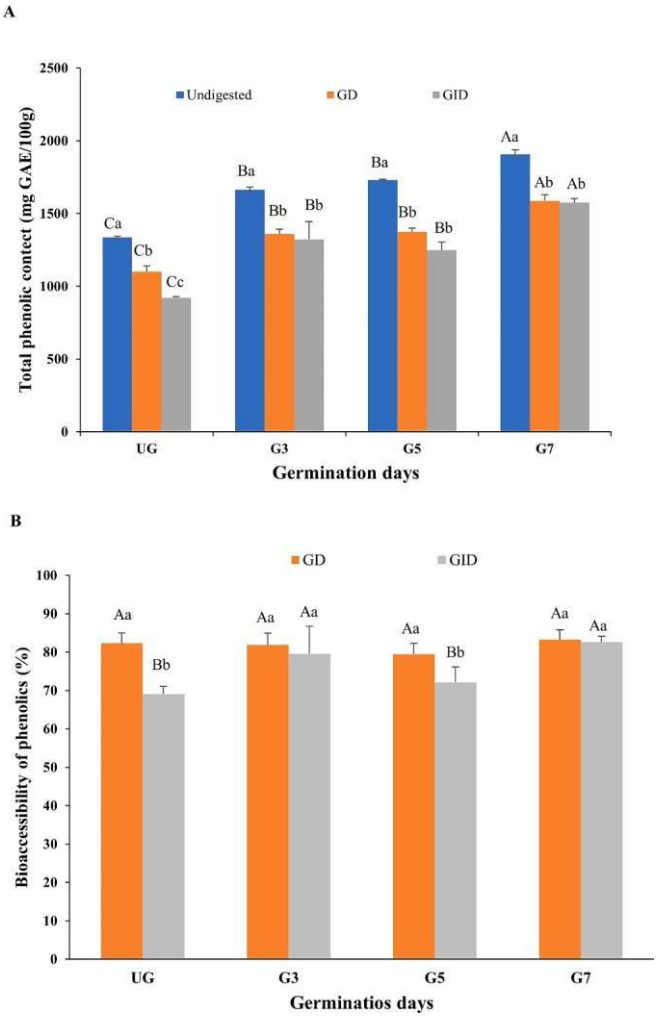

Fig. 1. A) Total phenolic content in lupin (*Lupinus angustifolius L.)* seeds and sprouts at different days of germination before and after gastric and intestinal digestion; B) Bioaccessibility of total phenolics after gastric and intestinal digestion. Abbreviations: UG, ungerminated lupin seeds; G3, lupin germinated for 3 days; G5, lupin germinated for 5 days; G7, lupin germinated for 7 days; GD, gastric digestion; GID, intestinal digestion. The values are expressed as the mean ± standard deviation. Different capital letters indicate significant differences between days of germination ($p < 0.05$, Tukey test). Different lowercase letters indicate significant differences between digestion endpoints ($p < 0.05$, Tukey test).

While the germination process contributes to an increased concentration of phenolic compounds, its biological efficacy depends on their bioaccessibility within the organism. Upon subjecting the samples to gastrointestinal digestion, a notable decrease in the bioaccessibility of

total polyphenols was observed (Fig. 1A). At the end of GD, the concentration was statistically lower in all samples compared to the undigested counterparts ($p < 0.05$). Specifically, the concentration of total polyphenols in the gastric digestates varied from 1099.4± 41.0 to 1587.3 4±1.9 mg GAE/100 g (UG < G3 < G5 < G7). Despite the increase in total polyphenol concentration with germination time, no significant differences in bioaccessibility were found. Bioaccessibility values ranged between 79.44±2.81 and 83.22 ±.62 %, with no significant differences ($p > 0.05$). This suggests that, although germination enhances the overall phenolic content, the bioaccessibility of these compounds remains consistent across different germination times during gastrointestinal digestion.

Upon completion of GID, the release of phenolic compounds in UG was the most restrained compared to gastric digestates and undigested sample. Interestingly, phenolic concentration in intestinal digestates from G3, G5 and G7 was similar to that found in the gastric phase. However, in this case, the bioaccessibility varied between 69.06± 2.02 and 82.56 ±.52 %. Notably, UG and G5 showed the lowest bioaccessibility in both GD and GID. In contrast, G3 and G7 exhibited the highest bioaccessibility of 79.53 ±.15 and 82.56 1.5± % respectively, showcasing statistical significance with respect to other samples ($p < 0.05$).

Although germination positively influenced the concentration of phenolic compounds, it did not exert a discernible impact on their bioaccessibility during gastrointestinal digestion (Fig. 1 B). This aligns with findings reported earlier, highlighting a decrease in total phenolic compounds during *in vitro* gastrointestinal digestion (Corona-Leo et al., 2021; Czubinski et al., 2019; Pereira et al., 2020). An increase in pH during digestion can cause a decrease in the concentration of phenolic compounds, since it generates instability in small molecules due to the decomposition of larger molecules during the digestion process (Pavan et al., 2014). This could cause phenolic compounds to bind to other compounds released in digestion, such as proteins, carbohydrates and minerals, forming complexes that affect their bioaccessibility (Chen et al., 2016; de Paulo Farias et al., 2021; Quan et al., 2018). Furthermore, fiber could influence the bioaccessibility of phenolic compounds, since it has been reported that the extraction of compounds is limited when they are retained in the fiber, which affects their solubility in gastrointestinal fluids (Lucas-González et al., 2018). Despite the decrease in phenolic compounds during gastrointestinal digestion, bioaccessibility improved when the seed was germinated. The functionality of phenolic compounds may also depend on the specific type of phenolic compound present.

3.3. *Effect of germination time on the concentration and bioaccessibility of flavonoids in lupin*

The concentration of flavonoids in both ungerminated and germinated lupin before and after gastrointestinal digestion is shown in Fig. 2A. The initial concentration of flavonoids in the UG was 11.75 mg quercetin equivalents (QE)/100 g dw. Through the germination process, the concentration increased up to 1.8 times. However, as the germination progressed from 3 to 7 days, there was no significant change observed in the total concentration of flavonoids ($p > 0.05$). This observed increase resembles with similar trends reported by (Wu et al., 2022), and Popoola, (2022), who also noted an increase in total flavonoid content during the germination process in rice, *Phaseolus vulgaris*, *Pisum sativum* and Nigerian Amaranthus viridis seed, respectively. The activation of enzymes, such as chalcone isomerase and phenylalanine ammonium lyase, during germination could explain this increase trend, since these enzymes participate in the synthesis of flavonoids (Singh et al., 2009), potentially accounting for the observed increase from the third day of germination.

Following GD, the concentration of flavonoids increased significantly ($p < 0.05$) compared to undigested samples. Notably, germination time also influenced the release of flavonoids in the gastric phase,

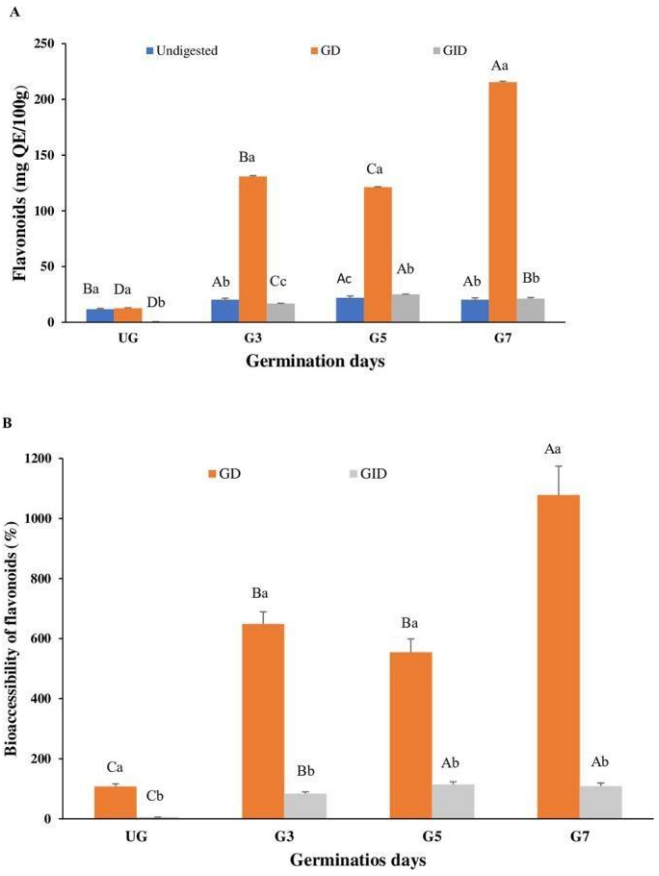

Fig. 2. A) Flavonoids content in ungerminated and germinated lupin (Lupinus angustifolius L.), before and after gastric and intestinal digestion; B) Bioaccessibility of flavonoids after gastric and intestinal digestion. Abbreviations: UG, ungerminated lupin seeds; G3, lupin germinated for 3 days; G5, lupin germinated for 5 days; G7, lupin germinated for 7 days; GD, gastric digestion; GID, intestinal digestion. The values are expressed as the mean ± standard deviation. Different capital letters indicate significant differences between days of germination ($p < 0.05$, Tukey test). Different lowercase letters indicate significant differences between digestion endpoints ($p < 0.05$, Tukey test).

since the concentration increased significantly ($p < 0.05$) up to 17 times in sample G7.

The rise in flavonoid concentration during GD contributed to an improvement of their bioaccessibility from 107.71 % in UG to 1078 % in G7. This suggests a potential release of bound flavonoids during gastric digestion. However, in the subsequent GID, the concentration of flavonoids significantly decreased compared to the GD (Fig. 2A). The flavonoid concentrations observed in the GID were similar to the undigested samples and lower than in the GD. Bioaccessibility in the GID mirrored the trend observed in the GD regarding germination time, albeit with lower percentages were (Fig. 2B). Bioaccessibility in the GID ranged from 5.37 % in UG to 114.20 % and 109.08 % in G5 and G7, respectively. It is noteworthy that the most substantial release of flavonoids occurred during GD compared to GID. This behaviour was favoured in gastric conditions characterized by an acidic pH and the presence of gastric enzymes. This aligns with findings from a study conducted by (Maduwanthi & Marapana, 2021), suggesting that pepsin could play a crucial role in releasing flavonoids bound to proteins and carbohydrates (Bouayed et al., 2011; Gumienna et al., 2011). This translated into an increase in the concentration of flavonoids in GD. Furthermore, it is essential to consider that these compounds may become entrapped within the lupin matrix or bind to other components during the digestive process, potentially hindering their intestinal absorption (Giuberti et al., 2018; Mas et al., 2022).

Also, it is important to highlight that determining bioaccessibility in the gastric fraction is crucial because it is the first point of contact between compounds and the digestive environment. The efficiency of the release and solubilization of the compounds at this stage directly influences their subsequent absorption in the small intestine. Some compounds may be more stable in acidic environments, while others may break down or precipitate, affecting their availability for absorption at later stages of the gastrointestinal tract. Understanding how compounds are released in the gastric phase is essential for the development of formulations that allow controlled release in the small intestine, where absorption is most efficient. This is especially relevant for extended-release formulations or nutritional supplements that require gradual absorption over time. Determining bioaccessibility in both phases ensures that it is fully understood how these molecules are released and absorbed throughout the gastrointestinal tract.

3.4. Characterization of free phenolic fraction in lupin seeds and 7-days-old sprouts

The germination process significantly enhanced the concentration of phenolic compounds and total flavonoids in lupin. Given that day 7 of germination exhibited the highest concentration of these compounds, G7 was specifically chosen for its elevated phenolic content. To delve deeper into the phenolic profile, both ungerminated (UG) and the day 7 germinated (G7) samples underwent analysis by HPLC-ESI-QTOF-MS/MS in negative ionization mode ([M—H]-) before and after gastrointestinal digestion. Phenolic compounds identification was performed by using reference standards or tentatively identified by comparison of retention time (RT), molecular formula, mass error between observed and theoretical mass, and fragmentation pattern with those reported in the literature for *Lupinus* (Czubinski et al., 2019; Farag et al., 2019; Stobiecki et al., 2010; Wojakowska et al., 2013). A total of 18 compounds were identified (Table 3): 3 phenolic acids, 11 flavonoids and 4 isoflavones (Fig. 1S y 2S).

3.4.1. Phenolic acids

Compounds 1, 2 and 3 were identified as phenolic acids. Compound 1 with a precursor ion [M – H]- at *m/z* 153, and daughter ion at *m/z* 109, was identified as 2,4-dihydroxybenzoic acid. Compound 2 was identified as 4-hydroxybenzoic acid with a mass [M– H]- at *m/z* 137 and compound 3 as vanillic acid with detected precursor ion [M — H]- at *m/z* 167. The identity of these three phenolic acids was further confirmed by the use of commercial standards. These compounds were observed in the UG sample and persisted throughout the germination process, with their identification confirmed in G7. However, during gastrointestinal digestion, their presence could not be detected. This absence could be attributed to the potential degradation or structural changes that acidic phenolic compounds may undergo due to the action of pancreatin and bile salts, joined to the neutral or slightly alkaline pH of the intestinal environment (Dutra et al., 2017; Goulas & Hadjisolomou, 2019; Ma et al., 2020; Peixoto et al., 2016). Also, during digestion, phenolic compounds can be degraded by enzymes such as glucosidases and esterases, which break the glycosidic and esterified bonds present in these compounds. On the other hand, its worth noting that phenolics acids may also form complexes with proteins in the gastrointestinal tract, consequently reducing their bioaccessibility (Bohn, 2014).

3.4.2. Flavonoids

Compounds 4 to 14 have been identified as flavonoids. In particular, compound 16 was identified as quercetin by comparison with a commercial standard, with a parent ion [M — H]- at *m/z* of 301, and MS/MS fragmentation pattern including daughter ions at *m/z* 286, 186 and 185. It is relevant to highlight that these flavonoid and phenolic acids (such as 2,4 dihydroxybenzoic, 4-hydroxybenzoic and vanillic acid) presented a similar behavior. They were exclusively detected in the UG and G7 samples, but were not observed after GID. The neutral pH of the intestinal phase generates an environment where quercetin is unstable and can be oxidized or degraded in the presence of digestive enzymes (Boyer et al., 2005). The literature indicates that quercetin, for instance, has low solubility in alkaline media, such as water and intestinal juice (Gao et al., 2009). It is probable that due to this characteristic, quercetin could not be detected after intestinal digestion.

Compounds 7, 9, 11, and 12 were only detected in germinated *Lupinus angustifolius* L. for 7 days, suggesting their de novo biosynthesis. The biosynthesis became apparent as these compounds were observed only in G7. In addition, they persisted following GID, since they were detected at the end of intestinal digestion. Identity of compound 7 was tentatively confirmed as kaempferol 3-O glucoside, with a parental ion [M – H]- at *m/z* of 447 and daughter ions at *m/z* 341 and 285 (Czubinski et al., 2019). Compound 9 with [M — H]- at *m/z* 461 and an MS/MS fragmentation pattern at *m/z* 461, 341, 270, 185 and 180 was characterized as chrysoeriol O-glucoside (Stobiecki et al., 2010). Compound 11 was assigned as isorhamnetin C-glucoside, with a precursor ion [M – H]- at *m/z* of 477 and MS/MS fragmentation at *m/z* 431, 311 and 269 (Wojakowska et al., 2013). Finally, compound 12 corresponded to apigenin, which was identified based on its parental ion [M–H]- at m / z 269, and main fragments generated at *m/z* 160, 227 and 133 (Czubinski et al., 2019). Compounds 7, 9, and 11 were identified as flavonoid glycosides present in 7-day germinated *Lupinus angustifolius*. This observation aligns with the phenomenon noted in germination, where some glycosylated flavonoids experience an initial increase in concentration. However, as the germination progresses, this concentration may decline due to the enzymatic activity of glucosidases, which release the aglycone forms (Dueñas et al., 2009; Eldin et al., 2023). This behavior is contingent on several factors in the germination process, including seed variety. Enzymes responsible for glycosylation, such as uridine diphosphate P-glucose flavonol glucosyltransferases, undergo increased gene expression during germination, leading to the generation of glycosylated forms, by transferring glucose molecules to aglycones (Taylor et al., 1998). The glycosylated forms, known for their increased stability and water solubility, can persist in the intestinal phase, enhancing their bioavailability in the organism. This behavior aligns with similar observations reported by Xie et al. (2022).

On the other hand, compounds 4, 5, 6, 8 and 10 were consistently present across all samples. They were detected both the UG and G7 samples before and after GID, except for compound 10, which was not identified in G7 after GID.

Compound 4, with a parental ion [M – H]- at *m/z* of 725, was identified as apigenin 7-apiosyl-6,8-di-C-glucoside, supported by fragmented ions at *m/z* 605, 533, 455, 335, 355 and 241(Czubinski et al., 2019). Compound 5 was tentatively identifed as chrysoirol diglucoside methylmalonylated, with precursor ion [M – H]- at *m/z* of 725 and daughter ions at *m/z* 682, 670, 639, 615, 605, 575, 542, 455, 353 and 335 (Wojakowska et al., 2013). Compounds 6 and 8 were identified by commercial standards, as vicenin and vitexin, with molecular ion masses [M –H] – at *m/z* 593 and 431, respectively. Certainly, the resistance of these compounds to gastrointestinal digestion may be due to the fact that the glucosylated groups are less susceptible to degradation by digestive enzymes, which allows them to be identified after gastrointestinal digestion. Compound 10, with a parental ion [M — H]- at *m/z* of 563 and an ion fragmentation pattern at *m/z* 413, 293, 500, 371, 413, 293 and 269, was identified as apigenin-7-apioglucoside (Dueñas et al., 2009). This apigenin derivative was not detected in G7 after GID, suggesting that its concentration decreases during germination and, therefore, is no longer available after digestion.

Finally, compound 13, belonging to the flavonoid group, with a precursor ion [M — H]- at *m/z* of 548, was tentatively identified as chrysoeriol O-glucoside malonylated (Wojakowska et al., 2013). Unlike apigenin-7-apioglucoside, this compound was not detected in UG after GID. This observation suffests that its maximum availability occurs during germination, limiting its identification in the non-germinated sample after digestion. These findings are consistent with the data

Table 3
Identification of free phenolic compounds by HPLC-ESI-QTOF-MS/MS in negative ion mode in ungerminated and 7-day germinated lupin before and after gastrointestinal digestion.

#	Compound	Formula	Retention time (min)	Calculated mass [M − H]⁻	Observed mass [M − H]⁻	Score	Error	Fragmentation [M − H]⁻ [m/z]	Treatment	Sample
	Phenolic acids									
1	2,4 DihidroXibenzoic acid	$C_7H_6O_4$	8.087	153.0193	153.018	60.6	6.68	109(10 0)	Undigested	UG
			8.116		153.019	47.05	2.04	nd		G7
					—	—	—	—	GID	UG
					—	—	—	—		G7
2	4-HydroXybenzoic acid	$C_7H_6O_3$	11.186	137.0244	137.0244	47.62	0.09	nd	Undigested	UG
			11.514		137.0253	42.71	—6.61	nd		G7
					—	—	—	—	GID	UG
					—	—	—	—		G7
3	Vanillic acid	$C_8H_8O_4$	13.435	167.035	167.0349	47.58	0.52	nd	Undigested	UG
			13.43		167.0334	69.86	8.69	nd		G7
					—	—	—	—	GID	UG
					—	—	—	—		G7
	Flavonoids									
4	Apigenin 7 apiosyl-6,8-di-C-glucoside	$C_{32}H_{38}O_{19}$	15.977	725.1935	725.1944	98.41	—1.16	605(2.42), 455(2.14), 355 (0.87)	Undigested	UG
			15.711		725.1933	98.09	—0.39	605(2.8), 455(2.54), 335 (0.78), 226(0.08)		G7
			15.79		725.1932	98.96	0.27	605(2.43), 455(1.22), 355 (0.57), 241(0.07)	GID	UG
			15.85		725.1943	97.04	—1.54	605)2.87), 533(0.19), 455 (2.2), 335(0.67)		G7
5	Chrysoirol diglucoside mrthylmalonylated	$C_{32}H_{38}O_{19}$	15.941	725.1935	725.1939	98.41	—0.7	682(0.07), 605(2.42), 455 (2.14), 335(0.87)	Undigested	UG
			15.882		725.1942	98.18	0.23	670(7.15), 615(6.98), 542 (30.93), 445(35.22)		G7
			15.657		725.1915	92.27	3.16	639(10.94), 447(6.7), 295 (0.85)	GID	UG
			15.942		725.1924	98.5	1.1	635(0.485), 575(2.56), 455 (1.73), 353(0.61)		G7
6	Vicenin	$C_{27}H_{30}O_{15}$	16.381	593.1512	593.151	98.57	0.99	593(100), 473(13.32), 353 (4.96), 168(0.2)	Undigested	UG
			16.401		593.1518	99.15	—0.96	593.1524(100), 473 (16.63), 353(5.53), 193 (3.93), 107(3.32)		G7
			16.346		593.1511	99.84	—0.22	593(10 0), 473(8.53), 353 (4.56), 257(0.65)	GID	UG
			16.3041		593.153	93.9	—2.48	593(10 0), 473(8.26), 353 (14.82), 257(2.51), 110 (2.17)		G7
7	Kaempferol 3-O glucoside	$C_{21}H_{20}O_{11}$	—	448.1011	—	—	—	—	Undigested	UG
			18.697		447.0945	89.6	—2.54	285(10 0)		G7
			—		—	—	—	—	GID	UG
			18.759		447.095	92.45	—2.81	341(4.74), 285(10 0)		G7
8	Vitexin	$C_{21}H_{20}O_{10}$	20.914	431.0982	431.0982	94.69	1.14	nd	Undigested	UG
			19.289		431.099	96.1	—2.15	431(10 0), 311(1.41), 260 (0.94), 112(0.41)		G7
			20.973		431.0974	67.91	0.34	nd	GID	UG
			19.29		431.0984	98.58	0.25	431(10 0), 311(54.54), 216 (2.87)		G7
9	Chrysoeriol *O*-glucoside	$C_{22}H_{22}O_{11}$	—	462.1168	—	—	—	—	Undigested	UG
			20.036		461.11	95.26	—2.5	461(10 0), 341(64.11), 270 (5.78), 180(3.55)		G7
			—		—	—	—	—	GID	UG
			20.095		461.1069	67.58	5.16	461(10 0), 341(55.02), 185 (8.69)		G7
10	Apigenin-7-apioglucoside	$C_{26}H_{28}O_{14}$	21.537	563.128	563.1399	96.22	1.84	413(13.3), 293(13.6)	Undigested	UG
			24.967		563.14	98.91	0.81	500(2.15), 371(1.67), 269 (66.54)		G7
			21.595		563.141	81.46	—2.87	413(8.03), 293(22.78)	GID	UG
11	Isorhamnetin 3-Glucoside	$C_{22}H_{22}O_{12}$	—	477.1038	—	—	—	—	Undigested	G7
			—		—	—	—	—		UG
			23.079		477.1036	98.98	0.69	431(10 0), 311(1.62), 269 (69.36)		G7
			—		—	—	—	—	GID	UG
			22.979		477.1018	91.21	3.61	431(93.88), 311(5.82), 269 (10 0)		G7
12	Apigenin	$C_{15}H_{10}O_5$	—	269.0455	—	—	—	—	Undigested	UG
			26.101		269.0457	98.55	—1.21	160/7.4)		G7
			—		—	—	—	—	GID	UG
			26.535		269.0462	97.13	—2.37	227(9.28), 133(14.78)		G7

(*continued on next page*)

Table 3 (*continued*)

#	Compound	Formula	Retention time (min)	Calculated mass [M ¡ H]-	Observed mass [M ¡ H]-	Score	Error	Fragmentation [M ¡ H]-[m/z]	Treatment	Sample
13	Chrysoeriol O-glucoside malonylated	$C_{25}H_{24}O_{14}$	30.601	548.1172	547.1086	89.76	0.78	437(8.25), 299(10 0), 188 (6.38), 99(2.4)	Undigested	UG
			30.578		547.1072	70.45	3.55	549(1.01), 445(1.17), 299 (10 0)		G7
			—		—	—	—	—	GID	UG
			30.478		547.1066	84.35	2.86	549(4.81), 425(3.85), 299 (10 0)		G7
14	Quercetin	$C_{15}H_{10}O_7$	31.527	301.0354	301.0355	84.03	—2.43	286(10 0), 185(10.91)	Undigested	UG
			31.507		301.0365	72.62	—4.68	286(10 0), 186(10.52)		G7
			—		—	—	—	—	GID	UG
			—		—	—	—	—		G7
	Isoflavones									
15	Genistein C-glucosylglucoside (II)	$C_{27}H_{31}O_{15}$	16.29	594.159	593.1514	97.2	1.83	503 (2.18), 473(7.92), 413 (0.55), 383(2.36), 353 (3.95), 221(0.2)	Undigested	UG
			—		—	—	—	—		G7
			16.232		593.1499	95.48	1.66	550(1.21), 473(6), 353 (5.08)	GID	UG
			—		—	—	—	—		G7
16	Genistein 6-C glucoside	C—H O		432.1062	—	—	—		Undigested	UG
			19.196		431.0976	98.69	1.42	311(97.46)		G7
			—		—	—	—	—	GID	UG
			19.181		431.0987	99.27	—0.55	311(81.09)		G7
17	Genistein 7-O-glucoside	C—H O		431.0984	—	—	—		Undigested	UG
			19.783		431.0984	62.54	—0.49	268(10 0)		G7
			—		—	—	—	—	GID	UG
			19.588		431.0998	89.58	—0.11	371(4.64), 268(83.19)		G7
18	Genistein	$C_{15}H_{10}O_5$	35.15	269.0455	269.0468	87.09	—5.72	133(19.25)	Undigested	UG
			35.091		269.0441	92.58	4.69	133(10.06)		G7
			—		—	—	—	—	GID	UG
			35.235		269.045	87.71	6.21	133(23.29)		G7

reported by Baranzelli et al. (2023), indicating that, in ungerminated wheat samples, the concentration of flavonoids decreased with gastrointestinal digestion.

3.4.3. *Isoflavones*

Compounds 15, 16, 17 and 18 were identified as isoflavones. Interestingly, were exclusively identified in G7 both before and after GID, similar to glycosylated compounds 7, 9 and 11, suggesting their biosynthesis during germination and their persistence through GID.

Compounds 15, 16 and 17 with molecular ion [M — H]- at *m/z* of 594, 431 and 432 respectively, were identified as glycosylated forms of genistein; genistein C-glucosylglucoside (II), genistein 6-C glucoside and genistein 7-O-glucoside, respectively (Wojakowska et al., 2013). Finally, compound 18 was assigned to genistein based on the detection of a precursor ion [M – H]- at *m/z* of 269 and a main fragment at *m/z* 133 (Sreerama et al., 2010). It is not worthy that genistein was detected in UG samples before and after GID, but in G7, it was not identified after digestion. This observation suggest that the concentration in the ungerminated sample is low and cannot withstand intestinal digestion. Qi et al. (2023) reported a decrease in isoflavone content in the intestinal phase of digestion, possibly due to the instability of isoflavones under alkaline pH conditions, reducing their bioavailability. Furthermore, isoflavones tend to bind to bile salts and form micelles that affect their bioaccessibility (Walsh et al., 2003). Most of the forms present in the samples were glycosylated forms, likely because the aglycone forms of isoflavones are absorbed more quickly and in greater quantities compared to the conjugated ones (Barnes et al., 2011). Glycosylated forms have a higher molecular weight, better water solubility, and polarity than aglycone forms (Raimondi et al., 2009), possibly explaining why more glycosylated forms of isoflavones were identified in GID in the germinated sample for 7 days.

These findings underscore the crucial role of germination in the synthesis of secondary metabolites, including phenolic acids, flavonoids and isoflavones and its profound impact on the bioaccessibility of these compounds in the gastrointestinal tract. The different chemical forms of these compounds can influence their stability and absorption capacity in the intestine, offering valuable insights into how biological processes and diet can impact the bioaccessibility of bioactive compounds in the body, thereby influencing overall health.

3.5. *Effect of germination time on antioxidant activity of Lupinus angustifolius before and after gastrointestinal digestion*

3.5.1. *Radical scavenging activity*

Antioxidant activity was measured in the ungerminated and germinated samples before and after gastrointestinal digestion using the ORAC, ABTS and DPPH assays (Table 4) each targeting different radical scavenging mechanisms. The ORAC assay relying on the quenching of peroxyl radicals through hydrogen atom transfer, demonstrated an increase in antioxidant activity with germination time. Specifically, the antioxidant activity increased up to 2.4 and 2.6 times in G5 and G7, respectively, compared to the UG lupin.

The ABTS assay measures the capacity to donate electrons and reduce the ABTS•+radical (Schaich et al., 2015). The ABTS assay, revealed that G3 displayed greater antioxidant activity compared to G5 and G7, showcasing 2.2-fold increase over UG lupin. Meanwhile, in the DPPH assay, based on an electron transfer mechanism (Huang et al., 2005), the highest activity was observed in G5 presenting a 1.2-fold increase compared to UG lupin. These variations could be attributed to the distinct mechanism of action employed by each method and the polarity of the bioactive compounds reacting with the radicals.

When subjected to gastric digestion, the antioxidant activity exhibited a behavior similar to that observed in undigested samples. Antioxidant activity increased with germination and after GD, the increase was significantly greater compared to undigested samples ($p < 0.05$). Specifically, in the ORAC assay, antioxidant activity increased within the range of 310.83–329.42 %, while ABTS assay demonstrated an increase of 161.83––269.52 % and with DPPH exhibited a rise of

Table 4
Radical scavenging activity of ungerminated and germinated lupin before and after gastrointestinal digestion as measured by ORAC, ABTS and DPPH assays (expressed in μmol TE/100 g).

	Germination days	Undigested	GD	Increase (%[1])	GID	Increase (%[1])
ORAC	UG	2492.9 ± 136.7 ^{Cc}	7748 ± 510 ^{Cb}	310.83 ± 11.56 ^{Ab}	21554 ± 1528 ^{Ba}	866.6 ± 76.4 ^{Aa}
	G3	5724 ± 479 ^{Bc}	18076 ± 1166 ^{Bb}	318.4 ± 41.8 ^{Ab}	34670 ± 2703 ^{Aa}	609.6 ± 72.4^{Ba}
	G5	6494 ± 358 ^{Ac}	20320 ± 998 ^{Ab}	313.42 ± 18.4 ^{Ab}	36296 ± 2909 ^{Aa}	556.0 ± 64.8^{Ba}
	G7	6103 ± 436 ^{Ac}	20045 ± 1102 ^{Ab}	329.42 ± 23.06^{Ab}	38172 ± 3737 ^{Aa}	641.2 ± 89.6^{Ba}
ABTS	UG	3694.24 ± 334.2 ^{Cc}	9941.28 ± 411.47 ^{Bb}	269.52 ± 25.76^{Ab}	17757.16 ± 663.6 ^{Ba}	487.4 ± 57.5^{Aa}
	G3	8273.03 ± 119.11 ^{Ac}	13386.87 ± 456.46 ^{Ab}	161.83 ± 5.66^{Bb}	20316.21 ± 556.88 ^{Aa}	245.58 ± 6.38^{Ba}
	G5	7419.53 ± 143.32 ^{Bc}	13112.24 ± 430.34 ^{Ab}	178.0 ± 5.84^{Bb}	19016.59 ± 215.70 ^{Ba}	256.34 ± 2.98^{Ba}
	G7	7541.20 ± 196.64 ^{Bc}	13287.70 ± 1188.93 ^{Ab}	176.33 ± 17.2^{Bb}	20649.93 ± 1523.92 ^{Aa}	273.83 ± 19.32^{Ba}
DPPH	UG	716.3 ± 58.0 ^{Bb}	229.3 ± 27.2 ^{Cc}	33.02 ± 2.1^{Ab}	1314.6 ± 74.0 ^{Ca}	196.06 ± 4.54^{Ba}
	G3	756.6 ± 69.3 ^{Bc}	1106.9 ± 108.6 ^{Bb}	152.21 ± 11.02^{Bb}	1787.8 ± 153.4 ^{Ba}	245.69 ± 17.61^{Aa}
	G5	908.0 ± 80.7 ^{Ac}	1233.2 ± 113.2 ^{Bb}	139.77 ± 14.45^{Bb}	2068.6 ± 122.1 ^{Aa}	219.73 ± 20.09^{Aa}
	G7	637.4 ± 63.1 ^{Cc}	1426.2 ± 122.9 ^{Ab}	221.3 ± 22.97^{Cb}	2295.3 ± 176.9 ^{Aa}	366.6 ± 37.2^{Aa}

The values are expressed as the mean ± standard deviation.
[1] Percentage increase of antioxidant activity observed at the end of gastric and intestinal digestion as compared to undigested lupin
Different capital letters in each column indicate significant differences between days of germination ($P < 0.05$, Tukey test).
Different lowercase letters in each column indicate significant differences between treatments ($P < 0.05$, Tukey test).

33.02–-152.21 %. At the end of GID, antioxidant activity inexperienced a significantly and further increase compared to GD and undigested samples ($p < 0.05$), with values surpassing those observed in GD. The increase in ORAC ranged from 556 to 866.6 %, ABTS showed a range of 245.58–487.4 % and DPPH showed an increase within the range of 196.06–366.6 %. Furthermore, the germination process promotes the release of bioactive compounds such as phenolic compounds, which influence the increase in antioxidant activity as germination time progresses. This is attributed to the activation of hydrolytic enzymes that can degrade the cell wall, leading to the release of these compounds (Wu et al., 2013). These secondary metabolites are expressed as a survival and adaptation mechanism against environmental factors during germination (Saltveit, 2017). The synthesis of phenolic compounds during germination depends on the activation or inhibition of multiple enzymes such as phenylalanine ammonia-lyase (PAL), chalcone isomerase (CHI), chalcone synthase (CHS), chalcone reductase (CHR), and rutin-degrading enzyme (Ling et al., 2018; Chen et al., 2022). However, it has been evidenced that the activation of PAL has a positive effect on the synthesis and accumulation of total phenolic compounds and flavonoids, thereby increasing antioxidant activity due to enhanced free radical scavenging capacity (Ren & Sun, 2014). The observed trend in antioxidant activity coincided with the concentration of phenolic compounds and flavonoids, which also increased with germination time, indicating that these metabolites contribute to the enhancement of antioxidant activity. This increase in antioxidant activity in the GID is in accordance with findings reported by other authors, demonstrated a similar pattern in the intestinal digestion phase (Chandrasekara & Shahidi, 2012; Liao et al., 2022).

Differences in the antioxidant capacity across various assays may result from the modification of certain metabolites into new structures during digestion, altering their reaction capacity and electron transfer ability. The transition from an acidic pH to an alkaline pH in the intestinal phase causes the deprotonation of hydroxyl groups present in the aromatic rings of the phenolic compounds, potentially contributing to increased antioxidant activity in GID (Seraglio et al., 2017). Furthermore, the number and position of hydrogen-donating hydroxyl groups in the aromatic rings of phenolic compounds may also influence antioxidant activity (Tagliazucchi et al., 2010). The lupin seed matrix is another factor influencing antioxidant activity, as complexes with minerals and proteins formed during digestion can alter the solubility of the compounds, resulting in an increase or decrease in the bioaccessibility of antioxidant compounds (Argyri et al., 2006).

3.5.2. *Modulation of intracellular ROS production in a model of oxidative stress*

ROS arise as a result of metabolic processes in the body, and their excessive production can lead to oxidative damage, necrotic or apoptotic cell death, and trigger various conditions, including kidney and cardiovascular diseases, among others (Honda et al., 2019; Serem & Bester, 2012; Tan et al., 2022). Neutralization and inhibition of ROS formation are possible through antioxidant compounds such as certain peptides, phenols and flavonoids. To assess the impact of germination on lupiń's ability to modulate intracellular ROS levels under oxidative stress, the initial step involved confirming absence cellular toxicity upon exposure to different doses (0.5–3 mg/mL) of lupin intestinal digestates. The intestinal digestates of G7 were chosen due to its high concentration of phenolic compounds, flavonoids and radical scavenging activity.

Fig. 3A illustrates the viability of RAW 264.7 macrophages (expressed as % of untreated cells) after 24 h treatment with different concentrations of G7. It was observed that concentrations of 0.5, 1 and 3 mg/mL of G7 resulted in cell viabilities of 81, 100 and 133 %, respectively. These findings indicate that G7 intestinal digestates did not exhibited cytotoxicity, as cell viability remained close to 100 % at all evaluated concentrations.

Under oxidative stress conditions, the intestinal digestate of sample G7 showcased remarkable antioxidant capacity, attributed to its high concentration of phenolic compounds and flavonoids, suggesting its potential to counteract ROS production and protect cells against oxidative damage. The inhibition of ROS production was evaluated over a period of 0 to 180 min, using different concentrations of the G7 intestinal digest (0.5, 1 and 3 mg/mL) (Fig. 3B). From the first minute, a 2.5 % inhibition was observed at a concentration of 3 mg/mL. Subsequently, the inhibitory potency of G7 increased after 30 min, with 3 mg/mL concentration consistently proving to be the most effective at each time interval. The efficiency of ROS inhibition depends on both the exposure time and the concentration of the G7 intestinal digest. However, no significant differences were observed between concentrations ($p > 0.05$), suggesting that inhibiting ROS production is achievable even at low concentration as 0.5 mg/mL.

Upon contact with ROS, it is conceivable that phenolic compounds, peptides and amino acids present in the intestinal digestate of G7 react with the generated ROS. Phenolic compounds, for instance, have the capacity to donate electrons to superoxide radicals, converting them into less reactive and less harmful forms. Certain amino acids, such as cysteine, and the peptides containing them, function as reducing agents by donating hydrogens/electrons to neutralize ROS free radicals, thereby stabilizing them (Sudhakar & Nazeer, 2015). This behavior aligns with the observations of (Apea-Bah et al., 2021) in their study on processed caupí beans and their digestion processes.

The inhibition of ROS formation was consistent with the results of antioxidant activity assays. Together, these outcomes indicate the ability of germinated lupin to inhibit reactive oxygen species even after

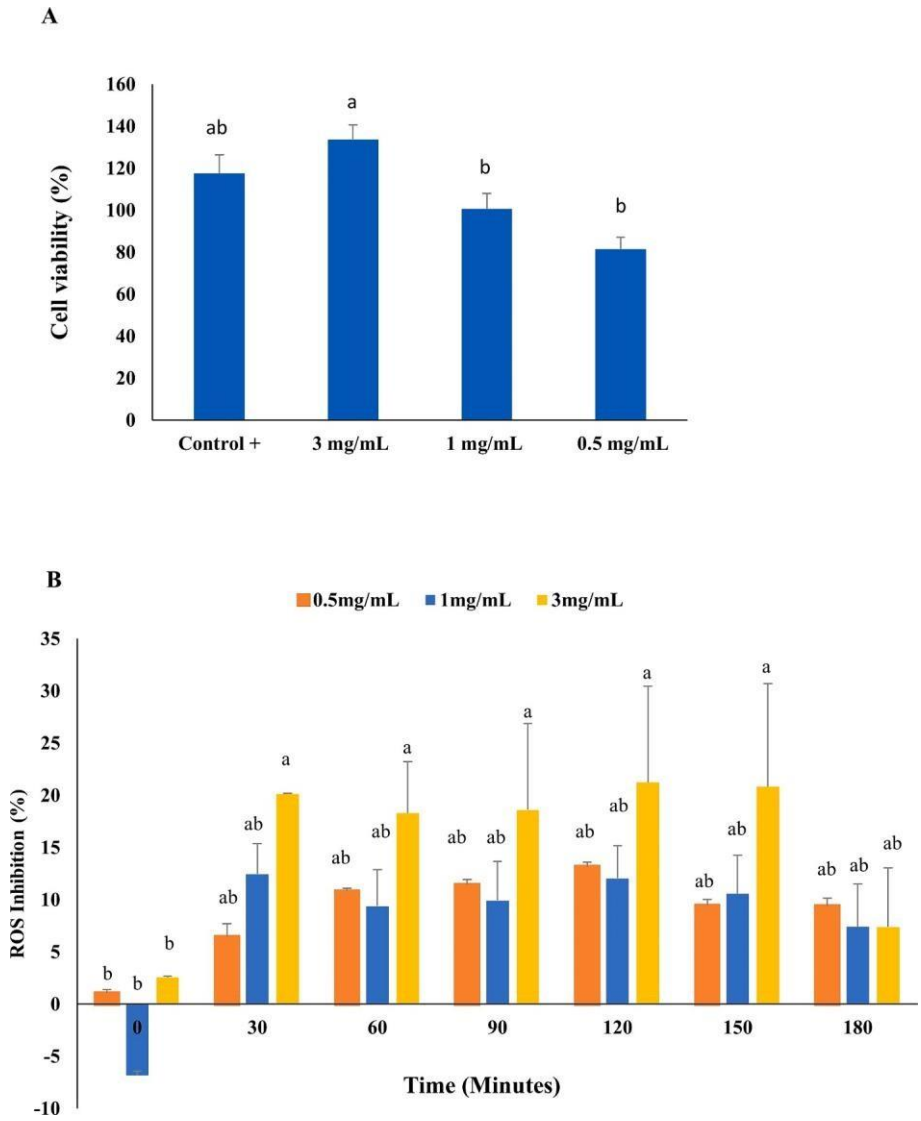

Fig. 3. Effect of intestinal digestates of *Lupinus angustifolius* germinated for 7 days (G7) at different concentrations: A) on viability of RAW 264.7 cells; and B) inhibition of reactive oxygen species in the presence of 2.5 mM t-BOOH for 3 h. The values are expressed as the mean ± standard deviation. Different letters indicate significant differences among treatments ($P < 0.05$, Tukey test).

intestinal digestion. Therefore, these results suggest that the consumption of germinated *Lupinus angustifolius* for 7 days may hold potential in preventing oxidative damage in the body. Furthermore, results also highlight the pivotal role of phenolic compounds and flavonoids bioaccessibility in mitigating the detrimental effects of ROS in the organism.

4. Conclusion

As germination of *Lupinus angustifolius* progressed, a gradual increase in the concentration of GABA, a neurotransmitter with beneficial properties for human health, was observed. This increase was especially noticeable after 7 days of germination. However, gastrointestinal digestion decreased the bioaccessibility of GABA, especially in the intestinal phase, possibly due to its degradation under acidic conditions. Germination time enhanced the concentration of total phenolic compounds in *Lupinus angustifolius*, with a bioaccessibility 82.56 % at the end of gastrointestinal digestion. The acidic pH of the stomach and the interaction with other digestive components may contribute to its bioaccessibility. Flavonoids showed an increase during germination time, with a bioaccessibility up to 114.20 % at the end of gastrointestinal digestion possibly linked to solubility and interaction with other digestion components. Phenolic compounds analysis unveiled intriguing findings. The presence of glycosylated flavonoids in the germinated samples suggested greater solubility and availability in the intestinal phase. Some isoflavones synthesized during germination persisted through gastrointestinal digestion, while others, like genistein, showed a significant decrease post-digestion. This observation is noteworthy in terms of biosynthesis, resistance to gastrointestinal digestion and predominant forms, contributing to antioxidant activity, which increased with germination time in undigested samples. Gastric digestion further improved antioxidant activity in the samples. *Lupinus angustifolius* germination emerges as a beneficial process to increase the concentration of bioactive compounds, such as GABA, phenolic compounds, flavonoids and isoflavones. However, gastrointestinal digestion significantly impacts the bioaccessibility and availability of these compounds, emphasizing the importance of understanding how gastrointestinal processes influence absorption and efficacy of these compounds in the body. These findings not only deepen our understanding of how germination and digestion impact the bioaccessibility of bioactive compounds but also underscores their potential effects on human health. Furthermore, the results highlight the potential of lupin germinated for

7 days in preventing oxidative damage in the body. The inhibition of ROS formation aligns with the results of antioxidant activity assays, underscoring the importance of bioaccessibility of phenolic compounds and flavonoids to mitigate the detrimental effects of ROS in the body.

CRediT authorship contribution statement

Fabiola Araceli Guzmán-Ortiz: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Ciro Baruchs Muñoz-Llandes:** Visualization, Data curation. **Cristina Martínez-Villaluenga:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114426.

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Conclusión

La germinación es una técnica eficiente que permite modificar la calidad nutricional de las semillas de *Lupinus angustifolius*. Durante la germinación de esta semilla se modifica el perfil electroforético generando fracciones de menos peso molecular con marcado interés farmacológico. Por otra parte, se disminuyen la concentración de compuestos antinutricionales como inhibidores de tripsina y ácido fítico; generando una alternativa de ingredientes modificados para su uso el desarrollo de nuevos alimentos con potenciales beneficios a la salud humana.

El uso de harinas de *Lupinus angustifolius* germinado en el desarrollo de botanas mediante la tecnología por extrusión es una alternativa viable que permite obtener botanas con características fisicoquímicas, nutricionales distintas dependiendo del tiempo de germinación.

Las botanas desarrolladas con harinas de Lupinus germinado a diferentes tiempos evidenciaron propiedades microestructurales especificas evidenciando interacciones entre proteínas y el almidón de maíz utilizado, modificando las propiedades de viscosidad y térmicas generando cambios en la digestibilidad *in vitro* del almidón y la proteína.

ANEXOS

Artículo de divulgación

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Germinación: un método de bioproceso que incrementa la calidad nutricional, biológica y funcional de harinas de leguminosas

Germination: a bioprocessing method that improves the nutritional, biological and functional quality of legume flours

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Resumen

La germinación de semillas de leguminosas es una técnica eficaz, rápida y económica que permite mejorar la calidad nutricional de las harinas obtenidas a partir de las mismas, se ha demostrado un aumento en la concentración de nutrientes, como proteínas, fibra y vitaminas. También se ha evidenciado una disminución de compuestos anti nutricionales como ácido fítico, lectinas, saponinas, alcaloides entre otros. Además, se ha reportado una mejora en la funcionalidad biológica, debido a la generación de péptidos de bajo peso molecular, con efecto antioxidante, antiinflamatorio, antidiabético, antiadipogénico, antimicrobiano e incluso anticancerígeno. De la misma forma se mejoran propiedades tecnofuncionales de las harinas (absorción de agua y aceite, propiedades de emulsificación y formación de espuma, densidad aparente, etc.) lo que las convierte en un ingrediente de interés para la industria alimentaria, potencializando su aplicación en el desarrollo de alimentos funcionales con beneficios a la salud humana.

Palabras Clave:
Germinación, Leguminosas, Calidad nutricional, Propiedades funcionales

Abstract

The germination of legume seeds is an effective, fast and economical technique that allows to improve the nutritional quality of the flours obtained from them, it has an increase in the concentration of nutrients, such as proteins, fiber and vitamins, it has also been evidenced a decrease in anti-nutritional compounds such as phytic acid, lectins, saponins, alkaloids, among others. An improvement in biological functionality has also been reported, due to the generation of low molecular weight peptides, with an anti-inflammatory, antidiabetic, antiadipogenic, antimicrobial and even anticancer effect. In the same way, the techno functional properties of flours are improved. (absorption of water and oil, emulsification and foam formation properties, bulk density, etc.) is achieved, making conversions an ingredient of interest to the food industry, enhancing their application in the development of functional foods with benefits to human health.

Keywords:
Germination, Legumes, Nutritional quality, Functional properties

1. Introducción

Las leguminosas son altamente consumidas en el mundo debido a su elevado aporte proteico, sin embargo, su calidad nutricional se encuentra limitada por una baja disponibilidad y digestibilidad de nutrientes, así como la presencia de compuestos antinutricionales, es por eso el interés en encontrar técnicas como la germinación, que mejoren dichas limitaciones para potenciar su aprovechamiento (Ghorphade and Kadam, 1989; Urbano *et al*., 2005; Vidal-Valverde *et al*., 2002). La germinación es la reanudación del ciclo de vida, donde la semilla bajo condiciones óptimas de humedad, tiempo y temperatura se convierte en plántula (Sangronis and Machado, 2007). Este proceso biológico comienza con la absorción de agua por la semilla, iniciando una serie de eventos metabólicos complejos; dicho método se ha utilizado para obtener harinas con características mejoradas en

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comparación a las obtenidas de semillas sin germinar, dicho efecto se ha estudiado en harinas obtenidas de semillas de leguminosas convencionales como haba, garbanzo, frijol, lenteja soya, lupinus etc., (Masood *et al*., 2014; Ghavidel and Prakash, 2007; Fouad and Rehab, 2015; Mostafa *et al*., 1987).

2. Incremento de la calidad nutricional

La germinación mejora la calidad nutricional de las harinas obtenidas a partir de leguminosas, sin embargo, el incremento o decremento de biomoléculas y compuestos nutricionales, dependerá del tipo de leguminosa tratada, así como de las condiciones de germinación principalmente tiempo y temperatura (Sangronis and Machado, 2007; Sibian *et al*., 2017).

La germinación aumenta la digestibilidad de proteínas y carbohidratos, el contenido de vitaminas del complejo B, fibra dietaria y minerales, la biodisponibilidad de aminoácidos, además reduce factores anti nutricionales (saponinas, alcaloides, polifenoles, alfa galactósidos, fitatos e inhibidores de tripsina) (Mbithi *et al*., 2001; Kaushal *et al*., 2012). El remojo de la semilla previo al proceso de germinación, también tiene un efecto sobre la reducción de sustancias no nutritivas (Mbithi *et al*., 2001).

Reducir de manera parcial o total los compuestos antinutricionales de semillas de leguminosas es de vital importancia, debido a la interacción que tienen con otros nutrientes impidiendo así su aprovechamiento. Aguilera et al., (2013) reportaron una disminución de lectinas cuando germinaron frijol durante 5 días hasta alcanzar una concentración de 2.5 mg / 100 g, mejorando el valor biológico y nutricional del frijol debido a que las lectinas son glucoproteínas que interactúan con carbohidratos formando aglutinaciones y por lo tanto impiden su absorción al cruzar el tracto gastrointestinal. Benítez *et al*., (2013) analizaron harinas de soja germinada y no germinada para cuantificar los fosfatos de inositol, demostrando que la germinación afecta positivamente el factor antinutricional, logrando una reducción en la concentración del 70%. En consecuencia, se elimina la capacidad quelante de los fitatos y el resto de los nutrientes tienen un mejor aprovechamiento.

Estudios similares han demostrado que los inhibidores de la tripsina del frijol se reducen en un 27 y 84% después de 2 y 3 días de germinación respectivamente (Hobday *et al*., 1973). En lentejas, se ha reportado reducciones entre 21 y 54 % después de 4 y 6 días de germinación, respectivamente (El-Mahdy *et al*., 1985). Esto es probable porque los inhibidores se utilizan como fuente de energía para el desarrollo de las plántulas (Vidal-Valverde and Frias 1992). Por otra parte, los porcentajes de reducción reportados para garbanzo germinado durante 3–6 días son 24–83% respectivamente (Guardado-Félix *et al*., 2017) y en soja, 17–23% de reducción de inhibidores de tripsina también se ha reportado en los mismos días de germinación (Warle *et al*., 2015). La reducción de compuestos antinutricionales de las legumbres podría atribuirse a la movilización y activación enzimática, así como a la degradación de proteínas, incluidos los inhibidores de la tripsina, durante la germinación de la semilla (Benítez *et al*., 2013).

3. Generación de compuestos con actividad biológica

Durante la germinación, se genera una proteólisis catalizada por las endopeptidasas presentes en los cuerpos proteicos promoviendo la degradación de las proteínas de almacenamiento (Hobday *et al*., 1973; El-Mahdy *et al*., 1985). Esto implica que a partir de las proteínas de almacenamiento se generan fracciones peptídicas de menor peso molecular, estos péptidos pueden tener propiedades fisiológicas específicas con potenciales beneficios para la salud, denominados péptidos bioactivos (Vidal-Valverde and Frias 1992). De tal manera que la germinación podría mejorar las propiedades nutricionales y nutraceúticas de las leguminosas al modificar el contenido de los diferentes metabolitos y, en particular, generando péptidos y aminoácidos con posible actividad biológica (Frias *et al*., 1995).

Vernaza *et al*., (2012) reportaron un aumento significativo ($p < 0.05$) en la capacidad antioxidante en harinas obtenidas a partir de soya geminada durante 72 h a 26°C en comparación a la harina de semillas no germinadas. También se ha reportado que el proceso de germinación de frijol común influye positivamente en la generación de péptidos bioactivos, con potentes propiedades antioxidantes capaces de inhibir la degeneración oxidativa causada por los radicales peroxilo, que desencadenan varias enfermedades, incluidas las enfermedades cardiovasculares y la diabetes (El-Mahdy *et al*., 1985; Satyanarayana *et al*., 2011).

De Souza Rocha *et al*., (2014) reportaron la generación de péptidos capaces de inhibir la expresión de genes involucrados en el desarrollo de diabetes tipo 2 a partir de semillas de frijol común (*P. vulgaris*) germinado durante 48 h a 25°C.

Por otra parte, González-Montoya *et al*., (2018) comprobaron que péptidos de entre 5-10 kDa obtenidos a partir de soya germinada durante seis días son más efectivos para inhibir la proliferación de cáncer de colón. La apoptosis podría ser uno de los mecanismos de acción que explican el efecto anti proliferativo de los péptidos de soya germinados en líneas celulares de colón humano.

4. Incremento en la calidad tecnofuncional

Cuando se activan enzimas hidrolíticas por efecto de la germinación, comienza la desintegración de macromoléculas, principalmente proteínas y carbohidratos, modificando las propiedades tecnofuncionales de las harinas de manera directa (Megat-Rusydi et al., 2011). Identificar y conocer las propiedades tecno-funcionales específicas de las harinas de leguminosas es esencial para determinar sus posibles usos como ingredientes alimentarios. Además, encontrar harinas con propiedades tecno funcionales ideales, a partir de leguminosas no convencionales es de interés para la industria alimentaria para desarrollar alimentos que no contengan proteínas alergénicas o bien sustituir las existentes (Kaur et al., 2015).

Con el fin de incrementar el perfil nutricional de leguminosas, así como su consumo, investigadores realizan esfuerzos continuos que permitan utilizar dichos ingredientes modificados, sin embargo, es necesario conocer las propiedades tecnofuncionales y caracterizar las harinas obtenidas a partir de las mismas (Granito et al., 2004). Se ha reportado que durante la germinación de leguminosas ocurren cambios importantes que afectan las propiedades tecnofuncionales, por ejemplo, la capacidad de absorción de agua se incrementa de manera positiva, y esto se debe a la desnaturalización de nutrientes como carbohidratos y proteínas, incrementando el número de moléculas que pueden interactuar con el agua disponible (Kaushal et al., 2012; Lawal, 2004). Mayor capacidad de absorción de agua se ha reportado en harinas de leguminosas con mayor contenido proteico y esto se

debe a la propia capacidad de las proteínas de interactuar con el agua (Seena & Sridhar, 2005).

Por otro lado, la capacidad de absorción de aceite es también la función propia de las proteínas de interaccionar con otros lípidos, sin embargo, dependerá del tipo de aminoácido presente en las moléculas de proteína y a los cambios generados por efecto de la germinación (Olelakan and Bosede, 2010). Los aminoácidos hidrofóbicos tienden a absorber más aceite y, por lo tanto, conducen a una alta capacidad de absorción de aceite. El incremento o decremento general en el contenido proteico de las harinas durante la germinación puede causar la exposición de aminoácidos hidrofóbicos e hidrofílicos, que a su vez aumentan la capacidad de absorción de aceite y agua después de la germinación (Rangel et al., 2003; Robertson et al., 2000).

La germinación mejora las propiedades funcionales de las proteínas, reduciendo la tensión superficial entre la interface agua y aire, mejorando así la capacidad de formación de espuma de las harinas resultantes. La capacidad emulsificante y la estabilidad de la emulsión formada, dependerá de la naturaleza anfifílica de la proteína en la harina obtenida, así como de los cambios en la concentración de lípidos y proteínas durante la germinación ya que se trata de una interacción única entre lípido-proteína (Seena and Sridhar, 2005).

5. Conclusión

La germinación resulta ser un método de bioproceso capaz de mejorar la calidad nutricional y funcional de las harinas obtenidas a partir de leguminosas en comparación a las resultantes de leguminosas sin germinar, convirtiendo dicho proceso en una alternativa económica y eficaz para generar ingredientes modificados con potencial uso en el desarrollo e innovación de alimentos funcionales con múltiples beneficios a la salud humana. Además, el incremento en las propiedades tecno funcionales podría permite la optimización de tiempos y costos de producción a gran escala.

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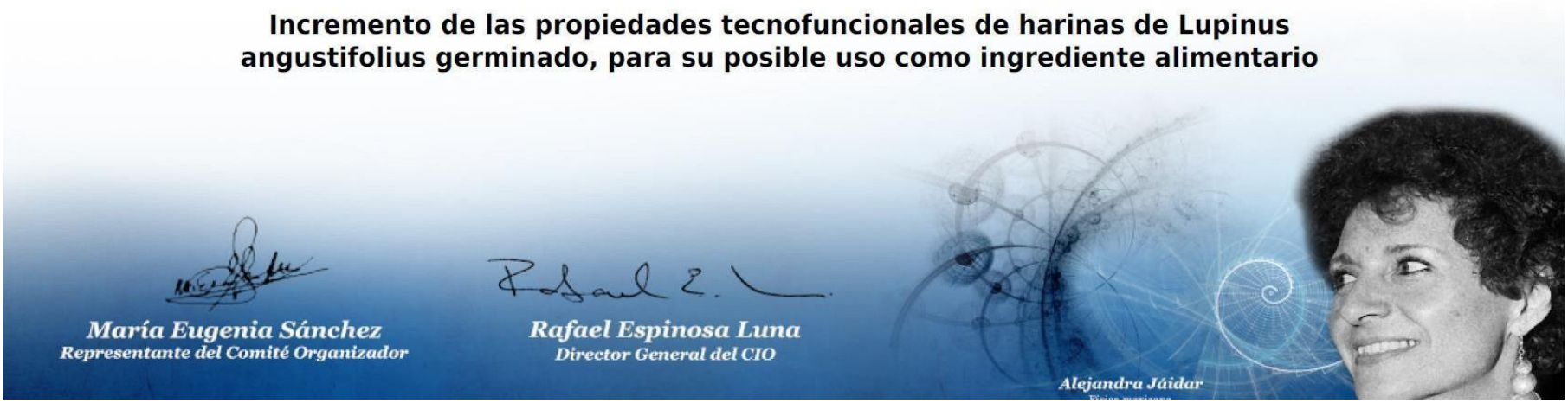
Por el trabajo:

Incremento de las propiedades tecnofuncionales de harinas de Lupinus angustifolius germinado, para su posible uso como ingrediente alimentario



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Candidato a doctor en el programa Ciencias de los Alimentos y Salud Humana de la UAEH

Por haber impartido la charla titulada es: " Semillas germinadas ¿buenas para la salud?" como una actividad de divulgación de la ciencia dentro del programa: ¡Una chela por la ciencia!

Miguel Severino Ángeles
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David Severino Ángeles
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Asunto: CONSTANCIA

A QUIEN CORRESPONDA

Por medio de la presente, se hace constar que el M.C. Ciro Baruchs Muñoz Llandes impartió el curso de "Uso de aguamiel en la producción de queso Oaxaca y panela". El curso de 8 horas fue dictado a productores de aguamiel de la región del Cardonal en el Estado de Hidalgo en las instalaciones de la planta piloto del edificio de química en alimentos del Área Académica de Química del Instituto de Ciencias Básicas e Ingeniería perteneciente a la Universidad Autónoma del Estado de Hidalgo. El M.C. Muñoz Llandes fue invitado en el marco de las actividades del segundo periodo técnico del proyecto de soberanía alimentaria de CONHACYT "Valorización del aguamiel producido en comunidades del Estado de Hidalgo: producción sustentable de jarabe rico en oligofructanos destinado a sectores económicos medio y medio alto", el día 23 de agosto de 2023.

Se extiende la presente a los siete días del mes de mayo de dos mil veinticuatro para los fines que al interesado convengan

Atentamente
"Amor Orden y Progreso"

Dr. Luis Guillermo González Olivares
Coordinador de Investigación y
Posgrado del ICBI

INSTITUTO DE CIENCIAS BASICAS E INGENIERIA
UAEH
INVESTIGACION

LGGO/SEPC

Ciudad del Conocimiento, Carretera Pachuca-Tulancingo Km. 4.5 Colonia Carboneras, Mineral de la Reforma, Hidalgo, México. C.P. 42184
Teléfono: 52 (771) 71 720 00 Ext. 2236 Fax 2109
invyposgrado_icbi@uaeh.edu.mx
uaeh.edu.mx

Col. Los naranjos, Mineral del Chico, Hidalgo, México

29 de septiembre de 2023

ASUNTO: CONSTANCIA

Por medio de la presente se hace constar que el M. C. Ciro Baruchs Muñoz Llandes impartió la capacitación "Desarrollo de productos lácteos enriquecidos con espirulina". El curso de 8 horas fue impartido a habitantes de la comunidad, Los Naranjos en Mineral del Chico, Hidalgo. El maestro Muñoz-Llandes fue invitado en el marco del proyecto "Impacto socioeconómico y nutrimental con incidencia social participativa en la producción de espirulina en municipios con alta carencia alimentaría"

Se extiende la presente constancia a los quince días de junio de 2024, Mineral del Chico, Hidalgo.

Vo.Bo.

C. Gumaro Godínez Hernández

Representante comunitario

Estancias

EDUCACIÓN
SECRETARÍA DE EDUCACIÓN PÚBLICA

Instituto Politécnico Nacional
"La Técnica al Servicio de la Patria"

Secretaría de Investigación y Posgrado
Centro de Nanociencias y Micro y Nanotecnologías

Folio
CNMN/STT/0147/2023.

Asunto
Carta de Término - UPS.

2023. Año de Francisco Villa
30 Aniversario de la Declaración sobre
la Eliminación de la Violencia contra la Mujer (ONU)
60 Aniversario del CECyT 7 "Cuauhtémoc" y del CENAC
90 Aniversario de la Escuela Superior de Ingeniería Textil
40 Aniversario del CIIDIR, Unidad Oaxaca

Ciudad de México, 04 de diciembre de 2023

Dra. Fabiola Araceli Guzmán Ortiz
Investigadora Cátedra CONAHCyT de la
Universidad Autónoma del Estado de Hidalgo
PRESENTE

Por este medio, se hace constar que, el alumno

Alumno: Ciro Baruchs Muñoz Llandes
Cursando el: Doctorado en Ciencias de los Alimentos y Salud Humana
Matrícula: 195771

Ha concluido satisfactoriamente su **Estadía de Investigación** en un período del **04 al 30 de septiembre del presente año**, con un horario de 09:00 a 17:00 horas de lunes a viernes.

Desarrollando el Proyecto denominado: *"Análisis microestructurales de la semilla de Lupinus a angustifolius sometida a diversos procesos, como germinación, extrusión y digestion gastrointestinal"*, a cargo de la Dra. María de Jesús Perea Flores, Especialista de Microscopía Confocal-Multifotónica y Preparación de Muestras del Laboratorio Nacional Multidisciplinario de Caracterización de Materiales y Nanoestructuras (LMCMN).

La estancia cumplió las tareas académicas y experimentales, así como la ejecución de los conocimientos teóricos-prácticos adquiridos en su formación académica.

Sin más por el momento, quedo de Usted como su atento y seguro servidor.

ATENTAMENTE
"La Técnica al Servicio de la Patria"

S.E.P.
INSTITUTO POLITECNICO NACIONAL
CENTRO DE NANOCIENCIAS Y
MICRO Y NANOTECNOLOGÍAS
DIRECCIÓN-

Dr. Oscar Camacho Nieto
Director

OCN/[illegible]

c.c.p. Dra. María de Jesús Perea Flores, Especialista de Microscopía Confocal-Multifotónica y Preparación de Muestras.

UNIVERSIDAD DEL PAPALOAPAN

DIVISIÓN DE ESTUDIOS DE POSGRADO

San Juan Bautista Tuxtepec, a 03 de abril de 2024
Asunto: **CARTA DE TERMINACIÓN**

Dra. Eva María Santos López
Dra. Esther Ramírez Moreno
Dra. Deyanira Ojeda Ramírez

Coordinadores DCASH

PRESENTE

Por medio de la presente, hago constar que el alumno **Ciro Baruchs Muñoz Llandes,** con número de **CVU 929811** y matrícula 195771 estudiante del **Doctorado en Ciencias de los Alimentos y Salud Humana (PNPC: 004298)** de la **Universidad Autónoma del Estado de Hidalgo** ha culminado de forma satisfactoria su estancia de investigación, en los laboratorios de la **Universidad del Papaloapan** con el proyecto de tesis titulado "Evaluación bioactiva de harinas de *Lupinus angustifolius* germinado".

La estancia comprendió el periodo del 2 de octubre del 2023 al 22 de marzo del presente año, culminando la tereas académicas y experimentales, así como los conocimientos adquiridos en su formación académica.

Sin más por el momento, les envió saludos cordiales.

ATENTAMENTE
Tuxtepec, Oaxaca, a 03 de abril de 2024

Dra. Rubí Guadalupe Utrilla Coello