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Integrated metabolomics and peptidomics to delineate characteristic metabolites in milk fermented with novel *Lactiplantibacillus plantarum* L3

Teng Wang [a,1], Guangqiang Wei [a,1], Faqiang Chen [a], Qingwen Ma [a,b], Aixiang Huang [a,*]

[a] College of Food Science and Technology, Yunnan Agricultural University, Kunming 650201, Yunnan, China
[b] Yunnan Normal University, Kunming 650092, Yunnan, China

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ABSTRACT

A novel wild-type *Lactiplantibacillus plantarum* (*L. plantarum*) L3 with good fermentation characteristics and protein degradation capacity was isolated from raw milk samples. In this study, the metabolites in milk fermented with *L. plantarum* L3 were investigated by metabolomic and peptidomics analyses. The metabolomics results revealed that the metabolites in milk fermented with *L. plantarum* L3 were Thr-Pro, Val-Lys, L-creatine, pyridoxine, and muramic acid, which improved the taste and nutritional qualities of the milk. Moreover, the water-soluble peptides derived from L3 fermented milk exhibited high antioxidant properties and angiotensin I-converting enzyme inhibitory (ACEI) activities. Additionally, 152 peptides were found using liquid chromatography-mass spectrometry (LC-MS/MS). Furthermore, endogenous enzymes secreted by *L. plantarum* L3 cleaved β- and α-casein to release six ACEI peptides (ACEIPs), nineteen antioxidant peptides (AOPs), and five antimicrobial peptides (AMPS). Overall, these findings could be valuable in improving the quality of fermented milk.

1. Introduction

Over the past few years, fermented milk has progressively become popular due to its appealing flavour, texture, and promising health benefits, mainly in individuals with special nutritional needs (Barros et al., 2020). Some studies have demonstrated that fermented milk provides satiety and additional health benefits, such as regulating the abundance and diversity of intestinal microbiota. Additionally, fermented milk has anti-cancer, antioxidant, and anti-diabetic properties, reduces high blood pressure, and reduces cognitive impairments (Farag et al., 2020). Therefore, fermented milk is a natural product that has been used to prevent or treat several health conditions (Hasab & Hzab, 2021). Interestingly, the health benefits of fermented milk are positively correlated with the functional metabolites produced during milk fermentation. Some reports have demonstrated that fermented milk contains small-molecular metabolites, including organic acids, peptides, amino acids, nucleotides, vitamins, fatty acids (conjugated linoleic acid and sphingolipids), and functional activators (such as folic acid, γ-aminobutyric acid, and bacteriocins), all of which improve the taste and nutritional qualities of milk (María, Jorge, María, Javier, & Inmaculada, 2020). All these metabolites are produced by the metabolism of proteins, fats, lactose, and citrate during milk fermentation (Baspinar, & Metin, 2020). On the other hand, lactic acid bacteria (LAB) can hydrolyze proteins into peptides, catalyzed by secreted peptidyl peptidase, aminopeptidase, and carboxypeptidase during milk fermentation (Law & Haandrikman, 1997). Several bioactive peptides (BPs), including angiotensin I-converting enzyme inhibitory peptides (ACEIPs), immunomodulatory peptides, antioxidant peptides (AOPs), casein (CN) -

Abbreviations: L3 Fermented milk, *Lactiplantibacillus plantarum* L3 fermented milk; LBST fermented milk, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* fermented milk; WSP, Water-soluble peptides; BPs, Bioactive peptides; AOPs, Antioxidant peptides; AMPs, Antimicrobial peptides; ACEIPs, Angiotensin I-converting enzyme inhibitory peptides; DPP - IVPs, Dipeptidyl peptidase - IV inhibitory peptides; LC–MS/MS, Liquid chromatography–Mass spectrometry; LAB, Lactic acid bacteria; UHPLC – QTOF MS, Ultra-high performance liquid chromatography – quadrupole time of flight mass spectrometry; ABTS•+, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); DPPH, 1,1-diphenyl-2-picrylhydrazylradical; FAPGG, N-[3-(2-)-Lphenyalanyl-glycyl-glycyl; IC_{50}, half maximal (50%) inhibitory concentration (IC) of a substance; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; MW, Molecular weight; PCA, Principal component analysis; OPLS - DA, Orthogonal partial least squares - discriminant analysis; VIP, Variable importance in the projection.

* Corresponding author.
E-mail address: aixianghuang@126.com (A. Huang).
[1] Contributed equally to this work.

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based phosphopeptides, and hypoglycemic peptides, are present in fermented milk (Ebringer, Ferencik, & Krajcovic, 2008). For instance, previous studies have shown that *Lactobacillus brevis* CGMCC15954, *Lactobacillus reuteri* WQ-Y1, and *Lactiplantibacillus plantarum* A3 hydrolyses CN into AOPs, including YLGYLEQLLR, VKEAMAPK, and YIPIQYVLSR (Cui et al., 2022). A related study found that milk fermented with *Streptococcus thermophilus* S10 contains dipeptides, tetrapeptides, and polypeptides (Li, Peng, Kwok, Zhang, & Sun, 2022). Therefore, BPs are important nutrients in fermented milk.

LAB are essential in fermented dairy products. Furthermore, the effectiveness of fermentation depends immensely on the fermenting agent. However, most excellent bacteria strains used in China for fermentation are imported, costly, and involve intellectual property rights. Therefore, screening and identifying local strains could solve these challenges. In recent years, several LAB with probiotic properties have been isolated. For example, the *L. plantarum* YW11 strain isolated from Tibetan Kefifir grains can biotransform linoleic acid (LA) to conjugated linoleic acid (CLA) (Aziz et al., 2020; Aziz et al., 2022) and is currently applied in the processing of fermented dairy products (Zhang et al., 2021). The *L. plantarum* 13-3 strain isolated from Kefifir grains in Tibet has demonstrated promising fermentation characteristics and can be used in the food industry (Aziz et al., 2022). The *L. plantarum* CCFM8610 was isolated from fermented vegetables and is used to process fermented milk (Huang et al., 2022). The Yunnan province of China is rich in microbial resources and is inhabited by 25 ethnic minorities. Additionally, 534 traditional foods are produced in varied ways by the different ethnic groups in Yunnan province. Interestingly, these foods are rich in LAB (Pan, Huang, Liu, & Xu, 2004). In a previous study, 10,400 LAB strain, were isolated from 1,008 traditional foods, animal feces, breast milk, and raw milk samples (Ma, Qian, Ji, Yang, & Huang, 2020). Subsequently, 300 LAB were randomly selected from the 10,400 strains to identify strains that yield high linoleic acid from the fermentation of Yunnan traditional foods (Ma, Qian, Ji, Yang, & Huang, 2020). In our previous study, we isolated *L. plantarum* L3 from raw milk samples. *L. plantarum* L3 exhibited very high acid production and protein degradation capacities (Chai, Ma, Nong, Mu, & Huang, 2023). Moreover, the *L. plantarum* L3 fermented milk had a high viscosity and unique aroma (Fig. S1), indicating that this strain has excellent fermentation capacity of dairy products. However, the useful metabolites in the milk fermented by *L. plantarum* L3 are unknown. Therefore, identifying the characteristic metabolites and BPs present in *L. plantarum* L3 fermented milk could be essential to evaluate its health benefits.

Metabolomics and peptidomics have been widely applied to study small-molecular metabolites and BPs in food. Metabolomic techniques focus on small molecules and are divided into two types. Targeted metabolomics quantifies targeted and untargeted metabolites to analyze the changes in metabolites (Cajka & Fiehn, 2016). For example, metabolomics technologies can trace the metabolic footprinting of fermented milk during the fermentation process (Li, Peng, Kwok, Zhang, & Sun, 2020), and it has also been applied to identify the metabolic profiles of different processed dairy products (Li et al., 2022). Peptidomics can reveal the role of microorganisms in the hydrolysis of food proteins and identify food-derived bioactive and biomarker peptides (Martini, Solieri, & Tagliazucchi, 2020). A previous study used peptidomics to analyze the peptide profile and potential BPs during *kefir* fermentation (Rocchetti, Michelini, Pizzamiglio, Masoero, & Lucini, 2021). Therefore, metabolomics combined with peptidomics is a reliable method to detect potentially functional metabolites in *L. plantarum* L3 fermented milk.

In this study, metabolomics and peptidomics were applied to identify the characteristic metabolites and potential BPs in milk fermented with *L. plantarum* L3. In addition, untargeted metabolomics combined with multivariate analysis was performed to identify the characteristic metabolites in milk fermented with *L. plantarum* L3. On the other hand, BPs were identified by liquid chromatography-mass spectrometry (LC–MS/MS) according to the data in the BIOPEP-UWM database. These findings provide a theoretical basis for applying *L. plantarum* L3 in milk fermentation. Particularly, *L. plantarum* L3 could be used for producing fermented milk with unique flavour, viscosity, active substances, and BPs.

2. Materials and methods

2.1. Materials

Directed Vat Set (DVS) commercial fermenters (*Lactobacillus bulgaricus and Streptococcus thermophiles* (LBST)) were purchased from the DSM Food Specialties (Helen, Australia). *Lactiplantibacillus plantarum* L3 (Gene bank accession number: PRJNA923166) was preserved at the Key Laboratory of Food Processing and Safety Control at Yunnan Agricultural University, Kunming, China. Primarily, the bacteria were characterized based on Gram stain reaction, morphological and physiological-biochemical characteristics, 16S rDNA sequencing, and whole genome sequencing. The bacteria were first activated in MRS broth before use. Additionally, the *L. plantarum* L3 density was standardized to 6×10^6 CFU/mL before use. The bacterial strains were preserved at −80 ℃ in glycerol supplemented with 20% (v/v) nutrient broth. Whole milk powder and sucrose were purchased from Devondale (Saputo dairy Australia pty Ltd., Aaronsford, Australia) and Angie Yeast Company Limited (Yichang, China), respectively. De Man, Rogosa, and Sharpe (MRS) broth medium was purchased from Sigma-Aldrich (3050 Spruce Street, St Louise, USA).

2.2. Sample preparation

In this study, milk powder (12%) and sucrose (7%) were dissolved in distilled water at 60 ℃ and stirred for 30 min. Next, the samples were heated at 95 ℃ for 10 min. Subsequently, the samples were cooled to the incubation temperature (37 ℃), and the *L. plantarum* L3 and DVS were added to the milk at 2% (V/V) and 0.014%, respectively. The milk was then fermented at 37 ℃ until the pH reached 4.3. The fermented milk (20 mL) was collected and stored at −80 ℃ for further analysis. The same method was used to ferment milk with LBST.

2.3. Acidity and viscosity of the fermented milk

Certain milk parameters, including acid level, pH and titratable acidity, in *L. plantarum* L3 fermented milk were evaluated. The pH was measured using an OHAUS ST3100 pH meter (Beijing Saibaio Technology Co., Beijing, China). The acidity was determined according to GB5009.239–2016 (National Standard of the People's Republic of China, 2016) guidelines and was expressed as Gilner degree °T (AOAC, 1997). Finally, a viscosimeter matching rotor 4 (171015, Jinghai Instruments Co., LTD, Shanghai, China) was used to measure the viscosity of the fermented milk samples. Before these analyses, the fermented milk was first stirred at 100 rpm for 30 s using a viscometer.

2.4. Metabolome profiling

2.4.1. Sample pretreatment

For metabolome analysis, 100 μL of fermented milk was transferred to an Eppendorf tube. After adding 400 μL of acetonitrile: methanol (1:1) containing isotopically-labelled internal standard mixture, the samples were vortexed for 30 s, sonicated for 10 min in an ice-water bath, and incubated for 1 h at −40 ℃ to precipitate proteins. Then, the sample was centrifuged at 13,000 rcf for 15 min at 4 ℃. The resulting supernatant was transferred to a fresh glass and then filtered through a 0.22 μm microporous membrane for further ultra-high performance liquid chromatography – quadrupole time of flight mass spectrometric (UHPLC-QTOF MS) analysis.

2.4.2. Analysis by ultra-high performance liquid chromatography-quadrupole time of flight mass spectrometric (UHPLC-QTOF MS)

A UHPLC-QTOF analysis was performed using a UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC BEH amide column (2.1 mm × 100 mm, 1.7 μm) coupled to a Q Exactive HFX (QE HFX) mass spectrometer (Orbitrap MS, Thermo). The mobile phase comprised 25 mmol/L of ammonium acetate and 25 mmol/L of ammonia hydroxide in water (pH = 9.75) (A) and acetonitrile (B). The auto-sampler temperature was set at 4 ℃, and the injection volume was 3 μL. In this experiment, the QE HFX mass spectrometer was applied due to its ability to acquire MS/MS (tandem mass spectrometry) spectra in information-dependent acquisition (IDA) mode under the data mining software (Xcalibur, Thermo). The acquisition software evaluates the full MS spectrum in this mode. The electron spray ionization (ESI) source conditions were set as follows: sheath gas flow rate at 30 Arb, Aux gas flow rate at 25 Arb, capillary temperature at 350 ℃, full MS resolution at 60,000, MS/MS resolution at 7,500, collision energy at 10/30/60 in NCE mode, and spray Voltage at 3.6 kV (positive) or −3.2 kV (negative).

2.5. Peptidomics profiling by liquid chromatography-mass spectrometry (LC-MS/MS)

The water-soluble peptides (WSPs) derived from fermented milk were prepared using a previous methodology (Cui et al., 2022) but with slight modifications. Firstly, the fermented milk was centrifuged (6000 rcf, 10 min), and the hydrolyzed protein supernatant was collected and passed through a 3 kDa ultrafiltration membrane to obtain peptides with molecular weights (MWs) <3 kDa. Then, the freeze-dried WSE with MW <3 kDa was stored at −80 ℃ to determine antioxidant activity, ACE inhibition activity, and for LC-MS/MS identification.

The WSP in L3 and LBST fermented milk was determined according to a previous method (Wei et al. 2022). Briefly, a lyophilized powder solution of the milk groups (MWs <3 kDa) was analyzed by Q Exactive™ Plus-Orbitrap mass spectrometer (Thermo Fisher Scientific, Germany) equipped with an EASY-nLC 1000 UPLC system. The mobile phases A and B comprised 0.1% formic acid in water and 0.1% formic acid in acetonitrile solution, respectively. Then, the samples (5 mg/mL) were separated in an analytical column (RP C18, 50 μm × 15 cm, Thermo Fisher) at a rate of 300 nL/min. The column was equilibrated with 95% A (0.1% formic acid), and the sample was loaded onto the Trap column by an autosampler. The gradient program used was as follows: (I) 5% B (0.1% formic acid-acetonitrile), 0–1 min; (II) 5%–30% B, 2–30 min; (III) 30% B, 31–40 min; (IV) 30% − 80% B, 41–45 min; (V) 30% − 80% B, 41–45 min; (VI) 80% B, 46–55 min; and (VII) 80%–5% B, 56–60 min. After, the MS/MS raw files were collected using the Proteome Discoverer 2.2 software (Thermo Fisher Scientific) and searched from the UniProt Bos taurus database by Mascot (version 2.3, Matrix Science, London, UK). The scan range was set at 400–4000 Da. Then, the screening criteria were as follows: full scan mass spectra were collected at 60 K resolution, and the first 20 ions were separated by collision-induced dissociation for MS/MS. The search parameters, including fragment mass tolerance, were 10 ppm and 0.02 Da. A false discovery rate (FDR) of 1% was adopted to validate the assay. Peptides were considered present when identified in all three fermented milk samples.

2.6. Determination of the biological activity of water-soluble peptide (WSP)

2.6.1. In vitro antioxidant activity assays

The *in vitro* antioxidant activity of WSP obtained from L3 and LBST fermented milk was determined by measuring the 1,1.-Diphenyl-2-pyridohydrazino (DPPH$^{\bullet}$) radical scavenging activity, 2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate (ABTS$^{\bullet+}$) radical scavenging activity, and the reducing capacity.

2.6.1.1. 1,1.-Diphenyl-2-pyridohydrazino (DPPH•) scavenging activity. The DPPH• scavenging activity was measured as described by Elfahri, Vasiljevic, Yeager, and Donkor (2016). Briefly, an aliquot of 200 μL of WSP solution was added to 800 μL of the DPPH solution (0.1 mM DPPH in 95% methanol). After incubation for 30 min, the absorbance of the incubated samples was determined at 517 nm. Methanol was used to blank the spectrophotometer. The DPPH• scavenging activity was calculated as follows:

$$DPPH^{\cdot}\,radical inhibition\,\% = (1 - Abs_{sample}/Abs_{blank}) \times 100\% \quad (1)$$

where Abs_{sample} is the absorbance of the sample group; Abs_{blank}, absorbance of the blank group.

2.6.1.2. 2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate (ABTS•+) scavenging activity. The ABTS•+ scavenging activity was measured as previously described by Sah, Vasiljevic, McKechnie, and Donkor (2014). Briefly, 20 mL WSP solutions were added to 2 mL ABTS•+ working solution and incubated at 30 °C for 6 min, and the absorbance of the mixture was measured at 734 nm. Similarly, 20 μL of dd-water was used as a blank sample. The percentage of ABTS•+ scavenging activity was expressed as the scavenging rate (%):

$$ABTS^{\cdot+}radical\ \ inhibition\,\% = (Abs_{blank} - Abs_{sample})/Abs_{blank} \times 100\% \quad (2)$$

where Abs_{sample} is the absorbance of the sample group; Abs_{blank} is the absorbance of the blank group.

2.6.1.3. Reducing power (RP) assay. The reducing power (RP) assay was performed according to the previous method of Kou et al. (2013). Briefly, 1 mL WSP solution was mixed with 2.5 mL phosphate buffer (pH 6.6, 200 mmol/L) and 2.5 mL potassium ferricyanide (1 g/100 mL). After incubation at 20 min for 50 °C, 2.5 mL of 10% trichloroacetic acid was added to the mixture, followed by centrifugation at 3,200 rcf for 10 min. The upper layer (5 mL) was mixed with 5 mL of distilled water and 1 mL of 0.1 g/100 mL ferric chloride, and the absorbance was measured at 700 nm.

2.6.2. Angiotensin I-converting enzyme inhibitory (ACEI) activity assay

Angiotensin I-converting enzyme inhibitory (ACEI) assays were performed according to a previous method but with slight modifications (Admassu, Gasmalla, Yang, & Zhao, 2017). Briefly, sample concentrations (0.5, 1.0, 1.5, 2.0, and 2.5 mg/mL) and 0.001 M N-[3-(2-furyl acryloyl)-Lphenyalanyl-glycyl-glycyl (FAPGG) solution was prepared with 0.08 M 4-(2-hydroxyethyl) piperazine-1-ethane sulfonic acid (HEPES) buffer (ultrapure water, 0.3 M NaCl, pH = 8.3). In each assay, 100 μL of the sample was added to a 96-well microplate and mixed with 50 μL of 0.001 M FAPGG and 50 μL of ACE (100 U/L). Subsequently, the plate was incubated at 37 °C for 5 min, and the absorbance of the samples was measured at 340 nm using an enzyme-labelled instrument (BioTek Instruments, Inc., Vermont, United States). In this assay, 100 μL of HEPES buffer was the blank solution. The initial absorbance (a_1 and b_1) of the control and sample wells was measured, and the absorbance was measured every 30 min at 37 °C (a_2 and b_2). Each analysis was performed in triplicate. The ACEI activity was calculated using Eq.no (3):

$$ACE - inhibitory\ activity\ /\% = [(A - B)/A] \times 100\%$$

where A is the absorbance reduction of the control wells, calculated as A = a_1-a_2, and B is the absorbance reduction of the sample wells, calculated as B = b_1-b_2.

2.7. Statistical analyses

All data were expressed as the mean of at least three independent experiments ± standard deviations (SD). Differences between multiple

groups were analyzed by ANOVA and Duncan's test. $P < 0.05$ was considered statically significant. Origin 2021b was used to visualize the statistical results (OriginLab Inc., CA, USA). Multivariate regression analyses, including principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), were performed using SIMCA software version 15.0.2. Unique metabolites in each set of groups were identified based on the variable importance in the projection (VIP) value in the OPLS - DA > 1 at $P < 0.05$ in single-dimensional statistical analysis. Pathways regulated by the unique metabolites in the L3 group were identified from the Kyoto Encyclopedia of Genes (KEGG) (https://www.genome.jp/kegg/) database. Descriptive and inferential statistics were performed using SPSS software (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Comparison of metabolites between L3 and LBST fermented milk

3.1.1. Chromatographic profiles of L3 and LBST fermented milk

In this study, the metabolites in the fermented milk were identified by the UPLC-Q-TOF-MS technique. The base peak intensity (BPI) chromatograms of L3 and LBST fermented milk in positive (A) and negative (B) ion modes were depicted in Fig. S2. Significant differences in the metabolite profiles were detected between the L3 and LBST fermented milk. Particularly, 848 metabolites were detected in positive and negative ion modes in the L3 and LBST fermented milk. Additionally, 574 metabolites were detected under the positive ion mode in the L3 and LBST fermented milk. Contrarily, 274 metabolites were detected under the negative ion mode in the L3 and LBST fermented milk.

3.1.2. Multivariate statistical analysis of metabolites in the L3 and LBST fermented milk

The PCA is the most used multivariate statistical method for the metabolomic analysis of samples. This study revealed a significant difference in the PCA scores between L3 and LBST fermented milk (Fig. S3A-B), indicating that the distribution of the metabolites between the two fermentation groups was significantly different.

OPLS-DA is a supervised statistical method that allows the exclusion of intragroup differences during the analysis and focuses on inter-group differences. In this study, the OPLS - DA analysis was performed to identify the differences in metabolites between L3 and LBST fermented milk. The results (Fig. S3C, E) demonstrated that the two fermentation milk differed in the type of metabolites present. The L3 fermented milk was located on the left side of the confidence interval, and LBST fermented milk was found on the right side of the confidence interval, and all samples were within the 95% confidence interval. In addition, 200 permutation tests on the mode were performed to prevent overfitting. As a result, the positive ion mode corresponded to $Q^2 = 0.934$, $R^2Y = 1$, and the negative ion mode corresponded to $Q^2 = 0.881$, $R^2Y = 0.998$ (Fig. S3D, F). Values for the mode's prediction rate (Q^2) and explanatory power (R^2Y) closer to 1 indicate higher credibility. The present results revealed that Q^2 and R^2Y were higher than 0.85, indicating that the mode exhibited good explanatory and predictive power. Thus, the potential marker compounds in L3 and LBST fermented milk were screened out.

3.1.3. Difference in metabolites between L3 and LBST fermented milk

This study also identified metabolites in LBST-fermented but not in L3 fermented milk. To achieve this, OPLS-DA mode was performed to determine the VIP, and VIP > 1.00 at $P < 0.05$ were screened for differential metabolites. The results revealed that L3 and LBST fermented milk differed in 101 metabolites, including amino acids, peptides, nucleotide metabolites, organic acids, and aromatic substances (Table S1).

To further study the different metabolites present in the L3 and LBST fermented milk, a heat map composed of 101 differential metabolites was constructed (Fig. S4). The hierarchical clustering analysis revealed that the unique metabolites in the two fermentation groups had significantly different properties. Among the unique metabolites between the two milk groups, 63 metabolites were more abundant in L3 fermented compared with LBST fermented milk. On the other hand, 38 metabolites were less abundant in L3 fermented milk.

Some reports have demonstrated that the type and quantity of free amino acids are closely related to the taste and nutritional quality of fermented milk. Some proteolytic enzymes secreted by *L. plantarum* degrade CN in bovine milk to produce free amino acids and short peptides. In the current study, the levels of *N*-acetyl-D-serine, L-histidine, L-arginine, 5-aminopentanoic acid, L-methionine, and hyperarginine were higher in L3 fermented milk than in LBST fermented milk. These metabolites could contribute to the taste and act as precursors for volatile compounds that can be converted into volatile flavour substances, such as alcohols, aldehydes, lipids, and sulfides, which enhances the flavour (Viana, Pérez-Martínez, Deutscher, & Monedero, 2005). Although the function of these free amino acids was not explored in this study, several previous studies have demonstrated the biological activity and beneficial function of free amino acids. Therefore, the higher levels of these amino acids enhance the nutritional quality of fermented milk. For instance, L-histidine is a functional amino acid with numerous physiological functions, such as anti-inflammatory, antioxidant, and immunomodulatory properties (Hasegawa et al., 2012). Additionally, L-arginine, an essential amino acid, exhibits diverse functions, including promoting cell division and wound recovery, improving body immunity, preventing viral infections, and replenishing energy (Klenner et al., 2015). L-arginine imparts the umami test to fermented milk. A previous study also identified L-arginine in *S. thermophilus* S10-fermented milk (Li et al., 2022). However, the present results demonstrated that the levels of 9 amino acids, including L-phenylalanine and *N*-acetylornithine, were lower in L3 fermented milk than in LBST fermented milk.

Most of the proteins are digested and absorbed as oligopeptides by the action of enzymes in the digestive tract. Oligopeptides are smaller MWs proteins that are absorbed and metabolized faster than free amino acids, providing the nutrients needed for human growth. In the present study, 18 dipeptides were identified in the two samples (Table S1), and these dipeptides were related to the physiological functions of fermented milk. The peptides differed in presence and abundant in the two fermentation groups included Val-Lys, His-Pro, Ala-Pro, Pro-Arg, Leu-Thr, Thr-Leu, and IsoSer. Val-Lys decreases the sugar levels in the blood and has an *in vivo* antagonistic activity against type 2 diabetes (Vu et al., 2015). It also stimulates glucose uptake by tissues and organs (Morifuji, Koga, Kawanaka, & Higuchi, 2009). In addition, *in vivo* studies have shown that L-creatine, composed of two amino acids (L-histidine and *β*-alanine), regulates antioxidation. The hypotensive activity of the dipeptide is better when Tyr, Phe, and Pro are at the carboxy end (Cushman, Cheung, Sabo, & Ondetti, 1982). Therefore, dipeptides detected in L3 and LBST fermented milk, including Thr-Pro, His-Pro, and Tyr-Phe, could have hypotensive effects. However, the functional properties of the peptides found in L3 fermented milk remain unclear, and more studies are needed to uncover this. In addition to physiological functions, certain dipeptides alter the flavour of fermented products. For example, peptides containing Gly-, Ala-, and Asp-residues impart sour flavours (Li, Peng, Kwok, Zhang, & Sun, 2022). Additionally, peptides, such as Ala-Pro, Ala-Val, Asp-Hyd, Asp-His, and Pro-Asp, give fermented milk a unique sour taste. Therefore, the results suggest that the presence of dipeptides in L3 fermented milk offers beneficial functions.

The presence and abundance of monosaccharides and polysaccharides in the two fermentation groups were also evaluated. A total of 13 monosaccharides and polysaccharides, including maltotetraose, L-galactose, maltotriose, fructan, D-galactose, maltopentaose, and D-xylitol, were significantly higher in the L3 than in the LBST fermented milk. Maltose, galactose, xylose, and *N*-acetylglucosamine are components of extracellular polysaccharides (Wu, et al., 2022). Therefore, the results suggest that *L. plantarum* L3 has better extracellular

polysaccharide production capacity than the commercial ferment LBST. Some reports have shown that the extracellular polysaccharides of *Lactobacillus* could enhance the texture of fermented milk (Korcz, & Varga, 2021). In this study, the viscosity of L3 and LBST fermented milk were determined. The results showed that the viscosity of L3 fermented milk was 5504.67 ± 252.85 MPa▪s, which was significantly higher than that of the LBST fermented milk (4653.00 ± 1287.81 MPa▪s ($P < 0.05$). The difference in viscosity could be related to the higher quantity of extracellular polysaccharides in L3 fermented milk. The extracellular polysaccharides secreted by *L. plantarum* L3 could be tightly linked to the proteins, effectively filling the three-dimensional network structure of casein colloids and thereby enhancing the viscosity of fermented milk (Ge et al., 2022). However, further studies are needed to determine the precise content of extracellular polysaccharides in L3 fermented milk.

In addition, nucleotides are the basic structural unit of nucleic acids in all organisms. The results demonstrated that the relative content of seven nucleotides, including pyridine, adenosine, 2-deoxy-D-ribose, deoxycytidine, deoxyadenosine, 5-methylcytosine, and cytosine, was higher in L3 fermented milk than in LBST fermented milk. Finally, pyridoxal, one of the components of vitamin B6, was also detected in L3 fermented milk. Previous studies have revealed that LAB can synthesize vitamin B (B2, B4, and B6). Moreover, fermented dairy products are an important source of vitamin B (Burgess, Smid, & Sinderen, 2009). Similarly, two studies identified vitamin B2 in milk fermented with *S. thermophilus* S10 and *Lacticaseibacillus casei* CGMCC1.5956 and B5 in milk fermented with *Levilactobacillus brevis* CGMCC1.5954 (Li, Peng, Kwok, Zhang, & Sun, 2022; Fan et al., 2022). In our analysis, sorbitol was identified in the L3 fermented milk. Studies have shown that sorbitol has an antioxidant function, which could positively affect the antioxidant capacity of L3 fermented milk (Fan et al., 2022). However, further *in vitro* and *in vivo* experiments are needed to validate the potential physiological functions of the micro and macro peptides in L3 fermented milk.

3.1.4. Pathways regulated by active metabolites in fermented milk

Metabolite set enrichment analysis identifies biologically relevant and significantly enriched patterns by utilizing metabolomics data. The metabolic pathway enrichment analysis results showed that fermented milk was significantly enriched in eight metabolic pathways, including phenylalanine, tyrosine and tryptophan biosynthesis, taurine metabolism, vitamin B6 metabolism, phenylalanine metabolism, thiamin metabolism, and aminyl-tRNA biosynthesis pathways (Fig. 1). Interestingly, proteolysis-related pathways including oligopeptide metabolites were also enriched. However, they could not be incorporated into the metabolic pathways since the metabolic pathways of peptides have not been reported. Studies have shown that milk fermented with *S. thermophilus* S10 is mainly enriched with metabolites that regulate fatty acid metabolism and biosynthesis, riboflavin metabolism, and

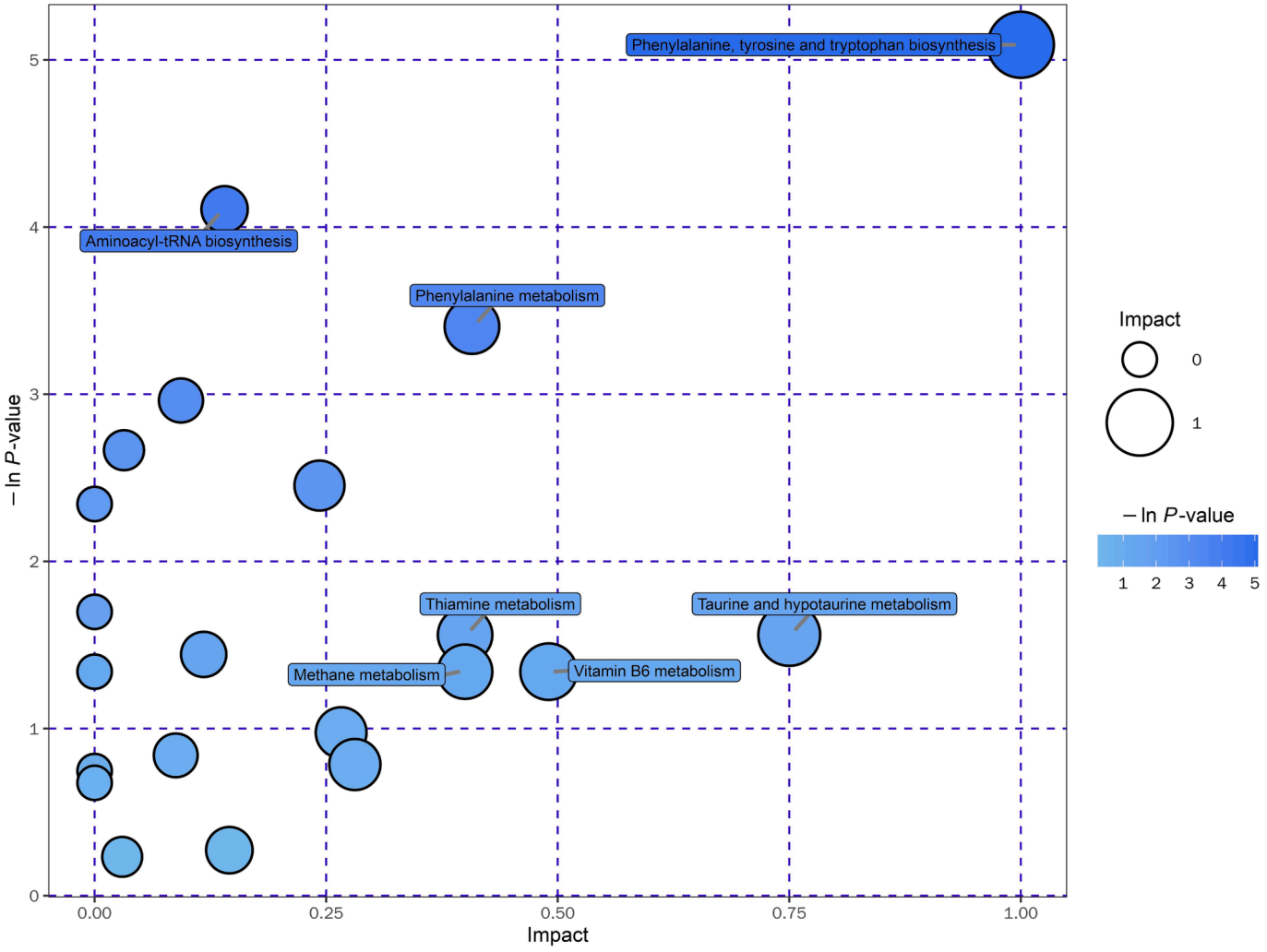

Fig. 1. Metabolic pathway regulated by metabolites significantly different between L3 and LBST fermented milk. Note: Each bubble in the graph represents a KEGG pathway; the horizontal axis indicates the relative importance of metabolites in the pathway; the vertical axis represents the enrichment significance of metabolites involved in the pathway-ln (*P*-value); the bubble size represents the impact value of a given metabolite.

amino acid metabolism (Li, Peng, Kwok, Zhang, & Sun, 2022). Furthermore, amino acids, such as arginine, leucine, lysine, tryptophan, and tyrosine, promote LAB growth (Rao, Pulusani, & Rao, 2010). In the current study, the differential metabolites found in L3 fermented milk mainly participated in amino acid metabolism, phenylalanine biosynthesis, and tyrosine, tryptophan, and phenylalanine metabolism, all of which are required for the *L. plantarum* L3 growth.

3.1.5. Characteristic components in L3 fermented milk

In the present study, the characteristic small-molecular metabolites in L3 fermented milk were evaluated using untargeted metabolomics. Therefore, a fingerprint of functional components of L3 fermented milk was designed to demonstrate the characteristic small-molecule metabolites present in L3 fermented milk (Fig. 2). The results demonstrated that L3 fermented milk contained several functional metabolites, including pyridoxine, functional amino acids, extracellular polysaccharides, muramic acid, and functional dipeptides (i.e., Val-Lys, Thr-Pro and His-Pro), and flavour peptides (i.e., Ala-Pro, Ala-Val, Pro-Asp). Overall, the characteristic functional metabolites might contribute to the good flavour and nutritional quality of L3 fermented milk.

3.2. Peptide profiles and bioactive peptides of L3 fermented milk

During milk fermentation, endopeptidases and exopeptidases secreted by *L. plantarum* L3 hydrolyze milk proteins to release peptides and amino acids. Interestingly, functional amino acids, including L-histidine, L-arginine, 5-aminopentanoic acid, L-methionine, and functional dipeptides, including Val-Lys, Thr-Pro, His-Pro, and Tyr-Phe, were found in L3 fermented milk. A previous study found that the 20 peptides in *S. thermophilus* S10 fermented milk were mainly peptides with more than two amino acids (i.e., SSVLGVAYL, LLGLSLE, and LLLDFPLILSLP) (Li, Peng, Kwok, Zhang, & Sun, 2022). However, metabolomics could not identify peptides (except dipeptides) in L3 fermented milk. Therefore, the peptide profiles and BPs of L3 fermented milk were analyzed by peptidomics.

3.2.1. WSP in L3 fermented milk

The L3 and LBST fermented milk were analyzed using LC-MS/MS, and the basic peak chromatogram of fermented milk peptides results are shown in Fig. S5. The peptidomics analysis identified 152 and 121 peptides derived from CN in L3 and LBST fermented milk, respectively. Interestingly, the number of WSP was substantially higher in L3 fermented milk than in LBST fermented milk, which might be related to the more abundant hydrolases in *L. plantarum* L3. Additionally, the characteristic peptides in L3 and LBST fermented milk were displayed using a Venn diagram (Fig. 3A), which showed that L3 and LBST fermented milk contained 84 common peptides. Furthermore, 68 and 37 unique peptides were identified in the L3 and LBST fermented milk, respectively. The peptides in L3 fermented milk and LBST fermented milk were derived from different CNs (Fig. 3B-C). For instance, WSP in L3 and LBST fermented milk were mainly derived from β-CN, α_{s1}-CN, and κ-CN. In addition, 49, 67, and 36 WSP in L3-fermented milk were derived from β-CN, α-CN, and κ-CN, respectively, while 34, 50, and 38 WSP were derived from β-CN, α-CN, and κ-CN, respectively, in LBST fermented

Fig. 2. The fingerprint of characteristic metabolites of *L. plantarum* L3 fermented milk.

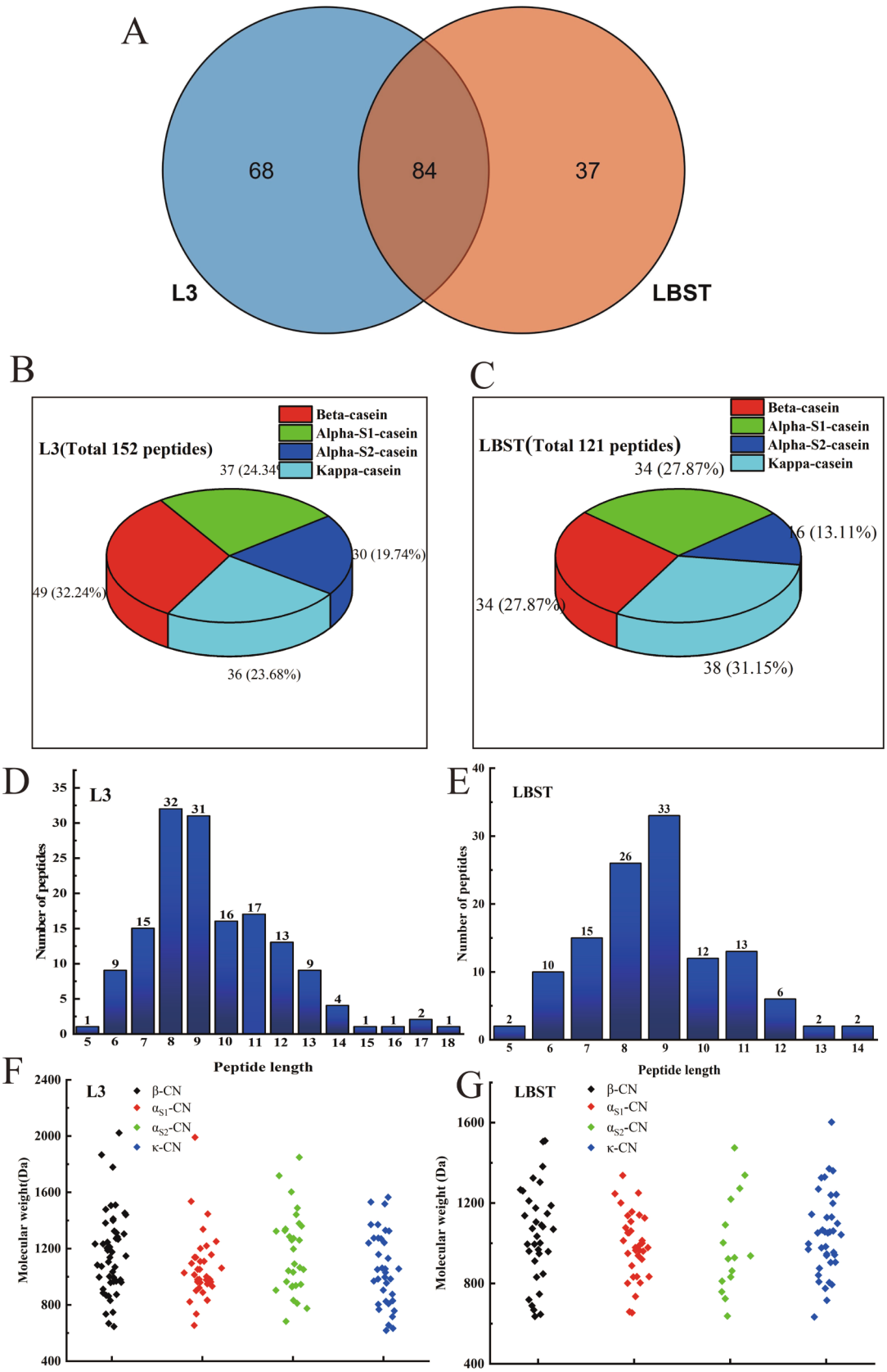

Fig. 3. The profile of WSP in L3 and LBST fermented milk. (A) Venn diagrams of WSP in L3 and LBST fermented milk. The peptides derived from the parent proteins α_{s1}, α_{s2}, β and κ-CN among peptides in milk fermented by L3 (B) and LBST (C); Amino acid composition of peptides identified in L3 (D) and LBST (E) group; MW distribution of the peptides identified in L3 (F) and LBST (G) group. L3: *L. plantarum* L3 fermented milk; LBST: *Lactobacillus bulgaricus* and *Streptococcus thermophilus* fermented milk.

milk. The difference in the number of peptides was substantially higher for β-CN and α-CN, indicating that *L. plantarum* L3 hydrolyses β-CN and α-CN better, leading to the release of more peptides. Furthermore, studies have shown that *Lactobacillus* secrete proteases and peptidases that hydrolyze κ-CN to release peptides (Pisanu et al., 2015). This result could explain the presence of 36 and 38 peptides from κ-CN in L3 and LBST fermented milk, respectively. In summary, *L. plantarum* L3 and LBST promote the degradation of milk proteins to release peptides. However, compared with LBST, proteases secreted by *L. plantarum* L3 catalyzes the release of more peptides from β-CN and α-CN.

The amino acid composition and the distribution of small molecular WSP in the L3 and LBST fermentation groups are shown in Fig. 3D-E. The results demonstrated that the WSP were mainly small peptides of 8 and 9 amino acids and were between 400 and 2000 Da (Fig. 3F-G), consistent with a previous finding (Jin et al., 2016) where 250 peptides, with molecular ranging between 400 and 2000 Da, were identified in yoghurt. The amino acid composition and MW distribution reflect the degree of protein hydrolysis and peptide bond breakage. The MW is closely related to the bioactivity and bioavailability of digested products (Luan, Feng, & Sun, 2021). In addition, the results demonstrated that both L3 and LBST groups contained many oligopeptides ($\leq$10 amino acids), which facilitate digestion and absorption in individuals.

3.2.2. Bioactive peptides in L3 fermented milk

Moreover, analysis of the BP database revealed 6 ACEIPs, 2 immunopeptides, 19 AOPs, 5 AMPs, and 1 dipeptidyl peptidase-IV inhibitory peptide in L3 fermented milk (Table 1). Furthermore, the ACEI peptides and AOPs were mainly derived from β-CN, α-CN, and κ-CN. This result suggests that CN is rich in proline, and LAB has a specific proline peptidase that cleaves CN, promoting the release of ACEIPs and AOPs. It is possible to observe that *L. plantarum* L3 and LBST promoted the degradation of milk proteins to produce BPs (Table 1). Interestingly, the content of BPs was substantially higher in the L3 than in the LBST fermented milk, indicating that L3 secretes specific proteases and peptidases. Overall, the results indicate L3 fermentation of milk releases BPs, such as ACEIPs, AOPs, and AMPs, with antioxidant, ACEI, and antimicrobial activities.

3.2.3. In vitro biological activity of WSP in L3 and LBST fermented milk

The antioxidant and ACEI activities of the WSP derived from L3 and LBST fermented milk were analyzed *in vitro*, and the results are shown in Fig. 4. The WSP with MW <3 kDa and >3 kDa in L3 and LBST fermented milk all exhibited *in vitro* antioxidant and ACEI activities. As shown in Fig. 4A–D, the half maximal (50%) inhibitory concentration (IC_{50}) values of $ABTS^{\bullet+}$ and DPPH• scavenging activities for L3-WSP were 9.09

Table 1
Potential bioactive peptides derived from L3 and LBST fermented milks.

No.	Protein	Peptide sequences	Molecular weight /Da	Bioactive peptides amino acid sequence	Bioactive	WSP L3	LBST
1	β-CN	KIHPFAQTQ	1069.579	KIHPFAQTQSLVYP	ACEI activity	√	√
2	β-CN	TPVVVPPFL	968.5816	TPVVVPPFLQP		√	√
3	α_{s2}-CN	ALPQYLK	832.4927	FALPQYLK		√	√
4	κ-CN	FSDKIAK	808.4563	FSDKIAK		√	√
5	κ-CN	YAKPAAVR	875.5097	YYAKPAAVR		√	√
6	β-CN	IPPLTQ	668.3978	IPPLTQTPV			√
7	β-CN	IPPLTQTPV	965.5666	IPPLTQTPV		√	
8	α_{s1}-CN	RPKHPIKH	1012.616	RPKHPIKHQ			√
9	α_{s1}-CN	KEDVPSE	803.3781	KEDVPSE			√
10	α_{s2}-CN	AMKPWIQPK	1098.613	AMKPWIQPK			√
11	β-CN	QEPVLGPVRGPFPI	1505.847	LLYQEPVLGPVRGPFPIIV	Immunoreactive	√	√
12	α_{s1}-CN	RPKHPIKHQGLPQEVLN	1991.13	RPKHPIKHQGLPQEVLNENLLRF		√	
13	β-CN	DELQDKIH	997.4949	EDELQDKIHPF	Antioxidant activity	√	
14	β-CN	EAMAPK	646.3228	VKEAMAPK		√	√
15	β-CN	EAMAPKHK	911.4767	VKEAMAPK		√	
16	β-CN	QDKIHPF	884.4625	EDELQDKIHPF		√	
17	β-CN	QSKVLPVPQKAVPYPQ	1779.016	SQSKVLPVPQKAVPYPQ		√	
18	α_{s1}-CN	IQKEDVPSER	1200.622	HIQKEDVPSER		√	√
19	α_{s1}-CN	HIQKEDVPS	1052.537	HIQKEDVPSER		√	√
20	α_{s1}-CN	HIQKEDVPSER	1337.681	HIQKEDVPSER		√	√
21	α_{s1}-CN	KEDVPSER	959.4792	HIQKEDVPSER		√	
22	α_{s2}-CN	ALPQYLK	832.4927	FALPQYLK		√	√
23	α_{s2}-CN	KISQRYQKF	1197.674	FLKKISQRYQKF		√	
24	α_{s2}-CN	IQPKTKVIPYVR	1441.889	IQPKTKVIPYVRYL		√	
25	κ-CN	RHPHPHLSFM	1274.621	ARHPHPHLSFM		√	
26	κ-CN	SPPEINTVQ	984.4997	VIESPPEINTVQ		√	
27	κ-CN	VIESPPEIN	997.52	VIESPPEINTVQ		√	√
28	κ-CN	VIESPPEINT	1098.568	VIESPPEINTVQ		√	
29	κ-CN	VIESPPEINTVQ	1325.695	VIESPPEINTVQ		√	√
30	κ-CN	ARHPHPHLS	1051.554	ARHPHPHLSFM		√	√
31	β-CN	SQSKVLPVPQKAVPYPQ	1866.048	SQSKVLPVPQKAVPYPQ		√	
32	α_{s1}-CN	KEDVPSE	803.3781	HIQKEDVPSER			√
33	κ-CN	ARHPHPHLSF	1198.623	ARHPHPHLSFM			√
34	κ-CN	ARHPHPHLSFM	1329.663	ARHPHPHLSFM			√
35	α_{s1}-CN	RPKHPIKHQGLPQEVLN	1991.13	RPKHPIKHQGLPQEVLNENLLRF	Antibacterial activity	√	
36	α_{s1}-CN	IPNPIGSENSE	1156.548	SDIPNPIGSENSEK		√	√
37	α_{s1}-CN	EQLLRLKK	1027.662	LEQLLRLKKY		√	
38	κ-CN	FSDKIAK	808.4563	FSDKIAK		√	√
39	κ-CN	AVRSPAQIL	954.5731	PAAVRSPAQILQ		√	√
40	β-CN	EAMAPKHK	911.4767	EAMAPKHK			√
41	β-CN	DELQDK	747.3519	TEDELQDKIHP			√
42	α_{s1}-CN	VAPFPE	659.3399	VVAPFPE			√
43	κ-CN	VQVTSTAV	804.4462	VQVTSTAV			√
44	β-CN	IPPLTQTPV	965.5666	IPPLTQTPV	DPP-IV Inhibitory activity	√	

WSP, water soluble peptide; L3, *L. plantarum* fermented milk; LBST, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* fermented milk; ACEI, Angiotensin I-converting enzyme inhibitory.

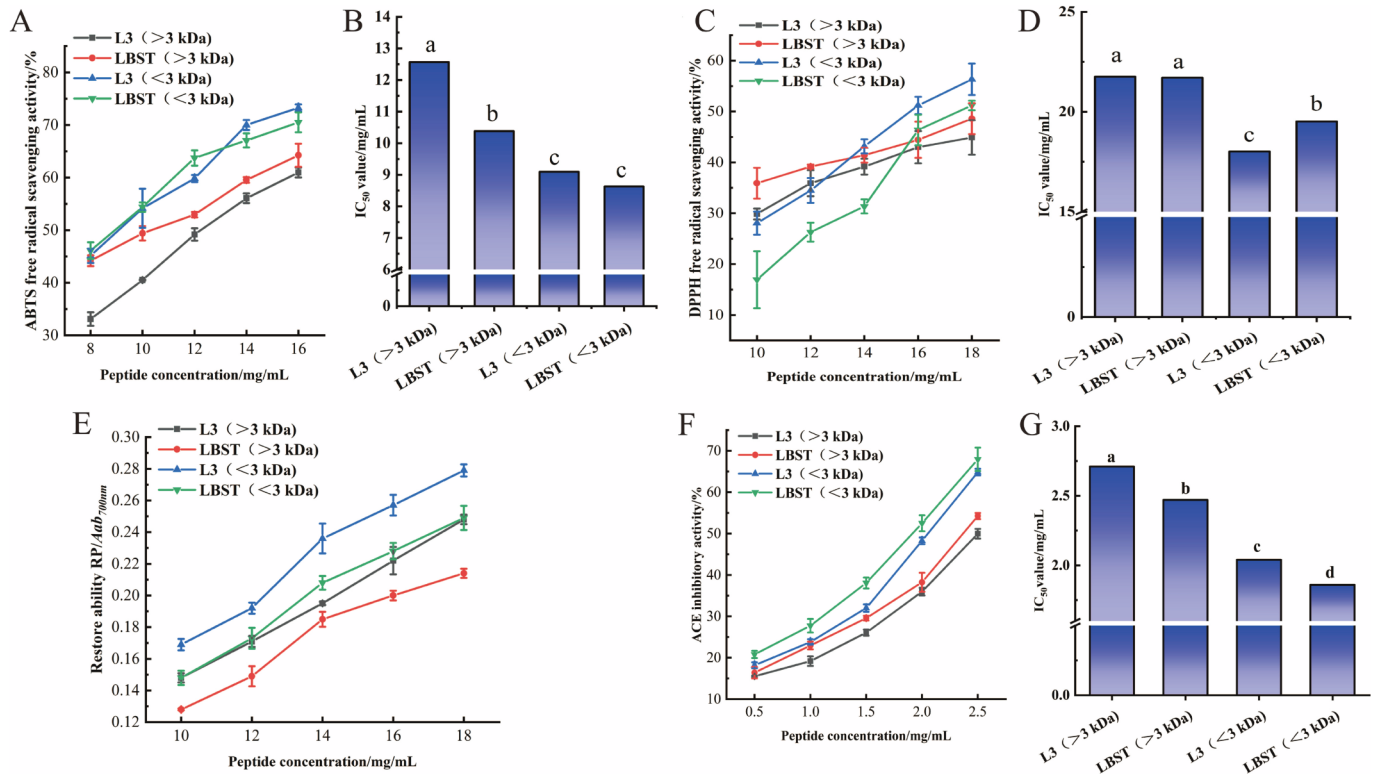

Fig. 4. In vitro biological activity of the WSP in L3 and LBST. (A-B) ABTS•+ scavenging activity; (C-D) DPPH• scavenging activity; (E) Restoration capacity RP; (F-G) ACE inhibition activity; Bars with different letters (a–c) are significantly different ($P < 0.05$). L3: *L. plantarum* L3 fermented milk; LBST: *Lactobacillus bulgaricus* and *Streptococcus thermophilus* fermented milk.

and 18.01 mg/mL and 8.63 and 19.52 mg/mL for LBST - WSP. On the other hand, the restored capacity restore point (RP) of WSP showed a concentration-dependent effect, and the restore capacity RP of L3 - WSP was higher than that of LBST-WSP (Fig. 4E). Overall, no significant differences were detected in the *in vitro* antioxidant activity of the two fermented milk WSPs ($P > 0.05$). Additionally, a previous study demonstrated that the IC_{50} of $ABTS^{\bullet+}$ and DPPH• scavenging activities of CN hydrolyzed by *L. plantarum* A3 and *L. brevis* CGMCC15954 were 13.63, 16.51, 9.37, and 14.94 mg/mL, respectively (Cui et al., 2022). This result indicates that the *in vitro* antioxidant activity of fermented milk could be related to the fermenting agent and the type of milk protein.

LAB releases ACEIPs through the proteolytic, envelope proteinases, transporter, and intracellular peptidase systems. As shown in Fig. 4F-G, all ultrafiltration components exhibited potential ACEI activity, and the IC_{50} of ACEI for the LBST – WSP with MW >3 kDa and MW <3 kDa were 2.47 and 1.86 mg/mL, respectively. Interestingly, these values were lower than 2.71 and 2.04 mg/mL for L3-WSP ($P < 0.05$), suggesting that LBST-WSP had higher ACEI activity than L3-WSP. One author (Ayyash, Al-Nuaimi, Al-Mahadin, & Liu, 2018) indicated that Lactobacillus. *reuteri* KX881777, *L. plantarum* KX881772, *L. plantarum* KX881779, and *L. plantarum*-fermented camel and cow milk showed ACEI activity. In addition, Nazila, Saeed, Mahta, & Mehran (2019) reported that the WSP (MW ≤ 3 kDa) derived from *Leuconostoc lactis* PTCC 1899 – fermented camel milk exhibited an ACEI activity (IC_{50} value = 1.61 ± 0.18 mg/mL). The IC_{50} values of ACEI in LBST-WSP and L3-WSP were 1.86 and 2.04 mg/mL, respectively, and were higher than 1.61 mg/mL of *L. lactis* PTCC 1899-fermented camel milk, indicating that the *L. lactis* exhibited higher ACEI activity. *In vitro* results showed that WSP derived from *L. plantarum* L3 fermented milk had high ACEI and antioxidant activities, which correlated with the reported potential ACEIPs and AOPs released during the milk fermentation.

3.2.4. Bioactive peptide formation pathway analysis

The BPs in fermented milk are derived from the enzymatic cleavage of proteins by peptidyl peptidases, aminopeptidases, and carboxypeptidases secreted by LAB. Studies revealed that changes in the enzymatic cleavage sites could alter the type and abundance of peptides in fermented milk. Therefore, the distribution of cleavage sites in CN at α_{s1}-CN, α_{s2}-CN, β-CN, and κ-CN for enzymes secreted by *L. plantarum* L3 was investigated based on the distribution of peptides found in L3-fermented milk (Fig. 5). The number of cleavage sites in L3 α_{s1}-CN, α_{s2}-CN, β-CN, and κ-CN by enzymes secreted by *L. plantarum* L3 was 33, 29, 35, and 30, respectively. This result indicates that *L. plantarum* L3 exhibits a complex enzymatic system and could cleavage the CN, mainly between amino acids 20–48, 69–87 for α_{s1}-CN, between amino acids 133–154 for α_{s2}-CN, and between amino acids 20–48 and 92–107 for β-CN. Furthermore, *L. plantarum* L3 multiplexed the Glu(E)-Asp(D) and Gln (Q)-Met(M) sites in α_{s1}-CN, the Glu(E)-Ser(S), Met(M)-Glu(E), and Lys (K)-Lys(K) sites in α_{s2}-CN, the Lys(K)-Lys(K), Glu(E)-Val(V), and Met (M)-Gly(G) sites in β-CN, and also Lys(K)-Ser(S) and Met(M)-Ala(A) sites in κ-CN.

The peptidase released during the fermentation of *L. plantarum* L3 could enzymatically cleave the D47-K48 and A53-Q54 sites of β-CN to produce the ACEI peptide KIHPFAQTQ (Fig. 5C and E). Moreover, the F174-A175 and K181-T182 cleavage in α_{s2}-CN could promote the production of the ACEI peptide ALPQYLK (Fig. 5B and E). In addition, *L. plantarum* L3 could enzymatically cleave the K79-H80 and Y90-Y91 sites of α_{s1}-CN, the K79-H80 and Y90-Y91 sites of α_{s1}-CN, W193-I194 and R106-Y107 sites of α_{s2}-CN, T54-Q55-, Q72-N73, L165-S166, and Q175-R176 sites of β-CN, and A87-R88, M97-A98, E152-V153, and Q164-V165 sites of κ-CN (Fig. 5A-D). Also, *L. plantarum* L3 enzymatically cleaved amino acids N17-E18, L94-E95, K103-Y104, D180-I182, and E189-N190 of α_{s1}-CN to release the AMPs RPKHPIKHQGLPQEVLN, EQLLRLKK, and IPNPIGSENSE (Fig. 5A and E). *L. plantarum L3* could also enzymatically cleave Y193-Q194 and P206-I207 to release the

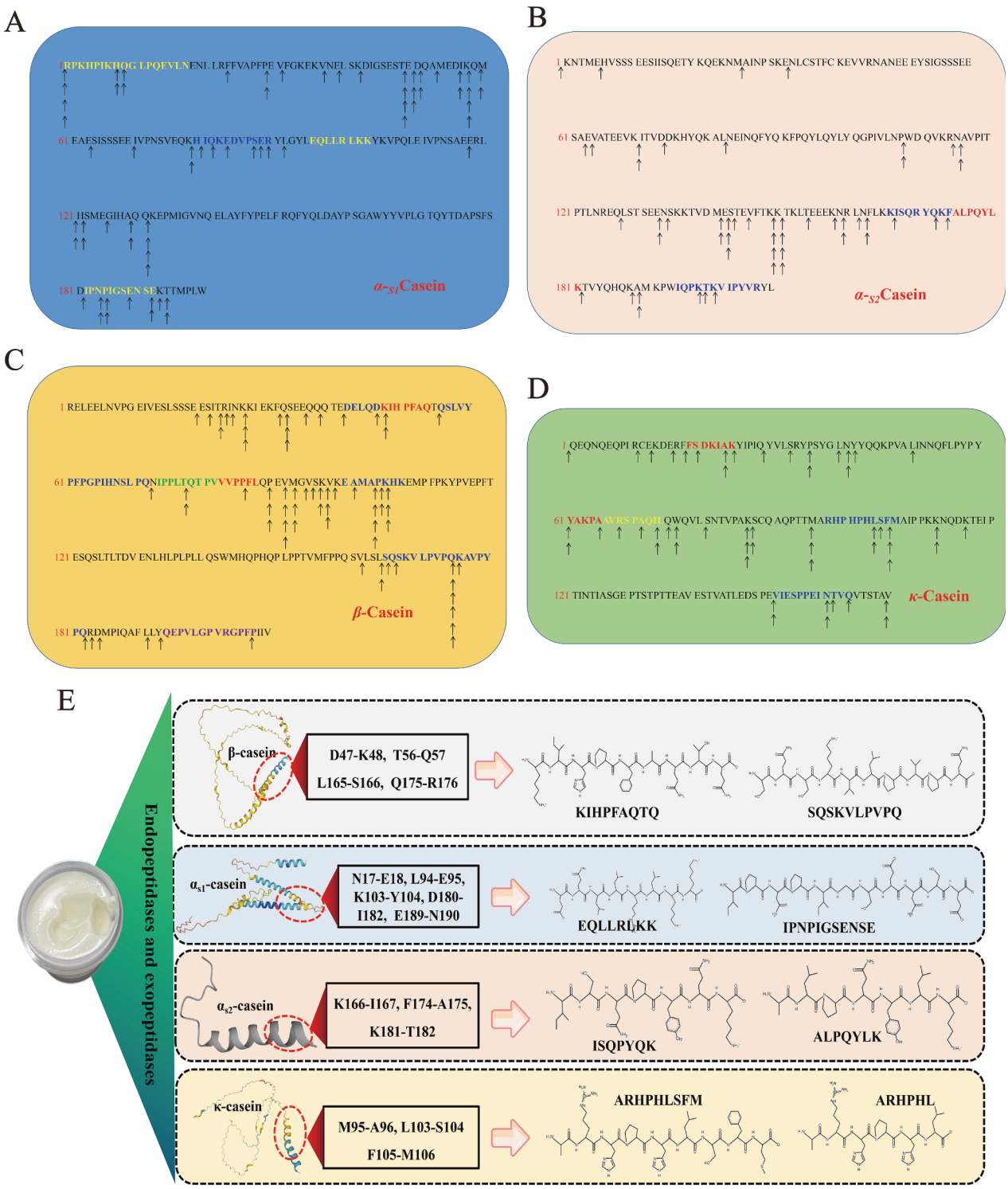

Fig. 5. The cleavage sites in α_{S1}- (A), α_{S2}- (B), β- (C) and α-CN by enzymes secreted by *L. plantarum* L3 (D); BPs in *L. plantarum* L3 fermented milk (E). → represents the digestion site, each → represents one digestion; bold red letters are angiotensin I-converting enzyme inhibitory peptides; bold blue letters are AOPs; bold yellow letters are antimicrobial peptides; bold green letters are dipeptidyl peptidase-IV inhibitory peptides; bold purple letters are immunomodulatory peptides.

immunomodulatory peptide QEPVLGPVRGPFPI found in dairy products, such as cheese and fermented milk. The κ-CN region near the chymosin-sensitive site (i.e., Phe105-Met106) was subjected to intense proteolytic phenomena, producing numerous peptides with antioxidant activity (i.e., ARHPHPHLSF and ARHPHPHLSFM) (Fig. 5D and E). Previous studies have shown that the *L. reuteri* WQ-Y1 protease system preferentially cleaves the peptide bonds of lysine (K) and histidine (H), arginine (R) and phenylalanine (F) to release BPs, while the protease systems of *L. plantarum* A3 and *L. brevis* CGMCC15954 preferentially cleave the peptide bonds of lysine (K) and threonine (T) to release BPs (Cui et al., 2022). These features demonstrate that the LAB strains have a similar enzyme system that cleaves bonds between lysine and other amino acids. Interestingly, peptides derived from β-lactoglobulin and α-lactalbumin were not detected. These findings confirmed that the stability of whey proteins to hydrolysis is higher than that of CN. The relative stability of whey proteins is attributed to the conformational

stability of globular proteins rather than the specificity of LAB proteases (Pinto, Picariello, Addeo, Chianese, Scaloni, & Caira, 2020). The above results indicated that *L. plantarum* L3 exhibited a good protein degradation capacity and its secreted peptidase hydrolyzed CN to release BPs.

4. Conclusion

The present study used untargeted metabolomic and peptidomics approaches to identify metabolites in *L. plantarum* L3 fermented milk and the pathways they regulate. The characteristic metabolites, including functional amino acids, functional dipeptides (i.e., Val-Lys, Thr-Pro and His-Pro), flavour peptides (i.e., Ala-Pro, Ala-Val, Pro-Asp), pyridoxine, extracellular polysaccharide composition, and muramic acid, could contribute to the unique flavour and nutritional qualities of *L. plantarum* L3 fermented milk. Furthermore, oligopeptide, taurine, vitamin B6, and thiamin metabolism were the main pathways regulated by the generated metabolites. Moreover, endogenous enzymes secreted by *L. plantarum* L3 cleaved β- and α-CN to release 6 potential ACEIPs (TPVVVVPPFL), 19 AOPs (DELQDKIH), and 5 AMPs (RPKHPIKHQGLPQEVLN). The BPs could offer health benefits, such as hypotension and free radical scavenging. Further studies are required to determine potential health benefits *in vivo*. Overall, our results create a valuable foundation for improving the quality of fermented milk using the *L. plantarum* L3 strain.

CRediT authorship contribution statement

Teng Wang: Methodology, Formal analysis, Investigation, Writing – original draft. **Guangqiang Wei:** Methodology, Formal analysis, Writing – original draft. **Faqiang Chen:** Data curation, Formal analysis. **Qingwen Ma:** Formal analysis, Software. **Aixiang Huang:** Conceptualization, Supervision, Investigation, Software, Writing – review & editing.

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

The authors do not have permission to share data.

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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.2023.100732.

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