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Revealing the formation mechanism of umami peptides in fermented broad bean paste based on peptidomics and macrogenomics

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ABSTRACT

Umami peptides play important roles in the flavor of fermented broad bean paste (FBBP), and proteases produced by microorganisms contributed to the production of umami peptides. In order to reveal the formation of umami peptides and their relationships with protease-producing microorganisms during the natural fermentation of FBBP, peptidomics and virtual screening were used to identify and screen umami peptides. Meanwhile, macrogenomics was used to analyze the abundance of microbial-derived protease genes during FBBP fermentation. Then, based on the Pearson correlation coefficient, the correlation network diagram of each protease-producing microorganism with umami peptides was constructed. The results showed that a total of two exopeptidases and four endopeptidases were annotated from FBBP. *Staphylococcus*, *Lactobacillus*, *Aspergillus*, and *Weissella* can produce most proteases. The species *Lactobacillus curvatus*, *Dyella jiangningensis*, *Erythrobacter* sp., and unclassified_g_*Pantoea* had strong correlation with umami peptides, and they may contribute to the process of protein hydrolysis to produce umami peptides. This study is expected to reveal the formation mechanism of umami peptides in FBBP, and the results of this study provided a better understanding of the relationship between proteases, microbiota, and core umami peptides in FBBP, which could help to improve the umami taste of Pixian Douban paste during fermentation.

1. Introduction

Pixian Douban paste (PDBP), as one of the most popular seasonings in China, is essential for Sichuan cuisine. It is made by chili peppers and fermented broad bean paste in a certain ratio, and then brewed through a fermentation process. Broad bean paste fermentation is an important step in the fermentation process of PDBP, which determines the flavor and product quality of PDBP[1]. The fermented broad bean paste (FBBP) is mainly based on broad bean and wheat flour as raw materials, using enzymes produced by microbial metabolism to break them down into flavor peptides, flavor amino acids, monosaccharides, and various volatile flavor substances such as acids, alcohols, and esters[2-3]. Among them, umami peptides have received a great deal of attention in recent years due to their good taste properties, high nutritional value, and being an important component of flavor[4]. The metabolism of microorganisms, including protein hydrolysis and lipolysis, determines the type and content of flavor substances during fermentation[5]. Microbiota played an irreplaceable role in the degradation of PDBP raw materials and the formation of flavor compounds[6].

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Fermenting microorganisms produce a variety of microbial enzymes through various metabolic pathways, among which proteases catalyze the formation of umami peptides from proteins, which contribute to the process of protein hydrolysis[7-9]. Most previous studies have focused on the relationship between key microbial communities and key flavor compounds during PDBP's fermentation. For example, Lin et al.[10] investigated the correlation between flavor and microorganisms by analyzing the changes in key aroma compounds, organic acids, free amino acids (FAAs), and microbial communities during different fermentation stages of PDBP. Liu et al.[11] analyzed the correlation of key microorganisms with FAAs, organic acids, and volatile aroma substances during post-fermentation. Yang et al.[12] determined the correlation between flavor compounds and microorganisms and demonstrated that *Aspergillus*, *Bacillus*, and *Lactobacillus* had a positive effects on some flavor compounds such as flavor amino acids. However, changes in umami peptides and their protease-producing microorganisms, as well as the correlation of the umami peptides and microbial community during broad bean fermentation have not been specified.

In recent years, many researchers have isolated and identified umami peptides from different fermented condiments. For example, Amin et al.[9] isolated four umami peptides from Indonesian fermented soybean by ultrafiltration, reversed-phase high-performance liquid chromatography (RP-HPLC), and electrospray ionization-mass spectrometry (ESI-MS), among which the peptide GENEEEDSGAIVTVK (GK-15) was the main source of the flavor of tempeh. Zhuang et al.[13] used sensory-guided fractionation, macroporous resin, medium-pressure liquid chromatography, RP-HPLC with ultra-high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) to isolate and identify five umami peptides from soy sauce, and found that the synthetic peptides had a much better sensory taste than the mixtures of their constituent amino acids, further suggested that the taste of peptides is controlled by their amino acid sequence rather than individual amino acids. However, the identification of peptides by traditional multiple chromatographic purification techniques is time-consuming, labor-intensive, and may lose some of the highly active peptides. Thus, the identification of peptides requires more efficient techniques and methods. Peptidomics is a non-targeted method for rapid identification of protein hydrolysates or crude peptide fractions[14]. Compared with the traditional umami peptide screening techniques, it can save a lot of time by simplifying sample handling and eliminating the need for separation and purification, as well as greatly reducing the possibility of missing flavor peptides during the assay[15-16]. Virtual screening is a class of screening techniques consisting of bioinformatics, molecular docking, and other techniques. The mutual combination of peptidomics and virtual screening techniques can rapidly screen bioactive peptides and improve experimental efficiency[14]. Macrogenomics has made an important contribution to the comprehensive analysis of microbial community structure and metabolic processes[17], and has been widely applied to the study of microbial communities in fermented foods such as white wine[18], kimchi[19], fish sauce[5], etc. Verce et al.[20] used macrogenomics and transcriptomics to explore the microbial diversity and assess the function and transcription of genes during cocoa fermentation in Costa Rica, and found that *Acetobacter*, *Komagataeibacter*, *Limosilactobacillus*, *Liquorilactobacillus*, *Lactiplantibacillus*, etc., were the major genera of microorganisms.

Therefore, in this study, peptidomics and virtual screening were used to identify free peptides from FBBP, and macrogenomics was used to analyze the abundance of protease genes produced by microorganisms during broad bean fermentation. The screened umami peptides and proteases were then subjected to Pearson correlation analysis, and correlation network diagrams were drawn to identify umami peptides extremely relevant proteases as well as microbiological sources. This study can provide information on major proteases and protease-producing microorganisms for the formation of FBBP core umami peptides, which contributed to the improvement of umami taste during fermentation of FBBP.

2. Materials and methods

2.1 Materials and chemicals

The koji was collected from Sichuan Pixiandouban Co., Ltd. (Pixian, China), and then mixed with water and salt (Jiuda Co., Sichuan, China) in the mass ratio of 100:75:38. Further, put them into a fermentation tank for natural fermentation for 90 days, regularly stir and replenish water during fermentation (stir every two days). Samples consisted of a 1:1:1 mass ratio mix of top, middle, and bottom layers and were collected monthly. Four separate sample were respectively collected at 0 (D0), 30 (D30), 60 (D60), and 90 days (D90) and preserved at –80 ℃ for further study.

Formic acid (HPLC gradient grade) and acetonitrile (HPLC gradient grade) were purchased from Sigma (USA). All solutions used were prepared in ultrapure water.

2.2 Determination of peptides by peptidomic analysis

2.2.1 Preparation of the peptide extract

Peptide extraction was performed as reported by our previous work with minor adjustments[21]. In brief, the freeze-dried bean broad paste was crushed to a constant weight and then passed through a 0.25 mm screen. FBBP was dissolved in deionized water at a ratio of 1:3 (g/mL), which was then agitated at 37 ℃ for an hour and centrifuged using centrifuge (Eppendorf 5810R, Germany) at 8 000 × *g* for 30 min at 4 ℃. The supernatant was filtered and the filtrate was passed through ultrafiltration centrifuge tubes to retain peptide fractions smaller than 3 kDa, and then lyophilized and placed in a refrigerator at –80 ℃ for further analysis.

2.2.2 Identification of peptides by UPLC-ESI-quadrupole time of flight (QTOF)-MS/MS

Peptides analysis of lyophilized fractions were performed in a Shimadzu UPLC system. Dissolve the sample using mobile phase A (0.1% formic acid) and load 1 μL of the peptide sample (5 mg/mL) with the auto-sampler. The separation gradient of mobile phase B (0.1% formic acid acetonitrile solution) increased from 5% to 95% within 16 min, and then increased from 95% to 5% during 16–20 min. High-resolution time-of-flight mass spectrometer (X500R, AB SCIEX, Framingham, MA, USA) was used for data collection. The molecular mass of peptides was identified using ESI-QTOF-MS/MS. Spectra in the *m*/*z* range of 50–900 were taken under a single charged

$[M + H]^+$ state. The acquisition mode was information correlation acquisition (IDA).

The data were processed and analyzed using PEAKS Studio version Xpro software (Bioinformatics Solutions Inc., Waterloo, Canada). Data processing and spectrum analysis were carried out including database search and *de novo* sequencing. The setting parameters were as follows: non-enzyme digestion, the tolerance of parent mass error was 1×10^{-5} and fragment mass error was 0.05 Da. Carbamidomethylation was the fixed modification, and methionine oxidation and acetylation (N-term) were the variable modifications. The credible peptide and protein that hit threshold (–lg *P*) set at ≥ 20 were selected.

2.3 Prediction of the activity of umami peptides

PeptideRanker and iUmami-SCM were used to predict the activity of the identified umami peptides based on the methods used by previous researchers to screen umami peptides[14]. iUmami-SCM is a novel sequence-based predictor that uses a scorecard approach with dipeptide propensity scores to predict and analyze umami peptides (https://camt.pythonanywhere.com/iUmami-SCM), and peptides were considered to have umami taste when the threshold value exceeded 588. PeptideRanker (https://distilldeep. ucd. ie/PeptideRanker/) classified peptides by predicting their probability of bioactivity, and peptides with a predicted score above a threshold of 0.5 were classified as biologically active[22].

2.4 Analysis of protease-producing microorganisms by metagenomic

2.4.1 DNA extraction and Illumina sequencing

DNA extraction and Illumina sequencing were carried out referring to previous studies[5,23]. The microbial metagenomic DNA of the broad bean paste was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Dallas, TX, USA). After using 1% agarose gel electrophoresis to detect DNA integrity, the genomic DNA was fragmented to approximately 400 bp in length using Covaris M220. The paired-end (PE) library was constructed using NEXTflex™ Rapid DNA-Seq (Bioo Scientific, Austin, TX, USA). The prepared library was double-ended sequenced on an Illumina HiSeq X Ten instrument (2 × 150 bp) (Illumina, USA).

To ensure the accuracy of the subsequent analysis results, the software FASTP was used to remove the reads with length less than 50 bp after mass clipping, average mass value less than 20 and N-base containing reads, and keep the high quality pair-end reads and single-end reads.

2.4.2 Species and function notes

The gene sets were compared with the Non-Redundant Protein (NR) database using Diamond software (parameters: blastp; E-value ≤ 1×10^{-5}). Species annotations at the domain, boundary, phylum, order, family, genus, and species levels were obtained from NR library, and then the sum of the gene abundances corresponding to the species was used to calculate the abundance of the substance. The gene set sequences were also compared with the gene library of Kyoto Encyclopedia of Gene and Genomes (KEGG), and the abundance of this functional class was calculated based on the sum of gene abundance corresponding to KEGG Orthology (KO), Enzyme Commission (EC), and Module, and all analyses were performed on MajorBio platform (http://www.majorbio.com/).

2.5 Statistical analysis

Data were reported as the mean ± standard deviation (SD). One-way ANOVA and Duncan's multiple range test were used to analyze differences with a level of $P < 0.05$. The non-redundant gene sets were annotated with each database for species and function, and all analyses were performed on the platform of Shanghai Meiji Biologicals (https://www. majorbio. com/). The correction heat map and correlation network diagram analysis were performed online https://www.omicstudio.cn/tool.

3. Result and discussion

3.1 Identification of peptides

3.1.1 Screening of umami peptides

The taste properties of peptides were closely related to their amino acid composition, amino acid arrangement, and the molecular weight of peptides[24]. Previous research confirmed that umami peptides were mostly small molecular weight, and peptides with molecular weights less than 1 000 Da usually have strong flavors[4,25]. Liu et al.[26] reported that the distilled segments of yeast extracts with molecular weights less than 1 000 Da had the strongest umami taste, and they had the best effect on umami and umami enhancement of chicken soup. Typically, their sensory properties were positively correlated with the taste properties of the constituent amino acids, and the amino acid composition of umami peptides often contains Asp (D), Glu (E), Asn (N) and Gln (Q) or a combination of them with each other[27-28]. Therefore, in this study, a total of 377 free peptides were screened based on molecular weight lower than 1 000 Da and containing ≥ 30% of D, E, N, Q from FBBP. As assessed by principal component analysis (PCA) (Fig. 1a), the differences between the D90 group and the other groups were more significant, with relatively small differences in the D0 and D30 groups. As shown in Fig. 1b, the unfermented group (D0) and the fermented group (D30, D60, D90), contained 98, 161, 250, and 352 peptides, respectively. The number of umami peptides increased with the increase of fermentation time, and the D60 and D90 groups contain the most common peptides. The molecular weight distribution of the identified peptides showed that peptides of all molecular weight chain segments increased with fermentation time and were mostly concentrated around 0.25–0.5 kDa (Fig. 1c).

iUmami-SCM shows excellent predictive performance in predicting umami peptides, outperforms other commonly used machine learning classifiers, and has been widely used for the screening of umami peptides[14,29]. PeptideRanker is a novel neural network based bioactive peptide prediction server. Zhu et al.[22] applied PeptideRanker and identified three umami peptides after further screening. Therefore, PeptideRanker is of significance in screening umami peptides. In order to include more potential umami peptides, peptides with iUmami-SCM scores > 588 or/and PeptideRanker scores > 0.5 were screened for further analyses. Thus, a total of 269

potential umami peptides were identified based on iUmami-SCM and PeptideRanker (Table 1), and their abundance was shown in Fig. 2. The abundance of free peptides in the fermented groups (D30, D60, and D90) was higher than those in the unfermented group (D0) and increased with fermentation time. This suggested that fermentation can increase the abundance of potential umami peptides of FBBP, and the accuracy of the potential umami peptides screened according to the above screening was 71.35%.

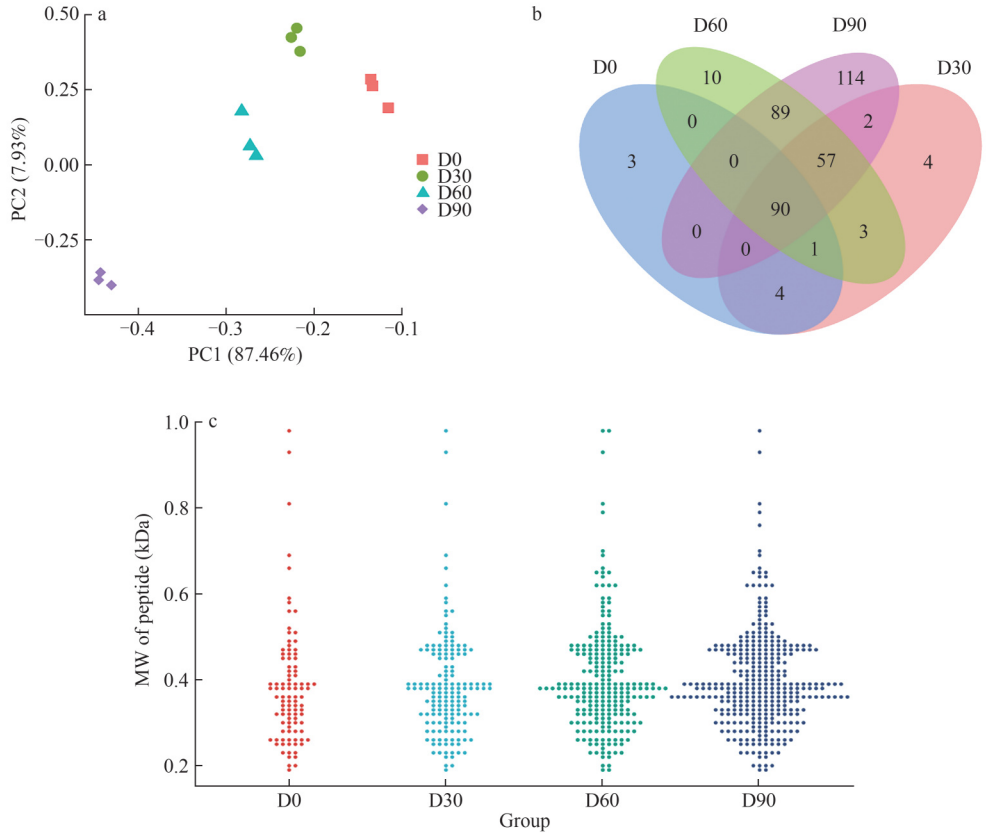

Fig. 1 Identification of umami peptides of FBBP with different fermentation time. (a) PCA and (b) Venn analysis of umami peptides from different fermentation groups. (c) Molecular weight (MW) distribution of umami peptides of groups D0, D30, D60, D90.

Table 1
Predictive score for peptides.

Peptide	Score (iUmami-SCM/ PeptideRanker)	Peptide	Score (iUmami-SCM/ PeptideRanker)	Peptide	Score (iUmami-SCM/ PeptideRanker)	Peptide	Score (iUmami-SCM/ PeptideRanker)
AANN	712/0.131	EAGD	621/0.097	EV	618/0.021	QE	618/0.035
AD	711/0.132	EAL	615/0.093	EVE	610/0.020	QEL	668/0.083
ADQF	637/0.650	EAN	615/0.052	EVG	641/0.052	QEY	611/0.080
ADQP	628/0.280	EAP	591/0.155	EVNV	590/0.026	QLEL	597/0.131
ADV	676/0.077	ED	711/0.034	EVQ	600/0.027	QNTP	601/0.188
AE	619/0.047	EDD	738/0.043	EVS	600/0.028	QVDD	673/0.068
AEL	668/0.097	EDE	784/0.029	EVTDSV	613/0.030	RNME	596/0.175
APD	595/0.368	EDEDE	784/0.036	EVV	602/0.021	SAE	607/0.066
AVE	595/0.037	EDL	680/0.083	EVVE	602/0.019	SDL	658/0.266
CDLSD	711/0.225	EDLP	664/0.194	EY	604/0.066	SDY	619/0.231
DA	642/0.131	EDMV	625/0.129	FDL	616/0.870	SE	696/0.044
DAA	623/0.150	EDP	657/0.135	FDR	593/0.846	SENP	640/0.138
DADL	667/0.270	EDPL	636/0.304	FE	604/0.589	SEY	650/0.094
DAEL	659/0.122	EDQ	665/0.041	FED	657/0.292	SNGDDE	704/0.169
DAF	607/0.804	EDSV	640/0.040	GD	650/0.387	SVE	615/0.037
DAL	622/0.287	EDTL	624/0.074	GDE	753/0.113	TDEE	815/0.026
DAP	599/0.392	EDV	676/0.031	GE	711/0.110	TDGE	665/0.060

Table 1 (*Continued*)

Peptide	Score (iUmami-SCM/ PeptideRanker)	Peptide	Score (iUmami-SCM/ PeptideRanker)	Peptide	Score (iUmami-SCM/ PeptideRanker)	Peptide	Score (iUmami-SCM/ PeptideRanker)
DAPN	600/0.319	EDVTSV	642/0.033	GEL	714/0.210	TDL	619/0.122
DAV	615/0.075	EDYTCQ	624/0.166	KE	619/0.025	TE	604/0.023
DAY	607/0.234	EE	1000/0.022	LAD	604/0.161	TEL	661/0.046
DD	765/0.098	EEAK	751/0.029	LDD	676/0.140	TEN	645/0.029
DDE	811/0.057	EED	855/0.026	LDDL	667/0.296	TFAD	624/0.281
DDL	707/0.260	EEGE	794/0.033	LDDLPNGMT	631/0.539	TGE	646/0.060
DDMT	643/0.333	EEL	859/0.039	LDE	722/0.065	TLED	732/0.044
DE	857/0.036	EEP	774/0.064	LDL	619/0.321	TVD	616/0.032
DEAY	685/0.103	EEPR	712/0.093	LDP	596/0.449	VAD	672/0.060
DED	784/0.046	EESV	789/0.027	LDV	615/0.087	VD	650/0.043
DESFQ	694/0.198	EETGTK	641/0.039	LDVE	611/0.047	VDL	650/0.114
DESH	727/0.064	EEV	809/0.020	LED	630/0.055	VDP	627/0.178
DETL	790/0.074	EFTL	697/0.306	LEE	774/0.034	VDQ	634/0.047
DG	696/0.391	EG	672/0.100	LEL	633/0.114	VDV	646/0.037
DGEPL	637/0.417	EGAE	644/0.058	LGDDN	618/0.207	VE	603/0.022
DGGNCV	613/0.345	EGDA	654/0.103	LGE	593/0.151	VEDL	654/0.061
DGNF	629/0.825	EGE	691/0.048	LGEN	624/0.117	VEE	801/0.020
DL	650/0.325	EGL	594/0.197	LLLDDL	603/0.342	VEL	660/0.045
DLD	619/0.190	EGV	606/0.056	LPE	636/0.215	VGE	688/0.057
DLDL	6290.323	EK	708/0.024	LSE	822/0.063	VQD	589/0.046
DLDLPFAAN	605/0.448	EKSD	659/0.039	LTE	588/0.041	VSN	593/0.055
DLE	599/0.083	EL	718/0.072	MCQE	601/0.276	YE	596/0.066
DLH	607/0.276	ELD	653/0.062	MDADA	644/0.217	YGD	607/0.392
DLK	623/0.146	ELE	633/0.038	MDEV	685/0.091	DDF	545/0.748
DLLPN	598/0.401	ELF	613/0.568	MDPD	606/0.564	DF	325/0.942
DLMEN	625/0.174	ELL	613/0.144	MPE	602/0.441	DFT	453/0.600
DLNL	603/0.315	ELN	657/0.067	MPNN	699/0.542	DGFN	560/0.770
DLNTVQ	602/0.058	ELNP	617/0.151	MQD	589/0.286	DLF	579/0.893
DLP	641/0.549	ELP	675/0.230	NASD	602/0.098	DM	582/0.607
DLQ	630/0.166	ELPN	651/0.164	NDL	611/0.207	DPPN	483/0.647
DLSESV	732/0.052	ELPT	741/0.129	NDP	588/0.321	DSW	582/0.733
DLY	607/0.375	ELQ	664/0.059	NDPL	590/0.553	DTF	415/0.571
DMAE	591/0.156	ELS	833/0.061	NF	596/0.941	DW	582/0.933
DMSD	617/0.238	ELT	645/0.049	NK	604/0.064	EFDGGP	455/0.577
DP	604/0.490	ELV	656/0.043	NLLE	595/0.101	EFP	404/0.719
DPAN	598/0.332	ELY	641/0.123	NLLPQAH	599/0.253	EGF	562/0.694
DPL	599/0.641	EN	687/0.036	NNDL	717/0.203	EPF	552/0.756
DQ	619/0.081	ENF	641/0.365	NNE	693/0.044	EW	582/0.585
DQDL	621/0.246	ENLP	628/0.180	NNP	733/0.319	FD	582/0.922
DQPT	683/0.197	EQ	702/0.033	NPE	589/0.141	FNP	535/0.938
DR	604/0.289	EQY	633/0.070	NTF	604/0.515	NW	582/0.934
DSGSD	631/0.176	ER	627/0.070	NTHF	589/0.465	WNP	560/0.946
DV	642/0.046	ERAP	588/0.177	NVA	611/0.059		
DVE	622/0.034	ESCN	642/0.158	NY	604/0.224		
DVF	599/0.551	ESD	704/0.045	PDNN	715/0.226		
DVL	590/0.126	ESE	719/0.031	PDPN	613/0.575		
DVV	614/0.039	ESMQ	706/0.131	PDYN	596/0.392		
DYAE	591/0.096	ESSD	678/0.056	PMDE	673/0.296		
DYEA	598/0.103	ESSHE	631/0.061	PN	603/0.480		
DYSV	594/0.109	ESVSD	654/0.044	PNPE	593/0.281		
EA	627/0.042	ESW	662/0.377	QD	596/0.084		
EAD	669/0.049	ETL	756/0.048	QDE	726/0.051		
EAEP	598/0.074	ETP	598/0.080	QDL	623/0.244		

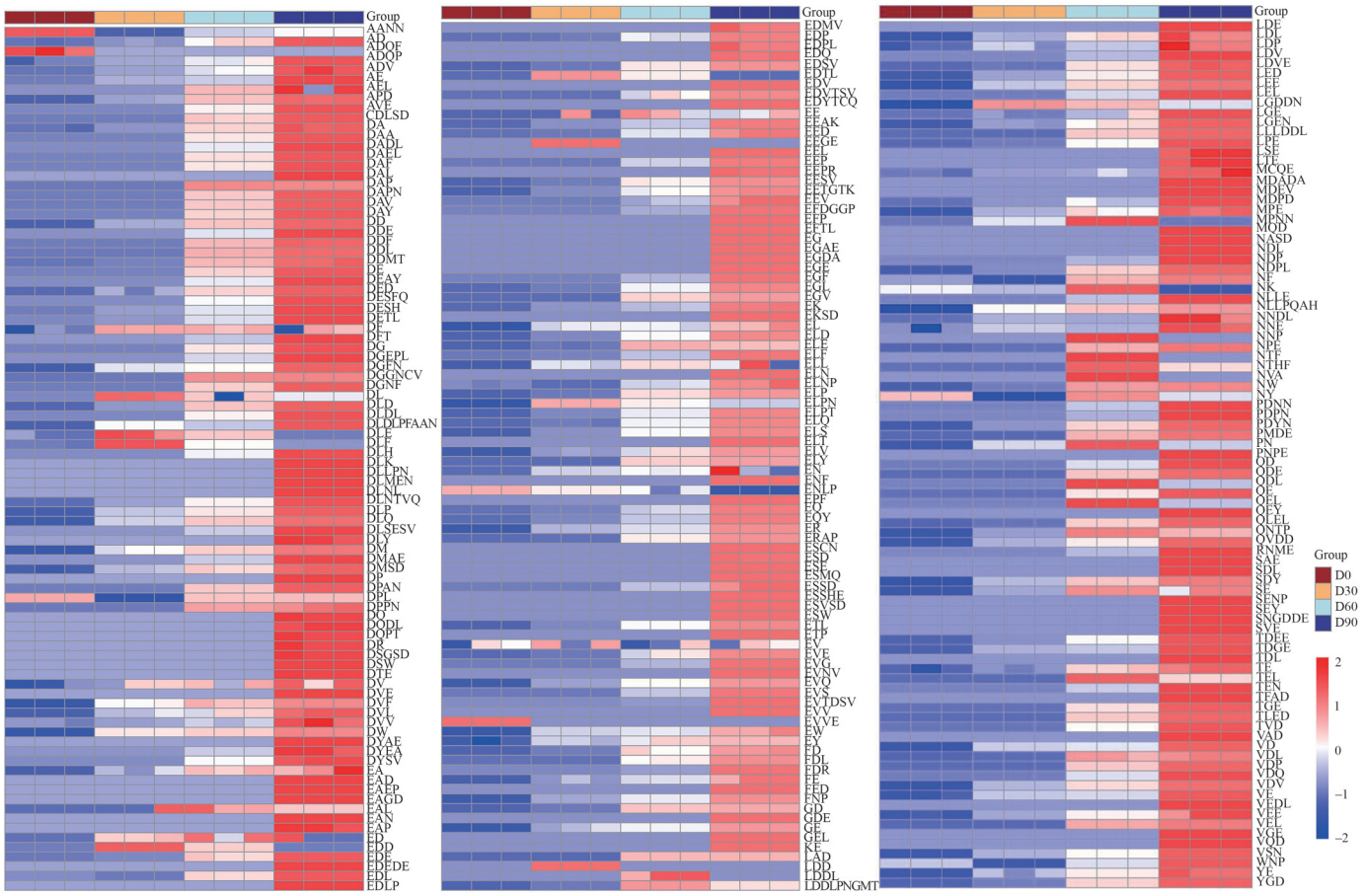

Fig. 2 Heat map analysis of umami peptides with different fermentation time.

3.1.2 Composition of screened umami peptides

In order to better understand the structural properties of umami peptides, the amino acid composition was analyzed. Fig. 3a showed the proportion of identified potential umami peptides grouped according to the number of amino acid residues. Among them, dipeptides and tetrapeptides accounted for about 15.99% and 27.88%, respectively, and the tripeptide is up to 47.21%, suggesting that the umami peptides were mainly composed of short peptides, which was in agreement with previous studies. For example, Song et al.[30] identified nine novel umami peptides from *Boletus edulis* by sensory analysis and molecular modeling methods, of which three were tripeptides and six were tetrapeptides. Yu et al.[24] isolated and identified four umami peptides from silkworm pupa hydrolysate, including three tripeptides and one tetrapeptide. Yu et al.[31] identified five umami peptides from myosin with the amino acid sequences DK, EEK, EDQK, SEGGR, and QDSIGS. Then, the amino acid composition of these peptides was further analyzed, results showed that D and E appeared with the highest frequency, followed by Leu (L), Asn (N), Val (V), Pro (P), Ala (A), Ser (S) and Gly (G) (Fig. 3b). Previous studies confirmed that V, P, and sweet amino acids (A, S, and G) also contribute to the umami taste of peptides in addition to D, E, N, and Q[28,32]. This may be the reason why these peptides have umami taste.

In addition, the frequency of each amino acid at the C-terminal and N-terminal positions was analyzed. As shown in Fig. 3c, D and E were the major amino acid residues both at the N-terminal and C-terminal ends, and a similar conclusion was reached by Wang et al.[33]. It was found that when positively charged basic amino acids (D or E) were located at the N-terminal end, negatively charged acidic amino acids (R or K) were located at the C-terminal end, which contributed to the peptide's umami properties[32,34]. A total of seven peptides with this structure were identified in this study, including DR, DK, EK, ER, EEAK, EEPR, and EETGTK. Additionally, previous studies have found that umami peptides with DE, DD, EE, and ED have strong umami taste after sensory evaluation[35-36]. A total of 15, 13, 13, 21 umami peptides containing these structures were found in this study, respectively. Most of umami peptides we screened have been recognized as having an umami taste by sensory evaluation in other studies. For example, Kong et al.[37] identified ED, AE, and VE from chicken soup and they exhibited strong umami taste. PN, EEL from Jinhua and Parma hams[38], EE, DA, DV, EV derived from chicken protein hydrolysate[36], and EGF, NNP, EGF, EDE, VDV in peanut protein isolate hydrolysate[7] and others has also been shown a umami taste.

3.2 Microbial proteases in FBBP

3.2.1 Protease in FBBP

Previous study has found that in addition to amino acids and organic acids, umami peptides were also the main substances that

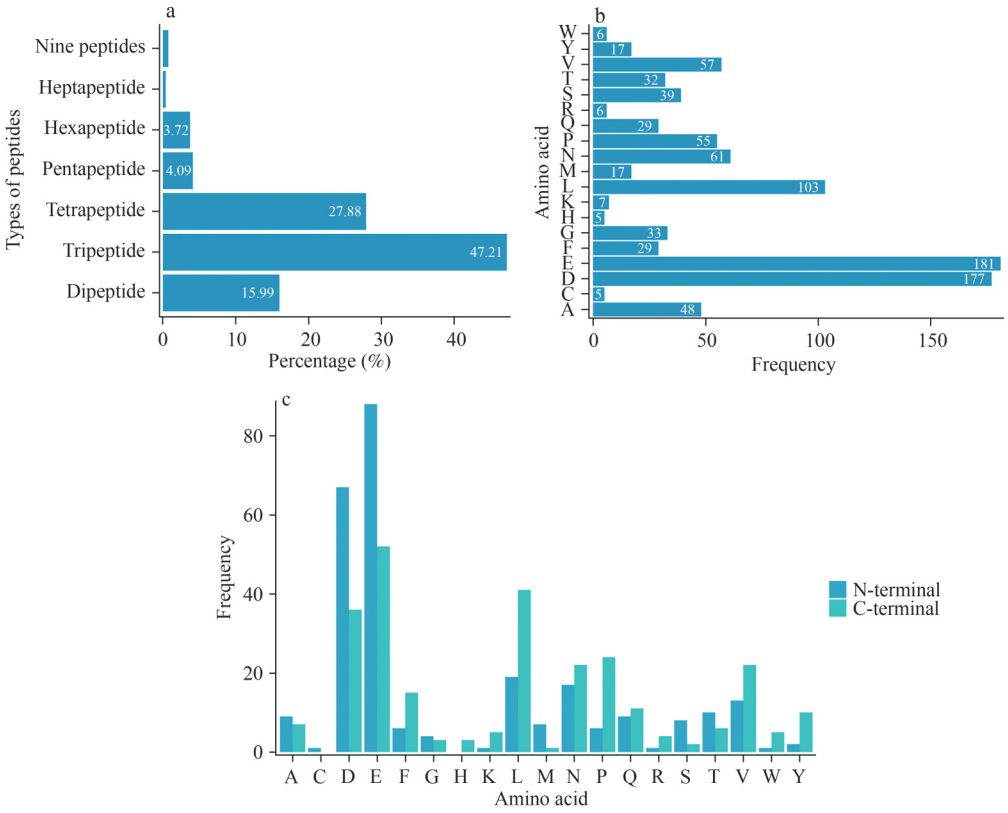

Fig. 3 Composition of screened umami peptides. (a) Percentage of specific types of peptides in the total sum of all umami peptides. (b) The amino acid composition of umami peptides. (c) C- and N-terminal amino acid composition. C, Cys; F, Phe; H, His; K, Lys; R, Arg; T, Thr; W, Trp; Y, Tyr.

form the flavour of FBBP[21], and proteases from microorganisms contribute to the production of umami peptides. However, there were few studies on protease and microbial information on the formation of umami peptides. Proteases can be classified into endopeptidases and exopeptidases according to their hydrolysis sites, such as non-terminal and amino-terminal or carboxy-terminal hydrolysis sites[39]. The combination of endopeptidases and exopeptidases hydrolyze both the non-terminal and amino-terminal sites of proteins to produce shorter chain peptides with lower molecular weight and more biological activity than a single protease[40]. In this study, abundant protease genes were identified from 39 microbial genera, as annotated by evolutionary genealogy of genes: Non-supervised Orthologous (eggNOG). These were mainly endopeptidases and exopeptidases. Exopeptidases were mainly categorized as aminopeptidases and carboxypeptidases, and endopeptidases were mainly categorized as serine endopeptidases, cysteine endopeptidases, metalloendopeptidases, and aspartic endopeptidases. The abundance of protease genes at different fermentation time was shown in Fig. 4. Aminopeptidase, carboxypeptidase, and serine endopeptidase were the major proteases after FBBP fermentation (D90), and their contents about accounted for 19%, 40%, and 25% of the total content, respectively. These same proteases are contained in the core proteases of fermented Chouguiyu[23]. As the fermentation time increased, the protease content gradually decreased from the beginning of fermentation to the 60th day, and returned to the peak at the 90th day, and the protease content was consistent with the changes in the content of related protease-producing microorganisms (Fig. 5).

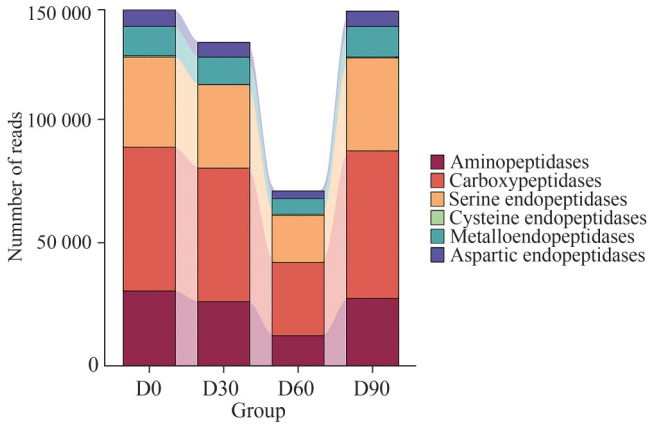

Fig. 4 Changes in proteases at different fermentation time.

3.2.2 *Microbial sources of exopeptidases and endopeptidases*

As the predominant proteases, aminopeptidases and carboxypeptidases were produced by similar genera of microorganisms (Fig. 5), including *Staphylococcus*, *Lactobacillus*, *Weissella*, *Bacillus*_f__Bacillaceae, *Streptococcus*, *Kocuria*, *Klebsiella*, *Rothia*_f__Micrococcaceae. In addition, *Pediococcus* also produced aminopeptidases. Endopeptidases mainly act on the peptide bonds within the polypeptide chains of proteins to break down long chains of proteins into short peptide fragments[41]. The active centers of metalloendopeptidases are metal ions such as Zn and Ca. The active centers of serine endopeptidases, aspartate endopeptidases, and cysteine endopeptidases were serine, cysteine, and aspartate,

respectively[23]. As one of the most prominent endopeptidases, serine endopeptidases were mainly produced by *Staphylococcus*, *Aspergillus*, *Bacillus*_f__Bacillaceae, *Triticum*, *Klebsiella*, *Aegilops*, *Leifsonia*, *Pseudomonas*, *Brachybacterium*, and *Glycine*. Metalloendopeptidases were produced by *Staphylococcus*, *Lactobacillus*, *Weissella*, and *Aspergillus*, *Pediococcus*, *Bacillus*_f__Bacillaceae, *Streptococcus*, *Kocuria*, *Triticum*, and *Aegilops*. Aspartate endopeptidase were produced only by *Bacillus*_f__Bacillaceae, *Aspergillus*, *Aegilops*, *Hordeum*, *Triticum*, and cysteine endopeptidases were produced only by *Aspergillus*, *Pichia*, *Medicago*, *Aegilops*, *Triticum*. Based on abundance analysis, *Staphylococcus*, *Lactobacillus*, *Aspergillus*, and *Weissella*, were the

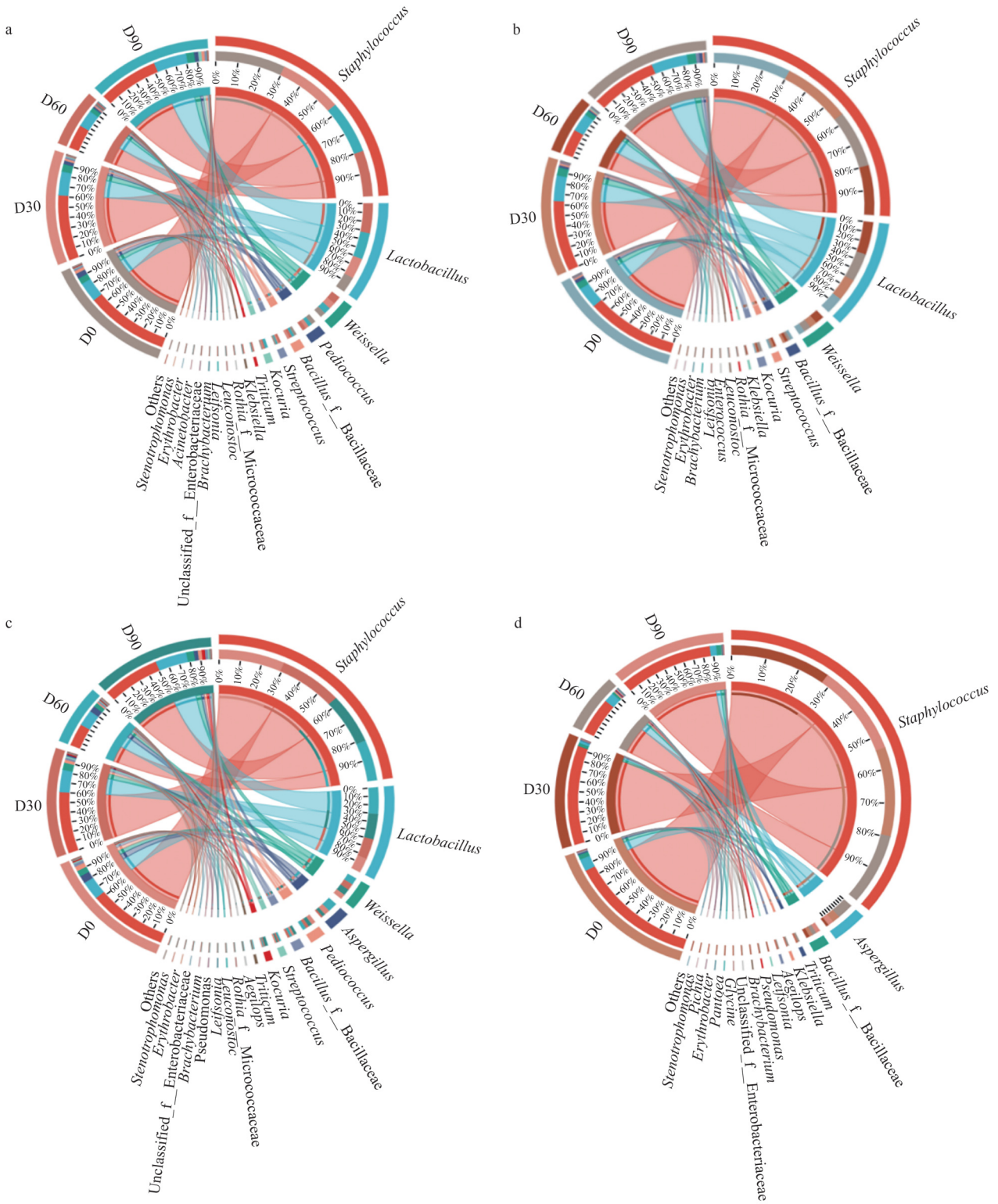

Fig. 5 Changes in abundance of different protease microbial sources in D0, D30, D60, and D90 groups. (a) Aminopeptidase; (b) carboxypeptidase; (c) metalloendopeptidase; (d) serine endopeptidase; (e) aspartic endopeptidase; (f) cysteine endopeptidase.

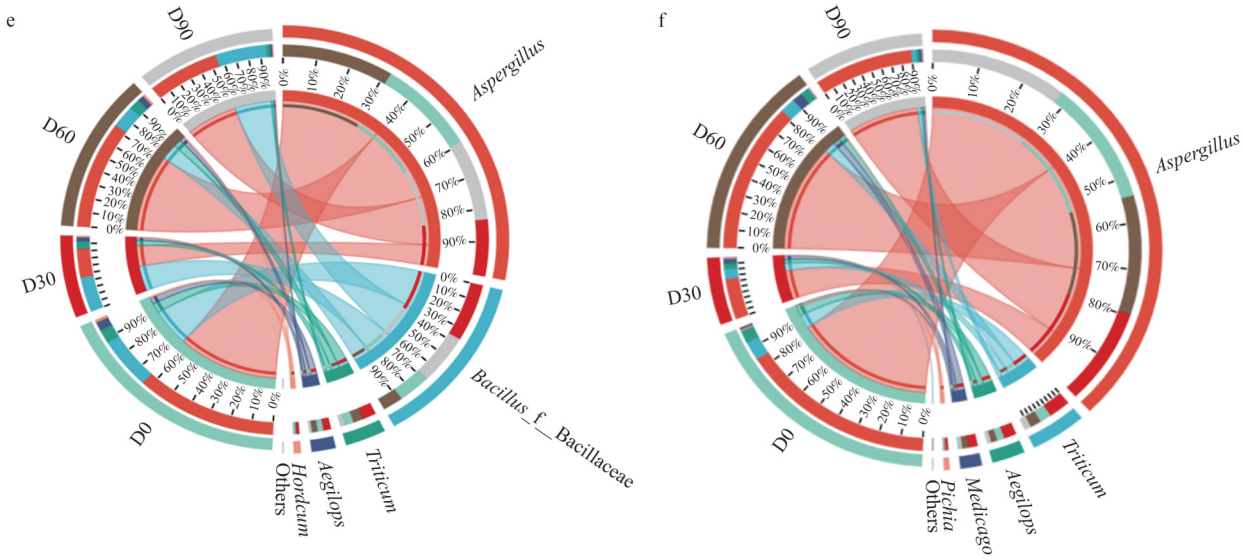

Fig. 5 (*Continued*)

genera of microorganisms that produced the majority of proteases. Previous studies have shown that the protease-producing microorganisms *Lactobacillus*, *Enterococcus*, *Weissella*, *Aspergillus*, and *Pichia* for PDBP fermentation process of the main microbial genera, they has a strong correlation with the flavor substance, contributing to the formation of PDBP flavor[10]. In addition, proteases produced by *A. oryzae* played an important role in the hydrolysis of soy sauce and peanut protein[42-43].

3.3 Correlation of FBBP umami peptides with proteases

To elucidate the association between microorganisms and umami peptides, the top 50 umami peptides in terms of abundance after screening were selected as the core umami peptides in the FBBP. Pearson correlation analyses of these umami peptides with the top 50 species of each enzyme-producing microorganism were performed, and correlation heat maps were plotted (Fig. 6). Significant

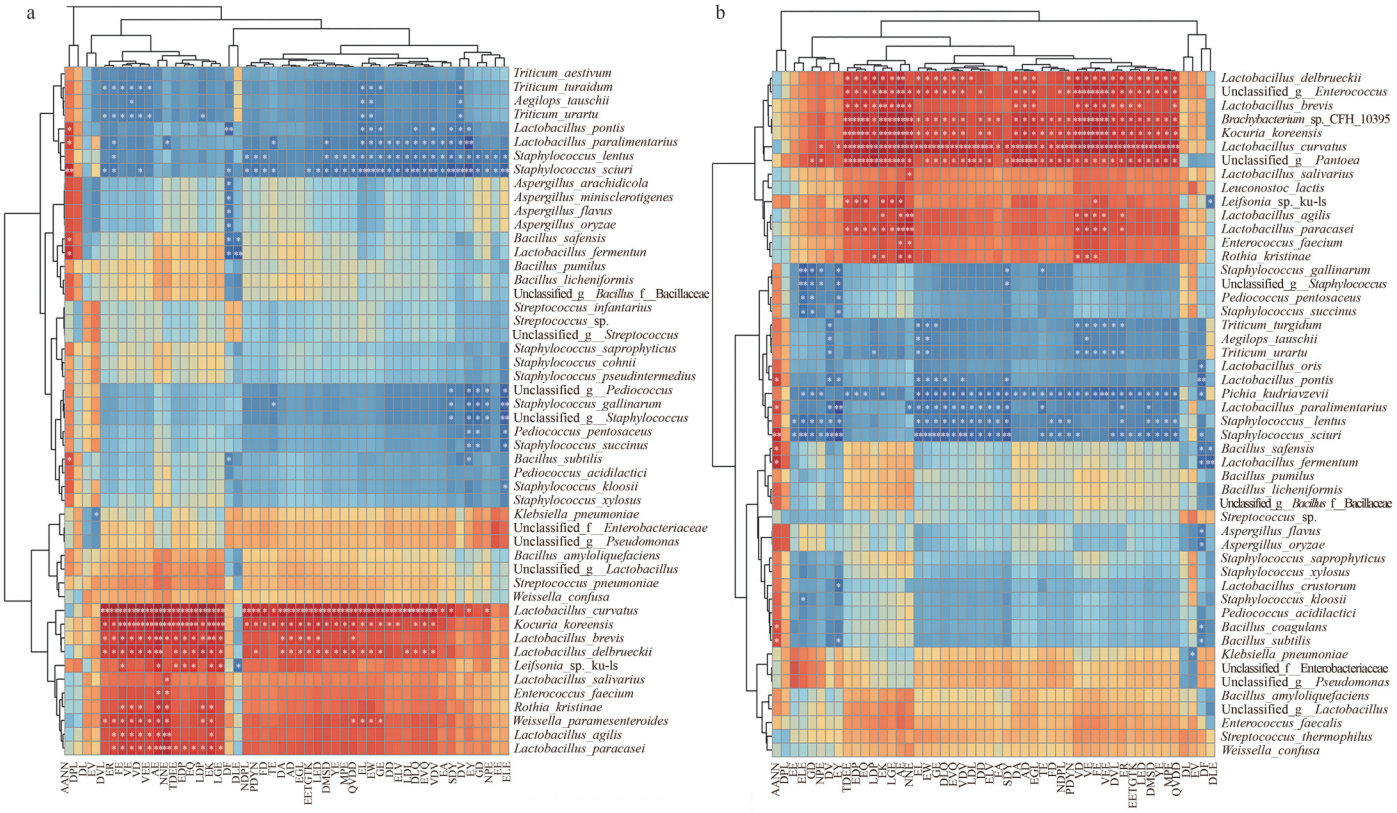

Fig. 6 Heat map of correlation clustering between core umami peptides and the top 50 species producing. (a) Aminopeptidase; (b) carboxypeptidase; (c) metalloendopeptidase; (d) serine endopeptidase; (e) aspartic endopeptidase; (f) cysteine endopeptidase.

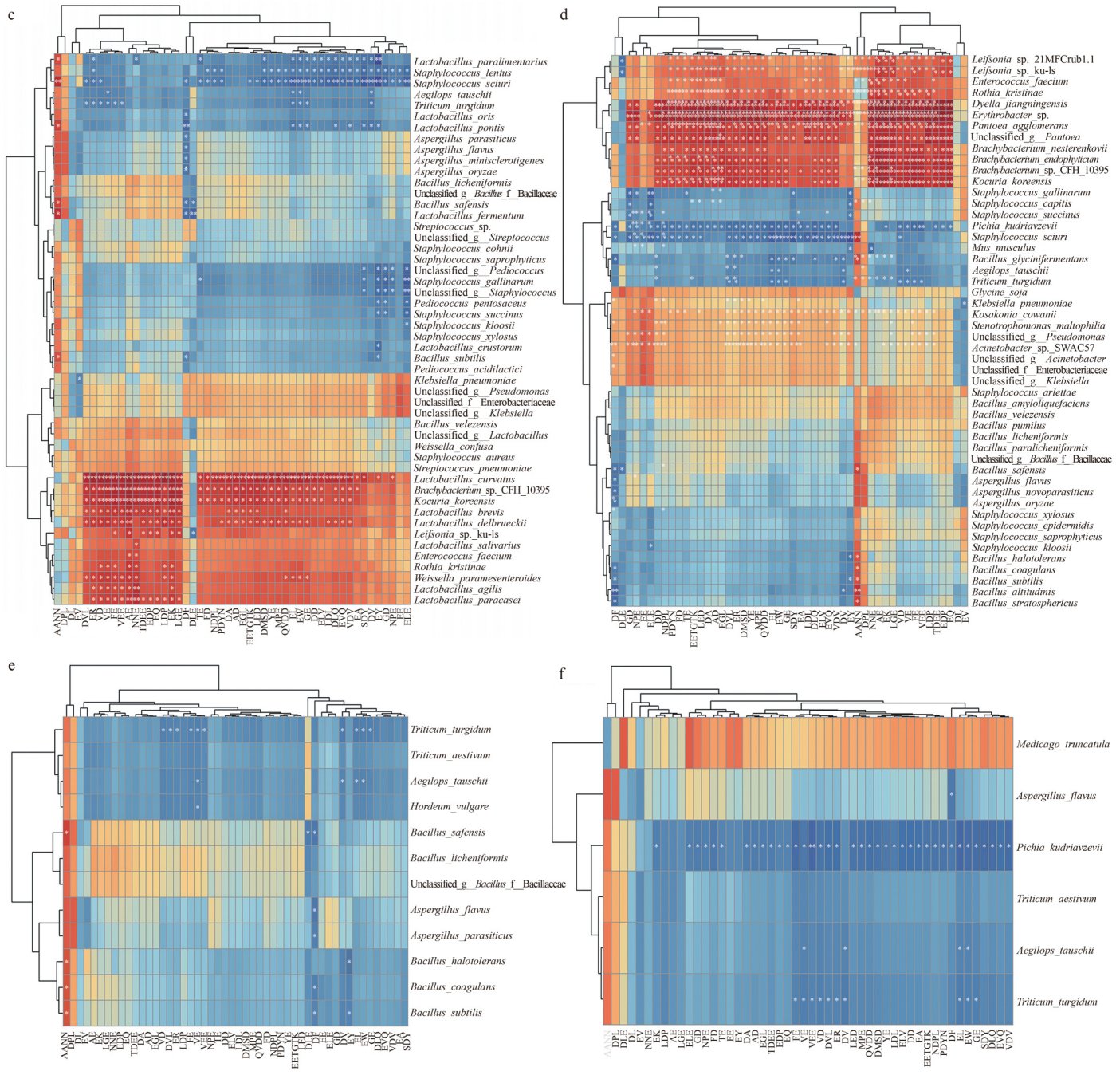

Fig. 6 (*Continued*)

correlations ($P < 0.05$ and $|r| > 0.7$) were identified and used to construct four correlation network graphs (Fig. 7), the correlation network diagram can show the relationships between microorganisms and umami peptides more visually. The size of each node indicates the abundance of the substance, and the strength of the correlation was indicated by the thickness of the line. For exopeptidases, the aminopeptidase-producing microorganisms *Lactobacillus curvatus*, *Kocuria koreensis*, *Lactobacillus paracasei*, *Lactobacillus delbrueckii*, *Lactobacillus brevis*, they were positively correlated with 34, 11, 2, 2, and 2 umami peptides, respectively. In contrast, *Lactobacillus agilis*, *Lactobacillus fermentum*, and *Staphylococcus sciuri* were all positively correlated with only one umami peptide (Fig. 7a). Carboxypeptidase-producing microorganisms *L. curvatus*, unclassified_g__*Pantoea*, *Brachybacterium*_sp._CFH_10395, *K. koreensis*, unclassified_g__*Enterococcus*, *L. paracasei*, *L. delbrueckii*, *L. brevis*, and *S. sciuri* were positively correlated with 34, 12, 11, 6, 2, 2, 2, 2, and 1 umami peptides, respectively (Fig. 7b). Metalloendopeptidase-producing microorganisms *L. curvatus*, *K. koreensis*, *Brachybacterium*_sp._CFH_10395, *L. brevis*, *L. delbrueckii*, *L. paracasei* were positively correlated with 34, 11, 11, 2, 2, and 2 umami peptides, respectively (Fig. 7c). Serine endopeptidase-producing microorganisms *Erythrobacter* sp., *Dyella jiangningensis*, unclassified_g__*Pantoea*, *Pantoea agglomerans*, *K. koreensis*, *Brachybacterium*_sp._CFH_10395, *Brachybacterium endophyticum*,

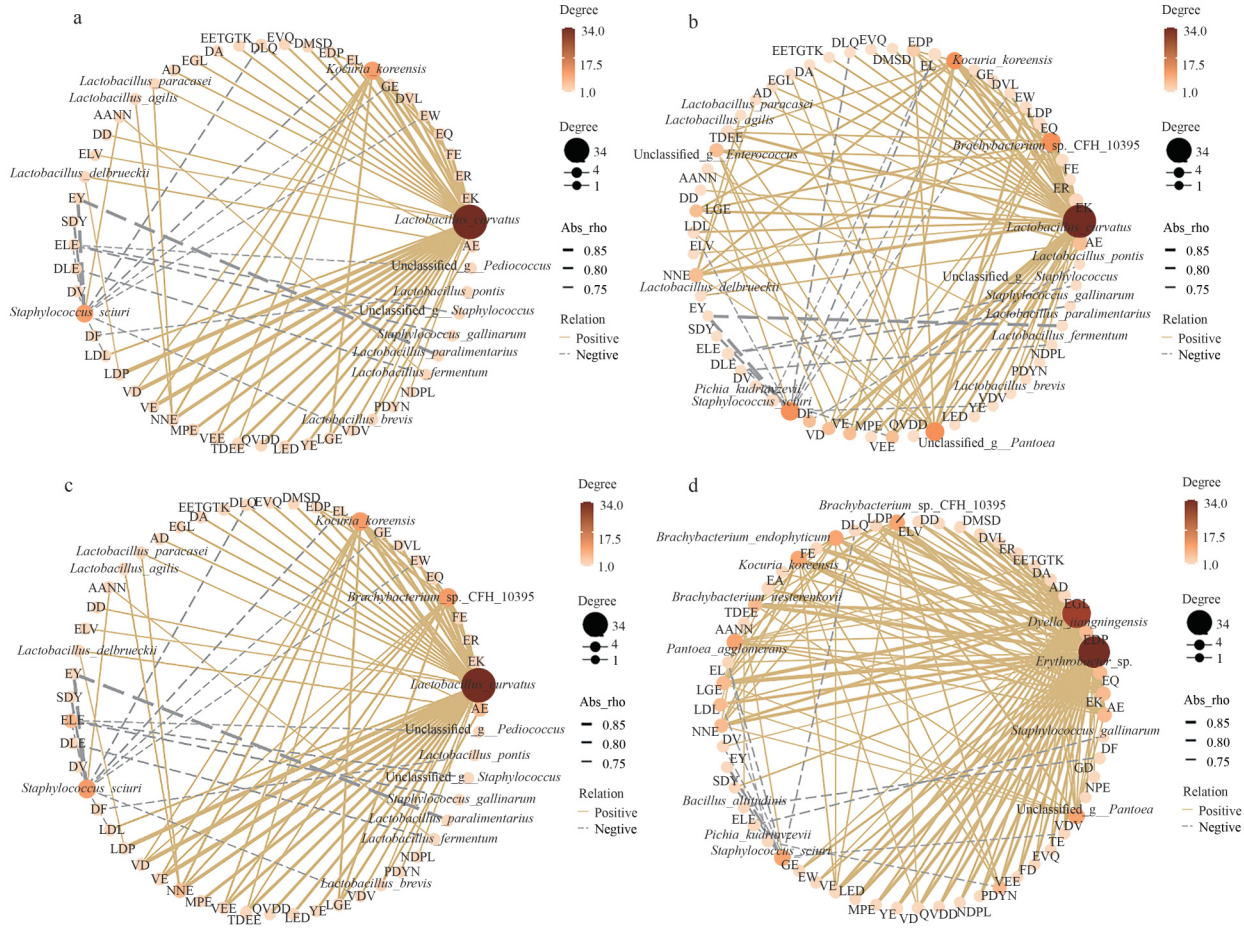

Fig. 7 Correlation network plot of core umami peptides with species producing. (a) Aminopeptidase; (b) carboxypeptidase; (c) metalloendopeptidase; (d) serine endopeptidase.

Brachybacterium nesterenkovii were positively correlated with 41, 35, 12, 12, 11, 11, 10, and 5 umami peptides respectively (Fig. 7d). In summary, we found that *K. koreensis* could produce all four of these proteases, this strain was found and isolated from traditional fermented seafood in Korea[44]. *L. curvatus*, *L. delbrueckii* can produce the above three proteases and *L. curvatus* had higher abundance among the protease-producing microorganisms, which was a dominant microorganism. The largest number of peptides were significantly and positively correlated with *Erythrobacter* sp., up to 41. A total of 24 protease-producing microorganisms were found to be closely associated with the umami peptides, among which *L. curvatus*, *D. jiangningensis*, *Erythrobacter* sp., unclassified_g__ *Pantoea*, and *P. agglomerans* were found to have a strong association with umami peptides, and they may play an important role in the hydrolytic preparation of the core umami peptides of FBBP. In addition, previous study has shown that *L. curvatus*, *K. koreensis*, and *L. delbrueckii* were also positively correlated with flavor compounds such as phenylethanol, phenylglyoxal, furfural, and various organic acids in FBBP[45]. Based on the relevance to umami peptides, *Lactobacillus*, *Staphylococcus*, *Brachybacterium* and *Kocuria* were the main genera that secrete various proteases, and their metabolism contributes to the formation of umami peptides in FBBP. Notably, there were too few microorganisms producing aspartic endopeptidases and cysteine endopeptidases were annotated, and there was no significant correlation between these microorganisms and umami peptides.

Previous study has shown that *Staphylococcus*, *Lactobacillus*, *Weissella*, *Pediococcus*, *Kocuria*, *Pantoea* etc. are the major microorganisms in FBBP[45]. *Lactobacillus*, *Weissella* and *Aspergillus* are also major microorganisms in PDBP[10]. In this study, it was also found that some of *Lactobacillus*, *Staphylococcus*, *Kocuria* and *Pantoea* had strong correlations with umami peptides. They played an important role in the hydrolytic preparation of core umami peptides. In addition, various proteases produced by *Brachybacterium*, *Dyella*, and *Erythrobacter* also contributed to the formation of umami peptides.

4. Conclusion

In this study, 377 peptides were preliminarily identified from fermented broad bean paste based on the proportion of umami amino acids D, E, N, and Q (≥ 30%) and molecular weight ≤ 1 000 Da. Virtual screening results revealed that 269 peptides were umami peptides, and the prediction results confirmed that the accuracy of the potential umami peptides screened by above approach was 71.35%. During the fermentation process, abundant protease genes have been identified mainly from 39 genera of microorganisms, among which aminopeptidase, carboxypeptidase, and serine endopeptidase were the major proteases in FBBP. *Staphylococcus*, *Lactobacillus*, *Weissella*, *Bacillus*_f__Bacillaceae, *Streptococcus*, *Kocuria*, *Klebsiella* and *Rothia*_f__Micrococcaceae can produce exopeptidases, and *Aegilops*, *Aspergillus*, and *Triticum* could produce endopeptidases. *Staphylococcus*,

Lactobacillus, *Aspergillus*, and *Weissella* were microbial genera that produced most proteases. The vast majority of them were also considered to be the main microbial genera in the fermentation process of PDBP, which also had a strong correlation with the formation of flavor substances. Core umami peptides interaction plots with protease-producing microorganisms at the species level showed that umami peptides were strongly associated with 24 protease-producing species. *K. koreensis* produced exopeptidases and some endopeptidases. *L. curvatus*, *D. jiangningensis*, *Erythrobacter* sp., and unclassified_g__*Pantoea* were strongly associated with umami peptides. They may play an important role in the hydrolysis preparation of FBBP core umami peptides. The results provide information on the major proteases and protease-producing microorganisms involved in the formation of FBBP umami peptides and help to reveal the formation mechanism of umami peptides, which can contribute to the improvement of the umami taste of PDBP's fermentation process. In this paper, the umami peptides identified by peptidomics and virtual screening will be further studied by sensory evaluation and molecular docking, so as to further verify their umami intensity and reveal their umami mechanism.

Conflict of interest

There are no conflicts to declare.

Acknowledgements

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