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Isolation and Characterization of the Microbiological and Physicochemical Qualities, the Protein and Amino Acid Profiles of Fermented Chicken and Duck Egg Ovalbumin

Nurliyani[a,*], M. Z. Abidin[a], & I. Kandarina[b]
[a]Department of Animal Product Technology, Faculty of Animal Science, Universitas Gadjah Mada
Jl. Fauna 3 Yogyakarta 55281 Indonesia
[b]Department of Biostatistics, Epidemiology, and Public Health, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada
Jl. Farmako Sekip Utara, Yogyakarta 55281 Indonesia
*Corresponding email: nurliyani@ugm.ac.id
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ABSTRACT

The objective of this study was to isolate and characterize the microbiological and physicochemical qualities, and the protein and amino acid profiles of fermented chicken and duck egg ovalbumin (OVA). The OVA was fermented using lactic acid bacteria (LAB) *Lactobacillus paracasei* M104 taken from goat milk and yeast *Kluyveromyces marxianus* KFA9 obtained from kefir. The OVA and fermented OVA were characterized by analyzing its electrophoresis and spectral profile on FTIR, amino acid profile, and microbiological and physicochemical properties. The findings indicated that the chicken and duck OVA showed a molecular weight between 45-55 kDa. The amount of LAB in OVA before and after fermentation was not significantly different. However, the amount of yeast decreased after fermentation. The concentrations of soluble protein, total free amino acids, and alcohol in the fermented chicken OVA were higher than those in fermented duck OVA. The OVA of chicken and duck eggs showed a comparable pattern in specific bands within the FTIR spectrum. However, there were several different bands in the FTIR spectra between OVA and fermented OVA. Specific carbohydrate-containing bands were notably absent in the fermented OVA. After fermentation, the chicken OVA indicated an increase in all types of amino acid concentrations. Conversely, the amino acid concentrations were constant before and after fermentation in duck OVA. The changes in the secondary structure of protein may affect its functional characteristics, which needs further studies. It is expected that fermented OVA produced using local starters can be used as an ingredient in functional foods.

Keywords: egg ovalbumin; fermentation; microbiological properties; physicochemical properties; protein profile

INTRODUCTION

Egg white is rich in high-quality protein compounds, making it highly ideal for children to consume during their growth. Ovalbumin constitutes over fifty percent of protein and is highly digestible, making it good to improve the nutritional status of malnourished persons and those with impaired digestion (Lee *et al.*, 2019). Apart from that, eggs also contain functional components such as foaming and emulsifying agents. However, eggs may also cause allergies, especially in children. Consequently, strategies are needed to reduce the allergenicity of eggs while simultaneously increasing the functional properties of eggs so that eggs can be used as ingredients in functional foods. Egg whites, also known as albumen, account for approximately 60% of the entire egg mass that consists primarily of water and protein. The protein in egg whites is mostly ovalbumin, with ovotransferrin and ovomucoid as primary proteins. Additional egg white protein, which regulates albumen viscosity, consists of lysozyme, avidin, cystatin, ovomacroglobulin (ovostatin), ovomucin, lysozyme, and avidin (Mine *et al.*, 2005). Egg white (EW) consists of about 88% water, 11% protein, 0.2% fat, and 0.8% ash. The important proteins found in egg whites are ovalbumin (54%), ovotransferrin (12%-13%), ovomucoid (11%), lysozyme (3.4%-3.5%), and ovomucin (1.5%-3.5%); the avidin content is only 0.05% (Li *et al.*, 2022a).

Ovalbumin, the most quantity protein in egg white, has a molecular weight of 45,000 Da and consists of a single chain of 385 amino acids with 105 titratable residues. OVA is a compound of three substances (A1, A2, and A3 in the ratio 85:12:3) with slightly different electrical characteristics. Of these, A1 has 2 mol of phosphate groups, A2 has 1 mol of phosphate group, and A3 has no phosphate group. All these phosphate groups are associated with the hydroxyl group of serine. The treatment with phosphatase on A1 and A2 resulted in phosphoryl-group free molecules (Li *et al.*, 2022a).

In addition to binding phosphate, ovalbumin is a single glycoprotein with an acetylated N-terminus. It contains four sulfhydryl groups and one disulfide bridge (Cys74-Cys121), which may not be accessed in its natural form (Kanaka *et al.,* 2018). According to Munjal & Khan (2018), the structure of ovalbumin consists of a single polypeptide chain of about 460 residues (about half of which are hydrophobic), a maximum of 2 phosphate residues per molecule, and an oligosaccharide side chain composed of only mannose and glucosamine residues (a single N-linked glycosylation site at Asn292) (Munjal & Khan, 2018; Shi *et al.,* 2018).

Egg white's foam, gel, and emulsifier characteristics are significantly affected by ovalbumin. It has nutritious properties due to its inclusion of all necessary amino acids. Additionally, it exhibits a remarkable antioxidant activity, antimicrobial, anticancer, and immunomodulator (Lee *et al.,* 2019; Li *et al.,* 2022b). A previous study reported that the ovalbumin peptides produced from the hydrolysis of ovalbumin with the papain enzyme show the strongest immunomodulatory activity compared to the hydrolyzed peptides with the other protease enzymes. The enzymatic breakdown of ovalbumin using papain can substantially enhance the ability of RAW 264.7 cells to engulf foreign particles and stimulate the formation of tumor necrosis factor-alpha (TNF-α), nitric oxide (NO), and interleukin-6 (IL-6) secretion (Choa *et al.,* 2023). Peptides can be produced from protein hydrolysis with protease enzymes or with microbes that have protease activity through fermentation processes. Hydrolysis of OVA through a fermentation process using local starter *Lactobacillus paracasei* M104 and *Kluyveromyces marxianus* KFA 9 has not been reported.

L. paracasei M104 was obtained from goat milk to induce acidification in goat milk by fermentation (Widodo *et al.,* 2019). According to Ghosh *et al.* (2019), *L. paracasei* are diverse Gram-positive bacteria that are very closely related to *Lactobacillus casei*, belonging to the *L. casei* group. In *L. casei*, fermentation is a crucial source of energy, which can ferment glucose, fructose, mannose, galactose, mannitol, N-acetylglucosamine, and tagatose. In addition, LAB species *Lacticaseibacillus casei* show a proteolytic activity that can hydrolyze proteins into peptides and amino acids (Satilmis *et al.,* 2023).

Moderate lactic acid fermentation using *Streptococcus thermophilus* and *Lactobacillus bulgaricus* provides egg whites with higher foaming activities (Jiang *et al.,* 2020). In addition, the 3-hour fermentation of ovalbumin using multiple Lactobacillus (*Lactobacillus delbruekii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, *Streptococcus thermophilus,* and *Bifidobacterium animalis)* increase the surface of the hydrophobicity and then it is gradually degraded by protease and eventually increase the foaming ability (Jia *et al.,* 2021). The study provided basic information on the application of fermentation approach in improving the functionalities of egg white in industries.

Kefir is a dairy product that undergoes fermentation using a starting culture called kefir grains. The process involves the interaction of different microorganisms found in kefir, resulting in the production of several bioactive substances that are beneficial for health (Vieira *et al.,* 2021). The molecular identification process has revealed that four isolates obtained from kefir, specifically KFA 3, KFA 7, KFA 9, and KFB 1, can be classified as *K. marxianus.* The kinetic modeling evaluation showed that KFA 9 had the most significant amount of biomass yield, product yield, and carrying capacity coefficient. Based on the comparison of the four types of *K. marxianus*, it is evident that KFA 9 stands out as the preferred option. This is primarily attributed to its faster growth rate and superior ethanol production efficiency (Kurniawati *et al.,* 2022).

K. marxianus KFA 9 can ferment glucose (Kurniawati *et al.,* 2022). Ethanol fermentation ability of the thermotolerant yeast *K. marxianus* can utilize various sugars, including glucose, mannose, galactose, xylose, and arabinose (Rodrussamee *et al.,* 2011). The proteolytic activity in milk fermented using *K. marxianus* produces an increase in peptide types compared with milk fermented using Lactobacillus (Zhang *et al.,* 2017). The strain *K. marxianus* JY-1 was safe and had high protease activity that could highly hydrolyze casein. The hydrolysate of casein hydrolyzed by *K. marxianus* JY-1 isolated from traditional fermented milk could promote the proliferation of RAW264.7 macrophages and the production of cytokines (IL-6, IL-1β, and TNF-α) compared with the control group, showing obvious immunomodulatory activity (Li *et al.,* 2023). Consequently, *K. marxianus* can enhance proteometabolism in milk when added with *Lactobacillus*, produce flavor components, and ameliorate the digestion and absorption properties of milk (Zhang *et al.,* 2017).

There have been no reports on the use of the local starter *L paracasei* M10 isolated from goat milk and *K. marxianus* KFA9 isolated from local kefir for ovalbumin fermentation. Therefore, this study aims to provide initial information about the isolation and characterization of the microbiological quality, physicochemical quality, as well as the protein and amino acids profile of the fermented ovalbumin using *L.paracasei* M10 and *K. marxianus KFA9*. The information above shows that *Lactobacillus casei* and *K. marxianus* can ferment mannose and have proteolytic activity. *L. paracasei* M104 and *K. marxianus* KFA 9, which are locally isolated, will grow on the OVA substrates containing mannose, which can produce specific product characteristics. The fermented OVA is expected to be used as an ingredient in functional foods.

MATERIALS AND METHODS

This study began with the purification of ovalbumin protein from chicken and duck egg whites. Then, the purified ovalbumin was fermented using a combination of a bacterial and yeast starter. The fermented ovalbumin was analyzed for its microbiological quality (total lactic acid bacteria and total yeast) and physicochemical quality (viscosity, pH, alcohol content, water, soluble protein, and total free amino acids). In addition, the ovalbumin and the fermented ovalbumin were characterized by their electrophoretic profile, protein secondary structure, and amino acid profile.

Ovalbumin Isolation and Purification

The protocols used to isolate ovalbumin have been reported by Geng *et al*. (2019) and Pereira *et al*. (2016). Initially, we isolated the egg white from the yolk. Then, the egg whites were agitated for 15 minutes, followed by a solution containing 21% polyethylene glycol (PEG) 8000 was introduced and subjected to centrifugation for 15 minutes at a low temperature. The liquid portion was collected, and its acidity was modified to a pH of 4.5 (which is the isoelectric point of OVA) using citric acid. Next, it was subjected to centrifugation at a low temperature for 15 minutes. The substance formed was ovalbumin. The OVA's purity was assessed using electrophoresis. The OVA that had been obtained underwent freeze-drying and subsequent characterization, which involved determining the soluble protein content and analyzing the protein's secondary structure using FTIR. High-performance liquid chromatography (HPLC) was employed to analyze amino acid profiles.

OVA Fermentation

Before the OVA fermentation process began, creating a yeast starter and a lactic acid bacteria starter was imperative. The yeast cultures of K. marxianus KFA9 and L. paracasei M104 were prepared by growing them in malt extract, De Man, Rogosa, and Sharpe (MRS) broth. Next, the cultures grew for 18 hours at ambient temperature. Next, 5% of the two cultures were transferred to a medium that contained an OVA mixture. The cultures were then incubated at room temperature for 18 hours.

As much as 26% of the purified OVA sample was taken and added with sterile distilled water for as much as 64%. The OVA solution was inoculated with the 5% yeast starter *K. marxianus KFA9* that had been obtained from the local kefir in Yogyakarta, Indonesia. Additionally, the starter *L. paracasei M104* that had been isolated from goat milk was also added at a concentration of 5%. The milk was from the Ettawah crossbred goats in Sleman, Yogyakarta, Indonesia. The process of fermentation was conducted for 18 hours at ambient temperature. An assessment was conducted on the quality of the fermentation products, including parameters such as pH value, viscosity, moisture content, alcohol concentration, total amino acid content, soluble protein, total lactic acid bacteria count, and total yeast count. The OVA underwent fermentation and was then freeze-dried. The protein profiles of the freeze-dried OVA and the fermented OVA were analyzed using SDS-PAGE, and the secondary structure of the protein profile was determined using FTIR. Additionally, the amino acid profile of the OVA was assessed using HPLC. Similarly, chicken and duck egg whites underwent fermentation using the same method as that of OVA fermentation.

Quality Evaluation

The analysis of the microbiological quality of the fermented OVA includes the total count of lactic acid bacteria and the yeast, and the count used the total plate count (TPC) method. The value of pH was measured using pH-meter (OHAUS), the viscosity was measured by Viscometer (NDJ-5S), the moisture analysis was carried out using oven dryer, the alcohol contents were analyzed using the Conway micro diffusion method, the soluble protein was analyzed using the Lowry method, and the total of free amino acids (FAAs) was analyzed using reagent ninhydrin with the glycine standards.

Electrophoretic Profile

The visualization of the purified OVA was conducted using the SDS-PAGE technique, as reported by Hashim *et al.* (2019), which is on a buffered system with a discontinuous setup. A denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Mini-Protein II cell (Bio-Rad) to assess the effectiveness of the separation and the amount of product obtained. A polyacrylamide gel containing 10% polyacrylamide and a stacking gel containing 5% polyacrylamide were used. The Coomassie Brilliant Blue R-250 from Sigma was used for staining. The ovalbumin sample with a concentration of 10 mg/mL and a volume of 5 µL was added to each well for the running process. To assess the purity of OVA, gel images were captured after de-staining.

Protein Secondary Structure using FTIR

Following the method by Kong *et al*. (2007), infrared spectroscopy, a well-established and highly regarded experimental technique, is used to analyze the polypeptides and protein's secondary structure. FTIR spectroscopy uses electromagnetic radiation to show the interactions among matters in the form of a spectrum. Every molecule possesses a distinctive spectrum fingerprint, which shows its differentiation from the other compounds (Fadlelmoula *et al.*, 2022).

An analysis was conducted on the secondary structure of OVA from egg whites obtained from commercial chicken and local ducks. We used the technique by Abd-Elaziz *et al*. (2018) as the basis for our study by making minor adjustments. Potassium bromide (KBr) was combined with the OVA. They were then put into a vibration mill to homogenize the sample. The resultant mixture was pulverized and subjected to a vacuum to form a pellet. The FTIR spectra were acquired using a Shimadzu Prestige 21 FTIR Spectrometer at a temperature of 25 °C. The particle was subjected to FTIR spectroscopy in the range of 300-4000 cm^{-1}, with a magnification of 16 cm^{-1}, with a total of 10 scans. The infrared second derivative amide spectra were used to quantify different ovalbumin protein secondary structures found in the egg white. The areas under the bands linked with specific substructures were determined through manual calculation.

Amino Acid Analysis of OVA

We used High-Performance Liquid Chromatography (HPLC) equipment from thermo to determine

the amino acids. The methodology described by Marino *et al*. (2010) was adopted with some modifications. In the process of sample preparation, a 60 mg sample was measured, put inside the tube, and then sealed. Next, 4 mL of 6N hydrochloric acid was added and mixed vigorously until a homogeneous solution was obtained. The sample would then undergo hydrolysis by subjecting it to an autoclave at a temperature of 110 ^{o}C for 24 hours. The specimen was cooled to ambient temperature and then neutralized using a 6 N solution of NaOH. Next, a solution of Pb $(CH_3COO)_4$ was combined with a 40% concentration and with a 15% oxalic acid solution, and the resulting mixture was transferred into a vial. The solution was then diluted with distilled water until the total volume reached 10 mL. We extracted an amount of 3 mL from the sample and filtered it using a Whatman filter with a 0.20 mm pore size. A 50 µL portion of the material was combined with 300 µL of ophthaldehyde (OPA) and agitated for 5 minutes. The sample was injected with a maximum volume of 10 µL into the HPLC system.

The HPLC condition involves a LiChrospher 100 RP-18 (5 µm) as the stationary phase. The eluent consists of two components: A) a mixture of methanol (MeOH), a 50 mM acetic buffer, and tetrahydro folic (THF) in a ratio of 80:15:5, with a pH of 6.8, and B) 65% methanol (MeOH). The detector used is the Thermo Ultimate 3000 RS Fluorescence Detector. The samples were eluted using gradient elution at a flow rate of 1.5 mL/min, and the entire process took 45 minutes. At minute 0.1, pump A was operating at 100% capacity while pump B was idle. At minute 15, pump A was idle while pump B was operating at 35% capacity. At minute 30, pump A was idle while pump B was operating at 100% capacity. At minute 40, both pumps were idle. At minute 45, the system was stopped. A standard solution mixture was created by using 50 ppm of a standard solution obtained from a stock solution. Afterward, the standard solution was diluted by a factor of 2 using distilled water. Then, 50µL of the diluted standard was added to 950 µL of OPA. The solution was combined, matured for 5 minutes, and then added to the HPLC system with a volume of 10 µL.

Statistical Analysis

The electrophoretic and protein secondary structure data of OVA were presented descriptively (electrophoretic profile, FTIR profile, and FTIR table). Meanwhile, the amino acid profile data, microbiological quality, and physicochemical quality of the fermented chicken and duck ovalbumin were presented as averages from the three replicate samples. An independent sample t-test was used to compare the data of the chicken and duck OVA. The paired sample t-test was employed to analyze the data of OVA before and after fermentation. This is to highlight the differences between the two. The mean values were presented along with their standard deviations. The SPSS ver. 15.0 software (SPSS Inc., Chicago. IL. USA) was used to conduct the statistical analysis.

RESULTS

Yield and the Electrophoretic Profile of OVA

Table 1 shows the values of the chicken and duck OVA. The yield of duck OVA was greater ($p<0.05$) compared to that of chicken OVA after purification. The average yield of duck OVA was 12.11%, and the yield of chicken OVA was 2.73%.

Figure 1 displays the purified OVA in the form of a precipitate-citrate. The analysis of the isolated duck OVA using a 21% PEG solution revealed the presence of impurities, as shown in Figure 1, lane 2. Nevertheless, when 25% PEG was used to isolate duck OVA, a distinct and uncontaminated band was observed at 45 kDa in lanes 5 and 6 of Figure 1.

As a comparison, the following shows the protein profiles of fresh egg whites and their fermentation products (Figure 2). Chicken or duck egg whites and their fermented products showed similar protein profiles. However, the duck OVA and the fermented duck OVA bands showed a higher molecular weight (MW) range than the chicken OVA and the fermented chicken OVA bands.

Microbiological Quality

The amount of LAB and yeast in fermented chicken and duck OVA can be seen in Table 2**.** The LAB quantities were not significantly different in OVA before and after fermentation. The average total LAB in the fermented OVA was 7.78 log cfu/mL. However, the amount of yeast decreased ($p<0.05$) after fermentation (Table 2). The average of yeast before and after fermentation in the chicken OVA were 6.81 and 6.24 log cfu/mL, respectively, and 7.24 and 5.99 log cfu/mL in duck OVA, respectively.

As a comparison, the quantities of lactic acid bacteria (LAB) and yeast found in the egg white both before and after fermentation are provided in Table 3. The amounts of LAB and yeast were not significantly different in egg whites before and after fermentation, which means that there was no increase during the 18 h fermentation.

Physicochemical Quality

After the fermentation process had been performed, it was found that there were no notable disparities in the viscosity or moisture levels between the chicken and duck OVA. The average values of the viscosity and the moisture levels of fermented OVA

Table 1. The yield of ovalbumin purification of chicken and duck eggs

Egg origin	Albumen (Egg white) (g)	Ovalbumin (g)	Yield (%)
Commercial chicken	33.45	0.90	2.74±0.68^{a}
Duck	31.81	3.85	12.11±1.57^{b}

Note: ^{a,b} Means in the same column with different superscripts differ significantly ($p<0.05$).

were 33.68 mPa.s and 86.74%, respectively. However, duck OVA had lower ($p<0.05$) soluble protein, amino acids, and alcohol than chicken OVA (Table 4). The average values of the soluble protein, the total free amino acids, and the alcohol content in the fermented duck OVA were 14.40%, 153.33 µg/mL, and 0.14%, respectively. As for the average values of the soluble protein, the total free amino acids, and the alcohol level in the fermented chicken OVA were 15.59%, 202.33 µg/mL, and 0.21%, respectively. When the pH value was measured, it was found that there was a decrease ($p<0.05$) in pH after the OVA fermentation process (Table 5). The average pH levels of the chicken OVA before and after fermentation were 4.70 and 4.43, respectively, and the average pH levels of the duck OVA before and after fermentation were 4.70 and 4.40, respectively.

When compared with egg white fermentation, based on Table 6, there was no change in moisture content and soluble protein, but there was a decrease ($p<0.05$) in pH after the fermentation of the egg white. As for the total amino acids and alcohol contents in the fermented chicken and duck egg white, as shown in Table 7, there was a significant difference ($p<0.05$) between the rates of the chicken egg and duck egg white free amino acid, but there was no significant difference between the alcohol contents found in chicken and duck egg white.

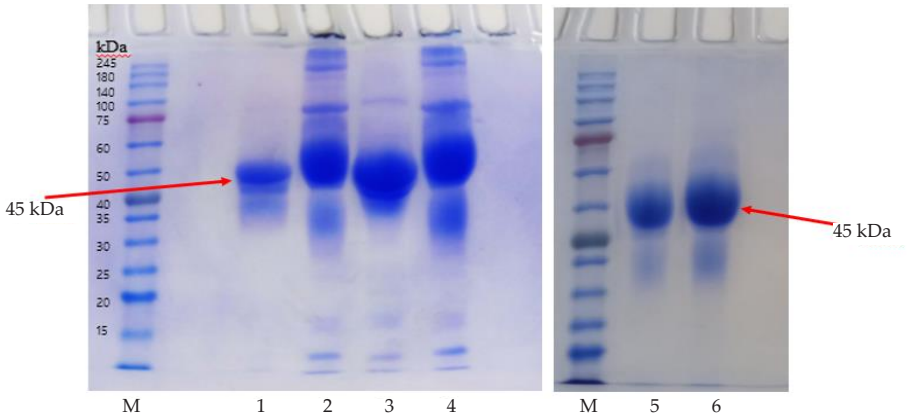

Figure 1. Ovalbumin (OVA) and fermented OVA profile on SDS-PAGE (M: protein marker, 1: chicken egg OVA. 2: duck egg OVA, 3: fermented chicken egg OVA, 4: fermented duck egg OVA using 21% PEG, 5 and 6: duck OVA using 25% PEG).

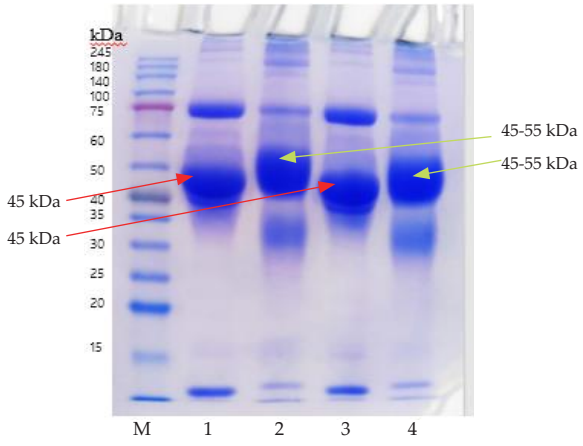

Figure 2. Egg white protein and fermented egg white protein profile on SDS-PAGE (M: protein marker, 1: chicken egg white protein, 2: duck egg white protein, 3: fermented chicken egg white protein, 4: fermented duck egg white protein).

Table 2. Total lactic acid bacteria and yeast of ovalbumin before and after fermentation

	Ovalbumin	Fermentation	
		Before	After
Lactic acid bacteria (Log CFU/mL)	Chicken	7.79±0.26	7.90±0.05
	Duck	7.81±0.41	7.63±0.15
Yeast (Log CFU/mL)	Chicken	6.81±0.18[a]	6.24±0.15[b]
	Duck	7.24±0.21[a]	5.99±0.11[b]

Note: [a,b] Means in the same row with different superscripts differ significantly ($p<0.05$).

Table 3. The average of total lactic acid bacteria and yeast of egg white before and after fermentation

	Egg white	Fermentation	
		Before	After
Lactic acid bacteria (Log CFU/mL)	Chicken	8.34±0.10	8.07±0.15
	Duck	8.95±0.50	8.10±0.76
Yeast (Log CFU/mL)	Chicken	7.11±0.06	7.17±0.13
	Duck	7.50±0.28	7.17±0 .07

Table 4. The average physicochemical properties of fermented chicken and duck ovalbumin

Ovalbumin	Physicochemical properties				
	Viscosity (mPa.s)	Moisture (%)	Soluble protein (%)	Total free amino acids (µg/mL)	Alcohol (%)
Chicken	40.66±4.59	86.67±0.13	15.59±0.38[a]	202.33±13.01[a]	0.21±00[a]
Duck	26.70±7.62	86.81±1.06	14.40±0.24[b]	153.33±2.08[b]	0.14±01[b]

Note: [a,b] Means in the same column with different superscripts differ significantly ($p<0.05$).

The Secondary Structure of OVA on the FTIR

The secondary structure of OVA and their fermented products are shown in Table 8. In general, the OVA of commercial chicken and duck eggs had similar characteristics in certain specific bands on FTIR spectra. These bands include 3294.42-3448.72 (Amide A), 1651 (Amide I), 1527 (Amide II), 1242 (Amide III), 964 (carbohydrate-containing band), and 617 cm^{-1} (Amide VI) as shown in Table 8 and Figures 3 and 4 (FTIR spectra for chicken and fermented OVA were not shown). There are several different bands in the FTIR spectra between OVA and fermented OVA. This study has identified that several types of fermented OVA bands are absent, particularly those that include carbohydrates (Table 8, Figures 3 and 4).

Table 5. The average pH of chicken and duck ovalbumin before and after fermentation

	Ovalbumin	Fermentation	
		Before	After
pH	Chicken	4.70±0.00[a]	4.43±050[b]
	Duck	4.70±0.00[a]	4.40±0.10[b]

Note: [a,b] Means in the same row with different superscripts differ significantly (p<0.05).

Table 6. Average physicochemical properties of chicken and duck egg white before and after fermentation

Physicochemical properties	Egg white	Fermentation	
		Before	After
pH	Chicken	7.93±0.05[a]	6.60±0.17[b]
	Duck	8.53±0.05[a]	6.63±0.25[b]
Viscosity (mPa.s)	Chicken	66.86±1.85	69.93±12.62
	Duck	43.83±4.31	103.56±66.75
Moisture (%)	Chicken	87.93±0.85	88.23±0.16
	Duck	87.15±0.58	87.38±0.30
Soluble protein (%)	Chicken	11.73±0.78	10.29±0.92
(Lowry method)	Duck	12.99±0.87	10.10±0.92

Note: [a,b] Means in the same row with different superscripts differ significantly (p<0.05).

Table 7. Total free amino acids and alcohol of fermented chicken and duck egg white

Albumen	Total free amino acids (μg/mL)	Alcohol
Chicken	316.66±58.32[a]	0.31±0.02
Duck	211.33±23.71[b]	0.30±0.08

Note: [a,b] Means in the same column with different superscripts differ significantly (p<0.05).

Amino Acid Profiles of Chicken and Duck OVA

A significant difference (p<0.000 – 0.018) in the amino acid compositions of chicken OVA and duck OVA is detected, and the data are presented in Table 9. After the OVA was fermented, there was also a difference in the amino acid profile between duck and chicken OVA. The amino acids found in the chicken OVA increased significantly after fermentation, but not for the duck OVA.

Table 10 presents the chicken OVA amino acid profile before and after fermentation. It shows that there was a significant difference (p<0.006- 0.038) in the chicken OVA amino acid before and after fermentation.

Table 11 shows the duck OVA amino acid profile before and after fermentation. This indicates that there

Table 8. Chicken and duck egg ovalbumin and fermented chicken and duck egg ovalbumin bands (cm^{-1}) on the fourier transform infrared

No	Bands	Chicken ovalbumin	Fermented chicken ovalbumin	Duck ovalbumin	Fermented duck ovalbumin
1	Amide A	3410.15	-	-	3448.72
	Amide A		3302.13	3294.42	3309.85
2	Amide I	1651.07	1651.07	1635.64-	1635.64
	Amide I	-	1627.62	-	-
3	Amide II	1527.62	-	1527.62	1543.05
	Amide II	1465.9	1450.47	1450.47	1450.47
	Amide II	-	-	-	1404.18
4	Amide III	1280.73	-	-	
	Amide III	1242.16*	1242.16*	1242.16*	1249.87*
5	Carbohydrate	1149..57*	-	-	-
	Carbohydrate	1111.00*	1103.28*	-	-
	Carbohydrate	1064.71*	-	1080.14*	1080.14*
	Carbohydrate	964.41*	964.41*	956.69*	-
	Carbohydrate	840.96*	-	-	-
6	Amide VI	586.36	617.22	617.22	-
	Amide VI	532.36	-	570.93	-

* The presence of carbohydrate bands (840-1242 cm^{-1})

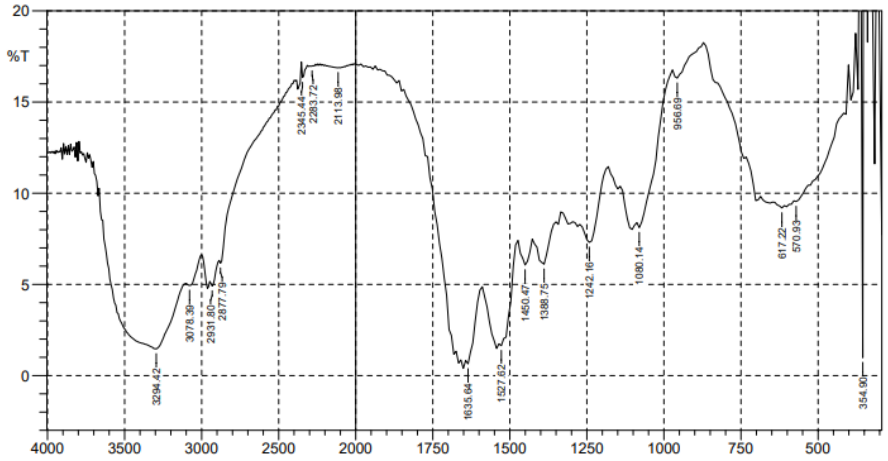

Figure 3. The fourier transform infrared (FTIR) spectra of duck ovalbumin

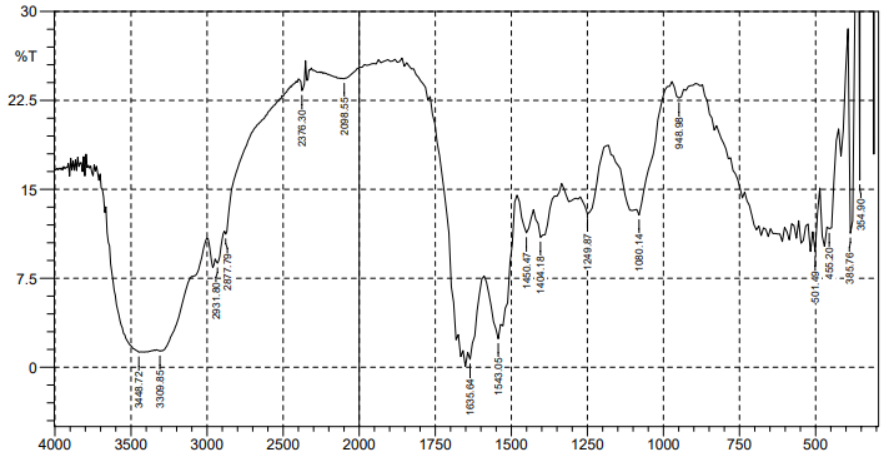

Figure 4. The fourier transform infrared (FTIR) spectra of fermented duck ovalbumin

Table 9. The chicken and duck ovalbumin amino acid profile

No	Amino acid	Amino acid concentration of ovalbumin (ppm)	
		Chicken egg	Duck egg
1	Aspartic acid	170.97±44.00[a]	409.76±73.08[b]
2	Glutamic acid	232.85±69.76[a]	638.37±62.36[b]
3	Asparagine	-	-
4	Serin	125.24±41.43[a]	397.90±17.57[b]
5	Glutamine	-	-
6	Histidine	49.60±15.70[a]	91.26±5.50[b]
7	Glycine	60.27±19.97[a]	159.93±6.44[b]
8	Threonine	73.02±19.74[a]	258.16±34.00[b]
9	Arginine	105.91±35.33[a]	248.60±11.48[b]
10	Alanine	97.50±32.40[a]	201.50±13.50[b]
11	Tyrosine	175.58±50.73[a]	529.81±36.71[b]
12	Methionine	55.58±18.02[a]	213.19±16.82[b]
13	Valin	103.27±26.94[a]	220.71±35.83[b]
14	Phenylalanine	105.13±33.47[a]	325.94±20.73[b]
15	Isoleucine	77.72±23.20[a]	143.91±18.50[b]
16	Leucine	145.55±45.05[a]	361.74±27.49[b]
17	Lysine	197.67±75.46[a]	500.17±64.91[b]

Note: [a,b] Means in the same row with different superscripts differ significantly ($p<0.05$).

Table 10. The chicken ovalbumin amino acid profiles before and after fermentation

No	Amino acid	Chicken ovalbumin amino acid (ppm)	
		Before fermentation	After fermentation
1	Aspartic acid	170.97±44.00[a]	399.07±41.22[b]
2	Glutamic acid	232.85±69.76[a]	585.83±28.84[b]
3	Asparagine	-	-
4	Serin	125.24±41.43[a]	310.98±4.69[b]
5	Glutamine	-	-
6	Histidine	49.60±15.70[a]	118.36±0.75[b]
7	Glycine	60.27±19.97[a]	139.62±2.59[b]
8	Threonine	73.02±19.74[a]	174.02±11.22[b]
9	Arginine	105.91±35.33[a]	266.11±4.75[b]
10	Alanine	97.50±32.40[a]	238.86±7.88[b]
11	Tyrosine	175.58±50.73[a]	437.08±11.26[b]
12	Methionine	55.58±18.02[a]	154.83±9.75[b]
13	Valin	103.27±26.94[a]	261.34±28.87[b]
14	Phenylalanine	105.13±33.47[a]	258.91±6.06[b]
15	Isoleucine	77.72±23.20[a]	199.19±15.08[b]
16	Leucine	145.55±45.05[a]	360.19±12.93[b]
17	Lysine	197.67±75.46[a]	450.16±37.03[b]

Note: [a,b] Means in the same row with different superscripts differ significantly ($p<0.05$).

was no significant difference in the amino acid content of the duck OVA before or after fermentation.

DISCUSSION

The difference in the percentage of PEG required in purifying chicken and duck OVA is possible due to the protein contents in the chicken and duck egg white when with different quantities. Since duck egg white has more protein content, a more significant percentage of PEG is needed to precipitate the non-OVA protein in the first stage and the OVA protein in the second stage of the purification process. In the context of this current research, Koga *et al*. (1969) have reported that the amount of ovomucoid protein, OVA, and globulin in duck egg whites is more than that found in chicken egg whites. Moreover, utilizing PEG precipitation at an appropriate concentration range enables the separation of OVA. In addition, a specific protein can be found in egg whites among other proteins in the solution. Most OVA may be dissolved in a solution containing 12% to 15% PEG-8000. However, ovomucin, another protein, precipitates at 5% PEG-8000, and then both ovotransferrin and lysozyme precipitates at concentrations from 3% to 12% PEG-8000 (Geng *et al*., 2019). According to Chen *et al*. (2021), polyethylene glycol (PEG) is a versatile and water-loving polymer that is commonly used to enhance the effectiveness of therapeutic molecules. It is physically linked to proteins, nanoparticles, peptides, liposomes, and nucleic acids to decrease their removal by the kidneys, prevent binding to antibodies and proteins, and improve their longevity and efficacy.

This study revealed that the OVA found in chicken eggs has MW of approximately 45 kDa. This finding confirms an earlier research report on the isolation of OVA from chicken eggs of an MW of 45 kDa (Geng *et al*., 2019). The chicken egg OVA found by Quan & Benjakul (2019) showed an MW of 44.50 kDa. Moreover, the

Table 11. The amino acid profiles of duck ovalbumin before and after fermentation

No	Amino acid	Duck ovalbumin amino acid (ppm)	
		Before fermentation	After fermentation
1	Aspartic acid	409.76±73.08	399.95±45.50
2	Glutamic acid	638.37±62.36	639.41±67.47
3	Asparagine	-	-
4	Serin	397.90±17.57	386.26±42.77
5	Glutamine	-	-
6	Histidine	91.26±5.50	88.32±8.81
7	Glycine	159.93±6.44	162.15±18.43
8	Threonine	258.16±34.00	252.63±26.18
9	Arginine	248.60±11.48	240.54±26.84
10	Alanine	201.50±13.50	195.49±21.31
11	Tyrosine	529.81±36.71	528.06±60.88
12	Methionine	213.19±16.82	210.61±21.31
13	Valin	220.71±35.83	220.15±19.57
14	Phenylalanine	325.94±20.73	320.61±37.41
15	Isoleucine	143.91±18.50	145.68±13.01
16	Leucine	361.74±27.49	356.31±38.04
17	Lysine	500.17±64.91	555.22±65.11

duck egg OVA in this study demonstrated an MW that ranges from 45-55 kDa. This differs from the previous study in which duck egg OVA had a MW of 43.49 kDa (Quan & Benjakul, 2019). The variation in the molecular weight range in various egg OVA can be attributed to the disparities in the carbohydrate/sugar composition. The sugar content (mannose and galactose) linked to duck OVA is greater than that of chicken OVA (Koga *et al.*, 1969). Furthermore, previous research by Yang *et al.* (2013) investigating glycan, specifically examining its composition and molecular weight attached to OVA, found that the molecular weight of OVA varied between 43.84 and 46.24 kDa.

It demonstrates that LAB and yeast can grow and survive on egg whites without adding external components because the egg whites contain protein as a source of N and sugar as a source of C for microbial growth. The presence of antimicrobial properties in egg whites is expected to enhance the LAB growth during fermentation, as well as the limited carbon supply in egg whites. An earlier investigation has reported a similar finding that the growth of *L. bulgaricus* increased at 6 hours of fermentation. After 9 hours of fermentation, there was a decrease in growth and a decrease in sugar levels during the egg white fermentation (Jiang *et al.*, 2020). Additionally, the egg white physicochemical features, such as its low nutrients, high viscosity, and alkaline pH, create a disadvantage for bacterial growth. In addition, the egg white also contains many antibacterial components. Egg white contains lysozyme and ovotransferrin, which are crucial components in the immune response to pathogens in eggs. Recently, it has been found that several small proteins and peptides have indicated potential functions to protect the embryo in an egg from bacterial invasion (Baron *et al.*, 2016).

The LAB (*L. paracasei* M105) could grow in an OVA medium without additional substances. This phenomenon is understandable since OVA serves as a nitrogen source (N) and contains sugar, which can be utilized for microbial growth. Due to the limited availability of sugar as a carbon source, sugar fails to adequately support microbial growth during fermentation, especially for yeast, which relies on sugar to produce alcohol. Ultimately, after the fermentation process, the number of yeasts experiences a substantial decline due to the reduction in the sugar substrate. Yeast primarily converts both fructose and glucose in grape juice into ethanol, carbon dioxide, and heat during alcoholic fermentation. In addition, many kinds of additional components are also produced. Yeast is essential in the process of brewing fermentation since it transforms sugars into alcohol, carbon dioxide, and various other compounds that impact the taste and scent of beer (Sun *et al.,* 2022). The findings of this current investigation are similar to a prior study in the population of *L. bulgaricus,* which exhibited a progressive increase over 6 hours during fermentation in an egg white solution. The percentage of *L. bulgaricus* started declining after being fermented for 9 hours. This can be attributed to the relatively low carbon concentration of the egg white. The glucose level gradually decreased with the duration of progressive fermentation (Jiang *et al.*, 2020).

The decrease in pH during egg white fermentation indicates the growth of microbes that convert the sugar in egg whites into acid, resulting in a decrease in pH (Table 4). In a previous study, it was reported that *L. bulgaricus* was able to use the glucose in egg whites (Jiang *et al.*, 2020), and *K. marxianus* KFA 9 was able to ferment glucose, lactose, fructose, and sucrose (Kurniawati *et al.*, 2022). Furthermore, the pH value of freshly obtained egg whites typically ranges from 7.6 to 9.7, with variations influenced by such factors as storage conditions and duration. A study was carried out to analyze the pH fluctuations during fermentation with the objective of assessing the influence of electrostatic screening effects. The pH at the start of fermentation was 9.27 and exhibited a linear drop to 8.94 after six hours. Lactobacillus experienced rapid growth during fermentation, transforming glucose into organic acids, primarily lactic acid, and causing a decrease in pH (Jiang *et al.*, 2020).

Table 5 of this research shows the absence of notable disparity in the alcohol levels between chicken and duck-fermented egg whites. *K. marxianus KFA 9,* when used in the form of a single culture, can ferment lactose and then convert it into ethanol (Kurniawati *et al.*, 2022). A recent study conducted by Arora *et al*. (2015) showed that two thermotolerant isolates, *K. marxianus* NIRE-K1 and *K. marxianus* NIRE-K3, could convert both glucose and xylose into ethanol through fermentation.

No substantial differences were detected within the viscosity, moisture content, and soluble protein concentrations. The protein degradation within egg white occurs during fermentation as caused by *L. paracasei,* which has been verified to possess a proteinase activity in its cell walls. In addition, it has been reported that *K. marxianus* can metabolize protein (Naes *et al.*, 1992; Zhang *et al.*, 2017). The proteinase activity appears to be inadequate because of the

existence of antimicrobial protein in the egg whites. Therefore, the deterioration of egg white protein did not substantially alter its physical and chemical properties. Nevertheless, the level of proteolytic activity observed in the fermentation of the chicken egg white exceeded that of duck egg white fermentation. Table 5 shows that the fermentation process of chicken egg white produces a higher amount of free amino acids compared to duck egg white, and this supports this study. It implies that chicken egg white is more easily degraded by microorganisms than duck egg white. Duck albumen is more difficult to degrade due to its higher concentration of conjugated sugars and larger amounts of ovalbumin and ovomucoid glycoproteins compared to chicken albumen (Koga *et al.,* 1969). The lower hydrolysis rate of duck egg whites during fermentation may be attributed to the higher protease inhibitor activity found in duck egg whites when compared to chicken egg whites. Previous research has found that the ovomucoid protease inhibitor activity in duck egg whites seems stronger than that of the ovomucoid in chicken and Muscovy duck egg whites (Nurliyani *et al*., 2023).

Table 4 indicates that the duck OVA is more difficult to degrade by LAB and yeast because the quantity of duck OVA is greater, and the OVA structure is more complex than that of chicken OVA. A previous study reported that duck OVA has a higher concentration of glycans (Koga *et al.,* 1969) and more disulfide bonds compared to other sources, such as chicken OVA (Sun *et al.,* 2002). Consequently, these glycans and disulfide bonds make it more difficult for protease enzymes to access OVA polypeptides. In addition, the variations of amino acid sequences and egg protein glycosylation show an impact on the characteristics of the protein, its ability to be digested, and its unique biological function (Yao *et al.,* 2022). Consequently, the amounts of soluble protein and free amino acids were lower in the fermented duck OVA than in the fermented chicken OVA. The ethanol concentration in the fermented duck OVA was similarly lower than that of the fermented chicken OVA, suggesting that the microbial proliferation in duck OVA was less pronounced than in chicken OVA. The current investigation confirmed this finding. The fermentation of duck OVA produced a smaller quantity of yeast and alcohol compared to chicken OVA.

The pH value of OVA before fermentation is low due to the utilization of citric acid during the purification process, which causes the precipitation of ovalbumin to its isoelectric point of 4.5. Chicken OVA and duck OVA show a sharp decrease in pH value during fermentation (Table 5). A previous study by Doblado *et al*. (2003) found that lactic acid–producing bacteria can convert hexoses (in oligosaccharides and complex sugars) into lactic acid during fermentation.

This study fairly matches the study by Veskovic *et al*. (2022), which analyzed the FTIR spectrum of OVA in solution, hydrogel, and solid forms. The results of the latter indicated the presence of particular group bands, including amides I (1634 cm^{-1}), II (1533 cm^{-1}), and III (1400-1200 cm^{-1}). The loss of some carbohydrate bands is possible because the bacteria used in the fermentation process caused the degradation of the carbohydrate group. The carbohydrate content of OVA acts as the source of carbon for the growth of microbes, resulting in lactic acid production and a decrease in pH (Table 5). Fermentation causes changes in the pH-modified protein structure or the breakdown of molecular structures of egg white protein, leading to modifications in the secondary and tertiary structures (Jiang *et al.,* 2020). A previous study also revealed that an analysis of whole egg white protein hydrolysate (WEWPH) by FTIR spectroscopy showed the digestion of egg white and the integrity of WEWPH in terms of the secondary structure (Johny *et al.,* 2022). Thus, it can be concluded that there are discernible disparities in the protein profiles observed in the FTIR spectra of OVA and fermented OVA, as indicated in Table 8.

Various approximations for the amide A band have been presented by Jiang *et al*. (2020), particularly within the range of 3300–3500 cm^{-1}. The OVA band observed in the present study falls within the range of 3294 to 3448 cm^{-1}, suggesting its amide A classification. The N-H stretching vibration is responsible for almost 95% of the observed signal, which is primarily associated with Amide A.

The existence of helical structures is indicated by the spectrum at 1651 cm^{-1}, as observed by Zhang *et al*. (2017). The OVA band Amide I observed in this work was found to range from 1627-1651 cm^{-1}, and this is in line with the previous study by Abd-Elaziz *et al*. (2018) that amide I band falls within the range of 100-1700 cm-1. In addition, the amide I band is associated with the stretching vibrations of the C=O bond in the amide molecule. The main factor influencing the amide I band of the protein is the stretching vibration of the C=O bond. This vibration provides information about the protein's secondary structure, which includes α-helix, β-sheet, β-turn, and random coil conformations. Previous research has reported that the distinct absorption peaks within the range of 1610–1640 cm^{-1} and correspond to β-sheet structures. Meanwhile, those falling within the range of 1640-1650 cm^{-1} correspond to β-turn structures. Similarly, the absorption peaks within the range of 1650-1662 cm^{-1} indicate α-helix structures, and those falling within the range of 1662-1695 cm^{-1} are associated with random coil structures. A previous study by Luo *et al*. (2022) reported that within this group, the α-helix and β-sheet are classified as highly organized structures, the β-turn as partially organized structures with less rigidity, and the random coil as disordered structures with low organization. Ovalbumin's secondary structure displays several motifs, including α-helix (41%), β-sheet (34%), β-turns (12%), and random coils (13%), among others. According to Kanaka *et al*. (2018), the ovalbumin protein exhibits a well-defined three-dimensional structure characterized by a prominent α-helical reactive loop extending from the protein's main body, connected to two peptide chains, and a primary β-sheet A. In their study, Veskovic *et al*. (2022) found out that the OVA native form consists of a combination of α-helix and β-sheet structures, along with an exposed helicoidal loop.

In this study, the chicken and duck OVA were observed to have a band between 1404-1543 cm^{-1}, as

mentioned in Joshi *et al*. (2012). This band falls within the range of 1400 to 1600 cm^{-1}, and this is characteristic of the amide II region. Amide II is the simultaneous presence of N-H stretching and bending vibrations, specifically referring to the N-H bond bending vibrations (Tarhan *et al*., 2020; Abd-Elaziz *et al*., 2018).

A band between 1242 and 1280 cm^{-1} is observed in the FTIR spectra, encompassing the amide III band. A study by Tarhan *et al*. (2020) attributed the presence of the amide III band in the range of 1220-1320 cm^{-1} to the combined vibrations of N-H bending and C-N stretching.

The spectral area between 840 and 1242 cm^{-1} was used to observe the carbohydrate band of OVA in the study. The strong intensity observed in the spectrum range falls between 1200-900 cm^{-1}, which matches to the carbohydrate and is similar to that of N-acetylglucosamine (GlcNAc) (Giosafto *et al*., 2016). A prior investigation discovered that the neutral sugars in chicken and duck OVA were mannose and galactose, and glucosamine was identified as the hexosamine (Koga *et al*., 1969). Kanaka *et al*. (2018) highlight the complexity of ovalbumin and note the variations in its chemical structure, including the differences in glycosylation, phosphorylation, and genetic variance. Two possible glycosylation sites have been identified at the amino acid residues Asn 317-319 (Asn-X-Ser) and Asn 293-295 (Asn-X-Thr). The diverse molecule mixture in the carbohydrate peptide chains has an identical base structure, including mannose β (1-4) glcNAc β (1-4) glcNAc.

Because OVA fermentation in this study has been observed to cause the carbohydrate-containing bands to disappear, it is possible that fermented OVA causes fewer sensitization reactions, thereby decreasing its allergenicity. The immunomodulatory activity of peptides might be associated with the amino acid composition, length, hydrophobicity, and charge, among which short hydrophobic peptides (2–10 residues) with more positive charge have better immune activity. Specific glycan in glycoproteins may serve as the targeted information carriers to stimulate immune cells and increase cytokine production. The chemical structure of glycans and their link to the peptide backbone have important impacts on the immunological function (He *et al*., 2024). It was also reported that removing glycans in wheat protein can reduce its immunogenicity and consequently, it can be developed into a hypoallergenic wheat product (Fu *et al*., 2023).

The hydrolysis of OVA during fermentation may change the protein conformation, which will affect its functional properties. Removing the OVA protein epitope due to the fermentation process can reduce its allergenicity. Yanga *et al.* (2017) reported that alterations in the conformational structure of a protein may result in changes in the IgG and IgE binding capabilities, which depend on the integrity of the IgG and IgE epitopes. According to Liu *et al*. (2018), enzymatic hydrolysis is an effective method to improve the functional characteristics of protein because of its high specificity, controllability, and minimal formation of by-products. Limited enzymatic hydrolysis could reduce molecular weight but expose hydrophobic groups previously buried in the protein, which might enhance the protein foaming capacity and emulsifying characteristics. In addition, protein hydrolysates with low DH (<10%) can be used as food texture enhancers and extensive protein hydrolysates are used as protein supplements or in such special medical diets as hypoallergenic foods. In ovalbumin, the removal of the 22 N-terminal residues (1–22) decreased its interfacial absorptivity (led to the destabilization of emulsions), whereas the removal of C-terminal residues (346–385) increased its interfacial absorptivity (Pokora *et al*., 2013).

The OVA band of both chicken and duck eggs showed a band ranging from 532 to 617 cm^{-1}, except for the fermented duck OVA. The spectral region spans from 537-606 cm^{-1} as the amide VI band, which is distinguished by an out-of-plane C=O bending structure (Kong *et al*., 2007).

Observably, the amino acid concentrations in duck OVA surpass those found in chicken OVA. This is likely because the high protein content in duck feed results in the higher levels of the OVA amino acid. Indonesian ducks typically graze in the rice fields for various food sources such as Periwinkle (*Tympanotomus fuscatus*), snails, crabs, grasses, grains, and other species. Attia *et al*. (2020) observed that factors such as the breed and species of poultry, the method of preparation, and the different components of the egg (whole, egg white, and egg yolk) have an impact on the egg amino acid compositions. According to Tumanggor *et al*. (2017), ducks consume various items while grazing, including seeds, grass, small crabs (*Cardiosoma armatum*), snails, small stones, sand, and unidentified substances. The Laguna crab, often referred to as the tiny crab, possesses a crude protein composition that varies from 33.2% to 39.8%. In contrast, the Periwinkle exhibits a greater crude protein, varying from 70.2% to 75.7% (Ogundiran *et al*., 2015).

The concentration of chicken OVA amino acids shows different levels before and after fermentation, as indicated in Tables 10 and 11. After fermentation, the chicken OVA demonstrates a significant increase in the levels of all amino acids. In contrast, the duck OVA exhibits an insignificant difference in the amino acid concentration before and after fermentation. The lactic acid bacteria and yeast used in the fermentation may have more easily-degraded chicken OVA compared to duck OVA, resulting in higher production of amino acids during the process of fermentation. Using the ninhydrin method, the analysis also indicates that the fermented chicken OVA amino acid levels exceed those of fermented duck OVA (Table 4). In addition, the amino acid levels in the fermented chicken egg white are also higher than those in the fermented duck egg white (Table 7). Similarly, the level of soluble protein in chicken OVA was observed to be greater than that in the fermented duck OVA, as shown in Table 6.

CONCLUSION

Even though the total number of lactic acid bacteria does not change, there is a decrease in the number of yeasts after the fermentation of OVA using the local

starter *Lactobacillus paracasei* M104 and *Kluyveromyces marxianus* KFA9. Furthermore, there are changes in the secondary structure of proteins and the removal of the carbohydrate-containing bands on the FTIR spectra in the fermented OVA. The changes in the protein structure may affect its functional characteristics, which demands further studies. It is expected that fermented OVA can be used as an ingredient in functional foods. The amount of all types of amino acids in the unfermented duck egg OVA is higher than that of the unfermented chicken OVA. However, the fermented chickens OVA showed an increase in all types of amino acids, total free amino acids, and soluble proteins.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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