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Characterization of collagen from Java barb (*Barbonymus gonionotus*) skin and its effect on selected properties of yogurt

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Abstract

Wastes of *Barbonymus gonionotus* (Java barb) skin can be processed as collagen as a food additive. Yogurt is a functional drink that has gained attention. This study aimed to characterize yogurt added with collagen from Java barb skin. The solutions used to extract the collagen were (A) 1:10 (w/v) for 24 h, (B) 1:20 (w/v) for 24 h, (C) 1:10 (w/v) for 36 h, and (D) 1:20 (w/v) for 36 h. The obtained collagen was added to yogurt at concentrations of 1%, 2%, and 3%. The yogurt was then examined for its protein content, pH, and titratable acidity. The highest yield of collagen was obtained from treatment D, with a value of 52.54% ± 0.94%. All the extracted collagens had triple-helix structures. The presence of α1 and α2 was also confirmed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis, and the amino acid patterns were found to be either Gly-Pro-Ala or Gly-Pro-Arg. The addition of collagen to yogurt at a concentration of 3% produced a significant increase in protein content (3.82%). In comparison, pH and titratable acidity did not show any significant changes. However, the titratable acidity at a collagen concentration of 3% showed a small increase of 1.14% ± 0.71% at the end of fermentation. In conclusion, collagen was successfully extracted with relatively high yields, and collagen-added yogurt met industry standards. The addition of collagen at a concentration of 3% increased the protein content of yogurt and only had a slight effect on its selected characteristics.

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Keywords
Collagen, Amino Acid, Java Barb, Yoghurt.

1. Introduction

Collagen is widely used in foods, cosmetics, film production, biomaterials, and pharmaceuticals. It is generally isolated from the skin tissues of terrestrial animals such as cattle and pigs. However, collagen from cows and pigs has concerns regarding the dangers of bovine spongiform encephalopathy disease and religious issues. For example, Muslims do not approve of pig-derived collagen because it is prohibited by Islamic Law (1-3). Therefore, alternative sources of collagen that can overcome these issues are needed. Fish skin can be used as an alternative collagen source. Java barbs (*B. gonionotus*) are abundant freshwater

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fish that live in tropical areas (4). The meat of Java barbs is an essential part, and other parts such as the head, bones, viscera, skin, and scales are discarded. These parts are potential sources of collagen because skin wastes from Java barbs have a collagen yield of 10%–15% of the total weight.

The two types of collagen extraction methods are acid-soluble collagen (*ASC*) and pepsin-soluble collagen (*PSC*) extraction. Collagen is generally soluble in acidic solvents. However, at a very low pH, its solubility slightly decreased (5). Therefore, collagen extraction is performed using low concentrations of weak acids such as acetic acid. Furthermore, the efficiency of ASC extraction was affected by the solution concentration ratio, presence of non-collagenous protein, material type, temperature, and soaking time (6). Therefore, collagen yields can vary because of acetic acid concentrations, extraction time, and temperature (7).

Recently, food consumers demand healthier food ingredients, especially concerning dietary requirements and improving quality of life (8). Therefore, the demand for more functional food and drink is growing. Yogurt is produced by milk fermentation using lactic acid bacteria. Yogurts have many benefits, for example, they help in digestion, prevent diarrhea, reduce cholesterol levels, and fight cancer (9-10). Several researchers made improvements in the functional effects of yogurt. Amaranth seeds, oat seeds, and whey were already added in yogurt (11,12). However, its quality can still be improved. The addition of collagen to yogurt is expected to increase the functional value of the product without affecting its physicochemical characteristics.

This study aimed to assess collagen extracted from Java barb (*B. gonionotus*) skin using different ratios of acetic acid concentration and extraction times and evaluate the effect of collagen addition on the physicochemical properties of yogurt.

2. Materials and Methods

2.1. Materials

Java barbs (*B. gonionotus*) were obtained from local farmers in Gresik Regency, East Java, Indonesia, and the average length of fish was 16 cm. Yogurt starters were *Streptococcus thermophillus*, *Lactobacillus bulgaricus,* and *Bifidobacterium lactis,* from a laboratory animal product technology collection, at the Faculty of Animal Husbandry, Brawijaya University. Full cream milk with 9% fat, 10% protein, and 3% carbohydrate was purchased from Diamond Cold Storage Co., Indonesia. All chemical reagents used in the study were reagent grade with a purity of >95%.

2.2. Collagen Extraction

Fish were skinned and washed two times in tap water. First, the skin was cut into 1×1 cm^2 sizes. The collagen extraction process was based on the method described by Kittiphattanabawon et al. (5), with slight modifications. To remove the non-collagen protein, the skin sample was soaked in a 0.1 M NaOH solution at a ratio of 1:10 (w/v) for 6 h. The solution was replaced every 3 h, and the sample was filtered and rinsed with running water until the pH reached 7. To remove any fat, the skin sample was soaked again, with an n-butanol ratio of 1:10 (w/v) for 18 h. The solution was replaced every 6 h, and collagen extraction was then carried out with two different ratios using 0.5 M acetic acid. The solutions used were (A) 1:10 (w/v) for 24 h, (B) 1:20 (w/v) for 24 h, (C) 1:10 (w/v) for 36 h, and (D) 1:20 (w/v) for 36 h. All these steps were performed at 4°C. The solutions were then filtered using

a 200-µm membrane and precipitated using 0.9 M NaCl. Finally, the filtrate was centrifuged at 5000 rpm for 30 min. The precipitate was then collected as collagen.

2.3. Collagen Characterization

2.3.1. Yield

Collagen yield was calculated by comparing the weight of collagen obtained with the weight of the skin samples used. The yield formula was calculated as follows:

$$yield(\%) = \frac{W1}{W0} x100 \quad (1)$$

where W0 is the initial weight of fish skin, and W1 is the final weight of collagen.

2.3.2. Proximate Analysis

A proximate analysis of the skin was performed in triplicate to assess the mass of crude protein, ash, moisture, and fat. Total nitrogen was determined using the Kjeldahl method, and ash content was determined using gravimetric analysis after heating the sample at 550°C for 24 h. Moisture was also analyzed using gravimetric analysis in an oven with a temperature of 105°C for 24–36 h. Finally, fat content was determined using the method described by Bligh and Dyer (13).

2.3.3. Fourier Transform Infrared (FTIR) Spectroscopy

Liquid collagen samples were vaporized at a temperature of 80°C for approximately 6 h, and FTIR analysis was performed using an FTIR spectrophotometer (8400S/Shimadzu). Collagen samples were analyzed by FTIR spectroscopy. Lyophilized (3 mg) collagen samples were mixed with 100 mg of dry KBr, pounded with a mortar and pestle, and then kept under a pressure of approximately 5×10^6 Pa. The samples were then placed in a mold to produce 13 × 1 mm clear transparent disks. The intensity of peak absorption was calculated by the baseline method, and the resulting spectra were analyzed using the ORIGIN 8.0 software (Thermo Nicolet, USA) (14).

2.3.4. Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed using the method of Laemmli et al. (15). The 50-µg sample was mixed with 100 µL of the sample buffer (Tris HCl 60 mM at pH 6.8, 2% SDS, and 25% glycerol) and 10% β mercaptoethanol. Samples were then heated at 100°C for 5 min, and a 15-µL aliquot was injected into each well. The separating and stacking gels were 7.5% and 4%, respectively. Electrophoresis was then performed at 15 mA using an AE-6540B (ATTO Co, Tokyo, Japan). Staining was done using Coomassie blue (0.04% Coomassie Blue R-250 in 25% v/v ethanol and 8% v/v acetic acid), and the excess stain was removed with a destaining solvent (25% v/v ethanol and 8% v/v acetic acid). Finally, quantitative analysis of protein band intensity was performed using Model GS-700 Imaging densitometer, with Molecular Analyst Version 1.4.

2.3.5. Amino Acid Analysis

Amino acids were analyzed after acid hydrolysis using 6N HCl, containing 1% w/v phenol. This was done under an inert atmosphere at a temperature of 110°C for 24 h. After hydrolysis, HCl was removed with a vacuum (16), and ultra performance liquid chromatography (UPLC) was employed for amino acid analysis. Collagen hydrolysis was

performed by adding 5–10 mL of 6N HCl to a sample of 0.1 g at 110°C for 22 h. The samples were then cooled at room temperature and transferred to a 500-mL measuring flask. Then, double-distilled H_2O was added to the boundary markers, along with a filter of 0.45 µm, and 10 µL of a sample was added with 70 µL of AccQ Fluor Borate and 20 µL of fluorine reagent. The aliquot was allowed to stand for 1 min, and 5 µL of aliquot was incubated at 55°C for 10 min and injected at UPLC connected with AccQ Tag ultra C18 column with a temperature of 490R. This is a mobile phase of the PDA detector gradient composition system, with a flow rate of 0.7 µL/mL and a wavelength of 260 nm.

2.3.6. Yogurt Starter

The mother starter was inoculated on skim milk broth media and incubated at 37°C overnight. A 1% isolate mixture was cultured in 50 mL of pasteurized skim milk. Additionally, the bulk starter was cultured at 37°C until the pH reached 4.6.

2.3.7. Yogurt Maker

Collagen was added to yogurt to reach 0%, 1%, and 3% concentrations. The milk was pasteurized at 85°C for 20 min, and before the inoculation, the temperature was lowered to 40°C. The milk was inoculated with 3% (v/v) with a bulk starter containing *Streptococcus thermophillus*, *Lactobacillus bulgaricus,* and *Bifidobacterium lactis*. The inoculated milk was incubated at 40°C until it reached a pH of 4.5–4.7 and then stored at 4°C.

2.3.8. pH

pH was measured using Horiba Laqua PH 1100-S pH meter (Horiba Ltd, Northampton, UK). Before measurement, the pH meter was calibrated first with a buffer solution of pH 7 and pH 10. The electrode tip of the pH meter was dipped in the yogurt sample, and the pH value was indicated on the meter screen (17).

2.3.9. Titratable Acid

In this study, 5 mL of diluted yogurt samples were mixed with 100 mL of boiling water, and the solution was titrated with 0.1 N NaOH. The titration was stopped when the sample turned pale pink. The acidity of yogurt products was expressed in percent, with values between 0.5% and 2% (18). AOAC methods No. 947.05 (17).

2.4. Statistical Analysis

Significant differences among treatments were assessed by analysis of variance, followed by a Duncan multiple comparison test at $P < 0.05$. All analysis was performed using SPSS 14.0 (SPSS Inc.; Chicago, USA), and data were expressed as mean values ± standard deviation.

3. Results and Discussion

3.1. Collagen Characterization

The characteristics of collagen determine its potential application. Primary criteria for collagen extraction include yield, functional groups, amino acid, and molecular weight (19).

3.1.1. Proximate

The analysis of the proximate composition of Java barb skin revealed that the moisture content on a dry-weight basis was 10.84%. The crude protein, crude lipid, and ash content of cod skin on a dry-weight basis were 82.06%, 2.60%, and 2.30%, respectively. The collagen from Java barb had higher protein content than that from *Catla catla* and *Cirrhinus mrigala* (20).

3.1.2. Yield

The results of the study using several treatment ratios and soaking times showed different yields (Figure 1). The average percentage of collagen yield obtained from Java barb was significantly affected by treatment ($p<0.05$). The highest yield was obtained from treatment D (52.54% ± 0.94%), and treatment A produced the lowest yield (42.12% ± 0.53%). Higher yields were obtained from more prolonged soaking.

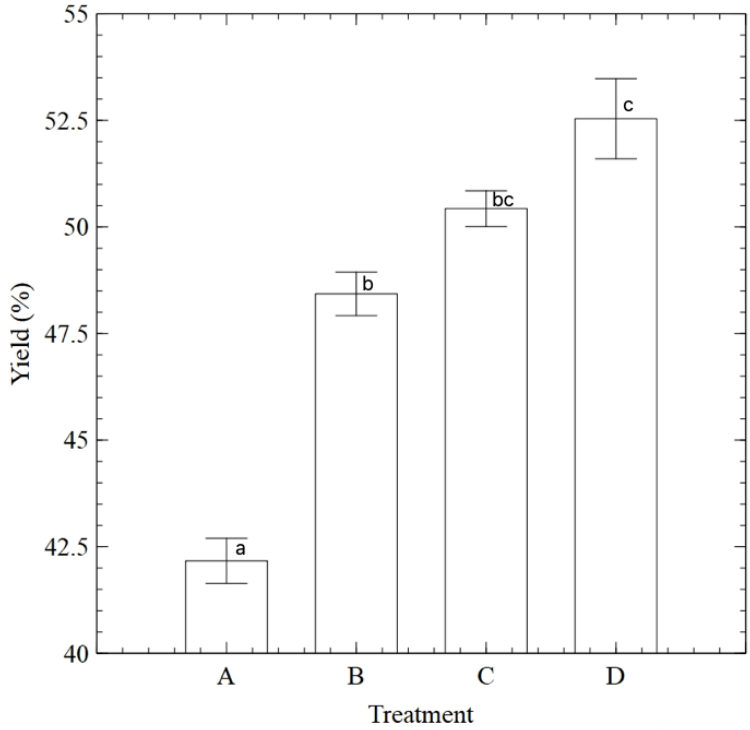

Figure 1. Yield of collagen from the skin of Java barb. (A) 1:10 (w/v) for 24 h, (B) 1:20 (w/v) for 24 h, (C) 1:10 (w/v) for 36 h, and (D) 1:20 (w/v) for 36 h.

The average yield of treatment D was higher than that reported by Noitup et al. (21), which produced collagen yields of 48.21% from Nile tilapia (*Oreochromis niloticus*). Furthermore, the lowest average yield obtained was higher than that reported by Duan et al. (7), who had collagen yields of 41.30% from carp (*Cyprinus carpio*). Collagen extraction produced different yields depending on the weight ratio of Java barb skin to different acetic acid concentrations. For example, a ratio of 1:20 (w/v) had a higher yield than a ratio of 1:10 (w/v). This was in line with the results of Sadowska et al. (22), who showed that the percentage ratio of Baltic cod (*Gadus morhua*) skin was greater at 1:20 (w/v) than at 1:10 (w/v). The yield of collagen dissolved in acetic acid increased with time, increasing after 24 h. For example, the highest collagen yield extracted from the skin of grass carp fish (*Ctenopharyngodon idella*) was 19.18 mg/g, observed after 36 h of immersion. After that, the

collagen yield decreased gradually (23). It appeared that the longer contact on the solvent extraction, the higher penetration to the cell. As a result, the yield also increased.

3.1.3. FTIR

The infrared spectra of collagen analysis showed that the peak absorption of treatment A resulted in a wavelength of 2925.81 cm^{-1}; treatment B, 2927.74 cm^{-1}; treatment C, 2925.81 cm^{-1}; and treatment D, 2927.74 cm^{-1}. These indicated the presence of typical collagen groups, namely, amide B (Figure 2 and Table 1). These results suggest that all treatments were included in the standard absorption range, meaning that amide B was detected at the wave number of each treatment. Additionally, the detection of the amide B group at wavelengths of 2915–2935 cm^{-1} indicated that amide B was formed from the asymmetric stretching of CH_2 (24,25).

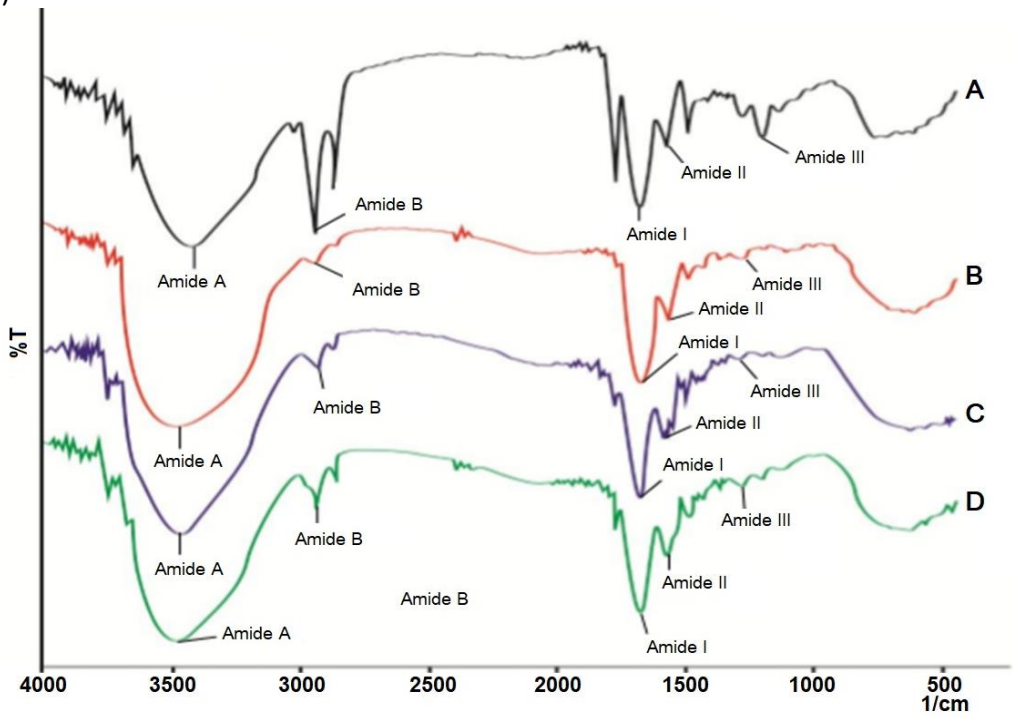

Figure 2. FTIR characters of the collagen. (A): 1:10 (w/v) for 24 h, (B): 1:20 (w/v) for 24 h, (C): 1:10 (w/v) for 36 h, and (D): 1:20 (w/v) for 36 h.

Table 1. Amide band position in the IR spectra.

Band	Amide band frequencies (cm^{-1})				Reference (cm^{-1})
	A	B	C	D	
Amide A	3427.27	3426.34	3423.41	3424.49	3400–3440[(1)]
Amide B	2925.81	2927.74	2925.81	2927.74	2915–2935[(2)]
Amide I	1650.95	1647.10	1647.1	1649.02	1600–1700[(3)]
Amide II	1544.88	1550.66	1546.8	1544.88	1480–1575[(3)]
Amide III	1240.14	1259.43	1244.00	1245.93	1229–1301[(3)]

Note: [(1)] Li et al. (26); [(2)] Coates (24); [(3)] Kong and Yu (25).

Amide was detected in all treatments at wavelengths of 1650.95 cm^{-1} for treatment A, 1647.10 cm^{-1} for treatment B, 1647.10 cm^{-1} for treatment C, and 1649.02 cm^{-1} for treatment D. Amide I is a typical functional group that makes up collagen and has four protein secondary structures, namely, α-helix, β-sheet, β-turn, and random coil. It is generally detected at

wavelength ranges of 1600–1690 cm^{-1} (19,25). This shows that the molecular compounds produced from the extraction process are collagen and have not been degraded to gelatin form. This is because the denaturation of collagen due to the heating process causes the complete transformation of the triple-helix collagen chain into a single α-helix (gelatin) chain (27).

Amide II, a typical functional group of collagens, was also detected in all treatments at wavelengths of 1544.88 cm^{-1} for treatment A, 1550.66 cm^{-1} for treatment B, 1546.80 cm^{-1} for treatment C, and 1544.88 cm^{-1} for treatment D. Amide II uptake is generally in the range of 1480–1575 cm^{-1} (25). Finally, amide III bands were detected at wavelengths of 1240.14 cm^{-1} for treatment A, 1259.43 cm^{-1} for treatment B, 1244.00 cm^{-1} for treatment C, and 1245.93 cm^{-1} for treatment D. Amide III has an absorption range of 1229–1301 cm^{-1}. The absorption regions in these results indicate the presence of amide III functional groups that show CH stretching and NH bending and are related to the triple-helix structure (19,25). All treatments resulted in collagen with amides A, B, I, II, and III.

3.1.4. SDS-PAGE

The SDS-PAGE profile of treatment A showed molecular weight values of 10.99–108.72 kDa. Treatment B obtained molecular weight values of 11.26–107.15 kDa, treatment C obtained 12.97–106.27 kDa, and treatment D obtained 10.92–107.82 kDa (Figure 3).

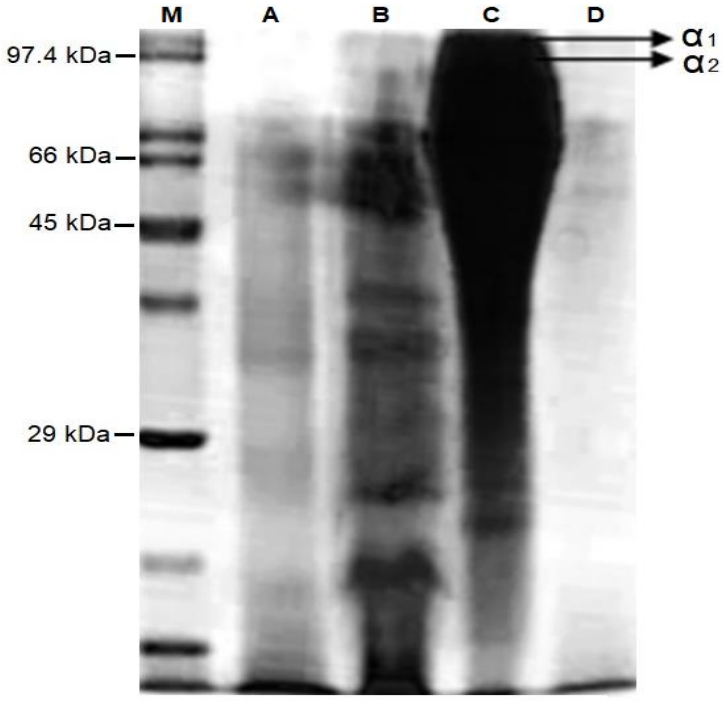

Figure 3. SDS PAGE of collagen. (A): 1:10 (w/v) for 24 h, (B): 1:20 (w/v) for 24 h, (C): 1:10 (w/v) for 36 h, and (D): 1:20 (w/v) for 36 h.

The molecular weight of Java barb collagen has α-1 and α-2 structures with values ranging from 99.45 to 108.72 kDa. These results are consistent with those of by Duan et al. (7), who stated that goldfish collagen (*Cyprinus carpio*) has α-1 and α-2 structures, with molecular weight values ranging from 97 kDa to 116 kDa. Therefore, collagen extracted from Bader fish skin is classified as type 1 collagen because two α structures are present, which are often referred to as (α-1) and (α-2) (23, 28).

3.1.5. Amino Acid

The amino acid analysis was only performed for the treatment with the highest yield. The result of the amino acid analysis and the comparison with other fish is depicted in Table 2.

Table 2. Amino acid compositions of collagen (residues/1000 residues).

Amino Acid	Java Barb[1]	Nile tilapia[2]	Bigeye Snapper[3]	Cod[4]	Leather Jacket[5]	Loach[6]	Grass Carp[7]
Ser	5.93	31	4	40.2	34.3	37	38.54
Glu	11.74	98	8.6	49.6	45.3	89	66.98
Phe	2.94	10	1.7	18.8	28.7	14	18.84
Ile	nd	11	0.6	10.2	12.8	11	12.81
Val	2.43	22	2.4	37.4	12.3	16	25.91
Ala	10.36	87	15.1	138.2	95.9	114	101.79
Arg	11.03	90	6.6	46.7	52	53	64.87
Gly	24.64	322	31.7	342.3	354.9	316	298.91
Lys	3.76	32	3.4	13.6	56	22	46.64
Asp	7.44	40	5.6	34.9	55.2	53	46.51
Leu	3.34	22	2.7	10.8	20.8	21	24.86
Tyr	nd	9	0.4		26.5	2	11.85
Pro	12.6	106	12.8	125.6	90.3	117	108.88
Thr	3.79	15	3.2	18.8	29.4	17	28.73
His	nd	10	1.1	7.3	7.5	5	5.66
Hyp	nd	86	nd	90.5	nd	95	nd

Note: [1] this study; [2] Song et al. (29); [3] Kittiphattanabawon et al. (5); [4] Shu et al. (30); [5] Muralidharan et al. (31); [6] Wang et al. (32); [7] Wu et al. (33).

The amino acid composition of the results, compared with several collagens from different fish species, is shown in Table 5. Glycine was the most abundant amino acid, with a value of 246.4 of the total 1000 residues. This was followed by proline, with 126.0. Undetected amino acids were isoleucine, tyrosine, histidine, and cysteine. Glycine is the most common amino acid in the overall composition of collagen and makes up the majority of the extraction residue, followed by proline and alanine (5, 29-33).

The most common amino acid sequence in collagen is Gly-X-X, in repeated triple-helix conformation (34). Proline is a unique amino acid in collagen because it plays a role in maintaining the structural integrity of proteins (35). The results of the amino acid analysis indicate that the likely pattern for collagen from Java barb (*B. gonionotus*) skin is Gly-Pro-Ala or Gly-Pro-Arg.

3.2. Yogurt Characterization

3.2.1. Proximate

The proximate analysis showed that adding more Java barb collagens significantly increased protein levels. However, no significant differences were found for fat, moisture, and ash levels (Table 3).

Table 3. Proximate content of yogurt.

Parameters	Treatments		
	1%	2%	3%
Protein	3.02 ± 0.73[a]	3.34 ± 0.23[ab]	3.82 ± 0.43[b]
Fat	3.48 ± 0.21[a]	3.53 ± 0.76[a]	3.48 ± 0.55[a]
Water	88.65 ± 1.02[a]	88.65 ± 1.01[a]	8.60 ± 0.93[a]
Ash	0.68 ± 0.22[a]	0.67 ± 0.17[a]	0.69 ± 0.21[a]

The addition of collagen significantly increased the protein content of yogurt, mostly because collagen is a macromolecule protein. Furthermore, high protein materials such as casein concentrate and milk protein concentrate can be used to significantly increase the protein content in yogurt, without increasing lactose levels (36, 37). The protein value was higher than that of yogurt added with amaranths and oats (12). Collagen-added yogurt is still in the range value of standard yogurt quality based on the National Indonesia Standard (SNI) (18). Moreover, according to the Codex Alimentarius Commission (38), yogurt should have a minimum protein content of 2.7% and a maximum fat content of up to 15%.

In this study, the fat content of the analyzed yogurt was between 3.48% and 3.53%. The fat content of yogurt varies but is typically between 0.5% and 3.5% (39). According to the US Food and Drug Administration (40), the yogurt criteria in regard to fat content classify non-fat products as having <0.5% fat; low-fat products, between 0.5% and 2.0%; and regular products, >3.25% fat. Based on this, the yogurt in this study was categorized as having regular fat levels.

3.2.2. pH and Titratable Acid

The pH value was assessed to determine the changes in the pH value of the yogurt before and after incubation. The decrease in pH in 0–18 h is shown in Figure 4a. At 12 h, yogurt almost reached the optimum pH, and after 18 h, the pH of the yogurt became too acidic, with a pH of 3.8. The decrease in pH is inversely proportional to the total amount of acid. In this study, the addition of collagen did not significantly change the pH and did not considerably affect the value of titratable acid. The highest titratable acid value was obtained with the addition of 3% collagen (Figure 4b).

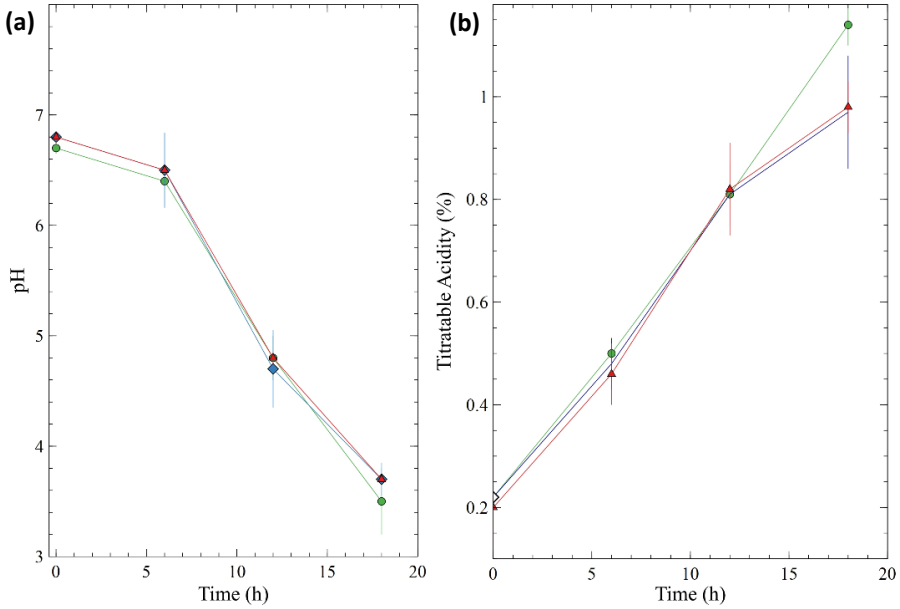

Figure 4. The value of pH. (a) and titratable acid; (b) during the fermentation process.

The decrease in pH is influenced by the activity and growth of lactic acid bacteria, which breaks down lactose into lactic acid. In addition, lactic acid bacteria excrete ß-galactosidase and lactic dehydrogenase enzymes, which produce lactic acid from lactose in fermentation (41). Therefore, the addition of high-protein materials, such collagen, and gelatin, can increase the acidity of yogurt (42). Typical yogurts have an average pH of 4.4. Therefore, yogurt–collagen needs to be harvested in 10–15 days.

4. Conclusions

Collagen from Java barb (*B. gonionotus*) skin processed using ASC methods was found to have similar characteristics to regular collagen. This protein has been confirmed to have a triple-helix structure, with a likely amino acid pattern of Gly-Pro-Ala or Gly-Pro-Arg. The proximate analysis showed that the addition of collagen at a concentration of up to 3% significantly increased its protein levels. However, it did not significantly affect pH or titratable acidity. A more detailed investigation of yogurt's sensory is needed. To the best of our knowledge, this is the first report on adding fish collagen to yogurt.

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Author Contributions

A.A.P, H.N, Y.D.J, F, K.D.M conceived and designed the experiments; A.A.P, H.M performed the experiments; H.N, Y.D.J, S.N.H.O, A.A.J analysed the data; All authors prepared the original draft; A.A.P, H.N, reviewed, and edited the manuscript final manuscript.

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Institutional Review Board Statement

Not applicable.

Data Availability Statement

The data supporting the findings of the article is available within the article.

Conflicts of Interest

All authors declare no conflict of interest, financial or otherwise.

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