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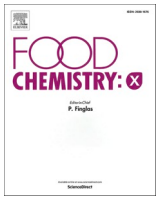

# Immunomodulatory peptides from sturgeon cartilage: Isolation, identification, molecular docking and effects on RAW264.7 cells

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ABSTRACT

Sturgeons (*Acipenseridae*), ancient fish known for their caviar, produce underutilized by-products like protein-rich cartilage, which is a source of high-quality bioactive peptides. This study investigates immunomodulatory peptides from sturgeon cartilage hydrolysates mechanisms. The study found that sturgeon cartilage hydrolysate F2–7 and its key peptides(DHVPLPLP and HVPLPLP)significantly promoted RAW267.4 cell proliferation, NO release, and phagocytosis ($P < 0.001$).Additionally, western blotting confirmed that F2–7 enhances immune response by increasing the expression of P-IKKα/β, IKK, p65, and P-p65 proteins in the NF-κB signalling pathway ($P < 0.01$). Molecular docking further demonstrated that DHVPLPLP and HVPLPLP bind to NF-κB pathway proteins via hydrogen bonding, with low estimated binding energies (−2.75 and −1.64; −6.04 and −4.75 kcal/mol), thus establishing their role as key immune peptides in F2–7. Therefore, DHVPLPLP and HVPLPLP have the potential to be developed as dietary supplements for immune enhancement. Their ability to enhance immune function provides a theoretical basis for novel immune supplements.

## 1. Introduction

The immune system plays a critical role in protecting organisms from viruses, bacteria, toxins, and other threats (Feng et al., 2021). Innate and adaptive immunity work in concert to guarantee that the body is capable of effectively defending itself against various pathogens (Nicole, 2022). However, when the immune system is dysfunctional, it may lead to the development of various immune-related diseases, including autoimmune and allergic diseases and immunodeficiency disorders (Grimbacher, Warnatz, Yong, Korganow, & Peter, 2016). Strengthening the body's immunity is important for preventing diseases and maintaining health. The immunomodulators market is currently experiencing rapid growth, particularly in therapeutic areas such as cancer, HIV, and autoimmune diseases (Bourinbaiar, Lee-Huang, Krasinski, & Borkowsky, 1994; Franzke et al., 2003; Wu, Yang, Cheng, Bi, & Chen, 2022). Despite the wide variety of immunomodulatory drugs on the market, existing drugs are often accompanied by side effects, including immunodeficiency and immune overreaction. Therefore, it is important to search for a novel natural immunomodulatory substance that is both mild and safe as well as effective.

Currently, food peptides from various sources have been reported with excellent immunomodulatory effects. Many studies have isolated immunologically active peptides from plant materials such as wheat germ (Wu et al., 2017), soybeans (Wen, Jiang, Zhou, Bi, & Yang, 2021), hericium erinaceus (Yu et al., 2021) and maca (He et al., 2022). For examples, Wu isolated a novel immunomodulatory peptide with the sequence Gln-Cys-Phe-Ser-Thr-Ala from wheat germ globulin (Wu et al., 2017). Wen identified the sequences of 51 peptides from the soybean digest and designated 46 peptides as immunomodulatory peptides (Wen et al., 2021). However, most of the immunoreactive peptides reported so far are from plant sources, and only some of them have been reported from animal immunoreactive peptides. Recent studies have revealed that peptides isolated from egg white (Chen et al., 2022), tuna (Cai et al., 2022) and other animal ingredients also have immune-boosting activity. Cai isolated the immunomodulatory peptide T1 from hydrolysis proteins of tuna, which demonstrated the optimal enhancement of the immunological activity (Cai et al., 2022). Therefore, animal sources of immunoreactive peptides are also important sources of immunoreactive peptides.

Several studies have investigated the potential immunomodulatory effects of marine-derived peptides (Kiewiet, Faas, & DeVos, 2018). For instance, the peptides DNSIAMESMK and LLQLGSGR purified from oyster hydrolysate have been shown to have immunomodulatory properties, both promoting macrophage proliferation and phagocytosis,

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as well as inducing the production of NO, TNF-α and IL-6 (Li et al., 2019; Li et al., 2019). Furthermore, six immunomodulatory peptides extracted from hydrolyze *Litopenaeus vannamei* head proteins exhibited strong binding capacity to TLR2 and TLR4/MD-2, suggesting their immunomodulatory effects (Jiang et al., 2022). Peptides derived from aquatic products have the potential to be commercially valuable in the food and pharmaceutical fields due to their low molecular weight and ease of absorption by the body (Mao et al., 2024). These studies indicate that peptides derived from marine fish may also possess excellent immune-enhancing properties.

Sturgeon is classified within the Acipenseriformes, Acipenseridae, and Acipenser taxonomic groups. As an ancient fish, it is regarded as a living fossil (Liang, 2015). In 2022, China's sturgeon aquaculture production reached 112,009 tons, representing 80 % of the global total (Tian, 2023). Current research focuses on sturgeon fish oils, acids, proteins, and chondroitin sulfate (Chen, Liu, et al., 2022; Chen, Zhang, et al., 2022). Sturgeon cartilage, a by-product of sturgeon processing, is typically used for animal feed or discarded, resulting in minimal added value. This cartilage constitutes about 10 % of the total weight of sturgeon by-products (Chen, Liu, et al., 2022; Chen, Zhang, et al., 2022). It is rich in type II collagen, which maintains its natural triple-helical structure and has potential as a substitute for mammalian collagen (Zhu et al., 2020). This makes it a promising source for collagen peptides with notable medical and nutritional benefits (Meng, Wei, Takagi, Dai, & Zhang, 2023). Despite this, research on the immunomodulatory effects of sturgeon cartilage-derived peptides is limited. This study aims to isolate, purify, and identify novel immunomodulatory peptides from sturgeon cartilage enzymatic digest and investigate the immune-enhancing mechanisms of peptide F2–7. The findings will offer new insights into immunologically active peptides of animal origin and explore the high-value utilization of sturgeon by-products. By elucidating the effects and mechanisms of peptide F2–7, this study will provide a theoretical foundation for developing new natural immunomodulators and advancing the application of animal-derived immunoactive peptides in food and medicine.

## 2. Materials and methods

### 2.1. Materials and reagents

Russian sturgeon was purchased from Yunnan Hai Wang Aquatic Products Co., Ltd. (Yunnan, China). BCA, NO, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) kits were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). The primary antibodies used in the experiments were as follows: IKK (Item No.: ab32041), P-IKKα/β (Item No.: #2697), p65 (Item No.: 10745–1-AP), P-p65 (Item No.: ab76302). Secondary antibodies (Goat anti-Mouse IgG [H + L]) and Goat anti-Rabbit IgG [H + L]) were purchased from Abiowell (China). All reagents used were of analytical grade or chromatographic purity.

### 2.2. Isolation, purification, and identification synthesis of sturgeon cartilage peptides

The protein content of sturgeon cartilage was 85.5 % (dry basis). The preparation process of sturgeon cartilage peptides: cartilage was boiled for 7 h to achieve a final material-liquid ratio of 1:30(g:mL), cooled and hydrolysed with 0.75 % (*w*/w) alkaline protease and 0.25 % (w/w) neutral protease(Guangzhou Bo Tang Trading Co., Ltd., China), hydrolysing time was 5.5 h, temperature was 55 °C, a pH of 9, and inactivation of the enzyme was carried out for 15 min, and the obtained samples were freeze-dried and stored at −20 °C. (The dry basis yield of the peptide was 65.7 %). The sturgeon chondroitin peptides were separated and purified by Sephadex G-15 dextran gel column(Beijing Solarbio Science and Technology Co., Ltd., China), DEAE-52 cellulose ion exchange column and RP-HPLC successively.

Finally, the peptides were identified and screened by LC-MS and molecular docking technique. DHVPLPLP and HVPLPLP (purity>95 %) were synthesized by Fmoc method (Micro Nano Biotechnology Co., Ltd., Shenzhen, China).

### 2.3. RAW 264.7 cells culture and cell viability

RAW 264.7 murine macrophage cells were provided by Procell Life Science and Technology Co., Ltd. (Wuhan, China). The cells were cultivated in a 37 °C incubator with 5 % $CO_2$ using high-glucose DMEM medium (Shanghai Basalmedia Technologies Co., Ltd., China), supplemented with 10 % FBS (Biowest, France), 1 % double antibody, and 1 % glutamine (Dong et al., 2023). In brief, cells in the logarithmic growth phase ($5 \times 10^4$ cells/well) were seeded into a 96 well plate, and cultured for 24 h. Subsequently, the cells were treated with varying concentrations of the sturgeon cartilage peptide for an additional 24 h. Then 100 μL of new medium containing CCK-8 assay solution (10 %, *v*/v) was added.

### 2.4. Detection of phagocytosis

The experimental group followed the same protocol as described in 2.3 Add 200 μL of medium (medium: neutral red = 10:1) and continue incubation in the incubator for 1–2 h. Aspirate the medium. Add 200 μL of cell lysate per well and shake for 15 min. Finally, absorbance was measured at 540 nm to calculate the phagocytic capacity of the cells (Shi & Zhao, 2022).

### 2.5. Measurement of NO and cytokines

The experimental group operates in the same way as 2.3 The supernatant was collected and centrifuged. Next, 50 μL of cell supernatant was added to each well and 50 μL of Griess Reagent I and II were added successively. NO content was calculated based on a standard curve.

Following 24 h of cell culture, the supernatant was collected and centrifuged. The IL-1β, IL-6, and TNF-α levels in the supernatant were determined according to the instructions of the ELISA kit (He et al., 2022).

### 2.6. Western blotting

The total protein of the cells was extracted with RIPA lysate and the protein concentration was determined by BCA kit (Hung et al., 2021), followed by SDS-PAGE electrophoresis, membrane transfer, closure, incubation. Incubate with primary antibodies overnight in a refrigerator at 4 °C, and then incubate with the corresponding secondary antibodies at room temperature for 2 h. Obtain protein bands and then perform grayscale value calculation using Image J.

### 2.7. Molecular docking

First, immunoreactive peptides were screened based on protein confidence. The PubChem Protein Data Bank was accessed to search for the 3D structures of the receptor proteins IKK and p65. Solvent and organic molecules were removed using PyMOL, and were saved in PDB format. Finally, molecular docking was conducted using AutoDock software with flexible ligand docking selected. Other settings included selecting the maximum output conformation to automatically generate the docking results (Wang et al., 2023).

### 2.8. Statistical analysis

All assays were performed in triplicate. Data were expressed as mean ± SD. Data analysis and graphical representation were conducted using Excel 2019, GraphPad Prism (version 8.00) and Image J (version 1.8.0.345). Statistical analysis was performed using analysis of variance

**Table 1**
Molecular weight distribution of Sturgeon cartilage peteties.

| Molecular weight distribution | ≤1000 Da | 1000–2000 Da | 2000–5000 Da | >5000 Da |
|---|---|---|---|---|
| Percentage | 82.0 ± 2.6 | 13.4 ± 2.31 | 4.50 ± 0.32 | < 0.1 |

Values are means ± SD.

(ANOVA) followed by Duncan's multiple comparisons to analyse differences between groups. A $p$-value <0.05 was considered significant. The level of statistical significance in the figures and tables was indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the LPS group.

## 3. Results

### 3.1. Basic components and molecular characteristics of sturgeon cartilage peptides

Surgeon cartilage is rich in protein, containing up to 85.5 %, making it a valuable source for bioactive peptides (Table S1). Furthermore, the free amino acid content reached 21.36 %, which could be readily absorbed by the human body. Sturgeon cartilage peptides, particularly rich in glutamic acid, glycine, leucine, arginine, hydroxyproline, and proline (Table S2), contribute to enhanced thermal stability, metabolism, and immune function (Hua & Jiang, 2021; Tziveleka, Ioannou, Tsiourvas, Berillis, & Foufa, 2017). As shown in Table 1, 82.0 ± 2.6 % of sturgeon cartilage peptides have molecular weights below 1000 Da, while 13.4 ± 2.31 % are within the 1000–2000 Da range, and 4.5 ± 0.32 % between 2000 to 5000 Da. This indicates that high-temperature cooking combined with enzymatic hydrolysis effectively produces small molecular peptides. These peptides, capable of crossing the intestinal barrier, are readily absorbed and exhibit various biological activities (Zhong, Li, Yao, & Lin, 2019). Therefore, sturgeon enzymatic products may possess significant bioactivity.

### 3.2. Purification of the immunomodulatory peptide from sturgeon cartilage

The purification process commenced with Sephadex G-15 chromatography resulting in a single peak that indicated the peptides exhibited similar molecular weights. Subsequently, DEAE 52 ion exchange chromatography was employed, yielding five distinct fractions (F1-F5). Notably, fraction F2 significantly enhanced the proliferation rate of RAW264.7 cells across various concentrations, demonstrating no cytotoxicity at levels below 200 μg/mL, with peak proliferation observed at a concentration of 100 μg/mL (Fig. 1 (D)). At this concentration, F2 also maximized NO secretion, with an increase of 21.41 μmol/mL relative to the NC group ($P < 0.001$, Fig. 1 (E)). Additionally, the phagocytic activity of RAW264.7 cells was markedly augmented at this concentration

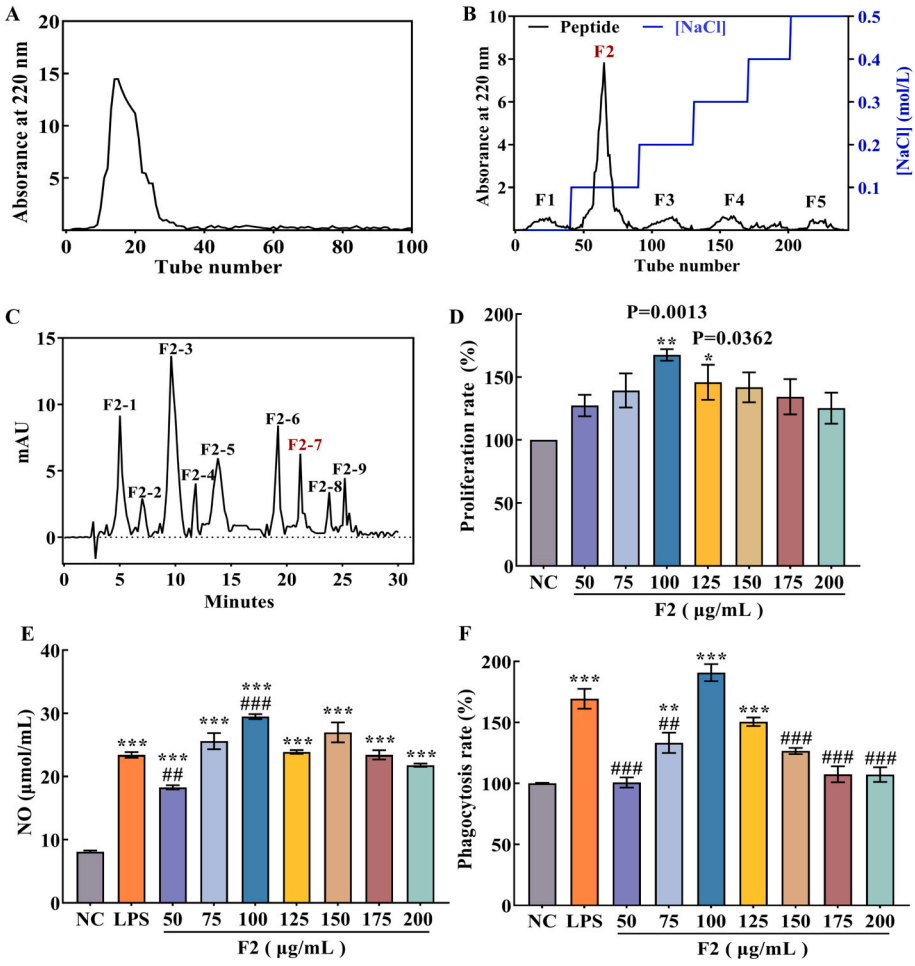


**Fig. 1.** (A) Sephadex G-15 filtration, (B) Ion-exchange chromatography, (C) RT-HPLC purification of fraction F2, (D) Effect of F2 on RAW264.7 cell viability, (E) NO production, and (F) neutral red phagocytosis. DMEM and LPS-treated groups served as negative and positive controls, respectively.Data are presented as the mean ± SD ($n = 5$). $^{*}P < 0.05$ vs. NC, $^{**}P < 0.01$ vs. NC, $^{***}P < 0.001$ vs. NC; $^{\#}P < 0.05$ vs. LPS, $^{\#\#}P < 0.01$ vs. LPS, $^{\#\#\#}P < 0.001$ vs. LPS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

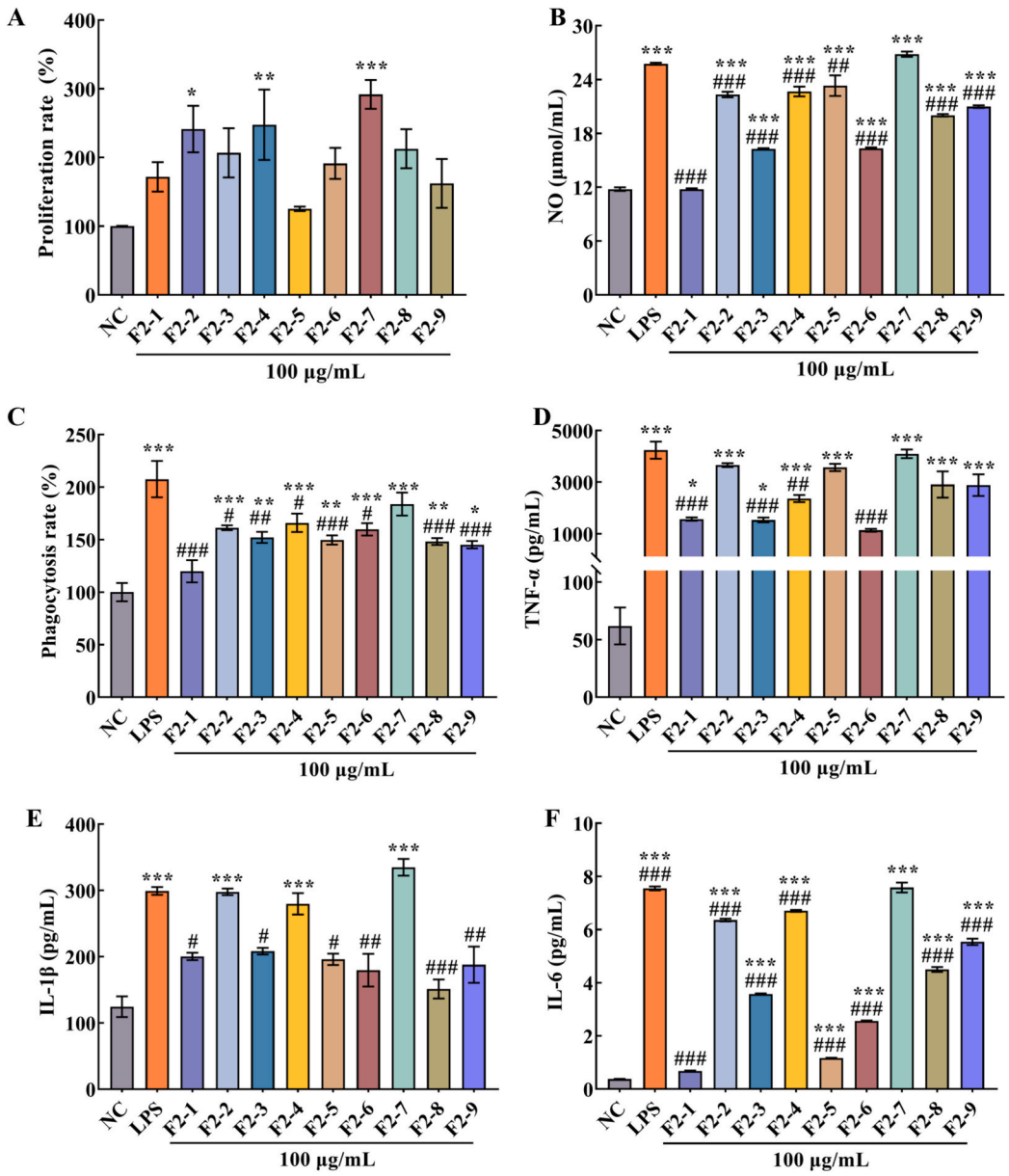


**Fig. 2.** Effects of RT-HPLC purified fractions on the (A) viability of RAW 264.7 cells, production of (B) NO, and (C) phagocytosis of neutral red, (D) TNF-α, (E) IL-1β, and (F) IL-6 production. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

($P < 0.001$, Fig. 1 (F)).

RP-HPLC serves as an effective technique for the separation of polar small peptides, particularly those with molecular weights below 1000 Da, and is commonly utilized for the final purification of peptides (Dauly, Perlman, Costello, & McComb, 2006). The incorporation of 10–16 % trifluoroethanol (TFE) into the mobile phase has been demonstrated to enhance peptide separation (Hara et al., 2015). Further purification of F2 utilizing RT-HPLC resulted in the isolation of nine fractions, among which fraction F2–7 exhibited the most pronounced immunomodulatory effects (Fig. 1(C)).Compared with NC group, the proliferation rate of RAW264.7 cells in F2–7 group was increased by 191.78 % ($P < 0.05$), Furthermore, As shown in Fig. 2 (B) and (C), the NO secretion level in F2–7 was 26.83 ± 0.67 μmol/mL ($P < 0.001$), and the phagocytosis ability was comparable to LPS group. In addition, F2–7 significantly promoted the secretion of TNF-α and IL-6 from RAW264.7 cells, with concentrations measured at 4092.00 ± 235.93 pg/mL and 7.58 ± 0.26 pg/mL, respectively ($P < 0.001$). Notably, the secretion of IL-1β in response to F2–7 surpassed that observed in the LPS-positive group, achieving a level of 334.86 ± 18.83 pg/mL. These findings indicate that F2–7 possesses robust immunostimulatory activity comparable to that of LPS. Consequently, an in-depth investigation into the immunomodulatory mechanisms of F2–7 is warranted.

### 3.3. Evaluation of immune activity of F2–7 components

#### 3.3.1. Effect of F2–7 on cells morphology

The growth state of the cells was observed with an optical microscope. As shown in Fig. 3 (A). The boundary of NC group cells was clear and uniform round or oval in size. In LPS group, the volume of cells increased, many vacuoles and long pseudopods, and their morphology was fusiform or irregular. It has been shown that macrophages are successfully activated when they have more and longer pseudopods, and that their pseudopods increase cell adhesion and phagocytosis (Chi, Chi, Huang, Zhou, & Tan, 2021). After stimulation of RAW264.7 cells with different concentrations of F2–7 in the experiments, the morphology was changed compared with that of NC normal cells, showing a spindle-

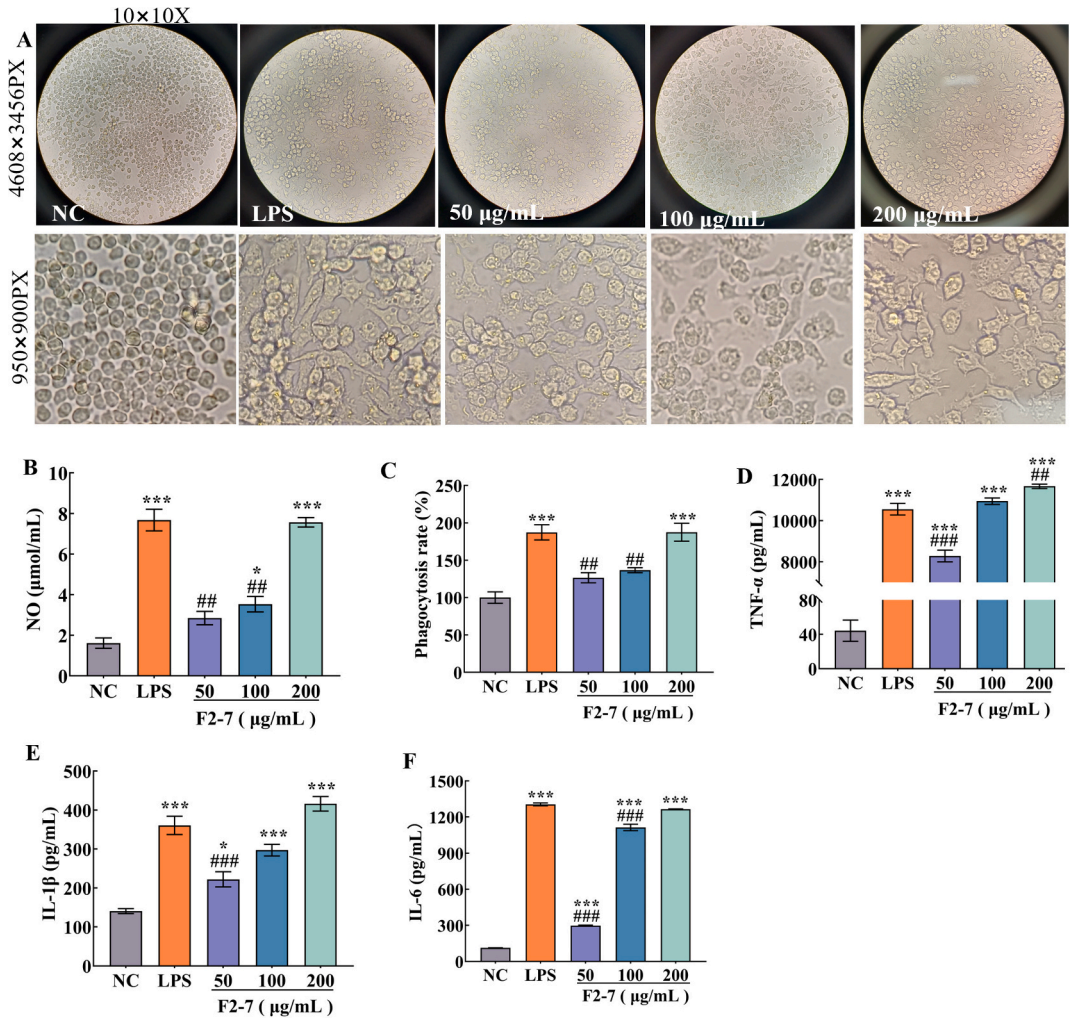


**Fig. 3.** Effects of F2–7 fraction on RAW 264.7 cells morphology (A), (B) NO secretion, (C) neutral red phagocytosis, (D) TNF-α, (E) IL-1β, and (F) IL-6 production. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

shaped and dendritic state, and there was a tendency for the pseudopods of the cells to gradually increase with the increase of concentration. It is shown that F2–7 successfully activates RAW264.7 cells, and pseudopods increase cell adhesion and phagocytosis, and enhance immunoreactivity.

### 3.3.2. *Effects of F2–7 on NO secretion in RAW264.7 cells*

NO is an intracellular signalling molecule with oxidative capacity, involved in various pathological and physiological processes, and plays a key role in regulating immune responses. Thus, it can be used as a quantitative marker of macrophage activation (Li, Mi, Duan, Ma, & Fan, 2020). As shown in Fig. 3(B), F2–7 induced a dose-dependent increase in NO secretion (50–200 μg/mL). NO secretion peaked at 7.56 ± 0.33 μmol/mL at 200 μg/mL ($P < 0.001$), comparable to the levels induced by LPS. These results suggest that F2–7 enhances NO secretion in macrophages.

### 3.3.3. *Effects of F2–7 on phagocytosis of RAW264.7 cells*

Phagocytosis is a fundamental function of macrophages, essential for clearing pathogens and tissue remodeling (Chen et al., 2023). Fig. 3 (C) demonstrates the effects of different levels of F2–7 on phagocytosis. The LPS group showed a significant increase ($P < 0.001$) in phagocytic ability of cells by 87.22 % when compared to the NC. Following treatment with distinct F2–7 concentrations, there was a dose-dependent increase in cells' phagocytosis of the neutral red dye. At a concentration of 200 μg/mL, the phagocytic capacity of the cells was 1.87 times greater than that of the NC ($P < 0.001$), which was like that of the LPS group. The results indicated that F2–7 improved the phagocytosis of RAW264.7 cells.

### 3.3.4. *Effects of F2–7 on cytokine secretion in RAW264.7 cells*

The measurement of inflammatory factors such as TNF-α, IL-6, and IL-1β is a crucial indicator of immune response strength and macrophage activation, reflecting the cells' ability to eliminate pathogens and regulate pro-inflammatory responses(Shraddha & Chandana, 2022).The secretion of cellular inflammatory factors in the cell supernatant is shown in Fig. 3 (D, E, F). When LPS stimulated the cells, the secretion of cytokines increased significantly ($P < 0.05$). In Fig. 3 (D), when the F2–7 concentration was 50 μg/mL, the secretion of TNF-α increased by 283.02 % compared with that of the NC ($P < 0.001$). In Fig. 3 (E), the secretion of IL-1β was significantly increased ($P < 0.05$) when the F2–7 concentration was 50 μg/mL compared with the NC, which was 1.5 times higher than that of the NC. As shown in Fig. 3 (F), when the F2–7 concentration was 50 μg/mL, the secretion of IL-6 was elevated by 163.59 % compared with that of the NC ($P < 0.001$). As the concentration of F2–7 increased, the levels of TNF-α, IL-1β and IL-6 secreted by the cells were almost close to those of the LPS group, and the concentration of 200 μg/mL showed a better effect. The results indicated that F2–7 could enhance the immune activity of the organism by promoting the secretion of cellular IL-6, TNF-α and IL-1β. Li, Xu, et al. (2019) and

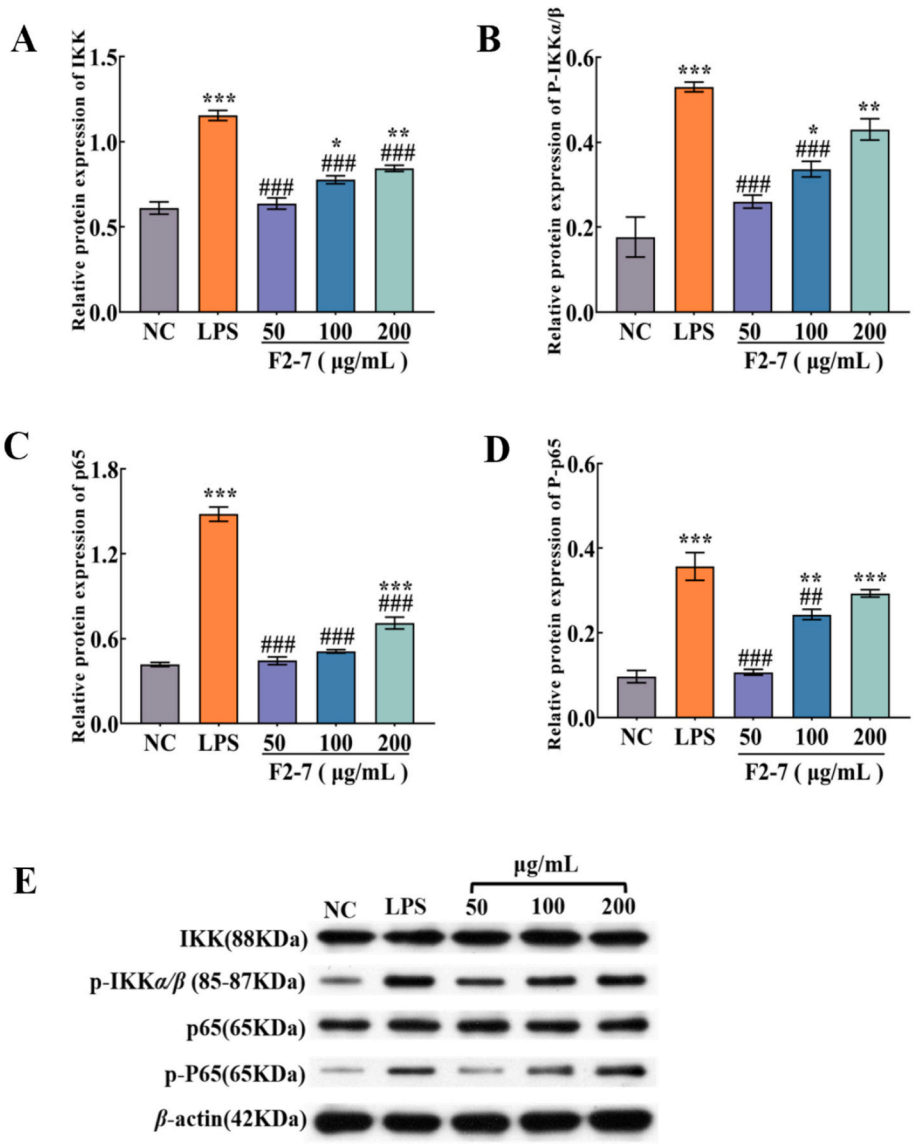


**Fig. 4.** (A) (B) (C) (D) RAW264.7 cell-related protein expression. (E) NF-κB-related protein blotting map.

Li, Ye, et al. (2019) found that both oyster peptides and the duck egg white hydrolysate studied by He et al. (2021) promoted the secretion of NO, IL-6, and TNF-α, demonstrating immunomodulatory effects. Therefore, the present study preliminarily investigated the immunological activity of F2–7.

### 3.3.5. *Study on the mechanism of F2–7 immunoreactivity*

The NF-κB signalling pathway is present in almost all animal cell types and is a key regulator and major transcription factor that regulates the expression of several cytokines, is involved in cellular responses to stimuli, and plays a key role in the immune response to infection (Manfred & Michael, 2007). As illustrated in Fig. 4 (A–D), the protein expression levels of IKK, p-IKK, P65 and p-P65 were markedly elevated ($P < 0.001$) in the LPS group relative to the NC group, indicative of RAW264.7 cell activation. The expression levels of related proteins were also significantly up-regulated after treating RAW264.7 cells with different concentrations of F2–7 fractions. The optimal effect was observed at a F2–7 concentration of 200 μg/mL, with a concentration-dependent effect.

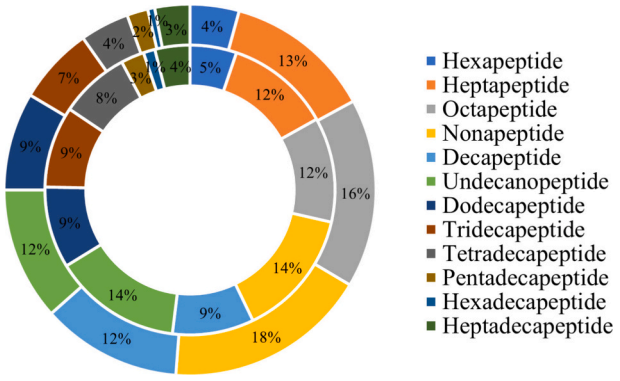


**Fig. 5.** The distribution of peptide length and quantity of Sturgeon cartilage peptides F2–7 in external layer pie plot, as well as the distribution of its bioactive peptides (score > 0.5) at internal layer pie plot.

**Table 2**
Peptide Identification Informations.

| Peptide sequence | *m/z*(Da) | −10lgP[a] | Hydrophobicity[b] (%) | PeptideRanker score[c] | Toxicity prediction[d] |
|---|---|---|---|---|---|
| LDHVPLP | 790.45 | 26.75 | 71.43 | 0.5458 | Non-Toxin |
| DHVPLPLP | 887.5 | 38.74 | 75.00 | 0.7107 | Non-Toxin |
| LDHVPLPLPQ | 1129.63 | 40.37 | 70.00 | 0.4500 | Non-Toxin |
| DHVPLPL | 790.44 | 29.17 | 71.43 | 0.6780 | Non-Toxin |
| DHVPLPLPQ | 1016.54 | 40.99 | 66.67 | 0.5230 | Non-Toxin |
| HVPLPLP | 772.47 | 38.51 | 85.71 | 0.7041 | Non-Toxin |

[a] −10lgP represents the protein confidence score, the larger the value the more confidence peptides the protein carries.
[b] According to "Peptide2.0" (http://peptide2.com).
[c] Bioactivity prediction was performed using PeptideRanker (http://distilldeep.ucd.ie/PeptideRanker/). Higher scores indicate better life activity of the peptide.
[d] Potential biotoxicity of target peptide sequences was assessed using Toxinpred prediction (https://webs.iiitd.edu.in/raghava/toxinpred/index.htm)

**Table 3**
Minimum binding energy in molecular docking.

| Receptor | Ligand | Minimum binding energy (kcal/mol) | Receptor | Ligand | Minimum binding energy (kcal/mol) |
|---|---|---|---|---|---|
| | LDHVPLP | −1.04 | | LDHVPLP | -0.56 |
| | DHVPLPLP | −2.75 | | DHVPLPLP | −1.64 |
| IKK | LDHVPLPLPQ | 1.71 | p65 | LDHVPLPLPQ | 2.28 |
| (PDB: 2VVY) | DHVPLPL | −1.63 | (PDB: 4EYT) | DHVPLPL | 1.04 |
| | DHVPLPLPQ | −0.47 | | DHVPLPLPQ | 0.82 |
| | HVPLPLP | −6.04 | | HVPLPLP | −4.75 |

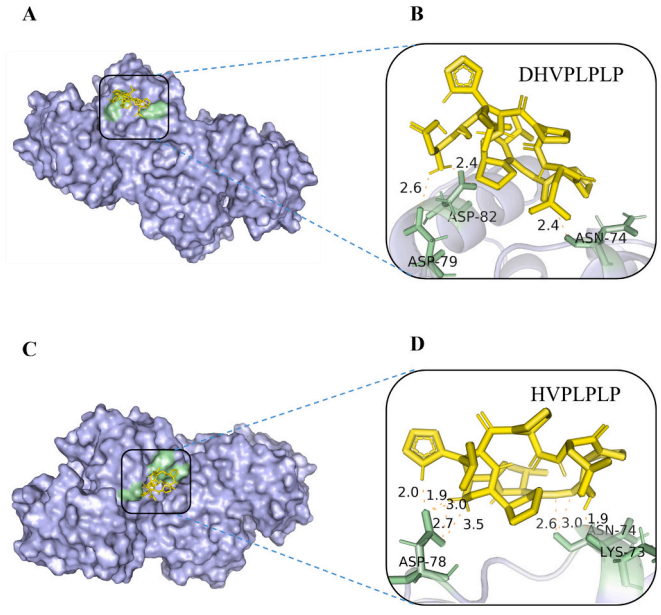


**Fig. 6.** The interaction of peptides identified from F2–7 with IKK by molecular docking. A, C are 3D diagrams of specific residues of the peptides interacting with IKK, and the orange dashed line indicates the hydrogen bonding.B, D show the active pockets formed between the peptide and the receptor proteins. (A, B) Molecular docking results of DHVPLPLP with IKK. (C, D) Molecular docking results of HVPLPLP with IKK.

### 3.4. Identification of the F2–7

In this study, F2–7 was analyzed by LC-MS/MS (Jiang et al., 2022), and PEAKS Studio 8.5 software was used for data processing and retrieval analysis. The number of amino acid residues in F2–7 was shown in Fig. 5. The length of F2–7 was mainly heptapeptide, octapeptide, nonapeptide, decapeptide, undecapeptide, dodecapeptide and tridecapeptide, accounting for 86 % of the total sequences. Setting PeptideRanker >0.5 as the threshold, there were 77peptides predicted with bioactivity, which accounting for 47 % of the identified sequences.It was found that most of the peptides in F2–7 contained the characteristic amino acids, up to 80 % or more. The most occurring characteristic amino acids were Pro, Leu, Asp, Ile, Val, Gln and Lys. the most abundant amino acid peptide sequences containing Pro and Leu were 95.12 % and 89.02 %, respectively. In addition, more than 50 % of the peptide sequences had hydrophobic amino acids present at the N and C termini. Therefore, based on the protein plausibility and the presence of more characteristic amino acids we selected six peptides(Table 2) with possible immune activity(LDHVPLP, DHVPLPLP, LDHVPLPLPQ, DHVPLPL, DHVPLPLPQ, HVPLPLP) and then further validated the immune activity by molecular docking and synthetic peptides. The identification information and secondary mass spectra of the six peptides are shown in Table 2 and Fig. S1.

**Table 4**
Interacting amino acid residues of targeted protein with peptides identified from F2–7.

| Targeted protein | Peptide sequence | Docking score | Number of hydrogen-bonds | Interacting residues | Distance (Å) |
|---|---|---|---|---|---|
| IKK | DHVPLPLP | −13.365 | 3 | Asp-79 | 2.6 |
| | | | | Asp-82 | 2.4 |
| | | | | Asn-74 | 2.4 |
| | HVPLPLP | −7.021 | 8 | Lys-73 | 1.9 |
| | | | | Asn-74 | 3.0 |
| | | | | | 2.6 |
| | | | | Asp-78 | 2.0 |
| | | | | | 1.9 |
| | | | | | 3.0 |
| | | | | | 2.7 |
| | | | | | 3.5 |
| p65 | DHVPLPLP | −12.654 | 2 | Gln-488 | 2.2 |
| | | | | Asp-497 | 1.9 |
| | HVPLPLP | −6.261 | 2 | Gly-403 | 3.0 |
| | | | | Glu-405 | 2.2 |

### 3.5. Molecular docking of immunomodulatory peptides in the F2–7 fraction

IKK regulates the transcriptional activity of NF-κB in response to cellular signals, and thus it has long been assumed that IKK dominates upstream in the NF-κB signalling pathway (Hwang & Ko, 2021; Jack, Kathryn, Jennifer, Hanneke, & Simon, 2022). Therefore, in this study,

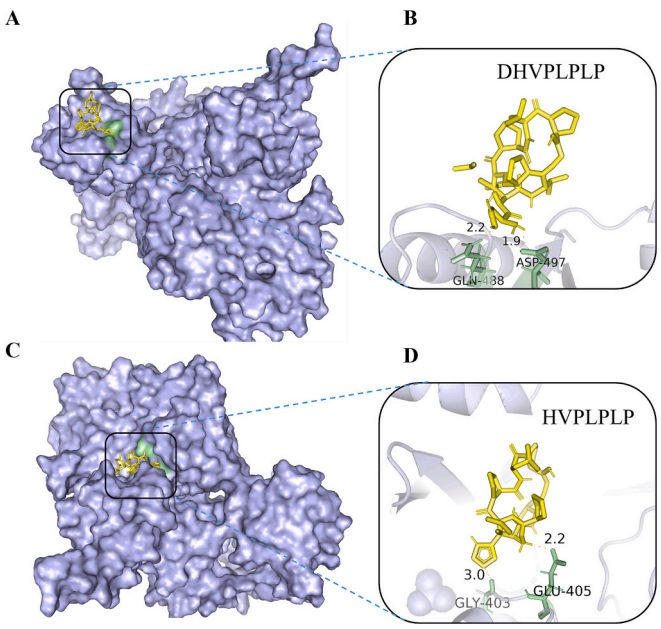


**Fig. 7.** The interaction of peptides identified fromF2–7 with p65 by molecular docking. (A, B) Molecular docking result of DHVPLPLP with p65. (C, D) Molecular docking result of HVPLPLP with p65.

IKK protein was chosen as receptor protein to study the binding ability of active peptides in F2–7 to determine whether active peptide activate the NF-κB signalling pathway. As shown in Table 3, DHVPLPLP and HVPLPLP exhibited the favorable interactions with targeted protein IKK (PDB: 2VVY), and the minimum binding energy was −2.75 and − 6.04 kcal/mol, respectively. Moreover, both DHVPLPLP and HVPLPLP were buried in the groove of IKK (Fig. 6). The DHVPLPLP formed three conventional hydrogen bonds (Asp-79, Asp-82 and Asn-74) with IKK residues. The HVPLPLP formed eight conventional hydrogen bonds with tree residues (Lys-73, Asn-74 and Asp-78). DHVPLPLP and HVPLPLP were stably attached to p65 (PDB:4EYT) by conventional hydrogen bonds with minimum binding energy of −1.64 and − 4.75 kcal/mol, respectively (Table 3). Among them, DHVPLPLP formed two conventional hydrogen bonds with the residue Gln-488 and Asp-497 at distances of 2.2 Å and 1.90 Å; HVPLPLP formed two conventional hydrogen bonds with Gly-403 and Glu-405 residues of p65 at distances of 3.0 Å and 2.2 Å (Table 4, Fig. 7). These findings suggested that DHVPLPLP and HVPLPLP were the possible cause of the enhanced immune activity of F-7.

### 3.6. The immunoreactivity of DHVPLPLP, HVPLPLP in RAW264.7 cells

DHVPLPLP and HVPLPLP were synthesized to verify their proliferative activity in RAW264.7 cells in vitro. The results were shown in Fig. 8(A), DHVPLPLP and HVPLPLP could enhance the proliferation of RAW264.7 cells. When the peptide concentration was 200 μg/mL, the most significant effect on cell proliferation was observed($P < 0.001$). The proliferation rates of DHVPLPLP and HVPLPLP were 222.86 ± 8.76 % and 169.53 ± 7.12 %, respectively. Therefore, the concentration of 200 μg/mL was chosen for the determination of immunoreactivity-related indexes.

The promotion of cellular NO secretion by DHVPLPLP and HVPLPLP was extremely significant ($P < 0.001$) compared with NC as shown in Fig. 8(B). Among them, the effect of DHVPLPLP on cellular NO release was 33.36 ± 0.28 μmol/mL, which was comparable to that of the LPS group. Both DHVPLPLP and HVPLPLP significantly increased macrophage phagocytosis by 89.93 % and 42.47 %($P < 0.01$), respectively, compared to the NC group as shown in Fig. 8(C). In summary, DHVPLPLP and HVPLPLP not only enhanced the proliferation of RAW264.7 cells but also significantly increased NO secretion and phagocytosis. These findings suggest that they have strong immunoreactivity and may play a crucial role in F2–7. This provides a solid foundation for further understanding the immune mechanism of F2–7.

## 4. Discussion

Sturgeon cartilage accounts for about 10 % of the body weight and is often discarded or processed into low-value feed. However, sturgeon cartilage is primarily composed of collagen, making it an excellent source for preparing bioactive peptides. Therefore, this study explores the bioactive peptides derived from sturgeon cartilage through enzymatic hydrolysis and investigates their effects on the proliferation of RAW264.7 macrophages, elucidating the potential mechanisms.

Molecular conformation, amino acid composition, peptide chain length, total charge, and other factors affect the biological activity of peptides (Mercier, Gauthier, & Fliss, 2004). Peptides with molecular weights less than 1000 Da exhibit good solubility and can effectively target enzyme cleavage sites. Additionally, they can directly cross the intestinal barrier and produce biological effects on immune cells in vivo (Vermont, Sofia, Ben, John, & Elvira, 2009; Zhong et al., 2019). Studies showed that 79.0 % of sturgeon cartilage peptides produced through neutral and alkaline protease treatment have molecular weights below 1000 Da, indicating their biological activity. To enrich the effective components of sturgeon cartilage peptides, these peptides were purified and evaluated using RAW264.7 cells.

When cells are activated, they enhance cell proliferation and phagocytosis, and promote the secretion of NO and cytokines, which

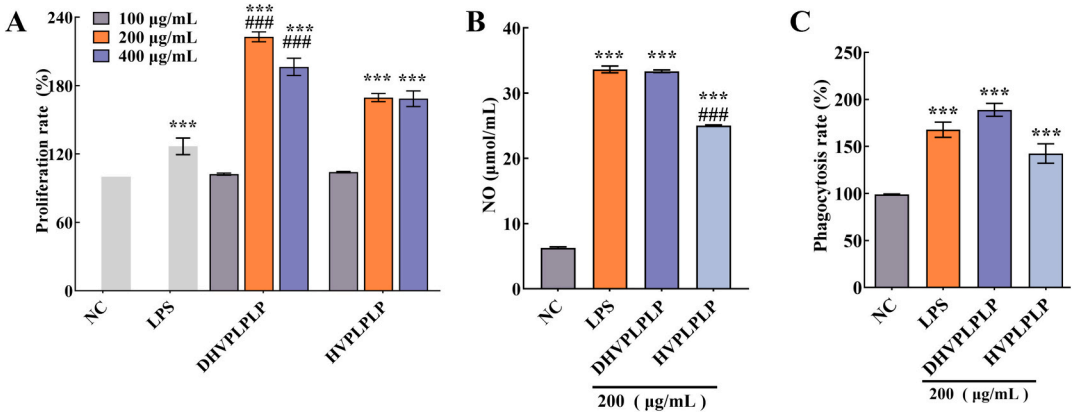


**Fig. 8.** (A) Effect of synthetic peptides on RAW264.7 cell viability, (B) NO secretion, (C) neutral red phagocytosis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

constitutes an important non-specific immune response in the body (Chen, Yuan, Du, Zhang, & Sun, 2019; Li et al., 2023). Macrophages phagocytose pathogens and emit signals that stimulate other immune cell responses (Linehan & Fitzgerald, 2015). Furthermore, macrophages produce NO, which serves both as an oxidising agent and as an indicator of macrophage activation (Beatriz et al., 2022; Li et al., 2020). The results of the experimental study demonstrate that F2–7 from sturgeon cartilage enzymatic digest significantly enhances the proliferation and phagocytosis of RAW264.7 cells , and promotes NO release. This indicates that the F2–7 fraction activates macrophage proliferation, enhances antigen phagocytosis, and secretes NO to eliminate pathogens. These findings are consistent with previous studies on the immunoreactive peptide SCSP in green clam digestive fluid (Li, Xu, et al., 2019; Li, Ye, et al., 2019). Cytokines play a pivotal role in immune responses, with a close association with immunomodulatory functions (Beatriz et al., 2022). Activated macrophages secrete TNF-α to recruit natural killer cells, neutrophils, and eosinophils (Kim et al., 2022), while IL-6 stimulates antibody production by B cells (Chen, Liu, et al., 2022; Chen, Zhang, et al., 2022). The F2–7 fraction has been demonstrated to significantly increase the secretion of cytokines by RAW264.7 cells, including TNF-α, IL-1β, and IL-6. This suggests that the F2–7 fraction enhances immune responses by directly activating RAW264.7 cells to produce immune factors, thereby achieving immune enhancement.

The NF-κB and MAPK signalling pathways are intimately connected with the immune system (Sun, 2017). The NF-κB signalling pathway regulates the expression of various cytokines, and its activity is regulated by κB inhibitory factors and κB kinase (IKK). Upon stimulation by upstream factors, IKK is activated and phosphorylates IκB, ultimately leading to the entry of NF-κB into the nucleus (Hwang & Ko, 2021; Tak & Firestein, 2001) The P65 protein activates target genes in the nucleus, where it binds to DNA sequences in the promoters of the target genes and regulates their transcription, ultimately generating effector molecules that are responsible for eliminating pathogens (Gilmore, 2006; Tergaonkar, 2006; Wang, Liu, Yue, Sun, & Zhang, 2020). The present study demonstrated that sturgeon chondropeptide F2–7 significantly enhanced the expression of IKK, P-IKKα/β, p65 and P-p65 proteins. (Please refer to Fig. 5(D–H).) The increase in P-IKKα/β indicates the activation of the IKK complex, which in turn phosphorylates IκB, prompting its degradation, release and subsequent activation of NF-κB into the nucleus. P-p65 represents a crucial step in the NF-κB signalling pathway, enhancing transcriptional activity. Furthermore, sturgeon chondroitin has been demonstrated to activate the NF-κB signalling pathway by increasing the level of P-p65, thereby exerting an immune response. Similarly, T1 purified from tuna hydrolysate also activates the NF-κB signalling pathway and promotes the immune response (Cai et al., 2022), consistent with previous studies. Consequently, it can be postulated that sturgeon chondroitin may play a role in enhancing immunity by activating the proliferation of RAW264.7 cells through the activation of the NF-κB pathway and the promotion of the expression of P-IKKα/β and P-p65.

To further elucidate the immune-enhancing mechanism of sturgeon chondropeptide F2–7, we performed molecular docking of peptides identified from F2–7 with NF-κB signalling pathway-related proteins (IKK and p65). Based on the minimum binding energy and docking scores, DHVPLPLP and HVPLPLP demonstrated high affinity for proteins related to immune response and inflammatory response, suggesting their potential for further analysis. In this study, DHVPLPLP and HVPLPLP, which were identified from F2–7, were found to be stably bound to IKK, primarily through conventional hydrogen bonding. This suggests that the stable binding of DHVPLPLP and HVPLPLP to IKK may affect the activity of IKK, promote the phosphorylation of IκB by IKK, release and activate NF-κB, and thus enhance the initiation of the NF-κB signalling pathway and enhance inflammatory and immune responses. Furthermore, DHVPLPLP and HVPLPLP interact with p65 through conventional hydrogen bonding, which also affects inflammatory and immune responses. This binding directly enhances the DNA-binding capacity and transcriptional activity of p65, which in turn promotes the expression of NF-κB-related genes and further enhances the immune response. Furthermore, studies have demonstrated that immunoreactive peptides enriched in hydrophobic amino acids exhibit enhanced immunoreactivity and facilitate intercellular membrane translocation (Chalamaiah, Yu, & Wu, 2018; Jacquot, Gauthier, Drouin, & Boutin, 2010; Rajasekaran, Kamalakannan, & Shin, 2015). The proportion of hydrophobic amino acids in DHVPLPLP and HVPLPLP is 75 % and 85.71 %, respectively, and has not been previously reported in the database (https://www.ncbi.nlm.nih.gov/, accessed on 25 September 2023). The present study further corroborates the significant immunoreactive potential of DHVPLPLP and HVPLPLP. The immunoenhancing effect of F2–7 on RAW267.4 macrophages was achieved primarily through the interaction of these two peptides with IKK and p65 proteins on the NF-κB signalling pathway.

## 5. Conclusion

This study evaluated the effects of sturgeon cartilage enzymatic hydrolysate on macrophage immune responses using an in vitro cellular model. The sturgeon cartilage hydrolysate was purified through Sephadex G-15, DEAE-52, and preparative liquid chromatography, resulting in the isolation of fraction F2–7, from which two novel peptides were identified: Asp-His-Val-Pro-Leu-Pro-Leu-Pro (DHVPLPLP) and His-Val-Pro-Leu-Pro-Leu-Pro (HVPLPLP). Among them, F2–7, along with peptides DHVPLPLP and HVPLPLP, significantly promoted the proliferation and phagocytic activity of RAW264.7 macrophages. Further investigation revealed that F2–7 enhanced the secretion of NO, TNF-α, IL-1β, and IL-6. Additionally, F2–7 activated the NF-κB signalling pathway by upregulating the expression and phosphorylation of IKK and p65, thereby significantly enhancing the immune response. These findings provide new insights into the potential applications of sturgeon cartilage. However, the bioavailability of DHVPLPLP and HVPLPLP and their immunomodulatory mechanisms in vivo require further exploration.

## CRediT authorship contribution statement

**Shuchan Li:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Miaoqing An:** Validation, Methodology, Investigation. **Yuxuan Zhao:** Validation, Methodology. **Wenjun Zhao:** Conceptualization. **Pan Li:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Bing Du:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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