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Effect of fermentation with Pleurotus ostreatus and processing in "Roget" bean (Phaseolus vulgaris L.) on its composition, anti-nutrient content, and digestibility

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EFFECT OF FERMENTATION WITH *PLEUROTUS OSTREATUS* AND PROCESSING IN "ROGET" BEAN (*PHASEOLUS VULGARIS* L.) ON ITS COMPOSITION, ANTI-NUTRIENT CONTENT, AND DIGESTIBILITY.

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ABSTRACT

The impact of solid-state fermentation of Roget beans with *Pleurotus ostreatus* on their nutritional profile and bioactive properties was assessed. Fermentation increased protein content, reduced total carbohydrates, and nearly eliminated phytates and trypsin inhibitors compared to soaking or autoclaving. Amino acid analysis showed a 25% increase in essential amino acids and a 15% rise in nonessential amino acids, particularly lysine and leucine levels in fermented beans. FTIR spectroscopy revealed enhanced α-helix and β-sheet structures while reducing protein aggregates, improving protein quality. Autoclaving increased total phenolic content and antioxidant activity more than fermentation, which slightly reduced these properties. Protein digestibility improved significantly from 78% in autoclaved beans to 93% post-fermentation. Fermentation also enhanced ACE inhibitory activity in the bioaccessible fraction, while slightly decreased glycolysis extent compared to autoclaving. These findings underscore that fermenting Roget beans with *P. ostreatus* enhance their nutritional value, making them a valuable alternative protein source and functional ingredient.

KEYWORDS: Roget beans, *Pleurotus ostreatus*, solid-state fermentation, nutritional profile, anti-nutritional factors, amino acids, protein digestibility, ACE inhibitory activity, antioxidant activity, glycaemic index.

RESUMEN

Se estudió la fermentación en estado sólido de las judías Roget con *Pleurotus ostreatus* para evaluar su impacto en el perfil nutricional y las propiedades bioactivas. En comparación con otros métodos de procesamiento como el remojo o la autoclave, la fermentación aumentó el contenido de proteínas, redujo los carbohidratos totales y casi eliminó fitatos e inhibidores de tripsina. El análisis de aminoácidos mostró un aumento del 25% en aminoácidos esenciales y un aumento del 15% en aminoácidos no esenciales, especialmente los niveles de lisina y leucina en los frijoles fermentados en comparación con los crudos o procesados. La espectroscopía FTIR reveló que la fermentación mejoró las estructuras de α-hélice y lámina β al tiempo que redujo los agregados de

proteínas, mejorando así la calidad proteica. La autoclave aumentó el contenido total de fenoles y la actividad antioxidante más que la fermentación, que redujo ligeramente estas propiedades. La digestibilidad de las proteínas mejoró significativamente del 78% en las judías autoclavadas al 93% post-fermentación. Además, la fermentación mejoró la actividad inhibidora de la enzima convertidora de angiotensina (ECA) en la fracción bioaccesible, mientras que la fermentación disminuyó ligeramente el grado de glicólisis en comparación con la autoclave. Estos hallazgos subrayan que la fermentación de los frijoles Roget con P. ostreatus mejora su valor nutricional, posicionándolos como una valiosa fuente alternativa de proteínas e ingrediente funcional.

PALABRAS CLAVE: Judías Roget, *Pleurotus ostreatus*, fermentación en estado sólido, perfil nutricional, factores antinutricionales, aminoácidos, digestibilidad de proteínas, actividad inhibidora de ECA, actividad antioxidante, Índice glucémico.

RESUM

S'ha estudiat la fermentació dels fesols Roget amb *Pleurotus ostreatus* per avaluar el seu impacte en el perfil nutricional i les propietats bioactives. En comparació amb altres mètodes de processament com el remull o l'autoclau, la fermentació va augmentar el contingut de proteïnes, va reduir els carbohidrats totals i gairebé va eliminar els fitats i els inhibidors de tripsina. L'anàlisi d'aminoàcids va mostrar un augment del 25% en aminoàcids essencials i un augment del 15% en aminoàcids no essencials, especialment els nivells de lisina i leucina en els fesols fermentats en comparació amb els crus o processats. L'espectroscòpia FTIR va revelar que la fermentació va millorar les estructures d'α-hèlix i làmina β mentre que reduïa els agregats de proteïnes, millorant així la qualitat proteica. L'autoclau va augmentar el contingut total de fenols i l'activitat antioxidant més que la fermentació, que va reduir lleugerament aquestes propietats. La digestibilitat de les proteïnes va millorar significativament del 78% en els fesols autoclauats al 93% post-fermentació. A més, la fermentació va millorar l'activitat inhibidora de l'enzim convertidor d'angiotensina (ECA) en la fracció bioaccessible mentre que la fermentació va disminuir lleugerament el grau de glicòlisi en comparació amb l'autoclau. Aquests resultats subratllen que la fermentació dels fesols Roget amb P. ostreatus millora el seu valor nutricional, posicionant-los com a una valuosa font alternativa de proteïnes i ingredient funcional.

PARAULES CLAU: Fesols Roget, *Pleurotus ostreatus*, Fermentació en estat sòlid, Perfil nutricional, Factors antinutricionals, Aminoàcids, Digestibilitat de proteïnes, Activitat inhibidora de l'ECA, Activitat antioxidant, Índex glucèmic.

1. INTRODUCTION

In recent years, the focus on providing healthier snack options for children has grown significantly, driven by concerns over childhood malnutrition and the rising prevalence of diet-related diseases. According to the World Health Organization (WHO), over 149 million children under the age of five were stunted in 2020, and 45 million were wasted, indicating severe malnutrition (WHO, 2021). In Europe, about 5.7% of children under five are overweight or obese, a trend that underscores the need for nutritious and balanced diets (WHO, 2020). In Spain, childhood obesity rates are alarming, with nearly one in three children considered overweight or obese (Moncho et al., 2022). Addressing these nutritional challenges is crucial for improving public health outcomes.

The current market for children's snacks is predominantly governed by multinational corporations that largely offer unhealthy options high in saturated fats and simple sugars. These products contribute to poor dietary habits and exacerbate issues such as obesity and malnutrition (Monteiro et al., 2018). Studies have shown that snacks marketed to children often contain excessive amounts of sugar and unhealthy fats, contributing to the growing rates of childhood obesity and related health problems (Elliott, 2019). Therefore, there is a pressing need to develop and promote healthier alternatives that can provide essential nutrients without compromising taste and convenience.

Feeding children healthy snacks from an early age is vital for fostering proper physical and intellectual growth. Adequate nutrition during childhood supports optimal brain development, cognitive function, and physical growth, setting the foundation for a healthy and productive life (Cusick & Georgieff, 2016). Consuming nutritious snacks can help prevent chronic diseases later in life and instill healthy eating habits that persist into adulthood. Evidence suggests that early dietary patterns have long-lasting impacts on health, emphasizing the importance of nutrient-rich diets in early childhood (Chaudhary et al., 2020).

Legumes are recognized as a vital component of a healthy diet due to their high nutritional value and numerous health benefits. They are rich in proteins, dietary fibers, vitamins, and minerals, making them excellent candidates for inclusion in children’s diets (Messina, 2014). The consumption of legumes is associated with a reduced risk of chronic diseases such as cardiovascular disease, diabetes, and certain cancers. Additionally, legumes are low in fat and have a low glycaemic index, making them suitable for maintaining healthy blood sugar levels (Becerra-Tomás et al., 2019). However, legumes also contain anti-nutritional factors (ANFs) such as tannins, phytates, and trypsin inhibitors, which can hinder the absorption of nutrients and affect digestibility. Therefore, processing methods that reduce ANFs while preserving or enhancing the nutritional profile of legumes are essential.

Fermentation, particularly solid-state fungal fermentation, is increasingly recognized for enhancing the nutritional and functional properties of legumes and cereals (Garrido et al., 2021). Studies have demonstrated that fermentation can significantly reduce the content of ANFs and increase the nutritional value of legumes, making them more suitable for consumption, especially for children (Tian et al., 2019; Espinosa-Páez et al., 2017). Moreover, fermentation can also improve the organoleptic properties (taste, texture, aroma) and functional characteristics (such as emulsification and water retention) of legumes, making them more appealing and versatile for use in various food products.

The use of edible fungi, such as *Pleurotus ostreatus*, in the fermentation process can enhance protein and carbohydrate digestibility, reduce ANFs, and increase the bioavailability of essential nutrients in legumes (Senanayake at al., 2023). Recent applications of *Pleurotus ostreatus* in improving the nutritional profiles of legumes like lentils, quinoa, and fava beans have shown promising results (Asensio-Grau et al., 2020; Sánchez-García et al., 2023; Muñoz-Pina at al., 2024). However, its effectiveness on other legumes, such as common beans, remains underexplored.

This study aims to investigate the impact of processing and fermentation with *Pleurotus ostreatus* on Roget beans' nutritional composition, anti-nutritional compounds, and digestibility. The Roget bean (*Phaseolus vulgaris* L.) is a variety known for its nutritional richness and culinary versatility. Originating from Valencia, Spain, this bean is widely cultivated for its unique characteristics and adaptation to the Mediterranean climate, making it a valuable crop in central-southern Spain.

By exploring these factors, the goal is to develop a nutritious and appealing ingredient suitable for healthy snack products, thereby contributing to sustainable food practices.

2. MATERIALS AND METHODS

2.1. Materials and reagents

Roget beans (*Phaseolus vulgaris* L.) were procured from local stores in Valencia, Spain. The *Pleurotus ostreatus* strain (CECT20311) was obtained from the Spanish Type Culture Collection. A variety of reagents were used for different determinations, including sulfuric acid, sodium hydroxide, sodium chloride, thioglycolic acid, phytic acid, 2,2'-bipyridine, acetylacetone, ascorbic acid, ethyl acetate, formic acid, DPPH, ABTS, Folin-Ciocalteu reagent, TPTZ, gallic acid, Trolox, mycopeptone, and chloramphenicol from Sigma-Aldrich Co. (St. Louis, MO, USA). For digestion, reagents such as porcine gastric mucosa pepsin (3200-

4500 U/mg, P6887), porcine pancreas pancreatin (8 x USP, P7545), p-toluenesulfonyl-L-arginine methyl ester, bovine bile (B3883), various analytical grade salts, boric acid, hydrochloric acid (37%), sulfuric acid (95-97%), sodium hydroxide, D-(+)-glucose (≥99.5%), ethanol (96%), and yeast invertase (Grade VII, ≥300 units/mg solid, I4504) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Additional reagents such as glacial acetic acid, concentrated hydrochloric acid, absolute ethanol, ethyl ether, sodium carbonate, ferric ammonium sulphate dodecahydrate, and calcium disodium EDTA salt were sourced from Panreac AppliChem (Barcelona, Spain). Nitric acid (70%), HPLC-grade acetonitrile, HPLC-grade methanol, ferric chloride hexahydrate, sodium acetate trihydrate, and potassium persulfate were obtained from Honeywell Fluka (Morris Plains, NJ, USA).

2.2. Fungal solid-state fermentation (SSF)

The solid-state fermentation was performed following the procedure described by Sanchez-García et al., 2023. Before inoculation with *Pleurotus ostreatus*, the beans were hydrated for 16 hours at room temperature at a 1:6 ratio. After hydration, they were blanched for 2 minutes and cooled with abundant water. The beans were then sterilized in an autoclave (JP Selecta™, Barcelona, Spain) for 20 minutes at 121°C. Subsequently, 50 g of beans were placed in fermentation jars with a T1 filter and inoculated with 5 mL of *Pleurotus ostreatus* starter culture. Incubation was maintained for 10 days at 25°C in a digital incubator (JP Selecta™). The starter culture was prepared in a liquid medium consisting of 2% glucose, 2% malt extract, and 0.1% mycopeptone, agitated horizontally at 25°C for 14 days in a digital incubator (JP Selecta™).

2.3. Total biomass content

To determine the final biomass content after fermentation (Sánchez-García et al., 2023), a 100 mg dry sample was mixed with 2.4 mL of 72% sulfuric acid at 25°C for 24 hours. The samples were then diluted with 55 mL of distilled water and sterilized at 121°C for 1.5 hours. A 1 mL aliquot of the neutralized hydrolysate was combined with 1 mL of acetylacetone, heated in a boiling water bath for 20 minutes, cooled, and mixed with 6 mL of ethanol and 1 mL of Ehrlich reagent. Absorbance was recorded at 530 nm after incubating at 65°C for 10 minutes and cooling. Results were expressed in mg of glucosamine per g of dry matter.

2.4. Proximal composition

To evaluate the nutritional changes due to fermentation or prior processing, five different samples were analysed: raw beans, RB; beans soaked for 16 hours (soaked beans, SB); beans blanched and sterilized in an autoclave for 20 minutes at 121°C (autoclaved beans, AB); beans blanched, sterilized in an autoclave for 20 minutes at 121°C, and incubated 10 days (10 days autoclaved beans, AB10)

and beans fermented (FB) under the same conditions as the AB10 but inoculated with *Pleurotus ostreatus*. The beans' moisture, fat, ash, and protein content were analysed according to official methods (AOAC, 2000). Moisture was assessed by measuring the weight change of samples before and after drying to a constant mass. Fat content was determined using the Soxhlet method with petroleum ether (60:40). Protein content was calculated from total nitrogen content using the Kjeldahl method with a conversion factor of 6.25. Ash content was measured by calcinating samples in a muffle furnace at 550°C for 10 hours to a constant weight. Carbohydrates were calculated by difference, and results were expressed in grams per 100 g of dry matter.

2.5. Amino acid profile

The amino acid profile of the beans was determined using both alkaline and acid hydrolysis methods, as done by Muñoz-Pina et al. (2024). For acid hydrolysis, 0.2 g of the sample was mixed with 10 mL of 6 M HCl and heated at 110 °C for 24 hours. After hydrolysis, the samples were evaporated and reconstituted with 20 mM HCl. For alkaline hydrolysis, 0.2 g of the sample was mixed with 10 mL of 4.3 M $LiOH \cdot H_2O$ and heated at 120 °C for 16 hours. Both hydrolysates were filtered through a 0.2 µm cellulose filter and then derivatized using the Waters AccQ-Tag commercial kit. The samples were analysed using an Agilent 1200 Series HPLC with a diode array detector (Agilent, Palo Alto, CA, USA). The identified compounds were quantified using a calibration curve, with results expressed in mg of free amino acids per gram of dry weight.

2.6. Fourier-transform infrared spectroscopy and protein secondary structure

The secondary structure compositions of bean protein isolates before digestion were determined using attenuated total reflectance (ATR) Fourier-transform infrared (FTIR) spectroscopy (Cary 630 spectrometer, Agilent Technologies Inc., USA) following the method outlined by Shrestha et al. (2023). Protein isolates were extracted from beans according to the procedure described by Sánchez-García et al., 2023, and then lyophilized using a freeze dryer (Telstar, Lyoquest-55, Terrassa, Spain).

For FTIR analysis, the dried protein isolates were placed in a diamond cell and scanned 120 times within the range of 4000 to 650 cm^{-1} at a resolution of 4 cm^{-1}. The Amide I region (1600-1700 cm^{-1}) was subjected to Fourier self-deconvolution, second derivative analysis, and curve fitting using OriginPro 2024. Gaussian peaks were identified and assigned to specific protein secondary structures based on literature values (Beck et al., 2017). Peaks were cantered at approximately 1638 cm^{-1} (β-sheet), 1654 cm^{-1} (α-helix), 1663 cm^{-1} (β-turn), and 1680 cm^{-1} (anti-parallel β-sheet). Additional peaks indicating protein aggregates

and amino acid sidechain absorptions were noted at 1625-1610 cm^{-1} (A1) and 1695-1690 cm^{-1} (A2).

2.7. Static *in vitro* gastrointestinal digestion

Autoclaved beans (AB) and fermented beans (FB) were digested, simulating oral, gastric, and intestinal phases for a healthy adult following the INFOGEST protocol described by Brodkorb et al. (2019).

Oral Phase: 5 g of sample was mixed with 5 mL of pre-warmed Simulated Saliva Fluid (SSF) at 37°C and incubated with shaking at 37°C for 2 minutes.

Gastric Phase: The oral phase mixture was combined with an equal volume of Simulated Gastric Fluid (SGF) containing porcine pepsin at 2,000 U/mL and adjusted to pH 3.0 with 1M HCl. Samples were incubated with shaking at 37°C for 2 hours, adjusting pH to 3.0 periodically.

Intestinal Phase: The gastric phase mixture was combined with an equal volume of Simulated Intestinal Fluid (SIF) and adjusted to pH 7.0 with 1M NaOH. SIF contained pancreatin and bile salts at final activities of 5.35 U/mg and 10 mM, respectively. Samples were incubated with shaking at 37°C for 2 hours, adjusting pH to 7.0 periodically. Post-incubation, 4 mL of Bowman-Birk inhibitor (0.05 g/L) was added to inhibit enzymatic activity. Samples were centrifuged at 8000 g for 10 minutes at 4°C, and aliquots of the supernatant were frozen at -20°C.

2.8. Quantitative analysis of total phenols, antioxidants, and tannins

For all undigested samples, an ethanolic extraction of phenolic compounds was performed (Caprioli et al., 2018). A 20 mL aliquot of 70% ethanol was added to 5 g of solid sample, adjusted to pH 2 with 2N HCl, and sonicated at 20°C for 120 minutes. After extraction, samples were centrifuged at 8000 g for 15 minutes. The extraction was repeated twice, and supernatants were combined.

Total phenol content (TPC) was determined using the Folin-Ciocalteu method as described by Muñoz-Pina et al. (2022). A 10 µL aliquot of each extract was mixed with 1.58 mL of distilled water, 100 µL of Folin-Ciocalteu reagent, and agitated for 3 minutes. Subsequently, 300 µL of 20% Na_2CO_3 was added, and the mixture was incubated at room temperature in the dark for 60 minutes. Absorbance was measured at 765 nm (UV/vis, Beckman Coulter DU 730). A standard curve of gallic acid (0 to 500 mg/L) was used, and results were expressed as mg of gallic acid equivalents (GAE) per g of dry matter.

Antioxidant activity was assessed using three different methods: ABTS, DPPH, and FRAP, following the procedure detailed by Thaipong et al., 2006. for all methods of antioxidant activity, The same ethanolic extract was used, and the

results were expressed in mg of Trolox per g on a dry basis, using a standard curve of Trolox as a reference (0 to 200 mg/L).

Tannin content was determined using the acidified vanillin assay (Zhang, Deng, et al., 2014). A 50 µL extract was mixed with 200 µL of vanillin reagent (3% vanillin and 14% HCl in MeOH) and incubated at room temperature for 20 minutes. Absorbance was measured at 500 nm (UV/vis, Beckman Coulter DU 730), and results were expressed as catechin equivalents (mg CAE /g dry matter).

The total phenolic content (TPC), ABTS, DPPH, FRAP, and tannins of the bioaccessible fraction after the intestinal digestion stage were analyzed following the described methodologies, using an aliquot of the digested sample instead of the extracts.

2.9. Phytic acid content

Phytic acid content was assessed before and after in vitro digestion using the method described by Sánchez-García et al., (2023). A sample of 50 mg of the undigested samples was mixed with 10 mL of 0.2 M HCl and left overnight at 4°C to extract the phytic acid. For the digested samples, the analysis was conducted on the bioaccessible fraction. An aliquot of 500 µL from the extract/digest was taken, and 1 mL of ferric solution (0.2 g of ammonium iron (III) sulphate dodecahydrate dissolved in 100 mL of 2 M hydrochloric acid and made up to 1 L with distilled water) was added. The mixture was incubated in a boiling water bath for 30 minutes, cooled to room temperature, and then centrifuged for 30 minutes at 3000 g. A 1 mL aliquot of the supernatant was mixed with 1.5 mL of 2,2'-bipyridine solution (10 g of 2,2'-bipyridine and 10 mL of thioglycolic acid dissolved in distilled water and made up to 1 L). The results were quantified as mg of phytic acid per gram of dry basis using a standard curve prepared from a stock solution of 1.3 mg/mL phytic acid concentration, diluted with 0.2 M hydrochloric acid (3.16 – 31.6 µg/mL).

2.10. Trypsin inhibitory activity

Trypsin inhibitory activity was conducted following the K.A. Millar, et al. 2019 method. Bean samples (1 g) were extracted with 50 mL of 0.01 M NaOH for 1 hour at room temperature, maintaining the pH between 8.4 and 10.0 during extraction. The extracts were then diluted to achieve 30–70% trypsin inhibition levels. In a test tube, 2 mL of trypsin solution (0.5 g of porcine pancreas trypsin in 500 mL of 0.001 M HCl) was added to 1 mL of the diluted extract and 1 mL of distilled water. The tubes were placed at 37°C. After 10 minutes, 5 mL of 37°C preheated BAPNA solution (freshly prepared by dissolving 80 mg of BAPNA (benzoyl-DL-arginine-p-nitroanilide hydrochloride) in 2 mL of DMSO, diluting it to 200 mL with 0.05 M Tris buffer) was added to each tube and vortexed. The samples were further incubated for 10 minutes at 37°C, followed by adding 1 mL

of 30% acetic acid to terminate the reaction. The absorbance due to the release of p-nitroaniline was measured at 410 nm. Trypsin inhibitory activity (TIA) was expressed as trypsin inhibition units (TIU) per milligram of dry matter.

2.11. Soluble protein (TCA)

Protein hydrolysis was evaluated by measuring the Trichloroacetic acid (TCA)-soluble protein fraction after digestion, following the procedure adopted by Sánchez-García et al., 2023. TCA solution (36% TCA) was added to digested samples to a final concentration of 12% and reacted for 15 minutes in an Eppendorf Thermomixer (Germany). Samples were centrifuged at 1000 rpm for 5 minutes (Eppendorf MiniSpin Plus, Germany), and the supernatant was diluted in the EDTA buffer (50 mM EDTA and 8 M Urea, pH 10). Absorbance was measured at 280 nm in quartz cuvettes, and results were expressed in g of tyrosine / 100 g of protein.

2.12. Reducing Sugars

The analysis of reducing sugars in the undigested samples was performed following the methodology proposed by Armellini et al., 2019. To extract sugars, 0.3 g of the sample was vigorously mixed with 2 mL of 80% ethanol and allowed to stand for 30 minutes. The mixture was then centrifuged at 5000 xg for 5 minutes, and the supernatant was collected. A 500 µL aliquot of the sample was combined with 1 mL of DNS reagent, prepared by combining 10 g/L of 3,5-dinitrosalicylic acid with 300 g of sodium potassium tartrate tetrahydrate and 16 g of NaOH. The mixture was heated at 100 °C for 5 minutes and then cooled to room temperature. The samples were diluted with 6 mL of distilled water, and the absorbance was measured at 530 nm. Results were expressed as mg of glucose per gram of dry sample.

For digested samples, 1 mL of the bioaccessible fraction was mixed with 4 mL of absolute ethanol. Then, 50 µL of this mixture was added to 250 µL of enzyme solution (1% amyloglucosidase and 1% invertase in acetate buffer, pH 5.2) and incubated at 37 °C for 10 minutes. Subsequently, 750 µL of DNS reagent was added, and the mixture was heated at 100 °C for 15 minutes. Finally, 4 mL of deionized water was added, and absorbance was measured at 530 nm. The extent of glycolysis was calculated using the equation 1:

$$Glycolysis\ extent\ (\%) = \frac{g\ free\ glucose\ Eq.\ bioaccessible\ fraction}{g\ starch\ (glucose\ Eq.)\ undigested\ beans} 100 \qquad (1)$$

2.13. Angiotensin-converting enzyme (ACE) inhibitory activity

The percentage inhibition of angiotensin-converting enzyme (ACE) for both undigested and digested samples was determined using the method detailed

by Hernández-Olivas et al. (2022). For undigested samples, Protein isolates were extracted from beans following the methodology described by Sánchez-García et al., 2023, and for digested samples, the determination was carried out on the bioaccessible fraction.

ACE reagent (25 mU/mL) and the substrate Hip-His-Leu (5 mM) were prepared dissolved in 0.15 M Tris base buffer containing 0.3 M NaCl at pH of 8.3. Three controls were included: (i) 100 µL of ACE + 40 µL of distilled water, (ii) 140 µL of distilled water, and (iii) 100 µL of distilled water + 40 µL of extract/digestion sample. The samples (100 µL of ACE + 40 µL of extract/digestion sample) were incubated at 37 °C for 5 minutes. Then, 100 µL of substrate was added to each tube, and the incubation continued for 30 minutes at the same temperature. The reaction was stopped by adding 150 µL of 1 M HCl, followed by 1 mL of ethyl acetate, and the mixture was vigorously shaken using a vortex mixer. The samples were then centrifuged at 1200 g for 10 minutes, and 750 µL of the supernatant was transferred to clean tubes. Ethyl acetate in the supernatant was evaporated by gentle shaking at 80 °C. The remaining hippuric acid in the tubes was dissolved in 1 mL of distilled water, and absorbance was recorded at 228 nm. The inhibition percentage was calculated using the equation 2:

$$ACE\ ia\ (\%) = 100 - \left\{100\ \frac{(C-D)}{(A-B)}\right\} \quad (2)$$

where A, B, C, and D represent the absorbances of ACE + distilled water, distilled water, ACE + extract/digestion sample, and distilled water + extract/digestion sample, respectively.

2.14. Statistical analysis

All experiments were conducted in triplicate, and the results are presented as mean ± standard deviation. Statistical analysis was performed using Statgraphics Centurion-XV software. One-way analysis of variance (ANOVA) with a 95% confidence interval ($p < 0.05$) was used to identify homogeneous groups.

3. RESULTS AND DISCUSSION

3.1. Effect of processing and fermentation on the proximate composition

Table 1 presents the proximate composition of Roget beans before and after solid-state fermentation (SSF) with *Pleurotus ostreatus*, detailing moisture, crude protein, lipids, total carbohydrates, reducing sugars, total starch, and ash. The table also includes total phenolic compounds, antioxidant

activity, and antinutrients such as tannins, phytates, and trypsin inhibitor activity.

Since SSF includes soaking and autoclaving—processes that can independently affect nutritional properties—samples of raw beans (RB), soaked beans (SB), autoclaved beans (AB), and 10-day incubated autoclaved beans (AB10) were also analysed as controls to discern changes specifically attributable to fungal activity. Results are expressed on a dry basis (DB).

Previous studies have demonstrated that *P. ostreatus* utilizes carbon, nitrogen, and sulphur from the substrate, leading to fungal mycelium growth (biomass) and altering the macro- and micro-nutrient profiles of fermented legumes. This process typically decreases carbohydrates while increasing reducing sugars and proteins (Espinosa-Paez et al., 2017; Asensio-Grau et al., 2020; Sánchez-García et al., 2023; Muñoz-Pina et al., 2024).

Consistent with these findings, we observed significant lipid, protein, and carbohydrate content changes between fermented and raw, soaked, and heated Roget beans. Fermentation increased the total protein content from 19% to 22%, a 16% increase surpassing the protein increments reported for other bean types (Espinosa-Páez et al., 2017). Meanwhile, total carbohydrates decreased by 8%, likely because carbohydrates serve as an energy source for mycelium growth, with some converting into complex proteins, peptides, or free amino acids. The amount of reducing sugars notably increased in the fermented sample compared to raw and processed beans, aligning with findings in other legumes and bean varieties. This increase is attributed to the fungal conversion of carbohydrates, primarily fiber, facilitated by fungal cellulase and hemicellulase activities, which hydrolyse fiber and elevate reducing sugar levels (Zamora et al., 2023).

Fermentation also resulted in increased ash content, contrasting with other beans where a decrease in total ash content is often observed due to autoclaving and subsequent fermentation. This variance is likely due to differences in the form of mineral constituents in ash, which depends considerably on their original form in the food, altering its weight and volatilization capacity.

The final biomass content was 26.12 mg of glucosamine/g DB, similar to that reported for Pardina lentils (Sánchez-García et al., 2023) but lower than values for other legumes like Castellana lentils (Sánchez-García et al., 2023) or fava beans (Muñoz-Pina et al., 2024). This suggests that *P. ostreatus* grows at different rates in different legume varieties, highlighting the influence of the substrate.

TABLE 1. Proximate composition, biomass, tannins, total phenolic compounds, antioxidant capacity (DPPH, ABTS, FRAP) and antinutrients (phytates and trypsin inhibitory activity) of raw (RB), soaked (SB), autoclaved (AB), 10 days incubated after autoclaving (AB10) and Pleurotus ostreatus fermented (FB) Roget beans.

	RB	SB	AB	AB10	FB
Major components					
Moisture (g/100 g DB)	7,84±0,10^{a}	183,2±0,7^{d}	102,3±3,3^{b}	104,0±1,6^{b}	169±2^{c}
Ashes (g/100 g DB)	4,41±0,05^{a}	5,68 ± 0,09^{b}	5,4±0,6^{b}	4,5±0,3^{a}	6,32±0,00^{c}
Protein (g/100 g DB)	18,9±0,2^{a}	19,5±0,9^{a}	19,6±1,6^{a}	17,6±0,7^{a}	22,2±1,2^{b}
Lipids (g/100 g DB)	1,29±0,14^{a}	1,25±0,07^{a}	2,6± 0.2^{c}	2,63±0,07^{c}	1,6±0,2^{b}
Carbohydrates (g/100 g DB)	75,4±0,4^{b}	74±1^{b}	74±2^{b}	73±1^{b}	69,9±1,2^{a}
Reducing sugar (mg glucose/ g DB)	1,8±0,2^{a}	6,32±0,04^{c}	4,2±0,5^{b}	4,6±0,6^{b}	45,3±0,7^{d}
Starch (g/100 g DB)	59,9±0,2^{a}	58,11±0,04^{a}	58,0±0,5^{a}	58,7±0,6^{a}	54,0±0,7^{a}
Biomass (mg glucosamine/g DB)	–	–	–	–	26±2
Minor components and antioxidant capacity					
TPC (mg GAE/ g DB)	4,7±0,2^{b}	5,4±0,2^{c}	2,2±0,2^{a}	2,3±0,3^{a}	2,8±0,3^{a}
DPPH (mg TE/ g DB)	3,96±0,12^{b}	5,6±0,9^{c}	1,55±0,17^{a}	1,7±0,7^{a}	1,8±0,3^{a}
ABTS (mg TE/ g DB)	15,6±0,8^{b}	18,5±0,2^{c}	5,7±0,5^{a}	5,2±0,4^{a}	6,5±0,8^{a}
FRAP (mg TE/ g DB)	4,24±0,18^{d}	3,60±0,02^{c}	2,14±0,16^{b}	1,68±0,02^{a}	1,70±0,07^{a}
Tannins (mg CAE/ g DB)	4,5±0,3^{b}	4,5±0,4^{b}	1,30±0,11^{a}	1,1±0,2^{a}	1,08±0,02^{a}
Phytates (mg phytic acid/ g DB)	14±2^{d}	10,8±0,5^{c}	6,9±0,3^{b}	6,16±0,01^{b}	0,32±1,15^{a}
Trypsin inhibitory activity (TUI/mg DB)	81,9±0,4^{c}	47±6^{b}	0,50±0,12^{a}	0,58±0,13^{a}	0,01±0,08^{a}

The results represent the mean of three repetitions. ^{a,b,c,d} Different lowercase letters indicate significant differences between beans ($p < 0.05$).

The total phenolic compounds and antioxidant activity of autoclaved and fermented beans differed from the raw samples, indicating that processing like soaking, cooking, or fermentation can alter phenolic compounds and antioxidant activity (see Table 1). The main mechanisms involved include ionic bond formation during the demethoxylation of pectin, cross-linking of phenolic compounds with the primary wall and middle lamella, polyphenol-protein interactions, and the interaction of phenolic compounds and fiber (M. García et al., 2021).

Autoclaving reduced the total phenolic content by 45%, and fermentation did not significantly alter the phenolic compounds or antioxidant activity compared to autoclaved samples, indicating no further influence from fermentation. Similarly, both thermal processing and fermentation negatively impacted antioxidant capacity to a similar extent, lowering the Trolox content (mg/g DB) measured by ABTS, FRAP, and DPPH methods. Compared to raw samples, soaking increased the antioxidant activity measured by DPPH and ABTS but showed lower values when measured using the FRAP method.

Raw beans contained moderate levels of antinutrients such as tannins (4.5 mg CAE/g DB) and phytates (14.4 mg phytate phosphorus/g DB), alongside high trypsin inhibitory activity (81.9 TUI/mg DB), consistent with existing literature (Carbas et al., 2020). Thermal treatments like autoclaving effectively reduced tannins due to their heat sensitivity, but fermentation did not further decrease tannin levels despite the presence of fungal tannase activity.

However, fermentation significantly reduced phytates and trypsin inhibitor activity beyond the reductions achieved by soaking or heat treatment alone. While soaking reduced phytates by approximately 25% and autoclaving by approximately 55%, fermentation nearly eliminated them, thanks to fungal enzymatic activity. Similar results were observed in the suppression of trypsin inhibitor activity. Soaking only reduced trypsin inhibitor activity by approximately 45%. Meanwhile, autoclaving almost eliminated trypsin inhibitor activity. However, trace amounts of TUI/mg DB were still detected (approximately 1%) after the heat treatment. No residual trypsin inhibitor activity was detected after fermentation.

3.2. Changes in amino acid profile after fermentation

Table 2 presents the amino acid composition of raw, soaked, autoclaved, and fermented Roget beans. Raw beans exhibited high levels of Glu, Asp, Leu, and Lys, followed by Arg and Gly. However, nonessential amino acids (NEAA) were present in much higher concentrations (60%) compared to essential amino acids (EAA).

Soaking had minimal impact, with only Thr increasing by about 35%. Similarly, autoclaving only resulted in a slight increase in the total amino acid content of about 15% without affecting the EAA/NEAA ratio.

Fermentation resulted in more substantial changes. The concentration of most amino acids increased, with EAA rising by 25% and NEAA by 15%. Compared to autoclaving, fermentation was especially efficient in boosting the levels of Leu, Lys, Phe, Gly, and Ser.

According to the WHO/FAO/UNU Expert Consultation (2007), lysine and leucine are crucial for children's growth. The increased levels of arginine and tyrosine, which are semi-essential for children's development (Hammarqvist et al., 2010), further highlight the nutritional benefits of fermented Roget beans. These findings are consistent with those reported for other beans (Espinosa-Páez et al., 2017; Muñoz-Pina et al., 2024). However, the varying levels of key amino acids like lysine across different bean types suggest that the fermentation effects significantly depend on the substrate.

TABLE 2. Amino acids profile (mg free AA/g dry basis) of raw (RB), soaked (SB), autoclaved (AB), 10 days incubated autoclaved (AB10) and fermented (FB) Roget beans.

Amino acid (mg free AA/ g DB)	**RB**	**SB**	**AB**	**AB10**	**FB**
Aspartic acid (Asp)	25±3^{a}	23,07±0,05^{a}	26,9±0,9^{a}	33±7^{b}	34,2±0,5^{b}
Serine (Ser)	10,2±1,2^{a}	10,3±0,3^{a}	11,3±0,8^{a}	12±1^{a}	15,3±0,7^{b}
Glutamic acid (Glu)	32±4^{ab}	30,5±0,3^{a}	34,2±1,3^{ab}	35±2^{ab}	39,5±0,8^{b}
Glycine (Gly)	10,2±1,3^{a}	10,0±0,2^{a}	11,3±0,8^{a}	11,6±0,9 ^{a}	15,5±0,6^{b}
Histidine (His)	6,6±0,7^{a}	6,4±0,2^{a}	7,80±0,15^{a}	7,5±0,6^{a}	10±3^{b}
Arginine (Arg)	11±1^{a}	9,86±0,10^{a}	10,4±0,4^{a}	10,7±0,8^{a}	13,2±0,8^{b}
Threonine (Thr)	6,6±0,9^{a}	6,50±0,12^{a}	7,3±0,6^{a}	7,7±0,7^{a}	10,6±0,5^{b}
Alanine (Ala)	6,2±0,7^{a}	6,30±0,04^{a}	6,9±0,8^{a}	7,1±0,5^{a}	10,0±0,4^{b}
Proline (Pro)	6,1±0,7^{a}	5,99±0,01^{a}	6,7±0,6^{a}	6,9±0,5^{a}	9,74±0,04^{b}
Cystine (Cys)	3,2±0,2^{a}	3,2±0,3^{a}	2,5±0,3^{a}	2,7±0,3^{a}	2,9±1,5^{a}
Tyrosine (Try)	3,9±0,4^{a}	4,05±0,14^{a}	4,1±0,2^{a}	4,4±0,4^{a}	6,2±0,8^{b}
Valine (Val)	9,14±1,02^{a}	9,03±0,05^{a}	10,0±0,5^{a}	10,0±0,4^{a}	13,0±0,5^{b}
Methionine (Met)	0,81±0,03^{ab}	0,65±0,01^{a}	0,79±0,07^{ab}	0,65±0,09^{a}	1,1±0,2^{b}
Lysine (Lys)	13,3±1,4^{a}	13,14±0,07^{a}	13,4±0,6^{a}	13,8±0,9^{a}	16,01±0,6^{b}
Isoleucine (Ile)	7,4±0,9^{a}	7,13±0,04^{a}	8,2±0,7^{a}	8,3±0,3^{a}	11,0±0,3^{b}

Amino acid (mg free AA/ g DB)	RB	SB	AB	AB10	FB
Leucine (Leu)	13,8±1,6^{a}	13,50±0,16^{a}	15,2±0,6^{a}	15,5±0,9^{a}	20,6±0,8^{b}
Phenylalanine (Phe)	9,82±1,09^{a}	9,79±0,16^{a}	11,0±0,7^{a}	11,2±0,7^{a}	14,3±0,5^{b}
Tryptophan (Trp)	2,64±0,10^{a}	3,5±0,2^{b}	3,05±0,00^{ab}	3,05±0,06^{ab}	3,1±0,3^{ab}
Total	178±20	173±2	191±10	200±18	246±12
EAA	70±8^{a}	69,67±1,02^{a}	77±4^{b}	78±5^{b}	100±7^{c}
NEAA	108±2^{a}	103,2±1,4^{a}	114±6^{b}	123±14^{b}	145±12^{c}
EAA/NEAA ratio	0,65±0,15^{a}	0,67±0,02^{a}	0,67±0,07^{a}	0,63±0,11^{a}	0,69±0,11^{a}

The results represent the mean of three repetitions. ^{a,b,c} Different lowercase letters indicate significant differences between beans ($p < 0.05$). Essential amino acids (EAA); Nonessential amino acids (NEAA).

3.2. Influence of fermentation on secondary protein structure

Protein quality and availability are influenced by secondary structures, which affect how proteins are accessible to hydrolytic enzymes and thus impact digestibility. Fourier-Transform Infrared (FTIR) spectroscopy was used to examine these structures in proteins.

The results are represented in Figure 1. In the secondary structure analysis of Roget bean proteins, raw beans predominantly exhibited β-sheet structures (49%), along with β-turns (26%), protein aggregates (20%), β-A (4%), and a minimal amount of α-helix (1%). These findings align with other studies showing beans to be rich in β-sheet structures due to their high 7S globulin content (Carbonaro et al., 2012).

Processing methods such as soaking, autoclaving, and fermentation caused notable structural changes. Soaking increased the α-helix (4%), β-sheet (58%) and β-A (9%) while decreased β-turn (20%). Autoclaving did not significantly affect β-sheet content but led to higher increases in β-turns (37%) and α-helix (7%) compared to raw and soaked samples. The heat treatment also reduced β-A content to 1%. Fermentation further modified the structure by noticeably increasing β-A (10%), α-helix (10%), and β-sheet (54%) structures, reaching the highest proportions among all analysed samples, while the lowest content of β-turns (16%) was observed after fermentation. Additionally, all processing methods resulted in a reduction in protein aggregates, with the most significant reduction observed in the 10-day incubated autoclaved sample.

These changes suggest that soaking, autoclaving, and fermentation influence the secondary structure of proteins in distinct ways. Fermentation, in

particular, enhances protein quality. The observed increase in β-A, α-helix, and β-sheet structures indicates that fungal activity during fermentation can disrupt hydrogen bonds and unfold protein structures (Zhao et al., 2023). This unfolding promotes better enzyme contact, potentially improving protein digestibility (Y. Wang et al., 2024).

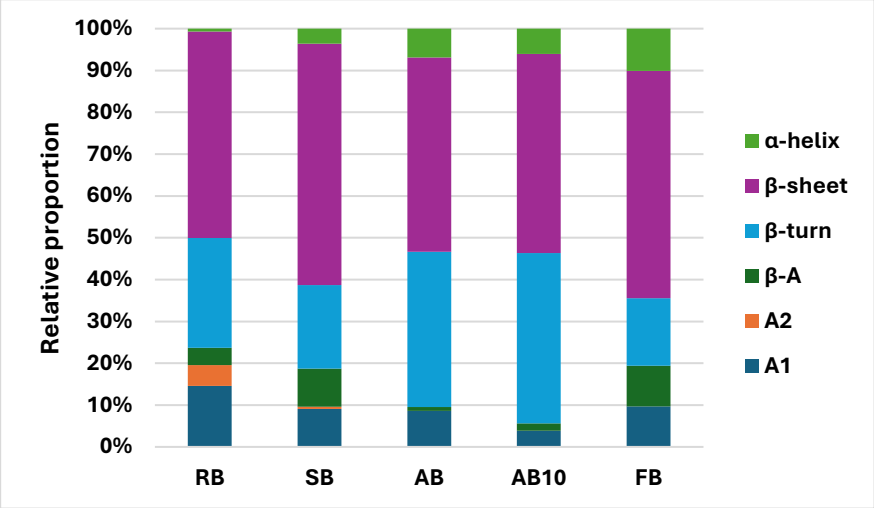

FIGURE 1. Fourier-Transform Infrared spectroscopy analysis: Relative proportion of secondary structure of protein isolates from the fava beans. Orange: aggregates A2, dark green: anti-parallel β-sheet (β-A), light blue: β-turn, light green: α-helix, purple: β-sheet, and dark blue: aggregates A1.

3.3. Effect of fermentation on protein digestibility

Protein digestibility was assessed between autoclaved and fermented Roget beans using the TCA-soluble protein test, which measures free small peptides and amino acids in the bioaccessible fraction after the intestinal digestion stage. The autoclaved sample was chosen as a reference since it mimics a typical cooking process. As shown in Figure 2, autoclaving resulted in a digestibility rate of 78%. This is higher compared to other legumes such as fava beans, black beans, kidney beans, and soybeans, which typically exhibit lower digestibility rates (40-60%) under similar digestion conditions (Espinosa-Páez et al., 2017; Hernández-Olivas et al., 2021, Muñoz-Pina et al., 2024).

Fermentation significantly increased protein hydrolysis to 93%. This aligns with the general understanding that fermentation enhances proteolysis due to the partial hydrolysis of proteins by fungal lytic mechanisms during fermentation (Sánchez-García et al., 2023). However, different patterns have been observed in other legumes, such as fava beans (Muñoz-Pina et al., 2024), where fermentation reduced protein hydrolysis, indicating that results are

highly substrate-dependent. The primary structure of the protein in different beans can lead to varying amounts of cross-linkers, like disulfide bonds, influencing resistance to proteolytic enzymes (Becker & Yu, 2013).

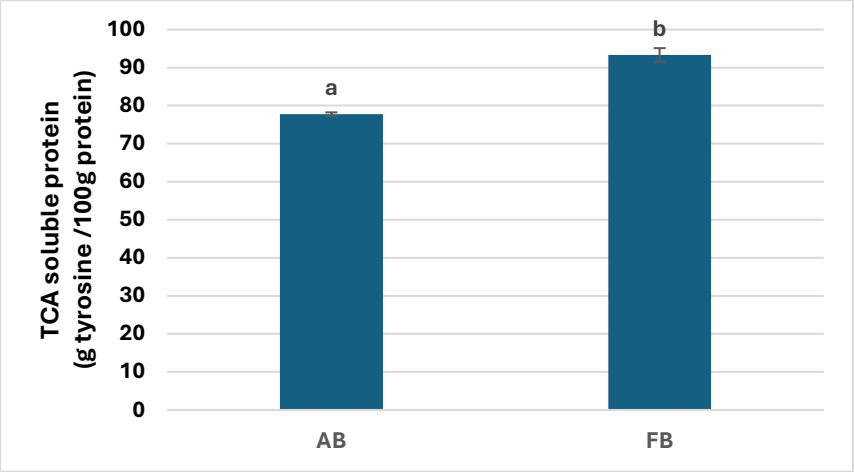

FIGURE 2. TCA soluble protein (g tyrosine /100 g protein) of the bioaccessible fraction on the intestinal digest of autoclaved (AB) and fermented (FB) Roget beans. ab Lowercase letters indicate significant differences between digestion models at a significance level of 95% ($p < 0.05$).

3.4. Effect of fermentation on carbohydrate digestibility

The extent of glycolysis was quantified to assess the breakdown of carbohydrates, particularly glucose, after autoclaving and fermentation with *P. ostreatus* (Figure 3). As previously observed in other legumes, the extent of glycolysis in autoclaved Roget beans was high (83%), primarily due to heat-induced starch gelatinization and the deactivation of enzyme inhibitors, enhancing hydrolysis by α-amylase. However, the extent of glycolysis drops to 77% when beans undergo fermentation with *Pleurotus ostreatus*. Although the initial content of total starch remains unchanged post-fermentation, changes in the ratios of rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) likely affect starch digestibility (Hooper et al., 2021).

Slower starch digestion is associated with a lower glycemic index, preventing abrupt insulin secretion in the blood, in contrast to fast starch digestion, which is linked to an increased risk of type II diabetes and obesity. Consequently, fermented beans could be a promising ingredient for developing children's snacks due to their lower starch digestion rate.

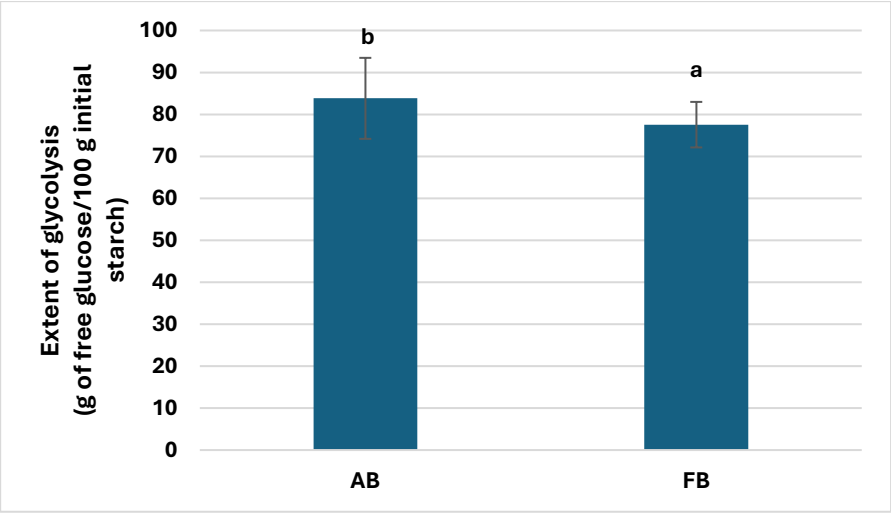

FIGURE 3. Extent of glycolysis (expressed as g of free glucose /100 g initial starch) of the bioaccessible fraction on intestinal digesta of autoclaved (AB) and fermented (FB) Roget beans. ab Lowercase letters indicate significant differences between digestion models at a significance level of 95% ($p < 0.05$).

3.5. Bioaccessibility of minor components and antioxidant capacity

Table 3 presents the results of the total phenol content (TPC) and the changes in antioxidant activity during digestion. After in vitro luminal digestion, there was a significant increase in bioaccessible phenolic compounds for autoclaved Roget beans, with a 600% increase. In contrast, fermented samples exhibited a lower increment in TPC bioaccessibility, reaching only 100%. Similar findings were reported by Tungmunnithum et al. (2022), who noted a substantial rise in TPC across various bean types post-digestion due to the breakdown of cell wall-bound phenols by digestive enzymes and pH changes.

The antioxidant activity followed a similar pattern for the autoclaved beans, displaying higher ABTS, DPPH, and FRAP antioxidant activity after digestion. However, digested fermented Roget beans had lower antioxidant activity compared to both the undigested fermented samples and the digested autoclaved samples. This aligns with previous research that observed comparable antioxidant activity in lentils (Sánchez-García et al., 2023) and fava beans (Muñoz-Pina et al., 2024) fermented with *P. ostreatus*.

The polyphenol content in legumes is largely influenced by polyphenol-protein interactions (M. García et al., 2021). The differences in antioxidant activity can be attributed to variations in protein digestion, amino acid release, and the partial hydrolysis and deprotonation of phenolic hydroxyl groups during digestion. Therefore, analysing free amino acids in the digested fermented sample could provide a better results interpretation.

Post-digestion, the amount of phytates was slightly lower for both samples compared to the undigested ones, while no tannins were detected in the bioaccessible fraction of either sample.

TABLE 3. Total phenolic content, antioxidant capacity (by DPPH, FRAP and ABTS tests) and antinutrients (phytates and tannins) in the bioaccessible fraction of autoclaved (AB) and fermented (FB) Roget beans digesta under standard in vitro GI conditions and in the corresponding undigested beans.

	Sample	TPC (mg GAE/ g DB)	DPPH (mg TE/ g DB)	FRAP (mg TE/ g DB)	ABTS (mg TE/ g DB)	Phytates (mg phytic acid/ g DB)	Tannins (mg CAE/ g DB)
AB	Digested	15 ± 1^{b}	$4,2\pm0,5^{b}$	$3,3\pm0,2^{b}$	33 ± 6^{b}	$5,6\pm0,5^{b}$	-
	Undigested	$2,17\pm0,17^{a}$	$1,55\pm0,17^{a}$	$2,14\pm0,16^{b}$	$5,7\pm0,5^{a}$	$6,90\pm0,26^{b}$	$1,3\pm0,1^{a}$
FB	Digested	$5,6\pm1,8^{a}$	$0,97\pm0,17^{a}$	$0,96\pm0,08^{a}$	$3,6\pm1,4^{a}$	$0,03\pm0,02^{a}$	-
	Undigested	$2,8\pm0,3^{a}$	$1,8\pm0,3^{a}$	$1,70\pm0,07^{a}$	$6,5\pm0,8^{a}$	$0,32\pm1,15^{a}$	$1,08\pm0,02^{a}$

Data shown are mean values from three replicates. ^{abc} Lowercase letters indicate significant differences between the beans at a significance level of 95% ($p < 0.05$).

3.6. Effect of fermentation on ACE inhibitory activity

Angiotensin Converting Enzyme (ACE) regulates blood pressure by converting angiotensin I to angiotensin II, which constricts blood vessels and raises blood pressure. ACE inhibitors lower blood pressure by preventing this conversion, aiding in hypertension and related conditions like heart failure and kidney disease. Fermenting beans and other protein-rich foods with fungi such as Pleurotus ostreatus significantly enhances their ACE inhibitory properties due to low molecular weight proteins and peptides (Muñoz-Pina et al., 2024).

Figure 4 illustrates the ACE inhibition levels of raw, soaked, autoclaved, and fermented Roget beans. Neither soaking nor autoclaving resulted in significant ACE inhibitory activity (approximately 20%). Autoclaving even decreased the ACE inhibitory activity compared to raw and soaked samples (less than 10%). In contrast, fermentation notably enhanced ACE inhibition, reaching 38%.

ACE inhibition was also measured for the bioaccessible fraction of autoclaved and fermented samples after in-vitro digestion. The fermented bean digest showed a higher ACE inhibition (49%) compared to the undigested fermented samples. Although an increase in ACE inhibition was observed for the digested autoclaved sample (22%) compared to the undigested one, it remained much lower than the bioaccessible fermented digested sample. These results suggest the potential benefits of fermented

Roget beans for hypertension management and reducing the likelihood of certain cardiovascular diseases.

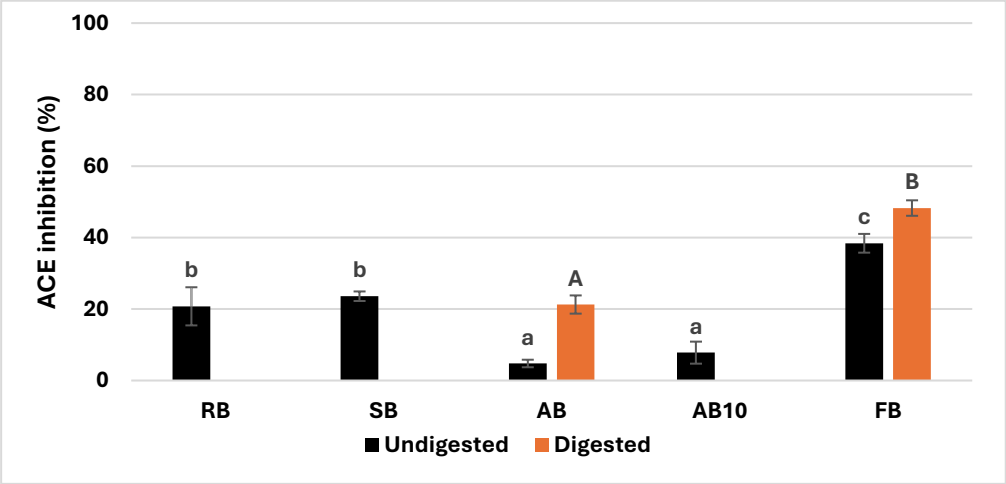

FIGURE 4. ACE inhibitory activity (%) of undigested raw (RB), soaked (SB), autoclaved (AB), 10 days incubated autoclaved (AB10) and fermented (FB) Roget beans and of AB and FB after GI digestion. a,b,c Different lowercase letters indicate significant differences between undigested beans ($p < 0.05$). A,B Different capital letters indicate significant differences between undigested and digested samples ($p < 0.05$).

4. CONCLUSIONS

Fermentation of Roget beans with *Pleurotus ostreatus* significantly enhances protein content, improves amino acid profile, and increases protein digestibility compared with other processing methods like soaking or heat treatments. Moreover, it effectively reduces antinutrients like phytates and trypsin inhibitors, thereby enhancing nutrient absorption. In addition, fermentation slightly reduces carbohydrate digestibility compared to autoclaving, supporting a lower glycaemic index. Importantly, fermentation markedly boosts ACE inhibitory activity to 38%, highlighting its potential for cardiovascular health. Overall, solid-state fermentation with *P. ostreatus* enhances the nutritional and functional properties of Roget beans, making them a promising ingredient for health-oriented foods.

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