




RESEARCH ARTICLE

Hypolipidemic activity of lactic acid bacteria: Adjunct therapy for potential probiotics

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Abstract

Background

Individuals with hyperlipidemia are two times more likely to develop atherosclerotic cardiovascular disease (ASCVD) as opposed to those with controlled serum total cholesterol (TC) levels. Considering the documented adverse events of the current lipid-lowering medications which ultimately affect patient's compliance, substantial efforts have been made to develop new therapeutic strategies. Probiotics, on the other hand, are reported to have lipid-lowering activity with the added benefit of being generally well-tolerated making it an appealing adjuvant therapy.

Methods

A total of fifty Lactic acid bacteria (LAB) were isolated from raw milk (human and animal) and dairy products. Isolates demonstrating promising *in vitro* cholesterol removal capabilities were morphologically and biochemically characterized. Lastly, two bacterial candidates were selected for evaluation of their potential hypolipidemic activity using a laboratory animal model. Statistical differences between the means were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A *p*-value < 0.05 was considered statistically significant.

Results

Most of the isolates demonstrated an *in vitro* cholesterol removal activity. The six LAB isolates showing the highest cholesterol removal activity (36.5–55.6%) were morphologically and biochemically identified as *Lactobacillus*, *Pediococcus*, and *Lactococcus* species. The results demonstrated two promising antihyperlipidemic candidates, a *Lactococcus lactis* ssp. *lactis* with an *in vivo* significant reduction of serum triglycerides (TG) levels by 34.3%, and a *Pediococcus* sp. that was able to significantly reduce both the serum TC and TG levels by 17.3% and 47.0%, respectively, as compared to the diet-induced hyperlipidemic animal group.

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Conclusion

This study further supports the growing evidence regarding the antihyperlipidemic activity among probiotics, presenting them as a promising therapeutic approach for the management of hyperlipidemia.

1. Introduction

According to the World Health Organization (WHO), cardiovascular diseases (CVDs) are the primary cause of death globally. In 2019, nearly 18.6 million people lost their lives to CVD worldwide (17.1% increase from 2010) with at least 75% of the world's CVDs-related deaths incidences occurring in low- and middle-income nations [1]. Such findings are highly alarming that non-communicable disease prevention, diagnosis, and treatment must be accelerated.

Hyperlipidemic individuals are nearly two times more likely to develop ASCVD in contrast to those with controlled serum total cholesterol levels [2]. Hyperlipidemia refers to a group of disorders characterized by elevated serum levels of cholesterol and triglycerides [3]. An increase in serum triglyceride concentrations is represented by an increase in the triglyceride-rich lipoproteins including chylomicrons, very low-density lipoprotein (VLDL-C), as well as their remnants. However, an increase in blood cholesterol levels is represented by an elevation in serum low-density lipoprotein (LDL-C) levels, with or without an increase in VLDL-C levels [4].

According to "The 3rd Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults"(ATP III), optimal LDL-C levels is <100 mg/dL, desirable total cholesterol level is <200 mg/dL, normal triglycerides level is <150 mg/dL and HDL-C level not below 40 mg/dL [5].

Cholesterol deposition in arterial walls ultimately leads to atherosclerosis in which narrowing, thickening, or blocking of the arterial lumen occurs and this is the hallmark of CVD [6]. TC reduction has been a vital aspect of public health strategies, and several effective pharmacological and non-pharmacological approaches have been implemented in this regard. As for the lipid-lowering medications, several pharmacokinetic interactions affect their activity and/ or toxicity. In addition, a lot of patients do not reach their lipid profile target levels using statins as monotherapy due to treatment resistance, abstinence due to drug-related adverse events, and poor compliance [7]. Consequently, many guidelines recommend an add-on therapy such as ezetimibe, fibrates, nicotinic acid, bile acid sequestrants, and PCSK9 drugs. However, these drugs have as well documented side effects and contraindications which puts further constraints regarding their use [8].

Mann and Spoerry have noticed the hypocholesterolemic activity of the fermented milk consumed by the people of the Massai tribes [9] and since then the connection between LAB and blood cholesterol levels has garnered substantial attention. Probiotics are living microorganisms that, when implemented appropriately, provide a variety of health benefits to the host like: enhanced digestion, increased immunity, cancer suppression, and decreased serum cholesterol level [10]. The majority of probiotics are commonly referred to as LAB because they generate lactic acid as a fermentation product. According to the morphological and phenotypic characteristics, LAB was categorized into several genera that include *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* sp. [11,12].

Lactobacillus bacteria are lactic acid-producing Gram-positive rods, obligate and/or facultative anaerobes that are mainly found in the human gastrointestinal and genitourinary tracts

[13,14]. *Bifidobacterium* is also Gram-positive rods but mostly straight anaerobes, pleomorphic, non-spore-forming bacteria and they produce yield lactic and acetic acids from carbohydrate fermentation [15,16]. Both *Lactobacilli* and *Bifidobacteria* have shown hypocholesterolemic effects in both animal models [17–19] and humans [20–23]. Additionally, several hypotheses were put to explain the mechanism by which probiotics produce their hypolipidemic activity [24–27]. Other studies, however, are paradoxical and fail to prove probiotics' hypolipidemic effects [28,29]. As a result, this subject remains a controversial issue and more research is needed [30].

Although basic and clinical studies on probiotics have evolved rapidly, probiotics still are not applied as drugs on a worldwide platform [31]. Yet, under the supervision of the US Food and Drug Administration (FDA), clinical physicians in America have proposed probiotics as evidence-based medical foods and dietary supplements [32]. Several probiotics are "Generally Recognized as Safe" (GRAS) for use in foods. Furthermore, the European Food Safety Authority (EFSA) ascertained that a number of traditional probiotic strains were safe for use in food through using "Qualified Presumption of Safety" (QPS) [33]. Interestingly, from October 2019 till March 2020, EFSA discovered that only six microorganisms out of 39 reports fit the criteria [34]. However, these strains are insufficient to meet the diverse needs of industry and humans [35]. Consequently, additional strains of bacteria must be discovered in order to broaden the application panel of probiotics and their implementations in the food industry [32]. Therefore, this study aimed to screen for a probiotic species with potential anti-hyperlipidemic activity from raw milk (human and animal) and dairy products.

2. Methods

2.1. Samples collection

2.1.1. Breast milk. A total of 25 Samples of breast milk were obtained from Egyptian lactating females attending the outpatient pediatric and/or obstetrics and gynecology clinics at different local hospitals. Subjects were chosen and milk samples were collected according to the criteria and procedures described by [36] with a slight modification of swabbing the areola with an alcohol swab before sample collection. The study was approved by the Ethics Committee of the Faculty of Pharmacy, Helwan University, and prior to their enrollment in the study, the mothers were informed of the investigational character of the study. The participant's consent was obtained verbally. No minors were included in the study.

2.1.2. Raw animal milk and dairy products. A total of twenty-five samples were obtained from dairy farms in Cairo, Egypt, and traditional commercial Egyptian dairy products (milk, cheese, yogurt, and cream).

All samples, the breast milk, raw animal milk, and dairy products, were transferred in sterile plastic bottles to the laboratory and immediately used for bacterial isolation.

2.2. Recovery of LAB

The pour plate technique was used to isolate the microorganisms [36]. For milk samples, 1 mL milk was inoculated into 9 mL of sterile saline solution (S.S.S) whereas, for the solid dairy products, 10 grams were aseptically homogenized in 90 mL of S.S.S. Samples were serially diluted (10 fold) then 1 mL aliquot of each dilution was plated into 9 mL of molten De Man, Rogosa, and Sharpe (MRS) medium agar (Sigma-Aldrich). Inoculated MRS plates were incubated anaerobically at 37°C for 48–72 hrs. in an anaerobic jar (GasPak System—Oxoid, Basingstoke Hampshire, England) using AnaeroPack™-Anaero anaerobic gas generator sachets (Mitsubishi, Japan). Typical colonies were selected according to the morphological characters described by [37], picked from the plates, and repeatedly sub-cultured to obtain pure isolates.

2.3. Maintenance and preservation of isolates

The different isolates were preserved in an MRS broth medium containing glycerol (20%) and stored at -80°C until further testing.

2.4. Testing the cholesterol removal activity of LAB isolates

Before each assay, the isolates were activated three times by successive sub-culturing in an MRS broth. The potential of the working isolates to remove cholesterol from the medium was assessed according to [38] with minor modification. Briefly, to prepare the cholesterol stock solution, 10 mg of cholesterol were dissolved in 1 mL of 96% ethyl alcohol [39]. Cholesterol was filter sterilized (0.20 µm, Genetix Biotech Asia, New Delhi, India) and added to the sterile MRS broth at a final concentration of approximately 100 mg/L. The freshly cultured bacterial isolates were inoculated into the sterile MRS broth at 1% (v/v) inoculum size and incubated anaerobically at 37°C for 18 hr. The un-inoculated broth was used as a control. Lastly, cultures were centrifuged at 10,000 g for 15 min at 4°C, and the resultant supernatants were taken to determine the residual cholesterol concentrations by capillary gas chromatography [40]. The experiment was repeated two more times for the isolates showing the highest cholesterol removal activity and they were selected for the following investigations.

2.5. Characterization and identification of the selected isolates

2.5.1 Morphological characterization. Microscopic examination following the Gram staining technique was used in accordance with [41] to identify the cell shape and arrangement of the isolates that demonstrated the highest cholesterol removal activity.

2.5.2. Biochemical characterization. The catalase test was carried out following the procedures described in [42]. API 50 CH test kit (BioMerieux, Marcy L'Etoile, France) in combination with 50 CHL liquid media were used to determine the carbohydrate fermentation patterns of the selected isolates [43].

2.6. Animal feeding trial: Testing the effect of two selected LAB isolates on serum lipid levels in a laboratory animal model

2.6.1. Inoculum preparation. Two LAB isolates out of the two selected genera (*Pediococcus* and *Lactococcus*) that showed the highest and most reproducible effect on cholesterol removal *in vitro* were used for the animal feeding trial. A standard calibration curve between colony-forming units (CFU) and their corresponding optical densities (O.D) for each test isolate was constructed [44]. Briefly, an overnight culture of each test isolate was prepared using a single colony from an 18hrs fresh agar culture, followed by a sequential decimal dilution in S.S.S. The O.D and the viable count of each resultant dilution were recorded. The viable count was carried out by plating 100 µL of the appropriate dilution in MRS-agar plates. Plates were incubated for 18hrs at 37°C under anaerobic conditions. The experiment was carried out 3 times, and the data obtained were averaged and a curve of O.D against CFU/mL was plotted (S7 Table (Supporting Information)). Lastly, the O.D of the two test isolates was adjusted to the corresponding required viable count of 1×10^9 CFU/mL for the animal feeding experiment [38] using the resultant equation $y = mx + c$, where "m" is the gradient and "c" is the y-intercept.

2.6.2. Preparation of atorvastatin. For humans, the initial treatment dosage of atorvastatin (Lipitor, Pfizer) is ten mg daily. The hamster's dose was determined by utilizing the human equivalent dose (HED) and body surface area (BSA). In brief, human daily dose per

kilogram multiplied by the variation coefficient of the BSA between humans and hamsters [45,46]:

$10 \text{ mg (human daily dose) / 60 kg (average human body weight) X 7.4 (variation coefficient in BSA) = 1.23 mg/kg.}$

2.6.3. Animal and study design. All animal studies were carried out following the Guide for the Care and Use of Laboratory Animals [47]. The procedures involving animals were approved by The Animal Ethics Committee of Helwan University. The study was designed following the procedures described in [38], with some modifications. Briefly, six-week-old male Golden Syrian hamsters were obtained from Theodor-Bilharz Research Institute (TBRI), Egypt. Hamsters were accommodated in a room with a humidity of $60 \pm 5\%$, a temperature of $22 \pm 2^\circ\text{C}$, a 12-hour light-dark cycle, and a standard food diet (Std). For the induction of hyperlipidemia, the animals were fed a high-fat diet (HFD) which was designed in accordance with the Paigen atherogenic diet [48].

After 1 week of acclimation, animals were randomly divided into five groups ($n = 6$). The first group (non-induced) was fed a standard food diet while the second group (diet-induced hyperlipidemia) was fed the HFD, these two groups received saline by oral gavage daily. The third group received the HFD + 1×10^9 CFU/mL/day of *Lactococcus lactis* ssp. *lactis*. The fourth group received the HFD + 1×10^9 CFU/mL/day of *Pediococcus* sp. The fifth group received the HFD + 1.23 mg/kg/day atorvastatin used as a positive control. During the feeding period, all animals had free access to water and their assigned food regime. The animals were kept on the previously stated regime for 28 days.

2.7. Measurement of serum lipid levels in tested laboratory animals

At the end of the twenty-eight days of the feeding trial, all tested hamsters were kept fasting for 12 hours then sacrificed. The whole blood was collected from the retro-orbital plexus then the serum was obtained by centrifugation at 3000 g for 15 minutes. The enzymatic colorimetric method and an AU680 automated biochemical analyzer (Beckman Coulter, Fullerton, CA, USA) were used for the determination of serum TC, LDL-C, HDL-C, and TG levels.

2.8. Statistical analysis

Data were expressed as mean \pm SEM and the analysis was performed using SPSS software (version 22.0, IBM Corporation, Armonk, NY, USA). Statistical differences between the means were analyzed by one-way analysis of variance followed by Tukey's post-hoc test. A p -value < 0.05 was considered statistically significant.

3. Results

3.1. Removal of cholesterol by the recovered LAB isolates

A total of 50 LAB were recovered from raw (human and animal) milk and other relevant dairy products in this study and analyzed *in vitro* for their cholesterol removal ability. Forty-nine out of 50 tested bacterial isolates were able to remove cholesterol from the fermentation broth during the 18 hours incubation period. The results are shown in Fig 1 and S1 Table (Supporting Information).

The cholesterol removal percentages varied among the LAB isolates, ranging from 2.2% to 71.7%. Isolate no.29 had no cholesterol removal ability (0.05%). The isolates that exhibited the highest cholesterol removal ability were of the following numbers: 1, 16, 17, 33, 34, and 35 with cholesterol removal percentages values of 68.6%, 59.6%, 51.2%, 71.7%, 46.4%, and 63.5%, respectively. These isolates were checked for their reproducible effect by repeating the same

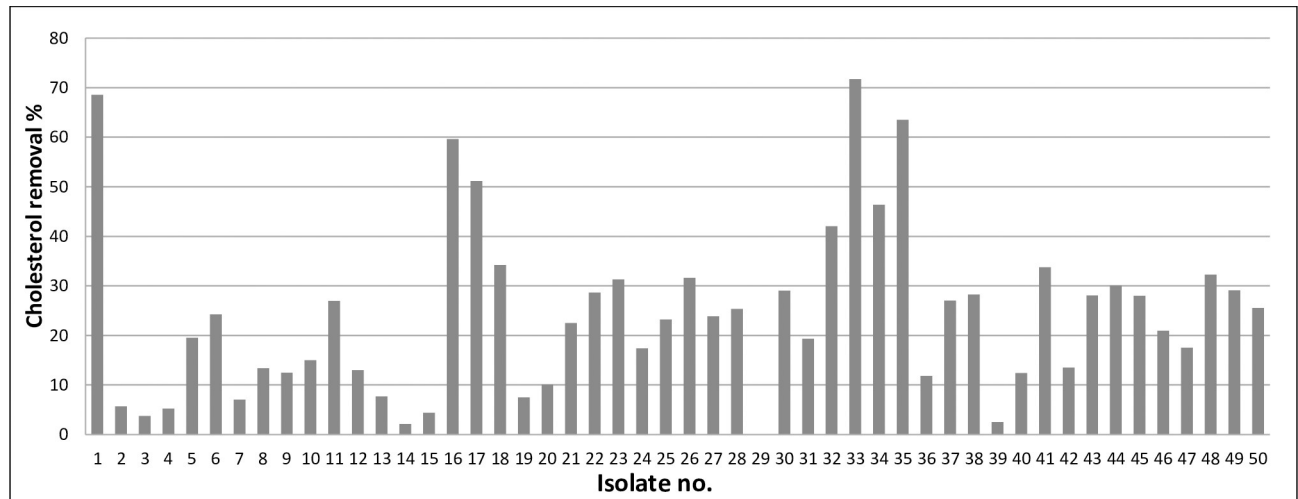


Fig 1. Preliminary screening of LAB isolates for their cholesterol removal capability.

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experiment two more times and the results are shown in Fig 2 and S2 Table (Supporting Information).

The order of cholesterol removal capabilities of the six tested isolates were as follow: isolate no. 1 ($55.6\% \pm \text{SEM} = 10.166$) > isolate no.16 ($47.1\% \pm \text{SEM} = 7.205$) > isolate no. 35 ($40.5\% \pm \text{SEM} = 13.2$) > isolate no.33 ($40.0\% \pm \text{SEM} = 15.88$) > isolate no. 34 ($39.0\% \pm \text{SEM} = 6.6$) > isolate no. 17 ($36.5\% \pm \text{SEM} = 7.4$).

3.2. Characterization and identification of the selected isolates

The six LAB isolates that produced the highest cholesterol removal activity were identified and the results were as the following:

3.2.1. Morphological features. Microscopical examination after Gram stain revealed that all isolates were Gram-positive, four out of six isolates (isolate no.1, 16, 17, and 35) had a cocci-shape while the remaining two isolates (Isolate no. 33 and 34) had a bacilli-shape.

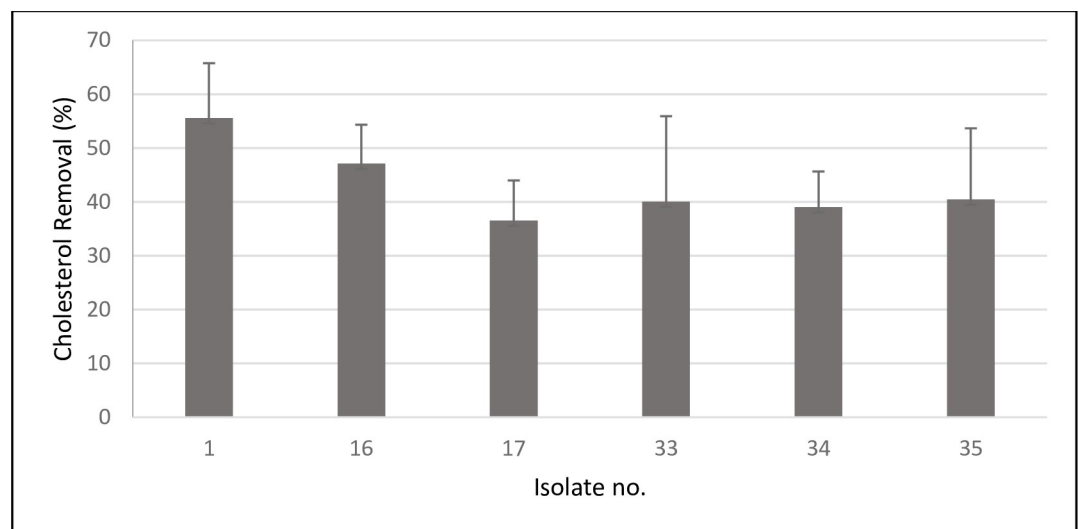


Fig 2. Comparison of cholesterol removal capabilities of six selected LAB isolates showing high activity in preliminary screening. Data are represented as the mean \pm SEM (n = 3).

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Table 1. Identification of the six LAB isolates based on the interpretation of the API 50 CH strips.

Isolate No.	Genus	Species
17	<i>Pediococcus</i>	N/A
35	<i>Pediococcus</i>	N/A
33	<i>Lactobacillus</i>	<i>acidophilus</i>
34	<i>Lactobacillus</i>	<i>acidophilus</i>
1	<i>Lactococcus</i>	<i>Lactis ssp. lactis</i>
16	<i>Lactococcus</i>	<i>lactis ssp. lactis</i>

N/A, data obtained were enough only for the identification of the genus level.

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3.2.2. Biochemical characterization. All isolates were catalase-negative. Carbohydrate assimilation and fermentation of 49 substances with one control were identified on API 50 CH strips and the results are shown in S 8 (Supporting Information). Following the manufacturer protocol, referring to the analytical profile index and by using the APIWEB'S microbial identification systems software, the isolates were identified as presented in Table 1.

The six LAB isolates belonged to three genera *Lactobacillus*, *Lactococcus*, and *Pediococcus*. Both isolates no. 17 and 35 were identified only to the genus level as *Pediococcus*. Isolates no. 33 and 34 were identified as *Lactobacillus acidophilus* (*L. acidophilus*) while isolates no. 1 and 16 belonged to the *Lactococcus* genus and were identified as *lactis ssp. lactis*.

3.3. Animal feeding trial: Effect of two selected LAB species on serum lipid levels of diet-induced hyperlipidemic hamsters

After a 28 days animal feeding trial, serum TC, TG, LDL-C, and HDL-C levels were determined in the different tested animal groups.

3.3.1 Effect of administration of two selected LAB isolates on serum TC levels. As shown in Fig 3 and S3 Table (Supporting Information), serum TC levels in the diet-induced hyperlipidemic group (166.5mg/dL) were significantly ($p < 0.05$) higher compared to the non-induced group (64 mg/dL) by 61.6%. TC levels was significantly ($p < 0.05$) lower by 39.2% and 17.3% in the atorvastatin (101.2mg/dL) and the *pediococcus* sp. (137. mg/dL) supplemented groups, respectively, in comparison to the diet-induced hyperlipidemic group (166.5mg/dL). Whereas, the TC levels of the *Lactococcus lactis ssp. lactis* supplemented group was 145.2mg/dL with no significant difference ($p > 0.05$) as compared to the diet-induced hyperlipidemic group.

3.3.2. Effect of administration of two selected LAB isolates on serum TG levels. The results in Fig 4 and S4 Table (Supporting Information) demonstrate that serum TG levels were significantly ($p < 0.05$) elevated by 58.1% in the diet-induced hyperlipidemic group (315.2 mg/dL) compared to the non-induced group (132 mg/dL). TG levels were significantly ($p < 0.05$) lower in all the supplemented groups, the decrease was 76.0%, 34.3% and 47.0% in atorvastatin (75.5mg/dL), *Lactococcus lactis ssp. lactis* (207.2 mg/dL), and *Pediococcus* sp. (167mg/dL) supplemented group, respectively, in contrast to the diet-induced hyperlipidemic group.

3.3.3. Effect of administration of two selected LAB isolates on serum HDL-C levels. As represented in Fig 5 and S5 Table (Supporting Information), serum HDL-C levels in the diet-induced hyperlipidemic group (42 mg/dL) were significantly ($p < 0.05$) higher by 38.1% compared to the non-induced group (26 mg/dL). Serum HDL-C levels were higher by 8.3%, 8.7%, and 9.7% in the *Lactococcus lactis ssp. lactis* (45.8 mg/dL), atorvastatin (46mg/dL), and *Pediococcus* sp. (46.5 mg/dl) supplemented groups, respectively, compared to the diet-induced

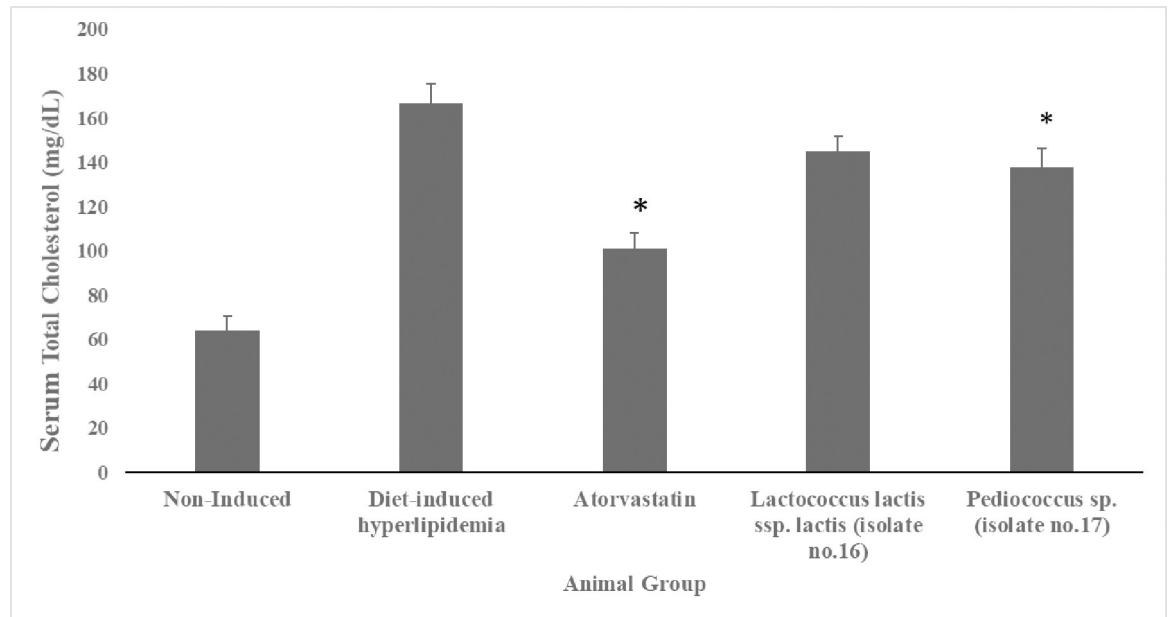


Fig 3. Serum total cholesterol levels (mg/dL) of hyperlipidemic hamsters. Non-induced group (standard food diet), diet-induced hyperlipidemic group (HFD-fed), *Lactococcus lactis* ssp. *lactis* group (HFD-fed + 1×10^9 CFU/mL /day), *Pediococcus* sp. group (HFD-fed + 1×10^9 CFU/mL /day) and atorvastatin group (HFD-fed + 1.23 mg/kg/day atorvastatin). Data are represented as the mean \pm SEM (n = 6). Asterisks indicate a significant difference ($p < 0.05$).

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hyperlipidemic group. However, this minimal rise was statistically non-significant ($p > 0.05$) in all the three supplemented groups.

3.3.4. Effect of administration of two selected LAB isolates on serum LDL-C levels.

Additionally, the levels of LDL-C were measured among the animal groups (data are illustrated in Fig 6 and S6 Table (Supporting Information)). Serum LDL-C levels in the diet-induced hyperlipidemic group (67.3 mg/dL) were significantly ($p < 0.05$) higher by 82.9% in contrast to the non-induced group (11.5 mg/dL). LDL-C levels were significantly ($p < 0.05$) lower by

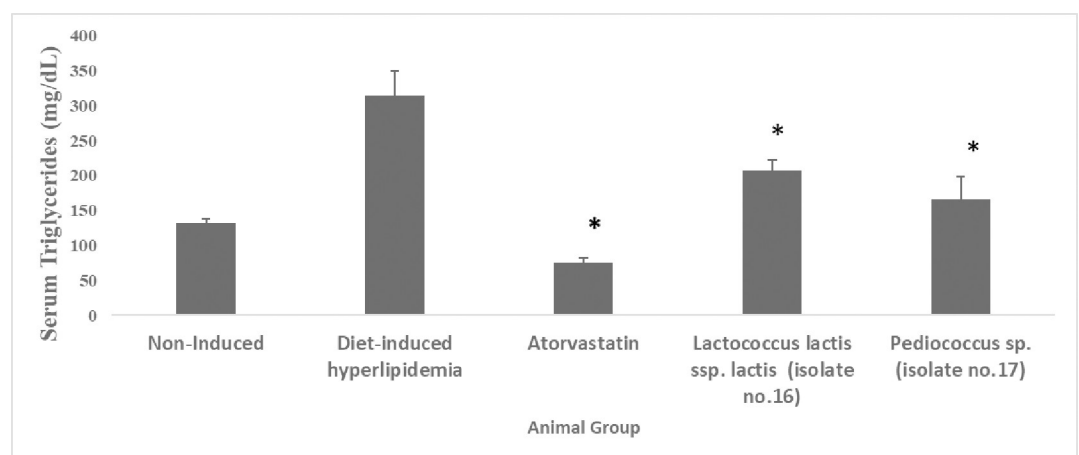


Fig 4. Serum triglycerides levels (mg/dL) of hyperlipidemic hamsters. Non-induced group (standard food diet), diet-induced hyperlipidemic group (HFD-fed), *Lactococcus lactis* ssp. *lactis* group (HFD-fed + 1×10^9 CFU/mL /day), *Pediococcus* sp. group (HFD-fed + 1×10^9 CFU/mL /day) and atorvastatin group (HFD-fed + 1.23 mg/kg/day atorvastatin). Data are represented as the mean \pm SEM (n = 6). Asterisks indicate a significant difference ($p < 0.05$).

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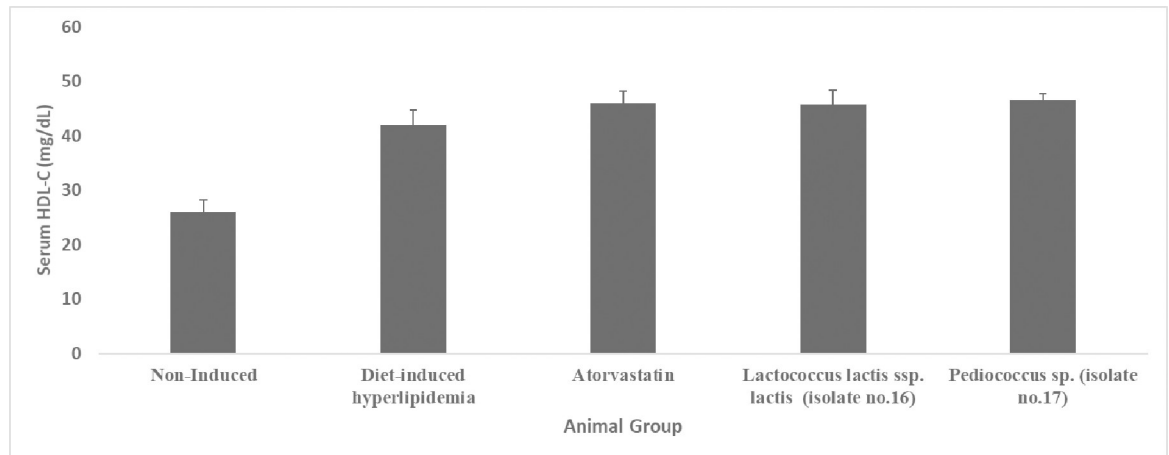


Fig 5. Serum HDL-C level (mg/dL) of hyperlipidemic hamsters. Non-induced group (standard food diet), diet-induced hyperlipidemic group (HFD-fed), *Lactococcus lactis* ssp. *lactis* group (HFD-fed + 1×10^9 CFU/mL/day), *Pediococcus* sp. group (HFD-fed + 1×10^9 CFU/mL/day) and atorvastatin group (HFD-fed + 1.23 mg/kg/day atorvastatin). Data are represented as the mean \pm SEM (n = 6). Asterisks indicate a significant difference ($p < 0.05$).

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40.3% in the atorvastatin-supplemented group (40.2 mg/dL) compared to the diet-induced hyperlipidemic group. However, LDL-C levels were none significantly decreased ($p > 0.05$) in both *Lactococcus lactis* ssp. *lactis* (58mg/dL) and *Pediococcus* sp. supplemented groups (58.2mg/dl) when compared to the diet-induced hyperlipidemic group.

4. Discussion

As hyperlipidemia causes an array of disorders [49], substantial effort has been expended in developing various medications against it. However, those lipid-lowering medications agents

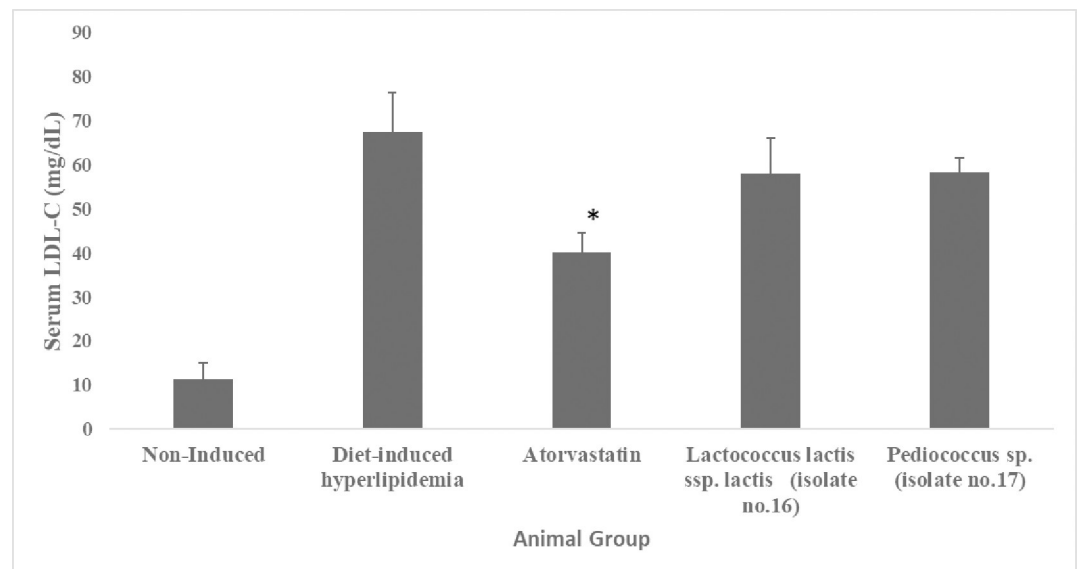


Fig 6. Serum LDL-C levels (mg/dL) of hyperlipidemic hamsters Non-induced group (standard food diet), diet-induced hyperlipidemic group (HFD-fed), *Lactococcus lactis* ssp. *lactis* group (HFD-fed + 1×10^9 CFU/mL/day), *Pediococcus* sp. group (HFD-fed + 1×10^9 CFU/mL/day) and atorvastatin group (HFD-fed + 1.23 mg/kg/day atorvastatin). Data are represented as the mean \pm SEM (n = 6). Asterisks indicate a significant difference ($p < 0.05$).

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have been reported to cause various adverse effects [7], which is why probiotics with their natural and safe characteristics offer a promising approach.

Exploring new *Lactococcal* probiotic strains would be favorable since they can grow well in milk, apart from *L. acidophilus* strains during the production of fermented milk products [50]. According to [51], developing or selecting a new probiotic necessitates a previous assessment of its safety, and *Lactococcus* sp. have a considerable advantage in this regard because they are frequently given the GRAS status, as are *Lactobacillus* species [50].

Lactobacillus and *Bifidobacterium* species are two examples of probiotics that have been well studied for their hypolipidemic effects in both human and animal studies [52]. Still, there is a gap between the number of discovered strains and the different needs of humans and industry [35]. That is why we ought to discover more bacterial strains and that is the target of our research, to discover other novel bacteria with potential anti-hypercholesterolemic activity.

4.1. Removal of cholesterol by the recovered LAB isolates

In this study, we isolated 50 LAB isolates and screened them for their *in vitro* cholesterol removal ability. Then we selected the six isolates that produced the highest cholesterol removal percentages to be identified and used in the rest of the study.

4.2. Characterization and identification of the selected isolates

Isolation of LAB is used to detect and ascertain the genus of the bacterial isolates. Isolation was carried out on MRS agar because it is a selective medium and considered as the most common medium used to grow LAB, including *Pediococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc* bacterial genera according to [53]. Macroscopic observation and microscopic examination were carried out after culturing on an MRS agar plate. Macroscopic observations of the isolated colonies revealed a circular shape with an entire margin, convex elevation, and milky white color. Such characters of LAB that we isolated are similar to what's described by [54–56]. As for the microscopic examination and catalase test, all the six isolates were catalase-negative, Gram-positive with isolates no. 1, 16, 17, and 35 a cocci-shape while isolates no. 33 and 34 having a bacilli-shape, such findings come in agreement with [57].

The carbohydrate fermentation pattern of the isolates was tested using the API 50 CH and analyzed by the APIWEB. Based on systematic databases, the API 50 CH is a cost-effective, easily operated, and well-recognized technique for the identification of microorganisms. The platform features a huge and reliable database that is obtainable through the internet-based APIWEB [58].

After identifying the isolates, we could state that according to the results in Fig 2, *pediococcus* sp. (isolates no. 17 and 35) had good cholesterol removal abilities of $36.5\% \pm 7.442$ and $40.5\% \pm 13.208$, respectively. Similar cholesterol removal ability was shown by the *L. acidophilus* species (isolates no.33 and 34) with the values of $40.0\% \pm 15.88$ and $39.0\% \pm 6.604$ respectively. Lastly, *Lactococcus lactis* ssp. *lactis* (isolates no.1 and 16) showed the highest cholesterol removal rates of $(55.6\% \pm 10.166$ and $47.1\% \pm 7.205)$, respectively.

4.3 Animal feeding trial

Lactococcus/Pediococcus are conventionally not thought to be natural inhabitants of the human gastrointestinal tract which is why limited studies exist on their probiotic activity, while *Lactobacillus* genera are extensively studied and considered as the most widely recognized probiotic [32,52,59]. That prompted us to use those less studied genera for the *in vivo* cholesterol removal activity study in order to produce better insights into them. Out of each of

these two selected genera, the species that had the most reproducible result in the *in vitro* cholesterol removal screening (Fig 2) were selected for the animal feeding trial.

4.3.1 Effect of two selected LAB isolates on serum lipid levels of hyperlipidemic hamsters. For the animal study, we have gone through two very important questions: the first one was which animal is better to be used? We found out that the golden Syrian hamsters have been increasingly used in the lipoprotein metabolism research, and the investigation of the hypolipidemic agents' effect, like PPAR agonists, statins, and CETP inhibitors [60]. The reason for that is that these hamsters resemble humans in terms of lipid utilization and high susceptibility to dietary cholesterol [61]. Upon isolation and characterization of the hamster LDL receptor gene [62], it showed a high similarity to that of humans. The synthesis of bile acids and hepatic cholesterol is also similar in both hamsters and humans, which makes them prone to hypercholesterolemia induced by an increased dietary cholesterol diet, on the other hand, rats are resistant to this [63]. For all the above-mentioned reasons, Syrian hamsters have already been used to observe many pharmacological agents that have different modes of action [64]. Moreover, hamsters are considered to be a better model than rats in the study of the potential probiotics cholesterol-lowering effect because hamsters show are similar to humans in bile acid composition and synthesis [65].

After we settled on the choice of the golden Syrian hamster as the animal model to be used in our study, we needed to answer the second question; when to supplement the LAB isolates or the standard drug? To get the answer we have gone through the literature and we found that the lipid-lowering effect could be measured using different study designs, one of them induces hyperlipidemia first then after a while introduces the LAB isolates [66,67]. The other approach induces hyperlipidemia simultaneously with the supplementation of LAB isolates. This latter approach was used in many researches like the screening for cholesterol-lowering probiotics from LAB isolated from corn silage [38], the investigation of the hypocholesterolemic activity of *Lactobacillus casei*-fermented milk in hamsters [68], the comparison between hamsters and rats as models for evaluation of the hypocholesterolemic efficacy of potential probiotics [65], the detection of the lipid-lowering effects of *Pediococcus acidilactici* in high fat diet-induced mice [69], investigation of the hypocholesterolemic effect of two *Lactococcus lactis* subspecies [70] and the detection of the effect of *Pediococcus pentosaceus* PP04 on high-fat diet-induced hyperlipidemia [71]. We chose to use that design in our work.

4.3.1.1 Effect on serum TC levels. As shown in Fig 3, *Pediococcus* sp. successfully reduced serum TC levels ($p < 0.05$) by 17.3% (137.7 mg/dL) 4 weeks post-administration in comparison to the diet-induced hyperlipidemic group. Our results come in agreement with [66,69]. Several hypotheses were put to explain the mechanism by which probiotics could produce this hypocholesterolemic effect: by the intestinal bacteria which consume cholesterol decreasing its available amount for absorption [24]. Cholesterol can bind to the bacterial cell surface [25], be implemented into bacterial membranes [26], or be transformed into coprostanol by the cholesterol reductase enzyme that is produced by *Lactobacilli* strains [27]. Another proposed mechanism is through suppression of micelle formation by a specific probiotic strain [72]. Also, short-chain fatty acids produced by gut microflora during selective fermentation of food may reduce blood cholesterol levels [73]. Lastly, some bacterial species secrete bile salt hydrolase, resulting in enhanced bile excretion in the stool [74].

As for *Lactococcus lactis* ssp. *Lactis* supplemented group, resulted in a modest decrease in the serum TC levels by 12.8%, however, this reduction was considered to be non-statistically significant ($p = 0.05$), although it demonstrated a good cholesterol removal ability *in vitro*.

The lack of the *in vivo* effect despite having an *in vitro* one could be due to the fact that inside the body in the duodenum and upper jejunum where cholesterol absorption mainly occurs [75] have high bile salt concentrations [76] that dramatically inhibit the growth of

bacteria and consequently inhibit their *in vivo* effect [38]. A study reported a contradicted result where supplementation of *Lactococcus lactis* ssp. *lactis* 527 (LACC-527) strain significantly decreased serum cholesterol levels in the Sprague-Dawley rats after 6 weeks post-administration [70]. This disagreement with our finding could be due to the difference in the used bacterial strain, for instance, the tolerance to bile salts has been shown to vary among *Lactococcus* strains [50], also could be due to differences in the animal model or the period of the experiment [70].

4.3.1.2. Effect on serum TG levels. The results in Fig 4 showed that both *Pediococcus* sp. and *Lactococcus lactis* ssp. *lactis* supplementation successfully reduced the serum TG levels ($p < 0.05$) by 47.0% (167 mg/dL) and 34.3% (207.2 mg/dL), respectively, 4 weeks post-administration as compared to the diet-induced hyperlipidemic group. In addition, these findings agree with [71] which reported a significant reduction in the serum TG levels following 8 weeks feeding period of *Pediococcus pentosaceus* in diet-induced hyperlipidemic mice. However, our results disagree with [69] which reported no significant difference in the serum TG levels between groups after supplementation of *Pediococcus acidilactici* M76 in high-fat diet-induced obese mice. Also, as for *Lactococcus lactis* ssp. *lactis* effect on TG level, similar results were reported by [70].

Figs 3 and 4 show that atorvastatin, a standard drug used in the market for the treatment of hypercholesterolemia had a higher reduction effect on the serum TC and TG levels than the two tested LAB isolates. The *Pediococcus* sp. supplemented group resulted in a 17.3% decrease in the TC level of the HFD-fed hamsters, in comparison to atorvastatin with a 39.2% decrease. On the other hand, the effect on serum TG levels was more pronounced; the *Pediococcus* sp. supplemented group demonstrated a 47.0% decrease while atorvastatin resulted in a 76.0% decrease. Still, given the reported side effects by atorvastatin [7], such differences in the hypolipidemic effect could be in favor of the use of probiotics as an adjuvant therapy to decrease the atorvastatin dose and consequently its side effects, which will ultimately improve the patient compliance.

4.3.1.3. Effect on serum HDL and LDL cholesterol levels. Neither *Pediococcus* sp. (isolate no.17) nor *Lactococcus lactis* ssp. *lactis* (isolate no.16) supplementation was able to significantly ($P > 0.05$) affect the serum HDL-C or LDL-C levels (Figs 5 and 6). Our results come in agreement with [70] who reported no significant difference in HDL-cholesterol levels between the control and *Lactococcus lactis* ssp. *lactis* 527 (LACC-527) supplemented groups. In addition, [71] reported that the supplementation of *Pediococcus* strain PP04 for 8 weeks had no effect on the serum HDL-C levels in mice.

Lactococcus lactis ssp. *lactis* 527 demonstrated similar results to our findings of no significant effect on serum LDL-C levels [70]. However, contradictory results were reported by [66] that demonstrated a significant reduction in the serum LDL-C levels caused by *Pediococcus pentosaceus* strain, KID7. Furthermore, another study reported an LDL-C reduction effect caused by *Pediococcus pentococcus* PP04 [71].

5. Conclusion

In this study, a total of 50 LAB were isolated from raw milk (human and animal) and dairy products. Preliminary screening was carried out to assess the cholesterol removal capabilities of the recovered LAB isolates *in vitro*. The six LAB isolates showing the highest cholesterol removal capabilities were morphologically and biochemically characterized. Finally, two bacterial candidates (*Pediococcus* and *Lactococcus*) were selected for evaluation of their potential hypolipidemic activity using a laboratory animal model. Both *Pediococcus* sp. and *Lactococcus lactis* ssp. *lactis* significantly ($p < 0.05$) reduced serum TG levels by 47.0% and 34.3%,

respectively, four weeks post-administration. Conversely, only *Pediococcus* sp. was able to significantly ($p < 0.05$) reduce serum TC levels by 17.3%. However, neither *Pediococcus* sp. nor *Lactococcus lactis* ssp. *lactis* was able to significantly affect serum HDL-C or LDL-C levels. Further investigations are needed to understand the mechanism by which these bacterial isolates exert their hypolipidemic activity. In addition, extensive research is urgently recommended for the production of probiotic-based therapeutic agents against hyperlipidemia.

Supporting information

S1 Table. Preliminary screening dataset.

(XLSX)

S2 Table. GC replicates dataset.

(XLSX)

S3 Table. Serum cholesterol dataset.

(XLSX)

S4 Table. Serum triglycerides dataset.

(XLSX)

S5 Table. Serum HDL-C dataset.

(XLSX)

S6 Table. Serum LDL-C dataset.

(XLSX)

S7 Table. Viable count dataset.

(XLSX)

S1 File.

(PDF)

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