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Standardization of processing parameters for production of protein hydrolysate from chicken liver by using *Lactobacilli* fermentation

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ABSTRACT

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This study aimed to optimize fermentation conditions for producing chicken liver hydrolysates with antioxidant properties using Lactobacilli, contributing to a more sustainable and resilient food and poultry industry. Chicken liver protein hydrolysates were prepared by fermentation using *Lactobacillus helveticus* and *Lactiplantibacillus plantarum,* and their concentrations and processing conditions were optimized according to pH and degree of hydrolysis. SDS-PAGE was performed to evaluate molecular weight of hydrolysates and then antioxidant activity was assessed. Two hydrolysates showing best antioxidant activities were selected for further physico-chemical characterization after freeze drying. The SDS-PAGE analysis of liver protein hydrolysates obtained by *L. helveticus* fermentation showed bands in range of 15-5 kDa, whereas those obtained by *L. plantarum* fermentation showed distinct bands in the range of 18-5 kDa. Hydrolysates fermented with a 2% inoculation rate for 16 hours exhibited a significantly ($p \leq 0.05$) higher degree of hydrolysis, as well as increased DPPH and ABTS values, before undergoing freeze-drying. The freeze-dried hydrolysates fermented with *L. helveticus* demonstrated a significantly ($p \leq 0.05$) higher yield and protein content. These findings conclude that Lactobacilli fermentation can be used effectively for the production of chicken liver hydrolysates with bioactivities such as antioxidant properties and effectively used in the food industry.

Keywords: Protein hydrolysate, Fermentation, Chicken liver, Lactobacillus, Antioxidant

INTRODUCTION

In recent years, the production and consumption of chicken meat have increased significantly. In India, the growth of the chicken meat industry has been driven by developments in both the public and private sectors (Churchil, 2022). Parallelly, the production of slaughterhouse by-products has also risen substantially. However, these by-products remain largely underutilized. Approximately 6-7% of the total weight of dressed poultry is composed of giblets, including the liver, heart, and gizzard, which are regarded significantly edible by-products of poultry (Biswas *et al.*, 2012). Chicken livers, which are rich in high-quality protein, can contribute to a generous amount of income, particularly when utilized as functional food ingredients. Recent studies suggest that chicken liver serves as an excellent source of protein hydrolysates, which exhibit various biological properties, including antimicrobial, antioxidant, and other functional activities (Romero-Garay *et al.*, 2022). Protein hydrolysates are the result of the fragmentation of proteins into smaller peptides or individual amino acids. This breakdown occurs through chemical or enzymatic processes that cleave peptide bonds of proteins. Protein hydrolysates are used in a wide range of sectors, including nutritional supplements, food formulations, pharmaceuticals, and animal feed sector, etc. The use of by-products from the poultry industry to produce protein hydrolysates can address issues related to environmental pollution, appropriate disposal, and economic losses. This has led to a growing focus on exploring and promoting them as a promising research objective.

Hydrolysates can be extracted through various methods, one of them being the chemical or acid-base method (Zhao *et al.*, 2020). Few studies have also used the pH method to recover chicken heart protein isolate with high yield (Mishra *et al.*, 2023). However, the employment of acid-base reagents raises environmental concerns owing to the chemical usage, posing a drawback to this method for industrial liver protein extraction. Furthermore, chemical hydrolysis can lead to products with diminished nutritional qualities and biological activities due to the possible generation of undesired byproducts during nonspecific chemical treatment. Alternatively, the hydrolysis of protein substrates using external proteolytic enzymes is a commonly employed method for processing protein hydrolysates (Kumari *et al.*, 2024) and peptides with desired biological properties, although it may not be very cost-effective. Enzymatic extraction breaks down larger molecules of raw material into smaller ones, enhancing both the hydrophilicity and solubility of proteins in the extraction solvent, thus reducing the usage of the solvent

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and production costs (Zou *et al.*, 2021). However, the presence of hydrophobic amino acids in certain peptides may contribute to bitterness. Fermenting food proteins with proteolytic microorganisms offers an alternative method for producing and processing protein hydrolysates on an industrial scale. Compared to traditional enzymatic hydrolysis, fermentation presents a more cost-effective approach for obtaining food-grade protein hydrolysates and bioactive peptides. Employing microorganisms eliminates the expense associated with the extraction and purification of enzymes for enzymatic production. Additionally, fermentation improves the nutritional value, taste, and physical characteristics of the final products (Tan *et al.*, 2019). Fermentation using lactic acid bacteria (LAB) is an eco-friendly and sustainable technology that helps preserve waste while extracting valuable biomolecules. Metabolic activities of *Lactobacillus* species, such as *L. plantarum* and *L. acidophilus*, have recently been explored for their antioxidant and anti-inflammatory properties (Monika *et al.*, 2022). Therefore, the fermentation process is a viable option for protein hydrolysate production.

While previous studies have delved into the enzymatic hydrolysis of porcine and chicken livers, the potential of Lactobacilli fermentation remains mostly untapped to enhance the antioxidant properties of chicken liver hydrolysates. Thus, this study aims to optimize the Lactobacilli fermentation technique to produce chicken liver hydrolysates with potent antioxidant capabilities.

MATERIALS AND METHODS

The freeze-dried *Lactobacillus helveticus*-NCDC-292 and *Latiplantibacillus plantarum*-NCDC-025 were sourced from the National Collection of Dairy Cultures (NCDC) at the ICAR-National Dairy Research Institute (ICAR-NDRI), Karnal, Haryana. Analytical grade chemicals were procured from firms such as LobaChemie Pvt. Ltd., Merck Life Science Pvt. Ltd., Sisco Research Laboratories Pvt. Ltd. and HiMedia Laboratories Pvt. Ltd. Chemicals used to analyze the antioxidant quality of hydrolysates such as 2, 2-azinobis 3-ethylbenzathiazoline-6-sulfonic acid (ABTS) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were obtained from Central Drug House (P) Ltd. The BLUelf-prestained protein ladder (245-5 kDa) was purchased from GeneDireX, Inc. (USA) for SDS-PAGE.

Preparation of sample

Fresh chicken liver was hygienically sourced from the Post-Harvest Technology (PHT) section of ICAR-Central Avian Research Institute (CARI) in Izatnagar, Uttar Pradesh. The birds were slaughtered humanely and hygienically. Organ samples were cleaned and then vacuum packed into low-density polyethylene (LDPE) packages of 0.2 mm thickness. Chicken liver samples were kept in the freezer (Blue Star, FS345, Denmark) at -20±2°C for further use.

Production of liver protein hydrolysates by fermentation of Lactobacilli

Freeze dried *Lactobacillus helveticus* -292 and *Lactobacillus plantarum* -025 cultures were revived using 12.5% sterilized skim milk. Activated cultures were kept in litmus milk by repeated subculturing every 10 days. The working cultures were prepared in 100 ml of sterilized skim milk from the mother culture. The bacterial concentration in the working cultures was standardized to 10w CFU/mL. The frozen liver samples were thawed for 12hr at refrigeration temperature (4±2°C). The samples were then diluted 1:1 with DDW and then homogenized using Ultra-Turrax T25 (Baden-Württemberg, Germany) in 13,500 min^{-1} speed till a uniform homogenate was obtained. The pH was checked and then the homogenates were heat treated using a water bath at 85°C for 15 min to eliminate any microbial contamination and inactivate any native/innate enzymes. Culture was added in each sample at 1% (10^7 CFU/mL) and 2% (2×10^7 CFU/mL) concentrations. After conducting a few preliminary tests, a 5% lactose concentration was set to be incorporated into each ground sample to act as substrate for fermentation to get the desired level of acidity (Kumar *et al.*, 2017). After adding lactose and culture aseptically to the homogenate within a laminar flow (ESCO Airstream Class II BCS, Singapore), the samples were kept inside an orbital shaking incubator (ORBITEK, India) at an optimal temperature of 37°C and a speed of 140 rpm for fermentation. The samples were drawn at 4, 8, 12, and 16 h. The controls were taken at 0 h.

The fermented products were immediately heated at 85°C to inactivate the cultures. The pH was recorded and then the fermentates were centrifuged (HERMLE Z 446 K, Germany) at 16,210 rcf for 25 min at 4°C to collect the fermented liquor supernatants containing protein hydrolysates. The hydrolysates were stored at -20±2°C for further analysis.

Determination of pH and DH (degree of hydrolysis)

The pH of the liver hydrolysates was measured using a digital pH meter (pH 700, Eutech Instruments, India). The degree of hydrolysis (%) was estimated following the method described by Maluf *et al.* (2020). To solubilize the protein, 500 µL of a 20% (w/v) trichloroacetic acid solution was mixed with a 500 µL sample aliquot, kept at room temperature for approximately 30 minutes, and then centrifuged at 1990 rcf for 15 minutes at 4°C. The protein content of the solubilized fraction was analyzed based on the method outlined by Rodrigues *et al.* (2021). Bovine serum albumin (BSA) was taken as a standard. DH % was calculated using Equation 1:

$$DH\ (\%) = \frac{\text{inbilized protein content in 20\% TCA (3mg)}}{\text{Total protein content}} \times 100 \quad \text{(Eqn.1)}$$

Estimation of molecular weight of liver protein hydrolysates using SDS PAGE

SDS-PAGE electrophoresis was conducted to analyze protein hydrolysates and assess the degree of hydrolysis, following the method described by Mohanty *et al.* (2021) with slight modifications. Polyacrylamide gels were prepared, consisting of a 16% resolving gel (1.5 M Tris-HCl, pH 8.8) and a 4% stacking gel (0.5 M Tris-HCl, pH 6.8). Protein hydrolysate samples were diluted at a 1:4 ratio using a sample buffer containing 20% mercaptoethanol, 4% SDS, 20% glycerol, 0.125 M Tris, and 0.02% bromophenol blue for staining. A BLUelf-prestained protein ladder (GeneDireX, Inc., USA) with a molecular weight range of 245 to 5 kDa was used in Tris-Glycine electrophoresis. Protein separation was carried out using a Bio-Rad Mini PROTEAN II System Cell (Bio-Rad Laboratories, Hercules, CA, USA) at a constant voltage of 100 V. After electrophoresis, the gels were stained with a 0.25% Coomassie Brilliant Blue R-250 solution (Bio-Rad, CA, USA) and agitated on a shaker for 45 minutes to visualize the protein bands.

DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity

The sample's antioxidant capacity was evaluated using the method outlined by Kumar *et al.* (2021). To begin, 1 mL of 100 µM DPPH reagent was combined with 250 µL of 0.1 M Tris-HCl buffer (pH 7.4) and 25 µL of the sample. After gentle stirring, the absorbance was measured at 517 nm at t = 0 min (tŽ). The mixture was then incubated in the dark at room temperature, and a second absorbance reading was taken at t = 20 min (t, Ž). Ethanol served as the blank. The calculation of free radical scavenging activity was determined by the reduction in absorbance using equation 2:

Scavenging activity (% inhibition) =100 - [(A_{20} /A_0) × 100] (Eqn.2)

where A_{20} denotes absorption at the 20th minute.; A_0 denotes absorption at 0th min

2-2-Azinobis-3-ethylbenthiazoline-6-sulfonic acid (ABTS) radical scavenging activity

The ABTSz scavenging activity was determined through spectrophotometric analysis following the method of Calderón-Chiu *et al.* (2021), with minor modifications. A 7 mM ABTSz solution was prepared by dissolving ABTSz in phosphate-buffered saline (PBS). To generate ABTSz radicals, the stock solution was mixed with an equal volume of 2.45 mM potassium persulfate (K, S, Oˆ) and left undisturbed in the dark for approximately 16 hours before use. The solution was diluted with double-distilled water (DDW) to achieve an absorbance of 0.70 at tŽ (t=0 min) and stabilized at precisely 30°C after six minutes of initial mixing. Then, 1 mL of the measured at 734 nm after 20 minutes (t, Ž) using an Eppendorf BioSpectrometer Basic. The $ABTS^+$ activity was then calculated using eqn. 3.

ABTS activity (% inhibition) = [(0.7- A_{20})/0.7] ×100 (Eqn.3)

Where, A_{20} denotes absorption at 20th min

Freeze-drying of liver protein hydrolysates

According to results of pH, DH% and antioxidant parameters of liver protein hydrolysates, two chicken liver protein hydrolysates were chosen for further analysis. Freeze-drying was carried out at a temperature below -50°C and a pressure lower than 150 mTorr using a Freeze Dryer (IlShin BioBase, South Korea).

Physico-chemical characterization of freeze-dried liver protein hydrolysates

The water activity (a_w) was calculated using 4TE Dew Point water activity meter, Aqua lab, Malaysia. Finely powdered lyophilized hydrolysate samples were loaded into a moisture-free sample cup to about 50% capacity. The cups werethen transferred inside instrument's holder, the lid was shut, and the instrument was secured. Each sample was left in the instrument for approximately 10-15 minutes to achieve a stable reading. Duplicate measurements were estimated for each sample.Moisture, protein, fat and ash content of the freeze-dried hydrolysates were determined according to Verma *et al.* (2017).

Statistical analysis

Two sets of identical samples were recorded for each parameter. Experiments were replicated three times to make the total six observations (n=6). The means were calculated using Duncan's Multiple Range Test (DMRT), and the data was analyzed with SPSS 26.0 software. The results were then presented in tabular format for interpretation. Statistical significance was set at p≤0.05 (5% level).

RESULTS AND DISCUSSION

Effect of fermentation on pH and degree of hydrolysis of chicken liver hydrolysates

The changes in pH and DH % of the hydrolysates were evaluated at 4 h intervals throughout the fermentation period until 16 h. There was a significant (p≤0.05) decrease in pH of all samples at the end of 16 h of fermentation (Table 1). Organic acids, lactic acid, mainly produced by the LABs, play a strong role in decreasing pH during fermentation period. Luan *et al.* (2021) observed similar results, where the pH of fermented pork sausage decreased to 4.96 after 24 h of fermentation with *L. plantarum* CD101. This low pH can help to keep an eye on spoilage bacteria (Yu *et al.*, 2020).

At 16 h, significant increase (p≤0.05) in DH % between the samples was observed (Table 1). Liver samples fermented with 2% *L. helveticus* showed the highest change in DH % with time (54.29%). This observation might be as due to higher proteolytic property of *L helveticus* resulting in production of smaller peptides

Table 1: Effect of fermentation on pH and degree of hydrolysis (%) of chicken liver protein hydrolysate

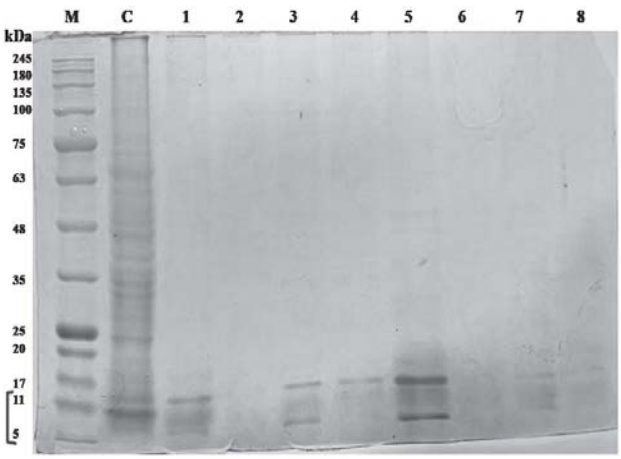
Treatments	Fermentation time (hours)				
	0 h	4 h	8 h	12 h	16 h
			pH		
1Lh	6.17 ± 0.02^{Aa}	5.77 ± 0.06^{Bb}	4.63 ± 0.07^{Bc}	4.44 ± 0.04^{Bcd}	4.38 ± 0.06^{Cd}
2Lh	6.15 ± 0.04^{Aa}	5.51 ± 0.03^{Cb}	4.44 ± 0.03^{Cc}	4.39 ± 0.02^{Bcd}	4.34 ± 0.02^{Cd}
1Lp	6.21 ± 0.03^{Aa}	5.96 ± 0.04^{Ab}	5.22 ± 0.03^{Ac}	4.91 ± 0.03^{Ad}	4.60 ± 0.1^{Ae}
2Lp	6.19 ± 0.03^{Aa}	5.83 ± 0.04^{Bb}	5.04 ± 0.11^{Ac}	4.85 ± 0.04^{Ad}	4.51 ± 0.04^{BCe}
			Degree of Hydrolysis (%)		
1Lh	14.17 ± 0.13^{Ad}	16.19 ± 0.69^{BCd}	23.65 ± 0.86^{Bc}	33.43 ± 0.96^{Bb}	40.55 ± 0.57^{Ca}
2Lh	14.27 ± 0.47^{Ae}	26.83 ± 0.9^{Ad}	34.44 ± 1.06^{Ac}	46.24 ± 0.99^{Ab}	54.29 ± 1.1^{Aa}
1Lp	14.23 ± 0.07^{Ad}	16.77 ± 0.33^{Dc}	18.03 ± 0.6^{Cc}	24.77 ± 1.04^{Cb}	37.78 ± 0.82^{Da}
2Lp	14.30 ± 0.04^{Ae}	18.16 ± 0.18^{Bd}	24.33 ± 0.49^{Bc}	35.11 ± 0.38^{Bb}	45.02 ± 0.98^{Ba}

n=6, Mean±Standard Error bearing different superscripts in uppercase letter within columns and lowercase letter within rows differ significantly ($p\leq0.05$). 1Lh- 1% *L. helveticus*, 2Lh- 2% *L. helveticus*, 1Lp- 1% *L. plantarum*, 2Lp- 2% *L. plantarum.*

and free amino acids. Agreeing with the observation, Anggraeni *et al*. (2022) reported 44.32% DH in hydrolysates of edamame protein (a legume) formed by 24 h fermentation with *Lactococcu slactis*.

SDS PAGE

The SDS PAGE profile was done for the liver hydrolysates by taking a raw liver sample as a control. Distinct bands were noticed for liver hydrolysates fermented with *L. helveticus* for periods of 4, 8, 12 and 16 h in molecular mass range of 15-5 kDa (Fig. 1a). Similarly, liver hydrolysates produced by fermentation with *L. plantarum* for periods of 4, 8, 12 and 16 h showed bands ranging from 18 to 5 kDa (Fig. 1b). The reduced molecular mass of the hydrolysates in comparison to that of the raw liver sample (control) is indicative of the modification in the structure of the protein during fermentation. This modification includes the unfolding of protein and the hydrolysis by intracellular protease enzymes produced by both *L. helveticus* and *L. plantarum* during fermentation process. This process results in the production of lower molecular weight peptides, which are more likely to cross the intestinal barrier and exhibit their biological effects (Chalamaiah *et al.,* 2015).



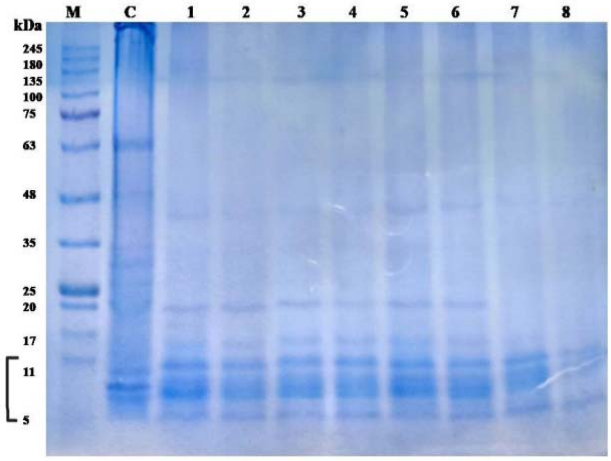


Fig. 1: SDS-PAGE profiles of chicken liver hydrolysates obtained **a)** after fermentation with *Lactobacillus helveticus* and **b)** after fermentation with *Lactiplantibacillus plantarum*
(Lane M = Molecular marker (GeneDireX), C= Raw liver, 1= 1% bacterial concentration and 4 h fermentation, 2= 1% bacterial concentration and 8 h fermentation, 3= 1% bacterial concentration and 12 h fermentation, 4= 1% bacterial concentration and 16 h fermentation, 5= 2% bacterial concentration and 4 h fermentation, 6= 2% bacterial concentration and 8 h fermentation, 7= 2% bacterial concentration and 12 h fermentation, 8= 2% bacterial concentration and 16 h fermentation.

Antioxidant activities of chicken liver hydrolysates

In the present study, DPPH and ABTS assays were assessed for the evaluation of the antioxidant activity of liver protein hydrolysates. The DPPH radical scavenging assay was used to assess the hydrogen-donating ability of antioxidant compounds, while the ABTS assay measured their single electron transfer capacity. There was a gradual increase in both the activity of DPPH (Fig. 2a) and ABTS (Fig. 2b) of the hydrolysates with an increase in fermentation time, regardless of the type or concentration of the Lactobacilli. However, samples fermented with 2% bacterial concentration exhibited

significantly (p≤0.05) higher antioxidant activity compared to those fermented with 1% bacterial concentration. This finding aligns with the study by Jain and Anal (2017), which reported that the antioxidant activity of eggshell hydrolysates fermented with Lactobacillus plantarum followed a dose-dependent pattern, showing a significant (p≤0.05) increase as the bacterial concentration increased. Studies indicate that the higher antioxidative properties of hydrolysates are a direct reflection of their lower molecular weight peptides (Kumar *et al.*, 2021). Cao *et al.* (2019) reported that *L. plantarum*can significantly improve the ABTS radical scavenging activity of fermented sausages. All hydrolysates exhibited increased radical scavenging efficacy according to the ABTS method, possibly due to their tendency to interact with the hydrophilic radical ABTS.

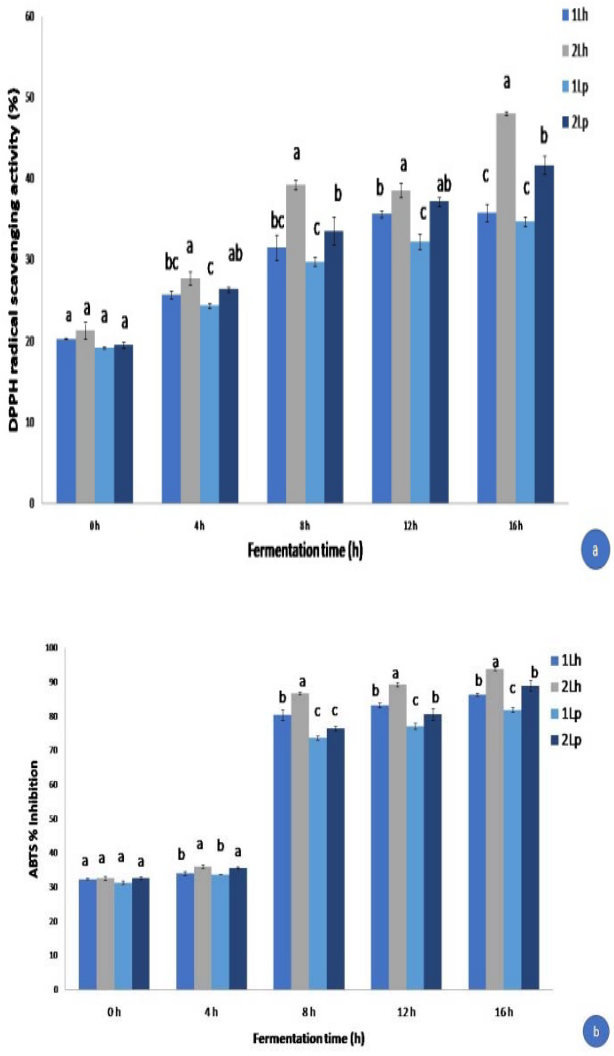


Fig. 2: Effect of fermentation on DPPH (a) and ABTS (b) activities. 1Lh- 1% *L. helveticus*, 2Lh- 2% *L. helveticus*, 1Lp- 1% *L. plantarum*, 2Lp- 2% *L. plantarum.*

Preparation of freeze-dried protein hydrolysate powder

Based upon the results of optimization, two hydrolysates namely, 2Lh16 (2% *L.helveticus* concentration, 16 hfermentation time) and 2Lp16 (2% *L. plantarum* concentration, 16 hfermentation time) were selected for further evaluation. These samples were freeze-dried and further characterized for their physico-chemical properties.

Physico-chemical characterization of protein hydrolysate powder

Powder yield and Water activity (a_w): The powder yield and water activity (a_w) of 2Lh16 and 2Lp16 were analyzed and then compared with freeze-dried raw liver (Lc). 2Lh16 had significantly (p≤0.05) higher powder yield than 2Lp16 (Table 2). Various factors such as temperature, pH, type of bacteria used and fermentation time affect degree of hydrolysis, which has an influence on yield of the hydrolysate. Thus, significantly (p≤0.05) higher degree of hydrolysis of 2Lh16 (54.29±1.1) compared to 2Lp16 (45.02±0.98), as indicated in Table 1, might be a reason for higher powder yield of 2Lh16. The drying method also influences yield of hydrolysate powder. Noman*et al.* (2020) reported yield of lyophilized Chinese sturgeon hydrolysate as 16.77 and 13.3%, hydrolyzed by papain and alcalase enzymes respectively. Kumar *et al.*, (2021) reported yield of spray dried and freeze-dried spent hen hydrolysate powder as 27.77% and 10.12% respectively, which aligning with the results of current study.

The water activity of powder samples significantly influences the stability, storage characteristics and various technical properties of the powder. Higher water activity results in increased free water content, adversely impacting the product's shelf life (Vardinand and Yasar, 2012). There was significant (p≤0.05) difference between a_w of all the samples with 2Lh16 showing the lowest a_w, which might lead to better shelf life (Table 2).

Proximate composition of freeze-dried protein hydrolysates:

The proximate composition of liver hydrolysates was analyzed and then compared with freeze-dried raw liver (Table 2). The crude protein percentage of raw chicken liver was found to be 75.56% on dry matter basis which indicated its suitability as a good source for producing hydrolysates. Liver hydrolysates fermented with *L. helveticus* exhibited a significantly (p≤0.05) higher protein percentage compared to those fermented with *L. plantarum*. The fat percentage of both the hydrolysates was less than that of raw liver, which might be as a result of destruction of structural lipids of cell membranes during the hydrolysis process. Therefore, liver protein hydrolysates with lower fat content might be able to contribute in producing food with higher oxidative stability (Chakka *et al.*, 2015).

Table 2: Physico-chemical characterization of freeze-dried raw liver (Lc) and liver hydrolysates (2Lh16 and 2Lp16)

Parameters	Lc	2Lh16	2Lp16
Yield (%)	15.45±0.33^{c}	24.62±0.87^{a}	20.57±0.56^{b}
Water activity (a_w)	0.48±0.005^{a}	0.44±0.007^{c}	0.46±0.004^{b}
Protein (%)	75.56±0.53^{c}	88.43±0.30^{a}	85.34±0.36^{b}
Moisture (%)	1.61±0.07^{a}	1.21±0.04^{c}	1.40±0.07^{b}
Fat (%)	14.29±0.16^{a}	4.48±0.04^{c}	5.57±0.02^{b}
Ash (%)	4.72±0.06^{c}	5.22±0.03^{b}	5.71±0.04^{a}

n=6, Mean±Standard Error bearing different superscripts within row differ significantly (P≤0.05). Lc- Freeze-dried raw liver; 2Lh16- 2% *L. helveticus*, 16 h; 2Lp16- 2% *L. plantarum*, 16 h.

CONCLUSION

This study focused on optimizing the fermentation process for producing chicken liver hydrolysates using *L. helveticus* and *L. plantarum.* The antioxidant activity of the hydrolysates was assessed through DPPH and ABTS assays, revealing that the antioxidant properties were influenced by the bacterial strain, its concentration, and fermentation duration. Notably, hydrolysates fermented with *L. helveticus* demonstrated superior antioxidant activity and yield compared to those produced with *L. plantarum*. These findings highlight Lactobacillus fermentation as a promising approach for generating protein hydrolysates from chicken liver, with potential applications in functional food development. The optimized process offers an effective way to utilize underutilized chicken liver, benefiting both the food and poultry industries.

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest, whether financial or otherwise.

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