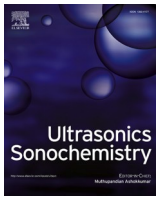
Ultrasonics Sonochemistry 118 (2025) 107359

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultson



Effect of ultrasonic treatment on the microstructure, antioxidant activities and metabolites of camellia bee pollen

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ARTICLE INFO

Keywords:
Camellia bee pollen
Ultrasound treatment
Microstructure
Bioactive components
Antioxidant activities
Metabolomics analysis

ABSTRACT

Ultrasound is an efficient and eco-friendly friendly non-thermal technology for enhancing the extraction of bioactive ingredients from food. This study explored the impact of ultrasound on the microstructure and antioxidant properties of camellia bee pollen. Additionally, the impact of key contributors to antioxidant activity was examined through non-targeted metabolomics analysis. The results showed that ultrasonic exposure progressively degraded the cell walls of bee pollen, resulting in severe collapse of the intine. Notably, this degradation concurrently facilitated the release of polyphenols and flavonoids. The DPPH and ABTS radical scavenging capacity reached the highest after 40 and 60 min of ultrasonic treatment. After 40 min of ultrasonic treatment, the MDA content in camellia bee pollen exhibited a significant rise of 33.47 % compared to the control group, while it further escalated by 57.07 % after 60 min of ultrasonic treatment. Non-targeted metabolomics analysis identified a total of 7 differential metabolites that serve as potential biomarkers for ultrasonic-treated camellia bee pollen. Further analysis of the purine and nucleotide metabolism pathway indicated that the antioxidant defense systems within camellia bee pollen were activated by ultrasonic treatment, leading to a significant enhancement in its antioxidant capacity. These findings establish a solid foundation for the advancement of ultrasound treatment as a novel and green technology to improve the biological activities and qualities of bee pollen.

1. Introduction

Bee pollen is a natural product made from a mixture of floral pollen and special glandular secretions (nectar and saliva) [1]. It is abundant in essential nutrients and bioactive compounds. The class of phenolic compounds found in bee pollen are considered significant bioactive substances, which is comprised mainly of phenolic acids, and flavonoids [2]. Phenolic acids have been reported to exhibit higher antioxidant activities against reactive oxygen species compared to vitamins C and E [3]. Additionally, flavonoids also have remarkable health-promoting effects, encompassing antioxidant, anti-inflammatory, and antimicrobial activities, as well as the prevention of cancer, cardiovascular and neurodegenerative diseases [4]. Many studies have confirmed that phenolic compounds are responsible for the wide spectrum of pharmacological activities attributed to bee pollen [5].

Camellia bee pollen is one of the prominent categories of bee pollen in China and is highly regarded as a functional food. It has been reported that camellia bee pollen has high contents of flavonoids and possesses higher antioxidant activities relative to other types of bee pollen [6]. However, the strong outer layer (called exine) and an inner layer (called intine) of bee pollen grains are highly resistant to acid, alkali, corrosion, biodegradation, and other environmental factors, which largely limit the bioavailability and digestion of bee pollen nutrients. Breaking through these layers is an effective method for enhancing the utilization

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https://doi.org/10.1016/j.ultsonch.2025.107359
Received 4 December 2024; Received in revised form 13 March 2025; Accepted 15 April 2025
Available online 19 April 2025

of intracellular bioactive compounds in bee pollen.

The disruption of cell walls can primarily be achieved through chemical, biological, and physical methods. The chemical methods disrupt the bee pollen wall through organic solvents, which has the disadvantages of cumbersome steps, low efficiency, environmental pollution, and harmful to human health [7]. Microbial fermentation and enzymes are the primary biological methods to break the pollen wall. They are cost-effective, but inefficient and easily contaminated with bacteria from bee pollen [8]. Physical methods commonly employed for disrupting bee pollen walls include radiation, microwaves, and ultrasound. The utilization of radiation and microwaves, despite their comparative simplicity and efficiency, entails significant capital investment and energy consumption. Additionally, the process generates excessive heat during wall-breaking, leading to the loss of heat-sensitive nutrients [9]. With the development of new "green and innovative" techniques, ultrasound has emerged as a promising non-thermal processing technology because of its inherent advantages of cost-effectiveness, energy efficiency, and environmental friendliness [10].

Ultrasound operates by inducing cavitations and microstreaming effects that break down the plant stromal cell walls and enhance the permeability of plant tissues, thus facilitating the release of bioactive compounds. Liu et al. (2015) examined the impact of physical methods on breaking the wall of *Pinus massoniana* pollen and found that ultrasonic treatment was significantly more effective than freeze–thaw treatment for this purpose. The ultrasonic extraction of various food bioactive compounds is mainly reported for 20 to 40 kHz with extraction durations ranging from 10 to 60 min, as higher ultrasonic frequencies make it more challenging to form cavitation bubbles [11]. Meanwhile, the ultrasound time is a key factor affecting the release of bioactive compounds. In the initial stage of ultrasonic extraction (approximately 10–20 min), there will be no significant increase in the extraction rate. This is because it takes a longer time for ultrasound to disrupt the material's structure and facilitate the release of internal components. While a lengthier ultrasonic treatment period gradually releases internal components outward, which hinders improvement in extraction rate [12]. Ghafoor et al. (2009) found that the duration of ultrasound-assisted extraction significantly influenced the extraction of phenolic compounds and antioxidants from grape seed, with optimal times being 29.03 min for phenolic compounds and 30.58 min for antioxidants [13]. Therefore, it is necessary to explore the ultrasound conditions for enhancing the bioactivities of camellia bee pollen and identify the potential markers that contribute to bioactivities.

Metabolomics is an emerging and powerful "omics" method with high throughput, high sensitivity and accuracy, and short analysis time. It can be used to detect thousands of metabolites simultaneously and quickly provide a visual analysis of differences between samples [14]. In recent years, metabolomics has been widely used to search for some potential characteristic metabolites for identifying contributors to biological activity. The global distribution of bee pollen metabolites after ultrasound treatment has not been reported in the literature.

Based on the above, this study comprehensively elucidated the effects of different ultrasonic times on the bioactive components and antioxidant activities of camellia bee pollen. The changes in the metabolites of camellia bee pollen at different ultrasonic times were studied by non-targeted metabolomics analysis and the main contributors to antioxidant activity were identified. This study provides the foundation for the utilization and deep processing of bee pollen.

2. Materials and methods

2.1. Chemicals and reagents

Folin-Ciocalteu, methanol, anhydrous ethanol, anhydrous sodium acetate, potassium acetate, sodium carbonate and aluminum nitrate were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China), with $\geq$ 99.7 % purity. Chemical standards including rutin and gallic acid were obtained from Yuanye Biological Technology Co., Ltd. (Shanghai, China), with $>$ 98 % purity. 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azinobis-3-ethylbenzthiazoline-6-sulphonate (ABTS) scavenging ability detection kits were supplied by Solarbio Science & Technology Co., Ltd. (Beijing, China). Malondialdehyde (MDA) assay kit was supplied by Solarbio Science & Technology Co., Ltd. (Beijing, China). Water was purified using a Milli-Q system, Millipore (Darmstadt, Germany). HPLC-grade acetonitrile and formic acid were obtained from Thermo Fisher Scientific (Waltham, USA).

2.2. Raw materials

Fresh camellia bee pollen was provided by the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences (Beijing, China), which was collected from Wuyi Street, Wuyishan City, Fujian province, China (27°70′N,118°00′E). The species of bee pollen samples were confirmed by scanning electron microscopy (SEM) (SU8100, Hitachi Inc., Japan) based on palynology. Samples with consistent size were carefully selected to ensure the uniformity of the material's physicochemical properties.

2.3. Ultrasound treatment

Ultrasound treatment of bee pollen was conducted in accordance with previously published methods with minor modification [15]. The mass of 100 g ground pollen was weighed into a beaker and 400 mL of ultrapure water was added and stirred to uniformity. The mixture sample was divided into three beakers and then the beakers were immersed in an ice bath to minimize thermal effects. The samples were sonicated at a constant frequency of 40 kHz and an output power of 400 W for 20, 40, and 60 min respectively by using an ultrasonic processor (KQ-500DE, Kunshan Ultrasonic Instruments Co., Ltd, China). The treated samples were dried to a final moisture content of $\leq$ 8 % in a vacuum oven (DZF-6090, Heng scientific instrument Co., Ltd, Shanghai, China) at 60 Pa, and the temperature of the heating plate and cold trap were 50 ℃ and −40 ℃, respectively. The dried samples were finely ground into a powder with an analytical grinder (IKA®A11 basic) and then passed through a 150-mesh sieve. All dried samples were hermetically sealed and stored under controlled conditions at −18℃ before the experiment.

2.4. Microstructure observation

Appropriate amounts of fresh and treated samples of camellia bee pollen were uniformly coated on the conductive adhesive respectively. Furthermore, the samples were placed in the sample chamber of an E-1045 ion sputtering apparatus and sprayed with gold at a current of 15 mA for 90 s. The structure morphology was performed using a tabletop scanning electron microscope (SU3500, Hitachi, Japan) at 10.0 kV.

2.5. Analysis of total phenol content (TPC) and total flavonoid content (TFC)

The contents of total polyphenols and total flavonoids of bee pollen samples were performed according to previously reported methods by Ren et al. (2023) with minor modifications [16]. The mass of 1.00 ± 0.05 g fresh and treated bee pollen samples was weighed separately, and extracted with 50 mL of 80 % methanol solution at 40 °C for 1h in a shaking water bath. Centrifugation was performed at 10,000 × *g* for 10 min. A volume of 2.5 mL collected supernatant was mixed with 2.5 mL of 20 % Folin-Ciocalteu and 5 mL of 20 % Na_2CO_3 solutions. The mixture was placed in the dark at room temperature for 30 min, after which the absorbance at 765 nm was measured with a spectrophotometer (Cary 3500, Agilent Technology Co., Ltd., USA). The results were expressed as milligrams of gallic acid equivalents (GAE) per gram (mg/g) with gallic acid curve as a standard ($R^2 = 0.9995$).

The flavonoid extraction was carried out by mixing 1.00 ± 0.05 g of bee pollen samples with 50 mL of 50 % methanol solution and shaking horizontally at 25℃ for 24 h. The mixture was centrifuged at 10,000 × *g* for 10 min. The volume of 1 mL of $Al(NO_3)_3$ (100 g/L) solution and 1 mL of $Al(NO_3)_3$ (9.8 g/L) were added to the supernatants. The absorbance was read at 415 nm. The results were expressed as milligrams of quercetin equivalents (QE) per gram (mg/g) with quercetin curve as a standard ($R^2 = 0.9989$).

2.6. Analysis of malondialdehyde (MDA)

According to the manufacturer's instructions, samples were homogenized and extracted with phosphate buffer solution (0.05 M, pH 7.8) at 4 °C. The supernatant was taken and 2.5 mL of thiobarbituric acid was added, then the tube was placed in a boiling water bath for 60 min and rapidly cooled in an ice bath. After centrifugation at 10,000 × *g* for 10 min at 4°C, the absorbance was measured at 532 nm and 600 nm, respectively.

2.7. Analysis of vitro antioxidant activity

The comparative antioxidant capacities of the different extracts were determined by DPPH and ABTS assays. These analyses were carried out following the manufacturer's instructions. Vitamin C was used as a positive control. The absorbance was detected at 517 nm for DPPH and at 734 nm for ABTS.

The radical scavenging activity (%) was expressed as follows:

$$(\%) = 1\text{–}100\ \% * (A_1 - A_2)/A_0.$$

where A_0 is the sum of absorbance values of negative control (DPPH/ABTS + ethanol solution); A_1 is the sum of absorbance values of reaction solution (DPPH/ABTS + sample); A_2 is the sum of absorbance values of blank control (sample + ethanol solution).

2.8. Analysis of metabolites

Metabolite extraction and UPLC-MS/MS analysis were performed following the methods reported by Zhang et al. (2023) with minor modifications [7]. The fresh and treated samples were crushed in an analytical grinder (IKA®A11 basic) for 1 min. Then, samples (100 mg for each) were precisely weighed and dissolved in 1.2 mL of 70 % methanol extract. After vortexing and centrifugation at 10,000 × *g* for 10 min at 4 °C. The supernatant solutions were filtered using 0.22 μm nylon membrane before UPLC-MS/MS analysis. Quality control (QC) samples were obtained by mixing 20 μL of each sample in a vial. The QC sample was inserted regularly and analyzed both before and after the group.

The extracts (4 μL for each) were respectively injected into a UPLC-MS/MS system (UPLC, SHIMADZU Nexera X2; MS, Applied Biosystems 4500 Q TRAP, USA). Waters ACQUITY UPLC HSS T3 column (1.8 μm, 2.1 mm × 100 mm) was selected and the column temperature was set at 40 °C. Samples were eluted using a step-wise gradient of water and acetonitrile (both A and B containing 0.1 % formic acid, v/v) as follows: 0–5 min, 95 % A; 5–6 min, 35 % A; 6–7.5 min, 1 % A; and 7.5–10 min, 95 % A, with a 0.4 mL/min flow rate. The MS parameters were as follows: ion source temperature, 550 °C; ion spray voltage, 5000 v (ESI +) and −4000 v (ESI-); nebulizer pressure, 50 psi. The acquisition ranges between *m/z* 25 and 1250.

2.9. Statistical analysis

All analyses were performed in triplicate and the experimental results were presented as means ± standard deviation. One-way analysis of variance followed by Duncan Multiple Comparison Test at 5 % probability level was conducted to determine the differences between samples. Statistical analysis was run using SPSS 26.0 (SPSS Inc., Chicago, USA) and Origin 2024 (OriginLab Inc., Northampton, USA) softwares. Analyst 1.6.1 software (AB SCIEX Pet. Ltd, Framingham, USA) was used for data filtration, alignment, and calculation. Metabolites were identified based on internal databases and public databases (MassBank, KNApSAck, HMDB, MoTo DB and METLIN). Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) was performed using SIMCA software version 14.1 (MKS Umetrics, Malmö, CH), volcano plot was using OmicShare tools (https://www. omicshare.com/tools), lollipop chart and flower plot were using ChiPlot tools (https://www.chiplot.omline). The screened metabolites with KEGG ID obtained from a volcano analysis were input into MetaboAnalyst 4.0 (https://www.metaboanalyst.ca) and then linked to KEGG (https://www.kegg.jp) to perform metabolic pathway analyses, and their significance was determined by the *P* value of the hypergeometric test. All remaining figures were drawn using GraphPad Prism 8.0 software (California, USA).

3. Results and discussion

3.1. Microstructure

The SEM images in Fig. 1 illustrate the morphological variations among camellia bee pollen spores treated with different ultrasonic treatments. Fresh camellia bee pollen (Fig. 1A) showed a spherical shape with a complete pollen coat and the cell surface exhibits three germinal furrows, characterized by a wider middle region and narrower ends. After undergoing different ultrasonic durations (20, 40, and 60 min), the integrity of bee pollen cells was compromised, leading to the occurrence of cell wall cracks, deformations, and structural loss. At 20 min of ultrasonic duration (Fig. 1B), the exine of bee pollen cells started to rupture from the germinal aperture. After 40 min of ultrasonic duration (Fig. 1C), the bee pollen was distorted and the width of the embryoid opening increased. The crack in the germinal aperture was observed to gradually enlarge and deepen. The cellular morphology became irregular, with the inner cell wall collapsing inward and the protoplast was observed to be sequentially extruded from gaps in the exine. At 60 min of ultrasonic duration (Fig. 1D), the cell walls of bee pollen were destroyed, accompanied by severe collapse of the intine.

The microstructure observation reveals that the germinal apertures of camellia bee pollen are situated in the opening of the thin-walled region at the exine, exhibiting the distinctive feature of low mechanical strength. The cavitation, mechanical, and thermal effects of ultrasound induce the pollen cells to dehisce easily from the germinal apertures, leading to the disruption of plasmalemma and tonoplast as well as the distortion and loosening of cell walls. These may lead to a dramatic decrease in cellular turgor pressure. When cells lose their turgidity, they are more prone to deformation. Additionally, the extension of ultrasonic time enhanced the expansion of micro-channels and rupture of the cell walls, which may result in the release of cell contents [17]. Some studies have demonstrated that the mechanical disruption of the pollen layer can effectively increase the bioavailability of bee pollen grains [2]. However, bioactive ingredients may also be degraded due to cavitation and sonochemical reactions occurring simultaneously or in isolation [18]. Therefore, the selection of optimum ultrasonic duration is crucial to avoid undesirable degradation of the extracted bioactive components in camellia bee pollen.

3.2. TPC and TFC

Polyphenols and flavonoids have been identified as the major compounds in bee pollen responsible for antioxidant activities [7]. In Fig. 2A and 2B, the polyphenols and flavonoids in camellia bee pollen were significantly increased by 97.01 % and 121.11 % after 40 min of ultrasonic treatment respectively ($P < 0.05$). This further confirmed that the cell walls of camellia bee pollen were destroyed by ultrasound treatment, as bioactive substances (such as polyphenols and flavonoids) were

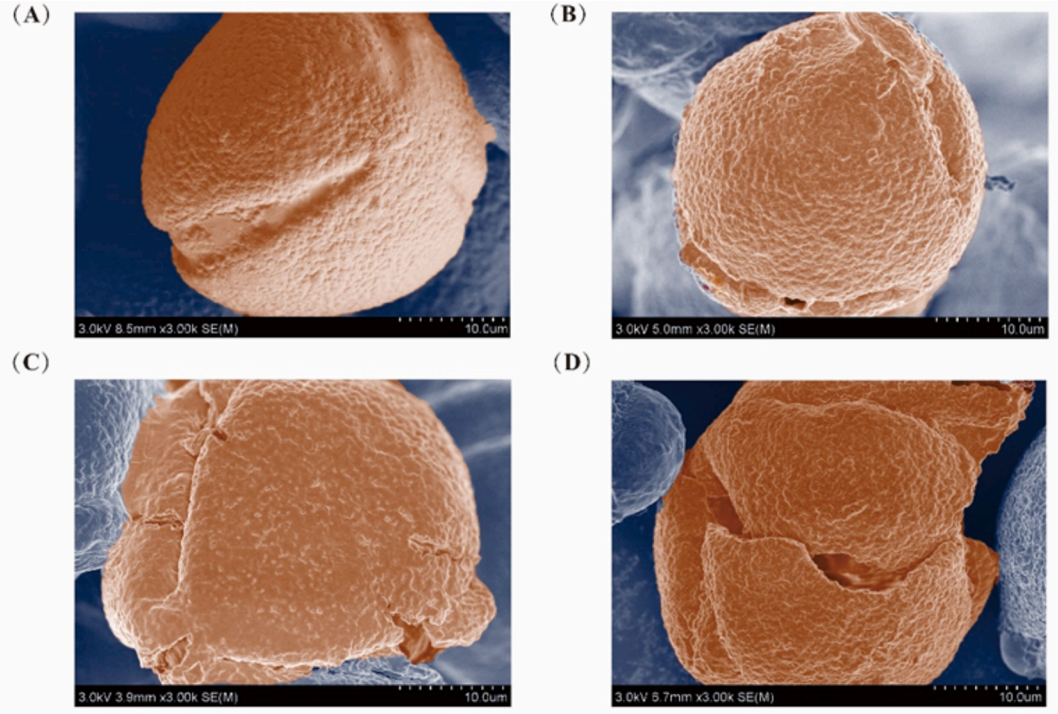

Fig. 1. The microstructure of camellia bee pollen at 3,000x under ultrasound treatment, CK (without ultrasound treatment) (A), ultrasound treatment for 20 min (B), ultrasound treatment for 40 min (C) and ultrasound treatment for 60 min (D).

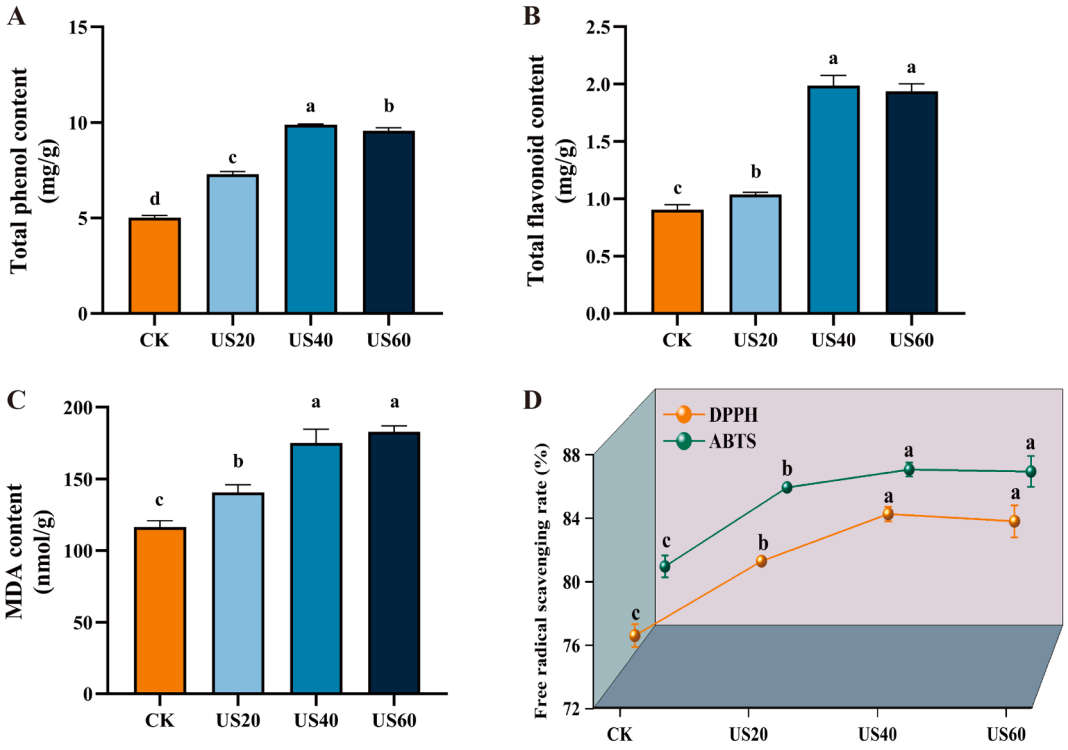

Fig. 2. The total phenol content (A), total flavonoid content (B), malondialdehyde content (C) and the ABTS and DPPH scavenging rate (D) of camellia bee pollen under ultrasound treatment.

usually stably bound to cellulose, hemicellulose, or cytoskeletal proteins in bee pollen cells [19]. On the one hand, ultrasonic cavitation led to the destruction of the bee pollen cell walls by fragmentation, erosion, and detexturation, resulting in the direct release of polyphenols and flavonoids initially present in intact cells [8]. On the other hand, the collapse of the cavitation bubbles led to the generation of microjets and strong shear forces, thereby potentially facilitating the conversion of bound phenolic compounds into free phenolic compounds and enhancing phenolic exudation [19–21]. Liu et al. (2015) also reported significant increases in phenolics and flavonoids of *Pinus massoniana* pollen under

sonication for 10, 30, and 50 min [11]. The TPC of the samples treated with ultrasound for 50 mins at an amplitude of 60 % was nearly doubled, while the TFC showed an approximate 50 % enhancement [11]. However, as the ultrasonic time increases beyond the optimal duration, the TPC of camellia bee pollen decreased significantly to 9.57 ± 0.15 mg GAE/g after 60 min of ultrasonic treatment compared to the group treated with ultrasound for 40 min, while there was no significant difference in TFC. The intensification of heat and pressure increases covalent bond cleavage or expedited oxidations, which has augmented the probability of compound degradation over time. Another potential factor could be the gradual increase in the number of cavitation microbubbles produced by ultrasound [22]. Long-time ultrasonic cavitation resulted in the generation of free radicals (reactive HO and hydrogen atoms), which caused the degradation of polyphenols and flavonoids by the hydroxylation in para-, meta-, and ortho positions [23].

3.3. MDA

Camellia bee pollen is rich in fatty acids such as α-linolenic acid and linoleic acid, and ultrasound treatment can release the fatty acids due to the wall-disruption [9]. MDA, as a byproduct of lipid peroxidation, reflects the degree of lipid deterioration and the damage of antioxidant capacity in camellia bee pollen [24]. It is generated during oxidative processes through the degradation of polyunsaturated fatty acids, particularly those containing two or more double bonds. As illustrated in Fig. 2C, the MDA content of camellia bee pollen was significantly increased by 33.47 % and 57.07 % after 40 and 60 min of ultrasonic treatment compared to CK ($P < 0.05$). This was attributed to the mechanical and chemical effects of ultrasound treatment. The ultrasound cavitation zone, characterized by high temperature and high pressure, where the MDA was more prone to be generated due to the decomposition and oxidation of polyunsaturated fatty acids [24]. Additionally, the reactive radicals (such as hydroxyl radicals, hydrogen radicals, and perhydroxyl radicals) produced by the decomposition of water vapor, accelerated the formation of MDA [25]. Similarly, Qi et al. (2023) reported that the lipid oxidation and the formation of MDA in chicken broth were accelerated attributed to the increase of free radicals with the extension of ultrasound time [26]. Bao et al. (2022) also found that the MDA contents of dry-cured yak meat after ultrasound treatment in 400 W were significantly higher compared with the control [27]. In contrast to our study, the MDA contents of fresh-cut cucumber and straw mushroom after 15–30 min ultrasound treatment were not significantly changed [28,29]. This was attributed to the fact that the ultrasound treatment for the short term can maintain better membrane integrity of fresh-cut cucumber and straw mushrooms. However, the cell walls of camellia bee pollen were severely destroyed after 40 min ultrasound treatment in this study. Furthermore, the MDA is also a biomarker for evaluating oxidative stress [30]. Thus, the antioxidant defense systems of camellia bee pollen might be activated by the increase of MDA.

3.4. Antioxidant capacity

The polyphenols exert their antioxidative and free radical scavenging effects in vivo through diverse mechanisms [19]. In this study, the DPPH and ABTS scavenging rates were used as the in vitro evaluation methods to evaluate the antioxidant and free radical effects of camellia bee pollen during the ultrasonic process, and the results are shown in Fig. 2(D). Both the camellia bee pollen treated with ultrasound for 40 and 60 min showed significant DPPH and ABTS radical scavenging effects. Specifically, the scavenging rate of DPPH and ABTS after ultrasound for 40 min reached the highest, increasing to 83.07 ± 0.46 % and 83.66 ± 0.12 %, respectively. There was no significant difference in DPPH radical scavenging rate between ultrasound treatment for 60 min and ultrasound treatment for 40 min ($P > 0.05$). It can be attributed to the mechanical and chemical impacts on the walls of camellia bee pollen as well as bioactive components such as peptides, amino acids, enzymes, and polyphenols. On the one hand, ultrasonic cavitation was able to break down the camellia bee walls, which accelerated the release of antioxidant compounds (polyphenols and flavonoids). The obtained results were consistent with the findings reported by Yang et al. (2019) and Liu et al. (2015), demonstrating that ultrasonic treatment significantly enhanced the DPPH and ABTS radical scavenging activity of rose and *pinus massoniana* bee pollen compared with CK due to the release of flavonoids and spermidine [11,31]. Our results also showed that the polyphenols and flavonoids content of camellia bee pollen exhibited a positive correlation with antioxidant capacity. On the other hand, ultrasound treatment can induce the formation of free radicals. Consequently, serving as a stressor, it triggered the activation of the antioxidant system of camellia bee pollen, thereby enhancing the activity of antioxidant enzymes such as peroxidase and facilitating the synthesis of secondary metabolites like polyphenols and flavonoids [32]. In addition, Qiao et al. (2024) also reported that ultrasound enhanced the antioxidant activity of soybean sprouts by inciting the antioxidant defense response system [33]. Ji et al. (2022) revealed that the increase of phenolic compounds and antioxidant enzyme activity in coffee leaves serves as a defense mechanism against ultrasound stimulation [34]. Therefore, it is necessary to further elucidate the mechanism in the antioxidant capacity of camellia bee pollen following ultrasonic treatment by non-targeted metabolomics analysis.

3.5. Metabolomic analysis

An untargeted UPLC-MS/MS metabolomics profiling was conducted to reveal the modification mechanisms in camellia bee pollen treated by ultrasonic treatment. By overlapping the total ion chromatography figure of the first and last acquisition of the QC sample in Fig. S1, two TIC lines highly coincidence that the instrument of high stability provides a basis for the repeatability and reliability of the data.

OPLS-DA model was used for discriminant analysis in this study, which can extract variable information. As shown in Fig. 3A and C, all samples fell within the 95 % confidence interval. The OPLS-DA model for CK and US treatment group showed a significant difference ($P < 0.001$). Then, the three treatment groups for US20, US40, and US60 were found to be separated obviously ($P < 0.05$). The aforementioned results indicated that the application of ultrasonic could impact the metabolic spectrum significantly of camellia bee pollen. Furthermore, the predictability of OPLS-DA model was confirmed by 200 permutation tests (CK vs US, $R^2Y = 0.983$, $Q^2Y = -0.482 < 0$; US20 vs US40 vs US60, $R^2Y = 0.704$, $Q^2Y = -0.432$) (Fig. 3B and D), suggesting that no obvious overfitting occurred and the model could be suitable in further screening different metabolites.

The ultrasonic treatment with different ultrasonic duration resulted in differences in the composition of metabolites in camellia bee pollen (Fig. 3). To further understand the metabolic differences, a volcano plot was generated to determine the number of significantly upregulated and downregulated metabolites in CK and US treated camellia bee pollen (Fig. 3E). There were 282 different compounds (194 upregulated and 88 downregulated) between CK and US20, 764 (534 upregulated and 230 downregulated) between CK and US40, 454 (143 upregulated and 311 downregulated) between CK and US60. Based on the number of different metabolites, US40 treatment group has the most significant difference compared with CK group. Thus, the subsequence analysis would be carried out on this result.

Among all the identified difference metabolites between US40 and CK treatment groups, we have filtrated the top 20 difference metabolites with the variable importance projection (VIP) > 1, which obtained from the OPLS-DA model between US40 and CK treatment groups in Fig. S2, included protoveratrine A, puerarin, Leu-Lys, and atractyloside I, et al. (Fig. 3(F)). According to Fold Change of difference metabolites from volcano plot (Fold Change ≤ 0.5 or ≥ 2, $P < 0.05$), the top 20 difference metabolites included 7 nucleotides and derivatives (guanine, xanthosine 5′-monophosphate, inosine, guanosine, adenosine, inosinic acid,

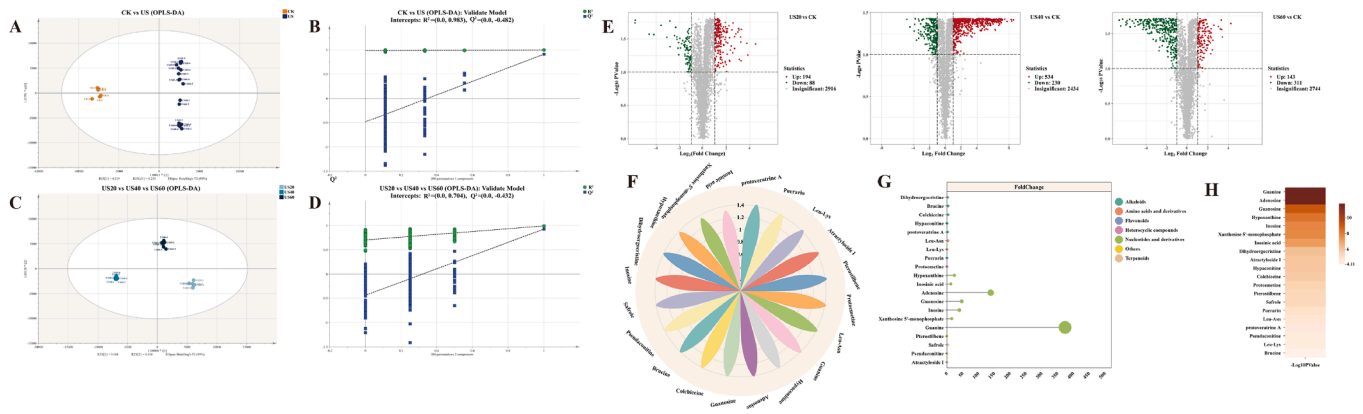

Fig. 3. Multivariate statistical analysis of camellia bee pollen under ultrasound treatment. The OPLS-DA score plot between CK and US treatment groups (A), and between US20, US40 and US60 treatment groups (C). The presentation of chance permutation at 200 times used for the discrimination between CK and US treatment groups (B), and between US20, US40 and US60 treatment groups (D). The volcano plots of differential metabolites in camellia bee pollen under ultrasound treatment (E). The top 20 different metabolites metabolites in camellia bee pollen under ultrasound treatment, overview (F), fold change (G) and heatmap visualization (H).

hypoxanthine), 5 alkaloids (protoveratrine a, hypaconitine, colchiceine, brucine, dihydroergocristine), 3 terpenoids (atractyloside I, pseudaconitine, safrole), 2 amino acids and derivatives (Leu-Lys, Leu-Asn), 1 heterocyclic compound (protoemetine) and 1 flavonoids (puerarin) (Fig. 3G). In addition, the different metabolites such as guanine, adenosine, guanosine, hypoxanthine, inosine, xanthosine 5′-monophosphate, and inosinic acid had a more significant contribution in the inter-group separation model according to Fig. 3H. This indicated that these metabolites were greatly affected by the physiological and metabolic activities of camellia bee pollen with the ultrasonic treatment.

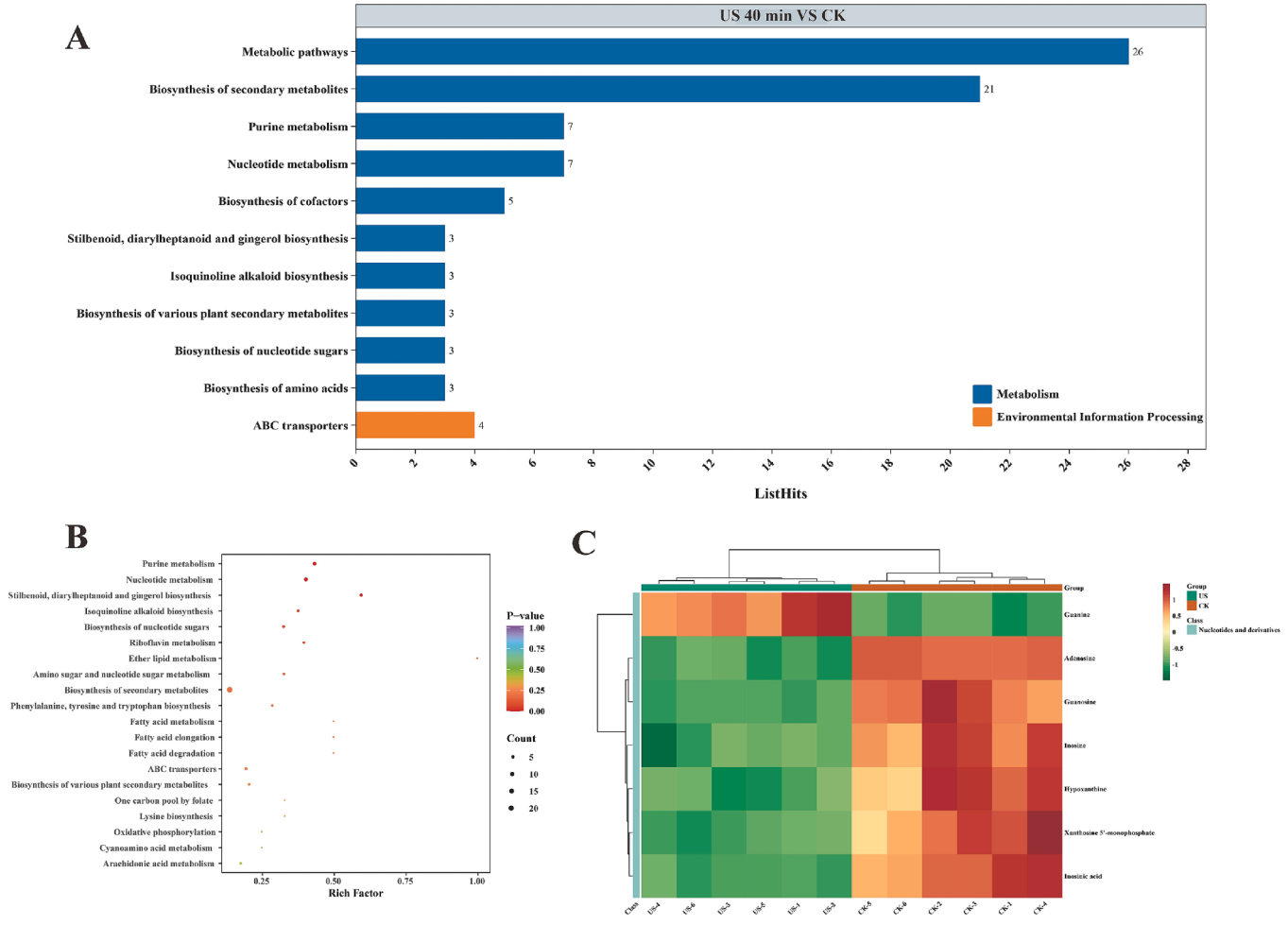

Fig. 4. Differential metabolites and KEGG pathway enrichment analysis of camellia bee pollen under ultrasound treatment. Mapping of differential metabolites between CK and US 40 min treatment groups onto KEGG metabolic pathways (A). The top 20 KEGG pathways enriched by the significantly different metabolites in camellia bee pollen under ultrasound treatment (B). The heatmap visualization of the identified metabolites on purine metabolism and nucleotide metabolism pathways (C).

Consistent with our results, the inosine monophosphate (IMP), is synthesized in a 12-step biosynthesis process that consumes ATP, which can be converted into adenosine monophosphate (AMP), xanthosine monophosphate (XMP) and guanylate (GMP) under the catalysis of adenylosuccinate synthase and inosine-5′-monophosphate dehydrogenase [35,36]. In addition, the purine nucleotides can be easily dephosphorylated into purine nucleosides catalyzed by nucleotidase, thereby purine nucleosides are converted to purines by purine nucleoside phosphorylase (PNP) such as the inosinic acid is easily degraded into inosine and hypoxanthine [35,36]. Therefore, the upregulation or downregulation of substances such as guanine, inosine, and nucleic acid might be used as a potential marker of camellia bee pollen response to ultrasonic treatment.

The KEGG database was used to infer the interconnectedness of the 20 differential metabolites in metabolic pathways. The results showed that the differential metabolites were mainly enriched in 2 classes of metabolic pathways, metabolism and environmental information processing (Fig. 4(A)). The top 20 enriched KEGG pathways for the metabolites in camellia bee pollen, treated with ultrasonic waves, are displayed in Fig. 4(B). Among them, the purine metabolism and nucleotide metabolism pathways enriched most of the differential metabolites, and the distribution of the identified metabolites on both pathways was displayed in Fig. 4(C). Ultimately, a total of 7 differential metabolites were identified as potential biomarkers for the camellia bee pollen treated by ultrasonic, including guanine, xanthosine 5′-monophosphate, inosine, guanosine, hypoxanthine, adenosine and inosinic acid (Fig. 5). Purine and nucleotide metabolism constitute a main pathway for organism [35]. They are involved not only in DNA and RNA synthesis and degradation but also in balancing adenosine triphosphate (ATP) demand and supply ATP for camellia bee pollen to tolerate the stress caused by environmental stress such as ultrasonic stress, osmotic pressure, and high temperature and pressure. The de novo biosynthetic pathway and the complementary salvage pathway are the 2 main pathways for nucleotide synthesis. The biosynthetic process of synthesizing

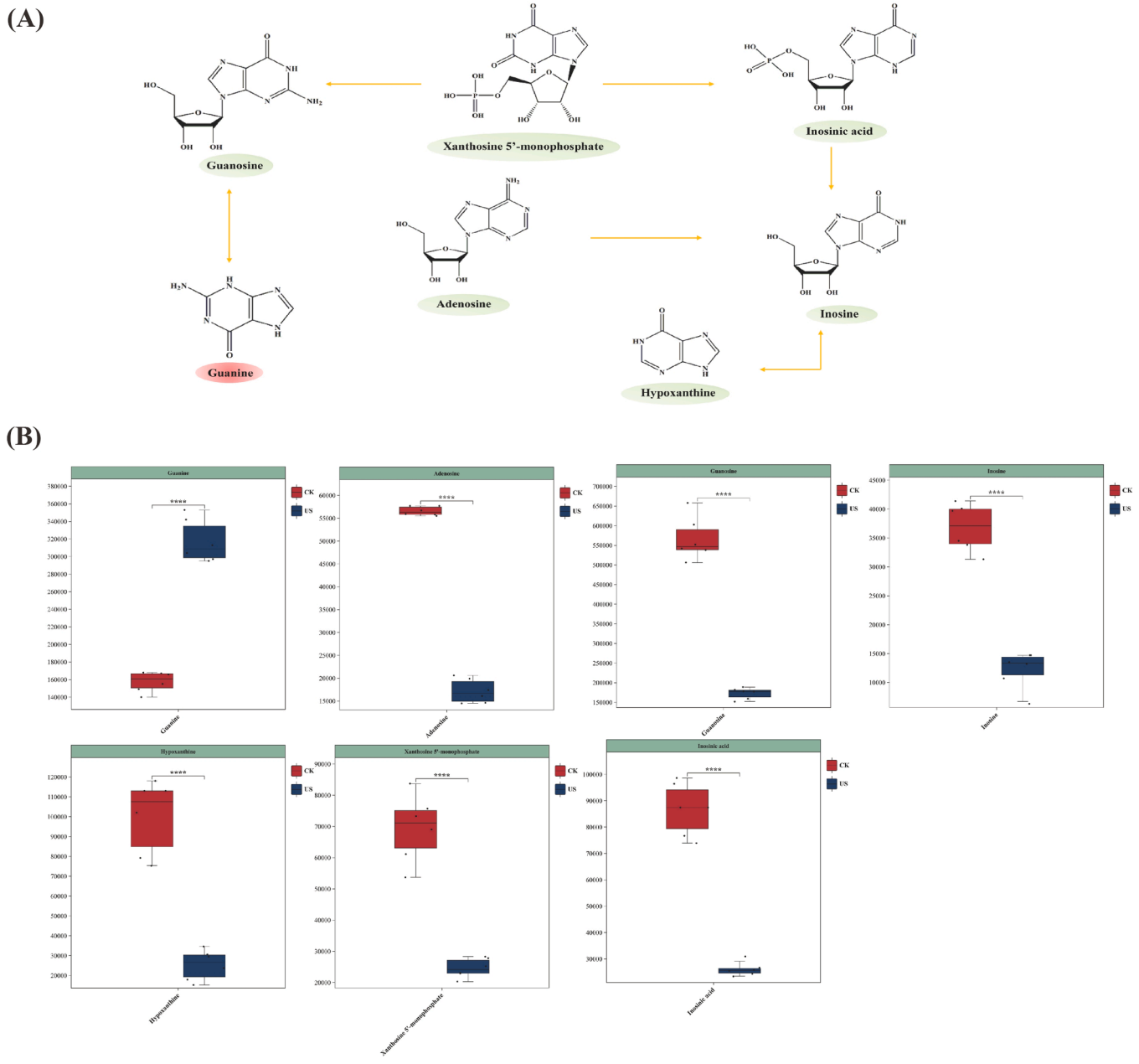

Fig. 5. The metabolism pathways (A) and boxplots of the primary differential metabolites of camellia bee pollen between CK and US treatment groups (B).

nucleotides from simple substances, including ribose phosphate, amino acids, one-carbon units, and CO_2 with consumption of ATP, is identified as the de novo biosynthetic pathway [35]. To avoid energetically costly processes, the complementary salvage pathway converts free purines or purine nucleosides into nucleotides. [35]. As shown in Fig. 5, the metabolites including xanthosine 5′-monophosphate, inosine, guanosine, hypoxanthine, adenosine, and inosinic acid associated with purine and nucleic acid metabolism were significantly reduced in camellia bee pollen cells subjected to ultrasonic stress. This was attributed to an adaptive response or the impaired DNA and RNA synthesis in camellia bee pollen cells. To ensure that camellia bee pollen has sufficient ATP to withstand ultrasonic stress, the metabolites shown in Fig. 5 (inosine, guanosine, hypoxanthine, and adenosine) play a role in the complementary salvage pathway, helping to reduce ATP consumption. On the other hand, the interaction of the reactive oxygen species (ROS) generated by ultrasonic treatment with DNA had the potential to induce DNA lesions and inhibit DNA biosynthesis [36]. Thus, the xanthosine 5′-monophosphate, inosine, guanosine, hypoxanthine, adenosine, and inosinic acid decline could also be attributed to the impaired DNA and RNA synthesis in camellia bee pollen cells. Xu et al. (2024) also found that *Listeria innocua* cells reduce nucleotide biosynthesis and conserve energy to enhance tolerance to the external environment [37]. Kuo et al. (2022) reported that the purine metabolism may balance ATP for shrimp to tolerate the stress caused by the environment or pathogen [35]. However, the guanine in camellia bee pollen cells subjected to ultrasonic treatment was significantly increased compared to CK. This may be attributed to the response to oxidative stress. Guanine, which has the lowest oxidation potential among the four DNA bases, is more prone to be oxidized [36]. Furthermore, the 8-hydroxy-2′-deoxyguanosine as the major oxidation product of guanosine is a key biomarker for evaluating oxidative stress [36]. Therefore, the decrease of guanosine might also be attributed to the ROS, which further confirmed that ultrasonic treatment induced oxidative stress in camellia bee pollen. As a defense against oxidative stress, more guanine was synthesized from simple substances such as amino acids and CO_2 or converted from guanosine under the catalysis of PNP in Fig. 5. In addition, Li et al. (2021) also confirmed that plants can activate the defense mechanisms to protect against external environmental stress, and the relevant response can be observed [38]. Zeng et al. (2023) also found that ultrasonic treatment significantly improved the activities of antioxidant enzymes (SOD, CAT, and POD) in sugarcane leaves, which is possibly due to the antioxidant defense systems activated by ROS [39]. In summary, the changes of the purine and nucleotide metabolism indicated that the antioxidant defense systems of camellia bee pollen activated by ultrasonic treatment, which significantly increased the antioxidant capacity might by balancing ATP, promoting the biosynthesis of polyphenols and flavonoids and improving the activities of antioxidant enzymes.

4. Conclusion

The ultrasonic treatment can effectively destroy the bee pollen walls and increase the antioxidant activities of camellia bee pollen. The microstructure analysis demonstrated that an increase in ultrasonic treatment time led to gradual destruction of the cell walls of bee pollen, accompanied by severe collapse of the intine. Meanwhile, the degradation of the bee pollen wall concurrently facilitated the release of polyphenols and flavonoids. The DPPH and ABTS radical scavenging capacity reached the highest after 40 and 60 min of ultrasonic treatment with no statistically significant difference observed ($P < 0.05$). Due to the mechanical and chemical effects of ultrasound treatment, the polyunsaturated fatty acids in camellia bee pollen were degraded to MDA, resulting in a significant increase of 33.47 % and 57.07 % after 40 and 60 min of ultrasonic treatment compared to CK. It is worth to note that the non-targeted metabolomics analysis indicated that a total of 7 differential metabolites were identified as potential biomarkers for the camellia bee pollen treated by ultrasonic, including guanine, xanthosine 5′-monophosphate, inosine, guanosine, hypoxanthine, adenosine and inosinic acid. Additionally, the purine and nucleotide metabolism pathway analysis further revealed that the antioxidant defense systems of camellia bee pollen were activated by ultrasonic treatment, which significantly increased the antioxidant capacity by balancing ATP and promoting the biosynthesis of polyphenols and flavonoids. In summary, these findings provide the theoretical basis for the advancement of ultrasound treatment as an emerging green and environment-friendly technology to improve the biological activities and qualities of bee pollen. Ultrasonic technology has great potential for industrial scale application in the processing of bee pollen. Future research will focus on investigating the impact of optimizing ultrasonic-assisted extraction parameters and elucidating the mechanisms of mechanical action on the bioactive compounds in bee pollen.

CRediT authorship contribution statement

Yanxiang Bi: Writing – original draft, Software, Methodology, Conceptualization. **Shiye Luo:** Writing – original draft, Software, Methodology, Conceptualization. **Jiabao Ni:** Writing – review & editing, Software, Methodology, Conceptualization. **Song Miao:** Writing – review & editing, Resources, Conceptualization. **Zhen Ning:** Methodology, Data curation. **Zhihao Zhang:** Writing – review & editing, Resources. **Sijia Xu:** Writing – review & editing. **Wenli Tian:** Writing – review & editing, Formal analysis. **Wenjun Peng:** Writing – review & editing, Funding acquisition, Conceptualization. **Xiaoming Fang:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by National Natural Science Foundations of China (Nos. 32472396, 31871861 and 31501548), The Apicultural Industry Technology System (NCYTI-43-KXJ17), and The Science and Technology Innovation Project of Chinese Academy of Agricultural Sciences (CAAS-ASTIP-2015-IAR). The authors also would like to express their gratitude to the anonymous referees for their valuable comments and suggestions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2025.107359.

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