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The aquatic product processing and by-product utilization

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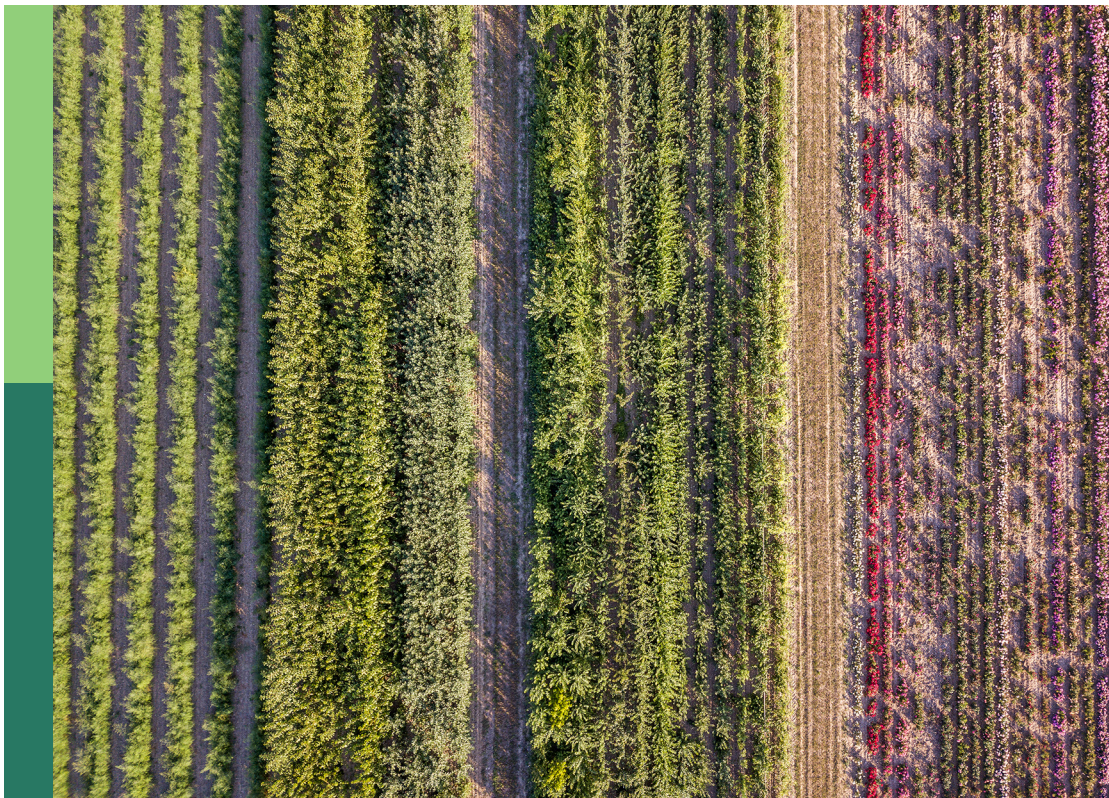

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The aquatic product processing and by-product utilization

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Editorial: The aquatic product processing and by-product utilization

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Editorial on the Research Topic
The aquatic product processing and by-product utilization

Aquatic products are extremely popular with consumers since they have a unique taste and rich content of essential nutrients required by the human body. However, they are highly susceptible to spoilage due to their high water content and fragile tissue. Consequently, various processing methods have been adopted to extend their freshness. The farmed rainbow trout (*Oncorhynchus mykiss*) was treated with high-voltage electrostatic fields which can reduce the growth rate of microorganisms to extend its shelf life (Cheng et al.(a)). With the growth of the global population and changes in consumption patterns, the demand for aquatic products continues to rise, and the types are becoming more diversified. For instance, rainbow trout (*Oncorhynchus mykiss*) filets were salted and smoked to extend their shelf life and enhance their commercial value (Cheng et al.(b)). Furthermore, surimi is a convenient and delicately flavored aquatic processed product that is easy to cook, and it is one of the highest volume aquatic processed products globally. The gel properties of surimi are crucial to the quality and texture of surimi products. Duan et al. presented CO_2 can alter the advanced structure of proteins to induce gelation, providing a theoretical basis for the development of aquatic products.

The aquatic product processing industry, while providing a rich variety of food products, also faces dual challenges of resource utilization efficiency and environmental impact. Traditional aquatic product processing methods often only utilize a portion of the edible components, with the remaining by-products often considered waste. This inefficient resource utilization mode not only leads to resource waste but also burdens the environment. By-products of aquatic product processing, such as fish bones, scales, and heads, which are often overlooked, are rich in proteins, lipids, minerals, and vitamins, and have great potential for development. Kendler, Sasidharan et al. reviewed the utilization of by-products generated from fileting side streams. These by-products can be transformed into high-value products, such as protein hydrolysates, fish oil, gelatin, chitosan, etc., widely used in food, pharmaceuticals, cosmetics, and biomaterials. Lian et al. obtained collagen peptide by enzymolysis of inedible fish to increase its economic value. Shibata et al. extracted fucoxanthin from Ishimozuku (*Sphaerotrichia firma*), which inhibited

fatty liver in obesity model mice and improved the production of short-chain fatty acids. Kotsoni et al. found that the protein content in the enzymatic hydrolysate can be increased after materials were pretreated by high-pressure. We can extract collagen and minerals from aquatic by-products, but the extraction method should be considered, as different extraction methods have different impacts. Green extraction methods should be considered to reduce energy consumption and chemical pollution. Kendler, Kobbenes, et al. found that microwave and ultrasound were green extraction procedures in the extraction of collagen from fish by-products. Yakti et al. reutilized leftover biomass from the marine environment as a feed of black soldier fly larvae, minimizing waste and benefiting various industries and society.

The comprehensive utilization of aquatic product processing and its by-products is an important way to achieve sustainable development of the aquatic industry. Faced with global resource and environmental challenges, we must strengthen technological innovation, optimize the industrial structure, expand international cooperation, promote industrial upgrading with scientific and technological innovation, and lead the future of the industry with green development.

Author contributions

SW: Conceptualization, Funding acquisition, Validation, Writing – original draft, Writing – review & editing. YC: Investigation, Supervision, Validation, Writing – review & editing. LS: Investigation, Supervision, Validation, Writing – review & editing. RD: Investigation, Validation, Writing – review & editing. HH: Investigation, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Creating added-value filet product from rainbow trout (*Oncorhynchus mykiss*) by salting and smoking method: physicochemical and textural attributes

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Rainbow trout (*Oncorhynchus mykiss*) are currently consumed as live fish, primarily for catering or consumers, as an alternative to salmon in sashimi or dishes. However, Covid-19 has hampered store and restaurant operations. Therefore, developing suitable processing conditions to extend its shelf life, such as online distribution specifications while enhancing the filets' commercial value, would raise its production value. In this study, we investigated the fish filets salted in a 5% salt solution for 2days and then smoked at 65°C for 4h under different storage conditions. As result, the higher rate of salt penetration and water loss in the resolved rigor mortis group was associated with tenderization of the meat compared to the rigor mortis group. Thermal-shrinkage and thermal-induced tissue destruction of the smoked fish filets during processing which affects the appearance, flavor, chewiness and overall acceptability. Nevertheless, according to the results of a consumer-type evaluation, the product characteristics of the fish filets from the resolution of rigor mortis group were consistent with those of the rigor mortis group, except for a weaker aroma. Thus, these results explain the relationship between frozen stored fish and the quality of processed products. The economic concept of regulating and distributing scheduling production between raw materials and finished products in the food industry conveys promising findings that will contribute to developing sustainable food processing systems.

KEYWORDS

rainbow trout, salted, smoked, circulation economy, sustainability

Highlights

- Flexibility in organizing stock and production schedules for bulk production.
- Maximizes the use of raw materials while reducing waste.
- Processing temperature and time must be strictly controlled.
- The smoked value of the fish remains even after storage at low temperatures.

1. Introduction

In 2020, international trade in world fisheries and aquaculture products yielded about $151 billion attributed to the COVID-19 explosion, below the record high of $165 billion in 2018 (FAO, 2022). Notably, aquaculture accounts for 91.6% of total production, and it is estimated that some 600 million people's livelihoods depend at least partly on fisheries and aquaculture (FAO, 2022). Fish is a significant contributor to macro- and micro-nutrient intake. Muscle foods, rich in valuable nutrients, such as high-quality protein, vitamins, and minerals, as well as n-3 polyunsaturated fatty acids (PUFAs), have long been appreciated by consumers (Sobral et al., 2018; Douny et al., 2021; Yemmen and Gargouri, 2022). It has been reported that about 60% of dietary protein requirements for adults are supplied by fish consumption (150 g) (Yemmen and Gargouri, 2022), and aquatic food provides about 17% of animal protein worldwide, reaching more than 50% in several countries in Asia and Africa (FAO, 2022).

Moreover, 80% of the total quality of fish meat is composed of water, which plays a crucial role in the texture and quality of the associated products (Sun et al., 2020). Consequently, it is essential to ensure the quality and safety of fish during processing while retaining the maximum quality of the original attributes, such as texture, flavor, and taste (Fomena Temgoua et al., 2022). Fisheries products (live, fresh, or frozen) account for 45% of global market consumption, while preserved products (dried, salted, brined, fermented, or smoked) account for 12% (Ekonomou et al., 2020; Gomes et al., 2021; Aksun Tümerkan, 2022). A significant increase in the production of rainbow trout (*Oncorhynchus mykiss*) has been realized, as this species has been widely cultured worldwide (Vásquez et al., 2022). The restaurant market favors sashimi as a rich source of PUFAs and the soft, delicate flavor (Zhao et al., 2022), which has the double benefit and provides sensory characteristics comparable to salmon at an affordable price for consumers with slightly more profitability for operators.

Smoking has been frequently used to store fish and meat. Smoking is an ancient food processing method that extends the shelf life of food by reducing moisture content and the antioxidant effects of phenols to minimize the microbial load (Iko Afé et al., 2021). It also improves sensory characteristics, including flavor, aroma, and appearance (Aksun Tümerkan, 2022; Praveen Kumar et al., 2022). The convenience of smoked fish products sold as ready-to-eat food has become popular (Bolívar et al., 2021). Nevertheless, these products have been recognized as high-risk in terms of potential contamination by foodborne pathogens, particularly *Listeria monocytogenes* (Iacumin et al., 2021), as the food requires no cooking before consumption. Thus, the initial microbial levels in food should be strictly controlled.

Smoking processes can be differentiated by temperature, and holding the temperature of the product at 30°C is called cold smoking. In comparison, hot smoking temperatures can reach 85°C at the center of the product to cook the food (Stołyhwo and Sikorski, 2005), but about 90% of the proteins are denatured at 60°C-65°C (Ünlüsayın et al., 2001). In addition, the smoke drying process involves hot smoking followed by a drying step in the equipment. Taken together, the food processing theory is that the smoke-drying process produces dried products with a water activity (AW) value≤0.75, thus, preserving the final product at room temperature and inhibiting microbial development (Iko Afé et al., 2021). Food products are smoked, dried, and heated *via* smoke obtained from the incomplete combustion of wood. Smoking causes more than 200 chemical compounds in the smoke to solidify, deposit on the product's surface, and infiltrate the inner layers. In particular, phenolic compounds contribute to the distinctive aroma and flavor of smoked products, while carbonyl groups yield sweet aromas and colors *via* the Maillard reaction (Bienkiewicz et al., 2022). The microbial growth and oxidation processes are delayed by the synergistic effects of salt, the smoke compounds, and dehydration, whether carried out using traditional or innovative smoking techniques, which facilitates preserving the smoked products (Ekonomou et al., 2020; Cunha et al., 2021; Gomes et al., 2021; Bienkiewicz et al., 2022). However, cooking and smoking (cold or hot) can reduce the risk of food hazards in raw fish; they may contribute to the formation of degradation products of unknown toxicity from existing pesticides or antibiotic residues (Mengden et al., 2015; Sobral et al., 2018). Therefore, processing time and temperature must be controlled during industrial production and home cooking. Hence, this study investigated the correlation between the degree of salinity, temperature, and smoking duration, which may help extend the shelf life of rainbow trout filets and increase the product's added value due to the smoke's unique flavor.

2. Materials and methods

2.1. Materials

The cultured rainbow trout (average weight 650 g) were purchased from a local market (Taichung, Taiwan). After removing the scales and viscera (average weight 500 g), the fish were rinsed in tap water, packed in polystyrene boxes covered with ice, and carried back to the laboratory within 30 min, where they were processed under different conditions for further analysis. All analytical grade reagents were purchased from Sigma-Aldrich® (Merck KGaA, Darmstadt, Germany).

2.2. Preparation for the smoking process

The smoking fish samples from each temperature (15 individuals) were transferred under a cold chain to the laboratory within 30 min. This study divided the rainbow trout filets (skinless) into groups according to the method described by Concollato et al. (2016), as detailed below. (1) Rigor mortis period (stored at 25°C for 2.5 h, 4°C and 0°C for 12 h). (2) Resolution of rigor mortis group (stored at 4°C and 0°C for 72 h), followed by the fish filets cut.

2.2.1. Evaluation of the smoked fresh rainbow trout processing conditions

The method followed that of Aksun Tümerkan (2022) with modifications. The rainbow trout filets stored under different conditions in section 2.2 were salted (5–20% NaCl, 5 days) and drained. The cured fish were washed with RO water, dried with tissue paper, and smoked (temperature 50°C- 80°C, 1–4 h) in a smokehouse ASR1297EL/WA (M&M Equipment Co., Skokie, IL, United States) until the filets reached an internal temperature of 72°C. Then, each group was packed separately under a vacuum and stored at 4°C to observe the filet quality changes. A sensory evaluation determined the optimal processing conditions for the final product.

2.2.2. Evaluation of the quality obtained with optimal salting and smoking

The rainbow trout filets stored for different times were salted (5% NaCl, 2 days) and smoked (temperature 65°C, 4 h) to evaluate the quality of the final product.

2.3. Determination of total viable count and psychrophilic bacteria

Microorganisms were detected based on the description of Lu et al. (2022) with modifications. Fish meat (10 g) was homogenized with 90 ml of sterile saline in the Stomacher® 400 Circulator Lab Blender (Seward Ltd., Worthington, United Kingdom) at low and high speeds for 2 min. Then 1 ml of the homogenate was added to 9 ml of sterile saline, followed by serial dilution, and 1 ml of the dilution was dispensed into culture dishes. The psychrophilic plate count agar (15 ml) was poured and then incubated at 37°C and 7°C for 2 and 10 days, respectively, and expressed as log CFU/g fish meat.

2.4. Determination of moisture content

Moisture was determined based on the method described in AOAC (2022) 930.15. The fish filets (3–5 g) were weighed in a flask (with constant weight) and dried in an oven at 105°C to constant weight. Moisture content was calculated using the following formula, expressed as moisture content (%).

$$Moisture\ content\ (\%) = {}^{W1}\!/_{W} \times 100$$

where W is the weight of the sample before baking (g) and W1 is the weight of the sample after drying (g).

2.5. Determination of salt content

A 5-g of salt-preserved sample was homogenized in 45 ml of distilled water, centrifuged at 1,000 × g for 5 min, and measured with a salinity meter (Atago Co., Ltd. Tokyo, Japan).

2.6. Determination of color

The color of the samples was measured as described by Huang et al. (2022). The samples' *L*, *a*, and *b* values were measured with a colorimeter (Nippon denshoku Co., Tokyo, Japan). The equipment was calibrated with a standard whiteboard (Y = 93.97, X = 91.96, and Z = 110.41) and measured by reflection, with three random measurements for each sample. The *L* value reflects the degree of lightness and darkness. A positive *a* value indicates a reddish color, while a negative value tends to be green. A positive *b* value indicates a yellowish color, while negative values tend to be blue.

2.7. Textural analysis

The textural analysis of the samples was performed using the methods described by Li et al. (2022) and Praveen Kumar et al. (2022), with some modifications. The samples were cut into about 1.5 cm^3 cubes, which were measured for hardness, elasticity, cohesion, and chewiness using the TA-XT2 Texture Analyzer (Stable Micro Systems Ltd., Godalming, U.K.) performed at 25°C room temperature. A P3 probe determined the conditions with 90% penetration of the measured sample at a 2 mm/s speed.

2.8. Sensory evaluation

The sensory evaluation was performed according to the method of Linhartová et al. (2019) and Lu et al. (2022), with modifications. In brief, the smoked fish filets were heated to a central temperature of 95°C in a microwave oven and then cooled for the hedonic scale test. A 7-point scale was used, including very much like = 7, neither like nor dislike = 4, and dislike = 1, for appearance, aroma, flavor, chewiness, and overall acceptability. The evaluation panel consisted of 60 experienced assessors, and the temperature was controlled at 25 ± 2°C. The panelists rinsed their mouths with drinking water after tasting each sample before proceeding to the next one.

2.9. Statistical analysis

All analyzes were performed in triplicate. The data were analyzed using SAS software (Version 9.0, SAS Institute, Cary, NC, United States) and the most minor square means (LS means). All data were statistically analyzed using one-way ANOVA, while as expressed as mean ± standard deviation. Duncan's multiple-range test was used to compare the differences between the means. A value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Evaluation of rainbow trout smoking conditions

The smoking process was divided into fleshing, cutting, salting, seasoning, smoking, and storage. The salting process is important to enhance storage, flavor, and moisture for drying (Estévez et al., 2021). The functional role of salt in the texture of fish products is to solubilize the fish proteins, initiate protein extraction, and improve hydration and water-holding capacity. Additionally, the protein structural changes facilitate interaction with other components (e.g., water and lipids), which helps hold the product together while preventing water and fat loss (Gomes et al., 2021). Variations in the salt content of rainbow trout meat for the different salt formulations are shown in Table 1 and Figure 1A. In this study, the concentration of the salting solution was 5–10%, and the salt penetration rate slowed to 2.9 and 4.8%, respectively, within 2 days of dipping. It has been reported that filets dipped in 10% salt water for 4 h heighten the perception of the salty and smoky flavors, thus minimizing the fish taste (Ruiz-Alonso

et al., 2021). However, the salt concentration was higher than 15%, thus showing an increasing trend in salt content with increased salting time, and did not reach the equilibrium concentration within 5 days. However, as the salinity of commercially available smoked products ranges from 2 to 6%, fish filets with a salt content >3% will be salty, and the salinity of fish filets that are salt dipped increases because of smoke drying. Therefore, this study selected 2 days of salt dipping in a 5% salt solution as the condition for salting the fish filets.

Moisture content changed during the salting of the rainbow trout filets in various concentrations of salt solution. The moisture content of the fresh fish filets (0 days) was 76.6%, and the moisture of the fish meat continuously trended downward with time at each salting concentration (Figure 1B). However, the moisture content of the fish filets decreased to about 75% within 2 days of dipping in the 5% salt solution. There was a greater tendency for moisture to decrease, as osmotic pressure increased with the higher salt content of the dipping solution (Delbarre-Ladrat et al., 2006). In addition, it has been reported that *Listeria monocytogenes* causes 11–60% of disease in cold-smoked fish and 4–12% of disease in heat-treated and cured seafood (Kołodziejska et al., 2002). Another investigation showed that the probability of *L. monocytogenes* in smoked fish is about 2.7% at the industrial level and 25% at the retail level (Mengden et al., 2015).

TABLE 1 Compositions of the brine pickling solution (%).

Additives (%)				
NaCl	5.00	10.00	15.00	20.00
$NaNO_2$	0.16			
Water	94.57	89.57	84.57	79.57
Vitamin C	0.26			

The total viable count (TVC) (Figure 1C) of psychrophilic bacteria (Figure 1D) in the rainbow trout filets after dipping in the different salt concentrations showed that the TVC was lower, and the high salt content of the dipping solution had a pronounced inhibitory effect on bacteria beginning on the second day of storage. A significant difference was observed between the groups ($p < 0.05$; Figure 1C), indicating that the osmotic pressure caused by the salt solution significantly discouraged the propagation of microorganisms. At the same time, it was attributed to the ability of salt to reduce the AW of food and affect enzyme activity, which has been reported in the case of seafood products, where increasing salt concentration reduces bacterial growth (Gram and Huss, 1996; Mariutti and Bragagnolo, 2017; Abel et al., 2020; Giannakourou et al., 2023). Moreover, a significant case in point for the usefulness and necessity of salt is the inhibition of *Clostridium botulinum* development and toxin production in processed meats and cheeses (Taormina, 2010). The growth of psychrophilic bacteria was weakly inhibited by the dipping solutions with less than 10% salt content, but the inhibitory effect decreased on the second day of dipping, and the growth of the

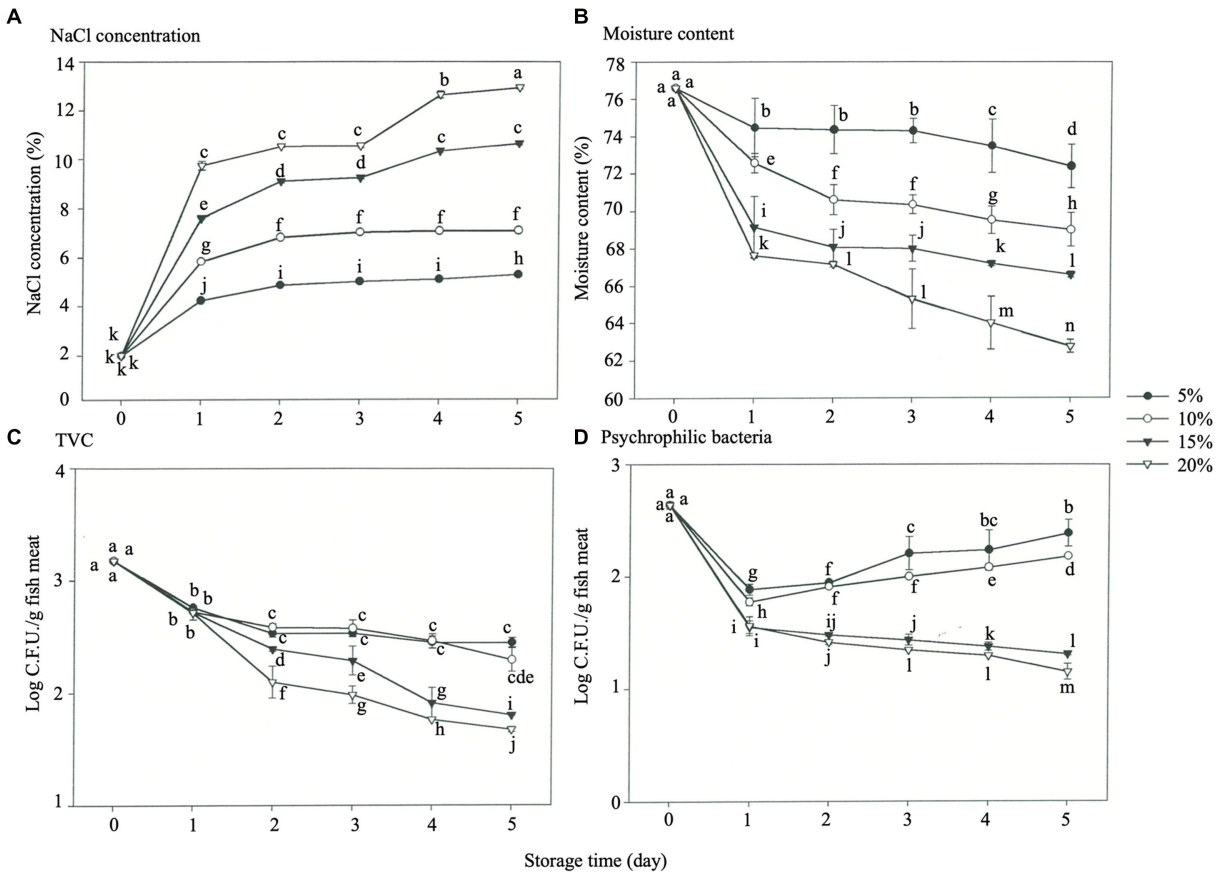

FIGURE 1
The evaluation of conditions suitable for smoking rainbow trout. **(A)** NaCl concentration; **(B)** Moisture content; **(C)** TVC; **(D)** Psychrophilic bacteria. Different lowercase letters indicate a significant difference ($p<0.05$).

psychrophilic bacteria increased with dipping time. However, the growth of psychrophilic bacteria was significantly inhibited in 15–20% salt content ($p < 0.05$; Figure 1D). However, it is worth mentioning that it indicates that seafood spoilage may be caused by lactic acid bacteria, *psychrotrophic Enterobacteriaceae,* and *Photobacterium phosphoreum* (Gram and Huss, 1996). Hence, NaCl will not ensure microbiological food safety (Kim et al., 2017).

The variations in the moisture content of rainbow trout during smoking showed that the moisture content decreased significantly at higher smoking temperatures ($p < 0.05$). However, the effect might also be related to the ability of NaCl, which increased the binding of water by causing swelling of proteins in the fish (Böcker et al., 2008; Abel et al., 2020). In addition, the moisture loss trend was consistent for 2 h at 65°C and 80°C (Figure 2A). We speculated that the high smoking temperatures probably caused the formation of a hard shell on the surface of the fish meat, which reduced drying efficiency during the subsequent stage.

Fresh fish products' apparent color and odor represent the two most important quality parameters for consumers (Linhartová et al., 2019). The chromatic changes in the *L*, *a*, and *b* values of rainbow trout during smoking indicated that the *L* value increased with increased smoking temperature and time, which was attributable to the denaturation of fish proteins at high temperatures. A similar trend was observed in the *a* and *b* values (Figures 2B–D). The variations in the *L* -value were attributed to moisture loss and protein degradation, but the product's surface underwent color changes after salting and smoking (Ruiz-Alonso et al., 2021). Furthermore, the formaldehyde, phenol, and cyclic hydrocarbon compounds in the smoke reacted with the Maillard reaction to the proteins on the fish surface, which could be the primary contributor to the color change (Bienkiewicz et al., 2022).

In contrast, given the delicate texture of rainbow trout meat, our goal was to maintain tenderness through smoking. Consequently, the salted rainbow trout filets were smoked at 50°C, 65°C, and 80°C for 1–4 h, respectively, followed by drying without post-heating, whereas the completed fresh rainbow trout filets were directly cooked in a microwave oven (900 W). The smoked rainbow trout filets were heated to a central temperature of 95°C and cooled to room temperature, followed by a consumer sensory evaluation. The sensory evaluation of the rainbow trout under different smoking conditions was conducted to assess the appearance, aroma, flavor, chewiness, and overall acceptability of the smoked filets (Table 2). The results revealed no significant difference in the chewiness or overall acceptability of the products smoked under different conditions. However, 4 h of smoking at 65°C provided the best appearance and flavor, and these parameters were used for smoking under different storage conditions.

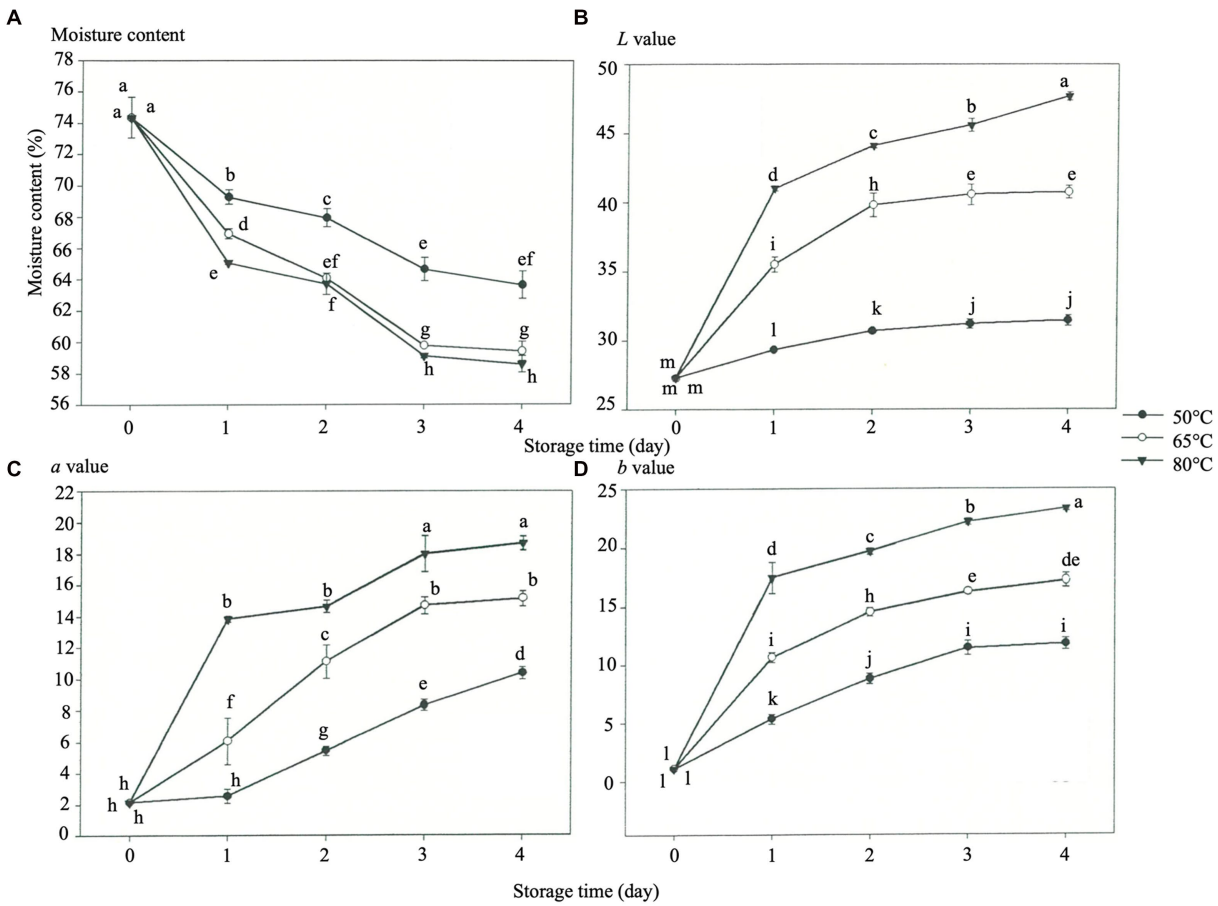

FIGURE 2
Effects of different smoking temperatures on the moisture content and color of smoked rainbow trout. **(A)** Mosture content; **(B)** *L* value; **(C)** *a* value; **(D)** *b* value. Different lowercase letters indicate a significant difference ($p<0.05$).

TABLE 2 Sensory evaluation of rainbow trout prepared with different smoking treatments.

Temperature (°C)	Time (h)	Appearance	Aroma	Flavor	Chewiness	Overall acceptability
50	1	4.7 ± 1.4c*	5.1 ± 1.1ab	5.2 ± 1.1ab	5.3 ± 1.0a	5.3 ± 0/9a
	2	4.9 ± 1.4bc	5.0 ± 1.1ab	5.2 ± 1.3ab	5.3 ± 1.1a	5.2 ± 1.1a
	3	5.2 ± 1.0abc	5.3 ± 1.1ab	5.2 ± 1.2ab	5.2 ± 1.3a	5.2 ± 1.1a
	4	5.4 ± 1.0ab	5.4 ± 1.1a	5.2 ± 1.2ab	5.4 ± 1.1a	5.4 ± 1.0a
65	1	5.2 ± 1.1abc	4.9 ± 1.2ab	4.9 ± 1.4ab	5.0 ± 1.4a	4.9 ± 1.5a
	2	5.2 ± 1.1abc	5.1 ± 1.0ab	5.3 ± 1.5ab	5.5 ± 1.0a	5.4 ± 0.9a
	3	5.1 ± 1.3abc	5.3 ± 1.3ab	5.7 ± 1.4ab	5.3 ± 1.4a	5.1 ± 1.3a
	4	5.5 ± 1.0a	5.0 ± 1.4ab	5.7 ± 0.9a	5.2 ± 1.6a	5.0 ± 1.5a
80	1	5.2 ± 1.0abc	4.7 ± 1.3b	5.0 ± 1.5ab	5.2 ± 1.4a	5.2 ± 1.3a
	2	5.1 ± 1.1abc	4.9 ± 1.3ab	5.1 ± 1.2ab	5.2 ± 1.4a	5.1 ± 1.1a
	3	5.0 ± 1.1abc	5.0 ± 2.0ab	5.0 ± 1.2ab	5.2 ± 1.1a	5.3 ± 1.1a
	4	4.9 ± 1.1abc	4.9 ± 1.1ab	4.9 ± 1.3b	5.0 ± 1.2a	5.0 ± 1.2a

*The lowercase letters mean in the same row with different subscripts are significantly different ($p < 0.05$).

3.2. Quality of rainbow trout products smoked under different conditions

The best way to assess the quality of a product is a sensory evaluation (Linhartová et al., 2019). Therefore, the sensory preference and overall acceptance of consumers regarding storage, aging, salting, and smoking conditions, with a positive effect on enhancing the quality of smoked products, is significant. In this study, the rainbow trout filets were cut during rigor mortis (2.5 h at 25°C, 12 h at 4°C, and 0°C), and the resolution of rigor mortis group (72 h at 4°C and 0°C), followed by salting and smoking to investigate the effects of the muscular state of the rainbow trout filets on the quality of the smoked product. In this study, variations in the salt content of rainbow trout were found in the resolution of rigor mortis (2.5 h at 25°C and 12 h at 4°C and 0°C) compared to the rigor mortis groups (72 h at 4°C and 0°C; Figure 3A). However, the salt content of the fresh fish was the lowest on the first day of dipping, whereas the salt concentration on the second day was approximately the same as that of the rigor mortis group, which may have been caused by the intact muscle structure of the fresh fish, as fish meat with intact cell membranes maintains a specific permeation pressure in response to different internal and external salt concentrations (Oliveira et al., 2017). Furthermore, endogenous enzymes damaged the fish tissue in the resolution of rigor mortis group, resulting in incomplete cell membranes and higher salt concentrations during the impregnation process.

Changes in the moisture content of rainbow trout under the different storage conditions in a 5% salt solution (Figure 3B) were detected for the rigor mortis and the resolution of rigor mortis groups. An increase in moisture content was associated with increased dipping time. However, the moisture content of the fish filets in the resolution of rigor mortis group was significantly higher than that of the rigor mortis group, which was attributed to the weakness of the muscle tissue after rigor mortis, which allowed the brine to enter gaps in the muscle tissue. As salt-soluble proteins dissolved, the degree of protein hydration increased, thus, increasing the moisture content of the fish. In contrast, fresh fish are continuously dehydrated, caused by intact muscle cells and the effect of osmotic pressure. Again, it was verified that NaCl serves preservation purposes rather than seasoning, which was attributed to the reduction of AW, and inhibited microbial growth, resulting in the extended shelf life (Abel et al., 2020; Ekonomou et al., 2020; Gomes et al., 2021; Giannakourou et al., 2023).

A food process must enhance the product's safety, reduce the formation of hazardous substances (chemical or microbial), and retain nutrients (Sobral et al., 2018; Praveen Kumar et al., 2022). Several studies have reported that lactic acid and yeast are most abundant in vacuum-packed hot-smoked fish stored at low temperatures, but appropriate storage maintains the organoleptic properties and inhibits microbial growth in products (Jemmi and Keusch, 1992; Mengden et al., 2015; Ekonomou et al., 2020). The most common species of psychrophilic bacteria are *Yersinia* pestis, *L. monocytogenes*, and *Pseudomonas* spp. The TVC (Figure 3C) and psychrophilic bacteria (Figure 3D) in rainbow trout under different storage conditions and a 5% salt dipping solution decreased during dipping in the rigor mortis and the resolution of rigor mortis groups. However, the TVC in the resolution of rigor mortis group was higher than that in the rigor mortis group, which was attributed to the higher bacterial count in the fish before dipping.

The psychrophilic bacteria increased during the dipping period in all groups. In particular, the resolution of rigor mortis groups (4°C and 72 h and 0°C and 72 h) showed a rapid rise in the number of psychrophilic bacteria on days 2 and 4 of dipping. It is well known that the combination of smoking, refrigeration, and reducing AW and antibacterial and antioxidant properties prolongs the shelf life of smoked fish, as similar results were observed in this study. Fortunately, regardless of the storage conditions, the microbes in the 5% salt-dipped rainbow trout for 2 days complied with the food hygiene standards. Although the levels of microorganisms are low after smoking and refrigeration, it is important to prevent contamination. Notably, *L. monocytogenes* can contaminate food during storage at refrigerated temperatures (Mengden et al., 2015). However, smoked fish can also become contaminated with microbes due to improper storage conditions and manufacturing practices before or after smoking (Douny et al., 2021). Thus, consumers may be at increased risk when storing smoked fish products for extended periods.

The changes in moisture content of the rainbow trout filets during smoking showed that the moisture content in the resolution of rigor

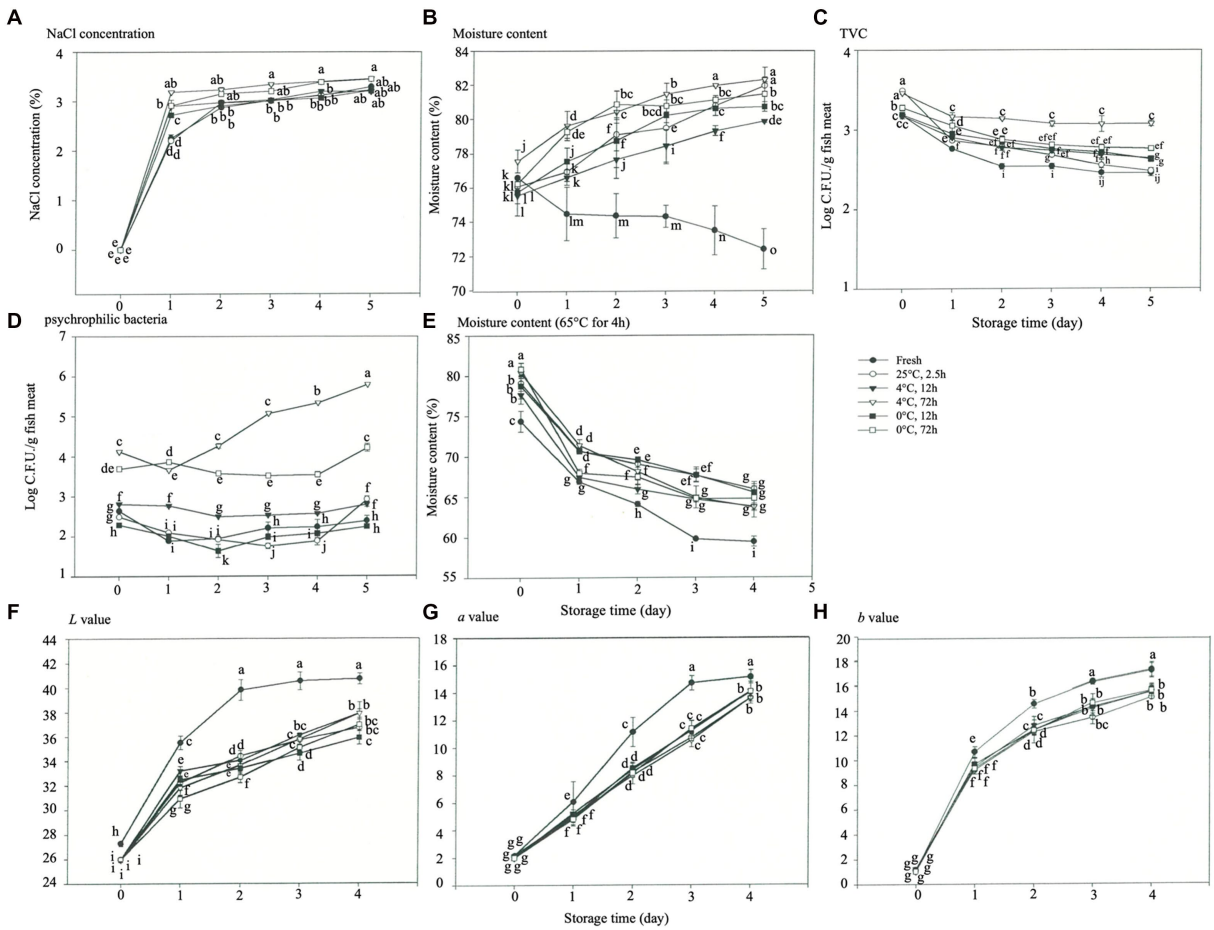

FIGURE 3
The physicochemical properties of smoked rainbow trout under different storage conditions. **(A)** NaCl concentration; **(B)** Moistue content; **(C)** TVC; **(D)** Psychrophilic bacterias; **(E)** Moisture content; **(F)** *L* value; **(G)** *a* value; **(H)** *b* value. Different lowercase letters indicate a significant difference ($p<0.05$).

mortis groups decreased faster than in the rigor mortis groups (Figure 3E). We hypothesized that the fish tissue in the rigor mortis group weakened due to forming more muscle fiber interstices, thereby releasing more moisture from the muscle. In contrast, moisture content decreased significantly in the fresh fish (control group) due to the lower moisture content post-salting. The *L*, *a*, and *b* values increased during the smoking of the rainbow trout filets under different storage conditions (Figures 3F–H). The muscle filet conditions have no significant effect on the chromatic changes during the smoking process.

The textural analysis included variations in the hardness, elasticity, cohesiveness, and chewiness of the rainbow trout filets during smoking (Table 3). As a result, hardness increased continuously in all groups during the smoking process, which was attributable to the constant denaturation of muscle protein. Contraction of beef during cooking is caused by the thermal denaturation ascribed to myofibrillar and connective tissue proteins (Vaskoska et al., 2021). The myofibrillar fraction or the structural proteins accounted for 64% of the total protein in rainbow trout white muscle, while soluble myoplasmic proteins accounted for 30% of the total protein (Delbarre-Ladrat et al., 2006). Moreover, the elasticity, cohesiveness, and chewiness of the fresh and rigor mortis groups increased during smoking, while these characteristics appeared to increase and then decrease in the resolution of rigor mortis groups. According to these results, the weakening of muscle tissues was more evident in the resolution of rigor mortis group than in the rigor mortis groups. Remarkably, the hardness of the filets increased by smoking and heating. It was hypothesized to be associated with sustained thermal exposure, which damages the muscle tissue, thereby altering the texture of the filets. Praveen Kumar et al. (2022) reported that fish meat texture softens during storage, which is caused by protein- degradation or hydrolysis, resulting in slightly reduced chewiness and gumminess.

Furthermore, protein is a key component in food that interacts with various functional components to form the sensory properties of the food (Tahergorabi et al., 2011). The sensory evaluation results revealed no differences in appearance, flavor, chewiness, or overall acceptability scores for the rainbow trout filets (Table 4) smoked at 65°C for 4 h and cooked in microwave heat. However, the rigor mortis group had higher aroma scores than the resolution of rigor mortis groups, except for the 25°C/2.5 h condition. In theory, there should be a significant difference in the taste of the smoked product resulting from rigor mortis, resolution of rigor mortis, and fresh material. However, the results showed that the resolution of rigor mortis groups had the same properties, except fewer aromas after smoking in the resolution of rigor mortis groups, indicating that rainbow trout stored at 4°C and 0°C for 3 days can be processed for smoking.

TABLE 3 Changes in hardness, elasticity, cohesiveness, and chewiness of rainbow trout stored under different conditions.

	Conditions	0	1	2	3	4
Hardness	Fresh	156.30 ± 10.30c	170.20 ± 10.00bc	184.00 ± 12.80b	225.70 ± 5.10a	242.70 ± 11.20a
	25°C, 2.5 h*	108.80 ± 4.70c	116.80 ± 6.80c	128.50 ± 5.50b	138.00 ± 5.60b	155.00 ± 13.20a
	4°C, 12 h	109.30 ± 13.20b	118.00 ± 8.50b	123.80 ± 6.80b	140.30 ± 7.80a	154.00 ± 7.90a
	4°C, 72 h	51.30 ± 11.90c	66.70 ± 4.20b	70.00 ± 2.70b	77.30 ± 2.50b	89.00 ± 3.60a
	0°C, 12 h	108.80 ± 12.50c	116.00 ± 4.80c	122.30 ± 8.70bc	131.50 ± 7.40b	159.70 ± 6.10a
	0°C, 72 h	52.70 ± 3.20c	63.70 ± 1.20b	65.00 ± 3.60b	78.30 ± 5.50a	82.30 ± 6.70a
Elasticity	Fresh	0.95 ± 0.00d	0.96 ± 0.00c	0.97 ± 0.01b	0.98 ± 0.00a	0.98 ± 0.00a
	25°C, 2.5 h	0.92 ± 0.01b	0.94 ± 0.01a	0.95 ± 0.02a	0.95 ± 0.01a	0.94 ± 0.01a
	4°C, 12 h	0.92 ± 0.01c	0.93 ± 0.00b	0.95 ± 0.01ab	0.96 ± 0.00a	0.96 ± 0.00a
	4°C, 72 h	0.91 ± 0.07bc	0.93 ± 0.01a	0.92 ± 0.00b	0.91 ± 0.00c	0.88 ± 0.01d
	0°C, 12 h	0.92 ± 0.01bc	0.93 ± 0.01bc	0.95 ± 0.01a	0.95 ± 0.01a	0.94 ± 0.01ab
	0°C, 72 h	0.92 ± 0.00b	0.93 ± 0.01a	0.92 ± 0.00b	0.91 ± 0.01c	0.88 ± 0.01d
Cohesiveness	Fresh	0.36 ± 0.00d	0.37 ± 0.01 cd	0.38 ± 0.00bc	0.40 ± 0.01a	0.40 ± 0.01a
	25°C, 2.5 h	0.35 ± 0.01b	0.37 ± 0.01a	0.38 ± 0.01a	0.38 ± 0.01a	0.37 ± 0.01a
	4°C, 12 h	0.35 ± 0.01b	0.36 ± 0.01b	0.38 ± 0.01a	0.38 ± 0.01a	0.37 ± 0.01a
	4°C, 72 h	0.32 ± 0.01b	0.34 ± 0.00a	0.34 ± 0.01a	0.32 ± 0.00b	0.31 ± 0.01b
	0°C, 12 h	0.34 ± 0.01c	0.35 ± 0.01bc	0.36 ± 0.01b	0.38 ± 0.01a	0.36 ± 0.01b
	0°C, 72 h	0.34 ± 0.02abc	0.34 ± 0.01ab	0.30 ± 0.00a	0.33 ± 0.01bc	0.32 ± 0.01c
Chewiness	Fresh	52.87 ± 4.09c	58.74 ± 3.74c	68.72 ± 5.47b	86.06 ± 4.00a	91.22 ± 3.62a
	25°C, 2.5 h	33.99 ± 1.41d	41.05 ± 4.04c	45.94 ± 2.56bc	49.81 ± 2.82ab	52.73 ± 4.56a
	4°C, 12 h	35.18 ± 3.45c	39.73 ± 3.54c	45.20 ± 2.86b	50.11 ± 2.85ab	54.17 ± 2.09a
	4°C, 72 h	15.30 ± 3.86c	20.23 ± 0.70b	22.06 ± 0.43a	22.52 ± 0.54a	21.30 ± 1.20ab
	0°C, 12 h	32.43 ± 1.97d	39.68 ± 1.40c	41.55 ± 1.98c	45.73 ± 2.03b	52.94 ± 2.74a
	0°C, 72 h	16.28 ± 0.76c	20.05 ± 0.99b	23.48 ± 1.73a	21.90 ± 1.49ab	20.81 ± 1.25ab

*The lowercase letters mean in the same row with different subscripts are significantly different ($p < 0.05$).

TABLE 4 Sensory evaluation of smoked fish meat of rainbow trout in different storage conditions.

Conditions	Appearance	Among	Flavor	Chewiness	Overall acceptability
25°C, 2.5 h	4.9 ± 1.1a*	4.9 ± 1.1a	5.0 ± 1.4a	5.1 ± 1.2a	5.1 ± 1.1a
4°C, 12 h	5.0 ± 1.1a	5.0 ± 1.0a	5.1 ± 1.2a	5.3 ± 1.2a	5.2 ± 1.1a
4°C, 72 h	5.1 ± 1.1a	4.8 ± 1.0ab	5.1 ± 1.2a	5.0 ± 1.3a	5.0 ± 1.1a
0°C, 12 h	4.9 ± 1.1a	5.0 ± 1.0a	4.9 ± 1.4a	5.0 ± 1.1a	5.1 ± 1.2a
0°C, 72 h	4.9 ± 1.2a	4.7 ± 1.1ab	4.7 ± 1.4a	5.0 ± 1.3a	4.8 ± 1.3a

*The lowercase letters mean in the same row with different subscripts are significantly different ($p < 0.05$).

4. Conclusion

This study found that the characteristics of rainbow trout filets with rigor mortis or resolution of rigor mortis were processed by salting and smoking at different storage temperatures were similar, except for less aroma in the resolution of rigor mortis groups. Therefore, rainbow trout stored at 4°C and 0°C for 3 days could be smoked, resulting in a good quality smoked product and a potential home application for the general public. It will be necessary to develop a process to extend the shelf life of rainbow trout to minimize the physical, chemical, and biological reactions that cause the deterioration of freshly harvested seafood. In cases of a lack of availability of fresh fish, a refrigerated stock (4°C and 0°C for 3 days) could be considered for production by smoking to relieve the inventory pressure. A risk assessment should be conducted on heat-induced toxic compounds in food (nitrosamines and polycyclic aromatic hydrocarbons) in the future.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

P-HH and P-HL: conceptualization. P-MW and P-HL: formal analysis. Y-TC and W-CK: funding acquisition and resources. Y-TC, S-CC, P-MW, and W-CK: investigation. P-HH, W-CL, S-CC, and P-HL: methodology. Y-TC: project administration. Y-TC, S-CC, P-MW, and P-HL: software. S-CC: supervision. Y-TC, W-CL, P-MW, and P-HL: validation. P-HH and W-CL: visualization. P-HH, W-CL, and P-HL: writing–original draft and writing–review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Changes in advanced protein structure during dense phase carbon dioxide induced gel formation in golden pompano surimi correlate with gel strength

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Changes in protein structure are closely related to gel strength. Dense phase carbon dioxide (DPCD) treatment is an excellent non-thermal food processing method that can be used to induce gel formation in surimi. The sensory, water holding capacity and gel strength of DPCD induced gels are superior to heat-induced gels. Fourier-transform infrared spectroscopy was used to investigate the role of DPCD in the quality of golden pompano surimi gels and changes in protein structure. The intermolecular forces of surimi gels were analyzed in terms of ionic and hydrogen bonds, disulfide covalent and non-disulfide covalent bonds, as well as hydrophobic interactions. Correlation analysis was used to investigate the relationship between the changes in advanced protein structure and gel strength during DPCD-induced gel formation in golden pompano surimi. The results showed that the α-helix and random coil levels of surimi gel were significantly decreased ($p<0.05$), while the β-sheet and β-turn content was significantly increased ($p<0.05$). The number of ionic and hydrogen bonds in gel proteins decreased significantly ($p<0.05$), while the hydrophobic interactions, and disulfide and non-disulfide covalent bonds increased significantly ($p<0.05$) after DPCD treatment. Correlation analysis showed that β-sheets, β-turns, hydrophobic interactions, and disulfide and non-disulfide covalent bonds were strongly positively correlated with gel strength, whereas α-helices, random coils, and ionic and hydrogen bonds were strongly negatively correlated with gel strength. Therefore, the α-helix and random coil structures of surimi gels were transformed into β-sheet and β-turn structures after DPCD treatment. Hydrophobic interactions, and disulfide and non-disulfide covalent bonds were the main intermolecular forces during the DPCD-induced gel formation of surimi. Ionic and hydrogen bonds were not the main intermolecular forces. The results provide fundamental data for elucidating the mechanism of DPCD-induced protein gel formation.

KEYWORDS

surimi processing, dense phase carbon dioxide, protein structure, gel strength, golden pompano

1. Introduction

Golden pompano (*Trachinotus ovatus*) is an important commercial marine fish inhabiting the coastal areas of southern China (Liu et al., 2019). It is a delicious, nutritious and fast-growing fish. The recent increase in market demand and the widespread application of cage culture technology, have increased the yield of golden pompano over the years (Bureau of Fisheries of the Ministry of Agriculture, 2021). As an important aquatic food, surimi is popular among consumers because of its convenience, high protein and low fat content. With the rapid development of the surimi industry, challenges such as unstable quality of surimi products, single processing method, severe homogenization of products, and lack of healthy new products have emerged (Jaziri et al., 2021; Monto et al., 2021). Therefore, effective improvement of the quality of surimi products and development of healthy surimi products is an important issue in the field of aquatic processing.

Currently, the processing of surimi products is mainly based on heat treatment. However, excessive heat treatment may result in the loss of heat-sensitive substances such as fatty acids, amino acids, and bioactive peptides in surimi. The poor heating and heat transfer rates, which prolong the temperatures treatment, result in gel degradation. Compared with a few non-thermal processing methods, heat treatment is associated with high energy consumption, and the waste generated during the treatment is not conducive to environmental protection (Nakamura et al., 2021). Dense phase carbon dioxide (DPCD) treatment is a new non-thermal processing method that couples CO_2 at a specific temperature (< 60°C) and pressure (< 50 MPa). DPCD treatment generates high-pressure acidic environment due to the pressure of carbon dioxide and its molecular action (Guo et al., 2017). Compared with conventional heat treatment, DPCD can be used under mild processing conditions. It is widely used for sterilization, enzyme inactivation, and improvement of textural and nutritional properties of food products (Damar et al., 2006). In addition, due to its low viscosity and high diffusivity, CO_2 penetrates bacterial cell membranes. Thus, DPCD can be used for maximum preservation of food quality while avoiding thermal damage.

As an elastic protein concentrate, the gel strength of surimi represents an important index for the evaluation of surimi products (Duan et al., 2023). During the gelation, the change in protein structure is closely related to the change in gel strength because the gelation is the result of protein denaturation and aggregation. Under the action of external factors, such as heat, acid, salt, and high pressure, the protein is first denatured. The intermolecular forces (hydrogen bonds, ionic bonds, hydrophobic interactions, disulfide and non-disulfide covalent bonds) that maintain the protein structure are disrupted. The secondary structures (α-helix, β-sheet, β-turn, and random coil) are also changed and the groups are exposed. The protein then aggregates and forms a stable gel three-dimensional gel network under the influence of covalent and non-covalent chemical interactions within the protein molecule (Liu et al., 2014). As reported previously by our team, DPCD treatment can denature proteins and form gels, while maintaining the color and water-holding qualities of food products. It can be used as an alternative to traditional thermal processing methods (Zheng et al., 2022; Duan et al., 2023). Accordingly, this study investigated the effects of DPCD treatment on the secondary structure and intermolecular forces of surimi gel proteins, and analyzed the correlation between the effects of DPCD on protein conformation and gel strength of golden pompano surimi. It provides fundamental data to elucidate the mechanism of DPCD-induced gel formation in pompano surimi, and thereby provides a theoretical basis for the development of aquatic gel products using DPCD technology.

2. Materials and methods

2.1. Surimi sample preparation

Golden pompano fish of average weight (750 ± 50 g) were purchased from Dongfeng Seafood Market (Zhanjiang, China). The fish were stored in oxygenated water and immediately transported to the laboratory within 1 h. The fish were rapidly immersed in ice water. The surimi was prepared according to the method proposed by Liu et al. (2021). Briefly, fresh fish were minced, washed and dewatered, following by chopping, mixing with salt, setting, cooking, and cooling. The prepared surimi was evacuated using a vacuum packaging machine (DZ500/2D, Wenzhou, China) and refrigerated at −35°C. Pure carbon dioxide (99.99%) was obtained from the Zhanjiang Oxygen Plant. All the chemicals and solvents used in this study were of analytical grade.

2.2. Experimental design

The process of DPCD treatment is based on methods previously reported by our team (Zhang et al., 2011). The surimi was placed in a custom-made cylindrical mold, before being processed by the DPCD equipment (Figure 1), this equipment diagram is taken from Duan et al. (2023). The main steps include equipment heating, sample placement, sealing, venting, pressurizing, pressure holding, and pressure release sampling. The treated samples are placed in sealed bags and the test indices are measured after 12 h at 4°C.

A single-factor experimental design was used. The detailed experimental conditions are shown in Table 1. Two control groups were set up: the untreated group representing the raw surimi group (Control), and the heat-treated group (WB) heated in a two-stage water bath at 40°C for 30 min (pre-gelation), followed by heating at 90°C for 30 min (gelation).

2.3. Fourier-transform infrared spectroscopy

The vacuum freeze-dried sample was thoroughly mixed with anhydrous potassium bromide, and ground and pressed into transparent flakes. It was scanned at the full wavelength (400 ~ 4,000 cm^{-1}) using an infrared spectrometer (FTIR Bruker Tensor 27; Ettlingen, Germany) with a resolution of 4 cm^{-1}; the scans were accumulated 32 times and repeated 3 times. Then, Omnic 9.2 and Peakfit V4.12 software programs were used to analyze the characteristic spectral peaks of the amide I band in the 1,600 ~ 1,700 cm^{-1} band. First, the baseline was corrected, followed by Gaussian deconvolution and fitting with second-order derivatives to maximize the residuals. The number of sub-peaks were obtained and

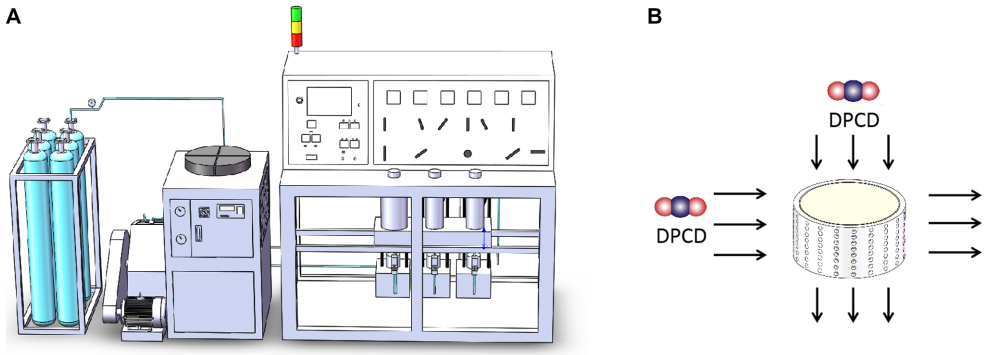

FIGURE 1
Dense phase carbon dioxide (DPCD) processing unit diagram **(A)** and mold drawing **(B)**: colorful balls is dense phase carbon dioxide.

TABLE 1 Test conditions.

Factor	Fixed condition	Factors
Pressure	Treatment temperature, 50°C; treatment time, 60 min	5, 10, 15, 20, 25, 30, and 35 MPa
Temperature	Treatment pressure, 20 MPa; treatment time, 60 min	30. 35, 40, 45, 50, 55, and 60°C
Time	Treatment pressure, 20 MPa; treatment temperature, 50°C	10, 20, 30, 40, 50, 60, and 70 min

the relative percentage of each sub-peak area was calculated to determine the levels of protein secondary structure.

2.4. Determination of intermolecular forces

The intermolecular forces of the protein were analyzed using a protein solubility method with slight modifications (Tan et al., 2010). The 2 g sample was treated with 10 mL B1 (0.6 mol/L NaCl), homogenized at 5,000 rpm/min for 5 min, transferred to 4°C for 1 h, centrifuged at 4°C and 18,600 × *g* for 25 min, and filtered through 1,000 mesh filter cloth, the supernatant S1 was stored at 4°C. Add 10 mL of B2 (1.5 mol/L urea, 0.6 mol/L NaCl) to the precipitate from B1, homogenized at 3,700 rpm/min for 2 min, transferred to 4°C for 1 h, centrifuged at 4°C, 18,600 × *g* for 25 min, stored the supernatant S2 at 4°C. Add 10 mL of B3 (8 mol/L urea, 0.6 mol/L NaCl) to the precipitate from B2, homogenized at 5,000 rpm/min for 5 min, transferred to 4°C for 1 h, centrifuged at 4°C, 18,600 × *g* for 25 min, stored the supernatant S3 at 4°C (repeated twice). Add 10 mL of B4 (0.5 mol/L β-mercaptoethanol, 0.6 mol/L NaCl, 8 mol/L urea) to the precipitate from B3, homogenized at 5,000 rpm/min for 5 min, transferred to 4°C for 1 h, centrifuged at 4°C, 18,600 × *g* for 25 min, store the supernatant S4 at 4°C. The final precipitate was dissolved in 10 mL B5 (1 mol/L NaOH solution). The supernatant obtained after centrifugation was added into the same volume (5 mL) of 20% trichloroacetic acid, and centrifuged at 3,950 × g for 15 min. The supernatant was discarded, and 1 mL of 1 mol/L NaOH solution was added into the precipitation and placed at 4°C (S5). The protein content of each supernatant was determined via Bradford method. The protein content of S1, S2, S3, S4, and S5 were accounted for the percentage of the total protein content that represented ionic bonds, hydrogen bonds, hydrophobic interactions, disulfide bonds, and non-disulfide covalent bonds in the whole system, respectively.

2.5. Determination of gel strength

The gel strength of the surimi was determined using a TMS-Pro analyzer (FTC Co., Ltd., Vienna, Virginia, United States). The probe = P/0.5 s, trigger force = − 5 g; pre-test speed = 5 mm s^{-1}; test speed = 1 mm·s^{-1}; compression deformation, 75% were set. The gel strength (g × mm) was obtained by multiplying the breaking strength (g) and the breaking distance (mm).

2.6. Statistical analysis

Experimental data were expressed as the mean ± standard deviation. Variance and Tukey's HSD multiple comparisons (with a 95% confidence interval) were obtained using JMP 16.0 software. The correlation between advanced protein structure and gel strength during DPCD-induced gel formation in surimi was analyzed via Pearson's correlation analysis using Origin 2022 software (Origin Lab, Hampton, NH, United States). Three batches of experiments were performed, with each batch containing three parallel samples.

3. Results and discussion

3.1. Fourier-transform infrared spectroscopy of golden pompano surimi under DPCD treatment

Fourier-transform infrared spectroscopy is easy to operate. Independent of functional groups, it can be used to quickly and accurately detect small structural changes in complex material systems. In addition, its low sample requirement and reproducible detection facilitate its widespread application for structural identification of materials in complex systems such as food

(Wan et al., 2022). In general, protein infrared spectra consist of amides I, II, III, A and B. They can be used to determine the secondary structure of complex proteins because they are almost independent of the nature of the side chains (Leng et al., 2022). They strongly contribute to hydrogen bonding, dipole–dipole interactions and the geometry of the protein peptide skeleton.

The effects of different DPCD treatments on the secondary structure of surimi are presented in Figure 2. With the increase in the intensity of different DPCD treatments, surimi proteins show typical absorptions at all major wave numbers in the FT-IR, with distinctive absorptions near the bands of amide A (~3,300 cm^{-1}), amide B (~3,100 cm^{-1}), amide I (1,700–1,600 cm^{-1}), amide II (1,600–1,500 cm^{-1}), and amide III (1,220–1,330 cm^{-1}; Xie et al., 2020). The red shift of the amide A band peak indicates N–H stretching and hydrogen bond formation. It suggests reduction of the intramolecular and intermolecular N–H stretching vibrations of surimi protein or weakening of hydrogen bonds by DPCD treatment. The amide I–III bands are strongly sensitive to the secondary structure of protein molecules, especially the amide I band located at 1,700–1,600 cm^{-1}, which is often used to analyze the secondary structure of proteins. Compared with the control group, the wave positions of the characteristic absorption peaks of amides B and II of surimi protein after DPCD treatment did not shift significantly after DPCD treatment. However, the wave peaks of the amide I band shifted to lower wave numbers, indicating a further decrease in the α-helical structure of surimi protein with the increased treatment intensity (Andonegi et al., 2020).

3.2. Effect of DPCD treatment on the secondary structure of golden pompano surimi

The protein secondary structure includes four main forms: α-helix, β-sheet, β-turn, and random coil, which correspond to the FT-IR wave number ranges of 1,650 ~ 1,660, 1,600 ~ 1,640, 1,660 ~ 1700, and 1,640 ~ 1,650 cm^{-1}, respectively (Yang et al., 2022). The effects of different DPCD treatments on the secondary structure levels of surimi protein are shown in Figure 3, based on the Gaussian fit of the protein secondary structure in the range of 1,700 ~ 1,600 cm^{-1}. The results showed a significant difference in the effect of different DPCD treatments on the secondary structure of surimi protein compared with the control group ($p < 0.05$). With increased intensity of DPCD treatment, the levels of α-helix and random coil decreased significantly ($p < 0.05$), and the content of β-sheet and β-turn increased significantly ($p < 0.05$). As the treatment intensity continued to increase, the concentration of each component gradually decreased. During the gelation of surimi protein, the formation of β-sheet is often accompanied by the decrease of α-helix. The β-sheet structure promotes the formation of a more ordered and compact network structure (Wei et al., 2018). This indicates that the protein structure of surimi protein undergoes reconstruction of secondary structure under the action of DPCD, mainly from α-helix and random coil to β-sheet and β-turn.

The secondary structure of surimi protein has a significant effect on its processing characteristics. The content of α-helical structure is closely related to the gel strength. The α-helix is the

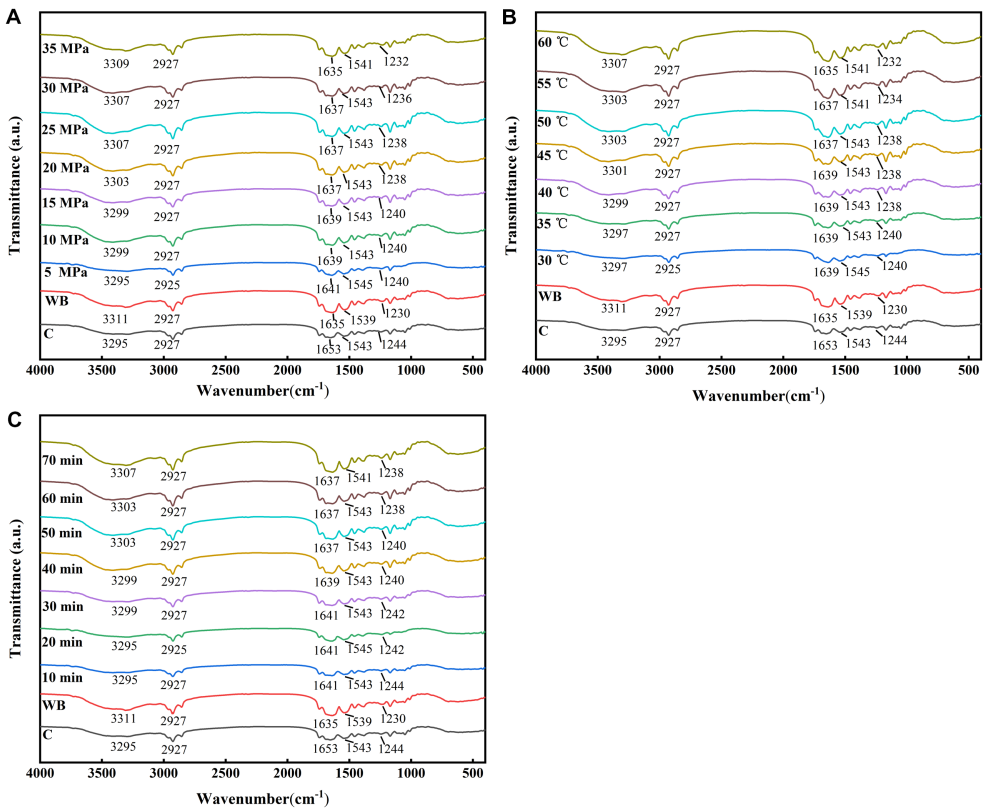

FIGURE 2
Fourier-transform infrared spectroscopy (FT-IR) spectra of protein in surimi with different DPCD treatments (**A**: Pressure, **B**: Temperature, **C**: Time).

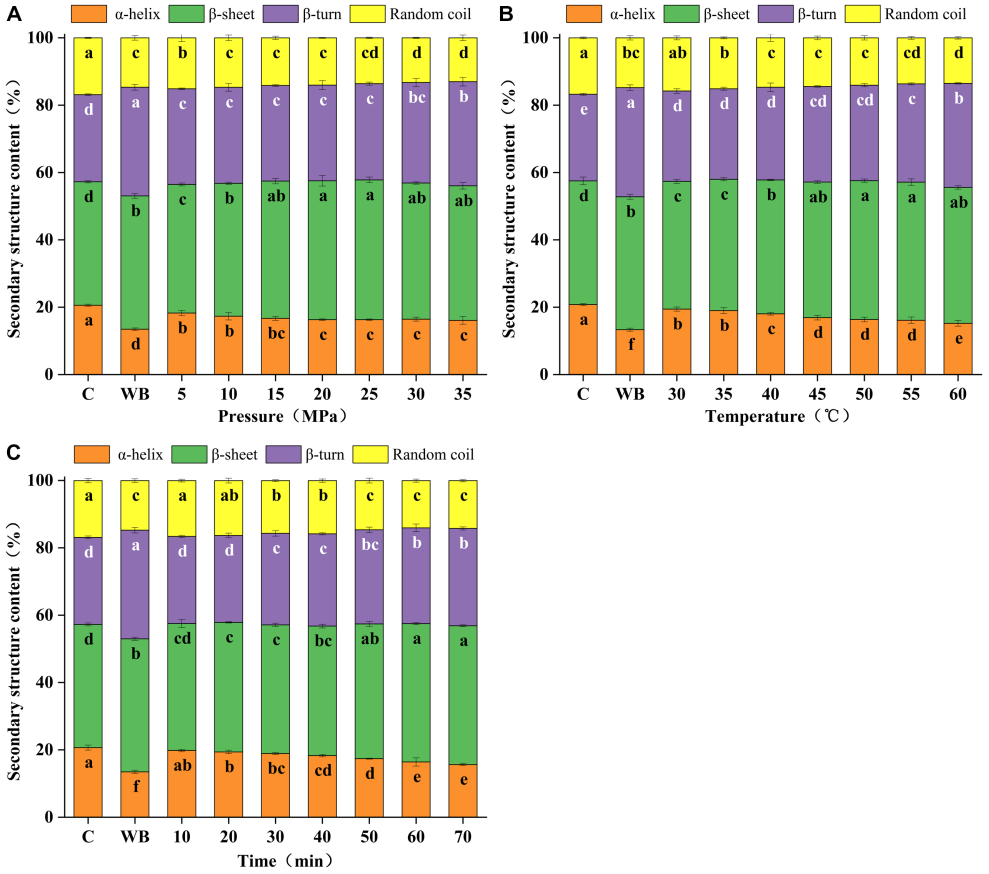

FIGURE 3
Effect of different DPCD treatments on the secondary structure content of surimi (**A**: Pressure, **B**: Temperature, **C**: Time). Different letters indicate statistically significant differences ($p < 0.05$).

major conformation of the secondary structure in natural proteins. During the processing of surimi into gels, the α-helix structure is partially uncoiled and transformed into β-sheet, β-turn or random coil. The β-sheet structure contributes to the hardness of surimi gels, while the β-turn and random coils cannot induce the formation of an ordered gel network (Ding et al., 2019). As shown in Figure 3, the content of α-helix and random coil was significantly lower ($p < 0.05$) and the level of β-sheet and β-turn was significantly higher ($p < 0.05$) in thermally-induced gel samples (13.3%) compared with the control group (20.8%). In addition, the α-helices were significantly fewer ($p < 0.05$) and the β-turns were significantly higher ($p < 0.05$) in the heat-treated gel samples than in the other sample groups. This indicates the destruction of additional α-helical structures and their transformation into β-turns under heat treatment.

3.3. Effect of DPCD treatment on intermolecular forces of golden pompano surimi

3.3.1. Effect of DPCD treatment on ionic bonds of golden pompano surimi

Under normal pH of surimi (near neutral), the ionic bond is the main force contributing to the natural structure of myogenic fibronectin. However, the ionic bond between protein molecules can be broken by external factors, such as heat, salt, and high pressure, resulting in protein aggregation and gelation (Hiromoto et al., 2022). As shown in Figure 4, compared with the control group (16.4%), the ionic bond of golden pompano surimi showed different degrees of decrease under different DPCD treatment intensities. The ionic bond content decreased significantly ($p < 0.05$) when the treatment pressure reached 5 MPa (9.3%). No significant change in the ionic bond content was detected when the treatment pressure continued to increase above 20 MPa. It indicates that at lower treatment pressure, the reduced pH of CO_2 and molecular effects break the ionic bonds between protein molecules, as well as between protein and salt ions (Ohashi et al., 1991). The ionic bond content of the surimi samples under different DPCD treatment times was similar to that of the different DPCD treatment pressure samples, with a significant decrease ($p < 0.05$) when the treatment time ranged between 10 min (14.1%) and 50 min (7.1%). No significant change in ionic bond content was found when the treatment time was extended beyond 50 min. The number of ionic bonds of the surimi samples at different DPCD treatment temperatures first decreased and then increased. The ionic bonds decreased significantly ($p < 0.05$) with increasing temperature below 50°C (6.7%), indicating that heating can disrupt the repulsion between protein molecules (Yang et al., 2020). However, when the temperature was above 50°C, the ionic bond content of the surimi increased slightly. Due to the addition of salts such as KCl and

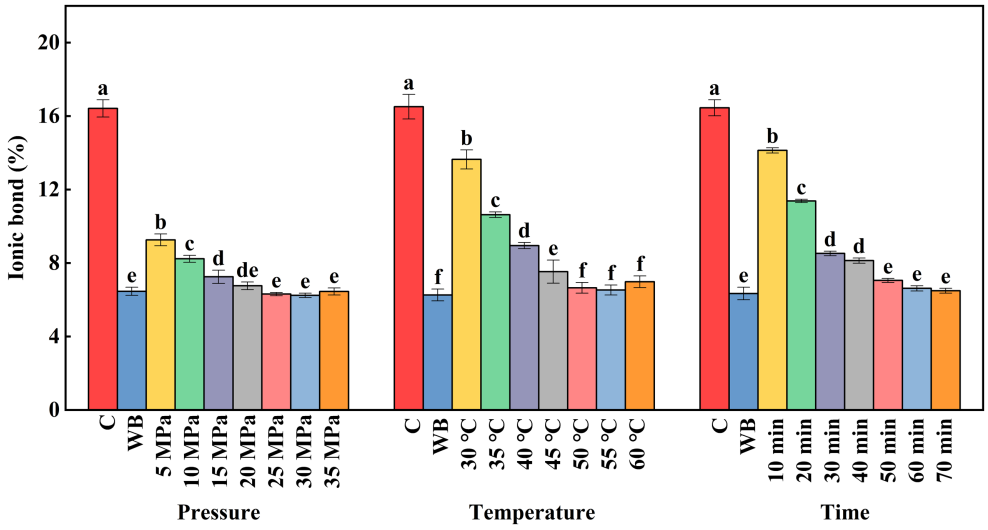

FIGURE 4
Effect of DPCD treatment on ionic bonds of golden pompano surimi. Different letters indicate statistically significant differences ($p < 0.05$).

NaCl during the preparation of surimi, the ionic bonds between protein molecules were broken and new ionic bonds were formed between proteins and salt ions when the treatment temperature reached 60°C. Two key factors contribute to the effect of DPCD on the ionic bonding of surimi. First, the dissolution of CO_2 in water decreases the pH of the system, which affects the ionization of proteins and the net charge value by adjusting the pH. Second, the heating facilitates the free movement of the ions without binding in a fixed lattice.

3.3.2. Effect of DPCD treatment on hydrogen bonds of golden pompano surimi

Hydrogen bond is a weak dipole bond and the main chemical force that maintains the secondary structure of natural proteins. As shown in Figure 5, the hydrogen bond content in untreated surimi was (15.6%). The different DPCD treatments significantly reduced the hydrogen bond content in surimi compared with the untreated group ($p < 0.05$). The hydrogen bonds of surimi showed a significant decrease with increasing pressure during DPCD treatment, especially below 15 MPa ($p < 0.05$). The DPCD denatures the surimi due to decreased pH, resulting in disruption of hydrogen bonds. When the DPCD treatment pressure was higher than 20 MPa, the hydrogen bonds decreased slowly, which may be attributed to the steady state after the prior disruption of a large number of hydrogen bonds in surimi (Xu et al., 2011). The trend of the hydrogen bond content of the surimi samples under different DPCD treatment times was similar to that of the stress group, which decreased significantly ($p < 0.05$) when the treatment time was less than 50 min. It did not change significantly when the treatment time was greater than 50 min. The hydrogen bonds in the surimi samples under different DPCD treatment temperatures decreased significantly ($p < 0.05$) below 50°C, because hydrogen bonding is more sensitive to temperature, and the higher the temperature, the weaker the hydrogen bonding (Wang et al., 2020). However, there was no significant change in the hydrogen bond content of the surimi when the treatment temperature was higher than 50°C. The thermal effect already induced severe damage in the protein. When the denaturation temperature was exceeded, the hydrogen bonds in surimi did not change significantly.

3.3.3. Effect of DPCD treatment on hydrophobic interaction of golden pompano surimi

Hydrophobic interaction occurs when natural proteins are exposed to external factors, such as heat, high pressure, and ions. Hydrophobic groups on the molecular surface are exposed and the hydrophobic components of neighboring proteins are bound tightly. Hydrophobic interactions cause protein aggregation, which is one of the factors facilitating gel formation in surimi (Gilleland et al., 1997). The hydrophobic interactions between protein molecules can be increased by heating. The hydrophobic interactions promote protein–protein binding to form an ordered three-dimensional gel network. As shown in Figure 6, all DPCD treatments significantly increased the hydrophobic interactions of surimi compared with the control group ($p < 0.05$). DPCD treatment induced denaturation of surimi, which exposed hydrophobic groups and increased hydrophobic interactions. CO_2 is a non-polar molecule, which promotes hydrophobic interactions between protein molecules. The solubility of CO_2 in water increases under pressure, which also promotes molecular effects of CO_2 (Duba and Fiori, 2016). However, the hydrophobic effect decreases at treatment pressures exceeding 20 MPa, which is attributed to the contraction of the protein structure under high pressure, resulting in buried hydrophobic groups. The hydrophobic interactions were increased by treatment temperatures in the range of 30–50°C. The thermal effects at this treatment temperature alter the protein structure slowly. The hydrophobic groups are gradually exposed, which increases hydrophobic interactions. In contrast, the hydrophobic interactions decreased significantly ($p < 0.05$) when the temperature was increased to 60°C. This is due to the weakening of the hydrophobic interactions of proteins in the gel under an acidic environment during DPCD treatment.

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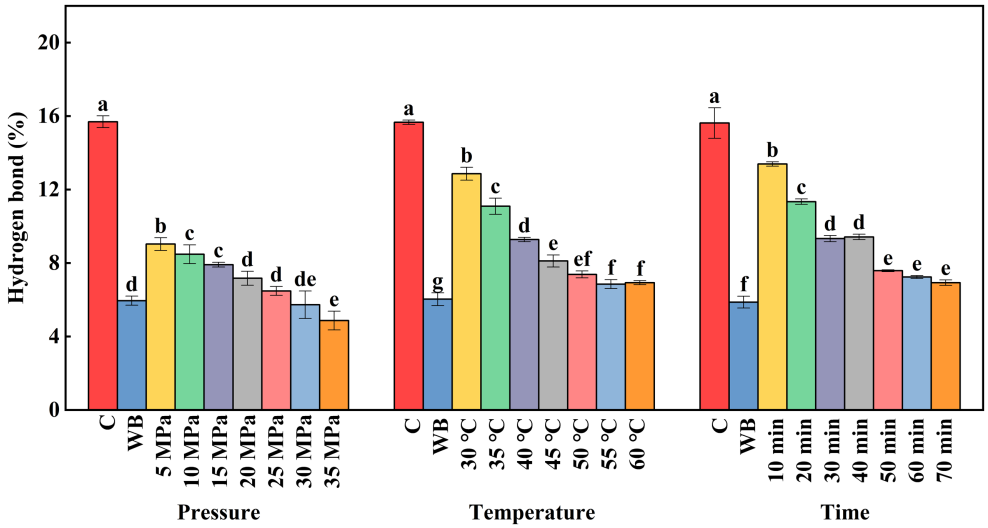

FIGURE 5
Effect of DPCD treatment on hydrogen bonds of golden pompano surimi. Different letters indicate statistically significant differences ($p < 0.05$).

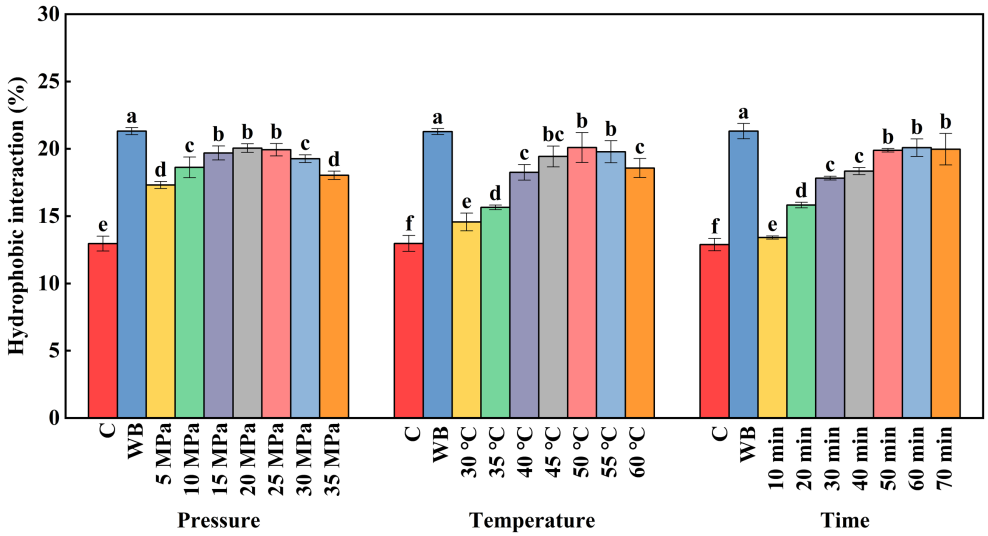

FIGURE 6
Effect of DPCD treatment on hydrophobic interaction of golden pompano surimi. Different letters indicate statistically significant differences ($p < 0.05$).

3.3.4. Effect of DPCD treatment on disulfide covalent bonds of golden pompano surimi

The intermolecular disulfide covalent bond is formed by the oxidation of two cysteine molecules with reactive sulfhydryl groups located on adjacent protein peptide chains. It is the most important covalent bond in thermally-induced protein gel (Chen et al., 2023). As shown in Figure 7, all DPCD treatments significantly increased the disulfide covalent bond levels of surimi compared with the control group ($p < 0.05$). Under treatment pressure below 15 MPa and treatment duration less than 50 min, the content of disulfide covalent bonds increased significantly, without additional changes following further increase in treatment intensity. This is because DPCD treatment has both thermal and molecular effects of CO_2, which can completely denature and stretch the protein to expose highly reactive sulfhydryl groups, resulting in the formation of additional disulfide covalent bonds. Compared with the control group, the number of disulfide covalent bonds gradually increased when the treatment temperature was increased to 45°C. The bond number did not change significantly when the treatment temperature exceeded 50°C. This is due to the limited total number of active sulfhydryl groups contained in the surimi, which are fully exposed and oxidized to disulfide covalent bonds when heated at 50°C. Their levels remained stable.

3.3.5. Effect of DPCD treatment on non-disulfide covalent bonds in golden pompano surimi

During protein gel formation, covalent cross-linking between proteins occurs mainly via non-disulfide covalent bonds. Non-disulfide covalent bonds are not only involved in protein

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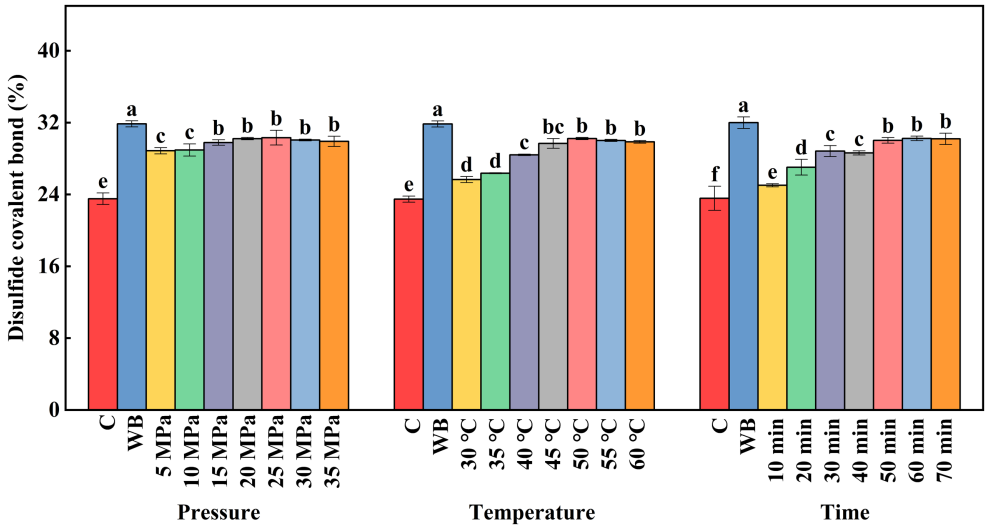

FIGURE 7
Effect of DPCD treatment on disulfide covalent bonds of golden pompano surimi. Different letters indicate statistically significant differences ($p < 0.05$).

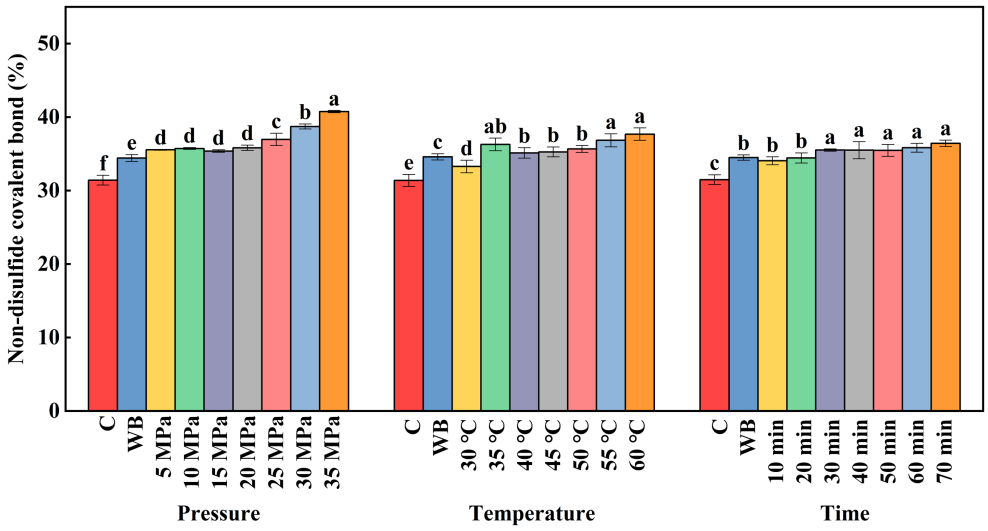

FIGURE 8
Effect of DPCD treatment on non-disulfide covalent bonds of golden pompano surimi. Different letters indicate statistically significant differences ($p < 0.05$).

gelation, but also play an important role in stabilizing the protein gel network (Du et al., 2022). As shown in Figure 8, compared with the control group, the increase of DPCD treatment pressure and treatment time can significantly increase the number of non-disulfide covalent bonds. The decreased pH of the system under high pressure and the molecular effect of CO_2 induced the formation of new non-disulfide covalent bonds in the surimi. The non-disulfide covalent bonds showed differential increase with the increase of DPCD treatment intensity. When the treatment pressure was above 25 MPa and the treatment time was greater than 30 min, there was no significant change in the non-disulfide covalent bond content. It is possible that within this treatment range, the molecular effect of CO_2 can induce the formation of a large number of non-disulfide covalent bonds in the gel. The non-disulfide covalent bond content increased with the increase in DPCD treatment temperature, which may be due to the increased molecular motion under increased thermal energy and the formation of a relatively large number of new covalent bonds.

3.4. Correlation between protein structure and gel strength during DPCD-induced gel formation

The protein structure and intermolecular forces and gel strength during DPCD-induced gel formation in golden pompano surimi were

correlated with gel strength. The effect of DPCD treatment on the gel strength of golden pompano surimi is shown in Appendix 1. As shown in Figure 9, the correlation heat map was color-coded using different intensities of red and blue. Red indicates positive correlation, blue denotes negative correlation. The intensity of the color is expressed by the Pearson correlation coefficient (P). Depending on the magnitude of P, the correlation can be classified as strong (0.6 ~ 1.0), medium (0.2 ~ 0.6), and weak (0.0 ~ 0.19; Marín et al., 2021).

As shown in Figure 9, β-sheets and β-turns exhibit a strong positive correlation with gel strength, whereas α-helix and R-coil showed a strong negative correlation with gel strength. Thus, as the treatment intensity of DPCD increases, the content of α-helix decreases, the content of β-sheet increases, resulting in enhanced gel strength. The β-sheet structure in proteins most likely interacts with CO_2 than the α-helix, because the β-sheet structure has fewer intramolecular hydrogen bonds and additional exposed side chain groups, which facilitate the reaction with CO_2. The interaction of CO_2 with the gel was further enhanced by increasing DPCD treatment pressure and temperature. Hydrophobic interactions, disulfide and non-disulfide covalent bonds were strongly positively correlated with gel strength. The ionic and hydrogen bonds were strongly negatively correlated with gel strength. Thus, the higher the number of hydrophobic interactions, disulfide and non-disulfide covalent bonds, the higher is the strength of DPCD-induced surimi gels. Therefore, hydrophobic interactions, disulfide and non-disulfide covalent bonds are the main intermolecular forces during the DPCD-induced gel formation in golden pompano surimi.

4. Discussion

The structure of proteins determines the properties of gels. The variation in protein secondary structure is closely related to the gel strength. The secondary structure of surimi protein forms specific spatial structures such as coils and folds by the side chain groups under the joint action of intermolecular forces (ionic bonds, hydrogen bonds, hydrophobic interactions, and disulfide and non-disulfide covalent bonds). The surimi gels are depolymerized, cross-linked, and aggregated to form a three-dimensional gel network structure (Ai et al., 2022; Xu et al., 2022). However, the intermolecular forces contribute to different stages of protein gel formation and induce protein gels in different ways.

The variation of advanced protein structure during DPCD-induced gel formation of golden pompano surimi are shown in Figure 10. Treatment of surimi with DPCD induces gelation. CO_2 in DPCD dissolves in water to form carbonic acid, which in turn dissociates to form H^+, HCO_3^- and CO_3^{2-} ions, lowering the pH of the system and thereby altering the advanced structure of proteins. The α-helix content in the secondary structure decreases and the β-sheet content increases. The β-sheet structure is a prerequisite for protein aggregation during the formation of gel network. The conversion of the α-helix structure to a more ordered β-sheet facilitates the formation of the gel network (Ju et al., 2022). The molecular effect of CO_2 in the temperature synergistic high-pressure state decreases the α-helix content of surimi gels and disrupts the hydrogen bonding. As a result, the protein is in a stretched state, which promotes the interaction between protein molecules (Sikka

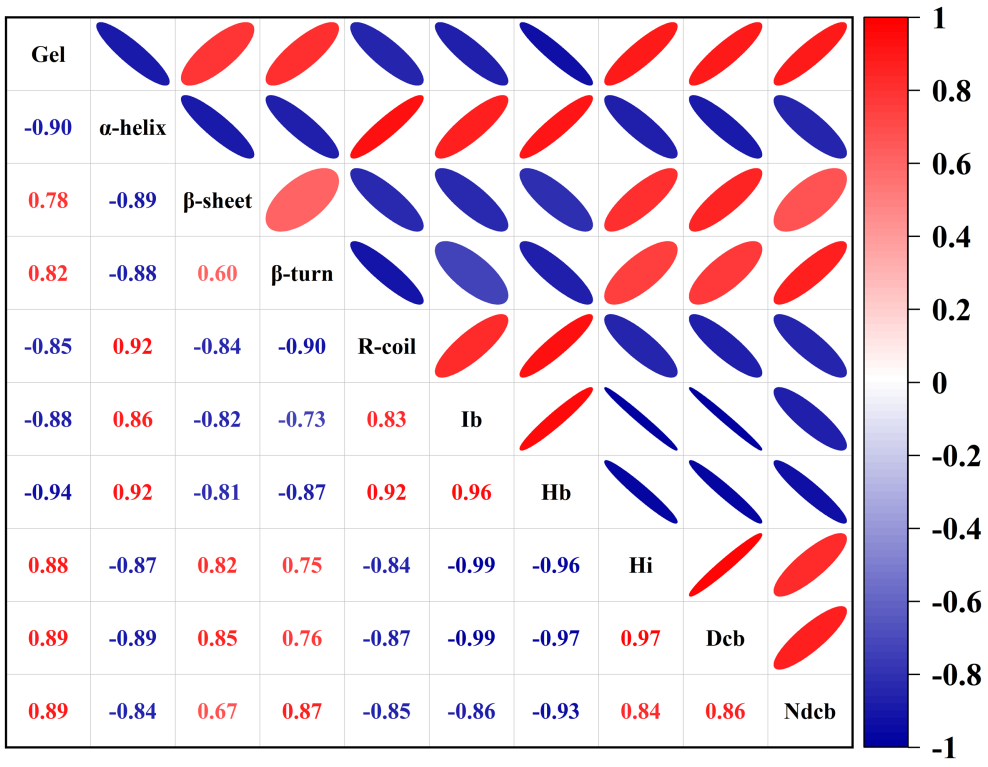

FIGURE 9
Correlation between protein structure and gel strength during DPCD-induced gel formation (Ib: Ionic bond, Hb: Hydrogen bond, Hi: Hydrophobic interaction, Dcb: Disulfide covalent bond, NDcb: Non-disulfide covalent bond).

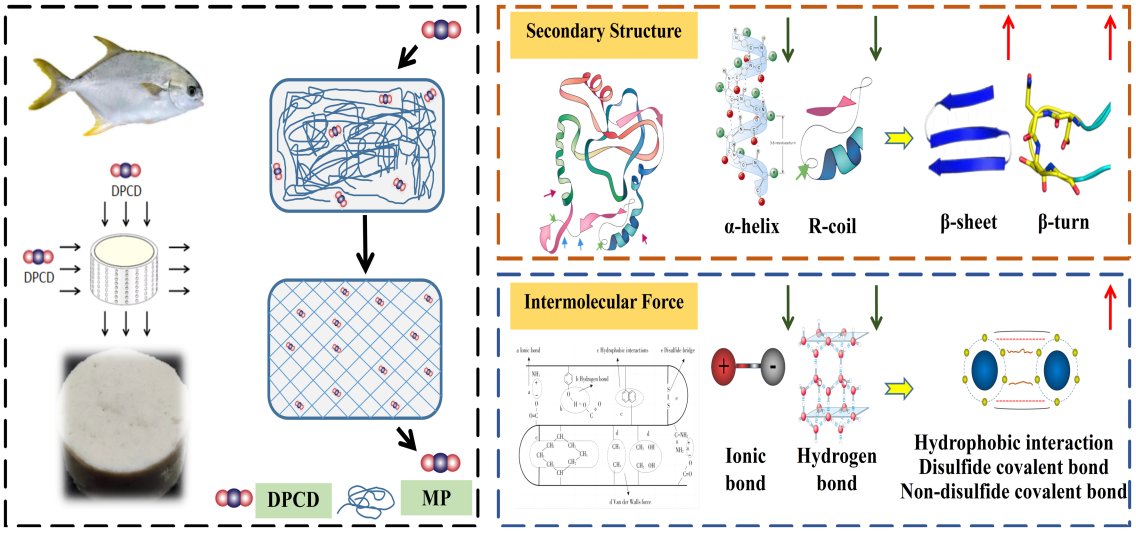

FIGURE 10
The variation of advanced protein structure during DPCD-induced gel formation of golden pompano surimi.

et al., 2008; Roy and Bandyopadhyay, 2023). The reduction in the number of hydrogen bonds also exposes additional hydrophobic groups. However, CO_2, as a hydrophobic solvent, interacts with the hydrophobic groups in the amino-terminal residues of surimi proteins, changing the aqueous environment around the protein (Duba and Fiori, 2016). Likewise, the hydrophobic groups are exposed, which further enhances the hydrophobic interactions. As the treatment intensity increases, additional sulfhydryl groups inside the protein are exposed and the free sulfhydryl groups oxidize to disulfide covalent bonds, resulting in protein polymerization (Chen et al., 2020). This indicates that the denaturation of proteins in the presence of DPCD may be induced by the combination of hydrophobic interactions, and disulfide and non-disulfide covalent bonds.

5. Conclusion

Dense phase carbon dioxide treatment induced gel formation in golden pompano surimi. Compared with the control group, different DPCD treatment conditions had significant effects on the protein secondary structure content and intermolecular forces in golden pompano surimi. The contents of α-helix and random coil were significantly decreased ($p < 0.05$), while the levels of β-sheet and β-turn increased significantly ($p < 0.05$). The number of ionic and hydrogen bonds decreased significantly ($p < 0.05$), and hydrophobic interactions, disulfide and non-disulfide covalent bonds increased significantly ($p < 0.05$). Correlation analysis showed that β-sheet, β-turn, hydrophobic interactions, disulfide covalent bonds and non-disulfide covalent bonds of surimi protein under DPCD treatment were strongly positively correlated with gel strength. The α-helix, random coil, ionic and hydrogen bonds were strongly negatively correlated with gel strength. Therefore, the secondary structure and intermolecular forces can be used as effective indicators of altered gel strength during gel formation induced by DPCD treatment in pompano surimi. In subsequent studies, we hope to develop novel techniques to evaluate gel quality based on such protein structure indicators. Meanwhile, the relationship between gel strength and protein structure can be explored at the microscopic level for rapid, efficient and comprehensive monitoring of surimi freshness.

Data availability statement

The data that support the findings of this study will be available upon request to the corresponding author.

Author contributions

WD and SL: conceptualization and methodology. WD and HQ: software and formal analysis. ZW and QX: validation. HQ and KH: investigation. SW: resources. QS and KH: data curation. WD: writing—original draft preparation and writing—review and editing. YL: visualization. ZH: supervision. SL: project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1189149/full#supplementary-material

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Physicochemical properties of rainbow trout (*Oncorhynchus mykiss*) filet treated with high-voltage electrostatic field under different storage temperatures

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The post-mortem rigid of farmed rainbow trout (*Oncorhynchus mykiss*) affects the texture of the meat and might even be risky for microorganisms, undermining the popularity of frozen filets among consumers. This study investigated the importance of different conditions (0°C, 4°C, and 25°C for 0–4 days) and physicochemical characteristics of rainbow trout filet storage on fish's characteristics, freshness, quality, and shelf-life. Results showed that the fish easily underwent rigor mortis and resolution of rigor mortis when stored at 25°C. However, rigor mortis was more likely to occur under storage at 0°C than at 4°C [with 100 kV/m high voltage electrostatic fields (HVEF)] due to low-temperature stimulation, but resolution of rigor mortis began to appear after 24 h of storage at either temperature. The protein solubility and gel strength of fish stored at 25°C continued to decrease due to thermal denaturation, and those of fish stored at 4°C and 0°C also showed a decrease owing to rigor mortis and then an increase when rigor mortis disappeared. The K values increased rapidly at 25°C and reached 61% at 14 h. Under storage at 4°C and 0°C, the K values only slightly increased before resolution of rigor mortis. However, the K-values did not exceed 40% for 4 days post-resolution of rigor mortis. Differential Scanning Calorimetry analysis and tissue sectioning showed that protein denaturation and separation between muscle fibers persisted during storage at various temperatures, most notably at 25°C. It is important to note that storing at 0°C for 96 h did not result in any significant changes in the molecular protein composition. Moreover, the muscle tissue remained in excellent condition compared to storage at 25°C and 4°C. Additionally, there was no significant difference in the total viable count (TVC) and psychrophilic bacteria between storage at 0°C and 4°C, considered safe for food consumption. These promising findings are conveyed in a circular economy based on the food industry, particularly adequate raw materials, stable prices, measures to reduce food loss and waste, and contributing to developing efficient, diverse, and sustainable food processing systems.

KEYWORDS

rainbow trout, freshness, rigor mortis, high voltage electrostatic field (HVEF), K value, protein denaturation

Highlights

- Constant low-temperature preservation is required to maintain the freshness of fish.
- Storage at 4°C with HVEF treatment may delay 12 h for reaching maximum rigor mortis.
- Preserved fish are also suitable for food processing.

1. Introduction

Rainbow trout (*Oncorhynchus mykiss*) has progressively gained a place in the market as an economically valuable species. It has been characterized as a fatty fish and is one of the most popular fish for farming (Rezaei and Hosseini, 2008; Barrientos et al., 2019; Bienkiewicz et al., 2019). Compared with wild (Pacific species) and cultured (Atlantic species) salmon, its prices are lower and its flesh color, mouthfeel, and intramuscular fat layer are substantially different (Fox et al., 2018). It has been cultivated for artificial farming in many countries and regions (Genç and Diler, 2019; Yin C. et al., 2022). Aquaculture farms have been established for local supplies to ensure the stability and safety of the raw material source for the food industry-related supply chain. In addition, rainbow trout have been widely used for fresh service distribution and as raw material for the food and catering industries. For Chinese dishes, it is suitable for steaming, deep-frying, and grilling, and for Western dishes, it is sold as ready-to-eat food (appetizers, salads, or sandwiches) in the form of smoked fish filets and frozen products. To date, rainbow trout is most commonly used by the catering industry as a substitute for salmon in preparing Japanese cuisine primarily to reduce the raw material cost; raw fish forms are typically sliced filets for direct consumption called *sashimi* (Xiong et al., 2021). In light of this situation, the U.S. Food and Drug Administration has explicitly stated that rainbow trout should not be labeled salmon on food packaging. Meanwhile, the European Union has established general rules to prevent food fraud. As stated in EU Regulation 1379/2013, correctly labeling species using scientific and commercial species names is mandatory (Monteiro et al., 2021).

The freshness of fishery products depends on the product's value, excluding the difference in fish species (Freitas et al., 2020; Li H. et al., 2022). Compared with livestock meat, fish tissues have fewer connective tissues, softer muscle tissues, and stronger self-digestion, making them prone to muscle protein hydrolysis and spoilage (Listrat et al., 2016; Prabhakar et al., 2020; Cheng H. et al., 2023; Hui et al., 2023; Ozogul et al., 2023). Therefore, preserving fish freshness is a typical application of low-temperature refrigeration (Freitas et al., 2020; Du et al., 2022). The muscles can retain good freshness during the post-mortem rigor mortis process, while the resolution of rigor mortis, the muscles soften, and the freshness decline (Warner, 2016). Thus, a remarkable relationship exists between freshness and the state of muscle protein (Yu et al., 2022).

In several surveys, it was found an "ambivalence" while analyzing the consumers' preference for fish properties; most of them seem to prefer wild fish to farmed fish, locally originated fish to imported fish, fresh fish to frozen fish, whole fish to processed fish, but the same consumers have opposite situations in terms of convenience, accessibility, low price, and environmental protection (Carlucci et al., 2015; Lopes and de Freitas, 2023; Pascoe et al., 2023; Rodriguez-Salvador and Calvo Dopico, 2023; Yuan et al., 2023). Despite that, frozen, canned fish products and ready-to-eat fresh fish filets are considered very suitable products (perfect for emerging markets) while most consumers tend to save the time and effort required to prepare food and eat fish, in addition to the perception that fresh fish products are considered challenging to prepare, which is higher among young people than elderly people, while people living in coastal areas prefer fresh fish (Carlucci et al., 2015). Rainbow trout are usually sold alive in Taiwan, but death frequently occurs during shipping caused of unsuitable conditions; sold on ice is less favored by consumers, presumably due to the post-mortem rigid storage affecting the meat quality. Therefore, knowing of the physical and chemical indices of the finished product is important for the sensory characteristics and shelf life. This study investigated the correlation among the changes in muscle protein solubility, water retention, enthalpy, K- value, TVBN- value, and bacterial count under different storage temperatures (where HVEF was used at 4°C). The findings will serve as a reference for the selection of storage conditions for fish.

2. Materials and methods

2.1. Materials

Cultured rainbow trout (*Oncorhynchus mykiss*) specimens, each weighing about 600–700 g was purchased from a local market (Taichung, Taiwan). After slaughtering and eviscerating, the fish were rinsed with tap water, packed in polystyrene boxes, covered with ice, and carried back to the laboratory within 30 min. The semi-dressed fish were cleaned, dried with towels, and individually placed in a PVC plastic bag and sealed. Finally, the fish were randomly grouped and stored at 0°C, 4°C, and 25°C and sampled for physiochemical property determination at different storage times (0, 6, 12, 24, 36, 48, 72, and 96 h). In particular, the 4°C group was stored in a refrigerator (75% relative humidity) with the 100 kV/m high-voltage electrostatic field (HVEF) by an output voltage of 50 kV DC electrostatic generator (SC-PME50, Cosmic International Co. Ltd., New Taipei City, Taiwan). The storage protocols and conditions were followed as described by Hsieh et al. (2011) with some modification. All analytical grade

reagents were purchased from Sigma-Aldrich® (Merck KGaA, Darmstadt, Germany).

2.2. Rigor mortis index

The fish rigor index was measured, following the method of Kiessling et al. (2006) and Le et al. (2020). Each fish was placed on the platform's edge at 1/2 length to determine the length of normal sagging (tail drop) and sagging during the rigor phase. The rigor index was then calculated using the following equation:

$$Rigor\ mortis\ index\ (\%) = \frac{D_0 - D}{D_0} \times 100$$

where, D_0 is the length of normal sagging before the rigid phase, and D is the length of sagging in the rigor phase.

2.3. Determination of protein concentration and solubility

The solubility of the proteins in the samples was determined as described by Mohsenpour et al. (2023) with some modifications. In brief, 40 g of NaOH and 1 g of glycerin were added to 200 mL of distilled water. In another beaker, 2 g of $CuSO_4$-$5H_2O$ was added to 200 mL of distilled water. Eventually, the two solutions were mixed, added with distilled water to a volume of 500 mL, and named Biuret reagent 1. Biuret reagent 2 was prepared by thoroughly mixing 40 g of NaOH and 1 g of glycerin with distilled water, followed by adding distilled water to a volume of 500 mL. The sample was separated into two tubes with 2 mL each, followed by the addition of 2 mL of Biuret 1 and 2, respectively, and then reacted at room temperature for 45 min. Absorbance at 550 mm was measured by a spectrophotometer (U-2000, Hitachi Ltd., Tokyo, Japan) and calculated by the following equation:

$$\text{Protein concentration}\ (mg/mL) = \left[(Ia - Ib) - (IIa - IIb)\right] \times F2$$

where, Ia is the absorbance of Biuret reagent 1 at 550 nm, Ib is the absorbance of the blank of Biuret reagent 1 at 550 nm, IIa is the absorbance of Biuret reagent 2 at 550 nm, IIb is the absorbance of the blank of Biuret reagent 2 at 550 nm, and F2 is the calibration coefficient for protein concentration.

Albumin fraction V was prepared by adding 10 mM tris–HCl buffer to 20 mg/mL as standard and then diluting to different concentrations. Biuret method and Kjeldahl were used for determination. The correlation and correction coefficients between the two methods were obtained and applied to the above Biuret method equation.

Water-soluble protein was extracted in accordance with the method of Sarma et al. (2000). The sample was homogenized for 3 min with 5 g of sample and 95 g of distilled water. Salt-soluble protein extract was performed as described by Eady et al. (2014). The sample was homogenized with 95 mL of 5% NaCl (pH regulated to 7.2 with 0.02 M NaHCO) and 5 g of the sample for 3 min. Both homogenates were centrifuged at 4,500 × g for 15 min. The supernatants were added with distilled water and 0.02 M NaHCO to a volume of 100 mL. Protein concentrations were determined as described in the previous section. Total protein solubility, namely, the sum of water-soluble and salt-soluble protein, was expressed in g/100 g of fish meat.

2.4. Preparation of surimi gelatin and determination of gel strength

Surimi gel was prepared following the method described by Huang et al. (2022). The fish meat was adjusted to 80% water, minced at 4°C for 1 min, added with 2.5% NaCl, and poured into a steel hollow cylindrical container (inner diameter and height both 13 mm), which was sealed, placed in a hot water bath at 90°C for 20 min, cooled for 15 min in an ice bath, and allowed to stand at room temperature for 30 min. Finally, a surimi gel sample was obtained. The measurement was performed at room temperature (about 25°C) with a tissue analyzer (CR-200D, Sun Scientific Co., Ltd., Japan) under the following conditions: using Model 1 and a spherical plunger (No. 2) of 5 mm diameter, loading table was raised at a speed of 150 mm/min, and the penetration level was 90% of the sample height. Meanwhile, the gel strength (g × mm) was obtained as breaking force × breaking strain.

2.5. Water holding capability

The WHC was determined using the method reported by Rybicka et al. (2022). In brief, 5 g of sample was wrapped with four sheets of Whatman grade 4 filter paper (150 mm circle, Whatman International Ltd., Giles, Buckinghamshire, United Kingdom), placed in a centrifuge tube, and centrifuged at 3,000 × g for 30 min at 4°C. The filter paper was removed, excess water from the sample's surface was aspirated, and the sample was weighed. The WHC was calculated with the following equation:

$$WHC\ (\%) = \frac{Sample\ Weight\ after\ centrifugation}{Sample\ weight\ before\ centrifugation} \times 100$$

2.6. Determination of K value, adenosine 5′-triphosphate (ATP), and its related compounds

The K value, ATP, and its related compounds were determined following the method described by Kuda et al. (2008) and Yin M. et al. (2022) with some modifications. In brief, 5 g of fish meat was homogenized with 25 mL of 6% perchloric acid (PCA) at 4°C for 3 min, followed by centrifugation at 3000 × g at 4°C for 10 min. NaOH was used to regulate the pH to 6.5–6.8, and 20 mL of supernatant was filtered through a 0.45 μm membrane in an ice bath for 30 min. In the end, the solution was added with deionized water to a volume of 50 mL and then frozen (−70°C) until use. A Hitachi Model L5000 high-performance liquid chromatography (HPLC) with a Might RP-18 column (5 μm, 250–4.6 mm end-capped, Kanto Chemical Co., Inc. Tokyo, Japan) was used. The mobile phase was 0.06 M K_2HPO_4–0.04 M KH_2PO_4 (pH 4.0), and the flow rate was 1 m/min. The detector

was a UV detector at a wavelength of 254 mm. The sample injection volume was 10 μL. The concentration of each product was calculated according to the relationship between the retention time, concentration, and peak area of the standard product. The percentage of H × R (inosine) and Hx (hypoxanthine) to the total amount of ATP-related compounds was determined. A low K value indicates the good freshness of the sample. The freshness index K value was calculated using the following equation:

$$K\ value\,(\%) = \frac{[HxR]+[Hx]}{[ATP]+[ADP]+[AMP]+[IMP]+[HxR]+[Hx]}$$

2.7. Determination of protein molecular composition with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein composition was analyzed following the method described by Bradford (1976). In brief, 0.2 g of fish sample was added to 5 mL of 62.5 mM Tris–HCl (pH 6. 8) solution (containing 8 M urea, 1% SDS, 1% 2-mercaptoethanol, 2 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride) and dissolved at room temperature (25°C) for 24 h. The sample was centrifuged at 900 g for 5 min, and 1 mL of the supernatant was added with two drops of tracking dye and heated at 100°C for 5 min to form the sample solution, which was then stored at −20°C until use. Each lane was loaded with 5 μL of the sample, followed by the chamber buffer, and the voltage was set at 80 V. The protein standards were myosin of rabbit skeletal muscle (200.0 kDa), *β*-galactosidase of *E. co1i* (116.3 kDa), phosphorylase B of rabbit muscle (97.4 kDa), serum albumin of bovine (66.2 kDa), ovalbumin of hen egg white (45.0 kDa), carbonic anhydrase of bovine (31.0 kDa), trypsin inhibitor of soybean (21.5 kDa), lysozyme of hen egg white (14.4 kDa), and aprotinin of bovine pancrease (6.5 kDa).

2.8. Determination of enthalpy (ΔH) change of actin and myosin in muscle

The method of Huang et al. (2022) and Nikoo et al. (2019) were adopted. In brief, 30–35 mg of the sample was loaded into a stainless-steel crucible. For the control group, the same amount of sample was added with 23–27 mg of distilled water [flesh fish's water content of 73–77% (Cheng Y.-T. et al., 2023)]. The enthalpy change of actin and myosin in muscle was measured with a Differential Scanning Calorimetry (DSC92, KEP Technologies, Mougins- Sophia Antipolis, France) at a temperature increase rate of 5°C/min in the range of 8°C–100°C.

2.9. Determination of total viable count (TVC) and psychrophilic bacteria

TVC and psychrophilic bacteria contents were performed using the Cheng Y.-T. et al. (2023) and ISO (2019) methods, respectively. In brief, 10 g of fish meat was homogenized with 90 mL of sterile saline in stomacher® 400 Circulator Lab Blender (Seward Ltd., United Kingdom) at low and high speeds for 2 min each, followed by the addition of 1 mL of the homogenate to 9 mL of sterile saline, serial dilution, and dispersion of 1 mL of dilution into culture dishes. Following the pour plate count method, 15 mL of psychrophilic plate count agar was poured and incubated at 37 and 7°C for 2 and 10 days, and the results were expressed as log CFU/g fish meat.

2.10. Determination of pH

In brief, 5 g of fish meat and 45 mL of distilled water were homogenized for 2 min at 2,000 × g in a homogenizer (Brinkmann Polytron Pt-3000, Kinematica AG, Switzerland) and then measured by a pH meter (MP220, Mettler Toledo, Columbus, United States).

2.11. Texture analysis

The texture of the sample was analyzed using the method described by Li Q. et al. (2022) with some modifications. The sample was cut into cubes of approximately 1.5 cm and measured for breaking stress (g), breaking strain (cm), and breaking strength (g × cm) of rainbow trout meat by a texture analyzer TA-XT2 (Stable Micro Systems Ltd., United Kingdom). The P/30C conical probe determined the conditions with 90% penetration of the sample at a speed of 2 mm/s.

2.12. Observation of tissue microstructure

The samples were cut into cubes (2 cm^3) and dipped in neutral formalin solution (10%, pH 7.0) at 4°C for 6 h, followed by paraffin embedding and cut into 4 μm thick sections of tissue slicing. The sliced samples were observed and photographed under a light microscope (Model: AFX-IIA, Nikon group Co. Tokyo, Japan) at a magnification of 10 × 10.

2.13. Statistical analysis

All the data in this study are presented using the mean ± standard deviation (SD), with three replicates for each condition ($n = 3$). The statistical analysis system (version 9.4, SAS Institute Inc., Cary, NC, United States) was used to analyze the differences between the means of each condition with a one-way ANOVA. Then, Duncan's new multiple-range test was used to compare the significant differences among each group ($p < 0.05$).

3. Results and discussion

3.1. Rigor mortis characteristics of rainbow trout and quality changes during storage

3.1.1. Rigor mortis index

The occurrence of rigor mortis was characterized by the initiation of a rigor mortis process caused by biochemically advanced ATP

depletion (below 2 μM). This test is a prerequisite for accurately controlling the quality of commercially available fish by determining adverse indicators (Delbarre-Ladrat et al., 2006). Improper treatment of different fish species in the pre-slaughter period possibly leads to the rapid deterioration of meat quality, decrease in muscle pH, WHC loss, protein denaturation, lipid oxidation, and increase in microbiota (Chytiri et al., 2004). The effects of storage temperature on the rigor mortis index of rainbow trout are shown in Figure 1A. Under 25°C storages, the maximum rigor mortis was obtained at 2.5 h, followed by the end of rigor mortis at 3.5 h. Only about 25% of rigor was available at 14 h. Under 4°C HVEF storage, the fish reached maximum rigor mortis between 12- and 24 h; resolution of rigor mortis release started after 24 h, and the degree of rigor was only about 43% after 96 h, and there were significant differences between all groups ($p < 0.05$). Under 0°C storage, the fish reached maximum rigor mortis in 6 h and lasted until 24 h, resolution of rigor mortis started afterward, and the degree of rigor mortis was about 57% after 96 h ($p < 0.05$). The above results showed that the rigor mortis occurred significantly faster at 25°C storage, while 4°C and 0°C declined after 36 h. This phenomenon can be attributed to the increased Mg-ATPase activity in myofibrils at 25°C, which contributed to the loss of sarcoplasmic reticulum (SR) function and resulted in the release of calcium ions that induced rigor mortis (Watabe et al., 1990). The fish stored at 0°C experienced the cold contraction effect, and the SR lost its function and released calcium ions, leading to rigor mortis. Notably, the 4°C group with HVEF storage obtained similar rigor mortis results as 0°C storage in a non-freezing condition ($p < 0.05$). The higher the storage temperature, the faster the rate of discharge of rigor mortis in the fish due to the release and activation of endogenous enzymes in the muscles that induce muscle tenderization (Moeller et al., 1977; Asghar and Bhatti, 1988). The meat of the rainbow trout slaughtered and fasted for 5 days had higher post-mortem pH at 0 h and earlier post-mortem rigor mortis compared with the trout that fasted for 3–4 days (Bermejo-Poza et al., 2017).

3.2. Changes in pH

The effect of storage temperature on the pH of rainbow trout muscle is shown in Figure 1B. The pH of the fresh fish muscle was about 6.67. In the absence of oxygen, the glycogen in muscles would undergo anaerobic glycolysis, and lactic acid would have decreased the pH after the death of the fish. Meanwhile, hydrogen ions might have been generated from ATP decomposition (Kumar et al., 2012). The decrease in fish pH at each storage temperature occurred at or after the maximum post-mortem rigor mortis, and the reduction was significant at high storage temperatures. Okuma and Abe (1992) reported that muscle glycogen content decreased to the lowest level during maximum rigor mortis, and the produced lactic acid reduced the pH. In addition, a pH increase was observed at 25°C storage for 10.5 h, which was assumed to result from microbial multiplication, muscle protein hydrolysis, and amine generation during ATP hydrolysis. Under storage at 0°C and 4°C, the pH decreased at 16-24 h, while the pH of the 4°C group was kept at pH 6.5 after 24 h with similar results to the 0°C group ($p < 0.05$). This also implies that HVEF can maintain pH stability to avoid a rapid drop in pH while achieving the same results as the 0°C storage, which this study's findings were consistent with those of Hsieh et al. (2011). Moreover, the pH decrease during storage was probably related either to the breakdown of glycogen, creatine phosphate, ATP, and lactic acid or the dissolution of carbon dioxide in fish muscles; while the carbon dioxide could be absorbed through the formation of carbonic acid and cause

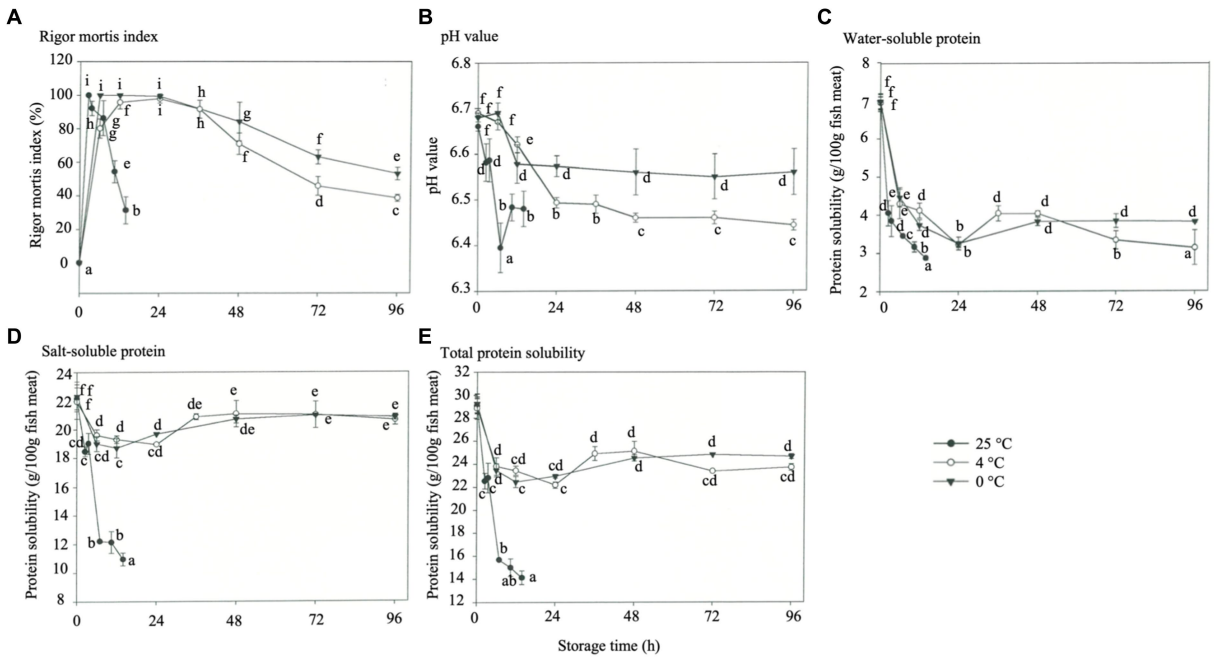

FIGURE 1
Effects of different storage temperatures on the physicochemical properties of rainbow trout. **(A)** Rigor mortis index. **(B)** pH value. **(C)** Water soluble protein. **(D)** Salt-soluble protein. **(E)** Total protein solubility.

acidification (Ekonomou et al., 2020). Meanwhile, the subsequent increase in pH during storage was probably attributed to the growth of lactic acid bacteria and the production of volatile base substances (ammonia, trimethylamine, and other biogenic amines) by microbial or endogenous enzymes due to protein degradation (Jemmi and Keusch, 1992; Hsieh et al., 2011; Ko et al., 2016).

3.3. Effects of storage temperatures on protein solubility

One of the most common methods for assessing protein denaturation and aggregation is determining protein solubility (Mohsenpour et al., 2023). The effect of storage temperature on water-soluble protein (Figure 1C), salt-soluble protein (Figure 1D), and total protein solubility (Figure 1E) of rainbow trout was examined. Proteolysis represents an essential biochemical phenomenon in post-mortem fish and is fundamental in developing the tenderness and flavor of processed products (Delbarre-Ladrat et al., 2006). In this study, the water-soluble protein showed a dramatic decrease in solubility at the maximum post-mortem rigor mortis less than three storage temperatures (Figure 1C). When rigor mortis began to disappear, the solubility of water-soluble proteins increased only under 25°C storage ($p < 0.05$). This phenomenon can be attributed to muscle contraction during rigor mortis. Because the ribosomes (composed of many ribosomal protein subunits) in myofibroblasts are subjected to mechanical damage, cold stress, free radical attack, and other factors during refrigeration, these proteins are degraded, dissociated, or oxidized, while leads to the formation of differentially abundant proteins (including myosin-binding proteins, filamins, actinin, troponin, and muscle-restricted coiled-coil protein) (Shui et al., 2022). With the release of muscle rigidity, the water-soluble protein solubility increased again because of the weakening of muscle tissues. Under 4 days of storage, the solubility of the protein decreased again. During storage, the muscles' free fatty acids and lipid oxides react hydrophobically with the protein surface, forming a hydrophobic zone that decreases the solubility (Giovambattista et al., 2008).

The solubility of salt-soluble proteins showed a decreasing trend during maximum rigor mortis under all three storage temperatures (Figure 1D). This phenomenon was presumably caused by forming a large molecular actin complex with myosin precipitated during centrifugation, leading to a decrease in solubility. However, the solubility of salt-soluble proteins showed a rebound at the initial stage of released rigor mortis, and this behavior can be attributed to the weakening of inter-actin bonding. Nevertheless, a slight decrease in solubility was observed at 4 days of storage, probably due to the initialization of protein denaturation. The solubility of salt-soluble proteins decreased under storage at 25°C while the muscles were unstiffened due to the denaturation of some proteins at this temperature. The solubility of total proteins decreased continuously under storage at 25°C, and that under 4°C and 0°C decreased during rigor mortis but increased at the beginning of resolution of rigor mortis (Figure 1E) ($p < 0.05$). However, this parameter decreased when the storage time increased, and the proteins denatured slightly, which was mainly evident under 4°C storage. Monovalent cations (such as Na^+ and K^+) have a decisive inhibitory effect on proteases (myostatin, dipeptidase, and aminopeptidase) that maintain muscle structure and preserve texture. Alternatively, transglutaminase activity was increased in NaCl, improving muscle firmness, cohesion, and elasticity in the product (Gomes et al., 2021). Some studies showed that high-voltage electric fields reduced the solubility of proteins in samples by affecting the structure of proteins (secondary and tertiary) or by degrading hydrogen bonds (Mohsenpour et al., 2023).

3.4. Gel strength properties

The gel was formed by the dissolution and dispersion of the actin of myofibril by salt, then hydrated in actin and denatured by heating, forming a 3D reticular gel (Levitsky et al., 2008). The effects of storage temperature on the gel breaking stress (Figure 2A), breaking strain (Figure 2B), and gel strength (Figure 2C) of rainbow trout gels were determined, and the results represented the hardness and elasticity of the gels. These two factors collectively indicate the strength of the gel. The gel-breaking stress, breaking strain, and gel strength decreased in all three storage temperatures when the muscles were rigid (Figures 2A–C), probably due to the rigid bonds formed by the muscle fibers. As a result, the actin and myosin were less soluble, which caused a poor gel structure with heating. The lowest gel strength was observed during the maximum rigor of rainbow trout stored at 25°C ($p < 0.05$), which was assumed to be caused by the synergistic effect of muscle rigor and thermal denaturation. For rainbow trout, the gel-breaking stress, breaking strain, and gel strength decreased slightly at three storage temperatures when the muscles started to unstiffen. This phenomenon can be attributed to the severe rigidity bonding between the myofibers and the weakening of muscle tissue during storage (Chan et al., 2022). Notably, HVEF treatment avoided degradation of proteins (myosin and actin) in the filets (Xie et al., 2021), and Qian et al. (2022) reported that treatment with 10 and 15 kV/m slowed the deterioration of texture properties (hardness and elasticity) during refrigeration, presumably related to protease activity inhibition.

Moreover, a slight increase in actin and myosin solubility was observed in the post-rigor period. The gel breaking stress, breaking strain, and gel strength decreased again at the end of rigor mortis, possibly due to endogenous enzymes and storage temperature causing the gradual denaturation of muscle proteins. The higher the storage temperature, the more evident is the phenomenon.

3.5. Water-holding capacity

The water content supports a range of biochemical, microbiological, and physical reactions that affect fish's sensory, nutritional, and functional properties during processing and storage. Thereby, the WHC serves as an indicator to assess the quality of fish (Chan et al., 2022; Mohsenpour et al., 2023). The effects of storage temperature on the WHC of rainbow trout were observed (Figure 2D). The results showed that the WHC of rainbow trout muscle decreased during rigor mortis at all three storage temperatures. The most significant decrease in WHC was observed at 25°C and 0°C due to heat and cold shrinkage ($p < 0.05$). When rigidification was resolved, the WHC at 25°C continued to decrease. By contrast, the WHC at 0°C and 4°C regained stability, except for the decrease caused by protein denaturation within 48 h ($p < 0.05$). In addition, Huang et al. (2020) reported improved WHC of catfish filets with HVEF treated during

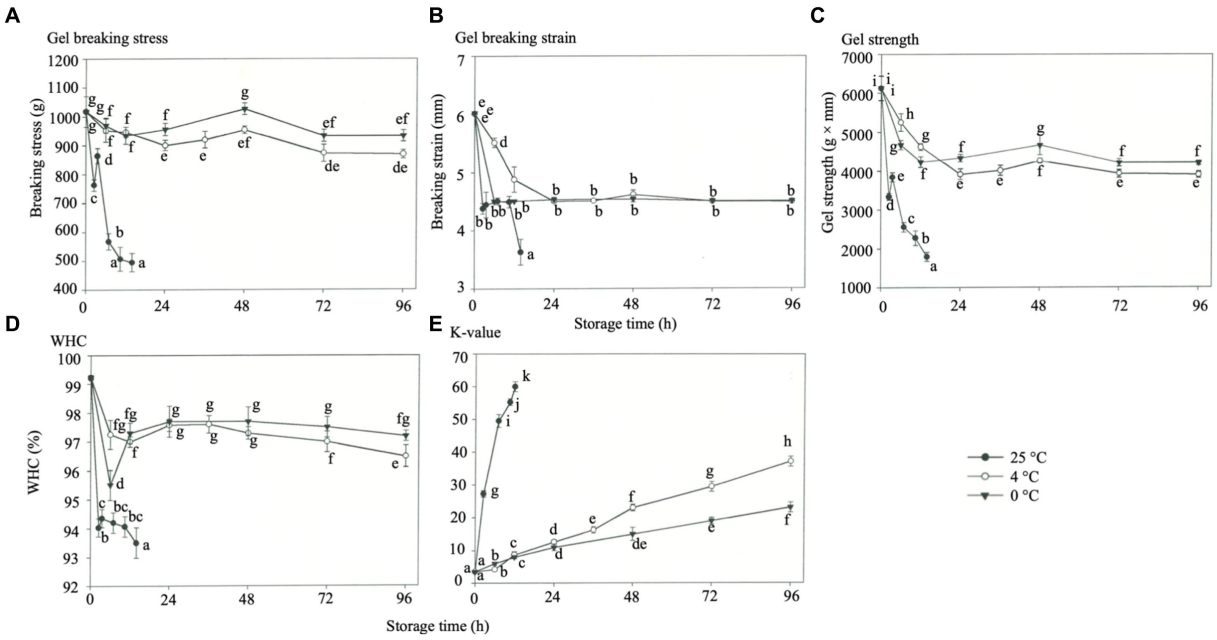

FIGURE 2
Effects of different storage temperatures on the gel strength, water holding capacity (WHC), and K-value of rainbow trout muscles. **(A)** Gel breaking stress. **(B)** Gel breaking strain. **(C)** Gel strength. **(D)** WHC. **(E)** K-value.

storage at 4°C, related to smaller overall gaps between muscle fibers. The principal fluid loss in fatty fish, such as salmon and rainbow trout, mainly comprises water because the WHC prevents fluid and potential nutrient loss while increasing product yield throughout the entire value chain, thereby improving quality (Ofstad et al., 1995). For producers, a high WHC results in low drip loss and great protein functionality and profitability, which can be translated to a good appearance, juiciness, and texture (Chan et al., 2022).

3.6. K-value and content of ATP with its related compounds

K-value determines the freshness and spoilage of aquatic products according to the variation of nucleotides in post-mortem fish (Prabhakar et al., 2020). Notably, less than 20% K-values would be considered "sashimi" quality, while values from 20 to 60% would be acceptable, while 60% represents an unacceptable threshold (Hsieh et al., 2011). The effect of storage temperature on the K value of rainbow trout was determined (Figure 2E), and the K-value of fresh rainbow trout was around 4.5%. The maximum K-value of rigor mortis was about 12% for 24 h storage at 4°C and 0°C, respectively, while increasing to 26.5% for storage at 25°C. Regardless of the storage temperature, the K-value of rainbow trout showed a significant increase at the end of rigor mortis. However, the K-value of rainbow trout increased to 61% when stored at 35°C after 14 h, reaching the level of spoilage, and increased to 23 and 37% when stored for 4 days at 0°C and 4°C, respectively ($p < 0.05$), while this study's results agreed with Hsieh et al. (2011). Despite treatment with the higher HVEF (300–900 kV/m), the fish's K-value deteriorated after the fourth day (Ko et al., 2016). Interestingly, the high HVEF (600–900 kV/m) treatment on fish filets has been reported to affect the metabolic reactions with reduced ATP degradation, protein denaturation, microbial growth, and oxidative degradation, which effectively extended the shelf life of fish filets at 4°C for at least 8 days (Ko et al., 2016). The above results showed that the K-value of rainbow trout increased slowly during rigor mortis when stored at 4°C and 0°C. A possible increase in endogenous enzymes caused by the weakening of muscle tissues enhanced nucleotide breakdown at the end of rigor mortis (Nie et al., 2022). The K-value showed a relatively significant increase after the fish reached maximum rigor mortis (Okuma and Abe, 1992). When stored at 25°C, the rainbow trout showed a linear, rapid increase in muscle status and K-value due to microbial growth, thermal denaturation, and endogenous enzyme activation.

Watabe et al. (1989) showed that the ATP content of fish at maximum post-mortem rigor mortis typically decreased to less than 1 μmol/g, and ADP and AMP showed a consistent decreasing trend. Moreover, IMP accumulated to the highest level at maximum rigor mortis in all the fish stored at 25°C. The ATP and ADP of fish disappeared rapidly, and IMP accumulated mainly within a short period post-mortem (Prabhakar et al., 2020). IMP contributes significantly to the taste of aquatic products (Sarower et al., 2012). The ATP content of rainbow trout decreased significantly at the initial stage at all three storage temperatures (Figures 3A–F). This study showed that rainbow trout stored at low temperatures of 0°C and 4°C had better taste during post-mortem rigor mortis than fish stored at other temperatures ($p < 0.05$). Therefore, applying an electric field to filets may influence their metabolic reactions and reduce the degradation of ATP (Ko et al., 2016). However, the IMP content decreased with the end of rigor mortis over 24 h of storage, and the content of spoilage factors inosine and hypoxanthine rapidly increased, indicating that the degree of freshness decreased quickly. Okuma and Abe (1992) reported that IP started degradation at maximum post-mortem rigor mortis with inosine and hypoxanthine

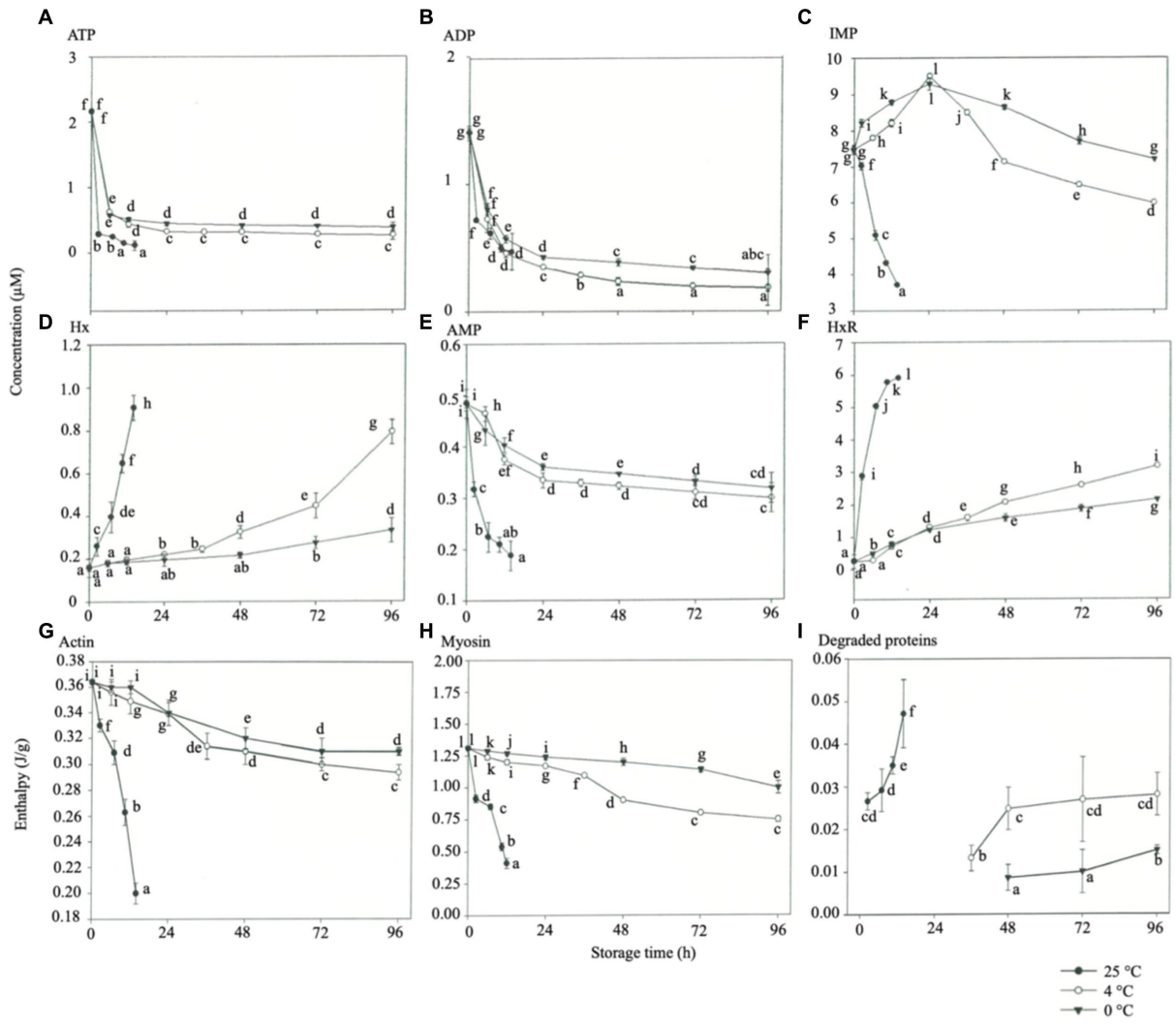

FIGURE 3
Effects of different storage temperatures on the content of ATP-related compounds and enthalpy of various proteins in rainbow trout. **(A)** ATP. **(B)** ADP. **(C)** IMP. **(D)** Hx. **(E)** AMP. **(F)** HxR. **(G)** Actin. **(H)** Mysoin. **(I)** Degraded proteins.

(Hx) formation. When the rainbow trout was stored at 25°C, the content of inosine and hypoxanthine increased rapidly and hence was the main reason for the rapid decrease in freshness.

3.7. The enthalpy changes in muscle

The DSC-measured enthalpy values express the net content of the protein structure (Minikel, 2014; LibreTexts, 2023). The majority of water in the muscle resides in the spaces of the muscle fibers (thin and thick filament interstices), so myosin and actin are associated with the WHC of the fish, the texture of the fish products, and the functional properties of the surimi (Offer and Trinick, 1983; Nikoo et al., 2019). The effects of storage temperature on the enthalpies of actin (Figure 3G), myosin (Figure 3H), and degraded proteins (Figure 3I) of rainbow trout muscle were observed. The thermal denaturation temperatures of actin and myosin were 75°C and 53°C, respectively, which gradually increased to 85°C and 63°C, respectively ($p < 0.05$), while the rainbow trout was in post-mortem rigor mortis. Regardless of the storage temperature for the rainbow trout, the heat absorption wave similar to that of degraded proteins appeared at 42°C. This finding may be attributed to the formed rigidity of the muscle fibers as a potent complex of actin and myosin following the increase of storage time and the arrival of multiple heat absorption peaks between 20°C and 50°C presumably due to the thermal denaturation and enzymatic hydrolysis of the proteins into small molecule proteins. The enthalpy of actin and myosin in the rainbow trout decreased smoothly during rigor mortis at 4°C and 0°C storage ($p < 0.05$). It was significantly reduced at the end of rigor mortis, with the most apparent protein denaturation occurring at 4°C storage and a rapid decrease in the enthalpy of actin and myosin at 25°C storages ($p < 0.05$). Based on the above results, the proteins are less likely to be denatured during rigor mortis, and protein denaturation would occur significantly as rigor mortis progressed. In addition, the degradation of proteins occurred at 2.5, 36, and 48 h under storage at 25°C, 4°C, and 0°C, respectively, and the heat absorption peaks of proteins increased with the storage temperature (Figure 3I) ($p < 0.05$). The possible reason might be the increased activity of endogenous enzymes in rainbow

trout at high temperatures, resulting in pronounced protein hydrolysis (Moeller et al., 1977). Kaneniwa et al. (2000) and Kaneniwa et al. (2004) showed that the enzymatic hydrolysis of muscle proteins in carp and rainbow trout occurred more significantly under storage at 20°C than at 0°C.

3.8. Protein molecular composition via SDS-PAGE

The protein compositions of rainbow trout stored at 25°C (Figure 4A), 4°C (Figure 4B), and 0°C (Figure 4C) were analyzed. The results showed that the molecular weight of rainbow trout's 66 kDa protein belt decreased after storage at 25°C for 14 h. The molecular weights of 37.9, 35.6, 33.3, 19.5, and 6.5 kDa protein belts also decreased after 10.5 h. The most significant disappearance was observed for the 6.5 kDa protein. Furthermore, the molecular weights of 66, 37.9, 35.6, and 33.3 kDa protein belts decreased after storage at 4°C for 96 h. At 72 h, the molecular weights of 19.5 and 6.5 kDa protein belts decreased, and the rate of the disappearance of 6.5 kDa proteins was the most significant. No significant changes in either protein belt were observed for storage at 0°C for 96 h. Bauchart et al. (2007) reported few protein hydrolyses in rainbow trout muscle, remarkably preserved at 0°C, which agreed with this study. Therefore, it is possible that the degeneration of the myofibrillar proteins was caused by the rigor mortis of the rainbow trout, in which the protein contents were best maintained at 0°C, followed by 4°C during preservation. However, the 30 kDa protein formed by troponin-T degradation in post-mortem muscle during storage has been reported (Ho et al., 1994; Gagaoua et al., 2021). Moreover, several studies have reported that the myofibrillar proteins degraded to myosin heavy chain (MHC) (~200 kDa), which gradually degraded or even disappeared with time, where the protein belts of 66 kDa and 33.3 kDa were probably the degradation products of MHC (Ge et al., 2018). In addition, the other proteins were para myosin (~100 KDa), tropomyosin (37.9 KDa), troponin-T (35.6 kDa), and Myosin Light Chain (MLC ~ 17 KDa) (Wang et al., 2011; Li et al., 2017), which were also consistent with this study. A similar phenomenon was observed for stored goat muscle in which MHC, troponin T, desmin, and actin were lost by the hydrolysis by endogenous enzyme in the muscles (Xiao et al., 2022). The disappearance of protein belts may be attributed to the hydrolysis of endogenous proteolytic enzymes in muscles, which was consistent with the findings of Alarcón et al. (2001) and Schreurs et al. (1995). Likewise, under refrigerated conditions, the dissociation and fragmentation of myofibrillar proteins occurred in muscle tissue resulting from endogenous cathepsin, calpain, several serine proteases, and bacterial growth (Ge et al., 2018; Xie et al., 2019; Shui et al., 2022). Alternatively, the high protein denaturation at high temperatures, even at low pH, induced high enzymatic hydrolysis activity (Shenouda, 1980; Xiong, 1997; Nielsen and Jørgensen, 2004; Quevedo et al., 2021). Incidentally, Zhang et al. (2021) have also reported that fresh ground pork at high salt (2 M KCl) raises the WHC of myofibrils such that the degeneration of myofibrillar proteins during freezing has not reproduced; in contrast, treatment with low pH and high salt decreased WHC; it increased the hydrophobicity of the surface, indicating that myofibrillar protein denaturation has occurred by a mechanism similar to freeze-thawing.

3.9. Observation of rainbow trout tissue sections

The tissue sections of rainbow trout during storage at 25°C (Figure 5A), 4°C (Figure 5B), and 0°C (Figure 5C) were observed. The fresh tissues were intact under storage at 25°C (0 h), while the gaps between the muscle bundles appeared after 2.5 h (post-mortem rigor mortis); subsequently, after 7 h (the end of rigor mortis), the gaps widened and increased, causing the separation of connections between the muscle bundles (Figure 5A). The rainbow trout showed small gaps in the muscle fibers when stored at 4°C for 24 h (rigor mortis stage). At the end of rigor mortis stage, the gaps increased gradually. After 96 h, the muscle fibers partially separated (Figure 5B), consistent with the above results (Figure 5A). The fish stored at 0°C for 24 h (rigor mortis phase) showed minimal gaps in the muscle fibers compared with those stored at 25°C and 4°C (Figure 5C). According to the report by Huang et al. (2020), the muscle fiber gap in catfish filets

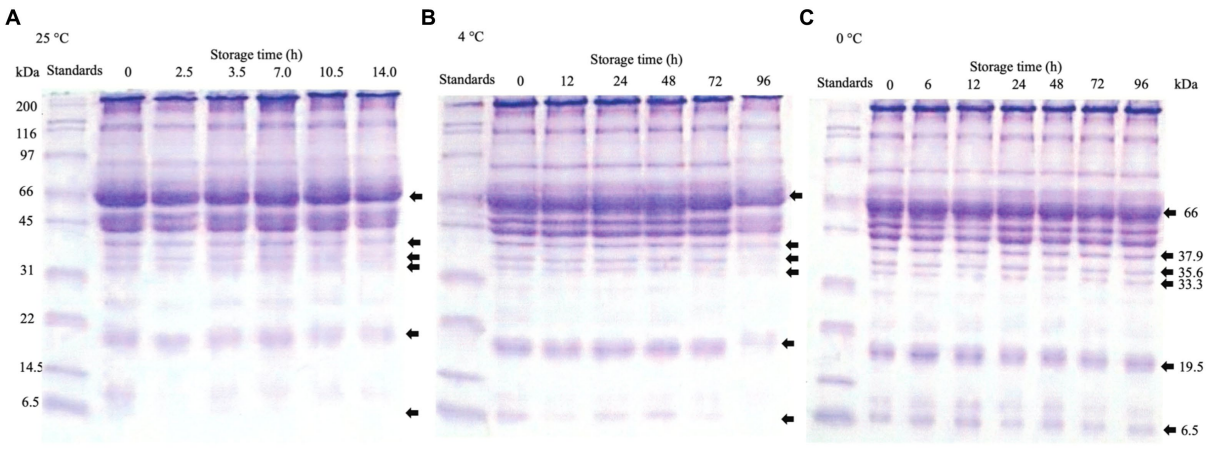

FIGURE 4
Effects of different storage temperatures on the SDS-PAGE analysis of rainbow trout proteins. **(A)** 25°C. **(B)** 4°C. **(C)** 0°C.

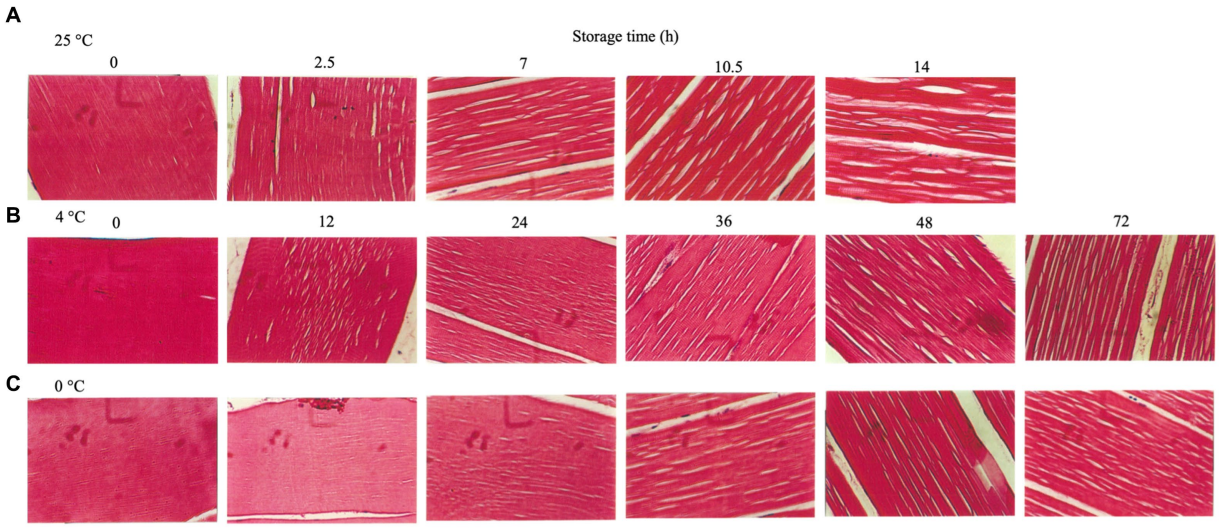

FIGURE 5
Muscle tissue sections of rainbow trout stored under different temperatures. **(A)** 25°C. **(B)** 4°C. **(C)** 0°C.

decreased significantly after undergoing HVEF treatment for 15 min and being stored at 4°C for 0, 3, and 7 days, while the discovery differs from this study, possibly due to differences in fish species, treatment intensity, and duration. Moreover, at the end of rigor mortis, the gaps in muscle fibers increased with storage time. Nevertheless, the muscle tissue integrity was significantly better when stored at 0°C than at 25°C and 4°C. The above results showed that the muscle trout in rigor mortis maintained its muscle tissues' integrity except for those stored at 25°C. However, spaces between the muscle fibers began to appear, which might be caused by the cavitation effect (Ma et al., 2021). With the end of rigor mortis, the separation of the muscle fibers became evident. The increase in muscle fiber's interstitial spaces was mainly associated with disrupting the Z-lines and related structures (Delbarre-Ladrat et al., 2006). The degradation of connective tissues in fish muscles occurs during storage, and the hypothesized primary cause of muscle fiber interstices and muscle tenderization is the action of endogenous muscle enzymes, which causes the degradation of fish structural proteins (Hansen et al., 1996; Paludan-Müller et al., 1998; Nielsen and Jørgensen, 2004). Therefore, the muscular endogenous enzymes begin operating as soon as the fish dies. In addition, the end of rigor mortis promotes the action of endogenous enzymes with increased muscle tissue disintegration and denaturation (Warner, 2016). Hence, preservation temperature should be an important factor.

3.10. Effects of storage temperature on the TVC and psychrophilic bacteria

The shelf life may be reduced by 50% when the storage temperature increases from 0°C to 4°C because bacterial growth, which causes fish spoilage, is significantly affected by the storage temperature (Lorentzen et al., 2020). Moreover, Genç and Diler (2019) reported the predicted shelf life of rainbow trout with different storage temperatures of 2°C, 10°C, and 20°C as 8–15.7 days, 2.3–4.5 days, and 1–1.8 days, respectively, while the actual shelf life shortened rapidly. The effect of storage temperature on the TVC (Figure 6A) and psychrophilic bacteria (Figure 6B). TVC of fresh fish (0 h) was about 3.2 log CFU/g. Under 25°C storage, this value increased sharply with the storage time, reaching 6.45 log CFU/g after 14 h ($p < 0.05$). TVC increased slightly for samples stored at 4°C and 0°C for 96 h, with recorded readings of 3.56 and 3.40 log CFU/g, respectively (Figure 6A). During storage for 24 h, psychrophilic bacteria exhibited outstanding proliferation in the fraction compared to 0°C, at 4°C, with a significant difference ($p < 0.05$). However, the proliferation of psychrophilic bacteria in rainbow trout showed an increasing trend after 36 h of storage at 4°C, with 3.72 and 4.13 log CFU/g at 48 and 96 h, respectively ($p < 0.05$) (Figure 6B), implying a significant difference at 36–96 h ($p < 0.05$). HVEF treatment cannot wholly inhibit microorganisms, but it effectively reduces the growth rate of microorganisms during the shelf life of fish filets (Hsieh et al., 2011; Ko et al., 2016). Compared with medium- and high-temperature bacteria, psychrophilic bacteria are sensitive to temperature change.

Chytiri et al. (2004) have been indicated that the mesophilic counts of fileted and uncut rainbow trout were over 7 log CFU/cm^2 after 10 and 18 days of ice storage. Meanwhile, more biotic bacteria were found in fileted fish during storage than in whole unshelled trout, probably due to the cross-contamination of trout samples during fileting; similar results were reported by Tavakoli et al. (2018). Temperature fluctuations also accelerate the loss of polyunsaturated fatty acids and increase unpleasant odors associated with water migration (Yu et al., 2020). In practice, this realization is difficult to achieve because the cold chain in transportation operations between the producer and the final consumer is regularly interrupted (Skawińska and Zalewski, 2022). Hence, attention should be paid to the temperature change during the low-temperature storage of rainbow trout.

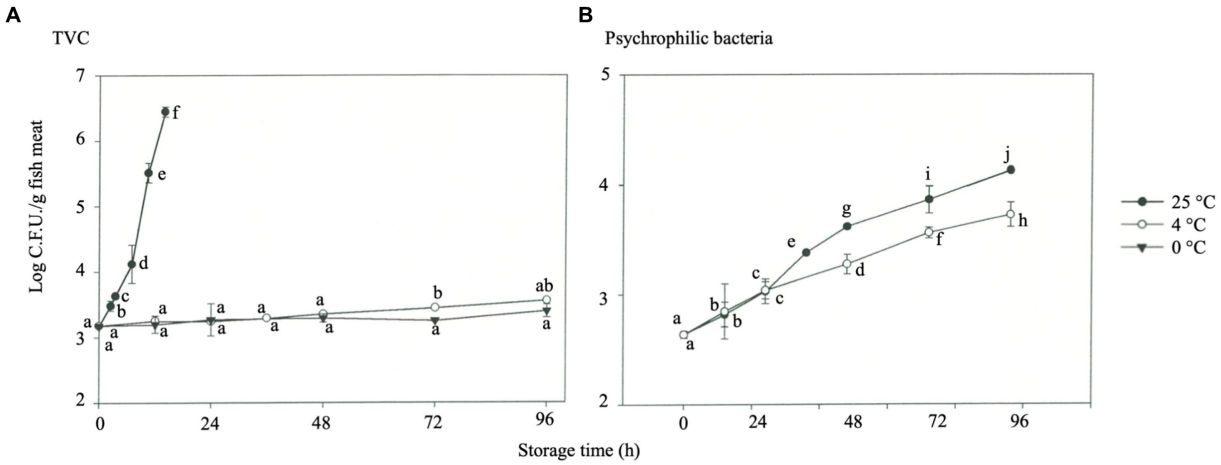

FIGURE 6
Microbial growth in rainbow trout stored under different temperatures. **(A)** TVC. **(B)** Psychrophilic bacteria.

4. Conclusion

This study found that rainbow trout stored at different temperatures underwent post-mortem rigor mortis and resolution of rigor mortis at different rates, leading to changes in rainbow trout muscles. The rainbow trout specimens stored at 25°C and 0°C were more susceptible to rigor mortis than those stored at 4°C due to shrinkage effects (heat and cold). In addition, both 4°C (treated with 100 kV/m HVEF) and 0°C storage only effectively inhibited the growth of TVC and psychrophilic bacteria. However, the post-mortem rigor mortis, which occurred under 4°C and 0°C storage, caused the degradation of the total protein solubility and WHC of the fish, serving as a warranty for good freshness and quality. Moreover, the endogenous enzymatic activity of rainbow trout at 25°C was higher than that at 4°C and 0°C storage, resulting in easy resolution of rigor mortis accompanied by microbial growth, while spoilage also occurred within 14 h at 25°C storages. By contrast, rainbow trout's freshness and hygienic quality were within the acceptable range when stored at 4°C (with HVEF at 100 kV/m) and 0°C for 4 days. This study revealed rainbow trout's physicochemical and microbiology after storage at different temperatures and treated methods. These findings provide researchers and producers with valuable information for scheduling production schedules for stable products, extending food shelf-life through processing (e.g., smoked or frozen after conditioning), and expanding the opportunities for aquaculture development.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

Y-TC, P-HH, W-CL, S-CC, P-MW, W-CK, and P-HL: conceptualization. P-HL: data curation. P-HH, W-CL, S-CC, P-MW, and P-HL: formal analysis and investigation. Y-TC and W-CK: funding acquisition. P-HH, W-CL, P-MW, and P-HL: methodology and validation. Y-TC and W-CK: project administration. Y-TC, W-CL, S-CC, and P-HL: resources. Y-TC, P-HH, W-CL, S-CC, P-MW, and P-HL: software. W-CK: supervision and visualization. P-HH and P-HL: writing – original draft and writing – review & editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The application of microwave and ultrasound technologies for extracting collagen from European plaice by-products

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This study's main aim was to utilize green extraction protocols to recover collagen from by-products originating from European plaice. Moreover, the objective was to evaluate pre-treatments, the composition of the up-cycled product as well as to identify the most promising by-product fraction. Microwave (MAE) and ultrasound-assisted extractions (UAE) were performed on untreated, pre-treated (salt-washed or enzymatic hydrolysis) fractions of backbones, skins, and heads. Both MAE and UAE were performed for 15 and 35 min. After pre-treatment and extraction, the quality and yield of products were evaluated. Protein and collagen concentration, as well as amino acid profiles, were measured. Skins deliver significantly higher yields of collagen and protein than heads and backbones ($p < 0.05$). Enzymatic hydrolysis resulted in the highest collagen yields for skins (77%), while salt-washing gave the highest results for backbones (43%) and heads (41%) regardless of extraction method and time. Total and free amino acid profiles differed between the three fractions, with backbones and heads showing overall more similarity in composition compared to skins. The study showed that MAE and UAE technologies are suitable for generating collagen from marine by-products. Additional research is recommended to optimize pre-treatment and extraction for skin, as most promising collagen supplier.

KEYWORDS

green extraction methods, marine collagen, by-product valorization, upcycling, side-streams utilization, innovative collagen extraction

1. Introduction

Global fish production reached a substantial 176 million tonnes in 2020, of which 157 million tonnes were used for direct human consumption and an increasing share of fish getting further processed and not sold as whole fish (FAO, 2022). This leads to a high amount of by-products, which can reach up to 70% of the total mass of the fish. By-products refer to the portions of the catch that are not considered the main saleable product, including skins, scales, heads, viscera, backbones, and fins (Ozogul et al., 2021). Europe has increased its by-product utilization significantly in the past year, however, the main share is used for low-value applications like fishmeal, fish oil, biogas or fertilizers (FAO, 2020). However, over the past few decades, extensive research has revealed that these by-product fractions contain valuable components that can be further up-cycled into products for human consumption (Al Khawli et al., 2019; Ozogul et al., 2021). Especially collagen has versatile fields of applications, such as a functional food ingredient or stabilizer in food, as biomaterial to regenerate skin and

bones as well as applications in cosmetics as skincare products, proven to exhibit bioactive properties (Avila Rodríguez et al., 2018; Lu et al., 2023). Moreover, collagen, in its application as a novel functional food ingredient, contains valuable essential amino acids which positively impact the nutritional quality of the product and moreover exhibit bioactive properties suitable for application as natural antioxidants (Pal and Suresh, 2016).

The demand for a more sustainable global food production system prompted the European Union (EU) Commission to launch the Circular Economy Action Plan in 2020, building upon strategies outlined in the European Green Deal of 2015. This plan aims to establish a framework that promotes the norm of sustainable products and contributes to achieving the 2030 Sustainable Development Goals (Commission and Communication, 2020). Especially novel technologies, such as green extraction procedures for obtaining bioactive components from marine by-products have attracted considerable interest in recent years, due to their reduced impact on the environment and decreased economic waste (Ozogul et al., 2021) Pal and Suresh (2016) list ultrasonic (ultrasound), microwave, super critical fluids and high pressure as emerging green extraction methods for marine collagen, but also states that these extractions require heating which potentially affect the structure of marine collagen. Previously, ultrasound-assisted extraction has been used to extract collagen from marine by-products (Ali et al., 2018; Shaik et al., 2021; Lu et al., 2023). Moreover, chitin and chitosan from shrimp shells have formerly been generated by microwave-assisted extraction (El Knidri et al., 2019; Santos et al., 2019). Ozogul et al. (2021) state that the extraction of marine collagen is most efficiently performed with organic acids or with enzymatic hydrolysis. However, to our knowledge, the research and knowledge on the utilization of ultrasound or microwave-assisted extraction on generating collagen from fish by-products has not been followed-up to date.

Thus, the present study aimed to investigate the utilization of green extraction technologies for valorizing three by-product fractions derived from European plaice (*Pleuronectes platessa*). Specifically, the focus was on extracting collagen from backbones, skins and heads using microwave or ultrasound-assisted extraction. Next to collagen, the total protein content and amino acid profiles were investigated. Additionally, the study examined the effects of enzymatic hydrolysis or salt-washing prior to extraction as suitable pre-treatments. By exploring these objectives, the study aimed to provide insights into optimal green extraction procedures and conditions for obtaining collagen from different by-product fractions of European plaice.

2. Materials and methods

2.1. Raw material and experimental design

Skins, heads and backbones from European plaice (*Pleuronectes platessa*), previously collected during the study of Kendler et al. (2023b), were separated, homogenized and kept frozen at −80°C until further analyses. Samples underwent pre-treatment by salt-washing or enzymatic hydrolysis or were used untreated for further extractions by microwave or ultrasound technologies, each for 15 and 35 min (MAE15, MAE35, UAE15, and UAE35). After extraction, samples got lyophilized for 24 h at −50°C and 13.3 Pa and frozen prior to further analyses.

2.2. Pre-treatments

2.2.1. Salt pre-treatment

The salt pre-treatment followed the method of Kołodziejska et al. (2008) with minor modifications. Minced raw material was thawed at 4°C. A 0.45 M NaCl-solution (6:1 v/w) was added and shaken with the material for 10 min at 4°C. The solution was filtered, and the salt-wash was discarded. Steps one and two were repeated two more times. The material was then washed in cold tap water. Next, the material was shaken in a 10% ethanol solution (6:1 v/w) for 30 min at 4°C, filtered again, and subsequently washed in cold tap water. The salt pre-treated material was stored at 4°C until extraction with microwave or ultrasound.

2.2.2. Enzymatic hydrolysis

Enzymatic hydrolysis was performed by following an in-house protocol, as described by Hjellnes et al. (2021, p. 26), using the peptidase Alcalase 2.4 L (provided by Novozymes AS, Denmark) as the catalyst. The hydrolysis was carried out in a laboratory-scale bioreactor (Model No. 2101000; Syrris Atlas) equipped with a stirring unit, thermostat, and pH meter.

Pre-heated water (50°C) and raw material (1:1% ww) were added and stirred in the reactor. Once the mixture reached 50°C, a portion was taken out as a 0-sample (prior to enzyme addition) and deactivated in a 90°C water bath for 5 min with stirring. Alcalase 2.4 L was then added to the remaining sample (0.1% of weight), and the stirring was initiated. Samples were taken out at 60 min and deactivated as described earlier. The pH was maintained at 7.0 throughout the hydrolysis, with NaOH added if necessary. After 60 min, the hydrolysis was stopped.

All samples were centrifuged and frozen overnight at −40°C. The following day, the frozen samples were divided into three fractions: fat, protein hydrolysate, and sediment. The weight of each fraction was recorded for yield calculation, and the sediment and protein hydrolysate fractions were further analyzed. To determine which fraction contained more collagen for subsequent extractions, both fractions were lyophilized and assessed for collagen content.

2.3. Green extraction technologies

Both untreated and pre-treated raw material was subjected to microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE).

2.3.1. Microwave-assisted extraction

Microwave-assisted extraction (MAE) was conducted with a MARS 6 microwave digestion system (CEM Corporation, NC), equipped with 30 digestion vessels. About 30–40 g (ww) raw material was homogenized (Polytron PT3100; Kinematica) at 10000 rpm for 1 min and subsequently mixed with deionized water (6:1 v/w). For hydrolysates, 2–3 g lyophilized samples were used and deionized water was added (6:1 v/w according to wet weight of hydrolysates). The water-sample mixture was shaken vigorously to mix water and hydrolysate. Finally, the material was added to the digestion vessels and following parameters were chosen for each extraction round: ramp time (5 min), hold time (15 or 35 min), temperature 40°C, power (350 W), stirring (medium speed).

2.3.2. Ultrasound-assisted extraction

For the Ultrasound-assisted extraction (UAE), an ultrasonic cleaning bath, of type USC-T (VWR) was used. The material was prepared, and same sample amount used as described in section 2.31. Basic 50 mL centrifuge tubes were used for extraction. The ultrasound water bath was pre-heated to 40°C and the centrifuge tubes were placed in the water bath when the temperature reached 35°C to obtain a ramp time of 5 min, like MAE.

After water, MAE or UAE extraction, the samples were centrifuged (34,000 g, 10 min, 20°C) and the two obtained phases, being the sediment and soluble protein phase were separated and weighted. The soluble protein phase was then lyophilized for further analyses.

2.4. Chemical analyses

2.4.1. Total protein and amino acid profile

The determination of total crude protein (%) was carried out using the Kjeldahl method (AOAC, 1990), using a Kjeldahl apparatus equipped with a digestion and auto titration unit (K-449 and K-375, Büchi Labortechnik, Schwitzerland). The procedure was carried out as previously described in Kendler et al. (2023a,b). A conversion factor of 6.25 x nitrogen (%) was applied to determine the protein content of samples (NMKL, 2003).

The determination of the total amino acid (TAA) distribution (%) followed the method of Blackburn (1978) utilizing acid hydrolysis. The procedure was previously described in detail by, Kendler et al. (2023a,b).

The method from Osnes and Mohr (1985) was followed to determine the free amino acid (FAA) distribution (%). The sample preparation was previously described in Kendler et al. (2023a,b).

The HPLC analysis for TAA and FAA distributions (%) was conducted by a ultra-high-performance liquid chromatography system (HPLC, UltiMate 300, Thermo Fisher Scientific, United States). The same HPLC system setup as previously described in detail by Kendler et al. (2023a,b) was used.

2.5. Collagen content

Collagen is a protein composed of repetitive triple helixes with proline and hydroxyprline as composites Zanaboni et al. (2000). Which is why the determination of hydroxyproline in section 2.5.1 gives information of the total collagen content.

2.5.1. Hydroxyproline

The determination of hydroxyproline content is based on the method of Leach (1960). An L-hydroxyproline stock solution was used (0.05 g hydroxyproline in 400 mL distilled water, 20 mL concentrated HCl, adding distilled water until volume reached 500 mL). From the stock solution, four standards of 2.5, 5.0, 10.0 and 15.0 µg hydroxyproline/mL were prepared.

The absorbance of the standard and sample solutions were measured against a blank using a GENESYS 10S UV–VIS Spectrophotometer (Thermo Fisher Scientific Inc., Unite States) at a wavelength of 555 nm.

A standard curve based on the absorbance of the standards was used to calculate the concentration of hydroxyproline in samples. The collagen content was then determined by using the equations 1–3 with the species specific collagen conversion factor of 9.6 for flounder (Sikorski et al., 1984).

$$Hyp_{content}\left(\frac{mg\ collagen}{ml\ hydrolysed\ sample}\right)=\frac{(OD-b)\cdot DF}{a} \quad (1)$$

$$Hyp\%of\ freeze\ dried\ sample=\frac{Hyp_{content}\cdot V}{m\cdot 10^{6}}\cdot 100\% \quad (2)$$

$$Collagen\%of\ freeze\ dried\ sample=Hyp\%\frac{100}{CF} \quad (3)$$

Where:

OD, Optical density (absorbance from spectrophotometer); DF, Dilution factor; a, A-value from standard curve (y = ax + b) (y = absorbance, x = hyp-content); b, B-value from standard curve; V, mL sample after acid hydrolysis; m, Mass of lyophilized sample for hydrolysis; *CF*, Conversion factor Statistics.

2.6. Statistics

All analyses were performed in triplicates, if not other stated (n = 3). The data from the analyses was tested by a General Linear Model (GLM) using the software IBM SPSS (release 28, IBM Corporation, United States). Where applicable, analysis of variance (ANOVA) was carried out, and when significance detected in the GLM or ANOVA, a Tukey HSD *post hoc* test was carried out to investigate the differences between groups. The α-level was set to 0.05.

3. Results and discussion

3.1. Pre-assessment of raw material quality and fractions

Analyses of the three raw material fractions prior to pre-treatment and extraction showed significant differences in their initial proximate composition. The skins protein content of 17.37 ± 1.4 g/100 g ww (wet weight) is significantly higher ($p < 0.001$) compared to backbones (11.88 ± 0.4 g/100 g ww) and heads (12.57 ± 0.8 g/100 g ww). Additionally, the skins ash content (1.31 ± 0.03 g/100 g ww) is significantly lower ($p < 0.001$) than the content of the backbones (5.44 ± 1.06 g/100 g ww) and heads (5.41 ± 1.5 g/100 g ww), which can be explained by the higher content of inorganic matter in backbones and heads. No significant differences in lipid and moisture content were observed. Moreover, the initial collagen content of skins (32.54 ± 0.4 g/100 g dw; dry weight) is significantly higher ($p < 0.001$) compared to backbones (18.71 ± 1.1 dw) and heads (12.73 ± 0.2 g/100 g dw) which is of special relevance for the extraction of collagen.

Analyses on the collagen content of sediment and protein hydrolysates generated through enzymatic hydrolysis showed that skin protein hydrolysates contain collagen of 52.07 ± 13.0% dw followed by heads protein hydrolysates with 34.63 ± 12.1% dw. Moreover, the collagen content in the sediment fractions of skins (28.3 ± 3.0% dw) and heads (13.2 ± 4.8%) were significantly lower ($p = 0.043$) than for

their respective protein fraction. The lowest collagen content was found in the sediment fraction of backbones (12.03 ± 1.7% dw). Following these results, merely the protein hydrolysate fractions were considered for further MAE and UAE, to guarantee the highest possible yield.

3.2. Total protein and collagen content after extraction

Analyzing the main effects of raw material, pre-treatments and extraction methods and times, it was found that regardless of pre-treatment, extraction and time, skins have the significantly ($p < 0.001$) highest collagen (%) and highest protein (%) concentrations of all three fractions. Moreover, pre-treatment could significantly increase collagen content ($p < 0.001$). In addition, the GLM demonstrated that both MAE and UAE, including both times, lead to a significant ($p < 0.001$) increase in the collagen (%) and protein content (%) compared to untreated raw material and without extraction.

Figure 1 shows the collagen and protein concentrations depending on pre-treatment (a, c) as well as after extractions with microwave or ultrasound for two different times (b, d). It is visible (Figure 1A) that skin yields the significantly highest collagen, regardless of pre-treatment ($p < 0.05$). Moreover, salt-washing is most effective in generating collagen for heads and backbones, whereas enzymatic hydrolysis yielded the most collagen for skins regardless of extraction method and time ($p < 0.05$). When looking at the different pre-treatments (Figure 1C) and their effect on the yield of protein of each raw material, it is visible that skins show significantly highest protein concentration for each pre-treatment. Thus, skins being the fraction with highest collagen and total protein yield regardless of pre-treatment. For both collagen and protein yield of skins, enzymatic hydrolysis was the most effective method. In addition, Figure 1B highlights that skins, regardless of extraction method and time, produce the highest collagen content ($p < 0.05$). Among the different extraction methods applied to backbones, MAE 35 has a significantly ($p < 0.05$) lower collagen content (27.96 ± 5.6%) compared to MAE 15, UAE 15, and UAE 35. No significant differences were observed among MAE 15, UAE 15, and UAE 35 for backbone as raw material. A different trend was observed for skins, where MAE 35 led to significantly higher collagen (75.58 ± 4.2%) compared to the lowest concentrations observed for UAE 15. Generally, the same trend for a suitable pre-treatment or extraction method and time was observed for backbones and heads, with enzymatic hydrolysis and UAE 35 yielding the highest protein concentrations for both fractions.

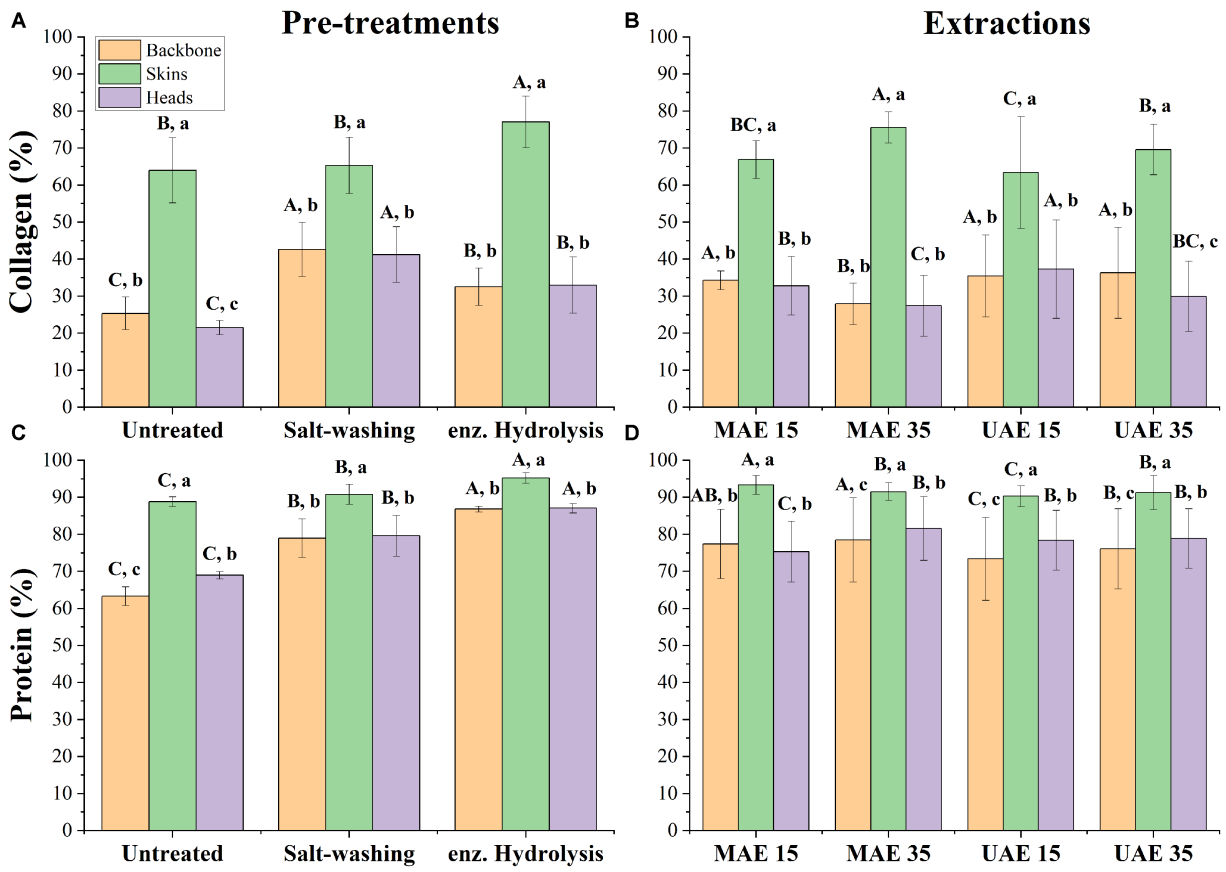

FIGURE 1
Overview of collagen and protein content (%) for backbones, skins and heads depending on pre-treatment in **(A,C)** and depending on extraction method in **(B,D)**; Error bars show SD. ANOVA was applied on raw materials for pre-treatments and extractions; where a significant difference between raw materials was detected ($\alpha < 0.05$), a Tukey HSD *post hoc* test was applied. Values with different small letters **(A,B)** are significantly different ($p < 0.05$) between raw material for each pre-treatment/ extraction; values with different big letters **(A,B)** indicate significant differences ($p < 0.05$) of pre-treatment/extraction on raw material.

3.3. Amino acid profile

Table 1 visualizes the TAA distribution of the three by-product fractions after extraction with MAE15, MAE35, UAE15, and UAE35. Pre-treatment by salt-washing or enzymatic hydrolysis both led to an increase in the TAA content of backbones ($p = 0.009$) and heads ($p < 0.001$) compared to no pre-treatment, while pre-treatments showed no difference in the composition for skins ($p = 0.418$). No between-subjects effects of extraction+time and pre-treatment for any of the three raw materials ($p > 0.05$) was observed. Moreover, the extraction method

TABLE 1 Total amino acid composition (mg/g) of backbones, skins and heads depending on extraction methods, regardless of pre-treatment; values presented as mean ± SD.

Raw material	Amino acids	MAE15	MAE35	UAE15	UAE35
	Essential AA	mg/g	mg/g	mg/g	mg/g
Backbones	Histidine	9.6 ± 2.2^{A}	9.6 ± 2.3^{A}	8.6 ± 2.0^{B}	9.1 ± 2.1^{AB}
Skins		$6.5 \pm 1.8^{b,B}$	$7.6 \pm 2.4^{ab,B}$	$7.8 \pm 1.3^{a,B}$	$8.2 \pm 2.6^{a,B}$
Heads		9.4 ± 1.5^{A}	9.9 ± 1.9^{A}	10.0 ± 1.2^{A}	10.5 ± 2.0^{A}
Backbones	Isoleucine	17.7 ± 5.0^{A}	17.5 ± 4.5^{A}	16.7 ± 4.0^{A}	15.3 ± 4.0^{A}
Skins		$10.4 \pm 4.4^{b,B}$	$11.3 \pm 3.3^{ab,B}$	$12.1 \pm 3.2^{ab,B}$	$12.8 \pm 3.1^{a,B}$
Heads		17.7 ± 4.5^{A}	17.2 ± 4.1^{A}	16.7 ± 4.2^{A}	17.7 ± 3.7^{A}
Backbones	Leucine	35.6 ± 7.6^{A}	34.4 ± 7.4^{A}	35.3 ± 7.3^{A}	33.8 ± 10.5^{A}
Skins		$23.3 \pm 8.1^{b,B}$	$24.9 \pm 9.9^{ab,B}$	$25.1 \pm 6.6^{ab,B}$	$28.7 \pm 8.1^{a,B}$
Heads		35.9 ± 5.8^{A}	33.3 ± 8.0^{A}	34.0 ± 9.2^{A}	35.6 ± 8.1^{A}
Backbones	Lysine	43.6 ± 16.0^{A}	40.4 ± 10.1^{A}	37.5 ± 8.5^{A}	35.8 ± 10.5
Skins		$24.3 \pm 11.1^{b,B}$	$32.2 \pm 8.6^{ab,B}$	$30.8 \pm 4.5^{ab,B}$	36.4 ± 9.2^{a}
Heads		38.9 ± 8.0^{A}	39.2 ± 10.5^{A}	38.3 ± 8.7^{A}	40.3 ± 10.4
Backbones	Methionine	$13.5 \pm 2.9^{A,B}$	13.2 ± 2.6	14.1 ± 2.8	$13.2 \pm 4.7^{A,B}$
Skins		12.2 ± 4.1^{B}	12.3 ± 2.9	12.5 ± 2.8	14.7 ± 3.1^{A}
Heads		$15.7 \pm 2.8^{a,A}$	13.5 ± 3.2^{ab}	14.6 ± 4.7^{a}	$10.5 \pm 6.5^{b,B}$
Backbones	Phenylalanine	20.0 ± 3.7	20.1 ± 4.0	18.7 ± 2.3	18.2 ± 3.5
Skins		17.2 ± 2.8^{b}	18.0 ± 3.3^{ab}	18.0 ± 2.6^{ab}	20.1 ± 4.3^{a}
Heads		20.4 ± 3.0	20.5 ± 3.5	19.9 ± 4.0	20.4 ± 3.0
Backbones	Threonine	25.3 ± 5.9	25.7 ± 6.7	25.3 ± 5.8	24.5 ± 7.2^{B}
Skins		23.4 ± 5.3^{b}	26.6 ± 7.0^{ab}	25.9 ± 4.4^{ab}	$29.8 \pm 8.1^{a,A}$
Heads		26.2 ± 3.3	25.5 ± 6.2	26.6 ± 8.3	$26.9 \pm 6.5^{A,B}$
Backbones	Valine	20.5 ± 4.6^{A}	19.5 ± 4.6^{A}	19.7 ± 3.5^{A}	21.4 ± 5.2
Skins		$15.1 \pm 4.2^{b,B}$	$17.2 \pm 4.4^{ab,B}$	$16.7 \pm 2.3^{ab,B}$	19.1 ± 4.5^{a}
Heads		20.4 ± 3.8^{A}	21.3 ± 4.7^{A}	20.5 ± 3.5^{A}	21.3 ± 5.1
	Non-essential AA				
Backbones	Asparagine	$0.01 \pm 0.01^{b,AB}$	$<0.01 \pm <0.01^{b}$	$0.01 \pm <0.01^{b}$	0.07 ± 0.1^{a}
Skins		0.02 ± 0.01^{A}	0.02 ± 0.02	$<0.01 \pm <0.01$	0.01 ± 0.01
Heads		$<0.01 \pm <0.01^{B}$	0.02 ± 0.04	0.01 ± 0.01	0.05 ± 0.1
Backbones	Glutamine	15.6 ± 25.8^{a}	$0.12 \pm 0.2^{b,B}$	$21.9 \pm 32.7^{a,AB}$	0.67 ± 0.7^{b}
Skins		11.1 ± 17.8^{b}	$0.87 \pm 07^{c,A}$	$31.6 \pm 29.3^{a,A}$	0.59 ± 0.6^{c}
Heads		16.4 ± 25.4^{a}	$<0.01 \pm <0.01^{b,B}$	$10.5 \pm 15.4^{a,B}$	0.29 ± 0.5^{b}
Backbones	Arg/Gly	173.4 ± 55.2^{B}	167.3 ± 72.0^{B}	160.2 ± 48.5^{B}	152.6 ± 51.4^{B}
Skins		$260.5 \pm 27.8^{b,A}$	$266.1 \pm 48.8^{ab,A}$	$257.2 \pm 26.9^{b,A}$	$298.9 \pm 72.1^{a,A}$
Heads		166.5 ± 36.2^{B}	169.9 ± 54.2^{B}	140.2 ± 45.2^{C}	161.4 ± 52.4^{B}
Backbones	Tyrosine	12.0 ± 2.0^{A}	11.3 ± 1.9^{A}	11.7 ± 2.4^{A}	10.5 ± 2.9^{A}
Skins		$6.1 \pm 2.1^{b,B}$	$6.0 \pm 1.3^{b,B}$	$6.9 \pm 1.6^{ab,B}$	$7.3 \pm 1.7^{a,B}$
Heads		12.7 ± 1.7^{A}	10.8 ± 2.4^{A}	13.0 ± 4.2^{A}	12.1 ± 2.6^{A}

(Continued)

TABLE 1 (Continued)

Raw material	Amino acids	MAE15	MAE35	UAE15	UAE35
Backbones	Alanine	46.8 ± 11.2^{B}	46.4 ± 15.4^{B}	44.5 ± 9.8^{B}	43.5 ± 12.4^{B}
Skins		$59.9 \pm 7.2^{ab,A}$	$59.5 \pm 11.5^{ab,A}$	$57.9 \pm 7.5^{b,A}$	$67.5 \pm 14.8^{a,A}$
Heads		46.1 ± 7.9^{B}	46.6 ± 7.9^{B}	42.1 ± 10.5^{B}	46.8 ± 12.9^{B}
Backbones	Aspartate	54.7 ± 11.3	53.2 ± 12.5	51.6 ± 9.6	50.0 ± 13.8
Skins		48.3 ± 8.8	50.3 ± 11.7	49.0 ± 6.5	55.1 ± 12.6
Heads		54.7 ± 7.7	52.8 ± 12.6	52.4 ± 12.3	54.5 ± 12.8
Backbones	Glutamate	72.9 ± 16.9	73.4 ± 19.0	68.7 ± 14.4^{AB}	72.8 ± 26.2
Skins		64.2 ± 9.5^{b}	71.0 ± 20.7^{ab}	$61.8 \pm 6.8^{b,B}$	78.0 ± 20.9^{a}
Heads		72.6 ± 11.2	72.0 ± 20.6	70.9 ± 22.8^{A}	74.6 ± 21.2
Backbones	Serine	34.5 ± 9.2	34.1 ± 10.8	32.8 ± 6.5^{B}	30.3 ± 8.2^{B}
Skins		38.3 ± 5.8^{b}	39.6 ± 7.6^{ab}	$39.7 \pm 5.1^{ab,A}$	$44.6 \pm 10.5^{a,A}$
Heads		34.6 ± 5.2	34.6 ± 8.8	31.5 ± 7.0^{B}	$35.49.2 \pm 8.1^{B}$
Backbones	$\sum$ Total-AA	463.3 ± 291	568.1 ± 167	567.3 ± 132	471.1 ± 228^{B}
Skins		551.8 ± 226	643.5 ± 140	580.3 ± 233	721.8 ± 169^{A}
Heads		588.2 ± 108	567.2 ± 149	541.0 ± 143	568.4 ± 146^{AB}

ANOVA was applied to detect differences between raw materials and between extraction methods; where a significant difference was detected ($\alpha < 0.05$), a Tukey HSD *post hoc* test was applied. Values with different superscript (a, b within a row; A, B within a column) are significantly different ($p<0.05$).

and time did not significantly influence the total amino acid contents ($p > 0.05$). Moreover, backbones and heads show significantly higher ($p < 0.05$) values for, e.g., histidine, isoleucine, leucine and valine, all essential amino acids, after extraction with MAE15 compared to skins. This pattern can also be observed for MAE35, UAE15, and UAE35. Nevertheless, when looking closer at the sum of all the TAA, a significant difference ($p < 0.05$) between the three by-products was only observed for UAE35, with skin having a significantly higher TAA content. This leads to the assumption that skins have a significantly different amino acid profile than the two other fractions, but all in all same total content. Skins showed significantly ($p < 0.05$) higher values in serine for UAE15 and UAE35. Moreover, the alanine concentrations in skins are higher in all four extraction methods compared to backbones and heads. In addition, Table 1 points out that some amino acids are more influenced by extraction method and time than other amino acids, which show no significant different concentrations. Moreover, arginine/glycine were identified as the highest concentrations in all three fractions, which was also observed (for glycine) by Ali et al. (2018); Tamilmozhi et al. (2013). This is reasonable because the strict repeating of Gly-X-Y creates the α-triple-helix of collagen (Zanaboni et al., 2000).

Table 2 shows the FAA profile of untreated by-products after extraction with MAE15, MAE35, UAE15, and UAE35. Overall, similar FAA profiles between backbones and heads were found, with significantly higher ($p < 0.05$) values of leucine and glutamine, regardless of extraction method. Moreover, the values of lysine are significantly ($p < 0.05$) lower in skins when compared to the two other fractions. The total FAA content differs significantly between the three by-products as well as between the four extractions. For backbones, no significant differences between the four extractions were found, whereas UAE15 was identified to deliver most FAA in skins, and UAE15 as well as UAE35 for heads. Similar to TAA, glycine (combined Arg/Gly) was also identified as the main FAA in all three fractions. Higher free glycine can be linked to a disruption of the α-triple-helix and potentially increase the interfacial properties, as previously described in the study of Feng et al. (2021) on microwave extraction of fish skin gelatine.

The study highlights the potential value of the three studied fractions for collagen extraction. The results support the findings of Mohamad Razali et al. (2023), stating that green extraction methods such as UAE and MAE increase the extraction efficiency of collagen. Moreover, Mohamad Razali et al. (2023) review the current progress of green extraction methods for collagen and state that MAE is a versatile technology, enabling the transfer of technology from small (lab) to big (industry) scale (Destandau et al., 2013). Furthermore, Mohamad Razali et al. (2023) highlights that UAE and MAE have been successfully applied in multiple studies to increase the extraction efficiency of collagen, hereby stressing the significance of these green extraction methods. The study carried out by Shaik et al. (2021) on UAE of stingray skin collagen found UAE to increase the collagen yield, which agrees with the present study, underlining skin as a high collagen and high protein fraction with great potential for further utilization.

4. Conclusion

The findings of the present study contribute to the development of sustainable extraction processes of marine collagen from plaice by-products. Furthermore, the results feature how pre-treatment and extraction techniques affect the final protein and collagen concentration of the three studied by-products, with skins being the most promising collagen supplier. However, to fully understand and interpret the impact of the results, the knowledge of collagen extraction from skin, should be expanded. In conclusion, the current study highlights the potential of the applied green extraction procedures suitable for application in food, ingredient and cosmetic industries and suggests further research on the characteristics of the collagen structure as the next step to understand the functionality and applicability of the gained product.

TABLE 2 Free amino acid composition (mg/g) of backbones, skins and heads depending on extraction methods, excluding effect of pre-treatment; values presented as mean ± SD.

Raw material	Amino acids	MAE15	MAE35	UAE15	UAE35
	Essential AA	mg/g	mg/g	mg/g	mg/g
Backbones	Histidine	$0.51 \pm 0.02^{a,A}$	$0.31 \pm 0.06^{b,B}$	$0.52 \pm 0.02^{a,B}$	$0.53 \pm 0.01^{a,B}$
Skins		$0.25 \pm 0.04^{a,B}$	$0.09 \pm 0.08^{b,C}$	$0.24 \pm 0.02^{a,C}$	$0.23 \pm 0.03^{a,C}$
Heads		$0.53 \pm 0.02^{b,A}$	$0.55 \pm 0.05^{b,A}$	$0.67 \pm 0.07^{a,A}$	$0.76 \pm 0.02^{a,A}$
Backbones	Isoleucine	0.42 ± 0.14^{A}	0.15 ± 0.01^{AB}	0.50 ± 0.24	0.30 ± 0.01^{A}
Skins		0.04 ± 0.01^{B}	$0.03 \pm <0.001^{B}$	0.13 ± 0.12	0.10 ± 0.09^{B}
Heads		0.33 ± 0.2^{AB}	0.39 ± 0.21^{A}	0.47 ± 0.27	0.17 ± 0.01^{B}
Backbones	Leucine	$0.53 \pm 0.17^{ab,A}$	$0.33 \pm 0.01^{b,A}$	$0.60 \pm 0.04^{a,A}$	$0.50 \pm 0.02^{ab,A}$
Skins		$0.06 \pm 0.02^{bc,B}$	$0.05 \pm 0.01^{c,B}$	$0.12 \pm 0.02^{a,B}$	$0.09 \pm 0.01^{ab,C}$
Heads		0.40 ± 0.09^{A}	0.44 ± 0.11^{A}	0.49 ± 0.10^{A}	0.28 ± 0.03^{B}
Backbones	Lysine	0.31 ± 0.34	0.21 ± 0.01^{B}	0.42 ± 0.03^{AB}	0.27 ± 0.01^{A}
Skins		0.03 ± 0.01	0.01 ± 0.01^{C}	0.04 ± 0.03^{B}	0.04 ± 0.02^{B}
Heads		0.37 ± 0.12^{ab}	$0.37 \pm 0.06^{ab,A}$	$0.67 \pm 0.27^{a,A}$	$0.25 \pm 0.03^{b,A}$
Backbones	Methionine	0.77 ± 0.2	0.54 ± 0.02	0.62 ± 0.14	0.59 ± 0.03
Skins		0.43 ± 0.03^{a}	0.34 ± 0.03^{b}	0.43 ± 0.02^{a}	0.34 ± 0.04^{b}
Heads		0.63 ± 0.4	0.63 ± 0.29	0.69 ± 0.34	0.80 ± 0.03
Backbones	Phenylalanine	$0.82 \pm 0.08^{a,A}$	$0.50 \pm 0.02^{b,AB}$	0.76 ± 0.02^{a}	$0.71 \pm 0.01^{a,A}$
Skins		0.13 ± 0.02^{B}	0.11 ± 0.01^{B}	0.28 ± 0.18	0.21 ± 0.16^{B}
Heads		0.57 ± 0.33^{AB}	0.57 ± 0.29^{A}	0.65 ± 0.32	0.33 ± 0.05^{B}
Backbones	Threonine	3.28 ± 3.0	1.37 ± 0.04^{A}	1.36 ± 0.02^{B}	1.71 ± 0.12^{B}
Skins		0.83 ± 0.01	0.40 ± 0.34^{B}	0.77 ± 0.04^{C}	0.73 ± 0.02^{C}
Heads		1.58 ± 0.12^{c}	$1.85 \pm 0.04^{b,A}$	$1.98 \pm 0.14^{b,A}$	$2.54 \pm 0.07^{a,A}$
Backbones	Valine	$0.58 \pm 0.07^{a,AB}$	$0.40 \pm 0.03^{b,AB}$	$0.66 \pm 0.07^{a,A}$	$0.61 \pm 0.03^{a,A}$
Skins		$0.12 \pm 0.01^{ab,B}$	$0.11 \pm 0.02^{b,B}$	$0.27 \pm 0.08^{a,B}$	$0.20 \pm 0.08^{ab,C}$
Heads		0.67 ± 0.3^{A}	$0.75 \pm 0.34^{A,}$	0.81 ± 0.24^{A}	$0.44 \pm 0.03^{a,B}$
	Non-essential AA				
Backbones	Asparagine	<0.001	<0.001	<0.001	<0.001
Skins					
Heads					
Backbones	Glutamine	$0.56 \pm 0.03^{a,A}$	$0.36 \pm 0.10^{b,B}$	$0.56 \pm 0.03^{a,B}$	$0.42 \pm 0.03^{ab,B}$
Skins		$0.36 \pm 0.02^{a,B}$	$0.23 \pm 0.02^{c,B}$	$0.34 \pm 0.01^{ab,V}$	$0.31 \pm 0.02^{b,C}$
Heads		$0.56 \pm 0.04^{b,A}$	$0.57 \pm 0.03^{b,A}$	$0.70 \pm 0.04^{a,A}$	$0.52 \pm 0.01^{b,A}$
Backbones	Arg/Gly	$6.60 \pm 0.09^{b,A}$	$5.9 \pm 0.24^{c,A}$	$6.67 \pm 0.22^{b,A}$	$7.71 \pm 0.20^{a,A}$
Skins		$4.55 \pm 0.03^{b,C}$	$3.13 \pm 0.14^{c,C}$	$4.78 \pm 0.08^{a,C}$	$4.45 \pm 0.06^{b,C}$
Heads		$5.01 \pm 0.07^{c,B}$	$4.51 \pm 0.12^{d,B}$	$5.66 \pm 0.14^{b,B}$	$6.75 \pm 0.16^{a,B}$
Backbones	Tyrosine	2.39 ± 3.1	0.86 ± 0.03^{A}	0.58 ± 0.01^{A}	1.00 ± 0.04^{A}
Skins		0.29 ± 0.08^{b}	$0.25 \pm 0.01^{b,C}$	$0.54 \pm 0.01^{a,B}$	$0.46 \pm 0.01^{a,B}$
Heads		0.26 ± 0.03	0.36 ± 0.02^{B}	0.39 ± 0.02^{C}	0.54 ± 0.26^{B}
Backbones	Alanine	$2.15 \pm 0.97^{ab,A}$	$1.10 \pm 0.04^{b,B}$	$2.39 \pm 0.03^{a,B}$	$2.60 \pm 0.01^{a,A}$
Skins		$0.74 \pm 0.03^{c,B}$	$0.79 \pm 0.04^{bc,C}$	$1.03 \pm 0.02^{a,C}$	$0.82 \pm 0.01^{b,C}$
Heads		$1.95 \pm 0.02^{b,AB}$	$2.66 \pm 0.08^{a,A}$	$2.75 \pm 0.03^{a,A}$	$1.98 \pm 0.21^{b,B}$
Backbones	Aspartate	0.41 ± 0.06^{B}	0.34 ± 0.05^{B}	0.50 ± 0.13^{B}	0.49 ± 0.05^{A}
Skins		$0.18 \pm 0.02^{b,C}$	$0.18 \pm 0.02^{b,C}$	$0.29 \pm 0.03^{a,C}$	$0.20 \pm 0.02^{b,B}$
Heads		$0.53 \pm 0.01^{bc,A}$	$0.54 \pm 0.05^{b,A}$	$0.73 \pm 0.04^{a,A}$	$0.45 \pm 0.01^{c,A}$

(Continued)

TABLE 2 (Continued)

Raw material	Amino acids	MAE15	MAE35	UAE15	UAE35
Backbones	Glutamate	$0.33 \pm 0.02^{b,A}$	$0.50 \pm 0.05^{a,A}$	$0.22 \pm 0.04^{c,B}$	$0.45 \pm 0.02^{a,B}$
Skins		$0.11 \pm 0.02^{a,B}$	$0.07 \pm 0.01^{b,C}$	$0.11 \pm 0.01^{a,C}$	$0.11 \pm 0.01^{a,C}$
Heads		$0.35 \pm 0.01^{d,A}$	$0.39 \pm 0.02^{c,B}$	$0.44 \pm 0.01^{b,A}$	$0.78 \pm 00.01^{a,A}$
Backbones	Serine	$1.26 \pm 0.04^{ab,B}$	$1.22 \pm 0.15^{b,B}$	$1.38 \pm 0.06^{ab,B}$	$1.46 \pm 0.05^{a,B}$
Skins		1.24 ± 0.04^{B}	1.01 ± 0.28^{B}	1.33 ± 0.03^{C}	1.22 ± 0.02^{C}
Heads		$1.86 \pm 0.02^{d,A}$	$2.16 \pm 0.08^{c,A}$	$2.71 \pm 0.08^{b,A}$	$3.27 \pm 0.10^{a,A}$
Backbones	∑ Free-AA	20.9 ± 5.7^{A}	14.1 ± 0.7^{B}	17.7 ± 0.5^{B}	19.3 ± 0.2^{A}
Skins		$9.3 \pm 0.3^{b,B}$	$6.81 \pm 0.2^{c,C}$	$10.7 \pm 0.5^{a,C}$	$9.5 \pm 0.4^{b,B}$
Heads		$15.6 \pm 0.2^{b,AB}$	$16.7 \pm 0.5^{b,A}$	$19.8 \pm 0.4^{a,A}$	$19.9 \pm 0.9^{a,A}$

ANOVA was applied to detect differences between raw materials and between extraction methods; where a significant difference was detected ($\alpha < 0.05$), a Tukey HSD *post hoc* test was applied. Values with different superscript (a, b within a row; A, B within a column) are significantly different ($p < 0.05$).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SKe: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. SKo: Conceptualization, Investigation, Formal analysis. KM: Validation, Methodology, Formal analysis. AJ: Conceptualization, Supervision, Validation, Writing – review & editing, Funding acquisition. JL: Conceptualization, Supervision, Validation, Writing – review & editing, Project administration, Resources, Funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Extraction of proteinaceous components and biominerals from cold water fish fileting side streams: a review

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Fileting is a popular form of processing methods and in addition to being sold fresh or frozen, filets are used for preparation products battered and breaded filets. This generates considerable amount of side streams like skin, frames, and cut-offs which forms around 30–70% of the total body weight. The European Waste Framework Directive 2008 and recent amendments [EU WFD (2018/851)] stipulates comprehensive regulations which the manufacturers must follow while handling the side streams generated during the processing. This demands a detailed compilation of information regarding the yield, classification and valorization potential of side streams associated with the fileting operations of the cold-water finfishes. The side streams are a rich source of proteins including structural proteins like collagen and biominerals such as hydroxyapatite which find multiple application in food and pharmaceutical industry. Previously, the recovery of these components was performed by extensive chemical treatment with acids or bases, including subsequent washing steps. Nowadays, green extraction methods, defined as technologies with reduced energy and chemical consumption, should be considered to achieve a green shift in the food industry. Nevertheless, industrial upscaling of green extraction methods and subsequent refinement of the isolated compounds must be further evaluated and improved in order to achieve a green shift in food industry by using side-stream derived compounds as ingredients. Moreover, legislations as well as national and international regulations must be considered and evaluated. Even though a number of articles are recently available regarding seafood side stream valorization, this review focus on side streams generated predominantly from cold water fish species and also discusses sustainable green technologies to be included during the recovery process.

KEYWORDS

fish fileting, side streams, collagen, hydroxyapatite, green technologies, extraction

1 Introduction

The utilization of marine resources has been shaping the lives of generations living along coastlines around the world. In 2020, the global fish production showed a total number of 176 million tons (FAO, 2022). Today, an increasing amount of fish is processed after capture, rather than being sold as whole fish. Further processing of fish into filets, which usually is the main product of fish, generates a high amount of by-products. These by-products can account for up to 70% of the fish weight (Ozogul et al., 2021). The post-harvest preparation of filets includes different steps, but involves the removal of intestines, heads, fins, backbones and skins (Ghaly et al., 2013; Maschmeyer et al., 2020). Moreover,

significant amounts of cut-offs are generated during automated fileting, and together with other by-products show the possibility to be more efficiently utilized, aiming for circular utilization and valorization. The major portion of the fish side streams generated today are utilized for production of undervalued products like animal feed, fertilizer and biofuel (Välimaa et al., 2019; FAO, 2020). Even though the utilization of side streams as a feed ingredient facilitates its re-entry into the food chain, it causes an energy loss of up to 90% toward its conversion to meet rendering the use of side streams for human nutrition more significant (Venslauskas et al., 2021). Depending on fish species, sex, maturity, season etc. the secondary products, referred to as side streams contain varying amounts of proteins, lipids, ash, and minerals (Maschmeyer et al., 2020). The different side stream fractions contain a large number of valuable compounds, such as oils, including valuable omega-3 fatty acids and proteins, composing of bioactive peptides, essential amino acids and collagen. Moreover, frames and bones are an excellent source of hydroxyapatite (HAp) (Rustad et al., 2011). Proteins derived from marine resources are highly digestible, with good amino acid profiles and when broken down into its peptides, show multiple bioactive properties (Ucak et al., 2021). In addition, collagen has received attention due to its broad range of possible applications, ranging from food ingredients, skin care to the utilization as biomedical material (Regenstein and Zhou, 2007; Välimaa et al., 2019; Subhan et al., 2021).

The focus of this review article lies within the proteinaceous compounds of fileting by-products as new biomaterials, hence valuable compounds within the oil fraction are not further considered, but have been discussed previously in e.g., Shahidi (2007), Wu and Bechtel (2008), and Rustad et al. (2011). Specifically, the objective of this review article is to highlight the potential of biomaterials such as hydroxyapatite, collagen and bioactive peptides as innovative biomaterials in various applications. In addition, extraction methods, both conventional and green procedures to isolate these valuable biomaterials from the by-product fractions are described. Special emphasis is placed on highlighting the potential of newer, environmentally friendly methods described as green methods in literature. These methods offer the opportunity to enhance the value of the waste resources, while simultaneously reducing energy and/or chemical consumption in extraction processes.

2 Biomaterials derived from fileting by-products

Fileting by-products offer several high-value compounds for further utilization in various fields of applications. The importance of marine protein and proteinaceous compounds is marked by the amount of protein available in different by-product fractions. As an example, the protein contents of Alaska pollock, pink salmon and Pacific cod by-products were reported to range from 13.9 to 16.4% in heads, 13.0 to 15.3% in viscera, around 25% in skins and 15.8 to 16.3% in frames on a wet weight basis (Bechtel, 2003). Marine proteins are highly valuable not only in their intact structure as a macronutrient, but more so when broken down into their peptide constitutes. By extracting and isolating valuable compounds such as peptides, collagen or hydroxyproline from the by-product structures, the major part of fileting by-products can be utilized and is available for valorization into products (Siddiqui et al., 2023). Hereby likewise, the remaining lipid fractions can be considered for further utilization. Figure 1 indicates the major biomaterials that could be potentially extracted from fileting side streams.

2.1 Peptides

Intensive research on bioactive peptides the past decades have lead to detection and characterization of a wide range of bioactive peptides (Pihlanto and Korhonen, 2003; Möller et al., 2008; Shahidi and Zhong, 2008). Bioactive peptides from marine origin with multiple positive effects on human health have been identified (Kim and Mendis, 2006; Kim and Wijesekara, 2010; Ozogul et al., 2021). In general, peptides display bioactivity at a chain length of 2–20 amino acids, and once released from the tightly packed protein structure, can exhibit their beneficial properties (Sun et al., 2021). Bioactive peptides were found to be rich in hydrophobic amino acids and determine their antioxidative and metal chelating activity (Mendis et al., 2005; Kim and Wijesekara, 2010). Moreover, the amino acid composition and distribution within the sequence influences functional properties such as the ability for emulsification and water holding capacity (Mendis et al., 2005). A commonly used method to acquire bioactive peptides from various by-product fractions is the application of enzymatic hydrolysis, hereby enzymatically deconstructing the proteins and release peptide chains. The possibility to generate fish protein hydrolysates including bioactive peptides from backbones, skins, heads and cut-offs from marine waste has been well-demonstrated previously (ŠliŽyte et al., 2009; Chalamaiah et al., 2012; Gajanan et al., 2016; Zamora-Sillero et al., 2018; Ozogul et al., 2021). A challenge for the application of marine peptides is their bitterness due to the presence of hydrophobic and alkaline amino acids (e.g., lysine, leucine) (Fan et al., 2019). Next to bitter taste, the bioactivity can be altered and decreased while passing through the gastrointestinal tract (GI), due to proteolytic degradation and the acidic environment. Here, innovative solutions such as the entrapment or encapsulation within different biomaterials (e.g., nanoliposomes) are applied to effective stabilize and increase the palatability of the peptides (Hosseini et al., 2017, 2021).

2.2 Collagen

Collagen is one of the most abundant proteins and have a triple helical structure, with three polypeptide α-chains. Up-to date a total of 28 classes within the collagen family have been discovered (type I-XXVIII) (Ricard-Blum, 2011). The collagen superfamily includes multiple types, but common to all is the repeating sequence of $(Gly\text{-}X\text{-}Y)_n$, indicating the repetition of proline (X) and hydroxyproline (Y) with Gly (glycine) being stringent in this sequence. Each member of the collagen superfamily includes at least one triple-helical domain (COL), being situated at the extracellular matrix (Zanaboni et al., 2000; Ricard-Blum and Ruggiero, 2005; Felician et al., 2018). Marine collagen is mainly found as type I collagen, whereas human collagen is prevalent (80–85%) in the form of type

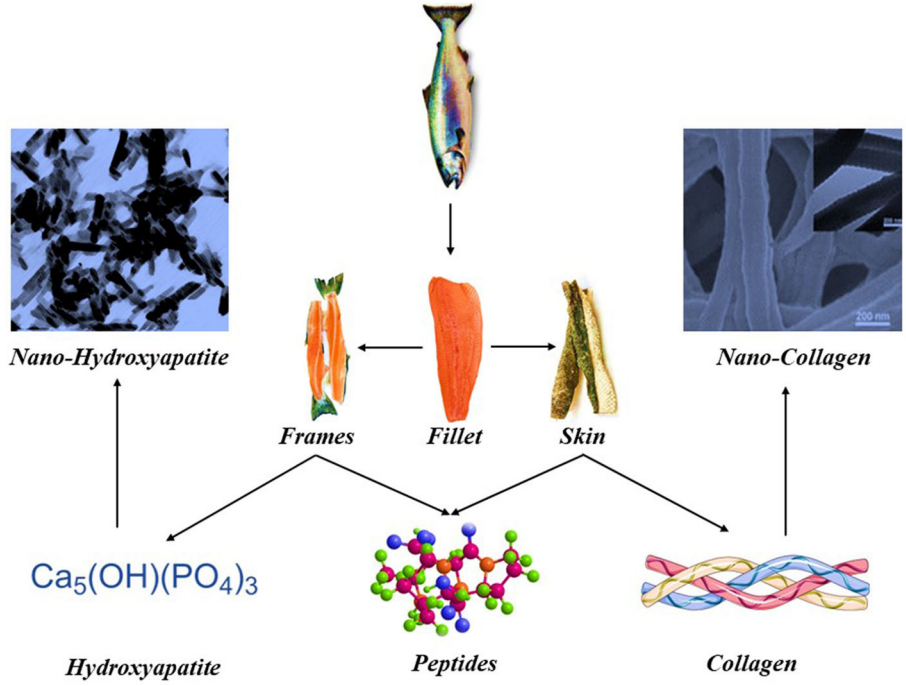

FIGURE 1
Major biomaterials potentially extracted from fileting side streams.

II (Felician et al., 2018). Marine collagen is regarded as a highly functional and useful material with potential for application in health-related sectors, including medical, pharmaceutical, cosmetic and the food industry (Salvatore et al., 2020). Especially marine collagen, in contrast to collagen from bovine or porcine origins, has the advantage of being appropriate for cultures restricting the consumption of bovine or porcine origin (Easterbrook and Maddern, 2008). This makes marine collagen attractive for a wide range of potential consumers (e.g., food applications) and patients (e.g., biomedical applications). Moreover, it is reported that the amino acid composition and biocompatibility of marine collagen is comparable to porcine or bovine origin, with glycine (<30%) and hydroxyproline (35–48%) being the dominating compounds in the molecule (Yamada et al., 2014). According to Subhan et al. (2021), around 30% of marine side stream is rich in collagen. However, this will highly depend on e.g., the fish species, processing regimes and quality of obtained by-products. Moreover, the physicochemical characteristics such as hydration behavior and thermal properties can differ between cold water fish (e.g., Atlantic or baltic halibut, cod) and warm water fish (e.g., Pacific big-eye tuna, tilapia), which has to be considered for applications (Rose et al., 1988; Gauza-Włodarczyk et al., 2017). Marine collagen has been extracted from cold water fish by-product fractions such as skins, frames, heads and bones. Particularly, fish skins have been reported to be a good source to extract high yields of collagen with great potential for further utilization (Nagai and Suzuki, 2000; Sadowska et al., 2003; Kittiphattanabawon et al., 2005; Zelechowska et al., 2010; Sousa et al., 2020; Martins et al., 2022; Kendler et al., 2023). The source of collagen, type, the pre-treatment parameters, and the extraction methodologies adopted are the main factors which determine the properties of collagen such as molecular weight, amino acid profile, structure of the molecule, solubility properties and functional characteristics (Rajabimashhadi et al., 2023). Even though the major raw material used for extracting type I collagen is bovine (Salvatore et al., 2020), different health preferences, cultural, religious, and social stigma, has resulted in the exploration of alternate collagen raw material sources such as fish skin (Rajabimashhadi et al., 2023). Whereas, collagen from fish could be extracted from different side streams such as scale, bone, head, air bladder and viscera, skin remains the most important source (Felician et al., 2018; Maschmeyer et al., 2020; Subhan et al., 2021).

2.3 Hydroxyapatite

Hydroxyapatite (HAp), chemically a calcium phosphate mineral with the structure of Ca_{10} $(PO_4)_6(OH)_2$ can be generated synthetically or is derived from biological sources such as bovine, coral, shells, algae or fish (Legeros and Legeros, 2008; Fernando et al., 2016; Mohd Pu'ad et al., 2019). HAp can be regarded as a bioceramic, showing great biocompatibility to human bones due to a similar Ca/P molar ratio of 1.67. This molar ratio indicates the amount of Ca^{+2} which is important for the growth and production of osteoblast cells, especially of significance for bone repair applications. HAp is hexagonal in its structure and when exposed to high temperatures (<1,200°C) transforms into tricalcium phosphate $(Ca_3(PO_4)_2)$ (Khamkongkaeo et al., 2021). An advantage of natural (marine-derived) HAp over the synthetically produced HAp is the micro- and nano scale

hierarchical structure, but also lower costs due to being generated out of waste materials. The chemical structure, characteristic and similarity to human hard tissue makes marine-derived HAp generated from fileting side streams an eco-friendly biomaterial (Khrunyk et al., 2020; Borciani et al., 2023). Fish are a good source of HAp, and previous studies on tilapia bones, fins and body parts have managed to generate HAp with similar Ca/P molar ratios to human HAp (Ahmad Fara and Abdullah, 2016; Khamkongkaeo et al., 2021). The primary by-product fractions after fileting are backbones, heads and fishbones, all of which contain HAp, which emphasizes the significant potential of further extracting HAp as a potential biomaterial.

3 Extraction of collagen

Collagen extraction methods generally consists of three major steps. (1) pre-treatment stage consisting of separation of the target tissue, reduction of raw material size for increasing the surface area available for reaction, isolation of collagenous matter from non-collagenous matter for increasing the process efficiency, (2) treatment stage consisting of extraction of collagen peptides through hydrolysis of the protein matrix using chemical and enzyme aided process and finally the (3) recovery stage consisting of salt precipitation or dehydration (Rajabimashhadi et al., 2023). For extraction of cold water fish collagen the temperature should be kept between 4 and 10°C for minimizing the risk of denaturation of collagen peptides (Araujo et al., 2021; Luo et al., 2022; Son et al., 2022).

3.1 Pre-treatment of collagen raw materials

Collagen rich raw materials like skin usually contain a significant quantity of non-collagenous materials such as, fat, pigments, and non-collagenous proteins. Other raw materials used such as scales and bones also contain inorganic components such as ash contributed by minerals like calcium, zinc etc. (Schmidt et al., 2016; Ahmed et al., 2020). The initial process in pre-treatment of the raw materials is removing non-collagenous tissue adhered using mechanical means and reducing the size of the solid parts to facilitate higher surface area to volume ratio for better efficiency of the treatments to be followed (Safiya and Casparus, 2020). The non-collagenous matter present in the raw material could, if not properly eliminated, interfere with the extraction treatments and prevent proper breaking down of the collagen peptide bonds. Hereby significantly reducing the solubility of collagen peptides into the acid, alkali or enzymatic media used (Ahmed et al., 2020). The removal of the non-collagenous components are usually done under moderate conditions to prevent unnecessary breakdown of the collagen. For instance, the removal of non-collagenous protein is most commonly done using 0.05–0.1 M NaOH. At these concentrations, the collagen is not disturbed while most of the non-target protein is dissolved in the alkali (Hukmi and Sarbon, 2018). NaOH also contributes toward swelling of the tissue, therefore accelerating the rate of mass transfer during the extraction process (Ahmed et al., 2020). Fat is another important non-collagenous material which needs to be removed especially in the case of side streams from high fat fishes. The removal of fat is usually done with chemical treatment including acetone, 10–15% butanol and other alcohols or non-ionic detergents (Schmidt et al., 2016; Hukmi and Sarbon, 2018). Hard raw materials like bones and scales contain high levels of ash, contributed by the mineral content, and these need to be removed through a de-mineralization process. Calcium being the prominent mineral in these components, chelating agents like EDTA (Ethylene diaminetetraacetic acid) (0.5 M) and acids like HCl (0.5–1 M) are commonly used with minimal destruction of collagen (Ahmed et al., 2020). The decalcification process has also been found to make the raw material more permeable, as it increase the surface area and also simultaneously partially hydrolyzing collagen breaking the cross links while maintaining the collagen peptide chains intact, aiding the extraction process (Schmidt et al., 2016). The pre-treatment procedures are also reported to cause leaching of collagen peptides resulting in loss of yield (Bai et al., 2017) and hence are performed in batches with different combinations of treatment time, temperature, reactant concentration and raw material to chemical ratio (Muralidharan et al., 2013; Hukmi and Sarbon, 2018). Table 1 indicates different pre-treatment conditions followed for cold water fish collagen extraction.

3.2 Conventional collagen extraction methods

The collagen extraction process depends on the solubility of collagen molecules into the extraction medium making it suitable for isolation and further recovery through filtration/dehydration. As the collagen molecule is water insoluble due to its triple helix structure, secured with inter/intra molecular hydrogen bonds, specific extraction techniques are required to solubilize and isolate collagen (Jafari et al., 2020). Depending on the solubility, collagen could be classified according to the extraction method utilized as acid, enzyme, or salt soluble (Oliveira et al., 2021).

3.2.1 Salt soluble collagen

Collagen extraction based on the saline solubility properties or salting out (SO) method utilizes neutral salts like NaCl and guanidine hydrochloride to precipitate collagen from the pre-treated raw material (Schmidt et al., 2016). In this method the salt concentration is gradually increased, which boosts the ionic strength of the solution, hereby increasing the solubility of the collagen (El Blidi et al., 2021). The collagen could be further isolated through dialysis or using ultrafiltration membranes (Saallah et al., 2021). The major drawback of this method is the lower yield, time consumption and resource dependency compared to other methods (Cao et al., 2019; Hong et al., 2019).

3.2.2 Acid soluble collagen

Acid hydrolysis can be employed for collagen extraction using organic acids such as acetic, lactic, citric, formic, and tartaric acid

TABLE 1 Conventional pre-treatment conditions followed for cold water fish collagen extraction.

Cold water species	Side-stream	Pre-treatment conditions		References
		De-fattening	De-proteinization	
Gadus macrocephalus (Cod)	Skin		0.1 M NaOH (20 volumes (v/m) constant agitation for 24 h at 4°C	Sun et al., 2017
Gadus macrocephalus (Cod)	Skin		0.1 M NaOH 1:10 (w/v), for 4 h 25°C. Centrifuged and washed with distilled water until neutral pH.	Coscueta et al., 2021
Gadus macrocephalus (Cod)	Skin		NaOH (0.02 M) 1:10 (w/v) for 18 h and washed with distilled water to neutral pH	Shu et al., 2017
Gadus macrocephalus (Cod)	Skin		0.1 M NaOH (1:10) 72 h, 4°C and washed with distilled water to neutral pH.	Carvalho et al., 2018
Salmo salar (Atlantic Salmon)	Skin	Soaked overnight using a solution with butanol to water ratio of 1:10.	8% NaCl solution.	Moreno et al., 2012
Salmo salar (Atlantic Salmon)	Skin	10% ethanol for 48 h	0.1 M NaOH with (1:10) 6 h 4°C. Washed with distilled water to neutral pH.	Alves et al., 2017
Salmo salar (Atlantic Salmon)	Skin		0.01 M NaOH. Rinsed with distilled water to neutral pH.	Woonnoi et al., 2021
Salmo salar (Atlantic Salmon)	Skin	30% isopropanol [1:10 (w/v)] Mixing with ultrasonicatation, d washed free of alcohol.	0.05 M NaOH [1:10 (w/v)] agitated at an rpm of 150 for 1 h @ 4–8°C	Nilsuwan et al., 2022b
Reinhardtius hippoglossoides (Greenland Halibut)	Skin	10% ethanol at a ratio of 1:10 (w/v) for 48 h	0.1 M NaOH at a ratio of 1:10 (w/v) for 72 h	Martins et al., 2022
Salmo salar (Atlantic Salmon) and *Gadus morhua* (Baltic Cod)	Skin	Distilled water (1:5 (w/v) ratio) at 4°C	0.1 M NaOH [1:6 (w/v)] for 48 h at chilled condition.	Tylingo et al., 2016
S. canicular (Small-spotted catshark)	Skin	10% ethanol at a ratio of 1:10 (w/v) for 48 h	0.1 M NaOH at a ratio of 1:10 (w/v) for 72 h.	Blanco et al., 2019

other than mineral acids like hydrochloric acid and sulfuric acid (Fassini et al., 2021). In this method, the acids involved, break down (depolymerize) the triple helix structure of the complex collagen molecules into shorter ones within a molecular weight range of 0.3–8 kDa (Bai et al., 2017). The acidic conditions also present an environment, predominant with positive charge, which further facilitates the aversion between the tropocollagen, accelerating the solubilization of collagen within the acid medium (Benjakul et al., 2012). Important parameters that influence the efficiency of the acid aided process are temperature, concentration, extraction period and solid to solvent ratio. The most common acid used for collagen extraction is acetic acid with a concentration of 0.5–1 M and for a time period of 24–48 h (Blanco et al., 2019; Jafari et al., 2020; Mohamad Razali et al., 2023). Blanco et al. (2019) also deliberated on the effect of acid concentration and process time on the choice of the optimum reaction temperature. Jafari et al. (2020) considered raw material to acid ratio as an important parameter to be considered while treating with acid for the extraction of collagen. They observed that an increase in the volume of the acidic solution enhances the free proton-amino acid interactions, which further speeds up fragmentation of the collagen molecules. The acid extraction method is also considered more effective than salt based extraction, resulting in better yield (Hong et al., 2019).

3.2.3 Enzyme soluble collagen

The low yield associated with the acid based method could be due to the partial solubility of collagen in the acid, including the likely formation of covalent crosslinks through condensation of aldehyde groups (Sinthusamran et al., 2013). This has led to the application of enzymes in collagen extraction which facilitate increased selectivity and lower deteriorative effect toward the amino acids in the collagen molecule (Hong et al., 2019). Different enzymes such as Flavourzyme, Alcalase®, papain, trypsin and pepsin could be employed (Schmidt et al., 2016). The most common type of enzyme employed for collagen extraction is pepsin (Nalinanon et al., 2010), which enables the breaking of peptide bonds among the collagen triple helix, facilitating the release of collagen molecules, increasing the total extraction yield (Schmidt et al., 2016; Bagus Fajar Pamungkas et al., 2019; Ahmed et al., 2020). The optimum temperature for pepsin reaction is estimated to be 60°C, and it is often used in combination with an acetic acid treatment to enhance the efficiency of the process resulting in a higher yield, quality, and free amine concentration with a lower denaturation temperature (Zhao et al., 2015; Delgado et al., 2017). Almost identical duration (24–48 h) and solvent to raw materials ratios (10–60 ml/g) are used in such combinations with an enzyme concentration between 1 and 1.5% (Hukmi and Sarbon, 2018; Cao et al., 2019). Even though the enzyme aided process is

safe and sustainable compared to other chemical methods (Zhang et al., 2020), it is considered expensive due to the cost of enzymes (Schmidt et al., 2016). Table 2 indicates the conventional collagen extraction conditions followed for cold water fish side streams.

3.3 Green processing methods for collagen extraction

The extraction methods play a significant role in determining the quality of the collagen. The general alkali-acid-enzyme based collagen isolation methods have some inherent shortcomings such as multistage operational requirement, energy consumption, water dependency, chemical residues, extremely low yield, and non-ecofriendly nature (Ameer et al., 2017; Jafari et al., 2020; Faraz et al., 2021; Phon et al., 2023). Application of industry 4.0 technologies such as big data analysis, artificial intelligence, sensor based monitoring and advanced image processing in combination with green recovery methods could enhance the seafood side stream valorization process with respect to efficiency and lower carbon footprint (Hassoun et al., 2023). In this scenario, advanced eco-friendly or green technologies are proposed for effective extraction and isolation of collagen and collagen form marine resources resulting in minimal impact to the environment with emphasis on sustainability (Venugopal et al., 2023). Some of the emerging technologies considered for green extraction of collagen are Ultrasound-assisted extraction (UAE), Subcritical water extraction (SWE), High-pressure processing (HPP), and Microwave-Assisted Extraction (MAE). Figure 2 indicates the potential green technologies that could be adapted for collagen extraction from fileting side streams.

3.3.1 Ultrasound-assisted extraction

UAE is a non-thermal physical method which utilizes ultrasonic sound waves above 20 kHz to disintegrate macromolecules to simpler forms and therefore enhancing the reaction interface between the solvents and the targeted substrates (Faraz et al., 2021). In collagen extraction processes, the applied UAE, generally in the range of 200–500 kHz, destroys the complex collagen matrix through cavitation and facilitate collagen production. The sound waves traveling through the solvent creates alterations in pressure and temperature, enhancing the transfer rate of the solvent into the matrix. Hereby resulting in the isolation of collagen molecules (Torres et al., 2017). UAE could enhance the collagen extraction efficiency when utilized in combination with acidic or enzymatic methods. An acidic pre-treatment is observed to weaken the collagen matrix by removing non-collagenous proteins and weakens the peptide bonds further increasing the efficiency of the ultrasound frequencies (Mhd Sarbon et al., 2013). Non-protein denaturing frequencies of ultrasound could be used in combination with enzymes to enhance the extraction efficiency as in the case of acidic process where the enzymes partially hydrolyzing and weakening the peptide bonds increasing the extraction efficiency of the process (Nadar and Rathod, 2017). Lee et al. (2022) successfully utilized UAE in combination with enzyme application for preparing collagen hydrolysate from Alaska pollock skin (*Theragra chalcogramma*). They used 1% Alcalase® along with an ultrasound frequency of 20 kHz with 80% amplitude, for a period of 30 min. The extracted collagen fraction exhibited enhanced antioxidant properties when compared to the control. Moreover, Kendler et al. (2023) utilized UAE with or without enzymatic hydrolysis prior the extraction to generate collagen from different European plaice by-product fractions. Several studies have demonstrated the feasibility of UAE to extract marine collagen from different origins (Ali et al., 2018; Shaik et al., 2021; Lu et al., 2023).

3.3.2 Subcritical water extraction

SWE uses the changes in properties of water, generated in response to variations in temperature and pressure. It is the state that water attains between the temperature 100–374°C (Boiling and critical temperature of water) under a corresponding pressure range of 1–22.1 MPa (Zhang et al., 2020). The subcritical stage of water is observed to be capable of disrupting the molecule-molecule interaction by reducing the activation energy for desorption and simultaneously enhancing water penetration into the protein matrix. Hereby resulting in separation of protein molecules like collagen, based on polarity (Pillot et al., 2019). These intricate subcritical pressure and temperature combinations could result in partial or complete hydrolysis of collagen, resulting in collagen within a limited period of time (Jung et al., 2014; Park et al., 2015). Melgosa et al. (2021) utilized SWE to extract collagen from Atlantic codfish frames with an extraction yield of 5-% and 100% of protein recovery rate at a temperature-pressure combination of 90°C and 100 bar. The SWE extracted collagen showed highest anti-inflammatory properties. Similar attempts were made by Sousa et al. (2020) from cod skin, where SWE was employed for collagen extraction with a yield of 13.8%.

3.3.3 High-pressure processing

HPP is considered to be a sustainable, non-thermal, green technology which utilizes hydrostatic pressure within a range of 100–1,500 MPa (Naderi et al., 2017). Intermolecular bonds are disrupted under high pressure affecting the structure of the proteins making them more susceptible to extraction procedures without compromising on quality (Yang and Powers, 2016). The entire HPP process is accomplished through a system comprising of pressure vessel, pressure generation system, temperature, device management, pressure valves and a material handling system (Naderi et al., 2017). HPP has been utilized in the pre-treatment stage during collagen extraction from fish skins where acid and skin where packed in plastic bags and then placed in the high-pressure chamber (Jaswir et al., 2017; Yusof et al., 2017). This facilitated better acid penetration into the skin, breaking the non-covalent bond in the gelatin molecule. Hereby enabling swelling and subsequent extraction (Yusof et al., 2017). Noor et al. (2021) observed that during the HPP pre-treatment process, acid treatment at 10°C speeds up the weakening of crosslinks within the acid molecule and further extraction at 45°C in water accelerates the collagen hydrolysis. The extraction potential from fish skin were enhanced up to 25% along with additional advantages such as a solid gel forming capacity and elevated melting point when HPP was employed in the pre-treatment

TABLE 2 Conventional collagen extraction methods adapted for cold water fishes.

Sl. No.	Cold water species	Side-stream	Extraction conditions	Collagen yield	References
I. Salt aided process					
	Common starfish *(Asterias rubens)* and Atlantic Lump fish *(Cyclopterus lumpus)*	Skin	2.5 M NaCl/0.05 M Tris dialysis against acetic acid	1.44% for star fish and 1.24% for lumpfish (w/wt.)	Vate et al., 2023
	Greenland halibut *(Reinhardtius hippoglossoides)*	Skin	2.6 M NaCl/0.05 M Tris-HCl, 0.7 M NaCl and 2.3 M NaCl/0.05 M Tris-HCl	2–4% (w/wt.)	Martins et al., 2022
	Salmon (*Oncorhynchus nerka*)	Skin	Precipitation with 2.6 M NaCl	36.73% (d/wt.)	Nilsuwan et al., 2022b
	Atlantic cod *(Gadus Morhua)*	Swim bladder	NaCl suspension and dialysis against acetic acid and distilled water	5.72–11.14%, (w/wt.)	Sousa et al., 2020
II. Acid aided process					
	Pacific cod *(Gadus macrocephalus)*	Skin	0.47 M acetic acid (1:33) for 90.2 h.	37.36% (d/wt.)	Shu et al., 2017
	Atlantic Cod *(Gadus morhua)* and Atlantic Salmon (*Salmo salar*)	Skin	Acetic acid 0.05 M (1: 10) for 72 h.	19.6% for salmon and 10.9% for the cod skin (d/wt.)	Alves et al., 2017
	Salmon *(Oncorhynchus nerka)*	Skin	Acetic acid (0.5 M) (1:10) for 72 h	20.30% (d/wt.)	Tarigan, 2022
	Salmon *(Oncorhynchus nerka)*	Skin	0.5 M acetic acid ratio 1:40 for 48 h.	25.95% (d/wt.)	Nilsuwan et al., 2022a
	Salmon *(Oncorhynchus nerka)*	Skin	0.5 M acetic acid (ratio 1:50) 48 h at 4°C.	36.73% (d/wt.)	Nilsuwan et al., 2022b
	Greenland Halibut *(Reinhardtius hippoglossoides)*	Skin	0.5 M acetic acid (1:20) for 4 days at 4°C.	3.8% (w/wt.)	Martins et al., 2022
III. Enzyme aided process					
	Atlantic Cod *(Gadus morhua)*	Skin	Skin/solvent ratio 1:31 (w/v), 17.6 h, pepsin: 850 unit/g.	55.96% (d/wt.)	Shu et al., 2017
	Atlantic cod *(Gadus morhua)*	Salt cured skin	Bromelain (0.5% w/w), 30°C, 1 h.	44% (d/wt.)	Coscueta et al., 2021
	Red mullet *(Mullus barbatus)*, *dusky grouper* (*Epinephelus marginatus*), gilt-head bream (*Sparus aurata*), Common seabream (*Pagrus pagrus*), Shi drum (*Umbrina cirrosa*) and Atlantic salmon (*Salmo salar*)	Fish scales	0.5 M acetic acid/with 0.1% (w/v) pepsin at a residue-to-solvent ratio of 1:20 (w/v) for 24 h.	1.04–2.03% (w/wt.)	Tziveleka et al., 2022
	Red drum fish *(Sciaenops ocellatus)*	Fish scales	1 M acetic acid solution containing 0.5% pepsin (w/w) for 8 h.	4.32% (w/wt.)	Chen et al., 2016

process (Jaswir et al., 2017). It was also observed that in the HPP aided collagen extraction process, despite of the extraction time and pressure conditions, for obtaining a high fish collagen yield, a higher volume of acid is required in comparison with mammalian sources due to the lower imino acid concentration resulting to its faster denaturation at lower temperatures (Yusof et al., 2017).

3.3.4 Microwave-assisted extraction

MAE is a thermal process which applies electromagnetic waves within a frequency of 300 MHz−300 GHz to elevate the temperature in order to evaporate fluids. Which causes cell deterioration releasing intracellular-bound compounds (Noor et al., 2021). In comparison with other green processes like ultrasound, MAE is observed to produce better yields with less

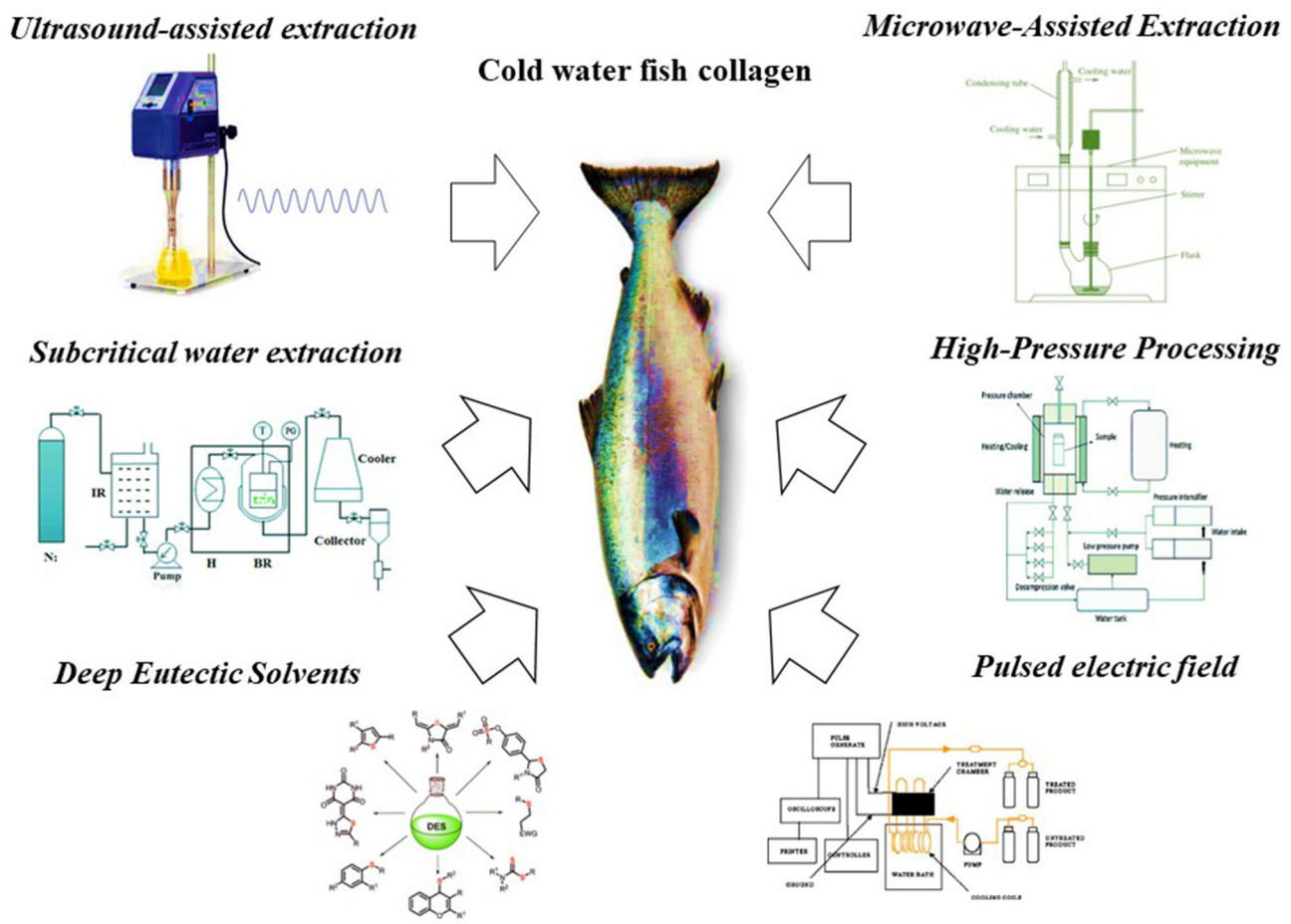

FIGURE 2
Potential green technologies for collagen extraction from fileting side streams.

consumption of solvents (Abdel-Aal et al., 2014). It also has advantages against conventional extraction processes such as reduced time of extraction, higher precision, and preservation of extract qualities (Delazar et al., 2012; Azmir et al., 2013). MAE has previously been successful in extracting collagen from European plaice by-products (Kendler et al., 2023) as well as chitin and chitosan from shrimp shells (El Knidri et al., 2019; Santos et al., 2019).

3.3.5 Deep eutectic solvents

DES are suggested as possible alternatives to traditional ionic fluids and organic solvents (de Morais et al., 2015; van Osch et al., 2015; Zhao et al., 2015). They have many advantages against the conventional ones like biocompatibility (Wagle et al., 2014), convenience in preparation (Passos et al., 2016) eco-friendliness (Pérez and Ramón, 2015) and economical (Li et al., 2013). DES could be prepared by mixing hydrogen bond donor compounds (HBD) or hydrogen bond acceptor (HBA) compounds (Gouveia et al., 2016; Li et al., 2016) which makes the melting point of the mixture lower than the individual constituents (Carriazo et al., 2012; Tang et al., 2012; García-Argüelles et al., 2013). Choline chloride (ChCl) is one such widely used DES which is biodegradable and non-hazardous (Florindo et al., 2014). Bisht et al. (2021) utilized a DES prepared by mixing a solution of Urea: Lactic acid (Molar ratio 1:2) for extracting type-I collagen from Atlantic codfish (*Gadus morhua*). Compared to acetic acid, DES improved the extraction yield and quality of the collagen.

3.3.6 Pulsed electric field

PEF is a non-thermal process which uses DC (Direct Current) voltage pulses in a range from micro to milliseconds through the intended medium placed between two electrodes, resulting in an electric field with an intensity proportional to the voltage applied and the distance between the electrodes (Luengo et al., 2016). The PEF process, when compared to conventional processes offers advantages such as swift and constant transmission rate, high penetration rate, reduced extraction time, reduced consumption of energy, operational continuity, and preservation of the innate properties of the extract (Dellarosa et al., 2017; Salvia-Trujillo et al., 2017). The PEF technology could be utilized for collagen extraction from seafood side streams as studies have demonstrated that a PEF ranging from 100–300 V/cm to 20–80 kV/cm can result in disintegrated cell membranes and thus enhance the extraction process (Barba et al., 2015; Puértolas and Barba, 2016). He et al.

(2019) successfully utilized PEF of field strength of 20 kV/cm, and pulse number of 8 in combination with pepsin (1%) and raw material to liquid ratio of 1:10 for extracting collagen from fish bone side streams.

4 Bionanomaterials from fish fileting side streams

Nanomaterials (<100 nm) are the key constituents in the cutting-edge science of nanotechnology which utilizes their properties such as high surface area, reactivity rate, absorption and strength compared to their massive counterparts enabling their incorporation into matrixes resulting in increased mechanical, physical and chemical properties of the respective composites (Khan et al., 2022). Evolution of bio-polymers for sustainable replacement of petroleum-based plastics has been fueled by the incorporation of nanomaterials into the biopolymer matrix for enhancing the barrier and physical properties (Garcia et al., 2018). But the drawbacks of inorganic nano-materials such as non-biodegradability and toxicity (Khalid et al., 2020) has inspired researchers to explore the possibilities of manufacturing nanomaterials from organic sources to overcome these obstacles. Seafood side streams like fileting discards such as frames and skin are an excellent source of nano-scalable biocomponents like collagen and hydroxyapatite. These biomaterials have a significant potential as nano-fillers in bio-polymer composites (Saini et al., 2018). Nano-collagen is basically the normal collagen reduced to nano-scale, which is below 100 nm. Such nano scaling of collagen enables it to be used as an ideal material for 3D printing bio-compatible materials due to the high surface area to size ratio and penetrability (Naskar and Kim, 2020). Nano-collagen fibers could be used for reinforcing biopolymers, making them breakage resistant with good elastic properties with potential for tissue engineering (Lo and Fauzi, 2021). As previously mentioned HAp is a potential material for biomedical applications. For scaffolings, bone fillers and orthopedic applications the formation into nano-scale is of particular interest. Here, powders, particles and rods in nano-scale generated from diverse marine by-products have been previously studied and listed in the review of Borciani et al. (2023).

4.1 Extraction of nano-collagen

Different techniques like Electrospinning (Law et al., 2017), Nano emulsion (Musazzi et al., 2018), Electrospray Deposition (Nagarajan et al., 2014), Milling (Sanz-Fraile et al., 2020) and Ultrasonication (Wu et al., 2021) are used for nano-scaling collagen molecules. Researchers have extracted nano-collagen from fish swim bladers by applying ultrasonication to the conventional acid extracted collagen (Trilaksani et al., 2020). Ultrasonication was applied at 200 watts, 20 kHz, under a temperature of 15°C with four different time periods such as 60, 90, 120, and 150 min followed by magnetic agitation while 96% ethanol being dripped at a ratio of 1:1 (v/v) at 1,500 rpm for 30 min at 15°C. The ultrasonication for 150 min produced collagen with a particle size of 404.1 nm with 0.446 polydispersity index. Dachi et al. (2020) applied ball milling process to extract nano-scale collagen from fish bone. Jia et al. (2020) used electrospinning technique to prepare fish collagen and polycaprolactone composite nanofibers in three different weight ratios (9:1, 7:3, and 5:5). The composite was prepared as a 12% solution in hexafluoro isopropanol and electrospun using a 1 mL syringe with a 27-gauge needle assisted by a syringe pump under 0.4 mL/h feeding rate with 45–55% humidity, electrospinning voltage of 9 kV DC and a temperature of 21–22°C.

4.2 Extraction of nano-hydroxyapatite

Nano-Hydroxyapatite (n-HAP) has been demonstrated to be synthesized from natural as well as synthetic resources (Sadat-Shojai et al., 2013). The nano-size and the associated morphological features like increased surface area enhances the absorption and other functions of n-HAP compared to its normal form (Yan et al., 2016; Hokmabad et al., 2018). But the fragile nature, hazardous manufacturing process and unbalanced stoichiometric ratio of the synthetic n-HAP has encouraged the industry to explore the natural resources like fish bone (Zhou and Lee, 2011). Tri et al. (2020) prepared n-HAP from salmon bones employing hydrothermal and calcination methods with hydrothermal time/temperature parameters such as 8 h/120°C and calcination time/temperature parameters such as 2 h/800°C. The prepared n-HAP were observed to possess a spherical shape with 30–60 nm particle size, 13.8 $m^2.g^{-1}$ of BET surface area, 0.018 $cm^3.g^{-1}$ pore volume and 24.8 Å pore diameter. The n-HAP also reported a Ca/P molar ratio of 1.64 which was identical with the natural. Venkatesan et al. (2015) utilized alkaline hydrolysis for extracting n-HAP from salmon bones. The pre-treated bones were treated with 2 M NaOH at 200°C for a time period of 1 h. The process was repeated several times and the resultant n-HAP was isolated through centrifugation, washing, and drying. The n-HAP was observed to be in the size range of 6–37 nm with good biocompatibility.

5 Innovative fields of application for the biomaterials extracted from fileting side streams

5.1 Encapsulation of bioactive peptides

The multitude of beneficial effects of marine derived bioactive peptides, such as e.g., their antithrombotic, anticancer, antimicrobial and antihypertensive properties have been previously identified by applying mainly *in vitro* biochemical assays (Ngo et al., 2012). To employ these properties in the human body, and being available at the cell location, peptides need to pass the gastrointestinal digestion (GI) in their intact form. However, peptides are highly susceptible to the GI digestion, hereby losing their structural integrity and further bioactive properties (Mohan et al., 2015). Encapsulation of susceptible compounds, such as bioactive peptides have been introduced to help preserving their functionality, in order to keep their physiological effects in the human body (Mohan et al., 2015; Sun et al., 2021). The encapsulation of bioactive peptides can increase multiple desired functions of the peptides, such as their bioaccessibility,

bioavailability as well as the general digestibility of those peptides. Moreover, sensory properties can be increased, due to camouflaging of bitter and general fishy taste of marine peptides which often occurs after hydrolysis (Hosseini et al., 2021). Hereby, their general applicability in food and nutraceutical products can be increased.

5.2 Biomedical applications of collagen

Due to its preferable biological properties, such as biodegradability and low antigenicity as well as biocompatibility and safety, collagen has become an important resource in medical applications (Lee et al., 2001; Zhang et al., 2022). In addition, Lim et al. (2019) reports especially good water solubility, extractability as well as low immunogenicity and production costs for marine collagen. Moreover, review articles have emphasized on the specific benefits of applying marine collagen in diverse biomedical applications, such as applications in skin, vascular, dental, corneal and cartilage tissue engineering and regeneration, wound healing and skin repair (Lee et al., 2001; Felician et al., 2018; Lim et al., 2019; Lin et al., 2021).

5.3 Cosmetic and nutricosmetic applications of collagen

Collagen's water holding capacity is essential for it being an effective and functional component in cosmetics, hereby contributing to the regulation of skin hydration (Alves et al., 2017). In their study, they concluded that collagen extracted from cod and salmon showed a good ability to retain water. In addition, they report that by exposing topical collagen in a human dermis reconstruction and investigating molecular markers for inflammation and irritation, no irritations were shown. Moreover, studies have shown that marine collagen peptides can be used for skin repair, showing positive effects skin texture, firmness and hydration (Lim et al., 2019).

5.4 Food applications of collagen

Marine collagen has found its way into the food and beverage market already and it can be expected that the number of products with marine collagen as ingredient will rise in the future due to its current popularity. When looking into current products on the market, marine collagen is found in drinks (e.g., hydra.sci; B'EAU; PureWild; COLLAB, etc. …) and in diverse food supplements (e.g., Vild Nord; INLIFE; Edible Earth; etc. …). Due to its functionality and bioactive properties, collagen can be applied as a natural food additive. Moreover, studies reported the benefits of nano-encapsulating marine collagen to preserve the biological functionality and increase the stability of the compounds, especially important when applied in food products (e.g., fluctuating temperature, pH, …) (Mosquera et al., 2014).

5.5 Application of hydroxyapatite

The main field of application and potential for increased utilization of marine-derived HAp lies within biomedical applications, including bone tissue engineering. Previous studies have shown HAp's effect as a coating material for bone implant fixations as well as its strong connection to bone, caused by the production of bone-like apatite (Soballe et al., 1993). The study of Fernando et al. (2016) on chitosan silver/hydroxyapatite composite coatings for biomedical applications reported good bioactive, biocompatible and high osteoconductive properties of HAp. Maschmeyer et al. (2020) refer to biogenic (biological) HAp, as bio-HA and describes fish bones to be the major natural source of calcium phosphate, which constitutes mainly of inorganic matter and thereof a majority in the form of HAp. Moreover, Piccirillo et al. (2015) extracted HAp derived from cod fishbones. They concluded that HAp-based materials are suitable for commercial biomedical products due to great density at high sintering temperatures (1,250°C), good bioactive properties, no cytotoxicity and being compatible to blood (hemocompatibility). Good overall properties, biocompatability and possibilities for applications within the biomedical field (e.g., bioceramics as bone graft substitute; scaffolding materials within dentistry or orthopedics, …) of HAp derived from different fish bones were reported in several studies (Pal et al., 2017; Mohd Pu'ad et al., 2019; Khamkongkaeo et al., 2021; Venkatesan and Anil, 2021; Öteyaka et al., 2023).

6 Potentials and challenges in future applications

As previously elaborated, a large number of highly valuable components are left as side streams after fileting of fish. So far, the marine seafood industry has been successful in implementing processes to further utilize those side streams as pet or animal feed, but has not yet managed to up-cycle these fractions into high-value products. To up-cycle side stream fractions by extraction of high-value components can be regarded as one of the greatest potentials, but also challenges in the marine seafood industry. Hanachi et al. (2022) refers to up-cycled products as second-degree valuated products, which includes the enhancement of biomaterials into products for human consumption, specifically pharmaceuticals, dietary or cosmetic products. The characteristics and positive effects of marine peptides on human health have been reported previously (Ngo et al., 2012; Ghanbari, 2019) and can be regarded as having a good potential in preventing diseases, as functional food ingredients and may help maintaining physical health when consumed frequently (Hanachi et al., 2022). Studies have also demonstrated through *in silico* analysis that the extracted peptides from seafood sources exhibits specific enzyme inhibiting bioactive properties which could be utilized in designing natural and non-toxic therapeutic measures (Jayant Singh et al., 2023). Moreover, further transformation of these up-cycled components into nano-biomaterials and encapsulated products offers the chance to enhance their functionality and positive effect on the human body. The bioactivity efficiency

of these bio-nanocomponents could be enhanced if emphasis on molecular modeling prior to *in-vivo* or *in-vitro* applications is given in future research (Javan Nikkhah and Thompson, 2021).

6.1 Industry scale-up and connected challenges

The scale-up for green extraction procedures from lab to pilot/industry-scale has previously been investigated especially for the extraction of plant bioactive compounds. Belwal et al. (2020) state an increasing trend of research focusing on the scale-up of so called non-conventional extraction techniques, including e.g., UAE as well as MAE. Moreover, Belwal et al. (2020) highlights that the scale-up from bench to pilot/industry-scale keeps the efficient extraction for most processes. However, this efficiency will be necessary in order to keep extractions as short as possible and as long as necessary to guarantee high quality extracts, while limiting the energy consumption. It is crucial that next to process scalability, economic analysis must be considered. One great challenge for the utilization of marine by-products remains the question of how to guarantee stable quality and supply of fractions. For industry to refine by-products into the desired biomaterials, it is of utmost importance to have a stable supply and the same quality of products (Herrero and Ibañez, 2018). The application of green technologies becomes more relevant in this aspect as it influences the quality and reliability of the extracted components, especially those finding application in biomedical fields (Ozogul et al., 2021). As previously highlighted by Rustad et al. (2011), the reproducibility, characteristics of the by-product fraction as well as feasibility for industrial processes are major challenges to further up-cycle by-products into high value compounds for the market. This leads to an inconstant supply, which makes it difficult to establish a market with competitive prices, compared to synthetically produced compounds, which often pose the cheaper option (Olsen et al., 2014). Only by overcoming these known obstacles, will it be possible to introduce marine by-products into the markets. More awareness of waste reduction as well as the high quality of these fractions has led the industry to slowly move toward the holistic utilization of marine resources. However, it requires all stakeholders (governments, fishing industries, consumers, producers and sellers) of the respective countries to pull together for an increased utilization of valorized products for human consumption.

7 Conclusion

The sustainability goals setforth by the United Nations insists on maximum utilization of available resources limiting discards from the point of generation to consumption. Re-entry of valuable nutrients as well as biomaterials into human consumption trail through valorization of the respective side streams is a significant procedure to be adapted by the fishing industry. The cold water fish fileting side streams is such an important resource which provides unlimited valorization options in the form of protein and biominerals. By utilizing fileting by-products to generate and up-cycle biomaterials, a significant amount of solid side streams is valorized and re-enters the value chain for humans. The conventional extraction methods of these biocomponents when gradually replaced by greener processes shall provide a complete sustainable technology frame work for the industry to adapt inlight of the growing sustainable and blue-bio-economy concept. The technologies and resources are available to generate environmentally friendly, high value products for diverse sectors. However, big investments to introduce these green technologies, including appropriate facilities, adequate storage and availability of raw materials pose a big challenge, which has yet not been overcome, but will be necessary to reach the UN sustainability goals.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ishimozuku (*Sphaerotrichia firma*) lipids containing fucoxanthin suppress fatty liver and improve short chain fatty acid production in obese model mice

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Ishimozuku (*Sphaerotrichia firma*) is one species of edible brown algae in Japan. The lipids contain rich of fucoxanthin and n-3 unsaturated fatty acids. This study analyzed dietary Ishimozuku lipid components and the effects on fatty liver and intestinal metabolite component production in obese model mice. Fatty acid composition and fucoxanthin contents of Ishimozuku lipids were analyzed by gas chromatography (GC) and high performance liquid chromatography (HPLC) analysis. Ishimozuku lipids contained fucoxanthin about 44.71 ± 0.02 mg/g. KK-A^y mice were fed with high-fat diet (Control) and a high-fat diet + 0.5% Ishimozuku lipid diet (Ishimozuku) for 4 weeks. Liver triglyceride contents and serum triglyceride concentrations were significantly lower in the Ishimozuku group than in the Control group. Results show that acetyl-CoA carboxylase (ACC) and stearoyl-CoA desaturase (SCD-1) mRNA expression were suppressed in the livers of Ishimozuku group mice. Furthermore, short chain fatty acid amounts in the cecal contents were greater in the Ishimozuku group mice. These results suggest that dietary Ishimozuku lipid components suppress liver lipid accumulation. Results also indicate Ishimozuku lipid components improve the balance of the intestinal microflora, which is disturbed by a high fat diet, and which promotes short chain fatty acid accumulation in the intestine. That accumulation is related to lipid and glucose metabolism.

KEYWORDS

fucoxanthin, obese, fatty liver, algae, short chain fatty acid, Ishimozuku, microflora

1 Introduction

Obesity is considered a major risk factor for type-2 diabetes, hypertension, and hyperlipidemia (Flier, 2004). The incidence of this cluster, which is known as metabolic syndrome, is a worldwide issue (Matsuzawa et al., 1999). Furthermore, Obesity leads to low-grade chronic inflammation, which is the cause of insulin resistance (Fernandez-Real and Ricart, 2003). Non-alcoholic steatohepatitis (NASH), a non-alcoholic fatty liver disease, is a condition by which inflammation and fibrosis occur in the liver in addition to fat accumulation (Musso et al., 2016). It in-duces more severe diseases such as hepatic cirrhosis or liver cancer. Although the mechanisms of NASH progress have not been

clarified even today, the "two-hit theory" is generally reported (Tiniakos et al., 2010). According to this theory, fat accumulation in the liver results from factors such as obesity and insulin resistance as a first hit (simple steatosis). Thereafter, simple steatosis progresses to steatohepatitis because of factors such as inflammatory cytokines and oxidative stress as the second hit. Therefore, when developing a therapeutic agent, this mechanism must be considered sufficiently. Investigating the site of the point which agents suppress is particularly important.

Undaria pinnatifida (Japanese Wakame), *Saccharina japonica* (Japanese Makonbu), and *Sargassum fulvellum* (Japanese Hondawara) are all known to contain fucoxanthin, which is a specific carotenoid in brown algae (Terasaki et al., 2021a). The structure of fucoxanthin is unique, with an unusual alliance bond and 5,6-monoepoxide in its molecule. According to reports, fucoxanthin has bioactivities that include anti-oxidant, anti-inflammatory, anticancer, anti-obesity, anti-diabetic, and anti-angiogenic activities (Peng et al., 2011). Dietary intake of fucoxanthin induces expression of uncoupling protein 1 and plays an important role in energy expenditure in white adipose tissue (WAT) (Maeda et al., 2005, 2007a). Moreover, it exhibits anti-inflammatory effects in adipose tissue, consequently ameliorating low-grade chronic inflammation induced by obesity (Hosokawa et al., 2010). Therefore, dietary fucoxanthin improves insulin resistance caused by obesity (Zhang et al., 2018). In addition, fucoxanthin has been proven to be the most potent inducer of apoptosis and anti-proliferation in human cancer cells (Kotake-Nara et al., 2001, 2005). Reportedly, fucoxanthin has anti-tumor properties sufficient to prevent carcinogenesis in mice (Terasaki et al., 2021b).

It is particularly interesting that dietary fucoxanthin has been reported to affect gut microbiota. Seaweed lipids containing fucoxanthin induce short-chain fatty acid production in intestines of colorectal cancer model mice (Rausch et al., 2021). Moreover, fucoxanthin has been associated with gut microbiota related to short-chain fatty acid production in colorectal cancer model mice (Terasaki et al., 2021a).

Earlier reports of some studies have described anti-obesity and anti-diabetes effects of dietary Wakame lipids in obesity-model mice. Wakame, consisting of brown algae, contains fucoxanthin. Moreover, brown algae, including Wakame, contain n-3 polyunsaturated fatty acids in marine products such as eicosapentaenoic acid (EPA) and stearidonic acid. These fatty acids reportedly improve arteriosclerosis and NASH (Scorletti et al., 2014). Dietary EPA and docosahexaenoic acid (DHA) improve the gut microbiome related to metabolism in diabetic model mice (Zhuang et al., 2021). Therefore, algae lipids contribute to prevention of these obesity-related disorders.

In Japan, Mozuku is well-known and familiar edible brown algae. The volume of Okinawa-mozuku (*Cladosiphon okamuranus*) distribution has become ~90% of the market in Japan. That amount includes aquaculture techniques, which have supported stable production. By contrast, Ishimozuku (*Sphaerotrichia firma*) is an edible brown algae harvested from Hokkaido to Kyushu in Japan. The production is low and it is all-natural. Its morphology resembles that of this Okinawa-mozuku (*Cladosiphon okamuranus*), but the species are different. Earlier reports have also described that Ishimozuku has higher fucoxanthin and polyphenol contents than those of Okinawa-mozuku (Maeda et al., 2018). For that reason, Ishimozuku shows higher anti-oxidant activity than Okinawa-mozuku.

This study evaluated the effects of lipid components of Ishimozuku on suppressing accumulation of fatty liver and hyperlipidemia. High fat diets containing Ishimozuku lipids were administered to obese model KK-A^y mice for 4 weeks. Subsequently, suppressive effects of hepatic lipid accumulation and gene expression of lipid synthesize was analyzed. Furthermore, as an effect on gut microbiota, changes in the productivity of short-chain fatty acids in the intestinal tract were evaluated.

2 Materials and methods

2.1 Samples and reagents

Ishimozuku was harvested in Fukaura town in Aomori, Japan. Plant samples were collected in 2019 during the summer. Ishimozuku lipid components were extracted using acetone. Then 4 kg wet weight algae were washed by water and were wiped off using a paper towel. After the lipid components were extracted using 4 L acetone for 12 h, they were filtrated using paper filter, and evaporated by rotary evaporator. About 10 g Ishimozuku lipids were obtained. All chemicals, which were guaranteed to be of reagent or tissue-culture grade, were from Sigma (MO, USA) or Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2 Fucoxanthin contents and fatty acid composition of Ishimozuku lipids

Fucoxanthin contents was analyzed by HPLC as previously reported (Maeda et al., 2018). The fatty acid composition of the Ishimozuku lipids was determined using a gas chromatograph (GC, GC-14B; Shimadzu Corp., Kyoto, Japan) equipped with an capillary column (Omegawax® 320; Merck KGaA, Darmstadt, Germany), as described in an earlier report (Maeda et al., 2007b).

2.3 Animal diet and care

Obesity-model mice (KK-A^y mice, male, 4 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and housed them in an air-controlled room (temperature, 23 ± 1°C) with a 12/12-h light/dark cycle. Mice were given free access to standard diets and tap water without any restrictions. After a week of adaptation, mice were divided control group (n = 8) and Ishimozuke group (n = 8), then fed an experimental diet for 4 weeks. The diet was made in accordance with AIN-93G's recommendations. The diet compositions are presented in Table 1. The mice were killed after they were anesthetized with isoflurane. Serum preparation for biochemical analysis was done after blood was collected from the abdominal aorta. The removal of liver, kidney, spleen, brown adipose tissue (BAT), and epididymal, mesenteric, inguinal, perirenal and retroperitoneal white adipose tissue (WAT) were

TABLE 1 Composition of diets used for animal experiments (grams/kg of diet).

Ingredients	Control	Ishimozuku
Lard[a]	130	130
Soybean oil[b]	70	70
Ishimozuku lipid		5
Corn starch[c]	277.4	277.4
Casein[c]	230	230
Dextrinized cornstarch[c]	92.1	92.1
Sucrose[d]	100	100
AIN-93 mineral mixture[c]	35	35
AIN93 vitamin mixture[c]	10	10
L-cystine[e]	3	3
Choline bitartrate[e]	2.5	2.5
Cellulose[c]	50	45
Tert-Butylhydroquinone[e]	0.014	0.014
Total	1000	1000

[a]Showa Chemical Co. Ltd., Tokyo, Japan. [b]Wako Pure Chemical Inds. Ltd., Osaka, Japan. [c]CLEA Japan, Inc. Tokyo, Japan. [d]Kanto Chemical Co. Inc., Tokyo, Japan. [e]Sigma-Aldrich Corp., St. Louis, USA.

carried out quickly, weighed, and frozen with liquid nitrogen. It was then stored at −70°C until analysis. To study mRNA expression, a small part of both the liver and epididymal WAT was stored in RNA LaterTM solution (Thermo Fisher Scientific K.K., Tokyo, Japan) at −70°C. An automatic analyzer (Olympus AU5431; Olympus Corporation, Tokyo, Japan) was used to analyze serum components (GOT, GPT, triglyceride) at the Japan Medical Laboratory (Osaka, Japan). The NIPRO Stat Strip XP2 (Nipro Corp., Osaka, Japan) was used to analyze blood glucose concentrations.

2.4 Measurement of hepatic lipid weights

Lipids were extracted according to the method explained by Folch et al. (1957). The 50 mg liver was homogenized using 2 mL chloroform: methanol (2:1 = v/v) and 2 mL water. Then it was centrifuged by 2000 rpm for 10 min. Next, the supernatant was transferred, 2 mL of chloroform : methanol (2:1 = v/v) was added to the residue again for extraction. Then, the supernatants were combined. The solvent was removed using an evaporator to obtain liver lipids.

Triglyceride E-test Wako (Fujifilm Wako Pure Chemical Corp., Osaka, Japan) was used to measure liver triglyceride. After 10 μL of 10 mg/mL dimethyl sulfoxide (0.1% Tween-20) liver lipids were plated in a 96 well plate, 200 μL of the enzyme reagent was added to each well. Then the reaction was conducted at 37°C for 15 min. The absorbance was measured at a main wavelength of 595 nm and at a sub-wavelength of 690 nm (Varioskan LUX; Thermo Fisher Scientific K.K., Tokyo Japan).

2.5 Quantitative real-time PCR analysis

The QuickGene RNA tissue kit S II (Kurabo Industries Ltd., Osaka, Japan) was used to extract total RNA from samples that had been treated with RNA later, according to the manufacturer's protocol. cDNA was generated from total RNA by following the manufacturer's protocol with a High-Capacity cDNA Reverse Transcription Kit provided by Thermo Fisher Scientific K.K., Tokyo, Japan. The AriaMx Real Time PCR system, which is owned by Agilent Technologies Japan, Ltd. in Tokyo, Japan, was used for real-time quantitative PCR analysis. The PCR solution comprised SYBER Green supermix, each primer (10 μM) and the cDNA sample. PCR cycling conditions were 95°C for 1 min and 40 cycles of 95°C for 15 s, 60°C for 1 min. The Primer sequences for ACC, ACL, SCD-1 and TNF-α were the following: ACC-F 5'-tgcctatgaactcaacagcg-3', ACC-R 5'-ctgtttagcgtggggatgtt-3'; ACL-F 5'-atcatggactcctacggcag—3', ACL-R 5'-gcttgggcttcatttctg ttt—3'; SCD-1-F 5'-agaaacatgcgtagacggatca-3', SCD-1-R 5'-agaaa catgcgtagacggatca-3'; TNF-α-F : 5'-tccaggcggtgcctatgt-3'; TNF-α-R : 5'-gcccctgccacaagca-3'; and GAPDH-F 5'-catggccttccgtgttccta-3', GAPDH-R 5'-gcggcacgtcagatcca-3'.

2.6 Analysis of metabolic parameters in cecal contents

After the cecum was suspended in cold saline, and weighed, the 0.1 g of cecal content mixed with 500 μL distilled water was mixed with a vortex mixer for 5 min. Then it was centrifuged 1000 rpm for 5 min. The pH of supernatant was analyzed using a compact pH meter LAQA twin (Horiba Advanced Techno, Co., Ltd., Kyoto, Japan).

The labeling reagents for long-chain and short-chain fatty acid analysis (YMC Co., Ltd., Kyoto, Japan) were utilized to analyze the contents of short chain fatty acids in cecum in accordance with the manufacturer's protocol. In a water bath (60°C), 1 mL of cecal content extract was mixed with 200 μL of reagent A and reagent B for 20 min. Subsequently, after mixing with 200 μL of reagent C and incubating at 60°C for 15 min, it was mixed with 4 mL of reagent D and 5 mL of hexane and centrifuged at 3000 rpm for 5 min. Nitrogen gas was used to correct and evaporate the supernatant. The 200 μL of methanol was used to dilute the residue and store it at −20°C until it is analyzed by HPLC. The labeling reagent was analyzed using an ACQUITY UPLC H-Class PLUS system (Nihon Waters K.K., Tokyo, Japan). The reverse-phase column used was a 2.1 mm × 100 mm column (ACQUITY UPLC HSS T3 1.8 μm; Nihon Waters K.K., Tokyo, Japan). The mobile phases were 0.1% formic acid water (solvent A) and 0.1% formic acid acetonitrile (solvent B). Linear gradient elution was performed as time (*t* min), (t, A%): (0 min, 100%), (3 min, 70%), (7 min, 60%), (10 min, 60%), and (10.1 min, 100%); with a 0.3 mL/min flow rate, 5 μL injection volume, at 400 and 210 nm wavelengths, with 30°C column temperature. The standard curve prepared for each standard reagent was used to calculate the concentrations of lactic acid, acetic acid, propionic acid, and butyric acid.

TABLE 2 Fatty acid contents in Ishimozuku lipids.

	Fatty acid	(wt %)
Saturated fatty acids	C12:0	1.23 ± 0.03
	C14:0	6.01 ± 0.06
	C16:0	29.64 ± 0.96
	C18:0	5.89 ± 0.15
	C20	0.91 ± 0.06
	C22:0	0.33 ± 0.03
Mono-unsaturated fatty acids	C16:1n-7	0.98 ± 0.05
	C18:1n-9	13.82 ± 0.22
	C22:1	0.16 ± 0.02
Poly-unsaturated fatty acids	C18:2n-6	10.43 ± 0.19
	C18:3n-6	1.15 ± 0.03
	C18:3n-3	3.74 ± 0.07
	C18:4n-3	6.04 ± 0.09
	C20:4n-6	7.77 ± 0.44
	C20:5n-3	6.97 ± 0.41
Saturated fatty acids		44.01 ± 1.34
Mono-unsaturated fatty acids		14.96 ± 0.20
Poly-unsaturated fatty acids		36.10 ± 1.19

TABLE 3 Tissue weights of KK-A^y mice fed experiment diets (g/100 g body weight; WAT, white adipose tissue).

	Control	Ishimozuku
Liver	6.27 ± 0.13	6.06 ± 0.10
Spleen	0.26 ± 0.07	0.26 ± 0.10
Kidney	1.41 ± 0.14	1.46 ± 0.12
Cecum	0.76 ± 0.04	0.68 ± 0.04
Epididymal WAT	4.62 ± 0.01	4.38 ± 0.02
Mesentery WAT	2.73 ± 0.14	2.70 ± 0.22
Perirenal and retroperitoneal WAT	1.59 ± 0.04	1.67 ± 0.06
Inguinal WAT	0.78 ± 0.02	0.82 ± 0.04
Total WAT	9.72 ± 0.18	9.57 ± 0.18

TABLE 4 Plasma parameters and leptin concentrations of KK-A^y mice fed experiment diets.

	Control	Ishimozuku
Triglyceride (mg/dl)	489 ± 72	306 ± 29*
GOT (IU/L)	81 ± 5	90 ± 4
GPT (IU/L)	33 ± 2	32 ± 1
Blood glucose level (mg/dl)	580 ± 21	557 ± 18

*$p < 0.05$ vs. Control.

2.7 Analysis of metabolic parameters in cecal contents

Results expressed as mean ± standard error (SE) are presented hereinafter. Statistical analyses between multiple groups were determined using ANOVA. Statistically significant differences were estimated using Fisher's PLSD test. Significant difference was inferred for $p < 0.05$. Statistical analyses were conducted using software (Stat View-J ver. 5.0; SAS Institute Inc., IL, USA).

3 Results

3.1 Fucoxanthin content and fatty acid composition of Ishimozuku lipids

Fucoxanthin contents of Ishimozuku lipid extract were analyzed using UHPLC. Results show that fucoxanthin contents were 44.71 ± 0.02 mg/g. Table 2 presents the fatty acid composition of the Ishimozuku lipid extracts. The percentages of saturated fatty acids were 44.01%. Monounsaturated fatty acids were 14.96%. Polyunsaturated fatty acids were 36.10%. The major fatty acids were palmitic acid (C16: 0, 29.64%), oleic acid (C18: 1n-9, 13.82%), and linoleic acid (C18: 2n-6, 10.43%). In addition, there were contents of stearidonic acid (C18: 4n-3, 6.04%) and eicosapentaenoic acid (C20: 5n-3, 6.97%).

3.2 Body weight gain, food intake, and tissue weight

The final body weight did not show any significant ($P>0.05$) difference (Control group, 42.4 ± 0.6 g; Ishimozuku group, 42.7 ± 0.8 g) and food intake (Control group, 164.4 ± 2.2 g; Ishimozuku group, 169.1 ± 4.5 g). Tissue weights found at the end of experimental diet feeding period are presented in Table 3. No significant difference ($P > 0.05$) was found between liver, spleen, kidney, or cecum weights, on between WAT weights.

3.3 Plasma parameter and blood glucose concentration

Table 4 shows plasma lipid parameters. Triglycerides were significantly lower in the Ishimozuku group ($p < 0.05$) than in the Control group. Glutamic-oxaloacetic transaminase (GOT) and glutamic-oxaloacetic transaminase (GPT) are indicators of liver inflammation. They were unaffected. Blood glucose concentrations did not show significant different between groups.

3.4 Hepatic lipid content

Figures 1A, B respectively portray the lipid and triacylglyceride contents of livers. Lipid contents of the Ishimozuku group tended to be lower than those of the Control group. Furthermore,

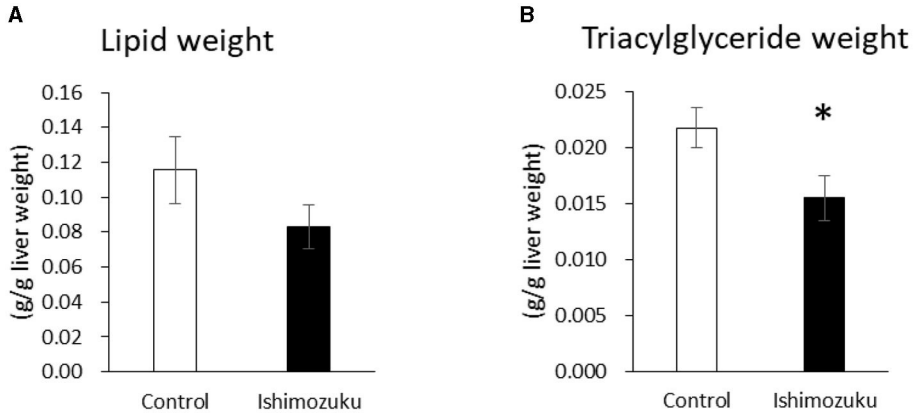

FIGURE 1
Lipid and triglyceride contents in the liver: **(A)** lipid weight and **(B)** triglyceride contents. $^{*}p < 0.05$ vs. Control.

TABLE 5 **Fecal weight and lipid content.**

	Control	Ishimozuku
Fecal weight (g/2 days)	2.11 ± 0.05	2.45 ± 0.20
Fecal triglyceride contents (mg/g dry weight)	0.39 ± 0.03	0.44 ± 0.03

the Ishimozuku group showed significantly more ameliorated triglyceride accumulation than the Control group.

3.5 Fecal weight and lipid content

Table 5 presents the fecal weight and lipid contents. The Ishimozuku group tended to show increased fecal weight and fecal triglyceride contents, but no significant difference was found.

3.6 Gene expression related to fatty acid synthesis and inflammation in the liver and WAT

The mRNA expressions associated with the synthesis of fatty acids in the liver were analyzed using real-time quantitative RT-PCR. Acetyl-CoA carboxylase (ACC) and ATP-citrate lyase (ACL) are rate limiting enzymes of fatty acid synthesis (Softic et al., 2016). After converting citrate to acetyl-CoA to acetyl Co A, ACL converts acetyl Co A to malonyl CoA. ACC mRNA expression was found to be suppressed significantly ($P < 0.05$) in the Ishimozuku group compared with the Control group (Figure 2A).

Stearoyl-CoA desaturase (SCD-1), an enzyme related to fatty acid metabolism, is responsible for forming unsaturated fatty acid. SCD-1 expression is up-regulated in the fatty liver. As a result, synthetic functions of oleic acid or palmitoleic acid are promoted (Ntambi et al., 2002). SCD-1 mRNA expression was significantly ($p < 0.05$) down-regulated in Ishimozuku groups (Figure 2A).

Tumor necrosis factor α (TNF-α) is a cytokine related to inflammation (Fernandez-Real and Ricart, 2003). After TNF-a is secreted from adipocytes in an obesity condition, it induces insulin resistance or low chronic inflammation in the body. TNF-α mRNA expression was found to be suppressed significantly more in the Ishimozuku group of WAT and liver tissue than in the Control group (Figure 2B).

3.7 Short chain fatty acid composition of cecal contents

Table 6 shows short chain fatty acid compositions of cecal contents. The Ishimozuku group mice were found to have significantly increased propionic acid and butyric acid contents compared to those of the Control group. Furthermore, pH of the cecal content was lower in the Ishimozuku group than in the Control group (Figure 3).

4 Discussion

This study analyzed anti-obesity effects of Ishimozuku lipid contents in obesity-model mice (KK-A^{y}). Results show that hepatic TG contents and serum TG concentration were suppressed significantly more in the Ishimozuku group than in the Control group (Table 4, Figure 1B). The mechanism of action was related to suppression of mRNA expression of ACC and ACL, which is a rate-limiting enzyme for fatty acid synthesis in the liver (Figure 2A). Moreover, the Ishimozuku group showed low mRNA expression of TNF-α in WAT and SCD-1 in the liver (Figure 2A). No significant difference was found in the lipid contents of feces. Results suggest that suppressing effects of absorption and promotion of excretion of dietary lipids in the intestine did not contribute to it (Table 5). This result suggests that chronic inflammation caused by obesity was ameliorated in WAT and liver.

Similar effects to those shown by this result have been reported for dietary brown algae lipids in animal experiments. For example, feeding with contained fucoxanthin diets (0.5 mg and 2.0 mg/g) suppressed serum TG concentration and hepatic TG contents (Woo et al., 2009, 2010). Fucoxanthin contents found through the

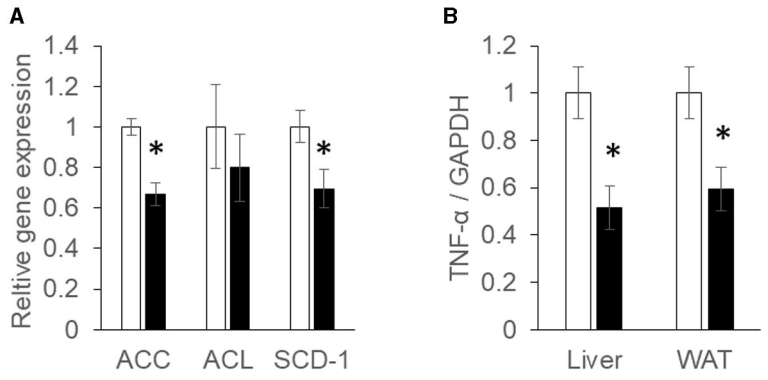

FIGURE 2
mRNA expression concentration of fatty acid synthesis and inflammation: **(A)** acetyl-CoA carboxylase (ACC), ATP-citrate lyase (ACL), and stearoyl-CoA desaturase (SCD-1) mRNA expression in the liver; **(B)** tumor necrosis factor α (TNF-α) mRNA expression concentration in the liver and white adipose tissue (WAT). Black bar represents the Control group. White bar represents the Ishimozuku group. *$p < 0.05$ vs. Control.

present study in the Ishimozuku group were 0.225 mg/g of diets, which was a slightly low level. Ishimozuku lipids contained EPA of about 6.97 ± 0.41% and stearidonic acid (C18: 4n-3) of about 6.04 ± 0.09% (Table 2). EPA is a typical n-3 polyunsaturated fatty acid that is abundant in marine products. Dietary fish oil high in EPA reportedly has suppressive effects of serum TG concentration and hepatic lipid concentration in animal and clinical studies (Dyerberg et al., 1975; Saini and Keum, 2018). Fatty acids contained in seaweed lipids exist mainly in the form of glycolipids. Presumably, the glycolipid-bound EPA contained in Ishimozuku lipids showed inhibitory effects on fatty acid biosynthesis.

The relationship between intestinal microflora and various diseases has been clarified by previous research (Kau et al., 2011). Acetic acid, butyric acid, propionic acid, and lactic acid are among the compounds these microbes secrete in large amounts (Shimizu et al., 2019). Short-chain fatty acids are responsible for maintaining acidic conditions in the intestinal tract and inhibiting the growth of harmful intestinal microbiota. Reportedly, they also reduce the risk of colorectal cancer in the large intestine (Ohigashi et al., 2013). Furthermore, these short chain fatty acids are involved in the regulation of appetite, lipid metabolism, and glucose homeostasis (Shimizu et al., 2019). Short-chain fatty acid receptors are present in the intestinal tract (Bouter et al., 2017). G-protein-coupled receptors GPR43 and GPR41 have been reported as major short-chain fatty acid receptors. Actually, GPR43, which is present in adipose tissue, promotes lipid metabolism (Kimura et al., 2013). GPR41 is present in the sympathetic nerve, where it promotes energy consumption by sympathetic nerve activation (Samuel et al., 2008). Also, GPR41 regulates the secretion of an intestinal hormone (PYY), which suppresses appetite (Degen et al., 2005).

By contrast, earlier reports indicate that type 2 diabetes, which is related to obesity, changes the intestinal microflora, especially decreasing the rate of short chain fatty acid production (Karlsson et al., 2013; Sato et al., 2014). Results show that the intestinal tract becomes alkaline, and that the host metabolism is downregulated, thereby increasing appetite. In these experiments, down-regulation of pH of the cecal contents was confirmed in the Ishimozuku group (Figure 3). In connection with that, contents of propionic acid and butyric acid were increased in the cecal contents (Table 6). This result suggests that short chain fatty acids in the gut stimulate GPR41 in the sympathetic nerve, might have caused lipid metabolism by stimulating sympathetic nerves in the Ishimozuku group.

TABLE 6 **Short chain fatty acid composition of cecal contents.**

	Control	Ishimozuku
Lactic acid (ng/g)	353.5 ± 64.9	360.2 ± 58.0
Acetic acid (ng/g)	1342.4 ± 174.5	1409.7 ± 76.2
Propionic acid (ng/g)	111.3 ± 13.2	223.2 ± 22.5*
Butyric acid (ng/g)	105.2 ± 8.0	191.6 ± 38.4*

*$p < 0.05$ vs. Control.

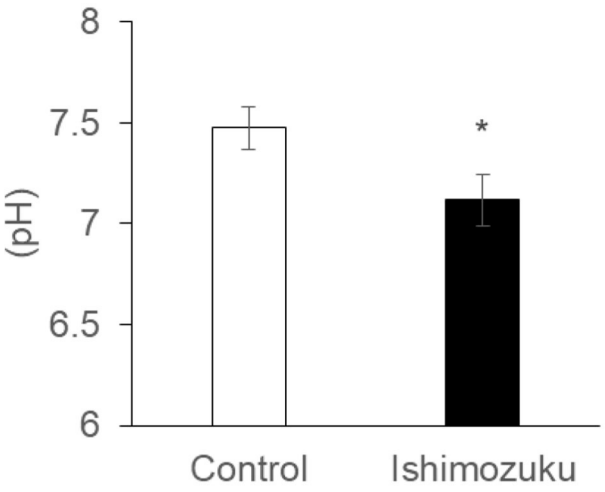

FIGURE 3
pH of cecal content extract *$p < 0.05$ vs. Control.

Gut bacteria ferment dietary fiber to produce short-chain fatty acids. Therefore, a diet that is high in fiber increases short-chain fatty acid production. However, in this experiment,

dietary algae lipid components promoted short chain fatty acid-production. Dietary fucoxanthin changes the gut microflora in colon cancer model mice (Terasaki et al., 2021a). It was reported *Lachnospiraceae*, which convert polysaccharide into short chain fatty acids, were increased in fed with fucoxanthin diets group. High fat diets are a risk factor affecting the incidence of colon cancer. Other reports suggest that dietary algae lipids containing fucoxanthin promote short chain fatty acid production (Sun et al., 2020). One report describes that dietary DHA, an n-3 polyunsaturated fatty acid, promote *Bifidobacterium*, *Lactobacillus*, and SCFA-producing species in gut microbiota, and upregulate short chain fatty acid production (Zhuang et al., 2021). Collectively, these results suggest that algae lipids increase the proportion of gut microbiota related to short chain fatty acid production and regulate lipid and glucose metabolism.

5 Conclusions

Dietary Ishimozuku lipid attenuated fatty liver and serum triglyceride concentrations in obesity-model mice. Results show that it promoted short chain fatty acid production in the intestine. Those short chain fatty acids are related to lipid metabolism. These results suggest that fucoxanthin contained in Ishimozuku lipids is useful as a food ingredient for controlling obesity-related diseases.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. The raw data were generated at Hirosaki University. Derived data supporting the findings of this study are available from the corresponding author HM on request.

Ethics statement

The animal studies were approved by the Animal Research Committee of Hirosaki University (approval number A11002). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MS: Conceptualization, Data curation, Methodology, Writing—original draft, Writing—review & editing. SF: Conceptualization, Funding acquisition, Supervision, Validation, Writing—review & editing. MT: Supervision, Validation, Writing—review & editing. HM: Funding acquisition, Supervision, Writing—original draft, Writing—review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Effect of high-pressure pretreatment on enzymatic hydrolysis of a mixture of rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) rest raw material

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Introduction: Fish rest raw material generated from the fish processing industry may be a useful resource for recovery of added value compounds. The application of non-thermal novel technologies can improve the extraction. High-pressure processing (HPP) has long been used for the preservation and extension of the shelf life of seafood. It also constitutes a promising technology for the increased recovery of valuable compounds, such as lipids and proteins. The objective of this study was to assess the yield and the chemical composition of the fractions obtained after enzymatic hydrolysis on a mixture of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) rest raw material pretreated by high-pressure (HP).

Methods: Six different pretreatments were applied prior to enzymatic hydrolysis; 600MPa x 8 min, 600MPa x 4 min, 400MPa x 8 min, 400 MPa x 4 min, 200MPa x 8 min, 200 MPa x 4 min.

Results and discusssion: These applied pretreatments did not yield higher oil extraction compared to the control. However, the fish protein hydrolysates (FPH) contained higher amount of protein when compared to the FPH obtained from the control.

KEYWORDS

fish protein hydrolysates, fish oil, high-pressure processing, fish rest raw material, salmon, trout

1 Introduction

Fishery and aquaculture global production came up to a record of 214 million tonnes in 2020, where 89% of it was directed to human consumption (FAO, 2022). Increased supplies, income growth, and improvement in applied technologies are among the factors contributing to the increased *per capita* consumption of aquatic food (20.2 kg) in 2020 compared to the previous years. Salmon and rainbow trout are species with high demand, especially in North America and Europe (FAO, 2022). However, as fisheries and aquaculture production expand, a proportional amount of fish rest raw material is generated. This consists of heads, bones,

trimmings, viscera, skin, frames, belly flaps, and intestines, which may reach up to 70% of the processed fish (Rustad et al., 2011).

Several studies have shown that the enzymatic hydrolysis of fish rest raw material, utilizing exogenous enzymes, provides a method to increase the revenue through the recovery of valuable compounds, such as lipids and proteins. In addition to nutritional properties these can display a pharmaceutical potential (Liaset et al., 2000; Šližyte et al., 2005; Kim and Mendis, 2006; Araujo et al., 2021; Bartolomei et al., 2023).

Novel non-thermal technologies, such as UV-light (UV), ultrasound (US), irradiation (IR), cold plasma (CPL), high-pressure processing (HPP), and pulsed electric field (PEF), have been studied extensively for their ability to preserve and extend the shelf-life of seafood avoiding nutritional loss while keeping their sensory quality (Olatunde and Benjakul, 2018; Ekonomou and Boziaris, 2021). Furthermore, the recent application of these technologies in fish rest raw materials appeared promising and gained high commercial interest as they may facilitate an increased recovery of value-added products compared to traditional extraction methods (Al Khawli et al., 2019; Ali et al., 2021; De Aguiar Saldanha Pinheiro et al., 2021). More specifically, HPP may disrupt the non-covalent interactions in biological tissues, leading to cell membrane destruction, increased cell permeability, and moderate denaturation and unfolding of proteins (Tao et al., 2014). Thus, when HPP is implemented as pretreatment before enzymatic hydrolysis, an improved enzyme-substrate interaction can be observed, causing a significant generation of bioactive compounds (Thoresen et al., 2020; Zhang et al., 2021). However, due to limited knowledge of the influence of the application of high-pressure treatment on the extraction of valuable compounds from seafood rest raw material, more research in this field is needed (Hassoun et al., 2023; Siddiqui et al., 2023).

The current study aims to assess the impact of high-pressure processing at varying pressure levels and hold times on a mixture of salmon and rainbow trout side streams. This pretreatment occurs before enzymatic hydrolysis and focuses on extraction yield and the characteristics of the resulting fractions.

2 Materials and methods

2.1 Preparation of samples

Fresh Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (4–6 h after slaughter) were purchased from Hofseth Aqua AS in Ålesund, Norway and transported on ice to the laboratory. Fish was immediately eviscerated by hand, and the rest raw material (heads, tails, skin, bones, and trimmings) was minced using a mincer (Hobart A200N) with a hole size of 9 mm. The minced raw material (1:1 w/w Atlantic salmon and rainbow trout) was placed in plastic bags of approximately 1.1 kg per unit before being transferred to −80°C. Further, the samples were sent frozen to Teagasc research center (Dublin, Ireland) for high-pressure (HP) treatment.

2.2 HP treatment

Frozen fish samples were thawed overnight (4°C) prior to HP treatment. The fish samples underwent a HP treatment using a commercial-scale Hyperbaric 420 High-Pressure Processor, with a pressure vessel of 200 L volume (HPP Tolling, St. Margaret's Dublin, Ireland), where pressure conditions of 200, 400, and 600 MPa were applied for hold times of 4 and 8 min, respectively. Sample temperature prior to HP treatment was 3.4°C and post-treatment temperature of the product was 6.5°C. The inlet water temperature throughout HPP was 9.0 ± 0.5°C. The pressurization rate for each treatment was 100 MPa/min. A control sample was also included, which did not undergo HP treatment, providing a reference for comparison purposes. Following the HP treatment, all the samples, including the control, were stored at −80°C and then sent back frozen to NTNU Ålesund for further analysis.

2.3 Enzymatic hydrolysis

Frozen raw material, both pretreated with HP and control (without pretreatment), was left to thaw in the cold room overnight at 6 ± 2°C prior to enzymatic hydrolysis. A small amount of the minced raw material, corresponding to each pretreatment and the control, was freeze-dried (Freezone 12 L, −84°C, Labconco Corporation, Kansas City, USA) and stored at −80°C for further analysis, while the rest was subjected to enzymatic hydrolysis (Figure 1). The hydrolysis was carried out in duplicate in 4 L closed glass vessels, where an equal proportion of raw material and warm water (50°C) was used. To enhance the quality of the final product, the hydrolysis was performed away from direct sunlight and flushed by nitrogen (Yorulmaz et al., 2011), which was inserted into the vessels for 30 s. Then, the vessels were placed in a water bath with a temperature of 53°C under stirring (150 rpm). When the mixture's temperature reached 50°C, natural antioxidants previously prepared at University of Zagreb (Dalsvåg et al., 2021) were added as a mix of chamomile and oregano extracts in a ratio of 1:1 which was added at an amount of 1.5 mL/kg raw material. After 10 min, the enzymes Papain FG and Bromelain 400 GDU/g (both from Enzybel) were introduced into the vessels in equal proportion, in a total amount of 0.1% (w/w) (1:1), and the hydrolysis was left to proceed for 1 h. Next, the mixture was filtered through a sieve with a hole size of 1.4 × 1.4 mm where solids/paste were removed, weighed, and stored at −80°C until analysis. Part of the solids/paste was freeze-dried prior to storage. The remaining hydrolysate mixture was equally divided into borosilicate glass beakers of 500 mL and transferred to a microwave oven where the inactivation of the enzymes took place at 90°C for 15 min. The hydrolysate mixture was left to chill before further centrifugation at 2250xg for 45 min. Centrifugation separated the hydrolysate mixture mass into four layers which were collected and analysed; the oil layer on the top, followed by the emulsion, the water-soluble fish protein hydrolysate (FPH), and the sludge at the bottom. After weighing, all the fractions were stored at −80°C for further analysis. Part of the emulsion, FPH, and sludge was freeze-dried and held at −80°C.

2.4 Product yield and recovery of protein and oil

The product yield of each fraction was calculated as a percentage of the total initial weight of the raw material (w/w) before enzymatic hydrolysis. The protein and oil recovery in each fraction obtained after enzymatic hydrolysis was determined as a percentage of the total content of protein and oil (w/w), respectively, present in the raw material prior to enzymatic hydrolysis.

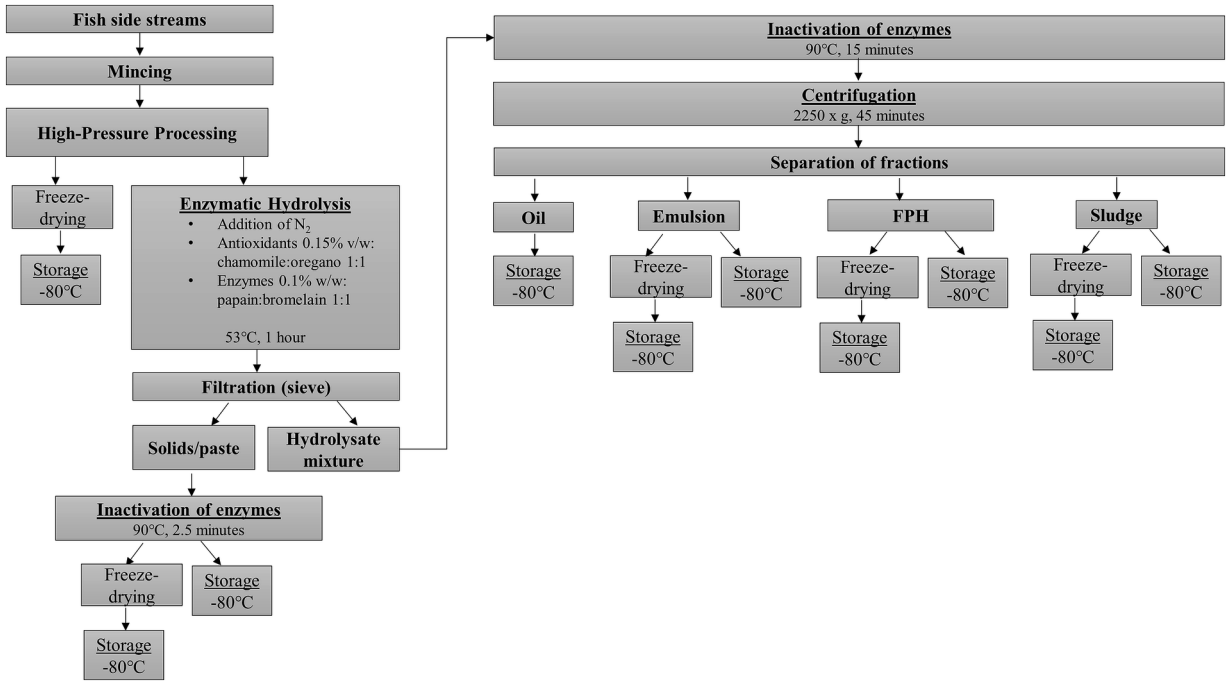

FIGURE 1
Schematic illustration of the procedure on applying HP pretreatment and enzymatic hydrolysis of a mixture of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) rest raw materials and the final products.

2.5 Chemical analysis

2.5.1 Moisture and ash content

The moisture content of raw material, solids/paste, protein, emulsion, and sludge, before and after the freeze-dryer, was determined by drying at 105°C until constant weight (24 h), according to (AOAC, 1990). The ash content of non-freeze-dried samples was determined by holding the samples at 550°C overnight (AOAC, 1990). Both moisture content and ash were determined in duplicate.

2.5.2 Crude protein content

The crude protein content was measured in triplicate in dried samples multiplying % total nitrogen by the nitrogen-to-protein conversion factor (NPCF) of 6.25, using the Dumas combustion principle, performed in an NDA 702 Dumas Nitrogen Analyser (Velp Scientifica, Italy). In cases where the measured crude protein content deviated from the expected value, a mathematical calculation was employed, taking into account the oil and ash content.

2.5.3 Total lipid content

Oil extraction was performed in duplicate using a mixture of water, methanol, and chloroform, as described by (Bligh and Dyer, 1959).

2.5.4 Determination of free amino acid (FAA) composition

The FAAs in the obtained protein hydrolysates was determined in triplicate, as described by Osnes and Mohr (1985) with modifications.

1 mL of water-soluble protein extract was transferred to Eppendorf tubes before adding 0.25 mL of 10% sulfosalicylic acid, followed by shaking. The solutions were stored in a cold room (4°C) for 30 min and then centrifuged for 10 min at 17400xg with an Eppendorf Microcentrifuge 5418R (Eppendorf AG, Germany). The obtained supernatant was suitably diluted and filtered before being inserted into vials and further analysed by high-pressure liquid chromatography (VHPLC Dionex Ultimate 3,000, Thermo Fisher Scientific Inc., USA) using a Nova Pak C18 column (Waters™, USA).

2.6 Degree of hydrolysis (DH)

The DH of fish protein hydrolysates was evaluated in duplicate by the formol titration method of Taylor (1957). Suitably diluted protein hydrolysate was titrated with NaOH after mixing with formaldehyde, using an automatic titrator (TitroLine® 7,800, Xylem Analytics, Mainz, Germany) coupled with a platinum electrode (Pt 62):

$$DH\% = \frac{\%\ free\ amino\ groups}{\%\ total\ nitrogen} \times 100$$

2.7 Statistical analysis

Effects of treatments on product yield and chemical composition was evaluated by ANOVA. A significance level of $p < 0.05$ was established to assess the statistical significance of observed differences between treatment groups and control. *Post hoc* analysis was performed using Tukey's honestly significant difference (HSD) test and Student's t-test to identify mean differences. The statistical analysis was conducted using SigmaPlot software, version 14 (Systat Software Inc., San Jose, California, USA).

3 Results and discussion

3.1 Product yield

The applied HPP conditions did not exhibit a significant influence on the yield of oil, emulsion, and FPH fractions obtained after enzymatic hydrolysis of samples consisting of a mixture of rainbow trout and Atlantic salmon rest raw material (Figure 2). However, between the quantity of oil and emulsion, and the applied pretreatment a correlation ($r = 0.833$ and $r = 0.845$, respectively) was observed. Specifically, higher applied pressure led to a decreased yield of oil and emulsion. The pretreatment conditions of 600 MPa x 8 min, 600 MPa x 4 min, 400 MPa x 8 min, and 400 MPa x 4 min resulted in significantly lower amounts of oil compared to the pretreatments 200 MPa x 8 min and 200 MPa x 4 min. The control demonstrated the highest oil yield. These findings are opposed to the results presented by Thoresen et al. (2020) in their study on HP pretreated residual materials from the chicken industry prior to enzymatic hydrolysis. Furthermore, the pretreatment 200 MPa x 4 min along with the control showed significantly higher emulsion yield compared to the other pretreatments. In contrast, the pretreatment 400 MPa x 4 min resulted in the lowest emulsion yield. However, it is worth highlighting that there was no significant difference in the emulsion yield among this pretreatment and the pretreatments 600 MPa x 4 min and 400 MPa x 8 min.

As displayed in Figure 2, apart from the sample pretreated at 200 MPa x 4 min, the remaining samples subjected to various HPP conditions showed a slightly higher yield of FPH compared to the control. The highest FPH yield was obtained from the sample pretreated at 600 MPa x 4 min. However, this difference was not statistically significant. This increase could be attributed to protein unfolding due to HPP leading to enhanced FPH volume (Thoresen et al., 2020) during enzymatic hydrolysis and the subsequent reduction in hydrophobic free amino acids, as shown in section 3.4.

The yield of sludge and solids/paste obtained after enzymatic hydrolysis was significantly affected by the applied HPP conditions and particularly pressure (Figure 2). The quantity of sludge obtained from samples subjected to pretreatment at 600 MPa x 8 min, 600 MPa x 4 min, 400 MPa x 8 min, and 400 MPa x 4 min was found to be significantly lower compared to the sludge quantity obtained from samples pretreated at 200 MPa x 8 min, 200 MPa x 4 min, or the control. The latter resulted into a significantly higher amount of sludge among all tested conditions suggesting that no HP pretreatment or application of lower pressures can result in greater sludge formation after enzymatic hydrolysis compared to the samples pretreated at higher pressures. These findings could confirm the assumption that the conformational changes in proteins due to HPP may have improved the availability of proteins for hydrolysis contributing to reduced sludge generation.

Regarding the yield of solids/paste fractions, the highest quantity was observed in samples pretreated at 600 MPa x 8 min, 600 MPa x 4 min, 400 MPa x 8 min, and 400 MPa x 4 min with no statistically significant difference among them. However, the sample pretreated at 200 MPa x 8 min demonstrated a significantly lower yield of solids/paste. Further, the sample pretreated at 200 MPa x 4 min and the control resulted in the lowest amount of solids/paste. These findings, highlight the significant correlation ($r = 0.938$) between the HP pretreatment conditions and the subsequent yield of solids/paste after enzymatic hydrolysis (Figure 3).

The increased quantity of solids/paste in samples subjected to higher pressures may be attributed to protein denaturation induced by HPP. It has been reported that higher pressures, specifically above 300 MPa, can lead to increased protein denaturation (Lullien-Pellerin and Balny, 2002), resulting in the formation of aggregates that are resistant to enzymatic degradation during hydrolysis (Wang et al., 2015; Joye, 2019). Consequently, the limited enzymatic breakdown of proteins, along with the possibility of denatured proteins encapsulating lipids within the protein matrix (Chapleau et al., 2004), may contribute

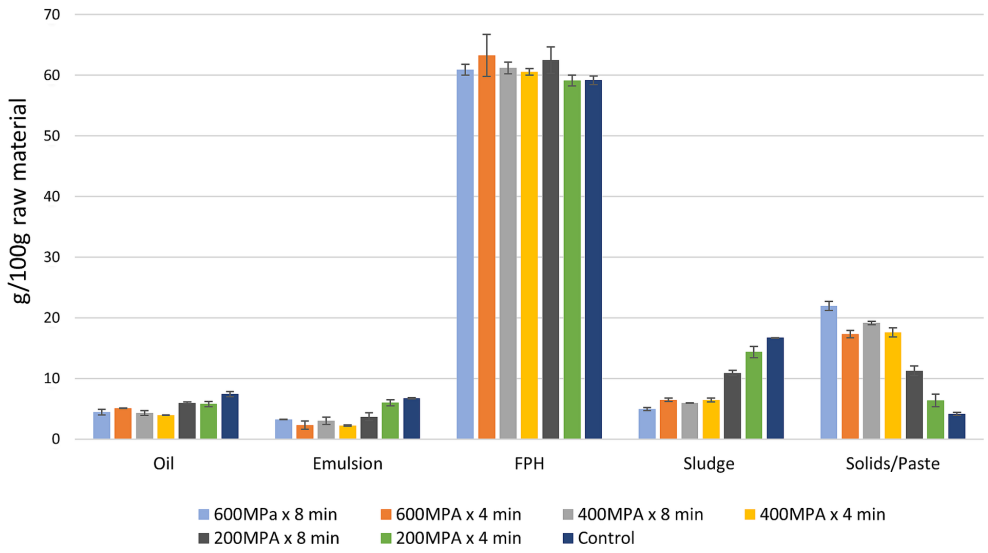

FIGURE 2
Quantification of the fractions obtained through enzymatic hydrolysis on samples consisting of a mixture of rainbow trout and Atlantic salmon rest raw material subjected to HPP and on the control after the addition of equal proportion of raw material and warm water in hydrolysis vessels expressed as g/100 g raw material (mean ± SD, $n = 2$).

to the elevated generation of solids/paste during enzymatic hydrolysis. This could also account for the lower oil and sludge yield obtained from samples pretreated by HPP compared to the control, as a correlation (r = 0.890) has been identified between the oil and solids/paste fractions. Furthermore, a significant correlation (r = 0.992) was observed between the sludge and solids/paste fractions. The solids/paste mass obtained from the control was dominated by fish bones.

3.2 Protein and oil recovery

The application of HPP had a significant impact on the protein recovery within the FPH, sludge, and solids/paste fractions. Moreover, HPP application significantly affected the recovery of oil within the sludge and solids/paste fractions. Concerning the FPH fractions, the protein recovery in the control (40.8 ± 0.1 g/100 g dry weight) was significantly higher than that recovered in samples subjected to HP pretreatment (Table 1). Among the pretreated samples, the FPH of the sample pretreated at 200 MPa x 4 min exhibited the highest protein recovery (39.5 ± 0.1 g/100 g dry weight), while the lowest was observed at 400 MPa x 8 min (32.5 ± 0.1 g/100 g dry weight). The oil recovery within FPH fractions was correlated (r = 0.837) with the applied pressure and time. The FPH of the control gave a significantly higher value (1.1 ± 0.1 g/100 g dry weight) compared to the FPH of samples pretreated by HP (Table 2). The values within the FPH fractions of the pretreated samples ranged between 0.3 ± 0.0 g/100 g dry weight and 0.8 ± 0.0 g/100 g dry weight, with the lowest oil recovery observed in FPH of the sample pretreated at 400 MPa x 8 min and the highest in FPH of the sample pretreated at 200 MPa x 8 min, respectively. The duration of the applied pretreatment influenced significantly the protein recovery within the FPH fractions, while it did not exhibit a notable effect on the oil recovery. Among samples subjected to the same pressure, the protein recovery within the FPH of samples pretreated for 4 min was significantly higher compared to those pretreated for 8 min. This observation could suggest that prolonged HP pretreatment could potentially result in decreased protein recovery in FPH.

In the sludge fractions, the values varied between 9.5 ± 0.1 g/100 g dry weight and 31.7 ± 0.6 g/100 g dry weight for the protein and 2.6 ± 0.1 g/100 g dry weight and 8.2 ± 0.5 g/100 g dry weight for the oil recovery (Tables 1, 2). The sludge fraction obtained from the sample pretreated at 600 MPa x 8 min displayed the lowest protein and oil recovery values, in contrast to the control, which demonstrated the highest recovery levels. As the applied pressure decreased, there was a noticeable increase in protein and oil recovery within the sludge fractions. It is worth noting that the protein and oil recovery in sludge showed a significant correlation (r = 0.942 and r = 0.939, respectively) with the sludge yield (Figure 2), indicating that higher sludge yield was associated with an increased protein and oil recovery within the sludge fractions.

A significantly decreasing trend was evident in both the protein and oil recovery within the solids/paste fractions as the applied pressure decreased or no pressure was applied, as illustrated in Tables 1, 2. This could be attributed to the observed increase in solids/paste yield with higher applied pressure. The protein and oil recovery in solids/paste displayed a significant correlation (r = 0.928 and r = 0.952, respectively) with the quantity of this fraction (Figure 2). Thus, a significantly higher protein recovery was observed in the solids/paste fraction acquired from the sample pretreated at 600 MPa x 8 min (55.4 ± 3.7 g/100 g dry weight) compared to the other pretreatments. The pretreatment 400 MPa x 4 min gave the highest oil recovery (42.6 ± 0.7 g/100 g dry weight), although no statistically significant difference was exhibited between this pretreatment and the solids/paste fraction of the pretreatments 600 MPa x 8 min (41.9 ± 3.6 g/100 g dry weight) and 400 MPa x 8 min (41.4 ± 1.1 g/100 g dry weight). Additionally, a significant correlation (r = 0.945) was noticed between the oil and protein recovery within the solids/paste fractions corresponding to various pretreatments, supporting the assumption that lipids could be potentially entrapped by denatured proteins within the protein matrix (Chapleau et al., 2004). Thus, for the pretreatments 600 MPa x 8 min, 400 MPa x 8 min, 400 MPa x 4 min, the solids/paste fraction emerged as the fraction with the highest protein recovery.

The protein and oil recovery in the emulsion was correlated (r = 0.879 and r = 0.801, respectively) with the pretreatment conditions (Tables 1, 2) and the emulsion yield (Figure 2). The control exhibited significantly higher protein (9.4 ± 0.1 g/100 g dry weight) and oil (6.8 ± 0.1 g/100 g dry weight) recovery in the emulsion compared to the samples pretreated by HP. Among the various HP pretreatment conditions studied, the emulsion obtained from the sample pretreated at 200 MPa x 4 min exhibited the highest protein and oil recovery.

The oil fraction acquired from the control displayed the highest oil recovery (69.6 ± 4.0 g/100 g dry weight). However, no statistically significant difference was observed between the oil recovery in the control and that of samples subjected to pretreatment at 200 MPa x 8 min (69.0 ± 1.4 g/100 g dry weight) and 200 MPa x 4 min (62.0 ± 4.6 g/100 g dry weight). These findings align with the results reported by Zhang et al. (2021) in their study regarding oil extraction from fish heads, where ultra-high pressure was employed as a pretreatment before enzymatic hydrolysis. Both studies demonstrated that, among the various pretreatment conditions tested, the highest oil recovery was observed in samples that underwent pretreatment at lower pressure levels.

3.3 Proximate composition

The protein, lipid, and ash content within the FPH, sludge, and paste/solids fractions did not appear to be influenced by the applied HPP conditions (Figure 4). Nevertheless, significantly higher protein content was observed in the FPH obtained from the samples pretreated at 600 MPa x 8 min (96.43 ± 0.4 g/100 g dry weight), 400 MPa x 4 min (97.2 ± 0.1 g/100 g dry weight), and 200 MPa x 4 min (91.4 ± 0.1 g/100 g dry weight) compared to the control (90.6 ± 0.2 g/100 g dry weight) (Figure 4A). However, there was no significant difference in protein content between the FPH of the sample pretreated at 600 MPa x 8 min and 400 MPa x 4 min. Although the protein yield across the FPH fractions did not show significant differences (Figure 2), there was a tendency for a slightly higher protein composition in the FPH derived from these specific pretreatments. This could confirm the assumption that HPP may facilitate protein unfolding and, due to thermal fluctuations, enhance protein solubility in the FPH fractions (Thoresen et al., 2020), leading to improved protein concentration in these. Regarding the lipid content in the FPH, a correlation emerges with the applied pressure and duration of pretreatment (r = 0.836). The lipid content in the FPH

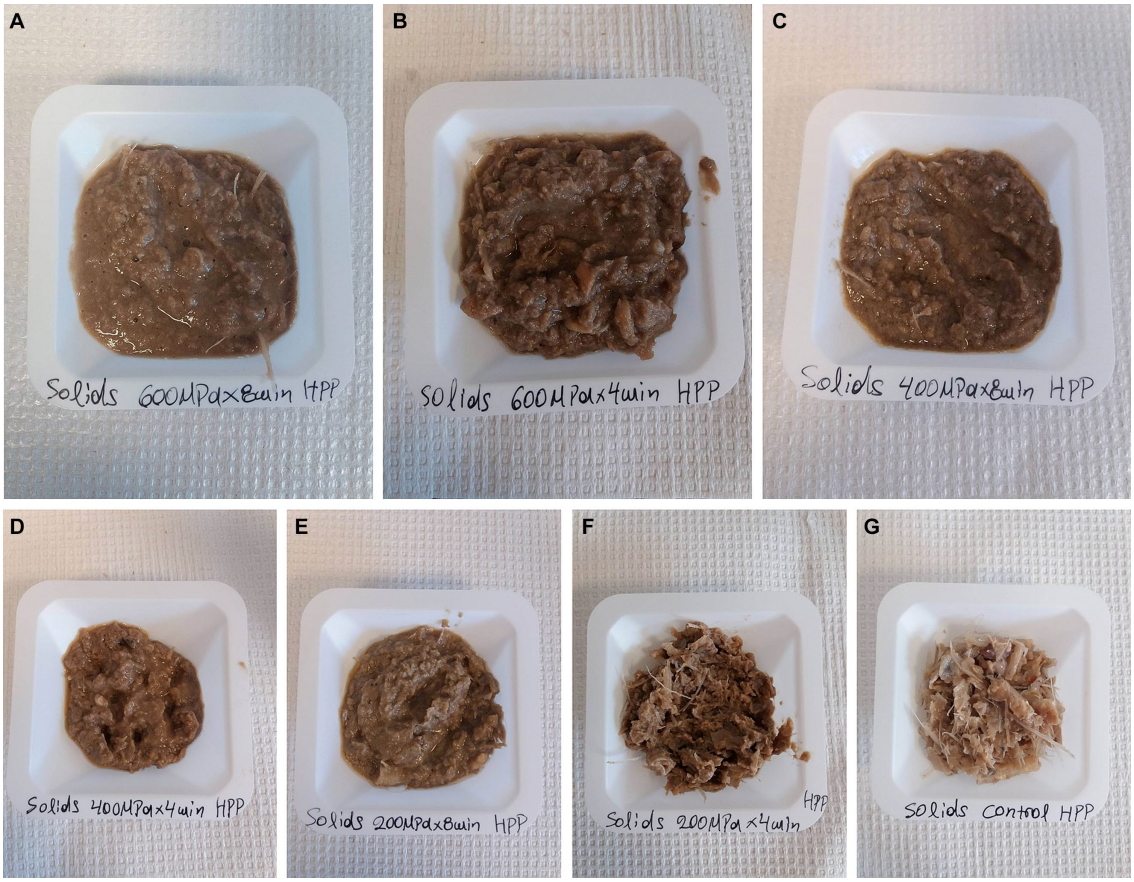

FIGURE 3
Illustration of the solids/paste fractions obtained after enzymatic hydrolysis on samples consisting of a mixture of rainbow trout and Atlantic salmon rest raw material subjected to HPP and on the control; **(A)** solids/paste corresponding to HPP 600 MPa x 8 min, **(B)** solids/paste corresponding to HPP 600 MPa x 4 min, **(C)** solids/paste corresponding to HPP 400 MPa x 8 min, **(D)** solids/paste corresponding to HPP 400 MPa x 4 min, **(E)** solids/paste corresponding to HPP 200 MPa x 8 min, **(F)** solids/paste corresponding to HPP 200 MPa x 4 min, **(G)** solids/paste corresponding to the control.

TABLE 1 Protein recovery in emulsion, FPH, sludge, and solids/paste fractions obtained after enzymatic hydrolysis on samples consisting of a mixture of rainbow trout and Atlantic salmon rest raw material subjected to HPP and on the control expressed as g/100 g dry weight (mean ± SD, $n = 2$).

Fractions	600 MPa		400 MPa		200 MPa		Control
	8 min	4 min	8 min	4 min	8 min	4 min	
Emulsion	3.1 ± 0.1	2.5 ± 0.1	2.7 ± 0.0	2.1 ± 0.1	3.2 ± 0.1	6.4 ± 0.1	9.4 ± 0.1
FPH	34.5 ± 0.1	35.4 ± 0.2	32.5 ± 0.1	37.8 ± 0.0	35.4 ± 0.1	39.5 ± 0.1	40.8 ± 0.1
Sludge	9.5 ± 0.1	11.2 ± 0.2	10.2 ± 0.0	11.1 ± 0.1	20.4 ± 0.2	25.5 ± 0.1	31.7 ± 0.6
Solids/paste	55.4 ± 3.7	24.6 ± 1.3	46.2 ± 1.0	42.3 ± 1.2	28.9 ± 0.3	14.5 ± 1.2	N/A*
SUM	102.4 ± 3.7	73.7 ± 1.3	91.5 ± 1.0	93.3 ± 1.2	87.9 ± 0.4	85.9 ± 1.2	81.8 ± 0.6

*N/A: not assessed.

from the control (2.83 ± 0.20 g/100 g dry weight) appeared significantly higher than that from HP pretreated samples. Among the various pretreated samples, the FPH derived from that subjected to 200 MPa x 4 min demonstrated the highest lipid content (1.8 ± 0.0 g/100 g dry weight). In contrast, the lowest lipid content was observed in the FPH obtained from the sample pretreated at 400 MPa x 8 min (0.7 ± 0.0 g/100 g dry weight). Regarding ash content, notable differences were observed, with significantly higher levels found in the FPH from the samples pretreated at 600 MPa x 4 min, 400 MPa x 8 min, and 200 MPa x 8 min compared to the other conditions.

DH in the FPH fractions did not appear to be influenced by the pretreatment conditions. As depicted in Figure 4A, the samples pretreated at 600 MPa x 4 min (22.6% ± 0.8), and 200 MPa x 8 min (23.1% ± 0.0) showed slightly higher DH in the obtained FPH compared to the FPH from the control (22.0% ± 1.6). However, this difference was not statistically significant.

The protein content within the paste/solids fractions did not exhibit significant difference among the pretreatments 600 MPa x 8 min (53.7 ± 3.6 g/100 g dry weight), 400 MPa x 8 min (50.8 ± 1.1 g/100 g dry weight), 400 MPa x 4 min (49.6 ± 1.4 g/100 g dry

TABLE 2 Oil recovery in oil, emulsion, FPH, sludge, and solids/paste fractions obtained after enzymatic hydrolysis on samples consisting of a mixture of rainbow trout and Atlantic salmon rest raw material subjected to HPP and on the control expressed as g/100 g dry weight (mean ± SD, $n = 2$).

Fractions	600 MPa		400 MPa		200 MPa		Control
	8 min	4 min	8 min	4 min	8 min	4 min	
Oil	46.5 ± 4.9	57.2 ± 0.5	49.1 ± 4.5	42.7 ± 0.7	69.0 ± 1.4	62.0 ± 4.6	69.6 ± 4.0
Emulsion	3.7 ± 0.1	3.3 ± 0.1	2.9 ± 0.0	2.3 ± 0.1	4.3 ± 0.1	6.2 ± 0.1	6.8 ± 0.1
FPH	0.4 ± 0.1	0.6 ± 0.2	0.3 ± 0.0	0.5 ± 0.0	0.8 ± 0.0	0.6 ± 0.1	1.1 ± 0.1
Sludge	2.6 ± 0.1	3.9 ± 0.2	3.1 ± 0.0	4.8 ± 0.0	6.7 ± 0.3	7.3 ± 0.2	8.2 ± 0.5
Solids/paste	41.9 ± 3.6	33.0 ± 1.3	41.4 ± 1.1	42.6 ± 0.7	27.2 ± 0.0	15.2 ± 0.8	N/A*
SUM	94.9 ± 6.1	98.0 ± 1.4	96.7 ± 4.6	93.0 ± 1.0	108.0 ± 1.4	91.4 ± 4.6	85.7 ± 4.0

*N/A: not assessed.

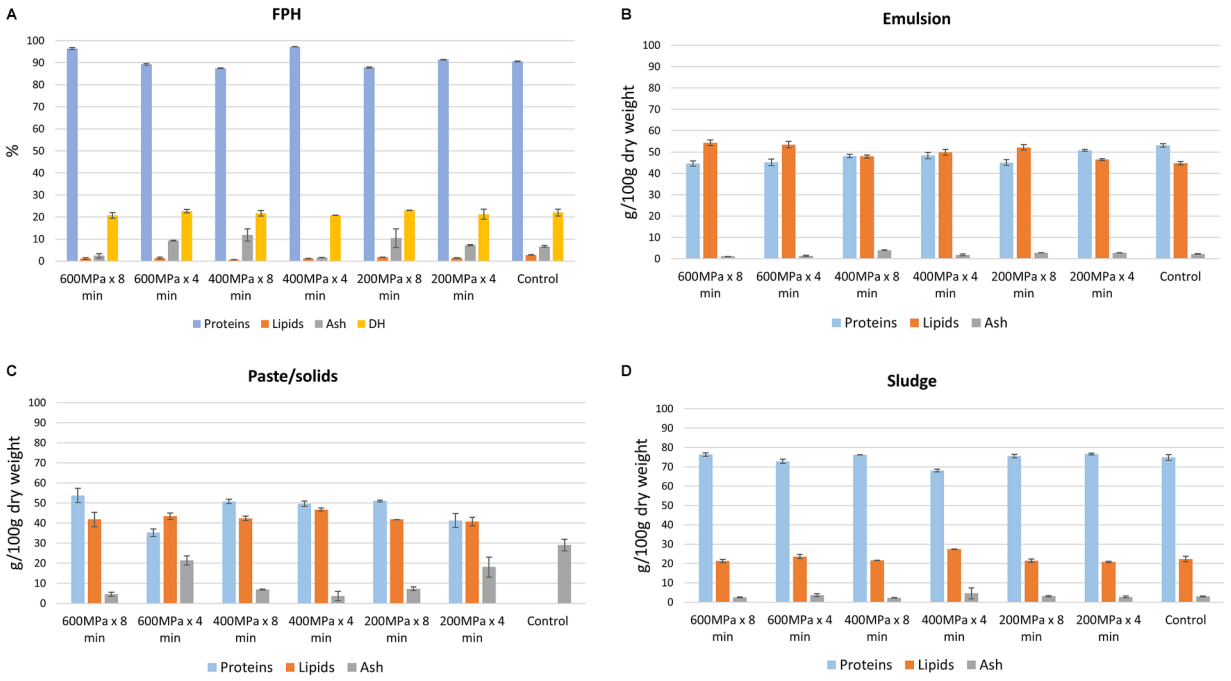

FIGURE 4
Chemical composition of different fractions obtained after enzymatic hydrolysis on samples pretreated by HPP and the control; **(A)** proximate composition (g/100 g dry weight, mean ± SD, $n = 2$), DH (%, mean ± SD, $n = 2$) of FPH **(B)** proximate composition of emulsion (g/100 g dry weight, mean ± SD, $n = 2$), **(C)** proximate composition of paste/solids (g/100 g dry weight, mean ± SD, $n = 2$), **(D)** proximate composition of sludge (g/100 g dry weight, mean ± SD, $n = 2$).

weight), and 200 MPa x 8 min (51.0 ± 0.5 g/100 g dry weight) (Figure 4C). On the other hand, the highest lipid content was observed in the paste/solids fractions obtained from the pretreatments 600 MPa x 4 min (43.4 ± 1.6 g/100 g dry weight) and 400 MPa x 4 min (46.7 ± 0.8 g/00 g dry weight), with no significant difference between these two conditions. As for the ash content, there was a correlation between the pretreatment conditions (applied pressure and time) and the ash content in the fractions (r = 0.816). The solids/paste fraction derived from the control, which primarily consisted of bones, exhibited the highest ash value (29.0 ± 2.90 g/100 g dry weight). In contrast, the fraction corresponding to the 400 MPa x 4 min pretreatment condition displayed the lowest (3.7 ± 2.3 g/100 g dry weight). One of the possible utilization of the solids/paste fraction could be its application as a nutrient-rich substrate for microalgae cultivation (Pleissner et al., 2023). This approach could represent a sustainable utilization of the fractions generated in this study.

The sludge fractions displayed a significant correlation between the protein and lipid content (r = 0.995). Sludge obtained from the pretreatments 600 MPa x 8 min (76.3 ± 0.9 g/100 g dry weight), 400 MPa x 8 min (76.2 ± 0.1 g/100 g dry weight), 200 MPa x 8 min (75.6 ± 0.9 g/100 g dry weight), 200 MPa x 4 min (76.6 ± 0.4 g/100 g dry weight), and the control (74.8 ± 1.5 g/100 g dry weight) exhibited higher protein content (Figure 4D). These pretreatments were associated with lower lipid content. Nevertheless, no significant difference was detected among these fractions concerning both protein and lipid content. Furthermore, there were no significant differences in the ash content among sludge fractions derived from the pretreated samples and the control.

In the emulsion fractions, a correlation was found between the protein and lipid content and the applied pretreatment conditions (r = 0.882 and r = 0.833, respectively). As the applied pressure increased, protein content decreased while lipid content increased (Figure 4B).

Thus, a significant correlation was observed between the protein and lipid content within the emulsion fractions (r = 0.967). The emulsion obtained from the control displayed a significantly higher protein content (53.1 ± 0.8 g/100 g dry weight) than the emulsion from samples exposed to HP pretreatment. Conversely, lower protein content was detected in emulsion fractions from the samples pretreated at 600 MPa x 8 min (44.6 ± 1.3 g/100 g dry weight), 600 MPa x 4 min (45.2 ± 1.5 g/100 g dry weight), and 200 MPa x 8 min (45.1 ± 1.3 g/100 g dry weight). The same pretreatment conditions corresponded to emulsion fractions with the highest observed lipid content, while the emulsion corresponding to the control demonstrated the lowest. The ash content values exhibited a range between 1.1 ± 0.0 g/100 g dry weight and 4.0 ± 0.1 g/100 g dry weight. The emulsion from the sample treated at 400 MPa x 8 min yielded the lowest ash content, while the highest ash content was observed in the emulsion from the sample subjected to pretreatment at 600 MPa x 8 min.

3.4 Free amino acid composition

The FAA composition of the FPH obtained from different HP pretreatments in a mixture of rainbow trout and Atlantic salmon rest raw material is presented in Table 3. Seventeen FAAs were identified and analyzed in this study. The results indicated that there was a significant reduction in the levels of all individual FAAs between the FPH derived from HP pretreated samples and the control. A significant correlation (r = 0.921) was observed between the applied HP pretreatment and the total amount of FAAs. Higher pressure levels resulted in lower total FAA levels, with the FPH pretreated at 600 MPa x 4 min exhibiting the lowest content (37.58 ± 0.64 mg/g dry weight), while the FPH from the control showed the highest content (72.06 ± 1.31 mg/g dry weight). However, significant differences were neither found between the samples pretreated at 600 and 400 MPa nor between the FPH from the control and the samples subjected to pretreatment at 200 MPa x 4 min. A similar trend was observed in the quantities of essential FAAs and non-essential FAAs. In contrast to our findings, the study conducted by Yue et al. (2016) did not observe a comparable decrease in the levels of essential FAAs on day 0 of storage during their investigation into the effects of HPP on squid muscles. In the current study, leucine and lysine emerged as the predominant essential FAAs, while glycine/arginine and alanine dominated the non-essential FAAs. A significant observation was made regarding the content of essential FAAs, as well as glutamine and alanine (non-essential FAA), in the FPH obtained from samples subjected to different levels of HPP. The data revealed a significant decrease in the concentration of these amino acids in FPH derived from samples pretreated at 600 and 400 MPa, with the content being approximately half of that observed in samples pretreated at 200 MPa or the control. These

TABLE 3 Effect of HPP treatments on the FAA composition within a mixture of rainbow trout and Atlantic salmon rest raw material, mg/g dry weight, in FPH (mean ± SD, n = 3).

Amino acids	600 MPa		400 MPa		200 MPa		Control
	8 min	4 min	8 min	4 min	8 min	4 min	
Aspartic acid	0.39 ± 0.03^{a}	0.34 ± 0.02^{a}	0.43 ± 0.01^{a}	0.61 ± 0.06^{b}	0.79 ± 0.02^{b}	1.08 ± 0.06^{c}	1.57 ± 0.11^{d}
Glutamic acid	2.68 ± 0.12^{ab}	2.69 ± 0.29^{ab}	2.44 ± 0.03^{a}	2.47 ± 0.21^{a}	3.06 ± 0.09^{b}	3.09 ± 0.20^{b}	3.84 ± 0.23^{c}
Asparagine	0.15 ± 0.01^{a}	0.18 ± 0.10^{a}	0.12 ± 0.00^{a}	0.24 ± 0.10^{b}	0.33 ± 0.02^{b}	0.28 ± 0.13^{b}	0.30 ± 0.04^{b}
Histidine	1.98 ± 0.08^{a}	2.14 ± 0.10^{ac}	2.13 ± 0.05^{ac}	2.25 ± 0.19^{ac}	2.60 ± 0.08^{bd}	2.41 ± 0.02^{cb}	2.76 ± 0.17^{d}
Serine	1.91 ± 0.05^{a}	3.02 ± 0.15^{b}	2.44 ± 0.05^{c}	2.47 ± 0.19^{c}	3.17 ± 0.12^{bf}	3.63 ± 0.06^{e}	3.54 ± 0.21^{fe}
Glutamine	0.91 ± 0.04^{a}	0.69 ± 0.05^{bc}	0.80 ± 0.01^{ba}	0.62 ± 0.05^{c}	1.44 ± 0.03^{d}	1.74 ± 0.01^{e}	1.83 ± 0.11^{e}
Glycine/Arginine	5.64 ± 0.20^{a}	5.26 ± 0.26^{a}	5.68 ± 0.15^{a}	6.86 ± 0.56^{b}	7.13 ± 0.47^{bc}	7.88 ± 0.10^{c}	7.42 ± 0.44^{bc}
Threonine	2.18 ± 0.08^{a}	1.83 ± 0.10^{a}	1.90 ± 0.05^{a}	2.06 ± 0.17^{a}	2.97 ± 0.33^{b}	3.70 ± 0.04^{c}	3.20 ± 0.20^{b}
Alanine	6.26 ± 0.41^{a}	7.53 ± 0.35^{b}	7.40 ± 0.17^{b}	7.48 ± 0.52^{b}	9.60 ± 0.28^{c}	10.40 ± 0.11^{cd}	11.09 ± 0.65^{d}
Tyrosine	2.21 ± 0.08^{a}	1.44 ± 0.13^{b}	1.45 ± 0.03^{b}	2.04 ± 0.14^{a}	2.93 ± 0.12^{c}	3.25 ± 0.09^{d}	3.30 ± 0.18^{d}
Aminobutyric acid	0.24 ± 0.01^{ab}	0.26 ± 0.02^{b}	0.35 ± 0.01^{c}	0.29 ± 0.04^{bc}	0.20 ± 0.02^{a}	0.24 ± 0.02^{ab}	0.17 ± 0.06^{a}
Methionine	1.71 ± 0.14^{a}	1.49 ± 0.09^{a}	1.82 ± 0.04^{ab}	2.31 ± 0.21^{b}	3.87 ± 0.16^{c}	4.67 ± 0.05^{d}	4.53 ± 0.38^{d}
Valine	1.16 ± 0.14^{a}	1.11 ± 0.02^{a}	1.35 ± 0.01^{ab}	1.62 ± 0.12^{b}	3.40 ± 0.14^{c}	3.94 ± 0.04^{d}	4.25 ± 0.19^{d}
Phenylalanine	2.07 ± 0.18^{a}	1.36 ± 0.07^{b}	1.53 ± 0.08^{b}	2.04 ± 0.19^{a}	3.39 ± 0.14^{c}	3.89 ± 0.05^{d}	3.75 ± 0.22^{dc}
Isoleucine	0.75 ± 0.02^{ab}	0.69 ± 0.04^{a}	0.79 ± 0.02^{ab}	0.93 ± 0.07^{b}	1.97 ± 0.06^{c}	2.24 ± 0.06^{d}	2.52 ± 0.19^{e}
Leucine	4.01 ± 0.13^{ab}	3.13 ± 0.18^{a}	3.86 ± 0.08^{a}	4.79 ± 0.33^{b}	9.27 ± 0.29^{c}	10.61 ± 0.17^{d}	10.45 ± 0.66^{d}
Lysine	5.43 ± 0.16^{a}	4.47 ± 0.19^{b}	4.76 ± 0.13^{ab}	5.34 ± 0.47^{a}	7.21 ± 0.19^{c}	8.04 ± 0.15^{d}	7.65 ± 0.42^{cd}
∑ESFAAs[1]	19.29 ± 0.36^{ab}	16.22 ± 0.32^{a}	18.14 ± 0.20^{ab}	21.33 ± 0.70^{b}	34.69 ± 0.55^{c}	39.49 ± 0.26^{d}	39.11 ± 0.98^{d}
∑NESFAAs[2]	20.39 ± 0.49^{a}	21.42 ± 0.56^{a}	21.10 ± 0.24^{a}	23.06 ± 0.83^{a}	28.63 ± 0.58^{b}	31.59 ± 0.29^{bc}	33.06 ± 0.87^{c}
∑TFAAs[3]	39.68 ± 0.60^{a}	37.58 ± 0.64^{a}	39.24 ± 0.31^{a}	44.45 ± 1.09^{a}	63.33 ± 0.80^{b}	71.15 ± 0.38^{c}	72.06 ± 1.31^{c}
Bitter[4]	9.70 ± 0.30^{ab}	7.77 ± 0.22^{a}	9.35 ± 0.12^{a}	11.68 ± 0.46^{b}	21.91 ± 0.39^{c}	25.34 ± 0.20^{d}	25.50 ± 0.84^{d}

Significant differences among the mean values within the same row are denoted by distinct letters, with a significance level of $p < 0.05$.
[1]ESFAAs: essential free amino acids; [2]NESFAAs: non-essential free amino acids; [3]TFAAs: total free amino acids; [4]Bitter: sum of methionine, phenylalanine, valine, isoleucine, leucine (Pérez-Santaescolástica et al., 2019).

results are consistent with the findings reported by Ahmed et al. (2021) in their study on HP treated hammour (*Epinephelus coioides*) fillets. The observed decrease in the concentration of the amino acids may be explained by the solubilization from muscle proteins which occurred at a slower rate compared to their conversion into biogenic amines through the process of decarboxylation (Ciampa et al., 2012). A slight increase in the concentration of serine, glycine/arginine, methionine, phenylalanine, leucine, and lysine was observed in FPH samples subjected to pretreatment at 200 MPa x 4 min compared to control, although the difference was not statistically significant. This observation can be attributed to the release of these amino acids (AAs) from degraded proteins as a result of improved proteolysis induced by HPP (Yue et al., 2016).

Alterations in the concentration of individual FAAs can significantly impact the taste properties of fish protein hydrolysates. Specifically, the presence of hydrophobic AAs, such as phenylalanine, valine, leucine, isoleucine, methionine, and proline, is closely associated with the perception of bitterness (Pérez-Santaescolástica et al., 2019). The findings of our study could indicate that the bitter taste of FPH could be influenced by HPP. Higher pressure levels applied during HPP could be associated with reduced bitterness in FPH (Table 3). Specifically, the FPH obtained from samples pretreated at 600 MPa x 4 min displayed the lowest content of hydrophobic FAAs, and therefore should probably have the lowest bitter taste, while the FPH from the control exhibited the highest amount of hydrophobic FAAs.

4 Conclusion

The study has shown that the applied HPP has a significant effect on the protein recovery, but not on the protein, lipid, and ash content within the FPH, sludge, and solids/paste fractions. Among the samples, the control exhibited the highest protein and oil recovery in the FPH and sludge, while the sample pretreated at 600 MPa x 8 min yielded sludge with the lowest protein and oil recovery. The samples pretreated at 600 MPa x 8 min and 400 MPa x 4 min produced FPH with the highest protein content. Degree of hydrolysis was found to be unaffected by the HPP. In addition, the application of HPP did not significantly impact the yield of oil, emulsion, and FPH fractions. However, the yield of sludge and solids/paste was notably influenced by the applied HPP conditions, especially the applied pressure levels. The control sample exhibited the highest oil, emulsion, and sludge yield, while the pretreatments at 400 MPa x 4 min and 600 MPa x 8 min resulted in the lowest emulsion yield. Furthermore, as the applied pressure increased, a decrease in the total FAAs of FPH fractions was observed, with an equal decrease in the amount of both essential and non-essential FAAs.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

EK: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Writing – original draft. ED: Formal analysis, Investigation, Resources, Supervision, Validation, Writing – review & editing. GA: Supervision, Writing – review & editing. TR: Investigation, Resources, Supervision, Writing – review & editing. BT: Investigation, Methodology, Resources, Writing – review & editing. JC: Conceptualization, Formal analysis, Investigation, Methodology, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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More sustainable use of aquaculture cleaner fish: collagen-rich protein hydrolysates from lumpfish (*Cyclopterus lumpus*) – effects of biomass, pretreatment, and enzyme choice

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Farmed lumpfish (*Cyclopterus lumpus*) are used as cleaner fish in Norwegian aquaculture. However, once the fish reaches sexual maturity, it becomes less effective in combating lice and is often wasted or turned into silage. This raises ethical, economic, and sustainability concerns regarding whether the use is sustainable without increasing its standard of living and further use of the fish to higher value products. To improve the utilization of lumpfish, a study was conducted to investigate the differences in extraction efficiency by comparing product yield, protein content, and peptide size distribution after enzymatic hydrolysis of lumpfish using several commercial proteases (Corolase 8,000, Corolase 7,089, Endocut 01 L, Flavourzyme, and Food Pro PNL), and different pretreatment methods (pulsed electric field and high-pressure processing). The choice of enzyme did not affect the amino acid contents of the different hydrolysates. Furthermore, varying enzyme concentrations had a minor impact on the final product's protein content. As anticipated, increased enzyme concentrations decreased the hydrolysates' average molecular weight. The study found that biomass treated either chemically (NaOH and butanol) or mechanically with a pulsed electric field before enzymatic hydrolysis resulted in higher protein content and relatively higher amounts of collagen peptides. Initial bioactivity testing indicated that the hydrolysates had no toxic effects on hepatocellular carcinoma and non-malignant lung fibroblast cells. Previous studies have shown that farmed lumpfish contains all the essential amino acids and has high levels of EPA, DHA, B12, and D3 vitamins. The contents of environmental pollutants and heavy metals were also below the EU maximum levels. This study's knowledge and results open for the potential use of lumpfish peptides and gelatin for, e.g., dietary supplements, feed, and biodegradable packaging.

KEYWORDS

lumpfish (*Cyclopterus lumpus*), protein hydrolysates, enzymatic hydrolysis, pretreatment, pulsed electric field, high-pressure processing

1 Introduction

Lumpfish (*Cyclopterus lumpus*) is a marine finned fish found in the North Atlantic and adjacent oceans (Jansson et al., 2023). In the wild, the fish are mainly harvested for their roe, which is used as lumpfish caviar (Powell et al., 2018). In Norway, farmed lumpfish are used as a treatment for sea lice (Brooker et al., 2018) [*Lepeophtheirus salmonis* (Krøyer, 1837)] infestations on Atlantic salmon in the aquaculture industry (Imsland et al., 2014, 2018). Sea lice are marine parasites that attach to the fish's body, feeding off their skin, mucus, and blood, making them more susceptible to other infections (e.g., bacteria, viruses, and fungi).[1] Juvenile lumpfish is transferred to the salmon net cage at approximately six months old (initial weight ~ 25 g) (Ageeva et al., 2021). When they increase in size and reach sexual maturity, there is a decrease in efficacy as lice eaters. This makes the reuse of lumpfish as cleaner fish not feasible (Brooker et al., 2018). In 2022, approximately 17.6 M lumpfish were distributed for use as salmon lice control.[2] After slaughtering the salmon stock in the net cage, the lumpfish is removed and destroyed (waste) or used for silage. The silage suitable for animal feed production can be sold for around 0.2 € per kilo. However, some salmon producers must pay to dispose of the lumpfish (Nøstvold et al., 2016).

The use of cleaner fish has raised ethical, economic, and sustainability concerns (Garcia de Leaniz et al., 2022) regarding whether the use of lumpfish is sustainable without increasing its standard of living and increasing the utilization of biomass to produce higher-value products. Usually, all applicable resources should follow the "food first"-principle and subsequently be used for the highest possible value (Vang et al., 2021). Recently, there has been a focus on new utilization areas for farmed cleaner fish. Zhuang et al. (2018) and Thong et al. (2023) tested the viability of lumpfish as food by exploring possible products that could be made in Vietnamese cuisine and understanding the stakeholders' perception and acceptance of the fish. Studies have shown (Ageeva et al., 2021) that farmed lumpfish contains all the essential amino acids and has high levels of EPA, DHA, B12, and D3 vitamins. Contents of environmental pollutants and heavy metals were also below the EU maximum levels, indicating that the lumpfish can be further exploited for human consumption. Some work has been published on collagen extraction from lumpfish (Zhuang et al., 2018; Vate et al., 2023), but there are few (if any) publications regarding protein hydrolysis of whole lumpfish.

Many studies have been conducted on producing and using collagen, gelatin, and enzymatically produced hydrolysates from other aquatic side streams. These proteins have various applications in biomedical engineering (Milan et al., 2021), food supplements (Lin et al., 2020), cosmetics (Amnuaikit et al., 2022), and food packaging materials.[3] Previous studies have shown that the skin and head of lumpfish can account for up to 54% of the biomass (Ageeva et al., 2021), and these parts are affluent in connective tissues, including collagen (Dave et al., 2019), possibly making it attractive for a protein source for further use in applications mentioned above. Leftover marine biomass has previously proven to be a potential source for generating natural bioactive peptides and proteins, focusing on bioactivities such as antimicrobial (Naghdi et al., 2023), anti-inflammatory (Giannetto et al., 2020), and ACE inhibition activities (Abbas et al., 2022).

This study aimed to explore differences in product yield, protein content, and peptide size distribution resulting from various commercial proteases and pretreatment techniques and find an efficient extraction method. The study is divided into three experimental setups: (1) extraction of lumpfish skin gelatin (LSG) and whole lumpfish gelatin (WLG), (2) testing commercial enzymes and concentrations for protein hydrolysis, and (3) testing chemical and mechanical pretreatment techniques in combination with enzymatic hydrolysis. Pretreatment of raw materials before enzymatic hydrolysis can significantly influence a hydrolysis process by providing more accessible sites for enzymes by increasing the surface area and by partially or fully unfolding the proteins (Asaithambi et al., 2022). The different pretreatments can also affect textural and sensory properties (Asaithambi et al., 2022). The hydrolysate's proximate composition and peptide size distribution were analyzed to find potential differences. Initial bioactivity assays were performed to assess any potential biological activities of the hydrolysates.

2 Materials and methods

2.1 Materials and reagents

The lumpfish used in this study were sampled from salmon net cages in March 2020 from Karanes (Karlsøy, Troms, and Finnmark county, Norway) as described in Ageeva et al. (2021). Whole lumpfish and lumpfish skin was cut into 2 × 2 cm pieces, packed in vacuum bags (Scancell, Kuppenheim, Baden-Wurttemberg, Germany), and frozen at −40°C until further processing.

The proteases used for enzymatic hydrolysis were Corolase 8,000 (batch number R205464ST, 100000 BPU/g, AB Enzymes, Darmstadt, Germany), Corolase 7,089 (batch number F181360ST, 840 Uhb/g, AB Enzymes, Darmstadt, Germany), Food Pro PNL (batch number 4863924615, 1,600 U/g, DANISCO, Copenhagen, Denmark), Flavourzyme (batch number HPN00549, 1000 LAPU/g, Novozymes, Bagsværd, Denmark) and Endocut 01 L (batch number 1043, 180 NU/g, Tailorzyme, Herlev, Denmark). All the enzymes used comply with the recommended purity specifications for food-grade enzymes issued by the joint FAO/WHO Expert Committee on Food Additives and the Food Chemicals Codex.

Peptide standards for molecular weight (MW) distribution were purchased from Sigma-Aldrich: Carbonic Anhydrase from bovine erythrocytes (C7025, 29 kDa), Lysozyme from chicken egg white (L7651, 14.3 kDa), Cytochrome c from the bovine heart (C2037, 12.3 kDa), Aprotinin from bovine lung (A1153, 6.51 kDa), Insulin Chain B oxidized from bovine pancreas (I6383, 3.5 kDa), Renin Substrate Tetradecapeptide porcine (R8129, 1.76 kDa), Angiotensin II human (A9525, 1.05 kDa), Bradykinin Fragment 1–7 (B1651, 0.757 kDa), [D-Ala^2]-Leucine enkephalin (E5008, 0.569 kDa), Val-Tyr-Val (V8376, 0.379 kDa), and L-Tryptophane (T0254, 0.204 kDa). All cell lines and microbes used for bioactivity screening were purchased at LGC standards: HepG2 (ATCC HB-8065™), MRC5 (ATCC CCL-171™), *Enterococcus faecalis* (ATCC 29212),

1 https://www.hi.no/en/hi/temasider/species/sea-lice

2 https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Akvakulturstatistikk-tidsserier/Rensefisk.

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Escherichia coli (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (ATCC 12386), *Candida albicans* (ATCC 90028) and *Staphylococcus epidermidis* (ATCC 35984).

All chemicals used in this study have been purchased from Sigma Aldrich and VWR and solutions for electrophoresis through Thermo Scientific.

2.2 Methods

2.2.1 Gelatin extraction and verification

Gelatin extraction was performed as described by Arnesen and Gildberg (2007), with slight modifications. The extraction was performed once for lumpfish skin (LS) and whole lumpfish (WL). LS and WL were processed as described in section 2.1 and performed in a closed 1 Liter high viscosity glass reactor (IKA, Staufen, Germany) with impeller and temperature control. The raw material to solvent ratio was 1:3.3 with the various solutions. The raw material was incubated twice in 0.05 M NaOH solution for 30 min before being drained in a sieve. After washing with cold water to remove excess NaOH, the biomass was neutralized with 0.067 M sulfuric acid before treatment with 5 mM citric acid. Each acid step was performed for 30 min. The biomass was further washed with cold water before gelatin was extracted with water for 2 h at 55°C. Before lyophilization, the LSG and WLG extract were filtrated with grade 4 filter paper (Whatman, Cytiva, Medemblik, Holland). The following formula calculated the mass yield for gelatin extraction:

$$Yield\,(\%) = \frac{Weight\ of\ dried\ gelatin\,(g)}{Weight\ of\ initial\ wet\ biomass\,(g)} \times 100$$

Protein recovery was measured by determining the amino acid content in the biomass and the dried WLG. Recovery is presented as percent hydroxyproline (Hyp) extracted compared to the Hyp content in the original biomass using the following formula:

$$Recovery\,(\%) = \frac{Hyp\ content\ of\ dried\ gelatin\,(\%) \times weight\ of\ gelatin\,(g)}{Hyp\ content\ of\ initial\ biomass\,(\%) \times weight\ of\ biomass\ used\,(g)} \times 100$$

To validate if gelatin had been extracted, SDS-PAGE was performed using Xcell SureLock Mini-Cell (Thermo Fisher Scientific, Waltham, United States) on NuPage 4–12% BisTris, 1.0 mm, mini protein gel in NuPage MOPS SDS running buffer. LSG and WLG were dissolved in water, and NuPage LDS Sample Buffer (4X) containing 5% 2-mercaptoethanol was boiled for 5 min. Samples (20 µg/well) were applied to sample wells and electrophoresed for 50 min at 200 V and 150 mA using SeeBlue Plus 2 pre-stained as a marker. The separated proteins were stained using NuPage Simply Blue Safe stain.

2.2.2 Enzymatic hydrolysis

Previous unpublished studies showed that the protein content (measured by the Kjeldahl method) and total amino acid content (Supplementary Tables S1, S2) after enzymatic hydrolysis on whole lumpfish vs. lumpfish skin was similar (ranging between 74 and 77%). Since filleting lumpfish is a time-consuming manual process (Figure 1) and therefore expensive, it was decided that the experimental work would be performed on whole lumpfish gutted with head. Firstly, the lumpfish was hydrolyzed using a selection of commercial proteases at different concentrations (Figure 2). Secondly, enzymatic hydrolysis was combined with mechanical and chemical pretreatment of the biomass (Figure 3), as described in section 2.2.3.

For enzymatic hydrolysis, the biomass was thawed at 4°C for 24 h before being mixed with tap water at a 1:1 ratio (w/v). All hydrolyses were carried out once for each enzyme concentration in closed 1 Liter high viscosity glass reactors (IKA, Staufen, Germany) with impeller and temperature control. The mixture was heated to the optimum temperature for each protease used: Flavourzyme (FL): 50°C, Endocut 01 L (E1): 50°C, Corolase 7,089 (C7): 55°C, Corolase 8,000 (C8): 65°C and FoodProPNL (FP): 55°C. The reaction was initiated by adding the enzyme of choice to the desired concentration (Figures 2, 3). The hydrolyses were conducted for 60 min at 40 rpm before inactivation at 90°C for 15 min. The samples were coarsely filtered through a sieve before centrifugation at 8,000 rpm for 20 min at 18°C in an Avanti

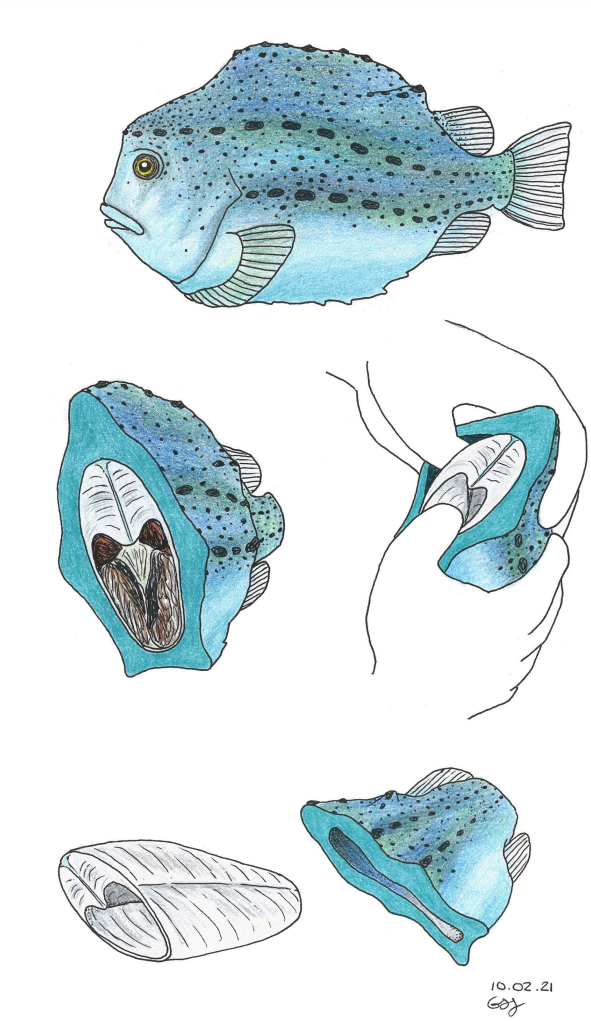

FIGURE 1
Illustration of manual lumpfish filleting. Illustration by Gunhild Seljehaug Johanson, Nofima, Tromsø, Norway.

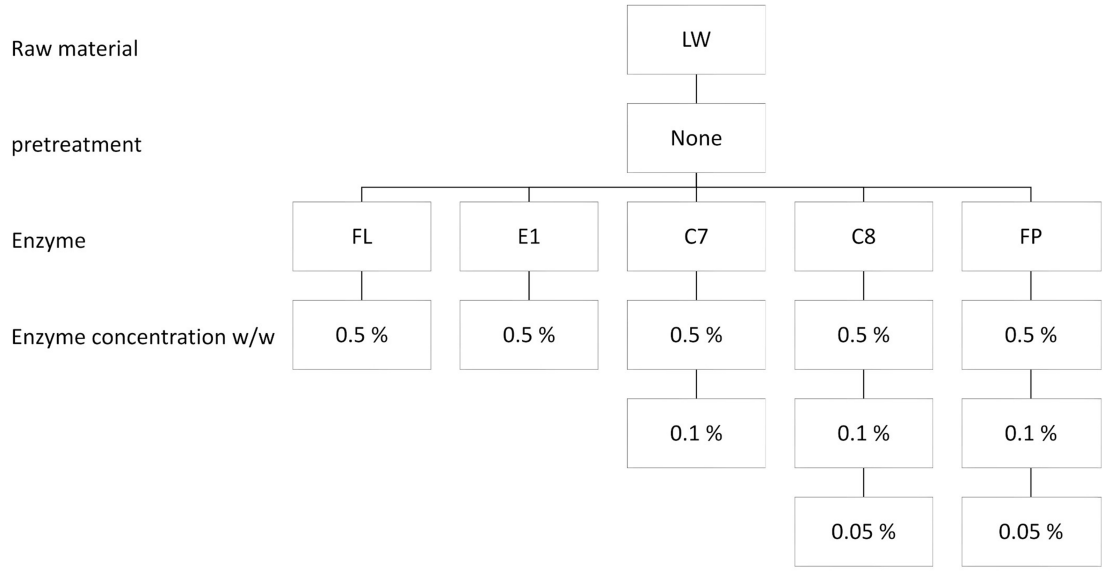

FIGURE 2
Experimental setup: Enzymatic hydrolysis using different enzymes and concentrations. Enzymatic hydrolysis of whole lumpfish (LW) using 5 proteases (Flavourzyme (FL), Endocut 01 L (E1), Corolase 7,089 (C7), Corolase 8000 (C8), and FoodProPNL (FP)) with enzyme concentration at 0.5% (w/w) was performed and evaluated. Subsequently, three enzymes were chosen based on protein yield from the first round (C7, C8, and FP), decreasing the enzyme concentration to 0.1% (w/w). Finally, two proteases were tested with an enzyme concentration of 0.05% (w/w) (C8 and FP).

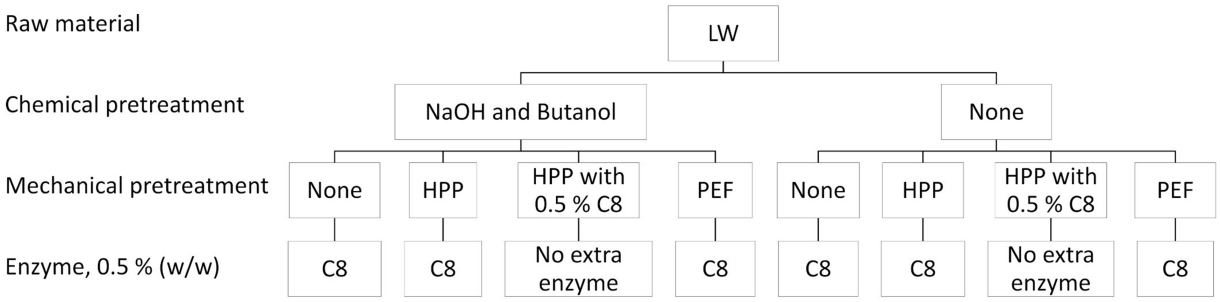

FIGURE 3
Experimental setup: Chemical and mechanical pretreatments and combinations of pretreatments, followed by enzymatic hydrolysis. Enzymatic hydrolysis of whole lumpfish (LW) chemical and mechanical pretreated using Corolase 8000 (C8) in all trials. Mechanical pretreatments are high-pressure processing (HPP) and pulsed electric field (PEF), and chemical pretreatments include lye (NaOH) and butanol steps.

JXN-26 B38623 high-speed centrifuge (Beckman, Indianapolis, United States). The supernatant containing water-soluble peptides was further filtered through depth filter sheets (Seitz T2600, PALL) to remove excess fat and any remaining particles. The solution was frozen at −80°C before lyophilization in a FreeZone Plus 12 Liter Cascade Console Freeze Dry System (LABCONCO, Kansas City, United States). The following formula calculated the mass yield for each hydrolysis:

$$Yield\left(\%\right)=\frac{Weight\ of\ dried\ hydrolysate\left(g\right)}{Weight\ of\ initial\ wet\ biomass\left(g\right)}\times 100$$

2.2.3 Pretreatment procedures

The biomass of lumpfish was subjected to various mechanical and chemical pretreatments and combinations of these treatments before enzymatic hydrolysis (as illustrated in Figure 3). These pretreatments were conducted to investigate if they could affect the product yield, protein content, and peptide size distribution following enzymatic hydrolysis of the biomass. The following enzymatic hydrolysis was performed as described in section 2.2.2. One batch of lumpfish (4 kg) underwent a chemical pretreatment using 0.1 M NaOH in 1:10-ratio (w/v) for three days to remove non-collagenous proteins to investigate if it was possible to obtain a product with higher collagen peptide content. The lumpfish was further washed with water until a neutral pH was achieved. The fish was mixed with 10% butyl alcohol in a ratio of 1:10 (w/v) for 24 h to remove lipids and thoroughly washed with water to remove excess butanol. The biomass was packed and stored at −20°C until further processing.

The pilot dual equipment (Elea Technology GmbH, Quakenbrück, Germany) was used for the lumpfish's mechanical pretreatment pulsed electric field (PEF). The treatment was done in a 10 L batch chamber

with an electrode distance of 24 cm. Tap water (20°C) was used in the process. The fish: water ratio was 1:3 (approximately 500 g fish to 1,500 g water). The water was changed between each run. The following conditions were applied: electrode voltage 24 kV; frequency 30 Hz; pulse count 800; pulse width 6 µs. The measured energy supplied to lumpfish and water was 4.5 ± 0.1 KJ/kg. After the treatment, the batch was placed in a sieve and dripped for 2 min. Then, it was placed in a clean, food-grade plastic bag, vacuum packed, and frozen at 18°C until further handling. A sample of the drip water was collected and lyophilized before the protein content was analyzed using the Kjeldahl method.

High-pressure processing (HPP) was used as a second mechanical pretreatment of the lumpfish. The fish was vacuum-packed (95% vacuum) in food-grade plastic bags. The HPP was performed at an ambient temperature of 200 MPa for 15 min in a high hydrostatic pressure machine QFP 2 L-700 (Avure Technologies Inc., Columbus, OH, United States). The come-up time was approximately 55 s, whereas the pressure release was immediate. The duration of treatment did not include the come-up time. The samples were frozen and stored at −18°C until further processing.

2.2.4 Chemical characterization

Moisture and ash contents were determined following the standard methods of the Nordic-Baltic Committee on Food Analysis (NMKL) 23 (NMKL, 2022) and 173 (NMKL, 2005), respectively. The Kjeldahl method (NMKL 6) (NMKL, 2003) was applied for protein content analysis, and crude protein was estimated based on N × 6.25. Total amino acid content was determined as described by Szkudzińska et al. (2017), with minor modifications. The run time was set to 32 min with a flow of 0.4 mL/min, giving 18 amino acids (including cysteine and taurine).

The peptide size distribution was determined by size exclusion chromatography (SEC), as previously described (Wubshet et al., 2017) with minor differences: The stationary phase was a BioSep-SEC-s2000 column (300 × 7.8 mm) (Phenomenex, Værløse, Denmark) on a Shimadzu HPLC system (Shimadzu, Nishinokyo Kuwabara-cho, Nakagyo-ku, Japan). The injection volume was 10 µL. Each injection was performed in two parallels and separated at 30°C. The mobile phase consisted of 30:70:0.05 acetonitrile: water: trifluoracetic acid. Isocratic elution was carried out at a 0.90 mL/min flow rate. After 17 min, the mobile phase was changed to 0.10 M NaH_2PO_4 and maintained for 3 min for column washing. Elution conditions were restored at 20 min, and the column was re-equilibrated for an additional 25 min. The column was calibrated using standards of known MW ranging from 0.2 to 29 kDa (details in 2.1 Materials and reagents). The parallels were compared in the interval 5–17 min (the window of compound elution according to standards) by calculating Pearson correlation based on retention time and intensity as measured by the PDA at 214 nm with 640 ms intervals.

2.2.5 Bioactivity studies

The five fish protein hydrolysates produced with 0.5% (w/w) enzyme (FP, C7, C8, E1, and FL) were evaluated for bioactivity in various assays. All bioactivity testing was performed by the analytical platform Marbio (UiT – the Arctic University of Norway, Tromsø, Norway). The hydrolysates were dissolved in ddH_2O to 10 mg/mL and screened for bioactivities at 100, 50, and 25 µg/mL. Potential growth-promoting or toxic/antiproliferative activities of the hydrolysates toward human cells were assayed using two cell lines, the hepatocellular carcinoma cell line HepG2 and the non-malignant lung fibroblast cell line MRC5, in an MTS *in vitro* cell proliferation assay as described previously (Hansen et al., 2019).

The hydrolysates were assayed for antimicrobial activities against five bacterial isolates and one fungal strain (*E. faecalis*, *E. coli*, *P. aeruginosa*, *S. aureus*, *S. agalactiae*, and *C. albicans*) in a minimal inhibitory concentration assay, as previously described (Jenssen et al., 2021). In addition, inhibition of biofilm formation against a biofilm-forming *S. epidermidis* isolate was assayed, as previously described (Jenssen et al., 2021). To evaluate the potential antioxidative activities of the hydrolysates, a ferric-reducing ability of plasma (FRAP) assay was performed according to the method of Benzie and Strain using Trolox as a reference Benzie and Strain (1996).

3 Results and discussion

3.1 Proximate composition of raw material

The proximate composition of the lumpfish used in this study has been published previously (Ageeva et al., 2021). The results showed that the lumpfish had high moisture levels (91.5% w/w) and low levels of protein (5.7%), fat (1.3%), and ash (1.6%). For comparison, a previous study by Dave et al. (2019) and (Lin et al. (2020) observed an almost identical proximate composition with 91.08% moisture, 5.02% protein, 1.59% fat, and 1.72% ash. The total amino acid content of whole lumpfish used in this study was 46.67 g/100 g (analyzed on dry weight), and it contained all essential amino acids (Supplementary Table 3).

3.2 Verification, yield, and amino acid composition of gelatin

Lumpfish skin and whole lumpfish were used as the starting point for gelatin extraction. The gelatin samples contained significant components in the form of α-chains and β-chains, as shown in Figure 4. The molecular weights of the α1- and α2-chains were larger than 100 kDa. The β-chain had a molecular weight > 200 kDa. This result suggests that the extraction conditions did not cause any degradation to the α-chains and β-chains. The mass yield of LSG and WLG were 1.61 and 0.63%, respectively. Vate et al. (2023) have previously shown a mass yield of 1.24% of native collagen isolated from lumpfish (gutted with head). The total amino acid content of LSG and WLG indicated higher protein content in the LSG than in the WLG sample (Table 1). LSG also has a higher amount of glycine (Gly), Hyp and proline (Pro) compared to WLG, which is expected since connective tissue (like skin) contains a more significant amount of collagen compared to filets (Liaset and Espe, 2008). Based on the amino acid composition of lumpfish biomass, the recovery of Hyp was 0.17 and 1.71% in extracted WLG and LSG, respectively. Based on the low mass yield and low recovery, it is believed that the process used for gelatin extraction is not satisfactory from an economic point of view. The continued work focused on complete protein extraction from whole lumpfish, using different pretreatments (both chemical and mechanical) and enzymatic hydrolysis.

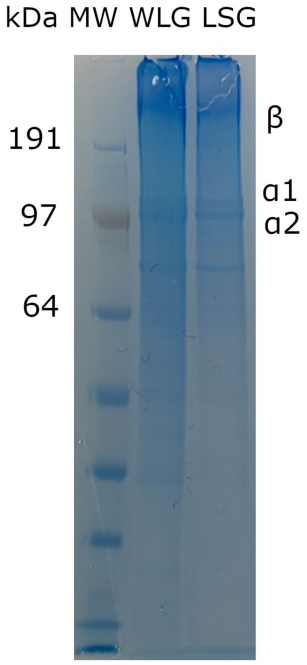

FIGURE 4
SDS-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of gelatin from whole lumpfish (WLG) and lumpfish skin (LSG). MW: molecular weight marker. Numbers represent the molecular weight of different protein bands when run in a MOPS buffer.

3.3 Chemical characterization of hydrolysates produced without pretreatment

The hydrolysis trials produced two fractions: fish protein hydrolysate and sediment. There were no visible lipid layers or emulsions in the hydrolysates, indicating that the amount of lipids not removed during the filtration is incorporated either in the fish protein hydrolysates or in the sediment (not analyzed).

Using the different commercial enzymes at a concentration of 0.5%, the mass yield ranged between 5.35 and 6.73% (Table 2). Reducing enzyme concentration led to a correlating decrease in mass yield. In a commercial setting, a reduction in yield and product loss must be evaluated against the costs saved on the reduced use of enzymes.

The lyophilized fish protein hydrolysates had between 4.9–7.4% moisture and 72–81% Kjeldahl protein (Table 3). There were no significant differences in Kjeldahl protein content when comparing the proteases used at 0.5% (w/w). Surprisingly, a reduction in enzyme concentration from 0.5 to 0.1% gave higher Kjeldahl protein for all enzymes tested (FP, C8, and C7). Decreasing the enzyme concentration to 0.05% showed a reduction in Kjeldahl protein content compared to 0.1% enzyme. However, the 10-fold decrease in the enzyme concentration did not significantly impact the Kjeldahl protein measurements. The sum of total amino acids is the most accurate estimate for protein content in a product (Mæhre et al., 2018), and the levels of total amino acids (Table 3) were lower than Kjeldahl protein in the samples. This is expected when using the protein factor 6.25 in Kjeldahl analysis, which was previously shown to be inaccurate for fish fractions. It has been found that using a protein factor of 5.6 is more accurate (Aspevik et al., 2021). When factor 5.6 is applied to the current results (data not shown), the levels are more similar than the sum of total amino acids.

However, the actual total amino acid content is expected to be higher than displayed in Table 3 since some amino acids, such as

TABLE 1 TOTAL amino acid composition (g/100 g) of gelatin extracted from whole lumpfish, not gutted (WLG), and lumpfish skin (LSG).

Parameter	WLG (g/100 g)	LSG (g/100 g)
EAA[a]		
Arginine	5.9	8.1
Histidine	0.68	0.85
Isoleucine	0.96	1.1
Leucine	2.1	2.5
Lysine	2.6	3.3
Methionine	1.4	1.8
Phenylalanine	1.6	2.1
Threonine	2.1	2.7
Valine	1.9	2.4
Sum EAA	19.24	24.85
DAA[b]		
Alanine	5.9	8.0
Aspargic acid	4.7	6.0
Glutamic acid	7.1	9.2
Glycine	16.2	22.43
Hydroxyproline	5.3	7.4
Proline	7.1	9.9
Serine	4.8	6.6
Tyrosine	0.42	0.36
Sum DAA	51.52	60.86
Sum AA	70.76	88.14

[a]Essential amino acids.
[b]Dispensable amino acids.

TABLE 2 Overview of hydrolysate yield influenced by protease and protease concentration using FoodProPNL (FP), Corolase 8,000 (C8), Corolase 7,089 (C7), Endocut 01 L (E1), and Flavourzyme (FL), $n = 1$.

Enzyme	Concentration, w/w (%)	Yield on wet weight (%)
FP	0.5	5.35
FP	0.1	4.42
FP	0.05	2.59
C8	0.5	6.73
C8	0.1	2.42
C8	0.05	2.27
C7	0.5	5.40
C7	0.1	2.49
E1	0.5	5.55
FL	0.5	5.66

TABLE 3 Proximate and total amino acid composition of lumpfish hydrolysates using Food Pro PNL (FP), Corolase 8,000 (C8), Corolase 7,089 (C7), Endocut 01 L (E1), and Flavourzyme (FL).

	FP			C8			C7		E1	FL
[Concentration, w/w] (%)	[0.5]	[0.1]	[0.05]	[0.5]	[0.1]	[0.05]	[0.5]	[0.1]	[0.5]	[0.5]
Parameter										
Moisture	7.4 ± 1.1	6.9 ± 1.0	5.1 ± 0.8	5.5 ± 0.8	5.2 ± 0.8	5.2 ± 0.8	6.9 ± 1.0	4.9 ± 0.7	6.7 ± 1.0	7.3 ± 1.1
Ash	6.7 ± 0.7	7.2 ± 0.7	16.0 ± 0.8	11.5 ± 0.6	16.3 ± 0.8	15.7 ± 0.8	16.2 ± 0.8	15.5 ± 0.8	13.0 ± 0.6	14.7 ± 0.7
Protein (N × 6.25)	77.8 ± 4.7	81.2 ± 4.9	76.1 ± 4.6	76.0 ± 4.6	78.3 ± 4.7	75.2 ± 4.5	75.7 ± 4.5	79.6 ± 4.8	73.4 ± 4.4	72.7 ± 4.4
Fat	0.6	<0.5	6.0	<0.5	4.2	4.9	3.0	4.2	4.9	<0.5
EAA[a]										
Arginine	5.3	5.4	4.7	4.8	4.6	4.6	4.8	4.8	4.5	4.4
Histidine	1.3	1.3	0.9	1.3	1.0	1.0	1.0	1.0	1.1	1.0
Isoleucine	2.4	2.3	1.7	2.4	1.9	1.8	1.9	2.0	2.1	2.1
Leucine	4.3	4.3	3.4	4.3	3.6	3.4	3.6	3.8	3.9	3.8
Lysine	4.8	4.8	4.0	4.8	3.8	3.9	4.0	3.9	4.5	4.2
Methionine	1.9	1.9	1.6	1.9	1.7	1.6	1.6	1.7	1.8	1.7
Phenylalanine	2.5	2.4	1.9	2.4	2.1	1.9	2.1	2.1	2.2	2.0
Threonine	3.1	3.1	2.5	3.0	2.6	2.5	2.6	2.7	2.8	2.6
Valine	3.2	3.1	2.5	3.2	3.0	2.5	2.6	3.1	2.8	2.8
Sum EAA	28.8	28.6	23.2	28.1	24.3	23.2	24.2	25.1	25.7	24.6
DAA[b]										
Alanine	5.0	5.2	4.8	4.8	5.0	4.8	4.6	5.1	4.7	4.7
Aspartic acid	6.4	6.4	5.3	6.2	5.4	5.4	5.5	5.6	5.8	5.7
Glutamic acid	9.3	9.5	8.0	8.8	8.2	7.8	8.1	8.5	8.4	8.2
Glycine	9.2	9.8	9.7	7.8	9.6	9.6	9.1	9.7	7.6	7.9
Hydroxyproline	2.3	2.5	2.4	1.7	2.4	2.4	2.3	2.4	1.8	1.9
Proline	5.0	5.2	4.8	4.3	4.9	4.7	4.7	4.9	4.2	4.3
Serine	4.1	4.3	3.8	3.7	3.6	3.8	3.8	3.7	3.6	3.6
Tyrosine	1.8	1.7	1.0	1.8	1.1	1.1	1.3	1.2	1.6	1.4
Sum DAA	43.1	44.6	39.8	39.1	40.2	39.6	39.4	41.1	37.7	37.7
Sum AA	71.9	73.2	63.0	67.2	64.5	62.8	63.6	66.2	63.4	62.3

All units of measurement are g/100 g.
[a]Essential amino acids.
[b]Dispensable amino acids.

asparagine and glutamine, are hydrolyzed to their aspartic and glutamic acid forms, respectively. In addition, other amino acids cannot be determined 100% correctly using this method. For example, tryptophan is completely degraded during the reaction, while sulfur-containing amino acids (e.g., cysteine, methionine) cannot be determined with certainty due to partial degradation of the amino acids. Furthermore, amino acids such as tyrosine, serine, and threonine may have lower recovery due to the nature of acid hydrolysis. The hydrolysates produced in this study contained all the essential amino acids. The choice of enzyme did not notably influence the amino acid contents in the different hydrolysates.

SEC is a method to elucidate the relative size of compounds in a complex sample, e.g., a proteinaceous sample after hydrolysis. The analysis can be used to find the hydrolysis process with the most desired peptide size distribution. Most bioactive peptides have molecular mass in the range of 400–2,000 Da (Zaky et al., 2022), and intestinal digestibility is affected by the size of the peptides (Korhonen and Pihlanto, 2006). In this study, SEC was used to evaluate the effect of enzyme choice and concentration on the peptide size distribution of the respective hydrolysates. All hydrolysates produced at different enzyme concentrations (without pretreatment) were analyzed, and the total average MW was calculated (Table 4). At 0.5% enzyme concentration, the average MW of the peptides in the hydrolysates ranged from 961 Da (FP) to 2,763 Da (FL). At 0.1% enzyme concentration, the same trend can be seen. However, at 0.05% enzyme concentration, C8 seems more efficient in peptide degradation than FP, with a total average MW of 2,318 and 2,670, respectively. The effect of enzyme concentration can be measured as the change in total average MW for peptides produced by the same enzyme. A reduction in total average MW can be observed for all tested enzymes with increased enzyme concentration. This can be expected as higher concentrations of enzymes typically increase protein hydrolysis. The

TABLE 4 Molecular weight (MW) distribution is categorized into six categories for all hydrolysates and concentrations.

Enzyme	C7		C8			FP			FL	E1
Concentration	[0.5]	[0.1]	[0.5]	[0.1]	[0.05]	[0.5]	[0.1]	[0.05]	[0.5]	[0.5]
Total average MW	1,076	2032	1,009	1955	2,318	961	1,190	2,670	2,763	1,507
>4,000	1.9%	13.9%	1.3%	9.8%	13.8%	1.6%	4.0%	21.2%	18.4%	6.9%
2000–4,000	11.6%	16.6%	7.6%	12.0%	14.6%	7.6%	12.0%	17.7%	14.9%	13.9%
1,000–2000	24.2%	27.2%	29.4%	29.3%	29.2%	27.0%	29.0%	23.8%	18.8%	30.6%
500–1,000	26.9%	18.0%	32.1%	22.8%	19.7%	31.9%	24.6%	14.6%	15.9%	22.4%
200–500	26.9%	15.5%	23.8%	17.9%	14.8%	21.3%	19.5%	13.7%	20.9%	17.2%
<200	8.6%	8.8%	5.9%	8.1%	7.8%	10.6%	11.0%	9.0%	11.2%	9.0%

The bottom row is the total average MW. MW -values are approximations based on retention time.

TABLE 5 Overview of hydrolysate yield influenced by mechanical and chemical pretreatment using Corolase 8,000 (C8).

Chemical pretreatment	HPP	HPP + 0.5% C8	PEF	Mass-yield w/w (%) (n = 1)
				9.42
	■			8.91
		■		10.65
			■	5.51
■				6.25
■	■			5.84
■		■		7.01
■			■	5.53

Mechanical pretreatments are high-pressure processing (HPP) and pulsed electric field (PEF), and chemical pretreatments include lye (NaOH) and butanol steps. Control: Hydrolyzed whole lumpfish.

same can be observed in the relative size distribution of peptides in each hydrolysate, shown in Table 4, where the peptides have been divided into categories based on size. Enzyme kinetics can be affected by concentrations (Juárez-Enríquez et al., 2022), possibly explaining this phenomenon.

3.4 Chemical characterization of hydrolysates produced using pretreatment and combinations of pretreatments

Samples treated chemically and using PEF pretreatment on non-chemically pretreated samples (Tables 5, 6) resulted in a higher overall protein content with relatively higher amounts of the amino acids Gly, Pro, and Hyp, which are characteristic of collagen (Vate et al., 2023). This indicates that these hydrolysates contain higher amounts of collagen peptides. The mass yield, however, is lower in these samples compared to non-chemically pretreated samples, which is expected as the chemicals used to remove compounds such as non-collagen proteins and fat. During PEF pretreatment, the water changes continuously, and the mass yield results indicate that some compounds are removed during this treatment. A test sample from the PEF-processing drip water showed high amounts of protein on a dry weight basis. Drip loss during preprocessing steps can give lower yields but might result in a product with higher collagen purity.

The samples that underwent chemical pretreatment have lower ash values compared to non-treated samples (Table 6). This implies that the chemical pretreatment removes some non-protein compounds, such as mineral residues. The biomass that was not chemically pretreated was not washed with fresh water before processing. Therefore, the remaining minerals from seawater could also be a factor in increasing the ash values.

3.5 Initial bioactivity assessment of hydrolysates

The five fish protein hydrolysates produced with 0.5% (w/w) enzyme (FP, C7, C8, E1, and FL) were evaluated for bioactivity in a selection of assays (Figure 5). The two human cell lines, HepG2 and MRC-5, were screened mainly to exclude any potential toxic activities of the hydrolysates and to investigate any potential growth-promoting effects. Compared to the HepG2 cells, an increase in cell survival (up to 134%) was observed, indicating growth-promoting effects on the cells. The assays were performed with one biological replicate and three technical replicates, so further studies must be conducted for confirmational data. The same was observed in the non-malignant cell line MRC-5 (up to 171% cell survival). The results strongly indicate that the hydrolysates do not exert any toxic effects on the cells. On the contrary, they are boosting cell growth and survival. Further studies investigating potential growth-promoting effects should be considered for an extended panel of relevant cell lines, including primary muscle cells. An increase in cell proliferation has been observed after treatment with protein hydrolysates in several previous studies against a variety of cell lines (Yang et al., 2019; Chotphruethipong et al., 2021; Jung et al., 2022). The hydrolysates were assayed for antioxidative

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TABLE 6 Proximate analysis and total amino acid composition of hydrolysates where lumpfish was pretreated before hydrolysis.

	No chemical pretreatment				Chemical pretreatment			
Parameter	Control	HPP	HPP + C8	PEF	Control	HPP	HPP + C8	PEF
Moisture	5.2 ± 0.1	4.0 ± 0.0	3.5 ± 0.1	3.4 ± 0.0	3.6 ± 0.2	2.5 ± 0.1	3.0 ± 0.0	2.8 ± 0.2
Ash	15.2 ± 0.15	14.4 ± 0.11	13.5 ± 0.13	9.6 ± 0.13	1.2 ± 0.06	1.2 ± 0.05	1.1 ± 0.04	1.2 ± 0.05
Protein (N × 6.25)	74.9 ± 0.4	76.2 ± 0.01	76.8 ± 0.2	83.0 ± 1.34	96.18 ± 0.75	96.70 ± 0.43	99.26 ± 0.53	97.98 0.12
Fat	0.3	0.5	0.3	0.6	0.8	0.8	0.6	0.8
EAA[a]								
Arginine	4.80	5.00	4.90	5.80	7.70	7.60	7.80	7.70
Histidine	1.20	1.20	1.30	1.10	1.00	1.00	1.10	1.10
Isoleucine	2.30	2.30	2.50	1.90	1.60	1.60	1.70	1.70
Leucine	4.20	4.20	4.50	3.90	3.40	3.40	3.50	3.60
Lysine	4.60	4.60	4.80	4.20	4.00	4.00	4.00	4.10
Methionine	1.80	1.80	1.90	1.80	2.00	2.00	2.00	2.00
Phenylalanine	2.30	2.30	2.50	2.20	2.30	2.30	2.40	2.40
Threonine	2.90	2.90	3.00	2.80	2.90	2.90	3.00	3.00
Valine	3.00	3.00	3.20	2.80	2.80	2.80	2.90	2.90
Sum EAA	27.1	27.3	28.6	26.5	27.7	27.6	28.4	28.5
DAA[b]								
Alanine	4.70	4.70	4.60	5.60	7.40	7.40	7.50	7.40
Aspargic acid	6.10	6.10	6.10	6.20	6.40	6.30	6.50	6.50
Glutamic acid	8.60	8.90	8.80	9.10	10.00	9.90	10.00	10.00
Glycine	8.50	8.60	7.90	12.3	19.20	19.10	19.50	18.90
Hydroxyproline	2.10	2.20	1.90	3.50	6.20	6.40	6.30	6.10
Proline	4.60	4.60	4.40	6.00	8.90	8.80	9.00	8.80
Serine	4.00	3.90	3.80	4.70	6.20	6.10	6.30	6.10
Tyrosine	1.60	1.50	1.70	1.20	0.82	0.81	0.83	0.89
Sum DAA	40.2	40.5	39.2	48.6	65.12	64.81	65.93	64.69
Sum AA	67.30	67.80	67.80	75.10	92.82	92.41	94.33	93.19

Chemical treatment included NaOH and Butanol treatment. HPP: high-pressure processing. HPP + C8: High-pressure processing where 0.5% C8 (w/w) was added to the biomass before processing. PEF: pulsed electric field. All hydrolysis′ are performed with 0.5% (w/w) C8.
All units of measurement are g/100 g.
[a]Essential amino acids.
[b]Dispensable amino acids.

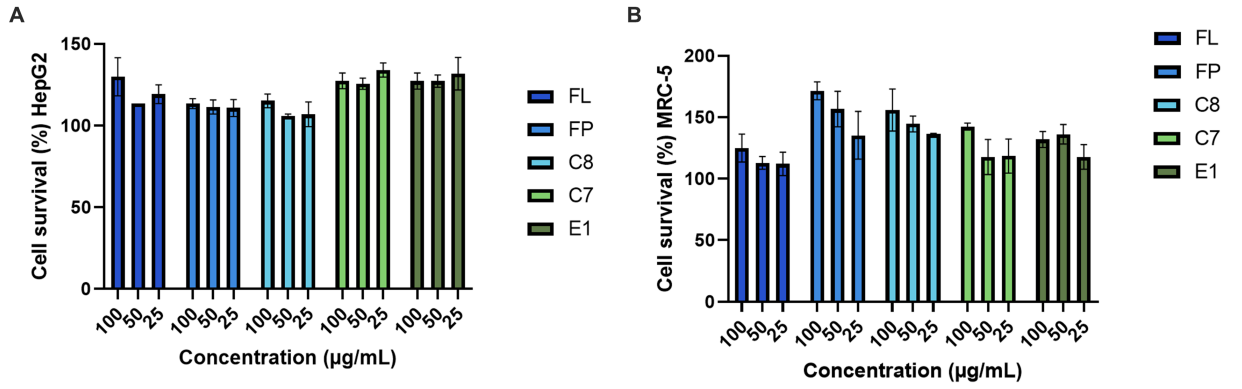

FIGURE 5
Cell survival of **(A)** HepG2 and **(B)** MRC-5 (n = 3) treated with the protein hydrolysates.

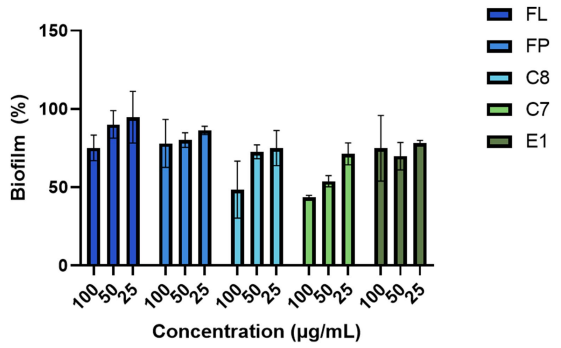

FIGURE 6
Inhibition of bacterial biofilm formation by the protein hydrolysates against the biofilm-producing *S. epidermidis* ($n = 3$).

activities in a FRAP assay, and no activity was observed (Supplementary Table 4).

The hydrolysates produced with 0.5% (w/w) enzyme (FP, C7, C8, E1, and FL) were screened against five bacterial strains, three Gram-positive and two Gram-negative, at 100, 50, and 25 µg/mL in a minimal inhibitory concentration assay. None of the hydrolysates gave any considerable growth inhibition against any tested bacteria (Supplementary Table 5). No growth inhibition was observed against *Candida albicans* at any assayed concentrations (Supplementary Table 5). In the assay for biofilm inhibition, some effects were observed for the hydrolysates produced using the Corolase enzymes (C7 and C8) (Figure 6). At the highest concentration (100 µg/mL), the C7 hydrolysate resulted in a biofilm formation of 43%. No growth reduction was observed for the bacterium, indicating that the activity was specific for biofilm formation, not targeting bacterial growth.

4 Conclusion

Farmed lumpfish is only partially utilized after being used in sea lice treatment in aquaculture, and it is essential to find a better sustainable alternative for this biomass. One way to achieve this is to use the leftover biomass as a source for proteins, oils, and other molecules that can be used in various products such as food, feed, cosmetics, and packaging.

However, the low yield and recovery from the gelatin extraction process of lumpfish raw material makes the current method economically unsound. LSG is, however, high in collagen-associated amino acids, and further studies on gel strength and biological and physical properties need to be analyzed to evaluate its potential in different products. The complete protein extraction from whole lumpfish has both benefits and disadvantages. Chemical pretreatment increases the protein content by higher collagen peptide content but reduces the mass yield. Different pretreatments and combinations will suit different product categories and should be adapted to the products being developed. Yield is vital in bulk productions, e.g., feed, while collagen peptides are essential for tissue engineering and wound healing. The processing price will also be affected and should be considered when establishing a production pipeline.

Initial bioactivity testing showed no toxic effects of the hydrolysates on hepatocellular carcinoma and non-malignant lung fibroblast cells. Additionally, the hydrolysates seemed to boost cell growth and survival. The hydrolysates should be assayed at higher concentrations and with more replicates to obtain more information about this activity.

Using cleaner fish has raised ethical, economic, and sustainability concerns, and this practice may disappear over the years. However, it is crucial to ensure that if lumpfish is used as cleaner fish, it will be utilized in the best possible way. Reutilizing leftover biomass from the marine environment as a source of proteins, oils, and other molecules with nutritional or technical properties can minimize waste and benefit various industries and society.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

KL: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. IM: Methodology, Resources, Writing – review & editing. TR: Methodology, Resources, Validation, Writing – original draft, Writing – review & editing. MJ: Formal analysis, Resources, Writing – original draft, Writing – review & editing. BV: Methodology, Writing – review & editing. RS: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted without any commercial or financial relationship that could be constructed as a potential conflict of interest.

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Supplementary material

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Tracing the journey of elements from fish feed to Nile tilapia faeces to black soldier fly larvae: a comparative approach

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Introduction: The circular bioeconomy concept revolves around biological production cycles that reintroduce products or waste from one production system to another, aiming to maximize resource utilization while minimising environmental impact. The purpose of this study was to evaluate and compare the element flow when integrating black soldier fly larvae (BSF) production with Nile tilapia production using varying experimental fish feed.

Methods: Tilapia (42.5 ± 11.2 g) were reared in recirculating aquaculture systems (RAS) at 25.5°C for 10 weeks and fed equal daily rations of four experimental diets containing fishmeal (FM), poultry blood meal (PBM), black soldier fly meal (BSF) and poultry by-product meal (PM) as the single main protein source, respectively. Faeces was collected daily from settling columns installed in the RAS and subsequently fed to BSF larvae.

Results and discussion: The fish exhibited the highest biomass gain when fed with FM (1,001 g) or PM (901 g). The growth was lowest for those fed with PBM (406 g). The fish fed with PBM also produced the highest amount of faeces (234 g). When the fish faeces were utilized as a substrate for rearing black soldier fly (BSF) larvae and although the biomass gain did not differ significantly, the feed conversion ratio (FCR) varied among larvae fed with different fish faeces, ranging from 8.36 to 25.04. Furthermore, the concentration of analysed elements (Al, B, Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Na, P, S, and Zn) varied based on the type of fish faeces provided. Results illustrate that a modulation of BSFL elemental composition is possible through manipulating the feed provided to the fish and emphasize the importance of fish feed composition for fish performance as well as the compositional quality of the larvae reared on the resulting fish faeces. Hence, if BSF larvae production is to be harnessed for the recycling of aquaculture sludge, ingredient choice and subsequent elemental composition of fish feeds are pivotal to larvae composition.

KEYWORDS

black soldier fly, fish faeces, waste management, fish feed, insect protein

1 Introduction

The circular bioeconomy concept embraces maximal utilization of biological resources, thereby addressing sustainability challenges and fulfilling societal demands. This approach considers biological production cycles that reintroduce products or wastes from a production system to the other in order to achieve maximal use of resources and minimal environmental impact (Zaman et al., 2023). Over the past years, the European Union has been actively promoting the responsible management and, when possible, the utilization of biowastes (Fava et al., 2015). This includes the appropriate processing of raw materials and biological resources, as well as the protection and conservation of environmental media such as water and soil (Schepelmann et al., 2006; Vehlow et al., 2007; Fava et al., 2015). These developments gave rise to "waste to value" business models that integrate different biorefinery techniques and biowaste processing methods to create value from wastes (Zaman et al., 2023).

The mass-rearing of insects has gained worldwide attention in recent years as a sustainable and efficient means of waste treatment and protein production (Gasco et al., 2020; Raksasat et al., 2020). This process offers several advantages over traditional waste treatment methods, including reduced environmental impact, resource recovery, and the production of valuable by-products (Salomone et al., 2017; Ojha et al., 2020; Sangiorgio et al., 2022).

The black soldier fly (*Hermetia illucens*) is one of the most commonly mass-reared insect species (Tomberlin and Van Huis, 2020). The larvae have a voracious appetite and can consume and thrive on different organic waste streams (Raksasat et al., 2020), including fruit and vegetable wastes (Borel et al., 2021), catering wastes (Candian et al., 2023), agricultural wastes (Beyers et al., 2023; Yakti et al., 2023), and human and animal manure (Sheppard et al., 1994; Diener et al., 2011; Lalander et al., 2013). The larvae efficiently convert organic substrates into a protein-rich larval biomass, which can be harvested and used as a sustainable feed component (Gasco et al., 2020). The bioconversion process also generates valuable by-products such as the rest substrate, also known as frass, which can be used a plant fertiliser (Beesigamukama et al., 2020).

In aquaculture, the black soldier fly larvae (BSFL) meal has been shown to enhance the nutrition and health of aquatic organisms. The incorporation of BSFL in the feed of Nile tilapia, for example, has led to improved growth, enhanced feed efficiency, and better development of liver and intestinal organs (Abdel-Tawwab et al., 2020; Limbu et al., 2022). Positive effects were also observed in other fish species such as rainbow trout (*Oncorhynchus mykiss*) (Elia et al., 2018), Atlantic salmon (*Salmo salar*) (Belghit et al., 2018), and Jian carp (*Cyprinus carpio*) (Li et al., 2017).

Generally, aquaculture produces wastes that can be categorized as solid or dissolved wastes. Solid wastes primarily consist of fecal droppings, unconsumed feed, and occasionally, fish carcasses when mortalities occur (Akinwole et al., 2016). The presence of solid wastes in the aquaculture system causes issues such as boosting aerobic bacterial activity leading to the depletion of oxygen and the reduction of biofilm formation (Michaud et al., 2006), ultimately reducing the biofilter efficiency and leading to the accumulation of fish-toxic ammonia. The adverse effects of elevated ammonia levels include decreased feed intake, reduced growth rates, vulnerability to diseases, and disruption of various physiological processes (Levit, 2010). In well-managed farms, approximately 30 percent of the feed given is expected to remain in the system as solid wastes, although this percentage may vary based on the system operation (Miller and Semmens, 2002). The use of recirculating aquaculture systems (RAS), for example, is gaining interest as they enable better solid waste removal compared to conventional flow-through systems (d'Orbcastel et al., 2009).

In theory, if black soldier fly larvae (BSFL) can consume waste materials obtained from aquaculture tanks, (i.e., fish faeces and feed leftovers), it would be possible to establish a feedback loop by incorporating BSFL into aquafeed. This feedback loop would confer an extra environmental advantage by reducing the pollution caused by aquaculture systems and generating value from waste.

However, currently the utilization of fish wastes as a substrate for black soldier fly larvae (BSFL) is prohibited in the European Union (EU) due to the associated risks related to the use of animal manure as a rearing substrate. Given that insects are farmed animals, their rearing should comply with legal frameworks like those established by the European Union (Commission Regulation (EU) 2017/893), and their feeding substrate should meet specific requirements. Aside from the legal issues with using fish faeces as a rearing substrate, the maximum allowed levels of heavy metals and other contaminants must not be exceeded in insects used for feed production.

Few studies have assessed the potential of utilising aquaculture sludge as feeding substrates for BSFL (Schmitt et al., 2019; Liland et al., 2023; Zhang et al., 2023). However, the properties and composition of fish faeces collected from aquaculture farms can hypothetically vary based on many factors such as the fish species and the feed provided leading to a variance in the composition of produced BSFL not considered in the above studies. We hypothesize that the composition of the fish feed, as the starting point of the nutrient flow, will influence the growth and elemental composition of the resulting BSFL. Furthermore, we anticipate that the accumulation of elements in the BSFL will correlate with the initial composition of the BSFL substrate, i.e., the fish faeces. To test these hypotheses, we coupled BSFL rearing to Nile tilapia production in RAS systems. The fish received four experimental feeds differing in their protein sources, i.e., fish meal (FM), black soldier fly larvae meal (BSFM), poultry blood meal (PBM), and poultry by-product meal (PM). The various types of fish faeces obtained were used as feeding substrates for BSFL, and their performance and composition were compared.

2 Materials and methods

2.1 Fish feeding trial

2.1.1 Experimental diets

Four experimental diets were formulated to be isonitrogenous (~40% crude protein) and isolipidic (~12% crude fat). The diets were formulated with fish meal (FM), black soldier fly larvae meal (BSFM), poultry blood meal (PBM), and poultry by-product meal (PM) as the single main protein source. BSFM, PBM, and PM have been used to replace FM in fish diets (Galkanda-Arachchige et al., 2020; Alfiko et al., 2022; Shaw et al., 2022a) and are commercially available as fish feed components. Diets were extruded at SPAROS I&D, Olhão, Portugal, (SPAROS) and stored at −20°C until use. Diet formulations and proximate composition are given in Table 1, and the mineral composition is given in Table 2.

TABLE 1 Fish diet formulation and proximate composition.

	Experimental Diets			
	FM	BSFM	PBM	PM
Ingredient composition (% incorporation)				
Fish meal[1]	51.0	–	–	–
Black soldier fly larvae meal[2]	–	61.6	–	–
Poultry blood meal[3]	–	–	37.2	–
Poultry meal[4]	–	–	–	56.4
Wheat bran[5]	29.8	19.9	39.4	26.0
Corn meal[6]	11.0	11.0	11.0	11.0
Vitamin and mineral premix[7]	1.0	1.0	1.0	1.0
Dicalcium phosphate (DCP)[8]	1.2	1.2	1.2	1.2
Fish oil[9]	3.0	3.0	3.0	3.0
Poultry fat[10]	3.0	2.3	7.2	1.4
Proximate composition (%—as fed)[11]				
Dry matter (DM)	91.90 ± 0.10	92.30 ± 0.10	91.60 ± 0.10	93.05 ± 0.15
Crude fat (*CF*)	11.55 ± 0.15	11.10 ± 0.20	11.60 ± 0.10	11.85 ± 0.25
Crude fibre (CFB)	2.85 ± 0.05	8.05 ± 0.15	4.20 ± 0.00	3.10 ± 0.00
Ash	11.65 ± 0.05	8.25 ± 0.05	4.35 ± 0.05	10.85 ± 0.05
Starch	12.05 ± 0.45	12.65 ± 0.25	13.45 ± 0.05	9.00 ± 1.00
Nitrogen-free extract (NFE)[12]	25.55 ± 0.05	24.55 ± 0.55	30.55 ± 0.35	23.55 ± 0.45
Gross energy (GE) (MJ/kg)[13]	18.48 ± 0.03	18.14 ± 0.00	19.49 ± 0.00	19.05 ± 0.05
Crude protein (CP) (% - N xS 6.25)	42.3 ± 1.10	43.8 ± 0.03	43.47 ± 1.02	45.1 ± 1.3

[1]Super Prime: 66.3% CP, 11.5% CF, Pesquera Diamante, Peru.
[2]Protein X (defatted Hermetia illucens meal): 58% CP, 9% CF, Protix, The Netherlands.
[3]Poultry blood meal: 89% CP, 0.47% CF, SONAC, The Netherlands.
[4]Poultry meal 65: 65% CP, 12% CF, SAVINOR, Portugal.
[5]Wheat bran: 14.8% CP, 4.7% CF, Ribeiro e Sousa, Portugal.
[6]Corn meal: 8.6% CP; 4.3% CF, Casa Lanchinha, Portugal.
[7]PREMIX, Portugal.
[8]DCP: 16.8% P, 20.9% Ca, PREMIX, Portugal.
[9]Sopropêche, France.
[10]SAVINOR, Portugal.
[11]Analysed in duplicate according to standard methods by the accredited laboratory SGS Analytics Germany, Augsburg, Germany; percentages given on as-fed basis; values represent means ± standard deviations.
[12]NFE = 100% – (% CP + % CF + % CFB + % ash + % moisture).
[13]Calculated using the factors 17.15, 23.64, 39.54 MJ/kg for NFE (carbohydrates), CP and CF, respectively (Kleiber, 1961).

2.1.2 Experimental system

The experimental system included four recirculating aquaculture systems (RAS) with a total water volume of 460 L, respectively. Each RAS featured a circular rearing tank with a conically shaped bottom (180 L), a settling column for faeces collection with a bottom outlet (10 L) and a biofilter (270 L). The biofilter was partitioned into three equally sized sections, with the first section comprising filter sponge (PPI 10, Schaumstoff-Meister, Straelen, Germany), the aerated centre section holding 30 L of biocarriers (Hel-X HXF12KLL, Christian Stöhr, Marktrodach, Germany) and the last section being a clear water chamber including a circulation pump (Universal 300, EHEIM, Deizisau, Germany). Water flow could be adjusted manually via a valve placed after the circulation pump. Water temperature was controlled by a titanium heater (600 W titanium tube heater, SCHEGO Schemel & Goetz, Offenbach, Germany) and a temperature controller (SCHEGO Schemel & Goetz, Offenbach, Germany). Apart from the aeration in the biofilter, further oxygenation of each RAS was achieved by an air stone placed in the rearing tanks and lighting was provided by overhead LED lamps. The biofilters of all RAS were matured and synchronized for 6 weeks before the start of the trial by stocking the systems with Nile tilapia and running the RAS in series, i.e., pumping from one RAS to the next and eventually back to the first. One day before introducing the experimental fish, the RAS were emptied, cleaned and refilled with tap water. From this point onward, they were again run separately from each other.

2.1.3 Experimental procedure

Mixed-sex Nile tilapia (*Oreochromis niloticus*) originating from the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB) in Berlin, Germany, were used in the trial. Prior to the trial, fish were reared in a RAS system at 25°C and fed a commercial tilapia diet (Aller Aqua Performa, 2 mm, 45% CP, 20% *CF*). At the start of the trial, a total number of 342 fish, of which 80 fish were individually weighed and measured (individual body weight: 42.5 ± 11.2 g;

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TABLE 2 Elemental composition of fish feed used in the fish feeding trial (dry matter basis).

Feed	Al	B	Ca	Co	Cu	Fe	K	Mg	Mn	Mo	Na	P	S	Zn	N
	mg/kg	mg/kg	g/kg	mg/kg	mg/kg	g/kg	g/kg	g/kg	mg/kg	mg/kg	g/kg	g/kg	g/kg	mg/kg	%
FM	243.77	4.54	31.17	0.59	17.19	0.37	11.00	2.86	68.53	1.07	4.63	22.48	6.41	100.29	6.77
BSFM	44.45	6.35	15.89	0.11	26.23	0.21	16.27	4.55	339.75	0.96	1.29	14.09	3.85	177.16	6.96
PM	19.65	5.69	30.46	0.45	21.78	0.20	9.83	2.18	64.22	0.73	3.94	21.22	5.55	109.00	7.38
PBM	15.72	5.78	5.27	0.11	21.05	1.00	7.55	1.90	82.57	0.65	1.82	9.13	4.51	68.69	7.02

individual total length: 132.4 ± 11.5 mm; $n = 80$), were randomly stocked into the four RAS (82–88 individuals per RAS) such that a starting biomass of 3,500 g per RAS system was achieved, which equated to a rearing density of 20 kg/m^3. The fish were hand-fed 80 g of the experimental diets in two equal portions daily for 14 days, except for the first day on which they received half the portion. After this period, the biomass was determined and the RAS were emptied, cleaned, and refilled with tap water again. The fish number in the batch of each dietary treatment was reduced by randomly removing individuals to reach a starting biomass of 3,500 g and batches were again randomly assigned to one of the RAS to be reared for the next 14 days. This procedure was replicated 5 times. Finally, 20 fish per dietary treatment were again weighed and measured individually.

During the trial, a daily water exchange of 7% of the RAS volume (32.2 L) was performed by removing water from the biofilter according to volumetrically defined markings and replenishing it with fresh tap water. Water recirculation in the RAS was set to 180 L/h, which was controlled and if necessary adjusted weekly. Before daily water exchange and feeding, oxygen, pH, temperature (HQ40d, Hach Lange, Berlin, Germany), and electrical conductivity (EC) (pH/Cond 740i, WTW, Weilheim in Oberbayern, Germany) were measured in each rearing tank. During each of the 14-day periods, the faeces were removed from the settling columns daily and the removed sludge water mix was filtered through 90 µm nylon mesh to separate the faeces from the water. The faeces were subsequently collected from the top of the mesh surface and pooled per dietary replicate for each 14-day period and stored at −80°C. This approach ensures the full collection of faeces from the system. Before passing the faeces on to the black soldier fly larvae trial, they were homogenized with a blender and freeze-dried until constant weight was achieved.

2.2 Black soldier fly larvae feeding trial

The black soldier fly (BSF) strain used in this study was obtained from the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB) in Berlin, Germany. The population was fed chicken feed (K (11 4) o.K., Agravis Raiffeisen AG, Velten, Germany) and was reared following the method described by Yakti et al. (2022). Prior to the experiment, eggs were manually collected from rearing cages and were allowed to hatch for 24 h on a substrate consisting of 30% dry chicken feed and 70% water. The neonates were let to grow for 6 days and then were separated from the substrate to be used in the BSF larvae feeding trial.

A feeding trial was conducted to assess the growth and mineral composition of black soldier fly larvae (BSFL) provided with the different faeces variants collected from the fish feeding trial. The trial had 5 treatments: The faeces of fish fed with fishmeal (FM), poultry blood meal (PBM), black soldier fly meal (BSFM), poultry by-product meal (PM), and a control. The control treatment consisted of 8% straw, 16% chicken feed (K (11 4) o.K., Agravis Raiffeisen AG, Velten, Germany) and 77% water, and was prepared as the other substrates. The substrates were prepared by adding water to each of the fish faeces variants to obtain a substrate with 23% dry matter content. The faeces collected from each fish replicate was handled separately without mixing it with other faeces batches obtained from the replicates of the same fish treatment. The substrates were mixed with water using a kitchen spatula and were let to soak in room temperature for 6 h, and then were homogenised and put in polyethylene boxes (area of

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12 × 17 cm). Each box contained 325 g of the wet mixture and 325 BSFL (6 days old with an average weight of 2.5 mg) were added on the top of the substrate. The composition of the control feed is shown in Table 3. Each treatment had 5 replicates and the larvae were grown in a chamber with 27°C and 50% relative humidity.

The single-larva weight was monitored daily by collecting and weighing 30–50 larvae per box, and the larvae were thereafter returned to the box. The harvest took place on the 10th day of the experiment. The PM treatment was harvested on day 7 due a loss of weight between day 6 and 7. In order to avoid the differential drying of substrates before full consumption, which is a known issue in using fish wastes as a BSFL substrate (Liland et al., 2023), the boxes were weighed daily, and water (27°C) was added to the boxes from day 4 to maintain a total weight of 150 g per box and ensure sufficient moisture (~ 50%) in the substrate.

The larvae were harvested and counted manually to calculate the mortality rate and dried at 60°C until no further weight reduction was recorded. The feed conversion ratio (FCR) was calculated on a dry basis by dividing the dry substrate reduction by the dry BSFL weight gain.

2.3 Chemical analyses

The feed provided to the fish in the fish trial, the faeces collected ($n = 5$), and the larval biomass produced ($n = 5$) were analysed for aluminium (Al), boron (B), calcium (Ca), cobalt (Co), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), phosphorus (P), sulphur (S), zinc (Zn), and nitrogen (N). All analyses were done as mentioned in Yakti et al. (2022) in two technical replicates. Briefly, for the elements other than N, a microwave digestion (MARS Xpress, CEM, Matthews, North Carolina) was conducted following LUFA methods Vol. III, 10.8.1.2. followed by multi-element analysis using ICP-OES (DIN EN ISO 11885) with an ICP emission spectrometer (iCAP 6,300 Duo MFC, Thermo, Waltham, MA, United States). N concentration was assessed using an elemental analyser (Vario MAX, Elementar Analysensysteme GmbH, Hanau, Germany), following LUFA Bd. III, 4.1.2. The crude protein in the produced BSFL biomass was calculated from the N content using a factor of 4.43 (Smets et al., 2021).

2.4 Statistical analyses

The data was analysed using SPSS (IBM Corp, Armonk, NY, USA). The parameters were analysed using one-way ANOVA ($p = 0.05$) followed by the consequent Tukey's HSD after confirming data normality and homogeneity of variance. Kruskal-Wallis test was used when the data did not meet the assumption of the parametric test also after applying common data transformations. Spearman's rank correlation was used to evaluate the linear relation between the elements' concentration in the BSFL and the fish faeces (BSFL substrate).

3 Results

3.1 Fish feeding trial

Rearing condition during the RAS trial were within acceptable limits for tilapia regarding mean oxygen levels (6.7–6.9 mg/L),

TABLE 3 The elemental composition of the control substrate (chicken feed and straw) used in the BSFL feeding trial (dry matter basis).

Al mg/kg	B mg/kg	Ca g/kg	Co mg/kg	Cu mg/kg	Fe g/kg	K g/kg	Mg g/kg	Mn mg/kg	Mo mg/kg	Na g/kg	P g/kg	S g/kg	Zn mg/kg	N %
400.47	10.25	19.90	0.29	13.25	0.39	10.83	1.65	90.08	0.96	0.98	3.21	1.76	62.72	1.38

TABLE 4 Fish growth performance and faeces collection.

	BSFM	FM	PBM	PM
% Survival[A]	99.8 ± 0.5	100.0 ± 0.0	100.0 ± 0.0	99.7 ± 0.6
Biomass gain (g)[A]	539[b] ± 63	1001[a] ± 70	416[c] ± 62	901[a] ± 115
Feed conversion ratio (FCR)[3A]	2.03[b] ± 0.22	1.08[c] ± 0.07	2.65[a] ± 0.38	1.22[c] ± 0.15
Feed administered over 14 days (g)	1,080	1,080	1,080	1,080
Faeces DM collected over 14 days (g)[A]	104.6[b] ± 10.9	97.2[b] ± 3.7	234.0[a] ± 14.9	105.0[b] ± 9.0

Values represent means ± standard deviations; means in rows with different superscript letters are significantly different ($n = 5$, $p < 0.05$). [A]$n = 5$; [3]FCR = total feed administered (g as fed)/ [final biomass (g)—initial biomass (g)].

temperature (25.5°C), water pH (6.9–7.8), and electrical conductivity (937–1,026 μS/cm) in all treatments and results are compiled in Table S1. The fish biomass gain differed based on the feed provided (ANOVA followed by Tukey's test on the log10 transformed data, $p < 0.001$, $F = 51.56$), and the values ranged from 415 g in the fish that received poultry blood meal (PBM) to 1 kg for the fish that were provided with fish meal (FM) (Table 4). No differences were observed between the growth of fish that received poultry meal (PM) and FM, but the growth decreased significantly in the black soldier fly meal (BSFM)-fed fish, and PBM-fed fish. Results for initial and final biomass, body weight and condition factor as well as specific growth rate can be found in Table S2.

The feed conversion ratio (FCR) of the fish was calculated and significantly differed among the treatments (ANOVA followed by Tukey's test on the log10 transformed data, $p < 0.001$, $F = 51.54$). Besides having the lowest biomass gain and producing the highest amount of faeces, the fish provided with the PBM had the highest FCR followed by the BSFM-fed fish. The best growing fish-treatments (FM and PM) had the lowest FCR values.

The amount of faeces collected also differed among the treatments (ANOVA followed by Tukey's test on the log10 transformed data, $p < 0.001$, $F = 121$). The PBM-fed fish produced the highest amount of faeces (234 g dry faeces), while the rest of treatments did not differ among each other and produced approximately 100 g dry faeces (Table 4).

In addition to the amounts of fish faeces, the chemical composition of the faeces varied significantly based on the feed provided (Table 5). The faeces collected from the FM-fed fish had the highest concentration of Al, B, Ca, Co, Mg, Mo, Na, and P. The faeces collected from the BSF-fed fish contained the highest concentration of Cu, Mn, and Zn. High Zn was also observed in the PM and FM faeces. The faeces collected from the PBM-fed fish contained the lowest concentration of Al, B, Ca, Co, Cu, K, Mn, Mo, Na, P, and Zn, but was the richest in Fe, S and N.

3.2 Black soldier fly larvae feeding trial

The performance of black soldier fly larvae (BSFL) was compared when cultivated on the different fish faeces and the control substrate. The peak weight of the BSFL was reached on day 6 for the PM treatment so the larvae had to be harvested on the seventh. The other treatments, including the control, were harvested on day 10. The single-larva weight at the end of the experiment was the highest in the larvae provided with the control diet and 0.088 g (SD = 0.003) ($p < 0.001$, $F = 272$,). No differences in the single-larva weight were found between the different faeces' variants, and they reached a weight from 0.0228 to 0.0219 with SD values ranging from 0.0024 to 0.0059. The total biomass gains of BSFL did not differ among the faeces treatments, and the highest growth was observed in the control treatment (Figure 1).

The harvested BSFL were manually counted to calculate the mortality rate and no differences were found (Figure 2).

The feed conversion ratio (FCR) was calculated and differed among the treatments. The control BSFL had the lowest FCR, and the values did not significantly differ from the BSFL that received the PBM faeces. Among the fish faeces treatments, differences were only observed between the PBM and the PM BSFL. (Figure 3).

The elemental composition of the BSFL differed significantly based on the fish faeces received (Table 6). Generally, the larvae that consumed fish faeces had lower Al, B, and Co concentration than the faeces (initial substrate). Higher concentrations of Ca, K, Mg, Mn, Na, S, and Zn were observed. The values of Cu, Fe, Mo, P were higher or lower based on the treatment (Table 6). Providing the BSFL with the control substrate led to higher Al and B, and lower Zn, Cu, and P in some fish faeces variants. The concentration of all analysed elements, namely Al, B, Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Na, P, S, and Zn differed based on the fish faeces provided. Providing the BSFL with FM faeces led to the highest values of Al, B, Ca, Co, Cu, Mo, and P while the values of other elements were closer to other treatments. The BSF-meal derived fish faeces led to the highest Mn and Zn values in the larvae. The BSFL given the PBM faeces accumulated the highest amounts of S and had the highest crude protein values.

A very strong significant correlation was found between the concentrations of Al, P, S, Zn, and N in the BSFL and their concentrations in the fish faeces they fed on (Spearman's $\rho > 0.75$, $p < 0.001$). Significant correlations (Spearman's $\rho > 0.5$, $p < 0.01$) were also found in the case of B, Ca, Cu, Fe, Mg, and Mo. No significant correlations were found in K, Mn, and Na (Figure 4).

4 Discussion

The rapid expansion of aquaculture is raising sustainability concerns due to two primary factors: the dependence on marine fish meal obtained from wild fish stocks for the production of fish feed (Stankus, 2021), and the environmental repercussions primarily associated with the generation of waste (Dauda et al., 2019). The high dependence on marine FM has led to mounting pressure on wild fish populations, resulting in the rapid depletion of wild fish stocks (Stankus, 2021). Research has been carried out to explore alternative protein sources that offer comparable nutritional value to marine fish meal (Daniel, 2018). These protein sources, among others, include poultry by-product meal

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TABLE 5 The elemental composition of the faeces collected from the fish provided with different feed.

Treatment		Al	B	Ca	Co	Cu	Fe	K	Mg	Mn	Mo	Na	P	S	Zn	N
		mg/kg	mg/kg	g/kg	mg/kg	mg/kg	g/kg	g/kg	g/kg	mg/kg	mg/kg	g/kg	g/kg	g/kg	mg/kg	%
FM	Mean	598.33^{b}	9.42^{b}	74.69^{b}	1.43^{b}	57.69^{ab}	0.80^{ab}	0.83^{c}	2.58^{c}	192.86^{ab}	2.02^{b}	1.17^{c}	35.68^{a}	3.54^{a}	323.88^{b}	2.55^{c}
	SD	72.24	0.79	2.90	0.31	3.78	0.16	0.07	0.12	15.03	0.07	0.09	1.28	0.06	14.21	0.09
BSF	Mean	133.23^{b}	8.55^{ab}	29.71^{a}	0.52^{a}	66.77^{b}	0.57^{bc}	0.83^{c}	2.57^{c}	581.27^{a}	1.82^{a}	0.63^{b}	15.08^{bc}	3.44^{a}	498.98^{a}	3.98^{ab}
	SD	8.41	0.99	1.43	0.09	0.59	0.09	0.05	0.13	67.24	0.08	0.03	0.69	0.13	8.46	0.051
PM	Mean	55.46^{ab}	8.14^{ab}	62.2^{d}	1.05^{b}	65.37^{b}	0.45^{a}	0.63^{b}	1.82^{b}	174.38^{bc}	1.43^{d}	1.05^{c}	30.49^{ab}	3.59^{a}	360.92^{d}	2.9^{bc}
	SD	6.76	1.50	7.39	0.13	8.91	0.02	0.07	0.06	21.80	0.07	0.09	3.13	0.07	12.41	0.084
PBM	Mean	26.52^{a}	7.22^{a}	11.02^{c}	0.38^{a}	34.88^{a}	1.36^{c}	0.21^{a}	1.25^{a}	87.48^{c}	0.65^{c}	0.33^{a}	8.59^{b}	6.74^{b}	122.66^{c}	9.94^{a}
	SD	4.73	0.71	0.86	0.04	3.05	0.59	0.05	0.14	13.22	0.02	0.06	0.41	0.20	7.38	0.72
F			3.81	259.82				122.99	149.73		446.64	148.90		812.56	1002.85	
H		17.85			16.14	14.72	16.28			16.71			17.86			17.86

Shown are the means and standard deviations (SD) of the elements quantified on a dry matter basis.
The different groups are expressed with the different letters above the mean values. The F-values are shown for one-way ANOVA, and the H- (test statistics) values are shown for Kruskal-Wallis test.

(Galkanda-Arachchige et al., 2020), insect meal (Oteri et al., 2021), and poultry blood meal (Luthada-Raswiswi et al., 2021). Such protein sources do not only serve as alternatives to FM but also adhere to the principles of the circular bioeconomy (Stegmann et al., 2020) in that they represent wastes streams or potential biowaste converting biomass (in case of insect meal) that can be upcycled into higher value protein sources for human consumption via aquaculture. Also, they are not subject to food-feed conflict as other feed raw materials such as FM from capture fisheries or many plant protein sources (Colombo and Turchini, 2021; Colombo et al., 2022). Accordingly, they can play an important role in achieving greater circularity in aquafeed production.

4.1 The fish feeding trial

The use of black soldier fly meal (BSFM) as a fish feed component has been widely investigated in different fish species (Alfiko et al., 2022). In the case of Nile tilapia, some studies have suggested that growth and feed intake do not differ between fish fed on commercial fish meal (FM) or black soldier fly meal (BSFM) (Tippayadara et al., 2021). Limbu et al. (2022) have demonstrated that replacing 75% of the FM with BSFM in an initial diet with 42% FM lead to an improved fish biomass gain and growth rate in Nile tilapia fry, and pointed to improved economic returns in the production system. In our study, replacing FM with BSFM led to an almost 50% reduction in biomass gain (Table 4). Both feeds, despite having minimal differences in nitrogen content, could have significantly differed in actual protein content since BSFM can contain certain amounts of mineral N or N integrated into the chitin skeleton, leading to a lower N-to-protein transformation factor compared to other protein sources (Smets et al., 2021). This may have contributed to the reduced fish growth. A meta-analysis on the inclusion level of insect meals in aquafeeds suggests maximum inclusion levels of 25–30% for unimpaired growth performance (Liland et al., 2021), and another meta-analysis suggests a maximum 29% BSFM inclusion in aquafeeds (Hua, 2021). In line with the results of the present study, other authors also found reduced growth performance above certain levels of FM replacement with BSFM for tilapia (Taufek et al., 2021). Considering that the BSFM was the sole dietary protein source in the BSF diet of the present study and BSFM inclusion level (61.6%) was higher than in the above studies (8–30%), it appears reasonable to assume that excessively high dietary BSFM inclusion, particularly if not complemented by other protein ingredients, is not conducive to maximum growth performance in tilapia. One reason for this could be chitin impairing protein digestibility, especially at high BSFM inclusion levels, as suggested by some authors (Shiau and Yu, 1999; Kroeckel et al., 2012; Rana et al., 2015).

The second FM alternative used in the experiment was poultry by-product meal (PM), which is generally known to be a valuable protein source and alternative to fish meal in aquafeeds. PM is made from the processed rendered parts of poultry carcasses, which include feet, heads and parts not typically consumed by humen. In a meta-analysis, Galkanda-Arachchige et al. (2020) showed that many freshwater fish species readily accept above 50% FM replacement with PM and 100% replacement can be possible without adverse effects. Although some studies have shown that PM can lead to lower protein use efficiency and digestibility in Nile tilapia (El-Sayed, 1998; Palupi et al., 2020). Hernández et al. (2010), and Yones and Metwalli (2015) observed similar growth performance and feed utilisation even at

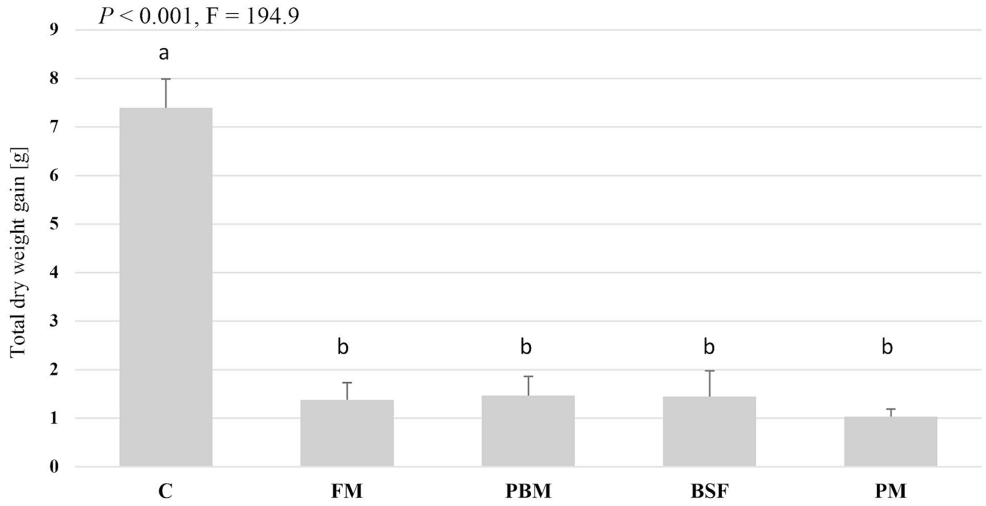

FIGURE 1
The dry biomass gain of black soldier fly larvae (BSFL) fed with different fish faeces. BSFL were grown on faeces collected from fish that received fishmeal (FM), blood meal (PBM), black soldier fly meal (BSF), poultry by-product meal (PBM), or a control substrate (chicken feed and straw), and the dry biomass gain was calculated. The analysis of variance ANOVA (n = 5, $p < 0.05$) revealed only a significant difference between the control treatment and the faeces treatments.

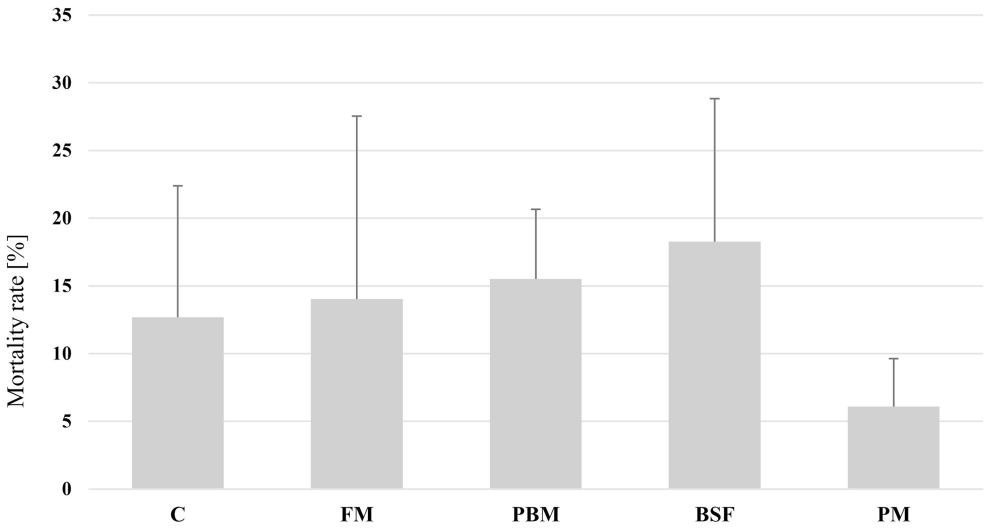

FIGURE 2
The mortality rate of black soldier fly larvae (BSFL) grown on different fish faeces. BSFL were grown on faeces collected from fish that received fishmeal (FM), blood meal (PBM), black soldier fly meal (BSF), poultry by-product meal (PBM), or a control substrate (chicken feed and straw). The larvae were harvested, and the survival was assessed. The analysis of variance ANOVA (n = 5, $p < 0.05$) revealed no difference between the treatments.

100% FM replacement with PM, which is consistent with the results of this study as the biomass gain and feed conversion ratio (FCR) were not compensated when FM was substituted with PM (Table 4). FCR is a critical parameter in evaluating livestock performance as it measures the efficiency with which animals convert feed into body mass. The partial or full replacement of fishmeal with a more sustainable or lower cost component without influencing the FCR can boost the profitability and sustainability in aquaculture systems.

PBM as the third protein source, although resulting in a diet which compared mostly favorably in terms of its essential amino acid profile to the other diets (Shaw et al., 2022a) led to the lowest biomass gain and the highest faeces production, and consequently the highest FCR (Table 4). PBM has been shown to negatively affect Nile tilapia growth when replacing FM (Otubusin, 1987; El-Sayed, 1998; Kirimi et al., 2016) and in the case of the present study, the comparably low Ca and P content of the PBM diet, which were both slightly below the requirements for Nile tilapia (Mjoun et al., 2010), supposedly played a role in suppressing the growth performance of the fish. Subsequently, mineral Ca and P supplementation or complementing PBM with other P-/Ca-rich protein sources, i.e., terrestrial animal by-products such as PM, would likely improve the suitability of PBM at high inclusion levels in diets for Nile tilapia. Despite the reduced growth and feed conversion performance achieved with the BSF and the PBM diet in this and prior studies (Shaw et al., 2022b; a), the nutritional value of BSFM and PBM should not be discounted, since in combination with complementary raw materials such as PM or catfish by-product meal they have the

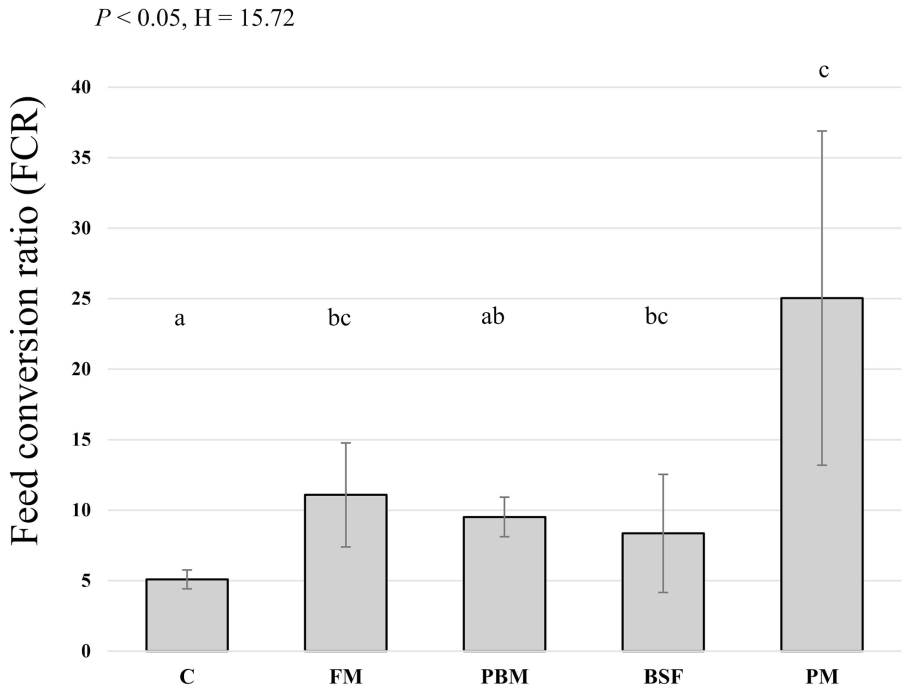

FIGURE 3
The feed conversion ratio (FCR) of black soldier fly larvae (BSFL) grown on different fish faeces. BSFL were grown on faeces collected from fish that received fishmeal (FM), blood meal (PBM), black soldier fly meal (BSF), poultry by-product meal (PBM), or a control substrate (chicken feed and straw), and the feed conversion ratio (FCR) was calculated. Kruskal-Wallis test ($n = 5$, $p < 0.05$) revealed significant differences between the treatments and are represented by the different letters above the columns.

potential to rival the growth performance achievable with commercially available feed (Shaw et al., 2023). To this effect, modern feed formulation in aquaculture should not be a search for FM or fish oil substitutes but rather have the goal of identifying combinations of complementary raw materials which together fulfil the nutritional requirements of the species in question as well as achieving further relevant objectives (waste reduction, low contaminant levels, cost, etc.).

Besides the above-described differences in fish growth, the experimental feeds resulted in differences in the elemental composition of the respective faeces (Table 5). Since fish excrete excess nutrients in dissolved forms via brachial and urinary excretion and in solid form via faeces, it is to be expected that dietary differences in elemental composition are at least partly reflected in the elemental composition of the respective faeces. This was apparent in the case of, e.g., Fe in this study, which showed the highest level in the PBM feed and subsequently in the PBM-derived faeces. Similar relations between elemental content in the fish feed and the resulting content in the faeces were for the most part found for Ca, K, Mg, Na, P, Zn, but not for S. In the case of N, the highest content as well as total amount was found in the PBM-derived faeces, while the opposite was observed in the faeces generated from the FM treatment. Considering that the PBM diet enabled the least favorable growth performance, this could indicate that the N in the PBM diet was not efficiently utilized by the fish. It can be assumed that growth inhibition was due to other limiting factors such as the low Ca and P content or lower digestibility of protein in the PBM compared to the other protein sources.

Overall, the results of the present fish rearing trial corroborate findings of a previous study in which the same diets were fed to Nile tilapia in a different experimental RAS setup (Shaw et al., 2022b), with similar differences found between the dietary treatments regarding fish growth and feed conversion performance, as well as faeces amounts and respective elemental compositions.

4.2 Black soldier fly larvae feeding trial

With the growing focus on circularity in food production systems, the recognition of fish faeces as a waste resource is increasing, necessitating strategic management practices. Generally, the assessment of aquaculture sludge as a substrate for insect production is limited and has been rarely discussed in literature (Schmitt et al., 2019; Liland et al., 2023). This could be due to the legal restrictions of using fish wastes as a substrate in production, the relatively poor growth of BSFL on fish faeces in comparison to many other organic waste streams, or the associated risks of contaminants. The accumulation of heavy metals in BSFL is considered to be a major issue when growing BSFL on waste streams and manure (Diener et al., 2015; Proc et al., 2020), and fish faeces, in particular, can feature high amounts of heavy metals (Lalander et al., 2013; Schmitt et al., 2019) and other unwanted elements which originate from the fish feed and are unabsorbed by the fish. The modulation of fish faeces composition by strategically choosing raw materials low in these contaminants and manipulating fish feed formulations accordingly, without compensating fish growth, can be a strategy to generate fish faeces that may be more suitable to be recycled by BSFL with reduced associated risks. To the best of our knowledge, the assessment of BSFL performance and composition on faeces stemming from fish fed different diets has not been done in published literature.

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TABLE 6 The elemental composition BSFL provided with different fish faeces or a control (C).

Treatment		Al [mg/kg]	B [mg/kg]	Ca [g/kg]	Co [mg/kg]	Cu [mg/kg]	Fe [g/kg]	K [g/kg]	Mg [g/kg]	Mn [mg/kg]	Mo [mg/kg]	Na [g/kg]	P [g/kg]	S [g/kg]	Zn [mg/kg]	Protein (N x 4.43)
FM	Mean	476.97^{bc}	4.24^{b}	77.02^{b}	1.15^{b}	69.13^{c}	0.80^{b}	14.09^{bc}	5.11^{b}	295.67^{b}	1.46^{c}	1.71^{b}	27.83^{c}	4.85^{b}	477.33^{b}	30^{ab}
	SD	35.68	0.54	7.35	0.05	9.38	0.06	1.29	0.35	12.10	0.10	0.09	3.74	0.17	18.96	2.54
BSF	Mean	86.40^{e}	2.98^{bc}	56.12^{ab}	0.22^{a}	58.85^{c}	0.45^{a}	12.18^{a}	4.81^{b}	1011.8^{a}	1.16^{b}	1.43^{a}	19.93^{a}	4.59^{a}	641.50^{c}	33.03^{b}
	SD	15.24	0.81	2.62	0.05	6.87	0.05	0.56	0.22	58.79	0.17	0.08	0.65	0.14	33.21	1.09
PM	Mean	41.08^{a}	2.15^{c}	73.22^{ab}	0.47^{b}	42.77^{b}	0.43^{a}	13.79^{bc}	4.63^{a}	313.83^{b}	0.85^{a}	1.75^{b}	23.94^{bc}	4.84^{b}	400.81^{b}	33.36^{b}
	SD	26.60	0.97	2.97	0.14	6.05	0.03	0.63	0.16	24.63	0.08	0.07	1.25	0.06	26.04	0.90
PBM	Mean	35.15^{a}	3.81^{bc}	46.49^{a}	0.23^{a}	33.93^{ab}	1.33^{b}	14.18^{ab}	5.27^{a}	336.02^{b}	0.78^{a}	1.83^{ab}	20.08^{ab}	7.00^{b}	231.64^{a}	38.7^{c}
	SD	10.89	1.51	15.65	0.11	11.86	0.41	4.89	1.69	86.40	0.23	0.61	6.93	2.58	91.88	4.19
C	Mean	799.32^{b}	6.38^{a}	70.94^{ab}	0.27^{a}	20.81^{a}	0.62^{a}	15.96^{c}	4.26^{a}	350.51^{ab}	1.42^{bc}	1.23^{a}	10.11^{a}	3.72^{a}	216.80^{a}	28.6^{a}
	SD	157.88	1.11	8.90	0.05	2.47	0.10	1.69	0.48	42.57	0.15	0.13	1.26	0.33	31.31	0.46
F			11.75			29.17					19.66				68.11	14.44
H		21.2		13.83	17.25		21.4	13.23	12.88	14.95		17.66	17.91	21.15		

Shown are the means and standard deviations (SD) of the elements quantified in the larvae.
The different groups are expressed with the different letters above the mean values. The *F*-values are shown for one-way ANOVA, and the *H*- (test statistics) values are shown for Kruskal-Wallis test.

As anticipated, the growth of BSFL on all faeces types was lower compared to the control treatment (a mixture of chicken feed and straw) (Figure 1), which was also reflected in the FCR values (Figure 3). Nevertheless, the fish faeces treatments did differ among each other in terms of insect biomass gain. The growth of the BSFL in this study, however, was considerably lower than that obtained by Liland et al. (2023) and Schmitt et al. (2019) who grew BSFL on aquaculture sludge obtained from commercial RAS facilities. In contrast to small and highly controlled experimental systems such as used in the present study, aquaculture sludge from commercial RAS can contain noteworthy amounts of unconsumed fish feed (Madariaga and Marín, 2017; Campanati et al., 2022) and perhaps even parts of fish carcasses, which may be factors explaining the difference in growth observed between the above mentioned studies and the presented trial which relied on pure faeces from an experimental RAS. In this sense, also the faeces collection method (e.g., sedimentation units as used in this trial vs. drum filters commonly used in commercial RAS) and residence time of sludge in the system water may influence not only the quantity but also the nutritional quality of the collected sludge. The FCR values observed for the fish faeces-fed BSFL (5.3 to 25.4) were comparable to the values obtained for pig manure (Newton et al., 2005), chicken manure (Sheppard et al., 1994) and are lower than those obtained from municipal organic wastes (Diener et al., 2011). The PBM-derived faeces contained the highest N value but, despite not differing from the control treatment in the FCR value (Figure 3), did not yield in a higher BSFL growth. This is likely due to a limiting nutrient other than protein, or the lower digestibility of proteins in PBM. However, the use of PBM-derived faeces in combination with high-carbohydrate low-protein components could potentially allow better utilisation of the N from the PBM-derived faeces.

The survival of BSFL did not differ among the treatments. It can be assumed that variance in the mineral composition and heavy metal content of the substrates (in the ranges observed) have no influence on mortality. BSFL are known to tolerate and survive on substrates with high concentrations of heavy metals (Cai et al., 2018; Hu et al., 2023; Siddiqui et al., 2023), but this can lead to the accumulation of certain heavy metals as shown for Cd, Pb and Zn from chicken feed and contaminated substrates (Diener et al., 2015; Van der Fels-Klerx et al., 2016). Generally, BSFL can accumulate higher concentrations of S, Ca, Mg, Mn, Zn, Fe and K than present in the rearing substrate when provided with a balanced diet such as chicken feed (Yakti et al., 2022). Such enrichment improves the nutritional value of larvae intended for the use as raw material for animal feeds (Shumo et al., 2019). In a study in which BSFL were given a substrate supplemented with very high concentrations of As, Cd, Cr, Hg, Ni, Pb, a reduction of growth was observed but only Cd and Pb accumulated (Purschke et al., 2017). Cai et al. (2018) have pointed at a link between the concentration of Zn and Cu in a municipal sewage sludge substrate and larval mortality. The concentrations were, however, higher than those found in the sampled faeces in this study.

In the present study, the concentration of analysed elements in the BSFL differed among treatments (Table 6) and the relation was assessed with a correlation analysis (Figure 4). Significant correlations in the case of Al, B, Fe, Cu, Ca, Co, Mg, Mo, P, S, Zn, and N were detected. Relations between initial concentration of elements in the feeding substrates and the produced BSFL biomass have been already observed in published studies that included gradients of wastes into BSFL substrate. Liland et al. (2017), for example, cultivated BSFL on

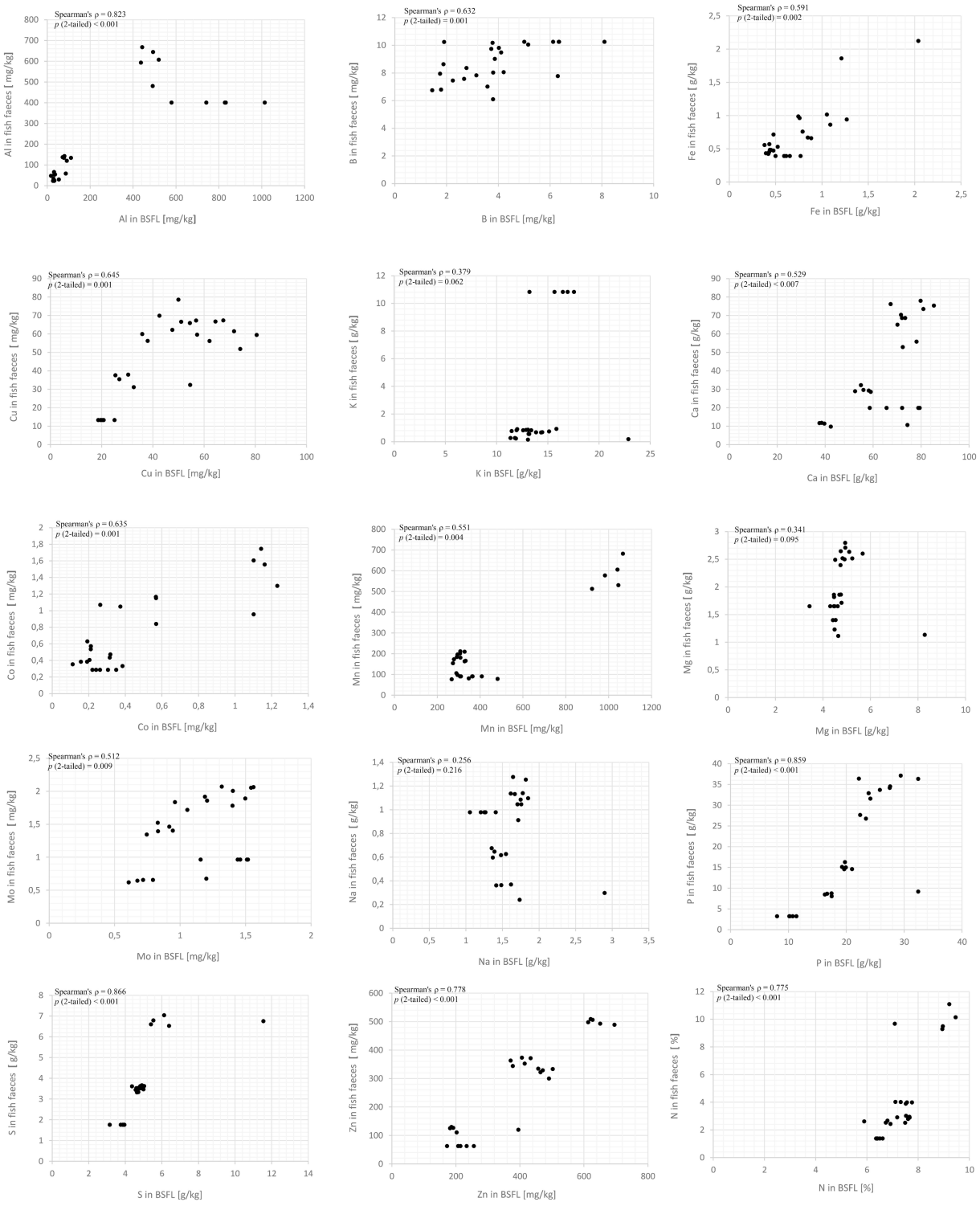

FIGURE 4
The relation between the concentration of elements in the insects rearing substrate (Fish faeces) and the concentration of the elements in the produced BSFL biomass. Shown are the plotted values, the spearman's correlation coefficient, and the *p*-values.

seaweed-enriched substrates and found an accumulation of Cd, Pb, Hg, and As in the BSFL. The concentrations of these elements in the larvae increased proportionally with increasing levels of seaweed inclusion in the substrate. In a similar approach, Liland et al. (2023) mixed chicken feed with different ratios of aquaculture sludge and showed that the content of individual minerals and metals was higher in the sludge-fed BSFL compared to those fed with chicken feed. Elements such as Fe, Zn, and Se, which were found in higher amounts in the aquaculture wastes, increased in the BSFL due the increased incorporation of fish faeces in the BSFL substrate.

5 Outlook

Circular production systems are gaining interest as they promote resource efficiency, waste reduction, economic benefits, and resilience in supply chains. The ability of insects to process different biowaste streams into higher value insect biomass means they could play a key role in closing nutrient cycles and linking food and agricultural production systems. Given that insects are farmed animals, their rearing should comply with legal frameworks like those established by the European Union (Commission Regulation (EU) 2017/893), and their feeding substrate should meet specific requirements. Aside from the legal issues with using aquaculture sludge as a rearing substrate, the maximum allowed levels of heavy metals and other contaminant must not be exceeded in insects destined for feed production. This study shows that ingredient choice in fish feed formulation cannot only significantly influence fish growth performance but also the elemental composition of the, respectively, produced solid waste streams, i.e., the fish faeces, and these differences in turn can be reflected in the elemental composition of the produced larvae. Along the lines of formulating more sustainable and circular aquaculture feeds and potentially upcycling the solid wastes excreted by the fish via insect larvae production, future research should focus on identifying combinations of complementary raw materials generated within the circular bioeconomy which enable good fish growth and health as well as result in solid wastes low in contaminants such as heavy metals. Considering the low growth performance of larvae reared on the fish faeces in this study, future research could identify complementary biowaste streams that can be complemented by the different elemental compositions and quantity of the collected faeces. Additionally, further studies should focus on altering the accumulation of contaminants such as Cd, Pb, Hg, and As which were not investigated in the current study.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by the Landesamt für Gesundheit und Soziales- Berlin, Germany. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WY: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. CS: Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – review & editing, Formal analysis. MM: Data curation, Methodology, Writing – review & editing. IM: Funding acquisition, Project administration, Resources, Writing – review & editing. WK: Funding acquisition, Resources, Supervision, Writing – review & editing. CU: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2024.1298885/full#supplementary-material

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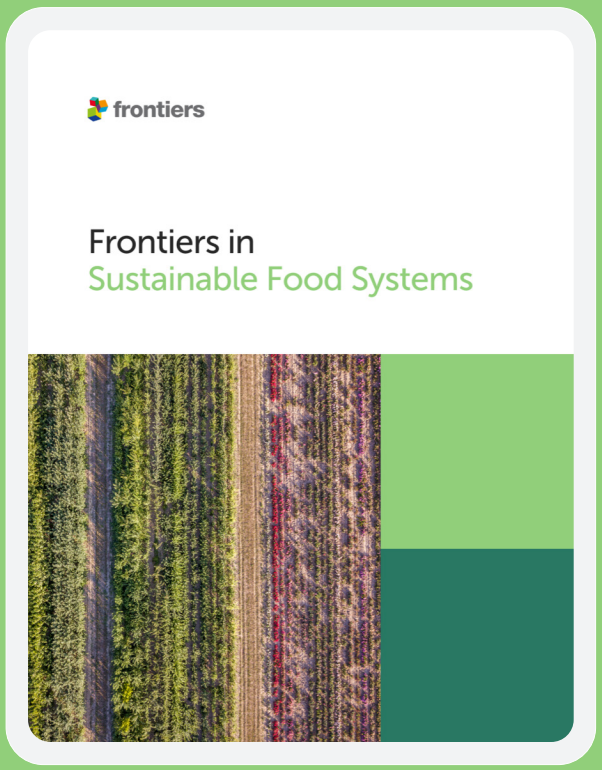

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