

## Cholesterol Lowering Capability of some *Lactobacillus* Strains and its Effect on Mice Fed a High Cholesterol Diet

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### Abstract

Elevated blood cholesterol is an important risk factor associated with Atherosclerosis and coronary heart disease. Several studies have reported a decrease in serum cholesterol during the consumption of large doses of fermented dairy products or *Lactobacillus* strains. Based on literature, *in vitro* cholesterol removal of lactic acid bacteria (LAB) has been accounted for their *in vivo* cholesterol reduction. But recently it has been proposed that such *in vitro* characteristic may not be directly relevant to their *in vivo* activity. The objective of this study was to find how much *in vitro* cholesterol reducing capability from bacterial culture media contain *Lactobacillus reutri* (ATCC 23272) and *Lactobacillus casei* (ATCC 393), As well as under vivo conditions. Bacteria used are a native strains isolated from a human and cheese origin respectively reflects its *in vivo* efficiency. Here, we investigate whether the given strains are capable of *in vitro* cholesterol assimilation or consumption using a colorimetric method. The proposed mechanism for this effect is the removal or assimilation of intestinal cholesterol by the bacteria, reducing cholesterol absorption. Although this effect was demonstrated *in vitro*, its relevance *in vivo* is still controversial. Furthermore, few studies have investigated the role of lactobacilli in atherogenesis *Lactobacillus reutri* (LA7) previously showed serum cholesterol reducing capability in mice subjected to fatty diet. Our in-vivo study was held as twenty male Swiss albino mice aged 4-6 weeks and weighing 25-30 g were orally ingested by *L. casei* and *L. reutri*. They were kept under a 12 hr light/dark cycle at 22-26°C and a relative humidity of 50%, in Cages (15 × 25 cm), three mice used as a control group. Samples are taken in eppendorf tubes and tested for total serum cholesterol concentration. However, more clinical evidence is needed to strengthen these proposals.

**Keywords** Assimilation; Cholesterol; *Lactobacillus casei*; *Lactobacillus reutri*

### Introduction

Cardiovascular disease is considered the principal global cause of morbidity and mortality by the World Health Organization, and therefore became a major scientific focus in both academic and industrial sectors. Atherosclerosis, an inflammatory disorder, is recognized as the essential cause of cardiovascular disease that accounts for approximately one-third of all deaths worldwide. Major advances in the understanding of atherosclerosis were made over the last decade and these discoveries provide insightful approaches that may help in the prevention of cardiovascular problems. Among the most important risk factors for atherosclerosis are high cholesterol, high blood pressure, and cigarette smoking [1].

People affected with hypercholesterolemia may avert the use of cholesterol-lowering drugs by practicing dietary control or supplementation of probiotics and/or prebiotics. Probiotics are defined as 'living microbial supplements that beneficially affect the host animals by improving its intestinal microbial balances.' Prebiotics are 'indigestible fermented food substrates that selectively stimulate the growth, composition and activity of micro flora in gastrointestinal tract and thus improve hosts' health and well-being. When probiotics and prebiotics are used in combination, they are known as 'symbiotic.' The use of probiotics and prebiotics has only acquired scientific recognition in recent years although their applications as functional foods have

been well-established throughout generations. In the interest of their promising effects on health and wellbeing, probiotics and prebiotics have become increasingly recognized as supplements for human consumption. In addition to improving gut health, probiotics have also been documented to exert other health promoting effects such as strengthening of the immune system, antihypertensive effects, prevention of cancer, antioxidative effects, reduction of dermatitis symptoms, facilitation of mineral absorption, amelioration of arthritis, and reduction of allergic symptoms and improvement of vulvovaginal candidiasis in women. Probiotics have also been studied for their Cholesterol-lowering effects [2].

### Aim of Work

The objectives of this study are to determine whether *L. casei*, *L. reutri* would assimilate cholesterol and to confirm whether consumption of selected strains would significantly prevent an increase of serum cholesterol in mice fed a high-cholesterol diet.

### Materials and Methods

#### Source and maintenance of cultures

Cultures of *Lactobacillus casei* (of cheese origin) and *Lactobacillus reutri* (of human origin) were obtained from Microbiological Resources Centre, Faculty of agriculture, Ain Shams University, (Cairo Mircn).

Cultures were maintained by sub culturing weekly with 1% inoculant in 10 ml portions of sterile de man, Rogosa, Sharpe lactobacilli MRS broth (HI-Media Laboratories, Mumbai, India) and incubating for 18 h at 37°C. Cultures were sub cultured twice in a like manner immediately before using. They were stored at 4°C between sub-cultures. Stock cultures were stored in 25% glycerol at -20°C until use.

Each subculture was used for not more than five passages from the original culture to ensure the genetic stability of the micro-organisms.

### Measurement of cholesterol uptake

Preliminary tests indicated that *Lactobacillus acidophilus* would during anaerobic growth, remove cholesterol from laboratory media.

Three different concentrations of standard cholesterol powder (50 mg/l, 100 mg/l, 150 mg/l) were prepared separately, added in a freshly prepared MRS broth, 10 ml taken from each concentration in 10 sterile test tubes, respectively, autoclaved for (20 min, 12°C), then *L. casei*, *L. reutri* was inoculated (1%), each strain its optical density adjusted at 0.5 spectrophotometric ally at 600 nm, in each test tube. The tube(s) was incubated anaerobically for 26 hr at 37°C in a Gaspak hydrogen-carbon dioxide anaerobic system. Samples taken from system each hour, cells were removed from the broth by ultra-centrifugation (Beckman, L77, ins) for 10 min at 12,000 X G and 1°C. The cell pellet was re-suspended in a volume of distilled water equal to that of the original broth culture.

The o-phthalaldehyde method for measuring cholesterol described by Rudel and Morris [3] was used to determine the amount of cholesterol in the re-suspended cells and spent broth. Un-inoculated sterile broth was also analyzed in some experiments. Since some modifications with respect to sample volume and reagent volumes were used, the procedure is described here for convenience. The sample (0.5 ml) was placed into a clean test tube (duplicates for each sample). Three milliliters of 95% ethanol was added to each tube, followed by 2 ml of 50% potassium hydroxide. The contents of all tubes were mixed thoroughly after addition of each component. Tubes were heated for 10 min in a 60°C water bath, and after cooling, 5 ml of hexane was dispensed into each tube. After mixing thoroughly with a vortex-Genie vibrator, on setting 5 for 20 seconds, 3 ml of distilled water was added, and the mixing was repeated. Tubes were allowed to stand for 15 min at room temperature to permit phase separation. Then 2.5 ml of the hexane layer was transferred into a clean test tube.

The hexane was evaporated from each tube at 600 C under the flow of nitrogen gas. O-phthalaldehyde reagent (4 ml) was added to each tube. The reagent contained 0.5 mg of o-phthalaldehyde (Sigma Chemical Co., St. Louis, Mo) per ml of glacial acetic acid. The tubes were allowed to stand at room temperature for 10 min, and then 2 ml of concentrated sulfuric acid was pipette slowly down the inside of each tube. The contents of each tube were immediately mixed thoroughly on the vortex mixer as described previously. After standing at room temperature for an additional 10 minute, A<sub>550</sub> was read against a reagent blank. The A<sub>550</sub> was compared with a standard curve to determine the concentration of cholesterol. Results were expressed as micrograms of cholesterol per milliliter.

### In Vitro: determination of cholesterol uptake directly from human blood sample

A freshly prepared MRS broth culture of *L. casei*, *L. reutri* (optical density 0.5) was inoculated (1%) into 10 ml of sterile MRS broth containing 1% of blood sample as the cholesterol source and incubated anaerobically for 25 hr at 37°C in a gaspak hydrogen-carbon dioxide anaerobic system. samples taken from system each hour. , cells were removed from the broth by ultra-centrifugation (Beckman, L77, ins) for 10 min at 12,000 X G and 1°C. The cell pellet was re-suspended in a volume of distilled water equal to that of the original broth culture. The o-phthalaldehyde method for measuring cholesterol described by Rudel and Morris is held. As mentioned above in the first experiment.

### Animal model for In vivo evaluation of cholesterol reduction by lactic acid bacteria

Administration of *Lactobacillus reuteri* CRL (104) cells/d) to mice for 7 d before inducing hypercholesterolemia, (by feeding mice with a fat-enriched diet for the subsequent 7 d) was evaluated. At this low dose, *L. reuteri* was effective in preventing hypercholesterolemia in mice [4]. Twenty male Swiss albino mice aged 4-6 weeks and weighing 25-30 g were orally ingested by *L. casei* and *L. reutri*. They were kept under a 12 hr light/dark cycle at 22-26°C and a relative humidity of 50%, in cages (15 × 25 cm), three mice used as a control group. Blood samples are taken in eppendorf tubes and tested for total cholesterol concentration at new kasr el ainy teaching hospital laboratory unit.

### Preparation of standard high cholesterol diet

1% of stabilized cholesterol powder added to solid commercial conventional diet.10 gm cholesterol powder weighted and added into mortar, 1-2 drops of tween 80 as emulsifier with gentle stirring then drop by drop hot water until form homogenous emulsion under water bath 60°C, reaching final volume 50-40 ml all this contend added directly on one kilo of solid commercial conventional diet.

### Results and Discussion

Culture identification the identity of the cultures of lactobacilli used in this study was confirmed as *L. acidophilus* as described in the 8<sup>th</sup> edition of Bergey's Manual of Determinative Bacteriology [5]. All strains were gram-positive, catalase-negative, non-spore forming rods and grew at 45°C but not at 15°C. None produced ammonia from arginine, whereas all hydrolyzed esculin. All strains fermented cellobiose, galactose, glucose, lactose, maltose, and sucrose. None fermented mannitol, melezitose, rhamnose, sorbitol, and xylose. Fermentation of melibiose and raffinose was variable, as expected. The identity characteristics of all strains fit those of *L. acidophilus* more closely than any other species.

**Total cholesterol:** The different strains in isolated culture were placed in the presence of a known quantity of cholesterol (100 mg/l), and after incubation in variable time, the analysis of cholesterol after ultracentrifugation was realized by the colorimetric method.

Results from the screening of cultures for cholesterol uptake *in vitro* are shown in Table 1. To measure the cholesterol removed with the cells, pellet cells obtained by centrifugation was re-suspended in distilled water to original volume of the culture was calculated from the equation  $A=(B/C) \times 100$

### Screening cultures for cholesterol uptake

Concentration for cholesterol in MRS broth spectrophotometrically						
Time	50 mg/liter		100 mg/liter		150 mg/liter	
	<i>L. casei</i>	<i>L. reutri</i>	<i>L. casei</i>	<i>L. reutri</i>	<i>L. casei</i>	<i>L. reutri</i>
0	14.89	8.7	19.4	30.24	28.94	10.51
1	22.19	20.8	6.4	33.25	27.5	0.136
2	44.39	7.3	1.4	31.056	36.17	5.39
3	47.4	5.8	33.5	35.77	28.455	36.95
4	30.6	16.8	26.7	39.83	27.479	42
22	58.6	39	29.8	5.6	3.821	3
23	37.36	10.8	42.6	4.634	36.341	10.99
24	79.23	61.4	47.6	18.13	11.3	84.42
25	21.44	57.2	24.1	5.2	0.813	55.12
26	16.39	27.2	66.4	8.617	29.18	20.42

**Table 1:** Effect of cholesterol concentration on cholesterol uptake by *L. casei* and *L. reutri*

Where A=cholesterol remained with the pellet (as percentage), B=absorbance of the sample containing the cells, C=absorbance of the sample without cells (blank). It was observed that, sample containing no cells has no pellet and cholesterol was determined in the whole system.

Concentration of cholesterol in 1% blood sample + MRS broth		
<i>L. reutri</i>	<i>L. casei</i>	Time (hr)
49.61	44.65	0
56.11	63.65	1
46.75	65.28	2
51.38	71.58	3
53.86	58.59	4
54.77	51.38	5
56.54	49.37	21
60.41	52.57	22
63.56	55.2	23
48.85	60.45	24
44.55	63.03	25
control=1.047		

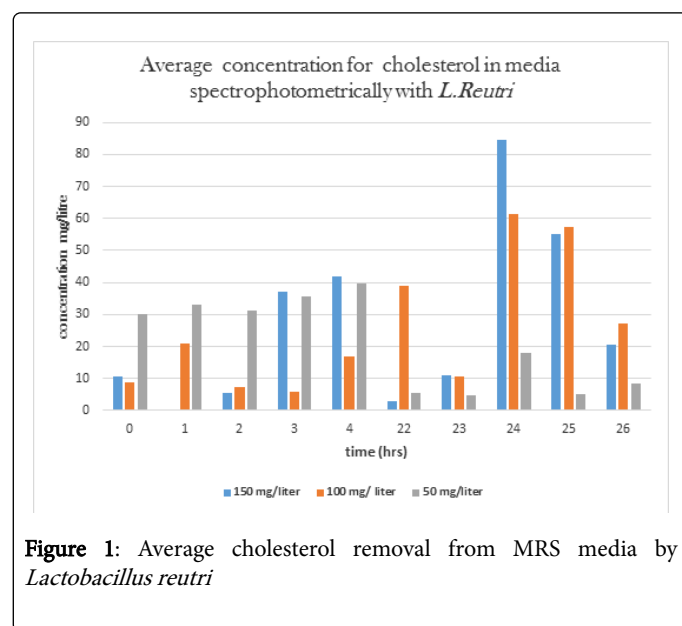
**Table 2:** Effect of total cholesterol concentration on human blood sample by *L. casei* and *L. reutri*

Results from the Assimilation of cholesterol by *L. casei*, *L. reutri* during anaerobic growth in MRS broth contain-ing human blood sample are shown in Table 2.

Oral ingestion of <i>L. reutri</i>		Oral ingestion of <i>L. casei</i>			
MIC E	cholesterol conc. mg/dl	Mice weight gm	MIC E	Mice weight gm	cholesterol conc. mg/dl
1	140	24	1	206	28.3
2	139	31	2	197	31.6
3	166	29	3	213	26
4	150	30	4	182	23
5	118	32	5	237	22
6	126	29	6	155	35
7	171	20	7	208	28.6
8	73	29	8	136	35
9	190	25	9	188	33.5
10	153	25.5	10	71	27

**Table 3:** Effect of and *L. reutri* on the prevention of hypercholesterolemia in mice

Influence of feeding *L. casei*, *L. reutri* on serum cholesterol levels in mice on high cholesterol diet, is summarized in Table 3.



**Figure 1:** Average cholesterol removal from MRS media by *Lactobacillus reutri*

Results from the screening of cultures for cholesterol uptake are shown in Table 1. Cholesterol concentrations in the cell suspensions ranged from 50 mg/liter to 150 mg/liter for both strains. After calculation we observed that *Lactobacillus reutri* had more cholesterol removal ability than *Lactobacillus casei* shown in Figures 1-4.

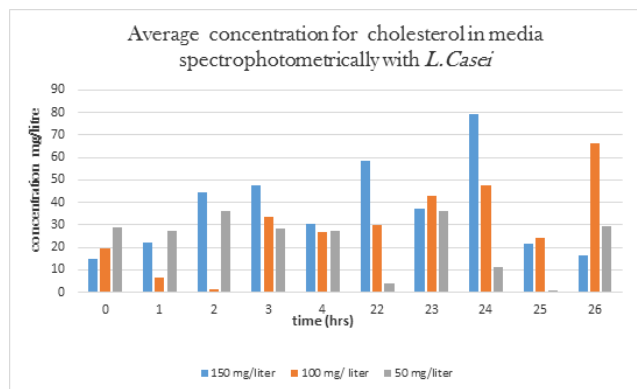


Figure 2: Average Cholesterol removal from MRS media by *L. casei*.

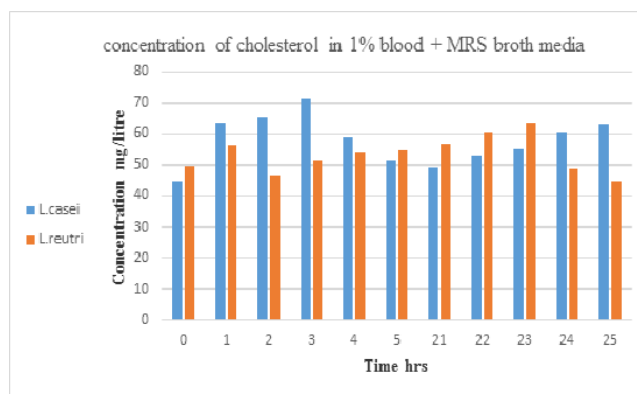


Figure 3: represent cholesterol concentration for *L. reutri*, *L. casei* into 1% direct Blood Sample

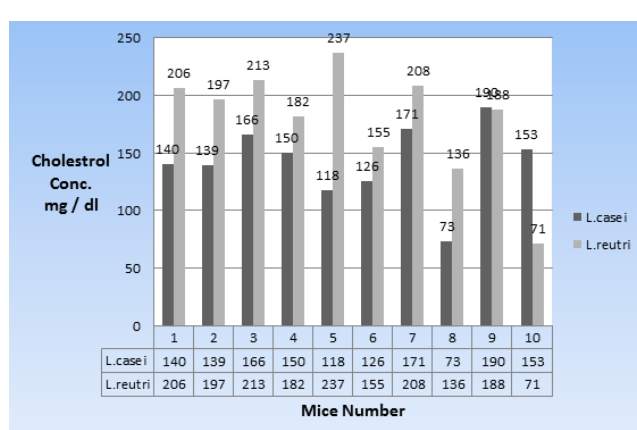


Figure 4: In vivo effect of *L. reutri*, *L. casei* on Mice cholesterol level (Influence of feeding)

## Conclusion

A high level of serum cholesterol in humans is generally considered to be a risk factor for coronary heart disease [6]. Much interest now exists to find ways to decrease the levels of serum cholesterol. Some reports have suggested the possibility of decreasing the serum cholesterol levels after ingestion of *L. acidophilus* [7-9], the mechanism by which the *in vivo* serum cholesterol level was lowered after pigs were fed with *L. acidophilus* was explained by a direct action of *L. acidophilus* on cholesterol which was found under *in vitro* conditions. *L. reutri*, *L. casei* and other lactic acid bacteria have been used as probiotics in various products. This study shows the potential of using *L. reutri* and *L. casei* as an adjunct to reduce serum cholesterol levels. Further *in vivo* study is necessary to prove the hypo-cholesteremic effect of these *Lactobacillus reutri* and *L. casei* strains in humans. Lactic acid bacteria including *L. reutri* are frequently associated with probiotics effects in humans [10].

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