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Hepatic Proteomics Analysis Reveals Attenuated Endoplasmic Reticulum Stress in *Lactiplantibacillus plantarum*-Treated Oxidatively Stressed Broilers

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ABSTRACT: Endoplasmic reticulum (ER) stress plays important roles in oxidative stress (OS), contributing to liver injury. *Lactiplantibacillus plantarum* P8 (P8) was reported to regulate broiler OS and the gut microbiota in broilers, but its roles in hepatic ER stress remain unclear. In the present study, the role of P8 in liver OS and ER stress was evaluated, and proteomics was performed to determine the mechanism. Results revealed that P8 treatment decreased liver OS and ER stress in dexamethasone (DEX)-induced oxidatively stressed broilers. Proteomics showed that differentially expressed proteins (DEPs) induced by DEX cover the "cellular response to unfold protein" term. Moreover, the DEPs (GGT5, TXNDC12, and SRM) between DEX- and DEX + P8-treated broilers were related to OS and ER stress and enriched in the glutathione metabolism pathway. RT-qPCR further confirmed the results of proteomics. In conclusion, P8 attenuates hepatic OS and ER stress by regulating GGT5, TXNDC12, SRM, and glutathione metabolism in broilers.

KEYWORDS: *Lactiplantibacillus plantarum, broiler liver, endoplasmic reticulum stress, oxidative stress, proteomics*

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■ INTRODUCTION

Liver is an important organ that has an important effect on nutrient homeostasis by metabolizing carbohydrates, proteins, and fats.[1] Moreover, it is also the main target of oxidative stress (OS), as the liver gets exposed to a variety of prooxidant toxic compounds and has the metabolic ability to transform xenobiotics into reactive toxic metabolites.[2] OS is a conjoint pathological mechanism, resulting in the initiation and progression of liver injury.[3]

OS is induced by the disequilibrium between reactive oxygen species (ROS) production and antioxidation. OS and ROS productions are considered integral components of endoplasmic reticulum (ER) stress. The redox status in the ER lumen can affect protein folding and the formation of disulfide.[4] ER stress has been characterized as an early or initial response of cells to injury or stress.[5] The unfolded protein response (UPR) can be induced to restore the protein folding capacity of ER via accumulating the unfolded proteins in the lumen of ER, which initiates cell death or cytoprotective responses.[6] Glucose-regulated protein 78 (GRP78), a main regulator for ER stress, is able to regulate the activation of transmembrane ER stress sensors, including protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6) through a binding−release mechanism.[7,8] Like other secretory cells, hepatocytes are also rich in ER. The strong protein synthesis capacity of hepatocytes makes them easy to be affected by UPR/ER stress, which leads to the prevention or the mediation of pathological changes in different liver diseases.[9]

Due to high temperatures and toxic substances in the environment, water, and feed, chickens are vulnerable to OS, leading to liver injury and ER stress.[10,11] Studies have proved that liver damage is directly correlated with growth retardation and diseases.[12−14] Thus, it is important to protect the liver against OS to enhance the health status of chickens. Portal vein blood, the main source of liver blood, flows from the intestine. When intestinal barrier dysfunction occurs and permeability is elevated, the liver can be exposed to various toxic factors and bacteria from the intestine.[15] The improvement of gut health, including the decrease of gut microbiota dysbiosis, can attenuate liver injury.[16] Probiotics are live microorganisms, which when given in sufficient quantities contribute to health benefits for the host.[17] It is suggested that probiotics have beneficial roles in intestinal disorder and protect the liver through the "gut−liver axis".[16,18,19] Dietary probiotics supplementation was shown to inhibit hepatic OS in chickens.[20−22] *Lactiplantibacillus plantarum* P8 (P8) is isolated from the natural fermented yogurt of herder's family.[23] Our previous study indicated that dietary supplementation of 1 × 10^8 CFU/g P8 improved the gut microbiota structure and elevated the intestinal antioxidant capacity and immune

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function of broilers.[23,24] Thus, it is hypothesized that P8 supplementation may also benefit the livers of broilers.

Although considerable research has investigated the antioxidant impacts of probiotics on broilers, there is still a scarcity of information about their impacts on ER stress. As a glucocorticoid analogue, dexamethasone (DEX) has been widely used to simulate OS in broilers.[25] Thus, in this study, OS was also induced by DEX injection, so as to explore the roles of dietary P8 administration in liver OS and ER stress of broilers. Moreover, the tandem mass tag (TMT)-based quantitative proteomic was used to determine differences in hepatic protein profiles between P8-treated and untreated broilers. Research results are expected to provide insights into the novel mechanism of the antioxidant and anti-ER stress roles of *L. plantarum* in chicken livers.

MATERIALS AND METHODS

Materials. The probiotic P8 (1×10^{11} CFU/g) powder and DEX injection were purchased from Beijing SciTop Biotechnology Co., Ltd. (Beijing, China) and Feilong Animal Pharmaceutical Factory of Beian City (Heilongjiang, China), respectively.

Experimental Design. A total of 400 one-day-old male Cobb 500 broilers with similar initial body weights (43.71 ± 0.26 g) were provided by the Henan Academy of Agricultural Sciences for a 21-day experimental period. The broilers were randomly allotted to four treatment groups (Con, DEX, P8, and DEX + P8 groups) with 10 replicates per group (10 broilers per replicate). Broilers in the Con and DEX groups were fed a basal diet, formulated in accordance with the National Research Council (NRC, 1994; Table 1). Broilers in the P8 and DEX + P8 groups were fed a basal diet containing 1×10^8 CFU/g P8. At 16 days of age, broilers in the DEX and DEX + P8 groups were injected with DEX (3 mg/kg, 200 μL),[26] whereas broilers in the Con group were injected with the same amount of saline. Broilers were fed ad libitum and had free access to fresh water and the management was performed as previously described.[23] The experiment was performed according to the guidelines of the Ethics and Animal Welfare Committee of Qingdao Agricultural University.

Table 1. Composition and Nutrient Levels of Basal Diets (Air-Dry Basis) %

item	content
ingredients	
corn	61.38
soybean meal	31.00
fish meal	2.00
soybean oil	2.00
limestone	1.50
$CaHPO_4$	1.30
DL-Met	0.22
NaCl	0.30
premix[a]	0.20
choline chloride	0.10
total	100.00
nutrient levels[b]	
ME/(MJ/kg)	12.47
CP (%)	20.55
CF (%)	4.59
Ca (%)	0.95
AP (%)	0.46
Lys (%)	1.10
Met (%)	0.55
total sulfur amino acids (%)	0.84
Try (%)	0.26
Thr (%)	0.79

[a]The premix provided the following per kg of diets: VA 8000 IU, VB_1 4.2 mg, VB_2 4 mg, VB_6 4.5 mg, VB_{12} 0.02 mg, VD_3 3000 IU, VE 20 IU, VK_3 2 mg, biotin 0.15 mg, folic acid 1.0 mg, D-pantothenic acid 11 mg, nicotinic acid 10 mg, Cu (as copper sulfate) 10 mg, Fe (as ferrous sulfate) 80 mg, Mn (as manganese sulfate) 80 mg, Zn (as zinc sulfate) 75 mg, I (as potassium iodide) 0.40 mg, Se (as sodium selenite) 0.30 mg. [b]The nutrient levels were calculated values.

Sample Collection. At day 22, one chicken from each replicate was randomly selected after 12 h fasting. Liver samples were collected, immediately snap-frozen in liquid nitrogen, and then stored at −80 °C.

Analysis of Biochemical Indices. The contents of hepatic malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), oxidized glutathione (GSSG), and glutathione peroxidase (GSH-Px) were detected using ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, China) according to the manufacturer's protocol.[27] The absorbance was read by a microplate reader (SpectraMax iD3, Molecular Devices, Shanghai, China).

Real-Time Quantitative PCR (RT-qPCR). Total RNA was extracted from the broiler liver by Trizol reagent (Tiangen Biochemical Technology Co., Ltd, Beijing, China), and the purity and concentration of RNA were measured by a spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA) and agarose gel electrophoresis. The mRNA expressions were determined by RT-qPCR using a TB Green Premix Ex Taq kit (TaKaRa) and a BioRad CFX96 Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA). Primer 5.0 and oligo 7.0 software were used for the PCR primer sequence design (Table 2). *β-actin* was used as the reference gene. The thermocycle protocol was: 95 °C for 30 s, 95 °C for 5 s with 40 cycles, and 60 °C for 34 s. Then, the purity of the PCR product was monitored by a melting curve. The $2^{-\Delta\Delta Cq}$ method was used to calculate target gene relative expressions.

Western Blotting. Proteins were isolated from the liver samples using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). Western blotting was then performed to measure the expressions of β-actin, GRP78, PERK, photoshop-PERK (p-PERK), and ATF4.[23] Protein samples were separated using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime Biotechnology, Shanghai, China). The protein was then transferred onto poly(vinylidene difluoride) (PVDF) membranes (Merck Millipore, Darmstadt, Germany). Thereafter, the PVDF membranes were incubated with primary antibodies against β-actin (Beyotime Institute of Biotechnology, Shanghai, China), GRP78 (Servicebio Technology Co., Ltd., Wuhan, China), PERK (Servicebio Technology Co., Ltd., Wuhan, China), p-PERK (Affinity Biosciences Co., Ltd., OH), and ATF4 (Servicebio Technology Co., Ltd., Wuhan, China). After rinsing with Tris-buffered saline−Tween 20, the membranes were further incubated with the HRP-labeled goat anti-rabbit IgG antibody (Beyotime Biotechnology, Shanghai, China). An electrochemiluminescence detection system (Invitrogen iBright FL1000, Thermo Fisher Scientific, New York) was used for protein detection. ImageJ software (National Institutes of Health) was used to quantitate relative protein expression.

TMT-Based Proteomic Analysis. The proteomic analysis was performed by Bioprofile Technology Co., Ltd. (Shanghai, China) as previously described.[28] Briefly, liver tissue was lysed in 200 μL of lysis buffer (4% SDS, 100 mM DTT, 150 mM Tris-HCl pH 8.0) and boiled. According to the FASP procedure, 200 μg of the protein from each sample was digested.[29] The peptide concentration was measured with OD_{280} by a spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA). Thereafter, the peptide was labeled with TMT (Thermo Fisher Scientific, Waltham, MA), and the TMT-labeled peptides were fractionated on an Agilent 1290 HPLC operating at 0.3 mL/min using a Waters XBridge BEH130 column (C18, 3.5 μm, 2.1 mm × 150 mm). The fractions were dried for nanoliquid chromatography−tandem mass spectrometry (LC−MS/MS) analysis according to a previous study.[28]

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Table 2. Primers Used for Real-Time PCR

gene	primer sequence (5′−3′)	product size (bp)	genebank no.
β-actin	F: TGATATTGCTGCGCTCGTTG R: AACCATCACACCCTGATGTCTG	127	NM_205518.1
GGT5	F: GAGAACCGAGAGCTTCCCAG R: GTTGAGTCAGGGTGACCAGG	134	XM_415237.7
SRM	F: GCACGTCGGAGATGGTTTTG R: ACCTTGGCAGCAGAGAATCC	169	XM_015297174.3
MT3	F: ACGTGTGGAGACAACTGC R: GCACACTTGGCACATCCT	195	NM_001097538.1
GPX3	F: CCTGCAGTACCTCGAACTGA R: CTTCAGTGCAGGGAGGATCT	133	NM_001163232.2
MAT1A	F: ACATTCAGGAGAATGGAGCGGTCA R: TCTCCAGCGTAACGGTTTCATCGT	87	NM_001199519.1
CPT1A	F: ACTCTTCTCGGGACGGAAGC R: GTGGCCGGACTGATTCCAGA	113	NM_001012898.1
ACOX1	F: CCAGTCAGCTTGGTAGAGGC R: AGTGACAGTGTGCCTCAGATG	16	NM_001006205
ORM1	F: GAAGTACGCGACCTTCACCC R: CTCAGGCCCACGTGGTTATC	237	NM_204541.3
TXNDC12	F: GGCTTGATGCCTTGACGCT R: TCAAAGACCACCACGACGAA	97	NM_001282295.2
TGM2	F: TACAACTCTGCCCACGACAC R: GCAGTAAACCCCTTCGCTCT	210	NM_001391996.2
GRP78	F: CGCGACGGTTCGTGTGT R: GGTTGGATGTCAGCTGGTTCT	294	NM_205491.2
eIF2α	F: CGGCCGTCGCCAGAATG R: GACCACGACACATTCGTTCC	233	NM_001006477.2
ATF4	F: TCTGCAACCATGGCGTTTT R: ACTTTCCTCAGCCACCGAAC	190	NM_204880.3
ATF6	F: CCCTCGCACTTAGCACAGAA R: ATTGCTCCTTGCCTTCCTCC	219	XM_422208.8
ERN1	F: AGCCCCGGGAACACCA R: AACTGCCATCGTTTGGGTCT	190	NM_001285499.2

The LC−MS/MS raw files were identified using Proteome Discoverer 3.0 software. Differentially expressed proteins (DEPs) were identified by fold change (FC) > 2 or <0.5 and $P < 0.05$. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used for functional enrichment analysis for DEPs. False discovery rate (FDR) correction for multiple testing was also performed. Data were available via ProteomeXchange with the identifier PXD040369.

Statistical Analysis. All data were analyzed and graphed by GraphPad Prism 8 (GraphPad Software, La Jolla, CA) and were expressed as the mean and standard error of the mean (SEM). SPSS (version 20.0) was used for one-way analysis of variance (ANOVA). When the data were significant ($P < 0.05$), Tukey's multiple comparison test was used to compare the mean values, and Duncan's multiple comparison test was used to compare the differences among the four groups.

■ RESULTS

Dietary P8 Supplementation Decreased the Liver OS of DEX-Treated Broilers. In comparison to the Con group, DEX treatment increased the concentrations of CAT, SOD, GSH-Px, GSH, GSSG, and MDA ($P < 0.01$) and decreased the GSH/GSSG ratio ($P < 0.01$); P8 treatment significantly increased the concentrations of SOD and GSH-Px ($P < 0.01$) and reduced the MDA level ($P < 0.01$). Moreover, DEX + P8 treatment significantly decreased the concentrations of SOD, GSH, GSSG, GSH-Px, and MDA ($P < 0.01$) and elevated the GSH/GSSG ratio compared to the DEX group ($P < 0.01$, Figure 1).

Dietary P8 Supplementation Decreased the Liver ER Stress of Oxidatively Stressed Broilers. The expressions of ER stress-related genes and proteins are shown in Figure 2. Compared to the Con group, DEX significantly increased the gene expressions of *GRP78*, *ATF4*, *ATF6*, *α* subunit of eukaryotic initiation factor 2 (*eIF2α*), and endoplasmic reticulum to nucleus signaling 1 (*ERN1*) ($P < 0.01$); P8 significantly decreased the gene expressions of *GRP78* and *eIF2α* ($P < 0.01$). Compared to the DEX group, DEX + P8 significantly downregulated the gene expressions of *GRP78*, *ATF4*, *ATF6*, *eIF2α*, and *ERN1* ($P < 0.01$) (Figure 2A). Western blotting further confirmed that the ER stress level was elevated in the DEX group, as reflected by the increased expressions of GRP78, PERK, p-PERK/PERK, and ATF4 ($P < 0.01$) in broilers exposure to DEX. Besides, compared to the DEX group, the GRP78, PERK, p-PERK/PERK, and ATF4 expressions were significantly decreased ($P < 0.01$, Figure 2B).

Proteome Changes in the Liver of P8-Treated Oxidatively Stressed Broilers. Proteomic analysis identified 5855 proteins. According to Figure 3, 749 DEPs between the DEX group and the Con group were identified, including 490 with decreased abundance and 259 with increased abundance. Moreover, 95 DEPs between the P8 group and the Con group were identified, including 53 with decreased abundance and 42 with increased abundance. Besides, 103 DEPs between the DEX + P8 group and the DEX group were identified, including 54 with decreased abundance and 49 with increased

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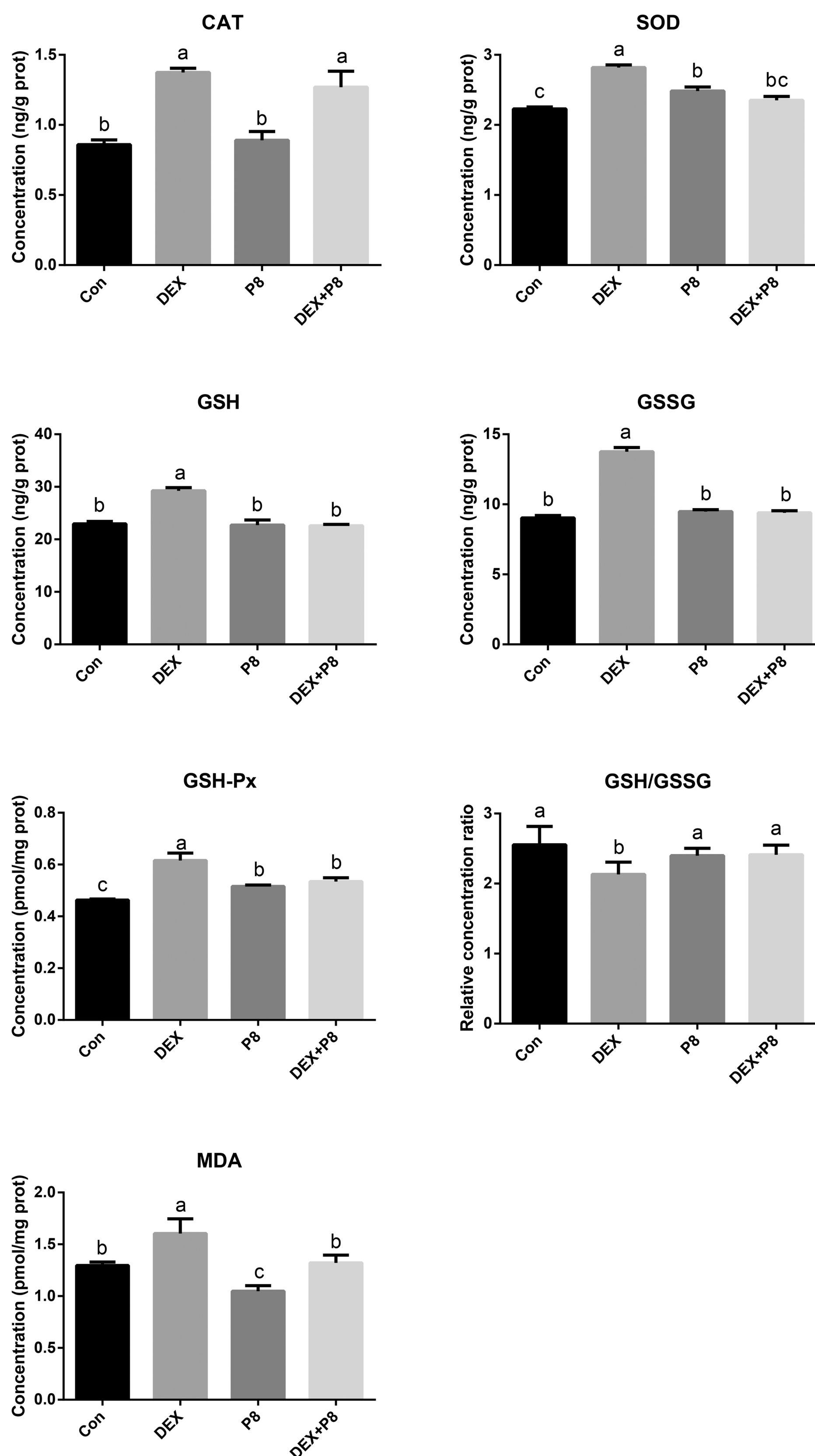

Figure 1. Effects of P8 on the hepatic antioxidant indices of oxidatively stressed broilers. [a,b,c]Mean values within a row with no common superscript differ significantly ($P < 0.05$). CAT = catalase, SOD = superoxide dismutase, GSH = glutathione, GSSG = oxidized glutathione, GSH-Px = glutathione peroxidase, MDA = malondialdehyde, Con = control diet, DEX = control diet plus dexamethasone injection, P8 = control diet containing 1×10^8 CFU/g *L. plantarum* P8, DEX + P8 = control diet containing 1×10^8 CFU/g *L. plantarum* P8 plus dexamethasone injection. n = 8 for each group.

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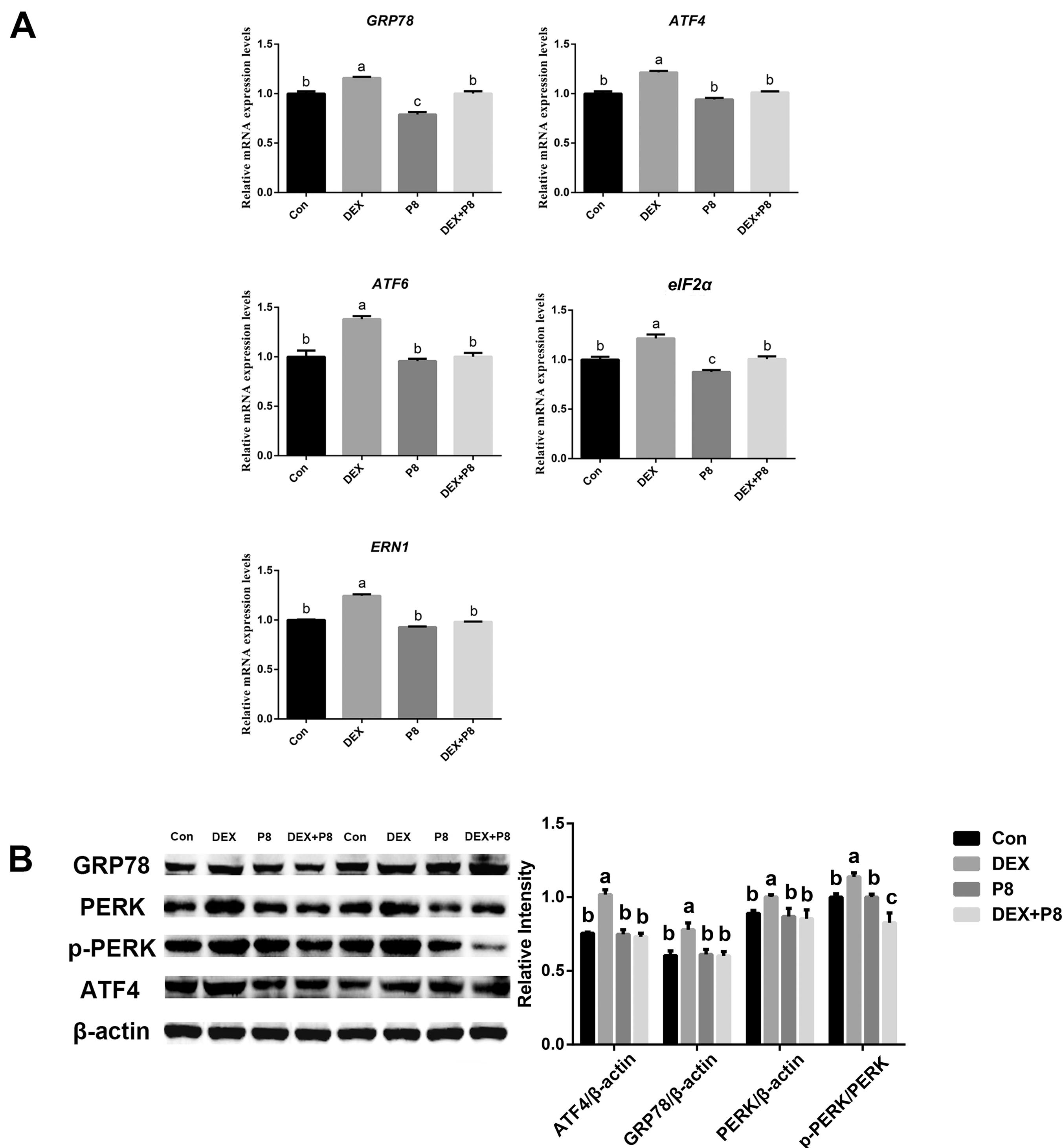

Figure 2. Effect of P8 on the expressions of ER stress-related genes and proteins of oxidatively stressed broilers. (A) The expressions of ER stress-related genes. (B) The expressions of ER stress-related proteins. [a,b,c]Mean values within a row with no common superscript differ significantly ($P < 0.05$). GRP78 = glucose-regulated protein 78, eIF2*α* = *α* subunit of eukaryotic initiation factor 2, ERN1 = endoplasmic reticulum to nucleus signaling 1, PERK = protein kinase RNA-like endoplasmic reticulum kinase, p-PERK = phospho-protein kinase RNA-like endoplasmic reticulum kinase, ATF4 = activating transcription factor 4, ATF6 = activating transcription factor 6, Con = control diet, DEX = control diet plus dexamethasone injection, P8 = control diet containing 1×10^8 CFU/g *L. plantarum* P8, DEX + P8 = control diet containing 1×10^8 CFU/g *L. plantarum* P8 plus dexamethasone injection. $n = 6$ for each group.

abundance. Here, the DEPs with FCs > 2 or <0.5 were selected. Compared to the Con group, the DEX injection upregulated the expressions of Q5ZJ02 (ZNF326, $P < 0.05$), A0A3Q8WI14 (GPD1, $P < 0.05$), A0A1L1RU05 (REEP6, $P < 0.01$), Q4ADJ6 (TF, $P < 0.01$), Q02020 (FGB, $P < 0.05$), Q5F3V6 (MFAP3, $P < 0.01$), Q8JIG5 (ORM1, $P < 0.05$), P14448 (FGA, $P < 0.05$), E1BQC2 (TF, $P < 0.01$), and E1C735 (MAT1A, $P < 0.05$) and downregulated the expressions F1NMN3 (LACTB, $P < 0.01$), E1BTQ4 (AVDL, $P < 0.05$), F1P296 (CGTL, $P < 0.01$),

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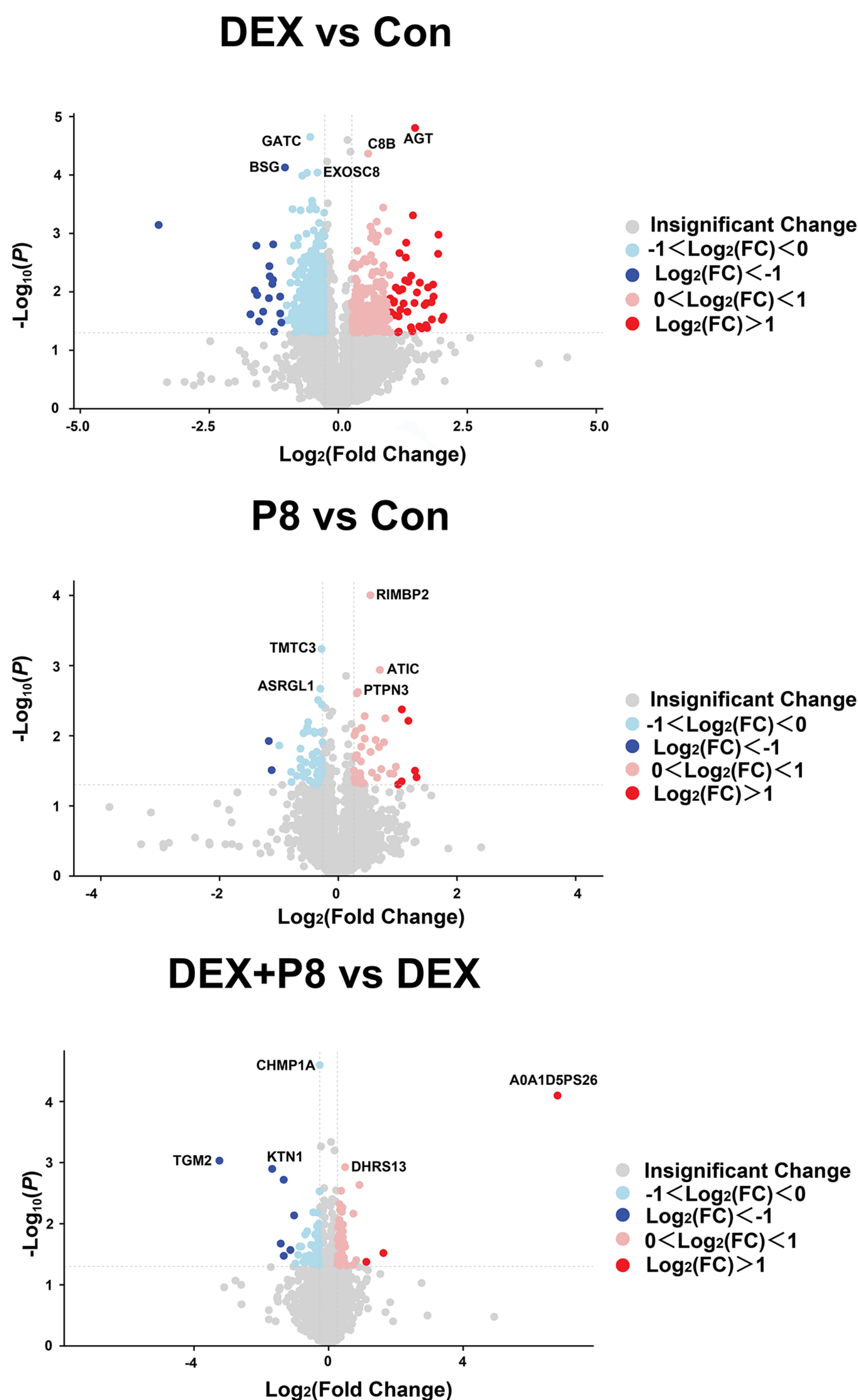

Figure 3. DEPs in the liver of broilers. DEPs = differentially expressed proteins, Con = control diet, DEX = control diet plus dexamethasone injection, P8 = control diet containing 1×10^8 CFU/g *L. plantarum* P8, DEX + P8 = control diet containing 1×10^8 CFU/g *L. plantarum* P8 plus dexamethasone injection. $n = 3$ for each group.

A0A1D5PHV2 (AVR2, $P < 0.05$), A0A1D5PVF8 (ACP5, $P < 0.05$), A0A1D5PQ51 (SLC22A5, $P < 0.05$), E1C9D2 (LOC416086, $P < 0.05$), Q6B842 (CPT1A, $P < 0.05$), P79787 (ACAN, $P < 0.01$), and P07354 (HAPLN1, $P < 0.01$) (Table 3); the P8 treatment significantly upregulated the expressions of F1CN05 ($P < 0.05$), R4GK63 (GBP, $P < 0.05$), E5G6H7 (BST1, $P < 0.01$), A0A3Q2TXQ0 ($P < 0.05$), A0A3Q2UGI9 (URAH, $P < 0.01$), and A0A1L1RS51 (LOC112531693, $P < 0.05$) and downregulated the expressions of A0A1D5PHV2 (AVR2, $P < 0.05$) and Q90864 (HBE1, $P < 0.05$) (Table 4). Compared to the DEX group, the DEX + P8 treatment upregulated the expressions of A0A1D5PS26 ($P < 0.01$), F1P490 (CLCA1, $P < 0.05$), and A4PBT0 (MT3, $P < 0.05$) and downregulated the expressions of Q01841 (TGM2, $P < 0.01$), E1C2P9 (KTN1, $P < 0.01$), E1BUH0 (LOC100857694, $P < 0.05$), A0A3Q2U474 ($P < 0.01$), A0A3Q2U7L8 ($P < 0.05$), A0A3Q2UJK8 ($P < 0.05$), and A2N883 (VH1, $P < 0.01$) (Table 5).

Bioinformatic Analysis of DEPs. GO terms were grouped into categories including biological processes (BP), molecular

Table 3. DEPs between DEX and Con Groups[a]

accession	gene symbol	log_2 FC	P value	threshold
Q5ZJ02	ZNF326	2.04	<0.05	up
A0A3Q8WI14	GPD1	2.01	<0.05	up
A0A1L1RU05	REEP6	1.94	<0.01	up
Q4ADJ6	TF	1.94	<0.01	up
Q02020	FGB	1.85	<0.05	up
Q5F3V6	MFAP3	1.84	<0.01	up
Q8JIG5	ORM1	1.82	<0.05	up
P14448	FGA	1.81	<0.05	up
E1BQC2	TF	1.74	<0.01	up
E1C735	MAT1A	1.72	<0.05	up
F1NMN3	LACTB	−3.48	<0.01	down
E1BTQ4	AVDL	−1.70	<0.05	down
F1P296	CGTL	−1.62	<0.01	down
A0A1D5PHV2	AVR2	−1.59	<0.05	down
A0A1D5PVF8	ACP5	−1.57	<0.05	down
A0A1D5PQ51	SLC22A5	−1.53	<0.05	down
E1C9D2	LOC416086	−1.46	<0.05	down
Q6B842	CPT1A	−1.34	<0.05	down
P79787	ACAN	−1.33	<0.01	down
P07354	HAPLN1	−1.33	<0.01	down

[a]DEPs = differentially expressed proteins, Con = control diet, DEX = control diet plus dexamethasone injection, FC = fold change, n = 3 for each group.

Table 4. DEPs between P8 and Con Groups[a]

accession	gene symbol	log_2 FC	P value	threshold
F1CN05		1.32	<0.05	up
R4GK63	GBP	1.29	<0.05	up
E5G6H7	BST1	1.18	<0.01	up
A0A3Q2TXQ0		1.07	<0.05	up
A0A3Q2UGI9	URAH	1.07	<0.01	up
A0A1L1RS51	LOC112531693	1.01	<0.05	up
A0A1D5PHV2	AVR2	−1.17	<0.05	down
Q90864	HBE1	−1.12	<0.05	down

[a]DEPs = differentially expressed proteins, Con = control diet, P8 = control diet containing 1 × 10^8 CFU/g *L. plantarum* P8, FC = fold change, n = 3 for each group.

Table 5. DEPs between DEX + P8 and DEX Groups[a]

accession	gene symbol	log_2 FC	P value	threshold
A0A1D5PS26		6.80	<0.01	up
F1P490	CLCA1	1.63	<0.05	up
A4PBT0	MT3	1.12	<0.05	up
Q01841	TGM2	−3.25	<0.01	down
E1C2P9	KTN1	−1.68	<0.01	down
E1BUH0	LOC100857694	−1.42	<0.05	down
A0A3Q2U474		−1.34	<0.01	down
A0A3Q2U7L8		−1.33	<0.05	down
A0A3Q2UJK8		−1.14	<0.05	down
A2N883	VH1	−1.02	<0.01	down

[a]DEPs = differentially expressed proteins, DEX = control diet plus dexamethasone injection, DEX + P8 = control diet containing 1 × 10^8 CFU/g *L. plantarum* P8 plus dexamethasone injection, FC = fold change, n = 3 for each group.

functions (MF), and cellular components (CC). As shown in Figure 4, the annotated GO terms of DEPs between the DEX group and the Con group mainly covered "cellular response to unfolded protein" (BP), "de novo protein folding" (BP), "nuclear pore" (CC), "microtubule" (CC), "site of DNA damage" (CC), "catalytic activity" (MF), "hydrolase activity" (MF), and "pyrophosphatase activity" (MF). The annotated GO terms of DEPs between the P8 group and the Con group mainly covered "regulation of gene expression, epigenetic" (BP), "chromatin organization involved in regulating of transcription" (BP), "organic anion transport" (BP), "flotillin complex" (CC), "condensed nuclear chromosome" (CC), "yolk" (CC), "phosphoribosylaminoimidazolecarboxamide formyltransferase activity" (MF), "protein heterodimerization activity" (MF), and "protein dimerization activity" (MF). The annotated GO terms of DEPs between the DEX + P8 group and the DEX group mainly covered "organelle fusion" (BP), "monocarboxylic acid metabolic process" (BP), "peptide cross-linking" (BP), "phagocytic vesicle" (CC), "endomembrane system" (CC), "trans-Golgi network membrane" (CC), "lyase activity" (MF), "transferase activity, transferring amino-acyl groups" (MF), and "protein-glutamine γ-glutamyltransferase activity" (MF).

KEGG pathway analysis for the DEPs showed significantly enriched pathways, as presented in Figure 5 and Table S1. The enriched pathways of DEPs (HEXA, ACOX1, HNRNPU, CTSC, CPT1A, etc.) between the DEX group and the Con group included "other glycan degradation", "peroxisome", "spliceosome", "lysosome", "mismatch repair", "fatty acid degradation", "glutathione metabolism", "valine, leucine and isoleucine degradation", "propanoate metabolism", and "glycosaminoglycan degradation". The enriched pathways of DEPs (ATIC, NTPCR, FABP1, etc.) between the P8 group and the Con group were "purine metabolism" and "PPAR signaling pathway". The enriched pathway of DEPs (GGT5, TXNDC12, SRM) between the DEX + P8 group and the DEX group was "glutathione metabolism".

RT-qPCR Validation. The gene expressions of some DEPs were verified by RT-qPCR. Results indicated that in comparison to the Con group, the expressions of *GGT5*, *MT3*, *TGM2*, *ACOX1*, and *CPT1A* decreased (P < 0.01), and the expressions of *SRM*, *TXNDC12*, *ORM1*, *GPX3*, and *MAT1A* upregulated (P < 0.01) in the DEX group. In comparison to the DEX group, the expressions of *GGT5*, *MT3*, *TGM2*, *ACOX1*, and *CPT1A* increased (P < 0.01), while the expressions of *SRM*, *TXNDC12*, *ORM1*, *GPX3*, and *MAT1A* decreased (P < 0.01) in the DEX + P8 group (Figure 6).

DISCUSSION

In modern poultry farming, broilers are exposed to a series of stressors, leading to liver injury.[30,31] OS and ER stress are involved in stress-induced liver injury,[10] greatly reducing the detoxification capacity, seriously affecting the animal's performance, and endangering the poultry industry.[32]

During OS, augmenting ROS can cause lipid peroxidation and DNA damage. MDA is the main product of the peroxidation of polyunsaturated fatty acids. MDA can be mutagenic and atherogenic by impairing DNA and proteins.[33] In the present study, the increased hepatic MDA content in the DEX group was decreased by the DEX + P8 treatment, indicating the decrease of OS in the broiler liver by P8 supplementation. With evolution, most living organisms possess an antioxidant defense system, including antioxidases and antioxidants. In the current study, with the injection of DEX, the concentrations of CAT, SOD, GSH-Px, GSH, and GSSG significantly increased, and the GSH/GSSG ratio decreased. Compared to the Con group, the P8 treatment

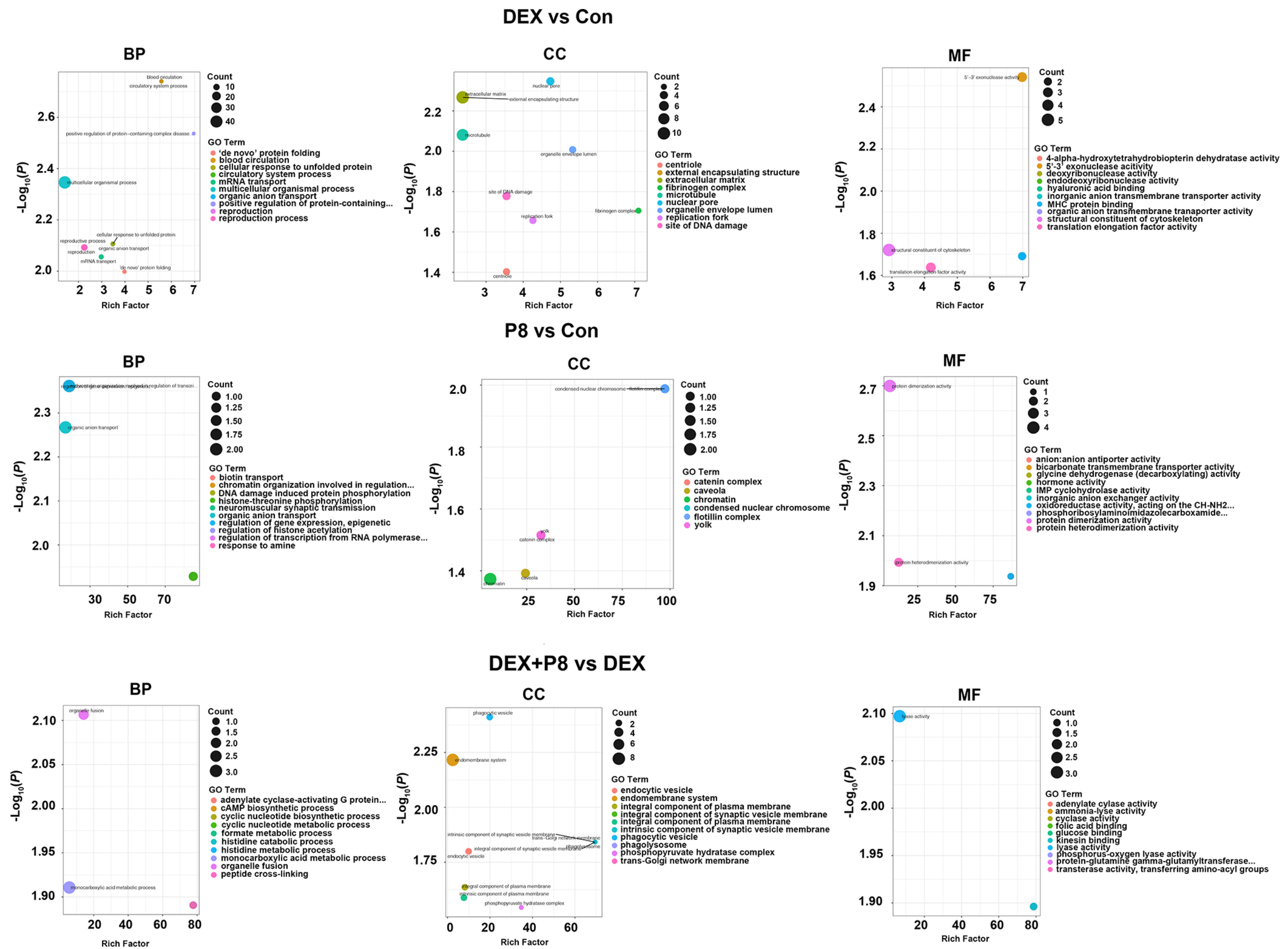

Figure 4. Top 10 GO terms. DEPs = differentially expressed proteins, BP = biological process, CC = cellular component, MF = molecular function, Con = control diet, DEX = control diet plus dexamethasone injection, P8 = control diet containing 1×10^8 CFU/g *L. plantarum* P8, DEX + P8 = control diet containing 1×10^8 CFU/g *L. plantarum* P8 plus dexamethasone injection. $n = 3$ for each group.

also increased the levels of GSH-Px and SOD. Moreover, the DEX + P8 treatment significantly reduced the concentrations of GSH-Px, SOD, GSH, and GSSG, and increased the GSH/GSSG ratio in comparison to the DEX group. Although studies demonstrated that DEX injection could result in decreased antioxidase activities,[25,34] it is shown that at the beginning of the ROS formation process, the antioxidases (including SOD, CAT, and GSH-Px) and antioxidants (including GSH) play important roles in ROS detoxification.[30] Thus, the increased levels of CAT, SOD, GSH-Px, GSH, and GSSG in the DEX group implied that broilers protect against DEX-induced OS through increasing concentrations of antioxidases. Judge et al.[35] also reported that exposure to oxidants could act as a signal to increase the activity and expression of antioxidases. In addition, the increased levels of antioxidases in broilers receiving P8 indicated the enhanced antioxidant capacity of broilers. Moreover, the decreased SOD, GSH-Px, and GSH contents in the DEX + P8 group suggested that dietary P8 supplementation renders broilers more resistant to subsequent stress; therefore, it is not necessary for broilers to express more antioxidases after DEX injection. Similarly, the study also demonstrated that with the pretreatment of probiotic *Bacillus amyloliquefaciens*, subsequent H_2O_2 induction downregulated antioxidase activities compared to the H_2O_2 treatment group.[36]

ER stress is a cell response to various stress factors.[5] It is unveiled that ER stress plays vital roles in the pathogenesis of liver diseases.[37] Yao et al.[10] also reported that OS and ER stress played an important role in chicken hepatocyte apoptosis caused by Se deficiency. The induction of ER stress is sensed by three transmembrane proteins, including PERK, inositol requiring 1 (IRE1), and ATF6, which activate the ER UPR through the upregulation of an ER-resident chaperon, termed GRP78.[38,39] Besides, the PERK-eIF2α-ATF4 pathway is also reported to mediate the ER stress response.[40] Moreover, ERN1 is the most evolutionary conversed and important sensor of the UPR to accumulate the misfolded proteins.[41] Hence, we measured the expressions of genes related to ER stress (*GRP78*, *eIF2α*, *ATF4*, *ATF6*, and *ERN1*). In comparison to the Con group, DEX injection significantly upregulated the expressions of the above genes, implying the elevation of ER stress; P8 significantly decreased the gene expressions of *GRP78* and *eIF2α*. DEX + P8 also significantly downregulated the gene expressions of *GRP78*, *eIF2α*, *ATF4*, *ATF6*, and *ERN1* in comparison to the DEX group. Similarly, the upregulated protein expressions of ATF4, GRP78, PERK, and p-PERK in the DEX group were also downregulated by the DEX + P8 treatment. These findings indicate that DEX injection caused hepatic ER stress, whereas the supplementa-

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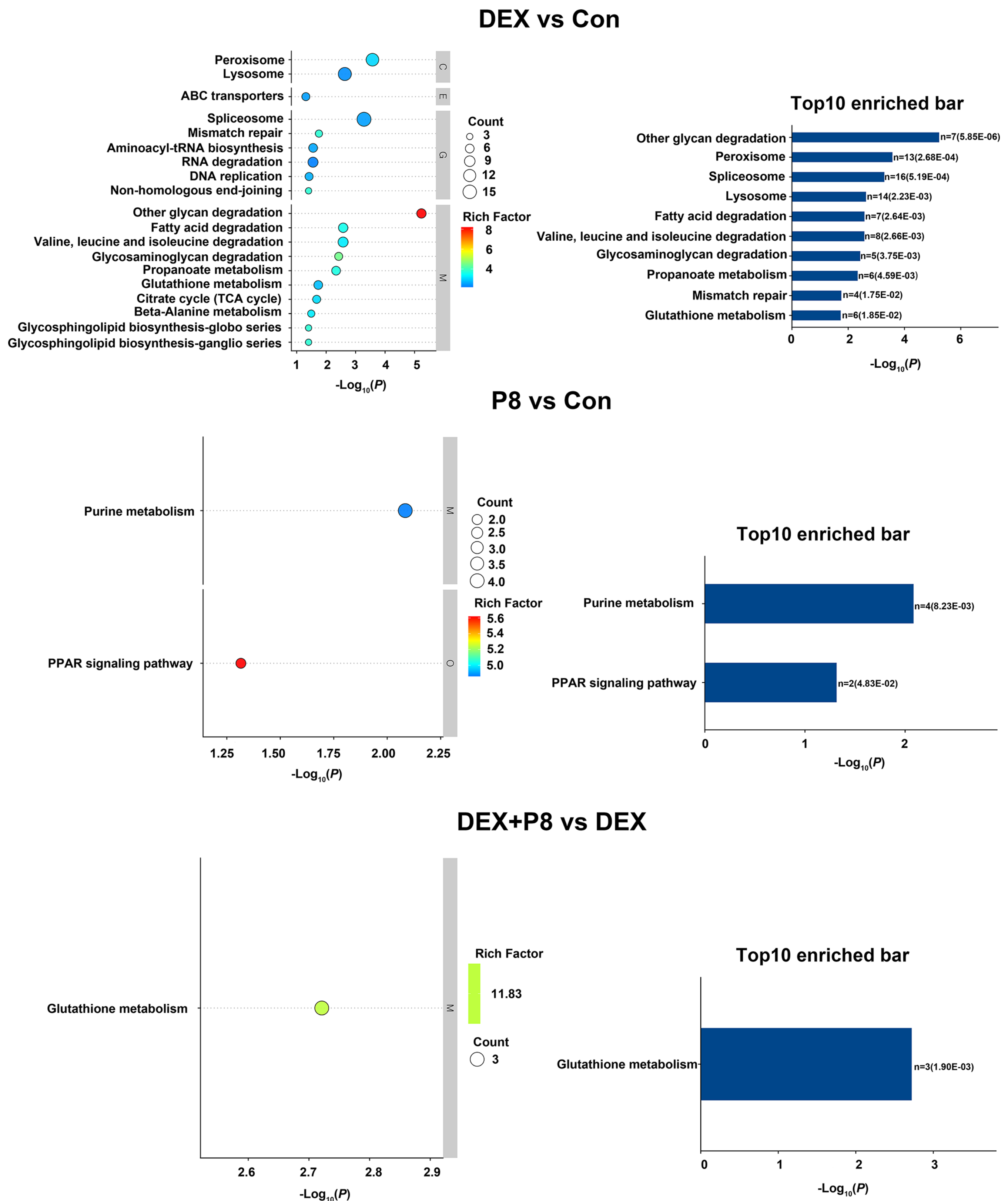

Figure 5. Top 10 KEGG pathways. DEPs = differentially expressed proteins, Con = control diet, DEX = control diet plus dexamethasone injection, P8 = control diet containing 1×10^8 CFU/g *L. plantarum* P8, DEX + P8 = control diet containing 1×10^8 CFU/g *L. plantarum* P8 plus dexamethasone injection. $n = 3$ for each group.

tion of P8 in broilers with or without DEX injection attenuated hepatic ER stress.

Proteins, approximately accounting for 18% of animal body weight, are vital cell components and have important effects on many biological activities.[42] Here, the liver proteins were

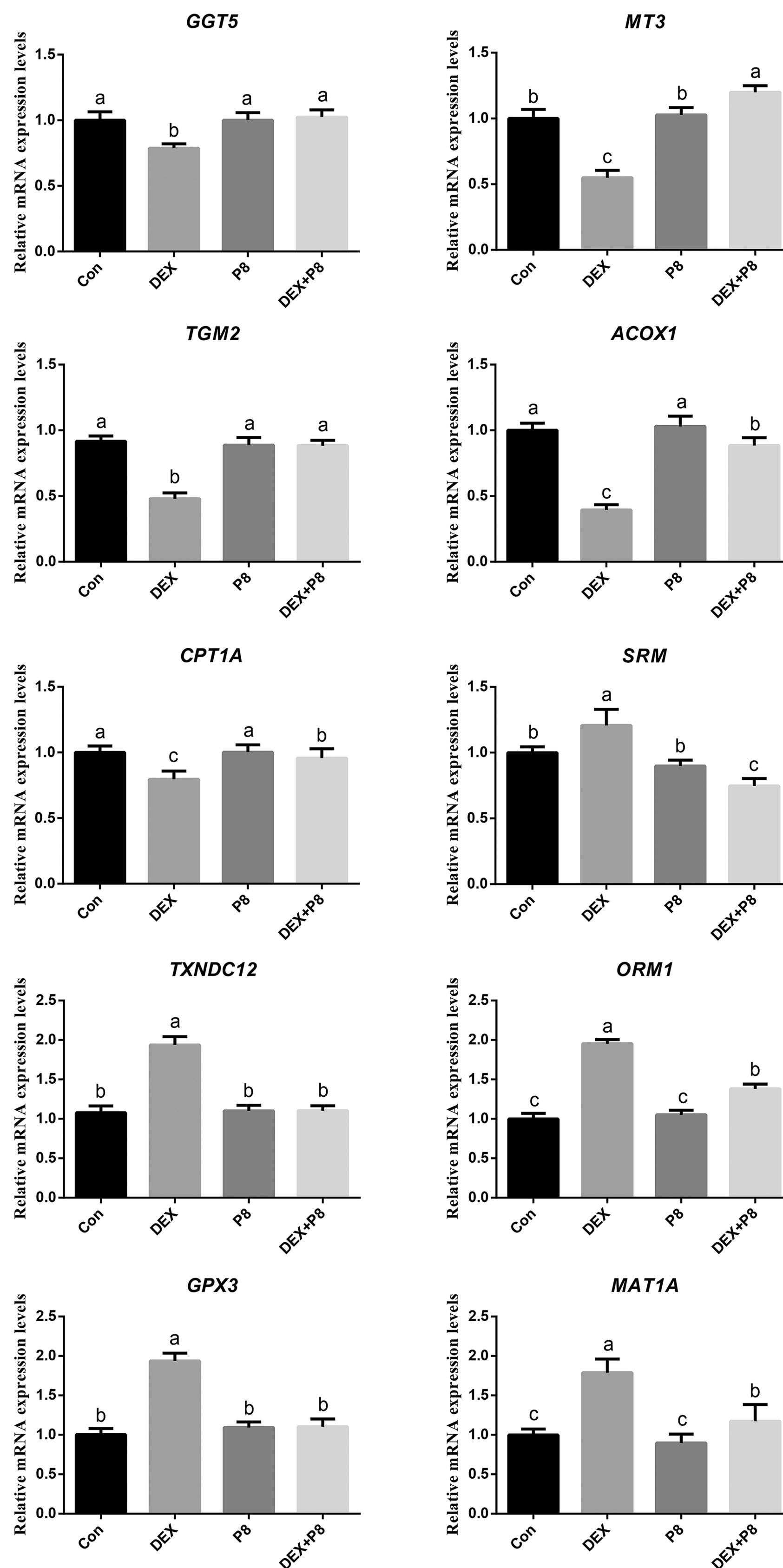

Figure 6. Validation of DEPs by RT-qPCR. GGT5 = γ-glutamyltransferase 5, MT3 = metallothionein 3, TGM2 = transglutaminase type 2, ACOX1 = acyl-coenzyme A oxidase 1, CPT1A = carnitine palmitoyl transferase 1A, SRM = spermidine synthase, TXNDC12 = thioredoxin domain-containing protein 12, ORM1 = α-1 acid glycoprotein 1, GPX3 = glutathione peroxidase 3, MAT1A = methionine adenosyltransferase 1A, Con = control diet, DEX = control diet plus dexamethasone injection, P8 = control diet containing 1×10^8 CFU/g *L. plantarum* P8, DEX + P8 = control diet containing 1×10^8 CFU/g *L. plantarum* P8 plus dexamethasone injection. $n = 6$ for each group.

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identified by TMT-labeled proteomics. A total of 5855 proteins were identified in broiler livers. Between DEX and Con groups, the DEPs mainly included GPD1, REEP6, ORM1, AVDL, CPT1A, etc. The DEPs between P8 and Con groups mainly included GBP, BST1, URAH, AVR2, HBE1, etc. The DEPs between DEX + P8 and DEX groups mainly included CLCA1, MT3, TGM2, VH1, etc. Some of the DEPs are associated with OS and ER stress. For instance, ORM1, also known as α-1 acid glycoprotein (AGP), is increased during inflammation and stresses. ORM1 is mainly synthesized in the liver and is induced by glucocorticoids and proinflammatory cytokines.[43] CPT1A, the liver isoform of carnitine palmitoyl transferase 1, is a rate-limiting enzyme of fatty acid β-oxidation, catalyzing the conversion of long-chain acyl-CoA into acylcarnitine, allowing the entry of long-chain acyl-CoA into mitochondria.[44] Namgaladze et al.[45] showed that CPT1A knockdown in macrophages exacerbated the proinflammation and ER stress after palmitate treatment. The increased ORM1 and decreased CPT1A expressions in the DEX group implied the increased OS and ER stress in the broiler liver after DEX injection. Besides, BST1 removes the inositol acyl group, which is required for quality control of the transport of glycosylated phosphatidylinositol-anchored proteins from the ER to the Golgi.[46] Labunskyy et al.[47] reported that the deletion of BST1 can activate UPR. The increased BST1 in the P8 group suggests that P8 supplementation may inactivate UPR, thereby regulating ER stress. Moreover, MT3 can act as electrophilic scavengers[48] and has a protective effect on liver injury induced by organic chemicals.[49] TGM2 is expressed by almost all cell types in the body and is mainly localized in the cytosol.[50] It is reported that TGM2 can be induced by ER stress and translocate to the ER.[51] Thus, the increased MT3 and decreased TGM2 in the DEX + P8 group indicated that the supplementation of P8 protected against DEX-induced ER stress and liver injury.

Further, GO analysis showed that the DEPs between the DEX and Con groups were mostly enriched in the "multi-cellular organismal process", "cellular response to unfolded protein", and "site of DNA damage", indicating that the DEX injection led to UPR and damage to hepatocytes. Most of the DEPs between P8 and Con were enriched in the "regulation of gene expression, epigenetic", "chromatin", and "protein dimerization activity", suggesting that P8 supplementation also had an effect on BP, CC, and MF. Moreover, the DEPs between the DEX + P8 and DEX groups were enriched in the "monocarboxylic acid metabolic process", "trans-Golgi network membrane", and "protein-glutamine γ-glutamyltransferase activity", suggesting that P8 may exert antioxidant and anti-ER stress capacities in DEX-induced oxidatively stressed broilers through regulating the glutamine system. Furthermore, KEGG analysis revealed that the DEPs between the DEX and Con groups were enriched in "other glycan degradation", "peroxisome", "glutathione metabolism", and so on. Among the significantly influenced pathways, glutathione metabolism was closely related to OS and ER stress.[52,53] The increased GPX3 expression in the enriched glutathione metabolism pathway was in line with the elevated hepatic GSH-Px concentration obtained by biochemical analyses in the current study. Moreover, the expression of ACOX1 in the peroxisome pathway was decreased by DEX treatment. It is suggested that ACOX1 knockout mice manifest hepatic metabolic impairment, leading to liver diseases, including steatohepatitis and hepatocellular carcinomas.[54] The decreased ACOX1 in the enriched peroxisome pathway implied liver damage by DEX injection. Furthermore, the DEPs between the P8 and Con groups were enriched in "purine metabolism" and "PPAR signaling pathway". Reports demonstrated that the OS and ER stress can be corrected by activating the PPAR signaling pathway.[55,56] The enriched PPAR signaling pathway in the P8 group indicated beneficial roles of P8 in antioxidation and anti-ER stress. Additionally, the DEPs (GGT5, TXNDC12, SRM) between the DEX + P8 and DEX groups were also enriched in "glutathione metabolism". Compared to the DEX group, the expression of GGT increased, and the expressions of TXNDC12 and SRM decreased in the DEX + P8 group. Although a study has suggested that GGT5 is associated with cancers,[57] analyses of *GGT5*-deficient mice showed that GGT5 is specifically responsible for the attenuation of the acute inflammatory response.[58] Moreover, GGT5 is also an essential component responsible for the catabolism of GSH;[59] thus, the increased GGT5 expression may be related to the GSH level alteration in the DEX + P8 group. TXNDC12, also known as ERp16, ERp18, ERp19, or hTLP19, is a member of the protein disulfide isomerase family that plays a vital role in cancer initiation and progression.[60,61] Jeong et al.[62] reported that TXNDC12 was able to aggravate apoptosis induced by ER stress. Upregulation of the SRM results in the increased content of polyamines, which are thought to have a significant effect on tumor development.[63] Therefore, the decreased expressions of TXNDC12 and SRM implied decreased ER stress and injury in the DEX + P8 group. Based on these findings, we found that the proteomics results were in good agreement with the biochemical results, further confirming that OS and ER stress induced by DEX can be attenuated by P8 supplementation. Moreover, glutathione metabolism played important roles in the antioxidant and anti-ER stress process in P8-treated oxidatively stressed broilers. The above-mentioned DEPs were verified by RT-qPCR, as a lack of antibodies against the target chicken proteins.

In conclusion, we demonstrated that P8 effectively decreased the hepatic OS and ER stress of DEX-induced broilers. Moreover, proteomics analysis indicated that GGT5, SRM, and TXNDC12, which were involved in the glutathione metabolism pathway, played important roles in the antioxidant and anti-ER stress process in P8-treated oxidatively stressed broilers.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c01534.

KEGG pathway enrichment analysis (PDF)

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Notes

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