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Baltic herring hydrolysates: Identification of peptides, *in silico* DPP-4 prediction, and their effects on an *in vivo* mice model of obesity

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ABSTRACT

Baltic herring is the main catch in the Baltic Sea; however, its usage could be improved due to the low processing rate. Previously we have shown that whole Baltic herring hydrolysates (BHH) and herring byproducts hydrolysates (BHBH) by commercial enzymes consisted of bioactive peptides and had moderate bioactivity in *in vitro* dipeptidyl peptidase (DPP)-4 assay. In this study, we identified the hydrolysate peptides by LC-MS/MS and predicted the potential bioactive DPP-4 inhibitory peptides using *in silico* tools. Based on abundance, peptide length and stability, 86 peptides from BHBH and 80 peptides from BHH were proposed to be novel DPP-4 inhibitory peptides. BHH was fed to a mice intervention of a high-fat, high-fructose diet to validate the bioactivity. The results of the glucose tolerance and insulin tolerance improved. Plasma DPP-4 activities, C-peptide levels, and HOMA-IR scores significantly decreased, while plasma glucagon-like peptide-1 content increased. In conclusion, BHH is an inexpensive and sustainable source of functional antidiabetic ingredients.

1. Introduction

Baltic herring (*Clupea harengus membras*) is the main fish species caught by commercial fisheries in the sea area of Finland. In the 2000 s, Baltic herring catches ranged from 63 to 136 tonnes per year. However, the value of the catch remains low due to the low processing rate, as a large part of the Baltic herring is used as raw material for fur animals' feed, while only about 4 % of the Baltic herring caught is used as food in Finland. It has been estimated that if Baltic herring were to be used more as a raw material for fishmeal or processed more for food, the value of the catch would increase by a factor of about 1.6 (Mäkinen et al., 2022) and by a factor of 17.9 times was estimated if Baltic herring could be processed into high-value-added products, such as food supplements and nutraceuticals (Manzoor et al., 2022).

The utilization of Baltic herring as a cost-effective and environmentally friendly source to produce bioactive peptides holds significant importance. Thus, exploring potential avenues for its incorporation in bioactive peptide manufacturing is imperative, aiming for commercial application in functional foods, nutraceuticals, and pharmaceuticals. Our earlier studies have shown that Baltic herring protein hydrolysates had antidiabetic bioactivity through *in vitro* dipeptidyl peptidase 4 (DPP-4) inhibition assay with a moderate value of IC_{50} 5.5–7.9 mg/mL (Mäkinen et al., 2022), however, *in vivo* antidiabetic studies of Baltic herring have been missing this far. Meanwhile, information on the molecular basis of the reported bioactivities is needed to proceed toward commercial product applications as functional ingredients.

Hydrolyzed proteins from fish species like Atlantic salmon, cod, blue whiting, halibut, tilapia and Atlantic herring have been demonstrated in

Abbreviations: AP1, Activator protein 1; BHBH, Baltic herring byproducts hydrolysates; BHH, Baltic herring hydrolysates; DPP-4, Dipeptidyl peptidase 4; ELISA, Enzyme-linked immunosorbent assay; GLP-1, Glucagon-like peptide-1; GTT, Glucose tolerance test; HbA1c, Glycated hemoglobin; HOMA-IR, Homeostatic Model Assessment-Insulin Resistance; IL, Interleukin; ITT, Insulin tolerance test; LC-MS/MS, Liquid chromatography with tandem mass spectrometry; RT-qPCR, Real-time – quantitative polymerase chain reaction; TNF-α, Tumour necrosis factor alpha.

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animal model studies to affect beneficially on glucose homeostasis (Benoit et al., 2021; Drotningsvik et al., 2016, 2018; Fleury et al., 2022), to reduce plasma cholesterol and triglyceride levels (Drotningsvik et al., 2016; Hosomi et al., 2009) on inflammation (Bjørndal et al., 2013; Durand et al., 2020) as well as on blood pressure (Drotningsvik et al., 2021; Vildmyren et al., 2022). The protective effect against kidney injury biomarkers in obese Zucker fa/fa rats was reported when the rats were fed diets containing protein hydrolysates made from raw by-products, such as those produced during salmon and herring processing (Drotningsvik et al., 2018).

In human intervention studies, a double-blind cross-over study with healthy volunteers, the effects of a single dose of 20 mg per kg weight of cod protein hydrolysate or casein on postprandial glucose metabolism was studied (Dale et al., 2018). In two other double-blind cross-over studies, similar cod protein hydrolysates were administered for 1 week to older healthy subjects in different doses (10, 20, 30, or 40 mg/kg body weight daily) (Jensen et al., 2019) or 4 g daily supplementation for 8 weeks did not have statistically significant effects on glucose metabolism (Jensen et al., 2020). Hovland et al., (2020) studied in a randomized, double-blind study, the effects of low doses of Atlantic herring and Atlantic salmon protein hydrolysate and cod protein on glucose regulation and markers of insulin sensitivity in overweight adult humans. After the test period, serum biomarkers of insulin sensitivity (alpha-hydroxy butyrate, acetoacetate and beta-hydroxybutyrate) were reduced within herring, cod, and casein-whey groups. The casein-whey and cod protein reduced the serum post proteins glucose levels in the glucose tolerance tests. Herring and salmon protein hydrolysates did not affect glucose or insulin levels. However, the effects of cod protein were not statistically different from salmon and herring groups. The authors concluded that 2.5 g/day of cod, herring or milk proteins may be sufficient to improve glucose regulation in overweight adults (Hovland et al., 2020).

Depending on the source of the raw fish material and the enzyme applied for hydrolysis, only a few DPP-4 inhibitory peptides from the hydrolysates have been identified (Välimaa et al., 2019). For instance, peptide sequences Gly-Pro-Ala-Glu and Gly-Pro-Gly-Ala from Atlantic salmon skin and gelatin have DPP4-inhibitory activity (Li-Chan et al., 2012). This is due to the complexity of hydrolysates where the enzyme cleavage sites, especially commercial enzymes, are hard to predict and the inadequate protein sequences of the raw materials. In addition, potent DPP-4 inhibitory peptides are usually short with a few amino acids and may only present in a small amount of the whole hydrolysate, which makes it challenging to identify (Nongonierma & FitzGerald, 2017). The conventional identification process would involve multi steps of labor-intensive chromatographic separation. Thus, several *in silico* tools have been applied to predict the potential DPP-4 peptides via molecular modelling of the structural interaction between the peptide and enzyme (Charoenkwan et al., 2020; Nongonierma & FitzGerald, 2015; M. Zhang et al., 2022). On the contrary, unlike this detailed peptide chemistry work, the *in vitro* or *in vivo* bioactivity investigation is often conducted with a whole dietary protein hydrolysate. There is a gap in interpreting those results and translating both data into effectiveness *in vivo* studies.

In the present study, we aimed to investigate the antidiabetic bioactivity of Baltic herring hydrolysates in a holistic approach. The peptide hydrolysates were identified by peptidomics and subjected to *in silico* prediction of DPP-4 inhibition bioactivity. After confirmation of the bioactivity by *in vitro* assay, novel DPP-4 inhibitory peptides were proposed. Meanwhile, the BHH was first time examined in an animal model with a high-fat, high-fructose diet of mice. The intervention study's outcomes were the glucose tolerance test, insulin tolerance test, and inflammatory factors.

2. Materials and methods

2.1. Materials

Baltic herring filleting by-products, including head, fins, skin, frame and bone and compromising approximately 60 % of the fish total weight, and the whole Baltic herrings were obtained from Martin Kala (Turku, Finland). Gly-Pro-p-nitroanilide was purchased from VWR (Helsinki, Finland). Dipeptidyl peptidase IV enzyme, Sitagliptin, Alcalase® and Flavourzyme® were purchased from Merck (Espoo, Finland).

2.2. Baltic herring hydrolysates

Whole Baltic herrings and herring filleting by-products were hydrolyzed according to (Mäkinen et al., 2022) using Alcalase and Flavourzyme in combination. Both hydrolysates were subjected to bioactivity analysis and peptide identification. For the *in vivo* studies in a mice model, the hydrolysate prepared from whole Baltic herrings (BHH) was selected. The selection was based on the raw material availability. Only a small percentage of the Baltic herring catch annually is used for food. Thus, considering the potential for industrial-scale production, whole Baltic herrings are a well-available raw material, while the quantities of filleting side streams are remarkably smaller.

2.3. Peptide identification by LC-MS/MS

2.3.1. Identification of fish hydrolysates by LC-MS/MS

Lyophilized BHH (10 mg) was dissolved in 1 mL (0.1 % v/v) formic acid and subjected to an Amicon filter with MWCO 10 kDa to remove large molecules. The filtrate (300 μL) was followed by SepPak tC18 cartridge (Waters, Milford, MA) clean-up and eluted by two steps firstly, 20 % (v/v) acetonitrile with 0.1 % (v/v) formic acid, and secondly, 80 % (v/v) acetonitrile with 0.1 % (v/v) formic acid. The cleaned peptide mixtures were dried under a nitrogen flow to evaporate acetonitrile and lyophilized. For identification, the dried peptide mixtures were reconstituted in 200 μL 5 % (v/v) acetonitrile with 0.1 % (v/v) formic acid and separated on a column (ACQUITY UPLC HSS C18, 1.8 μm, 2.1 × 150 mm, Waters, Milford, MA, USA) with the matching guard column (ACQUITY HSS C18, 1.8 μm Vanguard, Waters, Milford, MA, USA) at 45 °C. The flow rate was 0.4 mL/min, and the separation gradient was from 5 % to 35 % of Buffer B consisted of acetonitrile with 0.1 % (v/v) formic acids followed by a column cleanup. The liquid chromatography was coupled with SYNAPT G2-Si Q-Tof mass spectrometer (Waters, Milford, MA, USA), and data were acquired using MSe mode, a data-independent approach in positive ion mode over the mass range of m/z 50–2000 with a 0.5 s spectral acquisition time. The high energy collision energy was ramped from 4 to 50 V. Lockmass leucine enkephalin m/z 556.2771 was monitored across the run for mass correction. Each sample was injected in triplicates.

Raw files obtained from MS/MS were imported into Progenesis QI for proteomics V4.2. Data were analyzed in the ion accounting workflow. The imported data was searched against Atlantic herring *Clupea harengus* proteome (37476 protein sequences accessions, Uniprot accessed on 11.4.2022). As a subspecies of Atlantic herring, Baltic herring *C. harengus membras* is unavailable with a specific sequence database. For identification, non-specific enzyme cleavage was applied, and variable modifications were set to deamidation of asparagine or glutamine, hydroxylation of aspartic acid, lysine, asparagine and proline, oxidation of methionine, and N-pyroglutamic acids formation from either glutamine or glutamic acid. The mass tolerance for the peptides is 10 ppm, and the fragment tolerance is 20 ppm. The false discovery rate FDR was 1 %. To refine the identification, the peptides with a score < 5 and absolute mass error > 20 ppm were removed.

2.3.2. Selection of the most potential DPP-4 peptides

All the identified peptides were subjected to *in silico* DPP-4 prediction

tool iDPPIV-SCM (https://camt.pythonanywhere.com/iDPPIV-SCM, site accessed on 2.2023). iDPPIV-SCM is a scorecard method based on amino acid sequences to predict DPP-4 inhibition activity. Peptides with a score of > 294 were considered to have potential DPP-4 activity (Charoenkwan et al., 2020). Additionally, the *in silico* potential DPP-4 peptides of more than 10 (≥10) amino acids were considered too long and removed. According to the relative abundance from the Progenesis, the top 100 peptides were selected. Taking the stability through digestion into consideration, the peptides containing trypsin cleavage sites (lysine or arginine, but not before proline), or chymotrypsin cleavage sites (phenylalanine, tyrosine, or tryptophan, but not before proline), or cysteine residues (unstable and easily oxidized) were removed. The rest filtrated peptides were considered as novel DPP-4 inhibitory bioactive peptides.

2.3.3. *In vitro DPP-4 inhibition activity of selected peptides*

To confirm the *in silico* DPP-4 prediction, four peptides were selected and synthetized for *in vitro* DPP-4 activity measurement. Synthetic peptides with sequence PPVEEP (*in silico* score 456.6); GPAGDPA (*in silico* score 321.8), Hydroxyproline (HyP)-HyP-GRPGF (*in silico* score 355.3 with input of Pro), and GADPEDVIVS (*in silico* score 267.9, less than suggested threshold score 294, as a negative control) were purchased from Biomatik (ON, Canada) with purity over 95 %. These peptides were identified and relatively abundant from the hydrolysate. In the selected synthetic peptides, the ones without modification and have higher score than the threshold 294 had *in vitro* DPP4 activities, which confirmed the *in silico* activity prediction. The peptides were dissolved in 100 mM Tris-HCl pH 8.0 and serially diluted, and the *in vitro* DPP-4 activity assay was performed according to (Lacroix & Li-Chan, 2013). In brief, the reaction mixture included the substrate Gly-Pro-p-nitroanilide, the DPP-4 enzyme was from Sigma D4943, and the test solution (synthetic peptides, positive control with no inhibitor, negative control with no DPP-4 activity; and blank buffer control, respectively). The peptides' DPP-4 inhibition rates (%) were calculated according to Equation (1). The inhibition (%) was plotted against the concentration of the peptides by GraphPad Prism 6 (Boston, MA) and its IC_{50} value was calculated using non-linear regression.

$$DPP-4\ inhibition(\%) = 100 \times \left(1 - \frac{A_{405}(test\ peptide) - A_{405}(blank\ buffer)}{A_{405}(positive\ control) - A_{405}(negative\ control)}\right) \quad (1)$$

2.4. *Intervention in vivo in mice model*

2.4.1. *Animals and treatments*

Six-week-old male C57BL/6J mice were purchased from Changzhou SLAC Laboratory Animal Co. Ltd. (Changzhou, China). The standard diet (LFD, D12450B) and high-fat and high-fructose diet (HFD, D12451) were provided by Trophic Animal Feed High-Tech Co., Ltd. (Nantong, China). **Table S2** showed the compositions of the mice's diets. All animals were housed in a room with a temperature of 24 ± 2 °C, relative humidity of 50 ± 10 %, 12 h light/dark cycles, and free access to food and water *ad libitum*. The animal experiment was approved by the Institutional Animal Care and Use Committee of Jiangsu University of Science and Technology (ethical approval code: 20200302) and was carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Before the experiment, the mice were kept for 1 week to acclimatize to the conditions. To evaluate the hypoglycemic effect of Baltic herring hydrolysate (BHH) *in vivo*, forty male C57BL/6J mice were divided into five experimental groups (n = 8/group) as follows: LFD, HFD, HFD + low dose Baltic herring hydrolysate (LD-BHH), HFD + high dose Baltic herring hydrolysate (HD-BHH) and HFD + sitagliptin as the positive control (PC). The LFD group received a standard rodent diet and purified water as normal control. The HFD group received a high-fat and high-fructose diet and purified water. The LD-BHH group received the HFD and Baltic herring hydrolysate (0.6 mg/mL) in drinking water. The HD-BHH group received the HFD and Baltic herring hydrolysate (1.8 mg/mL) in drinking water. The PC group received the HFD and sitagliptin (0.1 mg/mL) in drinking water as the positive control. Drinking fluids were refreshed daily. Food intake, and water intake were monitored on a weekly basis in each of the groups. After estimation, each mouse of the two BHH intervention groups received 120 mg/kg and 240 mg/kg of Baltic herring hydrolysate per day, respectively. The intervention treatments of each group were carried out for 16 weeks and then were sacrificed by cervical dislocation. Plasma was obtained using anticoagulant tubes through centrifugation of blood samples at 3,000 rpm for 10 min and stored at − 80 °C until biochemical analysis. Each animal's liver was removed, then stored at − 80 °C until analysis.

2.4.2. *Weight gain and glucose metabolic evaluation*

The following parameters were evaluated in mice from all experimental groups: weight gain, fast blood glucose, and fast blood insulin. The body weight was monitored weekly. Before the glucose tolerance test (GTT) and insulin tolerance test (ITT) at week 15, the experimental mice were fasted for 4h, and blood was collected from the tail to detect glucose and insulin levels. The blood glucose concentration was measured using an Accu-Chek Advantage II glucometer (Roche Diagnostic, Switzerland). The blood insulin concentration was measured using an ELISA kit (R&D, Shanghai, China) according to the manufacturers' instructions. The Homeostatic Model Assessment-Insulin Resistance (HOMA-IR) score was calculated using fasting blood glucose and insulin concentrations. The glycated hemoglobin (HbA1c) content was measured using the HbA1c kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to manufacturers' instructions.

2.4.3. *Measurement of GTT and ITT*

At the end of 16 weeks of treatment, GTT and ITT were performed before the mice were sacrificed, as previously described (Xu et al., 2018) and during the same period during the day (9 a.m.–11 a.m.). Briefly, mice were fasted for 12 h, either were injected with glucose (1 g/kg body weight, Sigma-Aldrich, Shanghai, China) for GTT assay or with insulin (1.5 UI/kg body weight, Sigma-Aldrich, Shanghai, China) for ITT assay. The blood samples were taken from the mice's tail tip. The blood glucose concentration was measured using an Accu-Chek Advantage II glucometer (Roche Diagnostic, Switzerland) at 0, 15, 30, 45, 60, 90 and 120 min after injection. The GTT and ITT are expressed as area values under the curve (AUC).

2.4.4. *Measurement of DPP-4 activity, C-peptide, and GLP-1 levels*

Plasma C-peptide and active glucagon-like peptide-1 (GLP-1) levels were measured using the mouse C-peptide (Avivasysbio, San Diego, California, USA) and the mouse GLP-1 (active) ELISAs (Millipore, Billerica, Massachusetts, USA), respectively, according to manufacturers' instructions. Plasma DPP-4 level was measured using the mouse DDP4: a DPPIV/CD26 assay kit (Enzo Inc., Farmingdale, New York, USA) according to manufacturers' instructions.

2.4.5. *Measurement of inflammation markers*

To determine the tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1β, and IL-6 in the plasma, the mouse ELISA kit (No. BMS607-3, Invitrogen, CA, USA), the mouse ELISA kit (No. RAB0274, Sigma-Aldrich, MO, USA), and the mouse ELISA kit (No. 550950, BD Biosciences, CA, USA) were used, respectively, according to the manufacturer's protocols. The NF-κB P65 activity in the liver was assessed using the mouse NF-κB P65 ELISA kit (Cayman Chemical, Michigan, USA) in accordance with the guidelines provided by the manufacturer. Similarly, the activator protein 1 (AP1) activity was quantified through an ELISA-based assay (Cayman Chemical, Michigan, USA) following the instructions provided by the manufacturer.

2.4.6. Total RNA extraction and Real-Time quantitative PCR (RT-qPCR)

Total RNA was extracted from liver tissues using Trizol reagent (Takara Biotechnology Co. Ltd., Japan), followed by cDNA synthesis from 2 μg of total RNA using the Prime Script RT reagent kit (Takara Biotechnology Co. Ltd., Japan). Subsequently, RT-qPCR was conducted using SYBR Green real-time PCR Master Mix (Takara Biotechnology Co. Ltd., Japan) on a CFX quantitative PCR System (Bio-Rad, CA, USA). The following primer sequences that were designed by the National Center for Biotechnology Information (US National Library of Medicine, https://www.ncbi.nlm.nih.gov/nuccore/) and synthesized by Generay Biotechnology (Shanghai, China) are presented in **Table S3**. The relative expression of each targeted gene was normalized to the β-Actin threshold cycle (CT) value and quantified using the comparative threshold cycle $2^{-\Delta\Delta CT}$ method.

2.5. Statistical analysis

The *in vitro* experiments were repeated a minimum of three times, and the outcomes were presented as the mean value accompanied by the standard deviation. To identify any significant disparities ($p < 0.05$) between the samples, a one-way analysis of variances (ANOVA) was employed for comparing the treatments, followed by Tukey's test to distinguish the means. The statistical software TIBCO Statistica 13.3 (TIBCO Statistica Ltd, Palo Alto, CA, USA) was utilized for conducting the analyses.

3. Results and discussion

3.1. Identification of DPP-4 inhibitory peptides from the hydrolysates

For the whole Baltic herring hydrolysates (BHH), 1150 peptides were identified by LC-MS/MS. Of these, 499 peptides (43.4 %) were predicted with DPP-4 activity *in silico*. Short peptides of 6 amino acids were the most abundant 158 peptides, and 100 longer peptides (≥10 AA) were removed for further selection. After filtration with digestive stability, 80 peptides were considered abundant and of DPP-4 inhibition potential (Table 1). In this selection, we considered peptides released from the proteolytic hydrolysis relatively stable and do not contain trypsin/chymotrypsin cleavage sites. Of course, longer peptides after digestion could also release inhibitory peptides and contribute to the final bioactivity. Small peptides with less than 6 amino acids were not possible for identification through databased proteomic searching, because they may lose their peptide identity. Many identified peptides originated from various collagen types, for example, collagen alpha-1(II, V, XI, XIV, XVIII, XXVIII), alpha-2 (I, V, VI, VIII), alpha-5 (IV), and alpha-6 (IV) collagen, and other fish muscles related protein, for example, myosin heavy chain (**SI 1**). Metabolic proteins (such as creatine kinase, non-specific serine/threonine protein kinase) were identified as the source of protein contributed to the release of potential DPP4-inhibitory peptides (**SI 1**). *De novo* sequencing without a protein database is another approach for peptide identification, possibly even short peptides, in return, the origin of the identified peptides would stay unknown (Piovesana et al., 2018).

Table 1
Peptide identification and steps of DPP-4 inhibitory peptides selection.

Step	Baltic herring byproducts hydrolysates	Whole Baltic herring hydrolysates
1. Number of peptides peptides identified by LC-MS/MS	985	1150
2. Number of peptides peptides with potential *in silico* DPP4 activity prediction	457	499
3. Number of peptides peptides and their AA length from the *in silico* prediction	6 AA: 117 7 AA: 88 8 AA: 67 9 AA: 37 10 AA: 43 11 AA: 42 12 AA: 22 13 AA: 11 14 AA: 7 15 AA: 16 16 AA: 1 17 AA: 2 18 AA: 2 19 AA: 2	6 AA: 158 7 AA: 98 8 AA: 79 9 AA: 64 10 AA: 28 11 AA: 23 12 AA: 20 13 AA: 6 14 AA: 6 15 AA: 4 17 AA: 3 18 AA: 1 19 AA: 1 20 AA: 1 22 AA: 1
4.Number of peptides ≤ 9 AA	309	399
5. Select Top 100 abundant peptides	100	100
6.Remove peptides containing R/K (trypsin cleavage, not before P), and F/Y/W (chymotrypsin cleavage, not before P) and C residues.	86	80

For the Baltic herring byproducts hydrolysates (BHBH), 985 peptides were identified by LC-MS/MS. Of which, 457 peptides (46.4 %) were predicted with DPP-4 activity *in silico*. Short peptides of 6 amino acids were the most abundant 128 peptides, and 148 longer peptides (≥10 AA) were removed for further selection. Considering the abundance of peptides from MS relative ion intensity, 86 were considered potential DPP-4 inhibitory peptides (**SI 1**). Similarly, many peptides originated from various types of collagen proteins, for example, collagen alpha-1 (II, V, VII, VIII, IX, XI, XIX, XVII), alpha-2 (I, V), alpha-3 (V), alpha-5 (IV), alpha-6 (IV) and elastin (**SI 1**). Peptide side-chain modifications were also observed, such as oxidation of proline residues to hydroxyproline (HyP), and deamidation of glutamic acid residues during the process. Many animals, plants, and macroalgal proteins are used as substrates for generating DPP-4 inhibitory peptides. Especially animal proteins, such as bovine proteins (α-lactoalbumin, whey), and aquatic proteins (salmon gelatin, mussels) have been reported to have potent DPP-4 inhibition activity from enzymatic hydrolysis (Nongonierma & FitzGerald, 2017). Our results agreed with the previous proposal that collagen proteins were superior substrate for DPP-4 inhibitory peptides due to their high content of proline and/or hydroxyproline (R. Zhang et al., 2016; Y. Zhang et al., 2016).

3.2. DPP-4 inhibitory activities of the Baltic herring hydrolysates

The confirmed *in silico* bioactivity prediction with a score over 294 indicating potential DPP-4 inhibitory activity, peptides PPVEEP (score 456.6) and GPAGDPA (score 321.8) were selected from BHBH and synthetized, which had *in vitro* DPP-4 inhibitory activity IC_{50} value 936 μM and 1049 μM, respectively. From other identified peptides, peptide HyP-HyP-GRPGF (score 355.3) and GADPEDVIVS (score 267.9) did not have *in vitro* DPP-4 inhibitory activity or even being a substrate for DPP-4 enzymes (Fig. 1). To note, the iDPPIV-SCAM tool does not allow modified peptides; instead of HyP, the input was Pro residues. Potent DPP-4 inhibitory peptides have IC_{50} less than 100 μM, especially di-, tri-peptides have strong activity, for example, WP (IC_{50} = 45 μM), IA (IC_{50} = 88 μM), IPI (IC_{50} = 3.2 μM), IPA (IC_{50} = 49 μM) (Nongonierma & FitzGerald, 2017). However, dipeptides PP of IC_{50} 4344 μM and GF of IC_{50} 1547 μM were also reported (Neves et al., 2017) and longer peptides with strong IC_{50} value, for example, CAYQWQRPVDRIR of IC_{50} 78 μM, PACGGFYISGRPG of IC_{50} 96 μM (Huang et al., 2012). Nongonierma & FitzGerald (2014) revealed that the presence of the hydrophobic amino acids at the N terminus was an essential feature for DPP-4 inhibitory activity, rather than the overall hydrophobicity, molecular mass, charge, and isoelectric point. Because the DPP-4 enzyme prefers to cleave at Pro, Ala, and relatively less extent at Gly, Ser and Thr at position 2 (Nabeno et al., 2013; Nongonierma & FitzGerald, 2014), the inhibitory peptides with a competitive mechanism usually contain high content of these amino acids. From BHH, these amino acids taking up 63 % of total amino acids of the proposed DPP-4 inhibitory peptides, including Pro (25 %),

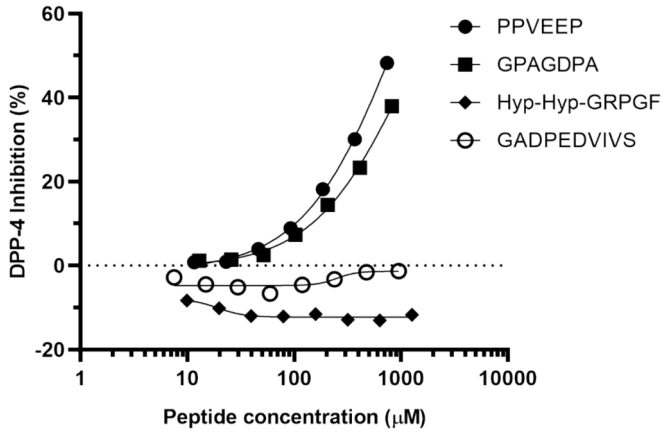

Fig. 1. DPP-4 inhibitory assay of selected synthetic peptides from Baltic herring hydrolysates.

Ala (10 %), Gly (18 %), Ser (5 %) and Thr (4 %); from BHBH, these amino acids taking up 71 %, including Pro (30 %), Ala (12 %), Gly (24 %), Ser (3 %) and Thr (2 %) (**SI 1**). Evaluating the IC_{50} value of all the proposed peptides would not be feasible. However, our results showed that the fish hydrolysates contained a high number of DPP-4 inhibitory peptides with moderate activity.

3.3. Body weight, glucose, and insulin

BHH, as a rich source of peptides, was supplemented in the diet of male C57BL/6J mice, where inflammation were induced through a high-fat, high-fructose diet. The effects of a low dose (LD-BHH) and high dose (HD-BHH) in comparison with the HFD + sitagliptin as the positive control (PC) were presented in Fig. 2. From Fig. 2**A** it was possible to observe that the experimental model successfully increased the mice's body weight in all groups except for the mice fed a standard (e.g., low-fat) diet. BHH was ineffective in preventing body weight gain in this high-fat, high-fructose model of obesity. This result contrasted those reported by (Lee et al., 2017), who used a high-fat model in ICR mice and supplemented the diet with hydrolyzed tuna collagen peptides (300

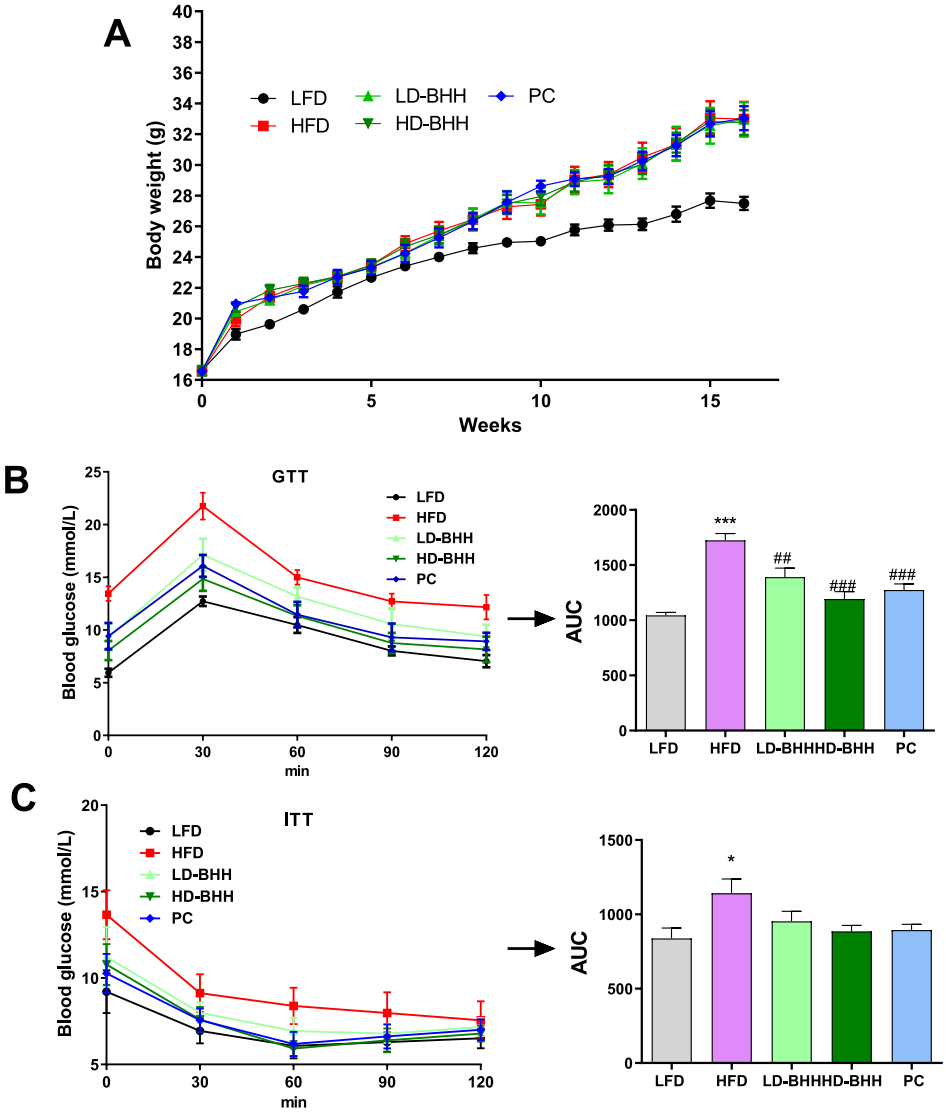

Fig. 2. The effect of Baltic herring hydrolysate on HFD-induced body weight gain and insulin resistance in C57BL/6J mice. Mice were fed a low-fat diet or high-fat and high-sucrose diet supplemented with Baltic herring hydrolysate (0.6 mg/mL and 1.8 mg/mL) or sitagliptin (0.1 mg/mL) as the positive control in drinking water for 16 weeks. **(A)** Body weight was monitored every week. **(B)** Glucose Tolerance Test (GTT) and **(C)** Insulin Tolerance Test (ITT) at 23-week-old mice. Data were presented as the mean ± SEM (n = 8/group). $^{*}p < 0.05$ or $^{***}p < 0.001$ *versus* LFD group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ or $^{\#\#\#}p < 0.001$ *versus* HFD group.

mg/kg/day). The authors found that the fish peptides could significantly reduce body weight gain and adipocyte size after 8 weeks of treatment. This inconsistency may indicate that the BHH does not have a regulatory effect on lipid metabolism *in vivo*, but further research is needed. In addition, there were no statistically significant variations in the consumption of food or water intakes across the different groups (Data not shown).

The effects of BHH on how quickly circulating glucose is cleared from the bloodstream (i.e., GTT) and ITT (a measure of insulin resistance) are shown in Fig. 2**B and 2C**, respectively. It is possible to conclude that both LD-BHH and HD-BHH were able to decrease ($p < 0.05$) glucose levels compared to the HFD group. Interestingly, the effects of LD-BHH and HD-BHH were similar ($p > 0.05$) to the positive control, showing the protective effects of peptides on glucose metabolism. Similarly, increased insulin resistance as measured by ITT was improved when mice consumed LD-BHH and HD-BHH, but the effects were not statistically significant compared to the HFD group. When considering fasting glucose (Fig. 3**A**), insulin (Fig. 3**B**), HOMA-IR (Fig. 3**C**), and HbA1c levels (Fig. 3**D**), a dose dependent effect was observed: while the HFD significantly increased the glucose and insulin levels, LD-BHH and HD-BHH groups had lower fasting glucose and insulin levels. Regarding the insulin levels, it is noteworthy that the HD-BHH group had approximately 50 % less fasting insulin than the HFD group, and statistically similar ($p > 0.05$) levels compared to the PC. Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) is a method for assessing β-cell function and insulin resistance from basal (fasting) glucose and insulin concentrations. Thus, assessing glucose, insulin, and glycated hemoglobin becomes central to understanding how the chronic consumption of BHH affected these biomarkers.

Research utilizing *in vivo* protocols (e.g., animal studies and human interventions) have shown that acute and, especially, chronic consumption of fish peptides can stimulate GLP-1 secretion, enhancing insulin release from pancreatic β-cells, increasing glucose uptake and tolerance and, consequently, reducing blood glucose levels (Chevrier et al., 2015; Pilon et al., 2011; R. Zhang et al., 2016). For example, Chevrier et al., (2015) supplemented male LDLR$^{-/-}$/ApoB$^{100/100}$ mice with peptides (10 g/kg) from Atlantic salmon frames for 12 weeks in a high-fat and sucrose obesity model. They observed improved GTT and ITT and glucose uptake. Similarly, Zhang et al., (2016) used KM mice with induced diabetes to study any beneficial effects of tilapia skin collagen peptides (0.85 or 1.7 g/kg) and found that, after 25 days of treatment, 30 % of blood glucose levels were decreased compared to the untreated group. This hypoglycemic effect of fish peptides was comparable to metformin, the gold standard drug used by diabetic individuals.

3.4. Plasma DPP-4 activity, GLP-1 content, and C-peptide level

We investigated the impact of BHH treatments on DPP-4 activity, C-peptide, and GLP levels in high-fat, high-fructose diet-fed mice (Fig. 4**A–C**). The activity of DPP-4 was found to be higher in the plasma of mice fed a high-fat diet compared to those fed a low-fat diet, as shown in Fig. 4**A**. Although LD-BHH treatment did not affect DPP-4 activity in the plasma of mice fed a high-fat, high-fructose diet, HD-BHH treatment significantly decreased DPP-4 activity in the plasma of high-fat, high-fructose-fed mice. Interestingly, the effects of HD-BHH were similar to the positive control (sitagliptin), showing the inhibitory effects of BHH on DPP-4 activity. The finding is consistent with the observation of *in vivo* DPP-4 inhibition activity in the plasma of Atlantic salmon skin gelatin hydrolysate from streptozotocin-induced diabetic rats (Hsieh et al., 2015). Following the conclusion of the 16-week experiment, it was observed that the plasma active GLP-1 level in normal mice measured 18.45 pM, a significantly higher value compared to the mice in the HFD group, whose levels measured 14.84 pM ($p < 0.05$). In contrast, the levels of active GLP-1 in the mice from the HFD and LD-BHH groups

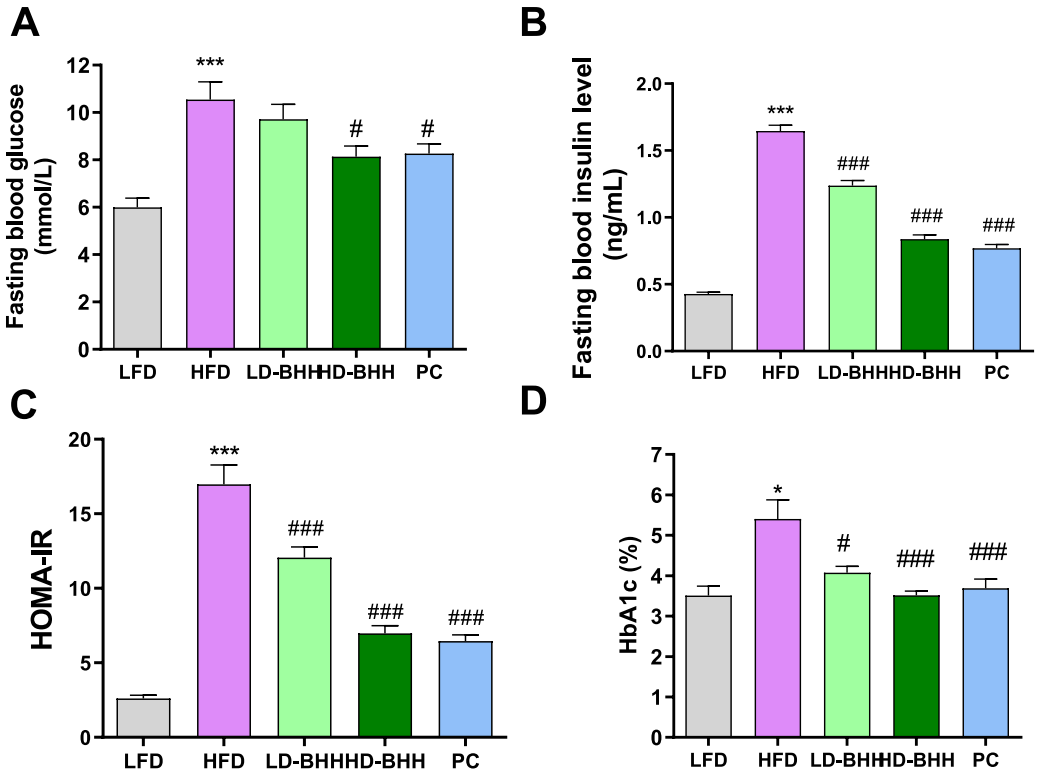

Fig. 3. The effect of Baltic herring hydrolysate on HFD-induced hyperglycemia in C57BL/6J mice. Mice were fed a low-fat diet or high-fat and high-sucrose diet supplemented with Baltic herring hydrolysate (0.6 mg/mL and 1.8 mg/mL) or sitagliptin (0.1 mg/mL) as the positive control in drinking water for 16 weeks. **(A)** Fasting blood glucose and **(B)** Insulin at 24-week-old mice. **(D)** Homeostatic Model Assessment-Insulin Resistance (HOMA-IR). **(D)** HbA1c content at 24-week-old mice. Data were presented as the mean ± SEM (n = 8/group). $^{*}p < 0.05$ or $^{***}p < 0.001$ *versus* LFD group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ or $^{\#\#\#}p < 0.001$ *versus* HFD group.

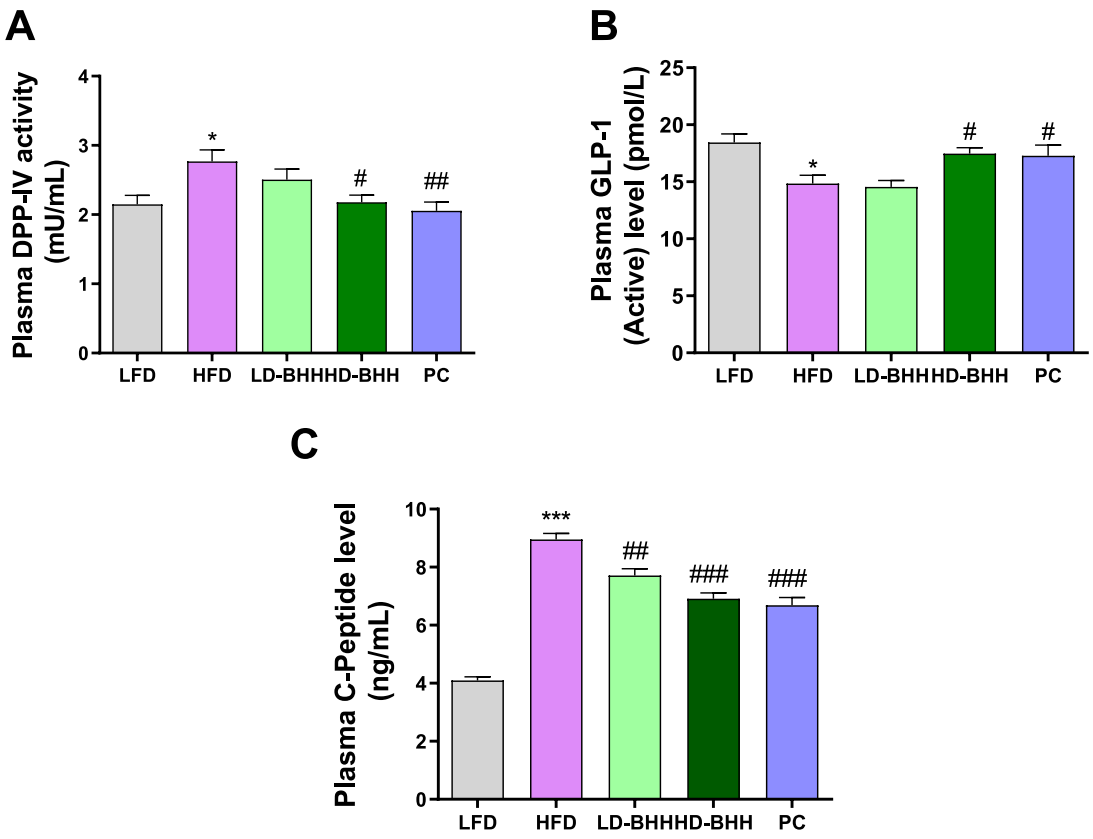

Fig. 4. The effect of Baltic herring hydrolysates on the plasma DPP-4 activity, GLP-1 content, and C-peptide levels of C57BL/6J mice. **(A)** Plasma DPP-4 activity. **(B)** Plasma glucagon-like peptide-1 (GLP-1) level. **(C)** Plasma C-peptide level. Data were presented as the mean ± SEM (n = 8/group). $^{*}p < 0.05$ or $^{***}p < 0.001$ *versus* LFD group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ or $^{\#\#\#}p < 0.001$ *versus* HFD group.

exhibited no statistically significant differences ($p > 0.05$). Moreover, the plasma active GLP-1 level of the HD-BHH group (17.47 pM) and positive control group (17.28 pM) mice were significantly higher than those of the HFD mice ($p < 0.05$) (Fig. 4**B**). Compared with a low-fat diet, a high-fat diet induces a decrease in plasma active GLP-1 levels in mice, which is consistent with the results reported by (Liu et al., 2021). This finding suggests that mice with HFD had higher clearance and degradation of GLP-1 by DPP-4.

The insulin secretion function was assessed by measuring the levels of C-peptide in the plasma of mice. As depicted in Fig. 4**C**, the LD-BHH and HD-BHH groups exhibited a significant decrease in plasma C-peptide levels after 16 weeks of dietary intervention, compared to the HFD group. These findings indicate an improvement in the mice's insulin secretion function following BHH dietary intervention. Additionally, the levels of C-peptide and HOMA-IR were notably reduced after the BHH dietary intervention, suggesting a decrease in insulin resistance and an enhancement in blood glucose stability among HFD mice. These results indicate that BHH also has good hypoglycemic activity.

3.5. Inflammation markers

It is becoming increasingly apparent that obesity is a form of inflammatory disorder that directly impacts insulin resistance and glucose and lipid metabolism (Jensen et al., 2020). The anti-inflammatory effect of BHH in high-fat diet-induced hyperglycemic mice was evaluated by measuring the levels of Inflammatory mediators in the plasma and liver in mice. As shown in Fig. 5**A–C**, the HFD group had fasting plasma TNF-α, IL-1β, and IL-6 levels higher than that of the LFD group. LD-BHH and HD-BHH reduced ($p < 0.01$ and $p < 0.001$) plasma TNF-α levels compared to the HFD group. Similar effects were also observed for the plasma IL-1β, and IL-6 levels. As shown in Fig. 5**D**, the high-fat diet induced hyperglycemia in mice, resulting in higher levels of hepatic inflammatory mediators (TNF-α, IL-1β, and IL-6) in the HFD compared to the LFD group. LD-BHH and HD-BHH reduced these hepatic inflammatory mediators' mRNA levels when compared to the HFD group.

Subsequently, we investigated the impact of BHH intervention on hepatic inflammation. Consistent with our expectations, the mRNA levels of Cd68, F4/80, and M1 marker (Cd11c) exhibited a significant decrease in the LD-BHH and HD-BHH groups compared to the HFD group (Fig. 5**E**). Additionally, there was a noticeable trend of elevated expression of the M2 marker Cd260, suggesting a reduction in the quantity of infiltrated macrophages and an alleviation of the M1/M2 polarization imbalance within the liver. To elucidate the molecular mechanism, we analyzed the activation of the NF-κB and AP1 pathways. Our findings indicated that the AP1-binding activity (Fig. 5**F**) was notably diminished in mice subjected to a HFD and treated with HD-BHH ($p < 0.05$), suggesting its potential role in inhibiting the activation of the inflammatory AP1 pathway. Furthermore, we observed a reduction in the activity of NF-κB p65 (Fig. 5**G**, $p < 0.05$) in the HD-BHH group, which further supports these findings. BHH treatment was shown to inhibit NF-κB activation, which is consistent with a previous study that FSGHF3 and peptides, prepared from fish skin gelatin prevented dextran sodium sulfate-induced elevations of pro-inflammatory cytokine production in mice, through suppression of NF-κB activation (Deng et al., 2020).

4. Conclusion

The current study presented an approach for identifying and selecting bioactive peptides, and linked peptide molecular information to the *in vivo* intervention study in an obesity model in mice. Due to the unique amino acid composition of Baltic herring proteins, DPP-4 inhibitory

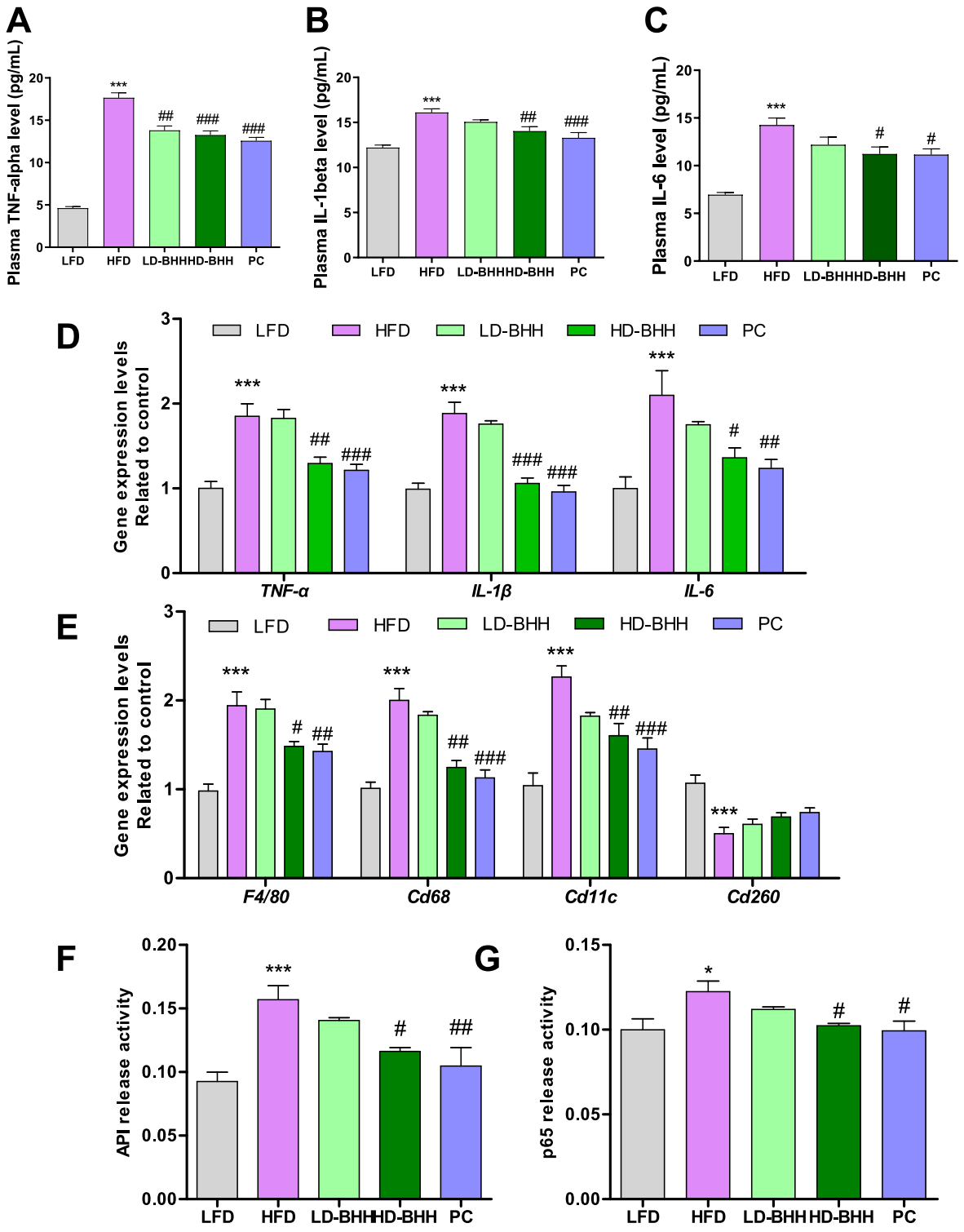

Fig. 5. The effect of Baltic herring hydrolysates on the inflammation markers of C57BL/6J mice. **(A)** plasma tumour necrosis factor-α (TNF-α) level. **(B)** Plasma IL-1β level. **(C)** Plasma IL-6 level. **(D)** mRNA expression of hepatic inflammatory mediators (TNF-α, IL-1β, and IL-6). **(E)** mRNA expressions of F4/80, Cd68, M1 marker Cd11c, and M2 marker Cd206. **(F)** Activator protein-1 (AP1)-binding activity. **(G)** NF-κB p65 level. Data were presented as the mean ± SEM (n = 8/group). $^{*}p < 0.05$ or $^{***}p < 0.001$ *versus* LFD group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ or $^{\#\#\#}p < 0.001$ *versus* HFD group.

peptides were liberated after proteolytic hydrolysis. This study proposed 86 peptides from Baltic herring byproducts hydrolysates and 80 from whole Baltic herring hydrolysates (BHH) as novel DPP-4 inhibitory peptides. In addition to *in vitro* assay and peptide identification, BHH was fed to a mice intervention of a high-fat, high-fructose diet to validate the bioactivity. Glucose tolerance and insulin tolerance improved when mice consumed the peptides. While plasma-like DPP-4 levels increased, plasma C-peptide, GLP-1 and HOMR-IR scores and inflammatory mediators significantly decreased. BHH is an inexpensive source for functional antidiabetic food applications.

CRediT authorship contribution statement

Dongxu Wang: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis. **Xin Huang:** Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pertti Marnila:** Writing – review & editing, Methodology. **Jaakko Hiidenhovi:** Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Anna-Liisa Välimaa:** Writing – review & editing. **Daniel Granato:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Sari Mäkinen:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, 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.org/10.1016/j.foodres.2024.114696.

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