



Uncovering proteome variations and concomitant quality changes of differently drying-treated rape (*Brassica napus*) bee pollen by label-free quantitative proteomics

Shuyue Wang, Liang Chen, Shanshan Li, Fuliang Hu*

Key Laboratory of Silkworm and Bee Resource Utilization and Innovation of Zhejiang Province, College of Animal Sciences, Zhejiang University, Hangzhou 310058, China

ARTICLE INFO

Keywords:

Rape bee pollen
Drying methods
Label-free quantitative proteomic
Functional quality

ABSTRACT

High moisture content of fresh bee pollen makes it difficult to preserve and thus makes drying a necessary process during the bee pollen production. Drying treatment will affect its quality and the effects of sun drying, hot-air drying and freeze drying on the proteome of rape (*Brassica napus*) bee pollen have been evaluated using label-free quantitative proteomics by liquid chromatography-tandem mass spectrometer (LC-MS/MS). A total of 8377 proteins are identified, among which the most abundant differential proteins were found in freeze drying-treated samples. Also freeze-drying treatment maximizes the content of antioxidant, antibacterial and anemic bioactive pollen protein. Besides, rape bee pollen is found to adjust its metabolism to protect itself during the drying process. These results can be favorable to evaluate the effects of drying treatment on the nutrition and function of processed rape bee pollen and insight into how rape bee pollen proteins respond to dehydration.

1. Introduction

Being one of the main bee products, bee pollen generally refers to the plant pollen grains collected by honeybees and aggregated by salivary gland secretions and flower nectar (Yin, Tao, Jiang, Meng, Zhao, Xue, et al., 2022). Owing to its high content in protein, fat, carbohydrates, vitamins and other special bioactive nutrients, bee pollen is well known as “omnipotent nutrition bank”. Moreover, studies have shown that proteins in the bee pollen possess a series of biological activities such as antioxidant, antibacterial, antithrombotic activities (Saisavoey, Sangtanoo, Chanchao, Reamtong, & Karnchanatat, 2021; Zhang, Lu, & Liu, 2022), contributing to the acknowledge that the bee pollen is a kind of natural high-quality food and its extensive use in medicine, health care and other industries. Notably, protein content of the bee pollen ranges

from 2.5 to 61 %, making it an important index to measure the quality of bee pollen (Frias, Barbosa, & Lourenco, 2016). However, due to the fact that the high moisture content of fresh bee pollen makes it highly hygroscopic, easy to bond into clumps and susceptible to microbial contamination (de Arruda, Santos Pereira, Estevinho, & de Almeida-Muradian, 2013), drying is a necessary process during the bee pollen production. It is without doubt that drying process makes difference to the bee pollen protein, not only the composition and content, but also various bioactive activities. In view of this, studying the drying method is of great significance in improving the quality of processed bee pollen.

Nowadays, the most common drying methods used in bee pollen industry are natural sun drying (SD), hot-air drying (HD) and freeze drying (FD), which have their pros and cons (J. Wang, Chen, Zhao, Zhang, & Fang, 2022). SD of which cost is the lowest, technology is the

Abbreviations: ACC, Acetyl-CoA carboxylase; AGPAT, 1-acylglycerol-3-phosphate O-acyltransferase; ANOVA, analysis of variance; BP, biological process; CC, cellular component; DAPs, differentially abundant proteins; DTT, dithiothreitol; FASP, filter-assisted sample-preparation; FBA, fructose-bisphosphate aldolase; FBP, fructose-1,6-bisphosphate; FC, fold change; FD, freeze drying; FDR, false discovery rate; GO, Gene Ontology; GPAT, glycerol-3-phosphate acyltransferase; HCD, higher-energy collisional dissociation; HD, hot-air drying; IAA, iodoacetamide; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS/MS, liquid chromatography-tandem mass spectrometer; LFQ, Label-free quantification; LPA, lysophosphatidic acid; MF, molecular function; MS, Mass spectrometry; OAA, HCO₃ to oxaloacetic acid; OPLS-DA, orthogonal partial least squares discriminant analysis; PCA, principal component analysis; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PRM, Parallel reaction monitoring; ROS, reactive oxygen species; SD, natural sun drying; SDS, Sodium dodecyl sulfate; SDT lysis buffer, 4% SDS, 100 mM DTT, 100 mM Tris-HCl pH 8.0; TFA, trifluoroacetic acid.

* Corresponding author at: Fuliang Hu, 866 Yuhangtang Road, Hangzhou 310058, China.

E-mail address: flhu@zju.edu.cn (F. Hu).

<https://doi.org/10.1016/j.foodchem.2023.137559>

Received 26 May 2023; Received in revised form 31 August 2023; Accepted 19 September 2023

Available online 21 September 2023

0308-8146/© 2023 Elsevier Ltd. All rights reserved.

simplest, not limited by equipment, but is easy to be affected by the environment, making the quality unstable. HD has advantages of low equipment investment and easy operation while makes low utilization of energy and relatively poor quality of products (Polat, Guclu, Kelebek, Keskin, & Selli, 2022). In conjunction with this, though FD is recognized as a drying method that might retain the primitive quality of food to the greatest extent as expected, it is expensive and time-consuming. That means, drying does change the quality of bee pollen. While relevant studies mainly focus on drying kinetics, general composition, physico-chemical properties and microstructure changes under the drying treatment (Bi, Zielinska, Ni, Li, Xue, Tian, et al., 2022; Isik, Ozdemir, & Doymaz, 2019; Keskin & Ozkok, 2020; S. Y. Wang, Bi, Zhou, Peng, Tian, Wang et al., 2022), as a food resource rich in protein, relatively few studies have been conducted on the changes of protein in bee pollen during drying processing.

Mass spectrometry (MS) is one of the most effective analytical platforms in the field of quantitative analysis, while label-free quantitative proteomics technology based on it has become an important method to identify sample proteins and accurately quantify their abundance. Recent study used this technology to identify the allergens and allergen hydrolysates of natural and fermented rape bee pollen and clarified fermentation could potentially alleviate bee pollen allergenicity (Tao, Yin, Fu, Wang, Meng, Li et al., 2022). Guo et al. were the first to use proteomics to study bee pollens, their study indicated rape bee pollen proteins have a range of activities like binding activity, enzyme activity and transporter activity (Guo, Yan, Guo, & Jin, 2014). Then series of studies were carried out by Navakauskiene et al. (Borutinskaite, Treigyte, Matuzevicius, Ceksteryte, Kurtinaitiene, Serackis, et al., 2019; Borutinskaite, Treigyte, Matuzevicius, Zaikova, Ceksteryte, Navakauskas, et al., 2017; Ceksteryte, Navakauskiene, Treigyte, Jansen, Kurtinaitiene, Dabkeviciene, et al., 2016). These studies demonstrated the proteomics of red clover (*Trifolium pratense*) pollen and oilseed rape (*Brassica napus* L.), as well as the proteomic of honeybee- and manually-collected pollen and their differences. However, there has been little discussion about the effect of drying methods on proteome of bee pollen.

Herein, label-free quantitative proteomic technology was used to analyze differently drying-treated bee pollen samples, to investigate the changes in protein composition and content as well as the influences on its functional and nutritional quality. This research aimed to provide new insights into the production and processing of bee pollen, simultaneously to provide reference for the consumers while purchasing.

2. Materials and methods

2.1. Materials

The fresh rape bee pollen was purchased from Jiangsu Beevip Biotechnology C., Ltd (Taizhou, China). Ammonium bicarbonate, dithiothreitol (DTT), iodoacetamide (IAA), and sodium carbonate were purchased from Sigma-Aldrich (St. Louis, MO). Urea and Sodium dodecyl sulfate (SDS) were purchased from Bio-Rad (Hercules, CA). Acetonitrile and water for nano-LC-MS/MS were purchased from J. T. Baker (Phillipsburg, NJ). Trypsin was purchased from Promega (Madison, WI). All other chemical reagents were purchased with analytical grade.

2.2. Sample preparation

The bee pollen was obtained from Menyuan County (Qinghai Province, China) in July 2022, stored at -18°C . In simple terms, the rape bee pollen was selected which means non-rape bee pollen was removed. Then the samples were evenly divided into four parts with three replicates each treatment: (1) Fresh, immediately stored in the refrigerator at -20°C ; (2) SD, sun drying treatment was produced outside directly under the sun from 10:00–16:00, about $24\text{--}30^{\circ}\text{C}$ and repeated for 4 days; (3) HD, hot-air drying treatment was produced at 3 m/s and 45°C

by an air blast drying oven (YIHENG, SH) for 24 h; (4) FD, freeze drying treatment was produced at 0.120 mBar and -80°C by a vacuum freeze dryer (LABCONCO, USA) for 48 h. All the rape bee pollen was dried with a thickness of 5 mm. The process was terminated when the moisture contents of the SD, HD and FD samples were 7.60 ± 0.11 , 7.04 ± 0.06 , 6.96 ± 0.25 , respectively. For chemical analysis, the dried sample was ground into powder, sieved (mesh 40), and kept at -20°C before analysis. Besides, fresh samples were pulverized into a paste before chemical analysis.

2.3. Protein extraction and digestion

Protein extraction referenced the methods of Yin et al (Yin, et al., 2022). Briefly, protein was extracted by using an SDT lysis buffer (4 % SDS, 100 mM DTT, 100 mM Tris-HCl pH 8.0). Samples were boiled for 3 min and further ultrasonicated 5 min. Undissolved cellular debris were removed by centrifugation at 16,000g at 4°C for 20 min. The supernatant was collected and quantified with a BCA Protein Assay Kit (Bio-Rad, USA).

Protein digestion was performed with the filter-assisted sample-preparation (FASP) procedure described by Wisniewski et al. (Wisniewski, Zougman, Nagaraj, & Mann, 2009). In short, 0.01 M DTT, IAA in UA buffer (8 M Urea, 150 mM Tris-HCl, pH 8.0) and the detergent was added to block reduced cysteine. Then the protein sample retained on the ultrafiltration tube were digested with trypsin buffer (6 μg Trypsin in 40 μL NH_4HCO_3) at a ratio of 50:1 overnight at 37°C . The peptides were collected by centrifugation at 12,000g for 10 min and acidified with 0.1 % (v/v) trifluoroacetic acid (TFA) before being desalted with C_{18} Cartridge. Finally, the samples were dried in a vacuum freeze-dryer and resuspended in 20 μL 0.1 % TFA then immediately go for LC-MS/MS analysis.

2.4. LC-MS/MS analysis

LC-MS/MS Analysis was performed on a Q Exacutive HFX mass spectrometer (Thermo Scientific, Germany) coupled with Easy 1200 nLC system (Thermo Scientific, Germany). Buffer A was aqueous formic acid (0.1 % (v/v) formic acid in water) and buffer B was 80 % (v/v) acetonitrile (0.1 % (v/v) formic acid). Peptides were first loaded to a trap column (100 $\mu\text{m} \times 20$ mm, 5 μm , C_{18} , Dr. Maisch GmbH, Ammerbuch, Germany) in buffer A and then separated by a self-packed column (75 $\mu\text{m} \times 150$ mm; 3 μm ReproSil-Pur C_{18} beads, 120 \AA , Dr. Maisch GmbH, Ammerbuch, Germany) at a flow rate of 300 nL/min. The optimized linear gradient was set as follows: 5–8 % (v/v) buffer B for 2 min, 8–23 % (v/v) buffer B for 88 min, 23–40 % (v/v) buffer B for 10 min, 40–100 % (v/v) buffer B for 8 min, and 100 % (v/v) buffer B for 12 min. MS data was obtained via a data-dependent top20 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for higher-energy collisional dissociation (HCD) fragmentation. Resolving power was 60,000 at m/z 200 for the MS scan, the automatic gain control target value was $3e6$, and maximum ion injection time 50 ms. Resolving power 15,000 at m/z 200 for the MS/MS scan, the automatic gain control target value was $1e5$, and maximum ion injection time 50 ms. These were selected with an isolation window of 1.6 m/z and fragmented via HCD with normalized collision energies of 28 eV.

2.5. Protein identification and quantitation

The MS data were analyzed using Sequest HT in Proteome Discoverer software (version 2.4, Thermo Scientific, Germany) and the database that searched against was the UniProt-*Brassica napus* (Rape) database (139490 total entries, downloaded 12/02/2022). The search parameters were set as follows: protein-cleaving enzyme, trypsin; maximum missed cleavages, 2; 1 peptide tolerance, 10 ppm; MS/MS tolerance, 0.02 Da. The fixed modification was carbamidomethyl (C), and the variable modifications were oxidation (M), deamidation (N, Q) and acetyl

(protein *N*-term). Both of the peptide and protein false discovery rate (FDR) values were less than or equal to 0.01. Label-free quantification (LFQ) was set as true, and the LFQ minimum ratio count was 1. The quantitative protein ratios were weighted and normalized by the median ratio in Maxquant software.

2.6. Parallel reaction monitoring (PRM) analysis

The abundance level of twenty-one selected proteins determined by label-free quantitative proteomics analysis were validated via PRM method. Based on the results of MS analysis, signature peptides for the target proteins which have reliable matching map of MS/MS and well chromatographic separation were selected for PRM analysis. The peptide mixture was separated via EASY-nLC 1200 system (Thermo Scientific, Germany). Buffer A consisted of 0.1 % (v/v) formic acid while buffer C consisted of 0.1 % (v/v) formic acid in 85 % (v/v) acetonitrile solution. After being equilibrated with 95 % (v/v) buffer A, the samples were loaded onto the Trap Column (100 $\mu\text{m} \times 20 \text{ mm}$, 5 μm , C₁₈, Dr. Maisch GmbH) and gradient eluted on chromatographic column (75 $\mu\text{m} \times 150 \text{ mm}$, 3 μm , C₁₈, Dr. Maisch GmbH) at a flow rate of 300 nL/min. And the separation gradient is as follows: 2–5 % (v/v) buffer B for 5 min, 5–23 % (v/v) buffer B for 40 min, 23–40 % (v/v) buffer B for 5 min, 40–100 % (v/v) buffer B for 2 min, and 100 % (v/v) buffer B for 8 min. Then the Q-Exactive HFX mass spectrometer (Thermo Scientific, Germany) was applied in PRM analysis in positive ion mode with a selected mass range of 350–1500 *m/z*. Data acquisition was set as follows: Resolving power was 60,000 at *m/z* 200 for the MS scan, the automatic gain control target value was 3e6, and maximum ion injection time 200 ms. Resolving power 15,000 at *m/z* 200 for the MS/MS scan, the automatic gain control target value was 1e6, and maximum ion injection time 100 ms. These were selected with an isolation window of 2.0 *m/z* and fragmented via higher energy collisional dissociation with normalized collision energies of 28 eV. Raw files of PRM analysis were processed using Skyline 4.1 (MacLean, Tomazela, Shulman, Chambers, Finney, Frewen, et al., 2010).

2.7. Bioinformatics and statistical analysis

Analyses of bioinformatics data were carried out with Perseus software, Microsoft Excel and R statistical computing software. R

(version:4.0.3) and R packages were used for principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). Hierarchical clustering analysis was performed with the pheatmap package, which is based on the open-source statistical language R25, using Euclidean distance as the distance metric and complete method as the agglomeration method. To annotate the sequences, information was extracted from UniProtKB/Swiss-Prot, Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). GO and KEGG enrichment analyses were carried out with the Fisher's exact test, and FDR correction for multiple testing was also performed. Enriched GO and KEGG pathways were nominally statistically significant at the $p < 0.05$ level.

3. Results and discussion

3.1. Protein identification and quantitation

Label-free quantitative proteomics analysis was performed to evaluate the protein expression level and understand the proteomic changes occurring in the rape bee pollen treated by different drying methods. A total of 53,663 unique peptides and 8,377 protein groups were identified, among which 8184, 8158, 8163, and 8173 proteins were identified in rape bee pollen samples of fresh as well as dehydrated by freezing, sun and hot air, respectively (Fig. 1A). Nearly three quarters (71.52 %) of the proteins showed a sequence coverage higher than 10 % (Fig. 1B), indicating the reliability of proteomic analysis. As the Venn diagram analysis of the identified proteins from each bee pollen group displayed (Fig. 1C), most of the identified proteins (7960) were shared in fresh and differently drying-treated rape bee pollen. Compared with identified proteins in fresh bee pollen, approximately 110 proteins were not identified in dried samples. Meanwhile, among the non-share proteins, 37 were special for fresh samples, 43 for HD samples, 45 for SD samples and 46 for FD samples. It seems that freeze-drying treatment has the greatest effect on protein numbers, maybe some proteins in rape bee pollen will be denatured at extreme temperatures but the temperatures under sun drying and hot-air drying is relatively normal to pollen as it is naturally produced in the such temperatures (Yoshida, Murakami, Niwa, Takeya, & Osanai, 2021). However, these two drying methods still reduced the amount of identified protein and sun drying did more. It is possible that the Maillard reaction occurs during the drying process, and

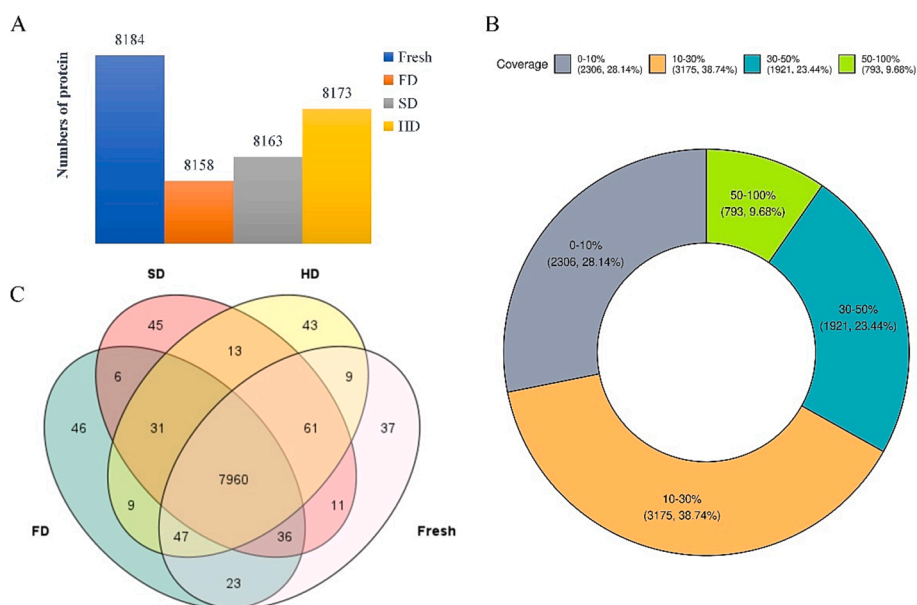


Fig. 1. (A) the number of identified proteins in rape bee pollen of fresh and differently drying-treated samples; (B) percentages of sequence coverage of proteins by identified peptides; (C) Venn diagram of identified protein distribution in fresh and differently drying-treated rape bee pollen.

the Maillard reaction will reduce the amino acids in the protein and lead to protein degradation. As the Maillard reaction is affected by water activity and irradiation (Shapla, Solayman, Alam, Khalil, & Gan, 2018; S. Y. Wang, et al., 2022), sun drying behaves a slower rate of water loss, thus the Maillard reaction may occur for a relatively long time. Besides, ultraviolet radiation will also affect the Maillard reaction thereby leading to greater protein variations.

In order to validate the differential amounts of proteins identified in the fresh and differently drying-treated rape bee pollen samples, 15 proteins related to the functional quality of rape bee pollen were selected and confirmed using PRM analysis. The information from the validation is presented in Table 1. The fold-changes of the selected proteins identified by PRM assay were almost consistent with the LFQ data, in which the fold-change of Superoxide dismutase [Cu-Zn] (A0A816KR61) and L-ascorbate peroxidase (Fragment) (A0A816PSV9) was slightly different between the data of LFQ and PRM.

3.2. Comparative analyses of fresh and differently drying-treated rape bee pollen protein samples

3.2.1. PCA and OPLS-DA

It was clear from the score plot of PCA (Fig. 2A) that the identified proteins in fresh and dried rape bee pollens could be separated, though still some overlap between the HD and SD, there was an obvious separation trend, with all samples located in the confidence ellipse of Hotelling T^2 . In the direction of the first principal component ($t [1]$), FD samples could first be distinguished from fresh and other dried ones without doubt. In spite of the almost complete overlap between Fresh, HD and SD samples in this direction, Fresh samples could be discerned subtle deviation, while clear discrimination was achieved in the direction of the second principal component ($t [2]$).

OPLS-DA was utilized as a supervised chemometric method to further analyze the differences between proteomic data of fresh and differently drying-treated samples, overcoming the limitations of PCA in finding variations. The score plot of the OPLS-DA model was showed in Fig. 2B, where the R^2X (cum), R^2Y (cum) and Q^2 (cum) values were 0.483, 0.988 and 0.624 respectively, indicating the reliability of the results. Similar to that observed in PCA modeling, data from fresh samples and differently drying-treated samples were all inside the confidence ellipse of Hotelling T^2 but with more condensed manner. Along $t [1]$ direction in OPLS-DA score plot, data of four samples were separated unambiguously from each other in contrast to the PCA results that Fresh, HD and SD samples could only be clearly distinguished in the $t [2]$ direction. Moreover, compared to FD and HD samples, the data from SD samples was further away from Fresh samples along the $t [1]$ direction,

indicating that under the evaluation criteria of OPLS-DA model, sun drying led to the greatest changes in proteomes among all these drying methods.

3.2.2. Hierarchical cluster analysis

Further hierarchical cluster analysis of quantified proteins was carried out and the studied rape bee pollen samples formed three major clusters (Fig. 2C). Proteins from HD and SD samples were not clearly distinguished and formed a sub-cluster that grouped with Fresh samples, having a similar proteomic profile, while the proteomic profile of FD samples is apart from others and independently formed the other sub-cluster. Hierarchical clustering analysis revealed that differences in the quantified proteins from fresh and differently drying-treated rape bee pollens, whereas the bee pollens dehydrate by sun or hot air were not obviously different. These clustering profiles were almost consistent with the PCA and OPLS-DA results.

3.3. Analysis of differentially abundant proteins (DAPs)

3.3.1. Daps and correlations

In this study, only proteins with fold change (FC) > 2 or FC < 0.5 and p value < 0.05 were considered as significantly differentially abundant proteins (DAPs) (see details in Table S1). There are 1350, 135, 152 proteins identified as DAPs in FD/Fresh, HD/Fresh and SD/Fresh groups, making a total of 1493 DAPs. As shown in the Upset graph (Fig. 3A), 14 DAPs were shared proteins among the three groups. There were 46, 48, 22 shared DAPs between the FD/Fresh group and HD/Fresh group, FD/Fresh group and SD/Fresh group, SD/Fresh group and HD/Fresh group in sequence. In addition, there were 1242, 53, 68 exclusive DAPs in the FD/Fresh group, HD/Fresh group and SD/Fresh group, respectively.

3.3.2. Bioinformatics analysis of DAPs and main functional DAPs

To further investigate the influences of different drying treatments on rape bee pollen proteins' functional and nutritional quality, Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were used to analyze the identified DAPs in pairwise comparison.

The expressional profiles of DAPs were analyzed through GO annotation (Fig. 3B–D), which classified these proteins into three categories: biological process (BP), cellular component (CC) and molecular function (MF). Within the BP category, metabolic process (GO: 0008152) and cellular process (GO: 0009987) were two dominant terms, localization (GO: 0051179) following closely behind. As for the CC domain, cellular component (GO:0005575) and cellular anatomical entity (GO: 0032991) were the most matched terms. And the top 3 terms associated

Table 1

Results of 15 selected differentiating proteins identified by both LFQ and PRM assays.

Protein Accession	Gene Name	LFQ			PRM		
		Log ₂ FC FD/Fre	Log ₂ FC HD/Fre	Log ₂ FC SD/Fre	Log ₂ FC FD/Fre	Log ₂ FC HD/Fre	Log ₂ FC SD/Fre
A0A816P2S6	Glutathione peroxidase	1.57	0.88	0.84	1.16	0.15	0.04
A0A816KR61	Superoxide dismutase [Cu-Zn]	1.04	0.56	0.58	0.70	0.14	-0.05
A0A816PSV9	L-ascorbate peroxidase (Fragment)	2.42	0.15	1.45	0.68	0.21	0.04
A0A816P1Z9	L-ascorbate peroxidase	1.39	0.70	0.59	0.92	0.29	0.29
A0A816ZG96	L-ascorbate peroxidase	1.57	0.76	0.56	1.5	0.75	0.49
A0A078GR87	Glutaredoxin-dependent peroxiredoxin	1.16	0.28	0.17	1.15	0.29	0.30
A0A816XT90	Peptide-methionine (R)-S-oxide reductase	1.85	0.93	0.78	1.38	0.54	0.52
P6277	Cytochrome c	1.21	0.60	0.67	0.92	0.34	0.57
A0A816PCC3	(rape) hypothetical protein	-1.12	-0.51	-0.45	-1.00	-0.21	-0.25
A0A816I831	Assimilatory sulfite reductase (ferredoxin)	-1.23	-0.17	-0.06	-0.49	-0.06	-0.07
A0A816VUU9	Ferroxidase	2.00	0.67	0.65	1.47	0.45	0.31
A0A078JZV4	BnaAnng40360D protein	1.60	0.67	1.31	1.83	0.82	1.22
A0A816WC94	(rape) hypothetical protein	2.12	1.04	0.92	1.05	0.26	0.40
A0A078G8L0	(rape) hypothetical protein	1.92	1.07	1.75	1.44	0.51	0.98
A0A816PTG8	(rape) hypothetical protein	1.53	1.17	1.42	0.92	0.17	0.54

Abbreviation: FC, fold change; LFQ, label-free quantitation; PRM, parallel reaction monitoring; FD, freeze drying-treated samples; HD, hot-air drying-treated samples; SD, sun drying-treated samples; Fre, fresh samples.

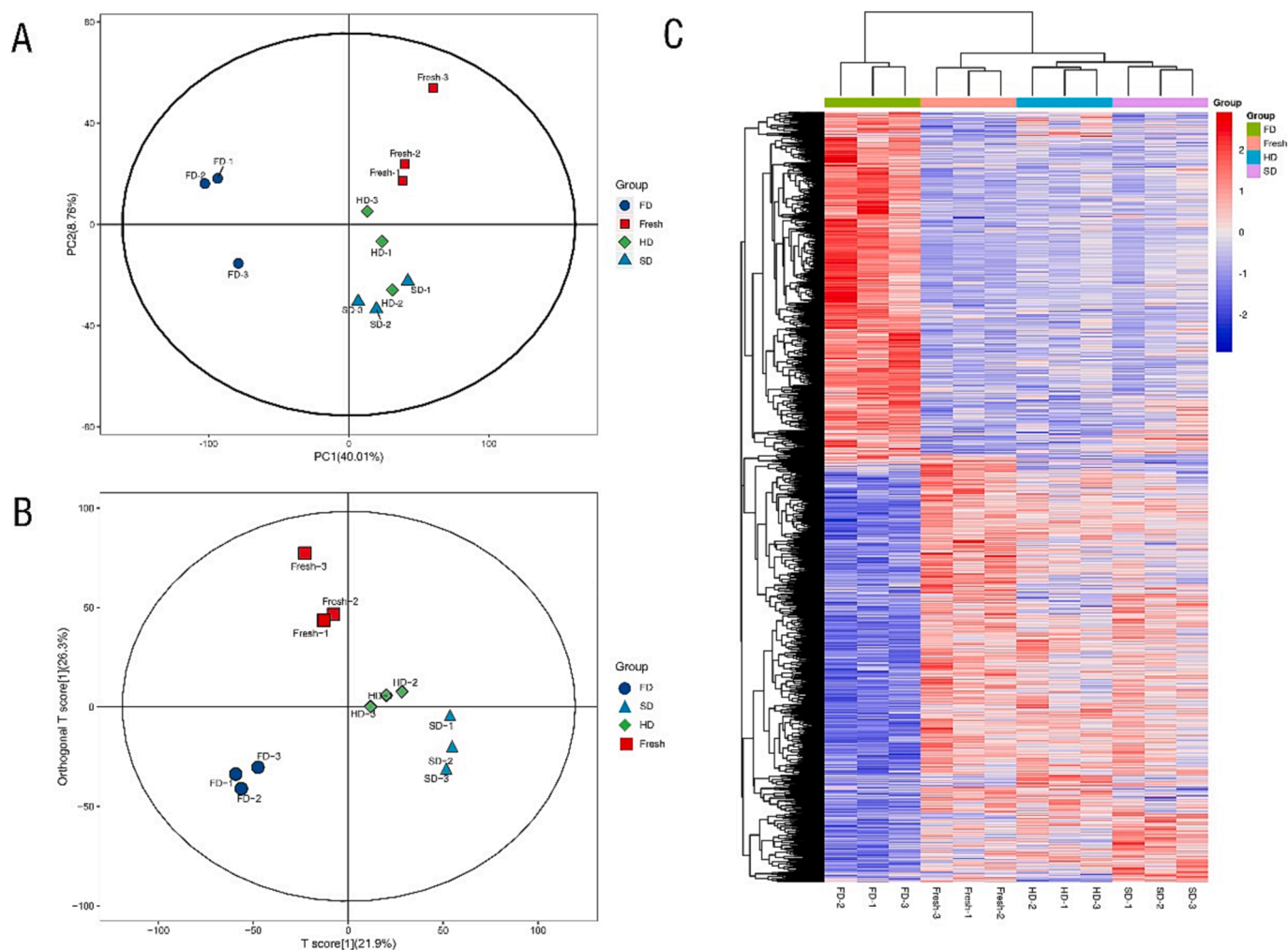


Fig. 2. (A) Score plot of PCA with R^2X (cum): 0.844, Q^2 (cum): 0.789; (B) OPLS-DA with R^2X (cum): 0.483, R^2Y (cum): 0.988 and Q^2 (cum): 0.624; (C) Hierarchical clustering of the quantified proteins from fresh and differently drying-treated rape bee pollens.

with gene counts in MF category were binding (GO: 0005488), catalytic activity (GO: 0003824) and transporter activity (GO: 0005215). Generally speaking, the GO annotations of DAPs is exhibited roughly the same enriched classifications, though in gene counts FD/Fresh had more quantities than others. With the focusing quality-concerns, DAPs with special biological functions were further discussed.

Antioxidant activity (GO:0016209), the most common biological activity of proteins, was found to be enriched in GO annotation (Fig. 3D) with 12 DAPs lumped into this term in total. Glutathione peroxidase (A0A078IXA9, A0A816P2S6), superoxide dismutase [Cu-Zn] (A0A817AVU7, A0A816KR61), l-ascorbate peroxidase (A0A816P1Z9, A0A816ZG96, A0A816PSV9), glutaredoxin-dependent peroxiredoxin (A0A078GR87, A0A816WU14), peptide-methionine (R)-S-oxide reductase (A0A816XT90) and peroxidase (A0A816WLG4) were identified as up-regulated DAPs in FD/Fresh group, while superoxide dismutase (Q09QU5) in HD samples and l-ascorbate peroxidase (A0A816PSV9) in SD samples were also significantly increased. To be precise, the contents of all the DAPs in these three pairwise comparisons that participate in antioxidant pathways have increased (Fig. 4). This may be due to the production of reactive oxygen species (ROS) during dehydration, while plants maintain redox homeostasis through antioxidant enzymes and low molecular weight osmolytes (Gill & Tuteja, 2010). Likewise, recent study found that leaves of chickpea seedlings up-regulated the expression of l-ascorbate peroxidase, superoxide dismutase and glutathione s-transferase, thereby reducing dehydration stress damage to membranes (Vessal, Arefian, & Siddique, 2020).

Some of the aforementioned proteins such as proxidase (A0A816WLG4) and l-ascorbate peroxidase (A0A816P1Z9, A0A816ZG96, A0A816PSV9) not only have the function of oxidation resistance, but also are able to bind heme. Meanwhile, previous studies have reported significant improvement in anemia symptoms after continuous bee pollen supplementation (Borutinskaite, et al., 2017; Haro, Lopez-Aliaga, Lisbona, Barrionuevo, Alferéz, & Campos, 2000), leading us to the anemic-related functions of bee pollen proteins, among which heme binding (GO:0020037) stood out for its enrichment with high significant level ($p < 0.01$). BnaA10g06850D protein (A0A078FV1), assimilatory sulfite reductase (ferredoxin) (A0A816I831), cytochrome C (P62772), cytochrome C oxidase subunit 1 (Q6YSM3) and seven (rape) hypothetical protein (A0A078FH37, A0A078J5R0, A0A816MXB0, A0A816STV0, A0A816WBI3, A0A816PCC3, A0A078IPQ1) all fell into this term and some have all been reported to be involved in anti-anemia activities (Cytochrome-C Levels in Iron Deficiency without Anemia, 1958; Liao, Allred, Keen, Mccune, Rucker, & Medeiros, 1995; Lill, Hoffmann, Molik, Pierik, Rietzschel, Stehling, et al., 2012). These proteins are benefit to iron deficiency anemia through binding to heme, a compound composed of iron complexed in a porphyrin (tetrapyrrole) ring, thus making the most of the available heme. On the other hand, ferroxidase (A0A816VUU9), participating in heme biosynthetic process (GO: 0006783), promotes Fe^{2+} one-electron oxidation coupled to the reduction of molecular oxygen directly to water (Gutteridge, 1983; Osaki & Walaas, 1967). This activity is known to be fundamental for iron incorporation into

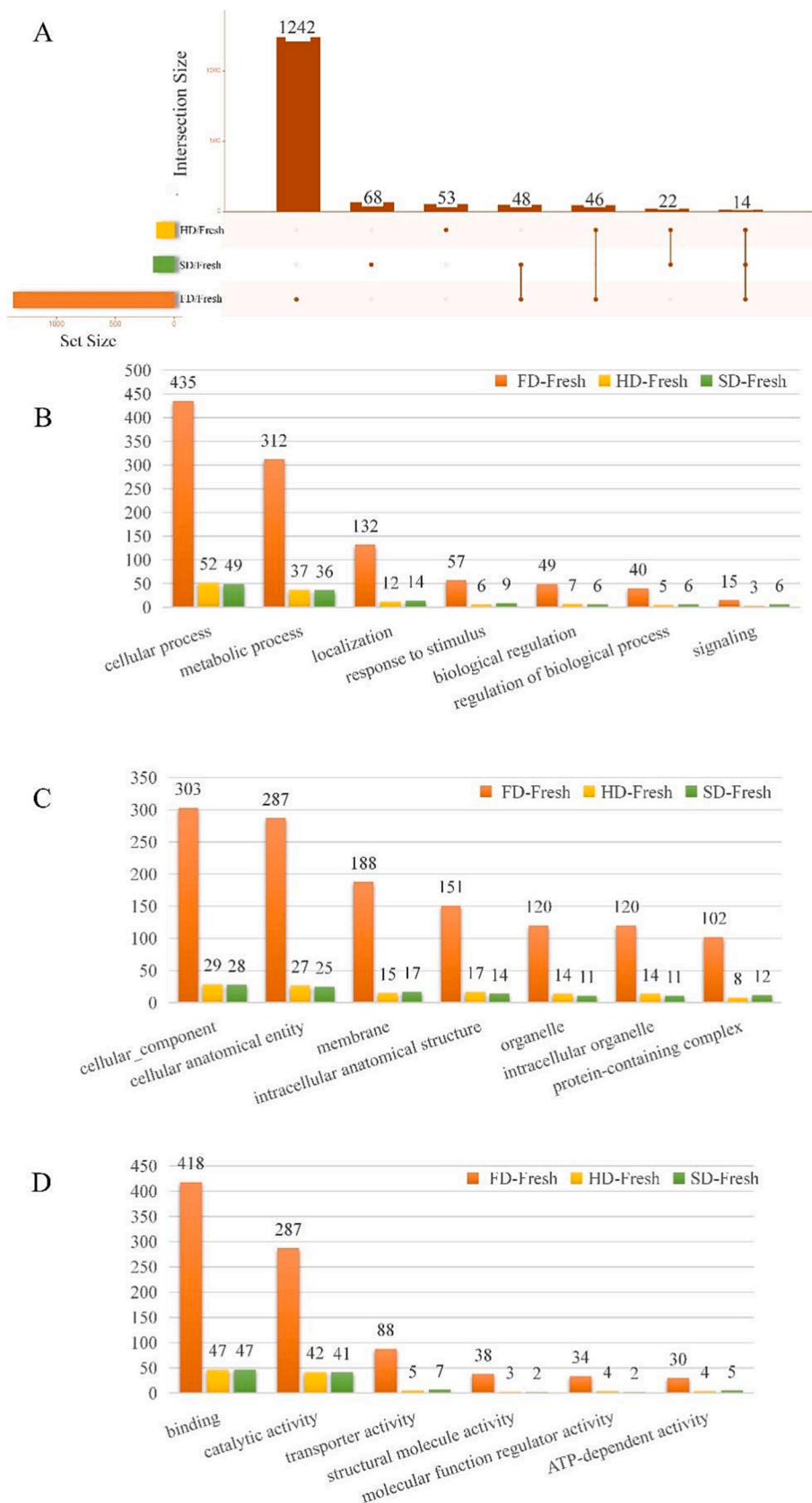


Fig. 3. (A) Upset diagram; (B-D) the GO analysis of DAPs in three pairwise comparison group, three categories: biological process, cellular component and molecular function were displayed respectively.

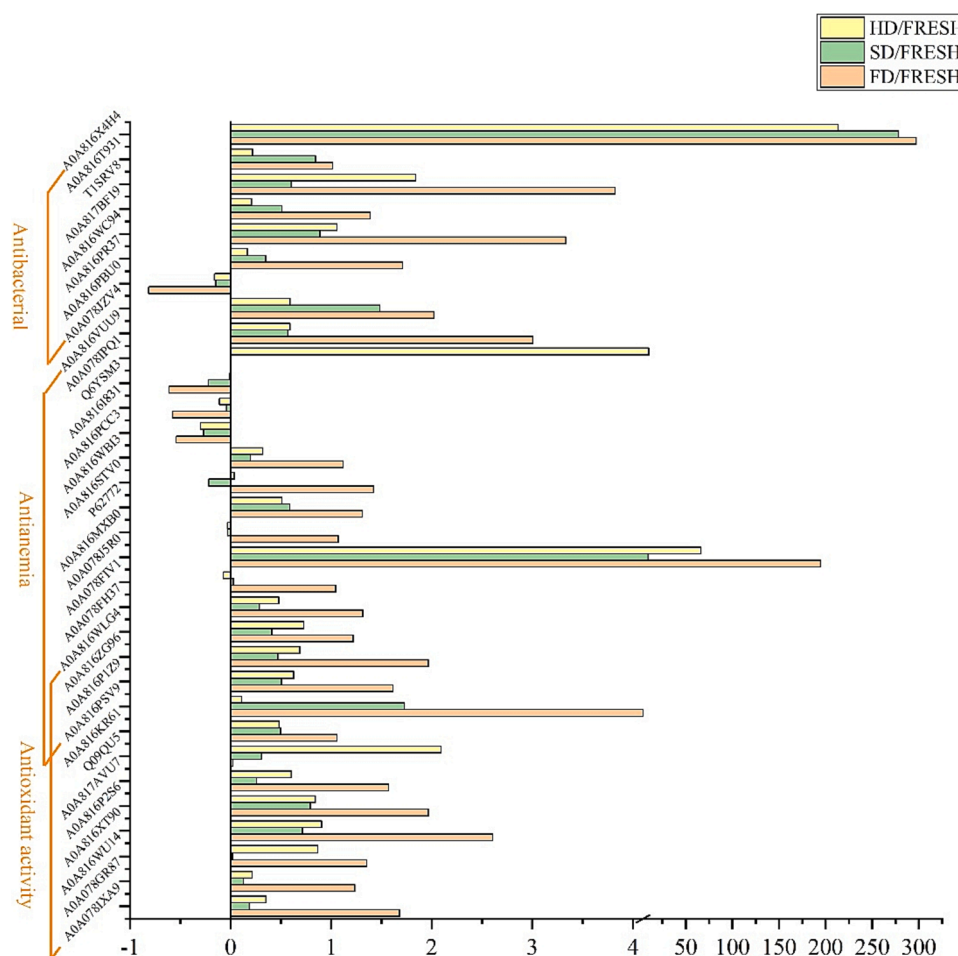


Fig. 4. Relative percentages of the abundance alterations of the functional differentiating proteins with reference to fresh samples. (Negative percentages indicate down-regulation and positive ones indicate up-regulation).

transferrin and, therefore, for iron homeostasis (Ganini, Canistro, Jiang, Stadler, Mason, & Kadiiska, 2012). As illustrated in Fig. 4, ferroxidase up-regulated about 300 % after the freezing drying treatment, while its content only getting almost a half more in other two drying-treated samples. In addition, our findings on heme binding proteins suggest a prominent advantage of freezing-drying treatment in the utilization of heme, among which (rape) hypothetical protein (A0A078J5R0) is the most convincing example on account of its extremely differentiated content of which three differently drying-treated samples (FD, HD, SD) are about 195, 66, 10 times more than fresh samples. Overall, this implies that the anti-anemic function of rape bee pollen is associated with the drying methods, meanwhile, freezing drying seems to perform best within the studied ones.

Antibacterial is another important bioactive function of identified protein, whose GO annotation can be categorized as defense response to fungus (GO:0050832), defense response to virus (GO:0050832) and killing of cells of another organism (GO: 0031640). BnaAnng40360D protein (A0A078JZV4), BnaA08g03260D protein (A0A078HVR6), 3-phosphoshikimate 1-carboxyvinyltransferase (A0A816T931) and (rape) hypothetical protein (T1SRV8, A0A816WC94, A0A078G8L0, A0A816PTG8, A0A816Y4U4, A0A078IVA0) were identified DAPs that exhibit the biological activity of antibacterial (Gaudet, Livstone, Lewis, & Thomas, 2011). While previous studies on the antibacterial activity of bee pollen mainly focused on the flavonoids, phenolic acids and fatty acids in bee pollen (Manning, 2001; Morais, Moreira, Feas, & Estevinho, 2011; Pereira, Oliveira, Sousa, Valentao, Andrade, Ferreira, et al., 2007), this study is the first report on the antibacterial activity of bee pollen proteins. Likewise, freezing-drying treatment resulted in a

substantial content increase of most antibacterial-related proteins (Fig. 4), potentially the defense capability of rape bee pollen proteins is enhanced after drying treatment, especially in freezing drying-treated samples.

Analysis based on the KEGG pathways could contribute to a better understanding of the biochemical metabolic pathways involving identified proteins. To further analyze the biological pathways in which DAPs of differently drying-treated rape bee pollen involved, the KEGG database was performed for enrichment analysis with the DAPs identified by either one-way analysis of variance (ANOVA) of four samples or pairwise comparison. According to Fig. 5, metabolism-related pathways and genetic Information processing-related pathways were two most enriched in ANOVA identified DAPs ($p < 0.05$). Since bee pollen is considered a nutritional and health food, it is comprehensible to more focus on its metabolism changes which related to its nutrient substance. Also, in order to clearly elucidate the KEGG pathway enrichment of protein that changes during the specific drying process, pairwise analysis results would be used in the following discussion (Table S2). As for FD/Fresh group, lipid metabolism was the relatively prominent class, including sphingolipid metabolism (ko00600), glycerolipid metabolism (ko00561), fatty acid biosynthesis (ko00061), and glycerophospholipid metabolism (ko00564), while the most common enriched pathway class in HD/Fresh and SD/Fresh group was carbohydrate metabolism. It is worth discussing these interesting facts revealed by the results of KEGG pathways.

It was said that plants under low temperature stress can reduce the stress damage through different metabolic responses, such as changing the content of membrane lipid or the degree of unsaturated fatty acid to

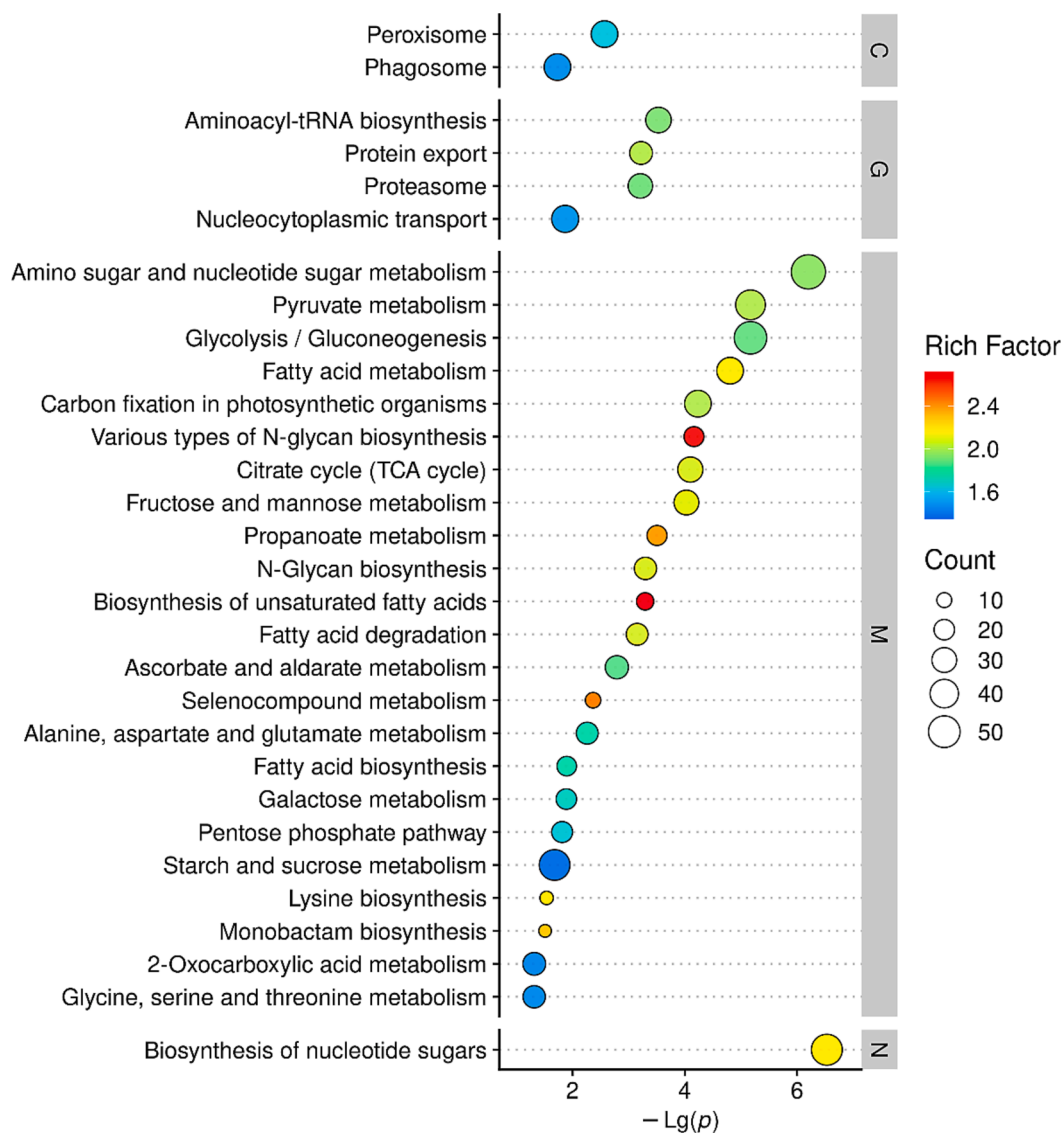


Fig. 5. Enrichment analysis of the KEGG pathway involving differentially abundant proteins identified by ANOVA of FD, SD, HD and Fresh samples.

maintain plasma membrane fluidity (Liu, Yang, & Mao, 2021; Teixeira, Coelho, Olsson, Brodelius, Carvalho, & Brodelius, 2009). In this study, we found that the expression of glycerol-3-phosphate acyltransferase (GPAT, A0A078J6S2) and biotin carboxyl carrier protein of acetyl-CoA carboxylase (G4WXE0) in chloroplast upregulated while almost all other lipid metabolism related DAPs down-regulated expression, including 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT, D3WFQ7), O-acyltransferase (A0A078J7P3, Q9XGR5), phospholipase D (T2C548) and so on. GPAT affects the unsaturation of fatty acids at the *sn*-1 position of glycerol-3-phosphate molecules by selecting the type of fatty acid, thus influences the cold tolerance of plant (Murata & Tasaka, 1997). The GPAT from chilling-resistant plants, such as rape, discriminates against non-fluid palmitic acid and prefers to select oleic acid and the up regulation of this enzyme suggested increased chilling resistance of rape bee pollen and more synthesis of lysophosphatidic acid (LPA). On the other hand, the down regulation of AGPAT and other correlative DAPs indicated the retain of LPA, one of the identified lipids that participates in hematopoiesis (Lin, Chiang, Ho, Yao, & Lee, 2020), which may further enhance the anti-anemic properties of freezing-drying treated bee pollen.

While carbohydrate metabolism was enriched in both HD/Fresh and SD/Fresh group, the enriched pathways were not the same where

ascorbate and aldarate metabolism (ko00053), pentose phosphate pathway (ko00030), fructose and mannose metabolism (ko00051) were shared enriched pathways, glycolysis/gluconeogenesis (ko00010) and inositol phosphate metabolism (ko00562) were special for HD/Fresh group and starch and sucrose metabolism (ko00500), pyruvate metabolism (ko00620), propanoate metabolism (ko00640) and galactose metabolism (ko00052) is specific to SD/Fresh group. Quite specially, fructose-bisphosphate aldolase (FBA, A0A078I1P4) was found to be participated in three enriched pathways and so likely to play an important role during the hot-air drying process. FBA is not only one of the essential enzymes in the glycolysis pathway that catalyzes the reversible aldol cleavage of fructose-1,6-bisphosphate (FBP) but also a non-regulated enzyme in the Calvin cycle controlling the flux of carbohydrates (Graciet, Lebreton, & Gontero, 2004; Sonnewald, Lerchl, Zrenner, & Frommer, 1994). Moreover, previous studies revealed FBA also involved in plant defense and response to various abiotic stress, including cold and heat stress (Lu, Tang, Huo, Xu, Qi, Huang, et al., 2012), drought stress (Fan, Zhang, & Zhang, 2009) and water-deficit stress (Khanna, Taxak, Jain, Saini, & Srinivasan, 2014). Combined with the conclusions from these studies, while suffer from hot-air drying, rape bee pollen may protect its membrane integrity and prevent protein degradation by up-regulated the expression of FBA to accumulate more

water-soluble carbohydrates like starch. As for sun dried rape bee pollen, the content of sucrose synthase (A0A816ITC9) and protein with fructokinase activity (A0A078JFK8) which is related to starch synthesis (Schaffer & Petreikov, 1997) decreased, meanwhile, the contents of phosphoenolpyruvate carboxylase (PEPC, A0A817AR13) and biotin carboxyl carrier protein of acetyl-CoA carboxylase (G4WXE0) decreased and increased, respectively. PEPC catalyzes the irreversible reaction of phosphoenolpyruvate (PEP) and HCO₃⁻ to oxaloacetic acid (OAA) and inorganic phosphoric acid to take part in the process of carbon fixation (Izui, Matsumura, Furumoto, & Kai, 2004), and it is also one of the key enzymes that control protein and fatty acid synthesis, to be specific, the inhibition of PEPC will change the direction of carbon atoms from carbohydrate synthesis to fatty acid synthesis by changing the metabolic pattern (Kubis, Pike, Everett, Hill, & Rawsthorne, 2004; Sugimoto, Tanaka, Monma, Kawamura, & Saio, 1989). Acetyl-CoA carboxylase (ACC) participates in the synthesis of fatty acids by catalyzing the carboxylation of acetyl-CoA to produce malonyl-CoA (Y. Wang, Yu, Li, Guo, He, & Wang, 2022) and therefore biotin carboxyl carrier protein of acetyl-CoA carboxylase affects the process of fatty acid biosynthesis. Hence, sun drying treatment led to the increased energy consumption of rape bee pollen and preference for the carbon metabolic pathway that produces higher energy.

It should be noted herein that some functional proteins inherently affected by drying treatments do not mean definite changes in bioactivity whether the DAPs were up-regulation or down-regulation in protein abundance. Further assessment caused by drying treatments from functional perspective should be proceeded.

4. Conclusions

Drying treatment is the most frequently processing step in bee pollen production, thus proteome variations caused by different drying treatments is an important quality-concerned issue and deserve an in-depth study in order to favor the quality maintaining. In this study, protein quantitation analysis for fresh and differently drying-treated rape bee pollen samples was successfully conducted by label-free quantitative proteomic technology. A total of 8377 protein groups were identified among which 1350, 135, 152 proteins were identified as DAPs in FD/Fresh, HD/Fresh and SD/Fresh groups respectively. More than that, comparative analyses regardless of PCA and OPLS-DA or hierarchical cluster analysis all indicated that compared to fresh samples, samples subjected to sun, hot-air and freeze drying experienced remarkable changes in proteomes, of which sun and hot-air drying treatments shared similar alterations and freeze-drying treatment resulted in the greatest variation that beyond expectation. GO annotation revealed that freeze-drying treated rape bee pollen proteins appeared to be outstanding in antioxidation and antianemia bioactive activities, while sun-drying treated samples showed the best in antibacterial activity. Moreover, KEGG pathway analysis evidenced that rape bee pollen would adjust its metabolism to protect itself during the drying process, though metabolism presented varying variations against different drying treatment.

Taken together, different drying methods could cause significant changes on proteomes of rape bee pollen, potentially affecting its nutritional and functional value. And this study could be considered as a preliminary investigation on drying-caused changes from a proteome perspective with the LC-MS/MS proteomic approach. Future study should focus on the optimization of each single drying method and further study functional-related pathways to guide the processing practice and maximize the utilization of bee pollen.

CRediT authorship contribution statement

Shuyue Wang: Investigation, Writing – original draft, Data curation. **Liang Chen:** Writing – review & editing, Visualization. **Shanshan Li:** Writing – review & editing. **Fuliang Hu:** Supervision.

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

No data was used for the research described in the article.

Acknowledgements

This work was supported by the earmarked fund for Modern Agro-industry Technology Research System from the Ministry of Agriculture of China (grant number CARS-44). Besides, we are grateful to Shanghai Bioprofile Technology Co., Ltd. for technical support in mass spectroscopy.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2023.137546>.

References

- Bi, Y. X., Zielinska, S., Ni, J. B., Li, X. X., Xue, X. F., Tian, W. L., ... Fang, X. M. (2022). Effects of hot-air drying temperature on drying characteristics and color deterioration of rape bee pollen. *Food Chemistry-X*, 16.
- Borutinskaite, V., Treigyte, G., Matuzevicius, D., Ceksteryte, V., Kurtinaitiene, B., Serackis, A., ... Navakauskiene, R. (2019). Proteomic studies of honeybee- and manually-collected pollen. *Zemdirbyste-Agriculture*, 106(2), 183–190.
- Borutinskaite, V., Treigyte, G., Matuzevicius, D., Zaikova, I., Ceksteryte, V., Navakauskas, D., ... Navakauskiene, R. (2017). Proteomic Analysis of Pollen and Blossom Honey from Rape Seed *Brassica napus* L. *Journal of Apicultural Science*, 61(1), 73–92.
- Ceksteryte, V., Navakauskiene, R., Treigyte, G., Jansen, E., Kurtinaitiene, B., Dabkeviciene, G., & Balzekas, J. (2016). Fatty acid profiles of monofloral clover beebread and pollen and proteomics of red clover (*Trifolium pratense*) pollen. *Bioscience Biotechnology and Biochemistry*, 80(11), 2100–2108.
- Cytochrome-C Levels in Iron Deficiency without Anemia. (1958). *Nutrition Reviews*, 16(5), 155–157.
- de Arruda, V. A., Santos Pereira, A. A., Estevinho, L. M., & de Almeida-Muradian, L. B. (2013). Presence and stability of B complex vitamins in bee pollen using different storage conditions. *Food and Chemical Toxicology*, 51, 143–148.
- Fan, W., Zhang, Z. L., & Zhang, Y. L. (2009). Cloning and molecular characterization of fructose-1,6-bisphosphate aldolase gene regulated by high-salinity and drought in *Sesuvium portulacastrum*. *Plant Cell Reports*, 28(6), 975–984.
- Frias, B. E. D., Barbosa, C. D., & Lourenco, A. P. (2016). Pollen nutrition in honey bees (*Apis mellifera*): Impact on adult health. *Apidologie*, 47(1), 15–25.
- Ganini, D., Canistro, D., Jiang, J., Stadler, K., Mason, R. P., & Kadiiska, M. B. (2012). Ceruloplasmin (ferroxidase) oxidizes hydroxylamine probes: Deceptive implications for free radical detection. *Free Radical Biology & Medicine*, 53(7), 1514–1521.
- Gaudet, P., Livstone, M. S., Lewis, S. E., & Thomas, P. D. (2011). Phylogenetic-based propagation of functional annotations within the Gene Ontology consortium. *Briefings in Bioinformatics*, 12(5), 449–462.
- Gill, S. S., & Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry*, 48(12), 909–930.
- Graciet, E., Lebreton, S., & Gontero, B. (2004). Emergence of new regulatory mechanisms in the Benson-Calvin pathway via protein-protein interactions: A glyceraldehyde-3-phosphate dehydrogenase/CP12/phosphoribulokinase complex. *Journal of Experimental Botany*, 55(400), 1245–1254.
- Guo, J., Yan, J., Guo, M., & Jin, Y. (2014). Application of reversed-phase liquid chromatography-tandem mass spectrometry in the identification of protein and bioactivity peptides from rape bee pollen. *Se Pu Chinese Journal of Chromatography*, 32(3), 284–289.
- Gutteridge, J. M. (1983). Antioxidant properties of caeruloplasmin towards iron- and copper-dependent oxygen radical formation. *FEBS Letters*, 157(1), 37–40.
- Haro, A., Lopez-Aliaga, I., Lisbona, F., Barrionuevo, M., Alferrez, M. J., & Campos, M. S. (2000). Beneficial effect of pollen and/or propolis on the metabolism of iron, calcium, phosphorus, and magnesium in rats with nutritional ferropenic anemia. *Journal of Agricultural and Food Chemistry*, 48(11), 5715–5722.
- Isik, A., Ozdemir, M., & Doymaz, I. (2019). Infrared drying of bee pollen: Effects and impacts on food components. *Czech Journal of Food Sciences*, 37(1), 69–74.
- Izui, K., Matsumura, H., Furumoto, T., & Kai, Y. (2004). Phosphoenolpyruvate carboxylase: A new era of structural biology. *Annual Review of Plant Biology*, 55, 69–84.

- Keskin, M., & Ozkok, A. (2020). Effects of drying techniques on chemical composition and volatile constituents of bee pollen. *Czech Journal of Food Sciences*, 38(4), 203–208.
- Khanna, S. M., Taxak, P. C., Jain, P. K., Saini, R., & Srinivasan, R. (2014). Glycolytic enzyme activities and gene expression in *Cicer arietinum* exposed to water-deficit stress. *Applied Biochemistry and Biotechnology*, 173(8), 2241–2253.
- Kubis, S. E., Pike, M. J., Everett, C. J., Hill, L. M., & Rawsthorne, S. (2004). The import of phosphoenolpyruvate by plastids from developing embryos of oilseed rape, *Brassica napus* (L.), and its potential as a substrate for fatty acid synthesis. *Journal of Experimental Botany*, 55(402), 1455–1462.
- Liao, Z. M., Allred, J., Keen, C. L., McCune, S. A., Rucker, R. B., & Medeiros, D. M. (1995). Copper deficiency alters isomyosin types and levels of laminin, fibronectin and cytochrome-C-oxidase subunits from rat hearts. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 111(1), 61–67.
- Lill, R., Hoffmann, B., Molik, S., Pierik, A. J., Rietzschel, N., Stehling, O., ... Muhlenhoff, U. (2012). The role of mitochondria in cellular iron-sulfur protein biogenesis and iron metabolism. *Biochimica Et Biophysica Acta-Molecular Cell Research*, 1823(9), 1491–1508.
- Lin, K. H., Chiang, J. C., Ho, Y. H., Yao, C. L., & Lee, H. (2020). Lysophosphatidic acid and hematopoiesis: From microenvironmental effects to intracellular signaling. *International Journal of Molecular Sciences*, 21(6), 2015.
- Liu, J. H., Yang, F., & Mao, S. (2021). Advances in the physiological functions of plant lipids in response to stress. *Chinese Journal of Biotechnology*, 37(08), 2658–2667.
- Lu, W., Tang, X. L., Huo, Y. Q., Xu, R., Qi, S. D., Huang, J. G., ... Wu, C. A. (2012). Identification and characterization of fructose 1,6-bisphosphate aldolase genes in *Arabidopsis* reveal a gene family with diverse responses to abiotic stresses. *Gene*, 503(1), 65–74.
- MacLean, B., Tomazela, D. M., Shulman, N., Chambers, M., Finney, G. L., Frewen, B., ... MacCoss, M. J. (2010). Skyline: An open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*, 26(7), 966–968.
- Manning, R. (2001). Fatty acids in pollen: A review of their importance for honey bees. *Bee World*, 82(2), 60–75.
- Morais, M., Moreira, L., Feas, X., & Estevinho, L. M. (2011). Honeybee-collected pollen from five Portuguese Natural Parks: Palynological origin, phenolic content, antioxidant properties and antimicrobial activity. *Food and Chemical Toxicology*, 49(5), 1096–1101.
- Murata, N., & Tasaka, Y. (1997). Glycerol-3-phosphate acyltransferase in plants. *Biochimica et Biophysica Acta, Gene Regulatory Mechanisms*, 1348(1–2), 10–16.
- Osaki, S., & Walaas, O. (1967). Kinetic studies of ferrous ion oxidation with crystalline human ferroxidase. II. Rate constants at various steps and formation of a possible enzyme-substrate complex. *The Journal of Biological Chemistry*, 242(11), 2653–2657.
- Pereira, J. A., Oliveira, I., Sousa, A., Valentao, P., Andrade, P. B., Ferreira, I. C., ... Estevinho, L. (2007). Walnut (*Juglans regia* L.) leaves: Phenolic compounds, antibacterial activity and antioxidant potential of different cultivars. *Food and Chemical Toxicology*, 45(11), 2287–2295.
- Polat, S., Guclu, G., Kelebek, H., Keskin, M., & Selli, S. (2022). Comparative elucidation of colour, volatile and phenolic profiles of black carrot (*Daucus carota* L.) pomace and powders prepared by five different drying methods. *Food Chemistry*, 369, Article 130941.
- Saisavoey, T., Sangtanoo, P., Chanchao, C., Reamtong, O., & Karnchanat, A. (2021). Identification of novel anti-inflammatory peptides from bee pollen (*Apis mellifera*) hydrolysate in lipopolysaccharide-stimulated RAW264.7 macrophages. *Journal of Apicultural Research*, 60(2), 280–289.
- Schaffer, A. A., & Petreikov, M. (1997). Sucrose-to-starch metabolism in tomato fruit undergoing transient starch accumulation. *Plant Physiology*, 113(3), 739–746.
- Shapla, U. M., Soleyman, M., Alam, N., Khalil, M. I., & Gan, S. H. (2018). 5-Hydroxymethylfurfural (HMF) levels in honey and other food products: Effects on bees and human health. *Chemistry Central Journal*, 12, 35.
- Sonneward, U., Lerchl, J., Zrenner, R., & Frommer, W. (1994). Manipulation of sink-source relations in transgenic plants. *Plant Cell and Environment*, 17(5), 649–658.
- Sugimoto, T., Tanaka, K., Monma, M., Kawamura, Y., & Saio, K. (1989). Phosphoenolpyruvate carboxylase level in soybean seed highly correlates to its contents of protein and lipid. *Agricultural and Biological Chemistry*, 53(3), 885–887.
- Tao, Y. X., Yin, S. T., Fu, L. L., Wang, M., Meng, L. F., Li, F. K., ... Li, Q. Q. (2022). Identification of allergens and allergen hydrolysates by proteomics and metabolomics: A comparative study of natural and enzymolytic bee pollen. *Food Research International*, 158, Article 111572.
- Teixeira, M. C., Coelho, N., Olsson, M. E., Brodelius, P. E., Carvalho, I. S., & Brodelius, M. (2009). Molecular cloning and expression analysis of three omega-6 desaturase genes from purslane (*Portulaca oleracea* L.). *Biotechnology Letters*, 31(7), 1089–1101.
- Vessal, S., Arefian, M., & Siddique, K. H. M. (2020). Proteomic responses to progressive dehydration stress in leaves of chickpea seedlings. *BMC Genomics*, 21(1), 523.
- Wang, J., Chen, Y., Zhao, L., Zhang, Y., & Fang, X. (2022). Lipidomics reveals the molecular mechanisms underlying the changes in lipid profiles and lipid oxidation in rape bee pollen dried by different methods. *Food Research International*, 162(Pt B), Article 112104.
- Wang, S. Y., Bi, Y. X., Zhou, Z. D., Peng, W. J., Tian, W. L., Wang, H., & Fang, X. M. (2022). Effects of pulsed vacuum drying temperature on drying kinetics, physicochemical properties and microstructure of bee pollen. *Lwt-Food Science and Technology*, 169.
- Wang, Y., Yu, W. X., Li, S., Guo, D. Y., He, J., & Wang, Y. G. (2022). Acetyl-CoA carboxylases and diseases. *Frontiers in Oncology*, 12.
- Wisniewski, J. R., Zougman, A., Nagaraj, N., & Mann, M. (2009). Universal sample preparation method for proteome analysis. *Nature Methods*, 6(5), 359–362.
- Yin, S., Tao, Y., Jiang, Y., Meng, L., Zhao, L., Xue, X., ... Wu, L. (2022). A combined proteomic and metabolomic strategy for allergens characterization in natural and fermented *Brassica napus* bee pollen. *Frontiers in Nutrition*, 9, Article 822033.
- Yoshida, C., Murakami, M., Niwa, A., Takeya, M., & Osanai, T. (2021). Efficient extraction and preservation of thermotolerant phycocyanins from red alga *Cyanidioschyzon merolae*. *Journal of Bioscience and Bioengineering*, 131(2), 161–167.
- Zhang, H. F., Lu, Q., & Liu, R. (2022). Widely targeted metabolomics analysis reveals the effect of fermentation on the chemical composition of bee pollen. *Food Chemistry*, 375, Article 131908.