Wiley
Aquaculture Research
Volume 2025, Article ID 6013553, 11 pages
https://doi.org/10.1155/are/6013553

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Research Article

Evaluation of the Immunomodulatory Effect of Fish Protein Hydrolysates Obtained From Atlantic Salmon (*Salmo salar*) by-Products Using *Dicentrarchus labrax* Brain Cell Line

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Received 23 December 2024; Revised 15 July 2025; Accepted 16 July 2025

This study investigates the immunomodulatory effects of fish protein hydrolysates (FPHs) derived from Atlantic salmon (*Salmo salar*) by-products using the *Dicentrarchus labrax* brain (DLB-1) cell line. Utilizing aquaculture by-products for FPH production offers significant potential in aquaculture, providing both economic benefits and a reduction on environmental impact. FPHs contain bioactive peptides with several biological properties, including health-promoting, antioxidant, anti-inflammatory, and antimicrobial activities. This study focused on the immunological properties of three FPHs, namely SS1, SS4, and SS5, obtained from salmon skin by-products using enzymatic hydrolysis with trypsin, α chymotrypsin, and bromelain, respectively. Cytotoxicity assays showed that SS5 hydrolysate exhibited no toxic effects on DLB-1 cells, even at high concentrations (up to 80 μg/mL), unlike SS1 and SS4 that showed a 50% cytotoxic concentration (CC_{50}) of 7.0 and 11.8 μg/mL, respectively. Gene expression analysis revealed that SS1, SS4, and SS5 hydrolysates significantly upregulated the proinflammatory gene *IL-1β* ($p < 0.05$), especially after LPS stimulation, indicating their potential to activate macrophages and modulate immune responses after bacterial infections. Interestingly, SS5 also significantly upregulated the anti-inflammatory gene *IL-10* when treated with LPS ($p < 0.05$), suggesting its ability to regulate inflammation and balance immune responses. These results highlight the role of hydrolysis conditions, such as enzyme selection and peptide molecular weight, in determining the bioactivity of FPH. Moreover, the study suggests that smaller peptides with mass >1.0 and <2.0 kDa, particularly those produced with the bromelain enzyme in SS5, exhibit enhanced anti-inflammatory properties. This research supports the use of FPH in promoting fish health by improving immune responses, and it contributes to sustainable aquaculture practices by transforming waste into valuable bioactive compounds, offering insights for future applications in functional feeds.

Keywords: aquaculture; bioactive peptides; DLB-1 cell line; fish protein hydrolysates; immunomodulation; *in vitro* model; salmon by-products

1. Introduction

Fish cell lines are becoming an increasingly important research resource, both to gain basic knowledge and to obtain useful tools for the aquaculture sector. Furthermore, the use of cell lines can greatly contribute to reducing the use of laboratory animals, accomplishing the 3Rs (reduction, refinement, and replacement) principles [1]. Ethical issues associated with

animal welfare and the European directive for the protection of animals used for scientific purposes (Directive 2010/63/EU) increase the interest in this research approach. Accordingly, fish cell lines represent a simple, effective option increasingly used to refine the use of animal models for several research sectors, such as toxicology, risk assessment, and immunity applications [2, 3]. In recent years, marine continuous fish cell lines have been developed [1, 4–6] to support research on molecular and physiological responses to stressors, depicting a valuable tool to study the effects and the molecular mechanism of action of emerging contaminants and accordingly contributing to reduce the need for *in vivo* laboratory studies [1, 3, 7, 8]. In parallel with advancements in fish cell line-based models, the attention on sustainability of aquaculture is increasing, particularly through the recovery and reuse of by-products generated during farming and processing activities. This aligns with the growing need to develop circular economy strategies and reduce the ecological impact of aquaculture. Indeed, aquaculture is the fastest-growing food production sector in the world, sustaining the increasing protein demand for the human population [9]. A large volume of waste is generated as a result of the biological activity of the cultured animals and during the processing of the resulting material [10]. Therefore, their further utilization can significantly reduce the impact on the environment generated by their release [11]. For example, they could be used for fish feed formulations in order to reduce wild fish captures to obtain protein and lipids as ingredients. Fish wastes are rich in resources, but they are still very poorly explored and characterized, so further research needs to be in practice to exploit their potential [12].

By-products originated by aquaculture companies, include skin, fins, head, trimmings, viscera, frames, and roe [13]. Most of the processing by-products are transformed into low-value products, such as animal feed, fish meal (FM), and fertilizer [14], but actually, these protein-rich waste materials have the potential to be converted into more valuable and bioavailable nutritional food products, such as fish protein hydrolysate (FPH) [15, 16]. Besides, one significant step towards the circular economy is represented by the potential to use the fish leftover and turn them into high-value products [12]. Thus, various physiological benefits, including antioxidant, antihypertensive, antimicrobial, immunomodulatory, and anticancer activities, have been already shown by peptides derived from FPH [16–18]. In recent years, increasing attention has been directed toward the use of FPHs in animal feed formulations. This approach not only contributes to lowering production costs but also helps reduce the environmental footprint of aquaculture activities [19]. Among the valorization strategies, the enzymatic hydrolysis of food proteins has emerged as a promising method for generating bioactive peptides with potential health-promoting properties, including anti-inflammatory effects [20]. Indeed, FPHs are gaining interest not only for their nutritional value but also for their possible bioactive functions. In particular, the potential immunostimulatory role of FPHs is particularly relevant. Enhancing the innate immune response of farmed fish through dietary immunostimulants has gained widespread interest as a strategy to improve resistance to disease and stress [21]. Nevertheless, identifying immune-boosting supplements that are affordable, nutritionally adequate, promote growth, and are readily accessible remains a significant challenge in aquaculture. Research into the biological effects of FPHs has incorporated *in vitro* approaches, alongside traditional *in vivo* experiments, to investigate their functional properties. In particular, cell lines derived from animal tissues are being used as alternative models to assess the potential bioactivity of FPHs. These systems provide an ethical and efficient platform for studying immunomodulatory activities and assessing the bioactivity of hydrolysates, with reduced reliance on live animal testing. However, in the aquaculture sector, the use of fish-derived cell lines—such as DLB-1—for screening the bioactive properties of FPHs remains limited, and their potential is still largely underexploited.

Nowadays, the Atlantic salmon (*Salmo salar*) is one of the leading aquaculture species in Europe, representing a significant resource in terms of production and economic value. A huge amount of waste, consisting mainly of bones, head, and offal, is produced from the salmon industry, reaching up to 40% of the total weight of the animal production [12]. About south Europe, European sea bass (*Dicentrarchus labrax*) is one of the most farmed species in the Mediterranean area and a crucial one for the Mediterranean aquaculture industry [1]. European production had reached 208,197 t and other Mediterranean countries, such as Egypt (24,498 t) and Tunisia (3500 t) undertake significant production of sea bass [22]. Despite its economic relevance, only a very limited number of cell lines have been developed from *D. labrax*, and among them, the *Dicentrarchus labrax* brain (DLB-1) cell line is currently the only one available [1, 23]. In particular the DLB-1 cell line was suitable for transcriptomic analysis of several genes related to immunity [24]; however, to date, DLB-1 has never been applied to investigate the immunomodulatory effects or the safety of dietary ingredients, such as FPH. Given the importance of identifying safe and functionally effective feed ingredients in aquaculture, and the lack of available *in vitro* models for European seabass, this study aims to evaluate the immunological activity and cytotoxicity of FPHs derived from Atlantic salmon by-products using the DLB-1 cell line. In this context, the present research proposes an *in vitro* method based on fish cell cultures to explore the possible immunomodulatory effects of FPHs. This approach offers a practical and ethical tool to screen the bioactivity of FPHs, supporting their potential application in aquaculture sector.

2. Materials and Methods

2.1. DLB-1 Cell Line. The immunomodulatory effect of FPHs was assessed on DLB-1 cell line derived from the European sea bass brain [1]. Continuous DLB-1 was established by Morcillo et al. [1] and kindly provided by Prof. Cuesta of the Immunobiology for Aquaculture Group of Murcia University. Cell monolayers were grown in culture medium composed by L-15 Leibovitz medium (Gibco) containing 0.16% NaCl (Merck), 7.5% foetal bovine serum (FBS, Gibco), 200 mM glutamine, penicillin (100 IU/mL, Gibco), and streptomycin (100 μg/mL, Gibco) at 25°C, and subcultured by trypsinization every week.

2.2. Hydrolysates Production. Salmon skin by-products were received frozen from Nutrimar AS (Norway) and stored at −80°C until use. Thawed skin sample were cut into small pieces before grinding them in a meat grinder for homogenized tissue samples. To 3 g of homogenized tissue samples, 5 mL of extraction buffer was added. Proteins were extracted using four different extraction buffers (SDS, RIPA, Tris lysis, and Urea buffer, Merck) to maximize the protein concentration from the tissue samples. Samples were subjected to sonication for 30 s except for the samples with Urea buffer which were vortexed for 1 min. All samples were left on ice for 15 min (except samples with SDS buffer were incubated at 95°C for 3 min) before centrifuging at 14.000× *g* for 15 min at 4°C. The supernatant from the different extraction methods were combined for further use. Filter aided sample preparation (FASP) was performed to clean the samples from impurities and exchange buffer.

Samples were further subjected to methanol/chloroform precipitation extraction adding 400 µL of methanol (Merck), 100 µL of chloroform (Merck), and 300 µL of water to 100 µL of sample and mixed well. The sample was centrifuged at 14.000 × *g* for 2 min. The top aqueous layer was carefully removed without disturbing the interface protein layer. Then, 400 µL of methanol was added to the sample and mixed well. The sample was centrifuged at 14.000 × *g* for 3 min. The supernatant was discarded, then the pellet was vacuum dried. All the protein pellets were resolubilized in 20 mM Triethylammonium bicarbonate buffer (TEAB)/7 M Urea (Merck). FASP was performed using Amicon Ultra 0.5 mL centrifugal filters with 10 kDa MWCO (Merck Millipore) to concentrate and exchange buffer. Protein concentration was measured using Pierce BCA protein assay (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin (BSA, Gibco) as standard. The samples were frozen at −20°C until enzyme digestion.

The enzymes used for hydrolysis included trypsin (Promega, Madison, USA), alpha-chymotrypsin (Promega, Madison, USA) and bromelain (Promega, Madison, USA). The samples were treated with 20 µL of 20 mM dithiothreitol (DTT, Merck) for 3 min at 37°C, and 15 µL of 20 mM iodoacetamide (IAA, Merck) in dark at RT for 1 h. Then, the sample was retreated with 10 µL of 20 mM DTT for neutralizing the remaining IAA and exchanged to respective buffers using FASP. For sample SS1, trypsin was employed at an enzyme-to-substrate (E/S) ratio of 1:100 in 50 mM ammonium bicarbonate/TEAB buffer (Merck), at 37 °C and pH 7.8. SS4 was hydrolyzed using α-chymotrypsin under a similar E/S ratio (1:100) in 50 mM ammonium bicarbonate buffer (Merck), at 25°C and pH 7.6. In the case of SS5, bromelain was used at a higher E/S ratio of 1:20 in 0.5 M sodium acetate buffer (Merck), with the reaction conducted at 25°C and pH 4.5. All hydrolysis reactions were carried out overnight to ensure adequate peptide release. After treatment, the enzyme was inactivated by heating at 95°C for 10 min, except for chymotrypsin that was inactivated by 5% formic acid (Merck), then the samples were dried using vacuum centrifuge. The obtained protein hydrolysates were resuspended in Dulbecco's Phosphate Buffered Saline (Gibco) at the concentration of 200 µg/mL (w/v).

2.3. Liquid Chromatography–Mass Spectrometry Analysis. The LC–MS analyses were performed on a UPLC system (Acquity, Quaternary Solvent Menager) coupled in line with a quadrupole time-of-flight hybrid mass spectrometer (Xevo G2-XS QTof, Waters) equipped with an electrospray ionization interface operating in positive ion mode. A C18 column (ACQUITY UPLC BEH C18, 130 Å, 1.7 µm, 2.1 mm × 150 mm) termostated at 60°C was used for the analyses. A gradient elution was developed with mobile phase A (water: formic acid; 100:0.1, v/v) and B (acetonitrile:formic acid; 100:0.1, v/v): mobile phase B was increased from 2% to 50% in 15 min and from 50% to 70% in 2 min. Column was equilibrated with the starting condition for 3 min before next injection. The flow rate was set at 0.3 mL/min and the injection volume was 10 µL. The mass spectrometer operated in high sensitivity mode using a capillary voltage of 3.0 kV and a cone voltage of 35 kV. Cone and desolvation gas flow was 50 and 600 L/h, respectively, while source and desolvation gas temperature was 120° and 300°C, respectively. Leucine enkephalin (0.1 ng/µL) was used as lock mass (m/z 556.2771). The data were acquired in positive total ion current (TIC) mode and in data dependent mode from m/z 50–2000. Raw data files were elaborated with Mascot Distiller software 2.8.5.0 and the peak list reporting the peptides molecular weight was achieved.

2.4. Cytotoxicity Assay on DLB-1 Cell Line. Cytotoxicity assay was performed in three replicates using DLB-1 cell line. 96-well tissue culture plates were seeded with 20,000 cells/cm^2 and incubated (24 h at 25°C). This cell concentration was established in order to obtain satisfactory absorbance values in the cytotoxic assay and preventing cell over-growth. After that, medium was replaced by 100 µL/well of the FPHs diluted in culture medium containing 2% FBS from 0.625 to 80 µg/mL. In all trials, cells were incubated for 48 h with FPHs dilutions at 25°C. Control samples received the same volume of culture medium with 2% FBS. After the incubation for 48 h at 25°C, cell vitality was determined using the MTT assay. The MTT assay relies on the reduction of the yellow soluble tetrazolium salt (3-(4,5 dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide) (MTT, Sigma–Aldrich, Saint Louis, USA) into a blue, insoluble formazan product by the mitochondrial succinate dehydrogenase [25–27]. After incubation with FPHs, DLB-1 cells were washed with phosphate buffer saline solution (PBS, Merck) and 50 µL/well of MTT (1 mg/mL) were added. After 4 h of incubation at 25°C, MTT were removed and cells were washed again, then the formazan crystals solubilized with 50 µL/well of 75% Ethanol (Merck). Plates were shacked (3 min, 300 rpm) in dark conditions and the absorbance at 570 nm determined in a microplate reader (Multiskan MS, LabSystems, USA). Cytotoxicity was expressed as the 50% cytotoxic concentration (CC_{50}), the concentration able to reduce cell growth by 50% in reference to the number of cells in the untreated control cell [28].

2.5. Immunomodulatory Activity Assay. Immunomodulatory effect of bioactive peptides was assessed measuring gene expression of selected molecular markers in cells exposed/not exposed

Table 1: Details of primers used for gene expression analyses.

Gene	Abbreviation	GenBank ID	Primer sequence (5'– 3')	References
18S rRNA	18S rRNA	AM490061	AGGGTGTTGGCAGACGTTAC CTTCTGCCTGTTGAGGAACC	[29]
Interleukin 1β	*IL-1β*	AJ311925	ATCTGGAGGTGGTGGACAAA AGGGTGCTGATGTTCAAACC	
Interleukin 8	*IL-8*	AM490063	GTCTGAGAAGCCTGGGAGTG GCAATGGGAGTTAGCAGGAA	
Interleukin 10	*IL-10*	DQ821114	CGACCAGCTCAAGAGTGATG AGAGGCTGCATGGTTTCTGT	
Tumor necrosis factor alfa	*TNF-α*	DQ200910	AGCCACAGGATCTGGAGCTA GTCCGCTTCTGTAGCTGTCC	

to the bioactive peptides. The assay was performed in three replicates using SS1, SS4, and SS5 hydrolysates. The DLB-1 cells were grown in 24-well tissue culture plates (20,000 cells/cm^2) with culture medium containing 7.5% FBS. After incubation at 25°C for 24 h, the medium was removed and replaced by SS1, SS4, or SS5 hydrolysates at 2 μg/mL or at at 4, 7, and 20 μg/mL, respectively in culture medium, based on the CC_{50} values. Control wells were filled with culture medium containing 2% FBS. After 24 h at 25°C, LPS (200 μg/mL) (from *Pseudomonas aeruginosa* 10; L-7018; Sigma–Aldrich) were added to half of the wells already treated with protein hydrolysates and to control wells. After 6 h at 25°C, all the wells were cleaned out and 350 μL of RA1 lysis buffer (Macherey-Nagel) were added to wells to carry out RNA extraction.

2.5.1. RNA Extraction. Total cellular RNA was isolated from 4 cm^2 of DLB-1 cells using NucleoSpin RNA (Macherey–Nagel) according to the manufacturer's instructions. To remove genomic DNA, DNAse I treatment was included into the RNA extraction protocol. Extracted RNA was stored at −80°C until use.

2.5.2. Cytokine Gene Expression Analyses by Real-Time Polymerase Chain Reaction. The GoScript Reverse Transcriptase (Promega) was used to reverse-transcribe the total RNA into cDNA. cDNA concentration was measured using a Qubit Fluorometer (ThermoFisher). Real-time PCR was carried out using BRYT Green GoTaq qPCR (Promega) with an ABI PRISM 7300 instrument (Applied Biosystems). The reaction mix consisted in 200 nM of each primer, 300 nM of CXR, GoTaq qPCR Master Mix (Promega) and 10 ng of each cDNA. The primers used for 18S ribosomal RNA (18S rRNA), interleukin 1β (*IL-1β*), 8 *(IL-8*), 10 (*IL-10*), and tumor necrosis factor α (*TNF-α*) are shown in Table 1. Reaction mixtures were incubated for 2 min at 95°C and then subjected to 50 cycles of 10 s at 95°C, 30 s at 60°C. Prior to the sample analysis, the specificity of each primer pair was assessed using positive and negative samples. The specificity of the primers was confirmed by a melting curve analysis of the amplified products. All cDNA samples were analyzed in triplicate along with negative controls without template. For each sample, gene expression was normalized against 18S ribosomal RNA gene and expressed as $2^{-\Delta\Delta Ct}$, where ΔCt is determined by subtracting the 18S rRNA Ct value from the Ct value of the target gene. Gene expression of treated samples was expressed as "fold changes" relative to untreated controls.

Table 2: Cytotoxicity values of skin salmon by-product hydrolysates on DLB-1 cell line.

Hydrolysate name	CC_{50} (μg/mL)	
	Mean	SD
SS1	7.0	4.8
SS4	11.8	2.0
SS5	>80	na

Note: The data represents the mean calculation from three replicates.
Abbreviations: na, not applicable; SD, standard deviation.

2.5.3. Statistical Analysis. The normality and/or homogeneity of variance assumptions was validated for all the data. The gene expression data were analyzed with one-way ANOVA and when significant were subjected to the Sidak's multiple comparison test (GraphPad 6 software).

3. Results

3.1. Cytotoxicity Assay on DLB-1 Cell Line. The effects of SS1, SS4, and SS5 hydrolysates on the vitality of DLB-1 cells was evaluated through MTT assay. No alterations were denoted in DLB-1 cells exposed to increasing concentrations of SS5 (0.625 to 80 μg/mL) until 48 h (Table 2). On the contrary, the exposure to increasing concentrations of SS1 and SS4 (from 0.625 to 80 μg/mL) significantly affected the vitality of DLB-1 cells. Particularly, hydrolysates SS1 and SS4 showed cytotoxicity values (CC_{50}) of 7.0 and 11.8 μg/mL, respectively. The cytotoxicity values belonging to all the tested hydrolysates were reported in Table 2.

3.2. Assessment of Peptide Content and Size. High-resolution mass spectrometry analysis enabled the detection of a large number of peptides in the SS1, SS4, and SS5 FPHs ($n >$ 1000). The number and molecular weight distribution of the detected peptides are summarized in Table 3 and graphically represented in the data plot in Figure 1. Previous studies [19] have reported that the anti-inflammatory activity of peptides from hydrolysates of salmon by-product is enhanced in those with molecular weights between 1.0 and 2.0 KDa. Table 3 highlights the subset of peptides falling within a potentially bioactive molecular weight range.

Table 3: Number and size analysis of detected peptides in skin salmon by-product hydrolysates.

Hydrolysate characteristics	SS1	SS4	SS5
Number of peptides	1570	1401	1032
Minimum peptide size	400.2	400.2	400.1
25% Percentile	531.1	499.4	515.3
Median peptide size	653.9	622.4	636.5
75% Percentile	816.6	789.3	827.8
Maximum peptide size	1636	1774	1569
Peptides (number and %) with mass >1.0 and <2.0 kDa	183 (11.7%)	172 (12.3%)	165 (16.0%)

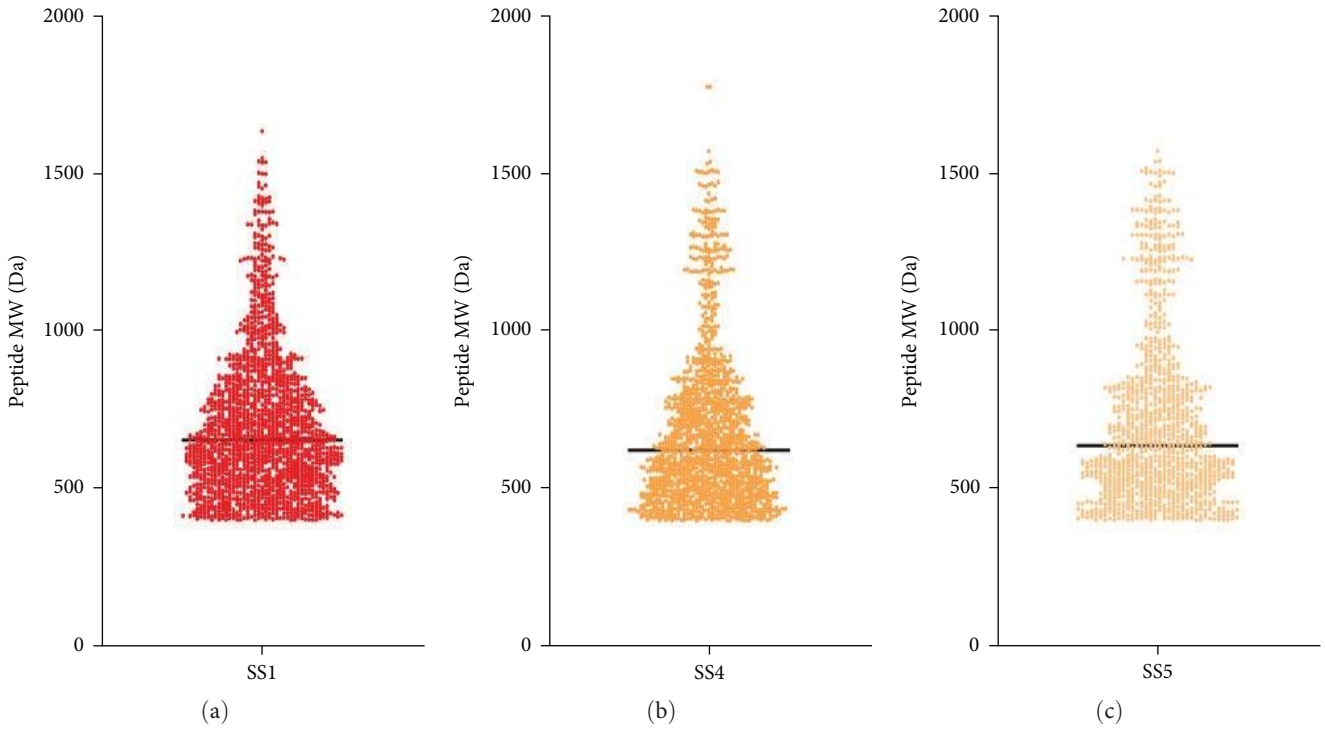

Figure 1: Size distribution of peptides in sample SS1 (a), SS4 (b), and SS5 (c).

3.3. Gene Expression Analyses. The cell culture treatment assays showed that SS1 and SS4 hydrolysates at 2 μg/mL were able to significantly upregulate *IL-1β* gene expression ($p < 0.05$) after LPS stimulation. Only for SS1 the upregulation was shown also without LPS stimulation (Figure 2).

Similarly, SS1 at 4 μg/mL, SS4 at 7 μg/mL, and SS5 at 20 μg/mL significantly upregulated ($p < 0.05$) *IL-1β* gene expression after LPS stimulation (Figure 3). The tests with high concentrations (4, 7, 20 μg/mL) confirmed the inflammatory effect for SS1 and SS4 and showed a similar effect for SS5 (Figure 3). However, SS5 also showed an anti-inflammatory effect due to a significant upregulation ($p < 0.05$) of *IL-10* when stimulated with LPS (Figure 3).

4. Discussion

The conversion of aquaculture by-products into valuable products like FPH supports sustainability, not only reducing the environmental impact but also opening opportunities for producing bioactive peptides with health benefits, finding wide applications on food and feed formulation [20].

To explore the immunomodulatory effects of Atlantic salmon (*Salmo salar*) by-products FPH, an in vitro model based on a *Dicentrarchus labrax* derived cell line was implemented in this study. The DLB-1 cell line applied in our study was suitable to assess the immunomodulatory effects of fish protein hydrolysates obtained from Atlantic salmon by-products expressing properly the panel of investigated immune-related genes and accordingly, allowing the analysis of their various bioactivities (inflammatory/anti-inflammatory). So far, *in vitro* approach has successfully been used to explore bioactivities of FPH mainly using mammal cell lines, especially, as human models [20, 30]. However, despite limited availability of fish cell lines, especially for some species, such as *Dicentrarchus labrax*, this approach proved to be useful also (as

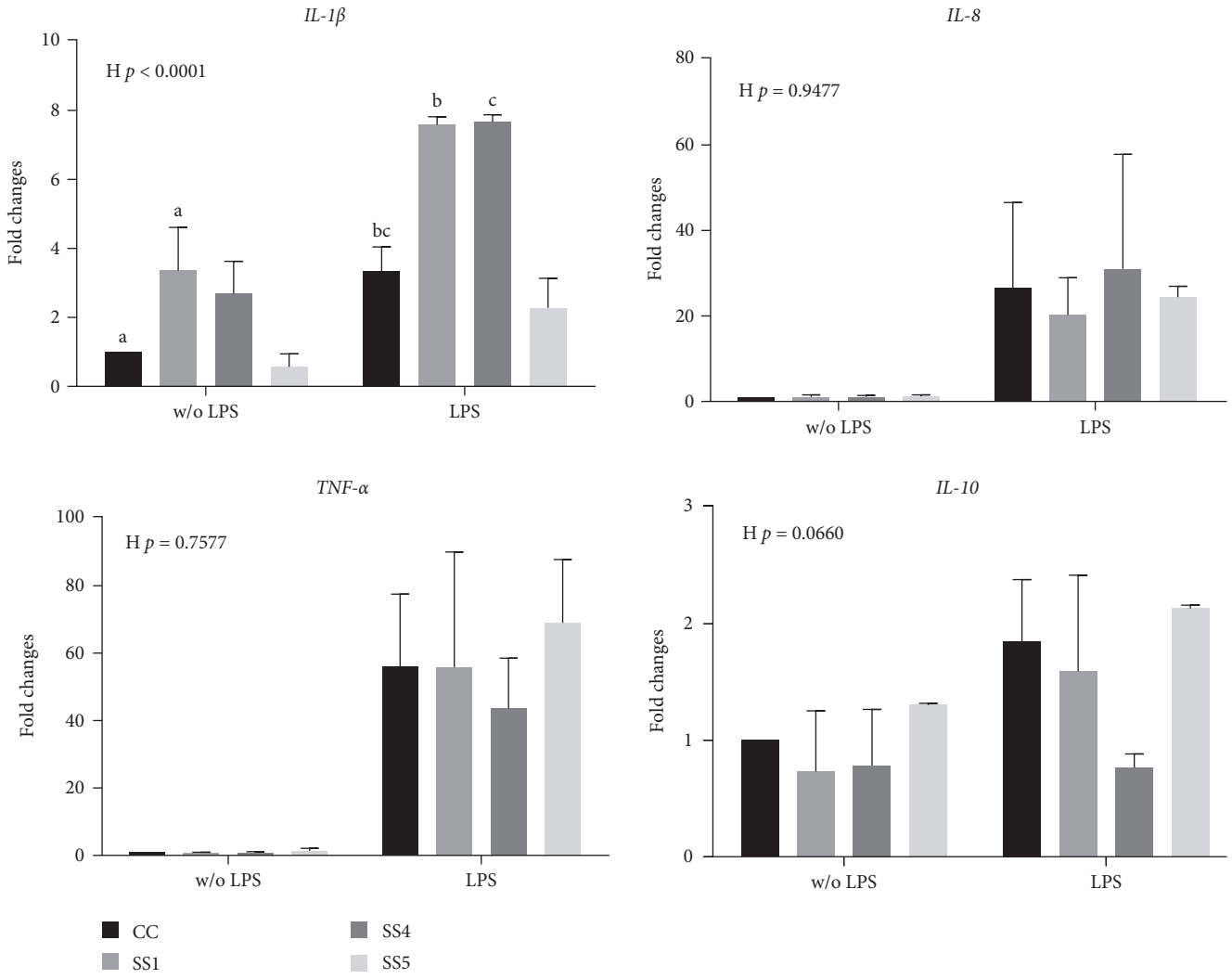

FIGURE 2: Gene expression analysis of SS1, SS4, and SS5 hydrolysates at 2 μg/mL. Bars represent the mean fold change relative to the control cells ($n = 3$) and the standard deviations. Same superscript letters mean significantly different values ($p < 0.05$).

a model) for fish. This model allows us to evaluate the immunomodulatory effects of FPH, providing insights into their biological impacts, while minimizing the use of live animals in experimental research. In particular, DLB-1 cell line has a glial origin [1], glial cells are particularly involved in immune function playing a key role in initiating inflammatory and immune responses and for this reason, this cell line suits very well for immunomodulatory investigations. Accordingly, in the present study, DLB-1 has been able to express immunological-related genes, including *IL-1β*, *IL-10*, *TGF-β*, and *TNF-α*, responding correctly to LPS stimulation and thus providing a suitable model to assess the FPH effect also under infection conditions. Furthermore, FPH have already shown the ability to modulate microglial activation markers and to decrease the expression of the proinflammatory cytokines *IL-6*, *IL-1β*, and *TNF-α* in the hippocampus of mice with LPS-induced inflammation, limiting brain inflammatory response, and improving the resolution of inflammation [31]. In this respect, a glial *in vitro* model could also be useful in future studies targeting neuroinflammation. However, so far, DLB-1 have been applied only to few *in vitro* research. In particular, concurrently with the development of the cell line Morcillo et al. [1] tested the toxic effects of MeHg, Pb, and As assessing their cytotoxicity and the induction of reactive oxygen species (ROS) production. Moreover, DLB-1 cell line have already been applied to test the cell viability, cell migration, and mitochondrial activities after the exposure to okra (*Abelmoschus esculentus*) ethanolic extracts and astaxanthin [27, 32]. Furthermore, the alteration of human pharmaceuticals cytotoxicity due to polystyrene nanoplastics exposure [3] and the permissiveness of DLB-1 to nervous necrosis virus, the most threatened viral pathogen of European sea bass, have been studied applying this cell line [24, 33]. In this respect, fish cell lines represent a valuable tool leading to the 3R approach application. Accordingly, in this study salmon protein hydrolysates have been screened and dosed using an *in vitro* method leading to a reduction of animals that could have been employed to assess the effects of the screened compounds with an *in vivo* study.

In previous studies, salmon FPH showed mainly an anti-inflammatory effect reducing the expression of several immune

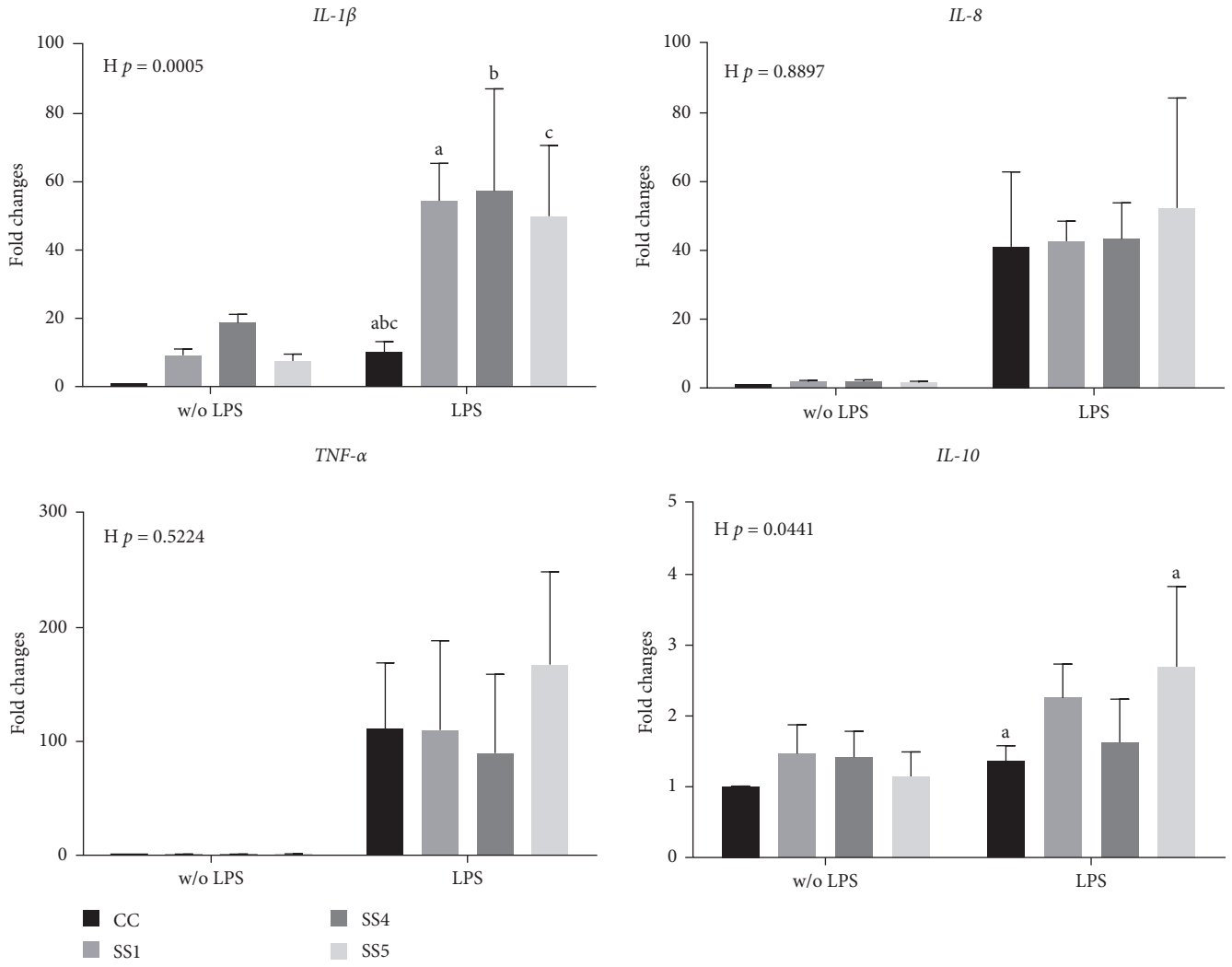

FIGURE 3: Gene expression analysis of SS1, SS4, and SS5 hydrolysates at 4, 7, and 20 μg/mL, respectively. Bars represent the mean fold change relative to the control cells ($n = 3$) and the standard deviations. Same superscript letters mean significantly different values ($p < 0.05$).

genes, including *TNF-α*, *IL-6*, and *IL-1β*, regardless of different proteases used for their preparation. However, a different panel of enzymes was used compared to our study [30]. In the present study, all the investigated protein hydrolysates upregulated the proinflammatory gene *IL-1β* after LPS stimulation, suggesting their ability to induce macrophage activation and lymphocyte reduction [24, 34]. Indeed, *IL-1β* is a key proinflammatory cytokine in fish that plays a critical role in the immune response. It is involved in initiating inflammation by activating immune cells, such as macrophages and promoting the production of other cytokines. Studies have shown that its regulation is crucial for maintaining a balanced immune response to prevent excessive inflammation [35, 36]. Similar to our results, FPH have demonstrated pro-inflammatory activity both *in vivo* and *in vitro* previous studies. An *in vivo* feeding trial showed an upregulation of pro-inflammatory cytokines, including *IL-1β* and *TNF-α* in distal intestine of juvenile barramundi (*Lates calcarifer*) fed a diet including tuna hydrolysates (TH) in association with fermented poultry by-product meal (PBM) [37]. In the same way, *TNF-α* was upregulated in head kidney of juvenile barramundi fed a diet supplemented with 10% TH and bioprocessed PBM and challenged with *Vibrio harveyi* [38]. *In vitro* activation of pro-inflammatory cytokines (*IL-1β* and *COX-2*) was shown by tilapia mince protein hydrolysate (100 and 800 μg/mL) in the human monocyte leukemia cell line THP-1 stimulated with LPS [39]. Similarly, papain hydrolyzed Nibea Japonica protein (200 μg/mL) rich in a low molecular weight peptides (<1 kDa) significantly increased production of proinflammatory cytokines *TNF-α*, *IL-6*, and *IL-1β* in LPS-activated RAW264.7 cells [40]. In our study, a different effect was observed among the tested FPHs, SS1, and SS4 were able to upregulate *IL-1β* at all tested concentration (SS1 2 and 4 μg/mL and SS4 2 and 7 μg/mL) whereas SS5 showed a similar effect only at higher concentration (20 μg/mL). Accordingly, a previous *in vivo* feeding trial study shows that the hydrolysate effect can be affected by dose, with different anti/pro-inflammatory action when administered at different doses [41].

Interestingly, the upregulation of the anti-inflammatory gene *IL-10* in cells exposed to SS5 hydrolysate and treated with LPS stimulation highlights the ability of this compound

to induce also an anti-inflammatory effect when bacterial interaction happened. Accordingly, in mammals *IL-10* is produced in response to viral, bacterial, fungal, and protozoan components [42]. *IL-10* expression has been modulated also after LPS injection in *in vivo* studies involving common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) [43, 44]. The fish cell line RTS-11, consisting of rainbow trout monocyte/macrophage, infected with *Piscirickettsia salmonis* showed an upregulation of *IL-10* during the early stages of infection, with values peaking at 36 h post infection [45] in accordance with the results obtained for SS5 group post-LPS stimulation. Moreover, SS5 hydrolysate did not produce any cytotoxic effects on DLB-1 cells leading to its utilization with a higher concentration (until 80 μg/mL) compared to SS1 and SS4 hydrolysates. FPH anti-inflammatory effect has been observed also *in vivo*, turbots (*Scophthalmus maximus*) fed FPH from pollock (*Theragra chalcogramma*) by-products showed a downloaded expression of interleukin-1β, *IL-6*, and *IL-8* after *Vibrio harveyi* challenge [46].

The use of aquaculture by-products for producing FPH not only offers economic and environmental benefits but also creates a source of biologically active peptides with health-promoting properties [19]. These peptides, particularly those generated through enzymatic hydrolysis, have shown significant potential in enhancing fish health by improving immune responses and disease resistance. By utilizing specific enzymes and optimizing the hydrolysis process, FPH can yield bioactive peptides of varying molecular weights, which are linked to specific biological activities, including anti-inflammatory and antimicrobial effects.

It has been demonstrated that adding FPH, which is rich in short-chain peptides, to the diet at the right amounts improves the fish's immunological response, growth performance, nutritional utilization, and antioxidant activity [41, 46–49], particularly in larvae and juveniles [50, 51]. Additionally, fish fed with FPH have demonstrated increased innate immunity and disease resistance against viral, bacterial, and parasitic infections [38, 51, 52].

The molecular weight of peptides significantly impacts the biological activities of FPH. Several studies have highlighted that fish-derived peptides exhibit a wide range of bioactive potentials, including antihypertensive, antioxidative, antimicrobial, and anti-inflammatory activities, which are dependent on the peptides' molecular weights [53, 54]. For instance, Najafian and Babji [55] found that peptides derived from animal muscles with molecular weights below 10.0 kDa and fewer than 50 amino acids exhibit antimicrobial activity. Additionally, a study by Ahn et al. [19] reported that salmon by-product protein hydrolysates with molecular weights between 1.0 and 2.0 kDa demonstrated the highest anti-inflammatory activity. Accordingly, Kotzamanis et al. [52] state that low molecular weight bioactive peptides in FPH may possess immune-stimulating and antibacterial properties and they hypothesize that FPH contains polypeptide fractions that could activate mechanisms in fish that are crucial for disease resistance. In this prospect, the anti-inflammatory propriety of the SS5 FPH could be due to its high amount (16%) of peptides with low molecular weight ranging between 1000 and 1569 Da. Moreover, enzyme type, concentration, temperature, pH, and time are all key factors that significantly influence the quality and functionality of the final product [56]. Each of these parameters plays a crucial role in determining the effectiveness of the hydrolysis process and, consequently, the bioactivity and yield of the peptides produced. Choosing the right enzyme is critical in the production process, as it provides greater control over the hydrolysis process and directly influences the characteristics of the final product. The enzyme selection not only determines the efficiency of the hydrolysis but also shapes the bioactivity and functionality of the peptides generated [16]. In this study the SS5 FPH, produced with bromelain enzyme, showed positive immunological effects in fact, according to Lin et al. [57], the degree of hydrolysis of bromelain hydrolysate was higher than those obtained with pepsin and papain enzymes. Higher degree of hydrolysis generally results in smaller peptides, which tend to exhibit enhanced bioactivity, including anti-inflammatory effects. Smaller peptides are more easily absorbed and can interact effectively with cellular receptors to inhibit the production of pro-inflammatory cytokines [19].

In conclusion, the transformation of aquaculture by-products into FPHs may offer economic and environmental benefits through the production of bioactive peptides with positive biological properties. These peptides, particularly those of lower molecular weight, have demonstrated anti-inflammatory and immune-stimulating effects using DLB-1 cell line. By optimizing hydrolysis conditions, including enzyme selection, FPH can be tailored to enhance fish health and disease resistance, offering a sustainable solution for both the aquaculture industry and fish health.

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

Enrico Volpe: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing – original draft, writing – review and editing. **Preethi Ramya Surendran**: investigation, methodology, writing – original draft, writing – review and editing. **Marina Naldi**: data curation, formal analysis, investigation, methodology, visualization, writing – original draft, writing – review and editing. **Francesca Errani**: methodology, writing – review and editing. **Alberto Cuesta**: resources, writing – review and editing. **Luca Parma**: conceptualization, writing – review and editing. **Elisa Benini**: writing – review and editing. **Alessio Bonaldo**: funding acquisition, writing – review and editing. **Sara Ciulli**: conceptualization, funding acquisition, resources, writing – original draft, writing – review and editing.

Funding

This study was supported by the Novofeed project, funded by Eranet – Marine Biotech, under the European Commission's 7th Framework Programme and the Research Council of Norway is acknowledged for the support to the Aquafeed Technology Center, ATC, with Grants 604814 and 245883/F50, respectively. The authors Enrico Volpe and Elisa Benini were supported by the Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022, adopted by the Italian Ministry of University and Research, CUP J33C22001190001, Project title "National Biodiversity Future Center —NBFC" under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4—Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of the Italian Ministry of University and Research funded by the European Union— NextGenerationEU.

Acknowledgments

This research was undertaken under the Novofeed project, funded by Eranet – Marine Biotech, under the European Commission's 7th Framework Programme and the Research Council of Norway is acknowledged for the support to the Aquafeed Technology Center, ATC, with Grants 604814 and 245883/F50, respectively. The authors Enrico Volpe and Elisa Benini were supported by the Project code CN_00000033, Concession Decree No.1034 of 17 June 2022, adopted by the Italian Ministry of University and Research, CUP J33C22001190001, Project title "National Biodiversity Future Center—NBFC" under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4—Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union—NextGenerationEU.

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