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HYPOCHOLESTEROLAEMIA AND PROBIOTIC ATTRIBUTES OF LACTIC ACID BACTERIA ISOLATED FROM INDIAN SUPERMARKET PROBIOTIC PRODUCTS: AN *IN VITRO* SCREENING STUDY

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ABSTRACT

Some proposed studies determined the ability of Lactic Acid Bacteria (LAB) to lower the *in vitro* cholesterol concentration by several mechanisms. Some important mechanisms involved include deconjugation of bile salts by bile-salt hydrolase (BSH) enzyme activity & *in vitro* cholesterol assimilation into bacterial cell membranes to produce short-chain fatty acids. The aim of the present study was to evaluate the possibility of LAB isolates in artificially made gastro intestinal tract environments and determine the ability of these LAB stains to lower the cholesterol concentration by carrying out *in vitro* assays. LAB strains were isolated from probiotic products purchased from different supermarkets in Pune (Maharashtra). Identification and characterization of selected strains was done by using guidelines for evaluation of candidate probiotic strains (ICMR and DBT). Quantitative Liebermann–Burchard colorimetric assay of cholesterol estimation was performed *in vitro* to detect cholesterol reduction ability of selected strains. Our results indicated that *Lactobacillus & Pediococcus* group members from LAB possessed good survival ability in artificial GIT fluids as well as good cholesterol removal ability in MRS broth supplemented with cholesterol and bile salts. The goal of this study was to develop a new consumable probiotic-containing product by using valid lactic acid bacterial combinations that could serve as cholesterol reducing pharmaceutical agent.

KEYWORDS: Lactic Acid Bacteria, probiotic, cholesterol, Liebermann–Burchard colorimetric assay.

1. INTRODUCTION

Dairy based fermented as well as non-fermented milk products like cheese, yogurt (sweet dahi), ice-creams, flavoured probiotic drinks and chocolates with probiotic functions are most popular among the market from many years. Exposure of high levels of cholesterol through dairy based products is very common to human consumers. Although cholesterol plays an important role in bile acid and steroid hormone synthesis in human body, excessively high level of cholesterol is cause of cardiovascular diseases in many populations. Now a day, up to 70% of the population is affected by lactose intolerance, familial hypercholesterolemia, dyslipidaemia, and vegetarianism issues all over the world. Use of milk-based products may be limited due to lactose intolerance and other health related conditions in cardiac and obese patients. Therefore, several raw materials have been extensively investigated to determine if they are suitable substrates to produce novel non-dairy functional foods (Perricone 2014). They may mediate a variety of health effects through numerous proposed mechanisms: inhibition of undesirable bacteria (El-Nagger 2004; Karska-Wysockib 2010), neutralization of toxins, increase of the immune response (Ghafoor 2005), antimutagenic and anticarcinogenic activities (Boutron-Ruault 2007; Liong 2008; Davis and Milner 2009; Baldwin 2010), reduction of cholesterol levels (Tamai 1996; Zhang 2008), control of diarrhea (Dylewski 2010; Gao 2010), alleviation of lactose intolerance (Guarner 2005), inflammatory bowel diseases (Kruis 2004; Matthes 2010) etc. Pharmacological agents that effectively reduce cholesterol levels are available for the treatment of high cholesterol; however, they are expensive and are known to have severe side effects (Bliznakov 2002). New strategies to market beneficial probiotic LAB strains in

therapeutic supplements and food additives are in progress day to day. These probiotics have received much attention on their proclaimed health benefits which include improvement in lactose intolerance, increase in natural resistance to infectious diseases in gastrointestinal tract, reduction in serum cholesterol level and suppression of cancer cells. LAB with active bile salt hydrolase (BSH) enzyme or products containing them have been suggested to lower cholesterol levels through interaction with host bile salt metabolism [De Smet 1998]. In past few years various types of probiotic capsules are recommended by clinicians to recover digestive flora during clinical treatments of many patients. Therefore, the interest has been increased regarding the commercial utilization of probiotic LAB strains isolated from different probiotic formulations available in market as probiotic super foods. The most commonly used probiotics belonging to the species of the genera *Lactobacillus, Lactococcus, Pediococcus, Streptococcus* etc. and the genus *Bifidobacteria*. Lactic acid bacteria, especially the species belonging to the genus *Lactobacillus*, such as *L. acidophilus, L. rhamnosus, L. gasseri, L.f ermentum*, and *L. plantarum* act as important probiotic because of their strain-specific properties that are beneficial to health [Giraffa 2010].

Most of the delicious, probiotic dairy drinks available in market, with billions of live and active probiotics claim that they may help support immune system and provide essential nutrients like calcium, iron & vitamins. The probiotic bacterial strains from these drinks survive and remain active in the digestive tract where about 70% of our immune system is located. It is very important to propose feasible alternatives to milk originated probiotic foods for those people suffering from lactose intolerance or hypercholesterolemia. For this reason, there is an increasing demand for vegetarian probiotic products in developed countries all over the world. To meet the growing demand for vegetarian probiotic products cholesterol lowering activity of some probiotic strains was studied by *in vitro* cholesterol assimilation assays. The use of Liebermann– Burchard reaction has been explored to develop a simple, reliable, and robust quantitative colorimetric method to assay cholesterol, and hence provide a good alternative to chromatographic methods (Joseph K. Adu 2019). It has been demonstrated that the Liebermann–Burchard reaction via colorimetric method could be used in successful analysis of cholesterol in dairy products (Joseph K. Adu 2019). Probiotic capsules, fermented cheese, curd, yogurt & probiotic drinks manufactured by different manufacturers were purchased from different local markets in Pune.

2. MATERIALS AND METHODS

2.1. Sample Collection

Commercial probiotic capsules, fermented cheese, curd, yogurt & probiotic sweet drinks (N =10) manufactured by different manufacturers were purchased from retail stores in supermarkets, Pune. Detail specifications from each product were recorded carefully in terms of name of manufacturer, manufacturing date, expiry date, specific health claims, storage temperature, etc. In addition, types and total number of probiotic strains were also recorded in million cells. It was noticed that most of the products did not have FDA's (Food and Drugs Authority) registration numbers on their labels, which is a governing constraint. It was also noted that manufacturers only stated the content of calories and total fats in the product but not the cholesterol content. The samples were stored at mentioned temperature for further analysis.

2.2 Isolation of Bacteria

All reagents, growth media and chemicals used in this study were analytical reagent grade procured from Hi Media Pvt Ltd. Mumbai, India unless otherwise mentioned. 10 grams (or 10 ml) of each sample was weighed aseptically and was homogenized using vortex in 90 ml sterile quarter–strength Ringer salt solution. 10^{-1} to 10^{-8} serial dilutions were made and from each dilution 100 µl of suspensions were spread on sterile nutrient agar medium plates. Complete micro biota from each sample was enumerated by using different media with the intent of pick out different groups of bacteria for novel probiotic formulation. Lactic acid bacteria were counted on sterile *Lactobacillus* MRS (De Man, Rogosa and Sharpe) agar medium plates added with 0.04 g/lit of bromocresol purple (Sisco Research Laboratories Pvt. Ltd, Mumbai) after incubation in $C0_2$ incubator [ESCO CelCulture $C0_2$ Incubator (CCL)] at 35°C in presence of 5% $C0_2$. Sterile modified Brewers bacteria agar plates were used for enumeration of facultative & obligate anaerobic bacterial strains. Sterile M17 & M17 plus cycloheximide medium agar (Biokar BK088) plates were used for counting of lactic *Streptococci* and *Enterococcus spp*. Specially designed MSE (Mayeux, Sandine & Elliker Agar-Biokar 087) agar plates were prepared for detection and enumeration of *Leuconostoc spp* in selected probiotic dairy products and probiotic sweet drinks. Bromocresol purple is a colorimetric indicator use to facilitate the differentiation of non-acid producing bacterial colonies from acid producing bacterial strains which lowers the pH of the medium and result into a yellow coloration of growth medium. Sterile nutrient agar plates with 5 % (v/v) sterile defibrinated sheep blood were used for the examination of haemolytic activity of the selected strains. Classical phenotypic tests based on different physiological parameters were conducted by using sterile MRS broth supplemented with bromocresol purple reagent.

2.3 Identification of Probiotic Strains

Viable numbers of bacteria were counted as CFU/ml (Colony Forming Unit) of colonies from each inoculated sample. Total 50 different colonies were purified twice by streaking on sterile MRS agar plates incubated at 35°C temperature for 48 hrs in CO_2 incubator. The colony morphology by Gram staining method as round shaped (*Pediococcus*) or rod shaped (*Lactobacillus*) on MRS agar media was taken for the presumptive identification. On MSE agar plates growth of colourless,

mucoid, gelatinous, 1 to 2 mm sized *Leuconostoc* colonies were observed within 48-72 hrs of incubation in presence of 5% CO_2. The isolated Gram-positive, catalase-negative cocci or rods, acid producing colonies were tentatively considered as *Pediococcus* and *Lactobacillus spp.* Apparent *Streptococcus* and *Lactococcus* counts were taken from M17 and M17 plus cycloheximide agar respectively. All isolated colonies were tested for their motility, acid fast stain morphology, catalase test, oxidase test, coagulase test, haemolytic activity and spore forming properties according to the Bergey's Manual of Systematic Bacteriology, 2nd edition vol.3, (2009). Out of 50 total 14 Gram-positive, nonmotile, oxidase negative, coagulase negative, lactic acid bacterial strains were selected for biochemical characterization study. IMViC, sugar fermentation test, starch hydrolysis test, gelatin hydrolysis test, arginine hydrolysis test, nitrate reduction test, H_2S production test, and lysine utilization test were employed to identify these isolated lactic acid bacteria (Aneja 2012). Each lactic acid bacterial colony was cultivated in MRS broth supplemented with 10 % (v/v) glycerol and was maintained at -20°C for further studies. Comparisons of probiotic potential properties of selected 10 isolates (with control) were also done by performing various tests in *vitro*. Salt tolerance of every isolate was checked in sterile MRS broth containing 2.5%, 4.5%, 6.5 % & 8.5% sodium chloride solutions. The pH of MRS broth was adjusted to 3 & 9 by adding sterile 2 N HCl & sterile 5 N NaOH after autoclaving of the medium. 10 ml of broth medium adjusted for defined parameters were inoculated with 100 µl of pure culture of selected strains. Incubation was done at 35°C in CO_2 incubator for 48 hrs. Bacterial growth was checked by measuring O.D. at 600 nm as well as by inoculating the test medium on sterile MRS agar medium plates. Total viable bacterial count was recorded in terms of CFU/ml for each above mentioned physicochemical parameter. 200 µl of washed cell suspension of every probiotic isolate was mixed with 1 ml of simulated gastric juice (pH range 1.5-3.5) and simulated intestinal fluid (pH range 6.3-7.3) in separate tubes and reaction mixture was incubated at 37°C in CO_2 incubator. 100 µl aliquots were removed after 0 min, 60 min, 90 min, and 180 min & % survival rate was calculated accordingly. TVC was performed by pour plate method on sterile MRS agar plates supplemented with L-cysteine hydrochloride (0.05 % w/v) incubated at 37°C for 48 hrs in CO_2 incubator. 150 µl of bacterial culture suspensions were also inoculated with 200 µg/ml of lysozyme and 0.4 % of phenol preparations in 5 ml sterile MRS broth for 2 hrs. Inoculation of isolates in sterile electrolyte solution without lysozyme or phenol was taken as control. 100 µl aliquots from each preparation were spread on sterile MRS agar plates incubated at 37°C for determination of total viable count in CFU/ml after 48 hrs. Cell surface hydrophobicity (CSH) plays a crucial role in the attachment to, or detachment from the surfaces. The adhesion ability of probiotic strains has been studied *in vitro* model systems, which are commonly used to select and assess probiotic strains for *in vivo* studies (Dunne 2001). Sterile modified TGYE (Tryptone glucose yeast extract) broth having pH 6.8 was prepared for preservation of fresh probiotic cultures of LAB isolates. Cells of exponential phase of selected strains were centrifuged at 8000 rpm for 10 mins at 4°C. Cell pellets were washed twice with 0.1 M PBS and resuspended into 5 ml of the same buffer (pH 6.8). The adhesion of probiotic strains varies among isolates and it was studied by auto aggregation assay described by Collado *et al* 2008 with some modifications. Initial absorbance (A600 nm) of cell pellet suspensions was adjusted to 0.25 ± 0.05 (approx. 10^7 to 10^8 CFU/ml). Initially optical density of a uniform bacterial suspension was recorded (A_0), then the same suspension left to rest for 24 hrs. at 37°C without vortexing. Again optical density was measured (A) and the aggregation percentage was expressed -

$$\text{Auto aggregation (\%)} = [(A-A_0)/A] \times 100$$

Where, A is the time represents the absorbance of the mixture at 24 h and A_0, absorbance at time 0

The bacterial adhesion to the hydrocarbons, which determines the bacterial cell surface hydrophobicity, was performed according to the method reported by Canzi, 2005. Selected probiotic strains were harvested in PUM (Phosphate urea magnesium sulphate) buffer at concentration of 10^8 CFU/ml & were added with 1 ml of different hydrocarbons viz. xylene, toluene, ethyl acetate and chloroform. (Initial absorbance of each mixture was recorded as A_0 at 630 nm). After mixing tubes were kept for 1 hr at 37°C to allow the phase separation. The aqueous (lower) phase was separated carefully and absorbance (A) was recorded at 630 nm. The decreased absorbance in aqueous phase was taken as measure of cell surface hydrophobicity (H%), calculated using following equation:

$$\text{\% Hydrophobicity (H \%)} = [(A_0-A)/A] \times 100$$

Where, Ao is the absorbance before extraction while A is the absorbance after extraction with respective hydrocarbons. Each experiment was replicated three times. *Lactobacillus plantarum* subsp. *Plantarum* ATCC 14917 was used as control strain for each test.

2.4 Preparation of Probiotic Samples

Quantitative cholesterol estimation of probiotic products was done by UV visible spectrophotometric method described by Joseph K. Adu et al 2019 based on determination of cholesterol by the Liebermann–Burchard reaction (Kenny 1952). 10 gm or / ml of each probiotic sample under investigation was saponified with 10 ml of 10% methanolic potassium hydroxide solution at 70°C for 30 minutes. Extraction of unsaponifiable fraction from previous step was done with a mixture of diethyl ether and distilled water (5: 2) by using a separating funnel. The process was repeated thrice. Evaporation of the crude extract was done in oven (Hamco, Hindustan Apparatus Mumbai) at 60 °C temp & dry crystals of the compound were obtained in petri dishes.

2.5 Spectrophotometric Assay of Cholesterol from Probiotic Products

1 mg/ml of working standard cholesterol (HiMedia GRM6048 Cholesterol, Pure) stock solution was prepared by dissolving 0.1 g cholesterol in 100 ml of chloroform. The standard solution was stored at 4°C for further study.

2.5.1 Preparation of Liebermann–Burchard Reagent

The Liebermann–Burchard reagent (LBR) was freshly prepared according to the method described by A.P. Kenny 1952. According to this method 20 ml of acetic anhydride in an amber glass bottle was kept in an ice bath for 30 minutes. 1 ml of concentrated sulphuric acid was added carefully to this acetic anhydride in amber bottle and stirred for 10 mins. In next step 10 ml of acetic acid was added to this mixture and was kept at room temperature. (Abel et al 1952)

2.5.2 Quantification of Cholesterol

From working standard solutions 10 ml of 0.1 to 0.9 mg/ml cholesterol solution was added to 2 ml of freshly prepared LBR reagent in a separate tube. The reaction mixture was incubated in the dark at room temperature for one and half hr. 10 ml chloroform solution added with 2 ml of LBR reagent was taken as a control in separate tube. Absorbance of the resultant solution was recorded in replicates (n=3), using LBR as the blank at 420 nm by using Wensar Double Beam UV-VIS Spectrophotometer, LMSP-UV1000B. Standard graph of cholesterol was plotted from standard solutions of the working standard and recorded absorbance at 420nm.

2.5.3 Cholesterol Estimation by LBR Reagent from Probiotic Products

The dried sample mass in the petri dish (from 10 gm or / ml of original sample) was redissolved in 10 ml of chloroform and added with 2 ml of the LBR into a test tube and mixed properly with shaking. The reaction was allowed to proceed for 90 minutes in dark place at room temperature. The change in colour of the reaction mixture from purplish, pink to light green and then to very dark green indicates detection of cholesterol from test sample. A blank solution of 10 ml of chloroform was treated separately by same procedure. Mean absorbance of test and blank solutions were recorded at 420 nm wavelength. Same procedure was repeated thrice for all selected probiotic samples.

2.5.4 *In vitro* Cholesterol Assimilation Activity of Probiotic Strains

Probiotic bacteria have demonstrated the potential to lower cholesterol levels by five different mechanisms, (Beena 1997 ; Fukushima 1995 ; Grunewald 1982; Hashimoto 1998; Rao 1981;Suzuki 1991) (a) fermentation products of lactic acid bacteria inhibit cholesterol synthesis enzymes and thus reduce cholesterol production; (b) the bacteria facilitate the elimination of cholesterol in feces; (c) the bacteria inhibit the absorption of cholesterol back into the body by binding with cholesterol; (d) the bacteria interfere with the recycling of bile salt (a metabolic product of cholesterol) and facilitate its elimination, which raises the demand for bile salt made from cholesterol and thus results in body cholesterol consumption; and, (e) due to the assimilation of lactic acid. Cholesterol assimilation was investigated in culture media and under simulated intestinal conditions. Investigation of studies related to cholesterol lowering ability of selected LAB strains from test probiotic samples was done as follows. Sterile 10 ml fresh MRS broth media added with 100µg/ml of filter-sterilized water soluble cholesterol (Hi-Media - 3b-Hydroxy-5-cholestene 5-Cholesten-3b-ol) was inoculated with 1% (v/v) inoculum of each strain and incubated at 37°C with 5% CO_2 for 24 and 48 hrs. After incubation 500 µl of these samples were streaked on sterile MRS-agar plates which were then incubated at 37°C and 5% CO_2 for 48 h. Colonies were counted from each plate and the CFU/ml were recorded. All viability tests were performed in triplicate to ensure accuracy. For cholesterol analysis, incubated tests suspensions were centrifuged at 8000 rpm for 10 min at 4°C using a centrifuge and the cells were removed from the broth and supernatants containing nonassimilated cholesterol were collected.500 µl of these samples were added with 500 µl of 33% (w/v) KOH and 1 ml absolute ethanol and vortexed for 1 min and incubated at 37°C for 15 min. 1 ml deionized water and 1.5 ml of hexane was added to the solutions and vortexed for 1 min. The two phases were then allowed to separate at room temperature. 1ml of the hexane layer was transferred into a glass tube and the solvent was evaporated by keeping the tubes in hot air oven at 60°C temp. LBR reagent method was used to determine the amount of cholesterol in resuspended cells and spent broth.

The selected strains were also investigated for their capability to assimilate cholesterol under simulated intestinal conditions. Sterile 10 ml fresh MRS broth media supplemented with 0.3% Oxgall (LobaChemie-05080) as bile salt added with 100µg/ml of filter-sterilized water soluble cholesterol was inoculated with 100 µl fresh test cultures of each isolate. Incubation was done at 37°C for 24 and 48 hrs in CO_2 incubator. After incubation viability of the selected strain was checked by streaking on sterile MRS agar plates. Inoculated plates were incubated at 37^{0}C for 24 hrs and TVC was measured in forms of CFU/ml. Simultaneously nonassimilated cholesterol content was also estimated from inoculated MRS-cholesterol plus bile salt broth. MRS broth without bile salt addition but only 100 µg/ml of filter-sterilized water soluble cholesterol was kept as control set. All experiments were performed in duplicates for confirmed results. From these results percentage cholesterol assimilation by each strain in MRS broth containing 100 μg/ml of cholesterol for 24 and 48 hrs was tabulated separately. The cholesterol assimilated by test strains was calculated as follows:

Cholesterol assimilated (µg /ml) = [cholesterol (µg /ml)] 0 h − [cholesterol (µg /ml)] 24h…… (a)

Cholesterol assimilated by each strain was calculated as percentage cholesterol assimilation:

$$\% \text{ cholesterol assimilated} = \frac{\text{Cholesterol assimilated (µg/ml)}}{\text{Cholesterol (µg/ml) 0 h}} \text{ X100} \quad \text{……..(b)}$$

OR

$$\% \text{ of cholesterol reducing rate} = \frac{[C_0 - C]}{C_0} \times 100 \quad \text{……..(b)}$$

Where, C_0 is the control broth and C is test broth.

2.6 Strain Identification by 16 S rRNA

The best five different probiotic LAB strains selected from this study were identified by 16 S rRNA gene sequencing method. Database parameters used for search were EzBioCloud (www.ezbiocloud.net) from Microbial Culture Collection NCCS, Pune. All these 5 strains were individually tested for cholesterol assimilation activity from original probiotic products by the same procedure.

2.7 Statistical Analysis

The results from the study were analysed using Graph Pad Prism 6 for Windows (Version 6.01, Graph Pad Software, 2012). Test results were expressed as Mean ± SD and relative standard deviations (RSD), and also analysed inferentially, using One-Way ANOVA (at 95% confidence level) from SPSS Statistics (IBM Corporation, version 20, 2011).

2.8 RESULTS AND DISCUSSION

Ten samples of probiotic capsules, fermented cheese, curd, yogurt & probiotic sweet drinks were studied for presence of probiotic strains. Out of fifty different colonies from MRS agar plates fourteen pure isolates were selected for detail morphological and microscopic studies along with catalase, oxidase, coagulase and haemolytic activity tests. All experiments were done according to the Bergey's Manual of Systematic Bacteriology, 2nd edition vol.3, (2009) (Table 1). On the basis of these results final nine different strains were randomly selected for carbohydrate fermentation tests and other classical biochemical tests (Table 2 & 3). Number of colonies per ml of each probiotic sample was calculated by formula.

$$\text{CFU /ml} = \frac{\text{Number of colonies (mean) x Dilution factor}}{\text{Volume plated (0.1ml)}}$$

All isolates were appeared as round, opaque, grey, creamy or milky white colonies on the surface of MRS agar plates. When viewed under oil immersion objective (100X) lens after Gram staining, they appeared as purple colour rods or cocci suggesting Gram positive bacteria. All isolates showed a negative result when tested for coagulase and oxidase activity tests. Glucose, fructose and galactose sugars were fermented by all selected strains irrespective of their source of origin. Variable results were noted for other sugar fermentations. Most of the isolates were incapable of fermenting rhamnose. Dairy isolates (P2La, P5Lp and P5Lc) did not fermented cellobiose, mannose, mannitol and sorbitol. All isolates tested negative for glycerol utilization (Negative control not shown in data) (Table 2). Indole, nitrate reduction, H_2S production, urease and ornithine utilization test results were negative for all nine isolates. The properties obligatory for exogenously derived benefits from probiotics are the ability to grow in the food or the media in which the organism is placed. An attempt was to evaluate selected LAB strains for their potential as functional probiotics. The selection was based on application of some biological techniques like tolerance to specific range of pH and salt concentrations, resistance to acid and bile, adherence to gut epithelial cell linings, ability to colonies the gastro intestinal tract, and the ability to influence metabolic activities such as cholesterol assimilation etc. An effective probiotic is expected to function and survive under a variety of physiological conditions. Upon consumption, probiotic bacteria should survive transit in the gastro-intestinal tract where it is open to challenges, such as the low pH environment of stomach and bile salts of the upper intestinal tract (Kailasapathy 2007 ; Kechagia 2013 ; Musikasang 2009; Shah 2000). Stomach pH can vary from as low as 1–2 under fasting conditions and up to 4–5 following ingestion of a meal (Chou 1999; Ranadheera 2012). At pH 1.5- 3.5, the viability of isolates decreased after 48 hrs, with bacterial isolates P7St (Probiotic drinks1) and P1Ef (Probiotic capsule1) recorded minimum growth. Four isolates, P2La (Fermented cheese1), P8Lb (Probiotic drinks1), P5Lp (Curd 1), P9Pp (Probiotic drinks2) recorded maximum tolerance to simulated gastric juice as well simulated intestinal fluid conditions. All isolates have exhibited maximum growth at 2.5-4.5 % of salt concentrations. P8Lb isolate from Probiotic drinks1 showed maximum growth at 6.5 as well as 8.5 % of NaCl concentrations. Our study showed that the acidic tolerance is not necessarily related to the species of LAB, but may also be strain-specific. In fact,

differences were observed among strains belonging to the same species, such as *E. faecalis* (P1Ef), *L. plantarum* (P5Lp), *L. mesenteroides* (P1Lm) & *P. pentosaceus* (P9Pp), in terms of acid and bile tolerance. Being salt sensitive in nature growth rate of *Leuconostoc spp* (P1Lm) was decreased along with increase of salt amounts. Isolates of *Leuconostoc* (P1Lm), *Bacillus* (P1Bc) & *Streptococcus* groups (P7St) showed poor survival in acidic conditions. However, the resistances of LAB cultures to low pH and bile in food matrices during passage through the digestive tract might be greater than those seen in the physiological solutions used in this qualitative experiment *in vitro*. Probiotics must also be safe, for example probiotic bacteria should not cause lysis or destruction of red blood cells. All nine isolates tested negative for haemolytic activity test. *L. monocytogenes* showing complete haemolysis on blood agar plate was used as positive control (data not shown). Deaminated aromatic amino acids derived from dietary protein source by gut bacteria may be converted into phenols which can inhibit the growth of LAB. Products of colonic protein degradation and metabolism include ammonia, phenols, indole and amines, all of which obviously exert toxic effects *in vitro* and in animal models. These compounds are present in faecal samples which suggest that they affect the gut mucosa (Hughes, 2000) and can exert a bacteriostatic effect against some lactobacilli strains. Therefore, study of resistance to phenol by probiotics strains is important for their survival in the gastrointestinal tract (Xanthopoulos 1997). All selected strains were further investigated for lysozyme resistance, as stress for probiotic organisms begins in saliva due to the presence of lysozyme. Average 40 % of all isolates were able to survive in broth medium supplemented with 200µg/ml lysozyme. In the present study, 80 % of *Lactobacillus* & *Pediococcus spp* (P2La, P5Lp, P8Lb, P9Pp) survived well and were completely unaffected by 2 hrs of incubation with 200µg/ml lysozyme & 0.4 % phenol. Growth resistance of these selected LAB isolates from different probiotic samples against simulated gastric and intestinal conditions, lysozyme and phenol (evaluated as % survival and CFU/ml after 48 hrs of incubation) is presented in Table 4.

Table 1: Colony morphology, Gram stain characters, catalase, oxidase, coagulase, spore forming and haemolysis activity test results and no. of total viable bacteria in CFU/ml.

Strain Name	Colony type	No. of colonies CFU/ml	Microscopic Appearance & Motile/ Nonmotile	Catalase	Oxidase	Coagulase	Sporulation	Haemolysis
P1Ef	small 0.5 mm with entire margin, convex, smooth white to cream colored	7.9 X 10^7	Gram positive cocci in short chains or arranged in pairs 0.6-2.0 µm by 0.6-2.5 µm in size	-	-	-	-	-
P1Lm	small < 1mm slimy, smooth, round, and colorless in nature	3.6X10^4	Gram positive cocci forming long chains or pairs 0.6 to 0.9 µm in size, coccobacilli with rounded ends	-	-	-	-	-
P1Bc	shiny, white, non-viscid, round, about 1 to 2 mm, with entire edge.	9.5X10^8	Gram-positive rods 0.9 by 3.0 to 5.0 µm in size	+	-	-	+	+
P2La	2-5mm in diameter, convex, entire, opaque, and without pigment.	8.3X10^7	Gram positive long slender rods, 0.5 to 0.8 µm across by 2 to 9 µm in length	-	-	-	-	-
P3St	Creamy white 0.5 – 3.0 mm, Circular Convex with Entire edge	3.7 X10^5	Gram-positive cocci in chains and pairs 0.7 to 0.9 um in size	-	-	-	-	-
P3Lc	milky white, 1 mm, rounded, smooth, and nontransparent	4.9 X10^5	Gram positive rods , 0.7 to 1.0 µm across by 2.0 to 3.5 µm in length	-	-	-	-	-
P4La	2-5mm, convex, entire, opaque, and without pigment.	6.2 X10^5	Gram positive long slender rods, 0.5 to 0.8 µm across by 2 to 9 µm in length	-	-	-	-	-
P5Lp	Creamy white, circular, smooth, low convex with entire margin.	7.1X 10^7	Gram positive rods 0.5-1.2 µm across by 1.0-10 µm, elongated assembled in pairs or chains	-	-	-	-	-
P5Lc	milky white, 1 mm, rounded, smooth, and nontransparent	5.1 X10^5	Gram positive rods , 0.7 to 1.0 µm across by 2.0 to 3.5 µm in length	-	-	-	-	-
P6Lr	grey 1-1.5 mm, smooth,opaque, circular, convex, entire edge	4.4 X10^7	Gram positive rods, single rods or in short chains , 0.8 to 1.0 µm in width and from 2.0 to 4.0 µm in length	-	-	-	-	-
P7St	Creamy white 0.5 – 3.0 mm Circular Convex with entire edge	6.0 X10^5	Gram-positive cocci in chains and pairs 0.7 to 0.9 um in size	-	-	-	-	-
P8Lb	Creamy gray, 1.0 – 5.0 mm Circular Raised with entire edge	7.6 X10^8	Gram-positive, rods, long and filamentous, 0.5-0.8 µm across by 2.0-9.0 µm	-	-	-	-	-

P9Pp	1.5-2.5 mm greyish-white, smooth, circular, low convex with entire margins	1.2 $X10^5$	Gram positive spherical, diameter from 0.36 to 1.43 µm arranged in clusters, tetrads, pairs or in singles	-	-	-	-	-
P10La	2-5mm, convex, entire, opaque, and without pigment.	$6.4X10^6$	Gram positive long slender rods, 0.5 to 0.8 µm across by 2 to 9 µm in length	-	-	-	-	-
Control	Creamy white, circular, smooth, low convex with entire margin.	7.0 $X\ 10^7$	Gram positive rods 0.5-1.2 µm across by 1.0-10 µm, elongated assembled in pairs or chains	-	-	-	-	-

mm: milimeter ; µm: micrometer ; - negative test ; + positive test

Table 2: Carbohydrate test results.

Strain	Sample	Arabinose	Cellobiose	Fructose	Glucose	Galactose	Rhamnose	Mannose	Mannitol	Sorbitol	sucrose	Maltose
P1Ef	Probiotic capsule1	-	-	+	+	+	-	+	+	+	+	+
P1Lm	Probiotic capsule1	+	+	+	+	+	-	+	+	+	+	+
P1Bc	Probiotic capsule2	+	-	+	+	+	-	+	+	+	+	+
P2La	Fermented cheese1	-	-	+	+	+	-	-	-	-	+	+
P5Lp	Curd 1	++	-	++	++	++	+	-	-	-	+	+
P5Lc	Yogurt1	-	-	+	+	+	-	-	-	-	+	v
P7St	Probiotic drinks1	-	+	+	+	+	-	-	+	-	+	-
P8Lb	Probiotic drinks1	-	+	+	+	+	+	+	+	+	-	-
P9Pp	Probiotic drinks2	-	+	+	+	+	-	+	-	-	+	+
Control	*Lactobacillus plantarum subsp. Plantarum* ATCC 14917	++	++	++	++	++	+	++	-	+	+	+

Table 3: Biochemical characterization tests results.

Strain	Indole	Methyl red	Voges Proskauers	Citrate utilization	Arginine hydrolysis	Starch hydrolysis	Blood Haemolysis	Nitrate reduction	Gelatin hydrolysis	H_2S production	Urease	Lysine utilization	Casein hydrolysis	Ornithine utilization
P1Ef	-	-	+	-	+	-	-	-	V	-	-	+	+	-
P1Lm	-	-	+	+	-	-	-	-	-	-	-	-	-	-
P1Bc	-	+	-	-	-	+	-	V	+	-	-	-	+	-
P2La	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P5Lp	-	-	+	+	-	-	-	-	-	-	-	-	-	-
P5Lc	-	+	-	-	-	-	-	-	-	-	-	-	-	-
P7St	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P8Lb	-	-	-	+	-	-	-	-	-	-	-	-	-	-
P9Pp	-	+	-	-	+	-	-	-	-	-	-	-	-	-
Control	-	-	+	+	-	-	-	-	-	-	-	-	-	-

+Positive result; - Negative result; V Variable reaction

Table 4: Tolerance tests of selected isolates.

Strain Name	Growth / % Survival											
	Sodium chloride solutions (%)				pH		Simulated gastric juice (% survival)	Simulated intestinal fluid (% survival)	Lysozyme concentration (200 µg/ml) (CFU/ml)		Phenol Concentration (0.4%) (CFU/ml)	
	2.5	4.5	6.5	8.5	3.0	9.0	pH 1.5-3.5	pH 6.3-7.3	0 hr	48 hrs	0 hr	48 hrs
P1Ef	++	++	++	-	-	+	30.91	47.09	6.18±0.4	2.84±0.6	6.18±0.4	2.64±0.02
P1Lm	+	-	-	-	++	-	29.84	52.82	6.42±0.5	2.28±0.4	6.42±0.5	2.60±0.06
P1Bc	++	++	++	+	++	+	32.33	60.87	6.54±0.4	2.59±0.4	6.54±0.4	2.87±0.04
P2La	++	++	+	-	++	-	77.45	55.24	6.55±0.8	5.16±0.4	6.55±0.8	5.59±0.03
P5Lp	++	+	-	-	++	-	62.82	49.84	6.47 ± 0.3	4.91±0.2	6.47 ± 0.3	4.81 ± 0.2
P5Lc	++	+	-	-	++	-	50.87	47.09	6.13 ± 0.3	2.60±0.6	6.13 ± 0.3	3.16±0.4
P7St	++	+	++	+	+	-	25.35	54.45	6.32 ± 0.2	2.54±0.6	6.32 ± 0.2	3.06±0.4
P8Lb	++	+	++	++	+	-	65.82	42.31	6.42±0.4	4.19±0.4	6.42±0.4	5.01± 0.9
P9Pp	++	+	+	-	++	-	69.61	50.24	6.12±0.4	5.68±0.5	6.12±0.4	5.19± 0.2
Control	++	+	-	-	++	-	63.00	50.84	6.60 ± 0.3	3.14±0.2	6.60 ± 0.3	3.74 ± 0.2

+ growth present ; ++ moderate growth ; - No growth ; *Bacteria applied at the dose of 10^5 CFU/ml of each selected strain significant at $P \leq 0.05$. CFU, colony forming units after 48 hrs of incubation at 37^0C under anaerobic condition in different time intervals; Values are represented as mean ± standard deviation (n = 3)

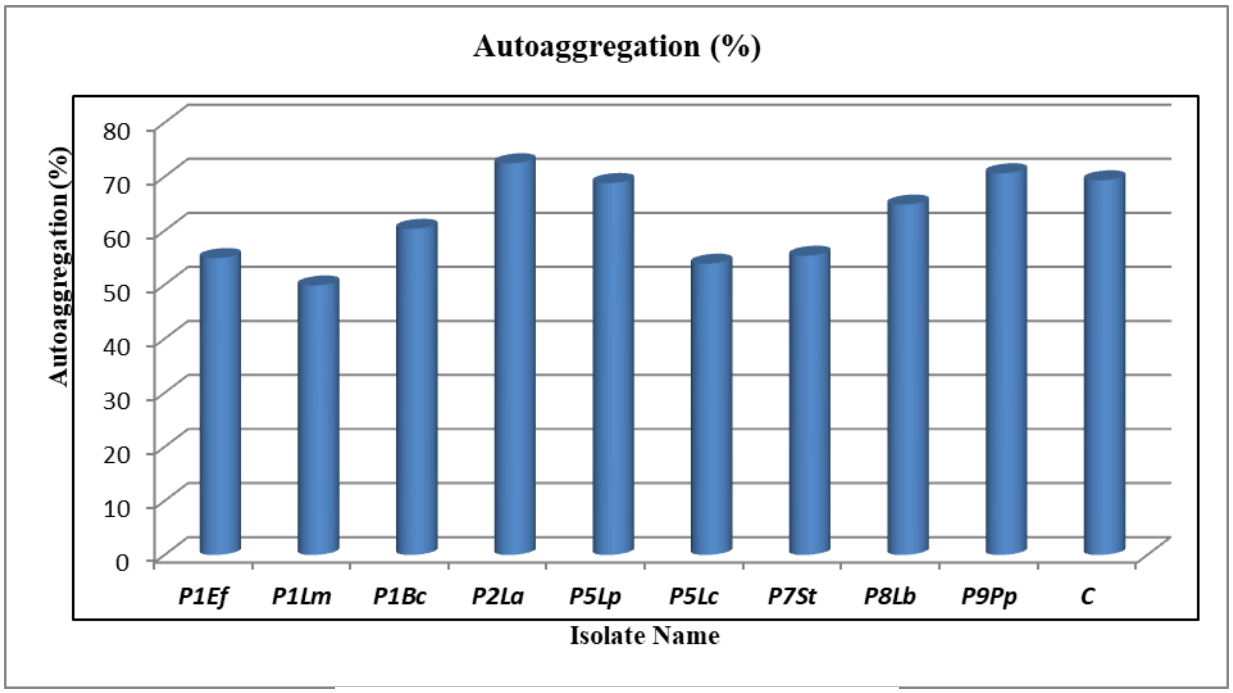

Figure 1: % Auto aggregation of selected isolates.

C: Control Auto-aggregation of cells re-suspended in PBS (pH 6.8) after 24 hrs incubation at 37˚C.

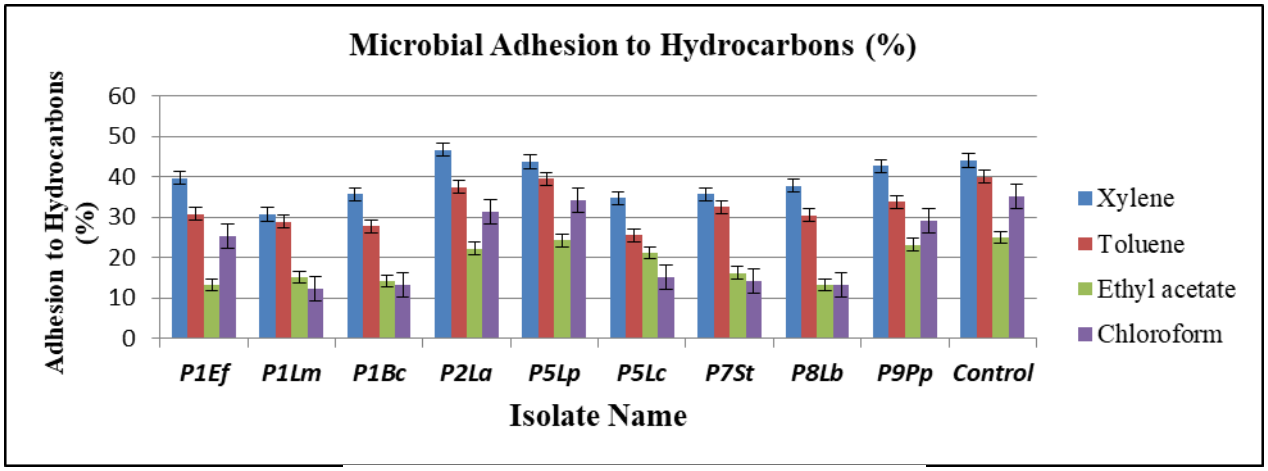

Figure 2: Microbial adhesion to hydrocarbons (%).

Bacteria applied at the dose of 10^5 CFU/ml of each selected strain significant at $P \leq 0.05$, All experiments were performed in triplicate and error bars indicate standard error.

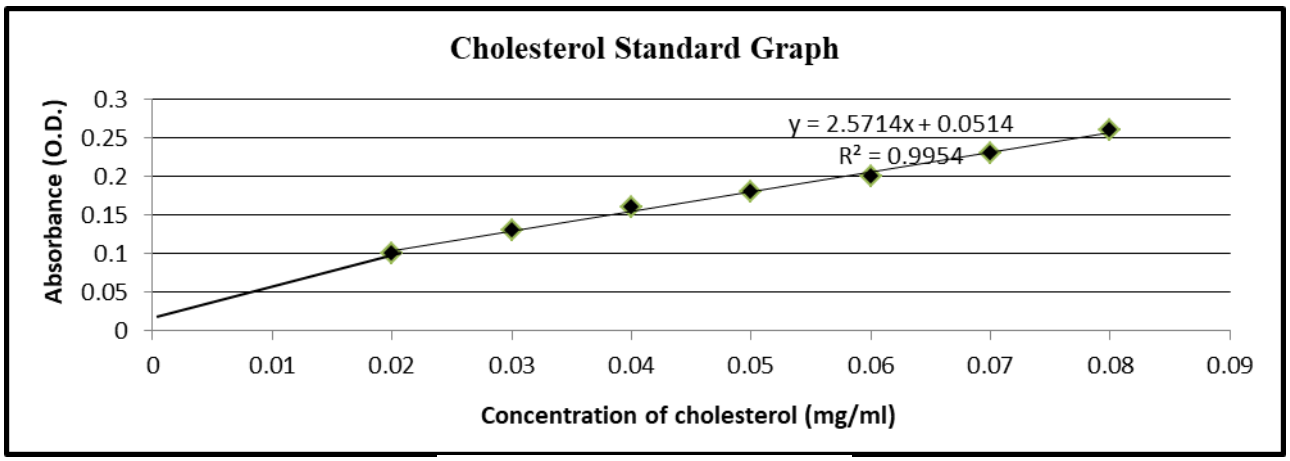

Fig 3: Standard curve of cholesterol.

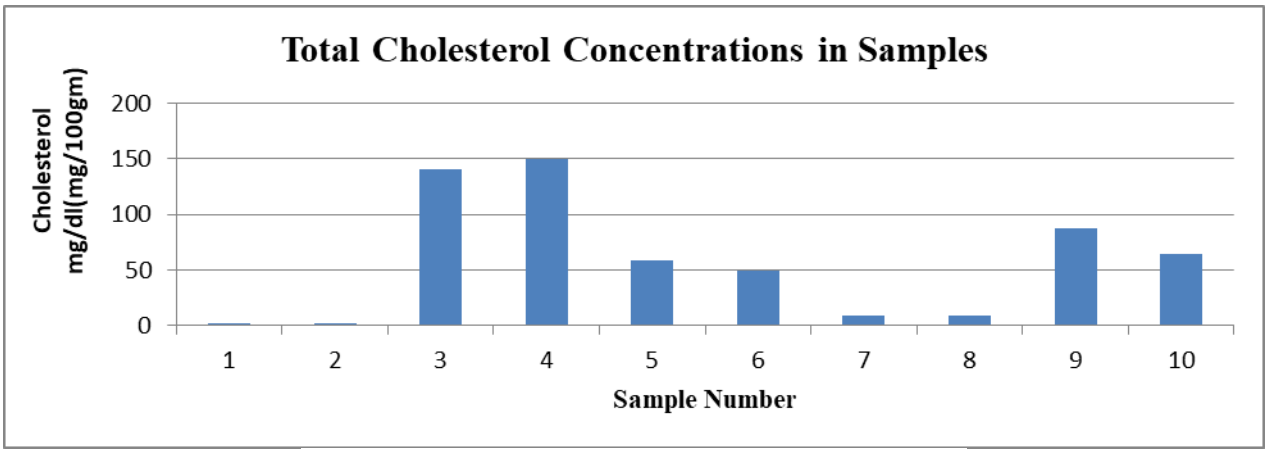

Fig 4: Total cholesterol concentrations in selected samples.

1: Probiotic capsule 1; 2: Probiotic capsule 2; 3: Fermented cheese 1; 4: Fermented cheese 2; 5: Curd 1; 6: Curd 2; 7:Yogurt1; 8: Yogurt 2; 9: Probiotic sweet drink1; 10: Probiotic sweet drinks2

Table 5: Cholesterol assimilation (%) in MRS broth by each lactic acid bacterial strain.

Strain Name	CFU/ml in MRS with cholesterol (100 µg/ml)	Cholesterol in spent broth (µg/ml)		Cholesterol assimilated %	CFU/ml (MRS with 0.3% Oxgall +100µg/ml cholesterol)	Cholesterol in spent broth under simulated conditions (µg/ml)		Cholesterol assimilated %
		24 hrs	48hrs			24 hrs	48hrs	
Control	0.00 ± 1.00				0.00± 1.11			
P1Ef	$1.82 \pm 0.02\times 10^4$	86.57	82.32	15	$1.50 \pm 0.02\times 10^4$	79.5	75.24	18
P1Lm	$3.17 \pm 0.17 \times 10^5$	69.78	64.12	23	$2.58 \pm 0.17 \times 10^5$	59.8	55.53	29
P1Bc	$2.62 \pm 0.02\times 10^8$	64.05	59.55	34	$2.72 \pm 0.02\times 10^8$	48.6	43.21	38
P2La	$2.81\pm 0.02\times 10^8$	44.10	39.65	62	$2.97\pm 0.02\times 10^8$	37.13	33.47	66
P5Lp	$1.18 \pm 0.05 \times 10^8$	54.42	48.0	53	$2.25 \pm 0.05 \times 10^8$	46.37	44.82	52
P5Lc	$2.93\pm0.02\times 10^7$	88.80	85.50	13	$4.12\pm0.08\times 10^7$	83.0	81.0	17
P7St	$1.43\pm0.02\times 10^8$	53.22	47.14	42	$1.10\pm0.05\times 10^8$	48.00	48.10	44
P8Lb	$2.49\pm0.02\times 10^6$	50.40	45.48	50	$2.86\pm0.03\times 10^6$	41.64	39.23	53
P9Pp	$2.08 \pm 0.09\times 10^8$	57.53	51.32	58	$4.27 \pm 0.05\times 10^8$	47.69	42.37	59
Control	$2.35\pm 0.02\times 10^8$	45.15	37.68	64	$2.47 \pm 0.05\times 10^8$	42.80	36.58	61

Probiotic bacteria must also be capable of adhering to intestinal epithelial lining in order to provide benefits in the host. Adherence enables the probiotic bacteria to persist for a longer time in the gut and enhances the host-bacteria interactions (Gueimonde 2006). Adherence to the host cell surface which determines the colonization capability of bacteria is a crucial step in the establishment of probiotics in the intestine. Microorganisms have developed on their cell surfaces a range of hydrophobic components referred to as adhesins, such as proteins, polysaccharides, teichoic acids and fatty acids that enable them to bind covalently to the surface of the intestinal epithelium and mucin. Through this adhesion property, probiotic microorganisms can prevent pathogen access by steric interactions or specific blockage on cell receptors. For this purpose, hydrophobicity ability of selected strains was determined by bacterial adherence to hydrocarbons (xylene, toluene , ethyl acetate & chloroform) test. Greater hydrophobicity of cells results in greater attractive forces and higher levels of adhesion (Rijnaarts 1993). Auto aggregation ability of probiotic bacteria correlates with adhesion to the oral cavity, gastrointestinal tract, and urogenital tract, which is a prerequisite for colonization and protection inside human body. *L. acidophilus* (P2La) exhibited a strong auto aggregating phenotype up to 70% and manifested a high degree of hydrophobicity determined by microbial adhesion to xylene up to 48%. Aggregation and hydrophobicity were abolished upon exposure of the cells to ethyl acetate and chloroform however all strains showed maximum affinity towards xylene. But *Lactobacillus* & *Pediococcus* strains showed maximum affinity towards chloroform (35% & 29%) than other isolates. (P5Lp) *L. plantarum* showed highest aggregation potential (70%) followed by (P9Pp) *P. pentosaceus* (69%). The values obtained with chloroform and ethyl acetate, were regarded as a measure of electron donor/basic and electron acceptor/acidic characteristics of bacterial surface, respectively (Bellon-Fontaine 1996). The fact that a high percentage of *L. acidophilus* (P2La) cells adhered to xylene, a polar solvent, demonstrated hydrophobic cell surface of this strain. Several small trials suggest that eating yogurt may lower harmful LDL cholesterol and blood markers of inflammation, both of which signal cardiovascular risk. Other fermented products, including probiotic drinks and beverages are also becoming more popular. Although the evidence seems to favour adding these products to diet, it should be also considered according to other ingredients added in that particular foods that might be less desirable for heart health. For example, dairy based fermented products like yogurt, cheese, butter, and kefir tend to be quite high in sodium, which can raise blood pressure. Some yogurts, sausages, juices and probiotic energy drinks contain three or more teaspoons of added sugar per serving. Regular and excessive consumption of foods high in salt, sugar and fat leads to obesity and is a risk factor for diabetes, high blood pressure and heart diseases. For these reasons, we set out to screen these selected market products for direct quantitative cholesterol estimations by Liebermann–Burchard assay. This simple assay is based on the strong colour developed when cholesterol is combined with strong acids. Extraction and quantification of cholesterol was performed according to method described by Abell et al. The cholesterol level in the fermented cheese products was very high about to 145 mg/100 gm while the cholesterol levels in the probiotic sweet drinks were 65mg /100 ml (probiotic sweet drink1) and 83 mg/100 ml (probiotic sweet drink 2). Curd samples which were tinned milk products were also observed to have high cholesterol levels up to 58 mg /100 gm of product. All these levels were above the dietary daily requirement of cholesterol in milk products (5 mg/100 g) recommended by United States Department of Agriculture Product. The Eat Right India mission of the Food Safety and Standards Authority of India (FSSAI) launched in August 2018 rightly focuses on reduction of salt, sugar and fat in diet. Salt is the main source of sodium in our diet. High sodium is a risk factor for high blood pressure which predisposes an individual to heart problems. Average Indian consumes around 10 gram of salt per day which is double the amount of salt recommended (5g/d). Sugar

provides only calories and no other nutrient to our body. High intake of sugar is a risk factor for obesity and predisposes the individual to diabetes and other diet related non-communicable diseases. Excess of fat intake is a risk factor for obesity and non-communicable diseases like diabetes, cardiovascular disease and cancer. Sample 6 and 7 (Yogurt1 and 2 both having cholesterol 10 mg/100ml) did not contain any information on the constituents, making it difficult to justify the high levels. In the case of the sample 6, it was noted that the product label claimed the presence of soya base, a plant-derived dairy source in the product. It was observed that cholesterol levels in the tinned milk products were higher than that in the fresh yoghurts (Figure 4). Whole buffalo milk or milk solids contain 26%–42% of fats, and so possess high contents of cholesterol. The use of such sources is expected to result in high cholesterol levels, as seen in sample no. 3, 4, 9 and 10. The use of milk solids, which contains fats accounts for high cholesterol levels and so, would require a defatting process during manufacturing of the dairy product. On the other hand, a thorough defatting process would lead to low cholesterol levels, as evident in sample no.7 and 8. Generally, it was observed that either low milk fats or skimmed milk was used in the manufacture of the yoghurt products, thereby accounting for their relatively low cholesterol levels. Furthermore, this study investigated the ability of the isolated strains in selected products to reduce cholesterol in MRS broth *in vitro*. All strains have demonstrated potential to lower cholesterol levels at variable concentrations. *Lactobacillus* strains showed maximum cholesterol removal (60-70%) in MRS broth. It is remarkable that at the end of the 24 and 48hr incubation periods, in the media containing 0.3 % oxgall, the P2La & P9Pp strain, which achieved maximum cholesterol removal to the values of 66% and 59%, respectively, Furthermore, the *Lactobacillus plantarum* ATCC 14917strain, which had the second highest cholesterol removal rate after the P9Pp strain. Serum cholesterol is a major risk factor for the development of atherosclerotic vascular disease. Drug therapy for hypercholesterolemia includes fibrates, statins and bile acid sequestrates; however, the undesirable side-effects of these compounds have caused concerns about their therapeutic use. Hence, there is a requirement for a more natural method to decrease serum cholesterol concentration in humans (Sridevi 2009). To provide health benefits, probiotics must overcome physical and chemical barriers such as acid and bile in the gastrointestinal tract (Ouwehand 2002). *Bacillus coagulans* has frequently been reported to be a contaminant and spoiling organism of milk products, vegetables, and fruits and processing of these contaminated products in foods may thus affect shelf lives. All the *Lactobacilli* isolates used in this study, which include species— *acidophilus, plantarum, casei, delbrueckii subsp. bulgaricus* are already approved by FSSAI as probiotics (live microorganisms) and GRAS - Generally Regarded As Safe. *P. pentosaceus* has been approved as an animal feed additive in the United States, European Union, China, Thailand, Australia, and New Zealand (Lim 2009). In recent years it has received increased attention and has been characterized for its possible utilization as probiotic in humans (Osmanagaoglu 2012; Zhao 2012; Borges 2013; Vidhyasagar and Jeevaratnam, 2013; Borges 2014; Garcia-Ruiz 2014; Lee 2014). Generally, members of LAB do not possess haemolytic activity. However, *Enterococcus* and *Bacillus* strains possess haemolytic potential and such strains should be adequately tested and confirmed for non-haemolytic activity (FAO-WHO, 2002). Recently, the assessment of *E. faecium* for qualified presumption of safety was performed, and this led to the conclusion that strains associated with clinical infections could be differentiated from commensal strains (EFSA Panel on Biological Hazards, 2012).

Expert bodies of health professionals make no attentions on probiotic species, strains, and doses that might alleviate the symptoms. The International Scientific Association for Probiotics and Prebiotics advises manufacturers to list the recommendations for or against probiotic use by healthy people. For people with various health conditions, however, published studies and reviews provide some guidance. The Association also advises manufacturers to list the total number of CFUs—ideally for each strain—on the "expiration" or "use by" date on the product label (International Scientific Association for Probiotics and Prebiotic 2017)

CONCLUSION

Our objective of this study was to develop a new consumable product by using suitable lactic acid bacterial combinations that could serve as a probiotic that reduces cholesterol levels in humans. It was observed that *Lactobacillus, Pediococcus* and *Enterococcus* group members exhibited hypocholesteremic effect. These strains possessed good survival ability in artificial GIT fluids as well as good cholesterol removal ability in MRS broth supplemented with cholesterol and bile salts. The minimal effective dose of these strains in terms of CFU/ml/day in the carrier non-dairy vegetable food can be monitored to develop a functional probiotic product to prevent hypercholesterolemia in cardiovascular diseases. A morphological analysis and 16S rRNA sequencing identified the isolate P5Lp as *Lactobacillus plantarum subsp. Plantarum* ATCC 14917(T) ACGZ01000098 100.00, isolate P9Pp as *Pediococcus pentosaceus* DSM 20336(T) and P1Ef as *Enterococcus faecium* ATCC 19434 (T) DQ 411813 100.00 . In conclusion *in vitro* study based on cholesterol uptake and survival during passage through the human GIT can be considered as key features for formulation of vegetable base probiotic formulation.

Applications of this study: To construct new probiotic formulations with nutraceutical and / bioceutical properties by means of functional food which can provide extra health benefits along with basic nutritional values.

Footnotes

Declarations

Availability of data and materials

The data that support the findings of this study are available from the corresponding author on request. Requests for the data from this study can be submitted via email to sarikamohol@gmail.com.

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

Conceptualization, Methodology, Investigation, Writing—original draft, by Sarika Bhosale Corresponding author. Writing—review and editing and supervision by. The authors read and approved the final manuscript.

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Consent for publication

Not applicable.

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