



Impact of Aerobic Training and Probiotic Intervention on Nrf2 and GLUT4 mRNA Levels in Non-Alcoholic Fatty Liver Disease: An Experimental Study in Male Wistar Rats

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Previous research has explored metabolic regulation improvements and therapeutic potential for treating fatty liver disease through various approaches. This study examines how the Impact of Aerobic Training and Probiotic Intervention on Nrf2 and GLUT4 mRNA Levels in Non-Alcoholic Fatty Liver Disease: An Experimental Study in Male Wistar Rats. We randomly assigned forty male Wistar rats (weighing 220 ± 20 g) to five distinct groups: Healthy Control (HC), NAFLD Control (NC), NAFLD with Aerobic Exercise (NAE), NAFLD with Lactobacillus Supplementation (NLS), and NAFLD with combined Aerobic Exercise and Lactobacillus Supplementation (NAELS). The healthy control group maintained a standard diet, while all other groups received oral tetracycline (140 mg/kg body weight dissolved in 2 ml water) through gavage for seven days to establish NAFLD conditions. The six-week intervention protocol included treadmill running at 18 m/min for exercise groups. Probiotic intervention groups received daily *L. Rhamnosus* GG doses (10^9 CFU/mL) via gavage for five weeks, administered five days per week. We conducted liver biopsies alongside these interventions and measured Nrf2 and GLUT4 expression in liver tissue using Real-Time PCR. Statistical analysis employed two-way ANOVA with Tukey post hoc tests, establishing significance at $p \leq 0.05$. We observed significant differences between the NC and HC groups ($p = 0.0000$) as well as between NC and NAELS groups ($p = 0.029$). The combined intervention group (NAELS) demonstrated the strongest effects on both Nrf2 ($\eta^2 = 0.35$) and GLUT4 ($\eta^2 = 0.52$) expression levels. These findings indicate that combining aerobic exercise with Lactobacillus supplementation substantially improves both gene expressions compared to individual interventions. Our findings suggest that combining aerobic exercise with Lactobacillus supplementation enhances Nrf2 and GLUT4 expression, potentially contributing to improved metabolic health in NAFLD patients. This combined approach may offer therapeutic advantages over single interventions for managing fatty liver disease.

Keywords: Aerobic Exercise, Lactobacillus Supplementation, Nrf2, GLUT4, Liver Tissue

1. Introduction

Aerobic exercise combined with *Lactobacillus* supplementation has been recognized as an effective strategy for improving metabolic regulation and managing non-alcoholic fatty liver disease (NAFLD) (1). Research suggests that the combination of physical activity and probiotic intake can modulate gene expression related to oxidative stress and glucose metabolism (2). Among these, the Nrf2 and GLUT4 genes play key roles in antioxidant defense and glucose uptake, respectively (3).

Evidence indicates that aerobic exercise reduces oxidative stress and improves liver function in animal models of NAFLD (4). This beneficial effect may be mediated through enhanced mitochondrial biogenesis and upregulation of antioxidant genes such as Nrf2 (5). Nrf2 acts as a master regulator of antioxidant enzyme transcription, protecting cells from oxidative damage—a critical mechanism in NAFLD, where oxidative stress significantly contributes to disease progression (6). Exercise-induced activation of Nrf2 has been shown to reduce lipid accumulation and inflammation in liver tissue, thereby improving hepatic health (7).

Lactobacillus supplementation complements the benefits of aerobic exercise by modulating gut microbiota and enhancing metabolic function (8). Probiotics have also been associated with improved glucose metabolism, partly through increased production of short-chain fatty acids (SCFAs) in the gastrointestinal tract (9). These SCFAs exert systemic effects, including upregulation of GLUT4 expression, which enhances glucose uptake in peripheral tissues such as skeletal muscle and liver (9). Therefore, combining *Lactobacillus* supplementation with aerobic exercise may synergistically enhance GLUT4 expression and improve glucose homeostasis in NAFLD (10).

Aerobic exercise alone has been shown to increase GLUT4 expression, contributing to improved insulin sensitivity and glucose transport (11). However, when combined with *Lactobacillus*, this effect may be amplified due to improved gut health and reduced systemic inflammation (12). By modulating anti-inflammatory pathways, probiotics may create a more favorable metabolic environment that further supports GLUT4 expression and overall metabolic improvement in NAFLD (13).

In addition to its impact on glucose metabolism, aerobic exercise has demonstrated potential in improving lipid metabolism among NAFLD models, including reductions in hepatic triglyceride levels (14). In this context, Nrf2 activation plays a crucial role by enhancing antioxidant defenses and reducing lipid peroxidation both essential for maintaining hepatocyte integrity. Moreover, *Lactobacillus*-supplemented exercise not only affects gene expression but also helps correct underlying metabolic disturbances in NAFLD (6, 15).

In summary, the combination of aerobic exercise and *Lactobacillus* supplementation represents a dual-targeted approach that influences key metabolic pathways. This strategy holds promise for enhancing Nrf2-mediated antioxidant defense and GLUT4-mediated glucose uptake, potentially offering therapeutic benefits for individuals with NAFLD. Further studies are needed to determine optimal dosing of *Lactobacillus* and to clarify the specific mechanisms involved. These findings support the development of lifestyle-based interventions for managing metabolic disorders associated with fatty liver disease.

2. Methods and Materials

2.1. Study Design and Animals

In the present experimental study, male Wistar rats were used to evaluate the effects of aerobic exercise and *Lactobacillus* supplementation on gene expression in liver tissue. A total of forty 8-week-old male Wistar rats (initial body weight: 220 ± 20 g) were obtained from the Laboratory Animal Breeding Center at the Razi Serum Institute.

The study was conducted on male Wistar rats with diet-induced obesity to investigate the impact of aerobic exercise combined with *Lactobacillus* supplementation on the expression of Nrf2 and GLUT4 genes in liver tissue. The animals were housed in transparent polycarbonate cages ($15 \times 15 \times 30$ cm) under controlled environmental conditions: temperature ($22 \pm 3^\circ\text{C}$), relative humidity (30–60%), and a 12:12 light-dark cycle. Rats had free access to water (10–12 mL/100 g body weight per day) via 500-mL laboratory animal bottles throughout the study.

After a two-week acclimatization period, the animals were randomly assigned to one of five experimental groups ($n = 8$ per group):

Healthy Control group (HC)

NAFLD Control group (NC)

NAFLD with Aerobic Exercise group (NAE)

NAFLD with Lactobacillus Supplementation group (NLS)

NAFLD with combined Aerobic Exercise and Lactobacillus Supplementation group (NAELS).

2.2. Fatty Liver Model Induction

Rats were administered tetracycline (140 mg/kg body weight, in 2 mL water) orally by gavage for 7 days. Fatty liver (steatosis) was verified by measuring liver enzymes and hematoxylin-eosin staining (16).

2.3. Bacterial Culture and Probiotic Administration

Lyophilized Lactobacillus rhamnosus GG (PTCC1637) was obtained from the Iranian Research Organization for Science and Technology (Tehran, Iran). Bacteria were cultivated in MRS medium (Zisti Gouya, Tehran, Iran) containing L-cysteine HCL and incubated at 37°C for 24 hours. Probiotic intervention groups were given daily doses of L. rhamnosus GG (109 CFU/mL) via gavage for 5 weeks, 5 days a week (17).

2.4. Aerobic Exercise Protocol

The aerobic exercise intervention consisted of a six-week treadmill running program, implemented after a one-week acclimatization period. During the familiarization week, rats were introduced to the treadmill by running at a speed of 5–10 m/min for 20–30 minutes per day, five days per week. The formal training program began at an intensity of 14 m/min for the first two weeks and was gradually increased to 18 m/min over the subsequent four weeks. The final two weeks of the protocol were conducted at a constant speed of 18 m/min. To minimize potential confounding effects of treadmill-related stress, control and Lactobacillus-supplemented groups remained under standard housing conditions without exposure to treadmill running. Prior to the start of the formal training, the exercise groups underwent a 5-day acclimatization period, during which they ran for 30 minutes daily at 5–10 m/min. Throughout the six-week experimental period, the aerobic exercise groups performed treadmill running at 18 m/min for 30 minutes,

five days per week. Non-exercised groups were maintained under normal cage conditions with unrestricted access to food and water. Exercise intensity was determined based on the percentage of maximum oxygen consumption (%VO₂max), as previously described (18). Each session included a 5-minute warm-up and cool-down phase at 5 m/min. Rats were gently encouraged to run using light tail stimulation; no electrical shocks or aversive stimuli were used (19). The VO₂max index was calculated using the following formula:

$$\text{VO}_2\text{max Index} = [\text{Maximum speed (m}\cdot\text{min}^{-1})] \times [\text{Grade (\%)} \times 100] \times [\text{Body weight (kg)}]$$

2.5. Tissue Collection and Processing

At the conclusion of the six-week experimental intervention, all animals were subjected to a 12–14 hour fasting period to ensure metabolic standardization prior to tissue collection. Following this preparatory phase, rats were anesthetized via intraperitoneal injection of ketamine and xylazine. Once deep anesthesia was confirmed, the animals were humanely euthanized, and liver tissues were promptly excised under sterile conditions. To minimize contamination and preserve sample integrity, the collected liver specimens were immediately rinsed in ice-cold phosphate-buffered saline (PBS, pH 7.0) to remove residual blood. Subsequently, the tissues were homogenized using a tissue homogenizer in PBS at 4°C to maintain RNA stability. Homogenized samples were then centrifuged at 12,000 rpm for 15 minutes at 4°C to separate cellular debris from the supernatant. The resulting supernatants were carefully collected and stored at –80°C until they were used for RNA extraction and subsequent gene expression analysis via real-time PCR.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from the previously stored liver tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's recommended protocol. RNA concentration and purity were assessed using a NanoDrop spectrophotometer or a comparable method. Only RNA samples with acceptable purity ratios (A260/A280 between 1.8 and 2.0) were selected for complementary DNA (cDNA) synthesis. Two micrograms of total RNA from each sample were reverse-transcribed into cDNA using an RNeasy kit (Parstous, Iran), according to the manufacturer's

instructions. Specific primers targeting Nrf2 and GLUT4 genes, along with the housekeeping gene GAPDH for

normalization, were used in the qRT-PCR analysis. Primer sequences are detailed below:

Table 1

Primer sequence of Real-time PCR

Primer Name	Sequence (5'-3')	Length
Nrf2	CAGCATAGAGCAGGACATGGAG	22
	GAACAGCGGTAGTATCAGC	19
GLUT4	CCATCTGATGACTGTGGCTCT	22
	GCCACGATGAACCAAGGAATG	21

Real-time PCR amplification was carried out using SYBR Green Real-Time PCR Master Mix (Parstous, Iran) on a real-time PCR system (e.g., ABI StepOnePlus or equivalent). The thermal cycling protocol included an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. Amplification specificity was verified through melt curve analysis. Gene expression levels were normalized to GAPDH, and relative mRNA expression levels of Nrf2 and GLUT4 were quantified using the $\Delta\Delta C_t$ method. Calculations were based on the following formulas:

$$\Delta C_t = C_{t \text{ target gene}} - C_{t \text{ reference gene}}$$

$$\Delta\Delta C_t = \Delta C_t \text{ treated group} - \Delta C_t \text{ control group}$$

$$\text{Fold Change} = 2^{-\Delta\Delta C_t}$$

2.6. Data Analysis

Statistical analyses were performed using SPSS 26 software. Additionally, graphs were drawn using

EXCEL2015 software. Data were presented as mean values \pm standard errors. The ANOVA test was used to determine differences between groups comparing the internal variability of group with the variability among all experimental groups. Multiple comparisons between the groups were performed using Tukey's method as a post-hoc test. P values <0.05 were considered statistically significant. All assays were performed in triplicate.

3. Findings and Results

NAFLD decreased Nrf2 and GLUT4 expression compared to healthy subjects. Both aerobic exercise and Lactobacillus independently improved these changes, Nrf2 in the NAE group showing the largest increase but still significantly different from the healthy group. The NAELS group showed levels similar to the healthy control group, with no significant difference observed.

Table 2

Descriptive statistic (mean \pm SD) of the groups

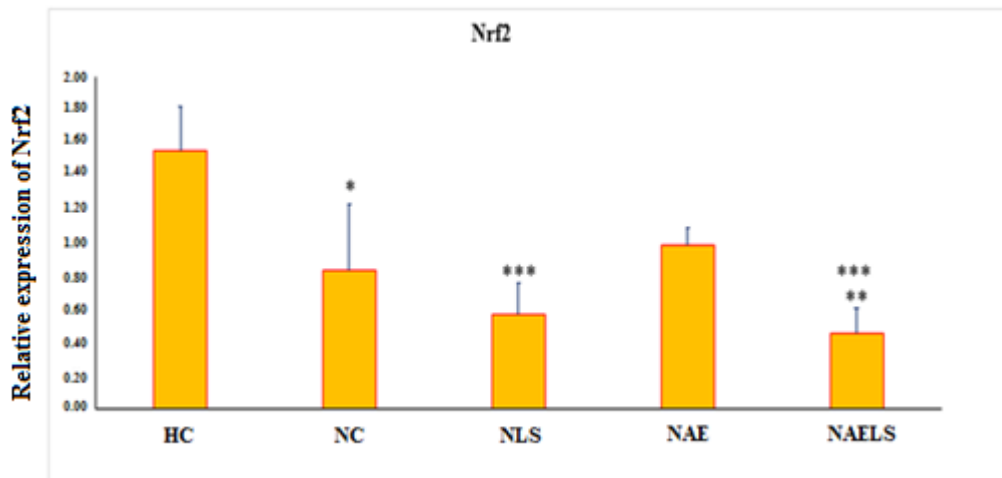
	HC	NC	NAE	NLS	NAELS
Nrf2	1.56 \pm 0.26	0.84 \pm 0.39	0.99 \pm 0.10	0.57 \pm 0.19	0.46 \pm 0.15
GLUT4	1.42 \pm 0.23	0.69 \pm 0.23	1.11 \pm 0.15	0.97 \pm 0.17	1.36 \pm 0.09

A significant difference in Nrf2 gene expression was observed between the NAFLD control group (NC) and both the healthy control group (HC; $p = 0.0000$) and the combined aerobic exercise and Lactobacillus supplementation group (NAELS; $p = 0.029$). Among the intervention groups, the NAELS group demonstrated the highest effect size ($\eta^2 = 0.35$), followed by the aerobic exercise group (NAE; $\eta^2 =$

0.21), while the Lactobacillus supplementation group (NLS) showed the smallest effect ($\eta^2 = 0.02$; [Figure 1](#)). These results indicate that the combination of aerobic exercise and Lactobacillus supplementation had the most substantial impact on enhancing Nrf2 expression compared to individual interventions.

Figure 1

Pairwise comparison of groups for *Nrf2* expression. Significant difference with the NAFLD control group. (*P* values <0.05).

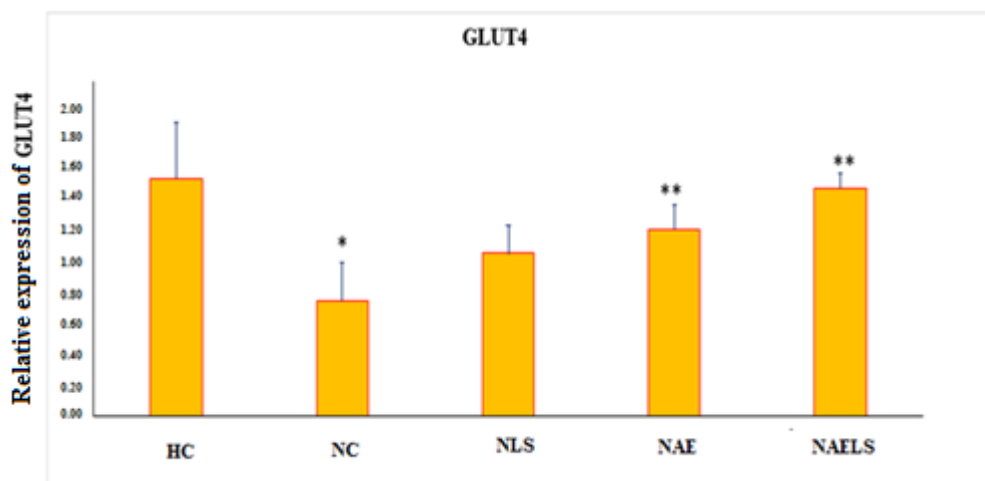


For GLUT4 gene expression, significant differences were also found between the NC group and the HC (*p* = 0.000), NAE (*p* = 0.003), and NAELS (*p* = 0.000) groups. The largest effect size was observed in the NAELS group (η^2 = 0.52), followed by the NAE group (η^2 = 0.27), and the NLS

group (η^2 = 0.13). This pattern suggests that the combined intervention not only significantly increased GLUT4 expression but also outperformed either treatment alone (Figure 2).

Figure 2

Pairwise comparison of groups for GLUT4 expression. Significant difference with the NAFLD control group (*P* values <0.05).



4. Discussion and Conclusion

Aerobic exercise, Lactobacillus supplementation, and the regulation of *Nrf2* and GLUT4 gene expression represent

interconnected areas of growing importance in metabolic health research, particularly in the context of metabolic disorders such as non-alcoholic fatty liver disease (NAFLD). Recent studies have demonstrated that NAFLD is associated with a significant reduction in the expression levels of both

Nrf2 and GLUT4 compared to healthy controls (20). Nrf2, recognized as a central regulator of antioxidant defense (21), plays a pivotal role in protecting cells from oxidative stress, while GLUT4 is essential for glucose uptake in skeletal muscle and adipose tissue (10).

Our findings indicate that both aerobic exercise and Lactobacillus supplementation independently enhance the expression of these genes, although their effects vary in magnitude. Aerobic exercise has been shown to induce Nrf2 expression across multiple tissues, an effect linked to improved antioxidant defenses and overall metabolic function (5). In rats with NAFLD, aerobic exercise alone significantly increased Nrf2 expression; however, it did not fully restore levels to those observed in healthy controls (22).

In contrast, when Lactobacillus supplementation was combined with aerobic exercise, Nrf2 expression reached levels comparable to those of the healthy control group. This synergistic effect highlights the therapeutic potential of combining lifestyle interventions for the management of metabolic disorders (23).

Similarly, aerobic exercise has a notable impact on GLUT4 gene expression. Exercise training is widely acknowledged as a strong stimulus for increasing GLUT4 content in skeletal muscle, which enhances insulin sensitivity and facilitates glucose transport (10). Both aerobic exercise and Lactobacillus supplementation were found to elevate GLUT4 expression in NAFLD models. However, the most pronounced improvements were observed in the combined intervention group, suggesting a synergistic enhancement of glucose metabolism (24).

Statistical comparisons among the experimental groups revealed significant differences in gene expression outcomes. Notably, the combination group (NAELS) exhibited the greatest effect sizes for both Nrf2 and GLUT4, surpassing those achieved by either intervention alone. These results imply that while each intervention offers individual benefits, a dual approach may be necessary to achieve optimal modulation of gene expression in NAFLD.

The underlying mechanisms are likely multifactorial. Endurance exercise activates signaling pathways that promote mitochondrial biogenesis and antioxidant responses, primarily through Nrf2 activation (5). Meanwhile, Lactobacillus supplementation modulates gut microbiota composition and influences metabolic signaling

pathways that further support Nrf2 activation and GLUT4 translocation to the cell membrane (25).

Beyond their direct effects on gene expression, these interventions also counteract several pathological features of NAFLD. By enhancing antioxidant protection via Nrf2 activation and improving glucose uptake through increased GLUT4 expression, both aerobic exercise and Lactobacillus supplementation contribute to improved metabolic profiles in affected individuals (26).

Overall, the combination of aerobic exercise and Lactobacillus supplementation is a promising therapeutic strategy to stimulate Nrf2 and GLUT4 gene expression in metabolic disease patients such as NAFLD. In addition to promoting the removal of oxidative stress with increased antioxidant defense, glucose metabolism is also improved with increased GLUT4 content. Future research will need to work to clarify the precise molecular mechanisms at work and consider optimal intensities and dosages of these interventions in terms of optimizing their health effects. The findings highlight the importance of lifestyle modification in metabolic disease treatment and pose a challenge to the pursuit of further study on multimodal therapy regimens.

Authors' Contributions

All authors equally contributed to this study.

Declaration

In order to correct and improve the academic writing of our paper, we have used the language model ChatGPT.

Transparency Statement

Data are available for research purposes upon reasonable request to the corresponding author.

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Declaration of Interest

The authors report no conflict of interest.

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Ethics Considerations

The experimental protocol was approved by the Ethics Committee of Islamic Azad University – Science and Research Branch (IR.IAU.SRB.REC.1403.408).

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