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Assessment of the antioxidant and antimicrobial activity of hydrolysates from lupine (*Lupinus mutabilis*) flour

Evaluación de la actividad antioxidante y antimicrobiana de hidrolizados de harina de chocho (*Lupinus mutabilis*)

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Abstract

Lupine (*Lupinus mutabilis*) is a seed commonly used as a staple food in the Mediterranean region and South America, particularly in Ecuador, where it has been consumed for centuries. Known for its high protein content (45%), lupine could serve as an important source for producing protein isolates, concentrates, and hydrolysates with bioactive capacities. To address this potential, our research aimed to evaluate the effect of the type of flour and hydrolysis conditions (enzyme and time) on the antioxidant and antimicrobial capacities of lupine hydrolysates. Thus, two types of flour were prepared: raw flour (RF) and debittered and defatted flour (DDF). Subsequently, enzymatic hydrolysis was conducted using three different enzymes (pancreatin, papain, and endopeptidase), and the hydrolysates were collected at various time points: 4 and 48 hours. Antioxidant capacity was assessed using the Oxygen Radical Absorption Capacity (ORAC) test and the Ferric Reducing Antioxidant Power (FRAP) test. Additionally, antimicrobial capacity was evaluated by measuring the inhibition of *Staphylococcus aureus* and *Escherichia coli*. The results showed hydrolysates treated with pancreatin at 4 hours from raw flour exhibited the highest antioxidant capacity, while debittered and defatted hydrolysates treated with pancreatin at 48 hours demonstrated the highest antimicrobial capacity. Overall, these findings highlight lupine as a valuable source for obtaining hydrolysates with antioxidant and antimicrobial properties. Furthermore, the type of flour and hydrolysis conditions (enzyme and time) significantly affected the bioactive capacities.

Keywords: Hydrolysis. Proteolytic enzyme. ORAC. FRAP. Antimicrobial inhibition.

Resumo
El chocho (*Lupinus mutabilis*) es una semilla comúnmente utilizada como alimento básico en la región Mediterránea y América del Sur, particularmente en Ecuador, donde se consume desde hace siglos. Conocido por su alto contenido de proteínas (45%), el lupino podría servir como una fuente importante para la producción de aislados, concentrados e hidrolizados de proteínas con capacidades bioactivas. Para abordar este potencial, nuestra investigación tuvo como objetivo evaluar el efecto del tipo de harina y las condiciones de hidrólisis (enzima y tiempo) sobre las capacidades antioxidantes y antimicrobianas de los hidrolizados del chocho. Así, se prepararon dos tipos de harina: harina cruda (RF) y harina desamargada y desgrasada (DDF). Posteriormente, se realizó una hidrólisis enzimática utilizando tres enzimas diferentes (pancreatina, papaína y endopeptidasa) y los hidrolizados se recogieron en varios momentos: 4 y 48 horas. La capacidad antioxidante se evaluó mediante la prueba de capacidad de absorción de radicales de oxígeno (ORAC) y la prueba de poder antioxidante reductor férrico (FRAP). Además, se evaluó la capacidad antimicrobiana midiendo la inhibición de *Staphylococcus aureus* y *Escherichia coli*. Los resultados mostraron que los hidrolizados de lupino crudo exhibieron la mayor capacidad antioxidante, mientras que los hidrolizados desamargados y desgrasados demostraron la mayor capacidad antimicrobiana. En particular, los hidrolizados obtenidos con pancreatina mostraron las actividades biológicas más prometedoras a las 48 horas. En general, estos hallazgos destacan al lupino como una fuente valiosa para la obtención de hidrolizados con propiedades antioxidantes y antimicrobianas. Además, el tipo de harina y las condiciones de hidrólisis (enzima y tiempo) afectaron significativamente las capacidades bioactivas.
Palavras-chave: Hidrólisis. Enzima proteolítica. ORAC. FRAP. Inhibición antimicrobiana.

1. Introduction

Lupine (*Lupinus mutabilis*) that is a food crop belongs to the Fabaceae family, which comprises more than 200 species, consumed for centuries in the Mediterranean region and South America (Tahmasian *et al*., 2022). Local communities traditionally consume lupine in the Andean region due to its health benefits and nutrient content. Over the years, researchers have explored the beneficial effects of this crop, resulting in increased cultivation and trade, both for animal feeding and human consumption (Boukid & Pasqualone, 2022).

Nutritionally, Lupinus seeds are characterized for a high protein and fat content (45% and 16% DM, respectively) (Czubinski *et al*., 2021). Thus, several studies have proved various techniques to produce lupine protein isolates, concentrates, and hydrolysates rich in bioactive peptides, used in healthy food development and nutraceuticals. Moreover, consuming lupine protein is linked to various health advantages, including lowering blood sugar, reducing cholesterol levels, providing antioxidants, and helping prevent cardiovascular diseases (Boukid & Pasqualone, 2022).

Hydrolysates can be produced by the enzymatic hydrolysis, which breaks out the protein structure to smaller fractions (containing between 2 and 20 amino acids), known as peptides (Montesano *et al*., 2020). Thus, the bioactivity capacity of the hydrolysates depends upon the peptides final structure (Wattanasiritham *et al*., 2016). The proteolytic enzyme (e.g., serine proteases, savinases proteases and cysteine proteases) are used commonly during the hydrolysis to generate peptides with different molecular weights and activate functional groups responsible for one or several bioactivities (Okagu *et al*., 2021).

The hydrolysis main issue is the interference of other molecules, which can hinder the accessibility of the enzymes to the specific amino acids sequence during the breakup of the protein. One of the lupine hydrolysis interferences is the seed fat content, which could hamper the release of bioactive peptides due to the formation of complexes of starch-lipid-protein (Wang *et al*., 2020). Additionally, the presence of anti-nutritional compounds (e.g., alkaloids) could be a drawback for consuming lupine-derived products (Cortés-Avendaño *et al*., 2020). However, several techniques such as boiling, soaking, and debittering can successfully remove both fat and alkaloids (Cortés-Avendaño *et al*., 2020; Teng *et al*., 2016).

Several literature reports demonstrated lupine seeds are a valuable source of protein associated with several health benefits. For instance, *Lupinus angustifolius L*. hydrolysates can promote insulin secretion and enhance glycolytic metabolism (Tapadia *et al*., 2019). Similarly, enzymatic protein hydrolysates derived from *L. albus*, *L. angustifolius*, and *L. luteus* were shown to possess ACE-inhibitory activity (Boschin *et al*., 2014). However, there remains a gap in research regarding the antioxidant and antimicrobial capacity of lupine hydrolysates. Therefore, the present study aimed to assess the effects of lupine flour pretreatment and hydrolysis conditions (enzyme and time) on the antioxidant and antimicrobial capacities of its hydrolysates.

2. Materials and methods

2.1 Sample preparation.

Lupine seeds were sourced from a local market in Quito, Pichincha, Ecuador. Upon arrival at the laboratory, the seeds were hand-washed to remove any impurities and stored at 8°C. The reagents used were of analytical grade.

2.2 Preparation of Raw flour (RF) and Debittered and Defatted flour (DDF)

To obtain the raw flour (RF), the lupine seeds were ground using a mill (Alpine 180UPZ Hosokawa, Germany) until an average particle size of 0.24 mm was achieved. The resulting flour was then hermetically stored in aluminum bags at 8°C.

2.2.1 Debittering process: The lupine seeds were soaked in distilled water at a ratio of 1:2.5 ($kg.L^{-1}$) for 18 hours at 18°C. Afterwards, the grains were boiled for 1 hour at 92°C. The seeds were rinsed in distilled water for six days, with the water changed every eight hours. Finally, the debittered seeds were dried at 40°C for 8 hours until they reached a moisture content of 10%. The seeds were ground to an average particle size of 0.24 mm, and the resulting flour was hermetically stored in aluminum bags at 8°C (Villacrés *et al*., 2008).

2.2.2 Defatting process: The debittered flour was mixed with hexane in a ratio of 1:5.7 ($kg.L^{-1}$) and sonicated using an Ultrasonic Processor GEX750 (Scientz, China) for 30 minutes at 400 W and 12 kHz. Subsequently, it was heated at 50°C in a water bath (Precision Scientific model 25, Scientific Senna, Mexico) for 2 hours and then centrifuged at 45,000 g (4,000 rpm) for 15 minutes at 10°C (Thermo Scientific IE-CL-31R, Scientific Senna, Mexico). Finally, the precipitate was transferred into a Laboratory Fume Hood and dried for 3 consecutive days at 18°C. The resulting debittered and defatted flour was hermetically stored in aluminum bags at 8°C (Castejón *et al*., 2018).

2.3 Characterization of RF and DDF

2.3.1 Chemical composition of RF and DDF: The moisture, ash, fat, protein, carbohydrates, and dietary fiber contents of the RF and DDF were determined according to AOAC methods (2005).

2.3.2 Total alkaloids quantification: The total alkaloid content was determined by colorimetry following the method described by Barlog (2014) with some modifications. Thus, 20 mg of flour was mixed with 40 µL of 15% NaOH for 15 minutes. Then, 2 mL of 99.4% chloroform was added and left in darkness for 24 hours at 20 °C. The next day, 2950 µL of chloroform was added to 50 µL of the mixture. Subsequently, 1 mL of BBT (a mixture of borax buffer, pH 8.0, and 3% bromothymol blue solution 0.001 M NaOH at a ratio of 24:1 $w.v^{-1}$) was added and shaken for 30 minutes. The aqueous phase (blue color) was removed by pipetting. Finally, 2 mL of 0.002 M NaOH (in methanol) was added to the organic phase, and the samples were measured at a wavelength of 595 nm using a spectrophotometer (Shimadzu UV-160A-VIS, Nepa Refurbishments, USA). A standard curve was prepared at concentrations between 1.25 and 10 µg to assess the quantity of alkaloids, which were expressed as alkaloids per gram of flour on a dry weight.

2.4 Hydrolysis of RF and DDF

The hydrolysis of both RF and DDF was performed according to methods described by Montoya-Rodríguez *et al*. (2014) and Salami *et al*. (2010). Initially, the flour was mixed with distilled water at a ratio of 1:10 ($w.v^{-1}$). Two consecutive hydrolysis steps were then carried out: first, cellulolytic hydrolysis to break down cellulose and hemicellulose in the matrix, followed by proteolytic hydrolysis to break down proteins. We used 150 ($mgEnzyme.kgSubstrate^{-1}$) of the enzyme at pH 4.5 (adjusted with 3 M citric acid) for 1 hour at 50°C. Subsequently, proteolytic hydrolysis was performed using 200 ($mgEnzyme.kgSubstrate^{-1}$) of each enzyme: pancreatin, papain, and endopeptidase. The conditions for each hydrolysis were as follows: 50°C and pH 7.40 for pancreatin, 40°C and pH 7.5 for papain, and 50°C and pH 5 for endopeptidase. The initial pH was adjusted using 3 M sodium hydroxide solution. The samples were taken at 4 and 48 hours to evaluate the antioxidant and antimicrobial capacities of the hydrolysates. The hydrolysates were dried using freeze-drying until they reached a moisture content of 2%.

2.5 Protein content of the hydrolysates

The protein content was determined by the Kjeldahl method.

2.6 Antioxidant capacity of hydrolyzed FR and DDF

The FRAP and ORAC methods were performed using aqueous extracts. For these assays, 500 mg of the concentrated hydrolysates were mixed with 25 mL of distilled water. They were placed in a shaker incubator (ACB LABOR, series 103457120, Electro lab, Brazil) set at 150 g (200 rpm) for 30 minutes at 5 °C. Subsequently, they were centrifuged at 220,000 g (9,000 rpm) for 15 minutes at -10 °C using a centrifuge (5810R, Eppendorf, Germany). Finally, the samples were filtered and refrigerated at -10 °C.

2.6.1 Ferric Reducing Antioxidant Power (FRAP): The FRAP assay was conducted according to the methodology described by Drummond e Silva *et al*. (2017). Initially, 30 µL of sample extract was mixed with 90 µL of water and 900 µL of FRAP reagent which consisted of 450 µL of acetate buffer (0.3 M, pH 3.6), 225 µL of 20 mmol HCl, and 225 µL of 20 mmol FeCl3, in an Eppendorf tube. The mixture was incubated for 30 minutes at 37°C. Finally, the absorbance at 595 nm was measured using a spectrophotometer (EpochTM, Gen5, BioTek, USA). To quantify the antioxidant capacity, a calibration curve was prepared with Trolox (0.5-3 mM). The results were expressed as mmol of Trolox equivalents per gram of protein ($\text{mmol TE} \cdot \text{g protein DryWeight}^{-1}$).

2.6.2 Oxygen Radical Absorbance Capacity (ORAC): The ORAC assay was performed according to the protocol developed by Drummond e Silva *et al*. (2017). Firstly, 20 µL of sample extract was mixed with 120 µL of sodium fluorescein in potassium phosphate buffer (final concentration: 0.3 µg/mL, pH 7.4). Afterward, 60 µL of AAPH was added. Potassium phosphate was used as a blank. The absorbance of the samples was measured at 485 nm (excitation wavelength) and 520 nm (emission wavelength) using a Multimode Plate Reader (Victor X, Perkin Elmer, USA). Trolox solutions (25−500 mM) were used as standards. The antioxidant capacity was expressed as mmol of Trolox Equivalent per gram of protein ($\text{mmol TE} \cdot \text{g protein DryWeight}^{-1}$).

2.7 Antimicrobial capacity of hydrolyzed FR and DDF

The antimicrobial capacity was evaluated according to the protocol developed by García-Ruiz *et al*. (2011). Staphylococcus aureus ATCC 12600 (SA) and Escherichia coli O517:H7 ATCC 43895 (EC) were used for assessing the antimicrobial activity. Tryptic Soy Broth was used as the medium. The hydrolysate was filtered through a membrane with a pore size of 0.45 µm. Then, the microplate was prepared. The first column remained empty, and 200 µL of medium was dispensed into the second and third columns. Starting from the fourth column, 200 µL of each dilution (160,

80, 40, 20, 10, 5, 2.5, and 1.25 µL/mL) was dispensed. Afterward, 20 µL of microorganisms was added to all microplate wells, except those in the first and second columns. Once completed, the absorbances were measured at 405 nm using a plate reader (Fluostar Omega, BMG-LABTECH, USA). Finally, the microplate was incubated at 37°C for 24 hours in an incubator (Instrument MBI-363, Lab Line, Brazil), and the absorbances were measured again at 405 nm. Antimicrobial inhibition was calculated using Equation 1.

$$\% \text{ Inhibition} = \left(1 - \frac{\text{Abs,sample,m,tf} - \text{Abs,smple,m,to}}{\text{Abs,m,tf} - \text{Abs,m,to}}\right) \times 100 \quad (1)$$

where: Abs,sample,m,tf:sample absorbance with microorganism at 24 h
Abs,sample,m,to:sample absorbance with microorganism at 0 h
Abs,m,tf: microorganism absorbance at 24 h
Abs,m,to:microorganism absorbance at 0 h.

2.8 Statistical analysis

The factorial design (2^3) was utilized in this research, comprising 3 variables each with 2, 3, and 2 levels, respectively. Consequently, 12 experiments were meticulously planned, incorporating the variables of flour type (RF and DDF), enzyme type (pancreatin, papain, and endopeptidase), and hydrolysis time (4 and 48 hours). The resulting hydrolysates were assessed for their antioxidant and antimicrobial capacities. Each sample underwent three independent runs. Statistical analysis of the obtained results was conducted using analysis of variance with STATGRAPHICS Centurion XVI.I and GraphPad PRISMA.

3. Result and Discussion

Chemical characterization of the raw materials, RF and DDF flour, was carried out. Additionally, protein content, antioxidant, and antimicrobial activities of the hydrolysates were evaluated. The protein content was determined using the Kjeldahl method. Antioxidant activity was assessed through FRAP and ORAC assays, while antimicrobial capacity was measured using the Kirby-Bauer assay. The results are detailed in the following sections.

3.1 Chemical Characterization of RF and DDF

The chemical characterization, including moisture, ash, proteins, fat, dietary fiber, carbohydrates, and alkaloids, of both the raw flour (RF) and the debittered and defatted flour (DDF), is presented in Table 1.

Table 1 – Chemical Composition of Raw Flour (RF) and Debittered and Defatted Flour (DDF).

	Sample	
Analyte	**RF** [%]	**DDF** [%]
Moisture	7.19 ± 0.00^{b}	7.70 ± 0.00^{a}
Ash	3.65 ± 0.03^{a}	1.70 ± 0.00^{b}
Protein	43.04 ± 0.68^{b}	54.10 ± 0.00^{a}
Fat	15.30 ± 0.05^{a}	2.27 ± 0.37^{b}
Dietary fiber	9.03 ± 0.93^{b}	12.30 ± 0.00^{a}
Carbohydrates	30.73 ± 0.89^{a}	23.2 ± 0.00^{b}
Alkaloids / [µg alkaloids/ g flour]	4.86 ± 0.11^{a}	2.81 ± 0.09^{b}

Results are the Mean ±Standard Deviation; ×±σ; N=3. Different letters in the same row indicate significant differences in the composition of the different flours.

Significant differences ($p < 0.05$) were observed in the chemical composition between RF and DDF, as seen in Table 1. Consequently, the results indicate potential decreases in the ash, fat, carbohydrates, and alkaloid content of DDF, attributed to the debittering and defatting processes. According to Martínez *et al*. (2020), blanching in boiling water can lead to reductions in ash, fats, carbohydrates, and alkaloid content due to leaching during the process, while Mansour *et al*., (2015) observed significant losses of soluble solids in cooked vegetables due to thermal breakdown and leaching into the water. In contrast, an increase in protein content in DDF was noted, potentially resulting from the defatting treatment, as the ultrasound phenomenon with hexane facilitates protein accessibility (Chen *et al*., 2011). Shen *et al*. (2020) further suggested the defatting process led to increased protein recovery from hemp seeds, attributed to the loss of terpenes and secondary lipid oxidation products.

3.2 Protein content of the hydrolysates

The protein contents in the raw flour, debittered and defatted flour, and hydrolysates at 4 and 48 hours are shown in Table 2.

Table 2 - The protein content measured after 4 hours and 48 hours of hydrolysis.

Flour	Protein content of flours [%]	Type of enzyme	Hydrolysis Time [hours]	Protein content of hydrolysates [%]
Debittered and defatted flour (DDF)	54.1± 0.00^{a}	Pancreatin	4	82.08±0.00^{b}
			48	82.86±0.03^{b}
		Papain	4	61.90±0.12^{c}
			48	63.85±0.34^{c}
		Endopeptidase	4	67.43±0.45^{c}
			48	58.36±0.43^{d}
Raw flour (RF)	43.04± 0.68^{b}	Pancreatin	4	54.60±0.10^{d}
			48	57.82±0.10^{d}
		Papain	4	49.97±0.20^{e}
			48	55.15±0.13^{d}
		Endopeptidase	4	55.54±0.20^{d}
			48	50.34±0.34^{d}

Results are expressed as Mean ± SD; n = 3. Different letters in the same row indicate significant differences in the composition of the different flours.

The protein content in debittered and defatted flour is higher than raw flour and its hydrolysates for any time, as seen in Table 2. This finding can be attributed to the increased protein accessibility resulting from the defatting treatment (Chen *et al*., 2011). Moreover, the protein content in the hydrolysates is higher than both the RF and the DDF. This increase could be explained by the release of proteins and peptides from the food matrix during hydrolysis (Mokni Ghribi *et al*., 2015). Additionally, pancreatin hydrolysates exhibited the higher protein content which can explain by its wide hydrolytic ability (Adamson & Reynolds, 1995). Moreover, Benítez *et al*. (2008) mentioned the highlighted pancreatin's blend of enzymes, such as trypsin and chymotrypsin, which offer diverse protein breakdown capabilities.

3.3 Antioxidant capacity of the RF and DDF

The antioxidant capacities of the hydrolysates from RF and DDF, obtained with pancreatin, papain, and endopeptidase at 4 and 48 hours, are shown in Figures 1.

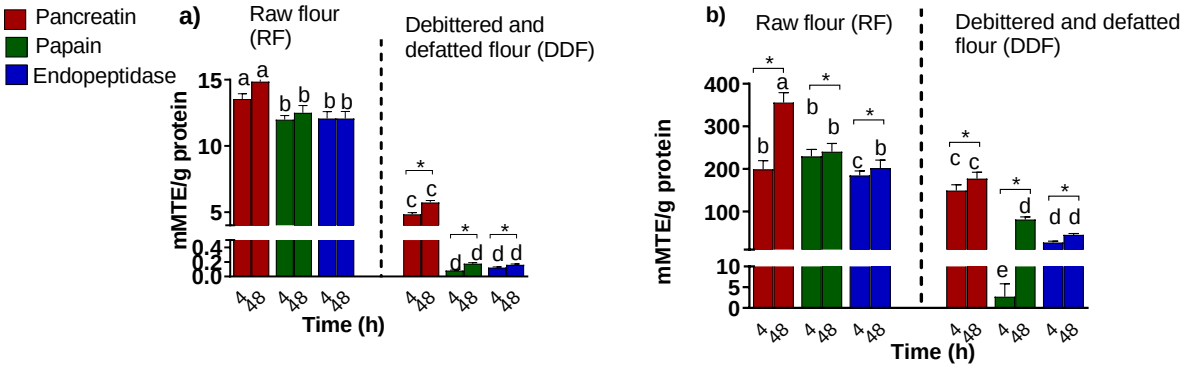

Figure 1 - Antioxidant capacity hydrolysates from RF and DDF at 24 and 48 hours evaluated for a) FRAP assay and b) ORAC assay.

$\bar{x} \pm \sigma$; n = 3. In each antioxidant assay, letters above bars indicate significant difference between treatments (LSD ≤ 0.05) and * indicate significant difference between time.

3.3.1 Effect of pretreatment: The antioxidant capacity of the hydrolysates from RF is higher than that of the pre-treated lupine flour, as seen in Figure 1. This finding can be explained by the removal of antioxidant compounds such as phenols during the debittering and defatting process (Shen *et al*., 2020). Additionally, the higher antioxidant capacity exhibited by the hydrolysates from RF can be explained by the alkaloid content present in the raw flour. Alkaloids, as secondary metabolites, are known to possess antioxidant properties, thus contributing to the overall antioxidant capacity of the hydrolysates (Al-Tamimi *et al*., 2021).

3.3.2 Effect of enzymatic hydrolysis: The antioxidant capacity of hydrolysates produced by pancreatin, papain, and endopeptidase is illustrated in Figure 1. Hydrolysates generated with pancreatin exhibited the highest antioxidant activity, a phenomenon attributed to its broad hydrolytic capacity (Adamson & Reynolds, 1995). Benítez *et al*. (2008) noted the combination of enzymes in pancreatin, including trypsin, chymotrypsin, elastase, and carboxypeptidase, contributes to its extensive specificity, enabling the hydrolysis of diverse protein substrates. This broad specificity likely explains the observed high antioxidant activity in pancreatin-derived hydrolysates. Additionally, Table 2 indicates pancreatin hydrolysates exhibited elevated protein content, which may facilitate the release of antioxidant peptides within the hydrolysates. Previous studies have shown pancreatin can cleave the C-terminal bond of aromatic amino acids (Phe, Tyr, and Trp), hydrophobic residues (Leu, Val, and Ile), as well as His residues, directly impacting antioxidant capacity (Tapadia *et al*., 2019).

Furthermore, while pancreatin hydrolysates exhibited the highest antioxidant activity, the antioxidant capacity of papain hydrolysates can be attributed to its preferential specificity in cleaving hydrophobic amino acids (Elias *et al*., 2008), which act as proton donors during redox reactions (Luo *et al*., 2014). However, it's worth noting papain may generate low molecular weight peptides during the hydrolysis process (Luo *et al*., 2014) resulting in an accumulation of shorter hydrophilic peptides inaccessible to free radicals (You *et al*., 2012). This aspect may directly influence the antioxidant capacity of papain hydrolysates.

Finally, endopeptidase-treated hydrolysates exhibited the lowest antioxidant capacity. These results could be explained by endopeptidase being a savinase characterized by releasing only His (Egerton *et al*., 2018). Additionally, the lowest antioxidant capacity may be due to the cleavage of short peptides (Garcia-Mora *et al*., 2015).

Overall, enzymatic hydrolysis significantly impacted the antioxidant capacity of the hydrolysates, primarily due to the increased protein content post-hydrolysis. This led to the liberation of various amino acids, such as Val, Leu, Ile, Ala, and Arg, known for their substantial contribution to antioxidant reactions (Wattanasiritham *et al*., 2016). Additionally, aromatic amino acids like Tyr and Phe act during antioxidant reactions by facilitating electron donation (Phongthai *et al*., 2018), while Trp and His neutralize free radicals through reduction via proton transfer (Babini *et al*., 2017), and Cys can cleave thiol groups participating in redox reactions (Leung *et al*., 2018). Moreover, antioxidant compounds such as phenols and alkaloids can be released during hydrolysis (Karamać *et al*., 2018; Magalhães *et al*., 2017). Moreover, research has demonstrated the complex interaction between proteins and phenolics exerts a positive effect on the antioxidant capacity of seed hydrolysates (Drummond e Silva *et al*., 2017).

3.3.3 Effect of hydrolysis time: The hydrolysates produced at 4 and 48 hours showed no significant difference in antioxidant activity, indicating time had no significant effects on the antioxidant capacity of the hydrolysates. Wafaa *et al*. (2022) observed during the initial hours (from zero to 5 hours), more peptides bonded within the protein molecules are broken, releasing smaller peptides that can exert antioxidant capacity. In contrast, based on Lapierre *et al*. (2019) research, the hydrolysates produced after 48 hours would exhibit a higher antioxidant capacity, as peptides containing antioxidant amino acids require extended periods to be separated from the original molecule. Overall, the close antioxidant capacity between hydrolysates at 4 and 48 hours can be explained by the inhibition of enzyme activity following intense hydrolysis during prolonged periods. This inhibition can lead to a decrease in the degree of hydrolysis, affecting the liberation of protein fractions (Wafaa *et al*., 2022). Additionally, Megías *et al*. (2008) stated the antioxidant activity of hydrolysates could decrease after 24 hours of hydrolysis, likely due to the release of short peptides, which cause an unstable antioxidant capacity mechanism.

3.4 Antimicrobial capacity of the RF and DDF

The antimicrobial capacities of the hydrolysates are presented in Figure 2.

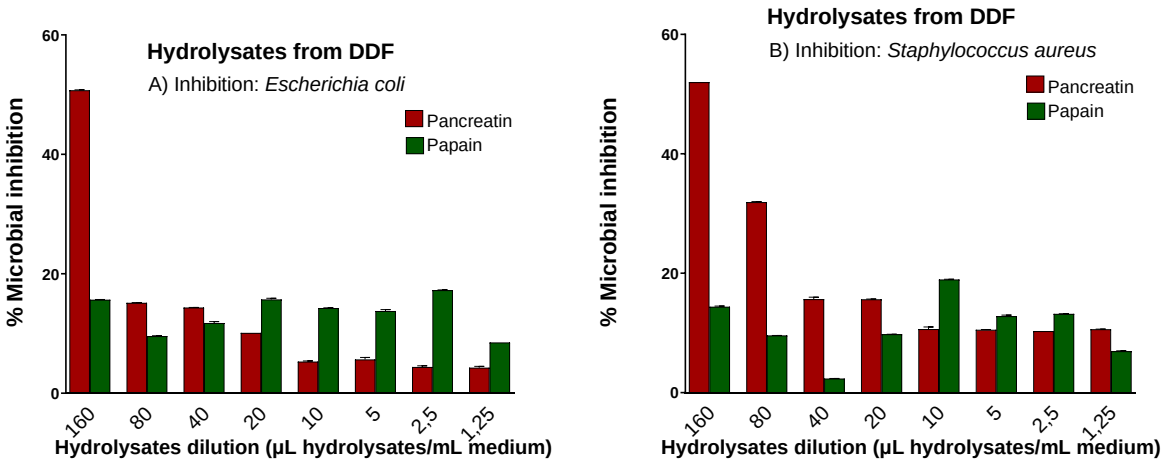

Figure 2 – % Microbial inactivation hydrolysates from DDF at 48 hours using different hydrolysate dilution $\bar{x} \pm \sigma$; N=3.

$\bar{x} \pm \sigma$; n = 3

A dilution of hydrolysates from DDF at 48 hours with pancreatin exhibited higher microbial inhibition: 51.99% against *Staphylococcus aureus* and 50.86% against *Escherichia coli* at a concentration of 160 µL/mL. Other dilutions (80, 40, 20, 10, 5, 2.5 and 1.25 µL/mL) from the same hydrolysates showed at least 10% microbial inhibition against both *Staphylococcus aureus* and *Escherichia coli,* as seen in Figure 2. On the other hand, hydrolysates from DFF obtained with papain-treated at 48 hours displayed microbial inhibition of at least 10% for any dilution (160, 80, 40, 20, 10, 5, 2.5, and 1.25 µL/mL), as seen in Figure 2. Conversely, endopeptidase-treated samples showed no antimicrobial capacity. Finally, hydrolysates from raw flour treated with any enzyme show no antimicrobial capacity. Overall, the results indicated the type of flour, enzyme, and hydrolysis time had a significant effect ($p < 0.05$) on the antimicrobial activity of the hydrolysates.

3.4.1 Effect of pretreatment: The findings showed DDF had a positive and significant effect ($p < 0.05$) on the hydrolysate's antimicrobial capacity. This could be attributed to the debittering and defatting process, which may have broken down several bonds within the matrix. This breakdown could have improved the accessibility of the proteases to specific sites within the protein chain (Chen *et al*., 2011), thereby enhancing the hydrolysate's antimicrobial properties. Moreover, Feyzi *et al*. (2017) found employing a defatting solvent (such as hexane) may cause protein disassociation and potentially increase the hydrophobic surface of the hydrolysates. Dziuba & Dziuba (2014) reported hydrophobic residues like Lys, Leu, Val, Pro, Cys, and Arg can inhibit microbial growth and development (Brogden, 2005; Nell *et al*., 2006). Additionally, studies have shown hydrophobic amino acids can alter the permeability of cell walls via electrostatic interactions, inhibit protein, DNA, and RNA synthesis, leading to a change in membrane permeability and, consequently, cell death (Bahar & Ren, 2013; Shang *et al*., 2016).

3.4.2 Effect of enzymatic hydrolysis: Based on the statistical analysis, pancreatin hydrolysates showed higher microbial inhibition which can be explained by the specificity of pancreatin and its complex composition, which facilitates more target cleavage sites in the protein (Su *et al*., 2012). Pancreatin can release peptides with hydrophobic residues like Leu, Val, and Ile, aromatic amino acids like Trp and Phe, and peptides containing Arg, which can contribute to the overall antimicrobial capacity (Sah *et al*., 2018). On the other hand, papain-treated hydrolysates showed antimicrobial capacity, likely accounted for by the release of hydrophobic amino acids such as Lys, Leu, and Val (Luo *et al*., 2014; Sah *et al*., 2018). However, the lowest antimicrobial values (< 50%) can be explained by papain producing low molecular weight peptides without defined amino acid sequences, resulting in unstable activities (Elias *et al*., 2008). Finally, endopeptidase-treated hydrolysates showed no antimicrobial capacity, likely due to the production of shorter peptides (Garcia-Mora *et al*., 2014), which can cause an unstable mechanism during the interaction between the protein fractions and the pathogen (Megías *et al*., 2008).

3.4.3 Effect of hydrolysis time: The results showed pancreatin-treated hydrolysates at 48 hours exhibited higher microbial inhibition ($p < 0.05$), likely caused by the release of Pro, Cys, Val, Ile, Leu, Arg, Trp, Phe, and Lys, which require more extended hydrolysis periods (Lapierre *et al*., 2019) and can inhibit microbial growth and development (Brogden, 2005; Nell *et al*., 2006).

4. Conclusion

The hydrolysates obtained in the present study developed promising antioxidant and antimicrobial capacities. Pretreatment methods such as debittering and defatting significantly influence the antioxidant capacity of lupine flour hydrolysates by removing antioxidant species such as phenols and alkaloids. Enzymatic hydrolysis, specifically with pancreatin, enhances antioxidant activity by releasing diverse antioxidant peptides. Moreover, longer hydrolysis times may seem beneficial, however, the enzyme activity inhibition and the release of short peptides can cause an unstable antioxidant capacity mechanism.

On the other hand, the study reveals the antimicrobial capacity of lupine flour hydrolysates is significantly influenced by flour type, enzyme choice, and hydrolysis duration. Debittering and defatting flour hydrolysates treated with pancreatin at 48 hours, exhibited notable antimicrobial activity, attributed to enhanced accessibility to proteases and increased hydrophobicity.

Overall, these findings showed the importance of enzyme specificity and hydrolysis conditions in optimizing the antioxidant and antimicrobial properties of lupine flour hydrolysates for potential applications in food preservation and health promotion.

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