Antioxidative and Metabolic Effects of *Lactobacillus plantarum*, Inulin, and Their Synbiotic on the Hypothalamus and Serum of Healthy Rats

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ABSTRACT: Nowadays, much attention has been paid to the link between gut microbiota and brain. The beneficial metabolic effects of probiotics and prebiotics in several diseases such as diabetes and obesity have been reported. However, studies bridging the association of gut microbiome with brain function in healthy states are rare. Therefore, it was hypothesized that the administration of *Lactobacillus plantarum* (*L plantarum*) and inulin may affect serum and hypothalamic metabolic parameters as well as oxidative markers in healthy male rats. Daily *L plantarum* (10^7 CFU/mL) and inulin (5% of daily food weight) or their combination (synbiotic) was given to healthy rats. Then, serum and hypothalamic levels of leptin, insulin, and oxidative markers were measured. Administration of synbiotic for 8 weeks led to significant changes in serum levels of high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, very low-density lipoprotein cholesterol, low-density lipoprotein/high-density lipoprotein ratio, triglyceride, and total cholesterol. The intake of synbiotic also resulted in a significantly reduced hypothalamic level of malondialdehyde and increased hypothalamic superoxide dismutase (SOD). Also, *L plantarum* could significantly increase hypothalamic SOD level. Furthermore, synbiotic administration insignificantly increased the hypothalamic and serum levels of insulin and leptin. These findings suggest that the synbiotic could significantly improve oxidative markers and lipid profile in healthy rats. Therefore, simultaneous intake of *L plantarum* and inulin appears to be more effective in the amelioration of metabolic and oxidative parameters.

KEYWORDS: Gut-brain axis, *Lactobacillus plantarum*, inulin, insulin, leptin, probiotic

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Introduction

Gut microflora in the gastrointestinal (GI) tract of healthy subjects differ from those found in patients[1] with irritable bowel syndrome, diabetes, or disorders of the central nervous system (CNS) such as Alzheimer and Parkinson diseases.[2,3] A balanced gut microbiota is important for health and normal brain development and function.[4,5] Evidence in this regard has revealed that there is a bidirectional communication between gut and brain, called microbiota-gut-brain axis. In fact, the intestine and brain are constantly communicating with and affecting each other.[6,7] The concept of functional foods, including prebiotic and probiotic supplements, implies their ability to beneficially influence body functions so as to enhance health and reduce the risk of some diseases.[8] However, studies on the disease state may not reflect and predict the antioxidative effects of probiotics and prebiotics in healthy individuals or those with suboptimal healthy state.

Leptin as a hormone is essentially produced by the adipose tissue and has important effects on the CNS.[9] It has a key role in controlling energy intake, glucose metabolism, and metabolic homeostasis.[10] The deficiency of leptin in the hypothalamus can be due to diseases such as obesity, insulin resistance (IR), and cardiovascular disease.[11] However, little progress has been made in determining the mechanisms for inducing insulin and leptin function in the hypothalamus.[12]

The balance between the production of reactive oxygen species (ROS) and antioxidant defenses plays a key role in cellular physiology and brain development.[13] On the contrary, studies have demonstrated that any disturbance in the level or function of leptin has been demonstrated to induce oxidative stress and the complications involving abnormalities of lipid metabolism.[14-16] The hypothalamus is an important region of the brain, the main task of which is dietary regulation and energy balance.[17] It might play a significant role in the early onset of obesity, IR, and diabetes due to its involvement in the control of energy balance and glucose homeostasis.[17,18] Impairment of insulin function in the hypothalamus dysregulates hepatic glucose production and food intake.

It may also cause inflammation, oxidative stress, and insulin receptor signaling disorders.[19] Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host.[20] They can improve the metabolic state and reduce the prevalence of obesity, IR, and oxidative stress through the effects they have on

the CNS.[21] Prebiotics are indigestible components that modulate the gut microbiome.[22] Prebiotics such as inulin have positive influence on lipid metabolism and controlling obesity and IR.[23] Synbiotics, as dietary supplements, are combinations of probiotics and prebiotics that have beneficial effects on lipid profile and leptin which could decrease IR due to their effect on insulin sensitivity as well[24]; they can also alleviate oxidative stress.[25-27] In addition, studies have shown that probiotics and prebiotics have beneficial effects on healthy subjects.[28,29]

In previous studies,[16,18] the beneficial effects of *L plantarum* ATCC 8014 and inulin on metabolic and oxidative markers in the amygdala and hypothalamus of diabetic rats were reported, which motivated this research group to conduct the present study. The knowledge of how different probiotics and prebiotics could affect the metabolic function in the absence of disease will gain mechanistic insights and help clarify the magnitude of their effects on metabolic function. Human studies can be suggested, if desirable results are achieved through the examination of the effects of these supplements in healthy animals. Therefore, the aim of this study was to investigate the effects of separate and concurrent administration of *L plantarum* and inulin on serum and hypothalamic levels of insulin, leptin, and oxidative markers in healthy male rats.

Methods

Animals and intervention

The experimental protocol was approved by the Institutional Animal Ethics Committee of Tabriz University of Medical Sciences (TBZMED). The study was carried out on 24 healthy male Wistar rats, aged 6 ± 1 weeks old (200 ± 20 g). The animals were obtained from the inbred animal colony of the central animal house, Faculty of Medicine, TBZMED. Before the onset of the study, the rats were fed standard laboratory chow diet for 7 days to adapt to the new environment. Then, they were randomly assigned to 4 groups. The animals were housed in standard cages and maintained under standard laboratory conditions at an ambient temperature of 22°C to 25°C, 12:12 hour light/dark cycle (starting from 07:00 to 19:00), with a relative humidity of 40% to 60% and free access to water and food. The weekly body weight of rats and their daily food intake were measured. The animals were divided into 4 groups, as follows: (1) healthy + *L plantarum* (HL, n = 6); (2) healthy + inulin (HI, n = 6); (3) healthy + *synbiotic* (*L plantarum* + inulin) (HLI, n = 6); (4) healthy sham (HSh, n = 6). All groups were fed a normal diet, containing 12% fat, 22% protein, and 66% carbohydrate (Table 1).

Supplementation

Lactobacillus plantarum ATCC 8014 was obtained from TBZMED Biotechnology Research Center (Tabriz, Iran). Ten milliliters was inoculated in MRS (Man-Rogosa-Sharpe) broth and cultured in aerobic conditions at 37°C for 48 hours in phosphate-buffered saline (PBS). Fresh bacterial suspensions were prepared at a concentration of 10^7 colony-forming units (CFU)/mL. One-milliliter gastric gavage was performed every 24 hours for each rat. The inulin content of the rat diet was calculated based on 5% of the daily food weight and was dissolved in drinking water.[18] Supplementations lasted for 8 weeks.

Table 1. Normal rat chow diet.

CONTENT	PER 100 G
Fat Soybean oil	12 g
Protein Casein L-Cystine	20 g
Carbohydrate Fiber Corn starch Maltodextrin Sucrose	62 g 3 g
Vitamin	1 g
Mineral	2 g
Energy	440 kcal/100 g

Anesthesia, surgery, and sampling

On the last day of the study, the animals were anesthetized with sodium pentobarbital (65 mg/kg body weight IP; Sigma Chemical Co., St. Louis, USA) for the manufacturer "Sigma." Then, 4 mL of blood sample (1 mL serum) was immediately obtained from cardiac puncture and centrifuged at 9000 rpm at 4°C for 20 minutes; the separated sera were then stored in an ultra-low-temperature freezer at −80°C, until assay. All rats were anesthetized and rapidly decapitated; then, the whole brain was removed. After that, the hypothalamic tissue samples were removed at once, then placed on ice, homogenized with 1 mL PBS, and centrifuged for 10 minutes at 9000 rpm at 4°C. Following centrifugation, the supernatant was separated and transferred to the microtubule. To normalize the data of the hypothalamus samples, the Bradford method was applied.[30]

Biochemical assays

Serum was analyzed for blood sugar (BS), triglyceride (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) by spectrophotometry method, using a diagnostic reagent kit.[16] Serum and hypothalamic levels of insulin and leptin were estimated by enzyme-linked immunosorbent assay (ELISA) kits (Crystal Day Biotech, Shanghai, China). The homeostatic model assessment of IR (HOMA-IR) as a measure of IR was calculated by a formula.[31] Hypothalamic levels of oxidative stress indices were measured. Superoxide dismutase (SOD) was determined using a RANSOD kit (Randox Laboratories, Crumlin, UK). *Glutathione peroxidase* (GPx) was evaluated by a RANSEL kit (Randox Laboratories).

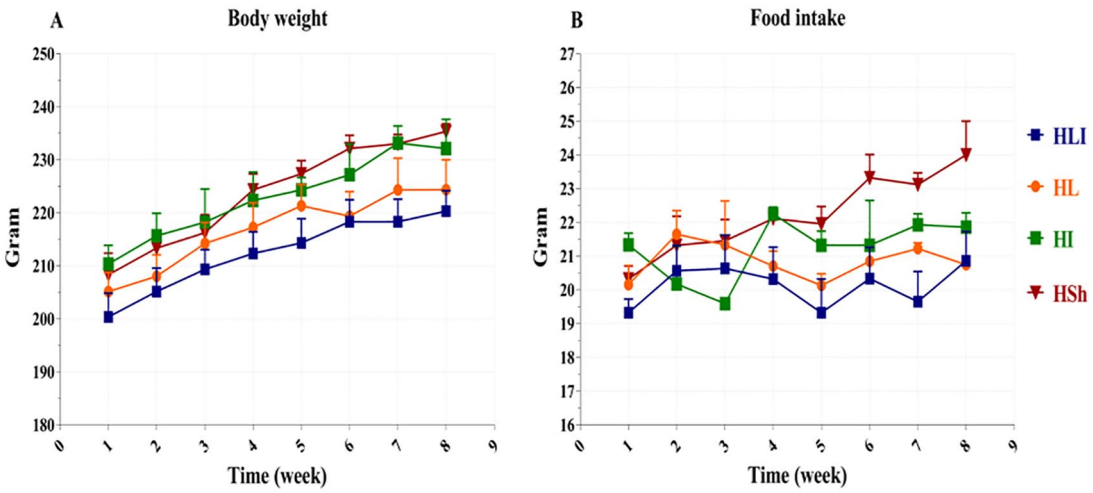

Figure 1. (A, B) Effects of *Lactobacillus plantarum* and inulin treatment on weight gain and food intake of control and treated rats over 8 weeks (n = 6 per group). (A) Changes of body weight during treatment. (B) Changes of food intake during treatment.The 1-way analysis of variance, followed by post hoc *Tukey* test, was used. Data are expressed as means ± SEM, and $P < .05$ is regarded as statistically significant. No significant effects were observed in weight gain and food intake. HI indicates healthy treated by inulin; HL, healthy treated by *plantarum*; HLI, healthy treated by *L plantarum* and inulin; HSh, healthy control (sham).

Lipid peroxidation was evaluated through measurement of malondialdehyde (MDA) levels as thiobarbituric acid–reactive substances. Total antioxidant status (TAS) was measured using Randox total antioxidant status kit, following the manufacturer's instructions (Randox Laboratories).

Statistical analysis

Data were analyzed using SPSS statistics software (version 23). The results are presented as means ± SEM for each group. We used the *Levene* and *Shapiro-Wilk* tests to assess the normality of data and equality of variances. One-way analysis of variance was used to determine the levels of significance among different groups. *Post hoc* analysis was performed using *Tukey* test. $P < .05$ was considered to be statistically significant.

Results

Changes in body weight and food intake

At the end of the intervention, in comparison with the HSh group, *L plantarum*, inulin, and synbiotic could not significantly reduce weight gain ($P = .09$, $P = .11$, and $P = .07$, respectively) of the treated rats. Furthermore, the administration of probiotic, prebiotic, and synbiotic did not significantly affect daily food intake (HL: $P = .06$, HI: $P = .09$, HLI: $P = .06$). The supplements (in particular, synbiotic) also helped to control desirable body weight and food intake in the treated groups, compared with the HSh group (Figure 1).

Changes in serum and hypothalamic levels of metabolic markers

Compared with the HSh group, after administration of the synbiotic, the levels of TC, TG, low-density lipoprotein cholesterol (LDL-C), and very low-density lipoprotein cholesterol (VLDL-C) reduced and HDL-C increased; in addition, the ratio of LDL/HDL ($P = .01$) decreased in the HL group (Figure 2). No significant differences were found in the serum or hypothalamic parameters between the HI and HSh groups. Indeed, the supplements had no significant effect on serum glucose, insulin, HOMA-IR, and leptin in the intervention groups, compared with the HSh group (Figure 3).

Changes in the hypothalamic oxidative markers

The results clarified that the administration of prebiotic had no significant effect on hypothalamic levels of SOD, GPx, MDA, and TAS, compared with the HSh group (Figure 4). Compared with the HSh group, SOD level had a significant elevation in the HLI ($P = .015$) and HL ($P = .042$) groups, regarding oxidative stress indices. However, there was a significant reduction in the MDA level in the HLI group ($P = .011$). GPx and TAS levels did not show significant changes, subsequent to supplementation. In addition, there were no significant differences among the intervention groups in terms of hypothalamic levels of insulin, leptin, and oxidative markers (Figure 4).

Discussion

This study is the first to demonstrate that oral concurrent supplementation of *L plantarum* and inulin (synbiotic) could improve lipid profile and hypothalamic oxidative markers in healthy male rats after an 8-week intervention. Synbiotic administration could not significantly increase serum and hypothalamic levels of insulin and leptin. Although mere inulin administration could not make significant changes to the study parameters, *L plantarum* could ameliorate HDL-C and hypothalamic SOD levels.

In this study, *L plantarum*, inulin, and their combination could not significantly change weight gain and food intake. In

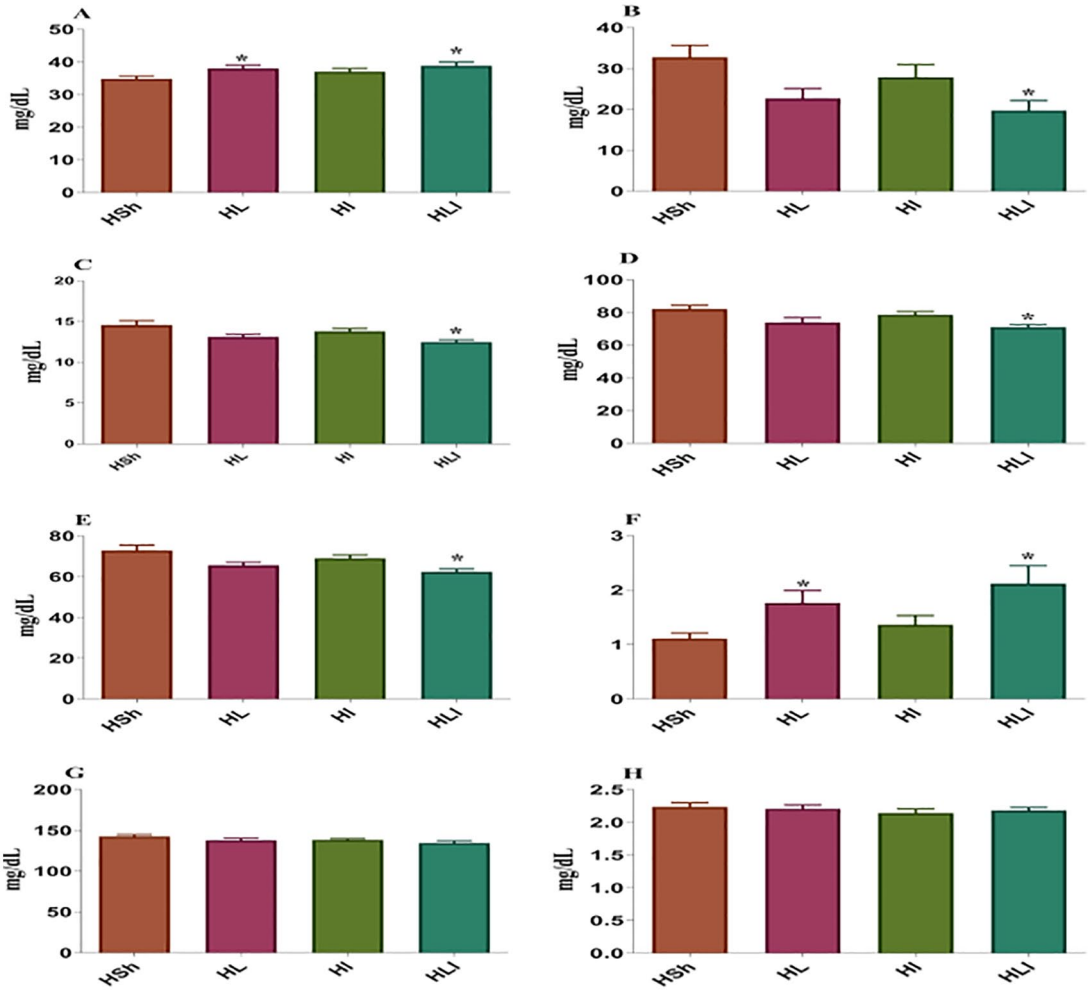

Figure 2. Effects of *Lactobacillus plantarum* and inulin on serum metabolic parameters of control and treated rats (n = 6 per group). The level of serum (A) HDL-C, (B) LDL-C, (C) VLDL, (D) total cholesterol, (E) triglycerides, (F) HDL/LDL, (G) blood glucose, and (H) HOMA-IR levels of control and treated rats. One-way analysis of variance, followed by post hoc *Tukey* test, was used. Data are expressed as means ± SEM, and $P < .05$ is regarded as statistically significant. *$P < .05$ compared with the HSh group. HDL indicates high-density lipoprotein; HDL-C, high-density lipoprotein cholesterol; HI, healthy treated by inulin; HL, healthy treated by *L plantarum*; HLI, healthy treated by *L plantarum* and inulin; HOMA-IR, homeostatic model assessment of insulin resistance; HSh, healthy control (sham); LDL, low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; VLDL, very low-density lipoprotein cholesterol.

our previous work,[16] it was indicated that *L plantarum*, inulin, and their synbiotic could decrease food intake and prevent weight loss in diabetic rats. In this regard, Shirouchi et al[32] demonstrated that *Lactobacillus gasseri* SBT2055 could reduce body weight in rats, although there was no significant difference in food intake among the study groups.[32] However, it was also shown that the administration of *Lactobacillus acidophilus, Lactobacillus fermentum*, and *Lactobacillus ingluviei* was associated with weight gain, whereas the administration of *L plantarum* and *L gasseri*is was accompanied with weight loss in obese humans and animals.[33] In another study, prebiotics including inulin could increase bacterial deconjugation of bile acids and affect food intake and body weight.[34] Low levels of serum leptin or insulin can result in the decrease in energy expenditure and increase in appetite.[35] In this study, the levels of insulin and leptin did not increase sufficiently to make significant changes in food intake or body weight.

Our findings showed that *L plantarum* and inulin could significantly improve the levels of serum lipid profile (LDL-C, HDL-C, VLD, TG, and TC), either in the probiotic or in the synbiotic group. Valenlia et al[16] reported that *L plantarum*, inulin, and their synbiotic improved lipid profile in diabetic rats. In line with our study, Li et al[36] also demonstrated that both *L plantarum* X1 and *L plantarum* CCFM30 could ameliorate lipid profile. In addition, they showed that *L plantarum* X1 could decrease lipid metabolism.[36] In contrast to our study, Takemura et al[37] reported that *L plantarum* No. 14 did not decrease serum lipid profile in mice fed a high-fat diet. Given the reported differences between this study and that of Li et al, it can be presumed that the type of diet (normal diet or high-fat diet) and animal gender (female mice or male rat) can have strong effects on the outcomes. Evidence has shown that prebiotic consumption has a positive effect on food intake (even in healthy animals) via reducing gastric volume and its effect on

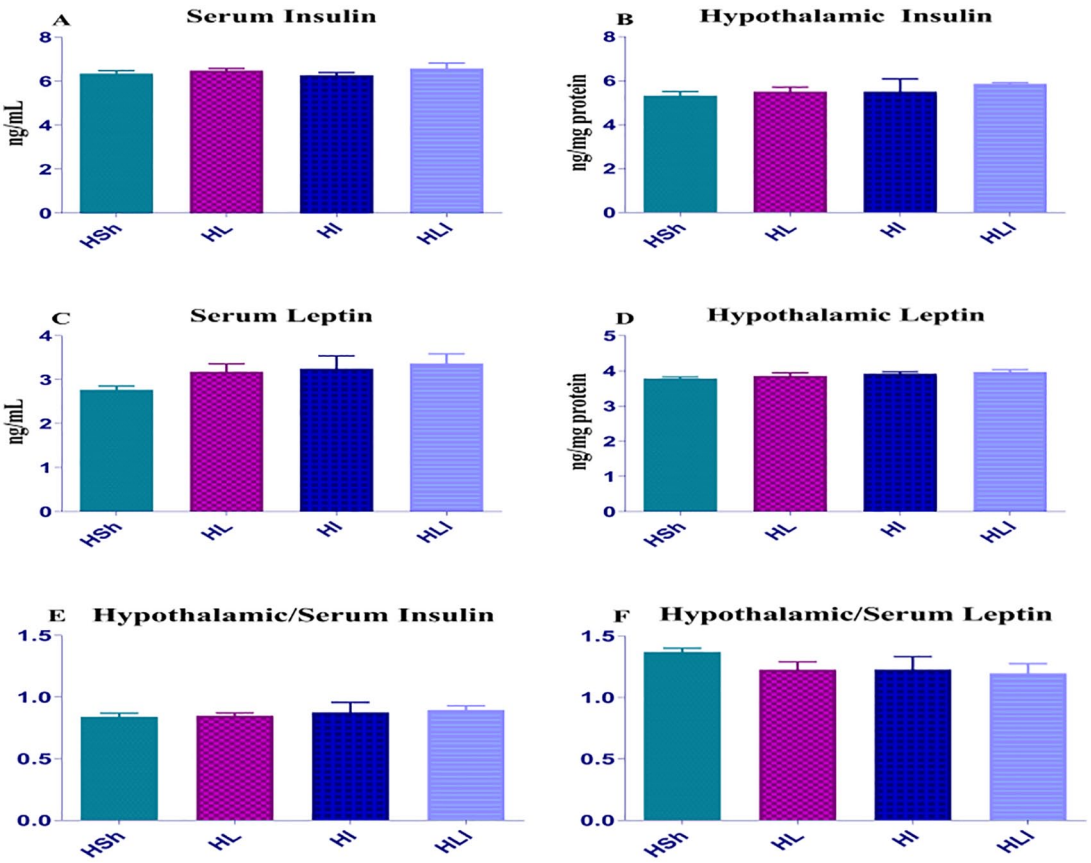

Figure 3. Effects of *Lactobacillus plantarum* and inulin on insulin and leptin levels in the hypothalamus and serum of rats (n = 6 per group). The levels of (A) serum and (B) hypothalamic insulin; the levels of (C) serum and (D) hypothalamic leptin; ratio of hypothalamic/serum (E) insulin and (F) leptin in control and treated rats. One-way analysis of variance, followed by post hoc *Tukey* test, was used. Data are expressed as means ± SEM, and $P < .05$ is regarded as statistically significant. No significant changes were observed in the levels of parameters. HI indicates healthy treated by inulin; HL, healthy treated by *L plantarum*; HLI, healthy treated by *L plantarum* and inulin; HSh, healthy control (sham).

appetite.[38,39] Although the changes in food intake in our study were not significant, they may be due to the low dose and, perhaps, short duration of the intervention.

Our results also revealed that the administration of the supplements could not reduce serum glucose through an increase in serum and hypothalamic insulin and leptin levels. In our previous work, it was indicated that *L plantarum*, inulin, and synbiotic improved fasting blood sugar as well as serum and hypothalamic levels of insulin and leptin in diabetic rats.[16] Unfortunately, similar research in human and animal samples is very limited. Therefore, it is not possible to interpret more clearly. A study reported that *L plantarum* can regulate the production of insulin and glucagon as well as insulin sensitivity in diabetic rats.[40] *Lactobacillus plantarum* also controls blood glucose metabolism through the improvement of insulin secretion and glucose tolerance.[41] In addition, prebiotics such as inulin could improve glycemic indices and IR in type 2 diabetes mellitus (T2DM) via stimulation of glucose uptake and metabolism in the brain, regulation of metabolism in peripheral tissues, and increase of insulin sensitivity in the body.[42,43] Nassar et al[44] reported that inulin significantly decreased blood levels of glucose, and consequently IR. Nevertheless, some studies have shown no effects of inulin on diabetes.[45] The mechanism by which insulin acts on the hypothalamus and its neurons is unclear.[46]

Leptin also plays an important role in the energy homeostasis as well as regulation of glucose metabolism.[47] Evidence demonstrates that hyperglycemia and IR are ameliorated by the improvement in the secretion and function of leptin and its receptors in the hypothalamus.[48] Improvement in hypothalamic leptin signaling could significantly improve insulin sensitivity in peripheral tissues.[47] Inulin and *Lactobacillus* supplementations were found to markedly improve leptin sensitivity.[34,49] Despite the beneficial effects of probiotics and prebiotics on insulin and leptin levels,[16] no significant changes were observed in our study. Probably the main reason for this finding is the body's regulatory systems to restore levels of glucose, insulin, and leptin in healthy states. Therefore, our supplements did not have the promising effect they had in diabetic rats In addition, as mentioned above, another reason for insignificant changes of insulin and leptin levels in our study may be lower dosage (10^7 CFU/mL) as well as the shorter length of the intervention.

Concerning oxidative stress indices, the results of this study showed that the synbiotic significantly improved hypothalamic SOD and MDA levels. Our previous works indicated that *L*

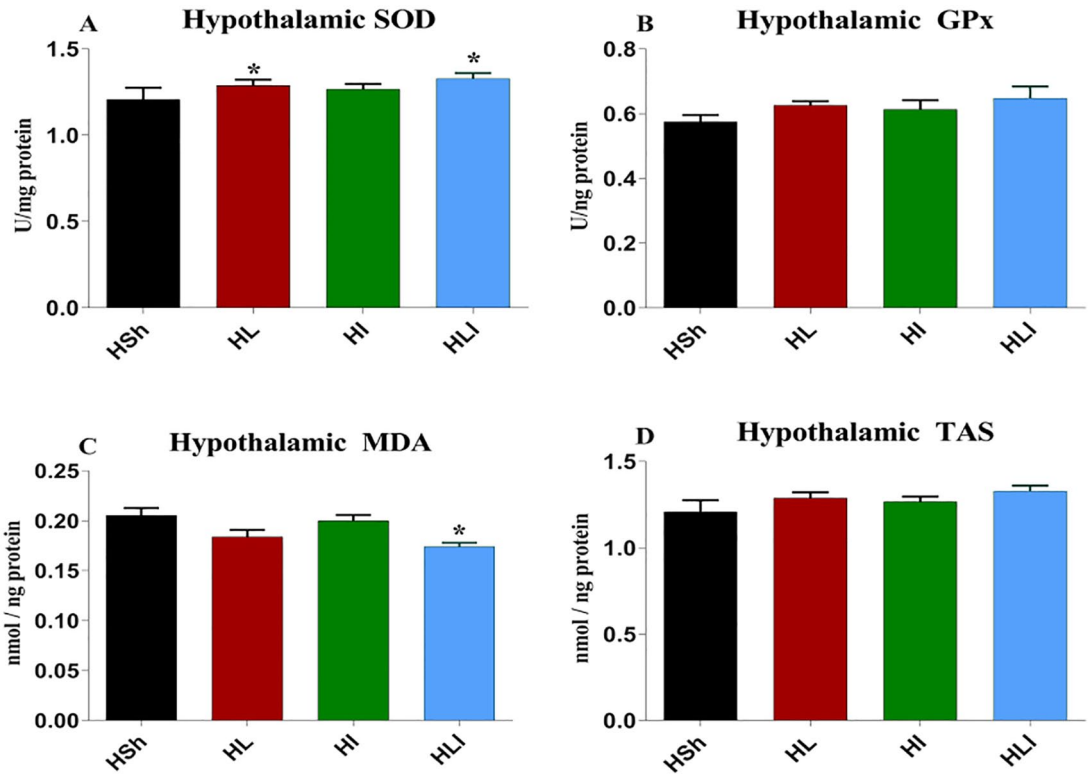

Figure 4. Effects of *Lactobacillus plantarum* and inulin on hypothalamic oxidative stress markers of rats (n = 6 per group). The level of hypothalamic (A) SOD, (B) GPx, (C) MDA, and (D) TAS levels of control and treated rats. One-way analysis of variance, followed by post hoc *Tukey* test, was used. Data are expressed as means ± SEM. *$P < .05$ compared with the HSh group. GPx indicates *glutathione peroxidase;* HI, healthy treated by inulin; HL, healthy treated by *L plantarum*; HLI, healthy treated by *L plantarum* and inulin; HSh, healthy control (sham); MDA, malondialdehyde; SOD, superoxide dismutase; TAS, total antioxidant capacity.

plantarum, inulin, and their synbiotic ameliorated the serum, amygdala, and hypothalamic levels of oxidative markers in diabetic rats.[16,18] *Lactobacillus plantarum* could also increase SOD concentration. Similar to our experiment, Li et al[36] showed that *L plantarum* X1 can partially increase the antioxidant capacity in diabetic mice. *Lactobacillus plantarum* X1 and *L plantarum* CCFM30 were reported to elevate SOD and GPx activity and ameliorate MDA level.[36] It is hypothesized that probiotics might produce some bioactive substances with free radical chelating ability (such as glutathione) that potentially prevents oxidative damage.[50] Antioxidant roles of probiotics, in particular lactic acid bacteria, are exerted via different mechanisms such as hunting ROS (improving the activity of antioxidant enzymes), chelating metal ions, blocking enzyme activity, and reducing ascorbate autoxidation.[51] Prebiotics like inulin-type fructans can improve oxidative stress,[43] albeit some studies showed that inulin did not have any significant effect on antioxidant (SOD and GPx) enzymes in women with T2DM.[52]

Oxidant agents are naturally and permanently produced even in the body of a perfectly healthy body. Therefore, it can be hoped that our supplements (especially the synbiotic) may prevent the damages caused by ROS production in healthy individuals even in a short period of time.

In general, our synbiotic could improve some of the metabolic markers and hypothalamic oxidative stress status. Furthermore, one of the main achievements of this study was that the effect of the synbiotic in the improvement of the metabolic state was stronger than mere *L plantarum* or inulin intake. Although the supplementation in our study provided promising results, it had some limitations. We could not evaluate gut microbiome composition after supplementation; therefore, it is highly recommended to be performed in future studies. Probably, more significant effects could be observed by increasing either the dosage or the duration of the intervention.

Conclusion

Our study demonstrated that synbiotic intake could contribute to improve some of the metabolic markers such as lipid profile and oxidative markers. Our findings suggest that specific *Lactobacillus* bacteria such as the *L plantarum* as well as inulin may have significant synergistic effects. Overall, due to their effective medicinal properties, both *L plantarum* and inulin may be considered as complementary options to prevent metabolic disorders in healthy rats. To achieve more reliable results, future studies are warranted.

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Authors' contributions

E-SH and MS-A designed and carried out the study, analyzed the data, performed the statistical analyses, and prepared the first draft of the manuscript. KB-V conceived the study and edited the manuscript. MM commented on the study design, data analyses, and inference of the results and critically edited the manuscript. All authors read and approved the final manuscript.

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