Multiomics analysis reveals flavonoid accumulation and biosynthesis across different cultivation years and localities of Gongronemopsis tenacissima (Dai-Bai-Jie) (#109469)

4th revision

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Multiomics analysis reveals flavonoid accumulation and biosynthesis across different cultivation years and localities of Gongronemopsis tenacissima (Dai-Bai-Jie)

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Background: The dried root of Gongronemopsis tenacissima (Roxb.) S. Reuss, Liede & Meve, traditionally known as Dai-Bai-Jie, is a medicinal plant used by the Dai ethnic group, primarily for detoxification. Due to extensive use, wild resources have become increasingly scarce, prompting domestication efforts in China. However, the accumulation patterns of secondary metabolites—particularly flavonoids, the main detoxifying components—and their biosynthetic mechanisms remain unclear.

Methods: This study investigated differences in flavonoid accumulation and transcriptional regulation in Dai-Bai-Jie cultivated for one, two, and three years at high altitudes, and three years at low altitudes. Transcriptome and widely targeted metabolome analyses were conducted. A total of 1,495 metabolites were identified using ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS), with 943 showing differential accumulation across the four groups. All flavonoids were classified into six clusters using k-means clustering. Flavonoid levels were generally higher in plants cultivated for two and three years, with total metabolite content also more abundant in these groups. Two-year cultivation was recommended as the optimal harvesting strategy.

Results: A regulatory relationship was observed between genes such as phenylalanine ammonia-lyase , CYP73A , 4-coumarate: coenzyme A ligase , and flavonol synthase , and the flavonoid components in Dai-Bai-Jie. No significant differences were found in Shannon, Chao1, or abundance coverage estimator (ACE) indices of rhizosphere microorganisms across different cultivation years and locations.

Conclusions: This study elucidates the mechanisms of flavonoid accumulation and supports the scientific rationale for optimal harvesting years of Dai-Bai-Jie. The findings provide a valuable foundation for guiding large-scale cultivation and reducing reliance on wild resources.

1 Multiomics analysis provides insights into flavonoids
2 accumulation and biosynthesis in different planting years
3 and localities of *Gongronemopsis tenacissima* (Dai-Bai-
4 Jie)

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20 **ABSTRACT**
21 The dried root of *Gongronemopsis tenacissima* (Roxb.) S.Reuss, Liede & Meve is a traditional
22 medicine utilized by the Dai ethnic group, commonly known as Dai-Bai-Jie, primarily for
23 detoxification purposes. Due to the extensive utilization, the wild resources are becoming
24 increasingly scarce. The plants have been domesticated in China. However, the accumulation
25 patterns of secondary metabolites, particularly flavonoids-the primary detoxifying components-
26 along with their biosynthesis, remain unclear.
27 The differences in flavonoid accumulation and transcriptional regulatory mechanisms underlying
28 the differential accumulation of flavonoids in Dai-Bai-Jie, cultivated for one, two, and three year
29 s at high altitudes, as well as three years at low altitudes, were investigated using transcriptome a
30 nd widely targeted metabolome methods. A total of 1,495 metabolites were identified through
31 Ultra Performance Liquid Chromatography coupled with Tandem Mass Spectrometry (UPLC-M
32 S/MS) from Dai-Bai-Jie, and 943 differential accumulation metabolites were detected among the
33 four groups. All the flavonoids were grouped into six clusters by k-means cluster analysis. The to
34 tal metabolite content in two and three years was relatively abundant, and flavonoid levels were
35 generally higher in two and three years. It is recommended that harvesting at two years of age be
36 considered the optimal strategy. All flavonoids were organized into six clusters through k-means
37 cluster analysis.

38 A regulatory relationship was observed between genes such as phenylalanine ammonia-lyase,
39 CYP73A, 4-Coumarate: Coenzyme A Ligase, and lavonol synthase and the flavonoid
40 components in Dai-Bai-Jie. However, significant differences in the Shannon, Chao1, or
41 Abundance Coverage Estimator (ACE) indices of rhizosphere microorganisms were detected
42 across different planting years and localities were not detected. This study elucidates the
43 accumulation mechanisms of flavonoids and the scientificity of harvesting years for Dai-Bai-Jie.
44 The results provide a crucial scientific foundation for guiding the large-scale introduction and
45 cultivation of Dai-Bai-Jie as a supplement or alternative to the use of wild resources.
46
47 Keywords: *Gongronemopsis tenacissima*; metabolome; transcriptome; rhizosphere microbes;
48 flavonoids
49

INTRODUCTION

51 *Gongronemopsis tenacissima* (Roxb.) ~~Moon~~ S.Reuss, Liede & Meve is a traditional medicine
52 utilized by the Dai ethnic group, commonly known as Dai-Bai-Jie, and holds significant value in
53 the ethnomedical traditions of Southeast Asia. In the Dai language, it is referred to as "Ya Jie
54 Xian Da," symbolizing its ability to purge the body of numerous toxins. This medicinal herb has
55 long been utilized in Dai-inhabited regions such as Xishuangbanna, Dehong, Ximeng, Menglian,
56 Xinping, Yuanjiang, Mojiang, and Puer in China, as well as neighbouring countries like Laos
57 and Myanmar (*Li et al., 1995*). The root of *G. tenacissima* is employed in folkloric medicine
58 named Dai-Bai-Jie for detoxification purposes. It is recognized for its efficacy in counteracting
59 toxicities resulting from various sources, including food, animals, and environmental factorssuch
60 as heat, water, and fire burns. Additionally, it is used to relieve throat discomfort and swelling
61 caused by excessive heat toxicity. With a rich historical background in traditional medicine, Dai-
62 Bai-Jie has been incorporated into contemporary hospital preparations at institutions like the
63 Xishuangbanna Dai Hospital. These formulations include Bai-jie Capsules, Ya-jie Gahan, and
64 Banna Coolant. Modern pharmacological research has shown that "Dai-Bai-Jie" possesses
65 inhibitory effects on cancer cells, protects against liver damage caused by certain drugs,
66 demonstrates anti-HIV activity, possesses antioxidant properties, and exhibits antibacterial
67 activities (*Gao et al., 2014; Li et al, 2021*).
68 Currently, various bioactive compounds have been isolated from Dai-Bai-Jie, including organic
69 acids, polyoxyprogesterone glycosides, volatile oils, and pyrrole alkaloids (*Liao et al., 2016;*
70 *Pang et al., 2018; Song et al., 2018; Song et al., 2021*). These discoveries not only deepen our
71 understanding of the medicinal properties of this herb but also open up potential avenues
72 fortherapeutic applications in modern medicine.
73 For a long time, Dai-Bai-Jie was incorrectly identified as the dried root of *Dregea sinensis*
74 Hemsl., which belongs to the genus *Dregea* of the Asclepiadaceae family (*Lin et al., 2003*).
75 However, a pivotal study conducted in 2014 revealed that Dai-Bai-Jie is, in fact, the dried root of
76 *Marsdenia tenacissima,* ~~a species within the genus *Marsdenia*~~ (*Li et al., 2014; Li et al., 2023*).
77 This identification was based on comprehensive molecular and morphological analyses utilizing
78 DNA fragments such as *psb*D-*trn*T, *trn*LF, and ITS, along with observations of leaf morphology

79 and floral characteristics. It is important to note that the "tong-guan-teng" mentioned in the
80 Chinese Pharmacopoeia, known for its broad-spectrum anticancer properties is associated with
81 *G. Cavalieri* (formerly known under *M. Cavalieri, Chen et al., 2022; Li et al., 2014*). Current
82 scientific investigations have revealed significant differences in the chemical composition and
83 therapeutic effects of these two species. Specifically, Dai-Bai-Jie is primarily indicated for
84 antidotal properties and management of gastrointestinal disease, while anticancer activity is
85 chiefly attributed to *G. cavalieri*. In 2022, *M. tenacissima* was reclassified into the genus
86 *Gongronemopsis* and is now referred to as *Gongronemopsis tenacissima* (Roxb.) (*Liede-*
87 *Schumann et al., 2022*).
88 Flavonoids are secondary metabolites found widely in plants, possessing a variety of functions
89 including antioxidant, anti-inflammatory, antitumor, antiviral, antibacterial, anti-vascular
90 sclerosis, and anti-liver fibrosis activities (*Fang et al., 2023; Wang et al., 2020; Zhang et al.,*
91 *2023*). Recent studies suggested that their protective effect on intestinal mucosal barrier function
92 may play a role in detoxification mechanisms (*Yang et al., 2020*). According to Dai medical
93 theory, the occurrence of disease is closely linked to imbalances among the four cosmic elements
94 within the body, which can be triggered by the presence of toxins (*Zhang et al., 2023*). Such
95 imbalances may stem from disturbances in antioxidant defences and disparities between pro- and
96 anti-inflammatory factors. Notably, recent studies have demonstrated a correlation between the
97 levels of total flavonoids and total polyphenols in Dai-Bai-Jie with its antioxidant and anti-
98 inflammatory activities (*Zhang et al., 2023*). Therefore, flavonoids may represent the most
99 significant active component for detoxification properties of Dai-Bai-Jie.
100 Due to the extensive utilization of Dai-Bai-Jie, wild resources are becoming increasingly scarce.
101 Fortunately, significant advancements have been made in the artificial cultivation technology for
102 *G. tenacissima*, leading to small-scale cultivation in Xishuangbanna, Yunnan. Under natural
103 conditions, the harvesting period for the roots of *G. tenacissima* is typically determined by
104 empirical knowledge and generally occurs after at least two years of growth. Similarly, under
105 cultivation conditions, the harvest period is usually 2-3 years, primarily considering the biomass
106 of the roots.
107 Despite these advancements, the accumulation patterns of flavonoids in Dai-Bai-Jie under
108 varying cultivation conditions remain unclear. To address this knowledge gap, this study
109 investigated the flavonoid accumulation patterns and influencing factors of Dai-Bai-Jie from a
110 multi-omics perspective, which may lead to a better understanding of the metabolic
111 accumulation mechanism of Dai-Bai-Jie and facilitate the scientific determination of optimal
112 harvesting years for this medicinal plant.
113
114 **MATERIALS AND METHODS**
115 **Plant materials and sampling**
116 The roots of one-year-old (CR1), two-year-old (CR2), and three-year-old (CR3) cultivated *G.*
117 *tenacissima* (Dai-Bai-Jie) were collected from Menghun County, Xishuangbanna Dai
118 Autonomous Prefecture, Yunnan, China (E 100.38°, N 21.82°; 1179 m) in November 2022(Fig.
119 1). Additionally, the roots of three-year-old Dai-Bai-Jie (CR4) cultivated in South Medicine

120   Garden (E100.79°, N22.00°; 533.57m) also located in Xishuangbanna Dai Autonomous
121   Prefecture, Yunnan Province, China, were gathered. Each plant was divided into two sections:
122   one for transcriptome sequencing and the other for metabolome analysis, with three biological
123   replicates per sample. Furthermore, the rhizosphere soil (CM1, CM2, CM3, CM4) corresponding
124   to each plant (CR1, CR2, CR3, CR4) was collected and utilized for 16S rRNA and ITS analysis.
125   **Metabolite Extraction and UPLC-MS/MS Analysis**
126   After the freeze-dried samples were crushed (30 Hz, 1.5 min), the extraction solution (70%
127   methanol water pre-cooled to -20℃) was added, and the mixture was vortexed for 30 s.
128   Subsequently, the samples were vortexed six times (once every 30 min) and centrifuged at
129   12,000 rpm for 3 min. The supernatant was then filtered through a microporous filter membrane
130   with a pore size of 0.22 μm and stored in an injection vial for Ultra Performance Liquid
131   Chromatography (UPLC-MS/MS) analysis.
132   Ultra High Performance Liquid Chromatography (ExionLC™ AD) was employed for sample
133   collection and analysis, utilizing an Agilent SB-C18 column (1.8 μm, 2.1 mm × 100 mm). The
134   mobile phase A consisted of 0.1% formic acid in water, while the mobile phase B was
135   acetonitrile containing 0.1% formic acid. The column temperature was maintained at 40°C, and
136   the automatic sampler temperature was set to 4°C. The flow rate was adjusted to 0.35 mL/min,
137   and the injection volume was 2 μL.
138   Applied Biosystems 6500 QTRAP was used for analysis. The typical ion source parameters were
139   as follows: electrospray ionization (ESI) temperature of 500°C; ion spray voltage (IS) of 5500 V
140   in positive ion mode and -4500 V in negative ion mode; ion source gas I (GSI), gas II (GSII),
141   and curtain gas (CUR) were set to 50, 60, and 25 psi, respectively. The collision-induced
142   dissociation parameters were set to high. SCIEX Analyst workstation software (version 1.6.3)
143   was used for Multiple Reaction Monitoring (MRM) data collection and processing.

Data processing, Metabolite Identification, and Statistical Analysis

144 **Data processing, Metabolite Identification, and Statistical Analysis**
145 Using MS-Converter, MS raw data files were converted into TXT format for further analysis. An
146 internal R program, along with a specialized database, was employed for peak detection and
147 annotation. In metabolite identification, the parameters matched include Q1 accurate molecular
148 weight, secondary fragments, retention time, and isotopes. The qualitative and quantitative mass
149 spectrometric analysis of metabolites in project samples is based on the MetWare Database
150 (MWDB) and Multiple Reaction Monitoring (MRM).
151 The identification of metabolites in the project samples is based on the precise mass of the
152 metabolites, MS2 fragments, the isotopic distribution of MS2 fragments, and retention time
153 (RT). By employing the intelligent secondary spectrum matching method developed in-house,
154 the secondary spectrum and RT of the metabolites in the project samples are intelligently
155 matched one by one with the secondary spectrum and RT in the company database. The MS
156 tolerance and MS2 tolerance are set to 20 ppm, while the RT tolerance is set to 0.2 min. The
157 level of substance identification is categorized into three levels: Level 1, Level 2, and Level 3,
158 with decreasing accuracy in that order. Level 1 represents the highest accuracy with MS/MS
159 spectrum and RT match score ≥ 0.7, MS/MS spectrum and RT match score between 0.5 and 0.7
160 of level 2. The level 3 indicates that the sample substance's Q1, Q3, RT, DP, and CE match
161 consistently with the database substance. The raw metabonomic data can be found in the denodo
162 platform (https://doi.org/10.5281/zenodo.17222367)
163 Prior to analysis, the raw data underwent preprocessing to filter out low-quality ion signals.
164 After obtaining the organized data, SIMCA (version 16.0.2) software was used for Principal
165 Component Analysis(PCA)and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-
166 DA), which were used to explore the metabolic patterns and identify differential metabolites
167 (DAMs) with p-values < 0.05 and VIP (variable importance in projection) > 1.

RNA-seq processing and data analysis

168 **RNA-seq processing and data analysis**
169 Total RNA was extracted and purified from the above samples. The extracted RNA was tested
170 for purity, concentration, and integrity. After the samples were qualified, the mRNA was isolated
171 and purified by Oligo (dt) for the construction of the cDNA library. Illumina Novaseq 6000
172 sequencing was performed after the library was qualified. Fastp software (*Chen et al., 2018*) was
173 used for quality control on the raw data.
174 After obtaining Clean Reads, Trinity assembly software is used to splice the Clean Reads to
175 obtain reference sequences for subsequent analysis, trinity assembly software was used to stitch
176 the clean reads to obtain reference sequences for subsequent analysis.
177 The RSeQC software (*Wang et al., 2012*) was used to evaluate the quality of transcriptome dataand
178 to analyze the sequencing data after passing the quality evaluation. Fragments per Kilobase Million
179 (FPKM) (*Trapnell et al., 2010*) was used to estimate gene expression level. The transcriptome
180 assembly was assessed in terms of their completeness and the percentage of complete, fragmented,
181 and missing fragments by using the BUSCO 5.3.2 (https://busco.ezlab.org, *Simão et al., 2015*).
182 DESeq2 (*Love et al., 2014; Varet et al., 2016*) was used for differential expression analysis
183 between samples. The corrected p-value and FDR (False Discovery Rate) were used as the key
184 indicators for the screening of differentially expressed genes (DEGs). Weighted Geneco-
185 expression Network Analysis (WGCNA) was used to find the gene modules that are co-expressed
186 and construct the hierarchical clustering tree. The statistical power of this experimental design,
187 calculated in RNASeqPower is 0.70.

188 The whole transcript data set can be found in the National Center for Biotechnology Information
189 (NCBI) database (BioProject ID: PRJNA996325).
190 **RT-qPCR validation**
191 We selected five genes associated with flavonoid synthesis for RT-qPCR according to FPKM
192 value (*Forkmann et al., 2001; Zou et al., 2016*). GAPDH was used as a reference gene and all
193 genes used in this study are listed in Table 1. cDNA was synthesized using MonScript™ RTIII
194 All-in-One Mix with ds DNase (Monad, China). According to the instructions of QuantiNova
195 SYBR Green PCR Kit (Qiangen, China), RT-qPCR was performed. The total volume of the system
196 was 10 μL, including 5μL 2x SYBR Green PCR Master Mix, 0.7 μL upstream Primer with 0.7
197 μM, 0.7 μL downstream primer with 0.7μM, 1μL cDNA with≤100ng/reaction, 2.55 μL RNase-
198 free water, 0.05 μL QN ROX Reference Dye.
199 **Microbial DNA extraction, 16S rRNA, and ITS gene sequencing**
200 Genomic DNA was extracted using CTAB (Nobleryder, China). 30 μL PCR amplification syste
201 m was as follows: Phusion® High-Fidelity PCR Master and high fidelity polymerase Mix (New
202 England Biolabs) 15μL, Primer 1 μL, DNA5-10 ng, ddH$_2$O. 16S V4 regional primer 515F (5'-G
203 TGCCAGCMGCGCGGGGGTAA-3') and 806R (5'- GGACTACHVGGGGTWTCTAAT-3') w
204 ere used to identify bacterial diversity. ITS5-1737F (5'- GGAAGTAAAAGTCGTAACAAGG-
205 3') and ITS2-2043R (5'- GCTGCGTTCTTCATCGATGC-3') were used to identify fungal diver
206 sity. Reaction procedure was set at 98 ℃ for 1 min, followed by 40 cycles at 98 ℃ for 10 s, 0℃ f
207 or 38 s, and 72 ℃ for 30 s, 72 ℃ extension for 5 min finally. PCR products were sequenced on th
208 e NovaSeq6000 platform (Maiwei Biotechnology Company).
209

210 # RESULTS
211 **RNA-seq analysis and DEGs identification**
212 We performed high-throughput transcriptome sequencing on the CR1, CR2, CR3, and CR4 of
213 Dai-Bai-Jie, with three biological replicates per sample. In total, we obtained 78.27 GB of clean
214 data. The clean Data of all samples were not less than 6 GB of clean data. The percentages of
215 bases with a Q30 quality score were greater than 90% for all samples. After assembling and
216 splicing, 85,346 unigenes were obtained. A BUSCO analysis was performed to evaluate the
217 completeness, recovering 253 out of 255 conserved eukaryotic genes (99.2%) (Fig. 2A).
218 Using the criteria of |log2Fold Change| ≥ 1 and FDR < 0.05, we screened for DEGs. The results
219 revealed that 15,255, 8,170, 10,529, and 8,225 DEGs were identified in the comparisons of CR1
220 vs. CR2, CR1 vs. CR3, CR2 vs. CR3, and CR3 vs. CR4, respectively. Among these, 654
221 common DEGs were shared across CR1, CR2, CR3, and CR4. Specifically, there were 6,043
222 unique DEGs identified in the comparison of CR1 vs. CR2, 1,243 unique DEGs in CR1 vs. CR3,
223 2,720 unique DEGs in CR2 vs. CR3, and 2,957 unique DEGs in CR3 vs. CR4 (Fig. 2B).
224 The DEGs in the four groups were analyzed using the Kyoto Encyclopedia of Genes and
225 Genomes (KEGG) metabolic pathway. The results showed that the DEGs of CR1 vs. CR2, CR1
226 vs. CR3, CR2 vs. CR3, and CR3 vs. CR4 were annotated to 144, 140,143, and 140 KEGG
227 metabolic and biosynthetic pathways, respectively. Notably, the "Metabolic pathways" category
228 emerged as the most frequently annotated, encompassing 2492, 1428, 1669, and 1432 genes in
229 each comparison, respectively. Closely following was the "biosynthesis of secondary
230 metabolites" category, which annotated 1375, 800, 930, and 808 genes, respectively. The "Plant-
231 pathogen interaction" pathway was annotated to 514, 271, 364, and 401 genes (Fig. 3).

232   WGCNA displayed that DEGs are divided into 27 co-expression modules of CR1, CR2, CR3,
233   and CR4. Among them, the turquoise module has the highest number of genes with 11313,
234   followed by the blue module with 5550 genes, and the least is the white module, which has 101
235   genes (Fig. 2C).
236   **RT-qPCR validation**
237   The RT-qPCR results for the five targeted genes indicated that four of them (excluding cluster-
238   60047.2) displayed a general consistency with the relative transcript abundance observed in the
239   transcriptome analysis. This concordance validates the reliability of the RNA-seq data (Fig. 4).
240   **Metabolomic profiling**
241   A total of 1495 metabolites were identified from Dai-Bai-Jie using UPLC-MS/MS. These
242   included 378 amino acids and their derivatives (25.28%), 265 phenolic acids (17.73%), 168
243   lipids (11.24%), 114 flavonoids (7.63%), 103 organic acids (6.89%), 92 alkaloids (6.15%), 80
244   nucleotides and their derivatives (5.35%), 55 lignans and coumarins (3.68%), and 42 terpenoids
245   (2.81%), 23 steroid (1.54%) and 75 metabolites belonging to other categories(11.71%)(Fig. 5A).
246   Notably, the flavonoid category was further subdivided into 9 chalcones, 17 dihydroflavonoids, 8
247   dihydroflavonols, 36 flavonoids, 40 flavonols, and 4 flavanols.
248   PCA was employed to illuminate the overall metabolite differences among the different groups.
249   The results showed that principal component 1(PC1, 38.39%) and principal component
250   (PC2,23.73%) accounted for 62.12% of the variance in the metabolic profile, indicating
251   significant differences across four groups. The three samples within each group demonstrated
252   high aggregation and good repeatability (Fig. 5B).
253   A total of 943 Differential metabolites (DAMs) were detected using FC ≥ 2 or ≤ 0.5 and VIP > 1
254   as screening conditions, including 255 amino acids and their derivatives, 174 phenolic acids, 45
255   nucleotides and their derivatives, 79 flavonoids, 42 lignans and coumarins, 64 alkaloids, 30
256   terpenoids, 44 organic acids, 20 steroids and 83 lipids. Among them, there were one common
257   DAMs shared of CR1, CR2, CR3, and CR4. Specifically, there were five unique DAMs in the
258   comparison of CR1 vs. CR2, 273 unique DAMs in CR1 vs. CR3, 172 unique DAMs in CR2 vs.
259   CR3, and 46 unique DAMs in CR3 vs. CR4 (Fig. 5D).
260   In the comparison of CR1 vs. CR2, a total of 627 DAMs were detected, of which 183 were
261   down-regulated and 444 were up-regulated. Compared to CR1, the metabolite that significantly
262   decreased in CR2 was gofruside, whereas the metabolite that significantly increased was 4-O-
263   (2"-O-acetyl-6"-P-coumaroyl-β-D-glucopyranosyl)-P-coumaric acid (Fig. 6A). The metabolite
264   protocatechuic acid 4-O-(2"-O-Vanilloyl) glucoside significantly decreased in CR3 compared to
265   CR1, while eugenol significantly increased (Fig. 6B). 449 DAMs were detected in CR2 vs CR3,
266   with 377 down-regulated and 72 up-regulated. The metabolite 6,7-dimethoxy-2-[2-(4'-hydroxy-
267   3'-methoxyphenyl)ethyl]chromone was significantly reduced in CR3 relative to CR2, while
268   sinapine was significantly increased（Fig. 6C). Lastly, a total of 259 DAMs were found in the
269   comparison between CR3 vs CR4, with 117 down-regulated and 142 up-regulated. The
270   metabolite that showed a significant decrease in CR4 was rutin, while exhibited a significant
271   increase when compared to CR3 (Fig. 6D). Cluster analysis was performed on the DAMs across
272   the four groups. The differences among the four groups were pronounced; specifically, the
273   phenolic acids were commonly more abundant in CR2, and flavonoids were commonly higher in
274   the CR1 and CR2 compared to in the other groups. Additionally, the levels of amino acids and

275   their derivatives were higher at CR3, while the contents of terpenes, nucleotides and their
276   derivatives were higher in CR4 (Fig. 5C).
277   To gain a deeper understanding of the accumulation patterns of metabolites in Dai-Bai-Jie across
278   different planting ages and altitudes, we employed k-means cluster analysis to categorize all the
279   metabolites. The analysis revealed that the metabolites clustered into six distinct groups (Fig.
280   6E). Notably, classes 1 and 6 exhibited the highest concentration of metabolites in CR2, with
281   class 6 containing the largest number of metabolites among all six classes. Classes 2 and 4, on
282   the other hand, demonstrated the highest abundance of metabolites in CR3. Class 3 was
283   characterized by the highest amount of metabolites in CR4, while class 5 displayed the highest
284   concentration of metabolites in CR1. This categorization provides valuable insights into the
285   specific patterns of metabolite accumulation within each growth year and altitude, enabling us to
286   further investigate their potential biological significance.
287   **Comparative metabolomic analysis aiming to flavonoids and flavonoid biosynthesis-related**
288   **genes among the different plantation age and locality.**
289   A total of 114 flavonoids were detected from Dai-Bai-Jie, including 34.21% flavonols, 31.58%
290   flavonoids, 14.91% dihydroflavonoids, 7.02% dihydroflavonols, 7.89% chalcone, 3.50%
291   flavanols, 0.88% flavonols, of which 79 flavonoids were differentially accumulated. Based on K-
292   means analysis, nine flavonoids, including 3′,5-Dihydroxy-4′,6,7-trimethoxyflavanone, acacitin-
293   7-O-galactide, robiniin-7-O-galactoside, phelamurin, huangbaioside, eriodictyol-7-O-glucoside,
294   exhibited a relatively high accumulation in class 2 for CR2. 15 flavonoids including 3 ', 4', 7-
295   trihydroxyflavone, cirsimaritin, hesperetin-7-O-glucoside, quercetin, exhibited a relatively high
296   accumulation in class 6 for CR2. Six flavonoids including kaempferol-7-O-glucuronid,
297   hesperetin-7-O-(6"-malonyl) glucoside, quercetin-3-O-(6"-O-galloyl) galactoside, myricetin-3-O-
298   rhamnoside (Myricitrin), diosmetin-7-O-glucuronide, syringetin-7-O-glucoside, exhibited a
299   relatively high accumulation in class 2 for CR3. Ten flavonoids including Rutin, hesperetin-5-O-
300   glucoside, isorhamnetin-3-O-rhamnoside, quercetin-3-O-robinobioside, exhibited a relatively
301   high accumulation in class 4 for CR3. Five flavonoids including 3-Hydroxy-4',5,7-
302   trimethoxyflavanone, aromadendrin-7-O-glucoside, eriodictyol-8-C-glucoside,
303   dihydromyricetin-3-O-glucoside, taxifolin-3'-O-glucoside, exhibited a relatively high
304   accumulation in class 3 for CR4. 34 flavonoids including rhamnazin , quercetin-3,4'-dimethyl
305   Ether, limocitrin-7-O-glucoside, kumatakenin, exhibited relatively high accumulation in class 5
306   for CR1.
307   To gain a deeper understanding of the molecular mechanisms underlying the differential
308   accumulation of flavonoids across various planting year and planting environments, we
309   conducted a comprehensive analysis of the expression patterns of genes involved in flavonoid
310   metabolism. KEGG analysis revealed that the 15 flavonoids exhibiting differential accumulation
311   were mapped to multiple biosynthetic pathways, including the flavonoid biosynthesis pathway
312   (KO00941), flavonol biosynthesis pathway (KO00944), as well as the broader metabolic
313   pathway (KO01100) and secondary metabolite biosynthesis pathway (KO01110)(Fig. 7A).
314   Correlation analysis was conducted between DAMs mapped to the KEGG pathway and the
315   corresponding DEGs on the pathway, and the correlation > 0.8 or < -0.8 and the P-value < 0.05
316   as the screening conditions. The analysis revealed complex regulatory relationship among
317   phenylalanine ammonia-lyase (PAL Cluster-63886.0, Cluster-63886.1), 4-Coumarate: Coenzyme
318   A Ligase (4CL, Cluster-58688.4, Cluster-62808.3), lavonol synthase (FLS, Cluster-46899.18,
319   Cluster-46899.5, Cluster-50957.2, Cluster-57391.0, C12RT1(Cluster-45854.0)and the

320 metabolites hyperin, lonicerin, vicenin-2, nicotiflorin, querceti, luteolin-7-O-(6"-malonyl)
321 glucoside, and hesperetin-7-O-glucoside (Fig. 7B).
322 **Taxonomic features of the rhizosphere microbes of Dai-Bai-Jie**
323 Plants recruit specific root-associated microbes that enable them to deliver photosynthates and
324 root exudates to their root microbiome, thereby stimulating plant growth and productivity
325 *(Lareen et al., 2016)*. Studies have indicated that the composition of microbial communities at
326 roots, the so-called root microbiome, can have significant impacts both on plant development
327 and their stress tolerance (*Mendes et al., 2011; Panke-Buisse et al., 2015*).
328 The coverage index between the bacterial and fungal sample groups exceeded 0.965, indicating
329 that the sequencing was representative and accurately reflected the bacterial and fungal diversity
330 of the samples. The four groups of rhizosphere soil bacteria involved a total of 40 phyla, 71
331 classes, 154 orders, 300 families, and 695 genera, and fungi comprised 13 phyla, 61 classes, 168
332 orders, 406 families, and 875 genera. The dominant bacterial phyla in the rhizosphere soils
333 included Crenarchaeota, Acidobacteriota, Chloroflexi, Firmicutes, Proteobacteria were the
334 dominant bacteria in the rhizosphere soils, whereas the predominant fungal phyla were
335 Ascomycota, Basidiomycota, Mortierellomycota, Glomeromycota, Chytridiomycota, and
336 Rozellomycota.
337 We investigated the richness indices (alpha diversity, ACE, Chao1) and the Shannon diversity
338 index of the microbial community, as well as the number of operational taxonomic units (OTUs)
339 across all samples. There were no significant differences in the Shannon, Chao1, and ACE indices
340 of rhizosphere microorganisms among the four groups (Table 2).
341 A total of 1952 bacterial operational taxonomic units (OTUs) and 5230 fungi were detected in
342 the rhizosphere microbiome. The co-possessed bacteria in the four rhizosphere soils are 2986
343 OTUs, 721 are unique to CM1, 406 are unique to CM2, 497 are unique to CM3, and 620 are
344 unique to CM4 (Fig. 8A). The co-possessed fungi in the four rhizosphere soils are 5677 OTUs,
345 383 are unique to CM1, 223 are unique to CM2, 263 are unique to CM3, and 406 are unique to
346 CM4 (Fig. 8B).
347 Community composition analysis revealed that the compositions were similar among all twelve
348 rhizosphere soils samples at the phylum level. Excluding CM3.3, the abundance of
349 Acidobacteriota in CM2 and CM3 was significantly higher than in CM1 and CM4 (Fig. 8C, D).
350 However, the community compositions presented some differences among all twelve rhizosphere
351 soils at the genus level (Fig. 8E, F).
352 **DISCUSSION**
353 The growth duration is the most critical factor affecting the quality of medicinal plants. Until
354 now, the harvesting period of Dai-Bai-Jie has primarily centered on biomass accumulation, with
355 the accumulation of bioactive components remaining unknown. Despite numerous research
356 reports have examined the metabolites and anti-tumor properties of *G. tenacissima*, majority of
357 these studies have not specifically targeted Dai-Bai-Jie, largely due to inaccuracies in plant
358 identification (*Li et al., 2014; Li et al., 2023*). Up to now, little is known about the chemical
359 composition and active ingredients of Dai-Bai-Jie (*Liao et al., 2016; Pang et al., 2018; Zhang et*
360 *al., 2016; Li et al., 2017*). This highlights the necessity for further scientific investigation to
361 comprehensively understand the growth patterns and accumulation of bioactive components in
362 Dai-Bai-Jie.
363 Plant-wide target detection was conducted using the high-resolution mass spectrometer AB Sciex
364 TripleTOF6600 for qualitative analysis of mixed samples. Subsequently, quantitative analysis
365 was performed using the AB Sciex 6500 QTRAP. This approach combines the advantages of

366 both non-targeted and targeted metabolomics, employing high-resolution mass spectrometry for
367 accurate qualitative detection and utilizing a triple quadrupole mass spectrometer with high
368 sensitivity, specificity, and excellent quantitative capabilities as a supplementary tool. In this
369 study, a comprehensive metabolic profiling of Dai-Bai-Jie was conducted using UPLC-MS/MS
370 widely-targeted metabolomics analysis. A total of 1,495 metabolites were successfully identified,
371 signifying the rich metabolite content of Dai-Bai-Jie. These metabolites are likely to form the
372 pharmacological material basis for the medicinal properties of Dai-Bai-Jie. Additionally, 943
373 DAMs were detected from four group samples obtained from distinct locations and three
374 different planting age, which suggests quality variations among them.
375 Flavonoids and total polyphenols were major contributors for detoxification of Dai-Bai-Jie
376 (*Zhang et al., 2023*). We detected a diverse array of secondary metabolites, including flavonoids,
377 phenolic acids, alkaloids, and terpenoids, which may potentially contribute to its antioxidant and
378 anti-inflammatory activities.
379 When comparing the accumulation of metabolites across different planting ages, it was observed
380 that the total metabolite content in CR2 and CR3 was relatively abundant. Additionally,
381 flavonoid levels were generally higher in CR1 and CR2. To achieve an optimal balance between
382 biomass, economic benefits, and the biological activity of Dai-Bai-Jie, it is recommended that
383 two-year harvesting serves as the optimal strategy.
384 Despite originating from the same planting age, samples CR3 and CR4 exhibited inconsistent
385 trends in metabolite accumulation, revealing a total of 259 DAMs. This variation can be
386 attributed to diverse environmental factors, including altitude, temperature, and soil conditions.
387 Although the number of DAMs identified was lower compared to those observed between
388 different years, it nonetheless underscores the significant impact of the environment on the
389 accumulation of secondary metabolites in Dai-Bai-Jie. Furthermore, it suggests that cultivation at
390 lower altitudes may result in a diminished abundance of secondary metabolites. This could be
391 due to the influence of lower temperatures at higher altitudes, which may induce the expression
392 of resistance genes, thereby promoting the accumulation of secondary metabolites.
393 Consequently, in the future large-scale introduction and cultivation of Dai-Bai-Jie, high-altitude
394 conditions should be carefully considered.
395 Based on Plant-wide target metabolome analysis, flavonoids are identified as the predominant
396 secondary metabolites in Dai-Bai-Jie. Notably, the flavonoid content is significantly greater in
397 plants cultivated for two and three years compared to those cultivated for one year. This finding
398 is generally consistent with the flavonoid accumulation patterns observed in most medicinal
399 plants (*Kuang et al., 2020*; *Yuan et al., 2022*).
400 Numerous flavonoids isolated from Dai-Bai-Jie have exhibited significant biological activities.
401 Specifically, hesperetin-7-O-glucoside has been demonstrated to effectively modulate the gut
402 microbiota composition and bile acid metabolism in murine models (*Wu et al., 2022*). The
403 antioxidative, antihypertensive, antidiabetic, anti-inflammatory, and cardioprotective activities of
404 rutin were reported, while rutin pretreatment before administration of ethanol can afford
405 significant protection against mucosal hyperemia, necrosis, edema, and mucosal or submucosal
406 hemorrhage (*Akash et al., 2024; Chua, 2013; Nicola et al., 2024*). Quercetin is known to possess
407 both mast cell stabilizing and gastrointestinal cytoprotective activity (*Anand David et al., 2016;*
408 *Catalina et al., 2016*).
409 The flavonoid content in Dai-Bai-Jie varies significantly with its plantation age, which may be the
410 result of DEGs patterns of genes involved in flavonoid biosynthesis. To date

411 , flavonoid biosynthetic pathway has been extensively studied, with the genes encoding enzymes
412 involved in this pathway and their respective functions having been verified in numerous plants.
413 Flavonoids, flavonols, and lignin are synthesized through various branching pathways originating
414 from the phenylpropane biosynthetic pathway (*Froemel et al., 1985*). We screened nine DEGs
415 related to flavonoid biosynthesis from Dai-Bai-Jie, *PAL, 4CL, FLS*, and *C12RT1* including. PAL
416 catalyzes the first step in the phenylpropanoid pathway and plays an important role in the
417 biosynthesis of phenylpropanoid and flavonoid compounds (*Levy et al., 2018*). 4CL is the last
418 enzyme in the general biosynthetic pathway of phenylpropane compounds, which catalyzes
419 cinnamic acid and its hydroxyl or methoxy derivatives to generate corresponding coenzyme A
420 esters (*Cao et al., 2023; Lavhale et al., 2018*). These intermediate products then enter the
421 biosynthetic pathway of phenylpropane derivatives (*Tian et al., 2017*). FLS is a key enzyme
422 specific to the flavonol pathway, which converts dihydroflavonol into the corresponding flavonol
423 by introducing a double bond between C-2 and C-3 of the C-ring (*Forkmann et al., 1986; Shi et
424 al., 2021*).
425 Correlation analysis conducted on flavonoid DAMs mapped to the KEGG pathway revealed that
426 the expression patterns of the genes *PAL, 4CL*, and *FLS* exhibited a consistent trend with the
427 accumulation of nicotiflorin and lonicerin. Similarly, hesperetin-7-O-glucoside displayed a
428 comparable trend with *C12RT1*. These DEGs may serve as key regulators of the distinct
429 accumulation patterns of flavonoid metabolites in Dai-Bai-Jie.
430 The RT-qPCR results showed that the expression trend of the key enzyme genes in the biosynthetic
431 pathway of flavonoids in Dai-Bai-Jie was consistent with the results of transcriptome sequencing,
432 thereby confirming the reliability of the transcriptome data.
433 In general, the age of plantation has been shown to induce changes in soil nutrient content and pH,
434 subsequently affecting the composition and diversity of soil bacterial and fungal communities. For
435 instance, *Na et al*. (*2016*) reported that fungal diversity decreased with the cultivation going on
436 from 5 a to 10 a of *Lycium bararum* L. whereas bacterial diversity remained relatively unchanged.
437 Conversely, *Li et al.* (*2020*) observed a significant increase in bacterial diversity and a decrease in
438 fungal diversity in lily soil with increasing planting years. However, in our study on Dai-Bai-Jie,
439 we did not detect any significant differences in the Shannon, Chao1, or ACE indices of rhizosphere
440 microorganisms across different plantation ages and localities. This inconsistency suggests that
441 the underlying mechanisms governing microbial community dynamics in the rhizospheres of Dai-
442 Bai-Jie may differ from those observed in other plant species, possibly due to the relatively short
443 introduction period of Dai-Bai-Jie.
444 The absence of significant changes in microbial diversity warrants further investigation,
445 particularly from the perspectives of soil nutrients, pH, and moisture content.
446 In summary, this study comprehensively characterized the disparities in flavonoid metabolite
447 profiles and abundances across varying cultivation environments and plantation age through
448 integrated transcriptome and metabolome analyses. Key genes intricately associated with the
449 differential accumulation of flavonoids were identified. The results laid a foundation for further
450 regulation of the effective components and support the formulation of scientifically harvesting
451 practices for Dai-Bai-Jie.

CONCLUSIONS

453 In summary, this study thoroughly characterised the disparities in metabolites and flavonoid
454 metabolite profiles and abundances across varying cultivation environments and plantation ages
455 through integrated transcriptome and metabolome analyses. A total of 1,495 metabolites were
456 identified using UPLC-MS/MS from Dai-Bai-Jie across three different planting durations (one

457   year, two years, and three years) at two distinct localities. Among these, 943 DAMs were detected.
458   A total of 114 flavonoids were identified, of which 79 exhibited differential accumulation. The
459   total metabolite content in CR2 and CR3 was relatively abundant, and flavonoid levels were
460   generally higher in CR2 and CR3. Therefore, it is recommended that harvesting at two years of
461   age be considered the optimal strategy. Key genes intricately associated with the differential
462   accumulation of flavonoids were identified. We found a complex regulatory relationship among
463   phenylalanine ammonia-lyase (PAL; Cluster-63886.0, Cluster-63886.1), 4-Coumarate: Coenzyme
464   A Ligase (4CL; Cluster-58688.4, Cluster-62808.3), flavonol synthase (FLS; Cluster-46899.18,
465   Cluster-46899.5, Cluster-50957.2, Cluster-57391.0, C12RT1; Cluster-45854.0), and the
466   metabolites hyperin, lonicerin, vicenin-2, nicotiflorin, quercetin, luteolin-7-O-(6"-malonyl)
467   glucoside, and hesperetin-7-O-glucoside. Different planting ages and localities did not result in
468   significant differences in the Shannon, Chao1, or ACE indices of the rhizosphere microorganisms
469   associated with Dai-Bai-Jie. The results establish a foundation for further regulation of
470   pharmacological components and provide support for the development of scientific harvesting
471   practices for Dai-Bai-Jie.

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481   ## REFERENCES

482   **Akash SR, Tabassum A, Aditee LM, Rahman A, Hossain MI, Hannan MA, Uddin MJ. 2024**.
483   Pharmacological insight of rutin as a potential candidate against peptic ulcer. *Biomedicine &*
484   *Pharmacotherapy* **177**:116961 DOI 10.1016/j.biopha.2024.116961.

485   **Anand David AV, Arulmoli R, Parasuraman S. 2016.** Overviews of Biological Importance of
486   Quercetin: A Bioactive Flavonoid. *Pharmacognosy Reviews* **10(20)**: 84-89 DOI 10.4103/0973-
487   7847.194044.

488   **Cao Y, Chen Y, Zhang L, Cai Y. 2023.** Two monolignoid biosynthetic genes, 4-coumarate:
489   coenzyme A ligase (4CL) and p-coumaric acid 3-hydroxylase (C3H) involved in lignin
490   accumulation in pear fruits. *Physiology and Molecular Biology of Plants* **29(6)**:791-798 DOI
491   10.1007/s12298-023-01329-1.

492   **Catalina CP, Caroll B, Alfonso M, Jocelyn F, Martin G. 2016.** Molecular mechanisms of
493   gastrointestinal protection by quercetin against indomethacin-induced damage: role of NF- κ B
494   and Nrf2 - ScienceDirect. *The Journal of Nutritional Biochemistry* **27**:289-298 DOI
495   10.1016/j.jnutbio.2015.09.016.

496   **Chen S, Zhou Y, Chen Y, Gu J. 2018.** fastp: an ultra-fast all-in-one FASTQ preprocessor.
497   *Bioinformatics* **34(17)**: i884-i890 DOI 10.1093/bioinformatics/bty560.

498   **Chen X, Luo Z, Liu X, Li X, Li Q, Zhang W, Liu Y, Cheng Z, Yang X, Liu Y, Jin R, ZhuD,**
499   **Wang F, Lu Q, Su Z, Guo H. 2022.** *Marsdenia tenacissima* (Roxb.) Moon injection exerts a
500   potential anti-tumor effect in prostate cancer through inhibiting ErbB2-GSK3β-HIF1α signaling
501   axis. *Journal of ethnopharmacology* **295**:115381-115381 DOI org/10.1016/j.jep.2022.115381.

502 **Chua LS. 2013.** A review on plant-based rutin extraction methods and its pharmacological
503 activities. *Journal of Ethnopharmacology* **150(3)**:805-17 DOI 10.1016/j.jep.2013.10.036.
504 **Fang H, Zhao X, Zhang M, Ma Y, Huang J, Zhou P. 2023**. Beneficial effects of flavonoids on
505 cardiovascular diseases by influencing NLRP3 inflammasome. *Inflammopharmacology*
506 **31(4)**:1715-1729 DOI 10.1007/s10787-023-01249-2.
507 **Forkmann G, Vlaming PD, Spribille R, Wiering H, Schram AW.1986.** Genetic and
508 Biochemical Studies on the Conversion of Dihydroflavonols to Flavonols in Flowers of Petunia
509 hybrida. *Zeitschrift für Naturforschung C* **41**:179-186 DOI 10.1515/znc-1986-1-227.
510 **Froemel S, de Vlaming P, Stotz G, Wiering H, Forkmann G, Schram AW.1985.** Genetic and
511 biochemical studies on the conversion of flavanones to dihydroflavonols in flowers of Petunia
512 hybrida. *Theoretical And Applied Genetics* **70(5)**:561-8. DOI 10.1007/bf00305991.
513 **Gao M, Wang SG, Ni K, Zhao Y, Zhang Y. 2014**. Protective Effect of Dai Baijie on Liver Injury
514 Induced by AIDS Antivirals. *Yunnan Journal of Traditional Chinese Medicine and Materia*
515 *Medica* **35(04)**:60-61 DOI 10.16254/j.cnki.53-1120/r.2014.04.032.
516 **Kuang CY, Jiang LH, Wang ZH. 2020.** Study on Dynamic Changes of Flavoind Contents in the
517 Tuber of *Smilax biumbellata* with Different Growth Years and Harvesting Periods**.** *Journal of*
518 *Chinese Midicinal Materials* **43(12):** 2905-2908 DOI 10.13863/j.issn1001-4454.2020.12.010.
519 **Lareen A, Burton F, Schäfer P. 2016.** Plant root-microbe communication in shaping root
520 microbiomes. *Plant Molecular Biology* **90(6)**: 575-87 DOI 10.1007/s11103-015-0417-8.
521 **Lavhale SG, Kalunke RM, Giri AP. 2018.** Structural, functional and evolutionary diversity of
522 4-coumarate-CoA ligase in plants. *Planta* **248(5)**:1063-1078 DOI 10.1007/s00425-018-2965-z.
523 **Levy HL, Sarkissian CN, Scriver CR. 2018**. Phenylalanine ammonia lyase (PAL): From
524 discovery to enzyme substitution therapy for phenylketonuria. *Molecular Genetics and*
525 *Metabolism* **124(4)**:223-229 DOI 10.1016/j.ymgme.2018.06.002.
526 **Li HT, Kang LP, Guo BL, Zhang ZL, Guan YH, Pang X, Peng CZ, Mai BP, Li X. Z. 2014.**
527 Textual Research on the Origin of the Commonly Used Dai Medicine "Dai Bai Jie". *China*
528 *Journal of Chinese Materia Medica* **39(8)**:5 DOI CNKI:SUN:ZGZY.0.2014-08-033.
529 **Li JY, Tan Yh, Li HT, Yan HD, Gong YX, Xiao YX, Yu WB. 2023**. Research on the Origin of
530 Dai Medicine "Dai Bai Jie" Based on Molecular and Morphological Evidence. *Guihaia* **43(01)**:32-
531 42 DOI 10.11931/guihaia.gxzw202110070.
532 **Li PT, Michael GG, Douglas SW.** Flora of China[M]. Science Press, Beijing, 1995, **16:** 189–
533 270
534 **Li XH, Li HT, Jin LY, Niu YF, Zeng QW, Zhang LX. 2021**. Study on Antioxidant and
535 Antibacterial Activities of Different Polar Fractions of Ethanol Extract from Dai Bai Jie in vitro.
536 *Yunnan Journal of Traditional Chinese Medicine and Materia Medica* **42(06)**:69-73 DOI
537 10.16254/j.cnki.53-1120/r.2021.06.023.
538 **Liao M, Zhang Y, Chen F, Yao Y. 2016.** Research on Chemical Constituents of Dai Bai Jie.
539 *Journal of South-Central University for Nationalities (Natural Science Edition)* **35(3)**:39-41 DOI
540 10.3969/j.issn.1672-4321.2016.03.009.
541 **Lin YF, Zhuan Y, Zhao YH. 2003.** Color Atlas of Dai Medicine in China. Kunming Yunnan
542 Publishing House of Minority Nationalities.
543 **Love LM, Wolfgang H, Simon A. 2014.** Moderated estimation of fold change and dispersion for
544 RNA-seq data with DESeq2. *Genome biology* **15(12)**:550 DOI 10.1186/s13059-014-0550-8
545 **Liede-Schumann S, Reuss SJ, Meve U, Gâteblé G, Livshultz T, Forster PI, Wanntorp L,**
546 **Rodda M. 2022.** Phylogeny of Marsdenieae (Apocynaceae, Asclepiadoideae) based on

547 chloroplast and nuclear loci, with a conspectus of the genera. *Taxon* **71 (4):** 833–875 DOI
548 org/10.1002/tax. 12713.
549 **Mendes R, Kruijt M, de Bruijn I, Dekkers E, van der Voort M, Schneider JH, Piceno YM,**
550 **DeSantis TZ, Andersen GL, Bakker PA, Raaijmakers JM. 2011.** Deciphering the rhizosphere
551 microbiome for disease-suppressive bacteria. *Science* **332(6033)**:1097-100 DOI 10.1126/science.
552 1203980.
553 **Nicola MA, Attaai AH, Abdel-Raheem MH, Mohammed AF, Abu-Elhassan YF. 2024.**
554 Neuroprotective effects of rutin against cuprizone-induced multiple sclerosis in mice.
555 *Inflammopharmacology* **32(2)**:1295-1315 DOI 10.1007/s10787-024-01442-x.
556 **Oyanagi M, Ozeki Y.2001**. Functional analysis of Phenylalanine ammonia-lyase gene promoter
557 of popular. *Progress in Biotechnology* **18(01)**:163-170 DOI 10.1016/S0921-0423(01)80069-1.
558 **Pang X, Kang LP, Fang XM, Yu HS, Han LF, Zhao Y, Zhang LX, Yu LY, Ma BP. 2018.**
559 C(21) steroid derivatives from the Dai herbal medicine Dai-Bai-Jie, the dried roots of *Marsdenia*
560 *tenacissima*, and their screening for anti-HIV activity. *Journal of Natural Medicines* **72(1)**:166-
561 180 DOI 10.1007/s11418-017-1126-1.
562 **Panke-Buisse K, Poole AC, Goodrich JK, Ley RE, Kao-Kniffin J. 2015.** Selection on soil
563 microbiomes reveals reproducible impacts on plant function. *Isme Journal* **9(4)**:980-9 DOI
564 10.1038/ismej.2014.196.
565 **Shi Y, Jiang X, Chen L, Li WW, Lai S, Fu Z, Liu Y, Qian Y, Gao L, Xia T. 2021.** Functional
566 Analyses of Flavonol Synthase Genes From *Camellia sinensis* Reveal Their Roles in Anther
567 Development. *Frontiers in Plant Science* **12**:753131 DOI 10.3389/fpls. 2021.753131.
568 **Song J, Dai R, Deng Y, Lv F. 2018.** Rapid structure prediction by HPLC-ESI-MS(n) of twenty-
569 five polyoxypregnane tetraglycosides from *Dregea sinensis* with NMR confirmation of eight
570 structures. *Phytochemistry* **147**:147-157 DOI 10.1016/j.phytochem.2017.12.021.
571 **Song J, Lv F, Dai RJ, Deng YL. 2021.** Rapid identification and structural characterization of
572 polyoxypregnane glycosides in Dregea sinensis by HPLC-MS(n) and HRMS. *Journal of Asian*
573 *Natural Products Research* **23(1)**:9-19 DOI 10.1080/10286020.2020.1715951.
574 **Tian XM, Yan LH, Xiang GF, LY J. 2017.** Research Progress on 4-Coumarate: Coenzyme A
575 Ligase(4CL) in Plants. *Biotechnology Bulletin* **33**:19-26 DOI
576 10.13560/j.cnki.biotech.bull.1985.2017.04.003.
577 **Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL,**
578 **Wold BJ Pachter L**. **2010.** Transcript assembly and quantification by RNA-Seq reveals
579 unannotated transcripts and isoform switching during cell differentiation. *Nature biotechnology*
580 **28(5)**:511-5 DOI 10.1038/nbt.1621.
581 **Varet H, Brillet-Guéguen L, Coppée JY, Dillies MA. 2016.** SARTools: A DESeq2- and EdgeR-
582 Based R Pipeline for Comprehensive Differential Analysis of RNA-Seq Data. *Plos One* **11(6)**:
583 e0157022 DOI 10.1371/journal.pone.0157022.
584 **Wang L, Wang S, Li W. 2012.** RSeQC: quality control of RNA-seq experiments. *Bioinformatics*
585 **28(16)**: 2184-5 DOI 10.1093/bioinformatics/bts356.
586 **Wang Q, Zhao H, Zhu M, Gao L, Cheng N, Cao W. 2020.** Spectroscopy characterization,
587 theoretical study and antioxidant activities of the flavonoids-Pb(II) complexes. *Journal of*
588 *Molecular Structure* **1209**:127919   DOI 10.1016/j.molstruc.2020.127919.
589 **Wu F, Lei HH, Chen G, Chen Ch, Song YC, Cao Z, Zhang C, Zhang C, Zhou JL, Lu YJ,**
590 **Zhang LM. 2022.** Multiomics Analyses Reveal That Long-Term Intake of Hesperetin-7-O-
591 glucoside Modulates the Gut Microbiota and Bile Acid Metabolism in Mice. *Journal of*
592 *agricultural and food chemistry* **70(47)**:14831-14840 DOI 10.1021/acs.jafc.2c05053.

593 **Yang LP, Chen P, Zhang GY, H. DX. 2020**. Protective Effect and Mechanism of Dai Bai Jie on
594 Intestinal Mucosal Barrier Function. *Journal of Yunnan University of Traditional Chinese Medicin*
595 *e***(004)**: 043 DOI 10.19288/j.cnki.issn.1000-2723.2020.04.001.
596 **Zhang P, Fan L, Zhang D, Zhang Z, Wang W. 2023.** In Vitro Anti-Tumor and Hypoglycemic
597 Effects of Total Flavonoids from Willow Buds. *Molecules* **28(22): 7557** DOI
598 10.3390/molecules28227557.
599 **Zhang XF, Zhao JK, Hao SQ, Ding Q, Yu SH, Yi ML, Wang J, Hu HY. 2023.** Biological
600 activities and detoxification mechanisms of Clerodendrum chinense var. simplex, Marsdenia
601 tenacissima and Arundina graminifolia: The Dai antidotes. *Journal of Sun Yat-sen University*
602 *(Natural Science Edition)* **62(3)**:89-99 DOI 10.13471/j.cnki.acta.snus.2022e027.
603 **Zou LQ, Wang CX, Kuang XJ, Li Y, Sun C. 2016.** Advance in flavonoids biosynthetic
604 pathway and synthetic biology. *China journal of chinese Materia Medica* **41(22)**: 4124-4128
605 DOI 10.4268/cjcmm20162207.

Figure 1

the sample used in this study

A, total plant of Dai-Bai-Jie. B, Root of cross-sections at different planting years. CR1: farmed for one year, CR2: farmed for two years, CR3, farmed for three years.
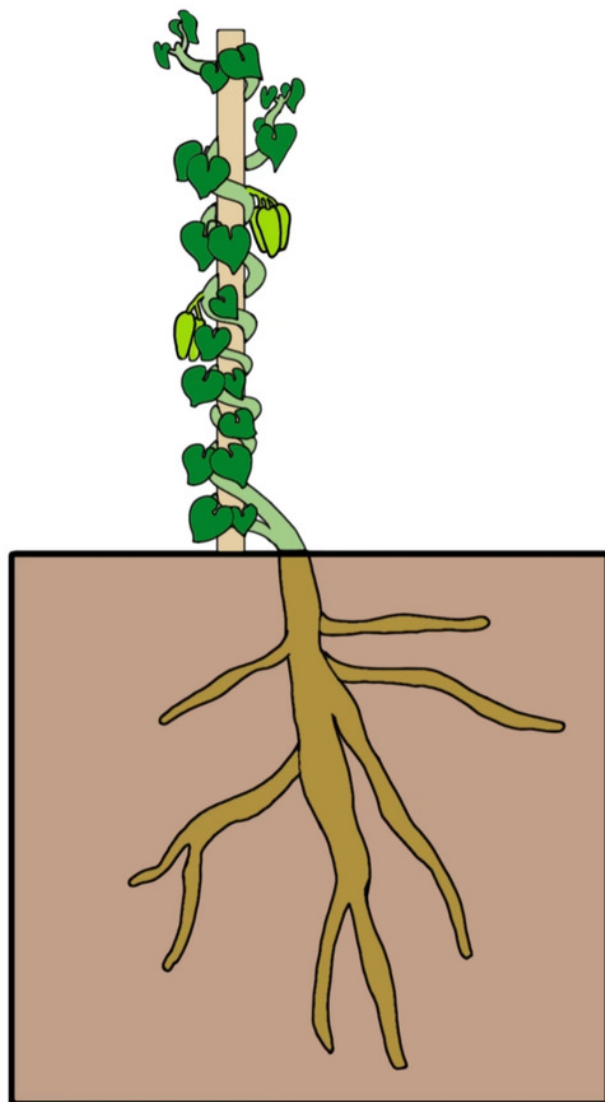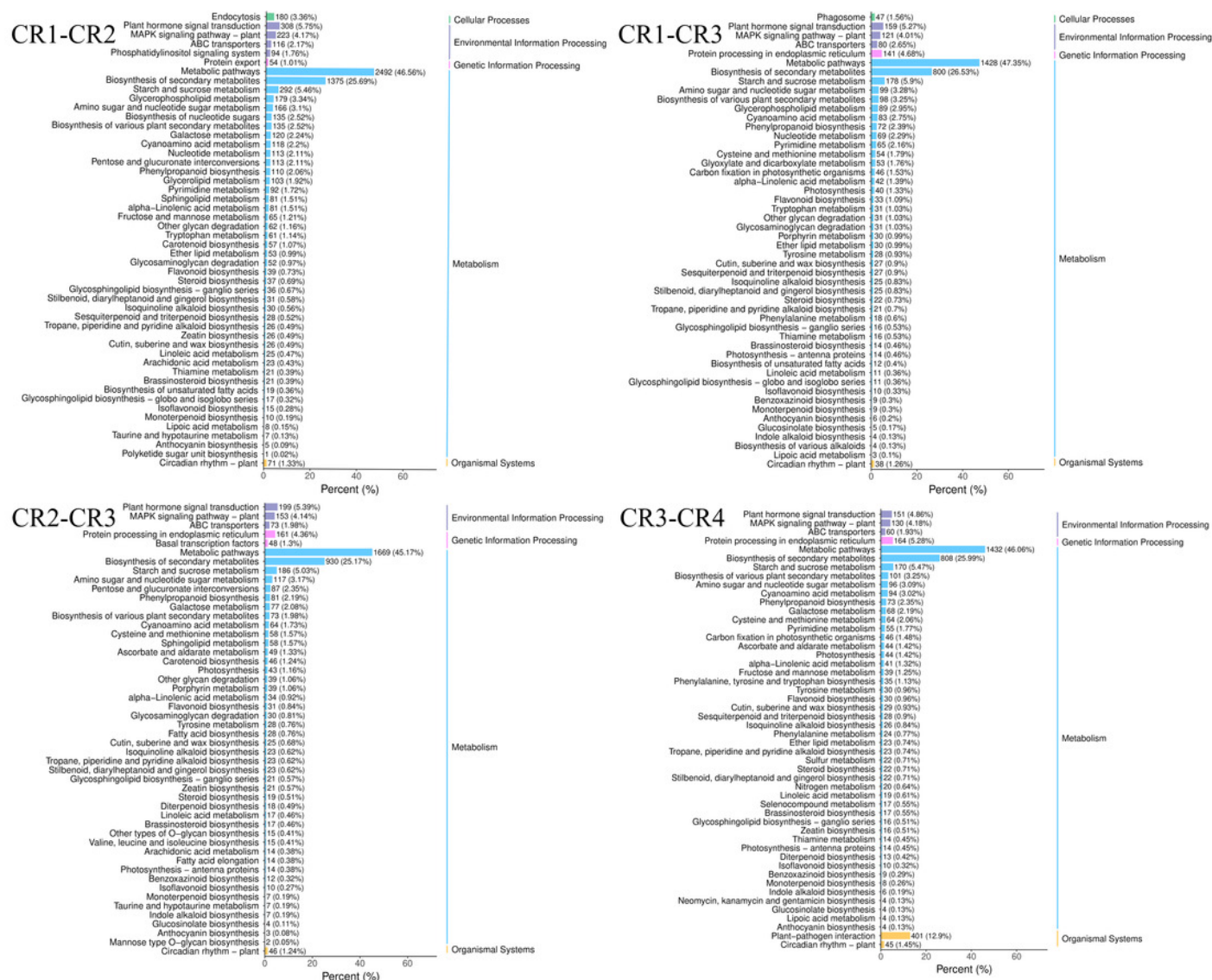
Figure 2

The DEGs in the four groups were analyzed by KEGG metabolic pathway

(A) CR1-CR2. (B) CR1-CR3. (C) CR2-CR3. (D)CR3-CR4.

Figure 3

Transcriptome analysis results

(A) BUSCO completeness assessments of the Dai-Bai-Jie transcriptome. (B) WGCNA clustering

tree. (C) Venn Diagram representing the number of DEGs among four group sample.
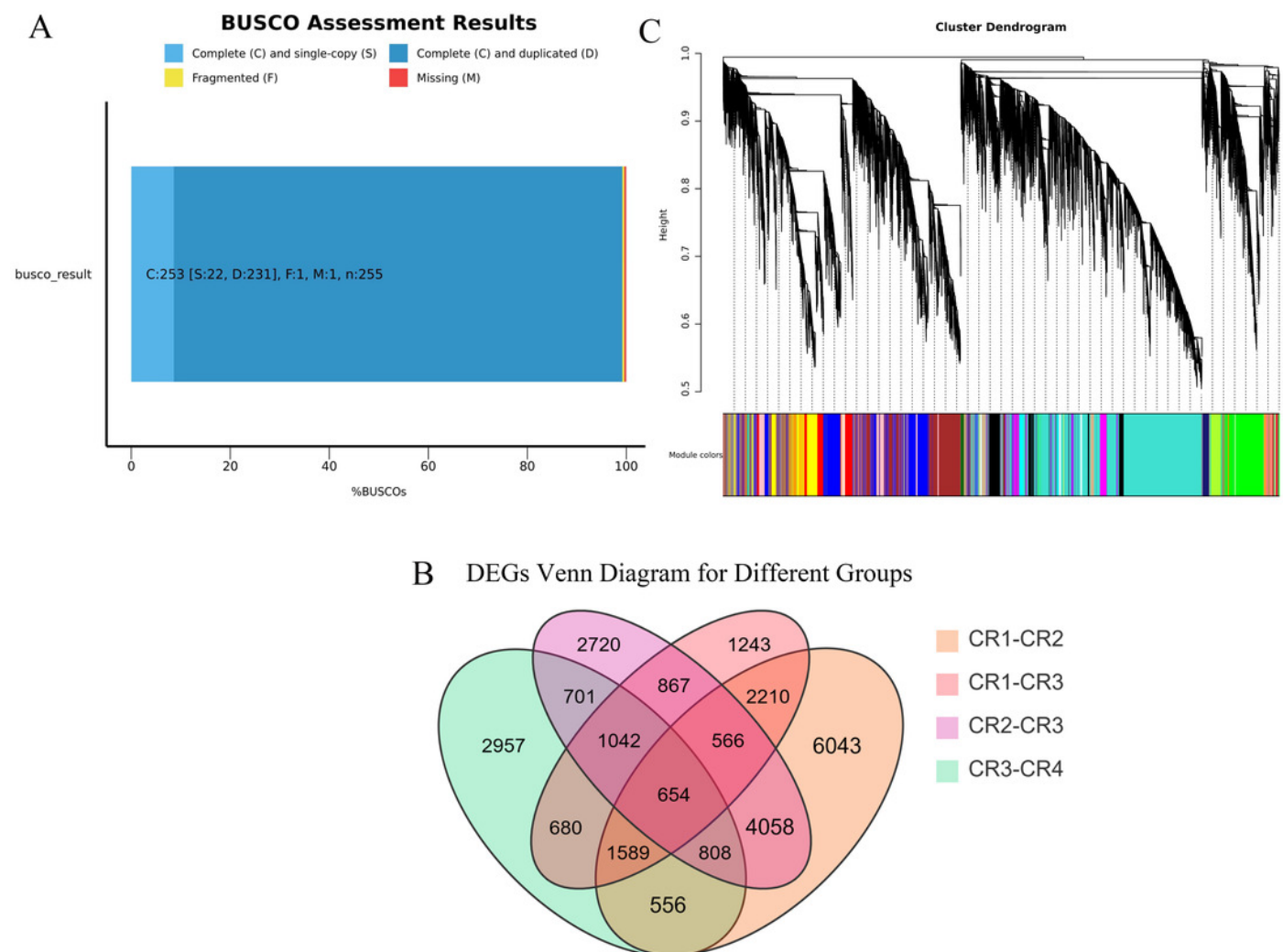
Figure 4

RNA-seq analysis of Dai-Bai-Jie and the qRT-PCR validation of five genes.

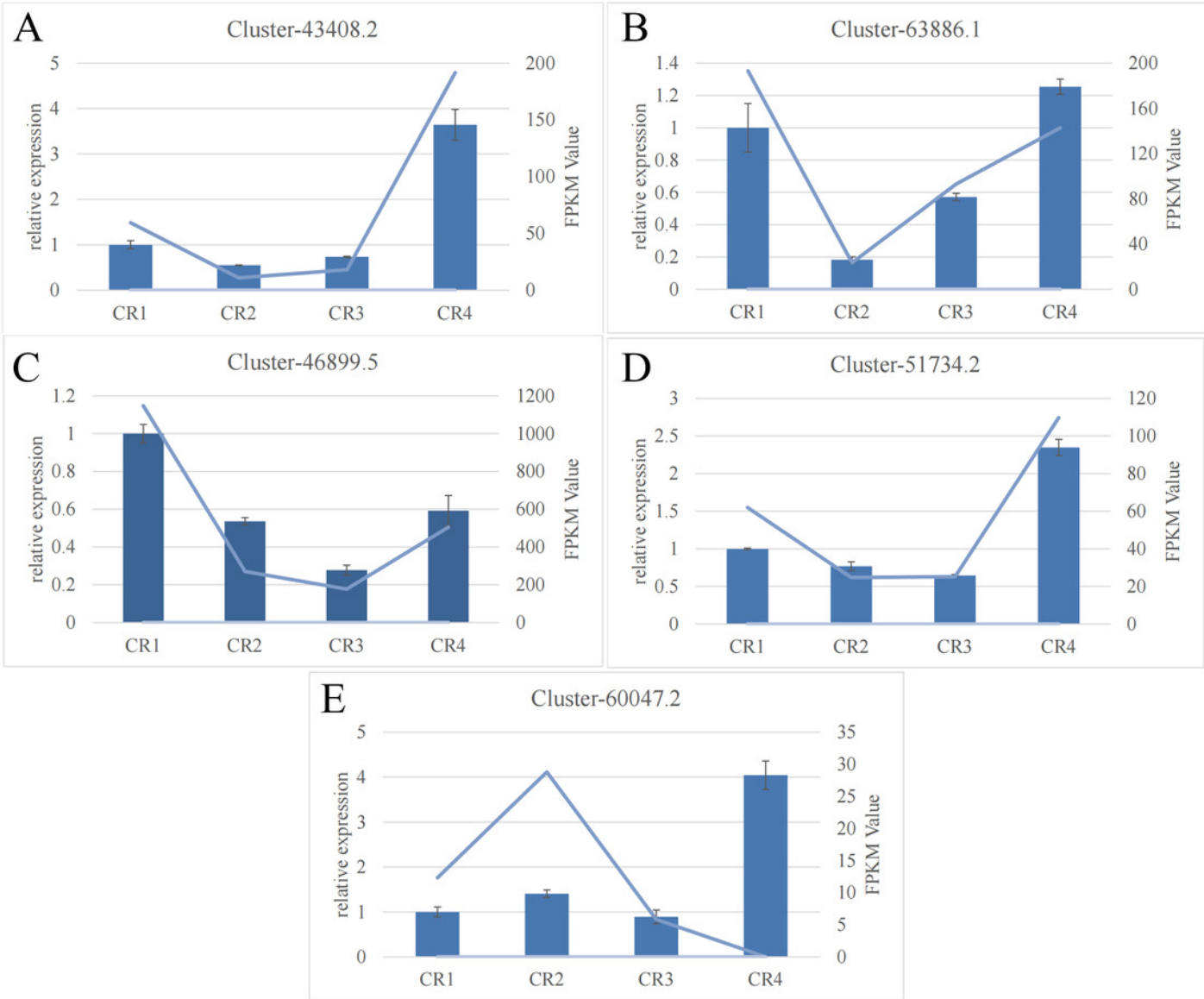RNA-seq analysis of Dai-Bai-Jie and the qRT-PCR validation of five genes.
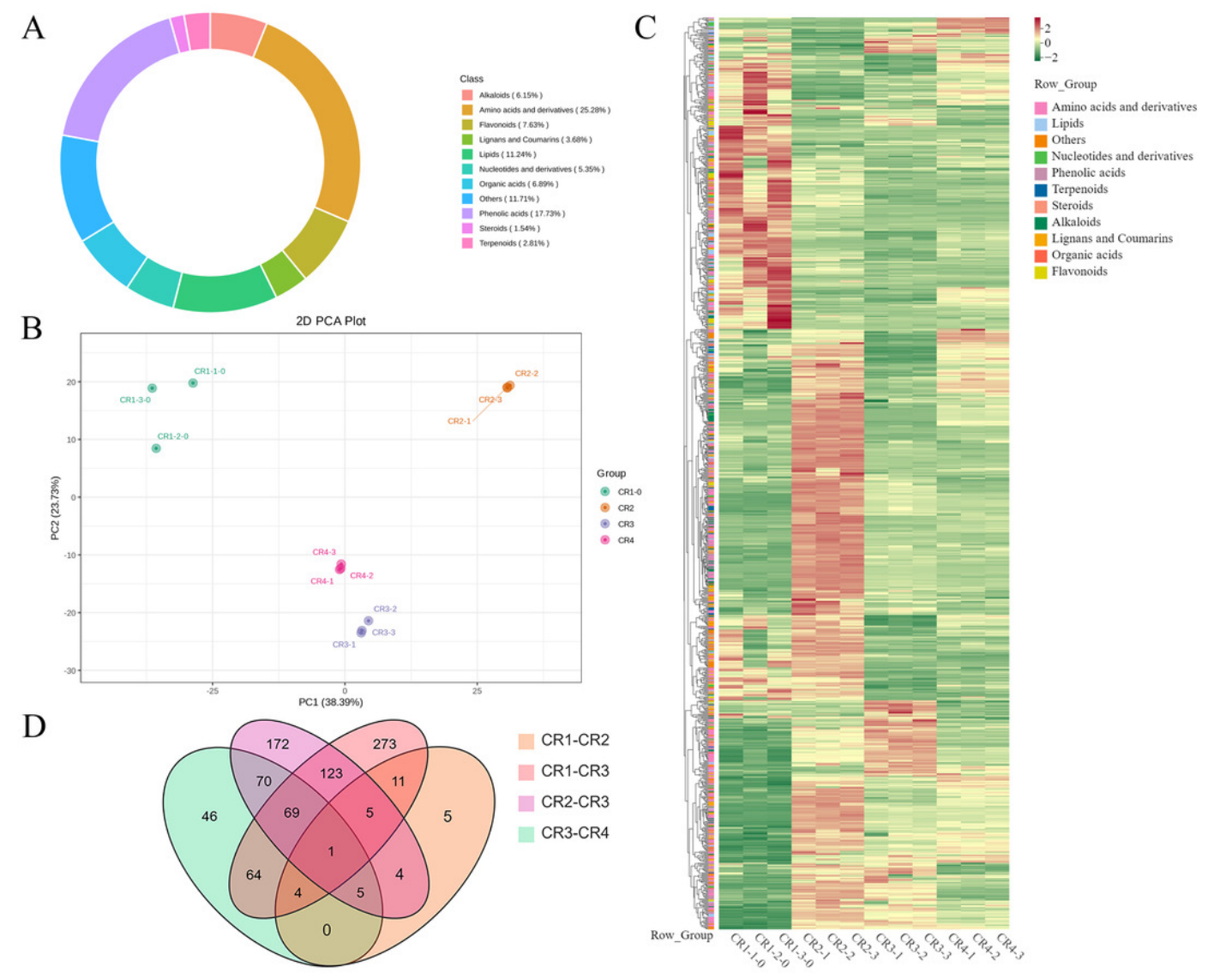
Figure 5

Metabolome analysis results

(A) Composition of metabolite in Dai-Bai-Jie. (B) PCA score plots for all samples. (C) Heat map of DAMs in four groups of samples. (D) Venn diagram of DAMs across groups.

Figure 6

The volcano diagram and the k-means diagram of Metabolites.

(A) Volcano diagram of DAMs (CR1 vs. CR2). (B)Volcano diagram of DAMs (CR1vs. CR3).
(C)Volcano diagram of DAMs（CR2 vs. CR3). (D)Volcano diagram of DAMs (CR3 vs.CR4). (E)
The K-means analysis of all Metabolites. The black line in the figure represents the average
pattern of all Metabolites in each class, and different colors represent different trend.
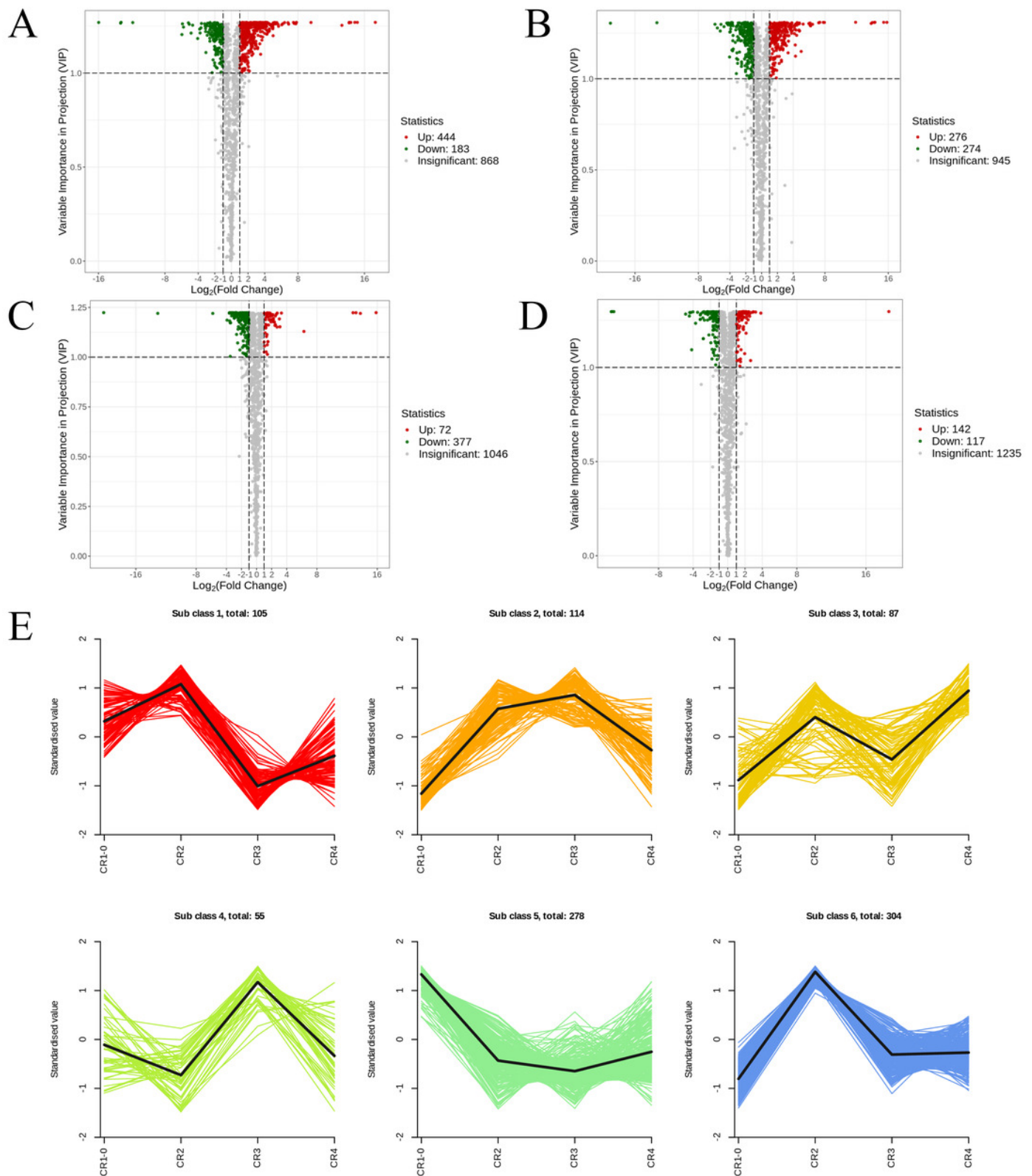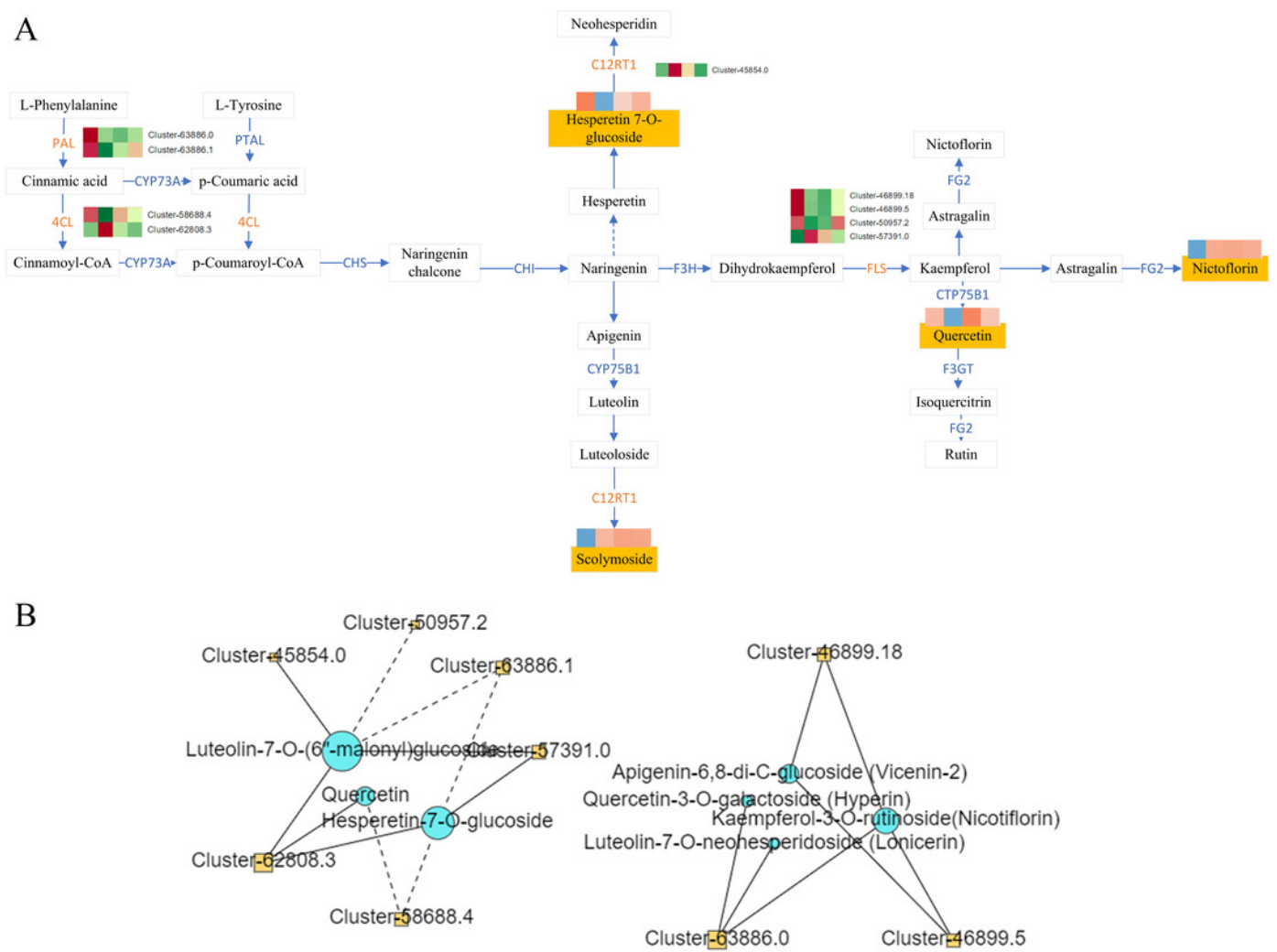
Figure 7

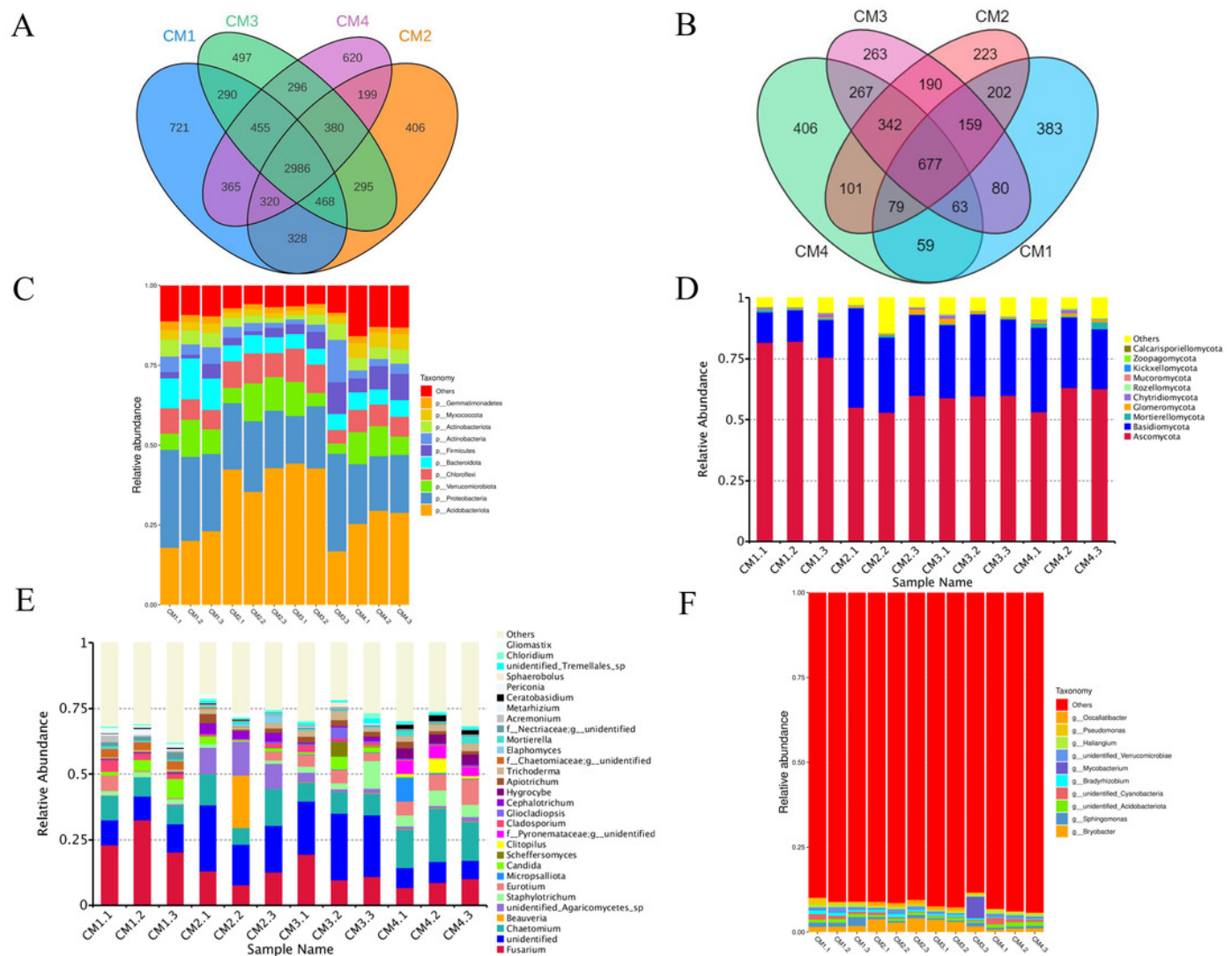Flavonoid synthesis pathway and Network diagram

(A)Flavonoid synthesis pathway. (B) Network diagram of flavonoids and differential genes.

Figure 8

Venn diagram and the relative abundance of phylum and genus among CM1, CM2, CM3, and CM4 in rhizosphere soil of Dai-Bai-Jie.

(A) Venn diagram of bacterial. (B) Venn diagram of fungus. (C) relative abundance of bacterial phylum. (D)relative abundance of fungal phylum. (E) relative abundance of bacterial genus. (F)relative abundance of fungal genus.

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Table 1(on next page)

primer of Five genes

1

Table 1 primer of Five genes

2

Gene	sequence（5'-3'）	product size
Cluster-43408.2 FLS	F: TGATGAATGGGAAGCCCGAG R: TAGCGGTCCTGTTTTGGCTT	175bp
Cluster-46899.5 FLS	F: AGCCCTTGAAGAATTTGGTTGT R: ATCTCTTGTAAAGGCCGATCAAA	114bp
Cluster-51734.2 CYP73A	F：GGACCTGGCTAAGGAAGTGT R: TGTGAAGAAAGGCACCGTCA	166bp
Cluster-60047.2 4CL	F: GCATCCGTGGCGATCAAATC R: TGCCACTTGGAACCCTTTGT	179bp
Cluster-63886.1 PAL	F: CATGCCCTCCTCAACAACGA R: GGACCTGCACTCCTTGATCC	171bp
GAPDH	F: GGCATTGTCGAGGGTCTCAT R: CCGGTGCTGCTGGGAATAAT	131bp

3

Table 2(on next page)

Diversity index of microbial communities in roots soils

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1

2

3

4   Table 2   Diversity index of microbial communities in roots soils（mean ± SD,n = 3）

Sample		Shannon	Chao1	ACE	Goods_coverage
16s	CM1	10.275±0.133	4844.719±755.638	4912.204±744.152	0.972±0.006
	CM2	9.546±0.128	4449.788±173.103	4563.577±259.590	0.972±0.002
	CM3	9.507±0.159	4580.545±122.909	4696.261±115.363	0.972±0.002
	CM4	10.039±0.067	4857.131±150.655	4936.003±163.463	0.971±0.002
ITS	CM1	5.894±0.317	1207.431±60.164	1227.965±58.934	0.997±0.001
	CM2	5.267±0.459	1152.941±259.092	1185.874±266.003	0.997±0.001
	CM3	5.722±0.276	1408.72±171.792	1445.749±164.948	0.996±0.001
	CM4	6.320±0.133	1424.764±70.520	1449.286±80.612	0.996±0.001