Supplemental Materials

Supplemental Materials and methods

Extraction of quinoa protein and in vitro simulated gastrointestinal digestion

The white quinoa was obtained from Qinghai Nongmai Food Co., LTD. (Haixi, Qinghai, China). To prepare the quinoa protein, a method described in previous studies was followed, with some modifications (You, Wu, Wang, Li, Liu, & Ding, 2022). The fresh quinoa was soaked to remove saponins, and then degreased using n-hexane. The resulting ground quinoa flour was resuspended in distilled water at pH 9.0 and centrifuged. The supernatant was collected and adjusted to pH 4.0. After another round of centrifugation, the pH of the solution was adjusted to 7.0, and it was then subjected to dialysis for desalination. The solution was subsequently subjected to vacuum freeze-drying, resulting in the quinoa protein freeze-dried powder. Dissolved the protein powder and heated it in a water bath, and adjusted the pH to 7.0 to prepare quinoa protein solution. In the simulated gastrointestinal digestion stage, the pH of the solution was initially adjusted to 2.0. Pepsin (Solarbio life sciences, Beijing, China) was added to digest the suspension at 37°C for 4 h. The pH was then adjusted to 7.6, and trypsin (Solarbio life sciences, Beijing, China) was added to simulate intestinal digestion for 6 h. After cooling the digestive solution to room temperature, it was centrifuged and the supernatant was retained. The solution was further subjected to dialysis and desalination, and then stored at 4°C.

Determination of degree of hydrolysis (DH)

The DH was measured using the pH-start method. To stabilize the pH, NaOH solution is added, and the volume of NaOH solution consumed was recorded to calculate the DH. The calculation formula for DH is as follows: $DH = v \times n/\alpha \times h \times m$. v is the volume of NaOH solution consumed during the enzymatic hydrolysis process, measured in mL; n is the concentration of the NaOH solution, measured in mol/L; α is the average dissociation degree of α-NH3; h is the number of peptide bonds broken by one mole of NaOH; m is the mass of protein in the sample, measured in g. By substituting the appropriate values into the formula, the DH can be calculated.

Ultrafiltration membrane separation and determination of polypeptide content

To prepare the enzymatic hydrolysate, follow the steps below: used a 0.45 μm

cellulose membrane filtration to remove insoluble substances from the digested enzymatic hydrolysate. Selected 3 kDa and 1 kDa ultrafiltration membranes and apply them at 4 ℃ under a pressure of 30-50 MPa. This will allow separation of the enzymatic hydrolysate into three components with different molecular weights. The content of polypeptides was determined using the Biuret method. The Biuret reagent was prepared as follows: copper sulfate and potassium sodium tartrate was weighed and dissolved in double distilled water and stir until fully mixed. 10% NaOH solution was added and diluted the solution with distilled water. Prepare a standard solution of the tetrapeptide Gly-Gly-Tyr-Arg. Create different concentrations ranging from 0.0 to 1.8 mg/mL. Take 2.5 mL of the sample solution and add 2.5 mL of 5% Trichloroacetic acid (TCA). Vortex the mixture to ensure proper mixing. After allowing the mixture to stand, separate the supernatant and bring it to volume using 5% TCA. Combine the prepared sample solution with the Biuret reagent in a ratio of 3:2 (sample solution: Biuret) and vortex the mixture. Centrifuge the mixture and collect the supernatant. Measure the optical density of the supernatant at 540 nm. This measurement will provide information about the concentration of polypeptides in the enzymatic hydrolysate. By following these steps, you will be able to prepare and determine the content of polypeptides in the enzymatic hydrolysate using the Biuret method.

Determination and synthesis of peptide sequences

To analyze the sample, the following steps were followed: the sample was dissolved in 200 μ In L Nano-HPLC Buffer A, mixed well and centrifuged to extract the supernatant. The supernatant was transferred to 0.22 μm rotary filter, the filtrate was collected. C18 membrane filled columns was prepared. The evaporated peptide sample was dissolved again in Nano-HPLC Buffer A, and then the sample was centrifuged through columns using methanol, Nano-HPLC Buffer A, and peptide samples respectively; Elution phase Buffer B was used to centrifuge. After desalination, the elution phase buffer B of the peptide containing sample is evaporated. The evaporated peptide sample was redissolved in Nano-HPLC Buffer A. The Nano-HPLC liquid phase system EASY-nLC1200 was separated. Liquid phase A was a 0.1% formic acid aqueous solution, while liquid B was a 0.1% formic acid acetonitrile solution. The sample was loaded onto the automatic sampler and adsorbed to 100 μ M × On a 20 mm RP-C18 chromatographic column, followed by 75 μ M × 150 mm RP-C18 chromatographic column separation. The enzymatic hydrolysis products were

separated by capillary HPLC and analyzed by MS using Q-Exactive MS. The detection method was calibrated with a standard calibration solution before use. The scanning range of the parent ion was 350-2000 m/z, and the mass spectrometry scanning method was information dependent collection mode. After each full scan, the strongest 20 fragments were collected. The fragmentation method was high-energy collision dissociation, with NCE energy of 28 and dynamic elimination time of 25 s. AGC target set to 3e6, maximum injection time of 100 ms, MS2 resolution set to 17 500, AGC target set to 1e5, maximum injection time of 50 ms. The identified peptides were synthesized by Jinsirui Biotechnology Co., Ltd (Nanjing, Jiangsu, China). The purity of the synthesized peptides was verified by Nano-LC-MS/MS. By following these steps, the sample was analyzed using UPLC and mass spectrometry, and the identified peptides were synthesized and stored at -20 ℃ for subsequent use.

Cell culture and detection of RAW 264.7 proliferation activity using MTT

RAW 264.7 cells were cultured in DMEM culture medium supplemented with 10% fetal bovine serum. Once the cells reached a stable state, they were washed with phosphate-buffered saline (PBS). Cell suspensions (100 μL) were added to the sample wells, while PBS was added to the blank control wells. Cell intervention solutions were prepared for each component peptide at various concentrations: 0, 20, 40, 80, 160, 320, and 640 μg/mL. These solutions were obtained after ultrafiltration. The prepared cell intervention solutions were added to the respective wells containing the cell suspensions and incubated in an incubator for 12 h. After the 12 h incubation period, the different concentrations of cell intervention solutions were added to the wells and further incubated for 24 h. After the incubation, MTT was added and incubated for 4 h. Following the MTT incubation, dimethyl sulfoxide was added and shaken in a constant temperature oscillation chamber at 37 ℃. The OD value at 570 nm was measured using a spectrophotometer.

References

You, H., Wu, T., Wang, W., Li, Y., Liu, X., & Ding, L. (2022). Preparation and identification of dipeptidyl peptidase IV inhibitory peptides from quinoa protein. *Food Research International, 156*, 111176.

Supplemental Tables

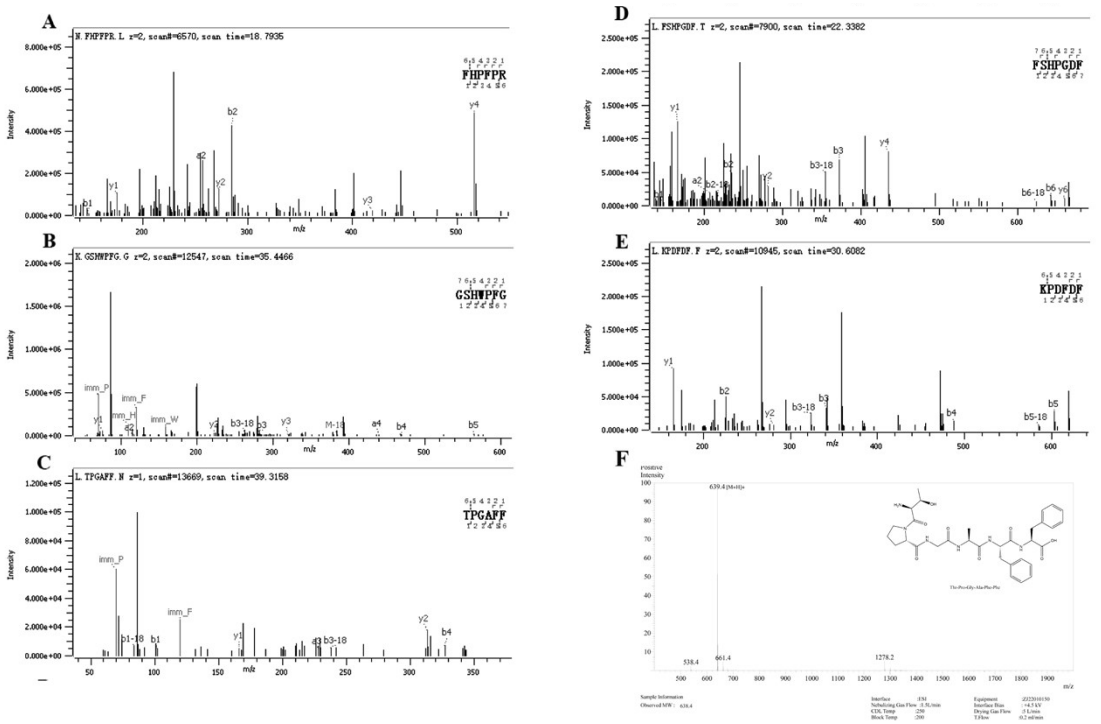
Supplemental Table 1. Primer sequences used for RT-qPCR analysis.

	Forward Primer	Reverse Primer
Muc2	CCATTGAGTTTGGGAACATGC	TTCGGCTCGGTGTTCAGAG
Tff3	CCTGGTTGCTGGGTCCTCTGG	GTCTCCTGCAGAGGTTTGAAGC
Klf4	AGCCACCCACACTTGTGACTATG	CAGTGGTAAGGTTTCTCGCCTGTG
Chst4	GGGTTCCCAGGTCATCGTTG	CCGAAAAGCTGTCCCACAAAA
Fut2	ACCTCCAGCAACGAATAGTGA	GCCGATGGAATTGATCGTGAA
Gal3st2	TTCCTCCTGGTTGGTTTCCT	TGGCTGTCTTGTGCGTCTTG
Trpv1	AGCTGCAGCGAGCCATCACCA	ATCCTTGCCGTCCGGCGTGA
Tac1	GGTCTGACCGCAAAATCGAAC	GATCTGGTCACTGTCGGACC
β-actin	GACATGGAGAAGATCTGGCA	GGTCTTTACGGATGTCAACG

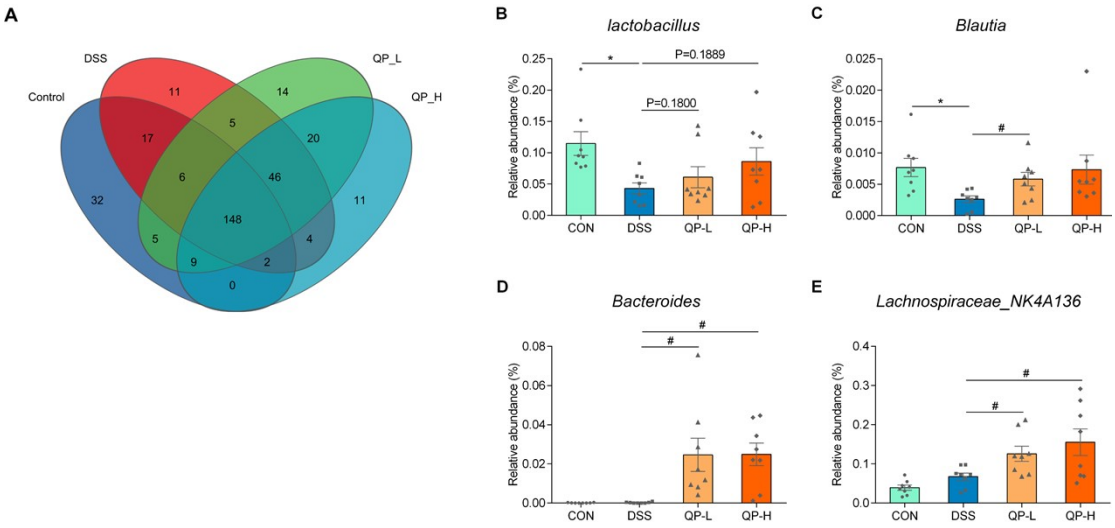
Supplemental Table 2. Identification of target peptide segments and prediction of their biological activity.

Serial Number	Sequence	m/z[Da]	Peptide Ranker Results
1	FHPFPR	800.420	0.9740
2	GSHWPFG	787.352	0.9726
3	TFF	414.203	0.9698
4	LGW	375.202	0.9641
5	APW	373.187	0.9588
6	SPGF	449.203	0.9490
7	FLR	435.271	0.9221
8	LGGF	393.213	0.9110
9	IGGF	393.213	0.9084
10	FPI	376.223	0.9069
11	TPGAFF	639.313	0.9016
12	FSHPGDF	806.347	0.9005
13	KPDFDF	768.356	0.8895
14	VMF	396.195	0.8868

Supplemental Figures



Supplemental Fig. 1. (A-E) Purity and identification of five quinoa peptides. (F) Identification of synthesized peptide TPGAFF.



Supplemental Fig. 2. Effects of TPGAFF on the composition of gut microbiota. (A) Venn diagrams of OTUs. (B-E) The relative abundance of beneficial microbes. Data are the mean ± SEM, n = 8. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the CON and DSS group, respectively.