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Preventive regulation of cellular heat stress injury by mung bean polyphenols revealed by multi-omics analysis

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Based on the Caco-2 cell heat stress model, the study explored the heat stress preventive regulatory mechanisms of key polyphenol fractions in mung bean by metabolomics and transcriptomics association analysis. Results Mung bean polyphenol intervention before heat stress significantly reduced the elevated expression level of heat shock protein 70 (HSP70) caused by 39 °C temperature. At the metabolic level, mung bean polyphenols could play a role in heat stress regulation by alleviating oxidative stress damage. At the gene level, mung bean polyphenols showed regulation of cell proliferation, differentiation, and DNA damage, with DUSP6 and NEURL3 as key regulatory genes. The correlation analysis showed that nucleotide metabolism, and oxidative phosphorylation metabolism were the key pathways in the regulation of mung bean polyphenols by heat stress. Then mung bean polyphenols can exert heat stress preventive activity through the regulation of cellular oxidative damage and energy metabolism. This study provides a good idea for the research and development of dietary intervention products for heat stress.

Heat is an important type of environmental stress. Heat stress is defined as the sum of non-specific responses of humans and animals to stimulation by excessively high temperatures that exceed their ability to regulate body temperature. Heatstroke is one of the main clinical manifestations of heat stress[1]. It is a consequence of the combined effects of environment and metabolism[2]. Heat stroke involves a complex series of processes such as dehydration, electrolyte disturbances, protein denaturation, and endothelial cell damage, which can cause damage to vital organs through nitrosative stress, oxidative stress, and endotoxemia[3]. The gut is an important digestive and immune organ, and dysfunction of the intestinal epithelial barrier plays an important role in the pathophysiology of heatstroke[4]. Protecting against heat stress-induced damage to the intestinal mucosal barrier is a treatment strategy for patients with heatstroke[5]. Mild cases of heatstroke can raise body temperature to 39 °C, with severe cases 41 °C reaching 42 °C, characterized by rapid progression and a high mortality rate[6,7]. The global threat of heat stress has raised concerns regarding heat stroke severity and incidence[8]. The impact of heat stress are expected to worsen in the future. Therefore, minimizing its effects has become a focus of scientific research today.

Recent studies have found that prevention strategies are more effective than currently available treatment strategies[4]. Dietary intervention is a potentially effective measure for preventing heat stress and intestinal injury[9–12]. In recent years, polyphenols have become the focus of research on heat stress regulation. Further, polyphenolic compounds, such as ferulic acid, chlorogenic acid, tea polyphenols, and resveratrol, can ameliorate heat-induced barrier disruption in livestock and poultry[13–16], yet research on the regulatory mechanisms of active compounds is still in its infancy.The mung bean (*Vigna radiata* L.) is a traditional Chinese legume used for food and medicine. Mung bean soup relieves summer heat, making mung beans a preferred ingredient for heat stress dietary intervention. Polyphenolic antioxidants have been proposed as the active ingredients responsible for the heat-protective effect of mung bean. Zingiberin and iso-zingiberin have also been proposed as heat stress-regulating components of mung beans. After cooking and processing, a large amount of polyphenols in the skin can be transferred to the soup. After feeding Mung bean soup orally to heat stressed rats and mice, the plasma oxidative stress level of heat stress model rats can be reduced, and the content of heat stress protein HSP70, glutathione, and immunoglobulin IgG in blood of mice can be significantly increased. These indexes showed significant correlation with the protein, flavonoids, polyphenols, and other groups in mung bean[17,18]. In our previous study, purified mung bean polyphenol was fed to Balb/c mice with heat stress, and it was found that the intervention of mung bean polyphenol diet before heat stress could improve the anal temperature rise and weight loss caused by heat stress at 41 °C. Significantly improved sweating and abnormal behavior during heat stress. Through the detection of serum IL-1β, TNF-α, GSH-Px, MDA, T-AOC, and cortisol, it was found that the intervention of mung bean polyphenol can significantly alleviate the inflammatory response and

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oxidative stress damage caused by heat stress. The results of HE staining in the intestinal sections of HS group mice showed goblet cells in the small intestine and mononuclear inflammatory cell hyperplasia in lamina propria. In the mung bean polyphenol group, the intestinal structure was intact, the intestinal villous mucosa epithelium was arranged neatly, and no inflammatory reaction was observed in the mucous lamina propria. The immune function damage of mice was effectively alleviated.

At the same time, vitexin, orientin, and caffeic acid were selected as key polyphenol components in mung bean based on content, in vitro antioxidant index, and regulation ability of heat shock protein 70 (HSP70). However, the mechanism of regulation of heat stress in mung bean remains unclear. Based on the above research basis, the preventive mechanism of the protective effect of key polyphenol components of mung bean on intestinal cells under heat stress was comprehensively discussed by using the 39 °C heat stress model of Caco-2 cells and the means of metabolomics and transcriptomic association analysis. The internal reason of mung bean polyphenols' heat-relieving effect was preliminarily revealed, and the theoretical reference was provided for the mechanism analysis of human dietary polyphenols' heat-stress regulation.

Results

HSP70 protein expression after heat stress and mung bean polyphenol modulation

HSP70 protein expression in Caco-2 cells after heat stress at 39 °C and treatment with mung bean polyphenols and positive drugs was investigated using westernblot experiments, and the results are shown in Fig. 1A (The uncropped raw western blot images can be found in Supplementary Fig. 1 for verification). The electrophoretic bands in the 39 °C HS group deepened in color compared with CON, and the addition of mung bean polyphenols and the positive drug treatment lightened the color of the bands, suggesting that both could alleviate the expression of HSP70 protein caused by heat stress, i.e., the two substances have a regulatory effect on heat stress.

Analysis of metabolomic effects of heat stress and mung bean polyphenol modulation on Caco-2 cells

Metabolite classification and PCA and PLS-DA analysis. A total of 631 metabolites were detected in the positive and negative ion modes for the four groups of samples, and the classification results of the Human Metabolism Database (HMDB, https://hmdb.ca) (Fig. 1B) revealed a large number of lipids and lipid-like molecules, in addition to organic acids and their derivatives, organic heterocyclic compounds, benzene ring-type compounds, nucleosides, nucleotides and analogs, organic oxides, and organic nitrogen compounds. Figure 1C shows the scatter score plots of PCA and PLS-DA, from which it can be seen that all the samples are within the 95% confidence interval. Each sample group had its own clustering area with a significant differentiation effect, suggesting that heat stress, mung bean polyphenol treatment, and positive drug treatment all had an effect on Caco-2 cell metabolism.

Screening for Differential Metabolites. Differential metabolite screening conditions were VIP > 1 (VIP value is the projection of variable significance obtained by OPLS-DA in the comparative analysis of the two groups, the larger the VIP value indicates that the more significant the difference is, and the more reliable the differential metabolite obtained by screening is), $p \le 0.05$. The CON-39 °C HS group, 39 °C HS -39 °C DF group, and 39 °C HS -39 °C HX group were screened for 129 (mainly nucleosides, nucleotides and analogs, lipid and lipid-like molecules, organic acids and their derivatives, and organic heterocyclic compounds), 121 (mainly nucleosides, nucleotides and analogs, organic acids and their derivatives, organic heterocyclic compounds, lipid and lipid-like molecules), 83 (mainly lipid and lipid-like molecules, benzene ring-type compounds, organic acids, and their derivatives) differential metabolites, respectively. Detailed information is provided in Supplementary Tables 1–3. As shown in the volcano plot of differentially abundant metabolites (Fig. 2A), these were mostly downregulated under heat stress and drug treatment, while mostly upregulated after mung bean polyphenol treatment. Hierarchical clustering analysis (Fig. 2B) revealed that the number of differentially abundant metabolites overlapping between the heat stress and polyphenol treatment conditions was 66, all being upregulated after heat stress and downregulated back to normal levels after mung bean polyphenol treatment. The number of overlapping differentially abundant metabolites between heat stress and drug treatment was 11, including both upregulated and downregulated ones. This suggests large differences in the metabolic regulation elicited by mung bean polyphenols and the positive control drug. Five overlapping differentially abundant metabolites, namely L-Carnitine, Guanosine, Magnolol, LysoPE (0:0/16:1(9Z)), and Honokiol, were found to be key metabolites involved in heat stress regulation.

KEGG functional pathway and pathway enrichment analysis. Functional pathway analysis of the differential metabolites revealed that differential metabolites during heat stress, mung bean polyphenols, and positive drug modulation were all predominantly enriched in metabolism, organismal systems, and human disease-related pathways. The enrichment analysis of the pathways showed that 20 significantly changed key pathways were enriched in the 39 °C HS-CON group, 39 °C HS-39 °C DF group, & 39 °C HS-39 °C HX group (Fig. 3), and the corresponding statistics are shown in Table 1. The primary pathways involved included metabolism, human diseases, organismal systems, and environmental information processing. Secondary pathways included signal transduction, nucleotide metabolism, cancer, sensory system, endocrine system, sensory system, substance dependence, cardiovascular disease, and neurodegenerative disease. In addition, pathways from the heat stress condition included drug resistance: antitumor drugs, aging, environmental adaptation, and digestion. Mung bean polyphenol regulation processes also included signaling molecules and interactions, energy metabolism, excretory system, and amino acid metabolism. The positive control drug regulation involved lipid metabolism, cell growth and death, infectious diseases: viral, infectious diseases: bacterial, glycine biosynthesis and metabolism.

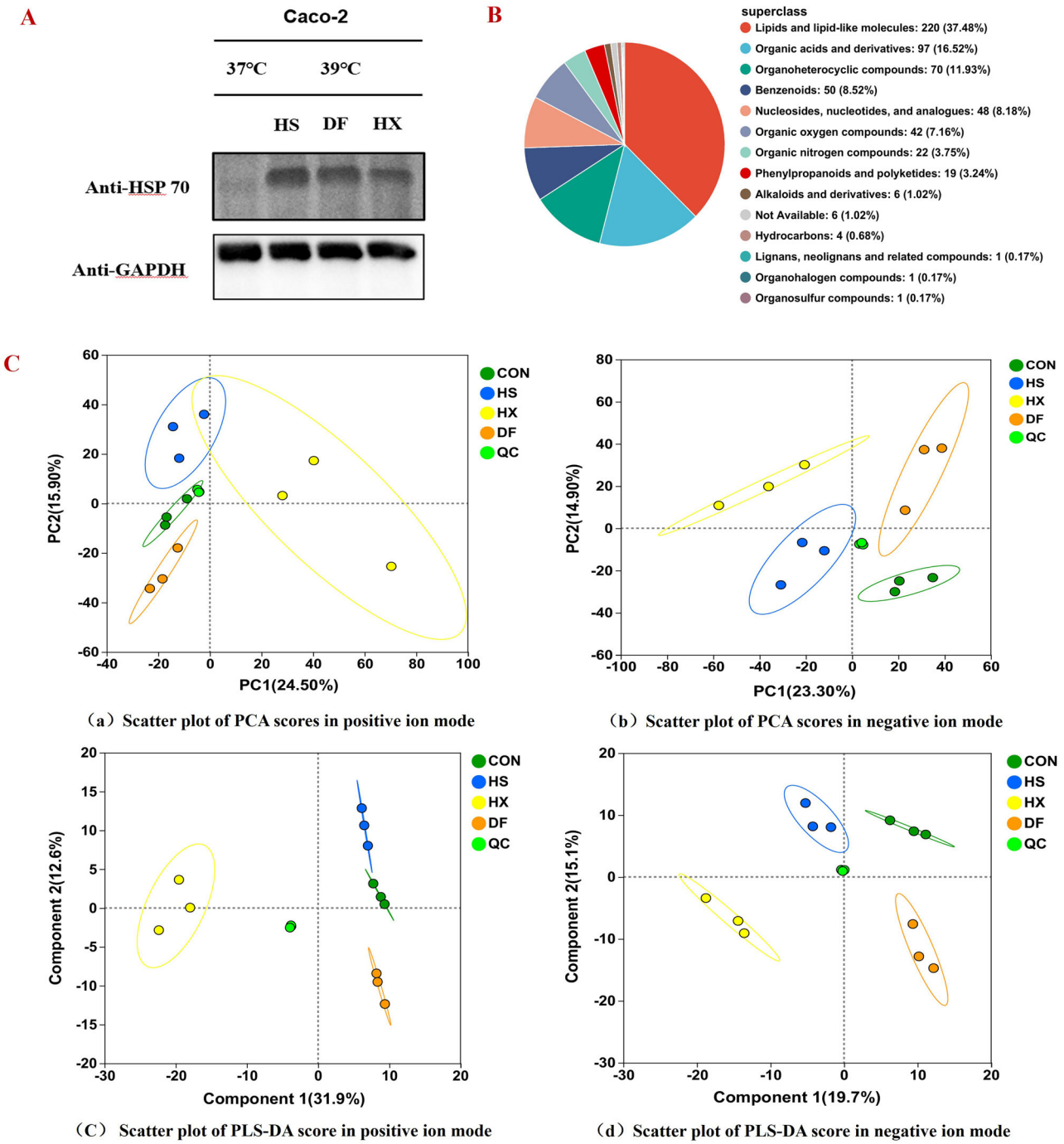
The common pathway among our three experimental groups was Diabetic cardiomyopathy (DC). However, there were differences in the metabolites involved and their regulation between the three groups. The metabolites involved in this pathway during heat stress were L-Carnitine, Adenosine triphosphate, Acetyl-CoA, Uridine diphosphate-N-acetylglucosamine, and NADPH, all of which were significantly upregulated. Metabolites involved in the mung bean polyphenol treatment-regulated processes included L-carnitine, oxidized glutathione, nicotinamide adenine dinucleotide phosphate (NADP +), uridine diphosphate-N-acetylglucosamine, and NADPH, whose levels were downregulated. Metabolites involved in of the processes regulated by positive control drug treatment were L-Carnitine (downregulated) and Sorbitol (upregulated). L-Carnitine is a key metabolite in the regulation of heat stress. In addition, cGMP-PKG signaling pathway, Nucleotide metabolism, Purine metabolism, Biosynthesis of cofactors, Taste transduction, Renin secretion, Aldosterone synthesis and secretion, and Aldosterone synthesis and secretion were also shared by the heat stress group and the mung bean polyphenol-treated group. Biosynthesis of cofactors, Taste transduction, Renin secretion, Aldosterone synthesis and secretion, Central carbon metabolism in cancer, and glucagon signaling pathway. The heat stress group shared two pathways with the positive drug group, namely, the FoxO signaling pathway and ABC transporters.

Effects of heat stress and mung bean polyphenol modulation on the Caco-2 cell transcriptome

A total of 97.64 Gb clean data were obtained, reaching more than 6.96 Gb per sample. The percentage of Q30 bases was above 91.44%. The clean reads of each sample were compared with the designated reference genome, with matching rates ranging from 95.2% to 96.81%. A total of 35,407 genes were detected, including 35,081 known genes and 326 new genes. A total of 181,780 expressed transcripts were detected, including 162,492 known and 19,288 new transcripts. Differential gene analysis was performed to obtain the genes that were differentially expressed between the two groups, and the screening thresholds were: |log2FC| >=1 and $P < 0.05$. A total of 26, 418,

and 348 genes exhibited significant differential expression after 39 °C heat stress, mung bean polyphenol, and positive drug modulation, respectively (Fig. 4A).

Principal Component Analysis and Functional Annotation of Differentially Expressed Genes. Figure 4B shows a plot of the PCA scatter scores for the four sample groups, all samples were within 95% confidence intervals, all had their own aggregation zones, and all four groups of samples were relatively distant from each other, with a significant differentiation effect. This suggested that heat stress, polyphenol treatment, and positive control drug treatment caused changes in Caco-2 cell gene expression, as shown in a Venn diagram (Fig. 4C). Two differentially expressed genes were shared after heat stress modulation with mung bean polyphenols and positive control drug treatment, namely dual-specificity phosphatase 6 (DUSP6) and neuralised E3 ubiquitin protein ligase 3 (NEURL3). DUSP6 was upregulated under both conditions, while NEURL3 was down-regulated by both, highlighting their roles as key genes in the response to heat regulation by polyphenols and control drug treatment.



（a）Scatter plot of PCA scores in positive ion mode

（b）Scatter plot of PCA scores in negative ion mode

（C） Scatter plot of PLS-DA score in positive ion mode

（d）Scatter plot of PLS-DA score in negative ion mode

Fig. 1 | Electrophoretic plots of HSP70 protein expression under heat stress, mung bean polyphenol modulation, and positive drug modulation treatments in Caco-2 cells, scatter score plots of PCA and PLS-DA. (1) $R^2X = 0.649$ in PCA positive ion mode and $R^2X = 0.615$ in negative ion mode. The model parameters in PLS-DA negative ion mode were $R^2X = 0.545$, $R^2Y = 0.723$, and $Q^2 = 6.231$, and the model parameters in positive ion mode were $R^2X = 0.764$, $R^2Y = 0.73$, and $Q^2 = 0.752$, which indicates that the model does not overfit. **A** Western blot analysis of heat shock protein (HSP70) expression; **B** Pie chart of metabolite classification; **C** PCA and OPLS-DA score scatter plots under positive/negative ion modes for four sample groups.

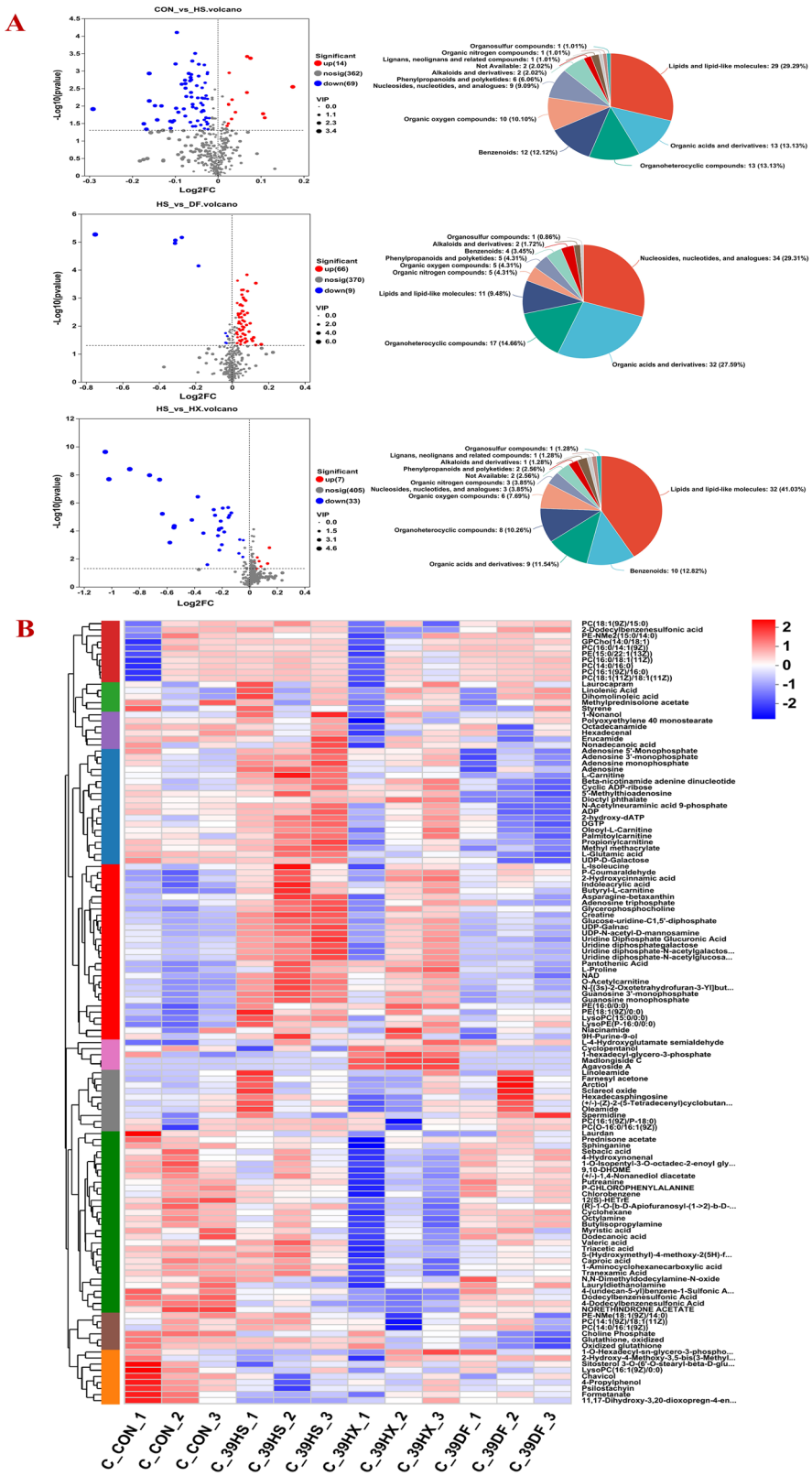


Fig. 2 | Volcano plots of differential metabolite regulation, and hierarchical clustering analysis heatmaps. A The horizontal axis represents the fold change in metabolite expression between two groups, and the vertical axis represents the statistical test value for the difference in metabolite expression levels. Each point in the graph represents a specific metabolite, and the size of the point represents the Vip value. The red dots represent significantly upregulated metabolites, the blue dots represent significantly downregulated metabolites, and the gray dots represent non significantly differentially expressed metabolites. The corresponding data can be found in the detailed table of differences. **B** Each column in the figure represents a sample, each row represents a metabolite, and the color in the figure indicates the relative level of the metabolite in the group of samples; the specific trend of level change can be seen in the number label under the color bar at the bottom right. The left side is the dendrogram of metabolite clustering, the right side is the name of the metabolites, the closer the two metabolite branches are, the closer their abundances are; the upper side is the dendrogram of sample clustering, and the lower side is the name of the samples. The closer the two sample branches are, the closer the abundance of all the metabolites in the two samples are, that is, the closer the trend of the metabolite abundance is.

GO functional enrichment revealed a predominance of biological process terms, followed by cellular components and relatively few molecular functions enriched under our experimental conditions (Fig. 5A). The biological process terms were all related to biological regulation, metabolic processes, multi-organism processes, movement, reproductive processes, organization of cellular components or biogenesis, biological adhesion, signalingbehavior, signaling, response to stimuli, and others. In contrast, enriched terms in the polyphenols and positive drug-regulated processes also included cell aggregation, cell population proliferation, development, and detoxification. Enriched cellular components included membrane-enclosed compartments, protein-containing complexes, membrane parts, extracellular parts, organelle parts, cell junctions, supramolecular

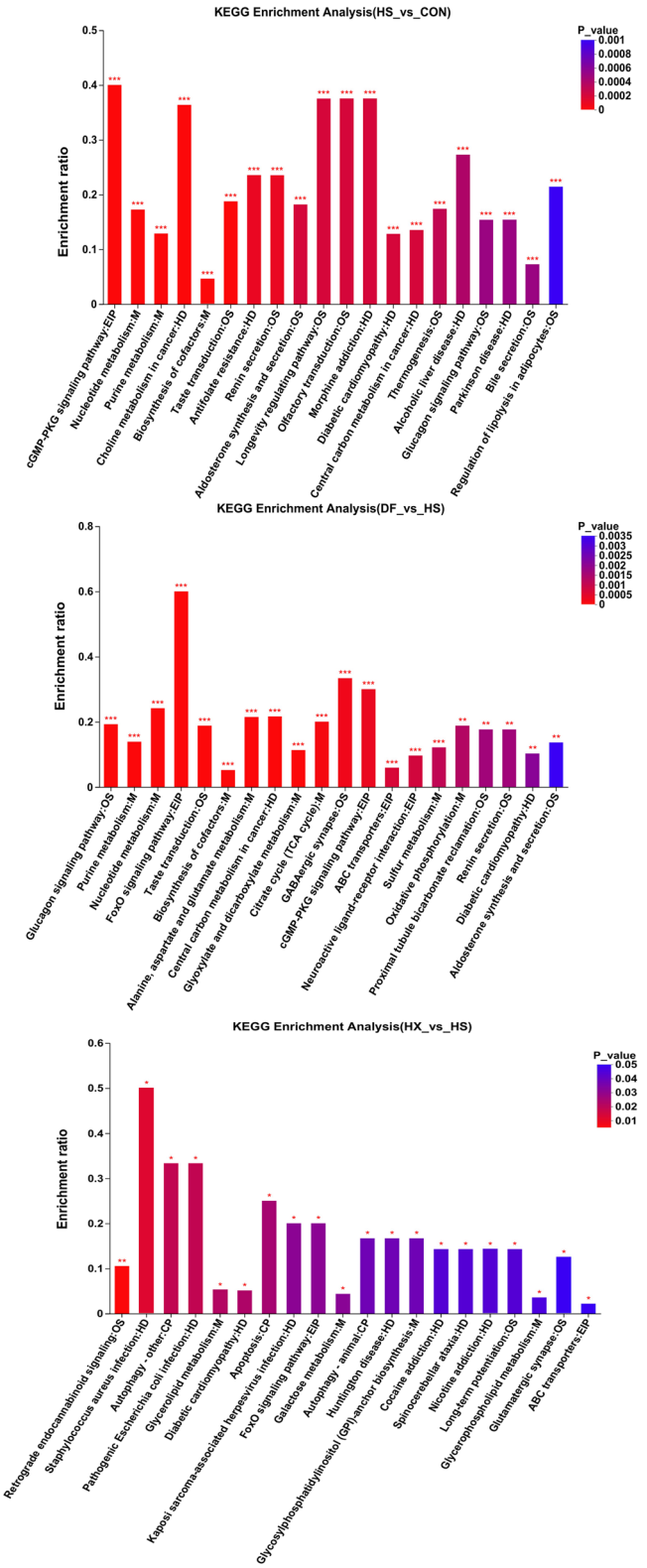


Fig. 3 | Pathway enrichment analysis of differentially abundant metabolites. The horizontal axis represents the pathway name, and the vertical axis represents the enrichment rate. It represents the ratio of the number of metabolites enriched in the pathway (metabolite number) to the number of metabolites annotated in the pathway (background number). The larger the ratio, the greater the degree of enrichment. The gradient of column colors represents the significance of enrichment, with darker default colors indicating more significant enrichment of the KEGG term. Items with *P* value or FDR < 0.001 are marked as ***, items with *P* value or FDR < 0.01 are marked as **, and items with *P* value or FDR < 0.05 are marked as *.

complexes, etc., with two types of substance regulation include synapses and nucleoid substances. Molecular functions included transcriptional regulation, structural molecular activity, transporter activity, modulators of molecular function, molecular transducer activity, binding, and catalytic activity. Heat stress-enriched processes also included translational regulator activity and protein folding chaperones. Polyphenol and positive control drug regulation also included receptor activity, molecular carrier activity, and antioxidant activity. These findings suggested that heat stress, mung bean polyphenols, and the positive control altered gene expression, thus regulating these processes, with the key altered genes shown in Supplementary Tables 4–6. KEGG enrichment analysis revealed that differentially expressed proteins in the heat stress group were mostly involved in human diseases and organismal system processes, while those in both the mung bean polyphenols and positive control drug group were mostly involved metabolism and human diseases (Fig. 5B). In addition, genes regulated by mung bean polyphenols were also involved in environmental information processing. The metabolic processes involved in heat stress, mung bean polyphenols, and positive drug differential genes included carbohydrate metabolism, environmental information, signal transduction, signaling molecules, and interactions.

GO and KEGG pathway enrichment analysis of differentially expressed genes. GO enrichment analysis of all differentially expressed genes revealed the 20 most significant pathways, shown in Fig. 6A. That there were no significantly enriched pathways based on differential expression after heat stress at 39 °C, but the affected pathways were all biological process-type pathways, including the cell's response to heat, to transforming growth factor β stimulation, to chemical stress, to growth factors, and stress to acidic pH. Enriched pathways under mung bean polyphenol treatment were involved in biological processes such as cellular signaling by Wnt, lymphatic vessel morphogenesis, reproductive developmental processes, development of lung epithelium, development of renal epithelium, renal tubules, and renal morphology, polycystic protein complex, and calcium-independent cell-matrix adhesion in cellular composition. Enriched pathways in the positive control drug group were mainly involved in flavonoid metabolic processes, energy metabolism, nuclear accounting transcriptional regulation, and other related pathways.

The 20 most significantly enriched KEGG pathways were displayed to identify the signal transduction pathways involving the differentially expressed genes between experimental groups (Fig. 6B). Pathway enrichment data is also summarized in Table 2. There was no significantly enriched pathway for differentially expressed genes in the heat stress mung bean polyphenol group. However, pathways with greater relative after heat stress at 39 °C included MAPK(mitogen-activated protein kinase), AGE-RAGE, estrogen and relaxin signaling pathways, in addition to other pathways involved in cell growth and proliferation, lipid regulation, protein processing in the endoplasmic reticulum, inflammation, and endocrine-related pathways. Differentially expressed genes in the mung bean polyphenols group were mainly involved in signal transduction pathways, such as the Rap1, cAMP, calcium signaling pathways, immune and disease-related pathways, as well as oxidative phosphorylation, pyrimidine metabolism, endocrine and hormone synthesis, and secretion-related pathways. In the positive control drug group, there were 13 enriched pathway terms, including IL-17, p53 signaling pathway, pathways involved in the metabolic conversion of saccharides, metabolism of exogenous substances by cytochrome P450, and metabolism of phenylalanine. Heat stress and mung bean polyphenol heat stress shared three key pathways: endocrine, growth hormone synthesis, secretion, and action as well as leukocyte migration across the endothelium.

Metabolomics-transcriptomics association analysis

O2PLS analysis and KEGG pathway enrichment. The structure of the O2PLS analysis is shown in Fig. 7A. It can be seen that the sample points of

Table 1 | Pathway enrichment analysis results for differentially abundant metabolites in Caco-2 cells

Serial number	Primary access	Secondary access	Pathway description	*P* value	Number of key metabolites
1	Environmental Information Processing	Signal Transduction	cGMP-PKG signaling pathway	0	4
2	Metabolism	Global and overview diagrams	Nucleotide Metabolism	0	10
3	Metabolism	Nucleotide Metabolism	Purine Metabolism	0	13
4	Human Diseases	Cancer: Overview	Choline metabolism in cancer	0	4
5	Metabolism	Global and Overview Charts	Cofactor biosynthesis	0	15
6	Organismal Systems	Sensory Systems	Taste transduction	0	6
7	Human Diseases	Drug Resistance: Antitumor Agents	Antifolate Resistance	0.0001	4
8	Organismal Systems	Endocrine System	Renin secretion	0.0001	4
9	Organismal Systems	Endocrine System	Aldosterone synthesis and secretion	0.0002	4
10	Living organism system	Aging	Lifespan regulatory pathways	0.0002	3
11	Organism system	Sensory system	Olfactory conduction	0.0002	3
12	Human Diseases	Substance dependence	Morphine addiction	0.0002	3
13	Human Disease	Cardiovascular Disease	Diabetic cardiomyopathy	0.0002	5
14	Human Disease	Cancer: An Overview	Central carbon metabolism in cancer	0.0002	5
15	Organism system	Environmental Adaptation	Thermogenesis	0.0003	4
16	Human Diseases	Endocrine Metabolic Diseases	Alcoholic Liver Disease	0.0004	3
17	Organism System	Endocrine System	Glucagon signaling pathway	0.0005	4
18	Human Diseases	Neurodegenerative Diseases	Parkinson's Disease	0.0005	4
19	Organism system	Digestive System	Bile secretion	0.0005	7
20	Organism system	Endocrine System	Regulation of adipocyte lipolysis	0.001	3

*P*_value is the *p* value, which represents whether the enriched result is statistically significant or not, the smaller the *p* value, the more statistically significant it is, and generally the *p* value is less than 0.05 to consider the function as a significant enrichment term.

the metabolic and gene sets exhibited the same trend of change, indicating a strong association. Figure 7B shows the annotated Venn diagrams and statistical graphs of KEGG pathways. These show the pathways shared by the heat stress, mung bean polyphenol treatment, and positive control drug treatment groups were 53, 56, and 38, respectively (For detailed information, please refer to Supplementary Tables 7–9).

In addition to the classification of pathways, it is clear that heat stress affects pathways of organismal systems, metabolism, human diseases, genetic information processing, and cellular processes, whereas mung bean polyphenol treatment affected the organismal systems, metabolism, human diseases, environmental information processing. Positive control drug treatment affected pathways of metabolism, human diseases, and environmental information processing.

The KEGG pathway enrichment Venn diagram and statistical graph are shown in Fig. 8. The pathway information is shown in Table 3. 39 °C heat stress caused significant changes in both the metabolic and transcriptional sets ($p < 0.05$) in one pathway, which was the longevity regulating pathway-multiple species, and there were two differentially expressed genes, HSPA1L and HSPA1A, and one differentially abundant metabolite, cAMP. The other pathways in which both metabolic and transcriptional profiles were significantly altered ($p < 0.05$) were the estrogen signaling pathway, MAPK signaling pathway, renin secretion, gustatory sensation, cGMP-PKG signalingsignaling pathway, MAPK signaling pathway, signalingrenin signaling pathway, signalingand cGMP-PKG signaling pathway. signalingsignalingThere were three pathways for which mung bean polyphenol treatment resulted in significant changes ($0.01 < p < 0.05$) in both the metabolic and transcriptional sets. These included pyrimidine metabolism, with four differentially expressed genes (*TYMP, NT5C, NME1-NME2, NME3*) and five differentially abundant metabolites (Uracil, L-Glutamine, Uridine-5'-Monophosphate, Uridine 5'-Diphosphate, and Uridine 5'-monophosphate). Another such pathway was nucleotide metabolism, with *TYMP, NT5C, NME1-NME2*, and *NME3* being differentially expressed and 18 differentially abundant metabolites (Guanine, Adenosine, Uracil, 5'-Guanylic Acid, Adenine, DGDP, L-Glutamine, Guanosine, N6-(1,2-Dicarboxyethyl)-AMP, Adenosine monophosphate, Adenylosuccinate, Adenosine 5'-Monophosphate, Uridine-5 '-Monophosphate, Uridine 5'-Diphosphate, Uridine 5'-monophosphate, ADP, Guanosine monophosphate, DGTP). Oxidative phosphorylation (OPP) was another example, with *COX5B, ATP5F1D, ATP6V0C, TCIRG1*, and *NDUFAB1* being differentially expressed, in addition to five differentially abundant metabolites (Beta-nicotinamide adenine dinucleotide, NAD, NAD +, Succinic Acid, and ADP). Detailed information on the differentially abundant metabolites and differentially expressed genes is presented in Table 3. Other pathways in which both metabolic and transcriptional sets were altered included glycosaminoglycan biosynthesis-acetylheparin/sulfate/heparin, purine metabolism, glyoxylate, and dicarboxylic acid metabolism, biosynthesis of cofactors, and the glucagon signaling pathway. In the positive control drug group. pathways with significant changes in both metabolic set and transcriptional set included Staphylococcus aureus infection, apoptosis, pathogenic Escherichia coli infection, galactose metabolism, Kaposi's sarcoma-associated herpesvirus infection, FoxO signaling pathway, glycerol metabolism, and diabetic cardiomyopathy, and retrograde endogenous cannabinoid signaling (Staphylococcus aureus infection、Apoptosis、Pathogenic Escherichia coli infection、Galactose metabolism、Kaposi sarcoma-associated herpesvirus infection、FoxO signaling pathway、Glycerolipid metabolism, and Diabetic cardiomyopathy and Retrograde endocannabinoid signaling).

Discussion

Through integrated analysis of HSP70 expression, metabolomics, and transcriptomics in a heat-stressed cellular model, we demonstrated that mung bean polyphenols possess preventive and regulatory activities against heat stress. Metabolomics analysis showed that different metabolites significantly altered by heat stress and mung bean polyphenols included

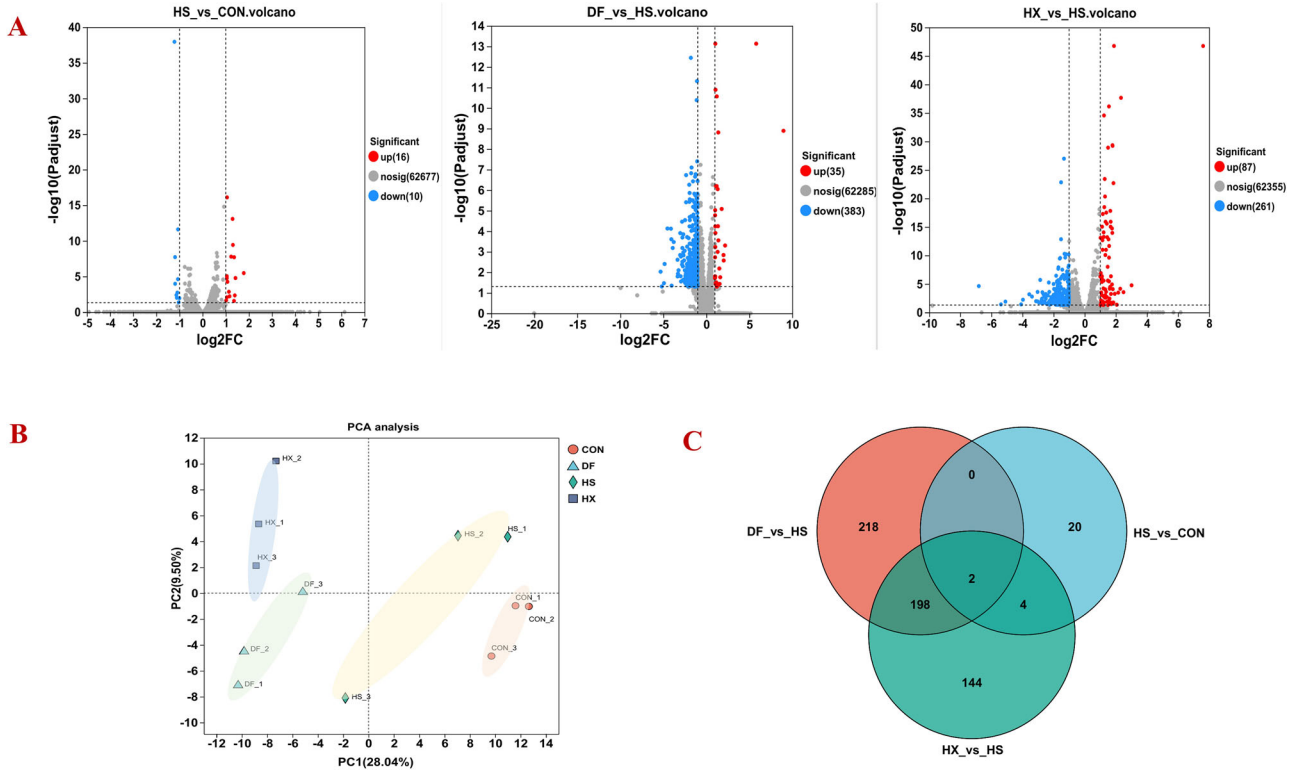


Fig. 4 | Differential gene expression in Caco-2 cells displayed via a PCA scatter score plot and Venn diagram. Circles of different colors in the Venn diagram represent different gene sets, and the numerical values represent the number of genes/transcripts that are shared and unique between different gene sets. **A** Volcano plot of differential metabolites across treatment groups; **B** PCA score scatter plot for simultaneous comparison of four sample groups; **C** Quantitative Venn diagram of differential metabolite counts among comparison groups.

nucleosides, nucleotides and analogs, lipids and lipid molecules, organic acids and their derivatives, and organic heterocyclic compounds. This reflects cellular defense mechanisms activated in response to damage to DNA, proteins, and membranes (lipids) by non-specific stressors[19]. From the differential metabolite abundance pattern, we observed that the positive control drug and mung bean polyphenols suppress heat stress process. Diabetic cardiomyopathy was a key metabolomic pathway for heat stress and polyphenol and positive drug modulation in Caco-2 cells. Studies have shown that this pathway is closely linked to cellular oxidative stress. Heat stress can lead to oxidative stress, which in turn causes cellular dysfunction and damage not only through protein oxidation, lipid peroxidation, DNA damage, and oxidative changes in microRNAs, but also through the activation of stress-sensitive pathways and redox regulation[20]. As indicated by the differentially abundant metabolite species and key pathways, mung bean polyphenols exhibited heat stress-modulating effects through alleviation of oxidative stress damage. This is in agreement with current reports showing that the modulation of intestinal heat stress by polyphenols is closely related to their antioxidant properties[15,21].

Our transcriptomic analyses revealed *DUSP6* and *NEURL3* as genes critical for the heat stress response and its regulation by polyphenols and the positive control drug. DUSP6 is an important member of the mitogen-activated protein kinase (MAPK) signaling pathway[22]. The MAPK pathway is highly conserved and involved in a variety of cellular activities, such as gene expression, growth, proliferation, differentiation, migration, development, inflammation, and apoptosis[23]. The extracellular signal-regulated kinase 1/2 (ERK1/2) pathway is a central regulator of cell proliferation that controls cell growth and cycle progression[24]. *DUSP6* is also an ERK1/2-specific dual phosphatase, and *DUSP6* interacts with ERK1/2 to indirectly control MAPK signaling, which is essential for cell proliferation and differentiation[25]. Currently, few studies have investigated the association between *DUSP6* and heat stress, which has a relevant regulatory role in inflammation, apoptosis, and neurological diseases[26,27]. *DUSP6* may affect cell proliferation and differentiation through the upregulation of its expression during heat stress and polyphenol heat stressUbiquitination is a type of stress protein. Ubiquitination is an important form of feedback in the adaptive program of eukaryotic cells to heat stress, targeting specific proteins associated with activities downregulated during heat stress, including nucleocytoplasmic transport and translation as well as stress granule components. Ubiquitination is necessary for the restoration of cellular activity and catabolism of stress particles, that is, it allows for cellular recovery after heat stress[28]. *NEURL3* is involved in the regulation of neuroinflammation and immune function, in addition to affecting mitochondrial function and a number of important intracellular physiological processes such as oxidative stress and apoptosis[29,30]. Studies on the role of *NEURL3* in heat stress and its regulatory mechanism are scarce. Based on the known functions of NEURL3 and our findings, we hypothesize that NEURL3 may mediate polyphenol-induced heat stress regulation by suppressing DNA damage and modulating nucleoplasmic processes.

The pathway that undergoes significant changes in both metabolic and transcriptional sets during heat stress is the Longevity regulating pathway-multiple species. Mitochondrial membrane potential measurements in Caco-2 cells at 39 °C showed that apoptosis increased from 0.57% in normal culture to 4.64%[31]. KEGG pathway enrichment revealed that down-regulation of heat shock protein genes affected cell viability. Further, we demonstrated that pyrimidine metabolism, nucleotide metabolism, and oxidative phosphorylation pathways were key differentially regulated pathways under polyphenol treatment. Pyrimidine and nucleotide metabolism were associated with the same four differentially expressed genes. The effect of heat stress on nucleotide metabolism was demonstrated in the 20th century, when heat stress was found to promote glycogen and phospholipid transport by stimulating RNA synthesis[32]. Nucleotide excision repair (NER) is the most common method used to repair DNA damage and

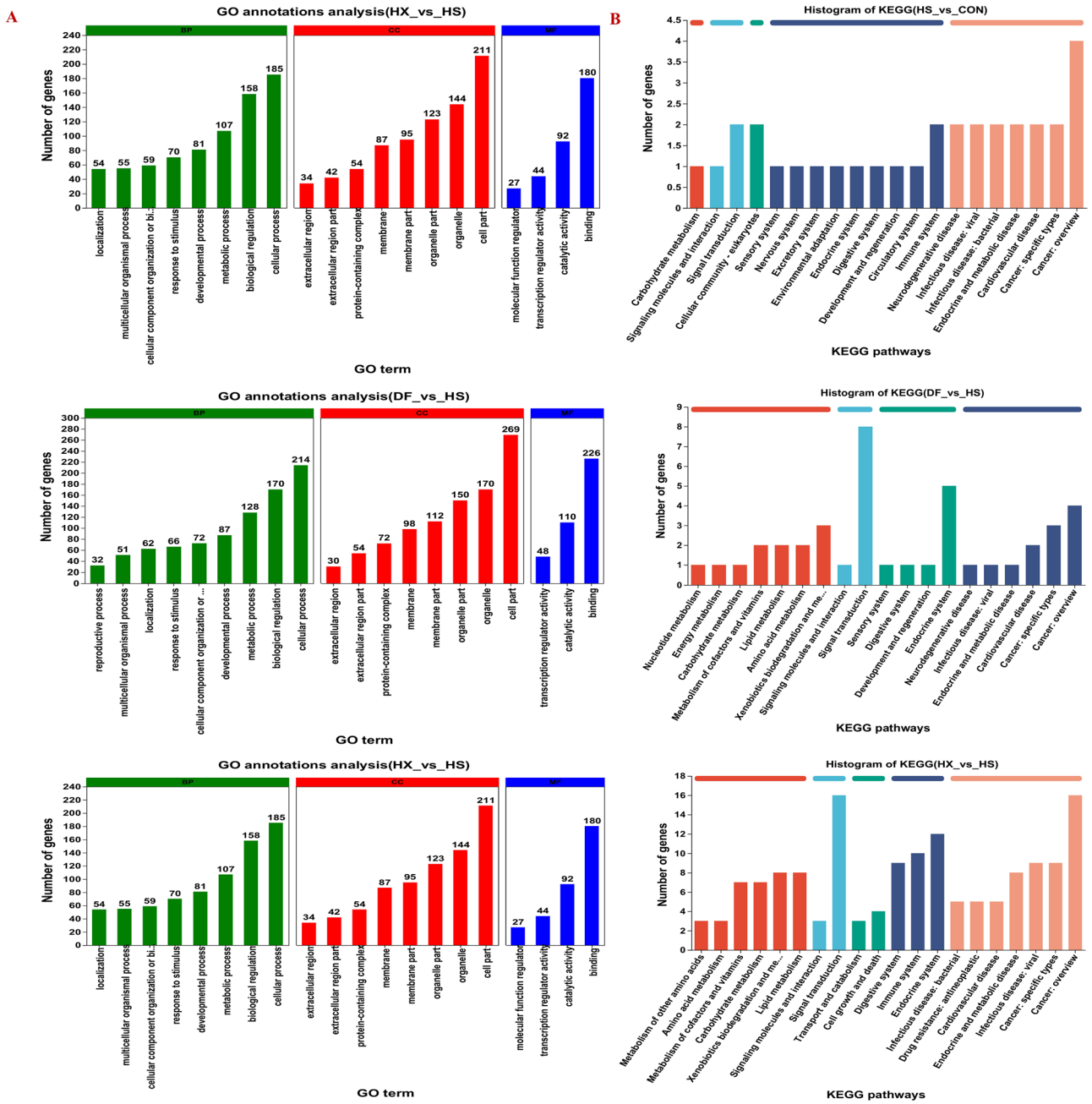


Fig. 5 | Differential gene expression in Caco-2 cells displayed via a functional annotation plot. Horizontal coordinates in the functional annotation diagram indicate the second-level classification terms of GO, vertical coordinates indicate the number of genes/transcripts in the second-level classification upon comparison, and the three colors indicate the three main classifications. **A** Bar chart of Gene Ontology (GO) functional enrichment analysis; **B** Bar chart of KEGG pathway enrichment analysis.

is inhibited by oxidative stress[33]. Dietary antioxidants significantly reduce oxidative DNA damage and prevent NER inhibition by oxidative stress[34]. Thus, mung bean polyphenols may exert heat stress-regulatory activity and promote the growth and repair of Caco-2 cells by decreasing oxidative DNA damage caused, thereby alleviating NER inhibition. Oxidative phosphorylation is a major pathway of cellular energy metabolism which provides direct energy for cellular activities through ATP production[35]. In addition to sugars and lipids, proteins (amino acids) and nucleic acids (nucleotides) can be oxidized to produce energy under certain physiological conditions. Almost all sugars, lipids, proteins, and nucleic acids can be converted into important and/or major oxidative energy-providing metabolites in the mitochondria[36]. It has also been shown that nucleotides can promote the rapid renewal of intestinal cells as well as tissue growth and repair[37], then the metabolism of nucleotide pathway and pyrimidine pathway may also be involved in the process of energy conversion, which, together with oxidative phosphorylation, provides energy for the repair following heat stress damage in Caco-2 cells subjected to treatment with mung bean polyphenols. That is, the heat stress-regulating activity of mung bean polyphenols is closely related to the regulation of energy metabolism.

Currently, most studies using dietary polyphenols for heat stress modulation have focused on livestock and poultry, and there are few studies on human dietary heat stress modulation. The use of polyphenols as active components in heat stress regulation is attributed to their antioxidant activity, but different polyphenols act via distinct mechanisms. Polyphenols have been shown to scavenge free radicals based on their chemical structure[38], thus eliminating oxidative damage

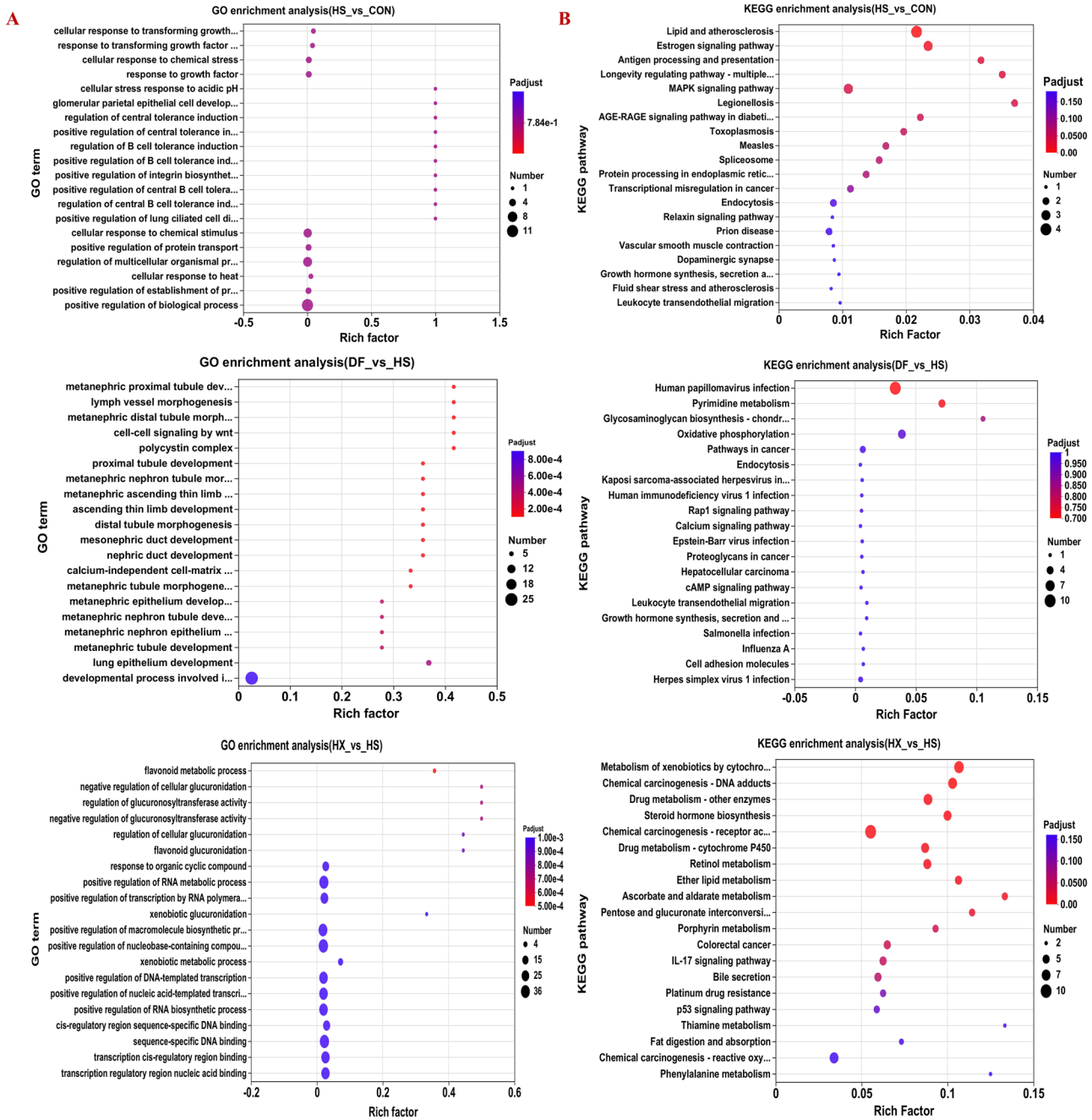


Fig. 6 | GO and KEGG enrichment analysis of differentially expressed genes. (1) GO enrichment analysis: The vertical axis represents the GO term, the horizontal axis represents the Rich factor (the ratio of the number of genes/transcripts enriched in the GO term to the number of annotated genes/transcripts [background number]; the larger the Rich factor, the greater the enrichment); the size of the dot indicates the number of genes/transcripts in the GO term, and the color of the dot corresponds to different Padjust ranges. The size of the dots indicates the number of genes/transcripts in the GO term, and the colors of the dots correspond to different Padjust ranges. (2) KEGG enrichment analysis: The vertical axis indicates the name of the KEGG pathway, the horizontal axis indicates the rich factor (the ratio of the number of genes/transcripts enriched in the KEGG pathway to the number of genes/transcripts in the annotated gene/transcripts [background number]), and the larger the rich factor, the greater the degree of enrichment]. The size of the dots indicates the number of genes/transcripts in the GO term. The size of the dots indicates the number of genes in the KEGG pathway, and the color of the dots corresponds to different Padjust ranges. **A** Bubble plot of Gene Ontology (GO) pathway enrichment analysis for intergroup comparison. **B** Bubble plot of KEGG pathway enrichment analysis for intergroup comparison.

caused by heat stress[39]. They have been also shown to block the action of enzymes that directly produce O_2^- (e.g., xanthine oxidase and protein kinase C)[40] or attenuate the effects of heat stress through the modulation of signaling pathways. For example, resveratrol, EGCG, and curcumin can inhibit NF-κB-driven transcription of inflammatory markers to cause inflammation[41–44], but also upregulate transcription factor Nrf2-mediated antioxidant enzymes and other antioxidant response pathways to stimulate the antioxidant enzyme system[45]. Polyphenols may also attenuate the effects of heat stress by regulating the expression of heat shock proteins that are directly involved in the immune response to heat stress injury[46]. A combined mechanism is also possible. For example, resveratrol can effectively scavenge ROS, regulate the activity of various antioxidant enzymes, reduce DNA damage, and significantly increase the expression level of various antioxidant enzymes and proteins[47]. In this study, association analysis of metabolite and transcript sets revealed that mung bean polyphenols

Table 2 | Results of transcriptomic significance pathway enrichment analysis in Caco-2 cells

Serial number	Primary access	Secondary access	Pathway description	*P* value	Number of key metabolites
1	Organism System	Endocrine System	Glucagon signaling pathway	0	5
2	Metabolism	Nucleotide Metabolism	Purine Metabolism	0	14
3	Metabolism	Global and overview diagrams	Nucleotide Metabolism	0	14
4	Environmental Information Processing	Signal Transduction	FoxO signaling pathway	0	3
5	Organismal Systems	Sensory System	Taste transduction	0	6
6	Metabolism	Global map and overview	Cofactor biosynthesis	0	17
7	Metabolism	Amino Acid Metabolism	Alanine, aspartate, and glutamate metabolism	0	6
8	Human Diseases	Cancer: Overview	Central carbon metabolism in cancer	0	8
9	Metabolism	Carbohydrate Metabolism	Glyoxalate and dicarboxylic acid metabolism	0	7
10	Metabolism	Carbohydrate Metabolism	Citric acid cycle (TCA cycle)	0.0001	4
11	Organismal Systems	Nervous System	GABAergic synapses	0.0002	3
12	Environmental Information Processing	Signal Transduction	cGMP-PKG signaling pathway	0.0003	3
13	Environmental Information Processing	Membrane Transport	ABC transporter proteins	0.0006	8
14	Environmental Information Processing	Signaling molecules and interactions	Neuroactive ligand-receptor interactions	0.0007	5
15	Metabolism	Energy Metabolism	Sulfur metabolism	0.001	4
16	Metabolism	Energy Metabolism	Oxidative phosphorylation	0.0012	3
17	Organismal systems	Excretory System	Proximal tubular bicarbonate recycling	0.0015	3
18	Organismal systems	Endocrine System	Renin secretion	0.0015	3
19	Human Disease	Cardiovascular Disease	Diabetic cardiomyopathy	0.0019	4
20	Organism system	Endocrine system	Aldosterone synthesis and secretion	0.0032	3

exert heat stress regulatory activity by reducing heat stress-induced oxidative DNA damage and regulating energy metabolism. There are some differences compared with previous studies.

The most important characteristic of polyphenols differs from other natural active substances in that they have synergistic activity, and there are differences in synergistic effects among different types of polyphenols[48]. The summer-relieving effect of mung bean is the characteristic that distinguishes it from other beans. In terms of the composition of monomer polyphenols, many kinds of beans contain the same monomer polyphenols, but only mung bean has the summer-relieving effect. We speculate that the active components of polyphenols in mung bean system may be the main substances that play the protective effect of heat stress, rather than the effect of single polyphenols. In other studies, vitexin and orientin were found to be common components of drugs with "heat-clearing" effect[49,50]. In the previous studies on the analysis of heat stress regulatory components of mung bean polyphenols, we found that the regulation effect of HSP70 mRNA level of vitexin, orientin, and caffeic acid was better than that of monomer polyphenols, which also confirmed our speculation. Therefore, through the development of this study, the regulation mechanism of heat stress is further explored from the perspective of multi-omics. At present, studies on the intervention of polyphenol diet in the regulation of heat stress mostly focus on the regulation effect of a single polyphenol on heat stress in livestock and poultry[51]. In recent years, there have been a few studies on the regulation effect of complex polyphenols, such as puerarin and hesperidin compound feeding[52]. In this study, the ratio of three polyphenols was based on their mass ratio in mung bean polyphenols, but the type, concentration and content of polyphenols significantly affected the difference in synergistic component efficacy, and there was a weak target effect between plant and human disease-related proteins, so the synergistic ratio of the three monomer polyphenols for optimal heat stress regulation is an important content worthy of discussion.

Regarding mechanistic insights, although key genes such as DUSP6 were identified through multi-omics correlation analysis, the upstream and downstream regulatory relationships of key pathways and key genes still need to be further confirmed by means of protein immunoblotting. Furthermore, as an in vitro cell-based study, addressing the intestinal microbiota-mediated biotransformation of polyphenols during digestion/absorption, along with clarifying the metabolic conversion and bioavailability of the three key mung bean polyphenols, would significantly enhance the translational relevance of dietary polyphenol interventions for heat stress regulation. Conducting in vivo validation studies using heat-stressed rodent models will constitute a primary focus of our subsequent research, particularly to assess systemic HSP70 modulation and gut-barrier function preservation.

Methods

Cells, instruments, and materials

Human colorectal adenocarcinoma Caco-2 cells were purchased from Bei Na Biotechnology Co. (Beijing, China). Vitexin, caffeic acid, Orientin from Shanghai Yuanye Biotechnology Co., LTD. (Shanghai, China). The following instruments were used: micropipettes (Sartorius, Germany); clean bench (SW-CJ-1FD; Layte, Nantong, China); CO_2 cell incubator (Thermo Fisher Scientific, WML); MF52-N inverted microscope (Guangzhou Mshot Optoelectronic Technology Co., Guangzhou, China); L3-5K low-speed centrifuge (Ke-Cheng Technology Co., Taiwan, China); HH-2water bath (Changzhou Aohua Instrument Co., Guangzhou, China); BioTek ELx800 enzyme marker (Turner BioSystems, Sunnyvale, CA); CytoFLEX flow cytometer (Beckman, Brea, CA); UHPLC-Q Exactive HF-X

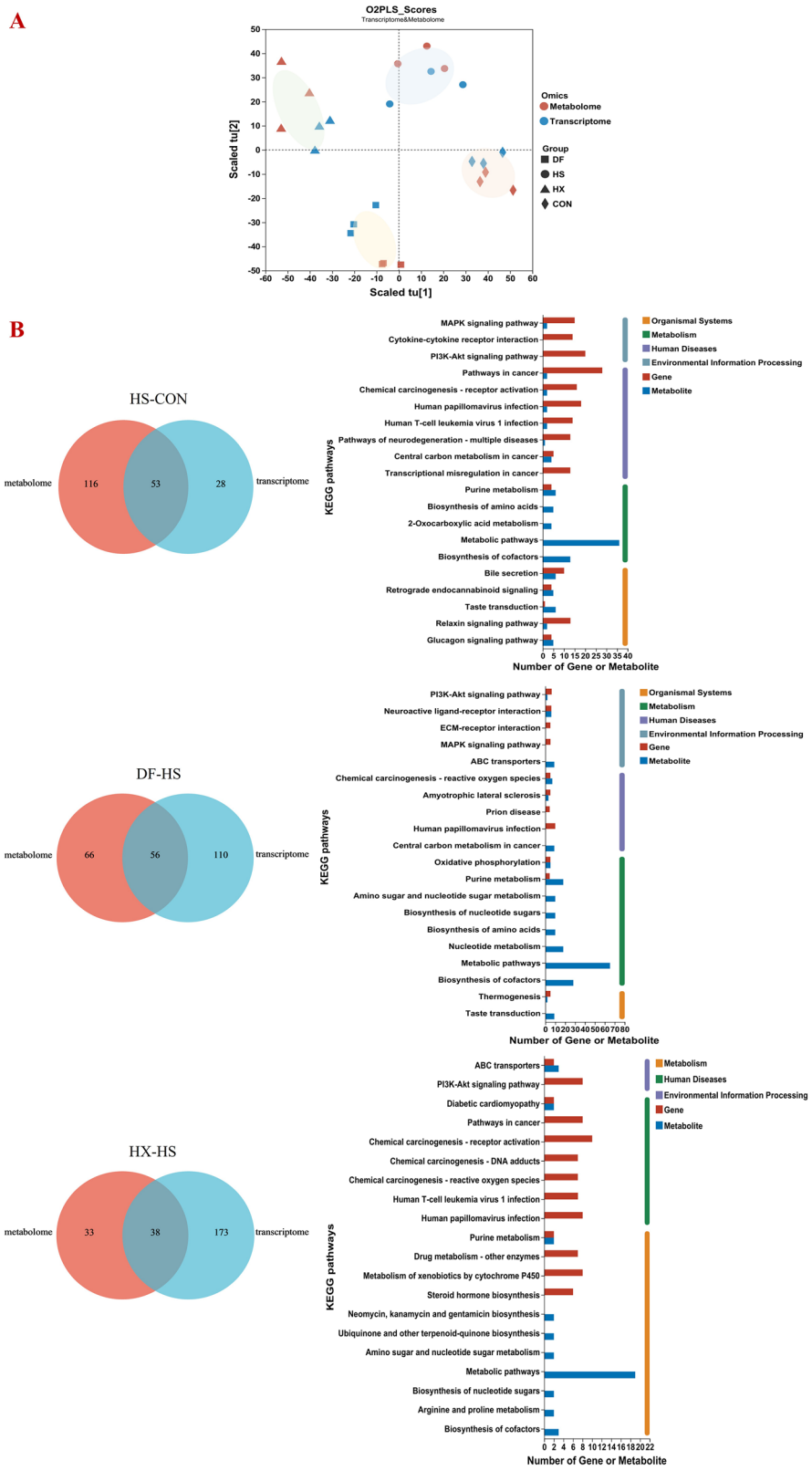


Fig. 7 | KEGG pathway enrichment analysis displayed via an annotated Venn diagram and annotation stats. Venn diagrams of enriched pathways annotated based on gene and metabolite sets. We focused on pathways in which the gene and metabolite sets were co-annotated. The horizontal coordinate of the graph represents the number of genes or metabolites in the pathway, where the blue bar represents the number of genes annotated to the pathway, the red bar represents the number of metabolites annotated to the pathway, and the vertical coordinate represents the KEGG pathway name. **A** O2PLS scatter plot for metabolite-gene set association analysis; **B** Venn diagram of KEGG pathway annotation counts and bar chart of functional enrichment.

ultraperformance liquid chromatography tandem Fourier transformed mass spectrometer (Thermo Fisher Scientific, Guangzhou, China); New-Classic MF MS105DU electronic balance (Mettler, Switzerland), GeneAmp® 9700 polymerase chain reaction (PCR) cycler (ABI, Austin); NovaSeq 6000 sequencer (Illumina, San Diego), and DYY-6C electrophoresis power supply (Beijing Liuyi Instrument Factory, Beijing, China).

The following materials were used: penicillin–streptomycin solution (100×); 0.25% trypsin solution (containing ethylenediaminetetraacetic acid dissolved in phosphate buffered saline [PBS]); Dulbecco's modified Eagle's medium (Wuhan Procell Life Sciences Co., Wuhan, China); anti-β-actin antibody (Beijing Boao Sen Biotechnology Co., Beijing, China); anti-HSP70 antibody (3A3) (Abcam, Cambridge, UK); horseradish peroxidase-labeled

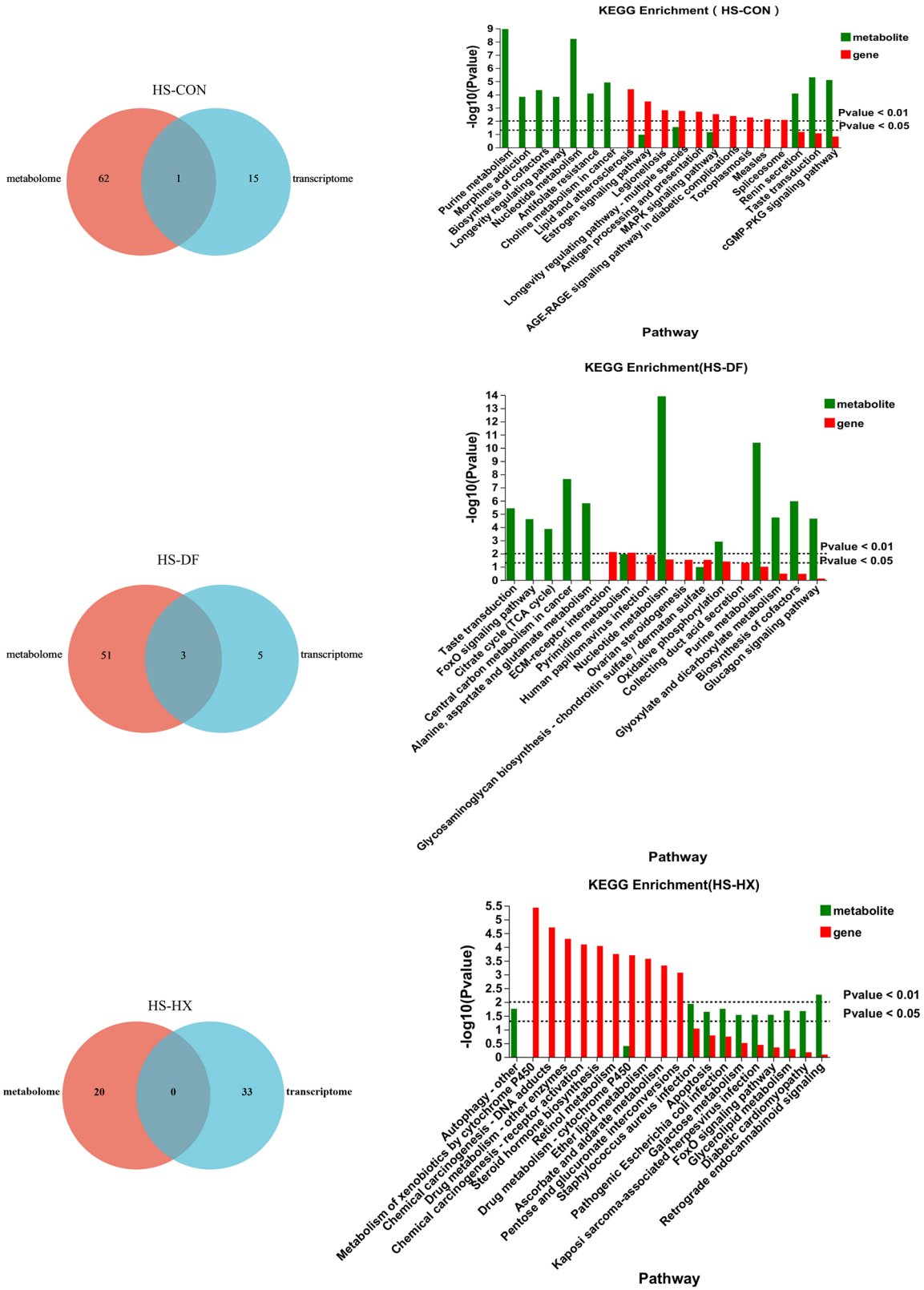


Fig. 8 | Venn diagram of KEGG pathway enrichment and statistics. Venn diagrams for pathways significantly enriched in the gene set (default $p < 0.05$) and the metabolic set (default $p < 0.05$). We focused on pathways significantly enriched in both gene and metabolic sets. The horizontal coordinate is the KEGG pathway, red bar is the result of gene enrichment, green bar is the result of metabolite annotation, and vertical coordinate is the negative logarithm of the enrichment significance *p*value with base 10; the smaller the *p*value, the more statistically significant it is, and generally $p < 0.05$ indicates a significantly enriched pathway.

Table 3 | KEGG pathway enrichment statistics for metabolomics and transcriptomics association analysis

Serial number	Primary access	Secondary access	Pathway description	*P* value	Number of key metabolites
1	Organism Systems	Nervous System	Retrograde endogenous cannabinoid signaling	0.0054	2
2	Human Diseases	Infectious Disease: Bacterial	Staphylococcus aureus infection	0.0118	1
3	Human Diseases	Infectious Disease: Bacterial	Pathogenic E. coli infection	0.0176	1
4	Cellular Processes	Transportation and catabolism	Autophagy-Other	0.0176	1
5	Metabolism	Lipid Metabolism	Glyceride Metabolism	0.0207	2
6	Human Diseases	Cardiovascular Disease	Diabetic cardiomyopathy	0.0218	2
7	Cellular Processes	Cell Growth and Death	Apoptosis	0.0234	1
8	Human Disease	Infectious Disease: Viral	Kaposi's sarcoma-associated herpesvirus infection	0.0292	1
9	Environmental Information Processing	Signal Transduction	FoxO signaling pathway	0.0292	1
10	Metabolism	Carbohydrate Metabolism	Galactose metabolism	0.0296	2
11	Metabolism	Glycine biosynthesis and metabolism	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	0.035	1
12	Cellular Processes	Transportation and Catabolism	Autophagy-Animal	0.035	1
13	Human Diseases	Neurodegenerative Diseases	Huntington's disease	0.035	1
14	Human Diseases	Substance Dependence	Nicotine addiction	0.0407	1
15	Human Diseases	Neurodegenerative diseases	Spinocerebellar ataxia	0.0407	1
16	Organismal Systems	Nervous System	Long-term potentiation	0.0407	1
17	Human Diseases	Substance-dependent	Cocaine addiction	0.0407	1
18	Metabolism	Lipid metabolism	Glycerophospholipid metabolism	0.0425	2
19	Organismal Systems	Nervous system	Glutamatergic synapses	0.0463	1
20	Environmental Information Processing	Membrane transport	ABC transporter protein	0.0464	3

IgG (Biosharp, Chongqing, China); fetal bovine serum (ExCell Bio, Suzhou, China); thiazolyl blue (MedChemExpress, Shanghai, China); dual antibody (Thermo Fisher Scientific, Guangzhou, China); vitexin, orientin and caffeic acid (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China); library building kits (NEXTFLEX Rapid DNA-Seq; Bio Scientific, AUS); sequencing kits (NovaSeq Reagent; Illumina, San Diego); pipettes (N13462C; Eppendorf, Germany), and cell culture dishes, 96-well cell culture plates, and cell culture flasks (Corning, NY).

Configuration of mung bean polyphenol solution

According to the effects of three kinds of mixed polyphenols on cell viability and HSP70 mRNA content in the preliminary experiment, a mixture of 50 μl/mL concentration was selected to study the regulation of cell heat stress. The preliminary experiment found that there was no effect on cell viability in the range of 10 μl/ ml-80 μl/mL. In the range of 30 μl/mL-70μl/mL, the regulatory ability of HSP70 mRNA was gradually enhanced, and 50 μl/mL had the best regulatory effect from the addition level to the regulation level. Finally, 50 μl/mL concentration was selected for mung bean polyphenol intervention test. Vitexin, orientin, and caffeic acid were prepared into 10 mg/mL standard solution with dimethyl sulfoxide solution, and then diluted step by step into 50 μl /mL solution with mass ratio of 10:3:6 for use.

Cell heat stress and mung bean polyphenol heat stress regulation methods

Cell culture and passaging methods. Caco-2 cell culture medium: DMEM + 10% FBS + 1% (Penicillin-Streptomycin Solution). Cell resuscitation methods: Caco-2 cells were removed from liquid nitrogen and quickly placed into a 37 °C water bath, and the storage tubes were gently shaken to dissolve the cryopreservative. After lysis, the cells were transferred to a centrifuge tube containing 5 mL of medium and centrifuged at room temperature at 1000 rm/p for 5 min. The supernatant was discarded and the cells were collected. Cells were suspended in complete medium containing 10% fetal bovine serum. The cells were inoculated into petri dishes, gently blown and mixed, and cultured at 37 °C with 5% CO_2 saturated humidity.

Cell passaging methods: The cells were passaged when the density of the cells reached 80%. Discard the culture medium and wash with PBS once. Add 1–2 mL of 0.25% trypsin to digest the cells, after digestion for 1–2 min, observe under the microscope, the cells separate from each other and become rounded that is, the digestion is completed, quickly discard the trypsin and add the complete medium, blow the cells to make a single cell suspension. The cells were passaged at a ratio of 1: 3 and cultured under 37 °C and 5% CO_2 saturated humidity. Construction of heat stress cell model. Caco-2 cells were added to DMEM high-glucose complete medium in an incubator at 37 °C with 5% CO_2. The test was carried out when the cell graft grew to about 80% density. The cells in the control group were incubated at 37 °C, and the cells in the heat stress group were incubated at 39 °C, and the treatment time was 6 h. The modeling time of heat stress was determined by the changes of cell morphology, cell vitality, HSP70 mRNA content, cell GSH-Px, LDH activity, T-AOC, SOD and MDA content. For detailed steps, refer to previous work[31] (Feng et al.).

Cell heat stress and polyphenol regulation program. Subgroups: In the 37 °C blank control group (CON), cells were cultured normally without any treatment, and the culture time was the same as that of 39 °C heat stress group. In the heat stress model group (HS), Caco-2 cells were grown in splice plates to a density of approximately 80%, and medium containing mung bean polyphenols was added for a 12-h incubation (Refer to Li et al. [53], for setting the culture time). Caco-2 cells were placed under heat at 39 °C for 6 h, then recovered at 37 °C for 6 h, following a method. Previously devised by our group. In the Patchouli positive drug group (HX), Caco-2 cells were grown to a density of approximately 80% in splice plates, and the optimal concentration (400-fold dilution of the original solution) of the patchouli-positive drug was added to the plate for 12 h. The cells were placed in heat stress culture at 39 °C for 6 h, and then recovered at 37 °C for 6 h. In the mung bean polyphenol heat stress regulation group (DF), Caco-2 cells were

Table 4 | Elution gradient of mobile phase

	Time (min)	Flow rate (mL/min)	A (%)	B (%)
Positive ion mode	0	0.4	100	0
	3	0.4	80	20
	4.5	0.4	65	35
	5	0.4	0	100
	6.3	0.4	0	100
	6.4	0.4	100	0
	8	0.4	100	0
Negative ion mode	0	0.4	100	0
	1.5	0.4	95	5
	2	0.4	90	10
	4.5	0.4	70	30
	5	0.4	0	100
	6.3	0.4	0	100
	6.4	0.4	100	0
	8	0.4	100	0

grown to a density of about 80%, and the mung bean polyphenols (ouabain, orientin, and caffeic acid concentrations were adjusted to 50 μL/mL at a mass ratio of 10:3:6) were added to the plate and cultured for 12 h. The cells were then incubated under heat stress at 39 °C for 6 h and then kept at 37 °C for 6 h.

Cells were collected for metabolomics and transcriptomics assays

HSP70 electrophoresis experiment. A total of 10^7 cells were collected, whereafter total cellular protein was extracted and subjected to denatured polyacrylamide gel electrophoresis. The proteins were transferred on to PVDF membranes via transfer electrophoresis. Membranes were blocked with TBS containing 5% skimmed milk powder for 2 h at room temperature. The membranes were reacted with diluted primary antibodies (mouse anti-human β-Actin antibody and HSP70 antibody) at 4 °C overnight. On the following day, after 3 washes with 0.5% Tween-20/TBS at room temperature, the membrane was incubated with a secondary antibody (horseradish peroxidase-labeled sheep anti-mouse IgG antibody) for 2 h. The membrane was then washed thrice with 0.5% Tween-20 in TBS for three times at room temperature. Specific bands were detected using an ECL chemiluminescence reagent kit.

Cellular metabolomics assay analysis methods. Metabolomic analysis. Metabolomic analysis was performed using the UHPLC-Q Exactive HF-X. Sample Preparation: 107 cells were added to a 2 mL centrifuge tube and a 6 mm diameter grinding bead was added. 400 μL of extraction solution (methanol: water = 4:1 (v:v)) containing 0.02 mg/mL of internal standard (L-2-chlorophenylalanine) was used for metabolite extraction. Cells were ground by the Wonbio-96c frozen tissue grinder for 6 min (−10 °C, 50 Hz), followed by low-temperature ultrasonic extraction for 30 min (5 °C, 40 kHz). The cells were left at −20 °C for 30 min, centrifuged for 15 min (4 °C, 13,000 × *g*), and the supernatant was transferred to the injection vial for LC-MS/MS analysis. In addition, 20 μL of supernatant was removed from each sample and mixed as a quality control sample. LC − MS Analysis: Chromatographic conditions: ACQUI-TYUPLCHSST3 (100 mm × 2.1 mm i.d., 1.8 μm; Waters, Milford,USA); The mobile phase A consisted of 95% water + 5% acetonitrile (containing 0.1% formic acid), and the mobile phase B consisted of 47.5% acetonitrile +47.5% isopropanol +5% water (con-taining 0.1% formic acid). The injection volume was 3 μL, and the column temperature was 40 °C, The elution gradient is shown in Table 4. MS conditions: Samples were ionized by electrospray, and MS signals were collected by positive and negative ion scanning modes, Scantype 70–1050 m/z, Sheathgasflowrate50 arb, Auxgasflowrate13 arb, Heatertemp 425 °C, Capillary temp 325 °C, Spray voltage (+) 3500 V, Spray voltage (-) -3500 V, S-Lens RF Level 50, Normalized collision energy 20,40,60 eV, Resolution 60000 Full MS, Resolution 7500 MS2.

Data processing. DataFor data preprocessing, the original data was filtered for missing values, simulation (missing value recoding), data normalization (normalization), QC validation, and data conversion. For metabolite annotation and classification, all metabolites identified via mass spectrometry were compared against the KEGG and HMDB databases to obtain annotations. The metabolites were classified in KEGG terms based on the biological functions they are involved in. Multivariate statistical analysis methods. The obtained metabolomic data were subjected to multivariate statistical analyses, such as principal component analysis (PCA) and OPLS-DA, using ROPLS (R software package). Screening of differential metabolites. Differential metabolites were screened using univariate statistical analysis (t-test) combined with multivariate statistical analysis (OPLS-DA/PLS-DA). The default screening conditions were $p < 0.05$, and VIP > 1 and (FC < 1 or FC > 1, no FC screening by default).

Cellular transcriptomics assay analysis methods. Extraction of total RNA. Total RNA was extracted from tissue samples. The concentration and purity of the extracted RNA were examined using Nanodrop2000. The integrity of total RNA was assessed via agarose gel electrophoresis, and the RIN value was determined using Agilent2100. A single library required total RNA ≥ 1 μg, concentration ≥35 ng/μL, OD260/280 ≥ 1.8, and OD260/230 ≥ 1.0. Oligo dT enrichment of mRNAs. Using magnetic beads with Oligo (dT) for A-T base pairing with poly(A), mRNA was isolated from total RNA for transcriptomic analysis. RNA fragmentation. The Illumina NovaSeq 6000 platform was used to sequence short fragments. The mRNAs obtained via enrichment were complete RNA sequences, with an average length of several kb. Therefore, they need to be randomly interrupted. By adding fragmentation buffer, mRNA can be randomly broken, and small fragments of approximately 300 bp. Reverse synthesis of cDNA. Under the action of reverse transcriptase, six-base random primers (random hexamers) were added, and mRNA was reverse-transcribed into cDNA, followed by two-stranded synthesis to form a stable double-stranded structure. Junction. The double-stranded cDNA structure has sticky ends, which were patched to flat ends by adding an End Repair Mix. Subsequently, an "A" base was added to the 3' end to connect the Y-shaped adapter.

Illumina platform on-line sequencing. For library enrichment, PCR amplification of 15 cycles was conducted, followed by 2% agarose gel recovery of target bands, TBS380 (Picogreen) quantification, and mixing onboard in proportion to the data, whereafter ridge PCR amplification on cBot was performed to generate clusters; Illumina platform sequencing was performed using the PE library, with a read length of 2×150 bp. Reference genome comparison. Reference gene source: Mus_musculus; Reference genome version: GRCm39; Reference genome source: http://asia.ensembl.org/Mus_musculus/Info/Index; Sequence comparison of Clean Reads of each sample with the specified reference genome was performed respectively. Expression difference analysis. Differential expression analyses were performed using the kallisto, Salmon, RSEM, DESeq2, edgeR, DEGSeq, Limma, and NOISeq. Then, based on the quantitative results of expression amount, differential gene analysis was performed between groups. Genes that were differentially expressed between the two groups were obtained, with a screening threshold of |log2FC| >=1 and $P < 0.05$.

Functional annotation genes were functionally annotated in nine major databases, including GO, KEGG, NR, EggNOG, Swiss-Prot, Pfam, Reactome, DO, and DisGeNet using Blast2go, HMMER, KOBAS, and goatools. Multivariate statistical analysis methods: ropls (R package) was used to perform multivariate statistical analyses, such as PCA, on the obtained metabolomics data.

Data availability

Data is provided within the manuscript or supplementary information files.

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Y.F. carried out the laboratory work and wrote the main manuscript text; S.Z. and T.F. conducted the initial draft writing; Y.F., X.F. and C.W. conceptualized and designed the study; S.Z. and X.L. performed data collection and analysis; D.S., Y.F., X.F. and C.W. conducted methodology analysis; Y.F., X.F. and C.W. supervised the project; X.F. and C.W. revised the manuscript; X.F. and C.W. acquired funding and also supervised the project. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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