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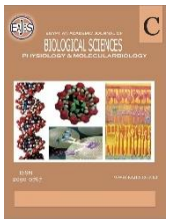

Impact of *Enterococcus Faecalis* KY072975 Supplementation as Probiotic on High Cholesterol Level *In-vivo*

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ABSTRACT

Elevated blood cholesterol levels are considered a significant and potentially fatal condition in humans. The current study sought to elucidate the effect of the Lactic acid bacteria (LAB) strain *Enterococcus faecalis*W7 as a probiotic on elevated cholesterol levels in hamsters. Ten male hamsters aged after acclimatization were randomly assigned to the control group (CG) and experimental groups high-fat group (HFG). Hamsters in CG were fed chow pellets only, while those in HFC were fed chow pellets supplemented with 40% beef tallow and 1% cholesterol for 30 days. After achieving obesity, hamsters in HFG were fed the same diet with isolate *E. faecalis* W7 lypholized powder (390 mg/kg BW/day) with high viable counts of LAB (4×10^{12} CFU/g) orally for two weeks (HFG+E.f. W7). The results demonstrated a significant drop in body weight in the HFG+E.f. W7 group (41.78%) compared to HFG rats (46.82%) and CG rats. The HFG+E.f. W7 group had significantly lower total cholesterol, low denisty lipoprotein (LDL), and triglyceride levels ($p<0.05$) compared to the HFG and CG groups. The HFG+E.f. W7 group showed substantial decreases ($p<0.05$) in liver enzymes SGPT and SGOT compared to the HFG and CG groups. While comparing rats in the HFG and CG groups, there was a small drop in HDL levels of 41mg/dL. In conclusion, using *E. faecalisW7* per os as a probiotic could be a potential intervention to reverse the adverse effects of excessive cholesterol levels in the blood.

INTRODUCTION

Cholesterol is an essential component of animal cell membranes, acting as a regulator of membrane fluidity and permeability. It is also regarded as an important precursor for the manufacture of steroid hormones, bile acids, and vitamin D (Wang *et al.*, 2012; Ribas, 2024). Long-term high blood cholesterol levels, on the other hand, can promote atherosclerosis and increase the risk of developing cardiovascular diseases (CVDs), which are a leading cause of mortality in many countries throughout the world (Ma *et al.*, 2019; Wazir *et al.*, 2023).

Probiotics are living bacteria that, when supplied in proper proportions, boost the host's health (FAO/WHO, 2002). Furthermore, probiotic LAB can induce a variety of health-promoting effects in their mammalian hosts, including immune modulation, anti-cholesterol, antidiabetic, and anticarcinogenic properties, as well as pathogen exclusion by interfering with pathogen adhesion and infectious agent growth inhibition through their generation of bactericidal compounds (Hatakka and Saxelin, 2008; Kumar *et al.*, 2011; Maftei *et al.*, 2024).

In addition to cholesterol-lowering prescription medicines, probiotics can reduce increased cholesterol levels by direct or indirect mechanisms (Fuller, 1989; Wang *et al.,* 2018; Ma *et al.,* 2019). A direct mechanism can be accomplished by inhibiting the de novo synthesis of cholesterol by hypocholesterolemia factors such as lactose, calcium hydroxyl methyl glutarate, Uric acid, whey proteins, and so on, or by reducing intestinal absorption of dietary cholesterol via assimilation, binding, or degradation. Bile salt deconjugation caused by the activity of bile salt hydrolase enzymes may indirectly lower cholesterol levels (Du Toit *et al.,* 1998; Liong and Shah, 2005; Li *et al.,* 2022). Deconjugated bile salts are less efficiently reabsorbed than conjugated versions due to their low water solubility. This event causes increased excretion of free bile acids in feces, increasing the need for cholesterol, which is a precursor for the production of bile salts. As a result, increased bile salt hydrolase (BSH) activity in the intestine may contribute to a decrease in serum cholesterol (Oner *et al.,* 2014; Choi *et al.,* 2015; Agolino *et al.,* 2024). It is also possible that BSH promotes the incorporation of the cholesterol moiety into bacterial membranes. This mechanism may modify the fluidity, permeability, and net charge of bacterial membranes and so boost the colonization ability and survival of these germs in the gut (Dambekodi and Gilliland 1998; Taranto *et al.,* 2003; Ziarno *et al.,* 2023).

Many research demonstrated the efficacy of probiotic lactic acid bacteria to lower serum cholesterol levels. Kawase *et al.* (2000) found that rats fed fermented milk with *Lactobacillus casei* TMC0409 and *Streptococcus thermophilus* TMC 1543 had considerably lower serum cholesterol than the control group. Wang *et al.* (2012) found that Lactobacillus strains isolated from traditional homemade koumiss products in China may effectively lower cholesterol in rats on a high-lipid diet. Tsai *et al.* (2016) found that applying a probiotic complex product containing *Pediococcus, Lactobacillus,* and Bifidobacterium to the lipid metabolism of hamsters fed a high-fat, high-cholesterol diet could reduce obesity, dyslipidemia, and lipid peroxidation. Hlivak *et al.* (2005) found that *Enterococcus faecium* effectively lowers serum cholesterol levels after a year of treatment. Albano *et al.* (2018) showed that 7 LAB strains may lower cholesterol concentrations in a medium containing cholesterol and bile acids by 42-55%. Ayyash *et al.* (2018) found that isolating lactic acid bacteria (LAB) can significantly decrease cholesterol in vitro.

In our earlier studies, Aboseidah *et al.* (2017a and b) found that probiotic lactic acid bacteria *Enterococcus faecalis*W7 isolated from healthy Egyptian newborns' stool had the highest cholesterol-lowering potential in vitro. The bacterial isolate W7 was also identified as the most potent strain for lactic acid production and was deposited in GenBank with the accession number KY072975. However, scientific research has raised concerns about the effects of *Enterococcus faecalis*W7 as a probiotic on cholesterol levels in vivo in Egypt. As a result, the current study sought to evaluate the effects of *E. faecalis*W7 in hamsters fed a high-fat, high-cholesterol diet in order to identify a promising treatment for hypercholesterolemia.

MATERIALS AND METHODS

1. Bacterial Culture:

The bacterial isolate used in the present study was *Enterococcus faecalis*W7 previously isolated from infants stool in Egypt and was stored on De Man Rogosa–Sharpe agar (MRS) slant at 4°C. A freeze-dried powder of this bacterium was prepared using Lypholizer for mixing with the animal food.

2. Experimental Design and Animal Groups:

Ten male hamsters were purchased from the National Research Center in Egypt. Individuals were housed in a controlled environment with a temperature of 20 ± 2°C, humidity of 55 ± 5%, and a 12-hour light-dark

cycle from 8 AM to 8 PM. During the first week of acclimatization, the animals were given chow pellets and water. They were then randomly divided into two groups: the control group (CG), which contained four animals, and the experimental group (HFG), which had six animals. Hamsters in the CG were given only chow pellets, while those in the HFG were provided chow pellets supplemented with 40% beef tallow and 1% cholesterol for 30 days. After feeding time and when obesity was detected in animals, HFG hamsters were fed chow pellets with 40% beef tallow and 1% cholesterol. They were also given (390 mg/kg BW/day) of isolate *E. faecalis* W7 lypholized powder with high viable counts of LAB (4 × 1012 CFU/g), known as HFC+ E. faecalisW7. The animals were given the LAB isolate *E. faecalis*W7 orally once a day for two weeks via a sterile orogastric tube.

3. Measurement of Body Weight And Blood Analysis (Tsai *et al.*, 2016):

The weights of the animals were recorded. Serum was taken by scarification from the medial canthus of the eye, centrifuged at 3000X rpm for 10 minutes, and kept at -20ºC in the refrigerator for subsequent analysis. Total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), serum glutamic pyruvic transaminase (SGPT), and serum glutamic oxaloacetic transaminase (SGOT) were all measured enzymatically using an enzymatic reagent kit (Sigma-Aldrich Chemical Co). At the conclusion of the experiment, the animals were slaughtered, and their livers were extracted, fixed in 10% neutral formalin, and kept for histology. According to Li *et al*. (2018), liver tissue samples were treated using dehydration, clarity, paraffin infusion, and embedding to produce paraffin tissue blocks. After that, the slices were stained with hematoxylin and eosin (H&E) and examined under a light microscope.

4. Statistical Analysis:

SPSS software for Windows version 20.0 (Statistical Package for Social Science, Armonk, NY: IBM Corp) was used to conduct statistical analysis. The data was analyzed using one-way ANOVA, followed by Duncan's multiple range test to identify significant differences between groups ($P < 0.05$). Data are presented as mean ± standard deviation from three independent replicates.

RESULTS

The mean body weight values are shown in Figure (1). The results revealed that the final body weight values of the hamsters from the HFG+E.f. W7 and HFG groups were significantly different from the CG group ($P < 0.05$). The weight percentage gain in HFG group was approximately 46.82%, while in HFG+E.f. W7 group the weight percentage gain was 41.78 % (Table 1). It was noticed that, the weight percentage gain in HFG+E.f. W7 decreased. The HFC diets resulted in overall increase of body weight and showed the highest increase in weight percentage gain in comparison to HFG+E.f. W7 and CG groups.

Table 1. Body weight gain of hamsters fed a high-fat plus high-cholesterol diet for 10 weeks, supplemented with or without probiotic W7.

Groups	Body weight (g)		Weight gain (%)	Daily weight gain (g)
	Initial	Final		
CG	61.60 ± 0.88 a	100.80 ± 13.90 b	36.74 ± 7.58 b	0.68 ±0.08 b
HFG	63.00 ± 0.01a	132.66 ±32.20 a	46.82 ± 11.03 a	0.98 ± 0.46 a
HFG+W7	66.00 ± 4.2 a	113.87 ± 4.40 a	41.78 ± 4.39 ab	0.55 ± 0.19 b

Data are expressed as means ± SE in the same column, Different letters in the same column mean significantly different and the highest take symbole a. Weight gain percentage = (final weight-initial weight) / final weight × 100. Daily weight gain = (final weight- initial weight) / days**.** CG, control group; HFG, high fat and cholesterol group, HFG+E.f.W7, high fat and cholesterol group fed with *E. faecalis* W7.

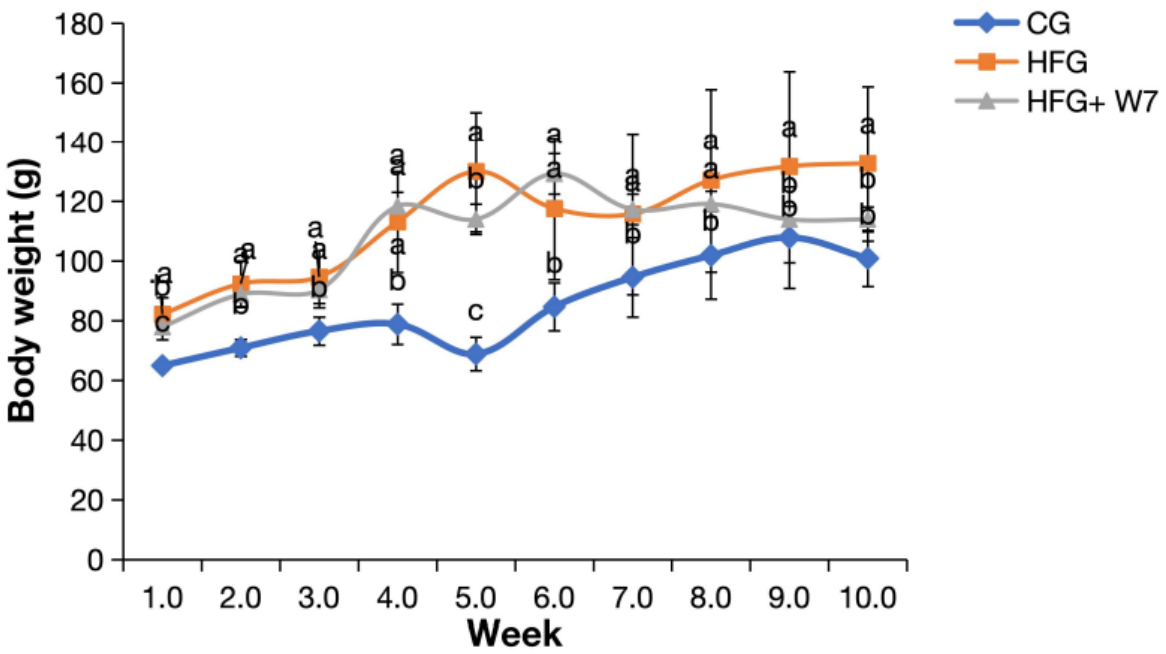

Fig. 1. Body weight of control, high fat plus high cholesterol diet (HFC), high-fat plus high-cholesterol diet and supplemented or not with LAB (W7) for 10 weeks

Throughout the 10-week experimental period, the levels of total cholesterol were significantly lower ($P < 0.05$) in the HFG+W7 group compared with those in the HFG group (Fig. 2). At the end of the trial, hamsters treated with W7 LAB showed total cholesterol levels in serum reduction by 50.17% ($P < 0.05$) (Figure 2A). The serum total cholesterol level was significantly decreased ($p<005$) in HFG+W7 compared to HFG group. There were significant differences ($P<0.05$) in triglyceride levels among hamsters in HFG+E.f. W7 and that in HFG group (Figure 2B). Where the triglyceride level of HFG+W7 group was 52.2 mg/dL but the HFG group showed 127.7 mg/dL.

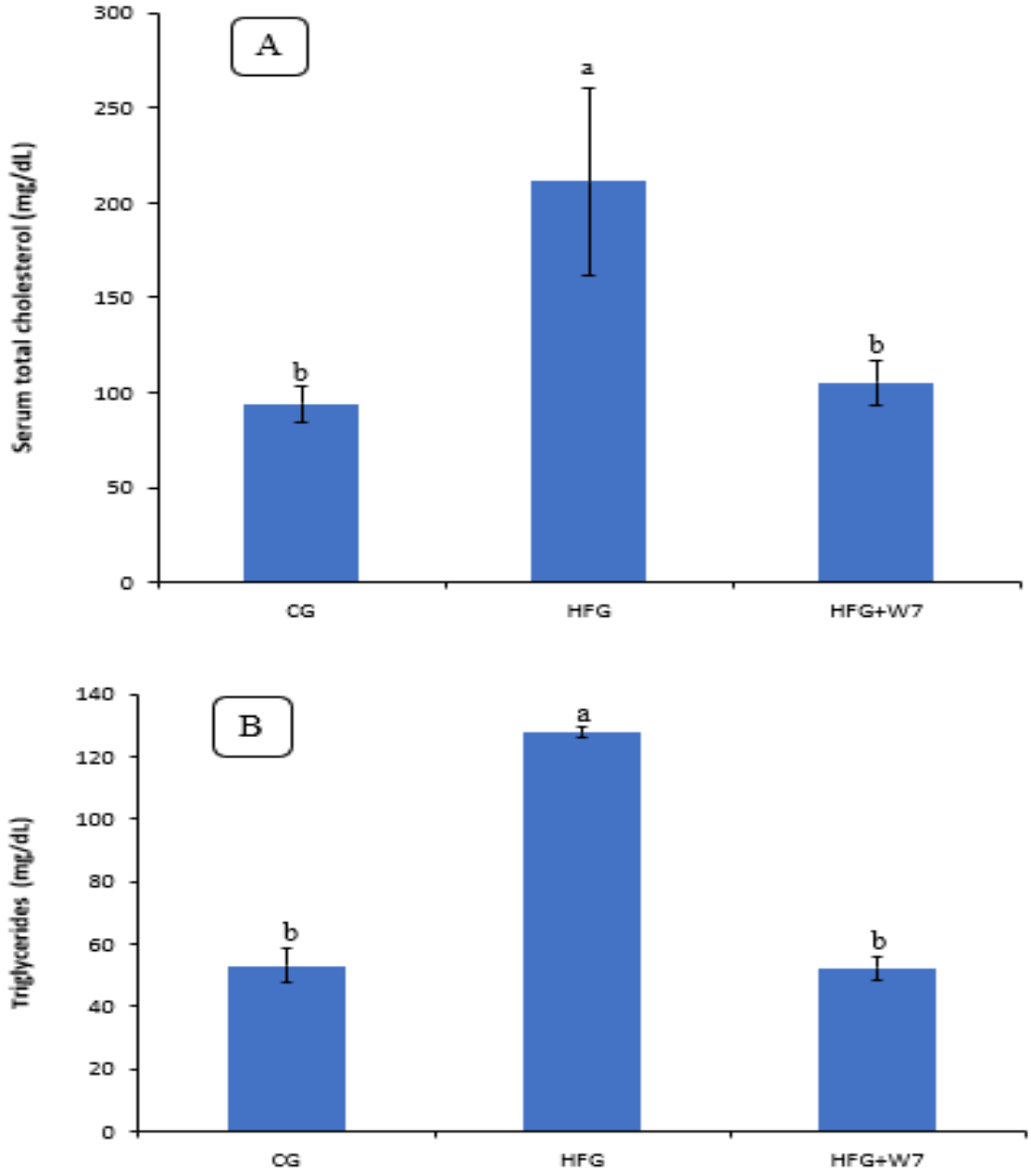

Fig. 2. Serum (A) total cholesterol and (B) triglyceride of hamsters fed a high-fat plus high-cholesterol diet and supplemented LAB product (W7) during 10 weeks of the study.

The levels of high-density lipoprotein cholesterol (HDL) were significantly lowered ($P < 0.05$) in the HFG+E.f. W7 group compared with the HFG group throughout the 10-week experimental period (Fig. 3). Hamsters supplemented with LAB showed HDL levels decreased by 25.8 % ($P < 0.05$) compared with the HFG group (Fig. 3A). Also, Hamsters treated with LAB showed reduced LDL levels and LDL /HDL ratio at 10 weeks compared with the HFG group (Figs. 3B and C). Compared with the HFG group, hamsters treated with LAB showed LDL levels (LDL /HDL ratio) in serum reduced by (58.7%) and (44.25%) ($P < 0.05$), at the end of the experimental period (Figs. 3B and C). The HFG+E.f. W7 group showed the greatest reduction in LDL levels 10 weeks (by 58.7 %) compared with the HFC group (Fig. 3B).

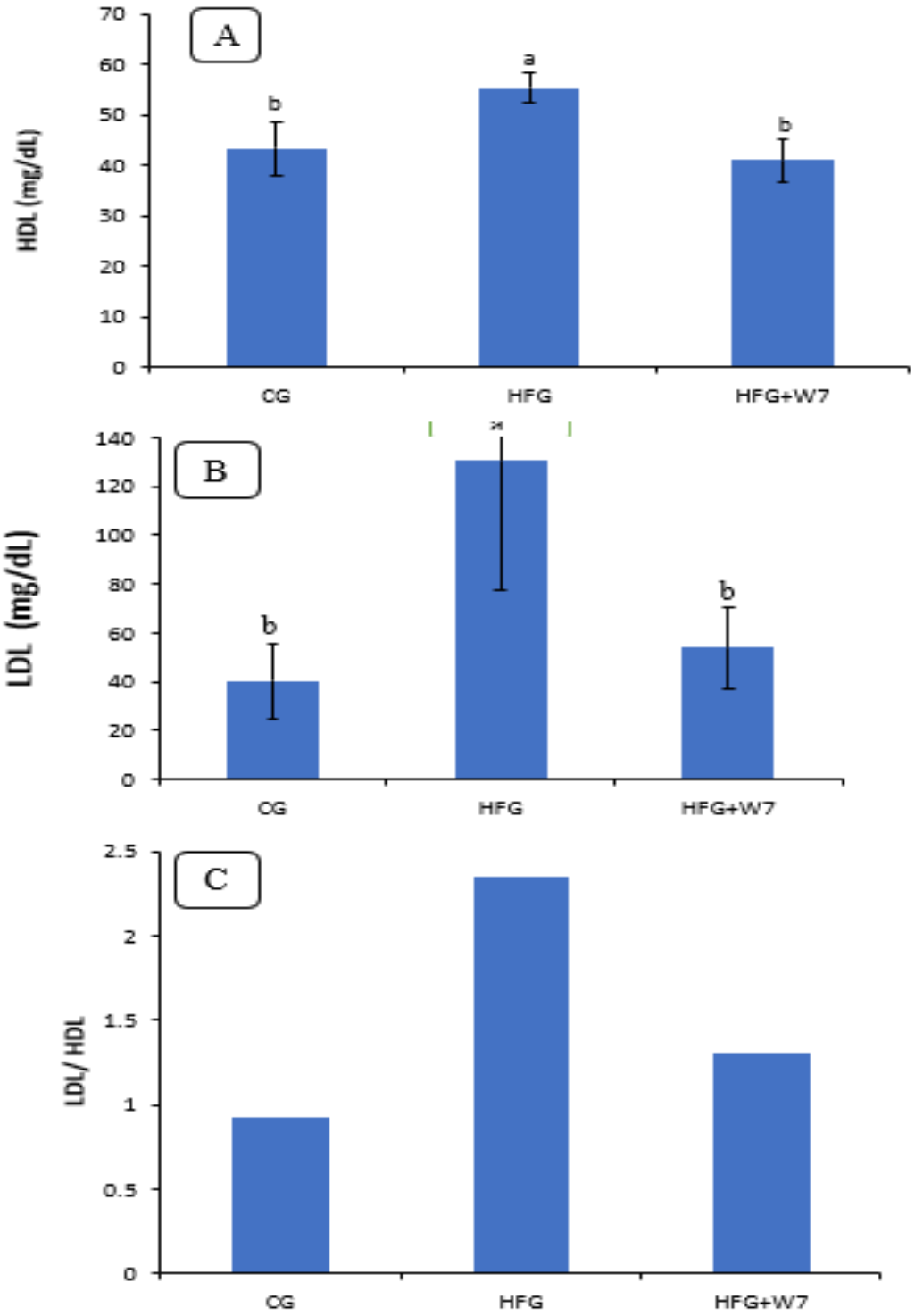

Fig. 3. Serum (A) HDL, (B) LDL and (C) LDL/HDL ratio of hamsters fed a high-fat plus high-cholesterol diet and supplemented or not with LAB (W7) during 10 weeks of the study.

The levels of SGPT and SGOT in the HFG group were significantly higher than that of the CG (1.7- fold and 16-fold increases for SGPT and SGOT, respectively, vs. control group). In contrast, hypercholesterolemic hamsters treated with W7 or significantly attenuated levels of hepatic SGPT and SGOT (51% decreases for SGPT; 44.23% decreases for SGOT in HFG+E.f. W7 group, respectively, vs. HFG group) (Fig. 4).

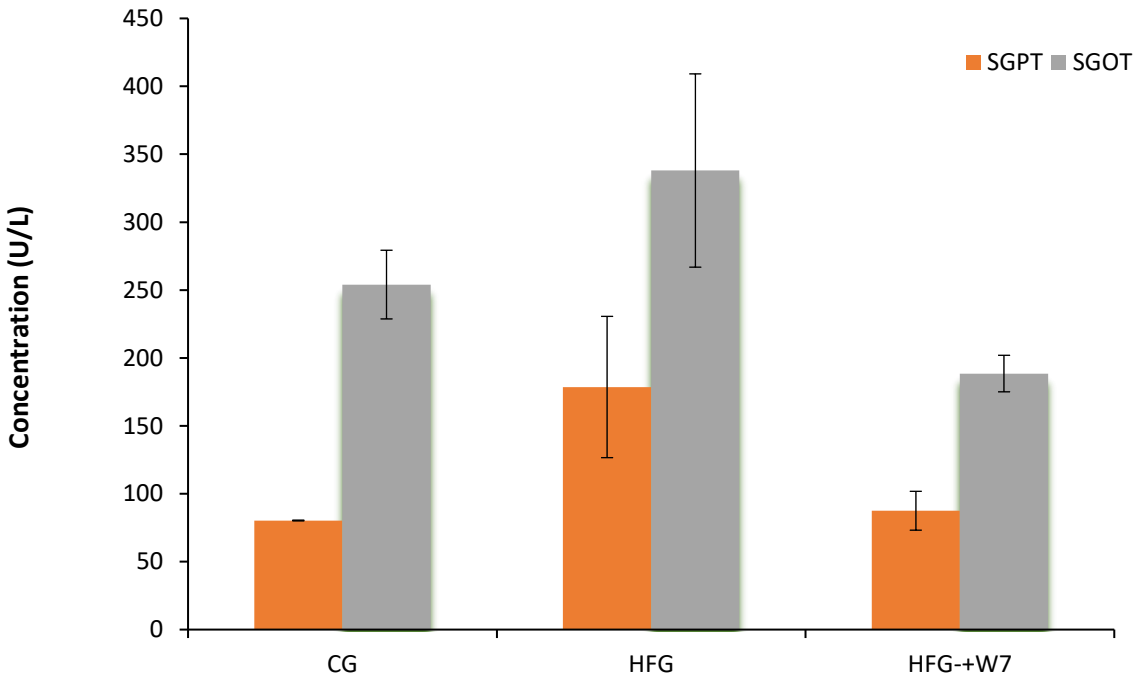

Fig. 4. Serum SGPT and SGOT level of hamsters fed a high-fat plus high-cholesterol diet and supplemented or not with LAB (W7) during 10 weeks of the study.

Histopathological Investigation:

At the end of the experiment, the liver cells of hamsters in the HFG group, appeared empty, swollen, and even exhibited necrosis in hepatic cells compared with CG group which had a well-organized structure. Hepatic sinusoids were clearly visible and hepatic cords were neatly arranged and distributed radially around the central veins (Fig. 5). In HFC+E.f.W7 group, although the tumescence of liver cells was not improved, the cavities and necrosis observed were relatively few. Additionally, staining suggested that many massive lipid droplets were accumulated in the liver tissues in HFG group and the lipid droplets had obviously decreased in HFG+E.f. W7 group.

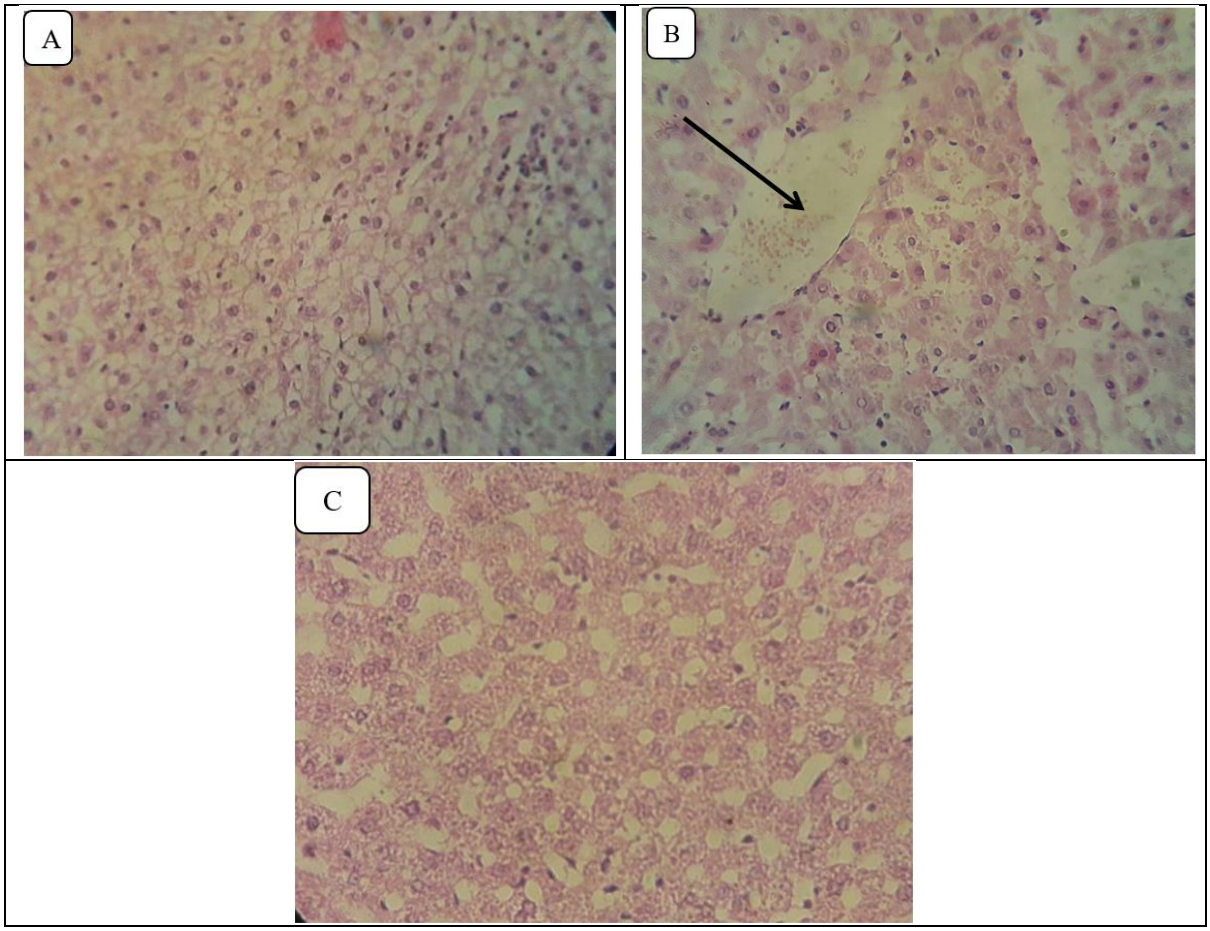

Fig. 5. Histopathological changes in livers of hamsters fed a high-fat plus high-cholesterol diet and supplemented or not with LAB (W7), during 10 weeks of the study. (A) Control, (B) HFG, (C) HFG+W7 (magnification 100×)

DISCUSSION

The current study highlighted the favorable benefits of *Enterococcus faecalis* W7 probiotic supplementation in lowering cholesterol levels in laboratory animals fed a high-fat diet (hamsters). The findings of this study on body weight growth were consistent with those of Huang *et al.* (2013), who employed L. plantarumLp09, an isolate from kefir grains, to complement a high-cholesterol diet. In their investigation, they found that the group given L. plantarumLp09 had lower weight growth and food efficiency than the group on a high-cholesterol diet.

The results revealed a significant decrease in both HDL and LDL concentrations in the group provided E. faecalisW7 (HFG+E.f. W7) compared to the HFG group. The serum HDL and LDL results were consistent with previous studies (Abd El-Gawad et al., 2005; Klein et al., 2008; Tsai et al., 2016), which showed that animals fed a cholesterol- and fat-rich diet showed lower total cholesterol levels and higher LDL and HDL concentrations when supplemented with a daily intake of LAB. Several studies have shown that animals fed cholesterol and fat showed a reduction in total cholesterol levels and LDL, but HDL concentrations did not increase significantly (Wang et al., 2009; Pan *et al.*, 2011; Jones *et al.*, 2012), while other studies did not observe a cholesterol-lowering effect following lactic acid bacteria consumption (De Roos *et al.*, 1999; St-Onge *et al.*, 2002, Lee *et al.*, 2020; Chen *et al.*, 2022). The varied characteristics of the LAB strains, such as acid resistance, bile tolerance, or distinct processes of cholesterol reduction in vitro, may be responsible for these contradictory results (Taranto *et al.*, 1998; Prete *et al.*, 2021; Abdi *et al.*, 2022).Other parameters that may be involved include diet

cholesterol content, LAB ingestion dosage, LAB combinations and their various ratios, animals used, and feeding period length (Wang *et al.*, 2009; Starovoitova *et al.*, 2012). In general, excess cholesterol in the body accumulates in hepatic cells, resulting in non-alcoholic fatty liver disease (NAFLD). Elevated levels of liver damage markers such as SGPT and SGOT are typically indicative of liver illness since these enzymes are exclusive to the liver (Kim *et al.*, 2017).

Consequently, the levels of SGPT and SGOT in our study showed a substantial decrease in rats fed *E. faecalisW*7 compared to rats fed a high cholesterol and fat diet. Furthermore, liver tissue analysis revealed serious damage in liver cells of rats fed a cholesterol-rich diet. However, rats in the HFC+E. faecalisW7 group had partially reduced liver damage, indicating that *E. faecalis*W7 plays a role in mitigating the negative effects of elevated cholesterol concentration on liver cells. Hu *et al.* (2013) reported similar findings, in which histopathological examinations revealed severe injuries in liver tissues of rats fed a cholesterol diet, but rats treated with LAB strains *Lactobacillus plantarum*NS5 and NS12 showed a significant ability to partially reduce these injuries.

CONCLUSIONS

The application of LAB isolate *E. faecalis*W7 for cholesterol reduction in rats fed a high cholesterol and high-fat diet is incredibly intriguing because of its contribution to reducing long-term hypercholesterolemia and, as a result, the risk of diseases connected with it. *Likewis*e, the Probiotic LAB E. faecalisW7 has been proposed for future research, which will be valuable due to its tendency to reduce cholesterol both in vitro and in vivo.

Declarations:

Ethical Approval: The animal experiments were approved by Suez Canal University's Scientific Research Ethics Committee (74/2022), and all experiments obeyed relevant standards and regulations.

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Availability of Data and Materials: The data presented in this study are available on request from the corresponding author.

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