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Original Article

Lactobacillus plantarum and *Lactobacillus helveticus* modulate SIRT1, Caspase3 and Bcl-2 in the testes of high-fructose-fed rats

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ABSTRACT

Background and Aims: The influence of a high-fructose diet and probiotics on the male reproductive system and the testicular apoptotic pathway has been poorly documented. In this study, we aimed to investigate the influence of *Lactobacillus plantarum* and *Lactobacillus helveticus* supplementation on apoptotic factors such as sirtuin1, caspase3 and bcl-2 on the testicular tissue of high-fructose-fed rats.
Methods: Fructose was given to the rats as a 20% solution in drinking water for 15 weeks. Gene expressions were established by real-time PCR. Protein levels were determined by Western blot analysis.
Results: Fructose consumption did not change mRNA expression of SIRT1, but did resulted in a decreased protein level. Dietary fructose reduced bcl-2 mRNA and protein expressions, whereas no changes were observed in the gene and protein expression levels of factor caspase-3. Both *Lactobacillus* supplementations increased SIRT1 protein expression without changing the mRNA levels in fructose-fed rats. The supplementations with both probiotics produced a significant down-regulation on caspase3 mRNA and protein levels. Bcl-2 proetin level increases with both probiotics supplementation while, mRNA level did not show difference in *L.plantarum*, but increased in *L. helveticus* supplementation.
Conclusion: Treatments with *L.plantarum* and *L.helveticus* can reduce testicular apoptosis induced by dietary high-fructose in rats via suppressing caspase3 and promoting sirt1 and bcl-2 protein expressions.

Keywords: Dietary fructose, *Lactobacillus plantarum*, *Lactobacillus helveticus*, apoptosis, testes

INTRODUCTION

Metabolic syndrome (MetS), which can be promoted by excessive fructose consumption, is the main health problem that affects people life quality along with the worldwide due to its many complications such as glucose intolerance, central adiposity, hyperlipidemia, hypertension, fatty liver disease and chronic low-grade inflammation (Dandona & Dhindsa, 2011; Morrison & Brannigan, 2015; Rastrelli, Filippi, Sforza, Maggi, & Corona, 2018; Tsai, Matsumoto, Fujimoto, & Boyko, 2004). In recent studies, it has been shown that low testosterone levels, one of the several factors responsible for male infertility, is commonly associated with metabolic syndrome, obesity, and Type-2 diabetes (Caldas, Porto, Motta, & Casulari, 2009; Dhindsa et al., 2010; Ebrahimi et al., 2017). Studies have shown that low testosterone levels may enhance oxidative stress and also trigger apoptosis of Germ cells and Sertoli cells (Chaki et al., 2006; Simoes et al., 2013). However, the roles of apoptosis in fructose-related MetS and testicular homeostasis has not been investigated. In recent years, studies in rodents showed that diabetes causes increased inflammation and testicular oxidative stress as well as elevat-

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ed Bax/Bcl2 ratio and caspase-dependent apoptosis (Nna, Bakar, Ahmad, & Mohamed, 2018; L. Zhao et al., 2017). The consumption of 10% fructose in drinking water for 8 weeks significantly increased apoptosis-associated speck-like protein (ASC) and caspase-1 levels of rat kidneys (Hu, Zhang, Pan, Li, & Kong, 2012). A recent study demonstrated that a high consumption of fructose (35% of daily calories) increased Bax / Bcl2 ratio and the number of apoptotic cells compared to the control in the liver of mice (Choi, Abdelmegeed, & Song, 2017). It has been reported that rats fed with a fructose diet have the activation of pro-apoptotic c-Jun N-terminal kinase (JNK) and apoptotic caspase3 in the liver and pancreatic tissues (Balakumar et al., 2016). We have previously shown that a high-fructose diet causes down-regulation of SIRT1 and up-regulation of iNOS protein expressions in the aorta of rats (Akar et al., 2012; Babacanoglu, Yildirim, Sadi, Pektas, & Akar, 2013). In rats administered with 30% fructose in drinking water, an induction of apoptosis was found and an increase in Bax/Bcl2 ratio and caspase-3 levels in the aorta were detected (Lu, Zhao, Yao, & Zhang, 2017). The consumption of 10% fructose in drinking water initiated apoptosis with increased Tumor necrosis factor-α (TNF-α) and p53 levels, while it suppressed SIRT1 of rat liver (L. Song et al., 2019). In another study, the administration of 30% fructose in drinking water caused structural abnormalities and increased apoptotic cell number in the testes of rats (Meydanli et al., 2018). In our previous study, dietary high-fructose enhanced mitogenic protein IGF-1R and inflammatory markers such as iNOS, IL-1β, and TNF-α expressions which are accompanied by low testosterone in the testes of rats. Besides, our histological examination demonstrated intratubular degeneration in the testes of fructose-fed rats (Yildirim et al., 2019).

Probiotics are living microorganisms that benefit their host and are used to prepare fermented products (Hotel & Cordoba, 2001; Rosa et al., 2016). The beneficial effects of probiotics on health are accepted worldwide. Probiotics may be useful in reducing cardiovascular risk factors from pathological conditions such as type 2 diabetes (Hendijani & Akbari, 2018; Markowiak & Slizewska, 2017). Increased adipocyte inflammation, liver fat accumulation, and apoptosis due to high-fat and fructose diets were significantly suppressed by *Lactobacillus* (*L.*) *rhamnosus* supplementation (Q. Liu et al., 2020). Another study reported that high-fructose-induced increases in plasma glucose, insulin and triglyceride levels, oxidative stress, and hepatic lipogenesis were reduced with *L. curvatus* and *L. plantarum* supplementations (Park, Ahn, Huh, McGregor, & Choi, 2013). In our recent study, an *L. plantarum* and *L. helveticus* supplementation in high-fructose-fed rats reduced plasma insulin levels and improved kidney antioxidant parameters (Korkmaz et al., 2019a). Also, the consumption of these probiotics improved the insulin signaling pathway in the kidney and liver of rats fed with fructose (Korkmaz et al., 2019b; Sumlu, Bostanci, Sadi, Alcigir, & Akar, 2020). In this study, we aimed to find the effectiveness of *L. plantarum* and *L. helveticus* on the apoptotic targets in the testes of high-fructose-fed rats.

MATERIALS AND METHODS

Animal and diets

The Ethical Animal Research Committee of Afyon Kocatepe University (Akuhadyek-49533702), in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council Committee 2011) approved the protocol for animal use. Three-week-old male Wistar rats were housed in temperature- and humidity-controlled rooms (20-22°C, 40-50% relative humidity) with a 12-h light-dark cycle. The rats were fed with a standard rodent chow diet composed of 62% starch, 23% protein, 4% fat, 7% cellulose, standard vitamins, and salt mixture. After acclimation for 1 week, the rats were randomly divided into 4 groups: control, fructose Fruc, fructose + *L. plantarum* (Fruc + LP), and fructose + *L. helveticus* (Fruc + LH). Fructose (Danisco Sweeteners OY, Finland) was given to the rats as a 20% solution (w/v) in drinking water, which was freshly prepared every day, *ad libitum* for 15 weeks. *L. helveticus* and *L. plantarum* were given by gastric gavage once a day for the final six weeks in 2 ml saline solution at appropriate dosing ($1x10^9$ CFU per 100 g of body weight of animal). The same volume of saline was given to the control and fructose groups by the gavage for the same period. At the end of the experiment, the animals were anesthetized with a mixture of ketamine-xylazine (100 and 10 mg/kg, respectively, i.p.). The testicular tissues were immediately dissected and blotted dry. Then they were frozen in liquid nitrogen and stored at −85 °C until the gene and protein expression studies.

Preparation of *Lactobacillus plantarum* and *Lactobacillus helveticus*

Lactobacillus helveticus and *L. plantarum* were cultured in De Man, Rogosa and Sharpe broth (MRS; Oxoid; Unipath Ltd., Basingstoke, Hampshire, England) at 30 ^{O}C in a rotary shaker at 150 rpm. Stock cultures were stored at -80 ^{O}C in MRS broth including 20% (v/v) glycerol. Erlenmeyer flasks containing 20 ml of MRS were inoculated with 1.5 ml of glycerol stock culture. The cultures were incubated at 35 ^{O}C ± 1 ^{O}C on a rotary shaker at 150 rpm and grown to an optical density of 1.0 at 600 nm (cell density corresponding to $1x10^8$ CFU/ml). The culture was divided into 10 ml tubes ($1x10^9$ CFU), then cells were harvested at 5000 g for 5 min at 4 ^{O}C. The cell pellets were washed by isotonic saline solution and lyophilized under a freeze dryer.

Determination of gene expressions with quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were isolated from the testicular tissues using RNeasy total RNA isolation kit (Qiagen, Venlo, The Netherlands) as described in the manufacturer's protocol. After isolation, the amount and the quality of the total RNA were determined by spectrophotometry at 260/280nm and agarose gel electrophoresis. Then, 1 µg total RNA was reverse transcribed to cDNA with a commercial first-strand cDNA synthesis kit (Thermo Fisher Scientific, USA). Sirt1, caspase3 and bcl-2 gene expression levels were determined by a real-time quantitative polymerase chain reaction (qRT-PCR, LightCycler480 II, Roche, Basel, Switzerland). mRNA expressions were determined by mixing 1 µl cDNA, 5 µl 2X SYBR Green Master Mix (Roche, Basel, Switzerland) and 2 µl primer pairs each (Table 1) at 0.5 µM final concentrations in a total volume of 10 µl. qRT-PCR was performed as follows: initial denaturation at 95 °C for 10 min, denaturation at 95 °C for 10 s, annealing at 58 °C for 15 s and extension at 72 °C for 15 s with 40 repeated thermal cycles measuring the green fluorescence at the end of each extension step. All samples were performed in

Table 1. Primer sequences of sirt1, caspase3, bcl-2 and internal standard gapdh used for the mRNA expression determination.

Gene	Forward Primer Sequence (5'→3')	Reverse Primer Sequence (5'→3')
sirt1	CGGTCTGTCAGCATCATCTTCC	CGCCTTATCCTCTAGTTCCTGTG
caspase3	GAGCTTGGAACGCGAAGAAA	CTCTGAGGTTAGCTGCATCG
bcl-2	TTCCTGCATCTCATGCCAAG	TACCAATAGCACTTCGCGTC
gapdh	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTGGGCCAT

triplicate and the specificity of the PCR products was confirmed using melt analysis. The relative expression of genes with respect to the internal control; glyceraldehyde 3-phosphate dehydrogenase (gapdh) was measured with the efficiency corrected advance relative quantification tool provided by the LightCycler® 480 SW 1.5.1 software.

Determination of protein expressions by Western blot

Testicular tissues were homogenized in 4-fold volumes of homogenization medium (50 mM Tris, 150 mM sodium chloride, 5 mM ethylenediaminetetraacetic acid, 1%(w/w) Triton X-100, 0.26% (w/v) sodium deoxycholate, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate and 0.2 mM phenylmethylsulfonyl fluoride, pH:7.4) with Tissue Ruptor™ (Qiagen, Venlo, Netherlands) homogenizer and then the homogenates were centrifuged at 1500g for 10 min at +4 °C. Then, the supernatants were collected and protein levels were determined using the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

All groups total proteins (50–100 µg) were separated by the SDS-PAGE method using Mini Protean Tetra electrophoresis (Bio-Rad Laboratories, Hercules CA, USA). The separated proteins were transferred onto the polyvinylidene fluoride membrane with a semi-dry electroblotting apparatus (TransBlot Turbo, BioRad, Germany) after which the membranes were blocked with 5% bovine serum albumin for 1 h. Primary antibodies were utilized for priming the respective SIRT1 (Anti-SIRT1 Rabbit IgG, Scbt sc-15404 1/500), Caspase3 (Anti-Caspase-3 Rabbit IgG, Sigma C8487 1/1000) and Bcl-2 (Anti-Bcl-2 Rabbit IgG, Scbt 1/ 1000) proteins overnight at +4 °C.

Membranes for normalization were labeled with an internal control Gapdh protein [Anti-Gapdh Rabbit IgG, Scbt sc-25778, 1/2000]. After the primary antibody incubation and washing step, Horse Radish Peroxidase (HRP) conjugated secondary antibody (Goat anti-rabbit IgG-HRP conjugate, Scbt sc-2030, 1/10000) was incubated for 1 hour. Then the blots were treated with Clarity™ Western ECL (Bio-Rad Laboratories, Hercules, USA) substrate solution for 5 min. Images of the blots were obtained using the ChemiDoc™ MP Chemiluminescence detection system (Bio-Rad Laboratories, USA) equipped with a CCD camera. The relative expression of the proteins with respect to Gapdh was calculated using the ImageLab 4.1 software.

Statistical analysis

The results are given as mean ± standard error of the mean (SEM); n is the number of rats. Statistical analyses were performed using the Student's t-test for unpaired data or one-way ANOVA followed by the Bonferroni post-hoc analysis. Values were evaluated with GraphPad Prism (version 6, GraphPad Software, La Jolla, CA). Data were considered to be significantly different when the P-value was less than 0.05.

RESULTS

Expression levels of genes related to testicular apoptosis sirt1, caspase3 and bcl-2 were measured by qRT-PCR. As shown in Figure 1a, neither dietary fructose nor the probiotic supplementations altered expression levels of SIRT1mRNA in the

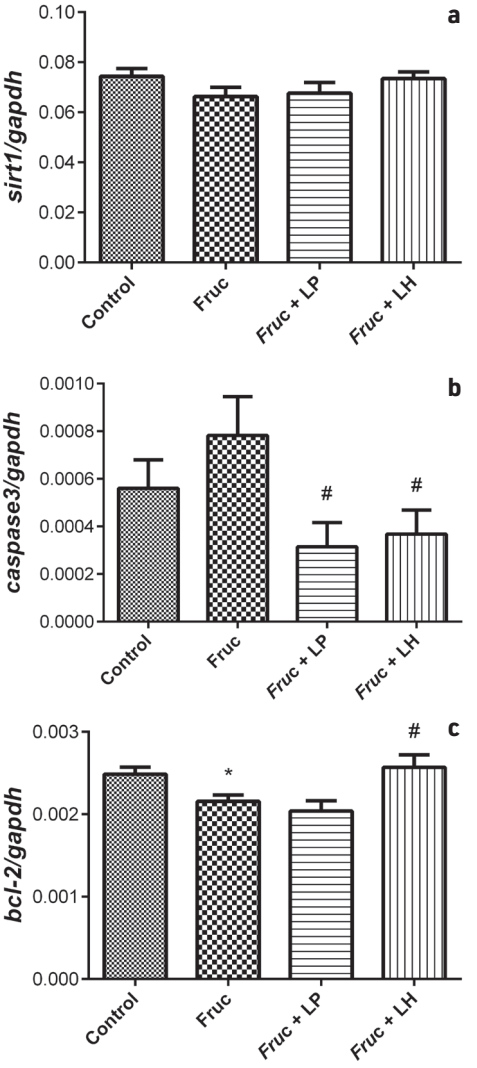

Figure 1. The mRNA expression levels of sirt1 (a), caspase3 (b) and bcl-2 (c) in the testes of Control, Fruc, Fruc + LP, and Fruc + LH groups. Data were normalized using *gapdh*. Values are expressed as mean ± SEM, n=6–8. *p<0.05 versus control group; #p<.05, significantly different from fructose group. Fruc: Fructose; LP: *L. plantarum*; LH: *L. helveticus*.

testicular samples of rats. There was a tendency toward an increase in the expression level of caspase3 mRNA in the fructose-treated rats compared to the controls, but the differences did not achieve a significance level. However, *L. plantarum* and *L. helveticus* supplementations decreased the expression of caspase3 mRNA in fructose-treated rats (Figure 1b). Importantly, the anti-apoptotic bcl-2 mRNA level was markedly decreased in fructose-fed rats, which was improved only with *L. helveticus* supplementation (Figure 1c).

The protein expression levels of SIRT1, caspase3 and bcl-2 in testes samples of rats were determined by Western Blot analysis. Dietary fructose did not change the level of caspase3. However, *L. helveticus* and *L. plantarum* supplementations decreased this protein in the testicular tissue of high-fructose-fed rats. The expressions of SIRT1 and bcl-2 proteins were significantly decreased with a high-fructose diet, however, *L. helveticus* and *L. plantarum* supplementations markedly upregulated these proteins (Figure 2).

DISCUSSION

A high-fructose diet may cause glucose intolerance, hypertension, hyperlipidemia, and chronic low-grade inflammation. The development of metabolic disorders might be worsened by the activation of proinflammatory cytokine and oxidative stress. We have previously shown that high-fructose consumption enhances the expression of inflammatory factors in the testis, blood vessels, liver or adipose tissue accompanied by low testosterone level in the rats (Akar et al., 2012; Babacanoglu et al., 2013; Pektas, Koca, Sadi, & Akar, 2016; Pektas, Sadi, & Akar, 2015; Pektas et al., 2017; Sadi et al., 2015; Yildirim et al.,

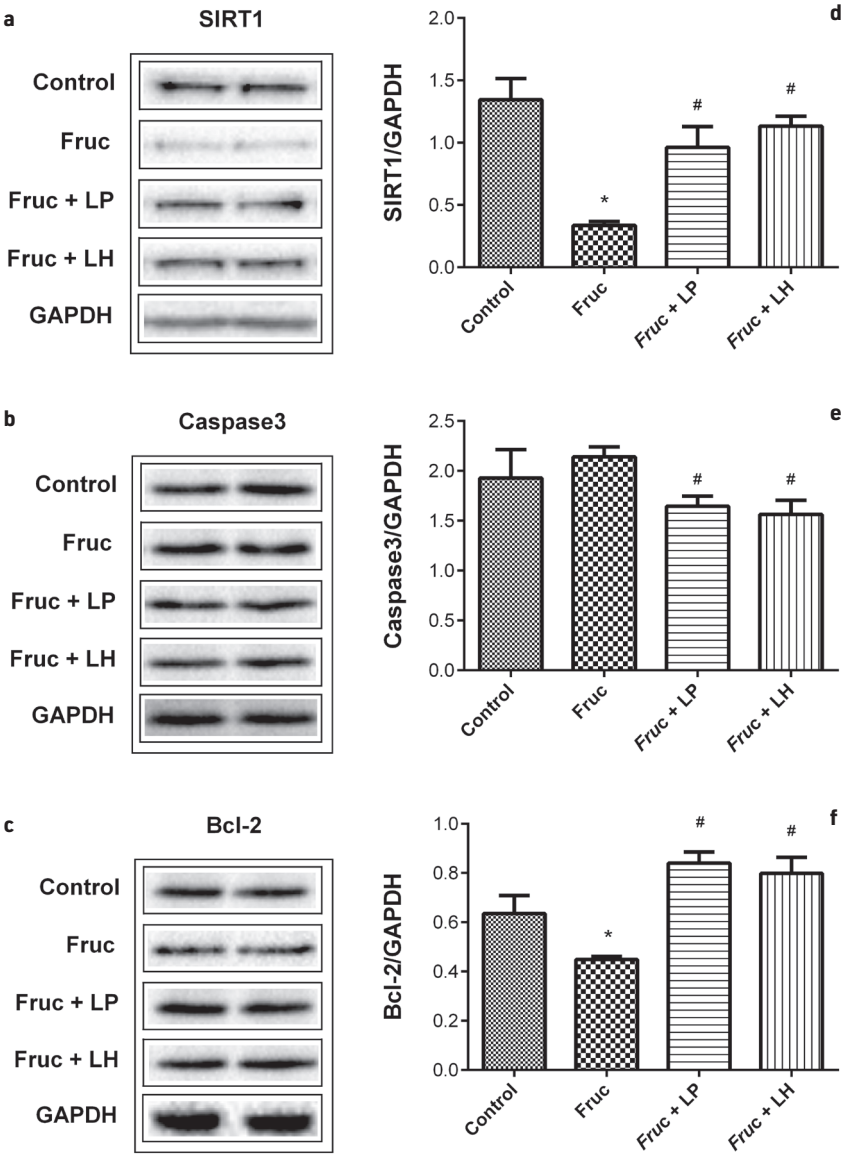

Figure 2. Representative Western blot bands (a-c) and relative protein expressions of SIRT1 (d), Caspase3 (e) and Bcl-2 (f) in the testes of Control, Fruc, Fruc + LP, and Fruc + LH groups. Data were normalized using Gapdh. Values are expressed as mean ± SEM, n=6–8. *p<0.05 versus control group; #p<.05, significantly different from fructose group: Fructose; LP: *L. plantarum*; LH: *L. helveticus*.

2019). Diseases such as MetS, type 2 diabetes, and obesity are associated with hypogonadism in men (Caldas et al., 2009; Dhindsa et al., 2010; Ebrahimi et al., 2017).

Apoptotic pathways are evolutionarily maintained and play a pivotal role in homeostasis in the testicular tissue. Although regular apoptosis of spermatogenic cells is necessary to maintain testicular homeostasis, excessive cell death can cause infertility due to damaged spermatogenesis (Passos et al., 2007). When cells are exposed to irreparable oxidative stress, the cells force proapoptotic signals to eliminate the damaging materials (Green & Llambi, 2015). Caspases and bcl-2 play important roles in cellular apoptotic processes. Previous studies showed that cell apoptosis can be suppressed due to caspase-3 inhibition with specific protease inhibitors (Cregan, Dawson, & Slack, 2004; Hayashi, Kojima, & Ito, 2006). SIRT1, which is a member of the nicotinamide adenine dinucleotide (NAD)-dependent protein deactylase family is associated with the regulatory control of diverse cellular process including energy homeostasis, inflammation, cell survival, apoptosis and DNA repair (Vachharajani et al., 2016; X.-l. Wang et al., 2016). Recent studies show that SIRT1 deficiency cause reproductive disorder due to diminished spermatogenesis and germ cell functions (Coussens, Maresh, Yanagimachi, Maeda, & Allsopp, 2008; McBurney et al., 2003). SIRT1 and bcl-2 have an inhibitory effect on cell apoptosis playing an important role in apoptosis. Bax belongs to the same protein family as the bcl-2 gene but has an opposite function, and their equilibrium determines the degree of cell apoptosis (Brady & Gil-Gomez, 1998; Cory, Huang, & Adams, 2003; H. Liu et al., 2018; W. Song et al., 2016).

Low testosterone levels in animals may cause oxidative stress and also trigger Germ and Sertoli cells (SCs) apoptosis by increasing Bax/Bcl-2 ratio and caspase3 activities (Chaki et al., 2006; Simoes et al., 2013). Streptozotocin-induced diabetes increases pro-apoptotic proteins, whereas Bax and Bad decrease the levels of SIRT1, bcl-2 and bcl-XL, which are antiapoptotic proteins in the rat testis. Besides, the weight of reproductive organs (testis and epididymis) and level of serum testosterone were decreased in the same diabetic rats compared to the control group (Koh, 2007a, 2007b; Tsounapi et al., 2012; Xu et al., 2014; Y. Zhao et al., 2012). In animal models, diabetes increases the expression of p53 and initiates apoptosis by activating caspase3 in rat testes (Alsemeh, Samak, & El-Fatah, 2020; Faid, Al-Hussaini, & Kilarkaje, 2015; Koh, 2007a; Y. Zhao et al., 2011). Additionally, the studies showed that diabetes increases testicular caspase3 mRNA expression and suppresses bcl-2 mRNA and protein levels (Du, Qiu, Wang, & Wang, 2018; Nna et al., 2018). Recent studies demonstrated that fructose consumption in rodents initiates apoptosis in several organs such as the kidney, pancreas, aorta, liver, and testes (Balakumar et al., 2016; Choi et al., 2017; Hu et al., 2012; Lu et al., 2017; Meydanli et al., 2018). In the present study, dietary fructose did not change testicular caspase3 mRNA and protein levels. However, this dietary intervention decreased anti-apoptotic bcl-2 mRNA expression and protein level. In addition, the testicular SIRT1 protein level, but not mRNA expression, was dramatically decreased in the fructose-fed group. In a study on mice, increased testicular oxidative and nitrosative stress were shown to induce apoptosis by stimulating the p53-mediated Bax/caspase3 pathway (Shahin, Singh, & Chaturvedi, 2018). In a previous study, we also showed that dietary high-fructose increased expression of iNOS, TNF-α, and NFkb mRNAs and caused testicular degeneration (Yildirim et al., 2019). All these results revealed that high-fructose consumption could activate testicular oxidative stress, inflammation and apoptosis in the rodents.

Studies in human and experimental animals proposed a link between metabolic diseases and intestinal microbiota (Backhed, Ley, Sonnenburg, Peterson, & Gordon, 2005; Ley et al., 2005). Supplementation with Lactobacillus (L.) species, which is one of the primary components of human intestinal microbiota, was shown to produce antioxidant, antihyperlipidemic, antidiabetic, antiapoptotic, and anti-inflammatory activities in the experimental studies (Choi et al., 2017; Honda, Moto, Uchida, He, & Hashizume, 2012; Korkmaz et al., 2019a; Mohammadi et al., 2019; Plaza-Diaz, Ruiz-Ojeda, Vilchez-Padial, & Gil, 2017; Sumlu et al., 2020; H. F. Wang et al., 2012; Y. Wang et al., 2017). Metabolic irregularities including hyperglycemia, hyperinsulinemia, and dyslipidemia due to high-fructose consumption in rats were found to improve with *L. acidophilus* and *L. casei* supplements (Yadav, Jain, & Sinha, 2007). Moreover, supplementations with *L. plantarum* and *L. helveticus* improved the insulin signaling pathway in the kidney and liver of rats fed with a high-fructose diet (Korkmaz et al., 2019a ; 2019b; Sumlu et al., 2020). The experiments experiments with probiotics, *L. helveticus* and *L. plantarum* supplementations were able to suppress apoptosis by decreasing caspase3 and improving bcl-2 levels in different organs of the animals (Bouhafs, Moudilou, Exbrayat, Lahouel, & Idoui, 2015; Girard et al., 2009; Huang et al., 2019; Mohammadi et al., 2019). Recently, a study showed that high-fat diet-induced adiposity, glucose intolerance and dyslipidemia were ameliorated by *L. plantarum* supplementation by increasing the expression of SIRT1 and PPARα in the liver and adipose tissues (Kwon et al., 2020). In our previous studies using *L. plantarum* and *L. helveticus* supplementations we determined that oral administration of these probiotics ($1x10^9$ CFU per 100 g of body weight of animal doses) for 6 weeks improved the deleterious effects of dietary fructose in the kidney and the liver (Korkmaz et al., 2019a; 2019b ; Sumlu et al., 2020).Therefore, in the current study we applied these two probiotics at the same dose for the same period. In this investigation, supplementation with *L. plantarum* and *L. helveticus* produced a marked downregulation on caspase3 mRNA and protein levels in testicular samples of high-fructose-fed rats. Also, the testicular protein level of SIRT1 was significantly increased by both prebiotic supplementations in fructose-fed rats. Moreover, the decline in testicular bcl-2 mRNA and protein levels due to high-fructose consumption was improved by *L. helveticus* supplementation. However, while *L. plantarum* supplementation increased bcl-2 protein level, it did not change mRNA expression, showing a posttranslational improving effect on the antiapoptotic factor.

In conclusion, our data indicated that treatment with *L. plantarum* and *L. helveticus* species can reduce testicular apoptosis induced by dietary high-fructose in rats by suppressing

caspase3 and promoting SIRT1 and bcl-2 protein expressions. In practice, formulations containing these probiotics could show beneficial effects in certain reproductive irregularities of males.

Peer-review: Externally peer-reviewed.

Ethics Committee Approval: The experiments reported here complied with the current laws and regulations of the Turkish Republic on the care and handling of experimental animals and the local ethics committee of experimental animals of Afyon Kocatepe University (Akuhadyek-49533702).

Informed Consent: Written consent was obtained from the participants.

Author Contributions: Conception/Design of Study- F.A.; Data Acquisition- O.G.Y., G.S.; Data Analysis/Interpretation- G.S., F.A.; Drafting Manuscript- O.G.Y., F.A.; Critical Revision of Manuscript- G.S., F.A.; Final Approval and Accountability- O.G.Y., F.A.; Technical or Material Support- O.G.Y., G.S.; Supervision- F.A.

Conflict of Interest: The authors have no conflict of interest to declare.

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