Study on the Mechanism of Salvia Miltiorrhiza Polysaccharides in Relieving Liver Injury of Broilers Induced by Fluorophenicol

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Abstract

In order to explore the transcriptomics and proteomics targets and pathways of Salvia miltiorrhiza polysaccharides (SMPs) alleviating florfenicol (FFC)-induced liver injury in broilers,60 1-day-old broilers were randomly divided into 3 groups: control group (GP1) was fed tap water, FFC model (GP2) was given tap water containing FFC 0.15 g/L, and SMPs treatment group (GP3) was given tap water containing FFC 0.15 g/L and SMPs 5 g/L.Starting from 1 day of age, the drug was administered continuously for 5 days. On the 6th day, blood was collected from the heart and the liver was taken. Then 3 chickens were randomly taken from each group, and their liver tissues were aseptically removed and placed in an enzyme-free tube. Using high-throughput mRNA sequencing and TMT-labeled quantitative proteomics technology, the transcriptome and proteome of the three groups of broiler liver were analyzed respectively. The results of the study showed that the liver tissue morphology of the chicks in the GP1 and GP3 groups was complete, and there were no obvious necrotic cells in the liver cells. The liver tissue cells in the GP2 group showed obvious damage, the intercellular space increased, and the liver cells showed extensive vacuolation and steatosis. Compared with the GP1 group, the daily gain of chicks in the GP2 group was significantly reduced ($P < 0.0\ 5$ or $P < 0.01$). Compared with the GP2 group, the GP3 group significantly increased the daily gain of chicks ($P < 0.0\ 5$ or $P < 0.01$). Compared with the GP1 group, the serum levels of ALT, AST, liver LPO, ROS and IL-6 in the GP2 group were significantly increased ($P < 0.0\ 5$ or $P < 0.01$), and the contents of T-AOC, GSH-PX, IL-4 and IL-10 in the liver were significantly decreased ($P < 0.0\ 5$ o r $P < 0.01$). After SMPs treatment, the serum levels of ALT, AST, liver LPO, ROS and IL-6 were significantly reduced ($P < 0.0\ 5$ or $P < 0.01$), and the contents of T-AOC, GSH-PX, IL-4 and IL-10 in the liver were significantly increased ($P < 0.0\ 5$ or $P < 0.01$). There were 380 mRNA and 178 protein differentially expressed between GP2 group and GP3 group. Part of DEGs was randomly selected for QPCR verification, and the expression results of randomly selected FABP1, SLC16A1, GPT2, AACS and other genes were verified by QPCR to be consistent with the sequencing results, which demonstrated the accuracy of transcriptation-associated proteomics sequencing. The results showed that SMPs could alleviate the oxidative stress and inflammatory damage caused by FFC in the liver of chicken and restore the normal function of the liver. SMPs may alleviate the liver damage caused by FFC by regulating the drug metabolism - cytopigment P450, PPAR signaling pathway, MAPK signaling pathway, glutathione metabolism and other pathways.

Introduction

FFC is a synthetic chloramphenicol fluorinated derivative, which is widely used in aquaculture industry due to its wide antibacterial range and accurate efficacy (Er and Dik. 2014; Lis et al. 2011). FFC inhibits the activity of peptide acyltransferase by binding to the A position on the 50S subunit of the bacterial 70S ribosome, thereby affecting the synthesis of bacterial protein and achieving antibacterial effect (Yang et al. 2004; Cannon et al. 1990). It has inhibitory effect on most Gram-positive and Gram-negative bacteria, including Escherichia coli, Salmonella, Pasteurella, Actinobacillus pleuropneumoniae, Staphylococcus aureus, etc (Syriopoulou P et al. 1981; Gharaibeh et al. 2010; Liu et al. 2003). In the process of poultry

breeding, antibiotics are widely used to prevent and treat infectious diseases, even as growth promoters (Landers et al. 2012). Studies have shown that FFC can affect the cardiovascular development and inhibit the growth of chicken embryos, resulting in the death of early embryos (Hu et al. 2020). FFC caused the reduction and apoptosis of bursa lymphocytes in chickens, reduced the transcription levels of interferon-related genes IRF-7, 2 '-5' OAS and cytokines IL-6, TNF- and IFN- in immune organs, and decreased the serum levels of NDV antibody and cytokines IL-1, IL-2 and IFN-γ (Meng 2020). After FFC treatment, the serum total protein and globulin levels of chickens were significantly reduced, macrophage activity was decreased, the metabolites NO and serum lysozyme of macrophages were decreased, so FFC could inhibit the humoral and cellular immune responses of chickens (El-Eela et al. 2016). FFC is an inhibitor of mitochondrial protein synthesis and can induce significant cytotoxicity (Hu et al. 2017). At present, new and complex drug resistance mechanisms of FFC are constantly discovered, and the drug resistance of FFC is becoming more and more serious, such as Escherichia coli, Salmonella, etc., the drug resistance generated by veterinary drugs can be transmitted to humans (Talebiyan et al. 2014; Liu & Wang 2018; Zhan et al. 2019). Excessive use of FFC can also lead to residual problems in chicken breeding. FFC remains in liver and chicken, which pose a serious threat to public health and human health.

In recent years, FFC is often used as an “open medicine” for chickens to prevent diarrhea and reduce the mortality rate of chickens.However, FFC can increase oxidative stress and accelerate hepatocyte apoptosis, thus affecting the liver function of broilers (Han et al. 2020; Nasim et al. 2016). With the overuse of FFC, there will be a high residue in the liver, which is the main site of drug metabolism and is vulnerable to the toxic damage of FFC.At present, the use of FFC still exists in farms, so it is urgent to find drugs that have antioxidant and liver protection effects and can be used together with FFC to alleviate its side effects.

Salvia miltiorrhiza can inhibit hepatic lipid peroxidation, improve hepatic microcirculation, and promote liver repair and regeneration (Xin 2013). SMPs are the effective ingredients of Salvia Miltiorrhiza, with liver protection, antioxidant, immune regulation and other pharmacological effects (Geng et al. 2015). Studies have shown that SMPs can effectively inhibit the TLR4/ MyD88 inflammatory signaling pathway in mice with liver injury induced by LPS/ D-Galn and alleviate the inflammatory response in mice (Wang et al. 2019). SMPs can significantly increase the content of TP and Alb, decrease the content of ALT, AST and MDA in chickens with liver injury caused by CCL4, improve the body's antioxidant capacity, and have a good therapeutic effect on liver injury caused by CCL4 (Ba et al. 2016). At present, there are few studies on FFC-induced liver damage in chickens and the mechanism of SMPs to alleviate it, and it is not clear. We use transcriptomics combined with proteomics to study in detail the genetic changes in the process of SMPs alleviating FFC-induced liver damage, which will help discover New potential molecular markers and further elucidate the molecular mechanism of SMPs alleviating FFC-induced liver damage in broilers.

Materials And Methods

Drugs and reagents

Fluorobenicol (purchased from Shandong Shenniu Biotechnology Co., Ltd.) Salvia Miltiorrhiza Polysaccharides (Hangzhou Zhengda Qingnianbao Pharmaceutical Co., Ltd., Hangzhou, China)

ALT, AST, T-AOC, GSH-PX, LPO and other kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).ROS, IL-4, IL-6, IL-10 and other ELISA kits were purchased from Shanghai Enzyme-Linked Biotechnology Co., Ltd. (Shanghai, China).

Animals and treatments

60 1-day-old AA broilers (purchased from Hebei Dawu Co., Ltd.) were randomly divided into 3 equal groups, which were respectively set as blank group (GP1) : feed tap water;FFC module (GP2) : give tap water containing 0.15 g/L FFC;SMPs treatment group (GP3) : Add FFC 0.15 g/L and SMPs 5 g/L to drinking water. The drug was administered for 5 consecutive days from 1 day of age.Each group was free to feed (purchased from Hebei Dawu Co., Ltd.). On the 6th day, the heart blood was collected, the broiler chickens were killed under general anesthesia, and the liver was immediately harvested. The separated serum samples are stored at -80℃ for later use.The liver tissue was rinsed with normal saline and stored at -80℃ for later use. In addition, 3 chickens were randomly selected from each group, and their liver tissues were aseptically removed and placed in an enzyme-free tube (labeled LA1, LA2, LA3 in the GP1 group, labeled LB1, LB2, LB3 in the GP2 group, and LC1 in the GP3 group. LC2, LC3.),The samples were transported on dry ice to Shanghai Parsons Biotechnology Co., Ltd. for transcriptomics and proteomics sequencing. All animal handling procedures were carried out in accordance with the NIH Laboratory Animal Care and Use Guidelines, and were approved by the Animal Welfare Committee of Hebei Agricultural University.

Analysis of daily weight gain and liver index of broilers

The weight of the chicks was recorded daily and the average weight gain was calculated.The livers of the chicks at 6 days old were collected and weighed to analyze the liver index.Liver index = liver weight (g)/ body weight (g).

HE staining to observe liver tissue

The liver tissue was fixed with 4% paraformaldehyde, dehydrated, transparent, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and finally mounted. The pathological changes of broiler livers in each group were observed under an optical microscope.

Detection of chicken liver function indexes

Strictly follow the instructions of the kit to detect the content of ALT and AST in the serum of each group of chickens.

Detection of antioxidant indexes of chicken liver

The spare chicken liver tissue was taken out from the cryogenic refrigerator at -80℃ and prepared into liver tissue homogenate by tissue homogenate machine in strict accordance with the kit instructions. The contents of T-AOC, GSH-PX, LPO and ROS of chicken liver in each group were tested.

Detection of inflammatory factor levels in the liver of chicks

In strict accordance with the kit instructions, the levels of IL-4, IL-6 and IL-10 in the liver of each group were detected by ELISA.

Preparation and quality determination of total RNA in the liver of broilers

Total RNA was extracted from liver tissue using Trizol reagent, and concentration, mass, and integrity were determined using NanoDrop spectrophotometer.

The expression of mRNA in liver tissues of each group was determined by transcriptome

The mRNA was purified from total RNA using magnetic beads with poly-T oligonucleotides attached. In Illumina's proprietary Fragmentation Buffer, Fragmentation is performed at high temperatures using divalent cations.The first cDNA was synthesized using random oligonucleotides and SuperScriptII.The second strand of cDNA was then synthesized using DNA polymerase I and RnaseH.The remaining protruding ends are converted to flat ends by exonuclease/polymerase activity and these enzymes are removed.The 3' ends of the DNA fragment are adenosylated to link the IlluminaPE adaptor oligonucleotides.The library fragments were purified using AMPUREXP system, and the cDNA fragments with length of 200bp were selected.Using IlluminaPCRPrimerCocktail selective enrichment in 15 cycles of PCR reaction on both ends of the connection cohesion molecules of DNA fragments.The product was purified by AMPUREXPsystem and quantified by Agilent Bioanalyzer 2100 system.Sequencing was performed on Novaseq platform by Shanghai Parsenol Biotechnology Co., Ltd.

Transcriptome analysis

Cutadapt (V1.15) software is used to filter connectors and low-quality Reads in the raw debutant data to obtain high-quality sequences for further analysis. Use HISAT2 (http://ccb.jhu.edu/software/hisat2/index.shtml) to locate the filtered reads to the reference genome, and the default mismatch does not exceed the distribution of the alignment area of the calculated reads. The Read Count value of each gene was counted and normalized using FPKM. According to $|log2FoldChange| > 1$, $P < 0.05$ as the standard, DESeq (1.30.0) was used to screen differentially expressed genes.

Protein extraction, digestion and TMT labeling

The sample was homogenized in the lysis buffer and the protein content was determined with BCA protein assay reagent (Beyotime). Add 200 μg sample to 30 μL SDT buffer, ultrafiltration of UA buffer, add 100 μL 0.05M iodoacetamide to UA buffer, and incubate for 20min in dark.After washing and digestion

for 12 hours, collect the filtrate.The peptide content was calculated by ultraviolet spectral density at 280nm.Each TMT reagent was dissolved in ethanol and then added to the corresponding peptide mixture for repeated drying.

Peptide Fractionation with Strong Cation Exchange (SCX) Chromatography

First, the TMT labeled peptides were separated by SCX chromatography, and the dry peptide mixture was reconstructed and acidified.Add to a 4.6 x 100 mm Polysulfoethyl column. The peptides were eluted with a gradient of 0%-10% buffer B (500 mM KCl in 25% ACN, 10 mM KH2PO4, pH 2.7) at a flow rate of 1 ml/min for 2 minutes, 10-20% buffer B lasts 25 minutes, 20%-45% buffer B lasts 5 minutes, and 50%-100% buffer B lasts 5 minutes. Elution monitored at 214nm, , and the eluted fractions were collected and combined every 1 minute, desalted on a C18 column, centrifuged, concentrated, and reconstituted. The samples are stored at -80°C.

Liquid Chromatography (LC) - Electrospray Ionization (ESI) Tandem MS (MS/MS) Analysis by Fusion orbitrap.

Using a fusion orbital rap mass spectrometer coupled with Easy nLC Proxeon Biosystems. Then Nanolc-MS/MS analysis was performed, Add the peptide mixture to the C18-reversed phase column containing RP-C18 5μm resin. The flow rate of separated with 80% acetonitrile and 0.1% Formic acid was 250 nl/min, and the time was over 90 minutes.The MS data was obtained using the data-dependent TOP20 method to dynamically select the most abundant precursor ion (300-1800 m/z) in the HCD fragments from the survey scan. Determination of the target value is based on predictive Automatic Gain Control.

Sequence Database Searching and Data Analysis

MS/MS spectra were searched using MASCOT engineembedded into Proteome Discoverer 1.4. against uniport Gallus gallus 32641 20190702 (32641 sequences, download at 20190702) and the decoy database. For protein identification, the following options were used. Peptide mass tolerance=20 ppm, MS/MS tolerance=0.1 Da, Enzyme=Trypsin, Missed cleavage=2, Fixed modification: Carbamidomethyl (C), TMT10(K), TMT10(N-term), Variable modification：Oxidation(M), FDR≤0.01.

Bioinformatics

GO functional Annotation: The process of GO Annotation to target mRNA collection by using the software Omicsbean (http://www.omicsbean.cn/) can broadly be reduced to four steps: Blast, GO item extraction, GO Annotation, and interscan supplemental Annotation.KEGG path annotation: The software Omicsbean was used to annotate the KEGG path to the target mRNA set.3. Enrichment analysis of GO annotation and KEGG annotation: Software Omicsbean was used to compare the distribution of each GO classification or KEGG pathway in the target mRNA set and the overall mRNA set, and enrichment analysis of GO annotation or KEGG pathway annotation was performed on the target mRNA set, and software R version 3.5.1 was used to generate KEGG enrichment analysis bubble diagram.

QRT-PCR was used to analyze the relative transcriptional level of mRNA in chicken liver

The RNA of chicken liver tissues was extracted with a total RNA extraction kit (Promega Biotechnology Co., China, Beijing, China). CDNA samples were obtained after total RNA reverse transcription. The primers used were synthesized by Takara Biotechnology Co., Ltd. (Dalian, China). MRNA levels of SLC16A1, FABP1, AACS, ACSBG2, GSTA3, HPGDS, GSTT1, ACACA, ACSL4 and GPT2 were detected by qPCR (**Table 1)**. First, the pre-denaturation treatment is 95°C for 120 s for one cycle, and then 95°C for 5 s, 61°C for 30 s, and 72°C for 30 s for 45 cycles. Using β-actin as a reference gene, And the relative transcriptional level of each gene was calculated by $2^{-\Delta\Delta CT}$.

Table. 1 Primer sequence

Gene	Primer sequence (5'-3')	Product length (bp)	Accession number
GSTA3	F:TTGGATAAGGCCGCAAACAGATA R:AATGCACGTCTGCTCTGCTCA	106	NM_001001777.1
AACS	F:GGCATGTTCCAAGCCTCATTC R:ACAACCCTTAAACACGTGGCTTC	200	NM_001006184.1
SLC16A1	F:CTGGAAGCTGCTGTGGTTTCA R:ACCTGGCTGTTGCCATCTTG	87	NM_001006323.1
ACACA	F:CTGATGGTCTTTGCCAACTGGA R:CACGATGTAGGCACCAAACTTGA	87	NM_205505.1
ACSL4	F:ACTGGAAATCGTAGGTGTGCTGAA R:CTCGGTTACTGCACGTCAAAGG	107	XM_004940805.3
GPT2	F:CGAACTTGCAGTGCGGTGTAG R:TCGGGTGCAAAGGCAAATATAA	147	XM_015292423.2
ACSBG2	F:AGCTGGGCCCAACAATGAA R:GCATCCGCGTAGAACTCATCAA	82	XM_015299814.2
β-actin	F: ATTGTCCACCGCAAATGCTTC R:AAATAAAGCCATGCCAATCTCGTC	124	NM_205518.1

Statistical analysis

The statistical analysis was performed using SPSS 21.0 software. One-way ANOVA was performed for comparison of variance among multiple groups. When the variance was not uniform, two independent sample T tests were used. The difference was significant at $P < 0.05$, and extremely significant at $P < 0.01$.

Results

Average daily weight gain and liver index of each group

As shown in **Table. 2**, compared with the control group, the daily gain of chicks in the FFC module group was significantly reduced. Compared with FFC module, SMPs can significantly increase the daily weight gain of chicks.There was no significant difference in liver index among each group.

Table.2. Average weight gain and liver index of chicks in each group

Group	Average weight gain (g)	Liver index (g/g)
GP1	$17.26\pm3.43^{\#\#}$	5.18%±0.74%
GP2	$4.84\pm2.23^{**}$	5.27%±0.67%
GP3	$9.70\pm1.95^{**\#\#}$	5.15%±0.48%

Notes: Data represented mean ± SD, n=10 per group.GP1: Control group, GP2: 0.15 g/L FFC group, GP3: 0.15 g/L FFC and 5 g/L SMPs group. Compared with GP1 group, * P<0.05, ** P<0.01; Compared with GP2 group, # P<0.05, ## P<0.01. (Same as below)

Effects of SMPs on the pathological changes of liver tissue in chickens with liver injury

As shown in **Figure. 1** the liver tissue structure of the blank group was complete and clear, the hepatocyte cords were neatly arranged, and there was no inflammatory cell infiltration. However, the liver tissue cells of the FFC model showed obvious damage, the intercellular space increased, the cell boundary was blurred, and the liver cells showed extensive vacuolation and steatosis, indicating that the florfenicol solution could cause serious damage to the liver of chickens. The liver tissue of the chicks in the SMPs treatment group was intact, and there were no obvious necrotic cells in the liver cells.

Liver function indexes of each group

As shown in **Table. 3**, the serum levels of ALT and AST in the FFC group were significantly higher than those in the control group. Compared with FFC model group, SMPs treatment group could significantly reduce ALT level and AST level.

Table.3. Contents of ALT and AST in serum of each group

Group	ALT（U/l）	AST（U/l）
GP1	3.01±1.20##	28.17±1.08##
GP2	6.97±2.75**	31.66±1.65**
GP3	3.20±1.23##	28.33±1.08##

Notes: ALT, Alanine aminotransferase; AST, Aspartate aminotransferase.

Liver oxidation index of each group

As shown in **Figure. 2**, compared with the control group, the GSH-PX enzyme activity and T-AOC level in the liver of FFC model chickens were extremely significantly reduced, while LPO content and ROS content were significantly increased. Compared with FFC model group, SMPs treatment group significantly increased GSH-PX enzyme activity and T-AOC level, and significantly reduced LPO and ROS content.

The levels of inflammatory factors in the liver of chickens in each group

As shown in **Figure. 3**, compared with the control group, the level of IL-6 in the liver of FFC model chicks was extremely significantly increased, the level of IL-4 was extremely significantly reduced, and the level of IL-10 was significantly reduced. Compared with the FFC model, the IL-6 level in the SMPs treatment group was extremely significantly reduced, the IL-4 level was extremely significantly increased, and the IL-10 level was significantly increased.

Transcriptome data summary

After filtering the sequences of GP1, GP2 and GP3, 40790366, 36813283 and 39873734 high-quality base sequences were obtained respectively, and high-quality sequences accounted for more than 93% of the total. The correlation coefficient of the samples was between 0.92 and 1, indicating that the differences between the samples were small and the sequencing data were very reliable. DESeq was used to analyze the differences in gene expression, and the conditions for screening the differentially expressed genes were as follows: expression differentially expressed multiple |log2FoldChange| > 1, with significance p-value <0.05. Compared with the GP1 group, there were a total of 1,989 differentially expressed genes in the GP2 group, among which 495 genes were up-regulated and 1494 genes were down-regulated. Compared with GP1 group, there were a total of 1278 differentially expressed genes in GP3 group, among which 344 genes were up-regulated and 934 genes were down-regulated. Compared with GP2 group, there were a total of 380 differentially expressed genes in GP3 group, among which 165 genes were up-regulated and 215 genes were down-regulated, the results were showed in （**Figure. 4A**）. Forty common differential genes were found in the three comparison groups (**Figure. 4B**). According to molecular function (MF), biological process (BP) and cell composition (CC), the GO enrichment analysis results of differentially expressed genes are classified. The results show that the top 20 GO enrichment families with the most significant enrichment include BP and CC. GP1 vs GP2 includes “regulation of immune

system process”,“extracellular region part”,“movement of cell or subcellular component”,“response to stimulus”,“cell migration”,“cell surface receptor signaling pathway”,“cell activation” and so on(**Figure. 4C**). GP2 vs GP3 includes“anoikis”,“circadian rhythm”“triglyceride metabolic process”,“response to oxygen-containing compound”,“RNA polymerase II transcription factor activity, sequence-specific DNA binding”,“small molecule metabolic process”and so on(**Figure. 4D**).

Summary of proteomic data

Differentially expressed proteins were screened for the criteria of up-regulation greater than 1.2 times or down-regulation less than 0.833 times. Compared with the GP1 group, 429 proteins were up-regulated and 488 proteins were down-regulated in the GP2 group. Compared with the GP1 group, 468 proteins were up-regulated and 402 proteins were down-regulated in the GP3 group. Compared with the GP2 group, 96 proteins were up-regulated and 82 proteins were down-regulated in the GP2 group(**Figure. 5A**). Figure 5b and Figure 5c respectively show the top 10 Level 2 terms in the enrichment analysis of BP, CC and MF in GP1vs GP2 and GP2 vs GP3. The terms of each category are ordered from left to right according to their -log(P-value) value, with the more to the left the more significant. The results showed that GP1vsGP2 differentially expressed proteins were significantly enriched in the “single-organism metabolic process”,“small molecule metabolic process”,“oxidation-reduction process”,“cytoplasm”,“cytoplasmic part”,“membrane-bounded organelle”,“oxidoreductase activity”,“catalytic activity”,“purine ribonucleoside binding”(**Figure. 5B**). GP2vsGP3 differentially expressed proteins were significantly enriched in the “single-organism metabolic process”,“small molecule metabolic process”“oxidation-reduction process”“fatty acid metabolic process”,“cytoplasmic part”,“intracellular part”,“catalytic activity” and so on(**Figure. 5C**).

Combined analysis of transcriptome and proteome

Next, we counted the number of differentially expressed proteins and their corresponding mRNAs in the GP2 vs GP3 comparison group. In the GP1 vs GP2 comparison group, there were 89 proteins with the same change trend as their corresponding mRNAs, of which 47 were up-regulated and 42 were down-regulated. In the GP1 vs GP3 comparison group, there were 55 proteins with the same change trend as their corresponding mRNAs, of which 35 were up-regulated and 20 were down-regulated. In the GP2 vs GP3 comparison group, there were 8 proteins with the same change trend as their corresponding mRNAs, of which 3 were up-regulated and 5 were down-regulated, as shown in **Figure. 6**.

GO enrichment analysis and KEGG enrichment analysis of differentially expressed transcripts and proteins

GO enrichment analysis was conducted for the differentially expressed proteins and their related mrnas of GP1 vs GP2 and GP2 vs GP3, In each GO category, select the top 10 GO terms with the smallest P value, namely the most significant enrichment, for display. The results showed that the differentially expressed proteins and transcripts of GP1 vs GP2 were mainly enriched in “small molecule metabolic process”“carboxylic acid metabolic process”“oxidoreductase activity”“catalytic activity”olic

process”、“cytoplasm”、“peroxisome”、“microbody”. GP2 vs GP3 was significantly enriched in “alpha-amino acid metabolic process”、“cellular amino acid biosynthetic process”、“organic acid metabolic process”、“cytoplasm”、“microtubule cytoskeleton”,“intrinsic component of plasma membrane”,“transaminase activity”、 “transferase activity”. KEGG database was used to analyze the biological metabolic pathways and signal transduction pathways involved in differentially expressed genes. The results showed that the differentially expressed proteins and transcripts of GP1 vs GP2 were mainly enriched in “Drug metabolism - cytochrome P450”,“Glutathione metabolism”,“PPAR signaling pathway”,“Metabolism of xenobiotics by cytochrome P450”,“Steroid biosynthesis”,“Drug metabolism - other enzymes” and other pathways. GP2 vs GP3 was significantly enriched in “Drug metabolism - cytochrome P450”, “Alanine, aspartate and glutamate metabolism”, “Arginine biosynthesis”, “Glycine, serine and threonine metabolism”, “Valine, leucine and isoleucine degradation”, “Cysteine and methionine metabolism”, “Vitamin B6 metabolism”, “Butanoate metabolism”, “PPAR signaling pathway” etc. As shown in **Figure. 7**.

Validation of differentially expressed genes using qRT -PCR

A qRT-PCR analysis was performed to investigate the transcriptional patterns of seven selected genes. As shown in **Figure. 8**, the expression levels of all target genes were consistent between the qRT-PCR and RNA-seq data. This showed that the quality of transcriptome data was reliable.

Discussion

Some studies have The liver is the main organ of animal metabolism and detoxification. So far, there are few studies on FFC's liver toxicity in chickens. Our study found that the therapeutic dose of FFC reduced the daily gain of chicks, which indicated that FFC inhibited the growth of chicks. The histopathological analysis of chicken liver showed that FFC can cause the increase of liver cell space, blurring of cell boundaries, extensive vacuolation and fatty degeneration of liver cells, indicating that FFC can seriously damage chicken liver. The liver tissue of the chicks in the SMPs treatment group was intact, and there were no obvious necrotic cells in the liver cells. It shows that SMPs can significantly improve liver damage in chickens caused by FFC. ALT and AST are often present in the cytoplasm of liver cells. Under normal circumstances, only a small amount of them are released into the blood. Both enzymes are released in large quantities into the blood when liver tissue is acutely damaged or cell membrane permeability is increased, causing a significant increase in serum levels, Therefore, ALT and AST are often used as reliable indicators to evaluate the degree of liver injury (Du et al. 2018; Liss et al. 2010). Our results showed that FFC significantly increased ALT and AST levels in broiler serum, It indicated that the broiler liver was damaged after taking the treatment dose (0.15g/L) of FFC, and SMPs could significantly inhibit the increase of ALT and AST in broiler serum caused by FFC, indicating that SMPs could alleviate the liver injury caused by FFC and restore the liver function of broiler chickens.

Under normal conditions, both enzymatic and non-enzymatic antioxidant systems in the body maintain a dynamic equilibrium state between the production and elimination of free radicals (Cui 2019). Oxidative

stress refers to the imbalance between the oxidation and antioxidant system in the cell, which leads to the redox balance tending to a peroxidation state, and a large amount of ROS is generated (Tao et al. 2018; Gautier et al. 2001). Excessive ROS can lead to lipid peroxidation, protein oxidation, enzyme inhibition and DNA damage, which in turn leads to cell cycle arrest and apoptosis (Liu et al. 2012; Zeng et al. 2014). The liver is the main site of biosynthesis, metabolism, clearance and host defense, so it is very vulnerable to ROS damage (Sánchez-Valle et al. 2012). GSH-PX and T-AOC are essential parts of the endogenous antioxidant defense system and play an crucial part in maintaining the redox equilibrium in the cell. GSH-PX is an important antioxidant enzyme, which can catalyze the reduction of hydrogen peroxide, reduce the peroxidation reaction, and effectively remove excess reactive oxygen species produced in the body. GSH-PX is an important oxidative stress marker (Jin et al. 2017). Glutathione is a major endogenous non-enzymatic antioxidant that helps prevent lipid peroxidation through GSH-PX catalyzed reactions. When gSH-PX activity weakens, the body produces too many free radicals to attack polyunsaturated fatty acids, leading to lipid peroxidation and forming lipid peroxidation (Fang et al. 2002). The level of T-AOC directly reflects the activity of antioxidant enzymes and the overall level of non-enzymatic antioxidants in the body (Scudamore et al. 2017). Lipid peroxidation is one of the main manifestations of oxidative damage. The LPO generated amplifies the reactive oxygen species, thus destroying the functional integrity of cells, leading to cell apoptosis, tissue necrosis and carcinogenesis (Sirajudeen et al. 2011; Zhong & Li 1990). In this study, the contents of GSH-PX and T-AOC in the liver of broilers in FFC group were significantly decreased, while the contents of LPO and ROS were significantly increased, which indicated that oxidative stress injury occurred in the liver of broilers. SMPs significantly increased the content of GSH-PX and T-AOC in broiler liver and inhibited the levels of LPO and ROS, indicating that SMPs could relieve the oxidative stress in broiler liver caused by FFC.

Cytokines are protein polypeptides produced by various cells induced by immunogen, mitogen or other stimulants, which have various functions such as regulating hemopoiesis, cell growth and damaged tissue repair.Interleukin is one of its components (Epanchintsev et al. 2015). IL-4 is a pleiotropic cytokine that can participate in a variety of physiological and pathological processes (Serhan et al. 2003). IL-6, also known as pre-inflammatory cytokines, is an important pro-inflammatory factor, mainly involved in the activation of T lymphocytes and B lymphocytes, and mostly plays a pro-inflammatory role in the early stage of inflammation, aggravating inflammatory damage (Xie et al. 2000; Ishihara & Hirano 2002). IL-10 is an immunomodulatory cytokine produced by a variety of cells. Its main biological function is to limit and stop inflammatory responses and regulate the differentiation and proliferation of some immune cells (Yang 2013). In this study, FFC increased IL-6 levels in the liver of chicks while decreasing IL-4 and IL-10 levels. SMPs restored the normal levels of IL-6, IL-4 and IL-10. This suggests that FFC may induce inflammation in the liver by regulating inflammatory cytokines, and SMPs can reduce inflammation in the liver.

According to the combined sequencing analysis of transcriptomics and proteomics, SMPs may alleviate the liver injury caused by FFC by regulating the expression of FMO, FABP1, SLC16A1, GPT2, AACS and other genes as well as drug metabolism-cytoplasmic P450, PPAR signaling pathway and glutathione metabolism pathway. CytochromeP450 enzyme is a family of hemoglobin proteins responsible for

detoxification.Plays a leading role in the oxidative metabolism of many exogenous and endogenous substrates, mainly catalyzing the phase I reaction of exogenous biotransformation, 90% of exogenous toxins are mainly metabolized and detoxified by CYP450 (Cong et al. 2019; Yang et al. 2003). GST is an important constituent enzyme in the metabolic enzyme system of the body, which catalyzes the combination of various exogenous substances or liver metabolites with glutathione to form metabolites easily excreted (Xu 2019). Our previous studies have shown that FFC can up-regulate the expression of GSTAL2, GSTT 1, GSTA3, FMO5, HPGDS and other genes in the Drug metabolism - Cytochrome P450 pathway. FFC mainly relies on GST to detoxify FFC and its harmful metabolites. The cause of liver damage caused by FFC may be due to the accelerated metabolism of drugs and the production of a large number of harmful metabolites, which increase the body's oxidative stress damage. SMPs alleviates FFC induced liver damage by regulating the expression of the related genes on Drug cytochrome P450.

The main site of fatty acid synthesis and lipid metabolism in poultry is the liver (Xu 2003). Studies have shown that in the process of fatty acid biosynthesis and metabolism, the activation of PPAR signaling pathway at the transcriptomic level is closely related to it. Studies have shown that the transcriptional regulation of FABP gene is related to the PPAR signaling pathway. As a key regulator of liver lipid metabolism, The way FABP1 affects lipid metabolism may be through the regulation of PPARα in chicken liver cells (He et al. 2013; Spann et al. 2006; Gao et al. 2015). Studies have shown that resveratrol can improve lipid deposition by regulating the expression of genes related to PPAR signaling pathway in chicken liver, thereby alleviating the damage of hepatocyte steatosis (Luo 2019). Previous studies have shown that AACS may be a biomarker for fatty liver in chickens (Tsai et al. 2017). In chickens, ACSBG2 gene plays an important role in lipid metabolism (D'andre et al. 2013). In this study, it was found that FFC resulted in a significant decrease in the mRNA and protein expressions of AACS and ACSBG2 in chicken liver, suggesting that FFC may cause liver damage in broiler chickens by affecting lipid metabolism. After SMPs treatment, mRNA and protein levels of FABP1, AACS and ACSBG2 could be significantly restored. It suggests that SMPs can regulate the expression of FABP1, AACS and other genes and proteins related to PPAR signaling pathway, so as to promote the normal metabolism of liver lipids and alleviate liver damage caused by FFC. In production, the metabolism of foreign organisms in broilers will affect its quality, and food safety is very important in poultry breeding. Our research shows that FFC can cause severe drug-induced liver damage as an "open medicine" for chicks, while SMPs can be used with FFC as a hepatoprotective agent and significantly relieve liver damage.

Conclusion

In conclusion, the Data presented here highlights the molecular mechanism by which SMP alleviates FFC-induced liver injury in broilers, and SMPs can regulate drug metabolism, lipid metabolism, amino acid metabolism, etc. SMPs alleviate the oxidative stress and lipid metabolism disorders in the liver of broilers, mainly through the regulation of Drug metabolism-cytochrome P450, PPAR signaling pathway and other pathways. It is hoped that this study can lay a foundation for the innovation of natural products in the treatment of drug-induced liver injury.

Declarations

Ethical Approval

The use of animals in this study was approved by Hebei Animal Protection Association.

Consent to Participate

Informed consent was obtained from all individual participants included in the study.

Consent to Publish

Consent was obtained from participants about publishing their data.

Authors Contributions

Wanyu Shi and Yongzhan Bao conceived and designed the study, and they rigorously revised the manuscript. Yumeng Geng performed most of the experiments and analyzed the results and drafted the manuscript. Chunyu Lu and Guozhong Jin helped collect samples and participated in the H&E. Shuying Li and Yuqing Cui participated in the qPCR experiment and analyzed the results. Chao Han participated in the detection and result analysis of biochemical indicators. All authors read and approved the final manuscript.

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Competing Interests

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

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Authors' conflicts

The authors have no conflicts of interest.

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Figures

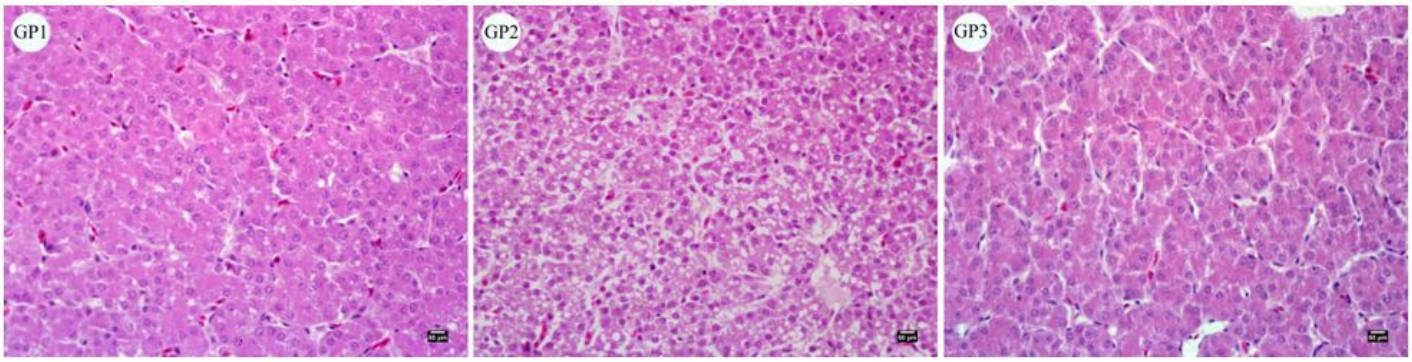

Figure 1

Histopathological changes of chicks liver (H&E staining, 400×).

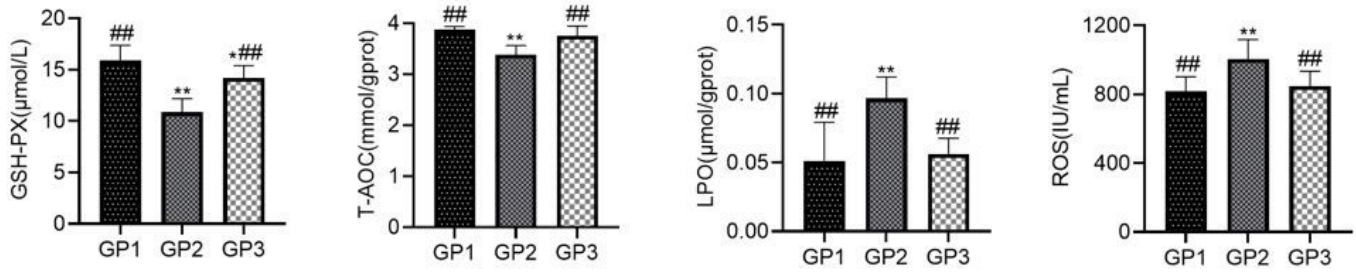

Figure 2

Contents of GSH-PX, T-AOC, LPO and ROS in chicken liver. GSH-PX, glutathione peroxidase; T-AOC, total antioxidant capacity; LPO, Lipid peroxide; ROS, reactive oxygen species.

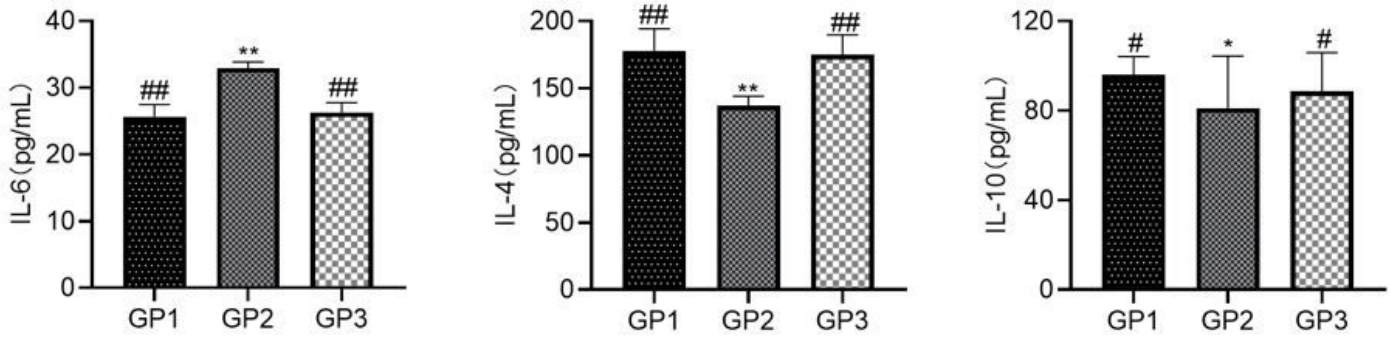

Figure 3

Levels of IL-6, IL-4 and IL-10 in the chicken liver. IL-6，Interleukin- 6; IL-4, Interleukin- 4; IL-10, Interleukin-10.

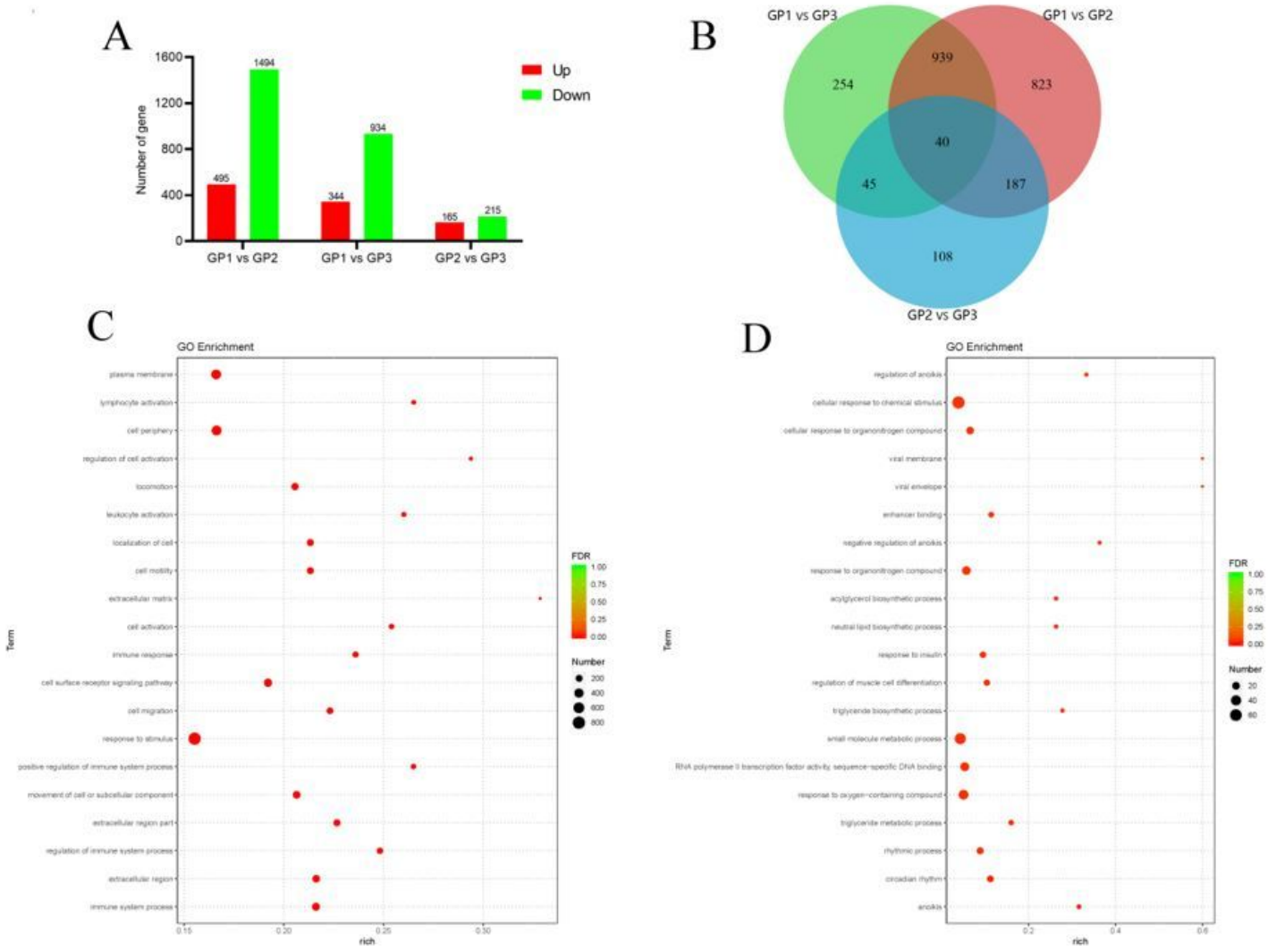

Figure 4

Differentially expressed genes (DEGs) results. A: Histogram of differentially expressed genes in GP1, GP2 and GP3. B: The Venn diagram of three groups of differentially expressed genes. C: GO enrichment bubble map of GP1vsGP2 comparison group of differentially expressed genes. D: GO enrichment bubble map of GP2vsGP3 comparison group of differentially expressed genes.

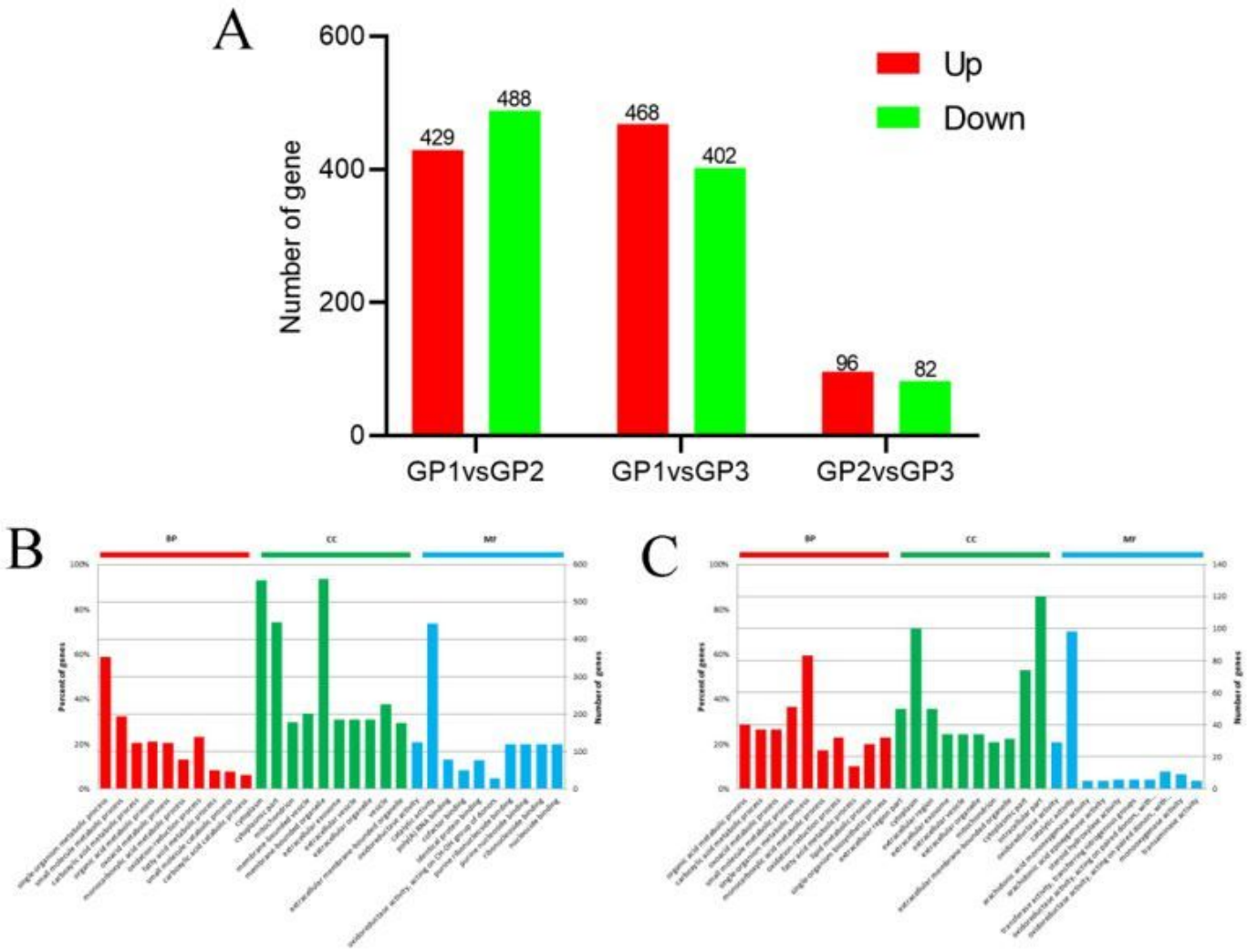

Figure 5

Differentially expressed proteins results. A: The histogram of differentially expressed proteins of GP1, GP2 and GP3. B: GO enrichment bubble diagram of GP1vsGP2 comparison group of differentially expressed proteins. C: GO enrichment bubble diagram of GP2 vs GP3 comparison group of differentially expressed proteins.

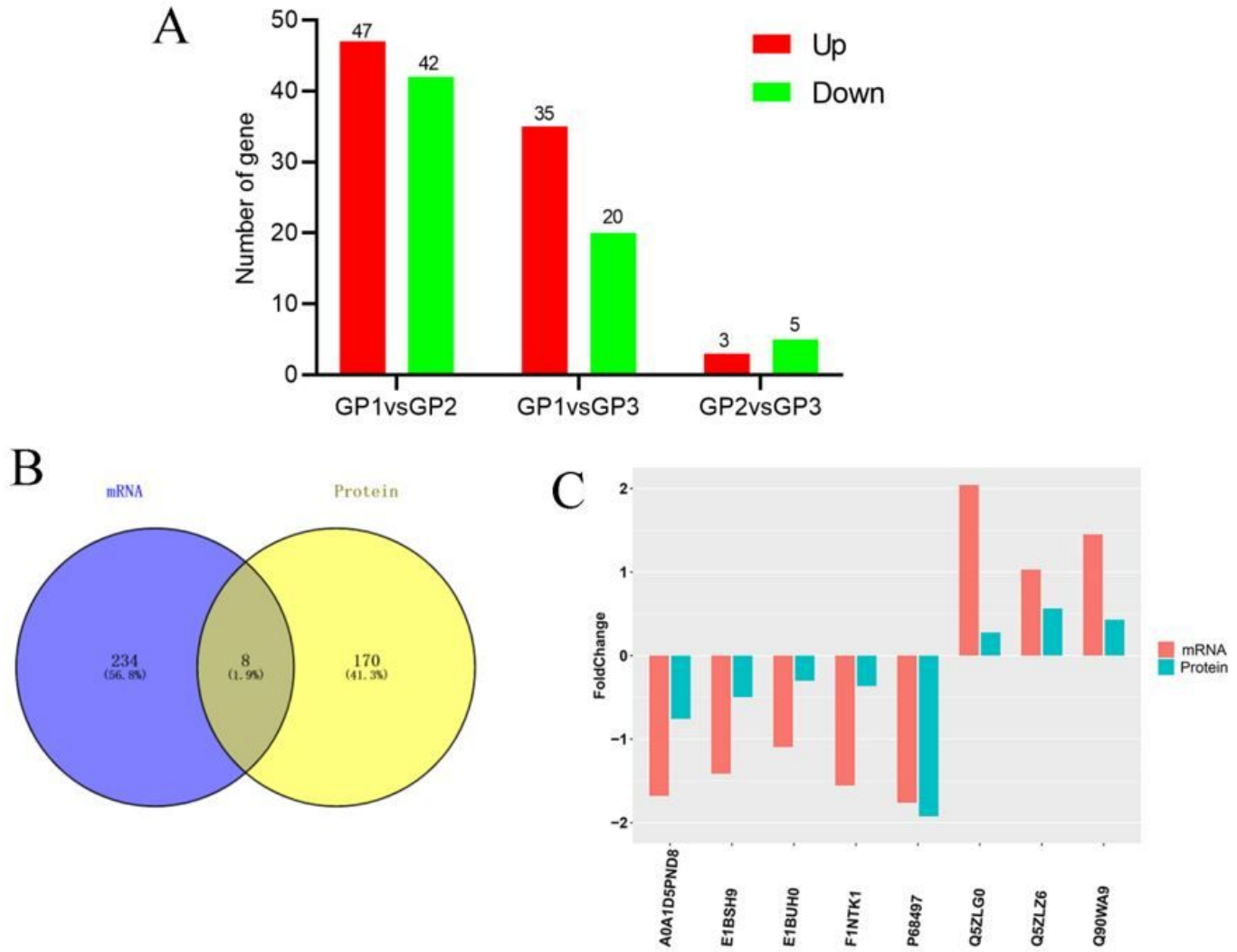

Figure 6

Results of differential expression of proteins and related transcripts. A: The histogram of GP1, GP2 and GP3 differentially expressed proteins and their related transcripts. B: The Venn diagram of GP2vsGP3 differentially expressed proteins and their related transcripts. C: GP2 vs GP3 differentially expressed proteins and related transcripts.

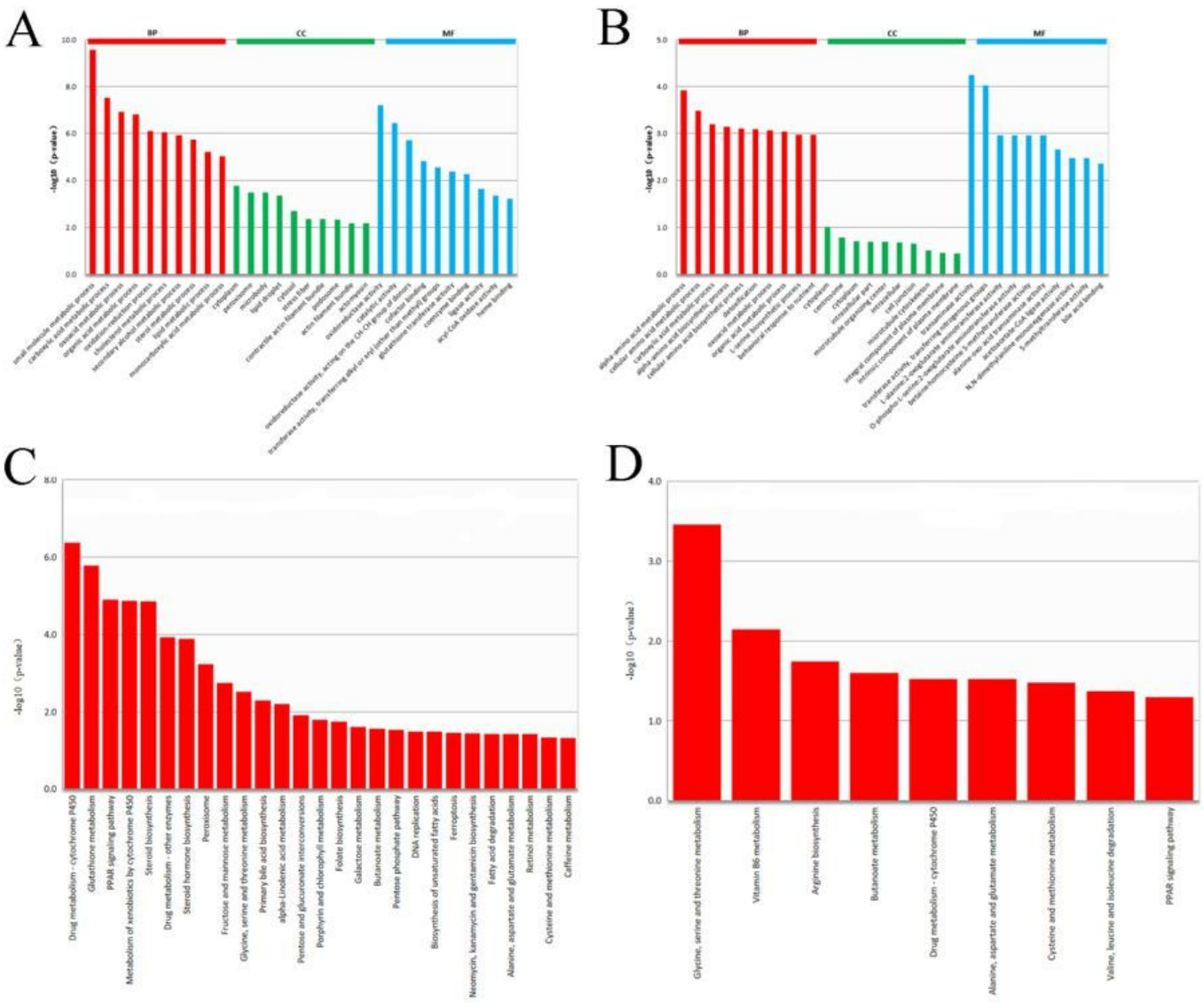

Figure 7

Histogram of GO enrichment and KEGG enrichment analysis. A: GO enrichment histogram of GP1vsGP2 differentially expressed proteins and their related transcripts. B: GO enrichment histogram of GP2vs GP3. C: KEGG enrichment histogram of GP1vsGP2 differentially expressed protein and its related transcripts. D: KEGG enrichment histogram of GP2 vs GP3.

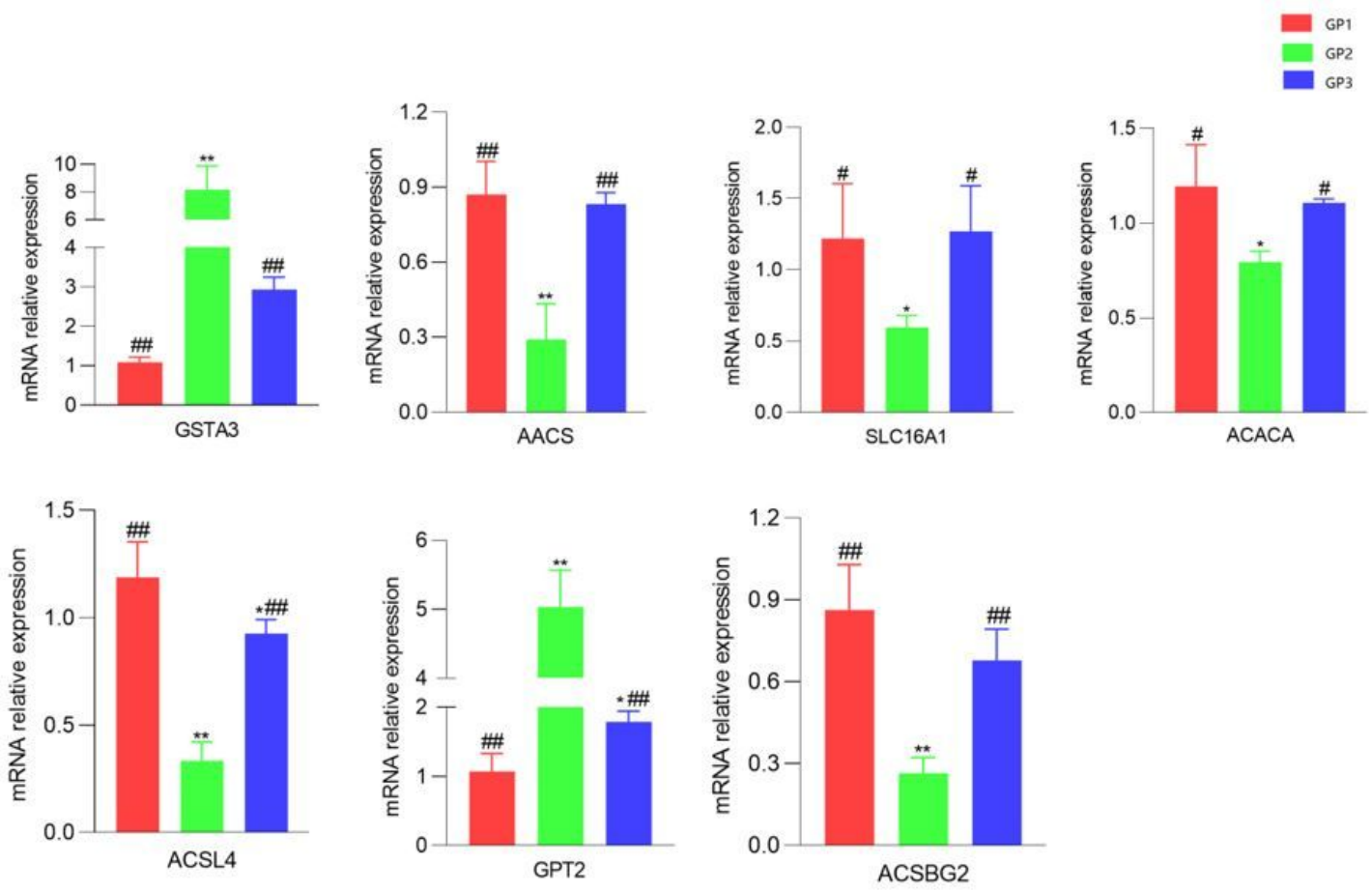

Figure 8

The relative expression levels of GSTA3, AACS, SLC16A1, ACACA, ACSL4, GPT2 and ACSBG2 in liver tissue of broilers of the three groups.