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A Proteomic Approach to Investigating the Antioxidant Properties of Passion Fruit Seed Oil on Broiler Liver under Cyclic Heat Stress

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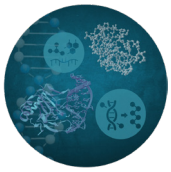
The impact of passion fruit seed oil (PFSO) supplementation on the liver proteome of broilers exposed to a cyclic heat stress protocol was the focus of this research. Five experimental diets (0, 0.30, 0.50, 0.70, and 0.90% PFSO) were fed to chickens, which underwent 8 h of daily thermal stress in a climate chamber throughout the trial. At 36 days, hepatic samples were procured from nine animals *per* group. The proteins were resolved via two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and the spots showing differential expression were identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The main discovery was that dietary supplementation with 0.30 and 0.90% PFSO led to increased levels of heat shock protein (HSPs), which provide essential cellular protection during stress. Simultaneously, a pronounced downregulation of antioxidant proteins was observed, suggesting a recalibration of the cellular defense mechanisms. Beyond stress proteins, significant changes were also noted in proteins governing lipid and carbohydrate metabolism, confirming the influence across multiple metabolic pathways of PFSO. These data indicate that PFSO boosted resistance to heat stress through the modulation of critical metabolic functions, presenting valuable implications for improving poultry welfare and performance in adverse environmental conditions.



Keywords: antioxidant proteins, broilers nutrition, carbohydrate metabolism, heat shock proteins, heat stress

Introduction

The industrial processing of passion fruit (*Passiflora* spp.) generates substantial amounts of by-product, primarily peels and seeds, which typically account for 65 to 70% of the total mass of the fruit.[1,2] Although often discarded as waste, these residues are known to contain important bioactive compounds capable of promoting health benefits, thereby positioning them as attractive ingredients for the development of functional foods.[3,4]

Earlier studies[5,6] have already highlighted the potential of utilizing passion fruit seed residues in the diets of commercial poultry, including broilers and laying hens, suggesting its role as a functional dietary constituent. Specifically, passion fruit seeds possess a considerable oil content recoverable through dedicated extraction methods. Passion fruit seed oil (PFSO) is remarkably rich in natural antioxidants and other bioactive compounds.[7-9] The strong antioxidant capacity of PFSO may offer protective benefits, particularly when animals face challenging and stressful conditions.

In industrial broiler production, high ambient temperatures represent a persistent challenge that culminates in heat stress, a condition that transcends the compromise of

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animal welfare to severely impact the qualitative attributes of the meat.[10,11] Whether acute or chronic in nature, this stress triggers metabolic disorders and alterations in blood amino acid profiles that directly affect *post-mortem* muscle biochemistry; for instance, the acceleration of anaerobic glycolysis promotes an abrupt drop in pH, resulting in anomalies such as PSE (pale, soft, exudative) meat, characterized by its pale coloration, flaccid texture, and low water-holding capacity. Concurrently, the exacerbation of reactive oxygen species (ROS) induces oxidative stress and subsequent cell membrane lipid peroxidation, which not only degrades the flavor and color of the cut but also accelerates oxidative rancidity, drastically reducing shelf life and the commercial value of the final product.[12-14]

The adverse effects of heat stress on poultry performance can be effectively managed by integrating natural feed ingredients rich in antioxidant compounds into the diets of exposed broilers.[15,16] To gain a thorough understanding of how these natural dietary components mediate molecular mechanisms and physiological adjustments during thermal stress, proteomic analysis offers a powerful and comprehensive investigative tool.[17,18]

In research[12,16,19,20] examining the impact of diet or exposure to stress conditions, the liver is a particularly crucial organ for proteomic investigations. This significance stems from its specialized functions within the avian organism: the liver plays a central role in maintaining systemic metabolism, encompassing protein and lipid synthesis, detoxification, and energy balance regulation in birds.

Consequently, this study was conducted to explore the effects of PFSO supplementation on the hepatic protein profile of broilers subjected to a model of cyclic heat stress. The analysis was performed using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Experimental

Reagents, standards, and experimental supplies

Ultrapure water (18.2 MΩ cm resistivity), obtained from an Elga LabWater PureLab Ultra system (Saint-Maurice, France), was used for all preparation and analysis steps. Key chemicals and standards included: acetone and sodium hydroxide (Merck, Rahway, NJ, USA); bovine serum albumin (BSA) and trypsin (Thermo Fisher Scientific, Waltham, MA, USA); dithiothreitol (DTT) and colloidal Coomassie Brilliant Blue G-250 (J.T. Baker, Phillipsburg, NJ, USA); and formic acid, trifluoroacetic acid, and high-performance liquid chromatography (HPLC)-grade acetonitrile (Sigma-Aldrich, St. Louis, MO, USA).

For the electrophoretic steps, prefabricated polyacrylamide gel strips (13 cm, pH 3-10), iodoacetamide, acrylamide, bis-acrylamide, tetramethylethylenediamine (TEMED), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), sodium dodecyl sulfate (SDS), and bromophenol blue were sourced from Amersham Biosciences (Amersham, UK). A protein molecular mass standard (14.4-97 kDa) was also obtained from Amersham Biosciences. For mass spectrometry quality control, [Glu1]-fibrinopeptide B (Thermo Fisher Scientific, Waltham, MA, USA) was used.

Animal experimentation: study design and sample procurement

All experimental protocols received prior approval from the Ethics Committee for the Use of Animals (CEUA) of the College of Veterinary Medicine and Animal Science (FMVZ) at São Paulo State University (UNESP), Botucatu, SP, under protocol 0106/2019. The study strictly adhered to the guidelines established by the National Council for the Control of Animal Experimentation (CONCEA).[21]

A total of 225 one-day-old male Cobb® 500 chicks were divided into 45 galvanized wire cages (0.4 × 0.5 × 0.6 m) equipped with feeders and nipple drinkers. All birds were vaccinated against infectious bursal disease, Marek's disease, and avian yaws disease upon hatching. Feed and water were provided *ad libitum*, and the lighting schedule followed the strain technical manual guidelines.[22]

The broilers were housed in a climatic chamber and exposed to cyclic heat stress (8 h daily) throughout the experiment. The thermal cycling regimen was based on adaptations of the method described by Zeferino *et al.*[23] The average maximum and minimum temperatures and relative humidity recorded during the trial are detailed in Table 1.

Table 1. Average temperature and relative humidity recorded within the climatic chamber during cyclic heat stress in broiler chickens

Bird age / days	Minimum temperature / °C	Maximum temperature / °C	Minimum relative humidity / %	Maximum relative humidity / %
1-7	30.1	36.5	45.1	63
8-21	29.2	34.2	49.4	63.2
22-36	27.8	33.7	48.9	64.2

Diets and treatments

The experimental diets were formulated using corn and soybean meal, following the nutritional recommendations proposed by Rostagno *et al*.[24] The study investigated five inclusion levels of PFSO: 0 (control), 0.30, 0.50, 0.70, and 0.90% (specific formulations can be found in Table S2, presented in the Supplementary Information (SI) section). These doses were adapted from the work of Zanetti *et al*.[25] The PFSO was a commercially sourced product.

The design comprised nine replicates *per* treatment, with five birds in each replicate, resulting in a total of 45 birds *per* dietary group. All diets were formulated without antibiotics as performance enhancers, but an anticoccidial agent was included in all treatments. The feeding program encompassed four phases: pre-initial (1-7 days), inicial (8-21 days), growth (22-33 days), and final (34-42 days of age).

Sample collection

At 36 days of age, after an eight-hour fasting period, the animals were individually identified and humanely euthanized by cervical dislocation. Immediately after slaughter, liver tissue samples were harvested from nine chickens *per* treatment (one bird *per* cage replicate), flash-frozen in liquid nitrogen, and stored at –80 °C until proteomic analysis. To minimize biological variation among individuals within a treatment group, the collected liver samples were pooled. Five separate pools were created for each treatment group by homogenizing samples from the nine animals, where each pool represented an experimental replicate for the proteomic analysis.

Proteomic analysis

Protein extraction, precipitation, and quantification

For protein extraction, 400 mg of pooled liver tissue were macerated in 2 mL of ultrapure water using an Omni Bead Ruptor 4 cell disruptor (Kennesaw, GA, USA). The resulting mixture was centrifuged at 10,000 rpm for 15 min at 4 °C using refrigerated centrifuge Hettich Universal 320R (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The proteins present in the supernatant were subsequently precipitated using ice-cold 80% (v/v) acetone at a 2:1 ratio (acetone:supernatant). After resting for 1.5 h at 4 °C, the samples were centrifuged again (15 min at 10,000 rpm), the supernatant was discarded, and the resulting protein pellets were collected for 2D-PAGE.

The protein pellet was resuspended in 0.50 mol L^{-1} NaOH for quantification. Total protein concentration was determined by the Biuret method,[26] using BSA as the standard. Absorbance was measured at 545 nm using a spectrophotometer (Evolution 60, Thermo Fisher Scientific, Waltham, MA, USA). A calibration curve was constructed using BSA standard solutions ranging from 10 to 100 g L^{-1}.

Liver proteome fractionation by 2D-PAGE

Liver proteins were fractionated by 2D-PAGE according to Braga *et al*.[27] For the first dimension, proteins were separated by their isoelectric point (pI) via isoelectric focusing (IEF). Protein aliquots were diluted to 1.50 μg μL^{-1} in a rehydration buffer containing 7 mol L^{-1} urea, 2 mol L^{-1} thiourea, 2% (m/v) CHAPS, 0.5% (v/v) ampholytes (pH 3-10), 0.002% (m/v) bromophenol blue, and 0.02 mol L^{-1} DTT. Three 13 cm IEF strips (pH 3-10) were loaded with 250 μL of this solution (375 μg total protein), covered with mineral oil, and hydrated for 12 h at room temperature. IEF was then performed using an Amersham Biosciences EPS 1001 system (Uppsala, Sweden) for 4.5 h.

Following IEF, strips were equilibrated in DTT and iodoacetamide solutions for 15 min each under gentle shaking. For the second dimension, strips were transferred onto 12.5% (m/v) polyacrylamide gels (180 × 160 × 1.5 mm) alongside a molecular mass marker (14.4-97 kDa). In this stage, proteins were separated by molecular mass (MM) as the presence of SDS imparted a uniform negative charge, ensuring migration toward the positive electrode. 2D-PAGE runs were performed using an Ettan™ DALTsix system (GE Healthcare, Uppsala, Sweden) at 100 V for 30 min, followed by 160 V for 4 h. The gels were then fixed for 1 h and stained with colloidal Coomassie G-250 for 72 h. For image analysis, the gels were scanned and processed using Image Master Platinum software (version 7.0, GeneBio, Geneva, Switzerland). This allowed for the determination of spot count, matching correlation, pI, MM, and differential expression. To ensure reproducibility, three biological replicates *per* group were analyzed through image superimposition.[27,28]

Protein spots characterization by LC-MS/MS

Protein spots that exhibited significant differences in expression between the treatments were excised from the gels. These spots underwent tryptic digestion using a 10 ng L^{-1} trypsin solution, following the methodology described by Braga *et al*.[27] The resulting peptide extracts were analyzed using the ultra efficiency liquid chromatography (UPLC®) system coupled to the Xevo Q-TOF G2 mass spectrometer (Waters, Manchester, UK) to characterize peptide sequences. The nanoAcquity UPLC® system was equipped with a nanoAcquity HSS T3

analytical column (75 µm internal diameter (i.d.) × 150 mm; Waters, Manchester, UK). The stationary phase consists of spherical high strength silica (HSS) with a 1.8 µm particle size and 100 Å pore size. The column was equilibrated with 93% mobile phase A (0.1% (v/v) formic acid in water) and 7% mobile phase B (0.1% (v/v) formic acid in acetonitrile). The peptides were separated using a linear gradient of 7-85% of mobile phase B over 70 min at a flow rate of 0.35 L^{-1}. The Xevo® G2 Q-TOF mass spectrometer (Waters) was operated in positive nanoelectrospray ionization mode, using the MSE (Data-Independent Acquisition) acquisition method with high-energy ramping (19-45 V) to enable simultaneous precursor and fragment ion detection in a single injection. Source conditions included a capillary voltage of 2.5 kV, sample cone and extraction cone voltages of 30 and 5.0 V, respectively, and a source temperature of 80 °C. Nitrogen was used as the desolvation (600 L h^{-1}) and cone gas (50 L h^{-1}), while argon served as the collision gas (0.20 L min^{-1}). Mass ranges were set at 20-100,000 *m/z* for the time-of-flight (TOF) analyzer and 20-4,000 *m/z* for the quadrupole in resolution mode (up to 20-16.000 *m/z* in non-resolving mode). Data were acquired over 70 min across the 50-2000 Da range. Real-time mass correction (lockspray) was performed using [Glu1]-fibrinopeptide B (1 pmol $µL^{-1}$; *m/z* 785.8427) at a flow rate of 1 µL min^{-1}. Raw data files were processed using Progenesis QI for Proteomics (version 4.0, Nonlinear Dynamics, Waters). Protein identification was performed by searching the UniProt database[21] specifically for *Gallus gallus domesticus*. Only proteins with scores exceeding 60 were considered identified.

Data analysis

The cage was defined as the experimental unit. The analysis compared protein spot expression across the five treatment groups (using three gel replicates *per* group). The ImageMaster Platinum software (version 7.0, GE Healthcare) was used to compare spot characteristics (volume, distribution, intensity, pI, and MM) across the gels via analysis of variance (ANOVA) and *t*-tests. Spots that showed statistically significant expression differences ($p < 0.05$) were selected for LC-MS/MS identification.[21,27,28]

Following identification, the FASTA sequences of the differentially expressed proteins were analyzed using Blast2GO software (version 6.0.3, Biobam, Spain), to categorize them by Gene Ontology (GO) terms (biological processes, cellular components, and molecular functions). Protein accessions were also submitted to the Reactome online database[27] for pathway enrichment analysis. Pathways with a false discovery rate (FDR) < 0.05 were considered significantly enriched. Finally, the STRING online tool, version 11.5, was employed to generate the protein-protein interaction network, using an average confidence level (0.400).[21,27,28]

Results

Figure 1 displays representative 2D-PAGE gels images derived from pooled liver tissue samples corresponding to each experimental condition. Protein spots that exhibited differential expression relative to the control group (C) are highlighted by red circles and indexed with numbers. The patterns of differential expression varied across the groups receiving PFSO supplementation. Specifically, the 0.3% PFSO group showed four differentially expressed spots. The 0.5%, 0.7%, and 0.9% PFSO groups displayed two, four, and a significantly higher count of fifteen differentially expressed spots, respectively.

The identity of the proteins associated with each of these spots is detailed in Table 2. In total, 129 proteins were successfully identified (see Table S2, SI section). From this set, 55 proteins were deemed differentially expressed. This selection was based on the highest scoring results and the closest approximation of theoretical pI and MM to the experimental values obtained from image analysis. Focusing on the biological context of the study, 22 of these differentially expressed proteins were systematically categorized into four functional groups based on their principal metabolic roles: heat shock proteins, antioxidant proteins, and proteins involved in lipid and carbohydrate metabolism (also shown in Table 2).

Figures 2-4 illustrates the findings of the Gene Ontology (GO) analysis, which organized the identified protein sequences into distinct categories spanning biological processes, molecular functions, and cellular components. Further analysis, specifically using Reactome, identified five metabolic pathways that were significantly enriched: gluconeogenesis, carbohydrate metabolism, metabolism (general), glycolysis, and detoxification of reactive oxygen species. These enriched pathways are summarized in Table 3. This outcome strongly suggests that the metabolic alterations observed are directly linked to the core functions of the proteins whose expression was modified.

The protein-protein interaction (PPI) network for the proteins impacted by PFSO supplementation was constructed using the STRING program against the *Gallus gallus* genome (Figure 5). This network helps visualize how these proteins cooperate toward shared biological functions. The network was resolved into three distinct clusters using k-means clustering, clearly indicating functional grouping: (*i*) blue cluster: contains heat shock

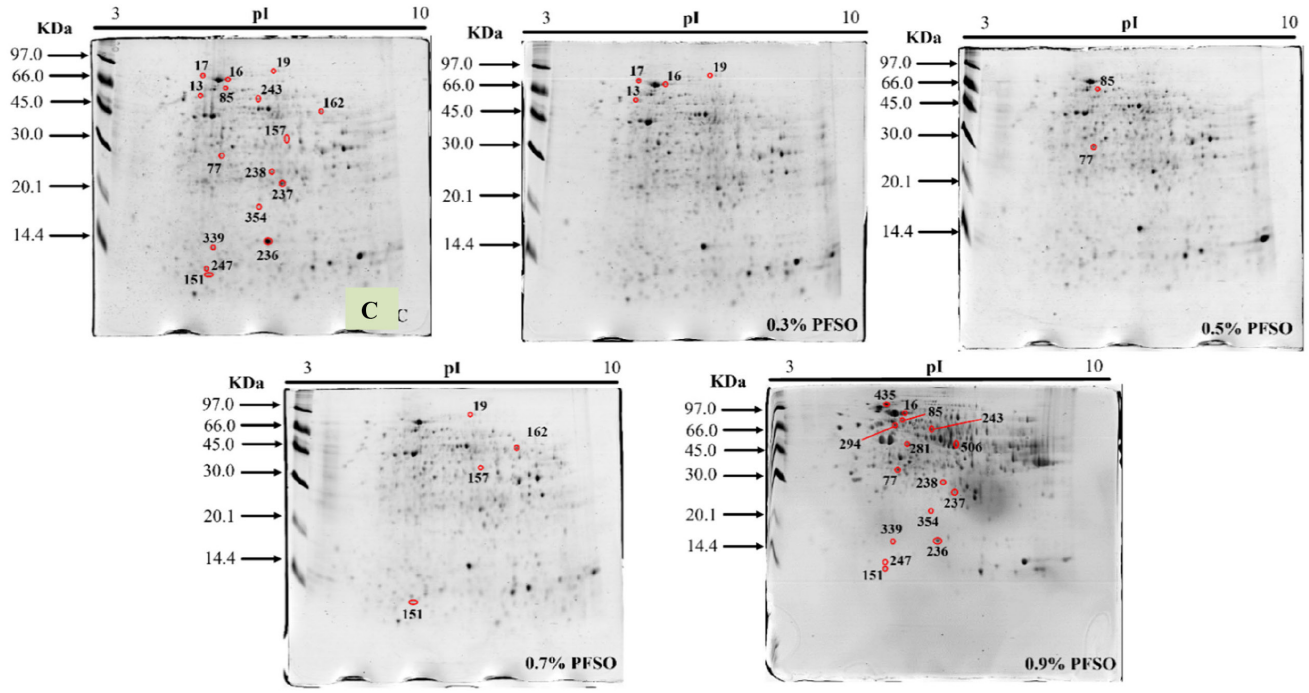

Figure 1. 12.5% (m/v) polyacrylamide gel (2D-PAGE, pH 3-10) of liver tissue from broilers supplemented with PFSO under cyclic heat stress. C: control group (0% PFSO); experimental groups: 0.3, 0.5, 0.7, and 0.9% PFSO. Circled spots indicate differential expression relative to the control group.

proteins primarily involved in the mechanism of misfolded protein repair; (*ii*) red cluster: composed of antioxidant proteins critical for the scavenging and removal of free radicals; (*iii*) green cluster: encompasses proteins dedicated to the metabolism of lipids and carbohydrates.

Discussion

Molecular chaperones

The current investigation revealed that supplementing broiler diets with PFSO, especially at concentrations of 0.3 and 0.9%, significantly enhanced the expression of multiple heat shock proteins (HSPs) when the birds were subjected to cyclic heat stress. This observation aligns with previous findings where other natural compounds, recognized for their antioxidant properties, influenced HSP levels under thermal challenges. For instance, Yang *et al.*[29] reported elevated messenger ribonucleic acid (mRNA) levels of HSP60, 70, and 90 in heat-stressed birds supplemented with resveratrol. Similarly, Tang *et al.*[15] demonstrated that rosemary extract in broiler feed induced higher HSP70 expression under heat stress conditions. Under non-stress conditions, HSPs are vital molecules for maintaining cellular homeostasis. Their functions include assisting in protein folding and transport, stabilizing the cytoskeleton, regulating the stress response, and providing a central defense mechanism against protein damage caused by various stressors.[30]

The specific mechanisms by which different dietary concentrations of PFSO modulate HSP expression are complex and not fully understood. This complexity is reflected in the significantly enriched metabolic pathways identified (Table 3) and the diverse protein-protein interactions observed (Figure 5), which demonstrate functional cooperation among HSPs, antioxidant proteins, and proteins involved in lipid and carbohydrate metabolism. The established presence of β-carotene in PFSO[25] may contribute to this effect by enhancing the antioxidant capacity in the liver of heat-stressed broilers. Foote and Denny[31] noted that β-carotene mitigates free radical damage by controlling the formation of singlet oxygen, thereby lowering the level of oxidative damage. The recruitment of several proteins observed in our study suggests an effort to re-establish homeostasis or initiate other biological processes (see Table S2 in the SI section). The increased expression of HSPs in the liver, particularly at both the lower and higher PFSO levels, signals the activation of a cytoprotective mechanism, which enhances the capacity of the cells to resist and mitigate the detrimental effects of thermal stress on the broiler liver.

Antioxidant proteins

The group supplemented with 0.9% PFSO displayed a reduced expression of several key antioxidant

Table 2. Differentially expressed proteins (*t*-test, $p < 0.05$) identified by LC-MS/MS from protein spots obtained by 2D-PAGE fractionation of the liver proteome of broilers supplemented with PFSO (0.3, 0.5, 0.7, and 0.9%) under cyclic heat stress relative to the control group (0% PFSO)

Spot	Accession number[a]	Protein name (gene)	Score	Sequence coverage / %	Matched peptides (n)	Relative abundance			
						0.3% PFSO	0.5% PFSO	0.7% PFSO	0.9% PFSO
Heat shock proteins									
13	Q5ZL72	60 kDa heat shock protein, mitochondrial (HSPD1)	3652.898	41.19	23	+1.33781			
16	Q5ZM98	stress-70 protein, mitochondrial (HSPA9)	5425.022	38.81	26	+1.11394			+3.20664
	O73885	heat shock cognate 71 kDa protein (HSPA8)	430.4864	19.81	11				
	P08106	heat shock 70 kDa protein (N/A)	332.263	15.3	9				
	Q90593	endoplasmic reticulum chaperone BiP (HSPA5)	95.1078	4.14	2				
17	P08106	heat shock 70 kDa protein (N/A)	739.1855	4.26	2	+1.50065			
	Q90593	endoplasmic reticulum chaperone BiP (HSPA5)	4219.087	43.4	26				
	O73885	heat shock cognate 71 kDa protein (HSPA8)	739.1855	4.18	2				
85	Q5ZL72	60 kDa heat shock protein, mitochondrial (HSPD1)	153.4286	11.34	5		+1.59668		+1.4106
	O73885	heat shock cognate 71 kDa protein (HSPA8)	364.3471	5.73	2				
435	Q04619	heat shock cognate protein HSP90-beta (HSP90AB1)	189.8523	6.21	4	absent[b]	absent[b]	absent[b]	present[c]
	P11501	heat shock protein HSP90-alpha (HSP90AA1)	371.9493	21.15	18				
Antioxidant proteins									
b151	P08629	thioredoxin (TXN)	1793.068	29.52	4			–1.41889	–1.93344
247	P08629	thioredoxin (TXN)	1764.833	38.1	5				–11.342
236	P80566	superoxide dismutase [Cu-Zn] (SOD1)	1125.452	16.23	3				–4.4163
237	Q5ZJF4	peroxiredoxin-6 (PRDX6)	1684.907	37.05	10				–2.32162
	P20136	glutathione *S*-transferase 2 (GSTM2)	1236.46	65.45	12				
354	P0CB50	peroxiredoxin-1 (PRDX1)	1126.749	37.19	8				–1.92674
	Q8UW59	protein/nucleic acid deglycase DJ-1 (PARK7)	112.5416	16.4	3				
Lipid metabolism									
77	P12276	fatty acid synthase (FASN)	292.6981	6.33	26		+1.51238		+1.31706
162	P12276	fatty acid synthase (FASN)	195.6263	4.74	12			–1.19717	
281	P08836	farnesyl pyrophosphate synthase (FDPS)	3869.996	43.05	18	absent[b]	absent[b]	absent[b]	present[c]
294	P23228	hydroxymethylglutaryl-CoA synthase, cytoplasmic (HMGCS1)	921.8295	19.92	12	absent[b]	absent[b]	absent[b]	present[c]
339	P80226	fatty acid-binding protein, liver (FABP1)	984.3133	37.3	4				–3.60731
Carbohydrate metabolism									
19	P21642	phosphoenolpyruvate carboxykinase [GTP], mitochondrial (PCK2)	491.4611	25.9	16	–1.41802		–1.39114	
157	Q5ZME2	malate dehydrogenase, cytoplasmic (MDH1)	5768.756	41.32	14			–3.72863	
238	Q5ZLN1	phosphoglycerate mutase 1 (PGAM1)	5953.544	39.76	10				–2.61189
243	Q91348	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (N/A)	215.5867	32.13	15				+1.55462
506	P0DMQ6	sorbitol dehydrogenase (SORD)	104.2482	9.86	3	absent[b]	absent[b]	absent[b]	present[c]

[a]Protein access code to the Uniprot database; [b]protein spots unidentified in 0.30, 0.50, and 0.70% treatments; [c]protein spots identified only in the 0.90% treatment. LC-MS/MS: liquid chromatography-tandem mass spectrometry; PFSO: passion fruit seed oil.

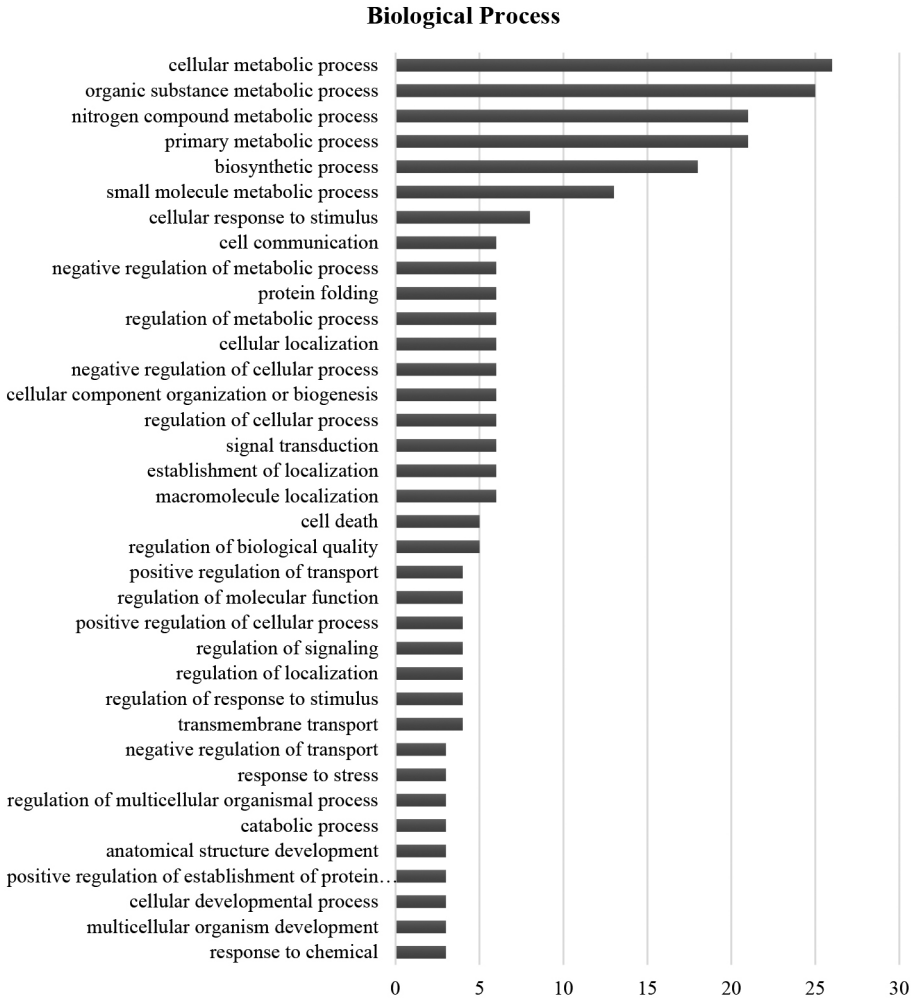

Figure 2. Classification of differentially expressed proteins (*t*-test, $p < 0.05$) identified by LC-MS/MS from 2D-PAGE liver proteome spots of broilers supplemented with PFSO (0.3, 0.5, 0.7, and 0.9%) under cyclic heat stress relative to the control group (0% PFSO). Proteins are categorized by Gene Ontology (GO) biological processes using Blast2GO software.

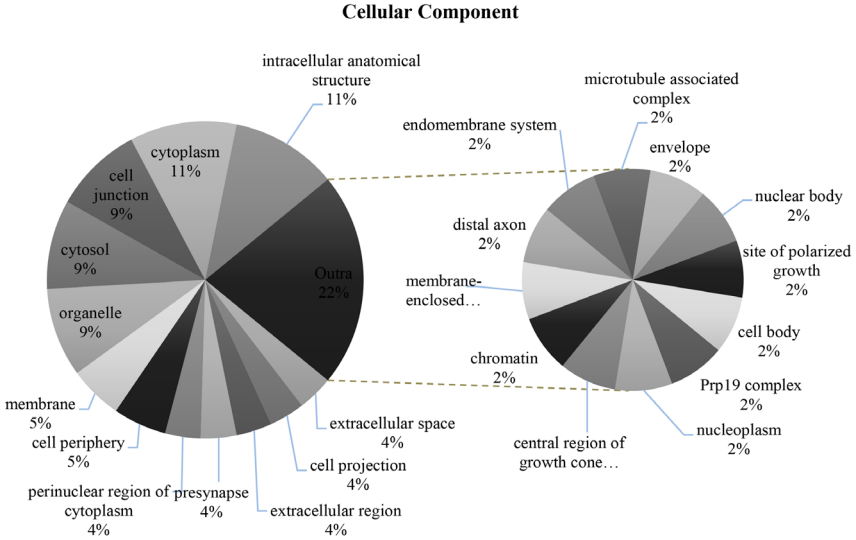

Figure 3. Classification of differentially expressed proteins (*t*-test, $p < 0.05$) identified by LC-MS/MS from 2D-PAGE liver proteome spots of broilers supplemented with PFSO (0.3, 0.5, 0.7, and 0.9%) under cyclic heat stress relative to the control group (0% PFSO). Proteins are categorized by Gene Ontology (GO) cellular component using Blast2GO software.

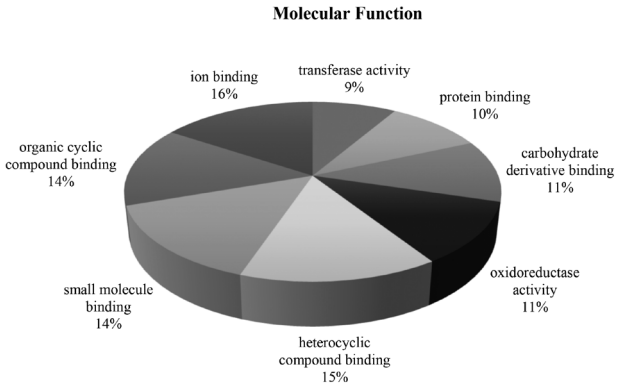

Figure 4. Classification of differentially expressed proteins (*t*-test, $p < 0.05$) identified by LC-MS/MS from 2D-PAGE liver proteome spots of broilers supplemented with PFSO (0.3, 0.5, 0.7, and 0.9%) under cyclic heat stress relative to the control group (0% PFSO). Proteins are categorized by Gene Ontology (GO) molecular function using Blast2GO software.

Table 3. Metabolic pathways significantly enriched (Reactome; FDR < 0.05) for differentially expressed proteins identified by LC-MS/MS from 2D-PAGE liver proteome spots of broilers supplemented with PFSO (0.3, 0.5, 0.7, and 0.9%) under cyclic heat stress relative to the control group (0% PFSO)

Pathway identifier	Pathway name	FDR
R-GGA-352875	gluconeogenesis	2.07^{-05}
R-GGA-353098	carbohydrate metabolism	4.49^{-05}
R-GGA-1660598	metabolism	4.97^{-04}
R-GGA-352882	glycolysis	0.009
R-GGA-3299685	detoxification of reactive oxygen species	0.015

FDR: false discovery rate; LC-MS/MS: liquid chromatography-tandem mass spectrometry; PFSO: passion fruit seed oil.

proteins, including thioredoxin, superoxide dismutase [Cu-Zn], peroxiredoxin-1, peroxiredoxin-6, glutathione *S*-transferase 2, and protein/nucleic acid deglycase DJ-1. Excessive reactive oxygen species (ROS) pose a threat to essential biological macromolecules. The cellular antioxidant network plays a critical role in preventing and minimizing this damage. However, oxidative stress occurs when ROS production surpasses the neutralizing capacity of the antioxidant defense system, leading to damage in lipids, proteins, and deoxyribonucleic acid (DNA), which subsequently impairs the health, growth, and development of poultry.[32]

Cellular antioxidant defense operates on three tiers. When the formation of free radicals is substantial, the first level (including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase) and the second level (including vitamin E, ascorbic acid, and glutathione - GSH) may be insufficient to prevent molecular damage. Consequently, the third level (involving methionine

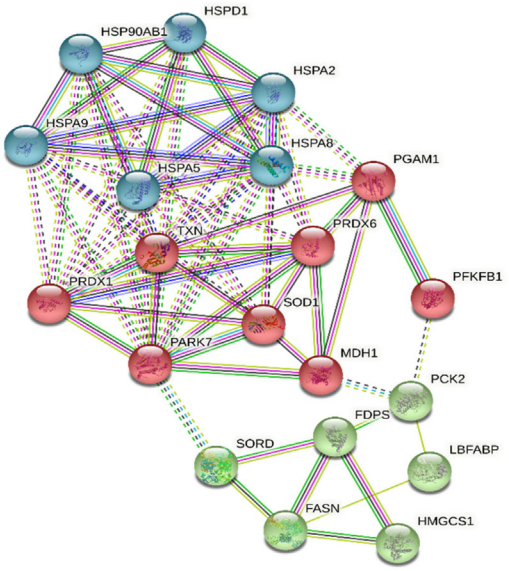

Figure 5. Protein-protein interaction network of differentially expressed liver proteins from broiler chickens supplemented with PFSO under cyclic heat stress. The interaction network was generated using the STRING online database, considering a minimum confidence score of 0.400. The interaction network was grouped into three clusters (blue, red and green) using k-means clustering. Each colored sphere (node) represents a protein identified by its gene. The lines represent evidence of interaction between connected proteins. Blue cluster: HSPD1 (60 kDa heat shock protein, mitochondrial), HSP90AB1 (heat shock cognate protein HSP 90-beta), HSPA2 (heat shock-related 70 kDa protein 2), HSPA9 (stress-70 protein, mitochondrial), HSPA5 (endoplasmic reticulum chaperone BiP) and HSPA8 (heat shock cognate 71 kDa protein). Red cluster: PRDX1 (peroxiredoxin-1), PRDX6 (peroxiredoxin-6), TXN (thioredoxin), PARK7 (protein/nucleic acid deglycase DJ-1), SOD1 (superoxide dismutase [Cu-Zn]), PGAM1 (phosphoglycerate mutase 1), MDH1 (malate dehydrogenase, cytoplasmic) and PFKB1 (6-phosphofructokinase 2). Green cluster: SORD (sorbitol dehydrogenase), FDPS (farnesyl pyrophosphate synthase), PCK2 (phosphoenolpyruvate carboxykinase [GTP], mitochondrial), FASN (fatty acid synthase), LBFABP (fatty acid-binding protein, liver) and HMGCS1 (hydroxymethylglutaryl-CoA synthase, cytoplasmic).

sulfoxide reductase and heat shock proteins) is activated to address the repair of damaged molecules.[32]

Our findings suggest that 0.9% PFSO supplementation, under cyclic heat stress, might bypass the full activation of the first and second levels of the antioxidant system, instead improving the overall antioxidant status by activating the third level through the enhanced expression of molecular chaperones. Furthermore, the higher PFSO concentration may have directly assisted in ROS elimination due to the inherent antioxidant properties of its components, such as β-carotene, α- and β-tocopherol, and polyphenols,[25,33] thereby reducing the need for high expression of the endogenous antioxidant enzymes observed.

Lipid metabolism

The liver is pivotal for lipid metabolism, performing essential roles in the absorption, synthesis, storage,

release, and breakdown of fatty acids and triglycerides.[34] In the context of lipid metabolism, the varied PFSO levels modulated the expression of four proteins: fatty acid synthase (FAS), fatty acid-binding protein (liver), farnesyl pyrophosphate synthase (FPPS), and hydroxymethylglutaryl-CoA synthase (HMGCS, cytoplasmic).

FAS is generally recognized as a central protein in diverse cellular functions, including fatty acid synthesis for energy storage, triglyceride secretion via lipoproteins, and the construction/repair of cell membranes.[34] Tang *et al*.[35] observed that heat stress negatively impacted lipid metabolism in broiler livers, specifically noting a downregulation of FAS. They proposed that this decrease likely served to conserve the energy necessary for basic cell survival. The upregulation of FAS observed in the 0.5 and 0.9% PFSO groups suggests that the overall energy demand of broilers was lower, possibly because PFSO alleviated the negative impact of cyclic heat stress, thereby allowing FAS activity to proceed normally. However, the observed downregulation of the same protein in the 0.7% PFSO group remains a point of ambiguity.

The majority of lipid synthesis begins with the mevalonate pathway, where FPPS acts early, providing initial substrates for multiple biosynthetic processes.[36] FPPS is a critical enzyme in the isoprenoid biosynthetic pathway, supplying precursors for the synthesis of steroids, terpenes, cholesterol, dolichol, and ubiquinones.[37] In a similar pivotal role within the isoprenoid pathway, cytoplasmic HMGCS catalyzes the condensation of acetyl-CoA and acetoacetyl-CoA to form HMG-CoA, which is then converted into mevalonate, a precursor for cholesterol and other isoprenoid compounds.[38] Although the precise physiological reasons require further exploration, the presence and modulation of these proteins in the liver of broilers supplemented with 0.9% PFSO indicate that PFSO favorably supported the biosynthesis of isoprenoids under heat stress. Isoprenoids are crucial for several cellular functions, including growth regulation, membrane structure, electron transport, and glycoprotein synthesis.[39]

Carbohydrate metabolism

This study showed a downregulation of phosphoenolpyruvate carboxykinase [GTP] (PEPCK), phosphoglycerate mutase 1 (PGAM1), and malate dehydrogenase (MDH) in the 0.3, 0.7, and 0.9% PFSO-supplemented groups. PEPCK [GTP] is central to gluconeogenesis, catalyzing the first committed step of this cycle, the production of glucose from non-carbohydrate sources (e.g., amino acids, propionate, glycerol).[40] Under heat stress, hepatic gluconeogenesis is often accelerated to supply energy, evidenced by increased mRNA expression of both cytosolic and mitochondrial PEPCK forms.[41] Likewise, increased expression of mitochondrial PEPCK has been noted in the liver proteome of broilers with ascites, suggesting an increased gluconeogenesis rate as a regulatory response to reduce susceptibility to the condition.[35]

The enzyme PGAM1 is involved in the glycolytic pathway, specifically catalyzing the conversion of 3-phosphoglycerate to 2-phosphoglycerate. Altered expression of PGAM1 is associated with metabolic diseases like pectoral myopathies in chickens[42,43] and was also found to be higher in chickens suffering from sudden death syndrome, affecting cardiac energy metabolism.[44] Malate dehydrogenase (MDH) plays a key role in facilitating the transport of malate and aspartate across the mitochondrial membrane and is a participant in the Krebs cycle.[45-49] Its presence in serum is considered an indicator of tissue damage and has been linked to liver damage in broilers.[46-48]

Collectively, the increased expression of these proteins is typically associated with the need of the cell to regulate homeostasis and meet elevated energy demands under abnormal physiological stress. Therefore, the reduced expression observed in the groups supplemented with different levels of PFSO strongly suggests that PFSO successfully mitigated the negative effects of cyclic heat stress, consequently lessening the extra energy required to combat oxidative stress.[47-54]

Finally, a downregulation of sorbitol dehydrogenase (SDH) can lead to the accumulation of sorbitol, causing oxidative stress, primarily by reducing non-enzymatic antioxidants (e.g., glutathione, ascorbic acid).[47,52-54] Kang and Shim[18] found lower SDH expression in the liver of heat-stressed broilers but noted its recovery upon early heat exposure. In our study, the presence (implying normal or near-normal expression compared to the stressed control) of SDH in the liver of broilers supplemented with 0.9% PFSO suggests that the PFSO provided a protective role against the effects of heat stress on the liver tissue.[53,54]

The text is based on the thesis of A.S.A.A., deposited in March 2023 in the public repository of the Universidade Estadual Paulista Júlio de Mesquita Filho (Unesp).[55]

Conclusions

Analysis of the hepatic proteome in broilers that received different levels of PFSO revealed that this supplementation successfully triggered several metabolic pathways aimed at maintaining homeostasis and enhancing thermal resilience under cyclic heat stress. This effect was particularly pronounced at the 0.9% supplementation level. The evidence strongly indicates

that PFSO plays a crucial role in modulating the expression of HSPs, thereby conferring a cytoprotective effect against the detrimental impacts of thermal challenge. Moreover, PFSO supplementation resulted in a favorable regulation of proteins governing key aspects of lipid and carbohydrate metabolism. This included an enhancement of the isoprenoid biosynthetic pathway within lipid metabolism and a reduction in metabolic processes like gluconeogenesis and glycolysis within carbohydrate metabolism, suggesting a decreased overall energy burden on the liver to cope with stress.

Supplementary Information

Supplementary data (Tables S1 and S2) are available free of charge at http://jbcs.sbq.org.br as PDF file.

Data Availability Statement

Data have not been deposited in an official repository. All datasets are available from the authors upon request.

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Artificial Intelligence GEMIN V3 was used to rewrite and edit the manuscript to maximize originality.

Author Contributions

A. S. A. A.: conceptualization, data curation, investigation; R. A. M.: conceptualization, investigation, writing original draft; J. C. S. V.: investigation, software, validation; F. K. L. K.: investigation, visualization; J. A. S.: data curation, software, writing original draft; L. B. G. A.: software, visualization; M. A. R. B.: resources, software, validation; J.A.: software, validation, writing original draft; J. R. S.: resources, formal analysis, funding acquisition, writing original draft; P. M. P.: conceptualization, formal analysis, funding acquisition, project administration, writing (review and editing).

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