ASSESSMENT AND IDENTIFICATION OF CHOLESTEROL-DEGRADING PROBIOTICS

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ABSTRACT: *This study aimed to isolate and identify probiotic Lactobacillus and evaluate their effects on cholesterol levels. Probiotic Lactobacillus was isolated from three samples butter, milk, spinach and egg yolk. MRS agar and MRS broth were used for the growth of Lactobacillus and identification of Lactobacillus were confirmed by gram staining and various biochemical tests. Growth and survival of Lactobacillus was evaluated by antibiotic resistance, acid tolerance in various pH (4, 7 & 9), Bile salt tolerance under different concentrations of bile salt (such as 0.1g, 0.2g, 0.3g, 0.4g & 0.5g) and in various temperatures such as (37℃, 50℃& 4℃). Cholesterol degradation capability of Lactobacillus was determined under three different concentrations of cholesterol (200µg/ml, 400µg/ml, and 600µg/ml). The results of cholesterol assimilation were recorded by the percentage of cholesterol degraded. Out of all three samples, Lactobacillus isolated from butter, milk showed the highest cholesterol degradation (26.68%) at concentration 600µg/ml. The present study showed that the isolated probiotic LAB was able to assimilate cholesterol which in turn can reduce the risk of cardiovascular diseases.*

Keywords: Acid tolerance, Antibiotic resistance, Bile tolerance, Cholesterol, Probiotic, and *Lactobacillus.*

INTRODUCTION

The human diet has widely evolved in the past few decades. Earlier fresh fruits and vegetables were part of diet more, but frequent consumption of fast food and sugary beverages lead to system and body organ damage, thus opening the doors of higher possibility of cardiovascular diseases, strokes due to plaque formation in vessels and high LDL levels. Our body is designed with a defense system to fight diseases including many of the natural flora that protects us from pathogen invasion called probiotics. In our GI tract, adequate amounts of probiotics are already present which can be consumed from dairy products also especially from fermented products [1]. Probiotics need prebiotics as a food source, to grow and eventually to perform its metabolic activity [2]. Previous studies suggest probiotics are favorable in treating gastrointestinal intestinal disorders such as diarrhea, dysentery, typhoid, etc. [3]. They also have the ability to resist the acidic pH of the gut and surviving in the condition that is harsh for many harmful bacteria. At normal levels, cholesterol is a vital substance for the body. But, if concentrations within the blood get too high, it acts as a major risk factor for coronary heart diseases, cardiovascular diseases, and hypercholesterolemia. The reduction of immoderate cholesterol levels in the blood reduces the risk of these diseases. Various cholesterol-lowering drugs have been produced, however, they all have acute side effects. Thus, various non-pharmacological approaches intended to lower cholesterol levels were designed and researched. Currently, there is growing enthusiasm for the utilization of probiotics and prebiotics, the natural microorganisms, which are present in the gut, that when consumed are believed to provide health benefits and are useful in reducing serum cholesterol [4]. Current studies show that probiotics are a potent and natural substitute for degrading LDL cholesterol levels in humans. The isolation of *Lactobacillus* from food and dairy products are of great interest as these are industrially important microorganisms having various beneficial effects [5].

Nowadays, research is going on the potential of probiotics to lower lipids and cholesterol levels. Most important of all is the Lactic acid bacteria found in the gastrointestinal tract, which has many beneficial effects in health including anti-cholesterol [5]. Some Lactic acid-producing bacteria contain bile salt, hydrolase, which interact with host bile concentrations and lowers the cholesterol [6].

In this study, *Lactobacillus* was isolated from three different sources and was identified using Gram staining, various biochemical tests for the assurance of *Lactobacillus*. Thus, the aim of this study was to identify and isolate strains of *Lactobacillus* and assess their ability to degrade cholesterol as well as the amount of cholesterol degraded. Our purpose was to produce a product that could serve as a cholesterol-reducing probiotic. This work provides the base for investigations in inventing new probiotics strains capable of assimilating [7]. Besides all advantages that various studies have currently proven, this study aims to investigate the interaction of probiotics with intestinal cholesterol re-absorption [8].

METHODOLOGY

Sample collection and Preparation:

Samples were examined for the probiotic hypocholesterolemic activity i.e butter, milk, spinach (pulp and water) and egg yolk. Samples were streaked on the MRS agar plates. Simultaneously, the samples were also inoculated in the MRS Broth and then incubated for 24 hours at 37℃.

Identification and characterization of *Lactobacillus*:

After the appearance of growth of the bacteria as small colonies in white color, further, phenotypic characterization was carried out by gram staining, and various biochemical tests such as Indole test, Methyl red, Voges Proskauer, Citrate utilization test, Urease test, Oxidase test, Catalase activity, and carbohydrate fermentation. The isolates were also examined for their antibiotic susceptibility, pH resistance, and bile tolerance.

Antibiotic susceptibility test:

For the antibiotic susceptibility test, the disc diffusion method and minimum inhibitory concentration test were used. The antibiotics which were used included Streptomycin, Penicillin, Tetracycline, Ampicillin and Erythromycin.

pH resistance:

Lactobacillus isolates from all four samples were tested against three different pH; 4, 7 and 9. Freshly grown *Lactobacillus* in the broth was taken and maintained to pH 4, 7 and 9 in 10ml test tubes. The test tubes were then incubated for 24 hours at 37℃.

Bile tolerance test:

To observe resistance of *Lactobacillus* in presence of bile salts, test tubes were filled with 10ml of broth and inoculum was prepared by adding *Lactobacillus* growth from each sample into test tubes; labeled with bile concentrations and sample name. Bile salts weighed at various concentrations such as 0.1, 0.2,0.3, 0.4 and 0.5g were then added into tubes for each sample. All the tubes were cotton plugged and incubated at 37℃ for 24-48 hours. Results were recorded by taking their optical density (OD).

Temperature Resistance:

A single colony of *Lactobacillus* was inoculated in MRS broth and incubated at three different temperatures 4℃, 37℃, and 50℃ for 24h to observe the survival and growth of *Lactobacillus* under different temperatures.

Cholesterol assimilation by using normal cholesterol:

The *Lactobacillus* strains were then enquired for their ability to degrade cholesterol. The whole procedure was carried for three days. For cholesterol assimilation, first cholesterol was made soluble by mixing it with warm ethanol and adding a few drops of chloroform while heating it. Then, it was evaporated, and crystals were formed.15 tubes were filled with freshly prepared MRS broth, 12 tubes supplemented with bile salt and water-soluble cholesterol at different concentrations such as 200µg/ml, 400µg/ml and 600µg/ml, were inoculated with 1% of *Lactobacillus* culture. While the remaining three tubes supplemented with different concentrations of water-soluble cholesterol were free of isolate and utilized as control. They were incubated at 37℃ for 24 hours [8]. Following incubation, the cells of *Lactobacillus* were harvested by centrifuging them at 10,000 rpm for 10 min and washed with distilled water twice. The cells were then added again in a mixture of broth containing 0.1g of bile salt along with different concentrations of water-soluble cholesterol (200µg/ml, 400µg/ml and 600µg/ml.) It was again incubated for 24h. After 24h of incubation, the cholesterol degradation capability of *Lactobacillus* was determined spectroscopically at 620nm and compared with un-inoculated controls. Cholesterol degradation was calculated as a percentage by the help of the following equation:

Cholesterol Assimilation (A) = 100 – (B/C) *100

Where,

A = % of cholesterol degraded

B = Absorbance of a sample containing cells

C =Absorbance of the sample without cells

RESULTS

Identification and characterization of *Lactobacillus*:

Gram staining of all the obtained isolates was done. Microscopic observations of stained slides lead to the visualization of clear rod-shaped, purple color non-motile Gram-positive bacteria which point out to phenotypic characteristics of *Lactobacillus*. Biochemical test results showed characteristics of *Lactobacillus* which are presented in Table 1.

Antibiotic Resistance:

Results obtained for the antibiotic susceptibility test by disc diffusion method showed that *Lactobacillus* isolated from buttermilk showed clear zones of inhibition against all 5 antibiotics whereas, *Lactobacillus* isolated from spinach showed no zones and were resistant to all antibiotics. Bacteria from egg yolk showed mix results. For the MIC test, some antibiotics showed a clear zone of inhibition around the MIC strip.

Minimum inhibitory concentration (MIC):

Results of MIC were recorded by observing the zone of inhibition around the MIC strips. The results recorded are mentioned in Table 3. Results showed clear zones of inhibition around the MIC strips but some of them are resistant and showed no zones as shown in Tables 2 and 3.

Cholesterol Assimilation by using normal cholesterol:

After 24h of incubation, the cholesterol degradation capability of *Lactobacillus* was determined spectroscopically at 620nm and compared with un-inoculated controls. Out of all three samples, *Lactobacillus* isolated from buttermilk showed the highest cholesterol degradation (26.68%) at concentration 600µg/ml. The results are shown in Table 4 to 7.

pH, Temperature Resistance, and Bile Tolerance:

Results for pH, Temperature Resistance and Bile Tolerance are shown in Table 8 to 10, respectively.

Table 1: Identification and Characterization of *Lactobacillus* strains

Biochemical Tests	Positive	Negative
Catalase Test	-	-VE
Indole Test	-	-VE
Voges Proskauer	-	-VE
Citrate Test	+VE	-
Urease Test	-	-VE
Glucose Test	+VE	-
Sucrose Test	+VE	-
Methyl Red	-	-VE

Table 2: Activity of *Lactobacillus* against antibiotics by disc diffusion method

Bacterial Culture Source	Antibiotics				
	Streptomycin	Ampicillin	Penicillin	Erythromycin	Tetracycline
Butter Milk	10mm	35mm	26mm	32mm	20mm
Spinach Pulp	5mm	7mm	5mm	5 mm	4mm
Spinach Water	6mm	6mm	7mm	6mm	5mm
Egg Yolk	5mm	10mm	10.2mm	13mm	11mm

Table 3: Activity of *Lactobacillus* against antibiotics by MIC

Bacterial Culture Source	Antibiotics				
	Streptomycin	Ampicillin	Penicillin	Erythromycin	Tetracycline
Butter Milk	32 µ*g*/ml	20µ*g*/ml	1.5µ*g*/ml	10 µ*g*/ml	15 µ*g*/ml
Spinach Pulp	25 µ*g*/ml	22µ*g*/ml	16 µ*g*/ml	15 µ*g*/ml	12 µ*g*/ml
Spinach Water	30 µ*g*/ml	28µ*g*/ml	20 µ*g*/ml	14 µ*g*/ml	10 µ*g*/ml
Egg Yolk	16µ*g*/ml	1.5µ*g*/ml	25 µ*g*/ml	2µ*g*/ml	1.0µ*g*/ml

Table 4: Effects of *Lactobacillus* on cholesterol degradation isolated from buttermilk

Sample	Concentration of water-soluble cholesterol (µ*g*/ml)	Optical Density	Percentage of cholesterol degradation (%)
Butter Milk	200µ*g*/ml	0.487	19.11%
	400µ*g*/ml	0.492	22.42%
	600µ*g*/ml	0.502	26.68%

Table 5: Effects of *Lactobacillus* on cholesterol degradation isolated from spinach water

Sample	Concentration of water-soluble cholesterol (µ*g*/ml)	Optical Density	Percentage of cholesterol degradation (%)
Spinach Water	200µ*g*/ml	0.572	4.99%
	400µ*g*/ml	0.582	9.74%
	600µ*g*/ml	0.584	13.12%

Table 6: Effects of *Lactobacillus* on cholesterol degradation isolated from spinach pulp

Sample	Concentration of water-soluble cholesterol (µ*g*/ml)	Optical Density	Percentage of cholesterol degradation (%)
Spinach Pulp	200µ*g*/ml	0.566	5.99%
	400µ*g*/ml	0.589	8.97%
	600µ*g*/ml	0.578	13.96%

Table 7: Effects of *Lactobacillus* on cholesterol degradation isolated from egg yolk

Sample	Concentration of water-soluble cholesterol (µ*g*/ml)	Optical Density	Percentage of cholesterol degradation (%)
Egg Yolk	200µ*g*/ml	0.583	3.16%
	400µ*g*/ml	0.617	4.64%
	600µ*g*/ml	0.621	7.46%

Table 8: Growth of *Lactobacillus* at various pH.

Sample	Various pH	Optical Density
Butter Milk	4	0.415
	7	0.789
	9	0.781
Spinach Water	4	0.559
	7	0.672
	9	0.657
Spinach Pulp	4	0.496
	7	0.897
	9	0.872
Egg Yolk	4	0.523
	7	0.630
	9	0.617

Table 9: Growth of *Lactobacillus* at different temperature.

Sample	Different temperature	Optical density
Butter Milk	4℃	0.019
	37℃	0.997
	50℃	0.762
Spinach water	4℃	0.321
	37℃	0.898
	50℃	0.748
Spinach Pulp	4℃	0.437
	37℃	0.892
	50℃	0.824
Egg Yolk	4℃	0.085
	37℃	0.792
	50℃	0.450

Table 10: Growth of *Lactobacillus* at various bile concentrations.

Sample	Concentration of bile salt	Optical Density
Butter Milk	0.1g	0.783
	0.2g	0.823
	0.3g	0.746
	0.4g	1.744
	0.5g	1.665
Spinach Water	0.1g	0.876
	0.2g	0.892
	0.3g	0.897
	0.4g	1.694
	0.5g	1.551
Spinach Pulp	0.1g	0.754
	0.2g	0.776
	0.3g	0.883
	0.4g	0.789
	0.5g	1.768
Egg Yolk	0.1g	0.982
	0.2g	0.872
	0.3g	0.765
	0.4g	1.761
	0.5g	1.675

DISCUSSION

Probiotics are live organisms that have shown tremendous health benefits proved by multiple studies, however, its clinical use for other purposes such as cholesterol degradation needs to be studied more and justified before its application. Previous *in vivo* studies suggest that probiotics maintain a

healthy lipid profile by reducing serum cholesterol [9]. This paper illustrates the study conducted on the basis of successfully accomplished previous studies. Further elaboration of the present study is done by selecting three different probiotic and prebiotic sources.

Buttermilk being a concrete probiotic source was selected. Spinach was selected for being prebiotic-rich leafy vegetables, moreover, egg yolk as selected as a sample because of its cholesterol/lipid levels. *Lactobacillus* identification from butter, milk, egg yolk, spinach pulp, and spinach water, was made possible by microscopic characterization and it was observed as purple color, Gram-positive, non- motile and rod-shaped, which shows the characteristics of *Lactobacillus* bacteria. Further identification was done on the basis of different biochemical tests such as the Indole test, methyl red, Vogesproskauer, citrate test, urease test, catalase test, Glucose and sucrose which showed negative results and this affirms the identification of *Lactobacillus*.

Further, *the Lactobacillus* growth rate was observed using various concentrations of bile salts (0.1, 0.2, 0.3, 0.4 and 0.5), under different pH (4, 7 and 9) and different temperatures (4°C, 37°C and 50°C). Antibiotic resistance activity of *Lactobacillus* was checked for different antibiotics (tetracycline, ampicillin, streptomycin, erythromycin, and penicillin) through disc diffusion and minimum inhibitory concentration. After the screening process of three days, cholesterol degrading activity was assimilated at a differently set concentration of water-soluble cholesterol ($200\mu g/ml$, $400\mu g/ml$ and $600\mu g/ml$).

In an effort to decrease reliance on antimicrobials, the time has clearly come to increase the exploration of the therapeutic applications of probiotics. There are too many reports describing the beneficial effects of probiotics. Probiotics offer dietary means to support the balance of the intestinal flora. They may be used to counteract immunological dysfunction, to prevent the infectious succession of pathogenic microorganisms and to influence intestinal metabolism [10].

Accordingly, owing to its good probiotic properties, this strain of *Lactobacillus* could be potentially used in functional food and health products especially where cholesterol reduction in food is the main target. Further in vivo study is necessary to prove the hypocholesterolemic effect of the isolated *Lactobacillus*. Moreover, in vitro studies are required to determine the mechanism involved in the reduction of cholesterol by such a promising isolate [11].

CONCLUSION

Lactobacillus was successfully isolated from three different sources including a prebiotic source and identified with the help of gram staining and various biochemical tests. The isolates showed resistance against three different pH and temperatures and tolerated bile salt concentrations. The further investigation aimed to check *Lactobacillus* for its cholesterol degradation property and to elaborate its reactivity to cholesterol, cholesterol assimilation test was performed which concluded that *Lactobacillus* isolated from buttermilk showed the highest cholesterol degradation (26.68%) at $600\mu g/ml$ concentration of cholesterol.

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