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Blue Whiting Protein Hydrolysates Exhibit Antioxidant and Immunomodulatory Activities in Stimulated Murine RAW264.7 Cells

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Abstract: This study investigated the antioxidant and immunomodulatory potential of six blue whiting soluble protein hydrolysates (BWSPHs, BW-SPH-A to -F) and their simulated gastrointestinal digests (SGID, BW-SPH-A-GI to -F-GI) in murine RAW264.7 macrophages. Hydrolysate BW-SPH-A, both pre- and post-SGID, increased endogenous antioxidant glutathione (GSH) in *tert*-butylhydroperoxide (*t*BOOH)-treated cells and reduced reactive oxygen species (ROS) in H_2O_2-challenged RAW264.7 cells compared with treated controls in the absence of BWSPHs ($p < 0.05$). BW-SPH-A-GI also exhibited higher ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) activities than the other BWSPHs tested ($p < 0.05$). All BWSPHs and SGID BWSPH samples induced immunostimulating effects in lipopolysaccharide (LPS)-activated RAW264.7 macrophages through the upregulation of NO production. BW-SPH-F-GI increased IL-6 and TNF-α levels compared with the LPS controls indicating the liberation of immunomodulatory peptide/amino acids during the SGID process. Therefore, BW-SPH-A and BW-SPH-F may have potential use against oxidative stress and immunosuppression-related diseases, respectively.

Keywords: blue whiting; protein hydrolysates; antioxidant; immunomodulatory; RAW264.7 cells

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1. Introduction

Cellular metabolic processes and environmental factors, i.e., atmospheric pollutants, cigarette smoke, and radiation, generate free radicals categorised as either reactive oxygen species (ROS) or reactive nitrogen species (RNS). An excess of free radicals, inducing oxidative stress, is detrimental to cell structures by way of DNA strand damage as well as lipid and protein peroxidation [1]. Long-term oxidative stress can accelerate the aging process as well as contribute to the development of a range of chronic diseases which include cancer, diabetes, and cardiovascular disease. Lifestyle and diet play an important role in the regulation of oxidative stress and can be modified to promote cellular redox balance, thereby potentially preventing damage and disease. Protection against oxidative stress in the body is provided via intracellular enzymatic antioxidant defence systems namely superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as non-enzymatic defence systems such as the glutathione system consisting of reduced (GSH) and oxidized (GSSG) forms of glutathione. The antioxidant enzyme, SOD, is the first line of defence against free radicals and converts the superoxide anion ($O_2^{-}\cdot$) to hydrogen peroxide (H_2O_2) and oxygen. Both CAT and GSH-PX then reduce H_2O_2 to water and oxygen, thereby preventing the formation of free radicals. GSH, which is a substrate

for antioxidant enzymes such as GSH-Px also directly scavenges free radicals, regenerates nutrient antioxidants (vitamins A and E) to their active forms and assists the transport of amino acids through the plasma membrane [2].

A number of antioxidative fish protein hydrolysates with potential to induce health benefits via promoting cellular redox balance have been identified [3–8]. However, the generation of antioxidant protein hydrolysates is dependent on hydrolysis conditions (protease source, temperature, pH, and degree of hydrolysis (DH)), which ultimately influences the peptide profile and amino acid composition of the resulting fractions [9]. Small molecular weight (MW) fractions rich in hydrophobic amino acids and hydrophobic di- and tripeptides are generally reported to exhibit effective antioxidant activity owing to the proton donating or electron/lipid radical scavenging ability of hydrophobic amino acids.

Fish protein hydrolysates have also demonstrated both anti-inflammatory activity [10–15] and proinflammatory activity [16,17] via modulation of nitric oxide (NO) and inflammatory cytokines such an interleukin (IL)-6 and tumour necrosis factor-alpha (TNF-α) in macrophage cell lines. The use of macrophage cells has become an increasingly popular approach to investigate the antioxidant potential of natural bioactive compounds. Although macrophages are responsible for the recognition and elimination of microbial pathogens, some virulent bacteria have been reported to induce macrophage apoptosis via stimulating ROS production [18]. Due to the adverse effects associated with some immunomodulatory drugs, protein hydrolysates generated from underutilised fish species may offer a sustainable and safe alternative for therapeutic immunomodulation.

Blue whiting (*Micromesistius poutassou*) protein hydrolysates (BWPH) have previously been demonstrated to exhibit antioxidant activity as measured by DPPH radical scavenging activity, ferrous chelating activity and reducing power [19,20]. In addition, BWPH have also displayed cardioprotective and antigenicity activity in vitro, antidiabetic activities in vitro and in vivo and antiobesity activities in vitro, in vivo, and in clinical trials [19,21–24]. Identification of bioactive fractions or peptides from blue whiting contributes to the sustainability ethos through reducing waste by converting an underutilised source of high-quality protein, harvested at high volumes into high-value functional food ingredients. To the best of our knowledge, no study to date has investigated the antioxidant potential of BWPH in cellular systems or their immunomodulatory potential.

In most cases, in order to exhibit effective bioactivity in vivo, bioactive peptides have to survive gut transit and depending on their target cell, may need to permeate the gut membrane. However, due to the large population of macrophages that exist along the entire length of the GI tract [25], it is possible that bioactive food components may interact with and modulate macrophages as they transit the gut. However, gut macrophage populations characteristically do not induce classic inflammatory responses so the ability of BWPH to modulate inflammation should initially be assessed in the well-established macrophage cell line RAW264.7, which can generate proinflammatory mediators and cytokines.

Recently our group generated distinct blue whiting soluble protein hydrolysates (BWSPHs) at commercial scale using various food-grade microbial-derived proteolytic enzyme preparations and hydrolysis conditions (enzyme:substrate (E:S) ratios ranging from 0.005% to 0.900% (*w*/*w*), 50 °C, 45–120 min, [26]). Although these hydrolysates differed in their DH and molecular mass distribution, all BWSPHs tested demonstrated in vitro antidiabetic activity through dipeptidyl peptidase-IV (DPP-IV) inhibitory and insulin secretory activity. The objective of this follow-on study was to research additional potential bioactivities of the six BWSPHs and their simulated gastrointestinal (GI) digests through noncellular and cellular antioxidant and immunomodulatory assays. The 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging, ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC) assays were used to assess noncellular antioxidant activity whilst the ability of the BWSPHs and simulated GI digests to modulate redox balance (GSH, CAT, and ROS) was assessed in oxidatively stressed RAW264.7 cells. The immunomodulatory potential of BWSPHs was also studied

via investigating their effect on NO production, and IL-6 and TNF-α cytokine levels in lipopolysaccharide (LPS)-activated RAW264.7 cells.

2. Materials and Methods

2.1. Materials

RAW264.7 cells were purchased from American Type Culture Collection (Manassas, Virginia). Foetal bovine serum (FBS) was purchased from Invitrogen (Paisley, Scotland). Corolase® PP was provided by AB Enzymes (Darmstadt, Germany) and BC pepsin was provided by Biocatalysts (Cardiff, UK). Cell culture plastics were supplied by Cruinn Diagnostics and Corning Incorporated. All other cell culture reagents and chemicals were purchased from Sigma-Aldrich Chemical Co. unless otherwise stated.

2.2. Sample Preparation

The six BWSPHs (BW-SPH-A to -F) samples provided by BioMarine Ingredients Ireland Ltd. (Lough Egish Food Park, Castleblaney, Co. Monaghan, Ireland) were generated from minced blue whiting as previously described by [26]. Simulated gastrointestinal digestion (SGID) of the BWSPHs (BW-SPH-A-GI to -F-GI) was performed as described in [26] using pepsin (pH 2, 37 °C, 90 min, E:S of 2.5% *w*/*w*) and Coralase PP (pH 7, 37 °C, 150 min, E:S of 1% *w*/*w*). Samples were heated at 85 °C for 15 min, freeze-dried and kept at −20 °C until used.

2.3. Cell Culture and Sample Preparation

RAW264.7 cells were grown in Dulbecco's Modified Eagles' Medium (DMEM) supplemented with 10% (*v*/*v*) FBS. Cells (between passage numbers 15–34) were cultured in an atmosphere of CO_2–air (5:95 (*v*/*v*)) at 37 °C and were maintained in the absence of antibiotics. The BWSPHs and SGID BWSPHs were diluted directly with sterile DMEM, unless stated otherwise, sterile-filtered using 0.45-micron filters, and stored at −20 °C. Reduced serum media (DMEM supplemented with 2.5% FBS) was used for cell-based experiments.

2.4. DPPH Activity

The DPPH assay was performed according to the method described by [27]. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), prepared in methanol with concentrations ranging from 0.04 to 0.40 µM, was used to prepare a standard curve for calibration. Briefly, 100 µL of blank (methanol), standards (0.04 to 0.40 µM Trolox prepared in methanol), and test samples (prepared in DMEM and tested in the range 1.5–4.0 mg/mL), were mixed with 0.06 mM DPPH/methanol solution (3.9 mL) and incubated for 30 min at room temperature (~20 °C). Colour blanks were included consisting of 100 µL DMEM and 3.9 mL methanol. Absorbance at 515 nm of all samples was measured (Lightwave II UV/Visible spectrophotometer, Biochrom Ltd., Cambridge, England). Results were expressed as % DPPH inhibition.

2.5. ORAC Activity

The ORAC activity was determined as previously described [28]. In brief, 50 µL of the blank (0.075 M sodium phosphate buffer pH 7.0), standards (0–120 µM Trolox prepared in assay buffers), and test samples (prepared in assay buffers and tested in the range 0.15–0.20 mg/mL), were mixed with 0.78 µM fluorescein (50 µL) and incubated for 15 min at 37 °C in a plate reader (BioTek Synergy HT, Winooski, VT, USA). The reaction was initiated by the addition of 0.221 M 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH) solution (25 µL) and maintained at 37 °C for 120 min. The fluorescence (Ex/Em wavelengths of 485/520 nm) signal was measured every 5 min for 2 h and the ORAC value was calculated with reference to a Trolox standard curve (0–120 µM) and expressed as µmol of Trolox equivalents per gram dry weight (µmol TE/g dw).

2.6. FRAP Activity

The FRAP activity was determined as described by [29]. In brief, the absorbance (590 nm) of the FRAP reagent ((150 µL) 0.3 M acetate buffer (pH 3.6), 0.01 M 2, 4, 6-tripyridyl-s- triazine (TPTZ), 0.02 M $FeCl_3.6H_2O$, 10:1:1) was determined using a plate reader. A 20 µL aliquot of 0.3 M acetate buffer pH 3.6 (blank), standards (0–200 µM Trolox prepared in assay buffers) and test samples (prepared in assay buffers and tested in the range 15–20 mg/mL) were added, mixed and the absorbance read after 30 min incubation at 37 °C. The FRAP value was expressed as µmol of Trolox equivalents per gram of freeze-dried powder (µmol of TE/g dw).

2.7. Cell Viability

RAW264.7 cells were seeded at a density of 1×10^5 cells/mL in DMEM supplemented with 10% FBS with a volume of 200 µL/well in a 96-well plate. After a 24 h incubation at 37 °C, media was aspirated and cells were either supplemented with increasing concentrations of BWSPHs (BW-SPH-A to -F) and SGID BWSPHs (BW-SPH-A-GI to -F-GI) with the final concentrations in the culture medium ranging from 0–1.0% (*w*/*v* dw), or DMEM only (control), for 24 h with a final volume of 200 µL. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrezolium bromide) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK), which consisted of a MTT reagent and a solubilisation solution. The well contents were aspirated and MTT reagent (10 µL) and DMEM (100 µL) were added to the wells and incubated for a further 4 h at 37 °C. The solubilisation solution (100 µL) was added and following overnight incubation, the absorbance was read at 570 nm using a microplate reader (VarioskanFlash, Thermo Scientific, Waltham, MA, USA). For subsequent assays, a nontoxic concentration of each sample was used, which induced greater than 80% cell viability.

2.8. ROS Production

Intracellular formation of ROS was determined via the oxidation-sensitive fluorogenic probe 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA). RAW264.7 cells were plated in 96-well plates (1×10^5 cells/mL, 200 µL/well) in DMEM supplemented with 10% FBS for 24 h and BWSPHs and SGID BWSPHs were added to the wells for a further 24 h at a final concentration of 0.5% (*w*/*v* dw). Cells were then washed with phosphate buffer saline (PBS) and exposed to DCFH-DA prepared in DMEM (20 µM, 200 µL/well) for 40 min at 37 °C in the dark. Intracellular esterases hydrolyse DCFH-DA to nonfluorescent 2′,7′-dichlorodihydrofluorescein (DCFH), which is then oxidised to fluorescent 2′,7′-dichlorofluorescein (DCF) upon reaction with ROS. DCFH-DA was aspirated, and cells were washed with PBS. The cells, except the negative control, were exposed to H_2O_2 (1 mM, 200 µL/well) for 60 min. The positive control was cells treated with H_2O_2. The negative control was cells exposed to DMEM only. Fluorescence of cells was measured at a wavelength of 485 nm followed by excitation at 530 nm every 5 min over the 60 min exposure period (VarioskanFlash, Thermo Scientific). Results were expressed as % of the positive control.

2.9. GSH Content

GSH content was determined in RAW264.7 cells (1×10^5 cells/mL, 200 µL/well). After 24 h incubation at 37 °C, cells were supplemented with BWSPHs and SGID BWSPHs in 6-well plates at a final concentration of 0.5% (*w*/*v* dw) (2 mL/well). Following 24 h, cells that were treated with BWSPHs were incubated in the presence of *t*BOOH prepared in DMEM (1 mM, 2 mL/well) for 3 h at 37 °C. The positive control was cells exposed to *t*BOOH only. The negative control was cells exposed to DMEM alone. The ability of the BWSPHs to protect against a *t*BOOH-induced reduction in cellular GSH was assessed. GSH was determined by the method of [30]. Briefly, cells were resuspended in phosphate EDTA buffer, sonicated (13 mA for 10 s, 3 times) (Soniprep 150, MSE, UK), centrifuged at 14,000 rpm × 30 min at 4 °C to remove cellular debris and supernatant was collected.

The final GSH assay mixture (2 mL) was made up of 100 µL cell supernatant, 1.8 mL sodium phosphate-ethylenediamine tetraacetic acid buffer, and 100 µL o-phthaldialdehyde (1 mg/mL). Fluorescence was determined at a wavelength of 430 nm followed by excitation at 360 nm (VarioskanFlash, Thermo Scientific). GSH content was expressed relative to total cellular protein content, which was calculated by the bicinchoninic acid (BCA) method [31]. Cell lysates (40 µL) were incubated in the presence of a BCA working solution (800 µL) for 1 h in 24-well plates and absorbance was subsequently read at 570 nm, from which the total cellular protein content (mg/mL) was calculated using bovine serum albumin (BSA) as a standard.

2.10. CAT Activity

CAT activity was determined in RAW264.7 cells (1×10^5 cells/mL, 200 µL/well). After 24 h incubation at 37 °C, cells were supplemented with BWSPHs and SGID BWSPHs in 6-well plates at a final concentration of 0.5% (*w*/*v* dw) (2 mL/well). Following 24 h, cells that were treated with BWSPHs were incubated in the presence of H_2O_2 prepared in DMEM (1 mM, 2 mL/well) for 3 h at 37 °C. The positive control was cells exposed to H_2O_2 only. The negative control was cells exposed to DMEM alone. Cells were resuspended in buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA), sonicated (13 mA for 10 s, × 3) (Soniprep 150, MSE), and centrifuged (14,000 rpm × 30 min at 4 °C) to remove cell pellets. Cell supernatant was collected for assay and stored at −80 °C. CAT activity was measured using a Calbiochem CAT Assay Kit (Merck Chemicals Ltd., Nottingham, UK) and performed according to the manufacturer's instructions. One unit of catalase activity was defined as the amount of catalase required to decompose 1 µmol H_2O_2 per minute at pH 7.5 and 25 °C. CAT activity was expressed relative to the total cellular protein content, which was calculated by the BCA method [31]. Cell lysates (40 µL) were incubated in the presence of a BCA working solution (800 µL) for 1 h in 24-well plates and absorbance was subsequently read at 570 nm, from which the total cellular protein content (mg/mL) was calculated using BSA as a standard.

2.11. NO Secretion

NO secretion was assessed in RAW264.7 cells using the Greiss assay as described in [32]. Briefly, cells were seeded in 96-well plates at 1×10^5 cells/mL (200 µL/well) and incubated for 48 h at 37°C. Cells were then simultaneously stimulated using LPS (2 µg/mL prepared in DMEM) and treated with BWSPHs or SGID BWSPHs at a final concentration of 0.5% (*w*/*v* dw) for 24 h at 37 °C. The positive control was cells incubated in the presence of LPS (2 µg/mL prepared in DMEM) alone and the negative control was cells exposed to DMEM alone. The cultured supernatant (50 µL) was plated on a 96-well plate and 50 µL Griess reagent (1:1 of 1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-1-naphtyl-ethylenediamine dichloride in water) was added. Sodium nitrite was used to generate a standard curve (0–100 µM). The mixture was incubated for 20 min at room temperature in darkness and absorbance was read at 550 nm (VarioskanFlash, Thermo Scientific).

2.12. Cytokine Secretion

RAW 264.7 cells (0.2×10^5 cells/mL, 200 µL/well) were seeded in 96-well plates and incubated for 24 h at 37 °C. Cells were then simultaneously stimulated using LPS (0.1 µg/mL prepared in DMEM) and treated with BWSPHs or SGID BWSPHs at a final concentration of 0.5% (*w*/*v* dw) for 24 h at 37 °C. The positive control was cells incubated in the presence of LPS (0.1 µg/mL prepared in DMEM) alone and the negative control was cells exposed to DMEM alone. After incubation, the culture medium was harvested and the secretion of cytokines IL-6 and TNF-α was measured by enzyme-linked immunosorbent assay (ELISA). Cytokine production was determined using eBioscience ELISA kits (Ready-SET-Go kit purchased from eBioscience, Hatfield, UK). Absorbance was measured at 450 nm on a microplate reader (VarioskanFlash, Thermo Scientific) and cytokine production was expressed as a percentage of LPS-stimulated RAW 264.7 cells (positive control). The

ELISA kits allow the detection of IL-6 and TNF-α with a minimum detection limit of 4.0 and 3.7 pg/mL, respectively, and intra-assay variation <6.5%.

2.13. Statistical Analysis

All experimental results of this study are expressed as the mean ± standard error of the mean (SEM) and data are from at least three independent experiments. A one-way analysis of variance (ANOVA) followed by Dunnett's test was used to compare significant differences between sample groups and control groups (Prism 5.0, GraphPad Inc. San Diego, CA, USA). The statistical software programs SPSS (Version 26, IBM Inc., Chicago, IL, USA) was used to perform statistical analyses on the data arising from the ORAC and FRAP analysis and an ANOVA followed by Tukey's and Games–Howell post-hoc tests was used to compare all values. Values before and after SGID were compared using Student's *t*-tests.

3. Results and Discussion

In this study, the in vitro antioxidant and immunomodulatory potential of six protein hydrolysates generated at industrial scale from the low-value underutilised species blue whiting, using different hydrolysis conditions, were assessed. All BWSPHs contained high protein contents ranging from 70.37 ± 0.33–73.60 ± 0.53 g/100 g [26]. Physicochemical data such as DH, molecular mass distribution, RP-UPLC, and free amino acid profiles demonstrated that the variation in hydrolysis conditions resulted in the generation of BWSPHs with distinctly different characteristics [26]. In summary, BW-SPH-A, BW-SPH-B, BW-SPH-E had significantly higher DH values (43.19–45.79%, $p < 0.05$) compared to all other samples with an abundance of low MW peptides, i.e., <1 kDa (69.74–77.86%). BW-SPH-C had the lowest DH value at 27.82% ± 1.11% with 55.55% ± 0.13% peptides <1 kDa [26]. The DH data, molecular mass distribution and RP-UPLC profiles and free amino acid composition show that all BWSPHs were further degraded during SGID [26] reaching similar levels at the end of the intestinal phase (57–65%). Hydrolysate BW-SPH-C which had the lowest DH (27.82% ± 1.11%), showed the highest level of hydrolysis during SGID with a resulting DH of 55.37% ± 1.83%. In vitro GI digestion is useful in predicting the metabolic fate and bioactive potential of food components during in vivo digestion. The SGID protocol employed herein is an example of a static digestion method whereby gastric enzyme pepsin and intestinal enzyme preparation Corolase PP (containing trypsin, chymotrypsin and elastase) were used to mimic gastric and intestinal digestion, respectively. While various protocols for static SGID exist [33], this method was chosen in order to compare the results of this study to previous works published by our group. No zone of inhibition was observed when SGID-treated samples were added on casein agar plates, which confirmed that there was no residual proteolytic activity associated with SGID-treated samples (data not shown).

3.1. Noncellular In Vitro Antioxidant Activity

The ability of food compounds to reduce DPPH radicals is often used as an indicator of their antioxidant potential. The results presented in Table 1 show that all BWSPHs demonstrated DPPH radical scavenging activity with half-maximal inhibitory concentration (IC_{50}) values ranging from 2.10 ± 0.12 to 2.47 ± 0.04 mg/mL, indicating all BWSPHs possess electron-donating abilities. The DPPH radical scavenging data obtained for BWSPHs (Table 1) are comparable with previous studies investigating the DPPH radical scavenging activity of protein hydrolysates from various fish species such as Klunzinger's mullet (IC_{50} = 2.08 ± 0.13 mg/mL), shortfin scad (IC_{50} = 1.89 mg/mL) and yellow-fin tuna waste (IC_{50} = 1.89 mg/mL) [34–36]. However, although BWSPHs possessed varying physicochemical properties [26], no significant differences in DPPH scavenging activity were observed ($p > 0.05$). Published studies have reported a variability in DPPH scavenging activity of BWPH depending on protease employed for hydrolysis [19,20]. BWPH produced with subtilisin, trypsin or a subtilisin-trypsin combination with varying DH (4–12%) inhibited

DPPH by 50% at concentrations ranging from 1.36–2.46 mg protein/mL [19]. Preparation of BWPH with Flavourzyme® 500L resulted in higher DPPH scavenging activity compared with BWPH generated with Protamex® or Savinase®16 L ($p < 0.001$) possibly due to the higher proportion of di- and tripeptides and free amino acids (mainly Leu, Phe + Tyr, and Glu) [20]. Amino acid residues Trp, Phe, Tyr, Cys, and His can reportedly contribute to antioxidant activity [37], however, no relationship was observed between the amino acid composition and the antioxidant activity of BWSPHs in this study.

Table 1. The DPPH radical scavenging activity of blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPHs).

	BW-SPH-A	BW-SPH-B	BW-SPH-C	BW-SPH-D	BW-SPH-E	BW-SPH-F
IC_{50} value (mg dw/mL)	2.10 ± 0.12	2.31 ± 0.13	2.14 ± 0.22	2.34 ± 0.30	2.11 ± 0.15	2.47 ± 0.04

Values are expressed as mean ± SEM of three independent experiments. DPPH: 2,2-diphenyl-1-picryl-hydrazyl-hydrate. IC_{50}: half-maximum inhibitory concentration. No significant difference was observed ($p > 0.05$).

The ORAC and FRAP activity of the BWSPHs and their simulated GI digests are presented in Table 2. Prior to SGID, ORAC values of 330.79 ± 9.76 to 393.32 ± 3.23 µmol TE/g dw were obtained for BWSPHs with BW-SPH-C and BW-SPH-D mediating the lowest and highest activity, respectively. A significant increase ($p < 0.05$) in ORAC activity was observed with samples BW-SPH-A-GI, -B-GI, -D-GI, and -F-GI following SGID (Table 2). As previously stated, the DH data, molecular mass distribution, and RP-UPLC profiles indicate that all BWSPHs were further hydrolysed during SGID, which would indicate that gut enzymes liberated peptides with superior ORAC activity from precursor peptides. Interestingly, the ORAC activity exhibited by BW-SPH-C-GI, the hydrolysate which was hydrolysed by the greatest extent during SGID (from 27.82 ± 1.11 pre-SGID to 55.37 ± 1.83 post-SGID [26], was similar to that prior to in vitro digestion. It is possible that bioactive peptides were not further hydrolysed by SGID or that peptides were further hydrolysed with the loss and gain of individual bioactivities, resulting in no overall change to the total bioactivity.

Table 2. In vitro ORAC and FRAP antioxidant activity of blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPHs) pre- and post-simulated gastrointestinal digestion (SGID).

Sample Code	ORAC Value (µmol TE/g dw)	FRAP Value (µmol TE/g dw)
BW-SPH-A	387.65 ± 9.97 ^{ab}	7.41 ± 0.15 ^{bc}
BW-SPH-A-GI	459.73 ± 8.72 ^{A*}	5.60 ± 0.03 ^{A*}
BW-SPH-B	350.65 ± 10.35 ^{bc}	7.25 ± 0.22 ^{c}
BW-SPH-B-GI	414.20 ± 4.68 *	5.06 ± 0.04 ^{B*}
BW-SPH-C	330.79 ± 9.76 ^{c}	7.67 ± 0.14 ^{bc}
BW-SPH-C-GI	348.49 ± 4.89 ^{C}	4.64 ± 0.09 ^{C*}
BW-SPH-D	393.32 ± 3.32 ^{a}	8.45 ± 0.08 ^{a}
BW-SPH-D-GI	409.00 ± 2.98 ^{B*}	4.75 ± 0.06 ^{BC*}
BW-SPH-E	365.88 ± 8.27 ^{abc}	7.57 ± 0.10 ^{bc}
BW-SPH-E-GI	386.50 ± 3.23 ^{B}	4.74 ± 0.09 ^{BC*}
BW-SPH-F	345.78 ± 4.26 ^{c}	8.03 ± 0.23 ^{ab}
BW-SPH-F-GI	385.01 ± 9.55 ^{B*}	4.48 ± 0.08 ^{C*}

Values are expressed as mean ± SEM of three independent experiments. The samples labelled -GI refer to the samples post-SGID. ORAC: oxygen radical absorbance capacity, FRAP: ferric reducing antioxidant power, TE: Trolox equivalents, dw: dry weight. Different lowercase letters (a,b,c) within a column indicate a significant difference between BWSPHs samples pre-SGID at $p < 0.05$. Different capital letters (A,B,C) within a column indicate a significant difference between BWSPHs post-SGID at $p < 0.05$. * indicates a significant difference at $p < 0.05$ between pre- and post-SGID values.

FRAP values ranging from 7.25 ± 0.22 to 8.45 ± 0.08 µmol TE/g dw were obtained for BWSPHs prior to SGID with BW-SPH-B and BW-SPH-D mediating the lowest and highest activity, respectively (Table 2). The FRAP activity of all samples was significantly decreased following SGID (Table 2). Similar findings were reported for a whey hydrolysate prepared with Alcalase™, which demonstrated reduced FRAP and ABTS inhibition and increased ORAC activity post-SGID compared to the undigested Alcalase-hydrolysate ($p < 0.05$) [38]. This is not unexpected as the ORAC and FRAP assays are based on different chemical reactions with the ORAC assay measuring the scavenging capacity of test compounds against peroxyl radicals through hydrogen atom transfer (HAT), whereas FRAP is categorized as an electron transfer (ET)-based, nonradical method. Due to the fact that SGID samples demonstrated increased ORAC values but reduced FRAP values compared with the corresponding undigested samples, it is possible that SGID samples scavenge radicals through HAT. The radical scavenging activity of BWSPHs and SGID BWSPHs was also assessed in a cell model (Table 3) which provides a better biological predictor for BWSPHs antioxidant ability.

Table 3. Cellular antioxidant activity of blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPHs) pre- and post-simulated gastrointestinal digestion (SGID).

Sample Code (0.5% *w/v* dw)	GSH Concentration (% tBOOH)	CAT Activity (% H_2O_2)	ROS Production (% H_2O_2)
Control	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
BW-SPH-A	140.3 ± 14.1 *	137.6 ± 7.4 **	86.4 ± 1.8 *
BW-SPH-A-GI	138.5 ± 12.7 *	133.2 ± 4.6	81.0 ± 3.7 *
BW-SPH-B	79.7 ± 11.1	123 ± 1.2	89.9 ± 2.1
BW-SPH-B-GI	124.8 ± 14.4 #	97.8 ± 4.2	90.7 ± 5.7
BW-SPH-C	102.3 ± 8.0	125.7 ± 3.1	89.7 ± 3.2
BW-SPH-C-GI	94.3 ± 13.2	138.3 ± 16.9	90.2 ± 3.0
BW-SPH-D	82.5 ± 12.2	125.6 ± 9.1	92.4 ± 3.0
BW-SPH-D-GI	108.7 ± 13.8	116.6 ± 9.3	104.3 ± 7.6
BW-SPH-E	108.6 ± 8.8	110.6 ± 11.6	94.7 ± 7.3
BW-SPH-E-GI	113.0 ± 9.4	146.4 ± 11.4 *	91.7 ± 3.5
BW-SPH-F	97.2 ± 13.4	128.4 ± 10.4	104.1 ± 6.5
BW-SPH-F-GI	113.7 ± 4.5	128.5 ± 13.8	96.1 ± 4.0

Antioxidant potential of BWSPHs (BW-SPH-A–F) and SGID BWSPHs (BW-SPH-A-GI—F-GI) at 0.5% (*w/v* dw (dry weight)) as assessed by their ability to increase glutathione (GSH) concentration in tertbutyl hydroperoxide (tBOOH)-treated RAW264.7 cells, increase catalase (CAT) activity in hydrogen peroxide (H_2O_2)-treated RAW264.7 cells and reduce reactive oxygen species (ROS) production in H_2O_2-treated RAW264.7 cells. For GSH and CAT assays, RAW264.7 cells (1×10^5 cells/mL, 2 mL/well) were exposed to BWSPHs and SGID BWSPHs for 24 h, followed by a tBOOH (1 mM, 3 h) or H_2O_2 challenge (1 mM, 3 h), respectively. ROS production was measured in RAW264.7 cells (1×10^5 cells/mL, 200 mL/well) exposed to BWSPHs and SGID BWSPHs for 24 h, followed by a H_2O_2 challenge (1 mM, 1 h). Values were expressed as a percentage relative to positive control (100%). Values represent the mean ± SEM of at least three independent experiments. Statistical significance between samples and the control was measured using ANOVA followed by Dunnett's test. * and ** denote statistically significant differences between samples and the control at $p < 0.05$ and $p < 0.01$, respectively. # indicates a significant difference at $p < 0.05$ between pre- and post-SGID values measured by *t*-test.

The peptide length and amino acid composition of peptides within the protein hydrolysate, which ultimately influence hydrolysate bioactivity, are dependent on DH. In this case, FRAP results indicate that bioactivity was attenuated during SGID. A correlation between a reduced DPPH scavenging activity and a reducing power of fish protein hydrolysate with increasing DH has been previously observed [39,40]. The highest activity for both ORAC (459.73 ± 8.72 µmol TE/g dw) and FRAP (5.60 ± 0.03 µmol TE/g dw) activity following SGID was observed with sample BW-SPH-A-GI (Table 2). The ORAC activity observed herein for the BWSPHs was slightly lower and higher than that reported for protein hydrolysates derived from salmon muscle (587.41 ± 26.50–882.58 ± 105.72 µmol TE/g sample) and mussel meat (66.40 ± 2.27–121.56 ± 3.96 µmol TE/g sample), respectively [41,42].

3.2. Effect of BWSPHs on RAW264.7 Cell Viability

The effect of 24 h incubation with six BWSPHs (BW-SPH-A to -F) and six SGID BWSPHs (BW-SPH-A-GI to -F-GI) with final concentrations ranging from 0–1.0% (*w*/*v* dw), on the viability of RAW264.7 cells was investigated. BW-SPH-A (0.05% (*w*/*v* dw)) was the only BWSPH to induce a significant proliferative effect on RAW264.7 cells ($p < 0.05$) (Supplementary Table S1). In contrast, BW-SPH-D (0.9% (*w*/*v* dw)) and BW-SPH-E (1.0% (*w*/*v* dw)) reduced the viability of RAW264.7 cells significantly compared with media alone (100%) ($p < 0.05$). SGID samples (up to 1.0% (*w*/*v* dw)) did not significantly alter the viability of RAW264.7 cells compared to the control ($p > 0.05$) (Supplementary Table S2). A BWSPHs concentration of 0.5% (*w*/*v* dw) was chosen for future cell-based experiments which induced greater than 80% cell viability. The same concentration was chosen for SGID BWSPHs to enable direct comparison between BWSPHs and simulated GI digests.

3.3. Cellular Antioxidant Activity

To further evaluate the antioxidant potential of BWSPHs, their ability to affect the endogenous antioxidant defence systems was assessed. The RAW264.7 cell line was selected due to its ability to generate intracellular oxidants as well as express enzymatic and nonenzymatic antioxidants [43]. Table 3 details the effects of BWSPHs and SGID BWSPHs on intracellular GSH concentration, CAT activity, and ROS production. Oxidative stress was induced via treatment with known oxidants *t*BOOH or H_2O_2. The organic peroxide, *t*BOOH, was chosen as the oxidative stressor to reduce GSH levels as H_2O_2 did not significantly reduce GSH concentration in RAW264.7 cells (data not shown). Unlike endogenous H_2O_2, the xenobiotic *t*BOOH, related to industrial air pollution, has no specific detoxifying element in the cell. It has been proposed that the glutathione system catalyses the decomposition of *t*BOOH to tert-butyl alcohol and glutathione disulphide, resulting in depletion of GSH [44–46].

Although BW-SPH-B, -C, -D, -E, and -F did not protect against the suppression of GSH in *t*BOOH-challenged cells, treatment with BW-SPH-A did increase GSH concentration significantly compared with the control ($p < 0.05$) (Table 3). Similarly, BW-SPH-A was the only hydrolysate to increase CAT activity significantly in H_2O_2-challenged RAW264.7 cells compared with the H_2O_2 control ($p < 0.01$) (Table 3). Similar to the BWSPHs tested in this study, which contained a high content of low MW peptides, large yellow croaker (*Pseudosciaena crocea*) protein hydrolysate (MW < 3 kDa) exhibited $O_2^{-}\cdot$ scavenging activity and DPPH scavenging activity in vitro and also effectively and dose-dependently (50–300 µg/mL) increased the activity of antioxidant enzymes GSH-Px, SOD, and CAT in H_2O_2-induced oxidative stress in HepG2 cells ($p < 0.05$) [3]. In addition, numerous fish-derived peptides have demonstrated ability to modulate oxidative stress pathways in vitro [4,5,9,47,48].

The simulated GI digest of BW-SPH-A also significantly increased GSH concentration in oxidatively stressed RAW264.7 cells compared to treated controls ($p < 0.05$), indicating that antioxidant peptides and/or antioxidant free amino acids were released from parent peptides during digestion (Table 3). SGID did not alter the ability of BW-SPH-A to increase GSH levels in oxidatively stressed RAW264.7 cells (Table 3) but did inhibit its ability to promote CAT activity (Table 3) indicating different components of the hydrolysate mediate GSH and CAT. In contrast, BW-SPH-E had no effect on CAT activity, whereas BW-SPH-E-GI did indeed enhance CAT activity compared with the H_2O_2 control ($p < 0.05$), possibly due to the release of encrypted antioxidant peptides/free amino acids during SGID. Interestingly, BW-SPH-A and BW-SPH-E were observed to have high DH values (43.19 ± 2.16 and 42.97 ± 3.30, respectively) and a high quantity of components <1 kDa (77.86 ± 0.16 and 69.74 ± 0.13, respectively) [26], therefore it is possible that the generation of short-chain peptides and free amino acids influenced subsequent antioxidant potential. Although BW-SPH-B and its simulated GI digest, BW-SPH-B-GI, did not alter GSH concentration or CAT activity significantly compared with controls, there was a significant difference between activities pre- and post-SGID ($p < 0.05$).

The fluorescence indicator DCFH-DA was used to detect H_2O_2-induced ROS production in RAW264.7 cells, which were pre-incubated with BWSPHs and SGID BWSPHs. RAW264.7 cells treated with H_2O_2 for 60 min significantly increased ROS generation ($p < 0.001$) (data not shown). Interestingly, cells pre-incubated with either BW-SPH-A or its digest BW-SPH-A-GI (0.5% (w/v dw)) significantly attenuated the production of ROS in H_2O_2-challenged RAW264.7 cells ($p < 0.05$). No significant differences in ROS production were observed between the hydrolysates and their corresponding simulated GI digests ($p > 0.05$). In contrast, ref. [49] reported that SGID soybean protein hydrolysate prepared with gastric proteases enhanced ROS inhibitory activity compared to the undigested soybean protein hydrolysate in H_2O_2 (1 mM, 6 h)-stimulated caco-2 cells. The soybean protein fraction obtained post-SGID had a higher DH and a higher content of short chain peptides than the pre-SGID fraction, which may have been responsible for its superior ROS reducing ability, possibly through enhanced cellular absorption. Subsequent isolation and characterisation of antioxidant peptides revealed each peptide sequence contained at least one of the following amino acid residues: Pro, Asp, Leu, Val, Arg, and His. The presence of hydrophobic amino acids is associated with high antioxidant activity through a radical scavenging mechanism. Interestingly, SGID BWSPHs, BW-SPH-A-GI, and BW-SPH-E-GI contained the highest levels of hydrophobic Met (1.01% and 1.03% w/w, respectively) and Phe (1.63% and 1.52% w/w, respectively) [26].

Arithmetical ranking of BWSPHs and SGID BWSPHs deemed BW-SPH-B, -C-GI and D-GI to be the lowest ranking of the hydrolysates tested for overall antioxidant potential, whereas BW-SPH-A and -A-GI were the top rankers (Supplementary Table S3). Results obtained for SGID BWSPHs demonstrate that BW-SPH-A and BW-SPH-E may have potential applications as antioxidant agents in a functional food offering.

3.4. Cellular Immunomodulatory Activity

The activation of macrophages is an important part of initiating defensive reactions ensuring effective innate and adaptive immunity. As observed in Figure 1, exposure of RAW264.7 cells to the endotoxin LPS for 24 h upregulated generation of the inflammatory mediator NO, compared with the untreated control ($p < 0.0001$). Figure 1 also demonstrates that all BWSPHs and SGID BWSPHs (0.5% (w/v dw)) increased NO production in LPS-induced RAW264.7 cells compared with the LPS control ($p < 0.05$). The NO producing ability of BW-SPH-F was the only hydrolysate which was significantly reduced by SGID ($p < 0.05$).

Upon exposure to invasive species, pathogen-recognition receptors (PRRs) of immune cells stimulate numerous signalling cascades resulting in the upregulation of inflammatory cytokines such as TNF-α and IL-6. TNF-α controls inflammatory response through upregulation of proinflammatory cytokines IL-6 and IL-1β as well as through the upregulation of endothelial cell adhesion molecules inducing leukocyte extravasation [50,51]. IL-6 plays an important role in immunity through terminal differentiation of B cells into immunoglobulin-secreting cells as well as regulating the balance between regulatory T cells and Th (T helper) 17 cells [52,53]. No signal for IL-6 or TNF-α protein expression was detected in untreated controls indicating DMEM and FBS were endotoxin free (data not shown). In addition to increasing NO production, hydrolysates BW-SPH-A, -B, -C and -F also increased IL-6 production in LPS-stimulated cells ($p < 0.05$), with hydrolysates BW-SPH-C, -D, and E increasing levels of TNF-α compared with the LPS control ($p < 0.05$). This immunostimulation is likely to be lost as the hydrolysates transit the gut, as SGID data show that BW-SPH-F-GI was the only digest to enhance IL-6 and TNF-α cytokine levels in LPS-activated macrophage ($p < 0.05$) (Figures 2 and 3) compared with the LPS control, indicating the release of immunostimulant peptides from BW-SPH-F during the digestion process. Arithmetical ranking demonstrated that BW-SPH-F and BW-SPH-F-GI were the highest-ranking hydrolysates of the BWSPHs and SGID BWSPHs tested with respect to individual immunomodulatory parameters (Supplementary Table S4). No relationship

between free amino acid content [26] and the immunomodulatory effect of BW-SPH-F-GI was observed, suggesting a role for peptides in its bioactivity.

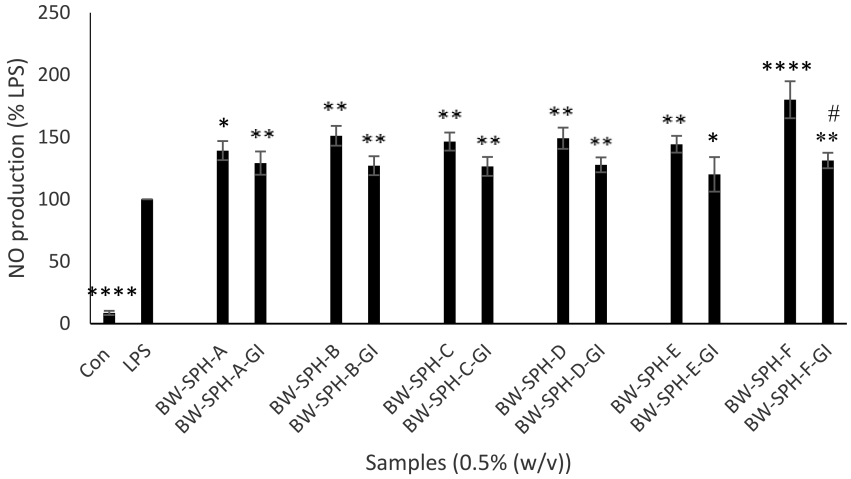

Figure 1. Effects of blue whiting soluble protein hydrolysates (BWSPHs) (BW-SPH-A–F) and simulated gastrointestinal digested (SGID) BWSPHs (BW-SPH-A-GI—F-GI) at 0.5% (*w*/*v* dry weight) on nitric oxide (NO) production in lipopolysaccharide (LPS)-challenged RAW264.7 mouse macrophage. RAW264.7 cells (1×10^5 cells/mL, 200 µL/well) were simultaneously exposed to 2 µg/mL LPS and BWSPHs or SGID BWSPHs for 24 h. NO was measured using Griess assay and values were expressed as a percentage relative to positive control (Con), cells treated with 2 µg/mL LPS alone (100% NO secretion). Values represent the mean ± SEM of three independent experiments. Significance was measured using ANOVA followed by Dunnett's test. *, **, and **** denote statistically significant differences between sample and LPS control at $p < 0.05$, $p < 0.01$, and $p < 0.0001$, respectively. # indicates a significant difference at $p < 0.05$ between pre- and post-SGID values measured by *t*-test.

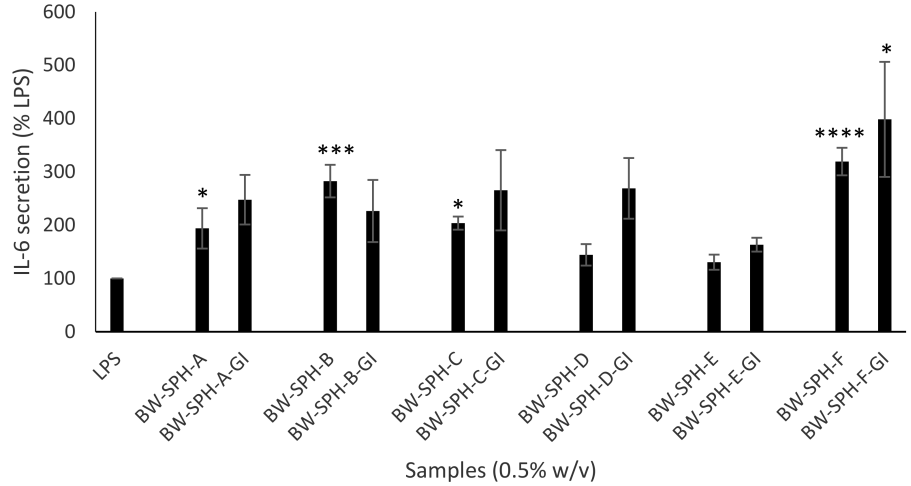

Figure 2. The effect of blue whiting soluble protein hydrolysates (BWSPHs) (BW-SPH-A–F) and simulated gastrointestinal digested (SGID) BWSPHs (BW-SPH-A-GI—F-GI) at 0.5% (*w*/*v* dry weight) on IL-6 production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. RAW264.7 cells (0.2×10^5 cells/mL, 200 µL/well) were simultaneously exposed to 0.1 µg/mL LPS and BWSPHs or SGID BWSPHs for 24 h. Values were expressed as a percentage relative to positive control, cells treated with 0.1 µg/mL LPS alone (100% IL-6 secretion). Values represent the mean ± SEM of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. *, ***, and **** denote statistically significant difference in IL-6 production between sample and LPS control at $p < 0.05$, $p < 0.001$, and $p < 0.0001$, respectively.

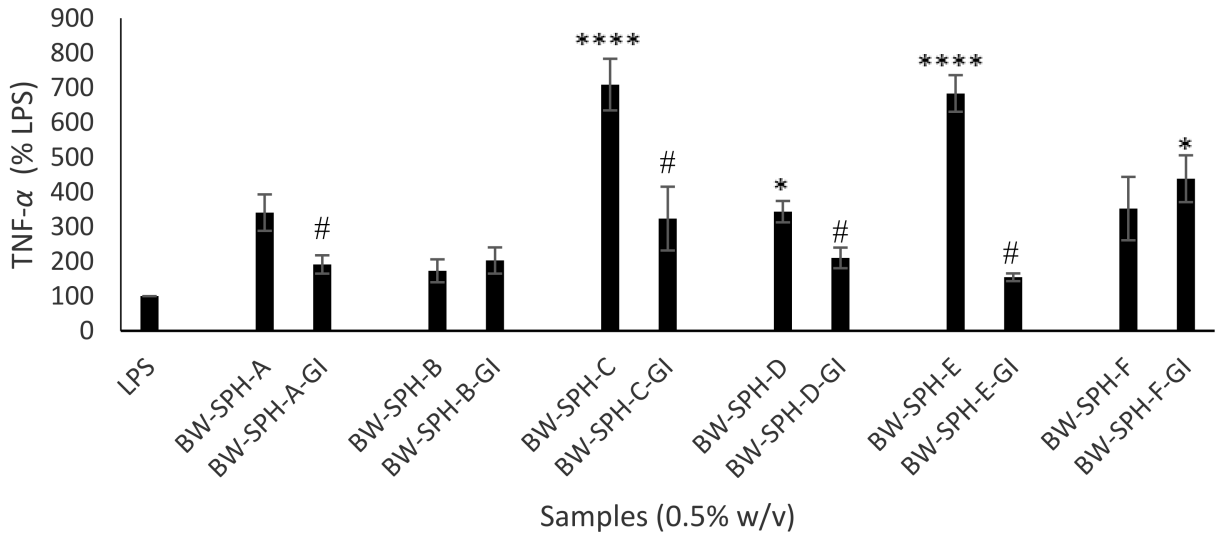

Figure 3. The effect of blue whiting soluble protein hydrolysates (BWSPHs) (BW-SPH-A–F) and simulated gastrointestinal digested (SGID) BWSPHs (BW-SPH-A-GI—F-GI) at 0.5% (*w*/*v* dry weight) on TNF-*α* production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. RAW264.7 cells (0.2×10^5 cells/mL, 200 µL/well) were simultaneously exposed to 0.1 µg/mL LPS and BWSPHs or SGID BWSPHs for 24 h. Values were expressed as a percentage relative to positive control, cells treated with 0.1 µg/mL LPS alone (100% TNF- *α* secretion). Values represent the mean ± SEM of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * and **** denote statistically significant difference in TNF-*α* production between sample and LPS control at $p < 0.05$ and $p < 0.0001$, respectively. # indicates a significant difference at $p < 0.05$ between pre- and post-SGID values measured by *t*-test.

In a similar cell model, exposure of wheatgrass to LPS-stimulated THP-1 monocytes increased NO production along with increasing levels of inflammatory cytokines TNF-α, IL-6, IL-1β compared with the LPS control ($p < 0.05$) [54]. Inflammatory compounds have been identified in numerous food components including fatty acids [55], polysaccharides [56], and protein sources [57–59]. Although the majority of immunomodulatory fish protein hydrolysates have been shown to mitigate proinflammatory mediators and cytokines, proinflammatory hydrolysates have been generated from giant croaker (*Nibea Japonica*) and tilapia fish with the ability to increase inflammation mediators and cytokines in cellular systems [16,17]. Intragastric administration of marine oligopeptide preparation from chum salmon (0, 0.22, 0.45, 1.35 g/kg bodyweight (BW), 4 weeks) did not activate macrophage cells, however innate and adaptive immunities were enhanced via the promotion of natural killer cell activity and stimulation of Th cells, thereby increasing the secretion of Th1 and Th2 cytokines [60]. In addition, a fermented fish protein concentration prepared from pacific whiting, which has been shown to increase immunoglobulin (Ig)A+ cells, secretory-IgA (S-IgA), and cytokines IL-4, IL-6, IL-10, IFNγ, and TNF-*α* upon oral administration to BALB/c mice (0.30 mg/mL, 7 days), is now commercially available as Seacure® [61]. The findings of the present study demonstrated that BW-SPH-F may act as a nonspecific immunostimulant (i.e., not antigen specific) upon oral administration, with the potential to stimulate immune cells for therapeutic use in chronic infections, immunodeficiency, autoimmunity, and neoplastic diseases. The production of inflammatory mediators and cytokines must be regulated carefully, however, as excess secretion may negatively impact human health [62].

While previous studies have reported fish protein hydrolysates with antioxidant and anti-inflammatory properties [11,63], we have identified hydrolysate BW-SPH-A herein, which exhibited antioxidant activities in oxidatively stressed RAW264.7 cells as well as proinflammatory effects in LPS-activated RAW264.7 cells. A study [64] concluded that tripeptide glutathione, which decreased LPS-induced ROS generation, also stimulated the production of NO and proinflammatory cytokines in RAW264.7 cells via nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK), and Notch signal pathways. Simi-

larly, bioavailable whey peptides obtained post-SGID inhibited free radicals in oxidatively stressed muscle and liver cells and increased the secretion of proinflammatory cytokine IL-1β from LPS-stimulated THP-1 macrophages [65]. However, in this study, the immunostimulating activity of BW-SPH-A was lost post-SGID.

4. Conclusions

The results presented herein are, to the best of our knowledge, the first evidence of a protein hydrolysate purified from blue whiting exhibiting antioxidant or immunomodulatory potential in a cellular model. Hydrolysate BW-SPH-A, which enhanced cellular redox status pre- and post-SGID, may have potential as an effective natural antioxidant. Hydrolysate BW-SPH-F, which increased the production of proinflammatory mediators and cytokines, also maintained its bioactivity post-SGID; therefore, it may have application as an immunostimulant with potential to improve the quality of life of immunosuppressed patients. The extraction and identification of biofunctional protein hydrolysates presents an opportunity to increase the value of low-value blue whiting through their applications as high-value functional food ingredients. Future experimentation should involve the identification and characterization of peptides responsible for the antioxidant and immunomodulatory activities exhibited by BW-SPH-A and BW-SPH-F, respectively. In vivo interventional studies should be used to assess the biofunctional activities of BWSPHs, as well as to examine the capacity of gut digestive enzymes to functionalise hydrolysates in addition to the study of potential bioactive peptide protection mechanisms, such as microencapsulation, for the fractions that lost activity during simulated gut transit.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11209762/s1, Figure S1: The antioxidant activity of blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPH) (1.5–4 mg/mL) on 2,2-diphenyl-2-picryl-hydrazyl (DPPH) free radicals. Table S1: The effects of blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPH) on the viability of murine RAW264.7 cells. Table S2: The effects of simulated gastrointestinal digested (SGID) blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPH) on the viability of murine RAW264.7 cells. Table S3. Arithmetical ranks of blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPH) pre- and post-simulated gastrointestinal digestion (SGID) with respect to individual antioxidant parameters. Supplementary Table S4. Arithmetical ranks of blue whiting (*Micromesistius poutassou*) soluble protein hydrolysates (BWSPH) pre- and post-simulated gastrointestinal digestion (SGID) with respect to individual immunomodulatory parameters.

Author Contributions: S.H. and N.M.O. conceptualized the research. S.G. and J.W. provided the BWSPH samples. P.A.H.-R. prepared the SGID digests and determined the FRAP and ORAC activity of all BWSPHs. S.H. determined the DPPH activity of BWSPHs and performed all cell-based assays. S.H. drafted the manuscript and P.A.H.-R., S.G., J.W., L.G., R.J.F. and N.M.O. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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