

Article

Comparative Assessment of Gill Cut and Water Perfusion Bleeding Methods on Freshness, Microbiological Stability, and Textural Properties of Asian Seabass (*Lates calcarifer*) Slices During Refrigerated Storage

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Abstract

Bleeding enhances fish quality by removing blood, which acts as a pro-oxidant and provides nutrients for microbes. However, the effects of bleeding techniques on freshness, quality, and fishy odor of Asian seabass remain unclear. Therefore, the effects of gill cut (GC) and water perfusion (WP) bleeding on slices during 12-day refrigerated storage were investigated. Freshness was retained in bled samples, particularly GC, as indicated by lower K-values during storage. Protein oxidation was higher in WP than GC samples, as shown by higher carbonyl content in the former during the storage. This coincided with lower sulfhydryl content, except during day 3–6 in which there was no difference between both samples. Similar fishy odor intensity between GC and WP was observed during storage, except GC showed lower intensity on day 6, whereas unbled samples (UB) exhibited higher intensity through the storage. Pearson correlation and principal component analysis (PCA) confirmed relationship among bleeding methods, microbial growth, and quality changes. Boxplot analysis supported the results by showing variation in quality indicators among treatments. GC therefore preserved freshness, retarded microbiological and biochemical changes, and retained textural properties. GC tended to reduce fishy odor formation more effectively than WP, particularly at intermediate storage stage.

Keywords: protein oxidation; water perfusion; shelf-life; texture analysis; gill cut



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1. Introduction

Postmortem biochemical and physicochemical changes, together with microbial proliferation, are major factors contributing to the rapid deterioration of fish quality during

storage. Glycogen reduction, lactic acid formation, a decline in muscle pH, and decomposition of adenosine triphosphate (ATP) occur postmortem in fish and contribute to biochemical deterioration and loss of freshness [1]. These changes can also promote protein denaturation and degradation of muscle components during storage [1,2]. In parallel, microbial growth contributes substantially to fish spoilage. Off-odor, off-flavor and soft or mushy texture may occur in fish fillets due to microbial growth as induced by high moisture and amino acid content [3]. Spoilage microorganisms can produce metabolic byproducts such as trimethylamine, ethanol, ketones, hydrogen sulphide, aldehydes and organic acids, which are responsible for undesirable odor and flavor and contribute to the deterioration of fish quality and reduction of shelf-life [3]. Thus, postmortem biochemical changes and microbial proliferation act concurrently to accelerate freshness loss and spoilage of fish during storage. With growing emphasis on high food quality and safety, the modern consumers prioritize freshness with prime quality when consuming fish. Despite this increasing demand, quality maintenance of fish is challenging since fish are perishable with a rapid spoilage rate. Among various quality indicators, fish freshness has consistently been the primary criterion used by consumers to evaluate product quality [4]. The freshness loss and spoilage in fish are complex and influenced by multiple factors, including harvesting practices, post-harvest handling, bleeding efficiency, storage temperature and species-specific physiology and amount of undigested food retained in the gastrointestinal tract [5]. Conventionally, fish freshness has been evaluated through sensory evaluation and standard testing methods [6]. With technological advancement, the innovative sensor systems such as electronic noses, tongues, and computer vision tools have been developed for monitoring fish quality [7]. Among the quality indices, K-value has gained wide recognition to indicate freshness. Numerous studies have validated its strong association with freshness levels in fish and shrimp [8,9].

Asian seabass (*Lates calcarifer*), commonly referred to as barramundi, holds considerable significance in global aquaculture, currently ranked 35th among individual finfish species and 42nd across all fish categories classified by the FAO [10]. Due to high-valued fish, Asian seabass had an approximate global production of 154,281 tons in 2022 [11], and it has been widely cultivated in Southeast Asia. Asian seabass is an economically important species because of its high-quality protein and essential amino acids [12]. Additionally, it is rich in health-promoting polyunsaturated fatty acids, e.g., n-3 fatty acids. Consumer demand for *L. calcarifer* is on an upward trajectory, with a 5.5% compound annual growth rate [13].

Bleeding is an essential post-harvest technology of fish, primarily employed to enhance product quality and prolong shelf-life. Residual blood in fish muscle is a significant source of hemoglobin and iron, which can accelerate oxidative reactions, leading to lipid deterioration [11]. This pro-oxidative activity is also attributed to the action of lipoxygenase and the release of non-heme iron during the storage [14]. Protein oxidation, particularly in the presence of lipid oxidation products, proceeds via free radical chain mechanisms, similar to those observed in the lipid oxidation pathway [15]. Protein oxidation is associated with fragmentation or aggregation, thus adversely affecting the quality and techno-functional properties of fish.

Our previous study demonstrated that GC bleeding maintained histological integrity and lipid oxidative stability more effectively than WP [2]. However, a comprehensive evaluation of their effects on protein oxidation, microbial dynamics, and sensory attributes during extended refrigerated storage remains unexplored, necessitating the current investigation. Accordingly, the present study aimed to further evaluate the influence of the WP and GC bleeding methods on the textural property, physicochemical properties (sulfhydryl group and carbonyl contents), quality indices (TVB-N, TMA-N contents, fishy odor inten-

sity), freshness index (K-value) and microbiological changes of Asian seabass slices during storage at 4 °C. This finding may assist the farmers and fishermen in optimizing bleeding practices to improve fish quality and extend the shelf-life.

2. Materials and Methods

2.1. Chemicals and Microbiological Media

All chemicals were of analytical grade. Adenosine 5'-triphosphate disodium salt (>99%), its derivatives, and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, ≥98%) were obtained from Sigma Aldrich (St. Louis, MO, USA). 2,4-Dinitrophenylhydrazine (DNPH) was acquired from Thermo Scientific Chemicals (Waltham, MA, USA). Other chemicals were sourced from Loba Chemie Pvt. Ltd. (Mumbai, Maharashtra, India). All microbiological media were acquired from Oxoid (Hampshire, UK).

2.2. Fish Sample and Preparation

Asian seabass (*Lates calcarifer*) with an average weight of 1.5–2 kg and an average total length of 42.0 ± 1.6 cm ($n = 9$) were captured live from Ko Yo Lake in Songkhla province, Thailand (7°09'09.4" N 100°32'56.6" E). The fish were transferred to the laboratory in oxygenated clean water within 45 min.

2.3. Bleeding

Fish were subjected to two different bleeding techniques: the gill cut (GC) method ($n = 3$) and the water perfusion (WP) method ($n = 3$), as tailored by Vijayakumar et al. [11]. For the gill cut method, fish were euthanized by brain spiking with an icepick, and the gill arches were severed. Thereafter, the fish were bled in iced water (30 min). They were subsequently placed on a draining rack (4 °C, 30 min) (Sandán intercool, Sandán corporation, Isesaki, Japan) to ensure the removal of excess blood. The water perfusion method began with euthanasia by brain spiking using an icepick, followed by cutting the primary dorsal arteries beneath the gill covers. After tail severance, distilled water was perfused into the caudal vein using a 1.1 mm diameter nozzle (flow rate 30 mL/s) through the largest visible blood vein (tail vein) for around 30 s. Subsequently, a 15 min immersion in iced water was done. To complete the process, the fish were placed in a head-down position at 4 °C for 30 min to ensure proper drainage. Fish that were not bled served as the control group ($n = 3$), which were euthanized using an ikejime stunning approach [16].

Following either bleeding or euthanasia, each group of fish was descaled, beheaded and eviscerated before being washed again to ensure the removal of any remaining debris or tissue. Dorsal fillets were excised and sliced into 2–3 cm thick portions. Slices (approximately 15 g) were placed in a polystyrene tray, sealed in a polyethylene bag, and stored under refrigeration. Sampling was done every three days over a period of 12 days for subsequent analyses.

2.4. Ethical Approval

All procedures involving Asian seabass (*Lates calcarifer*) were performed following the ethical guidelines for the care and use of experimental animals. The ethical approval was granted by the Institutional Animal Care and Use Committee (IACUC) of Prince of Songkla University, Thailand (Approval code: Ref.AQ040/2025).

2.5. Analyses

2.5.1. ATP, Its Derivatives and K-Value

Adenosine triphosphate (ATP) and its degradation products, including adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine 5'-monophosphate (IMP),

inosine (HxR), and hypoxanthine (Hx), were extracted from fish muscle using a simplified procedure adopted from Fan et al. [9]. Muscle samples (1.0 g) were collected after completion of euthanasia at time 0, 1, 2, 3, 4, 6, 12, 24, 36, 48, 72, 96, 120, 144, 216 and 288 h (12 days). To the sample, 5% perchloric acid (10 mL) was added and homogenized. After standing on ice for 15 min, 5.2 mL of 1 M potassium hydroxide and 4.8 mL of distilled water were added to adjust the pH to 2.5–3.0. After further incubation on ice for 15 min, the mixture was filtered through a 0.22 µm membrane to obtain a clear filtrate. ATP and its derivatives in the filtrate were analyzed using a HITACHI Chromaster Series 5000 HPLC system (Hitachi, Tokyo, Japan) equipped with a Spersil AQ-C18 column (5 µm, 4.6 mm I.D. × 250 mm). The mobile phase was 0.1 M phosphate buffer (pH 3.0). Detection was carried out at a wavelength of 254 nm. Standard solutions of ATP, ADP, AMP, IMP, HxR, and Hx were used to generate calibration curves by plotting concentration against peak area. The K-value was computed [17] as follows:

$$\text{K-value (\%)} = \frac{(\text{HxR} + \text{Hx})}{(\text{ATP} + \text{ADP} + \text{AMP} + \text{IMP} + \text{HxR} + \text{Hx})}$$

2.5.2. Microbiological Quality

Total viable count (TVC) and psychrophilic bacterial count (PBC) were analyzed following the APHA Compendium of Methods for Microbiological examination as modified by Palamae et al. [18]. Briefly, the sample (25 g) was transferred into a sterile stomacher bag with 225 mL of 0.85% saline. Homogenization was performed using a stomacher blender (M400, Seward, West Sussex, UK) at 220 rpm for 1 min. Serial dilution was prepared using sterile saline solution. Subsequently, aliquots of diluted samples (100 µL) were spread onto plate count agar. The plates were incubated at 35 ± 2 °C for 24 h for TVC determination, whereas PBC plates were counted after incubation at 4 ± 2 °C for 10 days.

2.5.3. Carbonyl Content

Carbonyl content was determined using 2,4-dinitrophenylhydrazine (DNPH) derivatization following the method of Palanisamy et al. [19]. Firstly, the homogenate was prepared. The sample (0.5 g) was homogenized in 10 mL of 0.05 M phosphate buffer (pH 7.2) for 30 s at a speed of 11,000 rpm using an IKA T 25 D homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany). The homogenate (300 µL) was precipitated with 0.5 mL of 30% (*w/v*) trichloroacetic acid (TCA), and the resulting pellet was dissolved in 200 µL of 10 mM DNPH. The mixture was incubated in the dark at room temperature for 1 h. After derivatization, protein was re-precipitated with 30% TCA and centrifuged at $12,000 \times g$ for 5 min. The pellet was washed thoroughly to eliminate unreacted DNPH and dissolved in 6 M guanidine hydrochloride in 20 mM potassium phosphate buffer (pH 2.3). Absorbance was read at 370 nm, and carbonyl level was calculated using an absorption coefficient of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$. The carbonyl content was expressed as µmoles per mg protein (µmol/mg protein).

2.5.4. Sulfhydryl Content

Total sulfhydryl content was determined using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), following the procedure described by Eymard et al. [20]. The homogenate prepared as previously described was homogenized in 10 mL of 0.05 M phosphate buffer (pH 7.2) for 30 s. One mL of homogenate was combined with 9 mL of 0.05 M phosphate buffer (pH 7.2) containing 0.6 M NaCl, 6 mM EDTA, and 8 M urea. After centrifugation at $14,000 \times g$ for 15 min at 5 °C, 3 mL of the supernatant were reacted with 0.04 mL of 0.01 M DTNB in 0.05 M sodium acetate buffer and incubated at 40 °C for 15 min. A blank solution was prepared by replacing the homogenate with the buffer solution containing NaCl, EDTA

and urea. The absorbance at 412 nm was recorded. Total sulfhydryl content ($\text{mol}/10^5 \text{ g}$ protein) was calculated using an extinction coefficient of $13,600 \text{ M}^{-1} \text{ cm}^{-1}$.

2.5.5. Total Volatile Base-Nitrogen (TVB-N) and Trimethylamine (TMA-N) Contents

Total volatile base nitrogen (TVB-N) levels were assessed using the classical Conway micro-diffusion method as outlined by Ahmad et al. [21]. The muscle (2 g) was homogenized with 8 mL of 4% (*w/v*) trichloroacetic acid (TCA) at 8000 rpm for 1 min. The homogenate was allowed to stand at room temperature for 30 min before filtration through Whatman no. 4 filter paper. The total volume of the filtrate was adjusted to 10 mL with 4% TCA. For TVB-N analysis, 1 mL of filtrate and 1 mL of boric acid indicator were added to the outer and inner ring respectively. Then, 1 mL of saturated potassium carbonate (K_2CO_3) was added to the outer ring, and the unit was sealed and gently mixed. For TMA-N determination, the same procedure was followed, except that 1 mL of formalin was added to the outer ring before the addition of K_2CO_3 . The Conway dish was incubated at 37°C for 60 min. After incubation, the inner solution was titrated with 0.02 N HCl until the color changed from green to pink. A blank was prepared using 4% TCA instead of sample. TVB-N and TMA-N contents were computed as follows:

$$\text{TVB-N or TMA-N content} = \frac{(N \times 14) \times (A - B) \times V \times 100}{\text{Weight of the sample}}$$

where N is normality of HCl, A and B are volume of HCl (mL) used for sample titration and for blank titration, respectively, and V is the total sample volume (mL). TVB-N and TMA-N contents were expressed as mg N/100 g sample.

2.5.6. Fishy Odor Intensity

Sensory evaluation was carried out as described by Maqsood and Benjakul [22], using 15 trained panelists. All panelists had prior experience with fish consumption. To standardize scoring, the reference samples were prepared by storing Asian seabass slices in polyethylene packaging at 4°C for 0, 3, 6, 9, and 12 days, which were assigned the odor scores of 0, 2.5, 5, 7.5 and 10, respectively. The training session was conducted twice a week for 2 weeks. During the evaluation, the samples were placed in glass containers (4 cm in diameter) and sealed with aluminum foil. The samples were coded using randomly assigned three-digit codes and presented to the panelists in a randomized order to minimize potential order effects. Before evaluation, the samples were allowed to equilibrate to room temperature and were presented to the panelists at room temperature. Panelists were instructed to open the containers, assess the odor by smelling the headspace, and assign an appropriate score based on their perception.

2.5.7. Textural Properties

Texture profile analysis (TPA) of the slice samples was performed using a texture analyzer Model TA-TX2 (Stable MicroSystems, Surrey, UK). The slice samples were uniformly cut into cubes ($1.5 \times 1.5 \times 1.5 \text{ cm}^3$) prior to analysis. TPA was performed at room temperature using a P/40R cylindrical probe with strain as the target mode and an automatic force trigger set to 10 g [23]. The parameters involving hardness, springiness, cohesiveness, and resilience were recorded.

2.6. Statistical Analysis

A completely randomized design (CRD) was applied throughout this study. For each treatment, the slices from three fish (slice thickness: 1.5–2 cm) were mixed and used as the composite sample. For each experiment, 6 slices were packaged in a bag, and the bag was

randomly taken for analysis for each treatment. Three slices were used for microbiological analysis, while another 3 slices were subjected to textural analysis. After textural measurement, the samples were ground and subsequently used for other analyses. The data represented the technical replicates ($n = 3$). Two-way analysis of variance (ANOVA) was performed to evaluate the effects of bleeding treatment, storage time, and their interaction (bleeding treatment $\times$ storage time), followed by Duncan's multiple range test when significant differences were detected ($p < 0.05$). Pearson correlation analysis was employed to determine the linear relationship between parameters, while principal component analysis (PCA) was conducted to identify the major source of variation and visualize the clustering of samples based on treatment and storage time. All statistical computations and graphical visualizations were performed using the SPSS software (version 28.0; IBM Corporation, Armonk, NY, USA) and R software (version 4.3.1; R Foundation for statistical computing, Vienna, Austria).

3. Results and Discussion

3.1. K-Value

Postmortem biochemical alterations in aquatic animals are marked by the degradation of ATP in muscle tissues. The K-value, which quantifies the degree of ATP degradation, has been established as a highly effective and widely adopted parameter for determining the freshness of fish [8,24]. K-values of samples with varying bleeding methods in comparison with unbled counterpart are shown in Figure 1. The initial K-value of Asian seabass slices was $5.66 \pm 0.30\%$ for unbled samples and increased gradually with storage time, reaching a value of 67.18 ± 0.24 in 288 h (12 days). For gill cut bled (GC) and water perfusion bled (WP) samples, the initial K-values were 5.00 ± 0.83 and 5.61 ± 0.88 , respectively. Over storage time, this value increased, and K-values of 54.06 ± 0.82 and 58.84 ± 1.99 were obtained, respectively. The K-value of the GC samples (40%) remained below the freshness threshold up to day 9 of storage. It has been well documented that a K-value higher than approximately 60% corresponds to advanced nucleotide degradation and is indicative of spoilage or quality rejection in fish products [25]. At the initial stage of storage, there was no significant difference in K-value among the UB, GC, and WP samples; however, as storage time upsurged, the UB samples exhibited a higher K-value than both GC and WP. The elevated K-value of unbled samples was likely associated with the presence of residual blood, which might contain the enzymes responsible for deamination and dephosphorylation. In addition, the blood could serve as nutrients for microbial growth. Some microorganisms, particularly spoilage bacteria, are capable of producing proteases, making ATP more susceptible to decomposition by enzymes inducing dephosphorylation or deamination [26]. When comparing K-values between the GC and WP samples, the latter showed a higher K-value, especially at the end of storage. This confirmed the adverse effect of WP on freshness stability of Asian seabass slices with prolonged storage. Vijayakumar et al. [11] also documented that the total viable count of the WP samples was higher than GC samples at the end of refrigerated storage. Bacteria at a higher load might induce the decomposition of ATP at a faster rate. WP samples showed a higher K-value than GC samples, particularly during the later stage of storage. The harsher conditions associated with water jet bleeding could physically disrupt muscle membranes and promote biochemical reaction, leading to oxidation of lipids and protein breakdown [11]. Nevertheless, the rate of increase in K-value is species dependent. Japanese seabream had a K-value of approximately 20% after 12 days of iced storage, whereas Pacific cod reached a K-value of around 45% within one day [27]. A strong and consistent linear relationship between K-value and the storage period of fish was reported [16].

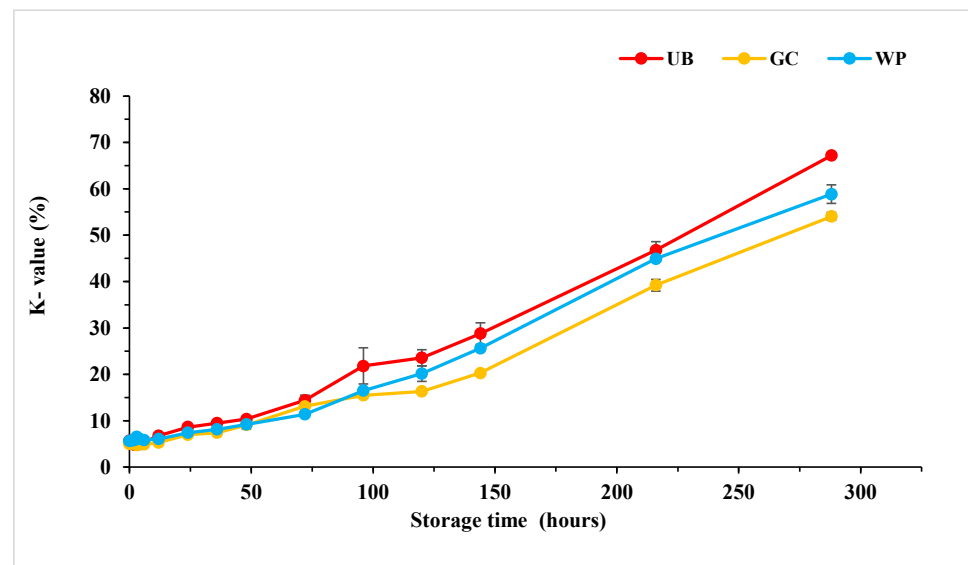


Figure 1. Changes in K-value in the slices from unbled (UB), gill cut bled (GC) and water perfusion bled (WP) Asian seabass during refrigerated storage of 12 days. Bars represent the standard deviation ($n = 3$).

Under normal physiological conditions, ATP and related nucleotides (ADP, AMP) form complexes with iron, maintaining a stable state and being less reactive [28]. Simultaneously, postmortem ATP degradation diminishes the stability of iron–nucleotide complexes, leading to the release of catalytically active iron. An elevation in free iron levels may promote Fenton-type reactions [11], where ferrous ions (Fe^{2+}) interact with hydrogen peroxide, resulting in the production of highly reactive hydroxyl radicals ($\bullet\text{OH}$) [29]. These radicals initiate oxidative damage to muscle proteins, promoting denaturation, cross-linking, and deamination [30]. Consequently, these findings highlight that inadequate bleeding was associated with enhanced ATP degradation, as indicated by an elevated K-value during storage.

3.2. Microbiological Quality

Total viable count (TVC) and psychrophilic bacterial count (PBC) of UB, GC, and WP samples during refrigerated storage are presented in Figure 2a,b. Significant effects of bleeding treatment, storage time, and their interaction on TVC and PBC were observed ($p < 0.05$). At the beginning of storage, TVCs of 3.76, 3.71 and 3.70 CFU/g were observed in UB, GC and WP samples, respectively. The relatively low initial counts indicate the freshness of fish and proper hygienic handling and processing, e.g., filleting or slicing. Nonetheless, the initial PBC was slightly lower (3.15–3.27 log CFU/g), and no significant differences were observed among the treatments on day 0 ($p > 0.05$). Asian seabass inhabit in the water of tropical region, and the contaminated bacteria were dominated by mesophilic bacteria. During the extended storage, both TVC and PBC increased with rising storage time. Under the refrigerated condition, psychrophilic bacteria were able to adapt and proliferate to a high extent. This was evidenced by the increase in PBC with augmenting storage time. Psychrophilic spoilage bacteria, particularly *Pseudomonas* spp., *Shewanella* spp., and *Aeromonas* spp., were able to adapt to chilled handling or storage conditions [31]. During the storage period, UB samples had higher TVC and PBC than the bled samples ($p < 0.05$), suggesting that the bleeding treatments contributed to the reduced bacterial growth. The presence of blood in fish tissues might favor microbial proliferation because it provides essential nutrients required for bacterial growth [24]. Both Gram-positive and Gram-negative bacteria possess specialized heme uptake systems that enable them to acquire

iron from host tissues [32]. These systems involve surface receptors and transport proteins that bind heme and facilitate its transport into the bacterial cell. Inside the cytoplasm, heme can be degraded to release iron or incorporated into bacterial proteins essential for metabolic processes, thereby promoting bacterial survival and proliferation [33]. Consistent with augmenting TVC and PBC, TVB-N and TMA-N contents in the UB samples were higher than bled samples during storage. These volatile basic compounds and TMA are produced by spoilage bacteria through the degradation of nitrogenous compounds, reflecting the progression of microbial spoilage [34]. Some bacteria, such as *S. putrefaciens*, *P. phosphoreum*, and *Aeromonas* spp., possessing TMAO reductase could reduce TMAO to TMA, a compound contributing to fishy odor [35]. The higher microbial counts observed during the late stage of storage in the water perfusion (WP) treatment might be attributed to tissue disruption caused by the high-pressure water flow during the perfusion process. Such mechanical damage to the muscle structure plausibly facilitated bacterial penetration and increased the availability of nutrients, thereby promoting microbial proliferation during storage [11]. Furthermore, the increased proliferation of microorganisms could enhance textural softening. During microbial growth, several nutrients, especially free amino acids, are necessary. As a result, proteases are secreted from microbial cells and hydrolyze muscle proteins [36]. As a consequence, the compactness or firmness of fish meat could be lowered, leading to an undesirable texture (Table 1). In general, spoilage bacteria, including *Shewanella* spp. and *Pseudomonas* spp., possess proteases, which can hydrolyze the muscle proteins to peptides or free amino acids [37]. Some microorganisms are able to hydrolyze collagenous protein or connective tissue, resulting in the weakening of meat texture [38].

Some microorganisms might have lipase, which is able to release free fatty acids in fish meat [39]. In general, free fatty acids are prone to oxidation [11], as indicated by augmented PV and TBARS values of fish meat when higher TVC and PBC were found. Furthermore, the partial hydrolysis of muscle proteins as induced by microorganisms might induce conformational changes of proteins in a way that favors their oxidation, such as disulfide bond formation as well as carbonyl formation [40]. This was reflected by the decreased sulfhydryl content and increased carbonyl content (Figures 3 and 4).

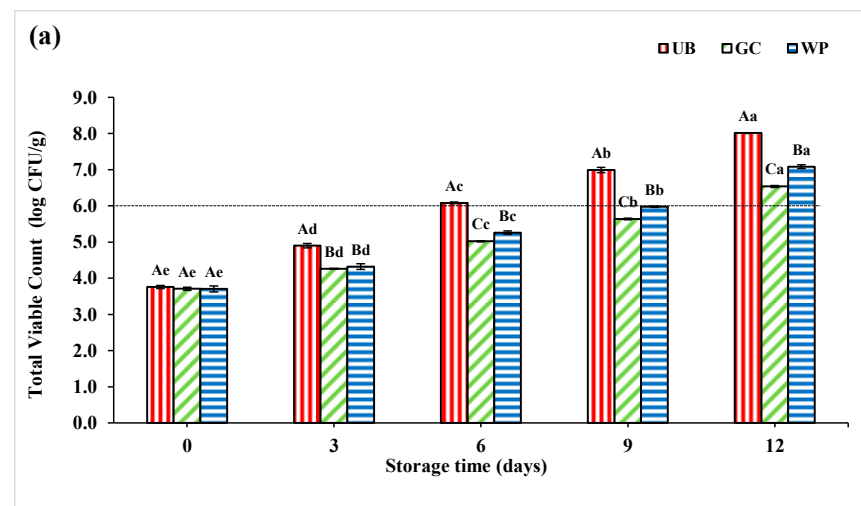


Figure 2. Cont.

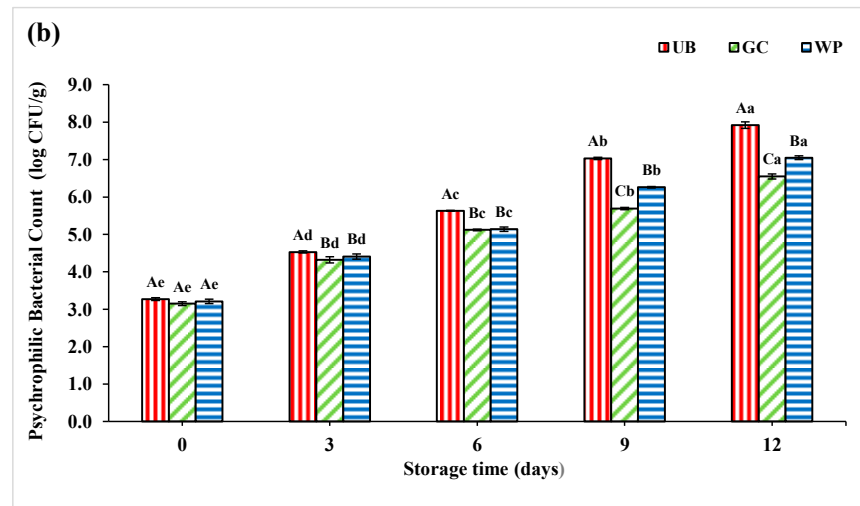


Figure 2. Changes in total viable count (TVC) (a) and psychrophilic bacterial count (PBC) (b) in the slices from unbled (UB), gill cut bled (GC) and water perfusion bled (WP) Asian seabass during refrigerated storage of 12 days. Bars represent the standard deviation ($n = 3$). Different uppercase letters within the same storage time denote significant differences among bleeding treatments, whereas different lowercase letters within the same sample at different storage times denote significant differences among storage times ($p < 0.05$).

Based on the microbiological limit of TVC (6 log CFU/g sample) established by ICMSF [41]), UB, GC and WP could be kept up to 3, 9 and 9 days, respectively. The result reconfirmed that the bleeding could extend the shelf-life of Asian seabass slices when kept under refrigerated conditions. A similar result was reported by Vijayakumar et al. [11] in which the slice from unbled Asian seabass showed the shortest shelf-life during refrigerated storage. Maqsood and Benjakul [22] also documented the positive impact of bleeding (tail cut method) on the shelf-life extension of Asian seabass. Therefore, controlling microbial growth through appropriate bleeding treatments could delay spoilage and maintain the quality and textural property of fish during refrigerated storage.

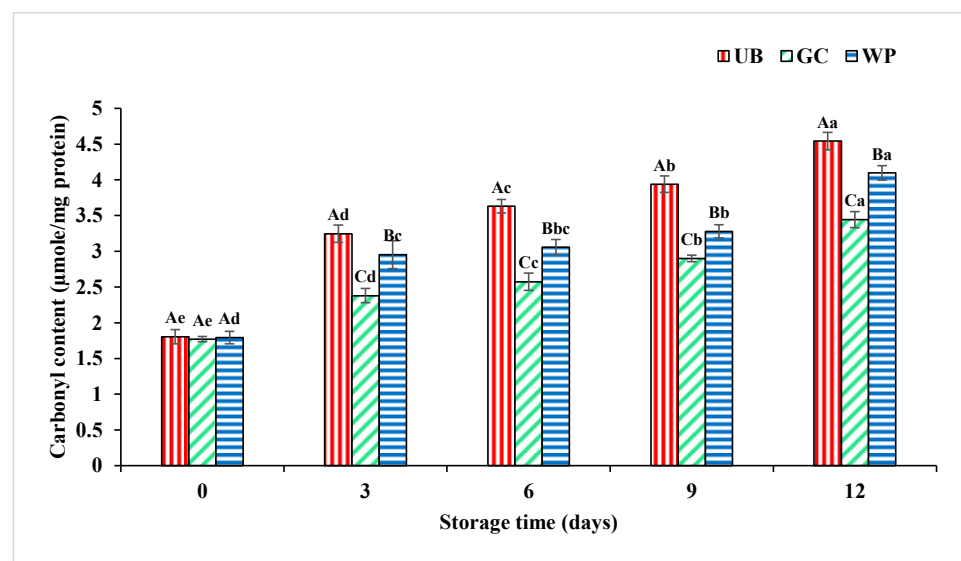


Figure 3. Changes in carbonyl content in the slices from unbled (UB), gill cut bled (GC) and water perfusion bled (WP) Asian seabass during refrigerated storage. Bars represent the standard deviation ($n = 3$). Different uppercase letters within the same storage time denote significant differences among bleeding treatments, whereas different lowercase letters within the same sample at different storage times denote significant differences among storage times ($p < 0.05$).

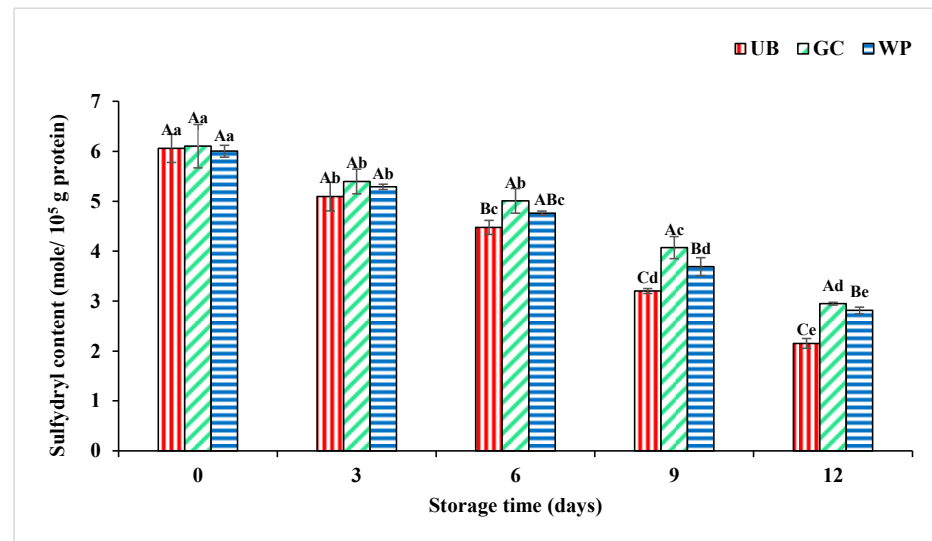


Figure 4. Changes in sulfhydryl content in the slices from unbled (UB), gill cut bled (GC) and water perfusion bled (WP) Asian seabass during refrigerated storage. Bars represent the standard deviation ($n = 3$). Different uppercase letters within the same storage time denote significant differences among bleeding treatments, whereas different lowercase letters within the same sample at different storage times denote significant differences among storage times ($p < 0.05$).

3.3. Carbonyl Content

Carbonyl contents of UB, GC, and WP samples increased ($p < 0.05$) with extended storage duration up to 12 days (Figure 3), reflecting progressive oxidation of muscle proteins and the subsequent transformation of amino groups into carbonyl derivatives. The analysis demonstrated that both bleeding treatments and storage duration, together with their interaction, had a significant effect on carbonyl content ($p < 0.05$). Protein oxidation can occur through multiple pathways, including direct oxidation of amino acid side chains such as proline, arginine and lysine [42]; glycation reaction via the Maillard process in the presence of reducing sugars [43]; oxidative cleavage of the peptide backbone through α -amidation or glutamyl residue oxidation [44]; and covalent interactions with reactive carbonyl compounds derived from lipid peroxidation [45]. Carbonyl content was significantly lower in the GC samples ($p < 0.05$) than other samples. The greater rate of carbonyl formation observed in unbled samples throughout storage was likely associated with the presence of blood, which is rich in heme-bound and non-heme iron capable of catalyzing oxidative reactions as supported by our previous study [11]. Residual Fe^{2+} and Fe^{3+} continuously interconvert through redox cycling reactions, such as the Fenton and Haber–Weiss reactions, generating reactive oxygen species including hydroxyl and ferryl radicals [46]. These radicals were able to oxidize susceptible amino acids (e.g., lysine, arginine, and proline) within muscle proteins, resulting in carbonyl group formation and loss of structural integrity [47]. In addition, as storage progresses, hemoglobin degradation releases heme iron, which can associate with muscle proteins and induce site specific oxidation, leading to pronounced protein carbonyl formation in unbled fish. The slightly higher carbonyl content observed in WP samples compared to GC samples during extended storage, plausibly resulted from oxidative coupling between lipids and proteins, triggered by tissue injury during water perfusion [11]. Membrane damage enhances lipid peroxidation, leading to the generation of reactive intermediates, including hydroperoxides and α , β -unsaturated aldehydes. These products can directly oxidize amino acid side chains or form Schiff-base adducts, thereby increasing protein carbonylation [48] during subsequent storage. Moreover, the reactive carbonyls derived from lipid oxidation can propagate secondary

oxidation reactions, accelerating oxidative deterioration in muscle proteins throughout storage [47]. Therefore, the GC method was shown to effectively retard protein oxidation of Asian seabass muscle.

3.4. Sulfhydryl Content

Sulfhydryl (SH) groups represent the most reactive functional groups in proteins, and their alterations play a crucial role in maintaining the stability of the three-dimensional structure of proteins. SH groups in proteins can undergo oxidation to form disulfide linkages. With increasing oxidative stress, more advanced oxidation products such as sulfoxides may be produced [49]. Therefore, the sulfhydryl content is considered a valuable indicator of protein oxidation [50]. Changes in the sulfhydryl content of Asian seabass samples, both unbled and bled samples using two different techniques, during storage are depicted in Figure 4. The changes in SH content were significantly associated with bleeding treatment, storage duration, and the interaction between these factors ($p < 0.05$). The initial SH content of the UB, GC, and WP samples was comparable ($p > 0.05$). The SH content of UB Asian seabass was lower than those of the GC and WP samples during day 6–12. The subsequent decline in SH levels was most likely driven by oxidation of cysteine residues. Reactive ferrous ions released from heme proteins promote the generation of reactive oxygen species, leading to the conversion of sulfhydryl groups into disulfide linkages [51]. Wang et al. [52] found that iron-triggered oxidation caused a notable decrease in sulfhydryl levels in yak myofibrillar proteins, indicating the pronounced reactivity of thiol groups to iron-related oxidative stress. However, WP seemed to promote protein oxidation more rapidly in the later stage of storage than GC (during 6–12 days). The observed effect may be attributed to the strong mechanical pressure exerted during the water jet treatment, which could have damaged cell membranes, thus promoting lipid and protein oxidation within the muscle tissue during subsequent storage. Babakhani et al. [53] reported a close relationship between the decrease in sulfhydryl (SH) content and the increase in lipid oxidation indices in minced Atlantic mackerel. Similarly, our previous study [11] reported that TBARS and PV in unbled Asian seabass were higher than those of bled counterparts during refrigerated storage. Those lipid peroxidation products could promote the oxidation of protein sulfhydryl groups. Thus, bleeding was involved in suppressing the formation of lipid oxidation products, which could enhance the generation of reactive species that subsequently induced protein oxidation during refrigerated storage.

3.5. TVB-N and TMA-N Contents

Proteinaceous and non-protein nitrogenous components in fish undergo decomposition as a result of enzymatic hydrolysis and microbial metabolism [54]. This degradation process generates total volatile basic nitrogenous compounds and trimethylamine (TMA), both of which are regarded as essential parameters indicating the degree of spoilage and quality deterioration in fish products [55]. TVB-N and TMA-N contents of Asian seabass subjected to two bleeding methods were lower than those of the unbled samples during the storage, as depicted in Figures 5a and 5b, respectively. Significant differences in the accumulation of TVB-N and TMA-N were evident among the bleeding treatments as storage progressed ($p < 0.05$). Within 12 days, the unbled group showed a progressive rise in nitrogenous volatile compounds, with TVB-N increasing from 3.9 to 21.9 mg N/100 g and TMA-N increasing from 0.7 to 7.2 mg N/100 g. The upsurge in both TVB-N and TMA-N concentrations was likely attributed to enzymatic deamination of amino acids, oxidative reaction of amines, and nucleotide deamination facilitated by both endogenous enzymes and microbial activity [56]. Overall, the unbled samples had higher ($p < 0.05$) TVB-N and TMA-N levels than the bled counterpart. The unbled samples, containing

higher residual blood and iron, supported greater microbial proliferation, as reflected by higher total viable counts.

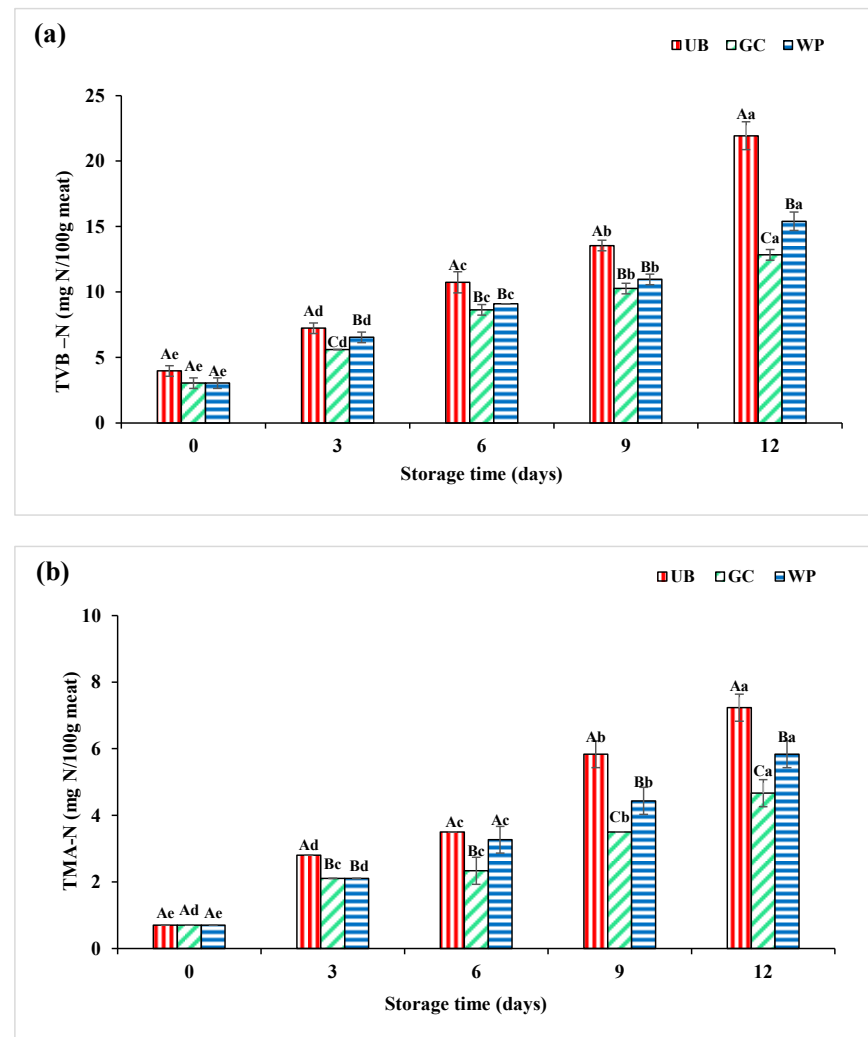


Figure 5. Changes in TVB-N (a) and TMA-N (b) contents in the slices from unbled (UB), gill cut bled (GC) and water perfusion bled (WP) Asian seabass during refrigerated storage of 12 days. Bars represent the standard deviation ($n = 3$). Different uppercase letters within the same storage time denote significant differences among bleeding treatments, whereas different lowercase letters within the same sample at different storage times denote significant differences among storage times ($p < 0.05$).

The increased availability of iron likely enhanced both microbial metabolism and the proteolytic activity of endogenous enzymes, thereby accelerating the breakdown of protein and non-protein nitrogenous compounds [57]. During the late storage period, WP samples exhibited higher TVB-N and TMA-N levels than GC samples. This was likely attributable to the harsh condition of the water jet, which disrupted fish muscle membranes and liberated phospholipids such as phosphatidylcholine and phosphatidylethanolamine [11]. These compounds along with L-carnitine, choline, and betaine serve as precursors for microbial production of TMA, thereby contributing to the elevated TVB-N and TMA-N contents [58]. Thus, bleeding effectively inhibited the decomposition of nitrogenous compounds, thus limiting the production of volatile nitrogen compounds.

3.6. Fishy Odor Intensity

The fishy odor intensity of Asian seabass slices stored at 4 °C, as influenced by different bleeding treatments (GC and WP) in comparison with UB, is presented in Figure 6. The differences in fishy odor intensity among the bleeding treatments became more pronounced with prolonged storage ($p < 0.05$). The UB samples exhibited significantly higher odor intensity than the GC and WP samples throughout 12 days ($p < 0.05$). This increased odor development in UB samples was closely associated with elevated lipid oxidation, particularly for the volatile secondary lipid oxidation products, and higher TMA-N concentration, as shown in Figure 5b. At refrigeration temperatures, bacterial metabolism of trimethylamine N-oxide (TMAO) via TMAO reductase results in the formation of volatile amines, chiefly TMA, possessing a fishy odor [59]. The accumulation of these nitrogenous volatiles imparts the characteristic fishy odor commonly associated with deteriorating fish [60].

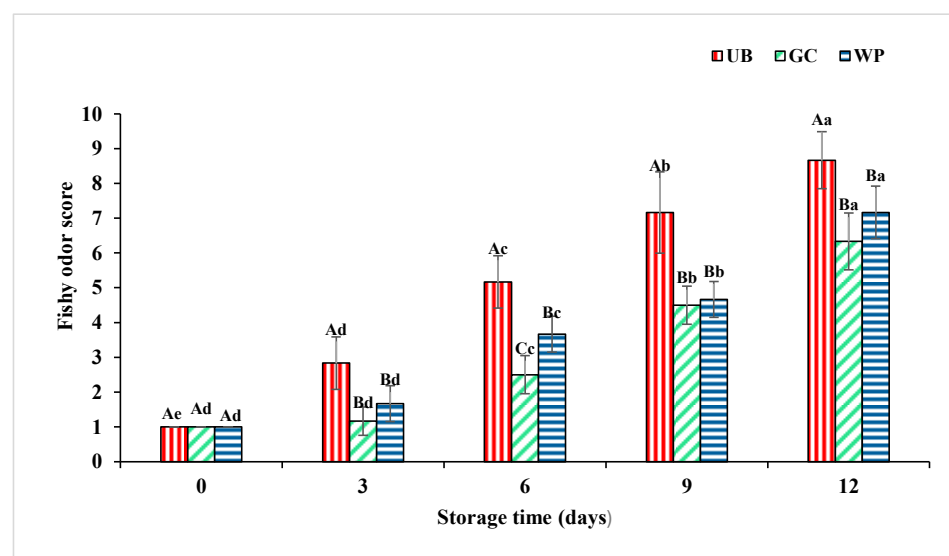


Figure 6. Changes in fishy odor in the slices from unbled (UB), gill cut bled (GC) and water perfusion bled (WP) Asian seabass during refrigerated storage. Bars represent the standard deviation ($n = 15$). Different uppercase letters within the same storage time denote significant differences among bleeding treatments, whereas different lowercase letters within the same sample at different storage times denote significant differences among storage times ($p < 0.05$).

Trimethylamine (TMA) can serve as an important chemical indicator associated with the development of fishy odor during refrigerated storage. In the present study, the fishy odor development could be governed by TMA formation. TMA is generally produced from trimethylamine oxide (TMAO) through microbial TMAO reductase. TMA has been known to contribute to the typical fishy odor of deteriorated fish. It was noted that the increase in TMA content (Figure 5b) was related to the increase in fishy odor intensity (Figure 6). The similarity in odor between fresh and 9-day-stored Asian seabass slices with bleeding indicated that bleeding was able to inhibit fishy odor development by minimizing lipid oxidation [22]. At day 12, the gradual increase in fishy odor scores of GC and WP bled samples could be the result of increased microbial growth, although the extent was lower than that observed in UB samples. Microbial proliferation plays a crucial role in fish deterioration by producing unpleasant odors and compromising overall quality [61].

Richard and Hultin [62] reported that unbled trout mince exhibited higher rancid scores than bled trout during 15 days of storage at 2 °C. During storage at 2 °C, bleeding effectively decreased rancidity in Atlantic mackerel, with only about 40% of bled minces

showing a rancid odor by day 8, in contrast to all unbled samples. Therefore, bleeding helps retard fishy odor formation related to lipid oxidation [62]. Although color parameters were not measured in the present study, the bleeding could increase the whiteness of muscle of Asian seabass due to the removal of blood rich in hemoglobin [11]. Such color deterioration associated with oxidation of hemoglobin in retained blood was linked to protein and lipid oxidation. Hemoglobin could act as a pro-oxidant, and the oxidation was closely related to the formation of volatile compounds contributing to off-odor, especially fishy odor. The result demonstrated that bleeding of Asian seabass slices contributed to a moderate reduction in fishy odor formation throughout the refrigerated storage period.

It was noted that there were no marked increases in oxidation products as well as microbial load as affected by WP at day 0. The early tissue disruption caused by WP [11] possibly remained subclinical at the beginning of storage. However, this phenomenon might facilitate oxidative chain reactions and increase nutrient availability for microbial proliferation during extended refrigeration, thereby manifesting as accelerated quality loss.

3.7. Textural Parameters

Texture is a vital quality parameter in fish flesh, playing a significant role in consumer acceptance and processing suitability in the seafood industry [63]. Textural properties, including hardness, springiness, cohesiveness, resilience, etc., have been used to represent the physical integrity of the tissue [64]. The texture profile of Asian sea bass slices during refrigerated storage (4 °C) is summarized in Table 1. The influence of bleeding treatment on textural characteristics varied significantly with storage duration ($p < 0.05$). Hardness, considered the main indicator of texture, progressively decreased during storage in both bled and unbled samples. The initial hardness was higher in UB and GC samples.

This was possibly due to some disruption of muscle tissue caused by water injection for WP samples. This might cause tissue disintegration associated with weakness of muscle [9]. During refrigerated storage, hardness gradually declined as myofibrillar proteins were degraded by endogenous proteases and microbial activity, producing softer and less cohesive muscle. The better retention of texture in GC samples might be associated with the more effective removal of residual blood, which reduced the presence of heme proteins and iron-containing components capable of promoting oxidative reaction during storage [11]. Free iron found in the blood might strongly induce protein oxidation of myofibrillar proteins in refrigerated fish muscle. Iron-mediated oxidative processes have been documented to contribute to structural deterioration during cold storage [65]. Such oxidative deterioration might affect the structural integrity of myofibrillar proteins and connective tissues, thereby facilitating muscle softening and loss of textural properties. Therefore, the reduced blood retention achieved by GC might have contributed to lower oxidative and structural deterioration, thus maintaining muscle integrity during refrigerated storage. For WP samples, the influx of water during perfusion increased muscle moisture and drip loss, weakening structural cohesion between fibers and diminishing tissue compactness [66]. This also facilitated the invasion/ entry of microorganisms capable of breaking down the muscle. The hardness of fillets from *Siniperca chuatsi* [23] and *Aristichthys nobilis* [63] decreased during storage.

This softening might result from the weakening of connective tissue like collagen [67], particularly the endomysium, degradation of Z-line in myofibrils [68] and a rise in microbial activity [69]. During extended storage, the differences ($p < 0.05$) were found in springiness, cohesiveness and resilience between bled and unbled Asian seabass slices. By day 12, UB fillets showed a pronounced decline in textural quality compared with GC and WP samples ($p < 0.05$) at 4 °C. Ayala et al. [70] reported that the loss of sea bream quality

over 22 days of chilled storage was primarily due to degradation of muscle protein by endogenous enzymes.

Table 1. Texture profiles of slices from unbled (UB), gill cut bled (GC) and water perfusion bled (WP) Asian seabass during refrigerated storage of 12 days.

Storage Time (Days)		0	3	6	9	12
Hardness (g)	UB	3466 ± 152 ^{Aa}	2874 ± 99 ^{Ab}	2642 ± 36 ^{Bc}	2099 ± 50 ^{Cd}	1679 ± 17 ^{Ce}
	GC	3641 ± 94 ^{Aa}	2886 ± 73 ^{Ab}	2759 ± 57 ^{Ac}	2489 ± 19 ^{Ad}	2238 ± 41 ^{Ae}
	WP	2975 ± 99 ^{Ba}	2694 ± 73 ^{Bb}	2656 ± 34 ^{Bb}	2324 ± 41 ^{Bc}	1878 ± 28 ^{Bd}
Springiness (cm)	UB	0.59 ± 0.02 ^{Aa}	0.48 ± 0.01 ^{Ab}	0.42 ± 0.01 ^{Bc}	0.34 ± 0.03 ^{Bd}	0.31 ± 0.00 ^{Cd}
	GC	0.54 ± 0.07 ^{Aa}	0.49 ± 0.02 ^{Aa}	0.48 ± 0.00 ^{Aab}	0.42 ± 0.03 ^{Abc}	0.39 ± 0.02 ^{Ac}
	WP	0.53 ± 0.04 ^{Aa}	0.43 ± 0.05 ^{Ab}	0.41 ± 0.00 ^{Bbc}	0.42 ± 0.03 ^{Abc}	0.34 ± 0.01 ^{Bc}
Cohesiveness (ratio)	UB	0.43 ± 0.06 ^{Aa}	0.43 ± 0.00 ^{ABa}	0.38 ± 0.01 ^{Aba}	0.27 ± 0.02 ^{Bb}	0.22 ± 0.02 ^{Bc}
	GC	0.42 ± 0.02 ^{Aab}	0.43 ± 0.01 ^{Aa}	0.41 ± 0.01 ^{Ab}	0.32 ± 0.01 ^{Ac}	0.28 ± 0.00 ^{Ad}
	WP	0.45 ± 0.02 ^{Aa}	0.42 ± 0.00 ^{Ba}	0.36 ± 0.02 ^{Bb}	0.30 ± 0.01 ^{Ac}	0.24 ± 0.02 ^{Bd}
Resilience (ratio)	UB	0.23 ± 0.03 ^{Aa}	0.19 ± 0.00 ^{Bb}	0.15 ± 0.01 ^{Bc}	0.13 ± 0.00 ^{Bcd}	0.12 ± 0.01 ^{Bd}
	GC	0.24 ± 0.01 ^{Aa}	0.22 ± 0.01 ^{Ab}	0.19 ± 0.02 ^{Ac}	0.15 ± 0.00 ^{Ad}	0.14 ± 0.01 ^{Ad}
	WP	0.23 ± 0.01 ^{Aa}	0.19 ± 0.01 ^{Bb}	0.16 ± 0.02 ^{ABc}	0.14 ± 0.01 ^{ABd}	0.13 ± 0.01 ^{ABd}

Values are presented as mean ± standard deviation ($n = 3$). Different uppercase superscripts within the same column under the same parameter tested denote significant differences ($p < 0.05$). Different lowercase superscripts within the same row denote significant differences ($p < 0.05$).

3.8. Principal Component Analysis and Pearson Correlation Analysis

Principal component analysis (PCA) was performed to visualize the relationships among bleeding treatments, storage time, microbiological indicators, spoilage indices, and protein oxidation parameters in Asian seabass during refrigerated storage (Figure 7). The first two dimensions of the PCA plot explained 98.8% of the total variance, with PCA1 and PCA2 accounting for 97.1% and 1.7%, respectively. Thus, most of the variation in the dataset was explained by PCA1, while PCA2 contributed relatively little to the overall variation. The sample scores showed a clear change along PCA1 with increasing storage time. Samples collected at the early stages of storage (Days 0 and 3) were mainly located on the negative side of PCA1, whereas samples from the later stages, particularly days 9 and 12, shifted toward the positive side. This pattern indicated that storage time was the main factor influencing the variation in the measured quality parameters.

The degree of this shift differed among the bleeding treatments. UB samples at days 9 and 12 were located further toward the positive side of PCA1, while GC samples were generally positioned closer to the center or negative side. WP samples showed an intermediate distribution between GC and UB. These patterns suggested that the bleeding treatments were associated with different changes in quality of slices during refrigerated storage. The loading vectors further illustrated the contribution of individual variables to the sample distribution. TVC, PBC, and TVB-N were oriented in a similar direction toward the positive side of PCA1, indicating a positive association between microbial growth and the accumulation of volatile nitrogenous compounds. TMA-N was also oriented toward the positive side of PCA1, suggesting that higher TMA-N values were associated with samples located in this region of the PCA plot. In contrast, sulfhydryl groups were oriented toward the negative sides of both PCA1 and PCA2, whereas carbonyl groups showed a strong contribution to PCA2 and a smaller contribution to PCA1. These patterns indicated that the changes in protein oxidation were also associated with the separation of samples in the

PCA plot. Samples from the later stages of storage, particularly UB at day 12, were located in the same general region as the loading vectors for TVC, PBC, TVB-N, and TMA-N. This distribution was consistent with the increase in microbial and spoilage-related indicators during refrigerated storage. In contrast, GC samples were generally located farther from the spoilage-related vectors and relatively closer to the sulfhydryl vector. This pattern suggested that GC samples had a different quality profile during storage, with a lower association with the microbial and spoilage variables appearing in the PCA. WP samples generally showed intermediate distribution.

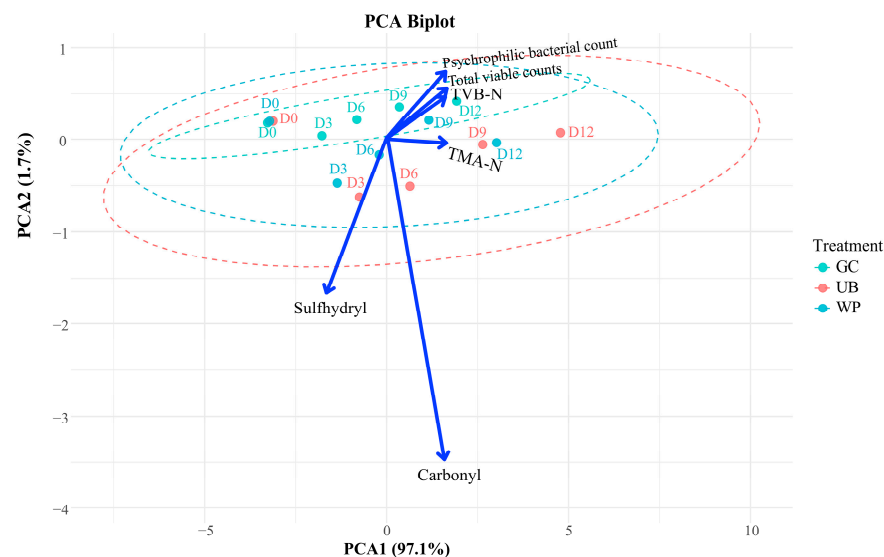


Figure 7. Principal component analysis (PCA) biplot illustrating the relationships among bleeding treatments, storage time, microbiological indicators (total viable count, TVC, and psychrophilic bacterial count, PBC), spoilage indices (trimethylamine nitrogen, TMA-N, and total volatile base nitrogen, TVB-N), and protein oxidation indicators (carbonyl and sulfhydryl groups) in unbled (UB), gill cut bled (GC), and water-perfusion bled (WP) Asian seabass during 12 days of refrigerated storage. Each point represents an individual sample, and the loading vectors indicate the direction and relative contribution of the measured variables to the principal components.

Overall, the PCA integrated microbiological, spoilage, and protein oxidation data and showed that refrigerated storage time was the main factor responsible for the separation of samples. The PCA also indicated that the bleeding treatments were associated with different patterns of quality change during storage. GC generally showed a smaller shift toward the spoilage-associated region than UB, while WP showed an intermediate pattern. However, PCA described relationships among variables and samples rather than causal effects. Therefore, these patterns should be considered together with the corresponding univariate results when interpreting the effects of bleeding treatments on quality deterioration.

To further evaluate structural and sensory property, texture profile analysis (TPA) and sensory testing were conducted (Figure 8). According to Figure 8a, GC samples showed persistently higher hardness, springiness, and resilience, suggesting superior firmness and elasticity across the storage period. GC samples also showed the lowest fishy odor intensity, which was related to the lower TMA-N content. TMA has been known to have a fishy odor [71]. UB had the most intense fishy odor, probably due to softer, corresponding to high TMA-N content. In addition, the TMA was produced by spoilage microorganisms, which were able to degrade proteins in the muscle, resulting in weaker firmness. WP samples consistently had tested parameters between UB and GC samples. Collectively, these results demonstrated a strong association between muscle structural integrity and odor retention, where firmer and more elastic tissues were related to lower fishy odor intensity.

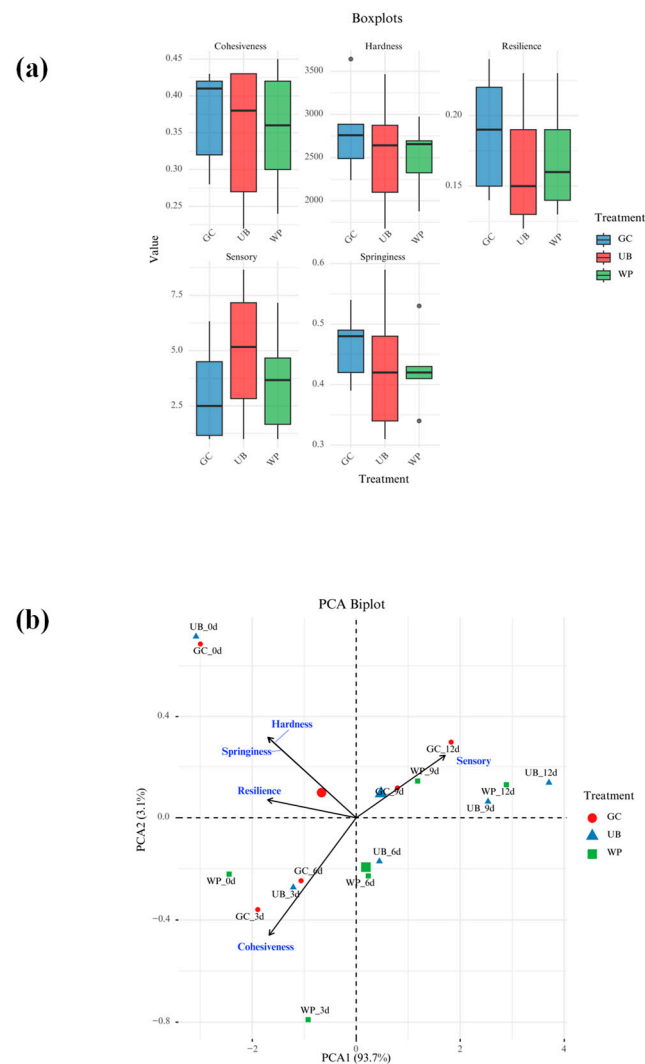


Figure 8. Boxplot distribution of textural parameters (hardness, springiness, cohesiveness, resilience) and sensory scores by treatment (a) and principal component analysis (PCA) biplot showing the relationships among these variables (b) in the slices from unbled (UB), gill cut bled (GC) and water perfusion bled (WP) Asian seabass during refrigerated storage of 12 days.

Figure 8b presents a PCA biplot integrating TPA parameters, sensory attributes, treatments, and storage time. PCA1 and PCA2 explained 96.9% of the total variance (PCA1: 93.7%, PCA2: 3.1%) and effectively distinguished the bleeding treatments in terms of texture and sensory responses. In the PCA plot, GC clustered in the positive PCA1 region, aligning with high springiness, hardness and superior sensory property, suggesting excellent maintenance of muscle integrity. UB showed intermediate shifts, while WP shifted toward negative PCA1, reflecting the reduced cohesiveness and structural weakening. Over the 12-day storage, samples shifted progressively along PCA1, which was in line with gradual textural degradation. Importantly, GC samples still maintained the superior textural and sensory quality to UB and WP counterparts. Overall, GC samples had lower TMA-N, TVB-N, and carbonyl levels, with coincidentally higher sulfhydryl content.

Basically, the combined evaluation of biochemical indicators, TPA results, Pearson correlation, sensory scores and PCA provided a comprehensive understanding of the impact of bleeding methods on Asian seabass storage stability. Gill cut bleeding not only effectively suppressed oxidative deterioration and nitrogenous spoilage but also maintained structural integrity and sensory quality, highlighting its potential as a preferred method for preserving fresh fish during refrigerated storage.

4. Conclusions

Both bleeding methods improved the quality of Asian seabass slices compared with unbled samples, as reflected by lower K-values, reduced protein oxidation, and lower TVB-N and TMA-N contents. GC slices consistently showed better texture preservation than WP and UB, indicating superior maintenance of muscle integrity and reduced development of fishy odor during refrigerated storage. Microbiological results supported these findings, in which TVC and PBC were lower in bled samples. PCA indicated that storage time and bleeding treatments mainly influenced microbial development, with unbled samples having higher microbial loads. Overall, GC bleeding provides superior preservation of freshness and overall quality of Asian seabass, in addition to its previously demonstrated benefits for enhancing lipid stability and histological integrity. From an industrial perspective, GC bleeding represents a practical intervention that can be integrated into pre-processing lines to improve the quality of Asian seabass during refrigerated storage. Scientifically, this study provides new insights into the role of bleeding methods in modulating biochemical changes and protein oxidation development, thereby advancing the understanding of post-harvest fish quality preservation. As a future perspective, combining an effective bleeding method with sustainable packaging or emerging non-thermal preservation technologies should be explored to further enhance the shelf-life, maintain the quality, and ensure the safety of bled fish products.

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Data Availability Statement: The original contributions presented in this study are included in the article; further inquiries can be directed to the corresponding author.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

GC	Gill cut
WP	Water perfusion
UB	Unbled
PCA	Principal component analysis
ATP	Adenosine triphosphate
TVB-N	Total volatile base nitrogen
TMA-N	Trimethylamine
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
DNPH	2,4-dinitrophenylhydrazine

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
IMP	Inosine 5'-monophosphate
HxR	Inosine
Hx	Hypoxanthine
TCA	Trichloroacetic acid
TPA	Texture profile analysis
TVC	Total viable count
PBC	Psychrophilic bacterial count
ANOVA	Analysis of variance
SH	Sulfhydryl
TMAO	Trimethyl ammine oxide
CFU	Colony forming unit

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