EVALUATION OF CHOLESTEROL DEGRADATION BY AN INDIGENOUSLY ISOLATED *Lactobacillus casei* WITH PROBIOTIC PROPERTIES

A

DISSERTATION REPORT

Submitted in partial fulfilment of the requirements for the award of degree of

Master of Science in Biotechnology

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled, **"EVALUATION OF CHOLESTEROL DEGRADATION BY AN INDIGENOUSLY ISOLATED *Lactobacillus casei* WITH PROBIOTIC PROPERTIES"** in partial fulfilment of the requirement for the award of the degree of Master in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work during the period of six months from January 2011 to June 2011, under the guidance of **Dr. Abhijit Ganguli**, Assistant Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

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CERTIFICATE

This is to certify that, the thesis entitled , **"EVALUATION OF CHOLESTEROL DEGRADATION BY AN INDIGENOUSLY ISOLATED *Lactobacillus casei* WITH PROBIOTIC PROPERTIES"** submitted by **Sushant Kumar Sharma (300901019)** in partial fulfillment of the requirements for the award of Degree of Master of Science in Biotechnology, to Thapar University, Patiala, is a record of the student's own work, carried out by him, under our supervision and guidance. The report has not been submitted for the award of any other degree or certificate, in this or any other University or Institute.

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ABSTRACT

A strain of *Lactobacillus casei* isolated previously from indigenously fermented food was characterized for its ability to reduce cholesterol. The strain could remove as much as 75% cholesterol under *in vitro* conditions when grown in MRS media; cholesterol could be used as a carbon source but growth was extremely poor. The culture parameters *viz*. pH, temperature and agitation and reduction kinetics were optimized for maximal cholesterol reduction. *L.casei* exhibited optimum activity at temperature 26ºC and a pH value 6.0 when incubated for 72 hours at 120 rpm. The percentage reduction of cholesterol by supernatant, *L. Casei* exoploysaccharide and extracellular enzyme was approximately 40%, 30% and 70% respectively. Investigations into the mechanism of cholesterol revealed that cholesterol bound partially to cellular exopolysaccharides and were catalyzed by an extracellularly produced enzyme.

List of Abbreviations

%	Percent
H	Hour
M	Molar
µM	Micro molar
nm	Nanometre
mg	Milligram
min	Minute
ml	Millilitre
L	Litre
MRS	de Man Rogosa Sharpe media
ºC	Degree centigrade
w/v	Weight by volume
v/v	Volume by volume
rpm	Revolution per minute
TLC	Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
Mau	Milli absorbance unit
WHO	World Health Organization

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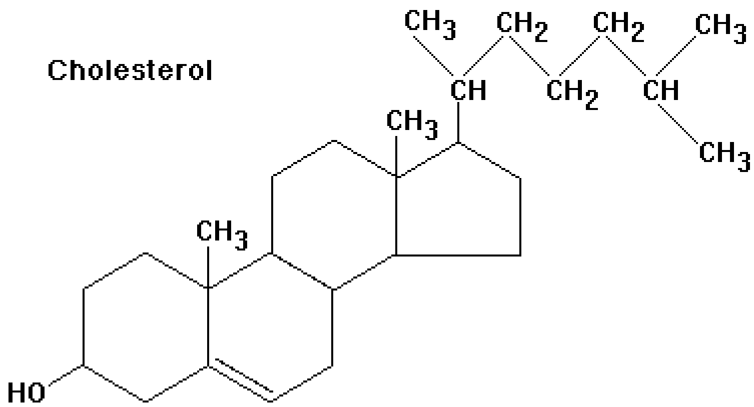
1. INTRODUCTION

Cholesterol is a waxy steroid of fat that is manufactured in the liver or intestines to produce hormones and cell membranes and transported in the blood plasma of all mammals. It is an essential structural component of mammalian cell membranes, where it is required to establish proper membrane permeability and fluidity. In addition, cholesterol is an important component for the manufacture of bile acids, steroid hormones, and vitamin D. Cholesterol is the principal sterol synthesized by animals, but small quantities are synthesized in other eukaryote, such as plants and fungi. It is almost completely absent among prokaryote, which include bacteria. Although cholesterol is an important and necessary molecule for mammals, high levels of cholesterol in the blood can clog arteries and potentially is linked to diseases such as heart disease.

Cholesterol has molecular formula - $C_{27}H_{46}O$, Molecular mass - 386.65g/mol, melting point - 148-150°C, boiling point- 360°C.



Francois Poulletier de la Salle first identified cholesterol in solid form in gallstone in 1769. However, it was only in 1815 that chemist Eugene Chevreul named the compound "cholesterine".

1.1 Physiology

Since cholesterol is essential for all animal life, it is primarily synthesized from simpler substances within the body. However, high levels in blood circulation, depending on how it is transported within lipoproteins, are strongly associated with progression of atherosclerosis. For a person of about 68 kg (150 pounds), typical total body cholesterol synthesis is about 1 g (1,000 mg) per day, and total body content is about 35 g. Typical daily additional dietary intake in the United States is 200–300 mg. The body compensates for cholesterol intake by reducing the amount synthesized.

1.2 Function

Cholesterol is required to build and maintain membranes. It modulates membrane fluidity over the range of physiological temperatures. The hydroxyl group on cholesterol interacts with the polar head groups of the membrane phospholipids and sphingolipids, while the bulky steroid and the hydrocarbon chain are embedded in the membrane, alongside the nonpolar fatty acid chain of the other lipids. In this structural role, cholesterol reduces the permeability of the plasma membrane to protons (positive hydrogen ions) and sodium ions.

Within the cell membrane, cholesterol also functions in intracellular transport, cell signaling and nerve conduction. Cholesterol is essential for the structure and function of invaginated caveolae and clathrin-coated pits, including caveol-dependent and clathrin-dependent endocytosis. The role of cholesterol in such endocytosis can be investigated by using methyl beta cyclodextrin (MβCD) to remove cholesterol from the plasma membrane. Recently, cholesterol has also been implicated in cell signaling processes, assisting in the formation of lipid rafts in the plasma membrane. In many neurons, a myelin sheath, rich in cholesterol, since it is derived from compacted layers of Schwann cell membrane, provides insulation for more efficient conduction of impulses.

1.3 Types of cholesterol

Three major classes of lipoproteins are found in the serum of a fasting individual- low density lipoproteins (LDL), high density lipoproteins (HDL), and very low density lipoproteins (VLDL). HDL cholesterol is considered "good" cholesterol, while LDL and especially VLDL cholesterol are considered the "bad" types of cholesterol that lead to heart disease if left untreated. Natural supplements such as Vasacor, and prescription medications such as Lipitor, are aimed at reducing the levels of the LDL and VLDL cholesterol that increase the risk of heart disease. LDL cholesterol makes up 60-70 percent of the total serum cholesterol. LDL is the major atherogenic lipoprotein and has been long ago identified as a primary target for cholesterol lowering therapy.

LDL is the fatty substance that builds up on the walls of arteries, damaging the arterial wall and blocking the proper flow of blood. This typically results in higher blood pressure and unnecessary strain on the heart muscle. HDL cholesterol makes up 20-30 percent of the total serum cholesterol. Clinical evidence indicates that HDL helps protect against development of atherosclerosis. It is advisable to check your HDL levels from time to time. Increases in HDL levels are usually positively associated with a decrease in LDL and VLDL cholesterol levels. The VLDL is triglyceride-rich lipoproteins and contains 10-15 percent of the total serum cholesterol .VLDL are produced by the liver and some VLDL remnants seem to promote atherosclerosis similar to LDL. VLDL is the "purest" form of the sticky artery-clogging cholesterol, and also the hardest to measure. VLDL cholesterol is usually estimated within a range based on the levels of free triglycerides circulating in the blood stream. Lowering triglycerides will have a direct and beneficial effect on VLDL levels as well.

1.4 Problems related to Cholesterol

1.4.1 Atherosclerosis

The biggest problem associated with high cholesterol is the development of atherosclerosis. Atherosclerosis is the narrowing of the arteries, primarily caused by a buildup of cholesterol that inhibits blood flow. Arteries are responsible for providing blood to all parts of body. Cholesterol can begin to accumulate inside arterial walls and block blood flow.

1.4.2 Heart Disease

High cholesterol levels impede blood flow to the heart and the heart muscle becomes starved for oxygen, causing chest pains (angina). If a blood clot obstructs a coronary artery affected by atherosclerosis, a heart attack (myocardial infarction) is likely to occur, and can often be fatal. Reducing cholesterol levels has become the primary method of reducing the risk of heart attacks in adults.

1.4.3 Stroke

Cholesterol buildup on the inside of the carotid arteries can contribute to risk of a stroke. The carotid arteries supply your brain cells with the blood, oxygen and nutrients brain needs. When normal blood flow to the brain is inhibited, certain areas of the brain that are affected can die. Brain cell death can lead to brain damage.

1.4.4 Diabetes and Blood Pressure

According to the American Diabetes Association, cholesterol, blood pressure and blood glucose are all interrelated. If cholesterol is high, it is likely that blood glucose levels and blood pressure are also high. This triad of chronic disease can wreck havoc on risk for heart disease. Taking steps to improve cholesterol may also improve glucose levels and blood pressure.

1.5 Cholesterol degrading organisms

The degradation and assimilation of cholesterol by various microorganisms is well known in natural environments. Cholesterol oxidase is known to be a key enzyme, which catalyses the oxidation of cholesterol to 4-cholesten-3-one and the reduction of oxygen to hydrogen peroxide

A great number of microbial strains have been reported to produce cholesterol oxidase, including *lactobacillus* , *Brevibacterium* , *Corynebacterium* , *Pseudomonas* , *Rhodococcus* , *Schizophyllum, Streptomyces* spp. , *Nocardia erythropolis* produced cholesten-3-one from cholesterol. A similar reaction has also been found in a great number of microorganisms, such as *Arthrobacter, Mycobacterium, Nocardia, Pseudomonas* sp. and *Bacillus sphaericus.*

1.6 Probiotic

The term Probiotic was derived from the Greek, meaning “for life.” An expert panel commissioned by WHO defined probiotics as “Live microorganisms when administered in adequate amount confering a health benefit on the host.” Probiotics are dietary supplements containing potentially beneficial bacteria or yeast, with lactic acid bacteria (LAB) as the most common microbes used. LAB has been used in the food industry for many years, because they are able to convert sugars (including lactose) and other carbohydrates into lactic acid. This not only provides the characteristic sour taste of fermented dairy foods such as yogurt, but acts as a preservative, by lowering the pH and creating fewer opportunities for spoilage organisms to grow. Probiotic bacterial cultures are intended to assist the body's naturally occurring gut flora to re-establish them. They are sometimes recommended by doctors and, more frequently, by nutritionists, after a course of antibiotics, or as part of the treatment for gut related candidiasis. Claims are made that probiotics strengthen the immune system. The rationale for probiotics is that the body contains miniature ecology of microbes, collectively known as the gut flora. The number of bacterial types can be thrown out of balance by a wide range of circumstances including the use of antibiotics or other drugs, excess alcohol, stress, disease, exposure to toxic substances, or even the use of antibacterial soap. In cases like these, the bacteria that work well with our bodies may decrease in number, an event which allows harmful competitors to thrive, to the detriment of our health.

Currently, the probiotics that are marketed as nutritional supplements and in functional foods, such as yogurts, are principally the *Bifidobacterium* species and the *Lactobacillus* species. Probiotics are sometimes called colonic foods. Most of the presently available probiotics are bacteria. *Saccharomyces boulardii* is an example of probiotic yeast.

1.6.1 Effects

There is no published evidence that probiotic supplements are able to replace the body’s natural flora when these have been killed off; indeed bacterial levels in faeces disappear within days when supplementation ceases. There is evidence, however, that probiotics do form beneficial temporary colonies which may assist the body in the same functions as the natural flora, while allowing the natural flora time to recover from depletion. The probiotic strains are then progressively replaced by a naturally developed gut flora.

Hence, probiotics have been defined as correctives of the eco-organ. If the conditions which originally caused damage to the natural gut flora persist, the benefits obtained from probiotic supplements will be short lived.

1.6.2 Side effects

Treatment with probiotics, like any medicine, may include a condition called excessive drainage syndrome, which includes headache, diarrhea, bloating, or constipation. Another commonly reported side effect is intestinal gas. To reduce the side effects it is recommended to lower the supplement dosage, or pretreat with colon hydrotherapy, or stool softeners and fiber as tolerated or advised by a healthcare professional.

1.6.3 Actions and Pharmacology

Probiotics may have antimicrobial, immunomodulatory, anticarcinogenic, antidiarrheal, antiallergenic and antioxidant activities.

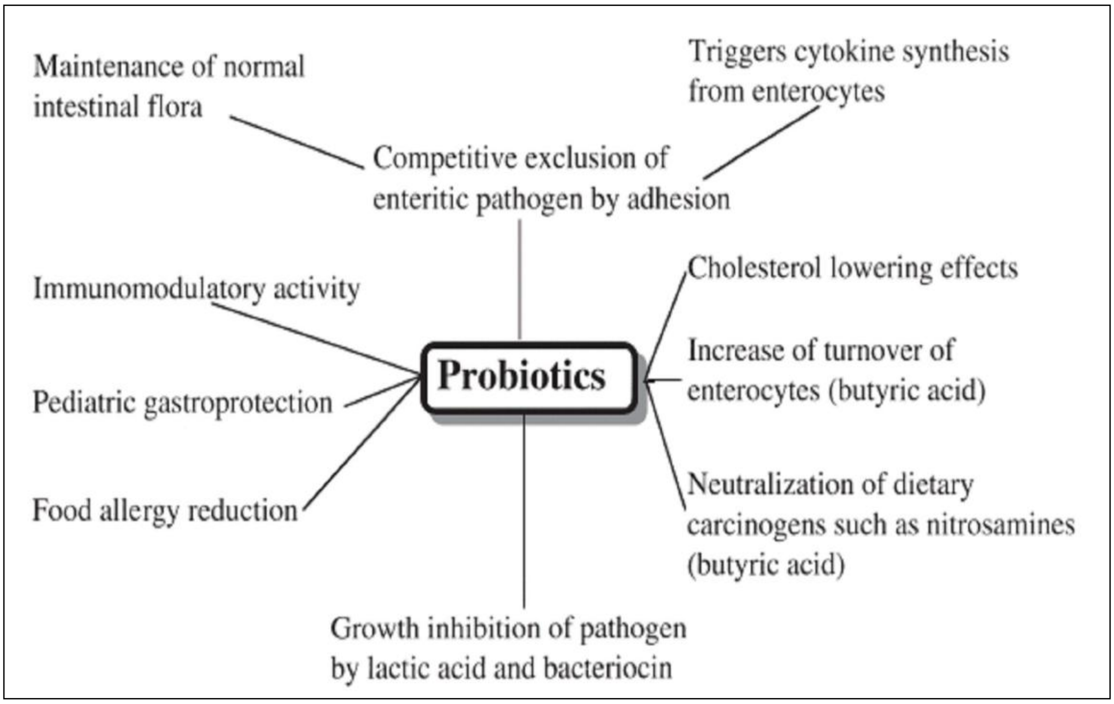


Fig. 1 Purported mechanisms of action of probiotics

1.7 *Lactobacillus* species

1.7.1 Cell structure and metabolism

Lactobacilli are rod-shaped, Gram-positive and catalase-negative bacteria that grow under microaerophilic to strictly anaerobic conditions, and do not form spores. They are usually straight, although they can form spiral or coccobacillary forms under certain conditions. They are often found in pairs or chains of varying length. *Lactobacilli* are classified as lactic acid bacteria, and derive almost all of their energy from the conversion of glucose to lactate during homolactic fermentation. In this process 85-90% of the sugar utilized is converted to lactic acid. They generate ATP by nonoxidative substrate-level 3 phosphorylation. Lactic acid bacteria (LAB) are common microflora in various fermented foods such as dairy products and processed vegetables, and show a wide variety of cell types and physiological and biochemical behaviour. In addition, LAB have been shown to inhibit in vitro growth of many enteric pathogens and have been used in both humans and animals to treat a broad range of gastrointestinal disorders .This inhibition may be due to the production of organic acids such as lactic acid, acetic acid, hydrogen peroxide, bacteriocins, bacteriocin-like substances and possibly biosurfactants.

1.7.2 Ecology

Lactobacilli are commonly associated with plant herbage. They have a generation time ranging from 25 minutes to several hundred minutes, and grow optimally between the temperatures of 30ºC and 40°C, although thermophilic strains can be comfortable at temperatures as high as 45°C. They are also commonly associated with the gastrointestinal tract of animals and humans. As natural Gastro intestinal microflora, they are believed to perform several beneficial roles including immunomodulation, interference with enteric pathogens, and maintenance of healthy intestinal microflora.

Lactobacillus casei is a species of genus *Lactobacillus* found in the human intestine and mouth. As a lactic acid producer, it has been found to assist in the propagation of desirable bacteria. This particular species of lactobacillus is documented to have a wide pH and temperature range, and complements the growth of *L. acidophilus*, a producer of the enzyme amylase (a carbohydrate-digesting enzyme). It is known to improve digestion and reduce lactose intolerance and constipation.

The most common application of *L.casei* is industrial, specifically for dairy production. *Lactobacillus* can also live in fermenting products, such as yogurt. *Lactobacillus casei* is typically the dominant species of non-starter lactic acid bacteria (NSLAB) present in ripening Cheddar cheese, and, recently, the complete genome sequence of *L. casei* ATCC 334 has become available. *L. casei* is also the dominant species in naturally fermented Sicilian green olives.

Among the best-documented, probiotics *L.casei*, *L. casei* DN-114001, and *L. Casei* Shirota have been extensively studied and are widely available as functional foods. In the past few years, there have been many studies in the decolorization of azo dyes by lactic acid bacteria such as *L. casei* TISTR 1500, *L. paracasei*, *Oenococcus oeni*. With the azoreductase activity, mono-, di- azo bonds are degraded completely, and generate other aromatic compounds as intermediates.

It produces lactic acid which helps lower pH levels in the digestive system and impedes the growth of harmful bacteria. *L. casei* may be found in “raw or fermented dairy and fresh or fermented plant products.” These sources may include yogurt, cheese, and other types of food sources such as fermented green olives. According to some reports, some strains of the bacteria help control diarrhea, while other strains have an anti-inflammatory effect on the gut. Other advantageous effects include reducing lactose intolerance, alleviating constipation, and even modulation of the immune system. Because friendly bacteria are vital to proper development of the immune system, to protection against microorganisms that could cause disease, and to the digestion and absorption of food and nutrients ensuring that the body has an appropriate amount of *L. casei* inhabiting the body is important. This may especially true for individuals suffering from Crohn's disease and critically ill children suffering from diarrohea.

1.7.3 Cholesterol Lowering

Animal studies have demonstrated the efficacy of a range of LAB to be able to lower serum cholesterol levels, presumably by breaking down bile in the gut, thus inhibiting its reabsorption (which enters the blood as cholesterol). Some, but not all human trials have shown that dairy foods fermented with LAB can produce modest reductions in total and LDL cholesterol levels in those with normal levels to begin with, however trials in hyperlipidemic subjects are needed.

2. REVIEW OF LITERATURE

2.1 Probiotic Effects of Lactic acid bacteria

At present, much effort is given to the maintenance of health through various approaches such as exercise and natural foods. Use of probiotics, in demand for improved well-being, in humans and animals has been widely discussed (Fuller et.al, 1991). In recent years, the probiotic effects of Lactic Acid Bacteria have gained interest in terms of their functional aspect. Probiotic, a growth promoting agent, is obtained from living strains to maintain a balance of intestinal microflora by decreasing the growth of coliform baillus. It has been also expected as a new type of antibacterial substance for resolving some of the complications in the use of antibiotics and the resistance of microorganism to antibiotic substances (Ahn et al., 1999, Cho et al., 2000). For commercial use, probiotics must satisfy safety as well as functional and technical issues, including viability, settlement, inhabitation, antibacterial agent creation, immunity hastening, antigenotoxic activation, pathogenic suppression, properties of organism, bacteriophage resistance and viability during the production process (Gilliland and Walker, 1990).

Numerous studies have been carried out on the stabilization of gastrointestinal microflora, reduction of saprogenic products, prevention of degenerative disease, activation of immunity, mediation of anticancer activities, lowering of cholesterol, reduction of lactose intolerance, and suppression and prevention of constipation. For probiotics bacteria to be effective they must survive the harsh environments in the stomach (low pH) and intestinal track, which contain bile acid (Lee et al., 2006).

Recent studies with *Lactobacillus plantarum* have indicated certain strains to have significant cholesterol lowering effect of up to 70%, (Lee et al., 1996).

2.2 Cholesterol reduction

Cholesterol is the major sterol constituent of eukaryotic organisms, involved in stabilization of membranes as well as being a factor in hormonal and other signaling pathways (Lamb et al. 1998). With the exception of the cell-wall deficient bacteria, *Mycoplasma*, the majority of prokaryotic organisms do not contain sterols in their membranes, and hapanoid is believed to fulfill the role of sterol in higher organisms. Nevertheless, cholesterol can be metabolized by a wide range of microorganisms as a carbon and energy source (Li and Beitz 1996).

The ability of actinomycetes, specifically mycobacteria, to bioconvert sterol is well documented, and has been utilized by the pharmaceutical industry to synthesize novel steroid compounds. In human macrophages, cholesterol is available as an integral part of the cell membrane, including the phagosomal membrane. Since mycobacteria reside in macrophages, an important issue is whether pathogenic mycobacteria can utilize the cholesterol located in the phagosomal membrane that surrounds the tubercle bacilli. Although it has been proposed that pathogenic mycobacteria use lecithin and not cholesterol (Kondo and Kanai 1976) *in vivo* as carbon source, a few reports indicate that cholesterol might be consumed by pathogenic mycobacteria. In addition, it has been shown that cholesterol is required for growth of pathogenic mycobacteria such as *M. scrofulaceum*, isolated from leprous tissues. Interestingly, the complete genomic sequence of *M. tuberculosis* suggests that it contains sterol biosynthetic enzymes as well as two putative cholesterol degradation enzymes. Supportive evidence for the ability of mycobacteria to synthesize cholesterol appeared in a recent report which demonstrates that *M. smegmatis* is able to synthesize trace amounts of cholesterol (Lamb et al. 1998).

The physiological role of cholesterol and the enzymes involved in its biodegradation by bacteria, and specifically mycobacteria, remain unknown. This study examines and compares the ability of various mycobacteria to take up and utilize cholesterol supplied in their growth media in an attempt to determine whether mycobacteria use cholesterol as a carbon source.

A study was conducted to determine the effect of diet supplementation with *Lactobacillus acidophilus* and *Lactobacillus casei* alone or in combination with water on total cholesterol and triglycerides concentrations in the blood serum and also, on growth performance of broilers. Two hundred one day old male Ross 308 broilers were randomly assigned to 5 treatments, with 4 replicate, 10 birds per each. Total cholesterol (Chol) and triglyceride (TG) were measured in blood samples of day 40. It was concluded that diet supplementation with *L. acidophilus* and *L. casei* in combination with water and or alone, significantly decreased total cholesterol and triglycerides concentrations in the blood serum of broiler chickens accompanying with improving feed conversion ratio, body weight gain and finally carcass yield (Hosseini et al., 2008).

In a study, fast-growing non-pathogenic mycobacteria degrade cholesterol from liquid media, and are able to grow on cholesterol as a sole carbon source. In contrast, slow-growing mycobacteria, including pathogenic *Mycobacterium tuberculosis* and bacillus Calmette-Guérin (BCG), do not degrade and use cholesterol as a carbon source. Nevertheless, pathogenic mycobacteria are able to uptake, modify, and accumulate cholesterol from liquid growth media, and form a zone of clearance around a colony when plated on solid media containing cholesterol. These data suggest that cholesterol may have a role in mycobacterial infection other than its use as carbon source (Av-Gay et al., 1997).

Cholesterol (5-cholesten-3-ol) and its oxides have been detected in a variety of foods and foodstuffs, especially, eggs, milks, meats, seafood and their processed products. Some of the cholesterol oxides have been hypothesized to be associated with colon cancer and to have potent angiotoxic effects suggesting that they have a likely role in cardiovascular diseases (Kauntiz et al., 1978), it was suggested that the bacterial degradation of cholesterol in cholesterol-containing foods may be useful for human health (Watanabe et al., 1986).

A great number of microbial strains have been reported to produce cholesterol oxidase, including *Brevibacterium*, *Corynebacterium* (Shirokano et al., 1977), *Pseudomonas* (Aono et al., 1994), *Rhodococcus* (Watanabe et al., 1986), *Schizophyllum* and *Streptomyces* spp. (Tomioka et al., 1976). *Nocardia erythropolis* produced cholesten-3-one from cholesterol (Turfitt et al., 1944). A similar reaction has also been found in a great number of microorganisms, such as *Arthrobacter*, *Mycobacterium*, *Nocardia* (Turfitt et al., 1944), *Pseudomonas* sp. (Talalay and Dobson 1953) and *Bacillus sphaericus*. However, knowledge of the cholesterol degradation pathway remains limited (Gilliland et al. 1985).

Transformation of cholesterol to 4-cholesten-3-one by cholesterol oxidase (EC 1.1.3.6, COD) has been widely reported 4-Cholesten-3-one has been characterized as a cholesterol derivative of interesting biological activities. COD is traditionally used as a key enzyme for detecting and quantifying cholesterol present in serum and food. It was first isolated from *Nocardia erythropolis* (Turfitt et al, 1944).

People are faced with a lot of health problems caused by their lifestyle. Human cardiovascular disease is the most important problem in many countries due to hypercholesterol. High cholesterol in serum and dietary are strongly associated with increased incidences of human cardiovascular diseases and colon cancer (Kim et al., 2008)

Reduction of total serum cholesterol of 1% can lower the risk of coronary heart disease by 2 to 3%. Lactic acid bacteria (LAB) have a long and safe history of application and consumption .LAB such as *Lactobacillus* spp. has been associated with several probiotic effects in humans and animals (Park et al., 2007). Probiotics have been considered to have potential health-promoting benefits as biotherapeutic agents. One of the health-promoting benefits of probiotics is their ability to reduce blood cholesterol (Gilliland, 1985; 2003; Lim et al., 2004).

Several studies have suggested that humans and animals origin isolated *Lactobacillus* spp. or their containing foods, influence cholesterol levels in laboratory media or living organisms (Taranto et al., 2003). Gilliland et al. (1985) have suggested that *Lactobacillus acidophilus* RP32 from fecal of pigs can grow well in the presence of bile and assimilate cholesterol from a laboratory growth medium. Thus, this species has the potential to inhibit increased serum cholesterol of pigs (Gilliland et al., 1985).

L. acidophilus isolated from human intestine can remove cholesterol from laboratory media due to assimilation property. Human intestinal isolated *Lactobacillus casei* can remove cholesterol from media by means of the destabilisation and co-precipitation of cholesterol micelles (Walker and Gilliland, 1993; Brashears et al., 1998)

A majority of *Lactobacillus* spp., which has cholesterol lowering ability, have been isolated from humans and animals. These species are considered to be starter culture for fermented foods, particularly milk or milk derived products (Caplice and Fitzgerald, 1999).

Probiotics are one of major food supplements for poultry industry. According to concerns about cholesterol, there are a lot of attempts to produce foods with low cholesterol. It has been reported that *L. acidophilus* can absorb cholesterol from in vitro system and this phenomenon can decrease the cholesterol level of medium. There are reports that probiotics can reduce the cholesterol level of blood in broiler chickens reduce the cholesterol level of blood in broiler chickens (Panda et al., 2003).

3. OBJECTIVES

Although cholesterol reduction by microorganism, example- Lactic acid bacteria assumes high importance, few studies have actually attempted to know the mechanisms specifically adopted by probiotic LAB. In this study, an attempt has been made to understand the mechanism of cholesterol reduction by a previously reported strain of *Lactobacillus casei.*

Following objectives were framed to carry out the above work

- To characterize cholesterol reduction by *L.casei*
- Optimize cultural parameters (pH, temp., agitation) and establish kinetics of *L.casei* for maximum reduction by culture.
- To understand cholesterol reduction mechanism.

4. MATERIALS & METHODS

4.1 Microorganism

The microorganism used was *L. casei*. It was pre-isolated from pickled mango samples (Dhillon et.al, 2007) and stored as 40% glycerol stock solution. The microorganism was revived in 5 ml MRS medium at 37ºC for 24 hours, prior to use.

4.2 Material

All the chemicals used were of analytical grade. Cholesterol (pure) used was purchased from HiMEDIA.

4.3 Qualitative methods of cholesterol detection

4.3.1. Preliminary characterization of cholesterol utilization

(A) Plate Method

L.casei was grown in MRS media, or in minimal medium containing the inorganic components of medium without any carbon source. In each case, cholesterol was added to the media when required. The cholesterol was suspended in 5 mL Tween-80, heated briefly, and added to the media (1 g/L). For plate preparation, cholesterol was suspended in 20 mL of warm Tween-80. The solution was filtered through a 0.22 µm sterile filter and added to the media. Plates were made by adding pure agarose to a final concentration of 2% as a solidifying agent (Av-Gay et al., 1997).

(B) TLC Method

Culture was grown overnight in MRS supplemented with cholesterol as carbon source and harvested; the supernatant was used to detect residual cholesterol by TLC. A hexane/ethyl acetate mixture (6:1.5 v/v) was used as eluent. Samples from different extraction mixtures were applied to the silica plates. For visualizing the TLC plates, the agent used was: 100 mg $MnCl_2$. 4 H_2O + 30 mL H_2O + 30 mL CH_3OH + 1 mL conc. H_2SO_4 (Abidi, et.al, 2001).

4.4 Optimization of culture conditions for maximum cholesterol reduction

Culture conditions were optimized for maximum cholesterol reduction by varying the pH, temp., incubation time & agitation for best results.

a. Effect of pH on cholesterol reduction

MRS Media containing cholesterol at initial pH values of 4.0, 6.0, 8.0, and 10.0 were used for cultivation of *L.casei* to study the effect of initial pH on the cholesterol reduction.

b. Effect of temperature on cholesterol reduction

MRS Media containing cholesterol at different temperatures ranging from 26ºC, 37ºC, 50ºC, 70ºC were used for cultivation of *L.casei* to study the effect of temperature on the cholesterol reduction.

c. Effect of agitation on cholesterol reduction

MRS Media containing cholesterol was used for cultivation of *L.casei* to study the effect of agitation on the cholesterol reduction.

4.5 Kinetics of cholesterol reduction

The estimation of the reduced cholesterol was performed according to the method of Lee et al., (1997). Based on the above optimized conditions, *L. casei was* incubated at 26ºC in the presence of cholesterol. Samples were collected at 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72 hours to measure the cholesterol concentration. For assay of cholesterol, a sample (2 mL) of culture was centrifuged (1519 x g, 10min) and washed with demineralized water (1 mL). The concentrations of total cholesterol in supernatants were analyzed by HPLC.

4.5.1 *Cholesterol reduction by culture supernatant*

Exopolysaccharides of Lactic acid culture was incubated with cholesterol and residual cholesterol was quantitated. To study reduction, concentrated cell free extracts and supernatant were incubated with cholesterol. The net decrease in cholesterol was observed for 3 hours.

Samples were removed after each hour and examined qualitatively and quantitatively for residual cholesterol. To confirm the nature of the principle, supernatant was boiled at 100ºC for 5min, separate aliquots were treated with proteinase. Treated aliquots were incubated as above for cholesterol degrading activity. Similar method was followed for cell lysate.

4.5.2 Quantitative method for cholesterol detection

High-Performance Liquid Chromatography (HPLC)

Cholesterol reduction was confirmed by HPLC. 2 ml of culture broth was collected and centrifuged at 1,500 ×g for 15 min. The supernatant was collected and then filtered through a 0.45-μm filter, and analyzed by HPLC. Cholesterol analysis was performed using a HPLC on Waters using C-18 column of dimensions 250mm x 4.6mm with particle size of 5μm using 20 μL loop, a HP 1100 series binary pump, column oven (34ºC), and a HP 1100 series UV detector (240 nm). Mobile phase used was acetonitrile/water 1:1 (v/v) wash was used to separate Cholesterol at 240 nm with isocratic flow rate of 1ml/min.

4.6 Cholesterol reduction by *L. casei* exopolysaccharide

4.6.1 Extraction of Exopolysaccharide of L.casei

0.1ml *L. casei* culture in 10ml MRS tube was inoculated and incubated overnight. Then 10ml of *L.casei* culture was inoculated in 1000ml FIB media and incubated for 60 hours at 37ºC. The culture was then centrifuged at 12,000 rpm for 10 mins. The supernatant was collected and added double the volume of ethanol and kept at 4ºC for 24 hours (Yanliang et al., 2010).

4.6.2 Recovery of Exopolysaccharide

The supernatant was centrifuged for 15 mins at 4ºC at 15,000 rpm and the pellet added to 100ml deionised water + 50ml cetyl pyridinium chloride (CPC), Left undisturbed for 3 hours (Yanliang et al., 2010).

4.6.3 Purification of Exopolysaccharide

After 3 hours, supernatant was centrifuged for 15 mins at 4ºC at 15,000rpm and the pellet added to 100 ml of 0.5 molar NaCl. And the earlier process was repeated. The exopolysaccharide was lyophilized and dialyzed (Yanliang et al., 2010).

4.6.4 Cholesterol Reduction

For cholesterol removal studies, 1mg/ml exopolysaccharide was incubated with cholesterol for 3 hr at room temperature in buffer. Samples were removed, centrifuged and supernatant checked qualitatively and estimated for residual cholesterol (Juan et al., 2010).

5. RESULTS AND DISCUSSION

5.1 Qualitative detection of cholesterol detection

5.1.1 Preliminary evidence of utilization

(A) Plate Method

Fig 2-Growth of *L.casei* in minimal media in presence and absence of Cholesterol

Figure 2 shows that in minimal media, in the presence of cholesterol, *L.casei* used cholesterol as carbon source and the growth was significantly high than in the minimal media without cholesterol. Tween-80 was used as the organic solvent for cholesterol (which by itself can be used as a carbon source).

(B)Thin Layer Chromatography

The fig 3 shows that when cholesterol (1 mg/ml) was added to the media inoculated with the culture and kept at 37ºC for 24 hours, shows the band on TLC plates whereas media inoculated with culture in absence of cholesterol did not show any band on TLC. This indicates that the culture degrades cholesterol.

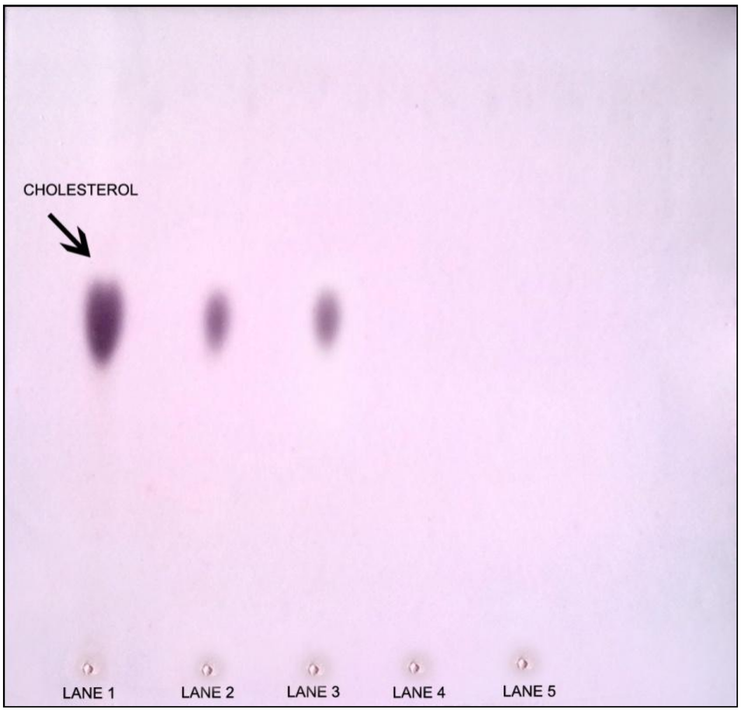


Fig 3- Thin layer chromatogram of cholesterol detection. LANE 1-standard cholesterol (1 mg/ml) (Rf value- 0.57), LANE 2,3-MRS+ Culture + cholesterol; LANE 4,5 --MRS+ Culture.

5.2 Optimization of culture conditions for maximal cholesterol reduction

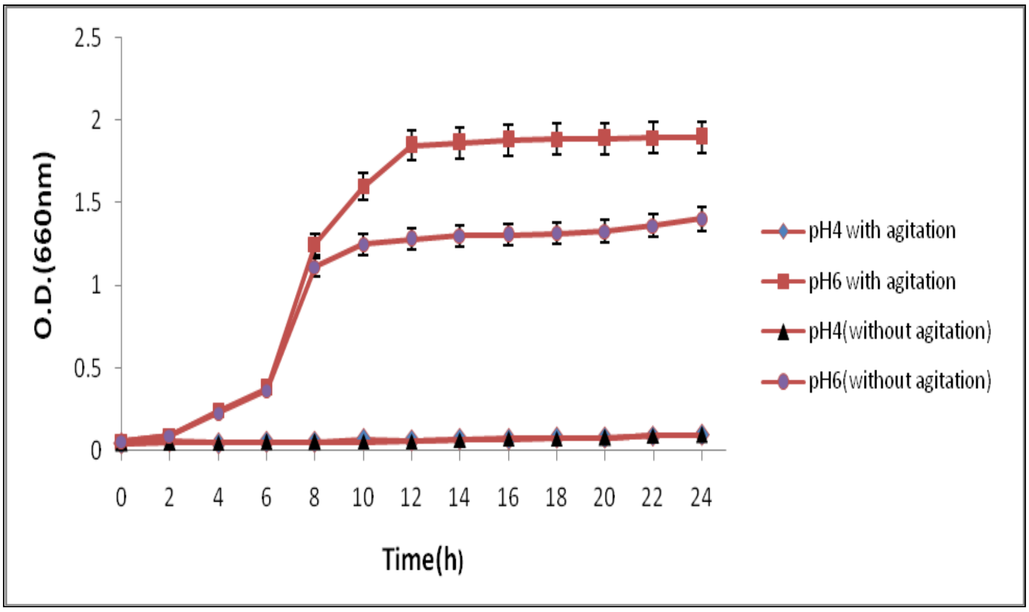


Fig 4-Growth profile of *L.casei* at different pH and agitation

Fig 4 shows the growth profile of *L.casei* at pH 4.0 and 6.0, with and without agitation. *L.casei* hardly grew at pH 4.0, resulting in low extracellular enzyme.

However, *L.casei* grew very well in the pH 6.0. The agitation speed markedly affected the growth profile of *L.casei*. As the agitation speed increased, the growth of *L.casei* noticeably increased. *L.casei* exhibited optimum activity at a pH value around 6.0.

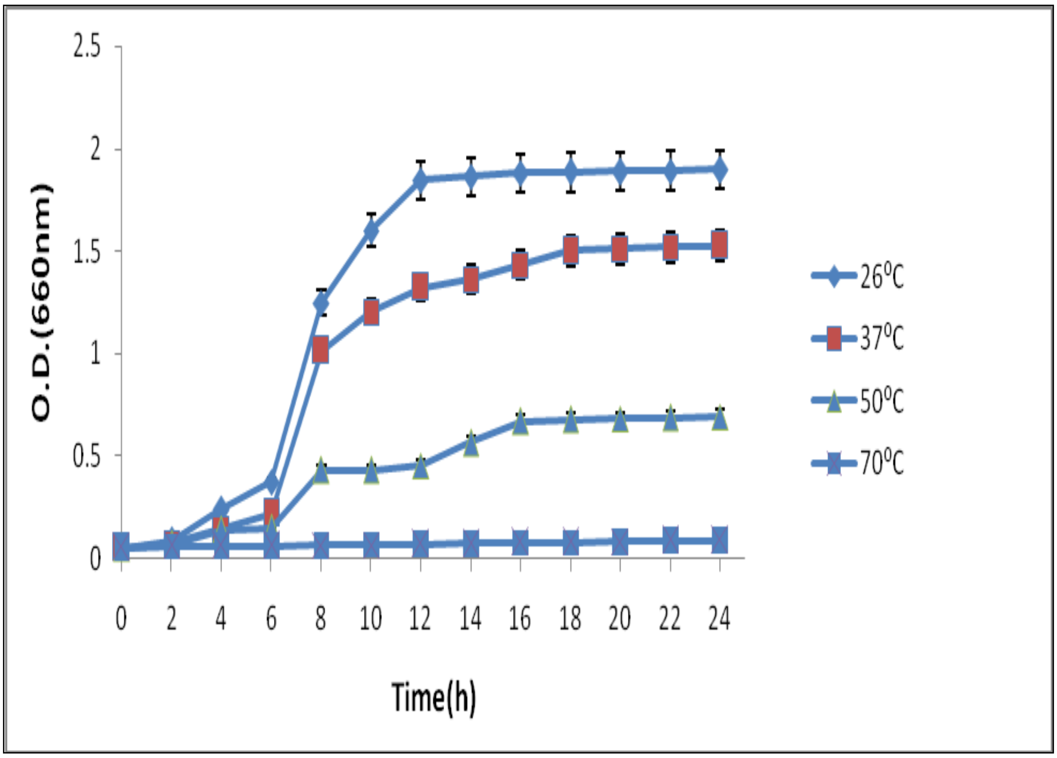


Fig 5- Growth profile of *L.casei* at different temperature

In Fig 5, Growth profile of *L.casei* at different temperatures is shown. *L.casei* grew very well at temperature 26ºC but also grew at temperature 37ºC. The agitation speed markedly affected the growth profile of *L.casei*. *L.casei* exhibited optimum activity at temperature 26ºC and a pH value 6.0.

5.3 Kinetics of cholesterol reduction

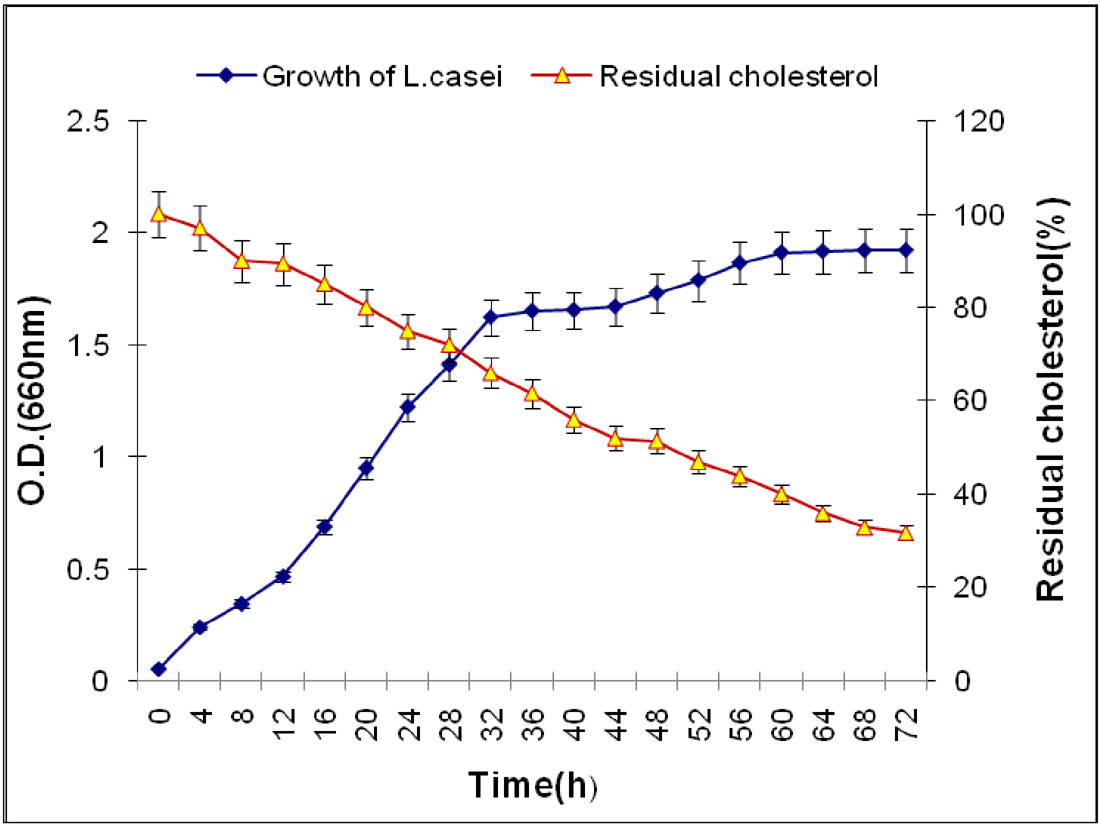


Fig 6 – Growth kinetics and cholesterol reduction by *L.casei*

During the 24 hours incubation, the bacterial number increased, whereas the cholesterol concentration decreased by 25%. No decrease in cholesterol occurred in MRS medium alone (data not shown). These results suggest *L.casei* has cholesterol lowering effect caused by physiological actions of the end products of short-chain fatty acid fermentation and cholesterol degradation by the bacteria.

L.casei grew slowly in the minimal medium with only cholesterol as the sole carbon source. Approximately 70% of the cholesterol (1 g/L) was reduced within 72 h of incubation.

5.3.1 Cholesterol Degradation by Supernatant

(A) Qualitative Analysis

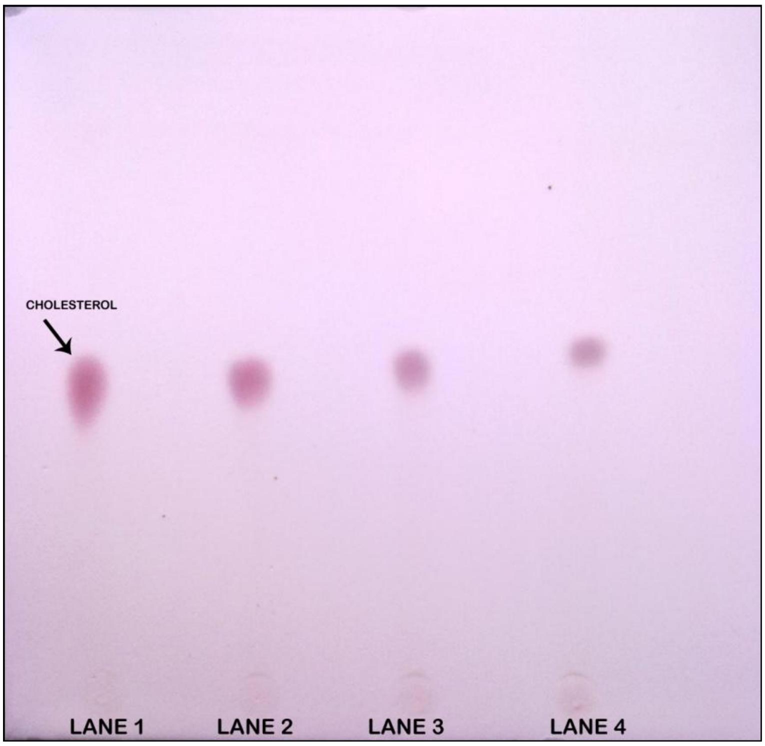


Fig 7- Thin layer chromatogram of cholesterol reduction by supernatant at different time intervals. LANE 1-standard cholesterol (Rf value- 0.57), LANE 2-1 hour, LANE 3 - 2 hours, LANE 4 - 3 hours.

Table 1 – Kinetics of cholesterol reduction by supernatant.

Time(h)	**Initial cholesterol (mg)**	**Residual Cholesterol (mg/ml supernatant)**	**% Reduction of Cholesterol**
0(Control)	0.340	0.340	0.0
1	0.340	0.295	13.24
2	0.340	0.236	31.59
3	0.340	0.205	39.71
4	0.340	0.204	40.00

Table 2 –Cholesterol reduction by boiled, proteinase K treated supernatant and cell lysate for 3 hours

Treatment	Initial cholesterol	Residual Cholesterol(mg/ml)	% Reduction of Cholesterol
Boil supernatant	0.34	0.205	39.71
Proteinase k + supernatant treatment	0.34	0.205	39.71
Cell lysate	0.34	0.205	39.71

(B) Quantitative Analysis - HPLC

The High Performance Liquid chromatograms shown below at various time intervals indicate reduction of cholesterol amounts.

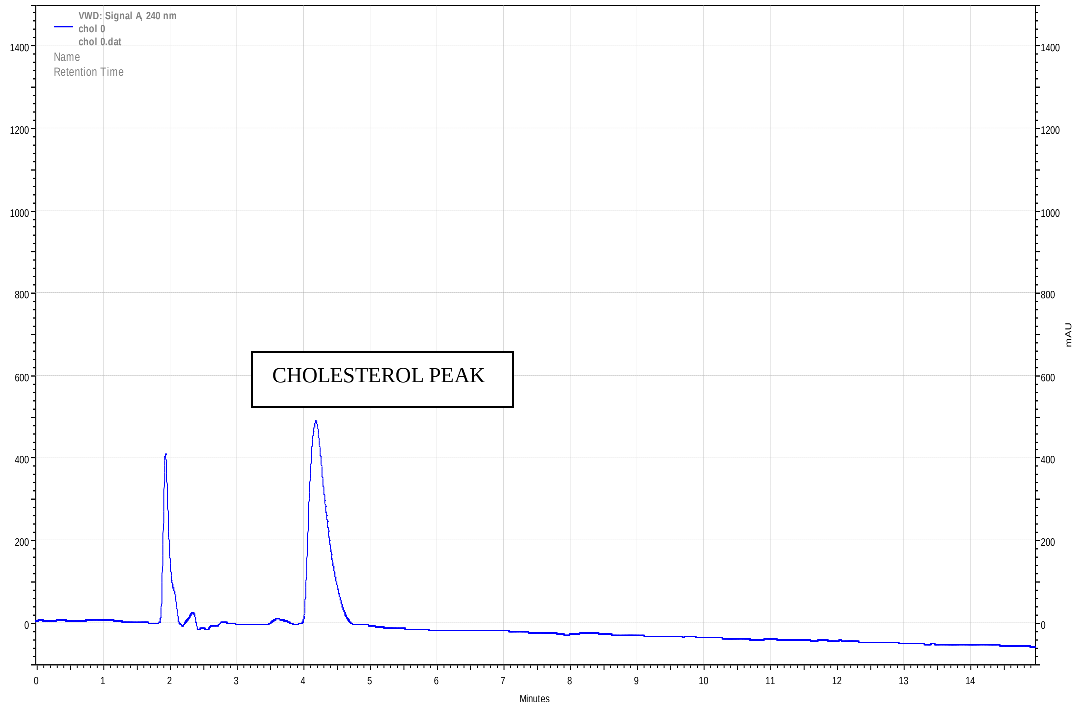


Fig 8- At 0 hour

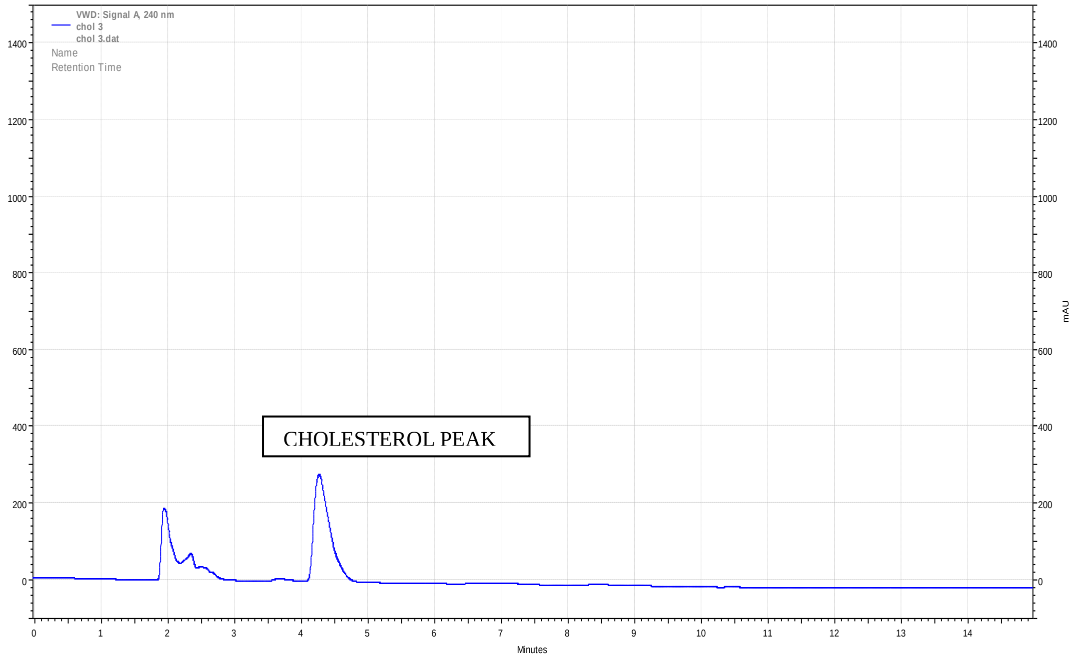


Fig 9- At 3 hours

Fig 8 & 9 shows the High Performance Liquid chromatograms of cholesterol with its retention time 4.4 minutes, however just after treating with supernatant of *L.casei*, *L.casei* have cholesterol peak height of 4.29 cm at 0 hours and at 3 hours peak height was 2.58 cm. According to calculation (given in appendix) resulting decrease in cholesterol is 39.71%. This high decrement in concentration of cholesterol is due to supernatant of *L.casei.*

5.3.2 Reduction Kinetics of Cholesterol by *L.casei*

(A) Qualitative Analysis

Cholesterol reduction kinetics was qualitatively analysed by thin layer chromatography. 10 µl sample was loaded on the silica gel TLC plate. It was run with hexane/ethyl acetate mixture (6:1.5 v/v), used as eluent, and developed by using 100 mg $MnCl_2$ .4 H_2O + 30 mL H_2O + 30 mL CH_3OH + 1 mL conc. H_2SO_4.

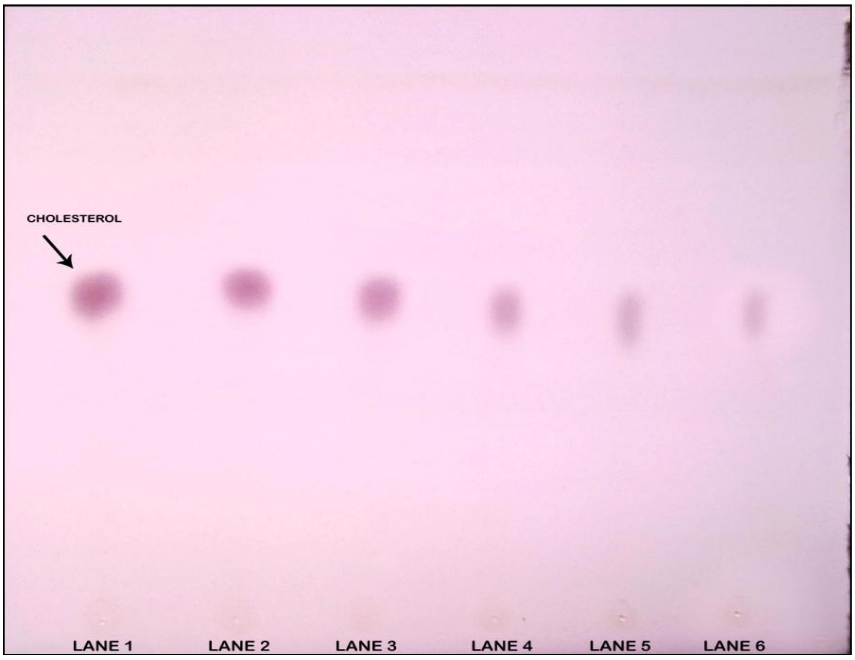


Fig 10- Thin layer chromatogram of cholesterol degradation at different time intervals by *L. casei*. LANE 1-standard cholesterol (Rf value- 0.57), LANE 2-12 hours, LANE 3 - 24 hours, LANE 4 - 48 hours, LANE 5 -72 hours, LANE 6 - 96 hours.

(B) Quantitative Analysis - HPLC

The High Performance Liquid chromatograms shown below at various time intervals indicate reduction of cholesterol amounts.

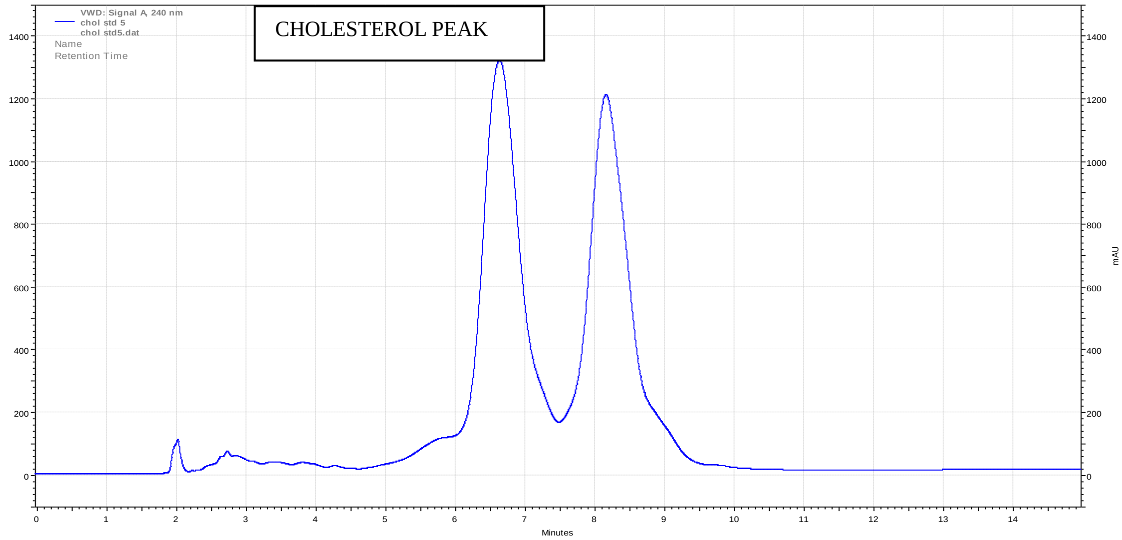


Fig 11- Chromatogram with Standard Cholesterol

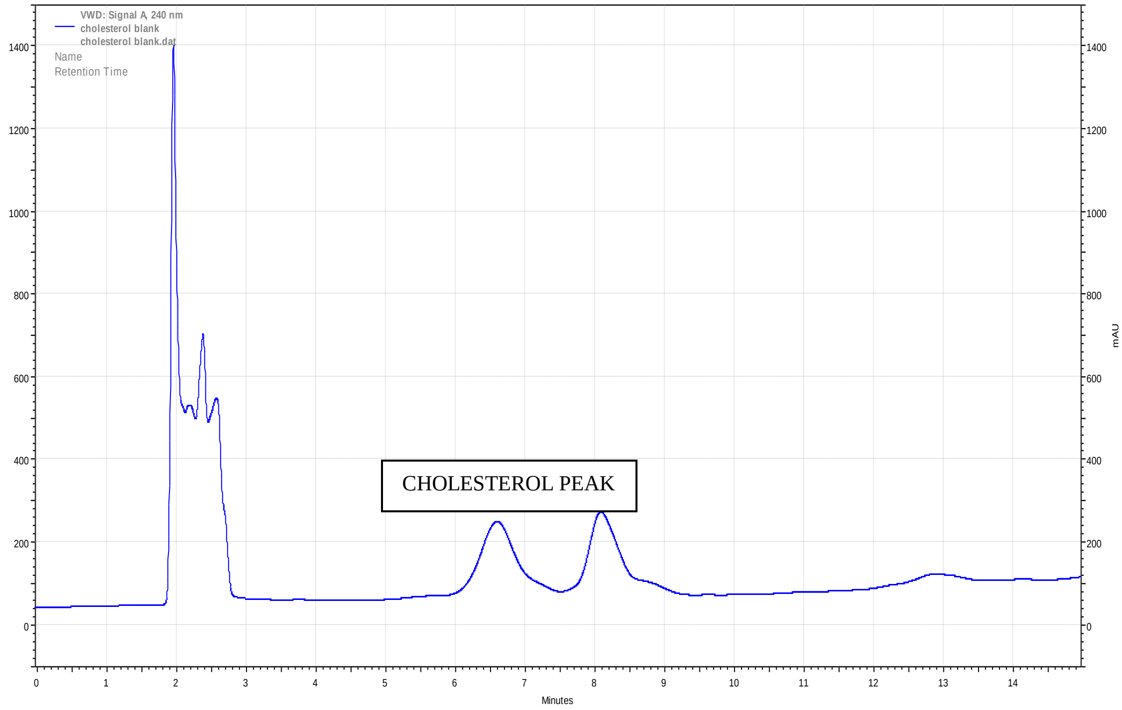


Fig 12- At 0 Hour

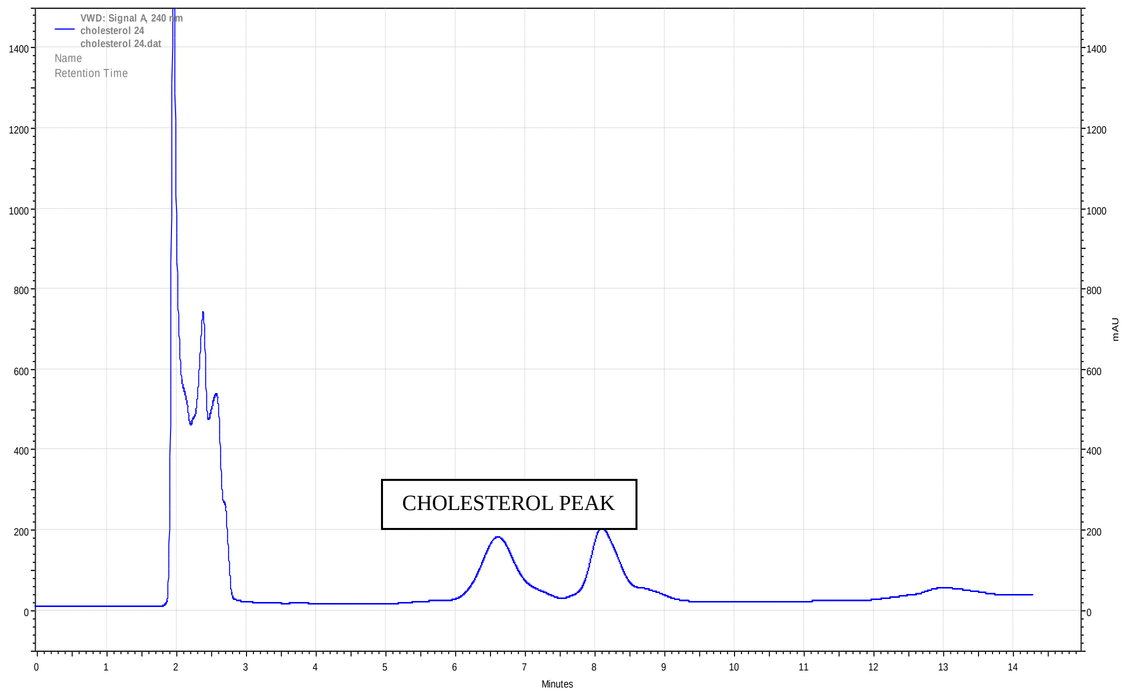


Fig 13- At 24 Hours

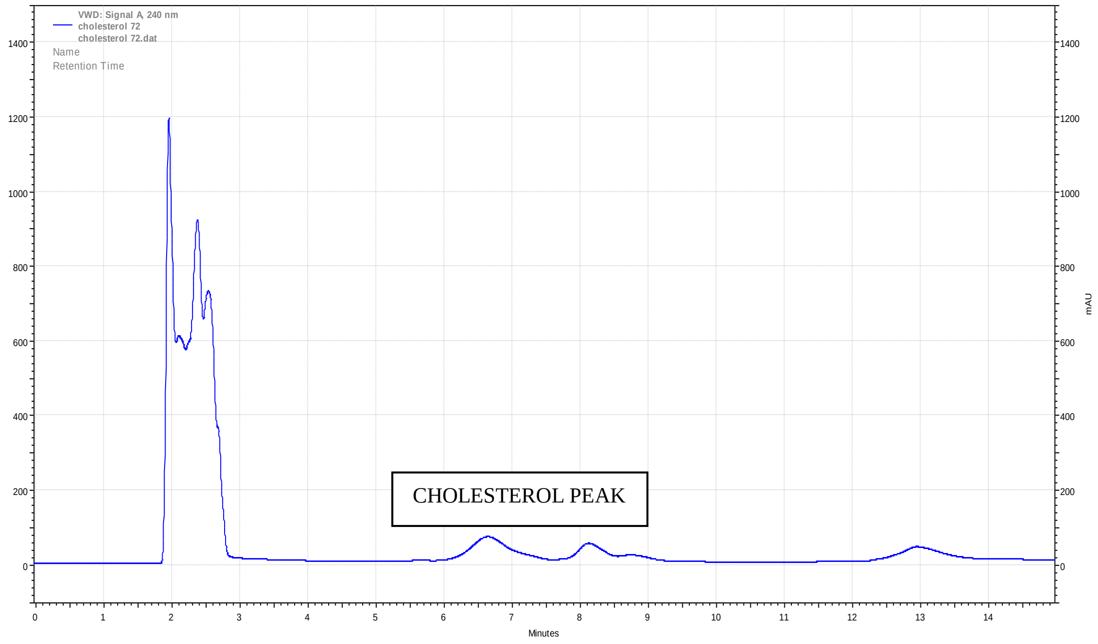


Fig 14- At 72 Hours

Fig 11, 12, 13 & 14 indicates the peaks in High Performance Liquid chromatograms of cholesterol with its retention time 6.6 minutes, however just after incubation of *L.casei,* it has cholesterol peak height of 2.199 cm, 1.649 cm and 0.699 cm at 0, 24 and 72 hours respectively. According to calculation (given in appendix) resulting decrease in cholesterol is 68.22 %. On keeping this for incubation up to 96 hours and analysed, it resulted in 69% decline of initial concentration of cholesterol which is not significant reduction after 72 hours. This high decrement in concentration of cholesterol is due to the extracellular enzyme of *L.casei.*

5.4 Cholesterol reduction by purified *L. casei* Exopolysaccharide

Table 3 – Kinetics of cholesterol reduction by exopolysaccharide

Treatment	**Residual Cholesterol (mg/mg Exopolysaccharide)**	**% Reduction of Cholesterol**
0.34 mg cholesterol (Control)	0.34	0.0
0.34 mg cholesterol + 1 mg Exopolysaccharide	0.24	29.42

The above results suggested that reduction of cholesterol occurs partially by exopolysaccharides (Gao et.al. 2006) produced by the *L.casei* and the remaining cholesterol is catalyzed by an extracellular enzyme produced in the supernatant (Yanliang et.al 2010). Qualitative results (TLC) obtained initially were confirmed by using HPLC method to ensure that cholesterol have been reduced.

6. CONCLUSION

In this study, an attempt was made to characterise the cholesterol reduction mechanism of previously identified strain of *L.casei* (Brashears et.al, 1998). Cholesterol reduction was maximum at the temperature of 26ºC, pH 6 and with agitation at 120 rpm for a period of 72 hours. The removal of cholesterol was presumptively detected by Thin Layer Chromatography (TLC) and confirmed by High Performance Liquid Chromatography (HPLC). Further examination of the principles involved in cholesterol reduction was revealed by the involvement of exopolysaccharide in binding of cholesterol. Further studies can be carried out on the enzyme characteristics and the nature of the degraded product.

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APPENDIX

de Man Rogosa Sharpe (MRS) Broth

Component	**Amount**
Proteose peptone	10.0 g
Beef Extract	10.0 g
Yeast Extract	5.0 g
Dextrose	20.0 g
Polysorbate 80 (Tween 80)	1.0 ml
Ammonium citrate	2.0 g
Magnesium sulphate	0.1 g
Manganese sulphate	0.05 g
Dipotassium phosphate	2.0 g
Sodium acetate	5.0 g
Distilled water	1000 ml
pH	6.6

Composition of Modified MRS medium

Component	**Amount**
Proteose peptone	10.0 g
Beef Extract	10.0 g
Yeast Extract	5.0 g
Dextrose	20.0 g
Polysorbate 80 (Tween 80)	1.0 ml
Ammonium citrate	2.0 g
Magnesium sulphate	0.1 g
Manganese sulphate	0.05 g
Dipotassium phosphate	2.0 g
Sodium acetate	5.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	6.8

Composition of Minimal medium

Component	Amount
Glucose	10.0 g
Sodium acetate	5.0 g
Dihydrogen potassium phosphate	3.0 g
Dip,otassium phosphate	3.0 g
Magnesium sulphate	0.2 g
Methionine	0.1 g
Distilled water	1000 ml
pH	6.8

Composition of FIB Media

Component	Amount
Peptone	5.0 g
Diammonium Sulphate	2.0 g
Yeast Extract	1.0 g
Calcium Chloride	0.7 g
Sodium Chloride	0.1 g
Magnesium sulphate	0.2 g
Dipotassium phosphate	1.0 g
Agar	3.0 g
Glucose	1.0 g
Distilled water	1000 ml
pH	6.8

Residual cholesterol amount after supernatant treatment (HPLC)

3.6 cm = 400 mau

So 1 cm=111.11 mau

Lets peak height of standard = 477.77 mau

Lets peak height of sample at 3 hours = 287.77 mau

% remain of cholesterol after 3 hours = 60.23 %

Residual cholesterol amount after *L.casei* treatment (HPLC)

3.6 cm = 400 mau

So 1 cm=111.11 mau

Let peak height of standard = 244.44 mau

Peak height at 24 hours = 183.33 mau

Cholesterol % remained after 24 hours = 75 %

Peak height at 72 hours = 77.7

Cholesterol % remained after 72 hours =31.78 %

Reaction mixture -

The reaction mixture of cholesterol degradation by exopolysaccharide and enzyme consisted of 50 mM sodium potassium phosphate buffer (pH7.0), 64 mM sodium cholate, 0.34% Triton X-100, 1.4 mM 4-aminoantipyrine, 21 mM phenol, 0.89 mM cholesterol.