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In vitro Cholesterol Assimilation and Functional Enzymatic activities of *Lactobacillus* sp.

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ABSTRACT: Probiotics are live microorganisms that are stated to have health benefits when consumed to the body. They can be found in various products such as yogurt and other fermented foods, dietary supplements, and beauty products. Experiments were carried out to determine some important health beneficial properties of probiotic isolates such as cholesterol lowering capacity and bile salt hydrolase activity. The potential probiotic lactic acid bacteria was isolated from various sources and determine efficient probiobic from acid tolerance and bile tolerance with the selection of potential probiotics with cholesterol assimilation property and to evaluate the effect of probiotic with anti-inflammatory property. Selected probiotics bacteria are found to produce bile salt hydrolase (BSH) that helps to reduce serum cholesterol and hence, the aim of the present study was to isolate, identify and screen for potential probioticlactic acid bacteria with bile salt hydrolase activity and cholesterol lowering capacity. The results elucidated that the *Lactobacillus* sp. has the potential probiotic activity and facilitated in vitro cholesterol assimilation and functional enzymatic activities.

Keywords: Probiotics, lactic acid bacteria, bile salts, cholesterol assimilation, anti-inflammatory.

INTRODUCTION

Probiotics are defined as live microorganisms that, when given to a host in sufficient quantities, improve their health. Probiotics are dietary supplements or food items that include good, friendly bacteria or yeasts that are often found in the human body. Probiotics are utilized as alternative supplements to promote health advantages, including impacts on decreasing cholesterol in humans (Lay and Liong 2010). According to Wang *et al*. (2012), probiotics are a key component of the gut microflora that guards against infections in the intestinal tract. According to various potential modes of action, including bile salt hydrolase activity, the probiotic cells have the capacity to lower lipid and cholesterol levels, hence lowering luminal cholesterol levels that are available for absorption (Jones *et al*., 2013). Probiotics are useful components that are added to food to improve their nutritional value. Customers often want the probiotic strains' health advantages to remain in the food they buy up until the point of consumption due to the increased acceptability of probiotic goods. It is difficult to employ probiotics as an active food component since they must have live bacteria of food consumed in order to be effective (Huang *et al*., 2017). Furthermore, *Lactobacillus* bacteria have the ability to create ferulic acid, which is used to inhibit hepatic HMG-CoA reductase and encourage the excretion of acidic sterol (Tomaro *et al*., 2012).

Cholesterol has a significant impact on heart health issues in humans. Probiotics' ability to lower lipid concentration and their hypocholesterolemic action has been the subject of numerous investigations (Shanthi *et al*., 2011). The effects of Lactic Acid Bacteria are reported to involve inhibition of pathogenic microorganisms, protection against gastrointestinal diseases, anti-mutagenic and anti-carcinogenic activities, and enhancement of the host immune response (Nguyen *et al*., 2007). These cholesterol-lowering effects were attributed to the deconjugation of bile acid salts by strains of bacteria so as to produce the enzyme bile acid salt hydrolase (BSH), as well as shortchain fatty acid fermentation, and cholesterol binding to the bacterial cell wall (Pereira and Gibson 2002).Cholesterol assimilation was carried out using probiotic bacteria *Lactobacillus* and assimilation process involving culture incubated at 30 °C for 24 h, in which Lactobacilli have five possible mechanisms to remove or reduce the cholesterol *in vitro* conditions (Amr *et al*., 2022). *Lactobacillus* strains were selected and their properties such as bile tolerance, acid resistance, cholesterol assimilation activity were assessed to determine their potential as probiotics (Song *et al*., 2015). Hence, the objective of the presented work is to evaluate acid resistance, bile tolerance and cholesterol-lowering ability of *Lactobacillus* strains and to investigate *Lactobacillus* strains for their potential to assimilate cholesterol in in-vitro conditions.

MATERIALS AND METHODS

A. Isolation of probiotic from different samples

Probiotics was isolated from raw milk, curd, yoghurt, cheese, and cow dung. The samples were diluted

serially 10- fold times and standard colony counting methods was performed. The pure isolate was later preserved in MRS slant at 4°C for further studies (Lin *et al*., 2006). The morphological, biochemical and β-galactosidase activity (Bhowmik and Marth 1989) was determined according to the standard methods.

B. Screening of efficient probiotic based on acid and bile tolerance

The isolates were screened for acid and bile tolerance as this is an important property of probiotics. The cells grown in MRS medium were harvested by centrifugation and mixed with stimulated gastric acid. Dilutions were made (up to 10) and cells plated in duplicate on MRS agar to obtain the CFU/ml. To study the bile tolerance, MRS broth containing bile salts was prepared. This suspension was inoculated (1%) into MRS broth and later viable counts on MRS agar plates and absorbance of the culture at 625 nm were determined (Hoque *et al*., 2010).

C. Cholesterol Assimilation

In-vitro cholesterol assimilation Cholesterol assimilation of LAB isolates was done by freshly prepared MRS broth was supplemented with 0.3% oxgall, cholic acid and taurocholic acid as bile salt and filters sterilized water soluble cholesterol (100 µg/ml) and incubated an aerobically at 37°C for 24 h. After incubation period, cells were removed by centrifugation (9000g for 15 min) and the remaining cholesterol in the spent broth was determined calorimetrically using o-phthalaldehyde method (Tomaro *et al*., 2014).

D. Anti-inflammatory property

The Human Red Blood Cell (HRBC) membrane stabilization has been used as a technique to study the in vitro anti inflammatory activity due to the fact the erythrocyte membrane has similarities to the lysosomal membrane and its stabilization means that the extract may also adequately stabilize lysosomal membranes (Yesmin *et al.,* 2020). By blocking the release of lysosomal components of activated neutrophils, such as bacterial enzymes and proteases, which induce further tissue inflammation and damage upon extracellular release, lysosomal stabilization is crucial in reducing the inflammatory response. The lysosomal enzymes unconstrained during inflammation which produces a variety of disorders and it's also leads to either acute or chronic inflammation. The non-steroidal medications either block these lysosomal enzymes or stabilize the lysosomal membrane in order to exert their effects.

RESULTS AND DISCUSSION

A. Isolation and Identification of Lactobacillus spp

Lactic acid bacteria (LAB) strains were isolated from various sources such as raw milk, curd, yoghurt, cheese, and cow dung (Table 1). Colonies with different morphologies on the MRS agar plate were selected, identified and further sub-cultured in order to obtain a pure colony (Plate 1) and the morphological, cultural and biochemical characteristics of *Lactobacillus* spp. was performed. The pure isolates were maintained at 4ºC in refrigerator for further studies. The cultures were subjected to routine morphological and biochemical tests using standard protocols. The microscopic view of isolated bacteria was gram positive, rod shaped coccobacilli, occurring singly or in chains. The gram staining results indicated that the isolated bacteria could be identified as *Lactobacillus acidophilus* (Plate 2). The non motile behavior characteristics were identified by Hanging-drop wet method and no bubble was observed in performing catalase test, indicating that the isolated bacterium is catalase negative (Plate 3). Thus, the results obtained coincided with *L. acidophilus* strain characteristics (Klaenhammer and Russell 2000). Supporting evidence indicated that one of the key techniques for identifying bacteria at the species level is through phenotypic and biochemical testing (Duha and Abdullah 2021). Furthermore, the isolated strain was a nonmotile, catalase-negative, Gram positive cocco-bacilli. It can ferment arabinose and sorbitol but not maltose, lactose, sucrose, or glucose, and it has been determined as belonging to the *Lactobacillus acidophilus* species (Hassan and Peh 2014).

The biochemical characteristics of *Lactobacillus* were obtained and the results were shown in Table 2. The activity of β- galactosidase was investigated because it is an essential enzyme for *Lactobacilli* to utilize lactose and has been shown to reduce the symptoms of lactose intolerance in animals. Lactobacillus produced blue colour colonies on X-Gal plates indicating the presence of β galactosidase enzyme. The ability to catalyze lactose hydrolysis is one potential of using *Lactobacillus* strains as a source of –galactosidase (Mishra *et al*., 2011).

Table 1: LAB isolated from various sources.

Genus of bacteria	Colony-forming unit (CFU/ml)				
	Raw milk	Cheese	Yoghurt	Curd	Cow dung
Lactobacillus	4.5×10^7	7.7×10^7	3.3×10^7	7.9×10^7	4.8×10^7

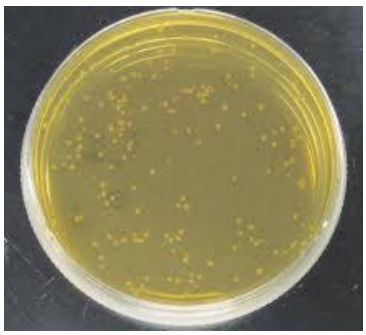

Plate 1. Growth of *Lactobacillus* sp. in MRS agar medium.

Plate 2. Microscopic view of *Lactobacillus* sp. Plate 3: Catalase test.

Table 2: Biochemical tests results.

Test	Observation

Catalase	Negative
Arabinose	No fermentation
Sorbitol	No fermentation
Maltose	Fermentation (acid production)
Lactose	Fermentation (acid production)
Sucrose	Fermentation (acid production)
Glucose (acid)	Fermentation (acid production)
Glucose (gas)	No gas formation (no bubble in Durham tube)

B. Analysis of Probiotic properties

The isolated *Lactobacillus* were taken from 24 hours cultured MRS broth and directly subjected to each of the two stress factors like low pH, bile salts to confirm the probiotic properties. The acid tolerance profile of *L. acidophilus* was tested in different pH values. At the lowest pH 2, the numbers of bacteria had decreased to an undetectable level after 2 h, however within 1 h treatment there still had more than 20% survival. The surviving percentage at the pH 3 was greater than at pH 2 in the whole process. After 3 h incubation, the viable rate of *L. acidophilus* was achieved to about 10%. When the test pH was increased to pH 4, a high survival was observed (Fig. 1). Hence, it was concluded that the *L. acidophilus* were tolerant to low pH.

The stomach pH acid tolerance of *L. acidophilus* NCFM protects viable cells from lethal acid stress and colonizes the intestine to perform a probiotic function (Wang *et al*., 2018). After 4 hours, *L. acidophilus* survived at pH 2.5 and had a high acid tolerance (Dixit *et al*., 2013). Similar to this, all of the isolated Lactobacillus strains were tolerant to pH 2 and 3 and had high survival ability in the studied acidic pH range (Srinu *et al*., 2013) and also the isolated strains of Lactobacillus tolerated pH levels of 2 and 3 (Shivram and Vishwanath 2012). The pH resistance among *L. acidophilus* strains obtained from chicken and rat exhibited minimal to no reduction in viable cell counts for up to 240 minutes at various pH ranges (Park *et al*., 2006).

The bile resistance of *L. acidophilus* was evaluated by supplement with bile (oxgall). The growth curves were observed in MRS broth containing 1%, 2%, 3% bile. The maximal viable count could rise when *L. acidophilus* was incubated in MRS broth without bile and the growth was significantly inhibited in MRS broth containing different amount of bile. With the increase of bile, the growth was obviously decreased and also showed good survivability at lower concentrations (Fig. 2). Thus, these results indicate that these isolate could be used as potential candidates as probiotics.

The *Lactobacillus* sp. will be employed as probiotics should be able to survive at low pH and in the presence of bile salt (Succi *et al*., 2005). The presence of similar processes in bacterial responses to numerous stressors and indicates that increasing probiotics bile tolerance might aid to generate more robust strains demonstrating greater resistance to additional technical or digestive factors affecting probiotics existence (Sanchez *et al*., 2012). The *L. acidophilus* LA1 cell number progressively decreased with rising bile levels and incubation times, and at all bile levels, there was a significant difference between the loss of free cells and encapsulated cells (Ameeta *et al*., 2023).

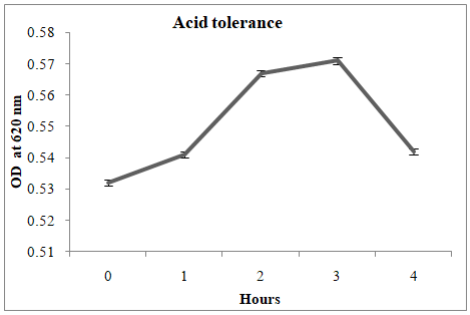

Fig. 1. Resistance to low pH at different time intervals.

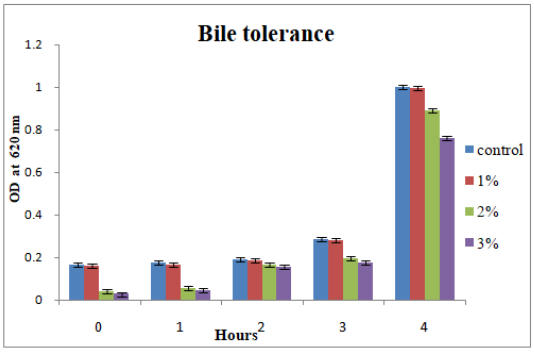

Fig. 2. Tolerance against bile salt at different time intervals.

C. In-vitro cholesterol assimilation

The in-vitro research on *L. acidophilus* ability to remove cholesterol has been regarded as a crucial factor in the selection of probiotic strains with a variety of health-promoting properties. Overall cholesterol removal was found to be considerably greater in the bile salts-containing broth than in the control. The amount of bile in the growth medium influenced the assimilation of cholesterol by *L. acidophilus*. The levels of cholesterol assimilation during 24 h of incubation at 37 ºC in MRS supplemented with 100 µg/mL water-soluble cholesterol. The overall cholesterol assimilation by *L.acidophilus* was observed to be higher in broth containing 0.3% oxgall (27.60 µg/mL) compared with other treatments. In broth containing taurocholic acid (14.69 µg/mL), the lowest cholesterol assimilation was observed and cholesterol assimilation in broth containing 0.3% cholic acid was observed to be 23.40 µg/mL (Fig. 3).

The probiotic bacteria's ability to produce bile salt hydrolase (BSH), which lowers serum cholesterol, can be utilized as a selection of probiotics. Several oral commercial probiotic bacteria, like *Lactobacillus acidophilus*, have been used to lower serum cholesterol (Wang *et al*., 2012). Supported report showed that certain Lactobacillus spp. cultures actively absorb cholesterol from laboratory medium, indicating that probiotic cultures can lower cholesterol levels (Hatice and Zubeyder 2006). *L. casei L1 exhibited a wide range of antagonistic activity and cholesterol assimilation capability* (Tulumoglu *et al*., 2018). The isolated strains from Chinese fermented rice have the potential to be employed as a probiotic and alternative medicinal

supplement due to their capacity to synthesize short-chain fatty acids and their ability to decrease cholesterol (Nageena *et al*., 2023). The findings demonstrated a strong correlation between high cholesterol assimilation ratio and bile salt resistance measures as well as bile salt hydrolase activity.

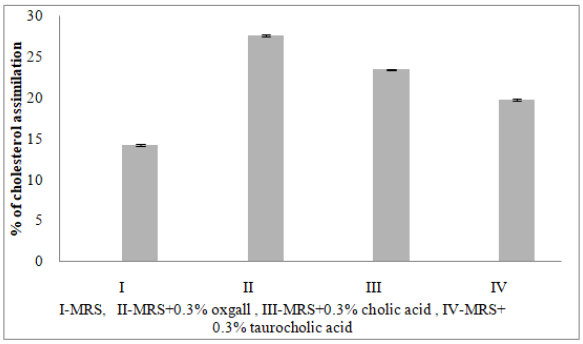

Fig. 3. Cholesterol assimilation (in µg/mL) by *L. acidophilus* in different bile media.

D. In vitro anti-inflammatory activity

In vitro Anti-inflammatory potential of *L. acidophilus* were tested with HRBC membrane Stabilization assay. The present study clearly indicates that there is no significant effect on blood agar plate for haemolytic activity and result obtained was shown in Plate 4. However, it can be confirmed that absence of haemolytic activity in *L. acidophilus* is a selection criteria for probiotic, indicating that these bacteria are non-virulent. *Lactobacillus* spp. displayed anti-inflammatory properties on HT-29 cells in addition, taking *Lactobacillus* spp. as a dietary supplement can both prevent and treat inflammation-related illnesses including inflammatory bowel disease (Shadi *et al.*, 2022). Probiotics show potential for application in the treatment of chronic inflammatory diseases since their extract was found to have anti-inflammatory action (Tjie Kok, 2023).

Plate 4. Negative haemolytic activity of *L. acidophilus.*

The anti-inflammatory properties of sesquiterpenes, spathulenol, and farnesol by membrane stabilizing and enzyme inhibition, as well as having a greater safety profile as environmentally friendly anti-inflammatory agents.

CONCLUSIONS

The present study demonstrated that the *Lactobacillus sp.* was isolated from different sources and identified as based on their morphological, biochemical and enzymatic characteristic features. The results confirmed that *Lactobacillus acidophilus* was found to have the most potential effectiveness as a probiotic based on its acid and bile tolerance, cholesterol-lowering effects and Anti-inflammatory property. From this study, it can be concluded that the capability to assimilate cholesterol and useful enzymatic activities of *Lactobacillus* sp. which have a potential to be used as a starter culture for the preparation of functional fermented probiotic foods. However, the production of these lesser-known unexplored ethnic food products could be commercialized for their health benefits and will help in formulating probiotics for therapeutic applications.

FUTURE SCOPE

The results of present investigation suggest future need to assess probiotic food products which are benefit to health for increasing therapeutic applications.

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Conflicts of Interest. None.

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