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In-vitro Assessment of Probiotic Potential of Lactic acid Bacteria

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ABSTRACT

Lactic Acid Bacteria (LAB) are the major constituents of the human intestinal micro flora. These have been considered as the major microbial group having probiotic potential. They are able to exert a wide range of beneficial health promoting effects that include inhibition of pathogen growth and production of antimicrobials and vitamins. In food industry, LAB have received considerable attention due to their probiotic activities. The probiotic strain's ability to resist unfavorable physiological conditions of the gastrointestinal tract (GIT) depends on various factors like tolerance to bile secretion and lysozyme resistance. The present study was conducted with the objectives of *in-vitro* screening of various indigenous LAB strains isolated from milk and yogurt in order to evaluate their probiotic potential. For probiotic potential, the pH sensitivity, bile resistance, H_2O_2 and lysozyme resistance of LAB strains will be determined. Our *in-vitro* studies concluded that PL5 (*Lactobacillus paracasei*), PL8 (*Enterococcus faecium*), PL13 (*L. delbrueckii*) and PL14 (*L. saekei*) proved to be most promising LAB strains among all that exhibited a high resistance towards low pH, bile, lysozyme and H_2O_2. In future, these *in-vitro* studies will facilitate scientists to select suitable LAB strains and evaluate their probiotic properties *in-vivo* to understand how they affect human host and cope with adverse conditions in human GI tract. In future, more potential properties of these strains may be checked, like folate & oxalate production, adhesion to mucin, production of β-galactosidase, cholesterol reduction mechanisms on LAB strains. It will be helpful to design new protocols for in-depth studies related to their potential.

Key words: Lactic Acid Bacteria, Probiotic Potential, GIT, Microflora

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1. INTRODUCTION

Gastrointestinal (GI) microbiota inhabits large number of microorganisms which include beneficial as well as pathogenic microbes. These microbes have well known effect on human and animal health. The variety of micro-flora develops with host age and gets more unstable with increasing age (1). In addition, the beneficial microorganisms provide a protection against pathogenic bacteria by competing with them for binding sites and nutrients (2) and by producing antimicrobials compounds such as bacteriocins. The beneficial bacteria are known as probiotics (3). In recent years, there is an increase interest in the research area of beneficial or probiotic bacteria and also the verification and characterization of health benefits related to the use of probiotics. The market of probiotic products is continuously increasing. The probiotics can be used to increase gastrointestinal microbiota and also for the treatment of cancer, allergies and infections of urogenital (4). The probiotic word is derived from Greek words "*pro*" and "*biotos*". In Greek, the word "*pro*" means for and "*biotos*" meaning life but in Latin's meaning of "*pro*" is for. So the entomology of word "probiotic" is hybrid and the correct sense of word probiotic is "for life" (5). LAB normally are inhabitants of intestine, vagina, animal gastrointestinal tract, oral cavity, food and sewage. Even some species of LAB are found in the intestine of honeybee such as *Bifidobacteria asteriodes, Bifidobacteria coryneform, Bifidobacteria indicum* (6, 7). In latest years, there are the various potential health benefits of increased research to characterize and validate the use of the LAB (8). LAB are colonized in GIT of human and animals and coordinate with other obligate anaerobic microorganism (9). As an indigenous bacterium, LAB establish a

symbiotic relationship with the host and gives benefit to it. They provide health effects to their host. Due to their beneficial effects, LAB are used as probiotics in different dairy products like cheese, milk, yoghurt and other nutritional additions (10-14). Survival of LAB species at low pH is an indicator for the efficacy of probiotic bacterial strain (15). The probiotic bacteria's ability to persist is mainly dependent upon the resistance for acid and bile (16). However, lysozyme resistance is also crucial for probiotics selection. The resistance to lysozyme (25-35 mg/L) is suggested for the choice of probiotics to be used in dairy industry (17).

2. MATERIALS AND METHODS

2.1. Isolation

For isolation of Lactic Acid Bacteria, the samples were collected from various dairy origins. The samples of raw milk and yoghurt were collected from the different areas of Faisalabad, Gujranwala, Okara and Jhang Districts (Table 1). Samples were shifted to the National Probiotic Laboratory (NPL), NIBGE Faisalabad in sterile conditions. Each isolate of Lactic Acid Bacteria was named as PL after the Probiotic Lab. The 14 strains named as PL1 (*Lactobacillus plantarum*), PL2 (*Lactobacillus plantarum*), PL3 (*Lactobacillus rahmnosus*), PL4 (*Lactobacillus delbrueckii*), PL5 (*Lactobacillus paracasei*), PL6 (*Weisella spp*), PL7 (*Enterococcus faecium*), PL8 (*Enterococcus faecium*), PL9 (*Lactobacillus delbrueckii*), PL10 (*Weissella paramesenteroids*), PL11 (*Lactobacillus delbrueckii*), PL12 (*Lactobacillus delbrueckii*), PL13 (*Lactobacillus delbrueckii)* and PL14 (*Lactobacillus saekei*) were selected from lab for the further evaluation of their probiotic potential properties of Lactic Acid Bacteria.

Table 1. List of Lactic Acid Bacteria strains and their origin

LAB strains	Origin	LAB strains	Origin
PL1 (*Lactobacillus plantarum*)	Milk	PL8 (*Enterococcus faecium*)	Milk
PL2 (*Lactobacillus plantarum)*	Probiotic tablet	PL9 (*Lactobacillus delbrueckii*),	Yogurt
PL3 (*Lactobacillus rahmnosus*)	Yogurt	PL10 (*Weissella paramesenteroids*)	Milk
PL4 (*Lactobacillus delbrueckii*)	Yogurt	PL11 (*Lactobacillus delbrueckii*)	Yogurt
PL5 (*Lactobacillus paracasei*)	Milk	PL12 (*Lactobacillus delbrueckii*)	Yogurt
PL6 (*Weisella spp*)	Milk	PL13 (*Lactobacillus delbrueckii*)	Yogurt
PL7 (*Enterococcus faecium*)	Milk	PL14 (*Lactobacillus saekei*)	Milk

The LAB strains PL1 (*Lactobacillus plantarum*), PL2 (*Lactobacillus plantarum*), PL3 (*Lactobacillus rahmnosus*), PL4 (*Lactobacillus delbrueckii*), PL5 (*Lactobacillus paracasei*), PL6 (*Weisella spp*), PL7 (*Enterococcus faecium*), PL8(*Enterococcus faecium*), PL9 (*Lactobacillus delbrueckii*), PL10 (*Weissella paramesenteroids*), PL11(*Lactobacillus delbrueckii*), PL12 (*Lactobacillus delbrueckii*), PL13 (*Lactobacillus delbrueckii)* and PL14 (*Lactobacillus saekei*) were cultured in sterile screw capped test tubes (PYREX®) containing 9 mL of MRS broth medium with 0.05% L-cysteine. The test tubes with LAB cultures were kept in sterile anaerobic jar (Oxoid™, UK) having anaerobic sachet (AnaeroGen™, Oxoid™, UK) which produced ascorbic acid for the removal of oxygen. The anaerobic jar containing test tubes of LAB strains was incubated at the 37^{0}C for 48 hours. After the required period of incubation, the growth of LAB in pellet form was observed at the bottom of the culture tubes.

2.2. Resistance to low pH

LAB cells from 48 hours old culture were collected by centrifugation at 4,000 rpm, 26^{0}C for 5 minutes. Pellet was put off in phosphate buffer saline. Then the suspension was diluted in fresh sterile MRS_C broth medium and pH was adjusted to 2.5, 3, 3.5 and 5.5. Microtiter plate wells were lablled with different pH values. Then 200µL of each suspension culture was transferred to labeled micro-titer plate (Kartell™) wells with respective pH values. Optical density was measured before incubation (OD_{630} at time=0 hours). Then, the micro-titer plate was incubated for 48 hours at 37^{0}C under anaerobic conditions. After the incubation (OD_{630} at time=48 hours) was measured by using Spectra max 384 plus. Spectra max 384 plus measures optical density by absorbance optical light at different wavelengths. The experimental treatments were performed in triplicates.

2.3. Resistance to Bile

The tolerance of LAB strains against bile salts was evaluated as described by Tsai et al. (18). First of all, 150µL of bacterial cultures with optimized optical density (i.e. OD_{630}=0.1) were dispensed in wells of micro-titer plate and then 50µL of different concentrations of bile (ox-gall, Difco) i.e. 0.30%, 0.50%, 1% and 1.5% were also added in each well. The Optical Density (OD) was measured at 630nm before incubation (at time=0). Then strains were incubated at 37 ^{o}C for 48 hours under anaerobic conditions. After 48 hours of incubation OD was measured at 630nm. All experiments were performed in triplicates.

2.4. Resistance to lysozyme

The stock cultures of LAB strains were grown in MRS broth medium supplemented with 0.05% L-cysteine. All Lactic Acid Bacterial cultures were incubated at 37^{0}C for 48 hours under anaerobiosis. 150µL of bacterial cultures

with optimized optical density (i.e. OD_{630}=0.1) were dispensed in wells of microtitre plate. Then 50µL of lysozyme (Merck™,Germany) concentrations i.e. 200µg/mL, 300µg/mL, 400µg/mL and 500µg/mL were added in each bacterial culture. The optical density (OD) of each well of microtitre plate (Kartell™) was measured at 630nm. Then it was incubated for 48 hours at 37^{0}C under anaerobic condition. The final OD was observed again after 48 hours. The minimum inhibitory concentration (MIC) of each strains of Lactic Acid Bacteria from PL1(*Lactobacillus plantarum*), PL2 (*Lactobacillus plantarum*), PL3 (*Lactobacillus rahmnosus*), PL4 (*Lactobacillus delbrueckii*), PL5 (*Lactobacillus paracasei*), PL6 (*Weisella spp*), PL7 (*Enterococcus faecium*), PL8 (*Enterococcus faecium*), PL9 (*Lactobacillus delbrueckii*), PL10 (*Weissella paramesenteroids*), PL11 (*Lactobacillus delbrueckii*), PL12 (*Lactobacillus delbrueckii*), PL13 (*Lactobacillus delbrueckii)* and PL14 (*Lactobacillus saekei*) was analyzed. The experiments were performed in triplicates.

2.5. Resistance to H_2O_2

Lactic Acid Bacteria strains were tested for their resistance to H_2O_2. The stock cultures of Lactic Acid Bacteria were incubated in MRS_C broth medium for 48 hours at 37^{0}C under anaerobic conditions. Different concentrations of H_2O_2 i.e., 40µg/L, 60 µg/L, 80µg/L and 100µg/L were prepared from 50% of pure H_2O_2 (Scharlau, China). 150µL of bacterial cultures with optimized optical density (i.e. OD_{630}=0.1) were dispensed in wells of micro-titer plate. Then 50µL of different concentrations of H_2O_2 were added. The optical density (OD) of each well of micro-titer plate was measured at 630nm. Then it was incubated for 48 hours at 37^{0}C under anaerobic condition. The OD was measured again after 48 hours. The minimum inhibitory concentration (MIC) of each LAB strains was established. The experiments were performed in triplicates.

3. RESULTS AND DISCUSSION

3.1. Resistance to low pH

In order to evaluate the resistance against pH, Lactic Acid Bacterial strains were grown in pH adjusted MRS_C broth medium and incubated at 37^{0}C for 48 hours under anaerobic conditions. The optical density (OD) of each Lactic Acid Bacterial isolate was measured at 630nm. A significant growth of all Lactic Acid Bacterial strains was observed at the pH 2.5, 3.0, 3.5, 5.5 respectively. All the Lactic Acid Bacterial strains exhibited their potential to survive even in acidic conditions. The PL5 (*Lactobacillus paracasei*), PL6 (*Weisella spp*), PL13 (*Lactobacillus delbrueckii)* and PL14 (*Lactobacillus saekei*) Lactic Acid Bacterial strains were able to grow at pH 3.0. But a decreased viability rate of Lactic Acid Bacteria was observed at that acidic pH 3.0. However, growth rate of PL1 (*Lactobacillus plantarum*), PL2 (*Lactobacillus plantarum*), PL3 (*Lactobacillus rahmnosus*), PL4 (*Lactobacillus delbrueckii* PL7 (*Enterococcus faecium*), PL8 (*Enterococcus faecium*), PL9 (*Lactobacillus delbrueckii*), PL10 (*Weissella paramesenteroids*), PL11 (*Lactobacillus delbrueckii*) and PL12 (*Lactobacillus delbrueckii*) was inhibited at pH 3.0. The maximum growth of all Lactic Acid Bacterial strains was observed in the medium at pH 5.5 as compared with positive control. All Lactic Acid Bacterial strains were able to grow well at pH 3.5 and 5.5 as shown in Diagram 1, Diagram 2, Diagram 3 and Diagram 4.

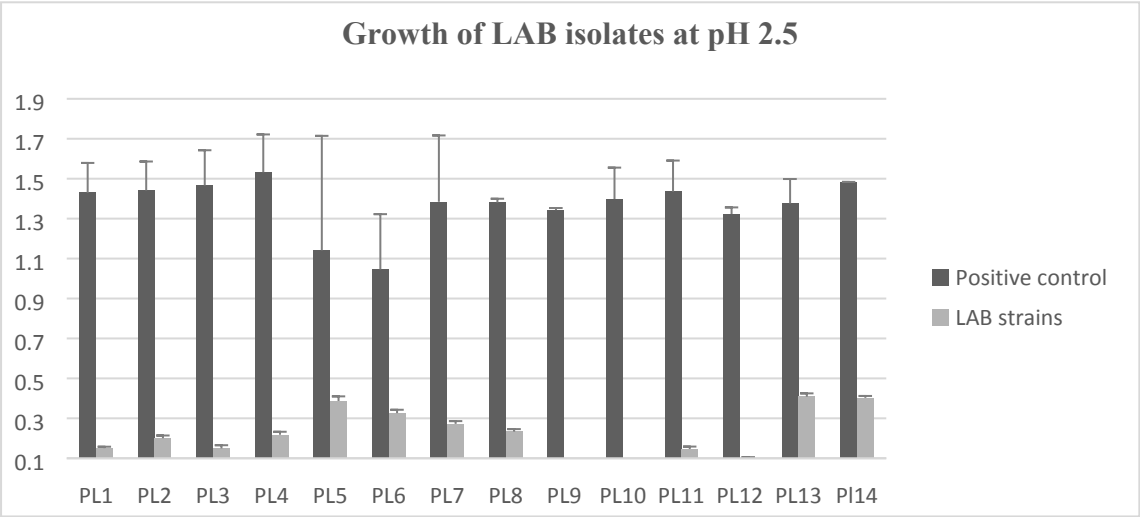

Diagram 1. Growth pattern of LAB strains at pH 2.5

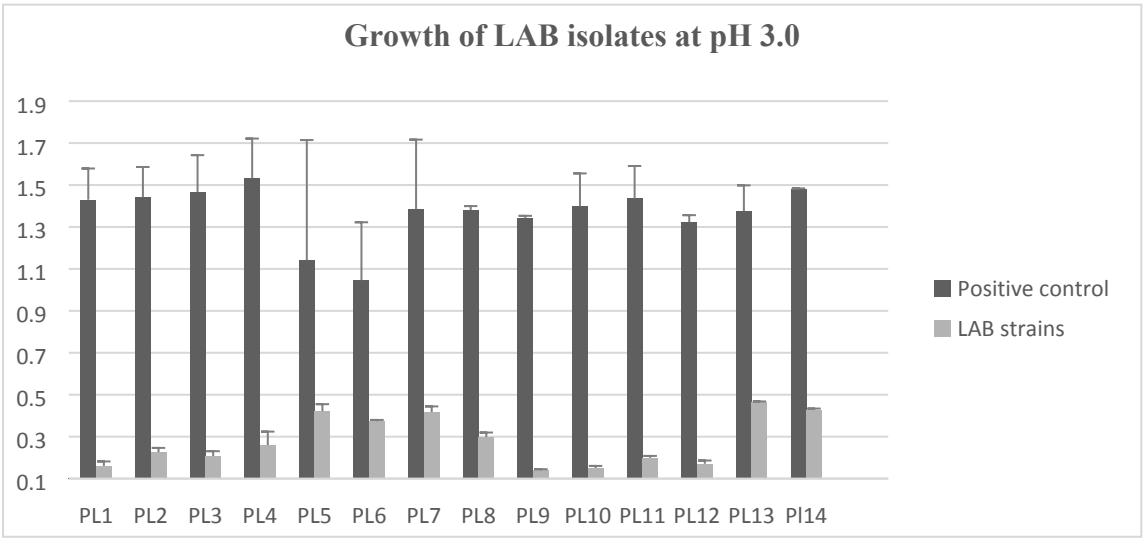

Diagram 2. Growth Pattern of LAB strains at pH 3.0

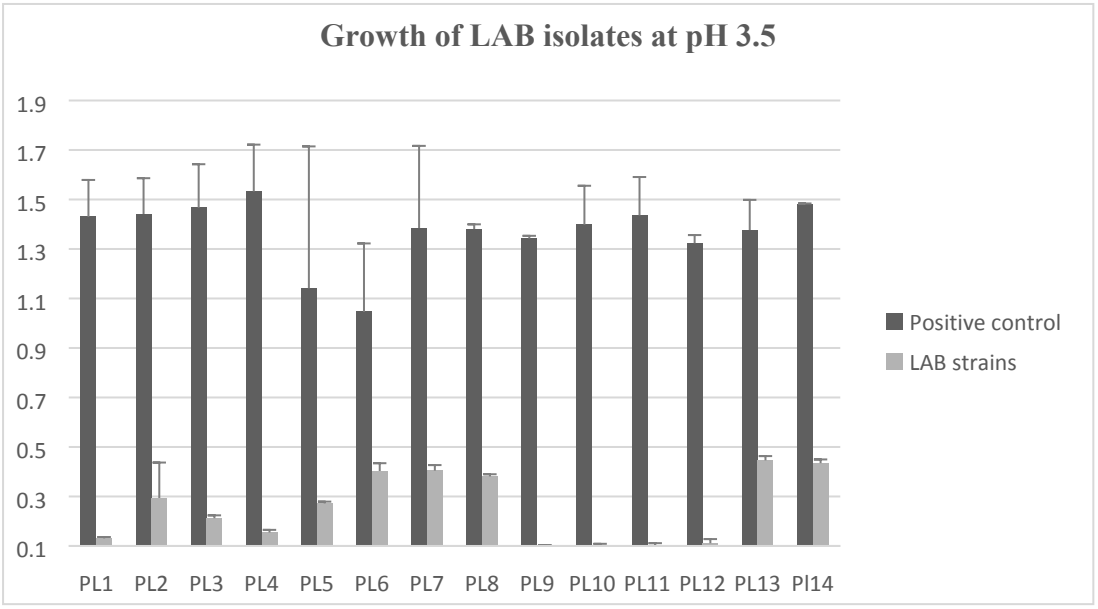

Diagram 3. Growth Pattern of LAB strains at pH 3.5

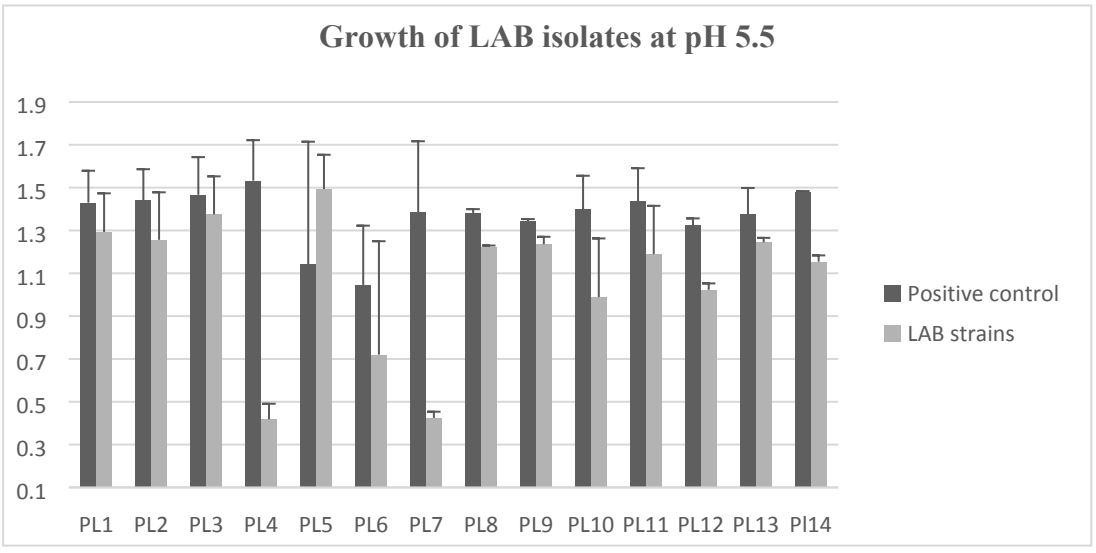

Diagram 4. Growth of LAB strains at pH 5.5

3.2. Tolerance against bile

The resistance of LAB strains against bile was evaluated. LAB cultures were inoculated in MRS_C broth containing different concentrations of bile i.e. 0.30%, 0.50%, 1% and 1.5% respectively. After 48 hours of incubation, the growth of LAB strains was observed by measuring the optical density at 630nm. The 0.30%, 0.50%, 1% and 1.5% concentrations of bile were used in the experiment but according to the results, the viability of LAB strains was decreased as concentrations of bile increased. All LAB strains were more resistance at 0.30% of bile as compared to other concentration of bile as shown in Diagram 5, Diagram 6, Diagram 7 and Diagram 8.

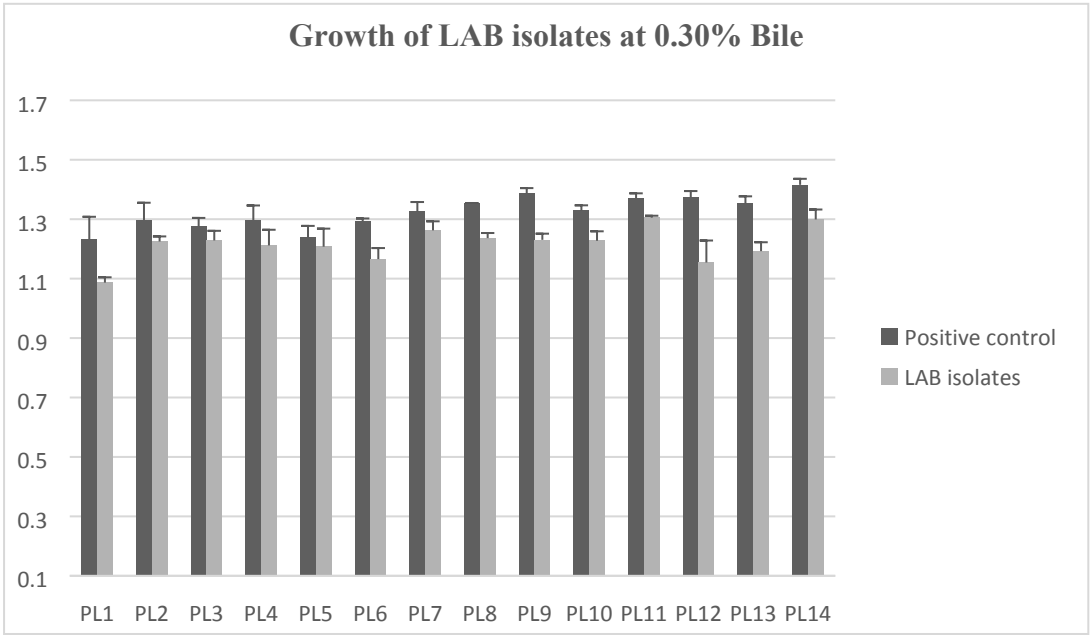

Diagram 5. Growth pattern of LAB strains at 0.30% bile

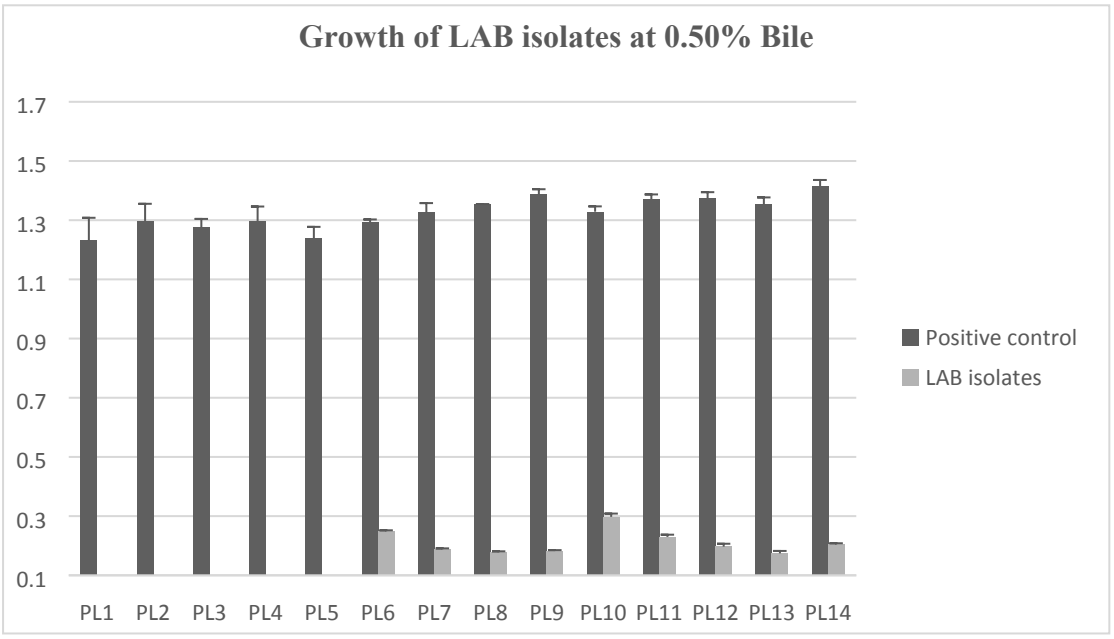

Diagram 6. Growth pattern of LAB strains at 0.50% bile

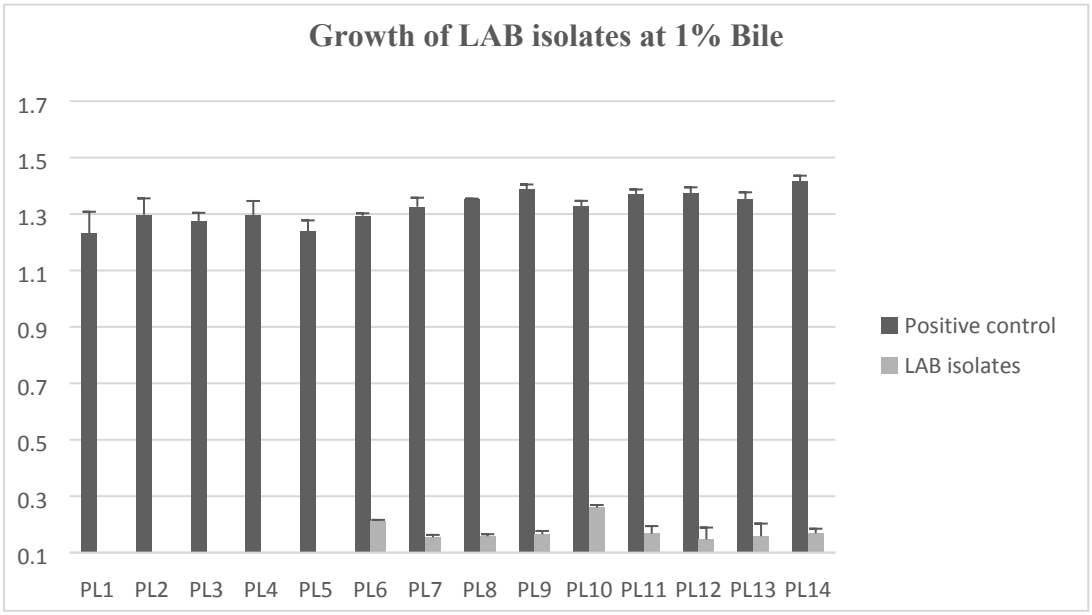

Diagram 7. Growth pattern of LAB strains at 1% bile

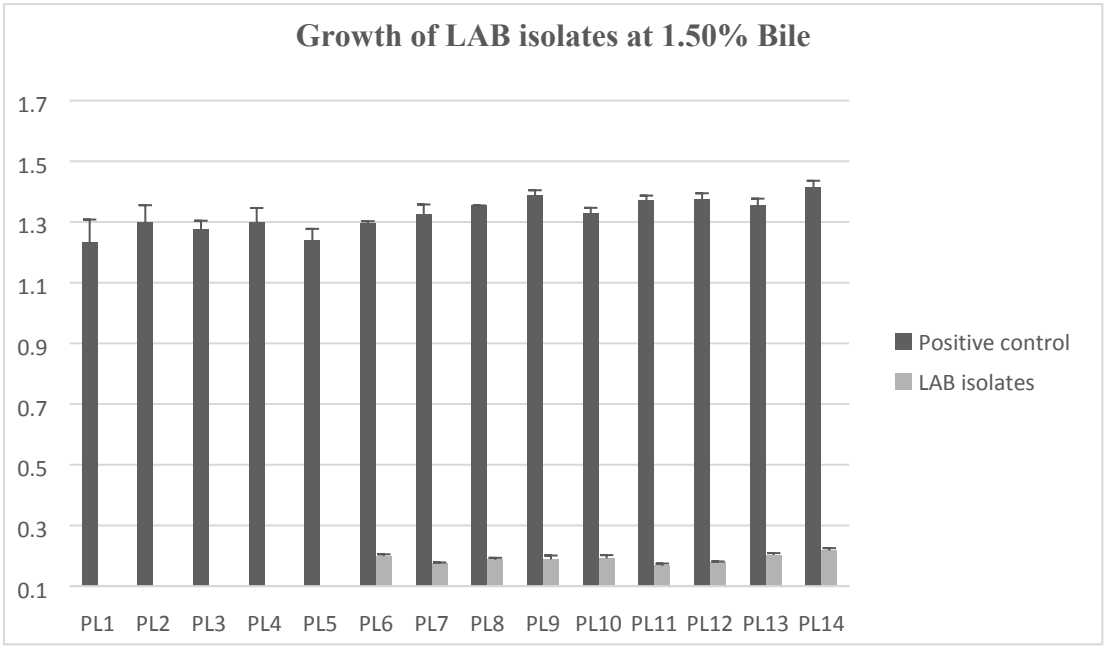

Diagram 8. Growth pattern of LAB strains at 1.50% bile Resistance to lysozyme

The resistance of LAB strains against lysozyme was evaluated. All the LAB strains were cultured in MRS_C broth and incubated at 37^0C for 48 hours under the anaerobiosis. The grown LAB cultures were treated with different concentrations of lysozyme i.e. 200µg/mL and 300µg/mL. After 48 hours of incubation, the resistance of LAB strains was observed by analyzing the optical density (OD) at 630nm. The resistance of each isolate was observed against different concentrations of the lysozyme such as 200µg/mL, 300µg/mL. All LAB strains were found to be resistant at all concentrations of lysozyme as shown in Diagram 9 and Diagram 10.

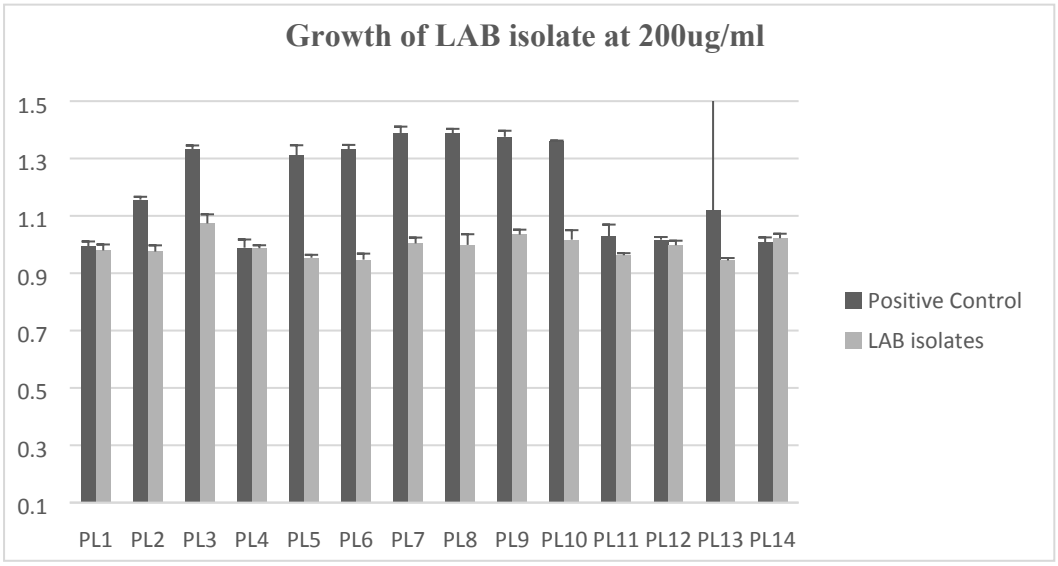

Diagram 9. Growth pattern of LAB strains at 200µg/mL

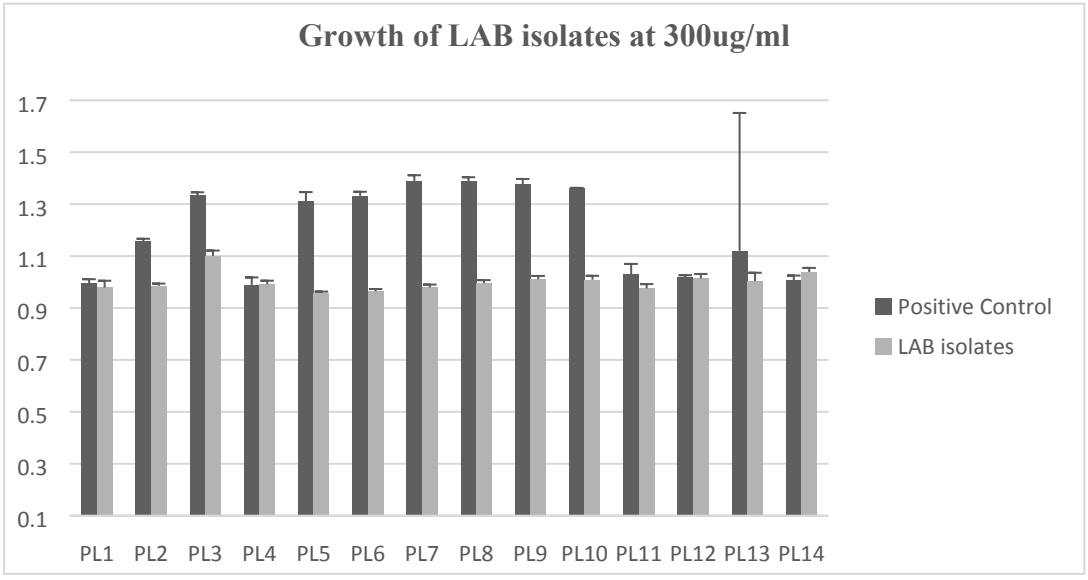

Diagram 10. Growth pattern of LAB strains at 300 µg/mL

3.3. Resistance to hydrogen peroxide (H_2O_2)

The resistance of LAB strains against H_2O_2 was evaluated. *In vitro,* to check the resistance of LAB against H_2O_2, LAB strains were treated with different concentrations of H_2O_2 and incubated at 37^0C for 48 hours under strict anaerobic conditions. After 48 hours, Optical Density (OD) at 630nm was observed. The resistance and sensitivity of each LAB isolate was evaluated. According to the results, the LAB strains PL7 (*Enterococcus faecium*), PL8 (*Enterococcus faecium*), PL9 (*Lactobacillus delbrueckii*), PL10 (*Weissella paramesenteroids*), PL11 (*Lactobacillus delbrueckii*), PL12 (*Lactobacillus delbrueckii*), PL13 (*Lactobacillus delbrueckii)* and PL14 (*Lactobacillus saekei*) were resistant against 40 µg/L, 60 µg/L of H_2O_2. However the LAB strains PL1 (*Lactobacillus plantarum*), PL2 (*Lactobacillus plantarum*), PL3 (*Lactobacillus rahmnosus*), PL4 (*Lactobacillus delbrueckii*), PL5 (*Lactobacillus paracasei*) and PL6 (*Weisella spp*) were sensitive against 40 µg/L, 60 µg/L of H_2O_2. Little growth of PL1 (*Lactobacillus plantarum*), PL4 (*Lactobacillus delbrueckii*) and PL5 (*Lactobacillus paracasei*) was observed at the 40µg/L concentration of the H_2O_2 as shown in Diagram 11 and Diagram 12.

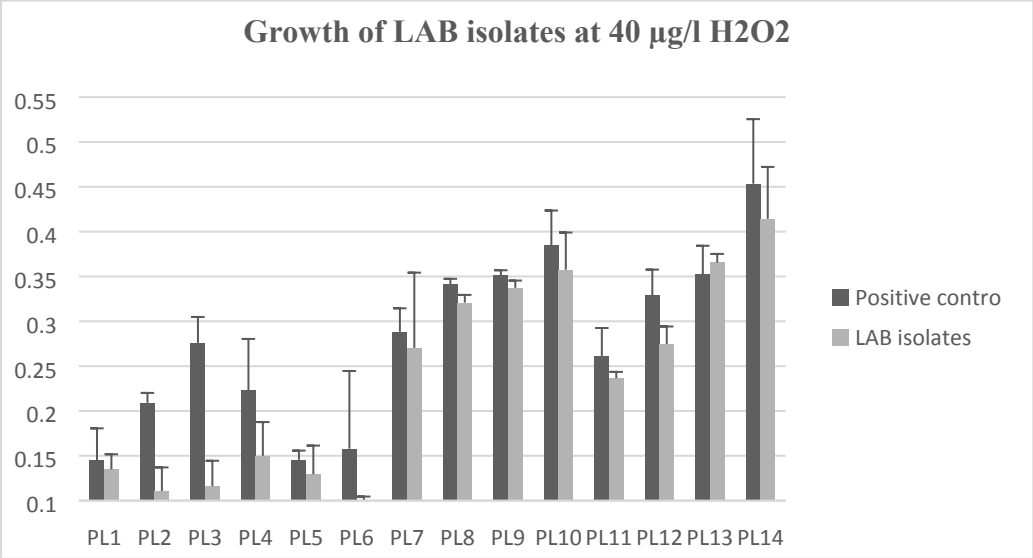

Diagram 11. Growth pattern of LAB strains at 40µg/L

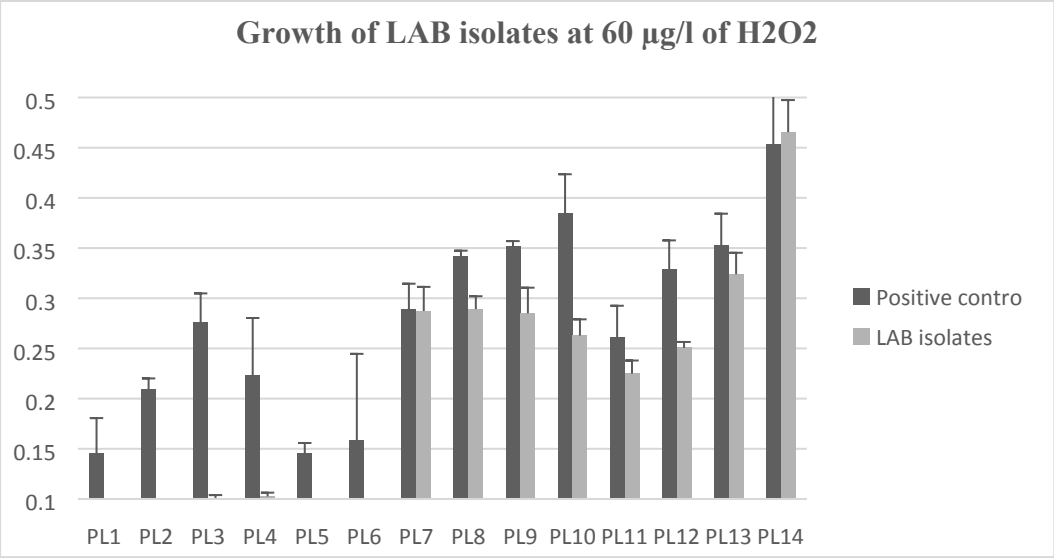

Diagram 12. Growth pattern of LAB strains at 40µg/L

Lactic Acid Bacteria can be found as components of the gastrointestinal micro-biota and as probiotics, they have potential to become an important means of enhancing digestive health and preventing diseases (19). So, their utility in food industry is increasing day by day. In order to estimate full potential of probiotics, research has been focused on Lactic Acid Bacterial strains that can withstand gastrointestinal transit i.e. gastric acidity and bile salts etc. (20). The aim of this study was to evaluate the probiotic potential of indigenous LAB strains, derived from different dairy origins such as raw milk and yoghurt. To determine the probiotic properties different tests were performed such as resistance to low pH, tolerance against bile, resistance to lysozyme and resistance to H_2O_2 (21, 22). To survive in the acidic environment, LAB maintain their alkaline pH. The growth of all the LAB strains was maximum at pH 5.5 and for each isolate, the growth decreased as the pH of their growth medium became more acidic. In the present study, LAB strains showed better growth at pH 5.5. Bile plays important role in the functioning of intestinal bacteria, predominantly for probiotic bacteria. We identified the actual behavior of LAB in the presence of bile. Sequentially, to employ a beneficial effect in the digestive tract, probiotic culture must tolerate the passage through the stomach and be tolerant to the bile concentrations in the small intestine. In the present study, we used concentration of the bile salts from 0.30% to 1.5%. Bile appeared to have a bacteriostatic rather than a bacteriocidal effect on the strains of LAB investigated in this study. However, 1.5% of bile was a crucial factor for the investigation of potential of LAB strains. The concentrations of human bile salts range from 0.3 to 0.5% in the stomach. In this study all the LAB strains were able to survive at the different bile concentrations. However, all tested Lactic Acid Bacteria did not tolerate 1.5% bile concentration. The resistance of LAB strains against lysozyme was also evaluated for probiotic potential. In the lysozyme assay, the lysozyme was used in different concentrations i.e. 200 µg/mL, 300µg/mL. The LAB strains from dairy origin were found to be resistant at all

concentration of lysozymes. The indigenous LAB strains were found to have growth in up to 300µg/mL concentration of lysozyme. *In-vitro*, the resistance of LAB strains was evaluated by applying different concentrations of H_2O_2. The PL7 to PL14 strains were resistant at 40µg/L, 60µg/L concentrations of H_2O_2, while the PL1 (*Lactobacillus plantarum*), PL2 (*Lactobacillus plantarum*), PL3 (*Lactobacillus rahmnosus*), PL4 (*Lactobacillus delbrueckii*), PL5 (*Lactobacillus paracasei*) and PL6 (*Weisella spp*) strains were sensitive against 40µg/L, 60µg/L concentrations of H_2O_2. The results of our study indicate that the LAB strains PL5 (*Lactobacillus paracasei*), PL6 (*Weisella spp*), PL8 (*Enterococcus faecium*), PL13 (*Lactobacillus delbrueckii)* and PL14 (*Lactobacillus saekei*) are the most promising probiotic LAB strains. They are able to grow well at acidic pH, at the all concentrations of lysozyme, H_2O_2 and bile. In future these strains can be used in various probiotics food products. In future, these *in-vitro* studies must facilitate others to select suitable LAB strains and evaluate their probiotic properties *in-vivo* to understand how they affect human host and cope with adverse conditions in human GI tract. More potential properties of these strains should be checked, like folate & oxalate production, adhesion to mucin, production of β-galactosidase, cholesterol reduction mechanisms. It will be helpful to design new protocols for in-depth studies related to their potential.

4. CONCLUSION

The results of our study indicate that the LAB strainsPL5 (*Lactobacillus paracasei*), PL6 (*Weisella spp*), PL8 (*Enterococcus faecium*), PL13 (*Lactobacillus delbrueckii)* and PL14 (*Lactobacillus saekei*) are the most promising probiotic LAB strains. They are able to grow well at acidic pH, at the all concentrations of lysozyme, H_2O_2 and bile. In future, these strains can be used in various probiotics food products.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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