

Research Article



α -Glucosidase Inhibitory and Antibacterial Activity of Fermented Mackerel By-Product Muscle and Skin Hydrolysates: *In Vitro* and *In Silico* Peptidomics Analysis

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ABSTRACT

Mackerel (*Scomberomorus commerson*) processing by-products represent an abundant yet underutilized protein source that can be valorized into bioactive peptides through microbial fermentation. This study investigated the α -glucosidase inhibitory and antibacterial activities of hydrolysates produced from fermented mackerel muscle, skin, and mixed by-products using *Pediococcus pentosaceus* IL13, with ultrafiltration applied to enrich low-molecular-weight peptides (<3 kDa). All hydrolysates exhibited concentration-dependent α -glucosidase inhibitory activity, with the mixed hydrolysate showing the lowest IC₅₀ value (34.692 mg/mL). The <3 kDa fractions consistently displayed the strongest antibacterial activity against both Gram-positive and Gram-negative bacteria, particularly *Listeria monocytogenes*, indicating enrichment of potent low-molecular-weight antibacterial peptides. LC-HRMS-based peptidomic analysis identified 211 peptides, from which 35 representative sequences were selected based on relative abundance for *in silico* evaluation. Several peptides were predicted to be novel, non-toxic, and non-allergenic. Among them, the collagen-derived peptide SPAMPVPGPMGPMGPR exhibited the highest predicted antidiabetic potential, whereas AVFPSIVGRPR was identified as the most promising antibacterial peptide based on consensus predictions from multiple antimicrobial peptide prediction platforms. These findings demonstrate that fermentation coupled with ultrafiltration effectively generates multifunctional bioactive peptides from mackerel by-products, supporting their sustainable utilization as promising ingredients for functional foods and nutraceutical applications.



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1. Introduction

The valorization of marine resources, particularly fisheries by-products, presents a dual solution to the challenges of waste management and the discovery of

novel therapeutics. The seafood processing industry generates substantial volumes of by-products, many of which remain underutilized, accounting for approximately 50-60% of total marine biomass, or an estimated 20-30 million metric tons annually (Rahaman *et al.* 2026). These materials, comprising muscles (10%), skin and trimmings (3.5%), bones (9-15%), heads (10%), guts (12.5%), and scales (3.5%),

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account for nearly 60% of total processed seafood waste (Jimenez-Champi *et al.* 2024). If not properly managed, the continuous accumulation of such by-products poses significant environmental risks; growing recognition of these challenges has therefore encouraged the fisheries sector to adopt zero-waste and circular economy principles as a strategy aligned with the United Nations Sustainable Development Goal 12 (Responsible Consumption and Production) for fostering sustainable and environmentally responsible production practices (Waqar *et al.* 2025; Khawari *et al.* 2026).

Given their rich biochemical composition and functional potential, fishery by-products are increasingly being valorized through fermentation, a sustainable and environmentally friendly bioprocessing approach. Fermentation utilizes microbial metabolic activities to convert complex substrates into valuable products, including enzymes and primary and secondary metabolites, and to produce unique flavors (Adebo *et al.* 2017; Wang *et al.* 2025). During this process, microorganisms secrete proteolytic enzymes that efficiently hydrolyze macromolecules, such as proteins, into smaller, more soluble peptides and free amino acids, thereby enabling an economical and effective approach to protein valorization (Aspevik *et al.* 2017; Cruz-Casas *et al.* 2023; Xu *et al.* 2023). These peptides, which are initially inactive within native protein structures, must be released through proteolysis or hydrolysis to exhibit their biological functions (Jimenez-Champi *et al.* 2024). Fish by-products represent an abundant source of bioactive peptides composed of 2-20 amino acids (500-3,000 Da) that can be generated through enzymatic digestion or microbial fermentation, with the latter considered a more natural and traditional approach that not only enhances flavor and preservation but also more effectively improves the nutraceutical value of the resulting hydrolysates (Nasri 2019; Waqar *et al.* 2025).

Previous studies demonstrated that hydrolysates derived from the muscle, skin, and mixed tissues of *Scomberomorus commerson* fermented by *Pediococcus pentosaceus* IL13 exhibited strong antioxidant and anti-inflammatory activities, with 211 peptide sequences identified using LC-HRMS (Ariandi *et al.* 2026). Building on these findings, the present study conducts an initial bioactivity screening, evaluating α -glucosidase inhibition as preliminary evidence of antidiabetic potential and assessing antibacterial activity against Gram-positive and Gram-negative pathogens. Fish-derived peptides have

previously demonstrated broad-spectrum antibacterial effects against pathogens (Coppola *et al.* 2021), while the collagen-derived peptide SIFIQRFTT from *Scomber scombrus* completely inhibited both Gram-positive and Gram-negative bacteria (Ennaas *et al.* 2015). Regarding antidiabetic activity, bioactive peptides VLLQKNNDT and LSMQRAQEE from Sturgeon muscle protein showed significant α -glucosidase inhibition (Liu *et al.* 2025), and collagen hydrolysates from *Aluterus monoceros* skin exhibited notable α -amylase inhibition of 71.17-80.45% (Kumar *et al.* 2019). Collectively, these findings highlight the promising potential of fish-derived peptides as multifunctional bioactive compounds for antidiabetic potential and antibacterial applications.

However, although *in vitro* assays provide direct evidence of biological activity, they do not identify the specific peptide sequences responsible for the observed effects or explain their underlying mechanisms of action. To address this limitation, liquid chromatography highresolution mass spectrometry (LC-HRMS) based peptidomics has emerged as a powerful analytical approach for the comprehensive identification of bioactive peptides generated during microbial fermentation (Hayes *et al.* 2024; Zhang *et al.* 2024). The identified peptide sequences can subsequently be subjected to *in silico* analyses to predict their bioactivity, toxicity, and allergenicity, thereby facilitating the rapid prioritization of promising candidates before experimental validation (Sharma *et al.* 2021; Rathore *et al.* 2024). In addition, specialized computational platforms, including AntiDMP-pred and AntiT2DMP-Pred, enable prediction of antidiabetic peptides targeting glucose metabolism-related pathways (Chen *et al.* 2022; Basith *et al.* 2025), whereas CAMP_{R4}, Antimicrobial Peptide Scanner v2, ADAM, and AntiBP3 are widely used to evaluate the antimicrobial potential of peptide sequences (Lee *et al.* 2015; Veltri *et al.* 2018; Gawde *et al.* 2023; Bajiya *et al.* 2024).

This study systematically investigated the antidiabetic and antibacterial potential of tissue-specific *Scomberomorus commerson* by-product hydrolysates using an integrated approach combining *in vitro* bioactivity evaluation of crude and ultrafiltered fractions, LC-HRMS-based peptidomic profiling, and *in silico* prediction of peptide bioactivity, toxicity, allergenicity, and antidiabetic and antimicrobial properties. This integrated workflow enabled the identification and prioritization of bioactive peptide candidates associated with the dual biological

activities of the fermented hydrolysates. To the best of our knowledge, this is the first study to comprehensively characterize the antidiabetic and antibacterial potential of fermented *S. commerson* by-product hydrolysates using an integrated *in vitro*, *in silico* peptidomics approach, providing molecular insights into peptide candidates with potential applications as multifunctional ingredients for functional foods and nutraceutical development.

2. Materials and Methods

2.1. Materials and Reagents

Mackerel (*S. commerson*) processing by-products, including skin and mechanically damaged muscle tissues such as fragmented flesh, softened meat, and ruptured abdominal portions, were obtained in January 2025 from the Muara Baru Fish Market, Penjaringan, Jakarta, Indonesia (6°06'36"S, 106°48'06"E). The collected materials were thoroughly washed and homogenized using a blender and a meat grinder prior to packaging and labeling. Samples were initially preserved at -4°C for 24-48 h, transported under chilled conditions in insulated ice boxes, and subsequently stored at -20°C until further analysis.

The lactic acid bacterium *P. pentosaceus* IL13 was obtained from the Microbial Bioprospecting Laboratory at the Research Center for Biotechnology, IPB University. Pathogenic bacterial strains, including *Listeria monocytogenes*, *Enterococcus faecalis*, *Escherichia coli*, and *Salmonella typhi*, were supplied by the Indonesian Culture Collection, National Research and Innovation Agency (BRIN), Indonesia. Cultivation of lactic acid bacteria and pathogenic isolates was carried out using De Man, Rogosa, and Sharpe broth (MRS Broth, HiMedia) and Luria-Bertani broth (LB, HiMedia), respectively. The chemicals and enzymes used in this study included α -glucosidase from *Saccharomyces cerevisiae* (Sigma; 100 U, Type I, ≥ 10 units/mg protein), MS-grade Pierce trypsin protease (Thermo Fisher Scientific), acarbose, chloramphenicol, formic acid, sodium carbonate, p-nitrophenyl- α -D-glucopyranoside (pNPG), p-nitrophenol, phosphate buffer, acetonitrile, and LC-MS-grade water (Fisher Scientific, Fair Lawn, NJ, USA). All additional reagents employed were of analytical grade.

2.2. Fermentation

A glycerol stock of *P. pentosaceus* IL13 was reactivated in MRS broth and cultured at 37°C for 24 h. The culture was then streaked onto MRS agar plates

and incubated at 37°C for 48 h. A single colony was subsequently transferred into 10 mL of MRS broth and grown at 37°C for 24 h. To prepare the starter culture, 1 mL of this pre-culture was inoculated into 100 mL of fresh MRS broth and incubated under identical conditions. Three experimental groups were prepared, consisting of mackerel muscle, mackerel skin, and a 1:1 (w/w) mixture of muscle and skin. For each group, 10 g of the sample was placed into a separate 50 mL Falcon tube, and 40 mL of 10% (w/v) glucose solution was added. The samples were sterilized at 121°C for 15 min and then inoculated with 400 μ L of the *P. pentosaceus* IL13 culture. Submerged fermentation was conducted for 4 days for the muscle and mixed (muscle + skin) samples, and for 5 days for the skin samples (Ariandi *et al.* 2026). The hydrolysates were harvested at the time point corresponding to the optimal growth curve of *P. pentosaceus* IL13. The supernatant was then separated by centrifugation at 14,000 $\times$ g for 30 minutes at 4°C, and the resulting liquid hydrolysate was stored at -20°C.

2.3. Ultrafiltration and Fractionation of Fish Protein Hydrolysates

Fish protein hydrolysates were separated into different molecular weight fractions using an ultrafiltration system equipped with 30, 10, and 3 kDa molecular weight cut-off (MWCO) membranes (Amicon Ultra-15, Millipore Co., Billerica, MA, USA). The fractionation process was carried out by centrifugation at 5,000 $\times$ g for 30 min at 10°C. Permeates obtained from sequential filtration were categorized into three peptide fractions, namely F-I (<30 kDa), F-II (<10 kDa), and F-III (<3 kDa), corresponding to distinct molecular weight distributions. All collected fractions were preserved at -20°C prior to subsequent bioactivity evaluations (Yaghoubzadeh *et al.* 2020).

2.4. α -Glucosidase Inhibitory Activity Assay

The antidiabetic potential of the samples was assessed through an α -glucosidase inhibition assay using a modified procedure based on the method reported by Sariwati *et al.* (2024). Briefly, 50 μ L of diluted sample solution was mixed with 30 μ L of phosphate buffer (pH 6.8) in a microplate to maintain optimal reaction conditions. The enzymatic reaction was initiated by adding 60 μ L of 4-nitrophenyl- α -D-glucopyranoside (pNPG; Sigma-Aldrich) as the chromogenic substrate, followed by 60 μ L of α -glucosidase enzyme solution. A control system without enzyme addition was prepared simultaneously to correct baseline absorbance values. The reaction

mixtures were incubated at 37°C for 30 min to allow substrate hydrolysis. The reaction was then terminated by adding 60 µL of 200 mM Na₂CO₃ to each well. Absorbance measurements were recorded at 405 nm using a microplate spectrophotometer after 10 s of shaking. Acarbose (100 ppm) was used as a positive control, and sterile distilled water as a negative control, to compare the inhibitory activity of the fish protein hydrolysates. The α-glucosidase inhibitory activity was assessed by measuring p-nitrophenol (pNP) released from pNPG. All assays were performed in 96-well microplates using a microplate reader. A pNP standard curve was prepared at concentrations ranging from 0 to 500 ppm in 0.1 M phosphate-buffered saline (PBS, pH 6.8). The absorbance was measured at 405 nm, and linear regression ($R^2 > 0.99$) was used to calculate pNP concentration. To determine the half-maximal inhibitory concentration (IC₅₀), freeze-dried crude hydrolysates were tested at concentrations ranging from 10 to 50 mg/mL. Dose-response curves were generated by plotting percentage inhibition against sample concentration, and IC₅₀ values were calculated using nonlinear regression analysis in GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA).

2.5. Antibacterial Activity Assay

The antibacterial activity of the crude and fractionated fish protein hydrolysates was evaluated against *L. monocytogenes*, *E. faecalis*, *E. coli*, and *S. typhi* using the agar well diffusion technique (Chalisya *et al.* 2025). Each bacterial isolate was first cultured in Luria-Bertani (LB) broth at 37°C for 24 h. The bacterial suspensions were subsequently adjusted to a turbidity equivalent to 0.5 McFarland standard, corresponding to approximately 1×10^8 CFU/mL. A 100 µL aliquot of the standardized culture was mixed with molten LB agar maintained at approximately 45°C and poured into sterile Petri plates. After agar solidification, wells with a diameter of 6 mm were aseptically prepared using a sterile corkborer. Filter-sterilized hydrolysate samples (0.22 µm membrane filtration) were then added into each well at a volume of 100 µL. The inoculated plates were kept at room temperature for 1 h to allow diffusion of the sample prior to incubation at 37°C for 24 h. Antibacterial activity was determined by measuring the diameter of the clear inhibition zones formed around the wells and expressed in millimeters. All experiments were conducted in triplicate. Sterile distilled water was used as a negative control, while

chloramphenicol solution at 100 ppm was employed as a positive control.

2.6. Proteomics Analysis using LC-HRMS

Peptide profiling was performed using Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) following Windarsih *et al.* (2022) and Molinari *et al.* (2023). 100 mg freeze-dried hydrolysate was suspended in 1 mL 50 mM ammonium bicarbonate buffer (pH 8.0) and centrifuged at $14,000 \times g$ for 20 min at 4°C. A 500 µL supernatant was mixed with an equal volume of acetone (−20°C), incubated at −20°C for 4 h, and centrifuged at $16,000 \times g$ for 20 min at 4°C. The pellet was dried at 45°C for 30 min and reconstituted in 500 µL buffer. Reduction was performed with 10 µL 10 mM dithiothreitol (DTT) at 56°C for 60 min, followed by alkylation with 10 µL 20 mM iodoacetamide (IAA) in the dark for 60 min. Digestion was performed with trypsin (0.1 mg/mL) for 17 h at 37°C. The reaction was terminated with 500 µL 1% trifluoroacetic acid (TFA), followed by centrifugation at $8,100 \times g$ for 5 min and filtration through a 0.2 µm PVDF membrane. Peptides were separated using a Vanquish™ Horizon UHPLC system with a PepMap™ 100 C18 column (150 mm × 1 mm, 3 µm) at 75 µL/min and 30°C, using water and acetonitrile containing 0.1% formic acid as solvents A and B. MS analysis was conducted on an Orbitrap Exploris 240 HRMS with an OptaMax™ NG H-ESI source in positive mode under Full MS/dd-MS² acquisition, at resolutions of 120,000 and 15,000 FWHM, respectively. Raw data were processed using Proteome Discoverer 2.5 with SequestHT against UniProt and NCBI databases, and label-free quantification (LFQ) was used to determine relative peptide abundance.

2.7. Assessment of Bioactive Potential of Identified Peptides

A total of 211 peptide sequences identified from the fish protein hydrolysates were ranked and selected based on their detected relative abundance for subsequent bioactivity evaluation. The biological potential of the selected peptides was assessed using several in silico prediction approaches. Briefly, peptide bioactivity probability was estimated using the PeptideRanker platform <http://distilldeep.ucd.ie/PeptideRanker/>, accessed on 22 November 2025. In addition, the novelty of the identified peptide sequences was investigated through literature mining and database comparison using BIOPEP-UWM (<https://biochemia.uwm.edu.pl/biopep-uwm/>), accessed on 20 November 2025.

2.8. Assessment of Toxicity and Allergenicity of Identified Peptides

Selected peptide sequences were subjected to *in silico* toxicity and allergenicity evaluation using several computational platforms. Toxicity prediction was performed using ToxinPred (<https://webs.iiitd.edu.in/raghava/toxinpred/ algo.php>, accessed on 23 November 2025), whereas allergenicity assessment was conducted using pLM4Alg-640, AlgPred2, and AllerCatPro accessed on 25-26 November 2025. For the pLM4Alg analysis (<https://f6wxpfd3sh.us-east-1.amazonaws.com>), the pLM4Alg-640 model was employed to classify peptide sequences as allergenic or non-allergenic. The numerical value associated with the model designation (pLM4Alg-640) represents the dimensionality of the embedding vectors utilized for sequence analysis (Du *et al.* 2024). This model was selected because it provides the highest-dimensional embedding architecture available within the platform. Allergenicity prediction using AlgPred2 (<https://webs.iiitd.edu.in/raghava/algpred2/>) was performed via the batch prediction module with the default hybrid configuration (RF + BLAST + MERCI) and a threshold of 0.3. In addition, peptide sequences were analyzed using AllerCatPro (<https://allercatpro.bii.a-star.edu.sg/>) to obtain detailed allergenicity profiles in comma-separated output format. Final predictions from all platforms were compiled and categorized as allergenic or non-allergenic based on the respective algorithm outputs.

2.9. *In Silico* Prediction of Antidiabetic and Antimicrobial Peptide Activities

The biological potential of the identified peptides was further investigated through *in silico* analysis to evaluate their predicted antidiabetic and antimicrobial properties. Antidiabetic activity was assessed using AntiDMP-pred (<https://i.uestc.edu.cn/AntiDMPpred/cgi-bin/AntiDMPpred.pl>) and AntiT2DMP-Pred (<https://balalab-skku.org/AntiT2DMP-Pred/>), accessed on 27 November 2025. Prediction scores generated by both platforms ranged from 0 to 1, with values exceeding 0.5 indicating potential antidiabetic activity. The antimicrobial potential of the peptides was subsequently estimated using multiple computational algorithms, including CAMP_{R4} (<http://camp.bicnirrh.res.in/> accessed on 27 November 2025). This platform assesses the probability that a peptide sequence exhibits antimicrobial activity using Random Forest, Support Vector Machine, and Artificial Neural Network

models. Each sequence is assigned a score from 0 to 1. Only peptides with a score ≥ 0.5 are considered potentially antimicrobial. Antimicrobial Peptide Scanner vr.2 (<https://www.dveltri.com/ascan/>, accessed on 28 November 2025), ADAM (ADAM <https://bioinformatics.cs.ntou.edu.tw/ADAM/>, accessed on 28 November 2025), and AntiBP3 (<https://webs.iiitd.edu.in/raghava/antibp3/predict.php>, accessed on 29 November 2025). These platforms were used to predict the probability that peptides exhibit antimicrobial activity based on sequence-derived physicochemical and structural characteristics.

2.10. Statistical Analysis

All experimental data were analyzed using Microsoft Excel, GraphPad Prism, and IBM SPSS Statistics version 27 (IBM Corp., Armonk, NY, USA). Statistical differences among treatment groups were evaluated by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test, with significance established at $P < 0.05$.

3. Results

3.1. α -Glucosidase Inhibitory Activity

The α -glucosidase inhibitory activity of crude and fractionated hydrolysates from fermented mackerel by-products is presented in Figure 1. All hydrolysate samples exhibited considerable antidiabetic activity, with inhibition values ranging from approximately 61.97 to 79.09%, and significant differences were observed among treatments ($p < 0.05$). In general, ultrafiltration enhanced the inhibitory activity of the hydrolysates, particularly in the lower-molecular-weight fractions. The <3 kDa fractions consistently showed the strongest α -glucosidase inhibition in Fish Muscle Hydrolysate (FMH), Fish Skin Hydrolysate (FSH), and Fish Mix Hydrolysate (FXH), reaching approximately 79.45%, 78.03%, and 79.03%, respectively, and were significantly higher than those of the corresponding crude hydrolysates. Similarly, the <10 kDa fractions exhibited high inhibitory activity, although slightly lower than the <3 kDa fractions. Among the crude hydrolysates, FXH exhibited the highest inhibitory activity compared with FMH and FSH. Notably, several ultrafiltered fractions demonstrated inhibitory activities comparable to or exceeding that of the positive control (acarbose 100 ppm), whereas the negative control showed minimal inhibition. These findings indicate that ultrafiltration

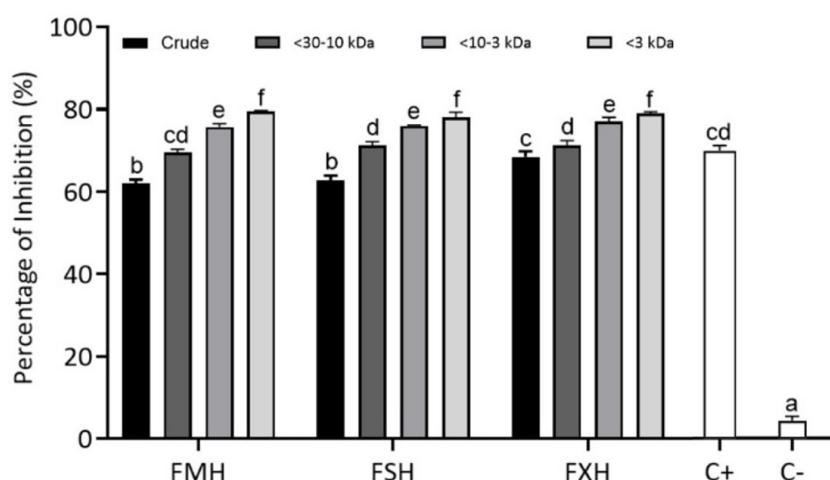


Figure 1. α -glucosidase inhibitory activity of crude and fractionated hydrolysates obtained by ultrafiltration (<30, <10, and <3 kDa) assessed by α -glucosidase inhibition. FMH (fish muscle hydrolysate), FSH (fish skin hydrolysate), FXH (fish mix hydrolysate), C+ (positive control, acarbose 100 ppm), and C- (negative control, aquades steril). Different superscript letters indicate significant differences among treatments ($p < 0.05$)

effectively concentrated low-molecular-weight bioactive peptides with potent α -glucosidase inhibitory properties.

Table 1 presents the α -glucosidase inhibitory activity of crude and ultrafiltered fish protein hydrolysates as evaluated by p-nitrophenol (pNP) concentration and pNP formation rate. In general, both pNP concentration and pNP formation rate decreased progressively following ultrafiltration, particularly in the low-molecular-weight fractions. Since lower pNP production reflects stronger inhibition of α -glucosidase activity, the reduction in pNP concentration and formation rate indicates enhanced antidiabetic potential of the hydrolysates after fractionation. Among all samples, the <3 kDa fractions consistently exhibited the lowest pNP concentrations and formation rates, suggesting the strongest inhibitory effects against α -glucosidase. The FMH <3 kDa fraction showed the lowest pNP concentration (37.323 ppm) and one of the lowest pNP formation rates ($0.00335 \mu\text{mol} \cdot \text{min}^{-1}$), followed closely by FXH <3 kDa and FSH <3 kDa fractions. Crude hydrolysates generally produced significantly higher pNP concentrations and reaction rates than their corresponding ultrafiltered fractions, indicating weaker inhibitory activity prior to fractionation. Notably, several low-molecular-weight fractions exhibited lower pNP formation rates than the positive control (acarbose), particularly FMH <3 kDa and FXH <3 kDa, while the negative control showed the highest pNP concentration (251.146 ppm) and

Table 1. α -glucosidase inhibitory activity of fish protein hydrolysates evaluated based on p-nitrophenol (pNP) concentration and pNP formation rate

Hydrolysates		pNP concentration (ppm)	pNP formation rate ($\mu\text{mol} \cdot \text{min}^{-1}$)
FMH	Crude	87.000 ^f	0.00782 ^f
	<30 kDa	65.292 ^{de}	0.00587 ^{de}
	<10 kDa	47.760 ^c	0.00429 ^c
	<3 kDa	37.323 ^a	0.00335 ^a
FSH	Crude	84.833 ^f	0.00762 ^f
	<30 kDa	60.844 ^d	0.00547 ^d
	<10 kDa	47.188 ^c	0.00424 ^c
	<3 kDa	41.344 ^{ab}	0.00372 ^{ab}
FXH	Crude	68.521 ^e	0.00616 ^e
	<30 kDa	60.833 ^d	0.00547 ^d
	<10 kDa	44.000 ^{bc}	0.00395 ^{bc}
	<3 kDa	38.333 ^a	0.00344 ^a
Control	C+ (acarbose)	64.063 ^{de}	0.00576 ^{de}
	C- (aquades)	251.146 ^g	0.02257 ^g

FMH (fish muscle hydrolysate), FSH (fish skin hydrolysate), FXH (fish mix hydrolysate), C+ (positive control), and C- (negative control). Different superscript letters indicate significant differences among treatments ($p < 0.05$)

reaction rate ($0.02257 \mu\text{mol} \cdot \text{min}^{-1}$), confirming the absence of inhibitory activity.

As shown in Figure 2, the freeze-dried crude hydrolysates exhibited concentration-dependent α -glucosidase inhibitory activity over the tested concentration range of 10-50 mg/mL. The percentage of inhibition increased progressively with increasing hydrolysate concentration, with FXH and FSH generally showing higher inhibition than FMH. At 50 mg/mL, FXH showed the highest inhibition,

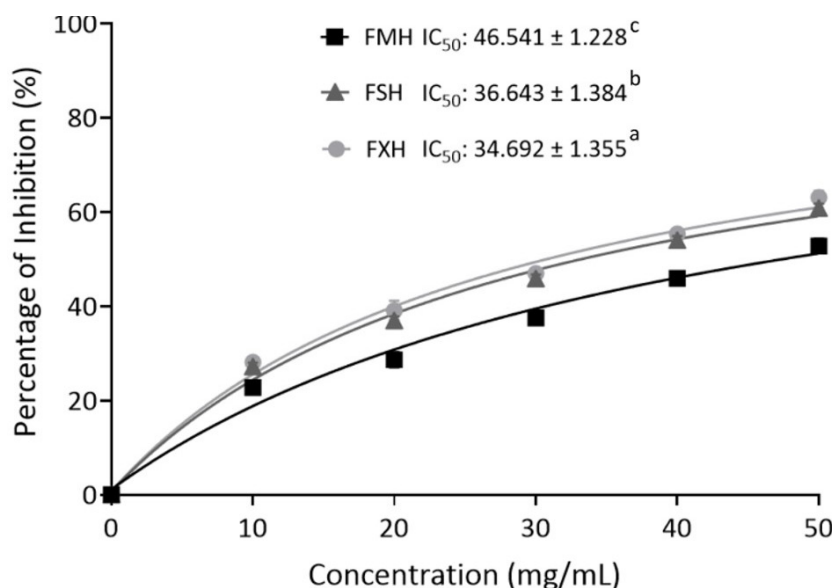


Figure 2. Dose-dependent α -glucosidase inhibitory activity and IC_{50} values of freeze-dried crude hydrolysates at concentrations of 10-50 mg/mL. FMH (fish muscle hydrolysate), FSH (fish skin hydrolysate), and FXH (fish mixed hydrolysate) exhibited varying inhibitory effects against α -glucosidase. IC_{50} values are presented as mean $\pm$ standard deviation. Different superscript letters indicate significant differences among treatments ($p < 0.05$)

followed by FSH and FMH. The IC_{50} values further demonstrated significant differences among the hydrolysates, as indicated by different superscript letters ($p < 0.05$). FXH exhibited the lowest IC_{50} value (34.692 ± 1.355 mg/mL), followed by FSH (36.643 ± 1.384 mg/mL) and FMH (46.541 ± 1.228 mg/mL). The lower IC_{50} value of FXH indicates a stronger α -glucosidase inhibitory potential compared with FSH and FMH under the conditions tested. These differences may be related to variations in peptide composition and molecular characteristics generated during fermentation, although the specific compounds responsible for the observed inhibition require further investigation.

3.2. Antibacterial Activity

The antibacterial activity of crude and ultrafiltration-fractionated hydrolysates derived from fermented mackerel by-products is summarized in Table 2. Overall, all hydrolysates exhibited inhibitory effects against both Gram-positive and Gram-negative bacteria, although activity varied by hydrolysate type and molecular weight fraction. For fish muscle hydrolysate (FMH), the inhibition zone increased progressively with decreasing molecular weight, with the <3 kDa fraction showing the highest activity against *L. monocytogenes* (22.50 ± 0.50

mm), followed by moderate increases against *E. faecalis* (15.67 ± 0.29 mm), *E. coli* (14.50 ± 0.50 mm), and *S. typhi* (8.67 ± 0.58 mm). Similarly, fish skin hydrolysate (FSH) demonstrated enhanced antibacterial activity after fractionation, particularly in the <3 kDa fraction, which exhibited the highest inhibition against *L. monocytogenes* (23.17 ± 1.89 mm) and moderate activity against *E. faecalis* and *E. coli*. However, FSH showed limited or no inhibitory activity against *S. typhi* in higher molecular weight fractions. Fish mixed hydrolysate (FXH) exhibited a consistent increase in antibacterial activity as molecular weight decreased, with the <3 kDa fraction showing relatively high inhibition across all tested bacteria. *L. monocytogenes* was the most susceptible, with the highest inhibition observed in the <3 kDa fractions across all hydrolysates: FMH (22.50 mm), FSH (23.17 mm), and FXH (21.83 mm). The positive control exhibited strong inhibition against most bacterial strains, while the negative control showed no activity. The positive control exhibited strong inhibition against most bacterial strains, while the negative control showed no activity.

Table 2. Antibacterial activity of fermented mackerel (*Scomberomorus commerson*) muscle and skin by-product hydrolysates against gram-negative and gram-positive bacteria

Hydrolysates		Bacteria strains			
		<i>Listeria monocytogenes</i>	<i>Enterococcus faecalis</i>	<i>Escherichia coli</i>	<i>Salmonella typhi</i>
		Inhibition zone diameter (mm)			
FMH	Crude	17.00±0.50 ^c	13.83±1.04 ^b	12.17±0.29 ^{de}	7.83±0.29 ^{cd}
	<30 kDa	17.17±0.76 ^c	14.33±1.26 ^{bcd}	13.00±0.50 ^{ef}	8.17±0.76 ^{cd}
	<10 kDa	19.67±1.89 ^d	15.00±0.50 ^{cdef}	13.83±1.04 ^{fg}	8.17±1.26 ^{cd}
	<3 kDa	22.50±0.50 ^{fg}	15.67±0.29 ^{fg}	14.50±0.50 ^g	8.67±0.58 ^d
FSH	Crude	16.67±0.29 ^c	14.17±0.76 ^{bc}	10.00±1.00 ^b	0.00±0.00 ^a
	<30 kDa	16.50±0.50 ^c	14.33±0.29 ^{bcd}	11.50±0.50 ^{cd}	0.00±0.00 ^a
	<10 kDa	21.33±0.76 ^{def}	15.17±0.29 ^{cdef}	11.00±1.00 ^{bc}	0.00±0.00 ^a
	<3 kDa	23.17±1.89 ^g	15.33±0.29 ^{defg}	12.50±0.50 ^{de}	6.17±0.29 ^b
FXH	Crude	16.83±0.76 ^c	14.83±0.76 ^{bcd}	12.17±0.29 ^{de}	7.67±0.58 ^c
	<30 kDa	16.67±0.29 ^c	14.50±0.50 ^{bcd}	12.83±0.29 ^{ef}	7.67±0.76 ^c
	<10 kDa	20.17±0.29 ^{de}	15.50±0.50 ^{cfg}	14.17±0.76 ^g	8.33±0.29 ^{cd}
	<3 kDa	21.83±1.76 ^{cfg}	15.83±0.29 ^{fg}	13.83±0.76 ^{fg}	8.17±0.29 ^{cd}
	C+	14.25±0.87 ^b	16.33±0.69 ^g	17.42±0.51 ^h	14.88±0.83 ^c
	C-	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a

FMH (fish muscle hydrolysate), FSH (fish skin hydrolysate), FXH (fish mix hydrolysate), C+ (positive control, Chloramphenicol 100 ppm), and C- (negative control, medium). Different superscript letters indicate significant differences among treatments ($p < 0.05$). The inhibition diameter for antimicrobial activity can be classified as very strong (inhibition diameter ≥ 20 mm), strong ($10 \text{ mm} \leq$ inhibition diameter < 20 mm), moderate ($5 \text{ mm} \leq$ inhibition diameter < 10 mm), and weak (inhibition diameter < 5 mm) (Davis & Stout 1971)

3.3. Peptide Profiling and *In Silico* Evaluation of Bioactivity, Toxicity, and Allergenicity

A total of 211 peptide sequences were previously identified from fermented mackerel by-product hydrolysates (FMH, FSH, and FXH) using LC-HRMS/MS. To enable focused *in silico* characterization, approximately 10% of the highest-abundance peptides from each hydrolysate type were selected, yielding a final set of 35 representative peptide sequences for further analysis. These 35 peptides were selected based on their relative abundance and coverage across hydrolysate types, with their molecular characteristics and *in silico* predictions for bioactivity, toxicity, and allergenicity summarized in Table 3. The identified peptides ranged from 8 to 18 amino acids in length, with the majority consisting of 10-14 residues. Based on hydrolysate source distribution, FMH contributed the highest number of unique peptides (20 sequences), followed by FSH (10 sequences), while five peptides were commonly identified across multiple hydrolysate types, particularly those originating from both FMH and FSH fractions.

PeptideRanker scores, which predict the probability of a peptide being bioactive, exhibited considerable variation across the identified sequences, ranging from 0.000 to 0.926. Notably, nine peptides achieved scores exceeding 0.5, indicating high predicted

bioactivity. The highest score (0.926) was observed for SPAMPVPGPMGPMGPR (peptide 28) from FSH and FXH, followed by GPAGASGPAGPR (peptide 30, score 0.782), GFPGSDGSAGPK (peptide 23, score 0.669), and AGEPGAAGLR (peptide 25, score 0.612). Among FMH-derived peptides, AVFPSIVGRPR (peptide 11, score 0.625) and IWHHTFYNELR (peptide 9, score 0.580) demonstrated the highest bioactivity potential. High-scoring peptides were mainly distributed in FSH and FXH, particularly those rich in glycine and proline residues, which are characteristic of collagen-derived sequences.

According to the BIOPEP-UWM database analysis, the vast majority of identified peptides (33 out of 35, 94.3%) were classified as novel, indicating they had not been previously reported in bioactive peptide databases. Only two peptides, IWHHTFYNELR (peptide 9) from FMH and AGFAGDDAPR (peptide 33) from FSH, were categorized as reported sequences. Toxicity assessment using ToxinPred3 revealed that the majority of peptides (32 of 35, 91.43%) were predicted to be non-toxic. However, two peptides, namely IEELEEEIEAEER (peptide 1) from FMH/FSH/FXH, TGPIGMPGAR (peptide 21) from FSH/FXH, and VEELEEEIEAR (peptide 35) from FSH, were classified as toxic, warranting caution for potential therapeutic applications. Notably, both toxic peptides

Table 3. Selected peptide sequences identified from fermented mackerel (*Scomberomorus commerson*) muscle and skin by-product hydrolysates and their *in silico* bioactivity, toxicity, and allergenicity predictions

Sequence peptides	Length	Hydrolysate	BIOPEP-UWM	Peptide Ranker	ToxinPred3			Allergenicity	
					Score	Prediction	pLM4Alg-640	AlgPred2	Allercatpro 2.0*
IEEEEEIEAER	12	FMH, FSH, FXH	Novel	0.044	0.505	Toxin	Allergen	Allergen	no evidence
LQDLVDKLQLK	11	FMH, FSH, FXH	Novel	0.224	0.315	Non-Toxin	Allergen	Allergen	no evidence
IQLVEELDRAQER	14	FMH, FSH, FXH	Novel	0.060	0.170	Non-Toxin	Allergen	Allergen	strong evidence
NLQQEISDLTEQIGETGK	18	FMH, FXH	Novel	0.082	0.255	Non-Toxin	Allergen	Allergen	no evidence
VIQYFATIAAIGAK	14	FMH	Novel	0.265	0.000	Non-Toxin	Allergen	Allergen	no evidence
LQGEVEDLMIDVER	14	FMH, FSH, FXH	Novel	0.140	0.130	Non-Toxin	Allergen	Allergen	no evidence
VAPEEHPTLLTEAPLNPK	18	FMH, FXH	Novel	0.241	0.000	Non-Toxin	Allergen	Allergen	weak evidence
VPTPNVSVVDLTVR	14	FMH	Novel	0.129	0.000	Non-Toxin	Allergen	Allergen	strong evidence
IWHHTFYNELR	11	FMH	Reported	0.580	0.315	Non-Toxin	Non-allergen	Non-Allergen	no evidence
SYELPDGQVITIGNER	16	FMH, FXH	Novel	0.206	0.100	Non-Toxin	Allergen	Allergen	weak evidence
AVFPSIVGRPR	11	FMH, FSH, FXH	Novel	0.625	0.255	Non-Toxin	Non-allergen	Allergen	strong evidence
VAEQELVDASER	12	FMH, FXH	Novel	0.062	0.190	Non-Toxin	Allergen	Allergen	no evidence
LDEAENLAMK	10	FMH	Novel	0.310	0.155	Non-Toxin	Allergen	Allergen	no evidence
GYSFVTTAER	10	FMH	Novel	0.189	0.105	Non-Toxin	Non-allergen	Non-Allergen	strong evidence
DLTDYLMK	8	FMH	Novel	0.430	0.240	Non-Toxin	Allergen	Non-Allergen	no evidence
TIEDQLSELK	10	FMH, FSH, FXH	Novel	0.097	0.190	Non-Toxin	Allergen	Allergen	no evidence
DIDDLEITLAK	11	FMH	Novel	0.206	0.000	Non-Toxin	Allergen	Allergen	no evidence
VQLELNQIK	9	FMH	Novel	0.082	0.365	Non-Toxin	Allergen	Non-Allergen	no evidence
LQDAEESIEAVNSK	14	FMH	Novel	0.100	0.090	Non-Toxin	Allergen	Allergen	no evidence
EITALAPSTMK	11	FMH	Novel	0.208	0.120	Non-Toxin	Allergen	Allergen	strong evidence
TGPIGMPGAR	10	FSH, FXH	Novel	0.562	0.395	Toxin	Non-allergen	Allergen	no evidence
GFSGLDGAK	9	FSH, FXH	Novel	0.500	0.215	Non-Toxin	Non-allergen	Allergen	strong evidence
GFPGSDGSAGPK	12	FSH, FXH	Novel	0.669	0.135	Non-Toxin	Allergen	Allergen	no evidence
GYTGLDGR	8	FSH, FXH	Novel	0.366	0.205	Non-Toxin	Non-allergen	Allergen	strong evidence
AGEPGAAGLR	10	FSH, FXH	Novel	0.612	0.160	Non-Toxin	Non-allergen	Non-Allergen	no evidence
DLEESTLQHEATAAALR	17	FSH, FXH	Novel	0.113	0.000	Non-Toxin	Allergen	Allergen	no evidence
SLSTELFK	8	FSH, FXH	Novel	0.383	0.000	Non-Toxin	Non-allergen	Allergen	no evidence
SPAMPVPGPMGPMGPR	16	FSH, FXH	Novel	0.926	0.200	Non-Toxin	Non-allergen	Non-Allergen	no evidence
GATGPTGLR	9	FSH	Novel	0.569	0.250	Non-Toxin	Non-allergen	Allergen	no evidence
GAPAGSGPAGPR	12	FSH	Novel	0.782	0.000	Non-Toxin	Non-allergen	Allergen	no evidence
GAAGPQGAR	9	FSH	Novel	0.505	0.265	Non-Toxin	Non-allergen	Allergen	no evidence
QADSVaelGEQIDNLQR	17	FSH	Novel	0.230	0.090	Non-Toxin	Allergen	Allergen	no evidence
AGFAGDDAPR	10	FSH	Reported	0.562	0.115	Non-Toxin	Allergen	Allergen	no evidence
VIDSMQSTLDSEVR	14	FXH	Novel	0.085	0.000	Non-Toxin	Allergen	Non-Allergen	no evidence
VEEEEEIEAER	12	FXH	Novel	0.038	0.385	Toxin	Allergen	Allergen	no evidence

BIO-PEP-UWM <https://biochemia.uwm.edu.pl/biopep-uwm/>, accessed on 20 November 2025; PeptideRanker <https://distilldeep.ucd.ie/PeptideRanker/>, accessed on 22 November 2025; ToxinPred3 <https://webs.iiitd.edu.in/raghava/toxinpred3/>, accessed on 23 November 2025; Allergenicity Predictions: pLM4Alg-640 <https://f6wxpfd3sh.us-east-1.awsapprunner.com/>, AlgPred2 <https://webs.iiitd.edu.in/raghava/algpred2/>, Allercatpro <https://allercatpro.bii.a-star.edu.sg/>, accessed on 25-26 November 2025 (*predicted evidence for allergenicity). FMH (fish muscle hydrolysate), FSH (fish skin hydrolysate), FXH (fish mix hydrolysate)

exhibited relatively low PeptideRanker scores (0.044, 0.562, and 0.038, respectively), suggesting limited bioactive potential.

Allergenicity predictions were performed using three complementary algorithms: pLM4Alg-640, AlgPred2, and Allercatpro 2.0, with the latter

providing additional evidence levels for predicted allergenicity. Considerable variability was observed across the prediction platforms. Only four peptides (IWHHTFYNELR, GYSFVTTAER, AGEPGAAGLR, SPAMPVPGPMGPMGPR) demonstrated consistent non-allergen predictions across at least two model

algorithms. Conversely, the majority of peptides exhibited discordant predictions, with many classified as allergens by one or more tools. Based on Allercatpro 2.0 analysis, seven peptides (IQLVEEELDRAQER, VTPPNVSVVDLTVR, AVFPSIVGRPR, GYSFVTTAER, EITALAPSTMK, GFSGLDGAG, and GYTGLDGR) showed strong evidence of allergenicity, while two peptides (VAPEEHPTLLTEAPLNPK and SYELPDGQVITIGNER) displayed weak evidence. The remaining peptides showed no evidence of allergenicity according to this platform. Overall, the results indicate that fermented mackerel by-product hydrolysates contain multiple peptides with favorable predicted bioactivity and safety profiles, supporting their potential as candidates for further functional and mechanistic evaluation.

3.4. *In Silico* Assessment of Antidiabetic Activities of Identified Peptides

The 35 identified peptide sequences from fermented mackerel by-product hydrolysates were further subjected to *in silico* prediction of antidiabetic potential using two complementary bioinformatics tools: AntiDMP-pred, which predicts general antidiabetic bioactive peptides (ADP), and AntiT2DMP-Pred, which specifically predicts type 2 antidiabetic peptides (T2ADP). The prediction probabilities for each peptide are presented in Table 4, revealing considerable variation in antidiabetic potential across the identified sequences. Based on AntiDMP-pred analysis, the majority of peptides exhibited moderate to high probabilities for general antidiabetic activity, with scores ranging from 0.30 to 0.83. Notably, 27 peptides (77.1%) achieved prediction probabilities exceeding the 0.5 threshold, indicating strong potential as antidiabetic bioactive peptides. The highest AntiDMP-pred scores were observed for VAELVDASER (peptide 12, probability 0.83), followed by LQGEVEDLMDIVER (peptide 6, 0.74), VAPEEHPTLLTEAPLNPK (peptide 7, 0.73), and GFPGSDGSAGPK (peptide 23, 0.72). Among FMH-derived peptides, LQGEVEDLMDIVER (peptide 6) and VAELVDASER (peptide 12) demonstrated the highest probabilities, while SPAMPVPGPMGPMGPR (peptide 28, 0.76) and DLEESTLQHEATAAALR (peptide 26, 0.79) represented the most promising candidates from FSH and FXH fractions. Only eight peptides (22.9%) exhibited probabilities below 0.5, with VIQYFATIAAGAK (peptide 5, 0.33) and IWHHTFYNEER (peptide 9, 0.30) showing the lowest scores.

Table 4. Selected peptide sequences identified from fermented mackerel (*Scomberomorus commerson*) muscle and skin by-product hydrolysates based on *in silico* analyses related to antidiabetic activity

Peptide sequences	Antidiabetic prediction (AntiDMP-pred) probability	Type 2 antidiabetic peptide prediction (AntiT2DMP-pred) probability
IEEEEEIEAER	0.67	ADP
LQDLVDKLQLK	0.52	ADP
IQLVEEELDRAQER	0.64	ADP
NLQQEISDLTEQIGETGK	0.53	ADP
VIQYFATIAAIGAK	0.33	non-ADP
LQGEVEDLMDIVER	0.74	ADP
VAPEEHPTLLTEAPLNPK	0.73	ADP
VTPPNVSVVDLTVR	0.43	non-ADP
IWHHTFYNELR	0.30	non-ADP
SYELPDGQVITIGNER	0.64	ADP
AVFPSIVGRPR	0.31	non-ADP
VAEQELVDASER	0.83	ADP
LDEAENLAMK	0.64	ADP
GYSFVTTAER	0.54	ADP
DLTDYLMK	0.40	non-ADP
TIEDQLSELK	0.69	ADP
DIDDLEITLAK	0.61	ADP
VQLELNQIK	0.56	ADP
LQDAEESIEAVNSK	0.67	ADP
EITALAPSTMK	0.65	ADP
TGPIGMPGAR	0.68	ADP
GFSGLDGAK	0.55	ADP
GFPGSDGSAGPK	0.72	ADP
GYTGLDGR	0.42	non-ADP
AGEPGAAGLR	0.62	ADP
DLEESTLQHEATAAALR	0.79	ADP
SLSTELFK	0.60	ADP
SPAMPVPGPMGPMGPR	0.76	ADP
GATGPTGLR	0.49	non-ADP
GPAGASGPAGPR	0.68	ADP
GAAGPQGAR	0.43	non-ADP
QADSVAEELGEQIDNLQR	0.62	ADP
AGFAGDDAPR	0.58	ADP
VIDSMQSTLDSEVR	0.56	ADP
VEEEEEIEAER	0.69	ADP

The antidiabetic prediction was evaluated using different tools:

AntiDMPpred <https://i.uestc.edu.cn/AntiDMPpred/cgi-bin/AntiDMPpred.pl>, and AntiT2DMP-Pred <https://balalab-skku.org/AntiT2DMP-Pred/>, accessed on 27 November 2025. ADP (anti-diabetic bioactive peptides), T2ADP (Type 2 anti-diabetic peptides), FMH (fish muscle hydrolysate), FSH (fish skin hydrolysate), FXH (fish mix hydrolysate)

In contrast, AntiT2DMP-Pred probabilities for type 2 antidiabetic peptide prediction were generally lower, with scores ranging from 0.010 to 0.811. Only two peptides (5.71%) had prediction probabilities exceeding 0.5: SPAMPVPGPMGPMGPR (peptide 28, 0.811) and AVFPSIVGRPR (peptide 11, 0.663). Notably, SPAMPVPGPMGPMGPR demonstrated

exceptional potential with the highest T2ADP probability (0.811), consistent with its outstanding PeptideRanker score (0.926) observed in Table 3. Several other peptides showed moderate T2ADP probabilities, including DLEESTLQHEATAAALR (peptide 26, 0.350), GAAGPQGAR (peptide 31, 0.222), QADSVAELGEQIDNLQR (peptide 32, 0.178), and AGEPGAAGLR (peptide 25, 0.104). The remaining 29 peptides (82.86%) exhibited low probabilities (<0.1) for type 2 antidiabetic activity, indicating that while many peptides may possess general antidiabetic properties, only a select few are specifically predicted to target type 2 diabetes-related pathways.

Comparative analysis revealed discordance between the two prediction tools for several peptides. For instance, VAEILDASER (peptide 12) showed the highest AntiDMP-pred probability (0.83) but minimal T2ADP potential (0.012). Conversely, AVFPSIVGRPR (peptide 11) demonstrated moderate AntiDMP-pred probability (0.31) but the second-highest T2ADP score (0.663). This divergence suggests that these tools capture different aspects of antidiabetic activity, with AntiDMP-pred potentially reflecting broader mechanisms, including α -glucosidase and DPP-IV inhibition, whereas AntiT2DMP-Pred may be more specifically tuned to pathways directly relevant to the pathophysiology of type 2 diabetes. Notably, peptides exhibiting consistently high prediction scores across both platforms, particularly SPAMPVPGPMGPMGPR, represent promising candidates for further experimental validation of antidiabetic activity.

3.5. *In Silico* Assessment of Antimicrobial Activities of Identified Peptides

The antimicrobial potential of the 35 identified peptides was comprehensively evaluated using four complementary *in silico* prediction tools: CAMP_{R4} (employing Random Forest, Support Vector Machine, and Artificial Neural Network algorithms), Antimicrobial Peptide Scanner vr.2, ADAM, and AntiBP3 (specifically predicting antibacterial activity against Gram-positive and Gram-negative bacteria). The prediction scores and classifications for each peptide are presented in Table 5, revealing considerable variation in antimicrobial potential across the identified sequences.

Based on CAMP_{R4} analysis employing three distinct machine learning algorithms, the predictions varied considerably depending on the model

used. Using the Random Forest (RF) algorithm, 6 peptides (17.14%) were classified as antimicrobial peptides (AMP), with prediction scores ranging from 0.50 to 0.78. The highest RF-based scores were observed for VIQYFAATIAAGAK (peptide 5, score 0.78), followed by AVFPSIVGRPR (peptide 11, 0.73), GPAGASGPAGPR (peptide 30, 0.55), and GAAGPQGAR (peptide 31, 0.54). The Support Vector Machine (SVM) algorithm classified 14 peptides (40.00%) as AMP, with the highest scores for IEEEEIEAER (peptide 1, 1.00), VEELEEIEAR (peptide 35, 1.00), and VAEILDASER (peptide 12, 0.99). The Artificial Neural Network (ANN) algorithm identified 9 peptides (25.71%) as AMP, with GAAGPQGAR (peptide 31, 0.91), AVFPSIVGRPR (peptide 11, 0.87) and GPAGASGPAGPR (peptide 30, 0.83) demonstrated the highest scores. Notably, several FSH and FXH-derived peptides, particularly those with collagen-typical glycine-proline-rich motifs (peptides 21-31), demonstrated consistent AMP predictions across at least two algorithms.

Antimicrobial Peptide Scanner vr.2 analysis revealed that 5 peptides (14.28%) were predicted as AMP, with scores exceeding the tool's threshold. The highest probabilities were observed for SPAMPVPGPMGPMGPR (peptide 23, 0.9347), AVFPSIVGRPR (peptide 11, 0.8824), TGPIGMPGAR (peptide 21, 0.7488), GFPDSDGSAGPK (peptide 23, 0.6088), and GPAGASGPAGPR (peptide 30, 0.5471). Several peptides previously identified as non-antimicrobial by certain CAMP_{R4} algorithms were classified as AMP by this tool, highlighting the value of employing multiple prediction platforms. Notably, IEEEEIEAER (peptide 1) and VEELEEIEAR (peptide 35) achieved perfect scores (1.00), despite their predicted toxicity noted in Table 3.

ADAM analysis, which predicts antimicrobial activity based on structural and physicochemical properties, classified 27 peptides (77.14%) as AMP, with scores ranging from 0.23 to 2.16. The highest ADAM scores were observed for GYTGLDGR (peptide 24, 2.16), IEEEEIEAER (peptide 1, 2.15), VEELEEIEAR (peptide 35, 2.13), LQDLVDKLQLK (peptide 2, 2.07), and GFSGLDGAK (peptide 22, 2.04). AntiBP3 analysis specifically predicted antibacterial activity using two complementary algorithms. Based on Support Vector Machine (SVM) predictions targeting Gram-positive and Gram-negative bacteria, only 4 peptides (11.42%) were classified as antibacterial peptides

Table 5. Selected peptide sequences identified from fermented mackerel (*Scomberomorus commerson*) muscle and skin by-product hydrolysates based on *in silico* analyses related to antimicrobial activity

Sequence peptides	CAMPR4 results with:						Antimicrobial		ADAM		AntiBP3			
	RF		SVM		SVM		Peptide Scanner vr.2				Targeting Gram positive Bacteria		Targeting Gram negative Bacteria	
IEEEEEIEAER	0.47	NAMP	1.00	AMP	0.05	NAMP	0.0048	Non-AMP	2.15	AMP	0.19	non-ABPs	0.29	non-ABPs
LQDLVDKLQLK	0.36	NAMP	0.61	AMP	0.16	NAMP	0.0011	Non-AMP	2.07	AMP	0.5	non-ABPs	0.52	ABPs
IQLVEEELDRAQER	0.41	NAMP	0.53	AMP	0.05	NAMP	0.0010	Non-AMP	-0.62	Non-AMP	0.27	non-ABPs	0.36	non-ABPs
NLQQEISDLTEQIGETGK	0.49	NAMP	0.88	AMP	0.06	NAMP	0.0013	Non-AMP	0.92	AMP	0.28	non-ABPs	0.19	non-ABPs
VIQYFATIAAIGAK	0.78	AMP	0.61	AMP	0.56	AMP	0.0545	Non-AMP	2.03	AMP	0.48	non-ABPs	0.32	non-ABPs
LQGEVEDLMIDVER	0.53	AMP	0.96	AMP	0.06	NAMP	0.0004	Non-AMP	-0.24	Non-AMP	0.39	non-ABPs	0.37	non-ABPs
VAPEEHPTLLTEAPLNPK	0.23	NAMP	0.05	NAMP	0.10	NAMP	0.0051	Non-AMP	0.55	AMP	0.31	non-ABPs	0.25	non-ABPs
VPTPNVSVVDLTVR	0.45	NAMP	0.93	AMP	0.70	AMP	0.0019	Non-AMP	0.74	AMP	0.38	non-ABPs	0.25	non-ABPs
IWHHTFYNELR	0.15	NAMP	0.06	NAMP	0.08	NAMP	0.0274	Non-AMP	1.39	AMP	0.61	ABPs	0.45	non-ABPs
SYELPDGQVITIGNER	0.14	NAMP	0.20	NAMP	0.07	NAMP	0.0006	Non-AMP	0.18	AMP	0.34	non-ABPs	0.38	non-ABPs
AVFPSIVGRPR	0.73	AMP	0.68	AMP	0.87	AMP	0.8824	AMP	0.29	AMP	0.35	non-ABPs	0.30	non-ABPs
VAEQELVDASER	0.44	NAMP	0.99	AMP	0.05	NAMP	0.0014	Non-AMP	-0.50	Non-AMP	0.29	non-ABPs	0.29	non-ABPs
LDEAENLAMK	0.43	NAMP	0.41	NAMP	0.08	NAMP	0.0302	Non-AMP	1.00	AMP	0.34	non-ABPs	0.26	non-ABPs
GYSFVTTAER	0.24	NAMP	0.08	NAMP	0.22	NAMP	0.0370	Non-AMP	-0.59	Non-AMP	0.50	non-ABPs	0.31	non-ABPs
DLTDYLMK	0.45	NAMP	0.82	AMP	0.21	NAMP	0.1598	Non-AMP *	1.03	AMP	0.63	ABPs	0.22	non-ABPs
TIEDQLSELK	0.38	NAMP	0.21	NAMP	0.05	NAMP	0.0029	Non-AMP	-0.01	Non-AMP	0.31	non-ABPs	0.24	non-ABPs
DIDDLEITLAK	0.45	NAMP	0.60	AMP	0.13	NAMP	0.0101	Non-AMP	0.14	AMP	0.47	non-ABPs	0.23	non-ABPs
VQLELNQIK	0.44	NAMP	0.57	AMP	0.18	NAMP	0.0154	Non-AMP *	0.76	AMP	0.49	non-ABPs	0.31	non-ABPs
LQDAEESIEAVNSK	0.43	NAMP	0.35	NAMP	0.06	NAMP	0.0029	Non-AMP	-0.37	Non-AMP	0.40	non-ABPs	0.27	non-ABPs
EITALAPSTMK	0.34	NAMP	0.07	NAMP	0.08	NAMP	0.0158	Non-AMP	0.41	AMP	0.36	non-ABPs	0.27	non-ABPs
TGPIGMPGAR	0.34	NAMP	0.15	NAMP	0.61	AMP	0.7488	AMP	1.70	AMP	0.30	non-ABPs	0.31	non-ABPs
GFSGLDGAK	0.45	NAMP	0.38	NAMP	0.78	AMP	0.0530	Non-AMP *	2.04	AMP	0.40	non-ABPs	0.27	non-ABPs
GFPGSDGSAGPK	0.39	NAMP	0.13	NAMP	0.76	AMP	0.6088	AMP	1.76	AMP	0.42	non-ABPs	0.21	non-ABPs
GYTGLDGR	0.31	NAMP	0.01	NAMP	0.11	NAMP	0.2885	Non-AMP *	2.16	AMP	0.42	non-ABPs	0.28	non-ABPs
AGEPGAAGLR	0.35	NAMP	0.00	NAMP	0.42	NAMP	0.2977	Non-AMP	1.43	AMP	0.21	non-ABPs	0.29	non-ABPs
DLEESTLQHEATAAALR	0.20	NAMP	0.01	NAMP	0.07	NAMP	0.0049	Non-AMP	-0.03	Non-AMP	0.38	non-ABPs	0.31	non-ABPs
SLSTELFK	0.32	NAMP	0.07	NAMP	0.18	NAMP	0.2440	Non-AMP *	1.30	AMP	0.58	ABPs	0.25	non-ABPs
SPAMPVPGPMGPMGPR	0.33	NAMP	0.21	NAMP	0.15	NAMP	0.9347	AMP	1.51	AMP	0.29	non-ABPs	0.28	non-ABPs
GATGPTGLR	0.50	AMP	0.05	NAMP	0.81	AMP	0.5724	AMP *	1.85	AMP	0.41	non-ABPs	0.26	non-ABPs
GPAGASGPAGPR	0.55	AMP	0.63	AMP	0.83	AMP	0.5471	AMP	1.73	AMP	0.30	non-ABPs	0.22	non-ABPs
GAAGPQGAR	0.54	AMP	0.46	NAMP	0.91	AMP	0.4149	Non-AMP *	1.78	AMP	0.31	non-ABPs	0.33	non-ABPs
QADSVAELGEQIDNLQR	0.47	NAMP	0.36	NAMP	0.06	NAMP	0.0005	Non-AMP	-0.53	Non-AMP	0.18	non-ABPs	0.23	non-ABPs
AGFAGDDAPR	0.31	NAMP	0.02	NAMP	0.35	NAMP	0.0154	Non-AMP	0.75	AMP	0.39	non-ABPs	0.25	non-ABPs
VIDSMQSTLDSEVR	0.23	NAMP	0.14	NAMP	0.06	NAMP	0.0008	Non-AMP	0.23	AMP	0.41	non-ABPs	0.25	non-ABPs
VEEEEEIEAER	0.48	NAMP	1.00	AMP	0.05	NAMP	0.0048	Non-AMP	2.13	AMP	0.16	non-ABPs	0.26	non-ABPs

The antimicrobial activity, general and specific to bacteria, viruses, and fungi, was evaluated using different tools. CAMPR4 <http://camp.bicnirrh.res.in/>, accessed on 27 November 2025; antimicrobial peptide scanner vr.2 <https://www.dveltri.com/ascan/>, accessed on 28 November 2025; ADAM <https://bioinformatics.cs.ntou.edu.tw/ADAM/>, accessed on 28 November 2025; AntiBP3 <https://webs.iitd.edu.in/raghava/antibp3/predict.php>, accessed on 29 November 2025. *Those marked with an asterisk indicate internal prediction inconsistencies. Each tool might have a different threshold, but a higher score indicates the greater the likelihood of the peptide being bioactive. RF (random forest), SVM (support vector machine (SVM)), ANN (artificial neural network), AMP (antimicrobial peptides), NAMP (non-antimicrobial peptides), ABPs (antibacterial peptides)

(ABPs): IWHHTFYNELR (peptide 9, score 0.61), DLTDYLMK (peptide 15, score 0.63), SLSTELFK (peptide 27, score 0.58), and LQDLVDKLQK (peptide 2, 0.52). The remaining 32 peptides (88.57%) were classified as non-antibacterial peptides (non-ABPs).

Integration of predictions across all four tools revealed that AVFPSIVGRPR (peptide 11) demonstrated the most consistent antimicrobial

potential, being classified as AMP by CAMP_{R4} across multiple algorithms (RF: 0.73, SVM: 0.68, ANN: 0.87), Antimicrobial Peptide Scanner vr.2 (0.68), and ADAM (0.87), while showing moderate antibacterial predictions by AntiBP3. Similarly, GPAGASGPAGPR (peptide 30) and GATGPTGLR (peptide 29) exhibited strong AMP predictions across multiple tools, supporting their potential as antimicrobial candidates. Several peptides, including TIEDQLSELK (peptide

16), DLEESTLQHEATAAALR (peptide 26), and QADSVAELGEQIDNLQR (peptide 32), were consistently classified as non-antimicrobial by all tools and algorithms, suggesting they lack inherent antimicrobial properties despite their antidiabetic potential noted in Table 4.

Internal prediction inconsistencies, marked with asterisks in Table 5, were observed for several peptides across different tools and algorithms. For instance, DLTDYLMK (peptide 15) and VQLELNQIK (peptide 18) showed discordant predictions between Antimicrobial Peptide Scanner vr.2 and other tools, while several peptides exhibited variability across the three CAMP_{R4} algorithms. These inconsistencies likely reflect differences in training datasets, algorithmic architectures, and the specific sequence features each tool prioritizes in antimicrobial prediction, underscoring the importance of consensus-based interpretation in peptide bioactivity assessment.

4. Discussion

The systematic evaluation of α -glucosidase inhibitory activity in this study demonstrated that fermentation of mackerel by-products generated hydrolysates with α -glucosidase inhibitory potential, while ultrafiltration effectively enriched the low-molecular-weight fractions associated with higher inhibitory activity. The consistent finding that the <3 kDa fractions exhibited the strongest inhibition across FMH, FSH, and FXH aligns with previous reports that low-molecular-weight peptides possess superior enzyme inhibitory capacity due to enhanced accessibility to the α -glucosidase active site and improved membrane permeability (Wan *et al.* 2023; Cagabhion *et al.* 2025; Liu *et al.* 2025), a pattern consistently observed across multiple protein sources, where ultrafiltration-enriched <3 kDa and 3-10 kDa fractions demonstrate the highest inhibitory capacity (Elisha *et al.* 2025). This enhanced accessibility is particularly relevant given that α -glucosidase harbors its catalytic pocket within an internal cavity, where smaller peptides can penetrate and interact more effectively with key residues than larger molecular species, while longer peptides experience steric hindrance that weakens their binding affinity (Wan *et al.* 2023; Liu *et al.* 2025). Furthermore, the lower pNP formation rates detected in the ultrafiltered fractions compared with acarbose confirm that fermentation coupled with ultrafiltration effectively enriched potent inhibitory compounds with strong antidiabetic potential.

The differential inhibitory activity observed among hydrolysate types reflects intrinsic differences in tissue-specific protein composition and the resulting peptide profiles. The superior activity of FMH over FSH is attributable to the abundance of sarcoplasmic and myofibrillar proteins in fish muscle tissue, which, upon fermentation, release a broad spectrum of hydrophobic and aromatic amino acid-containing peptides known to interact favorably with the α -glucosidase active site through hydrophobic contacts and hydrogen bonding (Rivero-Pino *et al.* 2020). In contrast, skin-derived proteins yield predominantly collagen-rich peptide profiles that, while bioactive, differ in binding affinity and conformational fit with the enzyme. This tissue-based variation is consistent with findings for Atlantic mackerel and sardine by-product hydrolysates, in which differences in raw materials and hydrolysis conditions substantially influenced the bioactive peptide profile and antidiabetic activity (Fuentes *et al.* 2025). Notably, the mixed hydrolysate FXH demonstrated the lowest IC₅₀ value (34.692 mg/mL), suggesting that combining muscle and skin-derived peptide pools generates a synergistic enhancement of inhibitory activity through complementary coverage of multiple interaction points across the α -glucosidase active site.

The IC₅₀ values obtained in this study compare favorably with those reported for other fish-derived hydrolysates, though direct comparisons are inherently limited by methodological heterogeneity across studies. Salmon gelatin (SG) and Tilapia gelatin (TG) exhibited IC₅₀ values of 483.97±8.10 µg/mL and 344.98±3.56 µg/mL, respectively (Munawaroh *et al.* 2023), while bone collagen hydrolysate from *Lamuru* (*Caranx ignobilis*) yielded 574 µg/mL (Nur *et al.* 2021), and sardine muscle hydrolysates showed an IC₅₀ of 48.7 mg/mL using pNPG as substrate (Matsui *et al.* 1999). As highlighted in a recent review, IC₅₀ values for α -glucosidase inhibition in fish-derived systems span 21.8 to 300 mg/mL, depending on species, tissue source, hydrolysis method, and assay substrate (Honrado *et al.* 2024). The variation across these studies therefore reflects not only species and tissue-specific differences in amino acid composition, but also the assay-dependent nature of IC₅₀ measurements, factors that underscore the importance of standardized methodology in comparative bioactivity assessments.

The antibacterial activity observed across all hydrolysates confirms that fermentation of mackerel by-products effectively releases antimicrobial peptides with broad-spectrum inhibitory capacity against both Gram-positive and Gram-negative pathogens, with

ultrafiltration-enriched low-molecular-weight fractions consistently demonstrating superior inhibition, a finding corroborated by Pezeshk *et al.* (2019), who demonstrated that fractionation of yellowfin tuna (*Thunnus albacores*) viscera hydrolysate yielded a <3 kDa fraction with the highest antibacterial activity against both Gram-positive (*Listeria* and *Staphylococcus*) and Gram-negative (*E. coli* and *Pseudomonas*) strains. Structurally, fish-derived antimicrobial peptides are typically amphipathic, cationic, and enriched in hydrophobic residues, features that facilitate electrostatic interaction with negatively charged bacterial membrane components, leading to pore formation, disruption of membrane integrity, impairment of cellular respiration, and ultimately cell lysis (Honrado *et al.* 2024). The mechanism of action of these potent low-MW fractions likely involves a multi-modal attack encompassing membrane depolarization, leakage of cellular contents, and induction of reactive oxygen species (ROS), while their superior activity over larger fractions reflects reduced steric and diffusional barriers at the bacterial cell surface (Nemati *et al.* 2024; Neelima *et al.* 2025).

The consistently higher susceptibility of *L. monocytogenes* relative to Gram-negative species across all hydrolysate types reflects the intrinsic structural vulnerability of Gram-positive bacteria to cationic antimicrobial peptides. Unlike Gram-negative bacteria, which are shielded by an outer membrane rich in lipopolysaccharides (LPS) that restricts peptide access to the cytoplasmic membrane, Gram-positive bacteria possess only a single lipid bilayer anchored by a peptidoglycan teichoic acid matrix that, while thick, is more permeable to small, positively charged peptides (Stephani *et al.* 2024). The strong inhibition against *L. monocytogenes* classified according to Davis and Stout (1971) recorded across all <3 kDa hydrolysate fractions is consistent with findings for Atlantic mackerel (*Scomber scombrus*) by-product hydrolysates, from which peptide SIFIQRFTT (P4) completely inhibited both *Listeria innocua* and *E. coli* growth at 200 µg/mL, while the GAPDH-derived peptide AMGAP preferentially targeted *Listeria* strains at minimal inhibitory concentrations (Ennaas *et al.* 2015; Offret *et al.* 2019). The markedly weaker and in several FSH fractions entirely absent inhibition of *S. typhi* is mechanistically consistent with the complex AMP resistance strategies evolved by *Salmonella* species, wherein LPS remodeling controlled by two component regulatory systems reduces surface negative charge and lowers AMP binding affinity, while additional resistance mechanisms including efflux systems and proteolytic degradation further compound

AMP evasion (Atef *et al.* 2021; Stephani *et al.* 2024; Kumar dan Choubey 2026).

The predominance of medium-chain peptides and the enrichment of glycine and proline-rich sequences among high-scoring fractions are consistent with a collagen-derived origin from fish skin and connective tissue proteins, whose release is facilitated by the synergistic action of endogenous and microbial proteases during fermentation (Cruz-Casas *et al.* 2023). PeptideRanker, widely adopted for bioactivity prioritization with a score threshold of 0.50, identified a meaningful subset of high-potential candidates; however, its predictions are best regarded as preliminary given its inherent limitations in capturing full biological context, necessitating integration with complementary bioinformatics tools and downstream experimental validation (Rivero-Pino *et al.* 2023; Huang *et al.* 2025). The high proportion of structurally novel sequences confirmed through BIOPEP-UWM cross-referencing underscores the underexplored peptide biodiversity of fermented fish by-products and highlights the value of LC-HRMS/MS-driven discovery workflows in expanding the functional peptide landscape beyond currently cataloged databases (Minkiewicz *et al.* 2022; Iwaniak *et al.* 2024).

The safety profiling of identified peptides was reinforced by ToxinPred3's ensemble architecture, integrating machine learning, deep learning, and motif-based classification with an AUROC of 0.98, enabling reliable *in silico* discrimination of non-toxic from potentially harmful sequences, with the co-occurrence of low PeptideRanker scores among flagged toxic peptides further supporting their exclusion from further consideration as bioactive candidates (Rathore *et al.* 2024). For allergenicity, the inter-platform discordance observed across prediction tools reflects well-recognized methodological heterogeneity in training datasets, feature representations, and classification thresholds rather than intrinsic peptide limitations (Limvipuvadh *et al.* 2025). This finding affirms the necessity of the multi-algorithm consensus approach employed, wherein pLM4Alg-640 addresses nonstandard sequences via protein language model architecture, AlgPred2 contributes ensemble-based prediction strength, and AllerCatPro 2.0 leverages three-dimensional structural similarity to detect conformational epitopes, yielding a reported 37-fold specificity improvement over sequence-only methods and substantially reducing false-positive classifications (Maurer-Stroh *et al.* 2019; Du *et al.* 2024; Nguyen *et al.* 2026). Collectively, this integrated multi-tiered pipeline provides a rigorous and efficient framework for identifying

functionally promising, safety-profiled peptide candidates warranting further experimental validation.

The complementary application of AntiDMPpred and AntiT2DMP-Pred facilitated mechanistically stratified screening of antidiabetic peptide candidates derived from fermented mackerel by-product hydrolysates. The distinct discordance observed between the two predictive platforms arises from fundamental differences in their training architectures and targeted mechanisms: AntiDMPpred, employing a random forest methodology, identifies peptides that inhibit carbohydrate-digesting enzymes such as α -glucosidase and α -amylase, which are pertinent to general postprandial glucose regulation (Cai *et al.* 2024; Hayes *et al.* 2024). In contrast, the markedly lower candidate yield under AntiT2DMP-Pred, the first machine learning tool specifically designed for type 2 diabetes mellitus (T2DM) peptide prediction via feature fusion of ten discriminative descriptors, achieving an accuracy of 0.957 on independent validation, which reflects its higher mechanistic specificity for T2DM-relevant pathways such as DPP-IV and α -glucosidase inhibition, rather than limitations of the peptide pool (Basith *et al.* 2025). Notably, the top ranked proline rich collagen derived sequence achieving the highest scores concurrently across both AntiDMPpred, AntiT2DMP-Pred, and PeptideRanker positions itself as the most promising candidate for targeted T2DM therapeutic development, a finding consistent with prior reports that hydrophobic and proline-rich residues facilitate stable binding interactions with key diabetic enzymatic targets such as α -glucosidase and DPP-IV (Basith *et al.* 2025; Liu *et al.* 2025).

The multi-algorithmic evaluation of antimicrobial potential across 35 peptides from fermented mackerel by-product hydrolysates revealed substantial inter-tool variability, reflecting a well-recognized challenge in *in silico* peptide discovery. CAMP_{R4}'s three machine learning algorithms produced divergent classifications, while AMP Scanner vr.2 and ADAM identified distinct subsets, underscoring how differences in training datasets and feature prioritization influence predictions (Veltri *et al.* 2018; Gawde *et al.* 2023). Notably, ADAM predicted antimicrobial activity for a majority of peptides, likely due to its sensitivity to broad physicochemical properties, whereas AntiBP3 trained specifically on antibacterial sequences remained far more conservative (Bajiya *et al.* 2024). This divergence reinforces the necessity of consensus-based interpretation, as reliance on any single algorithm would either overestimate or underestimate true antimicrobial potential. Several glycine-proline-rich

collagen-derived peptides from FSH/FXH fractions were consistently flagged by at least two CAMP_{R4} algorithms, aligning with emerging evidence that certain collagen fragments can exert membrane-active effects (Saleem *et al.* 2025). Integrating predictions across all four tools identified peptides with high-confidence antimicrobial signatures while filtering false positives from individual models. For example, AVFPSIVGRPR and GPAGASGPAGPR were classified as AMPs by CAMP_{R4} (multiple algorithms), AMP Scanner vr.2, and ADAM, with the former also showing moderate antibacterial probability in AntiBP3. Such convergent predictions across structurally and algorithmically distinct platforms elevate these peptides as priority candidates for experimental validation (Kanwal *et al.* 2025). Conversely, peptides are consistently non-antimicrobial by all tools despite possessing other bioactivities, suggesting functional specificity with minimal off-target effects.

In conclusion, this study demonstrates that microbial fermentation combined with ultrafiltration effectively valorizes *Scomberomorus commerson* processing by-products by generating hydrolysates enriched in low-molecular-weight peptides with α -glucosidase inhibitory and antibacterial activities. Peptidomic profiling and *in silico* analysis identified peptide candidates with predicted bioactivity and favorable safety profiles, providing a basis for further validation. These findings support fermented mackerel by-products as promising sources of bioactive peptides for potential functional food and nutraceutical applications. Further studies are required to validate the identified peptides through molecular interaction analysis, gastrointestinal stability, bioavailability, and *in vitro* and *in vivo* experiments.

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