DEVELOPMENT OF FUNCTIONAL FOODS FROM MARINE SOURCES

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DEVELOPMENT OF FUNCTIONAL FOODS FROM MARINE SOURCES

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Enrique Barrajón-Catalán Holds Patents in "Extraction of Bioactive Compounds From Wine Industrial Byproducts" (ES20150000423), "Modified Pectin Production From Critus" (ES2013-01183), "A Synergic Combination of Polyphenols With Antibiotic Properties" (ES201301181), "Ultrasounds Combined Instant-Vacuun Machine for Extraction" (PCT/ES2013/000191) and "Cistus Plants Extracts Enriched in Polyphenols With Biological Activities" (ES20090002106) and is a Co-Founder of Ilice Effitech SL. Jian Zhong Holds a Patent in "A Sorting Equipment for Fish Fillets With Bone and its Intelligent Control System"(ZL 201710367428.3). Jose Manuel Lorenzo Holds Patents in Meat and Meat Products. All Other Topic Editors Declare No Competing Interests With Regard to the Research Topic Subject.

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EDITORIAL
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Editorial: Development of Functional Foods From Marine Sources

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Keywords: marine foods, functional active substances, functional foods, aquatic products, extraction techniques, emerging technologies

Editorial on the Research Topic

Development of Functional Foods From Marine Sources

Ocean is a great treasure and can provide important food and nutrition resources for humans. With the increase in global population size, living standards and food demands of mankind, consumption, and demand of marine foods will continue to increase globally. Food production from the ocean is sustainable and has significantly lower environmental impact compared to terrestrial foods. Indeed, the ocean has potential to supply over 3 billion tons of marine food to feed 30 billion people. In addition, marine food raw materials can provide high quality sources of protein for human consumption. However, it is important to view the potential benefits of marine food consumption in the context of increasing concerns with ocean pollution (e.g., plastic debris, industrial waste) and its potential to damage marine life.

Research has shown that diets with increased consumption of marine foods are associated with reduced risk of diet-related chronic diseases. Marine foods are low in calories, and contain functional active substances such as bioactive peptides, terpenes, polyketides, alkaloids, unsaturated fatty acids, polysaccharides, vitamins and minerals, which have been shown to have positive health effects. As such, the extraction of functional active substances and the development of functional foods from marine resources is attracting increasing attention in the field of nutrition and health, and food science and technology.

To date, studies on marine foods have focused on three areas: (1) animal and plant-based sources of raw materials such as fish, shrimp, shellfish, and aquatic plants; (2) functional active ingredients such as bioactive peptides, unsaturated fatty acids, polysaccharides, vitamins and minerals which can be extracted from raw materials or their by-products; (3) preparation and development of functional ingredients such as fish oil emulsions, bioactive peptide powders, polysaccharide capsules, marine algae tablets, and shellfish-originated drinks. However, further studies related to sensory (e.g., taste) evaluation and molecular mechanisms and pathways of the different types of marine foods are important, to further advance discovery of new functional ingredients, product applications and nutritional and pharmaceutical therapeutics. In addition, with the increasing concern of ocean pollution and the risk it poses to marine sources, food safety aspects of marine foods (e.g., toxicity) also require further investigation.

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With the development and application of novel and emerging processing techniques (e.g., microwave, radio frequency, 3D printing), extraction techniques (e.g., ultrasonic extraction, microwave-assisted extraction) and analytical technologies (e.g., omic techniques, fast detection techniques), there is the potential for significant advances in our understanding. It is envisaged that these advances will provide the knowledge required to promote and optimize the development of marine-based products for human consumption.

This special issue is a platform to discuss development of functional foods from marine sources. Yu et al. explored the effect of cold chain logistic interruptions on lipid oxidation and volatile organic compounds of salmon (salmo salar) and their correlations with water dynamics. Their work suggested that the temperature fluctuation during cold chain logistics should be avoided to maintain the nutrients and freshness of salmon. Ma et al. analyzed the chemical localization, structural change, and antioxidant property of the colloidal particles in tuna head soup. Their results provided basic information to understand the colloidal particle formation in food soup and suggested food soup might be a potential high-value-added way for aquatic by-product tuna head. Wu et al. characterized a food grade emulsion stabilized by the by-product proteins extracted from the head of giant freshwater prawn (*Macrobrachium rosenbergii*). Their work suggested a potential high-value-added way for prawn by-product. Li et al. explored the shelf-life extension of large yellow croaker (*Larimichthys crocea*) by active coatings containing lemon verbena (*Lippa citriodora* kunth.) essential oil. Their results found that the locust bean gum-sodium alginate active coatings incorporated with lemon verbena (*Lippa citriodora* Kunth.) essential oil emulsions maintained the quality and extended the shelf life of large yellow croaker during refrigerated storage. Jia et al. explored the effect of oral nutritional formula with three different proteins on type 2 diabetes mellitus *in vivo*. Their work provided a potential way to develop and evaluate marine protein-based nutritional products. Huang et al. reviewed Marine bioactive compounds as nutraceutical and functional food ingredients for potential oral health. Poulose et al. analyzed the anti-diabetic potential of a stigmasterol from the seaweed *Gelidium spinosum* and its application in the formulation of nanoemulsion conjugate for the development of functional biscuits. Their work demonstrated the stigmasterol could be used as a supplement in diets for diabetic patients. Aspevik et al. studied the nutritional and sensory properties of protein hydrolysates based on salmon (*Salmo salar*), mackerel (*Scomber scombrus*), and herring (*Clupea harengus*) heads and backbones. Their work showed only minor variations were found in the final protein hydrolysate products. Xu et al. reviewed the application of marine-derived collagen as biomaterials for human health. Their work pointed out that marine-derived collagen will attract more and more attention in the fields of clinical, medicine, food, etc. All these works proved ocean could provide excellent marine sources for the development of functional foods.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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Effect of Cold Chain Logistic Interruptions on Lipid Oxidation and Volatile Organic Compounds of Salmon (*Salmo salar*) and Their Correlations With Water Dynamics

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Lipid oxidation and water migration are important factors in the quality changes of aquatic products. This study investigated the relationship between water migration and lipid oxidation in salmon filets under four different storage conditions (control: 0°C; T1: 4°C; T2 and T3: two temperature fluctuation groups) by detecting thiobarbituric acid–reactive substances, changes of fatty acids, and volatile organic compounds (VOCs), and other quality indicators including redness, microorganism, total volatile basic nitrogen (TVB-N), and water-holding capacity (WHC) were also measured. The results of low-field nuclear magnetic resonance (LF-NMR) showed that more trapped water (T_{22}) turned to form free water (T_{23}) in groups suffering temperature fluctuations. A more significant decrease in fatty acids was found in T2 and T1 groups, especially oleic acid (C18:1n9c), linoleic acid (C18:2n6c), and palmitic acid (C16:0). The VOCs with off-flavors (1-penten-3-ol, 2-penten-1-ol, (Z)-, 1-octen-3-ol, hexanal) in the groups suffered from simulated cold chain interruptions increased faster than the other two groups during storage. T_{22} was negatively correlated ($p < 0.05$) with stearic acid (C18:0), 1-penten-3-ol, hexanal, and nonanal, whereas T_{23} was positively correlated with 1-penten-3-ol, hexanal, and heptanal. Therefore, the temperature fluctuation accelerated the loss of polyunsaturated fatty acids and the increase of unpleasant odors related to water migration.

Keywords: salmon filets, cold storage, temperature fluctuations, low-field nuclear magnetic resonance (LF-NMR), fatty acid, volatile organic compounds (VOCs)

INTRODUCTION

Temperature is considered to be the most important environmental factor that inhibits the microbiological growth and thus influences the shelf life of fresh fish (1). However, it is very difficult to keep the temperature constant during the actual cold chain transportation, especially when loading and unloading. In recent years, more and more scientists have paid attention to the effect of temperature fluctuation on the quality of aquatic products. Several storage trials at

fluctuating temperature conditions on quality changes of fishery products have been conducted in recent years (2–4).

Salmon (*Salmon salar*) belongs to the family Salmonidae and is a high quality of considerable nutritional and economic importance because of its high content of polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (5, 6). However, salmon is highly perishable during transportation and storage, which are contributed not only by the growth of microorganisms and protein degradation, but also by lipid oxidation (7). Lipid oxidation in seafood may result in the production of volatile oxidation products, which will affect its odor, flavor, appearance, texture, and even the nutrients and the shelf life (8). Our previous study also found that salmon was rich in lipid; lipid oxidation not only affected the odor, but also affected the color appearance (9). Lipid oxidation in seafood is usually triggered by enzymes such as lipase, lipoxygenase, cycloxygenase, etc., which needs water as a medium. Unfortunately, water is the most abundant component in tissues of seafood. Although most water is trapped in muscle in fresh seafood, it may migrate because of the degradation of the cell membrane and myofibrillar proteins, leading to the spread of soluble enzymes (10). It is hypothesized that water migration may be correlated with the lipid oxidation and production of volatile organic compounds (VOCs).

Therefore, this article mainly studies the changes of water migration, lipid oxidation, and VOCs caused by temperature fluctuations in salmon during simulated cold chain logistics, as well as the growth of microorganisms, and their correlations were analyzed, so as to demonstrate the relationship between water migration, lipid oxidation, and off-odors.

MATERIALS AND METHODS

Sample Preparation

The fish in this study were fresh farmed Atlantic salmon (each gutted weighed about 5 kg) were purchased from an aquatic products market (Pudong District, Shanghai, China). The salmon samples were gutted after being caught in the local farms (brand: Super Salmon) and transported immediately in a closed box with ice to Shanghai by air within 3 days. On arrival, all the samples were transferred immediately to the laboratory. After washing with distilled water, the fish was cut into pieces of about 200 g and put them in polyethylene bags (Fanren Packaging Co., Ltd., Foshan, China). The samples were randomly divided into four groups and stored under four simulated logistics conditions for 8 days as shown in **Figure 1**. Sampling is performed daily. The sampling was carried out every day.

Sensory Evaluation

Ten persons from the laboratory staff were asked to assess the sensory evaluation of salmon. The total scores of five attributes include color, odor, tissue morphology, elasticity, and mucus, and the quality of the samples based on 5-point hedonic scales in descending order from 5 to 1 (9). The average of the total score of the five attributes is considered the final result, and a total score of lower than two is considered an unacceptable threshold.

Determination of Redness Value

The redness value (a*) of salmon was recorded by a portable colorimeter (CR-400; Konica, Tokyo, Japan). Each batch was conducted in triplicate.

Microbiological Analyses

Quantitative microbiological analysis was determined according to the method of Xie et al. (11). At each sampling, salmon filet samples (25 g) were homogenized with 225 mL of sterilized saline water (0.85 g/100 mL). One milliliter of these 10-fold dilutions by sterilized saline water was mixed with 15–20 mL of iron agar (no. HB8735; Qingdao Hope Biol-Technology Co., Ltd., Qingdao, PR China). Total mesophilic bacterial counts and psychrotrophic bacterial counts were determined by counting the total colonies on the plate after incubating at 30°C for 48 h and at 4°C for 10 days, respectively. For H_2S-producing bacteria, the black colonies were counted after 72 h of incubation at 25°C. Each dilution was performed in three parallels.

Determination of Total Volatile Basic Nitrogen and Thiobarbituric Acid–Reactive Substances

The total volatile basic nitrogen (TVB-N) value was determined according to the method of FOSS (12) by using an Automatic Kjeldahl Apparatus (Kjeltec Analyzer Unit; Foss Tecator AB, Hoganas, Sweden).

The thiobarbituric acid–reactive substances (TBARS) were determined according to the method of Ibrahim Sallam (5) with minor modifications. The fish samples (5 g) were homogenized with 25 mL of 20% (wt/vol) trichloroacetic acid (Sinopharm Chemical Reagent Co., Ltd., Shanghai, PR China), centrifuged, and incubated, and the absorbance was measured at 532 nm. The TBARS value was expressed as mg malondialdehyde equivalents per 100 g of samples (mg/100 g).

Determination of Water-Holding Capacity

The fish samples (M1) (3 g) were weighed accurately, and the fish samples (M2) were weighed after centrifugation at 3,000 r/min for 10 min. The formula for calculating water-holding capacity (WHC) (%) was as follows:

$$\mathrm{WHC\%} = \left(1 - \frac{\mathrm{M1} - \mathrm{M2}}{\mathrm{M1}}\right) \times 100\%.$$

Determination of Transverse Relaxation Time (T_2) of Low-Field Nuclear Magnetic Resonance and Proton Magnetic Resonance Imaging

T_2 relaxation measurements were performed using a low-field nuclear magnetic resonance (LF-NMR) analyzer minispec PQ 001 (Niumag, Ltd., Shanghai, China). The fish sample (2 × 2 × 2 cm) was put into the detecting tube (70-mm diameter). Using the Carr-Purcell-Meiboom-Gill sequence to set the T_2 measurement parameters (9), the analysis software (NMI20-030H-1 NMR analyzer: Suzhou Niumag Analytical Instrument Co., Suzhou, China) was used to iteratively invert the collected signals to obtain the transverse relaxation time

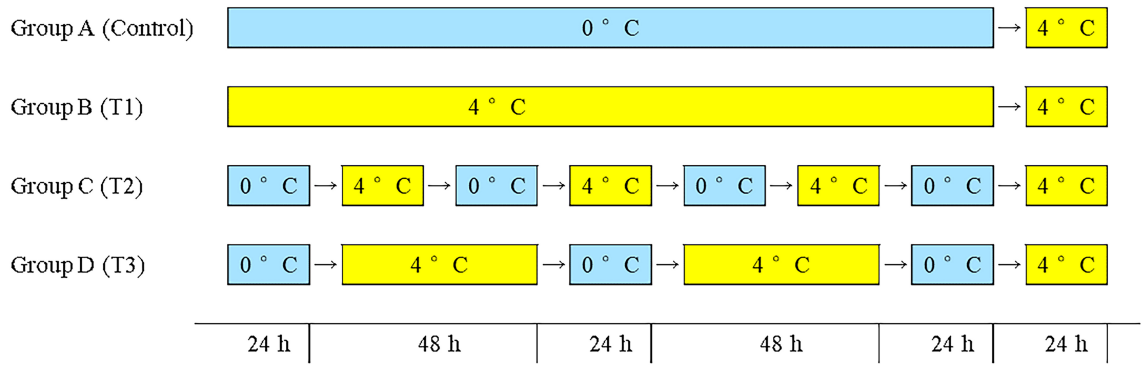

FIGURE 1 | Group A: 0°C constant (control group), group B: 4°C constant (T1 group), group C: seven shifts for 24 h from 0 to 4°C (T2 group), group D: five shifts for 0°C 24 h and 4°C 48 h (T3 group).

T_2 spectrum. Similarly, the proton density distribution of the sample is determined by magnetic resonance imaging (MRI) after setting the parameters. The gray-scale map of proton intensity was altered to be pseudocolor images by MATLAB software (MathWorks Inc., Natick, MA, USA).

Fatty Acid Profile

The total lipids of salmon were extracted by adding 10 g of flesh sample with chloroform/methanol (2:1, vol/vol) and then condensed using rotary evaporation according to the method of Folch et al. (13). The extracted sample (0.1 g) in a 100-mL round-bottomed flask and methylate the fatty acids by the boron trifluoride-methanol method according to the method of Zhang et al. (14) and analyze it by gas chromatography (GC) after methyl esterification. Qualitative analysis was performed according to the comparative retention time of fatty acid methyl ester mixed standards (Sigma–Aldrich Co. LLC, USA) and samples, and quantitative analysis was performed by the internal standard method. GC parameters were set according to the method of Merlo et al. (15).

VOC Determination by Headspace Solid Phase Microextraction Coupled With GC/Mass Spectrometry Analysis

The VOC analysis was measured according to the method of Parlapani et al. (16) with some modifications. Three grams of fish meat was weighed and added it to 20-mL headspace vials and equilibrate at 50°C for 15 min. Then the SPME fiber (DVB/CAR/PDMS 50/30 μm) was exposed to the headspace for additional 40 min. The fiber with VOCs was inserted into the injection port of the GC at 250°C for 5 min.

GC/mass spectrometry (MS) analysis was performed on an Agilent 7890A gas chromatograph coupled to an Agilent 5977A mass spectrometer (Agilent Technologies Co. Ltd., CA, USA). A capillary HP-5MS column (30 m × 0.25 mm × 0.25 μm) was used, and the GC oven temperature initially was maintained at 40°C for 3 min and then was raised to 100°C with a program of 3°C/min, and increase of 5°C/min to a final temperature of 230°C and kept for 5 min. The carrier gas (helium) flow rate was 0.8 mL/min, the analytical temperature was 250°C, and the injection was performed in splitless mode. The temperature of transfer lines was maintained at 280°C, the temperature of MS source and quadrupole was set at 230 and 150°C, respectively. The scan range from 30 to 550 m/z and ionization energy was 70 eV. The VOCs were identified by comparing their retention times with reference compounds or by comparing their mass spectra with those stored in the National Institute of Standards and Technology 11 spectral database. The amount of VOCs was expressed as the peak area of the deconvoluted component multiplied by any unit of 10^{-6}.

Statistical Analysis

At least three measurements of all experiments were performed. The data were performed in Excel 2010 software (Microsoft Corp., Redmond, WA, USA), and analysis of variance and mean comparison were carried out by using SPSS 20 (SPSS version 20.0; SPSS Inc., Chicago, IL, USA); the significant difference was set at $p < 0.05$, and the figures were performed using the Origin2018 software (OriginLab, USA).

RESULTS AND DISCUSSION

Sensory Evaluation

As shown in **Figure 2A**, the sensory scores of the four groups of fish samples decreased during storage, but the degradation rate of the T3 was significantly faster. On the third day, T3 began to produce mucus, but no off-odor was produced in all four groups. On the 6th day, the mucus in all groups increased significantly, whereas the elasticity decreased, and the color was darker than before; the three groups (control, T1, T2) started to exhibit a slight ammonia odor, but the unacceptable rancid odor began to appear in T3, which led to a significant decrease in sensory scores. On the 8th day, the sensory scores of all groups reached an unacceptable threshold. This phenomenon was similar to the research of Wang et al. (9). The sensory score of salmon at 0°C was significantly higher than the group that suffered temperature fluctuations, and the quality of salmon filets was

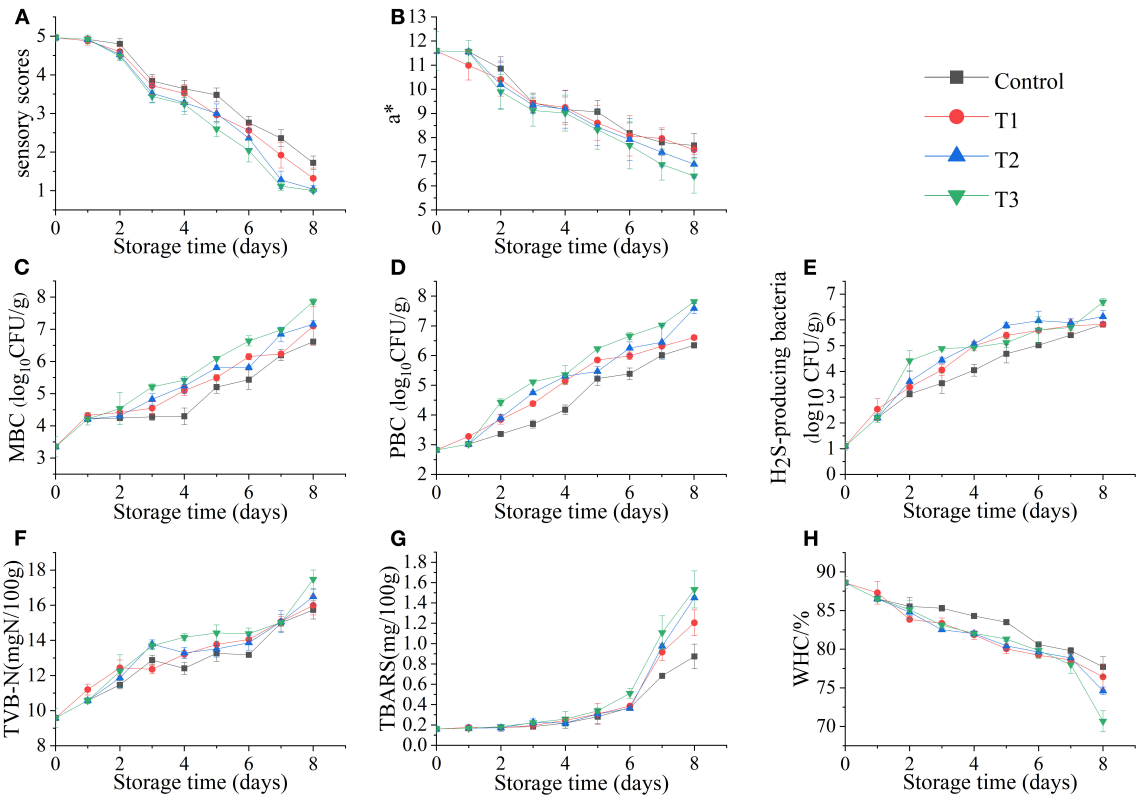

FIGURE 2 | Sensory score **(A)**, redness value (a*) **(B)**, total mesophilic bacteria count (MBC) **(C)**, total psychrotrophic bacteria count (PBC) **(D)**, hydrogen sulfide–producing bacteria count **(E)**, total volatile basic nitrogen (TVB-N) values **(F)**, thiobarbituric acid–reactive substances (TBARS) values **(G)**, and water-holding capacity (WHC) **(H)** of salmon filets under four different storage conditions.

rapidly decreased because of the temperature fluctuation, and the score was also significantly lower than that of the control group.

Determination of Redness Value (a*)

The fresh salmon filets exhibit bright red in color due to the high astaxanthin content. The change in redness value (a*) of salmon filets is shown in **Figure 2B**. The color of the four groups of fish samples changed significantly with the increase of time. Among them, the colors of T2 and T3 became significantly darker; especially the muscle of T3 was not bright. The redness value decreased with the increasing time, which might be due to the oxidation of astaxanthin (17). The color of salmon filets during storage is related to the astaxanthin state (18). And the decrease in the redness value was also one of the reasons for the decrease in sensory scores.

Microbiological Analyses

The changes in total mesophilic bacteria count (MBC), total psychrotrophic bacteria count (PBC), and H_2S-producing bacteria count in salmon filets stored at different storage conditions are shown in **Figures 2C–E**. In this study, the initial number of MBC was low [<4 log_{10} CFU/g] before the salmon filets were subjected to different treatments, indicating that the fish filets were fresh. It is shown that the MBC count of the control group was significantly lower than that of the other three groups, whereas the number of the T3 group was significantly higher, and the growth rates and numbers of the T1 and T2 groups were closer. The T3 group first reached the microbial limit [≤ 7 log_{10} CFU/g, ICMSF (19)] during storage. On the 8th day, the T3 group had reached 7.9 log_{10} CFU/g, and the T1 and T2 groups reached 7.1 and 7.2 log_{10} CFU/g, respectively, whereas the control group reached 6.6 log_{10} CFU/g, which did not exceed the microbial limit. The rapid reproduction of microorganisms should be caused by temperature fluctuations (20).

The psychrotrophic microorganisms are one of the main microorganisms in fishery products during refrigeration (21). It can be seen from **Figure 2D** that the growth trend of PBC is similar to that of MBC. The T3 group was significantly higher than the other three groups. Similarly, on the 8th day, the PBC of T3 was the highest, reaching 7.8 log_{10} CFU/g, followed by the T2 group also reaching 7.6 log_{10} CFU/g, whereas the control and T1 groups reached only 6.3 and 6.6 log_{10} CFU/g, respectively. The results confirmed that temperature fluctuations would accelerate bacterial growth in seafood.

Most H_2S-producing bacteria found in fishery products are gram-negative bacteria, such as *Shewanella* spp., *Serratia* spp., *Aeromonas* spp., etc. They can produce trimethylamine (TMA) and H_2S and amino acid decarboxylation and proteolytic activity

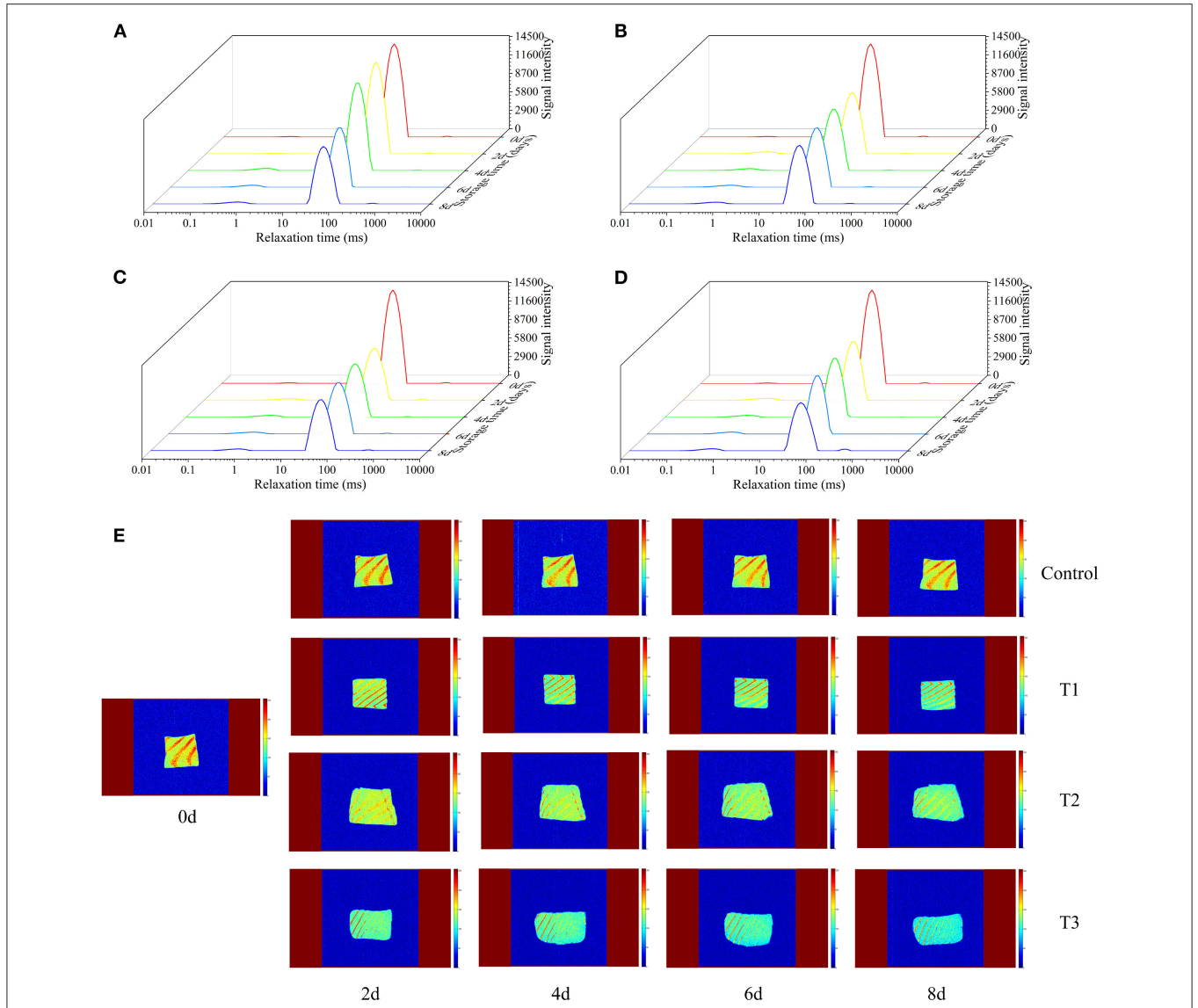

FIGURE 3 | Transverse relaxation time (T_2) of salmon filets under four different storage conditions **(A–D)** and magnetic resonance imaging (MRI) of salmon filets under four different storage conditions **(E)**.

(22). The counts of H_2S-producing bacteria in salmon filets under four storage conditions also increased with storage time, reaching the highest value on the 8th day. The T2 and T3 groups were 6.1 and 6.7 $\log_{10}$ CFU/g, respectively, whereas the control and T1 groups were only 5.8 $\log_{10}$ CFU/g.

Determination of TVB-N and TBARS

The TVB-N value is used as one of the important indicators to evaluate the freshness of fishery products (23). As shown in **Figure 2F**, the TVB-N value of salmon increased during four different storages. The TVB-N value of the T3 group was significantly higher than the other three groups, reaching 17.48 mg N/100 g at the end of storage, followed by the T2, T1, and the control groups in descending order. The increase of TVB-N is mainly related to the activities of spoilage microorganisms and endogenous enzymes in fish (24). The decomposition of protein and some non-protein nitrogen compounds in salmon will become basic nitrogen-containing substances, including ammonia, monoethyl amine, dimethylamine, and TMA, which are also contributing to the odor changes (25).

The TBARS value is one of the important indexes to evaluate the degree of lipid oxidation (26). Because salmon is rich in fat and PUFAs, the lipid oxidation may play an important role in deterioration by producing unpleasant odors and yellowish color (7). As shown in **Figure 2G**, the TBARS value of salmon increased slowly in the first 6 days, but rapidly in the following 2 days, indicating that lipid oxidation was accelerated at this time. After

TABLE 1 | The total fatty acids composition and relative contents of salmon filets under four different storage conditions.

Fatty acid (g/100 g lipids)	Storage time (days)								
	0 d	Con (4 d)	Con (8 d)	T1 (4 d)	T1 (8 d)	T2 (4 d)	T2 (8 d)	T3 (4 d)	T3 (8 d)
C12:0	0.1001	0.1084	0.1079	0.1193	0.0887	0.1084	0.0850	0.1152	0.0934
C13:0	0.0149	0.0156	0.0157	0.0166	0.0130	0.0181	0.0119	0.0137	0.0122
C14:0	2.2740	2.4776	2.4339	2.5620	2.0638	2.5555	1.8728	2.2322	1.9701
C14:1	0.0169	0.0312	0.0299	0.0321	0.0248	0.0308	0.0243	0.0283	0.0248
C15:0	0.2054	0.2417	0.2262	0.2381	0.1822	0.2392	0.1712	0.2088	0.1735
C16:0	12.3576	13.7579	13.5406	14.0297	11.4503	14.2928	10.7844	12.3254	10.7667
C16:1	3.0404	3.4349	3.2523	3.2391	2.8445	3.3210	2.4298	3.0094	2.6674
C17:0	0.3845	0.1947	0.1271	0.1593	0.0985	0.1789	0.1217	0.0906	0.4001
C17:1	0.1674	0.1260	0.1643	0.1784	0.1011	0.1887	0.1285	0.0760	0.1262
C18:0	4.5077	4.1606	3.4166	5.1688	4.1354	5.3129	3.9444	4.9316	4.1437
C18:1n9t	1.0567	0.9374	1.0206	1.1408	0.8064	1.2961	0.8763	0.8766	0.8664
C18:1n9c	34.5058	38.2825	37.3927	38.7214	32.5276	39.9953	29.5512	35.5687	30.5482
C18:2n6t	0.0479	0.0473	0.0741	0.0517	0.0439	0.0492	0.0266	0.0405	0.0331
C18:2n6c	17.7321	19.5138	19.0450	19.4899	16.5007	20.1636	14.9660	18.4532	15.6196
C20:0	0.4547	0.5338	0.4994	0.5583	0.4839	0.5113	0.3930	0.5333	0.4443
C18:3n6	0.2876	0.3393	0.3342	0.3514	0.3023	0.3494	0.2655	0.3390	0.3133
C20:1	5.2859	6.8235	6.1600	6.3603	6.0048	5.8658	4.9388	6.5716	5.3864
C18:3n3	3.4125	2.6435	3.1178	3.4409	2.0603	4.2061	2.5046	2.3758	2.3114
C21:0	0.0592	0.0670	0.0676	0.0759	0.0535	0.0520	0.0552	0.0645	0.0663
C20:2	1.9982	2.2326	2.0781	2.2625	1.9157	2.3525	1.7495	2.0407	1.7831
C22:0	0.2488	0.2745	0.2950	0.3244	0.2407	0.3228	0.2396	0.2575	0.2445
C20:3n6	0.6268	0.6862	0.6707	0.7027	0.5740	0.7035	0.5706	0.6497	0.5518
C22:1n9	0.3500	0.3948	0.3845	0.4113	0.3131	0.4173	0.3039	0.3734	0.3063
C20:3n3	0.6300	0.6487	0.6324	0.8561	0.5864	0.7823	0.6657	0.6309	0.5581
C20:4n6	0.4087	0.4770	0.4636	0.4895	0.3889	0.5001	0.4254	0.4628	0.3714
C23:0	0.0669	0.1059	0.0889	0.0883	0.0578	0.0781	0.0612	0.0757	0.0521
C22:2	0.2327	0.2560	0.2436	0.2789	0.2174	0.2674	0.2080	0.2390	0.2073
C20:5n3	1.8999	2.0425	1.9975	2.1745	1.9760	2.4936	1.8084	2.1709	1.7642
C24:1	0.2911	0.3606	0.3512	0.4076	0.2884	0.3889	0.2977	0.3259	0.2678
C22:6n3	3.7744	4.1343	4.0585	4.1417	3.2119	4.0720	3.7799	3.6447	3.1348
ΣSFA	20.6738	21.9377	20.8189	23.3407	18.8678	23.6700	17.7404	20.8485	18.3669
ΣMUFA	44.7142	50.3909	48.7555	50.4910	42.9107	51.5039	38.5505	46.8299	40.1935
ΣPUFA	31.0508	33.0212	32.7155	34.2398	27.7775	35.9397	26.9702	31.0472	26.6481
EPA+DHA	5.6743	6.1768	6.0560	6.3162	5.1879	6.5656	5.5883	5.8156	4.8990

8 days of storage, the TBARS values of T2 and T3 groups reached 1.45 and 1.53 mg/100 g, respectively, and the control and T1 groups reached 0.87 and 1.20 mg/100 g, respectively. The TBARS values of the temperature fluctuation group were significantly higher than that of the control group, indicating the temperature fluctuation accelerated the lipid oxidation of salmon.

Determination of WHC

The WHC of salmon filets decreased with storage time under different storage conditions (**Figure 2H**). The WHC of fresh salmon was 88.59%, and the WHC of four storage conditions decreased with the increasing time, especially at the end of storage. The WHC of the T3 group decreased to 70.67% on the 8th day, and those of the control, T1, and T2 groups were 77.71, 76.41, and 74.61%, respectively. The oxidation and decomposition of proteins could be the main cause of this phenomenon (27).

Transverse Relaxation Time (T_2) of LF-NMR

The transverse relaxation time (T_2) of the LF-NMR technique can determine the changes in water distribution and water migration of salmon filets under different storage conditions. The original contributions presented in the study are publicly available. These data can be found at https://doi.org/10.6084/m9.figshare.12610628.v1. According to the results of T_2 shown in **Figures 3A–D**, T_2 is closely related to the three states of salmon filets at different relaxation times: T_{21} (0.1–1 ms) represents bound water, which is closely related to proteins and other biological macromolecules, T_{22} (43–50 ms) represents the trapped water mainly existing in the tertiary or quaternary structure of the protein or existed between myofibrils and

TABLE 2 | Main volatile organic compounds (VOCs) and their relative contents (chromatographic peak area × 10^{-6}) in salmon filets under four different storage conditions.

VOCs	Relative concentration (Area × 10^{-6})								
	0 d	Con (4 d)	Con (8 d)	T1 (4 d)	T1 (8 d)	T2 (4 d)	T2 (8 d)	T3 (4 d)	T3 (8 d)
Alcohols									
1-Penten-3-ol	43.47	45.53	53.45	56.23	36.98	52.16	37.42	91.51	47.19
1-Pentanol	7.26	—	—	—	—	—	—	—	—
2-Penten-1-ol, (*Z*)-	11.24	27.07	16.94	23.13	—	35.52	8.13	38.71	15.02
1-Hexanol	1.55	2.55	8.81	3.36	8.91	4.70	10.78	13.08	3.47
1-Octen-3-ol	23.26	77.91	67.76	37.24	43.25	54.11	27.19	89.97	31.34
2-Octyn-1-ol	20.00	57.35	42.92	27.71	28.36	23.51	16.36	71.71	19.38
2-Hexadecanol	0.86	—	—	0.98	1.80	0.51	—	0.94	—
1-Heptanol	24.34	20.28	13.17	8.39	—	—	—	14.97	—
2-Ethyl-1-hexanol	—	—	13.82	7.07	12.17	—	11.20	—	4.89
3-Methyl-1-butanol	—	—	59.91	—	80.44	—	80.83	—	66.23
Phenylethyl alcohol	—	—	7.16	—	58.72	—	31.57	—	10.36
Aldehydes									
Hexanal	140.02	261.46	264.38	276.77	230.33	382.05	190.76	438.40	183.94
Heptanal	70.98	48.27	57.81	34.81	58.68	62.67	27.86	89.82	25.49
Benzaldehyde	12.10	15.97	72.14	31.29	51.24	30.60	42.53	46.79	37.00
Octanal	16.24	33.46	29.57	32.15	25.65	31.79	13.63	32.58	13.25
Nonanal	73.74	95.19	68.75	115.30	70.63	113.40	35.32	110.90	31.67
2,6-Nonadienal, (*E*, *Z*)-	1.76	—	—	—	—	—	—	—	—
Decanal	5.49	6.02	4.15	8.92	5.38	9.34	5.66	4.83	2.61
Propanal	—	6.46	5.27	25.68	10.11	22.70	7.90	—	—
Benzeneacetaldehyde	—	—	26.17	—	36.48	—	28.23	—	29.56
Ketones									
2,3-Pentanedione	7.52	—	6.93	—	8.30	39.53	8.32	—	8.08
2,3-Octanedione	46.68	6.17	30.58	12.11	20.70	16.01	11.20	30.45	12.78
2-Butanone	4.25	—	—	—	—	—	—	—	—
2,3-Butanedione	—	—	—	—	14.52	—	13.84	—	16.81
Esters									
Acetic acid isopentyl ester	—	—	—	—	—	—	—	—	2.39

— *Indicates not detected.*

membranes, and T_{23} (464–1,418 ms) represents the free water that has the longest relaxation time and exits outside the myofibrillar fibers (28). The changes of T_{21} were not obvious with the increasing storage time. As the storage time increases, the signal intensity of T_{22} decreased significantly, especially in the T3 group after 6 days of storage. On the other hand, the signal intensity of T_{23} increased rapidly, especially after 6 days of storage. This phenomenon indicated that the trapped water altered to free water during storage, due to the destruction of myofibril structure (29). The changes of T_{22} and T_{23} in the T3 group were more significant than other groups, in accordance with the changes of the TVB-N and TBARS values and bacterial counts.

Proton MRI

MRI was used to visualize the spatial distribution of moisture in salmon samples under four different storage conditions. In the MRI scan, red (bright) corresponds to regions of high water/proton density, and blue (dark) corresponds to regions of low water/proton density. Fresh salmon filets had the strongest internal signals and the highest moisture content, mainly red and bright yellow. As shown in **Figure 3E**, with the increasing storage time, the MRI scans of the four groups were gradually changed from bright yellow to blue, and the colors of the T2 and T3 groups were significantly bluer than the other two groups, indicating the decreased of moisture content, corresponding to the previously mentioned changes of T_{22}. Temperature fluctuations may promote the migration of water. More water migrates to the outside of the myofibrils, promoting the degradation and denaturation of proteins, resulting in the quality of salmon to decline faster.

Fatty Acid Profile

The composition and content of fatty acids in salmon are shown in **Table 1**. A total of 30 fatty acids were detected in salmon filets, including 11 saturated fatty acids (SFAs), eight monounsaturated fatty acids (MUFAs), and 11 PUFAs. The content of unsaturated fatty acids (UFAs) in salmon accounted for about 79% of the

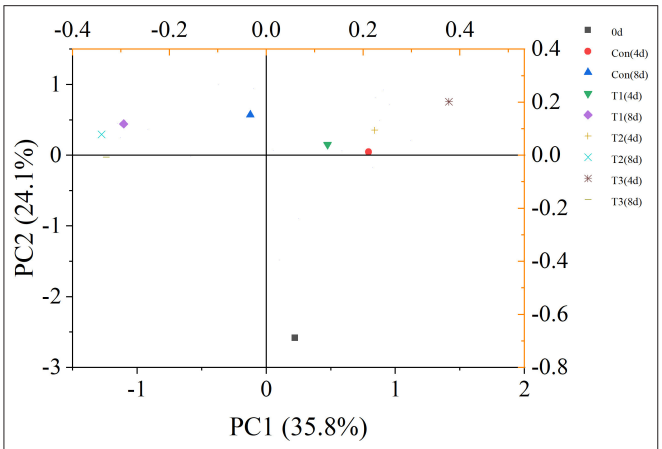

FIGURE 4 | Principal component analysis (PCA) score plot of volatile organic compounds (VOCs) of salmon filets under four different storage conditions.

total fatty acids. Generally, the higher the content of UFAs, the higher the nutritional value of the food is (6). Among the UFAs in salmon, PUFA accounts for about 32% of the total fatty acids, and MUFA accounts for about 47%. The most abundant fatty acid in salmon was oleic acid (C18:1n9c), followed by linoleic acid (C18:2n6c) and palmitic acid (C16:0).

During storage, the contents of total fatty acids, ΣSFA, ΣMUFA, and ΣPUFA of samples on the 8th day were all lower than those on the 4th day. The fatty acid content in the control samples was more stable than other groups, whose total fatty acids content decreased only by 2.90% when compared to the results on the 8th day with the 4th day. A more significant decrease in fatty acids was found in T2 and T1 groups. The predominant decreased fatty acids in all groups were oleic acid (C18:1n9c), linoleic acid (C18:2n6c), and palmitic acid (C16:0). Linolenic acid (C18:3n3) decreased by more than 40% in T2 and T1 group (4d VS 8d), but it did not change much in the T3 group. It was indicated that the degradation of fatty acid was correlated with the temperature and frequency of temperature fluctuation. The fatty acid is an important precursor of volatile compounds including aldehydes and ketones, leading to odor formation. Therefore, the VOCs were also detected in this study.

Determination of VOCs

The formations of VOCs are usually contributed by bacterial metabolisms, enzymatic reactions, or lipid auto-oxidation products (30). A total of 25 VOCs were identified in this study, including eleven alcohols, nine aldehydes, four ketones, and one ester (**Table 2**).

Among the volatile alcohol compounds of salmon filets, 1-penten-3-ol, 2-penten-1-ol, (*Z*)-, 1-hexanol, 1-octen-3-ol, and 2-octyn-1-ol increased during storage. 1-Penten-3-ol and 2-octyn-1-ol were the most abundant compounds in salmon filets, which are related to lipid oxidation (mainly caused by the oxidation of UFAs and arachidonic acid by lipoxygenase (31–33). 1-Octen-3-ol is one of the most important contributors of rancid off-flavors because of its low sensorial threshold value so that a little increment was enough for the contribution of lower sensory scores (34).

TABLE 3 | Pearson correlation analysis and levels of significance for correlations between TBARS values, fatty acid, and VOCs with T_{21}, T_{22}, and T_{23}.

Parameter	T_{21}	T_{22}	T_{23}
TBARS	0.477	0.574	−0.574
C16:0	−0.145	−0.521	0.216
C18:0	−0.062	−0.875**	0.343
C18:1n9c	−0.147	−0.576	0.309
C18:2n6c	−0.188	−0.575	0.378
C20:1	−0.074	−0.398	0.648
ΣSFA	−0.189	−0.691*	0.279
ΣMUFA	−0.135	−0.567	0.348
ΣPUFA	−0.172	−0.612	0.250
EPA+DHA	−0.022	−0.592	0.251
1-Penten-3-ol	0.079	−0.689*	0.837**
Hexanal	0.381	−0.807**	0.673*
Heptanal	−0.224	−0.337	0.703*
Nonanal	−0.041	−0.794*	0.595
2,3-Octanedione	−0.503	0.071	0.225

*Levels of significance are defined as *$p < 0.05$ and **$p < 0.01$.*

The highest content of aldehydes was hexanal in fresh salmon, followed by heptanal and nonanal. Hexanal and nonanal were related to the oxidation of linoleic acid and PUFA (35, 36), which were important contributors to meat flavor. In addition, in the later stage of storage, the content of benzaldehyde also increased significantly, and benzeneacetaldehyde also accumulated in the later stage of storage, which was related to the spoilage of salmon filets.

The ketones in salmon filets were much lower than alcohols and aldehydes. The mainly detected compounds of ketones were 2,3-pentanedione, 2,3-octanedione, 2-butanone, and 2,3-butanedione. 2,3-Octanedione was the predominant ketone in fresh salmon, and its content decreased with the increasing storage time. The content of 2,3-butanedione increased significantly during storage, which might be contributed by the metabolic activity of *Pseudomonas* (37).

The principal component analysis showed that VOC variations of different salmon samples were 35.8% [principal coordinates 1 (PC1)] and 24.1% [principal coordinates 2 (PC2)] (**Figure 4**). The dots of samples were generally distributed in three different quadrants by different storage time. The dot representing fresh salmon filet was far away from other samples. Therefore, the volatile compounds of fresh salmon were significantly different from the samples during storage. The dot of T3 samples on day 4 was also distinguished from other three groups, but the dots of samples became closer to each other after 8 days of storage, indicating that the differences of the content of VOCs between every four groups were much obvious on the 4th day than on the 8th day.

Relationship Between TBARS Values, Fatty Acid, VOCs, and Water Migration

Table 3 showed the correlation between TBARS values, fatty acids (C16:0, C18:0, C18:1n9c, C18:2n6c, C20:1, ΣSFA, ΣMUFA, ΣPUFA, and EPA + DHA), VOCs (1-penten-3-ol, hexanal, heptanal, nonanal, and 2,3-octanedione), and water migration in salmon filets. The relationship between T_{21} and the TBARS value, fatty acids, and VOCs was quite low, which is closely bound to proteins and has little impact on biological reactions (29). T_{22} was negatively significantly correlated ($p < 0.05$) with octadecanoic acid, 1-penten-3-ol, hexanal, and non-anal. In addition, T_{23} was positively correlated ($p < 0.05$) with 1-penten-3-ol, hexanal, and heptanal. During storage, the trapped water (T_{22}) gradually altered to be free water (T_{23}) because of the loss of WHC of myofibril protein. The migration of intramyofibrillar water might also lead to the leakage of enzymes such as lipase, lipoxygenase, and protease, which accelerated the degradation and oxidation of fatty acid and proteins and the proliferation of bacteria (38). Therefore, the results indicated that water dynamics had a significant correlation with the fatty acids and VOCs, which could be used to assess and detect the quality changes of salmon.

CONCLUSIONS

This study showed that the sensory score, redness value, and WHC of salmon filets under temperature fluctuation decreased faster than that of filets stored at constant temperature (0 and 4°C), whereas the MBC, PBC, H_2S-producing bacteria count, TVB-N, and TBARS values gradually increased. LF-NMR ^{1}H also showed that water migration was more significant because of temperature shifts. Meanwhile, the fatty acid degraded, and some VOCs including 1-penten-3-ol, hexanal, and 2,3-butanedione contributing to unpleasant rancid flavor increased, which could be a potential indicator of poor quality in salmon. The relationship between T_{22} or T_{23} and other quality indicators had a significant correlation, indicating that LF-NMR ^{1}H is a potential non-destructive method to evaluate the quality of seafood. The acceleration of fatty acid degradation and oxidation was observed in samples that suffered frequent temperature fluctuations, and the water migration accelerated the degradation and oxidation of fatty acids. Therefore, the temperature fluctuation during cold chain logistics should be avoided to maintain the nutrients and freshness of salmon.

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/s.

AUTHOR CONTRIBUTIONS

JX: conceptualization, project administration, and funding acquisition. Y-JY and Y-FQ: methodology. Y-JY: writing—original draft preparation, software, data curation, and visualization. Y-JY, S-PY, and TL: investigation. Y-JY, JX, and CH: validation. JX and Y-FQ: formal analysis, resources, and supervision. Y-FQ: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Colloidal Particles in Tuna Head Soup: Chemical Localization, Structural Change, and Antioxidant Property

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In this work, chemical localization, structural changes, and antioxidant properties of tuna colloidal particles (TCPs) in boiling tuna head soup were examined. The results demonstrated that TCPs might be core–shell colloidal spherical structures. The hydrophobic core consisted of triglycerides and chloride ions. The hydrophilic shell layer consisted of chloride ions, sodium ions, phospholipids, protein, and glycosyl molecules. Coalescence of TCPs occurred during the boiling process, and water may enter the hydrophobic core of TCPs after the boiling time of 60 min. TCPs had excellent antioxidant properties against H_2O_2-induced human umbilical vein endothelial cell injury. It might be resulted from that TCPs could decrease cell apoptosis proportion and downregulate mRNA levels of endoplasmic reticulum-bounded chaperone protein glucose-related protein (GRP78), C/EBP homologous protein (CHOP), and activating transcription factor-4 (ATF4). This work can provide useful basic information to understand the colloidal system in foods, especially in soup. In addition, it may also promote the potential high-value-added utilization of aquatic by-products.

Keywords: antioxidant property, core-shell structure, human umbilical vein endothelial cells, particle size, tuna colloidal particles

INTRODUCTION

Tuna is a diverse family of marine fish and widely distributed in the tropical and subtropical waters of the major oceans such as the North Atlantic and Indian Ocean (1). Among the tuna family, bigeye tuna (*Thunnus obesus*) is one of the major species on the global tuna market (2). Tuna is rich in protein, vitamins, minerals, and omega-3 unsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Omega-3 unsaturated fatty acids can lower blood lipids, regulate biochemical and physiological reactions, activate brain cells, enhance memory, promote brain development in infants, increase anti-inflammatory abilities, reduce the risk of coronary diseases, and prevent certain cancers (3, 4).

Tuna is commonly processed into raw sashimi and steak. The processing generates many by-products including head, viscera, gills, dark flesh, skin, and bone. These by-products compose almost 50–70% weight of the original tuna. High-value-added utilization of these by-products has attracted much attention in the field of food and aquatic products. Fish by-products have been explored and applied to produce fish oil, fishmeal, fertilizer, fish silage, profitable bioactive

compounds (i.e., bioactive peptides, oligosaccharides, fatty acids, enzymes, and water-soluble minerals) (5). Tuna by-products have also been explored and applied to produce fish oil (6), fishmeal (7), antioxidative peptides (8), collagen (9), sulfated polysaccharides (10), etc. These works significantly promoted the research and development of high-value-added utilization of tuna by-products. However, more researches are needed to deeply and widely explore the high-value-added utilization of tuna by-products.

Soup is one of the most popular diets in the world because of its significant advantages such as convenient processing, flavorful taste, and easy absorption to people (11). Soup can dissolve and retain many food nutrients from the food materials. It is especially suitable for the elderly, children, infants, and the infirm. Therefore, tuna head soup may be a simple way for high-value-added utilization of tuna head. Migration of nutritional components (e.g., lipid, protein, sugar, and other components) in tuna head soup and the effect of processing methods on them are necessary to be illustrated, which will be helpful to understanding the nutritional mechanism of tuna head soup for human beings. Our previous works found that micro/nanosized colloidal particles were formed in Atlantic salmon, bighead carp, and tuna head soups (12–14). After a series of chemical and physical reactions in the boiling process of tuna head soup, the chemical components included not only the original components migrated from the tuna head but also some newly formed components. These components could self-assemble to form micro-nano-colloidal particles during the boiling process. Further works are needed to deeply analyze the chemical localization and structural stability of colloidal particles during the boiling process and their potential antioxidant properties for human health.

Human umbilical vein endothelial cell (HUVEC) damage has been recognized to be associated with cardiovascular diseases. The simulation of H_2O_2, one of the most widely studied reactive oxygen species, or its intracellular production is responsible for the activation or deregulation of various signaling pathways, and participate in the initiation and development of cardiovascular diseases such as hypertension and atherosclerosis (15, 16). Therefore, H_2O_2-induced oxidative injury of HUVECs is a good cell model to study the antioxidant properties of some newly found substances (17–19).

Here, we examined the chemical localization and structural changes of tuna colloidal particles (TCPs) in boiling tuna head soup by laser scanning confocal microscopy, laser light scattering technique, and transmission electron microscopy. Further, we analyzed the antioxidant properties of TCPs against H_2O_2-induced oxidative injury of HUVECs using CCK-8 assay, real-time PCR, and flow cytometry assay. This work provided useful basic information to understanding the colloidal system in foods, especially in soup.

MATERIALS AND METHODS

Preparation of Tuna Head Soup

Tuna head soups were prepared according to our previous work with minor revision (12). Frozen half bigeye tuna heads (length: 27 ± 2 cm; width: 26 ± 2 cm; weight: 1.5 ± 0.3 kg; Dalian Xiangxiang Food Co., Ltd., Liaoning, China) were defrosted using a running-water thawing method for 1 h. Then, the tuna heads were washed with ultrapure water and cut into small pieces (5 × 3 × 2 cm). The tuna head pieces were drained to remove the ultrapure water. Subsequently, 400-g tuna head pieces were fried in 20 g cooked soybean oil at 120°C for 30 s on a home-use induction cooker (2,400 W). Then, 3,200 mL ultrapure water was immediately added to the fried tuna head pieces. After 10.5 min, the water was boiled. The tuna head piece–water mixtures were kept boiled at the temperature of 90°C for 150 min. The control sample was prepared using the same procedure without the addition of tuna head pieces. At the designed time points, 50 mL of soup was taken out and filtered for below research. Six parallel experiments were done for each sample.

Laser Scanning Confocal Microscopic Observation of TCPs

The LSCM observation was performed according to our and others' previous works with minor revision (20–24). Nile Red is a selective stain for lipids. N-[Ethoxycarbonylmethyl]-6-methoxy-quinolinium bromide (MQAE) is a fluorescent indicator for intracellular chloride ions (Cl^-). CoroNa™ Green is a sodium ion indicator that exhibits an increase in green fluorescence emission intensity upon binding sodium ions (Na^+), with little shift in wavelength. Therefore, Nile Red, MQAE, and CoroNa™ Green could specifically stain TG, chloride ions, and sodium ions, respectively. Rd-DOPE is a polar head group-labeled phospholipid probe. Nile Blue (Shanghai Macklin Biochemical Co., Ltd., China) might be in neutral form and could only bind to protein. Wheat germ agglutinin Alexa Fluor 488 (WGA488) can bind to sialic acid and N-acetylglucosaminyl residues to stain glycosyl molecules. Therefore, Rd-DOPE, Nile Blue, and WGA488 could specifically stain phospholipids (PL), proteins, and glycosyl molecules (GM), respectively.

In this work, freshly prepared tuna colloidal particle solutions (1 mL) were mixed with 100 μL of Nile Red (Sangon Biotech Shanghai Co., Ltd., Shanghai, China), 40 μL of MQAE (MedChemExpress, USA), and 40 μL of CoroNa™ Green (Thermo Fisher Scientific, USA) to stain the triglycerides, chloride ions, and sodium ions, respectively. Freshly prepared tuna colloidal particle solutions (1 mL) were mixed with 20 μL of Rd-DOPE (Avanti Polar Lipids, Inc. Alabaster, AL, USA), 10 μL of Nile Blue (Sangon Biotech Shanghai Co., Ltd., Shanghai, China), and 10 μL of WGA488 (Biotium, San Francisco, CA, USA) to stain the phospholipids, proteins, and glycosyl molecules, respectively. These samples were incubated in the dark for 30 min. Then, 10 μL of the stained samples was added onto glass microslides and square cover glasses were placed onto the samples. Finally, these samples were observed using a LSCM microscope (Zeiss LSM 710, Carl Zeiss, Jena, Germany) with a 63× (NA 1.4) oil immersion objective. LSCM observation was performed using an argon laser (excitation wavelength of 488 nm, emission wavelength of 500–535 nm), a He–Ne laser (excitation wavelength of 543 nm, emission wavelength of 565–615 nm), and a diode laser (excitation wavelength of 633 nm, emission wavelength of >650 nm).

Size Distribution of TCPs

The size distribution of TCPs in the soup was measured by laser light scattering technique using a Mastersizer 2000 instrument (Malvern, UK). The size range was set from 0.020 to 2,000 μm. The refractive indices of 1.520 and 1.330 were used for the particle and dispersant, respectively (25, 26). Standard parameters were calculated by the software (27): the surface weighted mean [$d_{32} = \sum v_i / \sum (\frac{v_i}{d_i})$, where v_i is the volume of particles in a size class of average diameter of d_i]; the volumic weighted mean [$d_{32} = \sum (v_i \times d_i) / \sum v_i$]; the specific surface area ($= 6 \times \rho^{-1} \times {d_{32}}^{-1}$, where ρ is the solution density); and the span distribution [$span = (d_{0.9} - d_{0.1})/d_{0.5}$, where $d_{0.1}$, $d_{0.5}$, and $d_{0.9}$ are the diameters below which lie 10, 50, and 90%, respectively, of the particle volumes].

Transmission Electron Microscopic Observation of TCPs

Freshly prepared tuna colloidal particle solutions were centrifuged at 3,821× g for 2 min at 4°C in an H1850R Table Top High-Speed Centrifuge (Hunan Xiangyi Laboratory Instrument Development Co. Ltd., Changsha City, Hunan Province, China). The supernatants were loaded on a 300-mesh copper grid and then were negatively stained by a droplet of 1% phosphotungstic acid. After drying the grid at room temperature for 5 min, the samples were observed by a transmission electron microscope (Tecnai G2 Spirit Bio Twin, FEI company, USA) at 120 kV acceleration voltage (28). The numbers of the black spots per square micron core part (oil phase) in the TEM images were calculated by analyzing three different TEM images from different samples.

Cell Culture and Treatments

HUVECs were acquired from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin–streptomycin liquid (Gibco, USA) at 37°C in a humidified atmosphere with 5% CO_2. The cells were digested with 0.25% trypsin, rinsed with PBS, centrifuged, and harvested in a centrifuge (150 × g at room temperature for 5 min) and resuspended in DMEM. The cells were seeded in 6-well plates at a density of about 1×10^6 cells per well. Then, the cells were cultured at 37°C with 5% CO_2 for 12 h. The cell wells were randomly divided into five groups (six wells per group) and incubated by fresh media with different substances: control group (control sample), H_2O_2 group (50 μM H_2O_2), low-dose group (1 μg/mL TCP + 50 μM H_2O_2), middle-dose group (10 μg/mL TCP + 50 μM H_2O_2), and high-dose group (100 μg/mL TCP + 50 μM H_2O_2). TCPs in tuna head soup at the boiling time of 150 min were applied in this assay. In this process, after 24 h incubation with TCP, the cells were incubated with H_2O_2 for 2 h.

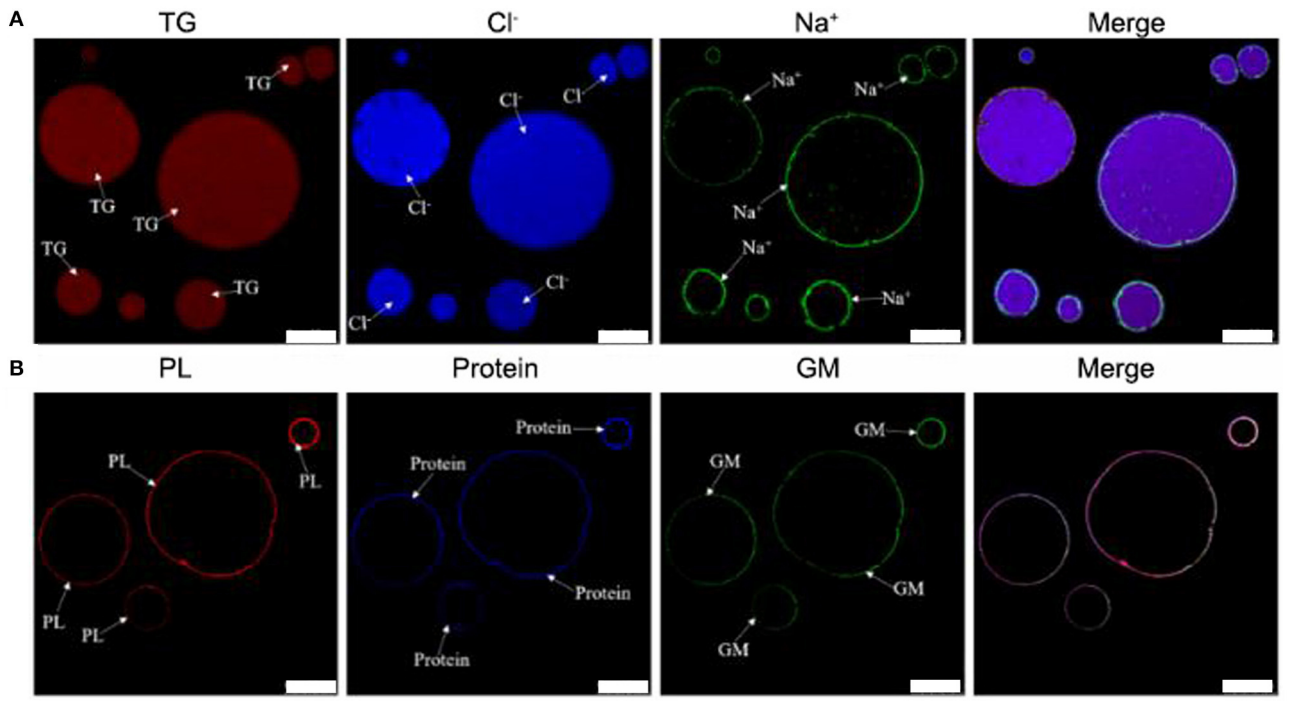

FIGURE 1 | LSCM images of TCPs in tuna head soup at 150 min boiling time. **(A)** Nile Red, MQAE, and CoroNa™ Green were used to stain the triglycerides (TG), chloride ions (Cl^-), and sodium ions (Na^+), respectively. **(B)** Rd-DOPE, Nile Blue, and WGA488 were used to stain the phospholipids (PL), proteins, and glycosyl molecules (GM), respectively. White scale bars indicate 10 μm.

After H_2O_2 incubation, the cells were analyzed by cell viability assay, real-time PCR assay, and flow cytometry assay.

Cell Viability Assay

The cell viability of HUVECs was examined by CCK-8 assay according to previous established methods (29, 30). Briefly, the culture media were removed and fresh media containing 10% CCK-8 solution were added to each well and incubated for 4 h at 37°C. After that, 100 μL of supernatant was transferred into a 96-well plate. The absorbances were measured at 450 nm using a microplate reader (Multiskan MK3, Thermo Labsystems, Finland). Six parallel experiments were performed for each group.

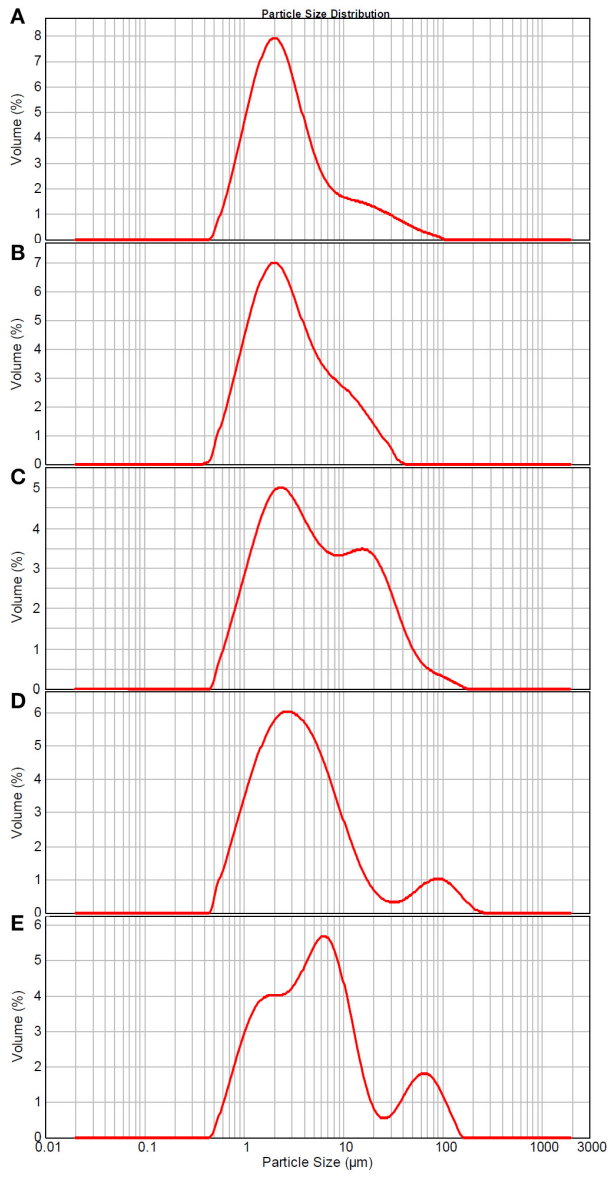

FIGURE 2 | Size distribution of TCPs in tuna head soup at different boiling times. **(A)** 5 min; **(B)** 15 min; **(C)** 30 min; **(D)** 60 min; and **(E)** 150 min.

Real-Time PCR

Total RNAs were isolated using a High Pure RNA Isolation Kit (Roche, Germany) according to the manufacturer's protocol. Then, cDNAs were synthesized using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA). The relative mRNA levels of the genes in HUVECs were measured using a Faststart Universal SYBR Green Master (Roche, Germany) on a LightCycler 480 instrument (Roche, Germany). All quantifications were performed with β-actin as an internal standard. The relative amount of mRNA was calculated using the relative quantification (ΔΔCT) method (31). The relative amount of each mRNA was normalized to that in control samples. Quantitative RT-PCR assay was performed using specific primers against the following target genes (32): GRP78 (forward, 5′-CACGTCCAACCCGGAGAA-3′; reverse, 5′-TTCC AAGTGCGTCCGATGA-3′); CHOP (forward, 5′-ACCAAGGG AGAACCAGGAAACG-3′; reverse, 5′-TCACCATTCGGTCAA TCAGAGC-3′); ATF4 (forward, 5′-AAACCTCATGGGTTCTC CAG-3′; reverse, 5′-GGCATGGTTTCCAGGTCATC-3′).

Flow Cytometry Assay

For apoptosis analysis, quantification of the cells was examined using the Annexin-V-FITC/Propidium Iodide (PI) Apoptosis Detection Kit (BD Company, Franklin Lakes, New Jersey, USA) according to the manufacturer's protocols (33). Briefly, after the treatment, the culture media were removed and the cells were digested with fresh media with 0.25% trypsin, rinsed with cold PBS twice, centrifuged and harvested in a centrifuge (150× g at room temperature for 5 min), and resuspended in fresh media with Annexin-V-FITC and PI at room temperature. After incubation for 15 min in the dark, these samples were analyzed using a flow cytometer (BD, USA).

Statistical Analysis

The data are expressed as the means ± standard deviations (SD). The statistical differences were calculated using the Student's *t*-test. The data were considered statistically significant when $P < 0.05$.

RESULTS

Chemical Localization of TCPs in Tuna Head Soup

Previous work suggested TCPs were micro/nano-sized colloidal structures (12). However, the location of chemical compositions was not systematically analyzed by co-localization technique of LSCM. In addition, the location of sodium and chloride ions was also not analyzed by LSCM. In order to deeply analyze the chemical localization of TCPs in tuna head soup, the co-localization technique of LSCM was applied in this work. As shown in **Figure 1**, the chemical localization of TCPs in tuna head soup at the boiling time of 150 min was analyzed by LSCM. According to the results, triglyceride ions were present in the inner core part of colloidal particles. Sodium ions, phospholipids,

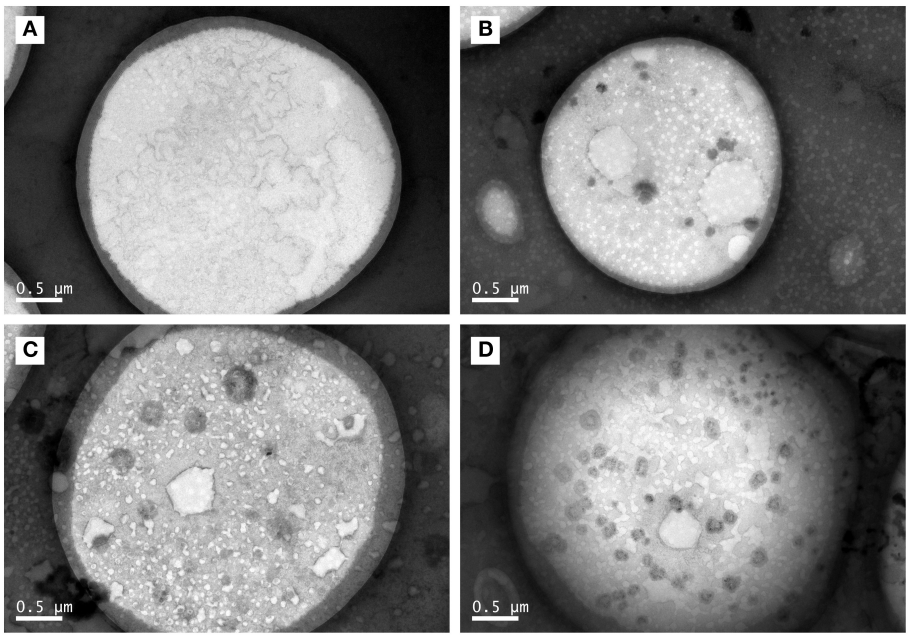

FIGURE 3 | TEM images of TCPs in tuna head soup at different boiling times. **(A)** 30 min; **(B)** 60 min; **(C)** 90 min; and **(D)** 150 min. Scale bars indicate 0.5 μm.

proteins, and glycosyl molecules were mainly present in the periphery part of colloidal particles. Chloride ions were present both in the inner core part and in the periphery part of colloidal particles. Therefore, the TCPs might be core–shell colloidal spherical structures, just like droplet structures in emulsions (20). The core part might be hydrophobic and consisted of TG and chloride ions. The shell layer might be hydrophilic and consisted of chloride ions, sodium ions, phospholipids, protein, and glycosyl molecules.

Size Distribution and Structure Changes of TCPs in Tuna Head Soup During the Boiling Process

The particle size distribution and structure changes of TCPs in tuna head soup during the boiling process were measured by the Malvern size analyzer and TEM, respectively. As shown in **Figure 2**, TCPs with diameters of 0.5–100 μm appeared in tuna head soup at the boiling time of 5 min. With the increase in boiling time, particle size increased and three peaks appeared in the size distribution image at the boiling time of 150 min (**Figure 2**).

As shown in **Figure 3**, the core–shell structures that were suggested by LSCM results (**Figure 1**) were confirmed by representative TEM images. Moreover, some black spots appeared in the core part of TCPs at the boiling time of 60 min and in the shell layer part of TCPs at the boiling time of 90 min. The numbers of the black spots per square micron core part (oil phase) in the TEM images were 0.04 ± 0.06, 1.60 ± 0.67, 2.26 ± 0.41, and 4.13 ± 0.84 for 30, 60, 90, and 150 min, respectively. Therefore, the amounts of black spots increased with the increase of boiling time.

Antioxidant Properties of TCPs Against H_2O_2-Induced HUVEC Injury

In order to analyze the antioxidant properties of TCPs against H_2O_2-induced HUVEC injury, CCK-8 cell viability assay was examined. As shown in **Figure 4A**, the cell viability assay showed that H_2O_2 treatment could induce significant HUVEC injury compared with the control group, which confirmed that H_2O_2-induced oxidative injury of HUVECs is a good cell model to study the antioxidant properties of some newly founded substances (17–19). TCP pretreatments with different concentrations could significantly decrease H_2O_2-induced oxidative injury. Moreover, middle-dose (10 μg/mL TCP + 50 μM H_2O_2) and high-dose (100 μg/mL TCP + 50 μM H_2O_2) groups showed no obvious differences to the control group, which suggested that middle-dose and high-dose TCP pretreatments could completely inhibit H_2O_2-induced oxidative injury. It demonstrated that TCPs had significant antioxidant properties against H_2O_2-induced HUVEC injury.

In this work, mRNA expression levels of GRP78, CHOP, and ATF4 of 10 μg/mL TCP-protected HUVECs against H_2O_2-induced injury were examined by real-time PCR assay (**Figures 4B–D**). H_2O_2 treatment could significantly upregulate these three mRNA levels compared with the control group, which confirmed that H_2O_2-induced oxidative injury of HUVECs was involved in the enhancement of endoplasmic reticulum stress. TCP pretreatments could significantly downregulate these three mRNA levels. However, TCP pretreatments could not decrease the mRNA levels to the values of the control group.

Finally, in order to analyze cell apoptosis, TCP-protected HUVECs against H_2O_2-induced injury were examined by flow cytometry assay (**Figure 5**). H_2O_2 treatment could significantly

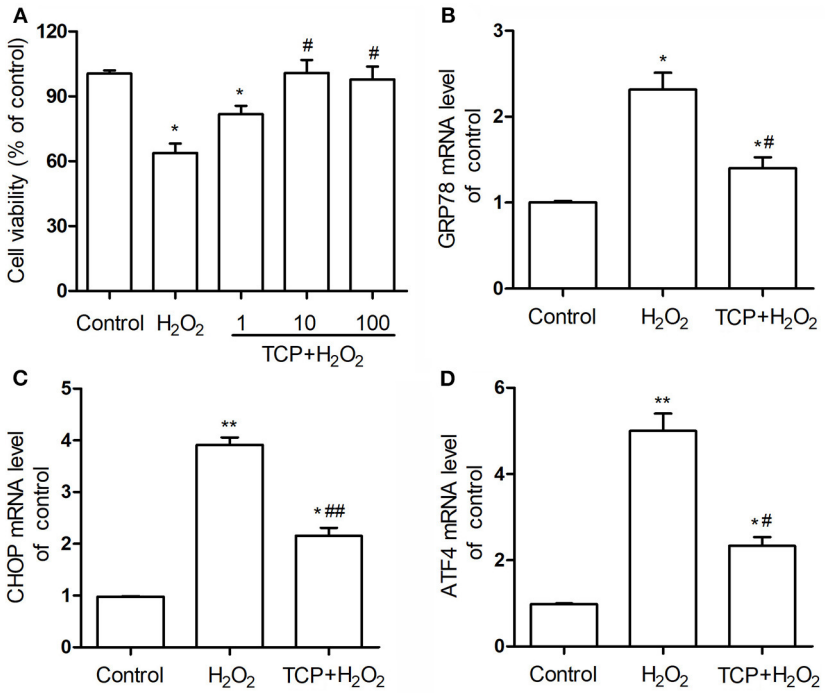

FIGURE 4 | Cell viability **(A)** of TCP-protected HUVECs with different TCP concentrations (1, 10, and 100 μg/mL) and mRNA expression levels of GRP78 **(B)**, CHOP **(C)**, and ATF4 **(D)** of 10 μg/mL TCP-protected HUVECs against H_2O_2-induced injury. The data are expressed as the means ± SD. Statistically significant differences among different groups: $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, compared to the H_2O_2 group. TCPs in tuna head soup at the boiling time of 150 min were applied in this assay.

increase apoptosis proportion (33.1 ± 2.4%) compared with the control group (5.5 ± 1.0%), which confirmed that H_2O_2 treatment could significantly induce cell apoptosis of HUVECs. TCP pretreatments could significantly decrease apoptosis proportion (16.8 ± 2.4%) compared with the H_2O_2 group. However, pretreatment of TCPs could not decrease the apoptosis proportion to the value of the control group, which is similar to mRNA results (**Figure 4**).

DISCUSSION

TCPs were suggested to be micro/nano-sized colloidal structures in previous work (12). However, the chemical localization of TCPs, structural change of TCPs during the boiling process, and antioxidant properties of TCPs have not been studied. In this work, we used LSCM to observe the chemical localization of TCPs (**Figure 1**). Then, we used the Malvern size analyzer (**Figure 2**) and TEM (**Figure 3**) to analyze the particle size distribution and structure changes of TCPs in the tuna head soup during the boiling process. Finally, we used CCK-8 cell viability assay (**Figure 4A**), real-time PCR assay (**Figures 4B–D**), and flow cytometry assay (**Figure 5**).

The LSCM results (**Figure 1**) suggested that the TCPs might be core–shell colloidal spherical structures, just like droplet structures in emulsions (20). It further confirmed a previous conclusion that TCPs were the micro/nano-sized colloidal structures in previous work (12). Moreover, the core part of TCPs might be hydrophobic and consisted of TG and chloride ions. The shell layer of TCPs might be hydrophilic and consisted of chloride ions, sodium ions, phospholipids, protein, and glycosyl molecules.

Malvern size analyzer results (**Figure 2**) showed that TCP particle size increased with the increase of boiling time. Moreover, three peaks appeared in the size distribution image at the boiling time of 150 min. The appearance of three peaks might be coalescence of colloidal particles, which is a common phenomenon of colloidal systems (34–36). Coalescence occurs when two or more colloidal particles come into close proximity and fuse together to form a large colloidal particle. It tends to occur when the attractive forces between two particles are larger than the repulsive forces, and then the interfacial layers rupture and fuse together.

Further, the core–shell structures were confirmed by representative TEM images (**Figure 3**). Moreover, the numbers of the black spots per square micro core part (core phase) in the TEM images increased with the increase of boiling time. LSCM images (**Figure 1**) of TCPs at the boiling time of 150 min showed that no hydrophilic substances (e.g., proteins, phospholipids, glycosyl molecules, sodium ions, and chloride ions) which were distributed into the core part of TCPs. Therefore, these black spots might not be these hydrophilic substances. Generally, in the process of boiling, the water is fully agitated and actively enter TCPs or passively be involved into the particle coalescence. Therefore, we could reasonably assume that the black spots might be water. It should be noted that this phenomenon only

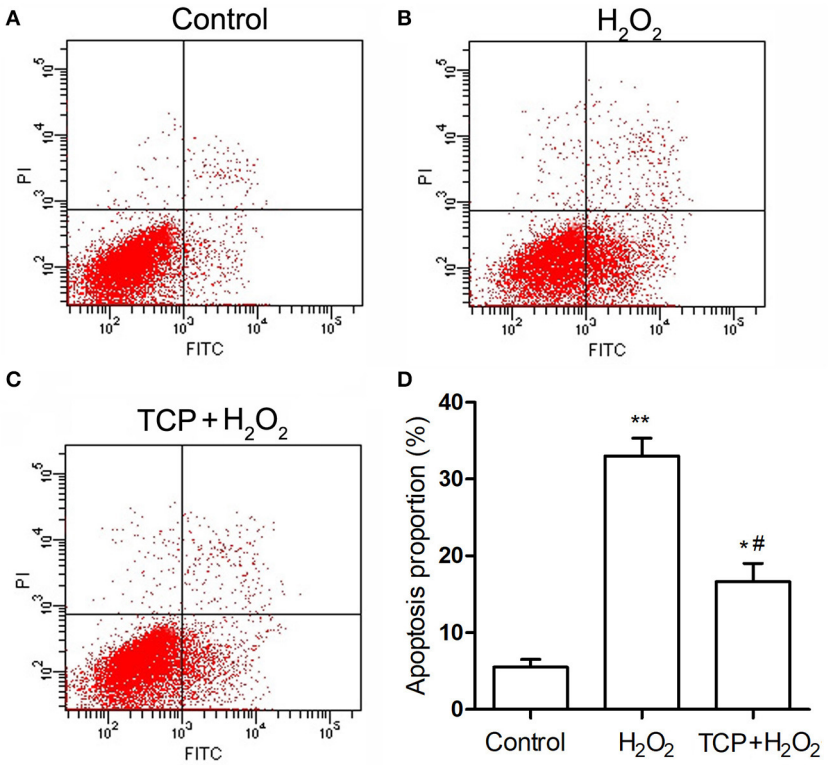

FIGURE 5 | Apoptosis of TCP-protected HUVECs against H_2O_2-induced injury. **(A)** Control group. **(B)** H_2O_2 group. **(C)** 10 μg/mL TCP + 50 μM H_2O_2 group. **(D)** Apoptosis proportion of TCP-protected HUVECs against H_2O_2-induced injury. The data are expressed as the means ± SD. Statistically significant differences among different groups: $^*P < 0.05$, $^{**}P < 0.01$, compared to the control group; $^{\#}P < 0.05$, compared to the H_2O_2 group. TCPs in tuna head soup at the boiling time of 150 min were applied in this assay.

occurred at and after the boiling time of 60 min. Further studies are needed to illustrate the underlying mechanism.

CCK-8 cell viability assay (**Figure 4A**) showed that TCP pretreatments with different concentrations could significantly decrease H_2O_2-induced oxidative injury. As the boiling time increases, the antioxidant performances of colloidal particles are expected to decrease, as they are oxidized over time. Therefore, we only used TCPs in tuna head soup at the boiling time of 150 min in this assay. Therefore, we may predict that TCPs in tuna head soup at the boiling times of 30–150 min had significant antioxidant properties against H_2O_2-induced HUVEC injury.

The physiological functioning of the endoplasmic reticulum is important for most cellular activities and survival. In response to certain stimuli, endoplasmic reticulum stress is activated in pathological vascular remodeling in cardiovascular diseases (37, 38). The excessive or prolonged stress may result in cell apoptosis and injuries (39). The enhanced expression of endoplasmic reticulum-bounded chaperone protein glucose-related protein (GRP78) is induced in response to endoplasmic reticulum dysfunction. Activating transcription factor-4 (ATF-4) is involved in the upstream signaling pathways in the process of endoplasmic reticulum stress. C/EBP homologous protein (CHOP) can initiate apoptotic events in the setting of severe or prolonged endoplasmic reticulum stress (38). Real-time PCR assay (**Figures 4B–D**) showed that TCP pretreatments could significantly downregulate the mRNA levels of GRP78, CHOP, and ATF4. Flow cytometry assay (**Figure 5**) demonstrated that TCP pretreatments could significantly decrease apoptosis proportion compared with the H_2O_2 group. Therefore, antioxidant properties of TCPs against H_2O_2-induced HUVEC injury resulted from the downregulation of mRNA levels of GRP78, CHOP, and ATF4 and significant decrease of apoptosis proportion. Previous work demonstrated that higher levels of oxidative stress contributed to the activation of endoplasmic reticulum stress, which mediated oxidative stress-induced apoptosis via an extrinsic pathway (40). Therefore, the TCP pretreatment might decrease the oxidative stress in HUVECs, which contributed to the deactivation of endoplasmic reticulum stress and further decreased oxidative stress-induced apoptosis.

CONCLUSION

This study analyzed the chemical localization, structural changes, and antioxidant properties of TCPs in tuna head soup. The chemical localization of TCPs was shown by co-localization technique of LSCM. The size distribution and structural changes of TCPs in the boiling process were analyzed by Malvern size analyzer and TEM. These results showed the chemical localization and core–shell structures of TCPs. Moreover, the sizes of TCPs increased with the increase of boiling time. Further, the antioxidant properties of TCPs against H_2O_2-induced HUVEC injury were studied by cell viability assay, real-time PCR

assay, and flow cytometry assay. TCPs had excellent antioxidant properties against H_2O_2-induced cell injury. TCP pretreatments could decrease mRNA levels of GRP78, CHOP, and ATF4 and decrease cell apoptosis proportion and, therefore, increase cell viability. This work provided useful basic information to understanding the colloidal system in foods. In addition, the excellent antioxidant properties of tuna head soup could also promote the potential high-value-added utilization of aquatic by-products.

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

CM: methodology, investigation, and writing—original draft preparation. PL: investigation. NT: conceptualization, project administration, formal analysis, resources, and funding acquisition. XW: conceptualization, project administration, formal analysis, resources, and writing—review and editing. SD: conceptualization and formal analysis. All authors contributed to the article and approved the submitted version.

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Characterization of a Novel Food Grade Emulsion Stabilized by the By-Product Proteins Extracted From the Head of Giant Freshwater Prawn (*Macrobrachium rosenbergii*)

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The aim of this work was to develop a food-grade emulsion that stabilized by the by-product proteins in the head of giant freshwater prawn. The physicochemical properties of protein particles were characterized, and the stability of proteins-stabilized emulsions under different environmental stresses was evaluated. Results showed that the proteins were relatively hydrophilic and preferentially resided in the aqueous phase to form oil/water emulsions. On this basis, the proteins exhibited superior ability to stabilize the emulsions, and remarkably, the emulsions stabilized by 2% proteins and 3:7 oil/water ratio efficiently resisted the freeze-thaw treatment and the change of pH (3–9), salt addition (NaCl, 50–400 mM), and storage temperatures (4–60°C). Furthermore, the emulsions showed a matched long-term stability with the existing biopolymers-stabilized emulsions. Consequently, this is the first finding of the by-product proteins in the head of giant freshwater prawn as an excellent emulsifier to stabilize emulsions, which help to improve the stability of food-grade emulsions and the added value of aquatic products.

Keywords: giant freshwater prawn, by-product, protein-stabilized emulsions, aggregation behavior, long-term stability, freeze-thaw stability

INTRODUCTION

Giant freshwater prawn (*Macrobrachium rosenbergii*) is one of the most significant freshwater species in the world. At present, its annual yield is about 250,000 tons (1). With the giant freshwater prawn farming in China, its aquaculture industry has been developing continuously because of its high commercial value. It is reported that the whole giant freshwater prawn contains 69–71% moisture, 25–26% protein, 1–2% lipid, and about 2% ash and carbohydrates (2).

Giant freshwater prawn is a good source of healthy food for human consumption and animal proteins for livestock (3). However, the head of giant freshwater prawn is often treated as a waste during the consumption and industrial processing. In fact, the head is also rich in proteins with high nutritional value and specific functional characteristics (4), but its utilization is rather low with only a small part being feed or fertilizer (5). Therefore, recycling the proteins in the head of giant freshwater prawn will facilitate the comprehensive utilization of aquatic resources.

Emulsions are stable systems formed by a certain proportion of oil phase, aqueous phase and emulsifiers. The emulsions have been widely used in food products, owing to their high stability, excellent antibacterial properties, and high nutritional value (6). The emulsifiers are used as surface-active substances and are dispersedly wrapped around the droplets. The emulsifiers contain both hydrophilic and oleophilic groups, which can adsorb at the water-oil interface to reduce the interfacial tension and hence maintain the stability of emulsions. Many chemicals are used as emulsifiers to prepare emulsions (7), but it is inappropriate to use them in the food industry because of their potential safety risks. Therefore, natural food grade emulsifiers show great advantages in stabilizing emulsions, promoting oil-aqueous phase dissolution, improving nutritional value and safety (8). Many studies prove that plant protein particles, such as corn protein (9), soybean protein (10), and pea protein (11), can be used as natural emulsifiers in food industry. However, plant proteins often lack key essential amino acids, and their high hydrophobicity makes them less likely to bind to water or oil (12). Generally, animal proteins generally contain eight essential amino acids, and their nutritional value is higher than plant proteins (13). Therefore, developing the proteins in the head of giant freshwater prawn as a food-grade emulsifier conforms to people's demand for "balanced diet," and the concept of sustainable development.

On this basis, the proteins were extracted from the head of giant freshwater prawn by isoelectric precipitation, and their physicochemical properties were analyzed by amino acid analyzer, contact angle measurement, particle size measurement and size exclusion high performance liquid chromatography. Moreover, the functional ability of the proteins to stabilize emulsions was also explored under the conditions of freeze-thaw treatment, pH (3–9), salt addition (NaCl, 50–400 mM), and storage temperature (4–60°C).

MATERIALS AND METHODS

Materials

Giant freshwater prawns were purchased from the Shanghai Aquaculture Base (Shanghai, China), and sunflower seed oil (Mighty, Suzhou, China) was purchased from a local supermarket (Mighty, Shanghai, China). All other materials and reagents were purchased from commercial suppliers and were used as received without any further purification.

Extraction and Analysis of the Head Proteins of Giant Freshwater Prawn

Preparation of Protein Samples

The proteins were extracted from the head of giant freshwater prawn by isoelectric precipitation (14). The head of giant freshwater prawn were homogenized by homogenizer (SUOTN, STSRH-200, 800 rpm) with deionized water at the solid-to-solvent ratio of 1:5 (w/v). pH value of the homogenate was adjusted to 12 with 1 M NaOH and then stirred for 2 h. After 15 min centrifugation at 7,500 × g (RCF), the supernatant was adjusted to pH 4 with 1 M HCl, to yield precipitated proteins. The above operation was repeated three times to remove impurities, and then the aqueous protein dispersions were adjusted to pH 7 before freeze-drying. The protein content was measured by Kjeldahl method, and 6.25 was used as Kjeldahl nitrogen-to-protein conversion factor.

Amino Acid Analysis

After the hydrolysis of samples with 6 M HCl for 24 h, amino acid composition was analyzed by an HPLC system with pico-tag column. Hitachi D-2850 chromatograph data processor was used to quantify the amino acids according to the peak area of known standard concentration (15).

Size Exclusion High Performance Liquid Chromatography

SE-HPLC was carried out on a HPLC system (Agilent Technologies, United States) with a TSKgel G3000SWxL column (TOSOH Bioscience, Japan). The protein solution (pH 12.0, 2 mg/ml) was filtered by using a 0.45 μm membrane before injection. A mobile phase consisting of 0.1 M NaCl (pH 12.0) was used to elute the sample at an equidistant flow rate of 0.5 ml/min, and the absorbance was determined at 280 nm. SDS (15 mM) and DTT (25 mM) were added to the protein samples to analyze the protein aggregation (16).

Contact Angle Measurement

OCA 20 AMP (Dataphysics Instruments GmbH, Germany) was used to measure the contact angle of the proteins. The freeze-dried protein powder was pressed into a circular sheet and placed in an optical glass colorimetric dish containing sunflower seed oil. Then, 5 μl distilled water was dropped onto the sample surface with a high-precision syringe. After standing for 4 min, the image of the droplet was captured by a high-speed camera, and the contact angle was calculated by Laplace-Young equation (17).

Particle Size Distribution

The particle size distribution of proteins in aqueous dispersions was determined by dynamic light scattering using a Nanosizer SZ90 analyzer (Malvern Instruments, United Kingdom). A sample solution of 0.3 mg/ml (1.1 ml, pH 7.0) prepared in distilled water was used for measurement in a quartz colorimeter with an optical path of 1 cm (18). The refractive indices of water and the particles were taken as 1.33 and 1.54, respectively.

Preparation of Emulsions

Emulsions were prepared using different protein solutions (1.0, 2.0, and 3.0%, wt) and different oil/aqueous phase ratios (2:8, 3:7, 4:6, and 5:5). Briefly, sunflower seed oil was added to protein solutions, homogenized at 16,000 rpm for 2 min using a rotor high speed blender (SUOTN, STSRH-200, China), and further treated by ultrasound processor (NingBo Scientz Biotechnology Co. Ltd., Ningbo, China) for 5 min (on 5 s, off 5 s, 300 W). The emulsions were prepared at pH 7.0 in the presence of sodium azide (0.02%, wt) to prevent the microbial contamination (19).

The effects of pH, salt addition and temperature on the stability of emulsions were investigated. The pH of protein solutions (2%, wt%) was adjusted to pH 3, 5, 7, and 9 with 1 M HCl or 1 M NaOH, and NaCl was added to the protein solutions (pH 7.0) to reach 50, 100, 200, and 400 mM, respectively.

According to the above method, the emulsions were prepared with 2% protein and 3:7 oil/water ratio. At the same time, the emulsions (pH 7.0) were stored at different temperatures (4, 25, 37, 50, and 60°C) to investigate their stability.

Creaming Index

The freshly prepared emulsions were stored at −20°C for 20 h, and then thawed in 20°C water bath for 2 h. After several freeze-thaw cycles, the height of emulsions and creaming layer was measured. Creaming index (CI) was calculated to reflect the freeze-thaw stability of emulsions by following equation (20).

$$CI = \frac{H_s}{H_t} \times 100\%$$

where, H_s is the height of the cream layer (cm); H_t is the total height of the emulsion (cm).

Optical Microscope and Confocal Laser Scanning Microscope (CLSM)

The emulsions were diluted and dropped onto the microscope slide, and then directly observed under an optical microscope using a 63 × oil immersion (MC02709, Carl Zelss, Germany).

The emulsions were dyed with fluorescent dyes of Nile Blue (0.1 wt%) and Nile Red (0.1 wt%, W/V). The samples were placed on a recessed sheet under dark conditions, and then observed with CLSM (TCS SP8, Leica, Germany) using Helium Neon laser (633 nm) and Argon Krypton laser (488 nm) (21).

Droplet Size Distribution and ζ-Potential of Emulsions

The droplet size distribution was determined by static light scattering using a Malvern Mastersizer 3000 analyzer (Malvern Instruments, United Kingdom). The samples were diluted with deionized water and stirred at a speed of 2,000 r/min to prevent multiple scattering effects. The refractive indices of water and sunflower seed oil were taken as 1.33 and 1.47, respectively. The Sauter, surface-weighted mean diameter ($d_{3,2}$) was calculated as:

$$d_{3,2} = \sum n_i d_i^3 / \sum n_i d_i^2$$

where, n_i is the number of droplets with diameter d_i. Nano ZS90 Zetasizer was then used to measure the ζ-potential potential of the emulsions after 100 times dilution (22).

Low-Field Nuclear Magnetic Resonance

The relaxation time of low field nuclear Magnetic resonance (LF-NMR) was measured using a MicroMR-22 MHz spectrometer (MicroMR, Shanghai, China). Five milliliters of the sample were placed into a 15 mm (diameter) NMR tube and the data was obtained from 6,000 echoes through 32 repeated scans. Carr-purcell-meiboom-gill sequence (CPMG) was used to determine the spin-spin relaxation time (23).

Rheology of Emulsions

The rheological behavior of the emulsions was determined by a rheometer (AR 1500, TA Instruments, United Kingdom) in the frequency range of 1–10 rad/s. The parallel plate (40 mm diameter) gap of the measurement system was fixed to 1 mm, and the temperature was 25°C. The static emulsion was injected into the gap of the plate to conduct strain scanning, and the scanning frequency was tested at 0.1–10 Hz. The changes of storage modulus (G') and loss modulus (G") were recorded (24).

TABLE 1 | Amino acid composition of the proteins in the head of giant freshwater prawn.

Amino acid	Percent amino acid (%)
Asp	11.36
Thr	4.94
Ser	4.81
Glu	16.71
Pro	3.90
Ala	4.82
Gly	5.11
Cys	0.58
Val	4.81
Met	2.58
Ile	4.38
Leu	8.51
Tyr	4.68
Phe	5.02
Lys	7.32
His	3.53
Arg	6.66
Trp	0.28

Data Analysis

The results were expressed as mean and standard deviation. SPSS software (IBM 20.0) was used to analyze the difference between the groups ($P < 0.05$).

RESULTS AND DISCUSSION

Characterization of the Proteins in the Head of Giant Freshwater Prawn

Amino Acid Composition

The amino acid composition of protein determines the structure and function of protein. Therefore, it is of great significance to clarify the protein content and amino acid composition. In this study, the protein content in the head of giant shrimp was 69.1%, and the essential amino acid (EAA) content was 41.09% (**Table 1**). On this basis, the proteins greatly satisfied human growth and maintenance, compared with the EAA of soybean protein isolate (36.12%) (15), zein (39.34%), and gliadin (25.13%) (25).

Physicochemical Properties

The particle size distribution of protein particles is shown in **Figure 1A**. It was monomodal, the particle size ranged from 100 to 500 nm and the *Z*-average was 141 nm. The particle size is very similar to those of Antarctic krill proteins (175 nm) (19), soy protein isolate-chitosan nanoparticles (148 nm) (18), novel zein

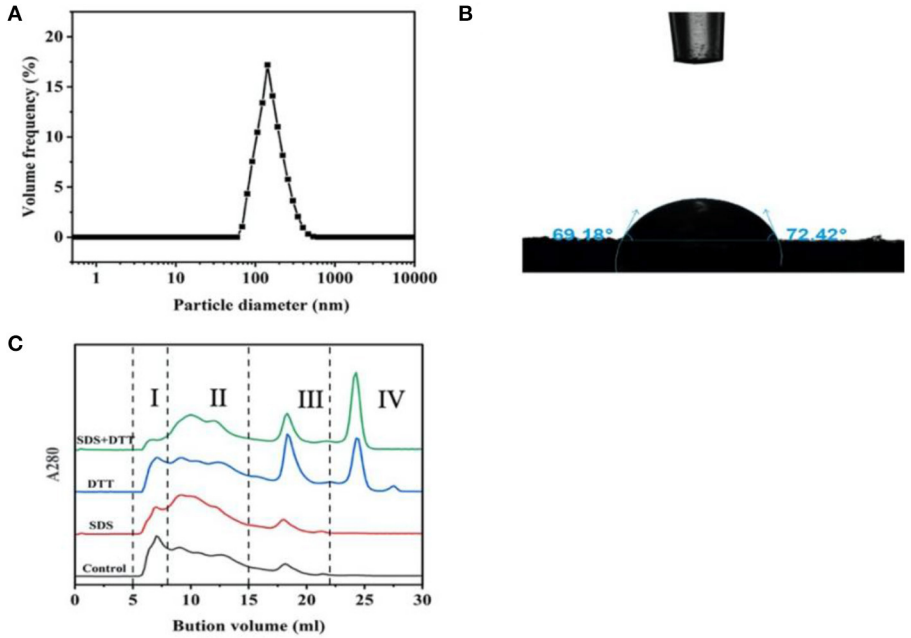

FIGURE 1 | Particle size distribution **(A)**, contact angle **(B)**, and aggregation behavior **(C)** of the proteins in the head of giant freshwater prawn.

particle (149 nm) (26) which possess a good ability to stabilize emulsions. Generally speaking, protein particles with smaller size present fast adsorption kinetics, which often quickly adsorb to the interface to form a more stable emulsion. In addition, the contact angle of protein particles was measured as ∼71° which was <90° (**Figure 1B**), indicating that the protein particles were relatively hydrophilic and preferentially resided in the aqueous phase to form O/W emulsions (17).

Aggregation Behavior

In **Figure 1C**, the role of hydrophobic interaction and disulfide bond in proteins aggregation was determined after treatment with hydrophobic interaction denaturant SDS and disulfide bond reducing agent DTT (16). The elution profile consisted of four components, including high molecular weight protein (HMW, I), large molecular weight protein (LAMW, II), medium molecular weight protein (MMW, III) and low molecular weight protein (LMW, IV). In the control sample, the peak value of HMW (I) was at ∼7 ml, and the peak value of LAMW (II) was at ∼17 ml (MMW, III). When SDS was added, the peak intensity of HMW (I) decreased and peak intensity (LAMW, II) appeared at ∼10ml. The peak intensity of MMW (III) had no obvious change. These results indicated that hydrophobic interactions contributed to the formation of protein aggregates, especially high molecular weight ones. After DTT treatment, the peak intensities of HMW (I) and LAMW (II) decreased slightly, while the peak intensities of MMW (III) and LMW (IV) increased sharply. In the presence of SDS and DTT, the peak of HMW (I) almost disappeared, and the corresponding LMW (IV) of monomer increased significantly, while the peak intensity of (LAMW, II) and MMW (III) decreased slightly. These results further indicated that hydrophobic interactions and disulfide bonds promoted the aggregation of HMW (I), and disulfide bonds were more important than hydrophobic interactions in maintaining the stability of protein conformation.

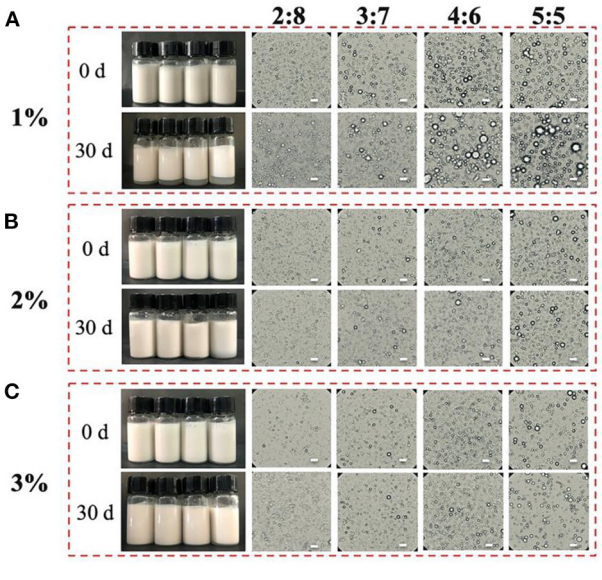

FIGURE 2 | Visual appearance and microstructures of the proteins-stabilized emulsions with different concentrations **(A–C)** at initial (0 days) and after 30 days of quiescent storage. Scale bar = 5 μm.

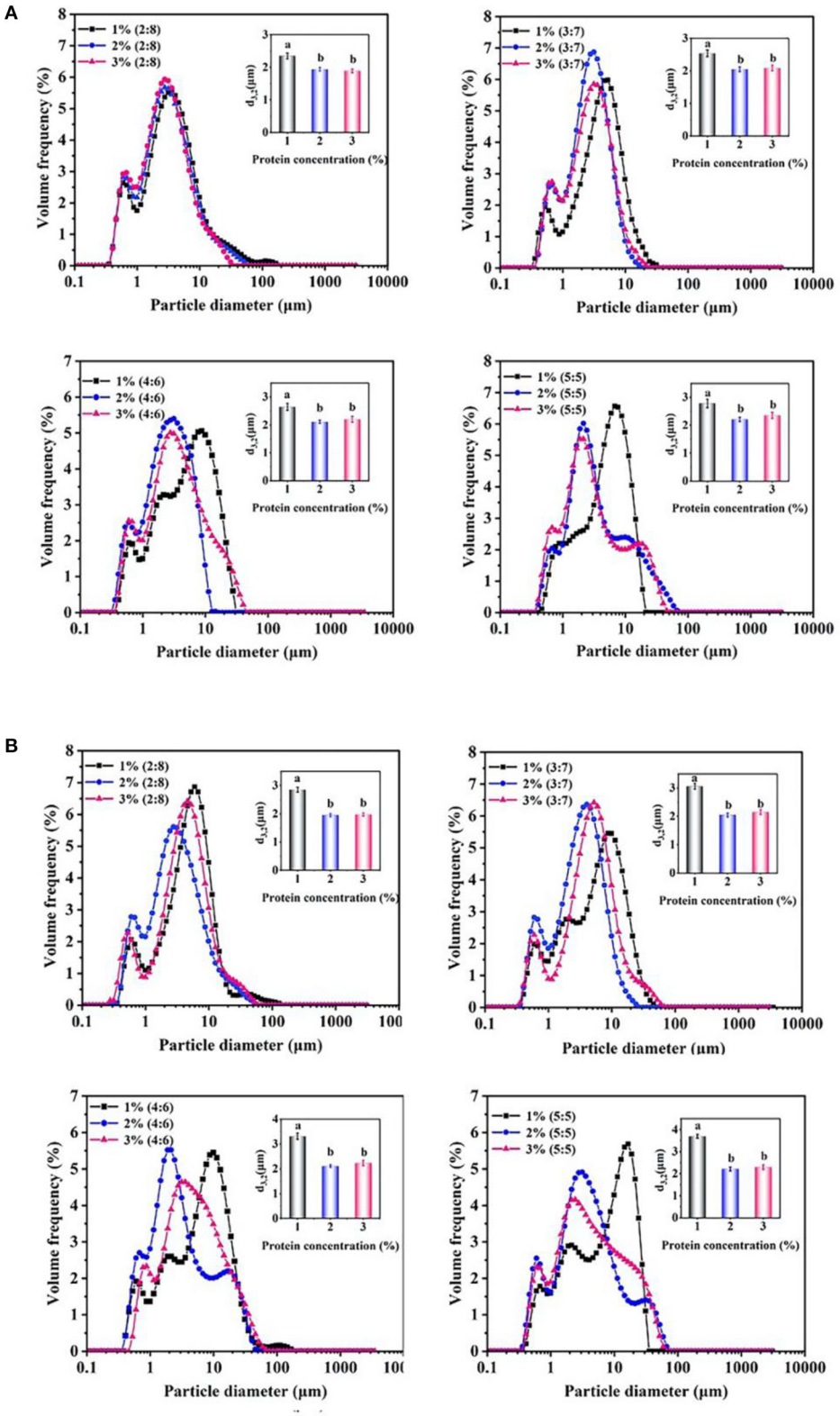

FIGURE 3 | Droplet size changes of the proteins-stabilized emulsions after 0 days **(A)** and 30 days **(B)**. Mean values followed by different letters are significantly different ($P < 0.05$).

Hydrophobic interactions occurred through the interaction of hydrophobic amino acids (including Gly, Ala, Val, Leu, Ile, Phe, Trp, Met, Pro), which is the driving force of protein folding (27). In this study, hydrophobic amino acids accounted for 39.41% of the total amino acids. Under these conditions, the hydrophobic side chains of the proteins were embedded in the hydrophobic core, exposing the hydrophilic group to the environment (28). Therefore, it could be highly inferred that this protein conformation determined the wettability of the head protein of giant freshwater shrimp (**Figure 1B**). In addition, the content of cysteine was 0.58%, which greatly promoted the formation of disulfide bond. Our previous study found that Antarctic krill protein contained 40.81% hydrophobic amino acids and 0.28% cysteine residues. Hydrophobic interaction played a greater role in promoting protein aggregation than disulfide bond (10). This difference is mainly due to the fact that the cysteine content in the head of giant freshwater shrimp is more than twice that of Antarctic krill.

Ability of the Proteins in the Head of Giant Freshwater Prawn to Stabilize Emulsions

The protein concentrations (1–3%, W/V) and oil/water ratios (2:8–5:5, V/V) were selected to prepare stable oil/water emulsions. **Figure 2** shows the appearance and microstructures of the proteins-stabilized emulsions. In **Figure 2A**, the emulsions prepared by 1% proteins displayed creaming after 30 days storage. Furthermore, the emulsions creamed more obviously with the increase of oil/water radio from 2:8 to 5:5. After the proteins was increased to 2 or 3%, the emulsions visually unchanged and did not present creaming (**Figures 2B,C**). From the viewpoint of microstructures, the droplet diameter of 1% proteins-stabilized emulsions significantly increased with the increase of oil/water radio after 30 days storage. However, the 2 or 3% proteins-stabilized emulsions displayed similar microstructures with each other during the whole storage. The increased diameter indicated that the coalescence occurred in the emulsions. The reason was that under low concentration of protein particles, the increase of oil/water radio caused insufficient protein particles to absorb to the surface of the droplets, thereby increasing the surface area of oil droplets to maintain the stability (29). With the increase of protein content, higher particle density resided on the O/W interface to achieve sufficient surface coverage of all droplets (18). Comparing with plant proteins including tea protein (30), soy protein isolate (31), zein (26), and animal proteins including Antarctic krill proteins (19), whey proteins (32), pork proteins (33), the emulsions stabilized by the proteins in the head of giant freshwater prawn also show excellent and comparable stability after a long-term storage.

As shown in **Figure 3A**, the average diameter ($d_{3,2}$, μm) of 1% proteins-stabilized emulsions was 2.6 μm at 2:8 oil/water ratio. When the oil/water ratio was 5:5, the droplet size increased to 2.8 μm. However, the emulsion prepared by 2 or 3% proteins remained ∼2.0 μm in diameter with the increase of oil/water radio from 2:8 to 5:5. After 30 days storage (**Figure 3B**), the droplet sizes of 1% proteins-stabilized emulsions greatly increased reaching 2.8, 3.1, 3.3, and 3.7 μm at oil/water ratios of 2:8, 3:7, 4:6, and 5:5, respectively. However, 2 or 3% proteins-stabilized emulsions almost showed no change in droplet sizes (∼2.0 μm) under different oil/water ratios. This further suggested that the emulsions prepared by 1% proteins underwent the coalescence during storage. Considering the stability of emulsions and the added amount of proteins, 2% proteins-stabilized emulsions were used to perform subsequent experiments.

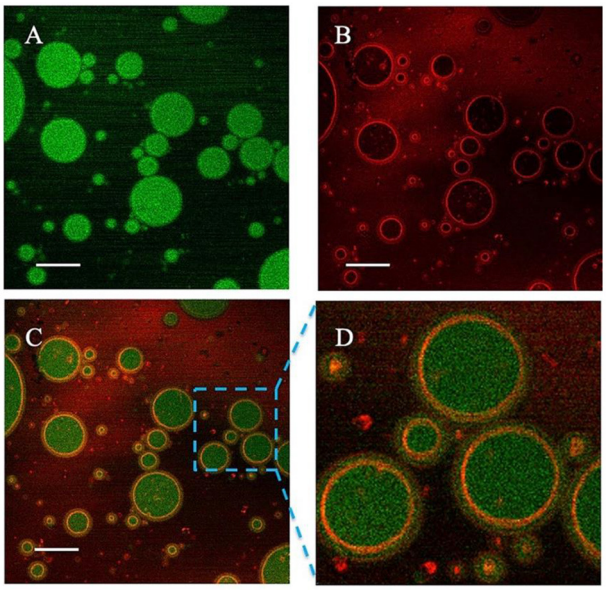

FIGURE 4 | Confocal laser scanning microscope (CLSM) images of the proteins-stabilized emulsions. **(A)** Nile red fluorescence (green) labeling Sunflower seed oil excited; **(B)** Nile Blue fluorescence (red) labeling proteins; **(C)** The overlapped image; **(D)** Enlarged image. Scale bar = 5 μm.

The emulsion microstructure was characterized by CSLM. **Figure 4A** shows the green fluorescence images of sunflower seed oil stained with Nile Red, and **Figure 4B** shows the red fluorescence images of protein stained with Nile Blue. Therefore, the green and red fluorescence represent oil phase and proteins particles, respectively. **Figures 4C,D** are the overlapped images of **Figures 4A,B**. It was clearly observed that the red fluorescence was wrapped around by green fluorescence, which supported the O/W type of emulsion. Moreover, the proteins stained by red color were uniformly and tightly distributed around the oil droplets, which effectively prevented the coalescence (32).

Effects of Different Environmental Stresses on the Stability of Emulsions

Effect of Freeze-Thaw Treatment

The emulsions prepared by 2% proteins were treated by the freeze-thaw treatment (**Figure 5A**), and their stability was further evaluated by the creaming index and $d_{3,2}$ values of the droplets. **Figure 5B** shows the changes in the stability of emulsions after two cycles freeze-thaw treatment. In detail, the emulsions at the oil/water ratios of 4:6 and 5:5 were creamed after one cycle, and

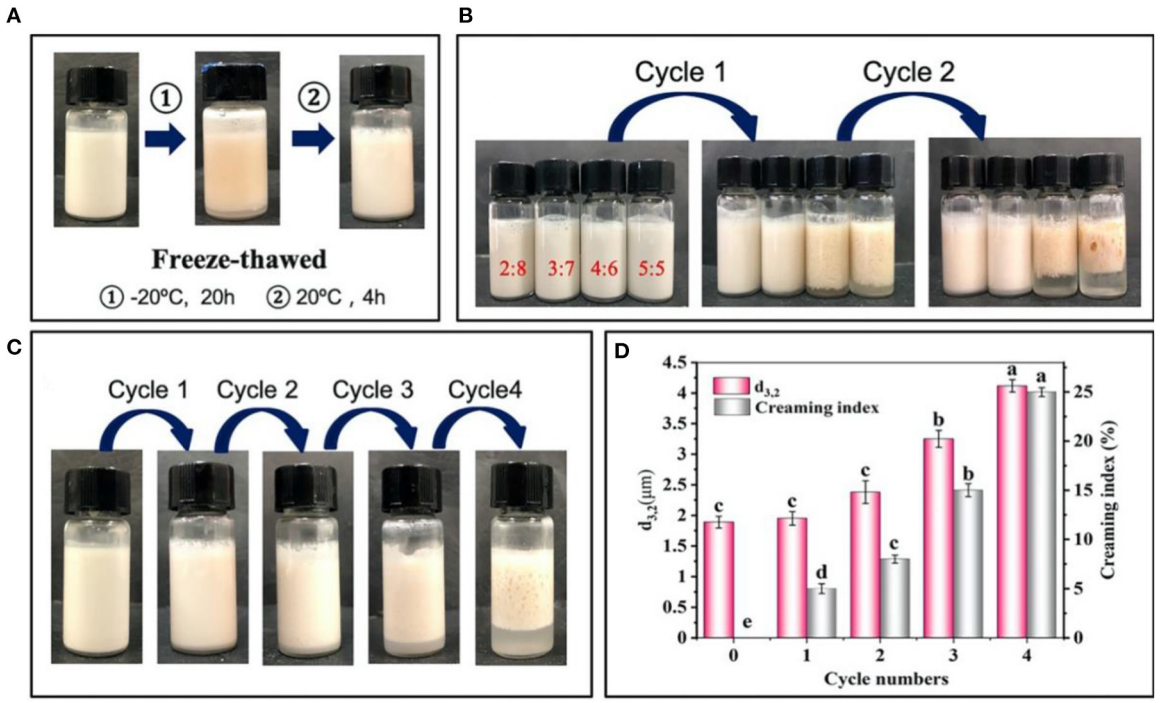

FIGURE 5 | Changes in visual appearance **(A,B)** of emulsions prepared by 2% proteins and 3:7 oil/water ratios **(C)** before and after freeze-thaw treatment. **(D)** Average diameter ($d_{3,2}$) and creaming index of emulsions prepared by 2% proteins and 3:7 oil/water ratio after freeze-thaw treatment. Mean values followed by different letters are significantly different ($P < 0.05$).

further displayed an obvious demulsification after two cycles, suggesting that the adsorbed protein particles greatly desorbed from the oil/water interface. With the emulsions being frozen, the water molecules formed ice crystals puncturing interfacial membrane, which induced the oil phase to penetrate each other and then gathered leading to the separation of oil phase and aqueous phase after thawing (34). On this basis, the emulsions at higher oil/water ratio (e.g., 4:6 and 5:5) formed larger droplets after being frozen, which was difficult to be completely covered by the limited proteins (35). Therefore, the emulsions at lower oil/water ratios of 2:8 and 3:7 exhibited higher freeze-thaw stability (**Figure 5B**). After further freeze-thaw treatment of the emulsions at 3:7 oil/water ratio (**Figure 5C**), obvious creaming was observed after 3 cycles and the creaming index maximally reached 25% after four cycles treatment. Moreover, a high degree of demulsification also occurred. Meanwhile, the $d_{3,2}$ values of the emulsions increased from initial 1.9–4.1 μm (**Figure 5D**). The results showed that the appropriate oil/water ratio and proteins concentration was 3:7 and 2%, which was selected to prepare the emulsions with a long-term stability and high freeze-thaw resistance.

Effect of pH

In general, the visual appearance of the freshly prepared emulsions was similar with each other at different pH values, and only a slight creaming occurred when pH was 5 (**Figure 6A**). The emulsions showed smaller $d_{3,2}$ values (∼2.0 μm) at pH 3, 7 and 9 compared with those (5.8 μm) at pH 5 (**Figure 6B**). Furthermore, the ζ-potential values at different pH were analyzed (**Figure 6C**). When the pH value was 3, 7, and 9, the ζ-potential values were >30 mV, and when the pH value was 5, a minimum 20.2 mV was reached. Previous studies have shown that pH 5 is close to the isoelectric point of giant shrimp protein, which reduces the surface charge of protein and forms aggregates (16). However, the proteins had a high positive charge at pH 3, and a high negative charge at pH 7 and 9, thus inhibiting the aggregation of proteins and maintaining the stability of emulsions. The relaxation time was rather sensitive to the mobility of water molecules, which was an important index reflecting the water migration during the formation of emulsions (**Figure 6D**). All of the emulsions showed two main relaxation peaks, which belonged to constitution water (10–100 ms) and movable water (100–1,000 ms), respectively. In contrast, the relaxation peaks shifted to the right at pH 5, suggesting that the mobility of the emulsions increased and its stability decreased (23). After 30 days storage, the visual appearance of emulsions did not change significantly at pH 3, 7, and 9 (**Figures 6E,F**), and the droplets size reached the minimum value of 1.9 μm at pH 3 and the maximum value of 6.5 μm at pH 5. Meanwhile, the ζ-potential of emulsions did not greatly change (**Figure 6G**). Such phenomenon was mainly because that when the pH reached the pI, the electrostatic repulsion between

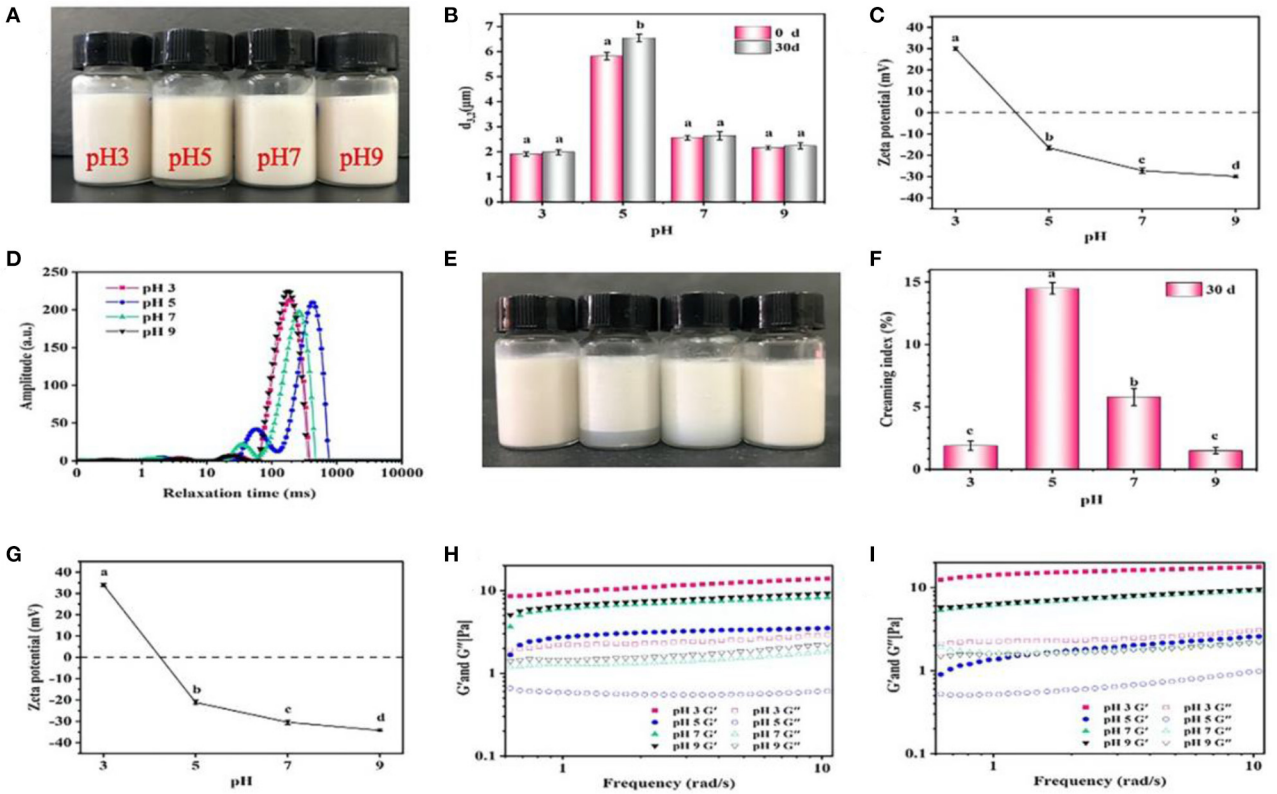

FIGURE 6 | Effects of pH on the visual appearance **(A,E)**, droplet size **(B)**, ζ-potential **(C,G)**, LF-NMR **(D)**, creaming index **(F)**, and rheological properties **(H,I)** of the emulsions prepared by 2% proteins and 3:7 oil/water ratio after 0 and 30 days storage. Mean values followed by different letters are significantly different ($P < 0.05$).

droplets decreased, which induced the aggregation of droplets and creaming (36).

The rheological properties of emulsions are shown in **Figure 6H**. Under different pH conditions, the storage modulus G' of all emulsions was higher than the loss modulus G", and all showed an increasing trend with the increase of shear frequency, which manifested that the gel-like networks were weak or similar to solid structure (20). Moreover, the G' and G" were lower at pH 5 than those at pH 3, 7, and 9. Generally, the higher the G' and G" values, the more stable the gel-like networks will be. These results were consistent with the LF-NMR results, because the increased mobility of emulsions weakened the gel-like networks (37). After 30 days storage, the G' and G" at pH 3, 7, and 9 further rose (**Figure 6I**). Such facts were mainly because that when the pH was far away from the pI, the proteins became charged and electrostatic repulsion increased, thereby forming small and flexible aggregates to stabilize emulsions with low $d_{3,2}$ values (19). Meanwhile, the volume of pores between emulsion droplets was decreased and the O/W interface contact area was increased, which induced the formation of more compact network structures and the increase of viscoelastic properties (24). In addition, the formation of gel-like networks was also contributed by the intermolecular interactions between proteins. For example, the hydrophobic interactions facilitated the adsorption and aggregation of protein particles at the O/W interface, and disulfide bonds could further stabilize protein molecules at the O/W interface to enhance their interactions, which facilitated the formation of the stabilized emulsions (38). Considering the results from SE-HPLC, it was inferred that hydrophobic interactions improved the formation of emulsions, and the disulfide bonds greatly maintained their stability.

Effect of Salt Addition and Temperature

The effect of salt addition on stability of emulsions is displayed in **Figure 7**. No obvious creaming occurred in the freshly prepared emulsions at different NaCl (50–400 mM) (**Figure 7A**), and the $d_{3,2}$ values of emulsions increased from 2.4 to 3.0 μm (**Figure 7B**). The emulsions with a higher concentration of NaCl (>200 mM) was more obviously creamed (**Figure 7C**), and the $d_{3,2}$ values also increased to 3.9 μm after 30 days storage (**Figure 7D**). **Figure 7E** shows the distribution of relaxation time of the emulsions with different NaCl. The relaxation time shifted to the left with the increase of NaCl, indicating that the addition of NaCl restrained the mobility of emulsion droplets. There was a negative correlation between NaCl concentration and relaxation time of the emulsions, possibly due to that the salt provided a electrostatic shielding of the charged protein particles to promote proteins aggregation and limit the mobility of emulsions (39). This argument was supported by the decrease of ζ-potential with increasing the NaCl concentration,

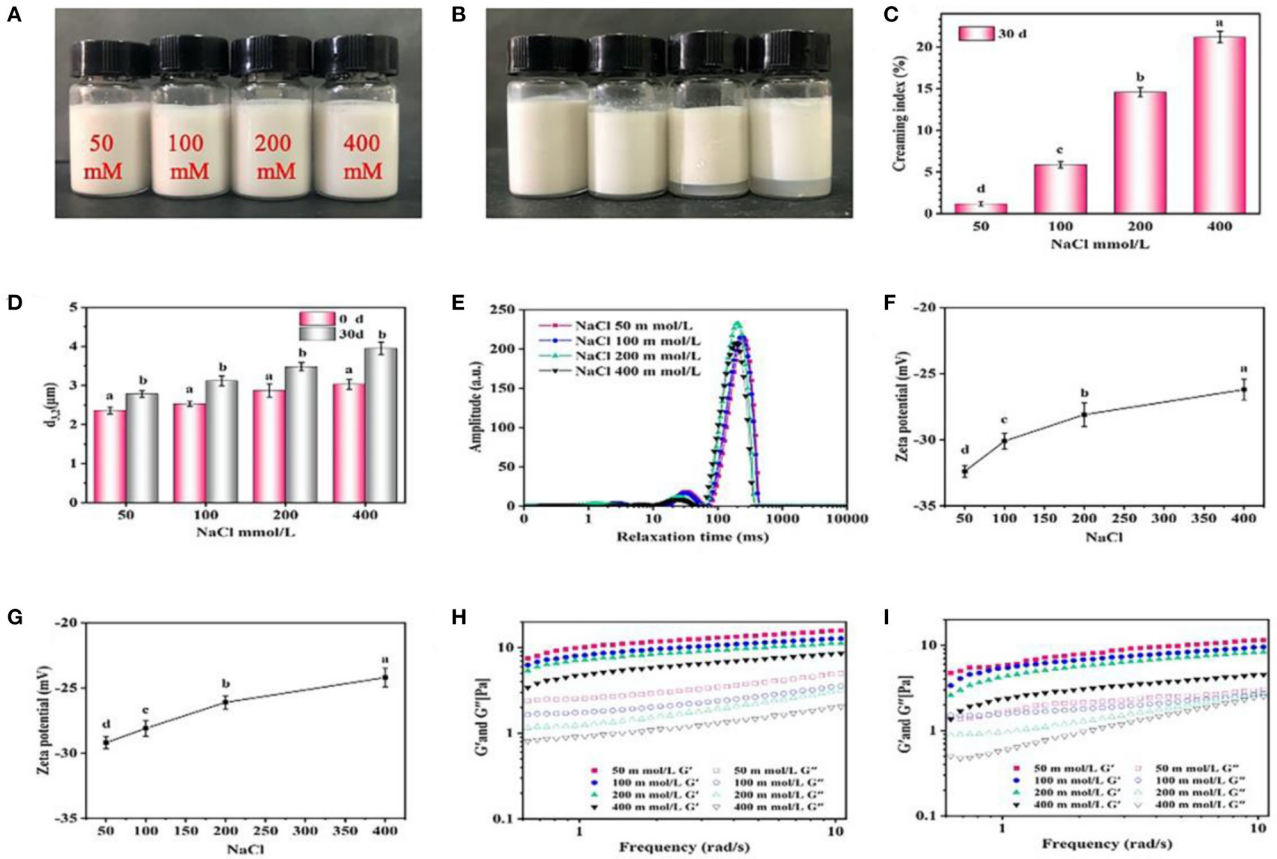

FIGURE 7 | Effects of NaCl concentration on the visual appearance **(A,B)**, creaming index **(C)**, droplet size **(D)**, LF-NMR **(E)**, ζ-potential **(F,G)** and rheological properties **(H,I)** of the emulsions prepared by 2% proteins and 3:7 oil/water ratio after 0 and 30 days storage. Mean values followed by different letters are significantly different ($P < 0.05$).

indicating that the electrostatic repulsion between droplets was weakened (**Figures 7F,G**). Furthermore, the values of G' and G" were greatly reduced with the increase of NaCl, and such phenomenon become more obvious compared with freshly prepared emulsions after 30 days storage (**Figures 7H,I**), suggesting that the electrostatic shielding of the charged particles weakened the formation of gel-like networks which led to the unstable emulsion.

The effect of temperature on the stability of emulsions was evaluated (**Figure 8**). There was no obvious creaming or phase separation in the emulsions after 10 days storage at different temperature (4–60°C) (**Figures 8A,B**). The droplet size of emulsions slightly increased from 1.8 μm at 4°C to 2.4 μm at 60°C (**Figure 8C**). In addition, the increase of temperature had a great influence on the rheological properties of emulsion. In detail, the emulsions presented higher G' and G" values at 50 and 60°C compared with those at 4, 25, and 37°C (**Figure 8D**), indicating that the formation of gel-like networks was enhanced at 50 and 60°C. Generally, heating induced the unfolding of the proteins, causing the exposure of the side chain including hydrophobic groups, charged groups and cysteine residues etc., and then the exposed groups would either re-fold or re-form covalent/non-covalent intermolecular interactions with other molecules (27). Such deduction was also highly supported by the results of ζ-potential, showing that the emulsions treated by 50 and 60°C has a higher ζ-potential than those treated by lower temperatures (**Figure 8E**). Therefore, it was concluded that the heating improved the intermolecular interactions between the protein particles, which finally enhanced the formation of gel-like networks in the emulsions.

Wu et al. (40) found that when the pH was 5, the whey protein isolate-stabilized emulsions greatly aggregated and a phase separation was evidenced. Hu et al. (41) reported that the emulsions stabilized by rice bran proteins became unstable under 0–300 mM NaCl treatment, and creaming was easy to occur. Ding et al. (42) discovered that fish oil-loaded crosslinked gelatin nanoparticle emulsions were sensitive to temperature changes, and obvious phase separation was observed after 37°C. Therefore, the proteins in the head of giant freshwater prawn would be a good food-grade emulsifier to stabilize emulsion in the food industry.

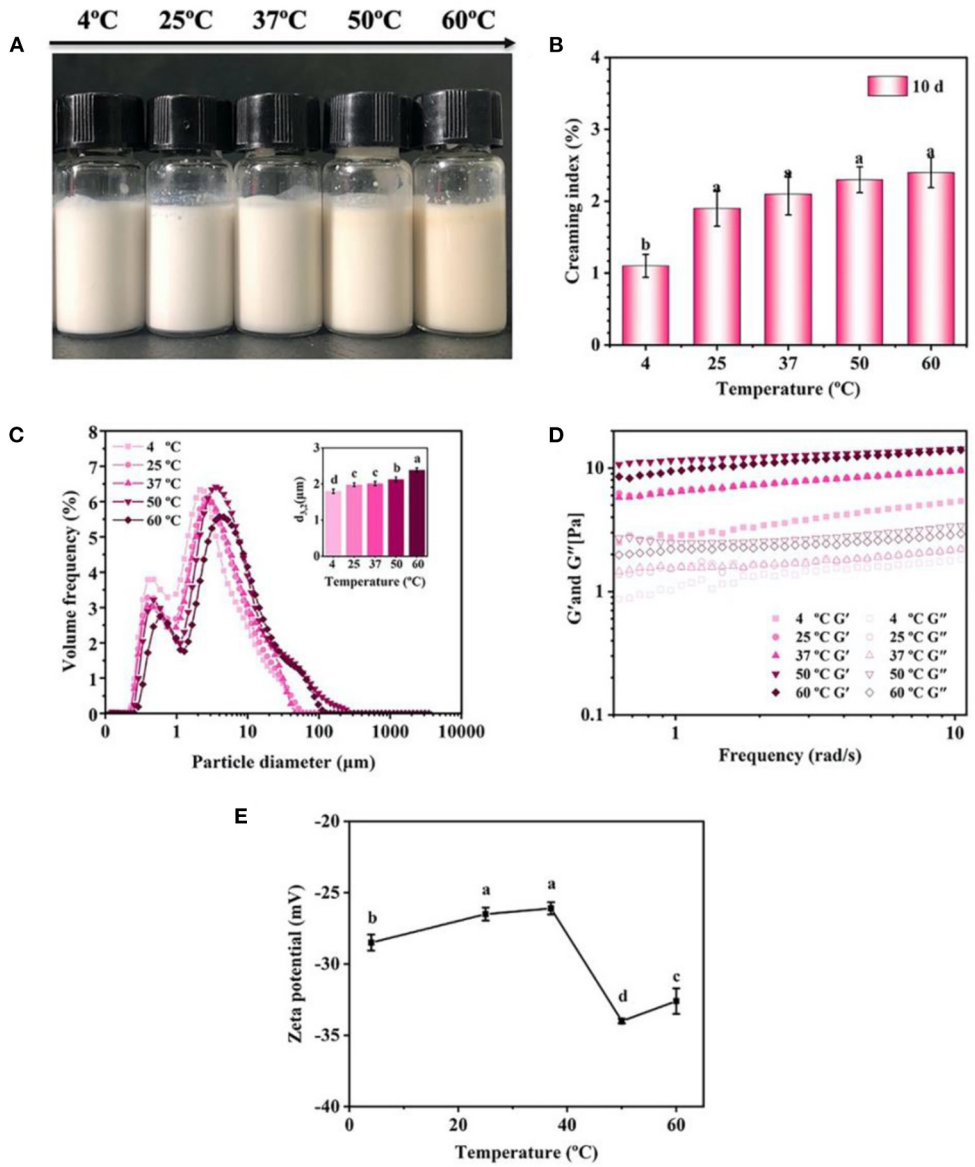

FIGURE 8 | Effects of storage temperature on the visual appearance **(A)**, creaming index **(B)**, droplet size **(C)**, rheological properties **(D)**, and ζ-potential **(E)** of the emulsions (pH 7) prepared by 2% proteins and 3:7 oil/water ratio after 10 days storage. Mean values followed by different letters are significantly different ($P < 0.05$).

CONCLUSION

There were a lot of balanced EAA in the proteins in the head of giant freshwater prawn, with small particle size, intermediate wettability, and preferential absorption on the interface to form stable emulsions. On this basis, a novel protein-stabilized emulsion was developed, and notably, the emulsions stabilized by 2% proteins and 3:7 oil/water ratio effectively resisted the environmental stresses (freeze-thaw treatment, pH, salt addition, storage temperature). Thereinto, the hydrophobic interactions improved the formation of the proteins-based emulsions, and the disulfide bonds greatly maintained the stability of emulsions. In comparison with the existing biopolymers-stabilized emulsions, the proteins-based emulsions showed an excellent long-term stability. Therefore, this study not only proves the ability of the proteins in the head of giant freshwater prawn in stabilizing the emulsions, but also provides a new idea to develop the by-product proteins of aquatic products.

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/s.

AUTHOR CONTRIBUTIONS

YW: investigation, software, resources, data curation, and writing—original draft. YL and RW: investigation, software, resources, and data curation. JW and YZ: conceptualization, funding acquisition, and writing—review and editing. HL: software, resources, and supervision. All authors contributed to the article and approved the submitted version.

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Shelf-Life Extension of Large Yellow Croaker (*Larimichthys crocea*) Using Active Coatings Containing Lemon Verbena (*Lippa citriodora* Kunth.) Essential Oil

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Active coating could improve the fish quality and extend the shelf life. This study investigates the effect of locust bean gum (LBG) and sodium alginate (SA) active coatings containing lemon verbena (*Lippa citriodora* Kunth.) essential oil (LVEO) emulsions on microbiological, physicochemical and organoleptic evaluation of large yellow croaker (*Larimichthys crocea*) samples during refrigerated storage at 4°C. Results showed that LBG-SA coatings incorporated with 0.30 or 0.60% LVEO emulsions significantly inhibited the growth of mesophile bacteria, *Pseudomonas* spp., H_2S-producing bacteria, lactic acid bacteria (LAB) and psychrophilic bacteria, and reduce the productions of trimethylamine (TMA), total volatile basic nitrogen (TVB-N) and ATP-related compounds. Further, the LVEO treatments also retarded the water migration and maintained the organoleptic evaluation results of large yellow croaker during storage at 4°C. In conclusion, the LBG-SA active coatings incorporated with LVEO emulsions maintained the quality and extended the shelf life of large yellow croaker during refrigerated storage.

Keywords: active coating, essential oil, large yellow croaker, total volatile basic nitrogen, shelf-life extension

INTRODUCTION

Large yellow croaker (*Larimichthys crocea*) is an important commercial marine fish in China and cultured extensively due to its flavor and commercial value (1, 2). However, fresh large yellow croaker is highly perishable and results in great economic losses (3, 4) due to lipid oxidation, protein degradation, and the production of undesirable compounds in the presence of microorganisms and related enzymes (5). Freshness is the most important issue relating to its quality and value (6). The fish spoilage could produce trimethylamines (TMA), organic acids, biogenic amines, alcohols, sulfides, ketones and aldehydes with unacceptable off-flavors. Fish spoilage is mainly related to the presence of Gram-negative proteolytic psychrotrophic bacteria, mainly *Pseudomonas* spp., *Shewanella* spp., and *Enterobacteriaceae* (7).

Some preservation techniques have applied to improve the quality and extend the shelf life (8). Using active coatings to delay the microbial growth on the fish surface could improve the fish quality and extend its shelf-life (9). Active coating mainly from food-grade natural materials including polysaccharides (e.g., alginates, gum, chitosan) has been developed as the coating for fish and fish products (10). Natural plant preservative has been paid more and more attention as it have little impact on human health or the environment to control spoilage organisms (11). Sodium alginate (SA) is an anionic polysaccharide and has good film forming properties (12). However, pure SA film still has relatively poor mechanical strength and antimicrobial activity, limiting its application to food packaging if modified properly (13). Locust bean gum (LBG) is a natural high molecular weight (300–1,200 kDa) branched polysaccharide (14). Being non-ionic, its aqueous solubility is not affected by pH or ionic strength of the liquid medium (15). SA could be used as an ion source (anionic) to promote the mucoadhesive property of non-ionic LBG (15, 16).

The incorporation of natural bioactive compounds in the active coating could improve the quality and performance, and essential oils (EOs) are the commonly used categories. Lemon verbena (*Lippa citriodora* Kunth.) is an aromatic plant native to South America and widely used for medicinal purposes, including antimicrobial, neuroprotective, anticonvulsant, cardioprotective, antigenotoxic, and anti-inflammatory activities (17, 18). Previous research has shown that high percentage of neral in LVEO exhibits antimicrobial and antioxidant activities (19). Nevertheless, the antimicrobial activity of neral against several spoilage organisms has been well-documented in *in-vitro* trials and applied in food storage (20, 21). However, some chemically active compounds of EOs are rarely present in food matrices, which have negative impact on the chemical food integrity, physical stableness, and the loss of bioactive activity of bioactive compounds (22). LVEO encapsulated by emulsion can conquer these problems by improving the oxidative stability of compounds, limiting the reaction of these compounds with food, protecting their constancy during process of food and maintenance, and providing controlled and targeted release conditions (22–24). Biopolymeric emulsions with high food compatibility could inhibit the microbial growth and lipid oxidation in fish during cold storage.

The research was to explore the effect of LBG and SA based coatings incorporated with LVEO on the quality of refrigerated large yellow croaker. The changes in microbial survival, total volatile basic nitrogen (TVB-N), trimethylamine (TMA), *K*-value, lipid oxidation, free fatty acids, hardness, and organoleptic evaluation of refrigerated large yellow croaker during storage for 18 days were tested to determine the preservative mechanism of each treatment.

MATERIALS AND METHODS

Essential Oil From Lemon Verbena

The leaves of lemon verbena were washed with deionized water and then hydro-distillated by a Clevenger-type apparatus for 3 h. LVEO was dried with sodium sulfate anhydrous and then kept at 4°C in sealed brown vials till being used. The LVEO components were analyzed by GC-MS with the method of Homayonpour et al. (10). The conditions were set as follow: Sample volume: 1 μL; Injection port temperature: 280°C; Ion source temperature: 230°C; Initial temperature: 60°C for 1 min; Program rate: 10°C/min; Final temperature: 290°C for 5 min; Septum purge with flow rate 2 mL/min. The LVEO components were recognized with confirmed with those of mass spectra and authentic samples with reference compounds in the NIST 2011. The relative content of each component of LVEO (%) was measured with area under peak.

Preparation of Active Films Incorporated With LVEO Emulsions

The LVEO/lecithin emulsions were prepared according to Liu et al. (16). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of LVEO for *Pseudomonas* spp. and *Shewanella* spp. are 0.30 and 0.60%, respectively. Therefore, different concentrations of LVEO (0.15, 0.30, and 0.60%, v/v considering the 1/2 MIC and MIC, as well the MBC concentrations, respectively) and 0.15% (w/v) lecithin were stirred mechanically in a beaker. Then the LVEO/lecithin emulsions were homogenized with a rotor-stator homogenizer (HR-6, Huxi Industrial co., LTD, Shanghai, China) at 15,000 rpm for 5 min. LBG-SA solution was prepared with SA (1.5% w/v, M/G = 2:1, Mw 2.1 × 10^6 g/mol, viscosity of 200 ± 20 mPa•s, specifications received from supplier) and LBG (0.5% w/v, from *Ceratonia siliqua* seeds, >75% galactomannan content, M/G = 4:1, Mw 320 kDa, specifications received from supplier). To obtain complete dispersion of LBG-SA, the solution was stirred at 60°C for 4 h. Glycerol (30% w/w based on LBG-SA) as a plasticizer was added to LBG-SA solution and stirred for 1 h. The pH value of 1.5% w/v sodium alginate and 0.5% w/v locust bean gum solutions were about 6.7 and 6.2, respectively. The resultant LBG/-SA coating solution was filtrated through a double-layer degreased gauze to remove any undissolved particles. The prepared LVEO/lecithin emulsions were separately added to the LBG-SA solution and stirred continuously for 2 h. Then, the LBG-SA active coatings solutions incorporated with LVEO emulsions were prepared with ultrasonic assisted treatments at 700 W using a ultrasonic assisted processor and degassed under vacuum.

Preparation of Large Yellow Croaker Samples

Fresh large yellow croaker (700 ± 25 g) were supplied by a local market and randomly divided into four batches. Each batch of samples was immersed in the corresponding freshly prepared active coating solutions for 20 min with a ratio of 1:3 (w/v). Then the large yellow croaker samples were taken out and air-dried at 4°C for 60 min to form the active coating. After that, each large yellow croaker sample was packaged in sterile polyethylene bag and stored at 4°C for the subsequent assessments at 3-day interval. The abbreviation was followed: (1) CK (large yellow croaker samples were treated with LBG-SA active coating without LVEO emulsion); (2) LYC-0.15%LVEO (large yellow croaker

samples were treated with LBG-SA active coating incorporated with 0.15% LVEO emulsion); (3) LYC-0.30%LVEO (large yellow croaker samples were treated with LBG-SA active coating incorporated with 0.30% LVEO emulsion); (4) LYC-0.60%LVEO (large yellow croaker samples were treated with LBG/-SA active coating incorporated with 0.60% LVEO emulsion).

Microbiological Analysis

Twenty-five grams fish flesh were fully blended with 225 mL normal saline and then subjected to gradient dilutions. The following microbiological analyses were carried out (25): (i) mesophile bacteria: plate count agar mediums were cultivated at 30°C for 48 h; (ii) *Pseudomonas* spp.: cetrimide agar mediums were cultivated at 30°C for 72 h; (iii) H_2S-producing bacteria: iron agar mediums were cultivated at 30°C for 72 h; lactic acid bacteria: MRS agars were cultivated at 30°C for 72 h; (v) psychrophilic bacteria: plate count agar mediums were cultivated at 30°C for 7 days. Each sample was measured in triplicates.

Total volatile Basic Nitrogen Determination

TVB-N determination was carried out with the method of Zhuang et al. (26). 5.0 g of minced fish flesh and 45 mL deionized water were homogenized and then centrifuged at 3,040 × g at 4°C for 5 min. 5.0 mL of the supernatant was taken to determine the content of TVB-N using steam distillation method with Kjeldahl equipment (Kjeltec 8400, Foss, Denmark) and TVB-N expressed as mg N/100 g of large yellow croaker muscle. Each sample was measured in triplicates.

Determination of Trimethylamine

TMA value was determined by picric acid colorimetric method according to Li et al. (27). 2.0 g of minced fish flesh and 18 mL trichloroacetic acid (7.5%, w/v) were homogenized and then centrifuged at 11,960 × g at 4°C for 10 min. After that, 5.0 mL of the supernatant were mixed successively with 1 mL of formaldehyde (10%, v/v), 10 mL of anhydrous toluene and 3 mL of saturated potassium carbonate solutions. Subsequently, 5 mL solution extracted from toluene layer was fully blended with 5 mL of picric acid solution (0.02%, w/v). The absorbance of the mixture was measured at 410 nm. TMA content was calculated according to TMA standard curve and expressed as mg 100 g^{-1} sample. Each sample was measured in triplicates.

Determination of *K*-Value

ATP-related compounds were determined by a RP-HPLC procedure with the method of Yu et al. (28). 2.0 g of minced fish flesh and 7.5 mL precooled perchloric acid solution (6%, v/v) were homogenized and then centrifuged at 11,960 × g at 4°C for 5 min. The precipitate was extracted again with the same condition. The supernatants were collected and neutralized with KOH solutions to the final pH ranges of 6.5–6.8. After that, the neutralized solution was centrifuged at 3,040 × g at 4°C for 5 min and the supernatant was made up to 25 mL with deionized water. Subsequently, the prepared solution was filtered through a 0.22-μm filter membrane and analysized using HPLC (Waters 2695, Milford, USA) furnished with a Shim-pack VP-ODS C18 column (150 × 46 mm). Each sample was measured in triplicates. *K*-value was calculated according to Equation (1).

$$K\ value\ (\%) = \frac{HxR + Hx}{ATP + ADP + AMP + IMP + HxR + Hx} \times 100 \quad (1)$$

where HxR, Hx, ATP, ADP, AMP, IMP, are hypoxanthine riboside, and hypoxanthine, adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, inosine monophosphate, respectively.

Determination of Peroxide Value

POV was determined by the method described by Quan et al. (29). 1.0 g of minced fish flesh and 11 mL of chloroform/methanol (2:1, v/v) were homogenized and then centrifuged at 11,960 × g at 4°C for 2 min. 7.0 mL of the supernatant and 2 mL of 0.5% sodium chloride solution were homogeneously mixed and then centrifuged at 3,040 × g at 4°C for 5 min to separate the solution into two phases. Three milliliter of lower phase, 2 mL of chloroform/methanol (2:1, v/v), 25 μL of ammonium thiocyanate and 25 μL of iron (II) chloride were mixed uniformly. The reaction mixture stood for 20 min at room temperature for 20 min and the absorbance was measured at 500 nm. Each sample was measured in triplicates. Results were expressed in mmol mequiv. peroxide/100 g sample:

$$POV = \frac{V \times N \times 1000}{W} \quad (2)$$

V is the thiosulphate for titration (mL); N is the normality of thiosulphate; W is the weight of lipid (g).

Evaluation of Thiobarbituric Acid Reactive Substances

TBARS was monitored with the method of Vale et al. (30) and expressed as mg of malonaldehyde (MDA)/kg of large yellow croaker sample. Five grams flesh and 20 mL of 20% TBA solution were homogeneously mixed and stood for 1 h. Then the mixture was centrifugated at 11,960 × g at 4°C for 10 min and collected the supernatants. Five milliliter collected supernatant was mixed with 5 mL TBA (0.02 M) and boiled for 40 min. Then the mixture was immediately transferred to ice bath and the absorbance was measured at 532 nm. Each sample was measured in triplicates.

Determination of Free Fatty Acids

The FFAs concentration of the large yellow croaker muscle lipid extract was measured by colorimetric reaction with cupric acetate-pyridine and the absorbance was determined at 715 nm according to Trigo et al. (31). The results were expressed as mmol·kg^{-1} muscle. Each sample was measured in triplicates.

Water Distribution and Migration

Low field nuclear magnetic resonance (LF-NMR) analysis was carried out according to Li et al. (32). Portions of 2 × 2 × 1.5 cm dorsal muscle was cut off and packaged with polyethylene film. Transverse relaxation (T2) was determined on the LF-NMR analyzer (MesoMR23-060H.I, Newmai co., Ltd., China)

with the proton resonance frequency of 20 MHz. The Carr-Purcell-Meiboom-Gill (CPMG) was used to obtain T_2 relaxation information to collect decay signals. The primary parameters were as following: SW (the receiver bandwidth frequency) = 100 kHz, RFD (the parameter to control the first data point that acquired) = 0.08, NS (the number of the scans) = 4, P1 (RF 90° pulse width) = 18 μs, P2 (RF 180° pulse width) = 36 μs, RG1 (analog gain) = 20 db, DRG1 (digital gain) = 6 db, PRG (preamplifier gain) = 0, delay DL1 = 0.2 ms and TW (the duration between successive scans) = 2,000 ms. Longitudinal relaxation (T_1) was measured by using the inversion-recovery (IR) sequence to confirm the MRI parameters followed the below parameters: P1 = 18 μs, P2 = 36 μs, SW = 200 KHz, RFD = 0.020 ms, RG1 = 20 db, DRG1 = 1, NS = 4, TW = 5,000 ms, PRG = 0, NTI = 20, and DL1 = 0.2 ms. Each measurement was performed in triplicate. Post-processing of NMR T_2 data distributed exponential fitting of CPMG decay curves were performed by Multi-Exp Inv Analysis software (Newmai Co., Ltd., China). From the multi-exponential fitting analysis, time constants for each process were calculated from the peak position, and the area under each peak (corresponding to the proportion of water molecules exhibiting that relaxation time) was determined by cumulative integration.

After the measurement of LF-NMR, ^{1}H MRI images of large yellow croaker samples were also determined on MesoMR23-060H.I NMR Analyzer (33). The MRI images, including T_1, T_2, and proton density weighted images, were acquired by using the spin-echo (SE) sequence. The MRI measurement was performed with time repetition (TR), slice width, and time echo (TE) being 500 ms, 3.0 mm, and 20 ms, respectively. Each sample was measured in triplicates. The MRI images were processed with two software: unified mapping and pseudocolor processing. After unified mapping and pseudocolor processing, the gray level images were converted to the color images.

Determination of Hardness

The hardness of large yellow croaker samples was determined using a TA.XT texture analyzer equipped with P/5 probe (34). Portions of 3 × 2 × 1.5 cm dorsal muscle (about 5.0 g) was cut off and the hardness was measured with the test speed of 1 m/s and sample deformation of 50%. Each sample was measured at least eight points.

Organoleptic Evaluation

The organoleptic evaluation of large yellow croaker samples was determined with the quality index method (QIM) described by Sun et al. (35). This method involves five important quality parameters, namely color, elasticty, mucus, muscular tissue and smell. Fifteen experienced panelists (trained by professional laboratory staff, eight women and seven men, 20–40 years old) conducted the organoleptic evaluation based on QIM with the score scale ranging from 1 to 10. All panelists were trained by professional laboratory staff and had a history in fish assessment and previously at Shanghai Ocean University, joined in another research displayed by Li et al. (36). They were designated based on their taste detection limit, sensitivity and smell of very low LVEO concentrations. Ten represents the best quality of large yellow croaker and the sample will not be accepted once the score is <4.

Statistical Analysis

Data analysis of the quality of large yellow croaker was performed in triplicate (except hardness determination and organoleptic evaluation). The date was analyzed using SPSS 22.0 through one-way ANOVA procedure followed by Duncan's-test. The results were expressed as means ± standard deviation. $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSIONS

Chemical Composition of LVEO

The important components of LVEO used included citral (31.79%), neral (23.75%), geraniol (22.01%), and D-limonene (10.36%) obtained from the MS libraries (**Supplementary Table 1**). The four most abundant components are responsible of the antimicrobial activity of LVEO (37, 38).

Microbiological Analyses

Figure 1 shows the corresponding growth data of TVC, *Pseudomonas* spp., H_2S-producing bacteria, LAB and psychrophilic bacteria of large yellow croaker samples during refrigerated storage at 4°C for 18 days. The low count (2.3 lg CFU/g) of TVC at the beginning indicated fish fresh (39). LVEO treatments could delay the spoilage microbial growth and the LVEO treated large yellow croaker samples had lower TVC than that of CK. On 15th day, CK exceeded the "shelf-life" limit of 7.0 lg CFU/g (40). Incorporation of LVEO to LBG-SA coatings showed the significant antimicrobial activity of coatings, which led to extend the shelf-life by inhibiting the growth of undesirable microorganisms. EOs have been widely used for the food preservation application as having potential antimicrobial activity in active coatings (41–43). The destructions of cell membrane structure and functional characteristics of the cell membrane are considered to be the most important mechanism of EOs against microorganisms (44, 45).

Pseudomonas spp. and H_2S-producing bacteria are both known as the specific spoilage organisms (SSOs) in spoiled fish during refrigerated storage (46). The two SSOs also showed similar increase results in this study (**Figures 1B,C**). Pseudomonas is the predominant aerobic microorganism related to the formation of undesirable odors (47). At the beginning, the number of *Pseudomonas* spp. was 1.0 lg CFU/g and increased in all large yellow croaker samples during refrigerated storage. There was a significant ($p < 0.05$) difference in the *Pseudomonas* spp. count between CK and LVEO treated samples at the end of storage and it was 8.7 lg CFU/g for CK. Therefore, it can be seen that LVEO were effective in delaying the growth of *Pseudomonas* spp. in large yellow croaker samples, and the inhibitory differences was related to the added concentration of LVEO. Nisar et al. (48) also reported that clove essential oil was also effective against *Pseudomonas* spp. and the inhibiting effects were increased with the increasing concentration of EO. Myszka et al. (49) reported that green pepper EO could inhibit the growth of *Pseudomonas* spp. and attenuate the bacterial virulence

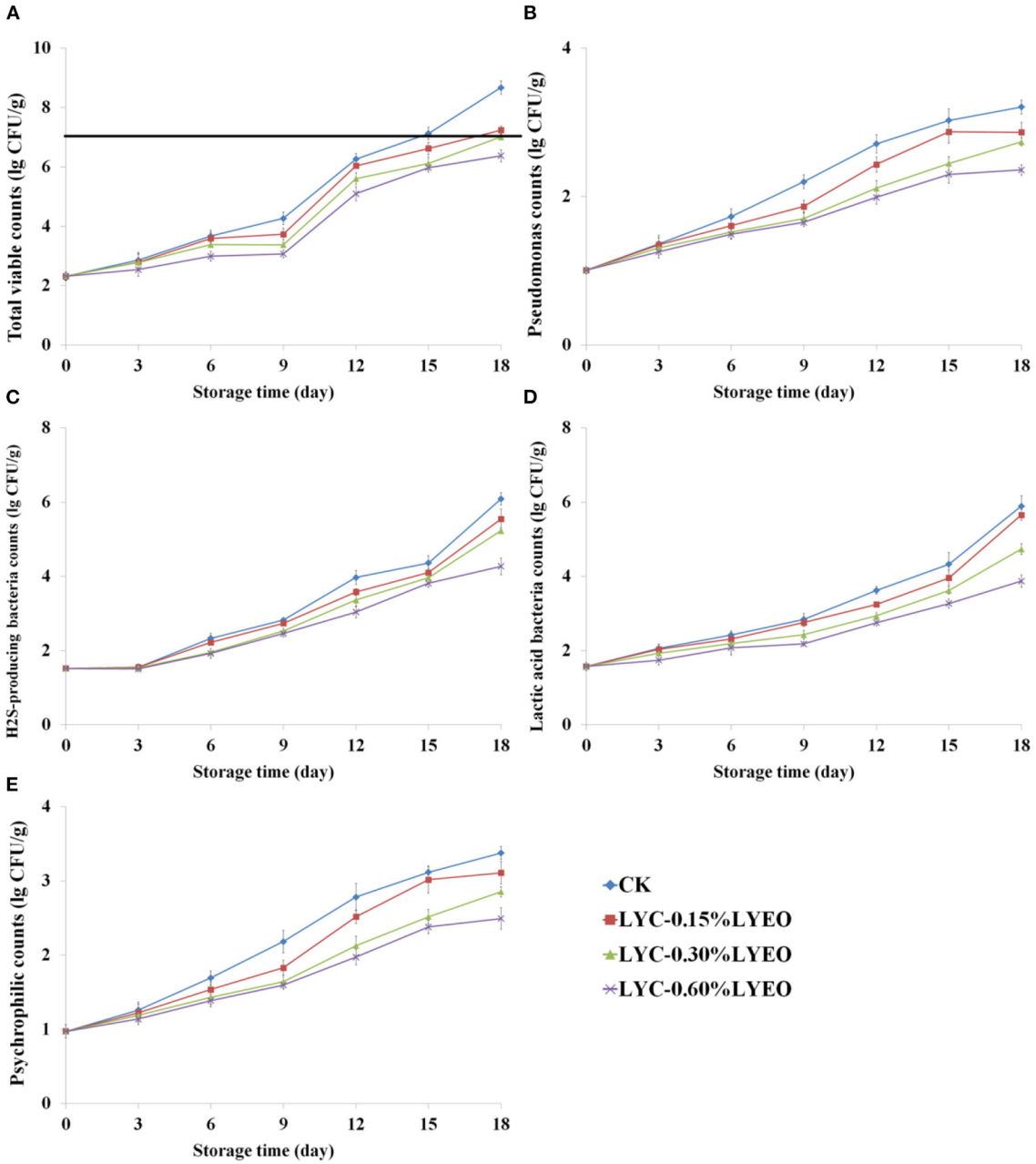

FIGURE 1 | Changes in total viable counts (TVC, **A**), *Pseudomonas* spp. counts **(B)**, H_2S-producing bacteria counts **(C)**, lactic acid bacteria counts (LAB, **D**), and psychrophilic counts **(E)** of large yellow croaker samples during refrigerated storage (CK, large yellow croaker samples were treated with LBG-SA active coating without LVEO emulsion; LYC-0.15%LVEO, large yellow croaker samples were treated with LBG-SA active coating incorporated with 0.15% LVEO emulsion; LYC-0.30%LVEO, large yellow croaker samples were treated with LBG-SA active coating incorporated with 0.30% LVEO emulsion; and LYC-0.60%LVEO, large yellow croaker samples were treated with LBG-SA active coating incorporated with 0.60% LVEO emulsion).

properties, such as pyocyanin production, elastase and alkaline protease activities.

The number of H_2S-producing bacteria (mainly *Shewanella* spp.) was 1.5 lg CFU/g at the beginning, which increased to 6.1, 5.5, 5.2, and 4.3 lg CFU/g, respectively, in CK, LYC-0.15%LVEO, LYC-0.30%LVEO, LYC-0.60%LVEO, at the end of storage. The number of H_2S-producing bacteria in LVEO treated samples were significantly ($p < 0.05$) lower than that in the CK samples. LYC-0.30%LVEO and LYC-0.60%LVEO samples had the lowest number of *Pseudomonas* spp. and H_2S-producing bacteria in all sampling times, which indicated LBG/SA coating incorporated with 0.30 or 0.60% LVEO could inhibit the growth of the two

SSOs in large yellow croaker samples during refrigerated storage. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of LVEO for *Pseudomonas* spp. and H_2S-producing bacteria are 0.30 and 0.60%, respectively. This is the reason that 0.30 and 0.60% of LVEO were used in the experiment. Zhang et al. (50) reported that cinnamon essential oil could also inhibit the growth of *Pseudomonas* and H_2S-producing bacteria in in vacuum-packaged common carp during refrigerated storage and the two microorganisms did not exceed the "shelf-life" limit of 7 lg CFU/g at the end of storage. H_2S-producing bacteria could produce fish off-odors even at low cell numbers and the spoilage action includes production of hydrogen sulfide, TMA, methyl mercaptan, and other characteristic compounds (51–53). It is reported that H_2S-producing bacteria was responsible for putrescine and cadaverine, however, Pseudomonas spp. contributed most to tyramine (54, 55). In our previous study, H_2S-producing bacteria grows from 2.1 to 8.9 lg CFU/g in cultured pufferfish after 18 days at 4°C, producing high levels of hexanal, 1-octen-3-ol, octanal, (E)-2-octenal and 2, 3-butanedione, which are volatile compounds causing strong fishy flavor (27).

The number of LAB was 1.6 lg CFU/g at the beginning (**Figure 1D**) and progressively increased in all samples during refrigerated storage but with slower rates in LYC-LVEO treated samples. The LAB number of CK, LYC-0.15%LVEO, LYC-0.30%LVEO, LYC-0.60%LVEO reached to 5.9, 5.7, 4.7, and 3.9 lg CFU/g, respectively, at the end of storage. This finding indicated that LAB was not primarily responsible for the spoilage in large yellow croaker samples during refrigerated storage, which is in consistence with trout filets packaged with probiotic carboxymethyl cellulose-sodium caseinate films (56). Although LAB was not the dominant microorganism of fish during refrigerated storage, they might cause the spoilage through producing a sour flavor and biogenic amines (47, 57).

Psychrophilic bacteria are the main microorganisms causing the spoilage of fish during refrigerated storage, thus decreasing the shelf life of fish (58). The number of psychrophilic bacteria was 1.0 lg CFU/g on 0 day and gradually increased with storage time (**Figure 1E**). The addition of LVEO significantly inhibited the growth of psychrophilic bacteria compared to CK ($p < 0.05$). Similar trend was found by Shokri et al. (59) with pectin based coatings containing clove essential oil.

In the current research, the counts of TVC, *Pseudomonas* spp., H_2S-producing bacteria, LAB and psychrophilic bacteria of large yellow croaker samples packaged with LVEO active coatings were significantly lower than that of CK during refrigerated storage ($p < 0.05$). Therefore, using LVEO treatments as an active coating could maintain the microbial quality of large yellow croaker samples during refrigerated storage.

Changes in TVB-N

Volatile nitrogen-containing compounds including ammonia, dimethylamine and TMA, known as TVB-N index, has been regarded as an indicator of spoilage in fish and fish products (60). The initial TVB-N value was determined as 8.17 mg/100 g (**Figure 2A**), indicting the raw fish being fresh. TVB-N contents of large yellow croaker samples progressively increased to 41.77, 27.72, and 25.26 mg N/100 g in the CK, LYC-0.15%LVEO and LYC-0.30%LVEO samples on 15th day exceeding the upper limit of 25 mg N/100 g (61). However, the LYC-0.60%LVEO samples were still under this limit at the end of storage as the 0.60% LVEO addition could inhibit the growth of SSOs or decrease the capacity of SSOs for oxidative deamination of non-protein nitrogen compounds (62). TVB-N results are consistent with TVC results. The effects of active coatings on TVB-N reduction of large yellow croaker have also been investigated. Shokri et al. (63) found that rainbow trout filets treated by chitosan with *Ferulago angulata* essential oil nanoemulsion could suppress the TVB-N growth and maintained acceptable freshness during a 16-day storage at 4°C. Dong et al. (64) also stated that active films containing Attapulgite loaded with Allium sativum essence oil pronounced lower TVB-N values in the preservation of large yellow croaker at 4°C under vacuum condition and extended shelf-life up to 9 days with 30 mg N/100 g fish as the TVB-N upper limit.

Changes in TMA

TMA is produced through the breakdown of trimethylamine oxide (TMAO) by bacterial and enzymatic activity; therefore, it can be used as an indicator of freshness for fish and fish products (65). Low initial TMA content (0.79 mg of TMA/100 g fish muscle, **Figure 2B**) indicates that the large yellow croaker samples were fresh, which was consistent with the relatively low TVC counts. Moreover, H_2S-producing bacteria could reduce TMAO to TMA and have low counts (1.5 lg CFU/g) at the beginning. The TMA of all the samples increased significantly ($p < 0.05$) during refrigerated storage. Jouki et al. suggested 5 mg N/100 g as an upper limit for rainbow trout (66). However, Klnc et al. (67) used 8 mg N/100 g as the limit of acceptability for sea bass. In the current research, the upper limit of TMA, as estimated by the TVB-N and TVC values, was 8 mg TMA/100 g for large yellow croaker samples. On the basis of this limit, CK, LYC-0.15%LVEO, LYC-0.30%LVEO, and LYC-0.60%LVEO samples exceeded the upper limit on 15th, 18th, 18th, and 18th day, respectively.

Changes in *K*-Values

K-value is widely used to quantify the fish freshness. The adenine nucleotides in degradation products promote spoilage and the formation of off-flavors, causing fish to lose their freshness (68). The *K*-value of large yellow croaker samples on 0 day was 11.73% (**Figure 2C**), staying at a very fresh level (*K*-value < 20%), and increased continuously during the whole storage time. The fish samples were considered very fresh till approximately on 6th day for the LYC-0.30%LVEO and LYC-0.60%LVEO samples, comparing with CK on 3th day, indicating that LVEO could inhibit the ATP degradation. The *K*-value of CK increased with greater speed compared with those of the LVEO treated samples. The CK, LYC-0.15%LVEO, LYC-0.30%LVEO and LYC-0.60%LVEO samples increased to 77.93, 71.33, 68.06, and 64.03% on 12th, 12th, 15th, and 18th day, respectively, exceeding the acceptable limit of 60% (69). The result was similar to Dong et al. (64), who corroborated that *Allium sativum* essence oil could

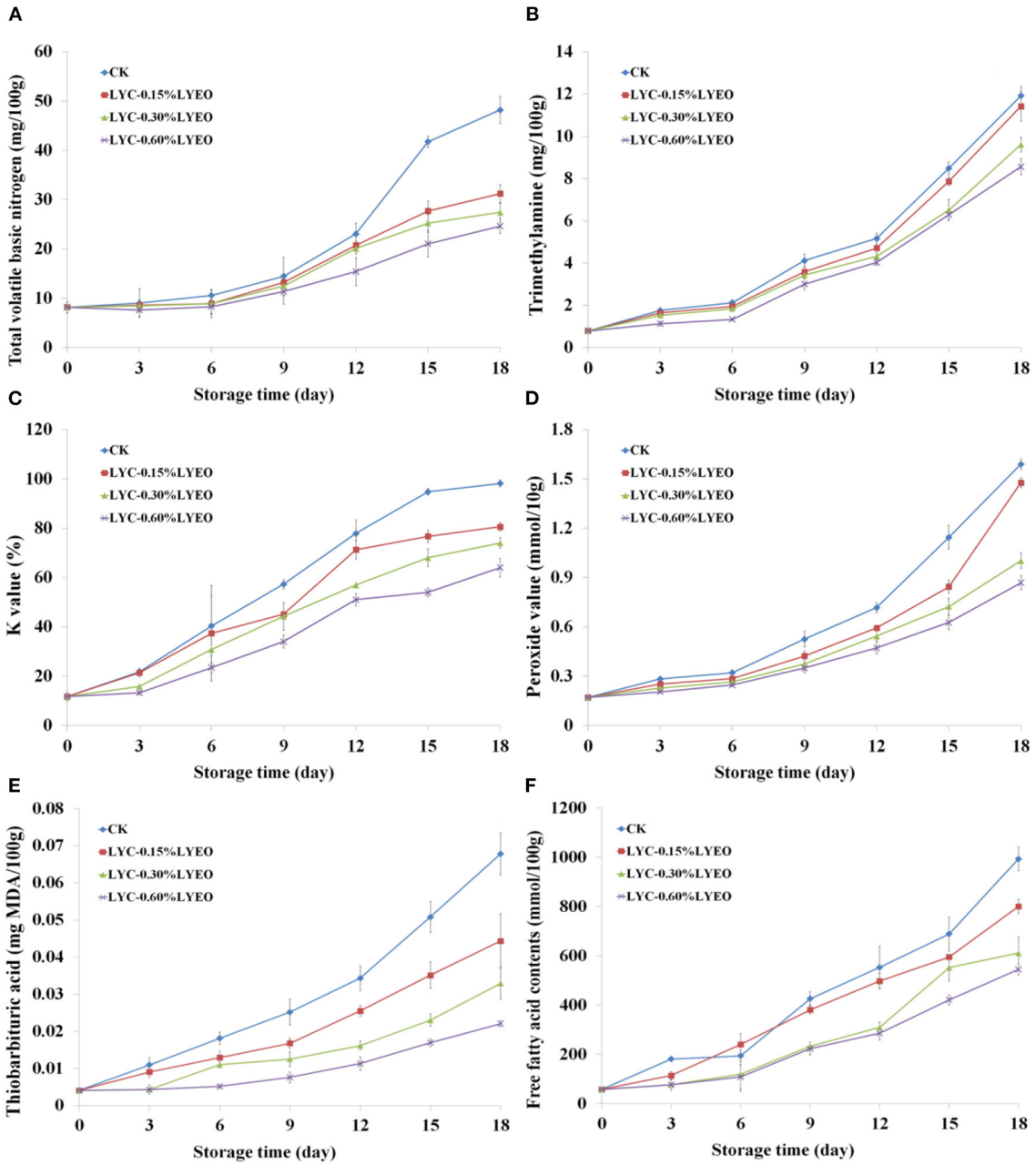

FIGURE 2 | Changes in total volatile basic nitrogen (TVB-N, **A**), trimethylamine (TMA, **B**), *K*-values **(C)**, peroxide value (POV, **D**), thiobarbituric acid reactive substance (TBARS, **E**), and free fatty acids contents (FFA, **F**) of large yellow croaker samples during refrigerated storage.

effectively inhibit the ATP degradation and maintain the high quality of large yellow croaker.

Changes in POV

POV is used to determine the formation of primary lipid oxidation products in fish and fish products during refrigerated storage (70). The POV on 0 day was 0.17 meq peroxide/kg fish (**Figure 2D**) and increased during refrigerated storage. The POV of all treated large yellow croaker samples increased during refrigerated storage, but at a slower rate in LVEO treated samples comparing with CK. The POV of the CK samples remarkably increased ($p < 0.05$) to 1.59 meq peroxide/kg fish at the end. This shows the CK sample was oxidized rapidly during refrigerated storage, while lipid oxidation in the LVEO treated large yellow croaker samples occurred more slowly. The delayed lipid oxidation was attributed to the release and diffusion of phenolic compounds presenting in the LBG-SA active coatings to the large yellow croaker samples. The phenolic compounds

TABLE 1 | Changes in water distribution in different treated large yellow croaker samples on 0 day, 9th day, and 18th day during refrigerated storage.

	Time	CK	LYC-0.15%LVEO	LYC-0.30%LVEO	LYC-0.60%LVEO
pT_{21}/%	0 day	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1
	9th day	2.2 ± 0.1a	2.2 ± 0.03a	2.1 ± 0.1a	2.2 ± 0.2a
	18th day	2.2 ± 0.04a	2.2 ± 0.1a	2.1 ± 0.1a	2.2 ± 0.1a
pT_{22}/%	0 day	95.4 ± 0.4	95.4 ± 0.4	95.4 ± 0.4	95.4 ± 0.4
	9th day	93.3 ± 0.2a	93.4 ± 0.2a	94.0 ± 0.1b	94.0 ± 0.2b
	18th day	88.3 ± 0.3a	89.3 ± 0.3b	90.3 ± 0.3c	90.2 ± 0.2c
pT_{23}/%	0 day	2.4 ± 0.3	2.4 ± 0.3	2.4 ± 0.3	2.4 ± 0.3
	9th day	4.4 ± 0.2a	4.4 ± 0.2a	3.9 ± 0.2b	3.8 ± 0.4b
	18th day	9.6 ± 0.3a	8.5 ± 0.2b	7.6 ± 0.4c	7.7 ± 0.2c

Different letters in same day from different groups indicate a significant difference ($p < 0.05$).

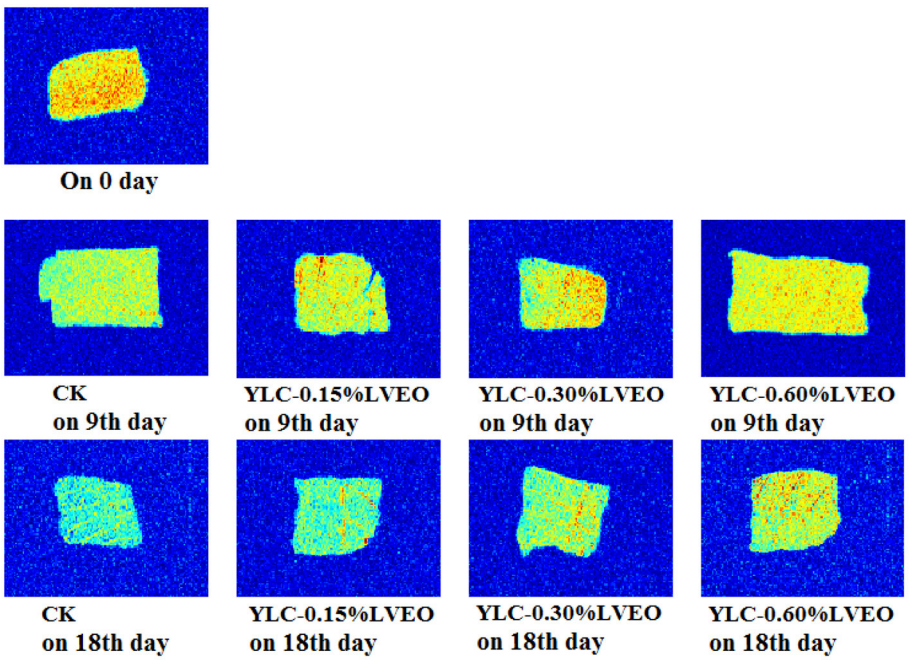

FIGURE 3 | Results of magnetic resonance image (MRI) of large yellow croaker samples under different treatment during refrigerated storage.

exhibited antioxidant activities, which are related with their free-radical scavenging ability and metal chelating capacities (71). These findings are consistent with those reported by Shadman et al. (72) who reported that *Zataria multiflora* Boiss. essential oil was effective in delaying the production of peroxide in rainbow trout (*Oncorhynchus mykiss*) filets during storage at refrigerated condition.

Changes in TBARS

Fish and other seafood are rich in unsaturated fatty acids, which are easily oxidized by heat, light and enzymes, resulting in undesirable rancid odor and poisoning (73). The increase in TBA may be described by the formation of secondary lipid oxidation products (74). As shown in **Figure 2E**, the initial amount of MDA in the large yellow croakers was 0.04 mg MDA/kg. TBA value increased in all samples until the end of storage; however, LVEO treated samples reached significantly ($p < 0.05$) lower TBA values of 0.22–0.44 mg MDA/kg of fish comparing with CK, which attained a higher level of 0.67 mg MDA/kg of fish. A TBA level of 5 mg MDA/kg of fish muscle comprises the threshold for detecting off-odors and off-taste at refrigerated storage (56). In this research, TBA values in all samples were lower than such recommended limits during the entire storage period, which probably LBG-SA active coating can reduce the diffusion of oxygen to the surface of the fish and act as a barrier between the fish and its surroundings, thus inhibiting lipid oxidation (13). It was shown that LVEO treatments had antioxidant activity, due to a high content of neral (75), as evidenced by lower TBA values in the large yellow croaker samples packaged with LVEO coatings. Perumalla and Hettiarachchy (76) reported that the antioxidant

activities of the EOs are mainly manifested in the binding of transition metal ion catalysts, the prevention of radical chain initiation, interaction with the free radicals and decomposition of peroxides.

Changes in FFAs

Lipid hydrolysis development was measured by the FFAs formation (31). A progressive FFA formation was observed in all large yellow croaker samples during refrigerated storage (**Figure 2F**). However, no significant changes in FFAs contents were observed in LYC-0.30%LVEO and LYC-0.60%LVEO samples within the first 3 days. Hydrolysis of glycerol-fatty acid esters is an important change in the lipid content of muscle after fish death resulting in the release of free fatty acids, which is catalyzed by lipases and phospholipases (77). The accumulation of FFAs could be related to the activities of lipase and phospholipase in fish muscle, digestive organs and microorganisms, which were enhanced with storage time. The LVEO presence could produce lower FFAs formation due to the modification of the lipase environment leading to a partial inhibition of its catalytic action (31). Consistent with this research, the employment of LVEO inhibited FFAs formation in the refrigerated large yellow croaker samples.

Water Distribution by LF NMR Analysis

LF-NMR is an effective way to evaluate the freshness of fish and MRI is also an assistive method to understand water migration in fish during storage (78). In this study the transverse relaxation time T_2 showed a multi-exponential behavior, which suggests that the water is divided into populations in the muscle tissue. T_{21} ranged from 11.2 to 17.5 ms represent the fraction of strongly bound water. T_{22} (generally 100–400 ms) relates to the water within the organized protein structures (intra-myofibrillar) and T_{23} is the water in the space between myofibrils (extra-myofibrillar), which can be more easily mobilized by dripping or cooking and therefore susceptible to fish spoilage (79). The pT_{21}, pT_{22}, and pT_{23} correspond to the relative amount of bound water, immobilized water and free water, respectively (**Table 1**). Variations in relaxation times over time were expected in view of the changes in protein structure during deterioration. The pT_{21} rarely changed for large yellow croaker samples during refrigerated storage as the bound water held within highly organized myofibril structures (36). Some changes in pT_2 suggested protein degradation in muscle tissue for large yellow croaker samples during refrigerated storage. The pT_{22} decreased progressively while pT_{23} increased for large yellow croaker samples during refrigerated storage. The CK had significant ($p < 0.05$) lower immobilized water content (from 95.35% at the beginning to 88.27% on 18th day) than that of other samples. However, no significant differences ($p > 0.05$) were shown in the immobilized water contents between LYC-0.30%LVEO and LYC-0.60%LVEO samples. The pT_{23} increased during refrigerated storage, however, the LVEO treated large yellow croaker samples had lower free water content than that of CK.

MRI has attracted more and more attention for providing the visual information of spatial, internal morphological organization and molecular distribution in food matrix (73).

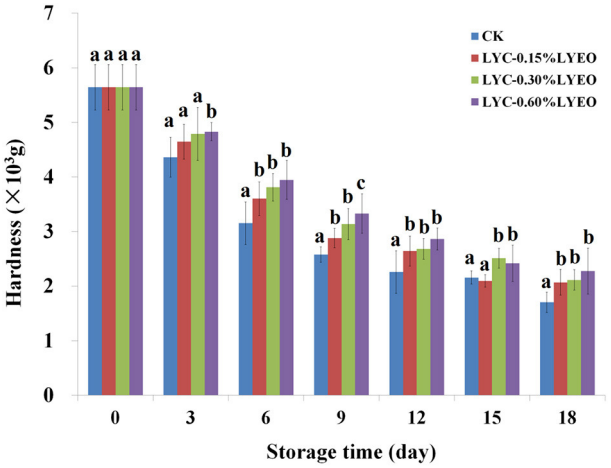

FIGURE 4 | Changes in hardness of large yellow croaker samples during refrigerated storage. Different letters in same day from different groups indicate a significant difference ($p < 0.05$).

Changes of water distribution of refrigerated large yellow croaker samples were investigated by T_1 and T_2 weighted images with MRI. Corresponding pseudo-color images are shown in **Figure 3**, in which red is the region with high proton signal density, and blue is the region with low proton signal density. There was no significant difference in MRI brightness of LVEO treated large yellow croaker samples on 9th day (**Figure 3**). Besides, the brightness of T_1 and T_2 images varied obscure and the brightness of the samples became darker and bluer during refrigerated storage. The color of CK samples was darker and bluer than other samples on 18th day and the brightness of LVEO treated samples were lighter compared with CK, which indicated the microstructure degradation and destruction of myofibril was more serious in CK sample (80).

Changes in Hardness

The hardness was 5.64×10^3 g on 0 day (**Figure 4**) and decreased significantly ($p < 0.05$) in all large yellow croaker samples because the muscle became softer probably due to the autolytic activity of enzymes, the hydrolysis of protein and the destruction of connective tissue (40). The CK samples showed the fastest softening rate, losing about 69.86% of its hardness at the end, and LVEO treated samples had higher values of hardness than CK, which suggested LBG-SA active coatings incorporated with LVEO could decrease the loss of large yellow croaker samples hardness during refrigerated storage. Decreases in hardness of large yellow croaker during refrigerated storage was related to the enzymatic degradation of muscle proteins and thereafter accelerated by microbial activity (53). In the current study, LVEO treated samples reduced the loss of hardness by inhibiting the microbial growth with LVEO.

Organoleptic Evaluation Results

The organoleptic evaluation results including color, elasticity, mucus, muscular tissue and smell of large yellow croaker samples

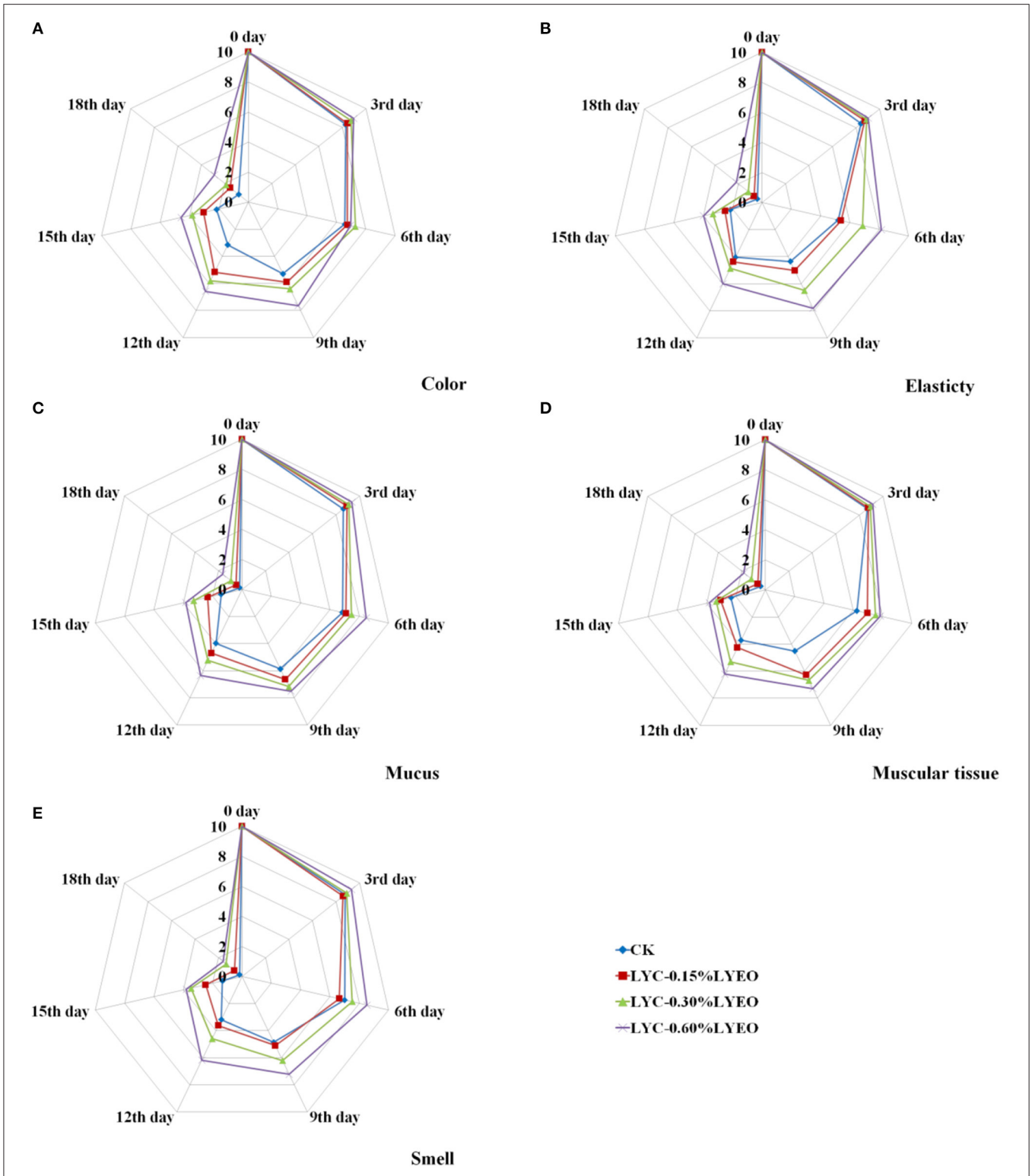

FIGURE 5 | Changes in color **(A)**, elasticty **(B)**, mucus **(C)**, muscular tissue **(D)**, and smell **(E)** of large yellow croaker samples during refrigerated storage.

during refrigerated storage at 4°C are shown in **Figure 5**. At the beginning, all samples had high scores proving the good quality and the scores decreased significantly ($p < 0.05$) with the prolonging of storage time. However, the organoleptic results showed that the scores of the LVEO treated samples were significantly higher than that of CK. Therefore, the method

of treating with the LBG-SA coating incorporated with LVEO could effectively delay the quality deterioration and maintain the organoleptic quality of large yellow croaker. At the end of storage, the scores of all the samples were lower than the limit value of 4 and they were considered as unacceptable for large yellow croaker samples in this research. The organoleptic results could directly show whether the large yellow croaker samples have gone spoiled during refrigerated storage. However, it took at least 2 or 3 days to get the chemical and microbiological results. The shelf life of perishable foods can be extended by reducing lipid oxidation and microbial reproduction. The inhibition of lipid oxidation can be attributed to the antioxidant properties of the active coatings, thereby reducing the production of unpleasant odors and flavors. The positive effects of EOs on the organoleptic properties of food have been demonstrated in some research (81). It should be noted that the smell of LVEO was also detected in the organoleptic evaluation; however, the influence on large yellow croaker was limited at this concentration. Besides, some of the observed changed could be due to inhomogeneities since the coating homogeneous and thickness was not measured.

CONCLUSIONS

The LBG-SA active coatings incorporated with different LVEO concentrations were applied to evaluate the effects on quality improvement of large yellow croaker samples spoilage during refrigerated storage at 4°C for 18 days. This research focused on exploring the effect of LVEO on the quality of large yellow croaker samples during refrigerated storage and the large yellow croaker without LBG-SA active coating was not considered in the experiment. The results of microbiological and physicochemical analyses showed that the LBG-SA films incorporated with 0.30% LVEO and 0.60% LVEO emulsions treated large yellow croaker samples maintained better quality during refrigerated storage, which mainly due to that LVEO could effectively inhibit the growth of SSOs and resist to oxidation to extend the shelf life. LYC-0.30%LVEO and LYC-0.60%LVEO had similar effects in slowing down the spoilage of large yellow croaker; however, 0.60% LVEO addition gave the active coating solution a strong flavor. Therefore, 0.30% LVEO addition combined with refrigerated storage at 4°C could be suitable for maintaining the freshness of large yellow croaker samples and extended the shelf life.

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/s.

AUTHOR CONTRIBUTIONS

BL, JM, and JX: conceptualization. BL, XW, XG, LZ, and XM: data curation. BL, XW, and JM: formal analysis. JX: funding acquisition and validation. BL, LZ, and JM: investigation. BL, LZ, JM, and JX: methodology. JM and JX: project administration, writing-review, and editing. BL and JM: software and writing-original draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.678643/full#supplementary-material

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REVIEW
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Marine-Derived Collagen as Biomaterials for Human Health

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Collagen is a kind of biocompatible protein material, which is widely used in medical tissue engineering, drug delivery, cosmetics, food and other fields. Because of its wide source, low extraction cost and good physical and chemical properties, it has attracted the attention of many researchers in recent years. However, the application of collagen derived from terrestrial organisms is limited due to the existence of diseases, religious beliefs and other problems. Therefore, exploring a wider range of sources of collagen has become one of the main topics for researchers. Marine-derived collagen (MDC) stands out because it comes from a variety of sources and avoids issues such as religion. On the one hand, this paper summarized the sources, extraction methods and characteristics of MDC, and on the other hand, it summarized the application of MDC in the above fields. And on the basis of the review, we found that MDC can not only be extracted from marine organisms, but also from the wastes of some marine organisms, such as fish scales. This makes further use of seafood resources and increases the application prospect of MDC.

Keywords: marine-derived collagen, tissue engineering, drug delivery system, cosmetics, food, health care product

INTRODUCTION

Collagen is a kind of biological macromolecule, which is the richest protein in the human body, accounting for more than 30% of the total body protein (1). It is the main material of extracellular matrix of skin, bone, ligament, cartilage and tendon. More than 85% of human collagen is type I, while other common types of collagens include type II, III, and IV. Collagen is a trimer composed of three polypeptide α chains (2). And it has a typical triple helix structure and glycine, proline and hydroxyproline residues is rich.

Collagen as a biomaterial is widely used in various fields due to its biocompatibility, biodegradability, accessibility and high throughput (3, 4). However, the health of collagen extracted from cattle and pigs is very worrying due to diseases (5). For example, outbreaks of bovine spongiform encephalopathy (BSE), infectious spongiform encephalopathy (TSE) and foot-and-mouth disease (FMD) have aroused wide health concerns about the use of collagen and collagen derived products in terrestrial animals (6). In addition, religious disputes are inevitable (7). At present, collagen has been extracted from many marine products. Marine-derived collagen (MDC) solves the problems of other animal diseases and pathogens. And, MDC has better chemical and physical durability and is abundant in quantity (8, 9).

In recent years, MDC in various fields has been widely used due to its extensive sources, simple extraction methods, good biocompatibility, edibility and so on. This paper summarizes the sources, extraction methods and characteristics of MDC. In addition, the application of MDC in medical tissue engineering, drug delivery, cosmetics, food and other fields was reviewed. On this basis, we preliminarily explored the biocompatibility of gill dolphin collagen and tilapia collagen as well as the application in skin tissue engineering.

Many researchers have been looking for alternative sources of collagen in aquatic animals (10, 11). With the extraction of MDC, fish skin, fish scale and other fishery wastes have been better utilized. Transforming waste into collagen solves the environmental problems related to fish (12, 13). The use of collagen derived from terrestrial animals is controversial due to the problems related to disease, religion and so on. However, as a biomaterial with a wide range of sources, MDC has attracted more and more researchers because of its good biocompatibility and degradation properties.

SOURCE, EXTRACTION, AND CHARACTERIZATION

With the increasing demand for collagen, new materials are needed as the source of collagen (**Tables 1**, **2**). Extracting collagen from marine organisms can not only avoid the problem of religious belief, but also has its unique properties. The efficiency and effectiveness of collagen extraction process has always been considered in the process of collagen extraction. Compared with the conventional acid assisted and pepsin assisted extraction of collagen, the collagen extracted by the improved physical assisted process retains a higher molecular weight, and the peptide spectrum is similar to that extracted only with acid (88). In addition, collagen extracted from dried jellyfish and squid has potential applications in biomedicine, medicine and health care products (89). As shown in **Table 3**, methods of extracting MDC are reported.

Electrodialysis is also a promising technology, but it has not been applied to the extraction of fish collagen. At present,

TABLE 1 | Characterization and amino acid characteristics of MDC.

Sources	Type	Characterization methods	Amino acids (composition, content, and characteristics)	References
The skin of Nile tilapia (*O. niloticus*)	Marine collagen peptides	Amino acid analysis	Seven essential amino acids (16.18%) and 10 non-essential amino acids (79.56%); Accounting for over 58% of the total residues in MCPs, were hydrophilic.	(14)
Jellyfish *Rhizostoma pulmo (jCOL)*	Collagen	Biochrome		(15)
Axinella cannabina; Suberites carnosus	Intercellular collagen (ICC)	UV or fluorometry		(16)
Mussel byssus	Collagen	High performance liquid chromatography (HPLC)	Amino acid composition of PSC obtained was similar regardless hydrolysis conditions	(17)
Tra catfish (*Pangasianodon hypophthalmus*), clown knifefish (*Chitala ornata*), and tilapia (*Oreochromis niloticus*)	Acid-soluble collagen (ASC)	Amino acid analysis	glycine 33.2–33.7%; The content of proline and hydroxyproline (imino acid) of collagen from three fish skins is 19.2–20%.	(18)
Takifugu flavidus	Collagen	Amino acid analysis	Gly was the most abundant residue; accounting for a quarter of the total amino acid components.	(6)
Eleven fish species inhabiting wide spectrum of temperatures	Acid Soluble Collagends (ASCs)	Circular Dichroism (CD)	Substitution from Hyp to Ser allows greater flexibility in the collagen triple helix; maintaining stability with seryl hydroxyl group driven hydrogen bonds.	(19)
Codfish skin	Collagen	Biochrom	Collagen type I consists of 20 different amino acids organized; three α-chains which wrap around each other; characteristic triple-helix conformation.	(20)
Sturgeon (*Acipenser schrencki* × *Huso dauricus*)	Type II collagens	Automated amino acid analyzer	The glycine abundant	(21)
Skipjack Tuna (*Katsuwonus pelamis*)	Scale gelatin (TG) and antioxidant peptides (APs)	SDS-PAGE; Fourier transform infrared spectroscopy (FTIR); electrospray ionization mass spectrometers (ESI-MS); radical scavenging assays	TG with a yield of 3.46 ± 0.27% contained Gly (327.9 ± 5.2 residues/1000 residues); content was 196.1 residues/1000 residues; TG was more unstable than that of type I.	(22)
Surf clam shell (*Coelomactra antiquata*)	Collagen	Hitachi L-8800 auto amino acid analyzer (Hitachi, Tokyo, Japan)	Guanidine hydrochloride soluble collagen (GSC) and pepsin soluble collagen (PSC) contained glycine as the major amino acid.	(23)

TABLE 2 | Sources of various marine-derived collagen (MDC).

Species	Tissue or organs	References
Tilapia	Skin	(14, 24–27)
	Scale	(28–35)
	Unknown	(36–39)
Jellyfish	Unknown	(40–47)
Shark	Skin	(48–51)
	Cartilage	(52)
Salmon	Skin	(53–59)
	Bone	(60)
	Scale	(60)
Sponge	Unknown	(27, 61–66)
Snakehead fish	Scale	(67)
	Unknown	(68)
Tuna	Skin	(69)
	Unknown	(70)
Others: Prionace glauca	Skin	(71)
Giant croaker (Nibea japonica)	Swim bladder	(72)
Sole fish	Skin	(73)
Codfish	Skin & bone	(74)
Sparidae	/	(75)
Sturgeon fish	/	(76)
Gadiformes	Skin	(77)
Mrigal fish	Scale	(78)
Flatfish	Skin	(79)
Weever	Skin	(80)
Seabass	Scale	(81)
Silver carp	Skin	(82)
Synodontidae fish	Scale	(83)
Eel	Skin	(84)
Codfish	/	(20)
Gadus morhua	/	(85)
Cyprinus Carpio	/	(86)
Grouper	Swim bladder	(87)

the physical and chemical properties of flavonoid collagen are retained by electrodialysis, which fully shows its advantages in the experiment. Therefore, we can assume that electrodialysis can also improve the production environment of fish collagen (95). The extraction of collagen from fish skin improves the value of marine by-products and avoids the pollution caused by large amounts of waste. Taking Atlantic cod as an example, the extraction rates of collagen by acetic acid and pepsin were 5.72 and 11.14%, respectively (96). Compared with the traditional organic acid solution extraction, the extraction rate of collagen and the properties of products are improved by CO_2 acidification water, which has potential value in the field of biomedicine and cosmetics (97).

Marine resources have great potential (**Figure 1**). When looking for natural moisturizing cosmetics, sea cucumber is finally selected. Pepsin soluble collagen was extracted from sea cucumber wall. Its moisture retention and moisture absorption with tilapia collagen are better than those of glycerol, which shows the potential application of MDC in cosmetics (98) (**Table 4**).

All of the fish collagen extracts were found to have high levels of imino acids (227-232/1000 residues). All collagen is soluble at acidic pH. In addition, the high collagen content, especially in the skin, and the good thermal properties [thermal transition temperature (31.6–33.7°C) and thermal denaturation temperature (31.1–32.2°C)] of the extracted collagen suggest that they have great potential as a collagen substitute in mammals (70). The low denaturation temperature of sponge collagen enables gelatin extraction at a lower temperature than that of mammalian gelatin. MDC is considered to be an equivalent biomaterial that is safer than the land-based biomaterials that currently dominate the market. The results showed that sponges A. cannabina and S. carnosus could be used as substitutes for collagen. If marine sponge is used as gelatin raw material in the food industry, it will bring high economic benefit (16). MDC also has promising applications *in vitro* 3D bioprinted models. But not the product of the modification of collagen and collagen denaturation gelatin easy rapid degradation. In order to solve this problem, in past research, scientists have developed a collagen and gelatin crosslinking of the chemical and physical methods, increasing the tunability of their mechanical properties. Marine collagen can be used as a biomaterial for tissue engineering and 3D bio-printing by controlling the content of methacrylate, as well as the intensity duration of ultraviolet light and the concentration of photoinitiator to control the required degree of cross-linking (122). The hydrogel with rheological characteristics was prepared by combining high-yield collagen with chondroitin sulfate. In addition, prionace glauca (PG) pepsin-soluble collagen (PSC) combined with shark-derived chondroitin sulfate produces a hydrogel with a cohesive polymer matrix that can be used for cartilage regeneration (91). The results showed that the best collagen yield was obtained when the papain concentration was 7,000 U/mg, and the pH value was 5.90, 22.79% collagen was hydrolyzed with alcalase and then separated by gel filtration chromatography. Compared with unhydrolyzed collagen, the four major components of the hydrolyzed product showed significant antioxidant and antiglycosylation activity (123). Based on previous studies, we summarized some characterization methods of MDC, aiming to understand the characteristics of the components of MDC (**Table 1**).

MDC IN MEDICAL TISSUE ENGINEERING

Tissue engineering and regenerative medicine is an emerging and rapidly growing life sciences domain. Using engineering and biological theory to create biomimetic tissues and organs on the basis of biological materials has become a common idea and hot topic among scientists in recent years. The excellent biocompatibility of MDC has stimulated its potential role in the design of biomaterial scaffolds in tissue engineering and regenerative medicine (**Figure 2**; **Table 4**).

TABLE 3 | Extraction methods of MDC and their advantages and disadvantages.

Sources	Extraction method	Principle	Advantages	Disadvantages	References
Mussel byssus	Pepsin solutions	Pepsin is typically indiscriminate in its digestion of proteins, with the notable exception of the triple helical domain of native collagen with further limited pepsin digestion, the cross-linked molecules at the telopeptide region are cleaved without damaging the integrity of the triple helix.	• Ensure the integrity of the collagen molecule		(17)
Axinella cannabina; Suberites carnosus	Alkaline solubilization, trypsin solubilization	The first method was initially introduced for the isolation of insoluble collagen (InSC) from *G. cydonium* and *C.reniformis* by employing an alkaline, both denaturing and reducing, homogenization buffer affording collagen in high yield; The second one utilizes a trypsin-containing extraction buffer, known to destroy the interfibrillar matrix and, therefore, releasing the collagen fibrils (ICC). After exhaustive water extraction, the remaining debris generally comprises the spongin/spongin-like collagen.		• Reagent residues in collagen; • Generate abundant waste liquid; • Resulting in environmental pollution	(16)
Surf clam shell (*Coelomactra antiquata*)	Guanidine hydrochloride and pepsin		• Safer; • Cheape; • More moderate; • Less destructive than acid hydrolysis		(23)
Indian major carp rohu (Labeo rohita)	Enzymatic method				(90)
Bigeye tuna	Acetic acid and pepsin				(70)
Shark (Prionace glauca) and ray (Zeachara chilensis and Bathyraja brachyurops)	Acidic and enzymatic extractions				(91)
Codfish skin	An acid-base procedure			• Ineffective with byssal threads	(20)
Salmon Byproducts	Bacterial extracellular proteases fermentation	The proteases secreted by marine bacteria play an important role in the decomposition of organic nitrogen in oceans.	• Potential bioactive peptides would be released; • The reaction time is shortened.		(92)
Nile tilapia (*Oreochromis niloticus*) skin	Collagen extraction after fermentation pretreatment		• Type I collagen with high purity; • Retained the integrity of their triple helical structure.		(93)
Jellyfish (Acromitus hardenbergi)	Physical-aided acid-assisted extraction method	Increase physical intervention.	• Similar amino acids composition; • Retained high molecular weight distributions;		(88)
Takifugu flavidus	Electrodialysis extraction	This method can purify charged proteins/peptides by ion-exchange membranes through a stimulated diffusion process under the influence of electric potential difference.	• High efficiency; • Large capacity; • High extraction yield; • Better environmental sustainability		(94)
	Freeze drying and electrospinning processes				(40)
Tilapia	Electrospinning		• Simple operation		(36)

Bone Tissue Engineering

MDC with its high hydrophilicity and amino acids provides the optimal extracellular microenvironment and has many applications in bone tissue. It can promote the proliferation and differentiation of osteoblasts, and the bone marrow mesenchymal stem cells (BMSCs) that induce osteoblastic differentiation retain their immunomodulatory function. For example, tilapia collagen can promote the growth and differentiation of osteoblasts without the use of any additional induction reagents (28, 49), just as human bone marrow mesenchymal stem cells (hMSCs)

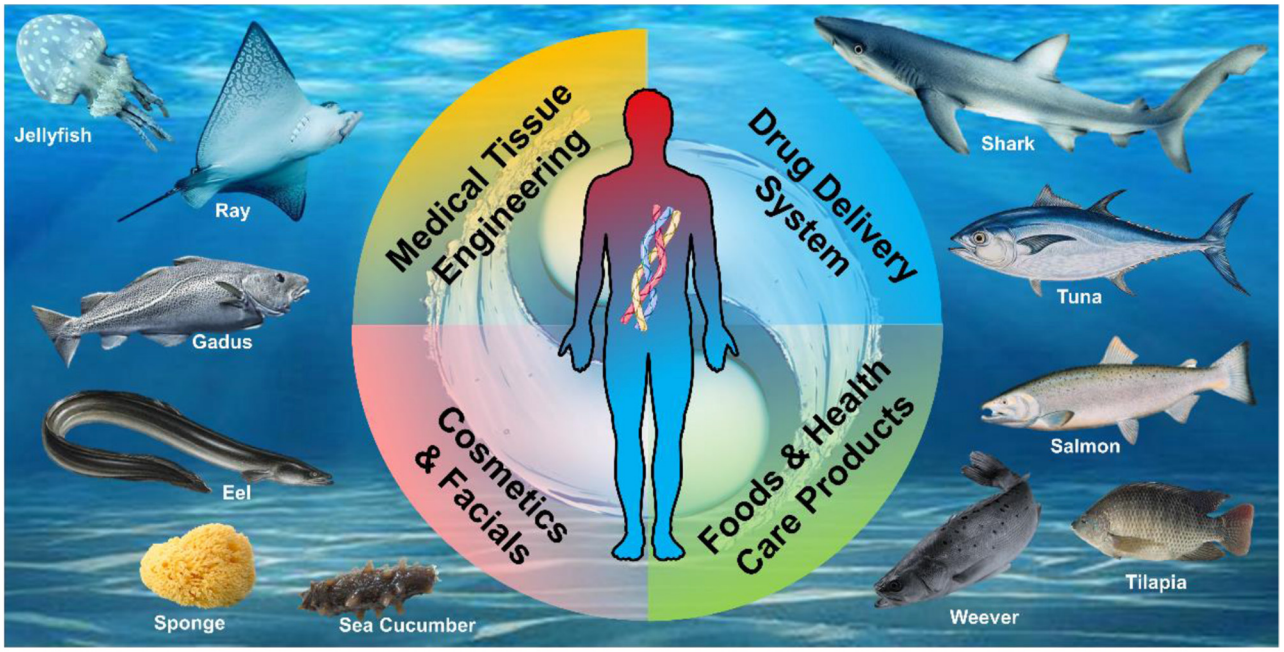

FIGURE 1 | Abundant marine-derived collagen (MDC) as biomaterials are extracted from various marine organisms for human health.

readily adhere to tilapia squamous collagen during cell culture *in vitro*, thus significantly accelerating the early differentiation of hMSCs into osteoblasts (75). Biphase scaffolds of biomimetic mineralized salmon collagen and fibrotic jellyfish collagen were prepared by combining lyophilized and cross-linked methods, indicating that they can support chondroblast and osteogenic differentiation of hMSCs *in vitro* (41). Shark skin collagen also promotes the growth of osteoblasts and the synthesis of collagen in bone cells (49). When this collagen was further mixed with calcium phosphate from shark teeth to form a 3D composite scaffold, it could support the attachment and proliferation of osteoblast-like cells (48). Some researchers also found that the collagen peptide extracted from the scales of two kinds of fish, Sephareidae, can promote the proliferation of osteoblasts and inhibit the proliferation of mature osteoclasts, which can be used to prevent osteoporosis and help bone remodeling (50). In Codfish, low concentration of fish collagen peptide (FCP) may promote the proliferation of cells, and also promote the expression and differentiation of apoptotic osteoblasts (74). Collagen in salmon skin can also significantly up-regulate gene expression of various collagen-modifying enzymes in mouse pre-osteoblastic cells (MC3T3-E1) osteoblasts, which has a positive effect on osteoblasts (59).

MDC can also play a great role in bone development and bone injury repair. They used a sponge-collagen-based (SPG) scaffold and photobiodularization (PBM) to test a model of skull defect in Wistar rats. The results showed that SPG/PBM treated rats showed more connective tissue and newly formed bone tissue in the defect area (66). Mixing sponge collagen with hydroxyapatite (HA) to form scaffolds has the potential to improve graft performance for bone regeneration applications (62). MDC peptide (MCP) extracted from salmon skin was used to study the femur of growing rats. The results showed that MCP supplementation could increase the femur volume, bone density, dry weight and ash content of growing male rats. Therefore, MCP supplementation could promote the development of long bone in growing male rats (104): The effects of MDC oligopeptides and calcium aspartate on bone mineral density in ovariectomized Wistar rats were studied. It was found that the combination of MDC oligopeptides and calcium aspartate could significantly improve bone mineral density, which also indicated that MDC oligopeptides could promote the absorption of calcium aspartate (103). All these indicate that MDC has a good effect on bone growth and development.

In future studies, MDC may also provide new options for bone grafting and regeneration. Researchers have successfully developed a novel collagen fiber wikestone hydrogel based on the dual network (DN) concept using fish swim bladder collagen (SBC) extracted from sturgeon. The gel was implanted into the osteochondral defect of rabbit knee joint and showed good biomechanical properties *in vivo*. Mixed with hydroxyapatite wrapped DN gel combined with bone also is good. This kind of new collagen matrix composite DN gel has good biomechanical properties and combined with bone, is a kind of soft, elastic ceramic material, to design the next generation of orthopedic implants as artificial cartilage, the body weight bearing area of bone defect repair material provides a new choice (76). For bone regeneration, low immunogenicity fish collagen protein and bioactive nano-hydroxyapatite (N-HA)-reinforced polylactide glycogen (PLGA) nanofiber membranes were prepared for

TABLE 4 | Applications of MDC in medical tissue engineering.

Applications	Manufacture technique	Forms	Additive materials	Biological evaluation	References
Bone Tissue Engineering	/	Scaffolds	Hydroxylapatite	/	(99)
	/	Scaffolds	Hydroxylapatite	/	(100)
	Freeze-drying and EDC cross-linked	Scaffolds	Alginate	hMSCs	(41)
	/	Solution	Moringa oleifera	Albino rats	(101)
	Freeze-drying/dehydrothermal treatment	Scaffolds	Glycosaminoglycan	MC3T3-E1	(48)
	/	Solution	/	Mouse	(49)
	Hydrolysis	Solution	/	BMSCs	(102)
	/	solution	/	Human osteoblasts	(74)
	/	Scaffolds	/	Wistar rats	(66)
	/	Peptide solution	Calcium aspartate	Rats	(103)
	Freeze-drying	Native collagen	/	Primary hMSCs	(28)
	Enzymatical hydrolysis	Peptide	/	Rats	(51)
	Enzymatical hydrolysis	Peptide	/	MG-63 cells	(75)
	Freeze-drying/EDC cross-linked	Scaffolds	/	NIH3T3, MG-63 cells and Mouse	(104)
	Freeze-drying/EDC/NHS or HMDI cross-linked	Scaffolds	/	Saos-2 cells	(50)
	Freeze-drying	Scaffolds	Chitosan/Hydroxyapatite	MG-63 cells	(61)
	Freeze-drying/Glutaraldehyde cross-linked	Scaffolds	Chitosan/Hydroxyappatite	6T-CEM cells	(52)
	Vacuum drying	Scaffolds	Hydroxyapatite/PMMA	MC3T3-E1 cells and L929 cells	(62)
	Glutaraldehyde cross-linked	Scaffolds	Poly (N, N'-dimethylacrylamide)	Rabbit bone defect model	(76)
	Electrospinning	Scaffolds	PLGA/Hydroxyapatite	Primary BMSC and Human gingiva fibroblasts	(6)
Cartilage tissue engineering	Freeze-drying/Chemical cross-linking.	Scaffolds	/	hMSCs	(105)
	Freeze-drying	Scaffolds	/	Rabbit	(106)
	Freeze-drying	Collagen solution	/	hMSCs	(42)
	Freeze-drying	Scaffolds	/	Rabbit chondrocytes and Rude mice	(107)
	Cryogelation	Scaffolds	/		(71)
	Freeze-drying	Peptide solution	/		(91)
	Enzymatical hydrolysis	Peptide	/	Primary horse adipose-derived stromal cells	(108)
	Enzymatical hydrolysis	Peptide	/	Rabbit osteoarthritis model	(77)
	Acid soluble	Native collagen	/	hMSCs	(29)
	Freeze-drying/EDC cross-linked	Scaffolds	/	Primary human and rat nasal septum chondrocytes and Rat septal cartilage defect model	(45)
	Freeze-drying/EDC cross-linked	Scaffolds	Alginate	hMSCs	(47)
	Enzymatical hydrolysis	Peptide	/	Human (clinic)	(109)
	/	Scaffolds	/	Rats	(45)
Dental tissue engineering	Enzymatical hydrolysis	Peptide	/	Rat odontoblast-like cells (MDPC-23)	(30)
	Enzymatical hydrolysis	Peptide	/	Primary human periodontal ligament cells	(31)
	Elecrospinning	Scaffolds	Bioactive glass/Chitosan	Primary human periodontal ligament cells and dog furcation defect model	(38)
Nerve regeneration	Enzymatical hydrolysis	Peptide solution	/	Rats	(53)
	Acid dissolution	tilapia collagen gel	/	hiPSCs	(37)
Skin tissue engineering	Enzymatical hydrolysis	Peptide solution	/	Rabbit	(14)

(Continued)

TABLE 4 | Continued

Applications	Manufacture technique	Forms	Additive materials	Biological evaluation	References
	Electrospinning	Nanofibers	/	Rats	(24)
	Hydrolyze	Peptide solution	/	/	(110)
	Solvent casting/Glutaraldehyde cross-linked	Scaffolds	/	L929 cells and Rat wound model	
	Freeze-drying/glutaraldehyde cross-linked	Scaffolds	/	Primary human fibroblasts and keratinocytes and Rat wound model	(78)
	Freeze-drying/Dehydrothermal treatment at 105°C	Scaffolds	Shrimp shell chitosan/glycerin	Primary human fibroblasts and keratinocytes	(35)
	EDC cross-linked	Scaffolds	/	NIH3T3 cells	(111)
	Freeze-drying/EDC cross-linked	Scaffolds	Alginate/Chitooligosaccharides	Primary human dermal cells	(79)
	Freeze-drying	Scaffolds	/	Hamster kidney fibroblasts (BHK21)	(112)
	Freeze-drying	Scaffolds	/	Rat wound model	(26)
	Freeze-drying/EDC cross-linked	Scaffolds	Chitosan	Mouse embryonic fibroblasts (MEF) and Rabbit wound model	(80)
	Enzymatical hydrolysis	Peptide	/	Human keratinocyte (HaCaT) and Rabbit scald wound model	(14)
	Enzymatical hydrolysis	Peptide	/	Rat wound model	(54)
	Electrospinning	Scaffolds	Bioactive glass	HaCaT cells, dermal fibroblasts and HUVECs	(36)
	Casting-solvent evaporation technique	Native collagen	/	Swelling behavior	
	Freeze-drying/ceftazidime cross-linked	Scaffolds	/	NIH3T3 cells	(113)
	Freeze-drying/Glutaraldehyde cross-linked	Scaffolds	/	NIH3T3 cells and HaCaT cells	(114)
	Electrospinning	Scaffolds	Chito oligosaccharides	Human skin fibroblasts	(115)
	Enzymatical hydrolysis	Peptide		Human (clinic)	(116)
	Enzymatical hydrolysis	Peptide	/	Human (clinic)	(117)
	Enzymatical hydrolysis	Peptide	/	L929 and HaCaT cells	(63)
Wound healing	/	Tilapia collagen extract	/	Rats	(24)
	/	Peptide solution	/	SD rats	(118)
	Enzymatical hydrolysis	Formulated into a cream	/	Male white rats (Rattus norvegicus)	(68)
	Electrospinning	Nanofibers	/	HaCaTs and SD rats	(39)
	/	Hydrogel	/	Albino rats	(119)
	Freeze-drying	Peptide solution	/	Mice	(44)
	Freeze-drying	Peptide solution	/	NIH3T3 cells	(72)
Corneal tissue engineering	Decellularization/Decalcification	Scaffolds	/	Rat ocular implantation model	(32)
	Drying at 25°C	Native collagen	/	Human limbal epithelial cells	(81)
Vascular tissue engineering	/	Peptide solution	/	CAVECs and Wistar rats	(120)
	Freeze-drying/Cold-pressing/1,4-butanediol diglycidyl ether cross-linked	Scaffolds	/	Mouse lymphatic endothelial cells	(67)
	Electrospinning	Scaffolds	PLGA	Primary rabbit aortic endothelial cells and smooth muscle cells	(40)
Oral mucosa regeneration	Freeze-drying/Dehydrothermal cross-linked	Scaffolds	Chitosan	Primary oral keratinocytes	(33)
Spinal cord injury repair	/	Double-layer collagen membrane	/	/	(121)

hiPSCs, human induced pluripotent stem cells; hMSCs, human marrow stromal cells; BMSC, bone marrow stromal cells; HUVECs, human umbilical vein endothelial cells; CAVECs, carotid artery vascular endothelial cells.

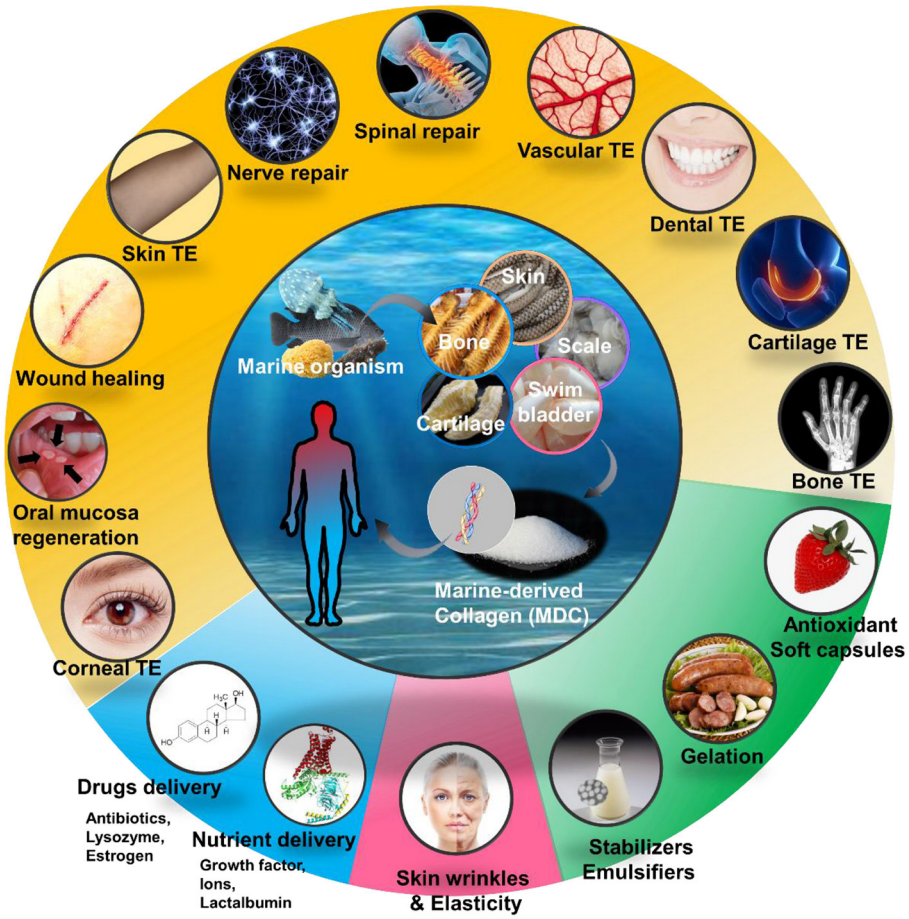

FIGURE 2 | Applications of marine-derived collagen (MDC) for various area in human health, mainly including medical tissue engineering (TE), drug delivery system, cosmetics and Facials, and foods and health care products.

electrospinning guided bone regeneration (GBR). It was found that the membrane had good cytocompatibility with bone marrow mesenchymal stem cells (BMSCs) and human gingival fibroblasts (HGF). The experimental results showed that the composite fibrous membrane has great potential to guide bone or tissue regeneration (6).

Cartilage Tissue Engineering

MDC plays a very important role in cartilage tissue and enhances chondroblast differentiation. For example, the researchers experimented with chondrogenic differentiation of human bone marrow mesenchymal stem cells (hMSCs) on the collagen fibers of tilapia scales and compared them with porcine collagen and uncoated culture dishes. The results showed that tilapia collagen fibrils in chondrogenic medium specifically enhanced chondrogenic differentiation of HMSCs. Therefore, collagen from tilapia scales can provide a suitable source of collagen for chondrogenic formation of hMSCs *in vitro* (29). Jellyfish collagen can also be used as a novel cartilage repair implant, using active growth factor nanoreservoir (TGF-β3), adult human mesenchymal stem cells derived from bone marrow. It was found that jellyfish type collagen implants led to chondrogenic differentiation of mesenchymal stem cells, and TGF-β3 as a nanoreservoir led to chondrogenic gene expression and chondrogenic differentiation (42). Using jellyfish collagen as a scaffold, for example, the researchers developed a three-dimensional porous scaffold with interconnected pores that can support and maintain chondrogenic differentiation of human mesenchymal stem cells (105). Porous scaffolds of jellyfish collagen fibers and sodium alginate hydrogels are also available (47). And it can stimulate the differentiation capacity of some other cells. For example, the researchers used the blue shark skin collagen with and without external stimuli induced human fat stem cells (hASC) their potential to differentiate into cartilage cells (71), and the sharks and rays of collagen combined shark chondroitin sulfate can be used to simulate human cartilage extracellular matrix. That suggests the MDC, a biomaterial, can be used as a template for cartilage regeneration (91). MDC stimulated the differentiation of chondroblasts and further promoted the formation of cartilage. The researchers prepared a three-dimensional porous fish collagen (FC) scaffold using MDC by freeze-drying technique. When rabbit auricle chondrocytes

were implanted into porous fish collagen, it was found that it promoted the formation of chondrospecific extracellular matrix (ECM) *in vivo* and *in vitro*, and thus promoted the formation of cartilage under the rabbit skin (106, 107). TGF-β1 can induce chondrogenesis of adipocyte stromal cells (ADSCs) by adding fish collagen to TGF-β1, which can induce chondrogenesis effectively (108).

In cartilage tissue repair, the MDC matrix provides excellent performance for cartilage tissue engineering through the experiments of nasal cartilage repair with MDC *in situ* model of rats (45). It also has a protective effect on cartilage (77).

Dental Tissue Engineering

In previous research, some researchers used type I collagen from tilapia scales in rat experiments to show that it has similar biocompatibility with pig skin collagen, which reminds us that tilapia scales collagen has the potential to replace mammalian type II collagen in oral and maxillofacial tissue regeneration. Soon after, the researchers carried out the periodontal membrane cell culture experiment of hydrolyzed tilapia collagen and proved that it had the function of periodontal tissue regeneration *in vitro*. The collagen of tilapia was extracted by electrospinning method, and the composite nanofiber membrane was prepared with bioactive glass and chitosan. The cell viability and osteogenic gene expression of human periodontal ligament cells (HPDLCs) were detected by the composite membrane in the canine class II bifection defect model experiment. It also promoted the expression of Runt-related transcription factor 2 (RUNX-2) and osteopontin (OPN) proteins (30, 31, 38). In conclusion, the application of MDC in teeth also has great potential.

Vascular Tissue Engineering

MDC also has some applications in vascular tissue. For example, MDC can promote the growth of vascular endothelial cells. Experimental studies have investigated the protective effect of MDC peptides (MCPs) on carotid vascular endothelial cells (CAVECs) in type 2 diabetes mellitus (T2DM) and its mechanism. They injected Wistar rats with different concentrations of MCPs. *In vitro*, the vascular/endothelial construction of human umbilical vein endothelial cells (HUVECs) was cultured. Then, inflammatory exudation and related molecular markers of the vena cava endothelial cells were detected and analyzed. The results showed that MCP treatment for 4 weeks significantly reduced blood glucose, endothelial thinning. And inflammatory exudation of carotid vascular endothelial cells was reduced in rats. *In vitro*, high glucose intervention increased apoptosis in HUVECs significantly. Moderate and high doses of MCPs partially improved this high glucose mediated apoptosis and reduced the level of apoptotic biomarkers. Therefore, moderate dose of MCP inhibits apoptosis and reduces the expression of coupling factor 6 and microparticles, suggesting that we can use MCP to prevent early cardiovascular complications of T2DM (120). Some researchers also used freeze-drying and electrospinning to prepare MDC and PLGA fiber tubular scaffolds for vascular transplantation, and the electrospinning fiber PLGA layer on the surface of porous tubular collagen scaffolds in dry and wet states improved the mechanical strength of collagen scaffolds. The results showed that co-culture of smooth muscle cells (SMCs) and endothelial cells (ECs) using a collagen-PLGA scaffold under a pulsating perfusion system enhanced the development of vascular EC and preserved the differentiated cell phenotype (40).

Due to the good biocompatibility of fish collagen, the researchers use extra methylation modification and 1, 4-butanediol diglycidyl ether (BDE) crosslinking steps to improve the scales of the collagen derived from the physical and chemical properties. It was found that collagen integration plaques with the surrounding tissue was good. The infiltration of cells, blood vessels and lymphatic vessels was good. This study demonstrates the collagen derived from fish scales as a promising scaffold material in various biomedical applications (67).

Spinal Cord Injury Repair and Nerve Regeneration

MDC has also been used in spinal cord regeneration. A new double-layer collagen membrane was designed and tested in a rat model of incomplete spinal cord injury. The previous research results showed that the transplantation of neural stem cells into a double-layer collagen membrane with different pore size promoted the differentiation of neural stem cells, alleviated the pathological injury, and improved the motor function of rats with incomplete spinal cord injury significantly (121).

Tilapia skin collagen was obtained by acid solution method and the stiffness of brain tissue was replicated for *in vitro* recombination experiments. By adding a cross-linker, a gel with a hardness similar to that of living brain tissue (150–1,500 Pa) was obtained, and the ability of the gel as a stem cell medium and the effect of hardness on neural lineage differentiation using human Induced pluripotent stem (iPS) cells were further investigated. It was found that exposure to a gel with a hardness of about 1,500 Pa promoted the production of neurons in the dorsal cortex during the early stages of neuroinduction (37).

To study the neuroprotective effects of MDC peptides (MCPs) isolated from salmon skin by enzymatic hydrolysis on perinatal asphyxia in male rats. Researchers found that MCPS promoted long-term learning and memory in perinatal asphyxia (PA) pups by decreasing oxidative damage and acetylcholinesterase (AChE) activity in the brain, and increasing the expression of p-CREB and brain-derived neurotrophic factor (BDNF) in the hippocampus (53).

Skin Tissue Engineering and Wound Healing

MDC has significant biological activity and plays an important role in skin tissue. MDC can promote wound healing. For example, the study used the MDC peptide (MCP) in Nile tilapia skin to carry out the burn wound experiment in deep part thickness of rabbits and the scratch experiment *in vitro* of rats (14). At the same time, there is also a research team, for example, using porous collagen sponge to conduct experiments on burned wounds in rats (114), using jellyfish collagen polypeptide to conduct oral experiments in rats and salmon skin wounds in rats (54), using ethylene amine and fish scale collagen to conduct

wound experiments in rats (113), all of which indicate that MCP can promote wound healing. Moreover, its suitability as a dermal substitute was found in the wound healing experiment of rat model (78). The researchers found that MDC could quickly and effectively promote the wound healing of rats (26). If it is made into scaffolds or nanofibers, it can also promote wound healing. The researchers prepared chitosan/sponge collagen/glycerin three-dimensional porous scaffolds and bionic electrospinning fish collagen/bioactive glass (COL/BG) nanofibers. The healing experiments on rat skin wounds also showed the ability of MDC to promote wound healing (35, 36). At the same time, MDC is also an excellent scaffold for skin tissue regeneration (79), and a potential wound dressing with antimicrobial properties (115).

In vivo experiments with MDC scaffolds from Cadfish and Weever showed that the scaffolds promoted the proliferation and migration of NIH/3T3 fibroblasts, and promoted tissue regeneration and healing (111, 124). Fibroblasts from small hamster kidney (BHK21) were inoculated on a three-dimensional collagen gel. The results showed that it could activate the proliferation of BHK-21 cells, so MDC could be used as a potential biomaterial extract for biomedical applications (112).

Hydrolyzed collagen is a kind of more and more popular health care products, its molecular weight is very low peptide, easy to be digested, absorbed and distributed by human body. Many clinical trials have been completed and current studies have shown the effects and benefits of collagen peptides on skin, such as hydration, elasticity and reduction of wrinkles. Therefore, hydrolyzed collagen can be considered an important weapon in the world every day in the fight against skin aging (116). Some researchers used hydrolyzed MDC to conduct experiments on the cheek skin of women aged 45–60 years old, and found that it could reduce skin wrinkles, enhance elasticity and tightness, improve gloss, and effectively improve the skin health (110). Orthosilicic acid, which hydrolyzes collagen and stabilizes it, which also has this effect (117).

MDC plays an important role in skin wound healing. Researchers used Nile tilapia skin collagen extract to promote skin wound healing in rats, and the experimental group showed obvious signs of skin healing. Moreover, the expression levels of vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1) were significantly increased, and the gene expressions of VEGF, basic fibroblast growth factor (bFGF) and Alpha-smooth muscle actin (α-SMA) were significantly up-regulated. These results indicate that local application of Nile tilapia collagen extract can promote skin wound healing in rats, which may be due to its stimulating effect on the recruitment and activation of macrophages to produce chemotactic growth factors, fibroblast proliferation and angiogenesis (24). The researchers also used Snakehead fish collagen, Queen Fish skin collagen, Rhizostoma pulmo jellyfish collagen, and Giant Croaker (Nibea japonica) swim Bladders Collagen Japan swim bladder was used for wound healing experiment. Results show that the snakehead ossein paste made of white male rats sewer rat wound healing the best dose of 3% concentration (68), preparation of fish skin collagen hydrogel promote epithelial regeneration, and no water gel processing rat inflammatory cells angiogenesis, collagen deposition and hexose amine content, epithelium and wound contraction increased significantly (119). At the same time, Jellyfish collagen promotes artificial wound formation on the monolayer of human umbilical vein endothelial cells (HUVECs) (44). Japanese loach swim bladder Acid-soluble collagen (ASC) and pepsin soluble collagen (PSC) have good application in wound healing of mouse *in vitro* scratches (72). The researchers also found that oligopeptide compounds derived from marine fish peptides (MFPs) have the potential to significantly increase uterine scar tension, reduce the risk of uterine rupture, and promote uterine wound healing in rats following cesarean section (CS). It is speculated that its promoting effect may be related to the formation of new capillaries in scar tissue, the growth and repair of collagen fiber and smooth muscle tissue (118).

Oral Mucosa Regeneration

MDC also plays a role in the repair of oral mucosa. Researchers prepared chitosan-collagen composite scaffolds (C3) to construct oral mucosal equivalents (EVPOME-C) *in vitro*, and compared EVPOME-C with oral mucosal equivalents (EVPOME-B) and natural oral mucosa constructed with Alloderm® (EVPOME-A) and Biomend®. The results showed that the C3 scaffold has a well-developed fiber network and a small enough porosity to prevent keratinocytes from growing in the scaffold after cell inoculation. The C3 scaffold has potential application value in epithelial tissue engineering, and provides a new treatment method for oral mucosal regeneration medicine (33).

Corneal Tissue Engineering

MDC has also been used in corneal tissue, in which fish scale-derived collagen matrix (FSCM) has been proposed as a substitute for human donor corneal tissue. To assess its biocompatibility, the FSCM was implanted as an anterior lamellar keratoplasty (ALK), placed in the interlamellar pouch (IL) and placed in the subconjunctiva (SC). The light transmittance was found to be similar to that through the human cornea. Implanting FSCM as an ALK resulted in only mild blurring, not pupil blurring, despite the presence of new blood vessels around the sutures; Interleukin placement causes moderate haze, partial occlusion of the pupil, and (partial) anterior lamella melting. The SC group showed local swelling and sclerosis, which decreased over time. Histology showed mild to moderate chronic inflammation in the ALK and IL groups, while severe inflammation was found in the SC group. Despite the technical difficulties, treatment of ALK with FSCM is feasible, while IL placement can cause anterior lamina melt. Further studies are needed to better understand its immunogenicity. The light scattering and transmission data suggest that the first version of the FSCM is comparable to human corneal tissue in this respect (32).

MDC IN DRUG DELIVERY SYSTEM

MDC plays an important role in the drug delivery system, as shown in **Table 5**. For example, the researchers report a simple method of preparing collagenous peptide-chelated calcium (CPCC) from marine fish scales and a novel CPCC-loaded nanoparticle to supplement calcium. Their experiments

TABLE 5 | Applications of MDC in drug delivery system.

Loaded drugs	Forms	Additive materials	Biological evaluation model	References
Antibiotic (ampicillin and tetracycline)	Powder and film	/	/	(64)
Lysozyme	Microparticles	/	/	(46)
Growth factor (bFGF)	Scaffolds	Chitosan/chondroitin sulfate/PLGA	Rat full-thickness skin wound model	(82)
Ion (Calcium)	Nanoparticle	Calcium alginate	Rats' femur	(83)
Ion (Calcium)	Injectable gel	Chitosan	Rats	(60)
α-lactalbumin	Microparticles	/	/	(46)
Estrogen (17-beta-estradiol-hemihydrate)	Nanoparticle	/	Postmenopausal women	(27)
Gastroresistant tablets	Enteric coating	/		(65)

showed that core-shell CPCC significantly increased bone mineral density and calcium content in the femur of rats, so the CPCC and core-shell CPCC nanoparticles are ideal choices for calcium supplementation (46). Acid-soluble collagen (ASC) and pepsin soluble collagen (PSC) isolated and identified from the waste skin of sea eel (Evenchely smacrura) can also be used for *in vitro* drug release experiments (83). The naturally keratinized sponges (Porous fungi, Dictyoceratida) are high in glycosaminoglycan content. It can be administered topically as a bio-based dressing and a biological active bionic carrier to regulate the process of wound healing (84). There are also spongy renal cartilages. A water-based gastric acid resistant coating dispersion was developed using renal sponge collagen 15% (W/W) as film forming agent. The results showed that the sponge collagen was resistant to drug for more than 2 h under the action of 0.1 M hydrochloric acid and disintegrated within 10 min in the phosphate buffer solution of pH 6.8. The coated tablets had good mechanical properties and could be stored for more than 6 months without loss of intestinal solubility (60). In hormone replacement therapy, transdermal administration of estradiol bypasses the liver system before metabolism, and therefore has better side effects than oral estrogen. Renal cartilage sponge collagen nanoparticles were used as an osmotic accelerator for transdermal delivery of 17β-estradiol-hemihydrate for hormone replacement therapy. The results showed that the hydrogels containing estradiol collagen nanoparticles could prolong the release time of estradiol and significantly improve the absorption of estradiol. Therefore, sponge collagen nanoparticles are a promising carrier for transdermal drug delivery (64).

The researchers mixed MDC with other biomaterials. The chitosan and chum salmon skin MDC composite gel materials. The compound gel was injected subcutaneously into the back of rats. The specimens were collected for histological examination and ELISA to detect tumor necrosis factor α (TNF-α). It was found that the composite gel could be used as a carrier of tissue filler and drug delivery system (65).

MDC also has potential as a microprotein delivery system. The microgranular protein delivery system was developed using collagen extracted from the jellyfish Catostylus tagi as a polymer matrix. The researchers extracted collagen microparticles by emulsification-gel-solvent, and the CMPs collagen microparticles was cross-linked with 1-ethyl-3-(3-dimethylaminopropyl) carbon diimine (EDC). *In vitro* experiments showed that cross-linking also resulted in greater stability of CMP in water, allowing for slow release of microgranular proteins. These show the potential use of MDC in the production of microparticles for the controlled release of therapeutic proteins (27).

MDC IN COSMETICS AND SKINCARE

MDC is a good moisturizer candidate, which has a wide range of functions in cosmetics. The researchers used MDC from the skin of salmon and cod as an ingredient in cosmetic formulas. Then the experimental results showed that collagen exhibited good water retention ability. Therefore, it is suitable as a moisturizer for skin application. Molecular markers of irritation and inflammation were analyzed that local exposure to collagen in the reconstructed human dermis was found to have no stimulating potential (85). The researchers also isolated collagen from grouper swim bladders and turned it into nanoscale collagen. To determine whether the chemical composition of collagen meets the quality standards of cosmetic raw materials, they did a lot of experiments. Finally, they found it have met the quality requirements of collagen standards as a cosmetic material based on Standar Nasional Indonesia (SNI) (87).

MDC IN FOODS AND HEALTH PRODUCTS

MDC is also widely used in the field of food science and health products (**Table 6**). Currently, MDC or other-sourced collagen can be used as an emulsion to modify food, such as fish oil. Fish oil is rich in omega-3 unsaturated fatty acids and has many important physiological functions and potential for disease prevention. However, there are many disadvantages about it, such as its double bonds are too unstable to rupture, its fishy taste, and poor water solubility. These limit the application. There is a need to develop new formulations, food-emulsions are a practical method, to encapsulate fish oils for protection, increase water solubility and isolate the fishy smell.

There are many influence factors in the use of food-emulsions. To improve the emulsion's stability, researchers need to keep our eyes on the temperature, pH, surface modification, storage time and so on. Emulsion stability mainly depends on droplet size and shell thickness (134). Higher storage temperatures (4–37°C) cause the fish oil emulsion to change from a liquid

TABLE 6 | Applications of MDC in foods and health care products.

Applications	Forms	Functions	References
Emulsion	Gelatin	Emulsion in food industry.	(125)
	Gelatin	Fish oil-loaded gelatin-stabilized emulsions in food.	(126)
	Gelatin	Optimal emulsion storage and transportation conditions in food.	(127)
	Gelatin and peptides	Decrease the creaming stability	(128)
Gelation	Peptides	Gelatins can increase the droplet stability and effect on the phase transition.	(129)
Antioxidant	Peptides	GPEGPMGLE, EGPFGPEG, and GFIGPTE, might serve as potential antioxidants applied in nutraceutical and pharmaceutical products.	(130)
	Gelatin	Antioxidative MCPs may increase life span and protection against tumor development.	(131)
	Peptides	Peptides serve as natural antioxidants in food and cosmetics.	(132)
Soft capsules	Gelatin	Electrospun nanofibers of MDC transport fish oi or nutrients to the stomach and intestines.	(133)

form to a redispersible gel form. It shows that increased temperatures decreased the creaming stability differences (128). The pH of gelatin solution, the speed of homogenizer and the homogenizing time also have important effects on the stability of the emulsion. The gelatin solution pH, speed of homogenizer and the homogenizing time also have important effects on the stability of droplet sizes linearly decreased with increased of solution pH and homogenizing times. Droplet sizes exponentially decreased with increased of homogenizing speeds (135). There are results demonstrating that Cooperative adsorption has better emulsion stability than competitive adsorption. In the work, they mainly explored the gelatin is combined with four surfactants [soybean lecithin (SL), sodium dodecyl sulfate (SDS), Span 80 and Tween 80], which adsorb each other at the oil-water interface, which can improve or decrease the stability of the emulsion (136). These results are connected with the changes of pH, too. The stability results of gelatin/surfactant co-stabilized (Span 80 and SL) or competitive stabilized (Tween 80 and SDS) were studied under different pH backgrounds (137).

Gelations can be modified by different surface modifications. Bovine and fish gelatins were modified by octenyl succinic anhydride (OSA) (125). The DS increase of OSA-modified bovine bone gelatins increases the droplet stability, but the DS increase of OSA-modified fish skin gelatins can only increases of the droplet stability and effects on the phase transition and creaming index of fish oil-loaded emulsions is very weak. The new formulation of oat β-glucan (OG)-MDC peptide mixed gels was researched. It has guiding significance for the formulation of low-fat meat products and is beneficial to improve food safety and nutritional value (129).

Collagen peptides may be used as a potential antioxidant in nutritional and pharmaceutical products. Prious research has shown that collagen peptides can serve as natural antioxidants in a variety of applications, such as food and cosmetics (138). Antioxidant Peptides from Gelatin Hydrolysate of Skipjack Tuna (Katsuwonus pelamis) might serve as potential antioxidants applied in health food industries (130). Antioxidant peptides from collagen hydrolysate showed that collagen peptides might serve as potential antioxidants applied in nutraceutical and pharmaceutical products (131). Previous studies have shown that MDC-prepared skin has two effects, namely extending the life span of rats and inhibiting the spontaneous occurrence of tumors. This result indirectly proves that the antioxidant properties of MCPs may be the cause, regarding the extension of life and protection of tumor development (132).

CONCLUSION AND OUTLOOK

Marine-derived collagen (MDC) has good biocompatibility and biodegradability. In recent years, scientists have made extensive exploration in food emulsions and biomedical applications. MDC can be extracted from fish waste products, which is an economical and sustainable source of collagen and can be used as an alternative to land-based collagen. Land-based collagen carries the risk of transmission of zoonotic diseases such as bovine sponge encephalopathy and hand, foot and mouth disease. For religious reasons, pig-derived collagen cannot be used in some foods. MDC protein has a very important application in food. MDC can be used as a food emulsion to encapsulate fish oil for protection. It has guiding significance for the formulation of low-fat meat products and is beneficial to improve food safety and nutritional value. In nutraceutical and pharmaceutical products, MDC might serve as potential antioxidants, even can inhibit the development of tumors.

Similar to materials such as polyhydroxyalkanoate (PHA) (139), PLGA (140), MDC is widely used in medical tissue, especially in bone tissue engineering, cartilage tissue engineering and functional repair of skin tissue. Good biocompatibility makes it the best template for cell growth. At the same time, scaffolds made of MDC can enable cells to live in 3D space (141, 142), thus improving the efficiency of culture, and collagen can induce cell differentiation in some specific environments, so as to produce specific functions. In addition, due to its biodegradability, MDC can be a good drug encapsulation and sustained-release system (141, 143) to improve the effectiveness of drug delivery. Of course, MDC also has some drawbacks. MDC is not strong enough, which makes its scaffold mechanical properties inadequate. In 2015, tilapia was proved to have good biocompatibility and can effectively induce skin regeneration (25). In order to explore the potential clinical application value of gill dolphin collagen materials, gill dolphin collagen extracted from gill dolphin skin was compared with tilapia collagen (144).

The gill dolphin collagen and tilapia collagen were dissolved in 0.5 mol/L acetic acid to form the membrane by casting method. The morphological structure, aqueous solubility and mechanical properties of gill dolphin and tilapia collagen membranes were characterized. The degradability and biocompatibility of the two materials were tested by subcutaneous implantation and cell culture (145, 146). The samples were detected at the experimentally specified time, and the application potential of the gill dolphin collagen membrane was evaluated by contrast with the tilapia collagen membrane. However, MDC also has the characteristics of low mechanical strength and rapid degradation *in vivo*, which can be solved by crosslinking with other natural or synthetic polymers. Therefore, 25% glutaraldehyde crosslinking can improve the mechanical strength and degradation characteristics of collagen membrane (147, 148). The residual glutaraldehyde after crosslinking was treated with glycine (149).

Based on this review, there are not many kinds of MDC available in the market at present, but there are abundant kinds of marine organisms with excellent physical and chemical properties. Therefore, the application prospect of all kinds of MDC is broad. As a new type of biomaterial, MDC egg has been widely recognized and attracted more and more attention from researchers in clinical, medicine, food and other fields.

AUTHOR CONTRIBUTIONS

NX, X-LP, and H-RL reviewed the literature and wrote this manuscript. J-XL and J-S-YC collected the data and critically reviewed this manuscript. X-YQ and S-JY reviewed the literature and wrote this manuscript. H-LG and X-HZ read and approved the final manuscript. JY and GX designed this manuscript. D-XW designed, reviewed the literature, and wrote this manuscript. All authors read and approved the final manuscript.

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Anti-diabetic Potential of a Stigmasterol From the Seaweed *Gelidium spinosum* and Its Application in the Formulation of Nanoemulsion Conjugate for the Development of Functional Biscuits

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The seaweed *Gelidium spinosum* was selected for the extraction of phytosterol by the Soxhlet method. The extracted phytosterol was chemically characterized as stigmasterol using Fourier-transform infrared spectrometry and gas chromatography–mass spectrometry analysis. The antioxidant and α-amylase inhibitory activity of stigmasterol has been confirmed by *in vitro* assays. The *in vivo* studies demonstrated an anti-diabetic effect in streptozotocin (STZ)—induced hyperglycemic rats. Biochemical analysis showed administration of stigmasterol reduced the blood sugar, urea, and creatinine level. The stigmasterol was then nano-emulsified and incorporated into dough for biscuit formulation. The stigmasterol incorporated biscuit showed higher proximate values, low moisture content, lighter color and the textural property revealed lower hardness. Sensorial results showed acceptability when compared to the control. This study demonstrated the stigmasterol reduced hyperglycemic effects and therefore could be used as a supplement in diets for diabetic patients.

Keywords: stigmasterol, antioxidants, antidiabetic, nanosized, *Gelidium spinosum*, glycogen synthesis

HIGHLIGHTS

- Stigmasterol isolated from *Gelidium spinosum* showed potent α amylase- inhibitory activity.
- The *in vivo* animal study showed stigmasterol reduce the blood sugar level in hyperglycemic rats.
- Nano-emulsified stigmasterol was incorporated into biscuit and its textural, sensorial and proximate analysis of the biscuits was determined.

INTRODUCTION

Seaweeds (macroalgae) constitute a significant source of bioactive compounds and can be exploited to yield a great variety of metabolites for pharmaceutical, cosmetics, food, and feed supplements. Bioactive compounds from seaweeds have a wider implication in pharmaceutical and medical scenarios. They are a rich source of antimicrobial, antibiotics, anticancer, antiviral compounds,

antidiabetic, anti-inflammatory, immunomodulatory effects, and also protect the cardiovascular system (1). The health benefits of seaweed gained attraction in the nutraceutical industry. The bioactive peptides from seaweed are antihypertensive agents by inhibiting angiotensin-converting enzyme (ACE) (2). Seaweeds are grouped based on their unique photosynthetic pigments, which give them their characteristic colors and unique properties. They are also rich in polysaccharides such as alginates, carrageenan fucoidan and laminarans and are likewise considered dietary fibers (3). Consumption of algal fiber helps in the development and protection of intestinal microflora in humans.

Phytosterols are a class of sterols principally synthesized by plants and algae and cannot be synthesized in the human body. Phytosterols are essential constituents of the cellular membrane, maintain membrane fluidity, stabilize phospholipid bilayers, and play an important role in signal transduction (4, 5). The type of seaweed, geographic origins and developmental phases of the living beings can add to various phytosterol profiles (6). Studies reported fucosterol is the most abundant sterol present in brown algae (7–10). The great diversity of marine algae may consequently be an exceptionally source of structurally different phytosterols. Phytosterol reduces LDL cholesterol and is essential to maintain the good cholesterol. It protects the heart from cardiovascular diseases. The European Food Safety Authority recommended that phytosterol consumption of 1.5–2.4 g/day reduces the risk of heart disease (11).

This study intends to explore phytosterols from the seaweed *G. spinosum* and the purified sterol was identified as stigmasterol. The extracted stigmasterol showed potent anti-diabetic activity on rats, the blood serum and biochemical analysis evident the anti-diabetic effect. Hence the stigmasterol was nano-emulsified and incorporated into the biscuit dough for the preparation of biscuits. The prepared stigmasterol incorporated biscuit showed improved textural quality including reduced hardness and fracturability, color and sensorial attributes compared to the control. Thus, the stigmasterol can be supplemented with food and can be used as an alternative strategy to treat diabetes.

MATERIALS AND METHODS

Seaweed Collection and Processing

The Seaweed *Gelidium spinosum* was collected from Mandapam coast, Tamilnadu, India. Collected seaweed was washed thoroughly to remove the salt, surface impurities, sand particles, and epiphytes from the sample's surface. The water was drained off and the seaweed sample was chopped and sun dried. The dried seaweed was powdered in the grinder and the samples were stored at 4°C for further studies.

Extraction and Purification of Phytosterols

The seaweed powder was taken in a thimble holder and then placed in a distillation flask. Extraction was performed using ethanol in a Soxhlet apparatus under steam for 6 h. The extract was cooled, and then water: petroleum ether (1:1) was added to a separating funnel. The layers were collected separately and the lipid containing fractions were mixed with 1 M ethanolic KOH and stirred overnight. The mixture was diluted with distilled water and extracted with 3 parts of diethyl ether. The extract was further dried over anhydrous sodium sulfate and deactivated alumina. The presence of phytosterols was confirmed by the Salkowski test (12). The organic phase of the extracted compound was then concentrated to dryness using a rotary evaporator (Yamato DC400, Japan), quantified and then purified using silica gel column chromatography using the solvent system methanol: water (4:1) followed by reverse phase HPLC on a C-18 column with a mobile phase of 1% ethanol in hexane, eluted fractions were then used for further analysis (13).

Characterization of Phytosterols

Thin layer chromatography was performed using the solvent system of methanol: water (4:1). The functional group of the phytosterol was determined by Fourier-transform infrared Spectroscopy (FTIR) analysis. To determine the functional groups, the analysis was carried out in the 4,000–450 cm^{-1} spectral region at a resolution of 1 cm^{-1} and 50 scans on a Perkin Elmer system. The samples were dispersed in KBr pellets the homogeneous mixture was prepared using a hydraulic press. Purified phytosterol extract was subjected to trimethylsilylation and then analyzed using gas chromatography–mass spectrometry (GC-MS). Briefly, dried extract was added with 100 µl of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) in a React-tube. The tube was heated at 90°C for 20 min and then cooled. The mixture was further dried at 60°C and then analyzed by GC-MS (Agilent 7890A - 240 MS with Ion Trap). The peaks obtained in the GC was subjected to mass spectral analysis and the individual components were identified by comparison with standard libraries.

Determining of Antioxidant Activity

For the antioxidant determination, stigmasterol was reconstituted in ethanol at the concentration of (100 mg/ml) and 25–500 µg/ml of extract were used for free radical scavenging activity (1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical assay. Scavenging activity was calculated by using ascorbic acid as standard.

$$\%\ Inhibition\ Antioxidant\ capacity = \frac{control\ absorbance - sample\ absorbance}{control\ absorbance} X\ 100 \quad (1)$$

The hydroxyl radical scavenging activity of stigmasterol was determined according to the protocol of (14). All the experiments were performed in triplicates. The hydroxyl radical scavenging activity was calculated by the following equation.

$$Hydroxyl\ radical\ scavenging\ activity = 1 - \frac{A_1 - A_2}{A_0} \times 100 \quad (2)$$

Alpha-Amylase and Alpha Glucosidase Inhibitory Assay

α- amylase inhibitory activity was evaluated as per the method of Jayasri et al. (15). Briefly, varying concentrations of stigmasterol (50–200 µg/ml) was added to 0.02 M sodium phosphate buffer of

pH 6.9. To this 0.5 mg/ml of α- amylase was added and incubated at 25°C for 10 min. To this 250 μl of starch solution (1%) was added. After incubation of 10 min, the reaction was stopped by adding 500 μl of dinitrosalicylic acid. The absorbance of the assay mixture was recorded at 540 nm. Alpha amylase inhibitory activity was calculated as percentage inhibition using the formula,

$$\%\ Inhibition = \frac{Abs_{control} - Abs_{Sample}}{Abs_{control}} \times 100 \quad (3)$$

Alpha glucosidase inhibitory potential was determined by the method of Javadi et al. (16). Briefly, different concentrations of stigmasterol were used as test, acarbose was used as positive control and solvent was set as a negative control. Samples and controls were added to reaction mixture containing 100 μL of 30 mM phosphate buffer and 15 μL of α-glucosidase enzyme (0.02 U/μL) from *Saccharomyces cerevisiae* (Sigma). The control and sample mixtures were then treated with 75 μL of PNPG (ρ-Nitrophenyl-ρ-D-glucopyranosidase, 0.3 mg/mL). The amount of ρ-nitrophenol released was measured using microplate reader at 405 nm. Alpha glucosidase inhibitory activity (%) was calculated using the formula,

$$\%\ Inhibition = \left(\frac{Abs_{control} - Abs_{Sample}}{Abs_{Control}}\right) \times 100 \quad (4)$$

Demonstration of Anti-diabetic Activity of Stigmasterol in Rats

Healthy male Sprague Dawley rats weighing between 130 and 160 g (5–6 weeks old) were procured from Biogen laboratory animal facility, Bangalore, India. The study was carried out in an air-conditioned room at an ambient temperature of about 25°C, relative humidity of 40–60% with 12 h light/ dark cycle, and provided free access to feed and water. To determine the anti-diabetic activity, the animals were grouped into four groups as given below:

Group 1: Normal control rats fed with normal pellet and sterile water orally for 4 weeks ($n = 10$).

Group 2: Rats treated with single dose of drug streptozotocin (35 mg/kg body weight) and fed with normal pellet and sterile water orally for 4 weeks ($n = 10$).

Group 3: Rats treated with single dose of drug streptozotocin (35 mg/kg body weight) and fed with normal pellet mixed with metformin 250 mg/kg body weight and sterile water orally for 4 weeks ($n = 10$). Metformin is a commonly used anti-diabetic drug and is used as positive control of (17).

Group 4: Rats treated with single dose of drug streptozotocin (35 mg/kg body weight) and fed with normal pellet mixed with stigmasterol 200 mg/kg body weight and sterile water orally for 4 weeks ($n = 10$). Three days after streptozotocin injection, the rats were examined for fasting blood glucose to confirm the diabetic stage. Fasting blood glucose was measured at 0, 7, and 14 days with glucometer (Dr. Morepen Gluco One Blood glucose monitoring system). The rats with fasting blood glucose above 200 mg/ dL were considered as diabetic. The dose of stigmasterol was selected based on the Human equivalent dose (HED) conversion to animal equivalent dose (AED) (18).

Determination of Biochemical Parameters

After the experimental regimen, the animals were sacrificed by cervical dislocation after giving mild anesthesia using chloroform. The blood samples were drawn from retro orbital site (under anesthetic condition) without anticoagulant and serum was separated by centrifugation at 2,500 rpm. The biochemical parameters include serum protein, urea, creatinine and glycogen content was determined using Biosystems kit, India.

Histopathological Analysis

The organs kidney and liver were dissected into small sections of 1 cm^3 and then fixed in 10% neutral formalin and then embedded in paraffin wax. The sections were cut into thin sections of 5 μm and then stained with haematoxylin -eosin, mounted in deparaffinated xylene, dried and then visualized under microscope (17).

Formulation of Stigmasterol Nano-Emulsion

To prepare stigmasterol nano-emulsion, 8 g of stigmasterol was added to 80 ml of water and heated at 30°C. To the mixture 6 g of lecithin and 6 ml of olive oil was added and vortexed for 5 min. The mixture was stirred using a magnetic hot plate set at a temperature of 50°C for 10 min and blended using a homogenizer at 6,000 rpm for 2 min and then fine nano emulsions were obtained using a high-pressure homogenizer. The formed emulsion was then freeze dried using a lyophilizer (Yamato, Japan). Lyophilized powder obtained was characterized for particle size analysis and its formulation into biscuit as a functional food ingredient.

Particle Size and XRD Analysis

Particle size of the stigmasterol emulsion was determined using a Particle size analyzer and performed using a laser diffraction particle size analyzer (Beckman-Coulter, LS-230, Miami, FL, USA). The degree of crystallinity was determined using a Rigaku (30 kv/25 mA) Geigerflex D/Mac, C series diffractometer (Tokyo, Japan) with Cu-kα radiation ($\lambda = 1.5406$ A°) at room temperature in glancing inclined angle mode.

Preparation of Biscuit

Biscuits were prepared as described in (19) with necessary modifications. The ingredients used were 100 g of whole wheat flour, 10 g of palm sugar, 50 g butter, 0.5 g baking powder and 2.0 g of egg white. In the test baking powder and egg were replaced with lyophilized powder of stigmasterol of 2.5 g. All the ingredients were mixed well and made into a smooth dough, which was then sheeted to a thickness of 4 mm and was cut using a cookie cutter. The biscuits were further baked in a preheated oven at 160°C for 20 ± 5 min. The baked biscuits were then stored in an air—tight container for further analysis.

Proximate Analysis of Biscuit

Proximate analysis of the control and the stigmasterol incorporated biscuit was analyzed and the ash, moisture, fiber,

fat, and protein content were determined by AOAC method (20). Carbohydrate content was obtained by subtraction method.

Texture Analysis of the Biscuit

Texture of the biscuit was analyzed using a Texture analyser (TA–HDplus, Stable Micro Systems, Surrey). The biscuit samples were pressurized with the probe until the biscuit broke. A three-point bending test was conducted using a three- point bending rig (HDP/BS) at a test speed of 2 mm/s and a distance of 5 mm. Calibration was carried using a load cell of 50 kg, where the hardness of the sample was analyzed (19, 20).

Color Analysis of Biscuit

Color analysis of the biscuits was performed using Hunter lab spectrophotometer (D-25, Hunter Associates Laboratory, Ruston, USA (21). Biscuits from each set were positioned in a glass sample cup of 5.8 cm internal diameter and were analyzed for color coordinates using L^*, a^*, b^* color space. The colors were analyzed in triplicates, and the mean was recorded.

Sensory Evaluation of Biscuit

A total of 15 volunteers, age 23–30 (seven males and eight female) evaluated the sensory characteristics of the biscuits The samples were analyzed for appearance, color, flavor, texture, crispiness, hardness and overall acceptability using a 9- point hedonic scale for sensory evaluation, varying from 9 (like extremely) to 1 (dislike extremely) (22).

Statistical Analysis

Statistical analysis used in this study represents average of triplicate experiments. The data were represented in terms of mean ± standard deviation. The data were analyzed using one-way ANOVA with Bonferroni posttest at $P < 0.05$ being significant using SPSS software.

RESULTS

Collection and Processing of Seaweeds

In this study, the seaweed *G. spinosum* was collected from the Gulf of Mannar, Mandapam coast, and processed for phytosterol extraction. The Salkowski test showed brown ring, which confirmed the presence of phytosterols in the seaweed extract. The yield of the crude extract of phytosterol was 3.56 mg/gm.

Determination of Antioxidant Activity by DPPH Assay and Hydroxyl Radical Scavenging Activity

DPPH radical scavenging assay is a method used to evaluate the free radical scavenging activity of the seaweed extracts. The assay results showed the phytosterol of *G. spinosum* showed significant effect in inhibition of DPPH free radical up to 95.33 ± 3.51 at 500 μg/ ml (**Figure 1**). The obtained results indicated the scavenging activity of phytosterol was concentration dependent. Ascorbic acid showed effective concentration (EC50) of 178.07 μg/ml, while phytosterol had an EC50 of 65.28 μg/ml. The hydroxyl scavenging assay showed phytosterol exhibited the inhibition of 92 ± 2.0 and the inhibition rate was higher than the standard ascorbic acid of 79.66 ± 1.15 (**Figure 1**). The EC50 of hydroxyl radical scavenging activity of ascorbic acid was found to be 146.53 μg/ml, whereas, stigmasterol showed 46.53 μg/ml.

Characterization of Phytosterols

The thin layer chromatography of the phytosterol extract showed spot in the solvent system methanol: water (4:1) with an Rf value of 0.3. FT-IR spectra showed characteristic absorption band at 3,430 cm^{-1} correspond to the -OH group. Peaks in the range of 2,955 to 2,326 cm^{-1} correspond to the presence of CH_2 and CH_3 groups. Band observed at 1,742 cm^{-1} may be due to the presence of carbonyl group C=O, peaks at 1,632 to 1,577 cm^{-1} corresponds to C=C and C-H vibrations, O-H bending and C-O stretching at 1,377 cm^{-1}, peak at 1,055 cm^{-1} corresponds to secondary C-O vibrations. GC-MS analysis of the TMS derivatives of the extract showed the presence of stigmasterol with a retention time of 20.82 min and a mass of 412.69 g/mol (**Figure 2**).

Determination of α- Amylase and α-Glucosidase Activity

Control of blood glucose levels is important in the control of micro and macro vascular complications. To control hyperglycemia, inhibition of starch hydrolysis was essential and stigmasterol extracted from the seaweed was found to possess α- amylase inhibitory activity. The assay results showed stigmasterol inhibited α- amylase (78 ± 1.0%), and the activity was higher when compared to the standard acarbose (62 ± 1.0%) at a concentration of 200 μg/ml (**Figure 3A**). Acarbose with an IC50 of 133.82 μg/ml, while stigmasterol with inhibitory concentration of 89.27 μg/ml. Alpha glucosidase mediates the breakdown of the oligo and disaccharide units of starch into glucose during digestion before it is absorbed into the bloodstream. Thus, inhibition of alpha glucosidase can be used as an ideal method to prevent diabetes. At a dose of 200 μg/ml, the stigmasterol alpha glucosidase inhibitory activity was found to be 80.3 ± 0.6%, which was higher than that of standard acarbose (75.18 ± 0.5%). The IC50 value of alpha glucosidase inhibition was found to be 89.98 μg/ml for stigmasterol and 120.18 μg/ml for standard acarbose (**Figure 3B**).

Anti-diabetic Effect of Stigmasterol

During the initial phase of experiment, the mean fasting blood sugar (FBG) level of groups 1- 4 were almost similar in the range of 84.12 ± 0.99 mg/dL. After 28 days, the FBG level was higher in the Group 2. The metformin- administered group showed reduction in the blood glucose level of 118.31 ± 1.1 mg/dL, while the diabetic group showed a glucose level of 376.12 ± 1.02. The stigmasterol administered rats (Group 4) showed a reduction in the FBG level of 137.73 ± 1.8 mg/dL when compared to the Group 2. The result obtained evidences when stigmasterol was administrated to the rats, lowering of blood glucose level was observed (**Figure 4A**).

The body weight of all the rat groups were in the range of 180 ± 5.2–197 ± 5.0 g at the starting day of treatment. The diabetic onset gradually reduced body weight in group-2, eventually to as low as 164 ± 4.8 g on an average after 28 days while the control

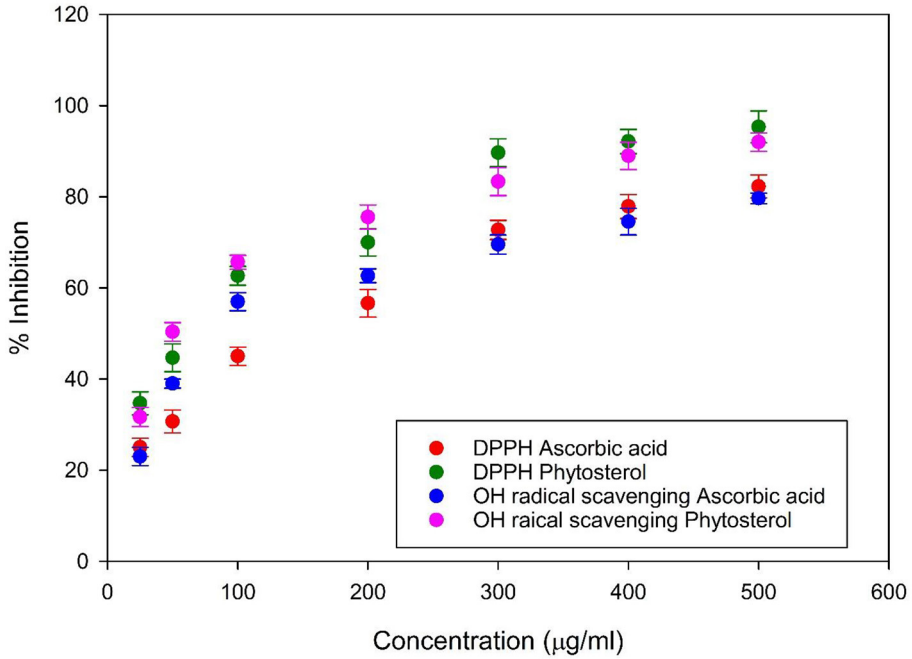

FIGURE 1 | Antioxidant activity by 1, 1-diphenyl-2-picrylhydrazyl and hydroxyl radical scavenging assay. The assay results evidence that antioxidant activity increases with the concentration of the stigmasterol, and the activity was higher than the control ascorbic acid.

rats weighed around 203 ± 4.9 g on an average. The Group 4 rats administrated with stigmasterol showed the average body weight of 252 ± 4.8 g, whilst the group 3 metformin administrated rats showed an average weight of 209 ± 5.9 g which was almost similar to that of control (Group 1) (**Figure 4B**).

The serum protein level was lower in diabetic rats (Group 2). Group 3 and Group 4 rats showed serum protein level almost nearer to control rats (7.92 ± 0.1 mg/24 h) (**Figure 4C**). Serum urea level was higher in the Group 2 rats with an average of 65.23 ± 0.2 mg/24 h which was much higher than the control (Group 1) rats of 30.21 ± 0.2 mg/24 h. The metformin (Group 3) and stigmasterol (Group 4) administrated rats showed the urea level of 37.56 ± 0.5 mg and 39.76 ± 0.3 mg/24 h (**Figure 4D**). Serum creatinine level for the control rats was on an average of 0.48 ± 0.02 mg/24 h while Group 2 showed an increased level of 1.53 ± 0.03 mg/24 h. The metformin and stigmasterol administrated groups showed almost similar values of 0.82 ± 0.04 mg and 0.85 ± 0.01 mg/24 h, respectively (**Figure 4E**). The average glycogen content was 53.1 ± 0.8 mg/g for control group. The untreated diabetic rats were 15.13 ± 0.7 mg/g which evidently depicts the glycogen storage was lower in diabetic rats due to their inefficiency in converting excess glucose to glycogen reserves due to impaired insulin secretion. The rats administrated with metformin and stigmasterol could reserve glucose as glycogen to a notable level of 44.25 ± 0.6 and 38.83 ± 0.8 mg/g, respectively. The administrations might have improved the insulin secretion level and thereby the glucose conversion to glycogen (**Figure 4F**).

Histological Examination

The effects of stigmasterol on kidney, and liver of rats were evaluated. The control rats showed normal histoarchitecture and the histopathological examinations confirm the protective effect of stigmasterol (**Supplementary Figure 1**).

Determination of Crystallinity and Particle Size Analysis of Nano-Emulsion

The crystallinity of the emulsion was confirmed by XRD analysis. Two high- intensity sharp peaks were obtained around 15°, 18.5° and small intensity peaks were observed at 11.9°, 12.19°, 16.2°, 19.2°, 21.9°, and 24° (**Figure 5A**). These peaks indicate the crystalline nature of the stigmasterol. Particle size analyzer showed the size distribution of particles of the stigmasterol was in the range of 50–500 nm (with a smaller intensity of 11.7 and 6.2%) (**Figure 5B**).

Textural Characterization of the Stigmasterol- Incorporated Biscuit

Stigmasterol- incorporated biscuits showed a significant reduction in hardness when compared with the control. This may be due to the emulsified stigmasterol incorporated into the dough, which made them softer and due to the effect of stigmasterol on the starch protein interactions. The control biscuit showed hardness of 12.803 ± 1.8, with the reduced hardness in the stigmasterol incorporated biscuit 8.876 ± 2.3.

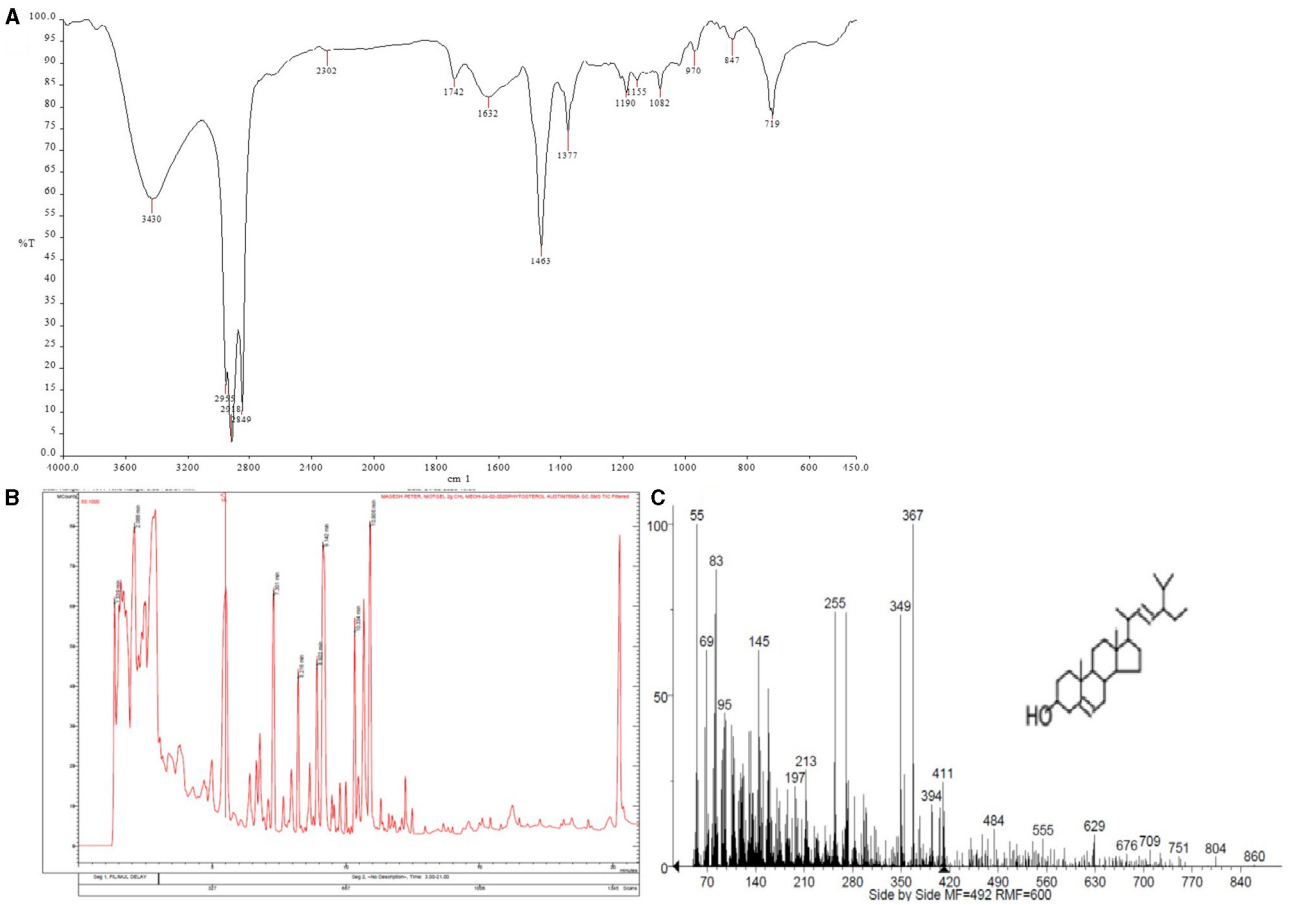

FIGURE 2 | (A) Fourier-transform infrared spectrometry spectrum showing the characteristic peaks of stigmasterol **(B,C)** Gas chromatography–mass spectrometry spectrum showing the presence of stigmasterol.

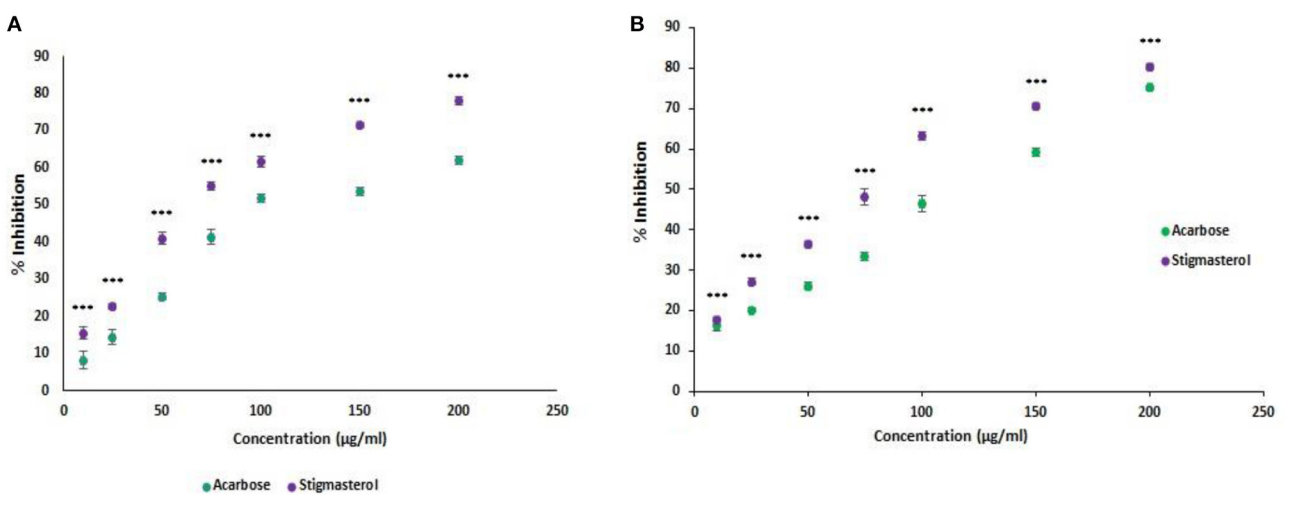

FIGURE 3 | (A) Alpha-amylase inhibition assay of the stigmasterol. The result shows that the alpha-amylase inhibition activity increases with the concentration of the stigmasterol, and the activity was higher than the control acarbose. **(B)** Alpha-glucosidase inhibition assay of the stigmasterol. Error bars indicate standard deviations, significance $^{***}P < 0.001$.

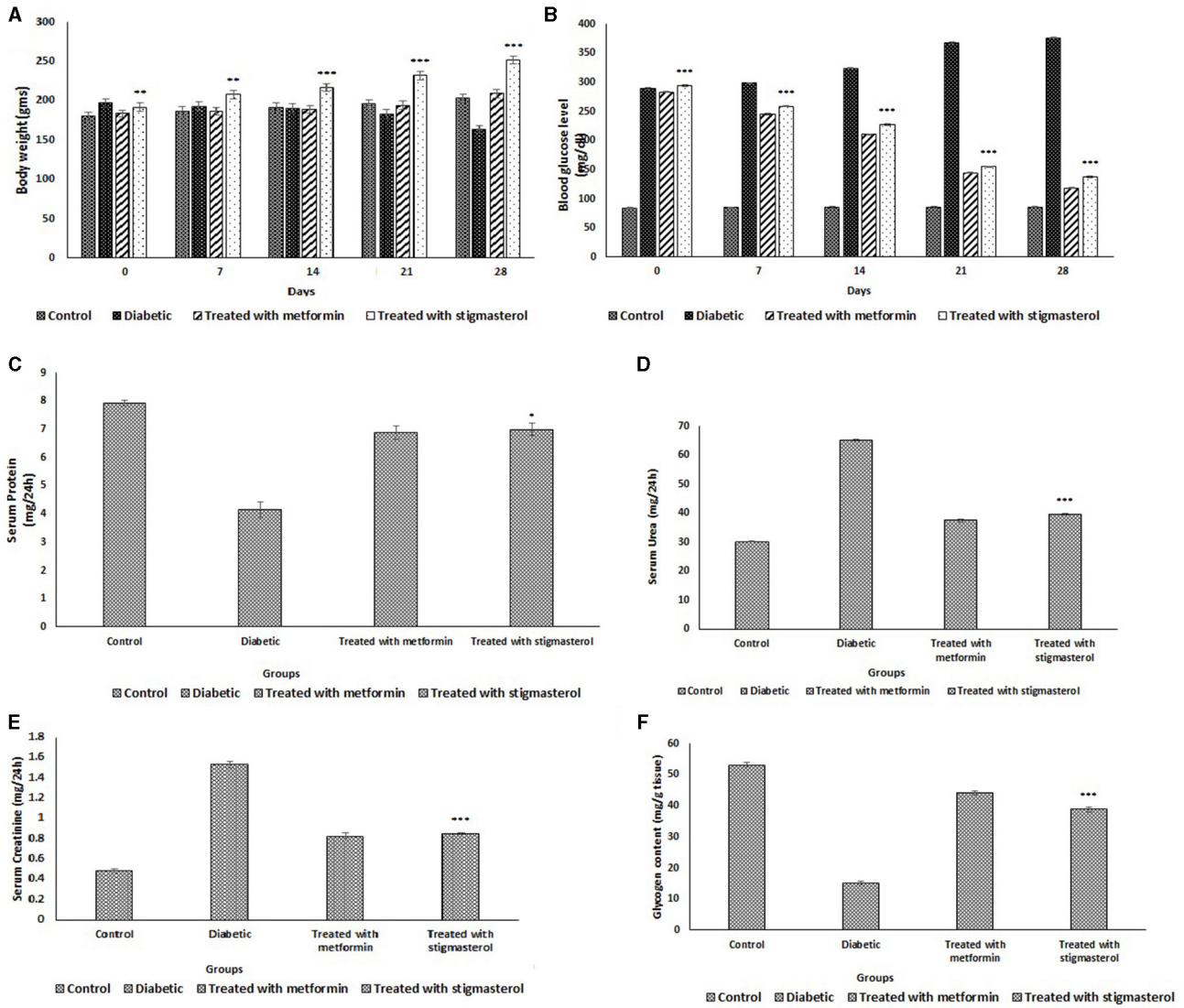

FIGURE 4 | Effect of stigmasterol on rats **(A)** body weight (g), **(B)** blood glucose level (mg/dL), **(C)** serum protein (mg/24 h), **(D)** serum urea (mg/24 h), **(E)** serum creatinine (mg/24 h), and **(F)** glycogen content in control and diabetic rats. Number of animals used $n = 10$, error bars indicate standard deviations, significance $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Color Analysis of Biscuits

The color parameters were expressed as tri-stimulus attributes of L*, a*, and b* values. The L* value depicting lightness of 44.68 ± 0.11 in stigmasterol- incorporated biscuit when compared to control. The a* value depicting red was almost similar for the control and stigmasterol- incorporated biscuit and the b* value for yellowness was higher in the stigmasterol- incorporated biscuit when compared with that of control. The obtained result showed lightness and yellowness in stigmasterol incorporated biscuits compared with the control (**Supplementary Table 1**).

Proximate Analysis of Biscuit

Stigmasterol incorporated biscuit showed reduced moisture and carbohydrate content. Higher values were observed in crude fiber, fat and protein content for the stigmasterol- incorporated biscuit (**Supplementary Table 2**).

Sensory Evaluation of Biscuit

The sensory analysis showed incorporation of stigmasterol in biscuit production enhanced acceptance of the product. Color and appearance of the stigmasterol incorporated biscuit were evaluated in the range of "like very much" in comparison with "like moderately" of the control. The flavor, texture, crispiness and hardness were more acceptable for the stigmasterol incorporated biscuits when compared to the control. Overall acceptability of the stigmasterol biscuits was "like very much" whereas control was "like moderately" (**Table 1**).

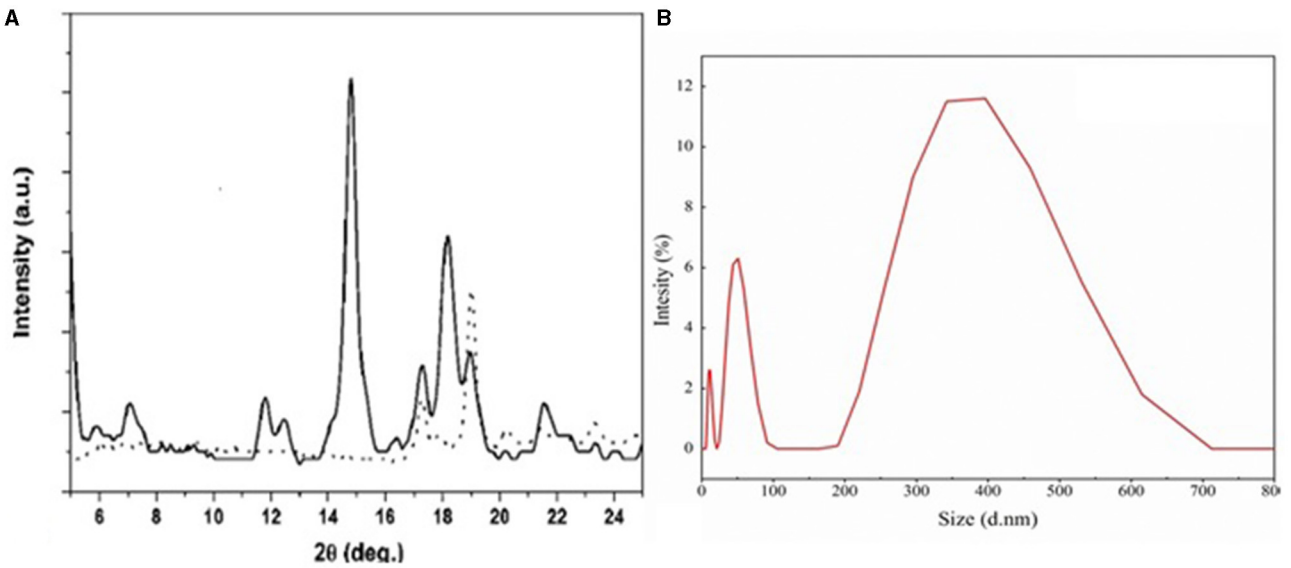

FIGURE 5 | (A) Shows the nature of the particle determination by XRD, and the peaks obtained showed the particle were crystalline in nature. **(B)** Particle size analysis by particle size analyzer.

TABLE 1 | Comparison of sensory evaluation results of the stigmasterol incorporated and control biscuits.

Sample	Color	Flavor	Texture	Appearance	Crispiness	Hardness	Overall acceptability
Control	7.5 ± 0.68	7.2 ± 0.58	7.6 ± 0.63	6.9 ± 0.78	6.7 ± 0.84	6.9 ± 0.74	7.8 ± 0.75
Stigmasterol incorporated biscuits	8.0 ± 0.79	7.4 ± 0.72	7.7 ± 0.72	7.3 ± 0.65	7.2 ± 0.42	7.4 ± 0.58	8.2 ± 0.67

DISCUSSION

The seaweed *G. spinosum* was selected for the phytosterol extraction based on the wide distribution and abundance in the Mandapam coast, Rameswaram, India. Phytosterols such as β-sitosterol, campesterol, and stigmasterol are noted to play significant effects on lowering low density lipoprotein in plasma and they were found to be a safe nutraceutical (23, 24). The presence of stigmasterol in the extract of *G. spinosum* was confirmed by FTIR spectra and GC-MS showed stigmasterol with a retention time of 20.82 min and mass of 412.68 g/mol. In this study, phytosterol extract of *G. spinosum* showed a significant radical scavenging activity Methylene cholesterol, ergosterol and stigmasterol extracted from the algae *S. aggregatum* showed antioxidant activity (25). α- amylase inhibitory activity of the stigmasterol was evaluated, and the activity was found to be higher when compared with standard acarbose. The -glucosidase enzymes in the epithelial tissue of the small intestine are involved in the final phase of carbohydrate hydrolysis to produce an absorbable monosaccharide. Inhibition of these enzymes reduces postprandial hyperglycemia by slowing the breakdown of dietary polysaccharides into simpler saccharides in the gastrointestinal tract (26). *In vivo,* an experimental study on animals proved the anti-diabetic potential of stigmasterol with reduced blood glucose, urea and creatinine level. Administration of stigmasterol extracted from plants induces insulin uptake from pancreatic α-cells resulting antihyperglycemic effect (27–30). A banana extract containing 21.91% stigmasterol was found to have a possible anti-diabetic effect in alloxan-induced diabetic mice in a recent study (17). Similar to our study, metformin was used as a control drug. The mechanism of stigmasterol's anti-diabetic effect is not fully understood. However, certain putative mechanisms in antidiabetic potential have been identified. There are primarily either one of the two mechanisms involved, or a combination of both. The first mechanism, it could be due to a decrease in intestinal glucose uptake or an increase in glycolytic and glycogenic systems with a subsequent reduction in glycogenolysis and gluconeogenesis pathways, implying effects on the glucose metabolic pathways. The second mechanism involves the activation or repair of -cells, followed by insulin release or insulin receptor stimulation (17, 31, 32). In the present study, the stigmasterol administration reduced the fasting blood glucose level significantly in the diabetic rats, similar to commercial drug metformin. The anti-diabetic activity of stigmasterol may be due to the regeneration of the β-cells of Langerhans of the pancreas and thereby secretion of insulin, thus controlling the blood glucose level (29, 30, 33). Due to high plasma glucose, renal alterations occur resulting in high protein metabolism that leads to impaired nitrogen balance and eventually elevated levels of creatinine and urea levels which serve as a marker for kidney dysfunction (17, 34). Stigmasterol administration showed reduced creatinine and urea level when

compared with the untreated diabetic rats, and the levels were almost similar to normal rats. There was a significant serum protein level reduction in diabetic rats when compared to other groups. This reduction might be due to the effect of insulin on maintaining the balance of protein by stimulating amino acid uptake and inhibition of degradation of protein (34, 35). Diabetic rats had low glycogen levels due to breakdown of glycogen reserves and absence of excess glucose conversion to glycogen due to impaired insulin production. Stigmasterol administration might have involved in the regeneration of pancreatic cells by releasing insulin and thereby controlling the glucose and glycogen levels to normal levels.

Stigmasterol has been reported to have potential with the possible mode of targeting the glucose transporter GLUT4 including increased translocation and expression of GLUT4 (36). The stigmasterol was emulsified and incorporated into the dough for biscuit preparation. Among the available types of functional foods, biscuits possess wider functions, and nutritional value. They absorb less moisture, ready to eat and easily available cereal and can be made into varying formulations, increased shelf life and they are economical. Biscuits are considered as the best nutraceutical for providing health benefits to the consumers in right proportions (37, 38). Stigmasterol added whole grain wheat biscuits lowered LDL cholesterol after 4 weeks of administration (39). Biscuit is a convenient food to deliver fortified food for daily intake and it is a food with wider acceptability for people of all ages. Stigmasterol added biscuits showed soft texture when compared to the control. This may be due to the emulsifier that weakens the gluten network resulting in reduction of dough cohesiveness and thus reduces the hardness. The sensory analysis evident that the stigmasterol- incorporated biscuit was much acceptable than the control.

CONCLUSION

The present study describes the stigmasterol extraction from *G. spinosum* and the presence of stigmasterol in the extract of *G. spinosum* was confirmed by FT-IR and GC-MS analysis. The anti-diabetic effect of stigmasterol was confirmed using *in vitro* and *in vivo* studies. Results obtained from this study showed stigmasterol isolated from *Gelidium* sp. are rich in antioxidants and exhibit anti-diabetic activity. The biscuit formulated using stigmasterol showed high acceptability, and thus it can be used as a functional food supplement and can also be used as an alternative strategy to treat diabetes.

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/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Ethics Committee of Pondicherry University.

AUTHOR CONTRIBUTIONS

NP, AR, and AC performed the experiment, GP performed statistical analysis and AS contributed in writing the manuscript. GK designed the experiments. JS designed the manuscript. All the authors approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.694362/full#supplementary-material

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The Effect of Oral Nutritional Formula With Three Different Proteins on Type 2 Diabetes Mellitus *in vivo*

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Oral nutritional (ON) products are an effective way to treat patients with type 2 diabetes mellitus (T2DM) whose gastrointestinal functions are normal. The influence of ON formula prepared with three different proteins on T2DM was studied. The hyperglycaemic mouse model using a high-fat diet (HFD) combined with an intraperitoneal injection of streptozotocin (STZ) was used to simulate T2DM. The study was done for 15 weeks using seven groups of mice: control group (CG, normal mice, and normal food), non-treated group (BG, diabetic mice, and normal food), positive control group (PG, diabetic mice, and HFD), soybean protein group (SPG, diabetic mice, and HFD), silkworm pupa protein group (SPPG, diabetic mice, and HFD), whey protein group (LPG, diabetic mice, and HFD), and whey protein combined with silkworm pupa protein group (LCSSPG, diabetic mice, and HFD). The plasma levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were analyzed on weeks 2, 10, 12, 14, and 15. The concentration of total protein (TP) and albumin (ALB) of the plasma was increased in SPG, SPPG, and PG comparing with BG ($p < 0.05$). The TC, TG, and LDL-C levels were decreased, and HDL-C level was increased in SPG, PG, SPPG, PG comparing with BG ($p < 0.05$). Blood glucose (BLG) levels were decreased 47, 34, 24, and 21% in SPG, LCSSPG, SPPG, and PG, respectively. While BLG was not significantly changed ($p \geq 0.05$) in LG after 5 weeks of treatment. Overall, the data suggested that consumption of SP, SPP, LCSSPG Oral-formula may be beneficial for the treatment of T2DM.

Keywords: type 2 diabetes mellitus, oral nutritional formula, soybean protein, whey protein, silkworm pupa protein

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a long-term chronic nutritional metabolic disorder disease that is characterized by high blood sugar, relative lack of insulin, and insulin resistance (1). The mechanism of diabetes is complex due to a variety of factors such as host genetic predisposition, diet, lifestyle changes, and different disease states (2). Oral nutritional (ON) formula is intended for patients with a limited or impaired capacity to intake, digest, absorb, metabolize, or excrete ordinary foods or certain nutrients, or with other medically determined nutrient requirements whose dietary management cannot be achieved by modification of the normal diet. Patients with T2DM have

different nutritional requirements compared with normal people. A recent study reported that relatively higher amounts of protein, total fat, monounsaturated fat, and polyunsaturated fat were consumed and relatively lower intakes of carbohydrates, non-milk sugars, and dietary fiber were observed in patients with T2DM compared with normal people (3). The importance of nutritional adjustments in the alleviation of T2DM by influencing weight and regulating metabolism has also been studied (3, 4). In addition, modifying carbohydrate and protein composition, adding monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), dietary fiber, and various vitamins and minerals have shown improved blood sugar, lipid, and protein metabolism comparing with a standard diet (5–7).

A diabetes-specific oral formula, which included whey protein (WP), soy protein, multiple carbohydrates, multiple sources of fiber, and various vitamins and minerals could improve postprandial blood glucose (BLG) profiles and 24 h BLG control comparing with a standard-fiber-containing formula based on a randomized, controlled, double-blind, or cross-over study (8). Glycated hemoglobin (HbA1C) was reduced, and hospitalization rates were lowered in patients with T2DM by combining nutritional therapy with other dietary components (9, 10). One of three isoenergetic ready-to-consume formulas showed decreased T2DM symptoms since the short-chain fatty acids in that diet promoted glucagon-like peptide 1 (GLP-1) level (6). Soluble dietary fiber significantly improved postprandial BLG control by delaying glucose absorption in the small intestine (11–13). A very high-protein and low-carbohydrate oral nutrition formula (protein provided 37% of total calories and carbohydrate provided 35% of total calories) also improved BLG with no significant statistical difference in insulin responses on T2DM (5).

L-Arginine, a biological precursor of nitric oxide, has an effect that improves skeletal muscle insulin sensitivity by NO or cGMP pathway to mediate increment of phosphorylation of Akt and AMPK-α (14). It was also found that an average daily intake of more than 800 IU of vitamin-D and more than 1,200 mg of calcium were inversely related to the incidence rate of T2DM when comparing with an intake of <400 IU of vitamin-D and <600 mg of calcium (15). In addition, prebiotics are non-digestible food ingredients that could improve host health by stimulating changes in the composition or activity of specific bacteria in the gut.

Insulin can increase the level of high-density lipoprotein cholesterol (HDL-C) and help people with T2DM to control blood sugar according to 20 randomized controlled trials with 607 adult participants (16). Researchers at Cornell University fed diabetic rats daily with *Lactobacillus rhamnosus* that were engineered to secrete GLP-1. Their results showed that GLP-1 secreting *lactobacilli* could increase plasma insulin levels and glucose tolerance in diabetic rats. Additionally, these rats developed insulin-producing pancreatic cells within the upper intestines up to about 25–33% of the insulin capacity of non-diabetic healthy rats (17).

The effects of various vitamins, minerals, proteins, dietary fiber, MUFA, PUFA, probiotics, prebiotics, and proteins, alone, have been investigated for several years. However, the use of different proteins as the variable in different ON formula *in vivo* was seldom studied. Since protein is an important bioactive molecule, protein may have a key role in the ON formula. Four formula with different proteins as the only difference between the diets were prepared. The three different proteins were soybean (SP), LP, and silkworm pupa protein (SPP) and a combination of LP and SPP. Lipid metabolite indices of plasma, protein metabolite index of plasma, postprandial plasma glucose, and body weight were evaluated. The scheme graph was shown in **Figure 1**. Results showed that the concentration of total protein (TP) and albumin (ALB) of the plasma was increased in SPG, SPPG, and PG comparing with BG ($p < 0.05$). The TC, TG, and LDL-C levels were decreased, and HDL-C level was increased in SPG, PG, SPPG, PG comparing with BG ($p < 0.05$). BLG levels were decreased 47, 34, 24, and 21% in SPG, LCSSPG, SPPG, and PG, respectively, while BLG was not significantly changed ($p \geq 0.05$) in LG after 5 weeks of treatment. Overall, the data suggested that consumption of SP, SPP, LCSSPG oral formula may be beneficial for the treatment of T2DM.

MATERIALS AND METHODS

Materials

Streptozotocin (STZ, Sigma Chemical St. Louis, MO, USA), chloral hydrate, sterilized saline water, citric acid, and sodium citrate (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) were purchased.

Preparation and Contents of the ON Formula

Three proteins, namely, SP, LP, and SPP were added as 23% (w/w) in SP ON formula, LP ON formula, and SPP ON formula, respectively. The proteins of LP and SPP were equally added (11.5% w/w of each) in the LCSSP ON formula. The rest of the formula was made up of 5% fish oil powder, 15% olive oil powder, 12% corn starch, 17.5% cassava starch, 9% oligosaccharide fructose, 5% isomaltose, 10% vanilla, 3.5% various vitamin, and mineral elements (**Table 1**). All raw materials for the formula were food-grade and purchased from Henan Jianjiu Industrial Co. (Zhengzhou, Henan, China). Formula tableting was done using a tablet machine (Mini PRESS-IISF, India).

Nutritional Analysis of the Formula

The methods and standards used to determine the component of the formula were listed in **Table 2**.

Experiment Design

Animals

A total of 84 specific-pathogen-free male (SPF) CD-1® (ICR) IGS mice, weighing 18–23 g (aged 6 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The animal room was maintained at an ambient temperature of 25 ± 4°C, relative humidity of 50 ± 15%, and a light/dark cycle of 12 h (fluorescent light). At the beginning of the experiment, free access to normal rodent chow (20 kJ/kg, 5% fat,

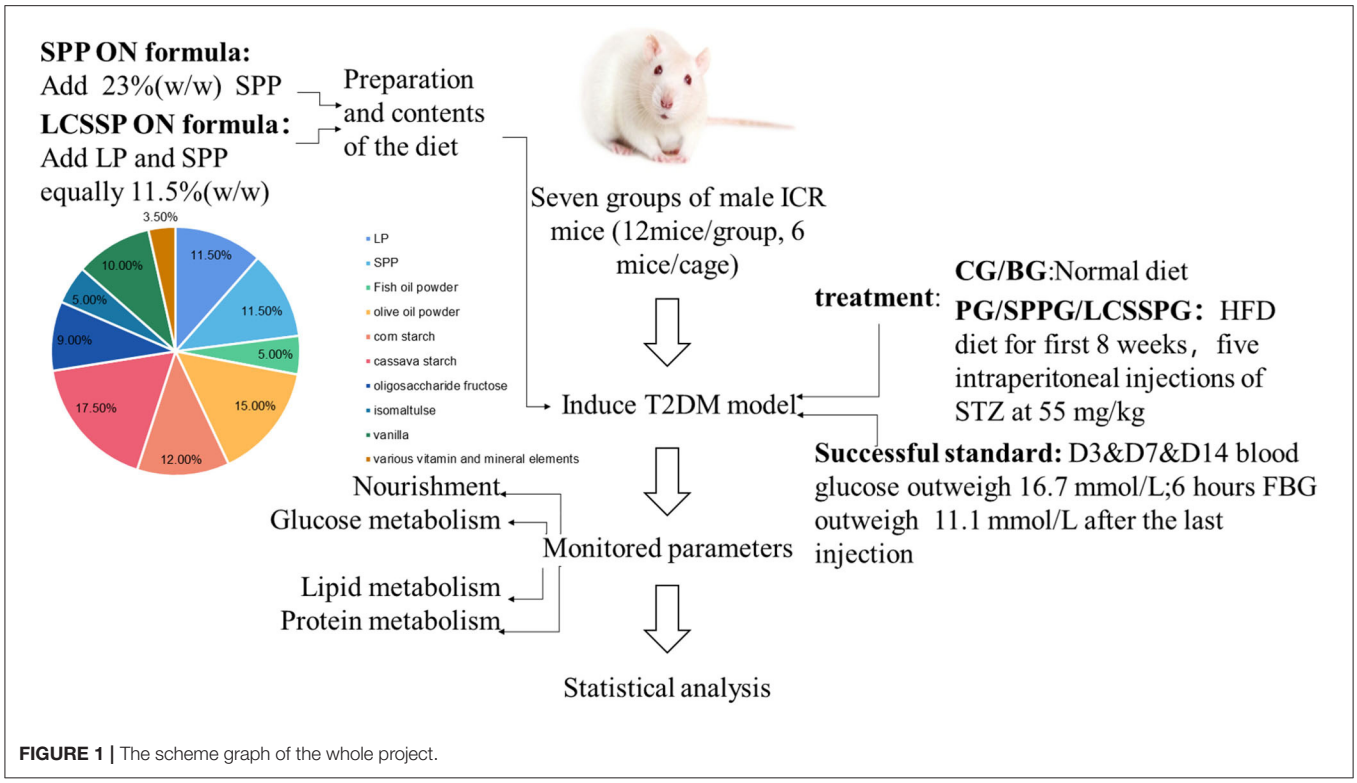

FIGURE 1 | The scheme graph of the whole project.

TABLE 1 | The enteral formula design of four special protein.

Nutrient component	Content(g/100g)			
	SP	LP	LCSPP	SPP
Protein				
Silkworm pupa Protein	0.0	0.0	11.50 ± 1%	23.0 ± 2%
Whey protein	0.0	23.0 ± 2%	11.50 ± 1%	0.0
Soybean protein	23.0 ± 2%	0.0	0.0	0.0
Fat				
Fish oil	5.0 ± 1%	5.0 ± 1%	5.0 ± 1%	5.0 ± 1%
Olive oil	15.0 ± 1%	15.0 ± 1%	15.0 ± 1%	15.0 ± 1%
Carbohydrate				
Tapioca starch	17.50 ± 1%	17.50 ± 2%	17.50 ± 2%	17.5 ± 2%
Isomaltulose	5.0 ± 2%	5.0 ± 2%	5.0 ± 2%	5.0 ± 2%
Oligosaccharide fructose	9.00 ± 2%	9.00 ± 2%	9.0 ± 2%	9.0 ± 2%
Corn starch	12.0 ± 2%	12.0 ± 2%	12.0 ± 2%	12.0 ± 2%
Vitamin and Mineral	Meet the standard of GB-2992-201318			
Dietary fiber	10.00 ± 1%	10.00 ± 1%	10.00 ± 1%	10.00 ± 1%
Essence	2.00×10^{-1}	2.00×10^{-1}	2.00×10^{-1}	2.00×10^{-1}

54% carbohydrate, 18% protein, purchased from Shanghai Jiesijie Experimental Animal Co. Ltd., Shanghai, China) and water for the first week was done to give time for acclimatization. The animal study followed ARRIVE (Animal Research: Reporting *in vivo* Experiments) guidelines and was approved by The Institutional Animal Care and Use Committee (IACUC), Shanghai Ocean University Center for Animal Experiment (Shanghai, China).

Grouping and Diabetes Modeling

The 84 mice were randomly divided into 7 groups ($n = 12$/group, 6 mice/cage) which included a control group (CG, normal mice, normal food), a non-treated group (BG, diabetic mice, normal food), a positive control group (PG, diabetic mice, HFD), soybean protein group (SPG, diabetic mice, HFD), silkworm pupa protein group (SPPG, diabetic mice, HFD), Whey protein group (LPG, diabetic mice, HFD), and Whey protein combined

TABLE 2 | The methods and standards for the analysis of enteral formula's nutrient composition.

Nutrient component	Measures	Standards
Protein	Kjeldahl (factor: 6.25)	GB50095-2010
Fat	Soxhlet extraction	GB/T 5009.6-2003
Carbohydrate	High performance liquid chromatograph	GB/T 8885-2017 GB5009.8-2016
Minerals	Inductively coupled plasma mass spectrometry	GB5009.268-2016
Sodium	mass spectrometry	GB 5009.91-2017
Potassium	mass spectrometry	GB 5009.91-2017
Iodine	Gas chromatography	GB 5009.267-2016
Vitamin		
Choline chloride	spectrophotometry	GB/T 17481-1998
Inositol D-quiral	Gas chromatography	GB5009.270-2016
VB3	plasma mass spectrometry	GB 5009.89-2016
Thiamine	High performance liquid chromatography	GB 5009.84-2016
Riboflavina	High performance liquid chromatography	GB 5009.85-2016
Pyridoxine Hydrochloride	High performance liquid chromatography	GB 5009.154-2016
Cyanocobalamin	High performance liquid chromatography	GB/T 17819-2017
Ascorbic acid	High performance liquid chromatography	GB5009. 86-2016
Palmitic acid VA	colorimetric	GB 5009.82-2016
VD3, cholecalciferol	High performance liquid chromatography	GB 5009.82-2016
Vitamin E	colorimetric	GB 5009.82-2016
Menaquinone	High performance liquid chromatography	GB 5009.158-2016
VB3	High performance liquid chromatography	GB/T 17813-2018
Calcium pantothenate	High performance liquid chromatography	GB 5009.210-2016
VH	spectrophotometry	GB 5009.259-2016
VB9	High performance liquid chromatography	GB/T 17813-2018
L-Arginine	infrared spectroscopy	GB 28306-2012
Taurine	High performance liquid chromatography	GB 5009.169-2016

with silkworm pupa protein group (LCSSPG, diabetic mice, HFD) (**Table 3**). The HFD (D12451, 47.3 kcal/100g, 45% fat, 35% carbohydrate, 20% protein) was purchased from Suzhou Shuangshi Experimental Animal Feed Technology Co. Ltd., (Suzhou, Jiangsu, China). All the groups were maintained on their diets for 8 weeks.

The study was conducted according to the method of Sun et al. (18). After 8 weeks, the mice fed with HFD were intraperitoneally given an injection of 55 mg/kg body weight STZ. Thereafter, they were given additional injections at the same time every other day for four additional shots. The STZ powder was dissolved immediately into 0.1 mol/L citrate acid buffers (pH 4.5) in an ice bath for 20 min (19). The CG mice were given an injection of an equivalent volume of the citric acid buffer. Mice were considered diabetic when at 3-, 7-, and 14-days BLG exceeded 16.7 mmol/L (20), and 6 h fasting BLG exceeded 11.1 mmol/L after the last injection (21). Twenty days after the first STZ shot, the feeding of the ON formula began. The BLG was determined using a BLG meter (Sannuo GA-3 type, Changsha, Hunan, China). All mice had free access to diet and water.

Measurements

Body Weight and Other Animal Measurements

Body weight was measured every week from the beginning of the experiment. The mice were sacrificed with chloral hydrate at 4.5 mg per 100 g body weight, and blood was drawn from the heart. One mouse per group was fasted for 6 h and sacrificed at weeks 2, 10, 11, and 14. All remaining mice were sacrificed by week 15. Approximately 1 ml of blood was obtained from each mouse in a 2 ml sterile citrate anticoagulant centrifuge tube and the plasma was separated by centrifugation at 4,000 g/min, for 15 min at 4°C. The serum was separated and stored at −80°C for further analysis, a maximum of 1 year (22).

Lipid Metabolite Indices of Plasma

Total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and HDL-C levels were measured using an automatic biochemical analyzer (AU5800 Clinical Chemistry System, Beckman Coulter, S.Kraemer Boulevard Brea, CA92821, USA).

Protein Metabolite Index of Plasma

The levels of TP and ALB were detected in the plasma of experimental animals using an automatic biochemical analyzer (AU5800 Clinical Chemistry System, Beckman Coulter, S. Kraemer Boulevard Brea, CA92821, USA).

Glucose Metabolite Index of Plasma

The fasting blood glucose (FBG) level was measured every week. Postprandial plasma glucose was measured at weeks 9, 11, 13, and 15 after mouse feeding. Blood samples were collected from the tail vein and BLG levels were determined using the glucometer.

Statistical Analysis

All data were analyzed as the mean ± SD and levels were compared among groups using one-way ANOVA. Statistical analysis was done using the Statistical Program for the Social Sciences Version 23.0 (SPSS, Chicago, IL, USA). Line chart and histogram were prepared using origin pro-2019 (Wellesley Hills, MA, USA). Differences were taken as significantly different when $p < 0.05$ and were considered very significant when $p < 0.01$.

RESULTS AND DISCUSSION

The Composition of Four ON Formula

The formulations of four kinds of the ON formula were analyzed according to the methods in **Table 2** and listed in **Table 4**. The nutrient compositions of the four ON formula were analyzed in SPG, LPG, LCSSPG, and SPPG. The protein contents were 19.9, 20.1, 21.3, and 21.8 g/100 g, respectively. Carbohydrate contents

TABLE 3 | Information's about the seven groups.

Groups	Groups	Nutrition support	STZ injection
BG	Blank group	Normal diet	Yes
PG	Positive control group	Abbott glucerna Slow Release powder	Yes
LCSSPG	Lactoalbumin Combined Silkworm Pupa Protein group	Lactoalbumin Combined Silkworm Pupa protein ON tablets	Yes
SPPG	Silkworm pupa protein group	Silkworm pupa protein ON tables	Yes
LPG	Lactoalbumin protein group	Lactoalbumin protein ON tablets	Yes
SPG	Soybean protein group	Soybean protein ON tablets	Yes
CG	Control group	Normal diets	No

were 43, 42.9, 43.6, and 42.1 g/100 g, respectively. Fat contents were 19.1, 19.3, 19.5, and 19.6 g/100g, respectively. The total energy of four ON formula was lower than 1870.5KJ. The fat-supplied energy was less than 35%, carbohydrate-supplied energy was less than 45%, and the proportion of protein-supplied energy was less than 23% in all self-made oral formula.

The compositions of the four ON formula were similar and protein type was the only way to distinguish them. The differences among those three proteins were, mainly, amino acid composition. Eighteen kinds of amino acids were detected in SP which included 1.8 g of methionine, 6.1 g of lysine 1.2 g of cysteine, 4.0 g of threonine, and 4.6 g/16 g N of alanine on average and so on (23). Stefan et al. demonstrated that the essential amino acids content of SP and LP was 27% and 43%, respectively (24). SPP, as a novel high-quality raw material for complementary medicine food and the mass ratio of essential or non-essential amino acids, was.77, which was higher than the FAO-WHO requirements (>0.6) (25).

Relatively lower essential amino acids content led to better protein metabolism according to the results. Nonetheless, the amino acid mixtures cannot reach identical results comparing with protein consumption (26). Plasma amino acid, closely related to muscle protein synthesis, was increased after protein intake (24, 25).

Diet can also lower the rate of developing diabetes in those with impaired glucose tolerance (7, 27, 28). Proper amounts of vitamins and minerals which include zinc, chromium, magnesium, vitamin B group, and inositol were added to alleviate glucose and lipid metabolism disorder in T2DM mice (28). An amount of 10 g/100 g dietary fiber was supplemented in the ON formula to prolong gastric emptying time and delay glucose absorption. A series of systematic reviews and meta-analyses suggested that the T2DM rate was decreased 15-30% when there is a 25–29 g dietary fiber intake according to a 2019 review report (29). Tapioca starch was added to the ON formula to stable postprandial BLG (30).

Fish oil- and olive oil-supplemented diets provided better glycaemic and lipid metabolic control in both human and animal experiments comparing with a normal diet. Eight grams of olive oil per day has a 5% T2DM risk-reducing effect according to a 22-year follow-up study (31). David et al. showed that an HFD, supplemented with fish oil for 30 weeks lowered the effects of liver cholesterol, cholesterol ester, and triacylglycerol in C57BL/6J mice compared with mice fed an HFD supplemented with lard (32). In this present study, the self-made ON formula combined all the features above.

The Regulation of Protein Metabolism by Four Oral Formula *in vivo*

The TP, ALB, and GLO levels in serum were analyzed to explore whether ON formula supplemented with specifically designed proteins had an effect on protein metabolism (**Table 5**). The TP, ALB, and ALB/GLO levels were decreased in BG when compared with CG. The concentration of TP in LCSSPG, SPPG, and SPG was decreased to 31, 39, and 39.5 mmol/L, respectively, which nearly reached normal levels after 4 weeks. The ALB level was significantly increased from 15.0 to 22 and 22.5 in SPPG and SPG, respectively. The TP, ALB, and GLO levels were increased in PG ($p < 0.05$) while no statistically significant difference in the ratio of ALB/GLO level was observed.

A decrease in the levels of TP, ALB, and GLO was verified both in T2DM humans and mice which may relate to the factor of insulin resistance in pancreatic β-cells (33). As expected, specially added proteins such as SPP and SP can release functional amino acids which can repair amino acid stimulated protein synthesis to further alleviate whole-body protein metabolism. Taken together, the results demonstrated that ON formula containing suitable protein such as SP or SPP at a proportion of 23 g/100 g could bring back protein levels to normal in plasma comparing with normal diet and even better than available ON formula products.

The Regulation of Lipid Metabolism by Four Oral Formula

The effects of ON formula consumption on lipid metabolism were shown in **Figure 2**. Statistical differences were conducted among seven groups which were shown in **Figure 1**. The TC, TG, LDL-C were increased significantly after 8 weeks high-fat diet (HFD) combined with STZ intraperitoneal injection. The HDL-C level was significant decreased in the BG comparing with CG ($p < 0.01$). The serum TC level was significantly reduced after consumption of SP, SPP, LCSSPP, PG, and LP comparing with BG (**Figure 2A**). The TG level was significantly decreased after administration of SP, SPP, LCSPP, and LP comparing with BG (**Figure 2B**). In the study, it was found that SP, SPP, LCSSP, and LP ON formula had a great role in decreasing the serum TG and TC level in HFD induced mice. The HDL-C levels were increased significantly on LCSSPPG, SPPG, SPG, and PG

TABLE 4 | Formulations of enteral nutritional formula.

Nutrient component	The Content(g/100g)			
	SPG	LPG	LCSSPG	SPPG
Protein	19.9 ± 0.4	20.1 ± 0.1	21.3 ± 0.3	21.8 ± 0.6
Fat	19.1 ± 0.22	19.3 ± 0.1	19.6 ± 0.5	19.6 ± 0.2
Carbohydrate	43.0 ± 0.1	42.9 ± 0.1	43.5 ± 0.4	42.1 ± 0.3
Mineral				
Sodium	0.41 ± 0.03	0.41 ± 0.02	0.38 ± 0.02	0.45 ± 0.1
Potassium	0.99 ± 0.2	0.94 ± 0.1	0.94 ± 0.1	0.95 ± 0.1
Chloride	0.78 ± 0.1	0.71 ± 0.2	0.78 ± 0.2	0.77 ± 0.2
Calcium	0.4 ± 0.04	0.38 ± 0.07	0.41 ± 0.4	0.42 ± 0.1
Phosphate	0.54 ± 0.05	0.51 ± 0.11	0.5 ± 0.1	0.52 ± 0.2
Magnesium	$(8. \pm 0.4)\times10^{-4}$	$(8.1 \pm 0.1\times10^{-4}$	$(8.3 \pm 0.1)\times10^{-4}$	$(8.2 \pm 0.2)\times10^{-4}$
Ferric	$(1.3 \pm 0.1)\times10^{-2}$	$(1.5 \pm 0.3)\times10^{-2}$	$(1.7 \pm 0.2)\times10^{-2}$	$2.0 \pm 0.2)\times10^{-2}$
Zinc	$6.8 \pm 0.4)\times10^{-3}$	$(7.0 \pm 0.2)\times10^{-3}$	$(7.1 \pm 0.2)\times10^{-3}$	$(6.9 \pm 0.2)\times10^{-3}$
Copper	$(8.5 \pm 0.3)\times10^{-4}$	$(8.7 \pm 0.2)\times10^{-4}$	$(8.3 \pm 0.24)\times10^{-4}$	$(8.8 \pm 0.1)\times10^{-4}$
Manganese	$(6.8 \pm 0.2)\times10^{-4}$	$(6.6 \pm 0.1)\times10^{-4}$	$(6.5 \pm 0.1)\times10^{-4}$	$(6.5 \pm 0.1)\times10^{-4}$
Molybdenum	$(7.1 \pm 0.3)\times10^{-4}$	$(7 \pm 0.2)\times10^{-4}$	$(6.9 \pm 0.3)\times10^{-4}$	$(7.2 \pm 0.2)\times10^{-4}$
Selenium	$(1.9 \pm 0.2)\times10^{-4}$	$(1.8 \pm 0.1)\times10^{-4}$	$(2 \pm 0.2)\times10^{-4}$	$(2 \pm 0.1)\times10^{-4}$
Vitamin				
Choline chloride	0.16 ± 0.03	0.14 ± 0.01	0.16 ± 0.2	0.14 ± 0.1
Inositol D-quiral	0.3 ± 0.04	0.32 ± 0.02	0.33 ± 0.1	0.2 ± 0.1
Tiamina	$(8.0 \pm 0.25)\times10^{-4}$	$(6.0 \pm 0.1)\times10^{-4}$	$(7.0 \pm 0.4)\times10^{-4}$	$(6.0 \pm 0.2)\times10^{-4}$
Riboflavina	$(3.0 \pm 0.4)\times10^{-4}$	$(4.0 \pm 0.2)\times10^{-4}$	$(5.0 \pm 0.3)\times10^{-4}$	$(5.0 \pm 0.4)\times10^{-4}$
Pyridoxine Hydrochloride	$(1.1 \pm 0.2)\times10^{-3}$	$(1 \pm 0.2)\times10^{-3}$	$(1.3 \pm 0.2)\times10^{-3}$	$(1.2 \pm 0.2)\times10^{-3}$
Ascorbic acid	$(3.0 \pm 0.5)\times10^{-2}$	$(4.0 \pm 0.1)\times10^{-2}$	$(3.5 \pm 0.3)\times10^{-2}$	$(3.8 \pm 0.1)\times10^{-2}$
Palmitic acid VA	$(5.0 \pm 0.3)\times10^{-4}$	$(6 \pm 0.2)\times10^{-4}$	$(9.0 \pm 0.1)\times10^{-4}$	$(8.0 \pm 0.2)\times10^{-4}$
VD3 cholecalciferol	$(1.2 \pm 0.3)\times10^{-5}$	$(1.6 \pm 0.2)\times10^{-5}$	$(1.1 \pm 0.1)\times10^{-5}$	$(1.5 \pm 0.2)\times10^{-5}$
Vitamin E	$(2 \pm 0.3)\times10^{-3}$	$(2.6 \pm 0.1)\times10^{-3}$	$(2.3 \pm 0.1)\times10^{-3}$	$(2.3 \pm 0.1)\times10^{-3}$
Phytonadione	$(4.0 \pm 0.4)\times10^{-5}$	$(6.0 \pm 0.1)\times10^{-5}$	$(9.0 \pm 0.2)\times10^{-5}$	$7.1 \pm 0.2)\times10^{-5}$
VB3	$(3.6 \pm 0.2)\times10^{-3}$	$(3.6 \pm 0.1)\times10^{-3}$	$(2.8 \pm 0.1)\times10^{-3}$	$(3.0 \pm 0.1)\times10^{-3}$
Calcium pantothenate	$(2.4 \pm 0.2)\times10^{-3}$	$(2.1 \pm 0.1)\times10^{-3}$	$(2.2 \pm 0.1)\times10^{-3}$	$(2.0 \pm 0.1)\times10^{-3}$
VH	$(3 \pm 0.2)\times10^{-4}$	$(3.3 \pm 0.1)\times10^{-4}$	$(3.1 \pm 0.1)\times10^{-4}$	$(3.2 \pm 0.2)\times10^{-4}$
VB9	$(1 \pm 0.3)\times10^{-4}$	$(1.2 \pm 0.2)\times10^{-4}$	$(1.1\times10\text{-}3)\times10^{-4}$	$(1.1 \pm 0.1)\times10^{-4}$
L-arginine	0.2 ± 0.03	0.21 ± 0.04	0.22 ± 0.1	0.21 ± 0.1
Taurine	$(3. \pm 0.5)\times10^{-2}$	$(3.4. \pm 0.2)\times10^{-2}$	$(3.9 \pm 0.2)\times10^{-2}$	$(3.1 \pm 0.1)\times10^{-2}$

comparing with the BG group indicating that LCSSPP, SPP, and SP oral formula may lower the risk of HDL-C loss in HFD-induced mice (**Figure 2C**). The level of serum LDL-C was greatly lowered after consumption of PG, SPPG, LCSSPG, and SPG while there was no significant difference in the LPG group which needed further analysis (**Figure 2D**). These data indicated that PG, SPPG, LCSSPG, and SPG had a good supporting role in decreasing the serum LDL-C level in HFD induced mice.

This study proved that the ON formula could significantly reduce serum TC, TG, HDL-C, and LDL-C levels in T2DM mice comparing with a normal diet. The results are consistent with previous research reported by Sung et al. (33). The proteins used in the ON formula are highly responsible for those changes in lipid metabolism. Sun He et al. found the anti-obesity activity of a silkworm pupa peptide in an HFD feeding in rats by inhibiting the differentiation of preadipocytes and adipogenesis (33). Rizaldy et al. verified the decreased weight and adiposity of LP in male obese rats due to decreased hepatic lipidosis compared to control, partly through downregulation of lipogenic and upregulation of β-oxidation transcripts in the liver (34). Based on literature reviews, a diet with soy protein containing isoflavones reduced low-density lipoprotein (LDL) (35), but without clear effects on TG or high-density lipoprotein (HDL). The results demonstrated similar results which alleviate lipid metabolism by high-quality protein addition.

The Regulation of BLG by Four ON Formula

The BLG and fasting BLG changes during the experiment are shown in **Figure 3**, respectively. The FBG and BG were enhanced from 7 to 10 wks and peaked in the tenth week. The FBG has a great reduction with the value of 46.7, 24.1, 21.8, and 33.9% in SPG, LCSSPG, SPPG, and PG group, respectively, after 5 wks of treatment. Continuous elevation of FBG and BLG in BG was observed, which is strong evidence for T2DM modeling

making. The FBG level was decreased to 16.8 to 8.9, 12.8, 13.2, and 11 in SPG, LCSSPG, SPPG, and PG groups at the end of the experiment. Furthermore, there were no wide fluctuations in BLG levels at the end of treatment, which needed to be further analyzed.

TABLE 5 | Effect of different enteral formula on TP, ALB and GLO in high fat diet induced mice.

Group	TP (mmol/L)	ALB (mmol/L)	GLO (mmol/L)	ALB/ GLO (%)
CG	46.6 ± 2.0**	26.5 ± 2.0**	20.4 ± 1.1**	1.31 ± 0.08**
BG	29.5 ± 3.0	15.0 ± 2.0	14.3 ± 0.7	1.07 ± 0.03
PG	32 ± 1.5	16 ± 1.0	13.5 ± 1.7	1.05 ± 0.03
LCSSPG	31.0 ± 2.0	18 ± 0.7	13 ± 0.7	1.35 ± 0.1*
SPPG	39.5.0 ± 1*	22.5 ± 0.3*	17 ± 0.7*	1.33 ± 0.03*
LPG	26.5 ± 1.0	15.5 ± 0.3	14 ± 0.7	1.11 ± 0.03
SPG	39.5 ± 0.3*	22.5 ± 1*	16.5 ± 0.3*	1.40 ± 0.08*

*Data represent as mean ± SEM. *Represents $p < 0.05$ vs. blank group. **$p < 0.01$ vs. blank group.*

The study, *in vivo*, indicated that the administration of the ON formula to the HFD-fed mice combined with an intraperitoneal injection of STZ produced a remarkable reduction of BLG and FBG after 5 weeks of treatment with SPPG, SPG, and LCSSPG. This was true except for the LPG and PG since they showed a low reduction on these parameters. The main proteins used in the formulations played an important role in the hypoglycaemic effect. Further, another study has shown that soybean supplementation would be helpful to control BLG and serum lipid in patients with diabetes (35). The soybean fiber contains compounds with high viscosity like pectin, galactomannans, and arabinogalactans that delay gastric emptying and glucose absorption. Novels peptides from silkworm pupae were found by Yu et al. (36) with high α-glucosidase-inhibiting activity so that this protein can retard glucose absorption and suppressing postprandial hyperglycaemia.

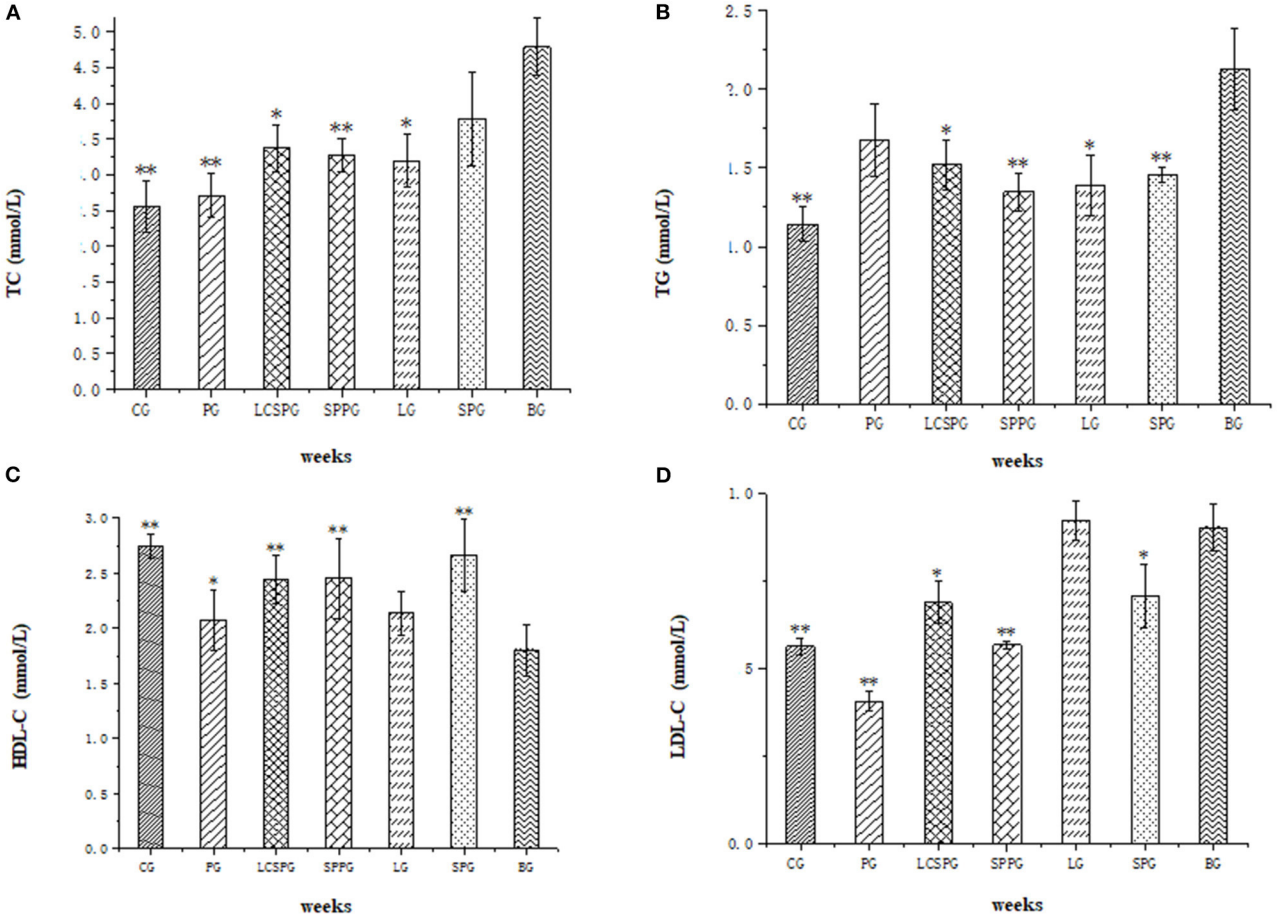

FIGURE 2 | (A–D) Total cholesterol concentration (TC). **(A)** triglycerides (TG), **(B)** high-density lipoprotein cholesterol (HDL-C), and **(C)** low-density lipoprotein cholesterol (LDL-C) **(D)** levels of seven groups. Data represented as mean ± SD *$p < 0.05$ and **$p < 0.01$: compared with a blank group (BG). BG was T2DM mice fed the normal diet; CG was normal mice fed a normal diet; PG was T2DM mice fed Abbott Glucerna Slow Release; LG was T2DM mice fed with LP ON formula; SPG was T2DM mice fed SP ON formula; SPPG was T2DM mice fed SPP ON formula; LCSSPG was T2DM mice fed LCSSP ON formula.

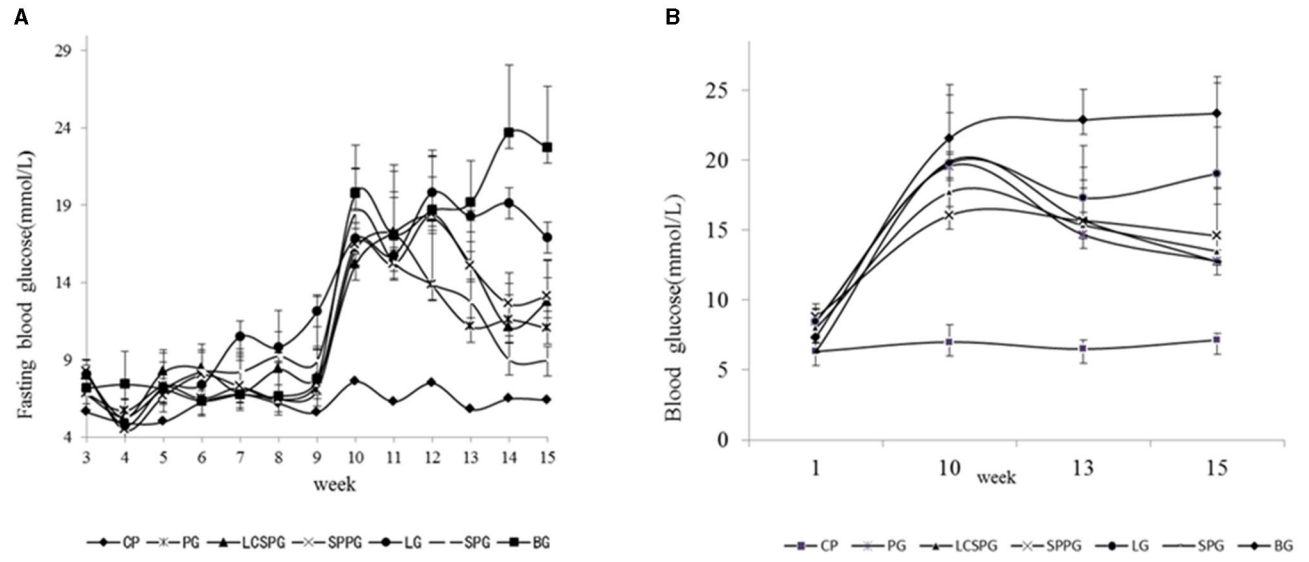

FIGURE 3 | Changes in blood glucose (BLG) **(B)** and fasting BLG **(A)** of the mice fed with a high-fat diet (HFD) combined with an intraperitoneal injection of streptozotocin (STZ) during the experiment.

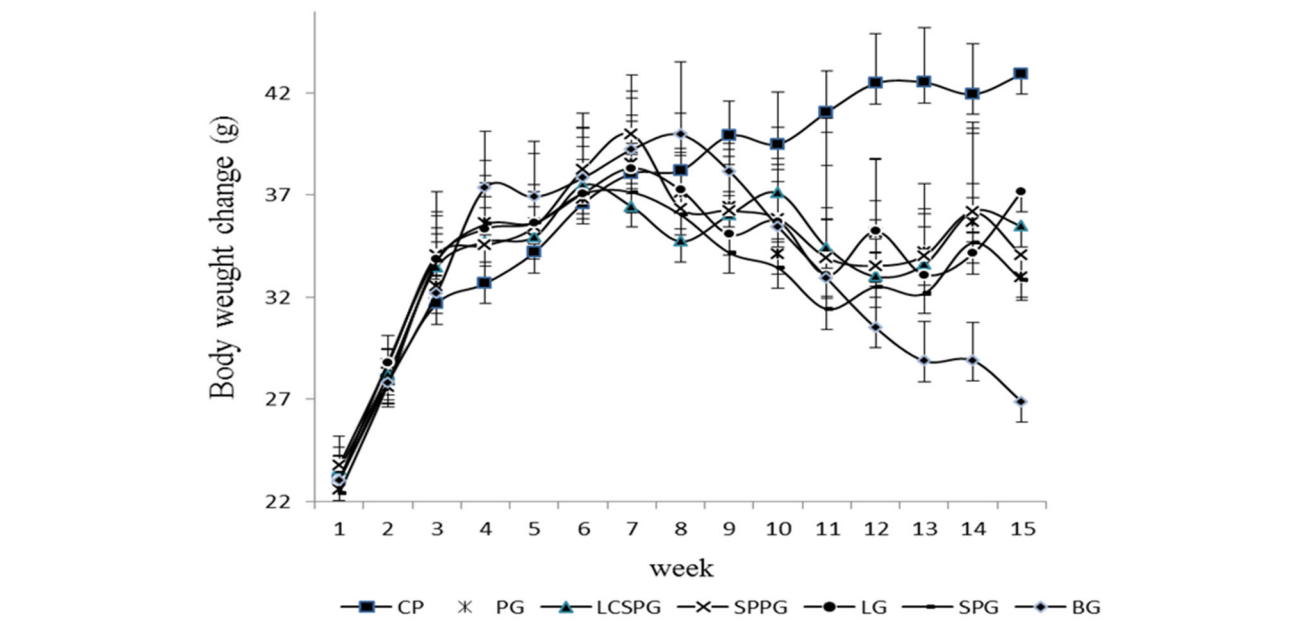

FIGURE 4 | Body weight change of mice when HFD, normal diet, and ON formula was given to corresponding groups for 15 weeks.

The Regulation of Body Weight by Four Oral Formula

The body weight changes after the 15-week feeding in the experiment are shown in **Figure 4**. Body weight gain rate was similar at the beginning of feeding in the HFD induced group (LPG, SPG, LCSSPG, SPPG, BG, and PG) and their body weight doubled after 8 weeks of HFD feeding. Increased thirst, frequent urination, and unexplained weight loss were the most common symptoms of patients with T2DM (37). The body weight decreased sharply (3.26 to 5.33 g per mouse) after T2DM models were successfully established in the 10th week in SPG, SPPG, LPG, CG, BG, and LCSSPG with no obvious differences among groups, which was consistent with the above mentioned. Body weight increased after 2 weeks of treatment and increased onward but slightly decreased (about 0.8–1.2 g per mice) after 5 weeks of treatment in all groups except CG and LG. Body weight loss was stopped after 2 weeks of treatment and increased onward until the end of treatment in LPG. The body weight loss in BG

was continued at the end of the experiment which claimed the successful development of T2DM mice.

Body weight, on behalf of whole-body nutrient metabolism index with losses over 10% could lead to metabolism dysfunction (38). Thus, the degree of weight stability and restoration was taken as a key factor in reversing T2DM symptoms. The body weight declined to the minimum value after 2 weeks of treatment and then plateaued and increased. The body weight gain occurred after a 2-week interval, which was similar to the previous study by Qingbo et al. (25). Over 10% of body weight loss was observed among all groups in the 2-week treatment of minimum weight. Therefore, a suitable amount of ON intervention in time is necessary to inhibit the deterioration of body weight loss. An obvious difference in body weight loss among groups was observed according to statistical analysis. The body weight gain was more rapid in the group fed with ON tablets compared with BG and PG. In the current study, at least a 3-week consumption of ON formula could help to reverse T2DM induced body weight loss according.

CONCLUSION

Dietary protein influences lipid, protein, and glucose metabolism (8). SP could lower cholesterol levels in animals and humans with unclear mechanisms (5, 9). Moriyama et al. suggested that the soybean β-conglycinin diet could reduce serum TG and glucose in genetically obese mice (37). The high content-release of essential amino acids, such as leucine, isoleucine, valine, lysine, and threonine, by LP after digestion, lead to increased secretion of insulin and decrease postprandial hyperglycaemia was verified (34). Emilia et al. pointed that branched short-chain fatty acids (isobutyric, isoleucine, and isovaleric) have an impact on glucose metabolism by improving insulin sensitivity with disturbed metabolism (39). LP can also promote the secretion of GLP-1 and gastric inhibitory peptide (GIP).

The GLP-1a incretin hormones, which can promote glucose-dependent insulin secretion, are the main drug targets in T2DM at present. Meanwhile, GIP is released from K cells after food intake. A greater weight gain, insulin resistance, and hepatic steatosis emerged in the HFD fed mice with GIP receptor deficiency (40). The mass ratio of essential amino acid to non-essential amino acid is superior to the reference mode proposed by FAO-WHO (41).

In this study, the effect of three different oral formula on T2DM *in vivo* was evaluated by some lipid, protein, and glucose metabolite index of plasma. Results showed that levels of TP, ALB, GLO, HDL-C, and ALB/GLO ratio were increased while levels of FBG, BLG, LDL-C, TC, and TG were decreased, all of which were associated with alleviating symptoms after administration of ON formula to SPG, SPPG, LCSSPG, and PG. Body weight, on behalf of whole-body nutrient metabolism, showed a significant different increasing trend between the treatment group and BG. Also, SP and SPP ON formula showed a greater alleviating effect comparing with another treatment group after 5 weeks of treatment. These specifically designed proteins are likely the key role for the restoration of T2DM in ON formula by enhancing a beneficial function and coinciding with the improvement of whole-body nutrient metabolism. Therefore, ON formula may present a novel nutrition therapeutic strategy for the treatment of T2DM.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Shanghai Ocean University Center for Animal Experiment (Shanghai, China).

AUTHOR CONTRIBUTIONS

PW and MM revising it critically and supporting this study. AC and YL carrying out the animal lab work and participating in data analysis. BB and WW design of the study and for drafting the manuscript partially. CB collecting field data. All the authors gave final approval for publication.

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REVIEW
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Marine Bioactive Compounds as Nutraceutical and Functional Food Ingredients for Potential Oral Health

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Oral diseases have received considerable attention worldwide as one of the major global public health problems. The development of oral diseases is influenced by socioeconomic, physiological, traumatic, biological, dietary and hygienic practices factors. Currently, the main prevention strategy for oral diseases is to inhibit the growth of biofilm-producing plaque bacteria. Tooth brushing is the most common method of cleaning plaque, aided by mouthwash and sugar-free chewing gum in the daily routine. As the global nutraceutical market grows, marine bioactive compounds are becoming increasingly popular among consumers for their antibacterial, anti-inflammatory and antitumor properties. However, to date, few systematic summaries and studies on the application of marine bioactive compounds in oral health exist. This review provides a comprehensive overview of different marine-sourced bioactive compounds and their health benefits in dental caries, gingivitis, periodontitis, halitosis, oral cancer, and their potential use as functional food ingredients for oral health. In addition, limitations and challenges of the application of these active ingredients are discussed and some observations on current work and future trends are presented in the conclusion section.

Keywords: marine bioactive compounds, oral health, dental caries, chewing gum, functional food

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INTRODUCTION

Oral and periodontal diseases can determine severe functional, phonatory and aesthetic impairments and are the main cause of adult tooth loss (1). According to statistics, the number of people suffering from untreated dental caries, severe periodontitis and oral diseases has reached 3.5 billion worldwide (2, 3). Moreover, the number of cancers occurring in the lips and oral cavity reached 377,713 in 2020, based on GLOBOCAN statistics (4). Oral diseases have become a major global public health problem. The growing awareness of the oral health benefits has compelled science and industry to conduct research on nutrients for the prevention and treatment of oral diseases, including isolated nutrients and compounds as dietary supplements. The marine-sourced compounds include protein and peptides, protein, ω-3 polyunsaturated fatty acids (PUFA), polysaccharides, polyphenolic compounds, enzymes, vitamins and pigments (5) with a wide range of physiological activities such as antitumor, antioxidant, antiviral, and immunomodulatory activities (6, 7). Thus, the wide variety of marine species is not only a source of important active compounds for the treatment and prevention of various diseases, but also a potential source of ingredients for oral health maintenance (8–10).

Indeed, bioactive materials and molecules from marine sources have been applied in many potential research areas in dental science. Shaikh et al. (11) conducted a review on the application of marine derived ingredients such as chitosan, bio-adhesives, tissue regeneration gel, calcium hydroxyapatite, gypsum and algal extracts in the field of dentistry. Ibrahim et al. (12) found that marine bioactive ingredients have inhibitory effects on *Streptococcus mutans* (*S. mutans*) and can be used to maintain dental health. In recent years, various scientific research publications on marine bioactive ingredients health benefits and the development of marine nutraceuticals have been published. Two hundred and ninety-eight papers related to marine bioactive compounds and oral disease published in the past 18 years were retrieved from Science Direct and analyzed by Vosviewer for keywords (marine bioactive compounds, oral disease/disorder/health). The results of the co-occurrence analysis showed that the research related to the effects of marine bioactive compounds on oral health gradually shifted to periodontitis, dental plaque, phytotherapy and functional food. **Figure 1** shows the overlay visualization of keyword statistics.

Published reviews with a comprehensive description of marine bioactive compounds as nutritional and functional food ingredients for potential oral health are limited. Therefore, it is necessary to systematically summarize the benefits, risks and prospects of marine bioactive compounds in oral health applications. This review provided a comprehensive overview of different marine bioactive compounds and their health benefits, as well as their potential to be used as functional food ingredients for oral health. In addition, the limitations and challenges of the application of these active ingredients were discussed and some perspectives on the current work and future trends were presented in the conclusion section.

ORAL HEALTH

Oral Health, Plaque and Microbiome

Oral health is an important indicator of people's overall health and quality of life. The World Health Organization (WHO) defines oral health as "a state of being free from chronic mouth and facial pain, oral and throat cancer, oral infection and sores, periodontal (gum) disease, tooth decay, tooth loss, and other diseases and disorders that limit an individual's capacity in biting, chewing, smiling, speaking, and psychosocial well-being" (13, 14). Recent years, oral epidemiology is shifting globally and the main oral disorders that plague people's lives include: dental caries, periodontal disease, oral cancer, oral manifestations of HIV, oro-dental trauma, cleft lip and palate, and Noma (13, 14). Oral diseases can lead to pain, impaired function and reduced quality of life, which can have a direct impact on an individual's quality of life (15).

Plaque in the oral cavity is considered to be the basis for the initiation and development of dental caries and periodontitis. Plaque consists of a large number of bacteria, intercellular material, a small amount of white blood cells, shed epithelial cells and food debris, and is a bacterial biofilm that cannot be washed away by water. In dentistry, biofilms are defined as microbial communities of surface-attached cells embedded in a self-produced extracellular matrix (16–19). Dental plaque biofilms on the tooth surface form a bacterial envelope that is coordinated and balanced with the surrounding tissues (16).

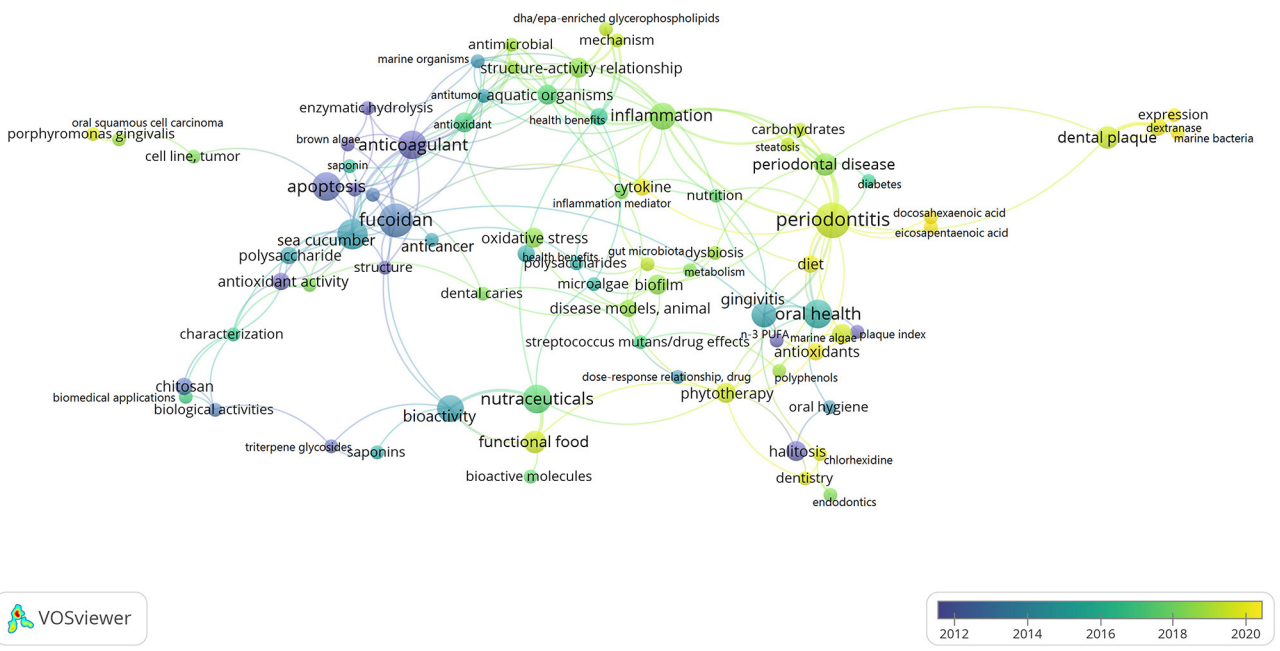

FIGURE 1 | Overlay visualization of keyword statistics.

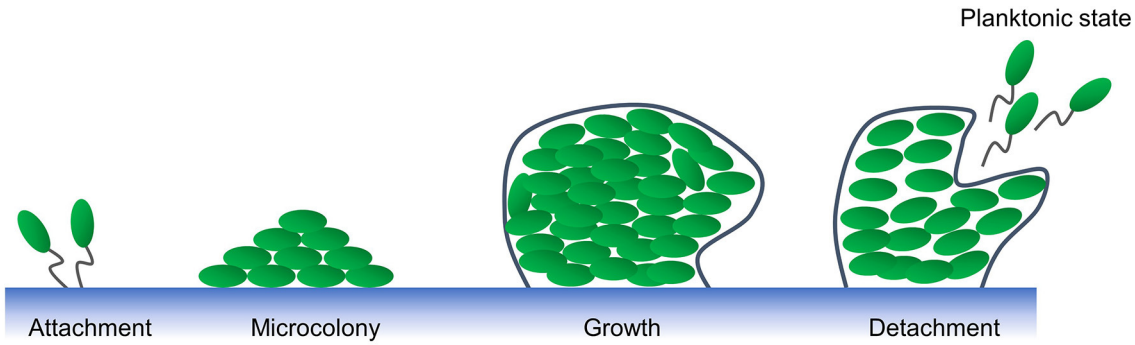

FIGURE 2 | Biofilm formation.

The development of biofilm formation was shown in **Figure 2** (16, 17). Dental plaque biofilms have the ability to interact in a complex interplay with the innate immune and inflammatory networks that ensure the maintenance of the host's health (20).

Indeed, the development and progression of oral disease is largely influenced by oral microbiome in the oral cavity (21–26). A variety of microorganisms collectively influence the microecological balance of the oral cavity through symbiotic "competitive" antagonism (15). Many of these microorganisms are harmless; a few, such as S. *mutans*, *Actinomyces sp., Treponema denticola, Tannerella forsythia, Bacteroides sp., A. actinomycetemcomitans, Staphylococcus intermedius*, *Porphyromonas gingivalis* and *Candida albicans,* can cause oral infections (15). Moreover, factors such as immunity weakening or steroid treatment can disturb the balance of oral bacteria and can also cause opportunistic infections. Nutritional deficiencies can also alter the composition of the oral microbiome and lead to oral health problems (27). Any process resulting in weakened immunity or a factor such as steroid therapy that disturbs the oral bacterial homeostasis can cause opportunistic infections (28). Deficient diet can also alter the composition of the oral microbiome leading to oral diseases such as dental caries and periodontitis. Therefore, to ensure oral health, the oral cavity needs regular care and maintenance.

Oral Health Maintenance

Mechanical oral hygiene aid is an important way to maintain oral health (29). The most common mechanical oral cleaning is toothbrushing, but this method is not appropriate for removing plaque from the gingival sulcus of normal dentition (30, 31). Hence, regular toothbrushing needs to be accompanied by some supplementary modalities for plaque removal. Mouth rinses and chewing gum are oral cleaning aids used in the daily routine (14, 32, 33). Rinses wash the mouth quickly by mechanically flushing, making it impossible for plaque to accumulate on the teeth. Anti-inflammatory compounds such as chlorhexidine, dextranase, menthol, and triclosan are used in the treatment of oral diseases, but can lead to side effects with long-term use (34). Calvo-Guirado et al. (35) compared the effectiveness of 0.20% chlorhexidine with Sea 4® Encias (seawater) oral rinse. Based on this study, the use of seawater mouthwash had similar antimicrobial activity to chlorhexidine. Despite the better clinical performance of chlorhexidine, seawater mouthwash can reduce side effects such as tooth staining and become a daily option for maintaining oral health. Chewing gum, on the other hand, increases the secretion of saliva in the mouth through the mechanical stimulation of chewing motion, rinsing the mouth and removing of dietary fermentable carbohydrates and plaque from the mouth. Moreover, the base of chewing gum is highly sticky, which further removes plaque from the teeth. Although sugar-free chewing gum can be used as an adjunct to brushing and can significantly and slightly reduce plaque production, there is no significant improvement in gingivitis (36). Based on these characteristics, if functional ingredients with the ability to resist *S. mutans* strains [e.g., quercetin (Qt)] are added to chewing gum formulations, chewing gum may be used as a delivery and retention of bioactive molecules in a particularly effective way, with potential anti-caries effects. Besides cleaning, the central role of diet, natural agents and nutrition is also considered to be indirectly responsible for the health of periodontal tissues and the fight against resorption of alveolar bone resorption (27, 37).

RESEARCH PROGRESS ON MARINE BIOACTIVE COMPOUNDS IN ORAL HEALTH

With the improvement of biological separation and purification technology and the in-depth research of marine biochemical drugs, an increasing number of marine bioactive substances have been isolated and identified (38–49). Now, more than 4,000 marine bioactive compounds have been isolated, but only a small number of them have been intensively studied and exploited (50). In terms of chemical structure, marine bioactive substances mainly include peptides, polysaccharides, alkaloids, terpenoids, macrocyclic polyesters, polyethers, polyenes, unsaturated fatty acids and steroids (6, 7, 51, 52). **Table 1** summarizes the various marine bioactive ingredients used to prevent and treat oral diseases ranging from dental caries to halitosis.

TABLE 1 | List of marine active ingredients for various oral diseases and their causative pathogens.

Objective	Compounds	Form	Source	Results	References
Antibacterial	*Chlorella vulgaris* and *Dunaliella salina* extract	/	/	The biofilms of *Streptococcus mutans* were more effectively prohibited by *D. salina* extract than *C. vulgaris* extract.	(51)
Articular cartilage regeneration using human dental pulp stem cells cultured	Alginate	Hydrogel	/	Dental pulp stem cells (hDPSCs) cultured in 3% alginate hydrogels may be useful for regeneration of articular cartilage.	(52)
Dental caries	Dextranase	/	Marine bacterium *Catenovulum sp*. (Cadex)	Cadex from a marine bacterium was shown to be an alkaline and cold-adapted endo-type dextranase suitable for development of a novel marine agent for the treatment of dental caries.	(53)
Dental plaque	Dextranase	/	*Catenovulum agarivorans* MNH15	Dextranase has high application potential in dental products such as toothpaste and mouthwash.	(54)
Dental plaque	Polysaccharide extract of sea cucumber *Stichopus horrens*	/	/	Polysaccharide extract of sea cucumber *S. horrens* had the potential to be further expanded into a beneficial substance with therapeutic feature that could be used in preventive and restorative dentistry	(11)
Dental pulp biomineralization and Differentiation	Alginate	Alginate/Hydroxyapatite-Based Nanocomposite Scaffolds	/	Alg/HAp scaffolds as feasible composite materials in tissue engineering, being capable of promoting a specific and successful tissue regeneration as well as mineralized matrix deposition and sustaining natural bone regeneration.	(55)
Dental pulp repair	Alginate	Hydrogel	/	Alginate hydrogels provide an appropriate matrix in which dental regeneration can take place and may also be useful for delivery of bioactive molecules, such as growth factors, to enhance the natural regenerative capacity of the dental pulp.	(56)
Encapsulation of periodontal ligament (PDLSCs) and gingival mesenchymal (GMSCs) stem cells system	Alginate	Hydrogel	/	Alginate is a promising candidate as a non-toxic scaffold for PDLSCs and GMSCs. It also has the ability to direct the differentiation of these stem cells to osteogenic and adipogenic tissues as compared to the control group *in vitro*.	(57)
Gingivitis	Sea cucumber extract	Toothpastes	/	Toothpaste containing sea cucumber extract produced statistically significant reduction in gingival inflammation.	(58)
Gingivitis	n-3 PUFA	/	/	n-3 PUFA induced a tendency toward reduced inflammation but it was not possible to conclude significant efficacy.	(59)
Gingivitis	Fucoidan	/	Brown algae (*Fucus vesiculosus (F85), Fucus vesiculosus (F95), Macrocytis pyrifera (M85), Undaria pinnatifida (U95), Hizikia fusiforme, Kjellmaniella crassifolia, Laminaria japonica, Sargassum honeri, Undaria pinnatifida*), Green algae (*Capsosiphon fulvescens, Codium fragile*), Red alga (*Grateloupia filicina*)	The fucoidan at the concentrations of above 250 μg mL^{-1} completely suppressed the biofilm formations and planktonic cell growths of *S. mutans* and *S. sobrinus*.	(60)
Gingivitis	*Enteromorpha linza* extract	Mouth rinse	/	The twice-daily use of an *E. linza* extract mouth rinse can inhibit and prevent gingivitis.	(61)
Halitosis	Three phlorotannins (eckol, dioxinodehydroeckol and dieckol)	/	Brown seaweed *Eisenia bicyclis*	Phlorotannins derived from *E. bicyclis* can be an effective deodorizing constituent in the food industry and pharmaceutical industries	(62)

(Continued)

TABLE 1 | Continued

Objective	Compounds	Form	Source	Results	References
Inducing mineralization of dental implants	Alginate	Poly-l-lysine/Sodium alginate coating	/	The composite coating could prevent bacterial infections and facilitate mineralization *in vivo* in the early postoperative period, and then, the mineralized surface could enhance the cytocompatibility	(63)
OSCC	*Enteromorpha compressa* solvent extracts	/	/	The presence of novel bioactive compounds in *E. compressa* has uncovered possible therapeutic value against OSCC by modulating antioxidant defense system, apoptosis and autophagy that could be used to explore very competent algal candidates for the development of potential alternative anticancer drugs.	(64)
OSCC	Prodigiosin (PG)	/	Alkaloid and natural red pigment as a secondary metabolite of *Serratia marcescens*	PG under various concentrations and time courses were shown to effectively cause cell death and cell-cycle arrest in OECM1 and SAS cells.	(65)
OSCC	Sandensolide	/	*Sinularia flexibilis*	Both the *in vitro* bioassay and the zebrafish xenograft model demonstrated the anti-oral cancer effect of sandensolide.	(66)
OSCC	11-dehydrosinulariolide	/	Soft coral *Sinularia leptoclados*	Treatment with 11-dehydrosinulariolide for 6 h significantly induced both early and late apoptosis of CAL-27 cells, observed by flow cytometric measurement and microscopic fluorescent observation.	(67)
OSCC	Pardaxin	/	*Pardachirus marmoratus*	Pardaxin shows antibacterial and antitumor activities. However, pardaxin-induced inhibition of oral cancer and the mechanism of tumor reduction in buccal pouch carcinogenesis after pardaxin painting remain undetermined.	(68)
Periodontitis	Sulfated polysaccharides (PLS)	/	Marine algae (*genus Gracilaria*)	The adjunct treatment with PLS from *Gracilaria caudata* could prevent the periodontal and hepatic tissue alteration caused by periodontitis.	(69)
Periodontitis	Chitosan	Drug delivery system (LDDS) with chitosan and poly vinyl alcohol (PVA)	/	CM-chitosan microsphere (Cs2-Ms) had better potentials used as core parts of the novel designed LDDS in the future developments.	(70)
Periodontitis	Sea cucumber extract	/	/	sea cucumber extract has an effect on periodontitis and can be an alternative to treating inflammation	(71)
Periodontitis	Omega-3 polyunsaturated fatty acids (PUFA)	/	/	Dietary intervention with high-dose of omega-3 PUFA during non-surgical therapy may have potential benefits in the management of periodontitis.	(72)
Periodontitis	Omega-3 polyunsaturated fatty acids (PUFA)	/	/	Both DHA and EPA have significant antimicrobial activity against the six bacterial species (*Streptococcus oralis, Actinomyces naeslundii, Veillonella parvula, Fusobacterium nucleatum, Porphyromonas gingivalis, and Aggregatibacter actinomycetemcomitans*) included in this biofilm model.	(73)
Periodontitis	Sulfated polysaccharides (PLS)	/	Marine algae of the *genus Gracilaria*	The adjunct treatment with PLS from *G. caudata* could prevent the periodontal and hepatic tissue alteration caused by periodontitis.	(69)
Supragingival calculus, plaque formation, and gingival health	Alga (*ascophyllum nodosum*)	/	/	Fifty-two participants showed less calculus formation in the alga group than in the control group. Plaque ($p = 0.008$) and gingival bleeding ($p = 0.02$) were also significantly less in the alga group. However, no significant difference was found between the groups for gingivitis ($p = 0.13$).	(74)

Delay the Development of Dental Caries by Inhibiting *S. mutans*

Dental caries, often referred to as cavities or tooth decay, can be defined as the localized chemical dissolution of tooth surfaces due to an imbalance in the oral microbial community (15, 53). According to the Global Oral Health Data Bank, the global prevalence of dental caries ranges from 49 to 83% (54), with ∼486 million children suffering from primary caries and 2.4 billion children suffering from permanent caries (55). *S. mutans*, the main causative agent in the development of dental caries, has the ability to synthesize extracellular glucans exclusively from dietary sucrose (56–58). The main marine bioactive ingredients that are currently used to slow down the development of dental caries by inhibiting the development of *S. mutans* are alginate, sulfated polysaccharides, microalgae extracts, and *glucanases* from marine bacteria. These bioactive components inhibit the development of *S. mutans* by reducing sugar utilization, antibacterial and eliminating biofilms.

Alginate is used to slow down the development of dental caries by reducing the use of sucrose by *S. mutans*. As a non-reducing sugar extracted from seaweed, alginate can be added to foods as a sucrose substitute to slow down the development of dental caries (59). However, alginate did not completely prevent the development of dental caries and showed no effect in disrupting the biofilm formation of *S. mutans* (60). In contrast, fucoidan (*Fucus vesiculosus* F85) possessed notable antimicrobial activities against *S. mutans*, but no biofilm elimination was observed in the study of Jun et al. (61). Microalgae such as *Dunaliella salina* and *Chlorella vulgaris* have antimicrobial activity and are widely used in food, coloring and dietary supplements. Jafari et al. (62) showed that certain concentrations of *C. vulgaris* and *D. salina* extracts had the ability to inhibit not only *S. mutans* but also biofilm formation. The mechanism for the anti-biofilm activity of *C. vulgaris* and *D. salina* may be due to the glucosyltransferase (GTF) inhibition of *C. vulgaris* and *D. salina* extracts activity, which prohibits the biosynthesis of water-insoluble glucans.

Dextranase from marine bacteria are also known to prevent biofilm formation in *S. mutans*. Ren et al. (63) showed that Cadex, an alkaline and cold-adapted endo-type dextranase from marine bacteria, impeded the formation of *S. mutans* biofilms to some extent by specifically cleaving the α-1,6 glycosidic bond. Meanwhile, Lai et al. (64) screened strains of *Catenovulum agarivorans* MNH15 from marine samples, which produced a dextranase with stable *S. mutans* inhibition, and its activity was not affected by sodium fluoride, xylitol, and sodium benzoate in dental care products. Therefore, more marine bacterial dextranases can be explored for the development of oral care products or novel marine agents for the treatment of dental caries to meet the vast demand for caries prevention.

Managing Gingivitis: Compounds, Underlying Mechanism

Gingivitis is one of the most common periodontal diseases, and clinically gingivitis can be divided into two categories (29, 65): (1) gingival diseases caused by non-plaque biofilms, which do not resolve even after plaque removal and may manifest as a systemic disease or in the local oral cavity. (2) Another class is plaque-induced gingivitis, caused by the interaction of plaque biofilm and the host's immune response. The infection remains contained in the gingiva and does not extend to the periodontium, but it can be reversed by reducing plaque at the gingiva and root canals (15, 65). Plaque-induced gingivitis has many clinical features and is mainly predicated on the presence or absence of bleeding at the time of probing. Hence, the main parameters for the determination of gingivitis are GI (Gingival Index) and BOP (Bleeding on probing) (65). The main marine bioactive ingredients that have been reported for anti-gingivitis are sea cucumber extract, algae extract and n-3 polyunsaturated fatty acids (PUFAs).

Research by Wen (66) showed that toothpastes containing sea cucumber extracts were effective in reducing gingivitis. Moreover, sea cucumber *Stichopus horrens* (SH) extracts can promote gingival tissue healing in addition to its anti-streptococcal ability. A study by Bakar et al. (67) showed that a 9% concentration of SH-containing toothpaste (SHCT) was effectively in reducing gingival inflammation (14 days of continuous use) and probe bleeding (30 days of continuous use). The anti-inflammatory effect was also observed with *Enteromorpha linza*, a green algae whose extract strongly inhibited the growth of *Prevotella intermedia* and *Porphyromonas gingivalis* (68). Cho (68) determined that the anti-inflammatory effect of *E. linza* was reduced by plaque index (PI), GI, and BOP measured the clinical effect of mouthwash with *E. linza* extract on gingivitis disease. The results indicated that mouthwash containing *E. linza* extract had a significant inhibitory effect on gingival abscesses *P. gingivalis* and *P. intermedia*, had a significant reduction in plaque, improved gingival tissues, and reduced bleeding. Hence, the measure of using *E. linza* extract mouthwash twice daily was recommended by this clinical study to manage gingivitis.

Furthermore, the anti-inflammatory effects of n-3 PUFAs from marine organisms such as *eicosapentaenoic acid* (EPA) and *docosahexaenoic acid* (DHA) (**Figure 3**) on gingiva have been demonstrated in animal models. Campan et al. (69) showed that n-3 PUFAs was able to induce a trend toward reduced inflammation. This is because EPA may show anti-inflammatory effects by competitively inhibiting the pro-inflammatory cyclooxygenase-1/2 (COX-1/2), forming PGE2 and producing PGE3. Notably, DHA inhibits *lipoxygenase* (LOX) to produce the pro-inflammatory COX metabolite D-series aminotransferases, thus exhibiting a more potent inhibitory effect on inflammation than EPA. Moreover, n-3 PUFAs has antimicrobial activity. Ribeiro-Vidal et al. (70) demonstrated that DHA and EPA were more effective in this biofilm model

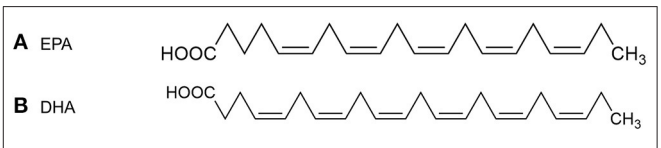

FIGURE 3 | The chemical structure of **(B)** DHA and **(A)** EPA.

of *Streptococcus oralis, Actinomyces naiveus, Veillonella parvula, Fusobacterium nucleatum, P. gingivalis,* and *Aggregatibacter actinomycetemcomitans* all exhibited significant antibacterial activity (71).

Periodontitis: Compounds, Primary Prevention, Underlying Mechanisms

Periodontitis is a chronic multifactorial inflammatory disease that may result from a host immune response triggered by bacterial biofilms and is characterized by inflammation of gingival tissue and resorption of alveolar bone (20, 65, 72). Periodontal disease can be treated with non-surgical as well as surgical therapies. Both supra- and subgingival plaque and calculus removal should be performed as an initial stage of the treatment of periodontal disease (65). Common pathogens of periodontitis are *Treponema denticola, P. gingivalis, Tannerella forsythia, Bacteroides sp., Actinomyces sp, Staphylococcus intermedius, Actinomyces actinomycetemcomitans,* and *Candida albicans* (72, 73). The main marine bioactive ingredients that have been reported to be beneficial for periodontal health are chitosan, sea cucumber extract, n-3 PUFAs, and algae extracts.

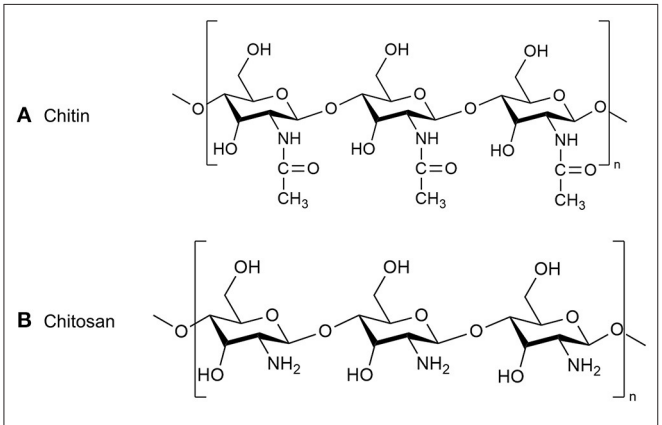

FIGURE 4 | The chemical structure of **(A)** chitin and **(B)** chitosan.

Chitosan is a chemically processed form of chitin (**Figure 4**) with the ability to *S. mutans, A. actinomycetemcomitans* and on *P. gingivalis* (74–76). Choi et al. (77) evaluated the *in vitro* antimicrobial properties of a chitosan (**Figure 5**) (72). A novel formulation based on the combination of chitosan and poly vinyl alcohol (PVA) was prepared by Wang et al. (78). Both *in-vivo* and *in-vitro* experiments demonstrated that the CM-chitosan microsphere (Cs2-Ms) is soft, more hydrophilic, and rapidly degradable by diffusion under physiological conditions. Besides, the authors pointed out that in the future, Cs2-Ms have more potential than Cs1-Ms to be used as core component of newly designed localized drug delivery system (LDDS) for the treatment of periodontitis. Sea cucumber extracts have been shown to have antibacterial effects and the compounds they contain with potential anti-inflammatory effects are saponins, which reduce the activity of COX-2, which plays a role in stimulating inflammatory mediators (12, 79–81). Hence, sea cucumber has a role in periodontitis and can be used as an alternative treatment for inflammation (81).

N-3 PUFAs has antibacterial and anti-inflammatory activity, which can be used for the prevention and treatment of periodontitis (82). Ribeiro-Vidal et al. (70) indicated that the results of DHA and EPA showed significant antimicrobial activity in the biofilm model of *Streptococcus oralis, Actinomyces naeslundii, V. parvula, Fusobacterium nucleatum, P. gingivalis,* and *Aggregatibacter actinomycetemcomitans* (71). As shown in **Figure 6**, the invasion of bacteria and their products into periodontal tissues leaded to the production of inflammatory mediators and enzymes such as IL-6, TNF-α, COX-2, etc. N-3 PUFAs were able to reduce the tendency of inflammation by competitively inhibiting the production of COX-2 and LOX (83). A study by Stańdo et al. (83) suggested that dietary intervention with high doses of n-3 PUFAs during non-surgical treatment may be helpful in the periodontitis treatment. By evaluating dietary intervention in periodontitis patients (stage III and IV), this study revealed that after 3 months of treatment, saliva samples from patients receiving n-3 PUFAs intervention showed significantly higher levels of the anti-inflammatory *interleukin-10* and significantly lower levels of the pro-inflammatory

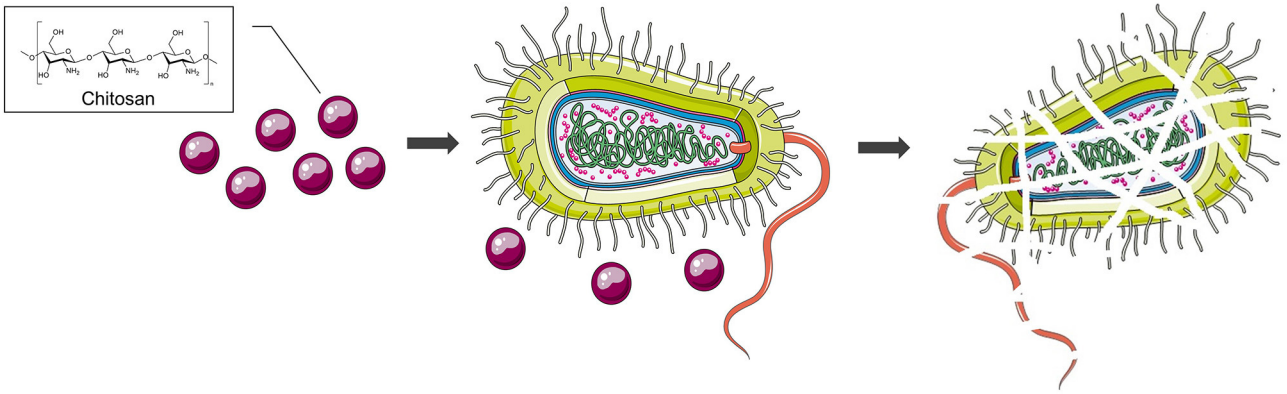

FIGURE 5 | Inhibition of bacterial activity by chitosan.

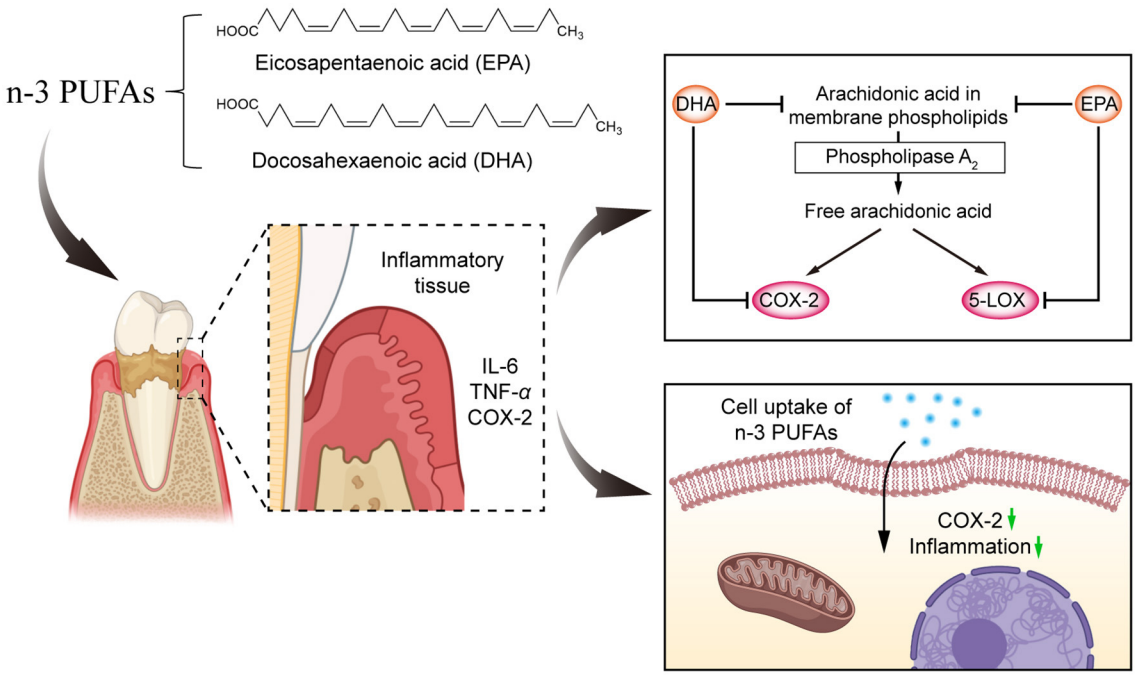

FIGURE 6 | The role of n-3 PUFAs in the reduction of periodontitis.

cytokines interleukin-8 and *interleukin-17* (83). Kruse et al. (84) used meta-analysis to evaluate the effect of n-3 PUFAs in periodontal treatment with adjunctive administration. The result showed that n-3 PUFAs appeared to have a positive effect on periodontal wound healing in terms of reduced clinical attachment level (CAL) and probing depth (PD). Based on this study, patients receiving periodontal therapy may benefit from nutritional counseling.

Marine algae have recently gained popularity for their bioactive molecules and their oral applications. For example, sulfated polysaccharides (PLS) has been reported to have anti-inflammatory effects and to prevent periodontal and liver tissue changes caused by periodontitis (85). *Ecklonia cava*, an edible marine brown algae, whose ethanol extract (ECE) can cause tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) gene downregulated (86, 87). An Indian red alga *Kappaphycus alvarezii* (KAB) is rich in marine bromophenols (MBs), which can downregulate the mRNA level in the gene of *P. gingivalis*, and has an inhibitory effect on it. Additionally, KAB has been reported to inhibit gingipain and hemagglutination activities. As a potent natural metabolite, MBs has potential for use in dental products (88). *Spirulina maxima* has also been reported to be biologically active against periodontitis. Kang et al. (89) indicated that in the presence of *S. maxima*, inflammatory cytokines such as TNF-α, IL-1β, IL-6, and the inflammatory transcription factor NF-κB were reduced in gingival tissues, the expression of myeloperoxidase (MPO) activity and matrix metallopr oteinases (MMPs) was decreased, and osteogenic-related factors were promoted. Thus, this study showed that *S. maxima* reduces gingivitis-induced periodontitis and consequently bone loss through anti-inflammatory effects (89).

Oral Squamous Cell Carcinomas: Compounds, Underlying Mechanisms

The most common oral cancers are found in the oral cavity and include the lips, tongue, gums, oral mucosa, floor of the mouth, hard palate, maxilla, mandible, nasopharynx, oropharynx, and hypopharynx (90). More than 90% of oral cancers are squamous cell carcinomas (91). It is estimated that 657,000 new cases of oral cavity and throat cancer are diagnosed each year, with more than 330,000 deaths (92). There are many factors that contribute to oral cancer. Besides tobacco, heavy consumption of alcoholic beverages, inappropriate dietary habits, poor oral hygiene, chronic inflammatory processes in the oral cavity, various viruses and bacteria, dentures, mucosal trauma, and mucosal trauma from restorations are also risk factors for the development of oral squamous cell carcinoma (OSCC) (93, 94). *P. gingivalis* causes oral inflammation and plays an important role in the development of OSCC. This is because *P. gingivalis* is able to convert nitrite in saliva to nitric oxide (NO), a metabolite that regulates different cancer-related manifestations such as apoptosis, cell cycle, angiogenesis, invasion and metastasis (68, 95). Therefore, early diagnosis and treatment of periodontitis is not only beneficial for the maintenance of patients' oral health, but also important for the prevention of OSCC (68, 96).

Some marine algae contain bioactive compounds that are potential agents for OSCC treatment due to their ability to modulate the antioxidant defense system, apoptosis and

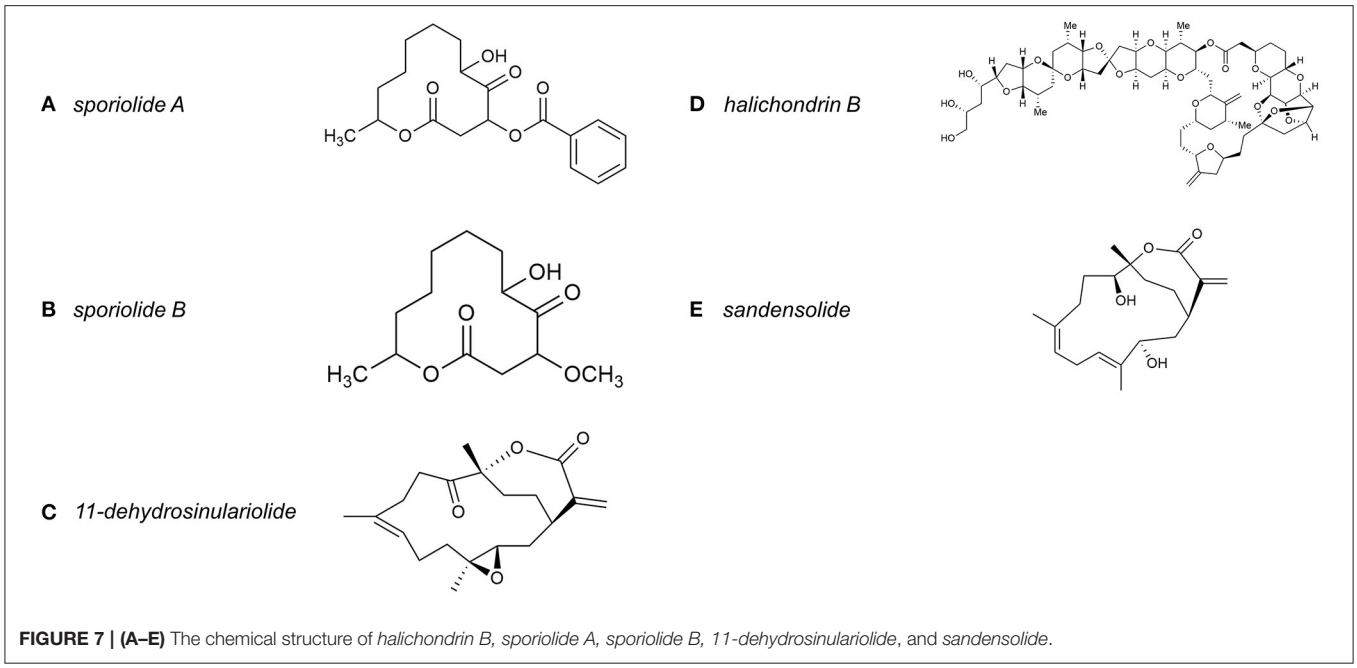

FIGURE 7 | (A–E) The chemical structure of *halichondrin B, sporiolide A, sporiolide B, 11-dehydrosinulariolide*, and *sandensolide*.

autophagy in oral cancer (94). For instance, blue-green microalga *Spirulina* has been reported to reduce the risk of OSCC by reversing oral leukoplakias (97). In studies of anti-cancer effects, at least in part, by generating high levels of intracellular reactive oxygen species (ROS) (98). In the example of red algae, a methanolic extract of the red algae *Luminacin* (a marine microbial extract) induced autophagy and cell death in head and neck cancer cells (99, 100). Furthermore, a study by Yeh et al. (98) indicated that methanolic extract of *Gracilaria tenuistipitata* (MEGT) from seaweed extracts had apoptotic to oral cancer cells through DNA damage, ROS induction and mitochondrial depolarization. However, the above studies did not further address the anti-cancer mechanism of the specific bioactive components in the methanolic extract of the seaweed. Sporiolides A and B (**Figures 7A,B**) from the brown algae *Actinotrichia fragilis*, two new dodecameric macrolides, showed cytotoxicity against L1210 cells (101, 102). In addition, Zhang et al. (103) showed that fucoidan inhibited OSCC development by targeting filamin A (FLNA)-derived circular RNA (circFLNA) to mediate the expression of key proteins associated with cell growth, apoptosis, migration and invasion.

Bioactive components extracted from sea hare, sponges, corals, and fish also have the potential to modulate OSCC (104). A family of cyclic and linear peptides known as dolastatins as well as depsipeptides have been isolated from sea hare (*Dolabella auricularia*) and have been reported to be active anticancer components. Dolastatin-H (*Dolabella auricularia*) and Isodolastatin-H (*Dolabella auricularia*) have been shown to be highly cytotoxic agents (105, 106). Other cytotoxic agents such as polypropionate, auripyrone-A and B, were also isolated from *Dolabella auricularia* (105). Discodermolide, isolated from sponges (*Discodermia dissolute*), is the most promising natural product discovered to date. Discodermolide has been shown to be more effective than taxol and is being tested for the treatment of solid tumors (107, 108).

Another antitumor marine bioactive compound is 11-dehydrosinulariolide (an active ingredient isolated from the soft coral *Sinularia leptoclados*), which is also a potential antitumor bioactive ingredient (109). Liu et al. (109) noted that treatment for 6 h significantly induced early and late apoptosis in CAL-27 cells, providing a clue to the biochemical mechanism of the antitumor effect of 11-dehydrosinulariolide (**Figure 7C**) on CAL-27 cells. Bioactive components extracted from fish, such as Lejimalides (A-D), are unique 24-membered polyene macrolides isolated from a marine tunicate *Eudistoma cf. rigida*, exhibit potent *in vitro* cytotoxic activity (110). Pardaxin (H- GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE-OH) is a 33 amino acid peptide, an antimicrobial peptide (AMP) isolated from marine fish species *Pardachirus marmoratus* (111–113). It is also a possible marine bioactive component for adjuvant chemotherapy in human OSCC care. Han et al. (114) pointed out that Pardaxin treatment caused cell cycle arrest in SCC-4 cells in the G2/M phase, thereby limiting cell proliferation.

Moreover, some cellular derivatives can be used to synthesize components with anticancer properties, such as E7389, a synthetic compound based on *halichondrin B* (**Figure 7D**), which was isolated from the Japanese sponge *Halicondria okadai* (115–117). Cheng et al. (118) investigated the effect of prodigiosin (PG), an alkaloid and natural red pigment as a secondary metabolite of *Serratia marcescens* on the growth inhibition of human oral squamous carcinoma cells. The results showed that PG induced autophagic cell death of OECM1 and SAS cells *in vitro* via the LC3-mediated P62/LC3-I/LC3-II pathway. This implied that PG might target the autophagic cell death pathway as a potential agent for cancer therapy. Furthermore, *sandensolide* (**Figure 7E**) isolated from *Sinularia flexibilis* also has

an inhibitory effect on the growth of human OSCC cells, and its possible mechanism of action is the induction of oxidative stress-mediated cell death pathway (119).

CHALLENGES OF MARINE BIOACTIVE COMPOUNDS IN ORAL HEALTH

The ocean is naturally one of the richest sources of bioactive compounds that can be used as functional ingredients in the development of products for oral health benefits. These bioactive compounds can also be incorporated into dietary supplements, nutritional products and pharmaceuticals to exploit their potential health benefits. Currently, marine based nutraceuticals mainly include fish oil (mainly n-3 PUFAs), algal oil, shark liver oil and squalene, seaweed (macroalgae) and its components, chitin, chitosan (monomers), chitosan (oligomers), enzymes, peptides, protein hydrolysates and other products (120). Indeed, dietary factors, such as nutrients with antioxidant effects, immunomodulatory effects and those related to bone metabolism, have been shown to affect periodontitis directly or interactively (121–123). The highly diverse and dynamic nature of marine ecosystems has led to the identification of marine resource compounds as an important resource for potential functional foods (8, 9). However, despite the health potential of these compounds, their bioavailability affects their effectiveness in the human organism (124). Indeed, bioactive compounds whose complex properties and absorption mechanisms are such as metabolism, digestibility, and activity after absorption, are a scientific challenge to be further investigated and clarified.

For the development of oral health products, the maturity of the preparation process, clinical studies, safety doses and risks of marine bioactive ingredients should be considered for the industrialization of marine bioactive ingredients. For example, marine derivatives such as secondary metabolites are trace amounts and natural reserves are too small to sustain widespread use and development (101). The current marine ingredients with more mature preparation processes and production procedures are n-3 PUFAs, which are effective in alleviating a wide range of health conditions (125). Therefore, n-3 PUFAs related functional foods and special diets have been at the forefront of research and development. Meanwhile, the preparation process of sea cucumber polysaccharides has been also relatively mature, the separation and purification technology has been developed, and the biological activity has been studied (42, 126). However, research on the relationship between biological activity and conformation of sea cucumber polysaccharides is still lacking, and the theoretical support as a functional food ingredient needs further improvement. Although there are more and more supporters of fucoidan as functional food ingredients so far, the structure-function relationship of rockweed polysaccharides is still controversial (127–130). The structural backbone of most fucoidan is unknown, and the location and branching sites of their specific sulfate groups have not been described, leading to structure-activity relationships for fucoidan that have yet to be elucidated. Even though the broad biological activity of fucoidan has been demonstrated, there is a lack of pharmacokinetic data and their clinical application is still limited and needs to be further promoted (131, 132).

The stability of the bioactive substance is another important consideration for its potential application. This is because various functional components in food products play an important role in improving food properties and efficacy. Bioactive substances with health benefits such as probiotics, vitamins, minerals, polyphenols, n-3 polyunsaturated fatty acids are sensitive to oxygen, light, heat, water, pH, etc., which affects the shelf life of the food and the effective release during application. Alginates is an important microcapsule for loading bioactive ingredients in the preparation of functional foods, acting as a unique emulsification, thickening, gelation, film formation and other properties have important applications in encapsulating functional food factors (127). Xiao et al. (133) investigated the encapsulation rate and slow release effect of three ratios of calcium alginate, chitosan-encapsulated calcium alginate, and chitosan-calcium alginate direct mixture on sweet orange oil. It was found that the polymerization of calcium alginate and chitosan encapsulated sweet orange oil could control the prolongation of sweet orange oil during chewing of chewing gum to the maximum extent and prolong the flavor action time of chewing gum. Overall, marine compounds have outstanding health potential can be used as a health strategy to prevent or treat diseases and benefit the maintenance of oral health in humans. Marine biological ingredients have been used in food products, but further *in vivo* studies and human clinical trials as well as industrial promotion are needed for more marine biological active ingredients.

CONCLUSION AND PERSPECTIVE

Oral diseases can cause pain, impaired function and reduced quality of life, resulting in considerable loss of productivity and financial burden to the patient. Dental health therefore has a direct impact on the normal life of people. Recent *in vitro* and *in vivo* studies have revealed the important role of marine bioactive components in the prevention of various oral health problems ranging from dental caries to halitosis and periodontal disease.

The studies mentioned in this review have shown that marine bioactive ingredients seaweed extracts, n-3 PUFAs, sea cucumber extracts, and marine bacterial metabolites have the ability to inhibit oral pathogens, repress their biofilms, and regulate the cancer cell cycle. Therefore, the marine bioactive ingredients mentioned in this paper such as n-3 PUFAs, sea cucumber extracts, and seaweed extracts can play a good role in inhibiting oral pathogenic bacteria, eliminating inflammation, and anti-tumor, which are good choices for developing oral functional foods, such as functional chewing gum or sugar-free tablets. However, before incorporating marine bioactive substances into functional food development, their industrial feasibility has to be evaluated and relevant studies have to be conducted to reveal the possible mechanisms of action of marine bioactive substances and long-term clinical trials.

As the global nutraceutical market grows, consumer interest in marine sourced ingredients is gradually increasing (10, 104).

The potential applications of marine bioactive compounds in food, functional food and supplement development are receiving more attention. Compared to systemic diseases, oral diseases have a strong specificity and the vast majority of treatments require surgical operations for treatment, routinely contacting the patient's saliva, blood and gingival sulcus fluid. When patients carry respiratory-transmitted pathogens such as pneumonia virus, influenza virus, *mycobacterium tuberculosis* and other microorganisms in the oral cavity and nasopharynx, the aerosols and droplets produced can pose a serious hazard. Especially during this COVID-19 pandemic situation, many people undergoing treatment for dental diseases can be affected. Therefore, adequate oral hygiene is very important. Furthermore, with the rising awareness of oral disease prevention and dental health maintenance, functional foods and dietary supplements for oral health are favored by consumers.

In fact, a number of marine bioactive compounds have been used in the food industry as functional food ingredients to enhance the functional properties of foods or as additives to improve certain properties of foods (stability, emulsification, texture improvement). In the industrialization of marine bioactive ingredients, their bioavailability, purity, environmental friendliness, cost effectiveness, etc. are aspects that need attention. The studies on marine bioactive ingredients with mature preparation processes have been mostly focused on bioactivity exploration. Future research on two aspects of the marine bioactive ingredients is therefore recommended. On the one hand, the stability and adaptability of the activity need to be further investigated, that is, the chemical modification or organic synthesis of the structure, while preserving its physiological activity, in order to expand its application in the food field. On the other hand, the comprehensive utilization of low-value products/byproducts should be further undertaken. For example, the comprehensive utilization of polysaccharide-rich processing waste to cut down the cost of industrial applications. For the newly discovered marine biological active ingredients, further research on their functional structure, active mechanism of action, especially clinical application and even pharmacokinetic data support is needed. Furthermore, studies on health promotion, safe dosage and side effects of newly discovered compounds will also provide favorable theoretical support for their industrialization.

AUTHOR CONTRIBUTIONS

Y-ZH: writing the article. X-PD, L-BQ, and SS: critical revision of the article. Y-ZH, X-PD, B-WZ, ZJ, and Z-MW: final approval of the article. All authors contributed to the article and approved the submitted version.

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Nutritional and Sensory Properties of Protein Hydrolysates Based on Salmon (*Salmo salar*), Mackerel (*Scomber scombrus*), and Herring (*Clupea harengus*) Heads and Backbones

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Protein hydrolysates based on salmon, mackerel, and herring heads and backbones were produced, and the sensory properties of the hydrolysates were evaluated by a highly trained sensory panel. The nutritional content of the products was evaluated, and the hydrolysates contained all the amino acids inherent to the raw material, including considerable levels of connective tissue amino acids glycine, proline, and hydroxyproline. Hydrolysates based on herring were the most flavor intense, whereas hydrolysates based on salmon were deemed more palatable. In this work, choice of fraction (heads vs. backbones) and enzyme had minor effects on sensory and nutritional properties, indicating that choice of raw material species was the major factor for flavor development in the produced protein hydrolysates. There were large variations in protein content and amino acid composition in the raw material fractions, but as expected, only minor variations were found in the final products.

Keywords: salmon (*Salmo salar*), mackerel (*Scomber scombrus*), herring (*Clupea harengus*), side-stream materials, protein hydrolysis, sensory properties

INTRODUCTION

Side-stream materials from fishing and aquaculture, such as heads, backbones, viscera, and trimmings may comprise up to 70% of the whole fish (1). In Norway, all side-stream materials obtained from salmon aquaculture, with the exception of blood, are utilized. The side streams are used for both feed and food products. Side stream materials from mackerel and herring are mainly used for lower-value feed ingredients (2). The Norwegian pelagic fisheries volumes in Norway have been rather stable the last decade, amounting to over 1.3 million tons. In 2019, 560,000 tons herring and ∼159,000 tons mackerel were landed (3). Most of the herring from Norwegian fisheries are fileted, whereas mackerel is mostly sold as round frozen (2). However, the growing share of mackerel that is fileted is generating increasing amounts of heads and backbones available for valorization [194,000 tons in 2019) (2)]. The raw materials have food grade quality after the primary processing and are suitable as food ingredients when handled correctly (4, 5).

The production of protein hydrolysates using commercial proteases is a promising approach for utilizing marine side stream materials. Enzymatic protein hydrolysis is a mild process that facilitates increased recovery of water-soluble peptides and oil from the raw material (6, 7). Protein hydrolysates based on marine sources are rich in essential amino acids, making such products suitable as food additives or nutritional supplements (8). Furthermore, the peptides in salmon, mackerel, and herring protein hydrolysates may have antioxidative, antimicrobial and/or antihypertensive activities (8, 9). Provided that the raw material can be converted to a product suitable for inclusion in foods, the additional processing costs may be justified, both regarding increased raw material valorization and environmental sustainability.

Off-flavors in the final product remain a challenge in the production of enzymatic protein hydrolysates from (10–14). Water-soluble molecules present in the raw material will follow the aqueous hydrolysate phase and influence the product sensory properties. These components include inorganic salts, trimethylamine, nucleotides, protein and non-protein amino acids, and possibly small amounts of lipid oxidation products (14–16). Bitter taste, which is ascribed to small hydrophobic peptides (11, 17), is one of the main off-flavors found as challenges in protein hydrolysates for human consumption. However, the choice of enzyme and processing conditions may significantly influence the bitter taste development in the final protein hydrolysate (14, 18–20). The enzymes Food Pro PNL and Bromelain were selected based on previous experience. Food Pro PNL have proven to be cost effective (21) and provide low bitterness (14) in protein hydrolysates. Bromelain has a broad specificity and efficient on connective tissue (22). The enzymes were also chosen because their optimal pH conditions and are in line with the natural pH of the raw material allowing a process upscaling to be done without laborious and cost-enhancing pH adjustments.

Several works have addressed the taste and flavor development of marine protein hydrolysates (10, 11, 13–15, 18, 19, 23). Quality assessment of food grade hydrolysates must include evaluation of sensory properties, preferably performed by a trained taste panel (24). Descriptive analysis is a comprehensive method which includes training of the panelists to quantify specific sensory attributes for appearance, flavor, texture, and aftertaste. The assessors are calibrated on samples that are considered the most different on the selected attributes typical for the samples to be tested. However, such study-to-study calibrations makes interstudy comparisons difficult. Thus, sensory evaluation of hydrolysates based on different raw materials, at otherwise similar processing conditions, are necessary for the assessment of products destined for human consumption. In this work, enzymatic protein hydrolysates from salmon, mackerel, and herring heads and backbones were produced using similar hydrolytic conditions. The aim was to evaluate their proximate and nutritional composition, and sensory properties to compare their suitability for inclusion in food formulations.

TABLE 1 | The sensory attributes used in the sensory evaluation of salmon, mackerel, and herring protein hydrolysates.

Attribute	Description
Total intensity of flavor	The intensity of all tastes and flavors
Sweet taste	Basic sweet taste
Salty taste	Basic salt taste (sodium chloride)
Acidic taste	Basic acidic taste
Bitter taste	Basic bitter taste
Umami taste	Basic umami taste
Fish flavor	Flavor of boiled white fish
Marine flavor	The flavor of fresh, salty sea
Shellfish flavor	The flavor of shellfish, a sweet teste of shrimp, crab, and crayfish
Burnt flavor	Related to a burnt flavor
Rancid flavor	All rancid flavors (grass, hay, stearin, paint)
Fermented fish flavor	Related to flavors that remind of a pier, bad and stale fish
Flavorless flavor	Related to water from boiling of potatoes
Cloying flavor	Related to a sickening flavor
Astringency	A complex feeling of contraction and dryness of the mouth
Fatness	The fattiness of the products
Aftertaste	The intensity of the tastes and flavors left in the mouth after 30 s

MATERIALS AND METHODS

Materials

Fresh farmed salmon (*Salmo salar*) was provided by Sotra Seafood, Øygarden, Norway. Frozen herring (*Clupea harengus*) and mackerel (*Scomber scombrus*) were provided by Pelagia, Norway. The raw materials were fileted, the heads and backbones were collected and ground using a kitchen grinder and kept frozen (−20°C) until hydrolysis. The proteases used were Bromelain BR1200 (EC 3.4.22.32, Enzybel, Waterloo, Belgium) and FoodPro PNL (EC 3.4.24.28, DuPont, Wilmington, DE). Peptide standards for analysis of molecular weight distribution were purchased from Sigma-Aldrich (Oslo, Norway) except lysozyme (Fluka biochemicals, Buchs, Switzerland) and Alberta standards (Alberta Peptide Institute, Department of Biochemistry, University of Alberta, Edmonton, Canada). All chemicals for analyses were of analytical grade.

Methods

Chemical Methods

Nitrogen was analyzed by the Kjeldahl method (25) and crude protein estimated based on N × 6.25. Ash was determined by combustion at 550°C (26). Dry matter was determined by drying at 103°C (27). Molecular weight distribution was measured by HPLC size exclusion chromatography (SEC) (1260 series HPLC Agilent Technologies) using a Superdex Peptide 10/300GL column (GE Healthcare, Uppsala, Sweden), acetonitrile with TFA as eluent, and UV detection at 190–600 nm (14). Total amino acid composition was quantified by HPLC with fluorescence detection

TABLE 2 | Proximate, lipid, and amino acid composition (g/100 g sample) of freeze-dried heads and backbones from salmon, mackerel, and herring[a].

	Salmon		Mackerel		Herring	
	Backbone	**Head**	**Backbone**	**Head**	**Backbone**	**Head**
Protein	46.4	34.6	46.6	34.6	51.1	38.5
Lipids	48.6	54.4	42.9	54.4	43.6	42.8
Ash	8.1	9.1	8.5	9.1	6.9	16.9
Dry matter	99.1	98.1	98.0	98.1	98.5	97.7
Fatty acids[b]						
Saturated FA	13.8	20.6	20.6	20.6	18.3	17.5
MUFA	51.4	37.6	38.8	37.6	48.1	51.7
PUFA	29.3	18.7	20.0	18.7	21.2	14.2
PUFA n-6	13.7	13.8	2.0	2.0	1.7	1.5
PUFA n-3	15.9	16.4	13.7	16.3	19.1	12.4
EPA	2.7	4.6	4.9	4.6	6.5	3.9
DHA	5.1	6.7	6.9	6.7	7.2	4.8
Amino acids						
Arginine	3.0	2.0	2.9	2.0	2.9	2.4
Histidine	2.1	0.7	1.2	0.7	1.5	0.8
Isoleucine	2.0	1.0	1.7	1.0	2.1	1.2
Leucine	3.3	1.8	2.9	1.8	3.7	2.2
Lysine	3.7	2.2	3.6	2.2	4.4	2.2
Methionine	1.3	0.9	1.4	0.9	1.6	1.2
Phenylalanine	1.8	0.9	1.5	0.9	1.8	1.3
Threonine	2.0	1.2	1.8	1.2	2.0	1.3
Valine	2.4	1.2	2.1	1.2	2.6	1.6
Sum IAA[c]	21.6	11.9	19.1	11.9	22.6	14.4
Alanine	2.8	2.0	2.9	2.0	3.0	2.4
Aspartic acid	4.2	2.7	4.1	2.7	4.6	2.8
Glutamic acid	6.0	3.9	6.0	3.9	6.6	4.2
Glycine	3.8	3.7	4.5	3.7	3.0	4.2
Hydroxyproline	0.7	0.9	0.9	0.9	0.3	1.1
Proline	2.2	1.9	2.3	1.9	1.9	2.3
Serine	2.1	1.4	2.0	1.4	2.0	1.7
Tyrosine	1.2	0.9	1.5	0.9	1.7	1.1
Sum DAA[d]	23.0	17.4	24.2	17.4	23.1	19.7
Sum amino acids	43.3	31.2	44.6	31.2	45.7	34.1

[a]*Allowed replicate variation: Protein: ≤0.3%, Lipids: ≤0.54%, Ash: ≤0.3%, Dry matter: ≤0.2%, Fatty acids: ≤5% of mean of dominating acids, Amino acids: RSD < 6% for 2/3 of amino acids.*

[b]*Lipid composition based on fat extracted from freeze dried raw material. FA: fatty acids, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.*

[c]*Indispensable amino acids.*

[d]*Dispensable amino acids.*

with excitation/emission at 250/395 nm. The proteins were hydrolyzed to free amino acids with 6N HCl, and amino acids were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate before HPLC analysis (Waters Accq Tag 3.9 × 150 mm) and detector (28). The lipid content was analyzed by the EU 152/2009 (29) method with acid hydrolysis. Fatty acid analyses were carried out by AOCS Official Method (Ce 1b-89) (30). All analyses were done in duplicate measurements.

Enzymatic Hydrolysis

In all experiments, enzymes were added at similar activity of 10 U/g protein, based on a casein-activity assay (14). Hydrolyses were conducted in a R10 Bear Varimixer (A/S Wodschow & Co. Brøndby, Denmark) or Distek 7100 Bathless Dissolution Tester (Distek Inc. North Brunswick, NJ). An equal mass of tap water was added to the ground raw material. The mixture was heated to 50°C and then the enzyme was added. Stirring was continued at 50°C for 60 min. The temperature was raised to 90°C and held at that temperature for 10 min (to inactivate the proteases). The mixture was centrifuged at 15,000 g for 10 min (Sorvall LYNX 6000, Thermo scientific, Waltham, MA). The liquid was decanted from the sediment into a separatory funnel and the aqueous and oil were separated. The aqueous phase was clarified by cross flow ultrafiltration through a membrane with nominal molecular

TABLE 3 | Proximate composition (g/100 g) of protein hydrolysates based on salmon (S), mackerel (M), and herring (H) backbones (B) and heads (H) using enzymes Food Pro (-FP) and Bromelain (-B)[a].

	Salmon				Mackerel				Herring			
	Backbone		Head		Backbone		Head		Backbone		Head	
	SB-FP	SB-B	SH-FP	SH-B	MB-FP	MB-B	MH-FP	MH-B	HB-FP	HB-B	HH-FP	HH-B
Protein	92.1	91.4	92.3	89.3	89.9	91.6	88.7	88.3	84.9	87.8	82.6	83.0
Dry matter	97.0	95.0	94.2	94.9	95.9	97.0	97.5	96.4	96.0	95.7	96.1	95.7
Ash	8.0	7.2	11.5	10.8	9.5	8.4	11.6	10.7	9.6	8.9	11.4	11.4

[a]*Allowed replicate variation: Protein: ≤0.3%, Ash: ≤0.3%, Dry matter: ≤0.2%.*

TABLE 4 | Amino acid composition (g/100 g sample)[a] of protein hydrolysates based on salmon (S), mackerel (M) and herring (H) backbones (B) and heads (H), using enzymes Food Pro (-FP) and Bromelain (-B).

	Salmon				Mackerel				Herring			
	Backbone		Head		Backbone		Head		Backbone		Head	
	SB-FP	SB-B	SH-FP	SH-B	MB-FP	MB-B	MH-FP	MH-B	HB-FP	HB-B	HH-FP	HH-B
IAA[2]												
Arginine	6.6	6.7	6.7	6.5	5.3	5.2	5.3	5.6	4.5	5.0	4.8	5.0
Histidine	2.2	2.2	1.9	1.8	3.8	3.8	2.7	2.6	2.0	2.0	1.5	1.5
Isoleucine	2.5	2.6	2.1	2.0	2.4	2.6	2.5	2.5	2.6	2.6	2.4	2.2
Leucine	5.1	5.3	4.2	4.2	4.9	5.2	5.0	5.1	5.6	5.6	5.1	5.0
Lysine	6.0	6.3	4.7	4.8	5.9	6.4	6.0	6.0	7.3	7.2	5.9	5.9
Methionine	2.2	2.3	2.2	2.1	2.0	1.9	2.0	1.9	2.3	2.2	2.3	2.2
Phenylalanine	2.2	2.4	2.1	2.0	2.0	2.1	2.2	2.2	2.3	2.3	2.3	2.1
Threonine	3.7	3.8	3.3	4.0	3.1	3.4	2.7	3.1	3.2	3.2	3.0	2.9
Valine	3.4	3.6	3.0	2.9	3.2	3.5	3.2	3.4	3.4	3.7	3.2	3.3
Sum IAA	33.9	35.2	30.2	30.3	32.6	34.1	31.6	32.4	33.2	33.8	30.5	30.1
DAA[c]												
Alanine	6.8	6.5	6.5	6.8	5.3	5.0	6.1	6.0	5.3	5.3	5.5	5.7
Asparagine	7.6	7.3	6.4	6.7	5.8	6.2	6.4	6.4	7.2	7.2	6.5	6.5
Glutamine	11.4	11.1	10.2	10.3	9.3	9.2	9.7	10.0	11.2	11.0	10.0	10.2
Glycine	10.6	9.6	12.8	12.7	6.9	5.0	8.8	8.9	5.6	5.7	7.8	7.8
Hydroxyproline	2.7	2.1	3.4	3.3	1.5	0.65	2.0	2.0	0.99	0.91	1.8	1.8
Proline	4.9	4.8	5.7	6.3	3.4	3.0	4.2	4.5	3.2	3.5	4.2	4.2
Serine	4.1	3.9	3.9	4.0	3.3	3.0	3.7	3.8	3.4	3.2	3.5	3.3
Tyrosine	1.6	1.8	1.4	1.1	1.4	1.8	1.6	1.7	1.9	1.9	1.8	1.8
Sum DAA	49.7	47.1	50.3	51.2	36.9	33.9	42.5	43.3	38.8	38.7	34.6	41.3
Sum protein AA	83.6	82.3	80.5	81.5	69.5	68.0	74.1	75.7	72.0	72.5	71.6	71.4

[a]*Allowed replicate variation: RSD < 6% for 2/3 of amino acids.*
[b]*Indispensable amino acids.*
[c]*Dispensable amino acids.*

weight cut-off of 100 kDa (Vivaflow 200, Sartorius, Goettingen, Germany). The ultrafiltration permeate was freeze dried and stored at −30°C until sensory assessment.

Sensory Analysis

The freeze-dried hydrolysates were dissolved in tap water at a concentration of 1.0 wt % before sensory analysis. A highly trained panel of 10 assessors (10 women; aged, 37–64 years) performed a sensory descriptive analysis according to the "Generic Descriptive Analysis," as described by Lawless and Heymann (24) and the ISO standard 13299 (31). The assessors were regularly tested and trained according to ISO standard 8586 (32), and the sensory laboratory followed the practice of ISO standard 8589 (33). The assessors agreed upon 17 attributes describing the hydrolysate samples (**Table 1**). Samples were served in glasses of plastic (20 ml) with a lid at a room temperature of 18°C ± 2°C. All attributes were evaluated on an unstructured 15 cm line scale with labeled end points going from "no intensity" (1) to "high intensity" (9). Each assessor evaluated all samples at individual speed on a computer system for direct

TABLE 5 | Molecular weight distribution (wt %) of hydrolysates based on salmon (S), mackerel, and herring (H) backbones (B) and heads (H) using the enzymes Food Pro PNL (-FP) and Bromelain (-B).

	Salmon				Mackerel				Herring			
	Backbone		Head		Backbone		Head		Backbone		Head	
MW (kDa)	SB-FP	SB-B	SH-FP	SH-B	MB-FP	MB-B	MH-FP	MH-B	HB-FP	HB-B	HH-FP	HH-B
>20	<0.01	0.1	0.1	<0.01	0.2	0.1	0.1	<0.01	<0.01	0.1	<0.01	<0.01
20–15	0.1	<0.01	0.1	<0.01	0.1	<0.01	0.5	<0.01	<0.01	<0.01	<0.01	<0.01
15–10	0.4	<0.01	0.5	0.1	0.3	0.1	2.3	<0.01	0.1	<0.01	<0.01	0.1
10–8	0.9	<0.01	1.1	0.2	0.6	0.1	2.7	<0.01	0.2	0.1	0.1	0.1
8–6	3.1	0.3	4.0	1.1	2.1	0.4	4.9	0.2	0.7	0.4	0.3	0.7
6–4	7.6	1.9	93	4.8	6.3	1.9	9.3	1.2	2.9	1.9	1.7	2.8
4–2	19.4	12.8	22.9	21.4	17.0	12.0	16.0	7.6	10.5	10.4	10.1	13.9
2–1	18.4	24.3	19.3	26.7	17.2	23.0	14.6	18.0	17.2	20.8	21.2	22.5
1–0.5	15.3	23.5	13.7	18.5	15.0	22.6	11.7	23.3	19.9	23.1	24.4	19.5
0.5–0.2	15.1	19.2	10.5	10.6	12.9	15.6	10.9	21.3	19.5	18.2	19.5	13.1
<0.2	19.7	17.9	18.5	16.3	28.3	24.1	27.1	28.3	29.1	25.1	22.5	27.3

recording of data (EyeQuestion, Software Logic8 BV, Utrecht, the Netherlands).

In a pretest session before the main test, the assessors were calibrated on samples that were considered the most different on the selected attributes typical for the hydrolysate samples to be tested. All samples were served to the panel coded with a three-digit number in a balanced block design. Tap water and unsalted crackers were available for palate cleansing, and red light was used in the sensory laboratory to masque differences in appearance between samples.

Statistical Analysis

Analysis of variance (ANOVA) of the sensory profiling data was performed using Minitab (v19.2, Pennsylvania State University, PA). First, a two-way mixed effects ANOVA model was conducted to assess differences between products for all sensory attributes. Product was set as a fixed variable, whereas assessor and interaction effects were set as random variables (34). Mixed effects ANOVA was used to evaluate the individual fixed effects of specie, fraction, and enzyme on sensory attributes, still treating assessor as a random variable. Tukey's pairwise comparison was applied where significant ($p < 0.05$) differences were found. Principal component analysis (PCA) was performed using Unscrambler v.10.4.1 (Camo, Oslo, Norway) to evaluate the association between sensory properties and molecular weight distribution of the hydrolysates. Prior to analysis, all variables were mean centered and standardized.

RESULTS AND DISCUSSION

Proximate Composition, Peptide Size Distribution, and Amino Acid Composition

All the raw materials contained high levels of protein and lipids (**Table 2**). The backbones contained most proteins and indispensable amino acids (IAA) relative to the heads for all species. As expected, all raw materials had high levels of the valuable polyunsaturated fatty acids (PUFAs). The salmon raw materials had much higher levels of *n*-6 PUFA than mackerel and herring, reflecting the amounts of plant oils used in salmon feed (35). All raw materials also contained high levels of the marine *n*-3 PUFA, EPA, and DHA, which are very susceptible to lipid oxidation and can influence the sensory properties of the final product (36). The proximate compositions were in agreement with other studies (9, 10), except the mackerel heads, which have slightly less ash content than previously reported (10), mainly ascribed to different batches of raw material and fileting methods used. The latter may give varying ratios of muscle tissue and bone, causing a displacement in the level of ash, which is mostly derived from the bone tissue.

All hydrolysates were high in protein and ash (**Table 3**) and especially, the head-based hydrolysates were richer in ash and lipids compared with the backbone-hydrolysates, in agreement with previous findings for salmon, mackerel (10), and herring (9). The difference in raw material proximate compositions did not affect the hydrolysate composition to a greater extent. Only minor variations in protein content could be found in the final dried products. This is as expected because the hydrolysate product only consisted of the filtrated water-soluble fraction containing mostly proteins, causing a displacement in composition. The sum of total amino acids is the most accurate estimate for protein content in a product (37), and the levels of total amino acids (**Table 4**) were slightly lower compared to that of analyzed total proteins (N × 6.25) (**Table 3**) for all hydrolysates. This provides evidence that the commonly used nitrogen-to-protein factor of 6.25 is inaccurate for the different fish fractions and substrates, and has been found to be closer to 5.2, and is of importance for the production of comparable hydrolysates (14). The hydrolysates contained all amino acids present in the raw material, with noticeable levels of glycine, proline, and hydroxyproline, predominant in fish

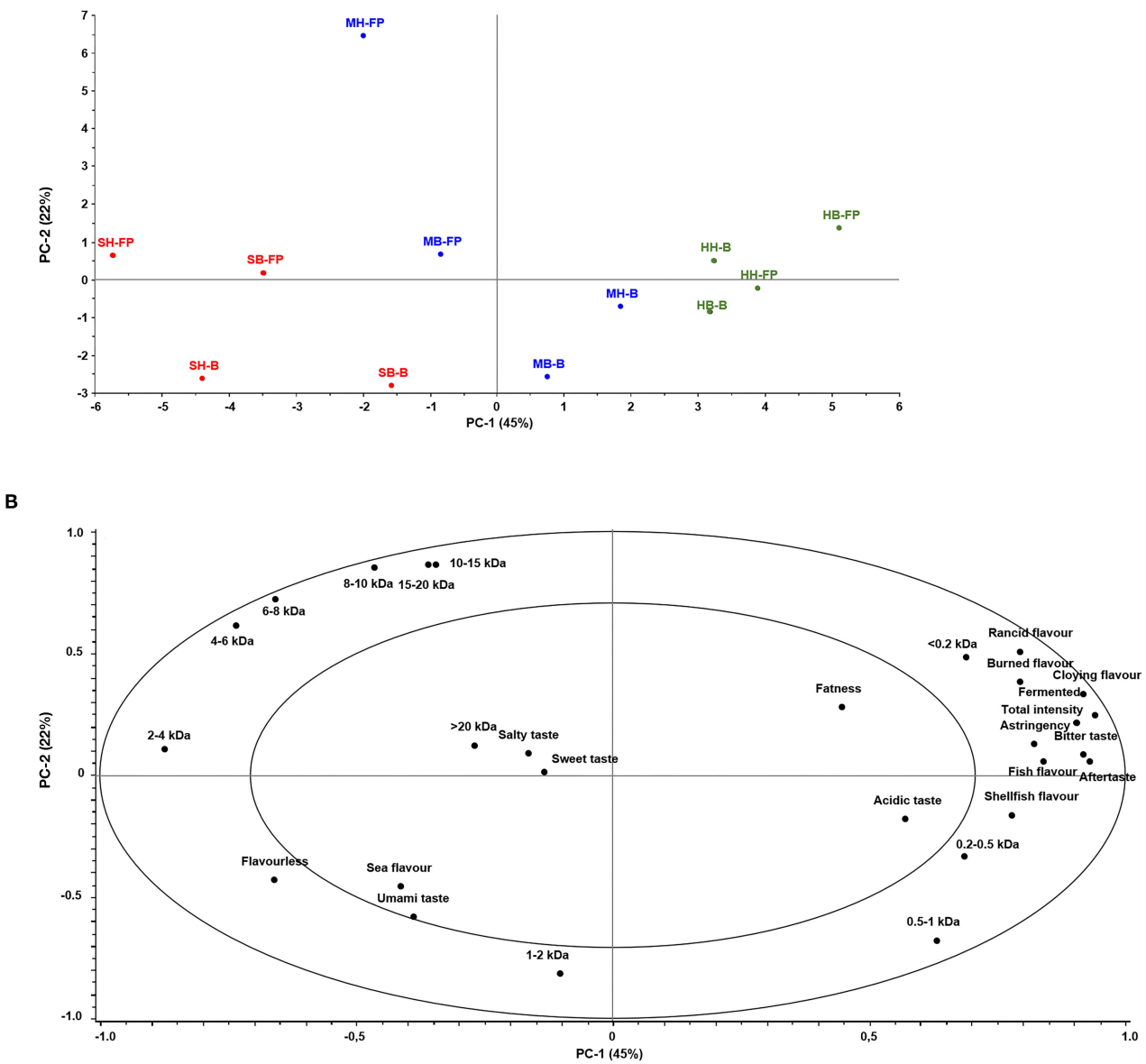

FIGURE 1 | Principal component analysis score plot of hydrolysates based on salmon (S; red), mackerel (M; blue), and herring (H; green) **(A)** and loading plot of sensory properties and molecular weight distribution **(B)**. Sample coding: H, heads; B, Backbones; -FP, Food Pro PNL; -B, Bromelain.

connective tissue. This is in accordance with Liaset and Espe (38), who reported higher levels of these amino acids in side-stream based hydrolysates compared with filets. No apparent effect of the used enzyme on amino acid distribution was observed, suggesting similar release of proteins from the raw material. Hydrolysates based on backbones had slightly higher levels of IAA for all three species tested. The differences were, however, small and all hydrolysates were considered of high nutritional protein quality, based on daily requirements for adults. On average, an adult of 70 kg requires about 6 g of IAA per day (39), and all hydrolysates contained >30 g IAA/100 g dried hydrolysate.

The molecular weight distribution (MWD) of the peptides (**Table 5**) showed that the hydrolysates mainly contained peptides <4 kDa, equivalent to peptides <20 amino acid units (40). Peptides of <25 amino acids may have bioactive properties (41), which adds to the nutritional value of the hydrolysates. Especially hydrolysates based on mackerel and herring contained high levels of molecules <0.2 kDa, mostly being small dipeptides and free amino acids. The enzymes were dosed to obtain

TABLE 6 | Mean sensory intensity values of protein hydrolysates based on salmon (S), mackerel (M), and herring backbones (B) and heads (H) using Food Pro PNL (-FB) and Bromelain (-B).

	Salmon				Mackerel				Herring				
	Backbone		Head		Backbone		Head		Backbone		Head		
	SB-FB	SB-B	SH-FP	SH-B	MB-FP	MB-B	MH-FP	MH-B	HB-FP	HB-B	HH-FP	HH-B	*p*-value
Total intensity of flavor	5.25^{cde}	5.59^{bcde}	4.96^{de}	4.72^{e}	6.23^{abcd}	6.33^{abc}	5.94^{abcde}	5.75^{abcde}	7.01^{a}	6.38^{abc}	6.39^{abc}	6.59^{ab}	<0.001
Sweet taste	2.94	2.48	2.47	2.60	2.53	2.95	2.70	2.67	2.57	2.44	2.57	2.57	0.559
Salty taste	2.67^{ab}	2.24^{b}	3.56^{a}	3.11^{ab}	2.91^{ab}	3.12^{ab}	2.88^{ab}	2.98^{ab}	2.67^{ab}	2.70^{ab}	3.23^{ab}	3.19^{ab}	0.01
Acidic taste	2.26^{b}	2.18^{b}	2.04^{b}	2.14^{b}	2.64^{ab}	3.09^{a}	2.19^{b}	2.17^{b}	2.69^{ab}	2.59^{ab}	2.42^{b}	2.50^{ab}	<0.001
Bitter taste	4.36^{bc}	4.74^{abc}	3.25^{c}	3.91^{bc}	4.71^{abc}	4.66^{abc}	4.72^{abc}	5.02^{ab}	6.05^{a}	5.98^{a}	5.23^{ab}	5.13^{ab}	<0.001
Umami taste	3.91^{ab}	3.59^{ab}	3.91^{ab}	3.71^{ab}	3.69^{ab}	4.58^{a}	2.87^{b}	3.21^{b}	3.02^{b}	3.28^{ab}	3.33^{ab}	3.62^{ab}	0.005
Fish flavor	3.20^{b}	3.17^{b}	3.31^{b}	4.22^{ab}	4.64^{ab}	5.17^{a}	4.32^{ab}	4.49^{ab}	5.30^{a}	5.29^{a}	5.45^{a}	5.56^{a}	<0.001
Marine flavor	1.63	1.45	1.61	1.99	2.03	2.06	1.35	1.49	1.34	1.62	1.34	1.32	0.05
Shellfish flavor	2.77	2.08	2.36	2.34	2.99	3.60	2.32	3.16	3.11	3.64	3.48	3.44	0.06
Burned flavor	1.68^{a}	1.51^{a}	1.22^{a}	1.17^{a}	1.40^{a}	1.57^{a}	2.06^{a}	1.50^{a}	2.53^{a}	2.11^{a}	2.56^{a}	2.09^{a}	0.009
Rancid flavor	1.10^{c}	1.11^{c}	1.18^{c}	1.12^{c}	1.62^{bc}	1.15^{c}	2.49^{abc}	2.03^{abc}	3.36^{a}	2.14^{abc}	2.88^{a}	2.66^{abc}	<0.001
Fermented fish flavor	1.32^{c}	1.66^{c}	1.29^{c}	1.55^{c}	3.23^{abc}	2.91^{bc}	3.14^{abc}	3.38^{abc}	5.21^{a}	4.34^{ab}	4.71^{ab}	4.85^{ab}	<0.001
Flavorless flavor	2.78^{abc}	4.30^{a}	3.27^{abc}	3.98^{ab}	3.15^{abc}	3.07^{abc}	2.97^{abc}	2.69^{abc}	2.29^{c}	2.98^{abc}	2.45^{bc}	2.15^{c}	0.001
Cloying flavor	1.58^{d}	2.02^{d}	1.73^{d}	1.92^{d}	3.34^{cd}	3.24^{cd}	4.24^{bc}	4.46^{abc}	6.31^{a}	4.74^{abc}	5.48^{ab}	5.51^{ab}	<0.001
Astringency	3.58^{abc}	4.00^{abc}	2.91^{c}	2.96^{c}	3.84^{abc}	3.52^{abc}	3.63^{abc}	3.38^{bc}	4.75^{a}	4.37^{ab}	4.14^{abc}	4.07^{abc}	<0.001
Fatness	2.72^{a}	2.90^{a}	2.74^{a}	2.81^{a}	2.87^{a}	3.06^{a}	3.24^{a}	3.31^{a}	2.88^{a}	2.74^{a}	3.16^{a}	3.45^{a}	0.008
Aftertaste	4.33^{c}	4.72^{bc}	4.32^{c}	4.21^{c}	5.31^{abc}	5.71^{ab}	4.89^{abc}	5.10^{abc}	6.00^{a}	5.61^{ab}	5.64^{ab}	5.66^{ab}	<0.001

Different letters indicate statistical difference ($p < 0.05$) among the hydrolysates by two-way ANOVA and Tukey's multiple comparison test.

comparable hydrolysis process for all raw materials, and the discrepancy in MWD may be explained with higher levels of free amino acids in the mackerel- and herring-based substrates. In addition, the effect of postmortem changes and presence of endogenous enzymes may add to the hydrolysis process, as the postmortem metabolomic activity of pelagic species is known to be high (42, 43). Furthermore, slightly higher levels of molecules <0.2 kDa were observed in Food Pro PNL-hydrolysates compared with those based on Bromelain. As the applied enzymes were declared endopeptidases, the amount of free amino acids should reflect upon the levels in the raw materials, and not be affected by hydrolysis. However, the increase in molecules <0.2 kDa has also been observed in previous research and indicate that Food Pro PNL has some exopeptidase activity (14).

Sensory Properties

The sensory properties of fish-based hydrolysates are of utmost importance to evaluate their potential use in products intended for human consumption. Generic descriptive analysis was performed on the hydrolysates to compare the effect of fish species, side stream fractions, and choice of enzyme on various sensory attributes (**Table 1**). Principal component analysis (PCA) was used to evaluate the association between sensory properties and MWD of the different hydrolysates. The first and the second principal components (PCs) explained 45 and 22% of the variance, respectively. The third and fourth PCs explained 8 and 5%, respectively (not shown). The PCA score plot (**Figure 1A**) shows that the hydrolysates were mostly separated based on specie; hydrolysates from salmon and herring were negatively correlated on PC1. PC2 mostly explains the effect of enzymes on the product variation, which has been found in several previous studies (8, 14). However, the higher variation explanation of the former PC illustrates the considerable raw material effect.

Based on the PCA loading plot (**Figure 1B**), it was evident that hydrolysates based on salmon were associated with larger peptides (>2 kDa), low flavor intensity, and pleasant flavors, such as sea flavor and umami taste. All herring hydrolysates were associated with small peptides (<1 kDa) and high sensory intensity of most of the tested attributes, including total intensity of flavor, bitter, rancid, burned, cloying, fermented fish, aftertaste, shellfish, and acidic tastes and flavors. It has been suggested that oxidation products play a part in the development of bitter tastes (12) and the positive association between rancid flavor and bitter taste with herring hydrolysates indicates that some oxidation has occurred during, or prior, to hydrolysis of the herring raw material. It was, however, found to have comparable or lower lipid contents compared with the other raw materials (**Table 2**), possibly indicating higher prooxidative activity from blood components of herring. A study on oxidation effects of hemolysate from trout, herring, and mackerel found the latter two to have more rapid deterioration effects on cod compared with the former (44). In the current study other factors may have added to the oxidation in herring, such as amount of remnant blood in the raw material, other blood components, and potential postmortem changes.

The mackerel hydrolysates were mostly in the center of the plot and associated with the lesser explained (<50%) attributes,

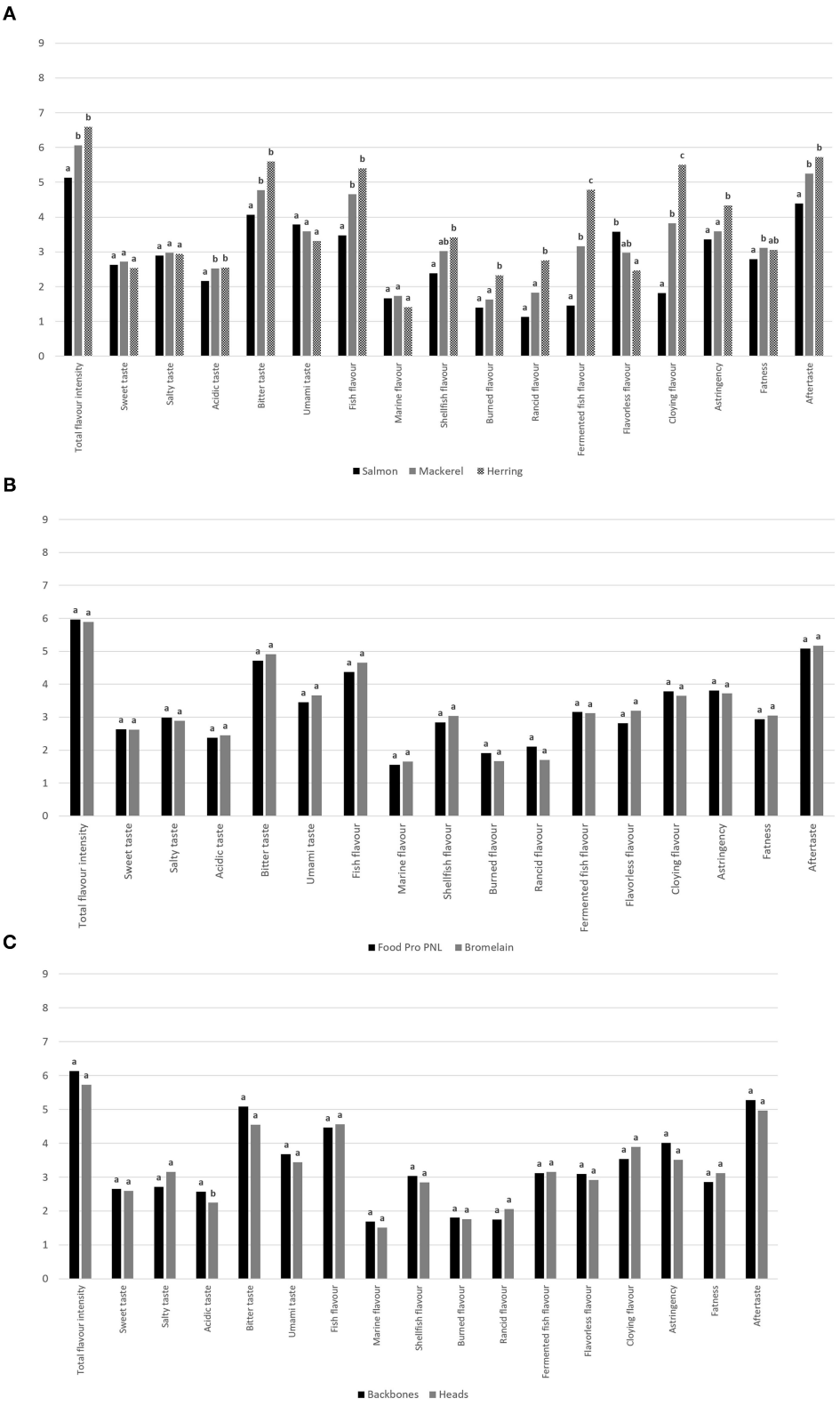

FIGURE 2 | Mean intensity of sensory attributes of protein hydrolysates based on **(A)** salmon, mackerel, and herring, **(B)** Food Pro PNL and Bromelain, and **(C)** heads and backbones. Different letters indicate statistical difference within each variable ($p < 0.05$) based on mixed model ANOVA and Tukey's multiple comparison test.

such as acidic, salty, sweet, sea flavor and fatness, and molecules <20 kDa. Furthermore, the mackerel hydrolysates based on Food Pro PNL were mostly similar to the salmon hydrolysates, whereas the mackerel hydrolysates based on Bromelain were more similar to herring hydrolysates (**Figure 1A**). This indicates that Bromelain produces hydrolysates of higher taste-intensity compared with Food Pro PNL in mackerel raw material.

The mean intensity values for the individual sensory attributes showed that, except for sweet taste and shellfish and marine flavors, all tested attributes varied significantly ($p < 0.05$) between the products (**Table 6**). In general, the products were taste and flavor intense, with total flavor intensity scores between 4.7 and 7. Hydrolysates based on herring were particularly flavor intense, bitter, and with high scores for fermented fish flavor, followed by mackerel and salmon hydrolysates. Fermented fish flavor is ascribed to "bad or stale fish" (**Table 1**), indicating that the herring hydrolysates were the most unpalatable of the assessed products. The hydrolysates were filtered post-hydrolysis to remove residual fat from the products and the overall intensity of rancid flavor was low. However, higher levels in the herring hydrolysates suggests some lipid oxidation in the raw material before or during the hydrolysis process, as discussed above. The unpalatability of hydrolysates from pelagic species may also be ascribed to their relatively high ratio of dark muscle and the accompanying prooxidative components such as copper and iron, compared with salmon (45). Mackerel and herring (and other pelagic species) are exempt from the regulatory requirement for bleeding upon loading (46). This may result in more blood in the side stream materials, affecting both the molecular composition and sensory properties of the resulting hydrolysate. Another possible explanation may be the difference in fatty acid composition of the raw materials (**Table 2**), with varying levels of fatty acids susceptible for oxidation during storage, with docosahexaenoic acid being the most susceptible (47). Although practically all lipids were removed in the downstream filtration process, there might have been oxidation products present in the final hydrolysates.

The individual effects of fish species, side stream fraction, and enzyme on the hydrolysates' sensory attributes were evaluated using mixed model ANOVA (**Figure 2**). Products based on herring and mackerel were significantly more flavor intense and had significantly higher intensity of the attributes, such as acidic, bitter and fish, and with a stronger aftertaste compared with hydrolysates based on salmon (**Figure 2A**). Although not significant at $p < 0.05$, the levels were higher for herring-hydrolysates compared with mackerel for the above-mentioned attributes. Further, hydrolysates based on herring were significantly more cloying and had a higher intensity of fermented fish flavor compared with both salmon and mackerel. Flavourlessness was the only attribute where salmon had the highest intensity, and this suggests that salmon raw material was the most applicable substrate for production of taste-acceptable and palatable hydrolysates.

No significant effects of neither enzyme (**Figure 2B**) nor fraction (**Figure 2C**) were observed, with the exception of acidic taste being higher in the backbone hydrolysates. The findings were in agreement with previous studies on salmon and mackerel raw materials (10), and indicate similar levels of peptides and molecules influencing sensory attributes in head- and backbone hydrolysates, both substrates being rich in muscle protein and connective tissues. Choice of enzyme is an important factor in the production of protein hydrolysates, as this will influence both processing costs (21) and product sensory properties (14, 19, 20, 30). Bromelain has been found to produce significantly higher bitter taste intensity compared with Food Pro PNL in fish hydrolysates (14); however, in the present study only small and insignificant differences between the two proteases were observed (**Figure 2B**). Further, Bromelain has been found to produce hydrolysates of less bitter taste compared with Alcalase in soy protein isolates (20), reflecting that both raw material composition and processing conditions are important for the development of bitter taste. The findings from this work indicate that choice of fish raw material and post-harvest handling may be the most important determinator for bitter and unpalatable taste development, where herring-based products were the least palatable compared with mackerel and salmon. Although fish-based protein hydrolysates are good sources of protein and IAA, they must be palatable to achieve consumer acceptance if they are to be used as food ingredients.

CONCLUSIONS

Hydrolysates based on herring were the most flavor-intense and bitter, followed by mackerel and salmon. Only small and insignificant effects of fraction (i.e., head vs. backbone) and choice of enzyme (i.e., Bromelain vs. Food Pro PNL) were observed on the tested sensory properties. All hydrolysates contained high levels of connective tissue amino acids glycine, proline, and a well-balanced amino acid composition. Hydrolysates based on heads were richer in ash compared with backbone-based hydrolysates, suggesting more bone material in the heads. The study indicates that salmon side stream materials, both heads and backbones, are more suited toward human consumption, compared with herring and mackerel. Additional raw material preparation and more focus on post-harvest handling to reduce potential prooxidative components should be investigated to produce palatable products from pelagic species.

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/s.

AUTHOR CONTRIBUTIONS

TA, SS, and KK: conceptualization. TA, SS, BV, MC, JA, and KK: methodology, validation, visualization, and writing—review and editing. TA and MC: software. SS, BV, MC, and JA: formal analysis and investigation. KK: resources. TA: data curation and writing—original draft preparation. TA and KK: supervision. KK: project administration and funding acquisition.

All authors have read and agreed to the published version of the manuscript.

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