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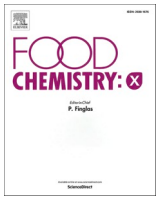

Insights into characteristic metabolites and potential bioactive peptides profiles of fresh cheese fermented with three novel probiotics based metabolomics and peptidomics

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ABSTRACT

The metabolite and peptide profiles of fresh cheese fermented by three novel probiotics, *Lacticaseibacillus rhamnosus* B6, *Limosylactobacillus fermentum* B44 and *Lacticaseibacillus rhamnosus* KF7, were investigated using LC-MS/MS-based metabolomics and peptidomics. The multivariate analysis revealed significant differences in metabolite composition between the probiotic fresh cheese and the control sample. The differential metabolites were primarily lipids and lipid-like molecules and organic oxygen compounds, which were associated with fatty acid and carbohydrate-related pathways. Among three probiotics, *L. rhamnosus* KF7 showed the highest effectiveness in sucrose decomposition. 147 potential bioactive peptides, mainly derived from casein, were identified in probiotic fresh cheese. Furthermore, 112 bioactive peptides were significantly up-regulated in probiotic fresh cheese. Molecular docking analysis indicated that two short peptides (LVYPFPGPIP and YPQRDMPIQ) in the B44 and KF7 groups exhibited low estimated binding energy values (−9.9 and −6.9 kcal/mol) with ACE. These findings provide a theoretical basis for developing novel probiotic-enriched fresh cheese.

1. Introduction

Fermented food is gaining popularity as a nutritious and functional food. The fermentation process involves complex microbial communities that trigger various biochemical reactions, leading to the release of bioactive compounds that are beneficial for human health (Diez-Ozaeta & Astiazaran, 2022). Fresh cheese, a fermented dairy product, is commonly known for its mild flavor profile and creamy texture. Some studies have demonstrated that fresh cheese has the potential to provide excellent nutritional and health benefits (Silva et al., 2019). It is important to note that the flavor and health characteristics of fresh cheese are influenced by small molecule metabolites and peptides produced during milk fermentation. Small molecule metabolites in fresh cheese, such as amino acids, organic acids, and fatty acids, contribute to the flavor and nutritional quality of cheese products (Zhang, Zheng, Zhou, & Ma, 2022). These metabolites may be derived from protein, fat and lactose metabolism during milk fermentation. Additionally, milk protein serves as a rich source of bioactive peptides (BPs). During milk fermentation, lactic acid bacteria (LAB) can hydrolyze proteins into peptides through the action of secreted peptidyl peptidase, aminopeptidase, and carboxypeptidase (Chourasia et al., 2021). Previous studies have shown that water-soluble peptides in fresh buffalo cheese exhibit various activities, including antibacterial, antioxidant and antihypertensive effects (Silva et al., 2019). Therefore, both small molecule metabolites and BPs play significant roles in determining the quality of fresh cheese.

Probiotics have gained substantial attention due to their potential health benefits and their contribution to overall well-being (Manzoor, Wani, Ahmad Mir, & Rizwan, 2022). Incorporating probiotics into food products, particularly fermented foods, has emerged as a promising strategy for enhancing nutritional value (Diez-Ozaeta & Astiazaran, 2022). For instance, ACE inhibitory peptides were discovered in milk fermented with wild *Lactococcus* (*L.*) *lactis* NRRL B-50571, which was isolated from hand-made dairy products (Beltrán-Barrientos et al., 2018). In previous studies, *Lacticaseibacillus rhamnosus* (*L. rhamnosus*) B6, *Limosilactobacillus fermentum* (*L. fermentum*) B44 and *L. rhamnosus* KF7 were isolated from Kefir and Bulgarian household hand-made yogurt. The *L. fermentum* B44 and *L. rhamnosus* B6 exhibited beneficial

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activity against dental caries; *L. rhamnosus* KF7 and *L. rhamnosus* B6 displayed antioxidant and cytoprotective abilities (Han, Wu, Wan, & Wu, 2022; Li, Zhang, & You, 2022; Yu, Wang, Wu, Zhang, & Wu, 2021). These findings suggest that these three bacterial strains have remarkable probiotic potential. Introducing probiotics into cheese could be a viable solution for diversifying dairy product offerings in a changing consumer market (Hao, Xia, Wang, Zhang, & Liu, 2023). Currently, the metabolites and peptides generated in fresh cheese fermented by *L. rhamnosus* B6, *L. fermentum* B44 and *L. rhamnosus* KF7 are unknown. It is crucial to identify the characteristic metabolites and bioactive peptides to evaluate the health benefits of these three probiotics and contribute to the development of novel dairy products with multiple probiotic properties.

Metabolomics and peptidomics have emerged as powerful approaches for comprehensively understanding the complex biochemical changes in food systems (Wang, Wei, Chen, Ma, & Huang, 2023). Metabolomics could provide insights into the overall changes in the metabolite composition of dairy products, offering valuable information about the biochemical responses to various interventions (Zhang et al., 2023). For example, Peng et al. (2022) investigated the effects of the probiotics (L. casei Zhang and B. lactis V9) on the growth and metabolomic profiles during milk fermentation and storage using metabolomics. On the other hand, peptidomics focuses on the systematic study of peptides, which may influence characteristics such as taste, aroma, and potential bioactivity of foods. Peptidomics can be used to understand the role of microorganisms in food proteolysis and to identify BPs and biomarker peptides derived from food (Martini, Solieri, & Tagliazucchi, 2021). Wang et al. (2023) analyzed BPs in milk fermented with *L. plantarum* L3 using peptidomics, and they identified 6 ACE inhibitory peptides, 19 antioxidant peptides and 5 antimicrobial peptides. Therefore, integrating non-targeted metabolomics and peptidomics approaches is a reliable method to explore the multifaceted effects of probiotics on fresh cheese, shedding light on characteristic metabolites and functional polypeptides.

This study aims to elucidate the impact of three novel probiotic (*L. rhamnosus* B6, *L. fermentum* B44 and *L. rhamnosus* KF7) incorporations on the characteristic metabolites and potential functional polypeptides present in fresh cheese. Non-targeted metabolomics and peptidomics were used to analyze the fresh cheese fermented with the probiotics. Through non-targeted metabolomics and multivariate analysis, the characteristic metabolites in the cheese were identified. On the other hand, polypeptides were identified and potential BPs were screened according to the database. Furthermore, the bioactive mechanism of these potential BPs was explained by analyzing the structure–activity relationship using molecular docking. This study aims to uncover the underlying mechanisms through which probiotics affect the metabolomic and peptidomic profiles of fresh cheese. Such insights are essential not only for improving our understanding of the probiotic-fresh cheese interaction but also for utilizing the potential of probiotics to enhance flavor and nutritional quality.

2. Materials and methods

2.1. Manufacturing of the fresh cheese

The pressure-driven filtration allows for the manufacture of cheese through liquid precheese technology. This process involves concentrating the milk into a retentate, which is directly converted to cheese through acidification and gelation without removing the whey (Chamberland, Brisson, Doyen, & Pouliot, 2022).

In this study, the fresh cheese was manufactured as follows: Defatted casein (CN) concentrated milk was prepared via filtration. Bulk pasteurized skim milk was obtained from Bright Dairy & Food Co., Ltd. The skim milk was concentrated using a filtration pilot plant (Tetra Pak, Denmark) equipped with a 1 m^2 tubular membrane to reach a concentration factor of 3. The skim milk was kept at a temperature below 10 °C throughout the process. The microfiltration process was carried out at a transmembrane pressure of 1.5 bar. The concentrate (80 %) was then mixed with cream (15 %), and sucrose (5 %) was dissolved into the mixture. Subsequently, the mixture was heated to 95 °C for 5 min and rapidly cooled to 34 ± 2 °C. The experiment was repeated six times under the same conditions to obtain six replicates for each treatment. A *Lc. Lactis* commercial starter (Danisco-China, Kunshan, China) and probiotics were added to the mixture at the concentration of 0.05 DCU/L and 3 × 10^9 CFU/L, respectively. The mixture was fermented at 34 °C until the pH reached 4.6 (about 12 h). The curd was then broken into small particles (about 1–3 mm) by simply stirring the coagulum. Finally, the final product was obtained after refrigeration at 4 °C for 12 h.

Three types of probiotic cultures incorporated in cheese consisted of *L. fermentum* B44 (CGMCC 17321), *L. rhamnosus* KF7 (CGMCC 6430) and *L. rhamnosus* B6 (CGMCC 13310), respectively, which were obtained in the freeze dried form from the State Key Laboratory of Dairy Biotechnology, Dairy Research Institute of Bright Dairy & Food Co., Ltd. The samples were labeled as: CK for cheese inoculated with only basic starter culture, B44 for basic starter culture and *L. fermentum* B44, KF7 for basic starter culture and *L. rhamnosus* KF7, and B6 for basic starter culture and *L. rhamnosus* B6. All the collected samples were stored at −80 °C until further analysis.

2.2. Metabolomics of fresh cheese

2.2.1. Metabolite extractions

The metabolites were extracted according to a previous method (Gu et al., 2020) with slight modifications. The frozen samples (0.4 g) were thawed at 4 °C and then mixed with 4 mL of extraction solvent at the same temperature. The extraction solvent consisted of methanol, acetonitrile, and water in a ratio of 2:2:1 (v/v/v). The mixture was vortexed adequately and then sonicated at 4 °C for 30 min. After that, it was left to stand still at −20 °C for 10 min. After centrifugation at 14,000×g for 20 min at 4 °C, the collected supernatant was vacuum dried. For LC-MS analysis, the samples were re-dissolved in 100 μL acetonitrile/water (1:1, v/v) solvent and centrifuged at 14,000g for 15 min at 4 °C, and the supernatant was transferred to LC vials for analysis. The quality control (QC) sample was prepared by pooling equal amounts (200 μL) of extracts from each sample.

2.2.2. UHPLC-Q-TOF-MS/MS analysis

The metabolites in samples were separated by using ultra-high performance liquid chromatography (UHPLC) (1290 Infinity LC, Agilent Technologies) equipped with an ACQUIY UPLC BEH column (particle size of 1.7 μm, 2.1 mm × 100 mm, Waters, Ireland). The mobile phase consisted of solvent A (25 mM ammonium acetate and 25 mM ammonium hydroxide in water) and solvent B (acetonitrile). The gradient elution program was as follows: 0–0.5 min, 95 % B; 0.5–7 min, 95 %–65 % B; 7–8 min, 65 %–40 % B; 8–9 min, 40 % B; 9–9.1 min, 40 %–95 % B; 9.1–12 min, 95 % B. The column temperature was maintained at 25 °C; the flow rate was set at 0.5 mL/min; and the injection volume was 2 μL. The samples were placed in an autosampler at 4 °C throughout the analysis. In order to avoid the influence of instrument detection signal fluctuations, a randomized sequence was applied for the continuous analysis of samples. QC samples are inserted into the sample queue to monitor and evaluate the stability of the system and the reliability of the experimental data.

The primary and secondary spectra were collected using a mass spectrometer (TripleTOF 6600, AB SCIEX, Singapore) in both electrospray ionization (ESI) positive and negative ion modes. The ESI source parameters were as follows: ion source gas1 (Gas1), 60 psi; ion source gas2 (Gas2), 60 psi; curtain gas (CUR), 30 psi; source temperature, 600 ℃; ion spray voltage floating (ISVF), ± 5500 V. In MS acquisition, the instrument was set to acquire data over the *m/z* range of 60–1000 Da with the accumulation time for the TOF MS scan of 0.20 s/spectra. In auto MS/MS acquisition, the instrument was set to acquire over the *m/z* range 25–1000 Da with accumulation time for product ion scan of 0.05

s/spectra. The MS/MS ion scan was obtained using information-dependent acquisition (IDA) with high sensitivity mode selected. The parameters were set as follows: the collision energy (CE) was kept at 35 V with ±15 eV; declustering potential (DP), 60 V (+) and −60 V (−); dynamically exclude isotopes within 4 Da, candidate ions to monitor per cycle: 10.

2.2.3. Metabolomics data processing and bioinformatics analysis

The initial MS data files in the form of wiff.scan were converted to MzXML format via ProteoWizard MSConvert. Peak picking, grouping and alignment were performed using XCMS software. The following parameters were used for peak picking: centWave m/z = 25 ppm, peakwidth = c (10, 60), prefilter = c (10, 100). For peak grouping, bw = 5, mzwid = 0.025 and minfrac = 0.5 were used. Only the ion features varied more than 50 % of the nonzero measurement values in at least one group were extracted. The metabolites were identified by comparing the MS/MS spectra with an in-house database created using available authentic standards.

After being normalized to total peak intensity, the processed data were imported into the online software MetaboAnalyst 5.0 (https://www.metaboanalyst.ca) for multivariate data analysis, including Pareto scaled principal component analysis (PCA) and partial least squares discriminant analyses (PLS-DA). The robustness of the model was evaluated using permutation testing. The variable importance in the projection (VIP) values >1 in the PLS-DA model and $p <$ 0.05 was considered statistically significant.

The metabolites were mapped to the pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/pathway.html). The enrichment analysis was conducted to explore the impact of differential metabolites further. The pathways with p-values below 0.05 were considered as significantly changed pathways.

2.3. Peptidomics of fresh cheese

2.3.1. Extraction of cheese peptides

The cheese samples were treated with digestive enzyme following the procedure by Hao et al. (2021), with some modifications. All the cheese samples were digested with pepsin solution adjusted to pH 2.0 and incubated at 37 °C for 2 h. After the reaction, the pH of the cheese hydrolysate was adjusted to 8 with 1 M NaOH to interrupt the gastric digestion phase (Wei et al., 2022) and digested with trypsin for 2 h at 37 °C. The peptide selection by ultrafiltration intercept was not preformed. The samples were centrifuged at 10,000×g and 4 °C. The flow-through liquid was vacuum-dried and combined with 0.1 % trifluoroacetic acid for reconstitution. After desalting the peptide using a C18 column, the peptides were vacuum-dried and reconstituted with 20 μL of 0.1 % formic acid solution. The peptide concentration was determined in OD280 to ensure that 2 μg of the peptides were available for mass spectrometry injection.

2.3.2. Identification of peptides

A previous method was followed (P. Liu, Guo, Mao, & Gu, 2020) with slight modifications. In brief, a Q-Exactive HF-X MS coupled with Easy-nLC 1200 (Thermo Fischer Scientific, Waltham, MA, USA) HPLC system was used for separating and detecting the peptide samples. The samples were injected into the trap column and then loaded onto a C18-reversed-phase column (75 μm × 25 cm, Thermo Fischer Scientific, USA) with a flow rate of 300 nL/min. The gradient phase contained of phase A of 0.1 % formic acid aqueous solution and phase B that was resolved using a linear gradient of 0.1 % formic acid in 84 % acetonitrile. The gradient procedure was as follows: 0–40 min, linear-gradient phase B (8–30 %); 40–50 min, B (30–100 %); 50–60 min, B (100 %).

Mass spectrometry acquisition was carried out in positive ion mode. The first-stage scanning range was set to 200–3000 m/z, achieving a resolution of 120,000 (m/z = 200). The automatic gain control (AGC) was set to 3e6; the maximum ion trapping (IT) was 200 ms. After each full scan, 20 MS2 scans were collected. The MS2 activation type was higher energy C-trap dissociation (HCD) and the isolation window was maintained at 1.5 m/z. MS2 scan resolution was fixed at 30,000 (m/z = 200) and normalized collision energy was maintained at 30 eV.

2.3.3. Data analysis

PEAKS Studio 8.0 software (Bioinformatics Solutions Inc, Waterloo, Canada) was used for data processing and spectral analysis of original MS/MS files, including peptide annotation and quantitative analysis.

The parameters were set as follows: pepsin and trypsin were used as enzymes; the precursor and fragment mass error tolerance for database search were set to 10 PPM and 0.05 Da, respectively. The fixed modifications were set to carbamidomethylation, while the variable modifications included Oxidation (M), Acetyl (Protein N-term) and Phospho (STY). The detected MS/MS data was matched to the UniPro database. A false discovery rate (FDR) of 1 % was used for validation.

2.4. Molecular docking

The crystal structure of ACE (PDB ID: 1O8A) was obtained from the RCSB Protein Data Bank (https://www.rcsb.org/). The polypeptide chain structure was drawn by using the Chem Office 2015 software (Cambridge Soft Co., Boston, USA), and the peptide geometry was subsequently optimized to minimal energy. The PyMOL software was used to remove water molecules and excess ligands, retain zinc ions and chloride ions. Additionally, total hydrogen was added to the ACE model before the docking process (Hao, Xia, Wang, Zhang, & Liu, 2023; Wu, Du, Jia, & Kuang, 2016). Docking simulations were performed using the Autodock Vina. The coordinates of the grid center were set as x:43.821, y:33.61, z:43.38, with a size of 80 × 80 × 80, and a reaction constraint box with a spacing of 0.375 Å (Hao, Xia, Wang, Zhang, & Liu, 2023). The docking results were visualized using PyMOL software (Guo et al., 2023); the interaction forces among the docked complexes were analyzed using Discovery Studio 4.0 software. The protein was displayed as a strip and the non-active amino acids were hidden.

3. Results and discussion

3.1. Comparison of metabolites

3.1.1. Metabolite profiles and PCA

In this study, the metabolites in fresh cheese were investigated using the LC-MS/MS-based non-targeted metabolomics. The base peak intensity chromatograms of fresh cheese fermented with various LABs were illustrated in Fig. S1. A total of 12,920 and 9,736 ion characteristics were detected in positive and negative ion modes, respectively (Table S1). Among these, 379 metabolites were identified by matching their mass spectrum with the database.

PCA analysis was performed to evaluate the overall difference between the control and probiotic-involved fermentation samples using an unsupervised model. Each sample in each group was depicted as a dot in the PCA score plot, indicating the variation between the samples based on their aggregation and separation trend. A higher aggregation of dots suggested a higher similarity in observed variables, while a greater dispersion of dots indicated a more significant difference in observed variables (Zhan et al., 2023). The relationship between cheese sample composition in positive and negative ion modes was illustrated in Fig. S2. All biological replicate dots were located within the 95 % confidence circle, and the QC samples were closely clustered, indicating good stability and reproducibility of the experiment (Liu et al., 2023). In the positive mode (Fig. S2A), PC1 (43.2 %) and PC2 (22.9 %) together explained 66.1 % of the total variance. In the negative ion mode (Fig. S2B), PC1 (77.2 %) and PC2 (11.5 %) together explained 88.7 % of the total variance. In both positive and negative ion modes, the samples from each group were clearly distinguished and separated, indicating

that the involvement of probiotics in fermentation significantly altered the metabolite composition of fresh cheese, providing a basis for identifying differential metabolites.

3.1.2. PLS-DA and comparative analysis of differential metabolites

The PLS-DA model was used to identify the most discriminative variables in the dataset. There were distinct separations between the cheese fermented by three novel probiotics and the CK group in PLS-DA score plots (Fig. 1A-C). The R^2Y and Q^2Y values of the PLS-DA model were all greater than 0.90, indicating a high level of interpretability and predictability in the model. All Q^2 values of the permutation test (N = 200) were lower than those of the original model, and the intercept of the Q^2 regression line relative to the longitudinal axis was negative (Fig. S3), suggesting that the model did not over-fit (Rong et al., 2023).

The metabolites were further screened based on the VIP value and *p*-value to identify differential metabolites. Metabolites that satisfied both VIP > 1 and p < 0.05 criteria were considered as differential metabolites. The differential metabolites of each comparison group were visualized in the form of volcano plots (Fig. 1D-F). A total of 46 (B6 VS CK), 103 (B44 VS CK), and 147 (KF7 VS CK) significant differential metabolites were selected, respectively. The lower number of differential metabolites in the B6 VS CK comparison group may be attributed to the antagonistic effect of biological control during the mixed fermentation of the two strains (Torres-Guardado, Esteve-Zarzoso, Reguant, & Bordons, 2022; Wang et al., 2023). Differential metabolites satisfying FC > 2 or FC < 0.5 were considered significantly up-regulated or down-regulated. In the B6 group, 6 metabolites were significantly up-regulated and 22 were significantly down-regulated. In the B44 group, 19 metabolites were up-regulated and 49 were down-regulated. In the KF7 group, 40 metabolites were up-regulated and 67 were down-regulated compared to the CK group.

The identified differential metabolites are presented in Table S1. The metabolites that showed the most significant differential accumulation among the three comparison groups were classified as lipids and lipid-

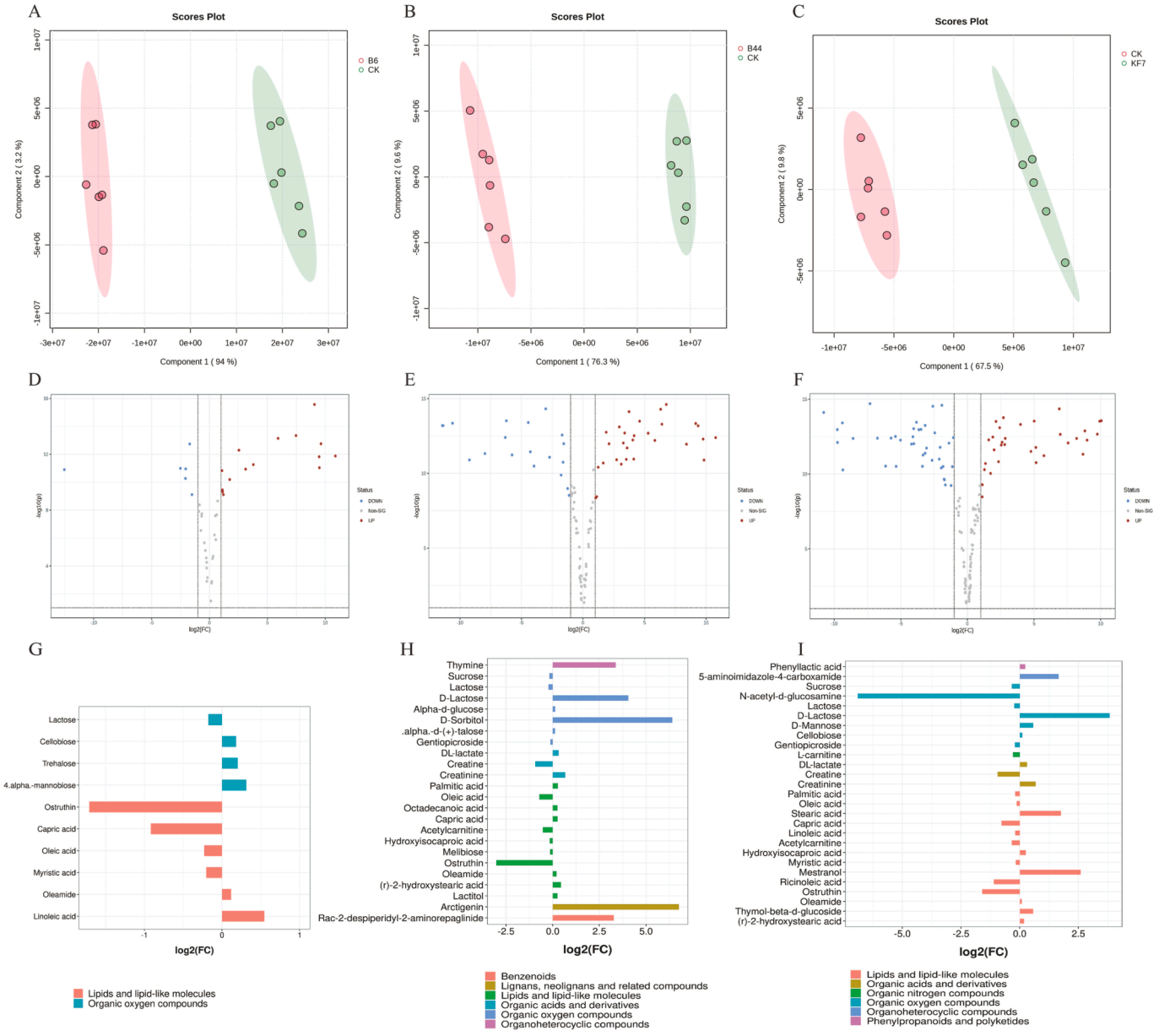

Fig. 1. PLS-DA score plots (A-C), volcano plots (D-F) and bar charts (G-I) of differential metabolites between B6/CK group, B44/CK group and KF7/CK group, respectively. Red dots, blue dots and gray dots in the volcano plot indicate significant upregulation, significant downregulation and insignificant changes of differential metabolites, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

like molecules, followed by organic oxygen compounds. The changes in these differential metabolites (VIP > 1, $p < 0.05$) between groups are illustrated in Fig. 1(G-I). Among the lipids and lipid-like molecules, the abundance of acetylcarnitine showed varying degrees of down-regulation in the B44/CK group and KF7/CK group (Fig. 1H-I). It is worth noting that acylcarnitine promotes fatty acid oxidation (Teren et al., 2020), suggesting that different levels of fatty acid oxidation may occur in B44 and KF7 groups. Additionally, the abundance of melibiose was down-regulated in the B44/CK group (Fig. 1H). Melibiose can be hydrolyzed into glucose and galactose through the catalytic action of α-galactosidase (Mital, Shallenberger, & Steinkraus, 1973). Among the differential metabolites, organic oxygen compounds were also observed. Lactose was down-regulated in the B6/CK group, B44/CK group, and KF7/CK group (Fig. 1G-I), indicating that the three probiotics have stronger lactose utilization ability. This suggests that adding these three probiotics is beneficial for lactose degradation during fermentation. In the comparison between the B44/CK group and the KF7/CK group, sucrose abundance displayed a reduction (Fig. 1H-I), with the down-regulation degree being greater in the KF7 group, indicating that the addition of *L. rhamnosus* KF7 was most effective among the three probiotics in sucrose decomposition. Furthermore, the B44/CK group and the KF7/CK group exhibited decreased creatine and increased creatinine levels. Creatine, a derivative of amino acid commonly found in milk and cheese, can be converted to creatinine by bacteria (Zhang, Zheng, Feng, Zhou, & Ma, 2022). Therefore, it is speculated that the transformation described above could occur during the fermentation process when *L. fermentum* B44 and *L. rhamnosus* KF7 were introduced. These observations suggested that the probiotics contributed to the breakdown of certain disaccharides and the conversion of fatty and organic acids in fresh cheese.

3.1.3. Metabolic pathway

Analysis of metabolite-related pathways provides further insights into the differences between groups (Wu et al., 2022). Differential metabolites were associated with specific metabolic pathways. The essential metabolic pathways of the differential metabolites were obtained by enrichment analysis based on the KEGG database to explore the changes in metabolic pathways of fresh cheese fermented by different probiotics. The key metabolic pathways involved in the metabolites are illustrated in Fig. 2. The vertical axis represents the metabolic pathway, while the color of the bubble in the figure indicates the *p*-value. A darker shade of red indicates a smaller *p*-value, indicating greater significance. The size

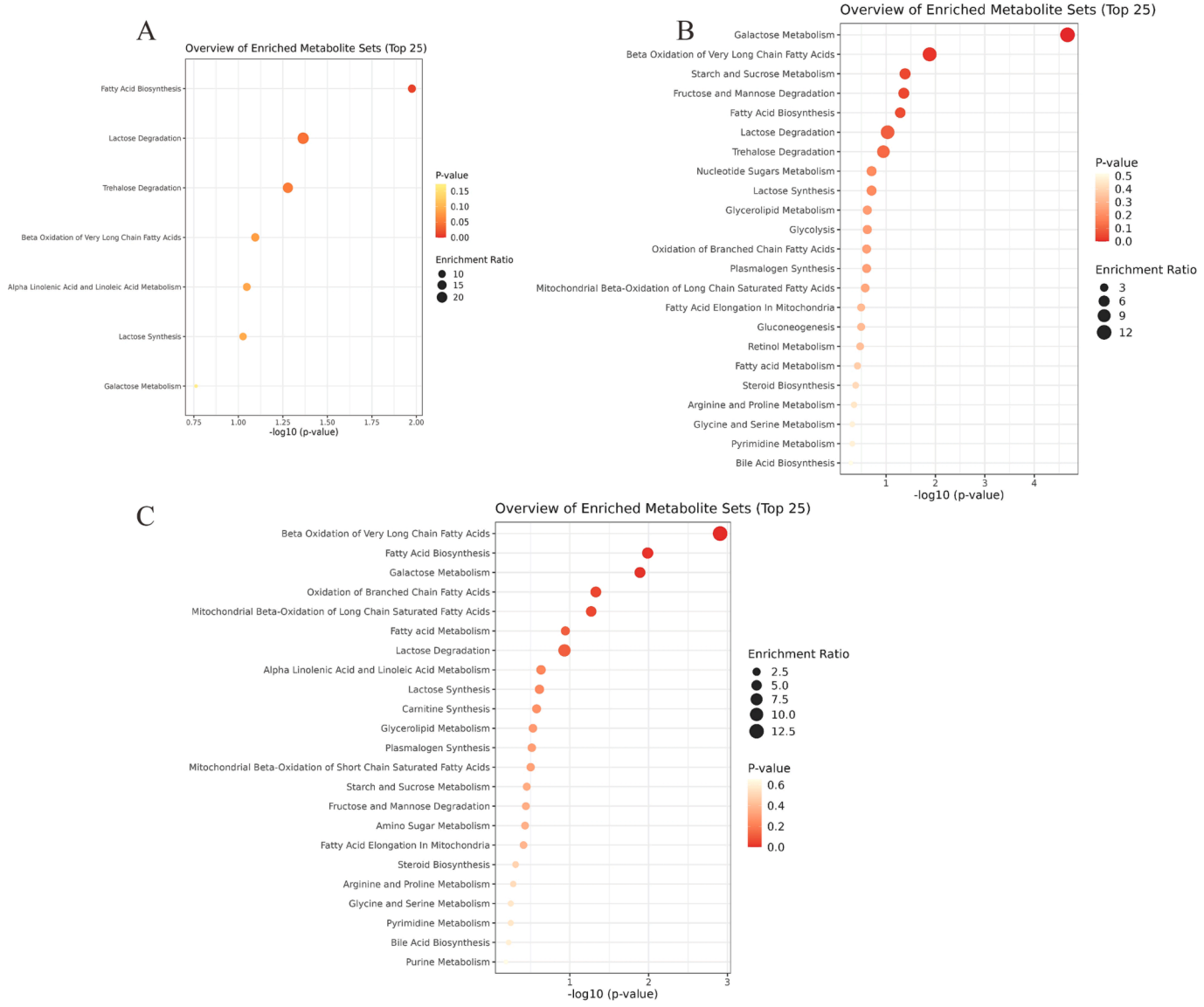

Fig. 2. Metabolic pathways with significant differences in metabolite enrichment in different comparison groups: B6/CK group (A), B44/CK group (B), and KF7/CK group (C).

of the bubble corresponds to the number of different metabolites in the pathway, with larger bubbles representing more mapped metabolites.

As depicted in Fig. 2A, the main pathways ($p < 0.05$) associated with the differential metabolites between the B6 and CK groups were fatty acid biosynthesis (involving capric acid and myristic acid) and lactose degradation (involving alpha-lactose). Galactose metabolism (involving melibiose, alpha-lactose, sorbitol, sucrose and alpha-D-glucose), starch and sucrose metabolism (involving sucrose and alpha-D-glucose) and fructose and mannose degradation (involving sorbitol and alpha-D-glucose) were the main pathways ($p < 0.05$) linked to the differential metabolites between the B44 and CK groups (Fig. 2B). Furthermore, L-acetylcarnitine and capric acid, which play a role in the beta oxidation of very long chain fatty acids, were affected in B44 group. The primary pathway ($p < 0.05$) related to the differential metabolites between the KF7 and CK groups was beta oxidation of very long chain fatty acids (involving L-carnitine, L-acetylcarnitine and capric acid), fatty acid biosynthesis (involving palmitic acid, capric acid and myristic acid), galactose metabolism (involving D-mannose, alpha-lactose and sucrose) and oxidation of branched chain fatty acids (involving L-carnitine and L-acetylcarnitine) (Fig. 2C). These findings were further supported by changes in the abundance of differential metabolites related to corresponding pathways.

The three probiotics involved in fermentation significantly impacted the metabolites in the fatty acid and carbohydrate-related metabolic pathways, as compared to the CK group. Specifically, more differential metabolites in carbohydrate-related pathways were discovered in the B44/CK comparison group, while more differential metabolites mapped to fatty acid-related metabolic pathways were discovered in the KF7/CK comparison group. Additionally, the lactose degradation pathway was only significant in the B6/CK comparison group. These phenomena might be attributed to the unique physiological characteristics of the three probiotics. In recent years, some studies have focused on the

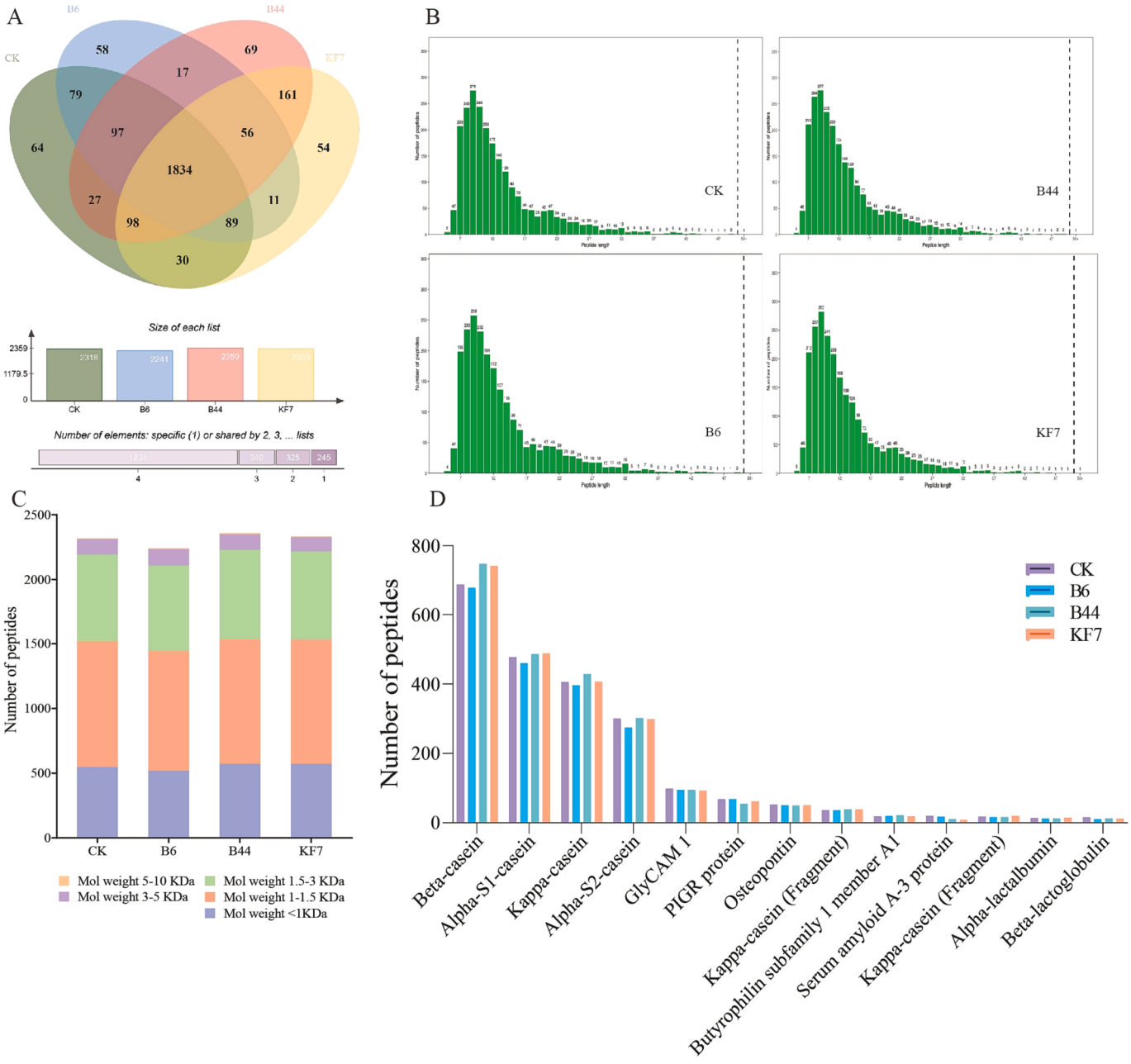

Fig. 3. The profile of peptides identified in fresh cheese treated with pepsin and trypsin. (A) venn diagrams of the number of peptides identified in four groups; (B) number of peptides categorized by length; (C) MW distribution of the peptides; (D) number of peptides derived from the different parent proteins.

changes in metabolic pathways observed in fermented foods. Wang et al. (2023) conducted a study on the impact of fermentation methods on the quality of Lycium barbarum and Polygonatum cyrtonema compound wine, which found that the tyrosine metabolism, phenylpropanoid biosynthesis, and 2-oxocarboxylic acid metabolism pathways were enriched with 17 different metabolites through KEGG analysis. Similarly, Zha et al. (2021) investigated the fermentation pathway of differential metabolites in *Lactobacillus plantarum* P9 fermented milk. They discovered significant changes in fatty acids such as stearic acid, 3-phenyllactic acid, 10-ketostearic acid, 10-hydroxystearic acid, as well as some bioactive substances. These changes have a substantial impact on the distinctive sensory quality and functional attributes of P9 fermented products. In general, these pathways (Fig. 2) may play an important role in the unique metabolite composition of probiotic fresh cheese. By understanding and manipulating these pathways, it is possible to create novel fresh cheese products with unique and desirable qualities.

3.2. Peptidomics of fresh cheese

3.2.1. Peptide profiling

LC-MS/MS was used to analyze the peptides in fresh cheese from each group after pepsin and trypsin hydrolysis. The difference in the number of peptides in the cheese samples was observed. The B44 and KF7 groups showed a higher total number of peptides than the CK group (Fig. 3A), indicating that the presence of probiotics during the fermentation process changed the properties of fresh cheese.

The Venn diagram (Fig. 3A) illustrates the number of common and characteristic peptides in fresh cheese fermented by different bacteria. After pepsin and trypsin hydrolysis, B6, B44, and KF7 group digests yielded 58, 69, and 54 unique peptides, respectively. In comparison to the CK group, 426 unique peptides were identified in the three samples fermented with probiotics. The resistance of peptides to enzymatic hydrolysis is an important factor in determining the bioavailability of peptides (Xu, Hong, Wu, & Yan, 2019). These peptides are resistant to enzymatic hydrolysis by pepsin and trypsin, suggesting their potential to be absorbed in the intestine.

The peptides in the four groups of samples mainly consist of 7–12 amino acids (Fig. 3B), with most of the peptide molecular weight (MW) being less than 3000 Da (Fig. 3C). The length and distribution of peptide molecular weight are associated with proteolysis and peptide bond cleavage (Wang et al., 2023). Peptides with lower molecular weights are easily broken down and absorbed by the gastrointestinal tract, allowing them to have potential physiological effects (Luan, Feng, & Sun, 2021). Consequently, the digests of fresh cheese in groups B44 and KF7 may contain a higher abundance of peptides that can be readily digested and absorbed by the gastrointestinal tract.

Fig. 3D illustrates the distribution of protein sources of peptides in fresh cheese. The peptides in digested cheese samples mainly originate from β-CN, α_{s1}-CN, κ-CN and α_{s2}-CN (Fig. 3D), which are the primary components of casein. β-CN, α_{s1}-CN and α_{s2}-CN have flexible structures that make them susceptible to digestion by pepsin and trypsin (Wei et al., 2022). Additionally, κ-CN is also a significant source of peptides in fresh cheese (Fig. 3D). Notably, the number of peptides derived from κ-CN in the B44 group was significantly higher than in other groups, suggesting that *L. fermentum* B44 exhibits better hydrolysis of κ-CN. The κ-CN has a rigid structure and is resistant to hydrolysis by pepsin and trypsin. However, LAB possesses extracellular proteases and peptidases, such as endopeptidase, dipeptidase, and aminopeptidase, which can selectively hydrolyze κ-CN and release active peptides (Pisanu et al., 2015). As a result, κ-CN in fresh cheese processed through fermentation is more easily hydrolyzed, leading to the release of peptides.

3.2.2. Bioactive peptides

The peptide sequence was searched in the general bioactive peptide database (BIOPEP) and the milk bioactive peptide database (MBPDB). A total of 147 BPs were found in the four groups of cheese samples, exhibiting ACE inhibition, antibacterial, antioxidant and anti-diabetic activities (Table S2). Previous studies have indicated that probiotics used in fermentation have the potential to release BPs in dairy products (Wang et al., 2023). In fresh cheese, the identified BPs were mainly derived from α-CN, β-CN, and κ-CN (Table S1). ACE inhibitory peptides were primarily released by α_{s2}-CN and β-CN; Antioxidant peptides were mainly released by κ-CN; while antimicrobial peptides were mainly released by α_{s1}-CN (Table S1). Additionally, a unique peptide DNIQGITKPAIR, produced in the B6 group, has shown immunomodulatory biological activity in previous studies (Qu et al., 2022). Two other unique peptides, NPWDQVKR and LVYPFPGPIP, were produced in the B44 and KF7 groups. NPWDQVKR was purified from goat cheese protein hydrolysate and demonstrated improved insulin resistance activity (Gong et al., 2020). LVYPFPGPIP and YPQRDMPIQ, another unique peptide produced in the B44 group, exhibited ACE inhibitory activity.

Assuming that peak intensity difference analytes for identical peptides accurately reflect the relative differences in their abundance (Solieri, De Vero, & Tagliazucchi, 2018), the relative amounts of identical peptides in different products from the same matrix were compared. The abundance of 139 BPs significantly differed between groups ($p < 0.05$). Among these, the abundance of 112 BPs exhibited a significant up-regulation in one or more groups of B6, B44 and KF7 compared to CK (Fig. 4). These results showed that probiotics promoted the release of BPs in fermented fresh cheese. The detailed list of peptides (Table S1) can be used to predict the peptide composition in the protease hydrolysates of fresh cheese fermented with *L. fermentum* B44, *L. rhamnosus* KF7 and *L. rhamnosus* B6. Furthermore, among 147 identified BPs, 91 had the sequence length of 6–10, 46 had the sequence length of 11–19; the molecular weight of 116 peptides was found to be less than 1600 Da, which is similar with the structural characteristics of the aforementioned transportable peptides in previous research (Karaś, 2019; Regazzo et al., 2010; B. Wang & Li, 2017).

3.2.3. Analysis of the CN cleavage site-specificity and unique BPs formation pathway

The composition of BPs in fresh cheese might result from the combined action of cleavage of proteins by peptidyl peptidase, aminopeptidase, and carboxypeptidase, which are secreted by LAB, as well as digestive enzymes. The BPs released from fresh cheese mainly originated from α_{s1}-CN, α_{s2}-CN, β-CN, and κ-CN (Table S1). The heat map demonstrates the frequency of occurrence of released BP amino acids in CN sequences, the distribution of digestive enzyme cleavage sites, and the regions that are more susceptible to proteolysis within the CN molecules (Fig. 5A-D). The red regions indicate a high frequency of occurrence, while the blue regions represent a low frequency. The swift color changes indicate the concentrated use of a single cleavage site.

The heat map reveals the presence of complex enzymatic systems that can break down CN during fermentation and enzymatic hydrolysis. The key regions for BPs were primarily f (16–49) and f (98–115) for α_{s1}-CN, f (204–222) for α_{s2}-CN, f (75–83) and f (208–224) for β-CN, and f (45–50) for κ-CN. Notably, a higher richness of peptides released from the region f (38–55) of α_{s1}-CN was shown in B6 group, while the relatively higher richness of peptides in the f (210–219) region of β-CN was found in B44 and KF7 groups. These findings indicate the specific hydrolysis patterns of CN by the three probiotics in conjunction with digestive enzymes.

The f (208–224) region from β-CN underwent significant proteolysis, generating peptides with ACE inhibitor activity (Fig. 5C; Table S2). The BP sequence hydrolyzed from β-CN in fresh cheese covered most of the protein sequence except for the first part of the sequence f (1–20) and f (176–180). This could be due to the presence of multiple pepsin and trypsin cleavage sites in these regions, leading to extensive hydrolysis. The regions f (75–83) and f (209–221) in β-CN showed higher peptide frequencies, indicating their susceptibility to proteolysis. Notably, these two regions do not contain a significant concentration of pepsin and trypsin cleavage sites. The peptide frequencies in f (75–83) of the KF7

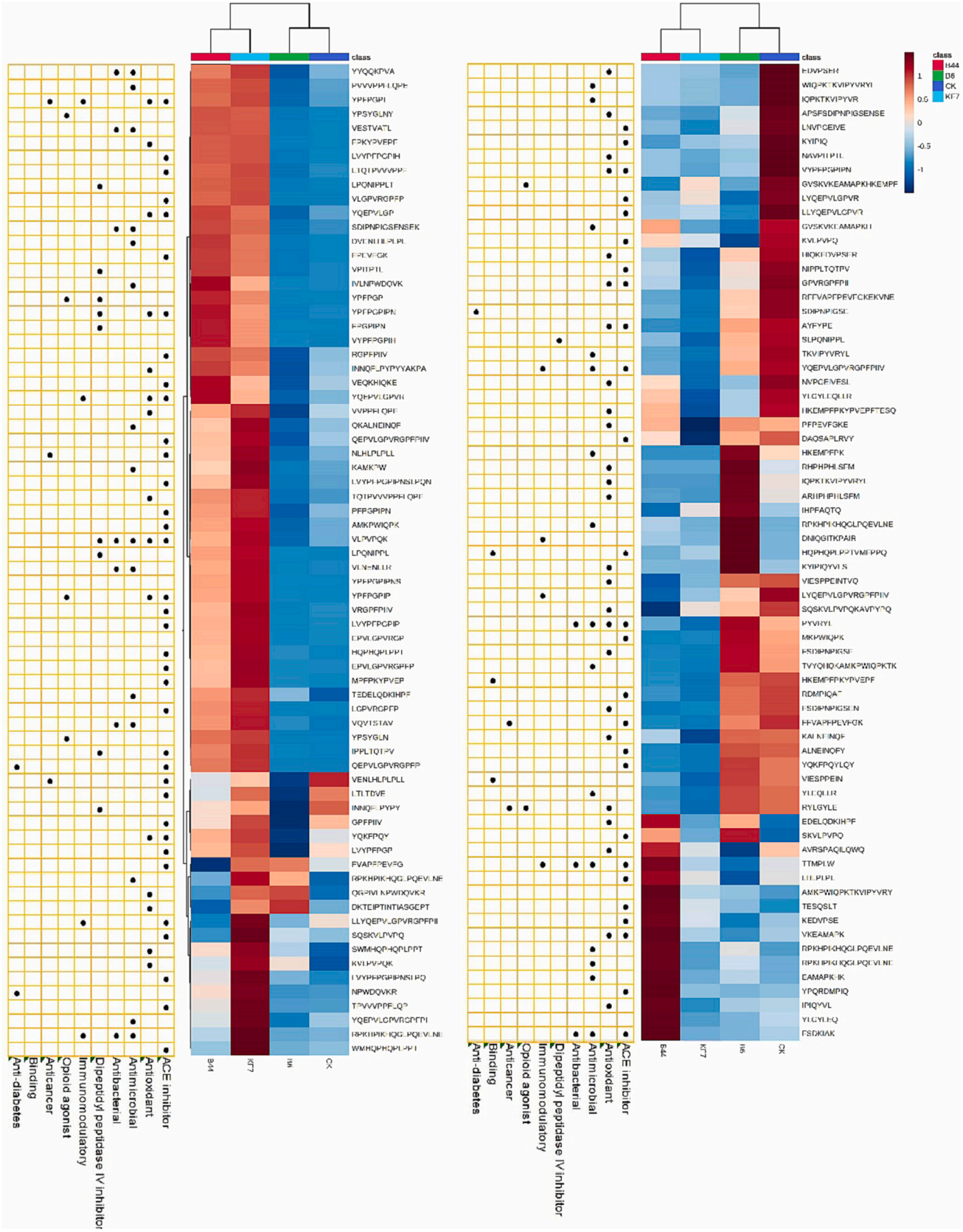

Fig. 4. Heatmap and annotate activity of bioactive peptides matched in fresh cheese. The peptides with higher levels are indicated in red, and those with lower levels are indicated in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

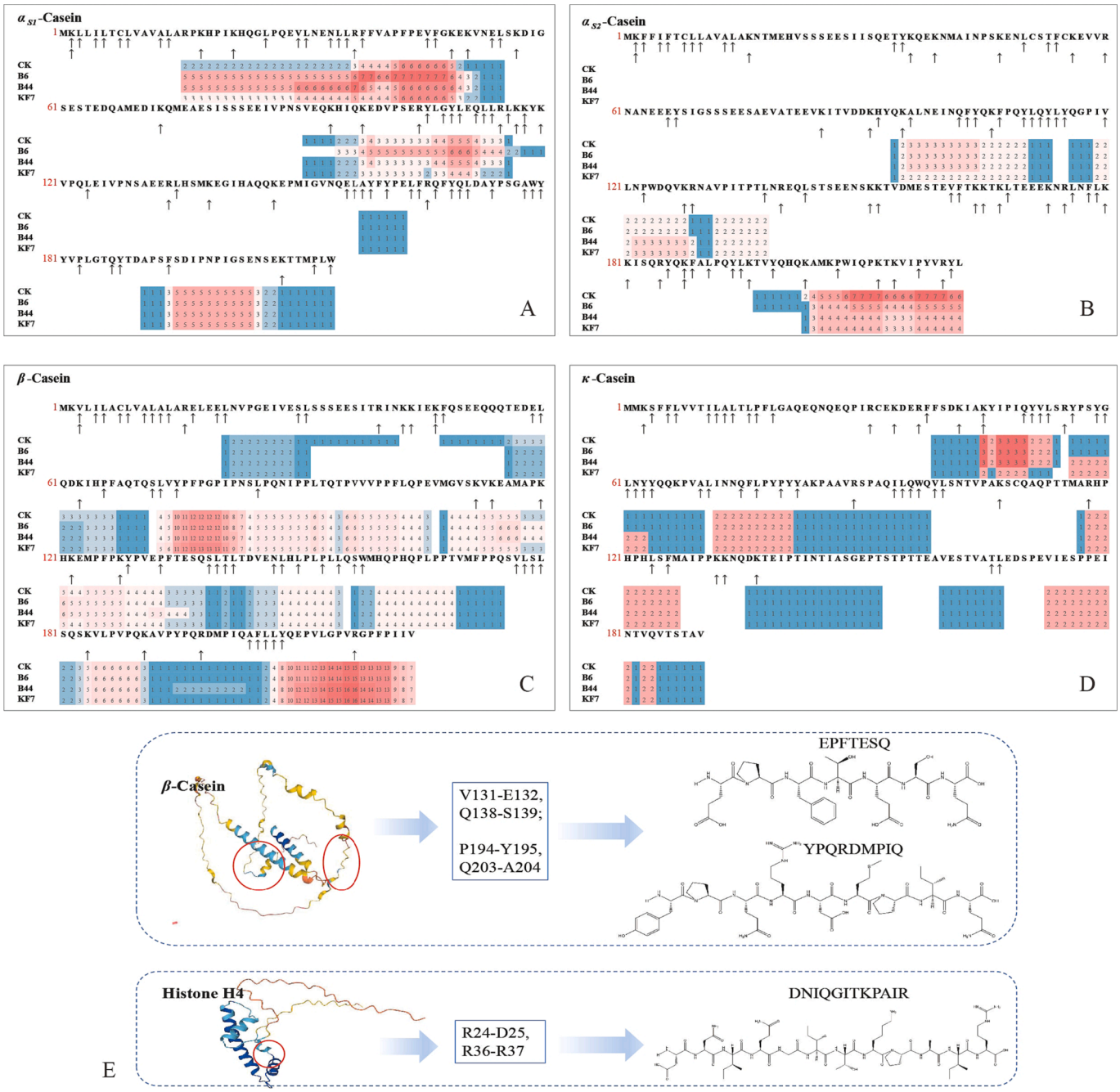

Fig. 5. Visual distribution representation of bioactive peptides derived from α_{s1}-CN (A), α_{s2}-CN (B), β-CN (C), κ-CN (D) and unique BPs in probiotics fermented cheese (E). The color (varying from blue to red) indicates the frequency of amino acids in the identified bioactive peptides. The first line and second line arrows represent the cleavage sites in CN by pepsin and trypsin, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

group were higher than those of the CK group; and the peptide frequencies in f (209–221) of both B44 and KF7 groups were higher than in the CK group (Fig. 5C). Therefore, it is hypothesized that *L. fermentum* B44 and *L. rhamnosus* KF7 aid in the hydrolysis of f (75–83) and f (209–221) of β-CN, resulting in the release of various BPs in the B44 and KF7 groups.

Although there was no significant difference in the frequency of amino acids of BPs in different groups of cheese samples in most regions of the protein sequence, the abundance of BPs in the samples changed significantly (Fig. 4). This finding suggests that the hydrolysis sites of different probiotics fermentation samples in each group are diverse even when the same protein sequence is hydrolyzed. As shown in Fig. 5E, specific short peptides with potential activity were found in fresh cheese fermented by probiotics. For example, in the fresh cheese fermented by *L. rhamnosus* B6, the R24-D25 and R36-R37 sites of Histone H4 protein were cleaved to release the immunoregulatory peptide DNIQGITKPAIR (Fig. 5E). In fresh cheese fermented by *L. fermentum* B44, the V131-E132 and Q138-S139, P194-Y195, and Q203-A204 sites of β-CN were cleaved to release the stimulating peptide EPFTESQ and the ACE inhibitory peptide YPQRDMPIQ. In summary, these results indicated that *L. fermentum* B44, *L. rhamnosus* KF7 and *L. rhamnosus* B6 exhibit diverse protein degradation capabilities and might secrete peptidase hydrolyzed CN to promote the release of more BPs.

3.3. Molecular docking simulation between inhibitor peptides and ACE

Molecular docking is an effective method for predicting the low-energy binding mode between a receptor's active site and a ligand by calculating the interaction energy and intermolecular force (Abedin, Chourasia, Chiring Phukon, Singh, & Kumar Rai, 2022; Peredo-Lovillo, Hernández-Mendoza, Vallejo-Cordoba, & Romero-Luna, 2022). A higher absolute value of the binding energy (BE) obtained from the docking results indicates a stronger binding between the ligand and the receptor.

The interaction sites and BE value between two ACE inhibitory peptides in B44 and KF7 groups and the receptor (ACE) were depicted in Fig. 6. Molecular docking analysis revealed that the BE of peptides LVYPFPGPIP and YPQRDMPIQ with ACE were −9.9 kcal/mol and −6.9 kcal/mol, respectively. These values were comparable to the BE of ACE inhibitory peptides reported in previous studies (−6.62 to −10.66 kcal/mol) (Li et al., 2022), indicating that peptides LVYPFPGPIP and YPQRDMPIQ could stably bind with ACE and effectively inhibit ACE activity. LVYPFPGPIP formed six hydrogen bonds with ACE residues, interacting with Glu_{384}, Ala_{354}, Tyr_{394}, Arg_{522}, Trp_{220}, and Ser_{517}, respectively. YPQRDMPIQ forms eight hydrogen bonds with ACE residues. The main active sites of ACE are divided into three pockets: S1, S2, and Ś1. The S1 pocket contains Ala_{354}, Glu_{384}, and Tyr_{523} residues, the S2 pocket contains Gln_{281}, His_{353}, Lys_{511}, His_{513}, and Tyr_{520} residues, and the Ś1 pocket contains Glu_{162} residues (Rohit, Sathisha, & Aparna, 2012). These findings suggested that LVYPFPGPIP binds to the S1 pocket of ACE, forming a hydrogen bond, which has played a role in exhibiting ACE inhibition. Similar studies demonstrated that the ACE inhibitory peptide HPHPHLS found in traditional Chinese cheese can form hydrogen bonds with ACE's Glu_{384} and Ala_{354} residues (Shi, Wei, & Huang, 2021). In conclusion, the characteristic peptides released in fresh cheese fermented by probiotics interacted with ACE through hydrogen bonds, specifically targeting the active sites. This provides a theoretical foundation for the development of fresh cheese as a potential source of ACE inhibitory peptides in the food industry.

4. Conclusion

The metabolic and peptide profiles of fresh cheese were significantly influenced by three probiotic strains (*L. rhamnosus* B6, *L. fermentum* B44 and *L. rhamnosus* KF7). A total of 379 metabolites were identified and categorized into 12 groups. Based on the PLS-DA, 46, 103 and 147 differential metabolites (VIP > 1.0, $p < 0.05$) were screened in the comparison of B6 vs CK, B44 vs CK, and KF7 vs CK, respectively. These differential metabolites primarily belonged to lipids and lipid-like molecules, organic oxygen compounds, and organic acids and derivatives. The abundance of disaccharides was down-regulated in samples fermented by probiotics, compared to the CK group. The fatty acid and carbohydrate-related metabolic pathways were the main pathways associated with the differential metabolites. Furthermore, a large number of peptides were detected in fresh cheese fermented by the probiotics. Among them, 147 peptides were found to have identified biological activities, such as ACE inhibition, antimicrobial and immunomodulatory effects. Furthermore, the abundance of 139 peptides was significantly different among the experimental groups, indicating that the probiotics contributed to the release of more peptides. A unique immunoregulatory peptide DNIQGITKPAIR was released in the fresh cheese fermented by *L. rhamnosus* B6, and a unique stimulating peptide EPFTESQ and a unique ACE inhibitory peptide YPQRDMPIQ were released in fresh cheese fermented by *L. fermentum* B44. The short peptide LVYPFPGPIP in B44 and KF7 groups exhibited the ability to bind to ACE active sites in molecular docking simulation. Our findings revealed significant differences in metabolic and peptide profiles of fresh cheese fermented with probiotics compared to the CK without probiotics. This study provides a theoretical basis for the further development of functional cheese. In the future, it would be valuable to explore the bioavailability of bioactive peptides in probiotic fresh cheese by static or dynamic in vitro digestion models coupled with cell models.

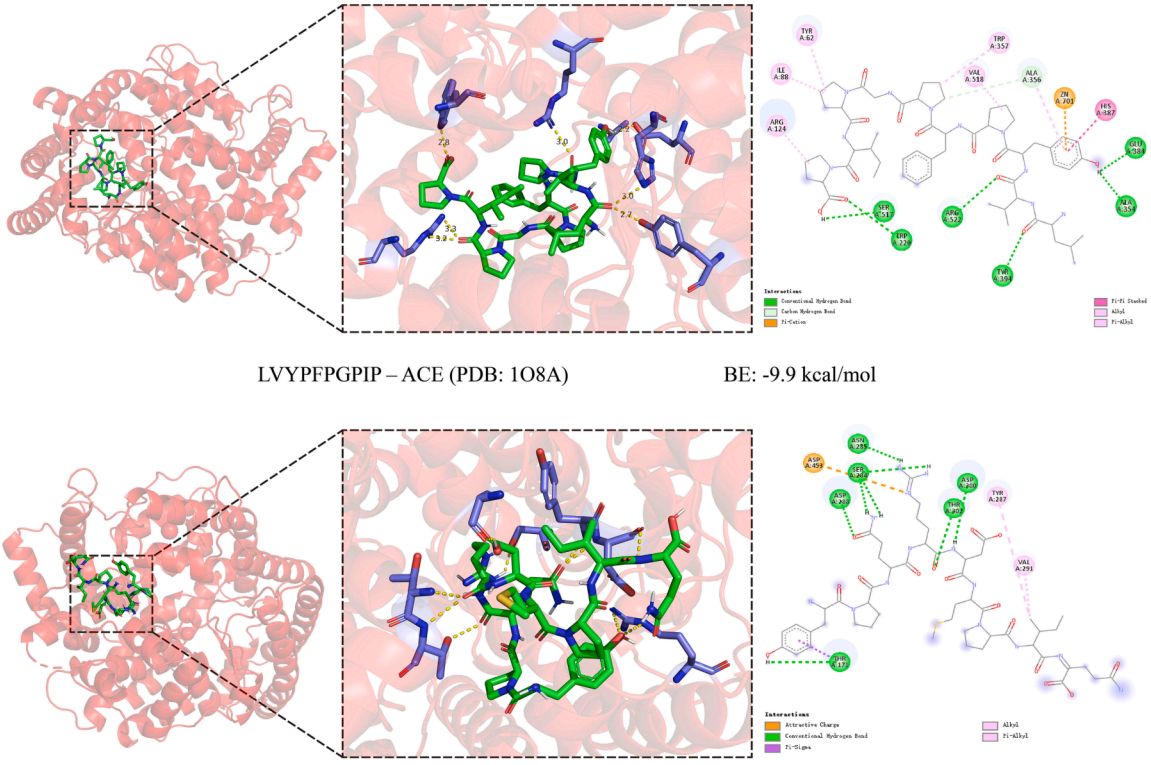

Fig. 6. Docking results of polypeptide chains LVYPFPGPIP and YPQRDMPIQ with ACE molecules (PDB: 1O8A).

CRediT authorship contribution statement

Xin Zhang: Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Yuanrong Zheng:** Conceptualization, Supervision, Writing – review & editing. **Zhenmin Liu:** Project administration, Conceptualization. **Miya Su:** Conceptualization, Supervision. **Zhengjun Wu:** Conceptualization. **Huanchang Zhang:** Conceptualization. **Chi Zhang:** Conceptualization. **Xingmin Xu:** 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.fochx.2024.101147.

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