THE EFFECTS OF A SYNBIOTIC PRODUCT ON THE BLOOD LIPID PROFILE AND RED BLOOD CELL MORPHOLOGY AMONG HYPERCHOLESTEROLEMIC SUBJECTS

by

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LIST OF ABBREVIATIONS

Abbreviations	Full Name
ANOVA	Analysis of variance
ANS	8-anilino-1-napthalenesulfonic acid
BMI	Body Mass Index
BSH	Bile-salt hydrolase
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CFU	Colony forming unit
COS	Chito-oligosaccharide
CVD	Cardiovascular diseases
DNA	Deoxyribonucleic acid
DPH	1, 6-diphenyl-1, 3, 5-hexatriene
F	Female
FAME	Fatty acid methyl ester
FAn	Fluorescence anisotropy
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FOS	Fructooligosaccharides

GC	Gas chromatography
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
GOS	Galactooligosaccharide
HCl	Hydrochloric acid
HDL	High-density lipoprotein
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low-density lipoprotein
IDL	Intermediate-density lipoprotein
M	Male
MRS broth	de Mann- Rogosa- Sharpe broth
NaCl-KBr	Sodium chloride-Potassium bromide
PCR	Polymerase chain reaction
RBC	Red blood cells
RCT	Reverse cholesterol transport
RSM	Response surface methodology

SCFAs	Short-chain fatty acids
SD	Standard deviation
SEM	Scanning electron microscopy
SFA	Saturated fatty acids
TMA-DPH	1-(4-trimethylammonium)-6-phenyl-1, 3, 5-hexatriene
UFA	Unsaturated fatty acids
VLDL	Very low-density lipoprotein
WHO	World Health Organization
XOS	Xylooligosaccharides

KESAN SUATU PRODUK SINBIOTIK KE ATAS PROFIL LIPID DARAH DAN MORFOLOGI SEL DARAH MERAH DIKALANGAN SUBJEK HIPERKOLESTEROLEMIA

ABSTRAK

Produk sinbiotik telah digunakan secara konvensional untuk meningkatkan kesihatan pencernaan. Meskipun banyak kajian telah menemui potensi baru produk sinbiotik, namun demikian maklumat yang sedia ada berkenaan dengan kesan sinbiotik ke atas penurunan kandungan kolesterol darah dan mekanisme yang terlibat adalah terhad. Kajian secara rawak, '*double-blind*' dengan placebo sebagai kawalan bertujuan untuk mengkaji kesan suatu produk sinbiotik ke atas profil lipid di kalangan subjek hiperkolesterolemia dan mekanisme yang terlibat. Tiga puluh dua subjek hiperkolesterolemia dengan aras kolesterol plasma awal pada 5.70 ± 0.32 mmol/L telah diagihkan secara rawak kepada dua kumpulan untuk menerima samada empat kapsul sinbiotik ('*Lactobacillus acidophilus*'-CHO 220 dan inulin) atau plasebo setiap hari. Sampel darah puasa diambilkan pada minggu ke-0, 6 dan 12 untuk analisis lipid, lipoprotein, asid hempedu dan sel-sel darah merah. Kumpulan sinbiotik menunjukkan penurunan signifikan keatas jumlah kolesterol plasma dan kolesterol lipoprotein kepadatan rendah plasma sebanyak 7.84% dan 9.27%, masing-masing pada minggu ke-12 ($P < 0.05$) manakala kumpulan kawalan tidak menunjukkan sebarang perbezaan yang signifikan. '*Cholesteryl ester*' ('*CE*') dalam zarah lipoprotein kepadatan tinggi bagi kumpulan sinbiotik adalah lebih tinggi daripada kumpulan kawalan ($P < 0.05$) menunjukkan bahawa peningkatan pengangkutan kolesterol dengan lipoprotein kepadatan tinggi dalam bentuk '*CE*' kepada hepar untuk dihidrolisis. Kumpulan sinbiotik juga mempunyai kepekatan '*CE*' dan kolesterol yang lebih rendah dalam zarah

lipoprotein kepadatan rendah dibandingkan dengan kawalan ($P < 0.05$). Meskipun '*Lactobacillus acidophilus*' CHO-220 boleh menyahkonjugasikan asid hempedu, namun perbezaan kepekatan asid hempedu dalam plasma antara kumpulan sinbiotik dan kawalan yang tidak signifikan ($P > 0.05$) dalam kajian kami telah menunjukkan bahawa produk sinbiotik tersebut bebas daripada isu ketoksikan asid hempedu. Kumpulan yang dibekalkan dengan produk sinbiotik telah menunjukkan penurunan aras jumlah kolesterol plasma dan kolesterol lipoprotein kepadatan rendah plasma, mungkin melalui sistem pengangkut lipid. Mikroskopi electron perskanan menunjukkan bahawa morfologi sel-sel darah merah telah diperbaiki melalui suplementasi sinbiotik. Suplementasi sinbiotik telah menurunkan nisbah kolesterol/fosfolipid dengan signifikan ($P < 0.05$) dalam membran sel-sel darah merah sebanyak 47.02% selepas 12 minggu, manakala kumpulan kawalan tidak menunjukkan sebarang perubahan yang signifikan. Kajian ini juga menunjukkan bahawa suplementasi sinbiotik mengurangkan kepekatan asid lemak tepu, peningkatan asid lemak tak tepu dan meningkatkan nisbah asid lemak tak tepu/asid lemak tepu secara signifikan ($P < 0.05$) selepas 12 minggu manakala kumpulan kawalan menunjukkan perubahan yang tidak signifikan. Perubahan membran sel-sel darah merah dikaji dengan menggunakan kaedah anisotropi pendafluoran dan proba pendafluoran dengan afiniti yang berbeza untuk bahagian fosfolipid yang berlainan. Penurunan bacaan bagi acid 8-anilino-1-naptalinsulfonik, 1, 6-difenil-1, 3, 5-hexatrin dan 1-(4-trimetilammonium)- 6-difenil-1, 3, 5-hexatrin secara signifikan ($P < 0.05$) dalam kumpulan sinbiotik selepas 12 minggu menunjukkan peningkatan kebendaliran membran dan pengurangan gumpalan kolesterol dalam membran sel-sel darah merah dalam kumpulan sinbiotik.

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ABSTRACT

Synbiotics have been conventionally used to improve gastrointestinal health. Although current researches have found new health potentials of synbiotics, little information is available on possible cholesterol-lowering effects and the mechanisms involved. This randomized, double-blind and placebo-controlled study investigated the effects of a synbiotic product on the lipid profiles of hypercholesterolemic subjects and the possible mechanisms entailed. Thirty-two hypercholesterolemic subjects with initial mean plasma cholesterol levels of 5.70 ± 0.32 mmol/L were randomly allocated to two groups and were given four capsules of either synbiotic (*Lactobacillus acidophilus* CHO-220 and inulin) or placebo daily. Fasting blood samples were collected at weeks 0, 6 and 12 for lipid, lipoprotein, bile and red blood cells (RBC) analyses. The synbiotic group showed plasma total- and LDL cholesterol reductions by 7.84% and 9.27%, respectively over 12 weeks ($P < 0.05$) while the control showed insignificant difference. Cholesteryl ester (CE) in the HDL subfraction in the synbiotic group was higher than the control ($P < 0.05$), indicating increased transport of cholesterol by HDL in the form of CE to the liver for hydrolysis. The synbiotic group also had lower CE and cholesterol concentrations in the LDL subfraction compared to the control ($P < 0.05$). Although *Lactobacillus acidophilus* CHO-220 could deconjugate bile, our results showed insignificant ($P > 0.05$) difference in bile acids concentrations between the synbiotic and the control groups, indicating that the synbiotic product is safe from bile-related toxicity. The synbiotic product lowered plasma total- and LDL-cholesterol levels, possibly via modifying the

interconnected pathways of lipid transporters. Scanning electron microscopy (SEM) showed spur RBC was improved upon supplementation of the synbiotic. The supplementation of synbiotic significantly ($P < 0.05$) reduced the cholesterol/phospholipids ratio of the RBC membrane by 47.02% over 12 weeks, while the control showed insignificant changes. Our present study also showed that the synbiotic supplementation reduced saturated fatty acids (SFA) concentration, increased unsaturated fatty acids (UFA) and increased UFA/SFA ratio ($P < 0.05$) over 12 weeks while the control showed insignificant changes. The alteration of RBC membrane was assessed using fluorescence anisotropy (FAn) and fluorescence probes with different affinities for varying sections of the membrane phospholipid bilayer. A significant ($P < 0.05$) decrease in FAn of 8-anilino-1-napthalenesulfonic acid (ANS), 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) and 1-(4-trimethylammonium)-6-phenyl-1, 3, 5-hexatriene (TMA-DPH) was observed in the synbiotic group compared to the control over 12 weeks, suggesting increased membrane fluidity and reduced cholesterol enrichment in the RBC membrane.

CHAPTER 1

INTRODUCTION

1.1 Background

Hypercholesterolemia is a metabolic derangement with the presence of high levels of cholesterol in the blood which can lead to many forms of diseases, most remarkably cardiovascular diseases (CVD) (Austin *et al.*, 2004). CVD is one of the most prevalent diseases in the developing countries and the urgency for its management is increasing as the incidence in these countries grows each year (Reddy and Yusuf, 1998). In 2003, according to the World Health Organization (WHO), CVD made up 16.7 million or 29.2% of total global deaths and CVD will be the leading cause of death in developing countries by 2010 (WHO 2003a). High blood cholesterol levels can be reduced by medication, exercise or dietary modification including the supplementation of probiotic and/or prebiotic (Neuhouser *et al.*, 2002). Probiotics are '*living microbial supplements that beneficially affect the host animals by improving its intestinal microbial balances*' (FAO and WHO, 2001). They are normally known as 'friendly bacteria' such as bifidobacteria and lactobacilli, and can be found in the human gut.

Prebiotics are '*indigestible fermented food substrates that selectively stimulate the growth, composition and activity of microflora in gastrointestinal tract and thus improve hosts' health and well-being*' (Roberfroid, 2007). When probiotics and prebiotics are used in combination, they are known as 'synbiotics'. Probiotics and prebiotics have been well-documented for their roles in enhancing gastrointestinal health. However, recent advances in research have documented new potentials of probiotics and prebiotics on other aspects of human health. This includes cholesterol-lowering effects and the prospective of establishing probiotics and prebiotics as non-drug alternatives for the management of hypercholesterolemia.

Past studies have shown that the administrations of probiotics and prebiotics are effective in improving lipid profiles such as the reduction of serum total cholesterol, triglycerides and LDL-cholesterol. Probiotic strains such as *Lactobacillus acidophilus* (Fukushima *et al.*, 1999; Lubbadeh *et al.*, 1999), *Lactobacillus plantarum* (Naruszewicz *et al.*, 2002; Ha *et al.*, 2006; Jeun *et al.*, 2010), *Bifidobacterium longum* (Xiao *et al.*, 2003; Abd El-Gawad *et al.*, 2005), *Lactobacillus casei* (Bertazzoni-Minelli *et al.*, 2004), *Enterococcus faecium* and *Streptococcus thermophilus* (Agerholm-Larsen *et al.*, 2000) have been found to positively improve blood lipid profiles, especially total cholesterol and LDL-cholesterol. Prebiotics such as inulin (Causey *et al.* 2000; Letexier *et al.*, 2003) and fructooligosaccharides (Alles *et al.*, 1999) have also been shown to positively modulate lipid profiles. Despite these findings, limited studies have emphasized on the use of synbiotics (probiotics and prebiotics in combination) to augment a cholesterol-lowering effect in humans.

In addition, past studies have associated cholesterol-enriched diets with hypercholesterolemia, which in turn causes morphological abnormality in red blood cells (RBC) leading to their decreased life span (Nayak *et al.*, 2008). Such abnormality can subsequently increase the risk of impairing the physical function and microcirculation of RBC. However, to our knowledge, the exact mechanisms of probiotics, prebiotics and synbiotics in lowering cholesterol and improvement of irregularities of RBC remain unclear. Most of the documented work emphasized on proving a cholesterol-lowering effect and little is known on the underlying mechanisms.

Although the combination of probiotic and/or prebiotics (synbiotic) had been developed and showed promising *in vivo* cholesterol-lowering effect in animal models (Lichtman *et al.*, 1999; Gallaher *et al.*, 2000; Lin *et al.*, 2004; Madsen *et al.*, 2008; Patterson *et al.*, 2008); such transferability of a similar effect in humans have yet to be evaluated, and the underlying mechanisms, elucidated. Considering that past studies have reported positive effects of probiotics, prebiotics and synbiotics on serum cholesterol (Nguyen *et al.*, 2007; Zhang *et al.*, 2007; Wong *et al.*, 2010), we hypothesized that these supplements could also positively improve morphological irregularities of the RBC brought about by hypercholesterolemia. Although many positive evidences have surfaced on the use of the probiotics, prebiotics and synbiotics to reduce blood cholesterol levels, to our knowledge, no attempt has been made to evaluate their effects on irregularities of RBC in humans.

1.2 Aim and objective of research

Many strains of *L. acidophilus* have been identified for use as dietary adjuncts, mainly attributed to their long history of safe use and *in-vitro* cholesterol-lowering effects (Gupta *et al.*, 1996). The usage of inulin has also been widely highlighted due to its safe consumption (Carabin and Flamm, 1999) and its application in cholesterol-lowering effects (Davidson *et al.*, 1998; Brighenti, 2007). The cholesterol-lowering effects of synbiotics containing *L. acidophilus* and inulin and/or inulin-type prebiotics have also been reported (Schaafsma *et al.*, 1998; Kießling *et al.*, 2002). In addition, strains of *L. acidophilus* have also been deposited as *L. casei*, *L. paracasei* and *L. johnsonii* elsewhere (ATCC, 2010). All of these strains have also shown cholesterol-

lowering effects in *in-vivo* models when used in combination with inulin and inulin-type prebiotics (Bertazzoni-Minelli *et al.*, 2004; Sarmiento-Rubiano *et al.*, 2007).

The aim of this study was to investigate the effects of a synbiotic product containing *L. acidophilus* CHO-220 and inulin on the plasma lipid profiles of hypercholesterolemic subjects. The effects of the synbiotic product on plasma lipid transporters and the possible mechanisms involved were evaluated. The effect of the synbiotic product on bile conversion was also assessed. In addition, this study was also aimed to evaluate the effect of this synbiotic product on the morphological irregularities of RBC induced by hypercholesterolemia. The morphology of RBC, ratio of cholesterol/phospholipids, membrane fluidity properties and fatty acid profiles of RBC were thus investigated.

The specific objectives of this study were:

1. To assess the effects of a synbiotic product on the human plasma lipid profiles such as plasma total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol.
2. To evaluate the changes in the compositions of triglycerides, cholesteryl ester, protein, total-cholesterol, free cholesterol and phospholipids that occurring in the subfractions of human lipoproteins such as Very Low Density Lipoprotein (VLDL), Intermediate Density Lipoprotein (IDL), Low Density Lipoprotein (LDL) and High Density Lipoprotein (HDL) upon the consumption of the synbiotic product.
3. To assess the effects of the synbiotic product on the hypercholesterolemia-induced morphological irregularities of the human RBC.

CHAPTER 2

LITERATURE REVIEW

2.1 Health facts

The World Health Organization (WHO) (2009) predicted that by 2030, cardiovascular diseases will remain the leading causes of death, affecting approximately 23.6 million people around the world. It was reported that hypercholesterolemia [a condition whereby it has been associated with higher than normal total cholesterol (≥ 5.2 mmol/L) and Low Density Lipoprotein-cholesterol (≥2.6 mmol/L)] contributed to 45% of heart attacks in Western Europe and 35% of heart attacks in Central and Eastern Europe from 1999 to 2003 (Yusuf *et al.,* 2004). The risk of heart attack is three times higher in those with hypercholesterolemia compared to those who have normal blood lipid profiles. The WHO (2003b) delineated that unhealthy diets that are high in fat, salt and simple sugar; and low in complex carbohydrates, fruits and vegetables lead to increased risk of cardiovascular diseases. Hypercholesterolemia is considered one of the major risk factors of cardiovascular disease, as approximately 90% of patients with cardiovascular problems have prior exposure to unfavorable blood cholesterol levels, especially high levels of total cholesterol and LDL-cholesterol, in addition to hypertension, obesity and diabetes (Greenland *et al.*, 2003). Clinical trials have indicated that the rates of coronary heart disease could be reduced by reducing blood cholesterol levels (Reese *et al.*, 2001). Population-based data have shown that a 10 percent decrease in total cholesterol levels could result in an estimated 30 percent reduction in the incidence of coronary heart disease (Reese *et al.*, 2001), while every one percent of reduction in LDL-cholesterol levels could reduce the relative risk for coronary heart disease by approximately one percent (Grundy *et al.*, 2004).

Parts of this literature review have been published:

1. Yeo, S. K., **Ooi, L. G.**, Lim, T. J., & Liong, M. T. (2009). Antihypertensive properties of plant-based prebiotics. Int J Mol Sci, 10, 3517-3530. (ISI; Published)
2. **Ooi, L. G.**, Ahmad, R., Yuen, K. H., & Liong, M. T. (2010). Hypocholesterolemic effects of probiotic-fermented dairy products. Milchwissenschaft (ISI; In-press)
3. **Ooi, L. G.**, & Liong, M. T. (2010). Cholesterol-lowering effects of probiotics and prebiotics: A review of *in vivo* and *in vitro* findings. Int J Mol Sci, 11, 2499-2522. (ISI; Published)

2.2 Probiotics, prebiotics, synbiotics and cholesterol

People affected with hypercholesterolemia may avert the use of cholesterol-lowering drugs by practising dietary control or supplementation of probiotics and/or prebiotics. Probiotics are '*living microbial supplements that beneficially affect the host animals by improving its intestinal microbial balances*' (FAO and WHO, 2001). Prebiotics are '*indigestible fermented food substrates that selectively stimulate the growth, composition and activity of microflora in gastrointestinal tract and thus improve hosts' health and well-being*' (Roberfroid, 2007). When probiotics and prebiotics are used in combination, they are known as 'synbiotics'. 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 well being, probiotics and prebiotics have become increasingly recognized as supplements for human consumption.

Lactobacillus and *Bifidobacterium* are common genera of probiotics and have been documented to exert health-promoting effects which include improvement of lipid profiles (Pereira and Gibson, 2002a, b), strengthening of the immune system (Galdeano *et al.*, 2007), alleviation of diarrhoea (Hickson *et al.*, 2007), improvement of lactose intolerance (Landon *et al.*, 2006), antihypertensive effects (Yeo and Liong, 2010) prevention of cancer (Hirayama and Rafter, 2000), antioxidative effects (Songisepp *et al.*, 2004), reduction of dermatitis symptoms (Weston *et al.*, 2005), facilitation of mineral absorption (Scholz-Ahrens *et al.*, 2007), amelioration of arthritis (Baharav *et al.*, 2004), reduction of allergic symptoms (Ouwehand, 2007) and improvement of vulvovaginal candidiasis in women (Falagas *et al.*, 2006).

Fructooligosaccharides (FOS), inulin, oligofructose, lactulose, and galactooligosaccharides have been identified as prebiotics due to characteristics such as resistance to gastric acidity, hydrolysis by mammalian enzymes and are fermented by gastrointestinal microflora to further selectively stimulate the growth and/or activity of beneficial intestinal bacteria. FOS contains 2 to 10 fructose units linked by glycosidic bonds, while inulin is a fructose polymer with β-(2-1) glycosidic linkages with chains of 3 to 60 units. Both FOS and inulin are found abundantly in chicory and artichokes. The major component of chicory root is inulin. Inulin belongs to the fructan family, and occurs naturally as important storage carbohydrates. Other than chicory, fructans are also found present in artichokes, salsify, asparagus and onions (Kim and Shin, 1998). Generally, prebiotics offer promising health benefits such as improvement of lipid profile (Mortensen *et al.*, 2002), exerting positive impacts on gastrointestinal microflora by promoting the growth of probiotics and/or inhibition of pathogenic microorganisms (Bielecka *et al.*, 2002), stimulation of the immune system (Schley *et al.*, 2002), cancer prevention (Klinder *et al.*, 2004), stimulation of mineral absorption and bone stability (Scholz-Ahrens *et al.*, 2002) and treatment of irritable bowel - associated diarrhoeas (Cummings and Macfarlane, 2002).

New compounds with gut resistant properties and selective fermentability by intestinal microorganisms are continuingly being identified and developed as prebiotics (Gibson and Fuller. 2000). These include oligosaccharides (isomaltooligosaccharides, lactosucrose, xylooligosaccharides and glucooligosaccharides), sugar alcohols and polysaccharides (starch, resistant starch and modified starch) (Cummings *et al.*, 2001). A combination of prebiotics and fermentable compounds are often used to strengthen

various health effects, including alteration of microbial population and production of short-chain fatty acids which may lead to the reduced incidence of gastrointestinal diseases (Topping and Clifton, 2001), cancers (Hinnebusch *et al.*, 2002) and cardiovascular diseases (Dewailly *et al.*, 2001), and improvement of lipid profiles (Wolever *et al.*, 2002).

Synbiotic products that are currently available on the market are often a combination of bifidobacteria or lactobacilli and fructooligosaccharides or inulins. There is a lack of studies relating to the lowering of cholesterol using synbiotics. Although studies have indicated that the administration of probiotics and/or prebiotics can reduce cholesterol level (Jeun *et al.*, 2010; Trautwein *et al.*, 1998), controversies surfaced when some studies did not demonstrate the cholesterol-lowering potential of probiotics and/or prebiotics (Alles *et al.*, 1999; Greany *et al.*, 2008).

Studies examining the efficacy of probiotics in reducing cholesterol often do not sufficiently address the mechanisms by which probiotics modulate cholesterol-lowering effects and the optimum dose, frequency, and duration of treatment for different probiotic strains. Several mechanisms have been hypothesized, which include enzymatic deconjugation of bile acids by bile-salt hydrolase of probiotics (Lambert *et al.*, 2008), cholesterol binding to cell walls of probiotics (Liong and Shah, 2005a), incorporation of cholesterol into the cellular membranes of probiotics during growth (Lye *et al.*, 2010a), conversion of cholesterol into coprostanol (Lye *et al.*, 2010b) and production of short-chain fatty acids upon fermentation by probiotics in the presence of prebiotics (De Preter *et al.*, 2007).

Probiotics are generally known to be nonpathogenic but they could be infectious especially in the debilitated and immuno-compromised populations (Peret-Filho *et al.*, 1998). Some species of *Lactobacillus*, *Bifidobacterium*, *Leuconostoc*, *Enterococcus* and *Pediococcus* have been isolated from the infection sites (Land *et al.*, 2005). Strains of probiotics have also been found to exhibit antibiotic resistance and have raised concerns on horizontal resistant genes transfer to the host and the pool of gastrointestinal pathogenic microflora (Huys *et al.*, 2006). Considering this, the safety verification of probiotics used industrially and commercially is of utmost importance.

2.3 Cholesterol-lowering potential of probiotics: *In vivo* evidence

The use of animals and humans models to evaluate the effects of probiotics and prebiotics on serum cholesterol levels has been emphasized over the years. Human studies have shown promising evidence that well-established probiotics and/or prebiotics possess cholesterol-lowering effects, while new strains of probiotics or new types of prebiotics were evaluated in animal models for their potential cholesterol-lowering effects. Many studies were using rats (Gallaher *et al.*, 2000; Shinnick *et al.*, 1988), mice (Lichtman *et al.*, 1999), hamsters (Lin *et al.*, 2004), guinea pigs (Madsen *et al.*, 2008) and pigs (Patterson *et al.*, 2008) 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 (Fernandez *et al.*, 2000). 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 (Patterson *et al.*, 2008). Hence, the positive cholesterol-

lowering effects shown in animal studies suggest a similar potential in humans. Human trial results that paralleled those obtained from animal studies further attested the transferability and reliability of results in selected animal models.

In a study evaluating the effect of *L. plantarum* PH04 (isolated from infant feces) on cholesterol, Nguyen *et al.* (2007) administered *L. plantarum* (at doses of 10^7 CFU/g per mouse per day) to twelve male hypercholesterolemic mice for 14 days. The authors found a significant ($P < 0.05$) reduction of total serum cholesterol (reduced by 7%) and triglycerides (reduced by 10%) compared to the control. In another study, Abd El-Gawad *et al.* (2005) 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 the concentration of total cholesterol by 50.3%, LDL-cholesterol by 56.3% and triglycerides by 51.2% compared to the control ($P < 0.05$). In another study, Fukushima *et al.* (1999) found that hypercholesterolemic male Fischer 344/Jcl 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*). 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. Similarly, Chiu *et al.* (2006) studied the effects of *Lactobacillus*-fermented milk on lipid metabolism using hamsters. The 8 weeks treatment involved a high-cholesterol diet plus: water (group

A/control group), sterilized milk (group B), milk fermented by *L. paracasei* subsp. *paracasei* NTU 101 (group C), milk fermented by *L. plantarum* NTU 102 (group D) or milk fermented *L. acidophilus* BCRC 17010 (group E). The fermented-milk-feeding groups (group C, D and E) showed a significantly ($P < 0.05$) reduced serum cholesterol and LDL-cholesterol level compared to those of control group (group A) and milk-feeding group (group B). These findings indicated that milk fermented by these three *Lactobacillus* strains were effective in reducing serum cholesterol concentration and LDL-cholesterol level.

The cholesterol-lowering potential of probiotics has also been evaluated using human subjects. Anderson *et al.* (1999) 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 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. In another study, Xiao *et al.* (2003) 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 of 220-280 mg/dl, body weight 55.4-81.8 kg, aged 28 to 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; $P < 0.05$). In a randomized, double-blinded,

placebo-controlled and crossover-designed trial involving twenty-six healthy volunteers, Klein *et al.* (2008) determined the effect of yoghurt consumption (300 g/day) containing probiotic strains *L. acidophilus* 74-2 and *Bifidobacterium animalis* subsp *lactis* DGCC 420 on blood lipids. In this 10-week study, the authors reported a significant ($P < 0.05$) reduction in serum triglycerides of 11.6% compared to the control (plain yogurt without *L. acidophilus* 74-2 and *Bifidobacterium animalis* subsp *lactis* DGCC 420). In another study, Agerholm-Larsen *et al.* (2000) conducted an 8-week randomized, double-blind, placebo-controlled and parallel study involving seventy subjects. In this study, the authors observed a significant ($P < 0.05$) decline in LDL-cholesterol of 8.4% upon consumption of 450 mL probiotics milk [containing of 6×10^7 CFU/mL of *Enterococcus faecium* (known probiotic isolated from human) and 1×10^9 CFU/mL of *St. thermophilus* (a common yogurt culture)].

2.4 Cholesterol-lowering potential of prebiotics: Animal and human studies

While the cholesterol-lowering effect of probiotics has been well-documented, prebiotics have also gained increasing attention in cholesterol studies, due to their role in promoting the growth of probiotics. Causey *et al.* (2000) conducted a randomized, double-blind and crossover study using hypercholesterolemic subjects to assess the effects of inulin from chicory root on blood cholesterol level. This study involved twelve men that were randomly assigned to two groups, namely the control group (consumed one pint of vanilla ice-cream without inulin daily) and the inulin group (consumed one pint of vanilla ice-cream containing 20 g of inulin daily). The 3-week study found that daily intake of 20 g of inulin significantly ($P < 0.05$) reduced serum triglycerides.

Similarly, another double-blind, randomized and placebo-controlled crossover study involving eight healthy volunteers with a daily consumption of 10 g of inulin for 3 weeks has also reached the same conclusion (Letexier *et al.*, 2003). Plasma triglycerides concentration was significantly ($P < 0.05$) lower in the treatment group (with inulin) compared to the placebo group (without inulin) (Letexier *et al.*, 2003). In another study, Brighenti *et al.* (1999) used a randomized, double-blind, placebo-controlled and parallel design trial involving twelve healthy male volunteers to study the effect of prebiotic on lipid profiles. In this 12-week trial, the authors found that the daily consumption of 50 g of a rice-based ready-to-eat cereal containing 18% inulin significantly ($P < 0.05$) reduced plasma total cholesterol and triglycerides by 7.9% (± 5.4) and 21.2% (± 7.8), respectively compared to the control. Similarly, Mortensen *et al.* (2002) found that forty male mice fed with a purified diet with 10% of long-chained fructan for 16 weeks showed that the fructan significantly reduced blood cholesterol by 29.7% ($P < 0.001$), LDL-cholesterol concentration by 25.9% ($P < 0.01$), IDL-cholesterol level by 39.4% ($P < 0.001$) and VLDL-cholesterol concentration by 37.3% ($P < 0.05$) compared to the control group. Davidson and Maki (1999) conducted a randomized, double-blind, placebo-controlled and crossover design trial for 12 weeks involving twenty-one healthy volunteers to study the effect of prebiotic on lipid profiles. The authors found that the daily consumption of 18 g/day of inulin-supplemented foods significantly reduced plasma total cholesterol ($P < 0.02$) and LDL-cholesterol ($P < 0.005$) by 8.7% (± 3.3) and 14.4% (± 4.3), respectively compared to the control. Daubioul *et al.* (2002) administered 10 g of fructan to male mice for eight weeks. The study involved sixteen male obese mice and the results showed that the fructan treatment significantly reduced ($P < 0.05$)

hepatic triacylgylcerol by 48% compared to the control group. In another study, Busserolles *et al.* (2003) found that male Wistar-Han rats fed with a diet containing oligofructose for four weeks showed a significant ($P < 0.05$) decline of 0.33% in hepatic triglycerides as compared to the control.

Other indigestible and fermentable compounds such as germinated barley, oligodextrans, gluconic acid, lactose, glutamine, hemicellulose-rich substrates, resistant starch and its derivatives, lactoferrin-derived peptide, and N-acetylchitooligosaccharides (Gibson *et al.*, 2004) have also been identified to exert prebiotic potentials with cholesterol-lowering effects. In a study evaluating the cholesterol-lowering effect of resistant starch, Fernandez *et al.* (2000) administered 10 g/100g of resistant starch into male Hartley guinea pigs for 4 weeks. This randomized, placebo-controlled and parallel designed study used sixteen male guinea pigs of 300-400 g body weight and the results showed that the resistant starch significantly reduced ($P < 0.01$) plasma cholesterol by 27.4% and LDL-cholesterol concentration by 28.0% compared to the control group. In another randomized, placebo-controlled and parallel designed study, Wang *et al.* (2008) found that ten male hypercholesterolemic Wistar rats (7-week-old; mean body weight of 210 ± 20 g) fed with starch from Chinese yam (*Dioscorea opposita* cv. Anguo) for 8 weeks showed a significantly lower plasma total cholesterol, LDL-cholesterol and triglyceride ($P < 0.05$) than the control (32.8%, 27.5% and 46.2% lower, respectively). Favier *et al.* (1995) evaluated the cholesterol-lowering effects of β- cyclodextrin in a randomized, placebo-controlled and parallel design trial involving ten male Wistar rats (mean body weight of 150 g). In this 21-day trial, the authors found that daily consumption of 25 g/kg of β-cyclodextrin significantly ($P < 0.05$) reduced plasma

cholesterol and triglycerides by 25.9% and 35.0%, respectively, compared to the control group.

2.5 Cholesterol-lowering potential of synbiotics: *In vivo* studies

Studies have presented evidence of independent cholesterol-lowering effects of probiotics and prebiotics, leading to subsequent evaluations on synbiotics. The administration of a synbiotic product (containing *L. acidophilus* ATCC 4962, fructooligosaccharides, mannitol and inulin) to twenty-four hypercholesterolemic male pigs yielded promising cholesterol-lowering effects (Liong *et al.*, 2007). The authors reported a significant reduction of plasma total cholesterol ($P < 0.001$), triglycerides ($P < 0.001$) and LDL-cholesterol ($P < 0.045$) in pigs consuming the synbiotic diet for 8 weeks compared to the control. Kieβling *et al.* (2002) evaluated the cholesterol-lowering effect of a synbiotic yoghurt (containing *L. acidophilus* 145, *B. longum* 913 and oligofructose) in a randomized, placebo-controlled and crossover study involving twenty-nine women. The authors found that the daily consumption of 300 g synbiotic yoghurt over 21 weeks significantly increased ($P < 0.002$) serum HDL-cholesterol by 0.3 mmol/L, leading to an improved ratio of LDL/HDL. In another study, Schaafsma *et al.* (1998) conducted a randomized, placebo-controlled, double blind and crossover designed study involving thirty volunteers (aged 33 to 64 years old; body weight of 66.5-98.0 kg) with mean total cholesterol of 5.23 ± 1.03 mmol/L and LDL-cholesterol of 3.42 ± 0.94 mmol/L. In this study, the authors observed that daily consumption of 375 mL synbiotic milk [containing of 10^7 - 10^8 CFU/g of *Lactobacillus acidophilus* and 2.5% (g/100g) of fructooligosaccharides] resulted in a significant decline in total

cholesterol ($P < 0.001$), LDL-cholesterol ($P < 0.005$) and LDL/HDL ratio ($P < 0.05$) of 4.4%, 5.4% and 5.3% respectively.

2.6 Cholesterol-lowering potential of probiotics, prebiotics and synbiotics: Contradictory findings

Although many studies have demonstrated convincing cholesterol-lowering effects of probiotics in both animals and humans, contradictory results have also surfaced. A study by Hatakka *et al.* (2008) refuted the purported cholesterol-lowering effect of probiotics, and reported that the administration of *L. rhamnosus* LC705 (10^{10} CFU/g per capsule; 2 capsules 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. In another study involving forty-six volunteers (aged 30 to 75 years old), Simons *et al.* (2006) found that the consumption of *Lactobacillus fermentum,* (2 x 10^9 CFU per capsule; 4 capsules daily) did not contribute to any lipid profile changes after 10 weeks. Lewis and Burmeister (2005) conducted a randomized, placebo-controlled double blind and crossover designed study to determine the effect of *Lactobacillus acidophilus* on human lipid profiles. In the study, eighty volunteers (aged 20 to 65 years old; baseline total cholesterol of > 5.0 mmol/L; mean Body Mass Index of 27.8 kg/m^2) consumed two capsules containing freeze-dried *L. acidophilus* (3 x 10^{10} CFU/2 capsules) three times daily for a duration of 6 weeks, and crossed over for another 6 weeks after a 6-week washout period. The authors found that *L. acidophilus* capsules did not significantly change plasma total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides of the subjects. Thompson *et al.* (1982) evaluated the effects of fermented dairy products

such as buttermilk fermented by *Streptococcus* (*St.*) *cremoris* and *St. lactis*, yogurt fermented by *L. bulgaricus* and *St. thermophilus* and milk inoculated with *L. acidophilus* on lipid profiles of sixty-eight healthy volunteers (26 male and 42 female). Lipid profiles of the subjects were not significantly changed after the 10-week trial. In another *in vivo* study involving seventy rats (with mean body weight of 146 g), Pulusani and Rao (1983) found that the feeding of milks fermented by *L. acidophilus*, *L. bulgaricus* or *St. thermophilus* did not contribute to any lipid profile changes after 4 weeks. The body lipids, liver lipids and liver cholesterol of the rats were also not affected. de Roos *et al.* (1999) conducted a randomized, placebo-controlled and parallel designed study to determine the effect of yogurt enriched with *Lactobacillus acidophilus* L-1 on human lipid profiles. In the study, seventy-eight male and female volunteers (aged 18 to 65 years old) with mean serum cholesterol levels of 3.9 to 7.8 mmol/L and mean Body Mass Index of 21.2 to 27.2 kg/m^2 were recruited. Subjects consumed 500 mL of either yogurt (enriched with *L. acidophilus* L-1 at concentration of 4.8×10^9 to 2.7×10^{10} CFU/500 mL) or control (plain yogurt without *L. acidophilus* L-1) daily for a duration of 6 weeks. The authors found that yogurt supplemented with *L. acidophilus* L-1 did not significantly affect blood lipid profiles of the subjects. Total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides were not changed after 6 weeks. Similar controversies were also raised from studies evaluating the cholesterol-lowering properties of prebiotics and also when probiotics and prebiotics were used together (synbiotic) (Table 2.1).

Table 2.1
Studies supporting the lack of significant improvements in lipid profiles by prebiotics / synbiotics supplementation

Compound(s)	Experimental design	Subjects	Dose; duration of the study	Effects	Ref
Inulin	Randomized, placebo-controlled, double - blind and crossover designed study.	8 volunteers	3-4 g/100 of inulin and wheat fiber daily for 12 weeks.	No significant improvement in lipid profiles.	Tarpila *et al.*, 2002.
Fructo-oligosaccharides (FOS)	Randomized, placebo-controlled, double-blind and crossover designed study.	10 diabetic patients (6 men and 4 women); with plasma total cholesterol 4.85-5.58 mmol/L.	20 g FOS/day for 4 weeks.	No significant improvement in lipid profiles.	Luo *et al.*, 2000.
Inulin	Randomized, placebo-controlled, double-blind and crossover designed study; with two six-week treatment periods, separated by a six-week washout period.	25 subjects; with baseline LDL-cholesterol ranging from 3.36 to 5.17 mmol/L.	45 g chocolate bar (containing of 18 g of inulin) daily during treatment period.	No significant improvement in lipid profiles.	Davidson *et al.*, 1998.
L. acidophilus and *B. longum* and fructo-oligosaccharides (FOS)	Randomized, single-blinded, placebo-controlled and parallel design trial.	55 normo-cholesterolemic volunteers.	3 capsules of synbiotics product (consisted of 10^9 CFU/g of *L. acidophilus* and *B. longum*, and 10-15 mg of FOS) once daily for 2 months.	No significant improvement in lipid profiles.	Greany *et al.*, 2008.

Such contradictory 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, dosage administered, analytical accuracy of lipid analyses, clinical characteristic of subjects, duration of treatment period, lack of statistical significant results, inadequate sample sizes, and lack of suitable control or placebo groups (Liong, 2007; Greany *et al.*, 2008). Although some of these studies failed to yield significant results, the reported cholesterol-lowering potential of probiotics and prebiotics supplementation warrants further research.

2.7 Dose-response effects

Although the cholesterol-lowering potential of probiotic and prebiotic has been widely studied, an accurate dosage administered has yet to be established. There is a lack of dose-response studies to determine the 'minimal effective dosage' of probiotics and/or prebiotics needed to reduce blood cholesterol levels. The concentration of probiotics in food products varies tremendously and there are currently no regulated standards for probiotic products to produce a cholesterol-lowering effect (FAO, 2006). 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 (Naruszewicz *et al.*, 2002) and 10^7 to 10^9 CFU/day in animals (Ha *et al.*, 2006; Lubbadeh *et al.*, 1999), some probiotics

have been shown to be efficacious at lower levels, while some require a substantially higher amount to exert a cholesterol-lowering effect.

The administration of *L. plantarum* 299v at a dosage of 5.0×10^7 CFU/mL (with consumption of 400 mL/d of a rose-hip drink) 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* (10^9 CFU/capsule with consumption of 3 capsules in the morning daily) did not produce significant changes in lipid profiles (Greany *et al.*, 2004). This suggests that higher dosage may not necessarily translate to better effects on cholesterol, as compared to lower dosage. Different strains need varying dosage to exhibit cholesterol-lowering effects (Table 2.2). Clinically effective dosage of probiotics should only be established based on studies of the specific strains conducted in humans.

Similar to probiotics, there is also no recommended daily dosage of prebiotics that specifically exert a cholesterol-lowering effect (FAO, 2007). Past studies have demonstrated the efficiency of various prebiotics and the combination of prebiotics and oligosaccharides in different dosages. While one study demonstrated the efficacy of lactulose and L-rhamnose in reducing fasting blood triglycerides, at dosages of 15 g/day and 25 g/day respectively (Vogt et. al., 2006), another study showed that arabinogalactan administered in dosages up to 30 g/day produced insignificant effect on lipid profiles (Robinson *et al.*, 2001). It appears that the cholesterol-lowering effect is specific to the types of prebiotics (Table 2.3). These inconsistent findings call for more in-depth studies to ascertain the proper dosage of prebiotics specifically targeting a cholesterol-lowering effect.

Table 2.2

Dose-response effects of different probiotic strains on lipid profiles

Models	Products/ Probiotics strains	Animals/Subjects	Dose; duration of the study	Effects	Ref
Animal	*L. plantarum* CK 102 (healthy human isolate)	*32 Sprague-Dawley male rats; 5 weeks old; induced hypercholesterolemic; mean BW of 129±1 g.	5.0×10^7 CFU/mL daily, 6 weeks.	TC: 27.9% decrease ($P < 0.05$) LDL-C: 28.7% decrease ($P < 0.05$) TG: 61.6% decrease ($P < 0.05$)	Ha *et al.*, 2006.
	L. acidophilus (wild chickens & human isolates)	*30 Awassi weaning lambs; hypercholesterolemic; mean BW of 55.1±3.4 & 57.9±4.7 kg for the treated & control groups, respectively.	1×10^9 CFU/ capsule, 2 capsules daily, 120 days.	TC: 22.6% decrease ($P < 0.05$) [treatment group with mean plasma TC of 72.8±5.7 mg/100 mL; control group with mean plasma TC of 94.0±7.8 mg/100 mL]	Lubbadeh *et al.*, 1999.
	L. plantarum KCTC3928 (Cellbiotech Co. Ltd, Korea)	*21 six-week-old C57BL/6 male mice; induced hypercholesterolemic.	1×10^9 CFU/mL of *L. plantarum* KCTC3928, 4 weeks.	TC: 33% decrease ($P < 0.05$) LDL-C: 42% decrease ($P < 0.05$) TG: 32% decrease ($P < 0.05$) HDL-C: 35% increase ($P < 0.05$)	Jeun *et al.*, 2009.
	Yogurt (with starter cultures); with *L. acidophilus* (Chr. Hansen Laboratorium, Denmark).	*60 white male mice; induced hypercholesterolemic; mean BW of 22 g.	1×10^7 CFU/mL daily for 56 days.	TC: 31.0% decrease ($P < 0.01$) LDL-C: 51.4% decrease ($P < 0.01$)	Akalin *et al.*, 1997.
	20% of skimmed buffalo milk coprecipitate; with *L. casei* (National Collection of Microorganisms Unit, National Dairy Research Institute, Karnal, India) and *Saccharomyces boulardii* (Department of Food Science and Human Nutrition, UNSW, Sydney, Australia).	*20 young Swiss male mice; induced hypercholesterolemic; mean BW of 20 ± 2 g.	1×10^6 cells/mL daily for 42 days.	TC: 17.9% decrease ($P < 0.05$) LDL-C: 35.6% decrease ($P < 0.05$) TG: 8.9% decrease ($P < 0.05$) HDL-C: 6.0% increase ($P < 0.05$)	Sindhu and Khetarpaul, 2003.
	Fermented skimmed milk; with *L. casei* strains (culture collection of the Department of Science and Technology, University of Verona, Italy).	*25 female Wistar rats with mean BW of 208.2 ± 8.3 g; induced hypercholesterolemic.	10 mL/kg body weight of fermented skimmed milk (containing 9.3 $\log_{10}$ CFU/mL of *L. casei*) daily for 10 days.	TC: 13.5% decrease TG: 39% decrease ($P < 0.05$) HDL-C: 10.7% increase	Bertazzoni-Minelli *et al.*, 2004.
	Fermented whole milk; with *L. acidophilus*,	*72 male Golden Syrian hamsters with age of 6-week-	Fermented whole milk (containing 1 x 10^{5-6}	TC: 37.5% decrease ($P < 0.001$) LDL-C: 50.9% decrease ($P <$	Chien *et al.*, 2010.

	Bifidobacterium lactis, *St. thermophilus* and *Lactobacillus bulgaricus*.	old; mean BW of 107.8 ± 2.8 g; induced hypercholesterolemic.	CFU/mL of probiotic mixture) daily for 28 days.	0.001)	
	Kefir; with *Lactococcus lactis* subsp. *lactis*,*Lactococcus lactis* subsp. *cremoris*, *St. lactis* subsp. *diacetyiactis*, *St. salivarius* subsp. *thermophilus*, *Leuconostoc cremoris*, *Lactobacillus delbrueckii* subsp. *bulgarics*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus acidophilus*, *Lactobacillus casei*,, *Lactobacillus helveticus* and *Saccharomyces cerevisiae*.	*18 male Wister rats weighing 280-300 g; induced hypercholesterolemic.	5% of mixed probiotic cultures in kefir daily for 14 days.	TC: 31.6% decrease ($P < 0.05$)	Tamai *et al.*, 1995.
Human	*L. plantarum* 299v (ProViva)	*36 healthy volunteers with moderately elevated fibrinogen concentrations (>3.0 g/L); 35-45 years old; mean TC of 5.59±0.88 mmol/L for treatment group & 5.51±0.75 mmol/L for control group.	400 mL of rose-hip drink containing 5.0×10^7 CFU/mL daily, 6 weeks.	TC: 2.5% decrease LDL-C: 7.9% decrease	Naruszewicz *et al.*, 2002.
	Enterococcus faecium & 2 strains of *Streptococcus thermophilus* (Causido®; Gaio®)	**32 patients; 36-65 years old; mean TC of 248.47±26.75 mg/dl, mean LDL-C of 172.22±21.17 mg/dl.	200 g of Gaio® containing 10^5-10^9 /mL of *E. faecium* & 5 - 20 x 10^8 /mL of *S. thermophilus* daily, 16 weeks.	TC: 5.3% decrease ($P = 0.004$) LDL-C: 6.15% decrease ($P = 0.012$)	Bertolami *et al.*, 1999.

TC: Total cholesterol; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; TG: triglycerides; BW: body weight

*Experimental design: Randomized, placebo-controlled, double-blind and parallel

**Experimental design: Randomized, placebo-controlled, double-blind and crossover