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Author: Jemeema Deborah Harikrishnan, Punsisi Rasara Weerasooriya

BMS, School of Science, Sri Lanka

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DETERMINATION OF CHOLESTEROL-LOWERING ABILITY OF LACTOBACILLUS ISOLATED FROM CURD

Jemeema Deborah Harikrishnan, Punsisi Rasara Weerasooriya

School of Science, Business Management School, Sri Lanka

ABSTRACT

Probiotics are live bacteria, considered safe and confer beneficial effects to the human when obtained in adequate amounts. Probiotics are common in fermented dairy products, including curd. Curd is a rich source of nutrients and probiotics. Lactobacillus is the most commonly found probiotics in curd. The current study aims to identify Lactobacillus from curd samples and to determine their cholesterol-lowering ability. To identify Lactobacillus, commercially available five different curd samples were cultured on MRS media specific for Lactobacillus growth. Lactobacillus was identified in all five samples by morphological and biochemical tests. The colony morphology of the isolates was single colonies with smooth, round and creamy white colour. The isolated Lactobacillus were characterized using gram staining, catalase test, acid-fast and endospore staining. The isolated bacteria were gram-positive, catalase-negative, acid-fast negative and non-endospore forming. The isolated pure colony of Lactobacillus was sub cultured in MRS broth for the cholesterol-lowering test. The cholesterol-lowering ability of Lactobacillus cell intact and cell free suspensions of all five samples were tested by ferric ammonium sulphate method. The cholesterol removal rate was measured spectrophotometrically at the 560 nm wavelength. The data analysis showed that cell intact suspension has significantly high ($p<0.05$) cholesterol-lowering ability than cell free suspension. Cholesterol-lowering ability of Lactobacillus found in curd would be a cost-effective alternative treatment with no side effects to reduce serum cholesterol levels compared to commercially available hypocholesterolemic pharmacological drugs that have side effects which could have an impact on the quality of life.

Keywords: Probiotics, Lactobacillus, Curd, Cholesterol-lowering ability

INSTRUCTION

Probiotics are living, non-pathogenic microorganisms, when administered in adequate amounts, confers health benefits to the host (Prabhurajeshwar and Chandrakanth, 2018). Probiotics, originated from the Greek language means 'for life' having the ability to express several health-promoting functions (Jain, Mehta and Bharti, 2017). Probiotics such as Lactobacillus, Streptococcus, and Enterococcus are the most commonly used bacteria in fermented dairy products (Azat et al., 2016). In 1907, a Russian scientist Elie Metchnikoff proposed that routine consumption of fermented milk could promote life expectancy observing from a Bulgarian population. In 1989 Fuller defined probiotics are live, non-pathogenic, and health-promoting microorganisms (Iranmanesh, Ezzatpanah, and Mojgani, 2013). Probiotics are commonly found in dairy products such as curd, yoghurt, fermented milk, fresh milk, cheese, and fermented food (Rezac et al., 2018).

Health benefits of probiotics

Probiotics are important in promoting gut immunity, reduction of serum cholesterol level and blood pressure (Kamal et al., 2015). Furthermore, it is used to treat allergic pathology conditions like allergic rhinitis and immunoglobulin E-sensitized eczema, inflammatory bowel disease, diarrhoea, viral infections, and type 2 diabetes (Sharma et al., 2017; Ozdemir, 2010). The probiotic bacteria express probiotic properties such as antiviral, antimicrobial, anticarcinogenic, and antimutagenic properties (Sanders, 2000; Saarela et al., 2000).

Characteristics of Lactobacillus

The genus Lactobacillus is a member of lactic acid bacteria which can cause rapid acidification on the raw materials producing lactic acid (Forouhandeh et al., 2010). Among the lactic acid bacteria, genus Lactobacillus has beneficial characteristics which are useful in industrial applications. There are more than 110 species found in genus Lactobacillus, mainly classified as obligate homofermentative, obligate heterofermentative, and facultative homofermentative (Amin et al., 2009). The genus Lactobacillus contains genetically and physiologically variable strains of Gram-positive, catalase negative, non-spore-forming and rod-shaped (Figure 1.) in various lengths (Shuhadha et al., 2017).

Figure 1. Scanning electron microscope image of Lactobacillus (Kokkinos et al., 1998).

Preclinical and clinical studies on curd Lactobacillus

Several studies have been carried out to understand the health benefits of Lactobacillus found in curd. Bacterially mediated dental caries occurs nearly 95% of the population and children are at moderate to high risk to have dental caries. Streptococcus mutans are major virulent caries-producing organisms which normally present in the oral cavity. A clinical study on Lactobacillus inhibitory property against salivary Streptococcus mutans was tested among 40 healthy caries free 10-12 years old children. The subjects were divided into two groups and blindly tested with normal and curd containing Lactobacillus. The study showed that Lactobacillus containing curd has the potential to reduced oral Streptococcus mutans counts compared to normal curd screening after 30 days. Thus the study confirmed consumption of probiotic curd could reduce the risk of developing Streptococcus mutans mediated dental caries (Sudhir et al., 2012). A study by Shandiliya et al., (2016) using rat models fed with curd Lactobacillus found, it has potential to treat IgE mediated food allergy promoting Th1 type immune response by suppressing the production of both IgE and IgG antibodies

Lactobacillus cholesterol-lowering ability

Cholesterol is a vital substance of the human body and a structural component of the human cell membrane (Liu et al., 2013). Cholesterol is required in the body for the biosynthesis of steroid hormones; to create cell membrane, and to insulate nerves (Anandharaj and Sivasankari, 2014; Hu et al., 2010). Moreover, it is essential in vitamin D synthesis and modulating membrane fluidity. The elevated level of total serum cholesterol or low-density lipoprotein (LDL) cholesterol is the major risk factor of cardiovascular diseases. The World Health Organization expects that by 2030 cardiovascular diseases may cause 23.6 billion deaths. (Suneeti et al., 2020). There are several commercially available hypocholesterolemic pharmacological drugs developed to treat hypercholesterolemia. Stains (3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors) Atorvastatin, Simvastatin, Rosuvastatin, and Lovastatin are the most commonly used statins (Anandharaj and Sivasankari, 2014). Apart from statins cholesterol absorption inhibitors (ezetimibe), and fibric acids, high-density lipoprotein stimulators (nicotinic acids) are also used (Tomaro-Duchesneau et al., 2014). Despite being effective to reduce the cholesterol levels, these drugs can cause side effects like gastrointestinal discomforts (Tsai et al., 2014). The predominant adverse side effects are statin-associated muscle symptoms causing muscle pain early within 4-6 weeks of starting a statin. Statin therapy is known to associate with a small surge in fasting blood glucose levels. A Randomized control trial (RTC) among subjects under statin treatment indicated an increased incident of Diabetes mellitus by 9%. Likewise, treatment of stain for the reduction of cholesterol levels found to be potentially harmful to cognitive functions (Mach et al., 2018). Hence, considering the side effects of statins, alternative methods using natural methods to reduce serum cholesterol levels are important. A meta-analysis of a randomized controlled clinical trial with only probiotic products demonstrated reduced total cholesterol and LDL cholesterol compared to the control group. There was no significant effect of probiotics on high-density lipoprotein (HDL) cholesterol or triglycerides. Therefore, probiotic Lactobacilli has been proposed having the ability to reduce serum cholesterol levels. (Cho and Kim, 2015).

Several mechanisms for cholesterol removal by probiotic Lactobacillus have been proposed, such as deconjugation of bile salts by bile-salt hydrolase (BSH). Bile salt hydrolase enzyme or products containing it have been found to reduce the serum cholesterol levels by interacting with host's bile salt metabolism via de conjugating bile acids enzymatically and increasing rate of elimination of cholesterol from the body (Figure 3.) (Yasiri et al., 2018).

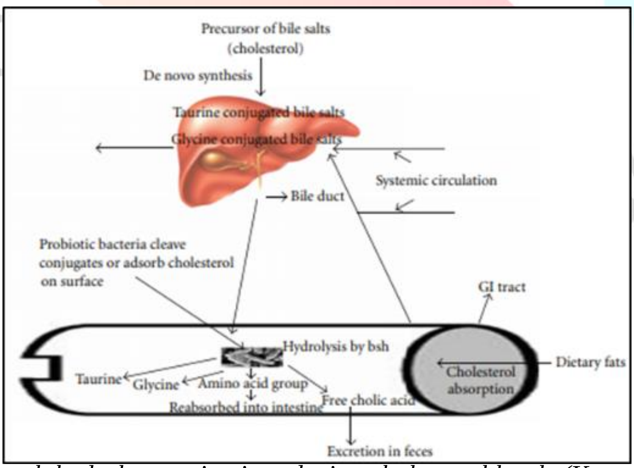


Figure 3. Bile salt hydrolase action in reducing cholesterol levels (Kumar et al., 2012)

Another way of reducing cholesterol absorption into gut enterocytes is via cholesterol assimilation by Lactobacillus (Figure 4.). This results in the elimination of non-metabolized cholesterol via faeces (Tomaro-Duchesneau et al., 2014).

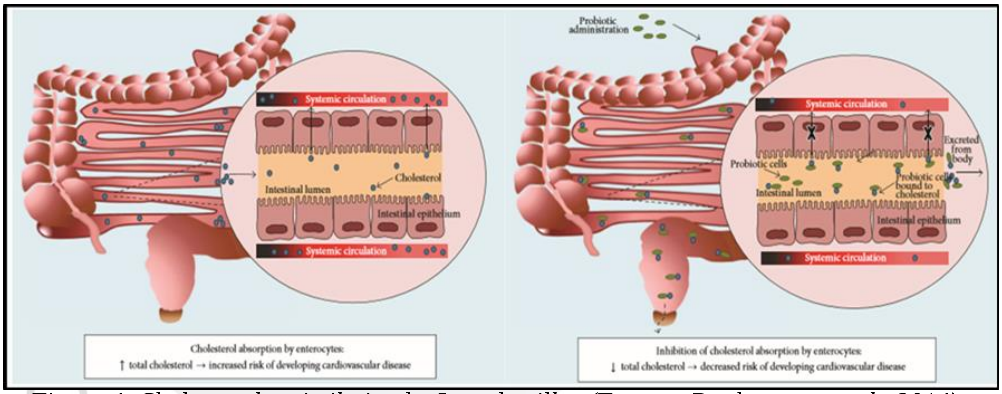


Figure 4. Cholesterol assimilation by Lactobacillus (Tomaro-Duchesneau et al., 2014)

Added mechanisms of reduction of cholesterol are via the production of short-chain fatty acids (SCFAs) during the growth of probiotics, and cholesterol conversion into coprostanol (Tsai et al., 2014).

Significance of Lactobacillus as biotherapeutics

The ability of Lactobacillus to survive in weak acidic environments, not producing toxin substance (Forouhandeh et al., 2010) and availability in the common type of foods make it more suitable to be used as biotherapeutics (Ascone et al.,2017). Poor dietary behaviour is one of the main risk factors of hypercholesteremia which mediate developing cardiovascular diseases. Probiotic Lactobacillus supplementation could improve the plasma lipid profile by altering the lipid transporters like very-low-density lipoprotein (VLDL), and LDL (Ooi et al., 2010). The consumption of probiotic Lactobacillus does not mediate any adverse effects, rather; it provides a beneficial impact to the host (Manzoor et al., 2016). Few studies had been carried out in Sri Lanka on Lactobacillus for the identification of morphological and biochemical characteristics. Studies on bile salt tolerance test to determine cholesterol reduction ability of Lactobacillus had also been carried out (Shuhadha et al., 2017). However, this study determines the cholesterol-lowering ability of Lactobacillus (cell intact) and metabolites (cell free) produced by the bacteria using ferric ammonium sulphate method. Aim of this study is to determine the cholesterol-lowering ability of cell intact and cell free Lactobacillus suspensions isolated from curd using ferric ammonium sulphate method.

METHODOLOGY

Sample collection and preparation

Five different commercially available brands of curd samples were purchased from local markets in Colombo vicinity.

All culturing techniques were carried out under aseptic condition.

Isolation of Lactobacillus on MRS agar

2g of each sample (1-5) was homogenized to a liquid consistency. A loop full of the sample was streaked using quadrant streak method on MRS agar. The streaked plates were incubated at 37°C for 24 hours.

Biochemical tests

Gram staining

A thin smear of bacteria was prepared on a glass slide by mixing bacteria with a drop of distilled water. Following air drying, the smear was heat fixed. The smear was initially stained with crystal violet for 45-60 seconds. Then Grams iodine was added for 90 seconds and followed by decolourizer for less than 5 seconds. Finally, Safranin was added and left for 20 seconds. After every staining step the excess stain was washed gently and allowed to air dry. The slide was observed under100X using a microscope.

Catalase test

A small amount of bacteria were mixed with a drop of hydrogen peroxide and observed for bubble formation.

Acid-fast staining

A thin smear of the bacteria was prepared on a sterile slide and heat-fixed. Then the smear covered with carbol fuchsin and heated for 5-10 minutes. Then the smear was covered with 20% sulphuric acid and then added methylene blue. After every staining step the slide was washed and air dried. The slide was observed under100X using a microscope.

Endospore staining

A thin smear was prepared on a slide and heat-fixed. A piece of filter paper was placed on the smear and flooded with malachite green and kept on the water bath using a stand without touching the water. The slide was heated gently till it started to evaporate. The above step was repeated removing from the heat. The filter paper was removed and allowed to cool down to room temperature. The slide was rinsed with water. Then the smear was stained with Safranin for 2 minutes and rinsed with water and blot and air-dried. It was observed under 100X using a microscope.

Sub culturing of pure colonies

The identified pure Lactobacillus was subcultured in 10ml of MRS broth and incubated at 37°C for 24-48 hours.

Cholesterol-lowering ability Test

A 200 µl of overnight Lactobacillus culture was added to 10ml of 0.1% MRS cholesterol broth and incubated for 48 hours at 37°C. A 0.2ml MRS Cholesterol broth was mixed with 4.8ml of ethanol and centrifuged at 3000rpm for 10 minutes. The 2ml supernatant/suspension was mixed with 2ml of ferric ammonium sulphate colour reagent and the absorbance was measured at 560nm. The cell-free supernatant was added to 10ml of 0.1% MRS Cholesterol broth and performed the same (Azat et al., 2016).

Triplicates were carried out for each sample of both cell intact and cell free suspensions to determine the cholesterol-lowering ability.

Cholesterol removal activity was calculated using the below equation

CRR= [(A (uninoculated) –A (inoculated))/A (uninoculated)] ×100%

Data analysis

The data obtained from cholesterol lowering ability was analyzed in one-way ANOVA using SPSS Statistics version 21 software.

RESULTS

Isolation of Lactobacillus

Colony morphological identification of inoculated bacteria on MRS agar after 24 hours incubation.

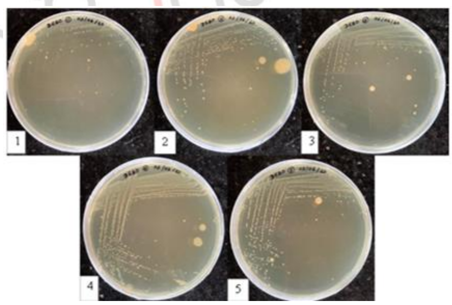


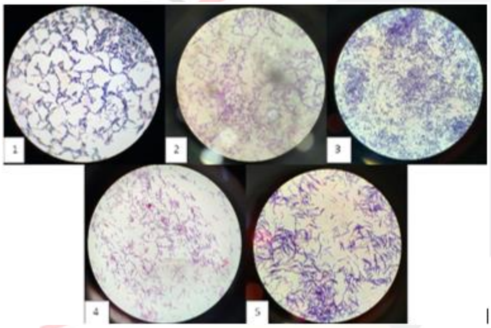
Figure 5. Morphological identification of Lactobacillus growth on MRS agar

A creamy white coloured smooth, with an entire margin of round colonies were observed in all five samples.

Biochemical tests for Lactobacillus

Gram staining

Morphological identification of isolated bacteria via Gram's staining



Cell wall characteristic determined by acid-fast staining

Figure 6. Gram stained Lactobacillus at 100X magnification.

Gram-positive (purple coloured), comparatively long, single rod shaped bacillus bacteria were observed in all samples.

Catalase test

The presence of catalase enzyme in the bacterial isolate was determined by the catalase test.

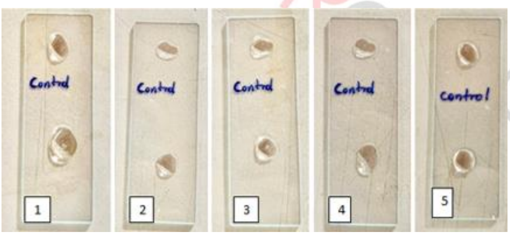


Figure 7. Slide method Catalase test.

No bubbles were observed in all five samples, confirming the bacteria are catalase-negative.

Acid-fast staining

Cell wall characteristic determined by acid-fast staining.

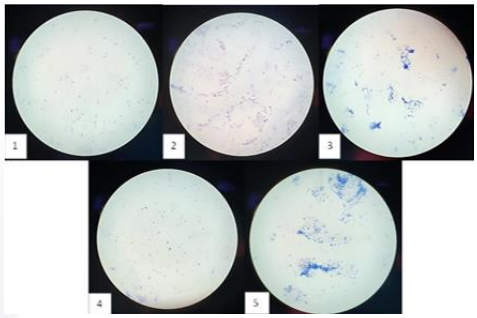


Figure 8. Acid-fast staining of Lactobacillus at 100X magnification.

Bacteria in all the samples were blue coloured (acid-fast negative).

Endospore staining

The inability to form endospore by Lactobacillus is determined by endospore staining

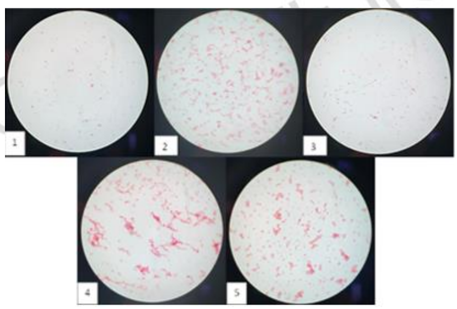


Figure 9. Endospore staining at 100X magnification.

Bacteria in all five samples were pink in colour (no endospore).

Results obtained confirmed that all the samples contain Lactobacillus.

Cholesterol-lowering ability Test

Table 1. Cholesterol-lowering test results

Samples	Cholesterol-lowering rate in percentage (%) Cell intact suspension Mean±SD	Cell free suspension Mean±SD
1	68.22±0.75	71.66±0.32
2	66.90±0.72	67.99±0.16
3	87.12±1.54	53.97±1.42
4	80.09±0.55	34.83±0.69
5	82.97±3.27	59.07±0.16

The cell intact suspension showed higher cholesterol-lowering percentage than cell free suspension

Table 2. One way ANOVA analysis between Lactobacillus cell intact and cell free suspensions.

ANOVA

Cholesterol-lowering Rate

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	956.484	1	956.484	6.563	.034
Within Groups	1165.993	8	145.749		
Total	2122.477	9			

As p-value is <0.05 there is a significant difference between cholesterol-lowering rate between cell free and cell intact suspensions.

DISCUSSION

Probiotics are beneficial bacteria which when administered in sufficient amounts contributes to several health benefits (Ooi et al., 2010). Lactobacillus bacteria are one of the mainly found probiotics in dairy products (Goyal et al., 2012). Curd is a healthy fermented dairy food with a rich source of minerals and vitamins (Balamurugan et al., 2014). This study aimed to determine the cholesterol-lowering ability of probiotic Lactobacillus in commercially available curd samples in Sri Lanka.

Initially, the Lactobacillus was identified phenotypically by culturing them on Lactobacillus specific MRS agar. All five samples cultured resulted in creamy white coloured, smooth circular-shaped colonies with an entire margin and opaque (Kumar and Kumar, 2014). Colonies of most of the samples were small but samples 2, 3, 4 and 5 had larger colonies (Figure 5.). This colony morphology is consistent with the results of a study carried out by Prabhurajeshwar and Chandrakanth (2018). Therefore the colonies resulted from different samples in this study could be Lactobacillus colonies.

The Gram staining is used to differentiate bacterial cell wall properties (Figure 6.). Observing purple coloured rods, confirmed that the bacteria are Gram-positive bacilli in different lengths occurring single or in chain forms (Somnath de et al., 2017). Difference observe in size of rods could be due to different species of Lactobacillus (Pena et al., 2004).

The catalase test for Gram-positive Lactobacillus resulted as catalase-negative because the isolates were not able to produce bubbles when mixed with hydrogen peroxide. This is due to the lack of catalase enzyme in Lactobacillus (Goyal et al., 2012).

In Acid-fast staining heat is used to penetrate the endospore coat to determine if the staining organism could form spores. In the current study, the isolated were stained to blue colour (Figure. 8) resulting in nonacid fast bacillus (Kusuma, Tjitraresmi, and Susanti, 2017). Endospore stain is used to differentiate an organism's ability to produce spores. The test resulted negative for all five isolates, showing red coloured rods (Figure 9) instead of green colour similar to the results of a study of Goyal et al., (2012).

The pure colonies of Lactobacillus were subcultured into MRS broth to obtain pure cultures for and determination of cholesterol-lowering ability.

Cholesterol is a vital substance required for body function. Elevated levels of cholesterol lead to cardiovascular diseases. Alternative methods using probiotics are effective. Among the many probiotics, Lactobacillus was found to be a promising candidate to improve blood lipid profiles (Ooi et al., 2010). A study of Viljoen and Wierzbicki (2008) has suggested that Lactobacillus could compete against cholesterol and inhibits the intestinal absorption. The current study also has shown 35% -87% (Table 1.) cholesterol removal rates. Therefore, confirms the cholesterol-lowering ability of Lactobacillus found in curd. Moreover, there is a significant difference between cholesterol-lowering ability between cell intact suspension and cell free suspension. The data analysis showed that cell intact suspension has significantly high ($p<0.05$) cholesterol-lowering ability than cell free suspension (Table 2.) The cell intact suspension has given the highest percentage and all the samples have shown above 68% of the cholesterol–lowering rate. The cholesterol removal rate in cell intact suspension is higher because the Lactobacillus could remove a significant amount of cholesterol only in the growing status. Also, cell intact suspension containing MRS media assists the growth of Lactobacillus and the ability of Lactobacillus to remove cholesterol is high. The viable bacteria in the cell intact suspension removes the cholesterol from the media via binding the cholesterol particles on Lactobacillus cell surface and incorporates to cellular membrane or transfers to Lactobacilli cytoplasm. The ability of incorporation of cholesterol to the bacterial cell wall also facilitates survival of Lactobacillus in the gastrointestinal tract when given to host (Ma et al., 2019; Kim et al., 2008). Another mechanism of cell intact Lactobacillus reducing the cholesterol is using the conversion of cholesterol to coprostanol by both intracellular and extracellular cholesterol reductase (Lye, Rusul, and Liong, 2010). The cell free suspension contains dead and resting cells of Lactobacillus and they have shown lower ability to remove cholesterol generally and also confirmed in this study. A study by Kim et al., (2008) using cell free supernatant of a Lactobacillus strain found to identify that cholesterol-lowering ability mediated by an active component with a proteinaceous nature stable under a variety of condition. In this current study, the cholesterol-lowering ability of the Lactobacillus cell free suspension mostly facilitated by the direct activity, with the action for cholesterol degradation otherwise inactivation (Azat et al., 2016). Lactobacillus cholesterol-lowering ability or effect of them on cholesterol concentration depends on the choice of strain, dosage, and delivery method (Cho and Kim, 2015).

CONCLUSION

The current study was aimed to isolate Lactobacillus from curd and to analyse their ability to reduce cholesterol level. Biochemical assays confirmed the presence of Lactobacillus in all five curd samples. The cell intact suspension of Lactobacillus exhibited higher cholesterol-lowering ability than cell free suspension. Understanding the cholesterol-lowering ability of Lactobacillus will be useful in developing novel treatments for hypercholesterolemia.

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