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ORIGINAL ARTICLE

Changes in hepatic thiol contents and regulation of glutathione S-transferase by high-fructose diet: Effects of kefir and some probiotic bacteria

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Abstract

In this study, thiol/disulfide homeostasis in the liver tissues of high-fructose-fed rats was investigated in conjunction with the changes in the main hepatic detoxification enzyme, glutathione S-transferase (GST). Additionally, the effects of well-known probiotics namely Kefir, *Lactobacillus helveticus,* and *Lactobacillus plantarum* supplementation on the thiol/disulfate contents and GST activity and gene expression levels were analyzed. Fructose, administered as a 20% solution in drinking water for 15 weeks, developed an animal model of metabolic syndrome in male Wistar rats. Kefir, *L. helveticus,* and *L. plantarum* supplementations were given by gastric gavage once a day during the final 6-weeks. The changes in hepatic GST were determined with kinetic-optimized spectrophotometric enzyme assays and qRT-PCR. Total thiol, native thiol, and disulfide levels were analyzed using (5,5-dithio-bis-(2-nitrobenzoic acid) as a chromogenic agent. High-fructose consumption reduced total and native thiol contents while increasing disulfide levels in the liver tissues of rats. Kefir and *L. plantarum* normalized the thiol levels and all probiotics reduced disulfide contents. High fructose augmented total GST activity but reduced the GST-Mu isoform. *L. helveticus* and *L. plantarum* normalized the total and GST-Mu activity, respectively. These results demonstrated a shift toward disulfide formation in the hepatic tissues of rats fed with high fructose. A possible reason would be the increase in total GST activity that uses the free glutathione, the main native thiol source in cells, as a substrate. Besides, probiotics such as Kefir, *L. helveticus,* and *L. plantarum* have an improving effect on thiol/disulfide homeostasis as well as main detoxification enzymes.

Keywords: Kefir, *Lactobacillus plantarum*, *Lactobacillus helveticus*, glutathione S-transferase, thiol/disulfide balance, fructose

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Introduction

Today, fructose is widely utilized in processed foods because it has a longer shelf life, dissolves quicker, and is sweeter than other carbohydrates [1]. Numerous studies have demonstrated that a high-fructose diet develops metabolic syndrome with insulin resistance, hypertriglyceridemia, endothelial dysfunction, oxidative stress, and hepatic steatosis [2-4]. As a result of hepatic stress caused by the load of fructose metabolism, it also showed hepatic oxidative damage and altered lipid metabolism [5].

Prooxidant and antioxidant imbalance might contribute to the onset of metabolic syndrome even though its etiology is extremely complex and not yet fully understood [6]. In comparison to healthy individuals, patients with metabolic syndrome have reduced plasma antioxidant enzyme activity and greater levels of oxidative damage indicators [7]. By encouraging the development of inflammation, thrombosis, and atherosclerosis and affecting vascular function, increases in oxidative stress have been linked to metabolic syndrome's pathogenesis [8].

Antioxidant defense is greatly aided by dynamic thiol-disulfide homeostasis, which is also engaged in critical processes like apoptosis, signal transmission, and enzyme activity regulation [8]. Organic molecules with a sulfhydryl group (-SH) are defined as thiols that compromise the majority of the body's non-enzymatic antioxidants. Intracellular and extracellular total thiol pool includes either free or oxidized (disulfide) forms or thiols attached to proteins. The reversible formation of mixed disulfides between two separate thiol-containing molecules might emerge from their oxidation. Covalent disulfide linkages that are produced when thiols undergo an oxidation reaction might be reduced via other antioxidant systems [9]. Thiols that are found in proteins as well as thiols that have a low molecular weight, such as cysteine, homocysteine, and glutathione make up the majority of the intracellular thiol pool and their levels decrease under conditions of high oxidative stress to neutralize free radicals, and the sulfhydryl groups of thiols play a crucial role in this process.

Among the several detoxification enzymes present in the liver, glutathione S-transferases (GSTs) play a vital role in the detoxification of xenobiotics and endobiotic compounds. To make electrophilic molecules more hydrophilic and less hazardous, they conjugate intracellular thiol-containing reduced glutathione (GSH) to the electrophilic substance. Therefore, GST activity fundamentally controls glutathione homeostasis, and the redox state of glutathione is crucial for several biological processes. Besides several foreign compounds and metabolites of oxidative stress are detoxified by the cytosolic GSTs, namely the Mu isoenzyme, which is particularly concentrated in the liver [10].

Probiotics are living bacteria that, when administered in sufficient doses, have a beneficial effect on a host. Their dietary supplements have been employed in the prevention and treatment of a number of illnesses, including gastrointestinal disorders, allergies, and diseases that are inflammatory in nature [11]. They have considerable antioxidant properties both in vivo and in vitro [12] and are naturally present in fermented dairy products including kefir, yogurt, cheese, and others. One of the key elements of the human microbiota, *Lactobacillus* species, has been shown to limit hyperinsulinemia, dyslipidemia, and hyperglycemia in high-fructose or high-fat-fed rats [13-15]. *L. plantarum* supplementation has been demonstrated to increase insulin resistance and antioxidant enzyme capacity, as well as to reduce pro-inflammatory cytokines [16]. Recently, we showed that supplementing with *L. plantarum* and *L. helveticus* mitigated the negative effects of dietary fructose on antioxidant enzymes such as superoxide dismutase and catalase [17]. Several lactic acid and acetic acid bacteria, as well as yeasts, are lodged in the polysaccharide matrix known as kefiran in the fermented milk product kefir, which have also possible health benefits in a variety of illness models [18,19]

Limited information in the literature exists regarding how probiotic therapy affects metabolic syndrome brought on by high fructose consumption. Therefore, the goal of the current investigation was to determine if supplementing fructose-fed rats with Kefir, *L. helveticus*, and *L.*

plantarum enhances thiol-disulfide equilibrium and modifies the primary detoxification enzymes namely glutathione S-transferase in the liver tissues of rats.

Materials and Methods

Animals and Diets

Three-week-old male Wistar rats were housed in climate- and humidity-controlled environments with a 12-hour light/dark cycle. The protocol to use animals was authorized by the Afyon Kocatepe University Ethical Animal Research Committee (approval number: 49533702-117). Standard rat chow, consisting of 62 percent starch, 23 percent protein, 4 percent fat, 7 percent cellulose, common vitamins, and salt, was fed to the rats.

The rats were randomly separated into five groups of eight rats each after one week of accommodation and the groups were as follows; Control, Fructose (F); Fructose + Kefir (F+K); Fructose + *L. plantarum* (F+LP) and Fructose + *L. helveticus* (F+LH). For 15 weeks, rats were fed a 20 percent (w/v) solution of fructose (Danisco Sweeteners OY, Kotka, Finland) in drinking water. During the last six weeks, rats were also administered to 1×10^9 CFU per 100 g of body weight of *L. plantarum* and *L. helveticus* (Horsholm, Denmark; ATCC: 14917 and ATCC: 15009, respectively) which were cultured in our laboratory through gastric gavage once a day. The rats were also gastric gavaged with kefir once daily for the final six weeks (1 ml per 100 g of animal weight). The kefir grains were obtained commercially (Sevdanem, Danem Kefir, Isparta, Türkiye) and fermented in our lab daily with pasteurized milk. Rats were sedated with a ketamine-xylazine cocktail (100 and 10 mg/kg, *i.e.*, respectively) at the end of the observational period and then decapitated. Liver tissues were dried, frozen in liquid nitrogen, and stored at -85 °C for further molecular analyses.

Preparation of Cytosolic Fractions from Rat Liver Tissues

After being washed with the ice-cold homogenization mixture (50 mM KH_2PO_4, 1.15% KCl, 0.5 mM PMSF, 5 mM EDTA, pH:7.0), the bladed homogenizer (Tissue Ruptor™, Qiagen, USA) was used to slice and homogenize rat liver tissues. The samples were then centrifuged at 1,500 g for 15 minutes to separate the nuclear component from the non-degradable cells. Supernatants were put into Eppendorf tubes and stored for subsequent use at -85°C. The Lowry technique [20] was used to determine the homogenates' total protein content.

Determination of Kinetic Parameters of Total and Mu Isoform of Glutathione S-transferase Activity

Total GST enzyme activity is quantified using 1 chloro-2,4 dinitrobenzene (CDNB), a common substrate for all GST isoforms, and reduced glutathione (GSH) as substrates. GST-Mu activity is differentiated from total by using l,2-dichloro-4-nitrobenzene (DCNB) instead of CDNB [21]. To begin, the kinetic characteristics of the liver GST enzymes were analyzed to find the most effective ways to measure their activity. This was accomplished by studying the GST enzymatic activity depending on GSH by maintaining a constant concentration of CDNB. In addition, variations in CDNB-dependent GST activity were tracked at constant GSH concentrations. The same experiments were also repeated with DCNB instead of CDNB as substrate. After calculating Km and Vmax values for the substrates of GST, the following optimized protocol was conducted to determine hepatic GST activities.

In a UV-transparent 96-well plate, 15 µl of 2 mg protein-containing homogenates were thoroughly mixed with 250 µl of phosphate buffer (50 mM, pH:7.0), 20 µl of GSH (50 mM), and 15 µl of CDNB (50 mM) or DCNB (20 mM). The changes in absorbance were then measured for 2 minutes at 340 nm using a spectrophotometric microplate reader (MultiScan GO™, Thermo Scientific, USA). Total GST and GST-Mu isoform activities were determined as the amount of product formed by The homogenate containing 1 mg protein per minute using the extinction coefficient of 9.6 $mM^{-1}.cm^{-1}$ for total GST and 8.5 $mM^{-1}.cm^{-1}$ for DCNB-dependent GST activities [22].

Determination of Total and Native Thiol Contents, Disulfide Amounts

Total thiol, native thiol, and disulfide contents of liver homogenates were measured using Ellman's reagent (5,5-dithio-bis-(2-nitrobenzoic acid)) as a chromogenic reagent [23]. For the measurement of total thiol contents, 120 µl of homogenates or different concentrations of GSH standards (0.10-0.25-0.50-0.75-1, 2, 4 mM) were mixed with 40 µl of 20% (w/v) TCA and centrifuged at 10,000 *g* for 20 minutes. Then, in a 96-well microplate, 220 µl of EDTA (10 mM, prepared in 500 mM Tris pH: 8.2), 20 µl of supernatant (or standards), and 20 µl of DTNB (10 mM, dissolved in methanol) were added. After 10 min incubation, absorbances at 415 nm were measured and the total thiol contents were calculated from the GSH standard calibration curve. Thiol contents of liver tissues were calculated as nmol thiol/mg protein [24,25].

Native thiol contents (GSH) were measured using the total thiol methodology with the exception that sodium borohydride ($NaBH_4$) was used to pre-reduce dynamic disulfide linkages (-S-S-) in the sample to free functional thiol groups [26]. Briefly, 600 µl of homogenate was mixed with 150 µl of reaction solution (3.5 M NaBH4 and 1.5 M NaOH, prepared absolute methanol) and incubated for 20 min at room temperature. After adding 70 µl of 12 M HCl, the samples were centrifuged at 10,000 *g* for 20 minutes. The thiol contents of the supernatant were determined by the abovementioned protocol as described. After the total thiol and native thiol measurements were completed, half of the difference between total and native thiols was calculated as disulfide contents as described previously [27].

Determination of gstm1 Gene Expressions by qRT-PCR

Total RNA was extracted from liver tissues by following the manufacturer's instructions for the Rneasy total RNA extraction kit (Qiagen, Hilden, Germany). Quantity and quality were determined using 260/280 nm spectrophotometer readings and agarose gel electrophoresis (Thermo Scientific, Waltham, MA, USA). One microgram of total RNA was converted to cDNA by reverse transcription using a Thermo Scientific kit (Thermo Scientific, Waltham, MA, USA). Gene expressions of *gstm1* isoform and internal standard *gapdh* were determined by qRT-PCR using the primer pairs for *gstm1* forward: 5′- AGAAGCAGAAGCCAGAGTTC and *gstm1* reverse: 5′-GGGGTGAGGTTGAGGAGATG, *gapdh* forward: 5′-TCCTTGGAGGCCATGTGGGCCAT and *gapdh* reverse: 5′-TGATGACATCAAGAAGGTGGTGAAG as we described previously in detail [28].

Statistical Analysis

Each experiment was carried out at least three times. The results are presented as mean ± standard errors of the mean (SEM). The enzyme kinetics module of GraphPad (version 8.0, GraphPad Software, La Jolla, CA, USA) software was used to determine Km and Vmax values from double reciprocal graphs. One-way ANOVA followed by the Bonferroni post-hoc test was used to conduct statistical comparisons using the same software. When the P values were less than 0.05, the differences in the groups were accepted as significant.

Results

The Changes in Metabolic Parameters

Our recent research [15,29] presented data on the metabolic indicators of the same animals, involving body weight, caloric consumption, plasma levels of insulin, glucose, and triglyceride levels. The use of high-fructose or probiotics did not affect the body weight of rats. All probiotics had no effect on the plasma glucose rise caused by a high-fructose diet. Fructose increased serum and liver fructose levels, as well as lipopolysaccharide contents, whereas the kefir intervention dramatically decreased fructose levels in rat hepatic tissues. Increased plasma insulin and triglyceride levels were normalized with kefir, *L. plantarum,* or *L. helveticus* supplementations. Herein, we used the same animal tissues to investigate the biochemical and molecular effects of fructose and probiotics on the liver.

Kinetic Parameters (Km and Vmax) of GST Enzymes

In this study, firstly we figured out the kinetic parameters of both the total and Mu-type

isozymes of GST enzymes using CDNB and DCNB as substrates in addition to GSH which is a common substrate of all GST isozymes. For this, the effect of CDNB on the enzyme activity of GST was studied by keeping the amount of GSH constant. Figures 1A and 1B show the Michaelis-Menten plot and the Lineweaver-Burk plot for the CDNB-dependent GST activity. Furthermore, GSH-dependent GST activity was investigated at constant CDNB levels, and variations in GST activity were tracked. The Michaelis-Menten plot (Figure 1C) and the Lineweaver-Burk plot (Figure 1D) show that the maximum velocity of total GST activity was calculated to be 481.80 ± 6.44 U/mg, and the Km value for GSH and CDNB was found to be 12.94 ± 0.71 mM and 8.85 ± 1.18 mM, respectively.

The kinetic characteristics of DCNB-specific GST-Mu activity were also established. The DCNB-dependent enzymatic activity of GST-Mu was investigated by maintaining the concentration of the GSH constant. Figures 2A and 2B show Michaelis-Menten and Lineweaver-Burk plots for DCNB-dependent GST activity. GSH-dependent GST-Mu activity was also investigated at constant DCNB levels, and fluctuations in GST-Mu activity were tracked. According to the Michaelis-Menten (Figure 2C) and Lineweaver-Burk (Figure 2D) plots, the maximum velocity of GST-Mu activity (Vmax) was calculated as 8.01±0.34 U/mg, Km value for GSH and DCNB were determined as 15.15±2.19 mM and 2.97±0.49 mM, respectively.

The Changes in Hepatic Total GST and GST-Mu with a High-fructose Diet and the Effects of Probiotics

Glutathione S-transferases catalyze glutathione conjugation to electrophilic molecules, mostly external xenobiotics but also endogenous chemicals. Glutathione conjugation is the initial stage and an essential detoxification mechanism. In addition to conjugation processes, some isoforms of this enzyme, such as Mu kinds, have peroxidase-like activity toward hydroperoxides [30] and hence could play a role in stress metabolism. Our results demonstrated a general increase in total GST activity in fructose-fed rats which was decreased by *L. helveticus* administration (Fig. 3A). On the contrary to total

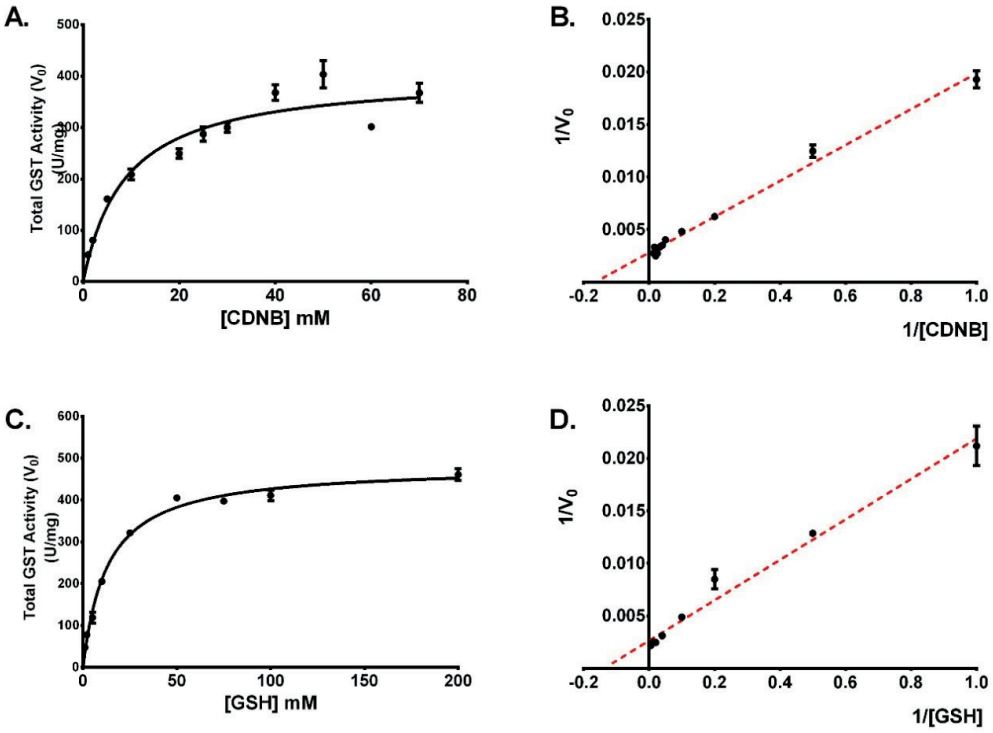

Figure 1. Michaelis-Menten and Lineweaver-Burk plots for CDNB-dependent GST activities (A and B), and GSH-dependent GST activities (C and D), respectively.

GST activity, GST-Mu enzyme activity and its gene expression levels were suppressed with fructose (Fig 3B and 3C). None of the probiotics have significant effects on gene expression levels but only *L. plantarum* upregulated GST-Mu activity towards the control group.

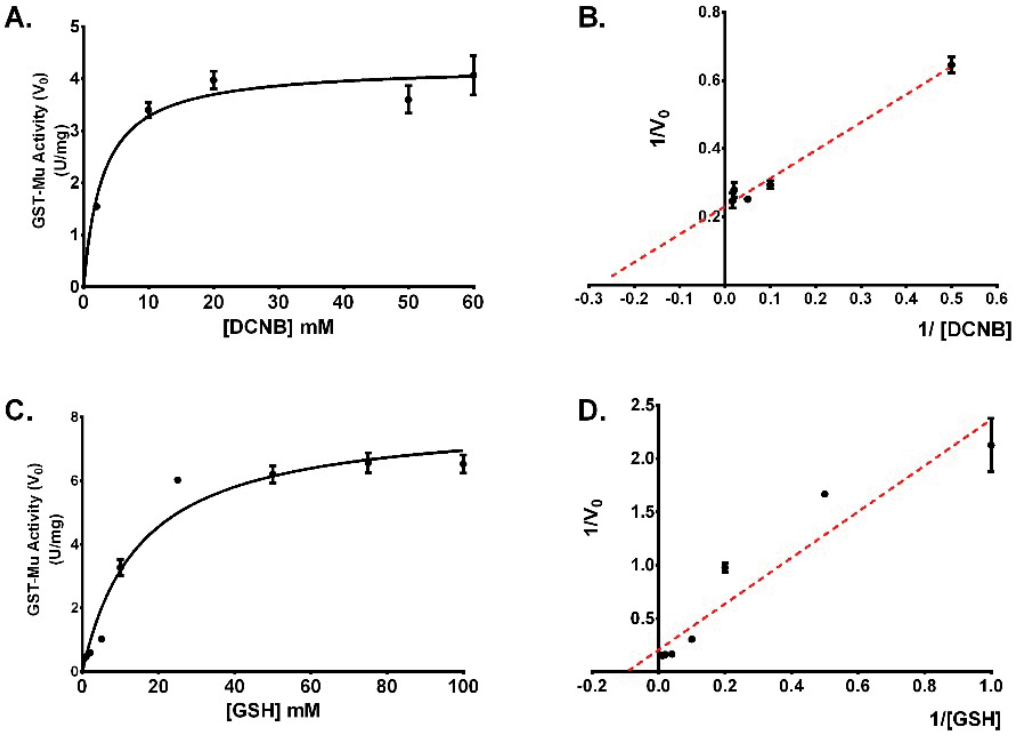

Figure 2. Michaelis-Menten and Lineweaver-Burk plots for DCNB-dependent GST-Mu activities (A and B), and GSH-dependent GST-Mu activities (C and D), respectively.

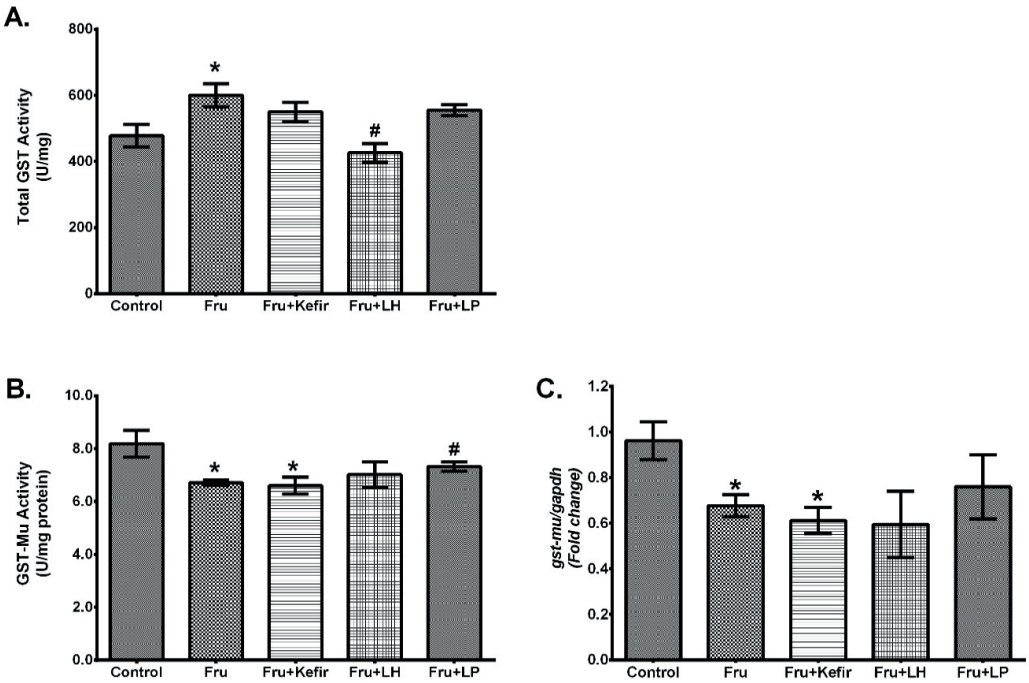

Figure 3. Effects of high-fructose diet and probiotics on hepatic total GST activities (A), hepatic GST-Mu activities (B), and GST-Mu gene expression levels (C). Differences from control (*) and fructose (#), $P < 0.05$. Results are given as mean ± SEM (n = 6–8). Fru: Fructose, LH: *Lactobacillus helveticus*, LP: *Lactobacillus plantarum*

Changes in Total Thiol, Native Thiol, and Disulfide Contents

Native, total and oxidized thiol levels in hepatic tissues were evaluated in this study to highlight dynamic variations in the thiol metabolism. In addition, the ratio of extant native thiol and disulfide concentrations to total thiols was evaluated to identify oxidative alterations thoroughly. Results clearly demonstrated the suppression of total and native thiol contents by high-fructose (Fig. 4A and 4B). However, oxidized forms of thiols, that is disulfide levels, were augmented with fructose (Fig. 4C). Kefir and *L. plantarum* have modulatory action on native thiol contents in such a way that they normalized the thiols up to the control group. In addition, the considerable elevation of disulfide content was identified in the liver tissues of rats fed a high-fructose diet. All probiotics significantly restored the disulfide levels ($P < 0.05$), indicating the suppression of oxidative stress-induced changes in thiol metabolism.

Discussion

High consumption of processed foods with excess fructose levels, reportedly has a

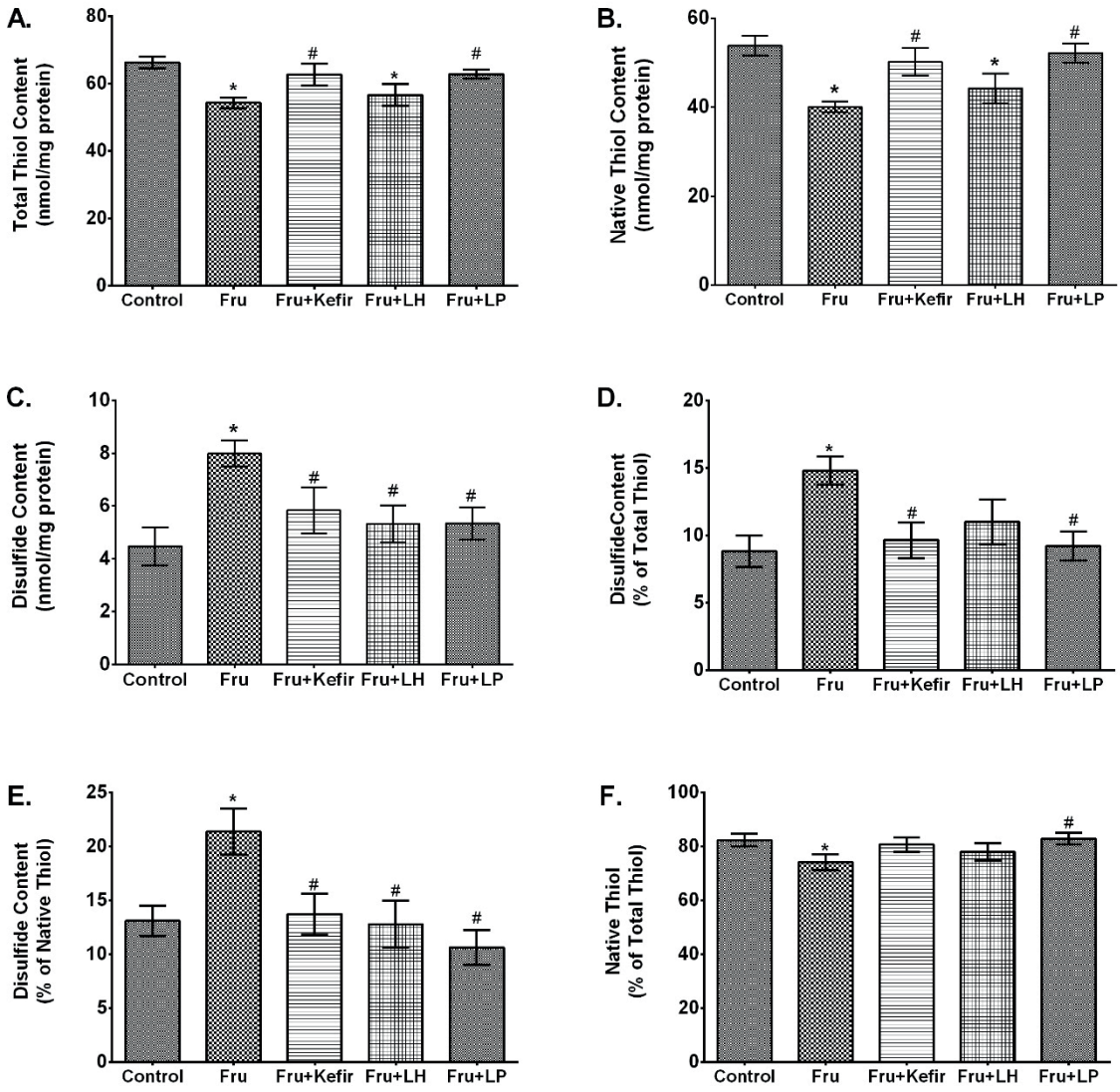

Figure 4. The changes in total thiol (A), native thiol (B), disulfide content (C), percent disulfide content against total thiol levels (D), percent disulfide content against native thiol levels (E), percent native thiol content against total thiol levels (F) in hepatic tissues of fructose- and probiotic-fed rats. Differences from control (*) and fructose (#), $P < 0.05$. Results are given as mean ± SEM (n = 6–8). Fru: Fructose, LH: *Lactobacillus helveticus*, LP: *Lactobacillus plantarum*

connection to the rising prevalence of insulin resistance, obesity, and metabolic syndrome [2]. On the other hand, the mechanism by which high-fructose consumption contributes to the development and worsening of metabolic disorders is still up for debate. One of the detrimental consequences of dietary fructose is the deterioration in the oxidant/antioxidant balance [31] and experiments have revealed that high-fructose ingestion causes oxidative stress in a variety of tissues and organs [32].

In cells, there exist specific oxidized/reduced ratios of free thiols and thiol-containing compounds such as cysteine, glutathione, and thiol proteins. This pool of cellular thiols is controlled by the mechanisms linked to their inherent antioxidative activity, as well as their dynamic synthesis and removal from cells via glutathione redox cycles and xenobiotic conjugation [33]. Dynamic thiol/disulfide homeostasis in organisms governs cellular antioxidant systems and signaling processes [34], and therefore, plays a vital role in maintaining the intracellular oxidation-reduction potential balance. GSTs, a major thiol metabolizing enzyme, react differentially under both normal and pathological situations to a number of chemicals and oxidative stress [35]. Both in vivo and in vitro studies have shown that GSTs are controlled by reactive oxygen species, including superoxides, hydrogen peroxide, and the byproducts of membrane lipid peroxidation [36,37]. The cytosolic Mu isoenzyme of GSTs is particularly high in the liver, and in addition to its involvement in the detoxification of various external substances and oxidative stress byproducts, it also plays a role in the regulation of gene expression [35]. Herein, we looked at how a high-fructose diet affects the hepatic thiol/disulfide balance and the major detoxification enzyme, glutathione S-transferase, which utilizes the cellular thiol pool for its function. Furthermore, we also investigated the effects of dietary kefir supplementation in conjunction with individual probiotic bacteria, *L. plantarum*, and *L. helveticus,* on thiol homeostasis and the main hepatic detoxification enzyme.

Our results showed that high-fructose consumption reduced hepatic total and native thiol levels by acting as an oxidative stress source in the liver tissues. The increased hepatic oxidative burden was further demonstrated by the increased disulfide concentration. The decrease in native thiol contents in the liver tissues most probably is the result of its increased utilization due to oxidative stress and enhanced total GST activities which use glutathione for its function. In contrast to overall GST activity, high-fructose diet decreased Mu-type GST isozyme expression and activity levels. The reduction in GST-Mu mRNA expressions suggests that its transcription is being controlled. The drop might possibly be related to a decrease in mRNA half-lives since increased oxidative stress can lead to mRNA instability [38].

Kefir, one of the well-known probiotics has been shown to exert anti-inflammatory, antiviral, and anti-allergic effects [39]. Its protective activities have also been established against metabolic diseases caused by fructose [19]. Recently, we have demonstrated favorable effects of kefir on the hepatic and intestinal abnormalities caused by excessive fructose consumption [29]. The expression of inflammatory markers and major lipogenic genes in the liver were both suppressed with kefir supplementation. In addition, kefir therapy enhanced insulin signaling elements and fructose transporters. Herein, while kefir did not significantly affect the activity or expression of GST enzymes, it did raise total and native thiol levels within the normal range. It also reduced the disulfide levels drastically in high-fructose-fed rats, which indicates that the oxidative pressure is being relieved in favor of a more reduced atmosphere.

Another recent study we performed also demonstrated the reduction of plasma insulin levels and improvement in the kidney antioxidant parameters with probiotic bacteria, *L. plantarum* and *L. helveticus* which were supplemented to the high-fructose-fed rats [17]. Hepatic weight, lipid content, and fatty acid synthase expression were all reduced in high-fructose-fed rats when *L. plantarum* was supplemented into the diet. *L. helveticus* had also a restorative impact on lipid accumulation by lowering fatty acid synthase expression and modulating insulin receptor substrate-1 [14]. According to the findings of this research, the effectiveness of *L. plantarum* in

restoring cellular total and native thiol contents and disulfide levels was comparable to that of kefir. *L. helveticus* was similarly efficient in reducing the increased levels of disulfide in rats that had been given fructose. *L. helveticus* is the only microorganism that significantly affects GST enzyme levels, decreasing total GST activity in the direction of the control group. Neither kefir nor *L. plantarum* substantially altered GST activity or expression levels. Taken together, the bases of the antioxidant effects of probiotics as indicated in this study would be the maintenance of cellular thiol contents in their reduced form. Previously, probiotic supplementation in rats has been shown to boost glutathione production in pancreatic cells [40] and to activate the transcription of genes involved in glutathione manufacture in the intestinal mucosa [41]. Additionally, supplementation with *L.casei*, *L. acidophilus*, and *Bifidobacterium lactis* was also effective in restoring glutathione levels in rats after oxidative stress [42]. These researches back up our hypothesis that probiotic bacteria would boost reduced glutathione and other thiol-containing substances in the presence of oxidative stress which is induced by high-fructose diet.

Overall, this study shows that high-fructose diet modulates the activities of total hepatic glutathione-dependent detoxification enzymes, mainly glutathione S-transferases. Because acute fructose intake causes a quick but transitory burst of damaging reactive oxygen species, this would have to be considered as a defensive reaction. The upregulation of the total GST enzyme activities by fructose observed in this study reduced free thiol levels and leads the formation of oxidized thiols (*i.e.*, disulfides). Because of this, the redox equilibrium that exists inside the liver tissues will ultimately become disrupted. Although more research is needed to explore the probable processes involved, the current study demonstrates the efficacy of chosen probiotics in the prevention of oxidative stress, particularly in terms of thiol/disulfide homeostasis. In this manner, antioxidant probiotic strains can be identified and studied as prospective candidates for the prevention and treatment of a variety of free radical-related disorders such as obesity, diabetes mellitus or metabolic syndrome.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability Statement

All data within this study has been presented and there is no other data for public repositories.

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