คุณสมบัติในการลดคอเลสเตอรอลของแลคโตบาซิลลัสสายพันธุ์ไทย

นางสาวภาวิณี ทรัพย์สมวงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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ปีการศึกษา 2555

HYPOCHOLESTEROLEMIC PROPERTY OF *LACTOBACILLUS* THAI ISOLATES

Miss Phawinee Subsomwong

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Microbiology
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Thesis Title	HYPOCHOLESTEROLEMIC PROPERTY OF *LACTOBACILLUS* THAI ISOLATES
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ภาวิณี ทรัพย์สมวงศ์ : คุณสมบัติในการลดคอเลสเตอรอลแลคโตบาซิลลัสสายพันธุ์ไทย (HYPOCHOLESTREROLEMIC PROPERTY OF *LACTOBACILLUS* THAI ISOLATES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.สมหญิง ธัมวาสร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.ดร. อัษฎาศ์ ลีพหนิชกุล, 64 หน้า

การมีระดับคอเลสเตอรอลในเลือดสูง เป็นสาเหตุของโรคหลายชนิดรวมทั้ง โรคหัวใจและหลอดเลือดซึ่งเป็นสาเหตุหลักของการเสียชีวิตในประชากรทั่วโลก การควบคุมอาหารและการใช้ยาเป็นวิธีที่ใช้ในการลดระดับคอเลสเตอรอลในเลือด มีรายงานแสดงให้เห็นว่าการใช้โพรไบโอติก เช่น แลคโตบาซิลลัส สามารถลดระดับคอเลสเตอรอลได้ การศึกษานี้มีวัตถุประสงค์เพื่อหาแลคโตบาซิลลัสที่มีความสามารถในการลดระดับคอเลสเตอรอล จากการทดสอบแลคโตบาซิลลัสที่แยกได้จากอุจจาระเด็กทารกและผู้ใหญ่สุขภาพดีจำนวน 51 สายพันธุ์ พบว่ามี 19 สายพันธุ์ สามารถสร้างเอนไซม์ bile salt hydrolase และใช้คอเลสเตอรอลในอาหารเลี้ยงเชื้อได้ จำนวน 6 สายพันธุ์ การศึกษาแลคโต-บาซิลลัส 19 สายพันธุ์ที่สร้างเอนไซม์ bile salt hydrolase พบว่า 9 สายพันธุ์ สามารถดีคอนจูเกท bile salt ชนิด TCA(sodium taurocholate) และ 10 สายพันธุ์ ดีคอนจูเกท TCA และ GCA (sodium glycocholate) การทดสอบแลคโตบาซิลลัสสายพันธุ์ที่ลดคอเลสเตอรอลในหลอดทดลองในการทนต่อเกลือน้ำดีและกรด พบว่า 2 สายพันธุ์ (แลตโตบาซิลลัส เฟอร์เมนทัม Lac31 และ และแลคโตบาซิลลัส แพลนทารัม L61-1) สามารถทน 0.3% เกลือน้ำดี และ พีเอช 3 การทดสอบในสัตว์ทดลอง พบว่าแลคโตบาซิลลัสเฟอร์เมนทัมไม่มีคุณสมบัติในการลดระดับคอเลสเตอรอลในหนู BALB/c แต่ไม่พบการแพร่กระจายของเชื้อไปที่เลือด ตับ และม้าม

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2555 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5387834120 : MAJOR MEDICAL MICROBIOLOGY
KEYWORDS : *LACTOBACILLUS*, HYPOCHOLESTEROLEMIC EFFECT, CHOLESTEROL ASSIMILATION, BILE SALT HYDROLASE

PHAWINEE SUBSOMWONG: HYPOCHOLESTREROLEMIC PROPERTY OF *LACTOBACILLUS* THAI ISOLATES.ADVISOR: SOMYING TUMWASORN, Ph.D., CO-ADVISOR: ASADA LEELAHAVANICHKUL, M.D., Ph.D., 64pp.

High level of cholesterol causes various kinds of disease including cardiovascular disease (CVD) which is the most major cause morbidity and mortality worldwide. Control of fat diet, exercise and medication are common method to reduce cholesterol. It has been reported that *Lactobacillus* probiotics can prevent and/or treat hypercholesterolemia in humans. This study aims to search for *Lactobacillus* Thai isolates with hypocholesterolemic property. Fifty –one *Lactobacillus* isolates from infant and adult feces were tested for bile salt hydrolase (BSH) production and cholesterol lowering activities by colorimetric assay. Nineteen isolates produced BSH and six isolates could assimilate cholesterol in vitro. Out of 19 BSH- producing *Lactobacillus*, 9 deconjugated sodium taurocholate (TCA) and 10 deconjugated both TCA and sodium glycocholate (GCA). Potential hypocholesterolemic *Lactobacillus* isolates were tested for bile and acid tolerance, 2 isolates (*L. fementum* Lac31 and *L. plantarum* L61-1) were found to survive in 0.3%oxgall and acidic pH 3 condition. *L. fermentum* Lac31 was tested for hypocholeterolemic effect in BALB/c mice but did not show the effect *L. fermentum* (Lac31) did not translocate to blood, liver and spleen.

Field of Study: …Medical Microbiology... Student's Signature……
Academic Year: ...2012..... Advisor's Signature……...
Co advisor's Signature……...

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LIST OF ABRIVIATIONS

µg	microgram
bp	base pair
cm	centimeter
DNA	deoxynucleic acid
dNTPs	deoxynucleotide triphosphates
g	gram
hr	hour
L	litter
mg	milligram
$MgCl_2$	magnesium chloride
ml	milliliter
mm	millimeter
mM	millimolar
NSS	Normal Saline Solution
^{o} C	degree Celcius
OD	optical density
PCR	polymerase chain reaction
rpm	revolutions per minute
TBE	Tris-boroate ethylenediamine tetraacetic acid
U	unit
UV	ultraviolet
V	voltage

CHAPTER I

INTRODUCTION

A high level of cholesterol in blood (hypercholesterolemia) can cause various kinds of disease, such as hypertension, hypercholesterolemia, cardiovascular disease (CVD) and atherosclerosis[1]. CVD is the most major cause of morbidity and mortality in many countries [2-4]. At the moment, there are many commonly used methods to reduce blood cholesterol such as diet control, exercising and taking medicines. Nevertheless it is noted that these mechanisms have some side effects to patients while the others may be ineffective for some patients.

In 1974 Mann and Spoerry reported that Maasai tribes' people who ingested fermented milk everyday had a lower serum cholesterol level in blood [5, 6]. There are many choices for reducing cholesterol level in blood such as diet modification [4], exercise, taking medicine and food supplement (probiotics). Probiotics are defined as "living organism that ingests appropriate amounts it will good for health and digestion" [1]. Numerous studies reported that probiotic such as *Lactobacillus* spp. can reduce cholesterol level in an in- vitro study and have hypocholesterolemic effect in vivo such as mice, rat, pig and human. It has been reported that some *Lactobacillus* strains can reduce a cholesterol level in vitro via bile salt hydrolase activity [7-12], assimilation of cholesterol into the cell [7, 9, 13-15], converting cholesterol to coprostanol [14] and binding with cholesterol [9, 10]. Hypocholesterolemic effect of *Lactobacillus* has been demonstrated in an animal model. Nguyen *et al*., reported that *L. plantarum* PH04 had a cholesterol lowering effects in mice [2]. Taranto *et al*., were investigated hypocholesterolemic effect of *L. reuteri* in hypercholesterolemic mice. They found that *L. reuteri* reduced triglyceride, and increased the ratio of HDL: LDL. Moreover, this strain has not only cholesterol lowering effect but also an ability to disappear the translocation of bacteria to spleen and liver. It is shown that this strain of *Lactobacillus* is a good quality probiotic to use [16]. Moreover, there is several reports show that it also has a hypocholesterolemic

effect in other animal models. Xie *et al*., reported that *L. plantarum* 9-41 and *L. fermentum* M1-16 had a hypocholesterolemic effect in Spraque-Dawley rats. Not only the cholesterol reducing effect in blood but they could increase the intestinal microbial balance as well [6]. Chul-gyu *et al*., reported that *L. plantarum* isolated from human feces had cholesterol lowering effect in Sprague-Dawley rats [5]. Rodas *et al*., investigated an activity of *L. acidophilus* ATCC43121 in swine with hypercholserolemia induced by diet [17]. Kiebling *et al.,* tested the hypocholesterolemic effect of yoghurt supplemented with *L. acidophilus* 145 and *B. longum* 913 in women. They found that the consumption of these bacteria resulted in the increase of HDL cholesterol level and may improve the LDL/HDL ratio in long term consumption of yoghurt's group [18]. In addition Baroutkoub *et al*., performed a clinical trial in study about hypocholesterolemic effect of *L.acidophilus* and *Bifidobacterium* in hypercholestromic patients by probiotic yoghurt with *L. acidophilus* and *Bifidobacterium* could reduce of total cholesterol and LDL cholesterol and rise HDL cholesterol [19]. The consumption of fermented products by the kind of bacteria could be an alternative way to increase the effectiveness of the treatment. This study aims to search for *Lactobacillus* Thai isolates with hypocholesterolemic effects.

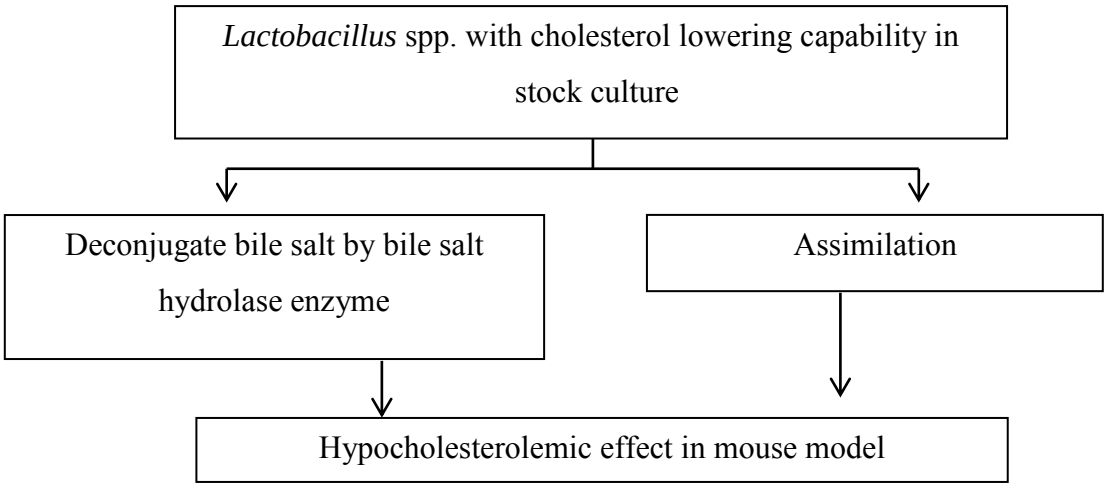
Hypothesis

Specific strains of *Lactobacillus* spp. Thai isolates have hypocholesterolemic properties to decrease the cholesterol both *in vitro* and *in vivo*.

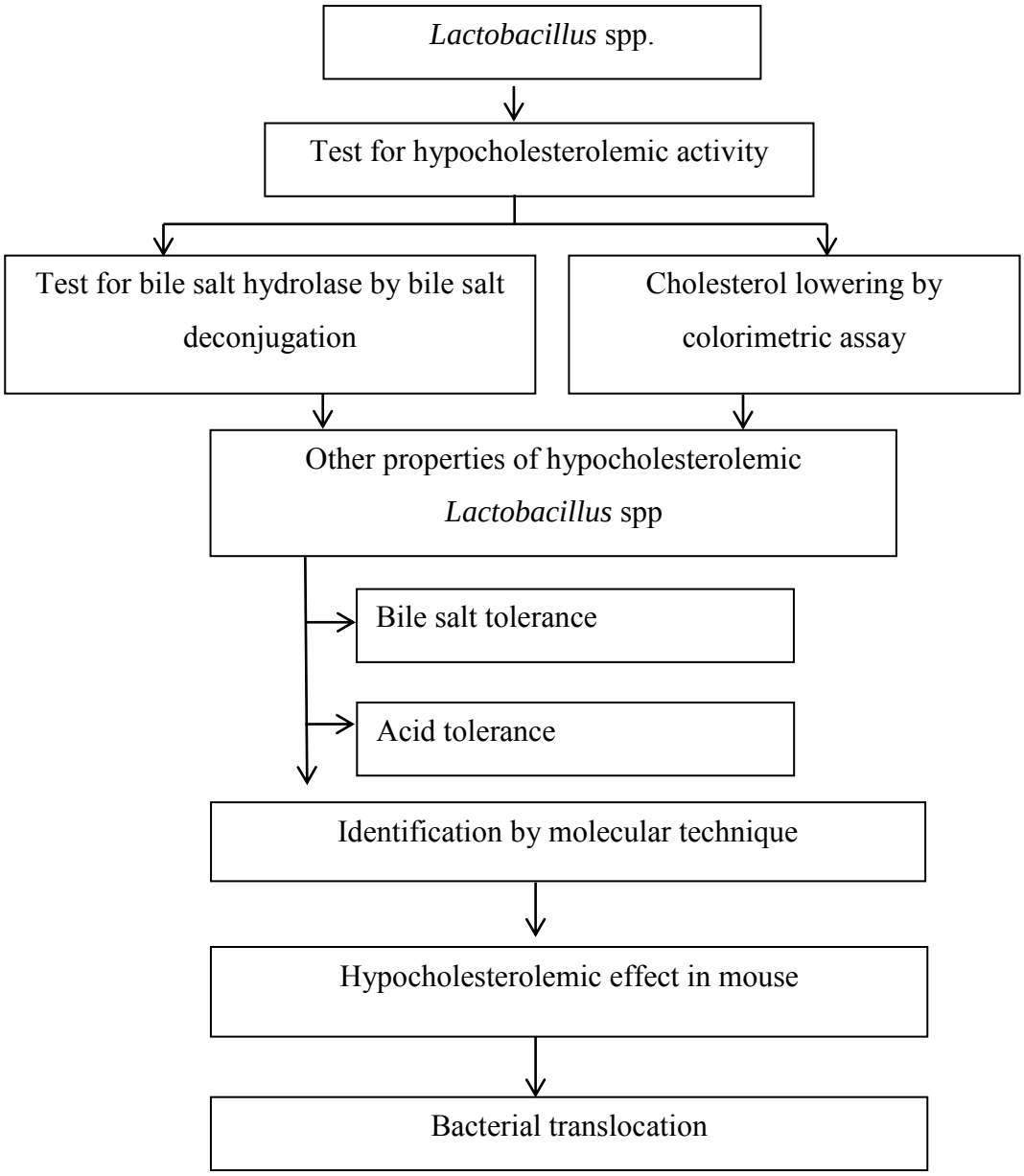
Objective

1. Determine *Lactobacillus* spp. for hypocholesterolemic activities
2. Determine other properties of hypocholesterolemic *Lactobacillus* spp. such as bile and acid tolerance.
3. Confirm hypocholesterolemic effect *of Lactobacillus* spp. in mouse model

Conceptual frame work



Work flow


Lactobacillus spp.
Test for hypocholesterolemic activity
Test for bile salt hydrolase by bile salt deconjugation
Cholesterol lowering by colorimetric assay
Other properties of hypocholesterolemic *Lactobacillus* spp
Bile salt tolerance
Acid tolerance
Identification by molecular technique
Hypocholesterolemic effect in mouse
Bacterial translocation

CHAPTER II

LITERATURE REVIEW

Cardiovascular disease (CVD)

Cardiovascular disease is the leading cause of deaths worldwide although, cardiovascular mortality rates have declined in many high-income countries since the 1970s. The mortality rate was estimated to be approximately 17.13 million deaths per year and growing to 23.6 million deaths in 2030[20]Low- and middle-income countries have very fast increase of cardiovascular deaths and diseases . Although cardiovascular disease mostly affects in older adults, atherosclerosis begins in early life. Healthy eating, exercise and no smoking are the prevention method to avoid risk factor of CVD.

CVD risk factors

a) Obesity

Obesity is defined as abnormal or excessive fat accumulation and it is a significant problem in developing and developed countries. Overweight is major risk factor for chronic disease such as diabetes, cvd, cancer, hypertension[1]. A person who has BMI(body mass index) over 30 kg/m^2 is considered to obese, while BMI equal or more 25 kg/m^2 is considered overweight[20]

b) Hypertension

Hypertension is defining high blood pressure. There are 2 type of blood pressure to measurement. One is systolic which depend on the heart muscle is containing and the other one is diastolic which the heart muscle relax. Hypertension was classified to 4 categories. First is normal blood pressure(BP) is defined as systolic blood pressure(SBP) is less than 120 mgHg and diastolic blood pressure(DBP) is less than 80 mmHg. Second is prehypertension is defined as 120-130 mmHg for SBP and 80-90 mmHg for DBP. Stage on progression hypertension is defined 140-159 mmHg and 90-99 mmHg for SBP and DBP, respectively. SBP over 160 mmHg and DBP over 100 mmHg are stage two of hypertension[14]

c) Hypercholesterolemia

Hypercholesterolemia means high level of cholesterol in the body. Cholesterol can be obtained from food and synthesized by organs. High cholesterol causes heart disease, heart attack, and stroke. High level cholesterol circulats in the blood and deposits called "plaque" at the artery wall. The vessel is narrow by blocking of the plaque. The normal range for total blood cholesterol is between 140-200 mg/dL. High density lipoprotein (HDL) cholesterol is good cholesterol. Low density lipoprotein (LDL) cholesterol is bad cholesterol. HDL can prevent CVD and atherosclerosis via extraction of LDL from artery wall to liver. When cholesterol is high,it meansthere are high levels of LDL cholesterol, normal or low HDL cholesterol and normal or high level of triglyceride. High cholesterol can be prevented by exercise and have a healthy diet. Some people could not change their life style and cholesterol is still high. So doctor will recommend the medications to cure the normal cholesterol.

d) Hypertriglyceridemia

Hypertriglyceridemia is defined as an abnormally high triglyceride concentration in the blood. A normal fasting plasma triglyceride concentration is considered below 150 mg/dl, borderline high at 150 to 199 mg/dl, high at 200 to 499 mg/dl and very high at 500 mg/dl or above. A high plasma triglyceride level is associated with increased risk factor cardiovascular disease, especially myocardial infraction. Hypertriglyceridemia may be caused by a genetic defect or secondarily by acquired factors, such as obesity, physical inactivity, ethanol consumption, diabetes mellitus, hypothyroidism, and drugs that either stimulate triacylglycerol synthesis or retard triacylglycerol catabolism.

Cholesterol

Cholesterol is the major sterol in humans. The sterol structure of cholesterol consists of four fused rings, three six-carbon and one five-carbon, designated A to D. Cholesterol has a hydroxyl group at C3, a C5-C6 carbon-carbon double bond, and two methyl groups, attached at positions C10 and C13 of the sterol ring. In addition, cholesterol has a branced eight-carbon hydrocarbon chain attached to the D ring at C17 (Figure 1)

cholesterol in the circulation does not cross the blood-brain barrier, all cholesterol in the brain must be synthesized within the central nervous system.

All of carbon atoms of cholesterol are derived from acetyl-CoA, which can be obtained from several sources, including the pyruvate dehydrogenase reaction, B-oxidation of fatty acids, oxidation of amino acids, and ethanol.

Cholesterol biosynthesis is regulated at the level of HMG-CoA reductase.

All 27 carbons of cholesterol are derived from the acetyl group in acetyl-CoA. The enzymes of the biosynthetic pathway, which number close to 30, are in the cytosol and the ER. The first reactions, up to 3-hydroxy-3 – methyl glutaryl-coenzyme A (HMG-CoA), are shared with the synthesis of ketone bodies. However, ketogenesis is mitochondrial, whereas the HMG-CoA synthase of cholesterol synthesis is cytoplasmic.

The NADPH-dependent formation of mevalonate by HMG-CoA reductase is the committed and regulated step of cholesterol synthesis. HMG-CoA reductase is feedback inhibited by free cholesterol. Cholesterol reduces the transcription of the down of the enzyme protein. HMG-CoA reductase has a lifespan of approximately 4 hours; therefore, a change in its rate of synthesis or degradation can affect cholesterol synthesis rather rapidly.

Insulin stimulates HMG-CoA reductase, most likely by inhibiting the AMP-activated protein kinase. Like acetyl-CoA carboxylase, HMG-CoA reductase is phosphorylated and inactivated by this insulin-inhibited enzyme.

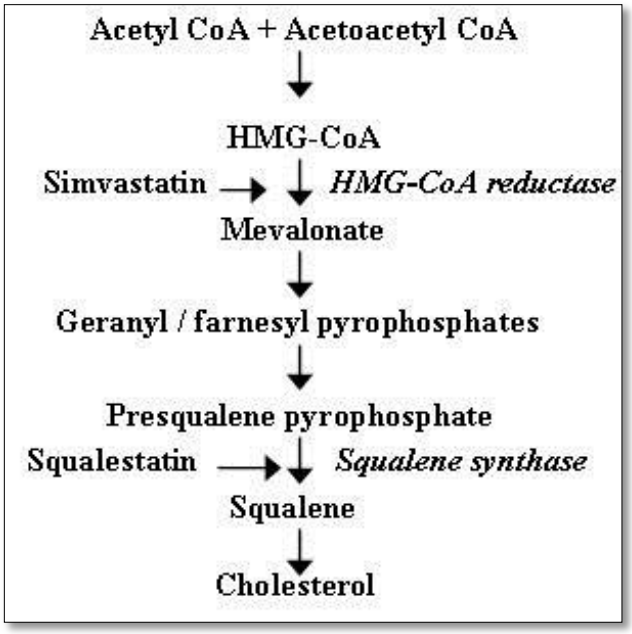


Figure2. Cholesterol Biosynthesis

Triglyceride

A triglyceride (TG, triacylglycerol, TAG,or triacylglyceride)is an ester derived from glycerol and three acids. Triglycerides are a blood lipid that help enable the bidirectional transference of adipose fat and blood glucose from the liver. There are many triglycerides: depending on the oil source, some are highly unsaturated, some less so.

The enzyme pancreatic lipase acts at the ester bond, hydrolysing the bond and "releasing" the fatty acid. In triglyceride form, lipids cannot be absorbed by the duodenum. Fatty acids, monoglycerides (one glycerol, one fatty acid), and some diglycerides are absorbed by the duodenum, once the triglycerides have been broken down.

In the intestine, following the secretion of lipases and bile, triglycerides are split into monoacylglycerol and free fatty acids in a process called lipolysis, which are subsequently moved to absorptive enterocytes, cells lining the intestines. The triglycerides are rebuilt in the enterocytes from their fragments and packaged together with cholesterol and proteins to form chylomicrons. These are excreted from the cells and collected by the lymph system and transported to the large vessels near the heart

before being mixed into the blood. Various tissues can capture the chylomicrons, releasing the triglycerides to be used as a source of energy. Fat and liver cells can synthesize and store triglycerides. When the body requires fatty acids as an energy source, the hormone glucagon signals the breakdown of the triglycerides by hormone-sensitive lipase to release free fatty acids. As the brain cannot utilize fatty acids as an energy source (unless converted to a ketone), the glycerol component of triglycerides can be converted into glucose, via glycolysis by conversion into Dihydroxyacetone phosphate and then into Glyceraldehyde 3-phosphate, for brain fuel when it is broken down. Fat cells may also be broken down for that reason, if the brain's needs ever outweigh the bodies.

Triglycerides cannot pass through cell membranes freely. Special enzymes on the walls of blood vessels called lipoprotein lipases must break down triglycerides into free fatty acids and glycerol. Fatty acids can then be taken up by cells via the fatty acid transporter (FAT). Triglycerides, as major components of very-low-density lipoprotein (VLDL) and chylomicrons, play an important role in metabolism as energy sources and transporters of dietary fat. They contain more than twice as much energy (approximately 9 kcal/g or 38 kJ/g) as carbohydrates (approximately 4 kcal/g or 17 kJ/g).

Bile acids

Cholesterol is 27-carbon lipid containing a fuse four-ring structure and a hydrocarbon chain. Except for the one hydroxyl group at C3, cholesterol is completing nonpolar. By contrast, bile acids contain 24 carbon atoms and are more polar than cholesterol: the steroid ring of bile acids contains one or more additional hydroxyl groups and the shorter hydrocarbon side chain terminates in a carboxyl group. In addition, the stereochemistry of the steroid nucleus is modified, resulting in a planar structure in which all of the hydroxyl groups are situated on the same side of the plane of the molecule.

The so-called “bile salts” are actually bile acids that contain an amino acid which is conjugated in amide linkage to the side chain of the carboxyl group of the bile acid. The two amino acids used most commonly by the liver to conjugate human bile acids are glycine which is predominant in adults, and the sulfur amino acid taurine, which is predominant in infants. Conjugated bile acids are more ionized at the slightly acidic pH

of the intestinal lumen than their nonconjugated counterparts and are therefore better emulsifying agents.

Bile salts play a major role in the digestion and absorption of triacylglycerols and cholesteryl esters. Bile salts emulsify dietary lipids in the gastrointestinal tract and stabilize the resulting mixed micelles. Along with phophatidylcholine, bile salts solubilize the cholesterol and bile pigments present in bile, preventing formation of precipitates (stones) of cholesterol or bilirubin in the gallbladder and bile ducts. In addition, formation of bile salts represents the only significant metabolic mechanism for eliminating excess cholesterol from the body.

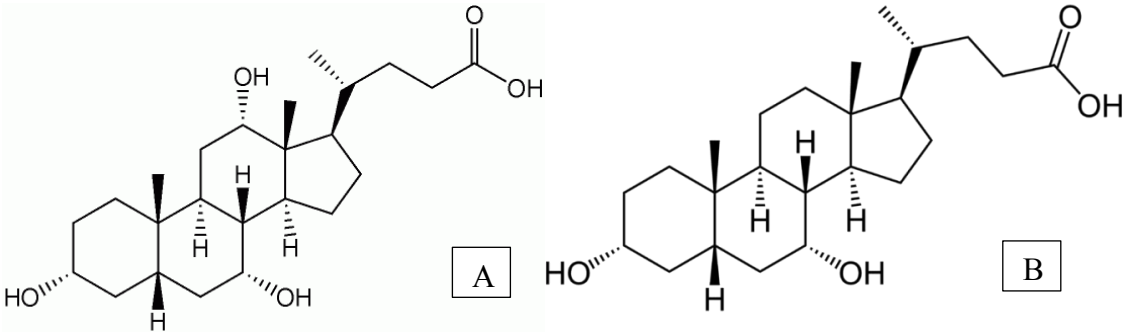


Figure 3. Primary bile acid ; A. cholic acid, B. chenodeoxycholic acid

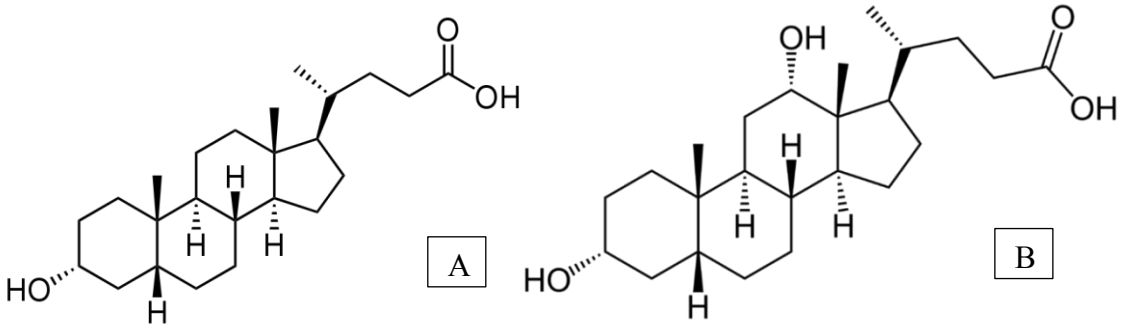


Figure 4. Secondary bile acid ; A. lithocholic acid, B. deoxycholic acid

Bile acids are synthesis from cholesterol

The steroid ring system of cholesterol cannot be degraded in the human body. Cholesterol has to be disposed of by the biliary system, either as such or after conversion to bile acids.

Bladder bile contains approximately 400 mg/dl of nonesterified cholesterol. Because only approximately half of this is absorbed by the intestine, nearly 500 mg of unmetabolized cholesterol can be eliminated from the body per day. Intestinal bacteria metabolize cholesterol to various “neutral sterols”.

Approximately half of the cholesterol eventually is metabolized to the primary bile acids in the liver. They include cholic acid and chenodeoxycholic acid, with cholic acid being the more abundant. The liver secretes the bile acids not in the free form but as conjugation products with glycine or taurine. The ratio of glycine conjugates to taurine conjugates is about 3:1. Bile acids are not useless excretory products; they serve a vital function in lipid absorption.

Bile acid synthesis is feedback-inhibited

The committed step in bile acid synthesis is catalyzed by the microsomal enzyme 7-α-hydroxylase. This monooxygenase reaction requires molecular oxygen, NADPH, and cytochrome P-450. Ascorbate also seems to be involved. Ascorbate deficiency (scurvy) impairs the formation of bile acids and causes cholesterol accumulation and atherosclerosis, at least in guinea pigs.

Bile acids reduce the level of 7α-hydroxylase by inhibiting transcription of its gene. Interesteringly, bile acids also reduce the activity of HMG-CoA reductase. Cholesterol, in contrast, induces 7α-hydroxylase synthesis in addition to inhibiting HMG-CoA reductase. These regulatory effects ensure the maintenance of an adequate pool of free cholesterol in the liver.

Thyroid hormones induce the synthesis of 7α-hydroxylase. This effect contributes to the increased plasma cholestererol level in patients with hypothyroidism.

Bile salt

Bile is yellow-green aqueous solution whose major constituents including bile acids, cholesterol, phospholipids and the pigment biliverdin[21, 22]. It is synthesized in the pericentral hepatocytes of the liver, stored and concentrates in the gallbladder interdigestively, and release into the duodenum after food intake. Bile functions as biololgical detergent that emulsifies and solubilized lipids, thereby playing an essential

role in the digestion. This detergent property of bile also confer potent antimicrobial activity, primarily through the dissolution of bacterial membrane [23, 24]

Bile salt has a membrane of functional roles, principally facilitating the intestinal digestion and absorption of dietary fats and fats soluble vitamins[25]. Bile salts are efficiently conserved in the enterohepatic circulation. After secretion into the intestine, the vast majority of bile salts are re-absorbed either through active transport in the terminal ileum and possibly the jejunum for conjugated bile salts or passive diffusion in the small and large intestine for unconjugated bile salts[26]. Following re-absorption, bile salts are transported through the portal circulation and returned to the liver, completing the enterohepatic circulation. In every bile cycle, approximately 4% of bile salts are lost in the feces and de novo bile salt synthesis from cholesterol is used to maintain the pool size under steady state conditions. Bile salts are essential in maintaining cholesterol homeostasis. Approximately 10% of cholesterol is excreted in an unmetabolized from and even less in utilized for other pathways, such as the production of steroid hormone and vitamin D[27]. Therefore, the conversion of cholesterol to bile salts in the liver and their subsequent secretion and fecal excretion provides the major route 90% for elimination of excess cholesterol.

Medication

There are many methods to reduced cholesterol in blood such as eating a well-balanced diet, getting regular exercise, and losing any excess weight. If these are not effective the doctor will order medicine to treatment. The main agents used clinically are statin, fibrates, inhibitors of cholesterol absorption, nicotinic acid or its derivatives and fish oil derivatives.

a) Statin (competitive inhibitors of HMG-CoA reductase)

The rate limiting enzyme in cholesterol synthesis is HMG-CoA reductase which catalyses the conversion of HMG-CoA to mevalonic acid. (Figure 5) These compounds are structural analogs of HMG-CoA. The medicines in this class are Lovastatin, atorvastatin, fluvastatin, pravastatin, simvastatin, rosuvastatin and pitastatin. (Figure 6) They are most effective in reducing LDL. Other effects include decreased oxidative stress and vascular inflammation with increased stability of artherolsclerosis lesion.

Statins are well tolerated; mild unwanted effects include myalgia, gastrointestinal disturbance, and raised concentration of liver enzymes in plasma, insomnia and rash.

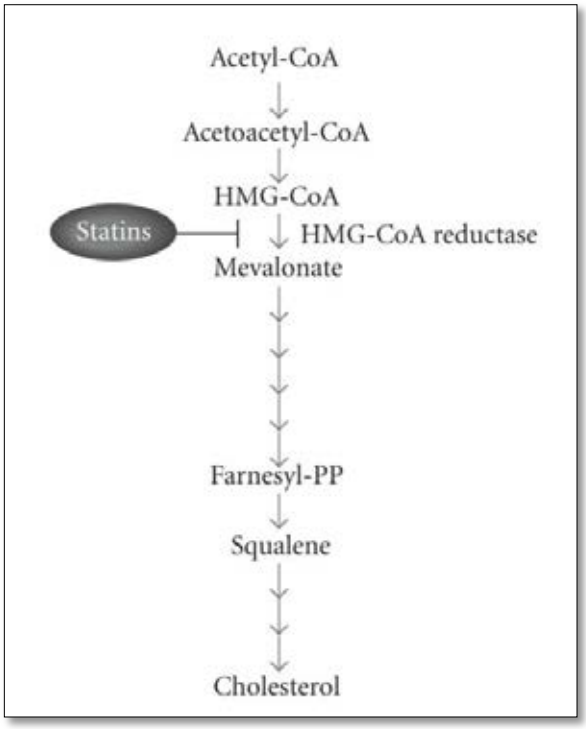


Figure 5. Site of action of Statins

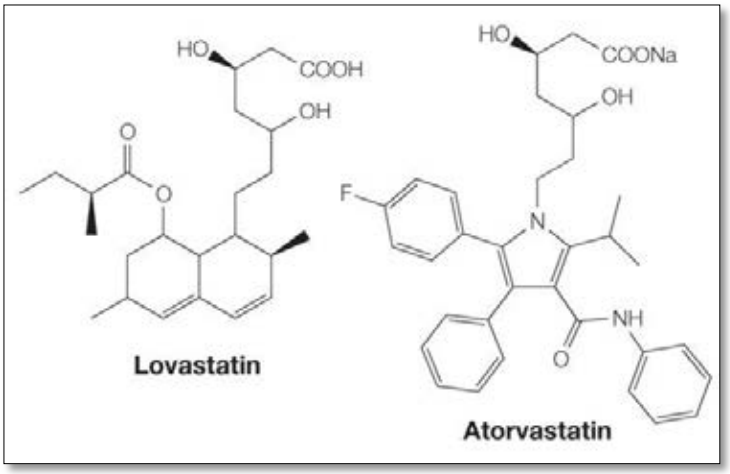


Figure 6. Statin derivatives.

b) Fibrates(Fabric acid derivatives)

Several fibric acid derivatives are available, including bezafibrate, ciprofibrate, gemfibrozil, fenofibrate and clofibrate.(Figure 7) These medicines decrease levels of VLDL and in some patients, LDL as well.

Myositis is unusual but can be severe with myoglobinuria and acute renal failure. It occurs particularly in patients with renal impairment, because of reduced protein binding and impaired drug elimination. Fibrates should be avoided in such patients and also in alcoholic individuals, who are predisposed hypertriglyceridemia but are at risk of rhabdomyolysis.

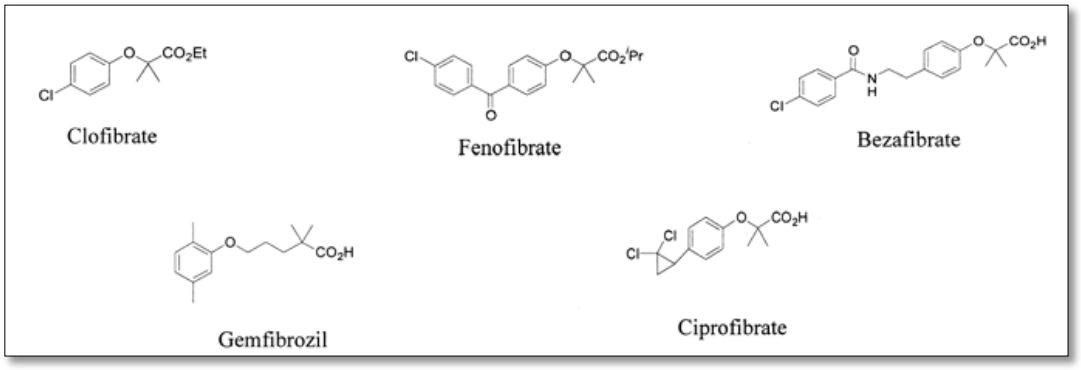


Figure 7. Fibrates derivative

c) Inhibition cholesterol absorbtion

Ezetimibe(Figure 8.) is the first member of a group of drugs that inhibit intestinal absorption of phytosterols and cholesterol.A transport protein, NPC1L1 appears to be the target of the drug.It is effective even in the absence of dietary cholesterol because it inhibits reabsorption of cholesterol excreated in the bile. Its primary clinical effect is reduction of LDL levels.

Ezetimibe is generally well tolerated but can cause diarrhea abdominal pain or headache; rash and angio-oedema.

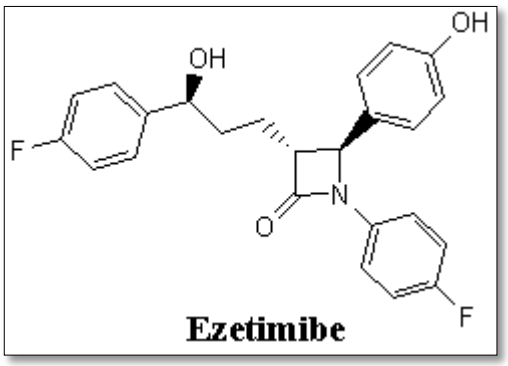


Figure 8. Structure of Ezetimibe

d) Bile acid binding resins

Colestyramine and colestipol are anion exchange resins. The bile acids, metabolites of cholesterol are normally efficiently reabsorbed in the jejunum and ileum. Excretion is increase up to tenfold when resins are given, resulting in enhanced conversion of cholesterol to bile acids in liver via 7α-hydroxylation, which is normally controlled by negative feedback by bile acids.(Figure 9)

Because resins are not absorbed, systemic toxicity is low but gastrointestinal symptoms especially diarrhea are common and dose related. Resins are bulky and unappetizing. They interfere with the absorption of fat soluble vitamins, and of drugs such as chlorothiazide, diagoxin and warfarin, which should therefore be taken at least 1 hour before or 4-6 hours after the resin.

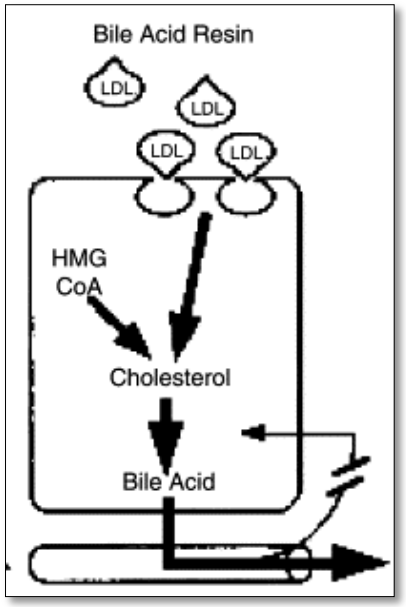


Figure 9. Mode of action of bile acid resins

e) Nicotinic acid derivatives

Nicotinic acid is a vitamin and as such is essential for many important metabolic processes. Quite separately from this, it has been used in gram quantities as a lipid-lowering agent. Nicotinamide inhibits hepatic triglyceride production and VLDL secretion with reductions in triglyceride and LDL-C including LP (a) and increase in HDL-C.

Flushing, patpitations and gastrointestinal disturbances are unwanted effects. Flushing is associated with production of PGD_2 and is reduced by taking the dose 30 minutes after aspirin. High dose can disturb liver function, impair glucose tolerance, and precipitate gout by increasing circulating urate concentration.

Combined drug therapy is useful when 1)VLDL leves are significantly increased during treatment of hypercholesterolemia with a resis 2) when LDL and VLDL leves are both elevated initially ; 3) when LDL or VLDL levels are not normalized with a single agent or 4) when an elevated level of Lp(a) or an HDL deficiency coexists with other hyperlipidemias. The lowest effective doses should be monitored more closely for evidence of toxicity.

Probiotics

Probiotics comes from 2 Greek words “Pro+bios” which means “for life” [28] WHO was defining probiotic as “live microorganism which administered in adequate amounts exerts health benefit on the host (FAO, WHO 2001).*Lactobacillus* and *Bifidobacterium* are the most popular to add for functional food[25].

Probiotics help balance normal flora in gut, lactose intolerance, reduce the risk of nosocomial diarrhea and rotavirus gastritis [29], reducing colonization of pathogen bacteria[30], improve immune system[31], antioxidative effect, reduction dermatitis and allergy symptom[32].

There are reports show probiotics have cholesterol lowering effects. More than 40 years Sharper *et al*., and Mann *et al*., reported men from the tribes of Samburu and Massai in Africa reduced serum cholesterol after consumption of large amounts of milk fermented with a wild *Lactobacillus* strain.[33, 34] Kieling *et al.*, reported hypocholesterolemic effect of yoghurt supplemented with *L. acidophilus* 145 and *B. longum* 913 in 29 healthy women[18]. Sindhu and Khetarpaul, reported hypocholesterol properties of *L. casei* NCDC19 (10^9 CFU/ml) and *Saccharomyces boulardii* (10^9) i n swiss mice. De Rodas *et al*. showed hypocholesterolemic effect of *L. acidophilus* ATCC43121 ($2.5x10^{11}$ cell/feeding) in swine[17].

Hypocholesterolemic mechanisms.

There are many mechanisms that probiotic used reduce cholesterol. These mechanisms are bile salt hydrolase production to deconjugation of bile salt, assimilation of cholesterol, and conversion of cholesterol to coprostanol by cholesterol reductase.

Deconjugation bile salt by BSH

The primary bile acids are cholic acid and chenodeoxycholic acid are synthesized de novo in the liver from cholesterol. The solubility of hydrophobic steroid nucleus is increased by conjugation as an N-acyl amidate with either glycine (glycoconjugate) and/or taurine(tauroconjugate) pior to secretion. The resulting molecules are therefore amphipathic and can solubilize lipids to form mixed micelles[35]. Deconjugation is catalyzed by bile salt hydrolase (BSH) enzyme (EC.3.5.1.24). Which hydrolyze the

amide bond and liberate the glycine/taurine moiety from the steroid core. The resulting acid are termed unconjugated or deconjuagated bile acids. (Figure 10)

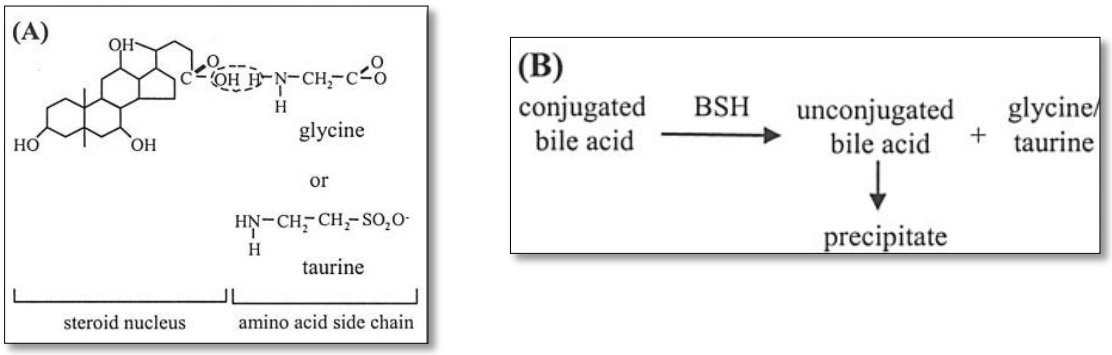


Figure 10. Structure of bile salt(A), BSH enzyme activity (B)

BSH activity is specific to the microbiota and is not present in Eukaryotic cells, substantiating the importance at the gut microbiota in cholesterol metabolism. BSH activity has been characterized in *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Enterococcus* and *Bacteriodes.*

Probiotic will be reduced cholesterol in bloods by BSH production to deconjuate bile salt. Deconjugate bile salts are less soluble and less efficiently reabsorbed from the intestinal lumen than their conjugated counterparts, which results in excretion of larger amounts of free bile acid in feces. Also, free bile salts are less efficient in the solubilization and absorption at lipids in the gut. So, the deconjugation of bile acids by *Lactobacillus* bacteria could lead towards a reduction in serum cholesterol either by increasing the demand of cholesterol for de novo synthesis of bile acids to replace that lost in feces or by reducing cholesterol solubility and absorption of cholesterol throughout the intestinal lumen.(Figure 11)[36]

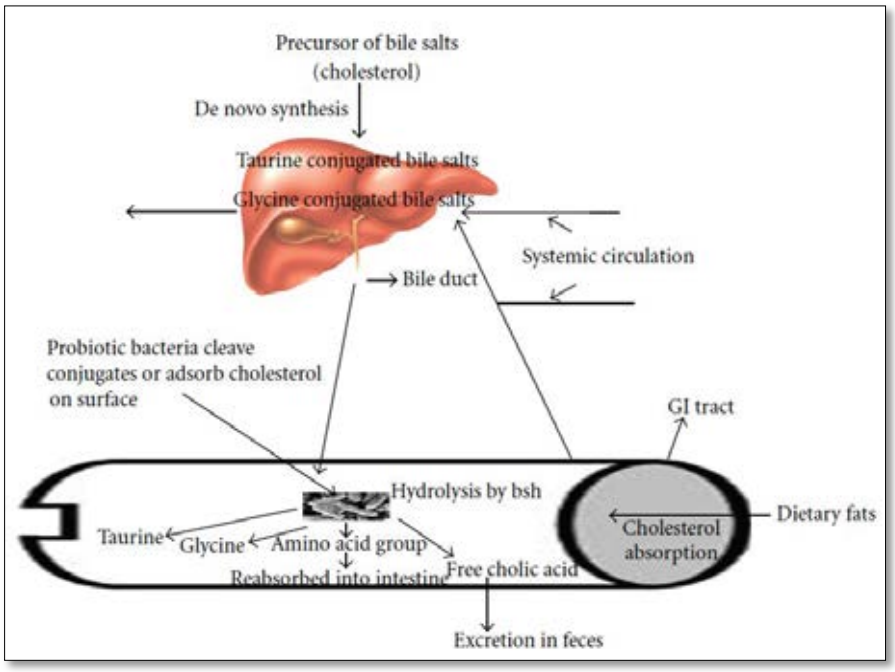


Figure 11. Cholesterol as the precursor for synthesis of new bile acids and the hypocholesterolemic role of bile salt hydrolase (BSH)

Micheal and Scott reported bile salt hydrolase activity in *Lactobacillus*. They were grown in MRS medium supplement with taurodeoxycholic, taurocholic or taurochenodeoxy cholic acid. There are two intensities 1) is the formation of precipitate holos around active colonies and 2) the formation of opaque granular white colonies[11]. Ahn *et al.* found bile salt hydrolase activity of *L.acidophilus* strains. These bacteria shown vary the formations such as precipitate halo, opaque granular white colonies, shiny precipitate halo or clearzone around active colonies on bile salt MRS agar plate[12].

a) Assimilation and/or incorporation of cholesterol to cell

Lactobacillus could assimilate cholesterol into the cell while growing and could bind cholesterol on cell while resting cell.

Vijendra and D.N. 2 showed *L. casei* reduced cholesterol in MRS broth medium supplement with cholesterol[35]. Their activity require strains specific. Moreover, lactic acid bacteria and *Bifidobacterium* could reduce cholesterol in media. *L. acidophilus* could assimilate cholesterol while growth and this strain will use as probiotic because it can

maintain viability for 2 h at pH2[15]. *L. buchneri* P2 could assimilate and incorporate cholesterol to cells. Growing and resting cell could reduce cholesterol more than killed cell[9]

b) Convert to coprostanol

Coprostanol is absorbed less in the human intestines compared with its parent cholesterol molecule, leading to increased excretion into feces and subsequently a reduced blood cholesterol level

Lye *et al.*, reported tested *Lactobacillus* could produce cholesterol reductase to change cholesterol to coprostranol[14].

Hypocholesterolemic effect in mice

Nowadays many studies have used animal model to confirm the effective of in vitro study. They study in rats, mice, hamster, guinea pig and pigs. Because these animals similarities with humans (cholesterol, bile acid metabolism, plasma lipoprotein distribution and regulation of hepatic cholesterol enzyme).

Nguyen *et al.*, reported hypocholesterolemic effect of *L. plantarum* PH04 in hypercholesterolemic mice for 14 days ($4x10^8$ CFU/ml, once a day). The results showed the significant reduction of total cholesterol and triglyceride compared to the control.[2] Taronto *et al.,* showed hypocholesterolemic effect of *L.reuteri* in hypercholesterolemic mice.They were received low dose of *L.reuteri* CRL1098 (10^4 CFU/ml per day) for 7 days. The result showed that low dose of *L.reuteri* could reduce 40% of triglyceride and 20% increase in ratio of high density lipoprotein to low density lipoprotein (ratio HDL: LDL)[16]. Moreover, this lactobacillus strain showed prevention effect because low dose of *L. reuteri* CRL1098 had effective in prevention of hypercholesterolemic mice. They reduced 22% total cholesterol, 33% triglyceride and increase the ratio HDL: LDL[38]. Wang *et al.,* investigated hypocholesterolemic properties of *L. paracasei* on lipid metabolism in hamster fed with a cholesterol enrich diet. They reported, hamster were received adlay milk (fermented with *Lactobacillus*) had low serum cholesterol level and ration LDL: HDL when compared with high cholesterol diet group[39].

Translocation of bacteria

Translocation is another important safety consideration as it may cause bacteremia, septicemia, and even multiple organ failure. M. Fernanda *et al*., [40] investigated *L .delbruckii* subsp. *lactis* UO004 for use as probiotic. There are many experiments to confirm that *Lactobacillus* was safety to use. But the simple method is translocation of bacteria to organ. In this study use BALB/c mice and divide the groups to received vary concentration of *Lactobacillus* (10^9, 10^8, 10^7 CFU/animal). Feed once a day for 4 days. The lympnode, liver, spleen were homogenize in PBS and plate on agar medium. The result show that no bacterial growth on agar plate. And they conclude that *L .delbrueckii* supsp. lactis UO 004 might be a good future candidate for use as a component in functional food.

Taranto *et al*., [16]investigate dose of *L.reuteri* CRL1098 (10^4,10^6, $10^7$and 10^8) that cause of translation to organ. Swiss albino mice were received this *Lactobacillus* via drinking water for 7 days. The result show *L. reuteri* did not induce side effect, the translocation of normal intestinal microflora in 10^4 CFU/ml. But 10^6-10^8 cell/day showed translocation of normal intestinal microflora to liver and spleen.

Nguyen *et al*., [2] reported *L .plantarum* PH04 reduced total serum cholesterol levels in mice and without any pathogenic effect and without bacterial translocation. They evaluated in blood, liver and spleen. All mice received 10^7 CFU of *Lactobacillus* for 14 days

CHAPTER III

MATERIALS AND METHODS

1. Bacterial strains and culture condition

Fifty- one *Lactobacillus* isolates from human feces of adults and infants stored in de Mann Rogosa-Sharpe (MRS) broth with 20% glycerol at -80 ^{0}C were cultured on MRS agar and incubated in an anaerobic condition (mixture of 80% N_2, 10% CO_2, and 10% H_2) at 37 ^{0}C for 48 h. The bacteria were subcultured twice before use in the experiment.

2. Hypocholesterolemic effect screening

2.1 Test for bile salt hydrolase (BSH) [5, 8]

A plate assay of Dash Kevicz and Feighner was used in the screening process[11]. MRS agar containing bile salt was prepared by adding either 0.5 mM sodium taurocholate (TCA) or sodium glycocholate (GCA). *Lactobacillus* was streaked on MRS agar supplemented with TCA or GCA. MRS agar without bile salt was used as a control. They were incubated at 37 ^{0} C in an anaerobic condition 72 h.

2.2 Test for cholesterol lowering by colorimetric assay [41]

Colorimetric assay as previously described (6) was used with minor modification as follow. Soluble cholesterol (polyoxyethanyl-cholesterol sebcate, Sigma, USA) was dissolved in sterile water to a concentration of 50 mg/ ml. MRS broth was sterilized by autoclaving at 121 ^{0}C for 15 min and then added with cholesterol to a final concentration of 0.5 mg/ml. *Lactobacillus* was added into MRS broth with cholesterol to a concentration of 10^8-10^9cfu/ml and incubated at 37 ^{0}C for 24 h in an anaerobic condition. An unfermented tube was used as a control.

Bacterial cells were removed by centrifugation at 2,000xg for15 min and 1 ml of supernatant was collected for cholesterol determination by colorimetric assay. One milliliter of 33% potassium hydroxide and 2 ml of absolute ethanol were added into supernatant tube which was mixed and incubated at 37 ^{0}C for 15 min in a water bath.

Distilled water (2 ml) and hexane (3 ml) were added and mixed for 1 min by vortex and 1 ml of hexane layer was transferred to a new test tube. Hexane was evaporated under the flow of nitrogen gas and 1 ml of op-thaldehyde was added and mixed together. After 10 minutes, 0.25 ml of concentrated sulfuric acid was added, mixed together and left for 30 min. The developed color was then be measured with spectrophotometer at 550 nm. Cholesterol reducing rate was calculated with the following formula.

Cholesterol reducing rate = $[(A_0\text{-}A)/A_0] \times 100$

(A_0 = unfermented tube)(A = fermented tube)

3. Other properties of cholesterolemic *Lactobacillus* spp.

3.1 Bile tolerance

MRS broth containing oxbile was prepared by adding 0.3% oxbile and 0.2% Thioglycolate. MRS-0.2%thio broth without 0.3% oxbile was used as control. *Lactobacillus* was suspended in 0.85% NSS to inoculum size as 10^9 CFU/ml. Fresh culture was inoculated (1%) into each media. They were incubated at 37^0 C in aerobic condition for 9 h. The growth was monitored every hour by using spectrophotometer at 620 nm. Percentage of survival was calculated with the following formula.

% survival = $[A_1/A_2] \times 100$

(A_1 = MRS with oxbile) (A_2 = control)

3.2 Acid tolerance [5]

MRS broth pH 1, 2 and 3 were prepared by adding hydrocholic acid (HCl) and with 0.2% Thioglycolate and MRS-0.2%thio broth alone was used as control. *Lactobacillus* was suspended in 0.85% NSS to inoculum size as 10^9 CFU/ml. Fresh culture was inoculated (10%) into each media. They were incubated at 37 ^{0}C in aerobic condition for 4 h. The viable cells were measure by plate count assay at 0, 1, 2, 3 and 4 h.

4. Genotypic identification of *Lactobacillus* isolates

Bacteria were sub cultured in MRS. They were incubated at 37 ^{0}C in an anaerobic condition for 48 h. Bacterial DNA was extracted from 2-3 colonies. They were picked into eppendorf with 200 µl of sterile distilled water inside. The suspension was mixed and spun down to removed supernatant and resuspened with 180 µl of sterile distill water. Solution of 20 µl 10X digestion buffer solution (5% tween20 and 10 mg/ml proteinase K in 0.2 M Tris pH 8.3) was added and incubated at 60 ^{0}C for 1 hour. Stop reaction by heating up to 100 ^{0}C 15 min. Supernatant was collected by centrifugation at 13,000 rpm 5 min and was collected for amplification and identification of *Lactobacillus* spp. by 16S rRNA gene sequencing.

Lactobacillus genus specific primers were L159F (5'-GGA AAC AG (A/G) TGC TAA TAC CG-3') and L677R (5'-CAC CGC TAC ACA TGG AG-3'[42]. The 25 µl of reaction mixture contains with 12.5 µl of Hot start master mix (GE Healthcare illustra, UK), 5 pmol of each primer, 5 µl of DNA template and 2.5 µl of water. Amplication consists of 95 ^{0}C for 5 min; 35 cycles of 95 ^{0}C for 30 s, 57 ^{0} C for 1 min, and 72 ^{0} C for min and a final extension of 72 ^{0}C for 5 min.

Bacterial isolate which gave positive result with genus-specific primers, was subjected to DNA sequencing. The 16S rRNA gene sequences was amplified by PCR using the universal primer 16S-8F (5'-AGA GTT TGA TCY TGG YTY AG-3') and 16S-1541R (5'-AAG GAG GTG WTC CAR CC-3')[43]. The 50 µl of reaction mixture contains with 25 µl of Hot start master mix (GE Healthcare illustra, UK), 5 pmol of each primer, 5 µl of DNA template and 15 µl of water. Amplication consists of 95^o C for 5 min; 35 cycles of 95 ^{0}C for 30 s, 57 ^{0}C for 1 min, and 72 ^{0}C for 10 min and a final extension of 72 ^{0}C for 10 min.

The PCR products were purified by QIA quick PCR purification kit (Qiagen Inc., USA). Sequencing was performed by using 10 ng purified PCR product with the same primer as in PCR amplification by the dideoxynucleotide chain termination method at the 1 st BASE Sequencing, Shan Alan, Malasia(http://www.base-asia.com) The nucleotide sequence was analyzed by using the sequence match program of Ribosomal Database Project II and GenBank DNA database search(www.ncbi.nlm.nih.gov/BLAST). The closest relative of the partial 16S rRNA gene sequences was evaluated. In general, when similarity values exceed 97% the strains were considered as the same species.

5. Test for hypocholesterolemic effect in mice

The male and female Balb/c mice were purchased from national laboratory animal center, Mahidol University (Nakhon Pathom, Thailand). These mice were kept in Laboratory animal room (Faculty of Medicine, Chulalongkorn University) at control temperature 25 ^{0}C with light/ dark 12 h. They were fed ad libitum for adaptive period (7 days).

Mice were randomly divided into 4 groups (Table 1) for prevention experiment. They were fed with two kinds of diets as shown in table 2. Bacterial suspension of $1.8x10^9$ CFU/500µl was oral administered once a day. Fresh water was given to all groups at all time. Their body weights were measured weekly. After 14 days, all mice were fast for 20 h and euthanized with isofluran. Blood sample was obtained from the retro-orbital vein, separated for serum by centrifugation at 12,000 xg for 3 min.[44] and kept at -80 ^{0}C until used. Serum samples were analyzed for the total cholesterol, triglyceride and LDL by commercial kit from Human Diagnostics Worldwide (Germany).

At the end of prevention experiment, these mice were divided into subgroups for treatment experiment (Table 3) and treated as described the prevention experiment. After 14 days, all mice were sacrificed by isofluran inhalation and cervical dislocation. Blood samples were collected by cardiac puncture. The liver and spleen were collected with aseptic technique for testing bacterial translocation. Serum was collected and processed as described above in the prevention treatment.

Table 1. Prevention experiment

1st week	2nd week
Chow + PBS	Chow + PBS
Chow +Hypo-*Lactobactillus*	High fat diet + Hypo-*Lactobactillus*
Chow + PBS	High fat diet + PBS
Chow + normal *Lactobacillus*	High fat diet + normal *Lactobacillus*

Hypo-*Lactobacillus*: *L. fermentum* (Lac31), normal *Lactobacillus: L. fermentum* (L34-1)

Table 2. The composition of diets

Chow	High fat diet
Rodent chow 50 g + 0.5 g of cassava flour	Rodent chow 50 g + 10 ml of pork oil + 0.5 g of cassava flour

Table 3. Treatment experiment

3-4st week
Chow + PBS
High fat diet + Hypo-*Lactobacillus*
High fat diet + PBS
High fat diet + normal *Lactobacillus*

Hypo-*Lactobacillus*: *L. fermentum* (Lac31), normal *Lactobacillus: L. fermentum* (L34-1)

6. Bacterial translocation

The bacterial translocation to blood and organs were determined. Liver and spleen were collected with aseptic technique, washed with sterile PBS and placed into 0.2 ml of brain heart infusion broth. After homogenization by using microtube grinder, 100 µl suspension of each organ suspension and 15 µl of blood were plated each on MRS agar. All plates were incubated for 72 h at 37 ^{0}C, anaerobically (80% N_2, 10% CO_2, and 10% H_2). The presence of 10 or more microorganisms on a plate was interpreted as bacterial translocation[2].

CHAPTER IV

RESULTS

1. Bile salt hydrolase (BSH) production by *Lactobacillus* spp.

Fifty-one *Lactobacillus* isolates from infant and adult feces were tested for bile salt hydrolase production via deconjugation of 2 types of bile salts, sodium taurocholate (TCA) and sodium glycocholate (GCA). Each bacterial isolate was streaked on MRS agar supplemented with 0.5% TCA or 0.5% GCA and MRS alone as a control. After 72 hr incubation, 19 *Lactobacillus* isolates deconjugated bile salts as shown by the precipitation of bile acid around *Lactobacillus* culture in streak line area (Figure 12). Nine *Lactobacillus* isolates could deconjugate TCA and 10 isolates could deconjugate both of TCA and GCA (Table 4).

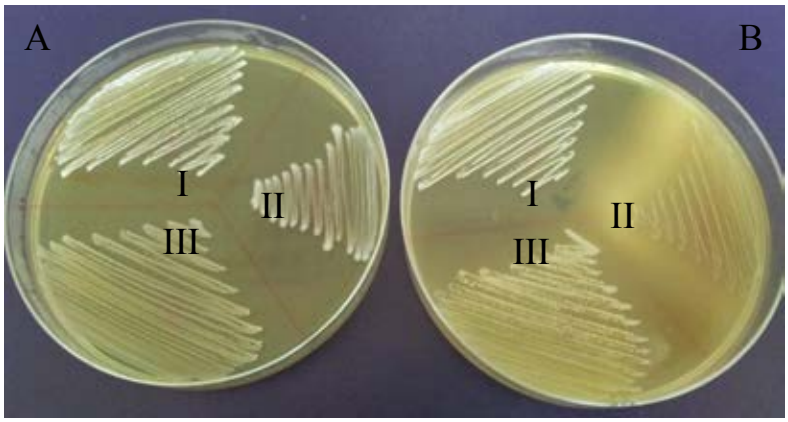


Figure 12. Detection of BSH production of *Lactobacillus*.
A: MRS as a control plate, B: MRS+bile salt
I; L61-1, II ; Lac31, III ; L62-1

2. Cholesterol lowering effect *in vitro*

All *Lactobacillus* isolates were also tested for hypocholesterolemic effect via cholesterol assimilation in media by colorimetric assay. Bacteria were inoculated in MRS broth supplemented with 0.5 mg/ml of soluble cholesterol. MRS broth without bacteria

was used as a control. Six *Lactobacillus* isolates could reduce cholesterol in media. The range of cholesterol lowering rate was 13.09% – 35.85%. (Table 4)

Table 4. Bile salt deconjugation and cholesterol lowering activity of *Lactobacillus* spp.

Code	Source	Deconjugation		%Cholesterol lowering	% survival rate
		TCA	GCA		
Lac31	Infant feces	+	-	26.44	20.83
L39-1	Adult feces	+	+	17.95	0
L41PR1	Adult feces	+	-	19.61	0
L45-1	Adult feces	-	-	13.09	20
L59-1	Adult feces	+	-	35.85	0
L62-1	Adult feces	+	+	24.54	100
L14-1	Adult feces	+	-	ND	0
L15-1	Adult feces	+	-	ND	0
L17-1	Adult feces	+	-	ND	0
L19-1	Adult feces	+	-	ND	0
L40-1	Adult feces	+	-	ND	0
L421	Adult feces	+	-	ND	44.44
L9-1	Adult feces	+	+	ND	10.49
L34-1	Adult feces	+	+	ND	23.33
L47-1	Adult feces	+	+	ND	50
L60-1	Adult feces	+	+	ND	23.73
L48-1	Adult feces	+	+	ND	42.86
L49-1	Adult feces	+	+	ND	34.12
L50-1	Adult feces	+	+	ND	0
L61-1	Adult feces	+	+	ND	25

ND=not detected

3. Other properties of hypocholesterolemic *Lactobacillus* spp.

a. Bile tolerance

Twenty *Lactobacillus* isolates which produced BSH and/or assimilated cholesterol in media were tested for the bile salt tolerance. Eleven isolates could survive in the presence of 0.3% oxbile with the rate of 10.9 – 100 % (Table 4)**.**

b. Acid tolerance

Three *Lactobacillus* isolates (Lac31, L62-1 and L61-1) were chosen for acid tolerance study on the basis of bile salt-tolerance, - deconjugation and - assimilation. Lac31 and L62-1 reduced cholesterol in media and produced BSH, and L61-1 produced only BSH. They were cultured in MRS supplement with hydrocholic acid to pH1, pH2, pH3 and MRS pH 6.4 as control. The survival curve of Lac31, L62-1 and L61-1 were shown in Figure 11. Lac31 was viable in MRS alone and pH3 after 4 h incubation. No viable cell was found in MRS pH2 after 3 h and MRS pH1 after 1 h. L62-1 cells were half decreased at pH3 after 3 h. No viable cell was found in MRS pH2 and pH1after 1 h. L61-1 was viable in MRS alone and pH3 after 4 h. No viable was found in MRS pH2 and pH1 after 1 h. (Figure 13)

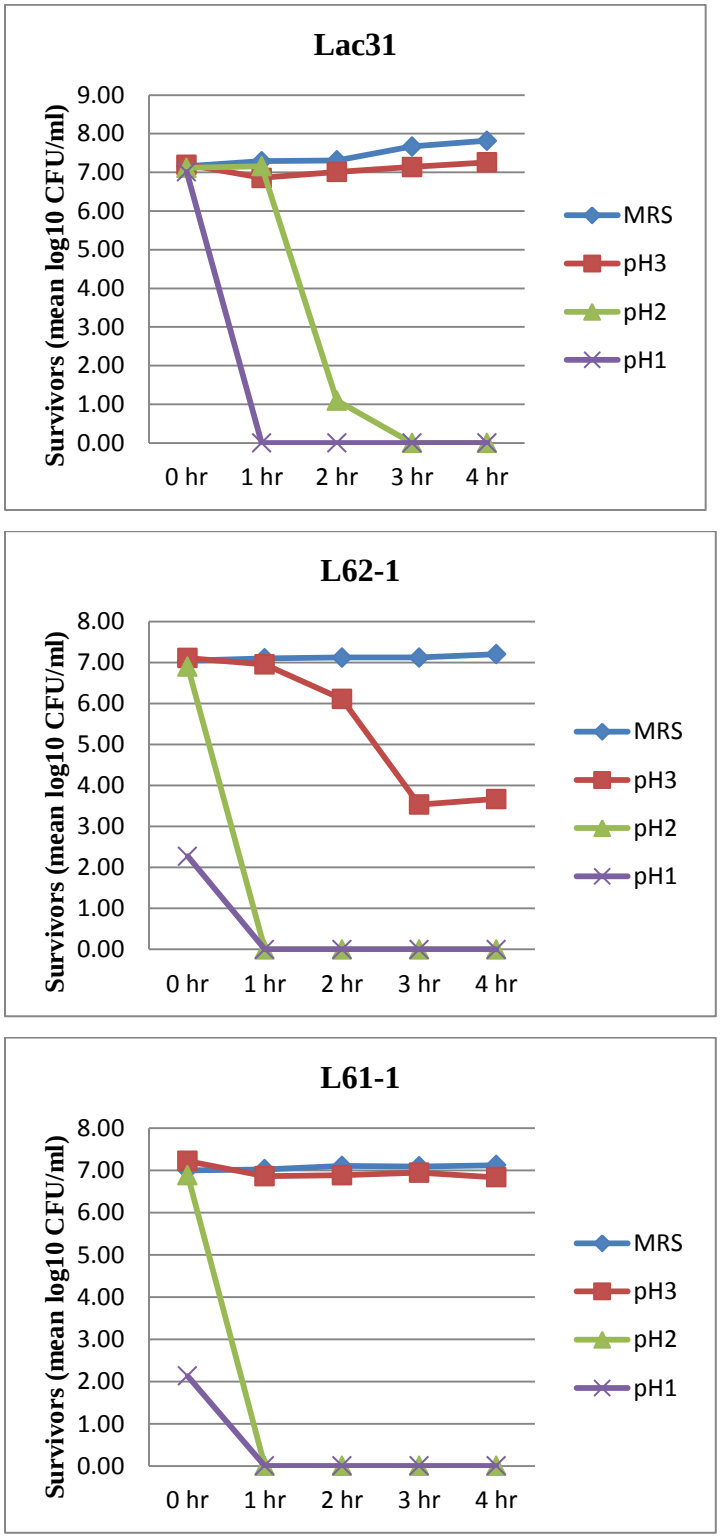


Figure 13. Survival curves of Lac31, L62-1 and L61-1

4. Identification by molecular technique

DNAs of 3 hypocholesterolemic *Lactobacillus* candidate were amplified by genus-specific primers. They were aligned with the 16S rRNA gene sequence of *Lactobacillus* spp. with Multalin program. These results confirmed that 3 isolates were *Lactobacillus.* Moreover, DNAs were amplified with universal primers. These universal primers were aligned with the 16S rRNA gene sequence of *Lactobacillus* spp. with Multalin program (Appendix D)

PCR products were sequenced and analysed with NCBI and RDP II database.Lac31, L62-1 and L61-1 were identified as *L. fermentum*, *L. ruminis* and *L. plantarum* , respectively(Table 5).

Table 5. Genotypic identification of *Lactobacillus* spp. based on 16S rRNA gene sequencing

No.	Code	Match organism	%identity (NCBI)	Length (bp)	Score (RDP)	Length (bp)
1	Lac31	*L. fermentum*	96	1481	0.886	1260
2	L62-1	*L. ruminis*	98	1605	0.847	1438
3	L61-1	*L. plantarum*	96	1498	0.801	1372

5. Hypocholesterolemic effect of *L. fermentum* Lac31 *in vivo* study

Since *L. fermentum* (Lac31) could deconjugate bile salt, reduce cholesterol level in media, survive in media with 0.3% oxgall and pH3, it was then chosen to test hypocholesterolemic effect *in vivo.*

Prevention experiment

Mice fed with chow and PBS (negative control) and high fat diet and PBS (high fat group) had total cholesterol of 104.33 mg/dl 139.38 mg/dl, respectively ($p < 0.01$). It showed that this high fat diet is suitable to induce normal mice to hypercholesterolemic mice. Mice fed with high fat diet and *L. fermentum* (Lac31) had total cholesterol 152.96 mg/dl and high fat diet and *L. fermentum* (L34-1) had 156.9 mg/dl (Table 6, Figure 14). They had total cholesterol more than high fat control group. We consume that *L. fermentum* (Lac31) no have prevent effect in mice model.

Mice fed with chow and PBS had triglyceride 174.22 mg/dl and high fat diet and PBS 140.19 mg/dl. In tested group, high fat diet and *L. fermentum*(Lac31) 141.87 mg/dl and high fat diet and *L. fermentum*(L34-1) 285.82 mg/dl(Table 6, Figure 15). We could not compare all groups because this model is not suitable for triglyceride induction.

Table 6. Effect of *L. fermentum* on total cholesterol and triglycerides (prevention)

1st week	2nd week	Total cholesterol (mg/dl)	Total triglyceride (mg/dl)
Chow + PBS	Chow + PBS	104.33**	174.22
Chow +Hypo-*Lactobactillus*	High fat diet + Hypo-*Lactobactillus*	152.96	141.87
Chow + PBS	High fat diet + PBS	139.38**	140.19
Chow + normal *Lactobacillus*	High fat diet + normal *Lactobacillus*	156.90	285.82

** mean in the same column are significantly different ($p < 0.01$)

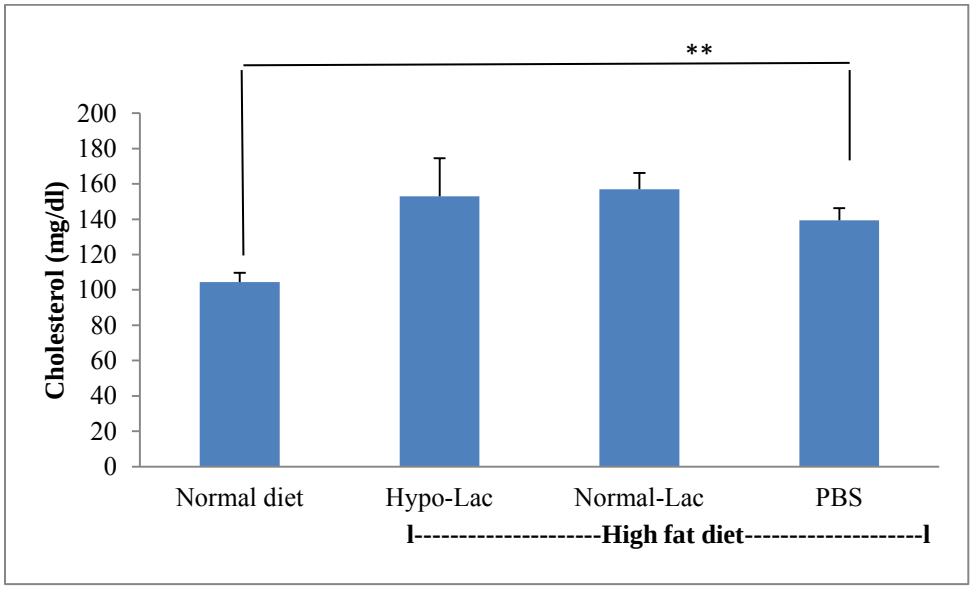


Figure 14. Prevention experiment for total cholesterol

** ($p<0.01$)

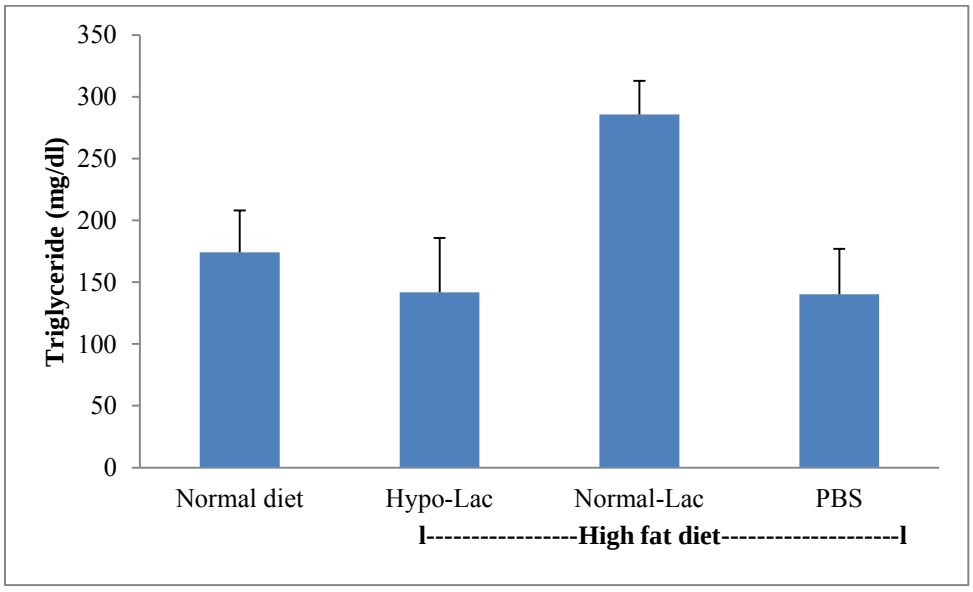


Figure 15. Prevention experiment for total triglyceride

Treatment group

Mice fed with chow and PBS, high fat diet- PBS, - *L. fermentum*(Lac31), and - *L. fermentum*(L34-1) had total cholesterol 106.11, 154.34, 133.41 and 90.55 mg/dl, respectively(Table 7, Figure 16). In group chow and PBS (negative control) and high fat and PBS (high fat control) were significantly different ($p < 0.01$) that mean this high fat diet could induce mice to hypercholesterolemic. For *L. fermentum* (L34-1) showed hypocholesterolemic effect compare with group high fat diet and PBS ($p < 0.05$). Other group not significant different ($p > 0.05$).

Mice fed with chow and PBS, high fat diet- PBS, - *L. fermentum*(Lac31), and - *L. fermentum*(L34-1) had total triglycerides 103.53, 133.46, 123.97 and 96.99 mg/dl, respectively. We could not compare all groups because this model is not suitable for triglyceride induction (Table 7, Figure 17).

Table 7. Effect of *L. fermentum* on total cholesterol and triglycerides (treatment)

3-4st week	Total cholesterol (mg/dL)	Total triglyceride (mg/dL)
Chow + PBS	106.11**	103.53
High fat diet + Hypo-*Lactobacillus*	154.34	133.46
High fat diet + PBS	133.41**,***	123.97
High fat diet + normal *Lactobacillus*	90.55***	96.99

mean in the same column are significantly different ($p < 0.01$). * ($p < 0.05$)

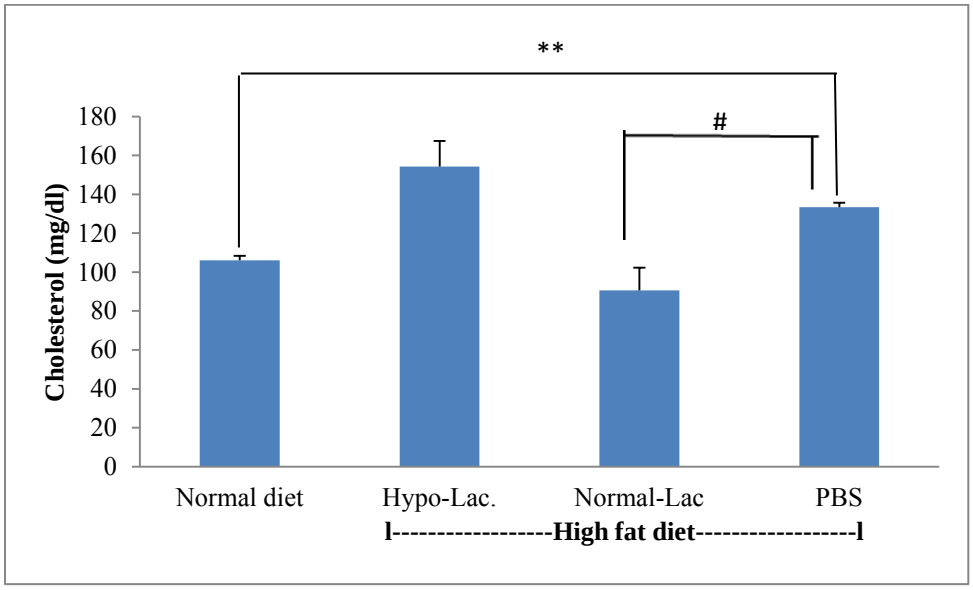


Figure 16. Treatment experiment for total cholesterol

** ($p<0.01$), # ($p<0.05$)

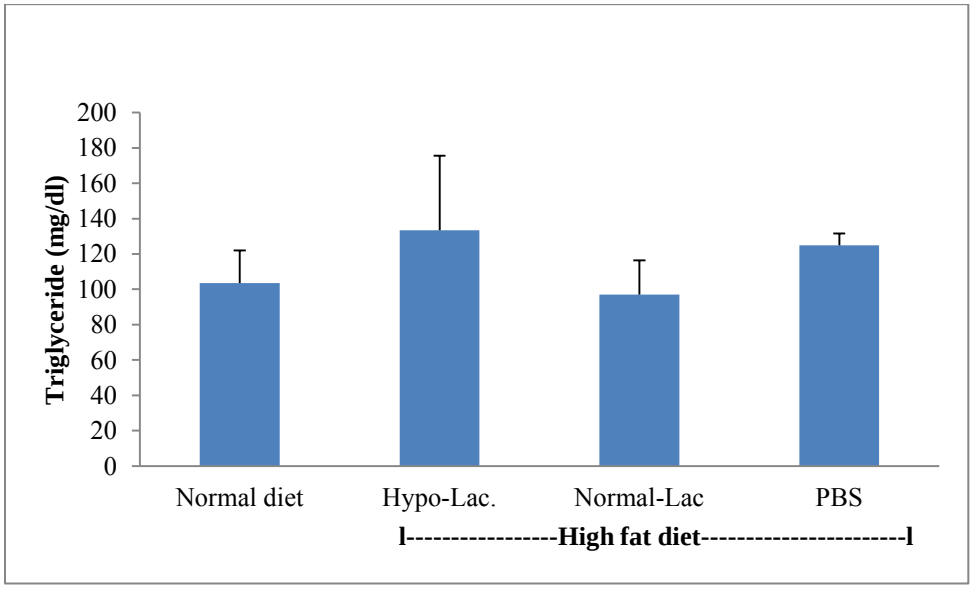


Figure 17. Treatment experiment for total triglyceride

6. Translocation of bacteria

Fifteen microliters of blood and 100 µl of liver and spleen suspensions were each plated on MRS agar and incubated at 37 ^{o}C for 72 hours in anaerobic condition. No bacterial growth was found on media. This suggested that there was no bacterial translocation to blood, spleen and liver in all mice.

CHAPTER V

DISCUSSION

Cholesterol is the essential component of cellular membrane in mammals and the precursor of primary bile acids and steroid hormones. The total body cholesterol pool is derived from both endogenous and exogenous sources and this pool is affected by the intestinal microflora, among many other factors[45]. Cholesterol is one of the causes of the development of atherosclerosis and coronary heart disease in humans. A high level of cholesterol in blood is considered to be a risk factor for coronary heart disease, cardiovascular disease, hypertension and artherosclerosis[9, 45-47]. In western countries, cardiovascular diseases are the major causes of morbidity and mortality in adults[36]. WHO has predicted that, by 2030, cardiovascular diseases will become the leading cause of death, affecting approximately 23.6 million people around the world. Nowadays, dietary management, behavior modification and drug therapy are used to reduce cholesterol in blood[48]. Currently, CVD can be treated by medication including statin, firbrates, niacin, cholesterol absorption inhibitors and bile acid sequestrants[25]. However, there is a limitation of treatment. Some patients were intolerant with medication and some medicines have side effect[49]. The used of probiotic bacteria is an alternative treatment because many reports show that probiotics have hypocholesterolemic effect in clinical trials[18, 50]. Probiotics are "live microorganisms which, when administered in adequate amounts, exert health benefit on the host" [1]. Probiotic strains belong mostly to the genera *Lactobacillus* and *Bifidobacterium*.

According to several studies, *Lactobacillus* has two major hypocholesterolemic mechanisms, which are bile salt hydrolase (BSH) production [11-12] and cholesterol assimilation [5, 7]. Therefore, 51 human- derived *Lactobacillus* spp. were tested for these mechanisms in this study and the result is that 19 isolates showed precipitate of bile acid around active colonies, which means *Lactobacillus* isolate could produce BSH enzyme [5, 11, 12, 51]. BSH enzyme cleaves the peptide linkage of bile acid, which results in removal of the amino acid group from the steroid core. The unconjugated bile acids precipitate at

low pH[35]. From 19 isolates as mentioned before, 9 isolates deconjugated only TCA and 10 isolates deconjugated both of TCA and GCA. This result is similar to the study of Ahn *et al*., [12] that reported *L. acidophilus* could deconjugate not only primary bile salt but also secondary bile salt. Besides BSH, 6 isolates of 51 (5 of them also had BSH mechanism) had cholesterol assimilation. These 6 isolates could reduce cholesterol in cholesterol broth with cholesterol reducing rate of 13.09% – 35.85%. Zeng *et al*., [9] also showed the same result, which is that *L. buchneri* P2 had high cholesterol reducing rate of 43.95%. Another study of Kimoto *et al*., [46] reported that *L. lactis* supsp. *lactis* bv. Diacetylactis N7 could remove cholesterol in media with high rate of 97%.

Other required characteristics of probiotics are bile and acid tolerance. Probiotic bacteria are mostly delivered via gastrointestinal system, where there is plenty of acid and bile. Therefore, probiotic needs bile and acid tolerance property in order to survive in the human gastrointestinal tract [52-54]. Usman and Hosono [10] suggested that probiotic must survive in pH3.0 for 2 h and in bile concentration of 1000 mg/L. In this study, we found 11 isolates could survive in 0.3% oxgall MRS for 9 h with % survival between 10.9 – 100 %. This result is supported by Mishra and Prasad's[37]study that reported *L. casei* NCDC63 and VT strain could be tolerant in1-2% bile concentration for 12 h. Pereira and Gibson[15] also reported that *L. fermentum* KC5b was able to maintain viability for 2 at pH2 and grow in a medium with 4,000 mg of bile acids per liter and able to remove 14.8 mg of cholesterol per gram of cells from the culture medium. We chose only 3 *Lactobacillus* isolates(Lac31, L62-1 and L61-1) that produced BSH enzyme and/or assimilated cholesterol and survive in 0.3% oxgall for 9 h to study acid tolerance in pH1-3 MRS broth. These isolates could not survive acid condition of pH2 and lower. Lac31and L61-1 could survive acid condition of pH3 for 4 h, whereas L62-1 could not. Zeng *et al*.,[9] reported that *L. buchneri* P2 could survive at pH3(control group at pH6.4 and no viable cell at pH2 after 4 h and 2 h at pH1. In addition, Mishra and Prasad[37] reported that all tested *Lactobacillus* strains were able to resist pH3 for 3 h, and *L. casei* NCDC17, C1 and Y could also resist pH2.

All 3 isolates of hypocholesterolemic *Lactobacillus* were genotypically identified by 16S rRNA gene sequencing. Lac 31 is identified as *L. fermentum*. L62-1 is identified as *L. ruminis* and L61-1 is identified as *L. plantarum*.

To confirm hypocholesterolemic effect *in vitro*, *in vivo* studies were conducted. Taranto *et al*., [8] reported the effect of *L. reuteri* on the prevention of hypercholesterolemia in mice. Mice were administered with *L. reuteri* CRL 1098 (10^4 cells/d) for 7 days before induction to hypercholesterolemia. It was found that tested group did not have high cholesterol in blood. This suggested that this strain can prevent mice from hypercholesterolemia. Moreover, it had hypercholesterolemia reducing property. Hypercholesterolemia-induced mice were fed with this *Lactobacillus* for 7 days and it was found that this *Lactobacillus* strain had effect on the reduction of total cholesterol and triglyceride as well as the increase of HDL: LDL ratio[16]. Additionally, Nguyen *et al*.,[2] found hypocholesterolemic effect of *L. plantarum* PH04 in high blood cholesterol mice when fed with 10^7 CFU/ml for 14 days. In this study, *L. fermentum* (Lac31) did not show hypocholesterolemic effect *in vivo* as expected. We plan to repeat the experiment by feeding *L.fermentum* (Lac31) twice a day and extend the feeding time to more than 2 weeks. *L. fermentum* (L34-1) which did not assimilate cholesterol and possess BSH enzymes in *in vitro* study, showed hypocholesterolemic effect in treatment model. . This suggests that this bacterium could reduce cholesterol in mice via other mechanism besides BSH production and cholesterol assimilation. In addition, However, this study showed that *L. fermentum* (Lac31) couldn't translocate to blood, liver and spleen. It is important to do a translocate study since probiotics must not cause bacteremia, septicemia or even multiple organ failure[55, 56]. This suggested that it is safe to use *L. fermentum* (Lac31) in animal and has a possibility to be developing to use in human as well. Our result is the same as those of other studies [2, 16, 40, 57].

CHAPTER VI

CONCLUSION

51 *Lactobacillus* isolates from the feces of healthy infants and adults, 19 isolates could produce BSH enzyme to deconjugate bile salt. Of these 19 isolates, 9 isolates deconjugated only TCA and 10 isolates deconjugated both TCA and GCA. Of 51 *Lactobacillus* isolates, 6 isolates reduced cholesterol. Cholesterol lowering rate ranged from 13.09% – 35.85%. Potential hypocholesterolemic *Lactobacillus* isolates were tested for bile and acid tolerance, 2 isolates (*L. fementum* Lac31 and *L. plantarum* L61-1) were found to survive in 0.3%oxgall and acidic pH 3 condition. *L. fermentum* Lac31 was tested for hypocholeterolemic effect in mice but did not show the effect. *L. fermentum*(Lac31) did not translocate to blood, liver and spleen.

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APPENDICES

APPENDIX A

MEDIA AND REAGENT

Media

1. M.R.S. Agar (de man, Rogasa, Sharpe)(Oxiod, Basingstroke, Hamps, Uk)
   Suspend 62 g in 1000 ml of distilled water. Boil to dissolve the medium. Dispense into tubes, bottles, or flasks and sterilize by autoclaving at 121 ^{0}c for 15 minutes.
2. M.R.S. Broth (Oxiod, Basingstroke, Hamps, Uk)
   Suspend 52 g in 1000 ml of distilled water at 60 ^{0}C. Mix until completely dissolved. Dispense into final containers and sterilize by autoclaving at 121 ^{0}C for 15 minutes.
3. M.R.S.broth + 20% glycerol(Merck, Germany) for stock culture
   Suspend 10.4 g in 160 ml of distilled water. Mix until completely dissolved. Adding 40 ml of glycerol and sterilized by autoclaving at 121 ^{0}C for 15.
4. M.R.S + 0.5 mM bile salt Agar
   autoclaving at 121 ^{0}C for 15 minutes. Keep this media warm in water bath at 50 ^{0}C. Dissolve sodium taurocholate (TCA) or sodium glycocholate (GCA) in 10 ml of sterile distrilled water. Mix media and bile salt solution together and pour in sterile petri dish.
5. M.R.S. broth + 0.5 mg/ml Cholesterol
   Soluble cholesterol (polyoxyethanyl-cholesterol sebcate, Sigma, USA) was dissolved in sterile water to a concentration of 50 mg/ ml. MRS broth was sterilized by autoclaving at 121^{0}C for 15 min and then added with cholesterol to a final concentration of 0.5 mg/ml.
6. M.R.S.+ 0.3% Oxgall (Dehydrated Fresh bile, Difco)
   Oxgall 0.3% was dissolved in 0.2 % thioglycolate MRS broth and then sterilized by autoclaving at 121^{0}C for 15 min.
7. Sterile 0.85% NaCl (Sigma, USA)
   NaCl 8.5 grams in 1 liter of distilled water. Sterilize by autoclaving at 121^{0}C for 15 minutes.

8. M.R.S + HCl to pH1, 2 and 3

   0.2% thioglycolate MRS broth supplement with hydrocholic acid to pH 1, 2 and 3 and sterilized by autoclaving at 121^0C for 15 min.
9. Brain Heart infusion broth (Difco, USA)
10. Gaspack (Anaerobe pack-Anaero, Mitsubishi, Japan)
11. Agar (Reagent organism, USA)
12. Gene ruler ^{TM} 100bp DNA ladder Plus (Fermentas, USA)
13. Proteinase K (Sigma, USA)
14. QIA quick PCR Purification Kit (Qiagen, Hildern, Germany)

Reagent

1. Reagent for colorimetric assay
   a. 33% Potassium hydroxy (33%KOH) 33 g of KOH was dissolved in 100 ml of sterile distilled water.
   b. Absolute ethanol – ready to use
   c. Hexane – ready to use
   d. Op-thaladehyde – Fresh prepared 0.015 g of opthaldehyde was dissolved in 30 ml of acetic acid. Mix until complete dissolved and keep away from light.
   e. Sulfuric acid – ready to use
2. Lipid profile kits
   a. Cholesterol liquicolor

      Method; the cholesterol is determined after enzymetic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminophenazone in the presence of phenol peroxidase.

<u>Reaction Principle</u>

$$\text{Cholesterolester} + H_20 \xrightarrow{\text{CHE}} \text{cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHO}} \text{cholesterol-3-one} + H_2O_2$$

$$2H_2O_2 + 4 \text{ amino phenazone} + \text{phenol} \xrightarrow{\text{POD}} \text{quinoneimine} + 4\ H_2O$$

Contents

RGT 4x30 ml, 3 x250 ml or 4x100 ml Enzyme reagent	
Phosphase buffer (pH6.5)	100 mmol/l
4-Aminophenazone	0.3 mmol/l
Phenol	5 mmol/l
Peroxidase	>5 KU/l
Cholesterolesterase	>150 U/l
Cholesteroloxidase	>100 U/l
Sodium azide	0.05%
STD 3 ml Standard	
Cholesterol 200 mg/dl or 5.17 mmol/l	

Reagent Preparation

RGT and the STD are ready for use

Reagent Stability

The reagents are stable up to the given expiry date, even after opening, when stored at 2-8 ^{0}C. The opened reagent is stable for 2 weeks at 15-25 ^{0}C . Contamination must be avoided.

Specimen

Serum, heparinised or EDTA-plasma.

Note:

Lipemic specimens usually generate turbidity of the sample/ reagent mixture which leads to falsely elevated results. The CHOLESTEROL liquicolor test avoids these falsely elevated results through its built-in Lipid Clearing Factor (LCF). The LCF clears up totally a turbidity caused by lipemic specimens.

Assay

Wavelenght: 500 nm, Hg 546 nm

Optical path: 1 cm

Temperature: 20-25 ^{0}C or 37 ^{0}C

Measurement: Against reagent blank. Only one reagent blank per series is required.

Pipetting Scheme

Pipette into cuvettes	Reagent blank	Sample or STD
Sample/ STD	-	10 μl
RGT	1000 μl	1000 μl
Mix, incubate 10 min. at 20-25 ^{0}C or 5 min. at 37 ^{0}C. Measure the absorbance of the sample/ STD against the reagent blank (ΔA) within 60 min.		

Calculation of the cholesterol concentration

1. With factor

Wavelength	C[mg/dl]	C[mmol/l]
Hg 546 nm	840 x ΔA	21.7 x ΔA
500 nm	553x ΔA	14.3 x ΔA

2. With Standard

Only the standard recommened by HUMAN (enclosed in kit or separately available, REF 10015) should be used.

$$C = 200 \times \frac{\Delta A sample}{\Delta A\ STD}\ [mg/dl] \quad or \quad C = 5.17 \times \frac{\Delta A sample}{\Delta A\ STD}\ [mmol/l]$$

b. Triglyceride liquicolor

Method

The triglycerides are determined after enzymatic hydrolysis with lipases. Indicator is quinoneimine formed from hydrogen peroxide, 4- amino- antipyrine and 4-chlorophenol under the catalytic influence of peroxidase.

Reaction Principle

$$\text{Triglycerides} \xrightarrow{\text{lipase}} \text{glycerol + fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{glycerol-3-phosphate +ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{dihydroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-aminoantipyrine} \xrightarrow{\text{POD}} \text{quinoneimine} + HCl + H_2O_2 + \text{4-chlorophenol}$$

Content

RGT 15 ml; 100 ml or 250 ml Monoreagent

PIPES buffer (pH 7.5)	50 mmol/l
4-chlorophenol	5 mmol/l
4-aminophenazone	0.25 mmol/l
Magnesium ions	0.45 mmol/l
ATP	2 mmol/l
Lipases	≥ 1300 U/l
Peroxidase	≥ 500 U/l
Glycerol kinase	≥ 400 U/l
Glycerol-3-phosphate oxidase	≥ 1500 U/l
Sodium azide	0.05 %

STD 3 ml Standard

Triglycerides 200 mg/dl or 2.28 mmol

Reagent Preparation and Stability

RGT and STD are ready for use.

The reagents are stable, even after opening, up to the stated expiry date when stored at 2-8 ^{0}C. At 20-25 ^{0}C the RGT is stable for 4 weeks.

Contamination must be avoided.

Protect from light

Specimen

Serum, heparinised plasma or EDTA plasma

Stability : 3 days at 2-8 ^{0}C., 4 months at -20 ^{0}C.

Note ; Lipemic specimens usually generate turbidity of the sample reagent mixture which leads to falsely elevated results. The TRIGLYCERIDES liquicolor mono test avoids

these falsely elevated resusts through its buil-in Lipid-Clearing Factor (LCF)The LCF clears up totally a turbidity caused by lipemic specimens.

Assay

Wavelenght: 500 nm, Hg 546 nm

Optical path: 1 cm

Temperature: 20-25 ^{0}C or 37 ^{0}C

Measurement: Against reagent blank. Only one reagent blank per series is required.

Pipetting Scheme

Please use only the HUMAN Triglycerids Standard provide with the test kits or separately available: REF 10163

Pipette into cuvettes	Reagent blank	Sample or STD
Sample/ STD	-	10 μl
RGT	1000 μl	1000 μl
Mix, incubate 10 min. at 20-25 ^{0}C or 5 min. at 37 ^{0}C. Measure the absorbance of the sample/ STD against the reagent blank (ΔA) within 60 min.		

Calculation of the triglycerides concentration

$$C = 200 \times \frac{\Delta A \text{sample}}{\Delta A \text{ STD}} \text{ [mg/dl]} \quad \text{or} \quad C = 2.28 \times \frac{\Delta A \text{sample}}{\Delta A \text{ STD}} \text{ [mmol/l]}$$

APPENDIX C

THE RESULTS OF ALL TESTS IN THIS STUDY

Table 8. Bile salt hydrolase, Cholesterol reducing rate, % survival and identification

No.	Code	Habitat	Deconjugate bile salt		Cholesterol reducing rate (%)	% survival in 0.3%oxbile	Identification
			TCA	GCA			
1	Lac31	IF	+	-	26.44	20.83	*L. fermentum*
2	Lac39	IF	-	-	17.95	53.03	NP
3	ST28	IF	-	-	19.61	0	NP
4	MT1	AD	-	-	13.09	0	NP
5	AT1	AD	-	-	35.85	0	NP
6	KT1	AD	-	-	24.54	0	NP
7	HNT1	AD	-	-	ND	0	NP
8	PT1	AD	-	-	ND	0	NP
9	L6-1	AD	-	-	ND	0	NP
10	L9-1	AD	+	+	ND	0	*L. mucosae*
11	L10-1	AD	-	-	ND	0	NP
12	L14-1	AD	+	-	ND	0	NP
13	L15-1	AD	+	-	ND	0	NP
14	L17-1	AD	+	-	ND	0	NP
15	L19-1	AD	+	-	ND	0	NP
16	L35-1	AD	-	-	ND	0	NP
17	L36-1	AD	-	-	ND	0	NP
18	L31-1	AD	-	-	ND	0	NP
19	L34-1	AD	+	+	ND	33.33	*L.plantarum*

IF; Infant feces, AD; Adult feces, ND; Not detected, NP; Non PCR product

Table 8. Bile salt hydrolase, Cholesterol reducing rate, % survival and identification

No.	Code	Habitat	Deconjugate Bile salt		Cholesterol reducing rate(%)	% survival in 0.3%oxbile	Identification
			TCA	GCA			
20	L29-1	AD	-	-	ND	0	NP
21	L32-1	AD	-	-	ND	0	NP
22	L24-1	AD	-	-	ND	0	NP
23	L27-1	AD	-	-	ND	0	NP
24	L22-1	AD	-	-	ND	0	NP
25	L37-1	AD	-	-	ND	0	NP
26	L21-1	AD	-	-	ND	0	NP
27	L23-1	AD	-	-	ND	0	NP
28	L38-1	AD	-	-	ND	0	NP
29	L39-1	AD	+	+	ND	0	NP
30	L40-1	AD	+	-	ND	0	NP
31	L41-PR1	AD	+	-	ND	0	*L. agilis*
32	LP383	AD	-	-	ND	0	NP
33	L421	AD	+	-	ND	44	*L.plantarum*
34	L44-1	AD	-	-	ND	0	NP
35	L45-1	AD	-	-	ND	29.1	NP
36	L46-1	AD	-	-	ND	0	NP
37	L47-1	AD	+	+	ND	50	*L. ruminis*
38	L59-1	AD	+	-	ND	0	*L. gilis*
39	L60-1	AD	+	+	ND	23.9	*L.fermentum*
40	L48-1	AD	+	+	ND	42.86	NP
41	L49-1	AD	+	+	ND	38.82	NP

IF; Infant feces, AD; Adult feces, ND; Not detected, NP; Non PCR product

Table 8. Bile salt hydrolase, Cholesterol reducing rate, % survival and identification

No.	Code	Habitat	Deconjugate bile salt		Cholesterol reducing rate (%)	% survival in 0.3%oxbile	Identification
			TCA	GCA			
42	L56-1	AD	-	-	ND	0	NP
43	L57-1	AD	-	-	ND	0	NP
44	L52-1	AD	-	-	ND	0	NP
45	L50-1	AD	+	+	ND	56.71	*L. ruminis*
46	L54-1	AD	-	-	ND	0	NP
47	L51-1	AD	-	-	ND	0	NP
48	L62-1	AD	+	+	ND	100	*L. ruminis*
49	L64-1	AD	-	-	ND	0	NP
50	L61-1	AD	+	+	ND	25	*L.plantarum*
51	L63-1	AD	-	-	ND	0	NP

IF; Infant feces, AD; Adult feces, ND; Not detected, NP; Non PCR product

APPENDIX D

NUCLEOTIDE SEQUENCES ALIGNMENT OF CANDIDATE LACTOBACILLUS STRAIN

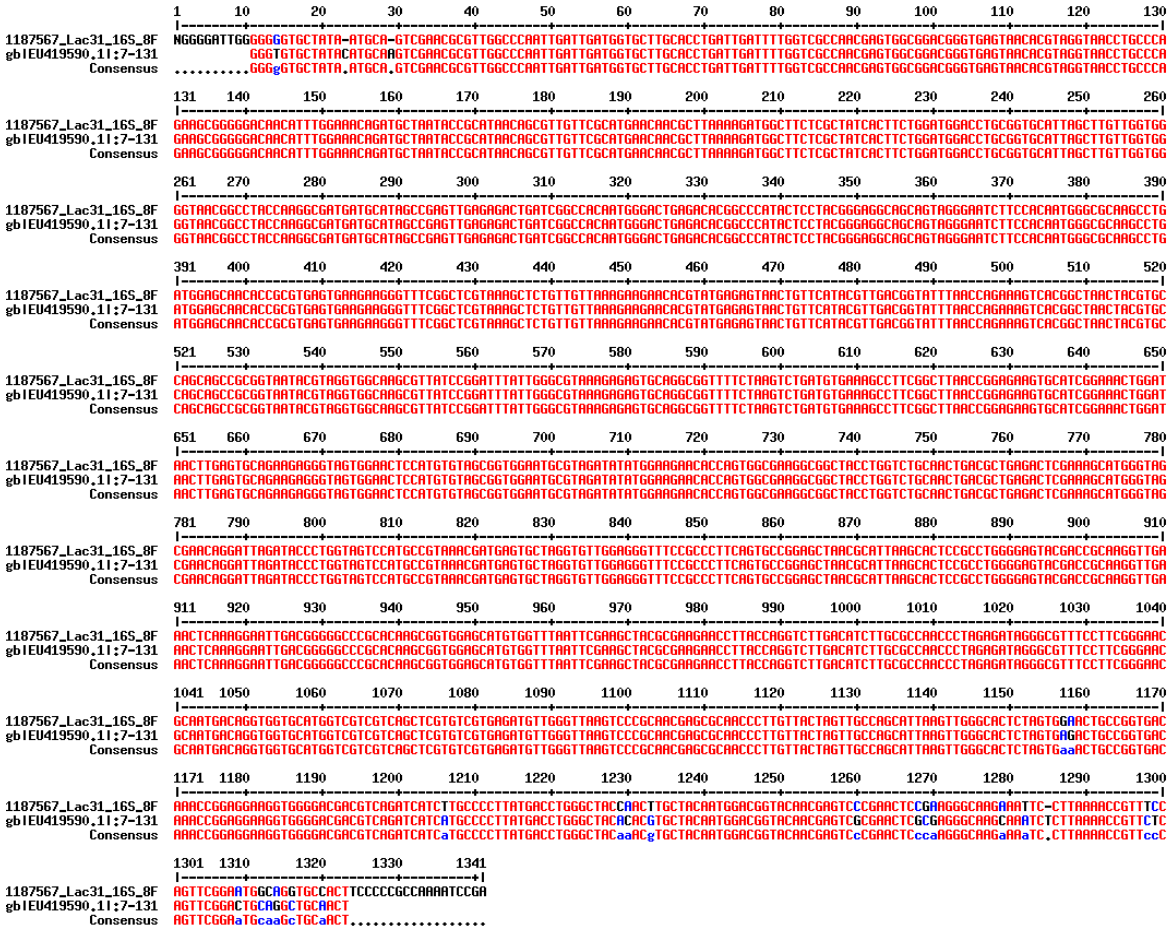

Figure 19. Nucleotide sequence alignment of *L. fermentum* (Lac31)

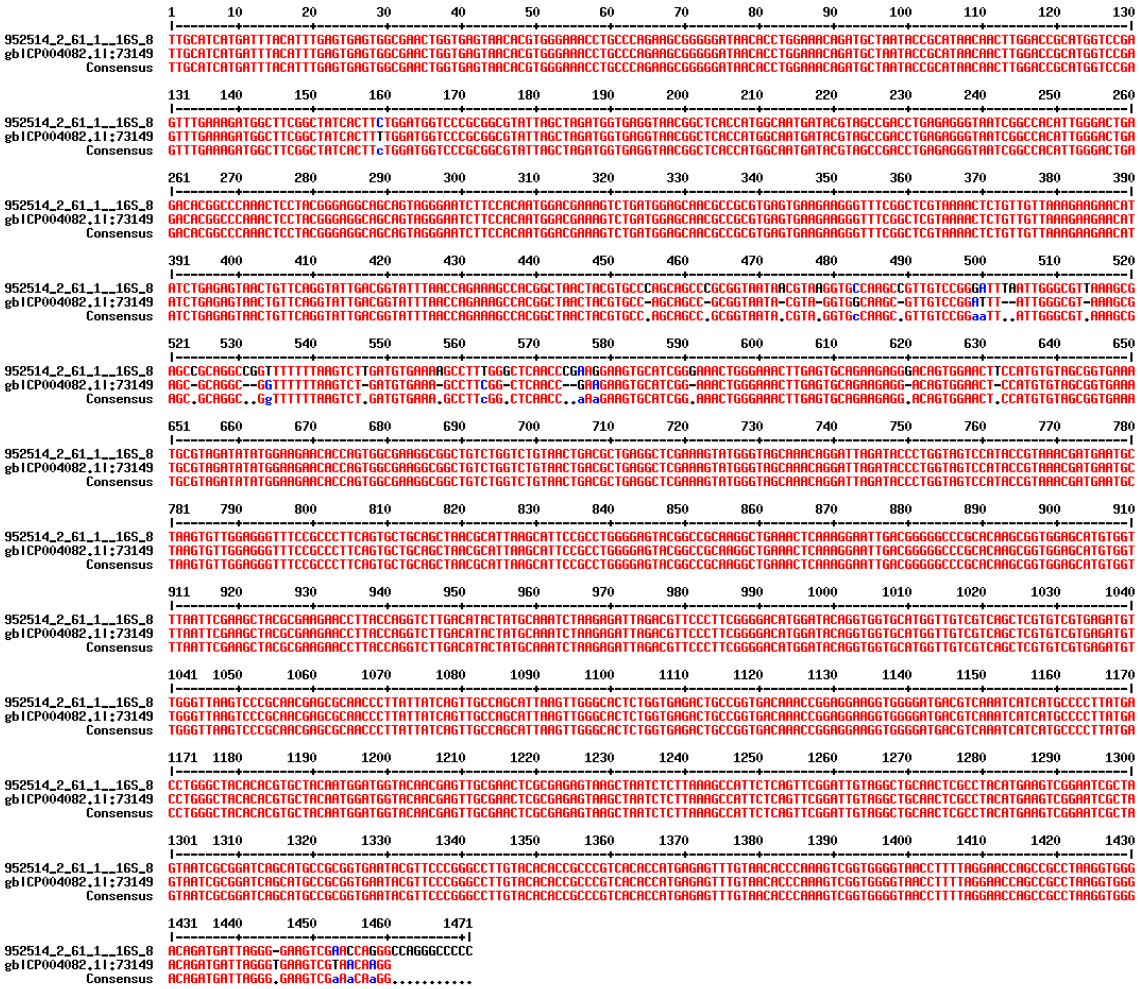

Figure20. Nucleotide sequence alignment of *L. plantarum* (L61-1)

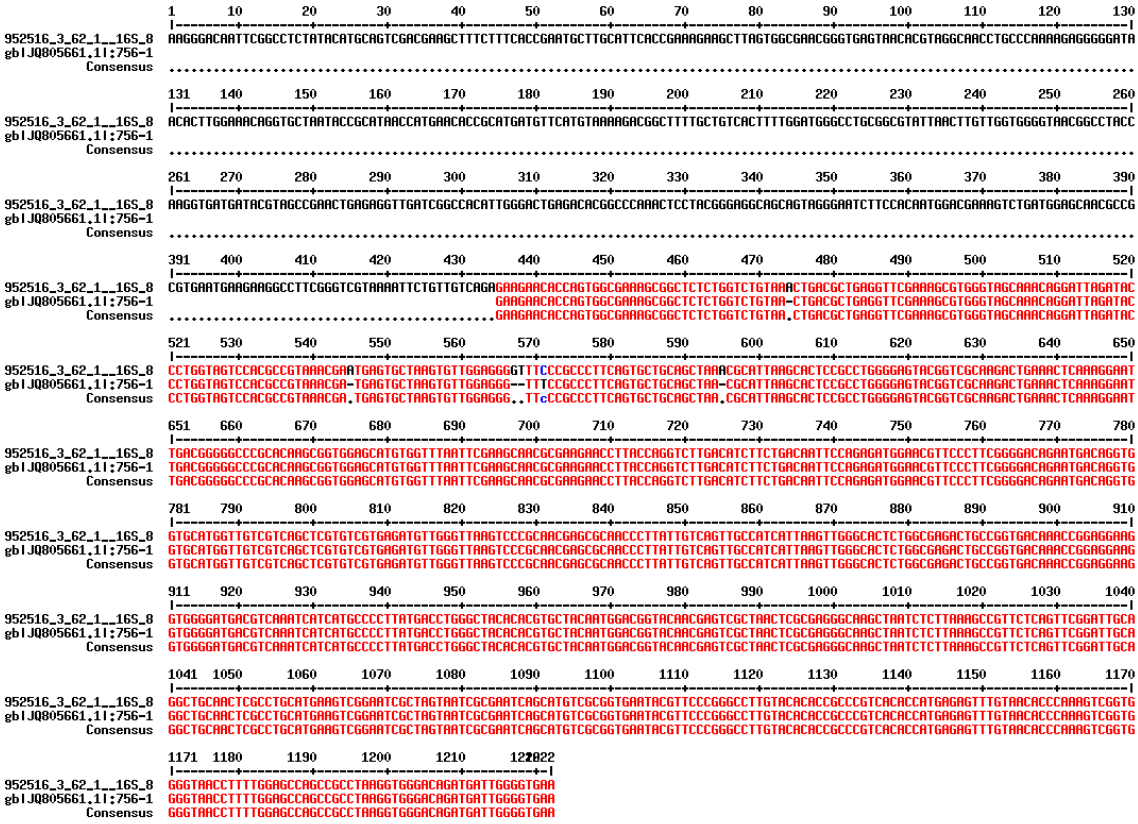

Figure21. Nucleotide sequence alignment of *L. ruminis*(L62-1)

APPENDIX E

DNA CODON

One- and Three-Letter symbols for the amino acids

A	Ala	Alanine
B	Asx	Asparagine or aspartic acid
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	le	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Trytophan
Y	Tyr	Tyrosine
Z	Glx	Gln or Glu

BIOGRAPHY

Miss Phawinee Subsomwong was born on September 10, 1987 in Uttaradit province, Thailand. She graduated with the Bachelor degree of Science (Microbiology) from the Faculty of Science, Burapha University Chonburi province 2009. She is currently a student in the Interdisciplinary Program of Medical Microbiology, Faculty of Graduate School, Chulalongkorn University since 2010.