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"Probiotics and Hypercholesterolemia"

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ABSTRACT

Probiotics are live microorganism which when consumed in enough amounts exert health effects on the host. Several animal and human studies showed that probiotics have beneficial effects in prevention and treatment of some diseases. In addition to improving gut health, immunity and protection against harmful microorganism, antihypertensive, antioxidative effects, anticarcinogenic properties, improving arthritis, reduction of dermatitis, obesity, probiotics have been also studied for their cholesterol reduction effects. The purpose of this study was to review recent researches in to lipid profile improving effect of probiotics in animals and humans. Probiotics exert lowering lipid profile through several mechanisms such as deconjugate bile acids through bile salt hydrolase catalysis, take up and assimilate cholesterol for stabilization of their cell membrane and binding cholesterol to cell walls of probiotics in intestine; conversion of cholesterol into coprostanol, inhibit hepatic cholesterol synthesis by short chain fatty acids such as propionate produced by probiotic bacteria and/or redistribution of cholesterol from plasma to the liver. Certain strains of probiotics have demonstrated cholesterol lowering properties while others did not. However, not all the trials have yielded conclusive results. Thus, more properly designed *in vivo* trials may bring additional understanding to eliminate the controversies, to better understand the underlying mechanisms and for safety assessment prior to consumption.

Keywords: Probiotics, Lactic Acid Bacteria, Cholesterol, Cardiovascular Disease, Health.

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INTRODUCTION

Cholesterol, the principle body sterol, is synthesized by various tissues including liver, intestine, adrenal cortex, skin and aorta and the remainder is provided by the average diet. It serves as a precursor of steroids in the body such as corticosteroids, sex hormones, bile acids, hormones and certain vitamins besides a component cell membranes and nerve cells. When amount of the total cholesterol synthesized and that obtained from the diet boosts the amount required for the synthesis of membranes, bile salts and steroids; pathological accumulation of cholesterol in blood vessels can occur, resulting in obstruction of the vessels. Heart failure from occluded arteries is the foremost cause of fatality in industrialized societies. However, prominent levels of total blood cholesterol (Hypercholesterolemia) or other blood lipids are considered to be a high risk factor for Coronary heart diseases (CHD) such as atherosclerosis, myocardial infarction and stroke, one of the chief causes of death and disability in the world [1, 2].

Global Scenario

India has been a phenomenal rise in cardiovascular diseases (CVD) in the last 40 years and according to WHO estimates by 2020 close to be 60 percent cardiac patients worldwide will be Indians. Globalization, explosive urbanization, profound lifestyle and food habits are the likely causes of increased CVD crisis in India. Cholesterol-lowering drugs like statins (3-hyroxy-3-methylglutaryl coenzyme A reductase inhibitors) are generally able to inhibit cholesterol synthesis in the liver and peripheral tissues. They have been extensively studied and found to possess better therapeutic effects than other lipid lowering drugs [3]. A recent meta-analysis that involved 14 studies with over 90000 patients for 5 years showed that statins reduced the risks of major cardiovascular events and overall mortality [4]. Yet, another meta-analysis of 11 studies showed no reduction in mortality with the use of statins [5]. However, statins are critical in patients with increased cardiovascular risk as opposed to low-risk patients [6]. This is probably due to the adverse effects related to the use of this class of drugs, which include myopathy[7] and cognitive impairment [8]. Other pharmacological agents that are used in the management of hypercholesterolemia are bile acid sequestrants, cholesterol absorption inhibitors, niacin, and fibrates [9]. Nevertheless, these drugs have also been associated with many adverse effects that limit treatment compliance as well as quality of life. An ideal strategy to control this disease is to lower cholesterol through a combination of lifestyle and pharmacologic approaches.

Health conscious society is now looking for safer and cost effective natural dietary inventions for the management of life style metabolic diseases including CVD as an alternative treatment to

prevent undesirable side effects of drug based therapy. Non-pharmacological treatment serves as the best supportive therapy to reduce cardiovascular risk in otherwise healthy people. The common recommendations are dietary modifications; exercise and weight control [10]. Functional foods are broadly defined as foods that provide additional physiological benefits to the consumer beyond basic nutrition. They are commercially available with health claims of reducing cholesterol levels include oat bran fiber, soy protein, fish oil fatty acids, plant sterols, probiotics and prebiotics [11]. One of the potential strategy which seems to be quite promising and recently stimulated a lot of interest is based on probiotic interventions since these 'magic bugs' hold great promise in the management of inflammatory metabolic disorders including cardiovascular diseases such as hypercholesterolemia, atherosclerosis, hypertension and stroke [12].

Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host [13]. It has a very long history on human health. In a Persian version of the Old Testament, it is said that 'Abraham owed his longevity to the consumption of sour milk [14]. The name probiotic comes from the Greek 'pro bios' which means 'for life' [15]. It is clear that, a number of definitions of the term 'probiotic' have been used over the years but the one derived by the Food and Agriculture Organization of the United Nations/ World Health Organization [16, 17] and approved by the International Scientific Association for Probiotics and Prebiotics [18] best exemplifies the breadth and scope of probiotics as they are known today: 'live microorganisms which, when administered in adequate amounts, exert health benefits on the host'. The primary probiotic bacteria associated with cholesterol lowering have been lactobacilli and bifidobacteria, although, other lactic acid bacteria, such as enterococci, are able to produce this effect [19].

Hypocholetserolemic Effects of Probiotics

There has been a growing interest on the beneficial effects of Lactic acid bacteria specially lipid metabolism. Increasing evidences suggest that few members of lactic acid bacteria, when consumed in sufficiently large numbers, exhibit prophylactic and therapeutic benefits. The early record of the influence of certain dairy products on blood lipids dates back more than 50 years on men from the tribes of Samburu & Maasai warriors in Africa, illustrated reduced serum cholesterol and a low incidence of coronary heart diseases despite of consumption of large amounts of milk fermented with a wild *Lactobacillus* strain [20]. This is often quoted as the basis to study potential hypocholesterolemic effect of fermented products containing lactobacilli and/or bifidobacteria for much of the animal and human studies subsequently carried out. However, some workers have failed to achieve any such activities in their trials. Till date, we have ample evidence that fermented product consumption may favourably affect the lipid metabolisms in animals as well as

humans. An attempt has been made to cover relevant *in vitro* and *in vivo* studies and to understand the mechanism through which lactic acid bacteria exert hypocholesterolemic effect.

Probiotic effect on cholesterol levels - *in vitro* tests

Several *in vitro* studies have demonstrated the cholesterol reducing ability of the lactobacilli [21, 22]. *L. acidophilus* when grew in medium containing cholesterol phosphotidylcholin micelles and recovered large amount of cholesterol in the cells after centrifugation of the medium, part of cholesterol that was assimilated was recovered in membrane fractions of cells, which explains the uptake of cholesterol by the cultures from the medium [23]. Deconjugation of bile acids occurs during enterohepatic circulation in healthy humans. *Lactobacillus* species of human origin have been reported to deconjugate both primary taurocholic and glycocholic acids under anaerobic conditions [24]. Rapid bile deconjugating ability was detected in three of nineteen test strains of *L. acidophilus*, when grown at 37°C in MRS broth supplemented with sodium thioglycollate, sodium taurocholate and cholesterol (Walker & Gilliland, 1993). This indicated the effect was strain specific.

In an in vitro study, bile tolerance, bile deconjugation and cholesterol reducing properties of four strains of lactobacilli (*L. acidophilus* V3, H3, I4 and C2) were tested [26]. The strain H3 released highest amount of free cholic acid amounting to 442.7 mg/ml. Cholesterol assimilation in the range of 13.1-25.3%. In a recent similar study, two probiotic strain *L. fermentum* i.e. PH5 and PD2 showed 52.34% and 54.67% human serum cholesterol reduction at the end of 24 of incubation at 37°C [27].

NPC1L1, which belong to the nuclear receptor super family, has been identified as a novel target gene of the liver X receptors (LXR α and β) that are crucial regulators of cholesterol homeostasis [28]. The activation of LXR reduces whole-body cholesterol and decreases atherosclerosis. In a study, using Caco-2 cell line, it was showed that *L. acidophilus* ATCC 4356 and *L. rhamnosus* GG were better inhibitors of NPC1L1 than the *B. lactis* 12, which demonstrate distinctions among probiotic strains [22]. In the same study, the cell supernatant of *L. acidophilus* ATCC 4356 grown without Caco-2 cells reduced NPC1L1 expression. These results indicate that soluble factors generated from bacteria can suppress NPC1L1 expression independent of contact between the bacteria and cells, may be possible to modify NPC1L1, a central player in cholesterol homeostasis, by manipulating the gut microbiota [29].

Probiotic effect on cholesterol levels - *in vivo* animal model

Many studies have used rats [30, 31], mice [32], hamsters [33], guinea pigs [34] and pigs [35] as models due to their similarities with humans in terms of cholesterol and bile acid metabolism, plasma lipoprotein

distribution, and regulation of hepatic cholesterol enzymes [36]. These animals also share an almost similar digestive anatomy and physiology, nutrient requirements, bioavailability and absorption, and metabolic processes with humans, making them useful experimental models for research applications [35]. Hence, the positive hypocholesterolemic effects shown in animal studies suggest a similar potential in humans.

Mice Study

Administration of *L. reuteri* to mice reduced the serum total cholesterol (TC) by 22%, triglycerides (33%) and increased the ratio of HDL-C to LDL-C by 17% [37]. In a study evaluating the effect on cholesterol, administration of *L. plantarum*, isolated from infant feces (4×10^8 CFU/ml dose per mouse in daily) to twelve male hypercholesterolemic mice for 14 days and found a significant ($P < 0.05$) reduction of total serum cholesterol (reduced by 7%) and triglycerides (reduced by 10%) compared to the control [38]. In another study, LDL-C was significantly ($P< 0.05$) lower (by 42%) in mice fed *L. plantarum* KCTC3928 and fecal bile acid excretion was accelerated (45%) [39]. Recently, *L. fermentum* SM-7 isolated from a fermented milk drink (koumiss) and it was found to exhibit acid and bile tolerance and exhibited antimicrobial activity against *E. coli* and *S. aureus in vitro*. In mice, *L. fermentum* SM-7 significantly reduced serum TC and LDL-C but did not increase HDL-C significantly [40]. The study also showed that there was no bacterial translocation in the liver, spleen, or kidney of the treated mice indicating safety of the *Lactobacillus* strain.

Rat Study

In a series of experiments, rats fed with 15% commercial yogurt reported to decrease the concentration of total cholesterol, HDL-cholesterol, triglycerides and phospholipids significantly in the experimental group than in the control group [41]. In another study, *L. acidophilus* or a mixture of probiotic microorganisms consistently reduced plasma lipids including the VLDL+LDL cholesterol and a decrease in HMG CO-A reductase activity in liver and increased neutral and acidic sterol excretion in feces of rats [42]. Hypercholesterolemic male Fischer rats (8 week old) fed with 30 g/kg of *L. acidophilus*-fermented rice bran significantly showed an improved lipid profile compared to the control (without *L. acidophilus*) [43]. In this 4-week study, the authors reported a significant ($P< 0.05$) reduction in serum total cholesterol and liver cholesterol of 21.3% and 22.9%, respectively compared to the control. In one more study [44], significant reduction in TC and LDL-C in rats fed *L. gasseri*. Abd El-Gawad [45] conducted a randomized, placebo-controlled and parallel designed study to assess the efficiency of buffalo milk-yogurts (fortified with *Bifidobacterium longum* Bb-46) in exerting a cholesterol-lowering effect. In the study, the authors fed forty-eight male albino hypercholesterolemic rats (average weight 80-100 g) with 50 g of

yogurt [contained 0.07% (w/v) *Bifidobacterium longum* Bb-46] daily for 35 days. The administration of *B. longum* Bb-46-fermented buffalo milk-yogurt significantly reduced concentration of total cholesterol by 50.3%, LDL cholesterol by 56.3% and triglycerides by 51.2% compared to the control ($P< 0.05$). Fazeli [46] showed that the consumption of *L. plantarum* A7 (10^8 CFU/ml) for 14 days is effective in lowering serum lipid levels in rats. A significant reduction by about 25% and 33% of serum TC and LDL-C respectively in rats fed *L. fermentum* 9-41-A [47]. This strain was also isolated from feces of healthy adults and selected for probiotic characteristics. In a latest study, Kumar et al., (2012) revealed a 23% reduction in plasma TC, 38% reduction in LDL-C and 19% increase in HDL-C of rats fed with *L. plantarum* Lp91, a bile salt hydrolase producing strain. The faecal excretion of cholic acid was also found to be significantly higher in the probiotic-fed rats.

Human Study

Gorbach [48] observed cholesterol reduction in 35 healthy volunteers for two weeks with a fermented product containing *Lactobacillus* GG, a strain isolated from human feces. Khedkar [49] selected 20 human volunteers aged 50-60 years randomly and gave them acidophilus milk in the dose 200 g/day for 30 days. The acidophilus milk was prepared from buffalo skim milk by incubating with *L. acidophilus* V3 showed significant reduction in serum cholesterol at the end of the study and 15 days after cessation of consumption. Anderson [50] explored the effect of fermented milk containing *L. acidophilus* L1 on serum cholesterol in hypercholesterolemic humans. This randomized, double-blind, placebo-controlled and crossover 10-week study was designed for forty-eight volunteers whose serum cholesterol values ranged from 5.40 mmol/l to 8.32 mmol/l. Daily consumption of 200 g of yogurt containing *L. acidophilus* L1 after each dinner contributed to a significant ($P< 0.05$) reduction in serum cholesterol concentration (2.4%) compared to the placebo group. A randomized feeding trial comprising 27 human volunteers with both normal lipid profile and hyperlipidemia was conducted [26]. The results showed wide variations among the volunteers. However, a significant reduction by 7.6% in total cholesterol and 15.7% in LDL cholesterol was noticed in the volunteers during study. The feeding favorably affected total serum cholesterol and LDL/HDL or total/HDL ratios. In a Dutch trial, Xiao [51] evaluated the effects of a low-fat yogurt containing 10^8 CFU/g of *B. longum* BL1 on lipid profiles of thirty-two subjects (baseline serum total cholesterol 220-280 mg/dl, body weight 55.4-81.8 kg, aged 28-60 years old). Results from this randomized, single-blind, placebo-controlled and parallel study showed a significant ($P < 0.05$) decline in serum total cholesterol, LDL-cholesterol and triglycerides after 4-weeks. The authors also observed a 14.5% increase in HDL-cholesterol when comparing to the control

(yoghurt without *B. longum* BL1). Pranami & Sheth [52] conducted a study on Indian fermented milk (Dahi) fortified with probiotic bacteria and inulin that improved serum lipid, blood glucose level and gut microflora in human subjects. Prajapati [53] evaluated the tolerance and beneficial effects of *L. helveticus* MTCC 5463 after daily consumption of milk based synbiotic product in human volunteers, randomly allocated to 2 groups (n=28) for 90 days: Control (C) that did not consume probiotic added fermented product and Experimental (T) that consumed a probiotic added fermented milk. Measurable indices of host physiological functions like IgM, IgA, IgE and IgG, total cholesterol, HDL, LDL and triglyceride were not altered although a change in the HDL/LDL ratio from 1.41 to 1.53 was observed among the test subjects and so, *L. helveticus* MTCC 5463 can be safely administrated to non-diseased individuals as a prophylactic daily dose of 10^8 cells/ml in the suggested milk based matrix. A recent trial [54] on 60 overweight (BMI>25), healthy adults, aged 40-60 years showed probiotic VSL#3 supplemented group had significant reduction in total cholesterol, triglycerides, LDL and VLDL and had increased HDL while evaluating the effects of VSL#3 and omega-3 fatty acids on insulin sensitivity, blood lipids and inflammation.

A meta-analysis based on six studies was conducted by Agerhol-Larsen [55] on the hypocholesterolemic effect of fermented dairy product on plasma cholesterol levels. The short term intervention study showed reductions in TC (-8.51 mg/dl) and LDL-C (-7.74 mg/dl) in subjects who consumed the fermented dairy product when compared to the control. Later, Guo [56] conducted another meta-analysis of randomized controlled trials that evaluated the effects of probiotics consumption on blood lipids and found subjects who received probiotics had significantly lower TC (-6.40 mg/dl) and LDL-C (-4.90 mg/dl) as compared to those taken placebo. Both the Meta analyses resulted in a similar observation. It seems that the hypocholesterolemic effects of probiotics in human clinical trials in the recent years have been more consistent.

Although many studies have documented convincing cholesterol-lowering effects of probiotics, controversial results have surfaced. Lewis & Burmeister [57] reported that freeze dried *L. acidophilus* supplementation (3×10^{10} CFU/2 capsules, three times daily) had no effect on elevated cholesterol subjects using a randomized, placebo controlled, crossover 6-week study. Pawan & Bhatia [58] showed that, probiotics given in the form of fermented milk product ‘Dahi’ and ‘Lassi’ in 60 male healthy volunteers had no significant reduction in cholesterol level and HDL-C in the human subjects. A study by Hatakka [59] refuted the purported hypocholesterolemic effect of probiotics, and reported that the administration of *L. rhamnosus* LC705 (10^{10} CFU/capsule daily) did not influence blood lipid profiles in thirty-eight men with mean cholesterol levels of 6.2 mmol/l after a 4-week

treatment period. Simons [60] in a double blind, placebo-controlled, parallel design trial also reported once again no beneficial effects on blood lipids after supplementation of *L. fermentum* for 10 weeks in volunteers (TC 4 mmol/l).

Such controversial findings may be attributed to various factors. Although *in vivo* trials utilize real life models with true representations of the actual pathological systems, these trials are also easily affected by external factors such as different strains of probiotics, varying types of prebiotics, administration dosage, analytical accuracy of lipid analyses, clinical characteristic of subjects, duration of treatment period, inadequate sample sizes, and lack of suitable controls or placebo groups [61, 62]. There is a lack of dosage-response studies to determine the 'minimal effective dosage' of probiotics needed to reduce blood cholesterol levels. A review of past studies has revealed that the effective administration dosages of probiotics vary greatly and is dependent on the strains used and the clinical characteristics of subjects, such as lipid profiles. Although probiotics have been delivered in the range of 10^7 to 10^{11} CFU/day in humans [63] and 10^7 to 10^9 CFU/day in animals [64, 65], some probiotics have been shown to be efficacious at lower levels, while some require a substantially higher amount to exert a hypocholesterolemic effect. The administration of *L. plantarum* 299 v at a dosage of 5.0×10^7 CFU/ml daily has been found sufficient to reduce LDL-cholesterol by 12% compared to the control (Naruszewicz et al., 2002). In contrast, the consumption of probiotic capsules containing *Lactobacillus acidophilus* DDS-1 and *Bifidobacterium longum* (3×10^9 CFU/capsule daily) did not produce significant changes in lipid profiles [66]. This suggests different strains need varying dosage to exhibit hypocholesterolemic effects.

MECHANISM OF CHOLESTEROL REDUCTION

In vitro

Different hypotheses have been advanced to explain the hypocholesterolemic effect of lactic acid bacteria *in vitro*. Many studies, hypothesized that lactic acid bacterial strains are able to remove cholesterol through a combination of two or more mechanisms which includes, assimilation of cholesterol during growth, binding of cholesterol to cellular membrane and deconjugation of bile salts by Bile Salt Hydrolase (BSH) [67, 68, 69, 70, 71, 72, 73, 74]

There was little or no information on the direct action of cultured milk products in reducing cholesterol until Gilliland [75] and Walker & Gilliland [25] reported that cholesterol removal *in vitro* could be due to the active up-take or assimilation of cholesterol by the bacterial cell. From then on, the cholesterol assimilation model was frequently used to explain the *in vivo* hypocholesterolemic effects. Gilliland [75] proposed that the uptake of cholesterol by *Lactobacillus acidophilus* strains

occurred only when the cultures were grown in the presence of bile under anaerobic conditions. They also found that uptake of cholesterol increased with increasing concentrations of bile salts in the media. Noh [23] noted that cholesterol removed from the culture supernatant of *L. acidophilus* incubated in the presence of bile salt was accompanied by an increase in the amount of cholesterol in the cell pellet. He also noticed that cells that were grown in the presence of cholesterol micelles and bile salts were more resistant to lysis by sonication, suggesting that assimilation of cholesterol into the cellular membrane resulted in sturdier bacterial cells. This lends support to the idea that cell membrane alteration occurred in the presence of both bile salts and cholesterol. They also observed that assimilation occurred both at pH 6.0 and without pH control. Similar results have been reported for bifidobacteria [76] and lactococci [77].

Usman [78] previously reported that strains of *Lactobacillus gasseri* could remove cholesterol from laboratory media via binding onto cellular surfaces. The ability of cholesterol-binding appeared to be growth and strain specific. Kimoto [77] strengthened a hypothesis that both live and heat-killed *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* N7 were able to remove cholesterol from growth media. However, the amount of cholesterol removed by live cells was significantly higher than that removed by dead cells. They found that cell density and dry weight were higher when the cells were grown in the presence of cholesterol, and the rate of cholesterol removal was more rapid during their exponential growth phase. Since only living cells can possibly uptake cholesterol into their membranes, they concluded that the mechanisms of cholesterol removal by the live strain were due to cholesterol assimilation and binding, while removal of cholesterol by dead cells was only due to binding onto bacterial cell surface. He also observed a difference in the fatty acid distribution pattern for *Lactococcus* grown with or without cholesterol. Later Taranto [37] reported modifications in the lipid profile of *L. reuteri* grown with cholesterol, while Liong & Shah [21] also observed alteration in the fatty acid profiles of lactobacilli grown in the presence of cholesterol in the growth medium. The incorporation of cholesterol into the cellular membrane increased the concentration of saturated and unsaturated fatty acids, leading to increased membrane strength and subsequently higher cellular resistance towards lysis [73]. He further evaluated this mechanism by determining the possible locations of the incorporated cholesterol within the membrane phospholipid bilayer of probiotic cells via incorporating fluorescence probes into the membrane bilayer of probiotic cells that were grown in the absence and presence of cholesterol.

Pigeon [79] suggested that cholesterol removal by *L. delbrueckii* and *Streptococcus thermophilus* strains were due to binding of free bile acids to their cell membranes through exocellular polysaccharide (EPS) and enhanced excretion of free bile acids through feces. They found that the

strains, which produced the most EPS, bound the greatest amount of bile acids, while the strains that produced the least amount of EPS only bound minimal amounts of bile acids including conjugated bile acids. Tok & Aslim [80] also demonstrated a correlation between cholesterol removal and EPS production of *L. delbrueckii* spp.

However, Klaver & Van der Meer [81] proposed another mechanism of cholesterol reduction by lactic acid bacteria. They proposed that the removal of cholesterol was due to the disruption of the cholesterol micelles caused by bile salt deconjugation and co-precipitation of cholesterol with free bile salts as the pH of the medium dropped because of acid production during growth of lactobacilli and bifidobacteria. This conclusion was based largely on their observation that no cholesterol was removed when the growth medium was maintained at pH 6.0, a pH at which free bile acids would remain in solution and prevent the precipitation of free bi3le salts. They associated these results to the decreased solubility of free bile acids under acidic conditions which in turn reduced the solubility of cholesterol (thus termed co-precipitation of cholesterol).

Taranto [82] & Ahn [83] also stressed that removal of cholesterol was closely related to bile salt deconjugation by bile salt hydrolase of probiotics at low pH. Bile, a water-soluble end product of cholesterol in the liver, is stored and concentrated in the gallbladder. The steroid is conjugated with an amide bond at the carboxyl C24 position to one of two amino acids, glycine and taurine [84], before it is excreted into the duodenum (small intestine) upon ingestion of food. It consists of cholesterol, phospholipids, conjugated bile acids, bile pigments and electrolytes. The conjugated bile salts are amphipathic in nature and form micelles that facilitate digestion, emulsification and absorption of lipids from the small intestine [85]. The conjugated bile salts are readily absorbed in the gastrointestinal tract by active transport mechanisms and are returned to the liver; this process is known as enterohepatic circulation. A large pool of bile acids accumulates and undergoes a number of enterohepatic cycles daily [86]. Once deconjugated, bile acids are less soluble and absorbed by the intestines, leading to their elimination in the feces. Cholesterol is used to synthesize new bile acids in a homeostatic response, resulting in lowering of serum cholesterol [87].

In an *in vitro* study, Jones [88] evaluated the role of bile salt hydrolase in cholesterol-lowering using *Lactobacillus plantarum* 80 (pCBH1). Bile salt hydrolase (BSH) is the enzyme responsible to hydrolyze conjugated glycodeoxycholic acid and taurodeoxycholic acid, leading to the deconjugation of glycol and tauro bile acids in the enterohepatic circulation. Thus, the conversion of cholesterol to bile acids is the major route by which cholesterol is metabolized. To date, only two studies have shown the ability of probiotics strains to be able to up-regulate CYP7A1 an enzyme that catalyzes the conversion of cholesterol to bile acids. An increase in CYP7A1 leads to

reduction in hepatic cholesterol levels [39, 89] and increase fecal cholesterol and bile acids excretion in hamsters and mice respectively.

Brashears [90] demonstrated that strains of *L. acidophilus* were able to deconjugate bile salts and remove cholesterol when grown at both pH 6.0 and without pH control. On the other hand, strains of *L. casei* grown at pH 6.0 removed very little cholesterol compared to the same strains grown at uncontrolled pH. However, examination of cellular membranes of *L. casei* grown under both conditions revealed no cholesterol deposits. Therefore, the authors concluded that removal of cholesterol by *L. casei* was most likely due to co-precipitation of cholesterol with deconjugated bile salts at pH less than 6.0, while removal of cholesterol by *L. acidophilus* was due to assimilation of cholesterol into cellular membranes. Cholesterol reduction may be strain specific. In an attempt to determine the validity of the hypothesis of assimilation and/or precipitation of cholesterol by *Lactobacillus* and *Bifidobacterium* species, Grill [91] cultured a strain of each species in a medium containing different bile salts. They found that the cholesterol removing ability varied according to the type of bile salts. In the presence of taurocholic acid, the removal of cholesterol was due to bacterial uptake and co-precipitation, but in the presence of ox gall, only co-precipitation was observed. It seems that the composition of bile salt is another important factor in determining the amount of cholesterol removed.

Cholesterol can also be converted in the intestines to coprostanol, which is directly excreted in feces. This decreases the amount of cholesterol being absorbed, leading to a reduced concentration in the physiological cholesterol pool. Possible conversion of cholesterol into coprostanol by bacteria has been evaluated [92]. In their study, the authors found that cholesterol dehydrogenase/isomerase produced by bacteria such as *Sterolibacterium denitrificans* was responsible for catalyzing the transformation of cholesterol to cholest-4-en-3-one, an intermediate cofactor in the conversion of cholesterol to coprostanol. This served as a fundamental for further evaluations using strains of probiotic bacteria. In a recent *in vitro* study, Lye [73] evaluated the conversion of cholesterol to coprostanol by strains of lactobacilli such as *Lactobacillus acidophilus, L. bulgaricus* and *L. casei* ATCC 393 via fluorometric assays. The authors detected both intracellular and extracellular cholesterol reductase in all strains of probiotics examined, indicating possible intracellular and extracellular conversion of cholesterol to coprostanol. The concentration of cholesterol in the medium also decreased upon fermentation by probiotics accompanied by increased concentrations of coprostanol. This mechanism warrants further evaluations as cholesterol reductase is also directly administered to humans to convert cholesterol to coprostanol in the small intestines for a bloodstream cholesterol-lowering effect.

More recently, another mechanism was hypothesized by Kim [29] who found the cell free supernatant of *L. acidophilus* ATCC 43121 to contain proteins that were able to significantly reduce cholesterol levels even after heat-treated or controlled at pH 6.0. The extract exhibited greatest cholesterol-reducing activity when maintained at pH 4.0. Subsequent analysis identified the up-regulated proteins to be associated with stress response, translation, and metabolic processes and also have functions related to the cell membranes. Huang & Zhang [22] reported that soluble factors produced by *L. acidophilus* have the ability to inhibit cholesterol absorption in Caco-2 cells by down-regulating the gene expression of Niemann-Pick C1-like 1 (NPC1L1). NCPC1L1 protein has been identified as a key player in cholesterol absorption and a promising target for cholesterol–lowering mechanisms [93]. These studies suggest the possibility to alter gut microbiota through supplementation of probiotics for reduction of cholesterol absorption. Lee [94] investigated cholesterol reducing activity of lactobacilli using genetic and proteomic analysis and reported that ccpA which encodes the catabolite control protein to play an important role in cholesterol reducing activity of probiotics. They also hypothesized that membrane associated proteins play an important role in probiotic cholesterol reduction.

In vivo

Most of the hypotheses raised to date are based on *in vitro* experiments, and few attempts have been made to evaluate the possible hypocholesterolemic mechanisms based on *in vivo* trials. Most of the *in vivo* trials conducted thus far have focused heavily on verifying the hypocholesterolemic effects of probiotics, rather than the mechanisms involved.

Fukushima & Nakano [95] suggested that the hypocholesterolemic effects could also be due to the ability of probiotic organisms to inhibit hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase. It is well documented that suppression of HMG CoA reductase is correlated with the inhibition of cholesterol synthesis. It has also been proposed that various milk components such as orotic acid, retentate, pyrimidine-like nucleotide, calcium or hydroxyl methyl glutaric acid reduces the *de-novo* synthesis of cholesterol through inhibition of NADPH formation (a reducing power required for biosynthesis of cholesterol) by HMG-CoA synthase (a rate limiting step in cholesterol biosynthesis) in liver and increases the cholesterol clearance from blood stream by enhancing the excretion of bile acids [96].

Liong [62] had evaluated the hypocholesterolemic effect of a synbiotic and the possible mechanisms involved by using 24 crossbred hypercholesterolemic pigs. In their parallel 8-week study, the authors found that the administration of a synbiotic containing *L. acidophilus* ATCC 4962, fructooligosaccharides, inulin and mannitol decreased plasma total cholesterol, LDL-cholesterol

and triacylglycerols compared to the control. These lipoproteins were subsequently subfractionated and characterized. Pigs supplemented with the synbiotic had a lower concentration of cholesteryl esters in the LDL particles, accompanied by a higher concentration of triacylglycerol. Triacylglycerol-enriched LDL particles are more susceptible to hydrolysis and removal from blood, while loss of cholesteryl esters forms smaller and denser LDL particles leading to a higher removal from blood compared to larger LDL particles. The authors also found that the administration of the synbiotic led to higher concentration of cholesteryl esters in the HDL particles. HDL is termed as the beneficial cholesterol attributed to its role of transporting cholesterol to the liver for further hydrolysis. Cholesterol is transported as cholesteryl esters in the core of HDL. Thus, synbiotic induced a hypocholesterolemic effect via altering the pathways of cholesteryl esters and lipoprotein transporters.

Prebiotics such as inulin and fructooligosaccharides are soluble, indigestible, viscous and fermentable compounds that contribute to hypocholesterolemia via two mechanisms: decreasing cholesterol absorption accompanied by enhanced cholesterol excretion via feces, and the production of short-chain fatty acids (SCFAs) upon selective fermentation by intestinal bacterial microflora [97]. Using Sprague-Dawley hypercholesterolemic-induced rats (n=32), Kim & Shin [98] found that the administration of inulin for 4-weeks decreased serum LDL-cholesterol with increased serum HDL cholesterol levels ($P<0.05$) compared to the control. Rats fed with inulin also showed higher excretions of fecal lipid and cholesterol compared to the control, mainly attributed to reduced cholesterol absorption. Similar to indigestible fibers, soluble indigestible prebiotics have been postulated to increase the viscosity of the digestive tract and increase the thickness of the unstirred layer in the small intestine, and thus inhibiting the uptake of cholesterol [99]. This may have led to a higher cholesterol catabolism in the liver that contributed to a hypocholesterolemic effect. Fermentation of prebiotics involves a variety of metabolic processes in the anaerobic microbial breakdown of organic compounds, yielding energy for microbial growth and the production of SCFAs [100]. Hara [101] observed that a dietary SCFA mixture and SCFA produced by the fermentation of sugar beet fibers significantly reduced plasma cholesterol levels in rats. Rossi [102] found that butyrate was the major fermentation product from inulin, inhibit liver cholesterol synthesis and provide a source of energy for human colon epithelial cells, meanwhile propionate may inhibit the synthesis of fatty acids in the liver, thereby lowering the rates of triacylglycerol secretion [103]. Propionate is also involved in the control of hepatic cholesterol synthesis and it reduces the rate of cholesterol synthesis which could lead to the lowering of plasma cholesterol levels [103].

In conclusion, the mechanisms proposed for mediating hypocholesterolemic effect by probiotics and/or prebiotics are numerous. Although those hypotheses were proved via *in vitro* studies, the mechanisms are not firmly established and demonstrated in *in vivo* studies. Therefore, more *in vivo* studies are needed to explore the underlying mechanism of cholesterol-lowering effects by probiotics and/or prebiotics in order to have a better understanding of the mechanisms and better formulations for human consumption.

CONCLUSION

The consumption of probiotics is gaining popularity especially in the maintenance of health and prevention of disease. In particular, the role of probiotics as a hypocholesterolemic agent has been explored extensively. This study has given an account of and the reasons for widespread use of probiotics for improving the lipid profile (Total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides) in animal and human studies. However, not all the trials yielded conclusive results. In order to justify the varying cholesterol-lowering effect exhibited by various strains of probiotics, researchers have endeavored to reveal the mechanisms of probiotics on hypocholesterolemic effect through *in vitro* and *in vivo* studies. Many of the proposed mechanisms and experimental evidences specifically targeting cholesterol-lowering effects remain controversial. Thus, more properly designed *in vivo* trials may disclose additional understanding and knowledge to eliminate the controversies.

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