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Investigation of the Antihyperlipidemic Mechanism of Potential Probiotics

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ABSTRACT

Background: Hypercholesterolemia leads to cardiovascular diseases that are almost the leading cause of death worldwide. The cholesterol-lowering effect of probiotics is getting increased attention especially that the chemical drugs produce several undesirable side effects. Understanding probiotics' potential effects and choosing the right way to use them requires research into the mechanisms through which they control serum cholesterol. **Methods:** In this study, we used two lactic acid bacteria (LAB) (*Lactococcus lactis* ssp. *lactis* and *Pediococcus* sp.) that were isolated in a previous work of ours from dairy products and had a high *in vitro* cholesterol removal activity together with promising antihyperlipidemic effect *in vivo*. In the laboratory animal experiment conducted on hamsters for studying the antihyperlipidemic effect of probiotics, the livers of the different groups were excised, and the levels of hepatic total cholesterol and Cytochrome P450 Family 7 Subfamily A Member 1 **(**CYP7A1) were determined. Statistical analyses were conducted by one-way analysis of variance (ANOVA) then post-hoc test. A probability of p-value < 0.05 was considered statistically significant. **Results:** *Lactococcus lactis* ssp. *lactis* and *Pediococcus* sp. significantly ($p < 0.05$) decreased the hepatic total cholesterol levels by 24.96 % and 35.80 %, respectively and increased hepatic CYP7A1 levels by about 49.60% and 37.84 %, respectively in comparison to the diet-induced hyperlipidemic control group. There was no significant difference between the effect of the two isolates and the reference drug, atorvastatin in the levels of both test parameters. **Conclusion:** We introduce two potential probiotics with similar effect as atorvastatin on both hepatic total cholesterol and hepatic CYP7A1 levels. Thus, these isolates can act as adjuvant therapy to decrease the atorvastatin dose and consequently its side effects. The possible mechanism for the antihyperlipidemic effect could be through upregulation of hepatic CYP7A1 genes that leads to more bile acid synthesis from cholesterol and consequently decreasing liver cholesterol level.

Keywords: *Total hepatic cholesterol; CYP7A1, Lactic acid bacteria; Probiotic; Antihyperlipidemic*

INTRODUCTION

The prevalence of cardiovascular diseases (CVDs) is reportedly increasing worldwide. The World Health Organization (WHO) reported that 17.9 million individual die annually from CVDs, an estimated 32% of all deaths worldwide with hyperlipidemia is a significant element in their emergence[1] . Hyperlipidemia is a clinical syndrome characterized by a rise in serum lipid levels including cholesterol as well as triglyceride [2]. Every 1% decrease in serum cholesterol levels reduces the risk of coronary heart disease (CHD) by 2%–3% [3]. Recent studies indicate that cholesterol buildup in the liver plays a role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) [4]. Accumulation of cholesterol in the liver results from an imbalance in the hepatic cholesterol homeostasis [5]. Cholesterol build-up causes liver damage by initiating intracellular signalling pathways that foster inflammation and fibrogenesis. Furthermore, cholesterol deposition in the hepatic mitochondria leads to the impairment of the mitochondria, which leads to increased production of reactive oxygen species (ROS), resulting in programmed cell death of the endoplasmic reticulum (ER) [6].

Even though statins are effective in treating hyperlipidemia, such drugs have documented several harmful side effects [7]. That's why there is a resurgence of interest in nutraceuticals with remedial properties that include: enforcing intestinal microbial balance, lowering serum lipid levels, ameliorating lactose intolerance symptoms, reducing the risk of developing colorectal cancer and boosting immunity[8] . In that respect, probiotics have drawn much interest [9] as they might have a potential effect to manage serum lipid levels as nutraceuticals [10]. Probiotics also showed beneficial effects on lipid levels and blood pressure in diabetic patients of type 2 diabetes mellitus [11]. Moreover, probiotic products are used highly by many clinicians [12].

Lactic acid bacteria (LAB), the most widely recognized probiotics in the human body [8], were shown to reduce serum lipid levels in humans, pigs, rats and hamsters. Consequently, LAB have attracted the attention of researchers, and the mechanism of lowering cholesterol has also been studied extensively [13]. Some mechanisms were proposed, like that probiotics remove the intestinal cholesterol, inhibit small-intestinal absorption by decreasing the expression of intestinal Niemann-Pick C1-Like 1 (NPC1L1) gene and that its bile salt hydrolase (BSH) deconjugates bile salt and consequently increases the faecal bile acid excretion [14]. Another mechanism came with a growing evidence suggested that LAB manipulate the expression of genes related to cholesterol metabolism [13]. The most critical regulatory gene in bile acids synthetic pathway is CYP7A1 because it is the first and rate-limiting enzyme in the classic synthesis of them. CYP7A1 is also known as cholesterol-7α-hydroxylase or cholesterol-7α-monooxygenase and cytochrome P450 7A1. It is a cytochrome P450 oxidoreductase enzyme responsible for monitoring of the cholesterol levels [15] .The increased of CYP7A1 expression in primary human hepatocytes led to an increase in bile acid synthesis [16]. While, fibroblast growth factor 15 (FGF15) and its human ortholog, FGF19 act as negative regulators of CYP7A1. FGF15 is transcribed and released in response to bile acid absorption and it plays a key role in the negative feedback inhibition of their hepatic synthesis[17]-[18]. Cholesterol diet induces CYP7A1, consequently bile acids synthesis and relative resistance to development of hypercholesterolemia in rats. While, high cholesterol diet fails to induce CYP7A1 in hamsters and most humans respond probably more similar to hamsters, so the excess cholesterol in them causes hypercholesterolemia and atherosclerosis [16] .

The exact mechanisms responsible for the cholesterol-lowering activity remain unclear [14]. The initial research regarding the lipid lowering properties relied on the isolation and screening of promising LAB strains. Lately, the research is also about determining the mechanism through which these probiotics exert their cholesterol-lowering properties [19].

In alignment with the current approach of research, comes the aim of our study. We have already isolated and characterized two LAB isolates of a highly promising lipid lowering effect in a previous work [2], and in the current one we aim to investigate the mechanism through which they exerted their action focusing on the hepatic cholesterol regulation possibly through CYP7A1.

MATERIALS AND METHODS

Source, Maintenance, and preservation of Lactic Acid Bacteria Strains

We used two LAB isolates, *Lactococcus lactis* ssp. *Lactis* and *Pediococcus* sp. that showed the uppermost *in vitro* cholesterol removal ability and promising antihyperlipidemic activity *in vivo* in our previous work [2]. The isolates were preserved in deMan, Rogosa, Sharpe (MRS) broth medium containing glycerol (20%) and stored at -80°C.

Testing the effect of two LAB isolates on Hepatic Cholesterol and Hepatic CYP7A1 levels in a laboratory animal model.

The procedures were carried out following the Guide for the Care and Use of Laboratory Animals [20]. The study was approved by the ethical committee of the Faculty of Pharmacy, Helwan University, Egypt

(Reference Number: 03A2019). The study (inoculum preparation, preparation of atorvastatin and animal study design) was conducted following the procedures described [2].

Determination of Hepatic Cholesterol Levels in tested laboratory hamsters

After 28 days of animal feeding with high fat diet with and without administering either atorvastatin or lactic acid isolates, animals were fasted overnight for 12 h, sacrificed and then their livers were excised [2]. Total lipids were extracted and purified from the liver tissue using the Folch method in which 2:1 chloroform: methanol (v/v) is used for lipid extraction [21]. The cholesterol level was measured with commercial assay kit, Total Cholesterol kit, cat no. GPL/SU011 (Reactivos GPL, Barcelona, Spain) according to the manufacturer's instructions.

Determination of Hepatic CYP7A1 levels in the livers of tested laboratory hamsters

Liver tissues were excised into small pieces, approximately 1 cm^2; they were washed with phosphate buffer saline (PBS) pH 7.4. PBS (2 mL) was then added to the washed tissue in a ratio of 3: 4 buffer/ tissues (V/V). In 5 mL test tube, the tissues were disrupted with tissue homogenizer for 1 minute until all solid tissues wer disrupted, followed by centrifugation at 6000 rpm for 10 min, then the supernatant was collected in 1.5 mL sterile Eppendorf tubes and stored at -20^{o}C until analysis. The CYP7A1 level was measured in hepatic tissue homogenates using Hamster CYP7A1 ELISA kit, cat no. ELK9712, ELK Biotechnology, Wuhan, China. The procedures were conducted according to the manufacturer protocol.

Statistical Analysis

All experiments were performed in triplicates except that the animal group included 6 hamsters and data was expressed as the mean ± SEM. Statistical analyses were performed using GraphPad InStat software version 3.05 (GraphPad Inc., La Jolla, CA, USA). Statistical analyses were conducted by one-way analysis of variance (ANOVA) followed by post-hoc test. A probability of p-value < 0.05 was considered statistically significant.

RESULTS

Effect of administering LAB isolates on Hepatic Cholesterol levels of diet-induced hyperlipidemic hamsters

The total cholesterol levels of the hamsters' liver were measured after 28 days of the experiment as described in 2.3 and the results are shown in **Figure 1.**

As shown in **Figure 1**, the hepatic total cholesterol level increased significantly in the diet-induced hyperlipidemic group, that were fed a high-fat diet (HFD), (HFD-fed) ($p < 0.05$) compared to the non-induced group by about 271.64%. The groups supplemented with atorvastatin, *Lactococcus lactis* ssp. *lactis* or *Pediococcus* sp. showed a significant decrease of the hepatic total cholesterol levels in comparison to the control diet-induced hyperlipidemic group 33.69 %, 24.96 %, and 35.80 %, respectively ($p < 0.05$). Moreover, *Pediococcus* sp. supplementation significantly ($p < 0.05$) decreased hepatic total cholesterol levels more than *Lactococcus lactis* ssp. *lactis* supplementation in hamsters by 10.83 % (please check). Remarkably, there was no significant difference between the reference drug atorvastatin and any of the two isolates' groups, Lactococcus *lactis* ssp. *lactis* or *Pediococcus* sp. supplemented group.

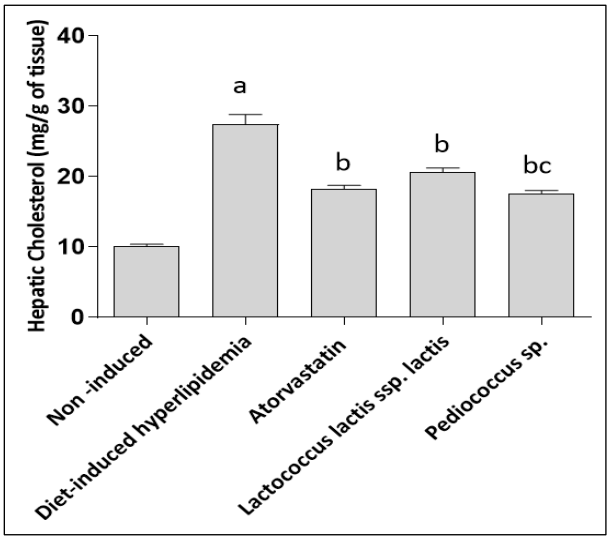

Figure 1. Hepatic total cholesterol levels (mg/g) of hyperlipidemic hamsters. Non- induced group (standard food diet), diet-induced hyperlipidemic group {high-fat diet (HFD), (HFD-fed)}, atorvastatin group (HFD-fed + 1.23 mg/kg/day atorvastatin), *Lactococcus lactis* ssp. *lactis* group (HFD-fed + 1×10^9 CFU/mL /day) and *Pediococcus* sp. group (HFD-fed + 1×10^9 CFU/mL /day). Data are represented as the mean ±SEM (n=6). Means not sharing a common letter differ significantly from each other ($p < 0.05$).

Effect of administering LAB isolates on Hepatic CYP7A1 levels of diet-induced hyperlipidemic hamsters

The hepatic CYP7A1 levels of five hamster-experimental groups were measured as described above in 2.4 after the 28 days experimentation period and the results are represented in **Figure 2**.

Results in **Figure 2** revealed that the administration of the HFD for 28 days, the diet-induced hyperlipidemic group, significantly ($p > 0.05$) decreased the hepatic CYP7A1 level in hamsters by about 78.79% compared to the non-induced group. On the contrary, atorvastatin, *Lactococcus lactis* ssp. *lactis* and *Pediococcus* sp. groups showed a significantly increased hepatic CYP7A1 levels ($p > 0.05$) by about 55.45%, 49.60%, and 37.84 %, respectively compared to the HFD-fed control group. However, there was no significant difference in the hepatic CYP7A1 levels among the three test groups.

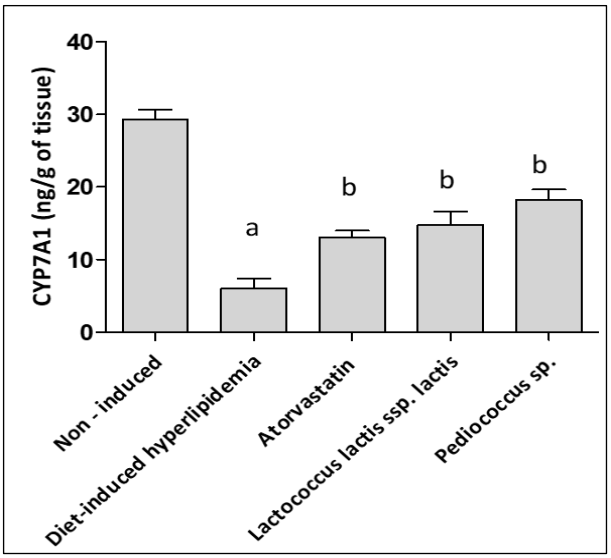

Figure 2. Hepatic CYP7A1 levels (ng/g) of hyperlipidemic hamsters. Non-induced group (standard food diet), diet-induced hyperlipidemic group (HFD-fed), atorvastatin group (HFD-fed + 1.23 mg/kg/day atorvastatin), *Lactococcus lactis* ssp. *lactis* group (HFD-fed + 1×10^9 CFU/mL /day) and *Pediococcus* sp. group (HFD-fed + 1×10^9 CFU/mL /day). Data are represented as the mean ±SEM (n=6). Means not sharing a common letter differ significantly from each other ($p < 0.05$).

DISCUSSION

Cholesterol level in blood is highly related to coronary heart diseases. A lot of studies have showed that probiotics are able to remove cholesterol and produce hypocholesterolemia. *Pediococci* can be found in a lot of foods like sausages and cheese. *Pediococcus* spp. are considered non pathogenic and safe as there were no adverse effects reported when taken in large quantities in food [22]. *Lactococcus lactis* has the probiotic properties and it was used for centuries in food industry, that's why it was rendered generally recognized as safe (GRAS) [23].

Effect of administration of LAB isolates on Hepatic Cholesterol levels of diet-induced hyperlipidemic hamsters

The results in **Figure 1** revealed that after 4 weeks of *Lactococcus lactis* ssp. *lactis* supplementation the hepatic total cholesterol levels were significantly lowered by 24.96 % in comparison to the diet-induced hyperlipidemic control group. This result is similar to the results obtained by a *Lactobacillus plantarum* CAAS 18008 that significantly reduced hepatic total cholesterol levels by 30.9% [14]. Another study about *L. plantarum* stated that the different strains of this species have different cholesterol-lowering capacities and different influencing factors [24].

Moreover, another study showed similar results where the supplementation of *Lactococcus lactis* ssp *cremoris* to female mice on a high-fat diet developed less liver fat [25].

As for Pediococcus sp. supplemented group, there was also a significant decrease of the hepatic total cholesterol level by 33.69 % compared to the diet-induced hyperlipidemic control group. Our result is similar to that reported about *Pediococcus acidilactici* M76 strain which decreased the hepatic total cholesterol significantly compared with HFD-fed mice group [26]. In addition, hepatic cholesterol content was also significantly decreased by a *Pediococcus pentosaceus* LP28 in a previous study [27]. Another similar result was documented where *Pediococcus acidilactici* FS2 strain reduced blood cholesterol levels when taken along with a prebiotic [22].

Another observation from **Figure 1**, is that the effect of *Pediococcus* sp. was significantly ($p < 0.05$) better than the effect of *Lactococcus lactis* ssp. *Lactis.* Interestingly, this latter result comes in accordance with our results about the two isolates where *Pediococcus sp.* reduced serum total cholesterol levels significantly while *Lactococcus lactis* ssp. *Lactis* couldn't. Moreover, in this study, *Pediococcus sp.* produced a higher reduction of serum triglyceride levels than *Lactococcus lactis ssp. Lactis* [2].

Finally, from **Figure 1** we can also see that the LAB isolates effect on hepatic total cholesterol levels were almost equal to that of atorvastatin ($p < 0.05$). Considering the fact that atorvastatin is the most widely used antihyperlipidemic [28], together with another highly important fact about its reported side effects [29], we can see how advantageous it could be to have LAB isolates producing the same effect as the reference drug without the side effects it causes.

Effect of administration of LAB isolates on Hepatic CYP7A1 levels of diet-induced hyperlipidemic hamsters

As shown in **Figure 2**, the effects of the LAB isolates were almost equal to the atorvastatin's one as

there was no significant difference in the hepatic CYP7A1 levels among the three groups. Again this can be a big advantage in favour of the LAB isolates as an adjuvant therapy to decrease the atorvastatin dose and consequently its side effects, which will ultimately improve the patient compliance [29].

The results in **Figure 2** also indicate the *absence of a significant difference of hepatic CYP7A1 levels after the* administration of *Lactococcus lactis* ssp. *Lactis or Pediococcus* sp. where both of them could significantly increase the enzyme levels by almost 50% and *37.84%*, respectively *compared* to the HFD-fed control group. The increase of CYP7A1 levels could be due to an upregulation of its gene expression caused by our LAB isolates because it is reported that a possible mechanism through which LAB can affect cholesterol levels is via manipulation of expression of genes related to cholesterol metabolism [13] . Similar results were reported about the mechanism by which *Lactobacillus plantarum* KLDS 1.0344 exerts its hypolipidemic activity where an upregulation of the hepatic CYP7A1 was shown with more faecal bile acids released [30]. Another study reported the effect of LAB as an upregulation of CYP7A1 expression and a downregulation of the farnesine X receptor gene [31]. In addition, the cholesterol-lowering effect of *L. rhamnosus* GG was attributed to the suppression of FGF15 signalling resulting in subsequent upregulation of the hepatic CYP7A1 [32]. The homeostatic response of cholesterol may imply that CYP7A1 overexpression in human could be a useful approach to decrease serum cholesterol [16]. A possible explanation of the increased hepatic CYP7A1 levels in response to our LAB isolates administration in comparison to the HFD-fed control group, can be found in a study that was conducted on hamsters using *Lactobacillus plantarum* CAAS 18008. The mentioned study reported similar results of ours as a decreased hepatic cholesterol level together with the increased CYP7A1 levels. The proposed explanation presented by the authors was that *L. plantarum* CAAS 18008 caused a deconjugation of bile acids and subsequently increased their excretion levels. Then more bile acids were synthesized from cholesterol in the hepatic tissues through catalysis of CYP7A1 and led to a significant decrease of serum cholesterol levels [14]. Moreover, reports mentioned that *Lactococcus lactis subsp. cremoris* and *Lactococcus lactis* may be resistant to bile acid which indicates that they could express BSH. BSH deconjugates primary bile acids and consequently they get metabolized to secondary bile acids. *Lactococcus lactis subsp. cremoris* and *Lactococcus lactis* don't produce secondary bile acids but could be involved in increased faecal secondary bile acids [33]. Another study tested the mechanism through which *Lactococcus lactis* subsp. *lactis* removes cholesterol and reported that it could incorporate cholesterol into the cell membrane, bind it to its cell membrane, deconjugate bile by bile salt hydrolase (BSH), precipitate cholesterol with deconjugated bile salts, and assimilate cholesterol [34].

Further experiments to confirm the mechanism of antihyperlipidemic effect of the used LAB isolates by detecting the BSH activity of them, the level of excretion of deconjugated bile acids and the transcription level of CYP7A1 gene in hamsters are needed.

CONCLUSION

In this work we used two lactic acid bacteria (LAB) (*Lactococcus lactis* ssp. *lactis* and *Pediococcus* sp.) that were isolated from dairy products and showed a high *in vitro* cholesterol removal activity and a good *in vivo* antihyperlipidemic effect in a previous study carried out by our team. A 4-week animal experiment for studying the antihyperlipidemic effect of probiotics was conducted and the livers of the different groups of hamsters were excised to determine their levels of hepatic total cholesterol and CYP7A1. Both of the LAB isolates (*Lactococcus lactis* ssp. *lactis* and *Pediococcus* sp.) significantly ($p < 0.05$) decreased the hepatic total cholesterol levels and increased hepatic CYP7A1 levels in comparison to the diet-induced hyperlipidemic group with no significant difference between their effect and that of atorvastatin on both levels. From this study, two potential probiotics with the same effect of atorvastatin on both the hepatic total cholesterol and hepatic CYP7A1 levels can be utilized for further exploitation. Their introduction gives a big advantage to the LAB isolates as an adjuvant therapy to decrease the atorvastatin dose, consequently its side effects and ultimately improve the patient compliance. The mechanism by which the two LAB isolates *(Lactococcus lactis* ssp. *lactis* and *Pediococcus* sp.) produce their antihyperlipidemic activity could be the up regulation of the gene expression of CYP7A1which is a key enzyme in bile acids synthesis from cholesterol so it ultimately can reduce the hypercholesterolemia.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Contributions

All authors read and approved the final version of the manuscript.

List of abbreviations

Full name	Abbreviation
Cytochrome P450 Family 7 Subfamily A Member 1	CYP7A1
Niemann-Pick C1-Like 1	NPC1L1
deMan, Rogosa, Sharpe	MRS
Bile salt hydrolase	BSH
Lactic acid bacteria	LAB
One-way analysis of variance	ANOVA
Cardiovascular diseases	CVDs
Coronary heart disease	CHD
Non-alcoholic fatty liver disease	NAFLD
Reactive oxygen species	ROS
Phosphate buffer saline	PBS
Endoplasmic reticulum	ER
High-fat diet	HFD

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