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*Research Article*

# Biological Properties of Red Macroalgal Protein Hydrolysates and Their Uses as Biopreservatives

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In this study, we investigate the effect of different enzyme/substrate (*E*/*S*) ratios on the antioxidant and antibacterial properties of protein hydrolysates from two unexplored red macroalgae, namely, *Sphaerococcus coronopifolius* (SCPH) and *Gelidium spinosum* (GSPH). Antioxidant properties were evaluated using DPPH radical scavenging capacity, reducing power, ferrous ion chelating ability, and total equivalent antioxidant capacity. On the other hand, antibacterial activities were evaluated using agar diffusion and minimum inhibitory concentration (MIC) methods against six bacterial strains (*Listeria monocytogenes*, *Staphylococcus aureus*, *Kocuria rhizophila*, *Micrococcus luteus*, *Escherichia coli*, and *Salmonella newport*). The biopreservative effect of SCPH and GSPH on minced meat quality during 11 days of storage at 4°C was also studied focusing on lipid oxidation status and microbial growth. Both protein hydrolysates have considerable antioxidant activities whatever the *E*/*S* ratio used, in terms of scavenging DPPH radical ($IC_{50}$ ranging from 0.12 to 3.22 mg/mL) and metal chelating activity ($IC_{50}$ ranging from 0.15 to 2.24 mg/mL). At 5 mg/mL, the reducing power of SCPH and GSPH varied from 1.16 to 2.87. Moreover, SCPH and GSPH exhibited strong antibacterial activity against all tested bacteria, with MIC values ranging from 1.56 to 25 mg/mL. In addition, the incorporation of SCPH and GSPH into minced beef meat significantly ($p < 0.05$) inhibited lipid peroxidation and microbial growth during 11 days of storage at 4°C. At the end of the storage period, the TBARS values measured in the groups treated with 1% (*w*/*w*) SCPH and GSPH were significantly reduced to $1.36 \pm 0.003$ and $1.34 \pm 0.01$ mg MDA/kg meat, respectively. Overall, the results showed that the addition of both protein hydrolysates has a positive effect on meat quality and suggest that SCPH and GSPH can serve as effective natural additives, enhancing the biopreservation and shelf life of refrigerated minced beef.

**Keywords:** antibacterials; antioxidants; beef meat; biopreservation; enzymatic hydrolysis

## 1. Introduction

Food contamination is a serious public health problem affecting many consumers around the world. Generally, foods serve as the best media for the growth of diverse microorganisms, and when they are contaminated, a loss of their original quality in particular flavor, texture, and appearance is accentuated. The degradative changes produced by microorganisms, such as putrefaction, fermentation, and rancidity, become unhealthy for consumption. Among food groups, beef meat has emerged as one of the most sought-after meat products globally, owing to its rich

nutritional profile characterized by high protein and low-fat content [1]. Refrigerated meat possesses a limited shelf life primarily due to enzymatic degradation, lipid peroxidation, and microbial spoilage [2]. Throughout the stages of slaughter, transportation, and packaging, beef stands susceptible to bacterial contamination from pathogens like *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella* spp. [3], as well as oxidation. These factors contribute to a reduction in shelf life, an increase in the occurrences of foodborne illnesses, and subsequent economic repercussions.

Therefore, food safety has become a constant concern for the food industry. The processing industry is regularly facing many serious challenges regarding the safety and hygiene of its products. To ensure food safety, the multiplication of spoilage and pathogenic microorganisms should be controlled. However, food industries use synthetic or chemical antioxidant and antimicrobial compounds to control microbial pathogens and extend the shelf life of food products [4]. To prevent oxidative alteration and microbial growth, chemical additives have been extensively used in the meat industry [5]. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are often used to stabilize foods by scavenging starting free radicals to combat lipid oxidation concerns in meat products [6, 7]. Moreover, some antioxidants may additionally exhibit antibacterial activities [8]. However, some of them are suspected to induce pathological and toxic effects on health. For this reason, scientists have been actively involved in the search for new alternatives to replace these synthetic compounds with effective analogs of natural origin [9].

In addition to their nutritional significance, foods are nowadays also selected for their content of health-promoting bioactive components. These biomolecules, when incorporated into our diets, have the potential to promote human well-being [10]. Among various reported biologically active compounds are carotenoids, polysaccharides, essential oils, polyphenols, phytosterols, biogenic amines, and peptides [10, 11]. Proteins not only serve as an essential source of nutrition and amino acids crucial for growth and development but also of peptides that, when consumed, can produce beneficial physiological effects [12]. Recently, peptides derived from macroalgal proteins, being faced as a superfood, have gained interest in different fields, namely, for functional foods, nutraceuticals, and cosmetic or pharmaceutical applications [13, 14]. This application interest has been justified by their nontoxicity and their capacity to protect the human body against free radicals and retard the development of many chronic diseases [15]. Moreover, protein hydrolysates and peptides prepared through enzymatic hydrolysis using food-grade enzymes are classified as GRAS (generally recognized as safe) [16].

In addition to their significant impact on human health, bioactive peptides have been shown to inhibit oxidation and microbial growth in several food model systems [17, 18]. In this context, food protein hydrolysates and bioactive peptides are given priority, and their biological activities make them ideal natural ingredients in the formulation of functional foods [19–21]. Notably, Japan was the first country to adopt a legal system in relation to allowable health claims on functional foods through the introduction of the FOSHU (Foods for Specific Health Use) licensing system in 1991 [22]. Although there is a growing demand for obtaining bioactive peptides from marine macroalgae [23], the number of biologically active peptides identified from this bioresource is still limited. In this context, peptidic hydrolysates from some red macroalgal species such as *Palmaria palmata*, *Gracilariopsis lemaneiformis*, *Porphyra columbina*, *Porphyra yezoensis*, *Pyropia suborbiculata*, *Porphyra purpurea*, and *Pyropia haitanensis* have shown potent antioxidant and antimicrobial activities [15, 24–27]. Other bioactivities of macroalgal protein–derived peptides were also demonstrated including anti-inflammatory activity, gastrointestinal absorption modulation, opioid action, and immune modulation [28, 29].

*Sphaerococcus coronopifolius* and *Gelidium spinosum* are two interesting red macroalgae that have gained importance for their remarkable richness in bioactive compounds with a wide range of valuable biological properties [30–35]. Furthermore, *S. coronopifolius* and *G. spinosum* are recognized as two protein-rich macroalgal biomasses, with contents up to 17.62% and 22.71%, respectively [36]. Recently, protein extracts have been successfully prepared from these two marine raw materials using ultrasonication as a cell destruction technology [36]. To the best of our knowledge, the enzymatic production of peptide-rich hydrolysates with antioxidant and antibacterial properties has not been previously demonstrated from these two species of red macroalgae.

In this context, we propose in this study a sustainable bioresource as a new alternative additive to chemical counterparts used in the field of food technology. Our main objective is the in vitro evaluation of the antioxidant and antibacterial properties of protein hydrolysates from *S. coronopifolius* and *G. spinosum*. Furthermore, we explored the possibility of practical applications of these hydrolysates in a model food system, particularly in meat-based formulations, as functional ingredients.

## 2. Materials and Methods

### 2.1. Materials

*2.1.1. Reagents.* Chemicals required for the assays including ferrous chloride ($FeCl_2$), ferrozine, potassium ferricyanide ($C_6N_6FeK_3$), trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), sodium hydroxide (NaOH), hydrochloric acid (HCl), ammonium sulfate ($(NH_4)_2SO_4$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), BHT, disodium salt of ethylenediaminetetraacetic acid (EDTA), 6-hydroxy-2,5,7,8-tetramethylychroman-2-carboxylic acid (Trolox), 2-thiobarbituric acid (TBA), and pepsin (P6887, 200–4500 units/mg protein lyophilized powder from porcine gastric mucosa) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). The ultrapure water was prepared using a Milli-Q system (Merck, Darmstadt, Germany) with a resistivity of approximately 18 MΩ.

*2.1.2. Bacterial Strains.* Cultures of *Micrococcus luteus* ATCC (American Type Culture Collection) 9341, *Listeria monocytogenes* ATCC 19112, *Staphylococcus aureus* ATCC 13709,

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*Escherichia coli* ATCC 8733, and *Salmonella newport* ATCC 6962 were from the ATCC (Rockville, Maryland, United States). Culture of *Kocuria rhizophila* CIP (Collection of Pasteur Institute) 5345 was from the CIP (Paris, France).

*2.1.3. Macroalgal Biomass.* Two red macroalgal species (S. *coronopifolius* and *G. spinosum*) were collected, respectively, on the cost of Menzel Abderrahmane City (Bizerte, located in the northwest of Tunisia, 37°13′48″ N and 9°51′36″ E) and from the Monastir coastal region (east of Tunisia, 35°46′ N and 10°49′ E) in January and February 2019. Then, the macroalgae were rinsed with tap water to remove epiphytes, sediment, and potential contaminants. After that, the fresh macroalgae were subsequently dried in a dark room at ambient temperature for a few weeks. The dried samples were then ground using a blender (Knife Mill Grindomix GM 200, Retsch, Haan, Germany) and kept in airtight glass jars in a refrigerator at 4°C for further analysis.

*2.2. Enzymatic Hydrolysis of Macroalgal Proteins.* Firstly, proteins were extracted using the ultrasonication method as described by Dhaouafi et al. [36]. Then, the preparation of red macroalgal protein hydrolysates was carried out according to the enzymatic procedure described by Dhaouafi et al. [14]. Briefly, *S. coronopifolius* and *G. spinosum* protein extracts at 1% (*w*/*v*) were separately digested by porcine pepsin at different enzyme/substrate (*E*/*S*) ratios (1/2, 1/4, 1/10, and 1/100 (weight/weight (*w*/*w*)) at 40°C and pH 3.5 for 24 h under constant stirring (150 rpm). The enzymatic hydrolysis was stopped by the addition of NaOH (2 M) to a final pH of 9. Then, the protein hydrolysates were centrifuged at 6000 × *g* for 20 min at 4°C. Finally, the peptidic fractions were freeze-dried (Lyovapor L-200; Büchi Labortechnik AG, Flawil, Switzerland) and stored at −20°C until further use. The lyophilized powders obtained were referred to SCPH and GSPH for *S. coronopifolius* and *G. spinosum* protein hydrolysates, respectively. Moreover, samples were prepared under the same conditions without the addition of enzymes to serve as negative controls and were designated, respectively, as SCUP and GSUP for *S. coronopifolius* and *G. spinosum* undigested proteins.

*2.3. Determination of Antioxidant Activities.* The obtained protein hydrolysates (SCPH and GSPH) and undigested proteins (SCUP and GSUP) at various concentrations (0.5–5 mg/mL) were evaluated for their antioxidant activities by using four in vitro tests, namely, DPPH radical scavenging capacity, iron chelating ability, reducing power, and total equivalent antioxidant capacity (TEAC).

*2.3.1. DPPH-Free Radical-Scavenging Assay.* The DPPH-free radical-scavenging activity of SCPH and GSPH (0.5–5 mg/mL) and undigested proteins was determined using the method described by Bersuder et al. [37]. Briefly, 500 μL of each sample was mixed with 375 μL of 99.5% ethanol and 125 μL of 0.02 mM DPPH ethanol solution. The mixtures were then shaken and kept for 30 min in the dark at room temperature. The absorbance was measured at 517 nm using a UV-visible spectrophotometer (SpectraMax ABS Plus, Shanghai, China). DPPH radical-scavenging activity was calculated according to the following equation (Equation (1)):

$$\text{DPPH radical-scavenging activity (\%)} = \left(1 - \frac{A_{517}\text{ of sample}}{A_{517}\text{ of control}}\right) \times 100. \quad (1)$$

The control was conducted in the same manner, except that ultrapure water was used instead of the sample. Trolox was used as a positive control. The test was carried out in triplicate.

*2.3.2. Reducing Power Assay.* The ability of the samples (0.5–5 mg/mL) to reduce iron (III) was determined according to the method of Yildirim et al. [38] with slight modifications. Briefly, 0.5 mL of different concentrations of each sample was mixed with 1.25 mL of 0.2 M phosphate buffer (pH 6.6) and 1.25 mL of 1% (*w*/*v*) potassium ferricyanide solutions. After 30 min of incubation at 50°C, 1.25 mL of 10% (*w*/*v*) trichloroacetic acid was added. The mixtures were then centrifuged at 3000 × *g* for 10 min, and 1.25 mL of supernatant was mixed with 1.25 mL of ultrapure water and 0.25 mL of 0.1% (*w*/*v*) ferric chloride. After 10 min of reaction, the absorbance of the resulting solution was measured at 700 nm using a UV-visible spectrophotometer. An increased absorbance of the reaction mixture indicates an increased reduction power. BHT was used as a positive control. The test was performed in triplicate.

*2.3.3. Ferrous Ion Chelating Ability Assay.* The chelating activity on $Fe^{2+}$ was determined following the method described by Decker and Welch [39]. Briefly, an aliquot of 100 μL of each sample solution (0.5–5 mg/mL) was mixed with 50 μL of 2 mM $FeCl_2$ and 450 μL of ultrapure water, followed by the addition of 200 μL of 5 mM ferrozine. After 10 min of reaction time at room temperature, the absorbance of the $Fe^{2+}$-ferrozine complex with red or violet color was measured at 562 nm using a UV-visible spectrophotometer. The chelating activity was then calculated as follows (Equation (2)):

$$\text{Chelating activity (\%)} = \left(1 - \frac{A_{562}\text{ of sample}}{A_{562}\text{ of control}}\right) \times 100. \quad (2)$$

The control was prepared in the same manner except that ultrapure water was used instead of the sample. EDTA was used as a positive control. The test was carried out in triplicate.

*2.3.4. TEAC Assay.* The TEAC of SCPH and GSPH (0.5–5 mg/mL) was assessed by the phosphomolybdenum technique. This method is based on the reduction of molybdenum present as molybdate $MoO4^{2-}$ ion to molybdenum $MoO^{2+}$ by antioxidants to form a green phosphate/molybdenum ($Mo_3O_{16}P_4$) complex [40]. Briefly, 100 μL of each sample was mixed in an Eppendorf tube with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Then, the tubes were incubated at 95°C for 90 min. After cooling at room temperature, the absorbance of each sample was measured at 695 nm against a blank, using a UV-visible spectrophotometer. The

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calibration curve was prepared by Trolox solution (0.5–5 mg/mL), and TEAC was expressed as milligram Trolox equivalent per milligram of sample. The test was carried out in triplicate.

### 2.4. Determination of Antibacterial Activity

*2.4.1. Agar Diffusion Method.* The evaluation of the antibacterial activity of all samples was tested against four Gram-positive bacteria (*L. monocytogenes*, *S. aureus*, *K. rhizophila*, and *M. luteus*) and two Gram-negative bacteria (*E. coli* and *S. newport*) following the method described by Abou-Diab et al. [41]. The bacterial strains were conserved at −20°C in glycerol-containing nutrient broth and were subcultured twice in Muller–Hinton (MH) broth (Biokar Diagnostics) under agitation (60 rpm) at 37°C, except for *M. luteus* which was subcultured at 30°C. After 24 h of incubation, the absorbances of precultures were measured at 620 nm against MH medium and used as a blank. A series of dilutions was then performed in Tryptone Salt at a final colony-forming unit (CFU/mL) of $10^6$. Then, 1 mL of inoculum was spread on MH agar for 20 min. After removing the excess inoculum, 20 $\mu$L of each sample (10 and 25 mg/mL) or the positive control (colistin, 0.1 mg/mL for *E. coli*, and ampicillin, 0.1 mg/mL for other strains) was deposited on the surface of the agar. Finally, the Petri plates were incubated for 24 h at the corresponding temperatures of the indicator bacteria used. Antibacterial activity was measured as the diameter of the clear zone of growth inhibition and recorded as the diameter of the inhibition zone in millimeters (mm). The test was carried out in triplicate.

*2.4.2. Minimum Inhibitory Concentration (MIC).* The MIC of undigested proteins and protein hydrolysates was determined in sterile 96-well microplates (Costar 3799, Corning Incorporated, United Kingdom) following the method described by Abou-Diab et al. [41]. Briefly, 100 $\mu$L of MH broth was mixed with 100 $\mu$L of samples at a concentration of 50 mg/mL serially diluted in half. Then, 100 $\mu$L of the tested strain prediluted at $10^5$ CFU/mL was added to each well. The absorbance was measured after 24 h at 620 nm using a microplate reader to verify the inhibition of bacterial growth. As a turbidity control, bacterial strains were incubated as described above without tested samples. The MIC was defined as the lowest sample concentration that completely inhibited the growth of the indicator bacterial species after 24 h of incubation at 30 or 37°C. The absorbance of the wells corresponding to decreasing concentrations of each sample was compared with those of the positive control wells (MH medium inoculated with bacteria) and with those of the negative control wells (containing only MH broth). The test was carried out in triplicate.

### 2.5. Preservation of Fresh Meat Product

*2.5.1. Meat Sample Preparation and Experimental Design.* Beef meat was freshly purchased from the local market in Lille (France). After the elimination of the excessive fats and all other impurities, beef patties were prepared. The minced meat was subdivided into seven treatments following the method described by Bougatef et al. [42]: Group 1: the control being the patty without additive; Groups 2 and 3: the patty with different BHT concentrations (0.5% and 1% *w*/*w*); Groups 4 and 5: the patty with SCPH prepared at *E*/*S* ratio of 1/10 (*w*/*w*) at two concentrations (0.5% and 1% *w*/*w*); and Groups 6 and 7: the patty with GSPH prepared at *E*/*S* ratio of 1/10 (*w*/*w*) at two concentrations (0.5% and 1% *w*/*w*). The preservative powder was added to the meat, and all were homogenized. Meat patties were shaped by hand and placed in Petri dishes, wrapped with polyethylene film, and kept at 4°C for 11 days. The meat sample preparation was achieved under strong aseptic conditions to avoid contaminations. Five samples were taken on days 0, 1, 3, 9, and 11 for quality monitoring.

*2.5.2. Lipid Oxidation Evaluation.* Lipid oxidation was evaluated by the measurement of the thiobarbituric acid-reactive substances (TBARSs) according to the method of Witte et al. [43] with slight modifications. In fact, TBARS assay values are generally reported in malonaldehyde (MDA) equivalents, a compound produced from the decomposition of polyunsaturated fatty lipid peroxides. Five grams of beef meat was added to 12.5 mL of solution containing trichloroacetic acid (20% *w*/*v*) and phosphoric acid (2 M). Thereafter, this solution was mixed with 25 mL of ultrapure water and filtered through the Whatman paper using a Büchner system. Then, 5 mL of the obtained filtrate was homogenized with 5 mL of 2-thiobarbituric acid (5 mM) at 4°C. After 17 h of incubation at room temperature, the absorbance of this mixture was measured at 530 nm, and the results were expressed as milligram MDA equivalents per kilogram of meat (mg MDA/kg meat). The test was carried out in triplicate.

*2.5.3. Metmyoglobin Analysis.* Metmyoglobin (MetMb) content was evaluated according to Fernández-López et al. [44]. Briefly, 0.5 g of samples was mixed with 5 mL of ice-cold phosphate buffer (40 mM, pH 6.8). The homogenates were centrifuged at 5000 × *g* for 30 min at 4°C. Then, the supernatants were filtered through Whatman filter paper, and the absorbance was recorded at 525, 572, and 730 nm. The MetMb (%) was calculated using the following formula (Equation (3)):

$$\text{MetMb}\,(\%) = 1.395 - \left[\frac{(A_{572} - A_{730})}{(A_{525} - A_{730})}\right] \times 100. \quad (3)$$

*2.5.4. Measurement of Heme Iron Content.* Heme iron content was determined as hemin, after extraction with acidified acetone solution, as described by Clark et al. [45]. Briefly, 10 g of meat samples was macerated in 50 mL of acidified acetone (40 mL acetone, 8 mL deionized water, and 2 mL HCl) at 25°C in the darkness for 1 h. After incubation, the mixtures were then centrifuged at 3000 × *g* for 10 min, and the supernatants were filtered through Whatman paper. Finally, the absorbance of the filtrate was read at 640 nm. The heme iron content was calculated and expressed as microgram of iron/gram of meat using the following formula (Equation (4)):

$$\text{Heme iron}\,(\mu\text{g fer/g meat}) = A_{640} \times 680 \times 0.0882. \quad (4)$$

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*2.5.5. Microbial Analysis.* The capacity of SCPH and GSPH to inhibit bacterial growth in the meat product was also tested. The microbial counts were carried out at 0, 3, 6, 9, and 11 days of storage at 4°C [42]. Briefly, 1 g of minced meat was mixed with 9 mL of peptone water (0.1%, *w*/*v*) in a sterile stomacher bag. A series of dilutions ($10^{-1}$ to $10^{-5}$) were performed, and 100 $\mu$L of each dilution was diffused on different selective media. Total viable counts (TVC) were enumerated using plate count agar (PCA). Potato dextrose agar (PDA) and violet red bile lactose (VRBL) were used for yeast and mold and coliform counts, respectively. All agar plates were then incubated at 37°C for 24 h, and the results of the microbial counts were expressed as the $\log_{10}$ of CFU per gram of patty ($\log_{10}$ CFU/g).

*2.6. Statistical Analysis.* All analyses were conducted in triplicate, and three independent repetitions were performed for each experimental condition. The statistical analyses were carried out using the GraphPad Prism 8.0.2 software package (GraphPad Software, Inc., San Diego, California, United States). Data were subjected to analysis of variance (ANOVA). A comparison of means was carried out by the post hoc Tukey test. The statistical significance was determined at a $p$-value < 0.05. All results were expressed as mean values with their standard deviations (SD).

## 3. Results and Discussion

### *3.1. Antioxidant Activity*

*3.1.1. DPPH-Free Radical-Scavenging Ability.* Antioxidants are known for their ability to interact with free radicals, forming stable species that delay or terminate the oxidation process. In this study, the DPPH-free radical-scavenging abilities of SCUP, SCPH, GSUP, and GSPH are presented in Figure 1. As shown in Figure 1, all protein hydrolysates (SCPH and GSPH) exhibited significant radical-scavenging abilities in a concentration-dependent manner. In contrast, undigested proteins (SCUP and GSUP) demonstrated significantly ($p < 0.05$) lower antioxidant activity compared to their protein hydrolysates. These results are consistent with previous research by Lassoued et al. [46], indicating that antioxidant activity increased proportionally depending on the concentrations used and that intact proteins do not have significant DPPH scavenging activity. The findings suggest that the hydrolysis of red macroalgal proteins by pepsin is capable of generating antioxidant peptides.

Moreover, the protein hydrolysates at an *E*/*S* ratio of 1/2 (*w*/*w*) displayed the highest radical-scavenging activity among all tested ratios. Specifically, at the 5 mg/mL concentration, GSPH showed the highest DPPH inhibition power (90.84 ± 3.36%), followed by SCPH (86.04 ± 0.95%). However, Trolox (used as the positive control) exhibited higher radical-scavenging capacities for all concentrations tested. Subsequently, SCPH and GSPH prepared at an *E*/*S* ratio of 1/4 and 1/10 (*w*/*w*) are positioned in the second and third in terms of antiradical activity. In this regard, antioxidant activity increases proportionally depending on the *E*/*S* ratios used. Using an *E*/*S* ratio of 1/10 (*w*/*w*) and a concentration of 5 mg/mL, the DPPH radical scavenging capacities of 69.66 ± 0.19 and 69.38 ± 0.21% were determined for SCPH and GSPH, respectively. Moreover, both protein hydrolysates prepared at an *E*/*S* ratio of 1/100 (*w*/*w*) exhibited slightly higher antioxidant activities than intact proteins (SCUP and GSUP). Despite this, the results obtained highlight the possibility of obtaining peptide hydrolysates with a respectable antioxidant power from the proteins of red macroalgae even at a low *E*/*S* ratio.

Furthermore, our findings indicate that the $IC_{50}$ values for different protein hydrolysates are consistently lower and inversely correlated to the *E*/*S* ratios (Table 1). Specifically, as the *E*/*S* ratio increases, the $IC_{50}$ value decreases. At an *E*/*S* ratio of 1/2 (*w*/*w*), SCPH and GSPH have recorded $IC_{50}$ values of 0.12 ± 0.02 and 0.26 ± 0.07 mg/mL, respectively. Importantly, no significant difference ($p > 0.05$) was observed between the $IC_{50}$ values of SCPH prepared at this *E*/*S* ratio and the standard antioxidant used, Trolox (0.12 ± 0.03 mg/mL). This result further confirms the antioxidant potential of the peptides contained in these hydrolysates and will raise the question of the possibility of a synergistic effect. In addition, our findings suggest that these specific peptides derived from proteins of *S. coronopifolius* and *G. spinosum* interact with free radicals to form stable molecules and halt the radical chain reaction.

Moreover, it has been widely reported that protein hydrolysates from different red macroalgal species, such as *Porphyra tenera*, *Porphyra columbina*, *Soleria chordalis*, and *P. palmata*, exhibit strong antioxidant activities [25, 47, 48]. For instance, Senevirathne et al. [48] produced protein hydrolysate from *P. tenera* with Alcalase exhibiting antioxidative activity with scavenging activities on DPPH about 94% at 2.5 mg/mL.

The antioxidant properties of protein hydrolysates depend on the characteristics of the contained peptides including size, amino acid sequence, and molecular structure [49–51]. Recently, the high antioxidant activity of low molecular weight (LMW) peptides has been associated with the exposure of hydrophobic groups after enzymatic hydrolysis [52, 53]. In addition, the presence of hydrophobic amino acids in the peptide sequence has been found to improve the solubility of peptides in lipophilic media, thereby facilitating their interaction with hydrophobic free radicals [54]. Previous studies have also suggested that aromatic and hydrophobic amino acid residues of bioactive peptides significantly enhance the antioxidant capacity [55]. Similar results were reported by Zarei et al. [56] showing that peptides containing more than 50% hydrophobic amino acids are the most antioxidants.

In this study, the antiradical capacity of protein hydrolysates (SCPH and GSPH) is influenced by several factors, including protease specificity, hydrolysis conditions, protein substrate used, and degree of hydrolysis (DH) [57]. Also, protein hydrolysates with high contents of LMW peptides are generally powerful in eliminating the DPPH radical compared to those rich in high molecular weight (HMW) peptides [49, 58]. In our previous study, we demonstrated that SCPH and GSPH prepared at *E*/*S* of 1/10 (*w*/*w*) contain a high number of LMW peptides (< 1000 Da) and a significant percentage of hydrophobic peptides (> 50%) [14].

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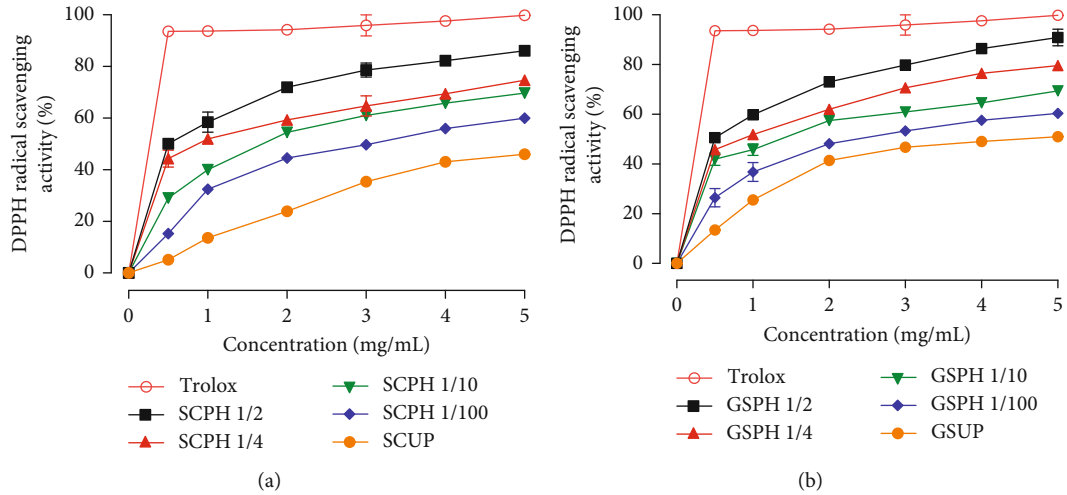


FIGURE 1: DPPH radical scavenging capacity of SCPH (a) and GSPH (b) prepared at various *E*/*S* ratios and tested at different concentrations. Trolox was used as the positive control. Values expressed are means of three independent determinations ($n = 3$). Bars represent the standard deviation (SD) from triplicate determinations.

**TABLE 1**: $IC_{50}$ values for DPPH radical scavenging capacity of SCPH and GSPH prepared at various *E*/*S* ratios.

| | | | | | | |
|---|---|---|---|---|---|---|
| $CI_{50}$ (mg/mL) | SCUP | SCPH 1/2 | SCPH 1/4 | SCPH 1/10 | SCPH 1/100 | Trolox |
| | $4.60 \pm 0.02^{a}$ | $0.12 \pm 0.02^{b}$ | $0.78 \pm 0.10^{c}$ | $1.85 \pm 0.03^{d}$ | $3.22 \pm 0.06^{e}$ | $0.12 \pm 0.03^{b}$ |
| | GSUP | GSPH 1/2 | GSPH 1/4 | GSPH 1/10 | GSPH 1/100 | Trolox |
| | $4.45 \pm 0.07^{a}$ | $0.26 \pm 0.07^{b}$ | $0.69 \pm 0.06^{c}$ | $1.39 \pm 0.16^{d}$ | $2.52 \pm 0.12^{e}$ | $0.12 \pm 0.03^{f}$ |

*Note:* Data are given as mean values ± standard deviation ($n = 3$). The different lowercase letters in the same row indicate significant differences ($p < 0.05$) between mean values.

*3.1.2. Reducing Power.* The reducing powers of all protein hydrolysates and BHT, used as a positive control, are illustrated in Figure 2. Notably, all prepared protein hydrolysates (SCPH and GSPH) reduced $Fe^{3+}$ to $Fe^{2+}$. However, as the *E*/*S* ratio increased, the reducing power increased also. Moreover, SCPH and GSPH exhibited significantly ($p < 0.05$) higher reducing powers than the undigested proteins (SCUP and GSUP), highlighting that peptic hydrolysis indeed improved this bioactive property. These results are in agreement with the study of Harnedy et al. [15] on the enzymatic hydrolysis of *P. palmata* protein isolate. This improvement could be attributed to the release of an increasing number of N- and C-terminal extremities containing electron-donor groups ($NH_2$ and COOH) during protein digestion [59]. Furthermore, Beaulieu et al. [60] demonstrated a significant increase of up to sixfold improvement in the reducing power following enzymatic hydrolysis of proteins from the red macroalga *P. palmata*. This underscores the efficacy of protein hydrolysis in enhancing antioxidant potential.

As shown in Figure 2, there is a correlation between the optical density at 700 nm ($OD_{700\,nm}$) and the concentration of the protein hydrolysates tested. Indeed, the results showed that both protein hydrolysates (SCPH and GSPH) displayed a steadily increasing tendency for reducing power. At the same concentration of 5 mg/mL, the reducing powers of SCPH and GSPH prepared at an *E*/*S* of 1/2 (*w*/*w*) ($2.78 \pm 0.005$ vs. $2.87 \pm 0.023$) were significantly ($p < 0.05$) higher compared to the *E*/*S* ratios of 1/4 (*w*/*w*) ($2.46 \pm 0.01$ vs. $2.06 \pm 0.06$) and 1/10 (*w*/*w*) ($1.34 \pm 0.01$ vs. $1.70 \pm 0.01$). However, BHT (5 mg/mL) showed slightly ($p > 0.05$) higher reducing power ($2.93 \pm 0.11$) than SCPH and GSPH prepared at an *E*/*S* ratio of 1/2 (*w*/*w*) and tested at the same concentration. The variability in reducing power observed in this study could be attributed to the differences in DH of protein hydrolysates [60]. Indeed, DH is a key parameter, and it is strongly correlated with the bioactivities of peptides generated during enzymatic hydrolysis [61].

The reducing power of bioactive peptides is closely related to their sequence, amino acid composition, and size [62]. Famuwagun et al. [62] have demonstrated that the composition of peptide mixtures contained in hydrolysates is influenced mainly by the protein source, enzyme specificity, and DH. Indeed, *P. palmata* protein hydrolysates prepared with Corolase and Alcalase consistently exhibited higher reducing power values compared to those prepared by Flavourzyme [63].

*3.1.3. Ferrous Ion Chelating Ability.* The chelation of $Fe^{2+}$ was used to evaluate the capacity of protein hydrolysates in metal chelating activity. The ferrous ion chelating activities of different protein hydrolysates prepared at various *E*/*S* ratios and EDTA, at concentrations ranging from 0.5 to 5 mg/mL, are depicted in Figure 3.

When comparing the different *E*/*S* ratios used for hydrolysis, a significantly ($p < 0.05$) higher $Fe^{2+}$ ion chelating activity was obtained when red macroalgal proteins were

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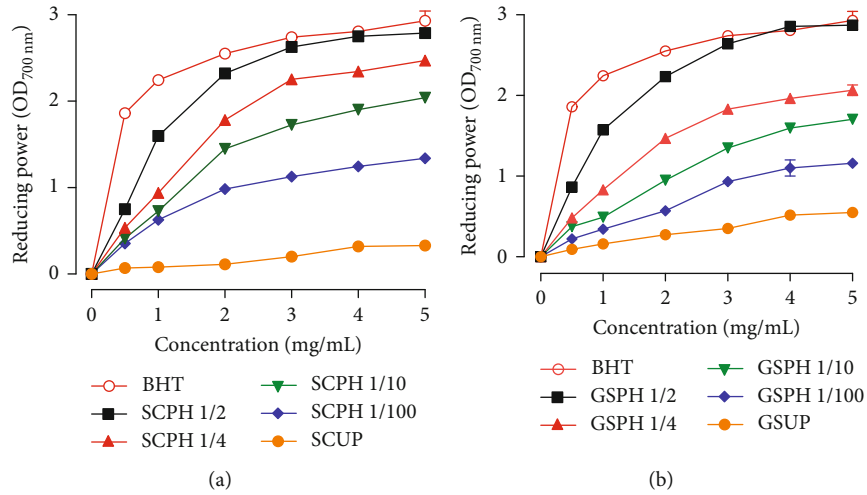


FIGURE 2: Reducing power of SCPH (a) and GSPH (b) prepared at various *E*/*S* ratios and tested at different concentrations. BHT was used as the positive control. Values expressed are means of three independent determinations ($n = 3$). Bars represent the standard deviation (SD) from triplicate determinations.

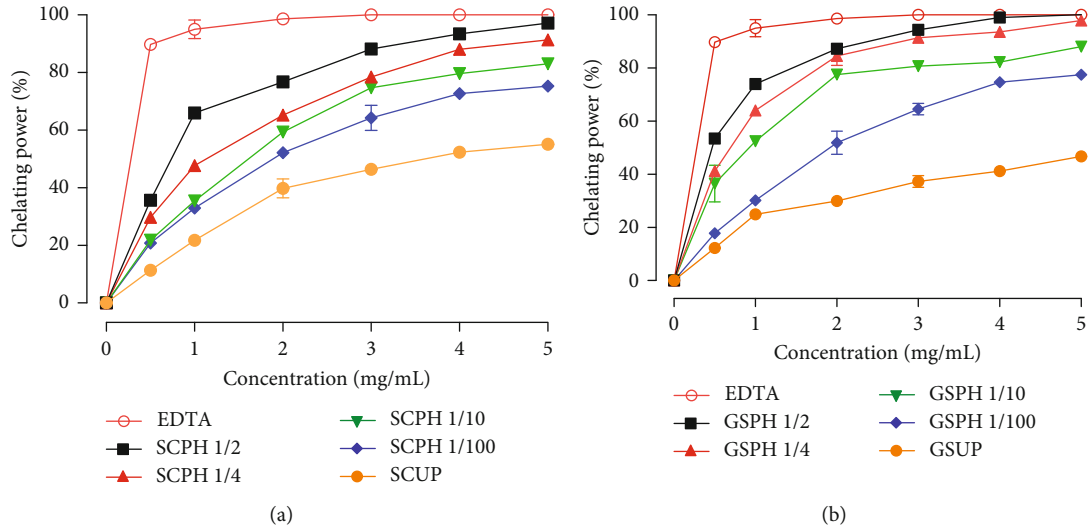


FIGURE 3: Ferrous ion chelating ability of SCPH (a) and GSPH (b) prepared at various *E*/*S* ratios and tested at different concentrations. EDTA was used as the positive control. Values expressed are means of three independent determinations ($n = 3$). Bars represent the standard deviation (SD) from triplicate determinations.

digested by pepsin at an *E*/*S* of 1/2 (*w*/*w*). On the other hand, the ferrous chelating capacity increased as a function of the concentration and the *E*/*S* ratio. Moreover, relatively high chelating capacities were recorded regardless of the concentration and/or *E*/*S* ratio tested. Specifically, at a concentration of 5 mg/mL and an *E*/*S* ratio of 1/2 (*w*/*w*), the ferrous chelating capacities of SCPH and GSPH were 97.17 ± 0.22 and 100 ± 0.14%, respectively. Under these conditions, SCPH and GSPH have $IC_{50}$ values estimated to be 0.59 ± 0.03 and 0.15 ± 0.02 mg/mL, respectively (Table 2). However, EDTA showed slightly higher ($p < 0.05$) ferrous chelating capacity (0.08 ± 0.01 mg/mL) than GSPH prepared at an *E*/*S* ratio of 1/2 (*w*/*w*). In addition, the *E*/*S* ratio of 1/10 (*w*/*w*) allowed the production of protein hydrolysates with similarly high ferrous ion chelating powers. Indeed, SCPH and GSPH, tested at 5 mg/mL, presented chelation levels of 83.03 ± 0.08 and 88.04 ± 0.79% and $IC_{50}$ values of 1.01 ± 0.09 and 0.94 ± 0.21 mg/mL, respectively.

The ferrous chelating capacity of bioactive peptides is related to their amino acid composition and molecular size [64]. Specifically, LMW peptides exhibit a significant ferrous chelating ability [65, 66]. Furthermore, amino acids such as histidine, cysteine, and glycine have been identified as excellent ferrous chelating residues [67].

*3.1.4. TEAC.* The antioxidant activity through the TEAC assay of all protein hydrolysates was measured at different concentrations (0.5–5 mg/mL) and compared with that of BHA (Figure 4). Obviously, all tested samples exhibited a concentration-dependent TEAC but with different potentialities depending on the *E*/*S* ratios used. However, high antioxidant activities were obtained for the majority of SCPH

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**TABLE 2**: $IC_{50}$ values for ferrous ion chelating ability of SCPH and GSPH prepared at various *E*/*S* ratios.

| | | | | | | |
|---|---|---|---|---|---|---|
| $IC_{50}$ (mg/mL) | SCUP | SCPH 1/2 | SCPH 1/4 | SCPH 1/10 | SCPH 1/100 | EDTA |
| | $3.76 \pm 0.14^{a}$ | $0.59 \pm 0.03^{b}$ | $0.95 \pm 0.12^{c}$ | $1.01 \pm 0.09^{d}$ | $2.08 \pm 0.08^{e}$ | $0.08 \pm 0.01^{f}$ |
| | GSUP | GSPH 1/2 | GSPH 1/4 | GSPH 1/10 | GSPH 1/100 | EDTA |
| | $5.53 \pm 0.22^{a}$ | $0.15 \pm 0.02^{b}$ | $0.69 \pm 0.02^{c}$ | $0.94 \pm 0.06^{d}$ | $2.24 \pm 0.06^{e}$ | $0.08 \pm 0.01^{b}$ |

*Note:* Data are given as mean values ± standard deviation ($n = 3$). The different lowercase letters in the same row indicate significant differences ($p < 0.05$) between mean values.

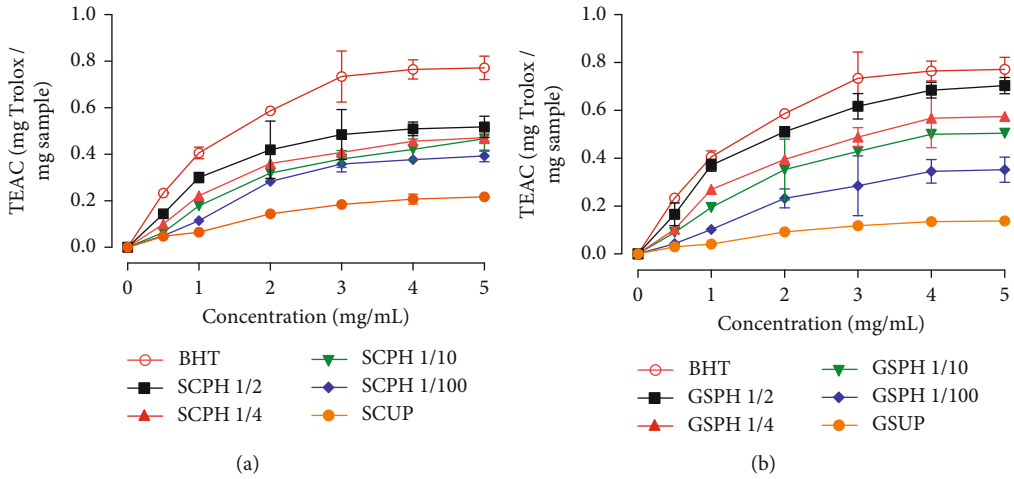


FIGURE 4: TEAC of SCPH (a) and GSPH (b) prepared at various *E*/*S* ratios and tested at different concentrations. BHT was used as the positive control. Values expressed are means of three independent determinations ($n = 3$). Bars represent the standard deviation (SD) from triplicate determinations.

and GSPH (Figure 4). Similar to the other tests, all SCPH and GSPH always showed a significantly higher antioxidant capacity than nonhydrolyzed proteins (SCUP and GSUP). Also as expected, SCPH and GSPH prepared with an *E*/*S* ratio of 1/2 (*w*/*w*) and tested at 5 mg/mL showed the highest TEAC. The TEAC under these conditions was estimated at $0.52 \pm 0.05$ and $0.70 \pm 0.04$ mg Trolox/milligram of sample for SCPH and GSPH, respectively. Respectable levels of this biological property were determined with SCPH and GSPH, prepared at *E*/*S* ratios of 1/4 and 1/10 (*w*/*w*). These results strongly suggest a correlation between DH and the concentration of bioactive peptides, as well as the TEAC. In this context, the antioxidant activity of hemoglobin hydrolysates was strongly influenced by DH [68]. The antioxidant activity of protein hydrolysates is dependent on the peptide size and also, to a greater extent on their sequences, evidenced by the specificity of the enzyme used. LMW peptides, in particular, are more readily absorbed by the body and exhibit stronger antioxidant activity [69]. BHA displayed a significantly higher TEAC than that of both hydrolysates (SCPH and GSPH) at all the concentrations tested.

The antioxidant activity of protein hydrolysates reflects the combined effects of various mechanisms. It is convenient to use multiple methods to obtain an overview of the antioxidant profile of the samples [70, 71]. Thus, after performing four antioxidant tests based on different mechanisms, our results demonstrate that SCPH and GSPH, regardless of the *E*/*S* ratio used, have significant antioxidant properties. Enzymatic hydrolysis produced peptidic mixtures with significant antioxidant capacity ($p < 0.05$), suggesting the release of bioactive peptides with antioxidant potential from the proteins of both red macroalgal species. This high antioxidant activity of SCPH and GSPH could be attributed to the ability of the bioactive peptides to reduce free radicals by hydrogen donation in a competitive reaction and/or their capacity to transfer an electron to reduce an oxidant. So far, many antioxidant peptides have been purified and identified from several red macroalgal species, namely, *Grateulopia lemaneiformis*, *Porphyra dioica*, *P. palmata*, and *Chondracanthus chamissoi* [24, 27, 72].

*3.2. Antibacterial Activity.* The in vitro antimicrobial activity of all protein hydrolysates (SCPH and GSPH) against pathogenic and nonpathogenic bacteria known to be involved in food spoilage and contamination was evaluated both qualitatively and quantitatively. The qualitative and quantitative analyses were carried out by the presence or absence of an inhibition zone (Figure 5 and Table 3) and the determination of the MIC values (Table 4), respectively.

In this study, the impact of using different *E*/*S* ratios on this biological property of the prepared protein hydrolysates was investigated against both $Gram^{+}$ and $Gram^{-}$ bacteria and compared to positive controls (colistin and ampicillin). Indeed, different diameters of growth inhibition zones were measured which vary depending on the *E*/*S* ratio and the bacterial strain tested (Figure 5).

According to the results given in Table 3, SCPH and GSPH, prepared at *E*/*S* ratios of 1/2, 1/4, and 1/10 (*w*/*w*),

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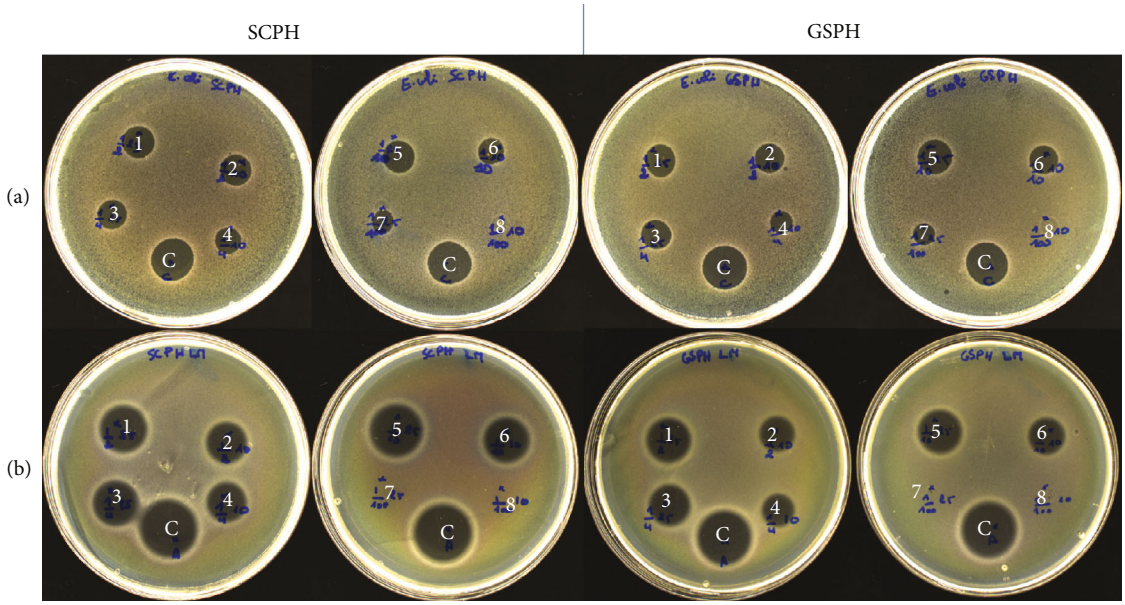


FIGURE 5: Inhibition zones of SCPH and GSPH tested at 10 and 25 mg/mL against *E. coli* (a) and *L. monocytogenes* (b). 1: *E*/*S* = 1/2 and 25 mg/mL; 2: *E*/*S* = 1/2 and 10 mg/mL; 3: *E*/*S* = 1/4 and 25 mg/mL; 4: *E*/*S* = 1/4 and 10 mg/mL; 5: *E*/*S* = 1/10 and 25 mg/mL; 6: *E*/*S* = 1/10 and 10 mg/mL; 7: *E*/*S* = 1/100 and 25 mg/mL; **8**: *E*/*S* = 1/100 and 10 mg/mL; C: positive control (colistin 0.1% for *E. coli* and ampicillin 0.1% for *L. monocytogenes*).

showed large inhibition zones against six bacterial strains. With the exception of *S. aureus*, SCPH and GSPH obtained with *E*/*S* ratio of 1/2 (*w*/*w*) and tested at 25 mg/mL demonstrated inhibition zones of approximately 20 mm against all indicator bacteria. In contrast, no statistically significant difference ($p > 0.05$) was observed between these inhibition diameters. This suggests that probably the difference in the nature and quantity of the peptide population contained in SCPH and GSPH does not seem to have a significant impact on the growth inhibition of the tested bacteria. SCPH prepared at an *E*/*S* ratio of 1/2 (*w*/*w*) and tested at a concentration of 25 mg/mL exhibited strong antibacterial activity, particularly against *E. coli* (28.66 ± 1.15 mm) and *M. luteus* (26.66 ± 3.05 mm). Interestingly, this antibacterial potency is comparable ($p > 0.05$) to that recorded for colistin (0.1 mg/mL).

Notably, SCPH obtained with *E*/*S* ratios of 1/4 and 1/10 (*w*/*w*) also showed broad inhibition zones against several bacterial strains (Figure 5). Indeed, the SCPH obtained with *E*/*S* ratio of 1/10 (*w*/*w*) measured inhibition zones against *E. coli* and *L. monocytogenes* of 15.66 ± 1.15 and 18.00 ± 1.73 mm, respectively. *L. monocytogenes* is a Gram-negative bacteria well known for its ability to contaminate several foods. This pathogenic bacteria causes one of the most serious foodborne diseases, called listeriosis [73]. In addition, GSPH (25 mg/mL) prepared at an *E*/*S* ratio of 1/10 (*w*/*w*) showed relatively interesting antibacterial activity, particularly against *L. monocytogenes* (16.83 ± 1.04 mm), *E. coli* (15.00 ± 2.00 mm), and *S. newport* (17.33 ± 3.05 mm). These results will highlight the possibility of using these two protein hydrolysates (SCPH and GSPH) in the preservation of food products.

Similarly, tested at 25 mg/mL and with *E*/*S* ratio of 1/2 (*w*/*w*), GSPH showed inhibition zones of 20.33 ± 2.08, 25.66 ± 2.08, and 21.00 ± 4.00 mm against *L. monocytogenes*, *E. coli*, and *S. newport*, respectively. Significantly, GSPH prepared at *E*/*S* ratios of 1/4 and 1/10 (*w*/*w*) also demonstrated antibacterial efficacy against *L. monocytogenes* (16.83 ± 1.04 mm) (Table 3) and *S. newport* (17.33 ± 3.05 mm). Furthermore, the inhibition zones of GSPH (10 and 25 mg/mL) prepared with *E*/*S* ratio of 1/10 (*w*/*w*) against *K. rhizophila* were also considerable, measuring 6.83 ± 1.25 and 16.33 ± 2.30 mm, respectively. On the other hand, only the growth of *E. coli* was inhibited by SCPH and GSPH (25 mg/mL) obtained with *E*/*S* ratio of 1/100 (*w*/*w*). Moreover, no antibacterial activity was demonstrated for both concentrations used of these protein hydrolysates against the other indicator strains tested (Table 3). In addition, undigested proteins (SCUP and GSUP) from the two red macroalgal species studied showed no growth inhibitory effect on any of the tested bacterial strains. This result proves the importance of enzymatic hydrolysis in the generation of antibacterial peptides.

Moreover, the MIC values highlighted variations in the antibacterial activity of all protein hydrolysates against the different bacterial strains (Table 4). Except for the undigested proteins (SCUP and GSUP) and SCPH and GSPH prepared at an *E*/*S* ratio of 1/100 (*w*/*w*), all other protein hydrolysates showed relatively low MIC values against both $Gram^+$ and $Gram^-$ indicator bacteria. These findings confirmed the results previously obtained by the agar diffusion method (Table 3).

In liquid medium, SCPH and GSPH prepared at *E*/*S* ratios of 1/2, 1/4, and 1/10 (*w*/*w*) were highly active against all bacterial strains tested, with MIC values ranging from 1.56 to 25 mg/mL. Importantly, no significant differences ($p > 0.05$) were detected between all protein hydrolysates, regardless of the *E*/*S* ratio chosen. Specifically, SCPH and GSPH prepared at *E*/*S* ratios of 1/2 and 1/4 (*w*/*w*) recorded MIC values of around 1.56 mg/mL against *K. rhizophila*.

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**TABLE 3**: Antibacterial activity of SCPH and GSPH prepared at various *E*/*S* ratios.

| | Concentrations (mg/mL) | *L. monocytogenes* | *S. aureus* | *K. rhizophila* | *M. luteus* | *E. coli* | *S. newport* |
|---|---|---|---|---|---|---|---|
| | | Inhibition zone diameter (mm) | | | | | |
| SCUP | 10 | — | — | — | — | — | — |
| | 25 | — | — | — | — | — | — |
| SCPH 1/2 | 10 | $13.66 \pm 1.52^{aA}$ | $11.00 \pm 1.00^{aA}$ | $13.33 \pm 4.04^{aA}$ | $14.00 \pm 4.00^{aA}$ | $15.33 \pm 1.52^{aA}$ | $11.00 \pm 2.64^{aA}$ |
| | 25 | $24.33 \pm 0.57^{aB}$ | $20.66 \pm 0.57^{aB}$ | $23.66 \pm 5.50^{aB}$ | $26.66 \pm 3.05^{aB}$ | $28.66 \pm 1.15^{bB}$ | $25.00 \pm 1.00^{aB}$ |
| SCPH 1/4 | 10 | $09.33 \pm 1.52^{aA}$ | $11.66 \pm 2.52^{aA}$ | $11.33 \pm 1.54^{aA}$ | $10.66 \pm 0.57^{aA}$ | $10.66 \pm 1.52^{aA}$ | $10.00 \pm 1.73^{aA}$ |
| | 25 | $22.33 \pm 1.52^{aB}$ | $20.33 \pm 1.53^{aB}$ | $19.66 \pm 2.08^{aB}$ | $23.33 \pm 2.10^{aB}$ | $24.00 \pm 1.73^{aB}$ | $19.33 \pm 1.52^{aB}$ |
| SCPH 1/10 | 10 | $10.33 \pm 0.57^{aA}$ | $13.00 \pm 1.73^{aA}$ | $10.33 \pm 0.57^{aA}$ | $09.33 \pm 1.15^{aA}$ | $11.33 \pm 0.57^{aA}$ | $11.66 \pm 2.51^{aA}$ |
| | 25 | $18.00 \pm 1.73^{aB}$ | $17.33 \pm 0.57^{aB}$ | $14.00 \pm 1.73^{bB}$ | $15.33 \pm 0.57^{abB}$ | $15.66 \pm 1.15^{abB}$ | $14.33 \pm 1.16^{abA}$ |
| SCPH 1/100 | 10 | — | — | — | — | — | — |
| | 25 | — | — | — | — | 13.33 ± 0.15 | — |
| GSUP | 10 | — | — | — | — | — | — |
| | 25 | — | — | — | — | — | — |
| GSPH 1/2 | 10 | $14.33 \pm 0.57^{aA}$ | $09.33 \pm 0.57^{aA}$ | $13.33 \pm 4.04^{aA}$ | $14.00 \pm 4.00^{aA}$ | $16.33 \pm 1.15^{aA}$ | $13.33 \pm 2.88^{aA}$ |
| | 25 | $20.33 \pm 2.08^{aB}$ | $13.66 \pm 2.03^{bB}$ | $23.66 \pm 5.50^{aB}$ | $26.66 \pm 3.05^{aB}$ | $25.66 \pm 2.08^{aBB}$ | $21.00 \pm 4.00^{aB}$ |
| GSPH 1/4 | 10 | $13.00 \pm 2.64^{aA}$ | $06.00 \pm 1.73^{bA}$ | $10.16 \pm 1.60^{abA}$ | $7.83 \pm 1.89^{abA}$ | $07.16 \pm 2.02^{abA}$ | $11.00 \pm 2.64^{abA}$ |
| | 25 | $19.33 \pm 2.08^{aB}$ | $18.00 \pm 1.73^{bA}$ | $15.33 \pm 0.57^{aB}$ | $13.00 \pm 2.64^{bB}$ | $16.33 \pm 2.30^{aB}$ | $17.66 \pm 2.51^{aB}$ |
| GSPH 1/10 | 10 | $08.80 \pm 1.31^{aA}$ | $08.33 \pm 2.08^{aA}$ | $06.83 \pm 1.25^{abA}$ | $12.00 \pm 1.00^{cA}$ | $12.00 \pm 2.00^{cA}$ | $09.66 \pm 2.51^{aA}$ |
| | 25 | $16.83 \pm 1.04^{aB}$ | $15.00 \pm 1.00^{aB}$ | $16.33 \pm 2.30^{aB}$ | $12.66 \pm 3.05^{aA}$ | $15.00 \pm 2.00^{aA}$ | $17.33 \pm 3.05^{aB}$ |
| GSPH 1/100 | 10 | — | — | — | — | — | — |
| | 25 | — | — | — | — | 12.33 ± 2.51 | — |
| Control | 0.1 | $30.00 \pm 2.00^{a}$ | $29.33 \pm 2.08^{a}$ | $27.33 \pm 1.52^{a}$ | $29.33 \pm 1.15^{a}$ | $28.16 \pm 1.60^{a}$ | $30.00 \pm 3.46^{a}$ |

*Note:* Values represent means ± standard deviations ($n = 3$) for triplicate experiments ($p < 0.05$). Colistin for *E. coli* and ampicillin for other bacterial strains were used as positive controls. "—" represents no activity. The different lowercase letters in the same row for each sample indicate significant differences ($p < 0.05$) between the inhibition zone diameter values against different bacterial strains. Different capital letters in the same column indicate significant differences ($p < 0.05$) between the studied concentrations.

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**TABLE 4**: MIC values of SCPH and GSPH prepared at various *E*/*S* ratios.

| | *L. monocytogenes* | *S. aureus* | *K. rhizophila* | *M. luteus* | *E. coli* | *S. newport* |
|---|---|---|---|---|---|---|
| | | | MIC (mg/mL) | | | |
| SCUP | > 25 | > 25 | > 25 | > 25 | > 25 | > 25 |
| SCPH 1/2 | $3.125 \pm 0.0^{aA}$ | $3.125 \pm 0.0^{aA}$ | $1.56 \pm 0.0^{bA}$ | $6.25 \pm 0.0^{cA}$ | $3.125 \pm 0.0^{aA}$ | $3.125 \pm 0.0^{aA}$ |
| SCPH 1/4 | $3.125 \pm 0.0^{aA}$ | $3.125 \pm 0.0^{aA}$ | $1.56 \pm 0.0^{bA}$ | $6.25 \pm 0.0^{cA}$ | $3.125 \pm 0.0^{aA}$ | $3.125 \pm 0.0^{aA}$ |
| SCPH 1/10 | $3.125 \pm 0.0^{aA}$ | $3.125 \pm 0.0^{aA}$ | $1.56 \pm 0.0^{bA}$ | $6.25 \pm 0.0^{aA}$ | $3.125 \pm 0.0^{aA}$ | $3.125 \pm 0.0^{aA}$ |
| SCPH 1/100 | > 25 | > 25 | > 25 | > 25 | > 25 | > 25 |
| GSUP | > 25 | > 25 | > 25 | > 25 | > 25 | > 25 |
| GSPH 1/2 | $3.125 \pm 0.0^{aA}$ | $3.125 \pm 0.0^{aA}$ | $1.56 \pm 0.0^{bA}$ | $6.25 \pm 0.0^{cA}$ | $3.125 \pm 0.0^{aA}$ | $3.125 \pm 0.0^{aA}$ |
| GSPH 1/4 | $3.125 \pm 0.0^{aA}$ | $3.125 \pm 0.0^{aA}$ | $1.56 \pm 0.0^{bA}$ | $6.25 \pm 0.0^{cA}$ | $3.125 \pm 0.0^{aA}$ | $3.125 \pm 0.0^{aA}$ |
| GSPH 1/10 | $3.125 \pm 0.0^{aA}$ | $3.125 \pm 0.0^{aA}$ | $3.125 \pm 0.0^{aB}$ | $6.25 \pm 0.0^{bA}$ | $3.125 \pm 0.0^{aA}$ | $3.125 \pm 0.0^{aA}$ |
| GSPH 1/100 | $25.00 \pm 0.0^{aB}$ | $25.00 \pm 0.0^{aB}$ | $12.50 \pm 0.0^{bC}$ | $25.00 \pm 0.0^{aB}$ | $25.00 \pm 0.0^{aB}$ | $25.00 \pm 0.0^{aB}$ |

*Note:* Values represent means ± standard deviations ($n = 3$) for triplicate experiments ($p < 0.05$). The different lowercase letters in the same row for each sample indicate significant differences ($p < 0.05$) between the MIC values against different bacterial strains. Different capital letters in the same column indicate significant differences ($p < 0.05$) between the MIC values of the samples against the same bacterial strains.

Both SCPH and GSPH, prepared at an *E*/*S* ratio of 1/10 (*w*/*w*), which contain several LMW peptides [55], have demonstrated potent antibacterial activity against pathogenic and nonpathogenic bacteria. Our results are in agreement with the few previous studies on marine protein hydrolysates of macro- and microalgae and fish coproducts which have shown that antimicrobial activity is strongly associated with the distribution of peptide sizes [74–76].

Moreover, the obtained results give significant importance to the protein hydrolysates (SCPH and GSPH) from the two red macroalgal species studied, particularly concerning *S. aureus*, which is well known to be resistant to various phytochemicals and its ability to produce multiple types of enterotoxins, which can cause gastroenteritis [77]. Currently, the mechanism by which marine macroalgal peptides affect bacterial cells involves cell wall disruption, which results in cytoplasmic leakage and ultimately leads to cell death [26].

Recently, the purification and identification of novel antimicrobial peptides from several marine sources have been described in the literature [78, 79]. Also, the isolation of antimicrobial peptides from some red macroalgal species, such as *P. yezoensis*, *Pyropia suborbiculata*, *Porphyra purpurea*, and *Pyropia haitanensis*, has been investigated [24]. However, to the best of our knowledge, this study marks the first attempt to produce protein hydrolysates with antimicrobial activities from *S. coronopifolius* and *G. spinosum*. Therefore, the present study proposes a novel unexplored biomass as a source of antibacterial peptides. This study could then provide a basis for the development of a new generation of natural antimicrobial agents that can be used as preservatives for food storage and distribution.

*3.3. Fresh Meat Product Preservation.* Protein hydrolysates (SCPH and GSPH) prepared with an *E*/*S* ratio of 1/10 (*w*/*w*) were selected to evaluate their effectiveness on the preservation of beef meat during refrigeration. The choice is based on the lowest *E*/*S* ratio for their production and their significant antioxidant and antibacterial activities. Consequently, meat samples were formulated with SCPH and GSPH at two concentrations of 0.5% and 1% (*w*/*w*) and preserved for 11 days at 4°C. Several quality indicators were evaluated during the cold storage period.

*3.3.1. Changes in TBARS Content.* Lipid oxidation is the main cause of food quality deterioration leading to rancidity and a shorter shelf life [80]. In this study, lipid peroxidation was assessed by quantification of TBARS content. The TBARS are generated through the second step of lipid oxidation during which peroxides are oxidized to aldehydes and ketones such as malondialdehyde (MDA). As shown in Figure 6, untreated meat (negative control) showed a significant increase ($p < 0.05$) in TBARS values from the third day until the end of the storage period. Initially, the TBARS level was $0.65 \pm 0.03$ mg MDA/kg meat, and it increased significantly ($p < 0.05$) in the untreated meat ($2.36 \pm 0.01$ mg MDA/kg meat) after 11 days of cold storage. However, no significant ($p > 0.05$) effect between the different treated groups has been observed on the first day of the experiment, with TBARS values around 0.7 mg MDA/kg meat.

At the end of the storage period, the TBARS values of the samples treated with SCPH (0.5% and 1% *w*/*w*) were $1.35 \pm 0.005$ and $1.36 \pm 0.003$ mg MDA/kg meat, respectively. Similarly, the meat samples treated with GSPH (0.5% and 1% *w*/*w*) presented TBARS values of $1.30 \pm 0.05$ and $1.34 \pm 0.01$ mg MDA/kg meat, respectively. In addition, the treated groups with the synthetic antioxidant BHT (0.5% and 1% *w*/*w*) showed TBARS contents of $1.61 \pm 0.01$ and $1.53 \pm 0.001$ mg MDA/kg meat, respectively. Our results revealed that meat groups treated with SCPH and GSPH have significantly ($p < 0.05$) lower TBARS contents than the negative control group and are very close to BHT, thus demonstrating a positive effect on meat preservation by reducing lipid peroxidation. This impact was observed dependent on the concentration of SCPH and GSPH (Figure 6). In addition, these results confirm the powerful antioxidant properties of protein hydrolysates from both red macroalgal species.

Protein hydrolysates prepared from bovine hemoglobin [81], eel by-products [42], and milk proteins [82] have been

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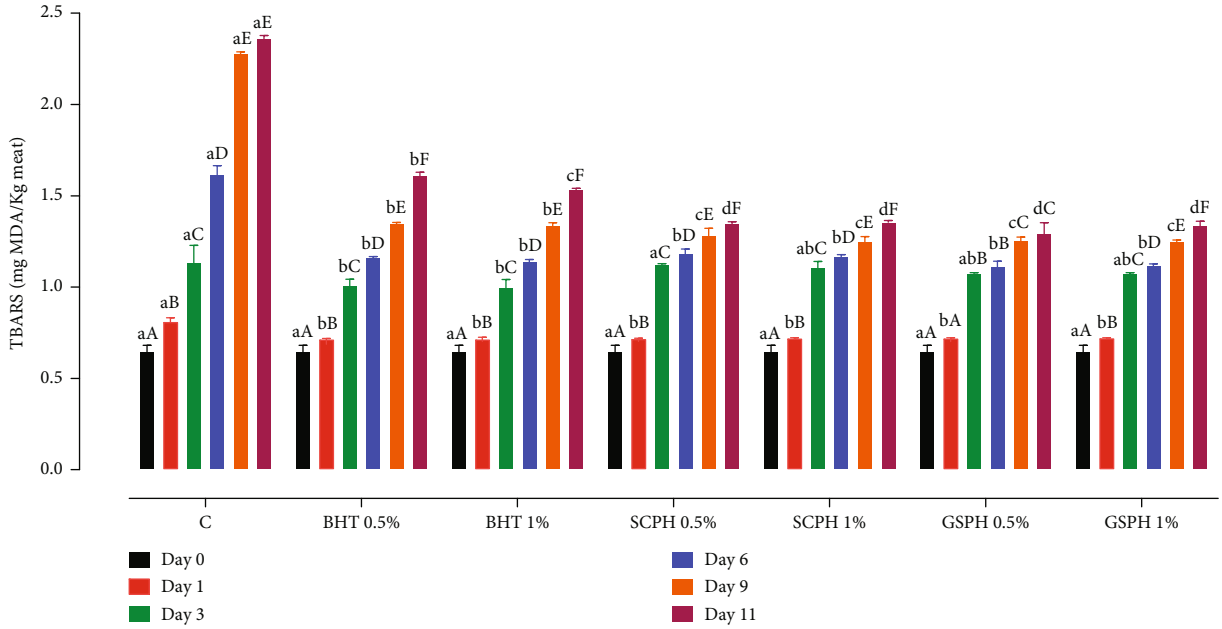


FIGURE 6: Changes in TBARS values of untreated and treated meat with SCPH and GSPH (0.5% and 1% *w*/*w*) during 11 days of refrigerated storage. C: negative control (meat without additive). BHT was used as the positive control. Bars represent the standard deviation from triplicate determinations. Different capital letters on the bars within the same treatment indicate significant differences ($p < 0.05$). The different lowercase letters on the bars within the same storage time indicate significant differences ($p < 0.05$).

successfully used as biopreservatives of meat during refrigeration. Furthermore, previous research has demonstrated the potential of bioactive peptides and protein hydrolysates as food antioxidants. These natural substances act through multiple mechanisms, such as reduction of hydroperoxides, scavenging of free radicals, and chelation of prooxidant transition metals [54]. Moreover, the antioxidant efficacy of bioactive peptides in mitigating lipid oxidation in meat and meat-based products throughout processing and storage contributes greatly to extending their shelf life [54]. Moreover, peptides derived from protein hydrolysates, due to their surface and interfacial properties, can be located at the oil–water interface in food emulsions. They can therefore form a physical barrier that reduces the contact of lipids with oxidizing agents, which contributes to reducing lipid peroxidation in such food systems [83]. Antioxidant peptides may have applications as natural food preservatives by preventing or minimizing lipid oxidation [84].

The efficacy of marine-derived antioxidant peptides and/or protein hydrolysates as oxidation inhibitors in model food systems has only recently been evaluated. For the first time, this study investigated the protective effect of peptides derived from red macroalgal proteins against meat quality deterioration, which broadens their application in the food field.

*3.3.2. Changes in MetMb Content.* MetMb levels of minced meat without and with the addition of protein hydrolysates and BHT during 11 days of cold storage are shown in Table 5. The initial MetMb level (21.43 ± 1.45%) was similar ($p > 0.05$) in all samples, with a significant increase ($p < 0.05$) throughout the storage period to reach a maximum value (41.91 ± 1.53%) in the untreated group on the 11th day of storage. Indeed, during a period of 3 days, while MetMb percentage increased rapidly ($p < 0.05$), attaining 31.10 ± 0.36% in the negative control sample, it varied between 26.37% and 27.61% (SCPH) and 24.79% and 26.92% (GSPH) for treated ones. Increasing MetMb levels in stored meat significantly affect the meat color because of the oxidation of myoglobin and the autoxidation of protein in the meat [85]. Our results are in line with those of Eljoudi et al. [86] who also detected a strong increase in MetMb content in the untreated meat group from approximately 25.39% on the first day to 36.92% on the ninth day of storage.

MetMb contents in samples treated with BHT, SCPH, and GSPH varied depending on the concentration used. At the end of the storage period, treated beef meat with 1% (*w*/*w*) SCPH and GSPH had the lowest MetMb contents of 28.27 ± 0.34 and 28.77 ± 0.77%, respectively. In addition, the group treated with BHT at the same concentration had a similarly low MetMb level (30.08 ± 0.91%). All obtained MetMb values for the different treated groups with SCPH or GSPH were significantly ($p < 0.05$) lower than those of untreated meat, regardless of the storage period and the concentration tested. In the current study, at different concentrations, protein hydrolysates from both red macroalgal species were efficient in controlling myoglobin oxidation by blocking oxygen penetration [85]. Generally, MetMb levels above 40% can negatively affect consumer acceptance, making the meat less appealing [87]. In our study, the control group slightly exceeded these limit damage. However, the meat samples formulated with BHT, SCPH, or GSPH at 0.5% and 1% (*w*/*w*) maintained MetMb levels below this critical limit even after 11 days of refrigerated storage.

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**TABLE 5**: Changes in MetMb and heme iron contents of untreated and treated meat with SCPH and GSPH (0.5% and 1% *w*/*w*) during 11 days of refrigerated storage.

| | MetMb (%) | | | | | |
|---|---|---|---|---|---|---|
| | **Day 0** | **Day 1** | **Day 3** | **Day 6** | **Day 9** | **Day 11** |
| Control | $21.43 \pm 1.45^{aA}$ | $24.90 \pm 1.85^{aA}$ | $31.10 \pm 0.36^{aB}$ | $32.58 \pm 1.08^{aB}$ | $38.79 \pm 0.92^{aC}$ | $41.94 \pm 1.53^{aC}$ |
| BHT 0.5% | $21.43 \pm 1.45^{aA}$ | $23.98 \pm 1.27^{aAB}$ | $26.41 \pm 0.34^{bB}$ | $29.86 \pm 0.18^{bC}$ | $31.33 \pm 1.10^{bD}$ | $33.09 \pm 0.63^{bD}$ |
| BHT 1% | $21.43 \pm 1.45^{aA}$ | $23.54 \pm 0.81^{aAB}$ | $24.89 \pm 0.46^{bBC}$ | $26.31 \pm 0.26^{cC}$ | $29.17 \pm 0.61^{bD}$ | $30.08 \pm 0.91^{bcD}$ |
| SCPH 0.5% | $21.43 \pm 1.45^{aA}$ | $25.84 \pm 2.62^{aAB}$ | $27.61 \pm 1.92^{aBC}$ | $28.83 \pm 1.06^{bcBD}$ | $29.84 \pm 1.05^{bCD}$ | $30.59 \pm 1.36^{bcD}$ |
| SCPH 1% | $21.43 \pm 1.45^{aA}$ | $23.80 \pm 1.64^{aAB}$ | $26.37 \pm 2.70^{bAB}$ | $26.98 \pm 1.67^{cBC}$ | $27.34 \pm 0.82^{bC}$ | $28.27 \pm 0.34^{cC}$ |
| GSPH 0.5% | $21.43 \pm 1.45^{aA}$ | $24.95 \pm 0.52^{aAB}$ | $26.92 \pm 2.22^{bBC}$ | $28.61 \pm 1.23^{bBD}$ | $29.03 \pm 0.43^{bCD}$ | $30.59 \pm 1.36^{bcCD}$ |
| GSPH 1% | $21.43 \pm 1.45^{aA}$ | $22.75 \pm 1.78^{aA}$ | $24.79 \pm 0.31^{bAB}$ | $27.08 \pm 1.32^{eBC}$ | $27.26 \pm 1.47^{bB}$ | $28.77 \pm 0.77^{cC}$ |
| | **Heme iron ($\mu$g/g)** | | | | | |
| | **Day 0** | **Day 1** | **Day 3** | **Day 6** | **Day 9** | **Day 11** |
| Control | $9.30 \pm 0.16^{aA}$ | $8.69 \pm 0.11^{aA}$ | $8.95 \pm 0.54^{aA}$ | $8.05 \pm 1.28^{aAB}$ | $7.66 \pm 1.13^{ab}$ | $7.55 \pm 0.15^{ab}$ |
| BHT 0.5% | $9.30 \pm 0.16^{aA}$ | $9.83 \pm 0.62^{abA}$ | $9.95 \pm 0.41^{abA}$ | $9.71 \pm 0.43^{abA}$ | $9.65 \pm 0.52^{bA}$ | $9.91 \pm 0.47^{bA}$ |
| BHT 1% | $9.30 \pm 0.16^{aA}$ | $9.79 \pm 0.64^{abA}$ | $9.99 \pm 0.50^{abA}$ | $10.01 \pm 0.63^{bA}$ | $10.15 \pm 0.69^{bA}$ | $9.77 \pm 0.57^{bA}$ |
| SCPH 0.5% | $9.30 \pm 0.16^{aA}$ | $10.19 \pm 0.29^{abA}$ | $9.75 \pm 0.46^{abAB}$ | $10.45 \pm 0.72^{bA}$ | $11.61 \pm 1.09^{bB}$ | $11.77 \pm 0.71^{cB}$ |
| SCPH 1% | $9.30 \pm 0.16^{aA}$ | $9.81 \pm 0.48^{bcA}$ | $9.85 \pm 0.36^{abA}$ | $11.41 \pm 0.39^{bAB}$ | $11.59 \pm 0.88^{bB}$ | $11.47 \pm 0.60^{cB}$ |
| GSPH 0.5% | $9.30 \pm 0.16^{aA}$ | $9.99 \pm 0.29^{abAc}$ | $10.39 \pm 0.43^{bcBC}$ | $10.17 \pm 0.30^{bAC}$ | $10.71 \pm 0.52^{bBC}$ | $10.35 \pm 0.28^{bcBC}$ |
| GSPH 1% | $9.30 \pm 0.16^{aA}$ | $9.83 \pm 0.51^{bcAC}$ | $10.19 \pm 0.37^{abAC}$ | $10.45 \pm 0.48^{bBC}$ | $10.75 \pm 0.51^{bBC}$ | $10.41 \pm 0.28^{bcAC}$ |

*Note:* Meat without additives was used as the negative control. BHT was used as the positive control. Values represent means ± standard deviations ($n = 3$) for triplicate experiments ($p < 0.05$). The different lowercase letters in the same column within the same storage time indicate significant differences ($p < 0.05$). Different capital letters in the same row within the same treatment indicate significant differences ($p < 0.05$).

*3.3.3. Changes in Heme Iron Content.* Myoglobin and hemoglobin, which are essential for oxygen storage and transport, are mainly associated with heme iron. The heme molecule plays a crucial role in physiological processes, but when separated from hemoproteins, it becomes dangerous because it can oxidize proteins and lipids and has the potential to initiate the oxidation reaction [88]. The impact of SCPH and GSPH on the heme iron content in beef meat is presented in Table 5.

The addition of SCPH and GSPH together with storage times displayed significant ($p < 0.05$) effects on heme iron content. For negative control samples, heme iron contents decreased significantly ($p < 0.05$) until 11 days, while a slight increase ($p < 0.05$) was observed for the SCPH and GSPH groups for all refrigerated storage days. The initial heme iron content was $9.30 \pm 0.16$ $\mu$g/g, and this obtained value was close to that reported in the literature (9.55 $\mu$g/g) for beef meat [86]. At the end of the storage (Table 5), untreated meat has a heme iron content of $7.55 \pm 0.15$ $\mu$g/g. This level indicates an alteration in the quality of the meat during the 11 days of storage at 4°C. The higher rate of decrease in heme iron content observed in beef meat with extended storage time suggested that higher disruption of heme proteins and higher release of iron from heme occurred in this food matrix.

The heme iron contents of the meat samples formulated with BHT (0.5% and 1% *w*/*w*) were estimated to be $9.91 \pm 0.47$ and $9.77 \pm 0.77$ $\mu$g/g, respectively. In addition, the addition of SCPH (0.5% and 1% *w*/*w*) allowed to reach at the end of the refrigerated storage period ($p < 0.05$) heme iron contents of $11.77 \pm 0.57$ and $11.47 \pm 0.60$ $\mu$g/g, respectively. For the same duration (11 days), the meat groups treated with GSPH (0.5% and 1% *w*/*w*) presented similar heme iron contents of $10.35 \pm 0.28$ and $10.41 \pm 0.28$ $\mu$g/g, respectively. Heme iron content is most likely correlated with total pigment content. A slight increase in heme iron content found at the end of storage of beef meat was possibly associated with the increased degradation of beef muscle, leading to the ease of heme extraction.

From the results, an extension of the shelf life of beef meat without further oxidative changes would be possible with the addition of protein hydrolysates from red macroalgae. The above-mentioned in vitro assays can provide insights into the potentialities of SCPH and GSPH against protein oxidation in real meat systems. In addition, it is claimed that enzymatic hydrolysis improves the capability of different antioxidant peptides, prevents early-stage lipid oxidation in meat products, and delays the onset of oxidation in meat during storage. Thus, the obtained peptides improve the quality of meat-based products by effectively preventing oxidative degradation in muscle foods [54].

*3.3.4. Microbiological Alteration.* Meat and meat products provide a favorable medium for microbial proliferation that sometimes becomes a health risk for the consumer even before the expiry date. To extend the shelf life of foods, natural antimicrobial substances such as bioactive peptides and protein hydrolysates are used as novel biopreservation strategies [89]. In this context, changes in microbial flora of treated and untreated meat during refrigerated storage are shown in Figure 7.

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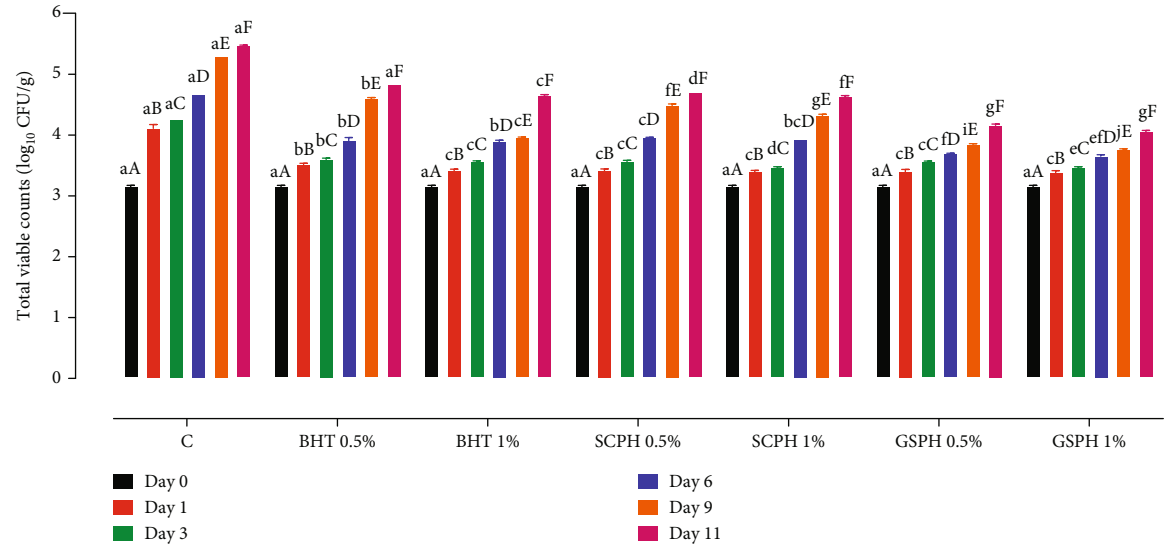
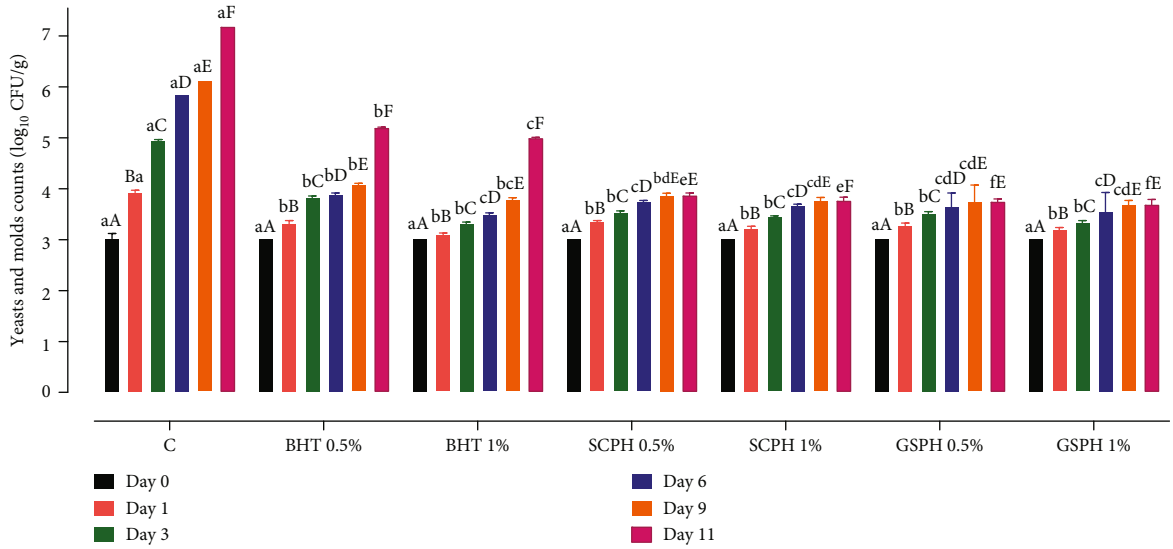



(a)




(b)

Figure 7: Continued.

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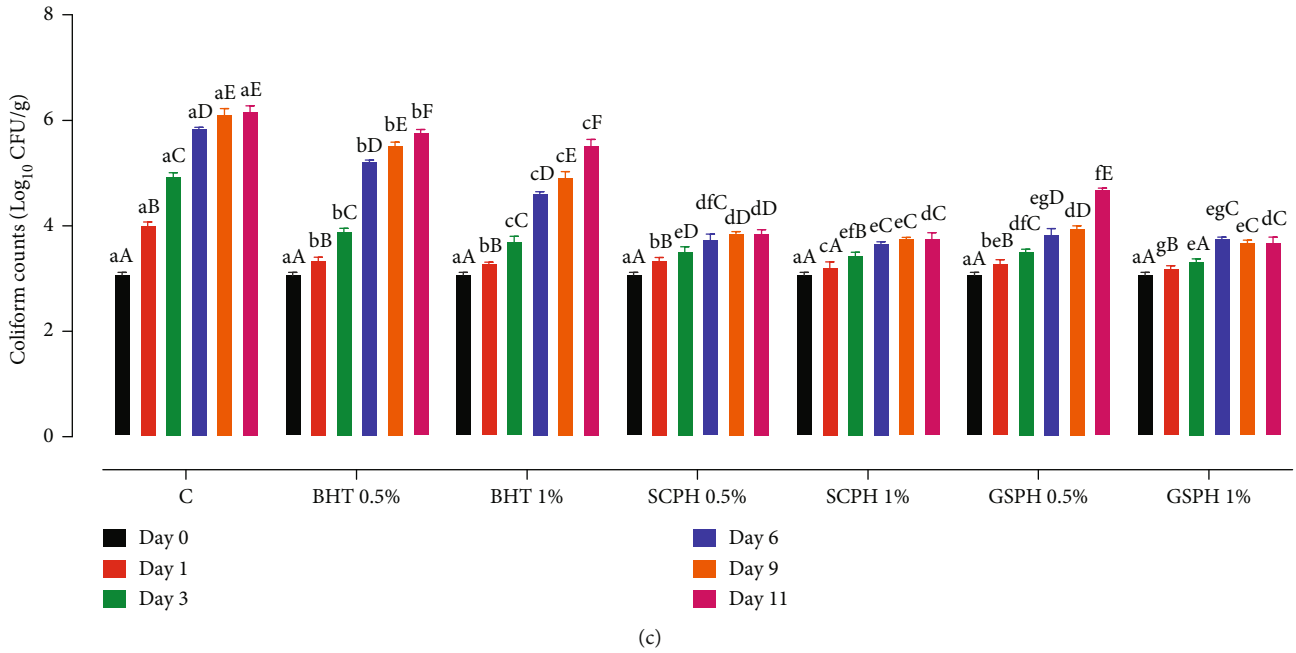


(c)

FIGURE 7: Changes in total viable (a), yeast and mold (b), and coliform (c) counts of untreated and treated meat during 11 days of refrigerated storage. C: negative control (meat without additive). BHT was used as the positive control. Bars represent the standard deviation from triplicate determinations. Different capital letters on the bars within the same treatment indicate significant differences ($p < 0.05$). The different lowercase letters on the bars within the same storage time indicate significant differences ($p < 0.05$).

Overall, all microbial indicators increase significantly ($p < 0.05$) during a cold storage period to reach the highest values on the 11th day. Among all experimental groups, the control group (untreated meat) presents the highest levels of TVC, yeast and mold, and coliform counts. However, the levels of all enumerated microorganisms decreased significantly ($p < 0.05$) with the presence of additives (BHT, SCPH, and GSPH).

As illustrated in Figure 7a, the starting minced meat has a relatively low TVC level (3.16 $\log_{10}$ CFU/g). This value remains close to that (~3.9 $\log_{10}$ CFU/g) obtained by Przybylski et al. [81]. In addition, TVC levels of all meats formulated with SCPH and GSPH increased significantly ($p < 0.05$ ) during a refrigerated storage period (11 days). According to the International Commission on Microbiological Specifications for Foods, the recommended TVC level should not exceed 7 $\log_{10}$ CFU/g [90]. A significant increase ($p < 0.05$) in TVC level was obtained in the untreated meat group to achieve its maximum (5.47 ± $\log_{10}$ CFU/g), without exceeding the damage limit, after 11 days of cold storage. Nevertheless, the TVC level decreased significantly ($p < 0.05$) with the increase in BHT, SCPH, and GSPH concentrations (0.5% and 1% *w*/*w*) throughout the storage period. TVC levels in treated meat samples with 1% (*w*/*w*) of SCPH or GSPH were 4.64 and 4.06 $\log_{10}$ CFU/g, respectively, on the last day of the cold storage period. Interestingly, until the end of storage, the TVC levels for SCPH and GSPH groups did not exceed the damage limit.

The findings indicated also that protein hydrolysates from both red macroalgal species displayed the slowest increase in TVC, and their values were quite similar ($p > 0.05$) than BHT at the same concentration. Przybylski et al. [81] hydrolyzed purified bovine hemoglobin with pepsin and studied the $\alpha$137–141 fragment of hemoglobin (TSKYR). Applying TSKYR on beef patties as a preservative (0.5%, *w*/*w*) reduced lipid oxidation (about 60%) and inhibited the growth of total viable colonies under refrigeration for 14 days. Additionally, chicken protein hydrolysates showed interesting effects against microbial proliferation in chicken breasts stored at 4°C for 8 days [91].

Yeasts and molds pose a significant threat to food products, as they can quickly contaminate and cause rapid spoilage. Thus, it is crucial to acknowledge their presence and include them in the sampling plans for general hygiene monitoring. On the first day (Day 0), the yeast and mold count of beef meat was relatively low estimated at 3.03 $\log_{10}$ CFU/g (Figure 7b). Similar results were reported by Przybylski et al. [81] for the same raw material (~3.5 $\log_{10}$ CFU/g). Moreover, a significant increase ($p < 0.05$) in yeast and mold growths was observed throughout the storage period in the untreated meat group (Figure 7b). Indeed, the microbial count of 7.19 $\log_{10}$ CFU/g was recorded on the last day of storage. Furthermore, the number of yeasts and molds in meat formulated with SCPH and GSPH was significantly ($p < 0.05$) reduced. Thus, the incorporation of different protein hydrolysates from two red macroalgal species effectively ($p < 0.05$) inhibits the growth of yeasts and molds compared to untreated meat. At the end of the storage period, the recorded yeast and mold levels were, respectively, 3.77 and 3.69 $\log_{10}$ CFU/g for SCPH and GSPH added at 1% (*w*/*w*). After 11 days, the SCPH- and GSPH-treated groups were more effective than the positive standard (BHT)

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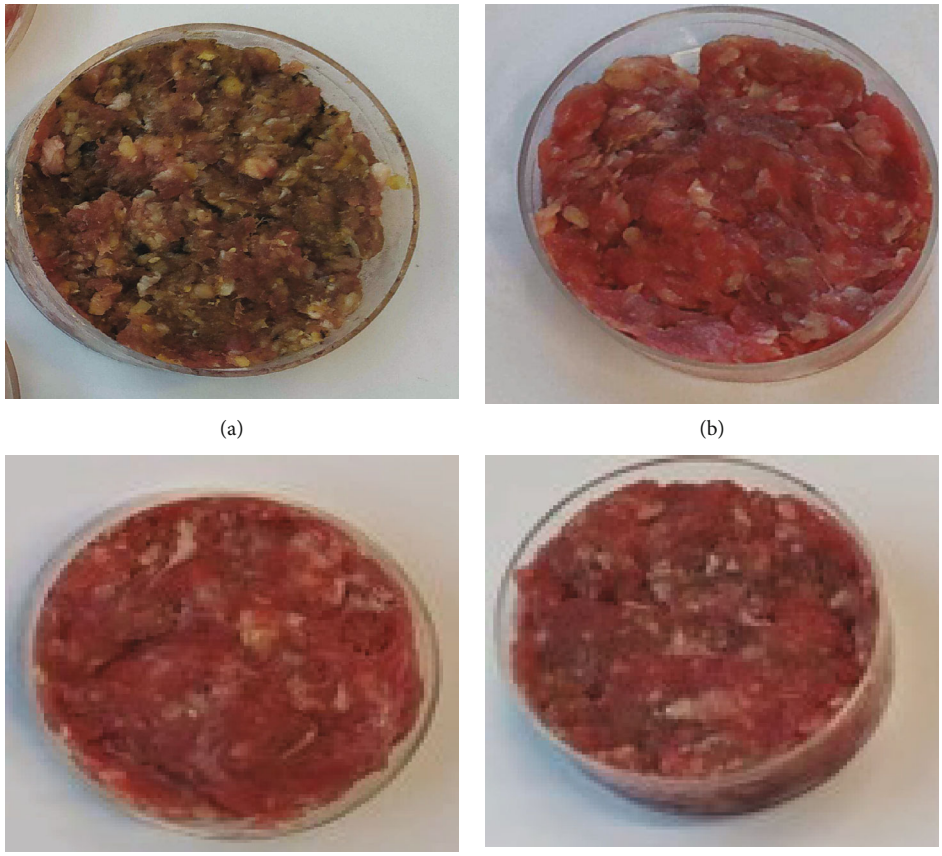


FIGURE 8: Visual meat appearance after 11 days of storage at 4°C. C: negative control (a), meat treated with BHT (b), SCPH (c), and GSPH (d) at a concentration of 1% (*w*/*w*).

groups. Przybylski et al. [81] have reported that the yeast and mold growths were not significantly inhibited from Day 0 to Day 9, except for $\alpha$137–141 at 0.5% (*w*/*w*) for the seventh day.

Also, coliforms are commonly used as indicators of overall hygiene and potential contamination in food products, including meat. Coliform bacteria increased considerably ($p < 0.05$), reaching higher values in negative control samples (3.08 $\log_{10}$ CFU/g (Day 0) to 6.19 $\log_{10}$ CFU/g (Day 11) compared to formulated ones (Figure 7c). The most effective additives were SCPH and GSPH at 1% (*w*/*w*), with the coliform counts of 3.77 and 3.69 $\log_{10}$ CFU/g, respectively (at the end of refrigerated storage). These effects were even more important ($p < 0.05$) than that of BHT at 0.5% and 1% (*w*/*w*). To conclude, both SCPH and GSPH presented important effects against microbial proliferation in meat during storage at 4°C, especially with the concentration of 1% (*w*/*w*).

These meat preservative effects are expected, given that SCPH and GSPH have already demonstrated significant in vitro antibacterial activity against six bacterial strains known to cause food spoilage (Tables 3 and 4). Several studies highlighted that antimicrobial peptides/protein hydrolysates were able to reduce effectively microbial proliferation in meat and can effectively serve as food biopreservatives [89]. Also, both red macroalgal protein hydrolysates (SCPH and GSPH) prolonged the shelf life of meat up to 11 days during refrigerated storage, likely attributable to their richness in antibacterial peptides. This quality improvement is evident in the meat color after 11 days of storage at 4°C (Figure 8). Overall, these results demonstrated that the addition of SCPH and GSPH, as biopreservative ingredients, significantly enhances the quality and extends the storage period of refrigerated minced beef meat by minimizing the growth rate of several microorganisms.

Various substances from marine algae, such as polysaccharides and polyphenols, have been used as additives to extend the shelf life of meat or meat products [11]. Indeed, sulfated exopolysaccharides extracted from red microalgae (*Porphyridium cruentum*) at 2% successfully improved the microbiological quality and oxidative stability of minced beef meat for 14 days at 4°C. Additionally, a protective effect of minced pork patties has been demonstrated for fucoidans from brown macroalga (*Laminaria digitata*) [92] and astaxanthin from a freshwater green microalga (*Haematococcus pluvialis*) [93]. To our knowledge, our study is the first investigation to demonstrate the positive effect of protein hydrolysates derived from two red macroalgae (*S. coronopifolius* and *G. Spinosum*) on extending the shelf life of beef meat. Indeed, SCPH and GSPH represent a promising alternative source of active and beneficial peptides that could be used as biopreservative agents.

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## 4. Conclusion

The present study has demonstrated that SCPH and GSPH, prepared at various *E*/*S* ratios, exhibited interesting antioxidant and antibacterial activities with different in vitro tests. Furthermore, the incorporation of SCPH and GSPH produced with an *E*/*S* of 1/10 (*w*/*w*) in stored minced beef meat significantly ($p < 0.05$) inhibits lipid peroxidation and reduces microbial growth. Overall, the two red macroalgal protein hydrolysates effectively extended the shelf life of beef meat and retained all quality attributes up to 11 days of refrigeration. Therefore, those protein hydrolysates can be a promising natural preservative ingredient for the meat processing industry.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Conflicts of Interest

The authors declare no conflicts of interest.

## Author Contributions

**Jihen Dhaouafi:** conceptualization, investigation, methodology, data curation, formal analysis, writing – original draft, writing – review and editing. **Montassar Romdhani**: methodology, formal analysis. **Ahlam Outman**: methodology, formal analysis. **Naima Nedjar:** conceptualization, methodology, data curation, validation, resources, project administration. **Rafik Balti:** conceptualization, methodology, data curation, validation, resources, writing – original draft, writing – review and editing, funding acquisition, project administration, supervision. All authors have reviewed and approved the submission of this manuscript version. Naima Nedjar and Rafik Balti contributed equally to this work.

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