Pediococcus acidilactici a Potential Probiotic Strain from Traditional Fermented Cheese, Kalarei

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Abstract:

Pediococcus acidilactici MZ375423 strain isolated from indigenous fermented cheese, Kalaeri, was showing following probiotic characteristics: tolerance to low pH, high ox-bile and NaCl concentrations. The therapeutic and nutritional aspects, as cholesterol lowering effect (20% reduction) in the presence of gelatinase ascertained its ability in improvising the nutritive value of functional foods. The negative test for catalase and oxidase, with a positive end production of acidic and neutral end products confirmed that the strain was heterofermentative. The biochemical tests viz. lipid, gelatin and starch hydrolysis, ImViC tests were performed at 37 °C. Growth studies and fermentation studies were optimized for the production of extracellular amylase (930.23 U/L) and lactic acid (14.53 mg/L) with an incubation period of 24 h at 37 °C. The properties for which the strain was tested positive was categorized as the microflora of Kalaeri, having nutritive value and therapeutic potential.

Keywords: Fermented foods; probiotics; *Pediococcus acidilactici*; enzymes

Introduction:

The presence of live probiotics in fermented foods and cultured milk has been widely used in the preparation of infant foods; the health friendly characteristics of these bacteria is attributed to the presence of beneficial properties associated with living cells (Shi et al. 2016). Prevention of bowl diseases, their studies for lactose intolerance and maintaining intestinal microbial balance while producing antibacterial peptides along with organic acids, antihypertensive and antihypercholestrolemic effects; alleviation of post menopausal disorders and reducing travellers' diarrhoea are some of the beneficial aspects associated with different genera, species and strain specific characteristics belonging to a group of probiotics which includes LAB (lactic acid producing bacteria) as one of the major group of bacteria. LAB belonging to Firmicutes, Bacteroides and Proteobacteria phyla have been associated with health benefits, their immense use in probiotic foods are becoming economically important (Jain et al. 2014). The market for probiotics is expected to reach US$ 64.02 billion by

2022 (PR Newswire, 2017). Most of the species exhibiting probiotic characteristics belong to *Lactobacillus* and *Bifidobacterium*.. But strains belonging to *Pediococcus* genera have gained tremendous importance for their biotherapeutic and nutritive potential. Although in the developed world, the association of LAB is mainly with dairy products such as yogurt, cheese and buttermilk.

Consumption of traditionally fermented foods like yogurt prepared from natural strains is one of the effective ways of increasing probiotic activity. Many traditional fermented foods prepared in various parts of the world have been reported to be having wide range of probiotics shaping the gut barrier function and curing diseases like dysbioses. *P. acidilactici* strain as a probiotic tends to treat constipation (Herndon et al. 2020), diarrhoea (Balgir et al. 2013), relieve stress (Castex et al. 2010), improve immune response in human and animals (Neissi et al. 2013) with a potential of producing enzymes like phytases (Bhagat et al. 2020); besides these are also the potential bacteriocin producing strains. *P. pentosaeus* KID7 a potential probiotic strain has been reported from finger millet having cholesterol lowering ability (Damodharan et al. 2015) too. The genomic analysis of *P. acidilactici* HN9 strain has emphasized their use as starter cultures for fermentation, as they can prevent fermentation failure from the bacteriophage and pathogen infection during the process of fermentation (Surachat et al. 2021).

Looking into immense potential of *Pediococcus* strains as a potential probiotic and their association with fermented dairy products, it will provide additional health benefits because of nutritional components present in the milk.

Our studies have tried to address viability of *P. acidilactici* strain at high bile salt, NaCl and low pH concentrations. The strain stored at -80 $^{\circ}$C in 50 % glycerol stocks had therapeutic and nutritive potential associated with it. Studies have reported that new functional probiotic foods will not only include baby formula, children food, fermented fruit juices, cereals and fermented soya bean products, but also disease specific clinical foods containing viable LAB, prebiotic precursors and/or pro-bioactive cellular components (Barbu et al. 2016). Fermented foods with probiotics have improvised the nutritional and ingredient values while producing various growth stimulating factors such as oligosaccharides, amino acid, and peptides. These foods can be used for the therapeutic properties associated with them and also considered for pharmaceutical preparations. The anti bacterial activity of LAB in fermented foods is mainly due to production of organic acids and compounds like bacteriocins. Most of the bacteria produce isomeric forms of lactic acid during glucose fermentation. From the time of Russian scientist Metchnikoff, to up till now the studies of LAB have been associated with health

benefits which have led to an increased use of commercial probiotic strains in food beverages and drinks leading to a rise in number of functional foods. LAB as commercial probiotics needs to be viable under simulated GIT so that bioactive molecules produced by these bacteria serve as health assets for the host. So, the viability of the LAB strains fulfilling all the probiotic characteristics viz. tolerance to low pH, high bile salt and NaCl concentrations, resistance to gastric juices, ability to grow at moderate temperatures, properties of aggregation and adherence to epithelial cells is of paramount importance. Such LAB should be devoid of any such virulence factors.

Material and Methods

Collection of bacterial samples:

Lactic acid bacterial (LAB) strain stored in 50% glycerol stocks at -80 $^{\circ}$C, isolated from fermented cheese, were selected for studying the probiotic characteristics (Bhagat et al. 2020). These bacterial strains were revived on MRS (de Man, Rogosa and Sharpe) broth (Hi-Media, India) at 37 $^{\circ}$C for 48 h (incubation period) under anaerobic conditions. Cultures were further streaked on MRS agar plates for single colony isolation, purification and maintenance. The isolated single colonies of following bacterial cultures were grown in MRS broth and streaked on nutrient agar plates to check their growth.

Characterization and identification of LAB cultures:

Selected bacterial isolates revived from glycerol stocks were subjected to different morphological, biochemical and physiological tests. Characterization studies included studies on colony formation, their size and shape. The colonies were studied for elevation, color, surface, margin and texture. Purified strain was streaked on MRS and observations were carried out following the standard protocols given by Kunchala et al. 2016. Presumptive LAB was subjected to physiological tests such as low pH tolerance, high bile salt tolerance, NaCl tolerance and growth characteristics were studied at different temperatures 4 $^{\circ}$C, 37 $^{\circ}$C and 45 $^{\circ}$C respectively. For tentative identification, biochemical tests were performed which included: Gram staining, catalase test, oxidase test, starch hydrolysis test, gelatin hydrolysis test, lipid hydrolysis test, IMViC (‘I’ is for indole test, ‘M’ is for methyl red test, ‘V’ is for Voges-Proskauer test and ‘C’ is for citrate test) and sugar fermentation test in the presence of glucose were performed.

Molecular identification of selected LAB isolate

Bacterial genomic DNA was extracted using DNA purification kit. Identification was based on 16S r RNA gene sequencing. The universal primers used were (lac1–27F 5'-AGAGTTTGATCCTGGCTCAG-3' and lac 1-1492R 5'-TACGGYTACCTTGTTACGACT-3'). The conditions of PCR programme were as follows: initial denaturation at 94 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR product obtained was sequenced by AgriGenome Labs Pvt. Ltd., Kerala. Basic Local Alignment Search Tool (BLAST) was used to analyse the homology of sequence and the sequence was submitted in NCBI GenBank with an accession number: SUB9831877 KRb MZ375423.

Microbial Growth, Amylase and Lactic acid production

Quantitative test for extracellular amylase was performed using starch hydrolysis test with 1% starch as the substrate. The enzymatic reaction mixture consisted of: 400 µl sodium acetate buffer (pH 6.0), crude enzyme extract (500 µL) and1% starch (100 µL). The reaction mixture was incubated at 37 °C for 30 min and the reaction was terminated by adding 10% trichloro acetic acid or by heating the mixture at 100 °C. Controls and blanks were also kept. Enzyme activity was calculated as 1mol of substrate converted into moles of product at 37 °C with an incubation period of 30 min expressed as U/mL/min. The end product of starch hydrolysis was determined by DNS assay method and absorption was measured at 575 nm (Shimadzu spectrophotometer MODEL UV-1800 UV-Vis).

Lactic acid production and effect of pH on the growth of the probiotic strain:

The titratable acidity is expressed as % lactic acid and is determined by titration of a known amount of reconstituted sample 0.1 N NaOH using phenolphthalein as an indicator.

Calculating the lactic acid production by LAB titration was carried out w.r.t 0.1NNaOH.

$$\% \text{ lactic acid [mg/mL]} = \frac{\text{mL NaOH} \times \text{N NaOH} \times \text{molecular weight}}{\text{Vol. of sample used}}$$

Titratable acidity is expressed as % lactic acid (CH_3-CHOH-COOH, M.W =90.08).The samples were withdrawn at regular intervals of 6 h to determine lactic acid produced by the culture and to ascertain the effect of pH on the growth of culture. Cell density was calculated by measuring absorbance at 600 nm.

Cholesterol lowering potential :

The amount of cholesterol dissolved in the broth was determined by the ortho-phthaldehyde method as described by Rudel and Morris 1973. Presummptive LAB was grown (48 h) in MRS broth containing water soluble cholesterol (100 mg/L). The medium was centrifuged at 8000g, 4 °C for 15 min) and the cell free supernatant (0.5 mL) was taken for determining the residual cholesterol. 2 mL of KOH (50% w/v) was added to 3 mL of absolute ethanol, vortexed for 1 min followed by heating at 70 °C for 15 min. After cooling up till room temperature for 3 min, 3 mL distilled water was added with 5 mL hexane and vortexed (1 min). 2.5 mL hexane was evaporated under nitrogen. The residue was dissolved in 4mL ortho phthaladehyde reagent (which was prepared by dissolving in 0.5 mg/mL acetic acid) and kept at room temperature for 10 min. Then 2 mL of concentrated sulfuric acid was added and vortexed for 1 min, keep at room temperature for 10 min. O.D. (Optical density) readings were taken at 550 nm. The reduction (%) in cholesterol was calculated by taking into consideration O.D readings of uninoculated MRS broth and test samples viz. CFS (cell free supernatant) of culture.

Physiological properties of LAB isolates:

Resistance to low pH, tolerance to high bile salt and NaCl concentrations

To determine the probiotic characteristics of the culture revived from 50% glycerol stocks kept at -80 °C. The strain was checked for survivability at low pH, as described by Tulumoglu et al. 2013. 24 h grown bacterial culture, at 37 °C was inoculated in MRS broth (100 mL) and 100 µL of the culture was added to 10 mL sterile phosphate-buffered saline (PBS) solution (NaCl: 9 g/L, $Na_2HPO_4.2H_2O$: 9 g/L, KH_2PO_4: 1.5 g/L, pH 6.2). Following pH values 2.0, 2.5, 3.0, and 6.2 (control) of PBS solution was adjusted using 10 M HCl, the culture was incubated for 24 h to 48 h at 37 °C and O.D readings (600nm) were taken at a regular interval of 12 h. The growth was observed for 5 days. The results were mean of triplicates.

Similarly, to check the bile tolerance of the culture, overnight grown bacteria was used. The cell pellets were collected after centrifugation for 10 min at 10,000 g, followed by washing for three times. The washed pellets were kept in PBS solution (NaCl: 9 g/L, $Na_2HPO_4.2H_2O$: 9 g/L, KH_2PO_4: 1.5 g/L, pH 6.2). 100 µL of bacterial solution was added to 900 µL of PBS solution containing 0.25%, 0.50% and 0.75% bile concentrations and a control sample without bile was also kept. The cell culture was spread on agar plates and incubated for a period of 24 h at 37 °C in order to determine the colony count (CFU/ mL).

In order to determine the NaCl tolerance, revived culture grown in MRS broth at varying concentrations of NaCl (i.e. 2.5%, 4.5%, 6.5% and 9.0% respectively) was incubated at 37 °C for 12 h. O.D readings were taken at 600 nm (Shimadzu spectrophotometer UV-1800 UV-Vis). The growth was observed for 5days.

Growth profile studies at different temperatures:

Optimal growth was determined at varying temperatures viz. 4 °C, 37 °C and 45 °C by inoculating 1% (v/v) of fresh overnight grown cultures in 10 mL MRS broth. O.D readings at 600 nm were taken after a regular time interval of 12 h for 5 consecutive days.

Results and Discussion:

Colony morphology studies:

For isolation and enumeration studies MRS broth was used and the procedure was carried out under anaerobic conditions at 37 °C with an incubation period of 48 h. The revived culture was checked for purity. Single colonies were obtained for the culture on MRS agar as well as NA medium. Colonies grown on MRS medium had colony morphology as described by Kunchala et al. 2016. Colonies found were: forms (circular/irregular), size (big/medium/punctiform), surface (rough/veined/glistening), texture (moist/mucoid/dry), color (cream/pink/pale-pink/translucent/yellow/white), elevation (flat/raised/umbonate) and margin (entire/lobate) (Suskovic et al. 1997; Kunchala et al. 2016). Phenotypical characterization revealed culture was Gram positive, cocci, non spore forming, anaerobic and aerotolerant, identified as *Pediococcus acidilactici* (KRb MZ375423) by 16srRNA. The phylogenetic tree shows 100% homology with aforementioned strain as shown in Figure 1.

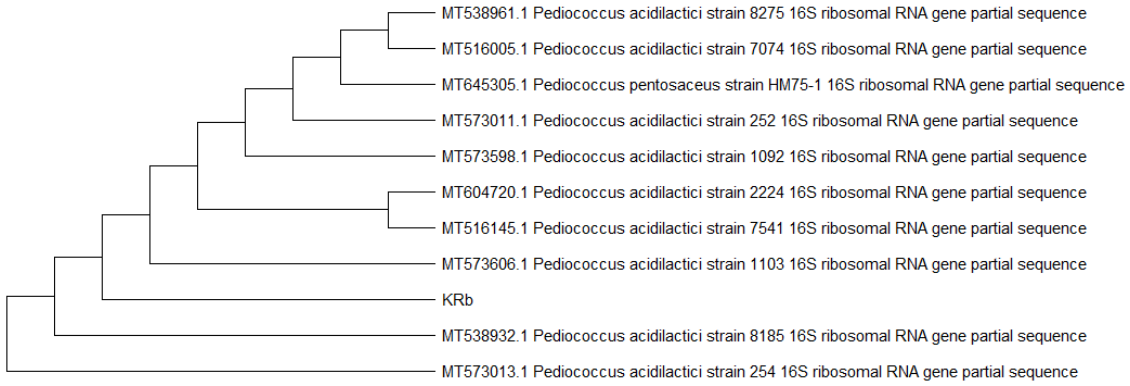

Figure 1 : Evolutionary analysis by maximum likelihood method conducted by Mega X2 method. The strain KRb was identified having 100% homology to *Pediococccus acidilactici* having accession no: MZ375423.

Studies for identification of the culture:

LAB considered as one of the major group of probiotics includes genera belonging to *Pediococcus, Lactobacillus, Streptococcus, Enterococcus* etc., although majority of the strains isolated from milk products have been reported to be belonging to *Lactobacillus* (Suskovic et al. 1997; Kunchala et al. 2016). In the present study, *P. acidilactici* reported from traditional fermented cheese, Kalaeri has been explored for morphological, biochemical characteristics showing similarity when compared to earlier reports by (Abbasiliasi et al. 2012). Bioinformatics approach was used to relate the strain to the phylogentic tree using Mega X software. The gene sequence with accession number: MZ375423 has shown 100% similarity confirming the identification of the strain. Biochemical tests performed for culture identification included: catalase test, oxidase test, indole test, methyl red test, voges proskauer test, citrate utilization test followed by starch, lipid and gelatin hydrolysis test (Cappucino & Sherman 1996). Culture in present study was negative for catalase, oxidase, indole, and citrate utilization test. The culture identified as *P. acidilactici* MZ375423 gave positive results for methyl red test confirming the production of acidic end products on fermentation of glucose present in the medium. Confirmatory test for Voges Proskauer (VP test) determined production of neutral and non-acidic end products stating that the culture is heterofermentative. The culture gave positive results for gelatin, starch and lipid hydrolysis. Production of extracellular dietary enzymes by *P. acidilactici* MZ375423 was confirmed by polysaccharide degrading enzymes (Figure 2), hydrolysis of 1% tributyrin and 1% starch in modified MRS medium respectively.

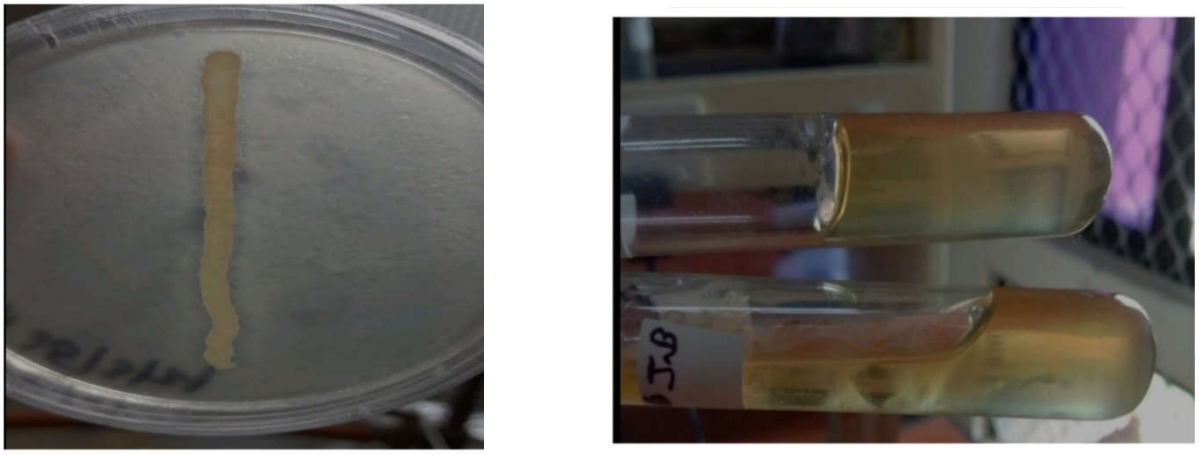

Figure 2: *P. acidilactici* revived from 50% glycerol stock showing (a) gelatin hydrolysis, polysaccharide degrading ability of LAB (b) zone of hydrolysis in (1%) tributyrin agar plate confirming presence of extracellular lipase, after an incubation period of 24 hours.

Enzymatic activity of amylase

Pediococcus strains are considered as good candidates among starter cultures in fermentation industry as they encode various genetic elements that are beneficial and promote fermentation process. They inhibit and prevent growth of spoilage bacteria and food borne pathogens (Surachat et al. 2021). Amylase produced by probiotics is very promising, highly stable having better economical productions with its uses expanded for medical, clinical and analytical purposes. Amylases (E.C.3.2.1.1) responsible for hydrolysis of starch yields oligosaccharides by cleaving α-1, 4 glycosidic bonds. Hydrolysis of starch with amylase enzymes tends to neutralize the acidic nature of medium and results in production of fructose syrup under high temperatures (Souza and Magalhães 2010). Commercial uses of enzymes does not require purification step but a high level of purity in pharmaceutical and clinical purposes is one of the essential features thus making the process costly and time consuming. Its applications in various industries: in starch liquefaction, in brewing and in textile industries for sizing, in paper industry for smoothness and in detergents to remove stains imparts different physicochemical properties to the biomolecule. Depending on the different physicochemical properties different methods for purification of bacterial amylase have been cited (Padmavathi et al. 2018). Production of amylase for food bioprocessing and preparation of fermented foods can be considered as one of the essential probiotic attributes of the strain (Sanni et al. 2002). The strain in the present study isolated from the fermented cheese was capable of showing starch hydrolysis confirmed by the clear zones observed around the colonies grown on MRS + (1%)

starch medium at 37 °C after an incubation period of 24 h, followed by flooding with iodine solution (Figure 3) confirming production of extracellular enzyme. Use of amylases in formulating lotions as well as ointments has been reported. Amylases from the group of bacteria which have lactate as an end product of fermentation (Singh et al. 2006).In fermentation industry where starch is used as an economical raw material and the presence of amylo-lactic acid bacteria will enhance the fermentation process (Tanyildizi et al. 2005) resulting in production of lactic acid in a single step thus reducing the cost of overall process of fermentation. Elmansy et al. 2018 reported a decrease in enzyme production with an increase in starch concentrations may be a result of release of excessive toxic metabolic wastes released with rapid consumption of starch thus suppressing the growth of bacteria as well as resulting in decline in α-amylase production. As reported by earlier studies (Hutkins 2018) the LAB isolates from later fermentation stages are often characterized by increased organic acid synthesis and the ability of the strain to increase acidification affects the antimicrobial properties of the strain inhibiting the growth of gram positive and gram negative bacteria (Szutowska and Gwiazdowska 2021).

Figure 3: Revived culture grown in (a) MRS medium at 37^0C for 48 hours (b) zone of hydrolysis in starch (1%) agar plate after iodine flooding.

Pediococcus acidilactici MZ375423 has showm maximum (930.23 U/mL) production of extracellular amylase in modified MRS medium (1% starch), after an incubation period of 48 h at 37 °C. Amylase activity by starch fermentation was studied at specific conditions viz at 37 °C for 30 min in 48 h grown bacterial culture. Enzyme unit (U) was defined as the quantity of substrate converted to sugars at an incubation period of 30 min at 37 °C.

Growth and Lactic acid production at different temperatures:

Determination of transient reducing sugars (by DNS method) and a growth linked lactic acid production studies were carried out at different temperatures of 4 °C, 37 °C and 45 °C in MRS medium. Samples were drawn after regular intervals of 24 hours as shown in Figure 4a. Growth profile studies were performed at different temperatures 4 °C, 37 °C and 45 °C to determine an optimum growth temperature of strain of *Pediococcus acidilactici* MZ375423. At 37 °C (Figure 4b) the culture was showing maximum (930.235 U/L) amylase production, in MRS medium. Fall in pH during the fermentation process at different temperatures (4 °C, 37 °C and 45 °C) revealed the ability of the strain to produce acidic end products while using glucose in the fermentation medium (MRS). As per reports, LAB capable of tolerating higher temperatures tend to have better growth rate along with higher yields in lactic acid production with lesser chances of contaminations during the fermentation process (Tanyildizi et al. 2005; Singh et al. 2006; Ibourahema et al. 2008).

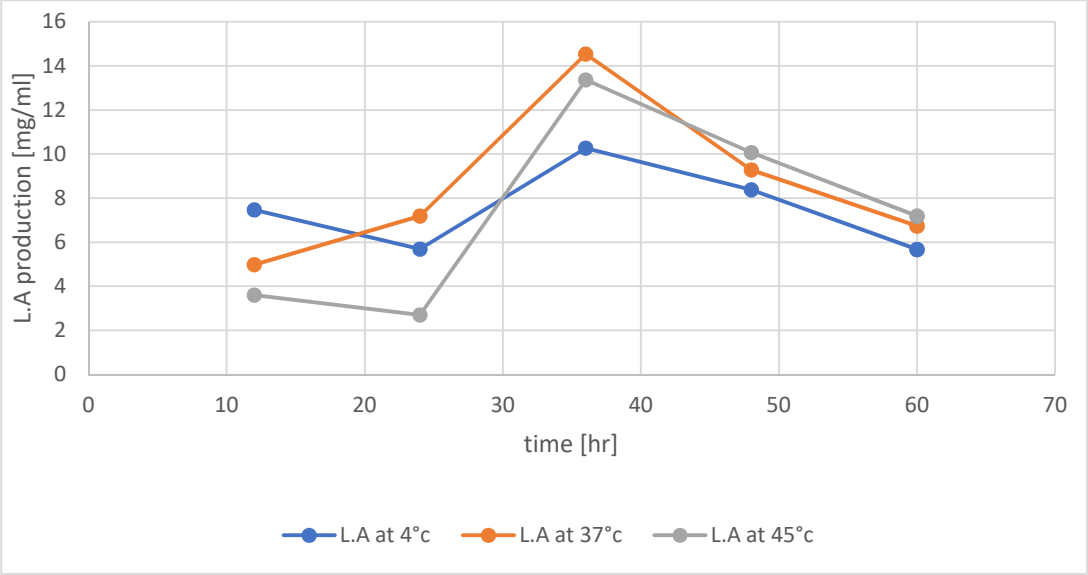

Figure 4a: Effect of temperature on lactic acid (LA) production in MRS medium determined at regular intervals of 12 hours. The result is mean of triplicates.

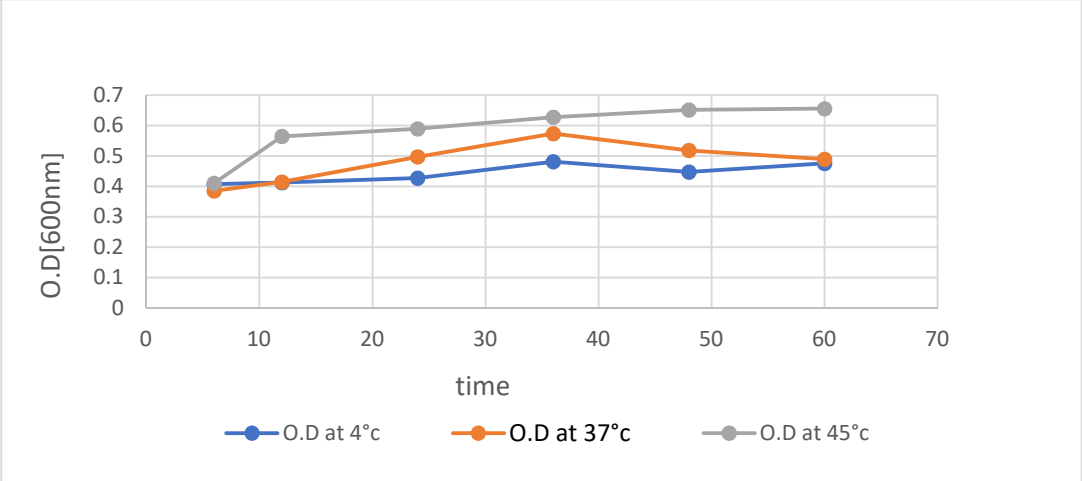

Figure 4b: Assessment of cell culturability in MRS medium through the examination of growth (*Pediococcus acidilactici*) at regular intervals of 12 hours. The result is mean of triplicates.

But in our case an optimum growth temperature was observed at 45°C after 36 h in MRS medium. The strain showed tolerance to high temperatures with an ability to produce lactic acid on fermentation of glucose in the medium (Figure 4 a, b). Growth and lactic acid production during glucose fermentation in MRS medium (dextrose: 20g/L) was reflected by changing pH after a regular interval of 12 h, at 4 °C, 37 °C and 45 °C respectively. Maximum (14.53 mg/mL) lactic acid production was achieved at 37 °C after an incubation period of 36 h in MRS medium. The strain was capable of producing lactic acid (4.186 mg/mL) at 4 °C after an incubation period of 36 h, thus showing the ability of the strain to be used in preservation of refrigerated food storage. Gutiérrez-Cortés et al. 2018 stated LAB isolated from dairy products belonging to genera *Lactobacillus, Enterococcus, Pediococcus*, and *Lactococcus* (Luiz et al. 2017) have important characteristics of producing organic acids, carbon dioxide, hydrogen peroxide, diacetyl, and bacteriocins (Ammor et al. 2006; Khan et al. 2010). These beneficial effects of probiotics along with the positive health effect of maintaining gastro-intestinal microbial balance while simultaneously suppressing growth of pathogens due to the production of lactic acid at 37 °C are the positive health effects of viable probiotic bacteria in gut. The inhibitory effect of lactic acid on LAB was considered to be more than inhibitory effect of fermentation activity. The accumulation of lactic acid tends to create osmotic pressure leading to inhibitory effect as compare to other fermentation products like sodium acetate, formic acid, acetic acid which have an individual inhibitory effect (Othman et al. 2018). So lactic acid bacteria fermentation is characterized by the kinetic product inhibition that affects growth rate and productivity. So the growth of the *P. acidilactici* MZ375423 strain was determined at regular intervals of 24 h.

Studies with such characteristics have served a potential to act as remedy for various gastrointestinal problems including IBD and colon cancer. However, it is not possible for a single probiotic strain to fulfil all the beneficial effects so an efficacy of the LAB and activity of LAB varies from strain to strain. For example, probiotic strains which are efficient against antibiotic-associated diarrhoea may not be having efficacy against IBD. In our studies we looked towards the cholesterol lowering ability of the strain isolated from traditional fermented cheese, Kalaeri. Cholesterol lowering effect in serum levels has been reported to lower the risk of coronary heart disease by 2-3% (Ishimwe et al. 2015). To overcome hypercholestermia, satin drugs are predominantly prescribed by the doctors having several adverse side effects as per the reports (Sultan and Hynes 2013). A 20% decrease in *in-vitro* cholesterol concentration in MRS-CHO broth was observed after the growth of culture, for 48 h at 37 °C. The reduction (%) of cholesterol level in our studies was comparatively lesser than the reports by Damodharan et al. 2015. The low levels of cholesterol in case of KID7 and *P. pentosaceus* KACC 12311 in the presence of 0.3 and 0.5% bile oxgal was attributed to be due to low cell growth while compared to MRS-CHO broth not supplied with bile had high levels of reduction. Such comparative studies are still awaiting in our case. Kumar et al. 2012 had reported that hypocholesteromic effect can be attributed to the cholestrol binding ability of probiotics in the small intestine. Cholesterol is also removed by probiotics by incorporation into cellular membranes of the growing bacteria.

Studies on essential probiotic criterion: low pH, high NaCl and bile concentration tolerance

As per literature studies a potential LAB can act as a probiotic strain if it fulfils one of the essential probiotic characteristics of surviving at low pH conditions prevailing in the gastrointestinal tract and maintaining its ability to carry out its metabolic activities even in transit state. So, present study focused on viability showing low pH tolerance and high bile salt as well as NaCl tolerance in in-vitro studies at 37 °C, so that they are able to carry out metabolic activities in the gut too. As per studies by Divisekera et al. 2019, LAB capable of tolerating NaCl concentrations up to 12% and showing survival at 6.5% NaCl concentration are categorized as osmo-tolerant. The LAB which are osmo-tolerant have an ability to produce bioactive compounds like lactic acid even when the concentration of NaCl is high in the gut (Menconi et al. 2014).

Studies on rate of survivability of the amylolytic *Pediococcus acidilactici* MZ375423 at low pH (2.0, 3.0), high bile salts as well as high NaCl concentrations (0.5% and 0.75%,) indicated that the strains can survive in gut conditions after being taken in traditional fermented dairy product, Kalaeri. pH resistance was observed in the growth of the organism after a regular interval of 12 h, as per O. D readings taken at 600 nm (Figure 5a). All the

three essential criterion as: high survivability at low pH, tolerance to high NaCl concentrations and bile salt concentrations was studied at 37 °C for 24 to 96 h incubation period. In our studies maximum growth was observed at pH 4 and 5; while pH 6.5 was kept as positive control (Figure 5a). Readings of the cell density at 600 nm were showing the viability of cells at pH 2.0 as well as 3.0 and lag phase was being followed by log phase. A decline in the growth of the strain at pH 2 as compared to pH 3 could not be related to earlier reports as maximum growth of bacteria at pH 4.0 and 5.0 was stated for *Lactobacillus* strains isolated from papaya. In our case *Pediococcus acidilactici* MZ375423 strain had shown maximum growth at pH 4.0 and 5.0and it was isolated from cheese product, Kalaei. Reports on the role of hydrogen ions and its concentrations on the growth of the bacteria were responsible for decline in growth rate. Studies by Divisekera et al. 2019 stated that the amount of probiotic bacteria in gastro intestinal conditions is with food matrix and so they are not directly exposed to the stomach HCl concentrations. The tolerance of these bacteria to acidic conditions is a prerequisite for being considered as a potential probiotic bacteria. The viability of these bacteria in the gut ensures that they can carry out their metabolic activities thus establishing their functionality in the gut (Dunne et al. 2001; Daoudou et al. 2011). As per physiological studies concentration of bile salt ranges from 0.2– 0.3%. in the small intestine, an increase of in bile concentration to 2% depends on the genotypic characteristic of the host and the type of food as well as amount of food ingested by an individual (Daoudou et al. 2011; Menconi et al. 2013). A normal healthy individual has a bile concentration of 0.3% and a commercial probiotic bacteria will be considered a potential strain if it tolerates up to 0.3% bile concentration.

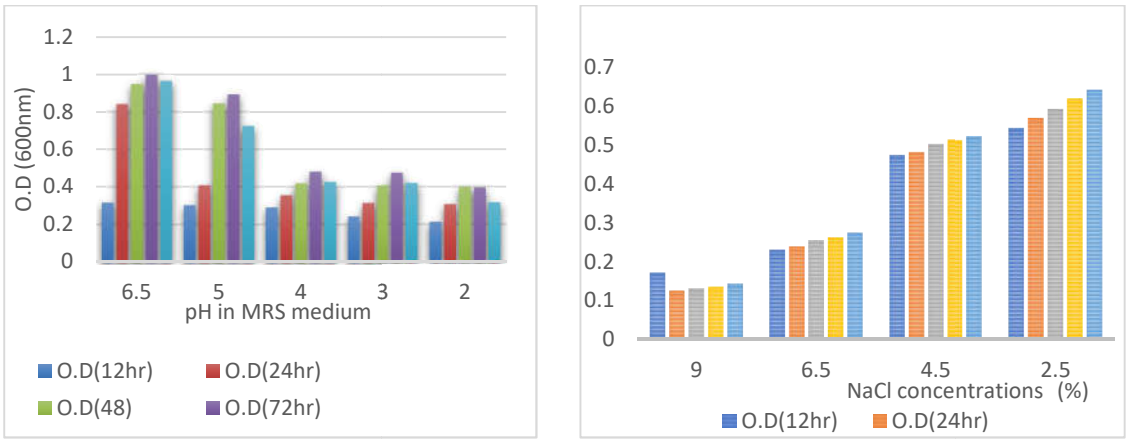

Figure 5: *Pediococcus acidilactici* showing tolerance to (a) pH concentrations at 37^0C in MRS medium when pH is adjusted with 0.1 N HCl prior to inoculation. The result is mean of triplicates (b) NaCl concentrations at 37^0C at a regular interval of 12 hours. The result is mean of triplicates

Reports have shown maximum growth of strains when the bile salt concentration was 0.8% and a decline in growth when bile salt concentration was 0.6% stating the variability of species at various bile salt concentrations. In present study tolerance to high bile concentration (0.75%) with CFU/ mL count of 8.8 X 10^{-4} (Figure 6) was observed, however a decrease in CFU/ mL was observed when compared to 0.5% bile salt concentrations (12.6 X 10^{-4}) after an incubation period of 48 h at 37 °C in MRS medium. An incline in survival rate was observed with an increase in bile salt concentrations. The role of bile is there in (specific and nonspecific) the defense mechanisms, in the gut of a healthy individual but the level of its inhibitory effect on LAB bacteria was determined primarily by the bile salt concentrations. Therefore, bile salt tolerance of LAB is considered to be an important characteristic ensuring their survival in the gut, capable of showing their metabolic activites while moving from gastric conditions to intestinal conditions (Szutowska and Gwiazdowska 2021). During food fermentation, probiotic LAB are exposed to a high salt and/or an acidic environment. Hence, the potential probiotic LAB strain was further studied for its tolerance to NaCl concentrations 0.25%, 0.45%, 0.65% and 0.9% respectively (Figure 5 b).

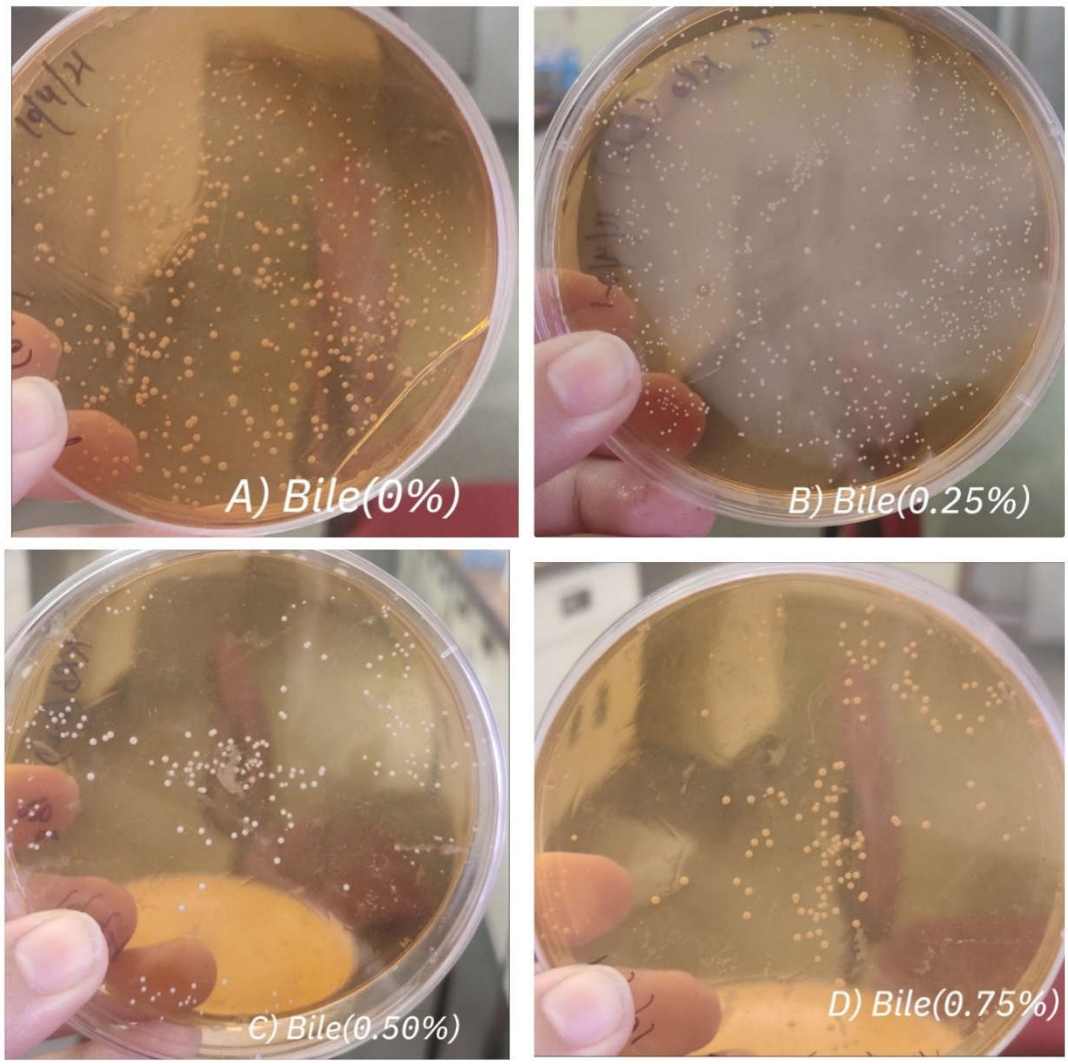

Figure 6: Effect of bile concentration on the culture (*Pediococcus acidilactici*) grown in MRS medium in order to determine colony count at 37^0C after an incubation period of 48 hour.

Conclusion:

Probiotics have demonstrated efficiency in preventing and treating various medical conditions, particularly those involved in the gastrointestinal tract. Studies have shown that strains vary in their probiotic characteristics, some strains which are having beneficial health effects may not be having therapeutic effects associated with them; so a search for commercial probiotic strains needs to be fuelled which are robust and can be an asset for nutraceutical as well as pharmaceutical companies.

Our studies have highlighted the importance of probiotic bacteria from traditional fermented cheese, Kalaeri, to have maximum survival rate in the gut even in the gastro intestinal transit. A high bile salt concentration and

low pH concentration have a tremendous role to play in protecting the gut from invasion of pathogenic bacteria but at the same time it is very important that normal microflora of the gut is maintained ensuring healthy diet of an individual. The cholesterol lowering effect imbibed in such strains can prevent individuals from coronary heart diseases too.The probiotic bacteria from fermented milk products can survive in the gut and produce various digestive enzymes like amylase, lipase and polysaccharide degrading enzymes; besides producing certain compounds like lactic acid, antimicrobial peptides like bacteriocins which will have an antagonistic effect on the pathogenic strains.

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