Antimicrobial activities of polyphenol-based metabolites present in lettuce and recent methods for their estimation

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Abstract

The tissue structure of fresh-cut lettuce is easy to be damaged during processing and transportation, which leads to the reduction of its edible quality and safety. Ultra-high-performance liquid chromatography (UHPLC) combined with time-of-flight tandem mass spectrometry (TMS) metabolomics was used to identify the differential metabolites related to the antibacterial activity in lettuce and explore their bacteriostatic mechanism. The experimental results revealed that the growth of *Escherichia coli* was quite different when different varieties of lettuce were used as the nutrient substrate. Between the varieties Beizisheng No. 3 (BZ3) and Shooter 101 (SS), 204 differential metabolites showed significant changes ($P < 0.05$), 86 metabolites showed changes between the varieties BZ3 and Beisansheng No. 1 (BS1). Among the metabolites, isoquercitrin was the upregulated differential metabolite in BZ3 compared to SS and BS1. The content of isoquercitrin in lettuce juice positively correlated with the antibacterial activity of polyphenol extract, but negatively correlated with the growth of *E. coli*. The bacteriostatic property of polyphenol extract destroys the morphological structure of *E. coli*. The higher the isoquercitrin content, the stronger the resistance of fresh-cut lettuce to *E. coli*.

Keywords: antibacterial activity; *Escherichia coli*; lettuce; metabolomics

Introduction

Lettuce, also known as *Lactuca sativa* L., is a biennial herb of the family *Asteraceae*. It is a very popular ready-to-eat vegetable grown worldwide (López *et al*., 2014). Lettuce contains various nutrients, such as phenols, vitamin C, folic acid, carotenoids, and chlorophyll (Yang *et al*., 2022). It has a good nutritional value, prevents cardiovascular diseases (Shi *et al*., 2022), is antidiabetic (Cheng *et al*., 2014b), increases gastrointestinal motility to aid weight loss (Cheng *et al*., 2014a), and prevents cancer (Sularz *et al*., 2021). Lettuce is abundant in phytochemicals, including flavonoids, anthocyanins, and terpenes. Dannehl *et al*. (2016) found that red leaf lettuce

contains one phenolic acid, five caffeic acid derivatives, three flavonols and their aglycones, and one anthocyanin. Llorach *et al*. (2008) identified the phytochemicals in different lettuce varieties based on HPLC-DAD-MS/MS and found two compounds, quercetin and luteolin rhamnosyl hexoside, which were not reported in lettuce previously.

Plants contain a variety of secondary metabolites, among which the higher contents of polyphenols (El Moussaoui *et al*., 2019), flavonoids (Muna *et al*., 2022; Soberón *et al*., 2020), and terpenoids (Ketut Srie and Purnawati, 2023) in lettuce have been proved to have antibacterial activities. Pepe *et al*. (2014) found that leaf lettuce contains 16 polyphenol compounds all of which have anti-inflammatory and antioxidant activities. Moodi *et al*. (2021) showed that quercetin extracted from *Ginkgo biloba* combined with metal ions can significantly improve the inhibition of *Staphylococcus aureus* and *Escherichia coli*. Anthocyanins are also widely used in the antibacterial field. Li *et al*. (2022) showed that the lowest inhibitory concentrations of acylated anthocyanins in legumes against *S. aureus* and *E. coli* were 0.312 mg/mL and 4.000 mg/mL, respectively. Lettuce and *Atractylodes macrocephala* belong to the *Asteraceae* family. Zhaoyong *et al*. (2024) showed that the essential oil of *A. macrocephala* had various inhibitory effects on the growth of *E. coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *S. aureus*, and *Bacillus subtilis*. van Treuren *et al*. (2018) determined the metabolic spectrum of phytochemicals in 150 lettuce samples. For many phytochemicals, the relative abundance was either positively or negatively correlated with available phenotypic data on resistance against pests and diseases, indicating their potential role in plant resistance.

Microorganisms have complex sources and easy reproduction characteristics, making them the primary risk factors for food raw materials, and processed and finished products (Zaman *et al*., 2024). In lettuce and its processed products, intestinal pathogens, such as *E. coli* (Tahir *et al*., 2022), *Salmonella* (Min *et al*., 2016), and *Listeria monocytogenes* (Shenoy *et al*., 2017), are the common causes of food poisoning. Mechanical damage during processing of fresh-cut lettuce often destroys the tissue, leading to microbial contamination (Lin *et al*., 2019). Microbial contamination of green leafy vegetables has caused at least 800 illnesses and eight deaths since 1993, particularly California-grown lettuce (iceberg, romaine, red leaf, and mesclun). Although cases of *L. monocytogenes* infection involving lettuce are rare, since 2010, eight recalls have been issued for green leafy vegetables infected with the microbe (Zeng *et al*., 2014). Due to the perishable nature of fresh-cut lettuce, air-conditioned packaging helps maintain its quality and safety (Islam *et al*., 2019). *E. coli* is a bacterium commonly found in the human gut and is a recognized marker of fecal contamination. It adheres to the surface of the lettuce through irrigation water (fertilizer), animal manure, or human pollution and reproduces through the plant juice lost during processing. The damages or wounds caused during processing invades the lettuce, causing contamination of the leaves as well as the entire processing line (Cuggino *et al*., 2023). The microorganisms also secrete toxins during their reproduction process, which could endanger the consumers' health (Cui *et al*., 2018; Tang *et al*., 2017), causing diarrhea, urinary tract infections, arthritis, and septic infections (van Hoek *et al*., 2019). However, research indicates that the number of *E. coli* growing on the wounds vary in different lettuce varieties. Therefore, it is important to determine the reasons for the differences in the antibacterial properties of lettuce and find the varieties that are suitable as fresh-cut vegetables.

During the growth of biological cells and tissues, a variety of metabolites with very low molecular weight were generated, of which 92 could be qualitatively and quantitatively analyzed by metabolomics (Li *et al*., 2010; Lindon *et al*., 2006; Sun *et al*., 2011). Metabolomics, a new technology, is based on a widely targeted metabolome database (Sawada *et al*., 2009) and adopts a multiple reaction–monitoring mode to qualitatively and quantitatively detect metabolites in samples (Chen *et al*., 2013). In recent years, metabolomic analysis based on LC–MS technology has been widely used in species identification and nutritional science (Gauthier *et al*., 2015; Steinmeyer *et al*., 2015). Liquid chromatography (LC) is predominantly employed to segregate the target and the matrix components, thereby facilitating the qualitative or quantitative detection of trace components within a sample. Xue *et al*. (2022) used 50 kinds of pollutants from drugs and personal care products as unknown and unexpected contaminants, and analyzed the contaminated lettuce and corn substrates using UHPLC-MS. Various parameters including S-plot, permutation test, and VIP in OPLS-DA were used for screening and identification of marker compounds, which proved the effectiveness of the metabolomics method for nontargeted screening of various unknown and unexpected drugs and personal care products in plant-derived foods. Yang *et al*. (2018) combined GC×GC-TOF/MS and UPLC-IMS-QTOF/MS to conduct nontargeted metabolomics analysis of 30 lettuce cultivars. The results revealed that the metabolomics differences between different varieties of lettuce were related to secondary metabolism. van Treuren *et al*. (2018) determined the phytochemical metabolite profiles of 150 *Lactuca* samples. The research results indicated that many plant chemicals had species specificity, some of which were typically associated with traits related to plant health or nutritional value. For a large number of phytochemicals, the relative abundance was either positively or negatively correlated with the

available phenotypic data on resistance to pests and diseases, indicating their potential role in plant resistance. Liu *et al*. (2021) used ultra-high-performance liquid chromatography–high resolution mass spectrometry (UHPLC-HRMS) to analyze the metabolites that may be related to browning in lettuce. Otify *et al*. (2023) used LC-QTOF-MS/MS metabolomics, assisted by molecular networking and integrated with metabolomics, to annotate 195 metabolites of six lettuce varieties, and evaluated the biological activity of lettuce against nonantibiotic and drug-resistant bacteria, contributing to lettuce breeding. Although several related studies have reported the metabolic profiles of lettuce polyphenols, they are still incomplete and lack relevant research on their antibacterial properties. This study aimed to screen lettuce varieties with high resistance to *E. coli* and adopt the broadly targeted metabolomics technology of UHPLC-MS. Unsupervised PCA, OPLS-DA, and other multivariate statistical analysis methods were used to identify the metabolites in different resistant lettuce varieties, determine the key factors related to their antibacterial properties, and provide a theoretical basis for the selection and comprehensive utilization of the different varieties.

Materials and Methods

Lettuce samples and chemicals

The lettuce cultivation facility is situated at the Crop Variety Test and Exhibition Base in Changping District, Beijing, positioned at a latitude of 40° North. The soil type of the facility is sandy loam, and the soil composition is as follows: 50% sandy soil, 20% clay, and 5% organic matter. The density of lettuce planting is 80 plants/bed, and the plant distance is 0.3 × 0.3 m (Zhong *et al*., 2009).

This study involved 10 lettuce varieties. For clear expression, the full names are abbreviated as follows: *Beisheng* No. 1: B1; *Beisheng* No. 2: B2; *Beisheng* No. 3: B3; *Beisheng* No. 4: B4; *Shooter* 101: SS; *Beisansheng* No. 1: BS1; *Beisansheng* No. 2: BS2; *Beizisheng* No. 1: BZ1; *Beizisheng* No. 2: BZ2; *Beizisheng* No. 3: BZ3. All chemicals used in this study were of LC–MS grade.

Strains

The gram-negative bacteria *E. coli* YS MN153456 used throughout the study was separated from the lettuce; the strains were stored in the Food Microbiology Laboratory (Beijing University of Agriculture, Beijing, China), and the bacteria were activated in nutrient broth (AOBOX, Beijing, China) at 37°C in shaker for 48 h. The prepared bacterial suspension was subcultured in Luria–Bertani (LB) broth (AOBOX, Beijing, China) at 37°C for 12 h and harvested in sterile saline water. The concentration of the bacterial suspension was monitored by its transmittance at $OD_{600\ nm}$ using a T6 spectrophotometer (Beijing Puxi General Instrument Co., Ltd., Beijing, China).

Screening of lettuce varieties with high resistance to *E. coli*

Fresh lettuce juice was centrifuged at 8000 rpm for 15 min in a large, refrigerated centrifuge (Thermo Scientific), and filtered through a 0.22 μm filter to obtain 50 mL of filtrate. The filtrate was stored in a sterile conical flask at 4°C for use. The cultured *E. coli* YS was diluted to 1×10^2 colony-forming units (CFU)/mL, inoculated into the lettuce juice of 2% inoculum, and cultured at 37 ± 1°C for 48 h. The *E. coli* YS was then counted in five parallel groups for each sample.

Lettuce metabolomics

Extraction of metabolites

According to a slightly modified method of Doppler *et al*. (2016) and Chen *et al*. (2013), 2 g of fresh lettuce leaves was taken, wrapped in tin foil, marked, and quickly put into liquid nitrogen for 15 min and subjected to vacuum freeze-drying for 24 h. The freeze-dried samples were then crushed with a mixer mill for 30 s at 45 Hz. A 50 mg aliquot of each sample was precisely weighed and transferred to an Eppendorf tube, after which 800 μL of extract solution was added (methanol/water 3:1, precooled at −40°C, containing the internal standard). The samples were vortexed for 30 s, homogenized at 35 Hz for 4 min, and sonicated for 5 min in an ice water bath. The homogenization and sonication processes were repeated thrice. The samples were then extracted overnight at 4°C on a shaker and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was carefully filtered through a 0.22 μm microporous membrane. 40 μL was taken from each sample and pooled as QC samples, which were stored at −80°C until the UHPLC-MS analysis. Four samples per group were tested in parallel.

UHPLC-MS analysis

Chromatographic conditions: The UHPLC separation was carried out in ExionLC™ System (Sciex) with a Waters Acquity UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm). Mobile phase A and B were 0.1% formic acid in water and acetonitrile, respectively. The column and the autosampler temperatures were set at 40°C and 4°C, respectively, and the injection volume was 2 μL.

In the initial state, the mobile phase A and B were 98% formic acid aqueous solution and 2% acetonitrile,

respectively. A flow rate of 400μL/min was maintained throughout the operation. Within 0.5–10 min after initiation, the aqueous solution of formic acid in mobile phase A decreased by 50%, while acetonitrile in mobile phase B increased by 50%. Subsequently, within the first 11–13 min, the aqueous solution of formic acid in mobile phase A reached 5%, while the acetonitrile in mobile phase B reached 98% and remained stable. Within the first 13–15 min, the aqueous solution of formic acid in mobile phase A recovered to 98%, and the acetonitrile in mobile phase B recovered to 2%, and eventually remained stable.

Mass spectrometry conditions: Sciex QTrap 6500 + instrument (Sciex Technologies) was used for data analysis in multireaction monitoring mode. Typical ion source parameters were used: ion spray voltage +5500/−4500 V; curtain gas 35 psi; temperature 400°C; ion source gas 1: 60 psi; ion source gas 2: 60 psi; DP ±100 V.

Antibacterial susceptibility tests

Antibacterial tests were performed using the Oxford cup method (Zhu *et al*., 2019). The extract was redissolved in ultrapure water to obtain a final concentration of 200 mg/mL. 100 μL of the prepared 1 × 10^6 CFU/mL *E. coli* YS strain was added to 100 mL of nutrient agar medium (AOBOX, Beijing, China) at approximately 50°C and shaken evenly, and 10 mL of the mixture was poured into a sterile petri dish. After solidification, 200 μL of the prepared extract solution was implanted on the inoculated agar through an Oxford cup. The DIZ was measured after 24 h of incubation at 37°C.

Effect of lettuce polyphenol extracts on the morphological structure of *E. coli*

Polyphenol extracts from lettuce were procured utilizing ultrasonic-assisted extraction techniques (Zhidi *et al*., 2022). *E. coli* was cultured in LB broth at 37°C for 8 h and washed off with PBS buffer to prepare *E. coli* suspension with a concentration of 10^6 CFU/mL. The polyphenol extract was added for 6 h, and the suspension was centrifuged. The precipitated cells of *E. coli* were washed twice with 0.1 M PBS (pH 7.4) and fixed overnight at 4°C with 2.5% (v/v) glutaraldehyde in 0.1 M PBS. The cells were then dehydrated by sequential exposure to ethanol with concentrations ranging from 30 to 100%, and the ethanol was finally replaced with tert-butanol. The centrifuged cells were dried at a "critical point" in liquid CO_2 at 95 bar pressure, and the samples were covered with gold by cathodic spray. Finally, the morphology of the bacterial cells was observed on a scanning electron microscope (Prysese Instrument).

Determination of the isoquercitrin content

The isoquercitrin content in the extracts was measured using HPLC (Agilent, Santa Clara, CA). The HPLC conditions were as follows: ZORBAX SB C18 column (250 × 4.6 mm, 5 μm); mobile phase A: 0.1% formic acid; mobile phase B: methanol; gradient elution: ~0–0.5 min, 11% B; ~0.5–5 min, ~11–25% B; ~5–6 min, ~25–50% B; ~6–20 min, 50% B; ~20–30 min, ~50–11% B; flow rate: 0.5 mL/min; injection volume: 5 μL; and column temperature: 30°C. Chromatographic data were scanned in the 360 nm wavelength range. Purified lettuce polyphenols were dissolved in methanol at a concentration of 1 mg/mL. The reference substance of isoquercitrin was accurately weighed and placed in a brown vial, fully dissolved in methanol. The reference substance reserve solutions were prepared at concentrations 0.01, 0.05, 0.1, 0.15, and 0.2 mg/mL, and the peak area was detected by HPLC. The standard curve was drawn with peak area as ordinate and concentration as abscissa. Isoflavone glycoside standard curve: y = 2E+07x-39366, R^2 = 0.9993 is a good linear relationship.

Data analysis

SCIEX Analyst Work Station software (version 1.6.3) was used for multiple reaction–monitoring data acquisition and processing. The raw MS data (.wiff) files were converted to TXT format using an MS converter. An in-house R program and database were applied for peak detection and annotation. SIMCA software (version 16.0.2, Sartorius Stedim Data Analytics AB, Umea, Sweden) was used to perform a multivariate statistical analysis on the UHPLC-MS data, including unsupervised PCA and OPLS-DA.

The PCA was mainly used to study the sample distribution, deviation characteristics, and common trends. The OPLS-DA was used to classify samples, identify the most discriminant variables, and verify them based on the goodness of fit (R^2Y) and goodness of prediction (Q^2Y) for their classification and prediction ability. The model was tested for overfitting by permutation test (n = 200) and the negative value of the intercept (Q^2 intercept) was obtained, indicating the robustness of the model. The VIP score showed the contribution of each variable to the model. The VIP scores in the predicted components were analyzed, and only those metabolites with a VIP score of ≥1 were considered categories between which there was discrimination. Finally, the metabolite information obtained was screened according to the VIP value, the P-value of Student's *t*-test, and the FC, with the log value of 2 as the base, so as to obtain the metabolites with significant differences.

Statistical analysis

All assays were analyzed in quadruplicate. A one-way analysis of variance was performed on the number of *E. coli* colonies, the DIZ, and the content of isoquercitrin. Significance of $P \leq 0.05$ and $P \leq 0.01$ levels was determined using SPSS 16.0 software.

Results

Screening of antibacterial lettuce varieties

After *E. coli* YS inoculation, the counts for different varieties of lettuce juice varied significantly. Figure 1 shows the results of *E. coli* YS growth in juice of different lettuce varieties. The number of *E. coli* colonies in SS was the highest, at 7.66 lg CFU/mL, and that in BZ3 was the lowest, with only 4.41 lg CFU/mL. The results showed that BZ3 had the highest inhibitory effect on *E. coli* and the strongest antibacterial activity, whereas SS had the poorest performance for both. At the same time, BS1, which ranked in the middle, was selected as the control for the metabolomic analysis.

Metabolomic analysis of lettuce

Analysis of the QC samples

Quality control samples were used to evaluate the stability and repeatability of the system during the test. As illustrated in Figure 2, results showed that the curves of the total ion current for metabolite detection had a high overlap, that is, the retention time and peak intensity

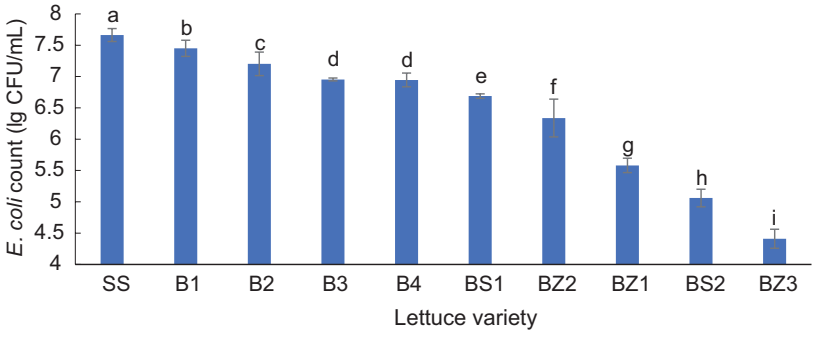

Figure 1. ***Escherichia coli*** **count results from different varieties of lettuce juice. Different letters in the figure indicate significant differences (*P* < 0.05).**

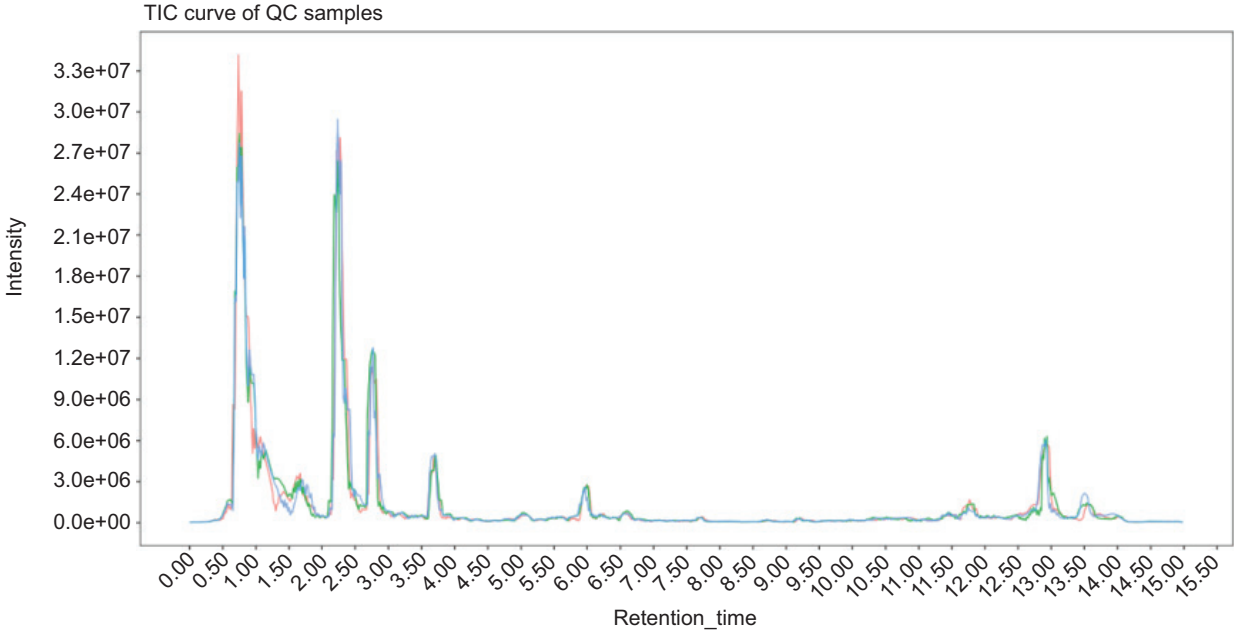

Figure 2. Overlay of the TIC of quality control (QC) samples by mass spectrometry detection.

were consistent, indicating that the signal stability was better when the mass spectrometer detected the same sample at different times.

Multivariate statistical analysis of metabolites in lettuce

To analyze the difference in metabolites between the two groups of lettuce samples, PCA analysis and OPLS-DA were used. The PCA score between the two groups is shown in Figure 3, and the OPLS-DA analysis and its model replacement test are summarized in Figure 4.

The PCA modeling method was used for the analysis because the antibacterial activities of BZ3 and SS were very different: when principal component (PC) 1 was 42.2%, PC2 was 12.4%, and BZ3 and SS could clearly be separated as having differences in metabolites. However, due to the small differences in antibacterial activity of BZ3 and BS1 with those of BZ3 and SS, the differences in metabolites of the former duo were smaller.

For the OPLS-DA model diagrams, it was often necessary to use permutation tests to verify the effectiveness of the model to prevent the overfitting phenomenon, which would lead to deviations. R^2Y represents the interpretation level of the model in the Y-axis direction, while Q^2 indicates its predictive ability (Xue *et al*., 2022). The closer R^2Y (cum) and Q^2 are to 1, the more stable and reliable the model is. $Q^2 > 0.5$ indicates better predictive ability of the model. In Figures 4A and B, the R^2Y (cumulative) and Q^2 values of BZ3 versus SS were 1 and 0.881, respectively, and the R^2Y (cumulative) and Q^2 values of BZ3 versus BS1 were 0.999 and 0.519, respectively, which demonstrated that the two OPLS-DA models were effective and there was no overfitting. Therefore, it could accurately analyze the differences in metabolites between the different antibacterial lettuce varieties. As shown in Figures 4A and B, the two different varieties of lettuce could clearly be distinguished in the score chart, which demonstrated that intermediate differences were present in the types and contents of metabolites.

Screening of differential metabolites in lettuce

The different metabolites between the groups were screened according to the VIP value of PC1 generated by OPLS-DA and the P-value of the *t*-test, (VIP ≥ 1 and P < 0.05). The higher the VIP value, the more it indicated the strength of the influence of differential metabolites on the antibacterial activity of different varieties of lettuce. As shown in Figure 5, 204 differential metabolites were identified in BZ3 and SS, 106 of which were upregulated metabolites, including 16 alkaloids, 11 terpenes, 6 phenols, 22 flavonoids, and 3 flavonoids. A total of 98 metabolites were downregulated, including 12 alkaloids, 7 phenols, and 21 amino acids and derivatives. Eighty-six differential metabolites were identified in BZ3 and BS1, of which 36 were upregulated and 50 were downregulated.

Cluster analysis of differential metabolites

A heatmap of the hierarchical clustering analysis method was used to analyze the differential metabolites in BZ3 versus SS and BZ3 versus BS1. The results are shown in Figure 6. A significant difference was found in the amounts of metabolites between the two groups, with the difference between the BZ3 and SS groups being more pronounced. The higher contents of BZ3 included cynaroside, delphinidin-3-*O*-glucoside, isoquercitrin, and glycitein methyl hesperidin, while adenosine 2′,3′-cyclic phosphate, guanosine 3′,5′-cyclic monophosphate, and (–)-cinchonidine were found in higher amounts in BS1. In contrast, l-glutamine, d-glutamine, and l-asparagine were higher in SS.

Analysis of the FC in the metabolite difference

By calculating the corresponding proportions of quantitative values for different metabolites, their significant changes are identified. To show the different metabolites with a large degree of change, we calculated the corresponding ratio of the quantitative value of the metabolites, expressed as FC, and took the logarithmic conversion with base as 2. The top 15 differential

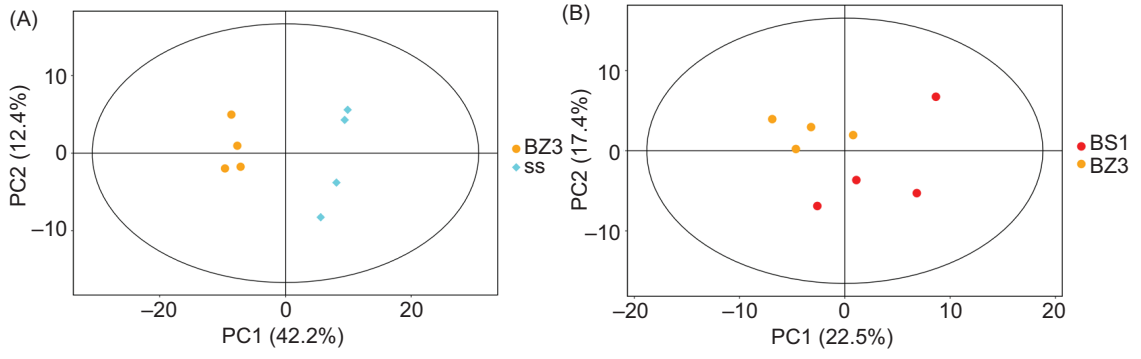

Figure 3. Principal components analysis for groups. Group A is BZ3 versus SS; group B is BZ3 versus BS1.

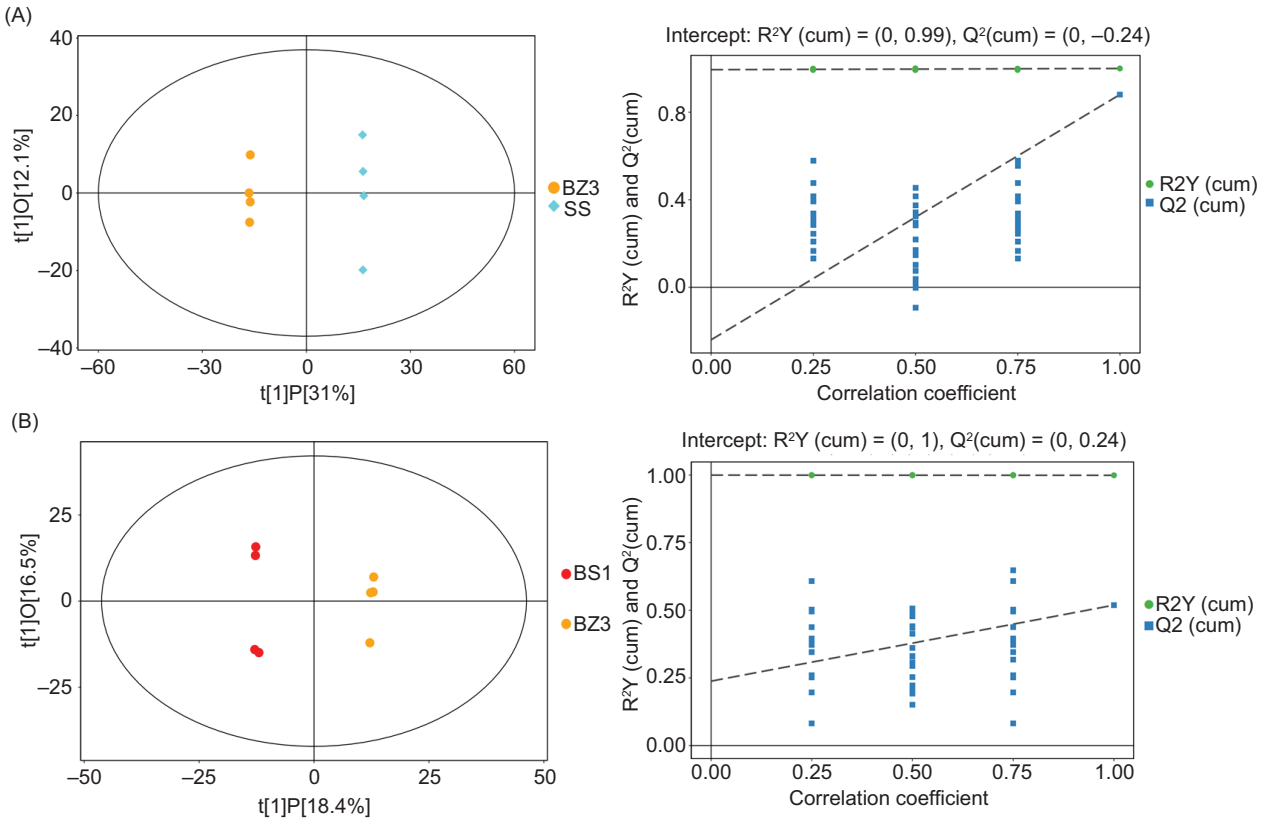

Figure 4. Orthogonal partial least squares discriminant analysis for groups. Groups A and A′ are BZ3 versus SS; groups B and B′ are BZ3 versus BS1.

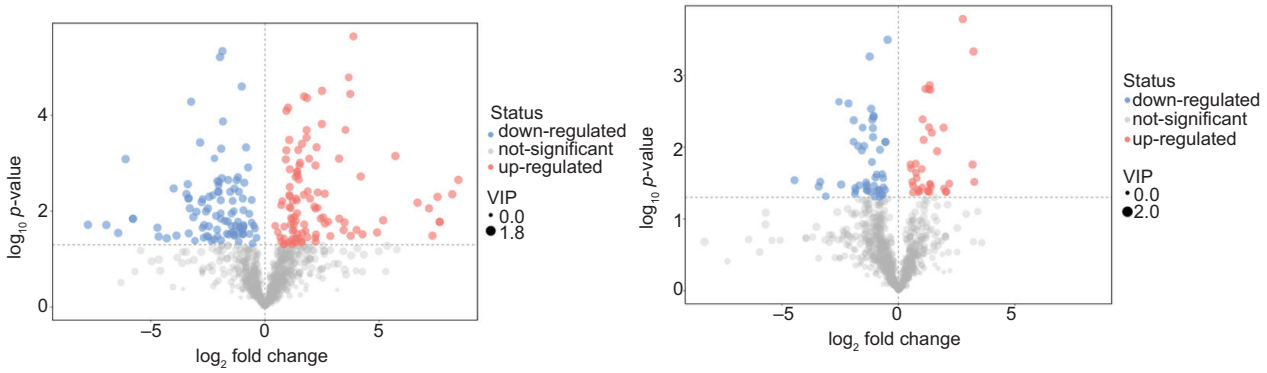

Figure 5. Volcano plot of different metabolite screening between groups. Group A is BZ3 versus SS; group B is BZ3 versus BS1.

metabolites according to their FC values are shown in Table S1. Figure 7 is a matchstick diagram of the two groups with significantly different metabolites. Because the metabolite contents in samples BZ3 and SS were quite different, the FC value was relatively large. Among the contents were 10 metabolites with $\log_2$FC ≥ 4, whereas in BZ3 and BS1, the FC value was small and the $\log_2$FC value was less than 4. Delphinidin-3-*O*-glucoside and isoquercitrin were the upregulated differential metabolites shared by the two groups, indicating that the contents of both metabolites in BZ3 were higher than those in BS1 and SS.

Analysis of the KEGG metabolic pathway

The screened-out differential metabolites were analyzed for metabolic pathway enrichment according to the KEGG

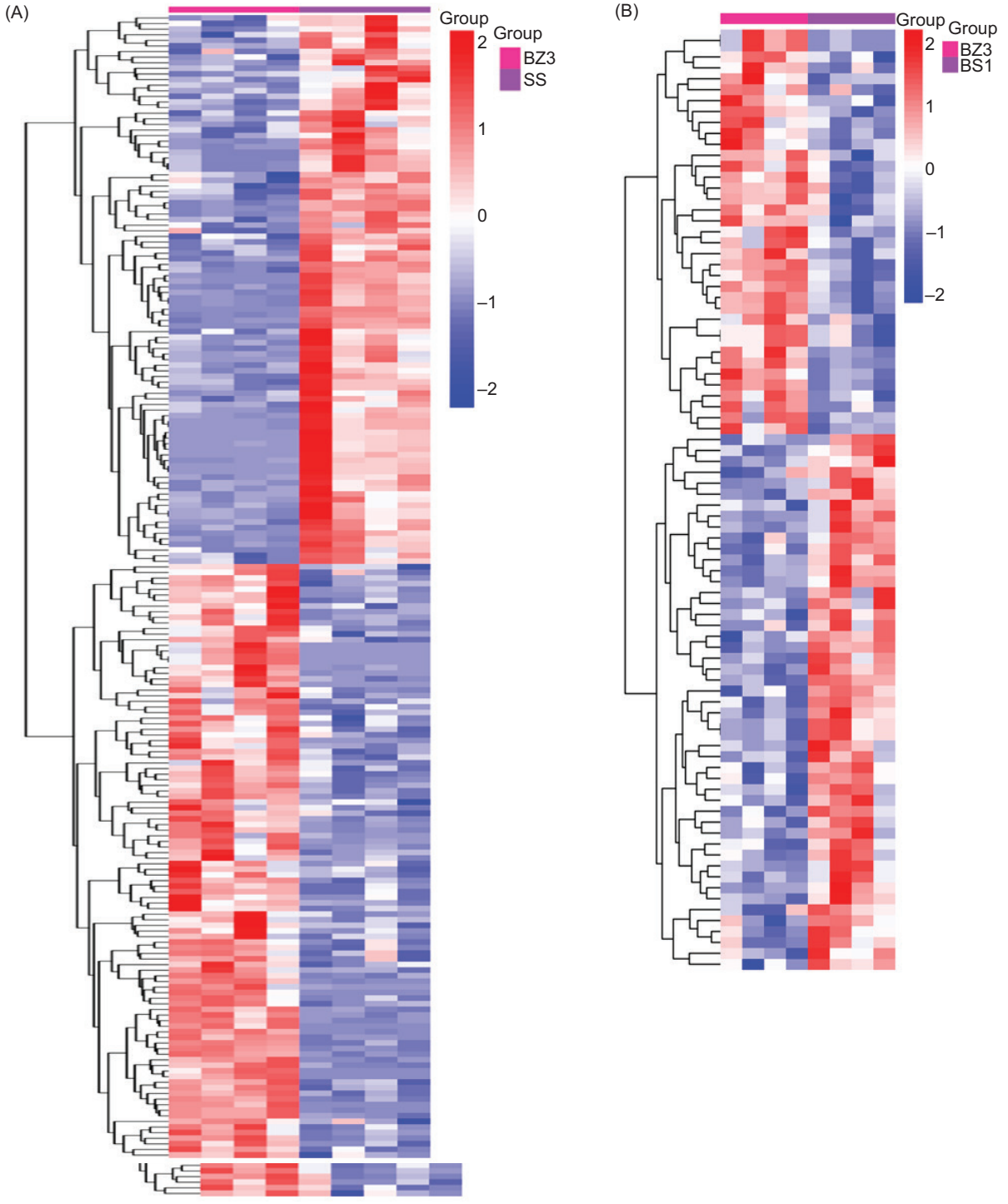

Figure 6. Heatmap of the hierarchical clustering analysis for differential metabolites. Group A is BZ3 versus SS; group B is BZ3 versus BS1. The color indicates the content: red indicates high expression, and blue indicates low expression.

data platform, and the results of the metabolic pathway analysis are displayed in a bubble chart (Figure 8). Forty-one metabolic pathways were enriched by BZ3 and SS. Among the metabolic pathways that were significantly enriched were monoterpenoid biosynthesis; alanine, aspartate and glutamate metabolism; valine, leucine, and isoleucine biosynthesis; α-linolenic acid metabolism; and arginine and proline metabolism. In contrast, 15 metabolic pathways were enriched by BZ3 and BS1. The pathways that were significantly enriched were tryptophan metabolism, α-linolenic acid metabolism, arginine and proline metabolism, phenylpropanoid biosynthesis, and pyrimidine metabolism.

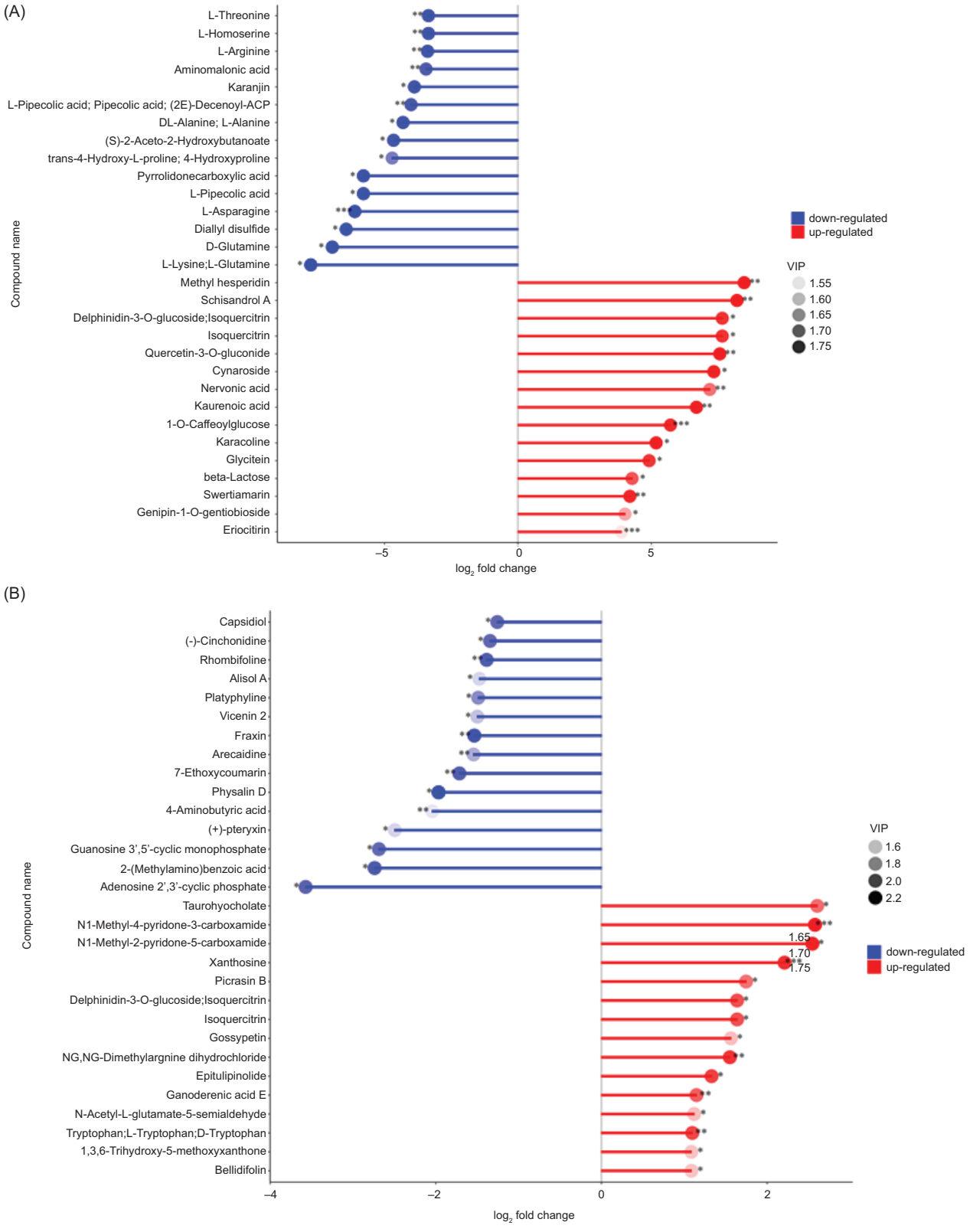

Figure 7. Matchstick analysis for significantly different metabolites. Group A is BZ3 versus SS; group B is BZ3 versus BS1. The abscissa shows the multiples of change after logarithmic transformation, and the shades of the dots represent the variable importance in projection (VIP) value. An asterisk (*) represents significance: *0.01 < *P* < 0.05, **0.001 < *P* < 0.01, ****P* < 0.001.

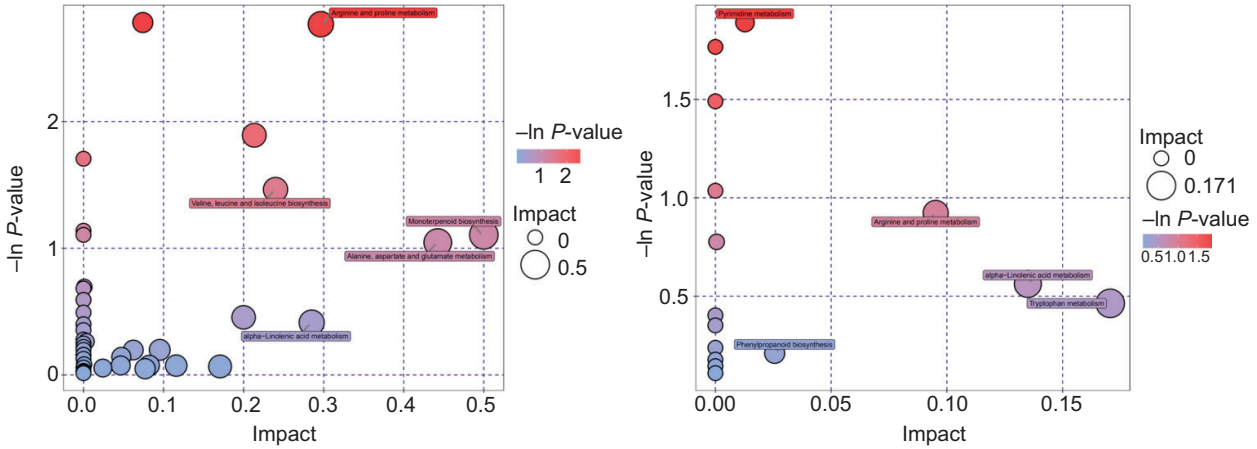

Figure 8. Pathway analysis for the two groups. Group A is BZ3 versus SS; group B is BZ3 versus BS1. Each bubble in the bubble chart represents a metabolic pathway. The abscissa of the bubble and the size of the bubble indicate the size of the enrichment and size of the influence factor of the pathway in the topology analysis. The larger the value, the greater the impact factor. The ordinate of the bubble and the bubble color indicate the *P*-value of the enrichment analysis (by taking the negative natural logarithm, i.e., −ln(*p*)). The darker the color, the smaller the P-value and the more significant the degree of enrichment.

Bacteriostatic activity

The polyphenol extracts of each variety had inhibitory effects on *E. coli*, and significant differences were found in their antibacterial effects ($P < 0.05$). The inhibition zone diameters of BZ3, BS1, and SS polyphenol extracts against *E. coli* were 18.61,16.33, and 11.23 mm, respectively. The inhibitory ability of BZ3 polyphenol extract to *E. coli* was significantly higher than that of other varieties.

Effect of lettuce polyphenol extracts on the morphological structure of *E. coli*

The micromorphology of *E. coli* in the control group and the lettuce polyphenol extract treatment group is shown in Figure 9. The morphology of *E. coli* in the control group (A) showed a complete and smooth rod-like structure. Upon introduction of lettuce polyphenol extract (B), there was a reduction of approximately 25% in the average bacterial length. Furthermore, the bacterial surface exhibited a rough and incomplete morphology, while the cellular surface was markedly depressed and wrinkled. The overall morphology of the bacteria was compromised, leading to cell disruptions and dissolution of their contents.

Content of isoquercitrin in the extract

The isoquercetin content in different varieties of lettuce was determined. There was a significant difference in its content in BZ3, BS1, and SS ($P < 0.05$), with BZ3 having the highest content, at 23.38 μg/g, and SS the lowest (11.26 μg/g). The content of BS1 fell in between the two (18.52 μg/g).

Correlation analysis

A correlation analysis of each index was carried out, and the results are shown in Table 1. The isoquercitrin content in lettuce samples positively correlated with the antibacterial activity of polyphenol extracts ($r = 0.995$) and negatively correlated with the growth of *E. coli* in lettuce juice ($r = -1$, $P < 0.01$). The results showed that the higher the isoquercitrin content, the stronger the antibacterial activity of the lettuce polyphenol extract, the less *E. coli*

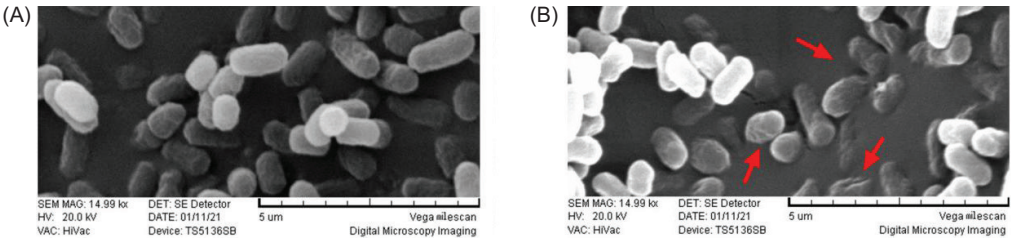

Figure 9. The effect of polyphenol extractson the *morphological* structure of *E. coli*. The cells indicated by the red arrow are those with folded morphology.

grew in the lettuce juice, and the stronger the lettuce antibacterial activity. Therefore, the isoquercitrin content was closely related to the antibacterial activity of the lettuce.

Discussion

Lettuce is rich in nutrients and has a crisp texture, which is greatly enjoyed by consumers in daily life. As a common fresh vegetable, its microbial safety has also attracted much attention. During the growth process of lettuce, metabolites are abundant, and there are interspecies differences as well. The types and contents of the metabolites are closely related to their antibacterial activities. Broadly targeted metabolomics has been widely used in profiling plant metabolites in recent years.

During the experiment, we found that the growth of *E. coli* YS differed when the bacterium was inoculated into lettuce juice samples. Therefore, a broadly targeted metabolome of lettuce metabolites was used to analyze their differences in different lettuce varieties. The PCA results of unsupervised pattern recognition showed significant differences in the metabolite content of different lettuce varieties. Up on comparison between BZ3 and SS, 204 differential metabolites were detected, and 86 differential metabolites were detected and between BZ3 and BS1. The results showed that metabolites differed across different varieties of lettuce, which were consistent with the findings by van Treuren *et al*. (2018). In addition, the study by Viacava *et al*. (2017) has shown that purple leaf lettuce has more phenolic compounds than the green leaf variety. Consistent with their experimental results, in our research, BZ3, a purple leaf lettuce, was richer in phenolic compounds than BS1 and SS. For example, in BZ3 and BS1, the FC value of isoquercitrin content was 3.11, while in SS, it was 201.65.

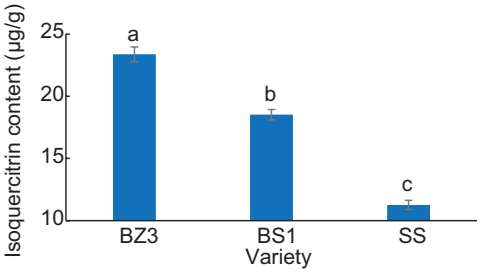

Figure 10. Contents of isoquercitrin in different varieties of lettuce. Different letters in the figure indicate significant differences (P < 0.05).

In the comparative analysis of BZ3 and SS, 106 upregulated differential metabolites were detected, most of which were flavonoids, alkaloids, and terpenes. Among them, 11 had $\log_2$FC > 4. In addition, 98 downregulated differential metabolites were detected. The content of antibacterial active substances in BZ3 was significantly higher than that in SS; thus, the antibacterial activity of the former was stronger than that of latter. The difference in antibacterial activity between BZ3 and BS1 was small, thus the number of different metabolites between them was significantly less than that between BZ3 and SS. Assefa *et al*. (2021) analyzed the metabolites of 113 red lettuce samples and found that the content of metabolites in them varied greatly. Cyanidin 3-*O*-(6″-*O*-malonyl) glucoside, 2,3-di-*O*-caffeoyltartaric acid, and quercetin 3-*O*-(6″-*O*-malonyl) glucoside predominated in red lettuce samples, and high levels of anthocyanins, flavonoids, and hydroxycinnamoyl derivatives were detected at the leaf incisions. The comparative analysis of BZ3 versus SS and BZ3 versus BS1 revealed delphinidin-3-*O*-glucoside and isoquercitrin as the significantly upregulated metabolites in the two groups. The isoquercitrin content of BZ3 was significantly higher than that of SS and BS1, and it had the greatest antibacterial effect. The results of correlation analysis showed that isoquercitrin content positively correlated with the antibacterial activity of lettuce, a key factor in the resistance of lettuce against *E. coli*. Zhang *et al*. (2024) extracted isoquercitrin from the antibacterial component of the fruit of *Amomum villosum* Lour. According to Yun *et al*. (2018), isoquercitrin is a flavonoid with good antibacterial activity. Tang *et al*. (2023) extracted isoquercitrin from the key antibacterial ingredient of an edible herb *Potentilla kleiniana* Wight et Arn. Fei *et al*. (2023) analyzed the metabolites of 50 kinds of lettuce by UPLC-Q-ToF-MS. The study revealed a positive correlation between the total phenols and total flavonoids content in lettuce and its antioxidant capacity. Furthermore, it was established that quercetin serves as a characteristic index for assessing the antioxidant activity of lettuce. In this study, only the resistance levels of 23 lettuce varieties to *E. coli* were

Table 1. Correlation analysis of each index.

	Isoquercitrin content	Bacteriostatic activity	*E. coli* counts in lettuce juice
Isoquercitrin content	1		
Bacteriostatic activity	0.995	1	
E. coli counts in lettuce juice	−1**	−0.993	1

**Indicates significant difference, P < 0.01.

compared and analyzed. However, it is imperative to broaden the scope of this research to encompass a more comprehensive analysis of the variability and correlation of lettuce metabolites. The analysis of correlational data pertaining to the characteristics of various lettuce varieties can offer valuable theoretical insights for the selection and breeding processes.

This study explored the impact of lettuce polyphenol extract on the cellular structure of *E. coli*. This study has demonstrated that the inhibition of polyphenol extracts on *E. coli* is achieved through the disruption of cellular structure, resulting in cell shrinkage and rupture. This process serves to inhibit the growth and reproduction of bacteria. Similar observation was reported by Jeyakumar and Lawrence (2021) with changes in the surface morphology of cells when treated with eugenol. It has been reported that flavonoids (Xu *et al*., 2019) and phenols (Bonechi *et al*., 2018) are secondary metabolites with antibacterial activity. Yu (2021) found that mulberry leaf polyphenols could significantly inhibit the motility of *E. coli* and the production of extracellular polymers. Birru *et al*. (2021) found that apple polyphenols can inhibit the growth of pathogenic bacteria and relieve the symptoms of chronic obstructive pulmonary disease. Taleb *et al*. (2016) showed that jujube pulp polyphenols inhibited the growth of *E. coli* and *S. aureus* by inducing oxidative stress in bacteria to produce hydrogen peroxide.

In the present study, lettuce polyphenol extracts had antibacterial effects on *E. coli*, and isoquercitrin content positively correlated with the antibacterial effect of the extract. The higher the content of isoquercitrin, the greater the antibacterial effect, which is consistent with the conclusion of bamboo leaf flavonoids inhibiting *E. coli* (Xiao *et al*., 2023). This finding will facilitate further investigation into the comprehensive utilization of lettuce. Moreover, lettuce polyphenols have good antioxidant activity (Xiao *et al*., 2015). Therefore, the selection of lettuce varieties with high polyphenol content as food raw materials can delay cell senescence and reduce the risks of cardiovascular disease and cell carcinogenesis to a certain extent (Hehuan *et al*., 2022). Consequently, the selection of lettuce varieties with elevated isoquercitrin content, such as BZ3, as raw materials in vegetable foods, such as salads and sandwiches, will significantly improve food safety.

Conclusion

Lettuce is a popular raw vegetable. Significant differences exist in the antimicrobial activity of and the contents and types of metabolites in different varieties of lettuce ($P < 0.05$). Purple leaf lettuce and loose-leaf lettuce have more metabolites than that of the green leaf variety and head lettuce, respectively. The greater the difference in the antibacterial activity of the lettuce, the greater the difference in the isoquercitrin content. The *E. coli* growth negatively correlated with the isoquercitrin content. The higher the isoquercitrin content, the stronger the antibacterial activity of the lettuce polyphenol extract, and the better the antibacterial activity of the lettuce.

Ethics Approval and Consent to Participate

Not applicable.

Consent for publication

Not applicable.

Competing Interests

The authors declare that there are no competing interests.

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Availability of Data and Materials

The data sets used and analyzed during the present study are available from the corresponding author on reasonable request.

Author Contributions

JL and ZC carried out the experiments and drafted a manuscript. FM, DL, and XC participated in its design and coordination. HL-B and YZ improved the writing of the manuscript. XG designed the experimental scheme and carried out the overall planning and improvement of the manuscript. All authors have read and approved the final manuscript.

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AI declaration statement

The author solemnly declares that the content of this article is original and has not been assisted by any artificial intelligence AI tools, including but not limited to ChatGPT, nor has it used software or online services such as automatically generated text.

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Supplementary

Table S1. The top 15 differential metabolites according to the fold change (FC) values.

BZ3 versus SS				BZ3 versus BS1				dem
Compound name	VIP	FC	log_2FC	Compound name	VIP	FC	log_2FC	
Eriocitrin	1.5478	14.6929	3.8770	Bellidifolin	1.5718	2.1222	1.0856	up
Genipin-1-*O*-gentiobioside	1.6151	16.1411	4.0127	1,3,6-Trihydroxy-5-methoxyxanthone	1.5718	2.1222	1.0856	up
Swertiamarin	1.7632	18.3111	4.1946	Tryptophan	1.9918	2.1383	1.0965	up
β-Lactose	1.7029	19.3753	4.2761	*N*-Acetyl-l-glutamate 5-semialdehyde	1.5892	2.1717	1.1188	up
Glycitein	1.7403	30.2168	4.9173	Ganoderenic acid E	1.8969	2.2145	1.1470	up
Karacoline	1.7779	36.2173	5.1786	Epitulipinolide	2.0084	2.5130	1.3294	up
1-*O*-Caffeoylglucose	1.7549	52.5024	5.7143	*NG*,*NG*-Dimethylarginine dihydrochloride	2.0537	2.9279	1.5499	up
Kaurenoic acid	1.7836	103.1271	6.6883	Gossypetin	1.5906	2.9542	1.5627	up
Nervonic acid	1.6701	145.8884	7.1887	Isoquercitrin	1.9579	3.1111	1.6374	up
Cynaroside	1.7707	162.0642	7.3404	Delphinidin-3-*O*-glucoside	1.9579	3.1111	1.6374	up
Quercetin-3-*O*-glucuronide	1.7900	189.2416	7.5641	Picrasin B	1.8347	3.3547	1.7462	up
Isoquercitrin	1.7447	201.6518	7.6557	Xanthosine	2.0793	4.6279	2.2104	up
Delphinidin-3-*O*-glucoside	1.7447	201.6518	7.6557	*N*,1-Methyl-2-pyridone-5-carboxamide	2.1440	5.8412	2.5463	up
Schisandrol A	1.7920	295.4953	8.2070	*N*,1-Methyl-4-pyridone-3-carboxamide	2.2080	5.9725	2.5783	up
Methyl hesperidin	1.7938	356.4401	8.4775	Taurohyocholate	1.8537	6.0823	2.6046	up
l-Lysine; l-glutamine	1.7885	0.0046	−7.7596	Adenosine 2′,3′-cyclic phosphate	1.9159	0.0842	−3.5709	down
d-Glutamine	1.7711	0.0081	−6.9547	2-(Methylamino)benzoic acid	2.0001	0.1499	−2.7380	down
Diallyl disulfide	1.7780	0.0116	−6.4337	Guanosine 3′,5′-cyclic monophosphate	1.8678	0.1554	−2.6859	down
l-Asparagine	1.7885	0.0145	−6.1112	(+)-Pteryxin	1.5186	0.1774	−2.4946	down
l-Pipecolic acid	1.7742	0.0181	−5.7878	4-Aminobutyric acid	1.4646	0.2427	−2.0429	down
Pyrrolidonecarboxylic acid	1.7742	0.0181	−5.7878	Physalin D	2.1688	0.2559	−1.9664	down
trans-4-Hydroxy-l-proline	1.6564	0.0382	−4.7115	7-Ethoxycoumarin	1.9572	0.3047	−1.7147	down
(*S*)-2-Aceto-2-hydroxybutanoate	1.7498	0.0396	−4.6586	Arecaidine	1.6429	0.3429	−1.5439	down
dl-Alanine; l-alanine	1.7611	0.0506	−4.3040	Fraxin	2.0829	0.3451	−1.5349	down
l-Pipecolic acid	1.7301	0.0623	−4.0035	Vicenin 2	1.5794	0.3541	−1.4979	down
Karanjin	1.7538	0.0680	−3.8786	Platyphylline	1.7442	0.3565	−1.4880	down
Aminomalonic acid	1.7217	0.0921	−3.4411	Alisol A	1.5144	0.3600	−1.4740	down
l-Arginine	1.7564	0.0958	−3.3844	Rhombifoline	1.9713	0.3827	−1.3857	down
l-Homoserine	1.7583	0.0980	−3.3507	(−)-Cinchonidine	1.8790	0.3938	−1.3445	down
l-Threonine	1.7583	0.0980	−3.3507	Capsidiol	1.8739	0.4183	−1.2574	down

dem, differentially expressed metabolites; VIP, variable importance in projection.