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Proteomic study of gamma-oryzanol preventive effect on a diet-induced non-alcoholic fatty liver disease model

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease associated with obesity and diabetes prevalence. The use of natural compounds has become an attractive approach to prevent NAFLD and its progression. Gamma-oryzanol (Orz) is a natural compound whose beneficial effects on chronic metabolic diseases have been reported. Therefore, we aimed to investigate the preventive effect of Orz on the hepatic proteome in a diet induced NAFLD model. Wistar rats were randomly distributed into three experimental groups (n=6/group) according to the diet received for 30 weeks: Control group, high sugar-fat (HSF) group, and HSF+Orz group. The isolated Orz was added to the chow at the dose of 0.5% (w/w). We evaluated the nutritional profile, characterized the presence of steatosis through histological analysis, triglyceride content in liver tissue and hepatic inflammation. Next, we performed label-free quantitative proteomics of hepatic tissue. Network analysis was performed to describe involved protein pathways. NAFLD induction was characterized by the presence of hepatic steatosis. Orz prevented lipid accumulation. The compound prevented alterations of the hepatic proteome, highlighted by the modulation of lipid metabolism, inflammation, oxidative stress, xenobiotic metabolism, and the sirtuin signaling pathway. It was possible to identify key altered pathways of NAFLD pathophysiology modulated by Orz which may provide insights into NAFLD treatment targets.

Keywords: Obesity; Label-free; Steatosis; Bioactive compound; Phytosterol.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a type of chronic liver disease widely spread around the globe. In fact, it is the most common liver disease nowadays surpassing viral hepatitis [1,2]. The prevalence of NAFLD is estimated in 25% to 30% of the population and is a potential risk of progression to cirrhosis and hepatocellular carcinoma (HCC) becoming a frequent cause of liver transplantation [1]. Associated with obesity and diabetes prevalence, the rapid increase in NAFLD epidemiology is reflected in a growing burden of end-stage liver diseases [3]. NAFLD is a multi-factorial disease linked to a complexity of metabolic changes. Comprising a broad spectrum of alterations, NAFLD ranges from simple steatosis to steatohepatitis (NASH), progressing to liver fibrosis which can reach a cirrhotic state and end in HCC development [4].

The pathogenic mechanisms are still not entirely understood and different hypotheses have been postulated in the literature. Hepatic steatosis is the onset of the pathophysiology and the main hallmark of the disease [5]. The increment of circulating free fatty acid from diet or adipose tissue lipolysis combined with hepatic fatty acid synthesis through de novo lipogenesis promotes lipid accumulation in lipid droplets (*i.e.*, steatosis). Glucose and fructose, dietary sugar, are the substrates for de novo lipogenesis, especially fructose since its uptake by hepatocytes is non-dependent on insulin levels [6,7].

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Steatosis creates an environment for the progression to NASH. Lipid-mediated overload can lead to several pathogenic processes, such as insulin resistance, lipotoxicity, apoptosis, ferroptosis, macrophage recruitment, pro-inflammatory cytokine release, reduction of antioxidant defense, and increase in reactive oxygen species (ROS) [5]. These events can activate hepatic stellate cells (HSCs), responsible for fibrinogenesis and liver stiffness. Moreover, the maintenance of hepatic damage is closely linked to oxidative stress induced by mitochondrial production or lipotoxicity [4,8].

Treatment of fibrosis is difficult to achieve [9]. Hereby, the search for effective alternatives to treat or prevent hepatic lipid accumulation is crucial. Treatment and preventive strategies for chronic liver diseases are extensively explored [10]. The current alternative is lifestyle changes improving eating habits and practicing physical activity [11]. Thus, using natural compounds has become an attractive approach to avoid NAFLD and its progression (*e.g.*, liver fibrosis) [11].

Gamma-oryzanol (Orz) is a natural compound structurally composed of multiple molecular species being a mixture of ferulic acid esters and phytosterols [12]. Present in brown rice, Orz is primarily found in rice subproducts such as rice bran and rice bran oil, the richest source of the compound. Interesting Orz protective effects have been reported and investigations in different pathologies were conducted, mainly in metabolic diseases and related disorders [13]. Orz presents activity in lowering plasma triglycerides and cholesterol levels, antioxidant, anti-inflammatory, and induction of transcriptional factors, such as nuclear factor erythroid 2-related factor 2 (NRF2) and peroxisome proliferator-activated receptors (PPARs) [13–15]. However, the role of Orz preventive effect on the hepatic proteome has not been described. Therefore, this study aimed to investigate the preventive effect of Orz on the hepatic proteome in a diet-induced NAFLD model.

2. Methods

2.1. Animal model and experimental protocol

Wistar rats weighing ±187 g were randomly distributed into three experimental groups (n=6/group) according to the diet received for 30 weeks. The Control group received a standard chow plus water and the high sugar-fat (HSF) diet group received an HSF chow plus 25% sucrose added to the water. The HSF+Orz group received the same diet as the HSF group plus the compound added to the chow. The animals were housed in individual cages in a controlled environment under a 12h light-dark cycle at a room temperature of 24±2°C and humidity of 55±5%, with a free supply of food and water. At the of the protocol, the rats were fasted for 8 h, anesthetized (50 mg/kg ketamine; 10 mg/kg xylazine; i.p.), and euthanized by decapitation. Blood samples were collected and the plasma was separated by centrifugation (800×g at 4°C for 10 min) for metabolic and hormonal analysis. The adipose tissue was isolated, dissected, and weighed for nutritional profile assessment. The liver was excised and weighed, and fragments were immediately frozen at the liquid nitrogen temperature and stored in a −80°C freezer for further analysis or kept in 4% paraformaldehyde for histological procedures. The study was performed according to the guidelines for animal research and approved by the Ethics Committee on Animal Experiments of the Botucatu Medical School, UNESP (protocol number 1309/2019).

2.2. Diets and Orz dose

The diets (Table 1) and the Orz dose were based on previous studies from our research group [16]. The isolated Orz (Tokyo Chemical Industry Co., Ltd., Toshima, Kita-ku, Tokyo) was added to the chow at the dose of 0.5% (w/w) during the manufacturing process and was homogeneously mixed with the other ingredients before pelleting [16,17].

Table 1
Diet composition.

Components	Control diet	HSF diet
Soybean meal (g/kg)	335	340
Sorghum (g/kg)	278	80
Soy hulls (g/kg)	188	116
Dextrin (g/kg)	146	20
Sucrose (g/kg)	-	80
Fructose (g/kg)	-	180
Soybean oil (g/kg)	14	-
Lard (g/kg)	-	154
Minerals (g/kg)	25	25
Salt (g/kg)	4	8
Nutritional values		
% Energy from protein	22.9	16.6
% Energy from carbohydrate	66.8	49.2
% Energy from fat	10.4	34.2
Energy (kcal/g)	3.59	4.35

HSF: high sugar-fat.

2.3. Nutritional profile

Caloric intake, final body weight, and adiposity index (AI) were assessed to evaluate the nutritional profile. Chow and water intake were daily calculated from the individual leftovers of each animal, and the caloric intake was determined by multiplying the energy value of each diet (g×Kcal) by the daily food consumption. For the groups fed with the HSF diet, the caloric intake also included the calories from the sucrose in drinking water. The body weight was measured weekly. The AI was obtained by the sum of epididymal, retroperitoneal, and visceral fat pad weights (total body fat) and calculated as follows: [body fat (g)/final weight (g)]×100 [18]. Plasmatic triglyceride (TG), glucose and very low-density lipoprotein (VLDL) levels were measured by a colorimetric enzymatic kit (BIOCLIN®, Belo Horizonte, MG, Brazil). The insulin level was measured using the enzyme-linked immunosorbent assay (ELISA) method (EMD Millipore Corporation, Billerica, MA, USA). To asses insulin resistance, Homeostatic model assessments—Insulin Resistance (HOMA-IR) and TyG index were calculated: HOMA-IR was calculated by the following formula: HOMA-IR=(fasting glucose (mmol/L)×fasting insulin (μU/mL))/22.5 [17] and the TYG index was calculated according to the following formula [Ln (fasting triglycerides (mg/dl)Xfasting glucose (mg/dl)]/2; Ln is the naperian logarithm [19].

2.4. Liver histology

Following liver excision, a transversal slice of the left lobe of each animal was stored in 4% paraformaldehyde with 0.1 M phosphate buffer (pH 7.4) for 24 h. Then, the tissue was transferred to 70% ethanol until paraffin waxing. Histological sections (5 μm) were obtained from a paraffin block and laid on glass slides which were stained with hematoxylin and eosin (H&E). To evaluate the presence of steatosis, the pattern of lipidosis was analyzed based on the percentage of affected hepatocytes per field and scored as 0 (<5%), 1 (5—33%), 2 (33—66%), or 3 (>66%). The final score was expressed by the sum of the 10 fields observed [20]. The analysis was assessed by an optical microscope (NIKON, Labophot, Japan).

2.5. Intrahepatic triglyceride levels

To evaluate the intracellular hepatic triglyceride content, liver tissue samples (~100 mg) were homogenized with 1 ml of phosphate-buffered saline. Lipids from thin layers were obtained and lipids from water-soluble components of the tissue extracts were partitioned by organic extraction. Then, were measured by colorimetric enzymatic kit (BIOCLIN®, Belo Horizonte, MG, Brazil). The tissue results were corrected by the grams of liver weight [21].

2.6. Label-free quantitative proteomics

2.6.1. Sample preparation

Left lobe liver samples (~50 mg) from 6 animals per group were homogenized in protein extraction buffer (urea 6M in 50 mM Tris-HCl, 50 mM NaCl, pH 8.5, and 1% of protease inhibitor) in 2.0-ml Protein LoBind® tubes prefilled with 0.5 mm silica beads using BeadBug™ (three shaking cycles of 30 s at 3500 rpm). Samples were centrifuged (10000xg, 30 min, at 4°C) and the Bradford assay measured total protein concentration. The samples were diluted 1:10 in ammonium bicarbonate (25 mM) and 20 μg of protein of the diluted solution was reduced with dithiothreitol (DTT) for 30 min at 56°C (in thermomixer) and alkylated with 15 mM iodoacetamide for 20 min at room temperature in the dark. After this, proteins were digested with trypsin 1:20 (enzyme: protein) overnight at 37°C with continuous mixing. Concentrated tryptic peptides were stored until further analysis at −20°C [22].

2.6.2. Liquid chromatography with tandem mass spectrometry (LC–MS/MS)

The analysis was performed by a Dionex Ultimate 3000 nano-LC system connected to an Orbitrap Fusion™ Tribrid™ Mass Spectrometer equipped with a nano-electrospray ion source (Thermo Fisher Scientific, Hemel Hempstead, UK). Tryptic peptides were separated on an EASY-Spray 15 cm×75 μm ID column packed with Thermo Scientific Acclaim PepMap RSLC C18, 3 μm, 100 Å particles. The mobile phases were 0.1% formic acid in water (Solvent A) and 0.1% formic acid in water/acetonitrile, 2:8/v:v (Solvent B). The elution gradient consisted of 4–28% of B for 90 min and then 28–40% for 10 min, followed by 95% within the next 6 min to rinse the column. The column was then re-equilibrated for 20 min. The total run time was 130 min. The flow rate was 300 nL/min and the temperature was set at 35°C. A blank sample was injected after each triplicate to avoid sample carryover. The operational m/z range to obtain MS spectra was set at 375–1500 Da at 120.000 resolution. The collision energy was set at 35 eV and the mass spectrometer was operated in data-dependent mode with 3s between each master scan. The analysis was performed in duplicate [22].

2.8. Inflammatory parameters

The levels of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) were measured in the liver tissue. The analysis was performed by the ELISA technique, using a commercial kit (R&D Systems, Minnesota, US, #DY510 #DY506).

2.9. Data analysis

The differences among the experimental groups in nutritional profile variables, micro and macrovesicular steatosis, intrahepatic triglyceride levels and inflammatory parameters were determined by a one-way ANOVA followed by Tukey's post-hoc test considering a α of 5%. The analyses were performed with SigmaStat 3.5 software, and the graphs were created using GraphPad Prism software version 10 for Mac. For the label-free quantitative proteomics, proteRaw files were analyzed with the MaxQuant software version 1.6.6.0 (https://maxquant.net/) (Max Planck Institute of Biochemistry). The peak list was searched against the UniProt database of Rattus norvegicus to identify the proteins and the false discovery rate (FDR) was set at 1%. Trypsin was the specified enzyme, carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine as a variable. Further parameters were set as follows: first and main search peptide tolerance=20 and 4.5 ppm, respectively; isotope and centroid match tolerance=2 and 8 ppm, respectively; the maximum number of missed cleavages=2. For LFQ data analysis, the match-between-runs option was activated, with the match time window set to 0.7 min and the initial alignment window to 20 min. Proteins identified were analyzed by the Perseus software 2.0.6.0 version https://maxquant.net/perseus/) (Max Planck Institute of Biochemistry, Germany). The filtering was applied only identified by site, reverse, contaminants, and at least 2 unique peptides. Analysis of variance was performed to identify the global significant proteins between all three experimental groups. Then, comparisons between 2 groups (HSF/Control or HSF+Orz/HSF) were applied to obtain the changes in protein expression of each condition and the fold change difference. The proteins were considered differentially expressed when the *P*-value was <.05 and the fold change threshold was 1.5 (log2 ratio>1.5 for up-regulated and <0.67 for down-regulated). The differential protein expression is displayed in the volcanos plot. Unsupervised multivariate principal component analysis (PCA) and heatmap with hierarchical clustering were generated by using the web tool ClustVis (https://biit.cs.ut.ee/clustvis/) [23]. To identify biological processes, diseases, and functions associated with the differentially expressed proteins in the experimental groups, data were analyzed by the software Ingenuity Pathway Analysis (last release; Qiagen) based on Gene Ontology database [24].

3. Results

The exposure to the diet efficiently promoted changes in the nutritional profile inducing obesity, a main feature of our experimental model. The caloric intake was higher in both groups that received the HSF diet, and there was no influence of Orz on the intake. The animals of the HSF group presented higher final body weight, adiposity index, plasma TG, glucose, VLDL and insulin levels, HOMA-IR and Tyg Index in relation to the Control group. The HSF+Orz group presented increased adiposity index, plasma TG levels and Tyg index in relation to the Control group. Otherwise, the use of 0.5% of Orz added to the diet prevented the diet-induced changes, interfering with the body weight, adiposity index, plasma TG, VLDL and insulin levels, HOMA-IR and Tyg Index in relation of the HSF group (Table 2).

The histological analysis of the liver was performed to verify the presence of steatosis, the main hallmark of NAFLD (Fig. 1A and C). The analysis detected microvesicular steatosis in the HSF group, characterized by several fine lipid droplets in the cytoplasm of the hepatocytes with a foamy aspect (Fig. 1B). Macrovesicular steatosis was very slight and not significant compared to the Control group. The Orz significantly prevented microvesicular steatosis compared to the HSF group in the score evaluation (Fig. 1D). There was no effect of Orz on macrovesicular steatosis (Fig. 1E). To confirm that vacuolization observed in the histological slides was lipid droplets, the intrahepatic TG content was assessed, validating the findings in H&E staining (Fig. 1F). Orz attenuated the intrahepatic TG content by 45%.

To investigate the influence of Orz on NAFLD in animals submitted to the HSF diet, we characterized the changes in the liver proteome. The label-free quantitative analysis identified 2194 proteins in the liver (Fig. 2A). Differentially expressed proteins are

Table 2
Nutritional profile.

Variables	Control	HSF	HSF+Orz
Caloric intake (g/day)	81.53±4.22	97.04±7.00*	93.35±7.10*
Initial body weight (g)	182.32±6.06	186.92±3.04	181.78±6.71
Final body weight (g)	462.36±23.86	634.04±30.06*	451.07±44.68&
Adiposity index (%)	4.24±0.30	8.16±0.56*	5.18±0.43&*
Plasma TG (mg/dl)	56.60±8.82	120.30±2.50*	79.80±12.92&*
Plasma VLDL (mg/dl)	12.33±3.72	24.00±0.82*	16.00±2.83&
Plasma glucose (mg/dl)	68.00±23.19	114.00±17.75*	94.67±15.82
Insulin (ng/mL)	0.87±0.27	3.71±1.62*	1.76±0.63&
HOMA-IR	5.90±2.32	33.72±13.52*	15.31±4.51&
Tyg Index	4.13±0.21	4.70±0.11*	4.47±0.07*,&

HSF: high sugar-fat, Orz: gamma-oryzanol, TG: triglycerides, VLDL: Very low-density lipoprotein and HOMA-IR – Homeostatic model assessment – Insulin Resistance. Values are shown as mean±SD and compared by one-way ANOVA followed by Tukey posthoc test. Differences were considered significant for $P<.05$: *vs Control and &vs HSF; (n=6/group).

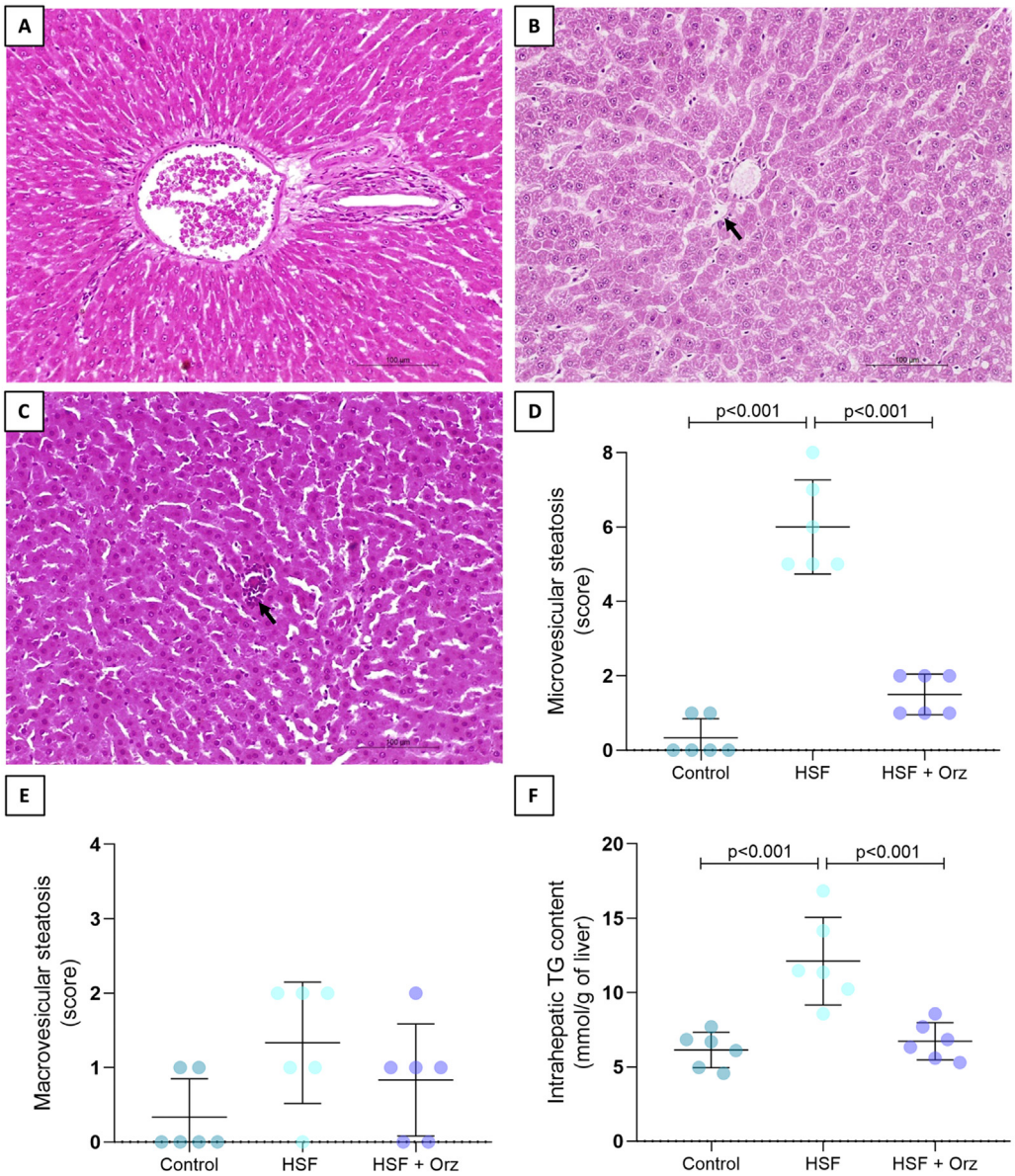

Fig. 1. Effect of Orz on hepatic steatosis. Representative images are presented from (A) Control group, (B) HSF group, and (C) HSF+Orz of liver slides staining with H&E. The presence of steatosis was evaluated by (D) microvesicular, (E) macrovesicular scores, and (F) Intrahepatic TG content. Black arrows in images B and C indicate the presence of inflammatory cells. HSF: high sugar-fat, Orz: gamma-oryzanol, and TG: triglycerides. Values are shown as mean±SD compared by one-way ANOVA followed by Tukey posthoc test, and differences were considered significant for $P<.05$ (n=6/group).

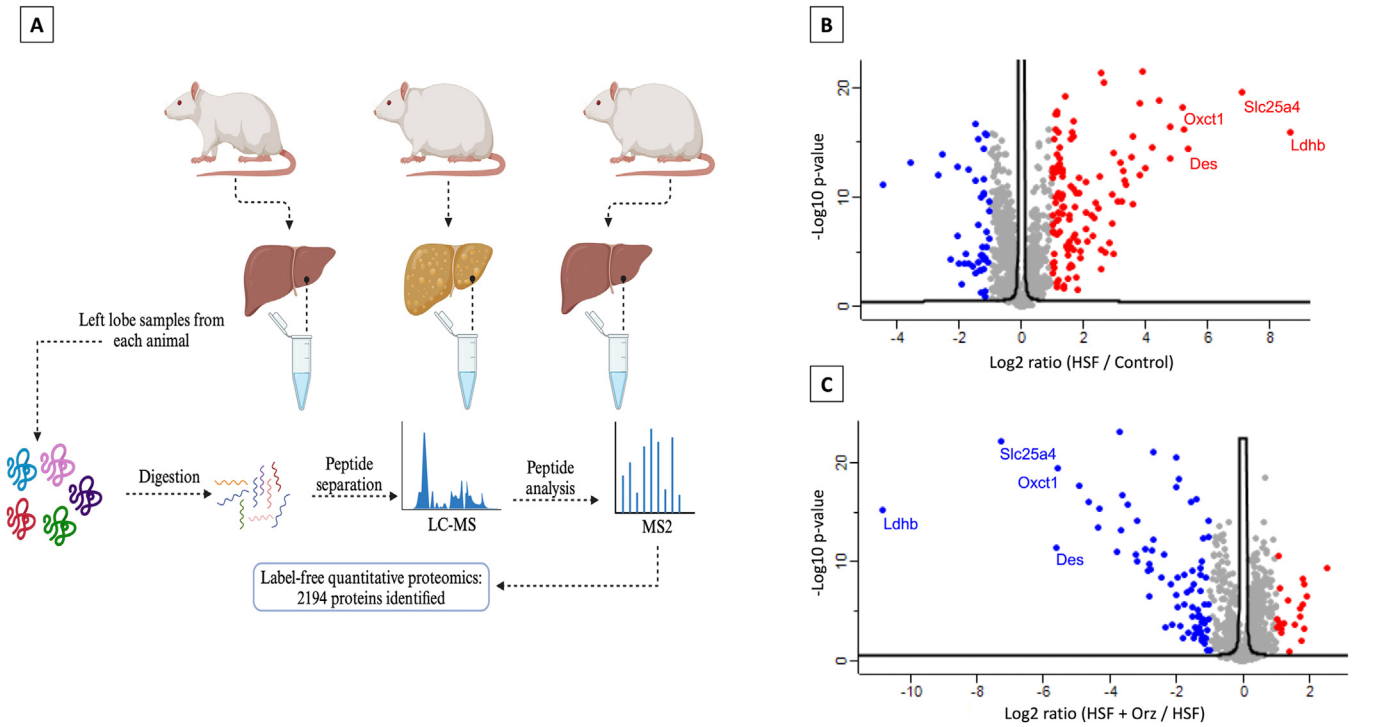

Fig. 2. Label-free quantitative proteomics workflow and data analysis. (A) Schematic representation of proteomics workflow and first steps of data analysis (created with BioRender.com). Volcano plots displaying the significance (log10 *P*-value) vs. ratio (log2) highlighting the differentially expressed proteins in (B) HSF / Control group and (C) HSF+Orz / HSF group. Red dots indicate up-regulated proteins and blue dots indicate down-regulated proteins. HSF: high sugar-fat; Orz: gamma-oryzanol; lactate dehydrogenase B (LDHB), ADP/ATP translocase 1 (SLC25A4), 3-oxoacid CoA-transferase 1 (OXCT1) and desmin (DES). The complete list of differentially expressed proteins observed in the volcanos is available in the supplementary material (Tables ST1).

displayed in the volcano plots. Red dots indicate up-regulation and blue dots indicate down-regulation of the proteins. There were 122 up-regulated proteins in the HSF group, and 43 down-regulated (Fig. 2B). In the HSF+Orz group, 18 proteins were up-regulated, and 78 were down-regulated (Fig. 2C). A subset of four proteins presented a notable high-fold change with a contrary expression in the comparison of the groups. Lactate dehydrogenase B (LDHB) log2 ratio=8.6 *vs.* log2 ratio=-10.8, solute carrier family 25 member 4 (SLC25A4) log2 ratio=7.1 *vs.* log2 ratio=-7.3, 3-oxoacid CoA-transferase 1 (OXCT1) log2 ratio=5.2 *vs.* log2 ratio=-5.5, and desmin (DES) log2 ratio=4.8 *vs.* log2 ratio=-5.6, up-regulated in HSF *vs.* down-regulated in HSF+Orz, respectively (Fig. 2B and C).

The PCA revealed groups well separated with replicates of the same group clustered together (Fig. 3A). The Control and HSF groups were distant from each other, and the HSF+Orz was placed between them. In addition, the hierarchical clustering of the differentially expressed proteins showed an evident separation of the groups, placing the HSF+Orz close to the Control group and far from the HSF group (Fig. 3B). Replicates were placed side by side with some variation of the expression profile. The largest number of proteins had a similar expression profile in the Control and HSF+Orz contrasting from the HSF group.

The network analyses presented on Table 3 describes the main diseases, functional annotation and canonical pathways involved during the HSF administration and Orz prevention. The function annotation, predicted to be affect in NAFLD, included the up-regulation of consumption of oxygen, synthesis of ATP, biosynthesis of nucleoside triphosphate, glycolysis, inflammation of body cavity, synthesis of purine nucleotide, concentration of triacylglycerol, metabolism of nucleotide, oxidative stress, hepatic steatosis, synthesis of fatty acid, fatty acid metabolism, synthesis of L-amino acid, and conversion of fatty acid. Simultaneously, there was a down-regulation of oxidation of lipid, synthesis of terpenoid, metabolism of reactive oxygen species, synthesis of reactive oxygen species, metabolism of amino acids and synthesis of amino acids. Furthermore, Orz demonstrated a significant impact on the regulation of these functional annotations by reversing the initially up-regulated functions into down-regulated functions, and the down-regulated functions were up-regulated after the prevention with Orz. In addition, several canonical pathways were differentially expressed, such as sirtuin signaling, xenobiotic metabolisms, and nicotine degradation II, that were negatively induced, and NRF2-mediated oxidative stress response, hepatic fibrosis signaling, tricarboxylic acid (TCA cycle), ILK signaling, actin cytoskeleton signaling, and oxidative phosphorylation that were positively induced. Otherwise, pretreatment with Orz was able to reverse the canonical path behavior.

Among the functional and disease modules regulated by the HSF diet and Orz pretreatment, the “hepatic steatosis” and “inflammation of absolute anatomical region” were positively induced in the HSF group in comparison to the Control group (Fig. 4A and B). Otherwise, the pretreatment with 0.5% of Orz promoted a negative induction on the “hepatic steatosis” and “inflammation of body cavity” modules (Fig. 4C and D).

The levels of TNF-α and IL-6 in the hepatic tissue are presented in Figure 5. The HSF group had higher levels of these cytokines when compared to the control group. Although IL-6 was higher in the HSF+Orz group compared to the Control group, the compound was able to reduce the rise when compared to the HSF group, indicating that Orz had a beneficial effect on reducing the elevation of this cytokine. In addition, the Orz was able to prevent increased levels of TNF-α when compared to the HSF group.

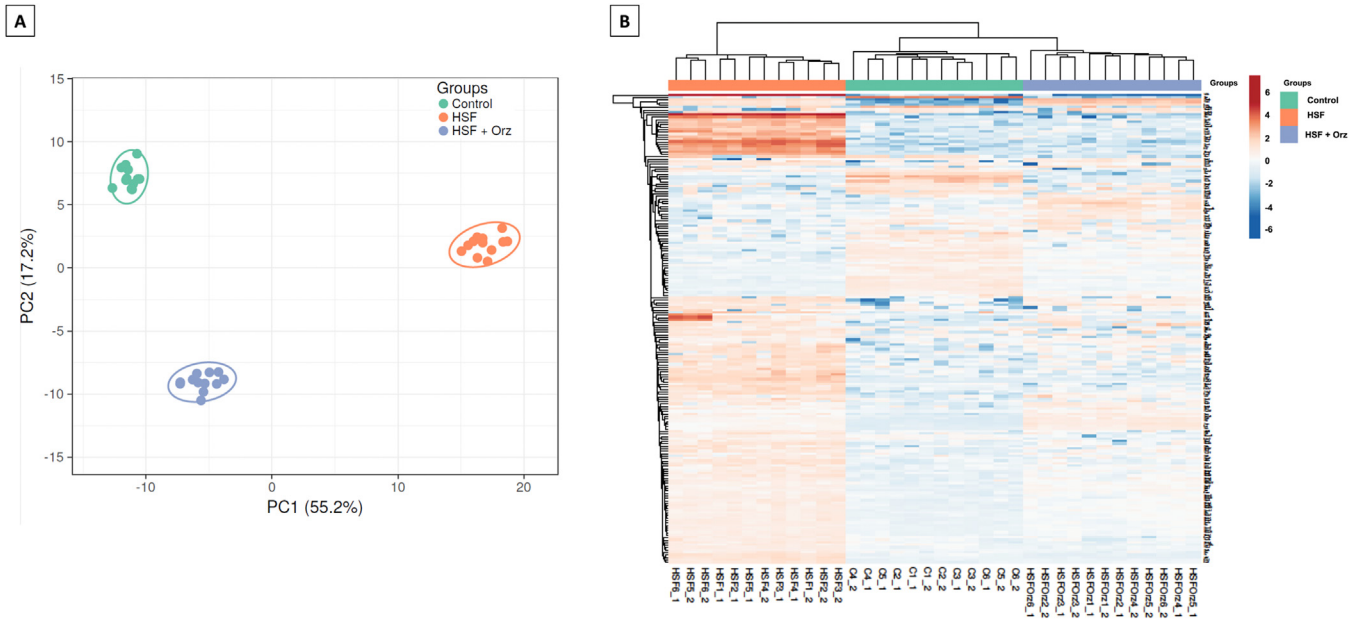

Fig. 3. Label-free quantitative proteomics data analysis. (A) Unsupervised multivariate Principal Component Analysis (PCA) of LFQ protein intensities. (B) Heatmap of differentially expressed proteins. Heatmap rows (proteins) and columns (samples) were clustered hierarchically by Euclidian distance. Red and blue correspond to maximum and minimum values relative to the row average. HSF: high sugar-fat, Orz: gamma-oryzanol.

4. Discussion

NAFLD represents a spectrum of disease states ranging from simple steatosis to NASH, which can lead to cirrhosis and HCC. NAFLD became a public health concern due to the rapid rise in the prevalence which brought scientific attention to the search for treatment and prevention of the disease. The Orz is considered an interesting alternative in the prevention of chronic metabolic diseases, and most recently NALFD due to its beneficial effects. The employees of proteomics have been an advance in understanding the pathophysiology of diseases and treatment targets. Consequently, in this study, we investigate the preventive effect of Orz on the hepatic proteome in a diet induced NAFLD model.

In our experimental design, the animals were fed with an HSF diet *ad libitium* to promote the diet-induced NAFLD. The results confirm the induction of NAFLD by the diet in accordance with the literature [25,26]. Orz had no influence on caloric intake, however, the compound prevented the increase of the final body weight and adiposity. A well-established effect of Orz is the reduction of fatty acids and cholesterol absorption in the gut enhancing excretion, leading to attenuation of adiposity gain [27]. This factor alone is not responsible for the effects of Orz, since the obesogenic stimulus of the diet is also provided by the high number of sugars. A previous investigation described Orz effect on steatosis preventing macro and microvesicular steatosis which was attributed to the improvement of dyslipidemia, insulin resistance, local inflammation, and oxidative stress [26].

In addition to the decreased absorption of lipids as an effect of Orz, molecular mechanisms are suggested to be involved in the beneficial effect of Orz, as the compound can present activities directly in the tissue.

We performed proteomics analysis to achieve a deep understanding of the biological effects of Orz in the hepatic tissue, and a total of ~2200 proteins were obtained. Based on the established fold change of 2, the HSF group presented 122 up-regulated proteins and 43 down-regulated, whereas the HSF+Orz group presented 18 and 78 up and down-regulated, respectively. The highlighted subset of four proteins presented opposite expressions between the groups with a high-fold up-regulation in HSF and down-regulation in HSF+Orz (LDHB, OXCT1, SLC25A4, and DES).

Among them, LDHB presented the highest fold differential expression, and specifically in the HSF+Orz group was 10.8-fold down-regulated. Lactate dehydrogenase (LDH) is a tetramer enzyme that conducts the reversible conversion of pyruvate to lactate and the reverse reaction. LDH was already described as a promising biomarker for acute liver disease by a proteomic study [28]. In HSF diet-induced NAFLD, the presence of insulin resistance and dyslipidemia favors the increased isoform LDHB, associated with pyruvate conversion from lactate (*i.e.,* lactate clearance) to gluconeogenesis processes from the breakdown of fat mediated by the transcription factor sterol regulatory element binding protein-1c (SREBP-1c), which is activated by insulin levels [29,30]. The hypoinsulinemic and hypocholesterolemic actions promoted by Orz may improve the uptake and use of dietary energy by peripheral tissues, reducing the expression of LDHB and consequently reducing the hepatic overload from the hypercaloric diet. Furthermore, Orz can inhibit gluconeogenesis and lipogenic enzymes, such as SREBP-1c, showing the influence of the compound on the availability of lipid substrate for gluconeogenesis and consequent decrease in LDHB [15,31]. Considering the high fold observed in our model, the enzyme may be a biomarker for NAFLD progression subject to the influence of Orz consumption.

Associated with LDHB role in the acetyl-CoA formation, OXCT1 was also up-regulated in the HSF group (5.2-fold). OXCT1 is an enzyme important in ketone metabolism and is normally highly expressed in the kidney, heart, and brain. In a healthy adult liver, the expression of OXCT1 is repressed to prevent the synthesis and catabolism of ketone bodies happening both in hepatic tissue [32]. Insulin resistance, a factor present in NAFLD pathogenesis, reduces glucose uptake and utilization favoring mitochondrial β-oxidation [33]. β-oxidation is also stimulated to compensate for the large influx and synthesis of lipids resulting in the accumulation of acetyl-CoA which is directed to the TCA cycle or ketogenesis [34,35]. The products of ketogenesis, ketone bodies, are then catabolized by the

Table 3
Functional modules regulated by HSF diet and Orz prevention.

Diseases or function annotation		z-score HSF *vs.* control	z-score HSF+Orzvs HSF
Energy production	Consumption of oxygen	2.16	-2.06
Energy production, nucleic acid metabolism, small molecule biochemistry	Synthesis of ATP	1.27	-1.98
Nucleic acid metabolism, small molecule biochemistry	Biosynthesis of nucleoside triphosphate	1.27	-1.98
Carbohydrate metabolism	Glycolysis	2.59	-1.94
Inflammatory response	Inflammation of body cavity	0.85	-1.39
Energy production, lipid metabolism, small molecule biochemistry	Oxidation of lipid	-1.42	-1.06
Nucleic acid metabolism, small molecule biochemistry	Synthesis of purine nucleotide	1.72	-0.90
Lipid metabolism, molecular transport, small molecule biochemistry	Concentration of triacylglycerol	1.11	-0.72
Nucleic acid metabolism, small molecule biochemistry	Metabolism of nucleotide	1.48	-0.65
Organismal injury and abnormalities	Oxidative stress	1.46	-0.45
Gastrointestinal disease, hepatic system disease, metabolic disease, organismal injury and abnormalities	Hepatic steatosis	1.55	-0.10
Lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism	Synthesis of terpenoid	-1.98	0.52
Lipid metabolism, small molecule biochemistry	Synthesis of fatty acid	1.58	0.66
Free radical scavenging	Metabolism of reactive oxygen species	-0.80	0.80
Free radical scavenging	Synthesis of reactive oxygen species	-0.56	1.04
Amino acid metabolism, small molecule biochemistry	Metabolism of amino acids	-2.45	1.20
Lipid metabolism, small molecule biochemistry	Fatty acid metabolism	0.65	1.95
Amino acid metabolism, small molecule biochemistry	Synthesis of amino acids	-2.21	1.98
Lipid metabolism, small molecule biochemistry	Conversion of fatty acid	0.18	2.20
Ingenuity canonical pathways		**z-score HSF *vs.* Control**	**z-score HSF+Orzvs HSF**
Sirtuin signaling pathway		-4.22	4.04
Xenobiotic metabolism AHR signaling pathway		-1.94	2.00
Xenobiotic metabolism CAR signaling pathway		-1.89	2.12
Xenobiotic metabolism PXR signaling pathway		-1.61	1.13
Nicotine degradation II		-0.82	1.34
NRF2-mediated oxidative stress response		1.34	2.00
Hepatic fibrosis signaling pathway		1.41	0.45
TCA cycle II (eukaryotic)		2.12	-2.45
ILK signaling		2.45	-1.89
Actin cytoskeleton signaling		2.65	-1.89
Oxidative phosphorylation		6.40	-5.39

HSF: high sugar-fat, Orz: gamma-oryzanol. Positive z-score indicates the activation pathway; negative z-score indicates the down-regulation of the pathway (performed by Ingenuity Pathway Analysis—IPA; Qiagen).

OXCT1 enzyme into acetyl-CoA as well [34]. Hepatic ketogenesis is increased in animal models and humans in NAFLD [34]. Our results show that the catabolism of ketone bodies is also increased, which indicates a futile circle establishment. The up-regulation of the OXCT1 enzyme has been described as involved in inflammation through nuclear factor kappa B (NF-κB) activation, however, the exact signaling mechanism is not clearly known [36]. The compound down-regulated OXCT1 in 5.5-fold. We hypothesized that the Orz prevents this metabolic circle by improving insulin resistance, reducing lipid influx, positive effects previously demonstrated in the literature [13,37].

Regarding energy metabolism, SLC25A4 is a transporter that imports ADP into the mitochondrial matrix and exports ATP, a step of oxidative phosphorylation [38]. The up-regulation of SLC25A4 (7.1-fold) is aligned with the increased expression of proteins of the mitochondrial respiratory chain in the HSF group. Based on the modulation of these proteins the mitochondrial function is still preserved. The preventive use of Orz down-regulated SLC25A4 in 7.3-fold. This appears to be a general effect of Orz related to the several proteins modulated that were involved in metabolism. We attributed no specific effect of the compound on the SLC25A4 expression. Taking together the three proteins above described (LDHB, OXCT1, and SLC25A4), the formation of acetyl-CoA is increased by different pathways which are expected in NAFLD. Although β-oxidation is stimulated, lipid influx and synthesis surpass the metabolism of substrates contributing to steatosis and Orz

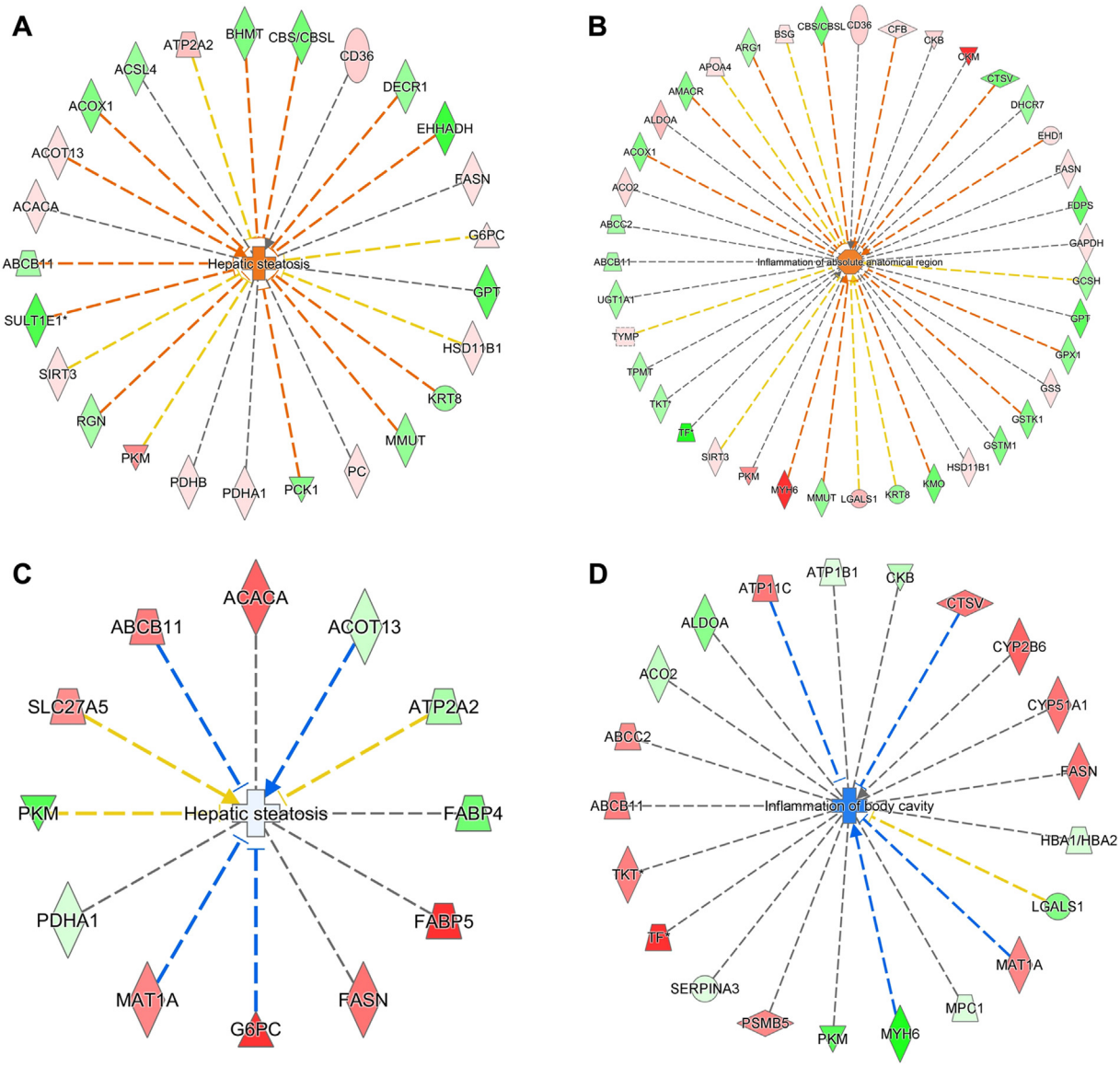

Fig. 4. Network Analyses of hepatic steatosis and inflammation among the groups. (A) Hepatic steatosis in the HSF group in relation to the Control group. (B) Inflammation of absolute anatomical region in the HSF group in relation to the Control group. (C) Hepatic steatosis in the Orz group in relation to the HSF group. (D) Inflammation of body cavity in the Orz group in relation to the HSF group. The orange and blue hubs indicate positive and negative-induced pathways, respectively. Gene up (red) or down (blue) regulation is positively correlated with color intensity; orange and blue lines indicate activation and deactivation, respectively; yellow lines are designated for inconsistent findings; and gray lines represent unexpected not predicted effects. HSF: high sugar-fat, Orz: gamma-oryzanol.

was able to prevent the substrate overload avoiding the lipid accumulation in the lipid droplets.

The last of the highly differentially expressed proteins subset, DES presented a 4.8-fold in the HSF group *vs.* -5.6-fold in HSF+Orz. DES is an intermediate filament protein found up-regulated in activated HSCs being a marker for fibrinogenesis [39,40]. Activated HSCs transdifferentiate into myofibroblasts which are the main effectors of the NAFLD progression to fibrosis, responsible for the deposition of collagen in the extracellular matrix, thereby driving the fibrosis process. HSCs activation, migration, and proliferation are controlled by a complex signaling network involving growth factors, lipotoxicity, inflammation, oxidative stress, and metabolic signals [41]. The activation of HSCs is especially discussed in advanced states of NAFLD with the presence of steatohepatitis, however, HSCs positively markedly for desmin was already reported in the NAFLD model [42]. Conversely, the inhibition of HSCs is a prerequisite for the resolution of fibrosis [41]. Therefore, is very interesting that Orz may act to prevent the HSCs activation and consequently the NAFLD progression. Some natural compounds showed effects inhibiting HSCs and hepatocyte damage, acting as antifibrotic treatments [43,44]. β-sitosterol, one of the main phytosterols present in Orz, is able to prevented collagen accumulation in activated HSC thought down-regulation of mRNA and protein expression levels of collagen [45].

Several canonical pathways are implicated in the pathogenesis of NAFLD, ranging from lipid metabolism, inflammation, oxidative stress, xenobiotic metabolism and the sirtuin signaling pathway. In this context, prevention with Orz was able to regulate some of the main pathways affected by the consumption of the HSF diet.

The greater influx of energy substrates through nutritional overload by the consumption of HSF diets is associated with increased TCA cycle activity, contributing to an increase in elec-

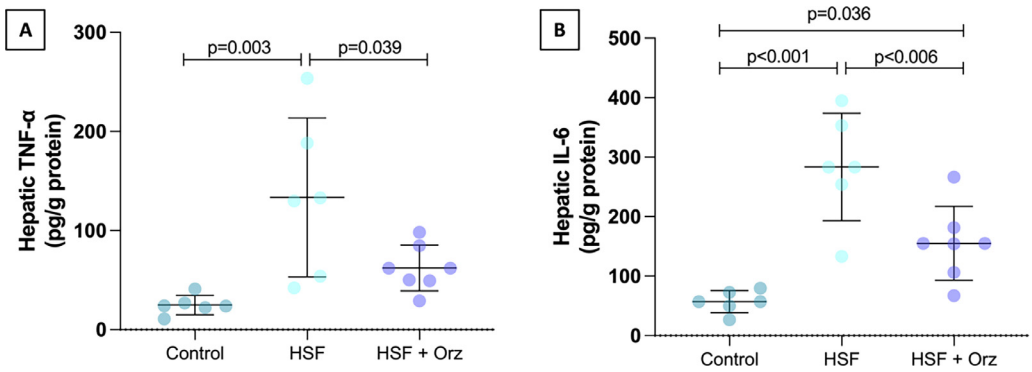

Fig. 5. Inflammatory parameters. (A) Hepatic tumoral necrosis factor alpha (TNF-α, pg/g protein). (B) Interleukin-6 (IL-6, pg/g protein). HSF: high sugar-fat, Orz: gamma-oryzanol. Values are shown as mean±SD compared by one-way ANOVA followed by Tukey posthoc test, and differences were considered significant for $P<.05$ ($n=6$/group).

tron deposition, high oxygen consumption and ROS generation in the respiratory chain, which may be an important element in the known link between oxidative stress, inflammation, and hepatic insulin resistance, pathophysiological pillars of NAFLD [46]. In addition, G6PC, which encodes the catalytic subunit of glucose-6-phosphatase, is essential for both glycolysis and gluconeogenesis processes to control glycogen levels, blood glucose, glycoprotein synthesis, and di- and monosaccharides that can act as structural components of cells [47]. These processes act by optimizing the turnover of blood glucose, triglycerides, amino acids, and lactate, inhibiting hepatic steatosis. In this study, pretreatment with Orz promoted up-regulation of the G6PC gene. The deregulation of energy metabolism and the pathological outcomes of hepatic steatosis in an obesogenic environment are characterized by the presence of oxidative stress markers generated by the lack of energy metabolism associated with the down-regulation of G6PC [47,48].

In this sense, many pathways are triggered to reduce oxidative stress. The antioxidant enzyme, glutathione 4 (GPX4) and glutathione (GSH) plays a role in the prevention of ferroptosis by eliminating ROS and consequently, lipid peroxides [49,50]. The literature reports that the formation of polyunsaturated fatty acids (PUFA) by acyl-CoA synthetase long-chain family member 4 (ACSL4) is an important factor in ferroptosis, as PUFA are prone to be oxidized and form lipoperoxides [51]. In this study, ACSL4 presented down-regulated in the HSF group and up-regulated in the HSF+Orz group. Orz may be able to promote fatty-acid oxidation mediated by ACSL4, increasing the phospholipid synthesis and reducing the production of lipid free radicals avoiding apoptotic signaling [52]. The antioxidant potential of Orz is explained by its ability to donate hydrogen from its ferulic acid constituent and to inhibit iron-driven hydroxyl radical formation [15]. In addition, Orz can activate NRF2 signaling pathway, which is responsible for the transcription of antioxidant genes, including GPX and ferritin in order to prevent and control the formation of free radicals [53].

NRF2 regulates the manufacture, use, and regeneration of GPX, thioredoxin, and NADPH, as well as the formation of ROS by mitochondria and NADPH oxidase [54]. The presence of oxidant factors induces NRF2 dissociation from Keap1 inhibition and nucleus translocation, leading to increased transcription of antioxidant genes, such as glutathione-S-transferase (GST) [54]. The GSTs activity regulates cellular antioxidant maintenance through the conjugation of electrophilic compounds to GSH [55,56], providing protection against oxidative stress and liver injury. The GSTs levels are suppressed in patients with steatosis and NASH [57]. Corroborating, our data shows a down-regulation of GST family proteins (GSTA1, GSTA5, GSTK1, GSTM1, GSTM2, GSTM5) and a positive induction of consumption of oxygen and oxidative phosphorylation in the HSF group that were reversed after Orz pretreatment. Rungratanawanich et al. (2018) suggest that the antioxidant effect of Orz may be through triggering the NRF2 pathway up-regulation and induction of defensive genes by reducing lipid peroxidation [58]. In our study, supplementation with Orz promoted an up-regulation of glutathione S-transferase mu 5 (GSTM5). Cycloartenyl ferulate and β-sitosteryl ferulate, the main steryl ferulates of Orz, suppressed spontaneous intracellular ROS in a similar manner to N-acetylcysteine (NAC) and α-tocopherol, which may be due to the ability to act as a precursor of GSH or acting directly as an antioxidant for some oxidizing species, such as nitrogen oxide (NO) [59,60].

Besides, among NRF2-regulated genes, ABCC2 encodes multidrug-resistance-associated proteins (MRPs) responsible for xenobiotic metabolism, defense, and protection of tissues and organs as well in GSH export and homeostasis, an essential non-enzymatic antioxidant [61]. ABC transporters are also associated with the transport of substrates such as flavonoids and phytoestrogens from fruits and vegetables and the excretion of molecules associated with the immune and inflammatory response, such as eicosanoids, Interferon-gamma (INF-γ), tumor necrosis factor-α (TNF-α) and interleukins [62]. TNF-α and IL-6-mediated inflammatory responses have been shown to induce oxidative stress and the inactivation of the Methionine adenosyl transferase 1A (MAT1A) enzyme, which is highly expressed in normal liver [63]. In rats, macro steatosis, inflammation, fibrosis and liver injury as well as reduced total and mitochondrial GSH levels development is associated with MAT1A activity loss [64]. The anti-inflammatory properties of Orz are a possible mechanism to explain the increased regulation of ABCC2 and MAT1A gene in comparison to the HSF group. Orz can reduce the mRNA expression of pro-inflammatory cytokines through the inhibition of NFκB and the tissue infiltration of inflammatory cells [12].

Among the xenobiotic receptors, the constitutive androstane receptor (CAR), the pregnane x receptor (PXR) and the aryl hydrocarbon receptor (AHR) are the most predominant in regulating hepatic responses to drugs and environmental chemicals, contributing to the regulation of pathophysiological conditions including obesity and diabetes by modulating hepatic energy homeostasis, insulin signaling, and cell proliferation [65–68]. CAR and PXR antagonism presented increased NFκB target genes in primary hepatocytes, highlighting its anti-inflammatory properties in NAFLD [69]. The up-regulation of phase I cytochromes P450 (CYPs) is one of the roles in the regulation of xenobiotic-metabolizing enzymes, converting target compounds into more soluble derivatives [70]. Also, inflammation is known to reduce CYP induction and may

be mediated through NFκB [71]. In our study, the HSF diet promoted down-regulation of some CYP subfamilies, such as CYP27a1, CYP1a2, CYP2b1, CYP51a1 and CYP3a18, while the pretreatment with Orz promoted up-regulation of these proteins. Among phase II, some transferases, such as sulfotransferase (SULT), GST, and UDP-glucuronosyltransferase (UGT), catalyze the conjugation of polar functional groups onto xenobiotics for excretion [72]. The hypercaloric diet promoted a down-regulation of UGTA1 and GST family proteins in the HSF group, while the pretreatment with Orz promoted up-regulation, evidencing the role of the compound on the xenobiotic metabolism pathway that may be a therapeutic target for metabolic disorders in addition to its known role as a xenobiotic sensor [65].

In addition, Orz prevention was able to modulate the sirtuin signaling pathway. The sirtuins act by regulating glucose, lipid metabolism and insulin sensitivity. Evidence has demonstrated that nutritional or pharmacological stimuli of hepatic sirtuin pathway signaling protect against the development of liver disease in rodents [73,74]. Furthermore, sirtuin activators provide a benefit in preventing lipogenesis, thus reducing the impact of fatty liver, improving hepatic steatosis, preventing inflammation, and inhibiting lipogenesis [75]. In our study, the HSF diet promoted a down-regulation of the sirtuin signaling pathway, while the pretreatment with Orz promoted activation. The sirtuin proteins family is able to activate some transcription factors, such as HIF-1 [76], which was suppressed by the compound through the down-regulation of some promoters (ENO3, PKFM, and ALDOA). Overall, HIF-1 activation is an adaptive response to mediate cell homeostasis under hypoxia; however, the response impairs glucose and lipid metabolism, alters autophagy, and increases inflammation-enhancing profibrotic mediators [77]. The down-regulation of some sirtuin pathway axis through HIF-1 can influence the intrinsic pathways of fatty acid catabolism in adipocytes, promoting a metabolic state that favors the development of obesity and associated pathologies, such as NAFLD [76,78,79].

As well, sirtuin 1 and sirtuin 6 prevented the activation of HSCs, counteracting liver fibrosis in a mouse model and may act as a metabolic sensor that controls the expression of transcription factors and co-activators involved in lipid metabolic homeostasis, promoting protection against liver diseases, including PPAR-α, PPAR-γ co-activator 1 alpha, and NF-κB [80–82]. In the liver, PPARα expression is relatively high in hepatocytes and plays a crucial role in fatty acid oxidation by elevating mitochondrial and peroxisomal β-oxidation rates [83]. PPARγ, mostly expressed in adipose tissue, is significantly induced in hepatocytes in metabolic diseases and regulates lipid metabolism. The up-regulation of PPARs is frequently found in NAFLD models as a response to exacerbated substrate influx and accumulation [83]. In the HSF group, up-regulation of PPARs inductors (PLIN2, CPT1B, FABP4, FABP3) was observed, as was down-regulation of the same proteins in the HSF+Orz group. The sought of natural PPAR agonists is explored in the literature, including studies with Orz in this context [84]. We hypothesized that the Orz may act as a PPAR agonist to treat obesity, hyperlipidemia, and insulin resistance.

In summary, the results of this study become relevant since liver disease is a major global problem, mainly because it is triggered by diets rich in sugar and fat from ultra-processed foods. It was observed that a simple association of Orz with the HSF diet provided relevant changes in the liver proteome. These discoveries are unprecedented and open a new avenue for exploration in future studies since Orz is a compound from brown rice, a food widely consumed in Western countries that consumes the most fats and sugars. We highlight that this was an exploratory proteomic study of the preventative effects of Orz. Although our results are interesting, mechanistic experiments are needed to validate the proteins and pathways modulated by the compound since there are few studies addressing these aspects. Therefore, future research is needed to overcome these limitations, including genomics, metabolomics, and transcriptomics studies.

5. Conclusion

This study brought to light the modulation of proteins and pathways related to the pathophysiology of NAFLD and its progression by the Orz compound. Orz appears to be a promising therapeutic agent for NAFLD as it was able to prevent multiple alterations on the hepatic proteome exhibiting a positive effect. However, it is important to note that, as a limitation, it requires further mechanistic validation of the Orz action on NAFLD through additional studies. These findings may provide insights into future studies aiming at NAFLD treatment targets.

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Declaration of Competing Interest

The authors declare no conflicts.

CRediT authorship contribution statement

Juliana Silva Siqueira: Writing – original draft, Methodology, Funding acquisition, Data curation, Conceptualization. **Jessica Leite Garcia:** Writing – original draft, Methodology, Data curation, Conceptualization. **Artur Junio Togneri Ferron:** Methodology. **Fernando Moreto:** Methodology, Conceptualization. **Luis Eduardo Sormani:** Methodology. **Mariane Rovero Costa:** Methodology. **Thiago Luiz Novaga Palacio:** Writing – review & editing, Methodology. **Gisele Alborghetti Nai:** Methodology. **Giancarlo Aldini:** Writing – original draft, Conceptualization. **Fabiane Valentini Francisqueti-Ferron:** Writing – review & editing, Methodology, Data curation. **Camila Renata Correa:** Supervision, Project administration, Funding acquisition, Conceptualization.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2024.109607.

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