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Analysis of protein components in blackberry wine and haze

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ABSTRACT

Wine haze seriously influences customers' purchasing decisions. Protein is an influencing factor in wine haze formation. In this study, the differentially expressed proteins (DEPs) in fresh blackberry wine (FBBW) and aged blackberry wine (ABBW) were analyzed by label-free quantitative proteomics, and the proteins in BBWH were identified by high-performance liquid chromatography-tandem mass spectrometry. A total of 178 proteins were confirmed in the FBBW and ABBW. Among them, 125 proteins were differentially expressed, while the other 53 proteins were not. Among the DEPs, 49 were from *Saccharomyces cerevisiae*, 37 were from *Aspergillus niger* and other microorganisms, and 23 were from the blackberries themselves. The clustering analysis of DEPs showed that most of the protein-forming haze with polyphenols in blackberry wine (BBW) came from *Saccharomyces cerevisiae*, mold, and blackberry raw materials. This is different from other studies on the source of protein in wine haze. This study is the first to investigate the composition and changes in protein in BBW and BBWH. The results will provide valuable information for solving BBWH caused by proteins.

1. Introduction

Blackberry is a small berry of the genus *Rubus* originating in tropical America (Oviedo-Arbeláez et al., 2018). Blackberry has excellent nutritional and health care characteristics, high economic benefits, and excellent varieties, which together have promoted the significant growth of the blackberry industry (Raseira & Franzon, 2012; Lin&Agehara, 2020). The annual commercial production of blackberries worldwide is approximately 154578 tons. The main production areas are North America, Europe, and Asia (Kaume et al., 2012). In the United States, blackberry is the fourth most economically important small fruit crop, with retail sales of US $697 million in 2019 (Lin & Agehara, 2021).

After picking, the physical, chemical and nutritional characteristics of blackberries have been found to change rapidly, and pathogens also grow rapidly (Liu et al., 2019). They must be washed and frozen within 4 h of picking; otherwise, they will ferment and deteriorate (Silva et al., 2020).

Processing of fresh blackberries can reduce this loss and reduce the energy consumption caused by freezing. Blackberry wine (BBW) is one of the most common processed blackberry products.

Traditionally, blackberry wine has been used as a popular drug for the adjuvant treatment of some diseases in Croatia (AmidžicKlaric, Klaric, Mornar, Velic, & Velic, 2015; Tomić et al., 2018). Over the past decade, its sales growth has been slow and its market share remains low (Klarić et al., 2016). However, due to its attractive red color, excellent antioxidant activity and preventive effect on chronic diseases, BBW has attracted increasing attention from researchers in many countries (Joh, 2014; Gao et al., 2012; Ortiz et al., 2013; Demetrashvili et al., 2021).

BBW, like wine, produces haze during aging. This has greatly affected consumers' purchasing decisions. In the process of winemaking, polyphenols interact with proteins through covalent and noncovalent bonding to produce invisible polymers, which form larger visible particles and gather at the bottom of the bottle (Charlton et al., 2002; McRae et al., 2018; Di Gaspero et al., 2020). Compounds including phenols interact with proteins to form haze in the wine, such as tannins, ellagic acid, caffeic acid, sulfate, ellagic acid and tartrate (Charlton et al., 2002; Lasanta & Gómez, 2012; McRae et al., 2018; Sommer et al., 2019).

Studies have shown that haze protein in wine mainly comes from insoluble protein in grape berries (Luguera et al., 1998; waters et al., 2005). These proteins are usually accumulated pathogenesis-related proteins (PRs) evolved by plants to resist fungal infection, including chitinases (PR-3 family) and TLPs (PR-5 family) (Van Sluyter et al., 2015).

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Blackberries (*Rubus* L.) and grapes (*Vitis* L.) do not belong to the same genera. There are differences in the composition of proteins and phenolic compounds in their fruits. The protein content of BBW is 1.1–1.4 g/L, which is much higher than that of wine (0.021–0.7 g/L) (Salazar et al., 2017; Tabilo-Munizaga et al., 2014; Kambiranda et al., 2016), and several times higher than that of cider, mulberry wine, strawberry wine and pear wine (Yuan & Zhao, 2017; Nwe & Nyein, 2019). To the best of our knowledge, the protein types in BBW and BBWH have not yet been identified and analyzed (Supplementary Fig. 1).

Herein, we explore the types and characteristics of FBBW, ABBW and BBWH proteins. This study involves basic research to analyze protein factors which caused the haze of blackberry wine and provide a reference method for analyzing precipitation protein factors of other fruit wine.

2. Materials and methods

2.1. Materials and chemicals

Acetone, sodium hydroxide, hydrochloric acid, gallic acid, and sodium carbonate were of analytical grade. Methanol, acetonitrile, ethanol, and formic acid were HPLC grade. Sucrose and potassium pyrosulfite were food grade. Folin-Ciocalteu's phenol reagent was purchased from Shanghai Macklin Biochemical Co.,Ltd (Shanghai, China). BCA reagent purchased from Nanjing Jiancheng Bioengineering Institute (JiangShu, China). An Ultrafiltration tube (Millipore, Germany), and *Saccharomyces cerevisiae* (LAlVIN DV10, Lallemand) were used. The water used to prepare all samples and standards was purified using the Synergetic system (Merck Millipore, Germany).

2.2. Extraction of proteins and peptides from BBWH

2.2.1. Winemaking method for BWW

Frozen blackberry fruits were purchased from Zhongliang Food industry (Nanjing, China) and stored at - 20 °C until use. Before the experiment, the frozen blackberries were thawed overnight at 20–25 °C. The winemaking of BBW was slightly modified according to Liu's method (Liu et al., 2017). Briefly, the blackberry fruits (5 kg) were crushed with a food processing machine (Midea, China), and then were moved into a stainless-steel barrel (7.5 L) with 500 mg of $K_2S_2O_5$ and 1 mL of pectinase. Sucrose was added to adjust the sugar content (24° Brix), which was measured by a hand-held refractometer (WZS-50, Shanghai Yidian). Yeast (0.2% w/v) was used as a starter. The wine was sealed and ferment at 20–25 °C. In the first 10 days of fermentation, the wine was stirred once a day. The precipitate and skins were removed on Day 15. The wine was then centrifuged at 5000 r/min for 20 min to remove residues. The BBW was aged in brown bottles (750 mL), which were sealed with corks and incubated for 14 months (20 °C). The same batch of raw materials was stored at −20 °C, and the FBBW was brewed after the previously made BBW was aged for 13 months (ABBW).

2.2.2. Collection haze from ABBW

ABBW (750 mL), was centrifuged at 5000 r/min for 10 min. The collected sediment was shaken and mixed with 25 mL of pure water and centrifuged for 10 min at 5000 r/min; this process was performed thrice. The haze was freeze-dried at −50 °C using an FDU-1200 freeze dryer (Tokyo, Japan), weighed, sealed, and stored at −4 °C.

2.2.3. Extraction of proteins and peptides from BBWH

The freeze-dried BBW haze was dissolved in 0.1 mol NaOH (1/1000 [w/v]) and incubated in a THZ-C-1 shaker (120 r/min, 20 min) (Tai-Cang, China). After centrifugation (10,000 r/min, 10 min), 4 mL of the supernatant was added to 40 mL of precooled acetone (−20 °C, 2 h), and the protein was precipitated overnight at −20 °C. The haze was collected after centrifugation (10,000 r/min, 10 min) and redissolved in 2 mL of pure water for further experiments.

2.3. Amino acid analysis

Next, 100 mL BBWH was added to 10 mL of 6.0 mol/L hydrochloric acid solution, and hydrolyzed at 95 °C for 14 h. After cooling to room temperature, the volume was 50 mL with water. Then, 2.0 mL of the filtrate was transferred to a 15 mL test tube and dried under reduced pressure. After dissolving with 2.0 mL of sodium citrate buffer solution (pH = 2.2) the solution was filtered with a 0.22 μm filter membrane, and the amino acid composition of the sample was analyzed by LA-8080 automatic amino acid analyzer (Hitachi, Japan).

ABBW and FBBW (50 mL) were concentrated by the nitrogen blowing method at 1:10. The concentrated solution was fixed to a volume of 5 mL and filtered with a 0.22 μm membrane. An LA-8080 automatic amino acid analyzer (Hitachi, Japan) was used to analyze the amino acid composition of the sample according to the method reported by Marcy, Carroll, and Young (1981) et al.

2.4. Identification of proteins from FBBW and ABBW

ABBW and FBBW (50 mL) were added to 100 mL SDT lysis buffer (4% SDS, 100 mM DTT, 100 mM Tris-HCl pH 8.0). Samples were boiled for 5 min and further ultrasonicated. Undissolved cellular debris were removed by centrifugation at 10,000×*g* for 15 min.

Protein (200 μL for each sample) digestion was performed with FASP method described by Wisniewski, Zougman, Nagaraj, and Mann (2009) et al. Briefly, the detergent, DTT and IAA in UA buffer were added to block reduced cysteine. Finally, the protein suspension was digested with trypsin (Promega) at ratio 50:1 overnight at 37 °C. The peptide was collected by centrifugation at 16,000 g for 15 min and content in the supernatant was quantified by Bradford method (Murphey et al., 1989).

The peptide was desalted with C18 StageTip for further LC–MS analysis using a Q-Exactive Plus mass spectrometer coupled with LC (Easy-nLC 1200, Thermo Fisher Scientific). The peptide was first loaded onto a trap column (100 μm × 20 mm, 5 μm, C18, Dr.Maisch GmbH, Ammerbuch, Germany). Reverse-phase high-performance liquid chromatography (RP-HPLC) separation was performed with the EASY-nLC system (Thermo Fisher Scientific, Bremen, Germany) using a self-packed column (75 μm × 150 mm; 3 μm ReproSil-Pur C18 beads, 120 Å, Dr. Maisch GmbH, Ammerbuch, Germany). Mobile phase A (H_2O mixture of 0.1% formic acid) and mobile phase B (acetonitrile mixture of 0.1% formic acid) were used to recover peptides for nLC-MSMS analysis. MS data were acquired using a data-dependent top 20 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for HCD fragmentation.

The MS data were analyzed using MaxQuant software (version 1.6.0.16) and searched against the fungi, bacteria, and Rosaceae database of UniProtKB/Swiss-Prot (36080 total entries, downloaded 08/14/2021). The trypsin was selected as proteincleaving enzyme, peptide tolerance was 10 ppm. Two missedcleavage was allowed and MS⁄MS tolerance was 0.02 Da. Carbamidomethylation of cysteines was defined as fixed modification, while acetylation of protein N-terminal, oxidation of Methionine were set as variable modifications for database searching. The database search results were filtered and exported with <1% false discovery rate (FDR) at peptide-spectrum-matched level, and protein level, respectively.

2.5. Identification of BBWH proteins

The molecular weights of the proteins were fractionated by an SDS–PAGE discontinuous system as described by Sadeghi-Nejad, Loridan, and Senior (1970). Then, 30 μL of each sample was placed into 30 μl SDS–PAGE sample buffer. After 30 min of mixing, the samples were denatured at 95 °C for 5 min, and then centrifuged at 10,000×*g* for 2 min. A 20 μL aliquot of each protein sample was then loaded into the

sample well. The samples and marker were applied at the top of the gel; both gels were polyacrylamide gels; and the top gel was a stacking gel. The gels were kept at a constant current of 90 mA until the marker dye reached the bottom of the gel. Protein fixation and staining were completed simultaneously using a solution of Coomassie brilliant blue. The gel destaining was accomplished by using 300 mL/L ethanol and 70 mL/L acetic acid solution.

The BBWH protein band at 30 kDa was cleaved and sent to a biology company for protein and peptide identification (Luming, China). In short, BBWH was enzymatically hydrolyzed and desalted. A Nano HPLC liquid phase system EASY-nLC1200 was equipped with a trap column (100 μm × 20 mm, RP-C18, Thermo Inc). The sample was adsorbed on the trap column, and then through the analysis column with 75 μm × 150 mm (RP-C18, Thermo Inc.) column separation.

The samples were further separated by capillary high performance liquid chromatography and analyzed by a Q-Exactive mass spectrometer (Thermo Scientific).

The data were analyzed by ProteomeDiscover 2.4 software processing, searching the library of raspberry, Rosaceae, and fungi (36080 total entries, downloaded 09/27/2021). The search parameters were set to enzyme: Trypsin, maximum missed cleavages = 2, MS/MS tolerance: 0.02 Da, main search peptide tolerance:10 ppm, fixed modifications: carbamidomethylation (C), variable modifications: oxidation(M), deamidation(N, Q).Proteins were only considered to be positively matched if they passed the scoring ($p \leq 0.05$).

2.6. Analysis of peptides using MS

Agilent Technologies 1260 Infinity II Ultra Performance Liquid Chromatography tandem mass spectrometry (Agilent Technologies 6420 Triple Quad LC/MS) was used to analyze small molecular peptides of BBWH. Mobile phase A was 0.1% formic acid aqueous solution, mobile phase B was acetonitrile solution, the ratio was 50%:50%, the flow rate was 0.200 mL/min, the electrospray ionization source (ESI) was used, the positive and negative ion modes were scanned, the electrospray capillary voltage was 4000 V, the acquisition mode was dynamic multireaction monitoring mode, the drying gas temperature was 350°, and the injection volume was 2 μL.

2.7. Detection of proteins by Fourier transform infrared (FTIR) spectroscopy

Acetone (twice the sample volume) was used for FBBW and ABBW. After removing pectin from blackberry juice with ethanol (Jiang et al., 2021), acetone (twice the sample volume), was added, incubated at 4 °C for 30 min, and centrifuged at 10000 r/min. The precipitate was dissolved with distilled water, freeze dried (−50 °C), and stored at 4 °C. The FTIR spectra in transmission mode were recorded using a Nicolet iS50 FTIR spectrometer (Thermo, USA) equipped with an MCT detector. Before Fourier transform, 32 scans were averaged with the water spectrum as the background. The spectral resolution was set to 4 cm^{-1} and the infrared spectrum was scanned.

2.8. Statistical analysis

Statistical analysis of analytical data was conducted on all samples using SPSS Statistics 20 Statistical Software (IBM, USA). One-way ANOVA in order to test for significant differences. Statistical significance was considered $p < 0.05$. All measurements were performed three times, and the results represent the mean ± standard deviation of parallel measurements.

3. Results and discussion

3.1. Analysis of the amino acid composition

The contents of the hydrolyzed amino acids of blackberry juice, FBBW, ABBW and BBWH are shown in Table 1.

Aspartate (Asp), alanine (Ala) and glutamate (Glu) were the main amino acids in blackberry juice, which were present at 22.31%, 20.51% and 15.27% respectively. They accounted for 12.91%, 14.32% and 14.56% of the total amino acids in FBBW, respectively. The proportion of amino acids in BBWH was closer to that of FBBW, which was 11.62%, 5.75% and 13.68%, respectively. Some studies have reported that proline (Pro) is an important amino acid for the formation of wine haze (Murray et al., 1994; Perutka et al., 2019; Dabalos et al., 2019). However, in this study, Pro in blackberry juice, FBBW, and BBWH accounted for only 2.85,5.08, and 3.92% of the total amino acid content, respectively. It accounted for 8.24% in ABBW, which was higher than that of blackberry juice and FBBW.

Pro accounted for 2.85% of the amino acid content in blackberry juice, 5.08% in FBBW and 3.92% in BBWH. It accounted for 8.24% of the ABBW, which was higher than that of blackberry juice and FBBW. Therefore, whether the protein (peptides) is rich in Pro has no strong correlation with the formation of BBWH. It should be noted that the amino acid content of other basic amino acids decreased notably after aging, which was related to the nutrient metabolism environment consumption in BBW (Martinez-Rodriguez et al., 2001).

3.2. Identification of FBBW and ABBW proteins

After label-free quantitative analysis, 503 peptides belonging to 178 proteins were identified in FBBW and ABBW.

The difference in proteins in the FBBW and ABBW groups is shown in Fig. 1.

The content of 19 DEPs in ABBW was higher than that in FBBW (Fig. 1). This shows that these DEPs can exist in wine with polyphenols for a long time. Therefore, these proteins are not the main proteins that constitute BBWH.

The content of 73 DEPs in FBBW was higher than that in ABBW. The content of these proteins decreased during aging. Some might have been degraded due to autolysis, while others might have formed haze with polyphenols in the wine. Among them, 34 proteins were from *Saccharomyces cerevisiae*, and 35 proteins were from molds (including 22

Table 1
Proportion of different amino acids of different samples.

Amino acid	Juice (%)	Fresh wine (%)	Aged wine (%)	Haze (%)
Glu	15.27 ± 1.19 b	14.56 ± 1.11 b	20.31 ± 1.32c	13.68 ± 1.17a
Asp	22.31 ± 2.15 b	12.91 ± 2.23 d	18.85 ± 3.28c	11.62 ± 2.11a
Lys	3.31 ± 0.02a	7.47 ± 0.9c	2.26 ± 0.01a	8.52 ± 0.03c
Leu	3.13 ± 0.09 b	4.79 ± 0.04c	2.42 ± 0.00a	5.94 ± 0.02 d
Val	3.52 ± 0.06 b	5.28 ± 0.3c	2.51 ± 0.01a	6.16 ± 0.03 d
Thr	3.27 ± 0.08 b	4.45 ± 0.03c	2.46 ± 0.01a	5.81 ± 0.02 d
Ala	20.51 ± 1.74c	14.32 ± 1.89 b	26.79 ± 2.34 d	5.75 ± 0.22a
Ile	2.72 ± 0.32 b	4.78 ± 0.05c	1.46 ± 0.00a	6.47 ± 0.03 d
Ser	5.24 ± 0.03 b	5.29 ± 0.03 b	3.68 ± 0.03a	5.48 ± 0.02 b
Gly	2.58 ± 0.02a	5.24 ± 0.03 b	2.88 ± 0.02a	5.35 ± 0.02 b
Phe	2.17 ± 0.01 b	3.61 ± 0.01c	1.25 ± 0.01a	5.21 ± 0.01 d
Arg	5.23 ± 0.08c	3.7 ± 0.02 b	1.03 ± 0.00a	5.15 ± 0.01c
Tyr	1.22 ± 0.01a	2.68 ± 0.01c	1.43 ± 0.01 b	3.88 ± 0.01 d
Pro	2.85 ± 0.02a	5.08 ± 0.27a	8.24 ± 0.07 b	3.92 ± 0.01a
His	1.27 ± 0.10 b	2.01 ± 0.02c	0.57 ± 0.00a	2.61 ± 0.01 d
Met	0.27 ± 0.02a	0.38 ± 0.00 b	0.22 ± 0.00a	1.45 ± 0.00c
Cys	0.1 ± 0.00a	0.46 ± 0.00a	0.24 ± 0.00a	0.23 ± 0.00a
NH_3	5.03 ± 0.02 d	2.98 ± 0.02 b	4.06 ± 0.02c	2.53 ± 0.01a

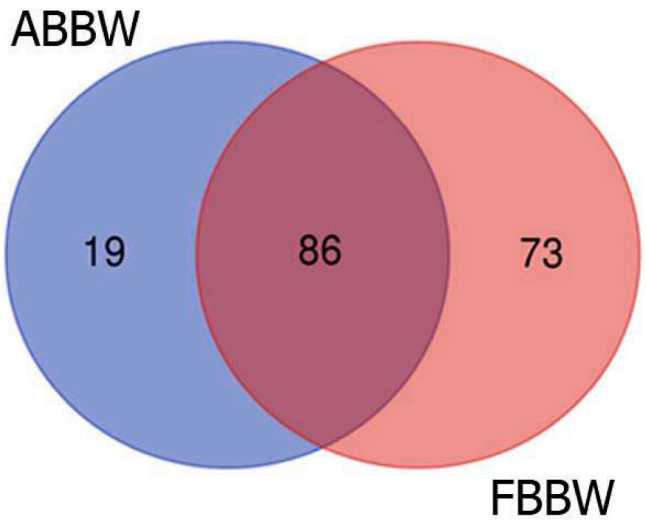

Fig. 1. Venn diagram of DEPs from FBBW and ABBW.

species from *Aspergillus niger*). Twenty-two proteins were from blackberry fruit (Supplementary Table 1).

Among these DEPs, 31 completely disappeared during aging. Among them, 6 belong to blackberry fruit, 8 belong to *Saccharomyces cerevisiae* and 8 belong to molds (5 from *Aspergillus niger*).

Protein content changes during aging, some of which are degraded due to autolysis, while others may form haze with phenolic compounds.

Waters and Vincenzi believe that TLP and chitinase are the main proteins in wine haze (Waters et al., 1995; Vincenzi et al., 2011). In this study, 80.5% of chitinase disappeared during aging, which is consistent with their research. However, TLP did not decrease in this experiment.

It was concluded that the protein composition of BBW was very different from that of grape wine. The main protein causing BBW haze is not TLP.

3.2.1. Analysis of DEPs in FBBW and ABBW

To understand their sources and functions more clearly, enrichment analysis of the DEPs in the FBBW and ABBW was carried out.

There were 125 DEPs and 53 non-DEPs. The non-DEP coexisted with phenolic compounds in the wine during the aging process of more than one year, indicating that they had weak interactions with phenolic compounds. Therefore, they are not the main proteins forming BBW precipitation. In this experiment, there was no difference in 53 proteins, of which 48 were from microorganisms and 5 were from plants (see Supplementary Table 1).

Protein clustering analysis of FBBW and ABBW was performed and significance analysis was conducted through different protein subsets, such as functions, biological pathways, and adjacent positions in the pathway.

Among the top 20 DEPs (Table 2) with the most significant reduction, 2 proteins were from plants, 4 were from *Saccharomyces cerevisiae* and 11 were from molds (among which 5 were from *Aspergillus niger*).

Some studies suggest that the interaction force between yeast and tannin is much stronger than that of the purified cell wall (Mekoue Nguela et al., 2016). Polyphenols can also interact with bacterial proteins (such as transporters and cell wall peptides) through covalent and noncovalent bonds (hydrogen bond, hydrophobic interactions, van der Waals forces) (Silva et al., 2021).

In this study, the protein involved in the haze formation of fruit wine comes not only from fruits but also from fermentation microorganisms to a greater extent. In other words, a variety of proteins are involved in the formation of fruit wine haze.

3.2.2. Functional annotation and classification of differential proteomes

Gene ontology (GO) analysis of DEPs includes three aspects: biological process (BP), molecular function (MF) and cellular component (CC), which reflects the role of proteins in multiple processes.

According to BP classification, the four most important protein processes in FBBW and ABBW are cell wall organization, external encapsulating structure organization, cell wall organization or biogenesis, and the carbohydrate metabolic process (Fig. 2). According to the CC classification, the top five cellular components with the most significant enrichment were cell periphery, extracellular region, external encapsulating structure, cell wall and fungal-type cell wall. According to MF classification, oxidoreductase activity, hydrolase activity, hydrolyzing *O*-glycosyl compounds, hydrolase activity, acting on glycosyl bonds and glucosidase activity were the most enriched.

According to these enrichment results, proteins related to cell degradation decreased significantly during aging. The difference between oxidoreductase and hydrolase in BBW should be due to the autolysis of protein. Among the DEPs, 5 proteins were pectin lyase, and 21 proteins were galactosidase. These proteins usually decompose

Table 2
Top 20 DEPs of FBBW and ABBW.

	Accession	Protein Name	*Source*	Coverage [%]	MW [kDa]	Abundance		
						202001	202104	D-value
1	Q05091	Polygalacturonase inhibitor	*Pyrus communis*	6	36.5	9513.3	9.01 E+09	9.01 E+09
2	Q12370	Seripauperin-17	*Saccharomyces cerevisiae*	11	13.1	9513.3	5.67 E+09	5.67 E+09
3	Q12708	Endo-polygalacturonase	*Sclerotinia sclerotiorum*	4	37.9	9513.3	5.59 E+09	5.59 E+09
4	A2RAY7	Putative galacturan 1,4-alpha-galacturonidase C	*Aspergillus niger*	33	45.9	1346501	4.7 E+09	4.7 E+09
5	Q9Y7F8	Probable arabinogalactan endo-beta-1,4-galactanase A	*Aspergillus tubingensis*	24	38.7	1444249	1.89 E+09	1.89 E+09
6	A2QK82	Probable pectinesterase A	*Aspergillus niger*	24	34.6	7995678	7.57 E+08	7.49 E+08
7	P19791	Ribonuclease M	*Aspergillus phoenicis*	42	26.6	5043797	6.52 E+08	6.47 E+08
8	A2QBB6	Probable endopolygalacturonase E	*Aspergillus niger*	24	39.6	1.34 E+08	5.78 E+08	5.55 E+08
9	Q8NK89	Alpha-L-arabinofuranosidase B	*Aspergillus kawachii*	33	52.6	22501503	5.09 E+08	5.08 E+08
10	P34755	3-phytase B	*Aspergillus awamori*	21	52.6	309686.3	5.89 E+08	4.55 E+08
11	P32939	Ypt/Rab-type GTPase YPT7	*Saccharomyces cerevisiae*	10	23	9513.3	3.53 E+08	3.53 E+08
12	P15703	Glucan 1,3-beta-glucosidase	*Saccharomyces cerevisiae*	23	34.1	12532680	3.41 E+08	3.28 E+08
13	Q4AEG8	Exo-1,4-beta-xylosidase xlnD	*Aspergillus awamori*	17	87.3	532541.7	2.36 E+08	2.36 E+08
14	P09435	Heat shock protein SSA3	*Saccharomyces cerevisiae*	9	70.5	1892937	2.26 E+08	2.24 E+08
15	O13287	6-phosphogluconate dehydrogenase, decarboxylating	*Candida albicans*	2	56.9	289792	2.04 E+08	2.04 E+08
16	B3PNH6	Enolase	*Mycoplasma arthritidis*	3	50.4	9513.3	1.39 E+08	1.39 E+08
17	D0E0C7	Major strawberry allergen Fra a 1-3	*Fragaria ananassa*	14	17.4	176623.5	1.27 E+08	1.27 E+08
18	A2R0Z6	Probable feruloyl esterase B	*Aspergillus niger*	10	57.2	9513.3	1.26 E+08	1.26 E+08
19	Q0CVX4	Probable alpha-galactosidase D	*Aspergillus terreus*	3	71.1	9513.3	1.11 E+08	1.11 E+08
20	A2QHG0	Probable exopolygalacturonase B	*Aspergillus niger*	7	48.4	322795	95708549	95385754

The data is the mean, n = 3.

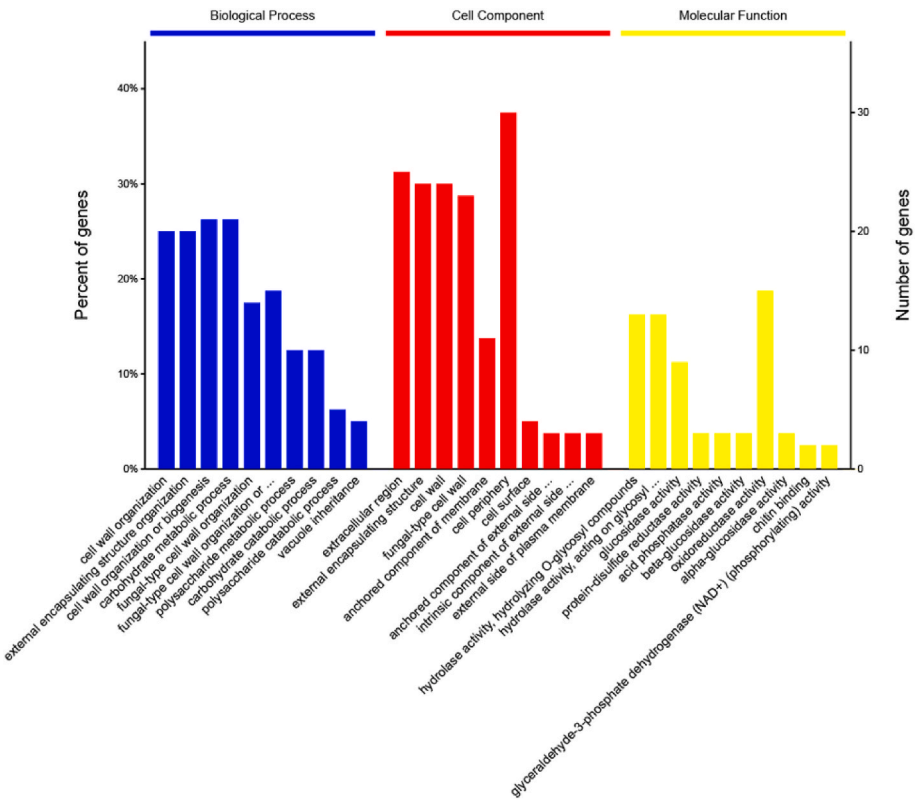

Fig. 2. GO annotation of DEPs from FBBW and ABBW.

pectin and improve the quality of wine (Romero-Cascales et al., 2012). They are also involved in the breakdown of plant cell walls. However, most of these enzymes come from microorganisms. In addition, 11 DEPs were identified as glucanases, 2 as mannosidases and 1 as chitinases. In the late stage of fermentation, the lack of a nitrogen source in the wine leads to the autolysis of *Saccharomyces cerevisiae* and *Aspergillus niger* (Charpentier et al., 2004).

3.2.3. KEGG pathway enrichment analysis and functional annotation of DEPs from FBBW and ABBW

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway can directly describe the interaction and response of metabolism, genetic information processing, environmental information processing, cellular processes, and tissue systems (Kanehisa et al., 2012).

The KEGG pathway of DEPs (Fig. 3, Supplementary Table 2), showed that the DEPs were mainly involved in the Biosynthesis of antibiotics, metabolic pathways, and starch and sucrose metabolism. The number of proteins involved in the biosynthesis of antibiotics was the largest. This was attributed to the synthesis of plant flavonoids and the metabolism of yeast flavonoids (sun et al., 2015).

Among the 10 KEGG signaling pathways with the most significant *p*-values, the following five pathways were most significant: starch and sucrose metabolism, glycolysis/gluconeogenesis, carbon metabolism, galactose metabolism and biosynthesis of antibiotics. These pathways are usually related to the fermentation of BBW.

3.2.4. Action network analysis of DEPs

Analyzing the interaction between proteins and the network formed by functional interactions is of great significance to reveal the function of proteins. For example, highly aggregated proteins have the same or similar functions; proteins with high connectivity are considered to be the key points affecting the metabolism or signal transduction pathway of the whole system. Through analysis, we found that the interaction networks involved in the DEPs of FBBW and ABBW were mainly concentrated in: glycolysis/gluconeogenesis, biosynthesis of antibiotics, carbon metabolism, metabolic pathways, protein processing in the endoplasmic reticulum, starch and sucrose metabolism (Supplementary Fig. 3).

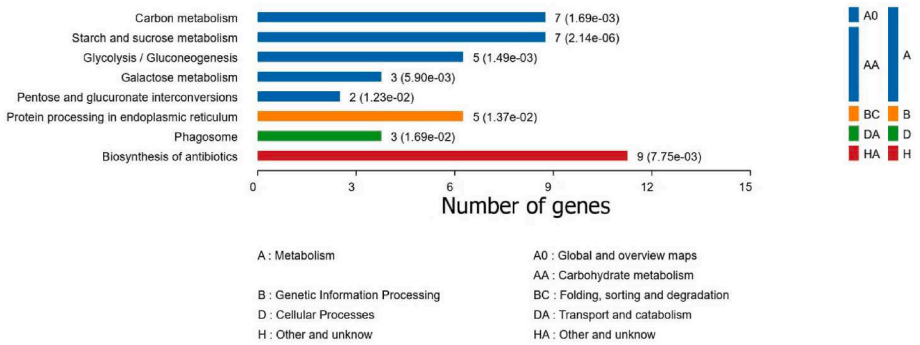

Fig. 3. Number of proteins involved in KEGG main pathway of DEPs from FBBW and ABBW.

The protein-protein interaction network (PPI) showed that the upregulated pathways were mainly related to antibiotic synthesis, carbon metabolism, glucose metabolism (Supplementary Table 3).

3.3. Identification of proteins in BBWH

SDS–PAGE was used to obtain bands with molecular weights between 14.4 kDa and 116 kDa for proteins in blackberry juice, FBBW, ABBW and BBWH (see Fig. 4).

At the bottom of the gel, there was a band below 14 kDa belonging to polypeptides. An observable band (30 kDa) was found on the BBWH lane in gel. A similar band was also observed in the lanes of blackberry juice, FBBW and ABBW. The protein band at approximately 35 kDa of BBWH was detected by HPLC–MS/MS after enzymolysis (Table 3).

In BBWH, 10 fruits, 20 yeast proteins, and 15 other bacterial proteins were identified. Among them, A0A2P6P1V1, M5W5K7, and A0A2P6R5D3 are noncharacteristic proteins. Furthermore, enzymes, such as A0A2P6Q3W0, A0A2P6SI73, and A0A314YGY8 were identified as polyphenol oxidase, glucan-1,3-β-D-glucosidase, and sucrose α-glucosidase, respectively.

A variety of oxidases were identified in BBWH protein. P00560, P00360, and P12709 are glycometabolism-related proteins; Q12083, P38788, P05750, and other proteins are component proteins of the yeast ribosome; P25334 is a polyphenol-oxidase-related protein; P11353 is an oxidase-related protein; P34227 is a peroxidase-related protein; and P22803 is a protein involved in redox reactions to provide hydrogen donors (Fornairon-Bonnefond & Salmon, 2003). These proteins were found in BBWH, suggesting that environmental degradation leads to yeast autolysis during the wine aging process (Schiavone et al., 2015).

In addition, there were 7 fungal proteins, including A2Q877, P17872, and Q12679, which are glucanase, pectinase, and cellulase, respectively. These proteins belong to *Aspergillus niger*. In this experiment, no protease activity (acidic and neutral) was found in FBBW or ABBW (the results are not listed). This is consistent with the results of Gazzola (2012) who believe that wine protein is highly resistant to low pH and protein hydrolysis.

3.4. Proportion of peptides in BBWH protein

Proteins with a molecular weight of less than 10 kDa are called peptides, and those larger than 10 kDa are called proteins (Moreno-Arribas et al., 2002). Most studies on wine haze protein mainly focused on protein molecular weights ranging from 11 kDa to 70 kDa (Waters et al., 1995; Nunes-Miranda et al., 2013), and less attention was given to the relationship between peptides in proteins and red wine haze (Blanco et al., 2004). In this experiment, peptides accounted for 41.61% of the total protein content of BBWH (Fig. 5).

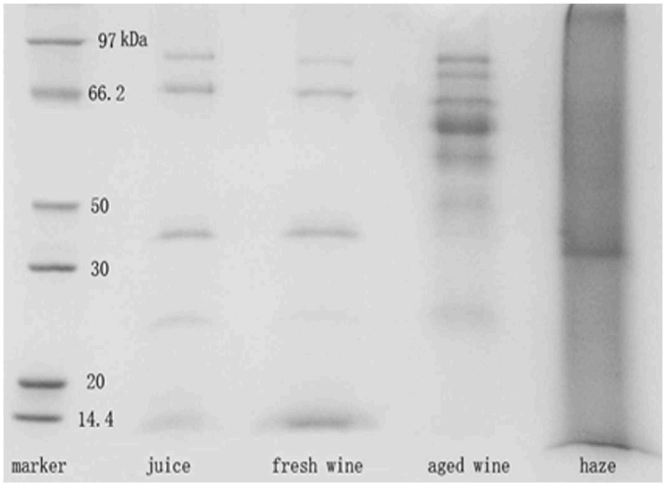

Fig. 4. SDS-PAGE diagrams of protein from different samples.

Such a high proportion of peptides may be due to the release of peptides during protein hydrolysis (Stivala et al., 2018). Under freezing cold stress, the activation of protease leads to protein degradation and increases the production of free radicals, thus releasing peptides (Porras-Agüera et al., 2019). The distribution of peptides less than 3 kDa in FBBW and ABBW is shown in Supplementary Fig. 5.

The molecular weight of these peptides is mainly concentrated at 1500 Da (approximately 15 amino acid residues). Among these peptides, those with amino acid residues less than 700 Da accounted for a large proportion. The results are consistent with those of Martinez-Rodriguez and Polo (2000) et al. They found that when wine yeast autolyzes, peptides (molecular weight between 10,000–700 Da) were first released. Then, these peptides are decomposed to produce peptides with molecular weights less than 700 Da, which are further decomposed into amino acids.

3.5. FTIR spectral analysis of protein in FBBW, ABBW, BBWH and blackberry juice

The infrared spectra of different sample proteins are shown in Fig. 6.

The absorption range of the infrared spectrum included the amide region (1700–1400 cm^{-1}), α-helix (1657–1651 cm^{-1}), β-sheet (1631–1621 and 1680–1694 cm^{-1}), and disordered region (1697–1670 cm^{-1}) (Cardamone, 2010). As shown in Fig. 6, the infrared spectra of FBBW protein and blackberry juice protein were significantly different. This is because the rich polyphenols in blackberry juice can loosen the protein structure, resulting in a decrease in α-helices and an increase of β-sheets (Cheng et al., 2019).

In BBWH protein, the characteristic peak of β-sheet is located at 1624 cm^{-1}, while there is a redshift in ABBW (1596 cm^{-1}), FBBW (1593 cm^{-1}) and fruit juice (1569 cm^{-1}), which is related to the different hydrogen bonding forces in the wine body (Shivu et al., 2013). The infrared absorption peak of juice strength is higher than that of ABBW, FBBW and BBWH. The strength of the infrared absorption peak in haze is the closest to that of the FBBW. After the fermentation of FBBW, the protein in the wine body is mainly derived from fermented microbial protein and plant protein. This confirmed that the protein in haze comes not only from blackberries, but also from fermentation related microorganisms in BBW.

To reduce the total amount of haze produced by blackberry wine during aging, we can start by reducing the content of fermentation microorganisms (such as *Saccharomyces cerevisiae* and *Aspergillus niger*) in the wine body. Controlling the fermentation factors to reduce the total number of colonies of microorganisms in fresh wine or removing as much protein as possible in the clarification process without affecting the content of polyphenols should be considered as methods to reduce BBWH. However, the effectiveness of these methods requires further experimental verification.

4. Conclusions

In this study, the types of proteins in FBBW, ABBW and BBWH were first identified. A total of 125 DEPs were confirmed in FBBW and ABBW, 86 of which were derived from microorganisms and 23 from plants. Most of the protein haze with polyphenols in BBW comes from *Saccharomyces cerevisiae*, mold, and blackberry raw materials. DEP, which predominantly included cell constituent proteins, oxidoreductase, and hydrolase, was mainly involved in the metabolic pathway of sucrose and the biosynthesis and metabolism of antibiotics.

The protein identification of BBWH demonstrated that the haze-forming proteins were mostly sourced from the fermented microbial proteins and a small part of plant proteins. And, the types of proteins in BBWH are different from those in wine haze. The findings of this study provide valuable information for analyzing the protein haze-forming

Table 3
Identification of protein in BBWH.

	UniProtKB	Protein	Coverage [%]	Length	Mass (kDa)	source
1	A0A314Z1F8	40 S ribosomal protein S21	18	82	9.2	*blackberry*
2	Q0Z8V0	Non-specific lipid-transfer protein	17	117	11.8	*blackberry*
3	A0A2P6PDD7	Putative rlpA-like protein, double-psi beta-barrel	15	135	14.4	*blackberry*
4	A0A2P6R3B4	Putative START-like domain-containing protein	11	152	17.1	*blackberry*
5	A0A2P6R5D3	Putative neprosin	7	443	49.6	*blackberry*
6	A0A2P6Q3W0	Putative catechol oxidase	4	585	64.9	*blackberry*
7	A0A2P6SI73	Putative glucan endo-1,3-beta-D-glucosidase	4	244	25.7	*blackberry*
8	A0A314YGY8	Beta-fructofuranosidase	3	637	70.5	*blackberry*
9	A0A2P6P1V1	Putative Golgin subfamily A member 5 protein	3	685	75.6	*blackberry*
10	M5W5K7	Uncharacterized protein	1	574	64.2	*blackberry*
11	P22803	Thioredoxin-2	13	104	11.2	*S. cerevisiae*
12	P00560	Phosphoglycerate kinase	11	416	44.7	*S. cerevisiae*
13	P25334	Peptidyl-prolyl *cis*-trans isomerase CPR4	10	318	35.8	*S. cerevisiae*
14	P00360	Glyceraldehyde-3-phosphate dehydrogenase 1	9	332	35.7	*S. cerevisiae*
15	P53066	Ankyrin repeat-containing protein YGL242C	9	181	20.1	*S. cerevisiae*
16	P00925	Enolase 2	8	437	46.9	*S. cerevisiae*
17	P00942	Triosephosphate isomerase	8	248	26.8	*S. cerevisiae*
18	P05750	40 S ribosomal protein S3	7	240	26.5	*S. cerevisiae*
19	Q08144	t-SNARE affecting a late Golgi compartment protein 2	7	397	45.8	*S. cerevisiae*
20	P16467	Pyruvate decarboxylase isozyme 2	6	563	61.9	*S. cerevisiae*
21	P11353	Oxygen-dependent coproporphyrinogen-III oxidase	6	328	37.7	*S. cerevisiae*
22	P34227	Peroxiredoxin PRX1, mitochondrial	6	261	29.5	*S. cerevisiae*
23	P60010	Actin	5	375	41.7	*S. cerevisiae*
24	P22202	Heat shock protein SSA4	5	642	69.6	*S. cerevisiae*
25	P40442	Secreted protein CSS1	4	995	99.7	*S. cerevisiae*
26	Q03558	NADPH dehydrogenase 2	4	400	45	*S. cerevisiae*
27	P38788	Ribosome-associated complex subunit SSZ1	4	538	58.2	*S. cerevisiae*
28	P40054	D-3-phosphoglycerate dehydrogenase 1	3	469	51.2	*S. cerevisiae*
29	P12709	Glucose-6-phosphate isomerase	3	554	61.3	*S. cerevisiae*
30	Q12083	DNA mismatch repair protein MLH3	2	715	81.9	*S. cerevisiae*
31	A2Q877	Probable xyloglucan-specific endo-beta-1,4-glucanase A	10	241	25.5	*A. niger*
32	A2QBB6	Probable endopolygalacturonase E	7	378	39.6	*A. niger*
33	A2R3I1	Probable pectin lyase A	6	379	39.8	*A. niger*
34	P17872	Pectinesterase	5	331	35.7	*A. niger*
35	A2R3I1	Probable pectin lyase A	3	379	39.8	*A. niger*
36	Q12679	Endoglucanase A	9	239	25.8	*A. kawachii (strain NBRC 4308)*
37	Q9P358	Endopolygalacturonase A	5	370	38.6	*A. awamori*
38	A0A0M8NZ10	Uncharacterized protein	2	1029	113.3	*Penicillium*
39	A0A0G4PHN3	Str. FM013	5	389	43.1	*Penicillium camemberti FM 013*
40	A0A1F5LN55	Uncharacterized protein	2	331	36.6	*Penicillium arizonense*
41	A0A1S9R9B0	Uncharacterized protein	6	541	60.2	*Penicillium brasilianum*
42	W6QZH1	Proteasome subunit alpha type	7	293	31.2	*Penicillium roqueforti*
43	A0A0C9MS75	Actin	5	375	41.7	*Mucor ambiguus*
44	S2KL29	D-3-phosphoglycerate dehydrogenase	3	436	47.6	*Mucor circinelloides f. circinelloides*
45	Q1GAQ0	Elongation factor Tu	10	396	43.3	*Lactobacillus delbrueckii* subsp. *bulgaricus*

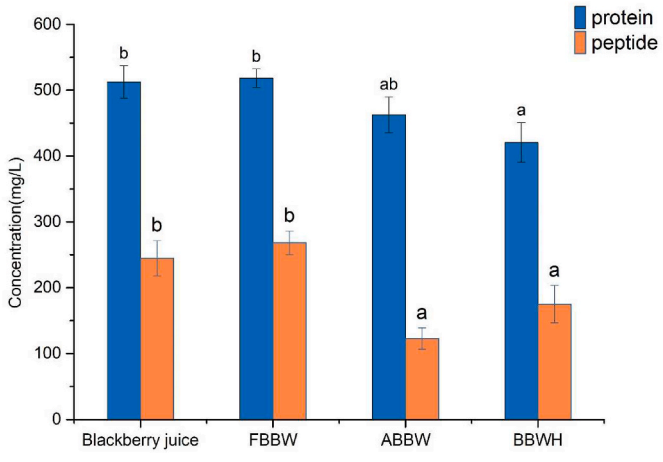

Fig. 5. Ratio of protein to peptides from different samples.

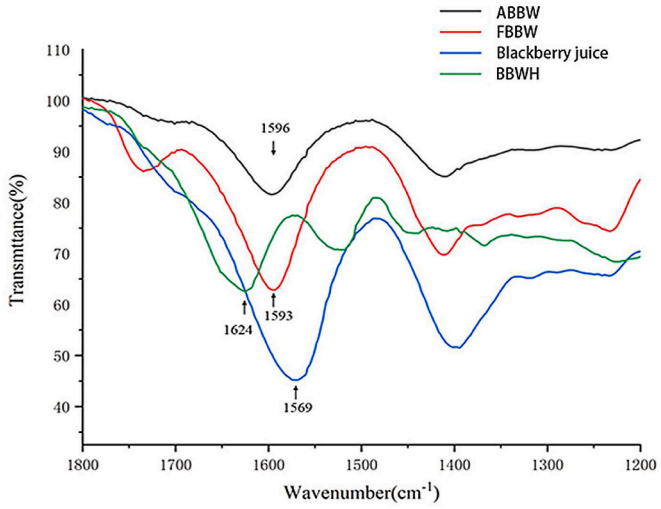

Fig. 6. FTIR spectra of the protein from different samples.

factors of BBWH, and they provide a reference method for analyzing the protein haze in other fruit wines.

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CRediT authorship contribution statement

Gang Wu: Investigation, Validation, Formal analysis, Data curation, Writing – original draft. **Jianzhong Zhou:** Conceptualization, Methodology, Formal analysis. **Linlin Fan:** Writing – review & editing, Supervision. **Xiaoli Liu:** Formal analysis, Data curation, Project administration, Funding acquisition. **Ying Wang:** Formal analysis, Investigation, Formal analysis, Data curation, Formal analysis. **Caie Wu:** Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

We declare that we have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113547.

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