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Combining widely targeted metabolomics and RNA-sequencing to reveal the function analysis of *Phyllanthus emblica* Linn. Juice-induced poultry macrophages

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ABSTRACT

This study explored the functional effects of cultivated and wild *Phyllanthus emblica* Linn juice (PEJ) in HD11 poultry macrophage lines, with the aim of potentially developing cultivated PE and its fruit residue as poultry feed additives. RNA-Seq was used to evaluate the functional differences between cultivated and wild PEJ induced HD11 cells. Both cultivated and wild PEJ could regulate cell replication by histone H1/H2 family genes and host immune response by Toll-like receptor 7 regulation. Wild PEJ inhibited M1-type polarization of host macrophages, while cultivated PEJ promoted M2-type polarization. Metabolites of cultivated and wild PE were identified by widely targeted metabolomics based on liquid chromatography-tandem mass spectrometry. Of the 911 metabolites, 238 differed functionally between cultivated and wild PE. The data provide a theoretical basis for the subsequent development of PE as a functional feed additive in poultry.

1. Introduction

Phyllanthus emblica Linn. (PE) is a perennial tree plant fruit of the family *Euphorbiaceae*, genus *Euphorbiaceae* (Gaire and Subedi 2014). It originated in India and has been widely distributed in India, Malaysia, Myanmar, Indonesia, and southern China (Huang et al. 2022). In China, it is most widely planted in the provinces of Guangdong, Guangxi, Yunnan, and Fujian (Ma et al. 2024). PE is rich in minerals (such as potassium, cesium, magnesium, copper, and others), amino acids (serine, glycine, phenylalanine, histidine, and others), and vitamins (C, B1, B2, and others) (Liu et al. 2013). The most attractive feature of PE is that after chewing, it goes from a brief sour taste to a sweet aftertaste, and can retain a pleasant fragrance in the mouth for a long time. In addition, PE generates saliva, relieves cough, strengthens the stomach, and reduces digestion (Li, Zhu, et al. 2020). In clinical medicine, PE is widely used to treat symptoms such as cough, malaria, inflammation, and excessive stomach acid (Saini et al. 2022). Due to its medical function, it is widely used in traditional Chinese medicine, Tibetan medicine, and Ayurvedic medicine in India. Due to its unique flavor and multiple health benefits, PE has been developed and processed into various foods such as preserved fruits, fruit wine, and health lozenges. PE is rich in moisture and is currently mainly used for processing into raw fruit juice, and further processing into various beverages. Production of PE in Guangdong, Guangxi, and Fujian provinces has also been increasing annually. The increases in PE production and processed products have also resulted in increasing processing by-product of PE fruit residue. During the processing of PE products, fruit residue accounts for 40 % of the fruit quality (the juice extraction rate is approximately 60 %) (Zheng et al. 2024). Effective use of PE residue could alleviate environmental pollution and increase its added value.

Fruit waste refers to defective fruits or waste by-products discarded during production, harvest, processing, transportation, or sales (Wang et al. 2024). Due to its rich nutritional components, it can promote animal production performance and increase feed conversion rate (Galvão et al. 2020). The active ingredients of different varieties of fruit waste can enhance animal immunity and improve intestinal health (Dhakal et al. 2022). Fruit pomace is widely used, and its development and utilization are mainly based on locally main cultivated varieties, showing regional characteristics. The utilization of pomace is mainly through fermentation, drying, and extraction of active ingredients (Günal-Köroğlu et al. 2023).

Adding PE extract to the feed of ruminants, pigs, and poultry can effectively reduce the number of toxic bacteria in the host, including *Proteus* spp., *Escherichia coli*, and *Staphylococcus aureus*, improve the

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antioxidant capacity of livestock (Nguse et al. 2022), and produce anticoccidian and antiviral effects (Sharma et al. 2021; Meng et al. 2021). Oral administration of the polyphenol extract of PE can down-regulate the activation of the NF-κB signaling pathway in White Leghorn chickens caused by *Escherichia coli*, thereby inhibiting the production of pro-inflammatory factors (Zhong et al. 2014). In addition, in vitro experiments have shown that the methanol extract of PE can inhibit the growth of Salmonella typhi and Salmonella enteritidis by direct effect (Nair et al. 2020). Most studies have focused on specific classes of metabolites, such as phenolic compounds (Li, Zhang, et al. 2020) and flavonoids (Karkon Varnosfaderani et al. 2018), resulting in functional of PE that can only be explained from a single perspective. The different extraction methods of PE active ingredients lead to significant differences in the yield and abundance of the active ingredients (Zhang et al. 2017), which cannot reflect the overall value of PE.

A study has shown that feeding fresh PE fruits to dairy cows on the basis of a total mixed ration. After a 28-day animal experiment, it was detected that PE fruits can reduce the total saturated fatty acids in dairy cows and increase the content of unsaturated fatty acids. Moreover, it can increase the biomarkers of antioxidant capacity in the blood of dairy cows, thereby protecting them from oxidative stress (Tilahun et al. 2022). However, in actual production, fresh fruits are first utilized by humans. To solve the relationship of competition between humans and livestock, the value utilization of by-products of fresh fruit processing is more meaningful. From the perspective of fruit residue waste utilization, reducing utilization costs and maximizing the medicinal value of PE fruit residue are feasible strategies for using PE fruit residue as a feed additive for livestock and poultry. Some components of cultivated PE, such as gallic acid and ellagic acid, have undergone significant changes during the breeding process, resulting in certain medicinal effects of cultivated PE being unable to meet medicinal standards (Huang, Chen, et al. 2021). Wild PE has characteristics such as small fruits, low yield, and a sour and astringent taste. These aspects hinder its consumer acceptance. During the selective breeding, varieties with large fruit, high yield, and sweet taste are the focus of cultivation.

Therefore, this study evaluates the utilization value of cultivated PE pomace as a poultry feed additive by exploring the similarities and differences in the functional regulation of poultry immune cell lines by cultivated PE juice (PEJ) and wild PEJ. The chicken macrophage cell line (HD11) is derived from chicken bone marrow and is well-suited for studying immune responses, including cytokine expression and cell signaling, due to its ability to effectively express various immune-related genes. As an immortalized avian immune cell line, the HD11 cell line is widely utilized in scientific research related to viral infections, immune modulation, and other related fields (Shi et al. 2024; Cui et al. 2021).

In this study, RNA-Seq was used to analyze the similarities and differences in the functional regulation of the whole transcriptome of HD11 poultry macrophages by cultivated PEJ and wild PEJ. Then, through widely targeted metabolomics technology, the similarities and differences in the overall metabolite content between cultivated PE and wild PE were analyzed. Finally, the feasibility of using cultivated PE pomace as a poultry feed additive was determined. We hope that this study can provide valuable theoretical basis and reference for the development and utilization of cultivated PE pomace.

2. Materials and methods

2.1. PE sample collection, preparation and cell line

Wild PE and cultivated PE were used in this study. Wild PE is a local uncultivated PE with small fruit and sour taste. Cultivated PE is a locally selected and cultivated edible variety with larger fruit and sweeter taste compared to wild PE. These two varieties of PEs were identified by senior agronomist Jiandong Ye from the Shanwei Academy of Agricultural Sciences.

Both wild and cultivated PEs are fresh fruits collected during the mature period in Guangdong, China. Three trees were randomly selected from each variety, and 50 ripe fruits were collected from each tree. The fruits from each group were washed and dried. They were then separately processed using a juicer (SUPOR, Zhejiang, China). The liquid of each preparation was filtered through a microporous membrane (0.45 μm) and stored at −80℃ for subsequent experimental use.

HD11 chicken bone marrow macrophages were a gift from the Key Laboratory for Prevention and Control of Avian Influenza and Other Major Poultry Diseases, Ministry of Agriculture and Rural Affairs. The cells were cultured in RPMI 1640 medium (HyClone, Logan, UT, USA) supplemented with 10 % fetal bovine serum (Gibco, Carlsbad, CA, USA) and maintained at 38 °C in an atmosphere of 5 % CO_2. The culture medium contained 100 units/mL penicillin and 100 μg/mL streptomycin.

2.2. Cell viability detection

The viability of HD11 cells after incubation with PEJ was detected using Cell Counting Kit-8 (CCK-8) (NCM Biotech, Suzhou, China). The HD11 cell line was seeded in a 96-well plate to achieve a density of 90 %. Different concentrations of PEJ (0, 0.1, 0.2, 0.4, 0.8, 1.6 μL/100 μL) were incubated in the 96-well plate respectively. Then the 96-well plate was placed in a cell incubator containing 5 % CO_2 and taken out after 48 h. According to the instructions of the CCK-8 kit, 10 μL of CCK-8 solution was added to each well. The 96-well plate was taken out after being placed in the cell incubator for 1 h. The absorbance at 450 nm was measured by a microplate reader (ThermoFisher Scientific, Waltham, MA, USA). The cell viability of HD11 was calculated as follows: Cell viability (%) = [(As − Ab) / (Ac − Ab)] × 100 %. As, absorbance of experimental wells (containing cells, medium, CCK-8 solution and PEJ); Ac, absorbance of control wells (containing cells, medium, CCK-8 solution, without PEJ); Ab, absorbance of blank wells (containing medium and CCK-8 solution, without cells and PEJ). Five parallel samples were set for each experimental group. The experiments were performed in triplicate.

2.3. RNA-seq

HD11 cells were cultured at a density of 90 % in T25 cell culture bottles, and incubated with corresponding doses of PEJ. The control group received an equal volume of PBS, rather than PEJ. Each group had three duplicate samples. After stimulation for 48 h, the cells were washed three times with PBS. Total RNA was extracted using TRIzol (TaKaRa Biotechnology, Dalian, China), according to the manufacturer's instructions. RNA integrity, library construction, and quantification of the library quality testing were performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). Detection was done as previously described (Liu et al. 2023). The HiSeq 2000 platform (Illumina, San Diego CA, USA) at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) was used to sequence the library preparation and generate paired end readings of 150 bp. The reference genome and version were *Gallus* (GenBank no. GCA_16699485.1).

2.4. Differentially expressed genes (DEGs), gene ontology (GO), and Kyoto Encyclopedia of genes and Genomes (KEGG) enrichment analysis

The original readings were normalized. Statistical models were used to calculate the hypothesis testing probability (p-value). The FDR (error detection rate) value was corrected through multiple hypothesis tests (Anders and Huber 2010). log2 (fold change) $| \geq 1$, and padj ≤ 0.05.

ClusterProfiler R software was used for GO and KEGG pathway analyses to correct for differences in DEGs. GO pathways with a corrected $p < 0.05$ were considered to significantly concentrate DEGs. KEGG is a database resource for understanding the advanced functions and practicality of biological systems (https://www.genome.jp/kegg/).

Protein–protein interaction (PPI) analysis of DEGs based on the

STRING database (https://cn.string-db.org/) was predicted based on known data.

2.5. Quantitative real-time PCR (qRT-PCR) validation

HD11 cells mRNA was extracted using TRIzol (TaKaRa Biotechnology, Dalian, China), according to the manufacturer's instructions. qRT-PCR was performed using a HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme, Nanjing, China), and a LightCycler96 Real-Time PCR System (Roche, Basal, Switzerland). Relative mRNA levels were calculated using the $2^{-\triangle\triangle}$Ct method (Jozefczuk and Adjaye 2011). The primers used in the validations are shown in Table 1.

2.6. Widely targeted metabolomic detection

Before ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) analysis, sample preparation, extraction, UPLC conditions, and electrospray ionization-quadrupole ion trap (ESI-Q TRAP)-MS/MS were performed as previously described (Li et al. 2024). Identification and quantification of the metabolites of the samples were performed by widely targeted metabolomics at Wuhan MetWare Biotechnology Co., Ltd. (Wuhan, China).

2.7. Widely targeted metabolomics analysis

Principal component analysis (PCA) is the observation of the degree of variation between different groups and within group samples. PCA was performed by the statistical function prcomp within R (www.R-project. org). The hierarchical clustering analysis (HCA) results of the samples and metabolites are presented as heatmaps with dendrograms. The original relative content of differential metabolites was normalized by row (Unit Variance Scaling, UV Scaling). A heatmap was drawn using the R software ComplexHeatmap package. Based on the orthogonal partial least squares-discriminant analysis (OPLS-DA) results, the variable importance in projection (VIP) of the obtained multivariate analysis OPLS-DA model was used to preliminarily screen metabolites with differences between different varieties (VIP$\geq$1). At the same time, metabolites with $p < 0.05$, fold change ≥ 2, or fold change ≤ 0.5 were identified as significantly different based on univariate analysis. The identified metabolites were annotated using the KEGG compound database (https://www.kegg.jp/kegg/compound/). The annotated metabolites were mapped to the KEGG Pathway database (https://www.kegg.jp/kegg/pathway.html).

2.8. Statistical analysis

Data were analyzed using GraphPad Prism software v5.01 (GraphPad Software Inc., San Diego, CA, USA). Results are presented as the mean ± SEM of at least three independent experiments. One-way analysis of variance followed by the Tukey–Kramer post-test was used. A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Global analysis of gene expression profiles in HD11 cells by cultivated and wild PEJ stimulation

Before studying the effect of PEJ on poultry immune cells, it was first necessary to determine the incubation dose of PEJ. HD11 cells were incubated with PEJ concentrations ranging from 0 to 1.6 μL/100 μL for 48 h. CCK-8 detection results show (Fig. 1) that when the PEJ concentration of cultivated (Fig. 1A) and wild (Fig. 1B) PEJ was 0.1 μL/100 μL, there was no difference from the control group (ns). When the concentration of PEJ was $\geq$ 0.2 μL/100 μL, the cell survival rate was extremely significantly different from that of the control group ($p < 0.01$ or $p < 0.0001$). When the PEJ concentration was $\leq$ 0.2 μL/100 μL, cell viability was > 80 % (Fig. 1). In order to induce the functional response of macrophages at the maximum dose without affecting cell viability, 0.2 μL/100 μL PEJ was selected for subsequent research.

This study successfully sequenced and constructed nine cDNA libraries, generating 447,661,746 original readings, of which 435,763,616 were clean readings. The percentage of bases with a phred value > 20 in total bases (Q20) and Q30 values were tested, with ranges of 97.14 % to 97.81 %, and 92.98 % to 94.39 %, respectively. The various data of each library are shown in Supplementary Table 1. The selection requirements for DEGs in this study were | log2 (fold change) | $\geq$ 1, with a p-value ≤ 0.05. The volcano plot in Fig. 2A shows that compared to the control sample, 453 genes were up-regulated and 293 genes were down-regulated in the wild PEJ stimulated cell samples. In the cell samples stimulated by cultivated PEJ, 467 genes were up-regulated and 319 genes were down-regulated (Fig. 2B). Fig. 2C shows that the expression patterns between samples were highly similar (R^2 > 0.97). The heatmap in Fig. 2D shows that each group of samples was significantly clustered together, with high intra group similarity.

3.2. GO and KEGG differential analyses in HD11 stimulated by cultivated and wild PEJ

In order to explore the gene regulation patterns of HD11 cells caused by different varieties of PEJ stimulation, GO analysis was performed on all DEGs in HD11 (Fig. 3A and 3B). GO enrichment results revealed that, in the wild PEJ stimulation group, DEGs were mainly involved in the response to cytokine, response to chemical, response to external biotic stimulus; nucleosome, DNA packaging complex, extracellular region part; and transmembrane signaling receptor activity, protein dimerization activity, signaling receptor activity functions (Fig. 3A). In the

Table 1
Primers used for qRT-PCR.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Accession number	Amplicon size (bp)
SMOC1	GTGCCATACCTACACTGGATAC	CCCTGGCTTGACGGTTTAT	XM_040672048.2	126
FUT7	CAGTGTGAAGGCAGTGGTAA	CTGTTGGATGAGCGGATGAA	XM_015279614.4	81
MARCO	CAGATGCAGAGGAGATGGAAAT	AGGCTCGCTTATGTGAAAGG	NM_204736.3	106
LRRC25	CCCGACTACGAGAACGTCTT	GTCGCTCTCCAGGAAATAGAGA	XM_040692919.2	138
TMEM119	GGCCCAGAACCTCAAATCTC	CCACTCCTTCAGCATCTTGATT	XM_040684687.2	134
ABCA2	CACTCTCCGTGCCTGAAATAG	CCGTTCCTCGTTGGCATAA	XM_040685624.2	113
RHPN1	TCGCATTGGAGCTGAGTTATG	GATGGACTCACTGTCATTCTGG	XM_040665208.2	107
NCAM2	GTGAAGCAGAAGGAGAACCTATT	TGCTGCCCTTTGACTTCTATAC	XM_052666931.1	115
ZFAND2A	TCGATTTCCTCCCTCTGAAATG	CTTCTTGTAGGCAGAGCTACAG	XM_040683521.2	98
LZTS3	GGGTGACAGAGAACAGAAAGAG	CCTCCCACTTGGTTTGTAGTC	XM_040671658.2	112
LIF	GGACCTCTTCAGCCTCTATCT	CTTGTACATGGCCACCATCA	XM_040685142.2	148
FGF14	CCTGGTTCTTGGGTCTGAATAA	CTTCCAATGGCTTGGGTAGAA	NM_204777.2	99
RET	TACCTCAGCAAGAGCCTAGATA	CTTGGAAAGGGTGAGATGAGAG	NM_205190.3	112
SLC13A3	GCCATCGAGGAGTGGAATTT	CGTCGTCAACATCATCCCTAAA	XM_040688599.2	102
18 s rRNA	TCAGATACCGTCGTAGTTCC	TTCCGTCAATTCCTTTAAGTT	AF173612	154

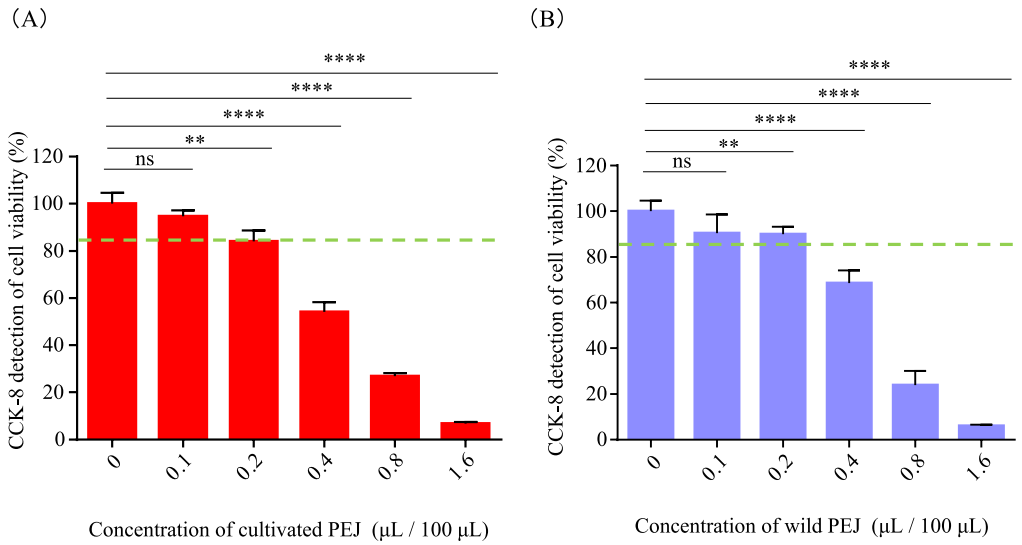

Fig. 1. CCK-8 assay detection of cell viability in HD11 cells stimulated by PEJ. HD11 cells were exposed to different concentrations of (A) cultivated and (B) wild PEJ for 48 h. Cell viability was then determined by the CCK-8 assay. The green dashed line indicates the allowed threshold for cell viability, values ≥ 80 %, indicate no effect on cell viability. Data are presented as mean ± SEM of three independent experiments. Indicate no significant difference denoted by "ns", statistically significant differences between groups are denoted by **p < 0.01, ****p < 0.0001. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cultivated PEJ stimulation group, DEGs mainly participated in defense response to other organism, nucleosome assembly, defense response; nucleosome, DNA packaging complex, protein–DNA complex; and signaling receptor activity, receptor ligand activity, receptor regulator activity (Fig. 3B).

From the KEGG enrichment results, 20 of the most important KEGG pathways were selected, and the up-regulated or down-regulated KEGG pathways were plotted and displayed. If there were fewer than 20 pathways, all pathways were drawn, as shown in Fig. 3C-F. Compared with the control group, the KEGG upregulated pathways in the wild PEJ stimulation group were significantly enriched in the extracellular membrane (ECM)–receptor interaction, cytokine–cytokine receptor interaction, and focal adhesion pathways (Fig. 3C), while the KEGG downregulated pathways in the wild PEJ stimulation group were significantly enriched in the influenza A, Toll-like receptor (TLR) signaling pathway, and NOD-like receptor signaling pathways (Fig. 3D). At the same time, compared with the control group, the KEGG up-regulation pathways in the cultivated PEJ stimulation group were significantly enriched in the cytokine–cytokine receptor, ECM-receptor, and neuroactive ligand-receptor interactions (Fig. 3E), while the KEGG down-regulation pathways in the cultivated PEJ stimulation group were significantly enriched in the phagosome, arginine biosynthesis, and cytokine–cytokine receptor interaction (Fig. 3F).

3.3. PEJs regulate cell replication by histone H1/H2 family genes, and inhibit host immune response by TLR7

In order to clearly distinguish the functional similarities and differences between cultivated and wild PE, we comprehensively investigated the difference between the two groups on macrophage activation. As shown in Fig. 4A, we selected the upregulated genes of wild and cultivated PEJ activated HD11 cells were selected to create a Venn diagram. The results showed that 285 genes were co-expressed upregulated, 152 genes were upregulated separately in the wild group, and 162 genes were separately up-regulated in cultivated group. Each of these three gene sets into the STRING database for PPI analysis to identify the central regulators of the molecular network. The PPI networks of the above three gene sets were constructed using Cytoscape, and the top 10 hub genes were identified using Cytoscape plug-in CytoHubba. The histone H1/H2 family was upregulated by both wild and cultivated groups (Fig. 4B). IL-10 was identified as the core of the top 10 hub genes in the gene set that was separately upregulated in the cultivated group (Fig. 4C), while there was no significant correlation between the top 10 hub genes identified in the gene set that was separately upregulated in the wild group (Fig. 4D).

Fig. 4E presents a Venn diagram was created by selecting the downregulated genes of wild and cultivated PEJ activated HD11 cells. The results showed that 167 genes were downregulated jointly, 129 genes were downregulated separately in the wild group and 148 genes were downregulated separately in the cultivated group. We treated each of these three gene sets in the same way as the upregulated gene sets. TLR7 was the key factor for the down-regulation of the wild and cultivated groups; its interaction factors included CSF1R, CCR2, C1QB, MMR1L4, VCAM1, SYK, RGS18, BTK, and CYBB (Fig. 4F). Integrin beta chain-2 (ITGB2) was identified as the core of the top 10 hub genes in the gene set that was separately downregulated in the cultivated group (Fig. 4G), while RAC2 was identified as the core of the top 10 hub genes in the wild group gene set that was downregulated separately (Fig. 4H).

PPI analysis using the STRING database revealed that the GO terms of the regulation of multicellular organismal process, nucleosome assembly, cell adhesion, tissue development, cellular response to organic substance, structural constituent of chromatin, protein heterodimerization activity, nucleosome, extracellular region, extracellular space, and cell periphery were enriched in the two PEJ stimulation groups (upregulated set; the top five false discovery rates were selected to display, with all rates listed when fewer than five were evident, which also applies below) (Supplementary Table 2-1). The results suggest that both varieties of PE have the function of promoting cell replication. DEG analysis of upregulated genes in the cultivated PEJ stimulation group showed that GO was enriched in positive regulation of cell population proliferation, regulation of cell population proliferation, positive regulation of molecular function, regulation of multicellular organismal process, and regulation of extracellular signal regulated kinase 1 (ERK1) and ERK2 cascade. Compared with the wild group, the cultivated PEJ group displayed stronger cell proliferation (Supplementary Table 2-2). In the upregulated gene set of the wild PEJ group, there were no significant pathway enrichments observed in the GO categories of Biological Process, Molecular Function, and Cellular Component (Supplementary Table 2-3).

Analysis of the downregulated gene set, revealed no obvious

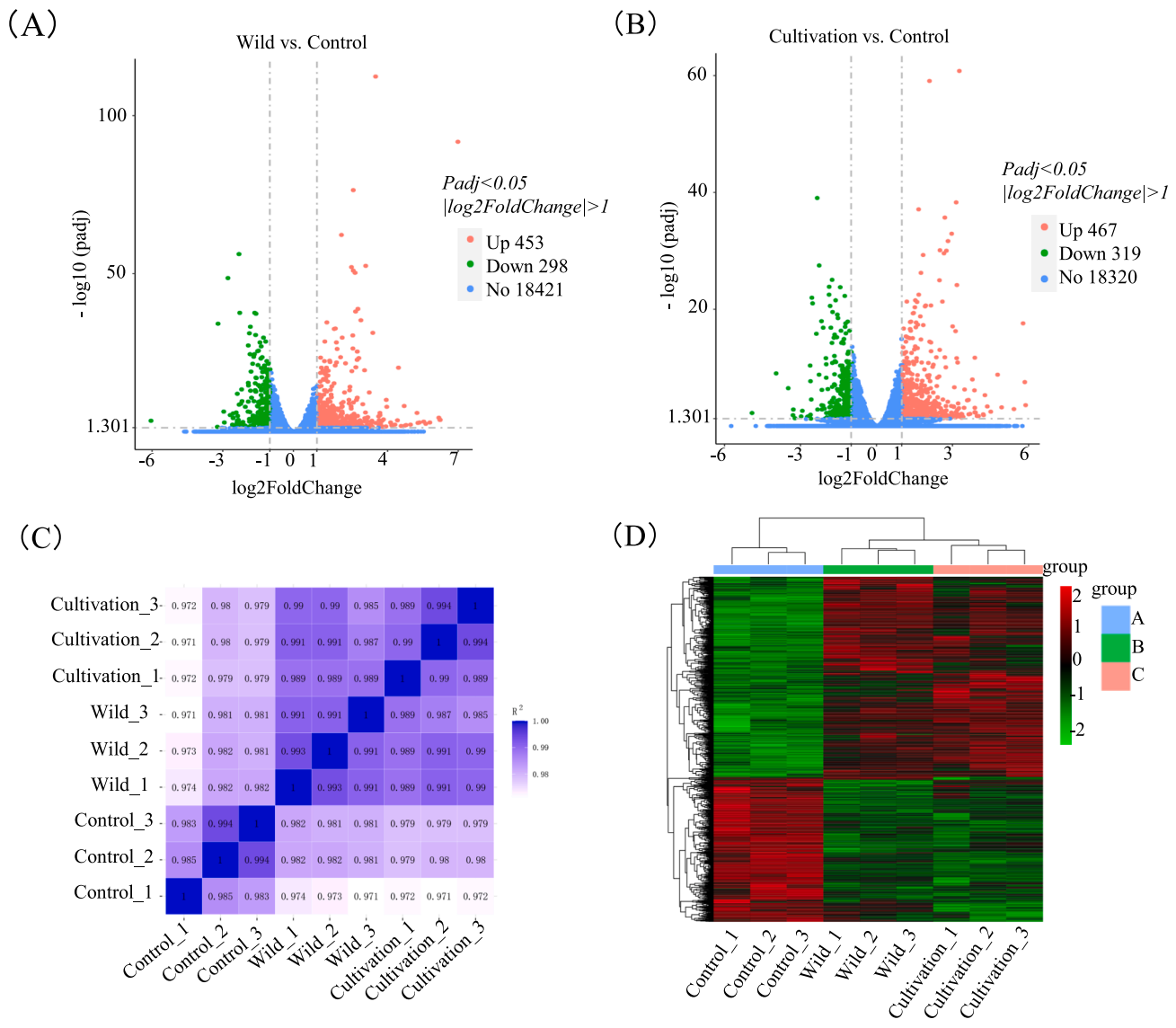

Fig. 2. Analysis of DEGs. Volcano plots showing the distribution of DEGs between (A) wild PEJ vs. control, and (B) cultivated PEJ vs. control. (C) Correlation analysis diagram of expressions of genes. (D) Heatmap analysis of differential genes clustering.

enrichment of functional signaling pathways in downregulated genes in the two PEJ stimulation groups and the downregulated genes separately regulated by the cultivated group (Supplementary Table 2-4; Supplementary Table 2-5). However, GO enrichment of the wild PEJ stimulation group revealed enrichments in immune response, defense response, response to enrichment, and defense response to other organization (Supplementary Table 2-6). Wild PE could inhibit immune response, and reduce host defense response pathways.

3.4. Quantitative real-time PCR analysis

To verify the results of transcriptome sequencing, 14 genes were selected for the qRT-PCR. As shown in Fig. 5, the expression levels of RET, ZFAND2A, LZTS3, NCAM2, SLC13A3, LIF, and GFG14 were upregulated in HD11 cell line by the wild and cultivated PEJ stimulated samples. RHPN1, ABCA2, TMEM119, LRRC25, MARCO, FUT7, and SMOC1 were downregulated in the same samples. The qRT-PCR validation showed that (Fig. 5) the expression levels of the selected genes were consistent with the RNA-Seq results, verifying the reliability of the transcriptome sequencing results.

3.5. Widely targeted metabolome analysis in PEJ

The difference between regulation of HD11 transcripts by wild and cultivated PEJ is due to differences in the two PE metabolites. To better explore the value of PE and clarify the similarities and differences of metabolites between cultivated and wild PE, a broadly targeted UPLC-MS/MS metabolite analysis was performed. A total of 911 metabolites were identified, including a large number of primary and secondary metabolites that may have medicinal functions (Supplementary Table 3).

The metabolites identified included 10.1 % amino acids and their derivatives, 19.21 % phenolic acids, 4,61 % nucleotides and their derivatives, 17.23 % flavonoids, 2.41 % Lignans and Coumarins, 4.17 % Tannins, 6.48 % Alkaloids, 3.62 % Terpenoids, 6.48 % Organic acids, 15.81 % Lipids, and 9.88 % other metabolites. Among the 157 identified flavonoids, two were isoflavones, one was biflavones, 16 were flavanols, five were flavonoid carbonoside, 83 were flavones, nine were chalcones, 25 were flavanones, nine were flavaononls, seven were anthocyanidins. Among the 59 identified alkaooids, 11 were plumerane, one was isoquinoline alkaloids, 29 were alkaloids, two were pyridine alkaloids, three were pyrrole alkaloids, one was benzylphhenylethylamine

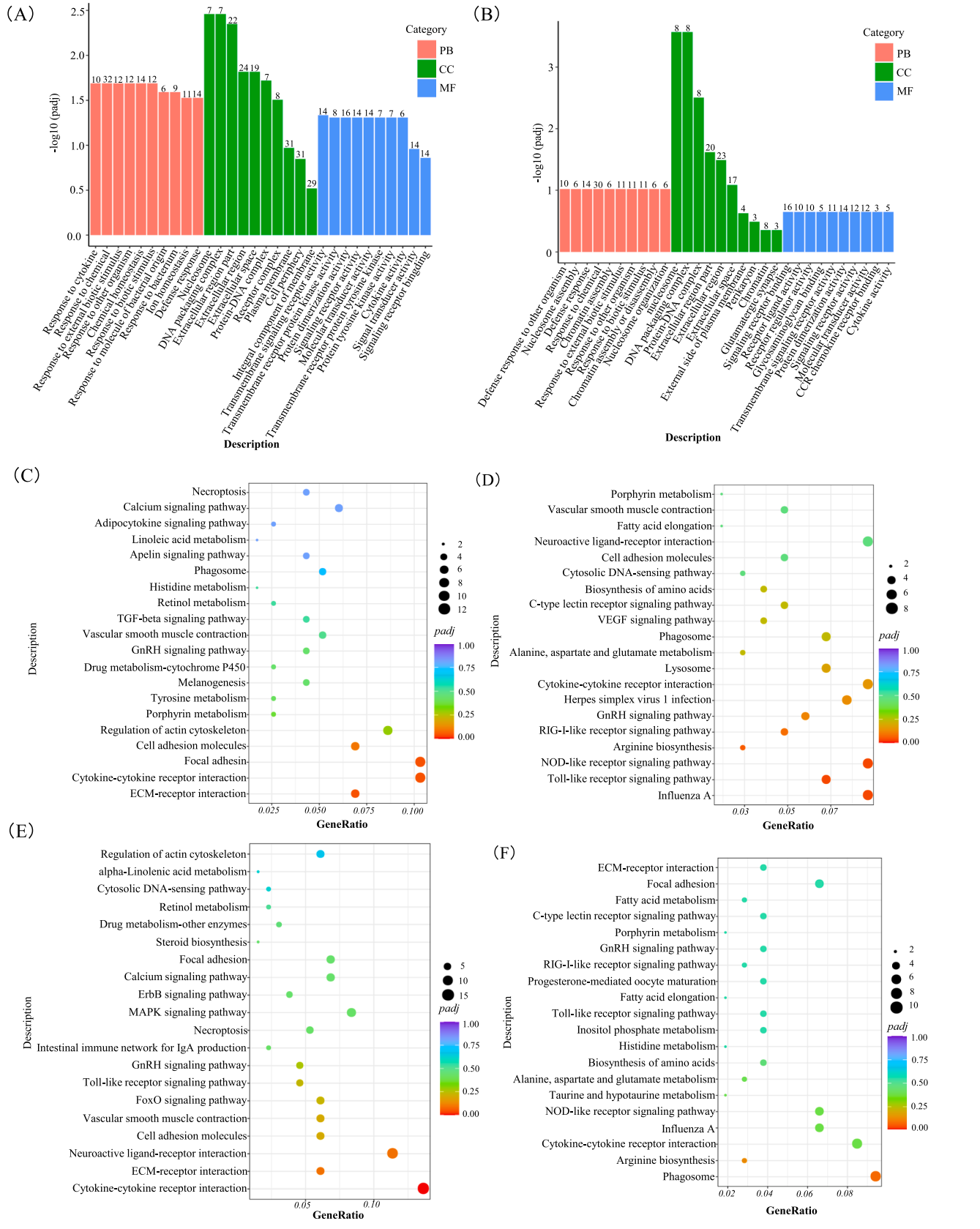

Fig. 3. GO and KEGG functional enrichment analyses of DEGs in HD11 cells. (A-B) GO enrichment analysis of differential expressed genes in HD11 by wild (A) or cultivated (B) PEJ stimulation relative to the control; (C-D) KEGG enrichment bubble diagram of upregulated DEGs (C) or downregulated DEGs (D) in wild PEJ group relative to the control. (E-F) KEGG enrichment bubble diagram of upregulated DEGs (E) or downregulated DEGs (F) in cultivated group relative to the control.

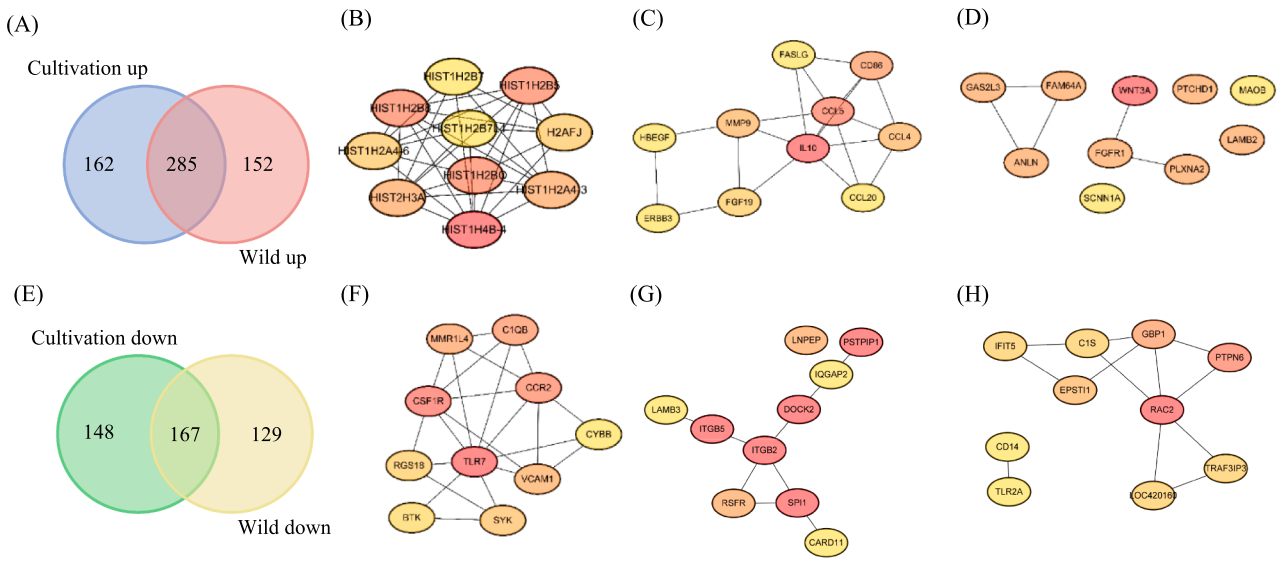

Fig. 4. Analysis of key genes and networks in the DEG sets. Venn diagram (A) represents the number of significantly upregulated genes in both the wild and cultivated groups. The intersection represents the number of upregulated genes in both groups. STRING was used in each DEG set analysis. The ranking of key genes was analyzed using Cytoscape's CytoHubba program, selecting the top 10 key genes for display. The redder the color, the higher and more significant the score. The key genes and their relationships in the DEGs set upregulated by (B) two groups coregulation, (C) the cultured group separately, and (D) the wild group separately. (E-H) represents down-regulation of DEGs, correspond to the above analysis.

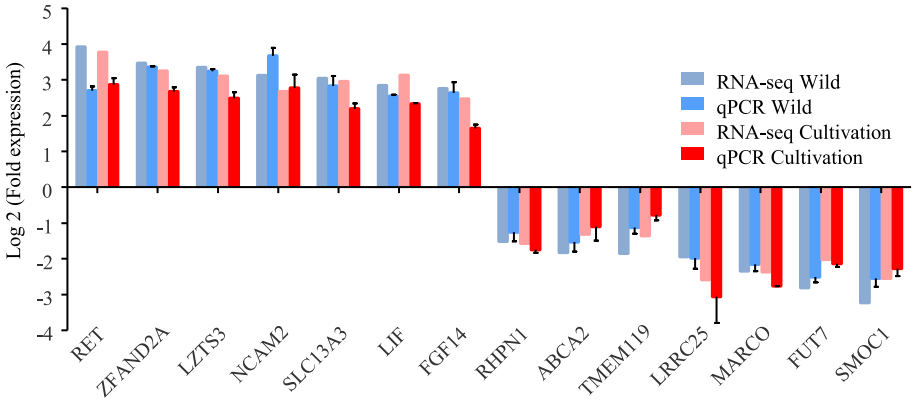

Fig. 5. qRT-PCR verification of the transcription genes. The transcriptional level of 14 selected genes was examined using qRT-PCR and compared with RNA-Seq data for validation. The expression levels were normalized to the expression of 18 s rRNA. Data are presented as mean ± SEM of three independent experiments.

alkalaids, eight were phenolamine, two were quinoline alkaloids and two were piperidine alkaloids. In addition, six kinds of secondary metabolites of lipids were detected, which were free fatty acids (66), glycerol ester (19), sphingolipids (2), LPC (29), LPE (25) and PC (3). Five kinds of secondary metabolites of terpenoids were also detected, including terpene (1), sesquiterpenoids (4), monoterpenoids (9), diterpenoids (1) and triterpene (18). Two secondary metabolites were those of coumarins (7) and lignans (15). Two secondary metabolites of tannins were proanthocyanidins (3) and tannin (35). Four secondary metabolites of others were saccharides and alcohols (54), vitamin (16), stilbene (3) and others (17).

3.6. Identification analysis of differential metabolites

PCA was performed on the metabolites of two groups of samples, and the two groups were significantly separated by a two-dimensional PCA plot (Fig. 6A). After data normalization, a cluster analysis was performed on all samples and a clustering heatmap was drawn using the R program script (Fig. 6B). Different expression patterns between wild and cultivated PEJ were identified in this mode. PCA and cluster analysis showed significant differences in metabolic products between wild and cultivated PE. In order to elucidate the differences in metabolites between the two groups, wild PEJ was used as the control in this study. In addition, cultivated PEJ vs. wild PEJ metabolites content with a fold change ≥ 2 (upregulated) or ≤ 0.5 (downregulated) were defined as significant differences. These metabolites were screened using VIP$\geq$1 of the OPLS-DA model, and 238 differential metabolites were screened between two PE varieties (Fig. 6C, Supplementary Table 4). Compared with wild PE, 72 metabolites were downregulated and 166 metabolites were upregulated in cultivated PE (Fig. 6C). These 238 differential metabolites were categorized into more than 11 distinct classes (Fig. 6D), with the most significant differences being amino acids, amino acid derivatives, phenolic acids, nucleotides, derivatives, flavonoids, alkaloids, organic acids, and lipids (Fig. 6D, Supplementary Table 4).

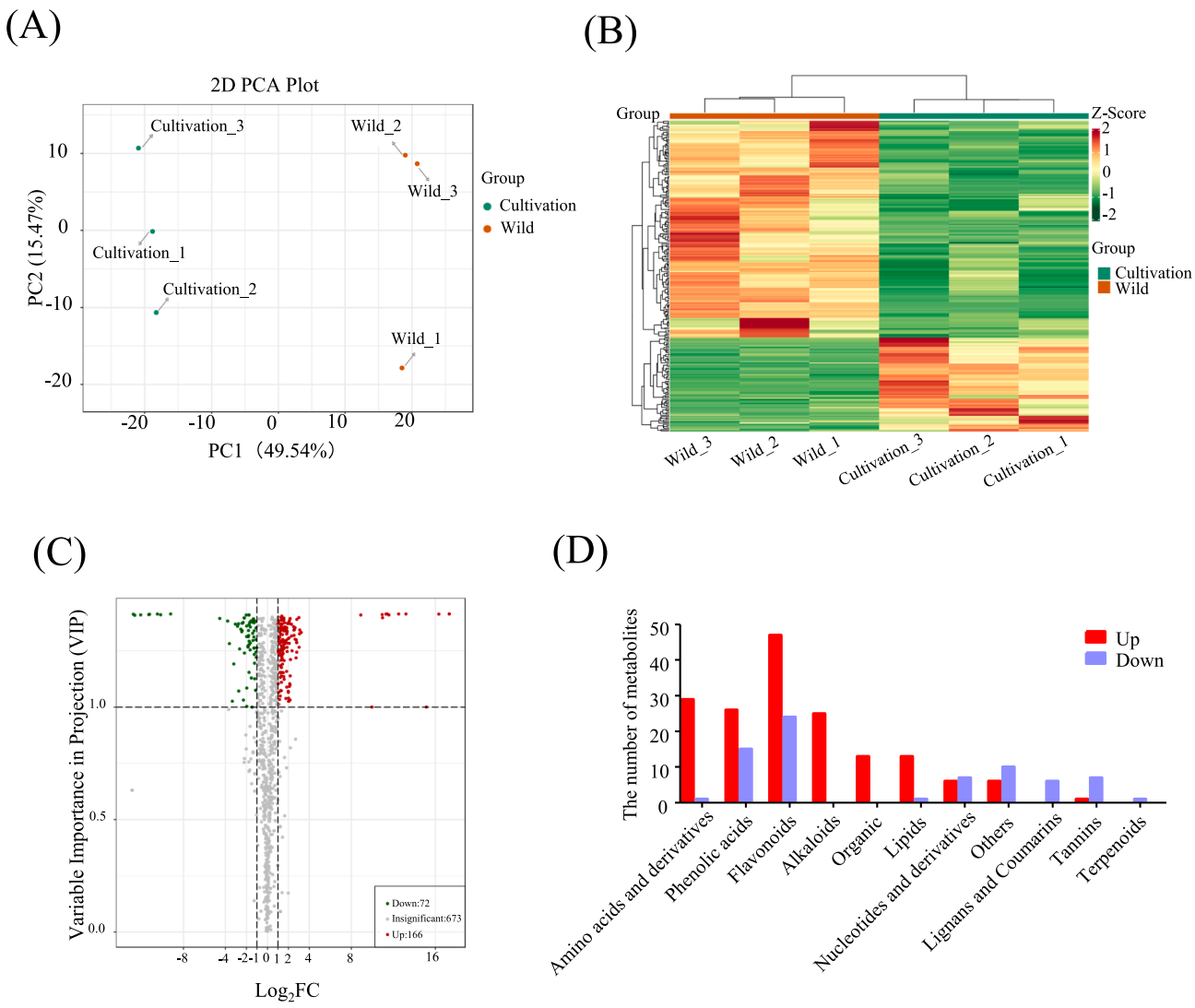

Fig. 6. Differential analysis of metabolites between cultivated and wild PE. (A) PCA analysis of metabolites identified from cultivated and wild PE. (B) Cluster analysis of metabolites from samples of cultivated and wild PE. The color indicates the level of accumulation of each metabolite, from low (green) to high (red). The Z-score represents the deviation from the mean by standard deviation units. The class represents the classification of metabolites. (C) Volcano plot of the 911 metabolites identified. Each point in the volcano plot represents a metabolite, with green dots representing downregulated differential metabolites, red dots representing upregulated differential metabolites, and gray dots representing detected but not significantly different metabolites. The abscissa represents the logarithmic value of the relative content difference of a metabolite between two groups of samples (log_2FC). The larger the absolute value of the abscissa, the greater the relative content difference between the two groups of samples. The vertical axis represents the VIP value, The larger the vertical axis value, the more significant the difference. (D) Analysis of the number of advantage metabolites for each PE using wild group as the control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.7. KEGG annotation and enrichment analyses of differential metabolites

In this research, 238 differential metabolites were identified between wild and cultivated PEs, including 30 amino acids and derivatives, 41 phenolic acids, 13 nucleotides and derivatives, 71 flavonoids, six lignans and coumarins, eight tannins, 25 alkaloids, 13 organic acids, 14 lipids, and 16 others (Supplementary Table 4). The key metabolic pathways and metabolite levels in the cultivated and wild groups were further analyzed. The closer the p value of the differential metabolic pathway was to 0, the more significant the enrichment was. Pathways of biosynthesis of various plant secondary metabolites, biosynthesis of amino acids, and 2-oxocarboxylic acid metabolism corresponded to the differential metabolites of wild and cultivated groups, including 33, 13, and 12 metabolites, respectively (Fig. 7A, Supplementary Table 5). The differential abundance score (DA Score) showed that, compared to the wild-type, only the anthocyanin biosynthesis, photosynthesis, and ascorbate and alate metabolism pathways tended to be downregulated overall, while other metabolic pathways tended to be upregulated (Fig. 7B).

4. Discussion

In China, non-grain crop resources are abundant, with an annual straw output of about 318 million tons, tail vegetables of 220 million tons, and other processing by-products of 820 million tons. Among them, the most widespread source is plant-based non-conventional raw materials (Zhao et al. 2023). As a fruit with high medicinal and edible value, PE is widely used in industries that include food, medicine, and healthy medical and food additives. There are many PE planting areas in southern China, which produce a large amount of PE processed fruit residue every year. Although the functions of various PE extracts have been discussed earlier, in actual production, PE residue is difficult to use effectively due to objective factors, such as high extraction cost of

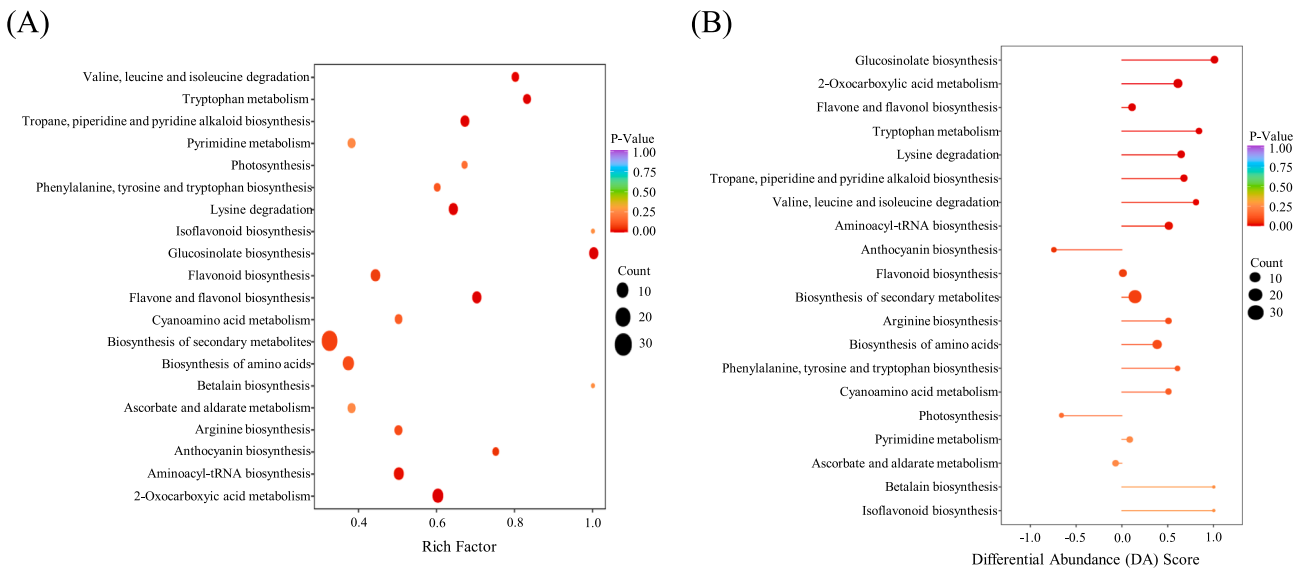

Fig. 7. Analysis of KEGG enrichment. (A) Enrichment analysis of differential KEGG metabolites. The horizontal axis represents the Rich Factor corresponding to each pathway, and the vertical axis represents the pathway name. The color of the point reflects the p-value size, and the redder the point, the more significant the enrichment. The size of the point represents the number of enriched differential metabolites. (B) Analysis of overall changes in KEGG metabolic pathways. The vertical axis represents the differential pathway name, and the horizontal axis represents the differential abundance score (DA Score).

effective ingredients and difficulty of centralized treatment of fruit residue. In view of this, this study used wild PEJ with significant medicinal effects as a control, and the functional regulation of cultivated PEJ on poultry immune cells was examined by comparison, thereby demonstrating the feasibility of using cultivated PE fruit residue as a whole as a poultry feed additive.

Macrophages alter their phenotype through polarization under different micro-environment stimulation. Macrophages typically polarize into two phenotypes, which perform different functions, M1 (pro-inflammatory) and M2 (anti-inflammatory). The markers of macrophage polarization into M1 phenotype are up-regulation of genes that include IL-1α, IL-1β, IL-6, NOS2, TLR2, TLR4, CD80 and CD86. Activation of M2 macrophages is associated with cytokines that include IL-4, IL-10, and IL-13 (Cui et al. 2021). In this study, the expression levels of IL-1β, IL-6, TGF-β, and IL-8 were significantly downregulated in the wild PEJ group, indicating that wild PE inhibits macrophage polarization to the M1 phenotype and inhibits the expression of inflammatory genes. However, the mRNA expression levels of IL-1β and IL-6 genes in the cultivated PEJ group were significantly downregulated, while the expression of IL-10 was significantly upregulated, indicating that the cultivated PEJ group promoted macrophage differentiation towards the M2 (anti-inflammatory) phenotype. Thus, wild PEJ inhibits pro-inflammatory responses by inhibiting the mRNA expression of inflammatory genes, while cultivated PEJ promotes anti-inflammatory responses by regulating anti-inflammatory factors. Although the polarization regulation mechanisms of cultivated and wild PEJ on HD11 were different, they both have the effect of reducing inflammatory responses.

In this study, LC-MS/MS based widely targeted metabolomics was used to analyze the metabolic differences between wild and cultivated PEs. A total of 911 metabolites were identified (Supplementary Table 3), of which 238 showed significant differences between wild and cultivated PEs (Supplementary Table 4). In the detection of metabolite of cultivated and wild PE, the largest difference in expression was of flavonoid metabolites (Fig. 6D) Forty-seven metabolites were upregulated and 24 metabolites were downregulated in cultivated PE compared with wild PE. Flavonoids are widely found in vegetables and fruits (Shen et al. 2022), have a wide range of biological effects, and have been proven to have a variety of anti-tumor effects (Yang et al. 2024), prevent obesity and diabetes (Dinda et al. 2020), function as antioxidants (Zhao et al. 2020), as well as other activities. In addition to flavonoids, the contents of tannins are the main indices for evaluating the differences of PE medicinal activity (Huang, Ran, et al. 2021). Although tannins have extensive applications in the fields of anti-inflammatory, antibacterial, antioxidant, and anticancer activities (Maugeri et al. 2022). However, tannins adversely affect nutritional by reducing the absorption of feed nutrients by animals (Duraiswamy et al. 2022). Fig. 6D shows that the number of highly expressed metabolites of tannins in cultivated PE is much lower than that in wild PE, indicating that although wild PE has higher medicinal value, cultivated PE is more suitable for utilization and development as a feed additive. The composition and abundance of amino acids, organic acids, alkaloids, and lipids are important indicators affecting taste (Zou et al. 2020). The results in Fig. 6D show that cultivated PE has a greater variety of metabolites affecting taste than wild PE. This may explain why cultivated PE tastes better than wild PE. Data of the detected metabolites indicates that both cultivated and wild PE are highly enriched in L-ascorbic acid (Vitamin C), with the abundance being greater in cultivated PE than that in wild PE (Supplementary Table 3). Due to the inability of humans and birds to synthesize Vitamin C on their own, which can only be obtained through food (Lykkesfeldt and Carr 2024), and the lack of Vitamin C can lead to the occurrence of various diseases (Maity et al. 2024; Jara et al. 2022), cultivated PE as a donor of Vitamin C has high utilization value. Therefore, considering all the above factors, cultivated PE has higher utilization value as a feed additive compared to wild PE.

Due to the crude juicing process of PE, there is a large amount of liquid in the PE pomace. The preliminary pre-experiment detection of this study confirmed that the PE pomace contains 67 % moisture (data not shown). Since PE contains a large number of volatile metabolites and water-soluble metabolites, we believe that when the PE pomace is directly fed to poultry as a feed additive, the liquid components in the pomace still have the same function as the PEJ. Due to the high organic acid level and high moisture content in the PE pomace, it is prone to spoilage and deterioration in summer, and it is extremely easy to have material conversion during storage (Li et al. 2023). In the future utilization of PE pomace as feed, we will use wheat bran or rice bran as a water-retaining material, in order to preserve the PE pomace for a longer time by means of anaerobic fermentation without affecting its medicinal value.

5. Conclusion

In this study, we successfully conducted RNA-Seq to analyze the transcriptional profile regulation similarities and differences in HD11 cells by two PEs. Metabolic analysis based on LC-MS/MS systematically compared the metabolic differences between wild and cultivated PEs. Both cultivated PE and wild PE inhibit the host immune response and regulate cell proliferation. However, wild PE more strongly regulates the host immune response than cultured PE, while cultured PE has a stronger ability to regulate cell proliferation and better palatability than wild PE. Functional differences may be caused by differences in the composition of flavonoids, lignans, coumarins, phenolic acids, and tannins, while taste differences may be caused by amino acids, alkaloids, organic acids, and lipids. In conclusion, cultivated PE pomace has high development and utilization value as a feed additive.

6. Ethics statement

Ethics approval was not required for this research.

CRediT authorship contribution statement

Chenggang Liu: Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Jin jin:** Investigation, Project administration, Validation. **Binyi Sun:** Visualization, Software, Data curation.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2024.100223.

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