Chemical and Biological Technologies in Agriculture

RESEARCH

Open Access

Metabolomics reveals the effects producing region and fermentation stage on substance conversion in cigar tobacco leaf

Guanghai Zhang[1], Heng Yao[1], Gaokun Zhao[1], Yuping Wu[1], Huachan Xia[1], Yongping Li[1] and Guanghui Kong[1*]

Abstract

Background The quality of cigar tobacco leaves was closely related to fermentation. To investigate the substance changes in cigar tobacco leaves during their fermentation, metabolomics was determined at different fermentation stages. In this study, the metabolic profile among different regions and fermentation periods of cigar tobacco leaf were evaluated.

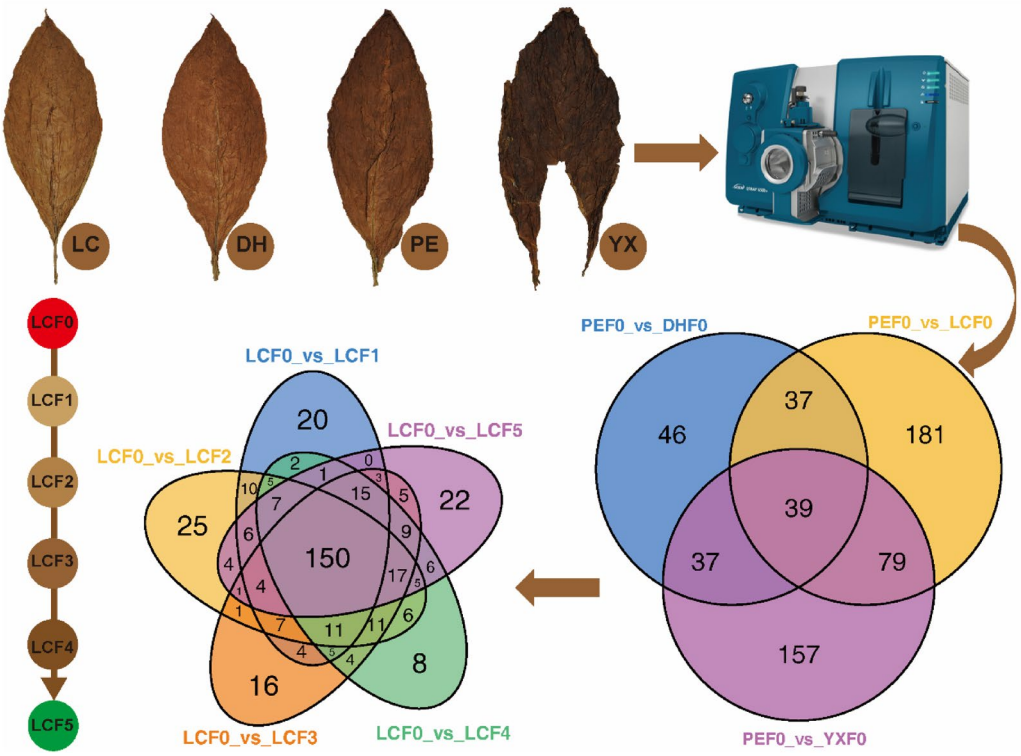
Results A total of 1103 metabolites were identified in cigar tobacco leaf samples. A total of 293, 105 and 199 metabolites showed differential accumulation in the cigar tobacco leaf among different regions (PEF0 vs. LCF0, PEF0 vs. DHF0, PEF0 vs. YXF0) and 216, 242, 220, 227 and 198 metabolites showed differential accumulation in the different fermentation (LCF0 vs. LCF1, LCF0 vs. LCF2, LCF0 vs. LCF3, LCF0 vs. LCF4, LCF0 vs. LCF5). The main upregulated compounds were flavonoids, phenolic acids and lignans and coumarins, and the main downregulated compounds were organic acids, phenolic acids and amino acids and derivatives in the fermentation comparison group.

Conclusions These results provide valuable information for accurately grasping the end time of fermentation and improve efficiency of cigar tobacco leaf fermentation.

Keywords Cigar tobacco leaf, Fermentation, Metabolomics

*Correspondence:
Guanghui Kong
13908776036@163.com
Full list of author information is available at the end of the article

Graphical Abstract



Introduction

High-quality cigar tobacco leaves are the basis of making high-quality cigars. Cigar tobacco leaves are still raw tobacco after drying. The tobacco leaves are green and variegative, poor toughness and easy to break. There are some defects in the quality of inhalation, such as lack of fragrance, heavy impurity, irritant, coarseness, pungency and bitterness [1, 2]. The tobacco leaves must undergo a certain period of fermentation or aging, so that its quality and processing properties can be significantly improved, in line with the requirements of industrial rolled cigars [3]. At the end of fermentation, the green gas, fishy gas and miscellaneous gas of tobacco leaves were significantly reduced or even eliminated [1, 4–6]. Cigar tobacco leaf fermentation is a process of biomass conversion in tobacco leaf under the combined action of enzymes, microorganisms and other factors under the condition of artificially controlled ambient temperature and humidity. However, due to the short development history and insufficient accumulation time, the cigar tobacco leaves fermentation technology is still the most difficult core technology in the cigar tobacco raw material production.

Tobacco fermentation is a process of biochemical transformation of organic compounds in tobacco leaves under the synergistic action of inorganic elements, enzymes and microorganisms. Chemical reaction effects mainly include REDOX reaction and Maillard reaction. Organic matter oxidizes with oxygen in the air under the catalysis of inorganic elements (Fe, Mg, etc.) [7, 8]. There are many enzymes in tobacco leaf cells, which are the main catalytic factors of various chemical transformation pathways in tobacco leaf fermentation [9–11]. Frankenburg proposed that tobacco leaf fermentation is a process of substance conversion catalyzed by enzyme (Frankenburg, [2]; Frankenburg, [1]).

After proper fermentation, the smoking quality, appearance quality and physical properties of cigar tobacco were improved obviously, which were closely related to the chemical changes of tobacco leaves during fermentation [12]. Its smell, color, texture, water retention and pH value have certain characteristics. Cigar tobacco leaf fermentation is a further metabolic process after air drying, including substance conversion, degradation, acidification, volatilization and so on [13]. So far, there has been no systematic study on the changes

of metabolome in cigar tobacco leaves during fermentation. In this study, broad target analysis was conducted on cigar tobacco leaves from different producing areas and cigar tobacco leaves from the same producing area at different fermentation periods, and the changes of metabolome of cigar tobacco leaves after treatment were systematically analyzed. The purpose of this study was to reveal the dynamic law of substance transformation during the fermentation process, so as to provide common reference for precise control of cigar tobacco leaves and their processing and breeding.

This study was designed mainly to address the following: (1) the metabolic variation of cigar tobacco leaf among different regions; (2) the metabolic variation of cigar tobacco leaf in different stages of fermentation. We hypothesized that (1) cigar tobacco leaf among different regions had different metabolomics profiles; (2) there were significant differences in metabolomics of cigar tobacco leaf in different stages of fermentation.

Materials and methods

Plant materials and experimental design

Metabolome detection was performed on cigar tobacco (*Nicotiana tabacum*) leaves from four different producing areas: Dehong (DH), Lincang (LC), Pu 'er (PE) and Yuxi (YX) before fermentation (F0, at 0 days), and 3 biological replicates were performed in each region.

The cigar tobacco leaves planted in LC were selected for fermentation experiments. The fermentation treatment was carried out in accordance with the Technical Regulations for Stacking and Fermentation of Cigar Tobacco Leaves, and the specific treatment process was as follows: (1) insecticidal storage (F1, at 4 days); (2) sorting (F2, at 14 days); (3) humidification and reduction (F3, at 24 days); (4) stacking and fermentation (stacking, turning over (5 times) (F4, at 34 days); (5) unstacking) (F5, at 25 days). A total of 9 handfuls of cigar tobacco leaves were taken from the upper, middle and lower layers when the stack was turned over each time. Six tobacco leaves with the same appearance and quality were selected for each hand. About 10 g of cigar tobacco leaves from the same part (avoiding the main vein) were cut with sterilizing scissors (about 2 g of cigar tobacco leaves per leaf) and placed in centrifugal tubes, which were frozen in liquid nitrogen and stored in a − 80 ℃ refrigerator. The metabolome was measured with 3 biological replicates per sample. Mixing sample extracts were used as quality control (QC) sample.

Sample preparation and extraction

Biological samples are freeze-dried by vacuum freeze-dryer (Scientz-100F). The freeze-dried sample was crushed using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. Dissolve 50 mg of lyophilized powder with 1.2 mL 70% methanol solution, vortex 30 s every 30 min for 6 times in total. Following centrifugation at 12000 rpm for 3 min, the extracts were filtrated (SCAA-104, 0.22 μm pore size; ANPEL, Shanghai, China, http://www.anpel.com.cn/) before UPLC–MS/MS analysis.

UPLC Conditions for metabolomics analysis

The sample extracts were analyzed using an UPLC–ESI–MS/MS system (UPLC, ExionLC™ AD, https://sciex.com.cn/; MS, Applied Biosystems 6500 Q TRAP, https://sciex.com.cn/). The analytical conditions were as follows, UPLC: column, Agilent SB-C18 (1.8 μm, 2.1 × 100 mm); The mobile phase was consisted of solvent A, pure water with 0.1% formic acid, and solvent B, acetonitrile with 0.1% formic acid. Sample measurements were performed with a gradient program that employed the starting conditions of 95% A, 5% B. Within 9 min, a linear gradient to 5% A, 95% B was programmed, and a composition of 5% A, 95% B was kept for 1 min. Subsequently, a composition of 95% A, 5.0% B was adjusted within 1.1 min and kept for 2.9 min. The flow velocity was set as 0.35 mL per minute; The column oven was set to 40 °C; The injection volume was 2 μL. The effluent was alternatively connected to an ESI–triple quadrupole–linear ion trap (QTRAP)–MS.

ESI-Q TRAP–MS/MS conditions for metabolomics analysis

The ESI source operation parameters were as follows: source temperature 500 °C; ion spray voltage (IS) 5500 V (positive ion mode)/-4500 V (negative ion mode); ion source gas I (GSI), gas II(GSII), curtain gas (CUR) were set at 50, 60, and 25 psi, respectively; the collision-activated dissociation (CAD) was high. QQQ scans were acquired as MRM experiments with collision gas (nitrogen) set to medium. DP (declustering potential) and CE (collision energy) for individual MRM transitions was done with further DP and CE optimization. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

Data analysis of metabolites in cigar tobacco leaves

According to the self-built database MWDB (Metware Biotechnology Co., Ltd. Wuhan, China) and the public database of metabolite information, primary and secondary mass spectrometry data were conducted to qualitative analysis via referencing existing mass spectrometry databases.

Unsupervised PCA (principal component analysis) was performed by statistics function prcomp within R (www.r-project.org). The data were unit variance scaled before unsupervised PCA. The HCA (hierarchical cluster

analysis) results of samples and metabolites were presented as heatmaps with dendrograms, while Pearson correlation coefficients (PCC) between samples were calculated by the cor function in R and presented as only heatmaps. Both HCA and PCC were carried out by R package Complex Heatmap. For HCA, normalized signal intensities of metabolites (unit variance scaling) are visualized as a color spectrum.

Differential metabolites selected in cigar tobacco leaves

For two-group analysis, differential metabolites were determined by VIP (VIP ≥ 1) and absolute Log2FC (|Log2FC| ≥ 1.0). VIP values were extracted from OPLS-DA result, which also contain score plots and permutation plots, was generated using R package MetaboAnalystR. The data were log transform (log2) and mean centering before OPLS-DA. To avoid overfitting, a permutation test (200 permutations) was performed.

KEGG annotation and enrichment analysis of differential metabolites

Identified metabolites were annotated using KEGG Compound database (http://www.kegg.jp/kegg/compound/), annotated metabolites were then mapped to KEGG Pathway database (http://www.kegg.jp/kegg/pathway.html). Pathways with significantly regulated metabolites mapped to were then fed into MSEA (metabolite sets enrichment analysis), their significance was determined by hypergeometric test's *p* values.

Results

Metabolic profiling in cigar tobacco leaf

Additional file 1: Fig S1A, B illustrate TIC plots of QC samples and multi-peak metabolites detected in MRM mode. TIC plots show intensity summed over time for all ions in a mass spectrum. In the multi-peak detection plot, peaks in different colors represent metabolites detected. According to the local metabolite database, a total of 1103 metabolites were identified via qualitative and quantitative analysis based on ion pair information of metabolites in cigar tobacco leaf (Table 1). The metabolites included 155 alkaloids, 139 amino acids and derivatives, 155 flavonoids, 37 lignans and coumarins, 133 lipids, 67 nucleotides and derivatives, 115 organic acids, 182 phenolic acids, 29 terpenoids and 91 others metabolites (Table 1).

Table 1 Overview of annotated metabolites

Metabolic type	Number	Percentage (%)
Alkaloids	155	14.05
Amino acids and derivatives	139	12.60
Flavonoids	155	14.05
Lignans and coumarins	37	3.35
Lipids	133	12.06
Nucleotides and derivatives	67	6.07
Organic acids	115	10.43
Phenolic acids	182	16.50
Terpenoids	29	2.63
Others metabolites	91	8.25

Multivariate analysis revealed differences among the metabolite profiles

Multivariate statistics were conducted to value the differences among the metabolic profiles of the various treatments in cigar tobacco leaf. The cigar tobacco leaves were clearly divided on the heatmap (Fig. 1A, B). In the PCA diagram, the first two principal components (PC1 and PC2) were shown to explain 27.48% and 23.14% of the data variance among different regions, respectively (Fig. 1C). In the PCA diagram, the first two principal components (PC1 and PC2) were shown to explain 34.87% and 21.44% of the data variance in different stages of fermentation, respectively. Quality control (QC) samples, a mixture of cigar tobacco leaf extracts, were grouped into the same area, indicating similar metabolic profiles and stable and repeatable data. The 12 samples from the four locations in cigar tobacco leaf were divided into four distinct groups, suggesting that each group had a relatively distinct metabolic profile (Fig. 1C). The 18 samples from the 6 fermentation periods in cigar tobacco leaf were divided into two distinct groups, suggesting that each group had a relatively distinct metabolic profile (Fig. 2B). Group 1 included LCF0. Group 2 included LCF1, LCF2, LCF3, LCF4, LCF5 and LCF6 (Fig. 1D). These two groups could be easily distinguished from each other. In addition, LCF1, LCF2, LCF3, LCF4, LCF5 and LCF6 clustered together. The PCA results showed that the metabolic profiles were different locations and various fermentation periods of cigar tobacco leaf (Fig. 1).

OPLS-DA was used to screen the variables contributing to differences among the three groups among different regions, we evaluated the differences using the OPLS-DA model between PEF0 and LCF0 ($R^2X = 0.748$, $R^2Y = 1$, $Q^2 = 0.972$), between PEF0 and DHF0 ($R^2X = 0.643$, $R^2Y = 1$, $Q^2 = 0.943$), between PEF0 and YXF0 ($R^2X = 0.697$, $R^2Y = 0.998$, $Q^2 = 0.948$) (Fig. 2); we also evaluated the differences in different stages of fermentation using the OPLS-DA model between LCF0 and LCF1 ($R^2X = 0.752$, $R^2Y = 0.999$, $Q^2 = 0.975$), between LCF0 and LCF2 ($R^2X = 0.755$, $R^2Y = 1$, $Q^2 = 0.979$), and between LCF0 and LCF3 ($R^2X = 0.758$, $R^2Y = 0.999$, $Q^2 = 0.968$), and between LCF0 and LCF4

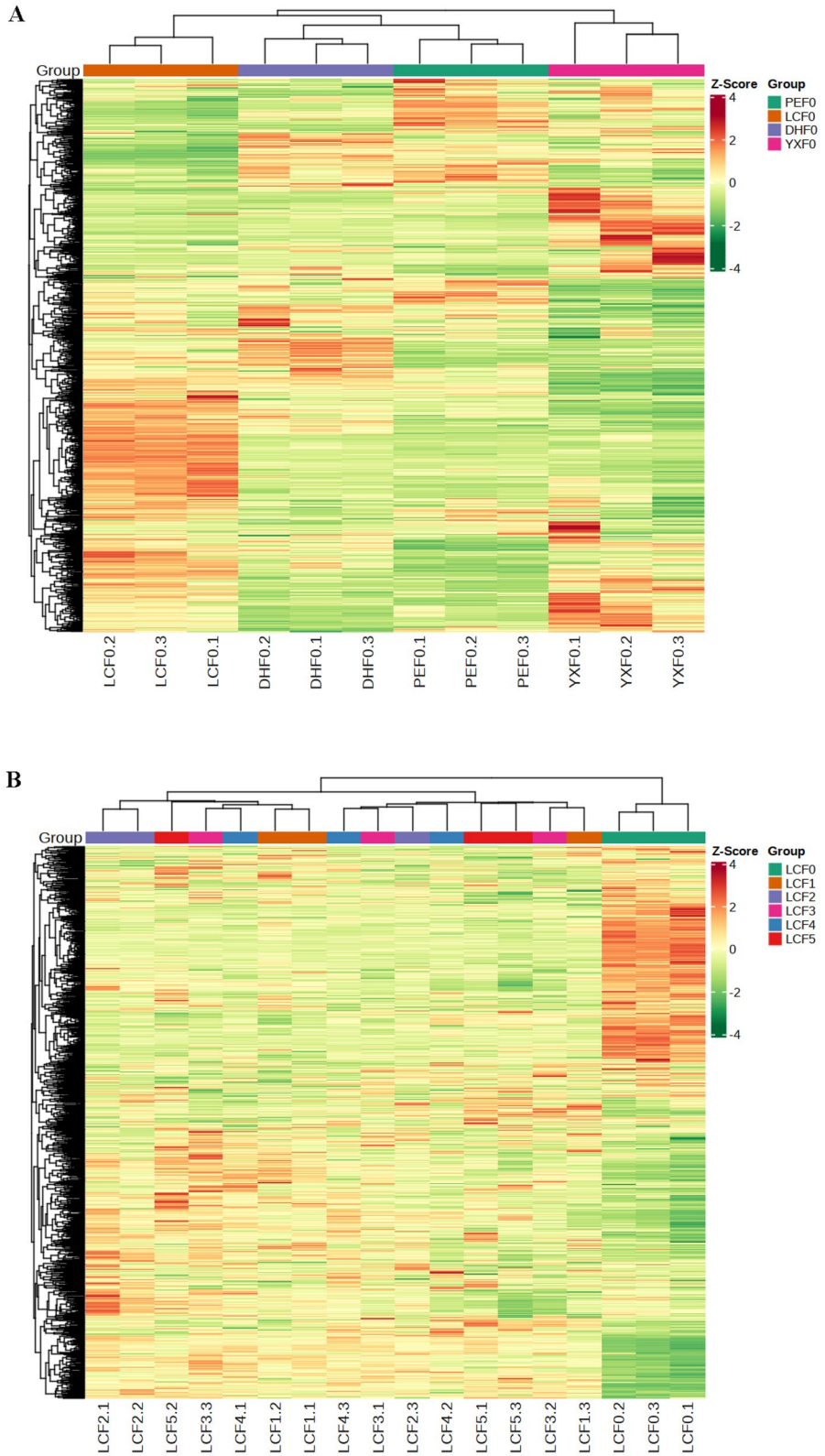


Fig. 1 Heatmap (**A**, **B**) and PCA plot (**C**, **D**) of metabolites among among different regions and in different stages of fermentation of cigar tobacco leaf. **A**, **B**: A column represents each sample, and a row represents each metabolite. Low and high abundance are indicated by green and red colors, respectively. **C**, **D**: PC1 and 2 indicate high cohesion within groups and good separation among the different treatments, respectively. DH represents Dehong; LC represents Lincang; PE represents Pu'er; YX represents Yuxi; F represents fermentation

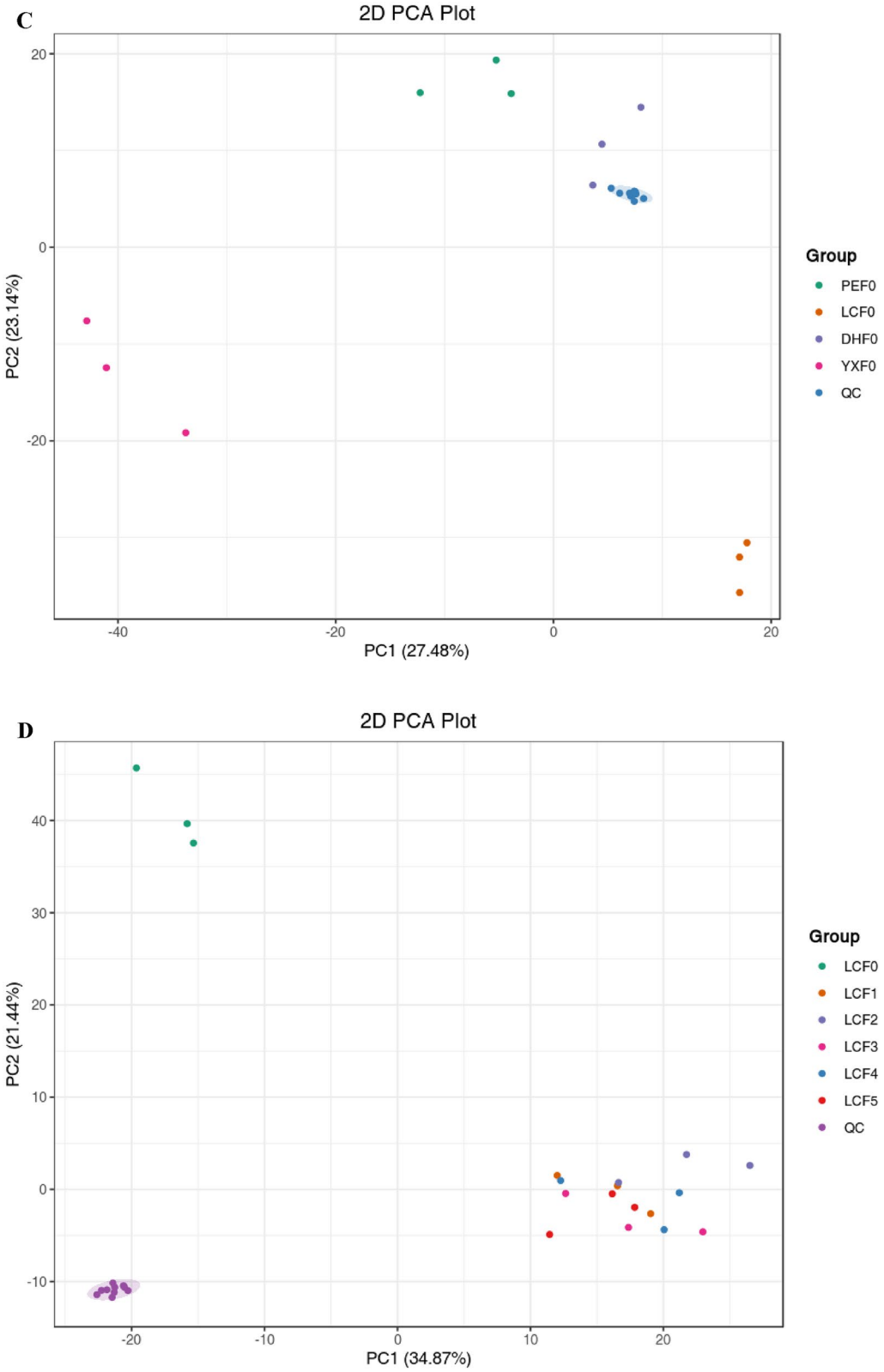


Fig. 1 continued

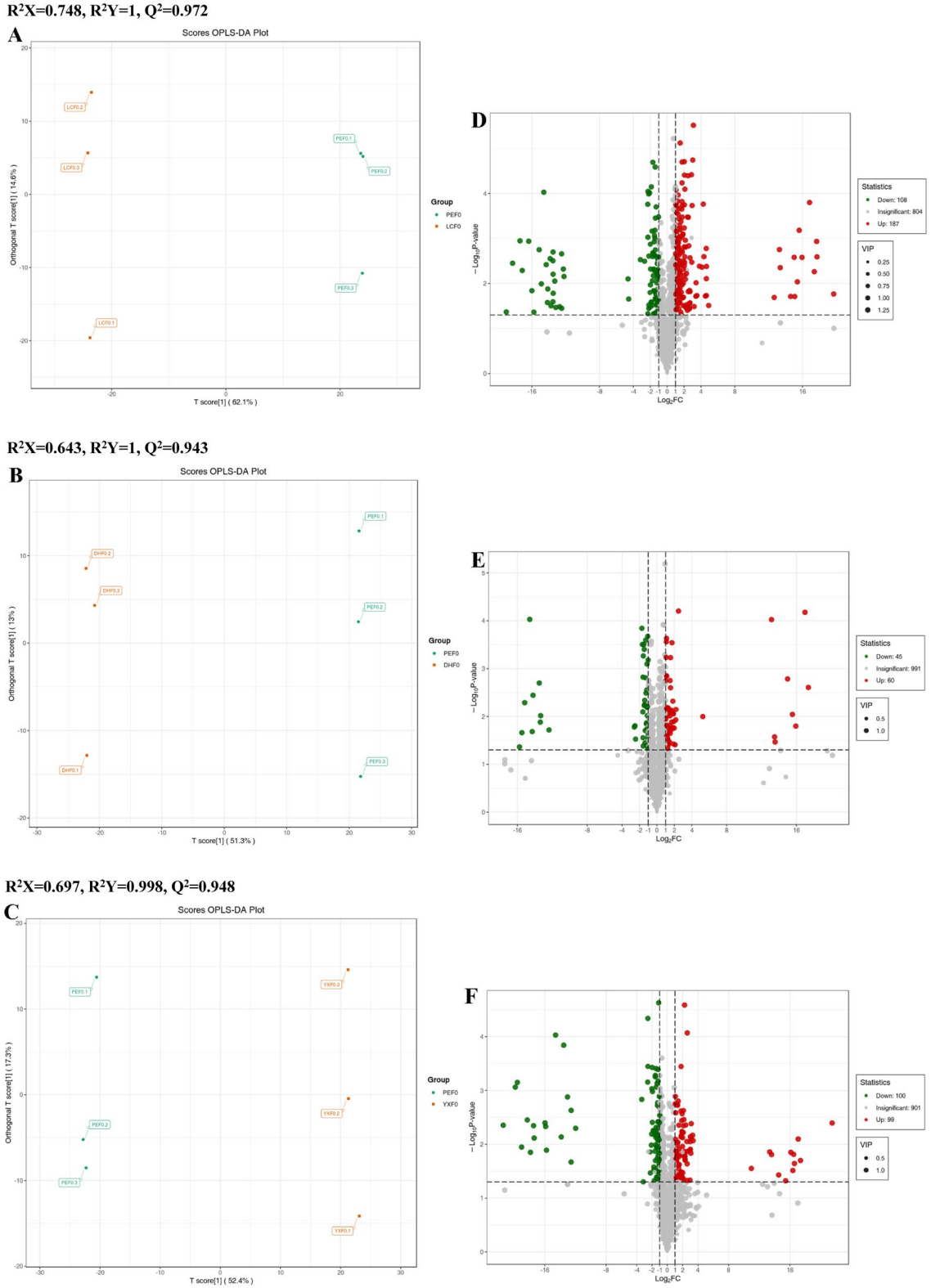


Fig. 2 Differential metabolomics analysis of cigar tobacco leaf from four regions. The loading (**A**-**C**) and volcano plots (**D**–**F**)from OPLS-DA model of PEF0 compared to LCF0, DHF0 and YXF0. Volcano plots show the differential metabolomics expression levels between PEF0, LCF0, DHF0 and YXF0. The downregulated differentially expressed metabolites and upregulated differentially expressed metabolites are illustrated by green and red spots, respectively; gray spots represent detected metabolites with nonsignificant differences

($R^2X = 0.748$, $R^2Y = 1$, $Q^2 = 0.973$), and between LCF0 and LCF5 ($R^2X = 0.652$, $R^2Y = 1$, $Q^2 = 0.976$) (Fig. 3).

Pairwise comparisons were carried out among different regions of cigar tobacco leaf samples and region to determine the metabolites that caused the observed differences. In the OPLS-DA models (Fig. 2A–C,), LCF0, DHF0 and YXF0 separated from PEF0, and LCF1, LCF2, LCF3, LCF4 and LCF5 were clearly separated from LCF0, suggesting major distinctions in the metabolic profiles between the region and different fermentation stages of cigar tobacco leaf. With respect to LCF1, LCF2, LCF3, LCF4 and LCF5 clustered closely together in the PCA plots (Fig. 1D), OPLS-DA models, however, show clearly distinct differences (Fig. 3A–E), indicating the different treatments are clearly distinct.

According to an FC ≥ 2 or ≤ 0.5 and VIP ≥ 1, there were 293 differential metabolites between PEF0 and LCF0 (upregulated = 187, downregulated = 106), 105 between PEF0 and DHF0 (upregulated = 60 downregulated = 45), and 199 between PEF0 and YXF0 (upregulated = 99, downregulated = 100) (Fig. 2D–F).

Regarding fermentation treatments, there were 216 differential metabolites between LCF0 and LCF1 (upregulated = 116, downregulated = 100), 242 between LCF0 and LCF2 (upregulated = 147, downregulated = 95), 220 between LCF0 and LCF3 (upregulated = 119, downregulated = 101), 227 between LCF0 and LCF4 (upregulated = 128, downregulated = 99), and 198 between LCF0 and LCF5 (upregulated = 100, downregulated = 98) (Fig. 3F–J).

Metabolite accumulation in general networks can be studied using the KEGG database. Our study enriched differential metabolites and classified them into different pathways for each comparison group. The significantly enriched metabolic pathways in the comparison of PEF0 vs. LCF0 were related to "biosynthesis of cofactors", "galactose metabolism", "caffeine metabolism", "ascorbate and aldarate metabolism" and "aminoacyl-tTNA biosynthesis" ($P < 0.05$) (Fig. 4A). The significantly enriched metabolic pathways in the comparison of PEF0 vs. DHF0 were related to "flavone and flavonol biosynthesis", "phosphonate and phosphinate metabolism", "purine metabolism", "fructose and mannose metabolism", "pantothenate and CoA biosynthesis", "biosynthesis of cofactors" and "vitamin B6 metabolism" ($P < 0.05$) (Fig. 4B). Metabolic pathways related to "biosynthesis of various alkaloids", "purine metabolism", "porphyrin metabolism", "metabolic pathways", "monobactam biosynthesis", "phenylalanine metabolism", "glyoxylate and dicarboxylate metabolism", "glycine, serine and threonine metabolism" and "tyrosine metabolism" were significantly enriched ($P < 0.05$) in the comparison of PEF0 vs. YXF0 (Fig. 4C). The results of the Venn diagram indicated that 39 metabolites were shared among the cigar tobacco leaf region comparison groups (Fig. 4D). According to these results, the metabolites responsible for the differences were significantly different. The characteristic metabolites of each cultivar comparison group were further studied, the top 20 metabolites with the highest FC in each comparison group were selected (Fig. 5), among which the 11 upregulated metabolites included three alkaloids, two others metabolites, three flavonoids, two phenolic acids and one organic acid, and the nine downregulated metabolite were two organic acids, one alkaloid, one terpenoid, two phenolic acids, one lipids and two lignans and coumarins in the comparison of PEF0 vs. LCF0 (Fig. 5A); the ten upregulated metabolites included three others metabolites, one terpenoid, one alkaloid, one phenolic acid, one organic acid, two flavonoids and one lignin and coumarin, and the ten downregulated metabolite were one others metabolite, three phenolic acids, three organic acids, one alkaloid, one lignin and coumarin and one amino acids and derivatives in the comparison of PEF0 vs. DHF0 (Fig. 5B); the eight upregulated metabolites included two others metabolites, one terpenoid, two alkaloids, and one flavonoid and two phenolic acids, and the 12 downregulated metabolite were five alkaloids, one other metabolite, two phenolic acids, one terpenoid, one organic acid, one flavonoid and one lignin and coumarin in the comparison of PEF0 vs. YXF0 (Fig. 5C); The FCs of these compounds were all greater than 13 in the different cultivar comparison groups.

In the different stages of cigar tobacco leaf fermentation, the significantly enriched metabolic pathways in the comparison of LCF0 vs. LCF1 were related to "aminoacyl-tRNA biosynthesis", "biosynthesis of amino acids", "ABC transporters", "cyanoamino acid metabolism", "thiamine metabolism", "D-Amino acid metabolism", "cysteine and methionine metabolism" and "glucosinolate biosynthesis" ($P < 0.05$) (Fig. 4E). The significantly enriched metabolic pathways in the comparison of LCF0 vs. LCF2 were related to "aminoacyl-tRNA biosynthesis", "ABC transporters", "glucosinolate biosynthesis", "cyanoamino acid metabolism", "biosynthesis of amino acids", "thiamine metabolism", "isoquinoline alkaloid biosynthesis" and "biosynthesis of various alkalois" ($P < 0.05$) (Fig. 4F). The significantly enriched metabolic pathways in the comparison of LCF0 vs. LCF3 were related to "aminoacyl-tRNA biosynthesis", "biosynthesis of amino acids", "glucosinolate biosynthesis", "cyanoamino acid metabolism", "ABC transporters" and "D-Amino acid metabolism" ($P < 0.05$) (Fig. 4G). The significantly enriched metabolic pathways in the comparison of LCF0 vs. LCF4 were related to "aminoacyl-tRNA biosynthesis", "glucosinolate biosynthesis", "biosynthesis of amino acids", "cyanoamino acid metabolism", "ABC transporters", "D-Amino acid

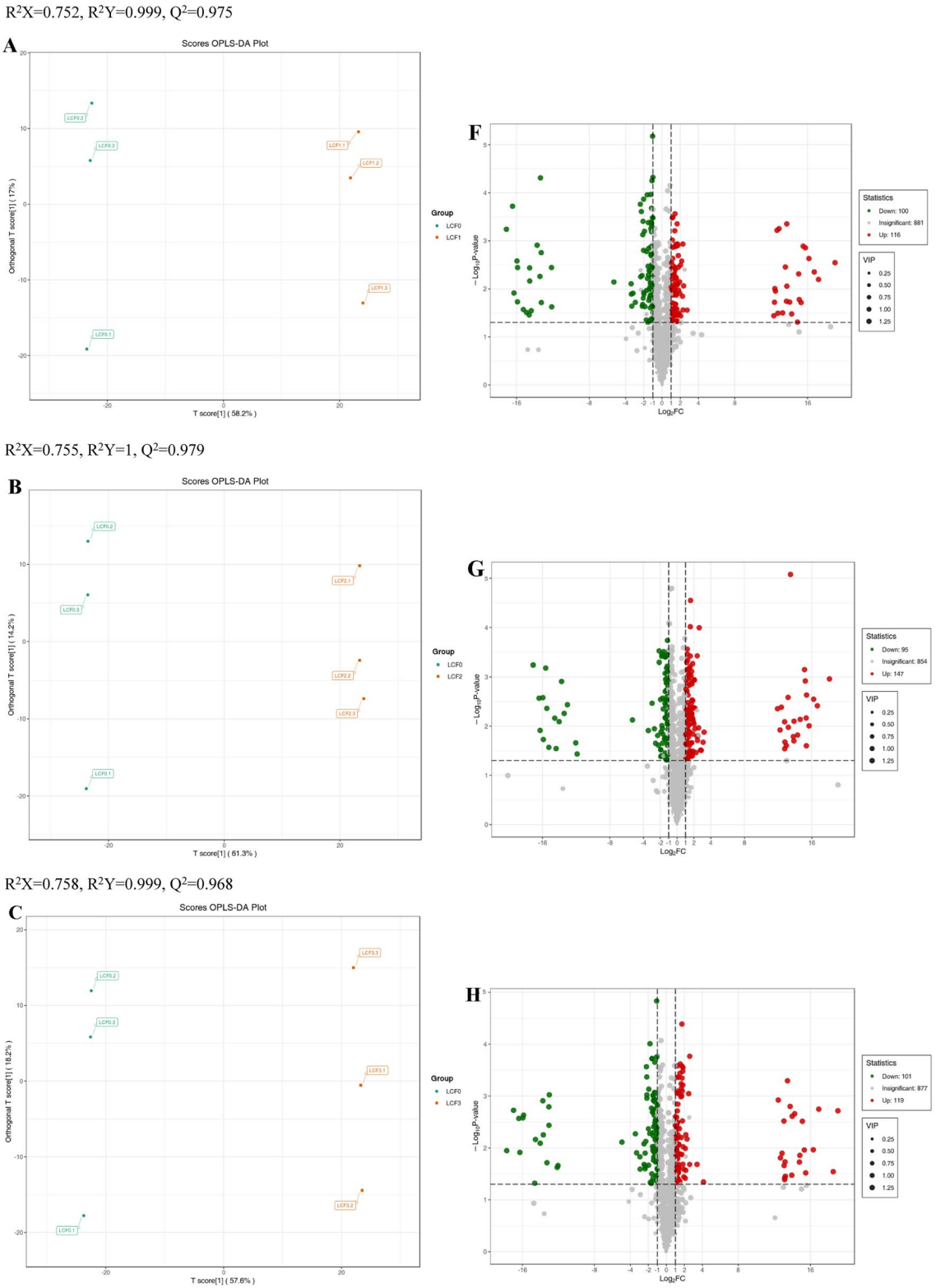


Fig. 3 Differential metabolomics analysis of the different fermentation stages of cigar tobacco leaf from LC. The loading (**A**-**E**) and volcano plots (**F**-**J**) from OPLS-DA model of LCF1, LCF2, LCF3, LCF4, LCF5 compared to LCF0. Volcano plots show the differential metabolomics expression levels between LCF1, LCF2, LCF3, LCF4, LCF5 and LCF0. The downregulated differentially expressed metabolites and upregulated differentially expressed metabolites are illustrated by green and red spots, respectively; gray spots represent detected metabolites with nonsignificant differences

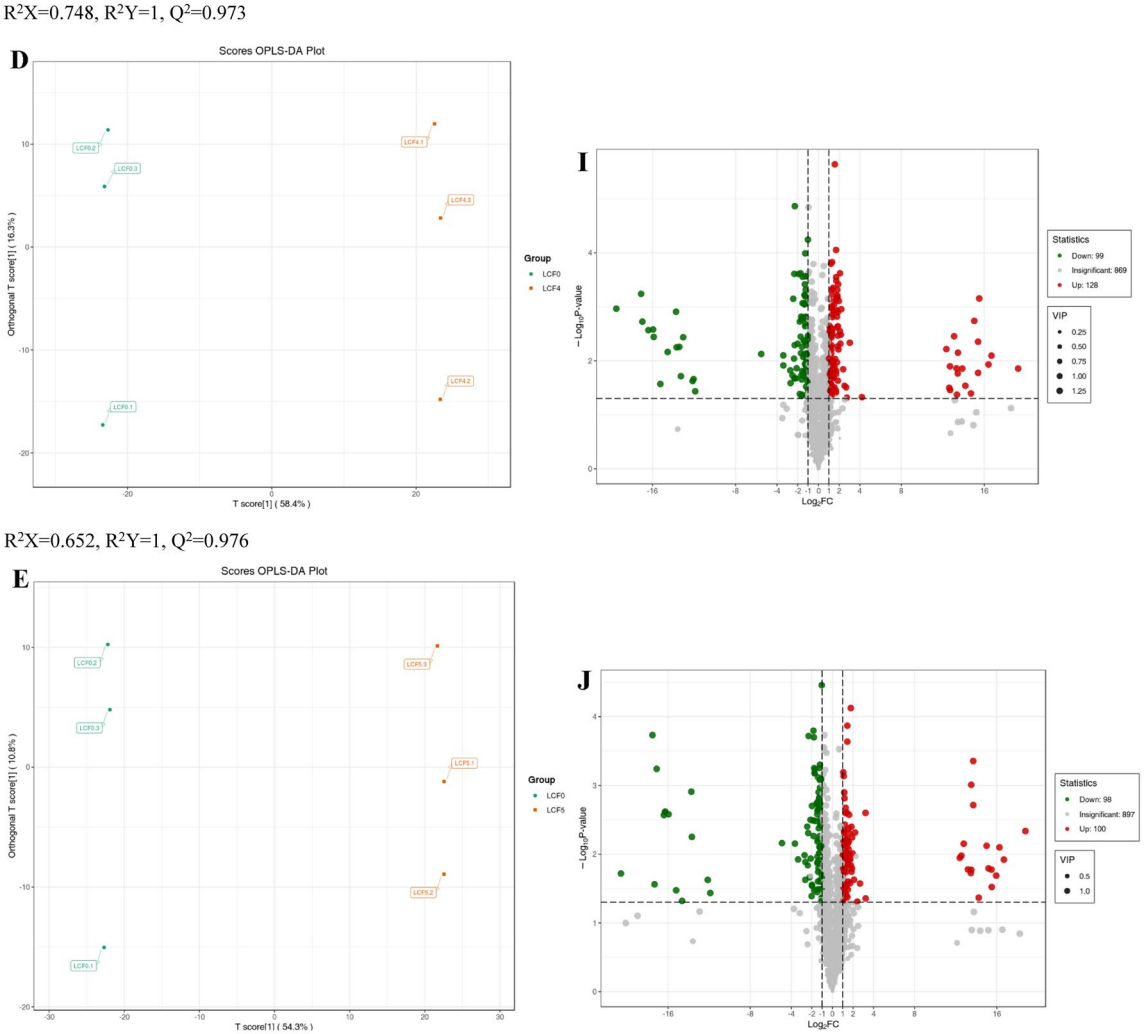


Fig. 3 continued

metabolism" and "2-Oxocarboxylic acid metabolism" ($P < 0.05$) (Fig. 4H). The significantly enriched metabolic pathways in the comparison of LCF0 vs. LCF5 were related to "aminoacyl-tRNA biosynthesis", "glucosinolate biosynthesis", "ABC transporters", "biosynthesis of amino acids", "isoquinoline alkaloid biosynthesis" and "biosynthesis of various alkaloids" ($P < 0.05$) (Fig. 4I).

According to the Venn diagram, 150 metabolites were shared between fermentation stages (Fig. 5H). Hence, the metabolites responsible for the differences in treatment were significantly different. We investigated the characteristic metabolites associated with each cultivar group, the top 20 metabolites with the highest FC in each comparison group were selected (Fig. 5), among which the 12 upregulated metabolites included one other metabolite, two lignin and coumarin, one phenolic acid, one lipid, one terpenoid, one amino acid and derivative, two flavonoids, three organic acids, and the eight downregulated metabolite were two organic acids, three phenolic acids, one other metabolites, one flavonoid and one alkaloid in the comparison of LCF0 vs. LCF1 (Fig. 5D); the 11 upregulated metabolites included one other metabolite, two lignins and coumarins, one lipid, one terpenoid, one amino acid and derivative, one lignin and coumarin, tree organic acids, one alkaloid, one flavonoid and one organic acid, and the nine downregulated metabolite were four phenolic acids, one lipid, one organic acid, one other metabolite, one flavonoid and one alkaloid in the comparison of LCF0 vs. LCF2 (Fig. 5E); the 11 upregulated metabolites included one other metabolite, two lignans and coumarins, one lipid, one terpenoid, one amino acid and derivative, three organic acids and two flavonoids, and the nine downregulated metabolite were one alkaloid, two amino acid and derivative, two other metabolites, one lipid, one flavonoid, one phenolic acid and one lignan and coumarin in the comparison of

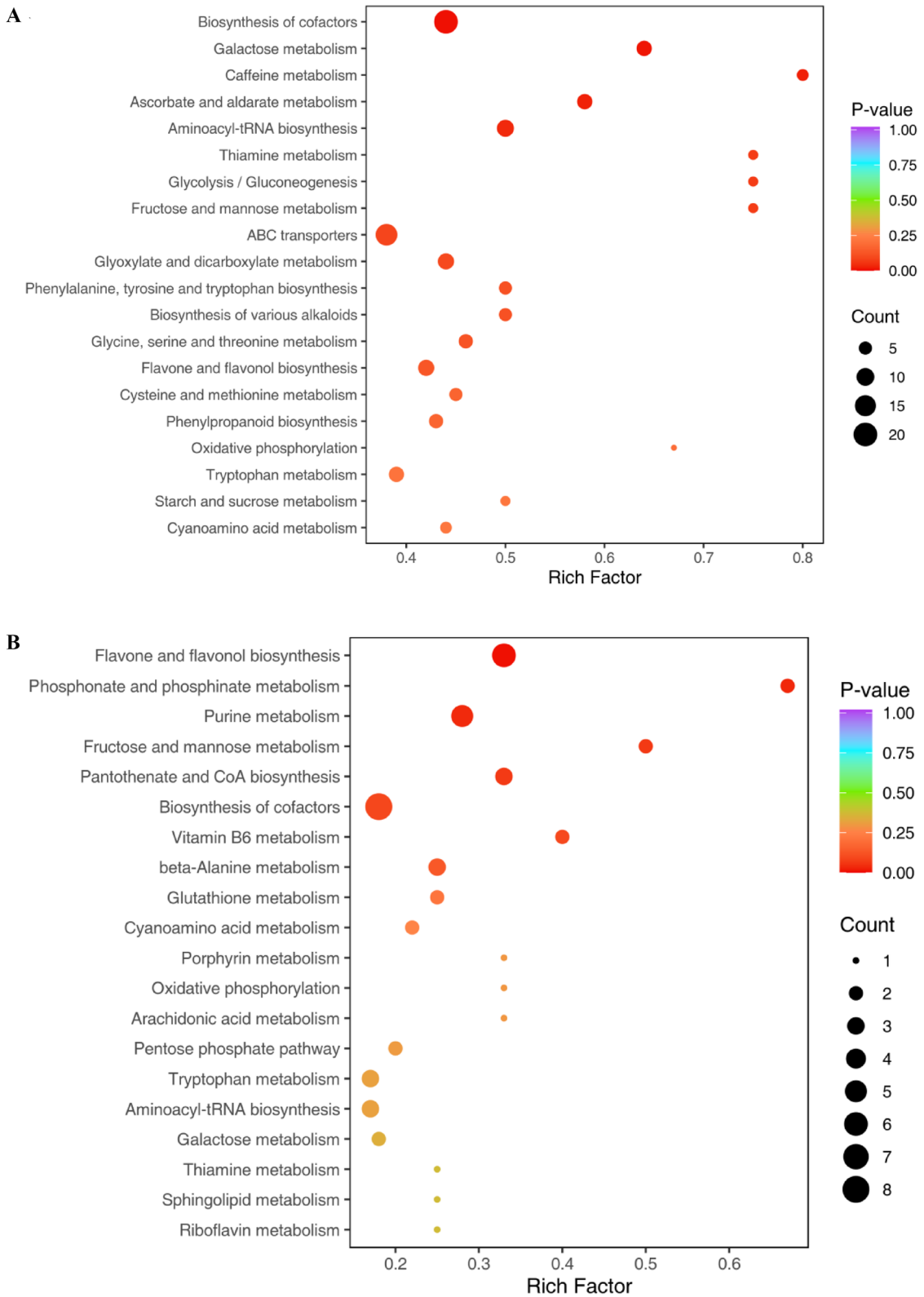
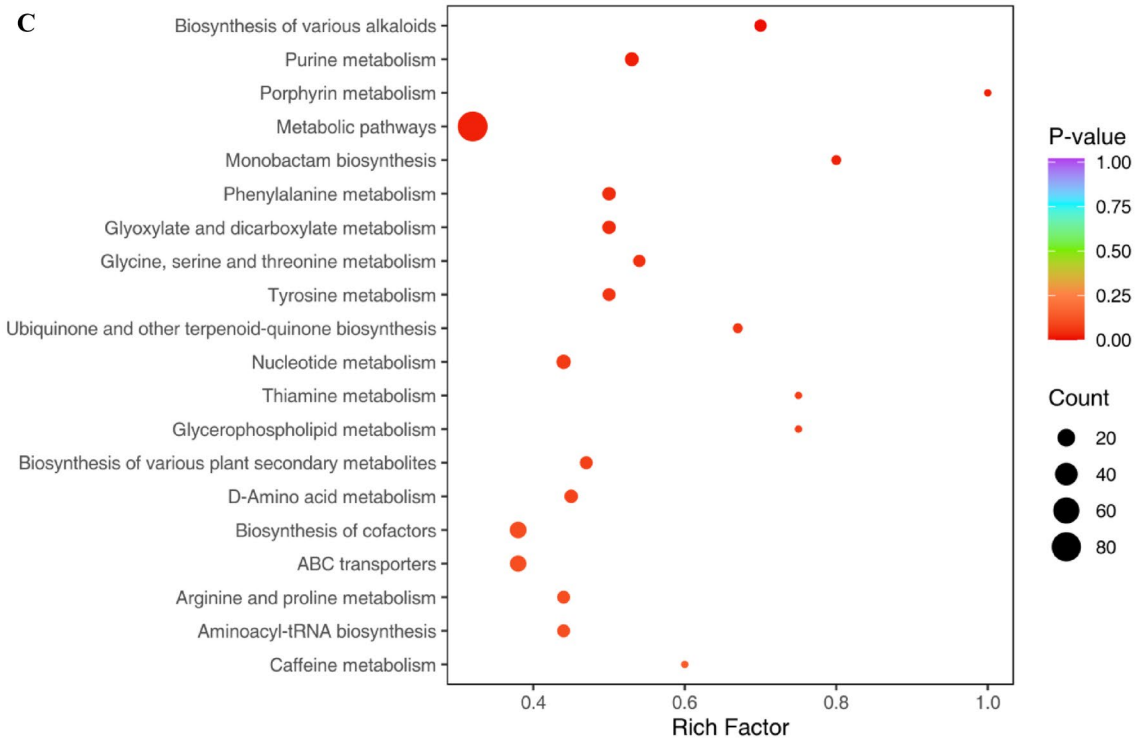


Fig. 4 Venn diagram and pathway analysis of differential metabolites among different regions and in different fermentation stages of cigar tobacco leaf. D, J: Venn diagram showing the overlapping and accession-specific differential metabolites among the comparison groups. **A**–**C**, **E**–**I**: KEGG pathway enrichment based on the differential metabolites between the two comparison groups. A metabolic pathway is represented by each bubble. Abscissa and bubble size jointly represent the influence factors of this pathway. A larger impact factor and *p* values of the enrichment analysis are represented by a larger bubble size and the bubble colors, respectively. The darker colors represent higher enrichment levels



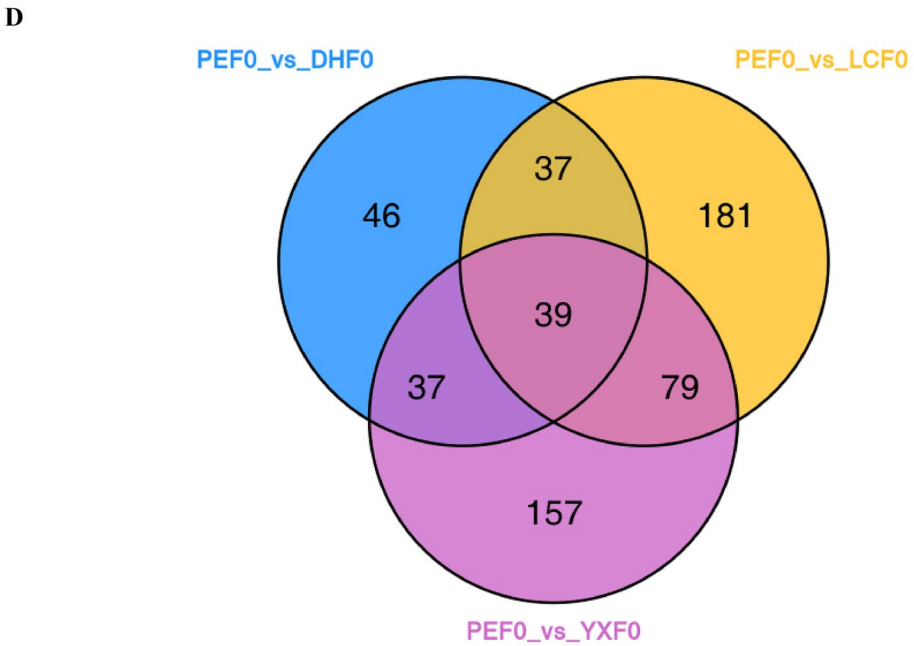
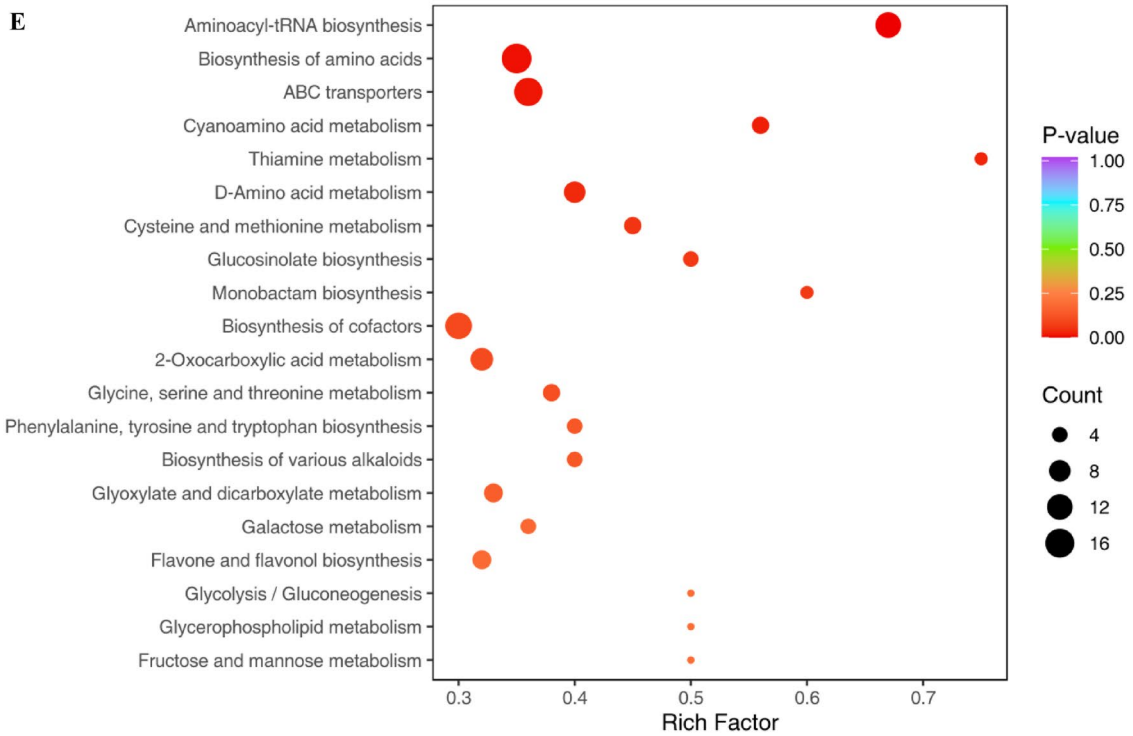


Fig. 4 continued



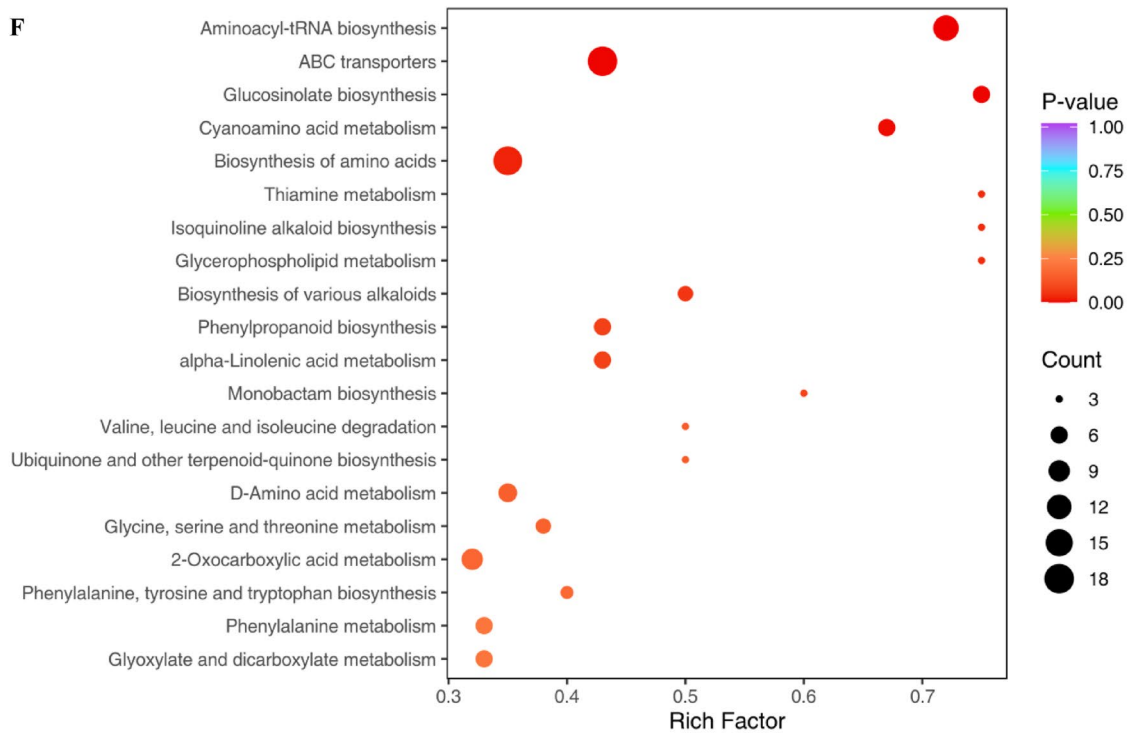
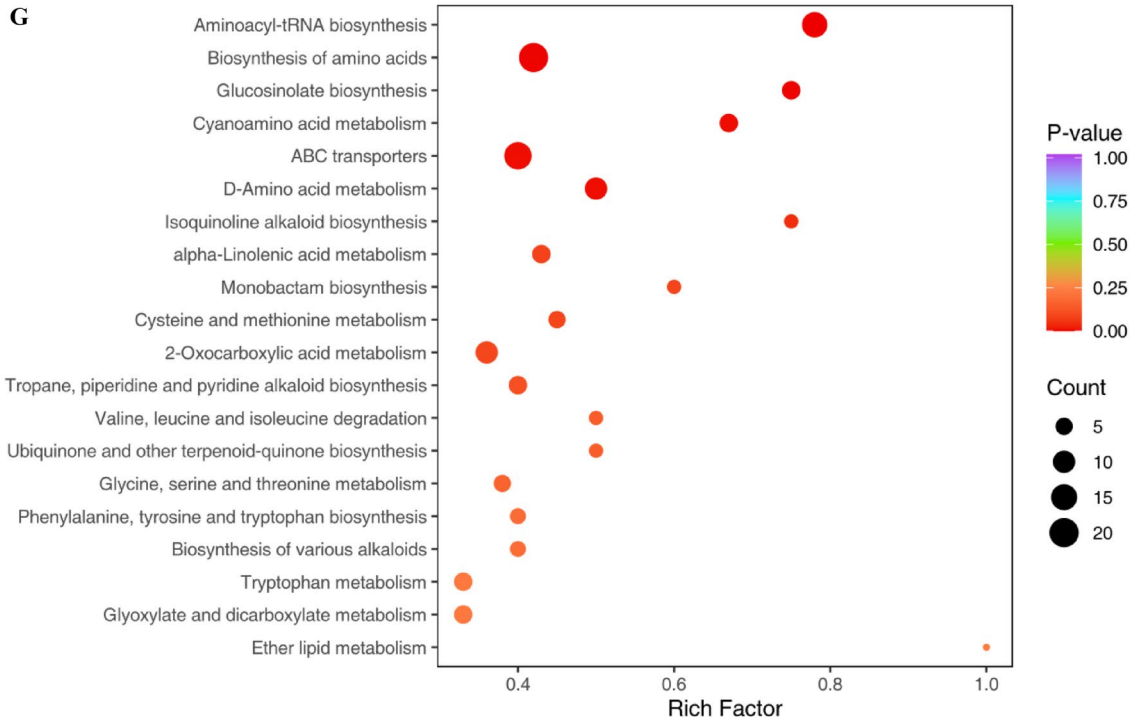


Fig. 4 continued



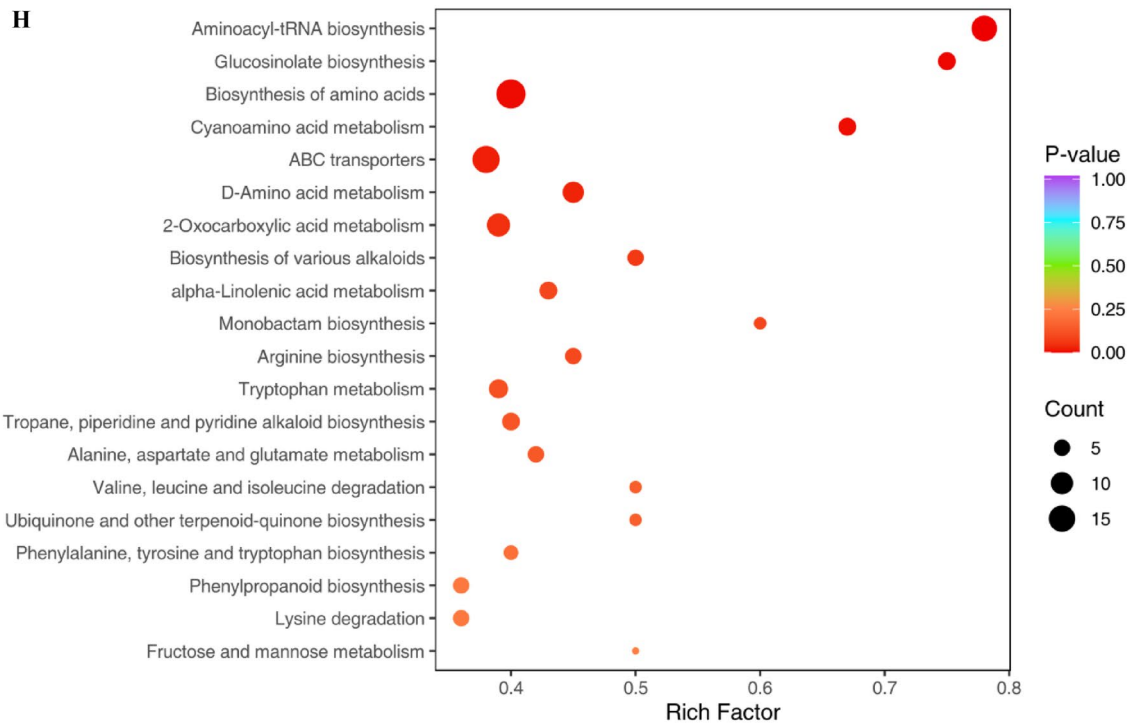
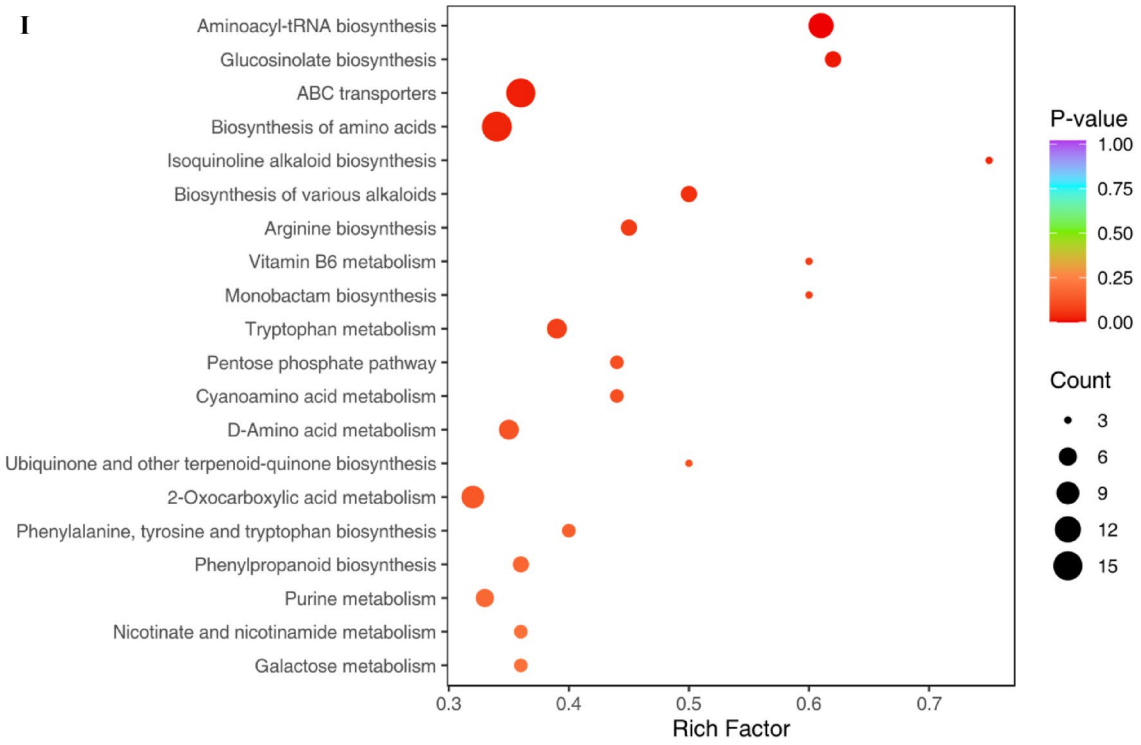


Fig. 4 continued



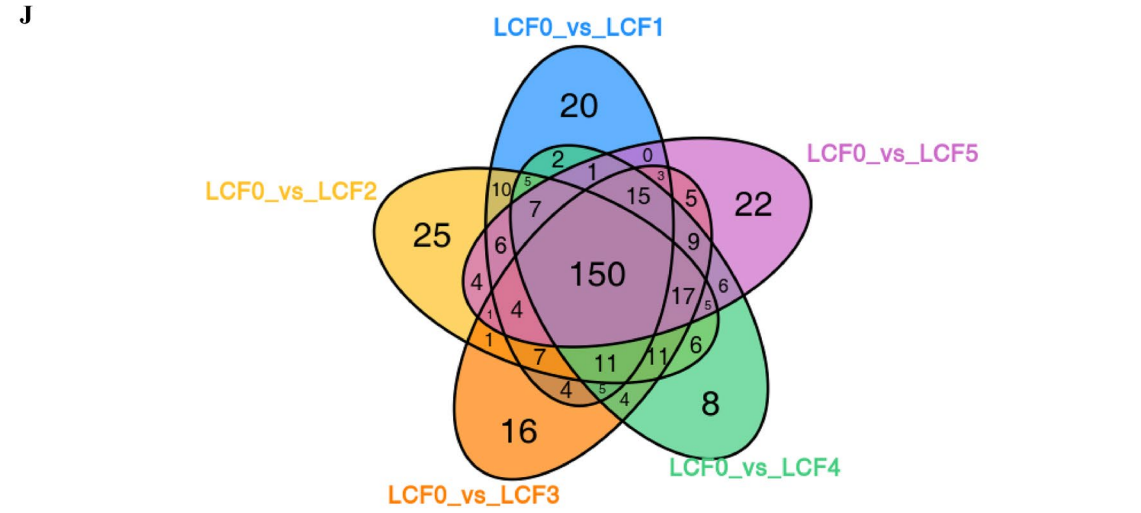


Fig. 4 continued

LCF0 vs. LCF3 (Fig. 5F); the 12 upregulated metabolites included one other metabolite, two lignans and coumarins, one lipid, one amino acid and derivative, three organic acids and three flavonoids, and the eight downregulated metabolites were three alkaloids, two phenolic acids, one phenolic acid, one other metabolite and one lignin and coumarin in the comparison of LCF0 vs. LCF4 (Fig. 5G); the nine upregulated metabolites included one other metabolite, two lignin coumarin, one lipid, two terpenoids, two organic acids and one amino acid and derivative, and the 11 downregulated metabolite were three alkaloids, one other metabolite, two flavonoids, four phenolic acids and one terpenoids in the comparison of LCF0 vs. LCF5 (Fig. 5H).

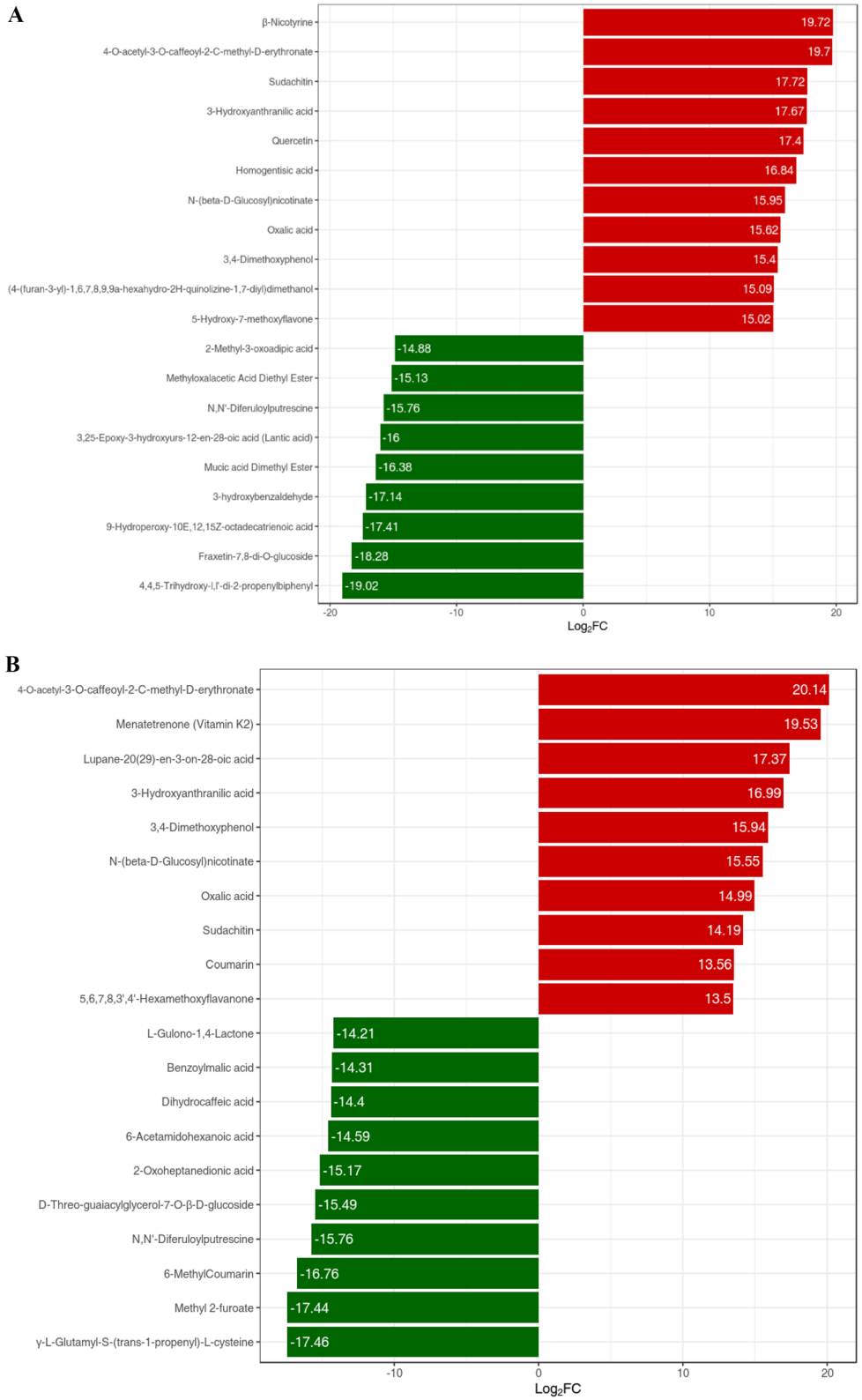


Fig. 5 Top 20 metabolites with the highest fold change in each comparison group. **A** is PEF0 vs. LCF0, **B** is PEF0 vs. DHF0, **C** is PEF0 vs. YXF0, **D** is LCF0 vs. LCF1, **D** is LCF0 vs. LCF2, **D** is LCF0 vs. LCF3, **D** is LCF0 vs. LCF4, **D** is LCF0 vs. LCF5. Red bar charts are the up-regulated metabolites, green bar charts are the down-regulated metabolites

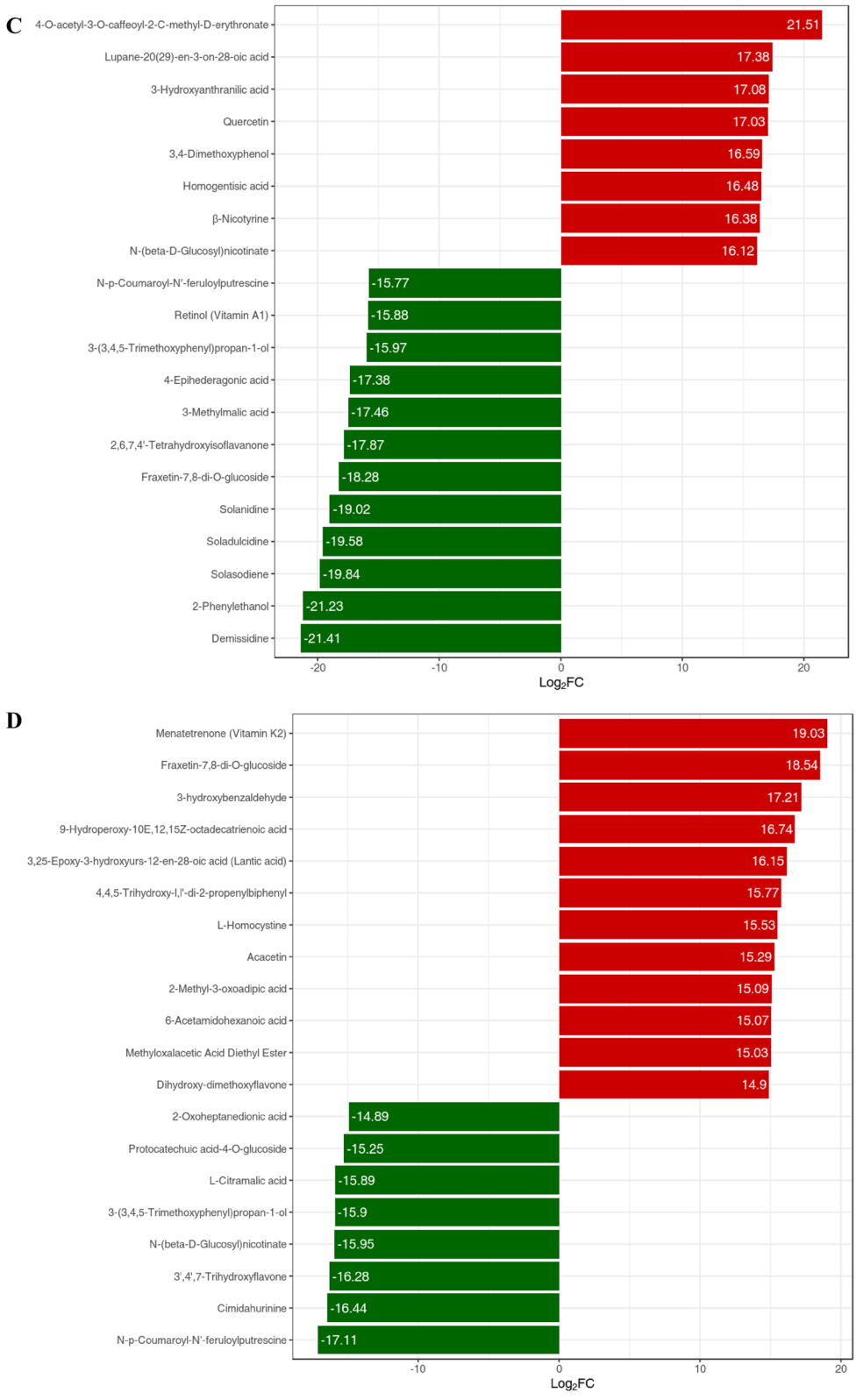


Fig. 5 continued

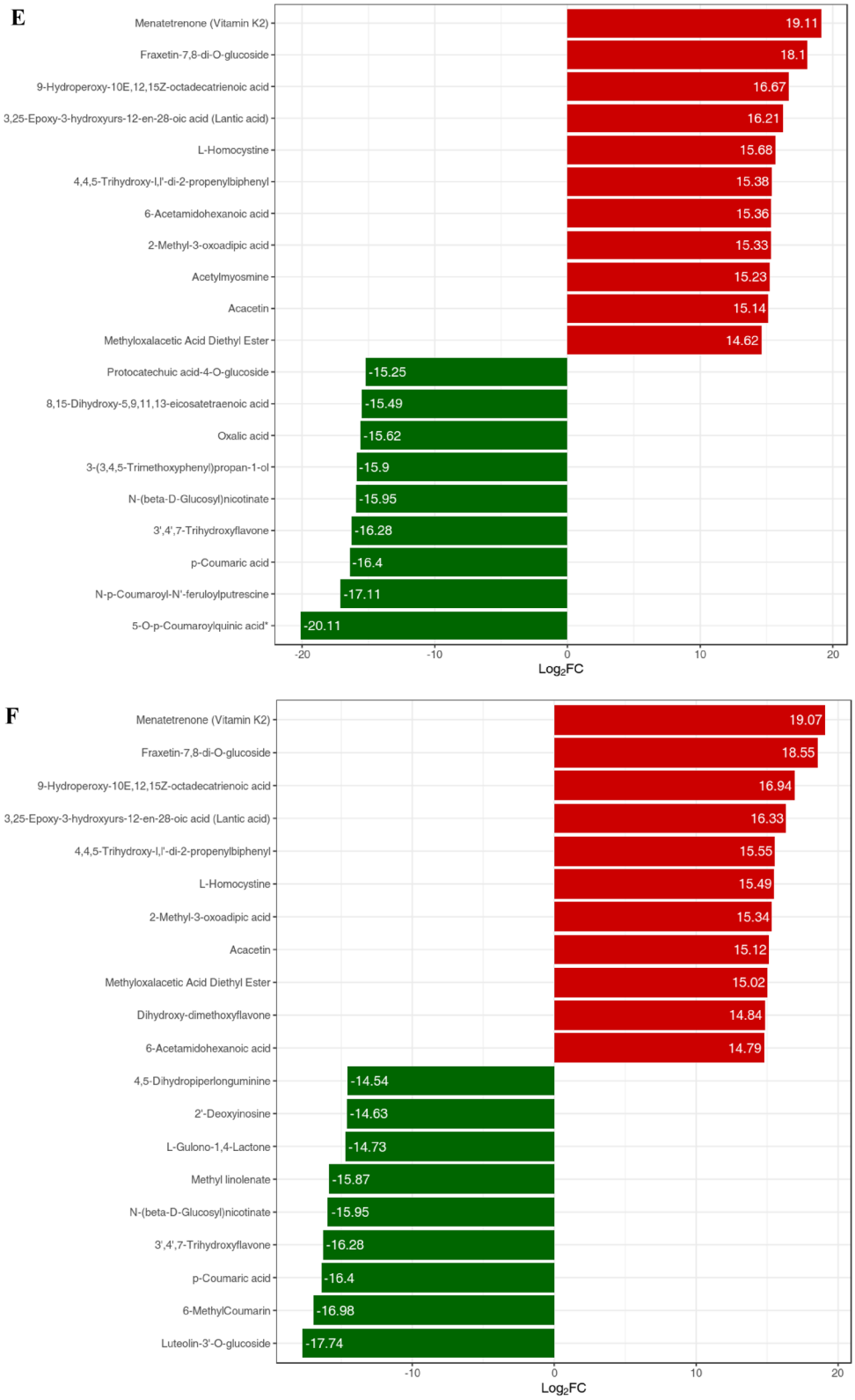


Fig. 5 continued

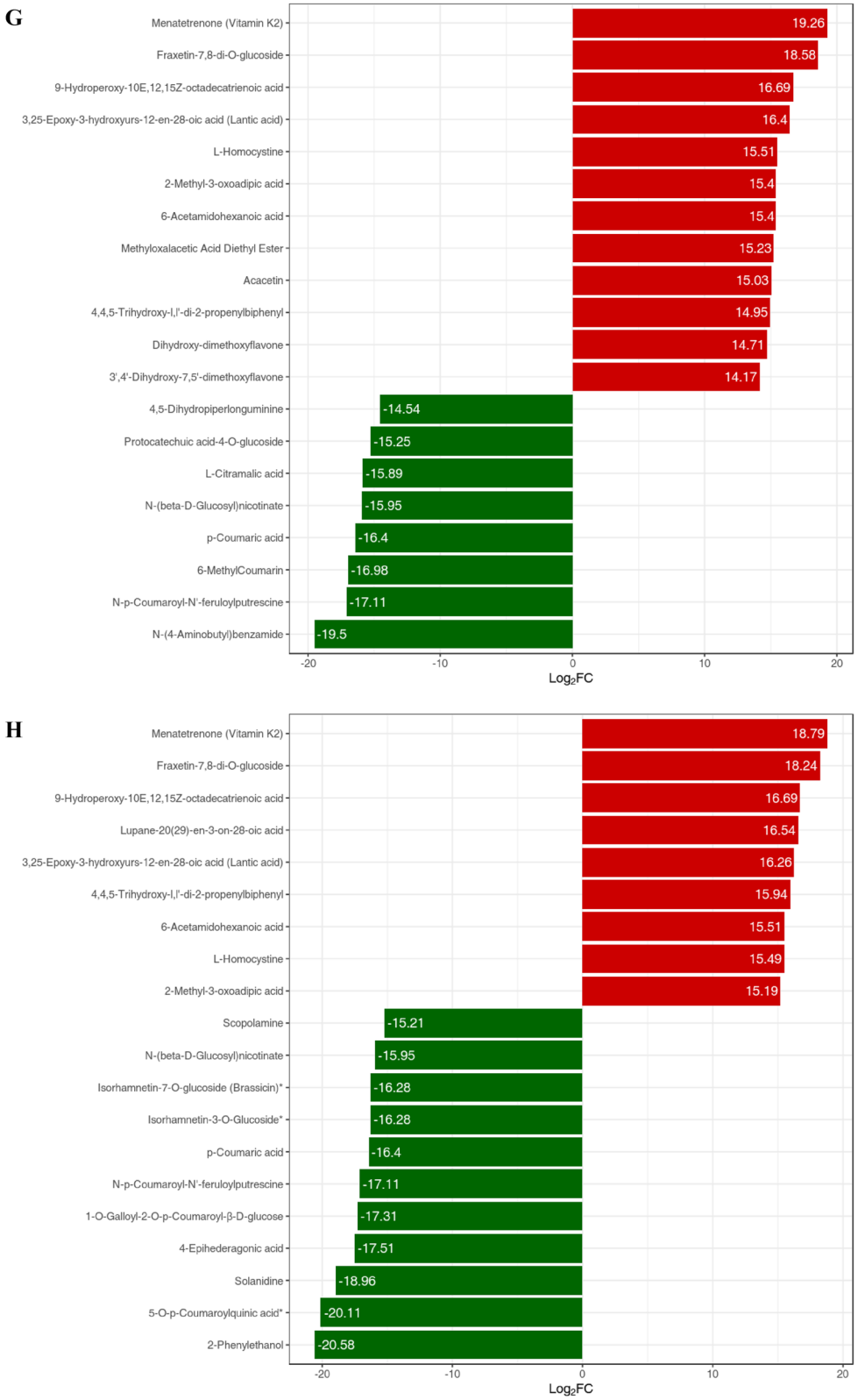


Fig. 5 continued

Discussion

Metabolites identified in cigar tobacco leaf

Cigar tobacco leaf fermentation is a further metabolic process after air drying, including substance conversion, degradation, acidification, volatilization and so on [13]. Therefore, Cigar tobacco leaves grown in four different locations were used for widely targeted metabolomics analysis. Meantime, cigar tobacco leaves from one of these places were selected for fermentation treatment, and then a widely targeted metabolomics was determined. These provided a comprehensive metabolic profile of cigar tobacco leaf. On the basis of metabolomics results, 1103 metabolites were identified by qualitative and quantitative analysis based on ion pair information of compounds in cigar tobacco leaf. The metabolites included 155 alkaloids, 139 amino acids and derivatives, 155 flavonoids, 37 lignans and coumarins, 133 lipids, 67 nucleotides and derivatives, 115 organic acids, 182 phenolic acids, 29 terpenoids and 91 others metabolites (Table 1). Most of metabolites were alkaloids, amino acids and derivatives, flavonoids, lipids and phenolic acids. A low percentage of metabolites were lignans and coumarins, nucleotides and derivatives, terpenoids. In addition to their antioxidant properties, flavonoids also have anticancer and anti-inflammatory properties [14]. Such as there are several anticancer, antioxidant, anti-inflammatory, and antiproliferative activities in naringenin [15, 16]. There are several anti-inflammatory, antioxidant, and protective properties in Kaempferol-3-*O*-glucoside [17]. Thus, this study provides important reference values for understanding the changes of functional substances in cigar tobacco leaf among different regions and in different stages of fermentation.

Differential metabolites among different regions

In this study, a total of 597 differential metabolites among different regions were found in the various comparison groups; however, only 39 metabolites were observed, suggesting that the metabolite profiles in cigar tobacco leaf among different regions were significantly different. The differential metabolite pathways in different comparison groups varied greatly. For example, the significantly enriched metabolic pathways in the comparison of PEF0 vs. LCF0 were main related to "biosynthesis of cofactors", "galactose metabolism", "caffeine metabolism", "ascorbate and aldarate metabolism" and "aminoacyl-tTNA biosynthesis" ($P < 0.05$) (Fig. 4A). The significantly enriched metabolic pathways in the comparison of PEF0 vs. DHF0 were main related to "flavone and flavonol biosynthesis", "phosphonate and phosphinate metabolism", "purine metabolism" ($P < 0.05$) (Fig. 4B). Metabolic pathways related to "metabolic pathways" were main markedly enriched ($P < 0.05$) in the comparison of PEF0 vs. YXF0 (Fig. 4C). These different metabolites may be responsible for ecological environment and climate factors [18]. Flavonoids play an important roles in the biomedical and health industries and act as plays an important role of free radical scavengers, reducing agents and singlet oxygen quenchers [14, 19]. However, the burning process of cigar tobacco leaf is complex and the substances in this process produced require further study. Therefore, it is very important to perform a metabolomics of cigar tobacco leaf among different regions to meet consumers' various needs for health care.

The top 20 metabolites with the highest FCs in each comparison group were selected (Fig. 5). The main upregulated compounds were alkaloids, flavonoids and phenolic acids, and the main downregulated compounds were organic acids and phenolic acids in the PEF0 vs. LCF0 comparison group. The main upregulated compounds were flavonoids and other metabolites, and the main downregulated compounds were organic acids and phenolic acids in the PEF0 vs. DHF0 comparison group. The main upregulated compounds were alkaloids and phenolic acids, and the main downregulated compounds were also alkaloids and phenolic acids in the PEF0 vs. YXF0 comparison group. These results indicated that the top 20 highest FCs were observed for alkaloids, flavonoids, organic acids and phenolic acids. The difference in metabolites among cigar tobacco leaf treatments indicates that the quality of these cigar tobacco leaves is different.

Differential metabolites in different stages of fermentation

It was found that the quality of cigar tobacco leaves was closely related to fermentation. Therefore, studying the metabolic mechanism underlying fermentation is very important for ensure the quality of cigar tobacco leaves and maintain the health of people. To investigate the substance changes in cigar tobacco leaves during their fermentation, metabolomics was determined at different fermentation stages. LCF was selected for further study. Our research showed that fermentation had significantly effects on metabolite content in cigar tobacco leaf. We found clearly that from Fig. 1D that LCF0 was easily distinguished from LCF1, LCF2, LCF3, LCF4, LCF5 and LCF6, and LCF1, LCF2, LCF3, LCF4, LCF5 and LCF6 clustered together. These results suggested that the metabolic profile changes of cigar tobacco leaves during the fermentation period could be used to accurately grasp the end time of fermentation and improve efficiency of cigar tobacco leaf fermentation. However, various

fermentation conditions of tobacco leaves will lead to different fermentation results and different transformation of related substances, so more comprehensive research is needed [20].

In our research, a total of 1103 differential metabolites were found in the different comparison groups (Fig. 5H). However, only 150 shared metabolites were found in these groups, indicating that the metabolite profiles in various treatments were significantly different. The results in fermentation treatments indicated that fermentation activated metabolic pathways, such as "aminoacyl-tRNA biosynthesis" and "ABC transporters". The changes in these substances may be related to enzymes [21]. The top 20 upregulated metabolites with the highest FCs in each comparison group were chosen (Fig. 5). The main upregulated compounds were flavonoids, phenolic acids and lignans and coumarins, and the main downregulated compounds were organic acids, phenolic acids and amino acids and derivatives in the fermentation comparison group. To analyze the formation and degradation of secondary metabolites during fermentation of cigar tobacco leaf at different stages, Isoquinoline alkaloid, phenylpropanoid and flavonoid biosynthesis were selected to reconstruct the metabolic network based on the KEGG pathway and their data (Fig. 6). According to the pathway analysis, hesperetin-7-O-glucoside content increased LCF1, LCF2 and LCF3 compared to LCF0, The increase in flavonoids during this period may be related to ensure the quality of cigar tobacco leaves, which needs to study by further research. These results indicated that the relatively unstable compounds in the process of cigar tobacco leaf fermentation changed. The results of our study were consistent with those of other studies. Researchers found that polyphenols were degraded during the fermentation of cigar tobacco leaf [13]. A previous researches indicated that flavonoids played physiological roles in protection against biotic or abiotic stresses [22, 23]. Therefore, the results further shown that the mechanism of protecting cigar tobacco leaf against biotic or abiotic stress may be that the variation of these metabolite contents. A compound reduced during fermentation may be associated with microorganisms. Microorganisms produce a variety of active enzymes during metabolism, which are secreted to the extracellular and degrade macromolecular organic compounds in tobacco leaves [5]. As is depicted the Fig. 6, we can see that p-coumaric acid, p-coumaroylquinic acid and caffeoylquinic acid could be the key metabolites of metabolic network regulation of cigar tobacco leaf fermentation, which need more comprehensive research by combining the techniques of molecular biology.

Conclusion

In this study, the metabolic profile among different regions and fermentation periods of cigar tobacco leaf were evaluated. A total of 1103 metabolites were identified in cigar tobacco leaf samples. A total of 293, 105 and 199 metabolites showed differential accumulation in the cigar tobacco leaf among different regions (PEF0 vs. LCF0, PEF0 vs. DHF0, PEF0 vs. YXF0) and 216, 242, 220, 227 and 198 metabolites showed differential accumulation in the different fermentation (LCF0 vs. LCF1, LCF0 vs. LCF2, LCF0 vs. LCF3, LCF0 vs. LCF4, LCF0 vs. LCF5). The main upregulated compounds were flavonoids, phenolic acids and lignans and coumarins, and

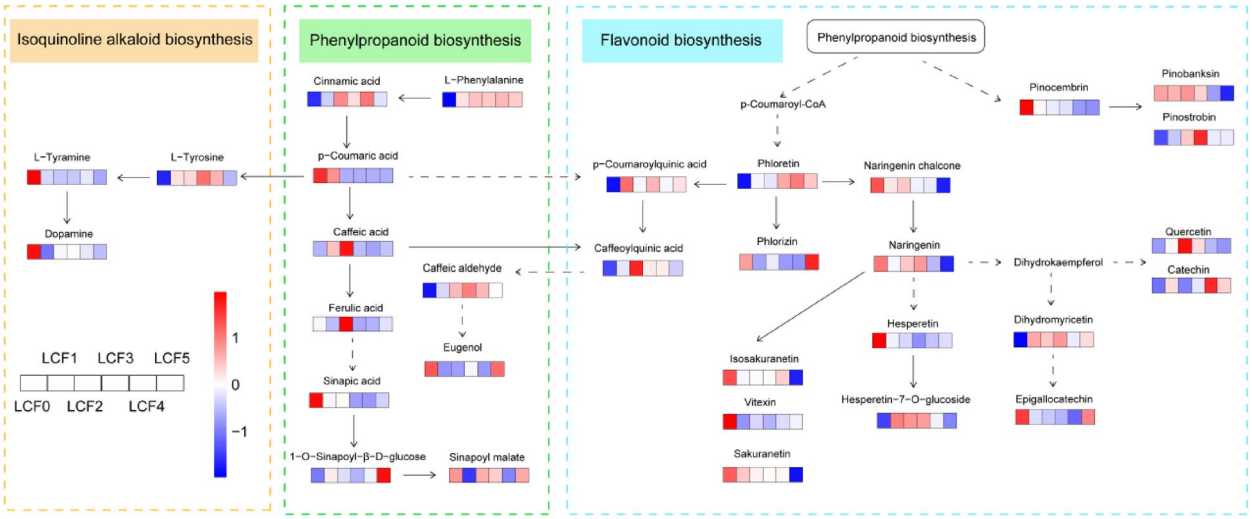


Fig. 6 Metabolic pathways of the main metabolites at different periods of cigar tobacco leaf fermentation. The bars show the different content of metabolites from left to right in LCF0, LCF1, LCF2, LCF3, LCF4 and LCF5

the main downregulated compounds were organic acids, phenolic acids and amino acids and derivatives in the fermentation comparison group. These results suggested that the metabolic profile changes of cigar tobacco leaves during the fermentation period could be used to accurately grasp the end time of fermentation and improve efficiency of cigar tobacco leaf fermentation.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-023-00444-1.

Additional file 1: Fig S1. Total ion current of one quality control sample by mass spectrometry detection (A) and multi-peak detection plot of metabolites in the multiple reaction monitoring mode (B).

Author contributions

GZ: investigation, writing. HY: writing—review and editing. GZ: resources. YW: investigation. HX: software. YL: investigation and formal analysis. GK: conceptualization, methodology, and writing—review and editing. All authors reviewed the manuscript.

Funding

This work was supported by the China Tobacco Monopoly Bureau Grants and Yunnan Provincial Tobacco Monopoly Bureau Grants (110202103018/2022530000241002), and Yunnan Provincial Tobacco Monopoly Bureau Grants (2020530000241001).

Data availability

The data underlying this paper will be shared on reasonable request to the corresponding author.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

This research has been confirmed for publication in the journal.

Competing interests

The authors have no competing interests.

Author details

[1]Yunnan Academy of Tobacco Agricultural Sciences, Kunming 650021, Yunnan, China.

Received: 23 May 2023 Accepted: 19 July 2023
Published online: 27 July 2023

References

1. Frankenburg WG. Chemical changes in the harvested tobacco leaf. II chemical and enzymic conversions during fermentation and aging. In: Nord FF, editor. Advances in enzymology and related areas of molecular biology: nord/advances. Hoboken: John Wiley & Sons, Inc.; 1950.
2. Frankenburg WG. Chemical changes in the harvested tobacco leaf: part I chemical and enzymic conversions during the curing process. In: Nord FF, editor. Advances in enzymology and related areas of molecular biology: nord/advances. Hoboken: John Wiley & Sons, Inc.; 1946.
3. Wen Z, Liu Y, Zeng D, Yang J, Xi H. The study and application of cigar filler stacking fermentation technology. Hans J Agric Sci. 2016;06:63–70. https://doi.org/10.1267/HJAS.2016.63010.
4. Liu F, Wu Z, Zhang X, Xi G, Zhao Z, Lai M, Zhao M. Microbial community and metabolic function analysis of cigar tobacco leaves during fermentation. MicrobiologyOpen. 2021. https://doi.org/10.1002/mbo3.1171.
5. Dai J, Dong A, Xiong G, Liu Y, Hossain MS, Liu S, Gao N, Li S, Wang J, Qiu D. Production of highly active extracellular amylase and cellulase from bacillus subtilis ZIM3 and a recombinant strain with a potential application in tobacco fermentation. Front Microbiol. 2020;11:1539. https://doi.org/10.3389/fmicb.2020.01539.
6. Giacomo M, Paolino M, Silvestro D, Vigliotta G, Imperi F, Visca P, Alifano P, Parente D. Microbial community structure and dynamics of dark fire-cured tobacco fermentation. Appl Environ Microbiol. 2007;73:825–37. https://doi.org/10.1128/AEM.02378-06.
7. Yang Y, Peng Q, Ou M, Wu Y, Jing F. Research progress in tobacco fermentation. Biol Sci Med. 2018;6:10. https://doi.org/10.4236/jbm.2018.66008.
8. Tao J, Chen Q, Chen S, Lu P, Chen Y, Jin J, Li J, Xu Y, He W, Long T, Deng X, Yin H, Li Z, Fan J, Cao P. Metagenomic insight into the microbial degradation of organic compounds in fermented plant leaves. Environ Res. 2022;214:113902. https://doi.org/10.1016/j.envres.2022.113902.
9. Zelitch I, Zucker M. Changes in Oxidative Enzyme Activity During the Curing of Connecticut Shade Tobacco. Plant Physiol. 1958;33(2):151–55. https://doi.org/10.1104/pp.33.2.151.
10. Jensen P. Fermentation of cigar-type tobacco. Ind Eng Chem. 1950;42:519–21.
11. Vigliotta G, Di Giacomo M, Carata E, Massardo DR, Tredici SM, Silvestro D, Paolino M, Pontieri P, Del Giudice L, Parente D, Alifano P. Nitrite metabolism in *Debaryomyces hansenii* TOB-Y7, a yeast strain involved in tobacco fermentation. Appl Microbiol Biotechnol. 2007;75:633–45. https://doi.org/10.1007/s00253-007-0867-2.
12. Zheng T, Zhang Q, Li P, Wu X, Liu Y, Yang Z, Li D, Zhang J, Du G. Analysis of microbial community, volatile flavor compounds, and flavor of cigar tobacco leaves from different regions. Front Microbiol. 2022;13:907270. https://doi.org/10.3389/fmicb.2022.907270.
13. Han FG. Tobacco chemistry (In chinese). Beijing: China Agriculture Press; 2010.
14. Ravishankar D, Rajora AK, Greco F, Osborn H. Flavonoids as prospective compounds for anti-cancer therapy. Int J Biochem Cell Biol. 2013;45:2821–31. https://doi.org/10.1016/j.biocel.2013.10.004.
15. Nyane NA, Tlaila TB, Malefane TG, Ndwandwe DE, Owira P. Metformin-like antidiabetic, cardio-protective and non-glycemic effects of naringenin: molecular and pharmacological insights. Eur J Pharmacol. 2017;803:103–11. https://doi.org/10.1016/j.ejphar.2017.03.042.
16. Zeng W, Jin L, Zhang F, Zhang C, Wei L. Naringenin as a potential immunomodulator in therapeutics. Pharmacol Res. 2018;135:122–6.
17. Kashyap D, Sharma A, Tuli HS, Sak K, Punia S, Mukherjee TK. Kaempferol—a dietary anticancer molecule with multiple mechanisms of action: recent trends and advancements. J Funct Foods. 2017;30:203–19. https://doi.org/10.1016/j.jff.2017.01.022.
18. Yang X, Jin D, Li A, Han L, Zheng Z. Analysis of quality characters of cigar tobacco and ecological factors in tobacco-growing areas in Wanyuan. Acta Tabacaria Sinica. 2017;23:69–76. https://doi.org/10.1647/j.chinatobacco.2016.312.
19. Shahidi F, Chandrasekara A. Millet grain phenolics and their role in disease risk reduction and health promotion: a review. J Funct Foods. 2013;5:570–81. https://doi.org/10.1016/j.jff.2013.02.004.
20. Tan SA, Tian YL, Liu XB. Research and application of humidifying and stacking fermentation technology of cigar tobacco. J Anhui Agri Sc. 2017;45(23):80–3. https://doi.org/10.1398/j.cnki.0517-6611.2017.23.027.
21. Bailey DG, Cisle JH, Schepartz AI. Peroxidase activity during curing and storage of cigar-filler tobacco. Phytochemistry. 1970;9:69–72. https://doi.org/10.1016/S0031-9422(00)86616-9.
22. Pang Z, Xu J, Yi C. Linking plant secondary metabolites and plant microbiomes: a review. Front Plant Sci. 2021;12:1–22.
23. Yang L, Wen KS, Ruan X, Zhao YX, Wei F, Wang Q. Response of plant secondary metabolites to environmental factors. Molecules. 2018;23:762.

Publisher's Note