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INVESTIGATING THE EFFECT OF TWO PROBIOTIC YOGHURT TREATMENTS BY INOCULATING *L. CASEI* AND *L. DELBRUECKII* STRAINS ON PREVENTION OR CURE OF ATHEROMA PLAQUES AND THEIR PHYSIOLOGIC CHANGES IN ADULT WISTAR MALE RATS

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ABSTRACT

Background and purpose: probiotic bacteria are among food supplements that have useful effects for human health. Hyperlipidemia is one of cardiovascular diseases factors and these diseases are among major mortality factors throughout the world. This study investigated the effects of two probiotic yoghurt treatments on the atherosclerosis and biochemical factors related to it in hypercholesterolemia rats.

Methodology: 30 white male Wistar rat with 250±16g weight in six groups were used. After treatment, taking blood sample and separating serums, triglyceride, LDL and HDL were measured by cholesterol biochemical factor diagnosis kit. Finally, histologic studies of aorta samples were studied regarding formation or non-formation of atheroma plaque.

Findings: in given yoghurts, after placing in oven, number of bacteria reached to 10^8cfu/ml and stayed the same during maintaining. Concentration of cholesterol and triglyceride in probiotic yoghurt lactobacillus GG and rats treated with it with has significantly reduced. Histologic

studies showed that treatment with probiotic yoghurt has prevented formation of atheroma plaque but it cannot cure the disease.

Discussion and conclusion: presence of lactobacillus GG in probiotic yoghurt reduces lipid metabolites and their higher activity in optimal pH and resistance to these bacteria to digestive enzymes which inhibits cholesterol synthesis in body and as a result, reduces cholesterol in blood.

Keywords: Cholesterol, triglyceride, LDL, HDL, probiotic, atherosclerosis

INTRODUCTION

Probiotics are microorganisms that can have useful effects by improving microbial balance of native micro-flora. Therefore, application of these probiotic bacteria in yoghurt production process leads to making probiotic yoghurt. Yoghurt is a fermented semi-solid product of milk which is produced by activity of two microorganism lactobacillus delbrueckiisubgenus bulgaricus and streptococcus thermophiles [1].

Atherosclerosis is always mortality cause in developed countries which involves aorta and coronary vessels [2]. Today, there are several assumptions about atherosclerosis which seems high concentration of plasma lipids is the main cause of atherosclerosis [3]. Atherosclerosis is derived from Greek meaning thickening the intimate layer of vessels and accumulation of lipid. Lipid is located in central part of plaque and it has fiber coating [4]. Lipoprotein causing atherosclerosis is LDL which is rich of cholesterol. This lipoprotein, because of penetration into lower endothelium or because of connection to extracellular matrix components lime proteoglycans, accumulates in vessels [5,6]. Direct relationship between reducing plasma lipids and lowering atherosclerosis development has been proved by repeated angiographies [7]. Probiotic bacteria have useful effects for health of host such that they have significant role in decreasing cholesterol, antimicrobial activity against pathogens, creating optimal balance in natural micro-flora, reducing lactose non-tolerance and other performances [8]. Since cholesterol is important in various actions like making plasma membrane, steroid hormones, bile salts formation and fetus development, its increase is very harmful and creates solid crystals in cells which leads to death of cell and its increase in blood causes atherosclerosis and cardio-vascular diseases; its increase in bile creates bile stones [9]. Regarding the effect of probiotics on reduction of lipid metabolites in serum and

using these microorganisms in yoghurt can make a valuable food product with high quality. It is hoped that by producing these products, we can create positive evolution in nutrition and health of people.

METHODOLOGY

In this research, first cultures were prepared from used bacteria in preparing probiotic yoghurts; then, for diagnosing bacteria, clones in 10^0_C and 45^0C were studied and test for fermenting sugars like calaboose, fructose, galactose, lactose, maltose, melibiose, raphiose, ribose and sucrose and trehalose were conducted. Probiotic yoghurts were prepared by microbial strains. Then, in order to evaluate survival of bacteria in probiotic yoghurt, number of bacteria was determined by serial dilution [6,7] and after that, pH of probiotic yoghurt was measured. Finally, concentration of cholesterol and triglyceride for probiotic yoghurt was determined. Laboratory animals were selected and classified using below characteristics:

In this research, 30 white male Wistar rats with 250±16g was used. Rats were kept in 21±3C and %50 moisture, 12 hours dark cycle and 12 hours light, with access to water and food in 4-5 rats in groups. Then, rats were randomly selected and divided to 6 groups (n=5): control group, common diet, scheme group that were get cholesterol (soluble in oleic acid), experimental group 1 and 2 with high cholesterol diet, have received 2ml probiotic yoghurt including L. Delbrueckii and L.Casei.

After 1 week, one rat was randomly selected from each group and their blood samples were taken in order to determine decrease or increase in lipid metabolites during yoghurt consumption and reduce error of test. After injection, blood samples were taken from all rats. After separating serums, using cholesterol kits, TG, HDL and LDL, their concentration were calculated and results of three replications of statistical tests were compared (after 8 weeks, one rat has biopsy from third and fourth groups). After week 12, aorta was separated using scalpel and washed with physiologic serum for histologic studies and formation of atheroma plaque. In order to fix samples, %10 formalin was used. Finally, slices from aorta with 6micron thickness were prepared and dyed with hematoxilin - eosin and five section of each sample was randomly selected and studied by microscope.

FINDINGS

Research has shown that in yoghurts prepared by different ratios of lactobacillus GG, L. Delbrueckii and streptococcus thermophiles, number of these bacteria has

reached 10^8cfu/ml and their number remained same during maintenance which shows the sustainability of bacteria in probiotic yoghurt. Optimal pH for bacteria in yoghurt is 4.2-4.6 that in statistical calculations related to pH changes, pH difference of probiotic yoghurts are compared with each other, and there is no significant difference in pH during different days which shows stability of pH during maintaining. This is an important issue in providing probiotic product because reduction of pH during maintaining product in lower values (pH<3) along with increase in acid production by bacteria which influences the taste of product and creates undesired conditions for product.

Results related to measuring cholesterol, triglyceride and other lipid metabolites concentration in rat's blood

Since effects of probiotic bacteria is reducing cholesterol and triglyceride in host, effects of two probiotic yoghurts was studied in vivo such that by evaluation of results, it was observed that cholesterol and triglyceride concentration in L. casei probiotic yoghurt significantly reduced that this reduction is more for cholesterol than triglyceride and higher in LDL than HDL.

In other section, difference between probiotic yoghurts were compared, and as tables and diagrams show, there is significant difference in reduction of cholesterol, triglyceride and other lipid metabolites which indicates effect of L.casei probiotic yoghurt in reducing cholesterol, triglyceride and lipid metabolites.

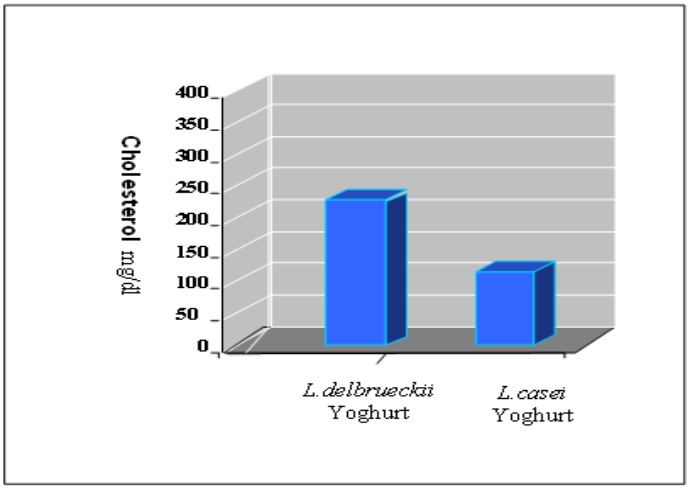
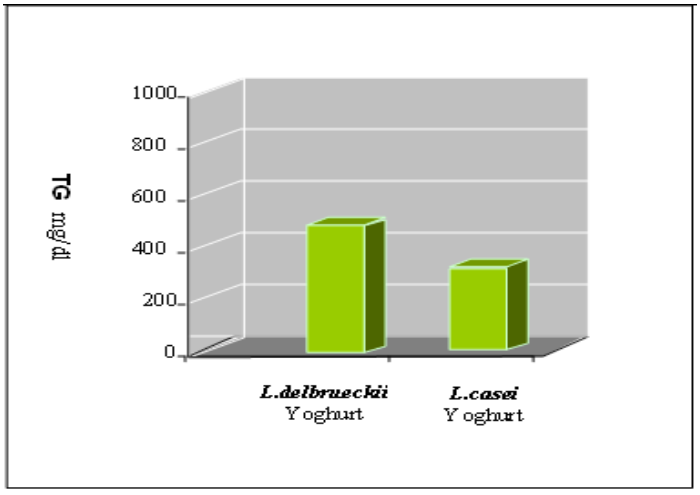
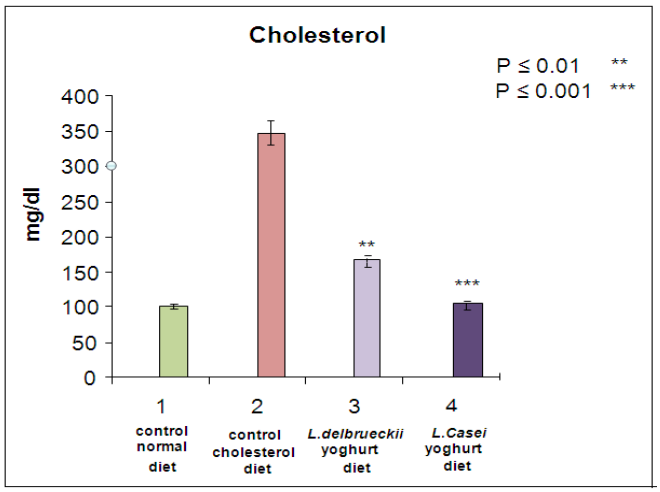


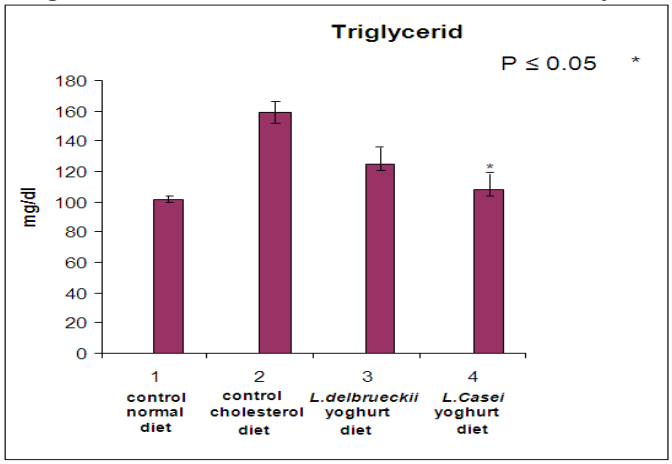
Diagram (1): cholesterol in probiotic yoghurts
L.casei ،L.delbrueckii



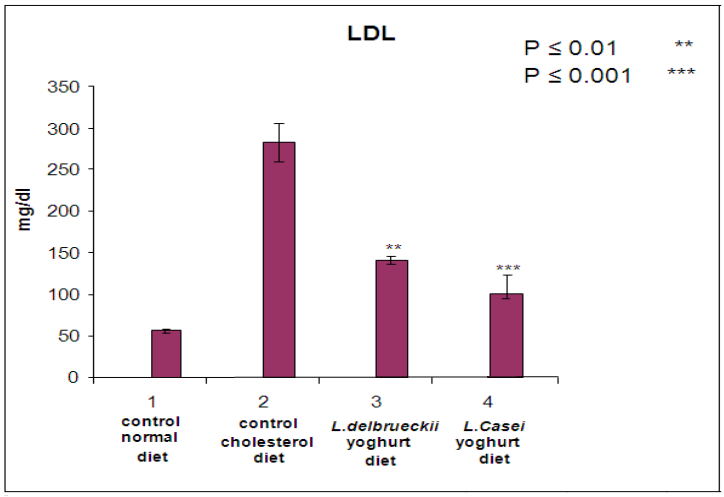
Diag 2: triglyceride concentration in probiotic yoghurt *L.casei* ،*L.delbrueckii*



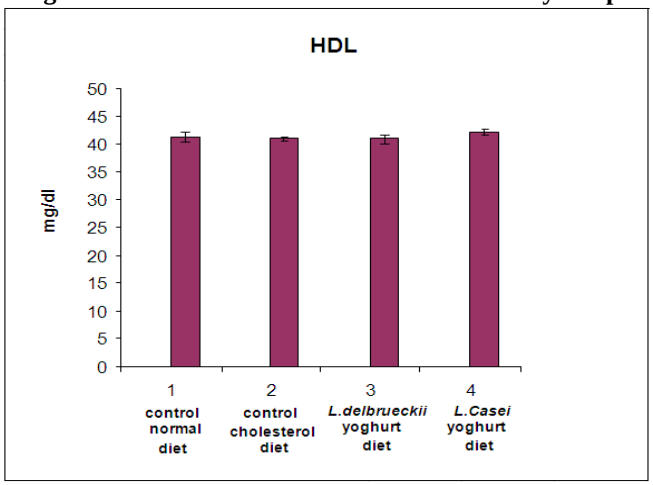
Diag 3: statistical variation of cholesterol in laboratory test



Diag 4: statistical variation of triglyceride in laboratory samples



Diag 5: statistical variations of LDL in laboratory samples



Diag 6: statistical variation of HDL in laboratory samples

Tissue section results

After preparing tissue section of all groups, dying with hemtoxilin-eosin and passing by microscope, wall structure and cell defense response of vessels were studied and finally, histologic studies were conducted which their results are as below:

1. Atheroma plaque in control group: in this group which eating and drinking was free, aorta tissue was healthy and there was no sign of forming atheroma plaque.

As figure 1 shows, vessel was natural in control group (common diet) and there is no lesion (fig 1).

2. Atheroma plaque in scheme (second): in this group with free eating and drinking and high cholesterol (%2 oleic acid), there was atheroma plaque in aorta tissue.

As figure 2 shows atheroma plaque is formed visibly in aorta of scheme group which have received high cholesterol regime (fig 2).

3. atheroma plaque in third group: in this group with free eating and drinking, high cholesterol regime (%2 oleic acid) along with probiotic yoghurt containing LDL, aorta tissue was healthier than scheme group and few effects of atheroma plaque is seen (fig 3).

4. Atheroma plaque in fourth group: this group, with free eating and drinking, received high cholesterol regime (%2 in oleic acid) along with probiotic yoghurt containing L.GG; aorta tissue was healthy and there was no sign of atheroma plaque (fig 4,5).

As figures show, it is clear that in third and fourth groups (high cholesterol + probiotic yoghurt), aorta is natural that this prevention by probiotic yoghurt containing L. Casei was better than yoghurt with L. delbruckeii.

5. atheroma plaque in fifth group: this group with free eating and drinking, high cholesterol regime (%2 in oleic acid) was received in 3 months and treated with probiotic yoghurt with probiotic yoghurt containing L.Casei. In this group, density and accumulation of cells in aorta and formation of atheroma plaque reduced compared to second group but it is not destroyed completely. In fact, we can tell that there is recovery but no treatment occurred (fig 6).

6. Atheroma plaque in sixth group: in this group, with free eating and drinking, high cholesterol regime (%2 in acid oleic) was received in three months and treated with probiotic yoghurt containing L.A. In this group, density and accumulation of cells in aorta and formation of atheroma plaque has decreased but not totally; in fact, we can say that there is recovery without cure (fig 7).

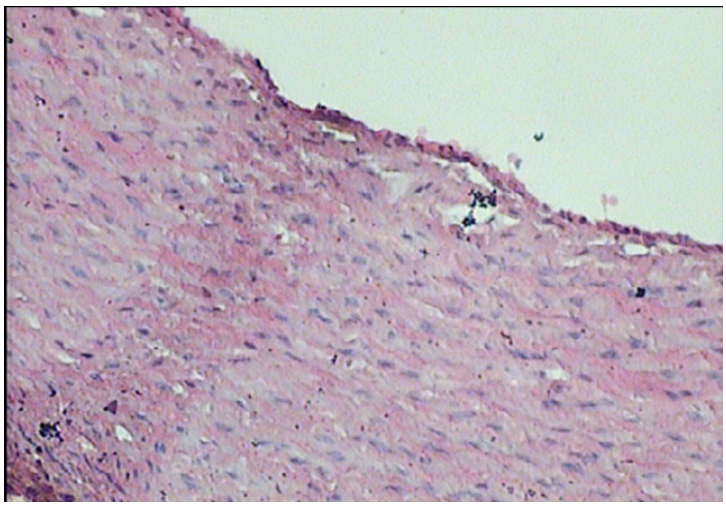


Fig 1: Aorta internal tissue in control group (*10) (hematoxilin-eosin dying)

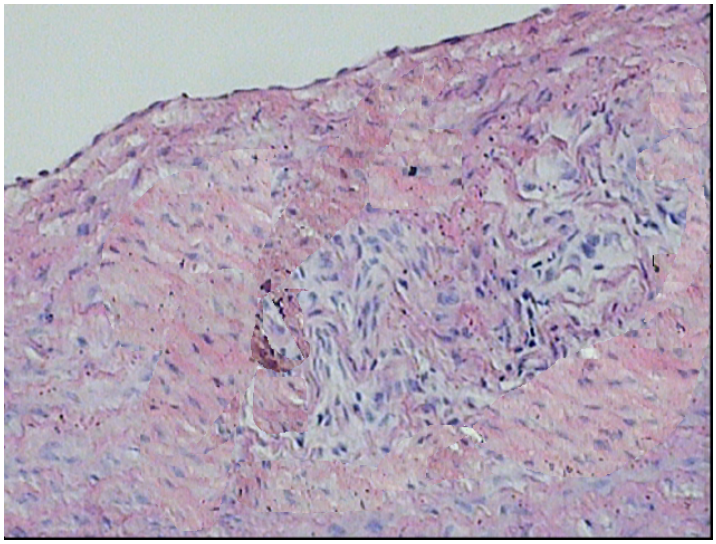


Fig 2: Aorta internal tissue in scheme group (*10) (hematoxilin-eosin dying)

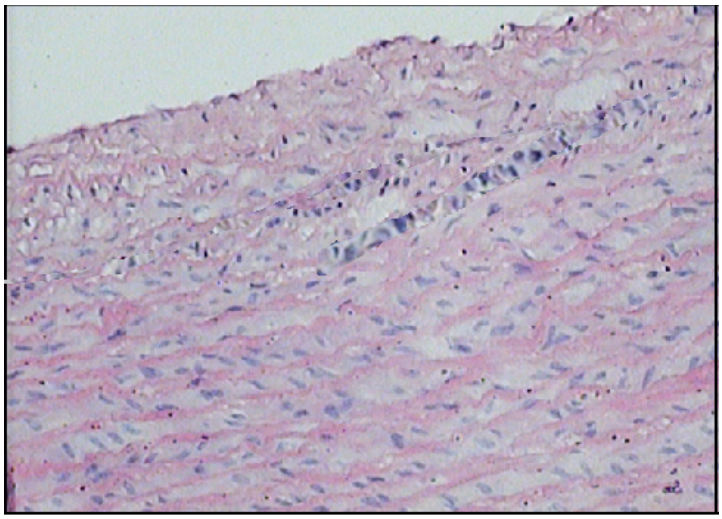


Fig 3: internal tissue of aorta in third group (*10) (hematoxilin-eosin dying)

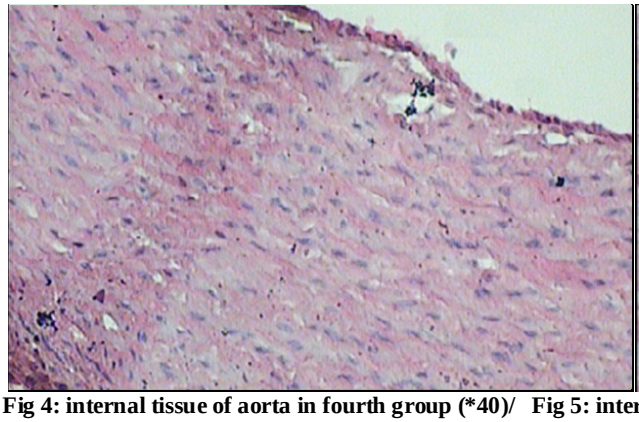


Fig 4: internal tissue of aorta in fourth group (*40)/

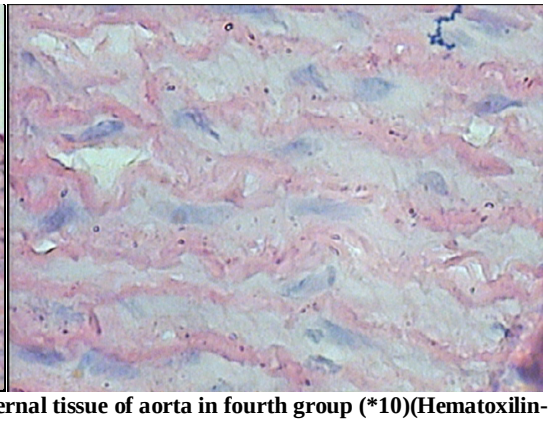


Fig 5: internal tissue of aorta in fourth group (*10)(Hematoxilin-eosin dying)

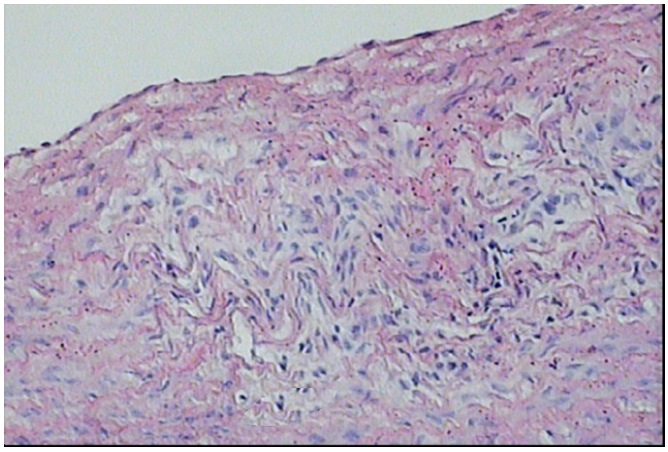


Fig 6: internal tissue of aorta in fifth group (*40) (hematoxilin-eosin dying)

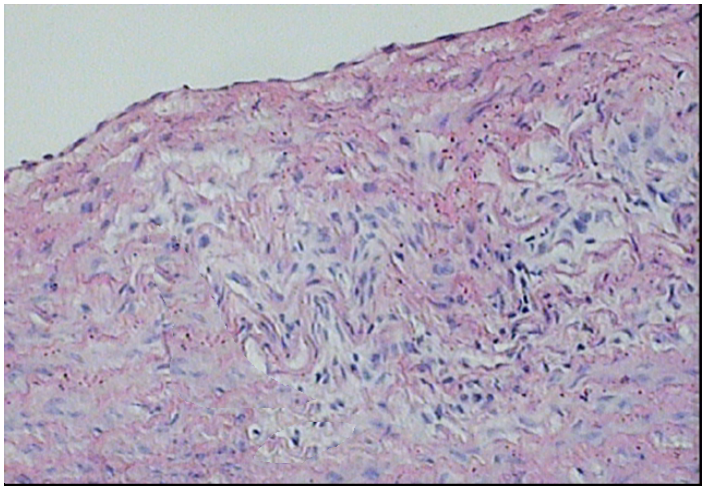


Fig 7: internal tissue of aorta in group 6 (*40) (Hematoxilin-eosin dying)

DISCUSSION

In this study, regarding reduction of cholesterol by lactobacillus, its concentration in probiotic yoghurt and milk used to make yoghurt was studied and their effects on lipid metabolites in serum was studied. In comparison of two yoghurt, probiotic yoghurt has lower cholesterol and triglyceride than common yoghurt; also it can reduce cholesterol, triglyceride and LDL more than HDL in blood serum of rats treated by probiotic yoghurt.

Marshall et.al (1998) conducted a study to test the hypothesis of reducing cholesterol by hydrolyze activity of probiotics such that consumption of probiotic products in test samples reduced serum cholesterol but non-consumption of these compounds for two weeks lead to high cholesterol [7].

De Rodas et.al (1996) stated that digestion of cholesterol and depositing bile salts which play role in reduction of cholesterol, is related to intestine-liver cycle [5,8] while in the case of increasing free bile salts, they exit the intestine and reduce cholesterol.

Marshall and Tamime (2005) reported that eliminating cholesterol by probiotic bacteria occurs in the presence or absence of bile salts. In culture of these bacteria, even in the absence of bile salts, probiotic bacteria can bind to cholesterol in few amounts and by increasing free bile salts, binding between cholesterol and bacteria increases; therefore, bacteria play their role in reducing cholesterol. But cholesterol deposition in low pH cannot eliminate cholesterol in-vitro conditions. They stated that there is a physical relationship between bacteria concentration and cholesterol which increases when bacteria has the ability to de-conjugate bile salts [9].

As results showed, lipid metabolites measured by laboratory kits in rat serum, had significant decrease which shows strong probiotic effects of probiotic yoghurt with continued use on reduction of cholesterol, triglyceride and LDL in rat serum. Results of this study showed that presence of probiotic bacteria lactobacillus GG in probiotic yoghurt has decreases lipid metabolites especially cholesterol which is the major cause for low concentration of cholesterol and triglyceride in probiotic yoghurt L. Casei. Second factor in pH of probiotic yoghurt and higher activity of these bacteria is growth of them. In common yoghurt, optimal pH of bacteria is not stable and varies due to inactivity and correct effect in reducing cholesterol, triglyceride and other lipid metabolites. Third factor is probiotic characteristics including high resistance of these bacteria to stomach acid and intestine, tolerance of pH (2-3), high bile concentration [10] and presence of hydroxyl methyl glutamatein probiotic yoghurt which is fermented by starter microorganisms and inhibits cholesterol synthesis in body and reduces it in blood [11,12]. Another point is that due to the presence of beta-galactosidase enzyme in probiotic bacteria, lactose in diet transforms to positive lactic acid which prevents accumulation of lipid in body.

One effective factor in reducing cholesterol, triglyceride and lipid metabolites by probiotic yoghurt is the presence of hydrolyze probiotic bacteria which are conjugated in intestine-liver cycle and influence bile salt metabolism. Therefore, these enzymes separate glycine and taurine from steroid and create free bile salts which reduce cholesterol and LDL in blood serum

[13] because free salts are defecated. After defecating salts, there is a need to synthesis of bile salts that cholesterol is their prerequisite and probiotic bacteria by hydrolase activity reduce lipid metabolites in body [12, 13].

Consumption of fermentation dairy products and probiotics reduces blood lipid. Consumption of these materials produces short-chain fatty acids which inhibits cholesterol synthesis in liver and moves cholesterol to liver. Bacteria prevent absorption of cholesterol be preventing its binding to bile salts [14].

Reduction of serum cholesterol is possible by three major methods including inhibition of producing it in body, elimination of produced cholesterol and elimination of its promoters. Major mechanisms for reduction of cholesterol by probiotics include:1) cholesterol absorption in cell and its digest or bind to bacteria cells. This method is done by L. Casei; 2) preventing the production of cholesterol by probiotic secreted products. For example, producing hydroxy methyl glutarate by lactobacillus on activity of coenzyme A reductase; 3) non-binding of bile acids to free acids by bifidobactrium and lactobacillus. Because cholesterol is promoter of bile acids, transforming cholesterol to these acids increases to compensate acids lost from body and to reduce serum cholesterol. Free bile acid bind to bacteria cells with fibers and this helps cholesterol exit from the body [15].

Atherosclerosis is seen in rats with high fat diet and low lipoprotein (HDL) and these rats can be used to study the relationship between atherosclerosis and blood lipid [16].

LDL modification by oxidation or enzyme invasion leads to phospholipid release. Patterns related to blood are common for atherosclerosis parts which leads to emergence of cohesive molecules and inflammatory genes by endothelial cells. Therefore, difficulty in circulation and lipid accumulation leads to inflammatory process in vessel [17].

Mechanisms by which probiotics can prevent damage to endothelium are their role in inhibiting cholesterol synthesis and platelet accumulation. Another mechanism is pleiotropic effects of probiotics like prevention of LDL-C peroxidation [18]. Also, as lipid redactor, it reduces LDL-C capability for lipid peroxidation with metal ion macrophage. LDL-C oxidation produces LDL-C; a molecule which induces inflammation and cytotoxic effects in endothelium and its absorption by macrophage is necessary [19, 20].

Probiotics increase HDL-C concentration in serum by decreasing LDL-C concentration. Increase in production of ApoA-I is a mechanism which increases HDL-C concentration [21].

Bacteria in yoghurt like lactobacillus are able to control high serum cholesterol. Therefore, they are sometimes called hypocholesteromic bacteria. L.Casei can absorb cholesterol and this is due to its action in intestine which reduces cholesterol absorption [22].

CONCLUSION

Consumption of probiotic yoghurt is considered as an effective treatment in reducing cardio-vascular diseases and reduction of cholesterol and triglyceride and it can be used as a medicine with low side-effects and other useful effects like antibacterial activity in intestine, curing acute inflammation of stomach and intestine, curing diarrhea, resistance against intestine enzymes and etc. it is hoped that by producing this dairy product and promoting its consumption in society, we can prevent risk of stroke, increase in blood cholesterol and triglyceride and its consequences.

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